Metabolism of Phenyl Carbon-14-Labeled *O*-Ethyl *O*-(4-Nitrophenyl) Phenylphosphonothioate in the Rat and in Hens at Toxic and Subtoxic Dose Levels

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Differences in the rate of metabolism of EPN in the hen and rat have been observed. Unmetabolized EPN was found in the feces of hens dosed at 4 and 50 mg/kg, whereas none could be found from a rat dosed at 17 mg/kg. When a single subtoxic oral dose was given to either rat or hen, most of the radioactivity was eliminated within 72 h as *O*-ethylphenylphosphonothioic acid, *O*-ethylphenylphosphonic acid, *O*-ethyl-4-hydroxyphenylphosphonic acid, *O*-ethyl-3-hydroxyphenylphosphonic acid, phenylphosphonothiolic acid, and phenylphosphonic acid. However, when a toxic dose of EPN was given to atropinized hens, unmetabolized EPN was found to represent a significant portion of the residual radioactivity in all tissues after 72 h. The synthesis of [^{14}C]EPN and its metabolites is also described.

EPN [*O*-ethyl *O*-(4-nitrophenyl) phenylphosphonothioate] insecticide has been used successfully on cotton for over a quarter of a century, and, during this period, no incidence of delayed neurotoxicity has been reported. Also, in studies with mammalian species, EPN has not been found to produce delayed neurotoxicity [e.g., in rats (Hodge et al., 1954; Suzuki, 1973) and in man (Moeller and Rider, 1962)]. However, delayed neurotoxicity has been reported in hens given daily subtoxic oral doses of EPN (Abou-Donia and Graham, 1978). The purpose of this study was to determine the fate of [^{14}C]EPN in hens and rats and to determine if metabolic differences might explain observed hen neurotoxicity. This paper also describes the synthesis of ^{14}C-labeled EPN and metabolites which were used in these studies.

EQUIPMENT AND METHODS

All radioactive liquid samples were measured by liquid scintillation counting (LSC) in premixed scintillation cocktail (Formula 947, New England Nuclear) using an Isocap 300 scintillation counter (Searle Analytic). Quenching corrections were made by internal standard procedures employing [^{14}C]toluene. The radioactivity of homogenized solid samples was determined by combustion analysis (CA) in a Model 306 sample oxidizer (Packard Intruments Co.), followed by LSC.

The ^{14}C-labeled metabolites in urine, feces, and tissue extracts were separated for quantitation by thin-layer chromatography (TLC) on 250-μm silica gel chromatoplates with fluorescent indicator UV_{254} (Brinkmann Instruments Co.) developed for 15 cm in the solvent systems hexane–ethyl acetate (1:1 v/v) and acetonitrile–water–concentrated ammonium hydroxide (40:9:1 v/v/v) (Abou-Donia and Ashry, 1978). Cochromatography with known EPN hydrolysis and oxidation products was used to indicate the nature of compounds found (Figure 1). Nonradioactive standards were visualized by UV irradiation. Radioactive bands were located with a Berthold Model 6000-2 automatic TLC radioscanner (Varian-Aerograph). Examination of the separated radioactive components was also performed by autoradiography using SB-5 X-ray film (Kodak). The amount of radioactivity in each polar band was determined by scraping the silica gel from the plate, eluting the radioactivity with distilled water, and counting aliquots by LSC. Nonpolar bands were eluted with ethyl acetate before counting aliquots by LSC. Silica gel was removed from the samples by centrifugation on a Universal Model UV centrifuge (International Equipment Co.).

Nuclear magnetic resonance (NMR) spectra were determined in deuterated chloroform by using tetramethylsilane as the internal standard, on a Varian T-60 spectrometer at 60 MHz.

Methylated derivatives of urine and feces metabolites were isolated by preparative TLC on 1000-μm silica gel GF chromatoplates (Brinkmann Instruments Co.) using the solvent ethyl acetate. Methylated standards were cochromatographed for comparison of R_f values. The ^{14}C-labeled materials were removed from the silica gel by successive washings with ethyl acetate.

Gas chromatography of methylated derivatives was performed with an F and M Model 810 gas chromatograph equipped with a flame ionization detector and a 2 to 1 splitter which permitted ^{14}C monitoring of the column effluent. The GC column was 1.8 m × 4 mm glass packed with 3% OV-17 on 80–100-mesh Chromosorb W-HP (Supelco, Inc., Bellefonte, PA). The temperature was programmed from 150 to 280 °C at 10 °C/min with a flow rate of 60 mL/min helium. All mass spectra were obtained with a Du Pont Model 21-492 mass spectrometer coupled to a Perkin-Elmer Model 990 gas chromatograph. Extracts containing EPN were analyzed by GC/MS using a 1.8 × 4 mm glass column packed with 10% OV-1 on Chromosorb W-HP under programmed temperature from 150 to 280 °C.

EXPERIMENTAL SECTION

Synthesis of Radiolabeled *O*-Ethyl *O*-(4-Nitrophenyl) [^{14}C]Phenylphosphonothioate ([^{14}C]EPN). The synthesis of [^{14}C]EPN is outlined in Figure 2 and consists of the following reactions.

(*A*) *Phenylphosphorus Thiodichloride* (*Jensen, 1953*). A mixture of [^{14}C]benzene (107 mg, 1.4 mmol; 23.5 mCi; New England Nuclear), nonradioactive dried benzene (750 mg, 9.7 mmol), phosphorus trichloride (4.8 g, 35 mmol), and anhydrous aluminum chloride (1.7 g, 1.3 mmol) was heated at reflux for 3 h and then cooled to 40 °C. Sublimed sulfur (380 mg, 11.9 mmol) was added to the mixture, which was heated at 80 °C for 10 min and then cooled to room temperature. This mixture was poured into a water–ice mixture (100 g) and the crude product extracted 3 times with 25-mL portions of methylene chloride. The extracts were combined, dried over anhydrous sodium sulfate, and evaporated to a residue with a stream of nitrogen. The crude product was extracted from the residue

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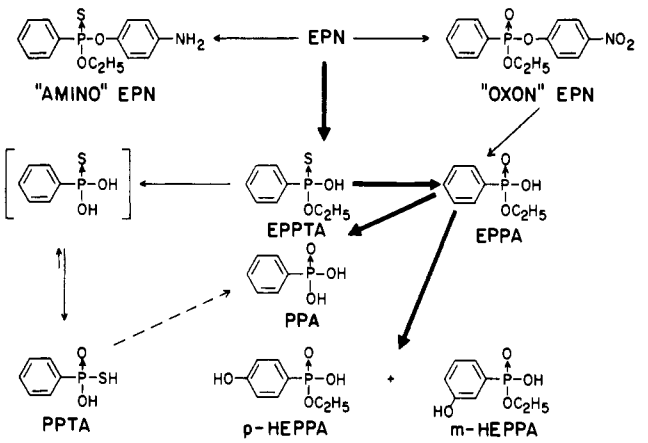

Figure 1. Metabolism of EPN insecticide in rats and hens.

