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The Absorption, Distribution, Excretion, and Metabolism of a Single Oral Dose of *O*-Ethyl *O*-4-Nitrophenyl Phenylphosphonothioate in Hens

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The Absorption, Distribution, Excretion, and Metabolism of a Single Oral Dose of *O*-Ethyl *O*-4-Nitrophenyl Phenylphosphonothioate in Hens. ABOU-DONIA, M. B., REICHERT, B. L., AND ASHRY, M. A. (1983). *Toxicol. Appl. Pharmacol.* **70,** 18–28. The disposition and metabolism of a single oral 10 mg/kg (LD50) of uniformly phenyl-labeled [^{14}C]EPN (*O*-ethyl *O*-4-nitrophenyl [^{14}C]phenylphosphonothioate) were studied in adult hens. The birds were protected from acute toxicity with atropine sulfate. Three treated hens were killed at each time interval (days): 0.5, 2, 4, 8, 12. Radioactivity was adsorbed from the gastrointestinal tract and distributed in all tissues. Most of the dose was excreted in the combined urinary–fecal excreta (74%). Only traces of the radioactivity (0.2%) were detected in expired CO_2. Most of the excreted radioactive materials were identified as phenylphosphonic acid (PPA), *O*-ethyl phenylphosphonic acid (EPPA), and *O*-ethyl phenylphosphonothioc acid (EPPTA). Radioactivity in tissues reached a peak of 11.8% in 12 days. The highest concentration of radioactivity was present in the liver followed by bile, kidney, adipose tissue, and muscle. EPN was the major compound identified in brain, spinal cord, sciatic nerve, kidney, and plasma. Most of the radioactivity in the liver was identified as EPPA followed by EPPTA and PPA. Kinetic studies showed that EPN disappeared exponentially from tissues. The half-life of the elimination of EPN from plasma was 16.5 days corresponding to a constant rate value of 0.04 day^{-1}. Relative residence (R_R) of EPN relative to plasma was shortest in liver and longest in adipose tissue followed by sciatic nerve and spinal cord.

Recently, interest in large-scale use of EPN (*O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate) as an insecticide has been renewed as a result of the ban on most chlorinated hydrocarbon insecticides. EPN belongs to the phenylphosphonothioate ester group that is recognized as causing delayed neurotoxicity in experimental animals (Abou-Donia, 1979a). This effect is characterized by leg weakness and degeneration of axons and myelin within the central and peripheral nervous systems (Abou-Donia, 1981). It has been demonstrated that EPN produces delayed neurotoxicity in chickens when given in feed (Durham *et al.*, 1956; Frawley *et al.*, 1956), by ip injection (Gaines, 1969), single po administration (Abou-Donia and Graham, 1979), subchronic daily po administration (Abou-Donia and Graham, 1978), or subchronic daily dermal administration (Abou-Donia *et al.*, 1983b). Similar results were observed in cats (Abou-Donia *et al.*, 1983d).

As early as 1930 it was recognized that not all species respond similarly to organophosphorus ester-induced delayed neurotoxicity (OPIDN); chickens and cats are susceptible while rodents are generally resistant (Smith *et al.*, 1930). Although the mechanism of selective delayed neurotoxicity among various species is still to be elucidated, recent studies on

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the structurally related leptophos (*O*-4-bromo-2,5-dichlorophenyl *O*-methyl phenylphosphonothioate) have indicated that pharmacokinetics and metabolism may play an important role in species selectivity. Leptophos is rapidly metabolized and excreted as degradation products mainly in the urine when given orally to nonsusceptible species, e.g., mice (Holmstead *et al.*, 1973) and rats (Whitacre *et al.*, 1976; Hassan *et al.*, 1977). This insecticide, however, is persistent and has a longer biologic half-life in the hen (Abou-Donia, 1976, 1979b, 1980).

Pharmacokinetics and metabolism of organophosphorus compounds, which affect the development of delayed neurotoxicity, are also influenced by the size of applied dose (Abou-Donia, 1983). Recently, a study has been undertaken to investigate the pharmacokinetics and metabolism of a daily dermal dose of 0.5 mg/kg of EPN in hens (Abou-Donia *et al.*, 1983a). The present report describes the pharmacokinetics and metabolism of a single oral 10 mg/kg (LD50; Abou-Donia and Graham, 1979), of uniformly phenyl-labeled [^{14}C]EPN in adult hens.

METHODS

Chemicals. EPN, its related compounds, and the radioactive EPN were provided by E. I. DuPont de Nemours and Co., Inc. (Wilmington, Del.). The following analytical grade compounds were used: EPN (*O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate), EPN oxon (*O*-ethyl *O*-4-nitrophenyl phenylphosphonate, EPNO), EPPTA (*O*-ethyl phenylphosphonothioic acid), EPPA (*O*-ethyl phenylphosphonic acid), PPA (phenylphosphonic acid), and PNP (*p*-nitrophenol). Desethyl EPN (*O*-4-nitro phenyl phenylphosphonothioate) and desethyl EPN oxon (*O*-4-nitrophenyl phenylphosphonate, desethyl EPNO), were prepared as previously described (Nomeir and Dauterman, 1979). Uniformly phenyl-labeled [^{14}C]EPN (*O*-4-nitrophenyl [^{14}C]phenylphosphonothioate) had a specific activity of 1.65 μCi/mmol. Radiochemical purity was found to be 99.58% as assayed by the sequential thin-layer chromatographic system described below (Abou-Donia and Ashry, 1978). Atropine sulfate was obtained from Sigma Chemical Company (St. Louis, Mo.). Pentobarbital sodium solution (Nembutal) was purchased from Abbott Laboratories, North Chicago, Illinois. All other chemicals used were of the highest grade commercially available.

