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FURTHER OBSERVATIONS ON THE NEUROTOXICITY OF ORGANOPHOSPHORUS COMPOUNDS

W. N. ALDRIDGE and J. M. BARNES

Toxicology Research Unit,
Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey

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Abstract—Twenty-two organophosphorus compounds have been given to adult hens to test their ability to produce a delayed neurotoxic effect like that produced by triorthocresyl phosphate. Fourteen compounds falling in several structurally homologous groups were active. All of them inhibited *in vitro* esterases of chicken CNS. No biochemical mechanism for this toxic effect can be put forward.

SINCE the discovery over 12 years ago[1] that certain organophosphorus compounds which were active anticholinesterase poisons could also produce a delayed neurotoxic effect in hens identical with that produced by tri*ortho*cresyl phosphate, work has continued in this laboratory in an attempt to discover the biochemical lesion produced by those organophosphorus compounds which induce this type of damage. The excellent work of Casida and his colleagues[2] which led to the identification of a metabolite of tri 2-tolyl phosphate (tri*ortho*cresyl phosphate) that was responsible for the neurotoxic action of the compound stimulated work on esterases (other than cholinesterase) present in the nervous system of the hen which might be sensitive to those organophosphorus compounds which produced a neurotoxic effect.[3, 4]

During the course of this work our attention was drawn to certain compounds studied by Becker[5–7] which had been synthesized with the aim of inhibiting tissue esterases other than cholinesterase. Another group of organophosphorus compounds which had been developed as anthelmintics for use in sheep[8] contained 2-chloroethyl groups and were found to be neurotoxic to hens.[9]

The work reported to date has not disclosed the nature of the biochemical lesion which is responsible for the development of the delayed neurotoxic effect produced by certain organophosphorus compounds. Nor is it yet possible to predict upon the basis of its chemical structure whether or not a given compound will have this neurotoxic effect. It may therefore serve a useful purpose to those who are concerned with the safety to man of organophosphorus compounds when used as pesticides to record some new examples of the type of compound that can produce the late neurotoxic damage in hens.

METHODS AND MATERIALS

Hens at least 6 months old, mainly of the Rhode Island Red × Light Sussex cross obtained from a local breeder, were kept in groups of 2–4 and fed a standard layer's mash. At the time of testing, their body weights ranged from 2–3 kg. When the compound to be tested was known to be an acute anticholinergic poison, atropine (20 mg

subcutaneously) and N-methyl pyridinium-2-aldoxime methanesulphonate (P2S, 100 mg intraperitoneally), were given 10–15 min before the organophosphorus compound. This was given in some instances by mouth, either undiluted or in arachis oil; other compounds were given by subcutaneous or intraperitoneal injection in a small (0·1–0·3 ml) quantity of ethanol. When the birds developed an acute poisoning with rapid collapse and convulsions an additional dose of atropine given intravenously sometimes saved them.

The bird were observed daily, except at weekends, for the next 14–21 days. The cholinergic effects especially after the subcutaneous injection of solutions of some compounds in ethanol might persist for several days and the birds would be weak and unable to stand or walk properly. More usually these signs cleared up within 48 hr and the birds appeared normal for the next 8–12 days. At this time signs of the late neurotoxic effects were sought. When the hens were held 2–3 feet above the floor and then dropped some unsteadiness after landing was a common early sign. Slight swaying or unsteadiness when walking were often difficult to establish with certainty at an early stage; if a true lesion was developing these signs would progress steadily during the ensuing 3–4 days after their existence was first suspected. When the disability did not increase, a second and if possible, larger dose was given.

In cases where the diagnosis remained in some doubt on simple clinical grounds, the birds were killed with nembutal and the spinal cord and sciatic nerves rapidly removed and fixed in formal saline and examined by Dr. J. B. Cavanagh.

