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Microbial Hydrocarbon Co-oxidation

I. Oxidation of Mono- and Dicyclic Hydrocarbons by Soil Isolates of the Genus *Nocardia*

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Nocardia cultures, isolated from soil by use of *n*-paraffins as the sole carbon source, have been shown to bring about significant oxidation of several methyl-substituted mono- and dicyclic aromatic hydrocarbons. Oxygen uptake by washed cell suspensions was not a reliable indicator of oxidation. Under co-oxidation conditions in shaken flasks, *o*- and *p*-xylenes were oxidized to their respective mono-aromatic acids, *o*-toluic and *p*-toluic acids. In addition, a new fermentation product, 2,3-dihydroxy-*p*-toluic acid, was found in the *p*-xylene oxidation system. Of 10 methyl-substituted naphthalenes tested (1-methyl, 2-methyl, 1,3-dimethyl, 1,4-dimethyl, 1,5-dimethyl, 1,8-dimethyl, 1,6-dimethyl, 2,3-dimethyl, 2,6-dimethyl, 2,7-dimethyl), only those containing a methyl group in the β position were oxidized at this position to the mono acid.

It is a generally accepted view among hydrocarbon microbiologists that the initial attack by microorganisms on aromatic hydrocarbons has received little attention. The literature is voluminous with respect to oxidation of aromatic non-hydrocarbon compounds, and it seems to us unfortunate that in many instances no distinction is made in the reporting of research between these two very different types of compounds. With few exceptions, most reported microbial aromatic hydrocarbon oxidations have centered on those cultures which bring about sufficient degradation of the molecule to provide energy and carbon for growth. What perhaps will be shown to have a more widespread occurrence in nature and will be more useful in industry is the partial oxidation of a compound, with the microorganism deriving its energy from carbon, and its growth from a cosubstrate. It is the purpose of this series of papers to demonstrate the extensive co-oxidation of aromatic hydrocarbons by the ubiquitous *Nocardia*.

Most of the pertinent literature concerning the oxidation of aromatic hydrocarbons has been adequately covered in a recent review by Van Der Linden and Thijsse (7). Recent studies by Claus and Walker (1) and Yamada et al. (8) have been concerned with monocyclic aromatic hydrocarbon oxidation by *Pseudomonas* and *Achromobacter*. Claus and Walker postulated that 3-methylcatechol was an early intermediate in the oxidation of toluene by strains capable of using toluene for growth. Yamada et al. found that several soil isolates closely related to *P. desmolytica* could grow and accumulate cumic acid from *p*-cymene. In contrast to the findings of Davis and Raymond (2), who observed cumic acid from *p*-cymene during growth of a *Nocardia* culture on *n*-alkanes, the pseudomonads isolated by Yamada et al. could not grow on *n*-alkanes. Their cultures were also observed to grow on toluene, *p*-xylene, ethylbenzene, and cumene, with no indication of an accumulation of products other than cells.

This paper concerns the efforts of our laboratory to determine the scope of aromatic hydrocarbon oxidation by certain selected soil isolates of the genus *Nocardia*. We have been primarily interested in what effect the position and number of methyl substituents on the aromatic nucleus has on the initial attack by these microorganisms. More specifically, we have endeavored to find which, if any, structures lead to significant accumulation of products, and further, what the products are.

Materials and Methods

Microorganisms. Most of the work described was carried out with a *Nocardia* culture designated *N. corallina* A-6. This culture and *N. corallina* A-11 were isolated from soil at the Marcus Hook, Pa., refinery with use of no. 2 fuel oil as the growth substrate. *N. salmonicolor* A-100 was isolated from an East Texas oil field top soil with use of ethane as the growth substrate. *N. albicans* A-116, *N. minima* A-138, and *Nocardia* sp. V-33 were isolated from field soils with use of *n*-hexadecane as the isolation substrate. Cul-

tures A-116 and V-33 were nonpigmented. The other cultures varied in color from pink to orange.

Identification of these soil isolates was carried out by methods described in the *Manual of Microbiological Methods* (6) and classified according to *Bergey's Manual*.

Media and culture conditions. Isolation and growth studies were carried out on basal medium of the following composition: 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% Na_2CO_3, 0.001% $CaCl_2 \cdot 2H_2O$, 0.002% $MnSO_4 \cdot H_2O$, 0.0005% $FeSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, 0.04% KH_2PO_4, 0.06% Na_2HPO_4, and 1.5% washed agar in distilled water. The *p*H of the medium was varied by adjusting the ratio of Na_2HPO_4-KH_2PO_4. This medium was further modified by the addition of phenol red, 0.001%, and urea, 0.04%, substituted for $(NH_4)_2SO_4$; for detecting acid production from the hydrocarbons investigated, the *p*H was adjusted to 7.3.

