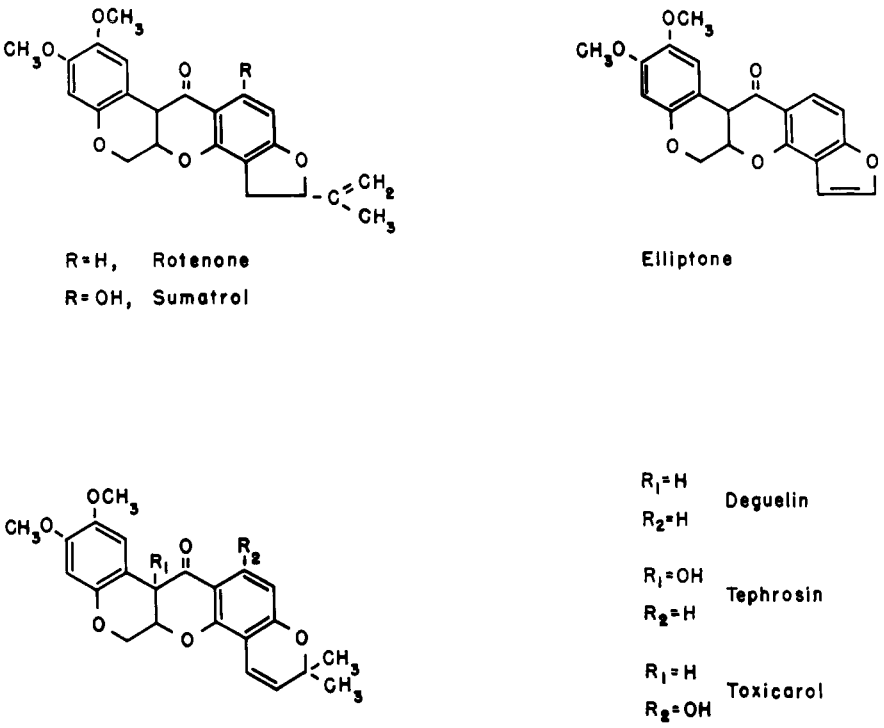

Figure 2. Structure of rotenoids used in this study

of rotenoids by any of the techniques presented above. A combination of systems in a two-dimensional chromatogram might be successful if the isolation of deguelin were necessary.

Elliptone is separated by solvents B and E, and by solvent B with silver nitrate plates. The first two are preferable to the latter because of the difficulty of visualizing the results on silver nitrate plates, as discussed earlier.

Tephrosin is the easiest of the rotenoids to separate. With the exception of solvents C and F, any solvent system presented here can be used. Solvents A and B are preferable.

Sumatrol can be separated with solvent A without resorting to chemical alteration of the molecule. As the mercuric acetate derivative, sumatrol is easily separated from the other compounds with solvent C. Solvent F precipitates sumatrol at the origin, whereas the other rotenoids give intermediate R_f values, but exhibit extensive tailing. Solvent F could be used to advantage as a second solvent in two-dimensional chromatography to separate sumatrol and toxicarol, for example.

Toxicarol is not completely separated from complex mixtures of rotenoids by any single solvent system used in this report. Two-dimensional chromatography might be used, or toxicarol and sumatrol could be separated first from the nonphenolic rotenoids by extraction with sodium hydroxide solution and then from one another by chromatography with solvent A, C (mercuric acetate derivatives), or F.

The absorption of sumatrol and toxicarol, both phenols, is enhanced in silica gel G layers impregnated with ferric chloride. Presumably, this is due to the complexing of the phenol with the ferric chloride in the plate. This technique has apparently not been used before. It may find application in the chromatography of other phenols. The plates are reddish brown after being heated for activation, but turn white when heated with the hydriodic acid spray reagent. The rotenoids give their customary colors with this reagent.

The techniques presented in this paper have been useful in separating and qualitatively estimating the amounts of rotenoids in *T. vogelii* leaf extracts at this station. Further studies are under way to develop a quantitative assay for rotenone based on thin-layer chromatography.

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Received for review August 13, 1965. Accepted November 6, 1965.

RESIDUE IN TISSUE

Esterase Inhibition in Pheasants Poisoned by *O,O*-Diethyl *S*-(Ethylthiomethyl)-phosphorodithioate (Thimet)

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The hazards involved, especially to wildlife, in the use of persistent organochlorine pesticides, are widely appreciated. This has led to a search for less persistent pesticides, and organophosphorus esters are now being used in increasing quantities. Although they are less likely to have chronic effects, many of these compounds are acutely poisonous, and methods are needed for examining wild birds and possibly other animals thought to have been killed by these compounds.

