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Distribution and Metabolism of *O*-Ethyl *O*-4-Nitrophenyl Phenylphosphonothioate after a Single Oral Dose in One-Week Old Chicks

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Abstract. The toxicokinetics and metabolism of a single 1 mg (2.7 μCi/kg) oral dose of uniformly phenyl-labeled [^{14}C]EPN (*O*-ethyl *O*-4-nitrophenyl [^{14}C]phenylphosphonothioate) have been studied in 1-week old chicks. One control and three treated chicks were killed at each of the following time intervals: 0.5, 2, 4, 8, and 12 days. Radioactivity was rapidly absorbed from the gastrointestinal tract and distributed in all tissues. ^{14}C in tissues reached a peak of 16.9% of the dose after 0.5 day and decreased to 0.6% at 4 days. The tissues of the gastrointestinal tract had the highest concentration of radioactivity, followed by bile and liver. Among nervous tissues, concentration of ^{14}C was highest in the peripheral nerves. The spinal cord had the next highest concentration, while the brain had the least. After 4 days 91.3% of the ^{14}C had been eliminated in the combined urinary-fecal excreta. By the end of the 12-day experiment this percentage reached 93.1%. No ^{14}C was detected in the expired CO_2. Following the oral administration of [14C]EPN, a monophasic body level curve was observed. The half-life for the elimination of ^{14}C from chick body was 16 h, corresponding to a rate constant of 0.04 h^{-1}. Most of the excreted ^{14}C materials were identified as *O*-ethyl phenylphosphonic acid, phenylphosphonic acid, and *O*-ethyl phenylphosphonothioic acid.

Key words: *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate – EPN – Chick – Metabolism – Delayed neurotoxicity

Introduction

Organophosphorus-induced delayed neurotoxicity (OPIDN) is characterized by ataxia and paralysis and accompanied by degeneration of axons and myelin in the central and peripheral nervous systems (Abou-Donia 1981). This effect was

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first seen in man (Smith et al. 1930); later other species (cats, dogs, farm, animals, and chickens) were found to be susceptible. Rodents and some primates are not sensitive. The adult chicken has become the animal of choice to study OPIDN because the clinical condition and the neuropathologic lesions are similar to those of humans (Anonymous 1978). By contrast, the young of susceptible species are not sensitive to OPIDN. Thus young chicks did not develop delayed neurotoxicity following a single oral dose of of 40 mg/kg mipafox (Barnes and Denz 1953), a single oral dose of 1,000 mg/kg TOCP (Bondy et al. 1960) or a subcutaneous injection of 2–5 mg/kg DFP (Johnson and Barnes 1970). As chicks grow older (90 days) they become sensitive to TOCP-induced delayed neurotoxicity (Abou-Donia et al. 1982).

Although the mechanism of selective delayed neurotoxicity among various species is still to be shown, recent studies on the neurotoxic organophosphorus esters leptophos (*O*-4-bromo-2,5-dichlorophenyl *O*-methyl phenylphosphonothioate) and EPN (*O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate) have suggested that toxicokinetics and metabolism may be determinant factors (Abou-Donia 1983). These insecticides were rapidly metabolized and excreted as degradation products mainly in the urine when administered orally to nonsusceptible species, e.g., mice (Holmstead et al. 1973) and rats (Whitacre et al. 1976; Hasson et al. 1977; Charles 1978; Chrzanowski and Jelinek 1981). On the other hand, these chemicals were persistent and had a longer biologic life in susceptible species, the hen (Abou-Donia 1976, 1979, 1980; Abou-Donia et al. 1983b) and the cat (Abou-Donia et al. 1983a, c).

Likewise, the insensitivity of chicks to a single dose of organophosphorus compounds may be attributed to the rapid metabolism and excretion of these compounds in young birds. This hypothesis is supported by the finding that repeated subcutaneous doses of DFP produced OPIDN in chicks (Johnson and Barnes 1970). These results suggest that perhaps the mechanisms of biotransformation and elimination of these chemicals may be saturable by prolong dosing in chicks. As a consequence, continuous administration of DFP may lead to its persistence in chicks' body and to the accumulation of this neurotoxic organophosphorus ester at the neurotoxicity target, at the threshold level to cause OPIDN.

This study was carried out to investigate the distribution and metabolism of a single oral dose of [^{14}C]EPN in the 1-week old chick. The results are discussed in relation to the insensitivity of chicks to OPIDN.

Methods

Birds. Leghorn chicks, were obtained the day they hatched from Featherdown Farm (Raleigh, NC, USA). They were housed in a humidity (40–60%) and temperature controlled (21–23° C) room with a 12-h light cycle before and during experimental procedures. They were supplied with feed (Layena Chicken Feed, Ralston Purina Co., St. Louis, MO, USA) and water ad libitum. Chicks were used after 1 week at which time they weighed (mean ± SE) 70.6 ± 2.1 g (68.7–81.4 g).

