THE TOXIC EFFECTS OF RONNEL FED TO RATS FOR THE CONTROL OF *Xenopsylla cheopis* (Rothsch.) (Siphonaptera, Pulicidae)

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INTRODUCTION

The purpose of this project was to study the potential of systemic insecticides in the control of fleas on rodents. A systemic insecticide is a chemical that when administered to an animal or plant will be absorbed and distributed in the tissues and fluids of the organism. Successful use of this pest control technique could possibly prove an important tool in preventing transmission of the diseases, sylvatic plague and murine typhus, to man and other susceptible animals. Until the development of DDT and other modern insecticides and anticoagulant rodenticides, transmission of these diseases to man was restricted through quarantines, exclusion of rodents from man's environment and rodent eradication campaigns. The use of antibiotic medication now limits the seriousness of these diseases in man. Because of the use of insecticides, rodenticides and antibiotics, sylvatic plague and murine typhus are not as important as in the past. However, there remain widely distributed foci of sylvatic plague in wild rodent populations and murine typhus in domestic rodents.

Foci of sylvatic plague remain a critical public health problem, even though the modern plague pandemic is on the decline (3). Kartman (18) considers that total eradication of sylvatic plague in urban and rural areas is not possible. Several sylvatic plague epizootics have been reported in California, and the foci observed were probably only a fraction of the many that may exist (29). With more people using recreational areas, opportunity for closer contact between man and reservoir animals and their ectoparasites is increased.

Rodent control may not immediately result in high levels of vector control, in fact, the hazard of human contact with potential vectors is likely to increase during rodent control campaigns. In a rodent poisoning program in California, the numbers of fleas increased greatly on animals that survived (28).

The establishment of more effective vector control techniques would be an important element in the development of an improved method of protecting man from sylvatic plague and murine typhus, and eventually would eliminate these diseases from their rodent hosts. The use of systemically acting insecticides could be a valuable adjunct to such a program. In parks, wildlife preserves, pastures, range lands and in the home, systemic insecticides may afford a means of protecting

man and other animals from disease vectors without introducing a rodenticide or a generally distributed insecticide into the habitat.

To study the systemic properties of ronnel in rats, fleas were allowed to feed on orally drenched rats restrained in flea-proof cages. Fleas confined in cells were exposed to rats drenched with or fed ronnel and restrained in squeeze cages. The toxic effect of feces from rats fed ronnel upon flea larvae was observed.

REVIEW OF LITERATURE

The concept of the use of insecticides acting systemically within animals is certainly not one of recent origin. Although not in the strict sense of systemic activity, as early as 1926, Gallagher (15) attempted to control the larvae of horn flies by administering compounds in drinking water of cattle. Two years later, Parman et al. (31) reported an extensive search for a chemical that when fed to poultry would kill or repel stick tight fleas, Echidnophaga gallinae Westwd., and other ectoparasitic arthropods. Both of these early attempts met with failure.

From 1929 to 1943 reports on animal experiments with systemically acting insecticides were not found. However, in 1938, Knipling (20) reported that feeding of phenothiazine to cattle prevented the development of horn fly larvae, Haematobia irritans L., in the animals' manure. Lindquist et al. (23) in 1944 showed that bed bugs, Cimex lectularis L. and C. hemipterus F., were killed when fed on rabbits orally dosed with DDT or pyrethrum. These results initiated extensive studies to determine the systemic properties of various chemicals. The systemic properties of several compounds were evaluated by feeding a variety of test insects on treated rabbits,

laboratory white mice, or guinea pigs (1,2,5,9,11,14,17, 21,24,25,26).

Much of the emphasis in recent years has been directed to the applications of chemotherapeutic agents for control of livestock pests. Several investigators (8,12,19,30,34,38) have demonstrated the effectiveness of Dow ET-57, Co-Ral,® Dowco 109® and dimethoate administered orally, subcutaneously, or dermally in reducing or controlling cattle grubs, Hypoderma spp. In some tests performed by these workers Co-Ral,® Dow ET-57 and Dowco 109® were lethal to at least 97 percent of the grubs. Although some of the cattle ticks, Boophilus microplus (Canestrini) and cattle lice, Solenopotes capillatus Enderl and Linognathus vituli (L.), were killed, the overall results obtained by Roulston (35) and Defoliart (8) using systemically acting chemicals were not favorable.

Kraemer and Furman (22) obtained a high level of control against the northern fowl mite, Ornithonyssus sylviarum (C. and F.), by feeding Sevin® to chickens.

Control of arthropod pests breeding in feces by means of pesticides incorporated in the feed of animals may constitute a convenient form of pest control if the value of the animal is unaffected. The feeding of phenothiazine to cattle to control horn fly larvae in

the manure was confirmed by Bruce (6). This worker also observed that phenothiazine was undesirable because it imparted a pinkish tinge to the milk of cows. Materials toxic to house fly larvae, Musca domestica (L.), and horn fly larvae, Siphona (=Haematobia) irritans (L.), were found in manure from cattle fed aldrin and dieldrin (16). Residues in manure from cattle fed Dowco 109 ® were not sufficient to bring about a significant reduction of horn fly larvae (12). The toxicity of droppings from young chickens fed any one of several compounds was evaluated with first instar house fly larvae (36). Moderate to high mortalities in the larvae were observed, but some of the insecticides were also lethal to the chicks.

Until the recent reports by Harvey (16) and Bennington (4), no publications have been found related to flea control in rodent populations by systemically acting insecticides. Harvey reported significant systemic activity for dimethoate administered orally to white Norway rats, but ronnel and Dowco 109 ® proved less effective on the oriental rat flea, Xenopsylla cheopis (Rothsch.). Bennington reported that oriental rat fleas were killed after feeding on rats fed ronnel in bait at a concentration of 4 grams per pound. Bennington also investigated the problem of acceptance of ronnel in

corn meal baits by domestic rats. He reported good acceptance of baits with 2.4 grams of ronnel per pound and poor acceptance of higher concentrations up to 12 grams per pound, unless sugar and liquid smoke were added as an attractant.

MATERIALS AND METHODS

Flea Culture. A culture of the oriental rat flea, Xenopsylla cheopis (Rothsch.) was reared by a technique similar to that described by Smith and Eddy (37). The equipment used is illustrated in Figure 1.

Four 7-gallon tubs each contained 2 quarts of larval substrate, consisting of 2 parts washed river sand and 1 part of fine sawdust and to which was added 1 cup of finely ground Friskies® dog food. This medium was found sufficient, and further supplementing with dried blood as suggested by Smith and Eddy (37) was not necessary. To facilitate subsequent harvesting of pupae, all the substrate ingredients were sifted through a 16-mesh screen before mixing.

A laboratory white rat was used as a host for fleas in the culture. It was confined to a 4.5 x 4.5 x 8 inch cage constructed of 16-guage, 2-mesh, welded galvanized hardware cloth. Beneath this cage, a galvanized sheet metal tray protected by paraffin was used to catch the urine, feces and uneaten particles of rat food. Urine in the tray was absorbed by paper toweling, but later experimentation showed that fine sawdust changed once or twice a week was as satisfactory. A few adult fleas and eggs were lost when the absorbent material in the tray was changed. In spite of this, ample fleas were reared.

To reduce the amount of urine excreted by the rat, Prince (33) and Smith and Eddy (37) used carrots or apples as a source of water; however, in this study the rat was allowed free choice of water. Several days' supply of rat food (Purina ® Dog Chow) in a self-feeder was available to the rat. Under the conditions of free choice of both water and food, rats used as host animals in the flea culture required little attention.

After the larval substrate and rat were placed in the tub, 500 to 1,000 newly emerged fleas were released on the rat. Nylon organdy was stretched over the tub to confine the fleas and still permit air circulation. Oriental rat fleas remain on the rat or in the larval substrate unless disturbed. The colony was kept in an air temperature of 70-74° F. and 65-70 percent relative humidity.

At the end of one week, the rat was removed from the tub since most of the fleas had dropped to the larval substrate. Mating took place on the rat or in the substrate, and the majority of the eggs were deposited in the larval medium. The exact time of oviposition is not known, but the majority of the eggs hatched between 10 and 17 days after the adults were placed on the rat.