There has been much research in the detection, identification, and determination of organochlorine residues in animal tissues, and methods for the routine examination of wildlife relicta are now well established (*20, 21*). However, the interpretation of the figures obtained is difficult, since residues found after death even in test-fed birds are not consistent (*23*), and acute poisoning may be aggravated by a number of factors, including residues accumulated over a long period or sudden physiological changes. No comparable analytical methods exist for organophosphorus pesticides in animal tissue, and indeed they would be difficult to set up since most of these compounds are readily broken down in the body even after death, thereby rendering res-

Pheasants have been dosed orally with varying amounts of Thimet, and post-mortem chemical and biochemical investigations have been carried out with a view to finding diagnostic features of poisoning. The most reliable indication of death due to Thimet poisoning was the complete inhibition of brain cholinesterase. Inhibition of cholinesterase in blood and of other esterases in brain and blood was more variable when measured by conventional assay procedures, but specific inhibition of esterase isozymes was demonstrated by electrophoresis. Chemical analysis for Thimet was of value only for the gut contents.

idues difficult to find and identify, and probably causing any quantitative figures to bear no relation to the dose received. However, since the danger from organophosphate esters is that of acute rather than chronic toxicity, any post-mortem examination should aim primarily at determining whether the animal died from organophosphate poisoning and, if so, which pesticide was responsible. The investigation described in this paper is one of a series being carried out in this laboratory which are designed to produce such a routine examination.

Although it is not yet possible to point with certainty to any specific biochemical lesions due to organochlorine pesticide poisoning, the primary effect of organophosphate poisoning in mammals has long been recognized as the inhibition of cholinesterase and the consequent disruption of cholinergic sites in the nervous system. However, organophosphates also inhibit some of the many nonspecific esterases and proteases found in body tissues, and this property was used to differentiate esterases broadly by selective inhibition with paraoxon (*1*). The introduction of zone-electrophoresis in starch gels (*17*), and the application of histochemical reagents to the gels (*9*) has revealed the presence of numerous molecular forms of carboxylic esterases, and, by incorporating inhibitors into the gel, made the demonstration of inhibition easier and more specific (*2*, *7*). This has led to a number of attempts in the field of agricultural chemistry to use this method to identify pesticide residues in plant extracts (*4*, *13*, *16*, *22*). However, no work appears to have been undertaken either on the addition of extracts of tissues from organophosphate poisoned animals to the gels or on the direct electrophoresis of suitable extracts of tissues from such animals.

Thimet was chosen as the first of a series of pesticides to be tested, as it is of high toxicity and is widely used in agriculture. In these preliminary experiments, pheasants were poisoned with varying amounts of Thimet and, if not already dead, were killed 18 hours after dosing. Tissue extracts and blood were then examined by electrophoresis, and cholinesterase and phenyl benzoate esterase (*8*) levels were assayed to determine changes suitable for the diagnosis of poisoning. Emphasis has been laid on tissue extracts rather than blood, which is the tissue normally used in human and veterinary medicine, since wildlife casualties to be examined are rarely fresh enough to allow blood to be collected. Spun tissue extracts were used rather than crude homogenates for enzyme assay since this allowed all the work, including electrophoresis, to be carried out with the same preparation with little loss of activity. Triton X-100 extracts were used for brain tissue since organophosphate-sensitive bound esterases are released by this method (*3*). Chemical examination of certain representative tissues were also undertaken.

Experimental

Animal. Pheasants (*Phasianus colchicus*) were purchased from animal suppliers and were of heterogeneous stock and unknown history. The birds were housed in a communal aviary until required, when they were weighed and transferred to individual cages immediately before dosing. Thimet (92.5% pure) was force-fed to the birds in gelatin capsules in doses ranging from 6.2 to 21.7 mg. per kg. After 18 hours, blood was collected into heparinized tubes from the brachial vein of birds which survived, and they were then killed by cervical dislocation. A small amount of blood was collected from the heart of birds which had died.

