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ENZYMATIC DETERMINATION OF TEPP RESIDUES ON RED-WINGED BLACKBIRDS

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ABSTRACT

In 1966 the U.S. Bureau of Sport Fisheries and Wildlife, since renamed the U.S. Fish and Wildlife Service, was considering the use of commercial tetraethylpyrophosphate (TEPP) for spraying problem bird roosts. Although TEPP was known to be a fast degrading organophosphate, the Bureau decided that a determination of TEPP residues on birds sprayed under simulated field conditions would be useful in reaching a decision on possible use of this material. The determination was done with an enzymatic Warburg manometric method for measuring the inhibition of acetylcholinesterase (AChE), a major cause of neurotoxication. It was found that at simulated roost temperatures of 7-8°C with relative humidity of 57-60%, the AChE inhibition activity of TEPP in red-winged blackbirds diminished rapidly in the first 2 days. However, after 19 days, an indicated 2.2% (2.8% statistically possible) remained as cholinergic inhibition residues that could be hazardous to humans or nontarget species, considerably more than 99% loss in 45.2 hr at 26°C that had been previously reported. This information, among others, was used by the Bureau in deciding not to pursue the use of TEPP in spraying problem bird roosts.

INTRODUCTION

Tetraethylpyrophosphate (TEPP) is a highly toxic, fast-acting organophosphate insecticide. The pure compound was reported by some workers to be 99% hydrolyzed in 45.2 hr at 25°C or 77°F (Giang and Hall, 1951), a rate faster than the other organophosphate pesticides. Because of this characteristic, it was considered in 1966 by the U.S. Bureau of Sport Fisheries and Wildlife (since renamed the U.S. Fish and Wildlife Service) for use as a lethal chemical to spray blackbird and starling (*Sturnus vulgaris*) roosts. It has been well documented that these roosts present serious problems to agriculture, safety, and health (Garner, 1978; Chick, 1980; Latham et al., 1980). However, TEPP residues on sprayed birds could pose a hazard to nontarget wildlife species or to persons who unknowingly or carelessly handle them because this chemical is highly toxic to neurosystems.

The Bureau realized that the hazards associated with the use of TEPP for blackbird and starling control must be evaluated by first providing knowledge of its residual properties. Despite the belief that TEPP would break down rapidly and become

harmless, in 1966 the Bureau requested that a study be conducted at its Gainesville, Florida laboratory to determine the potential hazards of TEPP residues on blackbirds. Based mainly on the results of this study and other studies, the Bureau decided against the use of TEPP.

Since pure TEPP would doubtless never be used in actual field application, we tested the popular commercial product TEPP-40 (purchased from a retail store, Ocala, FL) in our study to determine its residues on red-winged blackbirds (*Agelaius phoeniceus*) under laboratory simulated field conditions.

Because the major concern of the Bureau was the possible human hazard in contacting the dead or live birds sprayed with TEPP, we were interested only in the exterior TEPP residue on the whole birds. Therefore, a thorough extraction of TEPP with organic solvents was not used. Also, TEPP is among the most unstable of pesticides so that methods requiring prolonged processes of extraction, concentration, and purification could not be used. We instead used water as the extractant, taking advantage of TEPP's high solubility in water.

Although numerous analytical methods were being used successfully and routinely in the quantitative determination of the residues of many organophosphorus compounds, at the time this study was conducted the quantitative determination of TEPP had not been reported. In the comprehensive review by Bache and Lisk (1966) on the determination of organophosphorus insecticide residues by using Cook's emission spectroscopic detector, many insecticides were reported on but TEPP was not.

Gas chromatography had proved to be the most versatile and dominant technique in the quantitative determination of pesticide residues, but gas chromatographic methods, including those employing electron capture or microcoulometric detection, had not been reported for the determination of TEPP residues (Burchfield and Johnson, 1965). The Varian-Aerograph Company (Hartmann, 1966) reported the development of a phosphorus detector with high sensitivity; a number of organophosphorus pesticides were listed as test samples, but TEPP, again, was not among them.