The rat, cage and tray were placed in another tub similar to that described, and additional fleas were added. A strong colony of fleas was maintained by rotating the rat in this manner each week. The larval substrate could be used several months by periodically adding 1/4 cup of finely ground Friskies ® Dog Food. No additional sand was added, although some was lost in the cocoons.

Each week the cocoons containing prepupae and pupae were collected by first sifting the larval substrate through a 10-mesh screen in order to remove rat feces and uneaten rat food not caught by the tray under the rat cage. Then the substrate was sifted through a 16-mesh screen to remove the cocoons. After collection the cocoons were stored in quart size cartons (Figure 2) under temperature and humidity conditions common to the rest of the culture. This permitted the prepupal stage to complete metamorphosis within the cocoon. Like the larvae, this stage is unable to tolerate the low humidities that pupae and adults are able to withstand (27).

Between 8 and 13 days from the time the cocoons were collected emergence of the adults began. Fleas were at first selected from the cartons for use, but later a Berelese funnel, without lamp, was employed to separate adult fleas from the cocoons (Figure 2).

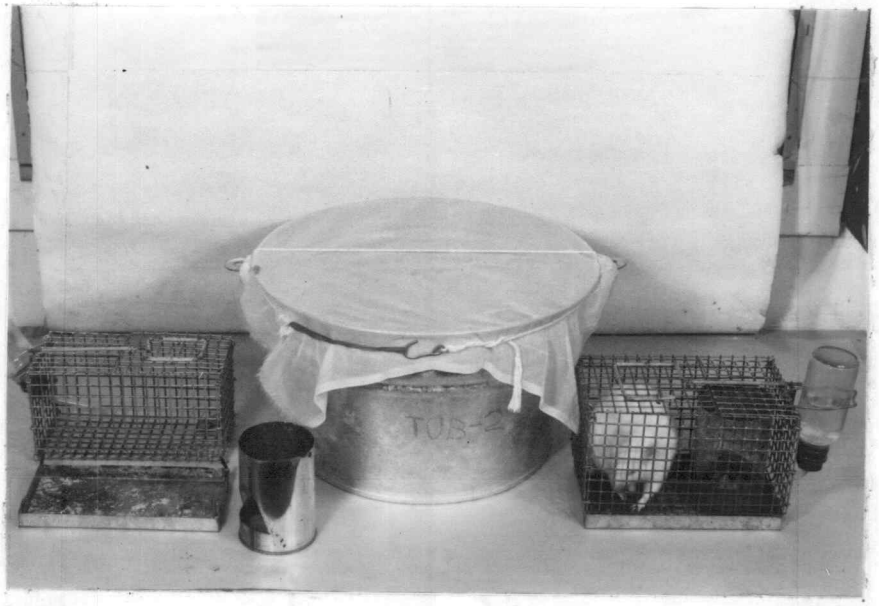


Figure 1. Equipment and rat used in rearing fleas. Components of rat cage to left of tub.

Figure 2. Carton for storing pupae, funnel for separating adults from cocoons and adult holding jar below funnel.

The upper reservoir was separated from the tapering lower portion of the funnel by a 16-mesh screen. The spout of the funnel was inserted through a jar top and soldered in place so that a quart jar could be screwed to the funnel. A thin layer of cocoons was sprinkled over the screen inside the funnel. As the fleas emerged, they moved about on the cocoons and screen and eventually fell through to the jar below. Emergence was stimulated by shaking the cocoons or by blowing gently on them.

A layer of vermiculite, 0.5 to 1 inch deep, was added to the quart jar to provide shelter and to minimize the effect of crowding. Less mortality was observed in vermiculite than in washed river sand. Adult fleas were aspirated from the jar to be used in various experiments. This method permitted collection of fleas in uniform age groups.

Rat Colony. Four- to five-week-old laboratory white rats were kept individually in holding cages 6 x 8 x 10 inches constructed of galvanized hardware cloth (Figure 3). They were fed ground dog food (Purina® Dog Chow) until reaching a weight of 100 to 200 grams and then were used in the various experiments. Rate of gain was between 5 and 10 grams a day as calculated from periodic weighings.

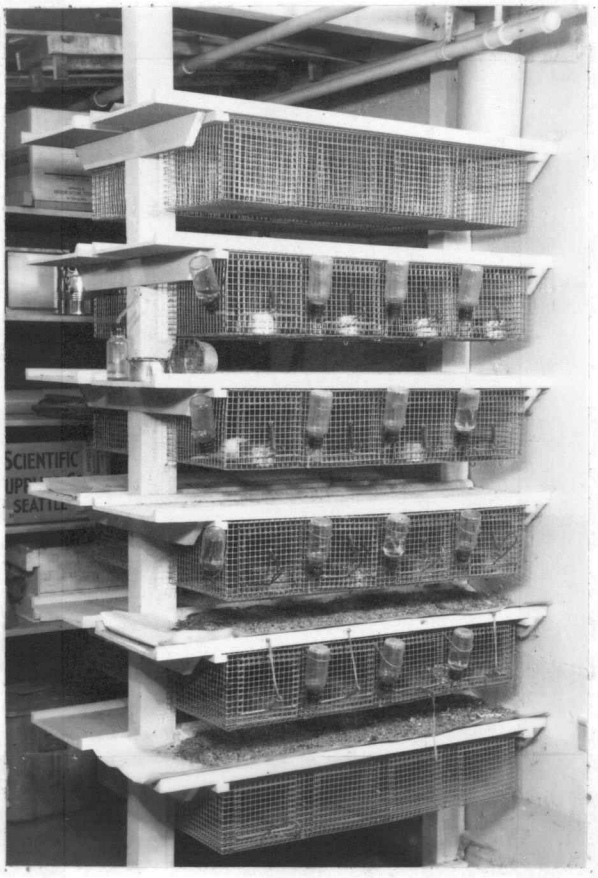
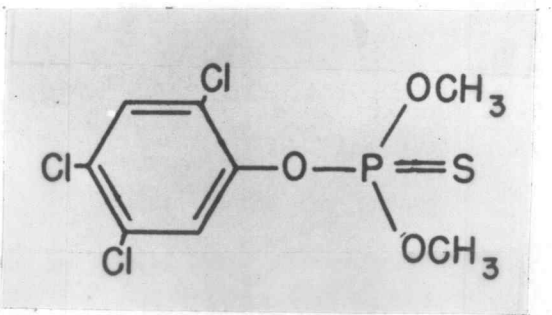


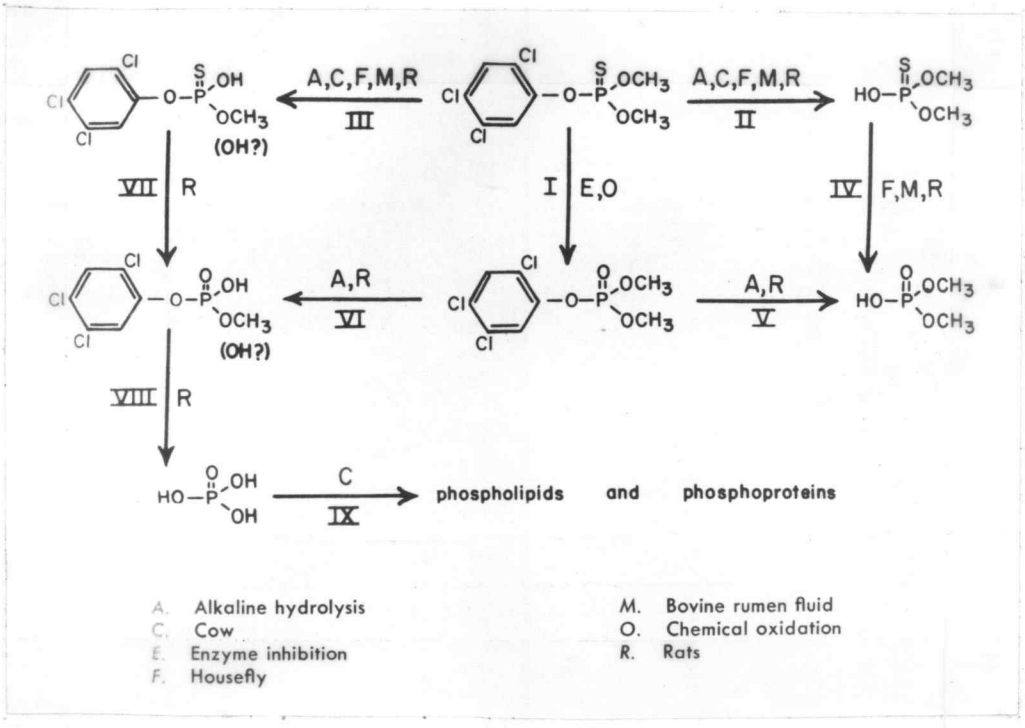
Figure 3. The rat colony cages.