Treatment of hens. Mature laying hens (*Gallus gallus domesticus*) each approximately 18 months old and weighing (mean ± SE) 1.5 ± 0.1 kg (SPAFAS, Norwich, Conn.) were used in this study. Each bird was administered a single dose of 10 mg/kg (2.73 μCi/kg) of [^{14}C]EPN in a gelatin capsule. Immediately before dosing each hen received 30 mg/kg of atropine sulfate to protect against the acute effect of EPN. After treatment each animal was placed in a separate metabolism chamber. The hens were allowed feed (Layena Chicken Feed, Ralston Purina Co., St. Louis, Mo.) and water *ad libitum* during the course of the experiment.

Sampling procedures. During the course of the experiment $^{14}CO_2$ was trapped continually in ethanolamine: ethylene glycol monomethyl ether (1:2, v/v). Excreta (combined urinary and fecal excretion) were collected daily. Three hens were killed at each of the following time intervals: 0.5, 2, 4, 8, and 12 days. Each time, blood samples were collected in heparinized syringes, tissues were excised, and gastrointestinal tract tissue and contents were separated. Radioactivity was measured in all tissues, gastrointestinal tract contents, and body fluids by combusting samples in a Packard Tri-Carb Oxidizer Model 306B (Packard Instrument Co. Inc., Downers Grove, Ill.) with 10 ml of the trapping solution Carbo-Sorb and 12 ml of the scintillation mixture Permafluor V. Scintillation counting was accomplished in a Packard Tri-Carb Model 3255 liquid scintillation spectrometer.

Extraction procedure. In a preliminary experiment it was found that no single solvent could extract all ^{14}C from all tissues. Thus, the following three solvents were utilized to extract EPN and its metabolites from the tissues: acetone for the liver, kidney, and muscle; acetonitrile for the brain, spinal cord, sciatic nerve, and adipose tissue; ethyl acetate for the plasma and bile. Following homogenization, samples were centrifuged at 2400 rpm for 15 min, and the organic layers were collected. The precipitated protein and the aqueous layer were reextracted with one additional volume of the solvent, and the organic layer was added to the original extract. The combined solvent extract was concentrated by a gentle blowing of nitrogen. Extracts of excreta and some tissues were cleaned from interfering materials by passing them through Fluorisil columns (Jones and Riddick, 1952; Mills, 1968).

Each sample of lyophilized excreta or fresh tissue was homogenized in 9 vol of ethyl acetate. Radioactivity extracted in the ethyl acetate was designated as "nonconjugated metabolites." The residual excreta were next subjected to hydrolysis with β-glucuronidase (EC 3.2.1.31) from bovine liver type B-1 (Sigma Chemical Co., St. Louis, Mo.) at pH 4.5 and 45°C for 18 h, followed by ethyl acetate extraction to yield the glucuronides. Then, excreta were incubated with sulfatase (EC 3.1.6.1) from limpets type V (Sigma Chemical Co.) at 37°C for 24 hr and subsequently extracted with ethyl acetate to produce the sulfate fraction. Exposure to hot sulfuric acid (2 N H_2SO_4) for 30 min, followed by ethyl acetate extraction after cooling, gave a hot acid-hydrolyzable fraction. Radioactivity remaining in the aqueous fraction was considered a water-

soluble fraction. The completeness of extraction of EPN and each of its metabolites by all solvent systems had been established in a preliminary experiment.

Chromatographic procedures. Gelman-type SA, ITLC silicic acid impregnated glass-fiber sheets were used in the chromatographic analyses. Aliquots of the ethyl acetate extracts were applied to the sheets and developed sequentially in the following solvent systems: acetonitrile:water:ammonium hydroxide (40:9:1) for 10 cm, and after drying with *n*-hexane:benzene:acetic acid (5:5:1) for 16 cm (Abou-Donia and Ashry, 1978). After development of the ITLC, strips were sectioned into 5-mm segments which were placed in scintillation vials for ^{14}C counting.

High-pressure liquid chromatography (HPLC) (Waters Associates, Milford, Mass.) was used to analyze EPN and its metabolites after elution on a microparticulate RP-8 column with a gradient of 1 to 95% methanol in water (initial solvent containing 5% glacial acetic acid) for 30 min following a 10-min isocratic delay (Lasker *et al.*, 1980). The flow rate was 1.2 ml/min at 25°C (280 nm). The data used for HPLC and the sequential thin-layer chromatography (STLC) are presented in Table 1.

Kinetic analysis. The kinetic analysis of ^{14}C in tissues and body fluids was carried out in a Tektronic 4052 graphic computer system. Half-lives of EPN were determined from the elimination rate constant, β, which was calculated by linear regression of the terminal linear exponential phase in EPN concentration, according to the equation

$$t_{½} = \frac{0.693}{\beta}$$

To determine various pharmacokinetic parameters, the total area under the EPN concentration against time curves for tissue, AUC_{tissue} and plasma, AUC_{plasma} was determined. Area under the curve was calculated by the trapezoidal rule and extrapolated to affinity with the last data point and the respective terminal linear exponential decline. Total clearance, *Cl*, and apparent volume of distribution, V_d area, were determined by the expression

$$Cl = \frac{DF}{AUC_{plasma}},$$

$$V_d = \frac{DF}{\beta(AUC)_{plasma}} = \frac{Cl}{\beta},$$

where *D* is dose and *F* is bioavailability. Both *Cl* and V_d may overestimate the actual values, since *F* is unknown.

