STUDIES ON THE MODE OF ACTION OF SYNERGIZED BAYER 21/199 AND ITS OXYGEN ANALOG IN THE HOUSE FLY, *Musca domestica* L.

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A LIST OF SYNONYMS AND GENERALLY ACCEPTED ABBREVIATIONS USED THROUGHOUT THIS PAPER

21/199 - - - - the parent compound or the phosphorothioate.

0-21/199 - - - the oxygen analog, corresponding phosphate, or phosphate of 21/199.

21/197 - - - - the 7-hydroxy-coumarin or 3-chloro, 4-methylumbelliferone moiety of 21/199 or 0-21/199.

PB - - - - - - - piperonyl butoxide.

ACh - - - - - acetylcholine.

Ch E - - - - - cholinesterase.

LD-50 - - - - the amount of toxicant necessary to kill 50 percent of the treated flies.

IN-50 - - - - The molar concentration of toxicant necessary to inhibit 50 percent of the enzyme cholinesterase.

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INTRODUCTION

Combinations of compounds having a higher biological activity than the sum of the activities of the individual components are said to be synergistic. Veldstra (26) defines synergism translated into molecular terms: "the combination effects a certain response with a smaller number of molecules than that required for the most active compound separately, or in the range of suboptimal concentrations, the effect of a certain number of molecules of the compound is enhanced in the mixture."

Synergism, literally a "working together", has been advantageously used in combination with many biologically active substances. Veldstra (26) in a recent review on synergism reports this phenomenon as occurring with plant growth substances, acetylcholine and related compounds, sympathomimetic amines, analgesics, hypnotics, narcotics, anticonvulsants, antibiotics, antimetabolites, vitamins, hormones, mitotic poisons, excretion mechanisms, and insecticides.

The concept of synergism in the field of insecticides dates back to the discovery of isobutylundecylenamide (IN-930) by Weed (27). This first effective synergist

of pyrethrum was later replaced by the development of more effective synergists based upon the discovery of Haller et al. (8, 9) that the methylenedioxyphenyl group was essential for the synergistic activity of sesamin. Thus, today, most synergists are derivatives of isosafrole, whose molecule bears a single methylenedioxyphenyl group (14, p.138).

The synergist by itself has relatively little toxicity to insects, but when used in combination with some insecticides, an increase in killing power may be noticed over the effect of the insecticides alone. Although most of our knowledge on synergism has been concerned with pyrethrum and allethrin (22) and halogen-containing insecticides (24), several workers have reported the synergism of organic phosphorus insecticides.

Hopkins and Hoffman (13) found that piperonyl butoxide increased the effectiveness of Bayer compound 21/199 from 30 to 95 percent of normal when used against Orlando DDT-resistant house flies. Eddy et al. (7) observed that synergists effective in combination with Bayer compounds 21/199, 21/200, and Potasan® were ineffective when used in combination with parathion, malathion, and EPN® in body louse studies. Hoffman et al. (12) reported that most chemicals known to

synergize pyrethrum will also improve the effectiveness of certain organic phosphorus insecticides, but some of the synergists were more effective with certain toxicants tested than with others. Bayer compounds 21/199, 21/200, and Potasan® were synergized more than the other toxicants tested. Rai et al. (19) found that piperonyl butoxide antagonized malathion's lethal action on two strains of house flies, but synergized the lethal effects of Diazinon and Bayer compound L-13/59.

Although many workers have investigated the mode of action of organic phosphorus compounds in insects and mammals, few have studied in any scope the mode of action of synergized phosphorus insecticides.

The foregoing discussion on the opposite effects of piperonyl butoxide on different organic phosphorus insecticides suggests differences in the mode of action of these compounds. This group of compounds, generally believed to have similar modes of action, are toxic to animals because they interfere in the normal mechanism of nerve impulse transmission through irreversible inhibition of the enzyme, acetyl cholinesterase (16, p.269, 18).

Among the many organophosphorus compounds tested by workers, the compounds showing the greatest degree of synergism were the O-(3-chloro,4-methylumbelliferone) or O (4-methylumbelliferone) esters of O,O dialkyl phosphorothioate (16, p.76). The experimental insecticide Bayer

21/199, O-(3-chloro-4-methylumbelliferone) 0,0 diethyl phosphorothioate, (Figure 2) is a member of this group. Research has indicated that this systemic insecticide may be effective against encysted cattle grubs (21, 23) or against migrating cattle grubs when applied as a spray (2).

No information is available on the mode of action and metabolism of a synergist with an ester of the O,O dialkyl phosphorothioate group in insects. This study was initiated to gain a better understanding of the mode of action of organophosphorus synergism by use of the pyrethrum synergist piperonyl butoxide, a [2-(2-N-butoxy-ethoxy)-ethoxy]-4:5 methylenedioxy-2-propyltoluene, in combination with Bayer 21/199, and its oxygen analog, the corresponding phosphate, O-(3-chloro-4-methylumbelliferone) 0,0 diethyl phosphate, (Figure 2) in the house fly, Musca domestica L.

MATERIALS AND METHODS

Experimental Insects. A strain of house flies, moderately resistant to DDT, was used in all of these tests. The LD-50 and LD-90 were 1.20 and 100 ugm of technical DDT per fly, respectively.

Oviposition media was prepared by filling a 50-ml beaker one-third full of spent larval media. A thin layer of moist absorbent cotton was placed over the media and pressed down so as to obtain a depression where oviposition could take place. Three to four drops of ammonia diluted with water were placed in the depression to act as an oviposition stimulant. The top flaps of the cotton were partially closed to darken the interior of the depression. Flies that did not readily oviposit could be induced to do so by removing all food and water from the cage, and placing a small portion of food in the depression of the oviposition beaker. Flies entering the depression would attract more individuals, and usually within one hour mass oviposition would occur. Eggs were removed from the cotton with a scalpel and placed in an 8-dram screw-cap vial three-fourths full of water. The eggs were then mixed and washed by gently shaking the vial, and allowed to settle to the bottom. A 0.25 ml aliquot, approximately 2000 eggs, was removed by pipette and placed in the larval media.

Larval media was prepared by thoroughly mixing three parts by volume of wheat bran with one part of alfalfa

meal. One quart of this media was then mixed with two quarts of sawdust. Seven hundred milliliters of water were added and stirring continued until a uniform mixture was obtained. The media was then placed in a four-quart stainless steel can. The tops of the media cans were covered with muslin cloth to prevent cross contamination of the cultures.

When the adults began to emerge the cans were placed in cages and the muslin covers removed. The peak of the adult emergence was recorded and the cans then removed.

Adult flies were fed a diet of a 1:1 mixture by volume of powdered nonfat milk and cane sugar. Sugar cubes were also placed in the cages. Water was supplied in quart jars with a paper towel folded inside to make the water accessible and to prevent drowning.

The rearing and holding rooms were maintained at 20 ± 2° C with a relative humidity of 45 to 50 percent. All tests except in vitro studies were conducted under the same environmental conditions in an adjacent room. Only three to four day old female flies were used in all of these studies.

Insecticidal Compounds. Compound Bayer 21/199 and its corresponding phosphate (O-21/199) and Bayer 21/197, 3-chloro, 4-methylumbelliferone, (Figure 2) were purified samples supplied by Geary Research Laboratory, New York. The piperonyl butoxide was an analytical standard received from Fairfield Chemical Company, Baltimore, Maryland.

Toxicity Studies. The flies were anesthetized with a minimum exposure to carbon dioxide and approximately one microliter of the appropriate toxicant in C.P. acetone was topically administered to the mesonotum of each fly. The microdrop apparatus used was fitted with a one-fourth milliliter tuberculin syringe calibrated to deliver 1.08 ul of acetone per one-fourth turn of a wheel attached to a screw-driven micrometer (Figure 1).

After 25 randomly chosen flies had been treated per level of toxicant, they were allowed to recover in holding cages made of one-quart waxed cartons fitted at both ends with screen lids. The treated flies were supplied with water by placing saturated cotton on the top screen lid. The moribund and/or dead flies were recorded 24 hours after treatment. Twenty-five flies treated with C.P. acetone only served as a control. After each test the holding cages were relined with clean paper and the screen lids thoroughly cleaned with acetone.

The LD-50 of the insecticides was determined by plotting the mean percent mortalities converted to probits versus log dosage. What was judged to be the best fitting straight line was then plotted through the probit points, and the LD-50 read directly in micrograms of toxicant per female fly.

