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Ruelene was orally administered to three female sheep at a dosage rate of 121.5 mg/kg. The cholinesterase (ChE) activity levels of blood plasma, red blood cells (RBC), and mosquitoes (Aedes aegypti) which had fed on the sheep were tested at various times before and after dosage. Maximum depression of the sheep blood ChE activity occurred at the fourth hour after dosage, while the major depression of mosquito ChE activity occurred at the fifth hour. Mosquito death occurred first at an average of 35% mosquito ChE activity inhibition and 100% mortality was observed at an average of 56% inhibition.

Mortality in Dermacentor andersoni was also tested. Groups of ticks which had fed on the treated sheep for seven hours or more after dosage sustained mortalities of 80-100%.

Some Toxicological Aspects of Ruelene, an Organophosophate, used Systemically to Control Ectoparasites

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SOME TOXICOLOGICAL ASPECTS OF RUELENE,* AN ORGANOPHOSPHATE, USED SYSTEMICALLY TO CONTROL ECTOPARASITES

INTRODUCTION

Parasites cause an inestimable loss of meat production each year from our valuable farm animals (Metcalf, Flint, and Metcalf, 1962). Endoparasites live on the host's digested food or on the host tissue, causing damage by decreasing the host's food supply, excreting toxic substances into the body and, in some cases, reducing the protein supplies of the host while increasing the protein requirements (Noble and Noble, 1964). Bloodsucking ectoparasites, such as ticks and mosquitoes, may cause losses in meat production not only by feeding on the host's blood, but also by irritating the host, causing the host to become hyperactive, and thus preventing it from efficient utilization of food. Therefore, a combination of ectoparasite and endoparasite control might significantly decrease the cost of meat production.

In attempts to control these parasites, man has resorted to the use of a multitude of pesticides. These pesticides have been formulated in many different ways such as dusts, wettable or dispersable powders, emulsive concentrates, solutions, and drenches.

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*4-tert-butyl-2-chlorophenyl methyl methylphosphoramidate. A product of Dow Chemical Company, Midland, Michigan.

The methods of application have also been varied: 1) sprays, dips, and dusts for ectoparasites, 2) oral applications -- drenches, capsules, boluses, or additives in feed concentrates--for endoparasites and some ectoparasites, and 3) topical application of organophosphates for systemic action against endoparasites, especially Hypoderma spp. Organophosphates are desirable as systemics because 1) they pose little residue problem in tissue and 2) they are rapidly absorbed through the skin and membranes of the animal. Annison, Hill and Lewis (1957) report that acetate base substances are absorbed from the rumen one and one half to two and one half hours after ingestion. As they are currently used, organophosphate systemics are not particularly practical in the control of ectoparasites because in many cases the organophosphates are completely and rapidly degraded by the animal, sometimes within a few hours. With this in mind, a desirable dosage system would be one that effected a prolonged, controlled release of the pesticide into the blood stream of the host. Perhaps this could be done in much the same manner as beef producers now implant female hormone in their beef animals. A recent study by Clifford, Yunker and Corwin (1967) indicates that this approach may have some promise.

Before a dosage system can be worked out, it is first necessary to see if a kill of ectoparasites can be effected by a systemically acting organophosphate. Some organophosphates have already been

established as dual control pesticides in that they killed both gastrointestinal parasites and Hypoderma spp. larvae in the dermis. Oral treatment was effective in both cases; topical application was less effective on gastrointestinal parasites than was oral application, although they were equally effective in control of Hypoderma larvae (Herlich, Porter, and Isenstein, 1961). If systemically acting pesticides could be made more effective against ectoparasites and accomplish a triple kill of parasites, our livestock industry would be materially aided.

Besides the purely practical side of this problem, the method of kill of these systemic organophosphates is another worthwhile consideration. In recent years, there seems to be some doubt as to whether organophosphates act by inhibiting cholinesterase (ChE) or some other enzyme (Chadwick, 1963). Any information along these lines would also prove helpful in making systemic control of ectoparasites feasible.

The purpose of this research was to study some of the toxicological principles of an organophosphate, Ruelene, acting systemically in the host-ectoparasite system. These aspects encompassed:

(1) The inhibition, by the pesticide, of cholinesterase (ChE) in the blood of the host, a sheep, which included analysis of blood plasma and red blood cell (RBC) fractions.

(2) The inhibition of ChE[1] in an arthropod ectoparasite, Aedes aegypti Meigen, a mosquito, feeding on the treated host.

(3) The correlation of the percent ChE activity inhibition in the mosquito with mortality.

(4) The correlation of mortality in the Rocky Mountain wood tick, Dermacentor andersoni Stiles, with ChE activity inhibition in the blood of the host.

(5) The time of first depression and the times of major depressions in ChE activity in both the homogenate of mosquito tissues and in the fractions of the host's blood.

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

LITERATURE REVIEW

Much work has been done using various formulations of Ruelene for the control of endoparasites. As a drench, wettable powder, and orally administered in polymer systems, Ruelene has proven to be effective against many gastrointestinal nematodes of sheep such as Haemonchus, Trichostrongylus, Ostertagia, Trichuris, Cooperia, Strongyloides, (Timmerman, 1963); Oesophogostomum (Dow Chemical 1959, 1960); Nematodirus (Douglas and Baker, 1959). In cattle, Ruelene, as a spray acting systemically, has proven very effective in killing cattle grubs, Hypoderma lineatum and H. bovis, presumably in the migrating stage (Kohler and Rogoff, 1961; McGregor et al., 1959; Dow Chemical, 1959, 1960). Periodically, unfavorable side effects have occurred, even after suggested dosage levels of Ruelene were used. However, the severity of the side reactions has not corresponded to the quantity of the compound administered (Dow Chemical, 1959, 1960). In work with cattle, it was demonstrated that these side reactions occurred if the Hypoderma larvae were killed when they were present in the neural canal of the host (McGregor et al., 1959). McGregor's experiment indicated that the time of application of Ruelene for control of cattle grubs was very important.

