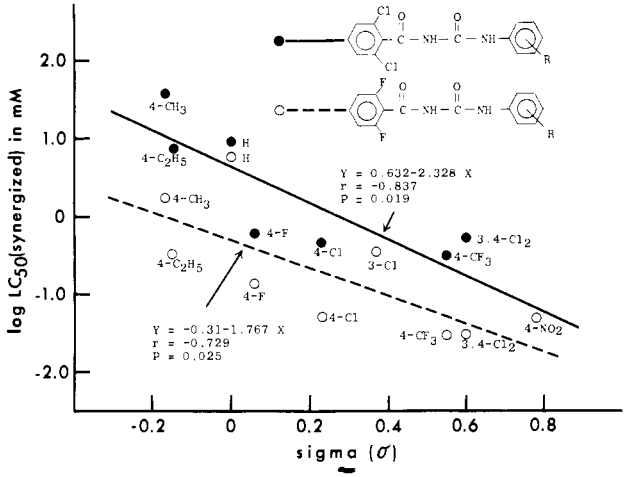

Figure 1. Correlation of insecticidal activity and Hammett's σ constant for substituted 1-phenyl-3-(2,6-dichlorobenzoyl)- and 1-phenyl-3-(2,6-difluorobenzoyl)-ureas.

moiety, produced the most active structures.

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Supplementary Material Available: Supplementary Tables I–III describing NMR and elemental analysis data, 4 pages. Ordering information is given on any current masthead page.

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Carbamate Poisoning. Effect of Certain Carbamate Pesticides on Esterase Levels in the Pheasant (*Phasianius colchicus*) and Pigeon (*Columba livia*)

Peter J. Bunyan and Derek M. Jennings*

Pheasants and pigeons were fed with lethal and sublethal doses of six widely used carbamates. Esterase levels were measured quantitatively in liver and brain and also by an electrophoretic method involving histochemical staining. It is suggested that the inhibition of esterase levels demonstrated by the quantitative measurement and the apparent elevation in esterase levels revealed by the electrophoretic method can be used to diagnose poisoning in birds with these compounds.

Previous communications from this laboratory (Bunyan and Taylor, 1966; Bunyan et al., 1968a,b, 1969, 1971) have demonstrated that esterase inhibition can be used for the diagnosis of organophosphorus poisoning in avian species, even under field conditions. Following increasing restrictions on the use of organochlorine pesticides and the concomitant increase in organophosphorus and carbamate pesticide usage, an investigation has now been undertaken into the possible use of the esterase inhibition method for the detection of carbamate pesticide poisoning in birds.

The pesticides chosen for this study include a number in full commercial use and additionally represent a wide range of chemical structures. Selection was also made to include compounds with a broad spread of toxicity, although at the time the experiments were carried out data were limited for avian species. For methiocarb and Zectran LD_{50} values were available for both pheasant and pigeon, but data for aldicarb, aminocarb, and pirimicarb were available only for the chicken. No data for avian species were available for propoxur.

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EXPERIMENTAL SECTION

Animals. The origin of the pheasants and pigeons and their treatment before and after dosing have been described previously (Bunyan and Taylor, 1966; Bunyan et al., 1968a). Relevant data on the birds and the dosage rate are given in Table I.

Pesticides. Six pesticides were chosen for study, namely: aldicarb (2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl) oxime), aminocarb (4-dimethylamino-3-methylphenyl methylcarbamate), methiocarb (3,5-dimethyl-4-methylthiophenyl methylcarbamate), pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate), propoxur (2-isopropoxyphenyl methylcarbamate), and Zectran (4-di-

Table I. Summary of the Experimental Treatment of Pheasants and Pigeons with Six Carbamate Pesticides[a]

Carbamate	Species	Male/female sex ratio	Dose, mg/kg	Approx. LD_{50}	Fate	
					Survived	Died
Aldicarb	Pheasant	1/1	6	0.5	2	0
		3/3	25-35	3	0	6 (2)
	Pigeon	2/2	25	2	0	4 (2)
Aminocarb	Pheasant	1/1	10	0.5	2	0
		1/1	60	3	1	1
Methiocarb	Pheasant	1/1	110	0.5	2	0
		4/4	400-525	2	6 (1)	2 (1)
	Pigeon	1/3	530-700	3	0	4 (2)
Pirimicarb	Pheasant	1/1	15	0.5	2	0
		3/3	110	3	0	6 (2)
	Pigeon	4/0	110	5	0	4 (2)
Propoxur	Pheasant	1/1	75	0.5	0	2
		3/3	330-450	3	0	6 (2)
	Pigeon	1/3	450	3	0	4 (2)
Zectran	Pheasant	2/2	2	0.5	2	0
			13	3	1	1

[a] Figures in parentheses refer to birds in which organ removal and esterase analysis were not carried out until 5 days post mortem.

methylamino-3,5-xylyl methylcarbamate).

Gifts of mainly technical nonformulated pesticides were obtained from the manufacturers. Aminocarb, methiocarb, and propoxur (all 99% pure) were obtained from the Baywood Chemical Co. Ltd., aldicarb from Murphy Chemical Co. Ltd., pirimicarb from Plant Protection Ltd., and Zectran from the Dow Chemical Co. Ltd. No further purification was carried out.

