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Effect of Nitrogen Source on End Products of Naphthalene Degradation

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Soil cultures, enrichment cultures, and pure culture isolates produced substantial quantities of salicylic acid from naphthalene in a mineral salts medium containing NH_4Cl as the nitrogen source. However, when KNO_3 was substituted for NH_4Cl, these same cultures failed to accumulate detectable quantities of salicylic acid but did turn the medium yellow. When an isolate identified as a *Pseudomonas* species was used, viable cell numbers were much greater in the medium containing KNO_3, but up to 94% of the naphthalene was utilized in both media. After 48 h of incubation in a 0.1% naphthalene-mineral salts medium, the cultures containing NH_4Cl showed irregular clumped cells, a pH of 4.7, 42 μg of salicylic acid per ml, and the production of 4.4 ml of CO_2. Under the same conditions, the cultures in the medium containing KNO_3 showed uniform cellular morphology, a pH of 7.3, no salicylic acid, the production of 29.7 ml of CO_2, and a distinct yellow coloration of the medium. The differences between nitrogen sources could not be accounted for by pH alone since results obtained using buffered media were similar. Growth with NH_4NO_3 displayed a pattern similar to that obtained when NH_4Cl was used. The yellow coloration in the medium containing KNO_3 was apparently due to more than one compound, none of which were 1,2-naphthoquinone or acidic in nature, as suggested by other investigators. Further attempts to identify the yellow compounds by high-pressure liquid chromatography, infrared analysis, and gas chromatography-mass spectrometry have been unsuccessful thus far.

The biodegradation of polynuclear aromatic hydrocarbons has been extensively studied in view of the potential carcinogenicity of the parent molecules or their degradation products. As early as 1950, Boyland (3) emphasized the carcinogenic character of the polynuclear aromatic hydrocarbons, and subsequently, numerous investigators have focused attention on the metabolism of these compounds. Naphthalene, being the simplest homolog in the polycyclic series, has received considerable attention.

The first intermediate to be isolated from a naphthalene-degrading microorganism was salicylic acid (10). Subsequent investigations focused on the pathway of naphthalene degradation (5, 6, 8, 11) and the effect of various cations on degradation (7). Interestingly, all of the studies employed the NH_4^+ ion in the medium and reported salicylic acid production. Additionally, Klausmeier and Strawinski (7) reported that an acidic yellow compound was also produced, and Murphy and Stone (8) identified a yellow product in the spent medium as 1,2-naphthoquinone. In the present investigation, use of NH_4Cl as the nitrogen source predictably yielded an accumulation of salicylic acid, whereas the use of KNO_3 in the medium failed to result in salicylic acid accumulation. This observation raised the possibility that the apparent differences were a consequence of the different nitrogen sources available during degradation.

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MATERIALS AND METHODS

Chemicals. The naphthalene employed in this study was scintillation grade, organic and inorganic chemicals were reagent grade, and organic solvents were pesticide grade. Ethanol, after distillation, was used for extraction purposes.

Media, microorganisms, and growth conditions. The mineral salts medium routinely used in this study contained 1.0 g of KNO_3 or NH_4Cl, 0.38 g of K_2HPO_4, 0.05 g of $FeCl_3 \cdot 6H_2O$, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per 1,000 ml of distilled water; the final pH was adjusted to 7.0 (4). The carbon source, dissolved in acetone, was individually dispensed into each culture vessel (0.25 g of naphthalene per 25 ml of medium), and the solvent was allowed to evaporate before inoculation with the microbial culture.

Naphthalene-utilizing microorganisms were isolated using a soil enrichment technique. Nine different samples of soil from a petroliferous area in Mississippi were collected in polyethylene whirl-pak bags. A 10-g

amount of each sample was homogenized in a Waring blender with 100 ml of mineral salts medium; 2 ml of the resultant slurry was added to 25 ml of mineral salts medium in 6-ounce (177-ml) prescription bottles containing either KNO_3 or NH_4Cl as the nitrogen source. The enrichment cultures were incubated under static conditions at ambient temperature. When the naphthalene flakes had visually disappeared from the enrichment medium, subcultures were made into growth medium containing the appropriate nitrogen source (NO_3-N and NH_4-N), using an 8% inoculum from the preceding enrichment culture.