Chemicals. Analytical grade (> 99%) and radioactive EPN and the following reference chemicals were obtained from E.I. DuPont de Nemours and Co., Inc., Wilmington, DE, USA: EPNO

(*O*-ethyl *O*-4-nitrophenyl phenylphosphonate), EPPTA (*O*-ethyl phenylphosphonothioic acid), and EPPA (*O*-ethyl phenylphosphonic acid). PPA (phenylphosphonic acid) and PNP (*p*-nitrophenol) were purchased from Aldrich Chemical Co., Milwaukee, WI, USA. Desethly EPN (DE-EPN, *O*-4-nitrophenyl phenylphosphonothioate) and desethyl EPNO (DE-EPNO, *O*-4-nitrophenyl phenylphosphonate) were prepared according to the published method of Nomeir and Dautermann (1979). Uniformly phenyl labeled [^{14}C]EPN (*O*-ethyl *O*-4-nitrophenyl [^{14}C]phenylphosphonothioate) had a specific activity of 1.65 mCi/mmol. EPN radiochemical purity was determined using the sequential thin-layer chromatographic system described below, and was found to be 99.58% (Abou-Donia and Ashry 1978). Gelman type SA, ITLC silicic acid impregnated glass fiber sheets and glass distilled high-performance liquid chromatographic grade methanol were purchased from Fisher Scientific Products Co., Relaigh, NC, USA. All other solvents and chemicals were analytical grade purity.

Treatment of Chicks. Radioactive and unlabeled EPN were dissolved in acetone to obtain the desired specific activity. Aliquots of acetone solution containing 1 mg (2.7 μCi)/kg were pipetted into gelatin capsules. After evaporation of acetone by a gentle blowing of nitrogen, a total of 15 1-week old chicks were given [^{14}C]EPN in a gelatin capsule. Immediately before dosing each chick received 15 mg/kg of atropine sulfate in water (0.1 ml) to protect against the acute effect of EPN. Five similar chicks given an empty gelatin capsule served as control. Three treated chicks were killed at time intervals of 0.5, 2, 4, 8, and 12 days after administration. An untreated control was killed at each time interval.

Sampling Procedures. Expired CO_2 was trapped continually by drawing room air (500 ml/min) through the cage and then bubbling it through two gas bottles to trap expired CO_2. Each bottle contained 200 ml of ethanolamine-ethylene glycol monomethyl ether (1 : 2 v/v). ^{14}C was measured by counting 1 ml of the trap solution with 10 ml of the scintillation medium: toluene-ethylene glycol monomethyl ether (2 : 1 v/v) containing 5 g of 2,5-diphenyloxazole (PPO) and 0.2 g of 1,4-bis[2-(phenyloxazolyl)benzene] (POPOP) per liter. Daily combined urinary-fecal excreta were freezedried using Labconco lyophilizer (Labconco Corp., Kansas City, MO, USA), then finely ground using an electric grinder, type SHG (Markson Scientific, Inc., Del Mar, CA, USA).

Measurement of ^{14}C Radioactivity. Duplicate samples from ground excreta, fresh tissues, and dried gastrointestinal contents were combusted in a Packard Tri-Carb Model 306B sample oxidizer, using 10 ml of the trapping solution Carbo-Sorb and 12 ml of the scintillation fluid Permafluor V (Packard Instrument Co., Downers Grove, IL, USA). Radioactivity was determined in a Tri-Carb Model 3255 liquid scintillation spectrometer (Packard Instrument Co.) at 5° C. Counts were corrected for dilution, quenching, background and counting efficiency. A series of quenched standards determined the counting efficiences to be above 70%.

Extraction of Tissues and Excreta. Preliminary studies showed that three solvents were required to extract EPN and its metabolites from the tissues: acetonitrile was used for brain and adipose tissue; acetone for liver, kidney, muscle, and skin; ethyl acetate for bile and lyophilized excreta. Samples were homogenized in nine volumes of solvent, then centrifuged at 2,400 rpm for 15 min, and the organic layers were collected. The aqueous layers and precipitated protein were re-extracted with one additional volume of the solvent, and the organic layer was added to the original extract. The combined organic extracts were dried over sodium sulfate and concentrated by a gentle blowing of nitrogen. Excreta and some tissue extracts were cleaned from interfering materials by passing them through Fluorisil Columns (Johns and Riddick 1952; Mills 1968).