Growth studies were carried out on mineral-agar plates. All hydrocarbons were used in the "vapor" phase. The more volatile compounds, hexane, benzene, toluene, and the xylenes, were introduced into vacuum-type desiccators from an attached reservoir as a vapor after closing the desiccator. This prevents driving out the air and creating a reduced oxygen environment. The remainder of the hydrocarbons (with the exception of ethane, which was added at a 40% concentration in oxygen and nitrogen in a desiccator) were added to filter-paper discs located in the top of the petri dishes. Cultures were incubated for 15 to 25 days at 30 C, after which time a positive result was recorded when growth exceeded a control plate incubated without hydrocarbon. Further confirmation was obtained by repeated transferring to new slants or plates incubated over the hydrocarbon in question.

Liquid medium for cell growth and product formation in shaken flasks was similar to the basal medium with the following exceptions. Total phosphate was increased to 0.5%, and 0.2% urea was substituted for $(NH_4)_2SO_4$. All shaken flask studies were carried out with 100 ml of medium in 500-ml Bellco no. 603 flasks, which have baffles in the bottom for increased agitation. A New Brunswick Incubator-Shaker model G-27 (New Brunswick Scientific Co., New Brunswick, N.J.) was maintained at 300 strokes per min at 30 C throughout the investigation.

Hydrocarbons were added as increments to all shaken flask systems. For cell production, additions to flasks were as follows: initial, 50 mg; at 24 hr, 100 mg; and at 48 hr, 250 mg.

After 72 hr, cells were recovered by centrifugation at room temperature, washed and centrifuged twice in 0.03 M phosphate buffer (*p*H 7.0), and resuspended to desired concentration in the same solution. Cell weights were determined by drying samples at 105 C to constant weight.

Manometric methods. Oxygen uptake by whole cells was measured with a Warburg apparatus in the conventional manner. Solid hydrocarbons were added to Warburg flasks as ether solutions, and the ether was removed by a gentle stream of air. Liquid hydrocarbons were added with microliter syringes (Hamilton Co., Whittier, Calif.).

Hydrocarbons. Benzene and toluene were reagent grade commercially available materials (Allied Chemical Co., Morristown, N.J.) and were used without further purification. The xylenes (*o*, *m*, *p*), ethane, and hexane were pure or research grade with a purity of 99% + (Phillips Petroleum Co., Bartlesville, Okla.). Naphthalene (Sun Oil Co., Philadelphia, Pa.) was 99% + pure. The 1- and 2-methylnaphthalenes (Humphrey Chemical Co., North Haven, Conn., and Distillation Products, Rochester, N.Y., respectively) assayed at 98.4 and 99%. *n*-Hexadecane was olefin-free (Humphrey Chemical Co., North Haven, Conn.). Except for 1,2-dimethylnaphthalene and 1,6-dimethylnaphthalene, the dimethylnaphthalenes were commercially available and purified by recrystallization from methanol when necessary (Aldrich Co., Fairfield, N.J.; Henley & Co., Inc., New York, N.Y.; and K & K Laboratories, Plainview, N.Y.) to obtain a purity of 99% +. The 1,2 isomer was assayed and found to be 96% pure (remainder, a non-dimethylnaphthalene impurity) and the 1,6 isomer, 91% pure (remainder, other dimethylnaphthalene isomers).

Product isolation. In the initial stages of this investigation, fermentation broths, after centrifugation to remove cells under neutral or slightly alkaline conditions, were acidified with 10% HCl to *p*H 2.0 and were subjected to continuous extraction with diethyl ether for periods up to 24 hr. Experience has shown that these conditions only complicate subsequent purification, since catechols, phenols, and many hydrocarbons form highly colored complexes and emulsions with the extracted cell debris. If significant oxidation occurs, we have found that ether extraction of the clarified, acidified beers employing a separatory funnel at ambient temperature is quite satisfactory.

After extraction, the ether was washed with a small quantity of distilled water to remove HCl. The wet ether was removed under a stream of dry nitrogen in a large beaker (200 ml of ether in a 1-liter beaker) on a steam table to facilitate rapid removal of ether and traces of water. The extract was redissolved by washing with small samples of dry ether and transferring to a small tared bottle. Removal of the ether in the same manner as above usually resulted in a product free of salts and residual aromatic hydrocarbons.

Product identification. Thin-layer chromatographic (TLC) plates of Silica Gel G (Arthur H. Thomas Co., Philadelphia, Pa.) were employed. Development of plates was carried out in a solvent system of methyl ethyl ketone–ethyl alcohol (100%)–ammonium hydroxide (28%), 35:60:5. After development and drying, plates were observed in a light box with long and short ultraviolet (UV) irradiation. Acids were detected with a neutral bromocresol green, 0.2%, in *n*-butanol spray; phenols and catechols, with a 5% $Fe(NO_3)_3$ in 1% HNO_3 spray. Unknowns were eluted from TLC plates for further study by UV (Beckman DK2-A), mass spectrometry (Consolidated 102) and infrared (Perkin-Elmer model 137) methods.

