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Microbial Degradation of ^{14}C-Diphenylamine in a Laboratory Model Sewage Sludge System

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Since the discovery of PCBs in fish in the 1960s (JENSEN 1966), the United States Food and Drug Administration has become increasingly concerned about contamination of the U.S. food supply by industrial chemicals. Accidental contamination of food by industrial chemicals has occurred during food preparation (JENSEN 1972) and during food storage (VILLENEUVE et al. 1973). Chemical pollutants in the environment also may contaminate food during the growing stages (LAWRENCE & TOSINE 1977). Sewage sludge is one source of chemical pollutants (ERICKSON & PELLIZZARI 1979); it is increasingly being used as a nutrient source for crops (BRAUDE 1980). Since certain industrial chemicals and their metabolites may be taken up by crops (MOZA et al. 1976), it is useful to know the fate of industrial chemicals in sewage sludge before a search is made for any of their by-products in foods.

Several organic industrial chemicals were selected for study with the objective of determining which metabolites produced in sludge would tend to bioaccumulate or which were more toxic than the parent material. This report describes the biodegradation of one of these chemicals, diphenylamine (DPA), in a laboratory model sewage system.

DPA, an aromatic amine, is used as a stabilizer in propellants and as an antioxidant in polymers and rubber. Thirty-six million pounds were produced in 1974 (LAYER & KEHE 1978).

MATERIALS AND METHOD

Samples and Reagents. Activated return sewage sludge was obtained from the Arlington County Water Pollution Treatment Plant, Arlington, VA. Nutrient medium (SOAP AND DETERGENT ASSOCIATION 1965) was composed of K_2HPO_4 (1 g/L), $MgSO_4.7H_2O$ (0.25 g/L), KCl (0.25 g/L), $FeSO_4.7H_2O$ (0.002 g/L), NH_4Cl (3 g/L), yeast (0.3 g/L), milk solids (0.15 g/L), and conc. HCl (sufficient to bring the pH to 7.0). The sludge mixed liquor was formed by dilution of sludge with nutrient medium until the absorbance was 1.4-2.0 at 600 nm. Incubations with test chemicals were begun within 1.5 h after sludge was obtained.

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Radiolabeled ^{14}C-DPA (2.19 mCi/mmol, uniformly ring-labeled) was obtained from New England Nuclear (Boston, MA). Unlabeled DPA was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). 4-Hydroxy-DPA was supplied by P-Y. Lu (Department of Entomology, University of Illinois, Urbana, IL). Ultrafluor (National Diagnostics, Inc., Somerville, NJ) was used as the liquid scintillation counting cocktail.

Apparatus. The incubation vessel was a 2 L resin flask (Arthur H. Thomas Co., Philadelphia, PA) modified by adding a spigot near the bottom to facilitate sampling. It was equipped with an airtight, four-port, glass cover through which compressed air was introduced by means of a sintered glass scrubber tube. Gases were flushed out through another port to a CO_2 trap containing 200 or 400 mL of ethanolamine in 2-methoxyethanol (1:2 v/v). The contents of the incubation flask and the trap were both magnetically stirred at room temperature. The air flow rate was maintained at 25-30 mL/min.

Sampling. Ten μCi ^{14}C-DPA and 7.5 mg unlabeled DPA in 5 mL acetone were added to 1.5 L sludge mixed liquor in the incubation flask to produce a solution containing DPA at the 5 ppm level. The water solubility of DPA is about 30 ppm (LAYER & KEHE 1978). Aliquots of the solutions in the trap and mixed liquor were taken after 5 min and after 1, 2, 3, 4, 5, 6, 7, 23, 27, 31, and 48 h. Duplicate 1.00 mL aliquots were taken from the trap and pipetted into liquid scintillation vials. Duplicate 25 mL aliquots of the sludge mixed liquor were stored frozen in 50 mL glass-stoppered Erlenmeyer flasks until they could be extracted and analyzed.

Extraction. Frozen sludge mixed liquors were thawed at room temperature and extracted with two 25 mL portions of peroxide-free ethyl ether. The volume of the ethyl ether phase was measured, and duplicate 1.00 mL aliquots were taken for liquid scintillation counting. Ether solutions were concentrated to dryness under a N_2 jet and taken up in 1.00 mL ethyl acetate for gas chromatographic (GC) and mass spectral (MS) analyses.

The volume of the extracted aqueous phase was measured. Solids were suspended by swirling, and duplicate 1.00 mL aliquots were taken for liquid scintillation counting. Solids were allowed to settle out, and the supernatant was poured off into open 15 x 140 mm petri dishes and evaporated to dryness at room temperature. Methanol-soluble metabolites were concentrated under a N_2 jet and reacted with ethereal diazomethane. The derivatives were analyzed by GC/MS.

