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# Toxicity of Short-Chain Fatty Acids and Alcohols Towards *Cladosporium resinae*

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Long-chain saturated fatty acids ($C_{13}$ to $C_{18}$) and fatty alcohols ($C_{12}$ to $C_{18}$) were well utilized by three different soil isolates of *Cladosporium resinae* as the sole carbon and energy sources in static liquid cultures. Shorter-chain compounds, down to $C_5$, did not support growth and were in fact toxic towards the fungus growing on glucose. Rapid and considerable potassium efflux, protein leakage, and inhibition of endogenous respiration were observed in the presence of the shorter fatty acids and alcohols. Possible mechanisms and significance of the toxicity are discussed.

Microbiological contamination of jet aircraft fuel systems is still a problem today, particularly in tropical areas (13, 17). The predominant species responsible is *Cladosporium resinae*, a filamentous fungus which can utilize intermediate- and long-chain *n*-alkanes as sole energy and carbon sources (6, 19). The major pathway for the initial oxidation of *n*-alkanes by this organism has been shown to proceed via homologous primary alcohol, aldehyde, and monocarboxylic acid intermediates (23). However, it is well known that fatty acids and fatty alcohols, particularly the lower members of a homologous series, are toxic towards bacteria (1, 2, 7, 11), yeasts (3, 8, 12, 16, 22), and some filamentous fungi (14, 15, 21). It was therefore of interest to investigate the utilization of fatty acids and alcohols by *C. resinae* and to study the effects, if any, of these compounds on the organism.

## MATERIALS AND METHODS

**Organism.** Three different soil isolates of *Cladosporium resinae*, 35A, 89A, and 102B, were obtained from D. G. Parbery, University of Melbourne, Victoria, Australia and maintained on Bushnell-Haas glucose-agar slants (5). Unless otherwise stated, potassium efflux data were reported for isolate 35A, since the other two isolates gave essentially similar results.

**Media and cultivation methods.** Bushnell and Haas mineral salts solution was used as the basal medium throughout. For the potassium efflux experiments, cells were harvested after growing for 3 days at 30 C in static 1-liter Fernbach flasks containing 180 ml of salt medium and 1% glucose.

Utilization of individual fatty acids and alcohols was determined under the following standard conditions. Duplicate 250-ml Erlenmeyer flasks, each containing 40 ml of salt medium and 0.4 g of the pure substrate, were autoclaved and inoculated with a spore suspension as described previously (19). Solid substrate was dispersed as fine particles in the medium by shaking the flask vigorously while the melted substrate solidified on cooling after autoclaving. The inoculated flasks were incubated statically at 30 C for 30 days. Cultures which showed no sign of growth were incubated for an additional 30 days. At the end of the growth period, 10 to 20 ml of petroleum ether (bp, 60 to 80 C) was added to each flask to dissolve the residual substrate. The cells were then harvested by vacuum filtration, and their dry weight was determined as described previously (19).

To investigate the effect of various fatty acids and alcohols on growth, 0.4 g of compound was added to 40 ml of salt medium containing 1% glucose in 250-ml Erlenmeyer flasks. After inoculation with a spore suspension, the flasks were incubated statically at 30 C for 21 days. Cells were harvested for dry weight determination.

**Potassium efflux.** Harvested cells were washed thoroughly with distilled water and suspended in 10 ml of water to give a final cell concentration of 0.5 to 1.0 mg (dry weight) per ml. Suspensions were preincubated for 5 min at 30 C with gentle shaking in a reciprocal shaker bath before the addition of the appropriate fatty acid or alcohol (0.1 g). Solid substrate was added as a fine powder. At the end of the incubation period, duplicate flasks were harvested by vacuum filtration, and the aqueous filtrates were analyzed for potassium with an EEL flame photometer (Evans Electroselenium Ltd., Essex, England). To determine the effects of pH and substrate concentration on potassium efflux, cells were suspended in an aqueous solution of the fatty acid or alcohol of known concentration, and the mixture was adjusted to the required pH with 1 N $H_2SO_4$. Total cellular potassium was determined by heating a washed cell suspension in 0.5 N perchloric acid at 100 C for 30 min, followed by analysis of the filtrate.

