

Side Effects of Organophosphorus Compounds: Delayed Neurotoxicity*

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Many organophosphorus compounds produce a chronic toxicity that has been classified as a dying-back process in which the long axons are particularly affected. Recent evidence indicates that an essential first step in the production of the lesion is the phosphorylation of a protein that is able to hydrolyse substrates such as phenyl phenylacetate. The esterase activity of the protein is inhibited by this phosphorylation, presumably at the active site. However, the toxic effect is related only to phosphorylation and not to loss of esterase activity.

Since 1930 (Smith & Elvore) it has been known that some organophosphorus compounds produce a chronic neurotoxicity. The compound originally studied was tri-*o*-tolyl phosphate, and the clinical signs appeared 8–14 days after administration. In this condition, ataxia is present and, if poisoning is severe, there is a permanent paralysis of the limbs. Other chronic conditions produced by organophosphorus compounds have been described (Durham et al., 1956; Gaines, 1969) but these must be produced by a different mechanism, since the clinical condition appears immediately after dosing and recovery is usually complete within 1 month. Until relatively recently the production of a permanent lesion was thought to be restricted to a few organophosphorus compounds. The sensitive species were man and the hen, but young chickens were not sensitive. During the last 10 years both the number of organophosphorus compounds active in this way and the number of sensitive species have been greatly extended and the age requirement has been explained (Aldridge, Barnes & Johnson, 1969, and Johnson, 1969a, for reviews; Johnson & Barnes, 1970). From pathological studies the lesion has been classified as a dying-back process in which the long axons are particularly affected; axons in the spinal cord and in the periphery, such as the sciatic nerve, are involved (Cavanagh, 1969, for review). Thus, the problem of delayed neurotoxicity of organophosphorus compounds is a general one, the solution of which may have important implications for our knowledge of how the integrity of long axons is maintained.

Over many years several enzymes have been eliminated from possible involvement in the genesis of the lesion. Almost all of these have been chosen for study by what might be called the “guess and test” approach. In recent years a more direct approach has been made and the proposition that phosphorylation of a protein in the central nervous system is an essential prerequisite for the development of the lesion has been examined. Analytical methods have been developed (Johnson, 1969b; Aldridge, Barnes & Johnson, 1969), to measure only a selected few of all the proteins in normal brain that can react with a radio-labelled neurotoxic agent, DF³²P.† Using this analytical method the brains of chickens treated with neurotoxic and non-neurotoxic organophosphorus compounds have been examined; neurotoxic compounds reduced appreciably the phosphorylation of the selected sites by DF³²P, whereas non-neurotoxic compounds have little effect (Johnson, 1969b; Tables 1 and 2). During the course of this investigation compounds were found that produced significant reduction in labelling, but had been classified by previous workers as non-neurotoxic. A reinvestigation with multiple dosing showed that a typical delayed neurotoxicity was produced. For neurotoxic effects to be produced more than 70% of the “neurotoxic protein” must be phosphorylated shortly after dosing.

The possibility that the “neurotoxic protein” was an esterase was considered. It is known that the rate of phosphorylation of an esterase by an organophosphorus compound is greatly reduced when a substrate is present. Numerous esters were

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† Names against which this symbol appears are identified in the Glossary on pages 445-446.

Table 1

The "phosphorylation site", "neurotoxic esterase", and neurotoxicity: non-neurotoxic compounds or doses *

Compound ^a	Phosphorylation site available (% of control) ^b	Esterase activity (% of control) ^b
diethyl <i>p</i> -nitrophenyl phosphate (paraoxon)	62, 92	88
tetraisopropyl pyrophosphoramidate	116	—
tetraethyl pyrophosphate (TEPP)	78	75
tri- <i>o</i> -ethylphenyl phosphate ^c	57, 62	—
<i>O</i> -(4-bromo-2,5-dichlorophenyl) <i>O</i> -methyl ethylphosphonothioate ^c	38, 73	—
2,2-dichlorovinyl dimethyl phosphate (dichlorvos)	54	64
bisdimethyl phosphorodiamidic fluoride	87	99
<i>O</i> -(4-bromo-2,5-dichlorophenyl) <i>O,O</i> -dimethyl phosphorothioate	97	100
ethyl <i>p</i> -nitrophenyl 4-phenylbutyl phosphonate	62	75
diisopropyl <i>p</i> -nitrophenyl phosphate	—	99
dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate (trichlorfon) ^c	—	32
<i>S,S,S</i> -tributyl phosphorotri-thioate ^c	—	65

* For dosing schedules, see Johnson (1969b, 1969c).

