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COMPARISON OF THE FUNCTIONAL EFFECTS OF DYFLOS, TRI-*o*-CRESYL PHOSPHATE AND TRI-*p*-ETHYLPHENYL PHOSPHATE IN CHICKENS

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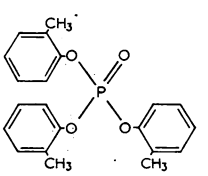
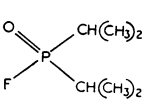
Tri-*p*-ethylphenyl phosphate is unique amongst the organophosphorus compounds which produce neurotoxic effects in not being an inhibitor of cholinesterase. The dysfunction it produces is also marked by some unusual features. Thus it produces a characteristic high-stepping gait which develops at varying periods after intramuscular injection but more regularly following oral administration. A careful comparison of the character, onset and development of the effects of diisopropyl phosphorofluoridate (dyflos), tri-*o*-cresyl phosphate and tri-*p*-ethylphenyl phosphate has illustrated the differences between the two former substances and tri-*p*-ethylphenyl phosphate. It has also confirmed a previous suggestion that this substance acts in a different manner from the other two, a suggestion supported by the histological evidence.

Despite marked differences in chemical structure certain triaryl phosphates, notably tri-*o*-cresyl phosphate (I), and some alkyl organophosphorus compounds, for example, dyflos (II), are known to produce closely similar neurotoxic effects in the hen (Barnes & Denz, 1953).



I



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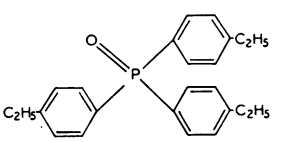

III

Recently it has been reported that tri-*p*-ethylphenyl phosphate (III) also possesses neurotoxic properties (Bondy, Field, Worden & Hughes, 1960), despite the fact that it is the only known triaryl phosphate with these properties which does not possess at least one phenyl group with a substituent in the ortho position. However, Silver (1960) has pointed out that the functional disturbance produced by this compound has some additional characteristics which further stress its anomalous position. In addition it has been claimed by Aldridge & Barnes (1961) that tri-*p*-ethylphenyl phosphate shows no significant anticholinesterase activity either *in vitro* or *in vivo*.

In an attempt to resolve the problems posed by these observations it was considered that a thorough comparison of the functional disturbances produced by tri-*o*-cresyl phosphate, dyflos and tri-*p*-ethylphenyl phosphate was necessary.

At the same time histological comparisons were made of the distribution of the lesions caused by these substances in the expectation that such differences might explain differences in functional effect.

METHODS

Tri-*o*-cresyl phosphate was obtained commercially from Kodak, but tri-*p*-ethylphenyl phosphate and dyflos were prepared at the Chemical Defence Experimental Establishment by Mr M. J. Rumens. Their purity, as judged by infra-red analysis, was not less than 95%. Tri-*o*-cresyl phosphate and tri-*p*-ethylphenyl phosphate were used undiluted, but dyflos was given in a 10% solution of ethanol in 0.9% sodium chloride solution. Adult hens (1 to 2 years of age), pure strain white leghorns, were used in all experiments. All agents were injected into the breast muscle 10 min after an intramuscular injection of 100 mg/kg of pralidoxime (2-hydroxyiminomethyl-*N*-methylpyridinium) methanesulphonate and 1 mg/kg atropine sulphate (Davies, Holland & Rumens, 1960).

In a separate series dyflos and tri-*p*-ethylphenyl phosphate were given orally to compare the influence of route of administration.

After the injections the birds were kept in cages for 6 days. They were then transferred to an open paddock and each was examined daily by at least two observers.

For the experiments in which the functional effects of these compounds were compared, the following rigid procedure was adopted. From the seventh day after injection, each bird was made to walk at varying rates and for varying times along a narrow earthed passage-way and the character of its gait assessed on the following scale: Slight but definite clumsiness or incoordination, 2 points; ataxia, implying a grosser degree of incoordination, but without serious incapacitation—"drunken gait," 4 points; marked ataxia with inability to maintain an upright stance for any length of time, a waddling or shuffling gait, the bird progressing on its hocks, 6 points; total inability to rise or walk, weak limb movements, 8 points.

If, particularly in the early stages, there was some doubt about the presence of incoordination, vigorous exercise generally served to resolve the difficulty. In the case of dubious signs the sign had to be at least suspected on three consecutive days before being regarded as real.

A high-stepping but often well-coordinated gait has been reported by Silver (1960) as characteristic of tri-*p*-ethylphenyl phosphate poisoning. It will be described in greater detail later. Its presence or absence has been recorded for all birds in the series.