Insecticide. Ronnel, O,O-dimethyl O-(2,4,5-trichlorophenyl) phosphorothioate, is manufactured by The Dow Chemical Company, Midland, Michigan. The experimental name assigned this material was Dow ET-57, and it is registered under the name Trolene ®. A less refined grade of ronnel has been designated as Dow ET-14 or Korlan ®.

Ronnel is a white crystaline chemical with a melting point of 35-37° C. and is stable at temperatures to 60° C. (10). The structural formula is:



It is hydrolysed *in vivo* and *in vitro* at both the alkyl-phosphate and aryl-phosphate bonds to 4 nontoxic, water-soluble phospho-diesters. The metabolic pathway proposed by Plapp and Casida (32) is:



In all of the experiments carried out technical grade ronnel of high purity was used. At the out-set of the project any water-soluble impurities formed during normal breakdown of the insecticide during storage were removed. The insecticide was dissolved in anhydrous ethyl ether, extracted with water and dried with Na_2CO_3. Not all of the ether could be evaporated

after drying and the remaining residue of ether-insecticide was dissolved in a small amount of warm 100 percent ethanol. Ronnel was recrystalized by chilling in a freezer to -10° C. and was stored under low temperatures to minimize further breakdown.

Experiment 1. In order to observe the effects of ronnel, three basic experiments were conducted in this project. Additional tests lending support to each basic experiment were necessary and are explained in conjunction with each experiment.

In Experiment 1, fleas were exposed to orally drenched white rats. The drench was prepared by dissolving ronnel in one part acetone and adding three parts of corn oil. The required amount of insecticide per 100 grams of rat was contained in 0.5 ml of acetone-corn oil solvent for treatment at the selected dosage levels. Rats not treated with insecticide were orally drenched with a 1:3 acetone-corn oil solution.

After treatment, each rat was restrained in a 4-mesh hardware cylinder. The rat and cylinder were placed in a glass cage with an absorbent bottom (Figure 4). Cages 8 x 8 x 10 inches were constructed from sheets of glass taped together from the outside. This provided a smooth inside surface on which fleas could not crawl.

The bottom of the cage consisted of a 9 x 11 inch sheet of 1/4 inch plywood over which was placed a single

layer of corrugated cardboard and several layers of paper towels for absorbing urine. The absorbent bottoms were held to the glass sides by 2 large rubber bands (Figure 6). Thin strips of modeling clay were pressed around the outside of the cage along the bottom of the glass and paper toweling as a precaution against loss of fleas. Nearly all of the fleas used in these tests were recovered. Small losses were probably due to ingestion by rats rather than by escape.

Adult fleas were aspirated from the holding jars as needed, anesthetized with carbon dioxide in an eight ounce plastic cup and counted from the cup using a small aspirator (Figure 5). Anesthetized fleas were allowed to recover before being placed on the rat. While on the host, they could move about freely or even leave the animal, but they were kept in the immediate area by the glass cage. After 24 hours, test fleas were collected from the cage and rat to be counted as alive, moribund or dead.

The results of these tests indicated a need for more uniformly aged fleas and a need to avoid surface contact of materials toxic to the fleas. Since fleas could be stimulated to emerge from the cocoons as needed, the Berelese funnel provided a simple means of collecting individuals of uniform age. To test the effect of age

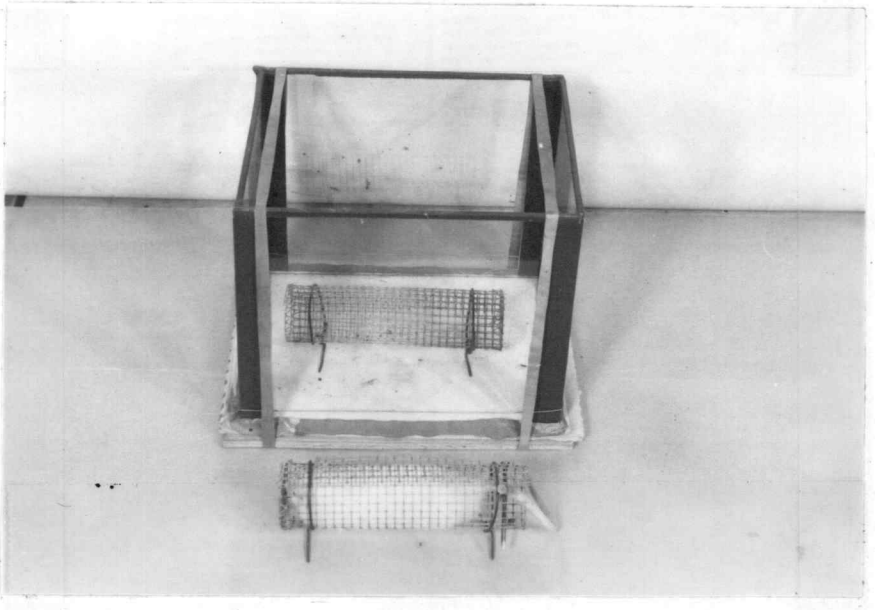


Figure 4. Glass cage and hardware-cloth rat cylinder used in Experiment 1.

Figure 5. Plastic cup and aspirator for anesthetizing and counting adult fleas. Aspirator was also used in counting larvae.

of fleas upon susceptibility to ronnel, a large sample was emerged and at 24-hour intervals 30 fleas were placed in pint jars treated with insecticide. Concentrations of 0.01 p.p.m., 0.1 p.p.m. and 1.0 p.p.m. of ronnel in acetone were made up, and 1 milliliter of each was added to different jars. One milliliter of acetone was added to jars to check toxicity of the solvent and natural mortality of fleas. After adding acetone or acetone-ronnel, the jars were swirled until the solvent had completely evaporated and all odors of acetone had disappeared. The amount of insecticide, in micrograms/square centimeter, at each treatment level was calculated to be 0, 1.2×10^{-4}, 1.2×10^{-3} and 1.2×10^{-2}. Mortality counts were made 24 hours and 48 hours after introduction, and statistical analysis is based on the final count.

Experiment 2. Following Experiment 1 a second series of tests were designed in which the host was restrained in a squeeze cage and test fleas were confined in a metal pill box or cell with silk or nylon organdy (Figure 6). A piece of paper, 0.5 x 1.0 inches, was folded to form a "V". This paper was placed in the cell as a means of increasing the inner surface area to reduce the effect of crowding.

Squeeze cages were constructed from 16-gauge hardware cloth. The bottom half, 2.5 x 10 inches, was flat except that the anterior end was bent up 1 inch. A rectangular hole, 1.5 x 2.0 inches, was cut 2.5 inches from the anterior end to allow exposure of a portion of the rat's ventral surface. The top half of the cage, 3 x 10 inches, was arched lengthwise and attached by hog rings to the bottom half along one side.

In the first series of tests, ronnel was formulated and administered as a drench as previously described. Immediately after treatment a portion of the rat's ventral region was shaved with a surgical clipper, and the rat was placed in a squeeze cage. Light metal pins prevented the rat from backing out of the cage. Rubber bands provided the necessary tension to keep the cage closed during use.

A cell containing fleas was later attached over the shaved ventral region protruding from the cage. Feeding took place through the organdy during a 24-hour exposure period. At the end of this interval, the fleas in each cell were anesthetized with CO_2, the organdy was removed and the fleas were transferred to large test tubes. Statistical analyses are based on the number of moribund-dead fleas recorded 24 hours after they were removed from the host.

To guard against contamination, after each test the equipment used in handling and holding the rats and fleas was washed with soap and water and rinsed in acetone.

In the next series of tests, the systemic effect of ronnel on adult fleas exposed to rats fed the insecticide in ground dog food was studied.