Three different weighings of the toxicants were made during the course of this study. New dilutions were made

from these weighings and compared with the preceding dilutions for replication of mortalities.

Bayer compounds 21/199, 0-21/199, and 21/197 were tested with and without the synergist, piperonyl butoxide, in the ratio of 1:10. Piperonyl butoxide was tested alone as a toxicant to house flies.

In vitro Cholinesterase Studies. The colorimetric method of Hestrin (11) for acetylcholine (ACh) was used in all enzyme studies, and the following reagents were employed in this technique:

1. Phosphate buffer pH 7.2 was prepared in 500 ml lots employing 6.6262 gm of anhydrous dibasic sodium phosphate and 2.7220 gm of monobasic potassium phosphate. This buffer is double strength and was diluted 1:1 by volume with distilled water before use.

2. Bicarbonate buffer (insect buffer) pH 7.6 to 7.8 was prepared in 500 ml lots employing 4.384 gm of sodium chloride, 1.050 gm of sodium bicarbonate, and 4.066 gm of magnesium chloride hexahydrate. Both buffers were held under refrigeration when not in use.

3. Acetylcholine chloride 0.05 molar was made with sodium acetate 0.001 molar. This reagent was refrigerated.

4. Hydrochloric acid reagent was made by mixing one part by volume of concentrated hydrochloric acid, specific gravity 1.18, with two parts of distilled water.

5. Sodium hydroxide 3.5 normal was prepared in distilled water.

6. Ferric chloride 0.37 molar was made in 0.1 normal hydrochloric acid.

7. Hydroxylamine hydrochloride 2.0 molar was prepared in distilled water. This reagent was refrigerated.

8. Alkaline hydroxylamine was prepared by mixing equal volumes of reagents number five and seven.

Groups of flies were anesthetized and decapitated with a small pair of cuticle scissors. The heads were homogenized for five minutes in a Potter-Elvehjem glass homogenizer with five milliliters of buffer solution per 25 fly heads. The technique of Chadwick et al. (4) of harvesting large numbers of fly heads and freezing them for indefinite periods of time was also utilized in these studies.

'Merthiolate' (Thimerosal, Lilly) previously utilized by Swell and Treadwell (25) to prevent the bacterial decomposition of tissue homogenates (brei) was employed in the in vitro studies. A concentration of 0.075 ml of a 1:10,000 solution per milliliter of fly head brei stabilized the refrigerated homogenate up to one week without affecting the enzyme activity.

The amount of brei to be used in all in vitro tests was dependent upon the rate of hydrolysis of ACh in a 15-minute control reaction period after the addition of 0.1 ml of C.P. acetone. In order to work in an area where

the amount of substrate hydrolyzed is directly proportional to the enzyme concentration, a curve was prepared by determining the amount of substrate hydrolyzed by various concentrations of brei in a constant reaction time of 15 minutes. The results illustrated in Figure 3 indicate linearity until 4.40 micromoles of ACh had been hydrolyzed, and then the reaction rate began to slow down. The concentration of fly brei used in these tests was adjusted to give 3.00 $\pm$ 5 percent micromoles of ACh hydrolyzed or five micromoles hydrolyzed per fly head per hour. This is in good agreement with the findings of Wolf and Smallman (28) who obtained 4 to 6.7 micromoles hydrolyzed per fly head per hour and with Metcalf and March (17) who reported an activity of 3.4 $\pm$ 0.7 micromoles hydrolyzed per fly head per hour.

Insect buffer was compared with phosphate buffer for stability and enzyme activity. Fly head homogenates prepared in phosphate buffer demonstrated a slightly higher Ch E activity than those prepared in insect buffer. In addition the phosphate buffer had a greater buffer capacity, and was found to be stable for an indefinite period of time when held under refrigeration. Insect buffer demonstrated a low buffer capacity and broke down readily even when refrigerated. Phosphate buffer was employed in all <u>in vitro</u> and <u>in vivo</u> Ch E studies.

The following procedure for *in vitro* enzyme analysis was employed. One milliliter of buffer solution was pipetted into standard 20 x 150 mm Pyrex test tubes. Seven-tenths milliliter of brei was pipetted only into the tubes employed for enzymic hydrolysis of ACh. The tubes were pre-incubated for four to five minutes in a clinical water bath set at 37° C., and then 0.1 ml of the inhibitor in C.P. acetone was added. C.P. acetone only was used in the controls. All the tubes were then replaced in the water bath and allowed to incubate for exactly 15 minutes after which 0.2 ml of substrate was added and the tubes incubated for an additional 15 minutes. The tubes were swirled approximately every five minutes during incubation. The reaction was stopped exactly 15 minutes after the addition of substrate by pipetting four milliliters of alkaline hydroxylamine into the tubes and swirling the mixture vigorously.

Following the addition of alkaline hydroxylamine an aliquot of brei equal to that used in the tubes for enzymic hydrolysis of ACh was added to the tubes employed for non-enzymic hydrolysis. After two minutes two milliliters of hydrochloric acid were added to the tubes and vigorously swirled. After one additional minute two milliliters of ferric chloride were added. The mixture was then swirled and filtered through Whatman No. 40 filter paper and transferred to a Pyrex cuvette (one

centimeter light path). The optical density was read at a wave length of 540 millimicrons 10 minutes after the addition of the ferric chloride reagent with a Beckman Model DU Spectrophotometer zeroed against a reagent blank. This reagent blank was made in the same manner as the preparation for non-enzymic hydrolysis except the order of the addition of the alkaline hydroxylamine and acid was reversed.

A standard curve for ACh concentration was prepared according to the method described by Metcalf (15) using the color development technique described above. The points used in the curve were 0.2, 0.4, 0.6, 0.8, and 1.0 micromole of ACh per milliliter final concentration after color development. Each concentration was run in duplicate five times. The mean optical density readings were plotted against the micromoles of ACh present resulting in a curve (Figure 4). This standard curve is used for the conversion of optical density values to micromoles of ACh.

The Ch E activity (micromoles of ACh hydrolyzed) was calculated by subtracting the micromoles of ACh remaining in the sample following incubation with the enzyme from the micromoles of ACh in the control sample following non-enzymic hydrolysis.

Bayer 21/199, 0-21/199, and 21/197 were tested <u>in vitro</u> with and without piperonyl butoxide at a 1:10 ratio.

Piperonyl butoxide was also tested alone as an anticholinesterase agent.

The molar concentration of the inhibitors tested was prepared in C.P. acetone. Each concentration was tested four times in duplicate for percent inhibition of house fly Ch E.

The In-50s were calculated by plotting the mean percent inhibition converted to probability units against log molar concentrations, and a straight line was fitted to the points by eye.

In vivo Cholinesterase Studies. The house flies were anesthetized with a minimum exposure to carbon dioxide and treated topically in groups of 25 with a microdrop as described under toxicity studies. Two concentrations (0.0325 and 0.045 ugm of toxicant per fly) with and without piperonyl butoxide 1:10 were employed in these tests. Only the toxicants plus piperonyl butoxide were previously found to cause significant mortality since the toxicant concentrations alone were such as to cause only four to eight percent mortality. The toxicants plus piperonyl butoxide at a 1:10 ratio were expected to cause 24 $\pm$ 15 percent mortality at 0.0325 ugm of toxicant per fly and 80 $\pm$ 8 percent mortality at 0.045 ugm of toxicant per fly.

After treatment the flies were allowed to recover from the anesthesia, and their heads harvested after a predetermined interval of time. The heads were washed in

three 50 ml beakers containing clean acetone in order to remove any unchanged material from the exterior of the head capsule. After drying at room temperature for 20 to 30 minutes the heads were homogenized in five milliliters of buffer solution. One group of 25 flies per test was treated with C.P. acetone to serve as a control.

In these studies 0.5 ml of brei plus 1.3 ml of buffer solution were pipetted into the reaction tubes. After a four to five minute pre-incubation period, 0.2 ml of substrate was added and the tubes incubated at 37° C for exactly 15 minutes. The reaction was stopped and the color developed by the same method employed in the in vitro studies. There is no method for calculating the control Ch E activity of a house fly before treatment under in vivo conditions. Therefore, percent of control activity, the relationship between the Ch E activity in the control flies and the Ch E activity in the treated flies, instead of percent Ch E inhibition was employed in the results. The control activity level was 3.00 $\pm$ 12 percent micromoles of ACh hydrolyzed. This figure is based on 14 groups of 25 flies each, the mean of all the controls employed in the in vivo tests.