TABLE 1. COMPOUNDS EXAMINED—CHEMICAL NAMES AND SOURCES

A.	Ethyl *p*-nitrophenyl phenylphosphonate	Dr. E. L. Becker
B.	Ethyl *p*-nitrophenyl phenylmethylphosphonate	Dr. E. L. Becker
C.	Ethyl *p*-nitrophenyl 2-phenylethylphosphonate	Dr. E. L. Becker
D.	Ethyl *p*-nitrophenyl 3-phenylpropylphosphonate	Dr. E. L. Becker
E.	Ethyl *p*-nitrophenyl 4-phenylbutylphosphonate	Dr. E. L. Becker
F.	Ethyl *p*-nitrophenyl *n*-amylphosphonate	Dr. E. L. Becker
G.	Ethyl *p*-nitrophenyl *n*-decylphosphonate	Dr. E. L. Becker
H.	Di(2-chloroethyl) 4-methylcoumarin-7-yl phosphate	Cooper Technical Bureau
I.	Di(2-chloroethyl) *p*-nitrophenyl phosphate	Cooper Technical Bureau
J.	Di(2-chloroethyl) 2:3:5-chlorophenyl phosphate	Cooper Technical Bureau
K.	Di(2-chloroethyl) 2:2-dichlorovinyl phosphate	Cooper Technical Bureau
L.	Di(2-chloroethyl) ethylphosphonothionate	Farbenfabriken Bayer AG
M.	Di(3-chloropropyl) 4-methylcoumarin-7-yl phosphate	Cooper Technical Bureau
N.	Di(2-chloropropyl) 4-methylcoumarin-7-yl phosphate	Cooper Technical Bureau
O.	2-Chloroethyl 2:2-dichlorovinyl methyl phosphate	Farbenfabriken Bayer AG
P.	2-Chloroethyl ethyl *p*-nitrophenyl phosphate	Cooper Technical Bureau
Q.	2-Chloroethyl 2:2-dichlorovinyl ethylphosphonate	Farbenfabriken Bayer AG
R.	2:2-Dichlorovinyl dimethyl phosphate	Shell Research Ltd.
S.	Ethyl 2:4:5-trichlorophenyl ethylphosphonothionate	Farbenfabriken Bayer AG
T.	2:4-Dichlorophenyl methyl methylphosphonothionate	Farbenfabriken Bayer AG
U.	Diphenyl 2-tolyl phosphate	Coalite Chemical Co.
V.	Phenyl saligenin phosphate	Albright & Wilson Ltd.

The activity of many of the compounds against esterases from the CNS was examined and the concentration producing 50 per cent inhibition after preincubation for 5 min at 37° without the substrate (phenyl 3-phenyl-propionate) was determined by methods previously described.[3] The compounds tested were gifts from various sources and their chemical names and origin are listed in Table 1.

RESULTS

The results of testing 22 compounds on hens and their activity against esterases are summarised in Table 2. Thirteen of the compounds were found capable of producing a characteristic delayed neurotoxic lesion in the hens. (Compounds A, B, C, D, H, I, J, K, O, S, T, U, V). The observations with Compounds H, U and V confirm the work of others.[2, 9]

Compound A which is the phosphate analogue of the insecticide EPN (ethyl *p*-nitrophenyl phenylphosphonothionate) is interesting in that it was not always possible to produce an effect after 10 mg/kg though in those birds affected the evolution of the ataxia was characteristic. The neurotoxic effects of EPN have been reported and the incidence of clinical effects was also somewhat irregular.[10] However, the pathological lesion in hens poisoned by EPN has been observed.[11] On the other hand the homologue, Compound C, which was given to a total of 9 hens always gave a characteristic response. Another modification of the classical picture was shown by Compounds S and T. These produced a prolonged cholinergic effect which only slowly abated. It had not completely disappeared when a characteristic ataxia made its appearance by 14 days. The response of hens to Compound S as to Compound A was not always consistent and this had been reported earlier.[12] However, the histological changes in the spinal cord and sciatic nerves of the ataxic birds were characteristic.

The negative findings with Compound P were confirmed on histological examination while those with Compound R confirm similar findings reported elsewhere.[10]

