Organophosphorus Poisoning

Diagnosis of Poisoning in Pheasants Owing to a Number of Common Pesticides

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Pheasants have been fed with lethal and sublethal doses of six common organophosphorus pesticides. Esterase levels have been measured in liver and brain extracts, and electrophoresis coupled with histochemical staining has been carried out on liver, kidney, and brain extracts to demonstrate inhibition. Chemical analysis of liver and gut contents was also undertaken. A discussion on the use of these procedures for the diagnosis of poisoning by organophosphorus pesticides is included.

An earlier communication (Bunyan and Taylor, 1966) demonstrated that changes in esterase levels in electrophoregrams stained for esterase activity following the oral dosing of pheasants by Thimet [*O,O*-diethyl-*S*-(ethylthiomethyl)phosphorodithioate; British Standard common name phorate] could be a useful indicator of poisoning by this pesticide. Further work reported in the preceding paper (Bunyan *et al.*, 1968) has shown that in general these esterase levels and patterns are consistent enough in both pheasants (*Phasianus colchicus*) and pigeons (*Columba livia*) to allow significance to be attached to any abnormal results which are encountered. Using the method of tissue extraction described, such abnormal results were still both detectable and reliable in Thimet-poisoned birds after 12 days post-mortem at 20° C.

This approach to the diagnosis of organophosphate poisoning has now been extended to a further six pesticides namely chlorfenvinphos [*O,O*-diethyl *O*-2-chloro-1-(2,4-dichlorophenyl)vinyl phosphate], demeton-methyl [a mixture of 70% demeton-*O*-methyl [*O,O*-dimethyl *O*-2-(ethylthio)ethyl phosphorothioate] and 30% demeton-*S*-methyl [*O,O*-dimethyl *S*-2-(ethylthio)ethyl phosphorothioate]], diazinon [*O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl)-phosphorothioate], dimethoate (*O,O*-dimethyl *S*-methyl carbamoylmethyl phosphorodithioate), ethion (*O,O,O',O'*-tetraethyl *S,S'*-methylene bisphosphorodithioate), and Guthion [*O,O*-dimethyl *S*-(4-oxo-benzotriazino-3-methyl)-phosphorodithioate; British Standard common name azinphos-methyl]. These six pesticides were chosen because they represent a fairly wide range of chemical structure. Further, demeton-methyl, dimethoate, and Guthion are estimated (Strickland, 1966) to be among the six most widely used organophosphorus pesticides in the United Kingdom, while chlorfenvinphos, diazinon, ethion, and Guthion have been suggested for use as seed-dressings to replace the more persistent organochlorine insecticides.

In this series of experiments, the effects of a single oral dose of each pesticide were investigated to coincide with data already obtained for Thimet. Information concerning the toxicity of these pesticides, both to birds in general and to pheasants in particular, was in most cases sparse or difficult to locate. In addition, with the larger number of pesticides under test and with a more thorough examination of each bird, it became impossible to investigate the effect of a range of doses as had been done with Thimet (Bunyan and Taylor, 1966). In an attempt to overcome these difficulties, the effects of each pesticide were investigated in a group of six birds treated in an arbitrary but standard manner. Based on the small amount of toxicity data available, a cock and a hen bird were dosed with one half of an LD_{50}, and the two other pairs were dosed with three LD_{50}'s. Adjustments were sometimes made to later doses in the light of unexpected results from the first pair of birds. The tissues of one of the latter two pairs were not removed and extracted until the body had hanged for 12 days at 20° C. to check that no spontaneous reactivation of inhibited cholinesterases occurred. All the birds were killed after 18 hours if they had not already died. Although statistically significant depressions cannot be demonstrated in all cases, the full range of esterases described in the previous paper (Bunyan *et al.*, 1968) was investigated in extracts of the liver, kidney, and brain of each bird in case any levels were raised by dosing. Plasma esterases were investigated in birds given a sublethal dose. Starch gel electrophoresis was performed on all extracts, and results were compared with those from polyacrylamide gel in a few cases. Chemical examination of liver tissue and gut contents was also undertaken. Some reactivation studies were also made on electrophoregrams stained for cholinesterase activity.

EXPERIMENTAL

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

Pesticides. Gifts of mainly technical grade pesticides were obtained as nonformulated concentrates from the manufacturers. Diazinon (95% pure) and dimethoate (95% pure) were obtained from Fisons Pest Control Ltd. Chlorfenvinphos (93% pure), ethion (95% pure), and Guthion (87% pure) were obtained from Shell Chemicals Ltd. Demeton-methyl (70 to 30; 100% pure) was obtained from Plant Protection Ltd. No further purification was undertaken.

Preparation of Tissue Extracts, Electrophoresis (Including Reactivation) and Enzyme Estimations. These operations were carried out on all birds as described (Bunyan *et al.*, 1968).

Chemical Methods. Chemical analysis was attempted

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Table I. Pesticide Dosage and Residues in Pheasants