The relative residence, R_R, of EPN in various tissues compared to plasma was determined as follows:

$$R_R = \frac{AUC_{tissue}}{AUC_{plasma}},$$

where the AUC's area is defined above. This parameter (R_R) represents the content of EPN in specific tissues relative to that of plasma which may be a reflection of the relative exposure of individual tissues to EPN. R_R is a time-independent parameter, which has recently been used to predict the ratio of average concentrations of EPN in specific tissues $\bar{C}_{tissue}$, to those in plasma, $\bar{C}_{plasma}$, (Abou-Donia *et al.*, 1983c), assuming linear kinetics or

$$R_R = \frac{\bar{C}_{tissue}}{\bar{C}_{plasma}}.$$

TABLE 1

SEQUENTIAL THIN-LAYER CHROMATOGRAPHIC (STLC) AND HIGH-PRESSURE LIQUID CHROMATOGRAPHIC (HPLC) DATA FOR EPN AND VARIOUS AUTHENTIC COMPOUNDS USED IN IDENTIFICATION OF EPN AND METABOLITES

Compound	STLC R_f[a]	HPLC RT[b]
O-Ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN)	0.89	28.0
O-Ethyl *O*-4-nitrophenyl phenylphosphonate (EPN oxon)	0.71	24.0
O-4-Nitrophenyl phenylphosphonothioate (desethyl EPN)	0.80	
O-4-Nitrophenyl phenylphosphonate (desethyl EPN oxon)	0.59	
Ethyl phenylphosphonothioate (EPPTA)	0.55	12.4
Ethyl phenylphophonate (EPPA)	0.35	9.1
Phenylphosphonic acid (PPA)	0.08	5.8
4-Nitrophenol (PNP)		20.0

[a] Gelman-type SA, silicic acid-impregnated glass-fiber sheets were sequentially developed with the primary solvent (acetonitrile–water–ammonia, 40:9:1) for 10 cm and the secondary solvent (*n*-hexane–benzene–acetic acid, 5:5:1) for 16 cm.

[b] Elution on a microparticulare RP-8 column with a gradient of 1 to 95% ethanol in water (the initial solvent also contained 5% glacial acetic acid) in 30 min following a 10-min isocratic delay. Flow rates of 1.2 ml/min at 25°C (280 nm).

RESULTS

Clinical signs and necropsy observations. Although atropine sulfate treatment protected the hens from the cholinergic effect of the 10-mg/kg single oral dose of EPN (LD50), none of these hens laid eggs, and none developed signs of delayed neurotoxicity. Postmortem

gross histologic observations revealed no differences in the size, shape, or color of various tissues of treated birds compared with the tissues of the control hens.

^{14}C in Expired Air. Oral administration of radiolabeled EPN resulted in a small amount of radioactivity in expired air (Fig. 1). Radioactivity measured on a daily basis reached a peak value on Day 5 and subsequently decreased. The cumulative percentage of ^{14}C recovered in expired air throughout the 12-day experiment was 0.17% of the administered dose (Table 2).

Radioactivity in excreta. The amount of radioactivity in the excreta was measured daily, beginning at 12 hr. The first sample of excreta contained 3.15% of the dose (cumulative recovery, Table 2). This percentage of the dose increased throughout the experimental period, but reached a peak on Day 3. The amount of radioactivity in excreta on Day 3 measured 23.5% of the dose. After Day 4, the rate of increase declined to a value of 0.3% of the dose by Day 10. This value remained steady until the end of the experiment at which time a total of 73.8% of the original dose had been recovered in the excreta (Fig. 1).

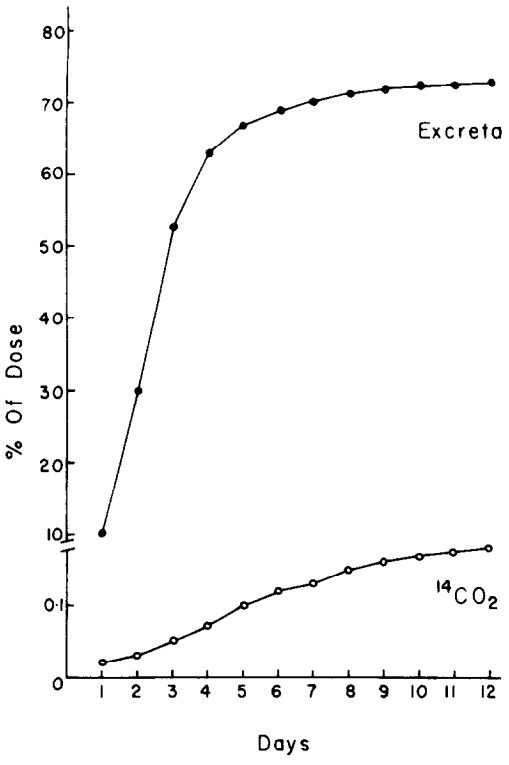

FIG. 1. Accumulated total ^{14}C in the excreta and expired $^{14}CO_2$ from hens given a single 10-mg/kg oral dose (2.73 μCi/kg) of [^{14}C]EPN.