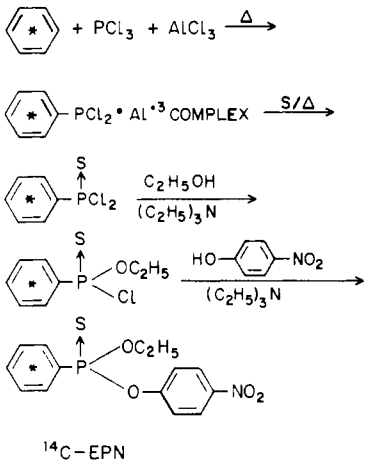

Figure 2. Synthesis of [^{14}C]EPN. (*) denotes position of ^{14}C label.

with cyclohexane to remove insoluble impurities, filtered, and evaporated to yield 1.65 g of a clear, colorless oil.

(*B*) [^{14}*C*]*EPN*. Triethylamine (0.9 g, 10.3 mmol) and 4.9 mL of dry benzene were added to this intermediate with constant stirring. The temperature was maintained at 28 ± 2 °C while a mixture of absolute ethanol (350 mg, 7.6 mmol) and 3 mL of dry benzene was added dropwise over 1 h. Stirring was continued at this temperature for another hour and then at 38 °C for an additional 15 min. *p*-Nitrophenol (1.04 g, 7.5 mmol) was added at once; then triethylamine (820 mg, 8.1 mmol) was added dropwise over 10 min. Finally, the mixture was heated at reflux with stirring for 45 min and cooled to room temperature.

Benzene was removed by evaporation at room temperature and ~10 mL each of methylene chloride and water was added. The organic layer was separated, washed twice with aqueous 5% potassium carbonate and once with water, and then dried with magnesium sulfate, filtered, and evaporated under vacuum to give 2.5 g of crude liquid product.

Purified [^{14}C]EPN (1.5 g, 4.85 mmol; sp act. 1.64 mCi/mmol) was obtained by preparative liquid chromatography using a 1 m × 15 mm i.d. column packed with 37–75-μm Porasil A silica particles and the solvent system hexane–dioxane (90:10 v/v) as the mobile phase. Radiochemical purity was greater than 99.5% as determined by TLC analysis on silica gel chromatoplates.

Synthesis of Postulated Metabolites. (*A*) *O-Ethyl O-(4-Nitrophenyl) Phenylphosphonate* ("*Oxon*" *EPN*). This compound was prepared by the method of Jelinek (1954). The product is a light amber colored oil, n^{27}_D = 1.5669 (lit. n^{27}_D = 1.5678).

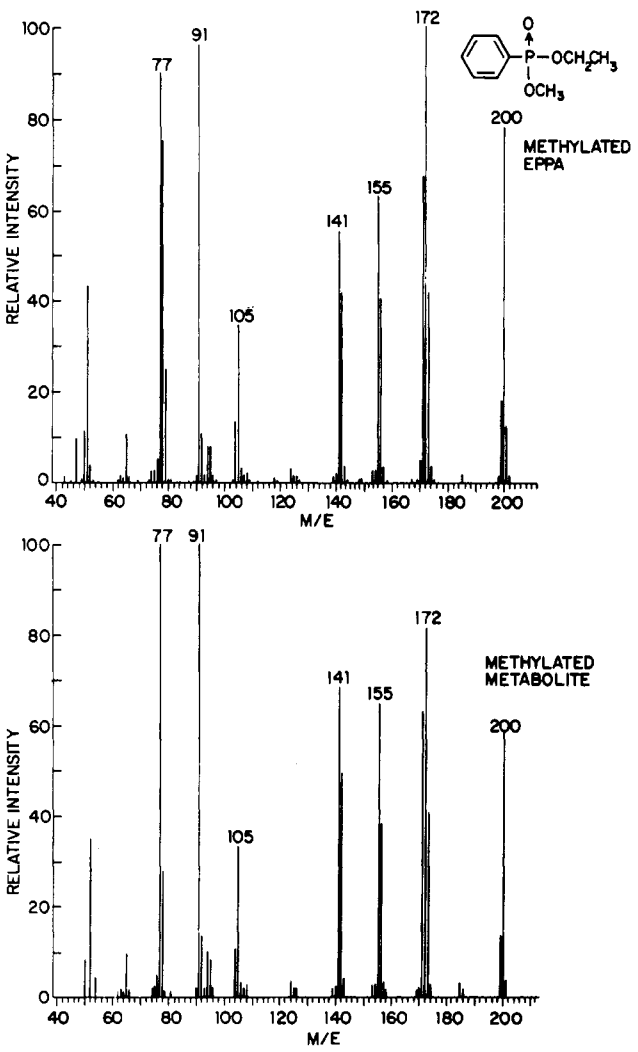

Figure 3. Mass spectra of *O*-ethyl *O*-methyl phenylphosphonate.

(*B*) *O-Ethyl O-(4-Aminophenyl) Phenylphosphonothioate* ("*Amino*" *EPN*). This compound was prepared by the reduction of EPN with zinc and hydrochloric acid as described by Ahmed et al. (1958).

(*C*) *O-Ethylphenylphosphonic Acid* (*EPPA*). Diethyl phenylphosphonate was hydrolyzed with excess of 10% aqueous sodium hydroxide according to the method of Rabinowitz (1960) to yield a coloreless oily product, n^{25}_D = 1.5224. The dicyclohexylamine salt gave mp 140–141.5 °C (lit. mp 140.7–141.8 °C).

(*D*) *O-Ethylphenylphosphonothioic Acid* (*EPPTA*). This compound was synthesized similarly by aqueous alkaline hydrolysis of diethyl phenylphosphonothioate (Rabinowitz, 1960), yielding a colorless oil, n^{25}_D = 1.5635. The dicyclohexylamine salt gave mp 148–151 °C (lit. mp 151.5–154.0 °C).