Preparation of Tissue Extract. The liver, kidney, brain, and a portion of the breast muscle were removed from each bird and placed on ice as rapidly as possible after death. The entire kidney and brain and a portion of the liver were washed in approximately 20 ml. of ice-cold water, and homogenized in a Potter-Elvehjem homogenizer equipped with a Teflon pestle. Liver and kidney were homogenized in ice-cold water to give a tissue suspension such that 1 gram ≡ 2 ml. and 1 gram ≡ 3 ml. of homogenate, respectively. Brain was homogenized in ice-cold 1% Triton X-100 (Lennig Chemicals Ltd.) such that 1 gram ≡ 3 ml. of homogenate. The suspensions were centrifuged at 0° to 3° C. and 4000 g for 15 minutes. The supernatants were centrifuged at 16,000 g for 40 minutes, and the supernatants were taken from the latter centrifugation. The protein concentration of these preparations were measured by the method of Warburg and Christian (*24*). In blood samples, activity was found only in the plasma, which was prepared by centrifuging heparinized samples at 1700 g for 10 minutes.

Electrophoresis. Starch gel electrophoresis was carried out according to Smithies (*18*) using hydrolyzed starch (BDH Ltd.) in 0.026*M* borate buffer, pH 8.97, in the amounts recommended by the manufacturer. Aliquots of tissue extracts (liver 10 μl.; kidney 25 μl.; brain 50 μl.) on strips of Whatman 3MM paper were inserted into slits in the starch, cut by a razor blade, and after sealing the samples in the gel with paraffin wax, the entire gel surface was covered with Parafilm. The gel was then subjected to a potential difference of 6 volts per cm. for 16 hours at room temperature. At the conclusion of the electrophoresis, the gel was trimmed and cut horizontally with a wire into three equal layers. Each layer was then cut vertically into four for ease of handling such that two samples were on each strip. The four strips from each layer were then all stained in one of the following three ways.

Electropherograms of protein were obtained by staining with a solution of Amidoblack 10B (1 gram) in methanol–water–glacial acetic acid (5 + 5 + 1; 250 ml.) for 2 hours and then removing the excess dye by continuous washing with the same solvent mixture.

Carboxylic Esterase Zymograms (*9*) were obtained by incubating the gel strips for 1 hour at room temperature in a freshly prepared filtered solution of Fast Blue RR salt (BDH Ltd.) (0.25 gram) in 1% α-naphthyl acetate in acetone (5 ml.), water (235 ml.), 19% magnesium sulfate (10 ml.) and 0.2*M* Tris buffer pH 7.0 (10 ml.).

Cholinesterase Zymograms were obtained by an adaptation of the method of Karnovsky and Roots (*11*). Acetylthiocholine iodide (Koch-Lights) (0.05 gram) was dissolved in 0.1*M* sodium maleate pH 6.0 (160 ml.), and 0.1*M* sodium citrate (10 ml.), 0.03*M* copper sulfate (20 ml.), and 0.0025*M* potassium ferricyanide (40 ml.) added in order with stirring. Gel strips were incubated at room temperature for 16 hours when cholinesterase activity could be seen as a brown stain on a white background. All gel strips were stored in 50% aqueous ethanol to ensure even shrinkage before measuring and recording.

Enzyme Estimations. The electrometric method of Michel (*14*) using Buffer II was satisfactory for the determination of cholinesterase levels in the plasma of several avian species including pheasant. Cholinesterase levels of the 1% Triton X-100 brain extracts were also measured by the electrometric method, using a newly designed buffer; 0.004*M* sodium barbitone (0.8245 gram); 0.0025*M* KH_2PO_4 (0.3402 gram); 0.3*M* sodium chloride (17.535 grams). For 1 liter of buffer, these reagents were dissolved in distilled water (900 ml.), and

the pH was adjusted to 8.1 with potassium hydroxide before making up to the mark. This buffer was also satisfactory for use with 1% Triton X-100 extracts of the brains of a number of other avian species. Phenyl benzoate esterase was measured according to the method of Gomori (*8*). For this purpose, serum samples were diluted 1 to 500, and brain extracts were diluted 1 to 100 with phosphate buffer, pH 6.3. Results are expressed as μmoles of phenol per hour.

Chemical Methods. Aromatic-free hexane (BDH Ltd.) (750 ml.) was further purified by being passed through silica gel (200 grams) previously heated at 250° for 4 hours. Alumina (P. Spence Ltd.) was converted to Brockmann activity V according to the method of Laws and Webley (*12*).