Metabolism of [^{14}C]EPN in excreta. In this study, excreta were subjected to sequential enzymatic and acid hydrolysis and ethyl acetate extraction to determine the nature of the ^{14}C conjugated materials (Table 3). Ethyl acetate extracted radioactivity prior to any treatment of the excreta (non-conjugated material) was 11% on the first day. This value decreased with time and was 1.8% on Day 5. It seems that nonconjugated ^{14}C materials on Day 1 were primarily the materials from the oral dose

TABLE 2

CUMULATIVE PERCENTAGE RECOVERY OF ^{14}C FROM HENS GIVEN A SINGLE 10 mg/kg ORAL DOSE (2.73 μCi/kg) OF [^{14}C]EPN

Days	Expired air[a]	Excreta	Tissues[b]	Contents of gastrointestinal tract	Total
0.5	0.01 ± 0.00	3.15 ± 0.31	10.29 ± 1.02	75.80 ± 5.37	89.25 ± 6.7
2	0.03 ± 0.01	31.13 ± 7.78	11.76 ± 1.20	42.40 ± 3.30	85.32 ± 12.29
4	0.07 ± 0.02	64.61 ± 13.25	10.20 ± 0.71	18.54 ± 1.66	93.42 ± 15.64
8	0.15 ± 0.02	73.29 ± 15.56	3.98 ± 0.39	1.31 ± 0.12	78.73 ± 16.09
12	0.17 ± 0.08	73.81 ± 16.11	1.92 ± 0.19	0.20 ± 0.02	76.10 ± 16.40

[a] Each value is the mean ± SE of the daily eliminated ^{14}C from birds used (15, 12, 9, 6, or 3 birds for 0.5, 2, 4, 8, or 12 days, respectively).

[b] Each value is the mean ± SE of the ^{14}C in tissue contents of three hens per time period.

TABLE 3

CLASSIFICATION OF METABOLITES[a] OF ^{14}C EXTRACTED FROM HEN EXCRETA

Day	Non-conjugated (Ethyl acetate extractable)	Hot-acid hydrolyzed	Water-soluble	Residual ^{14}C in excreta
1	11.8	32.2	5.7	51.0
2	6.8	5.1	7.9	80.1
3	5.9	6.6	6.6	80.8
4	2.8	0.4	2.0	94.8
5	1.8	0.5	2.6	95.1
6	5.3	1.1	7.8	85.8
7	10.2	0.9	6.3	82.6
8	14.5	1.7	10.1	73.7
9	26.1	0.8	26.0	47.1
10	24.1	3.2	33.2	39.5
11	52.8	2.5	38.7	6.0
12	48.6	2.0	49.4	0.0

[a] Extraction procedure is described under Methods. Results are expressed as percentage of total ^{14}C present in excreta.

that had not been absorbed from the gastrointestinal tract. This assumption is supported by the fact that 58% of nonconjugated ^{14}C recovered on Day 1 was identified as EPN (Table 4). The decrease in nonconjugated ^{14}C between Days 2 and 6 suggests that orally administered [^{14}C]EPN was absorbed, metabolized, and conjugated. However, as time passed, the nonconjugated ^{14}C materials increased and accounted for 48.6% of the ^{14}C excreted on Day 12. These results suggest that hydrolysis of the conjugated or bound ^{14}C materials must have taken place in the gastrointestinal tract. This conclusion is supported by the finding that EPN accounted for only 2% of the nonconjugated fraction on Day 12, and the remaining ^{14}C was identified as EPN metabolites (Table 4).

Only traces of conjugated sulfate (less than 0.1%) and no glucuronide were detected in the excreta. Hot-acid hydrolyzed ^{14}C materials that accounted for 32.2% of the ^{14}C in excreta on Day 1 sharply dropped on the following day and remained low throughout the experiment. It is plausable that these conjugates were protein- or peptide-bound materials containing phenylphosphonothioic acid and/or metabolites.

The water-soluble fraction initially accounted for a small amount of ^{14}C but increased beginning on Day 6 and accounted for 49.4% of ^{14}C on Day 12. These results agree with the hypothesis that the ^{14}C initially excreted is mostly the unabsorbed [^{14}C]EPN.

TABLE 4

TOTAL[a] EPN AND METABOLITES[b] IN NONCONJUGATED CLASS METABOLITES[b] OF ^{14}C EXTRACTED FROM HEN EXCRETA[c]

Days	Total μg excreted in intervals						
	PPA	EPPA	EPPTA	Desethyl EPNO	Desethyl EPN	EPNO	EPN
1	1.88 ± 0.2	4.6 ± 0.4	1.75 ± 0.2	3.05 ± 0.3	1.46 ± 0.1	4.63 ± 0.4	24.34 ± 2.1
2	13.04 ± 1.1	25.69 ± 2.4	23.71 ± 2.0	14.01 ± 1.2	15.02 ± 1.5	49.01 ± 4.5	55.73 ± 5.1
3	43.76 ± 4.1	13.42 ± 1.2	8.53 ± 0.7	40.59 ± 3.7	13.58 ± 1.1	30.95 ± 3.0	9.63 ± 0.9
4	50.21 ± 4.6	41.32 ± 3.9	17.97 ± 1.6	29.75 ± 2.8	2.08 ± 0.1	2.19 ± 0.2	1.48 ± 0.1
5	6.09 ± 0.6	3.17 ± 0.2	1.56 ± 0.1	2.08 ± 0.2	2.08 ± 0.1	2.19 ± 0.2	1.48 ± 0.1
6	1.83 ± 0.1	1.15 ± 0.1	1.01 ± 0.1	0.93 ± 0.1	0.68 ± 0.0	1.81 ± 0.1	1.54 ± 0.1
7	0.32 ± 0.0	9.35 ± 0.6	2.34 ± 0.2	1.89 ± 0.1	0.72 ± 0.0	1.28 ± 0.1	1.73 ± 0.1
8	3.57 ± 0.3	1.84 ± 0.1	1.01 ± 0.1	2.41 ± 0.2	1.66 ± 0.1	2.70 ± 0.2	1.01 ± 0.1
9	0.29 ± 0.0	0.35 ± 0.0	0.72 ± 0.0	3.62 ± 0.3	1.18 ± 0.1	0.66 ± 0.0	1.04 ± 0.1
10	3.07 ± 0.2	1.04 ± 0.1	0.86 ± 0.0	1.25 ± 0.1	1.08 ± 0.1	1.37 ± 0.1	0.92 ± 0.1
11	3.09 ± 0.3	2.51 ± 0.2	1.76 ± 0.1	1.56 ± 0.1	1.17 ± 0.1	1.69 ± 0.1	1.13 ± 0.1
12	1.09 ± 0.1	1.44 ± 0.1	0.99 ± 0.1	0.99 ± 0.1	1.08 ± 0.1	1.91 ± 0.2	0.11 ± 0.0

