LABORATORY EVALUATION OF TOXICANTS AND REPELLENTS AGAINST BITING FLIES

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AGAINST BITING FLIES

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PREFACE

One of the assigned responsibilities of the United States Department of Agriculture, Entomology Research Division, Insects Affecting Man and Animals, Biting Fly Laboratory, Kerrville, Texas is the evaluation of chemical insecticides and repellents that are candidates for use against insect pests affecting livestock. Since the pressure of other duties has obliged the author to seek methods of increasing the efficiency of that assignment, a critical review of past and current methods and an investigation of a possible new laboratory or "bench" method has been undertaken.

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INTRODUCTION

Biological methods of testing insecticides and repellents are utilized to evaluate promising materials, to supplement chemical analysis, to develop uses and formulations for practical application, and to make fundamental studies of relative toxicity, repellency, and mode of action. The physiological variability among individual insects of a species and the responses the insects exhibit to different environmental conditions make it imperative that evaluation methods be standardized to the greatest extent possible.

Each year several hundred chemical insecticide and/or repellent candidates are received at the United States Department of Agriculture, Entomology Research Division, Insects Affecting Man and Animals Laboratory, Kerrville, Texas, for evaluation against the biting flies of livestock. The initial evaluation of the chemical candidates is of upmost importance as it is the principal measure used to determine if a candidate is worthy of further testing. Therefore, the evaluation methods for processing the large numbers involved must be both dependable and efficient.

Over the years several evaluation methods for toxicants and repellents have been utilized at Kerrville and at other commercial, institutional, and agency laboratories. None, however, has been fully satisfactory in providing all required data from a single treatment and in a reasonable time period. An effective experimental design would make it possible to

secure data of: (1) repellency, (2) "knockdown," (3) initial or acute toxicity, and (4) residual toxicity, all in one application. The first three of these requirements can often be met by applying a candidate to an inanimate surface. Many modern toxicants, however, are retained on such substrates as glass, wire, and wood for extended periods and the results of bioassay tests on these residues as compared to tests on animals is seriously distorted. In the author's opinion the time required to complete a toxicant evaluation should be no more than ten days and preferably less than seven days for reasons of efficiency and economy.

The biting flies affecting livestock are represented by several species of diverse habits. Horn flies, *Haematobia irritans* (L.), remain on the host almost all of their adult life; stable flies, *Stomoxys calcitrans* (L.), feed and rest on the lower portions of the body and the legs intermittantly; horse flies, *Tabaninae*, deer flies, *Chrysopinae*, and mosquitoes, *Culicidae*, remain on the animal only long enough to secure a blood meal. The period of time that the insects come in contact with a residue applied to the host is therefore highly variable, particularly between species. Due to this variability and because of the ease of colonizing the stable fly, this species has been selected as the standard for toxicant and repellent evaluation studies at Kerrville.

The objectives of the present study are to re-examine past and present methods of evaluating toxicants and repellents for control of the biting flies of livestock, to investigate the feasibility of using an animal membrane laboratory method, and to recommend a suitable and efficient laboratory method for use at the Kerrville facility.

REVIEW OF THE LITERATURE

Although the assay of pesticide residues has been used for relatively few years, the principle of bioassay is not new. In pharmacy, for example, techniques have been developed, standardized, and have been used extensively for testing the potency of drugs. Like the bioassay of drugs, the evaluation of pesticide toxicity and length of residual action is founded on physiological responses of the insect or other test organism. Responses of insects that are usually considered relative to these studies are: (1) apparent death, (2) "knockdown," (3) irritability, (4) repellency, and (5) reduced feeding.

The toxicant may be administered as a spray, dip, dust, deposit or residue applied to a surface, microdrop treatment applied directly to the organism, an injection into the insect, or mixed in the feed. The most common method for biological assay of residual insecticides involves the use of insecticidal deposits on test surfaces such as filter paper, cloth, glass, vials, Mason jars, plywood, pressboard, and other absorptive materials (Nagasawa, 1959). Until about 1950 evaluation of insecticides for control of biting flies of livestock usually was carried out in the above manner or through treating either mosquitoes (*Culicidae*) or house flies *Musca domestica* (L.) with sprays by the Peet-Grady spray chamber method (NAIDM, 1951).

A dependable source of uniform test insects is required and the tests should be conducted under constant environmental conditions.

Multi-purpose evaluation techniques often of necessity are complex and just as often difficult to appraise, thus the general practice in bioassay studies has been to develop a separate technique for each division of information desires: e.g., one each, for speed of effectiveness, residual effectiveness, repellency, systemic effectiveness, et cetera. Each of the techniques usually requires its own specialized equipment.

Space Spray Equipment and Techniques. - Prior to the advent of the modern synthetic organic insecticides, transient mists and sprays were the common insect control agents for external livestock arthropod pests and thus equipment and methods developed for evaluating control agents stressed spray equipment. Tattersfield and Morris (1924) constructed a tabletop spray device consisting of a 44 X 19.5 cm glass cylinder and a spray atomizer. The test insects were placed in the petri dish at a fixed distance from an atomizer that was secured to a lid covering the top end of the cylinder. The atomizer was adjusted so spray fell as evenly as possible on the dish. After treatment the test insects were removed to clean holding cages and observed for mortality.

In 1928, C. H. Peet and H. G. Grady wrote a series of papers entitled Studies in Insecticidal Activity, and therein indicated the need for and suggested that a definite standard be adopted for time, temperature, humidity, insecticide concentration, spray concentration, pressure in sprayer, air condition and condition of insect. They described in detail a 6 X 6 X 6 ft chamber, the accessories required, and the procedure in conducting a test (Peet and Grady, 1928). Modifications of this method are still in use in 1965 by members of NAIDM (National Association of Insecticide and Disinfectant Manufacturers) as the standard evaluation technique for insecticide in oil sprays.

Campbell and Sullivan (1934, 1938) designed a settling mist apparatus for testing fly sprays that consisted of ten 17 X 8 inch aluminum cylinders mounted equidistantly around a 46-inch aluminum turntable. The cylinders had removable covers, each with a 3/4 inch hole in the center through which the insecticide was applied from a spray gun mounted on a movable arm attached to the center of the turntable. Screen covered petri dishes containing the test insects were placed in each of the ten cage holding "wells" located at the bottom center of the cylinders. The turntable was rotated so each cylinder in turn came beneath the spray gun. The insects were exposed to the settling mist for a specific time and then were removed to clean holding cages. Several authors, including Allen et al. (1943), Webb (1947), and Parr and Busvine (1948), have reported on modifications of the vertical settling tower but the principle in each case was essentially the same.

Probably the most versatile and extensively used of the settling tower designs was that described by Potter (1941, 1952). It was readily adapted to spraying insects directly or to the preparation of residual films. The apparatus consisted of a 27-inch long metal spray tower circular in cross section that was fixed in a stand over the spray table. The table consisted of a circular metal plate that could be lowered or raised beneath the tower. The distance between the spray table and the tower thus was adjustable and was a factor in the distribution of the spray. Insects could be sprayed by placing their container on the table, raising it into position, and then actuating the constant pressure air system. To prepare residual films the same procedure was used except the surface to be treated rather than the caged insects, was placed on the spray table. Insects were subsequently placed on the treated surface

for a given period of time or at intervals after treatment in order to evaluate either toxicity of the deposit or the residual life of the toxicant.

Hoskins and Caldwell (1947) designed a horizontal settling chamber that differs from the above chambers only in that the horizontal position allows fall out of the large spray droplets before they reach the test insects caged in a depression at the far end of the cylinder. Thus this technique attempts to treat the insects with droplets of a relatively constant and small size.

Roan and Kearns (1948) described a spray apparatus through which the spray was drawn by negative pressure. The spray was dispersed into a mixing chamber shaped to produce turbulence, then drawn through a 16 X 18 mesh wire screen cage containing the test insects. The insects then were removed from the spray chamber to clean holding containers. During the period 1950-1960 interest in space sprays for control of livestock insects lagged although several modifications of the Roan and Kearns design were reported. The present author and Mr. C. M. Gjullin constructed a similar device at Corvallis, Oregon in 1954 equipped with flow gauges and variable speed fan in order to control speed and amount of air flow. With this modification we were able to reduce air flow when working with fragile insects like mosquitoes or increase it when using stout insects like house flies or stable flies (Hoffman and Gjullin, 1954).

Potter and Way (1958) present numerous other examples of modifications of the several spray devices mentioned above.

Beginning about 1960 interest was revived in the use of space sprays for the control of livestock insects, particularly the use of concentrated, low volume sprays dispersed by automatic or semiautomatic devices

(Berry and Hoffman, 1963; Hoffman, Berry and Graham, 1965) but the tests, thus far, have been semifield studies utilizing cattle and sprayers set up as for practical use out-of-doors.

Measured Drop, Injection, and Dipping Methods. - Dipping individual or small groups of insects in insecticide suspensions of various concentrations has been reported by several authors but, as pointed out by McIntosh (1947), the data are highly variable depending on solvent, wetting agent, particle size of solute, and other physical and chemical variables. In general, it is not a method of choice for treating livestock insects at the laboratory evaluation level.

Treatment by injection often has been used to compare the toxicity of chemicals to insects when the chemicals involved varied markedly in their ability to penetrate the cuticle. Shafer (1915), in studies on how contact insecticides kill, compared the toxicity of several early insecticides applied to the surface and injected into the body cavity. Later McIndoo (1937) and Hansberry et al. (1940) used a micrometer, syringe, and hypodermic needle similar to that described by Trevan (1922). They treated adult blowflies and several species of lepidopteron larvae with nicotine.

Campbell (1926, 1932), in a survey of new insecticides, used for his injections a calibrated capillary tube on which was drawn a fine glass needle. He controlled the liquid in the pipette by air pressure on the insecticide column. Heal and Menusan (1948) modified Campbells micropipette by mounting it on a board in a vertical position adjacent to a vertical mercury column. The mercury column height was regulated by the manipulation of a screw controlled plunger and the two columns were connected by a U-tube. Delivery of the pipette was accurate to one microliter.

