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Title: THE COMPARATIVE PHARMACODYNAMICS OF THREE ORGANOPHOSPHATE PESTICIDES IN HOST RABBITS AND TWO ECTOPARASITES

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Proban, Famphur and Tiguvon were administered intravenously, subcutaneously, and orally at varying concentrations to 101 female rabbits. The cholinesterase (ChE) activity levels of both plasma and red blood cells were tested, as was tick and mosquito ChE, at various times before and after dosage. The determinations measured the inhibition of ChE due to pesticidal activity. Correlations between host blood ChE activity fluctuations and ectoparasite ChE activity were observed.

Ectoparasite mortality commenced at 32% ectoparasite ChE activity depression and there was 100% mortality at 68% ChE activity depression. No mortality of ectoparasites was observed to occur between the 24 and 96 hour postdosage period. Mated female ticks that had been exposed to sublethal doses of pesticide laid no eggs.

Residue studies indicated that Proban and Famphur were

degraded rapidly and excreted in the urine and feces. Tiguvon was more persistent and remained in certain tissues for approximately 28 days.

Therapeutic, subcutaneous dosages of all three pesticides eliminated ear mites from rabbits within 24 hours.

Reduction in temperature was fatal to mosquitoes that had received sublethal doses of pesticide in their blood meal.

Reproductive capabilities of rabbits were not impaired by therapeutic dosages of the pesticides.

The Comparative Pharmacodynamics of Three Organophosphate Pesticides on Host Rabbits and Two Ectoparasites

by

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THE COMPARATIVE PHARMACODYNAMICS OF THREE ORGANOPHOSPHATE PESTICIDES IN HOST RABBITS AND TWO ECTOPARASITES

INTRODUCTION

Ectoparasites of domestic animals cause medically and economically important diseases throughout the world. Many deaths of domestic animals may be attributed to the direct or indirect effects of these ectoparasites. The vitality of livestock is reduced by large infestations of ectoparasites thereby making the animals more susceptible to diseases. However, in parasitized animals, the greater economic losses are the result of reduced weight gains and feed utilization efficiency, thus increasing cost of livestock production while decreasing production of meat, milk, and fiber (Herms and James, 1961; Metcalf, Flint, and Metcalf, 1962). Recent estimates of the USDA set annual losses in livestock at approximately one billion dollars of which a considerable percentage was due to ectoparasites (Timmermann, 1963).

Ectoparasites are of great economic importance to livestock growers in the United States. The pasturing of livestock in rough terrain and in large acreage precludes the possibility of attempting to control these ectoparasites by range management. The ectoparasites must be controlled while they are on the host. This is especially true in the case of ixodid ticks that have a long, complex life cycle.

As the ectoparasites that have the most harmful effects are blood feeders, it would seem plausible that a way should be found to combat them through the host. It was therefore decided that investigation into the use of systemic pesticides was in order.

During the past several years, there has been an interest in the use of systemically acting organophosphorus compounds for control of both ectoparasites and endoparasites (Timmermann, 1963; Khan, 1969). The organophosphates are perhaps the most versatile group of pesticides currently used. For the most part, they pose little residue problem in tissue and they are readily and rapidly absorbed through the membranes of animals. As they are presently used, organophosphate systemics are impractical in control of ectoparasites as they are completely and rapidly degraded by the animal and eliminated, sometimes within a very few hours. Before these organophosphate systemics may become really effective, a system will have to be devised that will effect a prolonged, controlled release of pesticide into the bloodstream of the host. Any controlled release system must keep the pesticide in the blood stream at a level which is toxic to the ectoparasite but not to the host. This might be accomplished with an implant in much the same manner as a female hormone is implanted in cattle and sheep. The work by Clifford, Yunker, and Corwin (1967), indicates that the implant technique may have some promise. An external, controlled release device, such as

the PVC Vapona dog collar would be impractical in rough, brushy terrain as the animal could possibly be strangled by the collar's catching in the brush.

Before a controlled release system can be effected however, the pharmacodynamics of the organophosphorous compounds must be better understood. If these systemically acting organophosphates could be made truly effective against ectoparasites, the livestock industry would be materially aided. Besides the practical side to the study, the method of kill by the organophosphates was another worthwhile aspect. In recent years, there seems to be some doubt as to whether organophosphates actually kill by inhibiting cholinesterase (ChE) or some other enzyme (Chadwick, 1963).

The purpose of this study was to compare the pharmacodynamics of three organophosphate pesticides in the host-ectoparasite system. The pesticides were chosen for their wide range in toxicity to mammals. One of the pesticides, fenthion (Tiguvon), is well known, while the other two, Famphur and cythioate (Proban) are relatively new. During the course of the study particular emphasis was given to the following aspects:

(1) the average value for red blood cell (RBC) and plasma ChE[1]

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[1] The abbreviation ChE is used, in the broad sense, to encompass both acetylcholinesterase and pseudocholinesterase unless specifically designated.

activity levels in New Zealand and California strain rabbits.

(2) the effects of the three pesticides at three dosage rates for three different routes of application on the ChE activity levels of RBC and plasma of the rabbits. This included a comparison of initial ChE activity depression (both time and extent) and subsequent recovery.

(3) the effects of the lowered blood ChE activity level of the host on the ChE activity of two ectoparasites, Dermacentor andersoni Stiles and Aedes aegypti Meigen.

(4) the percent mortality of the ectoparasites feeding at different time increments after dosage of the host.

(5) the effect of ChE activity levels of the ectoparasites on the mortality of the ectoparasites.

(6) changes in behavior of the ticks and mosquitoes.

(7) effects of the sublethal doses on the behavior of the host.

(8) the disappearance of pesticide residues from selected host tissues.

(9) the best route of application on the basis of (a) ease of application, (b) safety to the host and (c) sustained toxic activity to the ectoparasites.

LITERATURE REVIEW

Systemic Control of Parasites

Much work has been done, using various formulations and routes of application, with the organophosphorous compounds. They have been used as drenches, sprays, wettable powders, orally in polymer systems, as topical applications, and as food additives. In these preparations they have been used against gastrointestinal parasites, dermal parasites, Hypoderma bovis (Linnaeus) and H. lineatum (Villers) and recently against ectoparasites, reviewed by Khan (1969).

As seen by a perusal of the literature, most of the attempted parasite control has been with obligatory parasites (those parasites which spend their whole parasitic life on one host). Very little literature is available concerning systemic control of facultative parasites such as mosquitoes and ticks.

Studies on mortality of A. aegypti and Stomoxys calcitrans (L.) that had fed on Ruelene-sprayed cattle were attempted by Roth[2] but were unsuccessful. The dipterans would not feed due to insufficient control of environmental conditions. There have been few published works on control attempts of D. andersoni with organophosphorous

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[2]Roth, A. R. 1966. Research Entomologist, U. S. Department of Agriculture, Entomology Research Division. Personal communication. Corvallis, Oregon.

compounds. Smith and Goulding (1968), using sheep as hosts and Ruelene as the organophosphorous pesticide, obtained good to excellent results in three different tests. Newton (1967) and Wharton (1967), working with the cattle tick, Boophilus microplus (Canestrini), and several organophosphorous compounds obtained fair results with sprays and dips. During these studies it was observed that the organophosphorous-resistant ticks, Ridgeland and Biarra types, showed varying degrees of resistance to the compounds used. The ChE activity levels of the two resistant types showed remarkable variations between each other and also when compared to a susceptible strain (Wharton, 1967).

Reports on the normal behavior, copulation, feeding, preoviposition, oviposition and other habits of D. andersoni (Cooley, 1932; Douglas, 1943; Herms and James, 1961; Evans, Sheals, and MacFarlane, 1961) proved useful in the study in determining any changes in normal patterns of behavior due to the pesticides.

Review of Literature on Tiguvon, Proban and Famphur

The three organophosphorous pesticides were chosen for their degree of mammalian toxicity, based on acute oral toxicity (AO = LD_{50}) of white rats. Tiguvon has a relatively low toxic effect on mammals (AO:178-310), Proban is intermediate (AO:100-160), and Famphur is relatively toxic (AO:35-60) (Kenaga, 1966).

After the pesticides were chosen, the chemical companies manufacturing the products (American Cyanamid and Chemagro) were contacted for information concerning dosage rates (Drain[3]; Burkhart[4]).

Tiguvon

A search of recent literature on the above mentioned pesticides was also undertaken. As Tiguvon is an older product, there was considerable literature concerning it and its usages. Tiguvon has been found to be effective when acting systemically in control of H. bovis and H. lineatum as a feed additive (Drummond, 1966; Cox, Mullee, and Allen, 1967; Hagen, 1967), a spray, a pour-on (Drummond and Graham, 1966; Hagen, 1967), and subcutaneously (Drummond, 1963a). Tiguvon was tested against resistant and susceptible house flies and mosquitoes with fair to excellent results (Metcalf, Fukudo, and Winton, 1963). It was not effective against mosquito larvae as it was rapidly decomposed into non-insecticidal compounds in water (Metcalf et al., 1963).

Tiguvon acted too slowly to be effective against adult mosquitoes,

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[3]Drain, J. J. 1968. Manager, Ruminant Program. American Cyanamid Company, Agricultural Division. Personal Communication. Princeton, New Jersey.

[4]Burkhart, R. L. 1968. D.V.M. American Cyanamid Company, Agricultural Division. Personal Communication. Princeton, New Jersey.

Aedes sp., when used alone as a low volume spray. However, excellent control was obtained when low volume application was made with equal parts of Baytex (Tiguvon) and Baygon, a carbamate (Stevens and Stroud, 1966). The Tiguvon-Baygon mixture was also found to control adult Aedes when applied as a thermal fog.

Tiguvon was found to be effective against larval and adult fleas (Xenopsylla cheopis (Rothschild))although the fleas exhibited a slight tolerance to the pesticide (Fox, Rivera, and Umpierra, 1966). Fenthion (Tiguvon), when used to control nasal bots in sheep, was effective when applied as a drench but not when applied as a pour-on or spray because it did not wet the skin (Pfadt, 1967).

Metabolites of fenthion (Tiguvon) in cattle were found to be phosphoric acid derivatives which were eliminated primarily in the urine. Residues were negligible in the muscle 14 days after a dermal application but were present 21 days after intramuscular application (Knowles, 1966).

An unsuccessful suicide attempt with Lebaycid (Tiguvon) presented an opportunity to determine systemic activity of this organophosphate in human beings. Samples of blood were prepared for thin layer chromatography. Extracts of the blood were lifted from the thin layer chromatograph and dissolved in a solvent. When these solvents were topically applied to Drosophila melanogaster Meigen, they were very lethal, indicating that good systemic activity

was possible (Clarmann and van Mallinckrodt, 1966).

Proban

Unlike Tiguvon, Proban is a new pesticide and there has been very little published concerning it. It has been used successfully as a systemically acting parasiticide to control ticks, mites, lice, and fleas when applied either orally or aurally to dogs and cats (Burkhart[5]). Proban (Cythioate) tablets have been given orally to dogs for treatment of fleas at 1.5 mg/lb (Yarborough and Yarborough, 1968). Proban in Silastic® silicone implanted in the peritoneal cavity of mice kept them louse free for from eight to eighteen weeks (Clifford et al., 1967).

Famphur

Either intramuscular or oral application of Famphur was found to be very effective against six species of intestinal helminths (Ara-Alera, Ramirez-Miller, and McGregor, 1965). Intramuscular injections of Warbex (Famphur) gave excellent control of Hypoderma larvae (Kutzer and Supperer, 1965; Drummond, 1962, 1964). Excellent results were obtained using Famphur against cattle grubs

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[5] Burkhart, R. L. 1968. D.V.M. American Cyanamid Company, Agricultural Division. Personal Communication. Princeton, New Jersey.

by subcutaneous injection (Kutzer and Supperer, 1965; Drummond, 1962), as feed additives (Drummond, 1964, 1966) and as pour-ons (Drummond, 1964). However, Famphur gave only 35% control against reindeer grub fly larvae although it was the most effective pesticide tested (Nordkvist, 1967).

Famphur given daily as a feed additive at 10 mg/kg was found to be effective against horn fly larvae in manure but only slightly effective against house fly larvae (Drummond, 1963b).

Many studies have been conducted concerning the metabolism and residues of Famphur in milk, urine, blood, and edible tissues (Knowles, 1966). Therapeutic doses of Famphur administered orally caused temporary depressions in blood ChE activity levels and left detectable residues in the milk and blood for a short period of time (Zacherl et al., 1965). Gas chromatograph determinations of Famphur and its oxygen analog, Famoxon, showed measurable residues in muscle, liver, kidney, fat, milk, and blood (Pasarela et al., 1967). Their results indicated that after chronic dosage was stopped, residues completely disappeared by the fourth day.

Gatterdam et al. (1967) found that metabolism of Famphur involved rupture of covalent chemical bonds to yield water soluble metabolites which were eliminated chiefly in the urine. They found that, depending on the route of application, there was a marked difference in metabolism and excretion of the pesticide. Intramuscular

injection provided higher sustained Famphur levels and lower Famoxon levels in the blood as compared to intravenous injection where the situation was reversed.

Mode of Action of Organophosphates

The organophosphorous pesticides share a fundamental characteristic: structurally they all contain the phosphorus radical in a combination which permits the compound to competitively inhibit AChE and other ChE's. Their success and value depends on the toxicity produced in the pest as opposed to that of the host.

The hydrolytic process by which AChE splits ACh takes place in two steps. In the first, an acetylated enzyme is formed and choline is eliminated. In the second step, the acetylated enzyme reacts with water to form acetate and restored enzyme. When this mechanism is understood, it is possible to explain the action of organophosphorous compounds. Even though there is a wide variety of organophosphorous compounds, they all have in their structure a group which can be split off by esterases, resulting in a phosphorylated enzyme instead of an acetylated one. The phosphorylated enzyme does not readily react with water so the enzyme becomes inactivated. When this inactivation process reduces the enzyme activity at the neural junctions to 20% or less, death or serious illness can be expected (Radeleff, 1964).

Since it has been postulated that insect ChE is different than mammalian ChE (Khan, 1969), there is a difference of opinion as to the mode of action of organophosphorous compounds in insects.

In early work with systemics, adverse side reactions in cattle were observed in cattle grub control experiments which did not correspond to the quantity of the compound administered (Dow Chemical 1959, 1960). This was found not to be due to the toxic activity of the pesticide, but was considered to be due to Hypoderma larvae killed in the neural canal or esophagus (McGregor, Ludwig, and Wade, 1959; Scharff, Sharmon, and Ludwig, 1962). Herms and James (1961) postulated that the larvae of Hypoderma spp. are in the neural canal only at certain times of the year and at these times, application of pesticide would be harmful.

Inhibition of an aliesterase has been suggested as an important cause of death (Chadwick, 1963). However, much more evidence suggests that the mode of action is acetylcholinesterase (AChE) inhibition. Van Asperan (1959) was able to demonstrate that house flies treated with organophosphates had their AChE inhibited 20-50% at knockdown, while the aliesterase was inhibited 80-90%. He stated that no conclusions could be drawn until the function of this inhibited aliesterase was determined. O'Brien (1961) found that aliesterase was inhibited more than AChE in those insects receiving sublethal doses of organophosphates, but it was inhibited less than AChE in

those insects that were killed. Van Asperan (1960) found that house flies resistant to organophosphates had very low aliesterase activity levels. Stegwee (1960) proved, with the use of triorthocresyl phosphate (considered to be nontoxic to insects), that the aliesterases could be inhibited 100% in the house fly with no deleterious effect.

Steward (1967) demonstrated by histochemical procedures that organophosphates applied to cattle grubs in vitro caused AChE inhibition and a high degree of aliesterase inhibition.

Organophosphate poisoning symptoms occurred only after significant ChE activity inhibition by the pesticide. Chadwick (1963) stated that the aliesterases, even though they were inhibited by the organophosphates, were apparently not the enzymes causing death since, when death occurred, the AChE was the enzyme most inhibited. O'Brien (1961, p. 1164) suggested a method for determining positively if organophosphates were inhibitors of AChE:

> . . . . If it could be shown with a variety of organophosphates that death always occurred when the ChE of some particular tissue was reduced below a certain critical level (and perhaps for some critical period of time), then we could accept as proven the hypothesis that organophosphates kill insects by inhibiting their cholinesterase.

Morallo and Sherman (1967) observed in their studies with flies and using topical application of pesticide that there was a great variation in the percent of ChE activity inhibition. They observed that in resistant flies the ChE activity was greatly depressed and little

mortality occurred. Conversely, in susceptible flies, there was little ChE activity inhibition, although very high mortality occurred. From this they concluded that there was little correlation between ChE activity inhibition and mortality in the flies. In their studies with the organophosphate Ruelene, Smith and Goulding (1968) found that there was a direct correlation between mosquito ChE activity inhibition and mortality in mosquitoes. They found that mortality commenced at approximately 33% insect ChE activity inhibition and that at 59% ChE activity inhibition there was approximately 100% mortality. Bigley (1966) found in his studies with bisected flies that body (abdomen and thorax) ChE activity was more closely correlated with pesticide effect than was head ChE activity. At death of the flies, it was found that body ChE activity was 90% inhibited whereas head ChE activity was only 50% inhibited.

Methods of ChE Determination

Some of the differences of opinion as to the function of organophosphorous compounds may be due to the various methods of determining ChE activity. These methods, reviewed recently by Witter (1963), Ganelin (1964), and Reed, Goto, and Wang (1966) include: (1) Warburg manometric method, which requires large amounts of tissue and limits the type of buffer that may be used; (2) titrimetric method, which is very time consuming and inaccurate if

an indicator instead of a potentiometer is used; (3) colorimetric method, in which any substance emitting the same wave length as the sample may cause possible interference; (4) electrometric method, in which the most common difficulty is the instability of the pH meter at intervals of one minute or less; (5) photometric method, in which activity is measured by change in absorbance of acid-base indicator.

Reed et al. (1966) perfected a direct radioisotopic method for assay of ChE that was both rapid and accurate with millimicromole quantites of acetylcholine (ACh) under varying pH, wide range of substrate concentrations, and various concentrations of enzyme source. Reed's method is especially useful since it is very sensitive and such small amounts of sample can be used. The high sensitivity is due to the fact that the acetic acid resulting from the enzyme action is the isotope labeled product and is measured directly. Frady and Knapp (1967) modified Reed's method slightly and thereby increased the speed and efficiency with which analyses for ChE activity could be made.

