Bull. Environ. Contam. Toxicol. (1984) 33:386–394

Bulletin of Environmental Contamination and Toxicology

Antidotes and Neuropathic Potential of Isofenphos

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Isofenphos (IFP, Amaze[4], Oftanol[4], O-ethyl-O-2-isopropoxycarbonyl phenyl isopropylphosphoramidothioate), the active organophosphate ester (OP) in a pesticide (Oftanol) registered by the EPA for home and garden use, was applied in the fall of 1983 to soil in a suburb of Sacramento, California to eradicate the Japanese Beetle. Questions as to the effectiveness of atropine and 2-pralidoxime (2-PAM), approved antidotes to OP poisoning, in treating acute exposure to IFP led to a multispecies study. This report of the first experiments on chickens and rats includes evidence that IFP produces organophosphate ester-induced delayed neuropathy (OPIDN) in the chicken.

MATERIALS AND METHODS

IFP (technical grade (92%), a gift to CDFA by Mobay Inc, Kansas City, MO) was administered in polyethylene glycol 400 (PEG-400, Sigma Chemicals). Atropine sulphate (Sigma Chemicals) and 2-PAM chloride (Aldrich Chemicals) were prepared in distilled water. Laying White Leghorn hens (Hyline, 1 1/2 years old) were given the OP by gavage after fasting and then treated therapeutically with the antidotes. These birds and 5, 6-month-old laying New Hampshire x White Leghorn crosses (developed by Dr. H. Abplanalp, UCD, Avian Sciences) were also treated prophylactically with atropine, given IFP s.c., and then treated for several days with atropine and 2-PAM administered either i.m. or s.c. Solutions of antidotes were prepared in H_2O and injected at maximum volumes of 1.0 ml (chicken) or 0.25 ml (rat). Birds were raised and cared for in the Department of Avian Sciences according to National Research Council standards. Male Sprague-Dawley rats, (220 to 240 grams, Simmonsen, Gilroy, CA) were housed in the campus Animal Resource Service facilities. Food was withdrawn the night before they were dosed by gavage. Protocols for animal care and sacrifice were submitted to and approved by the Campus Veterinarian.

[4]Amaze and Oftanol are TM's of Farbenfabriken Bayer GmbH, Leverkusen.

Table 1. Dose/Response to IFP by Hens

Dose mg/kg	Mortality 48 hours	Total Tested	Percent
7-20	6	6	100
5-6.75*	5	6	83
4.5	0	5	0
3.0	0	6	0
1.5	0	5	0
0	0	4	0

* 1/1 at 5.0; 4/5 at 6.75. IFP by gavage in 1 ml PEG to fasted White Leghorn hens. 5 experiments.

Table 2. Dose/Response to IFP by Rats

Dose mg/kg	Mortality 96 hours	Total Tested	Percent
80	4	4	100
55	5	5	100
50	4	4	100
45	3	5	60
40	10	18	56
35	1	5	20
30	0	5	0
25	0	5	0
20	0	5	0

IFP by gavage in 1 ml PEG to fasted animals. 4 experiments.

Homogenates of chicken brains were examined for inhibition of "neurotoxic esterase" (NTE) by a colorimetric assay (Ishikawa et al. 1983; Johnson 1977). After fixation by intracardiac perfusion with paraformaldehyde and glutaraldehyde, examination of nerves, spinal cord, brains and muscles of selected hens was performed on tissue embedded in paraffin or Epon 812-araldite (Mador et al. 1978), stained with hematoxylin-eosin (paraffin), Luxol fast blue and cresyl violet (paraffin), toluidine blue (TB, epon) and examined by light microscopy.

Motor activity of hens was scored according to: Stage 1, slight impairment; Stage 2, wobbles, trips, slips when walking or running; Stage 3, rests on tibio-tarsi, may scoot backwards instead of walking forwards; Stage 4, legs paralyzed, extended forward or sideways. Observers emphasized identifying impairments that accompanied the later stages of the disorder.

RESULTS AND DISCUSSION

The LD50 of IFP was between 3 and 5 mg/kg for White Leghorn hens (Table 1) and approximately 40 mg/kg for male rats (Table 2).

Table 3. Dose/Response to IFP by Hens After Antidotes

Dose mg/kg	Antidotes Total mg/kg	Mortality	Total Tested
100	85A 138P	3	5
75	75A 188P	1	5
50	75A 188P	2	5
25	30A 75P	0	5

A: atropine; P: 2-PAM. 20 mg/kg A, 50 mg/kg P 30 min. after IFP gavage; 1/2 doses at 7, 19, 30 hours; 1/4 doses at 43, 55, 67 hours for IFP>25 mg/kg. 2 experiments.

