Vol. 88, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1979 Pages 50-58

ALGAL OXIDATION OF AROMATIC HYDROCARBONS: FORMATION OF 1-NAPHTHOL FROM NAPHTHALENE BY *Agmenellum quadruplicatum*, strain PR-6

Carl E. Cerniglia and David T. Gibson

Department of Microbiology
The University of Texas at Austin
Austin, Texas 78712

Chase Van Baalen

Port Aransas Marine Science Laboratory
The University of Texas Marine Science Institute
Port Aransas, Texas 78373

Received March 24, 1979

SUMMARY

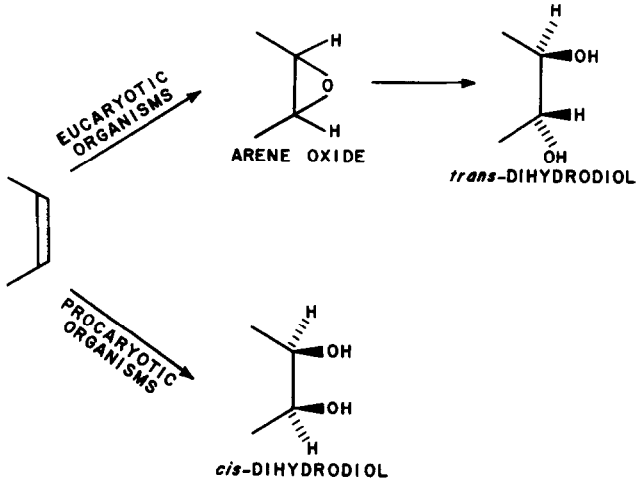
Agmenellum quadruplicatum, strain PR-6, *Coccochloris elabens*, strain 17A and *Oscillatoria* sp., strain JCM, grown photoautotrophically in the presence of [^{14}C]-naphthalene, produced six metabolites that were detected by radioautography. A large scale biotransformation experiment with *Agmenellum quadruplicatum*, strain PR-6 led to the isolation and identification of 1-naphthol as the major reaction product. Preliminary evidence was also obtained for the formation of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene.

INTRODUCTION

Interest in the metabolism of aromatic hydrocarbons stems from observations that some of these compounds exhibit toxic, mutagenic and carcinogenic properties (1). A major route for the metabolism of aromatic hydrocarbons by higher organisms involves the incorporation of one atom of molecular oxygen into the substrate to form an arene oxide. The latter compound can undergo isomerization to form 1-naphthol, enzymatic addition of water to form a trans-dihydrodiol and the enzymatic or non enzymatic addition of glutathione to form conjugated derivatives (1). In contrast, bacteria incorporate both atoms of molecular oxygen into unsubstituted aromatic hydrocarbons to form dihydrodiols with a cis relative stereochemistry (2,3). These observations have led to the suggestion that cis and trans hydroxylation of aromatic hydrocarbons represent basic differences

0006-291X/79/090050-09$01.00/0

in the metabolism of these substrates by procaryotic and eucaryotic organisms (4). The different reaction sequences are shown below.



Since blue-green algae are procaryotic organisms we decided to test the hypothesis that these organisms would catalyze a cis hydroxylation of aromatic hydrocarbons. Naphthalene was chosen as a model substrate since its metabolism by mammals, fungi and bacteria has been examined in detail (5,6,7,8,9,10).

MATERIALS AND METHODS

Organisms and Growth Conditions. *Agmenellum quadruplicatum*, strain PR-6, *Coccochloris elabens*, strain 17A, and *Oscillatoria* sp., strain JCM were each grown in Pyrex test tubes (175 x 22.5 mm) that contained 20 ml of ASP-2 medium (11,12). Illumination was provided by two fluorescent lamps (F48T12/CW/HO) placed on each side of a water bath that was maintained at 30 C. Air containing 1.0 ± 0.1% carbon dioxide was passed continually through the culture medium. Cells were harvested in the exponential phase of growth and resuspended in 30 ml of sterile ASP-2 medium. The final cell concentrations were approximately 0.3 mg/ml dry weight.