Radeleff and Woodard (1956) found little or no ChE activity in the plasma fraction of sheep blood when they used the electrometric method for their analyses. However, Smith and Goulding (1968), using the modified radioisotopic method on sheep blood, found that the plasma fraction contained approximately one tenth the amount of ChE activity as did the RBC fraction.

Supposed normal blood ChE activity levels have been assayed on cattle (Hermenze and Goodwin, 1959) and in sheep and cattle (Radeleff and Woodard, 1956) using the electrometric test. Although AChE activity was detected in the RBC fraction of blood, no ChE activity was detected in the plasma fraction. These findings have led to the use of whole blood rather than blood fractions for determining percent ChE activity inhibition (Rogoff et al., 1967). Use of whole blood for analysis of ChE may be misleading, as the red blood cells contain AChE which is bound to the cell membrane; the plasma ChE is a combination of several esterases, collectively termed pseudocholinesterases (Witter, 1963). With organophosphorous compounds, especially those with dimethyl esters, the red cell enzyme is inhibited first, followed shortly by the plasma enzyme. These effects are based on differences in the kinetics of inhibition and indicate that separate determination of red cell and plasma enzyme has a greater diagnostic value than does whole blood analysis (Gage, 1967). It is probably that in most whole blood methods of analyses, the plasma enzyme is dominant, although in methods based on unhydrolyzed acetylcholine (Winteringham method), the low substrate concentration used would imply that the activity was based almost entirely on the red cell enzyme (Gage, 1967).

Use of Controls in the Experiment

Gage (1967) found that in normal, healthy individuals, the variation of the blood ChE ranged as high as 25% from its established norm, with the plasma fraction indicating a slightly higher percentage variation than the RBC fraction. The great variation of ChE activity among individuals makes the use of a "norm" useless in a group or population of animals.

In this study, each animal served as its own control and a "norm" for each individual animal was established through analysis of several predosage samples of blood (Smith and Goulding, 1968).

METHODS AND MATERIALS

Three technical grade pesticides, Tiguvon[6] (98% pure), Proban cythioate[7] (98.4% pure), and Warbex famphur[8] (99% pure) were administered to 101, two to three month old female domestic rabbits, New Zealand and California strains. Eighty-one of the rabbits were used as hosts for ectoparasite studies and 20 were used for pesticide residue studies. A total of 127 rabbits was used to determine the normal blood ChE levels.

The rabbits used in the ectoparasite study were anesthetized with Somnopentyl[9] at a dosage rate of 0.44 ml/kg of body weight, injected intravenously into the ear. A plastic cage, 1.75 inches inside diameter with a screw top, was sewn directly onto a shorn spot on the rabbit's back (Figure 1) using "00" Vetafil suture thread and a #10 surgeon's needle with a 3/8" circle and cutting edge. The

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[6]Tiguvon 0,0-dimethyl 0-(4-(methylthio)-m-tolyl) phosphorothioate, donated by Chemagro Corporation, Kansas City, Mo.

[7]Proban 0,0-dimethyl 0-p-sulfamoyl phenyl phosphorothioate, donated by American Cyanamid Company, Princeton, New Jersey.

[8]Famphur 0-p-(dimethylsulfamoyl) phenyl 0,0-dimethyl phosphorothioate, donated by American Cyanamid Company, Princeton, New Jersey.

[9]Somnopentyl (Sodium pentobarbital injection), Pittman-Moore, Division of Dow Chemical Company, Indianapolis, Indiana.

Figure 1. Tick cage sewn to back of rabbit using "00" Vetafil suture thread. Rabbit anesthetized with Somnopentyl.

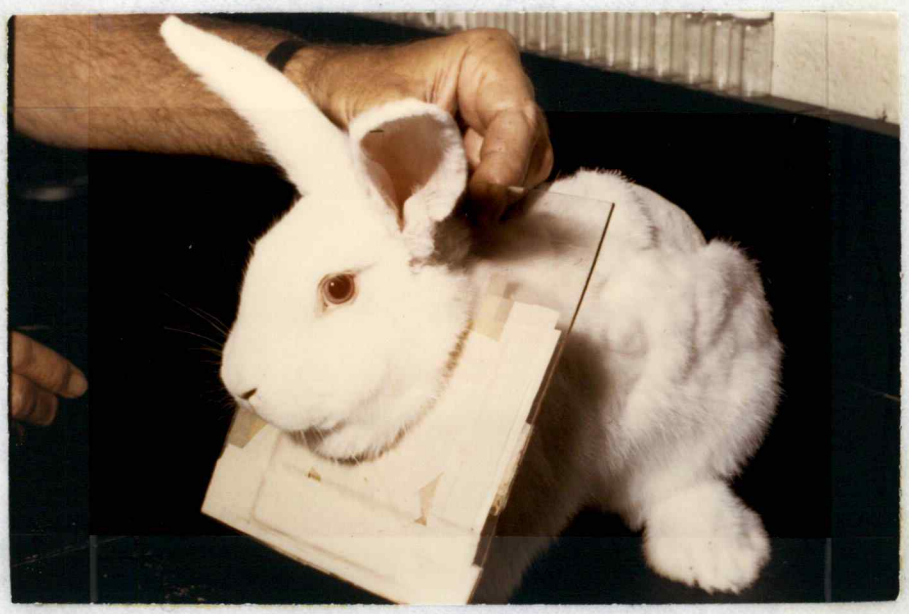

Figure 2. Plastic collar to keep rabbit from biting through sutures holding cage to back.

rabbits were then given an intramuscular injection of Streptillin[10] at a rate of 0.22 ml/kg of body weight to counteract any infection.

After the rabbits had recovered from the effects of the anesthetic, a collar constructed from a four inch square piece of 1/16 inch thick plastic was placed around each rabbit's neck (Figure 2). The collars prevented the rabbits from biting through the suture threads that held the cages to their backs.

Dosage

Three rabbits, each to receive a different dosage concentration of a particular pesticide, were placed in specially constructed restraining cages (Figure 3) and individually weighed. The dosage concentrations of the pesticides were then adjusted to body weight and route of application.

A solvent for the three pesticides was needed which would dissolve the powdered pesticides completely and in itself not be a cholinesterase depressant. Through experimentation, it was found that the best possible solvent was di-methyl sulfoxide (DMSO). To insure as few variables as possible, 0.2 ml DMSO were used as the solvent with the liquid Tiguvon as well as with Famphur and Proban.

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[10] Streptillin, Procaine Pencillin G in Crystalline Dihydrostreptomycin solution, Trico Pharmaceutical Company, Oregon City, Oregon.

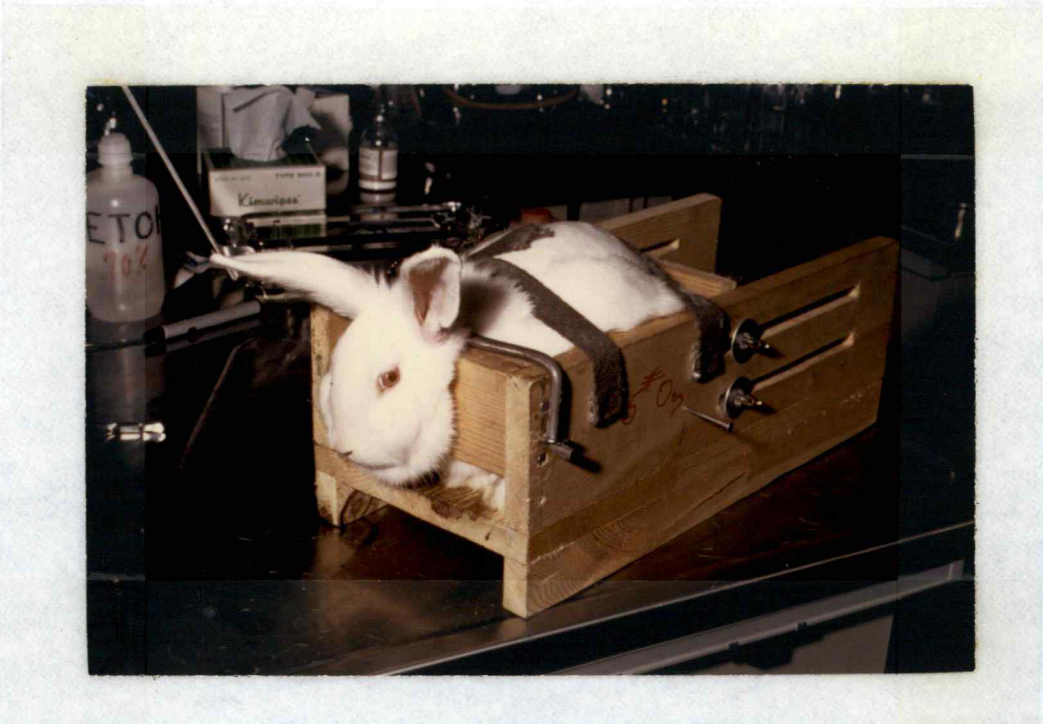


Figure 3. Adjustable restraining cage with contained rabbit.

The three pesticides were administered in three concentrations by three different routes. Oral applications were: Tiguvon 100, 125, and 150 mg/kg (since Tiguvon was in a liquid form, it was actually administered as 80, 100, and 120 microliters/kg of body weight); Proban 50, 75, and 100 mg/kg; Famphur 15, 30, and 50 mg/kg. The oral applications of the pesticides were accomplished using a five ml syringe, a bent three inch #23 Luer Lok needle with a one inch piece of #200 vinyl tubing slipped over the end (Figure 4). A speculum constructed of a piece of wood, 3/8 in. thick, 1 in. wide, and 2 and 1/2 in. long, with a 1/4 in. hole drilled in the middle, was placed behind the incisors to hold the rabbit's mouth open. The needle portion of the syringe was inserted through the hole in the speculum (Figure 5) and the prescribed dose of pesticide plus DMSO was slowly dripped onto the base of the rabbit's tongue.

Intravenous applications were Tiguvon 50, 75, and 100 mg/kg of body weight (40, 60, and 80 μliters/kg); Proban 15, 20, and 25 mg/kg; Famphur 5, 10, and 15 mg/kg. Intravenous application of the pesticide plus DMSO was accomplished using a disposable 2 1/2 cc syringe and a 26 gauge needle. The mixture was administered directly and slowly into a vein in the ear of the rabbit. Brisk rubbing of the ear caused the vein to be distended so that insertion of the needle therein was facilitated.

Subcutaneous applications were: Tiguvon 75, 100 and 125 mg/kg

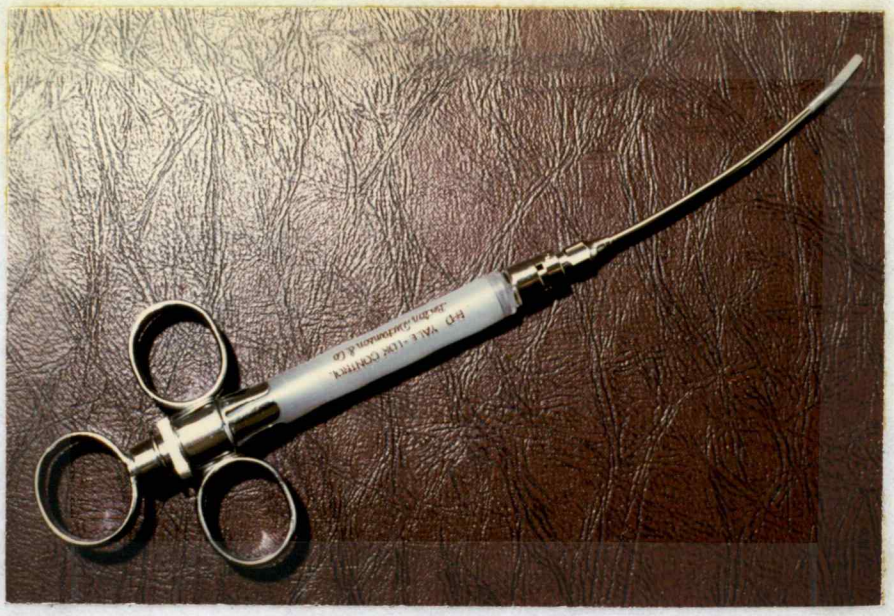


Figure 4. Apparatus used for oral dosage of rabbits, 5 cc B-D syringe, #23 Luer Lok needle with #200 vinyl tubing.

Figure 5. Speculum with needle through hole, for oral dosage of rabbits.

of body weight (60, 80, and 100 μliters/kg); Proban 25, 50, and 75 mg/kg; Famphur 10, 15, and 20 mg/kg. The subcutaneous injection was made over the left loin, using a disposable 2 1/2 cc syringe with a 26 gauge needle.

ChE Analysis

Blood

Two heparinized microhematocrit tubes (inside diameter 1.1-1.2 mm) of host blood were withdrawn from a vein in the ear at 48, 24, 3, 2, and 1 hours prior to dosage with pesticide. Further samples were taken hourly for a period of seven hours after dosage and then once daily for a period of 96 hours.

To facilitate separate assays of plasma ChE and RBC AChE, the hematocrit tubes were immediately centrifuged for 10 minutes at 1600 RPM using a Phillips-Drucker L-708 combination centrifuge employing an L-779F micro-hematocrit head.

Plasma

A 1.9 cm section, representing 20 μliters was cut from the plasma fraction of the centrifuged hematocrit tube. The contents of the cut section were added to 2 ml of phosphate buffer, pH 7.34 (Appendix I), to form a 1:100 v/v dilution. Fifty μliters of the dilution

were then added to fifty μliters of substrate consisting of 0.050 μmoles of acetylcholine bromide diluted with buffer of pH 7.34 and 0.005 μmoles of acetyl-1-C^{14}-choline iodide with an activity of 0.025 μcuries per assay. Analysis was made according to Reed et al. (1966), utilizing modifications made by Frady and Knapp (1967).

The assays were made in pairs, using 15 ml centrifuge tubes, and the reaction mixtures were incubated in a constant temperature oven at 37.5° C for 10 minutes. The reactions in the centrifuge tubes were stopped by the addition of two ml of ion-exchange resin-ethanol mixture (Appendix I) with a fast delivery pipette. Using a 50 ml burette, three ml of 100% ETOH were added to the centrifuge tubes and the tubes were then shaken vigorously. The tubes were then centrifuged at 1400×G for five minutes to settle the resin. After centrifugation, 4.0 ml of supernatant were pipetted into 20 ml counting vials containing 10.0 ml of scintillation counting solution (Appendix I) and the activity was then counted with a liquid scintillation spectrometer (Packard Tri-Carb, Model 314 EX).

Sample blanks were prepared in the same manner as those just described except that these blanks were boiled for 10 minutes prior to incubation. The blank sample readings provided a correction due to background activity and nonenzymatic hydrolysis of the substrate.

After the activity was counted, the counts per minute were calculated in terms of μmoles of substrate hydrolyzed per minute.

The lower confidence level was calculated for the RBC and plasma ChE activity of each animal from the predosage blood levels. The 95% limit was used, given by the formula (Mendenhall, 1967):

$$L.C.L. = \bar{x} - t\alpha\sqrt{s^2}$$

where

$\bar{x}$ = mean of predosage ChE activity

s^2 = variance of predosage ChE activity

$t\alpha$ = tabular value of Student's t at the 95% level

$(t_{.05})$

The 95% lower confidence limit represents the lowest point at which fluctuation can be attributed to the rabbit's normal variation. Any time the ChE activity level falls below the lower confidence limit, the depression is due to some other factor, in this case, pesticide inhibition.

Erythrocytes

To insure erythrocytic lysing, a 0.04% saponin-buffer solution (Appendix I) was utilized instead of buffer, but in all other respects, the procedures were identical with those for the plasma fractions.

Mosquitoes

Aedes aegypti, yellow fever mosquitos were obtained from an existing colony at Oregon State University, Corvallis, Oregon. A

large number of the mosquitoes were aspirated into a #15 pill vial, the vial (with mosquitoes) was taken into a -10°C cold room where the mosquitoes became immobilized in approximately 45 seconds. They were then taken into a room at 4° C where samples of 50 female mosquitoes were counted and placed in application cages made from screened #4 mason jar lids (Figure 6). The cages were then placed in a room maintained at 26.6° C where the mosquitoes were allowed to recover. The cages were then placed against a shaved spot on the back of the host and the contained mosquitoes were allowed to feed for 15 minutes. Samples of mosquitoes were permitted to feed on the host every hour for seven hours after dosage. After the fifteen minute feeding period, the cages were removed, the mosquitoes were quickly checked for mortality and the cages were put into the deep freeze where the samples of mosquitoes were quick frozen and later analyzed for ChE activity inhibition.

ChE Analysis

A base line or norm was established for the mosquitoes by analyzing, individually, 200 unfed mosquitoes. Each mosquito was weighed and placed in phosphate buffer, pH 7.34, that had been volumetrically adjusted to give a 1:100 w/v dilution. Mosquito and buffer were then placed in a ten ml Potter-Elvehjem tissue grinder and homogenized with a teflon pestle for five to ten minutes in an ice

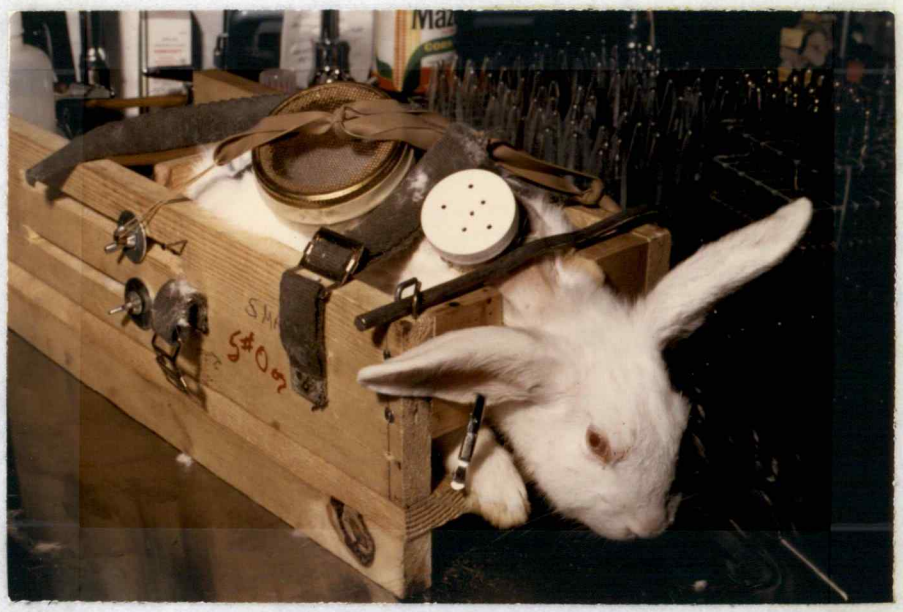


Figure 6. Mosquito cage (screened #4 mason jar lid) held to back of rabbit with rubber bands. Smaller tick cage is sewn to back of rabbit.

water bath.

