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Uptake of Vapour Phase [^{14}C]Dodecane by Whole Mycelia of *Cladosporium resinae*

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The kinetics of uptake of *n*-alkanes by a filamentous fungus, *Cladosporium resinae*, were shown to be similar to hydrocarbon uptake kinetics described for yeasts. Transport of *n*-alkanes across the cell membrane was accompanied by the partitioning of substantial amounts of hydrocarbon on to the cell surface. Metabolic inhibitors significantly reduced uptake rates. Most [^{14}C]dodecane was taken up by dodecane-grown mycelia; slower uptake was observed with mycelia grown on other *n*-alkanes or glucose. Uptake of dodecane was not restricted to specific regions of the mycelium, although higher concentrations of ^{14}C-labelled compounds were observed at the hyphal tips.

INTRODUCTION

The filamentous fungus *Cladosporium resinae* is commonly implicated in the biodeterioration of mineral oils (Parberry, 1971). Though there are many discussions of the kinetics of cell growth during hydrocarbon oxidation (Erickson, 1981) the mechanism of hydrocarbon uptake has not been sufficiently elucidated. A few bacteria and yeasts have been investigated, but no report has appeared on mechanisms of hydrocarbon uptake by filamentous fungi.

The low solubility of *n*-alkanes in water (McAuliffe, 1966) necessitates a special mechanism for substrate uptake to support appreciable growth. Two principal forms of alkane transfer have been proposed from work on unicellular organisms: (1) through direct contact of the cells with emulsified droplets of the hydrocarbon (Johnson, 1964; Käppeli & Fiechter, 1976), and (2) transport of solubilized hydrocarbons from hydrocarbon micelles (Goma *et al.*, 1973). In each case the cell wall played an important role as mediator between growth substrate and cell. The mechanism of transfer of hydrocarbon across the cell membrane remains obscure although some properties indicative of an active transport system have recently been described for *Candida tropicalis* (Käppeli & Fiechter, 1981).

An understanding of how the hydrocarbons are transported into the fungal cell would be of importance during the design of control measures to prevent the operational difficulties which occur as a result of microbiological contamination of fuel oils.

METHODS

Organism. Cladosporium resinae avellaneum was isolated from a contaminated water bottom sample obtained from a naval fuel tank and was identified by its morphology (Parberry, 1969).

Growth. The medium used throughout this study was the mineral salts solution of Turner *et al.* (1980), solidified with 1·5% (w/v) Difco bacto-agar. Glucose (0·2%, w/v), or *n*-alkanes (2%, v/v) were used as carbon sources. Hydrocarbons were incorporated into the medium bound to colloidal silica (Barauth *et al.*, 1967). An incubation temperature of 30 °C was used for all substrates.

In order to facilitate the rapid and simple removal of fungal mycelia from the agar surfaces, sterile cellophane discs were placed on the agar. Spore suspension (0·05 ml, 10^7 spores ml^{-1}) was added to the centre of each disc as an inoculum. The cellophane was incapable of supporting fungal growth in the absence of a carbon source, and did not have any detrimental effect upon growth rate.

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Uptake of [^{14}C]*dodecane*. Mycelial discs grown for 2 weeks on solid medium using cellophane overlays, were transferred to carbon-free medium for 18 h. After this period of starvation, 1 µl [1-^{14}C]-*n*-dodecane [0·1 mCi mmol^{-1} (3·7 MBq mmol^{-1})] was injected into the Petri dish lid. The plates were sealed with cellophane adhesive tape and returned to the 30 °C incubator. Mycelia were removed over a time course and labelled alkanes were immobilized by transferring to a salt-ice bath (−10 °C).

Half of each mycelial disc was placed in a vial containing 10 ml of a scintillation fluid (*p*-terphenyl, 4 g; 1-4-*bis*-2-(5-phenyloxazolyl)-benzene, 125 mg; xylene, 1 litre) to assay both the *n*-dodecane taken into the cell and that partitioned to the cell surface. To assess the amount of alkane transferred across the cell membrane, partitioned alkane was removed from the second half of each mycelium with petroleum ether washings before addition of the scintillant. Two washings with petroleum ether were sufficient to remove the extracellular alkane, i.e. only normal background radioactivity was found in a third wash. The radioactivity was assayed using a Beckman LS-100/C scintillation counter. Counting efficiency was determined at 95% by the addition of a [^{14}C]hexadecane internal standard.

Protein determination. Uptake concentrations were related to cell protein by the Lowry method using bovine plasma albumin as standard. The relationship between mean colony diameter and cell protein was determined for each growth substrate, enabling protein to be estimated by mycelial diameter measurements, taken immediately before the addition of [^{14}C]dodecane.