Methylation of Postulated Metabolites. A 500-mg sample of each postulated free acid metabolite was dissolved in 5 mL of dry methanol at 25 °C. Diazomethane (1.5 g) in ether at 0 °C was added to each sample and allowed to stand at ambient temperature for 2 h. Concentration under dry nitrogen gave 90+% yields of *O*-ethyl *O*-methyl phenylphosphonate (methylated EPPA) and *O*-ethyl *O*-methyl phenylphosphonothioate (methylated EPPTA). *O,O*-Dimethyl phenylphosphonate (methylated PPA) was synthesized by the methylation of phenylphosphonic acid (PPA) (Fisher Scientific). Combination gas chromatography/mass spectrometry (GC/MS) produced mass patterns consistent with the proposed compounds (Figures 3, 4, and 5).

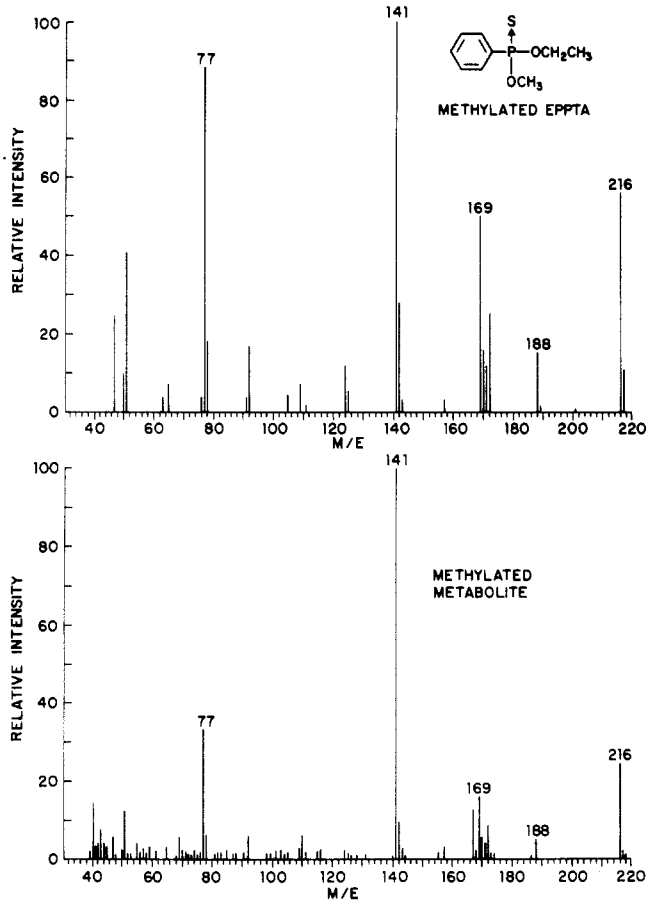

Figure 4. Mass spectra of *O*-ethyl *O*-methyl phenylphosphonothioate.

S-Methyl O-Methyl Phenylphosphonothiolate (Methylated PPTA). Methanol (7.1 g, 222 mmol) was added dropwise to a solution of dichlorophenylphosphine sulfide (18.5 g, 87 mmol) (Eastman, practical grade) and triethylamine (36 mL, 250 mmol) in 75 mL of dry benzene with constant stirring while maintaining the temperature at 30 ± 5 °C by external cooling. The mixture was heated under reflux for 15 h and then cooled to room temperature. A precipitate formed which was filtered from the cooled mixture and dissolved in 50 mL of water. This solution was made strongly alkaline (pH >10) by the dropwise addition of 50% aqueous sodium hydroxide and extracted twice with 50-mL portions of methylene chloride which was discarded. The aqueous phase was made acid (pH <3) with 6 N HCl and extracted 3 times with 50-mL portions of methylene chloride. The organic solvent extracts were combined, dried over anhydrous magnesium sulfate, and filtered, and the solvent was evaporated in a rotary evaporator, yielding a light amber oil of *S*-methyl phenylphosphonothiolic acid: NMR peaks at δ 1.95–2.18 (d, 3 H, SCH_3) and 7.3–8.0 (5 H, ArH). The dicyclohexylamine salt gave mp 176.5–178.5 °C. Anal. Calcd for $C_{19}H_{32}NO_2PS$: C, 61,8; H, 8.73; N, 3.80. Found: C, 61.8; H, 8.95; N, 3.74.

Methylation of this oil with diazomethane gave >90% yield of methylated PPTA. GC/MS produced a mass pattern consistent with the proposed structure, i.e., m/e^+ 202 and 155 ($m/e^+ - SCH_3$) (Figure 6).

O-Ethyl O-Methyl 4-Methoxyphenylphosphonate (Methylated p-HEPPA). Aqueous alkaline hydrolysis of diethyl 4-methoxyphenylphosphonate [prepared from 4-bromoanisole and triethyl phosphite by the procedure of Tavs (1970)] yielded a colorless, viscous oil product of *O*-ethyl-4-methoxyphenylphosphonic acid: NMR peaks

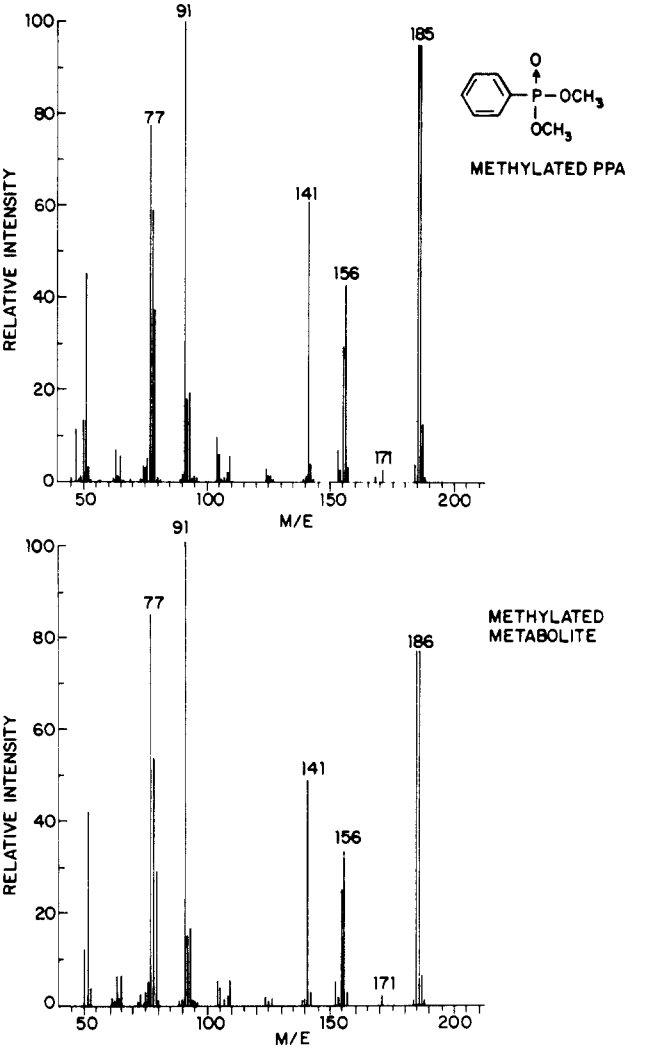

Figure 5. Mass spectra of *O,O*-dimethyl phenylphosphonate.