Synthesis of 2,3-dihydroxy-p-toluic acid (DHPT). This compound was prepared by a modification of the Kolbe-Schmitt (5) procedure. A 300-ml stainless-steel bomb containing a mixture of 15 g of 3-methylcatechol and 30 g of K_2CO_3 was pressurized to 685 psi with dry CO_2. The bomb and contents were heated

to a temperature of 225 C and maintained for 5 hr (pressure, 1,100 psi). After cooling, the reaction mixture was acidified with dilute HCl and extracted with diethyl ether. The ether was extracted with 5% $NaHCO_3$ to remove any unreacted 3-methylcatechol. After removal of the ether and drying, 18 g of DHPT was recovered.

RESULTS

Description of cultures. The classification of microorganisms in the order Actinomycetales isolated from soil apparently is open to question (4). In our opinion, cultures described in this paper belong to the genus *Nocardia*. They are nonacid-fast, gram-positive, and characteristically form long rods (10+ μ) in young cultures (<24 hr), which break up into short coccodial rods. They grow on dextrose, maltose, sucrose, lactose, and mannitol with no evidence of acid formation. Nitrites are produced from nitrates. Tests for gelatin liquification, starch hydrolysis, indole, H_2S, and urease are all negative. Colony color and consistency are highly variable properties within each culture, but in no instance have any of the cultures used in this study shown any tendency to form aerial hyphae.

Growth data on a solid medium by culture A-6 shown in Table 1 is fairly representative of *Nocardia* cultures isolated on *n*-paraffins from soils without a history of gross contamination with aromatic hydrocarbons. They grow readily on *n*-paraffins, routinely giving in shaken flasks a 75% conversion of *n*-hexadecane to cell material (dry weight basis) in a 72- to 96-hr period. Growth

TABLE 1. *Growth of Nocardia corallina, A-6 on hydrocarbons*

Hydrocarbon[a]	Growth
Ethane	+
Hexane	+
Hexadecane	+
Benzene	−
Toluene	+
p-Xylene	−
o-Xylene	−
m-Xylene	−
Durene	−
Naphthalene	−
1-Methylnaphthalene	−
2-Methylnaphthalene	−
1,3-Dimethylnaphthalene	−
1,5-Dimethylnaphthalene	−
1,6-Dimethylnaphthalene	−
1,8-Dimethylnaphthalene	−
2,3-Dimethylnaphthalene	−
2,6-Dimethylnaphthalene	−
2,7-Dimethylnaphthalene	−

[a] Hydrocarbon was sole source of carbon.

TABLE 2. *Effect of growth substrates on the conversion of 2,6-dimethylnaphthalene (2,6-DMN) → 6-methyl-2-naphthoic acid (6-M-2-NA) by whole cells of Nocardia corallina A-6*[a]

Growth substrates	O_2[b] uptake	Acid formed
	μM	μM
n-Hexadecane	6.7	2.8
n-Hexadecane + 2,6-DMN	2.2	1.5
n-Hexadecane + 6-M-2-NA	3.3	2.6
Nutrient broth	2.2	0.5
Nutrient Broth + 2,6-DMN	2.2	0.9

[a] Each Warburg vessel contained a cell suspension (1 ml; approximately 12 mg/ml, dry weight) and 2,6-DMN (3.19 μM). Center well contained 0.2 ml of 40% KOH. Length of experiment, 320 min at 30.3 C. Final volume was brought to 2.7 ml with 0.03 M PO_4 buffer, *p*H 7.1. Acid concentration was determined by UV adsorption measurements at 285 mμ.

[b] Corrected for endogenous.

on toluene-mineral-agar plates is profuse, but requires considerably more time to reach the same cell concentration in shaken flasks as that observed from *n*-paraffins.

Initial co-oxidation studies with several *Nocardia* cultures in shaken flasks containing aromatic hydrocarbons were essentially negative until it was observed that a nutrient medium containing high concentrations of nonhydrocarbon organic materials such as starch resulted in the oxidation of 2,6-dimethylnaphthalene to the 6-methyl-2-naphthoic acid (6-M-2-NA; J. D. Douros, *unpublished data*). This observation initiated a study of the factors controlling this conversion with pregrown cells in Warburg flasks.

