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The Degradation of $n$-Alkylcycloalkanes by a Mixed Bacterial Culture

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Mycobacterium rhodochrous strain 7EIC grows with dodecylcyclohexane at the expense of acetyl fragments released by $\beta$-oxidation of the side-chain. Cyclohexanecacetic acid, which is not amenable to $\beta$-oxidation and is not oxidized by this organism, accumulates in significant yield. In combination with Arthrobacter strain CA1, which degrades cyclohexanecacetic acid by a novel pathway, a stable mixed culture is established that is capable of the complete degradation of dodecylcyclohexane and related hydrocarbons.

INTRODUCTION

Cycloalkanes with long $n$-alkyl side-chains are components of crude oils. The extent to which this class of compounds is degraded in the biosphere is dependent on the nature of the side-chain. Degradation is typically initiated by hydroxylation of the terminal methyl group followed by oxidation of the primary alcohol to a fatty acid and subsequent $\beta$-oxidation.

$n$-Alkylcycloalkanes with an odd number of carbon atoms in the side-chain are susceptible to extended degradation by $\beta$-oxidation. The sequential removal of acetyl-CoA units ultimately forms cyclohexane carboxyl-CoA (Beam & Perry, 1974a) which may then be metabolized by continued $\beta$-oxidation (Blakley, 1978). In contrast, the microbial degradation of $n$-alkylcycloalkanes with an even number of carbon atoms in the side-chain is not so simple. Organisms capable of growth with dodecylcyclohexane ($C_{13}$ side-chain) as the sole source of carbon accumulate cyclohexanecacetic acid in the growth medium (Beam & Perry, 1974a). The recalcitrant nature of this acid (Davis & Raymond, 1961; Beam & Perry, 1974a) was assumed to result from the position of the ring with respect to the carboxyl group as this blocks $\beta$-oxidation. However, we have isolated a strain of Arthrobacter (CA1) capable of growth on cyclohexanecacetic acid as the sole source of carbon but incapable of growth on cyclohexanepropionic acid or cyclohexanecarboxylic acid. Degradation of cyclohexanecacetic acid is mediated by the initiation of a $\beta$-oxidation cycle which is blocked subsequent to the hydration step. The intervention of a (1'-hydroxy-cyclohex-1'-yl) acetyl-CoA lyase generates equimolar amounts of cyclohexanone and acetyl-CoA (Ougham & Trudgill, 1978). The cyclohexanone is then degraded by the pathway established for strains of Nocardia (Norris & Trudgill, 1971) and Acinetobacter (Donoghue & Trudgill, 1975).

We report here on the establishment of stable mixed cultures under laboratory conditions that are capable of the complete dissimilation of dodecylcyclohexane.
**METHODS**

Organisms and growth conditions. *Mycobacterium rhodochrous* strain 7EIC was a generous gift from Dr J. J. Perry (Department of Microbiology, North Carolina State University, Raleigh). *Arthrobacter* strain CA1 was isolated from a field in South Wales, contaminated with aviation fuel, by elective culture with cyclohexanacetic acid as sole source of carbon. Bacteria were grown in liquid culture on medium which contained in distilled water (g l⁻¹): KH₂PO₄, 2; Na₂HPO₄, 4; (NH₄)₂SO₄, 1; and 0.4 ml trace element solution (Rosenberger & Elsdon, 1960). Cyclohexanacetic acid was added at 1 g l⁻¹, the pH was adjusted to 7.1 and the medium was autoclaved at 103 kPa for 20 min. n-Alkylcycloalkanes were sterilized by membrane filtration and added aseptically to sterilized basal medium to give a final concentration of 2 g l⁻¹.

For experimental purposes, 250 ml conical flasks with side-arms, each containing 90 ml medium, were inoculated normally with 10 ml of a culture grown to early-stationary phase on either an n-alkylcycloalkane (*M. rhodochrous* 7EIC) or cyclohexanacetic acid (*Arthrobacter* CA1). For mixed culture studies, flasks were inoculated with 5 ml of an early-stationary phase culture of each organism. The cultures were grown at 30 °C and shaken at 100 rev. min⁻¹ on a rotary shaker. Culture absorbance was measured with a Klett–Summerson colorimeter using a green 54 filter (90% transmission between 510 and 535 nm). A reading of 16 Klett units is equivalent to about 1 × 10⁸ bacteria ml⁻¹.