Liver (5 grams), breast muscle (20 grams), and gut contents were examined for evidence of Thimet or its metabolites by admixture with one half of their weight of anhydrous sodium sulfate, and maceration with AnalaR chloroform (3 × 20 ml.), filtering through a Hartley Büchner funnel between each maceration. The combined chloroform filtrates were washed with 10% sodium chloride solution (2 × 20 ml.) and water, and were filtered through anhydrous sodium sulfate into a standard flask to be made up to 100 ml. with chloroform. Suitable duplicate aliquots of this solution were then taken almost to dryness on a rotary evaporator at ⩾50° C. and the residues transferred to an alumina column (100 × 10 mm.) and eluted with hexane (100 ml.). The hexane eluates were taken almost to dryness and the residues spotted onto silica gel H (Merck) coated thin-layer plates together with thimet standards, and after elution with a hexane–acetone mixture (9 + 1), the spots were visualized with the silver nitrate–bromophenol blue reagent or the blood plasma–bromothymol blue reagent previously described (*5*). Any Thimet was estimated by serial dilution.

Results

Electopherograms and zymograms of pheasant serum, liver, kidney, and brain are remarkably consistent. Minor variations in control tissue occurred only in relative band intensities, not in their position. Rises of temperature increase the over-all migration distances, but the relative distances moved by any pair of bands are unaltered, making comparisons possible. No cathodic bands are obtained. Only plasma yields electropherograms with discrete structure and exhibits nine bands. Other tissues exhibit a few indiscrete bands on a continuous background. With zymograms obtained using α-naphthyl acetate, liver exhibits eleven bands, kidney eight bands, and brain four bands. Plasma exhibits no reaction with α-naphthyl acetate. Cholinesterase zymograms are more simple; brain, kidney, and plasma each exhibit three slow-moving bands and liver has no reaction. In general, strong protein bands do not coincide with bands of enzyme activity. All bands are numbered from the sample slot.

Serum electropherograms show no differences from control to heavily poisoned birds (Figure 1), while serum cholinesterase zymograms give erratic results. Where inhibition was demonstrated in samples taken before and after poisoning from the same bird, it is complete only for the slowest band, while band 2 appears to be partially inhibited. In no case is band 3 affected. Zymograms of α-naphthyl acetate esterase activity in liver are also somewhat erratic. In moderately poisoned birds, bands 6 and 7 are diminished or completely suppressed, but in more heavily poisoned birds these bands appear much stronger than in the controls while bands 1 and 10 are completely inhibited (Figure 2). In one case, band 11 is inhibited. α-Naphthyl acetate esterase zymograms of kidney show no complete inhibition except for band 1 in the case of one heavily poisoned bird, but as with liver, there is again a strong increase of activity in the middle bands of birds which received large doses of Thimet (bands 5 and 6; Figure 3). All three bands in kidney cholinesterase zymograms are completely inhibited even with moderate doses of Thimet (Figure 4). α-Naphthyl acetate esterase zymograms of brain extract exhibit inhibition of the slowest moving band 1, even with moderate doses. No other changes occur unless the bird has died of poisoning, when band 2 is almost completely inhibited (Figure 5). A similar phenomenon is observed in brain cholinesterase zymograms which show no change from the controls unless the bird has died of poisoning, when bands 2 and 3 are completely inhibited and the intensity of band 1 considerably diminished (Figure 6). Band 1 of brain cholinesterase and band 2 of the brain α-naphthyl esterase zymogram occupy the same position on the gel.

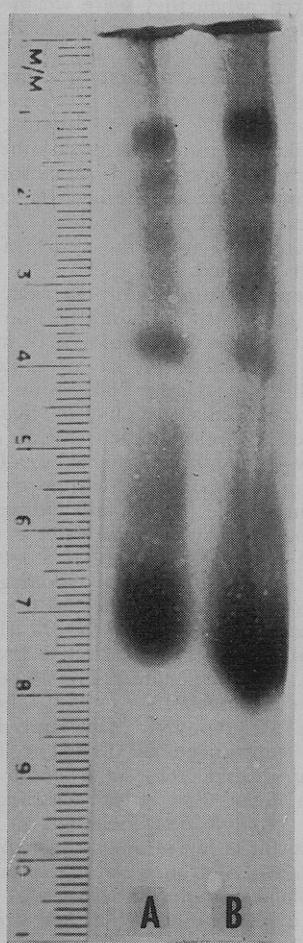

Figure 1. Electropherograms of pheasant plasma

A, After; *B*, Before dosing with 15.9 mg./kg. of Thimet

Results of the esterase assays carried out on the posioned birds are given in Table I together with dosages and other relevant information. Cholinesterase and phenyl benzoate esterase levels in plasma are rather erratic and, in this respect, mirror the electrophoretic results. Brain tissue extracts appear to give a clearer reflection of the condition of the bird. There is a rise in the phenyl benzoate esterase levels of both plasma and brain of the two moderately dosed birds.