Urinary-fecal excreta samples were extracted with ethyl acetate as described above. Radioactivity extracted with ethyl acetate was designated as "nonconjugated metabolites". The residual excreta were next subjected to hydrolysis with β-glucuronidase (EC 3.2.1.3 1) from bovine liver Type B-1 (Sigma Chemical Co., St. Louis, MO, USA) at pH 4.5 and 45° C for 18 h, followed by ethyl acetate to yield glucuronides. Then, excreta were incubated with sulfatase (EC 3.1.6.1) from limpets Type V (Sigma Chemical Co., St. Louis, USA) at 37° C for 24 h and subsequently extracted with ethyl acetate to produce the sulfate fraction. Hydrolysis of the residual excreta by 2N sulfuric acid on a steam bath for 30 min and extraction with ethyl acetate yielded hot-acid-hydrolyzed metabolites. Radioactivity remaining in the aqueous fraction was considered a water-soluble residue

unextracted radioactivity in the excreta samples was designated "residue". Aliquots of solvent extracts and water-soluble residues were analyzed for radioactivity and for EPN and metabolites by sequential TLC and reverse-phase HPLC. The recovery of extracted EPN and each of its metabolites by all solvent systems was determined in a preliminary experiment.

Identification and Quantification of Metabolites. EpN and its metabolites were analyzed by sequential thin-layer chromatography (STLC). In this system Gelman-type SA ITLC, silicic acid impregnated glass fiber sheets were first developed with the primary solvent acetonitrile : water : ammonia (40 : 9 : 1) for 10 cm. After drying, the plates were developed with the secondary solvent *n*-hexane : benzene : acetic acid (5 : 5 : 1) for 16 cm (Abou-Donia and Ashry 1978). EPN and its metabolites were identified by adding a mixture of EPN and structurally-related standards to the solvent extracts of tissue or excreta from [^{14}C]EPN-treated chicks. The chromatographic separation of the standards with the labeled unknown was determined on TLC sheets. The standards were detected by their color in iodine vapor. Next, the TLC sheets were cut into 5-mm strips, placed into scintillation vials, vigorously mixed with the scintillation medium described above, and radioactivity was measured in the scintillation spectrometer to quantify EPN and its metabolites. Concentrations of metabolites were calculated from the recovered radioactivity from the TLC sheets. This system separates EPN and six of its metabolites with the following R_f values: EPN, 0.89; EPNO, 0.71; DE-EPN, 0.80; DE-EPNO, 0.59; EPPTA, 0.55, EPPA, 0.35; and PPA 0.08.

Reverse-phase high-performance liquid chromatography (HPLC) was used to confirm STLC results in samples containing EPN related compounds at detectable levels (Lasker et al. 1980). HPLC analysis was carried out using a Waters Associates liquid chromatograph (Milford, MA, USA) consisting of two M6000A pumps, an M660 solvent programmer, an M440 UV detector, a U6-K universal injection system, and a Lichrosorb 10 μm RP-8 column, 25 × 4.6 mm (E. Merck, Darmstadt, FRG). Test chemicals or tissue extracts were injected in 5–25 μl at room temperature. The solvent system was methanol-water (the initial solvent also contained 5% glacial acetic acid) with gradient of 1 : 99 v/v to 95 : 5 v/v, in 30 min after a 10 min isocratic delay. The solvent inlet pressure was 2,000 ppm. Peak areas were measured with a Shimadzu Chromatopak E1A (Shimadzu, Kyoto, Japan) reporting integrator. The uv (280 nm) detection limit for EPN, EPNO, and PNP was 10 ng, and the limit for EPPTA, EPPA, and PPA was 100 ng. Retention times of these compounds were (in min): EPN, 28.0; EPNO, 24.0; PNP, 20.0; EPPTA, 12.4; EPPA, 9.1; and PPA, 5.8.

Results

Clinical and Necropsy Observations

In a preliminary experiment to establish the proper dosage of EPN, groups of six chicks were given a single oral dose of 1, 5, or 10 mg/kg EPN. Prior to these treatments, the chicks were given an oral administration of 0.1 ml containing 15 mg/kg atropine sulfate in water.

The results of this investigation show that the 1-week old chick is very sensitive to the acute cholinergic effect of EPN. Thus when 5 or 10 mg/kg oral dose of EPN was administered, to a group of six chicks all birds died within 24 h despite prior treatment with 15 mg/kg atropine sulfate. All chicks treated with 1 mg/kg single oral dose survived the experimental period. None of the 1-week old chicks developed clinical signs of delayed neurotoxicity or had histopathologic lesions in the central or peripheral nervous tissues. Post mortem gross histologic observations showed no difference in the size, shape of color of various tissues of treated birds compared with the tissues from the control chicks.

Radioactivity in Expired Air

No radioactivity was detected in expired carbon dioxide or air from chicks given a single oral dose of 1 mg/kg EPN.

