Table V. Dioxin Content in Blood, Body Fat, and Milk Fat of Cow 2 and Calf 165 Days after End of PCP Dosing[a]

tissue	dioxins, ppb 1,2,3,-6,7,8-HCDD	1,2,3,4,-6,7,8-HpCDD	OCDD	dioxins
blood				
cow	0.012	0.015	0.020	0.047
calf	0.027	0.014	0.006	0.047
body fat				
cow (shoulder)	4.8	11.1	6.1	22.0
calf (hind quarter)	2.3	1.9	0.5	4.7
milk fat (cow)	2.2	4.4	3.3	9.9

[a] Samples collected 14 days after calving (165 days after end of PCP dosing) at which time the animals were sacrificed.

thologic syndrome suggestive of dioxin and/or furan intoxication.

No clinical evidence of toxicosis associated with PCP administration was observed during the course of the study. Clinical findings will be discussed in a separate report.

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Identification of the Main Metabolite of Ethylenethiourea in Mice

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The degradation of 2-imidazolidinethione (ethylenethiourea) in mice involves oxidation at the sulfur atom, giving 2-imidazolin-2-yl sulfenate as the major product. This compound was synthesized by irradiation of an aqueous solution of ethylenethiourea with Co-60 γ rays and purified from the irradiation mixture by high-pressure liquid chromatography. The synthesis was verified by TLC, IR, NMR, and mass spectrometry methods. Other possible mechanisms for degradation are discussed.

The ethylenebis(dithiocarbamates), including Maneb, Zineb, Mangozeb, Nabam, and Amobam, constitute an important class of fungicides widely used for controlling crop diseases. Ethylenethiourea (ETU), which is a contaminant in these fungicides (Bontoyan and Looker, 1973) and a primary metabolite in environmental degradation and in test organisms (Engst and Schnaak, 1974; Watts et al., 1974; Marshall, 1977) has been reported to be goitrogenic (Seifter and Ehrich, 1948; Graham and Hansen, 1972; Graham et al., 1973, 1975), carcinogenic (Innes et al.,

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1969; Ulland et al., 1972; Graham et al., 1973), mutagenic (Seiler, 1974) and teratogenic (Khera, 1973; Ruddick and Khera, 1975; Lu and Staples, 1978). Concern has accordingly been expressed about the safety of ethylenebis(dithiocarbamates), and further studies, especially on their metabolic fate in animals, have been recommended (Engst, 1977). Metabolism of ETU in cows resulted in its breakdown into several products which appeared in the milk and urine (Lyman, 1971). In rats and guinea pigs about 66 and 48%, respectively, of the administered oral dose was excreted as ETU in the urine within 48 h (Newsome, 1974). Seventy–eighty percent of the activity of 4,5-^{14}C-labeled ETU administered orally to pregnant rats was eliminated in the urine within 24 h, and the tissue levels of radioactivity from [2-^{14}C]ETU reached a maximum within 2 h before falling back to negligible levels within 24 h (Kato et al., 1976; Ruddick et al., 1976).

Ethyleneurea and bis(imidazolin-2-yl) sulfide have been identified among the degradation products of UV irradiation of ETU on a solid substrate (Cruikshank and Jarrow, 1973), and ethyleneurea and glycine sulfate in aqueous solution in the presence of dissolved oxygen and sensitizers (Ross and Crosby, 1973).

This paper details the identification of the main metabolite of ETU in mice. Labeled compounds, administered to mice or incubated with liver microsome mixed function oxidase system, have been used to evaluate the degradation chemistry of ETU.

EXPERIMENTAL SECTION

Labeled Compounds. [2-^{14}C]ETU (sp act., 5.7 μCi/mmol), [4,5-^{14}C]ETU (sp act., 10.2 μCi/mmol) and [^{35}S]ETU (sp act., 3.1 μCi/mmol) were synthesized from carbon-14 disulfide (sp act., 62 mCi/mmol, Amersham) and ethylenediamine hydrochloride, from carbon disulfide and [1,2-^{14}C]ethylenediamine hydrochloride (sp act., 15.5 mCi/mmol, Amersham) and from carbon [^{35}S]disulfide (sp act., 10.2 mCi/mmol, Amersham) and ethylenediamine hydrochloride, respectively, according to the method of Allen et al. (1946). Chemical purities of the labeled compounds (mp 198 °C, lit. 198 °C) confirmed by ^{1}H NMR and MS were higher than 95% and radiochemical purities confirmed by thin-layer chromatography (TLC) and autoradiography were higher than 98%.