Burchfield and Johnson (1965), in the Guide to the Analysis of Pesticide Residues, tabulated methods used in the determination of all common pesticide residues. In all the references they listed, enzymatic methods were the only methods that could be used effectively in the quantitative determination of TEPP. Among the enzymatic methods, the Warburg manometric method was the most convenient and sensitive (Giang and Hall, 1951; Panosyan, 1958). Cook (1954) also described a quantitative enzymatic colorimetric method. Although he did not discuss the sensitivity of the method, his illustrated sample indicated it to be in the part per million range. A much more sensitive analytical procedure was needed for our purposes.

We now describe the determination of the TEPP residues on redwings with the enzymatic method based upon the inhibition of acetylcholinesterase (Chadwick and Hill, 1947; Metcalf and March, 1949; Brauer, 1949; Casida et al., 1954) using the Warburg method (Umbreit et al., 1964).

METHODS AND MATERIALS

Major equipment used was a Warburg apparatus (Precision Scientific Company*, Chicago, IL) with eighteen 15-ml reaction flasks, each with a venting plug. Specific chemicals used were: Acetylcholinesterase (AChE) in 20,000-unit ampules (one unit is defined as the amount of enzyme that will cause the hydrolysis of 1.0 μ mol of acetylcholine to choline and acetate per minute at pH 8.0 at 37°C), acetylcholine iodide (ACh) (Nutritional Biochemical Co., Cleveland, OH). We also used Ringer solution as modified by Augustinsson (1957), and multiplied for a factor of 3.0/2.6, consisting of 100 ml of 1.05% NaCl, 30 ml of 1.45% $NaHCO_3$, and 2 ml of 2.03% $MgCl_2 \cdot 6H_2O$ and 0.9% NaCl saline solution.

Warburg Manometric Procedure

The general procedure was described by Umbreit et al. (1964) with minor modifications. For each AChE inhibition reaction, 0.4 ml of 1.50% of AChE in the modified Ringer solution was employed. The AChE solution was prepared according to Giang and Hall (1951). One milliliter of the solution contained about 16 units of AChE. The total volume of the reaction solution was 3 ml. The substrate ACh solution in the side arm of the reaction flask was 0.4 ml. The 2.6 ml of the fluid in the main compartment of the flask consisted of 1.8 ml of the Ringer solution, 0.4 ml of the enzyme solution, and 0.4 ml of the TEPP solution. For the control sample, 0.4 ml of water was used instead of the TEPP solution. The reaction solution containing 0.4 ml of the enzyme solution liberated 149.3 μl of CO_2, and it contained the most suitable amount of enzyme for accurate measurement. The volume was therefore chosen as the standard volume of CO_2 evolved from control samples throughout our experiments. The relationship between 0.2 to 0.6 ml of the enzyme solution used and the respective volumes of CO_2 evolved was linear.

Preparation of Calibration Curve

Two milliliters (2.43 g) of TEPP-40 was dissolved in distilled water and made up to 500 ml. The TEPP-40 had been determined to contain 40.4% TEPP by standard procedure (Gunther and Blinn, 1955). The TEPP solution was passed through a column of weakly basic IR-45 ion exchange resin (Rohm & Haas Co., Philadelphia, PA) at a rate of ca 25 ml/min to remove acidic components (Horwitz, 1960). After that the column was washed with three 100-ml portions of water. The washings were combined with the effluent. The resulting solution was made up to 980 ml with water. The resulting TEPP concentration was 1,000 ppm. From this, test solutions containing 2, 5, 10, 20, 30, 40 and 50 ppb of TEPP were prepared.

Immediately after each preparation, two duplicate determinations of AChE inhibition were made. The average volumes of the two duplicate values of CO_2 evolved in 30 minutes were plotted against their corresponding concentrations of TEPP in the solutions. This resulted in the standard curve (Fig. 1). A plot of the mean volumes (μl) of CO_2 evolved in the first 30 minutes versus the concentrations of TEPP in parts per billion on semilogarithmic graph paper revealed that those plotted points lay close to a straight line (Draper and Smith, 1981). This characteristic indicated that the TEPP standard curve approximated an exponential curve.

Preparation of Redwing Carcasses for TEPP Residue Analyses

Treatment of redwing carcasses with TEPP — The carcasses were treated to approximate the maximum amount of TEPP residues that could possibly be found on birds sprayed with TEPP solution in the field.