Chemical investigation of samples of liver and muscle from all birds did not reveal the presence of Thimet in any case. The limit of detection was 1 μg.—i.e., 0.1 to 1 p.p.m. Thimet was easily demonstrated in the contents of three poisoned birds' guts whicl were examined and the levels were so high that the chloroform extract (about 100 μl.) could be spotted directly onto the plate without a column clean-up and the presence of Thimet demonstrated without too much interference. The total amount of pesticide in these gut contents could be roughly correlated to the dose given and these results are incorporated in Table I.

Discussion

A number of conclusions may be drawn from this preliminary investigation. Blood is not suitable as a tissue for diagnosing organophosphate poisoning in wild birds, since it is not easy to obtain and the levels of esterases and the electrophoretic results from both control and in vivo inhibited blood samples vary a good deal. Further work to be reported later using pigeons as the test bird has fully confirmed this observation. Brain tissue, however, is much more readily obtained and, being enclosed in the skull, is not subject to such rapid deterioration after death. Work now in progress has shown that esterase levels in brain tissue extracts do not fall to any large extent for long periods after death, even under simulated midsummer field conditions. Results obtained both by electrophoresis and cholinesterase assay

demonstrate the same complete inhibition of brain cholinesterase activity which occurs when a bird has died, and appears to be quite diagnostic of death by Thimet poisoning. Brain cholinesterase levels in poisoned birds which did not die are about 50% of control values. The mechanism involved in this partial or complete inhibition is obscure. The possibility that two enzymes are involved, one of which is inhibited more readily than the other, seems to be ruled out on the electrophoretic evidence, since no bands are lost unless the pheasant has died from the poisoning. A number of other special factors are generally recognized as operating in brain tissue. Among these are concentration of fat-soluble insecticides by the brain lipids and the subsequent excessive inhibition of cholinesterase upon liberation after homogenization (*15*), the apparent barrier to diffusion of ionic molecules into the brain, and nonuniform inhibition (*6*). Of these only the latter appears to offer a reasonable explanation of the present observation, and would require the postulate that one portion of the brain is inaccessible until a certain concentration of Thimet has built up, but that this effect is not seen in whole brain extracts. Some depression also occurs in the phenyl benzoate esterase levels, but death is not accompanied by complete inhibition of this esterase. The apparent slight rise in this esterase level after a small dose, which might be considered to be a defence mechanism which is overcome by larger quantities of inhibitor, has not been demonstrated in subsequent experiments on pigeons. The increases in intensity in certain bands in liver and kidney zymograms from poisoned birds also suggest the operation of a defense mechanism.

Of the three bands in the cholinesterase zymograms from brain tissue extract, bands 2 and 3, which are completely inhibited on death due to poisoning, have no counterpart in α-naphthyl acetate esterase zymograms, whereas band 1 which still appears faintly after death, migrates in exactly the same position as band 2 of the α-naphthyl acetate esterase zymograms. The latter also appears faintly after death due to poisoning. This suggests that bands 2 and 3 are true cholinesterase isozymes whereas band 1 is a nonspecific esterase capable of hydrolyzing acetylthiocholine. On this hypothesis, pheasant kidney contains three isozymes of true cholinesterase, whereas plasma contains three esterases acting on acetylthiocholine, only the slowest one of which appears to be inhibited by Thimet. Kaminsky (*10*) has also carried out electrophoretic studies and commented on the lack of specificity of esterases in the sera of avian species.

The more complex patterns obtained with α-naphthyl acetate zymograms of liver and kidney, and the changes which occur in them after Thimet poisoning are not easy to interpret in isolation. However, it is to be hoped that work on this and other species with a variety of