Animals. [2-^{14}C]ETU, [4,5-^{14}C]ETU, and [^{35}S]ETU in physiological saline were administered orally by stomach tube to clinically healthy male NMRI mice (30 g, Oy Orion Ab, Finland). The administered dose was 67 mg/kg, and 40 mice were treated with each labeled preparation. Immediately after treatment the mice were placed in metabolic cages for collection of urine and feces samples at 6 and 24 h. During the experiments the animals received normal laboratory feed and water ad libitum. The animals were anesthetized with diethyl ether and blood samples were taken from the jugular vein. Livers and kidneys were collected after death.

Isolation of Metabolites. For the separation and identification of ETU and its metabolites, the urine samples (about 20 mL at 6 h and 100 mL at 24 h) were evaporated to 1–2 mL on a rotating vacuum evaporator and suspended in ethyl acetate (2 × 10 mL). The filtered ethyl acetate extracts were concentrated under a stream of nitrogen gas to 0.1–0.2-mL volume. The ethyl acetate unextractable portion of the original urine was lyophilized to dryness and the residue suspended in methanol (10 mL). The suspension was filtered and the solution was evaporated under a stream of nitrogen to about 0.5–1.0 mL. Both the methanol and ethyl acetate fractions were analyzed by TLC in A and B solvent systems (see below). The main metabolite of ETU was purified from the methanol fraction by high-pressure liquid chromatography (LC). The metabolite was detected by absorption at 254 nm after chromatography on a 4 mm × 30 cm μ Bondapak C_{18} column. The Waters Model 440 absorption detector was equipped with 254- and 280-nm filters. The eluting solvent was water, delivered to the column by a Waters Model M-6000 A pump at a flow rate of 2 mL/min.

Feces, liver, and kidney samples were homogenized with five volumes of ethanol/water mixture (1:1, v/v) and centrifuged. Aliquots of the homogenate were concentrated on a rotating vacuum evaporator to dryness. The residue was suspended in ethyl acetate (2 × 10 mL). After filtration the ethyl acetate solution was concentrated to 0.1 mL by evaporation. The ethyl acetate unextractable portion of the original homogenate was lyophilized and suspended in methanol (10 mL). The methanol solution was then filtered and the filtrate evaporated under a stream of nitrogen to 0.5 mL. Both the ethyl acetate and methanol fractions were analyzed by TLC. After coagulation of the blood samples, serum was separated by centrifugation and subjected to cleanup on a Sephadex G-10 column (1.5 × 30 cm). The serum was eluted with water at a flow rate of 0.5 mL/min. Fractions of 1 mL were collected. The radioactivity of the fractions was measured with a LKB Ultrobeta 1210 liquid scintillation counter from 0.1 mL of sample added to 10 mL of scintillation liquid (Instagel, Packard). Radioactive fractions were concentrated and analyzed by TLC.

Microsomal Activation Tests. The microsomal fraction with necessary cofactors (S-9 mix) was prepared as described by Ames et al. (1973) except that the livers were homogenized in a blender instead of a Potter-Elvehjem apparatus. [2-^{14}C]ETU was incubated with the mice liver microsomes (1 mL of microsomal fraction and 4 mL of 0.15 M KCl) at 37 °C for 12 h, after which the incubated mixture was evaporated on a rotating vacuum evaporator to dryness. The residue was then suspended in methanol (10 mL) and the methanol suspension was filtered, concentrated under a stream of nitrogen, and spotted for TLC analysis.

Thin-Layer Chromatography. Thin-layer chromatography was carried out on silica gel plates (Merck, 0.25 mm, F_{254} fluorescence indicator) developed in (A) ethyl acetate/methanol/ammonia (90:6:6), (B) butanol/acetic acid/water (4:1:1), (C) chloroform/methanol (93:7), (D) methanol, and (E) methanol/acetic acid (9:1) systems. For the detection of radioactive metabolites the plates were covered with X-ray film (Kodak PE 4006) for 2 months. The metabolite products were visualized using fluorescence quenching, Grote's reagent, and Ehrlich's reagent. For the quantification of radioactive metabolites 3-mm sections were scraped off the plate and put into vials containing 10 mL of Instagel (Packard). The radioactivities were measured as described above.