Experiments with [^{14}C]-Naphthalene. Cells grown as described above were transferred to a closed growth system. A closed culture flask (simi-

lar to "Nephelo" flask, cat. no. 2574, Bellco Glass Co., Vineland, NJ) was used to prevent loss of the volatile aromatic hydrocarbon. The flask contained a 22 x 130 mm sidearm for turbidity measurements and the edges of the flask top and cleanout opening were flattened by polishing with fine carborundum. A small hole (No. 60) in the cleanout screw cap served for the addition and withdrawal of gas or liquid samples. The liner of the cleanout cap was replaced by a gas chromatography septum. A layer of aluminum foil and a teflon membrane were placed on top of the septum. The flask was clamped to the top of a variable speed rotating aluminum bar and incubated in a water bath at 30 ± 0.5 C. Illumination was provided by two fluorescent lamps (F48T12/CW/HO) positioned 30 cm below the water bath. The carbon dioxide level in the flask was maintained between 0.5 and 1.0% by periodic sampling and measurement on a Carle 8515 gas chromatograph. [1-^{14}C]-Naphthalene (2.2 µmole, sp. act. 3.67 mCi/mmole) in 100 µl of 95% ethanol and carbon dioxide were added to the flask which was allowed to equilibrate for 15 minutes before the lamps were turned on. After 12-24 hours growth in the presence of [^{14}C]-naphthalene, the cells were removed and the supernatant solution extracted with an equal volume of ethyl acetate. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo* at 45 C. The residue was taken up in a small volume of acetone and concentrated under nitrogen. Aliquots (50 µl) of the acetone solution were taken for analysis by radioautography. In an attempt to isolate sufficient quantitites of each metabolite, *Agmenellum quadruplicatum*, strain PR-6 was grown in 2 liters of ASP-2 medium. Eight culture tubes containing 250 ml of medium were used. Nonradioactive naphthalene (1 mg/ml) was added to the growing cultures. After 48 hours the cells were removed and the supernatant solution extracted with ethyl acetate. The solvent was removed *in vacuo* at 30 C. The residue (750 mg) was examined for metabolic products by thin-layer and silica gel column chromatography.

Analytical Methods. Thin-layer chromatography was carried out with silica gel 60F-254 glass plates (E. Merck, Dermstadt, Germany). Each chromato-

gram was developed three times in a solvent system consisting of chloroform: acetone (8:2). Multiple development was necessary for the separation of cis-naphthalene dihydrodiol from its trans-isomer. Naphthalene metabolites were located on chromatograms by radioautography. The developed chromatograms were exposed to Kodak X-ray film (Blue-sensitive SB-54) for 2 to 4 weeks.

High pressure liquid chromatography was performed on a component system consisting of a Waters Associate Model 6000 solvent delivery system, Model U-6K septumless injector and Model 440 absorbance detector at 254 nm or 280 nm. A μBondapak C_{18} column (3.9 mm x 30 cm) was used for the separation of polar metabolites which was achieved with a programmed methanol: water gradient (50-95%, 30 min).

A Finnegan model 3100 mass spectrometer coupled to a gas chromatograph and model 6000 computer system was used to identify 1-naphthol. A glass column (2 m x 1.5 mm I.D.) packed with 3% OV-1 on chromosorb W was used with the following operation conditions: injection temperature, 220 C; program 100-250 C at 8 C/min; flow rate of carrier gas (helium) 30 ml/min. The following conditions were used for mass spectrometry: molecular separator temperature, 250 C; ion source temperature, 100 C; ionization beam 70 eV; ionization current, 200 μA.

Absorption spectra were determined on a Beckman model 25 recording spectrophotometer.

Chemicals. Naphthalene (99.95%) was from Aldrich Chemical Company. [1-^{14}C]-Naphthalene (3.67 mCi/mmole) was purchased from Amersham/Searle, Arlington Heights, IL. *trans*-Naphthalene dihydrodiol was a generous gift from L. A. Kapicak, Union Carbide Corporation, Charleston, WV. *cis*-Naphthalene dihydrodiol was prepared as described previously (10). Other naphthalene derivatives were purified as described previously (6). Solvents for high pressure liquid chromatography were purchased from Burdick and Jackson Laboratories, Muskegon, MI.