[a] Results are expressed as μg of EPN and metabolites. Each value represents the mean ± SE of six determinations for excreta from three hens.

[b] Abbreviations are listed under Methods.

[c] Extraction procedure is described under Methods.

Later, EPN is absorbed, metabolized, and conjugated.

Considerable amounts of ^{14}C remained in the excreta following sequential hydrolysis and extraction. Although the nature of these ^{14}C-bound materials is not known, we suggest that the residual ^{14}C materials in the excreta represent organophosphorus bound peptides and/or proteins that were not hydrolyzed or extracted.

The total contents of EPN and some metabolites in the nonconjugated fraction of excreta extract are shown in Table 4. In addition to EPN, there were six metabolites which were identified. EPN comprised most of the nonconjugated fraction on Day 1, but declined to a small amount by Day 12. EPN oxon accounted for a small percentage of the nonconjugated fraction at Day 1 and generally increased. The general trend was not surprising; the amounts of the metabolites increased as the parent compound EPN decreased. Table 5 presents the total amounts of EPN and metabolites in the hot acid-hydrolyzable fraction of excreta extract. Here, as before, there were EPN and six metabolites. All metabolites mentioned in the nonconjugated fraction were also present in varying degrees. By contrast, in the hot acid-hydrolyzable fraction, the level of EPN was already low on Day 1 and decreased further to trace amounts by Day 2. The remainder of the radioactivity was distributed among the aforementioned metabolites with PPA and EPPA being the largest metabolites.

Radioactivity in tissues and gastrointestinal tract contents. Table 2 shows that the radioactivity in the tissues was about 10% at 12 hr and steadily declined to about 2% at Day 12. In the gastrointestinal tract contents, the amount at 12 hr was about 76%, decreasing to about 0.2% by Day 12.

The highest concentration of ^{14}C was found in the liver followed by bile, kidney, adipose tissue, and muscle (Table 6). Of nervous tissues, sciatic nerve contained the highest concentration of radioactivity and the spinal cord the least with the brain being intermediate. The concentration of ^{14}C in most tissues reached its maximum 2 days after administration.

The contents of the ventriculus and large and small intestines achieved the highest concentration on Day 2 (data not shown). The cecum content had its highest concentration of radioactivity on Day 4.

Pharmacokinetic data. While EPN disappeared exponentially from all analyzed tissues

TABLE 5

TOTAL[a] EPN AND METABOLITES[b] IN THE HOT ACID-HYDROLYZED CLASS METABOLITES OF ^{14}C EXTRACTED FROM HEN EXCRETA[c]

	Total μg excreted in intervals						
Days[d]	PPA	EPPA	EPPTA	Desethyl EPNO	Desethyl EPN	EPNO	EPN
1	66.24 ± 6.1	45.01 ± 4.3	1.71 ± 0.2	2.81 ± 0.2	3.79 ± 0.3	1.10 ± 0.1	1.59 ± 0.1
2	83.33 ± 8.1	60.51 ± 5.5	0.59 ± 0.0	0.88 ± 0.0	0.73 ± 0.0	0.27 ± 0.0	0.88 ± 0.0
3	33.03 ± 3.2	127.0 ± 10.1	4.97 ± 0.4	2.66 ± 0.2	4.62 ± 0.4	3.90 ± 0.3	1.77 ± 0.1
4	0.16 ± 0.0	0.13 ± 0.0	0.07 ± 0.0	0.52 ± 0.0	0.55 ± 0.0	1.22 ± 0.1	0.09 ± 0.0
5	2.86 ± 0.2	1.25 ± 0.1	0.23 ± 0.0	0.26 ± 0.0	0.26 ± 0.0	0.62 ± 0.0	0.14 ± 0.0
6	0.51 ± 0.0	0.51 ± 0.0	0.14 ± 0.0	0.25 ± 0.0	0.16 ± 0.0	0.27 ± 0.0	0.14 ± 0.0
7	0.36 ± 0.0	0.56 ± 0.0	0.09 ± 0.0	0.09 ± 0.0	0.25 ± 0.0	0.11 ± 0.0	0.05 ± 0.0
8	0.67 ± 0.0	0.34 ± 0.0	0.11 ± 0.0	0.20 ± 0.0	0.10 ± 0.0	0.20 ± 0.0	0.07 ± 0.0

[a] Results are expressed as μg of EPN and metabolites. Each value represents the mean ± SE of six determinations for extracta from three hens.