Synthesis of 2-Imidazolin-2-yl Sulfenate by γ Irradiation of ETU. Solutions of ETU (100 mg/50 mL) in deionized water were irradiated in nitrogen or oxygen atmosphere with Co-60 γ rays (dose rate 350 krad/h, total dose 1.5 Mrad, irradiation temperature +20 °C). The irradiated solutions were concentrated on a rotating vacuum evaporator. The amount of unaltered ETU after irradiation was determined by glass capillary gas–liquid chromatography (Hirvi et al., 1979). The radiolysis product, later identified as 2-imidazolin-2-yl sulfenate, was purified from the irradiation mixture by LC as described above.

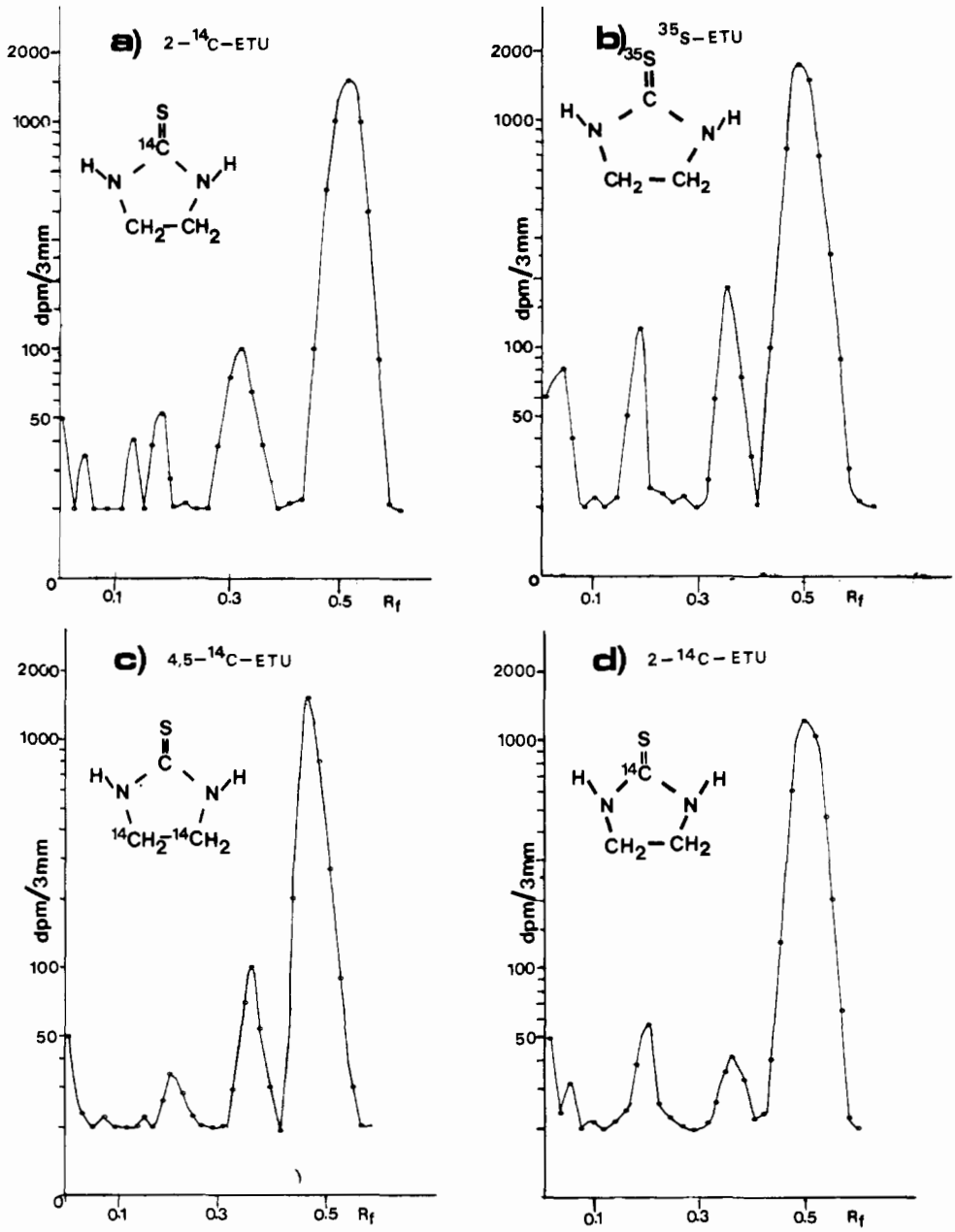

Figure 1. Thin-layer radiochromatograms of urine samples of mice (a–c) and mice liver microsomes (d). Solvent system EtAc/NH_3/H_2O (90:6:6).