In recent years most investigators have used some version of the micrometer and syringe for injection studies rather than the more sophisticated delivery systems. Kearns (1949), March and Metcalf (1949), and Buck (1949) have each demonstrated the reproducibility of the syringe application and have commented on its simplicity and availability.

The microdrop and micropipette equipment for applying a single measured drop to a particular location on an insect has been utilized extensively for critical evaluation of the acute toxicity of a chemical and in studies of the relative toxicity of several chemicals. The equipment is essentially the same as that described for injection use, ordinarily consisting of a 0.25 ml tuberculin syringe aligned between a micrometer and a bent, blunt 27 gauge needle (Dutky, 1942; Kearns, 1945; March and Metcalf, 1949; Bruce and Decker, 1950; Kerr, 1954; Dahm et al., 1961).

Application and Evaluation of Surface Deposits of Insecticides. - Potter (1938) evaluated residual applications of pyrethrins as a control for warehouse insects by treating sacking and placing the sacks in an infested building. He was able to demonstrate mortality of several species of moths up to 10 days. In 1941 Potter developed the previously described settling tower with which he could produce a consistent surface deposit and thus expect replicated data or comparisons to be reasonably reliable.

When DDT first became available Tattersfield and Potter (1943) impregnated filter papers with solutions of the insecticide in volatile solvents and evaluated the residues by bioassay using the red flour beetle *Tribolium castaneum* (Hbst). Morrison (1943) lined vials with DDT treated pieces of filter paper to which he exposed several species of insects. He varied both time of exposure and concentration of insecticide to

estimate effectiveness of the residues. Busvine and Barnes (1947) treated each of several 9 cm filter papers with 1 ml of a concentration series of DDT in a volatile solvent, then confined their test insects to the surface under petri dishes for a given length of time. With the dosage-mortality data obtained they were able to prepare a probit regression line and estimate the LD_{50} (LD = lethal dose).

In recent years the World Health Organization (WHO) has developed a test kit based on the treated paper concept. This kit has been used principally for testing mosquito resistance in the field. The device consists of two 5 X 1 3/4 inch screwtop, clear plastic cylinders secured in an end-to-end position by means of an intervening plastic panel that contains 2 sets of female threads and a shutoff slide. One of the pair of cylinders is lined with insecticide treated paper; the other one holds the insects before and after treatment. The convenient design and size has led several investigators to evaluate the kits usefulness as a bioassay tool for other insect species (Mount, 1964; Morgan, 1964).

Wood, particularly 6 X 6 inch plywood squares, often has been used as a substrate for laboratory testing procedures of residual insecticides. Teotia and Dahm (1950) reported on extensive studies concerning the residual toxicity of 5 organic insecticides sprayed on unpainted, painted and whitewashed plywood panels. They kept the panels out-of-doors between tests to simulate practical conditions and concluded that the residual effectiveness of insecticides is dependent upon at least four complex factors: the formulation used, the type substrata to which applied, the prevailing environmental conditions and the insecticide itself. Hopkins and Hoffman (1955) sprayed the toxicant Dilan and various candidate synergists on unpainted plywood panels with a simple bulb-type

atomizer in studies of the insecticidal activity toward resistant and nonresistant house flies and nonresistant stable flies. Flies were contained under petri dishes on the panels for constant, predetermined intervals of time one day each week for 6 weeks.

Residual tests also have been accomplished on glass panels in a manner similar to those conducted on wood (Teotia and Dahm, 1950; Bruce, 1949). Treatment has usually been by bulb or compressed air atomizers using a volatile solvent as the insecticide carrier. Results of tests on glass as reported by the above authors indicated the toxicity of dieldrin, parathion, chlordane, aldrin and lindane to house flies was greater when on glass than on plywood if weathering was not a factor, but when exposed to outdoor environmental conditions the glass surfaces lost their toxicity more rapidly than the absorptive wood surfaces.

Another glass surface used as a substrata for residual insecticide tests is the common fruit (or Mason) jar. Hoffman, Roth and Lindquist (1951) treated quart fruit jars with acetone solutions of several chlorinated hydrocarbon insecticides by introducing the amount of solution required to provide a desired mg/ft^2 deposit; they rotated the jars until the solvent evaporated thus producing a relatively equal distribution of toxicant over the entire inner surface of the jar. House flies or stable flies were used as bioassay organisms by confining flies to the jars for stipulated intervals of time, removing them to clean holding containers, and recording mortality 24 hours later. Hoffman and Lindquist (1949) utilized the same type of treatment in the investigation of the fumigating properties of several insecticides by placing the test flies in a clean quart jar and placing the clean jar in a mouth-to-mouth position over the treated jar.

Bushland et al. (1944, 1945) described a test method that consisted of treating small circles of woolen cloth by dipping the cloth in acetone-insecticide solutions or by spraying the cloth with a known amount of solution. Eddy and Bushland (1948) standardized the above method with woolen cloth cut to fit the bottom of a 50 ml beaker, used acetone as the solvent, and treated the cloth by dipping. This test was designed particularly for studies with lice.

Lindquist and McDuffie (1945) treated small, cylindrical screen-wire cages by dipping them in acetone solutions of toxicants and subsequently confined mosquitoes or biting flies within the cages to determine knock-down time and the length of effectiveness of the residue. A similar technique was employed by Eddy and McGregor (1947) for evaluation of experimental insecticides against the stable fly.

Eddy and McGregor (1949) described a method whereby white mice, sprayed with toxicants or repellents, were used as test animals in stable fly studies. The chemicals were applied to the mice as acetone solutions with an electric air-blowing paint sprayer. Twenty-four hours later the mice were exposed to hungry flies to determine the degree of toxicity and/or repellency of the chemicals to the feeding flies.

Roberts et al. (1960) developed a technique for evaluating toxicants and/or repellents on cattle that consisted of applying the materials to isolated, circular areas 6 inches in diameter on the upper half of the body of grown Hereford steers. The test material was dissolved in acetone and 5 ml applied with an atomizer using compressed air as a propellent. The spray was confined to each area with a metal cone shield.

Cages were made from Mason jar lids by soldering a piece of screen wire in place of the removable top. Each cage was supplied with a metal tray and the two elements were held together by flanged edges of the tray.

Stable flies that had not fed for 18 hours were placed in the cages and contained therein by sliding the cages onto a tray. When the investigator wished to expose the flies to the treated surface, he slipped the cage from the tray onto the treated spot and secured the cage in place on the animal by rubber bands and metal clips. After a 20-minute exposure the cage and flies were slid back onto the tray and then removed to a clean holding cage in the laboratory. Mortality was ascertained 24 hours later. Repellency was determined on the basis of the number of flies that took a blood meal as compared to the number that fed on an untreated control spot.

Methods of Evaluating Systemic Chemicals Against Blood Feeding Flies. - Rodhain et al. (1912) successfully fed tsetse flies through rat skin membranes during their studies on African trypanosomiasis. Since then many techniques and membranes have been used for the *in vitro* feeding of haematophagous arthropods in experiments directed toward disease or parasite transmission. Yoeli (1938), for example, was able to infect *Anopheles elutus* with *Plasmodium falciparum* by feeding the mosquitoes infected blood through rabbit skin membranes. Bishop and Gilchrist (1946) were able to infect *Aedes aegyti* with *Plasmodium gallinaceum* when these mosquitoes were fed infected blood through a chicken membrane.

Tarshis (1958) presented a comprehensive list of the various membranes and arthropods involved in *in vitro* feeding studies up to 1956 and the reader is referred to his paper for data on the materials employed by different investigators. It is of interest to note that only Totze (1934) and Ferris and Hanson (1952) included the stable fly as a test insect. The former was testing cellophane, the latter egg shell membrane as possible feeding membranes.

Studies on the effects of systemic chemicals utilizing the *in vitro* feeding concept were conducted by de Meillon et al. (1948) by allowing *Cimex lectularius* L. to feed through mouse skin to imbibe treated blood. Bar-Zeev and Smith (1959) used repellent treated ox caecum membranes as the bottom of dishes containing warm blood in their studies to develop an *in vitro* repellency test for mosquitoes. The cage containing the mosquitoes was brought into contact with the treated surface and repellency was estimated by the feeding response as compared to a similar cage of mosquitoes feeding through an untreated membrane.

Granett (1960) modified the Bar-Zeev and Smith equipment and procedure for his studies of repellents for stable flies. He used an animal-derived membrane treated on one side with repellent and secured so as to encompass the end of a 2½ inch length of Lucite tube. Stable flies were placed in a separate piece of tubing, enclosed by cheese cloth covers, and then released into the treated section of tubing by removing one piece of the cheese cloth. At the time of testing, citrated beef blood was warmed to 100 F. and 5 ml pipetted into a stendor dish. The membrane was brought into contact with the warm blood for 10 minutes after which repellency was determined by the number of stable flies that had not fed.

In vivo techniques of evaluating systemic toxicants commonly have used white mice, rats or guinea pigs as the host animal. Lindquist, Knipling and Jones (1944) demonstrated that bed bugs (*Cimex lectularius* L.) were killed by taking a blood meal from rabbits that had received oral doses of DDT 3 to 5 hours earlier. They were able to show similar results with stable flies after treating rabbits orally with pyrethrins. McGregor and Bushland (1956) and Drummond (1958) described a procedure for determining

systemic effectiveness of oral or injected toxicants administered to guinea pigs on screw-worm larvae, ticks and stable flies. Four hours after treatment the guinea pig was placed in a V-shaped restraining stock and exposed to stable fly feeding by bringing a screen cage containing flies in contact with a shaved area on the pig. The cage was left in contact until most of the flies had fed; at this time the cage was removed and placed in a constant temperature cabinet at 80 F Mortality was ascertained 24 hours later. Hewitt et al. (1958) reported a similar technique using white mice and mosquitoes.