Pure culture studies with a naphthalene-degrading pseudomonad were carried out with samples incubated on a rotary shaker. All experiments were performed with 25-ml volumes of media dispensed in 6-ounce bottles.

Analyses for naphthalene. Estimation of naphthalene utilization was undertaken, using high-pressure liquid chromatography. After naphthalene oxidation, the spent medium was extracted with hexane and analyzed by using a Water Associates model 202/401 liquid chromatograph with an ultraviolet detector (wavelength, 277 nm). A 25.4- by 0.63-cm outside diameter micro-Bondapak C_{18}/Corasil column was employed with a methanol-water (7:3) solvent system at a flow rate of 2.0 ml/min.

Identification and quantification of salicylic acid. Salicylic acid was identified by thin-layer chromatography of the spent medium on Kontes Quantum tlc systems K-416117-5051, using a benzene-methanol-acetic acid (45:8:8) solvent system. Additionally, its presence was confirmed by extraction of the lyophilized spent medium with benzene, followed by recrystallization and subsequent infrared analyses and melting point determination. Routinely, salicylic acid was quantified using a slight modification of the method described by Murphy (J. F. Murphy, Ph.D. thesis, The Pennsylvania State College, University Park, 1953). The method involved adding 0.1 ml of 5% $FeCl_3$ (wt/vol) to 7 ml of spent medium that had been clarified by filtration through a 450-nm membrane filter (Millipore Corp.) and measuring the intensity of the purple color at 550 nm using a Bausch and Lomb Spectronic 20 spectrophotometer. This method will detect 1 μg of salicylic acid per ml.

Quantification and characterization of water-soluble yellow material. For comparative purposes, the intensity of the yellow color was quantified as follows: the spent medium was clarified by centrifugation and filtered through a 450-nm Millipore filter, and the intensity of the color was evaluated at a wavelength of 425 nm, using a Bausch and Lomb Spectronic 20 spectrophotometer.

To determine whether the colored compound was 1,2-naphthoquinone, the following analyses were undertaken. The absorption spectrum of the ethanol-soluble fraction in the ultraviolet, visible, and infrared range was compared with that of 1,2-naphthoquinone; the spectra were determined on a Turner spectrophotometer model 350, a Bausch and Lomb Spectronic 20 spectrophotometer, and a Perkin-Elmer Infracord spectrophotometer, respectively. Additionally, the spent medium and an ethanol extract of the lyophilized spent medium were analyzed by thin-layer chromatography, using Kontes Quantum tlc systems K-416117-5051 plates and silica gel 7GF plates, respectively, developed in a chloroform-methanol (1:1) solvent system. An ethanol solution of 1,2-naphthoquinone was chromatographed on the same thin-layer chromatography plates for comparative purposes.

The bands from the test systems were eluted from the thin-layer chromatography plate into 400 ml of a chloroform-ethanol (9:1) mixture and analyzed by the Fourier-transform infrared technique, using a Nicolett model 7199 Interferometer.

Gas analyses. In studies concerned with the production of carbon dioxide from naphthalene, growth studies were undertaken in serum stoppered 6-ounce prescription bottles containing an atmosphere of Ar-O_2-N_2-CO_2 in proportions of 49:25:22:4. After incubation for 48 h, the contents were acidified and analyzed gas chromatographically, using a Fisher model 1200 Gas Partitioner equipped with a thermal conductivity dual-detector system.

RESULTS AND DISCUSSION

In this study, naphthalene was the sole carbon source in a minimal mineral salts solution containing either NH_4Cl or KNO_3 as the nitrogen source. Disappearance of naphthalene flakes from the medium and/or the detection of salicylic acid, a known product of naphthalene metabolism, was indicative of the presence of naphthalene-utilizing microorganisms in the enrichments. A total of nine separate soil samples were prepared with each medium.

