Biochemical Pharmacology, 1966, Vol. 15, pp. 1783–1789. Pergamon Press Ltd., Printed in Great Britain.

THE DELAYED NEUROTOXICITY OF PHOSPHORODIAMIDIC FLUORIDES

D. R. DAVIES, P. HOLLAND and M. J. RUMENS

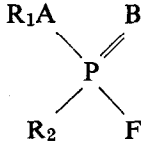
Ministry of Defence, Army Department, Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts

(*Received* 20 *April* 1966; *accepted* 30 *June* 1966)

Summary—Twelve phosphorodiamidic fluorides have been examined for neurotoxicity to hens. All were active. The character of the functional disorder and the distribution of the histological lesions were identical with those seen after poisoning by di-isopropylphosphorofluoridate or tri-*o*-cresylphosphate.

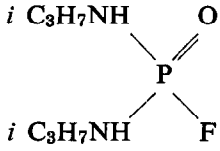
One of the substances examined is extremely neurotoxic. Intramuscularly, it produced severe effects at 50 μg/kg: percutaneously it is toxic at between 100–250 μg/kg and cumulatively 4–5 doses of 20 μg/kg (i.m.) spread over 21 days produce severe incapacitation in hens.

IT HAS been suggested that organophosphorus compounds of structure I would be neurotoxic.[1]



where R_1 is an alkyl group,
R_2 an alkyl, alkoxy or alkylamino group
A is oxygen or a secondary amino group
and B oxygen or sulphur.

The inclusion of an alkylamino or secondary amino group in the above depended solely upon the fact that II is known to be active in man[2] and chickens.[3]



(Mipafox)

II

As far as has been ascertained no other compounds of this type have even been tested for neurotoxicity. Twelve such substances have now been examined and all were active at very low doses.

MATERIALS AND METHODS

Animals

Chickens, white leghorns of both sexes, were used throughout and since age is

significant in the development of neurotoxic effects, only birds of 18 months or older were used.[2]

Toxic agents

These were prepared at C.D.E.E. by the following method. The appropriate primary amine in chloroform solution was added to phosphoryl chloride at −10°. A concentrated aqueous solution of potassium fluoride was then added and the reaction completed by raising the temperature to 40°. Amine hydrochloride was filtered off and the chloroform removed by distillation until the final product began to separate out. Low boiling point petroleum was then added and the mixture refluxed. On cooling the NN'dialkylphosphorodiamidic fluoride crystallized out.

All compounds were over 90 per cent pure as judged by nitrogen analyses.

Biological methods

For routine testing, the agents were given intramuscularly in isopropanol solution 10 min after the prophylactic intramuscular injection of 2-hydroxyiminomethyl-N-methylpyridinium methane sulphonate (P2S—100 mg/kg) and atropine sulphate (1 mg/kg).[1] In routine screening tests observations were carried out over 21 days, but in special circumstances the procedure was varied. These modifications are described in the appropriate place in the text.

The assessment of incapacitation was made as follows. From the seventh day after injection each bird was made to walk along a narrow earthed passage, for several minutes, and the character of its gait assessed as follows:

Slight, but definite clumsiness or incoordination	2 pts
Ataxia, implying a grosser degree of incoordination but without serious incapacitation—"drunken gait"	4 pts
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 pts
Total inability to rise or walk—weak leg movements	8 pts

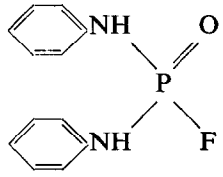
Observations were made by two observers independently and in the case of dubious signs the effect had to be confirmed on each of three consecutive days.

A number of birds were examined histologically for signs of demyelination and axon degeneration. The tissues were prepared as previously described,[4, 5] using the Swank Davenport method for demyelinating myelin and standard silver and dye methods for nervous tissues.

RESULTS

The results of screening 12 phosphorodiamidic fluorides are shown in Table 1. In general this class of compound exhibits the same order of neurotoxicity as the corresponding esters but there were two notable exceptions NN'-di-*n*-butylphosphorodiamidic fluoride produced 4 pts of incapacitation at 100 μg/kg and caused undoubted signs at 50 μg/kg. The other exception, NN'-di-iso-propylphosphorodiamidic fluoride was much less active and was fifty times less toxic than the corresponding oxygen analogue.

The substitution of an aryl group for an alkyl, as in III tends to reduce the neurotoxicity of the resulting compound, a tendency which is even stronger



III

when a methyl group is introduced into the ring. The position of the methyl group in the ring also appeared to be significant for the order of toxicity is:

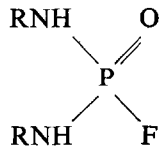
phenyl > *p*-cresyl > *o*-cresyl.