Spectral Studies. Infrared (IR) spectra were obtained with a Perkin-Elmer Model 421 instrument (1 mg of the sample in 300-mg KBr disk). Electron impact mass spectra were obtained with a JMS-D100 instrument having a direct inlet system and operating at an electron energy of 75 eV. Temperature of the ion source was 200 °C. Proton nuclear magnetic resonance (^{1}H NMR) spectrum at 60 MHz was obtained with JNM-FX 60 instrument. The sample was a 1% solution in acetone-d_6 in a 10-mm o.d. tube. The chemical shift values, measured with a JFA-100 data system attached to the spectrometer, are expressed in ppm downfield from internal tetramethylsilane.

RESULTS AND DISCUSSION

Figure 1 shows radiochromatograms of the ethyl acetate fractions of urine samples of mice fed with [2-^{14}C]ETU, [4,5-^{14}C]ETU, and [^{35}S]ETU. In addition to unaltered ETU, several metabolites can be detected. TLC analysis of the methanol fractions showed exactly the same peaks for all ETU samples. Because the main metabolite contained all the labeled atoms, was a little more polar than ETU, and gave a negative reaction with Ehrlich's reagent, we suspected it to be an S-oxidation product of ETU. It has earlier been proposed by Marshall and Singh (1977) that ETU is chemically oxidized by hydrogen peroxide and hypochlorite as described in Figure 2. The R_f values of the main metabolite found here (Table I) do not agree with the R_f values for sulfinate or sulfonate [R_f 0.13 (MeOH), 0.17 (MeOH/HAc), 0.57 (MeOH), 0.62 (MeOH/HAc), respectively] reported by Marshall and Singh (1977). The failure of the same authors to oxidize ETU to the level of sulfenate with hydrogen peroxide and hypochlorite encouraged us to try oxidation through γ irradiation. When ETU was irradiated in aqueous solution in nitrogen, only one main degradation product was detected by TLC. The irradiated mixture contained 79% of the original ETU according to GLC analysis. About 2 mg of the main degradation product was purified by LC from the irradiated mixture and was identified by MS, IR, and ^{1}H NMR techniques. Characteristic peaks in the infrared spectrum were (cm^{-1}): 3400–3000 s (N–H), 2980–2840 w (CH_2), 1580 s (C=N), 1470 s (CH_2), 1260 w

Table I. TLC Characteristics of 2-Imidazolidinethione, 2-Imidazolidone, and 2-Imidazolin-2-yl Sulfenate

compound	R_f values (solvent systems) A	B	C	D	E	detection using:[a,b] E	G	Q
2-imidazolidinethione	0.50	0.54	0.86	0.64	0.78	+	blue	+
2-imidazolidone	0.20	0.40	0.38	0.56	0.70	+	–	–
2-imidazolin-2-yl sulfenate	0.35	0.63	0.54	0.67	0.84	–	pale green	+

[a] E = Ehrlich's reagent, G = Grote's reagent, Q = fluorescence quench. [b] (+) positive reaction; (A) EtAc/NH_3/H_2O (90:6:6), (B) BuOH/HAc H_2O (4:1:1), (C) $CHCl_3$/MeOH (93:7), (D) MeOH, (E) MeOH/HAc (9:1).