Pregrown cell studies. Cells grown in shaken flasks, as described under Materials and Methods, gave varying oxygen uptake values per mole of acid from 2,6-dimethylnaphthalene, depending upon the substrate used for growth. Typical results are shown in Table 2. In almost every instance, oxygen uptake took place for at least 4 hr before any acid could be detected by UV methods. The effect of concentration of 2,6-dimethylnaphthalene and glucose on oxygen uptake and acid formation by hexadecane-grown cells is shown in Fig. 1. To eliminate the possibility that additional oxygen uptake was the result of oxidation of the acid, low concentrations of 6-M-2-NA were examined in the presence and absence of 2,6-dimethylnaphthalene. These results are shown in Table 3. From these and other data obtained in shaken flasks, we concluded that further oxidation does not occur even under growth conditions. What appears to be the problem is the stimulation

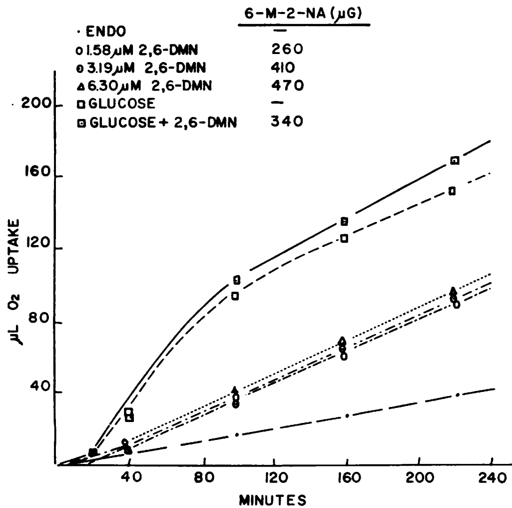

FIG. 1. *Effect of 2,6-dimethylnaphthalene and glucose concentration on oxygen uptake and 6-methyl-2-naphthoic acid formation. Oxygen uptake was measured at 30. 3 C. Each Warburg vessel contained 4.8 mg (dry weight) of cells suspended in 0.03 M phosphate buffer (pH 7.0), and 0.2 ml of 40% KOH in center well. Total volume, 2.7 ml. Concentration of acid was determined by UV at 286 mμ at end of experiment.*

TABLE 3. *Stability of 6-methyl-2-naphthoic acid (6-M-2-NA) in the presence of hexadecane-grown Nocardia corallina A-6*[a]

System	Substrate	Incubation time	Concentrated 6-M-2-NA recovered
		hr.	*μg*
1	100 μg of 6-M-2-NA	6	100
2	100 μg of 6-M-2-NA	27	98
3	828 μg of 6-M-2-NA	22	828
4	100 μg of 6-M-2-NA + 10 μg of 2,6-dimethylnaphthalene	12	111
5	10,000 μg of 2,6-dimethylnaphthalene	12	510

[a] Each Warburg vessel contained 10 mg (dry weight) of cells suspended in 0.03 M phosphate buffer, pH 7.0, and 0.2 ml of 40% KOH in center well. Total volume, 2.7 ml.

of endogenous respiration by the acid (Table 4). Examination of other hydrocarbons (*o, m, p*-xylenes and other dimethylnaphthalenes) by the oxygen uptake method gave very similar results, indicating that stimulation and repression of endogenous respiration makes this method a very unreliable indicator of partial oxidation.

Pregrown cells were also shown to oxidize 2,7-dimethylnaphthalene to 7 methyl-2-naphthoic acid (7-M-2-NA). A comparison of the rates of 2,6- and 2,7-dimethylnaphthalene oxidation under these conditions (Fig. 2) demonstrates that

TABLE 4. *Effect of 2,6-dimethylnaphthalene (2,6-DMN) and 6-methyl-2-naphthoic acid (6-M-2-NA) on oxygen uptake of hexadecane-grown Nocardia corallina A-6*[a]

Concn		Oxygen uptake[b] (μliters/7 hr)
2,6-DMN	6-M-2-NA	
μM	*μM*	
3		+12
6		+58
12		+152
24		+58
	2	+157
	10	+137
	30	−174

[a] Each Warburg vessel contained 4.5 mg (dry weight) of cells suspended in 0.03 M phosphate buffer, pH 7.0, and 0.2 ml of 40% KOH in center well. Total volume, 2.7 ml.
[b] Corrected for endogenous.

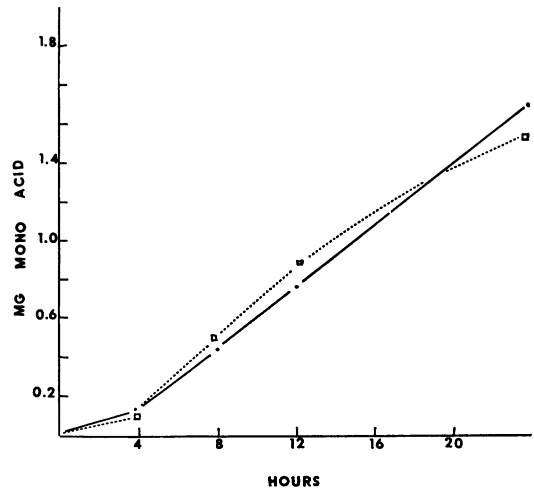

FIG. 2. *Comparison of rate of oxidation of 2,6- and 2, 7-dimethylnaphthalene by hexadecane-grown cells of Nocardia corallina, A-6. Each Warburg vessel contained 18 mg (dry weight) of cells suspended in 0.03 M phosphate buffer, 10 mg of dimethylnaphthalene, and 0.2 ml of 40% KOH in center well. Total volume, 2.7 ml. Symbols:* O, *7-methyl-2-naphthoic acid;* □, *6-methyl-2-naphthoic acid. Acids were determined by UV and concentrations shown are based on the 2.7-ml volume.*