at δ 1.10–1.35 (t, 3 H, OCH_2CH_3), 3.75 (s, 3 H, OCH_3), 3.80–4.15 (q, 2 H, OCH_2), and 6.8–7.9 (4 H, ArH). Dicyclohexylamine salt gave mp 126–128 °C. Anal. Calcd for $C_{21}H_{36}NO_4P$: C, 63.5; H, 9.07; N, 3.53. Found: C, 63.4; H, 9.12; N, 3.52.

Methylation of this oil with diazomethane gave >90% yield of methylated *p*-HEPPA: GC/MS mass pattern showed m/e^+ 230, 202 ($m/e^+ - C_2H_4$), 186 ($m/e^+ - C_2H_5 - CH_3$), and 171 ($m/e^+ - CH_2CH_3 - 2CH_3$) (Figure 7).

O-Ethyl O-Methyl 3-Methoxyphenylphosphonate (Methylated m-HEPPA). In a similar manner, diethyl 3-methoxyphenylphosphonate (bp 132–134 °C/1.0 mmHg, prepared from 3-bromoanisole and triethyl phosphite) was hydrolyzed with aqueous sodium hydroxide to give a colorless, viscous oil product of *O*-ethyl-3-methoxyphenylphosphonic acid: NMR peaks at δ 1.1–1.35 (t, 3 H, OCH_2CH_3), 3.70 (s, 3 H, OCH_3), 3.80–4.15 (q, 2 H, OCH_2), and 6.9–7.45 (4 H, ArH). Dicyclohexylamine salt gave mp 126–128 °C. Anal. Calcd for $C_{21}H_{36}NO_4P$: C, 63.5; H, 9.07; N, 3.53. Found: C, 63.4; H, 9.12; N, 3.52.

Methylation of this product with diazomethane gave >90% yield of methylated *m*-HEPPA. GC/MS showed a pattern consistent with this structure (Figure 8).

Animal Study. A male rat (Charles River—CD) was preconditioned for 21 days on a diet of ground Purina Laboratory Chow fortified with 450 ppm of technical (85% pure) EPN and 1% corn oil. The rat, weighing 318 g, was then dosed by gastric intubation with 17.3 mg/kg, 27.8 μCi [LD_{50} = 42 mg/kg (Hodge et al., 1954)], [^{14}C]EPN in 2 mL

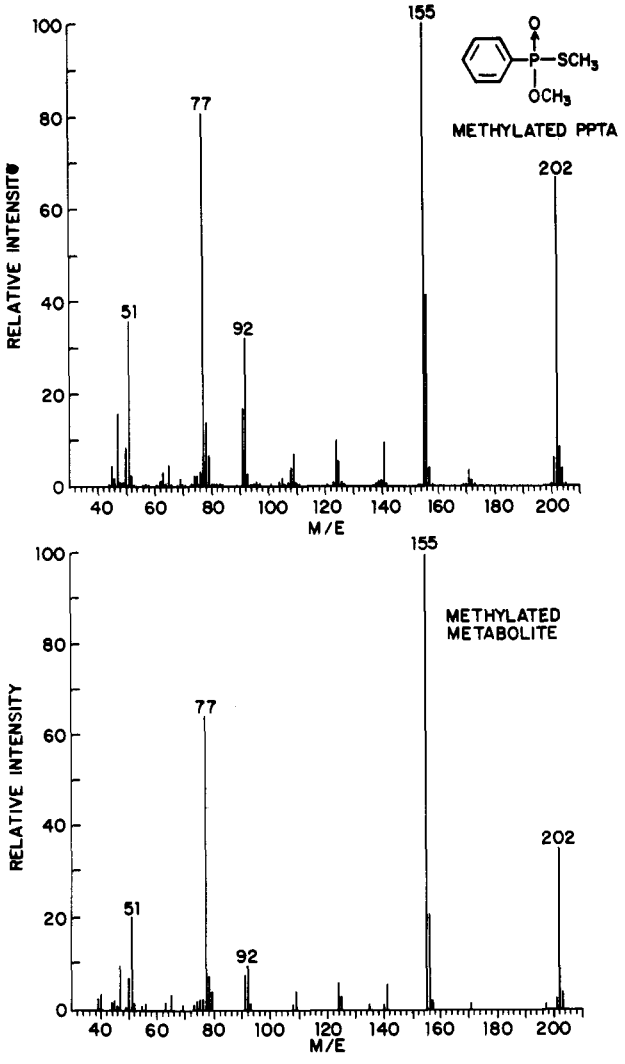

Figure 6. Mass spectra of *S*-methyl *O*-methyl phenylphosphonothiolate.

