Naphthalene Metabolism by Diatoms Isolated from the Kachemak Bay Region of Alaska

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(Received 20 July 1981)

Three pure cultures of diatoms – a *Navicula* sp., a *Nitzschia* sp. and a *Synedra* sp. – grown in the presence of naphthalene at 6 or 12 °C oxidized the naphthalene to ethyl acetate-soluble and water-soluble metabolites. The major ethyl acetate-soluble metabolite was identified as 1-naphthol by gas chromatographic and mass spectral analysis. Experiments with [¹⁴C]naphthalene indicated that the extent of naphthalene metabolism ranged from 0.7 to 1.4 %.

INTRODUCTION

Aromatic hydrocarbons are widely distributed in open ocean waters (Brown & Huffman, 1976). Many of these compounds and/or their metabolites have toxic properties, e.g. the initiation of tumour formation and cancer (Miller & Miller, 1976). In studies of the fate of hydrocarbons in aquatic ecosystems, a considerable amount of information is available on the bacterial and fungal degradation of these compounds and their derivatives (Atlas, 1981; Cerniglia, 1981). In view of the fact that cyanobacteria and microalgae are widely distributed in many aquatic environments and may be important in the catabolism of hydrocarbons, we initiated a research programme on the algal oxidation of aromatic hydrocarbons (Cerniglia *et al.*, 1979, 1980*a, b, c*).

Most studies on the microbial oxidation of hydrocarbons have been conducted at temperatures between 20 and 30 °C, but the expansion of oil exploration and transport of petroleum in cold waters has given rise to increased interest in the microbial degradation of crude oil at low water temperatures (Atlas, 1981). In this investigation, we report on three diatoms isolated from the Kachemak Bay region of Alaska which can metabolize the aromatic hydrocarbon naphthalene at low temperatures.

METHODS

Organisms and growth conditions. The diatoms K1A (*Navicula* sp.), K8A (*Nitzschia* sp.) and 4D (*Synedra* sp.) were isolated by enrichment culture at 6 to 10 °C from oblique net (20 µm Nitex nylon) tows made during August 1979 and April 1980, in the Kachemak Bay Region, south of Homer, Alaska. The enrichment medium was local sea water plus 5, 20 or 50% (v/v) ASP-2 medium (Van Baalen, 1962). The ASP-2 basal medium contained, per litre, 18 g NaCl, 5 g MgSO₄·7H₂O, 0.60 g KCl, 0.37 g CaCl₂·2H₂O, 1 g NaNO₃, 0.05 g KH₂PO₄, 1 g Tris, 0.03 g Na₂EDTA·2H₂O, 0.004 g FeCl₃·6H₂O, 0.034 g H₃BO₃, 0.004 g MnCl₂·4H₂O, 670 µg ZnSO₄·7H₂O, 38 µg Na₂MoO₄·2H₂O, 12 µg CoCl₂·6H₂O and 0.3 µg CuSO₄·5H₂O, supplemented with 0.125 g Na₂SiO₃·9H₂O, 250 µg thiamin and 4 µg vitamin B₁₂. Pure cultures were obtained by repeated streaking or by

treatment with ultraviolet radiation (254 nm, 15 W germicidal lamp) for several minutes and subsequent preparation of pour plates. Organism N-1 (*Cylindrotheca* sp.) was isolated from a water sample taken from the Pass adjacent to the Port Aransas Marine Laboratory (Estep *et al.*, 1978). The organisms were grown in 20 ml of supplemented ASP-2 medium (as above) in 22 × 175 mm Pyrex test tubes at 12 °C. The growth tubes were illuminated with two fluorescent lamps F20T12-WWX (Sylvania, Danvers, Mass., U.S.A.) on each side of the water bath, 8 cm from the front edge of the lamp to the tube centre. The cultures were continuously aerated with air enriched with $1 \pm 0.1\%$ (v/v) CO₂. The generation times under these conditions for the four organisms were about 24 h.