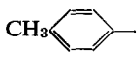
The neurotoxicity of NN'-di-*n-butylphosphorodiamidic fluoride*

The very high intramuscular neurotoxicity of this substance has prompted a more detailed examination of its toxic properties.

The intramuscular LD_{50} to chickens was approximately 2·0 mg/kg. This was thirty to forty times greater than the minimum dose required to cause histological nerve damage and incapacitation, viz. 50 μg/kg.

TABLE 1. THE NEUROTOXICITY OF PHOSPHORODIAMIDIC FLUORIDES OF TYPE:



R	Dose causing 4 pts incapacitation (mg/kg)	No. of birds examined	Range of doses examined (mg/kg)
CH_3	15·0	6	10–100
C_2H_5	3·0	6	0·75–3·0
C_3H_7	0·25	29	0·03–0·25
C_4H_9	0·10	36	0·03–100
C_5H_{11}	2·5	18	0·25–100
C_9H_{19}	100·0	6	25–100
iso C_3H_7	25·0	20	0·5 –100
iso C_4H_9	1·0		
	5·0	12	0·75–25
	10·0	15	1·0 –50
	20·0	6	10–50
	100·0	8	10–100

The agent was given intramuscularly 10 min after oxime and atropine.

The percutaneous toxicity of this substance was determined by placing discreet drops of an acetone solution, from an Agla syringe on the bare skin under the feathers and down on the left breast. Two birds were given 100 μg/kg and two, 250 μg/kg. At the lower dose neither bird showed any abnormal signs, but at the higher dose, both were severely incapacitated in 10 days.

TABLE 2. THE APPROXIMATE LD_{50} OF NN'-di-*n*-BUTYLPHOSPHORODIAMIDIC FLUORIDE

Dose (mg/kg)	Mortality
1·0	0/3
2·5	3/7
5·0	4/4

The effect of repeated small intramuscular doses was studied by the injection of 20 μg/kg every 3–4 days. Neither oxime nor atropine were administered. After 36 days when all birds had received a total of 180 μg/kg (or 9 doses), all were markedly affected (see Table 3). Unequivocal neurotoxic signs were apparent in all cases 27 days after the first dose and in two birds as soon as 21 days.

It is a characteristic feature of this type of poisoning that there is a latent period of 10–14 days between dosing and the onset of neurotoxic signs. It is a reasonable conclusion therefore that a bird which showed the first signs of incapacitation on the 27th day after receiving the first of a series of sub-toxic doses, had actually received a full neurotoxic dose by the 17th day or even earlier. At this time the total dose administered was only 120 μg/kg. The probable total neurotoxic dose administered has been calculated on this basis, the latent period being taken as 10 days. Thus it may be considered that five or six doses of 20 μg/kg given intramuscularly over a period of not more than 17 days will produce definite neurotoxic signs between 21–27 days after the first dose.

A separate experiment was carried out to test this suggestion, 5 × 20 μg/kg were given i.m. at 3-day intervals. Sixteen days after receiving the first dose two birds showed early signs of ataxia. All five birds in the group were affected 2 days later and three days later all showed unequivocal signs (see Table 4).

This confirms the implications of the previous experiment and might indicate that four doses of 20 μg/kg under these conditions are neurotoxic compared with a single dose of 50 μg/kg.

Histological changes. The histological changes produced by NN'-di-*n*-butylphosphorodiamidic fluoride are found in the long tracts of the spinal cord medulla and peripheral nerves. They consist of demyeclination in the nerve sheath with concomitant axon degeneration. They are similar both in character and in distribution to those seen after di-iso-propyl-phosphoro-phorofluoridate and tri-*o*-cresylphosphate.[2, 3]

Nervous tissue from birds was examined histologically; one after 100 μg/kg, one after 250 μg/kg and four after 70 μg/kg. The degree of incapacitation was severe in all six birds. Signs of Wallerian degeneration were found in the sciatic nerves of all birds and although the extent of the damage varied with the dose, even at 70 μg/kg quite a number of fibres were seen undergoing degeneration.