No work has been published concerning Ruelene, acting systemi-

cally, to control ectoparasites. McGregor et al. (1959) observed that spraying with 0.5% Ruelene controlled hog, goat, and cattle lice, horn flies, and screw worms. Studies on the mortality of Aedes aegypti and Stomoxys calcitrans feeding on Ruelene dosed cattle were attempted by Roth (personal communication) but were unsuccessful. The ectoparasites would not feed, due to insufficient control of environmental conditions. No work on control of D. andersoni with organophosphates has been published, but reports (Cooley, 1932; Herms and James, 1961) on their normal feeding, copulation, and preoviposition behavior have proved useful in determining any behavior changes due to Ruelene.

Presently a difference of opinion exists as to the mode of action of Ruelene, as well as other organophosphates, in mammals and insects. Inhibition of an aliesterase has been suggested by many authors as an important cause of death (Chadwick, 1963). Early work by Van Asperan (1959) showed that although house flies treated with organophosphates showed 20-50% cholinesterase inhibition at knockdown, the aliesterase was inhibited 80-90%. He stated that no conclusions could be drawn until the function of this aliesterase was known. O'Brien (1961) found that although aliesterases were inhibited more than cholinesterase in insects receiving sublethal doses of organophosphates, aliesterases were inhibited less than cholinesterase in insects killed by the organophosphates. Van

Asperan (1960) found that house flies resistant to organophosphates had low aliesterase activity levels. Stegwee (1960) proved, with the use of triorthocresyl phosphate, considered to be nontoxic to insects, that aliesterases could be inhibited 100% in the house fly with no effect. Organophosphate poisoning symptoms occurred only after significant ChE inhibition by the pesticide. As Chadwick (1963) pointed out, aliesterases apparently are not the enzymes inhibited by organophosphates because they are not produced in the insect nervous system. Steward (1967) showed by histochemical procedures that Ruelene applied to cattle grubs in vitro caused cholinesterase inhibition. O'Brien (1961, p. 1164) suggested a method for determining positively if organophosphates are inhibitors of ChE:

> . . . If it could be shown with a variety of organophosphates that death always occurs when the ChE of some particular tissue is 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.

Some of the differences of opinion as to the function of organophosphates 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 the following 1) Warburg manometric methods, which required a considerable amount of tissue and limited the type of buffer which could be

used, 2) titrimetric methods, which were very time consuming and inaccurate if an indicator rather than a potentiometer were used, 3) colorimetric methods (indicator pH method), in which other substances emitting the same wave length could have interfered, 4) electrometric methods, in which activity was measured by the change in pH and in which one of the most common difficulties was the instability of the pH meter at intervals of one minute or less, 5) photometric methods, in which activity was measured by change in absorbance of an acid-base indicator.

Reed et al. (1966) perfected a direct radioisotopic method for assay of ChE that is rapid and accurate with mμ mole quantities of acetylcholine (ACh) under varying pH, wide range of substrate concentrations, and various concentrations of the enzyme source. Frady and Knapp (1967) modified Reed's method slightly, thereby increasing the speed and efficiency with which analysis of ChE activity could be made in this study. Earlier authors (Radeleff and Woodard, 1956), using the electrometric method, found little or no ChE activity in the plasma fraction of sheep blood. Using the modified radioisotopic method, plasma fractions of sheep blood were found to contain approximately one tenth the ChE activity of the RBC fraction.

Normal blood ChE levels have been assayed on cattle (Hermenze and Goodwin, 1959) and on sheep and cattle (Radeleff and Woodard,

1956) using the electrometric test. Although acetylcholinesterase (AChE) activity was detected, no plasma ChE activity was shown by this method. This has led to the use of whole blood rather than blood fractions in determining percent ChE activity inhibition (Rogoff et al., 1967). The effects of Ruelene on blood of cattle have been tested by Eddy et al. (1959) and by Rogoff et al. (1967) on a 14 and 28 day basis. The whole blood ChE activity, of the animals used in both investigations, was depressed several days after dosage; the depressions could not be explained by the investigators.

Use of whole blood for analysis may be misleading, as the red blood cells contain AChE which is bound to the cell membrane; the plasma ChE is pseudocholinesterase (Witter, 1963). For an inhibitor to act upon RBC AChE, it must penetrate and break the bonding between the AChE molecule and the RBC membrane (O'Brien, 1963). No such limiting factor has been found for plasma ChE activity inhibition.

Ganelin (1964) measured RBC AChE and plasma ChE activity levels in human blood. He concluded that there was a variation of blood ChE activity levels among individuals. The individual must serve as his own control to demonstrate ChE depression after exposure to an inhibitor. Therefore each sheep used in this study served as its own control and base levels of ChE activity of the blood were determined prior to dosage.

MATERIALS AND METHODS

During three experiments, technical grade Ruelene* (99% pure, M. P. 56°C) was orally administered to three female sheep in #11 gelatin capsules at a dosage rate of 121.5 mg/kg. The sheep used as hosts in this study were Cheviot-Suffolk crosses, 8-10 months old. Five ml of blood were withdrawn from the jugular vein at 48, 24, 3, 2, and 1 hours prior to dosage and hourly thereafter for a period of 14 hours. Blood was then withdrawn at various times over a period of 82 hours.