Using a 100 μliter syringe, a 50 μliter sample was withdrawn from the 1:100 w/v dilution and added to 50 μliters of substrate. Subsequent analysis procedures were the same as those used with the plasma fraction of the blood. The mean of the 200 individual analyses was calculated and was used as an estimate of the average ChE activity level of yellow fever mosquitoes. The mean, as calculated, was 14.04×10^{-3} μmoles of substrate hydrolyzed/minute; standard deviation was 0.212×10^{-3} and variance was 0.049×10^{-6}.

Of those mosquitoes that were allowed to feed on treated hosts, only those that had engorged were analyzed for ChE. The analysis procedures were identical with those used with the unfed mosquitoes.

Ticks

The second ectoparasite used in the research was Dermacentor andersoni, the Rocky Mountain wood tick. The ticks were obtained from a colony maintained by the U. S. Public Health Service Laboratories at Hamilton, Montana.

Small insect cages were made from #20 pill vials and these were sewn directly to the host's back (Figure 1). Samples of ticks, both male and female, were placed in the cages 48 hours prior to dosage of the host (Figure 7). This procedure insured the attachment and feeding of all the ticks prior to dosage of the host. Two samples

Figure 7. Ticks within tick cage, attached firmly to rabbit's back in the feeding position.

of ticks were removed from the host each hour after dosage for a period of six hours. One of the samples was quick frozen for ChE analysis and the other was placed in a stoppered glass vial for behavioral and mortality observations.

ChE Analysis

As with the mosquitoes, a baseline or norm for the ticks had to be established. This was done using ticks that had not fed nor been exposed to pesticides. Seventy unfed ticks were individually weighed and each tick was placed in buffer solution that had been volumetrically adjusted to give a 1:100 w/v dilution. Each tick and its adjusted buffer were then placed in a seven ml Ten Broeck tissue grinder and ground for ten minutes in an ice water bath. Using the same procedure as with the mosquitoes, 50 μliters of the resultant homogenate was placed in 50 μliters of substrate and analyzed for ChE activity. The mean or norm established was 5.55×10^{-3} μmoles of substrate hydrolyzed/minute; standard deviation was 0.109×10^{-3} and the variance was 0.0118×10^{-6}.

Analysis of Organophosphorous Pesticide Residues

Samples of the heart, liver, kidney, muscle (loin), brain, urine, feces, fat, and blood were taken from host animals that had been dosed subcutaneously with therapeutic doses of Tiguvon, Famphur,

and Proban. The animals were sacrificed at 2, 5, 24, 72 hours and 7, 14, 22, and 28 days after dosage. The above mentioned organs and tissues were then removed, placed in small plastic bags and processed immediately for pesticide residues. The procedure used was modified from the work of St. John and Lisk (1968a, 1968b).

Standard Curve

A solvent blank and six pesticide fortified solvent blanks were prepared and used to determine standard curves for Tiguvon, Proban, and Famphur. The standard curve was used in each run to determine micrograms of residual pesticide in each experimental sample. The curve ran from 0-20 micrograms with a sensitivity of approximately 0.2 micrograms.

Primary Extraction

The blood and urine samples were extracted by hydrolysis. Samples of less than five ml of urine and two ml of blood were volumetrically adjusted to five ml with distilled water. To this were then added 0.5 ml of 10 N sodium hydroxide. The test tubes containing the samples were then placed in water in 400 ml beakers and heated on a steam bath at 65-80° C with agitation every half hour; after two hours the samples were removed and allowed to cool.

Tissue sample extraction was accomplished by homogenization

with a Sorvall Omnimixer in 35 ml of acetone which had been distilled over $KMnO_4$. The brei was then filtered under suction, through a #1 Buchner funnel fitted with a #1 Whatman paper, into a 250 ml suction flask containing approximately five grams anhydrous Na_2SO_4. The filtered samples were then transferred to 250 ml erlenmeyer flasks.

The size of the tissue sample extracted depended on the relative size of the organ. In the case of the liver, kidney, feces, and muscle, approximately ten grams of tissue were used. Depending on their relative size, the brain and the heart were extracted as whole entities or as 10 gram fractions of these organs. Ten grams of fat were extracted, then one gram aliquots of the extraction were used for analysis.

Evaporation

The acetone in the samples was then evaporated to approximately five ml over a warm (20°C) steam bath using a gentle air jet. The samples were then transferred to ten ml test tubes containing ether which had been distilled over Na and evaporated to two ml. After evaporation, the samples were volumetrically adjusted to five ml and then hydrolyzed in the same manner as the blood and urine.

Extraction

Each sample was then acidified with 0.75 ml concentrated hydrochloric acid and checked with pH paper to insure a pH of 2 or less. Five ml of distilled Na ether were then added to each of the samples and thoroughly mixed for one minute in a Vortex mixer.

Methylation

To each of the samples was added approximately 0.2 ml of redistilled methanol and one ml of the diazomethane solution in ether. If the color in the solution disappeared during a five minute period, more diazomethane was added. Color had to be retained to insure complete methylation. The samples were then allowed to set for ten minutes. The excess diazomethane was removed with air bubbled through H_2SO_4. Removal was complete when all the color of the diazomethane had disappeared. The sample tubes were then stoppered and analyzed by gas-liquid chromatography.

Sample Timing

All tissues were extracted the same day that they were removed from the animals. They were held overnight in a refrigerator at 0° C in the extracted form (acetone extracted). The following day evaporation was completed, the samples were hydrolyzed, acidified, and

measured aliquots were taken for methylation. All samples were methylated and analyzed the same day. If for any reason samples had to be retained for a longer period of time, they were held between acidification and methylation, never after methylation.

Gas Chromatography

The analyses were made using an Aerograph 600 machine equipped with an 8 ft 1/8 in aluminum column. The column was packed with 2% UCON Polar 50 HB 5100 on 60-80 mesh Gas Chrom Q. The detector used was an Aerograph Cesium Bromide Phosphorus detector and Milliflow micrometering regulators were used for precise control of hydrogen and air flow rates (H^2 = 16 cc/min; air 170 cc/min). The retention time for dimethyl thiophosphate was 2 1/2 minutes at 135° C with a carrier gas (N^2) flow of 40 cc/min. With these apparatus, as much as 4.5 microliters of ether solution could be injected. If dilutions were necessary they were made using ethyl acetate which had been distilled over P_2O_5.

Calculation

The peak height on the chromatogram due to the pesticide in the sample was measured in mm. All samples were standardized to give a reading in mm deflection per one microliter of sample per 10 ml of total volume at 16× attenuation. This was converted into

micrograms of pesticide present by the standard curves constructed from the normalized data and the results were expressed in parts of pesticide per million parts of tissue.

RESULTS AND DISCUSSION

Normal Rabbit ChE Activity

It was attempted from this study to establish an approximate "norm" for ChE activity levels in both the RBC and plasma fractions of the blood of New Zealand and California strain rabbits. The "norm" was established by analyzing five predosage blood samples from each of 127 rabbits. The activity was determined by the number of micromoles of substrate hydrolyzed by enzyme per minute. The norm for the RBC ChE activity was 5.22×10^{-3}, but the 95% confidence limits were from 3.26×10^{-3} to 7.18×10^{-3}. The norm for the plasma ChE activity was 2.83×10^{-3}, but the 95% confidence limits were from 1.74×10^{-3} to 3.92×10^{-3}. From this data it can be seen that using a norm for a population or a large group of animals is not feasible as the variation among the individuals of a group is too great.

Comparison of Routes of Application

Intravenous Administration

Following intravenous dosage of the pesticides, there was a drastic reduction in both RBC and plasma ChE activity within one-half hour; however the activity levels were somewhat recovered by one hour (Figures 8, 9, 10).

Figure 8. Comparison of the effect of three concentrations of Proban, administered intravenously, on rabbit RBC and plasma cholinesterase activity.

△ RBC

O Plasma

—△ 95% lower confidence limit of RBC ChE activity

—O 95% lower confidence limit of plasma ChE activity.

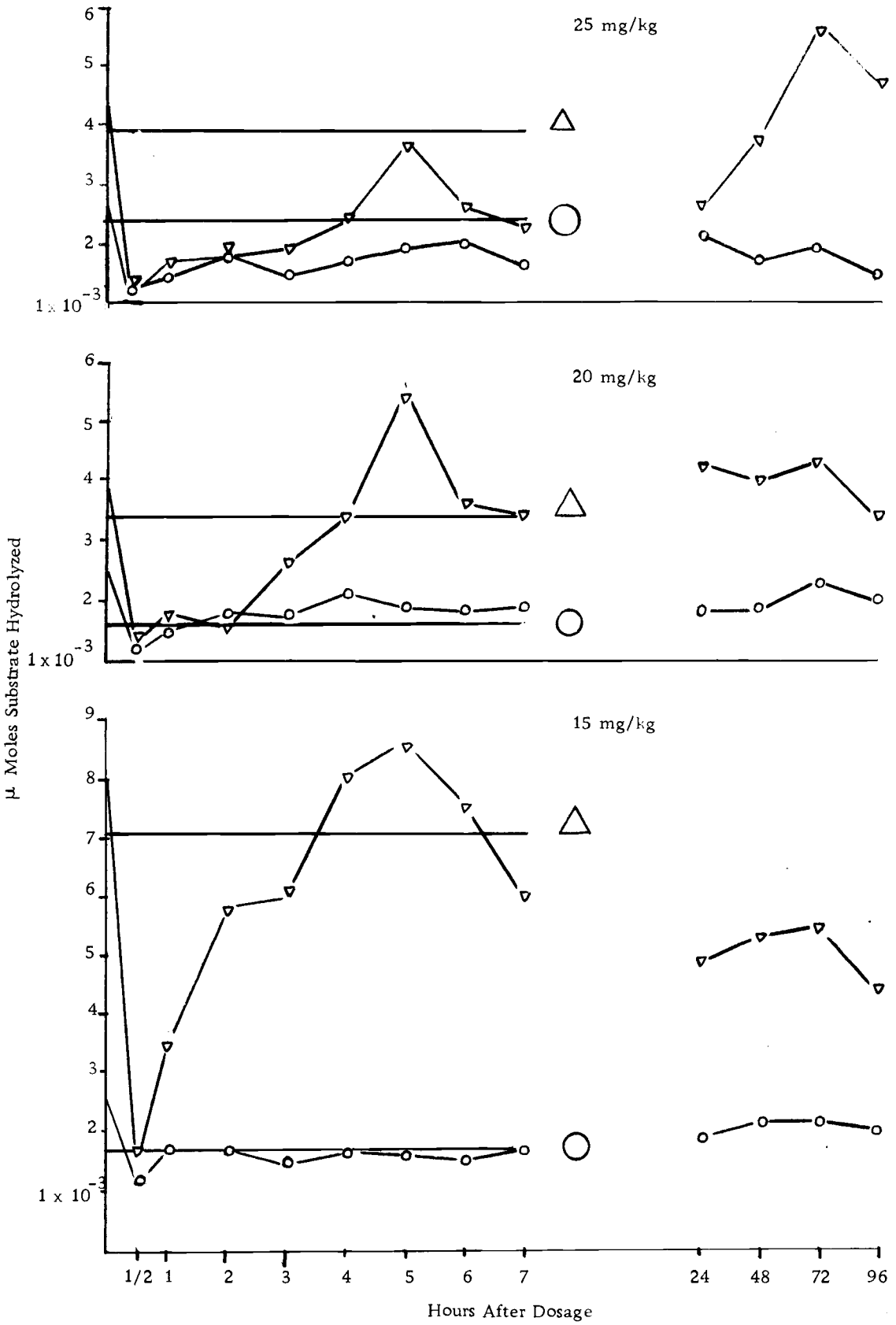
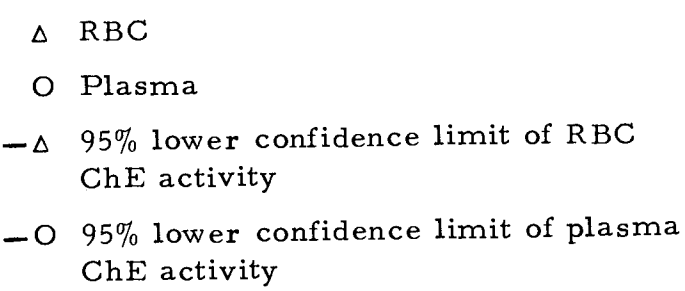

25 mg/kg
20 mg/kg
15 mg/kg
μ Moles Substrate Hydrolyzed
1 x 10⁻³
1/2 1 2 3 4 5 6 7 24 48 72 96
Hours After Dosage

Figure 9. Comparison of the effect of three concentrations of Famphur, administered intravenously, on rabbit RBC and plasma cholinesterase activity.



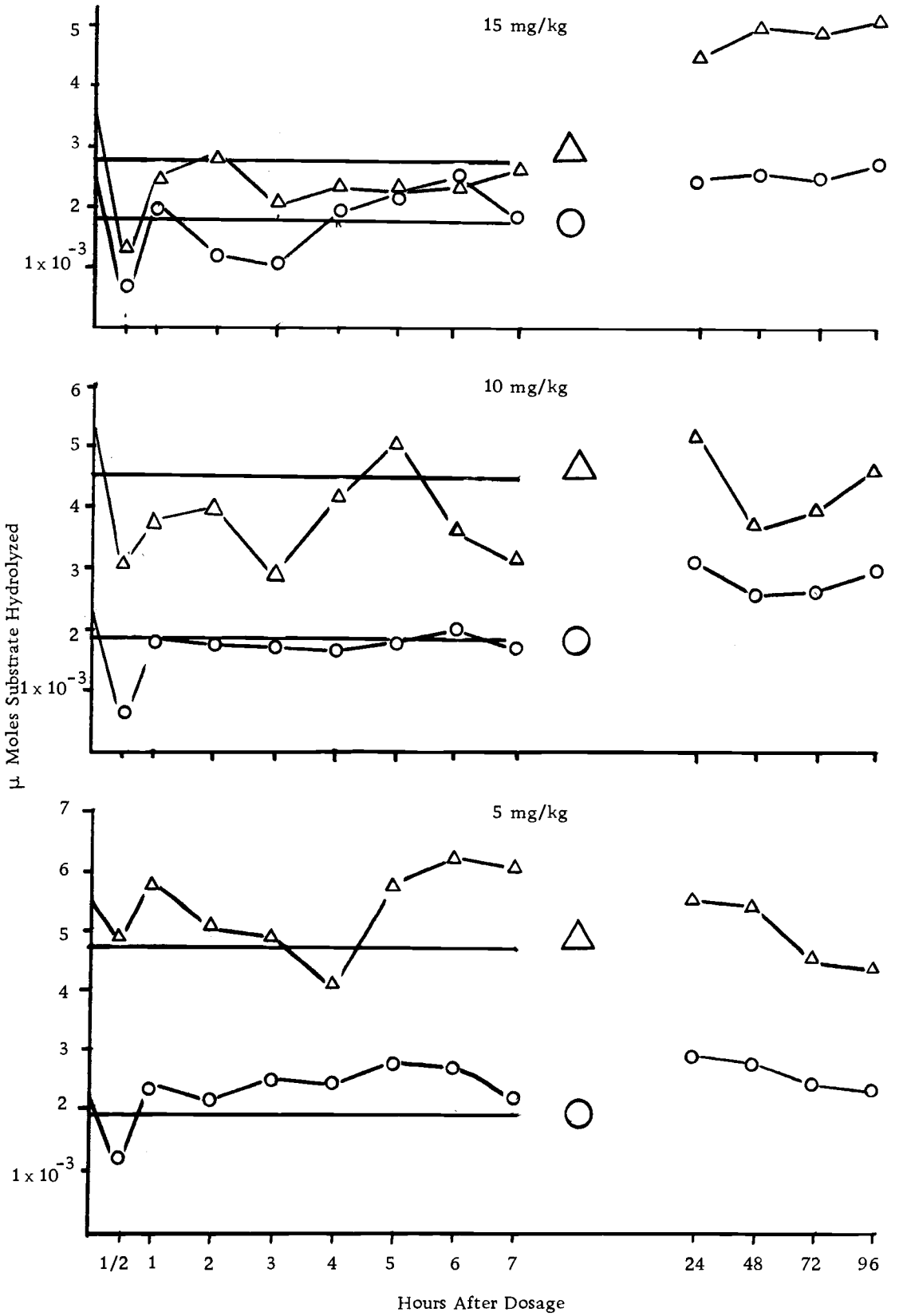

15 mg/kg
10 mg/kg
5 mg/kg
μ Moles Substrate Hydrolyzed
1 x 10⁻³
1/2 1 2 3 4 5 6 7 24 48 72 96
Hours After Dosage