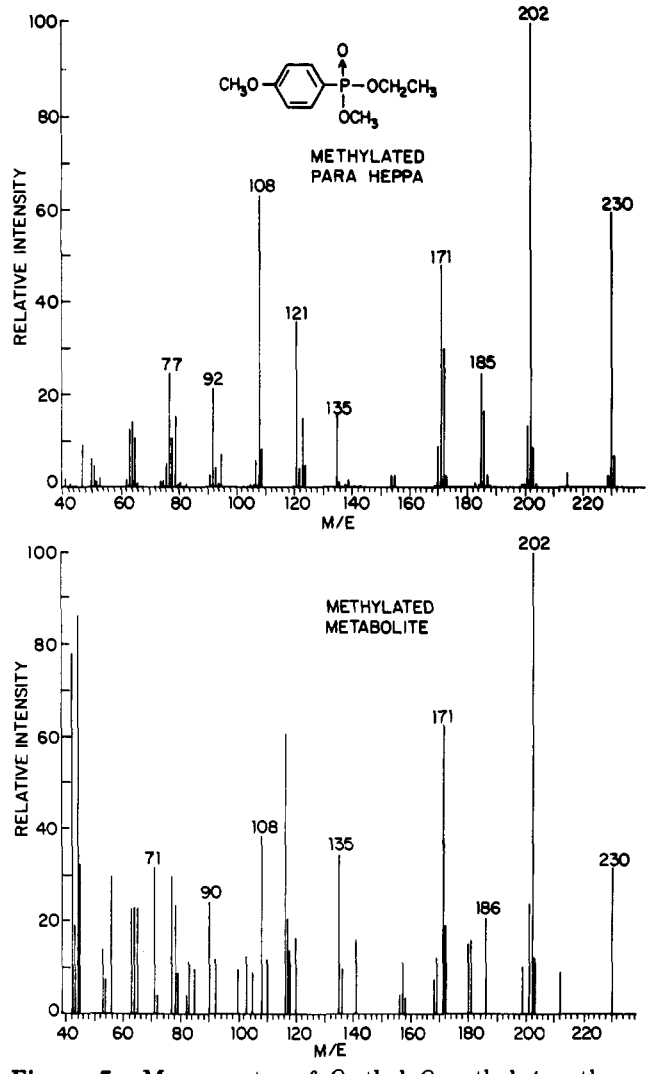

Figure 7. Mass spectra of *O*-ethyl *O*-methyl 4-methoxyphenylphosphonate.

of acetone–corn oil (10:90 v/v). After treatment, the rat was placed in an enclosed glass metabolism unit for 72 h, and respiratory gases, animal tissues, and excreta were sampled for analysis as described by Chrzanowski et al. (1979).

A White Leghorn hen, weighing ~1.5 kg, was given daily oral doses of technical EPN in capsules at the rate of 2.5 mg/kg [LD_{50} = 10 mg/kg (Abou-Donia and Graham, 1978)]. Twenty-one days later, this animal and a "fresh" (not preconditioned) hen were given a single oral encapsulated dose of [^{14}C]EPN (4 mg/kg; 30.6 μCi). In a comparative experiment, two atropinized (1 mg/kg intramuscularly) White Leghorn hens, weighing ~1.7 kg, were given a single oral encapsulated neurotoxic dose of [^{14}C]-EPN (50 mg/kg; 152.7 μCi). All hens used in these tests were 1 year old. The hens were immediately placed in separate open metabolism cages with stainless steel pans under them for the 72-h test period. Three 24-h feces samples were taken by scraping the pans daily. Collected feces were stored frozen until analysis.

After the 72-h test period, all animals were lightly anesthetized with chloroform, and blood was removed from the heart by a syringe and needle. The animals were sacrificed and the following organs were removed and weighed: brain, heart, lung, liver, spleen, kidney, and gastrointestinal tract. The spinal cord was excised from the backbone (chicken only), and the remaining carcasses were frozen with the tissues and feces. Metabolism cages were rinsed with a dilute detergent wash, and these samples were frozen along with the others pending ^{14}C distribution analysis.

Tissues and feces samples were lyophilized and homogenized by grinding with a mortar and pestle. Aliquots of the lyophilized tissue and excreta samples were analyzed for ^{14}C residues by CA.

Metabolite Identifications. Feces (5–20 g) were extracted 5 times with 100-mL portions of acetone–methanol–water (1:1:1 v/v/v) by using ultrasonic agitation. The extractions removed most of the radioactivity (87–96%). The extracts were combined and the unextracted residue was separated by centrifugation, air-dried, and assayed by CA. Total ^{14}C in the extract was determined by LSC, and aliquots were cochromatographed by TLC with standards of known EPN hydrolysis and oxidation products to indicate the identity of the metabolites found. Radiolabeled compounds from the extract, corresponding to the retention value (R_f) of the standards, were removed by scraping and extraction with the appropriate solvent and analyzed by LSC. The metabolities were isolated for structural confirmation by the purification and derivatization procedures that follow.

The feces extract was reduced to ~200 mL of volume under vacuum at 25 °C (mostly water) and partitioned 3 times with 200-mL portions of toluene.

The toluene extracts were combined, counted for total ^{14}C by LSC, and reduced to a few milliliters of volume

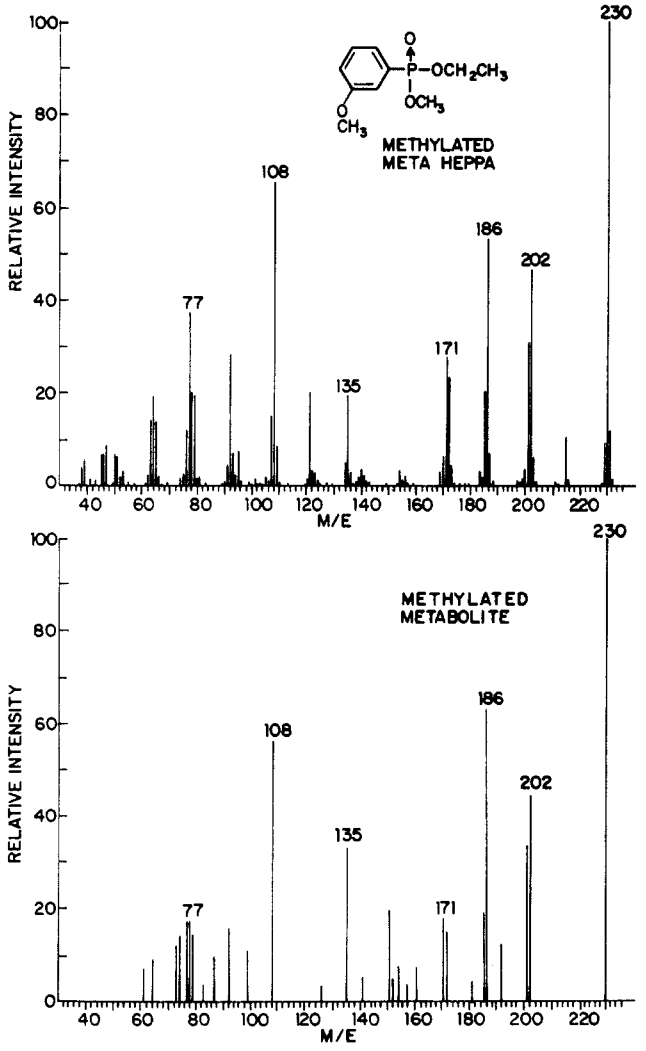

Figure 8. Mass spectra of *O*-ethyl *O*-methyl 3-methoxyphenylphosphonate.

under vacuum at 45 °C. Aliquots of the concentrate were cochromatographed on TLC with authentic standards of EPN, oxon EPN and amino EPN. [^{14}C]EPN was isolated from this fraction by preparative TLC on silica gel GF chromatoplates and the identity confirmed by GC/MS. Very polar ^{14}C-containing compounds (origin of TLC chromatoplates) were removed by scraping and extraction with water and then added to the above remaining aqueous feces extract.