Bird No.	Sex	Pesticide	Dose, Mg./Kg.	Condition after 18 Hours	Pesticide Content	
					Gut, total mg.	Liver, p.p.m.
1	♂	Chlorfenvinphos	101	Alive	0.02	0
2	♀	Chlorfenvinphos	100	Dead	2.0	0.1
3[a]	♂	Chlorfenvinphos	189	Alive	[b]	0
4[a]	♀	Chlorfenvinphos	197	Alive	[b]	0
5	♂	Chlorfenvinphos	297	Dead	20	120
6	♀	Chlorfenvinphos	52.6	Alive	0.5	40
7	♂	Demeton-methyl	45	Dead	[b]	[b]
8	♀	Demeton-methyl	44	Dead	[b]	[b]
9[a]	♂	Demeton-methyl	44	Dead	[b]	[b]
10[a]	♀	Demeton-methyl	44	Dead	[b]	[b]
11	♂	Demeton-methyl	8.3	Alive	[b]	[b]
12	♀	Demeton-methyl	8.3	Alive	[b]	[b]
13[a]	♂	Diazinon	44.2	Dead	[b]	0.1
14[a]	♀	Diazinon	48.8	Dead	[b]	0.1
15	♂	Diazinon	43	Alive	0.10	0
16	♀	Diazinon	45.5	Alive	0.01	0.1
17	♂	Diazinon	14.5	Dead	1.00	0
18	♀	Diazinon	14.7	Dead	0.20	0
19	♂	Dimethoate	43.2	Dead	0.02	0.4
20	♀	Dimethoate	42.4	Dead	0.02	0.4
21[a]	♂	Dimethoate	43.8	Dead	[b]	2.0
22[a]	♀	Dimethoate	44.1	Dead	[b]	2.0
23	♂	Dimethoate	8.2	Alive	0.004	0.02
24	♀	Dimethoate	9.0	Alive	0.004	0.02
25	♂	Ethion	248	Dead	2.0	0.8
26	♀	Ethion	271	Dead	4.0	4.0
27[a]	♂	Ethion	285	Alive	[b]	0
28[a]	♀	Ethion	257	Dead	[b]	19
29	♂	Ethion	31	Alive	0.20	2.0
30	♀	Ethion	31.5	Alive	0.20	0
31[a]	♂	Guthion	147	Dead	[b]	0.6
32[a]	♀	Guthion	155	Alive	[b]	0
33	♂	Guthion	30.6	Alive	0.01	0.3
34	♀	Guthion	30.6	Alive	0.02	0
35	♂	Guthion	192	Dead	10	0.5
36	♀	Guthion	177	Alive	10	0

[a] Birds whose tissues were not extracted until 12 days post-mortem. [b] No analysis was performed.

on all liver tissues and on the gut contents of freshly dissected birds. The basic operations were similar to those described for Thimet-treated birds (Bunyan and Taylor, 1966), except that experiments with standard compounds indicated that other solvents were required either to elute these pesticides from an alumina column (I) or to achieve a satisfactory R_f value on thin-layer plates (II). Changes were as follows: chlorfenvinphos I: 15% ether–*n*-hexane; diazinon I: 15% ether–*n*-hexane; dimethoate I: 15% *n*-hexane–chloroform; II: 20% acetone–benzene; ethion I: 10% ether–*n*-hexane; Guthion I: 15% chloroform–*n*-hexane.

All solvents were either AnalaR or purified before use. The final identification and estimation was made on thin-layer plates, where four of the pesticides were visualized by a blood plasma–bromothymol blue reagent on silica gel H (Bunyan, 1964). The two exceptions were dimethoate for which a silver nitrate–bromophenol blue reagent was used on alumina plates (Bunyan, 1964), and demeton-methyl for which no suitable method of detection could be found. A rough estimation of the amounts of pesticide present was obtained by serial dilution and comparison with standards.

RESULTS AND DISCUSSIONS

Results of electrophoresis and enzymes are given in Tables II and III employing previously used conventions (Bunyan and Taylor, 1966; Bunyan *et al.*, 1968). Some discussion of the results as they relate to the particular pesticide is included under each heading. A general discussion embracing both the biochemical and the chemical results (Table I) concludes the section. Depression or elevation of enzyme levels is expressed in Table III as a percentage of the control mean (Bunyan *et al.*, 1968) for comparative purposes. All quantitative results are expressed in units defined in the previous paper. Where desirable, esterases are referred to as follows: α-naphthyl acetate esterase = αNA; phenyl benzoate esterase = PB; triacetin esterase = TE; cholinesterase = ChE. This nomenclature is followed for both quantitative and qualitative (electrophoregram) results.

Chlorfenvinphos. The LD_{50} to pheasants of this pesticide has not been reported. The results of an experiment with a small number of pigeons suggested that it might possess high avian toxicity ($LD_{50} \simeq 10$ mg. per kg.). The six pheasants proved much more resistant to intoxication than the pigeons and did not exhibit an obvious correlation between dose and effect (Table I). As a result, dosages appear erratic and birds 1 and 6 are considered to receive the sublethal dose, and 2 and 5 the lethal one. Results for bird 1 (Table II and III) are not typical of a sublethal dose, and this bird may have been at the point of death when sacrificed. Birds 3 and 4 survived, despite receiving nearly

