necessary. At the usual rates of application (4 to 5 gallons per 1000 bushels), it is not possible to cover the entire surface area of the grain treated. Tests were made on shelled corn in which $^1/_2$, $^1/_4$, $^1/_8$, and $^1/_{16}$ of the test sample was sprayed with the dose required for the entire sample, then returned to and mixed with the unsprayed portion of the sample. The treated portion was sprayed on only one side of the kernels. When tested against rice weevils, a somewhat lower mortality was obtained when only $^1/_{16}$ of the sample was treated. When as much as $^1/_8$ of the sample was treated, the results were fully as good as when the entire sample was treated. In practice, spray should be applied to grain as thoroughly as possible, but it would seem equally important to provide for thorough mixing of the grain after the spray is applied, so that distribution of the treated kernels among the untreated ones is assured. For adequate mixing under conditions of actual use, the spray should be applied to grain at the lower end of the hiker or elevator. This takes full advantage of the auger or tumbler action for obtaining good distribution of the sprayed kernels.

Addition of Water to Grain During early experimental treatments, it was thought that the use of water sprays on grain might be objectionable because of a possible increase in the moisture content of the grain. A more careful consideration of the amounts of moisture added by emulsifiable sprays emphasized that the amount added is of little significance in relation to the amount of moisture usually present in grain—for example, 5 gallons (42 pounds) per 1000 bushels of wheat (60,000 pounds) is equal to 0.07%, a negligible amount that is soon adjusted through normal evaporation.

Summary and Conclusions

Tests made in the laboratory with sprays containing combinations of pyrethrum and piperonyl butoxide applied directly to grain showed that: (1) Water emulsions were more satisfactory and more lasting in their effect than sprays containing petroleum oil or vegetable oil as diluents. (2) Emulsion sprays were of the same order of effectiveness as dry protectant powders. (3) The concentration of an emulsion used to obtain a given deposit of active ingredients is not important. (4) Emulsions prepared from oil-free concentrates were as effective as those prepared from conventional concentrates containing petroleum oil. (5) The emulsifier MYRJ-45 was as effective biologically as Atlox 1045A. (6) Treatments were more effective when applied to screened grain than when applied to grain containing screenings. (7) An oil-free emulsion containing 2% of piperonyl butoxide and 0.2% of pyrethrins applied at the rate of 5 gallons per 1000 bushels of grain was adequate for the protection of stored grain.

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PESTICIDE SAFETY EVALUATION

Toxicity of Trinitrobenzene-Aniline Complex, a Rodent Repellent

R. W. FOGLEMAN, J. R. ELSEA, O. E. PAYNTER, and WALTER KUNDZINS

Hazleton Laboratories, Falls Church, Va.

COMPLEXES OF TRINITROBENZENE have been suggested as promising rodent repellents by the work of Welch and Duggan (*6*) and Welch (*5*). One such repellent is the trinitrobenzene-aniline complex (TNBAC), which was proposed for use in orchards and shelterbelt plantings to decrease the damage from rabbits during the winter months when feed becomes scarce. If solutions of TNBAC are either painted or sprayed on the trunks of trees in the fall, one application is effective throughout the dormant season. With this type of application the hazard to personnel handling the material is primarily one of dermal contact, and time of exposure is relatively brief.

Compounds of the nitrobenzene series are known as potent methemoglobin formers and may produce an effect on the blood-forming organs, especially the bone marrow (*2, 4*). Aniline is a potent methemoglobin former and is considered very toxic in pure solution (*2*). The chemistry of the complex of trinitrobenzene (TNB) and aniline is not fully understood; however, some of the physical characteristics of the individual components are not found in the complex.

DeWitt, Welch, and Bellack (*1*) have published criteria for a rodent repellent which include the requirement of safety for use. In preliminary tests at another laboratory where determinations of oral minimum lethal dose and studies of eye and dermal irritation were conducted, TNBAC was shown to have a low order of toxicity. Detailed toxicological studies, including comparison with TNB, were undertaken at Hazleton Laboratories to evaluate the safety of TNBAC more extensively.