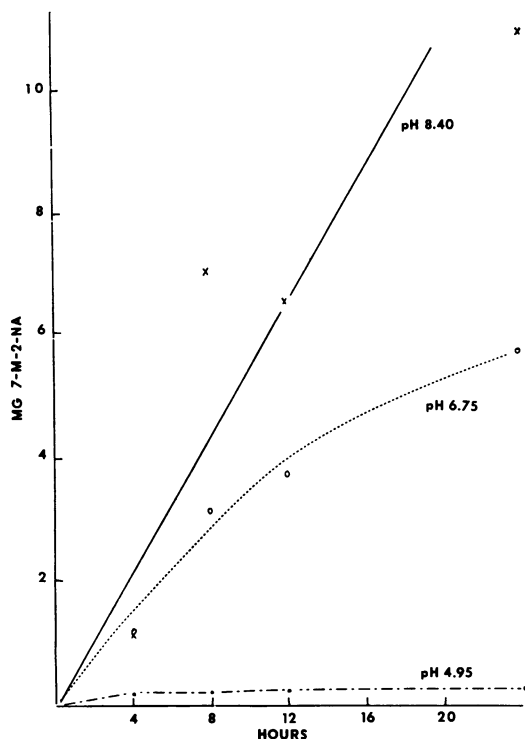

FIG. 3. *Effect of pH on oxidation of 2,7-dimethylnaphthalene by hexadecane-grown cells of Nocardia corallina A-6. Experiment was carried out in Warburg vessels at 30.3 C. Each vessel contained 30.5 mg (dry weight) of cells suspended in 5.0 ml of 0.03 M phosphate buffer and 20 mg of 2,7-dimethylnaphthalene. 7-Methyl-2-naphthoic acid was determined by UV.*

the organism can handle either molecule with equal facility. A high pH level favors this oxidation (Fig. 3). The results at the higher pH value are erratic, owing to a tendency of the cells to form emulsions with the residual 2,7-dimethylnaphthalene, which in turn complicates the acid analysis.

Co-oxidation in shaken flasks. The rather facile attack on the 2,6- and 2,7-dimethylnaphthalenes and negative results for other aromatics led to an examination of acid production from other aromatic hydrocarbons on mineral agar plates. Co-oxidation on plates has the advantage of better control of substrate. The incorporation of phenol red, as indicated under Materials and Methods,

TABLE 5. *Co-oxidation of p-xylene by Nocardia corallina A-6 growing on n-hexadecane*

System	No. of days in shaker	Total C_{16}[a] added	Total *p*-xylene	Cells recovered	Product recovered
		mg/ml	*mg/ml*	*mg/ml*	*mg/ml*
P43......	4	3.10	1.90	1.7	0.30
P44-3....	5	5.28	2.50	—[b]	0.66
P44-4....	5	5.28	2.98	—	0.33
P44-5....	5	5.28	3.84	—	1.74
P45-3....	4	5.28	2.98	3.3	0.65
P45-4....	6	6.16	4.70	4.2	0.92
P45-7....	4	6.38	2.98	4.8	0.75
P45-8....	6	6.38	4.96	5.0	0.57

[a] *n*-hexadecane.
[b] Cells lost during recovery process.