[b] Abbreviations are listed under Methods.

[c] Extraction procedure is described under Methods.

[d] Samples in missing days (9–12) did not contain enough radioactivity for STLC or HPLC analysis.

TABLE 6

CONCENTRATION OF RADIOACTIVITY[a] IN VARIOUS TISSUES AND BODY FLUID OF HENS GIVEN A SINGLE 10 mg/kg ORAL DOSE (2.73 μCi/kg) OF [^{14}C]EPN[a]

Specimen	Days after administration				
	0.5	2	4	8	12
Brain	244 ± 25	661 ± 79	606 ± 57	268 ± 27	63 ± 6
Spinal cord	177 ± 19	190 ± 21	145 ± 4	75 ± 11	53 ± 5
Sciatic nerve	363 ± 35	469 ± 52	359 ± 56	58 ± 9	57 ± 6
Lung	591 ± 60	586 ± 70	336 ± 34	62 ± 7	38 ± 4
Heart	94 ± 11	431 ± 43	236 ± 23	60 ± 6	29 ± 2
Red blood cells	51 ± 5	224 ± 39	165 ± 16	50 ± 4	30 ± 2
Plasma	82 ± 10	293 ± 35	234 ± 21	21 ± 3	6 ± 0
Liver	1574 ± 147	5,564 ± 561	5,587 ± 473	1035 ± 97	290 ± 30
Bile	969 ± 91	25,295 ± 2,391	15,179 ± 139	86 ± 8	35 ± 3
Kidney	934 ± 98	3,444 ± 361	2,296 ± 318	348 ± 35	1146 ± 14
Muscle	1094 ± 110	533 ± 28	373 ± 28	248 ± 25	121 ± 2
Adipose tissue	761 ± 2	847 ± 91	688 ± 70	506 ± 56	152 ± 5
Skin	570 ± 59	508 ± 29	420 ± 45	309 ± 15	165 ± 6

[a] Values are expressed as dpm/g fresh tissue, dpm/ml plasma and other body fluids. Each value is a mean ± SE of six samples from three hens.

(Table 7), it disappeared biexponentially from plasma. Half-life values for EPN were (days): brain, 2.58; spinal cord, 8.65; sciatic nerve, 7.7; plasma, 16.5; liver, 2.4; and kidney, 7.1. The total clearance of EPN was 2.63 ml/min, and the apparent volume of distribution was 90 liters or 60 liters/kg. The relative residences (R_R) of EPN in the various tissues were: brain, 0.08; spinal cord, 1.67; sciatic nerve, 2.09; muscle, 1.41; adipose tissue; 6.2; liver, 0.003; and kidney, 0.29.

EPN and metabolites in the tissues. In this study, EPN and its metabolites were identified in the purified extracts from the brain, spinal cord, sciatic nerve, plasma, liver, and kidney of birds killed 2 days after administration. The sequential thin-layer chromatography and high-performance liquid chromatography in combination with scintillation counting were used to identify EPN and its metabolites. Recovery of ^{14}C ranged from 93% in the sciatic nerve to 21% in the liver. Most of the ^{14}C in the nervous tissues was identified as EPN. All six metabolites identified in the excreta accounted for the remaining portion of ^{14}C. With the exception of the liver other tissues showed the same general trend. The liver exhibited extensive metabolism of EPN which accounted for only a small portion of the total ^{14}C. Most of the radioactive materials in the liver were identified as the polar metabolites EPPA, EPPTA, and PPA.

DISCUSSION

An investigation into the absorption, distribution, metabolism, and elimination of a single oral 10-mg/kg dose of [^{14}C]EPN in laying hens is reported. Although prophylactic treatment with atropine sulfate protected the hens from the acute effect of this LD_{50} dose, none of the treated hens laid eggs after the treatment. Similar results have been reported for the structurally related insecticide leptophos (Abou-Donia and Preissig, 1976). The fact that none of the birds developed delayed neurotoxicity agrees with our earlier finding that this dose does not cause delayed neurotoxicity in hens (Abou-Donia and Graham, 1978). It is realized, however, that the pharmacokinetic profile of smaller subtoxic doses