Oral Administration to Albino Rats

Procedure Male albino rats, weighing between 100 and 170 grams were intubated with a 5.0% weight per volume suspension of anhydrous TNBAC or TNB in 0.5% methylcellulose and observed for 7 days. Signs of toxicity were noted, and mortality was recorded daily. All survivors were sacrificed, and gross autopsies were performed at the completion of the study. The acute oral

A detailed toxicological study was made to evaluate the safety of the trinitrobenzene-aniline complex (TNBAC) as a rodent repellent for nonfood use. The acute oral LD_{50} in rats for TNBAC is 370 mg. per kg.; for trinitrobenzene (TNB), 505 mg. per kg. TNBAC included in the diet of rats at levels of 500 and 1000 p.p.m. suppressed growth; the diet containing 10,000 p.p.m. was rejected. Repeated oral doses in dogs at levels above 25 mg. of TNBAC per kg. per day produced gross signs of toxicity and hematological changes. Repeated dermal applications to dogs of formulations containing TNBAC produced hematological changes; there was no skin irritation. TNBAC appears to be only moderately toxic in single doses; repeated doses are cumulative. Operators should be cautioned against contamination of wearing apparel and unprotected skin.

LD_{50} was calculated by the method of Litchfield and Wilcoxon (*3*).

Results **TNBAC.** The acute oral LD_{50} for TNBAC was found to be 375 mg. per kg. of body weight, with confidence limits of 238 and 591 mg. per kg. Gross signs of intoxication were depression, hyperpnea, gasping, salivation, cyanosis, loss of normal reflexes, tachycardia, coma, and death. All deaths occurred within the first 48 hours after intubation. Necropsy findings of rats dying during the study included hemorrhagic lungs, stained kidneys, and a peculiar "rusty" coloration of the blood. Autopsies, which were performed on surviving rats at completion of the study, revealed necrotic areas over the surface of the spleen and rusty appearing blood.

TNB. The acute oral LD_{50} for TNB was found to be approximately 505 mg. per kg. The data were not suited for calculation of confidence limits. Signs of intoxication and findings at necropsy of animals dying were similar to those seen with TNBAC, except that the kidneys did not appear stained. The organs of the survivors at autopsy were essentially normal, with the exception of the physical appearance of the blood and the kidneys, which appeared darkened.

Subacute Feeding to Albino Rats

Procedure Male albino rats of the Carworth Farms strain were fed diets containing 500, 1000, or 10,000 p.p.m. of TNBAC. Group sizes were small. Two rats were used in each of the control and 500 p.p.m. levels, and four rats in each of the 1000 and 10,000 p.p.m. dietary levels. The diets were specially prepared by mixing TNBAC with Knox No. 1 unflavored gelatin. When gelled, a small portion of laboratory diet was added and the feed-gel mixture was whipped to dryness in a Waring Blendor. This primary mix was then added to the balance of the laboratory feed and blended in a Twin-Shell mixer. Control diets contained comparable amounts of the untreated gelatin. The rats were individually housed in wire-mesh cages elevated above the droppings, and water and the appropriate diets were available at all times. Body weights and food consumption were recorded daily. At completion of the study the rats were sacrificed by exsanguination, and autopsies were performed.

The two rats receiving 500 p.p.m. of TNBAC in the diet showed mild growth suppression over a 28-day period. The diet had been refused to some extent during the first week, but was accepted normally over the remainder of the study. These rats consumed an average of 15.3 grams of food and 7.68 mg. of TNBAC per day. The four rats receiving TNBAC at a dietary level of 1000 p.p.m. exhibited marked growth retardation during the 28-day study. The diet was not readily accepted and considerable spillage occurred, especially in the third and fourth experimental weeks. These rats consumed an average of 17.2 grams of food and 17.2 mg. of TNBAC per day. These values indicate marked spillage and do not reflect the true quantities of food and compound actually ingested. The four rats receiving 10,000 p.p.m. of TNBAC in the diet showed actual loss of weight during the first 14 days of the experiment and a considerable decrease in food consumption. A daily average of 11.8 grams of food and 118 mg. of TNBAC was consumed. One rat died on the 14th day and the remaining animals in this group showed cyanosis, depression, and decreased pain reflexes. This group was terminated and autopsies revealed darkened thyroids, spleens, and livers, and congested kidneys. A pooled sample of oxalated blood contained 14 grams weight % hemoglobin and 2% methemoglobinemia.