Four groups of flies, 25 per group, were treated per concentration per interval of time, and their breis tested in duplicate for activity.

Bayer 21/199 and O-21/199 with and without piperonyl butoxide were tested in these studies.

RESULTS

Toxicity Studies. Bayer 21/199 and its oxygen analog were equally toxic to house flies, the LD-50 of both compounds being 0.107 ugm per fly (Figures 5 and 6).

The parent compound and its oxygen analog plus piperonyl butoxide at a 1:10 ratio exhibited only a slight variation in their respective LD-50s (Figures 5 and 6). The LD-50 for 21/199 was 0.037 ugm per fly while the LD-50 for O-21/199 was 0.039 ugm per fly. Based on the LD-50s, synergized 21/199 and its corresponding phosphate are 2.8 times more toxic to flies than the toxicants used alone.

Bayer 21/197 tested at 10 ugm per fly was nontoxic. No mortality was obtained by the joint application of piperonyl butoxide with 21/197. Piperonyl butoxide alone at 10 ugm per fly was also nontoxic to flies.

The ranges, the variances, and the standard deviations of the mortalities at the concentrations of toxicants tested are given in Tables 1 through 4.

In vitro Cholinesterase Studies. The IN-50 of Bayer 21/199 was 2.3×10^{-5} molar (Figure 7). Bayer 21/199 plus piperonyl butoxide exhibited the same IN-50 as Bayer 21/199 alone (Figure 8).

The IN-50 of the oxygen analog of Bayer 21/199 was 1×10^{-8} molar (Figure 7). The phosphate plus piperonyl butoxide was slightly less potent than the phosphate alone with an IN-50 of 8×10^{-9} (Figure 8).

When the IN-50s of the parent compound and its corresponding phosphate were compared, the phosphate with or without piperonyl butoxide exhibited 435 times more anticholinesterase activity than the parent compound. The standard deviations of the mean percent inhibition of these compounds in vitro averaged ± 5 percent.

Piperonyl butoxide, when tested at a concentration equivalent to 2.5×10^{-2} molar of Bayer 21/199, did not inhibit fly Ch E. This concentration was 25 times greater than the maximum used in combination with this toxicant.

Bayer 21/197 was found to be a very poor anticholinesterase agent for fly Ch E. The IN-50 was 6×10^{-3} molar. Higher concentrations could not be prepared because of the insolubility of this compound in acetone. Bayer 21/197 plus piperonyl butoxide in a 1:10 ratio exhibited the same percent inhibition as 21/197 alone.

In vivo Cholinesterase Studies. The results of in vivo studies with 21/199 with and without piperonyl butoxide (1:10) at 0.0325 and 0.045 ugm of toxicant per fly are presented in Tables 5 and 6 and Figures 9 and 10. At the lower dosage (Table 5, Figure 9) the Ch E activity compared with that of the control flies at the one and two hour post-treatment intervals was about the same for 21/199 with or without piperonyl butoxide. The mean knockdown was 12 percent for 21/199 plus piperonyl butoxide at the two-hour

interval while no appreciable knockdown was observed for 21/199 alone.

After two hours there was very little increase in inhibition in the flies treated with 21/199. However, when 21/199 was administered with piperonyl butoxide the Ch E inhibition continued to increase at four and six hours until at the latter time interval the percent control activity was 44 percent or about one-half that found to occur at two hours. Fifty-nine and 44 percent knockdown occurred at four and six hours, respectively, in the flies treated with the synergized toxicant. At these time intervals less than three percent of the flies treated with 21/199 alone were severely affected. Compared with the six hour interval, the flies sacrificed at 12 hours exhibited less knockdown and percent Ch E inhibition following treatment with both the synergized and unsynergized toxicant.

At 0.045 ugm per fly the joint application of piperonyl butoxide and toxicant appeared to increase the anticholinesterase activity of the toxicant even at the early intervals (Table 6, Figure 10). The minima in control activity, 48 and 28 percent, for 21/199 alone and with piperonyl butoxide, respectively, was found in the flies sacrificed at two hours. At this time maximum knockdown (9 percent) occurred with 21/199 alone. The maximum knockdown for synergized 21/199 (83 percent) was found at

both four and six hours after treatment. During this interval the Ch E activity was approximately the same as that found for two hours. However, with 21/199 alone there is a marked decrease in Ch E inhibition in the flies sacrificed at four and six hours to approximately one-half that found at two hours. Compared with the six hour interval, less knockdown and percent Ch E inhibition occurred in flies 12 hours after treatment with both the synergized and unsynergized toxicant.

Preliminary *in vivo* studies with the corresponding phosphate of 21/199 when administered to house flies gave widely varying results. The Ch E inhibition ranged from 51 to 95 percent with a standard deviation greater than $\pm$ 21 percent at the lowest concentration of O-21/199 tested (0.030 ugm per fly) at a time interval of one hour. No flies were knocked down at this concentration. With the joint administration of piperonyl butoxide, O-21/199 at 0.030 ugm per fly exhibited from 92 to 100 percent inhibition after one hour with no flies knocked down.

DISCUSSION AND CONCLUSIONS

Although O-21/199 was 435 times more potent than 21/199 as an anticholinesterase agent against house fly head Ch E *in vitro*, the two compounds were equally toxic to house flies. When piperonyl butoxide was administered jointly with either of the two compounds it did not enhance the anticholinesterase activity *in vitro*, but did increase the anticholinesterase activity markedly *in vivo*. Concurrently the toxicity of both compounds in the house fly was increased 2.8 times when administered with piperonyl butoxide.

From this data it would appear that the parent compound is either nontoxic or slightly toxic to house flies and is converted to its oxygen analog *in vivo*.

The *in vivo* and *in vitro* conversion of a phosphorothioate to its oxygen analog has been demonstrated for several pesticides (3) including Bayer 21/199 (20). Since the toxicity of both 21/199 and its oxygen analog can be enhanced by the joint action of piperonyl butoxide it would not appear that the mode of action of this synergist is associated with the oxidative metabolism of this phosphorothioate.

Further data to support the theory that 21/199 is converted to its oxygen analog was demonstrated by preliminary tests with O-21/199 *in vivo*. Results indicated that only

five percent of a nontoxic dose (0.050 ugm per fly) of O-21/199 present in either the haemolymph or the cuticle of the head was sufficient to inhibit approximately 50 percent of the enzyme Ch E *in vitro*. This amount of contamination was found to be sufficient to mask the true *in vivo* activity of the compound. However, nearly 100 times this concentration of 21/199 (0.045 ugm per fly) would have to be present in the same conditions to inhibit only 23 percent of the enzyme *in vitro*. Yet, 53 percent inhibition occurred at this dosage rate *in vivo* two hours after treatment.

The *in vivo* inhibition data of fly head Ch E (Tables 4 and 5, Figures 9 and 10) indicate that apparent rapid recovery or enzyme reversibility occurs with 21/199. The data also show that fewer flies were knocked down 12 hours after treatment than at six hours after treatment. Return of Ch E activity after inhibition by organophosphorus compounds has been demonstrated by Davison (6). This worker claims that the stability of the phosphorylated enzyme depends upon the enzyme inhibited and the organophosphorus compound used, and that there may be two types of true Ch E differing in the stability of their phosphorylated active centers. It could be argued that the apparent return of the Ch E activity in the *in vivo* tests with 21/199 with and without piperonyl butoxide was due to differences in the Ch E activity of different

groups of house flies. However, the similar results obtained with this toxicant at two concentrations in regard to inhibition recovery and knockdown recovery support the theory that enzyme recovery probably occurs after its inhibition by Bayer 21/199.

Bayer 21/197 was non-toxic to house flies and could not be synergized with piperonyl butoxide. It was a poor anticholinesterase agent and it is a competitive inhibitor for the enzyme Ch E (10). These results, therefore, are of minor importance to this paper. Only the chemical structure is important in this discussion.

Organic phosphorothioates are generally less susceptible to hydrolysis than the corresponding phosphate, the latter being better phosphorylating agents, and, therefore, more potent in vitro anticholinesterase agents (3, 16, p.275-281). Therefore, the difference between in vivo and in vitro activity of 21/199 and O-21/199 may be due to either differences in rates and routes of absorption or in their relative stability in the biological system.

