Microbial Degradation of Cycloparaffinic Hydrocarbons via Co-metabolism and Commensalism*

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SUMMARY

Mineralization of a cycloparaffinic hydrocarbon in soil has been demonstrated. Addition of [U-¹⁴C]cyclohexane to marine mud, followed by incubation at 26 °C, resulted in the evolution of ¹⁴CO₂. All attempts in our laboratory to isolate organisms from soil which utilize cycloparaffins as a sole source of carbon and energy have proved unsuccessful. However, several hydrocarbon-utilizing bacteria oxygenated cycloparaffins to ketone derivatives that serve as the energy and carbon source for numerous other soil micro-organisms. The concerted attack of a mixed microbial population on cyclohexane has been demonstrated, suggesting that both co-metabolism and commensalism are associated with microbial degradation of cycloparaffinic hydrocarbons.

INTRODUCTION

In an earlier brief report (Beam & Perry, 1973) the probability that co-metabolism plays a role in the degradation of cycloparaffinic hydrocarbons in nature was discussed. The present paper provides additional information concerning our original hypothesis on cycloparaffin degradation.

The resistance of cycloparaffinic hydrocarbons to microbial attack has resulted in a paucity of reports concerning their biodegradation. Few reports (Imelik, 1948; Jones & Edington, 1968; Skarzynski & Czekalowski, 1946) claim utilization of unsubstituted cycloparaffins by pure cultures of micro-organisms. Cycloalkanones, however, appear to support the growth of numerous micro-organisms commonly found in soil (Shaw, 1966). Conversion of cycloalkanes to the corresponding cycloalkanone by microbial co-oxidation has been demonstrated (Ooyama & Foster, 1965). The presence of non-specific extracellular enzymes in soil that could oxygenate hydrocarbons has been reported (Skujins, 1967). These factors suggest that biodegradation of yet another group of environmentally important molecules, the cycloparaffins, may occur by the increasingly recognized process of co-metabolism as well as a commensal relationship between the micro-organisms involved.

METHODS

Organisms. The organisms utilized in this study were: *Mycobacterium rhodochrous* strain 7E1C (Dunlap & Perry, 1968); *Mycobacterium album* strain 7E1BIW (Blevins & Perry, 1972); *Mycobacterium vaccae* strain JOB5 (Vestal & Perry, 1969); *Mycobacterium rhodochrous* strain OFS (Dunlap & Perry, 1967); *Nocardia asteroides* strain A116; and strains R22

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(Blevins & Perry, 1972) and B58 which are short, Gram-positive, non-acid-fast, non-sporing rods similar to *Mycobacterium convolutum* (Gray & Thornton, 1928; Dr Ruth E. Gordon, Institute of Microbiology, Rutgers University, personal communication). Stock cultures were maintained on L-salts medium (Leadbetter & Foster, 1958) with propane (50/50, v/v, with air) as substrate. Strain CY6, a Gram-positive, non-acid-fast, ketone-utilizing rod, was isolated from soil by enrichment with cyclohexanone as substrate. This organism could not utilize cyclohexane, propane or normal alkanes (C_{12} to C_{18}) as substrate.

Substrates. All hydrocarbons and intermediates were the best obtainable from commercial sources and had a minimum purity of 99.0 %.

Culture conditions. The ability of organisms to utilize cycloparaffins was assessed by adding substrates at a concentration of 0.2 % (v/v or w/v) in 125 ml Erlenmeyer flasks containing 30 ml L-salts medium. Cultures were examined after incubation at 26 °C for 7 days on a rotary shaker.

Mineralization of [$U-^{14}C$]cyclohexane. The biodegradation of cyclohexane in soil was measured as previously described (Beam & Perry 1973). An apparatus similar to that described by Boggiolini & Bickel (1966) was employed except that the cold trap after the incubation vessel was maintained at -20 °C. This eliminated the significant radioactivity produced by the control in our earlier experiment.