Graham (1960) described a semi-field test for systemic insecticides in which 6-8 month old calves were treated by the oral route and at intervals after treatment captive stable flies were allowed to feed on the calves. To test dermally applied systemics, Graham secured a calf in a stanchion, fastened a canvas collar and shield about its neck, and sprayed the body of the calf. At intervals after treatment, he exposed stable flies to the untreated head of the animal, allowed the flies to feed, and 24 hours later determined mortality.

Rearing the Test Organism. - A uniform test organism is perhaps the first requirement of a successful bioassay technique. At the present time culture of the stable fly appears to have progressed to the point where the fly has attained this status; however, ready colonization has been a relatively recent accomplishment.

Newstead (1906) and Mitzmain (1913) successfully reared the stable fly, *Stomoxys calcitrans* (L.), in small numbers in the laboratory to study its life cycle and habits but were not able to colonize the species. Glaser (1924) reared 5 generations of *Stomoxys* in glass battery jars using fresh horse manure for a medium. He fed the adults on live

animals, as well as on defibrinated, whole horse blood. The latter he placed as drops onto the covers of the rearing jars.

Doty (1937) provided the first successful artificial media for rearing Stomoxys. His formulation was composed of wheat bran, alfalfa meal, water, Diamalt, and yeast and was constituted similarly to the C.S.M.A. (Chemical Specialists Manufacturing Association) medium used for rearing house fly larvae. He also found it necessary to add dry oat hulls to prevent packing of the medium. The flies were allowed to egg in trays of the same medium and he fed warm, citrated blood in petri dishes placed within the holding cages. Eagleson (1943) reported on a technique similar to that of Doty except that he used fermented, chopped alfalfa hay as his larval medium and added dry rolled oats to the medium each day during the larval development.

Campau et al. (1953) reported that he reared large numbers of stable fly by a simplified procedure having several points in common with the Peet-Grady method (1951) for rearing house fly. He used the same C.S.M.A. media, cages, and temperature and humidity conditions. He fed citrated blood in dishes in the cages and collected eggs on the partially dried excess blood. In 1954, Champlain, et al. modified the Campau technique by: (1) egging on moist cellulose sponges placed in the breeding cages, (2) use of sunlamps to stimulate oviposition, and (3) reduction of cage size to 14 X 12 X 12 inches. Also in 1954, Born reported on the use of sand in larval rearing pans to suppress mold and to localize site of pupation.

Between 1954 and the present, several investigators have reported modifications of the Campau rearing procedure; each modification designed to provide adequate numbers and simplify the rearing of a continuous,

hardy laboratory strain of stable flies under the conditions of the particular laboratory. At the United States Department of Agriculture, Agricultural Research Service, Entomology Research Division, Insects Affecting Man and Animals Laboratory, Kerrville, Texas, McGregor and Dreiss (1955) reported excellent results with a larval media consisting of 1 part of volume C.S.M.A. medium and 5 parts hardwood shavings blended together and moistened with water to the point that one drop of water could be squeezed from a handful of the mixture. No yeast or malt was added. Glass battery jars were half filled with the medium and the same volume of fly eggs was seeded into each jar by pipette. The jars were covered with cheese cloth and maintained at a constant temperature of 80 F. Emerging flies were caged each day and fed citrated blood from glass tubes pressed against the outside of the screen cages. Oviposition was obtained on black cotton cloth wrapped about a 1-inch ball of damp absorbent cotton to which a few drops of 5 per cent ammonia solution was added to stimulate the flies. This technique produced a uniform culture of stable flies with a 21-day cycle.

Roberts and Jones (1957) further altered the technique by using square 20 gallon wash tubs as larval rearing containers, fed the flies from blood soaked cotton pads through the screen holding cages, and collected eggs on a wet, black cotton cloth pressed against the underneath side of the cages. Except to remove flies, they eliminated the necessity of opening the cage. This technique was capable of supplying large numbers of flies, but handling and cleaning of the tubs required considerable manpower and production of larvae and pupae was less uniform than in colonies in which larvae were reared in smaller containers.

METHODS AND MATERIALS

Test Animals, Rearing, and Handling Techniques. - The Kerrville strain of the stable fly, *Stomoxys calcitrans* (L.), was used as the test insect throughout the current study. This strain had been reared continuously during the previous 10 years at the Kerrville, Texas, United States Department of Agriculture laboratory.

White mice of an unknown strain were obtained from a commercial source for use in tests of the Eddy and McGregor technique. The mice were housed in typical disposable plastic mouse cages before and between tests.

Eight 1500-1800 pound Hereford steers were selected as host animals for the spot test studies on the basis of hair color, length, diameter and density. Treated steers were confined to 9 X 10 foot, part-screen, large animal rooms under regulated temperature and lighting conditions. When not "in test" the animals were free to roam a 500 acre pasture.

The stable fly rearing technique in use at the time of the study was basically a modification of the Campau et al. (1953) procedure but was also influenced by the ideas of McGregor and Dreiss (1955) and of Roberts and Jones (1957). The facility that housed the stable fly colony, the equipment used in handling, and the specific details of rearing the flies at Kerrville were either designed or initiated by the present author and were directed to the purpose of providing a controllable environment and a uniform insect colony.

At Kerrville, a new biting fly laboratory was constructed in 1962 and the stable fly colony rooms were purposely located in a central position

of the building to reduce the possibility of other insects or parasites contaminating the cultures. The colony facilities consisted of two adjoining rooms, one 9 X 15 ft for larvae, and one 9 X 9 ft for adults and the equipment used in handling adults prior to and during test procedures. The rooms were maintained at a temperature of 78 F ± 2 and a relative humidity of 50-55 per cent. Overhead fluorescent lighting was controlled by a time clock programmed to provide 12 hours of light and 12 hours of darkness.

At Stillwater, all tests were conducted in one of the University insectary laboratories at room temperatures of 72-78 F and adult flies were held at all other times in constant temperature cabinets heated by electric lights and controlled by thermostats to 78 F ± 2. Relative humidity in the cabinets was kept at approximately 50 per cent by maintaining an excess of calcium nitrate [$Ca(NO_3)_2 \cdot 4H_2O$] in the cabinets.

At Kerrville, adult flies were housed in 12 X 12 X 18 inch cages consisting of 3/4-inch fir plywood ends, 18-mesh plastic screening, and four 1 X 2 X 18 inch wooden slats (Fig. 1). The screening was stapled to the plywood ends, stretched tight, and the entire unit made rigid by fastening the wooden slats between the ends on the outside of the plastic screening. A cotton cloth sleeve was stapled so as to enclose a 3 X 8 inch opening in one of the wood end pieces. This cage design had the advantages of: (1) low initial cost, (2) simplicity of construction, (3) ease in replacement of screening, (4) ease in cleaning, and (5) low physical injury to the flies. Each cage could house approximately 2000 flies without excessive mortality. At Stillwater, the adult flies were housed in a commercially manufactured 12 X 12 X 12 inch aluminum frame and screen cage (Fig. 2). This latter cage was capable of holding about 1000 flies.

Fig. 1.--Kerrville stable fly cage and vacuum powered aspirator tube.

Fig. 2.--Stillwater stable fly cage and mouth operated aspirator tube.

In both localities the flies were fed warm (85-95 F) citrated beef blood twice daily (8 AM and 4 PM) by wetting a 4 X 4 X 1 inch piece of synthetic sponge in warm water, squeezing out most of the water, and then rewetting the sponge with about 20 ml of warm blood. The sponge was then laid on the top, exterior of the cage and the flies readily fed through the screen mesh.

Eggs were collected on 4 X 4 inch wet, black-colored, cotton cloth that rested on a water saturated synthetic sponge. A petri dish containing the sponge and cloth was placed beneath a cage of flies and the flies readily deposited their eggs on the cloth through the screen mesh. Eggs were removed at approximately 8 AM and 4 PM daily and seeded into larval rearing medium (Fig. 3).

Larval rearing medium consisted of 1 part by volume standard C.S.M.A. media blended with 3 parts of wood shavings (Fig. 3). About 8 quarts of the mixture was placed in a 16 X 16 X 6 inch plastic dish pan and water was added to the point that only a drop could be squeezed out when the medium was compressed in the hand. From 0.3 to 0.4 ml of eggs were pipetted onto the surface of the medium and a double thickness of cheese cloth was securely fastened over the top of the pan to prevent entrance of other organisms and help retain medium moisture.

Larvae completed development and pupated on the 12th and 13th days after eggs were seeded. Pupae were removed from the medium by flooding the medium pans with water, disturbing the top 1 inch of the medium with the hands, and allowing the pupae to float to the surface through the holes of ½ inch mesh hardware cloth. The pupae were poured off into an 18-mesh wire screen tray and the tray of pupae placed into a forced-air drying box. Larvae that had not pupated by the 13th day were discarded.

Fig. 3.--Eggs on egg cloth and pan of stable fly larval medium.

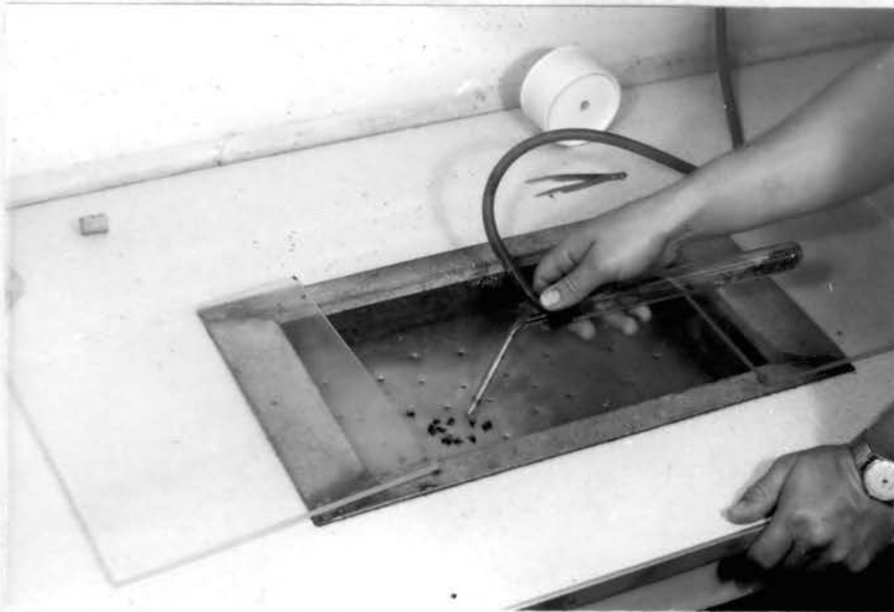

Fig. 4.--Stable fly females on cold tray being removed by vacuum aspirator.