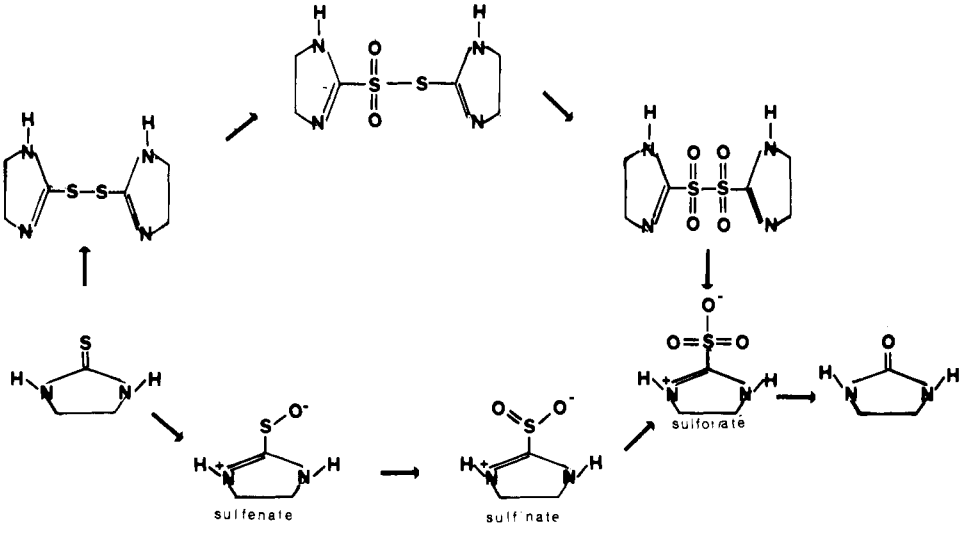

Figure 2. Chemical oxidation of ETU by hypochlorite and hydrogen peroxide proposed by Marshall and Singh (1977).

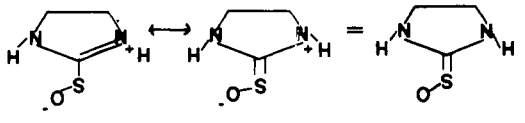

Figure 3. Structure of 2-imidazolin-2-yl sulfenate.

(S–C), 1215 m (S–C), 1065 m (S–O), 905 m, 780 w, 730 m (S–O). The greatest differences with the spectrum of ETU were the appearence of absorptions at 1580 (C=N), 1215 (S–C), and 1065, 905, 780, and 730 cm^{-1} (S–O) and the absence of absorptions at 1350, 1270, 1200 (N–C=S), and 1500 cm^{-1} (C=S).

The mass spectrum of the compound gave prominent ions at m/e 100 (100), m/e 102 (15%, $C_3N_2H_4{}^{34}S$). In a low-energy spectrum (15 eV) m/e 118 (M^+) and 117 (M^+ –1) were also observed.

In the ^1H NMR spectrum signals at δ 2.63 (s, CH_2) and 6.7 (s, NH) were detected. The mass spectrum gives a molecular formula $C_3N_2SOH_6$ for the studied compound. The abundant fragments at m/e 100 and at m/e 102 corresponding to the loss of water from the original molecule refer to a sulfur atom containing stable ions.

Furthermore, the ^1H NMR spectrum refers to the 2-imidazolin-2-yl sulfenate structure although it was not possible to determine the number of N-H protons with high accuracy. MS and ^1H NMR spectra support the ethylene thiourea *S*-oxide and the 2-imidazolin-2-yl sulfenate (Figure 3) structures. *S*-Oxides have been found in nature, for example in onions (Brodnitz and Pascale, 1971). Anal. Calcd for $C_3H_6N_2OS$: C, 30.49; H, 5.12; N, 23.71. Found: C, 30.46; H, 5.16; N, 23.00.

The main metabolite of ETU in mice had the same R_f values (in five different solvent systems, Table I), the same reactivities with Ehrlich's and Grote's reagents, and the same retention time in LC as 2-imidazolin-2-yl sulfenate. Likewise the mass spectrum of the main metabolite after purification by LC was identical with that of 2-imidazolin-2-yl sulfenate, showing fragments at m/e 100, 102, 117, and 118. Radioactivity measurements (Figure 1) showed the concentration of this metabolite to be about one-tenth that of ETU for every label. This same oxidation product of ETU was detected in serum and liver samples, though not in feces, after 6 h, by TLC. After 24 h the radioactivities in tissue samples were so low that neither ETU nor the main metabolite could be detected by TLC.

Incubation of ^{14}C-labeled ETU solution with mice liver microsome–NADPH system resulted in its breakdown to products identical with metabolites of ETU in living mice (Figure 1d). The enzyme system catalyzing the degradation of ETU is thus localized in the microsomes.

Ethyleneurea (EU) formation from ETU in mammals has been reported in many papers (Lyman, 1971; Kato et al., 1976; Ruddick et al., 1976). In Figure 1 the small radioactive peak of urine samples, present with 2-^{14}C and 4,5-^{14}C labels and absent with ^{35}S label, had the same R_f values (Table I) and reaction with Ehrlich's reagent as EU. When ETU in aqueous solution was γ irradiated under nitrogen atmosphere, the only oxidation product detected by TLC was sulfenate. However, in the presence of air, ethyleneurea was detected as well, indicating that the oxygen molecule of the sulfenate comes from the hydroxyl radical of water. To be oxidized to the level of EU, molecular oxygen is needed.