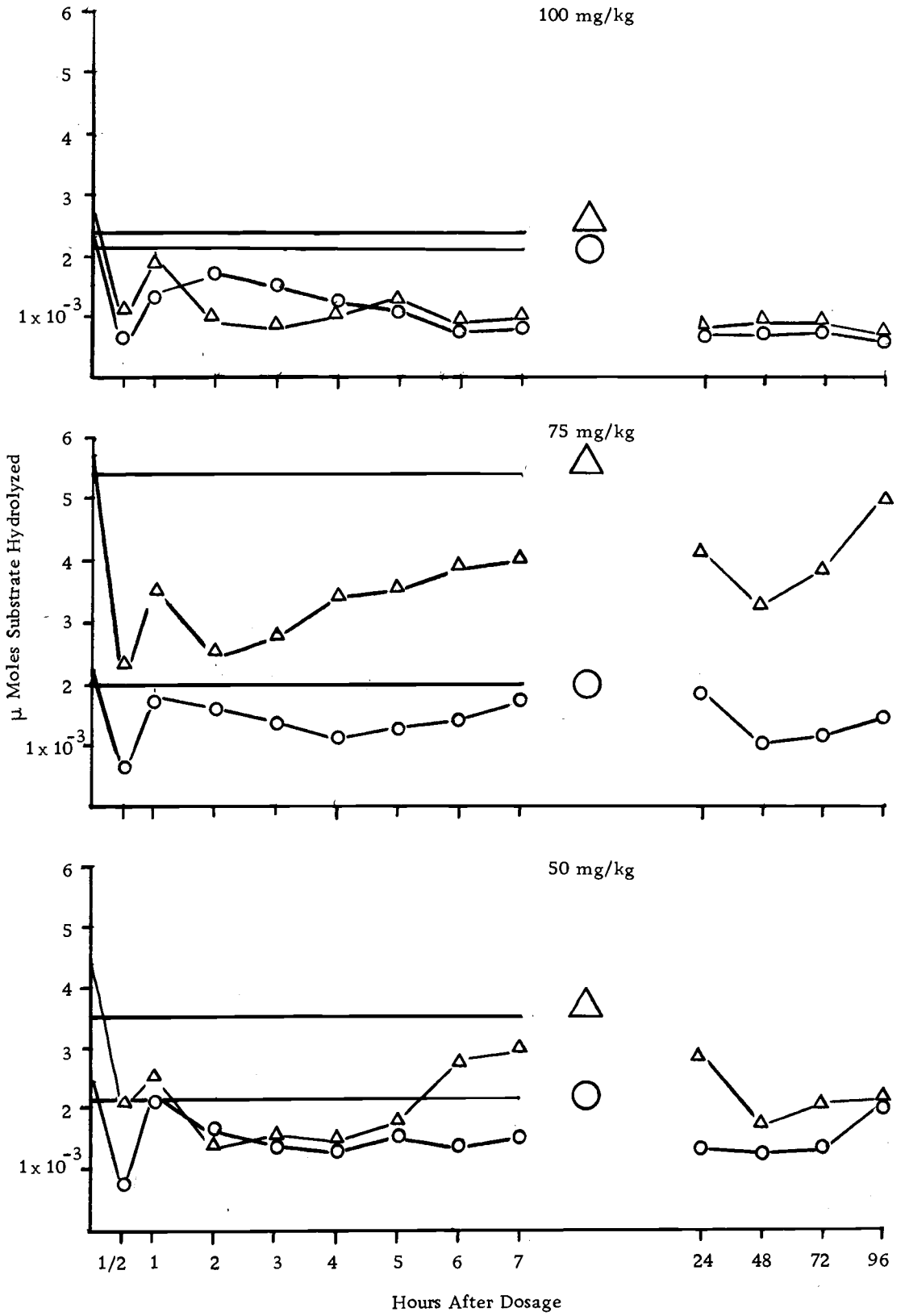
Figure 10. Comparison of the effect of three concentrations of Tiguvon, administered intravenously, on rabbit RBC and plasma cholinesterase activity.

△ RBC

O Plasma

— △ 95% lower confidence limit of RBC ChE activity

— O 95% lower confidence limit of plasma ChE activity


100 mg/kg
75 mg/kg
50 mg/kg
μ Moles Substrate Hydrolyzed
6
5
4
3
2
1 x 10⁻³
1/2
1
2
3
4
5
6
7
24
48
72
96
Hours After Dosage

Following Proban dosage the RBC ChE activity continued to recover, with maximum recovery reached at five hours. At this time, a second less drastic reduction was observed. The plasma and RBC ChE activity were not synchronized after the first hour. This can be explained by the fact that the plasma fraction is comprised of several esterases while the RBC fraction contains true AChE (Witter, 1963).

Using Famphur, there was a partial recovery of ChE activity at the end of the first hour and then a gradual depression of both RBC and plasma ChE activity until the third or fourth hour. Subsequent peaks and depressions depended on the dosage concentration and the metabolic rate of the individual animal (Figure 9).

With Tiguvon, the initial depression and recovery of ChE activity was demonstrated for all three dosage concentrations. However, the two lower dosages depressed the ChE activity again at the second hour, after which a gradual recovery took place to the seventh hour. At this time the activity was still depressed significantly below the mean. The ChE activity of the animal dosed with the greatest concentration of pesticide was depressed as with the lower dosages, but instead of recovering, it remained depressed until the seventh hour (Figure 10).

All three of the pesticides, when given intravenously, created the same reaction initially, but Tiguvon depressed the ChE activity the most and kept it depressed for the longest period of time.

Famphur depressed the ChE activity almost as much as Tiguvon but did not keep it depressed for as long a time (Figures 9 and 10). Of the three pesticides, Proban appeared to be least toxic to the host. The ChE activity levels were not depressed as much and were not held at critically low levels for as long a period of time. DuBois (1965) and Gage (1967) state that the rate of depression of ChE activity is more important than degree of depression. However, 50% inhibition of host blood ChE activity usually initiates toxic effects on the host. In this study it was found that in some instances the ChE activity level of the host blood was depressed as much as 70% with only minimal toxic effect.

In most cases, the blood ChE activity was depressed by the inhibitors to a point significantly (95% confidence limit) below the mean between 24 and 96 hours. These late depressions were not associated with ectoparasite mortality. This would indicate that the degree of initial ChE depression is the important factor.

Subcutaneous Administration

Regardless of concentration of the different pesticides, all inhibited the ChE activity of both the RBC and plasma fractions between one half and one hour after dosage. Maximum inhibition of ChE activity in all cases occurred between four and five hours (Figures 11, 12, 13). This would indicate maximum systemic activity

Figure 11. Comparison of the effect of three different concentrations of Proban, administered subcutaneously, on rabbit RBC and plasma cholinesterase activity.

△ RBC

O Plasma

— △ 95% lower confidence limit of RBC ChE activity

— O 95% lower confidence limit of plasma ChE activity

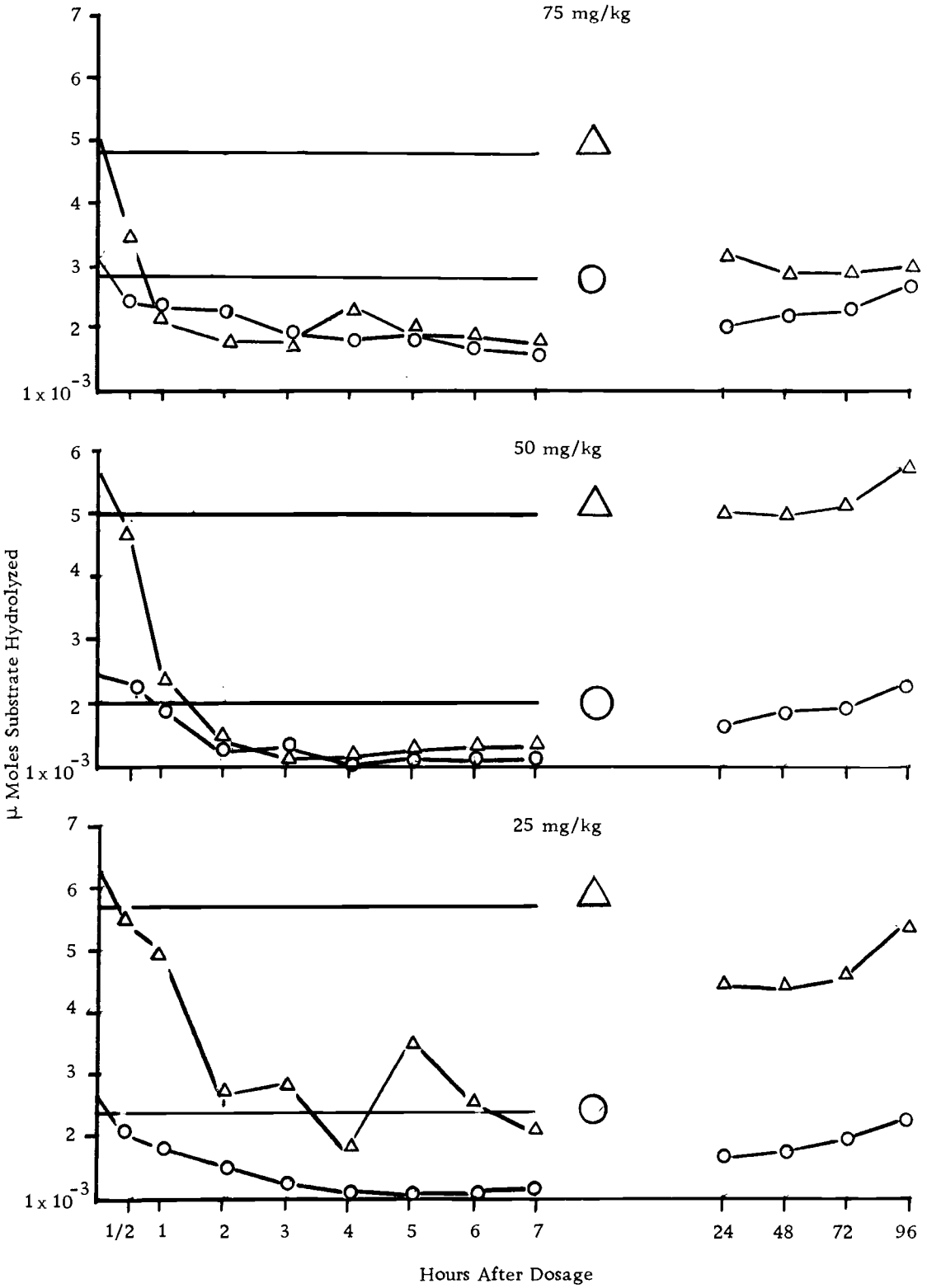

75 mg/kg
50 mg/kg
25 mg/kg
μ Moles Substrate Hydrolyzed
1 x 10⁻³
1/2
1
2
3
4
5
6
7
24
48
72
96
Hours After Dosage

Figure 12. Comparison of the effect of three different concentrations of Famphur, administered subcutaneously, on rabbit RBC and plasma cholinesterase activity.

Δ RBC

O Plasma

— Δ 95% lower confidence limit of RBC ChE activity

— O 95% lower confidence limit of plasma ChE activity

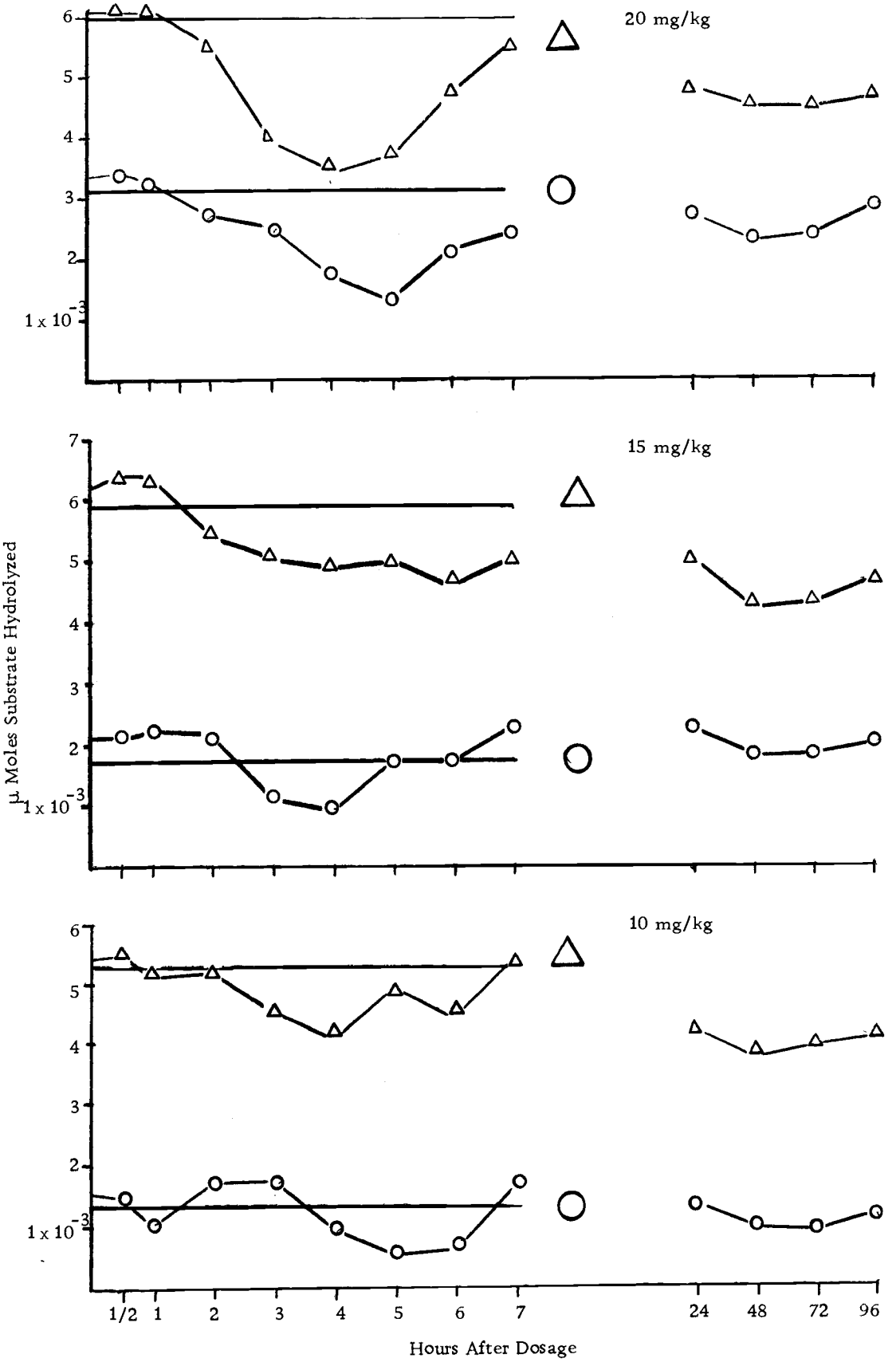

20 mg/kg
15 mg/kg
10 mg/kg
μ Moles Substrate Hydrolyzed
6
5
4
3
2
1 x 10⁻³
7
1/2
1
2
3
4
5
6
7
24
48
72
96
Hours After Dosage

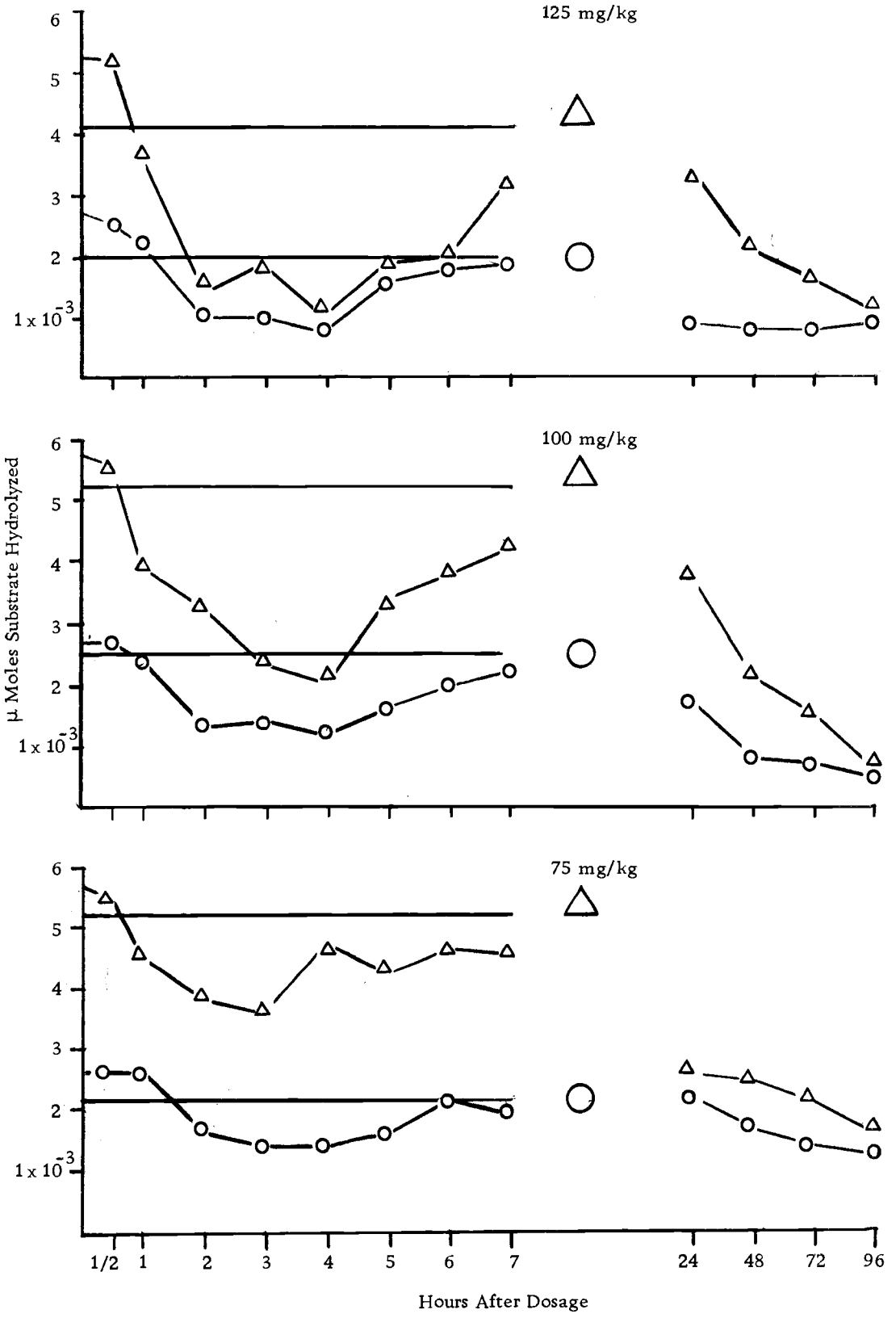
Figure 13. Comparison of the effect of three different concentrations of Tiguvon, administered subcutaneously, on rabbit RBC and plasma cholinesterase activity.