To facilitate separate assays of plasma ChE and RBC AChE, portions of the blood samples were placed in heparinized hematocrit tubes (Inside Diameter 1.1-1.2 mm) and centrifuged for eight minutes at 1600 rpm with a Phillips-Drucker L-708 combination centrifuge, using a micro-hematocrit head, L-779F.

Two insect cages, constructed from 1 1/2 inch seamless Teflon tubing, were sewn directly to a shorn spot on each sheep's back. One cage was used for ticks, Dermacentor andersoni, and the other for mosquitoes, Aedes aegypti. The ticks were obtained from the U. S. Public Health Service Laboratories, Hamilton, Montana, the mosquitoes from the Entomological Research Division, U. S. D. A., Corvallis, Oregon. The arthropods were weighed

*Donated by Dow Chemical Company, Midland, Michigan.

individually before and after feeding.

One hundred ticks (60 females and 40 males) were placed in one of the Teflon cages 48 hours prior to dosage of the sheep. They were removed, five at a time, (three females and two males) at various times during the span of the experiment and were placed in stoppered glass vials for mortality observations. Ticks were handled the same in all experiments.

Mosquitoes were first removed by aspiration from a large rearing cage, then taken to a cold storage room, temperature -4°C, where they became immobile in 45 seconds. They were then taken into a cold room, temperature 4°C, where samples of 25 females and 10 males were transferred to small cages made from #15 plastic vials. The samples were then placed in an environment maintained at 30°C temperature and 60% relative humidity.

In experiment 1, samples of mosquitoes were blown from the small cages into the cell on the sheep's back through a hole in the screened lid. They were allowed to feed for 15 minutes, lightly anesthetized with CO_2, and then transferred back into the small cages where they were allowed to recover. The application and removal of the samples were performed every 15 minutes during the span of the experiment. Temperature was maintained at 30°C by suspending an infrared lamp 18 inches above the sheep's back. Every other sample of mosquitoes was quick-frozen and later

analyzed for ChE activity inhibition, while the alternating samples were kept in their plastic vials at room temperature and observed for mortality.

The mosquitoes in experiment 2 were handled as in experiment 1 except that (a) they were placed in small cages which were held against the sheep's back, allowing the mosquitoes to feed directly through the screened ends of the small plastic cages, and (b) they were not anesthetized with CO_2.

Experiment 3 was a duplicate of experiment 2 with two exceptions: 1) the samples of mosquitoes consisted of 50 females instead of 25, and 2) the small cages were made of two screened #4 Mason jar lids taped together.

ChE Analysis

Analysis for ChE activity inhibition was identical in all the experiments.

Blood plasma: A 1.9 cm section representing 20 µl was cut from the plasma fraction of the centrifuged hematocrit tube. The contents of the cut section were added to two ml of phosphate buffer, pH 7.2 (Appendix I) to form a 1:100 v/v dilution. Fifty µl of this dilution were added to fifty µl of substrate consisting of 0.050 µ moles of acetyl choline bromide diluted with buffer of pH 7.2 and 0.005 µmoles of acetyl-1-C^{14}-choline iodide with an activity of 0.025 µC

per assay. Analysis was made according to Reed et al. (1966), utilizing the 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 water bath at 37°C for ten minutes. Reactions were then stopped by 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 then added, and the tubes shaken vigorously. The tubes were 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 counted with a liquid scintillation spectrometer (Packard Tri-Carb., Model 314 EX). Boiled enzyme was used to provide paired control samples; the blank sample readings provided a correction for the activity due to background and non enzymatic hydrolysis of the substrate.

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

Mosquitoes: Weighed, single, whole mosquitoes that had not fed, or that had fed on an untreated sheep, or that had fed on a treated sheep were individually placed in phosphate buffer that had been volumetrically adjusted to give a 1:100 w/v dilution. The buffer

and mosquito were then placed in a 10.0 ml Potter-Elvehjem tissue grinder and homogenized with a Teflon pestle for 10-15 minutes in an ice-water bath.

Using a 100 µl syringe, 50 µl samples were withdrawn from the 1:100 w/v dilution and added to 50 µl of substrate. Subsequent procedures were the same as those used with the blood plasma fractions.

RESULTS AND DISCUSSION

Rate of Inhibition of Host Blood ChE Activity Compared With Rate of Inhibition of Mosquito ChE Activity and Mortality

There was a slight depression of ChE activity in both the RBC and plasma fractions of the host's blood within one hour after oral dosage with Ruelene (Fig. 1-3). In the RBC fraction, the AChE activity tended to increase after the initial depression. The depression of the AChE activity level in experiment 1 was more pronounced than in experiments 2 and 3, but the per cent recovery in experiment 1 was not as great as in the other two. In experiments 2 and 3, the plasma ChE activity tended to stabilize at the end of the second hour but in no case did it tend to recover as it did in the RBC fraction.