**Protein leakage.** The conditions for the determination of protein leakage in the presence of the fatty acids and alcohols were similar to those for the potassium efflux experiments. Soluble protein in the

medium was estimated according to Lowry et al. (10) with crystalline bovine serum albumin as standard. Total cellular protein was determined by heating the cell suspension in 0.5 N sodium hydroxide at 100 C for 5 min followed by analysis of the alkaline extract.

**Endogenous respiration.** Cells were grown on D-[U-$^{14}$C]glucose (specific activity 10 $\mu$Ci/g) for 3 days at 30 C. The labeled cells were harvested, washed thoroughly, and suspended in Bushnell-Haas medium (2 mg [dry weight] of cells/ml). Expiration of $^{14}CO_2$ from the washed cells was measured in the absence and in the presence of fatty acids and alcohols. The cell suspension (3 ml) and the test compound (0.03 g) were added to the outer compartment of a 30-ml conical flask fitted with a center well. A small empty glass tube was placed inside the center wall. The flask was tightly stoppered with a rubber seal and incubated at 30 C with gentle shaking for 1 h. At the end of the incubation period, 1 ml of 40% trichloroacetic acid was injected into the cell suspension through the rubber seal. This was immediately followed by adding 1 ml of $CO_2$ trapping reagent (25) into the empty glass tube in the center well. After standing for 2 h, the glass tube was removed and added directly to 10 ml of scintillation fluid [4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene in 1 liter of toluene]. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

**Chemicals.** Fatty acids and alcohols (≥99% purity) were obtained from Sigma Chemical Co., St. Louis, Mo. D-[U-$^{14}$C]glucose was purchased from Radiochemical Centre, Amersham, England.

TABLE 1. *Growth of three isolates of Cladosporium resinae on normal fatty acids and alcohols*

| Carbon source | Total dry cell weight (mg) | | |
|---|---|---|---|
| | Isolate 35A | Isolate 89A | Isolate 102B |
| *n*-Pentanoic acid | 0 | 0 | 0 |
| *n*-Hexanoic acid | 0 | 0 | 0 |
| *n*-Heptanoic acid | 0 | 0 | 0 |
| *n*-Octanoic acid | 0 | 0 | 0 |
| *n*-Nonanoic acid | 0 | 0 | 0 |
| *n*-Decanoic acid | 0 | 0 | 0 |
| *n*-Undecanoic acid | 0 | 0 | 0 |
| *n*-Dodecanoic acid | 0 | 0 | 0 |
| *n*-Tridecanoic acid | 17 | 19 | 11 |
| *n*-Tetradecanoic acid | 30 | 24 | 27 |
| *n*-Hexadecanoic acid | 53 | 63 | 38 |
| *n*-Heptadecanoic acid | 31 | 32 | 17 |
| *n*-Octadecanoic acid | 14 | 10 | 7 |
| 1-Pentanol | 0 | 0 | 0 |
| 1-Hexanol | 0 | 0 | 0 |
| 1-Heptanol | 0 | 0 | 0 |
| 1-Octanol | 0 | 0 | 0 |
| 1-Nonanol | 0 | 0 | 0 |
| 1-Decanol | 0 | 0 | 0 |
| 1-Dodecanol | 5 | 10 | 1 |
| 1-Tridecanol | 32 | 16 | 12 |
| 1-Tetradecanol | 28 | 16 | 9 |
| 1-Pentadecanol | 27 | 17 | 11 |
| 1-Hexadecanol | 43 | 25 | 21 |
| 1-Octadecanol | 30 | 28 | 19 |
| Glucose | 76 | 70 | 72 |