The Ch E of the nervous system is the final site of action of organophosphorus compounds (1, 18). Synergism, however, does not have to occur at the site of action, but the synergist may increase the biological activity of the toxicant by competing with it for sites of loss through nonspecific absorption or by inhibiting and/or

competing with the enzyme sites which are active in the detoxification of the toxicant. This theory postulated by Veldstra (26) contains a complex, but acceptable, biochemical explanation for a possible mode of action of organophosphorus synergism. It is understood that the fate of a toxicant administered to an organism undergoes various processes of metabolism and elimination such as oxidation to the oxygen analog, phosphorylating reactions at the final site of action, hydrolysis to more polar degradation products, and excretion. When a toxicant is administered alone only a certain amount of the compound would be available to phosphorylate the Ch E enzyme at the final site of action before it was hydrolyzed or excreted. When a synergist is administered jointly with the toxicant it would be reasonable to assume that both compounds would be competing for identical sites of loss and thereby more of the toxicant would remain in the organism longer, and if necessary, undergo a hypertoxic change and ultimately more of it would phosphorylate the Ch E at the final site of action. Thus a higher mortality would result.

It could be argued that such a theory is invalid because all toxicants are not capable of being synergized. It is recognized, however, that compounds capable of synergism contain similar structures or ester linkages such as the 7-hydroxy-coumarin ring (Bayer 21/197) in Bayer compounds 21/199, 21/200, and Potasan®. Other organic

phosphorus compounds are either synergized to a lesser degree or not at all. A correlation between the structure of the synergist and the toxicant, therefore, may exist before synergism can occur. Such a correlation in regard to competition for sites of loss would explain in part why some pesticides are more readily synergized than others. However, there are no available data to support such speculation.

The inhibition of the esterase responsible for the detoxification of pyrethrum by hydrolysis of the ester linkage has been postulated as a possible mode of action of piperonyl butoxide in pyrethrum synergism (5). In studies with P^{32}-labeled 21/199 in the white mouse, Robbins et al. (20) found that joint oral administration with piperonyl butoxide in some manner prevents the enzymic hydrolysis of 21/199 to more polar degradation products. These workers advanced this theory because more of the toxicant was found in most of the tissues studied when the compound was administered jointly with piperonyl butoxide. The radioactive compounds found in mice treated with 21/199 alone were mainly more polar degradation products. This might also be explained by the possibility that the toxicant was competing with the synergist for sites of loss in the mice treated with 21/199 plus piperonyl butoxide, but was more easily metabolized by mice treated with 21/199 alone.

From the foregoing discussion on the results with 21/199 and O-21/199 in the house fly it might be concluded that the mode of action of synergized Bayer 21/199 includes the following mechanisms.

Bayer 21/199 appears to be converted to its oxygen analog *in vivo* in the house fly by a natural biochemical response of the organism to this pesticide. Piperonyl butoxide does not enhance the toxicity of 21/199 by association with the oxidative metabolism of this toxicant. Piperonyl butoxide, however, probably does enhance the toxicity of 21/199 by very complex biochemical reactions involving competition for sites of loss by the compounds *in vivo*. This would increase the toxicity of the compound because more of the toxicant would be available to inhibit the enzyme Ch E through phosphorylation. Structural similarities in organophosphorus compounds that can be readily synergized are probably a clue to the mode of action of phosphate synergism. It may be possible that such a correlation between the structure of the synergist and the toxicant in regard to competition for sites of loss exists before synergism can occur.

Cholinesterase enzyme recovery or reversibility probably occurs in house flies treated with 21/199 with or without piperonyl butoxide. Similarly, an apparent recovery of flies knocked down after treatment also occurred. This appears to be a natural detoxification mechanism peculiar to

this pesticide *in vivo* in the house fly, since it occurred with or without the joint application of piperonyl butoxide.

Table 1.--The range, variance, and standard deviation of the mortalities at the concentrations of 0-21/199 tested against house flies.

Dosage ug/ul	Mean percent mortality	Percent range	Variance	Standard deviation
0.075	22	12 - 40	68	8
0.1	44	12 - 76	251	16
0.125	55	32 - 72	177	13
0.150	82	68 - 88	44	7
0.175	86	48 - 96	233	15
0.2	91	76 - 100	63	8

Table 2.--The range, variance, and standard deviation of the mortalities at the concentrations of 21/199 tested against house flies.

Dosage ug/ul	Mean percent mortality	Percent range	Variance	Standard deviation
0.075	23	12 - 32	95	10
0.1	43	12 - 56	138	12
0.125	61	36 - 80	247	16
0.15	72	58 - 84	108	10
0.175	85	68 - 100	54	7
0.2	86	72 - 100	132	12
0.25	94	72 - 100	112	11

Table 3.--The range, variance, and standard deviation of the mortalities at the concentrations of 0-21/199 plus piperonyl butoxide 1:10 tested against house flies.

Dosage ug/ul	Mean percent mortality	Percent range	Variance	Standard deviation
0.030	26	12 - 40	96	10
0.040	48	24 - 64	224	15
0.050	76	40 - 100	493	22
0.075	96	80 - 100	352	19

Table 4.--The range, variance and standard deviation of the mortalities at the concentrations of 21/199 plus piperonyl butoxide 1:10 tested against house flies.

Dosage ug/ul	Mean percent mortality	Percent range	Variance	Standard deviation
0.025	6	0 - 12	17	4
0.0325	24	4 - 48	222	15
0.040	56	36 - 76	157	13
0.045	81	64 - 88	67	8
0.05	91	72 - 100	182	14

Table 5.--Percent knockdown and control Ch E activity at various intervals following application of 0.0325 ugm of 21/199 per fly with and without piperonyl butoxide (PB) 1:10.

Time in hours	Mean percent ± Standard Deviation			
	21/199		21/199 plus PB 1:10	
	Knockdown	Control activity	Knockdown	Control activity
1	0	80 ± 7	0	84 ± 7
2	2 ± 2	73 ± 7	12 ± 3	74 ± 1
4	3 ± 3	87 ± 4	59 ± 4	59 ± 8
6	2 ± 3	67 ± 5	44 ± 5	44 ± 10
12	0	81 ± 3	27 ± 13	60 ± 3

Table 6.--Percent knockdown and control Ch E activity at various intervals following application of 0.045 ugm of 21/199 per fly with and without piperonyl butoxide (PB) 1:10.

Time in hours	Mean percent ± standard deviation			
	21/199		21/199 plus PB 1:10	
	Knockdown	Control activity	Knockdown	Control activity
1	1 ± 2	76 ± 10	2 ± 2	66 ± 9
2	9 ± 3	48 ± 5	44 ± 3	28 ± 17
4	2 ± 3	73 ± 6	83 ± 4	28 ± 6
6	5 ± 2	66 ± 9	82 ± 7	29 ± 3
12	2 ± 3	79 ± 8	78 ± 2	43 ± 12