The gelatin capsules used as vehicles for the pesticide dissolved in the presence of sheep rumen contents within 15 minutes. The normal digestive processes and initial assimilation in the sheep rumen require 1.5-2.5 hours from the time of ingestion of food (Annison et al., 1957). This would suggest that fluctuations of ChE activity in the host's blood prior to the second hour were not due to Ruelene or its metabolites but to normal metabolic processes of the host. The earlier depression of the plasma ChE activity level in experiment 1 (Fig. 1) indicated that absorption of Ruelene into

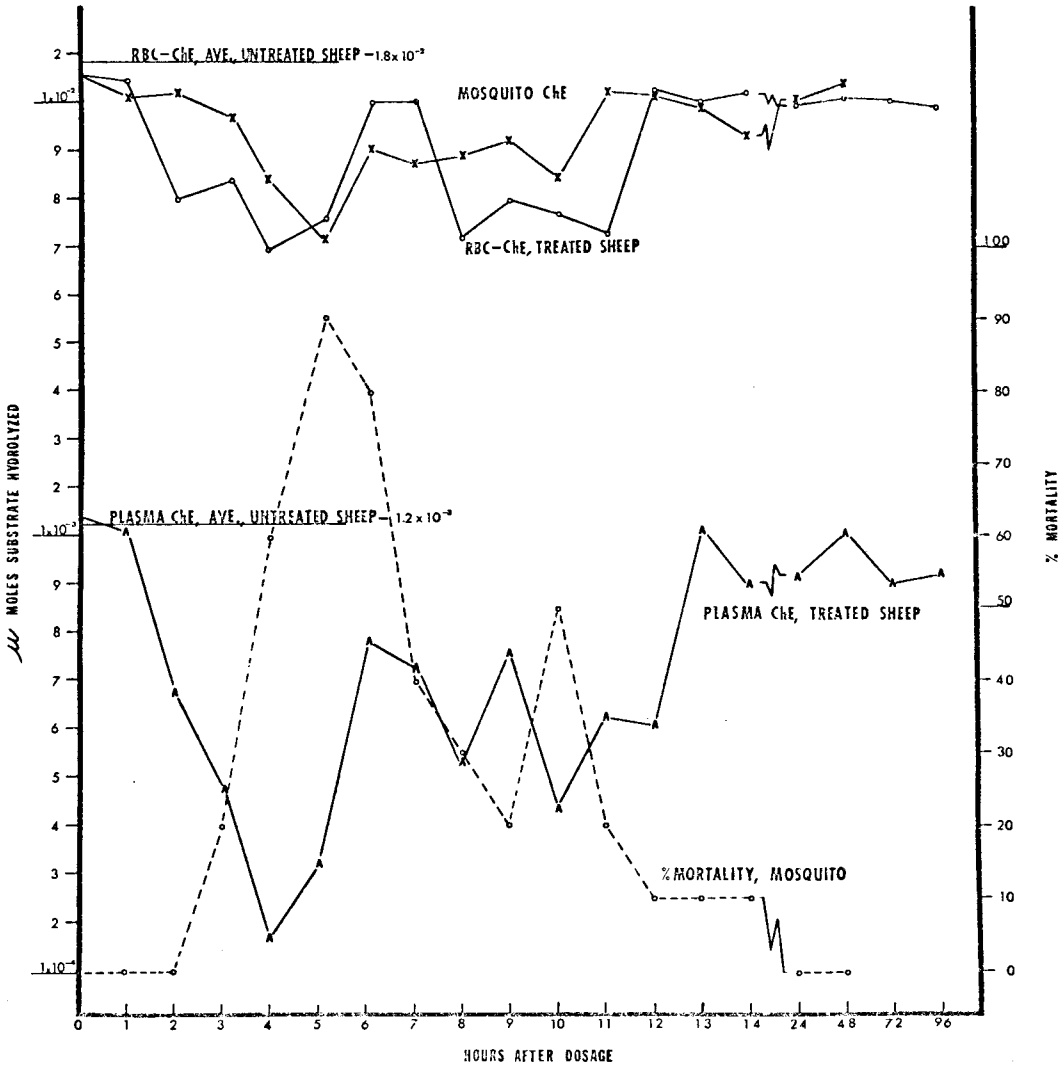

Fig 1— Experiment 1 Effects of orally administered Ruelene upon sheep blood ChE activity levels, mosquito ChE activity levels, and mosquito mortality.