TABLE 7

CONCENTRATION OF EPN[a] AND METABOLITES[a] IN TISSUES[b] OF HENS AFTER A SINGLE ORAL DOSE OF 20.0 mg/kg [^{14}C]EPN

Days	Concentration (ng/g fresh tissue)[b]							
	PPA	EPPA	EPPTA	DE-EPNO	DE-EPN	EPNO	EPN	% of EPN
Brain								
0.5	60.2 ± 6	49.6 ± 5	98.8 ± 10	27.3 ± 3	20.4 ± 2	63.3 ± 6	758.1 ± 81	70.34
2	88.5 ± 9	75.7 ± 8	43.4 ± 4	54.5 ± 6	62.4 ± 6	55.6 ± 7	621.8 ± 62	60.06
4	43.9 ± 5	86.6 ± 9	31.7 ± 3	50.5 ± 7	38.2 ± 4	45.2 ± 6	309.7 ± 39	51.12
8	38.8 ± 4	48.2 ± 5	42.1 ± 4	22.7 ± 3	22.5 ± 2	32.1 ± 4	118.3 ± 12	36.44
12	83.5 ± 9	48.8 ± 6	43.4 ± 5	34.8 ± 4	30.8 ± 3	22.6 ± 3	40.0 ± 5	14.93
Spinal cord								
0.5	77.6 ± 8	110.4 ± 12	105.4 ± 11	53.9 ± 5	19.4 ± 2	90.1 ± 10	609.9 ± 62	57.18
2	19.2 ± 2	45.8 ± 5	64.7 ± 6	53.7 ± 6	68.8 ± 8	85.9 ± 9	475.2 ± 48	58.44
4	94.0 ± 10	108.3 ± 11	18.0 ± 2	23.4 ± 3	42.0 ± 5	80.6 ± 9	1512.8 ± 160	80.51
8	102.3 ± 11	51.6 ± 5	50.9 ± 6	53.5 ± 6	70.1 ± 8	70.1 ± 7	1010.1 ± 112	71.71
12	74.7 ± 8	102.8 ± 12	44.8 ± 5	36.3 ± 4	84.1 ± 9	60.8 ± 7	797.3 ± 81	66.40
Sciatic nerve								
0.5	102.2 ± 11	48.4 ± 8	81.8 ± 8	83.9 ± 8	30.5 ± 31	28.9 ± 3	2126.7 ± 230	85.01
2	87.9 ± 9	116.9 ± 12	27.3 ± 3	128.0 ± 13	49.2 ± 5	64.5 ± 7	2487.1 ± 251	84.00
4	279.2 ± 30	261.8 ± 29	135.7 ± 14	133.7 ± 14	47.8 ± 5	116.6 ± 12	2200.6 ± 216	69.30
8	30.7 ± 4	58.8 ± 6	19.5 ± 2	36.9 ± 4	25.4 ± 3	43.3 ± 4	1634.1 ± 170	88.39
12	15.5 ± 2	16.0 ± 2	7.0 ± 1	14.4 ± 2	18.0 ± 2	13.8 + 2	1004.4 ± 111	92.74
Plasma								
0.5	166.9 ± 18	152.8 ± 19	37.5 ± 4	22.8 ± 3	26.5 ± 3	49.8 ± 6	724.3 ± 7	61.35
2	14.7 ± 2	35.5 ± 4	13.4 ± 2	25.2 ± 3	21.1 ± 2	19.2 ± 2	300.5 ± 31	69.95
4	6.3 ± 1	31.1 ± 4	8.2 ± 1	66.8 ± 7	4.2 ± 0	17.5 ± 2	280.1 ± 29	67.62
8	18.4 ± 2	44.0 ± 5	18.4 ± 2	27.2 ± 3	24.8 ± 3	13.8 ± 2	230.7 ± 24	61.14
12	12.4 ± 1	45.5 ± 5	12.8 ± 1	35.2 ± 4	21.2 ± 3	11.5 ± 2	119.3 ± 12	46.26
Liver								
0.5	56.5 ± 6	440.6 ± 45	341.6 ± 35	394.1 ± 41	343.0 ± 35	357.5 ± 36	20.7 ± 2	1.06
2	45.6 ± 5	294.8 ± 31	358.0 ± 36	401.0 ± 40	4.9 ± 0	92.0 ± 10	20.14 ± 2	1.68
4	31.2 ± 3	67.2 ± 7	80.2 ± 8	155.1 ± 16	37.8 ± 4	45.0 ± 5	13.5 ± 2	3.14
8	77.6 ± 8	72.7 ± 8	38.6 ± 4	43.2 ± 5	34.2 ± 4	12.7 ± 1	3.7 ± 0	1.31
12	204.6 ± 21	81.7 ± 9	17.6 ± 2	32.5 ± 4	12.1 ± 1	3.1 ± 0	1.2 ± 0	0.34
Kidney								
0.5	73.3 ± 8	95.3 ± 10	150.8 ± 16	262.1 ± 30	57.2 ± 6	42.0 ± 5	512.5 ± 53	42.95
2	7.8 ± 8	35.8 ± 4	71.3 ± 8	100.8 ± 11	13.3 ± 2	35.2 ± 4	406.3 ± 42	60.60
4	22.7 ± 3	23.0 ± 3	26.7 ± 3	28.9 ± 3	46.8 ± 5	30.5 ± 4	350.9 ± 36	66.27
8	15.4 ± 2	29.8 ± 3	45.5 ± 5	13.2 ± 2	5.4 ± 1	18.7 ± 2	259.8 ± 27	74.91
12	89.9 ± 9	27.1 ± 3	18.0 ± 2	14.5 ± 2	15.4 ± 2	12.1 ± 2	149.4 ± 16	60.88

[a] Abbreviations are listed under Methods.

[b] Extraction recoveries of EPN and metabolites were: brain, 52.4%; spinal cord, 52.0%; sciatic nerve, 93.2%; plasma, 60.7%; liver, 21.9%; and kidney, 24.3%.

[c] Concentrations were calculated from the recovered radioactivity from TLC plates and are expressed as nanograms of EPN or metabolite per gram of fresh tissues or milliliter of plasma. Each value represents the mean ± SE of six determinations from three animals.

[d] Percentage of all metabolites.

of EPN might be different than that found for LD_{50} (Chrzanowski and Jelinek, 1981; Abou-Donia *et al.*, 1983a).

