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NEUROTOXIC AND BIOCHEMICAL PROPERTIES OF SOME TRIARYL PHOSPHATES

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Abstract—A number of tri(alkyl-substituted phenyl) phosphates have been examined for neurotoxicity to chickens, and for inhibition *in vitro* and *in vivo* of pseudocholinesterase and esterase. No correlation was found between chemical structure and neurotoxicity. Inhibition *in vivo* of chicken plasma cholinesterase was caused by many of these compounds. It was possible to predict the degree of inhibition produced by the unsymmetrical esters from the results obtained with symmetrical tri-esters. Tri-4-methyl- and tri-4-ethylphenyl-phosphates were potent inhibitors *in vitro* of liver and intestinal phenyl butyrate esterase while the corresponding tri-2-methyl and tri-2-ethyl analogues were not. *In vivo* all four compounds caused inhibition of this esterase activity.

INTRODUCTION

OVER the last 60 years many cases of poisoning involving damage to the nervous system have occurred in men and women who have ingested tricresylphosphates, under a variety of circumstances. Smith *et al.*[1] first showed that tri-2-methylphenylphosphate (tri-*o*-cresylphosphate, tri-*o*-tolylphosphate, TOCP) was the toxic agent in these mixtures and demonstrated the value of the hen as a sensitive species for testing the neurotoxicity of these materials.

Recently, Hine *et al.*[2] and Henschler[3, 4] and Henschler and Bayer[5] have examined more of the analogues of TOCP including the tri-dimethylphenylphosphates (tri-xylenylphosphates). Bondy *et al.*[6], in a quest for a non-toxic tri-aryl phosphate plasticizer that could be produced commercially, examined a number of compounds that were present as impurities in commercial tolyl and xylenyl phosphate mixtures.

The work reported in this paper was carried out largely on the compounds prepared and tested by these workers. The observations on neurotoxicity have been supplemented by some studies on the biochemical activity of these compounds. The implications and the findings will be discussed but no attempt will be made in this paper to review the whole problem of the neurotoxicity of the organophosphorus compounds (see Barnes and Denz[7], Davies *et al.*[8]).

MATERIALS AND METHODS

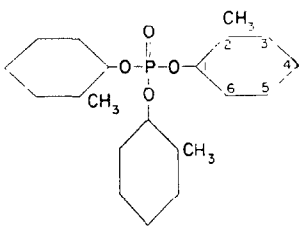
Hens

Young chicks are insensitive to the neurotoxic action of organo-phosphorus compounds (Barnes and Denz[7], Bondy *et al.*[6]), so that hens at least 4 months old and usually 6–12 months of age were used. Rhode Island Red, Rhode Island Red × Light Sussex and White Leghorns were obtained from commercial breeders. In addition F_1

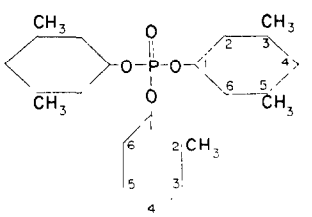
in-cross hens from two pure lines bred at the A.R.C. Station, Reeseheath, were used. These birds, when the same age, showed a smaller range of individual variation in their plasma cholinesterase levels than other birds even from a closed flock (Table 1). The pure line birds also showed a gradual fall of blood cholinesterase activity with age.

TABLE 1. THE PLASMA CHOLINESTERASE ACTIVITY (μL CO_2/MIN PER ML PLASMA AT 37 °C ACH SUBSTRATE) IN HENS FROM DIFFERENT SOURCES AND AT DIFFERENT AGES