Radioactivity in Excreta

Radioactivity in the excreta was measured daily, beginning at 12 h. The first sample of excreta contained 26.6% of the dose (Fig. 1). The daily rate of ^{14}C eliminated in the excreta reached a peak of 73.9% on the first day after administration, then sharply decreased until the fourth day when only 0.7% was excreted. By day 4, the total cumulative ^{14}C excreted in the excreta was 91.3% of the applied dose. After this time, daily rate of ^{14}C appearing in the excreta leveled off and the total ^{14}C eliminated in the excreta through the 12-day experimental period was 93.9% (Table 1).

Metabolism of [^{14}C]EPN in Excreta

Excreta samples collected after 0.5, 1, and 2 days were subjected to sequential enzymatic and acid hydrolysis and ethyl acetate extraction to characterize the nature of the ^{14}C eliminated materials (Table 2). Ethyl acetate extracted

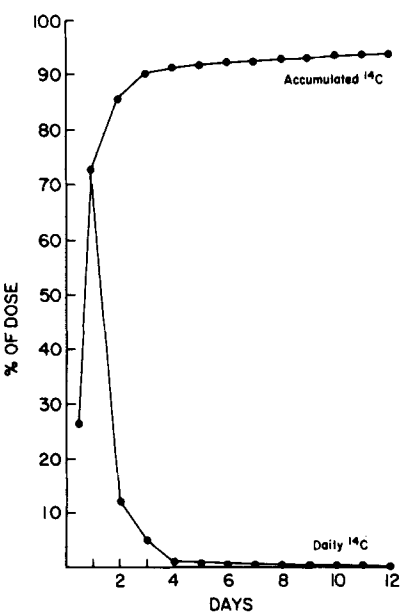

Fig. 1. Daily rate of ^{14}C and accumulated total ^{14}C in urinary-fecal excreta from 1-week old chicks after a single oral 1 mg/kg dose of [^{14}C]EPN

Table 1. Cumulative % recovery of ^{14}C radioactivity from chicks given a single 1 mg (2.7 μCi)/kg oral dose of [^{14}C] EPN

Day	Excreta[a]	Tissues[b]	Gastrointestinal tract contents[b]	Total
0.5	26.63 ± 3.15	16.32 ± 1.27	55.82 ± 5.63	99.37 ± 10.05
2	85.91 ± 9.67	3.95 ± 0.51	8.76 ± 0.87	98.62 ± 11.05
4	91.29 ± 9.80	0.63 ± 0.07	0.84 ± 0.08	92.76 ± 9.95
8	92.93 ± 9.85	0.02 ± 0.00	0.01 ± 0.00	92.96 ± 9.85
12	93.14 ± 9.91	0.00	0.00	93.14 ± 9.91

[a] Each value is a mean ± SE of the daily excretion values of ^{14}C radioactivity from all the birds used 15, 12, 6, 9, and 3 birds for 0.5, 2, 4, 8, and 20 days, respectively

[b] Each value is an average ± SE of the ^{14}C radioactivity of tissue or gastrointestinal tract contents from three birds

Table 2. Classes of metabolites of excreta from chicks given a single 1 mg (2.7 μCi)/kg oral dose of [^{14}C]EPN[a]

Days	Non-Conjugated	Glucu-ronides	Sulfates	Hot-acid hydrolysate	Water soluble	Residue	Recovery %
0.5	1.6 ± 0.15	3.7 ± 0.29	2.0 ± 0.21	17.3 ± 1.59	27.6 ± 2.67	45.2 ± 4.39	97.4 ± 9.30
1	1.4 ± 0.15	5.6 ± 0.49	3.3 ± 0.30	21.0 ± 2.19	27.0 ± 2.56	39.3 ± 3.87	97.6 ± 9.59
2	3.1 ± 0.39	8.3 ± 0.80	4.3 ± 0.38	21.5 ± 0.19	34.0 ± 2.95	25.1 ± 2.45	96.3 ± 7.16

[a] Values are percentages of total ^{14}C determined in excreta by oxygen combustion as described under Methods. Each value is a mean ± SE of six determinations from three chicks

radioactivity (nonconjugated materials) before any treatment of the excreta was only 1.6% after half a day. This value increased to 3.1% after 2 days. The amount of glucuronide conjugated materials were about twice those of the nonconjugated fractions. Hot-acid hydrolysate contained higher percentage of ^{14}C labeled materials that ranged from 17.3% to 21.5% at 0.5 and 2 days respectively. Water soluble ^{14}C materials accounted for more radioactivity than any of the previous fractions. Radioactivity remaining in the excreta after all sequential hydrolyses and extractions (residue) was initially higher than any other fraction but decreased with time, while all other fractions increased.