Table II. Changes in Pheasant Tissue Electrophoregrams Owing to Pesticides

Bird No.	Pesticide	Bands Inhibited in Electrophoregrams			
		Liver αNA	Kidney αNA	Brain αNA	Brain ChE
1[b]	Chlorfenvinphos	1, 6, 10	1, 8	...	3
2[a]	Chlorfenvinphos	1, 6, 10	...	...	2, 3
3[b,c]	Chlorfenvinphos	11	1	...	2, 3
4[b,c]	Chlorfenvinphos	11	6	...	2, 3
5[a]	Chlorfenvinphos	1, 4, 5, 10	1, 5, 8	...	2, 3
6[b]	Chlorfenvinphos	5, 6, 10	1, 8	...	...
7[a]	Demeton-methyl	1–6, 9–11	1–6, 8	...	2, 3
8[a]	Demeton-methyl	1–6, 9–11	1–6, 8	...	2, 3
9[a,c]	Demeton-methyl	1–11	1–5	1, 2	1, 2, 3
10[a,c]	Demeton-methyl	1–5	1–5	1, 2	1, 2, 3
11[b]	Demeton-methyl	4, 9, 10	1, 5, 6, 8	...	...
12[b]	Demeton-methyl	1, 4, 9, 10	1, 2, 8	...	...
13[a,c]	Diazinon	1, 6, 10, 11	...	2	2, 3
14[a,c]	Diazinon	10, 11	1	2	2, 3
15[b]	Diazinon	1–5, 10, 11	1, 8	...	...
16[b]	Diazinon	6, 10, 11	1, 8	...	...
17[a]	Diazinon	10, 11	1, 5, 6	1	...
18[a]	Diazinon	1, 2, 8, 9, 10, 11	1	...	...
19[a]	Dimethoate	1–4, 10, 11	1–6, 8	...	...
20[a]	Dimethoate	1–4, 10, 11	1–6, 8	...	...
21[a,c]	Dimethoate	1–5, 7–11	2, 5, 8	1, 2	1, 3
22[a,c]	Dimethoate	1–3, 5, 7–11	2, 5, 8	1, 2	1, 3
23[b]	Dimethoate	1, 4, 5, 10, 11	1, 2, 5	...	...
24[b]	Dimethoate	1, 4–7	1, 5	...	...
25[a]	Ethion	...	...	...	...
26[a]	Ethion	...	...	...	...
27[b,c]	Ethion	10	...	2	...
28[a,c]	Ethion	6, 10	...	...	...
29[b]	Ethion	...	...	2	...
30[b]	Ethion	7, 8	...	...	2, 3
31[a,c]	Guthion	1–11	1, 2, 6, 8	2	2, 3
32[b,c]	Guthion	1, 2, 4–11	1, 2, 6, 8	2	2, 3
33[b]	Guthion	1, 4, 6, 10	1–5	...	...
34[b]	Guthion	1, 6, 10	1–5	...	...
35[a]	Guthion	1–7, 9, 11	1–5	1	2
36[b]	Guthion	1–6, 9–11	1–5	...	...

[a] Birds which died from poisoning.
[b] Birds sacrificed following poisoning.
[c] Birds from which tissues were not removed until 12 days after death.

Table III. Tissue Enzyme Levels in Pheasants Dosed with Pesticides

Bird No.	Pesticide	Enzyme Level						
		Liver			Brain			
		TE	αNA	PB	ChE	TE	αNA	PB
1	Chlorfenvinphos	2.45 (−34%)	1.17 (−74%)	1.16[a] (−27%)	3.33 (−75%)	2.42 (−75%)	1.49 (−78%)	0.10[a] (−29%)
2	Chlorfenvinphos	1.02 (−72.5%)	1.19 (−74%)	0.88[a] (−55%)	1.37 (−90%)	1.41 (−86%)	1.24 (−82%)	0.12[a] (−14%)
3	Chlorfenvinphos	4.54[a] (+22%)	7.38 (+64%)	6.5 (+307%)	8.3 (−37.5%)	4.04 (−59%)	2.26 (67%)	0.17[a] (+21%)
4	Chlorfenvinphos	3.82[a] (+3%)	7.37 (+64%)	8.8 (+410%)	5.28 (−60%)	3.43 (−65%)	2.22 (−68%)	0.11[a] (−21%)
5	Chlorfenvinphos	1.36 (−63%)	1.73 (−62%)	0.48[a] (−70%)	0.70 (−95%)	1.46 (−85%)	1.54 (−77.5%)	0.07[a] (−50%)
6	Chlorfenvinphos	2.53 (−32%)	3.46[a] (−23%)	1.59[a] (0%)	10.45[a] (−21%)	5.78 (−41%)	4.21[a] (−38%)	0.12[a] (−14%)
7	Demeton-methyl	1.26 (−66%)	1.21 (−73%)	0.76[a] (−52%)	0.94 (−93%)	1.35 (−86%)	0.93 (−86%)	0.09[a] (−36%)
8	Demeton-methyl	0.75 (−80%)	1.25 (−72%)	0.45[a] (+72%)	0.77 (−94%)	1.17 (−88%)	1.19 (−83%)	0.08[a] (−43%)
9	Demeton-methyl	0.39 (−89.5%)	2.26[a] (−50%)	0.60[a] (−43%)	0.51 (−96%)	1.16 (−88%)	1.65 (−76%)	0.08[a] (−43%)
10	Demeton-methyl	2.9[a] (−22%)	5.20[a] (+15%)	4.07 (+154%)	0.36 (−97%)	0.91 (−91%)	1.82 (−73%)	0.10[a] (−29%)
11	Demeton-methyl	3.61[a] (−3%)	4.78[a] (+6%)	1.67[a] (+4%)	7.04 (−47%)	4.15 (−57.5%)	3.41[a] (−50%)	0.10[a] (−29%)
12	Demeton-methyl	2.75[a] (−26%)	5.38[a] (+20%)	1.93[a] (+20%)	8.21 (−37.5%)	4.07 (−58.5%)	4.27[a] (−38%)	0.18[a] (+29%)

Table III. (Continued)