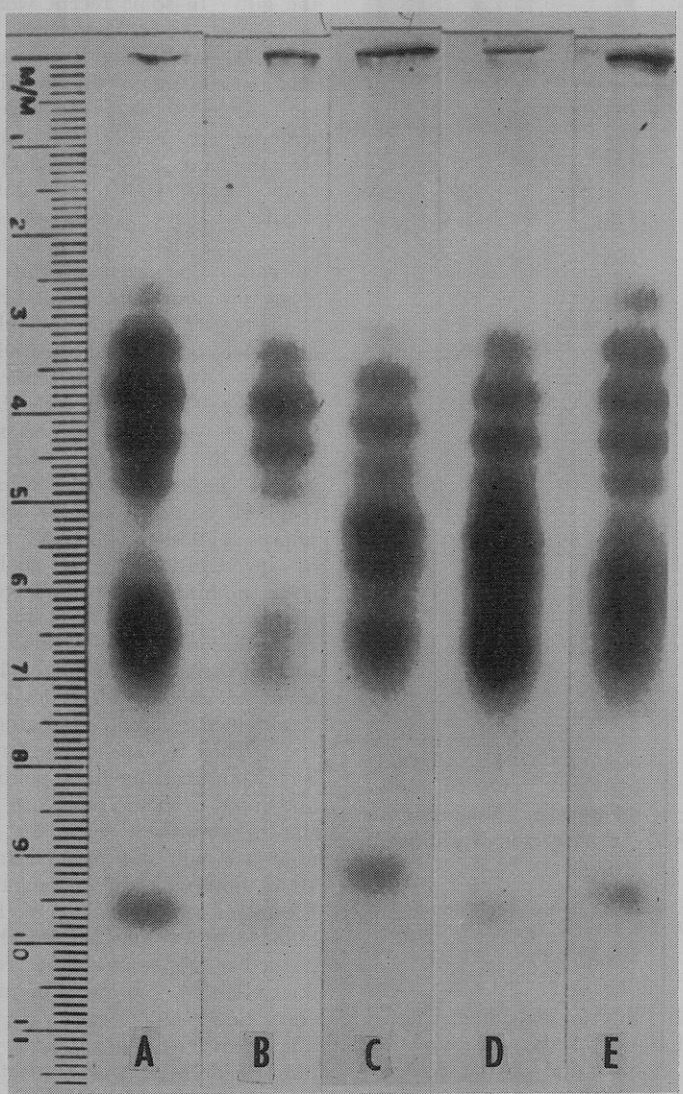

Figure 2. Carboxylic esterase zymograms of pheasant liver

The birds were dosed with Thimet: *A*, 6.2 mg./kg.; *B*, 13 mg./kg.; *C*, 15.9 mg./kg.; *D*, 20.6 mg./kg.; *E*, Control

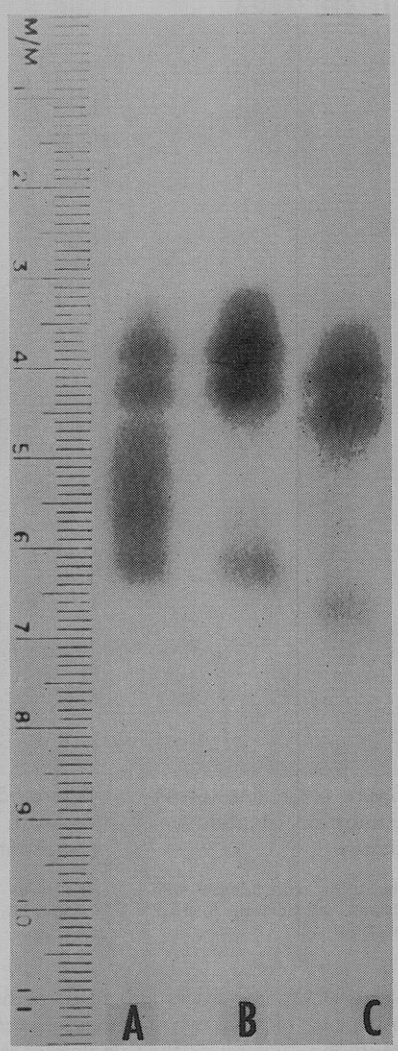

Figure 3. Carboxylic esterase zymograms of pheasant kidney

The birds were dosed with Thimet: *A*, 20.6 mg./kg.; *B*, 13 mg./kg.; *C*, Control

Table I. Effect of Treatment of Pheasants with Thimet on the Activity of Esterases

Pheasant		Thimet,		Activities of Enzymes[a]				
Sex	Wt., grams	Dosage, Mg./Kg.	Condition after 18 Hours	Plasma ChE[b]/ 0.1 ml.	Brain ChE[c]/ 0.1 ml. extract	Plasma PBE[d]/ml.	Brain PBE[e]/mg. protein	Total Thimet in Gut, Mg.
m	1080	6.2	Alive	8	96	12.8	0.147	...
m	1315	7.2	Alive	10	72	14.9	0.144	...
f	1325	10.3	Alive	2	97	2.06	0.085	...
m	1185	13.0	Alive	8	98	1.40	0.061	0.5
m	1533	15.9	Alive (barely)	0	57	2.13	0.072	3.5
m	1047	16.3	Alive	5	97	3.51	0.055	...
m	1109	18.8	Dead	0	2	...	...	...
f	852	20.6	Dead	6	2	4.04	0.051	...
m	1190	21.7	Dead	0	3	...	...	10

[a] Enzyme activities are expressed as follows: Cholinesterase (ChE) — Δ pH × 100/hour. Phenyl benzoate esterase (PBE) — μmoles phenol liberated per hour. Assay procedures are given in the text. Control values are given, with standard deviations (s) when six or more determinations were made.
[b] Mean control value ± s 31 ± 3.3.
[c] Mean control value ± s 184 ± 12.4.
[d] Mean control value 4.25.
[e] Mean control value ± s 0.080 ± 0.022.