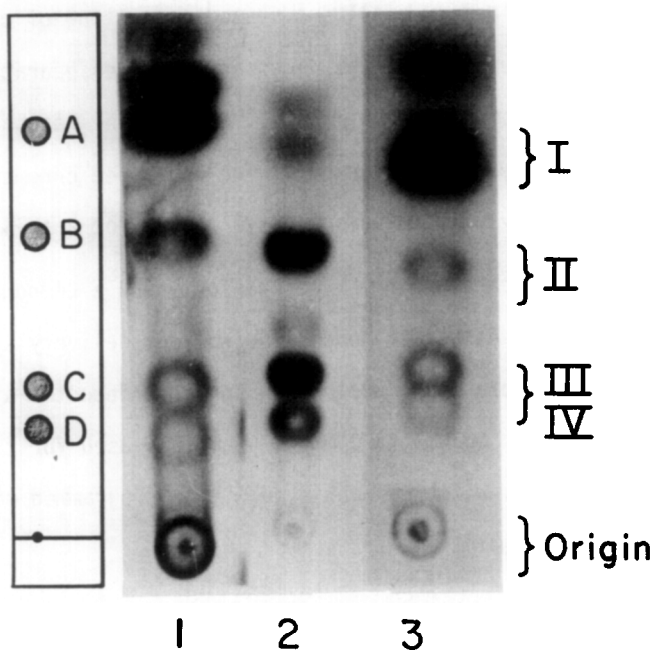


Figure 1: Radioautogram of metabolites formed from [^{14}C]-naphthalene by *Oscillatoria* sp., strain JCM (1), *Coccochloris elabens*, strain 17A (2) and *Agmenellum quadruplicatum*, strain PR-6 (3). The chromatographic mobilities of 1-naphthol, 4-hydroxy-1-tetralone, *cis*-naphthalene dihydrodiol and *trans*-naphthalene dihydrodiol are shown schematically and are designated A, B, C and D respectively.

RESULTS AND DISCUSSION

Cells of *Agmenellum quadruplicatum*, *Coccochloris elabens* and *Oscillatoria* sp. were grown photoautotrophically in the presence of [^{14}C]-naphthalene. Each organism produced at least six radioactive products that were detected by radioautography (Fig. 1). For comparative purposes the chromatographic mobilities of several known naphthalene metabolites are also shown in Figure 1. Although chromatographic mobilities are not conclusive for identification purposes the results indicated the possible formation of 1-naphthol, 4-hydroxy-1-tetralone, *cis*-1,2-dihydroxy-1,2-dihydronaphthalene

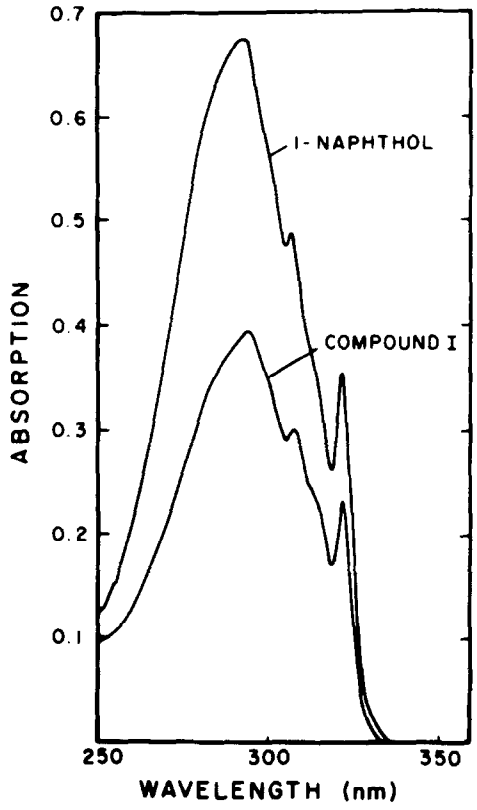


Figure 2. Absorption spectra of compound I formed from naphthalene by *Agmenellum quadruplicatum*, strain PR-6 and synthetic 1-naphthol. Each compound was dissolved in methanol and the spectra were recorded on a Beckman model 25 recording spectrophotometer.

(*cis*-naphthalene dihydrodiol) and *trans*-1,2-dihydroxy-1,2-dihydronaphthalene (*trans*-naphthalene dihydrodiol).

In order to obtain sufficient amounts of the metabolites for identification purposes a large scale experiment was conducted with cells of *Agmenellun quadruplicatum*. The residue (750 mg) obtained by ethyl acetate extraction of two liters of culture medium was dissolved in 1.5 ml of chloroform and applied to the top of a column (30 x 3.0 cm) of silica gel. The column was eluted with chloroform and 10.0 ml fractions were collected. Fractions

5-8 contained naphthalene (650 mg) which was identified by its characteristic absorption spectrum. The contents of tubes 18-27 were pooled, evaporated to dryness and the residue (~6.0 mg) was analyzed by high pressure liquid chromatography. The major component (Compound I) had an identical retention time to that given by 1-naphthol. The absorption spectrum of Compound I was identical to the spectrum given by synthetic 1-naphthol (Fig. 2). Further proof of the structure of Compound I was provided by mass spectral analysis. The algal product gave a parent ion at m/e 144 and a fragmentation pattern that was identical to that given by 1-naphthol.

Fractions 82-88 (Compound III) were pooled, evaporated to dryness and the residue analyzed by high pressure liquid chromatography. The retention time and absorption spectrum of the product were identical to those given by *cis*-naphthalene dihydrodiol (Fig. 3) although the amount of compound obtained was not sufficient for further characterization. Although metabolites II and IV were detected by radioautography they were not detected in the large scale biotransformation experiment.