Feeding in the rat colony cages (Figure 3) was unsatisfactory because of the probability of surface contamination to the area where test fleas would be exposed. Also, at times food was scratched out of the container and made it difficult to calculate the amount of insecticide ingested. Feeding cages designed for separate collection of urine and feces and described in another section were also used in these tests.

In a feeding test, ronnel was fed to 4 rats at the rate of 325 mg./kg. for 6 days. At the end of the last feeding period fleas were exposed to the rats in squeeze cages. The rats used in this test were from a feeding test to determine toxicity of feces to flea larvae. Six days was considered an unnecessarily long feeding interval and in the next test 4 rats were fed at the rate of 500 mg./kg. for 4 days. The fleas were handled as before.

A third test was carried out by feeding ronnel to 3 rats at a dosage level of 100 mg./kg., 3 rats at 250 mg./kg. and 3 rats at 500 mg./kg. for 4 days. In this test the daily feeding period was less than 24 hours. Because of this, not all of the food was ingested which resulted in a lower dose than was desired and actual dose was based on the total daily food consumption. At the end of the daily feeding period during the last 3 days of the test, the rats were transferred to and held in squeeze cages for 5 to 6 hours each day. A single cell containing 50 48-hour old fleas was assigned to each rat and exposed during this time each day. Fleas were also exposed, under similar conditions, to 3 rats fed no insecticide. Mortality counts of adult fleas were made 24 hours, 48 hours and 72 hours after the last exposure to the rats.

Experiment 3. Plapp and Casida (32) showed that after treating a cow at the rate of 100 mg./kg. 7 percent of the total dose of ronnel was voided with the manure within 24 hours. It was thought that unabsorbed insecticide may also be voided from the rat and, if so, would be a means of further distributing insecticide in a rodent's habitat.

To test this possibility, feces from rats used in the preliminary experiment were collected, dried, ground and evaluated for toxicity to larval fleas. Using this substrate it was possible to conclude only that ronnel or toxic derivatives were present in the urine and/or feces of these rats. Feces collected for these tests were invariably contaminated with urine.

Feces free of contamination with urine or spilled feed could be collected from rats held in squeeze cages, but usually the amount defecated was insufficient for testing with flea larvae.

Some modification of the Comar cage (7, p. 130) for metabolism studies made it useful in feeding insecticide without the hazard of contaminating animal, feces or urine and also permitted separate collection of feces and urine. As illustrated in Figure 7, a 4-mesh hardware cloth cylinder was inserted into a hole in the side of a 10.5 fluid ounce tin can.

A cloth baffle was placed around the cylinder as a precaution against any feed being scratched out of the can and contaminating the feces or urine. Test food was placed in the bottom of the can, and water was supplied by means of a tube. Water that might drip and wet the food was carried away by a metal channel fixed under

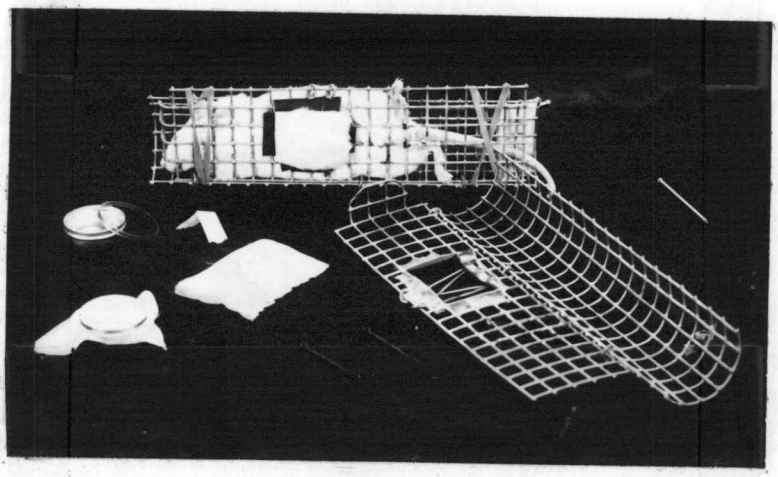


Figure 6. Squeeze cages with and without rat. Flea cell assembled in lower left and unassembled in upper left.

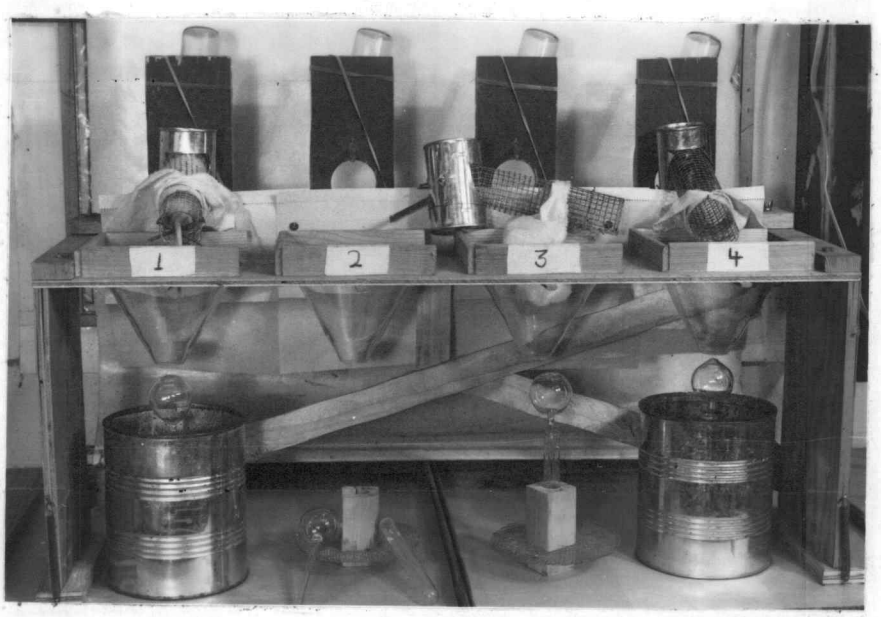


Figure 7. Feeding cages. Numbers 1 and 4 assembled, number 1 with rat. Numbers 2 and 3 disassembled to show components.

the tube. A stemmed ball in a test tube was used to separate urine and feces voided by the test animal. A 10-mesh hardware cloth skirt attached to the tube support caught the feces as it was deflected from the ball.

For feeding experiments, a stock formulation of ronnel and ground dog food was thoroughly blended in a small tumble mixer. This concentrate, ranging from 2-10 mg. per gram, was further diluted to desired dosage levels with the rat food when needed. The usual procedure was to make up enough of the concentrate to last 3-4 days of the experiment.

Reproducible results were possible when ronnel was mixed in rat food and fed to rats in the modified Comar apparatus. Test animals were given one pretreatment feeding based on the individual's weight. This was an attempt to minimize variation in the volume of the gastro-intestinal tract contents of each animal. Prior to this, tests had been conducted to determine the amount of food consumed by rats in a 24-hour feeding period. During pretreatment and treatment the amount of food was held to slightly less than the amount the rat was expected to consume. Daily weights of rats were kept to determine if losses were occurring and to adjust feeding levels.

Every 24 hours the feces from each rat were collected and kept separate in a 1 ounce souffle cup and allowed to dry. Feces clinging to the side of the funnel or to the ball, and possibly contaminated with urine, were discarded. When dry, usually 24 to 48 hours after collection, each fecal sample was ground in a mortar and sifted through a 40-mesh screen. The fine fraction was saved in order to observe the toxic effect on flea larvae. Second instar larvae were selected from a flea colony tub having the greatest uniformity as to development.

With the fecal residue study, feces of animals not on treatment were collected, dried, ground, and sifted as described above. To 100 grams of the feces, 100 milligrams of ronnel were added and thoroughly blended in a small tumble mixer. The stock, with a concentration of 1,000 p.p.m. by weight, was diluted with additional ground feces free of insecticide to 100, 150, 200, 300, 400 and 500 p.p.m. and bioassayed with second instar larvae in order to establish a standard dosage-mortality curve.

Mortality counts in larvae used in bioassays were accomplished by sprinkling the ground feces very thinly over a white surface and aspirating the live larvae as they moved. After a preliminary 24-hour exposure count, a final count was made at the end of 48 hours.