Naphthalene metabolism. Experiments using [1-¹⁴C]naphthalene were done to determine the amount of naphthalene oxidized by each organism. Cells (0.5–0.8 mg) were pooled from the growth tubes by allowing them to settle for several minutes and decanting the supernatant, and transferred to a 22 × 175 mm screw-cap tube (final volume 10 ml). [¹⁴C]Naphthalene (1 µCi in 20 µl ethanol, 6.9 mg l⁻¹) was added just before closing the tube with a plastic top lined with a chromatography septum, aluminium foil and 1 mil (0.001 in) Teflon film. CO₂ was added through a small hole in the plastic top with a gas-tight syringe to an initial concentration of 1% (v/v). The screw-cap tubes were clamped to a glass rod and rotated slowly in the same illuminated water bath as used for growing the cultures. The tubes were incubated at either 6 or 12 °C. After 22 h incubation, cells were removed by centrifugation and each supernatant was extracted with five 30 ml volumes of ethyl acetate. The organic extracts were dried over anhydrous Na₂SO₄ and the solvent was removed *in vacuo* at 42 °C. Each residue was redissolved in methanol and analysed by high pressure liquid chromatography (h.p.l.c.). To determine the ratio of ethyl acetate-soluble metabolites to water-soluble metabolites, each organic-soluble extract was redissolved in 50 µl acetone, and 10 µl portions were added to vials containing 10 ml scintillation fluid; 1 ml of the aqueous phase was added to 10 ml scintillation fluid. The radioactivity present was determined in a liquid scintillation counter. Corrections were made for machine efficiency and quenching.

An experiment with unlabelled naphthalene was conducted with organism K8A (*Nitzschia* sp.) in order to obtain sufficient material for the isolation and structure elucidation of the products of naphthalene metabolism. Four 10 ml samples of organism K8A were incubated in screw-cap tubes as described above with 6.9 mg naphthalene l⁻¹ at 12 °C. After 22 h the cells were centrifuged and the supernatant was extracted and concentrated as described above. The residue was redissolved in methanol and analysed by gas chromatography and mass spectrometry (GC-MS).

Analysis of metabolic products. All h.p.l.c. analyses were performed on a Beckman model 332 instrument with a model 155-10 variable wavelength absorbance detector (Beckman Instruments, Berkeley, Calif., U.S.A.) operated at 254 nm. An Altex Ultrasphere-ODS column (25 cm × 4.6 mm i.d.; Altex Scientific, Berkeley, Calif., U.S.A.) was used for the separation of naphthalene metabolites, which was achieved with a programmed methanol/water gradient (50–95%, v/v; 30 min) with a flow rate of 1 ml min⁻¹. In experiments with [¹⁴C]naphthalene, 0.5 ml fractions were collected at 0.5 min intervals in scintillation vials and 5.0 ml Aquasol-2 (New England Nuclear Corp., Boston, Mass., U.S.A.) was added to each vial. The radioactivity present in each fraction was determined in a Beckman LS-250 liquid scintillation counter.

GC-MS analysis of naphthalene metabolites was performed on a Finnigan model 3100 mass spectrometer coupled to a gas chromatograph equipped with a glass column (2 m × 1.5 mm i.d.) packed with 3% OV-1 on Chromosorb Q. The injection temperature was 50 °C with a temperature programme of 100–250 °C at 8 °C min⁻¹. The carrier gas was helium, with a flow rate of 30 ml min⁻¹. The following conditions were used for mass spectrometry: molecular separator temperature, 350 °C; ion source temperature, 100 °C; ionization beam, 70 eV; ionization current, 200 µA.

Chemicals. Naphthalene (99.9%) was from Aldrich Chemical Co., Milwaukee Wis., U.S.A. [1(4,5,8)-¹⁴C]Naphthalene [5 mCi mmol⁻¹; 185 MBq mmol⁻¹] was from Amersham Searle, Arlington Heights, Ill. U.S.A. All naphthalene derivatives were purified as described previously (Cerniglia & Gibson, 1977). Solvents for h.p.l.c. were purchased from Burdick and Jackson Laboratories, Muskegon, Mich., U.S.A.

