Analytical Studies of Metabolism of Terraclor in Beagle Dogs, Rats, and Plants

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Tissues from beagle dogs fed Terraclor in food (PCNB, daily for up to two years) were analyzed by gas chromatography for residual PCNB, impurities, and metabolites. The data obtained are indicative that PCNB is not present in fat or tissue and the metabolic products of PCNB are pentachloroaniline (PCA) and methyl pentachlorophenyl sulfide. The presence of PCA and methyl pentachlorophenyl sulfide has been established by infrared and mass spectroscopy. Chromatographic analyses of extracts of rat tissues from rats fed PCNB and from plants grown in PCNB treated soil are indicative of an identical metabolism.

Terraclor, or pentachloronitrobenzene (PCNB), manufactured and sold by Olin Mathieson Chemical Corporation as a soil fungicide, was incorporated in animal food and fed daily to beagle dogs and rats in a two-year chronic test conducted at the Medical College of Virginia. The animal tissues, as well as the excretion products, were analyzed by gas chromatography employing electron capture detection for PCNB, impurities such as hexachlorobenzene (HCB), pentachlorobenzene (PCB), and metabolites of PCNB.

The metabolism products of PCNB in plants were followed by analyzing young plants grown in soil treated with PCNB.

EXPERIMENTAL

Apparatus. A Jarrell-Ash 26-700 Gas Chromatograph with electron affinity detector was used.

Column. Four feet of stainless steel tubing (U-shaped), 1/8-inch I.D., with 1/8-inch walls, containing 2% SE-30 on Chromosorb G DMCS treated; acid-washed; 80 to 100 mesh was used. The column was conditioned at 210° C. with N_2 flow of 100 ml. per minute for about two days and exit disconnected from the detector.

Instrument conditions used were: injection port, 210° C.; detector, 210° C; column, 170° C; nitrogen, 100 to 155 cc. per minute; detector standing current, 5×10^{-9} amp. at 15–20 volts; chart speed, 1/2 inch per minute.

Reagents. *n*-Hexane. Distill reagent grade hexane from 50% sodium dispersion in mineral oil. Use 1 ml. of dispersed sodium per liter of hexane.

Acetonitrile, Mallinckrodt Nanograde Reagent; use as received.

Pentachloronitrobenzene (PCNB), Olin Mathieson Technical Grade, 98% PCNB.

Pentachlorobenzene (PCB), obtained from PCNB process; recrystallized, and structure confirmed by infrared spectroscopy.

Pentachloroaniline (PCA), Prepared from PCNB by reduction with Zn in ethanol-HCl and recrystallized from ethanol.

Hexachlorobenzene (HCB), as pentachlorobenzene.

2,3,4,5-Tetrachloronitrobenzene, as with PCA.

Methyl pentachlorophenyl sulfide, prepared from PCNB by reacting with Na_2S followed by CH_3I with subsequent recrystallization from ethanol; M.P. 95 to 96° C. Structure confirmed by mass spectroscopy.

Standard solution, solutions of PCNB, PCA, HCB, and methyl pentachlorophenyl sulfide employed were in concentrations of 0.01 μg. per ml. and PCB at 0.005 μg. per ml. These concentrations are in the linear range peak height *vs.* μl. injected when 3 to 8 μl. are employed. Relative retention data *vs.* PCNB are PCB = 0.42; 2,3,4,5-tetrachloronitrobenzene = 0.67; HCB = 0.85; PCA = 1.4; methyl pentachlorophenyl sulfide = 2.0.

Extraction Procedures. Fat, liver, kidney, brain, and spleen. Ten grams of representative samples are osterized in an 8-ounce osterizer for two minutes with 100 ml. of acetonitrile containing 50 grams of Na_2SO_4. Ten milliliters of the acetonitrile is then added to a centrifuge tube along with 10 ml. of water. The contents are mixed and then extracted with 10 ml. of hexane for two minutes. The contents are then centrifuged and the hexane layer is dried with 5 grams of Na_2SO_4 in a separatory funnel. This hexane is then chromatographed for PCNB, etc.

Blood, bile, and urine. Five milliliters of sample plus 1 ml. of acetone is extracted with 10 ml. of hexane for two minutes in a 50-ml. centrifuge tube. The contents of the tube are centrifuged and the hexane layer is dried with 5 grams of Na_2SO_4 and chromatographed for PCNB, etc. Ten-gram samples were taken for the urine analyses; 5 grams for blood and bile.