Table 4. Dose/Response to IFP by Rats After Antidotes

Dose mg/kg	Mortality 6 days	Total Tested
135	3	6
90	4	5
80	1	4
45	1	5
40	0	4

135, 90, 45 mg/kg IFP rats given 100 mg/kg A and 50 mg/kg P twice a day for 3 days. Last shot A only; 45 mg/kg received 1/2 dose on day one.

Symptoms were those expected of an OP, i.e., tremors, weakness, and gasping. Chickens exhibited extensive salivation; rats showed piloerection. Death did not always occur rapidly; some rats died up to 4 days after treatment.

In both animals, repeated doses of atropine and 2-PAM raised the levels of the lethal doses of IFP (chicken, Table 3; rat, Table 4). For example, in two experiments in which IFP was given by gavage to White Leghorn hens (Table 3), 4/5 hens survived 75 mg/kg of IFP (15 times the LD50) and 2/5 hens survived 100 mg/kg of the OP (20 times the LD50). Leg weakness and lethargy were two common symptoms of hens dosed with IFP. Some birds did not recover movement of their legs for several days. A few never completely recovered; e.g. one bird gavaged with 100 mg/kg died after 11 days.

Treatment of rats with atropine and 2-PAM for two and a half days following gavage of IFP raised the LD50 from 45 mg/kg to above 80 mg/kg (Table 4). We suspect the antidotes would have been even more effective if given for another day or two since more than half (5/9) of the mortality occurred after the antidote injections ceased.

Subcutaneous injection of IFP and prophylactic administration of atropine led to the survival of hens at 100 mg/kg and above. For

Table 5. Symptoms Of OPIDN In Hybrid Hens

Stage	Days To Most Extreme Stage
Two	36
Three	13 14 15 36
Four	13 16 17 17 20 20 28

Days for single hen to reach stage indicated.
Stages explained in text.

example, injection of 20 mg/kg atropine 30 minutes before and 50 mg/kg 2-PAM simultaneous with s.c. injection of IFP followed by periodic injections of the antidotes resulted in the survival of all (14/14) hybrid hens tested at 100 mg/kg. In addition, pronounced symptoms of OPIDN were observed under these conditions (Table 5). At first the hens were unable to stand; most regained use of their limbs within several days. Starting on days 10-14 many of the hens that had regained their mobility showed progressive symptoms of leg ataxia and paralysis. In an early stage, birds were able to stand in the cages, but sank down onto their hocks (tibio-tarsi) when placed on a flat surface. Often the animals were unable to walk forward, scooting backward whenever they tried to move (Stage 3). A few days later many birds became unable to stand in their cages; later, the legs of some became permanently extended forward or sideways (Stage 4). Wings remained functional; the animals were alert and ate and drank water when the cages were modified to make them available. (Some antidote injected hybrid birds exhibited mild behavioral signs of leg weakness; they stood and moved normally in the cages, but sat and moved little when placed on the floor.)

White Leghorn hens also showed the symptoms of OPIDN, especially at 150 mg/kg. For example, in one experiment after prophylactic dosing with atropine and 2-PAM, two of three birds at 100 mg/kg and three of three birds at 150 mg/kg IFP, s.c. survived past the first week. All of the birds at 150 mg/kg and one bird at 100 mg/kg were noticeably ataxic by 15 days; one bird at 150 mg/kg was dead, one was at Stage 4 and one was at Stage 3 at 25 days. Antidote injected controls exhibited no signs of leg weakness.

Strong evidence of the neuropathic potential of IFP was obtained when the brains of treated animals were examined for NTE activity. Inhibitions as great as 85% were found in the NTE activity of brains of hybrid birds exposed 3 days before to 100 mg/kg of IFP (Table 6). The NTE of injected controls was not affected. The inhibition of NTE of the Leghorns (data not shown) was 64% at 100 mg/kg one day after treatment, consistent with the decreased sensitivity to OPIDN of the birds. Inhibition of NTE in the brain of chickens treated with OP's has been shown to be a reliable indicator of potential neurotoxicity (Johnson, 1982). Loss of 70% or greater of NTE activity is usually followed by symptoms of the neuropathy several weeks later. Preliminary data

Table 6. NTE Levels of Hybrid Hens

Day	Treatment	Total Absorbance/min/g brain	NTE
1	None	32.3	6.31
	Control	36.8	6.35
	IFP	23.0	1.72
3	None	34.7	5.04
	Control	39.2	7.08
	IFP	24.5	1.05
7	None	48.3	6.53
	Control	42.4	7.47
	IFP	24.3	1.83
		26.4	1.89

None: uninjected. Control: injected with A,P, and PEG. IFP: 100 mg/kg s.c. Averages of 2 (IFP) or 3 (none, control) replicates. Total: hydrolysis of phenyl valerate. NTE: hydrolysis due to 50 uM mipafox after 40 uM paraoxon.