Δ RBC

O Plasma

— Δ 95% lower confidence limit of RBC ChE activity

— O 95% lower confidence limit of plasma ChE activity


125 mg/kg
100 mg/kg
75 mg/kg
μ Moles Substrate Hydrolyzed
6
5
4
3
2
1 x 10⁻³
1/2
1
2
3
4
5
6
7
24
48
72
96
Hours After Dosage

of the pesticide at this particular time. This low level of activity was maintained to the seventh hour with Proban while with Famphur and Tiguvon, there was a gradual recovery of ChE activity until the seventh hour.

Oral Administration

When the pesticides were applied orally there was no significant change in the ChE activity in either plasma or RBC fractions of the blood for at least one hour. Proban (Figure 14) and Tiguvon (Figure 16) caused a very sharp ChE activity depression between one and two hours after dosage. Inhibition of ChE activity due to Proban stabilized somewhat after the second hour, but inhibition due to Tiguvon indicated a gradual decrease through the sixth hour. When Famphur (Figure 15) was applied orally, the reaction was different than with the other two pesticides in that the original depression of blood ChE was more gradual, reaching a low level at the fourth or fifth hour and gradually increasing after that time.

When comparing the three routes of application, it was obvious that when the organophosphates were administered either subcutaneously or orally, they depressed the blood ChE activity more than when applied intravenously, and maintained this low level of inhibition for a longer period of time (Figures 11-16). When administered orally or subcutaneously the pesticides were released

Figure 14. Comparison of the effect of three different concentrations of Proban, administered orally, on rabbit RBC and plasma cholinesterase activity.

△ RBC

O Plasma

— △ 95% lower confidence limit of RBC ChE activity

— O 95% lower confidence limit of plasma ChE activity

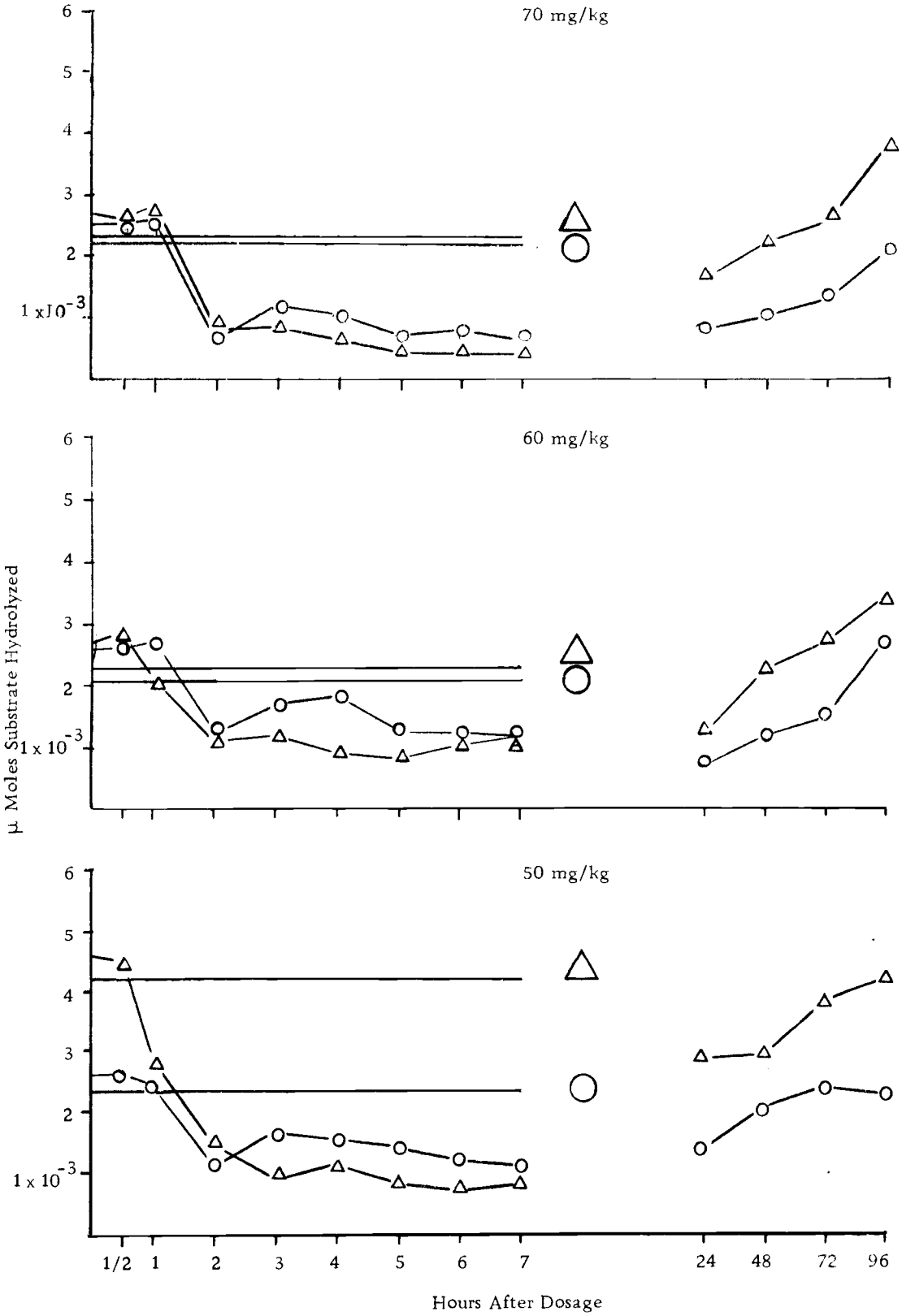

70 mg/kg
60 mg/kg
50 mg/kg
μ Moles Substrate Hydrolyzed
1 x10⁻³
1 x 10⁻³
1 x 10⁻³
6
5
4
3
2
1/2
1
2
3
4
5
6
7
24
48
72
96
Hours After Dosage

Figure 15. Comparison of the effect of three different concentrations of Famphur, administered orally, on rabbit RBC and plasma cholinesterase activity.

Δ RBC

O Plasma

— Δ 95% lower confidence limit of RBC ChE activity

— O 95% lower confidence limit of plasma ChE activity

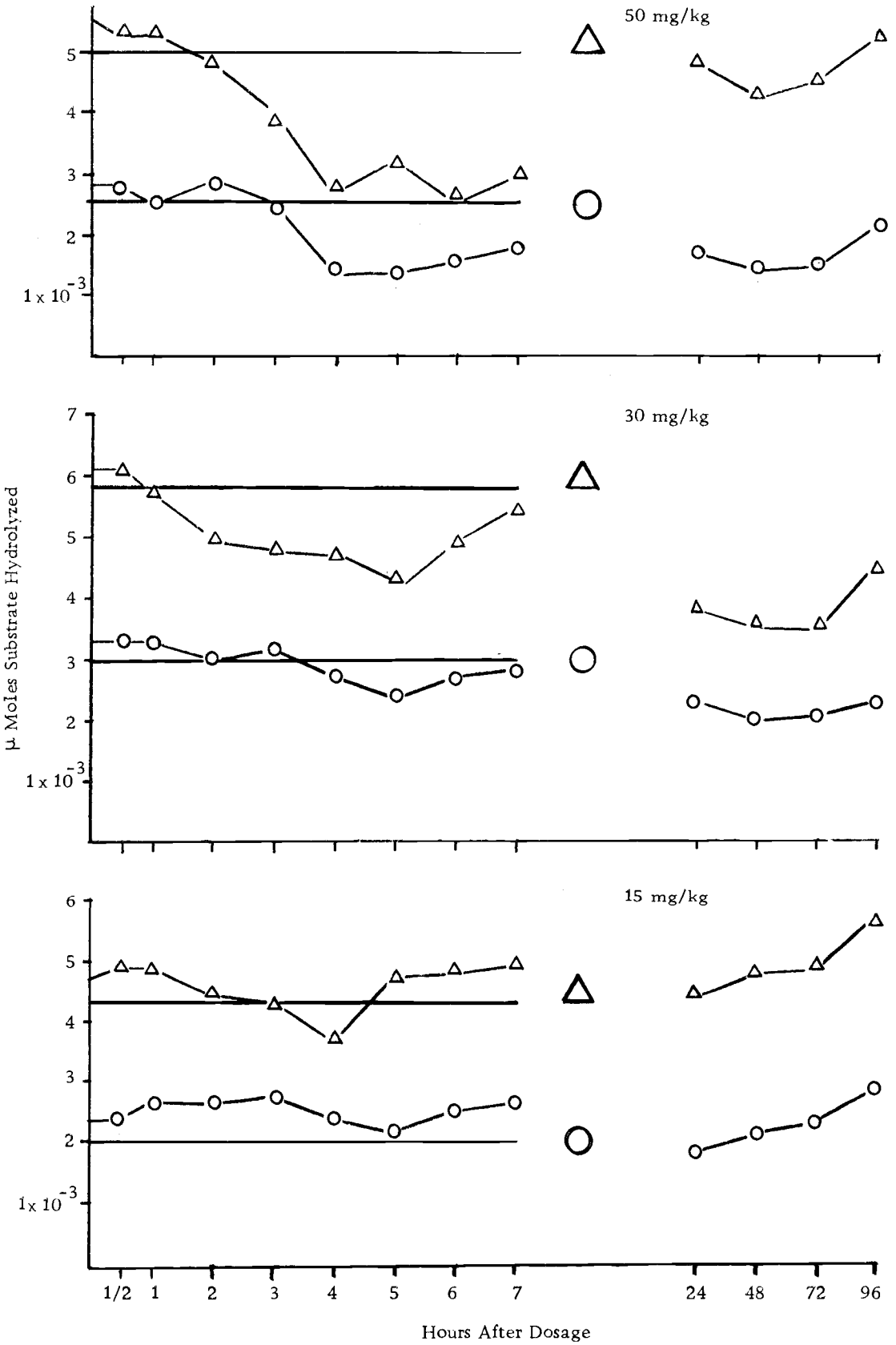

50 mg/kg
30 mg/kg
15 mg/kg
μ Moles Substrate Hydrolyzed
1 x 10⁻³
1/2
1
2
3
4
5
6
7
24
48
72
96
Hours After Dosage

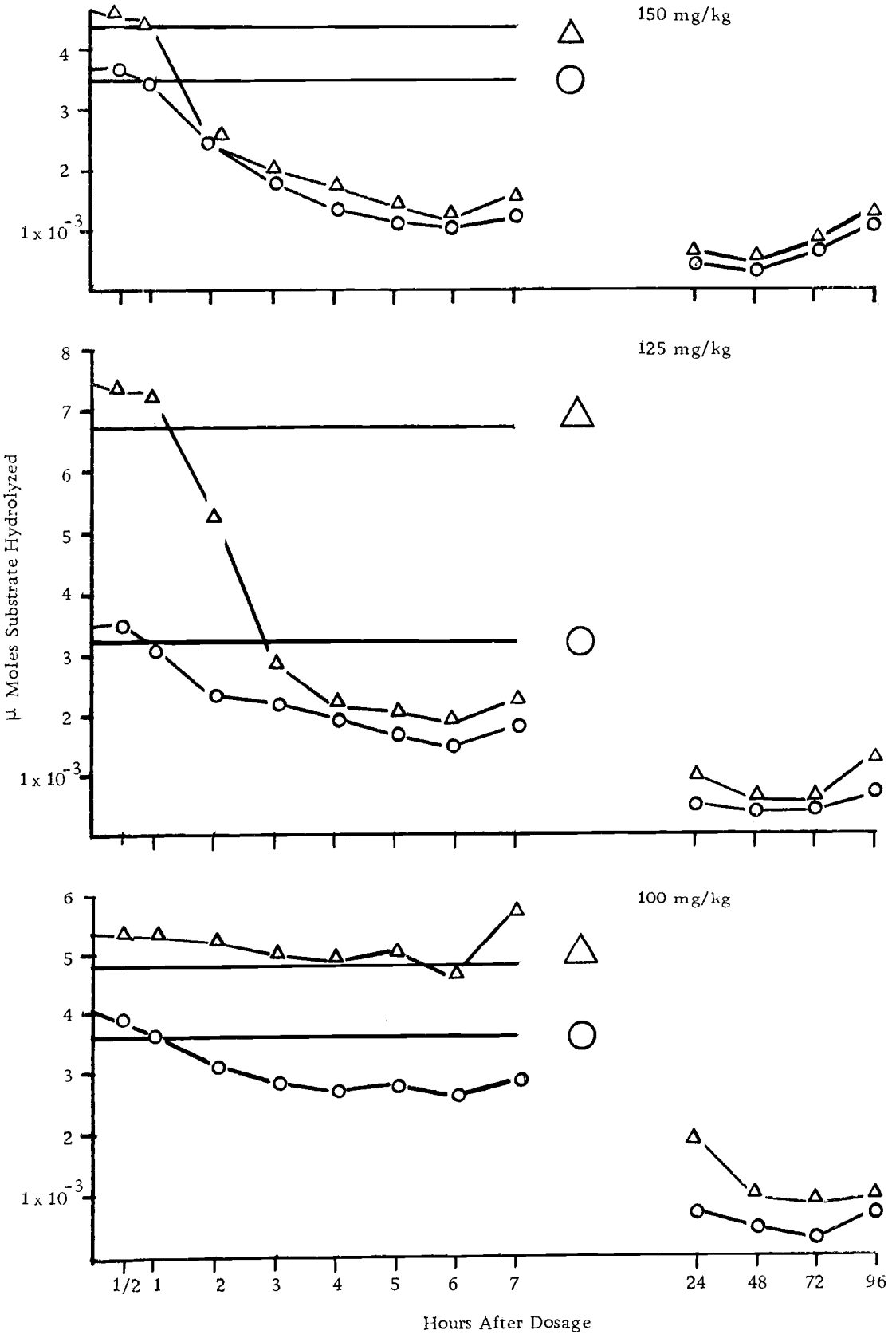
Figure 16. Comparison of the effect of three different concentrations of Tiguvon, administered orally, on rabbit RBC and plasma cholinesterase activity.

Δ RBC

O Plasma

— Δ 95% lower confidence limit of RBC ChE activity

— O 95% lower confidence limit of plasma ChE activity


150 mg/kg
125 mg/kg
100 mg/kg
μ Moles Substrate Hydrolyzed
1 x 10⁻³
Hours After Dosage
1/2
1
2
3
4
5
6
7
24
48
72
96

into the blood stream slowly, insuring a longer systemic activity and therefore a more prolonged toxic effect on blood sucking ectoparasites than when administered intravenously. However, the oral dosage was more time consuming to administer and the necessary handling of the recipient caused unnecessary agitation, thereby increasing the possibility of recipient self injury. Therefore, of the three routes used for administration of pesticide, it was found that the subcutaneous route was the safest to the host and the easiest to administer. It maintained systemically active levels of pesticide in the blood for reasonable amounts of time, and enabled one to treat the animals with the least possible handling.

Pesticidal Effect on Tick and Mosquito ChE Activity Levels

When reference is made to application or administration in this section, it is to be understood that the author is referring to administration to the host and not to the ticks or mosquitoes.

With intravenous administration of the pesticides, the depressions of mosquito and tick ChE activity corresponded with depressions and recoveries of RBC and plasma ChE activity levels. This was best demonstrated with the results from tests using Proban and Famphur (Figures 8, 9, 17, 18). Tiguvon affected ChE activity of the mosquitoes even at the lowest dosage level (Figure 19) but affected the ChE activity of the ticks only at the highest dosage

Figure 17. Comparison of the effects of three concentrations of Proban, administered intravenously, on % tick and mosquito cholinesterase activity and mortality.

— Δ — tick

— O — mosquito

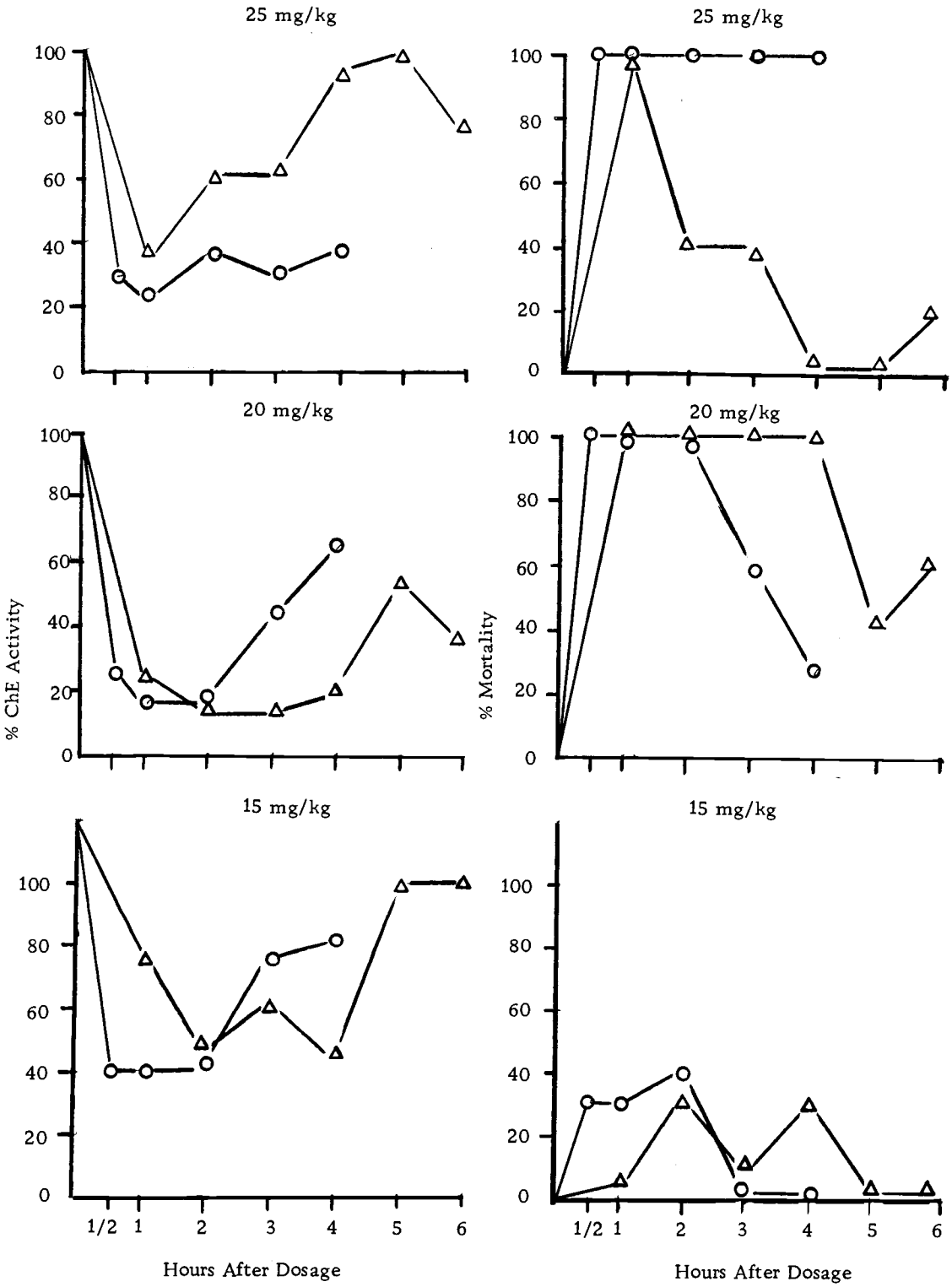

25 mg/kg
25 mg/kg
20 mg/kg
20 mg/kg
15 mg/kg
15 mg/kg
% ChE Activity
% Mortality
100
80
60
40
20
0
1/2 1 2 3 4 5 6
Hours After Dosage
Hours After Dosage

Figure 18. Comparison of the effects of three concentrations of Famphur, administered intravenously, on % tick and mosquito cholinesterase activity and mortality.