Feces and Muscle. Ten grams of representative sample are osterized in an 8-ounce osterizer for two minutes with 100 ml. of hexane containing 30 grams of Na_2SO_4. The hexane is dried with additional Na_2SO_4 (5 grams) in a separatory funnel and then chromatographed for PCNB, etc.

Acid Hydrolyzed Samples. Liver, 5 to 10 grams of liver are refluxed in a 125-ml. ₸ 24/40 Erlenmeyer flask with 10 ml. of water and 10 ml. of 1:1 aqueous H_2SO_4 for one hour. The contents are cooled and 50 ml. of hexane are added down the condenser and the flask is shaken for two minutes. The hexane layer is then dried with 5 grams of Na_2SO_4 and chromatographed for PCNB, etc.

Urine. The procedure for urine is identical to the liver method with the exception that 10 grams of sample were employed and no additional water was added.

Plant Material. Procedure employed is identical to that of feces and muscle.

CHROMATOGRAPHY OF PCNB, IMPURITIES, AND METABOLITES

The hexane extract of the tissues, excretion products, and plant material is chromatographed and each component calculated by the procedure of Methratta *et al.* (1967). The amount of methyl pentachlorophenyl sulfide found in the tissues was based on the PCA standard in that the majority of the tissues had been analyzed before the metabolite was identified. The sensitivity to electron capture of methyl

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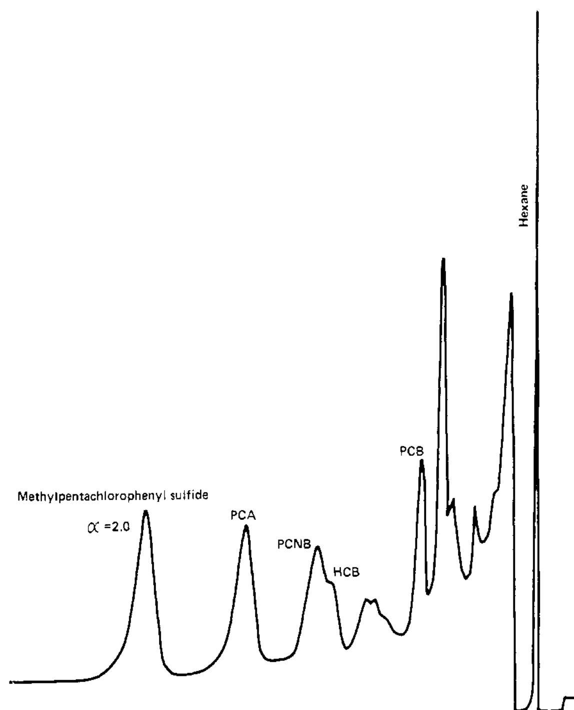

Figure 1. Separation of compounds in steam distillate from dog feces

Varian Aerograph 1521. Thermal conductivity column, 2 meter 1/4'' O.D. aluminum tubing. 15% SE 30 silcone gum on 80-100 mesh Chromosorb W @ 237° C. Injection temperature, 205° C. Detector temperature, 225° C. Helium at 60 cc./min.

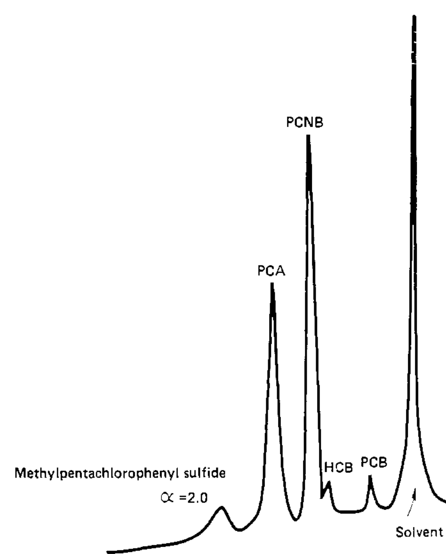

Figure 2. Chromatogram of hexane extract of dog feces with an electron capture detector

pentachlorophenyl sulfide has since been established as about 17% more sensitive than PCA. Therefore, the data presented for methyl pentachlorophenyl sulfide are high by approximately 17%.

RESULTS AND DISCUSSION

The beagle dog tissues were received frozen and were obtained from dogs which had been fed food containing 0 to 1080 p.p.m. PCNB for two years. The tissues were maintained frozen until the analyses were made. The PCNB used in this study assayed 97.8% and contained 1.8% HCB; <0.1% PCB, and 0.4% 2,3,4,5-tetrachloronitrobenzene.