Treatment. In line with previous experiments, and in order to deal with the range of pesticides and conditions chosen, treatments were given with each compound to a small group of birds in an arbitrary but standard manner. Two series of treatments were undertaken. In one involving only pheasants, a pair (male and female) were treated with each pesticide at approximately three times the LD_{50} dose and a second pair were given approximately one-half the LD_{50} dose. For methiocarb, due to the high LD_{50} value, twice the LD_{50} doses were given. All birds were sacrificed after 16 hr if not already dead and esterase levels were determined on brain and liver extracts within 3 hr in order to minimize the possibility of enzyme reactivation. Starch gel electrophoretic analyses were also performed on the same extracts at 8 hr post mortem. In the light of the results from these experiments, in which little or no esterase reactivation was apparent, a second series of treatments was undertaken in which aldicarb, methiocarb, pirimicarb, and propoxur were each fed to two pairs of pheasants and two pairs of pigeons at high doses ($3 \times LD_{50}$). Esterase levels were measured and electrophoresis carried out on extracts from one pair of birds from each species as soon as possible after death, and on extracts from the remaining pairs 5 days post mortem. In the latter, bodies remained at ambient temperatures until tissues were removed and extracted just prior to examination.

Preparation of Tissue Extracts, Enzyme Estimations, and Electrophoresis. α-Naphthyl acetate esterase and cholinesterase were measured quantitatively and electrophoretically as previously described (Bunyan and Taylor, 1966; Bunyan et al., 1968a). Due to the possibility of spontaneous reactivation of esterases inhibited by carbamates all esterase determinations were undertaken as rapidly as possible and at standard times post mortem, to allow meaningful comparison between the various

Table II. Control Esterase Levels in Pheasants and Pigeons[a]

Esterase[b]	Pheasant		Pigeon	
	Brain	Liver	Brain	Liver
Cholinesterase	7.48		14.13	
Triacetin esterase	4.38	4.79	6.99	3.65
α-Naphthyl acetate esterase	3.00	5.56	2.48	5.24

[a] Esterase activities are expressed as micromoles of substrate hydrolyzed per hour per milligram of protein. [b] Mean esterase levels from 24 pheasants and 24 pigeons, respectively, taken as control (i.e., 100%).

treatments. During measurement of esterase levels where carbamate inhibition has occurred it has been extensively reported (Winteringham and Disney, 1964; Disney, 1966; Winteringham and Fowler, 1966) that high substrate concentrations and dilution of the esterase preparation can lead to a reversal of carbamate inhibition. However, in order to allow comparison to be made with the results obtained from previous organophosphorus feeding experiments no changes were made in the assays previously described (Bunyan et al., 1968a). The final dilutions for tissue extracts which were used were 60-fold for liver and 90-fold for brain extracts, while the final substrate concentration used was 2.2×10^{-2} *M*. Gels were scanned after staining with a Joyce Loebl densitometer in the reflectance mode and results were quantified as previously described (Bunyan et al., 1971).

RESULTS AND DISCUSSION

A summary of the experimental treatments is given in Table I. In previous communications the quantitative results have been listed individually both as absolute values and as the percentage difference from the control mean value of each esterase in each species. Because of the complexity of the conditions used in this investigation results are presented in the form of a modified scattergram of percentage variation from the mean control values shown in Table II and only selected typical results are shown.

Figures 1–6 show the brain cholinesterase and both brain and liver triacetin esterase and α-naphthyl acetate esterase levels in pheasants and pigeons as a percentage of the control mean level, after dosing with carbamates. In an earlier communication (Bunyan et al., 1968a) the authors postulated that in the absence of larger numbers of results, which are seldom available in the field, a measured esterase value of more than two standard deviations ($2 \times SD$) from the control mean value may be considered statistically significant. Figures 1–6 include the $2 \times SD$ levels shown as percentage variations from the control mean value, for each esterase measured.

Figures 1–6 also show esterase levels obtained electrophoretically, enabling a comparison to be made of the results obtained for pheasant and pigeon esterase levels by the conventional method and by quantitative scanning of starch gel electrophoregrams of the same extracts. For the latter, total integral values normalized for background are also plotted as variations from control mean values.

Some discussion of results for each pesticide is included below and a more general discussion concludes the section. For convenience esterases are referred to as follows: cholinesterase, ChE; triacetin esterase, TE; and α-naphthyl acetate esterase, NA. These abbreviations are used for both quantitative and electrophoregram results.

Aldicarb. The only avian toxicity data available were a reported value of 9 mg/kg for the cockerel (Union Carbide UK Ltd., 1971) although recently a value of 15.2