TABLE 3. THE CUMULATIVE EFFECT OF NN′-di-*n*-BUTYLPHOSPHORODIAMIDIC FLUORIDE

Bird No.	Days after commencement of dosing																							Remarks
	1*	4*	7*	9*	14*	17*	21*	22	23	24*	25	26	27*	28	29	30	31	32	33	34	35	36	37	38	
59												2	3	2	1	1	2			2	4	4	4	4	
60												4	1	4	3	2	1			4	3	5	4	4	
61							2	4	4	4	5	5	5	5	5	6	5			4	5	4	5	6	Dosing ceased on 21st day
62													7	2	2	2	3			1	3	4	4	6	
63							2	3	4	3	2	2	2	4	3	4	4								Dosing ceased on 21st day
64													3	2	2	2	2			1	3	3	3	2	

Nos. 1–8 indicate increasing severity of signs as indicated in the text.

* Days on which drug was administered (i.m., 20 μg/kg).

Damage was extensive in the spinal cord and consisted of degeneration of spinocerebellar tracts at the cervical and to a lesser extent at the thoracic levels. At all dose levels degeneration was seen in the ventral tracts, being evident at lumbar thoracic and cervical bulb level.

TABLE 4. THE EFFECT OF $5 \times 20\,\mu g/kg$ OF NN'-di-*n*-BUTYLPHOSPHORODIAMIDIC FLUORIDE GIVEN i.m. AT 3-DAY INTERVALS*

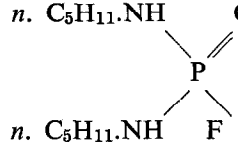
Bird No.	Days after commencement of dosing		
	16	18	21
287	1	2	4
288	1	2	4
289		1	4
290		1	4
291		2	3

* Drug administered at 3, 6, 9 and 12 days after first dose.

Degeneration in the brain occurred in the spinocerebellar tracts and at higher doses there were signs of hyaline degeneration of nerve cells in the deep cerebellar nucleii of the pons.

A typical behaviour of a single bird poisoned with NN'-di-*n-amylphosphorodiamidic fluoride*

The usual pattern of behaviour following poisoning by IV is similar to that after di-iso-propylphosphorofluoridate and tri-*o*-cresyl phosphate. One bird which received 2·5 mg/kg i.m., behaved atypically and exhibited signs



IV

which were closely reminiscent of those seen after poisoning with tri-*p*-ethylphenyl-phosphate.[3] The remaining birds in this group which exhibited signs and all other birds which received larger doses behaved as described above.

Fourteen days after poisoning, the atypical bird walked with a high stepping gait. The first signs consisted of an exaggerated but well controlled upward and forward movement of the leg and the bird walked "on tip toe". However, there were no signs of weakness. Four days later there was considerable improvement although signs of high stepping gait still remained. Improvement was not maintained and a week later the high stepping stiff legged gait was even more pronounced. At this stage weakness was apparent. Fourteen days later improvement again occurred and this time was progressive, until after a further 6 weeks, functional recovery was virtually complete.

DISCUSSION

The substitution of a secondary amino group for the ester oxygens of dialkylphosphorofluoridates does not, in general modify the neurotoxicity of the basic compounds.

There are two exceptions to this, but the present work provides no clue as to why this should be. Straight chain derivatives exhibit a peak of neurotoxicity at C_4, but the C_3 homologue is also very toxic. At C_5 there is a considerable reduction in toxicity, 25–50 fold, and this is even greater at C_9 which is 400 times less toxic than the C_5 compound. Branching of the chain results in a reduction of neurotoxicity relative to the straight chain compounds.

The high toxicity of NN'-di-*n*-butylphosphorodiamidic fluoride was unexpected. The effects of 70 μg/kg were, both functionally and histologically comparable with those produced by 1 mg/kg di-*iso*-propylphosphorofluoridate or 200 mg/kg tri-*o*-cresylphosphate. Very little loss of toxicity occurs when the compound is administered in divided doses, for as few as five doses of 20 μg/kg spread over 2 weeks can cause effects as severe as a single dose of 70 μg/kg.

Acknowledgement—Dr. J. B. Cavanagh, Institute of Neurology, Queen's Square, carried out the histological examinations and provided the report which is included in the text.

REFERENCES

1. D. R. Davies, P. Holland and M. J. Rumens, *Br. J. Pharmac.* **15**, 271 (1960).
2. P. L. Bidstrup and D. Hunter, *Br. med. J.* 277 (1952).
3. J. M. Barnes and F. A. Denz, *J. Path. Bact.* LXV, 597 (1953).
4. J. B. Cavanagh, *J. Neurol. Psychiat., Lond.* **17**, 163 (1954).
5. M. Lancaster, *Br. J. Pharmac.* **15**, 279–281 (1960).