## RESULTS AND DISCUSSION

Although fatty acids and alcohols are known to be intermediates in the oxidation of *n*-alkanes by *C. resinae* (23), to date there has been no comprehensive study on the utilization of these intermediates by the organism (18, 23). In the present work, the growth of three isolates of *C. resinae* on saturated fatty acids and 1-alkanols ranging from the $C_5$ to $C_{18}$ members as sole carbon and energy sources was investigated. Results are summarized in Table 1. The lower fatty acids from *n*-pentanoic acid to *n*-dodecanoic acid did not support any visible growth, even on prolonged incubation. The long-chain fatty acids ($C_{13}$ to $C_{18}$) were well utilized by all three isolates. Similarly, the lower alcohols ($C_5$ to $C_{10}$) were not utilized, whereas the long-chain alcohols ($C_{12}$ to $C_{18}$) supported moderate to good growth of the isolates. In both the fatty acid and alcohol series, the $C_{16}$ member appeared to give the highest dry cell weight. Walker and Cooney (23) reported recently that two isolates of *C. resinae* could not grow on 1-hexanol or *n*-hexanoic acid, whereas 1-dodecanol and *n*-dodecanoic acid supported limited growth to no growth. On the other hand, *n*-hexadecanoic acid and 1-hexadecanol gave 4 to 5 times more cell mass than glucose. However, it should be pointed out that in their study purity of the substrates was not high (93 to 97%) and the cultural conditions were not defined.

The inability of the fungus to make use of the lower fatty acids and alcohols is probably due to the toxicity of these compounds towards intact cells, since both 1-hexanol and 1-dodecanol are known to be oxidized by the cell-free extracts (24). This hypothesis was tested by growing the same isolates of *C. resinae* on glucose in the presence of excess (1%) fatty acid or alcohol. The $C_5$ to $C_{12}$ fatty acids inhibited growth completely, whereas $C_{13}$ and all higher fatty acids had no significant effect on growth (Table 2). Similar results were obtained for the corresponding fatty alcohols, except that 1-dodecanol did not inhibit growth completely. In addition, the endogenous respiration of a washed suspension of isolated 35A cells was severely inhibited by the lower fatty acids (*n*-hexanoic, *n*-heptanoic, and *n*-octanoic) and lower fatty alcohols (1-hexanol and 1-octanol), whereas the long-chain fatty acids and alcohols only slightly inhibited the endogenous respiration (Table 3). The short-chain compounds also affected another cellular property, the selective

permeability of cell membrane. Rapid and considerable loss of cellular potassium, one of the first indications of the changes in the selective permeability of microbial membranes (9), occurred in the presence of excess lower fatty acids and alcohols (Table 4; Fig. 1 and 2). The rate and extent of the potassium efflux were dependent on the chain length of the compounds. Thus, after 5 h of incubation with the lower fatty acids, the potassium loss from washed cells ranged from 100% for *n*-pentanoic acid to a slight 17% for *n*-tridecanoic acid. A similar pattern of potassium efflux was obtained in the presence of the lower fatty alcohols up to 1-dodecanol. On the other hand, all the higher fatty acids and alcohols tested had practically no effect. In general, the amount of potassium efflux was dependent on the concentration of the short-chain compound in the aqueous medium. Moreover, the effect of the fatty acids (but not the alcohols) appeared to be enhanced by a lower pH value of the medium. These properties are illustrated by *n*-heptanoic acid and 1-heptanol (Fig. 3). The loss of potassium in the presence of the short-chain compounds was accompanied by the leakage of

TABLE 2. *Effect of various fatty acids and alcohols on the growth of Cladosporium resinae in 1% glucose*