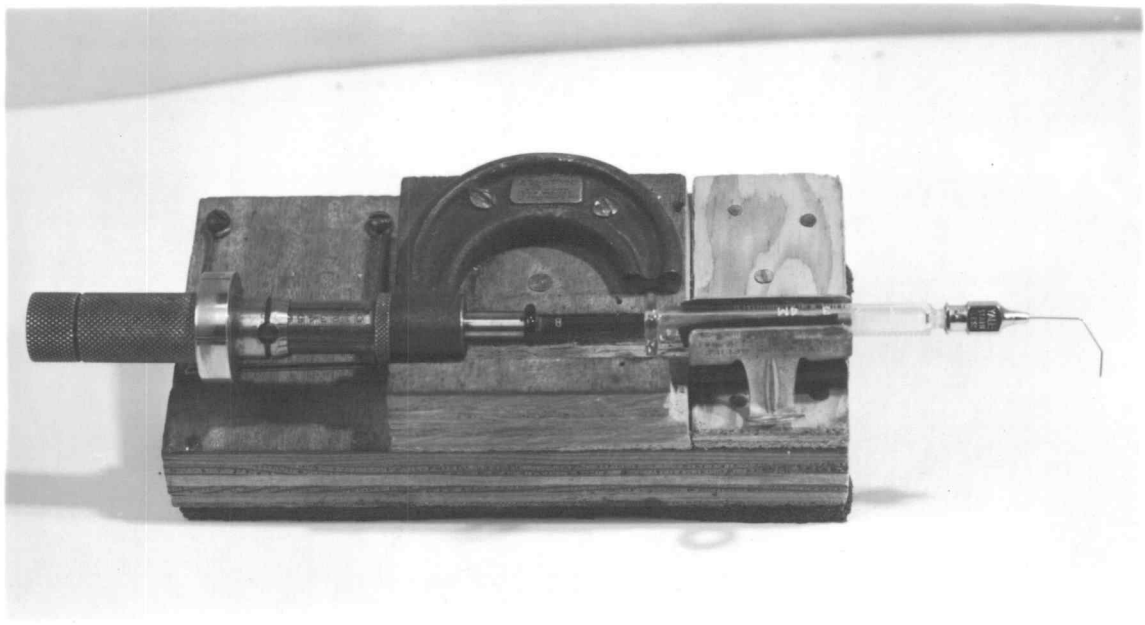

Figure 1. Microdrop apparatus

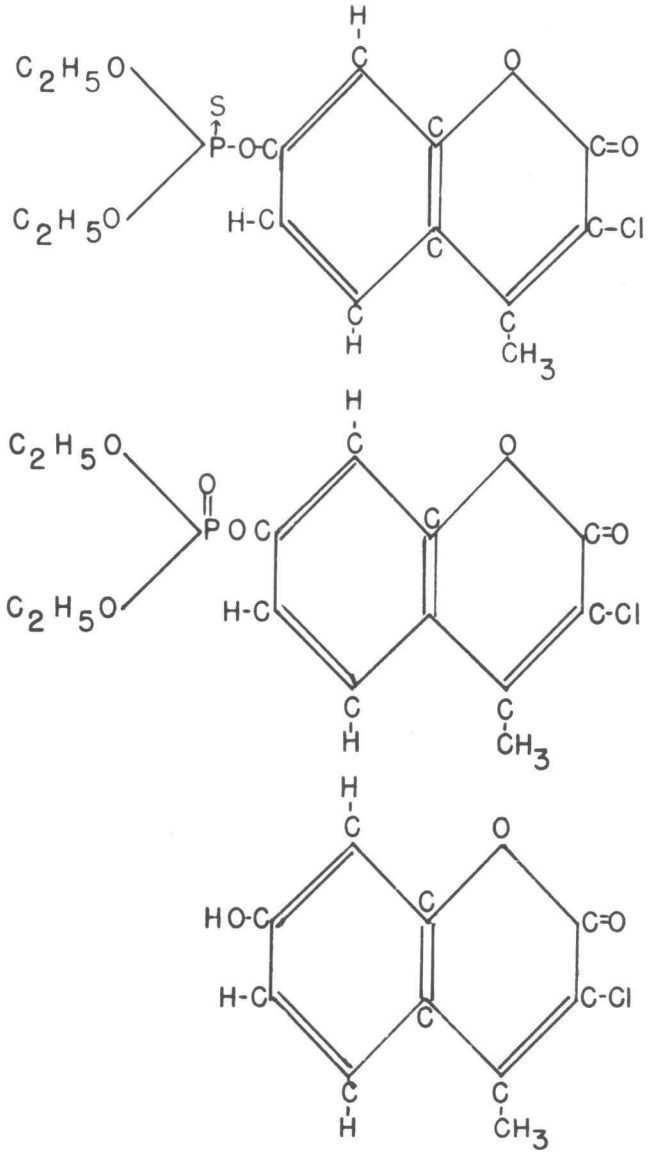

Figure 2. The chemical structures of Bayer 21/199, O-21/199, and 21/197

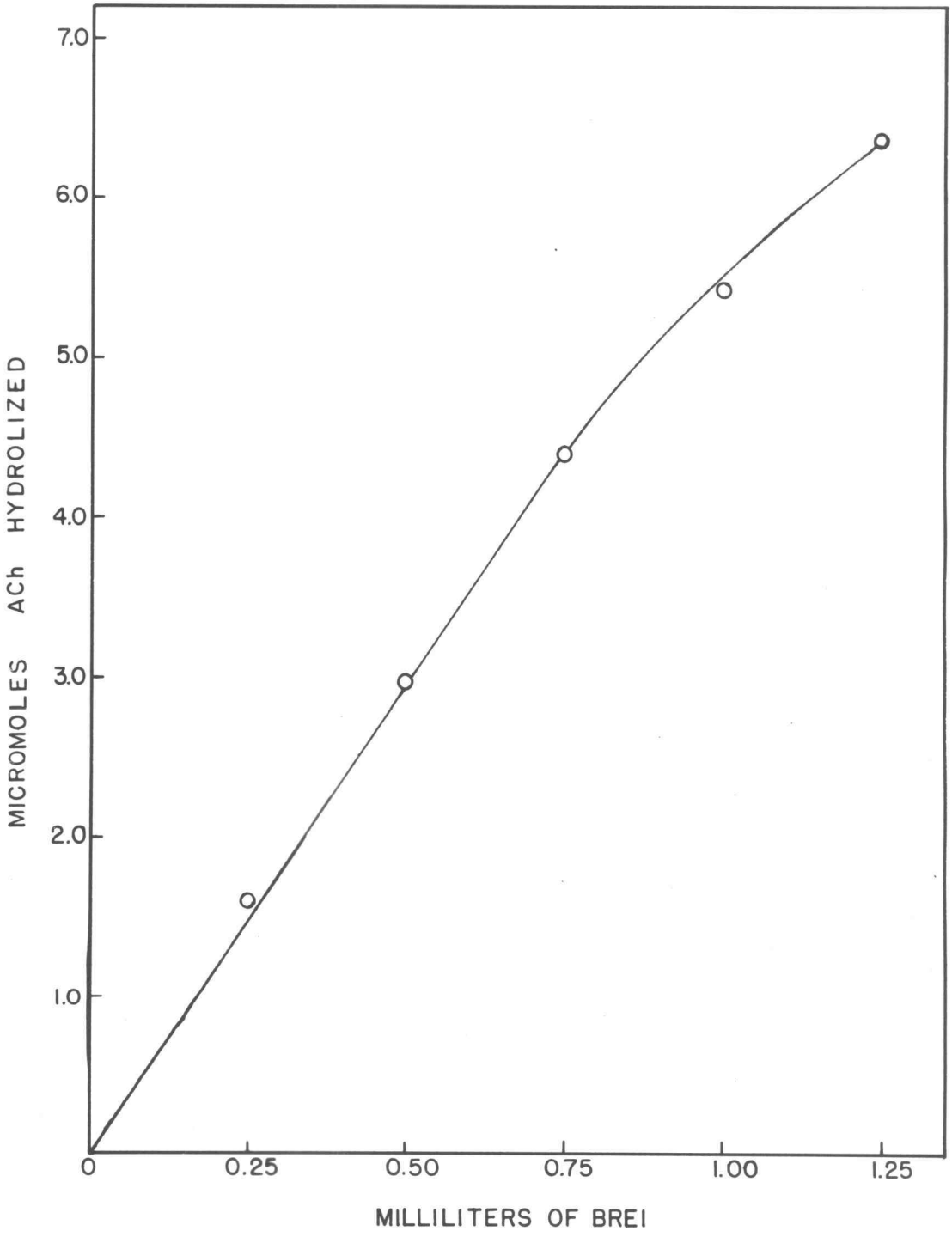

Figure 3. Relation of Ch E activity to quantity of fly head brei employed