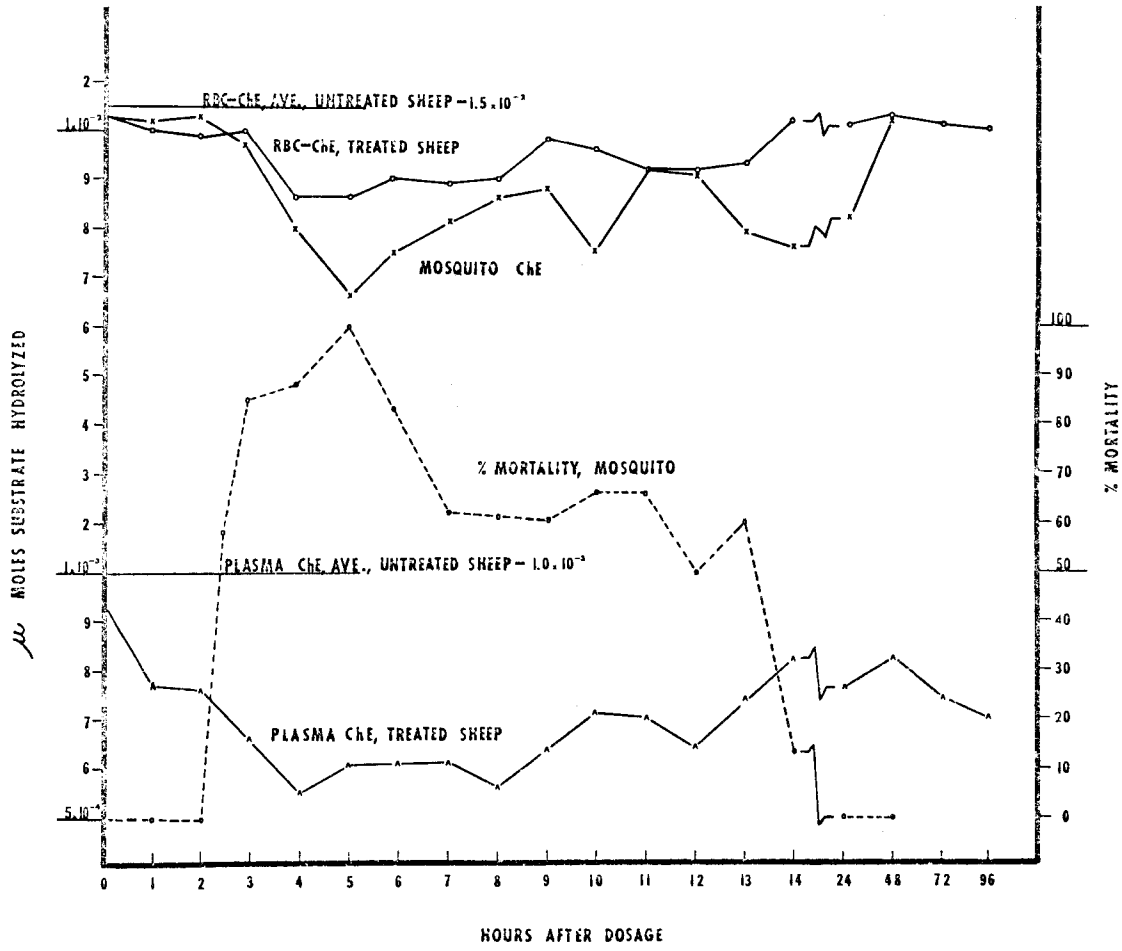

FIG. 2 – Experiment 2 Effects of orally administered Ruelene upon sheep blood ChE activity levels, mosquito ChE activity levels, and mosquito mortality.

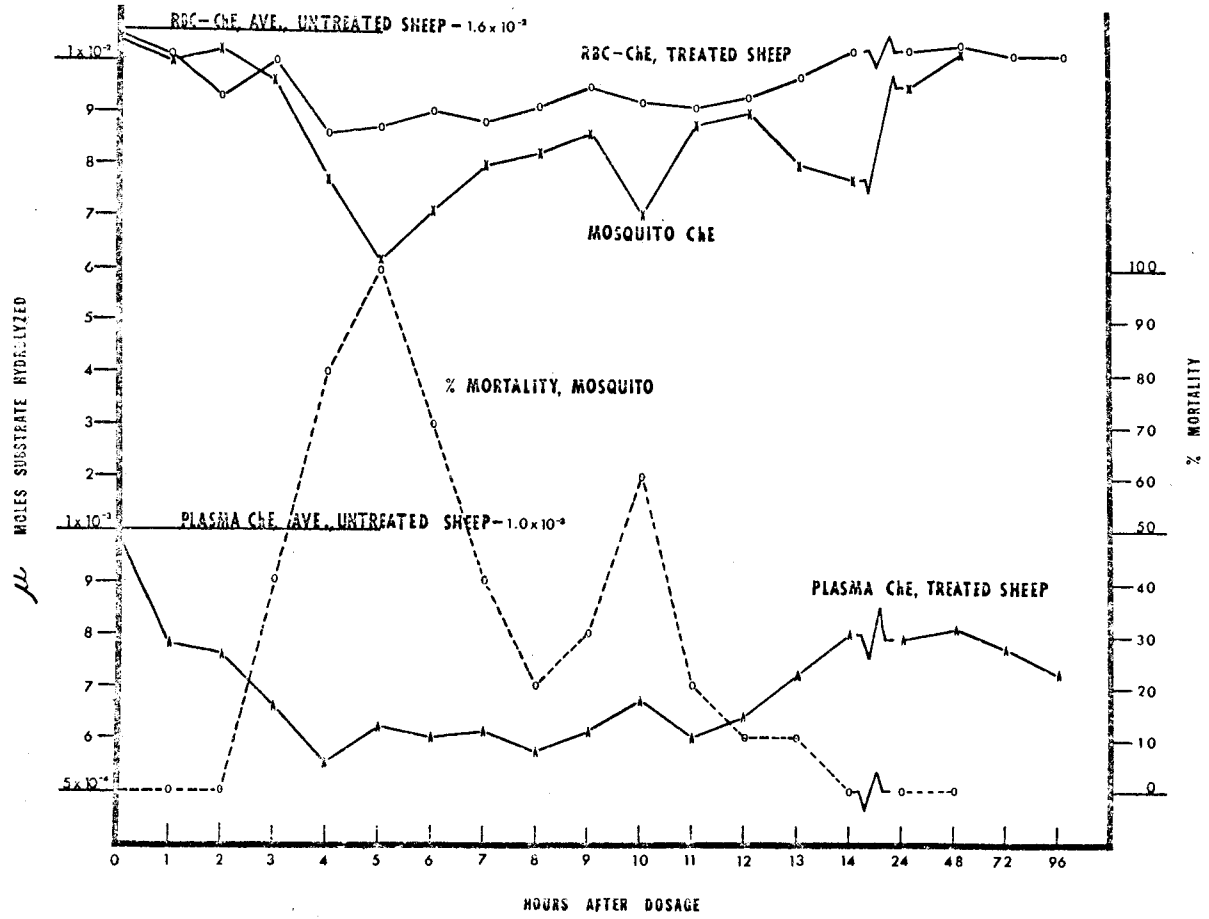

FIG. 3—Experiment 3 Effects of orally administered Ruelene upon sheep blood ChE activity levels, mosquito ChE activity levels, and mosquito mortality.

the blood stream of the host occurred more readily in this experiment than in the others. According to Annison et al. (1957), this early absorption may be due to variation in the rate of absorption among individuals of the same species. The earlier depression might also explain the lack of stabilization of the plasma ChE activity level.