Ketogenesis determinations. Non-proliferating suspensions of JOB5, OFS, 7EIC, R22, and CY6 were examined by methods previously described (Beam & Perry, 1973) for their ability to oxidize 5-8 carbon ring cycloalkanes to the homologous cycloalkanone. All organisms were grown on propane (Perry, 1968) except CY6 which was grown on cyclohexanone. After incubation with the cycloalkane the bacteria were removed by centrifugation and the supernatant was adjusted to pH 7.7 (NaOH) and extracted twice with equal volumes (500 ml) of diethyl ether; the diethyl ether was removed under a stream of nitrogen. The residue was dissolved in 200 μ l of diethyl ether and subjected to gas-liquid chromatographic analysis (Beam & Perry, 1973). The presence of cycloalkanone in the extracts was confirmed by comparing the retention times with those of standard cyclic ketones and by functional group analysis (Hoff & Feit, 1963). Total nitrogen was determined by the method of Um-breit, Burris & Stauffer (1964).

Co-metabolism and commensalism in the degradation of cyclohexane. Strain CY6 and *Mycobacterium vaccae* strain JOB5 were grown at the expense of cyclohexanone and propane, respectively. The bacteria were harvested by centrifugation and washed three times in 0.07M-phosphate buffer (pH 7.2). Approximately 50 mg (dry wt) of each organism were placed in separate 1 l filtering flasks containing 250 ml L-salts medium. As substrates, propane (50/50, v/v, with air) and [$U-^{14}C$]cyclohexane (2.5×10^5 c.p.m. in 150 mg carrier cyclohexane) were introduced into each flask. To a third flask containing the same substrates, 50 mg strain CY6 and 50 mg strain JOB5 were added. After 5 days' incubation in shake culture at 26 °C, the bacteria were harvested by centrifugation, washed with buffer, and fractionated according to the method of Roberts *et al.* (1957) to determine if carbon derived from the labelled substrate had been incorporated. Protein was determined by the u.v.-adsorption method of Warburg & Christian (1941). In each case, the protein fraction was acid hydrolysed and the constituent amino acids separated by paper chromatography (Block, Durrum & Zweig, 1956). The chromatogram was sprayed lightly with ninhydrin to identify the amino acids, the spots were cut out and radioactivity determined.

Influence of co-substrate on degradation of [$U-^{14}C$]cyclohexane. Strains OFS, JOB5, R22, and CY6 were employed in cyclohexane co-substrate systems with hexadecane or glucose as growth substrate. Substrates were added at 0.2 % (w/v or v/v) to litre flasks containing

Table 1. Ability of stock cultures to utilize cycloparaffins and related substrates as sources of carbon and energy

	Organism							
	<i>M. con-</i> <i>volutum</i> B58	<i>M. vaccae</i> JOB5	<i>M. rhodo-</i> <i>chrous</i> 7E1C	<i>M. album</i> 7E1BIW	<i>N. astero-</i> <i>ides</i> A116	Strain CY6	<i>M. con-</i> <i>volutum</i> R22	<i>M. rhodo-</i> <i>chrous</i> OFS
Cyclopentane	—	—	—	—	—	—	—	—
Cyclohexane	—	—	—	—	—	—	—	—
Cycloheptane	—	—	—	—	—	—	—	—
Cyclooctane	—	—	—	—	—	—	—	—
Dodecylcyclo- hexane	+	+	+	—	+	—	+	+
Heptadecylcyclo- hexane	+	+	+	—	+	—	+	+
Cyclopentanone	+	—	—	—	—	—	+	—
Cyclohexanone	+	—	—	—	—	+	+	—
Cycloheptanone	—	—	—	—	—	—	+	—
Cyclooctanone	—	—	—	—	—	—	—	—
Cyclohexanol	—	—	—	—	—	—	+	—
1,2-Cyclo- hexanediol	—	—	—	—	—	—	+	—
1,3-Cyclo- hexanediol	—	—	—	—	—	—	—	—
1,4-Cyclo- hexanediol	—	—	—	—	—	—	—	—
<i>n</i> -Tetradecane	+	+	+	+	+	—	+	+

500 ml L-salts medium. A third flask, receiving no growth substrate, constituted a control. Radiolabelled cyclohexane (10^6 c.p.m.) in 20 μ l cyclohexanone was added to each flask. Cultures were continuously aerated and were agitated by magnetic stirrers. $^{14}\text{CO}_2$ in expelled air from each system was trapped in 2N-NaOH, precipitated as BaCO_3 , and the radioactivity determined by liquid scintillation counting. A thixotropic gel powder (Packard Instrument Co.) was added to the scintillation fluid to keep the carbonate in suspension.