Chromatographic data obtained on blood samples taken in the early stages of the feeding studies were indicative of materials that were not present in commercial PCNB. Early investigators (Betts *et al.* 1955; St. John *et al.*, 1965) had identified pentachloroaniline as a metabolite of PCNB in rabbits and cows, respectively. Therefore, PCA was chromatographed and compared *vs.* peaks in the extracts from treated dogs, and by retention time it was established that PCA could be a metabolite in the dog.

Subsequent chromatographic examination of other tissues such as fat and liver and excretion products were indicative of the presence of PCB, HCB, PCNB, PCA, and a metabolite at $\alpha = 2.0$ *vs.* PCNB.

Positive identification of PCA and the metabolite $\alpha = 2.0$ in extracts of dog feces was attempted by employing thin-layer chromatographic techniques by an essentially identical procedure to that of Gorbach and Wagner (1967). Zones on the thin-layer plate were qualitatively established by UV fluorescence and the material was removed with hexane. The material in the hexane solution was then concentrated by the "wick-stick" method (Garner and Packer, 1968) and examined by infrared spectroscopy. The presence of PCA was established conclusively. However, the metabolite at $\alpha = 2.0$, which was contaminated with PCA and HCB, could not be identified. Information obtained from the infrared spectrum was indicative of a compound that did not contain nitrogen.

A separation employing steam distillation of about 800 grams of feces from treated dogs followed by hexane extraction of the water distillate proved to be a more workable technique for isolating milligram quantities of the metabolite $\alpha = 2.0$. The hexane extract obtained in this manner contained about 1.6 mg. $\alpha = 2.0$, in addition to PCNB, PCB, HCB, and PCA and large amounts of foreign material. This extract was passed through a short column (~10 cm. in length and 1.5 cm. in diameter) of silica gel. This treatment removed the majority of the foreign material and allowed for chromatographic trapping of $\alpha = 2.0$. This separation is illustrated in Figure 1. The trapped material was then analyzed by mass spectroscopy (CEC 21-103C) and the mass spectrum indicated an apparent molecular ion at m/e 294. Peaks at m/e values of 296, 298, 300, and 302, representing the isotopic distribution of chlorine and sulfur, are in the ratio of five chlorines and one sulfur. The mass spectrum is indicative of methyl pentachlorophenyl sulfide and, subsequently, the mass spectrum of $\alpha = 2.0$ was compared to a synthetically prepared sample of methyl pentachlorophenyl sulfide, and the spectra were identical.

The presence of PCA suggests a reduction mechanism and methyl pentachlorophenyl sulfide a possible alkaline hydrolysis of PCNB to form a salt of a thiophenol which is subsequently methylated. Typical analytical data on dog liver, fat, feces, urine, kidney, and muscle are tabulated in Table I. The data presented are the averages of three male dogs fed at the daily levels of five and 1080 p.p.m. PCNB in dog food. Over the two-year feeding period, the average total consumption of PCNB was 1.05 and 233.8 grams, respectively. The average standard recovery for PCNB was 96% (0.04–10 p.p.m.); PCB = 107% (0.02–9 p.p.m.); HCB = 84% (0.04–9 p.p.m.); PCA = 93% (0.01–11 p.p.m.). The sensitivity of the methods employed for each component is about 0.005 p.p.m. Although extensive recovery data are not available for methyl pentachlorophenyl sulfide, it was established that recoveries of 87–105% were obtained at the 0.1-p.p.m. level

Table I. Terraclor Studies on Twenty-Four Month Male Beagle Dog Tissues

Data in p.p.m.

	PCNB	PCB	HCB	PCA	α = 2.0[a]
Muscle					
5 p.p.m. PCNB	ND	<0.003	0.016	ND	ND
1080 p.p.m. PCNB	ND	0.234	7.28	ND	0.227
Kidney					
5 p.p.m. PCNB	ND	0.012	0.035	ND	ND
1080 p.p.m. PCNB	ND	0.214	6.41	ND	1.08
Fat					
5 p.p.m. PCNB	ND	0.093	0.452	0.010	0.030
1080 p.p.m. PCNB	ND	5.15	194	0.643	2.50
Liver					
5 p.p.m. PCNB	ND	0.007	0.039	0.057	0.039
1080 p.p.m. PCNB	ND	0.387	5.92	0.037	0.322
Urine[b]					
5 p.p.m. PCNB	ND	ND	ND	<0.002	<0.001
1080 p.p.m. PCNB	<0.004	ND	<0.001	0.092	<0.001
Feces[b]					
5 p.p.m. PCNB	0.059	0.007	0.009	0.188	0.134
1080 p.p.m. PCNB	14.1	0.422	1.37	16.7	3.64

[a] Methyl pentachlorophenyl sulfide.
[b] Twenty-four hour samples before sacrifice.
ND = None Detected.