RESULTS AND DISCUSSION

EXPERIMENT 1

Drench Studies. Experiment 1 was designed primarily to explore the toxic effects of ronnel when administered to rats in an oral drench and challenged with adult fleas. Table 1 shows that a high level of kill in fleas resulted whether ronnel was given as a single or multiple treatment, or whether fleas were placed on the animals 2 or 24 hours after treatment. There was much variation within and among tests, and in some tests numbers of fleas killed appeared independent of dose to rats. From these observations, it might be suspected that not all flea mortality was due to systemic effects alone. At least three factors may have been responsible for the variations encountered. The effects of flea age on susceptibility to ronnel and ability to feed were considered as important as contamination with ronnel or toxic derivatives voided by the dosed rat.

Effects of Flea Age. Before the emergence funnel (Figure 2) was devised, fleas that emerged from cocoons within the pupal containers were selected at random and used. Under this circumstance, age of fleas ranged from a few hours to a week or more. When subjecting fleas to ronnel in jars it was observed that regardless of

Table 1.--Mortality in fleas on rats under restraint in flea proof cages. Dosage by oral drench. Age of fleas not uniform.

Test number	Number of fleas per rat	Percent flea mortality at indicated dosage. Mg./kg. of rat body weight 0	50	100	250	500	1000
	Four successive treatments. Fleas exposed 24 hours after last treatment						
1	50	0	-	90	43	84	100
2	50	0	-	78	87	97	100
	One treatment. Fleas exposed 24 hours later						
1	50	0	-	2	74	93	98
2	50	0	-	4	39	61	94
3	25	0	-	55	48	100	100
4	25	0	-	38	96	100	96
	One treatment. Fleas exposed 2 hours later						
1	25	0	0	100	100	94	-
2	25	0	0	54	92	100	-

treatment level, as the age of fleas increased there was an increasing susceptibility to ronnel (Table 2). Also, as might be expected, there is an increased mortality response at any age group to increased concentrations of ronnel.

There are three exceptions in this table where mortalities in 24-hour, 48-hour and 72-hour old fleas were greater at 1.2×10^{-4} ugm./cm.^{2} than at 1.2×10^{-3} ugm./cm.^{2}. It is not possible to say with so few replications which is correct, but it might be speculated that the mortalities at the higher treatment level are more accurate. Since there are such minute quantities of insecticide involved, it is evident that errors could have easily occurred when diluting higher concentrations to desired concentrations. After the age of 5 days and under the conditions of this experiment, there was natural mortality in adult fleas.

It is not meant that the observed increasing susceptibility with age to ronnel under contact conditions can be directly related to toxic effects of this material acting systemically. It does, however, illustrate the importance of uniform flea age in these studies. Moreover, in Experiment 1 test fleas could and probably did come in direct contact with ronnel or a toxic derivative in the feces and/or urine of treated rats.

Table 2.--Effect of age on mortality in adult fleas in direct contact with ronnel plated on inner surface of pint jars.

Age of fleas in hours	Number of tests	Mean percent mortality to fleas at indicated concentration $\mu gm/cm^2 \times 10^{-3}$ 1/			
		0	0.12	1.2	12.0
24	2	0	2	0	8
48	2	0	3	2	35
72	2	0	22	12	60
96	2	0	28	32	75
120	1	0	27	37	87
144	1	44	50	50	94

1/ 30 fleas at each treatment level in each test.

Thus, old fleas in the test population coming in contact with these toxic materials would exhibit a high order of kill, perhaps without ingesting any insecticide in a blood meal.

Using a supply of adults, which had emerged over a 15-20 minute period, samples were taken at intervals and placed in cells which were attached to the experimenter's arm. It was observed that more adult fleas fed or tried to feed in the age groups of 36 hours, 48 hours and 72 hours from time of emergence. It was noted that when the integument of freshly emerged fleas was light in color and soft feeding did not take place. Old fleas that appeared shriveled and lacked vigor did attempt to feed, but they were unable to penetrate the skin with their blade-like mouthparts.

More uniform populations of fleas were secured for the experiments that followed by using fleas that had emerged over a period of time of less than one hour and by arranging the experiment so that the fleas would be between 48 and 72 hours old when first exposed to the test animals. The technique of immobilizing the animal in a squeeze cage and feeding the fleas through a thin nylon or silk cloth eliminated for practical purposes, the possibility of test fleas coming in surface contact with ronnel or its derivatives.

EXPERIMENT 2

Drench Studies. Ronnel was administered as an oral drench to rats in squeeze cages and challenged for systemic activity with 2-day to 3-day old fleas in cells. The results (Table 3) indicated that a high level of kill to fleas was possible with ronnel acting as a systemic insecticide in rats. Mean flea mortalities ranged from 15 percent in rats treated at 100 mg. of ronnel/kg. of body weight to 81 percent at the 1000 mg./kg. level. Further testing at 50 mg./kg. in rats was abandoned because of the low levels of flea mortality encountered.

It is estimated by using the dosage-mortality curve established in Figure 8 that a dose to rats of about 350 mg./kg. would induce a 50 percent mortality in 48-hour to 72-hour old oriental rat fleas within 24 hours after removal from the host.

Table 3 shows the ranges of mortalities in the test fleas at the various dosage levels. The mean of the maximum percent mortalities between the dosage levels of 100 mg./kg. and 1000 mg./kg. was 73 percent with a standard deviation of 10 percent. Between these dosage levels the mean of the minimum mortalities was 23 percent with a standard deviation of 6 percent. This indicated that minimum mortalities at the various dosage levels are more nearly alike than the maximum mortalities.

Table 3.--Mortality in 100 fleas per cell held 24 hours on rats in squeeze cages. Single oral drench. 48-72 hour old fleas exposed to rats 2 hours after treatment.

Dosage to rats Mg./kg.	Number of rats per test	Mortality 24 hours after removal from rat		Standard deviation
		Mean	Range	
0	12	0	0	
50	7	3	1 - 4	± 1
100	11	15	2 - 40	± 15
250	12	42	21 - 63	± 13
500	11	63	36 - 87	± 20
1000	11	81	32 - 100	± 21