RESULTS

The ability of various organisms to utilize cycloparaffinic hydrocarbons is depicted in Table 1. These results are typical of those obtained with a number of hydrocarbon-utilizing organisms in our collection. *Mycobacterium album* (strain 7E1BIW) could utilize propane (Blevins & Perry, 1972) and normal alkanes as sole source of carbon and energy but could not utilize heptadecyclohexane. Some organisms isolated on cyclohexanone could utilize propane and other hydrocarbons as a source of carbon and energy while others, e.g. strain CY6, could not.

Figure 1 depicts the release of $^{14}\text{CO}_2$ from a marine mud incubated with $[\text{U-}^{14}\text{C}]$ -cyclohexane. These results suggest that most of the CO_2 was released within 5 days of incubation. $^{14}\text{CO}_2$ release was confirmed by trapping the gas in 2N-NaOH and precipitating the labelled carbon as BaCO_3 .

The ability to oxidize alicyclics to the corresponding cycloalkanones was widespread among the organisms tested. Non-proliferating suspensions of strains JOB5, OFS, 7E1C and R22 readily oxidized the alicyclic rings of 5–8 carbons after growth on propane. Strain CY6, which does not grow at the expense of hydrocarbons, lacked this oxidative ability.

Fig. 1. Evolution of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]$ cyclohexane incubated with marine mud. One μCi labelled substrate added to 100 g (wet wt) soil.

Table 2. *Cycloalkane oxygenation by non-proliferating suspensions of strains R22 and OFS*

The organisms were harvested after growth on propane, washed three times with Sorensens' buffer (pH 7.2), and exposed to 0.2 % (v/v) cycloparaffin in the same buffer for 60 h.

Organism	Substrate	Ketone re-covered (μg)	Total bacterial N	$\mu\text{g Ketone}/\mu\text{g N}$
R22	Cycloheptane	819	5944	0.44
	Cyclooctane	970	6288	0.15
OFS	Cycloheptane	2358	3234	0.73
	Cyclooctane	860	3380	0.25

Table 2 depicts representative recovery of two cycloalkanones following their formation from cycloheptane and cyclooctane by non-proliferating strains of R22 and OFS. Strain OFS produced more of the ketones as measured by the ratio of ketone recovered to bacterial nitrogen present in the system.

The concerted attack of two organisms on a cycloparaffinic ring was demonstrated with *Mycobacterium vaccae* strain JOB5, strain CY6, and $[\text{U-}^{14}\text{C}]$ cyclohexane. In a 50/50 propane: air atmosphere, strains JOB5 and CY6 individually contained only 61 and 71 c.p.m. respectively of the labelled substrate/mg protein. However, when added together, the resultant bacteria contained 598 c.p.m. labelled carbon/mg protein (Table 3). After acid hydrolysis of this protein fraction, constituent amino acids (aspartic, alanine, glycine, glutamate, isoleucine, and leucine) were found to contain radiolabelled carbon. The low-level radioactivity found when the micro-organisms were added separately was probably due to $[\text{U-}^{14}\text{C}]$ cyclohexane adsorption by the bacteria.

Degradation of cyclohexane by a mixed culture of micro-organisms was demonstrated by using a co-substrate technique. A mixed culture of strains JOB5, OFS, R22 and CY6 was

Table 3. *Co-operative metabolism of [U-¹⁴C]cyclohexane*

Cultures were established in 1 l flasks containing 250 ml L-salts medium. Vessel contained propane: air atmosphere (50/50, v/v) and 2.5×10^6 c.p.m. [U-¹⁴C]cyclohexane.

Organism	Protein, specific activity (c.p.m./mg)
JOB5	61
CY6	71
JOB5 + CY6	598

Table 4. *Influence of co-substrate on degradation of [U-¹⁴C]cyclohexane by a mixed culture of four micro-organisms of soil origin*

Mixed culture of *Mycobacterium vaccae* strain JOB5, *M. rhodochrous* strain OFS, *M. convolutum* strain R22, and strain CY6 was established in litre flasks containing 500 ml-salts medium. Substrate concentration 0.2 % (w/v or v/v). [U-¹⁴C]cyclohexane (10^6 c.p.m.) added in 20 μ l carrier.