After the pupae were dry, they were placed in ½ pint paper cartons and put into a clean cage. Adult emergence occurred from the 2nd through the 5th day thereafter but the bulk of the flies emerged on the 3rd and 4th days. Thus the egg to adult cycle averaged 17 days. During the Spring of 1965, pupae were shipped one day each week from Kerrville for use at Stillwater,

Efficient equipment and methods for handling the test organisms is an important phase of any evaluation technique. At Kerrville, flies were removed from cages with a 1.5 inch O.D. plastic aspirator tube attached to the air intake of a toy, tank-type vacuum cleaner. Carbon dioxide was used to immobilize the flies temporarily in the aspirator tube before they were spread out on the 38 F surface of a refrigerated cold tray (Harris, Hoffman and Frazer, 1965). The flies were separated according to sex and the females were aspirated into a glass tube (Fig. 4) by the negative pressure of a vacuum pump and then distributed according to the needs of the particular test procedure.

At Stillwater, flies were removed from the cages by mouth controlled aspirator tube (Fig. 2), lightly anesthetized with carbon dioxide, poured into a ½ pint cardboard carton, and kept under carbon dioxide anesthesia during the time required to select and transfer the females to pretreatment holding cylinders.

Tests on Plywood and Glass Surfaces. - At Kerrville, 6 X 6 X ¼ inch pieces of unpainted fir plywood were sprayed with 5 ml aliquots of acetone solutions of 12 candidate toxicants and/or repellents (Table 2). The solution concentrations were adjusted to provide deposits of 25 mg/ft^2 of the technical chemical except pyrethrins was applied at 2.5 mg/ft^2. The sprays were applied through an artists air brush and using an air

supply adjusted to deliver 5 psi. After treatment the panels were aligned on a table in the laboratory and remained in the same position throughout 6 weeks of test procedure.

Twenty-four hours after treatment 25 female stable flies were confined to each panel under 90 mm petri dishes (Fig. 5). The flies were exposed for 20 minutes, during which time the flies were observed for knockdown. They were then immobilized with carbon dioxide, transferred to clean ½ pint cardboard holding cartons, and held for 24 hours in the stable fly adult room before mortality was assessed. The above procedure was repeated with the same panels 7, 14, 21, 28, 35, and 42 days following treatment in order to evaluate the residual effectiveness of the deposits. The test was replicated once and the data presented are an average of the 2 tests. Additional replications were not deemed necessary for the purposes of this evaluation, as the data obtained from the 2 tests compared favorably with data the author had obtained in previous testing of these materials in other years.

Treatment of quart glass Mason jars was accomplished by introducing acetone solutions of the technical materials (Table 3) into the jars and rotating the jars until the solvent evaporated. This procedure left a film of toxicant crystals over the inner surface of the jars and likewise contaminated the screen lids that had been allowed to tumble in the jar as it rotated (Fig. 6). Treatment rate, test conditions, and data recorded were the same as for the plywood panel tests.

Tests Using White Mice. - Adult white mice weighing 25 to 30 grams were used as the test animals. Each mouse was treated with 10 ml of an acetone solution of one of the candidate toxicants and/or repellents (Table 4). Treatment of the mice was accomplished by placing a mouse in a 1-inch

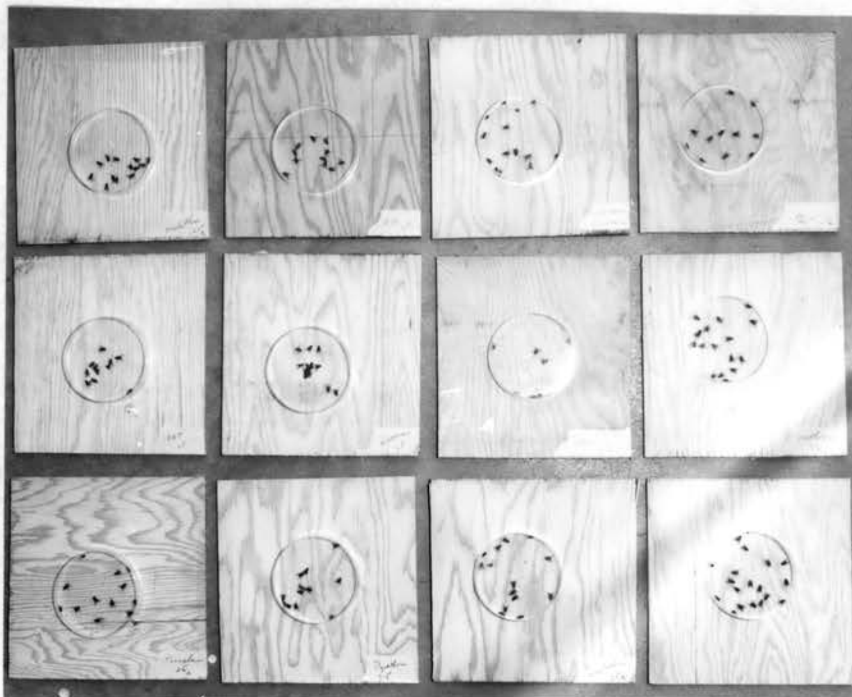

Fig. 5.--Typical plywood residual deposit test setup.

Fig. 6.--Quart fruit jars treated with acetone solutions of toxicants.

diameter, 4-mesh hardware cloth tube, attaching the tube to a variable speed laboratory mixer geared to rotate at approximately 10 revolutions per minute, enclosing the rotating cylinder and mouse in a slotted glass spray tube, and applying the spray through the slot with an artists air brush. Following treatment each mouse was placed in a separate holding cage.

Twenty-four hours later the mice were confined to clean hardware cloth restraining tubes, the tubes put into 3 X 10 inch, cylindrical 18-mesh screen cages, and 20 starved female stable flies released therein (Fig. 7 and 8). The flies were exposed for 20 minutes, then were removed to clean holding cages and the numbers of fed and unfed determined. The next day 24 hour mortalities of stable flies were ascertained. Exposure to stable flies was repeated with each mouse on the 3rd, 5th, and 7th days to determine the residual effectiveness of the chemicals.

Spot Tests on Cattle. - The spot test was conducted basically as described by Roberts et al. (1960), except for certain refinements of technique associated with improved facilities and equipment and the use of only female flies as test insects. The principle refinements were: (1) sprays applied through an artists air brush at a constant 10 psi air pressure, (2) treated steers confined to part-screen, large animal rooms at all times during the 8-day test period, (3) two sun lamps turned on for 4 hours each day in each animal room, (4) flies immobilized by cold temperature rather than carbon dioxide during most of the pretreatment handling, and (5) the use of female flies only.

Toxicity was measured by the per cent mortality of the flies 24 hours after exposure, and knockdown by the number of the flies unable to fly or walk immediately after they were removed from the test area. Repellency

Fig. 7.--Mouse confined to restraining tube being attacked by stable flies. Illustration in glass for better photographic demonstration.

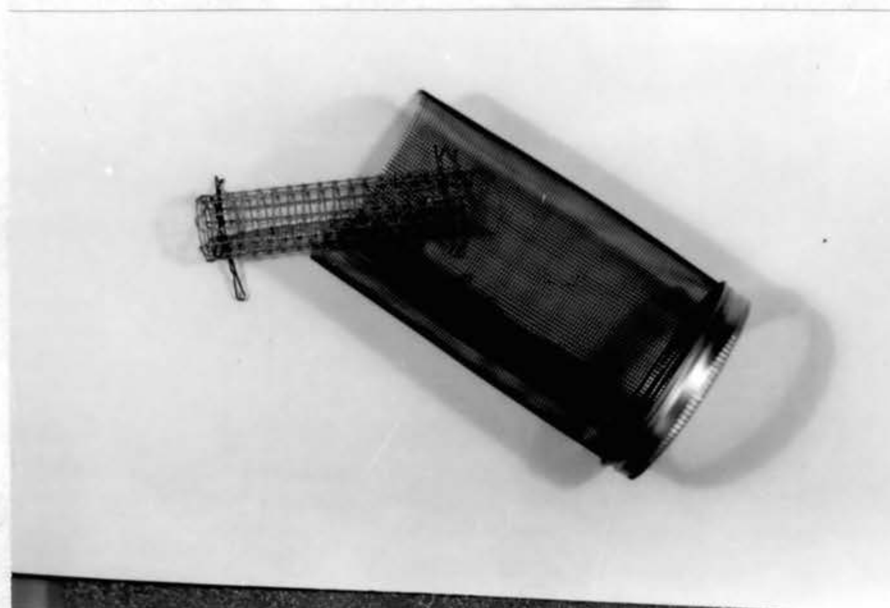

Fig. 8.--Mouse restraining cage, Bobby pins and typical wire screen cylinder used in mouse-insecticide evaluation technique.

was measured by subtracting the per cent of flies that fed from 100 per cent. When any of the values fell below 90 per cent, the material was considered to have failed for that effect.

Figures 9 and 10 illustrate a treated spot, spot test fly cage and tray, clips and bands for securing the cage in place, and the manner the cage was placed in treatment position. The toxicants and/or repellent candidates evaluated for purposes of this paper are listed in Table 5.