EPN and six metabolites were identified in all excreta fractions (Table 3). EPN accounted for most of the radioactivity in the non-conjugated fraction. In the glucuronide fraction, the polar metabolites EPPA had the highest concentration followed by DE-EPN oxon and EPN. Also, in the sulfate fraction, EPPA accounted for the largest concentration of EPN metabolites followed by EPN. In both the hot-acid and water soluble fractions most of the radiolabeled materials was identified as EPPA followed by PPA and EPPTA. EPN oxon, the only metabolite with toxicity higher than that of the parent compound EPN, was present in all fractions only in small concentrations.

Table 3. Total[a] EPN[b] and metabolites in excreta from chicks given a single 1 mg (2.7 μCi)/kg oral dose of [^{14}C]EPN

Fraction	Days	Total μg excreted in intervals						
		PPA	EPPA	EPPTA	DE-EPNO	DE-EPN	EPNO	EPN
Non-conjugated	0.5	2.6 ± 0.3	1.7 ± 0.2	7.0 ± 0.7	9.0 ± 0.9	5.2 ± 0.5	17.9 ± 2.1	350.3 ± 36.0
	1	2.1 ± 0.2	2.9 ± 0.3	3.9 ± 0.4	6.8 ± 0.7	2.5 ± 0.3	4.8 ± 0.5	162.0 ± 17.0
	2	1.1 ± 0.1	1.7 ± 0.1	1.8 ± 0.2	3.4 ± 0.4	1.3 ± 0.1	2.5 ± 0.2	70.0 ± 7.0
Glucuronides	0.5	18.7 ± 2.0	310.0 ± 32.0	51.8 ± 5.2	189.6 ± 20.0	26.3 ± 3.0	28.8 ± 2.9	151.8 ± 16.0
	1	7.6 ± 7.1	165.7 ± 17.1	59.4 ± 6.0	109.5 ± 11.1	18.6 ± 7.9	32.1 ± 3.5	270.2 ± 31.0
	2	1.5 ± 0.2	6.0 ± 0.7	2.6 ± 3.0	9.0 ± 0.9	4.4 ± 0.5	1.7 ± 0.2	9.4 ± 1.0
Sulfates	0.5	15.3 ± 1.7	100.1 ± 21.0	17.7 ± 1.8	35.4 ± 4.1	5.6 ± 0.6	10.4 ± 1.1	148.5 ± 15.0
	1	23.6 ± 2.7	210.8 ± 22.0	14.4 ± 1.5	45.6 ± 5.1	7.8 ± 0.8	9.0 ± 1.0	77.4 ± 8.0
	2	1.9 ± 2.0	4.3 ± 0.5	1.2 ± 0.1	1.7 ± 0.2	0.5 ± 0.0	0.9 ± 0.0	7.2 ± 0.5
Hot acid	0.5	229.0 ± 31.0	2,841.0 ± 290.0	236.0 ± 24.0	158.0 ± 15.0	54.0 ± 6.0	58.0 ± 0.6	113.0 ± 12.0
	1	268.0 ± 27.0	1,796.0 ± 180.0	157.0 ± 16.0	74.0 ± 8.0	41.0 ± 5.0	54.0 ± 5.7	89.0 ± 9.0
	2	4.0 ± 0.5	46.0 ± 5.1	23.0 ± 2.5	5.0 ± 0.6	2.0 ± 0.2	3.0 ± 0.3	8.0 ± 0.9
Water-soluble	0.5	158.0 ± 16.0	1,201.0 ± 125	92.0 ± 9.1	100.0 ± 11.0	19.0 ± 2.1	23.0 ± 2.5	20.0 ± 2.1
	1	561.5 ± 58.0	2,470.0 ± 250	47.4 ± 5.0	56.4 ± 6.0	1.8 ± 0.2	11.1 ± 1.2	31.6 ± 3.5
	2	230.1 ± 25.0	1,258.0 ± 130.0	23.2 ± 2.5	25.2 ± 2.7	0.9 ± 0.0	5.6 ± 0.7	16.3 ± 2.0

[a] Each value is mean ± SE of six determinations from three chicks
[b] Abbreviations are listed under Methods

Radioactivity in Tissues

Table 1 shows that the radioactivity reached a peak of 16.3% at 0.5 days and sharply dropped to about 0.02 at day 8. In the gastrointestinal tract contents 55.8% of the dose was determined at 12 h, decreasing to about 0.01% by day 8. No radioactivity was detected in tissues or gastrointestinal tract contents by day