Co-substrate	¹⁴ CO ₂ recovered (c.p.m.)
Hexadecane	1758
Glucose	73
No addition	28

unable to metabolize [U-¹⁴C]cyclohexane as the sole carbon and energy source (Table 4). Addition of glucose as cosubstrate did not appreciably enhance ¹⁴CO₂ evolution from the cycloparaffin. Addition of hexadecane as co-substrate resulted in a 63-fold increase in CO₂ liberation from the labelled substrate.

DISCUSSION

Despite the widespread occurrence of cycloparaffinic compounds in nature, no micro-organisms capable of utilizing these compounds as sole carbon and energy sources have been isolated. Stock culture organisms, capable of utilizing a wide variety of hydrocarbons, are unable to break the pristine alicyclic molecule and use its stored carbon and energy. Numerous organisms can use unsubstituted cycloalkanones for growth but the ability to use cycloketones as a carbon and energy source is not indicative of the capacity for general hydrocarbon utilization. Strain CY6, isolated on cyclohexanone, would not grow on any saturated hydrocarbons tested. Similar results were obtained by Dunlap & Perry (1968) with an organism isolated by enrichment culture on 2-undecanone that was incapable of hydrocarbon degradation. However, most of the organisms (24 of 30) isolated in pure culture by enrichment with cyclohexanone could grow on propane or other *n*-alkanes. Perry & Scheld (1968) previously reported that 80 % of the organisms isolated on cyclopentanone and cyclohexanone could use *n*-tridecane as a carbon and energy source.

The results with strains JOB5, OFS, 71EC and R22 indicate that the ability to oxidize cycloparaffinic hydrocarbons to the homologous cycloketone is a common attribute of hydrocarbon-utilizing bacteria. This oxidation could also result from contact of a cycloparaffin in the soil environment with enzymes such as a peroxidase, polyphenol oxidase, catechol oxidase or diphenol oxidase of rather broad substrate specificity.

The degradation of cyclohexane in a marine mud has been demonstrated (Fig. 1). Establishment of a commensalism (Table 3) between two organisms under laboratory

conditions helps to explain the degradability of cycloparaffins in fertile soil. *Mycobacterium vaccae* strain JOB5 co-oxidizes cyclohexane to the corresponding cycloalkanone while growing at the expense of propane. However, JOB5 cannot utilize the cyclohexanone as growth substrate. Strain CY6, although unable to grow on propane, can utilize the cyclohexanone produced as a co-metabolic product from cyclohexane by JOB5. Since the only labelled substrate present was cyclohexane, these data show a definite enhancement of cycloparaffin degradation by microbial co-metabolism and commensalism. A similar co-operative action by two organisms (a bacterium and a fungus) has been reported by Gunner & Zuckerman (1968) for degradation of the pyrimidine ring of the insecticide diazinon.

Further evidence for co-metabolic degradation of cycloparaffins was gained through dual substrate studies with a mixed microbial population. Increased mineralization of cyclohexane, as evidenced by increased $^{14}\text{CO}_2$ evolution (Table 4), in the presence of a compound which requires a molecular oxygenase suggests that oxygenation of the cycloparaffin may be accomplished via the same oxidative process. Far less degradation of cyclohexane occurred while glucose served as the co-substrate. Glucose has been reported to be an effective co-substrate for decomposition of vanillin (Kunc, 1971) and *p*-anisidine (Alexander & Lustigman, 1966) by soil micro-organisms, alkyl benzene sulphonate by *Pseudomonas* sp. (Benarde, Koft, Horvath, & Shaulis, 1965; Horvath & Koft, 1972), and diazinon by *Arthrobacter* sp. (Sethunathan & Pathak, 1971). Unlike compounds such as *m*- or *p*-chlorobenzoate (Horvath 1973), cyclohexane may lack the capacity to induce the requisite oxidative enzymes responsible for its degradation. Studies are being continued on this aspect of cycloparaffin metabolism.

The inability to isolate organisms from soil that can utilize cycloparaffinic hydrocarbons as carbon and energy sources cannot be taken as proof that such organisms are not present in the environment. The relative abundance of cycloalkanone utilizers and the widespread capacity of cycloalkanes to be oxidized to the homologous ketone by micro-organisms as well as extracellular enzymes in soil strongly suggest that co-metabolism and commensalism do play an important role in the biodegradation of these compounds in nature. Thus, cycloparaffinic hydrocarbons should be considered likely additions to the increasing list of compounds (Horvath, 1972) whose degradation involves co-metabolism.

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