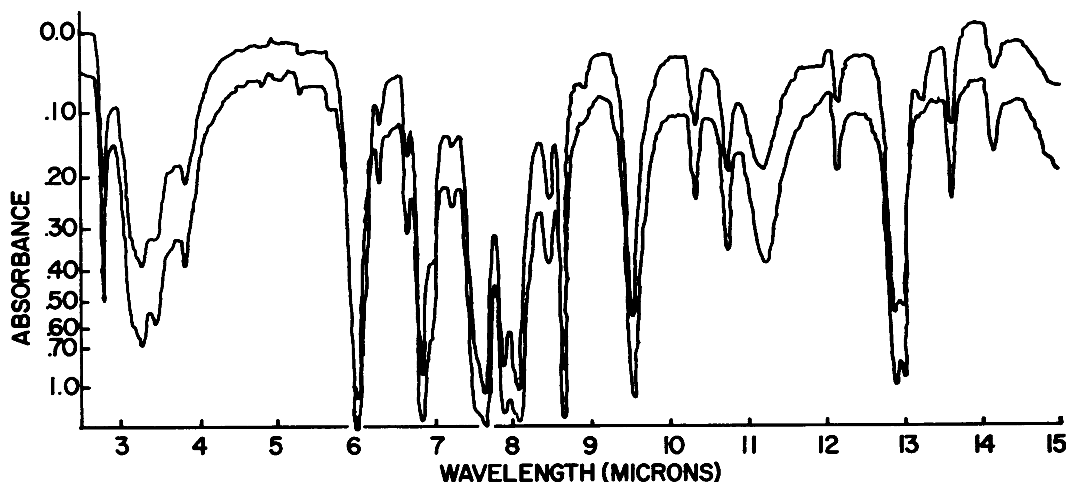

FIG. 4. *Infrared spectrum of synthetic (lower curve) and biological (upper curve) 2,3-dihydroxy-p-toluic acid.*

provided a sensitive detection system. With such a system, we observed that yellow zones formed around colonies of culture A-6 growing rapidly on *n*-hexadecane in the presence of several different aromatic hydrocarbons. These observations, coupled with the long induction period observed in Warburg studies, led to a series of trials in which the time of initial addition and quantity of the aromatic to be oxidized were varied relative to the growth stage. These studies led to a feeding schedule in which the aromatic hydrocarbon was not added until cell concentrations reached 100 to

TABLE 6. *Effect of nitrogen source and concentration on growth and acid production from p-xylene by Nocardia corallina A-6*

System	C_{16}[a] added	*p*-Xylene added	Nitrogen		*p*H		Acid	Cells
			Urea	NH_4NO_3	Initial	Final		
	mg/100 ml	*mg/100 ml*	%	%			*mg/100 ml*	*mg/100 ml*
P48-10	524	602	0.2		7.0	6.6	86	415
P48-14	525	602	0.2		7.4	6.8	124	441
P48-15	524	602	0.35		7.4	7.0	144	423
P48-16	524	602		0.2	7.4	5.9	107	267
P49-4	444	344		0.1	7.0	6.2	54	223
P49-5	444	400		0.1	7.4	6.6	151	373
P49-6	444	400		0.2	7.4	6.6	94	245
P49-7	444	400		0.3	7.4	6.1	113	302
P49-8	444	400		0.4	7.4	6.5	57	182

[a] *n*-Hexadecane.

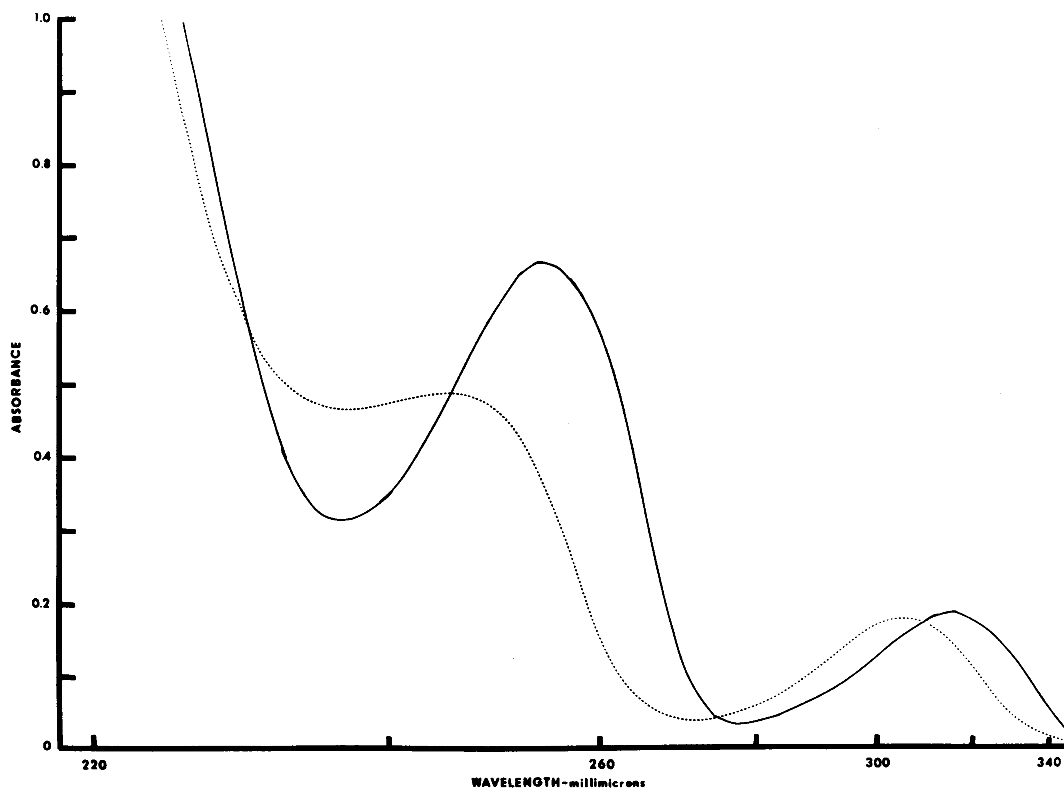

FIG. 5. *Ultraviolet spectrum of 2,3-dihydroxy-p-toluic acid in methanol. Dashed line, alkaline; solid line, acid.*

150 mg (dry weight per 100 ml of medium). For most of the *Nocardia* cultures studied, this required 24 to 48 hr, starting with a 10% inoculum in shaken flasks.