Each experiment was continued until the functional condition of individual birds ceased to deteriorate. Selected birds were taken for histological examination and tissues were prepared as previously described (Cavanagh, 1954; Lancaster, 1960), using the Swank Davenport method for degenerating myelin and standard silver and dye methods for nervous tissues.

RESULTS

*The neurotoxic effects of intramuscular tri-*p*-ethylphenyl phosphate.* With doses of 500 to 1,000 mg/kg, ataxia and paralysis may occur at any time between 10 and 20 days, and in their manner of progression and final manifestations are virtually indistinguishable from those produced by dyflos or tri-*o*-cresyl phosphate.

The lowest dose causing disturbance of gait was 150 mg/kg. Above this and up to 500 mg/kg the onset of ataxia and weakness when they occurred was always delayed until 20 to 30 days and was nearly always preceded by a characteristic high-stepping gait. This began with an exaggerated but well-controlled upward

and forward movement of the leg which caused the foot frequently to brush the breast. The stride then continued with a rapid and powerful forward movement, the toes being fully extended as the movement progressed. Because the movement is a rapid one it can only be fully appreciated in a " slow-motion " film, but the general impression when actually observing the bird is of a long, graceful springy stride. In a few birds this type of gait disappeared only to reappear 5 to 10 days later, when it generally heralded ataxia during subsequent days (Table 1).

Occasionally with the onset of clumsiness a further striking but transient feature developed in which the high-stepping gait gave way to a stiff-legged stumbling run invariably ending after a few steps in the bird collapsing on its hocks. During this running phase the bird makes violent wing movements apparently to maintain its balance.

These features are no longer seen with the onset of ataxia and weakness, and the condition of the bird thus tends to merge into the state seen with higher doses. At threshold doses, individual birds not severely incapacitated (up to 4 points) may eventually show complete functional recovery.

The oral toxicity of tri-p-ethylphenyl phosphate. When tri-*p*-ethylphenyl phosphate was given orally all the characteristic features described above were seen. The onset and development of ataxia, as distinct from high-stepping gait, were, however, less variable and ultimately indistinguishable from those seen with tri-*o*-cresyl phosphate. High-stepping gait was always combined with some degree of ataxia and was never seen alone. Thus the controlled striding action seen after intramuscular dosing was replaced by a stiff-legged stumbling run that had also been seen occasionally in the injected birds during the transition period to the ataxic state.

With this method of dosing, ataxia developed after 8 to 10 days and its progress thereafter was rapid. The maximum effect was reached in a further 3 to 4 days, as is shown in Table 2.

Structural changes in the nervous system of hens poisoned with tri-p-ethylphenyl phosphate. Birds given 0.2 to 1.0 mg/kg either orally or intramuscularly were examined. In general, the overall distribution and pattern of neuronal damage were the same as those following dyflos or tri-*o*-cresyl phosphate (Cavanagh, 1954 ; Fenton, 1955). However, certain differences were recognized, but these were differences in emphasis rather than in the character of the lesions.

Thus the sciatic nerves were consistently and severely affected by tri-*p*-ethylphenyl phosphate. With high doses the degree of damage was indistinguishable from that due to tri-*o*-cresyl phosphate and dyflos. With lower doses the damage was more severe with tri-*p*-ethylphenyl phosphate than with low doses of either of the others. On the other hand, the ventral tract of the spinal cord was relatively little affected by tri-*p*-ethylphenyl phosphate. For instance, at the higher doses only about 12 degenerating and swollen axons were visible in a random section at the lumbar level, while at 0.5 mg/kg only one or two could be seen.

The damage to the spinocerebellar and ventral pathways was similar to that caused by tri-*o*-cresyl phosphate and dyflos, and no additional changes occurred in other tracts.

One difference between birds dosed orally and those dosed intramuscularly should be mentioned, since it confirms the conclusions discussed elsewhere concerning the effect of route of administration upon the manner of development of the damage. Orally dosed birds all showed changes that were in approximately the same stage of development at a given time, while intramuscularly injected birds, even a month later, showed axonal degeneration in an early stage side by side with more advanced changes such as gliosis. These findings imply a more gradual cumulative intoxication with the intramuscular route and are consistent with a slow release of toxic agent from a depot.

Comparison of the functional changes produced by intramuscular dyflos, tri-o-cresyl phosphate, and tri-p-ethylphenyl phosphate. To determine whether the features of tri-*p*-ethylphenyl phosphate poisoning described earlier were in fact unique to this substance, all three compounds were compared at various dose levels. These doses were chosen on the basis of previous experience so as to produce (*a*) severe, (*b*) moderate and (*c*) minimal effects. The birds were examined simultaneously. Table 1 shows that considerable variation in effect occurs both with the compound and with the dose.