Source	Age	No.	Plasma cholinesterase	Coefficient of variation (%)
1. Commercial birds	Adult	62	16·5 ± 4·81	29·2
2. Closed flock	Adult	6	18·3 ± 4·47	24·4
3. F_1 incross hens (A.R.C. Station, Reeseheath)	126–136 days	13	21·3 ± 2·42	11·3
	143–154 days	13	20·3 ± 1·53	7·5
	156–161 days	10	18·4 ± 2·18	11·8
	180 days	12	17·5 ± 1·59	9·1
	200–210 days	12	13·9 ± 1·45	10·4
	250–280 days	10	16·1 ± 1·85	11·5
Total	4–8 months	70	18·02 ± 3·12	17·2



Tri 2-methylphenylphosphate



Mono 2-methylphenyl, di 3:5-dimethylphenylphosphate

FIG. 1. Examples of structural formulae of a symmetrical and unsymmetrical tri(alkyl substituted phenyl) phosphate.

Compounds

The compounds studied in this paper are either symmetrical tri(alkyl-substituted phenyl)phosphates or phosphate esters containing two identical phenyl residues (B) and one other (A). The elemental analysis and physical characteristics of these compounds have been reported by Bondy *et al.*[6] Since no methods are available for the determination of the molecules as a whole the possibility of impurities must be deduced

from the methods of synthesis. The symmetrical tri(substituted phenyl)phosphates were prepared from the highly purified phenols by reaction with $POCl_3$ followed by isolation and fractionation by distillation or crystallization. For the preparation of the mixed esters (mono-A-, di-B-phosphates) the general method was to react phenol-B with $POCl_3$, fractionate the product to obtain di-B-phosphoryl chloride and then react this with the Na salt of phenol-A. The product was then finally purified, usually by distillation. The purity of the final product depends upon the degree of fractionation obtained at the various stages. The following example (Bondy, unpublished) gives the boiling points of the various intermediates which might be formed by reaction of 2-ethylphenol with $POCl_3$. It is clear that $POCl_3$ will not be present after

TABLE 2. BOILING POINTS OF POSSIBLE PRODUCTS OF THE REACTION OF 2-ETHYLPHENOL WITH $POCl_3$

Compound	B.P. (°C)	Pressure (mm Hg)
$POCl_3$	107·2	760
Mono-2-ethylphenyl phosphoryldichloride	approx. 80	0·5
Di-2-ethylphenyl phosphorylchloride	approx. 165	0·5
Tri-2-ethylphenylphosphate	221–223	1·0

fractionation of di-B-phosphoryl chloride and therefore the final product will not contain tri-A-phosphate. The boiling points of the remaining intermediates are sufficiently far apart for fractionation to separate almost completely di-B-phosphoryl chloride from mono-B-phosphoryldichloride and tri-B-phosphate but traces might remain. The most likely impurities by this route of preparation of mono-A-, di-B-phosphate are therefore mono-B-, di-A-phosphate and tri-B-phosphate. When prepared by the alternative route through mono-A-phosphoryl dichloride (only mono-2-methylphenyl-, di-3:5-dimethylphenylphosphate and mono-2-ethylphenyl-, di-3:5-dimethylphenylphosphate were prepared by this route), a possible impurity is mono-B-, di-A-phosphate. Such assumptions are only valid if no rearrangement of the groups takes place during the preparation and final fractionation.

Many specimens of tri-2-methylphenylphosphate (TOCP) inhibit plasma cholinesterase *in vitro*.[9–11] This inhibition is not due to the parent compound but to an impurity.[12] All the compounds used in this paper have therefore been tested for inhibitory power *in vitro* against chicken plasma cholinesterase. Plasma (0·5 ml) was incubated for 15 min at 37 °C with 9×10^{-5} M compound (this concentration in water gives a faintly opalescent solution and is therefore saturated). The range of the control cholinesterase was 15·6–16·4 μl CO_2/min per ml plasma (5 per cent range). With two exceptions all the compounds gave less than 5 per cent inhibition. Tri-3-methylphenylphosphate gave 5 per cent and tri-2-methylphenylphosphate 6 per cent inhibition. It may be concluded that these compounds are substantially free from impurities inhibitory to chicken plasma cholinesterase.