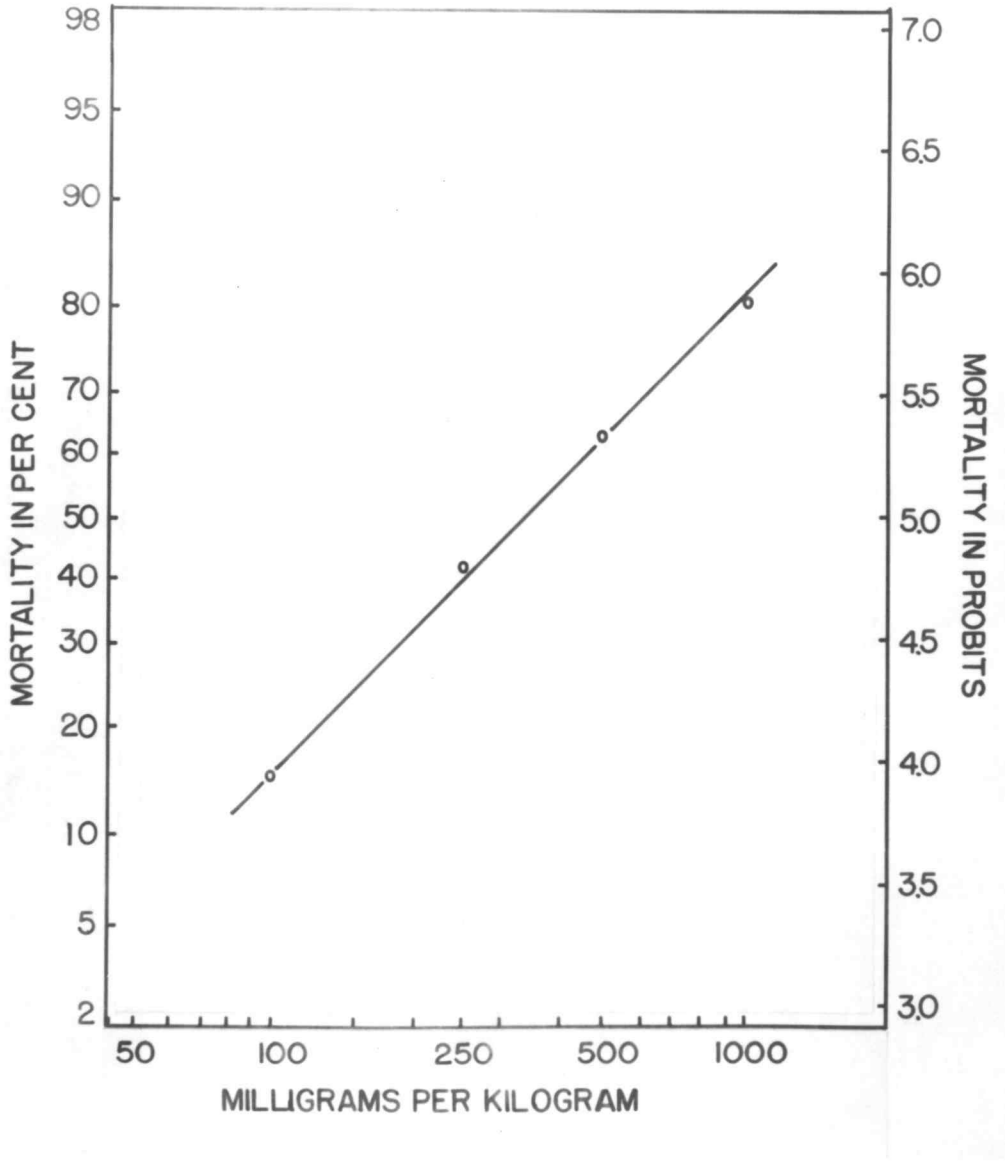


Figure 8. The effect of ronnel administered to rats as oral drenches at rates of milligrams per kilogram of body weight, upon mean mortalities in fleas.

Low mortalities may have been due to improper positioning or slipping of flea cell, or slight rotation of the rat so that the cell came in contact with a poorly shaved area.

The level of mortality in 48-hour old oriental rat fleas was increased from 36 percent to 88 percent by waiting 2 hours to expose fleas to test rats (Table 4). Plapp and Casida (32) demonstrated that ronnel is at a maximum level in the blood of a cow 8 hours after treatment, but the cow, compared to rats, metabolized and excreted it at a slower rate. Since these workers also showed that similar metabolic pathways are followed for either animal, it would be reasonable to assume that ronnel reaches a peak level in the blood of rats prior to 8 hours. It was known that fleas fed shortly after exposure to animals. For these two reasons a delay between exposure of fleas to test animals was important. This delay would allow a sufficient level of ronnel, after a single oral drench, to develop in the tissues of the host prior to the feeding of the fleas. Mortalities that occurred in fleas exposed within 2 minutes to treated rats were most likely due to their feeding more than once during the 24-hour exposure period and after sufficient ronnel had entered the tissues of the host.

Table 4.--Mortality in 100 fleas per cell exposed 24 hours to rats in squeeze cages. Single oral drench at 1000 mg./kg. Age of fleas 48 hours.

Minutes between treatment of rats and exposure of fleas to rats	Number of rats per test	Mortality 24 hours after removal from rat		Standard deviation
		Mean	Range	
2	7	36	28 - 47	± 8
120	7	88	71 - 97	± 9

Some fleas may have delayed feeding or were disturbed and did not feed until after the insecticide appeared in the tissues of the rats. No tests were carried out to determine if waiting longer than 2 hours to expose fleas would result in an increase or a decrease of the level of mortality.

Feeding Studies. Ronnel was fed to 4 rats at the rate of 325 mg./kg. for 6 days and to another group of 4 rats at the rate of 500 mg./kg. for 4 days. At the end of the last feeding period for each group of rats, 72-hour old fleas in cells were placed on each animal. Table 5 shows mean mortalities of 8 percent and 22 percent for the respective dosage levels.

Because ronnel diminishes in the blood stream after a few hours (32) and dosage levels much above 500 mg./kg. by feeding were not feasible, another approach was necessary in order to increase the number of fleas killed. In Table 6 are summarized the results obtained by feeding 12 rats for 4 days at various levels (3 each at 0, 100, 250 and 500 mg./kg.) in feeding cages. The last 3 days of the test the rats were placed in squeeze cages for 5 to 6 hours each day and 50 fleas were assigned to each rat and placed on it daily during this time. Although the total exposure of fleas was only 16 hours compared to the single 24 hour exposure period in the preliminary tests, there was a 4-fold increase in flea mortality.

Table 5.--Mortality in 100 fleas per cell to rats in squeeze cages. Ronnel fed to 4 rats for 6 days at 325 mg./kg. and to 4 rats for 4 days at 500 mg./kg. prior to exposing fleas.

Dosage to rats mg./kg.	Percent mortality 24 hours after removal from rat		Standard deviation
	Mean	Range	
0 1/	1	0 - 2	± 1
325	8	2 - 11	± 4
500	22	7 - 44	± 17

1/ Two rats used at this level.

Table 6.--Mortality in 50 fleas in cells on rats in squeeze cages. Rats fed ronnel for 4 days. Fleas exposed to rats last 3 days of feeding program.

Desired	Daily dose, mg./kg. Actual 1/				Mean dose mg./kg.	Percent flea mortality after final exposure		
	Day 1	Day 2 2/	Day 3	Day 4		24 hours	48 hours	72 hours
0	0	0	0	0	0	2	2	2
0	0	0	0	0	0	0	0	2
0	0	0	0	0	0	2	6	12
100	100	96	62	89	87	68	92	-
100	100	100	90	100	98	18	40	58
100	100	100	83	100	96	0	10	44
250	250	250	163	232	224	92	96	-
250	173	141	143	250	177	90	98	-
250	250	234	170	250	226	88	100	-
500	299	71	320	374	268	100	-	-
500	235	307	291	500	334	94	98	-
500	500	321	219	500	385	82	92	-

1/ Duration of rat feeding period for day 1, 20 hours; day 2, 13 hours; day 3, 10 hours, and day 4, 15 hours.

2/ Fleas exposed to rats for 5 to 6 hours on days 2 through 4.

A comparison may be made of the effect of ronnel on fleas when administered as a drench or in the feed to rats at the rate of 500 mg./kg. An average of 63 percent of fleas in cells died within 48 hours after being exposed 24 hours to orally drenched rats (Table 3). When ronnel was fed to rats at this level over a period of 4 days, only 22 percent of the fleas were killed under similar conditions (Table 5). However, 92 percent died when exposed on 3 consecutive days on rats ingesting the insecticide at an average rate of 329 mg./kg. over a 4-day feeding period (Table 6). These variations in flea mortalities could be expected as under drench conditions the fleas fed when ronnel was approaching or at the maximum level in the tissues. Since rats do not ingest their total meal within a short period as a dog or cat might, it then is likely that ronnel never reaches as high a level in the tissues as under drench conditions although the dose (mg./kg.) may be the same. However, because of the duration of the feeding period ronnel may be expected to be present in the tissues over a longer period of time than if it were administered as a single dose.

In the feeding tests it was found that a dose of 500 mg./kg. or a level of 5000 p.p.m. of ronnel in rat food was near the maximum level that was acceptable

to the rats. Bennington (4) reported similar observations in domestic rats, even where there was a free choice of food available.

Effects of Ronnel on Rats. In Experiments 1 and 2 no apparent toxic symptoms of ronnel were noted in treated rats. Three rats were drenched with ronnel at 1500 mg./kg. Of these, two died within 4 days and the third rat survived. The acute oral LD_{50} dose in rats is 1740 mg./kg. of ronnel of body weight (10).

EXPERIMENT 3

Effect on Flea Larvae. Early in the project several tests were carried out to determine insecticidal activity in the feces of orally drenched rats. The tests showed positive but inconsistent results (Tables 7 and 8). This was apparently due to at least three factors. Feces of animals orally drenched with the acetone-corn oil solution were oily and appeared to interfere with normal development of the larvae. The age or stage of development of the larvae used was not uniform. At time of treatment, residual food contents of the gastro-intestinal tract apparently varied from animal to animal in the same treatment level.

Because it was found that considerable natural mortality occurred in larvae before their first moult, a more critical selection of larvae was undertaken. However, little improvement in consistency in response to treatment was noted (Table 8).