Instrumental. Radioactive extracts were counted with a Packard 3375 Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, IL). Extracts were chromatographed on a Varian 3700 gas chromatograph (Varian

Associates, Inc., Palo Alto, CA) equipped with a flame ionization detector (FID) and a 1.83 m x 2 mm i.d. column packed with 3% OV-101 on 80/100 mesh Chromosorb W HP. The column temperature was held at 60°C for 5 min and then programmed at 10°/min to 260°C and held for 10 min at the final temperature. Injection port temperature was 200°C; detector temperature was 270°C. N_2 carrier gas flow rate was 30 mL/min. Mass spectra were obtained with a Hewlett-Packard 5992B gas chromatograph/mass spectrometer (Hewlett-Packard Co., Palo Alto, CA) using Autotune and Peakfinder software. Programmed and isothermal column temperatures are given in Table 1.

RESULTS AND DISCUSSION

DPA was degraded in the model sewage sludge system; the results using 3 different 1.5 L batches of sludge were virtually identical. With our extraction procedure, the average recovery of added radioactivity was 82%. When 300 mL of the sludge mixed liquor was boiled for 5 min before 24 h incubation with 5 ppm DPA, 98% of the radioactivity, as unchanged DPA, was recovered. Ninety-seven percent of the radioactivity, as unchanged DPA, was recovered from 300 mL of nutrient medium alone after 24 h incubation with DPA. These data indicate that the sludge microorganisms alone caused the degradation of DPA.

Figure 1a shows the difference between the amount of remaining DPA, as determined by FID/GC, and the amount of radioactivity in the ether phase during one incubation experiment. This difference represents the ether-soluble metabolites of DPA. The metabolic intermediates were present at the first sampling (5 min) and maintained a relatively steady level during the first 24 h of incubation. DPA decreased to less than 4% of the added level within 24 h.

Figure 1b shows the change with time in the amounts of radioactivity in the ether extract, in the trap and in the extracted aqueous phase. Water-soluble metabolites accumulated rapidly within the first 7 h and leveled off or decreased slightly after 24 h. Significant production of CO_2, as shown by the radioactivity in the trap, began after 7 h.

Additional incubations of 5 ppm DPA in 300 mL of sludge mixed liquor were run for 7-24 h in order to obtain sufficient quantities of metabolites for analysis. Table 1 gives GC and MS data for standards and for four DPA metabolites found in the ether extracts. Metabolites were absent in extracts of sludge mixed liquor incubated without DPA. Three of the metabolites were identified as 4-hydroxy-DPA, aniline and indole. Indole was observed in only one experiment. The structure of the fourth metabolite, an isomer of 4-hydroxy-DPA, was not determined. FID/GC indicated that individual metabolite levels were low at any one time -- always less than 5% of the initial