Bird No.	Pesticide	Enzyme Level						
		Liver			Brain			
		TE	αNA	PB	ChE	TE	αNA	PB
13	Diazinon	7.98 (+115%)	5.13[a] (+14%)	2.55[a] (+59%)	16.05[a] (+21%)	8.90[a] (−9%)	3.8[a] (−44%)	0.12[a] (−14%)
14	Diazinon	5.81 (+56%)	6.10 (+35%)	2.88[a] (+80%)	4.60 (−65%)	2.88 (−71%)	2.36 (−66%)	0.07 (−50%)
15	Diazinon	1.97[a] (−47%)	0.90 (−80%)	0.13[a] (−92%)	5.93 (−55%)	4.03 (−49%)	3.66[a]	0.07 (−50%)
16	Diazinon	2.26[a] (−39%)	2.01 (−55%)	2.57[a] (+60%)	8.23 (−38%)	5.64 (−42.5%)	5.10[a] (−26%)	0.13[a] (−7%)
17	Diazinon	1.72[a] (−54%)	3.11[a] (−31%)	1.04[a] (−35%)	3.01 (−77%)	2.44 (−75%)	1.89 (−72%)	0.07 (−50%)
18	Diazinon	1.09 (−71%)	0.93 (−79%)	0.27[a] (−73%)	8.15 (−38.5%)	6.07 (−38%)	4.57[a] (−33%)	0.07 (−50%)
19	Dimethoate	3.94[a] (+6%)	4.00[a] (−11%)	2.88[a] (+80%)	0.86 (−93.5%)	1.65 (−83%)	5.38[a] (−21%)	0.12[a] (−14%)
20	Dimethoate	1.20 (−68%)	1.13 (−75%)	0.24[a] (−86%)	0.80 (−94%)	1.71 (−82.5%)	6.33[a] (−8%)	0.05 (−61%)
21	Dimethoate	0.64 (−83%)	1.20 (−73%)	0.83[a] (−48%)	0 (100%)	1.20 (−88%)	0.61 (−91%)	0.18[a] (+29%)
22	Dimethoate	0.73 (−81%)	1.01 (−78%)	1.13[a] (−29%)	0 (100%)	0.92 (91%)	0.49 (−93%)	0.17[a] (+21%)
23	Dimethoate	2.68[a] (−28%)	2.80[a] (−38%)	1.74[a] (+9%)	6.46 (−51%)	4.70 (−52%)	1.68 (−75.5%)	0.11[a] (−21%)
24	Dimethoate	1.13 (−69.5%)	1.26 (−72%)	0.77[a] (−52%)	7.41 (−44%)	5.03 (−48.5%)	2.16 (−68.5%)	0.11[a] (−21%)
25	Ethion	0.88 (−76%)	2.91[a] (−35%)	0.71[a] (−56%)	2.23 (−83%)	1.27 (−87%)	2.04 (−70%)	0.08[a] (−43%)
26	Ethion	1.47 (−60%)	3.92[a] (−13%)	1.08[a] (−33%)	3.73 (−72%)	3.27 (−67%)	4.67[a] (−32%)	0.06 (−55%)
27	Ethion	2.75[a] (−26%)	4.26[a] (−5%)	2.05[a] (+28%)	7.55 (−43%)	4.25 (−57%)	2.82 (−59%)	0.08[a] (−43%)
28	Ethion	2.22[a] (−40%)	4.88[a] (+8%)	4.89 (+206%)	14.3[a] (+8%)	8.08[a] (−16%)	3.42[a] (−50%)	0.14[a] (0%)
29	Ethion	2.52[a] (−32%)	4.18[a] (−7%)	2.99[a] (+87%)	14.66[a] (+10%)	8.68[a] (−11%)	4.64[a] (−48%)	0.19[a] (−36%)
30	Ethion	3.61[a] (−3%)	5.78[a] (+28%)	3.39 (+111%)	13.42[a] (+1%)	9.33[a] (−4%)	5.26[a] (−23%)	0.19[a] (+36%)
31	Guthion	0.56 (−85%)	1.18 (−74%)	0.27[a] (−73%)	1.52 (−89%)	1.71 (−83%)	1.18 (−83%)	0.028 (−80%)
32	Guthion	2.77[a] (−25%)	5.11[a] (+14%)	1.98[a] (+24%)	2.10 (−84%)	2.10 (−79%)	1.42 (−79%)	0.043 (−69%)
33	Guthion	1.59[a] (−57%)	2.37[a] (−47%)	1.13[a] (−29%)	8.30 (−37%)	4.99 (−49%)	2.68 (−61%)	0.095[a] (−32%)
34	Guthion	1.84[a] (−50%)	2.67[a] (−41%)	1.13[a] (−29%)	7.80 (−41%)	6.00 (−39%)	2.73 (−60%)	0.099[a] (−30%)
35	Guthion	0.47 (−87%)	0.82 (−82%)	0.10[a] (−94%)	0.97 (−93%)	1.32 (−87%)	1.01 (−85%)	0.041 (−71%)
36	Guthion	1.03 (−72%)	1.18 (−74%)	0.63[a] (−61%)	4.00 (−67%)	3.54 (−64%)	2.11 (−69%)	0.068 (−52%)
37	Diazinon	1.62[a] (−56%)	3.81[a] (+3%)	1.55[a] (−3%)	6.23 (−53%)	6.75[a] (−31%)	3.27[a] (−52%)	0.09[a] (−36%)
38	Diazinon	3.17[a] (−15%)	6.31[a] (+4%)	1.72[a] (+7%)	5.75 (−57%)	4.58 (−53%)	2.91 (−56%)	0.06 (−50%)

[a] No significant difference from the control mean.

200 mg. per kg., and had to be killed before the bodies were hanged for 12 days at 20° C.

Electrophoretic strips of α-naphthyl acetate esterase activity on starch gel were generally less intense but otherwise not widely different from controls. Brain in particular was unaffected by any dosage level. In the liver and kidney, only the slowest and fastest bands were consistently affected. Cholinesterase electrophoregrams exhibited complete inhibition of bands 2 and 3 in the birds that died from poisoning and also in birds 3 and 4, but in the latter they could be reactivated by preincubation in 2-PAM ($10^{-4}M$, Table II).

The electrophoretic results were not mirrored by the enzyme estimations. In particular, the liver α-naphthyl acetate esterase levels of birds 3 and 4 were significantly elevated (+64%) as were the liver phenyl benzoate levels (+307 and +410%, respectively). In addition the brain esterases, with the exception of phenyl benzoate esterase in general and those of the least poisoned bird (6) in particular, were significantly depressed. Esterase levels of liver and brain extracts are summarized in Table III.