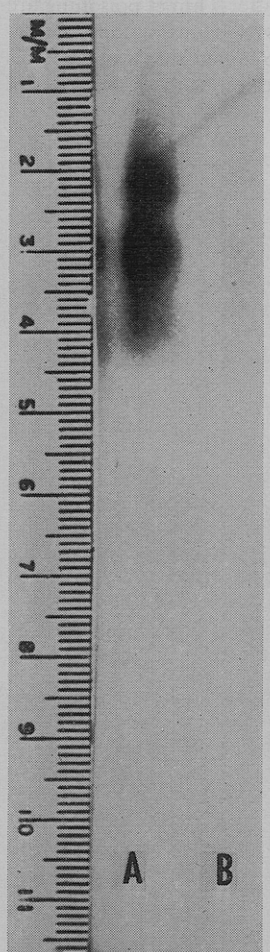

Figure 4. Cholinesterase zymogram of pheasant kidney

The birds were dosed with Thimet: A, Control; B, 15.9 mg./kg.

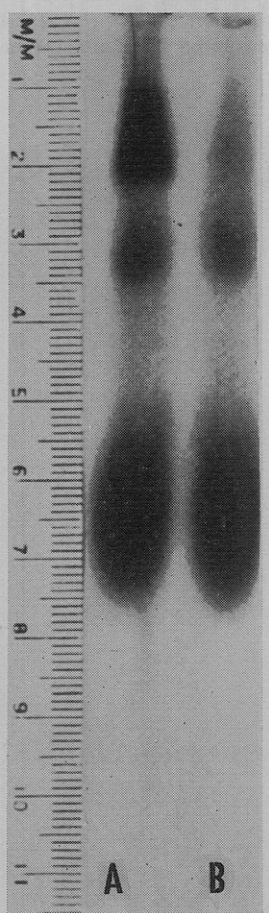

Figure 5. Carboxylic esterase zymograms of pheasant brain

The birds were dosed with Thimet: A, Control; B, 15.9 mg./kg.

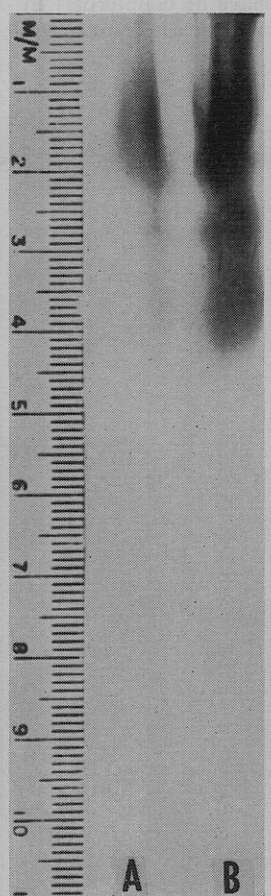

Figure 6. Cholinesterase zymograms of pheasant brain

The birds were dosed with Thimet: A, 15.9 mg./kg.; B, Control

organophosphate esters will lead to the emergence of a pattern of activation and inhibition whereby the pesticide responsible for poisoning can either be positively identified or identified as one of a group.

The chemical investigation of tissue was of no value for detecting Thimet using techniques of the sensitivity described. Examination of the contents of the digestive organs was more promising, although it is noteworthy that all experimental birds were killed within 18 hours of dosing. Under natural conditions birds may survive for longer periods before dying after ingesting lethal doses of Thimet or other organophosphate insecticides. In this event all traces of the poison may be cleared from the gut. In some recent work on the poisoning of rabbits with schradan, Stransky and Benes (*19*) also concluded that the contents of the digestive organs and the urine were the most suitable materials for analysis. It may be that the application of gas-liquid chromatography could lead to the detection of smaller amounts of organophosphates or their metabolites in poisoned tissue extracts if they were present, although many problems would still arise owing to the large number of pesticides in use, and wide variation of sensitivity to detection. In this event, confirmation of poisoning of the type provided by esterase inhibition would still be necessary.