Depression of the mosquito and plasma ChE activity between the second and third hours after dosage was the first indication of inhibition by Ruelene or its metabolites. The depression of the RBC AChE activity was delayed for one hour. Reasons for this delay are considered to be: the time required for the organophosphate to phosphorylate the AChE which is bound to the RBC membrane, and the time required for the inhibitor to break the bonds between the AChE molecule and the RBC membrane (Witter, 1963).

It was demonstrated that maximum ChE activity inhibition in the host's blood (average for the three experiments, 47% RBC and 48% plasma) was reached by the fourth hour (Fig. 1-3). Maximum mosquito ChE activity inhibition (average of the three experiments, 56%) was delayed one hour; this was due in part to the time involved in applying the sample of mosquitoes, allowing them to feed, and removing them. The mortality of the mosquitoes as indicated by Figures 1, 2, and 3 followed the depression of the mosquito ChE more closely than it did the depression of the blood

fraction ChE. Secondary and tertiary depressions of ChE activity levels in the host's blood, probably caused by the metabolic processes of the host, occurred from approximately 6-9 and 10-13 hours after dosage. These depressions were closely followed by depressions in the ChE activity levels of the mosquitoes and increases in their mortality. A direct correlation was indicated between host blood ChE activity level, ChE activity level of the mosquitoes that had fed on the blood of the treated host, and mortality of the mosquitoes. Reduction of inhibitor to nontoxic levels in the blood of the host (24-48 hours) was indicated by the mosquitoes in 1) the lack of deaths in the samples and 2) the lack of ChE inhibition at 48 hours. The depression of ChE activity in the blood of the host between 48 and 96 hours could not be explained but was not unique, as Eddy et al. (1959) encountered the same phenomenon.

Figure 1 indicates that 20% mortality occurred at 33% mosquito ChE activity inhibition; in Figure 2, 57% mortality occurred at 33% inhibition; in Figure 3, 40% mortality occurred at 40% inhibition. Maximum mortality (100%) was reached at 56% and 59%, respectively (Experiments 2 and 3). The mortality of the mosquitoes in the experiments indicated that inhibition of their ChE was lethal, and the lethal range was very narrow. The variations in mosquito mortality among the experiments were assumed to be due to biological variation among the mosquitoes and to minor differences in the

experiments. In comparison, the hosts in the study showed no signs of intoxication at any level of blood ChE activity inhibition.

During major depressions of host blood ChE activity, the mosquitoes were reluctant to feed. They had to be constantly agitated at these times or they would remain motionless on the side of the cage, staying as far away as possible from the exposed skin of the host. This reluctance to feed may indicate a repellent effect at maximum ChE activity inhibition by the pesticide.

Tick Mortality

Samples of D. andersoni that had attached to the backs of experimental sheep and engorged during the course of Ruelene dosage were removed at prescribed times and observed periodically for mortality. Samples of ticks removed five minutes prior to dosage of the sheep served as controls. All the control ticks engorged. Copulation took place, and the females in the samples began ovipositing at the end of the eighth day. The samples of ticks removed prior to lowest depression level of ChE activity in the host's blood (four hours, Fig. 1-3) sustained no deaths by the 192 hour post-dosage period (Tables 1 and 2).

In the samples that were removed after five hours of exposure to dosed host's blood, one female tick from experiment 1 died by the end of one hour, another female had died before the 21st hour

Table 1 Experiment 1. Number of ticks dead per sample on sheep dosed with Ruelene.

Ticks Removed from Host			Ticks Dead at Indicated Hours after Dosage								
Number Removed	Hours after Dosage	Number Dead 1 Hour after Removal	21	30	45	69	96	120	144	168	192
10	0	0	0	0	0	0	0	0	0	0	0
5	3	0	0	0	0	0	0	0	0	0	0
5	5	1	2	2	2	2	2	2	2	2	2
5	7	1	1	2	3	3	3	3	3	3	3
5	9	1	2	3	4	4	4	4	4	4	4
5	11	1	2	4	4	4	4	4	4	4	4
5	25	1	-	3	4	4	4	4	4	4	4
5	55	2	-	-	-	3	3	4	4	5	5

Table 2 Experiment 2. Number of ticks dead per sample on sheep dosed with Ruelene.

Ticks Removed from Host			Ticks Dead at Indicated Hours after Dosage								
Number Removed	Hours after Dosage	Number Dead 1 Hour after Removal	21	30	45	69	96	120	144	168	192
10	0	0	0	0	0	0	0	0	0	0	0
5	3	0	0	0	0	0	0	0	0	0	0
5	5	0	1	1	2	2	2	2	2	2	2
5	7	0	3	3	4	4	4	4	4	4	4
5	9	1	3	4	4	4	4	4	4	4	4
5	11	1	2	3	4	4	4	4	4	5	5
5	13	0	2	2	3	3	3	4	4	4	4
5	25	2	-	2	3	3	4	4	4	5	5
5	27	1	-	3	3	4	4	4	4	4	5

(Table 1) and the remainder survived. Of the ticks from experiment 2, one female died before the 21st hour and another, a male, between 30 and 45 hours; the remainder of the sample survived. The deaths that occurred in these samples indicated that the ticks had fed at a period when the toxicant in the host's peripheral blood was at high levels and the ChE activity low. The surviving ticks in both samples either fed prior to critical toxicant level in host's blood or did not feed. The early death of the tick in experiment 1 was probably due to the more rapid absorption of toxicant into the blood stream of the host (Fig. 1) and consequently an earlier appearance of toxicant in host blood at levels critical to ticks.