Autoradiography. Fungal mycelia grown in dodecane vapour on cellophane discs were incubated with [^{14}C]dodecane vapour for 30 min. Discs were then mounted on cardboard supports and pressed against X-ray sheet film (Kodak X-Omat RP) for two weeks at −20 °C in the dark.

Chemicals. All unlabelled *n*-alkanes were purchased from Aldrich Chemical Co. and were guaranteed to be at least 98% pure. Radioactive [1-^{14}C]-*n*-dodecane (98+%) was obtained from ICN Pharmaceuticals Inc. and [^{14}C]hexadecane from Amersham. Photographic film and processing chemicals were by Kodak.

RESULTS AND DISCUSSION

The ability of *C. resinae* hyphae to take up *n*-alkanes was shown to be constant throughout the mycelium; but somewhat enhanced at the hyphal tips (Fig. 1). Whether the higher levels of [^{14}C]dodecane within the metabolically active terminal cells represent higher rates of *n*-alkane uptake, or reflect a degree of intercellular transport of the hydrocarbons was not determined, but the less defined wall structure associated with the hyphal tips may enable more rapid transport of *n*-alkanes into the cell. Cooney *et al.* (1980) showed that most of the alkane taken into the cell was stored prior to oxidation, possibly in large, membrane bound vesicles, so intercellular transport would seem unlikely. However, intracellular transport of various enzymes and wall precursor compounds is generally known to occur towards hyphal tips via membrane-bound vesicles so the possibility of some form of intercellular transport should not be overlooked.

Monitoring of the amounts of [^{14}C]dodecane bound to and taken up by mycelia over time (Fig. 2) showed that in fungus grown on a solid medium a substantial proportion of alkane was located on the outer cell wall. The concentration of this partitioned hydrocarbon remained constant at approximately 8 nmol (mg cell protein)$^{-1}$ over a 24 h period of incubation and represents the amount of dodecane necessary to saturate the binding sites. The use of vaporized alkane overcame the problem of uptake being limited by partitioning. The binding capacity of the outer cell wall was sufficiently high to enable radioactivity to be measured within 5 min of adding the [^{14}C]dodecane. Saturation of the cell surface took approximately 2 h. This length of period could have been due to the relatively low concentration of dodecane in the vapour phase compared with that obtained in liquid culture. A high binding affinity for hydrocarbon was as important a component of the fungal *n*-alkane transport system as it is for other hydrocarbon-utilizing micro-organisms (Miura *et al.*, 1977; Käppeli & Fiechter, 1976; Rosenberg & Rosenberg, 1981). The accumulation of partitioned hydrocarbon on, or in the cell surface was an essential step in the movement of alkanes into the cell, serving by creating a diffusion gradient to facilitate passive diffusion into the cell, or as a reservoir for an active transport system.

When mycelia inactivated by formaldehyde (0·33 M) were used, the concentration of partitioned dodecane was lessened by 31%, but intracellular uptake rates were 84% less than those obtained using untreated mycelia. Similar results were obtained when a metabolic inhibitor, cyanide or azide, was included in the assay (Table 1). These results may indicate that partitioning was a passive process, whilst transport across the cell membrane was an active

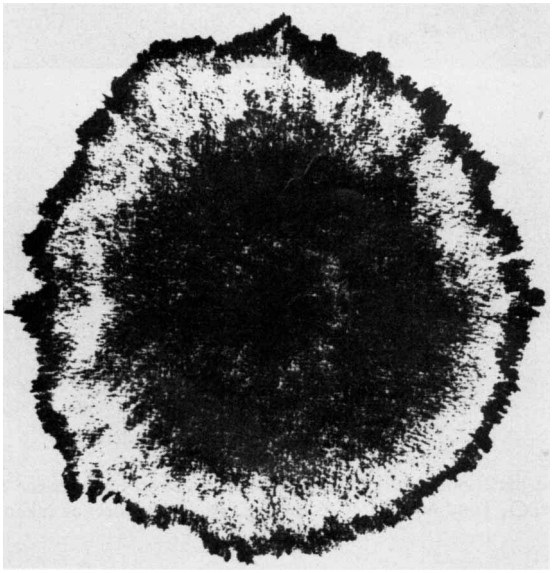

Fig. 1. Autoradiograph of a whole mycelium of *Cladosporium resinae* following 30 min exposure to [^{14}C]dodecane vapour.