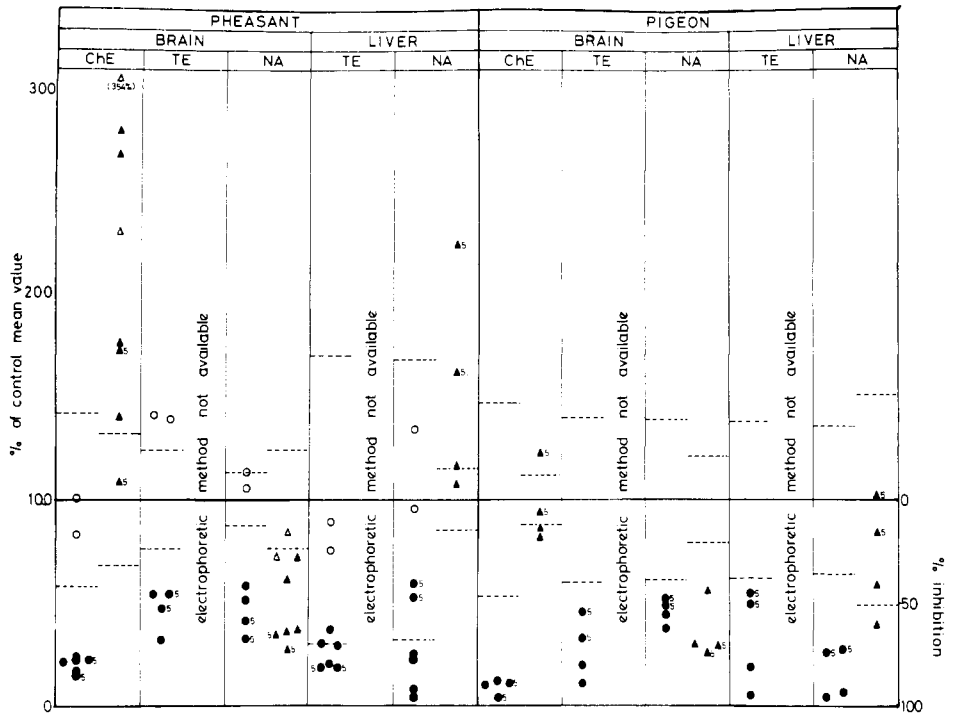

Figure 1. The percentage variation from the control mean in pheasant and pigeon esterase levels measured by a quantitative method and an electrophoretic method following forced feeding of aldicarb with acute and subacute doses. Esterase levels were measured at 0 and 5 days (indicated by 5) post mortem. Two × SD for each control mean level is indicated by a dashed line. Individual results are shown as follows: (○) level measured quantitatively in birds surviving to sacrifice at 16 hr; (●) level measured quantitatively in birds dead at 16 hr; (△) level measured electrophoretically in birds surviving to sacrifice at 16 hr; (▲) level measured electrophoretically in birds dead at 16 hr. Mean esterase levels from 24 control pheasants and 24 control pigeons taken as 100%.

mg/kg has been reported for the pheasant (Union Carbide UK Ltd., 1975). To pheasants, 5 and 25 mg/kg doses were administered and 25 mg/kg was administered to pigeons. All birds given the higher dose died while the others survived. Brain ChE levels measured conventionally in all lethally poisoned birds are massively inhibited (Figure 1), ranging between 75 and 86% in pheasant brain extract examined either immediately or 5 days post mortem. Greater inhibition (93–98%) occurs in the pigeons and, significantly, the greatest inhibition (98%) is observed in a "day 5" pigeon brain. Quantitative brain ChE levels in the subacutely dosed pheasants are very close to control mean levels. Pheasant brain TE and NA levels exhibit a similar but less marked inhibition. Pheasant liver TE levels are remarkably similar to the brain ChE pattern but liver NA levels are more variable. In the pigeon (Figure 1) other esterase levels show less inhibition than the brain ChE levels but are well below the 2 × SD level. Brain and liver TE and liver NA levels measured at day 5 exhibit less inhibition than day 0.

Comparisons of the results obtained by conventional assay methods with those from starch gel electrophoresis (SGE) reveal remarkable differences. Acutely dosed pheasant brain ChE (Figure 1) obtained by SGE shows an average *elevation* in activity of 105% above control mean values in tissue extracts from lethally dosed "day 0" birds and an *elevation* of 40% at day 5, compared to significant inhibition measured conventionally. Subacutely dosed pheasants show brain ChE *elevations* of approximately 140% while liver NA levels (Figure 1) from lethally dosed pheasants show similar if less marked differences. Other measured levels are similar by both methods.

Comparison of the esterase activity by both methods in pigeon brain and liver extracts reveals a similar though less marked discrepancy (Figure 1). Both methods indicate a brain ChE and liver NA inhibition but this is greater by conventional measurement. Brain NA results obtained by both methods show similar reductions of between 42 and 78%.

Aminocarb. No data for single dose oral LD_{50} levels to avian species were available and an estimate of 20 mg/kg was made based on values quoted for the rat. Only two pairs of pheasants were used. Subacute and active doses of 10 and 60 mg/kg, respectively, were administered. One of the acutely dosed cock pheasants survived.

Conventionally assayed brain ChE levels in the acutely dosed birds are significantly inhibited (60%) while levels in subacutely dosed birds are nearer normal. The brain TE levels in the lethally dosed bird are depressed by just over 50% but levels from the surviving birds are near normal. The liver TE and NA levels are depressed between 46 and 78% at both dose levels but brain NA values are near normal.

Comparison of brain ChE levels obtained by SGE shows very large differences. Elevations of approximately 200% in electrophoretic measurements of acutely dosed birds compare with inhibitions of 50% measured quantitatively (Figure 2). Electrophoretic measurements from the subacutely dosed birds are also highly elevated (+174%; +197%). By contrast liver NA levels show the least difference between the two methods among all six of the carbamates studied (Figure 2). Brain NA levels by both methods in the acutely dosed birds are generally similar.