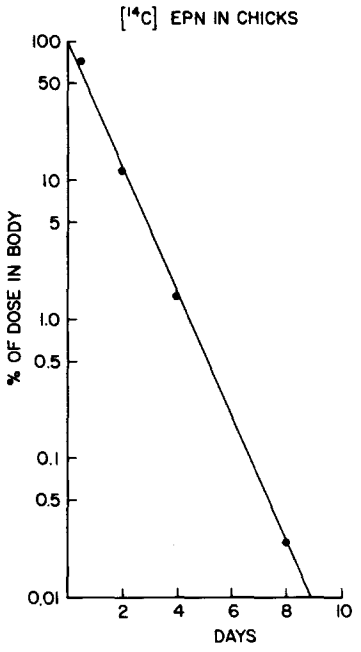

Fig. 2. Change in body burden of ^{14}C in 1-week old chicks after a single oral 1 mg/kg dose of [^{14}C]EPN

Table 4. Concentration[a] of radioactive material in tissues and bile of 1-week old chicks given a single oral dose of 1 mg (2.7 μCi/kg) of [^{14}C]EPN

Specimen	Days		
	0.5	2	4
Brain	18.9 ± 3.0	8.7 ± 0.8	2.2 ± 0.3
Spinal cord	57.5 ± 10.2	20.5 ± 2.0	0
Peripheral Nerves	106.3 ± 4.8	14.7 ± 2.4	0
Lung	66.6 ± 13.6	19.8 ± 2.0	0
Heart	35.4 ± 4.8	10.5 ± 1.1	0.4 ± 0.0
Liver	147.7 ± 13.5	46.6 ± 4.5	7.1 ± 0.5
Bile	252.1 ± 27.0	100.9 ± 10.1	0
Kidney	125.5 ± 10.9	39.7 ± 3.5	2.8 ± 0.2
Muscle	71.8 ± 7.0	7.5 ± 0.6	0.9 ± 0.0
Skin	92.8 ± 8.8	50.4 ± 5.1	5.8 ± 0.5

[a] Concentrations are expressed as ng of EPN equivalents per gram of fresh tissue or milliliter bile. Each value represents the mean ± SE for six samples from three birds

Table 5. Concentration[a] (ng/g) of EPN[b] and metabolites in selected tissues and bile from chicks given a single 1 mg (2.7 μCi)/kg oral dose of [^{14}C]EPN

Tissue	Days	Concentration (ng/g)						
		PPA	EPPA	EPPTA	DE-EPNO	DE-EPN	EPNO	EPN
Brain	0.5	1.3 ± 0.2	8.3 ± 0.9	0.8 ± 0.1	0.6 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.7 ± 0.1
Liver	0.5	4.3 ± 0.4	12.0 ± 1.2	76.8 ± 7.7	6.8 ± 0.7	1.4 ± 0.2	4.0 ± 0.5	15.0 ± 1.3
	2	4.3 ± 0.5	7.5 ± 7.9	2.7 ± 0.3	7.2 ± 0.8	2.0 ± 0.2	3.0 ± 0.4	5.0 ± 0.5
Kidney	0.5	5.4 ± 0.6	20.9 ± 2.2	23.7 ± 2.5	5.0 ± 0.5	0.8 ± 0.1	2.6 ± 0.3	7.9 ± 0.8
	2	3.4 ± 0.4	2.9 ± 0.3	5.6 ± 0.4	13.6 ± 1.4	1.5 ± 0.2	2.6 ± 0.3	46.3 ± 5.1
Muscle	0.5	0.4 ± 0.0	2.0 ± 0.2	0.3 ± 0.0	2.5 ± 0.3	0.5 ± 0.1	0.8 ± 0.1	7.4 ± 0.8
	2	0.8 ± 0.1	0.6 ± 0.3	0.7 ± 0.1	1.1 ± 0.1	0.4 ± 0.0	1.4 ± 0.1	35.9 ± 3.6
Skin	0.5	0.4 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	2.0 ± 0.2	0.8 ± 0.1	1.3 ± 0.1	13.9 ± 1.4
	2	0.6 ± 0.0	0.7 ± 0.1	3.4 ± 0.3	8.6 ± 0.9	2.4 ± 0.3	4.6 ± 0.5	8.0 ± 0.8
Bile	0.5	14.4 ± 1.5	7.0 ± 0.7	7.7 ± 7.9	16.3 ± 1.6	0.5 ± 0.0	14.2 ± 1.5	92.4 ± 9.5
	2	29.1 ± 2.9	29.1 ± 2.9	23.9 ± 2.5	15.2 ± 1.5	7.1 ± 0.7	11.6 ± 1.3	96.7 ± 10.0

[a] Values are expressed as ng/g fresh tissue or ng/ml bile. Each value is a mean ± SE of six determinations from three chicks
[b] Abbreviations are listed under Methods

12. The data in Table 1, when plotted as log% ^{14}C in the chick's body versus days after administration, gave a straight line (Fig. 2). The half-life for the elimination of ^{14}C from chick body was calculated from this plot to be 16 h, corresponding to an apparent rate constant of 0.04 h^{-1}.