A priori, birds 3 and 4 probably survived massive doses owing to an elevation of liver esterases, but the levels measured may be due to post-mortem reactivation. How-

ever, since these birds did not die as expected, deductions are tentative. The enzyme assay figures show that all brain esterases are affected by sublethal doses of chlorfenvinphos, but only in the birds which died from poisoning does brain cholinesterase inhibition exceed 90% of the control, and bands 2 and 3 of the cholinesterase electrophoregram become irreversibly inhibited.

Demeton-Methyl. The oral LD_{50} to pheasants is reported to be 15 mg. per kg. (May and Baker Ltd., 1967). All four birds (7, 8, 9, and 10) given three LD_{50}'s died, while the pair (11 and 12) given one-half an LD_{50} survived. Tissues from birds 9 and 10 were not extracted until 12 days after death.

The effect of demeton-methyl on α-naphthyl acetate electrophoregrams is extremely marked. In the four birds receiving lethal doses, bands 7 and 8 in the liver, band 7 in the kidney, and bands 4 and 5 in the brain are the only ones to appear consistently. In liver and kidney, most of the electrophoregrams of 12-day-old tissue show one or two extra bands, which suggest a small amount of spontaneous reactivation. The reverse is true of the fresh brain tissue suggesting that some inhibition may occur by diffusion after death. This also applies to brain cholinesterase. Reactivation by 2-PAM was possible on the cholinesterase electrophoregrams of fresh poisoned brain tissue but not on older extracts, which also suggests a slow post-mortem change, and no spontaneous reactivation. In the sublethally dosed birds, fewer bands are completely inhibited, but all are weaker than the controls. The brain cholinesterase electrophoregram is normal (Table II).

Compared with chlorfenvinphos, there are fewer discrepancies between the electrophoretic and the enzyme assay results. Liver triacetin and α-naphthyl acetate esterase levels are significantly depressed in birds 7 and 8, but with one exception appear to be reactivated in 9 and 10. This was also shown by electrophoresis. The liver esterases of birds 11 and 12 are not affected. All the brain esterase levels are significantly depressed, with the exception of phenyl benzoate esterase, and there is no spontaneous reactivation. There is an obvious difference in extent between the lethal and sublethally poisoned birds and again death due to poisoning seems to be accompanied by more than 90% inhibition of brain cholinesterase (Table III).

Diazinon. The oral LD_{50} to pheasants has now been given as 6 mg. per kg. (Noakes, 1965), which accords with the author's later experience. At the start of this experiment, the only guidance available was the figure of 15 mg. per kg. for the duck (Sanderson and Edson, 1964), and in consequence some high dosages were given. As with chlorfenvinphos, dose and effect were not always related, and while two birds (17 and 18) receiving 15 mg. per kg. died, two others (15 and 16) receiving 45 mg. per kg. survived and are taken as the sublethally dosed pair. The tissues of birds 13 and 14 (given 44 and 49 mg. per kg., respectively) were not extracted until 12 days after death.

α-Naphthyl acetate electrophoregrams are moderately affected by diazinon. In the liver, bands 10 and 11 are always inhibited, and bands 1 and 2 tend to be affected, while in the kidney only band 1 is always inhibited. The brain is scarcely affected. All the bands which appear are only slightly less intense than the controls, and there is distinct evidence of spontaneous reactivation in all the α-naphthyl acetate electrophoregrams of birds 13 and 14 when compared with birds 17 and 18. Brain cholinesterase electrophoregrams are also generally unaffected, except in birds 13 and 14, where band 1 is much more intense, and bands 2 and 3 are completely inhibited (Table II).

The main feature of the enzyme assay results is the spontaneous reactivation which appears to have occurred to a variable extent in all the enzymes of the two birds hanged for 12 days. Further, this effect initially operates rapidly following diazinon poisoning, since despite the fact that this pesticide has one of the highest avian toxicities among the group investigated, measured esterase inhibition is generally low even in freshly extracted birds. The alternative explanation that reactivation occurs during extraction cannot be ruled out. Thus in the liver, very few enzyme levels are abnormal. Of the exceptions, the two sublethally poisoned birds (15 and 16) had received massive doses, and it is possible that recovery had not proceeded to a level of "insignificant inhibition." Brain esterases however are widely affected, but in general the inhibition is far less than is the case with those other pesticides of the group which exhibit high avian toxicity. Brain cholinesterase inhibiton never exceeds 90% and in only one case approaches this figure. Brain α-naphthyl acetate esterase levels are also less affected, but a number of the phenyl benzoate levels are significantly inhibited. Table III summarizes these results and includes levels measured on two other birds (37♂; 38♀) dosed 10 mg. per kg. of diazinon. Both these birds died, but the tissues were not removed and extracted until 5 days later, in an unsuccessful attempt to discern a trend in the spontaneous reactivation rate. The effects noted above are also exhibited by these birds.

Dimethoate. The oral LD_{50} to pheasants is reported as 15 mg. per kg. (Sanderson and Edson, 1964). All four birds (19, 20, 21, and 22) given three LD_{50}'s died, while the pair (23 and 24) given one-half an LD_{50} survived. Tissues from 21 and 22 were not extracted until 12 days after death.

α-Naphthyl acetate electrophoregrams appear to be strongly affected by dimethoate poisoning, although this effect is not quite so large as that encountered with demeton-methyl. In the liver, bands 1, 4, 10, and 11 are nearly always affected, with 2 and 3 when the birds had died of poisoning, while in the kidney, bands 1, 2, and 5 are nearly always inhibited, with band 8 if the bird died. The brain is relatively unaffected, except for a slight over-all decrease in intensity and the inhibition of band 1 in birds 21 and 22. Brain cholinesterase electrophoregrams are completely inhibited after 12 days (21 and 22) but only weakened in the freshly extracted brains from the birds which had died of poisoning. One usual feature of the latter is that the strongest band (1) appears to lose most in intensity, suggesting that dimethoate may be preferentially inhibiting pseudocholinesterase. In the sublethally poisoned birds, brain cholinesterase band intensities are enhanced. No reactivation was possible (Table II).