Table II. Terraclor Studies on Rat Fat

Data in p.p.m.

PCNB Level in Food, p.p.m.	PCNB	PCB	HCB	PCA	α = 2.0[a]
	Male Rats Fed PCNB Seven Months, Sacrificed, and Fat Analyzed				
50	ND	0.019	10.8	0.019	0.46
500	ND	0.304	117	1.11	4.74
	Male Rats Fed PCNB Seven Months, Then on Control Diet For Two Months, Fat Analyzed				
50	ND	ND	3.67	ND	ND
500	ND	ND	22.3	ND	ND

[a] Methyl pentachlorophenyl sulfide.
ND = None Detected.

Table III. Acid Hydrolysis *vs.* Direct Solvent Extraction

Data in p.p.m.

	PCA	
Urine	H^-	Direct Solvent
Male Dog 56	0.097	<0.005
(1080 p.p.m. PCNB)	0.079	<0.004
Male Dog 59	1.14	0.354
(1080 p.p.m. PCNB)	2.92	0.164
Liver		
Male Dog 56	0.111	0.028
(1080 p.p.m. PCNB)	0.092	0.034
Male Dog 59	0.195	0.044
(1080 p.p.m. PCNB)	0.220	

from dog fat. The extraction efficiency of the solvent systems employed for the tissues is greater than 90%.

A typical chromatogram illustrating the components extracted from dog feces is presented in Figure 2.

Data obtained on the fat tissues of both dogs and rats are indicative of fat storage of the chlorinated impurities such as PCB and HCB. The chlorinated species containing functional groups such as $—NH_2$ and $—SCH_3$ are also present, but are nonpersistent, whereas PCNB is not detected at all in the fat tissue. Earlier data obtained by Finnegan *et al.* (1958) on fat obtained from rats fed PCNB in their diets were indicative of the presence of apparent PCNB; however, the method of detection was neutron activation for chlorine and was not specific for PCNB. In the present study, fat obtained from rats which had been fed PCNB in their diets for seven months at levels up to 500 p.p.m. and then put on control diet for two months showed complete absence of PCB, PCNB, PCA, and methyl pentachlorophenyl sulfide. HCB remained after two months at a decreased level. These data are in Table II for male rats fed at levels of 50 and 500 p.p.m. PCNB.

The possibility of further reaction of PCA to form conjugates of glucuronic and sulfuric acid (Williams, 1959) was indicated by data obtained from sulfuric acid hydrolysis, followed by conventional extraction of liver tissue and urine obtained from male beagle dogs fed ppm PCNB for two years in their diet. These data are presented in Table III. In addition to the increase in PCA content after acid hydrolysis, it was established by retention time on selected samples of urine that 2,3,4,5-tetrachloroaniline was present, whereas none was detected in the unhydrolyzed samples. Experimental work has not been done to determine if any mercapturic acid derivatives of PCNB are present as found by Betts *et al.* (1955) in the rabbit study. It is conceivable that methyl pentachlorophenyl sulfide could be oxidized and converted to other products; however, work has not been done to determine if these products are present.

Studies conducted at Olin on the metabolic route of PCNB in plants are indicative of the same mechanism as in animals. Experiments were made in which cotton seed was planted in soil containing 300 p.p.m. PCNB. The young cotton plants were then monitored for PCNB, impurities, and metabolites.

Table IV. Residues from Young Cotton Plants in Soil Treated with 300 p.p.m. PCNB

Component	Found, p.p.m.	
	One Week	Two Weeks
Pentachlorobenzene	2.0	3.96
2,3,4,5-Tetrachloronitrobenzene	0.009	0.018
Hexachlorobenzene	2.77	4.61
Pentachloronitrobenzene	91	155
Pentachloroaniline	0.65	1.11
Methyl pentachlorophenyl sulfide	1.36	2.95

The data in Table IV were obtained on one- and two-week old plants (excluding the root portion). Pentachloroaniline and methyl pentachlorophenyl sulfide have also been detected in young corn and soybean plants grown in soil treated with PCNB. In addition, PCA had been identified previously by Gorbach and Wagner (1967) as a metabolite in potatoes. These investigators also indicated the presence of unidentified metabolites in addition to PCA at relative retention volumes of 0.9 and 1.8. From the chromatographic retention data of extracts from the animal tissues and young plants, it can be stated that the material at 0.9 is not a metabolite but, in fact, is HCB and that at 1.8 is the metabolite methyl pentachlorophenyl sulfide.

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