At 4 weeks the 500 and 1000 p.p.m. dietary levels were terminated. While no significant gross pathology was found at autopsy, blood smears from animals in both groups showed a macrocytic anemia, marked basophilia, Howell-Jolly bodies, and a large number of normoblasts.

Subacute Administration to Dogs

Procedure Four male and four female mongrel dogs received orally, 5 days a week, gelatin capsules containing either 25 or 75 mg. of TNBAC per kg. of body weight, or 25 or 100 mg. of TNB per kg. Two additional dogs served as controls. Each animal was individually housed and fed the basic laboratory diet. Complete blood counts, urinalyses, bromosulfalein liver function tests, determinations of blood urea nitrogen, and methemoglobin determinations were made at intervals throughout the study. The dogs were observed daily for signs of intoxication and body weights were recorded weekly. At termination all groups were sacrificed by exsanguination under pentobarbital anesthesia, and autopsies were performed. Sections of various tissues were examined for evidence of histological changes.

Results **TNBAC.** The two dogs receiving 75 mg. of TNBAC per kg. showed anorexia, vomiting, and weight loss after four doses of the compound. Changes in erythrocyte morphology were marked and similar to those seen in the rats. There was 18% methemoglobinemia in the female and 12.3% in the male, and a reduction of total hemoglobin to approximately 9 grams weight % in both animals. The dogs were removed from the study for 30 days and returned at the level of 10 mg. per kg. per day. Subsequently they received 13 doses over a 19-day period with no clinical signs of toxicity. Methemoglobin at termination was 1.6% in the female and 4.1% in the male. Total hemoglobin was reduced in the male. All other clinical laboratory tests were within normal limits.

Two dogs received 37 doses of TNBAC at a dosage level of 25 mg. per kg. over a period of 54 days. The male developed ataxia after 30 doses; this persisted to termination. The female was asymptomatic. Clinical laboratory findings in these animals indicated up to 9.1% methemoglobinemia, and mild changes in morphology of the erythrocyte series were observed. The other clinical laboratory tests were normal. Methemoglobinemia decreased to as low as 0.9% at 43 days and was 4.5% at termination.

At autopsy of the two groups of dogs receiving TNBAC the spleens were enlarged. No other grossly observable

changes were noted in any of the dogs. Microscopically, splenic sections showed thickened trabeculae, indicative of passive congestion. Sections of liver, kidney, gonads, and bone marrow from the experimental dogs were comparable to the controls.

TNB. Two dogs received four doses of 100 mg. of TNB per kg. of body weight over a 4-day period. The female developed convulsions, anorexia, and soft feces. Blood smears indicated a marked derangement of the erythrocyte morphology, and there was 11.4% methemoglobinemia in the female and 21.8% in the male. Hemoglobin values were within normal limits, as were the results of other clinical laboratory tests. These dogs were removed from the study for 30 days, allowed to recover, and then returned to the study at a daily dosage level of 10 mg. per kg. After receiving 13 doses over a 19-day period, the dogs appeared clinically normal and the study was terminated. Terminal methemoglobin values were 1.0% in the female and 5.9% in the male. All other clinical laboratory tests were within normal limits.

Two dogs received 37 doses of 25 mg. of TNB per kg. over a 54-day period. The female showed anorexia, depression, and emesia during the first 30 days of the study, but returned to normal during the remainder of the period. The male became ataxic on the 39th experimental day after receiving 29 doses of TNB. After 34 doses this dog showed generalized muscular stiffness with ataxia, seeming to fall to the left. This syndrome persisted until termination of the study.

Morphological changes in the erythrocyte series were noted as early as 4 days and persisted throughout the study. Methemoglobinemia was erratic over the experimental period. Values varied from a maximum of 24.4% in the female and 33.8% in the male at 30 days, to a minimum of 0.4% in the female and 0.9% in the male at 43 days; terminal values were 9.4% for the female and 4.9% for the male. The male developed a mild anemia. All other tests were normal.