In general, the esterase assays reflect the electrophoretic findings, and correlate well with the dosage given and the final condition of the birds. Noteworthy exceptions are values in the livers of birds 19 and 23, where all esterase levels are very near the control mean values. Triacetin and α-naphthyl acetate esterase levels are significantly de-

pressed in the livers of all other birds. Brain cholinesterase levels are inhibited in excess of 90% in the four birds which died but considerably less in the other two. As is generally the case, this inhibition pattern is followed very closely for brain triacetin esterase, but not for brain α-naphthyl acetate esterase. In this respect, dimethoate poisoning is similar to diazinon. However, only one brain phenyl benzoate esterase level is depressed (Table III).

Ethion. The oral LD_{50} to pheasants is reported as 250 mg. per kg. (May and Baker Ltd., 1967), which places ethion as the pesticide with the lowest lethal toxicity in the group examined. Owing to the large quantities which would have been involved in the dose rates originally planned, the four pheasants to be lethally dosed (25, 26, 27, and 28) were only given slightly more than one LD_{50}, and three died. Because of the lowering of the lethal dosage, the sublethal dose to birds 29 and 30 was also lowered by a similar factor to one-eighth of one LD_{50}. Tissues from 27 (survived dose) and 28 were not extracted until 12 days after death.

α-Naphthyl acetate electrophoregrams are virtually unaffected by poisoning. Over-all intensities are generally higher after 12 days at 20° C., suggesting some reactivation after an over-all partial inhibition. Brain cholinesterase electrophoregrams are also unaffected with the exception of 30, in which bands 2 and 3 are inhibited, although this is accompanied by a considerable increase in the intensity of band 1 (Table II).

The pattern of enzyme inhibition is similar to that exhibited by diazinon. The liver esterase levels appear generally unaffected by treatment with ethion. The exceptions are the triacetin levels in birds 25 and 26, but spontaneous reactivation of this cholinesterase-related enzyme seems to have occurred in 27 and 28. Brain esterase in lethally dosed birds are more affected, although again there is evidence of reactivation in 27 and 28. Even in the freshly extracted lethally dosed birds, inhibition, while large, is generally less than that with other pesticides excepting diazinon. Spontaneous reactivation or reactivation during extraction thus seems likely (Table III).

Guthion. The oral LD_{50} to pheasants is reported as 15 mg. per kg. (Baywood Chemicals Ltd., 1967). This figure is similar to that for the ethyl analog in both pheasants (Baywood Chemicals Ltd., 1967) and rats (Murphy and Dubois, 1958). Pheasants 33 and 34 given two LD_{50}'s based on the above figures survived the dose with very little effect even on blood esterases. As a result, these birds were considered as sublethally poisoned and the other four were given "lethal" doses of between five and six times this quantity. The two cock birds (31 and 35) died, while the two hens (32 and 36) survived. Tissues from birds 31 and 32 were not extracted until 12 days after death.

Liver and kidney α-naphthyl acetate electrophoregrams are heavily affected in all the lethally dosed birds, regardless of whether death had occurred. In liver electrophoregrams, the effect is drastic and uniform, but in the kidney, the effect is slightly erratic and appears mainly on the slower running bands. The inhibition is still marked in birds 33 and 34, especially in the kidney. In the liver of these two birds, bands 1,6, and 10 are affected. However, all brain α-naphthyl acetate electrophoregrams are virtually unaffected by treatment with Guthion. Cholinesterase electrophoregrams show the customary inhibition of bands 2 and in one case band 3 in birds which died, and also in bird 32 which survived a lethal dose. No reactivation was possible with 2-PAM (Table II).

Guthion has certain similarities to diazinon and ethion in the pattern of measured enzyme inhibition following poisoning. There is little correlation between the electrophoretic and the quantitative examination. The liver enzymes are not drastically affected, although in three of the lethally dosed birds (31, 35, and 36), liver triacetin esterase and α-naphthyl acetate esterase are significantly inhibited. All brain esterases are significantly inhibited with the exception of phenyl benzoate esterase levels in the sublethally dosed birds. The inhibition of brain phenyl benzoate levels is only exhibited in the case of Thimet (Bunyan and Taylor, 1966), diazinon, and Guthion. Despite the widespread depression of brain esterases following Guthion administration, the inhibition levels are slightly lower than those produced by the more poisonous organophosphates. Unlike diazinon, brain cholinesterase levels in the two birds which died are of the order of 90%, and depression is also fairly severe in the surviving massively dosed birds. There is no evidence of spontaneous reactivation (Table III).

Plasma Enzyme Levels. Plasma enzyme levels were measured both before and after dosing all sublethally poisoned birds, with the exception of those birds that received diazinon. In general, the figures obtained are erratic, especially for cholinesterase levels, and are not quoted in detail, but cholinesterase inhibition is normally in excess of 70%. α-Naphthyl acetate esterase levels are less inhibited (ca. 50%) and phenyl benzoate esterase levels inhibited least of all (ca. 10 to 30%). An exception arises with the two birds dosed Guthion, where the inhibition pattern is reversed (25, 36, and 80%, respectively).