Methods

All the compounds studied are virtually insoluble in water. After some trials with intraperitoneal injections it was decided to give them all by mouth. Solid esters were

dissolved either in arachis oil or, where this was not possible, in a mixture of tri-4-methylphenylphosphate and tri-3-methylphenylphosphate (20:80 or 40:60). Evidence of absorption after dosing by mouth was obtained:

(a) By estimating the total ether-soluble P in dried faeces collected for 36 hr after dosing. Results with a range of compounds indicated that at least 50–80 per cent of the dose was absorbed, or (less likely) broken down in the gut. The exact nature of the excreted P was not determined.

(b) By estimating the blood cholinesterase activity before and after a single dose. The fall which was seen with the majority of compounds studied was evidence of absorption.

Tri-2-methylphenylphosphate was shown to be equally effective in producing ataxia whether given as solutions in arachis oil or in the inert aryl phosphate solvent.

Determination of enzymes

The cholinesterase activity of heparinized plasma (0·5 ml) was determined by a manometric method using acetylcholine (0·0138 M) as substrate and in a medium (total volume 4 ml) containing NaCl (0·131 M), $MgCl_2$ (0·035 M) and $NaHCO_3$ (0·0312 M) and gassed with 5 per cent CO_2 in N_2 at 37 °C.[13, 14] Brain pseudo cholinesterase activity was calculated from the rates of hydrolysis of acetylcholine (0·0138 M) and *n*-butyryl choline (0·03 M). From the known ratios of the rates of hydrolysis of these substrates by true and pseudocholinesterase of chicken brain, the activity of each enzyme against either substrate may be calculated.[15, 16]

Esterase activity of chickens' liver and intestine was determined using phenylbutyrate (1·7 per cent, v/v) as substrate in the medium described above for cholinesterase determination but containing in addition 0·5 per cent (w/v) gelatin. Tissue was homogenized in the medium (without gelatin) to give a 10 per cent (wet w/v) homogenate. Esterase activities were determined on suitable dilution and were expressed as μl CO_2/min per mg protein. The protein content of the homogenates was determined by a modification[17] of the biuret method of Robinson and Hogden[18]. Protein was precipitated by adding the homogenate (1–2 ml), to approx. 10 ml alcohol–ether mixture (50:50 v/v). After precipitation and centrifugation the pellet was resuspended in alcohol–ether and recentrifuged. The pellet was finally dissolved in NaOH, the protein determined by the biuret method and the results expressed as mg of albumin.

For experiments *in vitro* the compounds were dissolved in dimethylformamide. Various dilutions (0·03 ml) were added to the enzyme (3 or 3·5 ml). The concentration of dimethylformamide during the incubation with inhibitor never exceeded 1 per cent (v/v). This concentration had no effect upon chicken plasma cholinesterase or liver and intestinal esterase. Since the inhibition of esterase is dependent upon the time of incubation, enzymes were always incubated at 37 °C for 15 min prior to the addition of substrate.

Plasma cholinesterase

Determinations were made before, 24 hr and 4 days after a single dose in the majority of instances. There was evidence of absorption within a few hours of a dose by mouth. In every case activity had returned to normal levels within 4 days of an oral dose. However, in four birds given a non-neurotoxic compound (tri-2-ethylphenylphosphate) by intraperitoneal injection, return of plasma cholinesterase activity

did not take place for many weeks, indicating that the compound must have been absorbed very slowly from this site.