Male redwings were killed five minutes before being dipped. A 5% TEPP solution was prepared by mixing 125 ml of TEPP-40 solution with 875 ml of distilled water. Groups of five birds were each placed on an all-glass holder and dipped simultaneously for five seconds in the dipping tubes (Fig. 2), each of which contained 400 ml of 5% TEPP solution. Birds were left on holders to drain for five minutes, and then placed on a glass tray. Twenty-five birds so treated were arranged into five new groupings so that each new grouping contained one bird from each of the five original groups that were dipped together. This arrangement of birds was designed to improve the uniformity of the TEPP treatment received by each final group of five birds. Each new group of five birds was placed in a separate glass tray lined with one sheet of folded Whatman #1 filter paper. Birds not used immediately for residue analysis were refrigerated.

Residue extraction and cleaning — Five TEPP-treated redwings were each strapped to a holder and washed simultaneously and vigorously in the tubes, each containing 400 ml of water, 14 times (Fig. 2). Each wash took three minutes. For speedy washing, the tubes were not rinsed after each wash. After each of the first 13 washes, 50 ml of the extract was pipetted from each tube and placed in a beaker. A total of 650 ml of the extract (A) was collected. Five minutes were allowed for birds to drain between

washings. The 14th washing was kept separate from the first 13; 100 ml of this extract was taken from each tube for a total of 500 ml. Washing of five control birds was done in the same manner. One hundred milliliters of Extract A was poured into a beaker and mixed with 0.25 g of analytical grade Celite. The extract was stirred and filtered by suction through a glass Buchner funnel with a medium fritted disk. The filtrate was passed through an ion exchange column that contained 9 ml IR-45 resin, which was previously washed repeatedly with water until the washing no longer changed the color of phenolphthalein indicator to red. The extract, after passing through the column, was collected for Warburg analysis. The extracts from the 14th washing and the washings of the control birds were treated in the same manner before analysis.

To determine the volume of water needed in washing birds for residue analysis, we retained five TEPP-treated birds on bird holders and washed them vigorously 16 times, as described in the previous section. The AChE inhibition activity of each extract was determined as previously described. Total inhibition occurred up to the 8th washing. The TEPP residue per bird determined from eighteen 14th washings was (17.5 ± 10.6 μg) and was negligible, and the 13th washing was almost free of inhibition effects. Therefore, the first 13 washings were combined in the determination of the residue on birds. The combined 14 washings of control birds did not inhibit AChE.

Calculating residues on birds — From the volume (μl) of CO_2 evolved, the concentration of TEPP (in ppb) in the extract was located on the standard curve (Fig. 1). Then, the average TEPP residue on one bird was calculated as follows:

(Total vol. of extract in ml x TEPP conc. in ppb)/10^{-9}/number of birds = TEPP mg/bird.

Five birds were washed 13 times in five tubes, each tube containing 400 ml of water, and the solution was changed after each washing, the total volume of washing was 400 x 13 x 5 = 26,000 or 26.0 x 10^3 ml. This volume would increase upon dilution to obtain proper concentration of TEPP for AChE inhibition that could be read on the standard curve. If the solution was diluted from 1 to 1,000 ml, then the final volume was 2.6 x 10^6 ml. Let *C* be the reading of ppb of TEPP in the diluted solution on the standard curve, then the amount (in grams) of TEPP residue on one bird would be as follows:

26.0 x 10^6 x (*C* x 10^{-9}) 10^{-3} g/5 birds = 5.2 *C* mg/bird.

Since the average redwing weight was 46 g in this test, the TEPP residue on each gram of bird then would be

5.2 *C*/46 g body weight = 0.113 *C* mg/g body weight.

Recording temperature and humidity — The temperature and relative humidity in the refrigerator, where the treated birds were stored, were recorded in a separate run with a hygrothermograph (Model 594, Bendix Corp., Baltimore, MD). In this run, the hygrothermograph was placed in the refrigerator with trays of birds that were treated in the same manner as the TEPP-treated birds except that the TEPP solution was replaced with water containing 0.1% Tween-20 (Atlas Chemical Co., Wilmington, DE). This wetting agent was used to make the water penetrate the feathers of the birds similar to the TEPP solution. The door of the refrigerator was opened for one minute each day to simulate the daily removal of samples. The temperature and relative humidity of the refrigerator were recorded daily under the same conditions as when TEPP-treated birds were stored.