General Discussion. The thesis developed earlier (Bunyan and Taylor, 1966; Bunyan *et al.*, 1968) that measurement of esterase levels, particularly those in brain tissue, would be a reliable diagnostic tool for organophosphate poisoning in wildlife at risk from the agricultural use of these pesticides, is moderately well supported by the experimental work presented. As with Thimet, brain cholinesterase inhibition in birds dying from chlorfenvinphos, demeton-methyl, dimethoate, and Guthion are in excess of 90% of the normal mean value, and there is no evidence of reactivation. Diazinon is the major exception in the group so far investigated, where one bird examined 12 days post-mortem did not show any significant inhibition, and another freshly dead bird only showed inhibition similar to most sublethally poisoned birds. Although results were erratic, consideration of two extra diazinon poisoned birds 5 days post-mortem suggests that reactivation is time dependent and therefore not due to the extraction technique. Ethion exhibits a similar reactivation, although with about 90% inhibition, it is less marked in the tissue from recently dead birds. Most sublethally poisoned birds and a few which survived massive doses show brain cholinesterase inhibition of less than 50%.

There is a striking similarity between brain cholinesterase and brain triacetin esterase inhibition in all the birds examined, reinforcing the author's view (Bunyan and Taylor, 1966) that these enzymes are related, and that estimation of the latter in the liver is a useful substitute for cholinesterase

estimation. Dimethoate and Guthion inhibit brain α-naphthyl acetate esterase more than they do brain cholinesterase and triacetin esterase. Chlorfenvinphos inhibits all esterases to about the same extent, while demeton-methyl, diazinon, and ethion inhibit α-naphthyl acetate esterases less. Only Thimet, diazinon, and Guthion inhibit brain phenyl benzoate levels.

Examination of esterase levels in the liver after poisoning reveals sharp differences in the effects produced by the various pesticides. Dimethoate is particularly severe, significantly inhibiting liver triacetin and α-naphthyl acetate esterases in most of the birds examined. Chlorfenvinphos and Guthion also produced significant depression of these two esterases in birds which had died. Diazinon and ethion produced virtually no effects in liver esterase levels. Demeton-methyl produces significant inhibition of triacetin and α-naphthyl acetate esterase in the liver of newly dead birds but there is a recovery with time. Chlorfenvinphos caused a significant rise in liver α-naphthyl acetate and phenyl benzoate esterase levels when given in large quantities to two birds (3 and 4) which did not subsequently die.

A breakdown of the electrophoretic results has been presented earlier for each pesticide, but a general comparative examination also reveals some interesting points. Chlorfenvinphos and Guthion present an over-all electrophoretic picture which suggests far greater inhibition than was measured, while with diazinon a certain lack of correlation was apparent. With the other three pesticides the agreement between electrophoretic and quantitative work was much better, with the exception of brain α-naphthyl acetate esterase levels, where there is seldom any effect visible on the electrophoregram. Most of these pesticides produce a large inhibition in some if not all of the birds dosed, when measured quantitatively, suggesting an over-all rather than a selective inhibition of isoesterases which is not readily picked up by the less sensitive electrophoretic method.

The tacit assumption based on Thimet dosing (Bunyan and Taylor, 1966), that death due to a cholinesterase inhibitor would be accompanied by inhibition of bands 2 and 3 of the brain cholinesterase electrophoregram, as well as by 90% inhibition of brain cholinesterase, is not substantiated by this work. Only with chlorfenvinphos is the situation as simple as that. Demeton-methyl, which is chemically similar to Thimet, shows inhibition of bands 2 and 3 in all dead birds, but also inhibition of band 1, 12 days post-mortem, while dimethoate (bands 1, 2, and 3) and diazinon (bands 2 and 3) only show inhibition in 12-day post-mortem extracts. Electrophoregrams from freshly dead dimethoate poisoned birds show a distinct weakening of band 1, suggesting a preference for nonspecific esterases. Guthion exhibits inhibition of bands 2 and 3 in 12-day extracts, and of bands 1 and 2 in birds examined immediately after death. Ethion shows virtually no effects on brain cholinesterase electrophoregrams despite some large measured inhibitions. Reactivation of cholinesterase electrophoregrams by 2-PAM ($10^{-4}M$), which was suggested (Bunyan *et al.*, 1968) as a more "positive" diagnosis of organophosphorus poisoning was only possible in the fresh extracts from demeton-methyl poisoned birds, and in two aged extracts of chlorfenvinphos-treated birds, although the latter had not died from poisoning.

As with the quantitative estimations, sharper differences between the pesticides are again revealed by examinations of liver and kidney α-naphthyl acetate esterase electrophoregrams (Figures 1 and 2). Demeton-methyl, dimethoate, and Guthion all exhibit extensive inhibition of α-naphthyl acetate isoesterases, particularly the fastest and the slowest bands. This effect is most severe in birds dying from poisoning. There is some evidence of spontaneous reactivation in the faster liver esterases following demeton-methyl poisoning and in the kidney esterases following Guthion poisoning. Chlorfenvinphos produces a moderate inhibition, removing individual bands rather than blocks of bands, from both the fastest and slowest isoesterases. Diazinon is similar to chlorfenvinphos in the production of moderately severe inhibition, but is rather more specific in its point of attack. Thus bands 10 and 11 in the liver and band 1 (with one exception) in the kidney are always inhibited. A few other bands are inhibited in a rather erratic fashion, and there is slight evidence of spontaneous reactivation in the kidney. Ethion produces no effect on liver and kidney α-naphthyl acetate esterase electrophoregrams.

Although results from electrophoresis appear rather more erratic than those obtained with Thimet (Bunyan and Taylor, 1966), this is primarily due to the wide range of pesticides investigated and the difficulty in finding a reliable LD_{50}. As a result, the coverage of dose range around the LD_{50} used with Thimet was not possible with these pesticides.