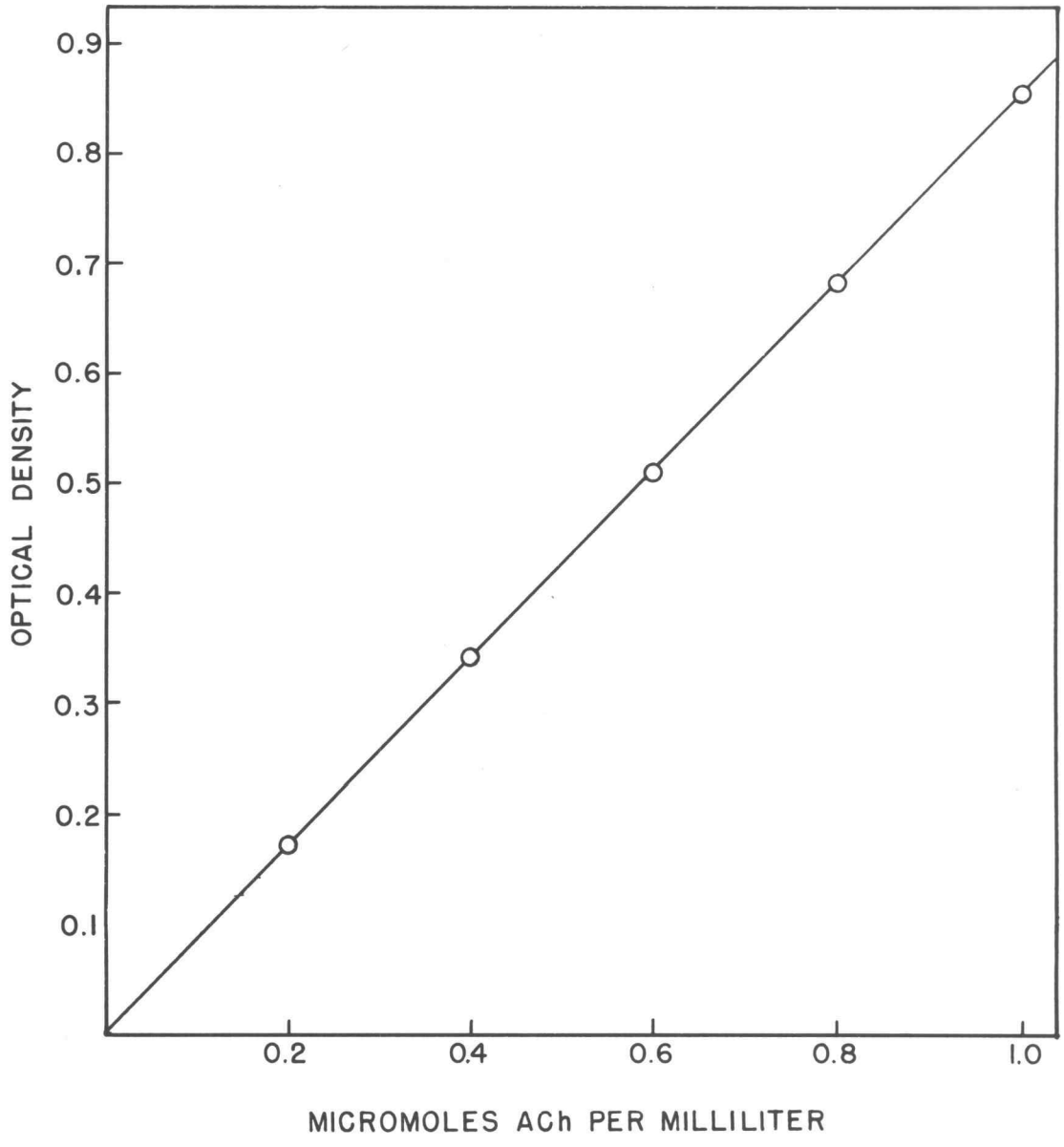

Figure 4. The density of color as a function of acetylcholine concentration

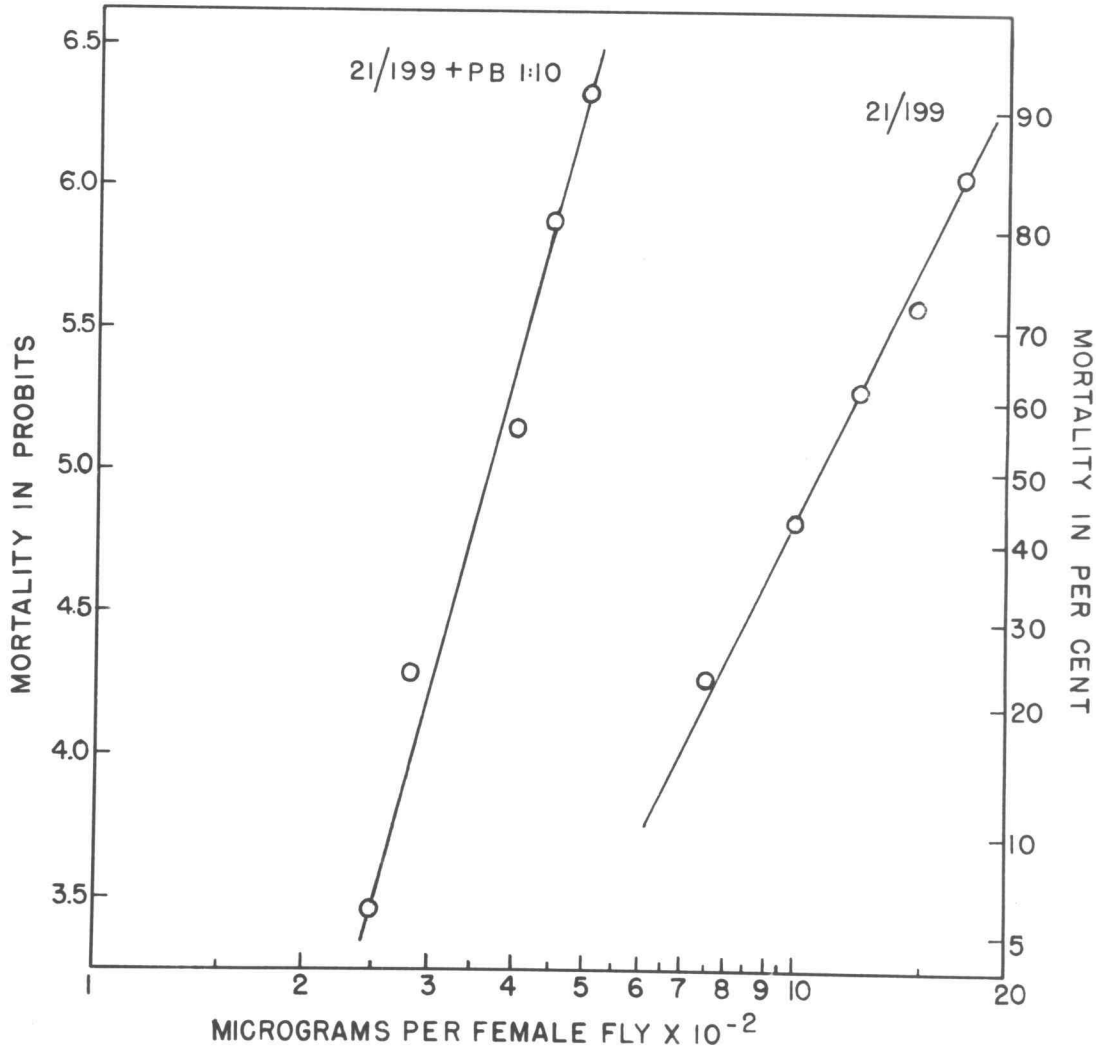

Figure 5. The toxicity of 21/199 with and without piperonyl butoxide 1:10 to house flies