RESULTS AND DISCUSSION

Sensitivity and Precision of Measurement of CO_2 Evolved

The volumes of CO_2 evolved, as the result of enzymatic reaction of AChE, were determined with 15 replicates of samples that were prepared in the same way as control as previously described. The means of the volumes of CO_2 evolved (after correction for the thermobarometric change) was 184.4 μl with standard error of 1.00. A 99% confidence interval was 181.4 - 187.1 μl and 95% confidence interval was 182.2 - 86.5 μl, showing good reproducibility of evolved CO_2 volume.

An experiment was also run to determine if there was a significant difference among the volumes of CO_2 evolved in the first 30 minutes when there was no TEPP, TEPP at 1 ppb, and at 2 ppb in the solutions, respectively. Four replicates of TEPP solutions were determined for the volume of CO_2 evolved. Each set had a control solution, a 1-ppb TEPP solution, and a 2-ppb TEPP solution. The results, after correction for thermobarometric change and CO_2 evolved due to spontaneous decomposition, indicated that we were able to detect TEPP in a 1-ppb solution using a volume of only 0.4 ml. Statistically, by using Duncan's test (Duncan, 1955), significant differences between control and TEPP at 1 ppb, and control and TEPP at 2 ppb were noted at $\alpha = 0.10$ level of significance; whereas the difference between TEPP at 1 ppb and 2 ppb was significant at $\alpha = 0.19$ level. This indicated that a significant difference did exist between samples containing no TEPP and TEPP at 1 ppb, and between samples containing TEPP at 1 ppb and TEPP at 2 ppb under the conditions of the experiment.

Determination of Degradation of TEPP Residues on Birds

Residue determinations were conducted on three runs. Sixty-one days elapsed between the first and second run, and one day between the second and third runs. Redwings were sacrificed and dipped in TEPP solution as previously described. In the first run, five birds were used as a control and 75 birds were treated with the TEPP solution and then placed in 15 glass trays, immediately extracted, and the amount of TEPP on the birds determined. The remaining 14 trays were stored in a refrigerator. On each of the nine subsequent consecutive storage days, one tray was removed from the refrigerator and TEPP residues were determined. Residues also were determined for birds stored 13 and 19 days. On the 13th storage day the birds had already become soft and were deteriorating. In both the second and third runs, 25 birds were treated with TEPP.

The temperatures at which the birds were stored varied between 45 and 47°F and the relative humidity was about 60% on the first six days and then declined to about 57%. The TEPP residues found on birds are given in Table 1. The residues were also calculated in mg of TEPP per kg of bird. The data in Table 1 were plotted to show the TEPP residue per redwing and per kilogram of redwing versus days in storage (Fig 3).

Spontaneous Degradation of TEPP in Dilute Water Solution

Several dilute water solutions of TEPP were prepared to determine the general degradation pattern of TEPP. About 2 ml (2.43 g) of 40.4% TEPP was dissolved in about 500 ml of water. The solution was passed through an IR-45 ion exchange column and made up to the volume of 980 ml as described previously. The TEPP concentration in this original solution was 1,000 ppm. From this we prepared a series of 50-ml solution with respective TEPP concentrations of 1,000, 500, 250, 125, and 63 ppb and stored them in 100-ml volumetric flasks. The five flasks were left open to the air in the laboratory at the ambient room temperature for six days. The laboratory temperature ranged from 15 to 22°C (59-72°F) each day. To simulate field conditions, we made no attempt to control the temperature of the solutions.

The extent of AChE inhibition of each solution was determined daily. All volumes of CO_2 evolved were corrected, by using 149.3 μl for the first 30 minutes as a standard of control as previously described. The total volumes of CO_2 evolved and the corresponding TEPP concentrations in the solutions were plotted into degradation-pattern curves as shown in Figure 4. The general pattern of TEPP degradation seemed to be that at low concentrations the degradation proceeded rapidly during the first day and then gradually leveled off. At higher concentrations, TEPP decomposed first to the detectable range of our procedure and then followed the same pattern of degradation as that of the low-concentration solutions. As a whole, the pattern of degradation, when converted to decrease in TEPP concentrations, was similar to that of TEPP residue on birds (Fig. 4).

