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Use of Reactivation Techniques for the Differential Diagnosis of Organophosphorus and Carbamate Pesticide Poisoning in Birds

A. D. Martin, G. Norman, P. I. Stanley, and G. E. Westlake

Tolworth Laboratory, Agricultural Science Service, Ministry of Agriculture, Fisheries and Food, Surbiton, Surrey, UK

The inhibition of brain acetylcholinesterase activity has been regarded as being indicative of poisoning by organophosphorus pesticides (BUNYAN *et al.*, 1968, 1969, LUDKE *et al.*, 1975, BROWN *et al.*, 1977). However the diagnostic value of assaying brain acetylcholinesterase for this purpose has become limited by the introduction into agricultural usage of a number of toxic carbamate pesticides which also inhibit this enzyme system (BUNYAN and JENNINGS, 1976). By utilising the difference in the facility with which esterases inhibited by these two groups of compounds reactivate both spontaneously and by chemical means (ALDRIDGE and RIENER 1972, REED and FUKUTO 1973) a procedure has been established which can differentiate between organophosphate and carbamate inhibited brain acetylcholinesterase. The rapid and simple nature of the procedure make it suitable for use in the investigation of alleged pesticide poisoning incidents.

MATERIALS AND METHODS

The organophosphorus and carbamate pesticides selected for study, their purity and sources of supply were: carbophenothion [S-(4-chlorophenylthio)methyl 0,0-diethyl phosphorodithioate] analytical grade, Stauffer Chemical Co. Ltd.; chlorfenvinphos [2-chloro-1-(2,4-dichlorophenyl) vinyl diethyl phosphate], technical grade, Shell Research Ltd.; dimethoate [0,0-dimethyl S-methylcarbamoylmethyl phosphorodithioate] technical grade, Murphy Chemical Co. Ltd.; mevinphos E [2-methoxycarbonyl-1-methylvinyl dimethyl phosphate], analytical grade, Shell Research Ltd.; pirimiphos-methyl [0-2-diethylamino-6-methylpyrimidin-4-yl 0,0-dimethyl phosphorothioate] analytical grade, Plant Protection Ltd.; aldicarb [2-methyl-2-(methylthio) propionaldehyde 0-methylcarbamoyloxime] analytical grade, Union Carbide Corporation; bendiocarb [2,3-isopropylidenedioxyphenyl methylcarbamate], technical grade, Fisons Ltd.; methiocarb [4-methylthio-3,5-xylyl methylcarbamate], technical grade, Bayer Ltd.; oxamyl [N,N-dimethyl-2-methylcarbamoyloxyimino-2-(methylthio) acetamide], analytical grade, Du Pont de Nemours and Co. Inc.; pirimicarb [2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate], technical grade, Plant Protection Ltd. and thiofanox [3,3-dimethyl-1-(methylthio)butanone 0-methylcarbamoyloxime], analytical grade, Diamond Shamrock, Europe. All other chemicals were obtained from normal commercial sources.

Batches of 21 day old female Japanese quail (*Coturnix coturnix japonica*) were housed 4 to a cage and maintained on Spillers turkey starter crumbs and water *ad libitum*. The birds were kept in a controlled environment of 19°C ± 1°C and 60% ± 5% relative humidity with a 16h long day light period. Body weights were determined at 28 days of age when groups of birds were dosed with an amount of each pesticide calculated to be 3 times the LD_{50}. All compounds were administered in corn oil solution by gelatin capsule except dimethoate which was given as an

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aqueous solution by intubation. At the same time a control group of birds was dosed with the appropriate solvent vehicle and sacrificed 2h later. Immediately after death or sacrifice brains were removed from up to 4 control and 8 treated birds while the bodies of up to 4 control and 4 treated birds were kept at ambient temperature for 7 days before dissection. All brains were homogenised in 3 volumes of ice cold 1% Triton X-100 immediately after removal. The post mitochondrial supernatant (PMS) was prepared by centrifuging the homogenate at 17,000g for 45 min at 4°C. The PMS was stored overnight at -18°C before assay and reactivation.