Methiocarb. The single dose oral LD_{50} of methiocarb to pigeons and the estimated LD_{50} value to pheasants are 50 and 225 mg/kg, respectively (Bayer UK Ltd., 1971). Because of the relatively low toxicity of the compound to pheasants a quantity equivalent to 2 × LD_{50} was employed for acute dosing to eight pheasants. Two birds died and the subsequent total brain ChE levels determined showed that the greatest enzyme inhibition (75–85%) occurs in these two birds, one of which was not assayed until 5 days

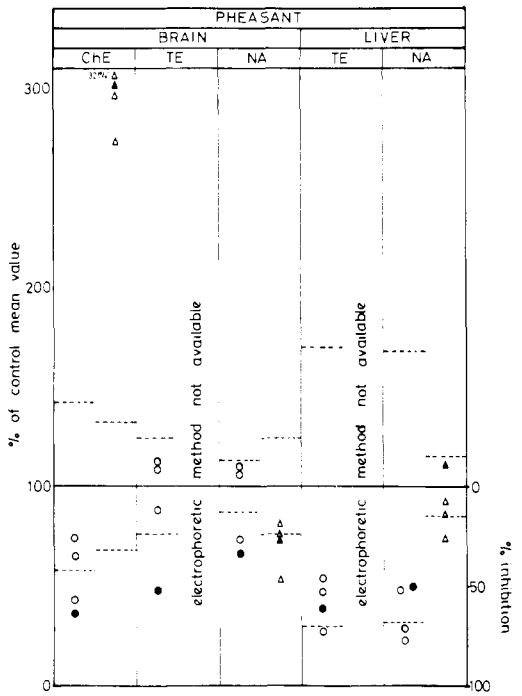

Figure 2. The percentage variation from the control mean in pheasant esterase levels measured by a quantitative method and an electrophoretic method following forced feeding of aminocarb with acute and subacute doses. Esterase levels were measured immediately post mortem (day 0). Two × SD for each control mean level is indicated by a dashed line. Individual results are shown as follows: (○) level measured quantitatively in birds surviving to sacrifice at 16 hr; (●) level measured quantitatively in birds dead at 16 hr; (△) level measured electrophoretically in birds surviving to sacrifice at 16 hr; (▲) level measured electrophoretically in birds dead at 16 hr. Mean esterase levels from 24 control pheasants taken as 100%.

post mortem. Inhibition in the other six birds ranged from 52 to 73%. Brain and liver TE and NA show similar but less marked inhibition. In the subacutely dosed pheasants, inhibition is generally much less although brain ChE and especially NA levels (Figure 1) are significantly affected.

Following acute (3 × LD_{50}) dosing of methiocarb to the four pigeons, all the birds died and brain ChE levels measured at both day 0 and day 5 are inhibited 85–90% (Figure 3). Other esterase levels are significantly depressed.

Comparison of the results obtained by total esterase measurements with those from SGE show remarkable differences. Pheasant brain ChE levels on starch gel are elevated by up to 186% (Figure 3) compared to a depression of 52% in the same bird when measured by the pH stat method. Brain ChE levels in the other pheasants show similar but smaller differences. The difference is also shown to a lesser degree in the liver NA levels of acutely dosed birds. Little difference occurs in levels measured in subacutely dosed birds or in any of the brain NA measurements. Similar but smaller differences occur for pigeon brain ChE and liver NA measurements.

Pirimicarb. The only recorded avian toxicity datum was an LD_{50} of 25–50 mg/kg for the hen (I.C.I. Plant Protection Division, 1971). Subsequent data from the same source after these experiments had been completed included values of 8.2 mg/kg for the Japanese quail and 19.5 mg/kg for the pigeon. Two pheasants were dosed at an estimated subacute level of 15 mg/kg and survived, while a further four birds of each species were given acute doses of 110 mg/kg, and died. In general, total esterase

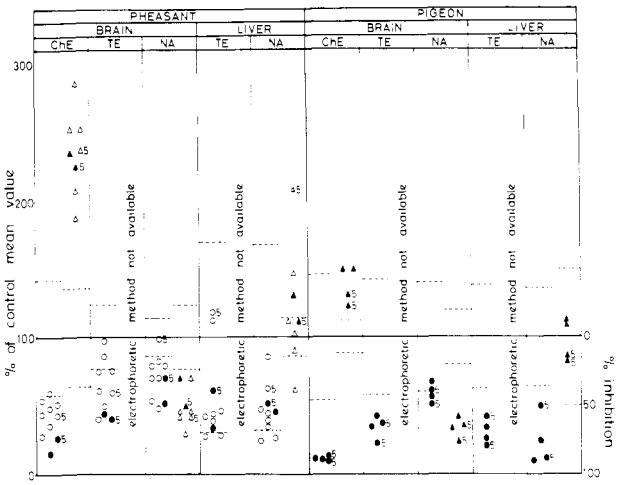

Figure 3. The percentage variation from the control mean in pheasant and pigeon esterase levels measured by a quantitative method and an electrophoretic method following forced feeding of methiocarb with acute and subacute doses. Esterase levels were measured at 0 and 5 days (indicated by 5) post mortem. Two × SD for each control mean level is indicated by a dashed line. Individual results are shown as follows: (○) level measured quantitatively in birds surviving to sacrifice at 16 hr; (●) level measured quantitatively in birds dead at 16 hr; (△) level measured electrophoretically in birds surviving to sacrifice at 16 hr; (▲) level measured electrophoretically in birds dead at 16 hr. Mean esterase levels from 24 control pheasants and 24 control pigeons taken as 100%.

analysis in pheasants and pigeons gave results similar to those discussed above, although pheasant brain ChE results show greater variation (Figure 4). While ChE activity in the lethally dosed pheasants assayed immediately post mortem is inhibited 95–97%, less inhibition occurs in the two birds in which activity was measured at day 5. Other measured levels are near normal.