RESULTS

1. *Neurotoxic action*

This was judged entirely by inspection of the birds and no pathological examination was carried out on animals used in these experiments. Birds were observed for at least 21 days after a single dose and evidence of ataxia sought. The earliest signs were usually some unsteadiness when standing after the birds had been made to walk. This might be difficult to judge at an early stage but in true poisoning the disability would

TABLE 3. THE NEUROTOXIC ACTION OF TRI(SUBSTITUTED)PHENYLPHOSPHATES GIVEN BY MOUTH TO ADULT HENS

Substituents	Dose (mg/kg)	Neurotoxicity
None	500	0/4
Tri-2-Me	200–250	4/6
	100	0/9
Mono-3-Me, Di-2-Me	250	2/2
	50	0/2
Mono-2-Me, Di-4-Me	250	2/2
	50	3/4
Mono-2-Me, Di-3:5-di-Me	1000	2/2
	500	0/2
Tri-3-Me	2000	0/2
Tri-4-Me	15 × 500	0/2
Tri-2-Et	1000	0/4
Mono-4-Me, Di-2-Et	500	2/2
Mono-2-Et, Di-4-Me	200	2/2
Mono-2-Et, Di-3:5-di-Me	1000	2/2
	500	1/2
Tri-3-Et	1000	0/1
Mono-4-Et, Di-3-Et	500	0/2
Tri-4-Et	1000	3/3
	200	1/1
Mono-4-Me, Di-4-Et	500	0/2
Mono-4-Et, Di-4-Me	5 × 500	0/2
Mono-4-Et, Di-3:5-di-Me	5 × 500	0/2
Tri-2-*n*-Pro	1000	0/2
Mono-4-Me, Di-2-*n*-Pro	4 × 500	0/2
Mono-2-*n*-Pro, Di-4-Et	1000	2/2
	200	1/2
Mono-2-*n*-Pro, Di-3:5-di-Me	1000	0/2
Tri-2-Cl	1000	0/2
Tri-2-Me-4-Et	500	0/2
Miscellaneous compounds		
Tetra-2-Me-phenylpyrophosphate	1000	0/2
Di-*n*-propoxy, Mono-2-Me-phenylphosphate	1000	0/2

invariably progress significantly within 2–3 days of its onset being first suspected. Ataxia never first appeared later than 14 days after a single dose.

In severe cases the bird became unable to stand within a few days of the onset of signs of poisoning. In milder cases ataxia would not progress to this stage but remain conspicuous for several weeks and then slowly improve. Weight loss was not consistently seen until the birds became very helpless. In Table 3 the compounds studied

have been listed together with the doses shown to be either toxic or non-toxic. In addition to these, the five symmetrical tri-dimethylphenylphosphate esters (2:3, 2:5, 2:6, 3:4, 3:5) were found to be non-toxic in doses of 500 mg/kg. As there seemed to be no overall correlation between chemical structure and neurotoxicity it seemed to be worthwhile looking for some correlation between biochemical properties and either chemical structure or neurotoxic activity.

2. *Cholinesterase inhibition and toxicity*

The hypothesis put forward by Earl and Thompson[11, 19] based on their study of tri-2-methylphenylphosphate (TOCP) was that the inhibition of pseudocholinesterase was probably related to the myelin damage seen in those birds poisoned with TOCP Davison[15] showed that for the alkyl phosphate esters as a whole no such correlation could be established since compounds that were not neurotoxic could depress brain pseudocholinesterase to the same degree and for as long as the neurotoxic compounds. We had found that the triaryl phosphate ester, tri-4-ethylphenylphosphate which was neurotoxic produced little depression of pseudocholinesterase whereas the non-neurotoxic ester tri-2:5-dimethylphenylphosphate inhibited pseudocholinesterase.

TABLE 4. A COMPARISON OF THE EFFECTS ON PLASMA AND BRAIN PSEUDO-ChE ACTIVITY AND THE NEUROTOXICITY OF THREE TRI(SUBSTITUTED)PHENYLPHOSPHATES GIVEN BY MOUTH TO TWO HENS

(Dose 300 mg/kg of solution in arachis oil.)