At autopsy the spleens and livers from all the dogs receiving TNB were heavier than from the controls; however, no grossly observable pathological changes were noted. Microscopically the spleens showed thickened trabeculae but were otherwise normal, indicating that the gross enlargement was due to passive congestion. Sections of kidney showed definite indication of a toxic reaction. Generally, both endothelial and epithelial cells of the glomerular tufts were undergoing proliferation and cloudy swelling. In the dogs receiving the dosage levels of 100 and 10 mg. per kg. the cells of the loops of Henle were undergoing fatty metamorphosis. In the dogs at the dosage level of 25 mg. per kg., these changes were more severe. The loops of Henle were undergoing degeneration and the toxic process involved the distal convoluted tubules, where the cells appeared swollen and coarsely granular. Sections of liver, gonads, and bone marrow from the experimental animals were comparable to the controls.

Dermal Application to Dogs

Procedure Three formulations—(1) an acetone solution containing 6% TNBAC, (2) an emulsifiable concentrate containing 12% TNBAC, and (3) a dispersion formulation containing 20% TNBAC—were studied for dermal irritation. The emulsifiable concentrate and dispersion formulation were diluted with distilled water to contain 60 mg. of TNBAC per ml. prior to use. Each formulation was applied to the clipped thoracic skin of one male and one female mongrel dog daily, 5 days per week, at the rate of 60 mg. of TNBAC per kg. of body weight, for ten applications.

Blood was drawn from the jugular vein at intervals for clinical laboratory studies, including methemoglobin determinations. Observations were made for signs of local skin changes and systemic toxicity during the experimental period.

Results There were no signs of local irritation produced on the skin of the dogs receiving any of the three formulations of TNBAC tested. The aniline color of the materials was not found around the lips, nares, or hind paws, indicating the dogs had made no attempt to lick or scratch the treated areas. Body weights were maintained during the study by all animals.

Methemoglobin formation was noted in all dogs 24 hours after the second application of the test material; values ranged from 1.6 to 4.1%. During the remainder of the study methemoglobinemia appeared to be cumulative, with levels ranging from 4.9 to 9.6%. Following a 2-day rest period methemoglobin values were slightly lowered over values following five daily applications of the materials. The data indicate that TNBAC from the formulations was dermally absorbed and produced systemic effects in dogs.

Discussion

These data are not intended to comprise a complete toxicological study of TNBAC. They are sufficient, however, to evaluate the safety of this compound for nonfood use.

Acute and subacute toxicity studies in rats and dogs place TNBAC in the moderately toxic class of compounds. The primary signs of intoxication noted in the experimental animals were methemoglobin formation, alteration in erythrocyte morphology, and anemia. These physiological changes were seen following both ingestion and dermal applications of the compound.

There are three common routes by which a compound may gain access into the body: inhalation, ingestion, and dermal absorption. Only the latter appears to be of significance in the evaluation of safety for use of TNBAC. In the field intoxication by inhalation would not be expected, as the repellent formulations are to be used out of doors, and toxic quantities of TNBAC would not build up in the atmosphere. The repellent formulations are, at present, not being recommended for use on or around feed or foodstuffs. Until more experimental data are available, applicators are warned against using TNBAC in granaries or barns where animal or human food is stored.

Serious considerations should be given to dermal absorption. Accidental spillage of the formulations onto shoes, clothing, gloves, or unprotected skin can lead to definite toxicity if adequate precautions are not taken. Contaminated clothing should be removed immediately and the underlying skin should be washed thoroughly with soap and water.

Experimental studies show methemoglobin formation in the dog following repeated exposure to small quantities of TNBAC, and similar results could be expected to occur in humans. Hamblin (*2*) refers to the insidiousness of onset of the toxic signs and symptoms in the human following exposure to nitrogen compounds and to aniline. A sensation of euphoria concommitant with methemoglobin build-up gives a false sense of security and can hamper the recognition of intoxication by the individual. Individuals using this material over an extended period of time can be exposed to accumulating quantities of the formulation. Should contaminated garments be worn for several days, it is possible that a toxic quantity of TNBAC could be dermally absorbed with no apparent debilitating effects. Therefore, applicators should be particularly cautioned against wearing previously contaminated clothing, especially shoes and gloves, until all traces of TNBAC have been removed.

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PESTICIDE RESIDUES

Colorimetric Determination of Ethyl 4,4′-Dichlorobenzilate (Chlorobenzilate) as a Spray Residue

H. J. HARRIS

Research Laboratory, Geigy Agricultural Chemicals, Division of Geigy Chemical Corp., Bayonne, N. J.