^a The compounds are either non-neurotoxic or were administered in non-neurotoxic doses.

^b The controls were (mean \pm S.D.) 37 ± 8 pmol of P per gram (wet weight) of brain or 72 ± 9 nmol of phenol/min/g.

^c Non-neurotoxic dose. Higher or repeated doses lead to delayed neurotoxicity.

therefore examined for their influence on the rate of phosphorylation of the "neurotoxic protein". Only phenyl phenylacetate significantly diminished the rate (Johnson, 1969c), and the "neurotoxic protein" was subsequently shown to hydrolyse this ester. Highly active brain enzymes hydrolysing this ester had previously been identified (Poulsen & Aldridge, 1964) but they were highly sensitive to some non-neurotoxic inhibitors (paraoxon and TEPP) and were shown not to be associated with delayed neurotoxicity (Aldridge & Barnes, 1966b). The presence of the esterase activity of the "neurotoxic protein" can therefore be detected only after selective inhibi-

Table 2

The "phosphorylation site", "neurotoxic esterase", and neurotoxicity: neurotoxic compounds *

Compound	Phosphorylation site available (% of control)	Esterase activity (% of control)
tri- <i>o</i> -ethylphenyl phosphate ^a	13	—
bis(2-chloroethyl) <i>p</i> -nitrophenyl phosphate	27	8
<i>N,N'</i> -diisopropyl phosphorodiamidic fluoride (mipafox)	8, 11	0
<i>N,N'</i> -dibutyl phosphorodiamidic fluoride	8	—
diisopropyl phosphorofluoridate (DFP)	8, 8	5, 10
tri- <i>o</i> -tolyl phosphate (TOCP)	8	—
diphenyl <i>o</i> -tolyl phosphate	5, 13	7
<i>O</i> -(4-bromo-2,5-dichlorophenyl) <i>O</i> -isopropyl methylphosphonothioate	15, 11	—
cyclic methylene- <i>o</i> -phenylene phenyl phosphate ^b	5-24	0
ethyl <i>p</i> -nitrophenyl 2-phenethylphosphonate	35	14
<i>S,S,S</i> -tributyl phosphorotri-thioate ^a	—	23
<i>O</i> -(4-bromo-2,5-dichlorophenyl) <i>O</i> -methyl ethylphosphonothioate ^a	11	14
dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate (trichlorfon) ^a	—	32
2,2-dichlorovinyl diethyl phosphate	—	26

* For dosing schedules and description of controls, see the footnotes to Table 1.

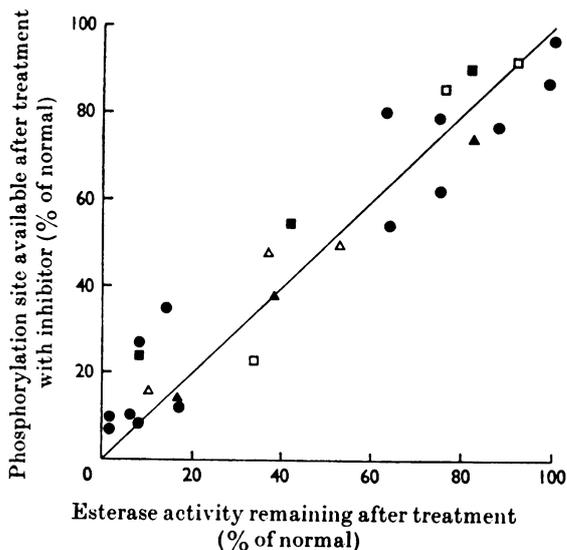
^a Results observed 17-24 h after the last of repeated doses.

^b "Phenyl saligenin phosphate".

tion of similar but irrelevant enzymes. This esterase activity was shown to be inhibited *in vivo* by neurotoxic compounds and not inhibited by non-neurotoxic compounds (Johnson, 1969c; Tables 1 and 2). There is also an excellent correlation between inhibition by a wide variety of compounds both *in vivo* and *in vitro* of the "phosphorylation site" and the esterase activity (see the accompanying figure).