Comparison of the total and electrophoretic esterase measurements shows considerable differences especially for pheasant brain ChE and liver NA, although the elevation of brain ChE levels apparent from electrophoresis is considerably less than with the other carbamates discussed above. The largest elevation is apparent in one of the subacutely dosed birds. Pheasant liver NA levels obtained by both methods (Figure 4) are also variable. Most pheasant brain NA levels are similar when measured by either method.

Results obtained electrophoretically for pigeon esterase levels are much closer to those obtained quantitatively showing inhibition rather than elevation. However, there is still a marked discrepancy between the two methods in the degree of inhibition.

Propoxur. The single dose oral LD_{50} of this carbamate was reported as 150 mg/kg in the chicken (Bayer UK Ltd., 1971). Reports subsequent to this investigation (Tucker and Haegele, 1971; Schafer, 1972) show that toxicity in a wide range of avian species is generally in the range 4–40 mg/kg. Acute and subacute doses of 450 and 75 mg/kg, respectively, were administered to three pairs of pheasants and two pairs of pigeons. All the birds died which accords with the recent LD_{50} values. Results from the birds dosed as supposedly subacute levels are not differentiated from the others in this case. Brain total ChE levels in pheasants are, with one exception (74%), inhibited in excess of 85% (Figure 5). Brain and liver TE levels are similarly inhibited (60–90%). Brain NA levels are also significantly depressed (30–70%). Pheasant liver NA levels exhibited more variation.

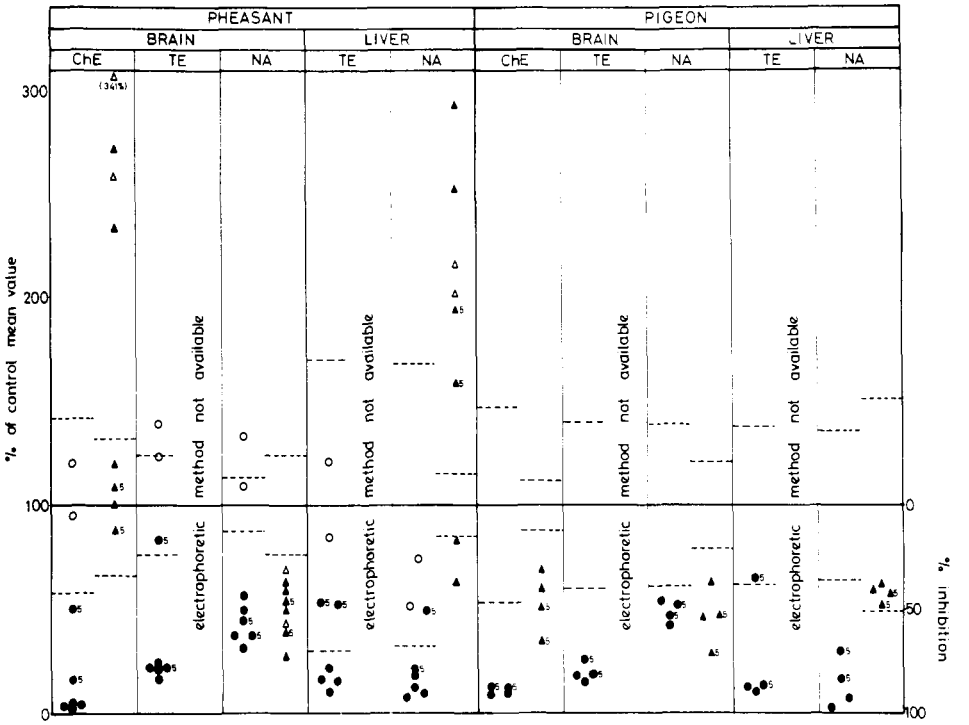

Figure 4. The percentage variation from the control mean in pheasant and pigeon esterase levels measured by a quantitative method and an electrophoretic method following forced feeding of pirimicarb with acute and subacute doses. Esterase levels were measured at 0 and 5 days (indicated by 5) post mortem. Two × SD for each control mean level is indicated by a dashed line. Individual results are shown as follows: (○) level measured quantitatively in birds surviving to sacrifice at 16 hr; (●) level measured quantitatively in birds dead at 16 hr; (△) level measured electrophoretically in birds surviving to sacrifice at 16 hr; (▲) level measured electrophoretically in birds dead at 16 hr. Mean esterase levels from 24 control pheasants and 24 control pigeons taken as 100%.