The combined water extract was reduced to ~10 mL and acidified by passing it through a cation-exchange column (50–100-mesh Dowex 50-W, X-8, Bio-Rad Laboratories). The 4.5 × 50 cm column had been activated by washing with excess 1 N HCl, followed by distilled water. The acidic metabolites were eluted with 1 L of distilled water, evaporated to dryness under vacuum at 40 °C, and redissolved in 10 mL of methanol. Diazomethane (1.5 g) in ether at 0 °C was added and the mixture allowed to stand at ambient temperature for 2 h. The mixture was then evaporated to dryness under dry nitrogen, and the methylated ^{14}C-containing metabolities were separated by preparative TLC on silica gel chromatoplates. Purified fractions were quantitated by LSC and structures confirmed by GC/MS.

Urine was analyzed in the same manner as the feces except that solvent extraction was unnecessary. The samples were applied directly to TLC plates and ion-exchange columns for quantitation and metabolite identification.

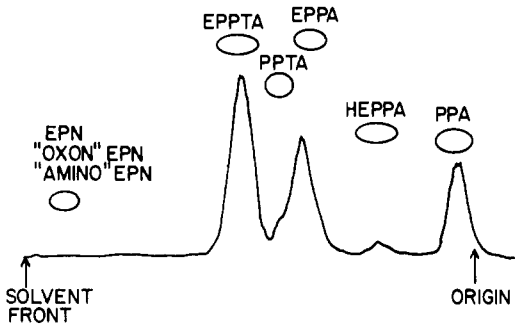

Figure 9. Rat urine 24-h sample; radioscan of TLC plates (silica gel G; acetonitrile–water–concentrated ammonium hydroxide, 80:18:2 v/v/v).

Tissue Analysis. Whole lyophilized lung, kidney, and liver and 10-g aliquots of fat and muscle were ultrasonically extracted 3 times with 50-mL portions of acetone–methanol–water (1:1:1 v/v/v). The combined extracts were centrifuged to remove solids which were combusted to determine unextracted ^{14}C. A 1-mL aliquot of the combined extract was counted by LSC, and the remainder of the extract was concentrated to a residue under vacuum at 35 °C. The residue was redissolved in 50 mL of water and partitioned with two 50-mL portions of ethyl acetate. The ^{14}C-labeled metabolites in these fractions were characterized by comparing their TLC behavior on silica gel with that of authentic standards of the candidate metabolites.

RESULTS AND DISCUSSION

When a male rat, which had been preconditioned on a diet containing 450 ppm of EPN, was dosed by intragastric intubation with 17 mg/kg (28 μCi) radiolabeled EPN, 87% of the dose was eliminated within 72 h. Approximately 29% of the administered dose was recovered from the feces and 58% from the urine (Table I). The greatest amount (53.4%) was excreted during the first 24 h. No radioactivity (<0.01%) was found in the exhaled air, and only small amounts of radioactivity were detected in the gastrointestinal tract (0.4%), carcass (1.1%), hide (5.6%), and internal organs (0.2%). Total recovery of applied radioactivity was 95%.

Thin-layer cochromatographic analysis of first-day rat urine showed five major, polar metabolites, three of which corresponded to standards of EPPTA (44%), EPPA (26%), and PPA (23%). The remaining two metabolites are most likely PPTA (3.4%) and HEPPA (2.8%), although no standards were available to confirm their TLC behavior. Their positions on TLC plates are conjectural. No radioactivity could be located at the R_f of standard EPN, oxon EPN, or amino EPN. A representative TLC radioscan of rat urine is shown in Figure 9.

Isolation and methylation of the urinary metabolites followed by TLC analysis produced a pattern of radioactivity shown in Figure 10. These areas of radioactivity cochromatographed with methylated standards of EPPTA, EPPA, PPTA, and PPA. After isolation of these methylated metabolites from preparative TLC plates, GC analysis of the radioactive material coupled with counting the column effluent showed retention times of 4.3 min for *O,O*-dimethyl phenylphosphonate, 4.9 min for *O*-ethyl *O*-methyl phenylphosphonate, 6.7 min for *S*-methyl *O*-methyl phenylphosphonothiolate, and 7.1 min for *O*-ethyl *O*-methyl phenylphosphonothioate. The identity of these metabolites was confirmed by combination GC/MS with standard reference compounds for comparison. Mass spectral data are shown in Figures 3, 4, 5, and 6. The identity of *O*-ethyl *O*-methyl methoxyphenylphosphonate

Table I. Distribution of Radioactivity after Treatment of Animals with [^{14}C]EPN

location	% of original treatment: rat (17 mg/kg)	fresh hen (4 mg/kg)	preconditioned hen (4 mg/kg)	atropinized hens (50 mg/kg) 1	atropinized hens (50 mg/kg) 2
external fractions					
prefurnace gas trap	<0.01				
postfurnace gas trap	<0.01				
subtotal	(<0.01)				
urine, 0-24 h	49.7				
urine, 24-48 h	7.08				
urine, 48-72 h	1.37				
subtotal	(58.10)				
feces, 0-24 h	3.74	62.9	76.8	6.66	10.1
feces, 24-48 h	9.74	18.6	9.44	17.9	19.2
feces, 48-72 h	12.61	5.27	6.47	8.42	20.2
cage wash	3.27	7.18	7.18	29.7	18.3
subtotal	(29.4)	(93.9)	(99.9)	(62.7)	(67.8)
body fractions					
hide	5.64				
carcass	1.08	0.35	0.26	4.39	4.13
G.I. tract	0.40	0.97	2.12	14.0	17.7
lungs	0.01	<0.01	<0.01	0.31	0.87
liver	0.14	1.43	0.75	0.64	0.57
kidney	0.03	0.26	0.03	0.06	0.07
spleen	<0.01	<0.01	<0.01	0.01	0.01
blood	<0.01	<0.01	<0.01	0.05	0.03
internal eggs				0.01	0.01
brain	<0.01	<0.01	<0.01	<0.01	<0.01
spinal cord		<0.01	<0.01	<0.01	<0.01
heart	<0.01	0.01	<0.01	0.02	0.01
testes	0.03				
subtotal	(7.33)	(3.01)	(3.17)	(19.5)	(23.4)
total recovery	94.8	96.9	103	82.2	91.2