Although naphthalene disappearance was observed in soil enrichments, only those prepared with the medium containing NH_4Cl showed an accumulation of salicylic acid. Enrichments prepared with the medium containing KNO_3 exhibited a distinct yellow coloration. The rate of substrate utilization increased with each subculture. After two subcultures, all nine of the enrichments using NO_3-N as the sole nitrogen source had a visible yellow color within 43 h but no salicylic acid was detected, even after 221 h. Conversely, all nine of the enrichments using NH_4-N as the sole nitrogen source had detectable salicylic acid within 43 h. After 62 h, the number of enrichments exhibiting a positive test for salicylic acid diminished until only one culture was positive after 221 h and two of the negative cultures had developed a slight yellow coloration.

After growth, all of the enrichments were streaked on nutrient agar, and 25 colonies (selected for colonial dissimilarity) were subjected to purification procedures. Irrespective of the soil from which they were obtained or the nitrogen source employed in the enrichment procedure, all cultures responded identically to the nitrogen source employed in the medium; namely, transfer of an isolate into growth me-

dium containing KNO_3 resulted in yellow color production and no salicylic acid accumulation. Conversely, transfer into medium containing NH_4Cl resulted in salicylic acid accumulation and no yellow color production.

From the 25 naphthalene-degrading organisms isolated, a single isolate was selected for further study. It was classified as a *Pseudomonas* species on the basis of the following characteristics: it was a gram-negative, aerobic, nonsporeforming rod, motile by means of a polar flagellum. Nutrient agar colonies were translucent, mucoid, circular, and convex with a slightly irregular margin. The culture was negative for indole formation, nitrate reduction, gelatin liquefaction, and starch hydrolysis. Litmus milk showed no change. The carbohydrates glucose, lactose, and sucrose were not fermented. The isolate was catalase positive, oxidase positive, and citrate positive.

Pure culture studies on naphthalene degradation. The growth of the pseudomonad in media containing either NO_3-N or NH_4-N was accompanied by differences in cellular morphology. Cells grown in media containing NO_3-N were short rods, whereas cells from media containing NH_4-N were morphologically highly irregular and clumped. This may be due to differences in the final pH or to the presence of phenolic compounds which have been shown to induce swelling in bacterial cells (2).

Naphthalene utilization by cultures grown in mineral salts medium containing NH_4-N was accompanied by a concomitant decrease in pH to below 5.0; in the presence of NO_3-N, the pH stayed near neutral to slightly alkaline.

Cell numbers during growth in media containing NO_3-N ranged from 8×10^7 colony-forming units per ml after 48 h to 1×10^9 colony-forming units per ml after 96 h of incubation. In spite of evident turbidity and substrate utilization, viable cell numbers in media containing NH_4-N never exceeded 10^4 colony-forming units per ml. In both media, 94% of the naphthalene had disappeared as determined by high-pressure liquid chromatography analyses. As suggested by Klausmeier and Strawinski (7), the increase in acidity of the culture medium may have stopped growth or the swelling of the cells caused by phenolic compounds (2) may have destroyed viability.

Accumulation of salicylic acid (up to 42 μg/ml in 24 h and 48 μg/ml in 72 h) was demonstrated only in media containing NH_4-N. In media containing KNO_3, only a trace (<10 μg/ml) of salicylic acid was detected early in the growth cycle (less than 8 h), indicating that the compound was produced but did not accumulate in the medium.

Spectrophotometric assessment of the water-soluble yellow color indicated a considerable increase in intensity in media containing KNO_3 during the first 48 h, after which it began to decrease (Fig. 1). No color was evident in media containing naphthalene and NH_4Cl. Furthermore, no yellow color was produced from glucose when either NO_3-N or NH_4-N was employed as the nitrogen source.