Gross examination of the feces at time of grinding indicated that some feces appeared more oily than others, but no record of this was kept to correlate with larval mortality. It was later shown that flea larvae could not live in dog food that was free of insecticide but which analysed 7 percent fat. Flea larvae could complete their life cycle in the same dog food that was diluted 1:1 or

Table 7.--Toxicity to flea larvae of feces from rats in Experiment 1. Stage of development of flea larvae not uniform.

Number of test	Percent larval mortality 1/ in 48 hours at indicated dosage to rats. Mg./kg.					
	0	50	100	250	500	1000
1	20	-	0	10	100	100
2	10	-	60	100	100	100
3	0	-	0	0	0	100
4	30	30	20	100	100	
5	0	30	10	100	100	
6	0	10				
7	0	10				
8	15	85				
9	10	50				
10	35	100				
11	0	10				
12	0	70				
13	40	100				
14	0	100				
15	10	80				

1/ 20 larvae used at each treatment in each test.

Table 8.--Percent mortality in flea larvae in feces from rats receiving an oral drench at 50 mg./kg.

Number of test 1/	Mortality in 48 hours 2/		Standard deviation
	Mean	Range	
1	77	10 - 100	35
2	98	85 - 100	6
3	97	80 - 100	8
4	53	0 - 100	48
5	92	50 - 100	20

1/ 6 rats used in each test.

2/ 20 second instar larvae per fecal sample.

more with fine vermiculite. Since it is likely that digestive tract residual contents varied in amount from rat to rat, it was finally considered possible that a wide range in concentration of unabsorbed ronnel was occurring in the feces. It seemed possible that higher concentrations of ronnel would appear in the feces of dosed, starved rats than in the feces of animals recently fed.

Further tests were conducted using the feeding cages. An attempt was made to equalize the digestive tract contents of the rats by giving one pretreatment or conditioning feeding. In most cases after the first collection of feces from the rats fed ronnel, the mortalities in flea larvae were greater and more consistent within each dosage level. This indicated that residual contents of the digestive tract varied the concentrations of toxic materials appearing in the feces and influenced the levels of larval mortality.

The daily mean percent mortality of flea larvae from fecal samples of days 3 through 6 (Table 9) were averaged and were plotted on log-dosage probit scale as shown in Figure 9. It was determined from this curve that the feces from rats fed at the rate of 130 mg./kg. would be lethal to 50 percent of the flea larvae within 48 hours.

Table 9.--Mortality in flea larvae held 48 hours in feces from rats fed daily on ronnel in feeding cages. Feces collected daily.

Feces from day	Mean percent mortality in larvae 1/ at indicated dosage to rats. 2/ Mg./kg. 0	50	90	150	200	325
1	0	0	3.75	65.00	16.25	40.00
2	0	2.50	17.50	95.00	62.50	76.25
3	0	2.50	5.00	71.25	82.50	98.75
4	0	-	21.25	50.00	85.00	96.25
5	0	-	18.75	63.75	88.75	98.75
6	0	-	15.00	73.75	97.50	98.75
Mean of days 3 through 6			15	65	88	98
Standard deviation			± 7	± 11	± 7	± 1

1/ 20 second instar larvae per daily fecal sample.

2/ 4 rats used at each treatment level.

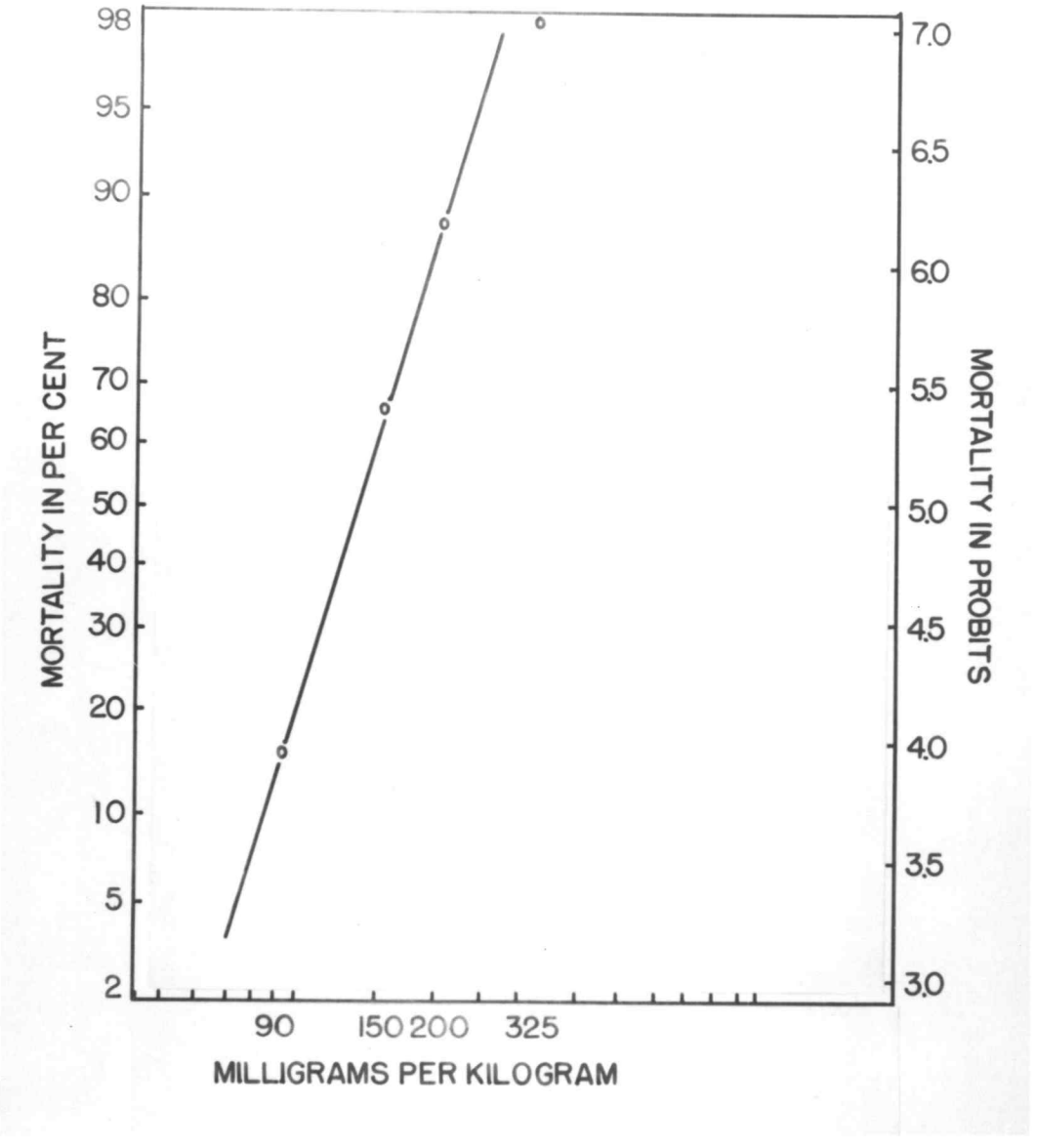


Figure 9. The relationship between doses of ronnel in milligrams per kilogram of rat body weight, administered in the feed of rats and mortality levels in flea larvae exposed to rat feces.

Standard Dosage-Mortality Curve. Ground and screened feces from rats never treated with insecticide were fortified with ronnel and bioassayed with flea larvae. The results of these tests are summarized in Table 10. There is no explanation as to why there was such a high standard deviation at the 200 p.p.m. level. The mean percent mortalities of the 7 replications at each concentration were plotted on log-dosage probit scale and are shown in Figure 10. It was determined from the curve that feces fortified with ronnel at a level of 190 p.p.m. by weight will kill 50 percent of the flea larvae within 48 hours.

The average concentration of ronnel in the food of each of 4 rats being fed a dose of 150 mg./kg. on 4 successive days was known to be about 1800 p.p.m. by weight. Feces from these rats induced larval mortalities of 65 percent (Table 9). The standard dosage-mortality curve (Figure 10) indicated a concentration of 220 p.p.m. ronnel for this mortality level. Since the slope of the standard curve and that of the feeding test (Figure 2) are very similar, it may be that this insecticide has not been chemically altered. If this is true, then it would appear that about 12 percent of the ronnel ingested passed through the gastro-intestinal tract unaltered. However, the physical state and distribution of

Table 10.--Bioassay with flea larvae of feces fortified with ronnel.