Concentrations of radioactivity in some tissues and bile from chicks are listed in Table 4. The highest concentration was found in the bile. ^{14}C, however, was depleted fast from bile and no radioactivity was detected by day 4. Liver and kidney, organs concerned with the metabolism and excretion of xenobiotics had the next highest concentrations of radioactivity. Among the storage tissues, muscle, and skin initially contained high ^{14}C concentration, that depleted very fast. Lung and heart had considerable concentrations of radioactivity at 12 h, but no ^{14}C was detected at 4 days. Among nervous tissues, peripheral nerves contained the highest concentration of ^{14}C followed by spinal cord and with the brain having the least. ^{14}C concentration in these tissues dropped very rapidly with time and no ^{14}C was detected in the spinal cord or peripheral nerves at 4 days.

EPN and Metabolites in the Tissues

In this study, EPN and its metabolites were identified in the purified extracts from brain, liver, kidney, muscle, skin, and bile of birds sacrificed 12 h and 2 days after administration (brain at 12 h only). Recovery of total ^{14}C ranged from 69% in the sciatic nerve to 12% in the liver (Table 5). Most of the ^{14}C in the muscle, skin, and bile was identified as EPN. All six metabolites identified in the excreta accounted for the remaining portion of ^{14}C. EPN accounted for only 6.5% of the ^{14}C in the brain 12 h after administration. The liver exhibited extensive metabolism of EPN which accounted for only 12.5% and 5.1% of the total ^{14}C at 0.5 and 2 days respectively. Kidney also contained all metabolites.

Discussion

This study reports the absorption, tissue disposition, elimination, and metabolism of a single oral dose of 1 mg/kg [^{14}C]EPN in the 1-week old chick. The results show that young chicks are very sensitive to the acute effect of orally administered EPN, since all chicks given a single oral dose of 5 or 10 mg/kg died despite prophylactic treatment with atropine sulfate. These results indicate that young chicks are more sensitive to the cholinergic effect of EPN than adult chickens which had an oral LD_{50} of 10 mg/kg (Abou-Donia and Graham 1979). The results that none of the surviving chicks developed delayed neurotoxicity is in agreement with earlier reports (Barnes and Denz 1953; Bondy et al. 1960; Johnson and Barnes 1970).

EPN and/or metabolites was eliminated rapidly through combined fecal-urinary excreta, thus 86% of the oral dose was excreted within 48 h of the administration. Only a small fraction of ^{14}C was eliminated as nonconjugated

substances. It seems that the nonconjugated ^{14}C materials in the urinary-fecal excreta were from the administered EPN that had not been absorbed from the gastrointestinal tract and eliminated via the feces. This assumption is supported by the finding that most of the nonconjugated ^{14}C materials in the excreta were identified as the parent chemical EPN. On the other hand, most of the ^{14}C labeled substances in the conjugated fractions were identified as polar metabolites of EPN. This was particularly evident in the hot-acid hydrolysate and water-soluble residue that contained only trace amounts of EPN. The nature of the residual unextracted ^{14}C materials in the excreta after sequential hydrolysis and ethyl acetate extraction is not known. It is plausable, however, that these conjugates were proteins or peptides bound to phenylphosphonothioic acid and/or metabolites.

The highest ^{14}C concentration was always present in the bile, liver, and kidney, reflecting their excretory functions (Table 4). The high concentration of EPN in the kidney may be explained by the extensive binding of EPN to plasma and tissues and the consequent low glomerular filtration of this compound (Weiner et al. 1960). Perhaps nonionic absorption and passive reabsorption of EPN in the renal tubule (Milne et al. 1958) is enhanced by the compound's high lipid solubility (partition between octanol and water is 200,000, Davies et al. 1975). It seems that EPN was metabolized primarily in the liver, since this tissue had most of the ^{14}C content of all visceral tissues.

Nervous tissues were among the tissues that contained low concentration of ^{14}C radioactivity. These tissues lost radioactivity rapidly; thus 4 days after the administration spinal cord and peripheral nerves were voided of ^{14}C while the brain contained only traces of radioactivity. Also, EPN accounted only for 6.5% of ^{14}C in the brain. This rapid depletion of EPN and/or metabolites from nervous tissues may have contributed to the resistance of chicks to EPN-induced delayed neurotoxicity. By contrast, in adult hens, which are susceptible to OPIDN, nervous tissues contained small but persistent concentrations of ^{14}C, most of which was identified as EPN throughout the 12-day experiment (Abou-Donia et al. 1983b).