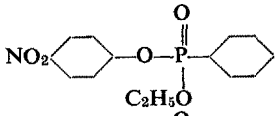
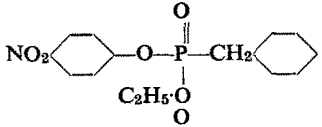
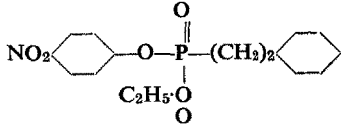
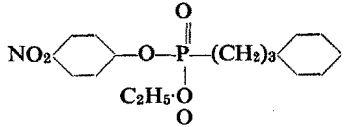
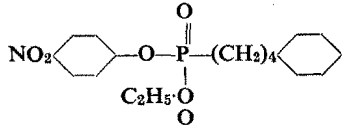
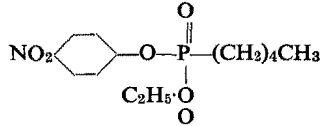
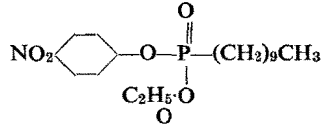
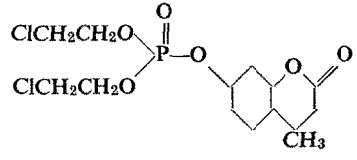
DISCUSSION

It is very difficult to be certain that those compounds with which it was not possible to produce clinical ataxia were truly without a neurotoxic effect. After Compound F, given in the largest doses the bird would tolerate, histological changes were found but were unaccompanied by clinical signs. Several of the other compounds that did not produce ataxia were so acutely toxic that it was not possible to give bigger doses since atropine and oxime (P2S) gave only limited protection against the acute cholinergic effects. Limited supplies of some compounds made it impossible to repeat some of the tests and obtain material for histology. These unavoidable restrictions and difficulties in proving whether a compound is intrinsically inactive limit the interpretations which can be made.

The identification of the saligenin phosphates as toxic metabolites of certain neurotoxic triaryl phosphates[2] led to the development of a working hypothesis that esterases which hydrolyse substrates with a structural resemblance to these toxic metabolites might be inhibited prior to the onset of the neurotoxic damage.[3] Esterases which were active against such substrates were found in the nervous system of the hen but these esterases were subsequently shown to be inhibited *in vivo* equally by organophosphorus compounds that produced neurotoxic damage as well as some that do not produce such lesions.[13]

Although the inhibition of such esterases clearly is not the whole story as an explanation of the origin of the neurotoxic lesion the hypothesis did make it possible to predict for the first time that a completely new type of organophosphorus anticholinesterase would also have neurotoxic activity (Compounds B, C, and D).

TABLE 2. RESULTS OF TESTS FOR ACUTE TOXICITY, NEUROTOXICITY AND ANTI-ESTERASE ACTIVITY OF SELECTED ORGANOPHOSPHORUS COMPOUNDS

No.	Formula	Acute toxicity	Dose (mg/kg)	No. of doses	Route	Neurotoxicity	Histology	Anti esterase activity (conc. for 50% inhibition in 5 min at 37°C, μM),
A.		++	10	1	s.c.	+	+	0·14
B.		++	10	1	s.c.	+	+	0·35
C.		++	6	1	i.p.	+	+	0·072
D.		+++	4	4	i.p.	+	+	0·08
E.		+++	4	8	i.p.	−		0·48
F.		+++	2	12	i.p.	−	+	0·18
G.		++	16	1	i.p.	−		0·08
H.		+	200	3	*p.o.*	+		2·4

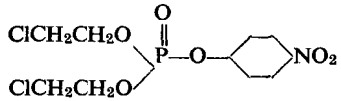
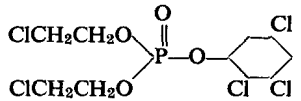
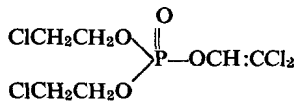
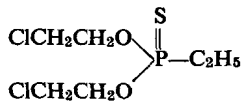
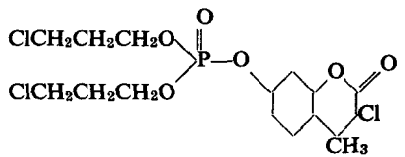
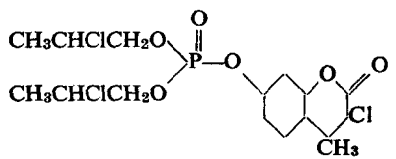
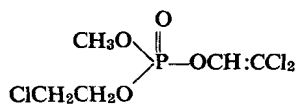
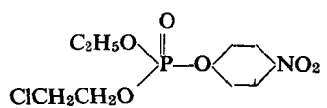
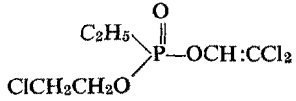
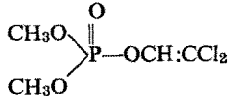
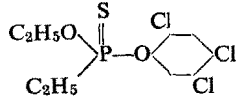
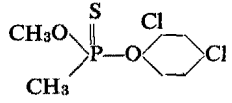
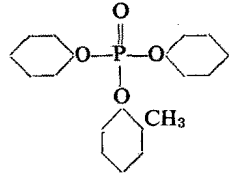
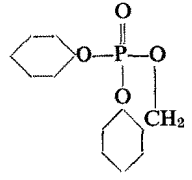
I.		++	100	1	*p.o.*	+	+	0·6
J.		+	500	1	*p.o.*	+		1·0
K.		++	25	1	s.c.	+	+	0·3
L.		++	40	1	s.c.	−		
M.		+	400	1	*p.o.*	−		
N.		0	2000	1	*p.o.*	−		
O.		++	5	1	s.c.	+	+	0·57
P.		++	20	1	s.c.	−	−	

