Stereospecificity in Toxicity of the Optical Isomers of EPN

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EPN(ethyl p-nitrophenyl phenylphosphonothionate) is a phosphonate-type insecticide useful to control many insect pests. It has rather high mammalian toxicity: the acute oral LD_{50} value for the rat is 14 to 42 mg/kg. It is known that EPN potentiates the toxicity of the insecticide malathion (ETO, 1974). Also, EPN produced paralysis of the legs in chickens(DURHAM et al., 1956; WITTER and GAINES, 1963; GAINES, 1969). However, the paralytic effect of EPN is different from that produced by TOCP(tri-o-cresyl phosphate), since the syndrome appears immediately after administration.

EPN contains an asymmetric phosphorus atom and optical isomers are therefore possible. Since the optical isomers of organophosphorus compounds often show differences in their biological activities, the EPN isomers were prepared and tested for toxicity to mice, hens and insects. Furthermore, racemic, (+)- and (-)-EPN were examined for delayed neurotoxicity to hens.

MATERIALS AND METHODS

Preparation of the Optical Isomers of EPN

The resolved isomers of O-ethyl phenylphosphonochloridothioate were prepared as reported previously(OHKAWA et al., 1976b): $[\alpha]_D^{20}$ -72.5°(c 3.4, $CHCl_3$) and $[\alpha]_D^{20}$ +71.3°(c 3.2, $CHCl_3$). An equimolar mixture of the (-)-chloride(382 mg) and p-nitrophenol(265 mg) in 2 ml of toluene was refluxed for 3 hours in the presence of two equivalents of anhydrous K_2CO_3 (450 mg). The reaction mixture was concentrated, and the obtained crude product was purified on a silicic acid column (20 x 2 cm, i.d., 100 mesh silicic acid, Mallinchrodt Chemical Works) by eluting with n-hexane-benzene(1/1, v/v). The yield of (+)-EPN was approximately 90%; $[\alpha]_D^{20}$ +29.7°(c 7.9, $CHCl_3$). (-)-EPN was prepared from the (+)-chloride in the same manner; $[\alpha]_D^{20}$ -27.7°(c 7.0, $CHCl_3$). Both isomers were identical in

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nuclear magnetic resonance spectra(nmr), infrared spectra(ir) and electron impact mass spectra(ms): nmr(CCl_4, int. TMS) δ 1.35(3H,t,CH_3), 4.25(2H,q,CH_2) and 7.00-8.30ppm(9H,m,aromatic); ir(direct) 1580 and 1490(aromatic ring), 1520 and 1350(NO_2), 1220(P-O-aryl) and 1030cm^{-1}(P-O-alkyl); ms m/e 323(M), 293(M-NO), 185, 169, 157(base peak) and 141. No impurities were detected by nmr and thin-layer chromatography(silica gel 60F-254 chromatoplates, E.Merck) in n-hexane-acetone(5/2, v/v).

Toxicity Testing

Insecticidal activity was determined on a susceptible strain of housefly(Musca domestica, WHO) and rice stem borer larvae(Chilo supressalis) according to the procedure reported previously(OHKAWA et al., 1976a). Each test chemical was dissolved in acetone and 0.4 µl of the solution was applied topically to 3- to 4-day old adult houseflies and 15- to 20-day old larvae of rice stem borer(4th instar, 30 to 40 mg of body weight). Two replications of 20 to 40 insects each were used at each dose level. Mortalities were determined 24 hours after treatment for houseflies or 48 hours for rice stem borers.

Toxicity was also determined on male dd strain mice weighing 18 to 20 g. Each chemical was dissolved in 10% Tween 80 solution and 0.4 ml of the solution was injected intraperitoneally(ip) to mice. Twenty or more mice were used in determining each LD_{50} value. Mortality was evaluated 24 hours after dosing.

White leghorn hens aged 12 to 18 months and 1.7 to 2.2 kg in weight were used for the present study. All test compounds were administered ip as a dimethyl sulfoxide solution. For each dose, 3 to 5 hens were used. The dose killing 50% of the hens within 24 hours is reported as LD_{50}. Furthermore, hens were dosed subcutaneously with atropine sulfate in saline at the rate of 20 mg/kg immediately, 6 and 24 hours after ip administration of the test compound. The hens were checked daily for signs of paralysis by placing them on the ground and observing their ability to walk for 4 weeks or until they died(KADOTA et al., 1975).

Histological Methods

After the observation period, some of the treated hens were sacrificed, and their spinal cords and sciatic nerves were dissected. The tissues were fixed for a minimum of 7 days in 10% formol-saline. The sciatic nerves and cervical,

thoracic and lumbar regions of the spinal cord were cut transversely, and cross sections and longitudinal sections were obtained. Tissues were dehydrated in ethanol and embedded in paraffin wax. Sections(5-10 μm) from all tissues were stained with hematoxylin and eosin, GREES silver solution (MARSLAND et al., 1954), and Luxol fast blue and cresyl violet. Tissues were also stained with SWANK-DAVENPORT's solution(SWANK and DAVENPORT, 1935).