In the chick EPN and six metabolites were identified in the excreta and analyzed tissues. The reactions involved in EPN metabolism are oxidation and hydrolysis (Fig. 3). Oxidation of EPN yielded EPNO that was subsequently hydrolyzed to ethanol and desethyl EPN. Hydrolysis of EPN produced desethyl EPN which was further oxidized to desethyl EPNO. Hydrolysis of EPN produced PNP and EPPTA. The latter compound could be oxidized to EPPA which was subsequently oxidized to PPA. PPA may also form through the hydrolysis of desethyl EPNO which also produces PNP. *p*-Nitrophenol and phosphonothioc and phosphonic acid derivatives may be conjugated to form glucuronides, ethereal sulfates, and hybrids. Of all of these biotransformation products, only EPNO has more acute and delayed neurotoxic effects than EPN. This metabolite was always present in minor quantities in tissues and excreta. Thus, metabolic pathways of EPN in the chick were detoxification mechanisms since they mostly led to the formation of polar and water soluble metabolites that accelerated the excretion of EPN from bird's body and rendered it less toxic. These metabolic biotransformation products may be formed by one or more of

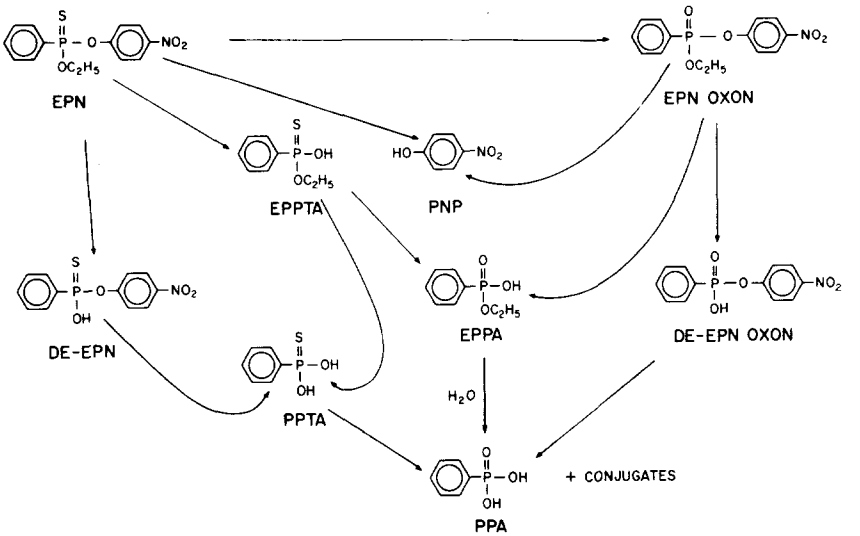

Fig. 3. Suggested metabolic pathways of EPN administered orally to 1-week old chicks

the following enzymes: mixed-function oxidase, hydrolase, and glutathion *S*-alkyl transferase (Dauterman 1971). Previous studies, have shown that oxidation of EPN to EPNO takes place by rat and chicken hepatic mixed-function oxidase systems. By contrast hydrolysis of EPN and EPNO by rat and chicken hepatic microsomes neither required NADPH nor was it inhibited by SKF 525A or by anaerobic conditions (Lasker et al. 1982).

Toxicokinetics analysis of a single oral dose of [^{14}C]EPN in chicks' body indicates first order kinetics and suggests a single compartment model for ^{14}C elimination. These results suggest that orally administered [^{14}C]EPN in the chick is absorbed from the gastrointestinal tract, distributed in the tissues, and eliminated from the body without significant delay due to storage. These results might have been attributed, at least in part, to the absence of adipose tissue in the abdomen and the gastrointestinal tract in young chicks. Hence EPN storage was not extensive and was circulated and exposed to xenobiotic metabolizing enzymes in the liver leading to extensive metabolism of EPN. Similar results were obtained with leptophos (Konno and Kinebuchi 1978).

The present results are in contrast to the metabolism and pharmacokinetics of [^{14}C]EPN in the adult hen. In the hen, which is susceptible to OPIDN, EPN persisted in the body tissues and its biologic half-life was 54 h (Abou-Donia et al. 1983b). On the other hand this value in the rat (an insensitive species), was 16 h (Chrzanowski and Jelinek 1981). The present and previous studies have shown that EPN was rapidly metabolized and excreted as degradation product in nonsusceptible species and insensitive young chicks, while it was metabolized to lesser degree by adult chickens (a susceptible species) and persisted in their tissues (Abou-Donia et al. 1983b). This study suggests that perhaps the rate of metabolism and excretion of delayed neurotoxic organophosphorus esters is a contributing factor in age sensitivity as well as species susceptibility.

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