TABLE 1

A COMPARISON OF THE INTRAMUSCULAR NEUROTOXICITY OF DYFLOS, TRI-*o*-CRESYL PHOSPHATE AND TRI-*p*-ETHYLPHENYL PHOSPHATE IN CHICKENS

Numbers indicate relative intensity of ataxia; – indicates absence of any signs of ataxia or paralysis; parentheses indicate the presence of high-stepping gait. All birds were given protective doses of oxime and atropine (see Methods)

	Dose mg/kg	Days after injection																	
		8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Dyflos	1·0	–	2	4	6	6	6	7	7	8	8								
			4	5	6	7	7	8											
	0·6	2	4		6														
		1	1	2	3	6	6	6	6	6	6								
	0·3				–	–	–	–	–	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
		–	–	–	2	–	2	2	2	–	2	1	3	2	3	3	3	2	2
		–	–	–	2	2	2	–	–	–	–	–	1	1	0	1	1	1	1
Tri-*o*-cresyl phosphate	400	–	–	–	2	4	6	8											
		–	–	–	2	4	6	8											
		–	–	(1)	(2)	4	4	4	5	5	5	7	7	7					
	150	–	–	–	–	–	–	–	2	2	2	3	4	6					
		–	–	–	–	1	1	1	3	4	5	5	6	5	6	6	6	6	
	75	–	–	(1)	–	4	5	6	6	7	7								
		–	–	–	–	(2)	(–)	(–)	(–)	(2)	(2)	2	3	4	5	5	5	6	
		–	–	–	–	–	–	–	–	3	4	4	5	6					
	50	–	–	–	–	–	–	–	1	1	1	1	–	2	1	–			
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			
		–	–	–	–	–	–	–	2	2	2	5							
	30	–	–	–	–	–	–	–	1	–	–	1	1	1					
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			
		–	–	–	–	–	–	–	–	–	2	2	–	2	1	1			
Tri-*p*-ethyl-phenyl phosphate	750	–	–	–	–	–	–	–	–	(–)	(–)	(–)	3	3	4	4	5	6	
		–	–	–	–	–	–	–	2	3	4	6	6	7					
		–	–	–	–	3	3	3	(4)	6	7								
	300	–	(–)	(–)	(–)	(–)	–	–	(–)	(–)	(–)	(–)	(1)	1	(–)	(–)	(2)	(2)	
		–	–	–	–	–	–	–	–	–	–	–	–	1	2	2	–	(1)	
		–	(–)	(–)	–	(–)	–	–	–	(–)	(–)	(–)	(–)	(2)	(3)	(4)	(4)	5	
	150	–	–	–	–	–	–	–	–	(–)	(–)	–	(–)	(1)	2	2	5	6	
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	(–)	(–)	(–)	(–)	(–)	(–)	(1)	(2)

With dyflos 6 to 8 points of dysfunction were obtained with doses of 1.0 and 0.6 mg/kg, but with 0.3 mg/kg only 2 out of 3 birds became ataxic, and these were but mildly affected. The third bird developed a high-stepping gait on the 16th day, which appeared to be very similar to, but not identical with, that seen in tri-*p*-ethyl-phenyl-phosphate-poisoned birds. In the latter, the high-stepping gait following intramuscular injection is characterized by a long, graceful springy stride. This is not so in the high-stepping gait following dyflos.

Tri-*o*-cresyl phosphate (400 mg/kg) produced severe effects in all birds (7 to 8 points). The severity of the effects was less at the lower doses (150 and 75 mg/kg). Three out of 16 birds (1 at 400 mg/kg and 2 at 75 mg/kg) showed a high-stepping gait, but, as with dyflos, the signs were not identical with those seen after tri-*p*-ethylphenyl phosphate.

The onset and rate of progress of neurotoxic signs were dose-dependent with all three compounds, although this was less obvious with dyflos. With this compound the delay in onset varied from 8 to 11 days, being longer delayed at the smallest dose tested. With tri-*o*-cresyl phosphate the first signs did not occur until 11 days after poisoning, and at threshold doses these were delayed for up to 17 days.

In contrast to this regular pattern the onset of signs in tri-*p*-ethylphenyl phosphate poisoning was most irregular. Thus even at 750 mg/kg only one bird showed signs of ataxia on the 12th day, whilst the remaining two showed no such signs until the 15th. At 300 to 150 mg/kg ataxia was not apparent until 19 to 24 days.