An analytical method is presented for the microdetermination of the acaricide, ethyl 4,4′-dichlorobenzilate (Chlorobenzilate), as a spray residue. Essentially, the method involves nitration of the compound, followed by the interaction of the nitrated product with sodium methylate to produce a red color which is measured spectrophotometrically at 538 mμ. The method has been further developed to determine the compound in the presence of DDT. This involves saponification of the Chlorobenzilate to 4,4′-dichlorobenzilic acid, followed by its separation by extraction from the dehydrochlorinated DDT, and its subsequent nitration. The nitrated 4,4′-dichlorobenzilic acid gives the same colored complex with sodium methylate as the ester, with maximum absorption at 538 mμ.

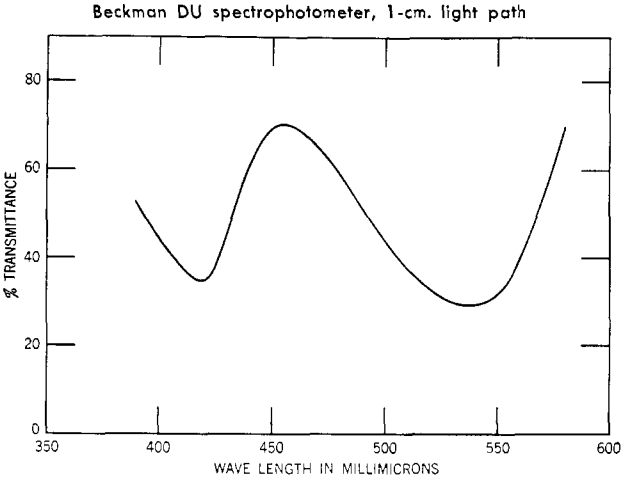
CHLOROBENZILATE has been thoroughly tested in the field by experimental workers and found to be a potent weapon in the control of various species of mites. Already accepted for certain agricultural crops, the compound should find wide-spread uses as an acaricide. This necessitates the development of an accurate microanalytical method, in order to check on the magnitude of the residues remaining on crops at harvest time.

The structural similarity of Chlorobenzilate to DDT suggested the use of the Schechter-Haller (*4*) procedure for the determination of the compound. Investigation showed that the method is suitable for Chlorobenzilate. When the compound is nitrated, and the nitrated product made to react with sodium methylate, a red colored complex is formed which shows maximum absorption at 418 and 538 mμ. The absorption curve for this complex is reproduced in Figure 1.

This adaptation of the Schechter-Haller procedure was tested at the University of California Citrus Experiment Station for the microdetermination of Chlorobenzilate in the presence of citrus extractives, and it was reported (*1*) that extensive isolative procedures are required for its successful use. Two satisfactory alternative methods for citrus have been developed by Blinn, Gunther, and Kolbezen (*1*) based on the hydrolysis of Chlorobenzilate to 4,4′-dichlorobenzilic acid, which is then selectively oxidized to 4,4′-dichlorobenzophenone. The latter is determined either by its absorption at 264 mμ or by the absorption of its 2,4-dinitrophenylhydrazone derivative at 510 mμ. Gunther and Blinn (*2*) have also suggested an adaptation of a total chlorine method, although this procedure is nonspecific for Chlorobenzilate.

The modified Schechter-Haller procedure described in this paper has proved to be satisfactory for the determination of Chlorobenzilate on a variety of crops. Because of the widespread use of DDT in pest control, some way of eliminating this interfering substance prior to nitration was desirable. A satisfactory procedure is based on a method for determining DDT in biological materials by Prickett, Kunze, and Laug (*3*), in which the DDT is dehydrochlorinated with alcoholic potassium hydroxide and extracted with petroleum ether. Under these conditions, Chlorobenzilate is saponified to 4,4′-dichlorobenzilic acid and remains in the aqueous phase as the potassium salt. Upon acidification, the salt is converted to the acid, which is then removed by extraction with ethyl ether. The 4,4′-dichlorobenzilic acid is then determined colorimetrically using the modified Schechter-Haller procedure. The acid gives the same red colored complex as the ester with maximum absorption at 418 and 538 mμ.

Figure 1. Transmittance curve for complex resulting from interaction of nitrated Chlorobenzilate and sodium methylate



Reagents

All chemicals are analytical reagent grade. Where solvents were distilled, it was done in an all-glass apparatus. Distilled solvents are