— Δ — tick

— O — mosquito

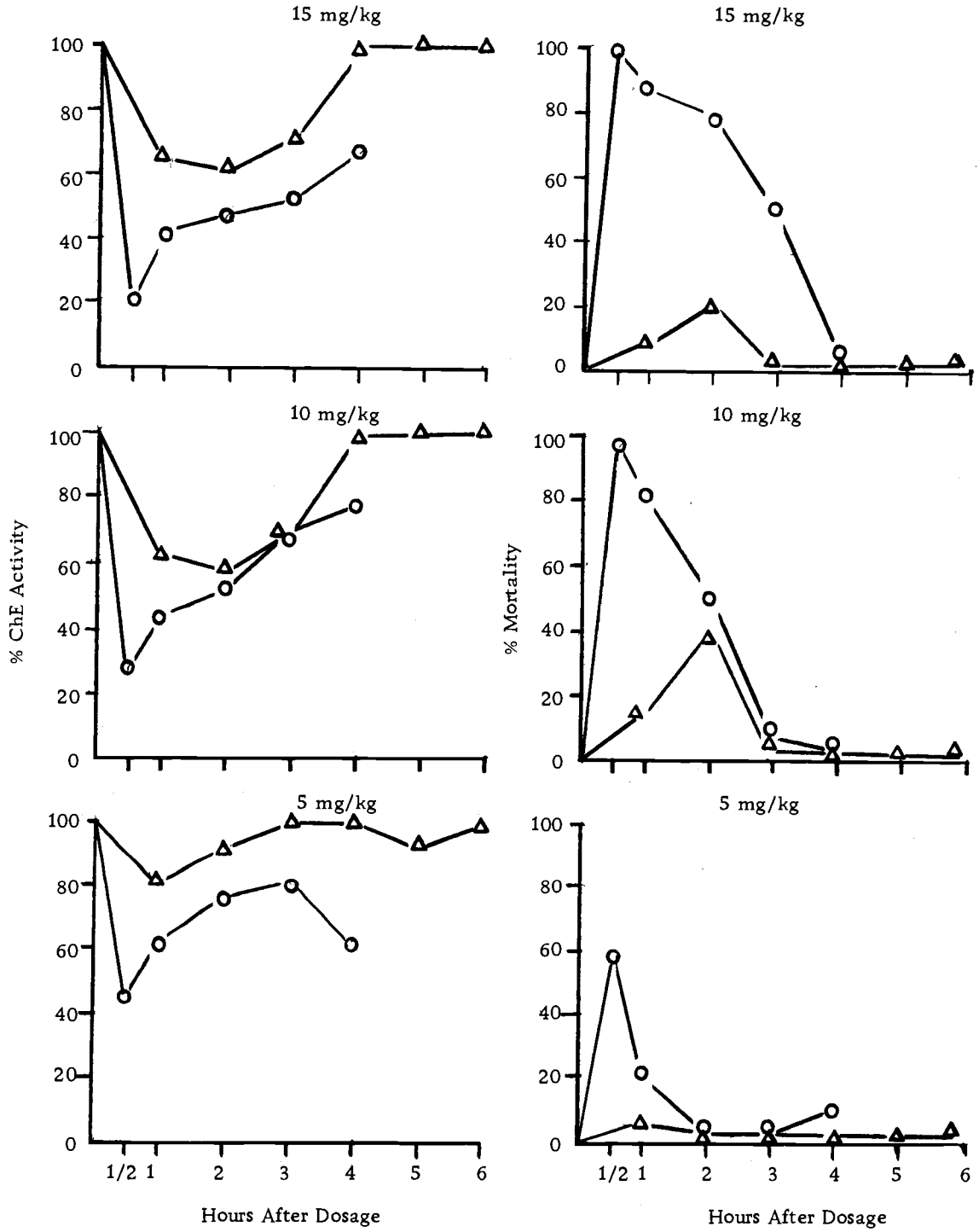
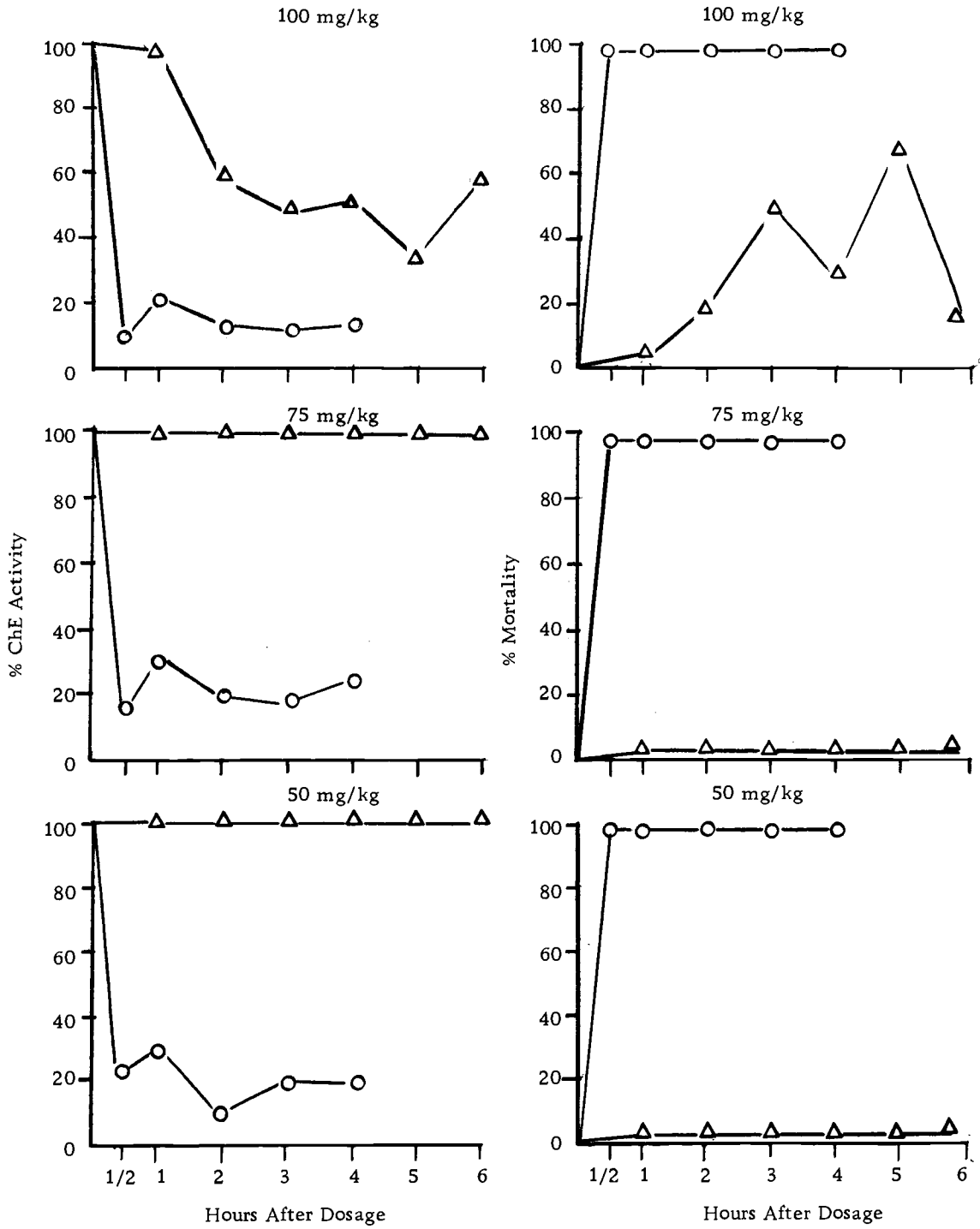

15 mg/kg
15 mg/kg
10 mg/kg
10 mg/kg
5 mg/kg
5 mg/kg
% ChE Activity
% Mortality
100
80
60
40
20
0
1/2 1 2 3 4 5 6
Hours After Dosage
Hours After Dosage

Figure 19. Comparison of the effects of three concentrations of Tiguvon, administered intravenously, on % tick and mosquito cholinesterase activity and mortality.

— Δ — tick

— O — mosquito


100 mg/kg
100 mg/kg
75 mg/kg
75 mg/kg
50 mg/kg
50 mg/kg
% ChE Activity
% Mortality
0
20
40
60
80
100
1/2
1
2
3
4
5
6
Hours After Dosage
Hours After Dosage

level.

When Proban was administered subcutaneously, there was good correlation between host blood ChE activity levels (Figure 11) and mosquito ChE activity levels (Figure 20). The depression of tick ChE activity was delayed until approximately two hours after dosage (Figure 20).

Famphur, applied subcutaneously, induced initial depression of both tick and mosquito ChE activity at the same time that it caused depression of host ChE activity levels (Figures 12 and 21). However, the subsequent peaks and depressions of ChE activity of the ectoparasites could not be correlated to each other nor to host ChE activity. The feeding behavior of the ectoparasites may be the explanation for these observed variations.

With subcutaneous injection of Tiguvon, there was a close correlation between depression of host ChE activity levels (Figure 13) and mosquito ChE activity (Figure 22). However, the tick ChE activity depressions were delayed for one hour (Figure 22).

Generally speaking, the ChE activity levels of the ectoparasites in the orally administered Proban studies were well coordinated with blood ChE activity levels (Figure 23). However, the most drastic depression of mosquito ChE activity occurred one hour after that of the host (Figures 14, 23). The tick ChE activity levels were significantly depressed at the same time as depression of host RBC

Figure 20. Comparison of the effects of three concentrations of Proban, administered subcutaneously, on % tick and mosquito cholinesterase activity and mortality.

— Δ — tick

— O — mosquito

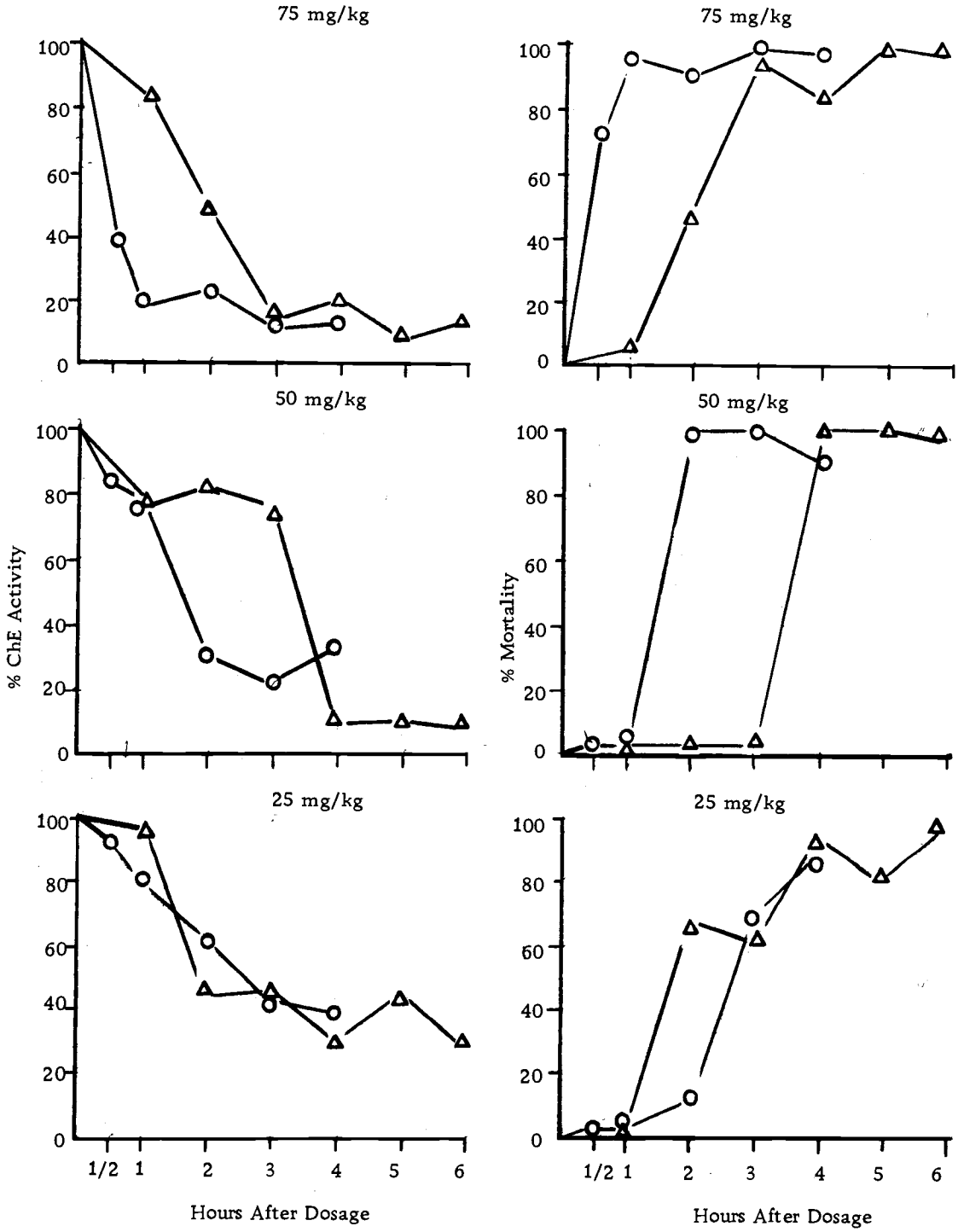

75 mg/kg
75 mg/kg
50 mg/kg
50 mg/kg
25 mg/kg
25 mg/kg
% ChE Activity
% Mortality
100
80
60
40
20
0
1/2
1
2
3
4
5
6
Hours After Dosage
Hours After Dosage

Figure 21. Comparison of the effects of three concentrations of Famphur, administered subcutaneously, on % tick and mosquito cholinesterase activity and mortality.

— Δ — tick

— O — mosquito

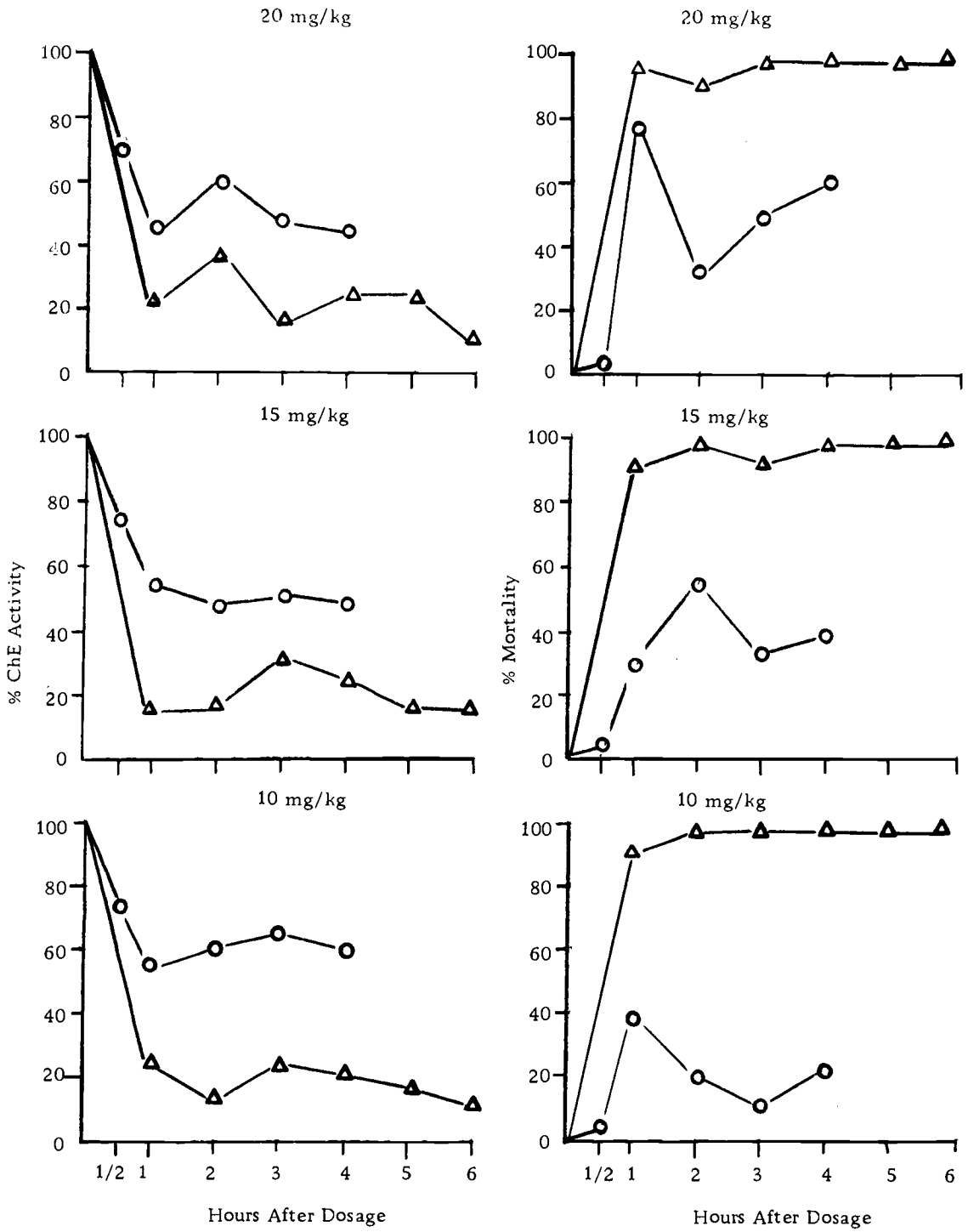

20 mg/kg
20 mg/kg
15 mg/kg
15 mg/kg
10 mg/kg
10 mg/kg
% ChE Activity
% Mortality
100
80
60
40
20
0
1/2
1
2
3
4
5
6
Hours After Dosage
Hours After Dosage

Figure 22. Comparison of the effects of three concentrations of Tiguvon, administered subcutaneously, on % tick and mosquito cholinesterase activity and mortality.