	Percentage normal activity				
Time after dosing	24 hr		14–21 days		Condition
Substituents	Blood	Brain	Blood	Brain	
Tri-2:5-dimethyl	15	55	115	70	Normal
	29	69	110	48	Normal
Tri-4-ethyl	92	88	117	86	Ataxic
	105	88	130	90	Ataxic
Tri-2-methyl	0	25	105	75	Ataxic
	0	23	140	57	Ataxic

The data with three compounds is given in Table 4. It is clear that there is no correlation among the triarylphosphates between the power to inhibit cholinesterase and the ability to produce neurotoxic damage.

3. *Cholinesterase inhibition and chemical structure*

Although tri-2-methylphenylphosphate is able to reduce blood and tissue cholinesterase activities to very low levels in the animal, it was shown earlier[12] that purified specimens have no such activity *in vitro*. All the compounds considered here are free of any anticholinesterase inhibitory activity *in vitro*. Many, but not all, produce cholinesterase inhibition in the whole animal. The data for symmetrical tri-arylphosphates are summarized in Table 5, and it has been possible to divide the compounds into three groups depending on the degree of inhibition produced by a comparable single oral dose. In every case this inhibition must have been produced by a metabolite and the change must involve one or more of the phenyl rings. No

suggestion about the nature of this change can be offered but the properties of the mixed esters show some interesting features. Where the symmetrical ester is active in the hen as an inhibitor of cholinesterase so also will any unsymmetrical ester show inhibitory activity provided it contains at least one aryl group of an active symmetrical tri-ester (Table 6).

TABLE 5. THE EFFECT OF SYMMETRICAL TRI(SUBSTITUTED)PHENYLPHOSPHATES UPON BLOOD CHOLINESTERASE OF HENS 24 HR AFTER A SINGLE ORAL DOSE

(Group A, compound producing <25 per cent inhibition; group B, compound producing 25–75 per cent inhibition; group C, compound producing >75 per cent inhibition. Figures in brackets indicate the number of chickens examined.)

Substituents	Dose (mg/kg)	Inhibition %
A. Tri-3-Me/tri-4-Me (60/40)	1000	8 (2)
Tri-2:6-di-Me	500	18 (2)
Tri-3:5-di-Me	500	24 (3)
Tri-3-Me	500	3 (1)
Tri-4-Me	1000–500	21 (3)
Tri-2-Cl	1000	4 (2)
B. None	500	60 (4)
Tri-3-Et	500–1000	69 (2)
Tri-2:3-di-Me	500	28 (2)
Tri-3:4-di-Me	500	37 (2)
Tri-2-Me-4-Et	500	38 (2)
C. Tri-2-Me	500	83 (7)
Tri-2-Et	200–1000	81 (4)
Tri-2-*n*-Pro	200–1000	76 (4)
Tri-2:5-di-Me	500	80 (4)

TABLE 6. THE EFFECT OF ASYMMETRICAL TRI(SUBSTITUTED)PHENYLPHOSPHATES ON CHICKEN BLOOD CHOLINESTERASE ACTIVITY 24 HR AFTER A SINGLE DOSE BY MOUTH

(Group (1) Combinations from group A (Table 5). Group (2) Combinations from groups A and B (Table 5). Group (3) Combinations from groups A and C (Table 5). Group (4) Combinations from groups B and C (Table 5). Figures in brackets indicate the number of chickens examined.)

Substituents	Dose (mg/kg)	Inhibition %
(1) Mono-4-Et, Di-3:5-di-Me	500	15 (2)
Mono-4-Et, Di-4-Me	500	15 (2)
Mono-4-Me, Di-4-Et	500	14 (2)
(2) Mono-4-Et, Di-3-Et	500	42 (2)
(3) Mono-2-Me, Di-4-Me	500	77 (2)
Mono-2-Me, Di-3:5-di-Me	1000	84 (2)
Mono-2-Et, Di-4-Me	500	91 (2)
Mono-2-*n*-Pro, Di-3:5-di-Me	500	84 (2)
Mono-2-*n*-Pro, Di-4-Et	1000	92 (4)
Mono-4-Me, Di-2-Et	200	86 (2)
Mono-2-Et, Di-3:5-di-Me	500	92 (2)
Mono-4-Me, Di-2-*n*-Pro	500	75 (2)
(4) Mono-3-Me, Di-2-Me	500	87 (2)