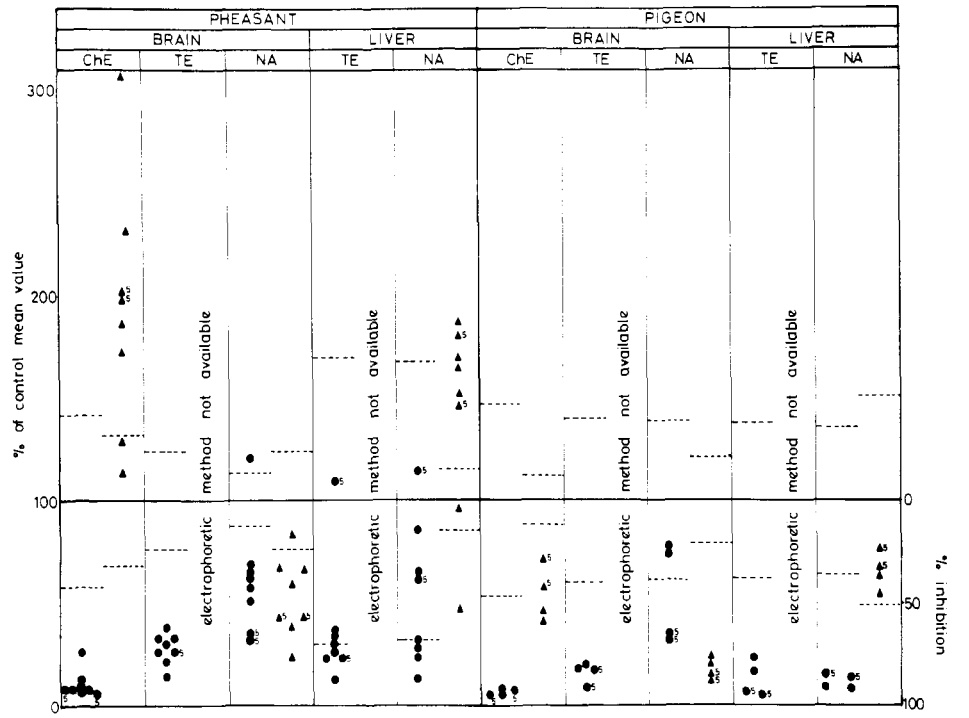

Figure 5. The percentage variation from the control mean in pheasant and pigeon esterase levels measured by a quantitative method and an electrophoretic method following forced feeding of propoxur with acute and subacute doses. Esterase levels were measured at 0 and 5 days (indicated by 5) post mortem. Two × SD for each control mean level is indicated by a dashed line. Individual results are shown as follows: (●) level measured quantitatively in birds dead at 16 hr; (▲) level measured electrophoretically in birds dead at 16 hr. Mean esterase levels from 24 control pheasants and 24 control pigeons taken as 100%.

Total esterase levels in the pigeons are generally comparable to those in the pheasants. Brain ChE measured at both days 0 and 5 is inhibited 92–95% while brain and liver TE and liver NA are inhibited greater than 75%. Total brain NA levels show smaller but still considerable inhibition. NA levels measured in pigeons on day 5 are notable since they are inhibited to a greater extent than those measured at day 0.

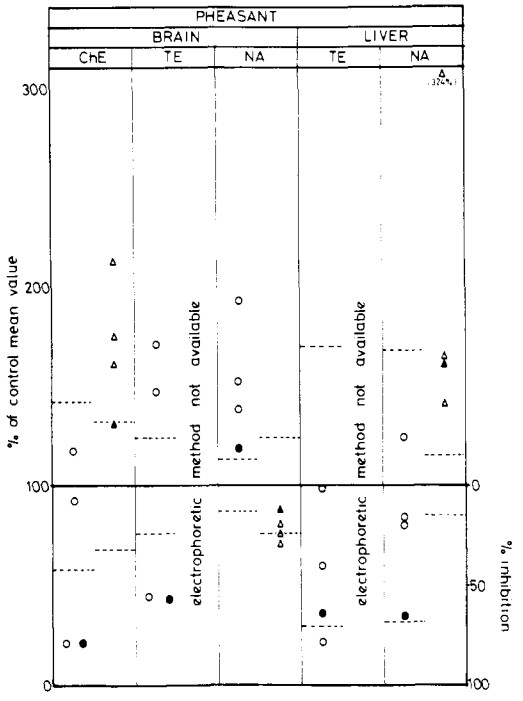

Figure 6. The percentage variation from the control mean in pheasant esterase levels measured by a quantitative method and an electrophoretic method following forced feeding of Zectran with acute and subacute doses. Esterase levels were measured immediately post mortem (day 0). Two × SD for each control mean level is indicated by a dashed line. Individual results are shown as follows: (○) level measured quantitatively in birds surviving to sacrifice at 16 hr; (●) level measured quantitatively in birds dead at 16 hr; (△) level measured electrophoretically in birds surviving to sacrifice at 16 hr; (▲) level measured electrophoretically in birds dead at 16 hr. Mean esterase levels from 24 control pheasants taken as 100%.

In contrast to the massive inhibition observed in total esterases, electrophoretic measurements of pheasant brain ChE and liver NA reveal elevated levels similar to those observed in birds dosed aldicarb and methiocarb. However, pigeon electrophoretic results (Figure 5) do not reveal elevated levels. All three enzyme levels are below the control mean values, but brain ChE and liver NA inhibition are much less than that measured quantitatively.

Zectran. Comprehensive data on the single dose oral LD_{50} of this pesticide to several avian species are available (Tucker and Crabtree, 1969). A value of 4.5 mg/kg is quoted for the ring-necked pheasant. Only two pairs of pheasants were used. Doses of 13.5 mg/kg were given to one pair and 2.5 mg/kg to the other. One bird (female) survived the larger dose, but for convenience is classed with and referred to as a lethally dosed bird. Brain ChE levels measured conventionally in the lethally dosed pair are strongly inhibited (80%; Figure 6). Levels in the subacutely poisoned birds are nearer normal. The other esterase levels measured in the acutely poisoned birds generally show less drastic inhibition and levels in the subacutely poisoned birds are normal or elevated by up to 70%. Brain NA levels are exceptional, since they are all elevated (18–93%).

A comparison of the esterase levels measured by conventional methods with those obtained after electrophoresis reveals striking discrepancies. Differences of over 100% are obtained in brain ChE and liver NA levels measured in acutely dosed birds (Figure 6). As with aminocarb the effect is reversed for brain NA levels where electrophoretic measurements show evidence of some inhibition but total esterase measurements suggest elevation.