RESULTS AND DISCUSSION

Three pure cultures of diatoms isolated from Alaskan waters (strains K8A, 4D and K1A) were incubated with [¹⁴C]naphthalene at either 6 or 12 °C. The h.p.l.c. elution profiles of the ethyl acetate-soluble naphthalene metabolites formed by each diatom are shown in Fig. 1 (*b*, *c*, *d*). For comparative purposes, the chromatographic properties of synthetic naphthalene derivatives are shown in Fig. 1 (*a*). All of the organisms oxidized naphthalene to a compound which co-chromatographed with 1-naphthol. These results are similar to our earlier studies on the oxidation of naphthalene by cyanobacteria and microalgae (Cerniglia *et al.*, 1980*b*).

Fig. 1. H.p.l.c. elution profiles of ethyl acetate-soluble metabolites formed from $[1-^{14}\text{C}]$ naphthalene by different diatoms. (a) Resolution of a mixture of synthetic naphthalene derivatives. Metabolites formed by: (b) *Nitzschia* sp. strain K8A; (c) *Synedra* sp. strain 4D; (d) *Navicula* sp. strain K1A. H.p.l.c. conditions were as described in Methods. Excess naphthalene was lost during the evaporation of ethyl acetate extracts of flask contents.

Table 1. *Distribution of radioactivity in the ethyl acetate-soluble and water-soluble metabolites formed from $[^{14}\text{C}]$ naphthalene by diatoms*

The $[1-^{14}\text{C}]$ naphthalene concentration was 6.9 mg l^{-1} ($1 \mu\text{Ci}$). Incubation was done in closed tubes. Naphthalene oxidation to CO_2 was not determined. Medium and boiled organism controls were negative.

Organism	Distribution of radioactivity [d.p.m. (mg cell dry wt) $^{-1}$]			Percentage metabolism of naphthalene
	Organic-soluble	Water-soluble	Total	
<i>Nitzschia</i> sp. strain K8A	8965 (49)*	9311 (51)	18276	0.8
<i>Synedra</i> sp. strain 4D	18044 (58)	13332 (42)	31376	1.4
<i>Navicula</i> sp. strain K1A	9658 (55)	7987 (45)	17645	0.8
<i>Cylindrotheca</i> sp. strain N-1	6550 (43)	8784 (57)	15334	0.7

* Numbers in parentheses indicate the percentage of total metabolites.

In order to confirm that 1-naphthol was the major metabolite in the oxidation of naphthalene, cells of *Nitzschia* sp. strain K8A were incubated for 22 h in the presence of naphthalene and the ethyl acetate-soluble extract was analysed by GC-MS. The GC-MS analysis of this extract showed a compound that had a similar retention time (9.5 min) and mass spectrum (m/e 144) to that of authentic 1-naphthol.

Table 1 shows that these diatoms oxidized naphthalene to both organic-soluble and water-soluble derivatives. The amount of naphthalene oxidized to recoverable products ranged

from 0.7 to 1.4%. It is interesting to note that *Cylindrotheca* sp. strain N-1 grown at 12 °C, conditions in which it had a similar growth rate to organism 4D, oxidized less naphthalene (Table 1). These data suggest that cold-adapted microalgae may prove to be more metabolically active than might be thought from their slow growth rates.

In an earlier study we showed that the cyanobacterium *Oscillatoria* sp. strain JCM oxidized 4.8% of the added naphthalene (Cerniglia *et al.*, 1980a). The ratio of ethyl acetate-soluble metabolites to water-soluble metabolites was 41:59. Table 1 shows that all of the diatoms formed water-soluble products. The identity of these products remains to be determined but the results suggest that diatoms may be able to convert naphthalene to ring cleavage products or to form conjugated compounds.

These results extend the original observations on the oxidation of naphthalene by temperate forms (Cerniglia *et al.*, 1980b) to cold-adapted diatoms and reinforce the view that the capacity for oxidation of aromatic compounds is a general metabolic feature in the microalgae. Algal rates of aromatic oxidation compared with rates for the aerobic heterotrophic microbial populations in the photic zone are unknown. However, the photic zone in the sea may prove to be a major sink for transformations of aromatic compounds in nature. Whether this will increase or decrease their toxicity for zooplankton and higher trophic levels is unknown.

We are grateful to Drs Rita Horner and Robert A. Gibson for the identification of the cultures. This study was funded in part by the Bureau of Land Management through interagency agreement with the National Oceanic and Atmospheric Administration, as part of the Outer Continental Shelf Environmental Assessment Program.

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