Quantitative measurements on the amount of radioactivity recovered as ethyl acetate soluble products showed that over a 24 hour period only 1.4 percent of the added naphthalene was metabolized by *Agmenellum quadruplicatum*. This low rate of oxidation of naphthalene by the organisms used in the present study has hindered our attempts to isolate and identify all of the reaction products. Nevertheless the preliminary results reported in this manuscript demonstrate that blue-green algae can oxidize aromatic hydrocarbons. The tentative identification of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene suggests similarities with the mechanisms used by bacteria for the oxidation of this hydrocarbon (10). However, the amount of 1-naphthol formed seems too large to account for this being the sole pathway in *Agmenellum quadruplicatum*, strain PR-6. 1-Naphthol could arise by the isomerization of 1,2-naphthalene oxide as has been reported for the mammalian (5) and suggested for the fungal (6-8) oxidation of naphthalene. Alternatively 1-

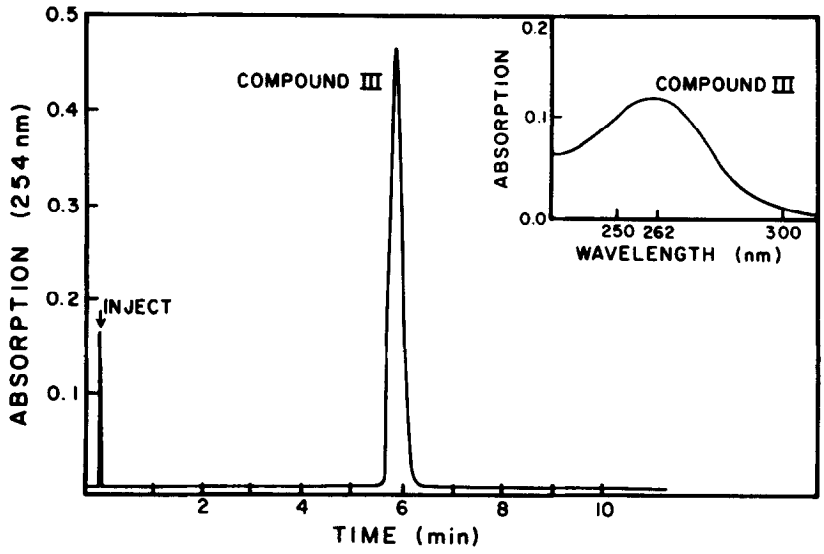


Figure 3. Purification of compound III formed from naphthalene by *Agmenellum quadruplicatum*, by high pressure liquid chromatography. The inset shows the absorption spectrum of compound III after purification by high pressure liquid chromatography.

naphthol could possibly be formed by an oxidizing species generated during photosynthesis. The demonstration of alternative routes of naphthalene oxidation awaits the conclusive identification of the other metabolites and detailed enzymological studies.

Acknowledgements

This research was supported by Public Health Service Grant ES-00537 from the National Institute of Environmental Health Sciences. C.E.C. is a postdoctoral trainee supported by Grant Number T32 CA09182 awarded by the National Cancer Institute, DHEW. We thank Rita O'Donnell and Joseph C. Morgan for technical assistance and Roberta DeAngelis for assistance in preparing the manuscript.

REFERENCES

1. Daly, J.W., Jerina, D.M. and Witkop, B., Experientia, 28, 1129 (1972).
2. Gibson, D.T., Crit. Rev. Microbiol., 1, 199 (1971).
3. Gibson, D.T. in Fate and Effects of Petroleum Hydrocarbons in Marine Ecosystems and Organisms. D.A. Wolfe, ed., pp. 36-46. Pergammon Press (1977).
4. Gibson, D.T., Zbl. Bakt. Hyg. I. Abt. Orig. B, 162, 157 (1976).
5. Jerina, D.M., Daly, J.W., Witkop, B., Zaltzman-Nirenberg, P. and Udenfriend, S., Biochemistry, 9, 147 (1970).
6. Cerniglia, C.E. and Gibson, D.T., Appl. Environ. Microbiol., 34, 363 (1977).
7. Cerniglia, C.E. and Gibson, D.T., Arch. Biochem. Biophys., 186, 121 (1978).
8. Cerniglia, C.E., Hebert, R.L., Szaniszlo, P.J. and Gibson, D.T., Arch. Microbiol., 117, 135 (1978).
9. Davies, J.I. and Evans, W.C., Biochem. J., 91, 251 (1964).
10. Jeffrey, A.M., Yeh, H.J.C., Jerina, D.M., Patel, T.R., Davey, J.F. and Gibson, D.T., Biochemistry, 14, 575 (1975).
11. Provasoli, L., McLaughlin, J.J. and Droop, M.R., Arch. Mikrobiol., 25, 392 (1957).
12. Van Baalen, C., Botanica Mar., 4, 129 (1962).