Of the five ticks from experiment 1 removed after seven hours exposure, one female died one hour after removal, a second female died between 21 and 30 hours and a third, a male, between 30 and 45 hours. The other two ticks in the sample showed no signs of intoxication at 192 hours (Table 1). The light engorgement of the tick which died one hour after removal indicated that it had fed at a time when toxicant was at high levels in the blood of the host (between three and five hours). The second and third ticks that died were more engorged than the first, indicating that they had fed later, yet had engorged enough to receive a lethal dose of toxicant from the host's blood. The two surviving ticks, a male and a female, were not engorged and apparently had not fed for some time.

Three of the five ticks, all females, in experiment 2 that were removed after seven hours exposure died before the 21st hour and a fourth, a male, died between 30 and 45 hours. Only one tick, a male, which apparently did not feed, survived. The slightly engorged state of the three female ticks that died prior to the 21st hour was an indication that they had fed after the highest concentration of toxicant in host's blood (three to five hours, Fig. 2). The male tick that died between 30 and 45 hours was more engorged than those which died before the 21st hour, which indicated that it probably had fed heavily between five and seven hours.

In experiment 1, one of the female ticks removed after nine hours exposure was heavily engorged and died within an hour after removal (Table 1), which showed that it had fed heavily during the time when toxicant level in host blood was declining but still at levels critical to ticks (Fig. 1). The other three ticks, two females and a male, that died were less engorged than the first, which indicated that they had fed most heavily during the six to eight hour period (Fig. 1) when toxicant level in the host's blood was again increasing. The male tick that survived was not engorged and apparently had not fed.

In experiment 2, as in experiment 1, one tick, a female, in the sample removed nine hours after exposure was heavily engorged and died within an hour after removal from the host (Table 2), which

indicated that it had fed between four and seven hours. Two more ticks, both females, died within 21 hours and a fourth, a male, died between 21 and 30 hours. The engorgement states of the two female ticks that died within 21 hours indicated that they had fed most heavily during the five to seven hour period when toxicant level in host blood had decreased but was systemically active (Fig. 2). The fourth tick that succumbed was the most lightly engorged of the four that died, which indicated that it had fed during the six to nine hour period when toxicant level in host's blood was increasing (Fig. 2).

In the sample of ticks from experiment 1 removed after 11 hours exposure, one female tick was lightly engorged; yet it died within an hour after removal (Table 1). The death, in spite of the light engorgement state of this tick, indicated that it had fed during the nine to eleven hour period when systemic activity of the toxicant was increasing (Fig. 1) and the tick acquired a lethal dose of toxicant with very little ingested blood. A second tick, a female, died within 21 hours and two more, a male and a female, between 21 and 30 hours. The engorgement state of the tick which died within 21 hours after host dosage indicated that it had fed heavily during the six to eight hour period when systemic action of the toxicant in host's blood was relatively low. The male and female ticks that died between 21 and 30 hours were not as heavily engorged as the

tick that died within 21 hours but were more completely engorged than the one that died within one hour. The deaths and engorgement states of the ticks between 21 and 30 hours would indicate that they had fed most heavily between 8.5 and 9.5 hours. The male tick that survived was more heavily engorged than the ones that died which indicated that it had probably fed heavily during the predosage to one hour period (Fig. 1, Methods and Materials) when no toxicant was present in the host's blood.

The sample of ticks from experiment 2 which were removed from the host at a comparable time (11 hours) demonstrated increasing numbers dead from 1 to 168 hours, at which time the entire sample of ticks had succumbed (Table 2). The male tick that died within one hour after removal was heavily engorged while the female tick that died at 168 hours was lightly engorged. The engorgement states of these two ticks and their deaths apparently indicated that the tick that died within one hour had fed heavily during the eight to ten hour period when levels of systemically acting toxicant were fluctuating. The female tick which died at 168 hours had fed most heavily during the ten to eleven hour period when toxicant in the host's blood was at relatively high levels (Fig. 2). The engorgement states of the ticks that died within 21 hours, between 21 and 30 hours, and between 30 and 45 hours were such that they indicated different rates and different times of feeding during the six to eleven

hour period (Fig. 2).

No sample of ticks was taken for experiment 1 at 13 hours after host dosage for comparison with the sample from experiment 2. In the sample of ticks which was removed after 13 hours exposure, two females died within 21 hours after host dosage, another female between 30 and 45 hours, and a fourth, a male, between 96 and 120 hours (Table 2). The surviving male tick was only lightly engorged, which indicated that it had only begun to feed and had not ingested enough host blood to receive a lethal dose of toxicant. The two ticks that died within 21 hours were heavily engorged, which indicated that they had fed heavily during the seven to eleven hour period when systemic action of the toxicant was fluctuating (Fig. 2). The tick that died between 30 and 45 hours was engorged, but not as much as was the one which died between 14 and 21 hours, which indicated that it had fed during the 11 to 13 hour period when the toxicant was at high levels in the host's blood. The death of the fourth tick between 96 and 120 hours has two possible explanations: 1) death occurred from a metabolite of the Ruelene degraded by the sheep or the tick and/or 2) intoxication processes of Ruelene had proceeded at a slower rate than in previous ticks.