Co-oxidation of p-xylene. Phenol red-containing mineral agar plates streaked with *N. corallina* A-6 and incubated over a mixture of *n*-hexadecane and *p*-xylene gave very intense yellow zones around colonies in 24 to 48 hr. In the absence of phenol red, the agar took on a reddish brown color after several days. Because of this indication of product on plates, further study was carried out in shaken flasks to obtain enough material for identification. Tables 5 and 6 show data obtained in shaken flasks with culture A-6 in the presence of *p*-xylene and *n*-hexadecane. The total acid production shown in Table 5 obtained with varying rates and ratios of hexadecane–*p*-xylene additions indicate the very high degree of variability encountered in these systems, most of which can be accounted for by unfavorable growth conditions. Table 6 indicates that urea or NH_4NO_3 are equally satisfactory nitrogen sources.

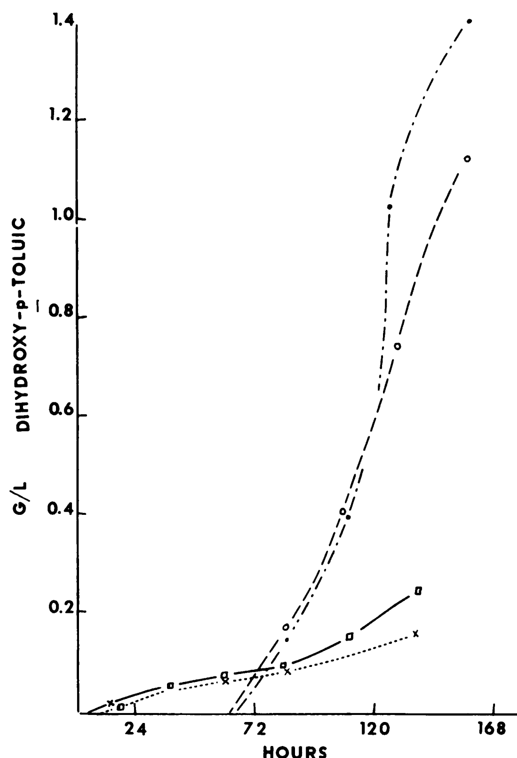

FIG. 6. *Influence of initial p-xylene addition on 2,3-dihydroxy-p-toluic acid formation in a co-oxidation system of Nocardia corallina A-6 growing on n-hexadecane with either urea or* NH_4NO_3 *as a nitrogen source. Curves 1 and 2, p-xylene addition started at time of inoculation;* ×, *urea;* □, NH_4NO_3. *Curves 3 and 4, p-xylene addition started at 60 hr;* ●, *urea;* ○, NH_4NO_3.

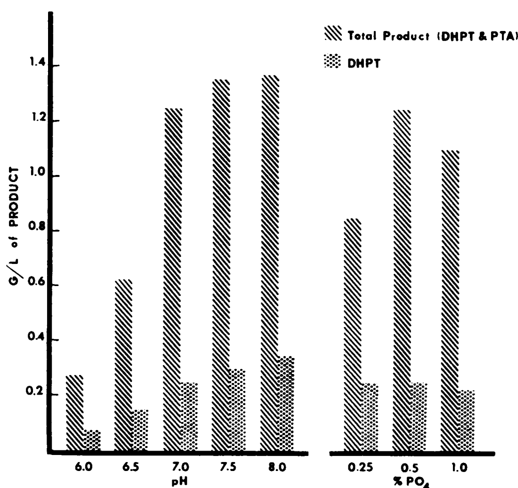

FIG. 7. *Co-oxidation of p-xylene and n-hexadecane by Nocardia corallina, A-6 in shaken flasks at different pH levels and phosphate concentrations. Media and growth conditions are those described under Materials and Methods. The phosphate concentration for the pH study was 0.5%. The pH of the phosphate study was 7.0. Flasks were analyzed after 6 days.*

Examination of the isolated acids by TLC gave two acidic spots (bromocresol green) with R_F 0.35 and 0.59 with use of methyl ethyl ketone-ethyl alcohol (100%)-NH_4OH (28%) (35:60:5). An additional spray with $Fe(NO_3)_3$ gave a black spot at R_F 0.59. Extraction of the R_F 0.35 spot and examination by UV methods indicated this to be *p*-toluic acid. This was further confirmed by comparison with authentic *p*-toluic acid purchased from a commercial source.