The route of administration influenced the neurotoxicity of these substances, as can be seen from Table 2. Thus dyflos was much less neurotoxic orally than intramuscularly. On the other hand, tri-*p*-ethylphenyl phosphate was more toxic orally and the effects came on earlier and were more consistent and more severe than when it was given by intramuscular injection.

TABLE 2

A COMPARISON OF THE ORAL NEUROTOXICITY OF DYFLOS AND TRI-*p*-ETHYL PHENYL PHOSPHATE IN CHICKENS

Numbers indicate relative intensity of ataxia; – indicates absence of any signs of ataxia or paralysis; parentheses indicate the presence of high-stepping gait. All birds were given protective doses of oxime and atropine (see Methods)

Compound	Dose mg/kg	Days after injection							
		7	8	9	10	11	12	13	14
Dyflos	0·6		–	–	–	–	–	–	–
		–	–	1	–	1	1	1	1
		–	–	1	–	–	–	–	–
	0·3	–	–	–	1	1	–	–	–
		–	–	–	–	–	–	–	–
		–	1	1	1	–	–	–	–
Tri-*p*-ethylphenyl phosphate	750		2	2	(5)	(5)	7	7	
		–	–	1		Died			
		–	(2)	(2)	(3)	(4)	(5)	7	
	300	–	–	(1)	4	4	5	6	
		–	–	1	(5)	(5)	7	7	
				1	4	5	5	6	

DISCUSSION

The observations reported here confirm those of Silver (1960) that the functional picture in tri-*p*-ethylphenyl phosphate poisoning shows characteristics which are not obvious in either dyflos or tri-*o*-cresyl phosphate poisoning. Thus the high-stepping gait which is such a feature after tri-*p*-ethylphenyl phosphate is seldom seen after the other two, although with them a similar, though not identical, sign is observed in occasional birds.

One of the most constant features in dyflos or tri-*o*-cresyl phosphate neurotoxicity is the interval to onset. With dyflos it is very regular and even at threshold doses only slightly prolonged. The delay period with tri-*o*-cresyl phosphate is rather more dose-dependent, but the pattern of onset is still quite marked. By contrast, the onset of ataxia in tri-*p*-ethylphenyl phosphate is very inconstant and markedly delayed.

These anomalous features of tri-*p*-ethylphenyl phosphate poisoning are influenced by the route of administration. Thus slow release from a depot following intramuscular injection has the effect of extending the time scale during which the train of symptoms develop. Oral dosing and increase of dose have, on the other hand, the effect of condensing the sequence of events and blurring the outlines of their components.

Even so, the characteristic high-stepping gait described above still dominates the picture whatever the route of administration. With the other two compounds, lowering the dose or giving it intramuscularly does not produce these characteristic signs, so that it does not seem unreasonable to agree with Silver (1960) that tri-*p*-ethylphenyl phosphate is not acting in the same manner on the nervous system as the other organophosphorus compounds.

The histological evidence gives further support to this conclusion. Reducing the dose of dyflos (Lancaster, 1960) and tri-*o*-cresyl phosphate (Cavanagh, unpublished results) reduces the extent of damage to the peripheral nerves to a greater degree that it does in the long tracts of the spinal cord. With tri-*p*-ethylphenyl phosphate the reverse would seem to hold, and furthermore there is a relative sparing of the ventral tracts. The effect is thus for this compound to produce a predominantly peripheral neuropathy at all dose levels.

The most compelling evidence in favour of the individuality of tri-*p*-ethylphenyl phosphate intoxication is its relative absence of anticholinesterase activity either *in vitro* or *in vivo* (Aldridge & Barnes, 1961). Up to the present time all the dialkyl phosphorofluoridates (Davies *et al.*, 1960) and all the triaryl phosphates (Hine, Dunlap, Rice, Coursey, Gross & Anderson, 1956) that produce the neurotoxic syndrome are anticholinesterases *in vivo*, although the converse does not hold.

This must therefore unequivocally set tri-*p*-ethylphenyl phosphate apart from the other neurotoxic organophosphorus compounds. Nevertheless the fact that in general the same neurone systems are attacked by tri-*p*-ethylphenyl phosphate as are damaged by the other organophosphorus compounds suggests that the final metabolic pathway disturbed by the former may well be the same as that disturbed by the latter. In view of its inertness towards cholinesterases, however, the intermediary

role of these enzymes as suggested for the alkyl phosphates by Davies *et al.* (1960) cannot be invoked. The interesting speculation thus suggests itself that the differing emphasis of the tri-*p*-ethylphenyl phosphate lesions may conceivably be an expression of its own as yet unknown intermediary metabolism. It is thus with much interest that further biochemical data about this substance are awaited.

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