Esters with mono-4-alkyl substituted phenols are not inhibitory. On the other hand dimethylphenyl esters with or without 4-substitution showed similar (mild) inhibitory activity (Table 4). The most active tri-dimethylphenyl ester was that with the 2:5-substitution. Although there are some correlations between chemical structure, and cholinesterase inhibitory activity, the exact relationship remains uncertain because the inhibitory activity must depend upon metabolites of the original compounds and the structure of these is at present quite unknown.

4. *Esterase inhibition and neurotoxic action*

It has been known for some years that tri-2-methylphenylphosphate was a very active inhibitor of liver and gut esterases in the rat.[20] Recent work on the potentiation of the organophosphorus compound malathion (O:O-dimethyl-S-diethylsuccinylthiophosphonate) has confirmed this in the case of TOCP[21] and some other triaryl phosphates (Casida[22]; A. N. Worden, unpublished). It seemed therefore worth while trying to find out whether this ability to inhibit esterase could be linked with either chemical structure of these aryl phosphates or their neurotoxicity. For this purpose two pairs of compounds in which chemical structure and neurotoxicity were uncorrelated have been studied.

The behaviour of the four compounds against an esterase preparation *in vitro* are given in Table 7. The pair of 2-substituted phenyl phosphates are very much less

TABLE 7. THE INHIBITORY ACTION OF FOUR TRI(SUBSTITUTED)PHENYLPHOSPHATES AGAINST THE ESTERASE HYDROLYSING PHENYL BUTYRATE IN CHICKEN LIVER HOMOGENATE

(All inhibitors were incubated for 15 min at 37 °C prior to the addition of substrate. The mean total esterase activity was 528 μl CO_2/ml of 10 per cent homogenate/min with an A-esterase activity of 114, giving a B-esterase activity of 414. The concentration of inhibitor necessary to produce 50 per cent inhibition was determined graphically using results corrected for A-esterase activity. When a saturated solution of the inhibitor did not produce 50 per cent inhibition, the inhibition obtained is given in brackets. Incubation of chicken plasma cholinesterase for 15 min at 37 °C with 9×10^{-5} M of any of these inhibitors produced no more than 6 per cent inhibition.)

Substituents	Neurotoxicity	Concentration for 50% inhibition
Tri-2-Me	+	1×10^{-4} M (41%)
Tri-4-Me	—	8×10^{-8} M
Tri-2-Et	—	1×10^{-4} M (27%)
Tri-4-Et	+	3×10^{-7} M

active than the 4-substituted analogues but there is no correlation of this activity with neurotoxicity to hens.

When the esterase activity in hens is examined after the compounds have been given by mouth no differences are seen among the four compounds. These observations are set out in Table 8 where the effect on pseudocholinesterase is also noted 24 hr after the dose and neurotoxicity at 10 days. Again, no correlation between biochemical activity and neurotoxic action can be seen but it is interesting to note that the tri(4-substituted

phenyl) phosphates show a specific action against phenylbutyrate esterase that they do not have against plasma (pseudo) cholinesterase.

DISCUSSION

The initial purpose of this study was to confirm the findings of Bondy *et al.*[6] on the neurotoxicity or otherwise of a series of symmetrical and unsymmetrical tri(substituted phenyl) phosphates many of which had not been previously examined. We agree with

TABLE 8. THE ACTIVITY OF THE LIVER AND INTESTINAL ESTERASE HYDROLYSIS OF PHENYL BUTYRATE IN HENS 24 HR AND 10–15 DAYS AFTER GIVING A SINGLE DOSE OF FOUR TRI(SUBSTITUTED)PHENYLPHOSPHATES

(The effect on plasma cholinesterase and the neurotoxicity of each compound is also recorded for comparison. Dose: 500 mg/kg by mouth.)