P.P.M. of ronnel	Percent mortality 1/ in 48 hours		Standard deviation
	Mean 2/	Range	
0	0	0	
100	4	0 - 8	2
150	24	12 - 32	7
200	57	24 - 84	22
300	90	76 - 100	9
400	98	96 - 100	2

1/ 25 second instar larvae per sample.

2/ Mean of 7 samples per treatment level.

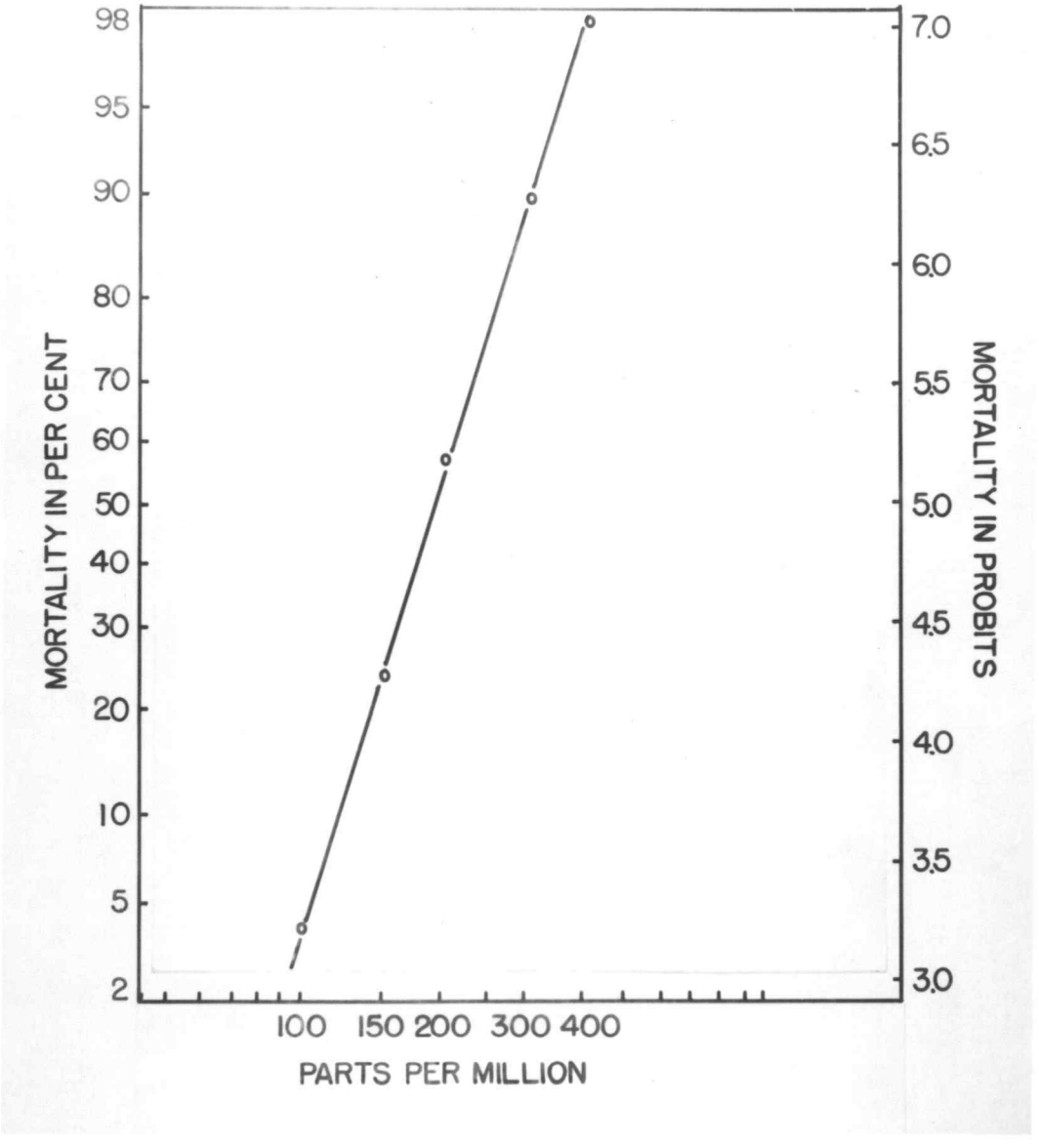


Figure 10. Mean percent mortality in flea larvae exposed to rat feces fortified with ronnel at 100 to 400 parts per million by weight.

insecticide in the feces from treated rats and in feces fortified with ronnel may not be the same. This would be at best only an estimate of loss of insecticide in transit.

Duration of Toxic Residues in Rat Feces. The feces from 5 rats given a single feeding of ronnel at the rate of 100 mg./kg. was collected during the 24-hour feeding period and at two 24-hour intervals thereafter. It was processed as previously described and challenged with flea larvae for toxic residues. A mean of 26 percent of the larvae were killed in 48 hours in the feces collected during the feeding period. The levels of mortality dropped to 4 percent and 0 percent for the following collection intervals (Table 11).

At a later date, 6 rats were given a single feeding of ronnel at the rate of 250 mg./kg. The feces were collected, processed and tested with flea larvae as before. A mean of 77 percent of the larvae were killed in the first fecal collection and 3 percent resulted in the second collection (Table 11). Since the level of kill on the second day was so low, the feces of the third day were not tested.

Table 11.--Mortality in flea larvae held 48 hours in feces from rats given a single feeding of ronnel in feeding cages. Feces collected at 24 hour intervals.

Feces from day	Percent larval mortality 1/						Mean mortality
	Rats treated at 100 mg./kg.						
1	5	35	20	60	10	-	26
2	0	5	15	0	0	-	4
3	0	0	0	0	-	-	0
	Rats treated at 250 mg./kg.						
1	65	85	95	80	45	90	77
2	5	5	0	0	10	0	3

CONCLUSIONS

The overall effect of ronnel acting as a systemic insecticide and as a contact poison in feces may have considerable value in controlling fleas and other disease vectors on wild and domestic rodents.

The preliminary test in Experiment 1 indicated that ronnel, when administered as an oral drench to rats, was toxic to oriental rat fleas. Further tests established that there was a relationship between the age of fleas and susceptibility to ronnel under contact conditions; newly emerged fleas were less susceptible to ronnel than aged fleas. This relationship, however, was not directly established for ronnel acting systemically. Mortality levels in fleas of a uniform age were more consistent than those in fleas where the ages ranged from a few hours to a week or more.

It was concluded from the data of Experiment 2 that ronnel, when administered to rats, was an effective systemically acting compound against oriental rat fleas. In single oral drenches timing of exposure of fleas was important. A delay must be allowed for sufficient levels of ronnel to appear in the tissues of the host after drenching. When fleas were exposed for 24 hours to rats fed ronnel at rates comparable to drench doses, low mortalities in fleas were observed. A significant increase

in mortality was recorded when fleas were allowed to feed for a short period on consecutive days on rats that received daily doses of ronnel in their food. These observations indicate that under conditions of practical usage, flea mortality may be as dependent upon the frequency with which ronnel is administered to rats as it is upon dosage level.

Preliminary observations on the presence of toxic materials in the feces from rats pointed out a high level of kill in flea larvae. However, mortality levels were erratic in feces from treated rats and there was mortality in larvae in feces from untreated rats. Further tests showed natural mortalities in larvae before their first moult and that oil in the feces of drenched rats was detrimental to the larvae. Residual food in the digestive tract at time of treatment acted as a diluent of the test food and therefore caused variations in mortality levels early in the tests.

The similarity of the slope of the feeding test of Experiment 3 and slope of the standard dosage-mortality curve suggest that ronnel passed through the digestive tract unaltered. The amount that passed through was calculated to be approximately 12 percent when the rats were fed ronnel at a rate of 150 mg./kg. This is at best only an estimate as neither the physical state nor the

distribution of ronnel in the feces was known. While the appearance of ronnel in rodent feces may have considerable effect upon flea larvae, the total effect upon flea populations will likely be minimized by the fact that after feeding has been discontinued, ronnel levels rapidly diminish in the feces.

The techniques and equipment designed for this problem may be of value in other toxicological studies. Oriental rat fleas were shown to be suitable for determining systemic properties of compounds in mammals. Flea larvae may be useful in bioassay techniques for directly determining insecticide in media suitable to the larvae.

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