(not shown) indicated that neither technical grade IFP or analytical grade IFP-oxon inhibited chicken brain NTE in vitro, as if IFP was activated within the body to a neuropathic compound. (Although there is no evidence to suggest it, experiments are needed to formally exclude the possibility that a chemical other than IFP in the technical grade sample is the neuropathic agent or precursor, Hammond et al., 1982.)

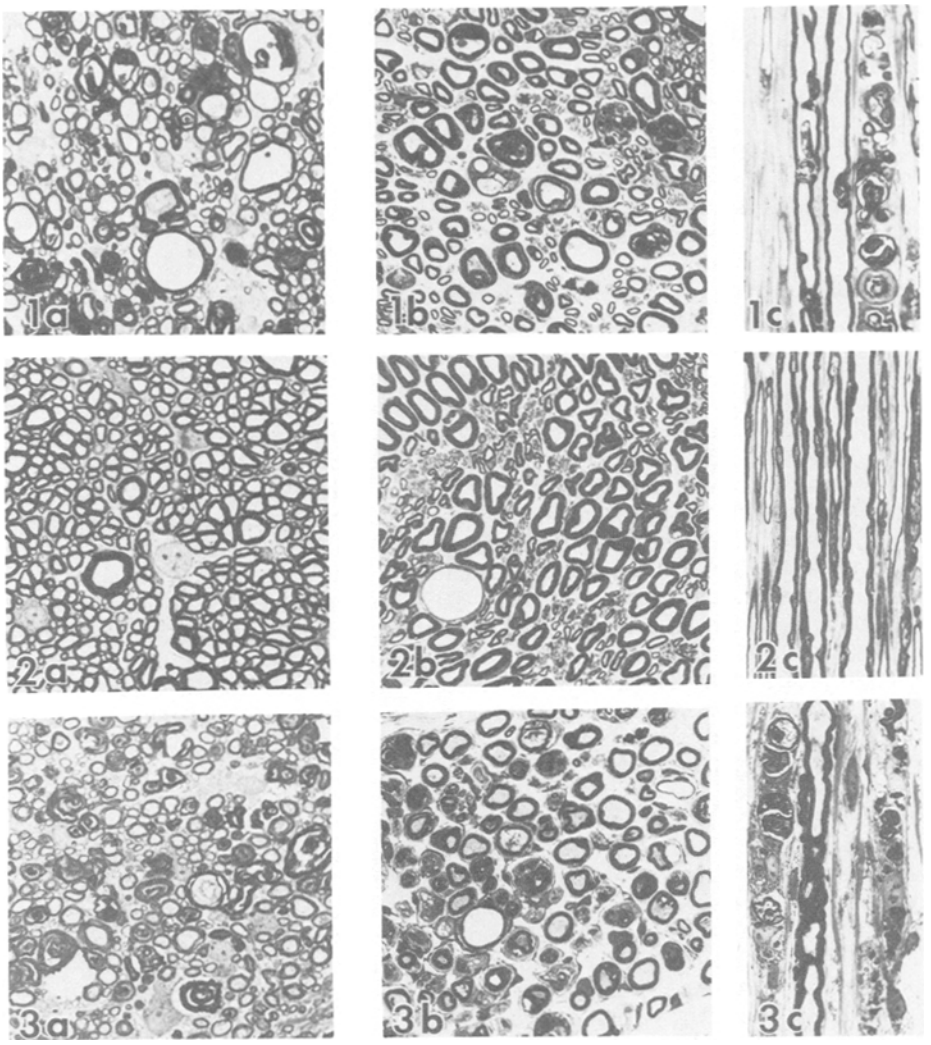
Histologic examination of the ischiatic nerve and its distal branches, the spinal cord and the brain of hybrid chickens confirmed the presence of nerve damage in two TOCP (200 mg/kg) and four IFP (100 mg/kg) exposed birds with Stage 4 symptoms of leg paralysis. The microscopic lesions were similar both in their nature and their bilaterally symmetrical topographical sites to those characteristic of OPIDN (Abou-Donia, 1982). Lesions in the IFP exposed birds consisted of axonal swelling, spheroid formation and axonal necrosis, together with degeneration and phagocytosis of the associated myelin sheath. Lesions were most severe in the lateral metatarsal nerve (LMN, Figs. 1b,1c), throughout the length of the spinal cord, particularly in ventral motor, gracilis (Fig. 1a), cuneate and spinocerebellar tracts with involvement of the latter into the medulla oblongata of the brain. Similar lesions were found in the TOCP exposed positive control birds (Figs. 3a,b,c). Incidence of lesions in the axons of the ischiatic nerve increased with their distance from the spinal cord; for example, there was approximately a 1% incidence of axonal damage in the ischiatic nerve and 40% involvement in the distal LMN branch. No significant lesions were found in either antidote injected (Figs. 2a,b,c) or untreated control