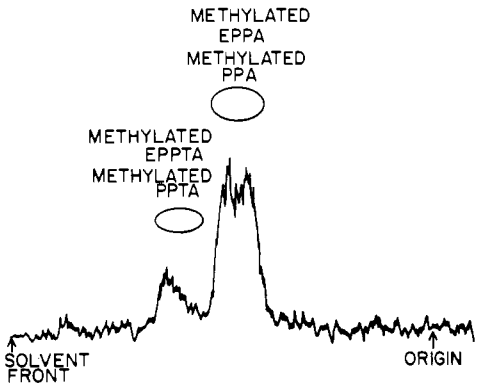

Figure 10. Methylated ^{14}C-labeled metabolites from rat urine 24-h sample; radioscan of TLC plates (silica gel G; ethyl acetate).

in methylated rat urine was not confirmed by GC/MS although the presence of the parent compound HEPPA was indicated by TLC analysis of fresh urine.

During this study, EPPTA was found to be somewhat unstable and was shown to partially oxidize to EPPA during isolation procedures. The conversion was slow, however, and did not greatly influence the metabolite distribution found.

Cochromatography of the extractable feces metabolites with standards again showed only five metabolites which had behavior identical with those isolated from the urine. No EPN, oxon EPN, or amino EPN (<0.01%) was found. Examination of the isolated, derivatized fecal metabolites by GC/MS confirmed four of the compounds as methylated EPPTA, PPTA, EPPA, and PPA.

The quantitative fecal and urinary excretion pattern for all sampling intervals is summarized in Table II. The administered [^{14}C]EPN was completely hydrolyzed and oxidized to polar acidic metabolites which were rapidly eliminated from the rat (at least 91%) within 72 h.

In a similar study, Bradway et al. (1977) reported that no detectable amounts of EPN (<0.01 ppm) were found after 3 days in urine and blood, or after 8 days in adipose tissue, of rats dosed orally with EPN (0.1 of the LD_{50}).

When hens, either fresh or preconditioned, were treated orally with a subtoxic dose (4 mg/kg) of radiolabeled EPN, 94–100% of the administered radioactivity was eliminated within 72 h (Table I). The bulk of this ^{14}C (~70%) was excreted during the first 24 h after dosing. No significant differences were found in the excretion pattern for fresh and preconditioned hens.

Small amounts of residual radioactivity (~3%) were found in the carcasses and internal organs of both hens after 72 h with the majority being in the GI tract and liver. Only trace amounts of radioactivity were found in the brains (~0.028 ppm) and spinal cords (0.012 ppm), which were comparable to the levels found in muscle as shown in Table III.

At a toxic dose level (50 mg/kg), an average of 65% of the administered radioactivity was found in the excreta and cage washes after 72 h (Table I). The excretion pattern was altered somewhat from the low-dose animals in that the administered dose was not entirely eliminated during the 72-h posttreatment period. Most of the remaining radioactivity (~16%) was found in the gastrointestinal tract (Table I).

Separation of the solvent-extractable ^{14}C-labeled metabolites from hen excreta by TLC showed the same five polar metabolites found in the previous rat study (49–60% of original dose), in addition to intact EPN (4–10%) and small amounts of oxon EPN and/or amino EPN (0.1–0.8%). Table IV summarizes the quantitative excretion pattern obtained for the hens.

Table II. Radioactivity Identified in Rat Feces and Urine

fraction	% of original dose						
	EPN	oxon EPN and/ or amino EPN	EPPTA	PPTA	EPPA	*m*-HEPPA and/ or *p*-HEPPA	PPA
urine, 0–24 h	<0.01	<0.01	22	1.7	13	1.4	11.6
feces, 0–24 h	<0.01	<0.01	2.5	0.31	0.5	0.05	0.76
subtotal	(<0.01)	(<0.01)	(24.5)	(2.01)	(13.5)	(1.45)	(12.4)
urine, 24–48 h	<0.01	<0.01	0.05	0.03	1.8	1.2	4.0
feces, 24–48 h	<0.01	<0.01	4.2	0.22	0.35	0.35	1.7
subtotal	(<0.01)	(<0.01)	(4.25)	(0.25)	(2.15)	(1.55)	(5.7)
urine, 48–72 h	<0.01	<0.01	0.02	0.01	0.30	0.48	0.56
feces, 48–72 h	<0.01	<0.01	5.5	0.06	0.52	0.59	1.90
subtotal	(<0.01)	(<0.01)	(5.52)	(0.07)	(0.82)	(1.07)	(2.46)
total recovery	<0.01	<0.01	34.3	2.33	16.5	4.07	20.6

Table III. Radioactive Residue Level in Selected Hen Tissues

tissue	residue level, ppm of EPN			
	fresh (4 mg/kg)	preconditioned (4 mg/kg)	atropinized hen no. 1 (50 mg/kg)	atropinized hen no. 2 (50 mg/kg)
fat	0.002	0.001	2.34	2.22
breast muscle	0.011	0.015	0.47	0.59
leg muscle	0.016	0.016	1.28	0.81
brain	0.021	0.035	0.81	0.78
spinal cord	0.010	0.014	1.28	1.93
lungs	0.120	0.109	20.6	57.7
liver	5.71	3.10	23.1	21.7
kidney	6.55	2.85	13.9	9.10

Representative TLC radioscans of excreta extracts (50 mg/kg dose) are shown in Figures 11 and 12. The unidentified nonpolar ^{14}C-labeled compounds (2.9%) observed in these TLC scans are thought to be hydroxylated phenyl analogues of EPN based on TLC behavior although no standards were available to confirm their identity. Also, no differences in the types of metabolites formed at the two dose levels could be demonstrated; only quantitative differences were found.