**Bacterial counts.** Reliable bacterial counts could only be made on late-exponential and early-stationary phase cultures when aggregates of *M. rhodochrous* 7EIC initially formed in the presence of the hydrocarbon were dispersed. Flasks were removed from the shaker and given a vigorous lateral shaking before samples (0.5 ml) were aseptically removed and serially diluted in 50 ml sodium/potassium phosphate buffer pH 7.1. Samples (0.1 ml) of appropriate dilutions were spread on nutrient agar. After incubation at 30 °C for 48 h, Petri dishes were exposed to artificial light (1.6 klx) for 12 to 24 h which promoted the production of an orange pigment by *M. rhodochrous* 7EIC. This enabled identification and enumeration of individual colonies of each species.

**Extraction of alkylcyclohexane fatty acids from culture medium.** Samples of culture medium (6 ml) were aseptically removed and freed from bacteria by centrifugation at 27000 g for 15 min at 2 °C. Undecanoic acid (0.5 ml of a 10 mg ml⁻¹ solution in 0.1 M-NaOH) was added to 5 ml of the culture supernatant to act as an internal standard. After acidification by addition of 0.5 ml of 2.5 M-HCl, fatty acids were extracted by shaking with two separate volumes (3 ml) of peroxide-free diethyl ether. The pooled ether extracts were dried over anhydrous Na₂SO₄, evaporated to a small volume under a stream of N₂ and methylated with BCl₃/methanol according to Metcalfe & Schmitz (1961).

**Gas–liquid chromatography.** A Pye series 104 gas chromatograph equipped with a flame ionization detector and a 1.5 m × 4 mm i.d. glass column containing 1.5 % (w/w) diglycerol on Chromosorb W (60 to 80 mesh) was used. A carrier gas flow-rate of 45 ml min⁻¹ coupled with a temperature programme in which the temperature was maintained at 80 °C for 2 min and then increased by 15 °C min⁻¹ to 120 °C allowed all standard alkylcyclohexane fatty acid methyl esters of potential interest to be separated from each other and from the internal standard. Results were quantified by means of manual peak area measurement and reference to the internal standard.

**RESULTS**

Initial experiments confirmed the ability of *M. rhodochrous* 7EIC to grow with dodecylcyclohexane as the sole source of carbon and that cyclohexanacetic acid was the only cyclohexyl fatty acid which accumulated in the medium. No growth was observed when cyclohexanacetic acid was provided as the sole carbon source. In contrast, *Arthrobacter* CA1 was capable of growth with cyclohexanacetic acid as sole source of carbon but incapable of growth on dodecylcyclohexane and cycloalkyl hydrocarbons generally.

Two additional factors indicated the suitability of *Arthrobacter* CA1 for inclusion in a putative mixed culture system. Firstly, it is hydrocarbon-tolerant and is capable of growth on cyclohexanacetic acid in the presence of dodecylcyclohexane (0.2 %, v/v) with no significant change in specific growth rate (0.107 to 0.115 h⁻¹). Secondly, it grew well in culture supernatant from *M. rhodochrous* 7EIC grown to stationary phase with dodecylcyclohexane; the supernatant was supplemented with (NH₄)₂SO₄ (1 g l⁻¹), re-adjusted to pH 7.1 and re-sterilized.

When dodecylcyclohexane medium was inoculated with equal volumes of early-stationary phase cultures of *M. rhodochrous* 7EIC and *Arthrobacter* CA1, grown respectively with
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Fig. 1. Growth of *M. rhodochrous* 7EIC and *Arthrobacter* CA1 singly and in mixed culture as described in Methods. Growth of *M. rhodochrous* 7EIC on 0.2% (v/v) dodecylcyclohexane (●), *Arthrobacter* CA1 on 0.1% (w/v) cyclohexaneacetic acid (▲) and the mixed culture on 0.2% (v/v) dodecylcyclohexane (○). Accumulation of cyclohexaneacetic acid in the medium by *M. rhodochrous* 7EIC (■) and by the mixed culture (□).

dodecylcyclohexane and cyclohexaneacetic acid, the involvement of both organisms in extended degradation of the hydrocarbon was indicated by the observed increase in turbidity over that obtained with *M. rhodochrous* 7EIC alone (Fig. 1). In addition, the culture of *M. rhodochrous* 7EIC accumulated cyclohexaneacetic acid throughout the growth phase, the maximum accumulation (0.54 mg ml⁻¹) being maintained after 45 h in the stationary phase. In marked contrast, the mixed culture showed only a transient accumulation of cyclohexaneacetic acid during the first 48 h of growth (Fig. 1).

Analysis of the relative abundance of the two organisms in early-stationary phase culture showed the ratio *M. rhodochrous* 7EIC to *Arthrobacter* CA1 was 1:2:1. The stability of this mixed culture system is indicated from the results of regular transfer of a 10% (v/v) inoculum to fresh medium at approximately 4 d intervals. The mean ratio of the two organisms determined from five sequential duplicate samplings was 1:33:1.