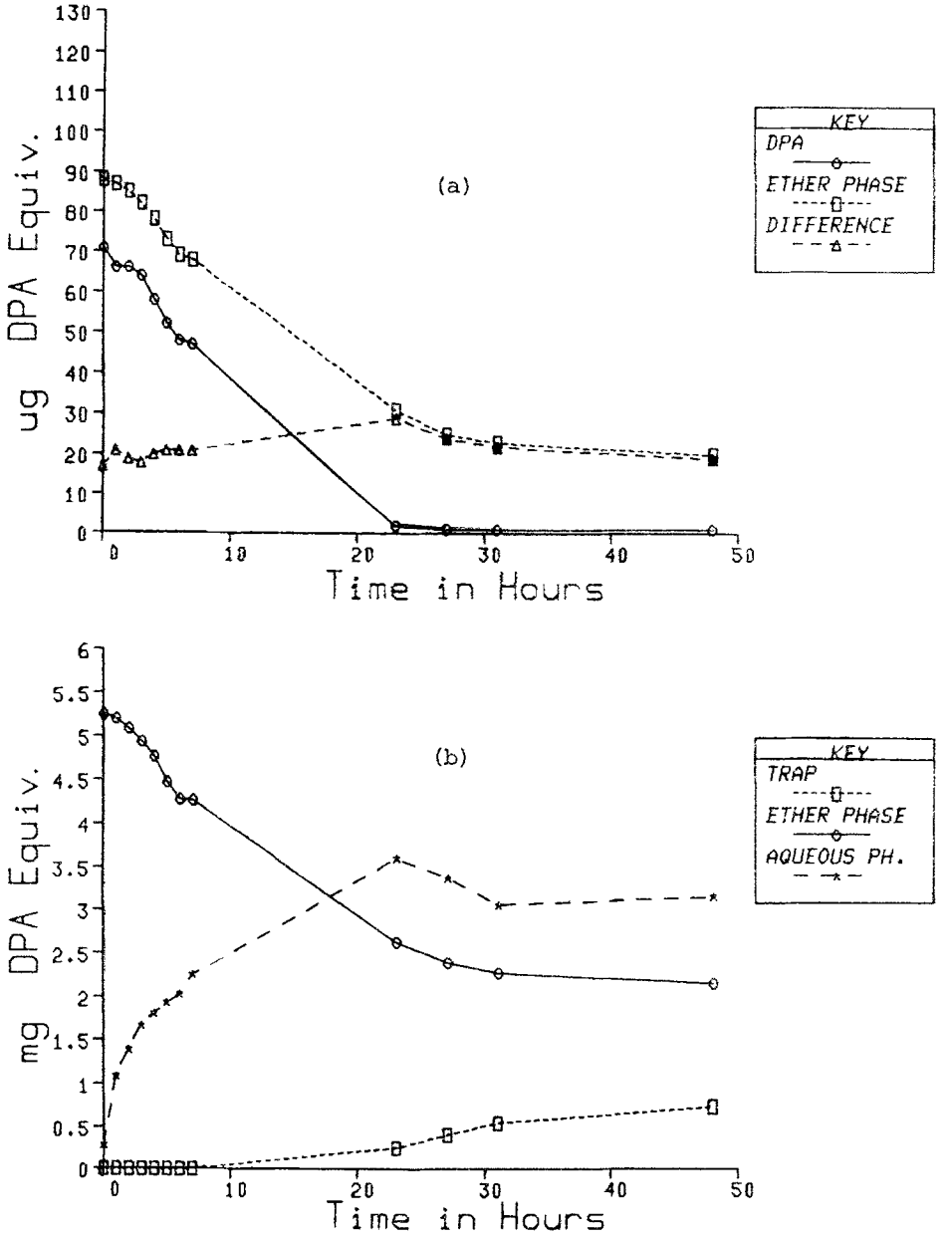

Figure 1. a) DPA (o), total radioactivity (□) and metabolites (△) in the ether extracts of 25 mL aliquots of sludge mixed liquor. b) Distribution of total radioactivity among trap (□), ether phase (o) and extracted aqueous phase (x). Mg DPA equivalents = [(corrected cpm in aliquot t_x) x (volume sludge mixed liquor, or trap, at t_x) ÷ cpm per mg DPA] + [cpm in aliquots removed ÷ cpm per mg DPA] where cpm is counts per minute and t_x is time of sampling.

TABLE 1

GC Retention Values and Molecular Ions
of Some Aromatic Amines and DPA Metabolites

Compound	GC Retention Time (min)	MS Molecular Ion (m/z)
4-Hydroxy-DPA	12.3[a]	185
Metabolite 1	12.3[a]	185
Metabolite 2	10.4[a]	185
Aniline	7.4[b]	93[c]
Metabolite 3	7.4[b]	93
Indole	5.7[d]	117[c]
Metabolite 4	5.8[d]	117

[a] Hold at 100°C for 4 min, program at 16°/min to 250°C.
[b] Isothermal, 60°C.
[c] EPA/NIH Mass Spectral Data Base, Vol. 1, S. R. Heller and G.W.A. Milne, editors, U.S. Government Printing Office, Washington, DC, 1978, pp. 58 & 177.
[d] Isothermal, 110°C.

DPA level. The absence of significant metabolite peaks in the FID/GC chromatograms and the fact that there is a relatively large difference between the actual amount of DPA and the amount of radioactivity (see Figure 1a) indicate that other nonchromatographable metabolites may have been formed.

Sludge microorganisms produced some DPA metabolites which were more acutely toxic than the parent material. The acute oral LD_{50} values of the DPA metabolites, 4-hydroxy-DPA, indole and aniline, in rats are 1220, 1000 and 440 mg/kg, respectively. The LD_{Lo} value of DPA in rats is 3000 mg/kg (LEWIS & TATKEN 1980).

After extraction with ether, the aqueous phase contained three methanol-soluble compounds which reacted with diazomethane. GC/MS analysis showed molecular ions at m/z = 179, 193 and 207 and base peaks at M-91 (probably C_6H_5N) in all cases. Loss of m/z = 91 and m/z = 59 (CO_2CH_3) suggested that the derivatized DPA metabolites were methyl esters of carboxylic acids with the anilino moiety intact. These compounds were not investigated further.

The metabolites found in this study suggest that DPA undergoes progressive oxidative metabolism in the sewage treatment process, as outlined by HUGHES & STAFFORD (1976). DPA is probably first oxidized to 4-hydroxy-DPA and an isomeric compound. The oxidized ring is then cleaved, forming water-soluble acids which are degraded further to aniline and CO_2.

Sewage in treatment plants is usually digested aerobically for 2-6 h (HUGHES & STAFFORD 1976). After 6 h incubation under our conditions, the microbial degradation of DPA was incomplete, with more than 35% undegraded. BAIRD et al. (1977) have shown that at least eight other aromatic amines also were incompletely degraded by activated sludge within 6 h. Our work shows that DPA behaves similarly and indicates a possible degradation route for aromatic amines in activated sludge. The identified metabolites are more toxic than DPA but are not persistent in the sludge mixed liquor after they are formed.

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