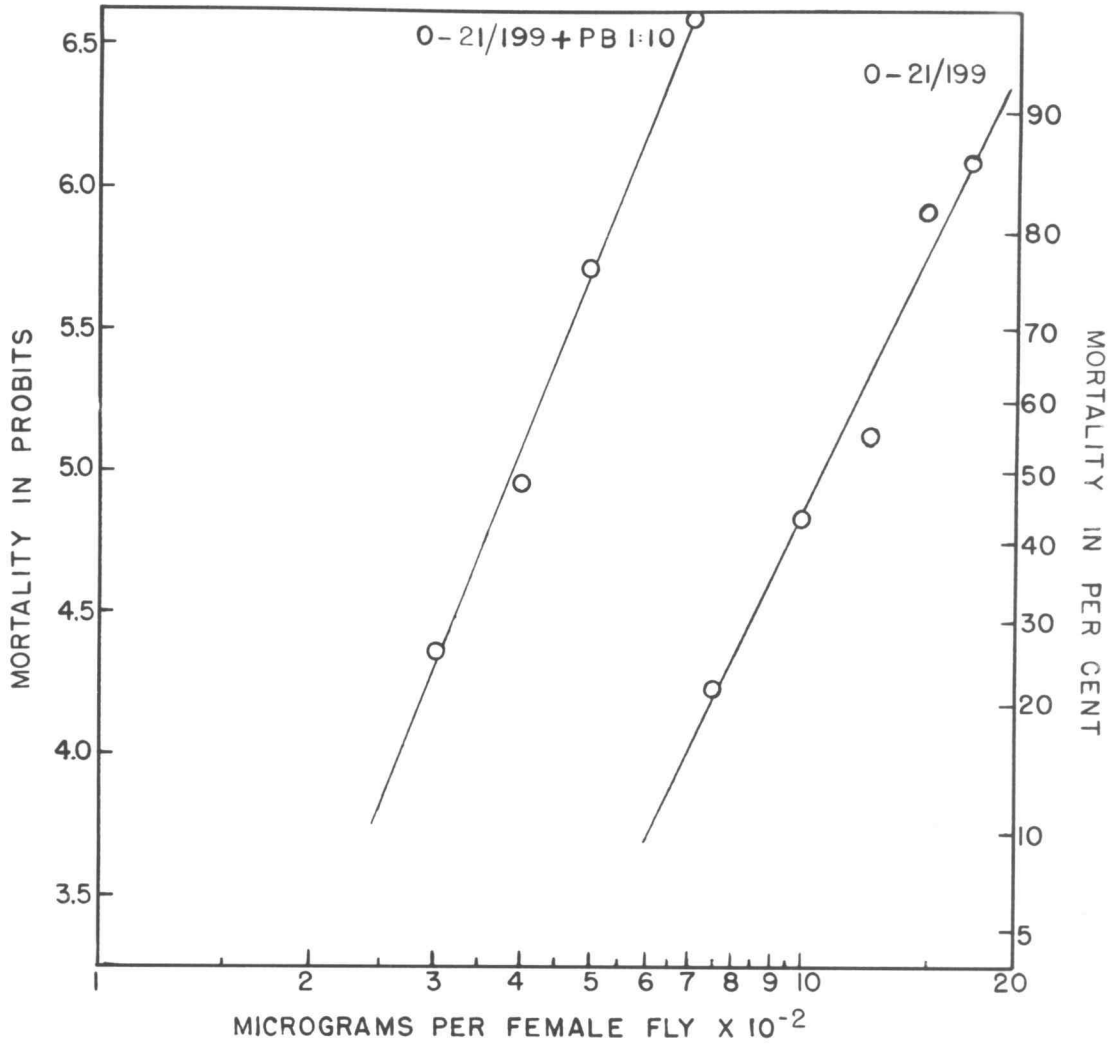

Figure 6. The toxicity of 0-21/199 with and without piperonyl butoxide 1:10 to house flies

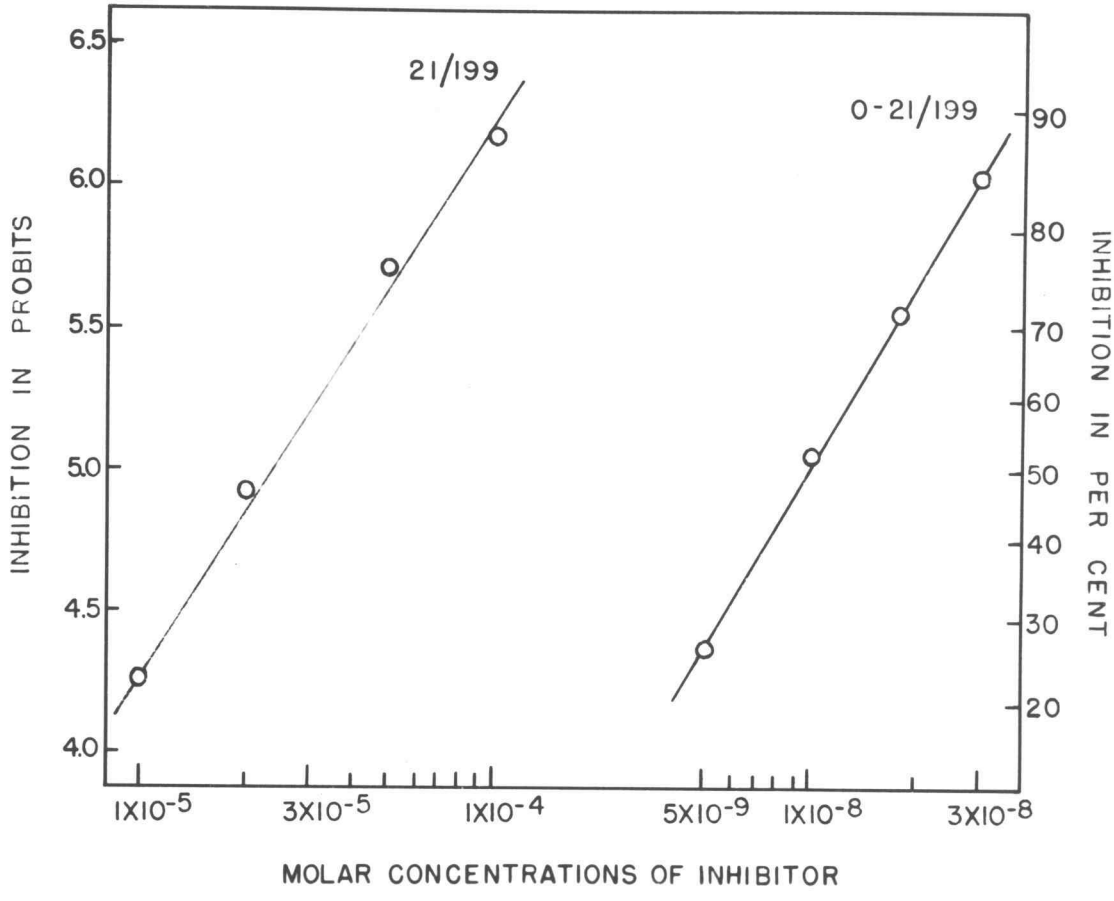

Figure 7. In vitro inhibition of fly head Ch E by 21/199 and 0-21/199

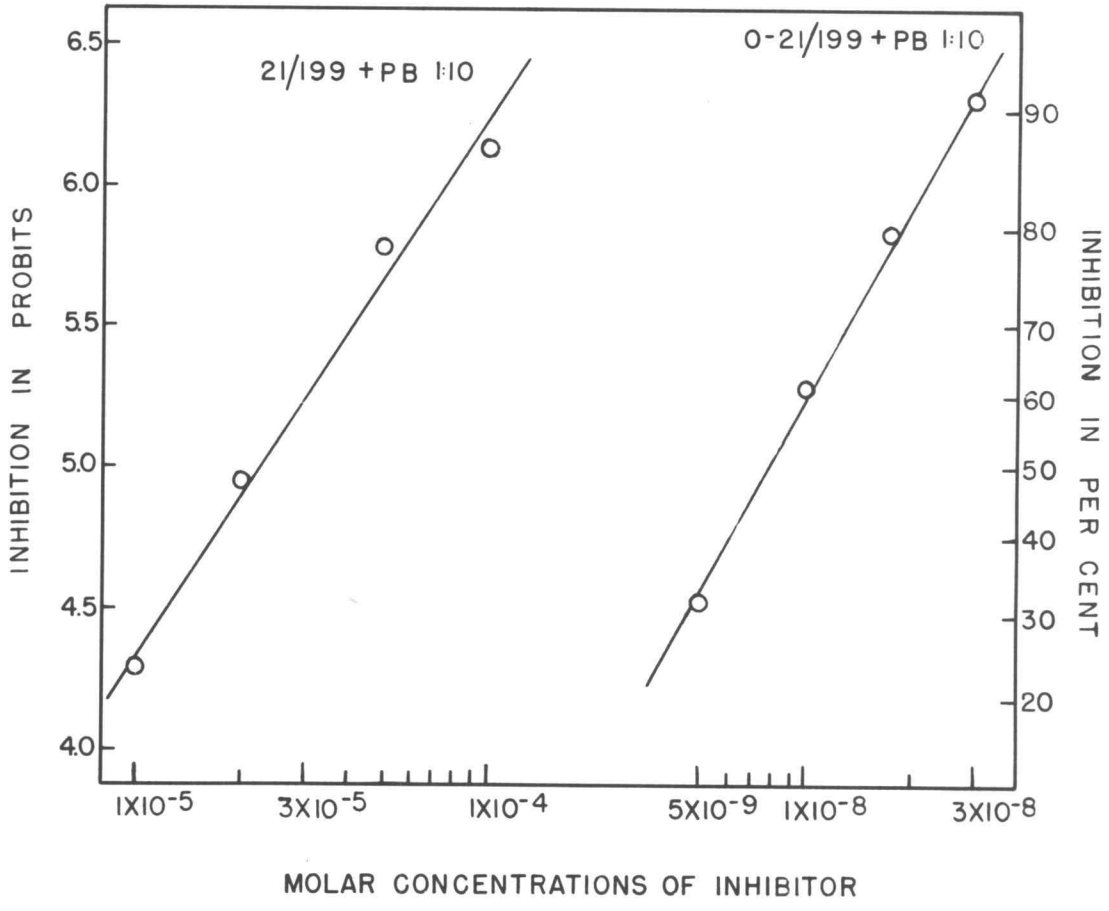

Figure 8. <u>In vitro</u> inhibition of fly head Ch E by 21/199 and O-21/199 plus piperonyl butoxide 1:10