In the sample of ticks from experiment 1 with 25 hours of exposure to the treated host's blood, one female died approximately 15 minutes after removal, two others, both females, died between

26 and 30 hours, while a fourth, a male, succumbed between 30 and 45 hours (Table 1). There was a direct relation between the degree of engorgement and the time before death in this sample, which indicated that the ticks had fed heavily during the 10 to 13 hour period when the toxicant level in the host's blood was declining but still at levels critical to ticks when ingested in large quantities. One male tick in the sample survived and, as in the 13 hour sample of experiment 2, it had apparently only begun to feed.

In experiment 2, the sample removed at 25 hours after exposure had two dead within one hour, one male and one female. A third, a male, died between 30 and 45 hours, a fourth, a female, between 69 and 96 hours, and the fifth, a female, between 144 and 168 hours. The increasing engorgement states of the ticks that died between 1 and 96 hours indicated that they had fed heavily during a decreasing, but critical, toxicant level in host's blood (10 to 13 hours, Fig. 2). The death of the fifth tick in this sample probably occurred from the same causes as the death of the tick between 96 and 120 hours in the 13 hour sample (Table 2).

All ticks removed after 27 hours exposure (experiment 2) were heavily engorged. The mortality of the ticks between removal and 45 hours (Table 2) indicated that they had engorged heavily during the 10 to 13 hour period (Fig. 2) when toxicant level in the host blood was declining but still at levels critical to ticks if ingested in large

quantities. The death of the fifth tick, a female, between 168 and 192 hours is assumed to be due to a metabolite of Ruelene.

Among the ticks removed 55 hours after exposure (experiment 1), two died within one hour, a third died between 56 and 69 hours, a fourth died between 96 and 120 hours, and the fifth died within 168 hours (Table 1). The times of death of the ticks in this sample would suggest that they had died of a metabolite of Ruelene degraded either by themselves or by the host.

Herms and James (1961) stated that ixodid ticks attach and feed uninterruptedly to repletion. Cooley (1932) stated that if undisturbed they fully engorge in nine to ten days, at which time they detach and fall from the host to the ground. Results of this study suggested, however, that the female ixodid tick, D. andersoni, attaches, remains attached, and feeds intermittently rather than continuously until completely engorged.

Copulation took place while ticks were attached to their host, but no eggs were laid when females were later removed and placed under observation. Normally the preoviposition period occupies about five days (Cooley, 1932), but surviving females observed for 12 days failed to oviposit. The failure of the females to oviposit may be an indication of the disruption of some part of the reproductive cycle due to sublethal doses of Ruelene.

SUMMARY

Reduction in rates of cholinesterase activity during the first two hours after dosage of sheep with Ruelene was not due to an inhibitor. Inhibition of ChE activity began at the end of the second hour and was evidenced first by a depression of plasma and mosquito ChE activity, then by depression of RBC AChE activity. Maximum inhibition of ChE activity in the host blood was reached four hours after dosage with Ruelene. The maximum mosquito ChE activity inhibition was delayed one hour.

Inhibitor was present in the peripheral blood of the dosed sheep, which in turn depressed the ChE activity of the mosquitoes which had fed upon the sheep. Secondary and tertiary maximum depression points were thought to be caused by host metabolic processes (Ganelin, 1964). Rapid decline of inhibitor in the host's blood between 24 and 48 hours after dosage was indicated by decreased inhibition of mosquito ChE activity. Depression of host blood ChE activity between 48 and 96 hours was not explained but was not unique.

Mosquito mortality first occurred when ChE activity inhibition increased above an average of 35%, and 100% mortality occurred at an average of 56% inhibition, which indicated a correlation between ChE activity inhibition and per cent death in a sample. In comparison,

the host showed no toxic effects when ChE activity inhibition of its blood had been depressed 45%. Mosquitoes were reluctant to feed during major depressions in ChE activity in the host's blood, which possibly indicated a repellent effect at high systemic activity levels of the inhibitor.

Ticks were removed from the test animals at various times after Ruelene dosage and observed for mortality. Those ticks removed before lowest ChE activity levels in host blood sustained no deaths. Those ticks in samples which were removed after lowest ChE activity levels of host blood indicated varying engorgement states and consequently varying death rates. Ticks in samples removed at 25 and 27 hours after dosage were fully engorged and received a declining but systemically active dose of toxicant from the peripheral blood of the host. All ticks in the sample removed 55 hours after dosage succumbed to a metabolite of Ruelene degraded by the host and/or by the ticks themselves. Delays in death beyond the 96 hour postdosage period were assumed to be due to a metabolite of Ruelene degraded by the host and/or the ticks.

Surviving, mated females laid no eggs after removal from their host.

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APPENDIX

APPENDIX

Reagents Used for ChE Activity Analysis

1. Phosphate Buffer - pH 7.2 (Gomori, 1955)

   (a) Mono-sodium Phosphate - $NaH_2PO_4.H_2O$

   For a O.2M solution, 13.9 g of $NaH_2PO_4.H_2O$ were placed in a 500 ml volumetric flask and brought to volume with glass distilled water.

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

   For a O.2 M solution, 35.85 g of $Na_2HPO_4.12H_2O$ 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 7.2, 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 7.2).

   (b) Radioactive Material

A total of 5 ml of hot substrate was prepared which contained 2.5 μC. 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 7.2) were added.

3. Saponin - 0.04%

To facilitate lysing of the red blood cells, 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.

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.