From the preliminary work reported in this paper it would seem that a diagnosis of death due to organophosphorus poisoning in birds may be possible by investigation of the cholinesterase levels in brain tissues. Electrophoresis could be used particularly where a species has not been met before. In more common species, mean cholinesterase levels and their standard deviations may be obtained gradually, and a significance test applied to low cholinesterase levels both from experimentally poisoned birds and those from the field. In many instances, however, such an elaborate approach will not be necessary, since 100% inhibition will accompany death. Electrophoretic patterns of liver and kidney extracts may help to identify the particular organophosphate responsible for poisoning. Work is in progress to check how esterase levels vary with time after death in control birds, and also how rapidly spontaneous reactivation of esterases occurs after death in poisoned birds. The latter is dependent on the inhibitor involved, and in the case of Thimet there is no reactivation. Chemi-

cal extraction of the gut contents may lead to the identification of the pesticide by physical methods such as chromatography, or by biochemical methods such as the addition of the extract to a standard tissue preparation before electrophoresis, and subsequent demonstration of inhibition in the resulting zymogram.

Acknowledgment

The authors wish to thank E. N. Wright and M. R. Boulton for technical assistance, and R. R. Page for photographing the gels. Gifts of pheasant tissue for control purposes from J. S. Ash, Game Research Association, Fordingbridge, Hants., and Thimet from British Cyanamide, Ltd., are also gratefully acknowledged.

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Received for review August 31, 1965. Accepted December 28, 1965.

RESIDUE RECOVERY

Determination of Translocated Tetramine in Foliage by Hydrogen-Flame Gas Chromatography

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An analytical method for the quantitative determination of translocated tetramine (tetramethylenedisulfotetramine) in plant foliage, techniques for sample preparation, extraction, cleanup, and analysis by hydrogen-flame gas chromatography are reported. Different quantities of tetramine were added to Douglas fir foliage and then recovered. When the values of tetramine added *vs.* tetramine recovered were plotted, a straight-line relationship with a slope of 1.20 was found. This relationship was applied to the analysis of translocated samples. The procedure was applicable to recovery of added tetramine from representative species of five plant genera.

Effective reforestation in many areas of the United States has been seriously limited because of animals' feeding on seed and young seedlings. Research directed at minimizing these losses through the development of chemical repellents or toxicants is being carried out by the Denver Wildlife Research Center (*3, 5*). A chemical, either repellent or toxicant that is also plant systemic, is especially sought. Tetramine (tetramethylenedisulfotetramine) is classified as a systemic toxicant (*3, 5, 6*).

The synthesis of tetramine from formaldehyde and sulfamide was reported by Hecht and Henecka in 1949 (*1*). In 1953, a U. S. patent for the procedure was issued to Hecht, Henecka, and Meisenheimer, who claimed their product as a rodenticide (*2*). In 1952, Spencer and Kverno found that plants growing from tetramine-treated seed were toxic to meadow mice (*Microtus* spp.) and concluded that the compound had been translocated (*6*). Subsequently, Kverno and Campbell found that foliage from Douglas fir [*Pseudotsuga menziesii* (Mirb) Franco] seedlings growing in soil treated once with tetramine was toxic to hare (*Lepus americanus*) for as long as 4 years (*4*).

An analytical method for the detection of tetramine in plant foliage was needed to facilitate study of the translocation characteristics of the compound. Previously, mouse bioassay was the only analytical technique available, and was considered inadequate because tetramine concentrations of less than 20 p.p.m. could not be detected in vegetation, and because of individual variation in the test animals.

This paper describes a method for the quantitative detection of translocated tetramine in plant foliage by a process of extraction, cleanup, and subsequent analysis by hydrogen-flame gas chromatography.

Methods and Materials

The analytical instrument was a Wilkens Aerograph Hy-Fi Model 600-C gas chromatograph, incorporating a hydrogen - flame ionization detector, equipped with a $^1/_8$-inch o.d. × 10-foot stainless steel column packed with 10% Dow 11 Silicone on Fluoropak 80. A Wilkens Aerograph Model 650 hydrogen generator served as the hydrogen source, and the recorder was a 1-mv. Honeywell Electronik 15 with Disc chart integrator.

The tetramine used to establish the analytical procedure was purified by recrystallization from an acetone solution, followed by washing with water. The solubility of tetramine in eight common solvents at room temperature was established by analyzing the filtrates of the respective saturated solutions by gas