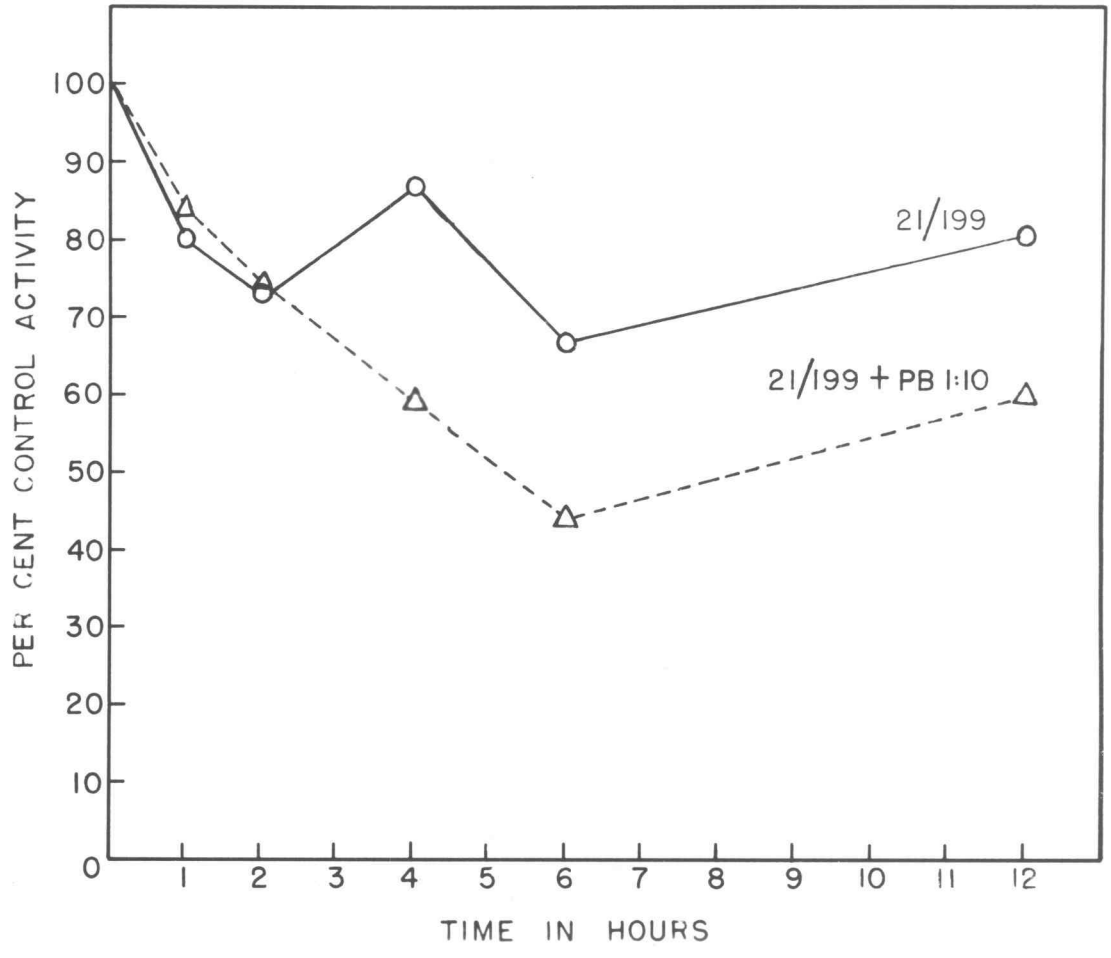

Figure 9. *In vivo* inhibition of fly head Ch E by 0.0325 ugm of 21/199 per fly with and without piperonyl butoxide 1:10

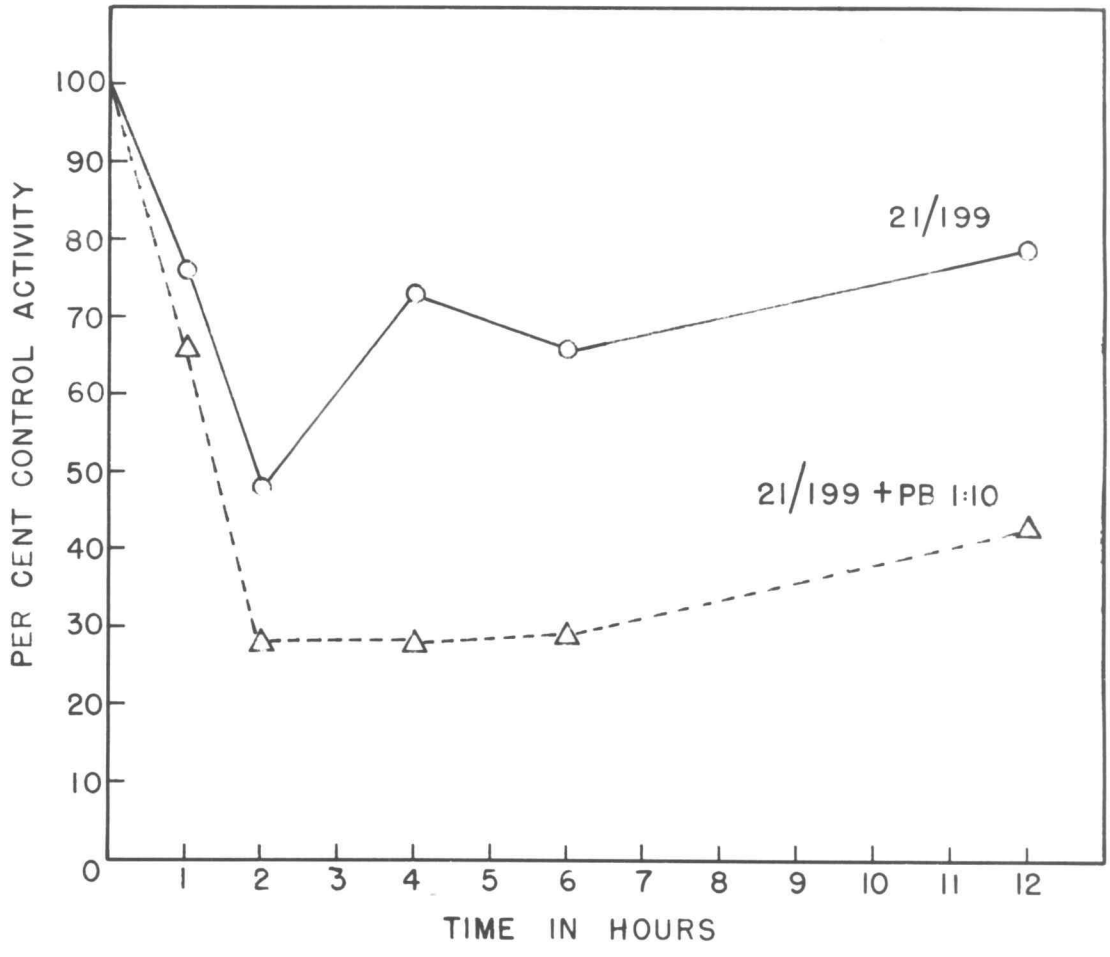

Figure 10. In vivo inhibition of fly head Ch E by 0.045 ugm of 21/199 per fly with and without piperonyl butoxide 1:10

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