Transfer of a 2% (v/v) inoculum of the mixed culture to cyclohexaneacetic acid medium followed by growth to stationary phase and two further 2% (v/v) transfers resulted in the total elimination of *M. rhodochrous* 7EIC from the culture.

**Effects of n-alkyl chain length on the ratio of the two organisms**

If the predominant role of *Arthrobacter* CA1 is the sequestration and oxidation of cyclohexaneacetic acid rather than a more general scavenging of intermediary metabolites generated by *M. rhodochrous* 7EIC then alteration of the side-chain length of the n-alkylcycloalkane should have marked and predictable effects on the ratio of the two organisms.

Mixed cultures were therefore established with hexylcyclohexane (C₆ side-chain) and heptadecylcyclohexane (C₁₇ side-chain) as sole sources of carbon. *Mycobacterium rhodochrous* 7EIC was capable of growth with both hydrocarbons. In the former case cell yields were low but in the latter extended β-oxidation resulted in ring cleavage, high cell yields and negligible accumulation of alkylcyclohexane fatty acid intermediates.

We have established that, as with dodecylcyclohexane as carbon source, *M. rhodochrous* 7EIC growing on hexylcyclohexane accumulated cyclohexaneacetic acid. In mixed cultures, as one would predict, the availability of a much larger proportion of the organic carbon
to *Arthrobacter CA1* only was reflected in the ratio of *M. rhodochrous* 7EIC to *Arthrobacter CA1* of 0.5:1 obtained as the mean of five duplicated samples taken from cultures transferred at regular 4 d intervals.

In contrast, the proportion of *Arthrobacter CA1* rapidly declined to a stable, low proportion in a mixed culture when heptadecylcyclohexane was provided as the carbon source, the ratio of *M. rhodochrous* 7EIC to *Arthrobacter CA1* rising from an initial value of 1:2:1 to 4:6:1. The persistence of *Arthrobacter CA1* at a low level was probably the result of a degree of indiscriminate scavenging of metabolites and the possible formation of trace amounts of cyclohexaneacetic acid from heptadecylcyclohexane by *M. rhodochrous* 7EIC (Beam & Perry, 1974a).

**DISCUSSION**

The observation by Beam & Perry (1974b) that the complete oxidation of the recalcitrant hydrocarbon cyclohexane was achieved by the complex mixture of organisms present in marine mud has indicated the importance of commensal and co-oxidative attack on compounds that prove resistant under laboratory conditions where the emphasis is on the capabilities of pure cultures. Simulation of natural mixed substrate and mixed culture conditions (Beam & Perry, 1974a; de Klerk & van der Linden, 1974) allowed the complete degradation of cyclohexane to be demonstrated in the laboratory, although it was shown subsequently (Stirling et al., 1977) that degradation of this compound can also be mediated by a single organism.

No single organism has yet been isolated and shown to degrade completely *n*-alkylcycloalkanes with an even number of carbon atoms in the side-chain. The two-organism system that we have constructed has a parallel in the degradation of hexaethyleneglycolmonophenylether and related compounds (Baggi et al., 1978). A strain of *Nocardia*, isolated by enrichment culture with polyethyleneglycol as the substrate, was capable of hydrolytic fragmentation of the hexaethyleneglycolmonophenylether, utilizing the ethylene glycol released and accumulating the monoethyleneglycolmonophenylether. The isolation of a strain of *Cylindrocarpon* by enrichment culture with the accumulated metabolite would suggest that a two-organism system could also be constructed in this instance.

The yield of cyclohexaneacetic acid produced from dodecylcyclohexane by *M. rhodochrous* 7EIC was about 50% of that theoretically obtainable (Fig. 1). In principle, \( \alpha \)-oxidation would provide an effective means of shortening the side-chain by one carbon atom and, while there is evidence that it occurs from the structures of alkylcyclohexane fatty acids in the phospholipids of dodecylcyclohexane-grown *Mycobacterium* strains (Beam & Perry, 1974a), the quantitative contribution of this and incorporation of some alkylcyclohexane fatty acid intermediates into cellular lipids (Beam & Perry, 1974a) is unknown.

There is no evidence that cyclohexaneacetic acid formed by *M. rhodochrous* 7EIC was further metabolized by that organism (Fig. 1). However, mixed cultures in which the unique isolate *Arthrobacter CA1* degraded cyclohexaneacetic acid formed from the hydrocarbon provided an effective system for the degradation of dodecylcyclohexane. It remains to be seen whether or not such a two-membered community functions in nature.

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REFERENCES