The acetylcholinesterase activity of the PMS was assayed at pH 7.9 by the method of ELLMAN *et al.* (1961) using a final assay volume of 3.0 mL (WESTLAKE *et al.*, 1980). Spontaneous reactivation was achieved by gel filtration and dilution (REED and FUKUTO, 1973). PMS (0.3 mL) was applied to a 10 mL column of Sephadex G-25 (PD10 Column, Pharmacia Fine Chemicals) and eluted with 5.7 mL of 0.1M phosphate buffer pH 7.9. The eluate was further diluted with buffer and kept at 4°C for up to 120h before assay when a volume of 2.5 mL containing the equivalent of 10 µL of the original supernatant, was used in the final assay volume of 3.0 mL. Chemical reactivation was initiated by mixing equal volumes of PMS and 0.5 mM aqueous pyridine 2-aldoxime methiodide (2-PAM) solution. Samples (20 µL) were taken for assay at periods of up to 60 min.

RESULTS

All birds receiving either an organophosphorus or a carbamate pesticide died within 3h. As expected, brain acetylcholinesterase was severely inhibited in all those birds which had received an organophosphorus pesticide (Table 1) although inhibition did not always reach the 90% level reported by BUNYAN *et al.* 1968, 1969. The six carbamate pesticides included in this study also caused a similar reduction in brain acetylcholinesterase activity when assayed immediately after death.

Immediately after gel filtration and dilution the activity of carbamate inhibited enzyme was increased approximately 2-3 fold and had returned to normal levels within 24h, with the exception of pirimicarb which required up to 96h. In some instances further small increases were recorded between 24h and 120h after filtration. At no time was any spontaneous reactivation recorded for the organophosphorus inhibited enzymes (Table 1).

In contrast chemical reactivation with 2-PAM increased the activity of both carbamate and organophosphate inhibited enzymes (Table 1) although there was considerable variation between compounds in the amount of activity recovered. While the enzymes inhibited by carbophenothion and chlorfenvinphos were reactivated to normal levels within 15 min, reactivation of the enzymes inhibited by dimethoate, mevinphos or pirimiphos-methyl was less marked. All the carbamate inhibited enzymes showed some enhancement in activity, with increases of between 2 and 4 fold being reached in 60 min. As this reactivation was of a more progressive nature than that obtained with the organophosphate inhibited enzymes it is probable that it was due to spontaneous reactivation brought about by dilution rather than direct chemical reactivation by 2-PAM.

TABLE 1

Brain acetylcholinesterase activity[a] assayed immediately after death or sacrifice

Compound	Dose mg/kg	Group Size	Activity at death/ sacrifice	Activity 24 hours after gel filtration	Activity 30 min after chemical reactivation
Control	-	37	22.8 ± 3.3	15.6 ± 3.9	16.9 ± 2.7
Carbophenothion	170	8	1.1 ± 0.2[b]	1.0 ± 0.2	16.4 ± 1.8[c]
Chlorfenvinphos	444	8	0.8 ± 0.1[b]	0.9 ± 0.2	17.1 ± 1.8[c]
Dimethoate	75	8	2.4 ± 0.5[b]	1.9 ± 0.4	5.7 ± 1.1[c]
Mevinphos	30	7	1.3 ± 0.6[b]	1.7 ± 0.1	11.5 ± 2.4[c]
Pirimiphos-methyl	520	8	4.0 ± 1.1[b]	3.1 ± 0.7	6.3 ± 2.5
Aldicarb	30	8	3.9 ± 1.1[b]	15.8 ± 1.0[c]	8.4 ± 1.3[c]
Bendiocarb	50	8	1.4 ± 0.3[b]	16.9 ± 3.4[c]	3.8 ± 1.1[c]
Methiocarb	75	8	3.9 ± 0.9[b]	15.0 ± 1.7[c]	9.7 ± 0.6[c]
Oxamyl	14	8	3.3 ± 0.9[b]	14.2 ± 1.4[c]	7.5 ± 0.7[c]
Pirimicarb	160	8	1.4 ± 0.3[b]	10.4 ± 0.7[c]	5.6 ± 0.5[c]
Thiofanox	3.6	8	3.0 ± 0.3[b]	16.0 ± 0.8[c]	8.2 ± 0.6[c]

a μmoles/min/g. brain ± SD
b. inhibition significant at P <0.001
c. reactivation significant at P <0.001