GENERAL DISCUSSION

This laboratory has developed the concept that the measurement of esterase levels in wild bird tissues can be used as an indicator of poisoning by organophosphorus pesticides (Bunyan et al., 1968a,b; Bunyan and Taylor, 1966). The method has been used successfully in a number of investigations of poisoning in the field (Bailey et al., 1970; Pest Infestation Control, 1973) where "aged" tissues were involved. The present work explores the possibility of using the same concept to recognize carbamate pesticide poisoning in wild birds should it occur with the increasing use of these pesticides in agriculture. Similar experimental methods were employed to those used previously (Bunyan et al., 1968a) when it was demonstrated that normal levels of avian esterases were consistent enough to allow gross changes in an individual to be considered significant. A possible limitation which we recognized was the widely reported spontaneous reactivation of inhibited esterases following this type of poisoning.

Despite the lack of reliable avian toxicity data for most of the compounds we investigated, the initial objective of feeding sublethal doses to pheasants and lethal doses to pheasants and pigeons was generally achieved with the exception of propoxur where all the birds died.

The primary criterion for assessing organophosphorus poisoning is the inhibition of brain ChE in excess of 90% while significant inhibition (>2 × SD) is considered to indicate exposure. The results from the series of feedings described here indicate rather more variation of brain ChE inhibition in birds poisoned with carbamates. Only four carbamates were fed to pigeons, but in all cases the inhibition of brain ChE exceeds 90% when measured by our method either immediately or 5 days post mortem. For the same compounds fed to pheasant, inhibition is generally slightly less but always significant and in excess of 75% at day 0. For pirimicarb there is some evidence for reactivation after 5 days post mortem, but inhibition is still significant. For Zectran and aminocarb which were fed only to pheasants, the levels of inhibition are 80 and 60%, respectively. With the exception of methiocarb, brain ChE levels in birds given subacute doses are near to normal levels.

The finding of gross inhibition of brain ChE in these birds is of interest. It is now generally accepted that ChE inhibition by carbamates is a two-stage process involving the initial formation of an enzyme–inhibitor complex, followed by the formation of a carbamylated enzyme intermediate (O'Brien, 1967; Aldridge and Reiner, 1972). Since the carbamylated intermediate often breaks down spontaneously, many methods of measurement can underestimate the degree of inhibition which occurs in vivo especially if the formation or maintenance of equilibrium levels of the complex is discouraged by dilution of the tissue extracts whose activity is to be measured, by the use of high substrate concentrations or by carrying out the assay slowly. The dependence of the results on these factors has been amply demonstrated (Winteringham and Disney, 1964; Disney, 1966; Winteringham and Fowler, 1966). We chose to retain our original ChE assay (Bunyan et al., 1968a) since we seek to maintain a standard approach to field investigations, and although it employs a high substrate concentration and a diluted tissue extract it has the advantage of being a dynamic method which can be applied rapidly to the extract. The massive inhibition which we observed suggests that in lethally poisoned birds there is a sufficient excess of carbamate present until the

dilution stage at the start of the assay to maintain the equilibrium level of the complex and hence the level of inhibition despite probable continuous breakdown of the carbamylated enzyme. Aldridge and Reiner (1972) suggest an error of approximately 6% if the assay is carried out within a short time. Since we are interested in gross changes in enzyme levels 5–10% errors are tolerable. The assay therefore appears to give the definitive answer which we seek. The slightly lower inhibition levels we observed here than in those birds poisoned by organophosphorus esters in a similar experiment (Bunyan et al., 1968b) may be an indication of reactivation during the assay, although no obvious changes of rate of acetylcholine breakdown with time were ever observed.

It is also notable that in most lethally dosed birds no reactivation appears to have occurred in extracts of tissue from the intact animal left for 5 days post mortem. It seems likely that similar considerations apply as above, i.e. that a sufficient excess of carbamate exists in the tissue to continue to maintain the equilibrium value of the enzyme–carbamate complex and hence the inhibition level, even though the carbamylated enzyme may have undergone spontaneous reactivation during this period.

Unlike the results from birds poisoned by organophosphorus esterase, brain TE inhibition is not similar to that of ChE. In general it is less although still significantly inhibited, but the relative levels of inhibition by the various pesticides are maintained. Since the assay method is similar to that for ChE this probably represents a fundamentally different interaction between the esterase and the inhibitor rather than reactivation.

NA esterase levels are extremely variable, but those in lethally poisoned birds generally show even less inhibition than TE discussed above. In brain, but not liver, the inhibition is generally significant. Although no firm conclusions can be drawn from the figures, the assay conditions would favor reactivation if inhibition of these esterases by carbamates is similar in mechanism to that for ChE. One important observation is that many esterase levels in subacutely dosed pheasants are normal or elevated. This applied particularly to brain TE and liver TE and NA levels. In brain the elevations are generally above the 2 × SD level. This effect did not occur in brain tissues of birds given similar relative doses of organophosphorus pesticide (Bunyan et al., 1968b) when inhibition was generally large and may be due to a combination of metabolic breakdown and spontaneous reactivation. The variations in the isoesterase patterns obtained by histochemical staining of electrophoregrams of tissue extracts in this series cannot be correlated with the carbamates which poisoned the birds. However, comparison of the overall esterase activity with control mean values as measured by the total integral of the densitometric scans reveals an interesting situation. For pheasants, both lethally and sublethally dosed, the electrophoretic measurements generally show extensive elevation of brain ChE and liver NA levels and near normal levels of brain NA, whereas, as discussed above, except for certain sublethally dosed birds, conventional esterase measurements indicate significant inhibition. A similar effect is discernible in the lethally dosed pigeons. However, in the large majority of these cases, while the electrophoretic values are considerably higher than those obtained conventionally for all three tissue enzymes, they are either inhibited or normal by comparison with control mean values. Only brain ChE levels after methiocarb dosing are elevated. The explanation of this phenomenon is not possible on the basis of the evidence from these experiments. However, gel filtration with Sephadex, a modified dextran, has recently been shown to remove excess inhibitor from carbamylated ChE, thereby allowing spontaneous reactivation to occur (Reed and Fukuto, 1973). Starch gel may possess the same properties since it is known to act as a molecular sieve during electrophoresis (Smithies, 1955), and borate ions present in the gel may also have a reactivation effect. This could account for the return of some esterase levels to normal after removal of excess inhibitor.