— Δ — tick

— O — mosquito

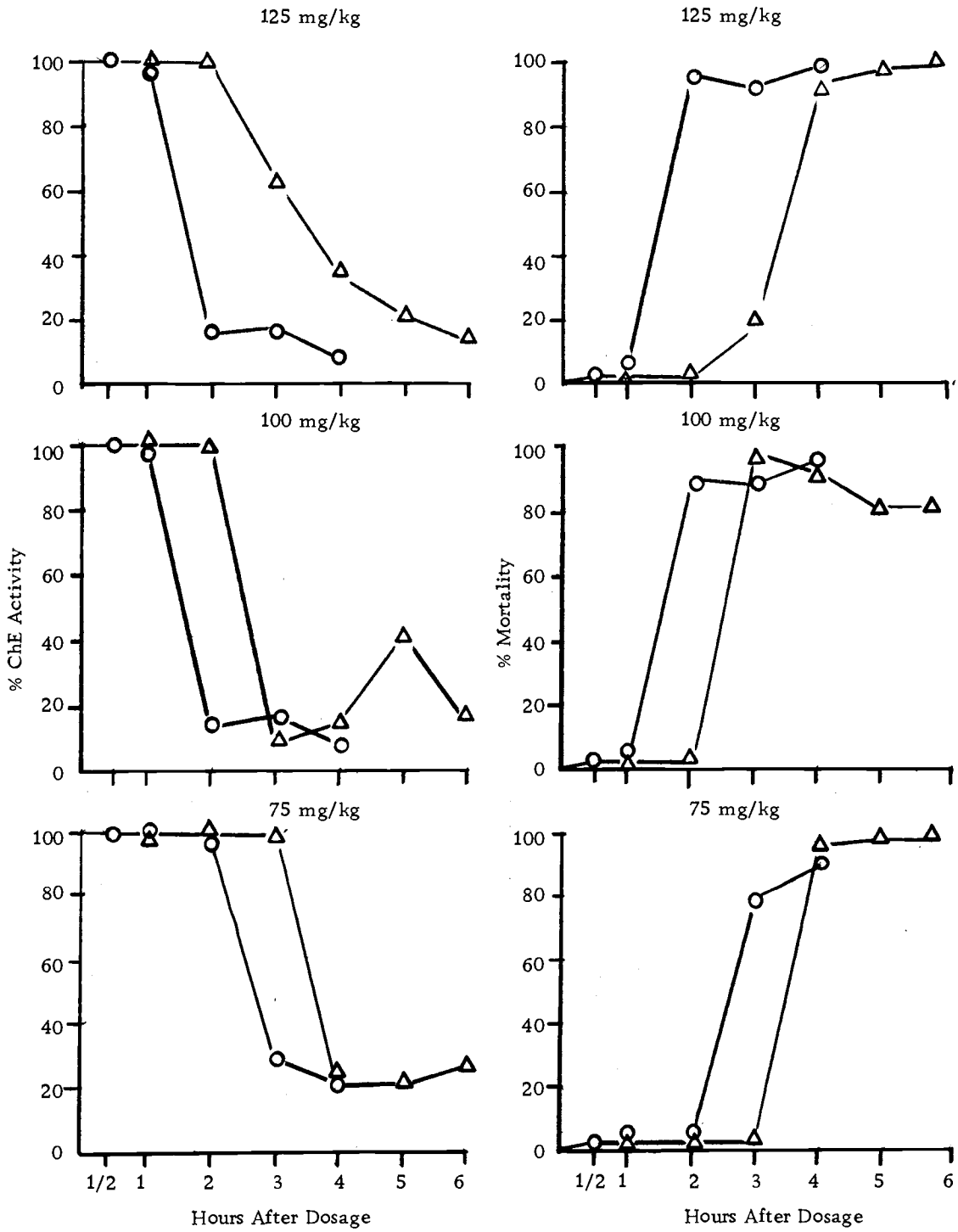
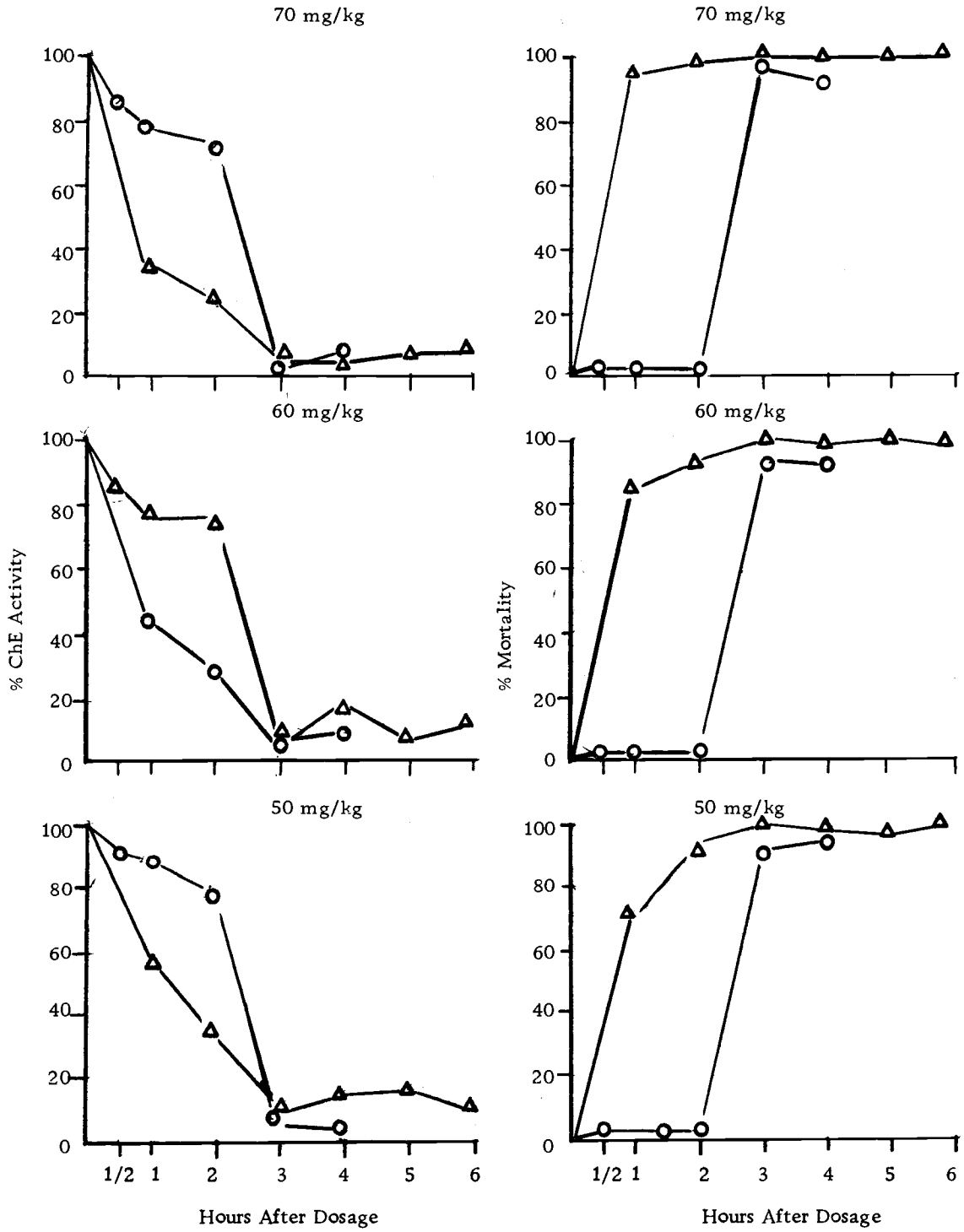

125 mg/kg
125 mg/kg
100 mg/kg
100 mg/kg
75 mg/kg
75 mg/kg
% ChE Activity
% Mortality
100
80
60
40
20
0
1/2
1
2
3
4
5
6
Hours After Dosage
Hours After Dosage

Figure 23. Comparison of the effects of three concentrations of Proban, administered orally, on % tick and mosquito cholinesterase activity and mortality.

— Δ — tick

— O — mosquito


70 mg/kg
70 mg/kg
60 mg/kg
60 mg/kg
50 mg/kg
50 mg/kg
% ChE Activity
% Mortality
100
80
60
40
20
0
1/2 1 2 3 4 5 6
Hours After Dosage
Hours After Dosage

ChE activity (Figures 16, 23). Maximum inhibition of ectoparasite ChE activity occurred at the three hour post dosage period (Figure 23), which was one hour after maximum depression of host blood ChE activity.

There was a close correlation between parasite ChE activity levels and host blood ChE activity levels at the highest concentration of orally administered Famphur. With the two lower dosages, a much greater percent of ectoparasite ChE activity depression was observed than was expected on the basis of the host blood ChE activity levels (Figures 15, 24). This would indicate high metabolic activity of the host and rapid degradation of the pesticide as it was being slowly absorbed into the blood stream.

Oral dosage of Tiguvon had no effect upon tick ChE activity levels (Figure 25). The ticks were observed ingesting blood during peak systemic activity levels of pesticide in host blood, yet there was no resultant depression in tick ChE activity levels. From this observation it was assumed that when orally administered the Tiguvon was relatively inefficient as a systemic against ticks. The mosquito ChE activity level was slightly depressed at one-half hour but did not correlate closely with the fluctuations in host blood ChE activity levels (Figures 16, 25).

Figure 24. Comparison of the effects of three concentrations of Famphur, administered orally, on % tick and mosquito cholinesterase activity and mortality.

— Δ — tick

— O — mosquito

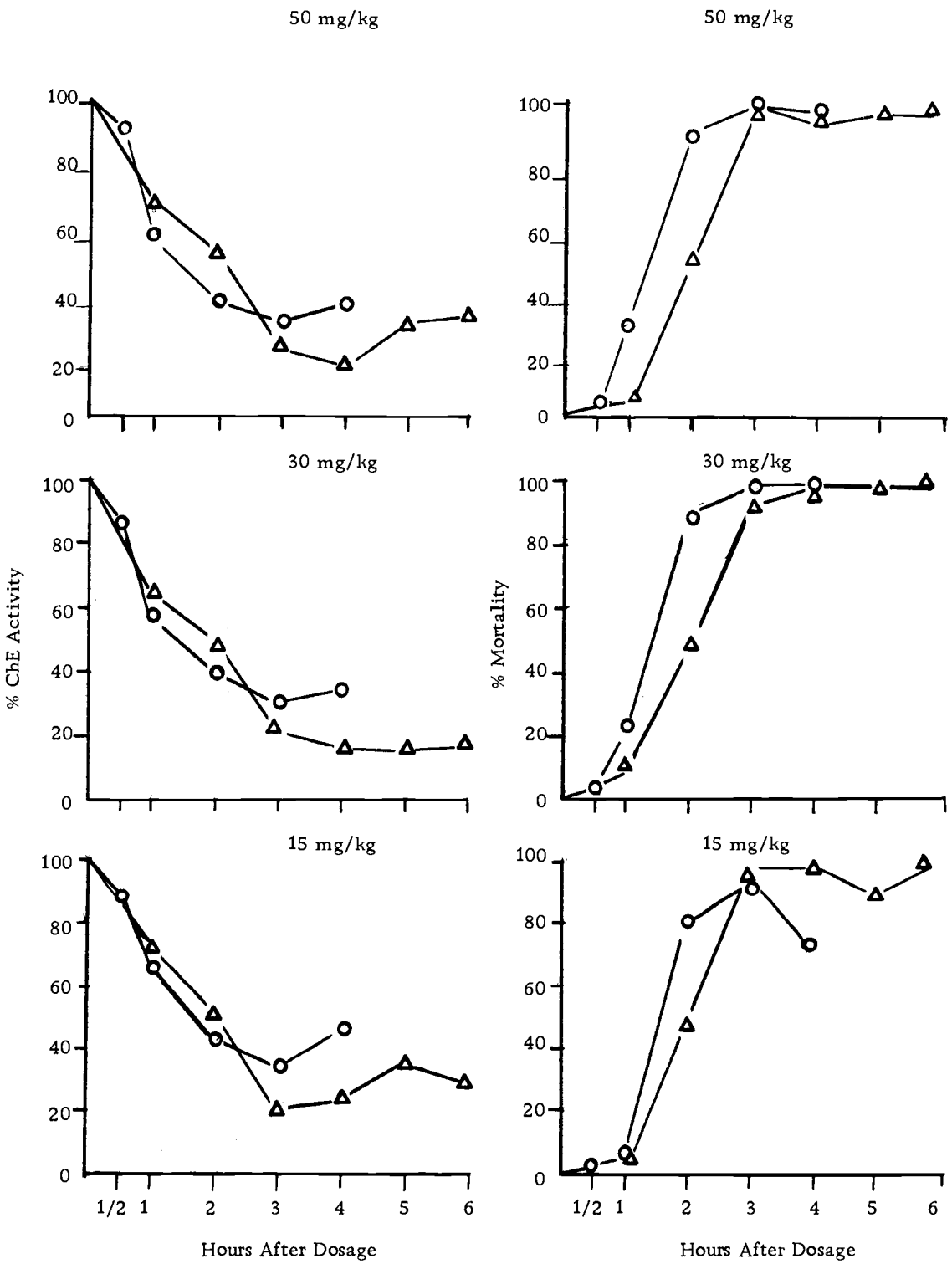
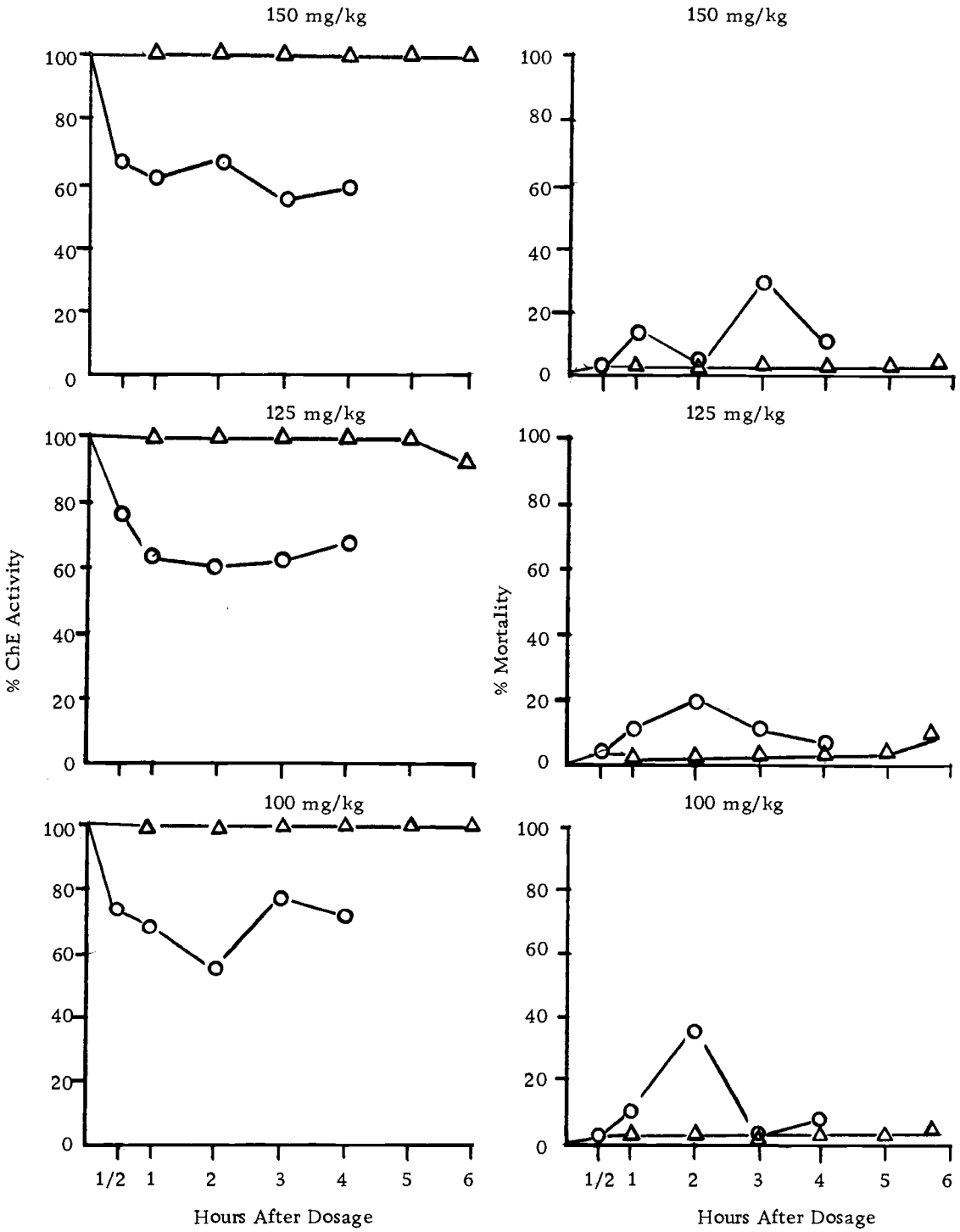

50 mg/kg
50 mg/kg
30 mg/kg
30 mg/kg
15 mg/kg
15 mg/kg
% ChE Activity
% Mortality
100
80
60
40
20
0
1/2
1
2
3
4
5
6
Hours After Dosage
Hours After Dosage

Figure 25. Comparison of the effects of three concentrations of Tiguvon, administered orally, on % tick and mosquito cholinesterase activity and mortality.