TABLE 2

Brain acetylcholinesterase activity[a] in respect of bodies kept at room temperature for 7 days after death or sacrifice.

Compound	Dose mg/kg	Group Size	Activity at 7 days after death or sacrifice	Activity 24 hours after gel filtration	Activity 30 min after chemical reactivation
Control	-	35	14.8 ± 2.9	10.0 ± 3.0	13.0 ± 3.3
Carbophenothion	170	4	3.1 ± 0.7[b]	3.2 ± 0.6	10.6 ± 0.4[d]
Chlorfenvinphos	444	4	0.6 ± 0.1[b]	0.5 ± 0.2	6.0 ± 2.8[e]
Dimethoate	75	4	0.9 ± 0.1[b]	0.8 ± 0.2	1.5 ± 0.1[d]
Mevinphos	30	4	2.1 ± 0.7[b]	1.2 ± 0.4	2.4 ± 0.6
Pirimiphos-methyl	520	4	4.8 ± 2.6[b]	2.9 ± 1.2	4.0 ± 1.4
Aldicarb	30	4	2.1 ± 1.1[b]	10.8 ± 0.9[d]	3.9 ± 1.3
Bendiocarb	50	4	5.8 ± 1.5[b]	12.4 ± 1.2[d]	9.4 ± 2.4[f]
Methiocarb	75	4	13.8 ± 0.7	12.1 ± 1.3	12.7 ± 1.3
Oxamyl	14	4	15.2 ± 1.0	12.4 ± 1.1	12.8 ± 1.1
Pirimicarb	160	4	5.9 ± 1.5[b]	8.6 ± 0.7[e]	8.4 ± 1.3[f]
Thiofanox	3.6	4	9.5 ± 3.1[c]	10.1 ± 1.3	10.7 ± 1.7

a μ moles/min/g tissue ± S.D.
b inhibition significant at P <0.001
c inhibition significant at P <0.01
d reactivation significant at P <0.001
e reactivation significant at P <0.01
f reactivation significant at P <0.05

When brain acetylcholinesterase was assayed and reactivated after the bodies of the poisoned birds had been kept at room temperature for 7 days a more variable situation was revealed (Table 2). While organophosphates were found to be still severely inhibiting brain acetylcholinesterase, inhibition by the carbamates, with the exception of aldicarb, was less than that recorded immediately after death. In the case of methiocarb and oxamyl the activities were indistinguishable from those found in the control group. Spontaneous reactiviation led to an increase in the activity of those enzymes inhibited by aldicarb, bendiocarb and pirimicarb. As found previously none of the organophosphorus inhibited enzymes was susceptible to spontaneous reactivation. When chemically reactivated with 2-PAM only the carbophenothion and chlorfenvinphos inhibited enzymes showed any marked increase in activity and even this was less pronounced than found previously. The carbamate inhibited enzymes again showed small and progressive increases in activity after dilution with the 2-PAM solution.

DISCUSSION

Inhibition of esterases by certain esters of organophosphorus and carbamic acids giving rise to phosphorylated or carbamoylated enzyme intermediates and their reactivation to the parent enzyme have been the subject of many studies (see ALDRIDGE and RIENER, 1972). As a result it has become established that the carbamoylated enzymes are generally less stable and more susceptible to hydrolysis. They therefore tend to reactivate spontaneously. Conversely the phosphorylated enzyme, is more readily reactivated chemically by nucleophilic reagents such as 2-PAM, due to the electrophilic nature of the phosphorus atom. In addition some phosphorylated enzymes can spontaneously dealkylate (age) and in this form they remain unaffected by nucleophilic reactivators. These differences are the basis of the procedure described here for distinguishing between organophosphorus and carbamate poisoning in birds.