A question of obvious practical and theoretical importance that arose is whether other acylating inhibitors such as carbamates are neurotoxic. Carbamates that inhibit this "neurotoxic esterase" have been found (Johnson & Lauwerys, 1969), but the

Effects of different compounds on the "neurotoxic site" and the "neurotoxic esterase" *in vivo* and *in vitro*



inhibited enzyme is unstable and activity returns fairly quickly. However, even repeated administration of these carbamates does not produce delayed neurotoxicity (Johnson, 1970). Esters of sulfur-containing acids produce a stable sulfonylated enzyme and they are not neurotoxic (Johnson, 1970). These findings might be thought to indicate, despite all the positive correlations, that this protein and its esterase activity are irrelevant to neurotoxicity. This view is incorrect because Johnson also showed that while the protein remained sulfonylated *in vivo*, administration of DFP † did not cause any delayed neurotoxic effect. Inhibitory carbamates were similarly protective (Johnson & Lauwerys, 1969; Johnson, 1970). Therefore the genesis of the lesion depends on the nature of the acyl group covalently bound to the esterase active site: it does not depend simply on inhibition of the esterase.

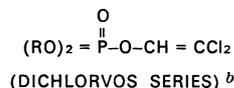
It now becomes legitimate to ask whether all organophosphorus compounds that react with the "phosphorylation site" or that inhibit the neurotoxic esterase produce delayed neurotoxicity. Recent work on a series of analogues of dichlorvos¹ has produced some highly active compounds (Table 3). However, all attempts to produce the lesion with

Table 3

Effect of varying alkyl substituents on activity of neurotoxic organophosphorus compounds

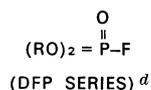
R substituent	Neurotoxic dose <i>in vivo</i> (mg/kg)	Neurotoxic esterase I ₅₀ ^a (M)
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GENERAL FORMULA



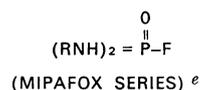
methyl	c	~ 6 × 10 ⁻⁵
ethyl	18	~ 3 × 10 ⁻⁶
isopropyl	1.5	2.5 × 10 ⁻⁸
pentyl	1.5	3 × 10 ⁻⁹

GENERAL FORMULA



methyl	30	—
ethyl	0.75	—
propyl	0.25	—
isopropyl	0.3	7 × 10 ⁻⁷
butyl	0.5	—
pentyl	2.5	—

GENERAL FORMULA



methyl	15	—
ethyl	3	—
propyl	0.25	—
isopropyl	25	5 × 10 ⁻⁶
butyl	0.1	< 10 ⁻⁷
isobutyl	1	—
pentyl	2.5	—

^a Activity determined *in vitro* after incubation for 20 min at 37°C.

^b The definition of neurotoxicity for this series is more severe than the criteria used by Davies, Holland & Rumens (1960, 1966).

^c Not neurotoxic at a dose of 100 mg/kg (more than 80% of the neurotoxic esterase activity was inhibited at this dosage).

^d Toxicity data taken from Davies, Holland & Rumens (1960).

^e Toxicity data taken from Davies, Holland & Rumens (1966).

¹ Supplied by Shell Research Ltd., Sittingbourne, England.

dichlorvos have failed, even though 70% or more of the esterase is inhibited *in vivo*. All other compounds in this series are neurotoxic. Thus, it would appear that dimethylphosphorylation of this esterase does not lead to delayed neurotoxicity. However, dimethyl phosphorofluoridate has been reported to produce the lesion (Davies, Holland & Rumens, 1960), but only one chicken was used.

Neurotoxicity tests on a considerable number of other dimethyl phosphates and methyl methylphosphonates have all given negative results. Measurements in our laboratory have shown the neurotoxic esterase to be markedly resistant to most of the methyl compounds available. Table 3 shows a marked increase of neurotoxic activity with an increase in substituent chain-length in three homologous series. It seems possible to generalize, therefore, that the methyl compounds may present fewer neurotoxic hazards than higher homologues. Neurotoxic methyl

methylphosphonothioates are known (Aldridge & Barnes, 1966a), but these compounds may well need metabolic conversion to an active agent, and the nature of the acylating group is not known. However, the methyl homologue in the mipafox series (Table 3) is reported to be neurotoxic and in this instance there is little doubt of the identity of the acylating group.