RESULTS

Toxicity to Mice, Hens and Insects

The LD_{50} values for mice, houseflies and rice stem borer larvae are presented in Table 1. Racemic, (+)- and (-)-EPN were equally toxic to mice by the ip route. In contrast, the (+)-isomer was 3.9 fold more toxic to hens than the (-)-isomer. The toxicity of the racemic compound was close to that of the (+)-isomer. Also, the three forms of EPN showed a marked difference in insecticidal activity. The (+)-isomer was 2.9 fold and 4.0 fold more toxic to adult houseflies and rice stem borer larvae, respectively, than the (-)-isomer. The activity of the racemic compound was close to the mean of the two resolved isomers for rice stem borer, and close to that of the (+)-isomer for housefly. The data indicate that (+)-EPN is more toxic to hens, houseflies and rice stem borer larvae than the (-)-isomer, whereas both isomers are equally toxic to mice.

Table 1

Toxicity of Racemic, (+)- and (-)-EPN against Mice, Hens, Houseflies and Rice Stem Borer Larvae

compound	LD_{50}			
	mg/kg		μg/g	
	mouse (ip)	hen (ip)	housefly (topical)	rice stem borer (topical)
(+)-EPN	17	12	1.1	2.9
(-)-EPN	16	47	3.2	11.7
(±)-EPN	16	17	1.3	5.9

Table 2

The Delayed Neurotoxic Effect of Racemic, (+)- and (-)-EPN in Atropinized Hens

dose (mg/kg)	(+)-EPN			(-)-EPN			(±)-EPN		
	hens dosed, No.	death No.	delayed paralysis No.	hens dosed, No.	death No.	delayed paralysis No.	hens dosed, No.	death No.	delayed paralysis No.
31.2	3	0	0	3[a)]	0	0	5	0	0
40.6	3	0	0	3[a)]	1	2	5	0	0
52.8	3	0	0	3[a)]	2	1	5	1	1
68.6	3	0	0	3	0	3	3	0	1
89.2	3	2	0	3	0	3	3	1	2

a); Not atropinized.

Paralytic Effects of Racemic, (+)- and (-)-EPN in Hens

Racemic, (+)- and (-)-EPN were tested for paralytic effects in hens. The data are presented in Table 2. Three forms of EPN caused moderate acute cholinergic effects immediately after administration, and these signs disappeared completely within one week. Then, the hens dosed with racemic and (-)-EPN developed paralysis of the legs about 10 to 14 days after administration. This delayed paralysis appeared to be irreversible, and the sign persisted during the observation period. With (-)-EPN, the delayed paralysis was observed in all of the surviving hens dosed at 40.6, 52.8, 68.6 and 89.2 mg/kg. The lowest (-)-EPN dosage giving this sign was 40.6 mg/kg. Racemic EPN produced the paralysis in one of each of the 52.8 and 68.6 mg/kg groups, and in two of the 89.2 mg/kg group. The minimum dosage giving this effect was 52.8 mg/kg. No such paralytic effects were seen in the hens dosed with the (+)-isomer even at the highest level.

The (-)-EPN-poisoned hens showed degenerating myelin sheaths as well as swollen and fragmented axons both in the sciatic nerve and cervical, thoracic and lumbar regions of the spinal cord. These changes were not seen in the hens treated with (+)-EPN.

DISCUSSION

While racemic, (+)- and (-)-EPN were equally toxic to mice, the three forms of EPN showed a marked difference in insecticidal activity. (+)-EPN was more toxic to houseflies and rice stem borer larvae than the (-)-isomer. Also, differences of the three forms of EPN both in acute toxicity and delayed neurotoxicity in the hen were apparent. (-)-EPN was low toxic, but produced delayed paralysis in the hen, whereas (+)-EPN caused no paralytic effects. Therefore, (+)-EPN appears to be a more appropriate insecticide than the racemic compound, since it combines high toxicity to insects and no delayed paralytic effect in the hen.

It was reported that the paralysis produced in the hen with EPN differs from that noted with TOCP(DURHAM *et al.*, 1956; WITTER and GAINES, 1963; GAINES, 1969). The onset of ataxia usually appears 10 to 14 days after dosing with TOCP, whereas with EPN, the syndrome develops immediately after dosing. However, the present study demonstrated that the hens dosed with racemic and (-)-EPN develop paralysis after the other acute symptoms had disappeared. This delayed neurotoxic sign was clearly observed with (-)-EPN, since it produced slight acute toxic symptoms.

The EPN-poisoned hens were reported to show the pathological lesions in the sciatic nerve similar to those obtained with TOCP, although unlike TOCP, the spinal cord of the EPN-poisoned hens was normal(FRAWLEY, 1976). However, the present study revealed that both sciatic nerves and spinal cords were damaged in the hens dosed with (-)-EPN but not with (+)-EPN. These changes observed were virtually identical with those caused by TOCP.

Although the mechanism of delayed neurotoxicity by organophosphorus compounds is unknown, it has been suggested by JOHNSON(1975) that the delayed neurotoxic effect of some organophosphorus compounds is associated with phosphorylation of a nervous tissue protein capable of hydrolyzing phenyl valerate, which is called "neurotoxic esterase". It is of interest to examine whether or not the optical isomers of EPN show a difference in inhibition of neurotoxic esterase of hen brain *in vivo*.

SUMMARY

While the optical isomers of EPN were equally toxic to mice, (+)-EPN was 2.9 fold and 4.0 fold more toxic to houseflies and rice stem borer larvae, respectively, than the (-)-isomer. In addition, (-)-EPN produced paralysis of the legs in hens about 10 to 14 days after dosing, whereas (+)-EPN caused no paralytic effects. Thus, (+)-EPN appears to be a more appropriate insecticide than the racemic compound, since it combines high toxicity to insects and no delayed neurotoxicity in hens.

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