Tests with Treated Membranes. - During the early experimental work at Kerrville several membranes of synthetic and animal origin were investigated as surfaces through which stable flies could feed (Table 1). The membranes were stretched over 55 mm petri dishes of warm, citrated beef blood and placed beneath inverted pint fruit jars that contained 20 starved stable flies. Ten replications were completed with each membrane, thus providing a total of 200 flies per membrane. The extent of fly feeding and the physical characteristics of the different membranes were observed closely. At Stillwater, 3 additional membranes were obtained and were explored using the equipment described below.

As liquid blood soaked through some membranes or formed droplets at the site of stable fly punctures, a feeding technique was devised that used blood-saturated, synthetic sponges set into 55 mm petri dishes (Fig. 11). This modification proved satisfactory and was adopted as the standard feeding method for all membrane feeding studies. Blood in the sponges was kept warm by placing the petri dishes over small electric heating wafers adjusted to 95 F with thermostatic controls. In later tests, at Stillwater, heat was provided by 7-watt electric bulbs installed under the petri dishes.

The test chamber developed to hold flies before and during tests consisted of a 125 mm length of 60 mm inside diameter, clear, rigid,

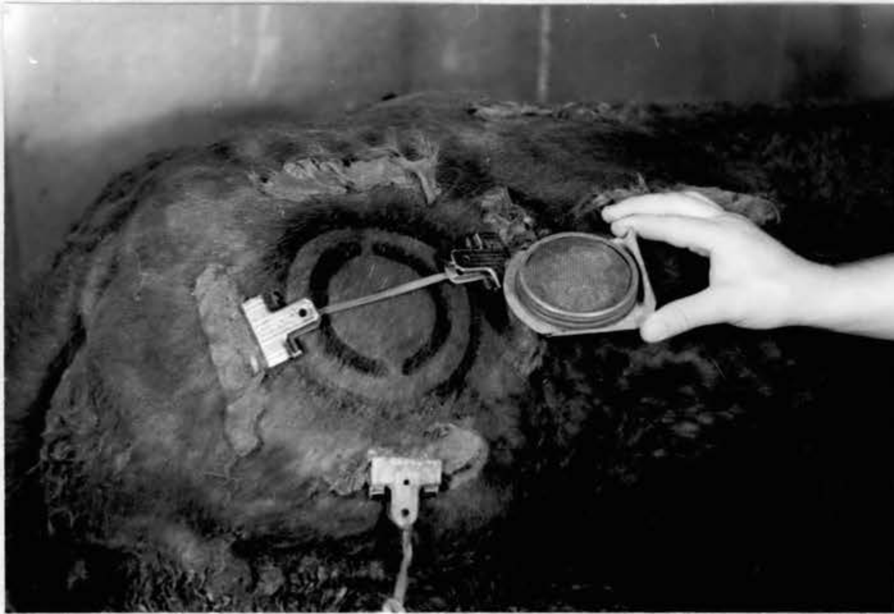

Fig. 9.--Spot test cage in closed position and a treated spot with associated clips and bands.

Fig. 10.--Placing the spot test cage in position under one of the bands that hold the cage during exposure.

plastic tubing similar to the chamber devised by Granett (1960). A transverse slot was cut in the tube 50 mm from one end and a removable, clear plastic shutoff (or slide) was fitted into the slot. A 4-inch piece of clean cheese cloth, held in place by a rubber band, covered the end of the tube furthest from the slot (Figs. 12 and 13).

A platform constructed to support the test chambers consisted of a 16 inch diameter circular turntable, mounted on the shaft of a 1¼ revolutions per minute window display gear box and motor. The platform had six 56 mm holes (or wells) drilled equidistantly around its perimeter to contain the petri dishes and blood sponges during tests (Fig. 14). Sixteen mesh copper screening was fastened beneath the wells to help conduct the heat and to keep the petri dishes in place. A sheet of asbestos was glued to the entire underside of the platform except at the 6 wells. Thus, heat applied below the platform was conveyed only through the openings, eliminating the possible attractancy of heat at other areas of the platform.

A 16 inch diameter clear plastic cake cover was utilized as a canopy over the platform when the unit was used to evaluate candidate attractants (Fig. 15).

Membranes were cut into 4 X 4 inch squares, stretched over an 80 mm heavy cardboard ring, and secured in place with 2 rubber bands. A 60 mm circle was drawn on the membrane and the area within the circle was treated with 0.2 mm of an acetone solution of the technical chemical dispensed from a 1 ml pipette. The treatment was allowed to air-dry 24 hours before it was used in any test procedure.

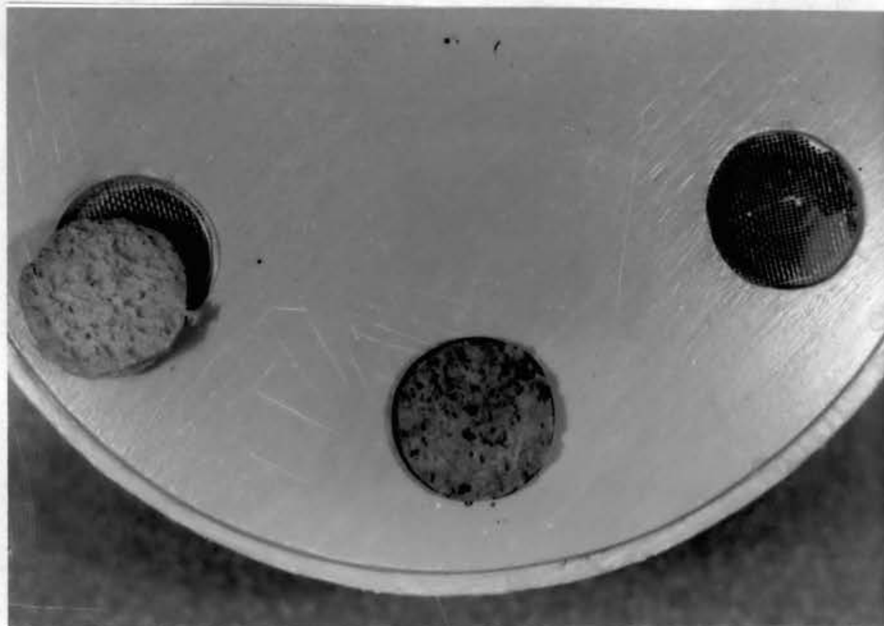

Fig. 11.--Platform, wells, petri dishes, blood sponges, and heating light used in membrane tests.

Fig. 12.--Test cylinder, shutoff slide, slot, cheese cloth cover, and treated membrane.

Fig. 13.--Test chamber with shutoff slide assembled and membrane treated area marked.

Fig. 14.--Platform, gear box and motor, lighted heating system, and treatment chamber all ready to begin a test.

The test procedure ultimately settled upon for purposes of the evaluations presented in this paper included the following steps:

1. flies were starved 24 hours then removed from cage by aspiration.
2. flies were sexed under cold temperature or carbon dioxide anesthesis.
3. 12 female flies were counted into holding area of each test cylinder.
4. approximately 1 hour later test cylinders were placed on treated membranes over blood sponges.
5. turntable was started and slides were removed from slots to start exposure.
6. operator observed activity of the flies; space and contact repellency responses were noted during the first 5 minutes of exposure.
7. after 10 minutes the number of fed and knocked down was recorded.
8. after 20 minutes knockdown was again recorded, test chambers were inverted, the flies were immobilized with carbon dioxide, and the number fed again recorded.
9. flies were immediately transferred to clean ½ pint, screen-top paper cartons and placed in constant temperature room or cabinet.
10. 24 hours after exposure the dead and live flies were ascertained for mortality data.
11. the above procedure was repeated for 5 consecutive days to establish residual activity of the compound.
12. standards of comparison were 0.25 per cent methoxychlor and 0.05 per cent pyrethrins. A compound was considered to have failed as a toxicant or an anti-feeding repellent when results for that event fell below 80 per cent.

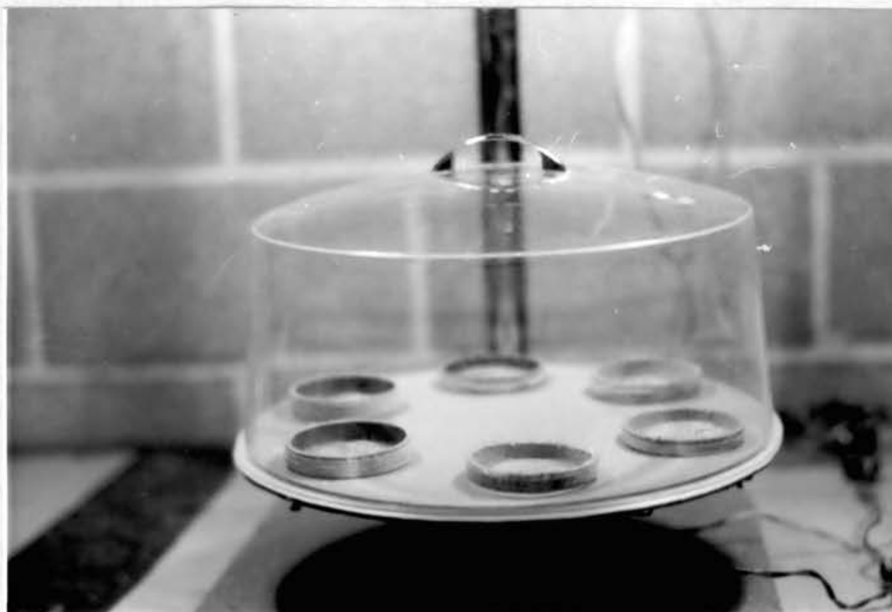

Fig. 15.--Canopy in place on turntable for attractancy test.

Fig. 16.--Engorged flies in test chamber feeding through Baudruche membrane.