A comparison of the relative enzyme activities recorded before and after the application of procedures for the spontaneous and chemical reactivation of brain acetylcholinesterase immediately after the death of poisoned birds clearly demonstrates the difference in response of these two types of compound (Table 1). The carbamate inhibited enzymes were more readily reactivated by gel filtration and dilution than they were by admixture with 2-PAM, while the converse was true for these enzymes inhibited by the organophosphorus esters. In addition smaller variations were found within each group which can largely be accounted for on the basis of differences in their chemical structures. Thus the dimethyl substituted carbamate, pirimicarb, gave rise to a carbamoylated enzyme which spontaneously reactivated more slowly than that derived from the other, monomethyl, substituted compounds. Similarly acetylcholinesterase inhibited by the organophosphorus dimethyl esters, dimethoate, mevinphos and pirimiphos-methyl was reactivated by 2-PAM to a lesser extent than when inhibited by the diethyl esters carbophenothion and chlorfenvinphos. That this is probably due to the greater tendency of the dimethyl phosphorylated brain acetylcholinesterase of the Japanese quail to age as has been demonstrated in the rat (DAVIES and GREEN, 1956) is confirmed by the inability of 2-PAM to bring about any noticeable increase in the activity of this enzyme in those birds which had been left for 7 days (Table 2).

Conversely the differences in the activities recorded for the carbamate inhibited enzymes when assayed 7 days after death (table 2) cannot be explained on the basis of differences in their chemical structure as, with the exception of pirimicarb, all the compounds used would have given rise to the same methyl carbamoylated enzyme.

Whether or not this inhibited enzyme reactivates spontaneously *in situ* depends on the amount of free carbamate present (ALDRIDGE and RIENER, 1972). It must therefore be concluded that methiocarb and oxamyl are present in lower concentrations in the brain after death than are aldicarb, bendiocrab and thiofanox.

This study has shown that when brain acetylcholinesterase is assayed and reactivated immediately after death, inhibition by carbamate and organaphosphorus pesticides can be readily differentiated (Table 1). At this time it seems probable that sufficient carbamate is present in the brain tissue as a result of the ingestion of a lethal amount of the compound, to ensure a significant reduction in acetylcholinesterase activities. Such a situation would be expected to occur in the event of a wildlife poisoning incident. When the enzyme is separated from the free inhibitor by gel filtration followed by dilution, conditions for spontaneous reactivation are created. If the assay and reactivation of brain acetylcholinesterase is delayed for several days after death, as may happen when a poisoning incident occurs during the normal agricultural use of a pesticide, it is still possible to clearly identify enzyme inhibited by an organophosporus compound (Table 2). However, it appears that for some carbamates the amount of excess compound present must be small, either because large quantities do not reach the brain or because they are chemically or enzymically broken down after death allowing the brain acetylcholinesterase to reactivate to normal levels.

Although this study has been confined to an investigation of the inhibition and reactivation of brain acetylcholinesterase by organophosphorus and carbamate pesticides of limited variation in chemical structure and in a single avian species it is likely that the results obtained are of a wider application. As a result, the procedure used in this laboratory for the investigation of wildlife deaths in which agricultural chemicals are implicated (BROWN *et al* 1977) has been modified. If the routine assay of brain acetylcholinesterase activity suggests that this enzyme is inhibited then spontaneous and chemical reactivation by the techniques described is attempted. On the basis of the relative activities determined before and after reactivation, subsequent residue analysis, on which the confirmation of any pesticide involvement ultimately depends, is concentrated on procedures appropriate to the type of compound indicated.

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