TABLE 2—*continued*

No.	Formula	Acute toxicity	Dose (mg/kg)	No. of doses	Route	Neurotoxicity	Histology	Anti esterase activity (conc. for 50% inhibition in 5 min at 37°C, μM)
Q.		+++	1	2	s.c.	–		
R.		++	30	1	s.c.	–		
S.		++	100	1	i.p.	+	+	
T.		++	10	1	s.c.	+	+	
U.		0	50	1	*p.o.*	+		
V.		+	2	1	i.p.	+	+	0·54

Acute toxicity	+++	lethal dose less than 10 mg/kg
	++	lethal dose between 10–100 mg/kg
	+	lethal dose between 100–1000 mg/kg
	0	over 1000 mg/kg
Route	s–c	subcutaneous
	i–p	intraperitoneal
	p–o	by mouth
Neurotoxicity	+	a persistent weakness and ataxia coming on 10–16 days after dosing
Histology	+	lesions as described by Cavanagh[16]
	—	no lesions seen

Anti-esterase activity as described in following paper.[13]

However, even if the hypothesis of a selective inhibition of the X and Y esterases had been supported by the work described in the following paper, it was difficult to see what structural resemblance there was between the substrates used to demonstrate the esterases and a neurotoxic compound like di*iso*propyl phosphorofluoridate.

The discovery that phosphates containing the 2-chloroethyl group can be neurotoxic introduces a new family of compounds into consideration. Whereas diethyl phosphates are not neurotoxic (e.g. paraoxon) the di(2-chloroethyl) phosphates are (Compounds H, I, J and K). If the phosphate contains 2-chloroethyl alkyl it may be neurotoxic where the alkyl is methyl (Compound O) but not when the alkyl is ethyl (Compound P). Nor is a corresponding ethylphosphonate (Compound Q) toxic.

Clearly there are many variations of the structure of these molecules to be tested before any reasonable structure–activity relations can be delineated as has been done with the phosphorofluoridates.[14, 15] It is worth emphasising how very small may be the modification in the molecule needed to convert the undoubtedly non-neurotoxic compound dichlorvos (Compound R) into a highly neurotoxic one (Compound O) i.e. the substitution of $—CH_2CH_2Cl$ for $—CH_3$.

In considering what sort of enzyme is likely to be inhibited by these neurotoxic compounds it will be necessary to discover what moiety of the original inhibitor is responsible for the phosphorylation of the enzyme; this may not be obvious at first glance (for instance cf Compound O).

The production of the delayed neurotoxic effect may depend upon the inhibition of one particular enzyme but it may also be determined by the rapidity and degree to which inhibition is reversed or new enzyme is resynthesized. Both such characteristics for neurotoxic and non-neurotoxic organophosphorus compounds require to be studied against any enzyme(s) suspected of being involved.

At present we can only state that all the neurotoxic organophosphorus compounds, which vary greatly in chemical structure, are active inhibitors of some esterases other than cholinesterase. Until such time as an organophosphorus compound is discovered which is actively neurotoxic but inactive both *in vitro* and *in vivo* against esterases we will continue to regard the search for sensitive esterases as a logical way of trying to make progress.

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