A knowledge of the degradation pattern of organophosphate pesticides determined by AChE-inhibition is of much more practical importance than is determination by a chemical means (Giang and Hall, 1951), because the *in vitro* AChE inhibition is directly

related with the cholinergic toxicity. Since TEPP-40 used in the field is a mixture containing closely related longer lasting organophosphorus compounds that could contribute to AChE inhibition, it was only logical for us to consider that the TEPP residue as a whole had not completely decomposed. Although thin-layer, gas chromatography, gas liquid chromatography, and thin-layer plus cholinesterase inhibition have been reported for the qualitative and quantitative determination of TEPP residue (Askew et al., 1969; Crossley, 1970; and Shafik et al., 1971), the use of enzymatic method based on AChE inhibition will probably remain the preferred method because a chemical determination alone may not demonstrate the magnitude of the hazard. Furthermore, with the use of the nonhygroscopic ACh iodide, which allowed accurate weighing, instead of the hygroscopic ACh chloride as employed in Cook (1954) contributed to the high sensitivity of this enzymatic procedure.

Our experiments both on the TEPP residues on birds and on the spontaneous degradation of TEPP have shown that although AChE inhibition diminished rapidly with days in storage, it lingered on for a much longer period of time than was previously believed. The belief that TEPP residue was very short-lived might have stemmed from the finding by Giang and Hall (1951) that at 25°C (77°F), 99% of the chemically pure tetraethylpyrophosphate hydrolyzed in 45.2 hr. However, under the more realistic conditions for spraying birds at simulated field temperature of 7-8°C (45-46°F) and relative humidity of 57-60%, TEPP-40 residues diminished rapidly in the first days but gradually leveled off to the 19th day when over 2.2% (2.8% statistically possible) remained unhydrolyzed. What other circumstances, in addition to low temperature, may contribute to prolonging the residual AChE inhibition, and how this AChE-inhibition residue can affect humans and other animals are not known. But as long as *in vitro* cholinesterase inhibition is directly related to cholinergic toxicity, such lingering cholinesterase inhibitors are the real hazard and cannot be neglected.

Our findings have shown that TEPP residues would not disappear soon after a spraying, but would linger on dangerously for a longer period of time than previously believed. Any plans for the use of TEPP in the spraying of bird roosts must take this fact into consideration. By and large, this experiment has also demonstrated that for a meaningful determination of a pesticide residue interpretable for the toxicity, it should not only be the analysis of the chemically identifiable applicant but should also include the entire toxicological, but chemically unidentified, components. This is especially apparent for cholinergic neurotoxic pesticides. This report tells how our Fish and Wildlife Service deliberated before reaching a decision of general concern.

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* Use of company and trade names does not signify endorsement by the U.S. Government.

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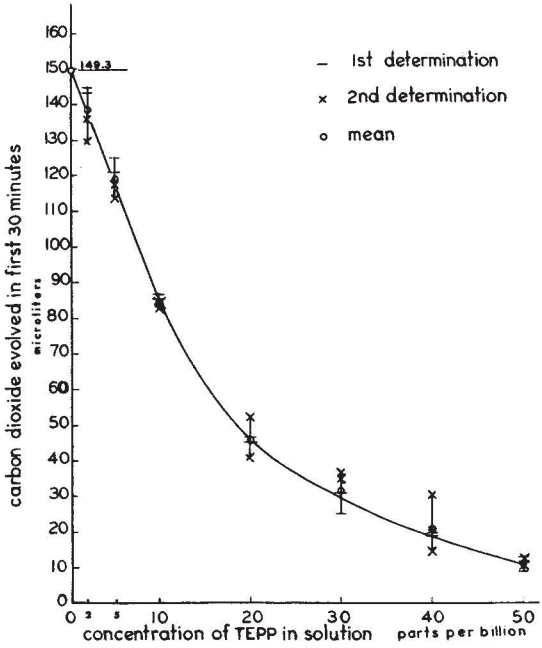

FIGURE 1. Standard curve for evaluation of CO_2 from TEPP standard solutions. Reaction conditions: bath temperature, 25°C; strokes per minute, 130; pH of reaction mixture, 7.4; gas phase, N_2-CO_2 (95-5%).