RESULTS AND DISCUSSION

Evaluation of Membranes. - At Kerrville, 11 membranes were evaluated to determine their suitability as surfaces that could accept and retain deposits of chemicals and also permit stable flies to feed on blood placed beneath the membranes. Table 1 presents results of feeding through various membranes. Only canvas cloth and lens paper were readily penetrated by the flies and both of these materials had the disadvantage of wetting throughout their thickness. Four animal derived membranes were penetrated by 52 to 66 per cent of the flies and were considered promising as they accepted and retained the chemical deposits satisfactorily. However, the feeding results of the replicates varied widely and observations indicated many of the flies had difficulty penetrating the membranes due to thickness or toughness of these products. Unfortunately, an ultra-thin beef caecum membrane, Silverlight, that had been used successfully by other investigators was no longer available.

Samples of cellulose dialysis tubing and animal derived osmosis demonstration membranes were obtained at Stillwater, but results with these products were similar to those obtained previously. The dialysis tubing proved too tough for the flies to penetrate although they were persistent in their attempts.

Finally, a commercial source of Baudruche transparent membrane--the outer lining of the intestine of beeves--was located and samples obtained. This membrane was of a semipermeable nature and averaged 0.0008 inch in

Table 1.--Evaluation of membranes for the *in vitro* feeding of stable flies.

Membranes	Description	Per cent fed
At Kerrville		
Hog-gut casings	Intestine of hog (salted)	54
Sausage casings (synthetic)	Plastic tubing	21
Lens paper	Rice paper	94
Latex	Synthetic rubber	0
Parchment	Animal skin, sheep	58
Parchment, synthetic	Heavy paper	33
Canvas cloth	Tight weave cotton cloth	87
Chamois skin	Sheep skin	13
Toy balloon rubber	Probably latex	6
Saran wrap	Plastic sheeting	0
Osmosis demonstration membrane	Caecum sheeting, opaque	52
At Stillwater		
Cellulose dialysis tubing	Clear, tough, synthetic tubing	10
Osmosis demonstration membrane	Caecum sheeting, opaque and of irregular thickness	66
Baudruche	Outer layer of intestine of cattle, 0.0008 inch	93

thickness. In feeding tests with Baudruche membranes approximately 93 per cent of the flies penetrated and obtained a blood meal, the blood was not absorbed by the membrane, and acetone solutions were easily applied and, at least, visually appeared to produce a satisfactory deposit. On the basis of these data the Baudruche membrane was accepted as the test surface.

Plywood Panel and Glass Jar Tests. - Data for wood and glass residues was limited to per cent knockdown after 20 minutes exposure in the treated chambers and mortality of the same flies 24 hours later. The test procedures do not lend themselves readily to an assessment of repellency. Further, it should be recognized that in the glass jar technique all surfaces are treated, while in the wood panel technique the flies have a choice of contacting treated wood or untreated petri dish. In both tests, but especially the wood panel test, a chemical that has fumigant qualities would, no doubt, be more effective than one whose action was restricted to contact effect.

Table 2 presents the average results of 2 tests of chemical deposits on plywood panels. DDT and methoxychlor were the only toxicants providing 90 per cent or greater mortality on or after 5 weeks. Toxaphene provided 72 per cent or greater mortality for 3 weeks but never demonstrated a high knockdown during the exposure period. Pyrethrins gave a very fast knockdown 1 day after treatment but did not demonstrate persistence beyond 1 week.

In general, the number of flies knocked down and the mortalities produced by a specific chemical at any given interval of time were greater for the tests on glass than for the tests on wood panels. However, in comparing results of the 2 techniques, it is noted that the

Table 2. Knockdown and mortality of stable flies exposed to chemical deposits on unpainted plywood. All residues applied at 25 mg/ft^2 except pyrethrins at 2.5 mg/ft^2. Average 2 tests.

Compound	Per cent KD/mortality at indicated time post-treatment (Days) 1	(Weeks) 1	2	3	4	5	6
Lethane 384	6/6	0/0	0/0	0/2	0/0	0/0	0/0
Thanite	10/8	0/0	0/0	0/0	0/0	0/6	0/0
DDT	80/100	68/100	88/96	76/100	46/88	54/96	56/88
Methoxychlor	100/100	100/100	96/100	94/100	82/96	84/100	84/98
Toxaphene	24/90	20/92	14/84	18/72	22/36	4/42	6/46
Malathion	40/100	46/94	22/96	26/82	36/88	10/68	6/52
Coumaphos	96/100	76/98	60/86	36/90	44/84	20/62	24/48
Ciodrin	82/100	68/94	62/90	18/78	34/80	12/44	18/50
Stauffer R-3413	12/0	0/0	0/0	0/6	0/0	0/0	0/0
Shell SD-7393	20/6	0/0	0/0	0/0	0/0	0/0	0/0
Pyrethrins	100/100	68/92	8/14	0/0	0/0	0/4	0/0
Acetone control	0/0	0/0	0/2	0/4	0/0	0/0	0/0

toxicants were aligned in about the same relative position as regards to their toxicity to stable flies. Data for the glass jar tests are presented in Table 3.

These 2 techniques were used extensively during the early years of development of the chlorinated hydrocarbon insecticides and are still useful to establish residual toxicity of chemical deposits applied to inanimate surfaces. However, as both techniques require several weeks to complete and neither provides repellency information, each appears deficient as a practical method of evaluating chemicals for biting fly studies.

Tests with Sprayed White Mice. - Seven of the 13 chemicals sprayed on white mice apparently prevented stable flies from feeding 1 day after treatment, but only 2, methoxychlor and pyrethrins, provided 100 per cent mortalities (Table 4). Effectiveness of all toxicants decreased rapidly, apparently because of the activity of the mice in cleaning themselves. On the 3rd day post-treatment only methoxychlor (0.25%), malathion (0.5%), and Ent. 28087 (1.0%) continued to discourage feeding. However, the per cent of feeding by stable flies was extremely irregular and even with untreated controls a range of 15 to 95 per cent was recorded with different mice. Thus, the data from the present tests, are not considered to be conclusive evidence of the candidate chemicals true effectiveness.

Unless standardization of technique could be developed to a higher level than that utilized by the author, the mouse technique would not fulfill our requirements.

Spot Tests on Hereford Cattle. - Roberts et al. (1960) devised a rating or classification whereby a residual toxicant evaluated by the spot test was rated as follows: (1) not effective 1 day-Class I; (2) 90 per cent effective at 1 day-Class II; (3) 90 per cent effective 2 thru 7

Table 3. Knockdown and mortality of stable flies exposed to chemical deposits in quart glass jars. All residues applied at 25 mg/ft^2 except pyrethrins at 2.5 mg/ft^2. Average 2 tests.

	Per cent KD/mortality at indicated time post-treatment						
	(Days)	(Weeks)					
Compound	1	1	2	3	4	5	6
Lethane 384	8/24	0/0	0/6	4/2	0/0	0/4	0/0
Thanite	18/2	0/4	0/2	0/0	0/2	0/0	0/6
DDT	92/100	96/100	100/100	88/100	86/100	70/96	74/96
Methoxychlor	100/100	100/100	100/100	82/98	86/98	92/100	84/94
Toxaphene	36/100	46/96	34/90	20/66	16/74	14/66	16/54
Malathion	48/100	26/100	38/100	48/96	26/90	30/88	22/80
Coumaphos	68/100	86/100	86/100	42/78	54/86	12/70	16/78
Ciodrin	98/100	72/100	74/100	62/94	66/88	40/64	24/56
Stauffer R-3413	10/10	2/0	0/0	0/0	0/8	0/0	0/0
Shell SD-7393	28/36	10/6	0/2	0/0	0/0	0/0	0/0
Pyrethrins	100/100	100/100	82/96	40/36	14/4	4/0	0/2
Acetone control	0/0	0/2	0/0	0/2	0/0	0/0	0/2

Table 4. Per cent feeding and mortality of stable flies exposed to insecticide treated white mice. Each mouse sprayed with 10 ml of an acetone solution of the candidate.

Compound[a]	Per cent concen-tration	Per cent feeding/mortality on indicated days post-treatment (Days) 1	3	5	7
Lethane 384	0.5	35/25	60/5	50/0	55/0
DDT	0.25	0/95	15/80	35/40	20/5
Methoxychlor	0.25	0/100	0/100	15/55	35/35
Toxaphene	0.5	5/85	20/40	40/10	25/15
Malathion	0.25	0/35	10/35	25/10	70/10
	0.5	0/60	0/50	15/35	50/15
R-3413 (Stauffer)	0.5	35/15	50/0	85/0	65/0
SD-7393 (Shell)	0.5	10/20	45/0	45/0	55/0
E-28085	0.5	40/10	45/0	25/10	55/0
E-28087	0.5	20/15	20/10	45/10	55/0
	1.0	0/20	0/0	(mice died before 5 days)	
E-28092	0.5	0/35	55/10	60/0	75/0
E-28093	0.5	0/20	15/0	40/0	45/0
	1.0	(mice died within 1 hour)			
Phillips 949	0.5	15/0	45/0	35/0	60/0
Pyrethrins	0.05	0/100	85/30	60/10	85/15
Control (untreated)	--	55/0	80/0	65/0	55/0

[a] Compounds identified in Table 9.

days-Class III; (4) effective 8 or more days-Class IV. They classified repellents on the basis of 90 per cent or more not feeding as follows: (1) not effective 1 day-Class I; (2) effective at 1 day-Class II; (3) effective 2 or 3 days-Class III; (4) effective 4 or more days-Class IV. Methoxychlor (0.5%) and pyrethrins (0.05%) were selected as standards for these classifications.