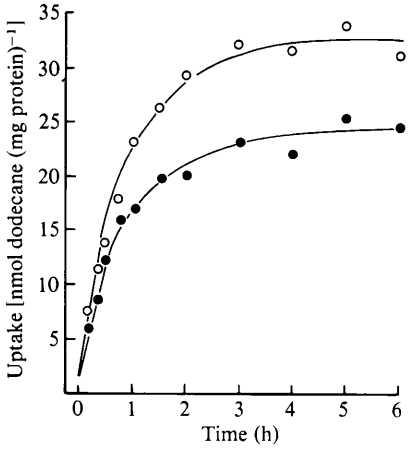

Fig. 2. Time course of [^{14}C]dodecane uptake by *Cladosporium resinae*. ○, Total [^{14}C]dodecane bound to mycelia; ●, [^{14}C]dodecane taken into cells (not washed off by petroleum ether).

Table 1. *Effect of growth substrate and assay conditions upon [^{14}C]dodecane uptake rate and partitioning by whole mycelia of Cladosporium resinae*

Mycelia were assayed for radioactivity at 10 min intervals over a period of 1 h. The partitioning rate was expressed as total bound [^{14}C]dodecane minus that taken into the cell, i.e. that portion of the [^{14}C]dodecane removed by washing with petroleum ether. Values in parentheses represent percentages of rates obtained for dodecane-grown mycelia.

Growth substrate	Assay condition	Uptake rate [nmol (mg protein)$^{-1}$ h^{-1}]	Partitioning rate [nmol (mg protein)$^{-1}$ h^{-1}]
Dodecane	NA	21·02 (100%)	9·86 (100%)
Glucose	NA	6·04 (29%)	3·20 (33%)
Dodecane	HCHO inactivated	3·07 (16%)	6·79 (69%)
Dodecane	KCN (10 mM)	4·90 (23%)	7·76 (79%)
Dodecane	NaN_3 (10 mM)	2·53 (12%)	7·04 (72%)

NA, No addition of inhibitors to mycelia prior to assay.

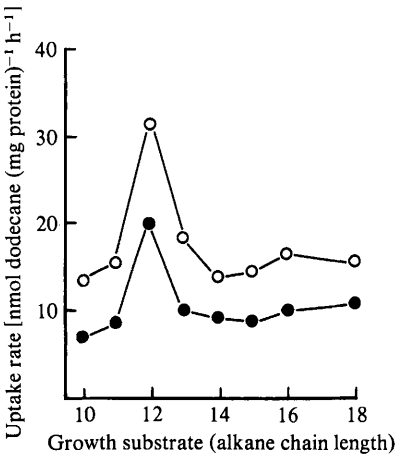

Fig. 3. Uptake and partitioning of [^{14}C]dodecane by *Cladosporium resinae* following growth on a variety of *n*-alkanes. ○, Total bound [^{14}C]dodecane; ●, [^{14}C]dodecane taken up (not washed off by petroleum ether).

process. However, the low rates of uptake in the presence of inhibitors could be attributed either to the specific inhibition of one or more of the enzymes involved in alkane oxidation or to the blocking of active transport processes. Work with yeasts (Käppeli & Fiechter, 1981) published whilst this work was in progress noted similar doubts regarding the interpretation of inhibitor studies. It has yet to be resolved whether or not hydrocarbon transport can be said to be a truly active process.

The lower rates of both partitioning and uptake of [^{14}C]dodecane obtained using glucose-grown mycelia (Table 1) reflect the more lipophilic nature of fungus during growth on hydrocarbons. *Cladosporium resinae* contains more lipids when grown on hydrocarbons than when grown on glucose (Walker & Cooney, 1973). The higher lipid levels of alkane-grown yeasts were shown by Hug *et al.* (1974) to be not merely a reflection of the lipophilic nature of the substrate, but necessary for metabolism, though no specific role was assigned to them. Käppeli & Fiechter (1977) showed the role of lipid components found in the cell walls of hydrocarbon-grown yeasts in changing the binding affinity of the cell for alkanes. The lipids found in large amounts in alkane-grown *C. resinae* may play a similar role, both increasing the alkane-binding affinity of the cell walls and possibly dissolving the alkane after uptake.

Previous studies have shown little or no specificity of the alkane uptake process. We have shown that uptake of dodecane was considerably faster into mycelia grown on dodecane than into mycelia grown on other *n*-alkanes (Fig. 3). This we attribute to the nature of the lipid material within the cell, which has been shown by many workers to reflect the chain length of the alkane substrate. The faster uptake was enabled by the significantly better binding efficiency of the dodecane-grown cells. We consider it unlikely that an active uptake mechanism could be specific for a single *n*-alkane, but quite possible that the chemical nature of the cell could exert sufficient influence to allow more favourable uptake of specific alkanes under certain conditions. It should be noted that the uptake rates obtained for mycelia grown on *n*-alkanes other than dodecane were still significantly higher than for glucose-grown mycelia.

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