To establish that the observed differences in media containing NH_4-N or NO_3-N were a response to the nitrogen source and not a manifestation of the change in pH, growth studies were undertaken in a buffered medium. The mineral salts medium routinely used was replaced by phosphate-buffered medium (0.067 M phosphate buffer) buffered at pH 5, 6, 7, and 8. The pH remained unchanged throughout the experiment. Growth was slower and appeared to be less with the increased buffering capacity, similar to the findings of Klausmeier and Strawinski (7); nevertheless, use of NH_4-N resulted in some salicylic acid accumulation, particularly at pH 5.0, and no yellow color production; conversely, the presence of NO_3-N in the medium resulted in the production of a yellow color and no salicylic acid accumulation. In the nitrate-containing media, yellow color was more intense at pH 7.0 and 8.0. This was not unexpected since other experiments with spent medium had shown that within the pH range of 4.5 to 8.0, the intensity of the color was reduced by 34% at the lower pH but never disappeared. Thus, pH alone did not account for the absence of the color in cultures containing NH_4Cl.

The first report of the production of a yellow compound from naphthalene was made by Murphy and Stone (8), who identified 1,2-naphtho-

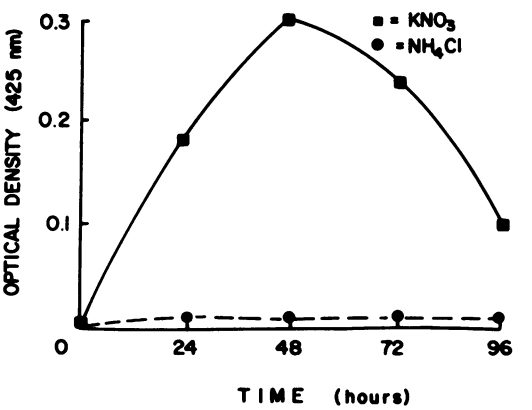

FIG. 1. *Influence of potassium nitrate and ammonium chloride on the production of a water-soluble yellow compound during the microbial metabolism of naphthalene.*

quinone from a medium containing NH_4NO_3. Since 1,2-naphthoquinone is soluble in ethanol, the absorption spectrum of the ethanol extract of lyophilized spent medium containing KNO_3 was determined. The absorption maximum was found to be at 300 nm (Fig. 2), whereas 1,2-naphthoquinone has absorption maxima at 250, 340, and 405 nm. The infrared spectrum of this ethanol extract showed bands at 1,570, 1,500, 1,460, and 1,400 cm^{-1}, which indicated that the material is aromatic. Absorption bands in the regions which would correspond to phenolic or acidic groups were absent. The lack of acidic groups in the infrared spectra coupled with the near neutral pH of the spent nitrate medium argues against the yellow coloration being caused by an acidic yellow-pigmented material, as reported by Klausmeier and Strawinski (7). To further confirm the absence of 1,2-naphthoquinone in the ethanol-soluble yellow material, thin-layer chromatograms were made from the ethanol extract of the lyophilized spent medium. Although four distinctly yellow bands and one slightly yellow band were obtained, none corresponded to the 1,2-naphthoquinone standard. Under the conditions of the test, the R_f values of the four yellowest bands were 0.07, 0.17, 0.51, and 0.90, as compared with 0.85 for 1,2-naphthoquinone.

To gain some insight into the nature of the materials in the four yellowest bands, eluates of the bands were subjected to infrared analyses. The spectra for all bands were suggestive of highly conjugated carbonyl systems but the spectra did not match 1,2-naphthoquinone.

Further attempts to characterize the water-soluble yellow materials by using high-pressure liquid chromatography, infrared analysis, and gas chromatography-mass spectrometry have thus far proved unsuccessful, in part due to the limited amount of material available for analyses. At the present time, the thin-layer chromatography data suggest that the yellow color produced in the nitrate medium is caused by more than one compound.