Tables 5 and 6 present data for 15 chemicals evaluated as residual toxicants, knockdown agents and repellents by the spot test technique. Four of the chemicals, methoxychlor, N-2404, R-5723-A, and SD-8436, illustrate data typical of Class IV residual toxicants in that 90 per cent or greater mortality was obtained following 20 minute exposures to treated spots on cattle thru 8 days. These data further indicate the above named toxicants possess an ability to penetrate the insect rapidly although it does not necessarily correlate directly with speed of knockdown. For example, SD-8436 provided only 21 per cent knockdown at 1 day but was one of the better residual toxicants of the group. Pyrethrins, on the other hand, often gives a 100 per cent knockdown but less than 100 per cent mortality, as illustrated by the second day data in Tables 5 and 6.

Speed of knockdown and toxicity (as evidenced by mortality) often do show some correlation (methoxychlor and N-2404, for example) and the knockdown can be valuable information to the experienced investigator in evaluating a candidate. Often the first days results will indicate to the investigator whether knockdown can be used as an indicator of toxicity for subsequent days tests with the same treatment.

Class IV repellents are illustrated by pyrethrins, E-28086, E-28087, and E-28093. The actions of pyrethrins are apparently manifold and the limits of repellency and toxicity in chemicals possessing such qualities

Table 5. Average mortality of stable flies 24 hours after a 20 minute exposure to treated spots on Hereford steers. Residual toxicity classification awarded by Roberts system. Average of 3 or more tests.

Compound[a]	Per cent concen- tration	Per cent mortality at indicated days after treatment					Residual toxicity class
		1	2	4	7	8	
Lethane 384	0.5	25	12	--	--	--	I
Thanite	0.5	0	--	--	--	--	I
	5.0	24	0	--	--	--	I
Methoxychlor	0.5	100	100	100	98	91	IV
Pyrethrins	0.05	100	78	10	0	--	II
	0.1	100	94	63	17	0	III
Malathion	0.25	74	26	12	--	--	I
	0.5	100	93	89	67	45	III
Coumaphos	0.5	100	93	87	54	62	III
Phillips 949	0.5	0	0	--	--	--	I
	5.0	8	0	--	--	--	I
N-2404	0.25	100	100	82	74	48	III
(Stauffer)	0.5	100	100	100	90	96	IV
R-5723-A	0.25	100	100	91	96	91	IV
(Stauffer)	0.5	100	100	100	98	98	IV
SD-8436	0.25	100	96	91	76	42	III
(Shell)	0.5	100	100	100	98	98	IV
PCRB-26774	0.5	12	0	--	--	--	I
E-28085	0.5	0	0	--	--	--	I
	5.0	12	0	0	--	--	I
E-28086	0.5	91	60	0	--	--	II
	5.0	94	94	62	21	--	III
E-28087	0.5	0	0	--	--	--	I
	5.0	96	91	46	49	--	III
E-28093	0.5	0	0	--	--	--	I
	5.0	18	0	--	--	--	I
Controls	--	0	0	0	0	0	--

[a] Compounds identified in Table 9.

Table 6. Knockdown and feeding repellency of stable flies exposed to treated spots on Hereford steers. Average of 3 or more tests.

Compound[a]	Per cent concen- tration	Feeding repellency classification	Per cent KD after 20 minutes exposure on days post-treatment 1	2	4
Lethane 384	5.0	II	0	0	0
Thanite	5.0	III	0	0	0
Methoxychlor	5.0	III	100	100	92
Pyrethrins	0.05	IV	100	100	24
	0.1	IV	100	100	80
Malathion	5.0	II	96	82	46
Coumaphos	0.5	I	10	0	0
	5.0	II	91	76	54
Phillips 949	5.0	II	60	21	0
N-2404	0.5	I	26	8	0
(Stauffer)	5.0	III	100	100	74
R-5723-A	0.5	II	96	84	63
(Stauffer)	5.0	III	100	100	96
SD-8436	0.5	I	21	30	14
(Shell)	5.0	I	62	42	10
PCRB-26774	5.0	I	0	0	0
E-28085	0.5	I	0	0	0
	5.0	I	12	0	0
E-28086	0.5	III	5	0	0
	5.0	IV	18	24	0
E-28087	0.5	I	0	0	0
	5.0	IV	32	10	0
E-28093	0.5	I	0	0	0
	5.0	IV	12	0	0
Control	--	I	0	0	0

[a] Compounds identified in Table 9.

are not easily resolved. The other 3 repellents listed above apparently affect stable flies by a different mode as their toxicity was not outstanding and, in fact, was essentially nil with E-28093 even though in the spot test technique flies are confined in close contact to the animal and the flies cannot entirely avoid the treated hair.

Although the spot test is a very desirable bioassay technique in that it makes use of the natural host and thus provides the insect with a desired environment, it also has certain deficiencies that limit its application. Of particular concern are the variables that exist between different host animals and between different locations on the same animal. Between animals, there are differences in hair coat, sensitivity of individuals to the biting of flies, extent an individual will lick itself, and extent an individual will rub or otherwise abrade itself. On a single animal, the areas reached by the tongue or tail are subject to a more rapid loss of chemical than other areas of the body and thus location on an animal is, no doubt, important to the successful evaluation of the chemical. Further, the handling, care and feeding of a herd of full-grown steers is an undesirable and expensive undertaking.

Tests with Treated Baudruche Membranes. - As a preliminary in the development of the membrane technique, tests of methoxychlor were conducted at several concentrations between 0.05 and 0.5 per cent to establish a standard of comparison for residual toxicants. Likewise, pyrethrins was investigated at concentrations of 0.025 to 0.25 per cent to provide a repellent standard for feeding, tactile and space repellency effects. The following tabulations give the terminal day averages of 5 tests of the various concentrations:

Residual toxicity (methoxychlor)	
Per cent conc.	Avg. per cent mort. 5th day
0.05	0
0.1	21
0.15	65
0.25	84
0.5	100

Feeding repellency (pyrethrins)	
Per cent conc.	Avg. per cent flies feeding 4th day
0.025	44
0.05	14
0.1	0
0.25	0

Based on the above data 0.25 per cent methoxychlor and 0.05 per cent pyrethrins were adopted as standards for comparison and the following classifications were proposed for:

(a) residual toxicants,

Class I - less than 80 per cent mortality 1 day post-treatment

Class II - 80 per cent or greater mortality 1 day post-treatment

Class III - 80 per cent or greater mortality 2 thru 4 days post-treatment

Class IV - 80 per cent or greater mortality 5 days post-treatment

(b) feeding repellents,

Class I - over 20 per cent of flies fed 1 day post-treatment

Class II - 20 per cent or fewer flies fed 1 day post-treatment

Class III - 20 per cent or fewer flies fed 2 or 3 days post-treatment

Class IV - 20 per cent or fewer flies fed 4 days post-treatment

(c) tactile and space repellents,

Class I, II, III, or IV awarded on basis of the same 4 day standard as for feeding repellents but determined by the investigators observations of the insects response to the chemical

during the first 5 minutes of exposure. A chemical was considered to have failed as a space repellent at the test concentration on the day that, at least, 20 per cent of the flies contacted the treated membrane. Tactile repellency was considered to have failed at the test concentration on the day that, at least, 20 per cent of the flies remained on the membrane in an attempt to feed.

In Table 7 data are presented of membrane tests for the same chemicals reported in Table 5 for the spot test. The 4 toxicants that ranked Class IV in the spot tests also ranked Class IV on membranes. The toxicity of pyrethrins persisted longer on the membranes than on spots and thus ranked higher by the former test technique. Coumaphos treated spots were more toxic than treated membranes and it is thought that length of contact time was responsible. In general, however, the rankings awarded a given toxicant at the basic test concentrations were similar.

Of particular interest are the repellency responses presented in Table 8, in that they help explain some of the variable results of the toxicity data. Malathion gave only an average of 33 per cent mortality at 0.5 per cent concentration on membranes 1 day after treatment while at the same concentration on spot animals 100 per cent kill was obtained. In neither case did any of the flies obtain a blood meal, but in the spot test the flies were quickly incapacitated because of their close confinement to the treated hair, whereas in the membrane tests the flies could escape the treated surfaces. In this response the flies demonstrated both tactile and space repellency to the fresh deposit of malathion. On the 2nd day post-treatment the residues were no longer repellent on either surface; the flies fed equally well and the mortality data were

Table 7. Average 24 hour mortalities of stable flies following 20 minute exposures to treated Baudruche membranes and the classification awarded each candidate as a residual toxicant. Average 3 tests.

Compound[a]	Per cent concen-tration	Per cent mortality at indicated days after treatment 1	2	3	4	5	Residual toxicity class
Lethane 384	0.25	0	10	8	--	--	I
Thanite	0.25	18	10	25	0	--	I
Methoxychlor	0.1	100	73	33	10	0	II
	0.25	95	97	80	88	80	IV
Pyrethrins	0.05	100	92	66	25	8	III
	0.1	100	100	100	100	100	IV
Malathion	0.25	83	58	25	0	--	II
	0.5	33	92	92	58	67	III
Coumaphos	0.25	42	67	92	64	23	I
Phillips 949	0.1	0	0	0	--	--	I
	0.25	0	0	0	--	--	I
N-2404 (Stauffer)	0.1	100	70	50	0	0	II
	0.25	100	100	92	92	83	IV
R-5723-A (Stauffer)	0.1	100	70	42	60	0	II
	0.25	92	100	92	83	83	IV
SD-8436 (Shell)	0.25	100	100	100	100	100	IV
PCRB-26774	0.25	0	0	--	--	--	I
E-28085	5.0	8	0	0	--	--	I
E-28086	1.0	60	33	17	--	--	I
	5.0	100	58	50	75	58	II
E-28087	1.0	0	0	0	0	0	I
	5.0	100	42	33	17	25	II
E-28093	1.0	25	0	0	--	--	I
	5.0	56	0	0	8	17	I
Controls	--	0	0	0	0	0	--

[a] Compounds identified in Table 9.

Table 8. Knockdown and repellency of stable flies exposed to treated Baudruche membranes. Average 3 tests.