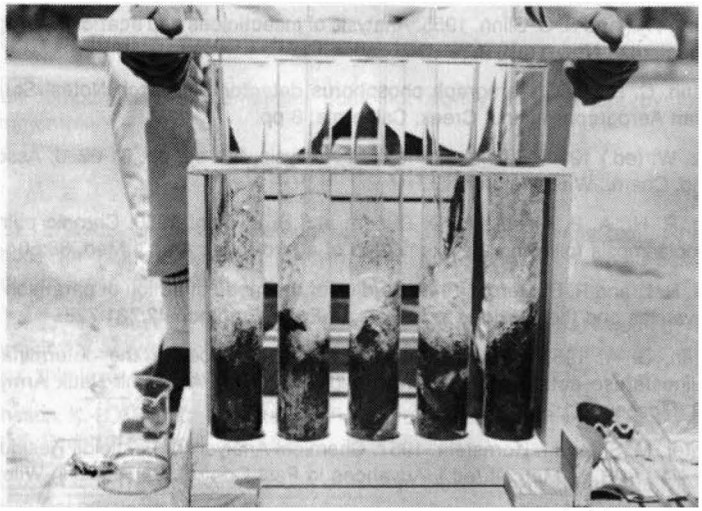

FIGURE 2. Stripping TEPP-treated redwings on glass holders.

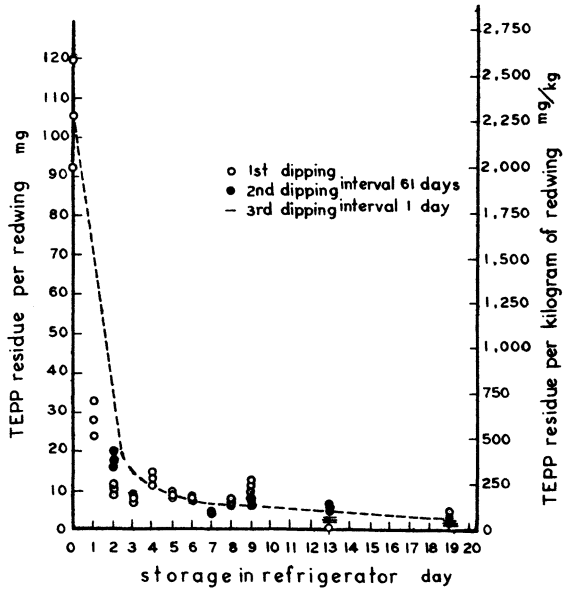

FIGURE 3. TEPP residues on TEPP-treated redwings after various days of storage in refrigerator. Conditions of storage: temperature, 45-47°F relative humidity, 57-62%.

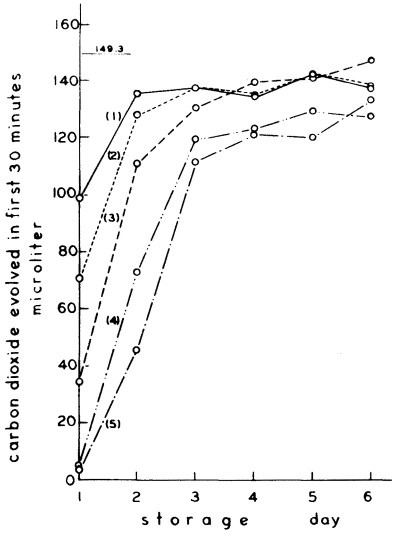

FIGURE 4. Volumes of CO_2 evolved from five different TEPP water solutions at 1 through 6 days in storage at room temperatures*. Initial concentrations of TEPP (ppb) in the solutions from which the five curves were plotted.:**

(1) 63; (2) 125; (3) 250; (4) 500; (5) 1,000.

*** In order to simulate field conditions, no attempt was made to control the temperature of the solutions, which were exposed to the natural winter room temperature of 15-22°C during the experiment.**

**** CO_2 evolved from solution without TEPP was 149.3 μ l (standardized).**