birds. (Some of the OP treated birds exhibited gross and microscopic lesions in upper leg muscles attributed to pressure-induced muscle necrosis associated with the prolonged recumbency of the paralyzed birds.)
These findings differ from the conclusions presented in the FAO Summary Report of confidential data on IFP (1982) in which it was said that neither atropine, 2-PAM or toxigonin "was effective as an antidote..." to rats in situations where atropine sulphate and 2-PAM were both given at 50 mg/kg, 45 to 120 minutes after single oral toxic doses of IFP. The FAO Summary also reported that the oral LD50 for IFP in the chicken was 21 mg/kg (four times greater than in our studies) and that prophylactic administration of atropine elevated the LD50 to 74 mg/kg (less than in our results). One probable reason for the differences in our work and the FAO Report is that we gave the animals repeated doses of the antidotes extending over several days.

The FAO summary also concluded that IFP was not neuropathic based on the finding that atropine-treated hens that survived 75 mg/kg of IFP did not develop OPIDN. The greater effectiveness of atropine plus 2-PAM over atropine alone as an antidote to acute poisoning by IFP enabled us to introduce doses greater than 75 mg/kg of IFP s.c. to the hens resulting in OPIDN in two strains of chickens. It is interesting that the hybrid hens were apparently more sensitive to IFP than the White Leghorn type hens so far as developing OPIDN was concerned. Cisson and Wilson (1982) found that another strain, the scaleless chicken, was less sensitive to TOCP than a New Hampshire line, probably because of differences in the rate at which TOCP was converted to its toxic metabolites in the body. The commercial White Leghorn type chickens used for OP testing are not a standard genetically homogeneous "strain"; each breeder develops and selectively breeds their own strains. Perhaps such genetic differences may explain the discrepancy in LD50 values between our findings and those of the manufacturer.

The levels of antidotes used in the study were established for the commercial White Leghorn hens; some signs of mild leg weakness were noted when the same dose regime was applied to the hybrid chickens. However, NTE was not inhibited; no lesions in excess of those in uninjected controls were found in the nerves of antidote-treated hybrid hens, and all the symptoms of OPIDN appeared in the White Leghorn birds, without leg weakness in the controls indicating that any symptoms of leg weakness shown by the hybrid controls were unrelated to those caused by the OP.

The findings presented here should alleviate some of the concerns about IFP and antidotes; rats and chickens were both protected by the antidotes licensed for use in the US. However, the protection given to rats was not great, and some animals died days after exposure. The response of the rats may be a "worst case" study; they are noted for not responding well to such agents (Fleisher et al., 1970).



Figures 1-3. Microphotographs of cross sections of cervical cord (a), cross (b) and longitudinal (c) sections of LMN from IFP (Fig. 1), antidote-treated (Fig.2) and TOCP-treated hens (Fig. 3). Note similar severe axonal swelling and necrosis with myelin degeneration and phagocytosis of individual myelinated axons in both TOCP and IFP samples. Tissues embedded in epoxy and TB stained. Magnification: 160X.

There is no general agreement on the use of neuropathic OPs. One, TOCP (a plasticizer) has been responsible for the paralysis of many thousands of people since the turn of the century. Others, like EPN have been used widely in places like cotton fields without either great public outcry or confirmed cases of paralysis in humans. Some toxicologists favor banning all neuropathic OP's, others advocate assessing their risks after determining no-effect-levels.

Why has IFP not been recognized as neuropathic by others considering the extensive testing required before an OP is licensed? Perhaps it was because EPA regulations do not require that OPs be tested at their highest protected levels. The fact that the neuropathy appeared only at high dose levels of IFP does not mean that there is little danger of contracting the disorder; nerve damage is known to occur with low level repeated exposures to neuropathic OPs. For example, Abou-Donia and Graham (1978,1979) found that although EPN caused OPIDN in hens at an acute single dose of 25 mg/kg, its no-effect-level for repeated exposure was between 0.1 and 0.01 mg/kg, more than 100 fold less. A study of the lowest dosage level of IFP that will cause the neuropathy after repeated doses is needed. (EPA regulations (1982) require that 90 day chronic trials be carried out on OPs known to cause OPIDN in order to establish reasonable no-effect-levels.)

There is neither cure nor approved antidote for OPIDN. Immediate treatment of experimental animals with carbamates (Johnson and Lauwerys, 1969), glucocorticoids (Baker et al., 1982) and other chemicals have been reported to block its occurrence. However, the insidious onset of OPIDN makes it difficult to imagine the effective use of such treatments in the clinic.

Acknowledgements. Supported by a contract from CDFA and NIH Grant ES 00202 to BWW. The authors acknowledge the assistance of C. Ackerman, J. Henderson and J. Sonoda, and the courteous advice of G. Rapmund and D. Hilmas, RAM V, US Army.

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Received January 31, 1984; Accepted February 10, 1984.