Compound[a]	Per cent conc.	Repellency Feeding	Repellency Tactile	Repellency Space	Per cent KD after 20 minute exposure on days post-treatment 1	2	3	4
Lethane 384	0.25	I	I	I	0	0	0	0
Thanite	0.25	I	I	I	0	0	0	0
Methoxychlor	0.1	IV	I	I	100	25	8	17
	0.25	IV	I	I	75	83	46	79
Pyrethrins	0.05	IV	IV	b	100	92	75	83
	0.1	IV	IV	b	100	100	100	100
Malathion	0.25	I	I	I	25	8	0	0
	0.5	IV	II	II	50	34	17	25
Coumaphos	0.25	I	I	I	0	0	0	0
Phillips 949	0.25	I	I	I	0	0	0	0
N-2404 (Stauffer)	0.25	I	I	I	0	8	0	0
R-5723-A (Stauffer)	0.25	I	I	I	50	33	0	0
SD-8436 (Shell)	0.25	I	I	I	50	17	17	8
PCRB-26774	0.25	I	I	I	0	0	0	0
E-28085	5.0	I	I	I	0	0	0	0
E-28086	5.0	IV	IV	I	0	0	8	25
E-28087	5.0	IV	IV	II	0	0	0	0
E-28093	5.0	IV	IV	IV	0	0	0	0

[a] Compounds identified in Table 9.
[b] Fumigation effects Knockdown rapidly, masking repellency.

similar. It is the belief of the author that the latter test more nearly demonstrates the response the flies would exhibit and thus presents a truer picture of the chemicals action.

An outstanding illustration of space and tactile repellency was provided by E-28093 when tested at the 5 per cent concentration level. Flies confined over membranes treated with this material remained at the opposite end of the cylinder on the cheese cloth cover during the first 3 days exposures, thus demonstrating a definite negative response or spatial repellency. On the 4th day an occasional fly would venture onto the membrane but immediately withdraw, demonstrating tactile repellency. No feeding was attempted at any time and, except for the first day, mortality was very low. A continuous, rapid vibration of the wings was evidence of the insects irritation during exposure to this chemical.

Table 9. Common name, Entomology Research Division code number, and identification of the chemicals.[a]

Common name	Code number	Chemical identification
Lethane 384	6	2(2-butoxyethoxy) ethyl thiocyanate
Thanite	92	a mixture of isobornyl thiocyanoacetate and related compounds
Methoxychlor	1716	1,1,1-trichloro-2,2-bis(p-methoxyphenyl)ethane
DDT	1506	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
Pyrethrins	3107	an extract of *Chrysanthemum cinerariaefolium*
Toxaphene	9735	chlorinated camphene containing 67 to 69 % chlorine
Malathion	17034	*O*,*O*-dimethyl dithiophosphate of diethyl succinate
Coumaphos	17957	*O*,*O*-diethyl *O*-(3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-2-yl)phosphorothioate
Phillips 949	25029	2-hydroxypropyl *n*-octyl sulfide
N-2404	25775	Confidential--Stauffer Chemical Corp.
R-5723-A	25865	Phosphorodithioic acid, *O*-ethyl, *O*-methyl, *S*-phthalimidomethyl ester
SD-8436	25840	2-chloro-1-(2,4-dibromophenyl)vinyl dimethyl phosphate
PCRB-26774	26774	Confidential--Pesticides Chemicals Research
Ciodrin	24717	alpha-methylbenzyl 3-hydroxycrotonate dimethyl phosphate
R-3413	25737	Phosphorodithioic acid, *S*-(4,6-dimethyl-2-pyrimidinyl) *O*,*O*-diethyl ester
SD-7393	25744	Confidential--Shell Development Company
E-28085	28085	Confidential--Commercial Solvents Corp.
E-28086	28086	Confidential--Commercial Solvents Corp.
E-28087	28087	Confidential--Commercial Solvents Corp.
E-28093	28093	Confidential--Commercial Solvents Corp.

[a] Confidential compounds will be identified after patent rights clarified.

SUMMARY AND CONCLUSIONS

A review of the literature provides a background of the history and development of bioassay techniques and equipment used in the application of chemicals to substrata or directly to insects. The development of laboratory cultures of *Stomoxys calcitrans* (L.) is traced.

Considering the importance of a uniform test insect in bioassay, the current rearing technique, equipment, and facility of the Kerrville laboratory of the USDA-ARS-ERD are described. Facilities and materials that are important to this laboratories rearing procedure are: (1) 78 F constant temperature rearing rooms; (2) plastic screen holding cages; (3) exterior of cage feeding and egging; (4) C.S.M.A. and wood shaving media; (5) cold-tray anesthesia; and (6) vacuum system handling devices. A consistent 17-day cycle has been accomplished.

Bioassay techniques previously used at the Kerrville laboratory, and reconsidered in this paper, include spray treated plywood panels, acetone solution treated quart glass fruit jars, acetone solution sprays applied to white mice, and acetone solution sprays applied to spots on live Hereford steers. Each of these techniques is described, but basically they consist of applying a known quantity of the technical chemical in a volatile solvent by sprayer or pipette, allowing 24 hours for the application to dry, placing stable flies in glass or screen exposure chambers in such a position that the flies have an opportunity to contact the chemical deposit for a specified interval of time, and then

recording knockdown at the end of the exposure period and mortality 24 hours later. Of the above procedures, the mouse and spot tests also considered repellency. This effect was estimated by counting the number of flies that did not take a blood meal during exposure.

A new _in vitro_ membrane technique is described that utilizes a 16 inch motorized turntable to support petri dishes of heated beef blood soaked sponges. Ultra-thin animal derived intestinal membranes, treated with 0.2 ml of acetone solutions are used as the substrata and flies are exposed in 125 X 60 ml rigid plastic tubing. Knockdown, mortality, residual toxicity, and feeding, tactile and space repellency are recorded.

Fourteen natural and synthetic membranes were evaluated as _in vitro_ feeding membranes but only animal derived materials demonstrated promise, and of these only the ultra-thin outer intestinal lining of beeves (Baudruche membrane) afforded the flies easy access to the blood, did not readily leak, and retained the chemical deposit satisfactorily.

Using the Baudruche membrane, concentration series of methoxychlor (0.05 to 0.5 per cent) and pyrethrins (0.025 to 0.25 per cent) were conducted to establish standards for residual toxicity and repellency, respectively. From these data a classification system was proposed similar to that used by Roberts et al. (1960) for the spot test. However, for the membrane technique, a 5 day termination was selected for residual toxicity and a 4 day termination for repellency. Repellency was divided into 3 elements: feeding, tactile and spatial. An 80 per cent level was adopted as the breaking point in determining success or failure for any effect.

In residue tests conducted on plywood panels, DDT and methoxychlor at the rate of 25 mg/ft^2 continued to kill 88 to 100 per cent of the

flies thru 6 weeks. Methoxychlor and pyethrins were indicated to be the better knockdown agents tested, but pyrethrins was not effective beyond 1 week at the evaluation level of 2.5 mg/ft^2.

The same chemicals deposited in glass jars provided data similar to that obtained on wood panels but residual toxicity was extended in most cases. In addition to DDT and methoxychlor, malathion, coumaphos and Ciodrin provided residual mortalities for 4 to 6 weeks when applied to glass jars. It is probable that the toxicant deposit is more available on glass then on the absorptive surface of wood. Further, in the glass method all surfaces are treated whereas in the wood panel test the panels are covered with untreated petri dishes. Nevertheless, it is believed that either of the above techniques can be utilized effectively to give a rapid estimate of the toxicity of a chemical deposit by disregarding the residual aspect of the test procedure.

Although mortality data obtained from exposure of stable flies to treated white mice roughly suggests toxicities similar to those obtained on wood and glass, the contact and feeding of flies was so erratic the procedure was not considered satisfactory for our purposes. In addition, a problem was experienced with mortality of the hosts.

Stable flies exposed to treated spots on Hereford steers clearly indicated the fast knockdown character of methoxychlor and pyrethrins and thereby demonstrated "feeding repellency" due to incapacitation. Other chemicals, including N-2404 and SD-8436, did not cause knockdown of the flies during exposure but were equally effective in producing mortality. Tests with Lethane 384, Thanite and PCRB-26744 consistently were negative for all effects. Usually above 90 per cent of the untreated control flies fed.

In general the results of knockdown and residual toxicity tests on membranes were similar to the data obtained in the spot tests. Pyrethrins, however, persisted for a greater time on membranes while coumaphos was less effective. It is suggested that pyrethrins degraded more rapidly when on the animal and that the flies fed through and departed from the membrane treated with the relatively slow acting coumaphos before receiving enough toxicant to cause mortality.

All 3 elements of repellency were readily demonstrated by the membrane technique. Flies exposed to 0.5 per cent malathion 1 day after treatment avoided the treated membrane and sustained only a 33 per cent mortality, while a similar exposure on steers killed all the insects by contact and was thus recorded as 100 per cent feeding repellency according to the spot test definition of repellency. On the 2nd day after treatment no repellency was exhibited in either test and the mortality data were similar. Flies exposed to 5.0 per cent E-28093 completely avoided the membrane for 3 days, then on the 4th day an occasional fly would contact and retreat; thus demonstrating spatial, then contact repellency. As feeding was not accomplished, the effect could also be considered feeding repellency.

It is the authors opinion that each of the test procedures considered, except perhaps the mouse test, could be utilized in some aspect of bioassay of toxicants and repellents. Limitations, of course, are evident and have already been considered for wood panel, glass jar and mouse tests. Both the spot test on animals and the *in vitro* animal membrane tests appear to be satisfactory multi-purpose bioassay techniques for preliminary evaluation of toxicants and/or repellents against biting flies. However, the convenience and degree of standardization possible in a laboratory technique, such as the membrane test, are strong

recommendations for its adoption, other things being equal. After certain minor modifications of the existing equipment are achieved, I believe the membrane test can be utilized to advantage as a bioassay method at the Kerrville laboratory.

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Master of Science

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Major Field: Entomology

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