		Esterase activity (μl CO_2/mg protein per hr)			
		2-Me	4-Me	2-Et	4-Et
Liver esterase	Control 25·5 ± 13·6 (7)				
	24 hr	1·5	1·5	2·7	1·2
	10 days	30·6	4·1	23·3	24·5
Intestinal esterase	Control 20·5 ± 7·4 (6)				
	24 hr	0·44	2·2	0·7	1·5
	10 days	17·0	12·9	22·1	23·3
Plasma ChE % inhibition	24 hr	78	5	81	21
Neurotoxicity	10 days	+	−	−	+

those authors in being unable to find an overall correlation between chemical structure and neurotoxicity. Among small groups of compounds some rules appear to emerge. Thus compounds containing one, two or three 2-methylphenyl residues are toxic. The findings of others with different compounds containing these groups confirm this generalization.[2, 4] However, other substituents affect the toxicity in so far as mono-2-methylphenyl, di-4-methylphenylphosphate is more toxic than mono-2-methylphenyl, di-3:5-dimethylphenylphosphate. This is the only limited generalization that can be made for the series of compounds studied here. For instance 2-methyl substitution alone does not confer neurotoxicity for tri-2:3-, tri-2:5- and tri-2:6-dimethylphenyl-phosphates are not toxic; it appears therefore that the presence of a 2-methylphenyl residue is the essential feature. No similar generalization may be made for the presence of 2-ethylphenyl or 2-*n*-propylphenyl residues and in addition the neurotoxicity of tri-4-ethylphenylphosphate[6] is completely unexpected.

Metabolism of these compounds may account for the lack of correlation between structure of the administered compounds and their neurotoxicity. Several years ago it was clearly shown that tri-2-methylphenylphosphate was not an inhibitor of pseudo-cholinesterase *in vitro* The inhibition found *in vivo* after its administration indicated that it must be a metabolite causing the inhibition and it was shown that liver slices *in vitro* can produce an inhibitor.[12] This indicated the possibility that the neurotoxicity might be caused by a metabolite instead of the parent compound. The present work does not allow us to decide whether a metabolite or the parent compound is

neurotoxic. However, the experiments so far carried out illustrate some of the difficulties in reaching even a tentative hypothesis.

A major difficulty is knowing whether any metabolic transformation affects one, two or three of the phenyl rings. For example, tri-2-methylphenylphosphate and tri-2-ethylphenylphosphate do not inhibit plasma cholinesterase *in vitro* and have only a low inhibitory activity against the esterase hydrolysing phenylbutyrate. After administration, both cholinesterase and the esterase are inhibited and therefore this inhibition must be due to a metabolite. Although the results in this paper show that only one 2-methylphenyl or 2-ethylphenyl residue is necessary for the production of a cholinesterase inhibitor, nevertheless we have no evidence that the inhibition of cholinesterase and esterase is caused by the same metabolite.

Tri-4-methylphenyl- and tri-4-ethylphenyl-phosphates do not inhibit plasma cholinesterase *in vitro* but are potent inhibitors of esterase. *In vivo* tri-4-methylphenylphosphate produces no inhibition of cholinesterase but caused almost complete inhibition of esterase (phenylbutyrate as substrate) in liver and intestine. These results are entirely consistent with the *in vitro* findings and it is not necessary to postulate metabolic conversion. Tri-4-ethylphenylphosphate likewise causes complete inhibition of esterase but only slight inhibition of plasma cholinesterase. Nevertheless this inhibition of cholinesterase although slight is more than is expected since it is completely inactive *in vitro*. If this is taken at its face value it must be concluded that tri-4-ethylphenylphosphate is also metabolized *in vivo*. However, the question of purity here is vital. Tri-2-ethylphenyl- or tri-3-ethylphenyl phosphates both cause inhibition of cholinesterase *in vivo*. Tri-2-ethylphenylphosphate is particularly active as are compounds with one or two 2-ethylphenyl residues. Consequently a small amount of 2-ethylphenol in the original 4-ethylphenol used for the synthesis would result in this inhibition. With tri-4-methylphenylphosphate impurities are not likely to be present. It is a solid (m.p. 76 °C) and may be readily purified by recrystallization. Tri-2-ethylphenylphosphate is a liquid (b.p. 221–223 °C at 1·0 mm Hg pressure, cf. Table 2). The question whether tri-4-ethylphenylphosphate is metabolized to an inhibitor of cholinesterase in the chicken is therefore unsettled.