Chemical examination of the gut contents from freshly dead birds and the liver from all birds was carried out with the aid of thin-layer chromatography, using detection methods (Bunyan, 1964) which vary widely in their sensitivity to the six pesticides examined. No successful system was found for demeton-methyl. All guts examined contained the administered pesticide in amounts ranging from about 7% of the original dose with chlorfenvinphos and Guthion, where dosage was massive, to 0.05% of the original dose for dimethoate. This is however an unreal situation, since birds in the field are unlikely to receive a single fatal dose of insecticide, but instead will absorb

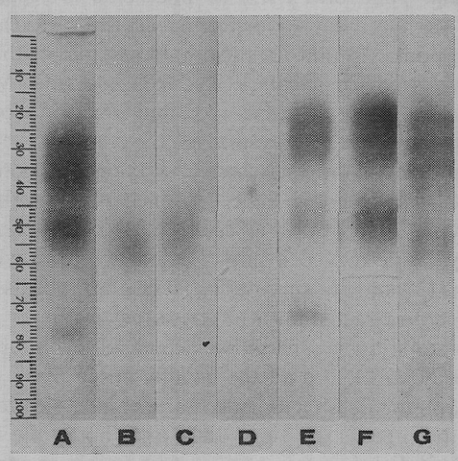

Figure 1. α-Naphthyl acetate esterase electrophoregrams of pheasant livers after acute organophosphorus poisoning

A. Control *B.* Demeton-methyl *C.* Dimethoate *D.* Guthion *E.* Chlorfenvinphos *F.* Diazinon *G.* Ethion

small quantities of the insecticide on food over a longer period. Furthermore, analysis within such a short period of time after death is unlikely. These two factors decrease the changes of successful analysis, although experience on field samples both in this laboratory and others (Stransky and Benes, 1961) suggests that changes of finding ingested organophosphates are highest in gut contents.

The rapid rate at which organophosphates are either absorbed irreversibly on protein or metabolized and excreted may be gaged by a review of the results from analysis of liver tissue. The majority of birds had levels of 0 to 0.5 p.p.m., which represents a total liver burden of approximately 0 to 12 μg. following ingestion of 50 to 200 mg. of pesticide 18 hours previously. Such levels are unlikely to be exceeded in field samples for the reasons already given. These small residues, even under favorable laboratory conditions, do not lend themselves readily to cleanup and analysis by conventional chemical means, nor by most chromatographic methods. The recent introduction of a variety of detectors sensitive to phosphorus (Karmen and Guiffrida, 1964; Burchfield *et al.*, 1965; Bache and Lisk, 1965), will allow analysis of organophosphorus compounds in the nanogram range by gas-liquid chromatography, although most reports to date deal only with plant material. Use of the thermionic detector (Karmen and Guiffrida, 1964) in this laboratory is currently aimed at finding small residues in avian tissue, but confirmation of results by other techniques is still required. Experiments to be reported later on chronic feeding (100 p.p.m.) of some of these organophosphate pesticides to pheasants and pigeons has revealed that especially in the latter, residues in liver, muscle, and fat are smaller and even more difficult to demonstrate by the methods described above than with acutely poisoned birds.

The inhibition of esterases, particularly cholinesterase, detected either quantitatively or electrophoretically in liver, kidney, or brain can provide either a useful initial indication, or a confirmation of poisoning by organophosphates. Such observations are, within reason, not affected by the port-mortem lag before analysis. Diagnosis of death as a result of poisoning can confidently be made where brain inhibition exceeds 90% of the normal mean value, and diagnosis of sublethal poisoning can be made where brain inhibition is significant. Some indication of the pesticide involved can be obtained by a study of the relative inhibition produced in liver and brain and of the pattern of alteration of liver and kidney electrophoregrams. Misleading results may be produced if spontaneous reactivation occurs as is the case with diazinon.

This type of approach, which utilizes the difference in biochemical effects produced by two pesticides in a particular organ or by a particular pesticide in two organs, has recently been advocated by Korolev (1965), who also found that the brain was unaffected when blood and liver cholinesterases were seriously inhibited following sublethal acethion poisoning.

Esterase levels and electrophoretic patterns from control birds are required to apply the method successfully. The latter can usually be reliably obtained from one control bird, although the former require a larger control group. There is some indication that esterase levels and patterns may be similar within families of birds, and that once reliable results are obtained for one member they may be extrapolated to others, especially since many of the changes described are large. Further confirmation of the identity of an organophosphate pesticide may be possible by extraction of the residue from poisoned tissue and addition of this extract to a standard tissue extract with subsequent demonstration of typical inhibition in the resulting electrophoregram. Such a procedure without the extraction has been demonstrated in human (Ecobichon and Kalow, 1963) and rat (Bulmer and Fisher, 1967) liver extracts.

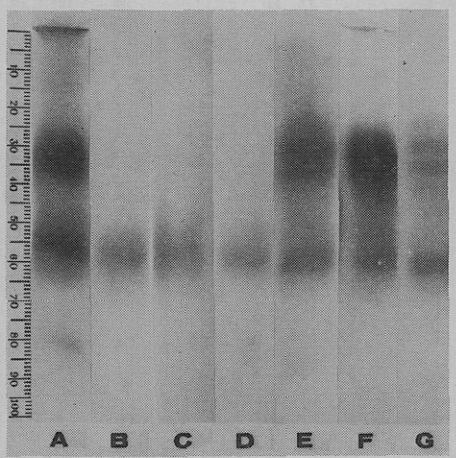

Figure 2. α-Naphthyl acetate esterase electrophoregrams of pheasant kidneys after acute organophosphorus poisoning

A. Control *B.* Demeton-methyl *C.* Dimethoate *D.* Guthion *E.* Chlorfenvinphos *F.* Diazinon *G.* Ethion

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