Residual EPN and metabolites EPPTA, EPPA, meta and para isomers of HEPPA, and PPA were isolated from excreta, derivatized, and confirmed by GC/MS as previously described. The retention time for *O*-ethyl *O*-methyl 4-methoxyphenylphosphonate is 7.6 min and for *O*-ethyl *O*-methyl 3-methoxyphenylphosphonate is 7.4 min. The main peaks in the mass spectra are consistent with ions produced from authentic standards of the two methylated isomers of hydroxyphenylphosphonic acid (Figures 7 and 8).

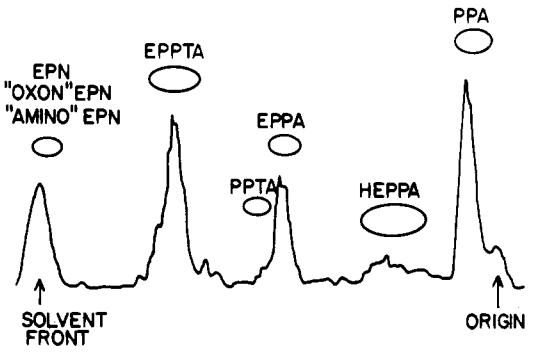

Figure 11. Acetone–methanol–water extract of 24–48-h hen excreta; radioscan of TLC plate (silica gel G; Acetonitrile–water–ammonium hydroxide, 80:18:2 v/v/v).

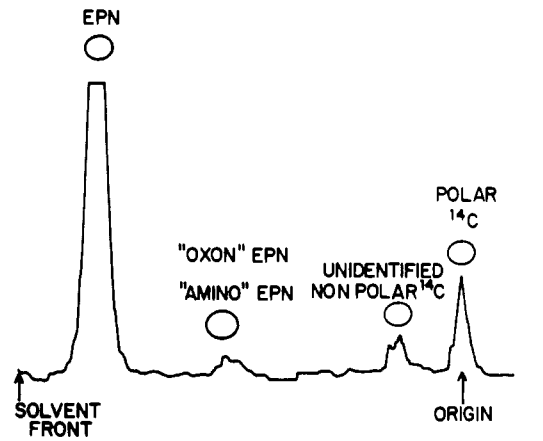

Figure 12. Toluene extract of 24–48-h hen excreta; radioscan of TLC plate (silica gel; hexane–ethyl acetate, 50:50 v/v).

The levels of oxon EPN and/or amino EPN and PPTA found (1.1% total) were too low to allow confirmation by

Table IV. Radioactivity Identified in Hen Excreta

fraction	% of original dose						
	EPN	oxon EPN and/or amino EPN	EPPTA	PPTA	EPPA	*m*- and *p*-HEPPA	PPA
fresh (4 mg/kg)							
0–24-h excreta	4.53	0.14	17.0	1.32	15.1	4.54	10.8
preconditioned (4 mg/kg)							
0–24-h excreta	3.87	0.17	1.31	1.69	35.7	5.56	15.5
atropinized hen no. 2 (50 mg/kg)							
0–24-h excreta	1.36	0.04	2.37	0.01	2.33	0.47	2.17
24–48-h excreta	5.22	0.24	4.33	0.02	2.22	1.11	3.95
48–72-h excreta	2.41	0.17	5.77	0.02	2.39	1.50	6.03
cage wash	1.37	0.34	6.39	0.27	1.45	1.92	6.56
total recovery	10.4	0.79	18.9	0.32	8.39	5.0	18.71

Table V. Characterization of ^{14}C-Labeled Residues in Atropinized Hen No. 2 Tissues

compound	% of ^{14}C-labeled residual				
	fat	muscle	lungs	liver	kidney
EPN	65	21	75	0.1	0.9
oxon and/or amino EPN	0.1	0.1	0.9	0.2	0.2
unidentified nonpolar ^{14}C	0.1	0.1	1.8	0.9	0.9
unidentified polar ^{14}C	0.1	55	3.0	0.1	0.1
EPPTA	4.7		1.0	3.7	10
PPTA	<0.1	<0.1	<0.1	<0.1	<0.1
EPPA	11		0.3	12	4.0
m- and *p*-HEPPA	2.5		0.1	0.1	0.1
PPA	13		3.8	31	50
unextracted residue	3.6	24	14	52	34
total	100	100	100	100	100

GC/MS. Therefore, their TLC behavior was used exclusively to establish identity.

Cochromatographic analysis of extracts of tissues and organs was restricted to those from the high-dose hens since they contained practical working levels of ^{14}C. Characterization of the ^{14}C-labeled residues by TLC cochromatography is given in Table V. The major ^{14}C-labeled compound found in fat and lung tissue was EPN (~70%). Only trace amounts (<1%) of EPN were found in liver or kidneys. Most ^{14}C-labeled residues in these two organs were polar or bound (unextracted) metabolites. The bulk of the ^{14}C-labeled metabolites extracted from muscle appeared to be substituted phenylphosphonic acid analogues of EPN, but due to matrix effects, poor resolution of individual metabolites was achieved. Consequently, all polar radioactivity found in muscle was listed in Table V as "unidentified polar ^{14}C".

Higher than proportional levels of radioactive residues were found in hen tissues and organs at the high-dose level compared to the low dose (Table III). The greatest increases occurred in the fat (~1500-fold increase) and lungs (~340-fold increase) while most other tissues had from 28- to 123-fold increases of ^{14}C-labeled residue. The livers and kidneys of the hens, however, showed comparable levels of ^{14}C-labeled residues at both dose levels. This finding, coupled with the slower excretory rate at the high-dose level, indicated that the 50 mg/kg dose exceeded the metabolic capacity of the liver for detoxification of EPN. Thus, EPN remained long enough in the high-dosed hen to enter the fat and other tissues and organs. Although no proportional buildup of ^{14}C-labeled residues could be demonstrated in nerve tissue compared to buildup in other tissues, the ^{14}C levels which were observed (~0.8 ppm in brain and ~1.6 ppm in spinal cord) in the high-dose hens may contribute to the delayed neurotoxicity reported by Abou-Donia and Graham (1978).

CONCLUSIONS

No differences were found in the types of metabolites formed in rats or hens dosed with [^{14}C]EPN. However, greatly different rates of metabolism were demonstrated with respect to dose level in the hen. Whereas a sublethal dose was very quickly eliminated by either rat or hen as polar nontoxic metabolites, a single lethal dose in atropinized hens was retained much longer. As a consequence of this apparent saturation of the detoxification mechanisms, EPN remained in circulation for a sufficient time to be found in the hen tissues after 72 h.

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