In addition there is the further complication that we have no information of the intrinsic activity of the metabolites which are produced and therefore no idea of the amounts of the administered dose which have to be metabolized to produce the observed inhibitions. Therefore although it is clear that many of these triaryl phosphates are metabolized *in vitro*, it must be appreciated that the only criterion used here for metabolism is the demonstration of inhibition of cholinesterase or esterase. Compounds might well be produced which are inactive in this respect. It is therefore a completely open question whether the parent compound or a metabolite is causing the neurotoxicity. It might be thought that the lack of correlation between structure and neurotoxicity was suggestive that a metabolite was more likely to be the active substance. However, it could be that those compounds which produce neurotoxicity are those which are slowly metabolized.

The relation between cholinesterase inhibition and structure of the triaryl phosphate administered is more straightforward. All compounds which produce more than 75 per cent inhibition of cholinesterase after administration of 0·5 g/kg orally contain at least one phenyl residue with the only substituent in the ring in the 2-position. More than one substituent in the ring often lowers the inhibition obtained as is shown by

tri-2:6-dimethylphenyl- and tri-2:3-dimethylphenyl-phosphate. Further, if we have tri-A-phosphate producing more than 75 per cent inhibition and tri-B-phosphate producing less than 25 per cent inhibition (when A and B are alkyl substituted phenyl residues) then any combination of these residues in one molecule such as mono-A-, di-B- or mono-B-, di-A-phosphate will produce more than 75 per cent inhibition. This generalization has been found to hold for all the compounds studied in this paper. Since all are inactive *in vitro* as inhibitors of cholinesterase, they must be metabolized *in vivo*. This correlation must indicate that metabolism of the group associated with high inhibition (A groups) is not affected by the nature of the other phenyl residues (B groups).

Suggestive evidence that the metabolic change might occur at the alkyl group itself is that tri-2-chlorophenylphosphate does not cause any inhibition of plasma cholinesterase after oral administration of 1·0 ml/kg. However, tri-phenylphosphates produce considerable inhibition and this may mean that substitution in the phenyl ring can play a part in converting these compounds to inhibitors of cholinesterase. Myers *et al.*[23] suggested that *p*-hydroxylation was a likely step and this might explain why tri-3-methyl- and tri-4-methylphenyl-phosphates cannot be converted to inhibitors. However, our findings with the tri-dimethylphenylphosphates are not consistent with his hypothesis. The tri-2:6-dimethylphenylphosphates instead of being the most active is inactive while the 2:5-substituted analogue is the most active and the 3:4 is more active than the 2:6.

Recent work has revealed a further series of phosphorofluoridates which produce the same signs of neurotoxic damage as tri-2-methylphenylphosphate[7] though in doses of one-hundredth or less of those required for the triaryl phosphates. No hypothesis of the mechanism of this lesion can be seriously entertained unless it takes into account these facts. The only common link between these two groups is phosphorus, but of course many tri-esters of phosphoric acid are not neurotoxic. Even in a closed series, however, no link between the two groups of compounds has been found. For instance although di-*n*-propylphosphorofluoridate is neurotoxic at 0·25 mg/kg[7] and tri-2-methylphenylphosphate at 250 mg/kg, di-*n*-propoxy-mono-2-methylphenylphosphate is not neurotoxic in doses up to 1000 mg/kg.

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