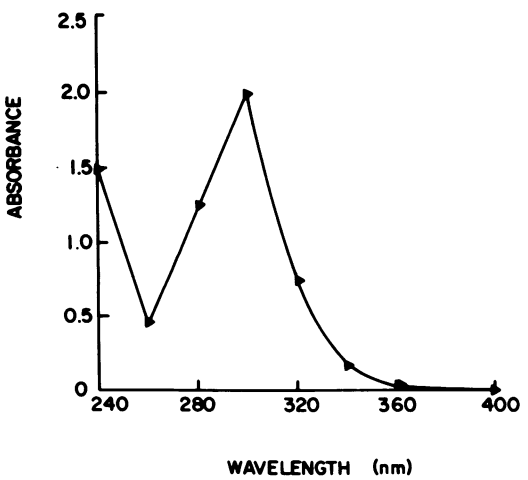

FIG. 2. *Absorption spectrum of the water-soluble yellow compound as a function of wavelength.*

When the culture was grown in a closed system (serum stoppered, 6-ounce bottle) containing an atmosphere of Ar-O_2-N_2-CO_2 (49:25:22:4) and a naphthalene concentration of 0.1%, another major difference caused by the nitrogen source of the medium was observed. Naphthalene oxidation in media containing NO_3-N was accompanied by a sevenfold increase in carbon dioxide evolution as compared with oxidation in media containing NH_4-N (Table 1). Oxygen utilization in the NO_3-N cultures was likewise essentially seven times greater than it was in the NH_4-N cultures, and thus, the ratios of CO_2 produced to O_2 consumed were essentially the same in both media (respiratory quotient $\simeq$ 1.0). Estimation of the residual amount of naphthalene in the culture media revealed that essentially all of the naphthalene (>90%) had disappeared from both the NO_3-N culture and the NH_4-N culture. These data suggest that naphthalene degradation is more complete in the presence of NO_3-N as the sole nitrogen source.

When the organism was grown in media containing NH_4NO_3, as employed by Murphy and Stone (8) and Strawinski and Stone (11), the growth characteristics closely resembled those of cells grown in the presence of NH_4-N. Naphthalene degradation for 48 h in media containing NH_4NO_3 was accompanied by a reduction in pH (5.3), no observable yellow color production, and accumulation of salicylic acid (35 μg/ml), and the amounts of carbon dioxide evolution (6.2 ml) and oxygen utilization were similar to those obtained from NH_4-N-containing media. These re-

TABLE 1. *Effect of different nitrogen sources on pH, production of salicylic acid, yellow color, and carbon dioxide accumulation during naphthalene dissimilation*[a]

Growth medium containing:	pH	Salicylic acid (μg/ml)[b]	Yellow color (absorbance unit)[c]	CO_2 (ml)[d]
KNO_3	7.3	ND[e]	0.3	29.7
NH_4Cl	4.7	42.0	0.1	4.4

[a] Data recorded after 48 h of incubation.
[b] Quantified by using the color reaction with $FeCl_3$.
[c] Assessed spectrophotometrically at 425 nm after removal of the cells by centrifugation and filtration.
[d] Quantified by gas chromatography.
[e] ND, Not detected (<1 μg/ml).

sults suggest that when available simultaneously, the effect of NH_4-N is dominant over the effect of NO_3-N during the degradation of naphthalene.

To our knowledge, this is the first report suggesting that the mineral nitrogen source may have a major influence on the accumulation of end products or intermediates resulting from naphthalene dissimilation. Though no particular attention has been given to the nitrogen source during studies on hydrocarbon degradation, the effect of nitrogen has been determined in other biological systems including plants (9), algae (1), and fungi (12). In this study, provision of different nitrogen sources during naphthalene oxidation resulted in differences in cellular morphology, salicylic acid accumulation, carbon dioxide evolution, and the production of a yellow coloration in the medium. The relevance of these findings to in situ polynuclear aromatic hydrocarbons remains to be determined.

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