In addition to a phenol reaction, the R_F 0.59 material gave a positive Evans test (3) for catechols. A study of available mono- and dihydroxy aromatic acids by UV methods (shift in adsorption peaks versus concentration) tentatively identified the unknown as 2,3-dihydroxy-*p*-toluic acid (DHPT). This compound was not commercially available and had not been described in the literature. Synthesis as outlined under Materials and Methods led to the following similarities between the unknown and the synthesized DHPT. Elemental analysis: synthetic: —C, 57.0; H, 5.08; O, 37.92; unknown: —C, 57.55; H, 5.06; O, 37.39; required for $C_8H_8O_4$: —C, 57.1; H, 4.8; O, 38.1.

Infrared and ultraviolet spectra were identical, as shown in Fig. 4 and 5.

The effect of timing on the initial addition of

p-xylene is shown in Fig. 6. Addition of *p*-xylene from the time of inoculation prevents the development of satisfactory growth. This can be overcome somewhat by continuous addition of the *p*-xylene at very low rates, a procedure which is very difficult to carry out in shaken flask systems. Figure 7 shows that the optimal *p*H for production of both acids in shaken flasks by culture A-6 is probably above 7.5. A phosphate concentration of 0.5% was found to be optimal for the production of both acids.

Table 7 compares several *Nocardia* cultures in their ability to oxidize *p*-xylene to *p*-toluic acid and DHPT. The significance of the inability of the nonpigmented species to oxidize *p*-xylene is under investigation.

Oxidation of other aromatics hydrocarbons. Of the other monocyclic hydrocarbons tested, benzene, toluene, *m*-xylene, and *o*-xylene, only *o*-xylene gave a significant accumulation of products. This product was shown to be *o*-toluic acid by comparison with authentic material with use of TLC, UV, and infrared methods. Traces of a muconic acid were detected by UV methods in the toluene growth medium.

TABLE 7. *Conversion of p-xylene to 2,3-dihydroxy p-toluic acid (DHPT) and p-toluic acid (PTA) by several Nocardia species in co-oxidation systems*[a]

Nocardia species	Yield (mg/100 ml)	
	DHPT	PTA
N. corallina A-6	22	46
N. corallina A-11	23	110
N. salmonicolor A-100	33	111
N. albicans A-116[b]	0	0
N. minima A-138	15	43
Nocardia sp. V-33[b]	0	0

[a] Growth substrate was *n*-hexadecane.
[b] Nonpigmented species.

TABLE 8. *Co-oxidation of methylnaphthalenes by Nocardia corallina A-6*[a]

Substrate	Aromatic acid yield (mg/100 ml)
1-methylnaphthalene	4.6
2-methylnaphthalene	159.4
2,3-dimethylnaphthalene	19.2
1,3-dimethylnaphthalene	94.6
1,6-dimethylnaphthalene	33.6

[a] Growth substrate was *n*-hexadecane (0.5 ml/flask). Total aromatic hydrocarbon added to each flask was 250 mg (except for 2,3-DMN, 400 mg). Total incubation time, 6 days.

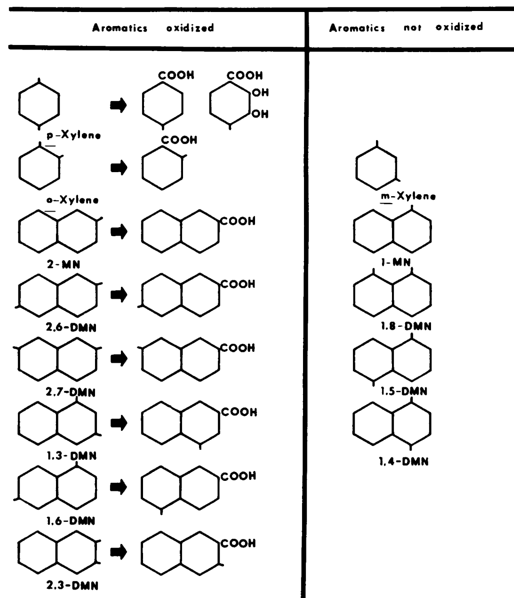

FIG. 8. *Summary of co-oxidation studies with Nocardia isolated on n-paraffins.*

In addition to 2,6- and 2,7-dimethylnaphthalene oxidation, *N. corallina* A-6 oxidized 2-methylnaphthalene and 1,3-, 1,6-, and 2,3-dimethylnaphthalenes. Yields of acidic products from shaken flasks are shown in Table 8. All products were shown to be mono acids. Nuclear magnetic resonance studies indicate that all oxidations took place in the β position.

A resume of the hydrocarbons tested and products identified are shown in Fig. 8. In summary, it is very apparent that many aromatic hydrocarbons which will not support growth can be partially oxidized by soil isolates. This is particularly significant, not only in finding cultures which can accumulate products, but as an additional avenue of decomposition of these compounds in nature.

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