The apparent elevation above control mean levels in pheasant tissue may be a combination of this effect with induction of esterases. Many examples of induction of microsomal enzymes by zenobiotics are now known (O'Brien, 1967) and in some the elevation of aliesterases has been demonstrated (Kay, 1966; Puyear and Paulson, 1972). Welch and Coon (1964) have demonstrated that chlorcyclizine and phenobarbital cause elevation of aliesterase levels with subsequent protection against poisoning by parathion, malathion, and eserine. In many instances, induction follows very rapidly after a single large dose. If the massive doses of carbamate given to the pheasants in this experiment also trigger the induction mechanism, any rise in esterase levels would probably be masked by the concomitant inhibition. Subsequent reactivation of the type postulated above would then reveal it. Work is now in hand to test this theory. Differences in the extent of inhibition or elevation observed between the species, the carbamates, and the esterases examined could be rationalized as a result of the combined effect of the relative dose, the chemical structure of the inhibitor, and the enzyme of the overall kinetics of the interaction together with the extent of any degradation of the inhibitor or induction of the enzyme which occurs.

On the practical level, the measurement of esterase levels continues to provide a useful indication or confirmation of certain types of poisoning on the basis of these results. While massive brain ChE inhibition can indicate organophosphorus or carbamate poisoning, a recovery or increase of esterase activity after electrophoresis may generally differentiate between the two types of poisoning (Bunyan, 1973). Other minor and more specialized differences in the relative levels of esterases in various tissues will, as explained in this and previous work (Bunyan et al., 1968b), help to support the diagnosis. Since analytical methods are now generally available for organophosphorus pesticide residues in tissue (Pest Infestation Control Laboratory Triennial Report, 1976; Jennings et al., 1976), but not for carbamates, the diagnostic approach outlined above can yield useful results in combination with analyses. Work is now in hand to demonstrate other utilizable reactivation methods for this type of diagnosis.

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Metabolism of *O*-Ethyl *O*-[4-(Methylthio)phenyl] *S*-Propyl Phosphorodithioate (BAY NTN 9306) by White Rats

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A 10 mg/kg dose of phenyl-^{14}C-labeled BAY NTN 9306 (*O*-ethyl *O*-[4-(methylthio)phenyl] *S*-propyl phosphorodithioate), administered orally to female white rats, was metabolized rapidly and excreted primarily in the urine (ca. 92% in 24 hr). No $^{14}CO_2$ was detected and essentially all the radioactivity in urine consisted of water-soluble materials that were converted to three substituted free phenolic derivatives by hydrolysis with glucuronidase–aryl sulfatase or acid. Analyses of tissues taken from rats 3 hr after treatment with an oral dose of 70 mg/kg revealed the presence of BAY NTN 9306, five phosphorus-containing metabolites formed by oxidations of thiono and thioether sulfur groups of the molecule, and three substituted phenols, both free and conjugated.

The experimental organophosphorus (OP) insecticide *O*-ethyl *O*-[4-(methylthio)phenyl] *S*-propyl phosphorodithioate (BAY NTN 9306, Mobay Chemical Corp.) appears unusually promising for use in controlling *Heliothis* spp. pests of field crops, including the tobacco budworm, *Heliothis virescens* (F.), which has become resistant to most OP compounds in many areas where it is a major pest of cotton. With the added advantage that the mammalian toxicity is relatively low (acute oral LD_{50} for rats is 227 mg/kg), there is considerable interest in the development of this insecticide for commercial use.

The present report describes the fate of radiolabeled BAY NTN 9306 after oral treatment of white rats.

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EXPERIMENTAL SECTION

Chemicals. The Chemagro Agricultural Division of Mobay Chemical Corp., Kansas City, Mo., provided pure samples of BAY NTN 9306 (hereafter designated 9306), including some that was radiolabeled uniformly with ^{14}C in the phenyl ring of the molecule (>99% radiochemical purity, specific activity 7.06 mCi/mmol), and certain chemicals considered as potential metabolic derivatives (Table I).

Rats and Their Treatment. Female white rats (Sprague-Dawley strain, 150–170 g) were each treated orally with sublethal doses of ^{14}C-labeled 9306 in 0.5 ml of corn oil. The dose was administered with a syringe and stomach tube after light anesthesia of the rats with ether. Animals were fed immediately before treatment but were provided only water through the next 24 hr; those held longer were fed each day.

For most tests the rats were treated with a dose of 10 mg/kg, using ^{14}C-labeled 9306 that was diluted with 4 parts of nonradioactive 9306. Those used for studies of the distribution of radioactive compounds in tissues were treated with a dose of 70 mg/kg, using ^{14}C-labeled 9306