The very polar metabolites, which did not move in the A solvent system, were rechromatographed in the B system of BuOH/HAc/H_2O (4:1:1). The results obtained for [2-^{14}C]ETU and [4,5-^{14}C]ETU (Figure 4) show clearly two different radioactive peaks. The ring opening and formation of glycine suggested by Lyman (1971) was not detected in this study. The two metabolites moving in the same band as amino acids contained both ^{14}C labels.

Further study of the biological activity of 2-imidazo-

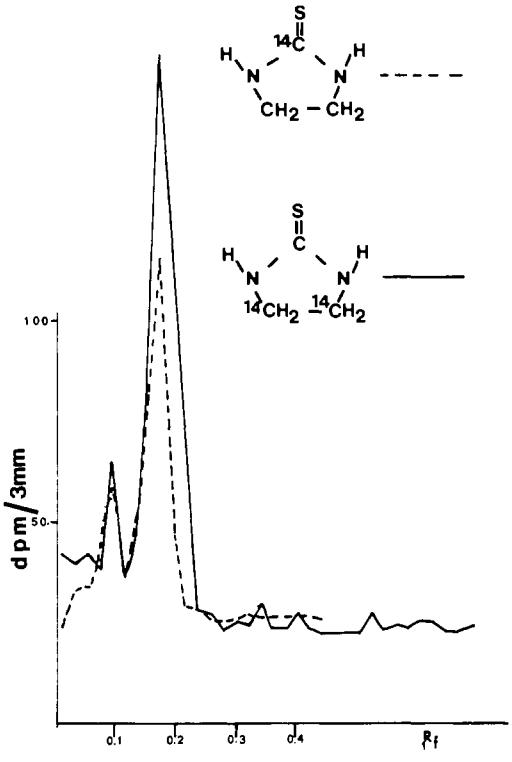

Figure 4. Thin-layer radiochromatograms of urine samples of mice, which did not move in A solvent system and were rechromatographed in B solvent system.

lin-2-yl sulfenate and other metabolites of ETU is needed to clarify the toxicity of ETU.

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Fate and Effects of Pentachloronitrobenzene in Rhesus Monkeys

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Pure pentachloronitrobenzene (PCNB) was administered to rhesus monkeys as single oral doses of 0.5, 2, or 91 mg/kg bodyweight and in a 70-day feeding study at a level of 2 ppm in the daily diet. PCNB was readily absorbed from the gastrointestinal tract, metabolized, and excreted rapidly by both the urinary and the fecal route. Pentachloroaniline and pentachlorothioanisole were the major metabolites, accompanied by a variety of other biotransformation products containing nitrogen reduced to anilines and sulfur as thiophenols, thioanisoles, or sulfoxide. Feeding PCNB for 70 days did not result in significant accumulation of PCNB or its metabolites. Parameters of clinical chemistry, hematology, endocrinology, and histopathology remained within the limits of variation which are normal for rhesus monkeys.

Pentachloronitrobenzene (PCNB) is the active ingredient of a variety of fungicides, e.g., Quintozen, Terraclor, or Brassicol, used for seed and soil treatment in agriculture and horticulture. It can be produced by chlorination of nitrobenzene (Schlör, 1970) or by nitration of pentachlorobenzene (Häfner, 1976). The technical product may contain up to 6% of hexachlorobenzene (HCB) (Zimmerli and Marek, 1972; Stijve, 1971; Casanova and Dubroca, 1972), as well as pentachlorobenzene (QCB) and tetrachloronitrobenzenes (Beck and Hansen, 1974). In earlier studies, Kuchar et al. (1969) and Borzelleca et al. (1971) investigated the toxicokinetic behavior, biotransformation, and toxicological effects of PCNB in dogs, rats, and cows using Terraclor, which contained 1.8% of hexachlorobenzene, 0.1% pentachlorobenzene, and 0.4% 2,3,4,5-tetrachloronitrobenzene. Analyzing hexane extracts of feces, urine, organs, fat, and milk of the exposed animals, they found residues of hexa- and pentachlorobenzene as

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