The explanation of these findings in terms of function of the central nervous system evades us. The "neurotoxic protein" is located on membranous material. We do not know where this membrane is, or how it is involved in the maintenance of long axons. Why there should be an absolute difference between a sulfonylated and a phosphorylated protein is a current mystery to be solved. Dimethyl phosphates deserve more attention to establish whether the non-neurotoxicity of dichlorvos is typical of this class of compounds.

REFERENCES

- Aldridge, W. N. & Barnes, J. M. (1966a) *Biochem. Pharmacol.*, **15**, 541
 Aldridge, W. N. & Barnes, J. M. (1966b) *Biochem. Pharmacol.*, **15**, 549
 Aldridge, W. N., Barnes, J. M. & Johnson, M. K. (1969) *Ann. N. Y. Acad. Sci.*, **160**, 314
 Cavanagh, J. B. (1969) *Brit. Med. Bull.*, **25**, 268
 Davies, D. R., Holland, P. & Rumens, M. J. (1960) *Brit. J. Pharmacol.*, **15**, 271
 Davies, D. R., Holland, P. & Rumens, M. J. (1966) *Biochem. Pharmacol.*, **15**, 1783
 Durham, W. F., Gaines, T. B. & Hayes, W. J. (1956) *Arch. industr. Hlth*, **13**, 326
 Gaines, T. B. (1969) *Toxicol. appl. Pharmacol.*, **14**, 515
 Johnson, M. K. (1969a) *Brit. Med. Bull.*, **25**, 231
 Johnson, M. K. (1969b) *Biochem. J.*, **111**, 487
 Johnson, M. K. (1969c) *Biochem. J.*, **114**, 711
 Johnson, M. K. (1970) *Biochem. J.*, **120**, 523
 Johnson, M. K. & Barnes, J. M. (1970) *Biochem. Pharmacol.*, **19**, 3045
 Johnson, M. K. & Lauwerys, R. (1969) *Nature (Lond.)*, **222**, 1066
 Poulsen, E. & Aldridge, W. N. (1964) *Biochem. J.*, **90**, 182
 Smith, M. I. & Elvore, E. (1930) *Prelim. Rep. Publ. Hlth Rep. (Wash.)*, **45**, 1703

DISCUSSION

NAMBA: Why was the brain used for this study? In persistent neuropathy caused by organophosphorus compounds in man, signs of lower motor neurone lesions predominate, and there is no sign of effects on the central nervous system, except for upper motor neurone signs that occur in some patients in very late stages.

ALDRIDGE: Initially, the brain was used for convenience, but control experiments have shown that effects on the spinal cord are similar. We consider that the biochemical reaction occurs throughout the central nervous system, but that in some way the long axons are vulnerable.

AUGUSTINSSON: What is the background for using phenyl phenylacetate as a substrate for the "neurotoxic esterase"?

ALDRIDGE: This substance was used on the basis of an inhibitor-substrate analogue. Since tri-*o*-tolyl phosphate is converted to *o*-tolyl saligenin phosphate [cyclic methylene-*o*-phenylene-*o*-tolyl phosphate], it was deduced that an enzyme that was inhibited by this compound should hydrolyse structurally similar substrates such as phenyl phenylacetate and phenyl 3-phenylpropionate. This was found to be true (Poulsen & Aldridge, 1964). These substrates are hydrolysed by chicken brain as rapidly as is acetylcholine, but are not hydrolysed by the cholinesterases. Two enzymes are responsible for most of the hydrolysis; the activity that is relevant to delayed neurotoxicity forms only about 3% of the total hydrolysis of phenyl phenylacetate.

HOLMSTEDT: Would you elaborate on the experiments in which one carbamate was administered before a neurotoxic organophosphorus compound. Were single doses used? What was the relationship between the times of administration of the two compounds?

ALDRIDGE: In Dr Johnson's experiments *in vivo*, the dose of DFP † was one producing delayed neurotoxicity in all control animals. The single dose of the carbamate or of the sulfonyl fluoride was one that produced 90% inhibition of the esterase activity. In one series of

chickens, the degree of inhibition of the esterase was determined. Each of a similarly-dosed series of chickens was treated with one dose of DFP. While the esterase was inhibited by 50% or more, a dose of DFP did not produce delayed neurotoxicity; this was true when the chickens had been pretreated with either the phenyl benzyl carbamate or the benzyl sulfonyl fluoride. When esterase activity was 70% or more of the control value, the chickens were only partially protected or quite unprotected against a dose of DFP that would be neurotoxic in control birds.