— Δ — tick

— O — mosquito


150 mg/kg
150 mg/kg
125 mg/kg
125 mg/kg
100 mg/kg
100 mg/kg
% ChE Activity
% Mortality
100
80
60
40
20
0
1/2
1
2
3
4
5
6
Hours After Dosage
Hours After Dosage

Comparison of Ectoparasite ChE Activity and Ectoparasite Mortality

In comparing ectoparasite ChE activity levels and mortality, it was found that mortality first occurred in both species of ectoparasite whenever their ChE activity level was depressed below 68% of normal. When ectoparasite ChE activity was 38% of normal, there was approximately 100% mortality in the samples. The only major exception to this observation was with the lowest dosage of Proban, applied intravenously (Figure 17). In this case, low mortality was observed instead of high mortality, which was expected since the ectoparasite ChE activity was at low levels. In all cases the ectoparasites' ChE activity levels corresponded much more closely to their mortality than they did to host blood ChE activity levels. In general, however, there was a correlation among all three factors (host blood ChE depression - parasite ChE depression - death of parasite) when the host was exposed to systemically acting organophosphorous pesticides.

When the efficacies of the three pesticides were compared, it was found that Famphur administered subcutaneously gave a good kill of ticks but not of mosquitoes (Figure 21). At their highest concentrations, both Tiguvon and Proban promoted sustained kills on both parasites for the full test period. However high mortality of both ticks and mosquitoes occurred one hour earlier with Proban than

with Tiguvon (Figures 20, 22). With intermediate and low concentrations of pesticide administered subcutaneously, there was relatively little difference between either Proban or Tiguvon.

When the pesticides were administered intravenously, there was 100% mortality of mosquitoes within one half hour after application with the two highest concentrations of Proban and Famphur; this rapid, high mortality was evident when using all three concentrations of Tiguvon. Sustained high kills of mosquitoes was demonstrated by both Proban and Tiguvon (Figures 17, 19); while Famphur-exposed mosquitoes showed a steady reduction in mortality (Figure 18). The ticks were susceptible to systemically acting Proban and good to fair mortality results ensued for the entire test period with the two higher concentrations (Figure 17). However, at the highest concentration of intravenously administered Proban, very high mortality occurred only at one hour after dosage. Since Proban was very toxic to ticks at the intermediate concentration, it was assumed that the low mortality was due to poor feeding by the ticks at the time of high systemic activity of the pesticide in the blood of the host. Famphur had little effect on ticks, with never more than 40% mortality even at the higher concentrations (Figure 18). Tiguvon had no effect on the ticks at the two lower concentrations and only 60% mortality was in evidence at the highest concentrations (Figure 19).

When the pesticides were administered orally, Tiguvon had little

or no effect on either ectoparasite (Figure 25). Proban was very effective against ticks, as high mortality rates were established within one hour with all three concentrations of pesticide and these mortalities were sustained for the entire test period (Figure 23). Proban produced high mortality among the mosquitoes from the third hour (Figure 23). Famphur, when acting systemically, demonstrated exceptional effectiveness against mosquitoes from the second hour and against ticks from the third hour (Figure 24).

It may therefore be concluded that the ectoparasites were killed due to pesticidal inhibition of the enzyme cholinesterase. It may be further concluded that with both ticks and mosquitoes mortality commences at approximately 32% ChE inhibition and 100% mortality occurs at approximately 68% ChE inhibition.

Best Route, Ease, and Safety of Administration

Through experimentation, it was found that the best route of application of the pesticides was subcutaneous. This route was found to be superior to the other two application routes in that when the pesticide was applied there was no escape attempted by the rabbits and therefore less chance of harm to them through handling. The pesticides were diffused more slowly through the bloodstream of the host thereby maintaining the systemic activity of the pesticide over a longer period of time. The toxic effect of the pesticide on the host

was much reduced due to the slower absorption of the pesticide into the blood stream thereby giving the host's detoxication systems more time to detoxify the pesticide. The slower, more even absorption of the pesticide into the blood stream of the host was very advantageous as one of the ectoparasites, the tick, was an intermittent feeder and the slow absorption insured that the tick would eventually get a toxic dose of pesticide. By keeping systemic activity of the pesticide at higher levels in the blood for longer periods, kills of both ticks and mosquitoes could be assured.

Behavior

Mosquito

The feeding behavior of the mosquitoes was such that they fed to repletion within five minutes of the time that they were applied to the host's back. At times when systemic activity of the pesticides were at maximum in the host's blood, the mosquitoes were reluctant to feed. At these times they would alight on the part of the cage that was furthest from the skin of the host and had to be agitated constantly to insure feeding. This behavior indicated a repellent action, possibly brought on by the activity of the pesticide in the blood of the host. There was evidently no toxic component being excreted through the epidermis of the host, as no mortality occurred among unfed

mosquitoes when the open ends of the cages were sealed and the cages left on the host for thirty minutes.

Ticks

The ticks were observed to be intermittent feeders, but they consumed large quantities of blood during each feeding period. According to Evans, Sheals, and MacFarlane (1961), they attach to a host and for a period of a few hours take in blood slowly and then stop for approximately 48 hours. They then commence feeding once more and feed intermittently for a period of approximately nine days at which time they fall from the host (Cooley, 1932). In these experiments, the ticks were not allowed to feed to repletion, but were removed from the host on the fourth day after attachment.

The males were observed to feed for a short period, detach themselves and wander around the cages. When in their wandering they chanced upon a female they attached themselves venter to venter with her and mating took place. In many instances the males mated with more than one female and a few females were visited by more than one male.

Once attached, the females did not release and fed until they were removed from the host. According to Cooley (1932) and Douglas (1943), after a full blood meal of approximately nine days, the females fall from the host and go through a period of metabolic change and

ovary development, usually lasting about five days. They then lay eggs for a period of approximately three weeks, at the end of which they are spent and die.

The control females (those removed at time 0 in the experiment) all laid viable eggs five to seven days after removal from the host. In the observation samples those surviving females that had been exposed to the pesticides, regardless of concentration or route of application, laid no eggs during a 32 day post removal observation period.

When the systemic activity of the pesticide was at its maximum in the blood of the host, the attached ticks became hyperactive, in many instances detaching themselves and crawling about in their cages. Hyperactivity continued one hour after maximum systemic activity of pesticide in host blood. At this time the ticks reattached and fed until such time as they were removed or died from effects of the pesticide.

Residue Studies

Studies were conducted using gas-liquid chromatography to determine the persistency of the three pesticides in selected organs and tissues.

Residues of two of the pesticides, Proban and Famphur, were very short-lived in the tissues of the host. Proban residues were essentially gone by the 3 day post dosage period, only 0.05 PPM being

present in the feces and 3.31 PPM in the muscle at this time (Table 1). Slightly more Famphur residue was present at the 3 day period. Parent compound was found in detectable amounts at three sites in the rabbit: feces, kidney, and muscle. The disappearance of Famphur residues in host tissue was complete after seven days (Table 2). Tiguvon, however, demonstrated a high residue persistency in host tissues at the one week period. At this time, all the tissues but the liver, fat, and blood had considerable amounts of pesticide in them. Gas chromatograph results for the two week period indicated that there was considerable pesticide present in the urine and muscle tissue. The 22 day analysis showed a faint but detectable residue of the Tiguvon in the urine and the muscle. Residues of Tiguvon, in host tissue, had completely disappeared by the fourth week after dosage (Table 3).

Table 1. Residues in PPM of Proban[a] in several rabbit tissues for 7 days after dosage.

Tissue	2 hr	5 hr	1 day	3 day	7 day
Urine	----	480	100	0	0
Liver	21.8	13.05	2.54	0	0
Feces	4.97	0.87	2.4	0.05	0
Fat	2.51	2.04	1.6	0	0
Kidney	13.9	17.3	1.06	0	0
Muscle	5.39	2.52	6.36	3.31	0
Brain	4.78	5.42	0.17	0	0
Heart	6.58	7.90	0.45	0	0
Blood	2.39	1.32	.06	0	0

[a]Treated subcutaneously at 50 mg/kg of body wt.

Table 2. Residues in PPM of Famphur[a] in several rabbit tissues for 7 days after dosage.

Tissue	2 hr	5 hr	1 day	3 day	7 days
Urine	68.8	195	32	0.90	0
Liver	.58	.53	.54	0	0
Feces	.64	.48	.81	.04	0
Fat	1.3	1.74	1.82	.04	0
Kidney	2.52	1.80	.92	.32	0
Muscle	.58	.74	.40	.24	0
Brain	.85	.39	.54	0	0
Heart	----	----	1.24	0	0
Blood	1.46	1.1	----	0	0

[a]Treated subcutaneously at 20 mg/kg of body wt.

Table 3. Residues in PPM of Tiguvon[a] in several rabbit tissues for 28 days after dosage.

Tissue	2 hr	5 hr	1 day	3 day	7 day	14 day	22 day	28 day
Urine	5.76	80.0	71.6	43	28.7	4.37	0.45	0
Liver	0.13	0.706	0.71	0	0	----	0	0
Feces	0.11	0.10	1.96	0.63	0.159	----	0	0
Fat	0.291	0.4	1.00	0	0	----	.27	0
Kidney	0.5	2.3	1.65	1.4	0.442	----	0	0
Muscle	0.07	18	0.15	0.5	0.161	0	0.040	0
Brain	0.16	1.93	1.04	0.49	0.046	----	0	0
Heart	----	----	----	----	0.144	----	0	0
Blood	0.145	1.2	----	----	----	----	0	0

[a]Treated subcutaneously at 125 mg/kg of body wt.

The lack of pesticide in the blood after the twenty-four hour period could possibly be the reason that there were no mortalities among ectoparasites that were allowed to feed during this time (24-96 hours).

Muscle tissue retained the pesticide for the longest period of time (Tables 1-3). There was no apparent difference in retention time of pesticide in muscle removed from the point of injection and muscle far removed from the site. Pesticide residues were present in greatest amounts and were detected for the longest periods of time in the host's urine. This would be expected as long as there were any residues of pesticide present in any of the host's body tissues and as these stored pesticides were metabolized by the host they would be excreted in the urine. Parent compounds were of primary importance in this study and metabolite identifications were not attempted.

It would appear from the amounts of Tiguvon eliminated in the feces that it could be used as a larvicide against fly larvae in host feces. Both Famphur and Proban were too short-lived to be considered for this purpose.

Other Observations

Ear Mites

The ears of several rabbits were so badly infested with ear mites Otodectes cynotis (Hering) that the auditory canals were badly

inflamed and swollen closed. Three of the infected rabbits were selected and subcutaneous doses of Tiguvon at 100 mg/kg, Proban at 50 mg/kg, and Famphur 15 mg/kg were administered to them. In all cases, the mites were completely eliminated within 24 hours, the swelling had disappeared within 48 hours and the ears appeared normal. Upon inspection, the auditory canals were open and no inflammation was visible. From this, it would appear that any of the pesticides used in this study could be used for control of ear mites. However, additional observations on effective dosage levels are needed.

Temperature Effects

It was noticed during the experiments that, when cages of mosquitoes which had fed on pesticide-dosed hosts were subjected to a rapid reduction in ambient temperature, that mortality ensued within twenty seconds. To substantiate this observation, mosquitoes were allowed to feed upon rabbits at various times after dosage. Prior to any observed mortality, the mosquitoes were exposed to a 15° C reduction in temperature. In all instances, knockdown occurred immediately (within 20 seconds) and there were no recoveries among those mosquitoes that had fed. Among those mosquitoes that had not fed or had fed upon undosed rabbits, there was a 1 1/2 minute time interval between exposure to the cold and knockdown. In all instances there was recovery when they were exposed to room

temperatures. Further investigation was beyond the scope of this study.

Host Reproduction Studies

The experimental female rabbits were bred and all gave birth to healthy living young. The average number of young per litter was four. There were no anomalies in any of the young and there were no still births. Selected individuals from the F_1 generation were bred and these gave birth to normal, healthy individuals. It was concluded from the reproduction study that sublethal dosages of Tiguvon, Proban, or Famphur were in no way harmful to the reproductive capabilities of these rabbits.

SUMMARY

A "norm" for rabbit blood ChE was determined by analyzing five predosage samples of blood from each of 127 rabbits. The norm for RBC ChE activity was 5.22×10^{-3} and for plasma ChE activity it was 2.83×10^{-3}. These norms could not be used due to too great a variation between individuals, therefore norms for each animal were determined.

There was immediate inhibition and early partial recovery of blood ChE activity when the pesticides were administered intravenously. Tiguvon depressed blood ChE activity the furthest and kept it depressed for the longest period of time. Proban appeared to be least toxic to the host as it depressed blood ChE activity levels the least and for the shortest period of time. Rate of blood ChE depression is more important than degree of depression. Toxic symptoms begin to appear in the host when its blood ChE activity is depressed more than 50% of normal.

When administered subcutaneously, all the pesticides inhibited blood ChE activity by one hour after dosage and maximum depressions were reached by five hours after dosage.

When pesticide was administered orally, there was no significant change in blood ChE activity for more than one hour after dosage. Tiguvon and Proban depressed ChE activity sharply with depression of

ChE due to Tiguvon remaining depressed while that depressed by Proban gradually recovered. Famphur depressed ChE activity gradually with maximum depression being reached between four and five hours after dosage.

The degree of depression and length of time that subcutaneous and oral administrations depressed ChE activity indicated that these routes of administration would expose blood sucking ectoparasites to a systemically acting pesticide for a longer period of time. However, oral dosage was time consuming and posed problems for the host. Therefore the subcutaneous route of application was deemed the best of the three.

When pesticide was administered intravenously, tick and mosquito ChE activity corresponded with host blood ChE activity depressions and recoveries. However, Tiguvon affected tick ChE only at high dosages and was delayed for a time. Feeding behavior was believed to be a major factor in ChE activity variations.

With orally administered pesticide there was a correlation between host ChE activity depressions and ectoparasite ChE activity. However, with this dosage route, Tiguvon had no effect on tick ChE activity, indicating that Tiguvon was not a good systemic for ticks when given orally.

There was a close correlation between host ChE activity and parasite ChE activity when route of application of pesticide was

subcutaneous.

Ectoparasite mortality began whenever their ChE activity was depressed 32%, and 100% mortality was reached when their ChE activity was depressed 62%. Famphur was found to be effective against ticks and not mosquitoes. Proban was effective against both parasites by all dosage routes. Tiguvon was effective against both parasites by intravenous and subcutaneous application routes, but was not effective against either parasite when administered orally.

When mortality experiments with mosquitoes were performed between 24 and 96 hours after dosage, there were no deaths.

Due to ease of application, slowness of diffusion, longer systemic activity of pesticide in host blood, and safety to the host, subcutaneous dosage route was considered to be the best route of administration.

Hyperactivity of both ticks and mosquitoes at maximum systemic activity of pesticide, in host blood, led to the belief that some repellent or toxic compound was being excreted through the skin of the host. However, closure of mosquito cages resulted in no mortality.

Ticks were found to be intermittent feeders. Mated females that were subjected to sublethal doses of pesticide laid no eggs whereas controls laid viable eggs.

Results from residue studies indicated that, of the pesticides

studied, Proban was the least residual and Tiguvon the most residual. Residues from Tiguvon persisted for approximately 28 days.

When therapeutic doses of all three pesticides were given subcutaneously they ridded rabbits of ear mites within 24 hours. When mosquitoes that had been exposed to sublethal doses of pesticide were exposed to a reduction in temperature of 15° C, there was mortality within 20 seconds.

Reproductive capabilities of rabbits subjected to sublethal doses of the three pesticides were not affected. Mated females gave birth to healthy individuals averaging four per litter. The F_1 generation was bred and also gave birth to normal, healthy young.

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APPENDIX

Reagents Used for ChE Activity Analysis

1. Phosphate Buffer - pH 7.34

   (a) Mono-sodium Phosphate - $NaH_2PO_4 \cdot H_2O$

   For a 0.2M solution, 13.9 g of $NaH_2PO_4 \cdot H_2O$ were placed in a 500 ml volumetric flask and brought to volume with glass distilled water.

   (b) Di-sodium Phosphate - $Na_2HPO_4 \cdot 12H_2O$

   For a 0.2 M solution, 35.85 g of $Na_2HPO_4 \cdot 12H_2O$ were placed in a 500 ml volumetric flask and brought to volume with glass distilled water.

   (c) For one liter of a buffer solution of pH 7.34, 140 ml of mono-sodium phosphate solution (a) were mixed with 360 ml of di-sodium phosphate solution (b) and 500 ml of glass distilled water.

2. Substrate

   (a) Non Radioactive Material

   A cold (carrier) substrate solution was made by placing 226.12 mg of acetylcholine bromide (Eastman Kodak Co.) in a 100 ml volumetric flask and was brought to volume with phosphate buffer, (pH 7.34).

   (b) Radioactive Material

A total of 5 ml of hot substrate was prepared which contained 2.5 μC of activity. Using a microsyringe, 62.5 μl of acetyl-1-C^{14}-choline iodide (New England Nuclear Corp.) from a stock solution containing 40 μC/ml was placed in 0.5 ml of cold substrate. To this mixture, 4.44 ml of phosphate buffer (pH 7.34) were added.

3. Saponin - 0.04%

A 0.04% saponin solution was made by placing 0.1 g of saponin in a 250 ml volumetric flask and bringing it to volume by addition of Gomori buffer. The saponin solution was beneficial in lysing red blood cells.

4. Scintillation Counting Solution

The scintillation counting solution was made by placing 3.0 g of ϕ-p-terphenyl and 30 mg of POPOP (1, 4-bis-2-(5-phenyloxazolyl) benzene) (Packard Instrument Co.) in a one liter volumetric flask and bringing it to volume by the addition of toluene (reagent grade).

5. Amberlite CG-120, sodium salt, 200-400 mesh (Mallinckrodt Chemical Co.), analytical-reagent grade.

An amberlite, CG-120, resin-ethanol suspension for stopping the hydrolysis reaction was made in the following steps:

1. The amberlite, CG-120, was washed by filtering

100% ethanol through 100 g of amberlite with the aid of a Buchner funnel.

2. The white film on the top of the washed amberlite, CG-120, was scraped off and discarded.

3. The washed amberlite, CG-120, was dried in a dessicator for at least 24 hours and stored therein until used.

4. The suspension was prepared by placing 37.5 g of washed and dried amberlite, CG-120, in a 250 ml volumetric flask and bringing it to volume by adding 100% ethanol.