The results indicate that metabolism was the major route of clearance of orally administered EPN because little unchanged EPN was found in combined urinary–fecal excreta. The liver seems to play a major role in the metabolism of EPN, since this organ contained most of the ^{14}C content of all visceral tissues. EPN was metabolized with great efficiency since it was removed rapidly from the liver. Although the radioactivity in the urine alone was not determined, the results suggest that in the hen, bile plays a more important role than the kidney in the excretion of EPN and metabolites. This conclusion is supported by the results that the bile contained more concentration of ^{14}C than the kidney at most time intervals. The relatively high concentration of EPN in the kidney may be attributed to extensive binding of EPN to plasma that results in low glomerular filtration of the insecticide (Weiner *et al.*, 1960). Also, nonionic absorption and passive reabsorption of EPN in the renal tubule (Milne *et al.*, 1958) are perhaps increased by the insecticide's high lipid solubility (partition coefficient between octanol and water is 200,000, Davies *et al.*, 1975).

The disappearance of EPN from plasma, unlike that from most tissue, was apparently biexponential with a terminal half-life of 16.5 days. The large value for apparent volume of distribution (60 liters/kg) indicates extensive extravascular distribution and suggests high tissue affinity for the insecticide.

The accumulation and persistence of EPN, as well as its relative pool size in various tissues compared to the plasma, are indicated by its relative residence (R_R) values in these organs relative to plasma. Adipose tissue had the highest R_R value, followed by the muscle which is consistent with the function of these tissues as temporary depots for EPN in the chicken's body (Abou-Donia, 1981). EPN is released, with time, from the storage tissues to the plasma, and transferred to liver for metabolism, then excreted through the kidney. As a result, both kidney and liver had low R_R values, reflecting their function in the metabolism and elimination of EPN.

Among the nervous tissues, EPN in the spinal cord had the longest apparent half-life followed by the sciatic nerve, then the brain. R_R values, however, were largest for sciatic nerve, followed closely by the spinal cord, and the brain had the least value. This difference in the R_R values is consistent with the differences in the sensitivity of nervous tissues to histopathologic lesions produced by a neurotoxic dose of EPN. Most damage is seen in the sciatic nerve and spinal cord. In the brain, histopathologic lesions mostly occur in the medulla; the upper brain stem, cerebellum, and cerebrum are unchanged (Abou-Donia. *et al.*, 1983b). The large R_R value found for the sciatic nerve may explain the vulnerability of this nervous tissue to EPN-induced lesions.

Metabolism of EPN is carried out by oxidation and/or hydrolysis. All of the metabolites shown in Fig. 2 were detected in the tissues and excreta. EPN was oxidized to EPN oxon, the only known metabolite to be more potent than EPN, both as a cholinergic and as a delayed neurotoxic chemical (Abou-Donia, 1981). EPN oxon may be deethylated to desethyl EPN oxon, then hydrolyzed to phenylphosphonic acid (PPA) as it has been shown *in vitro* (Lasker *et al.*, 1982). PPA may also be formed by the hydrolysis of EPN and the oxidation of the hydrolytic product.

Considerable amounts of ^{14}C in excreta cannot be attributed to glucuronides or sulfates since only a very small percentage was found to belong to these two classes of conjugates. Most conjugated ^{14}C was hydrolyzed with hot sulfuric acid or remained in the aqueous solution as water soluble metabolites.

EPN, like many other aromatic organophosphorus neurotoxicants (Abou-Donia, 1981), needs metabolic activation and undergoes biochemical detoxification. In the rat (a nonsusceptible species), EPN was rapidly detoxified (Chrzanowski and Jelinek, 1981), while it was metabolized to a lesser extent by the cat (a susceptible species) (Abou-Donia *et*

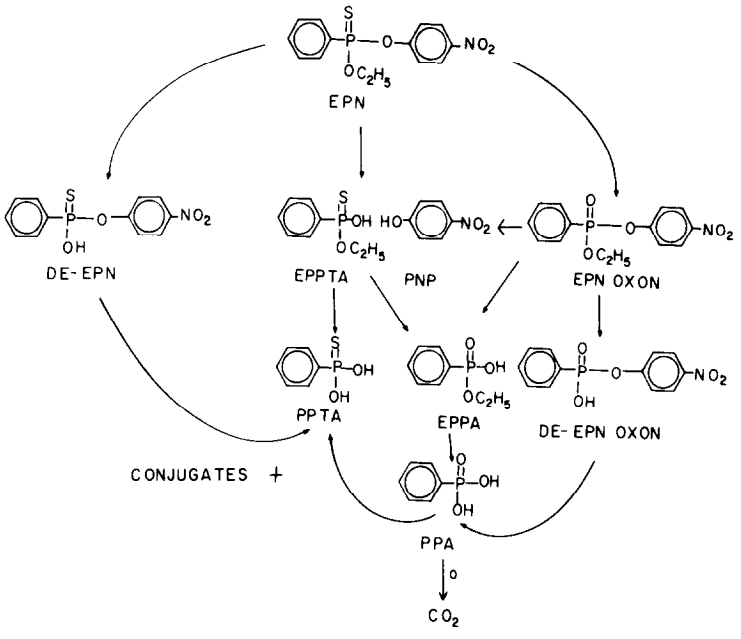

FIG. 2. Suggested EPN metabolic pathways in hens following a single oral dose of 10 mg/kg EPN.

al., 1983c, e). Furthermore, the mouse (Nomeir and Dauterman, 1979) and rat (Lasker *et al.*, 1982) hepatic microsomes metabolized EPN *in vitro* faster than the chicken (Lasker *et al.*, 1982). This study indicates that a single oral toxic dose of EPN has a long elimination half-life and exhibits high relative residence (R_r) of EPN in tissues relative to plasma. This report confirms the earlier hypothesis that pharmacokinetics and metabolism of organophosphorus compounds may play a prominent role in species selectivity to OPIDN (Abou-Donia, 1981, 1983).

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