| Fatty acid or alcohol added | Total dry cell weight (mg) | | |
|---|---|---|---|
| | Isolate 35A | Isolate 89A | Isolate 102B |
| *n*-Pentanoic acid | 0 | 0 | 0 |
| *n*-Hexanoic acid | 0 | 0 | 0 |
| *n*-Heptanoic acid | 0 | 0 | 0 |
| *n*-Octanoic acid | 0 | 0 | 0 |
| *n*-Nonanoic acid | 0 | 0 | 0 |
| *n*-Decanoic acid | 0 | 0 | 0 |
| *n*-Undecanoic acid | 0 | 0 | 0 |
| *n*-Dodecanoic acid | 0 | 0 | 0 |
| *n*-Tridecanoic acid | 58 | 53 | 57 |
| *n*-Tetradecanoic acid | 71 | 67 | 57 |
| *n*-Hexadecanoic acid | 79 | 76 | 79 |
| *n*-Heptadecanoic acid | 61 | 63 | 65 |
| *n*-Octadecanoic acid | 60 | 57 | 68 |
| 1-Pentanol | 0 | 0 | 0 |
| 1-Hexanol | 0 | 0 | 0 |
| 1-Heptanol | 0 | 0 | 0 |
| 1-Octanol | 0 | 0 | 0 |
| 1-Nonanol | 0 | 0 | 0 |
| 1-Decanol | 0 | 0 | 0 |
| 1-Dodecanol | 34 | 38 | 22 |
| 1-Tridecanol | 63 | 59 | 65 |
| 1-Tetradecanol | 67 | 58 | 70 |
| 1-Pentadecanol | 66 | 63 | 64 |
| 1-Hexadecanol | 67 | 64 | 66 |
| 1-Octadecanol | 57 | 56 | 64 |
| None (control) | 65 | 60 | 67 |

TABLE 3. *Effect of fatty acids and alcohols on the endogenous respiration of Cladosporium resinae isolate 35A*

| Substrate added | Endogenous respiration (% of control[a]) |
|---|---|
| *n*-Hexanoic acid | 25 |
| *n*-Heptanoic acid | 32 |
| *n*-Octanoic acid | 40 |
| *n*-Decanoic acid | 71 |
| *n*-Dodecanoic acid | 87 |
| *n*-Tetradecanoic acid | 92 |
| *n*-Hexadecanoic acid | 97 |
| 1-Hexanol | 31 |
| 1-Octanol | 47 |
| 1-Decanol | 86 |
| 1-Dodecanol | 95 |
| 1-Tetradecanol | 93 |
| 1-Hexadecanol | 98 |

[a] Rate of $^{14}CO_2$ expiration in the absence of added substrate (850 counts/min per mg of dried cells per hour).

TABLE 4. *Potassium efflux and protein leakage from whole cells after 5 h of incubation with various fatty acids and alcohols at 30 C*

| Substrate added | Potassium loss[a] | Protein leakage[b] |
|---|---|---|
| *n*-Pentanoic acid | 100.0 | 16.5 |
| *n*-Hexanoic acid | 85.5 | 16.0 |
| *n*-Heptanoic acid | 61.0 | 17.8 |
| *n*-Octanoic acid | 58.4 | 22.8 |
| *n*-Nonanoic acid | 58.1 | 34.2 |
| *n*-Decanoic acid | 59.2 | 42.3 |
| *n*-Undecanoic acid | 54.0 | 40.1 |
| *n*-Dodecanoic acid | 34.2 | 8.4 |
| *n*-Tridecanoic acid | 17.0 | 4.0 |
| *n*-Tetradecanoic acid | 2.9 | 4.2 |
| *n*-Hexadecanoic acid | 2.3 | 3.8 |
| *n*-Heptadecanoic acid | 2.5 | 4.7 |
| *n*-Octadecanoic acid | 3.0 | 4.4 |
| 1-Pentanol | 97.9 | 50.8 |
| 1-Hexanol | 65.0 | 51.0 |
| 1-Heptanol | 60.2 | 49.1 |
| 1-Octanol | 60.7 | 49.6 |
| 1-Nonanol | 56.0 | 42.6 |
| 1-Decanol | 52.5 | 40.5 |
| 1-Dodecanol | 25.9 | 5.6 |
| 1-Tridecanol | 3.1 | 4.9 |
| 1-Tetradecanol | 2.9 | 4.0 |
| 1-Pentadecanol | 2.5 | 4.0 |
| 1-Hexadecanol | 2.5 | 3.9 |
| 1-Octadecanol | 2.8 | 5.1 |
| None (control) | 2.5 | 4.4 |

[a] Expressed as the percentage of total cellular potassium.

[b] Expressed as the percentage of total cellular protein.

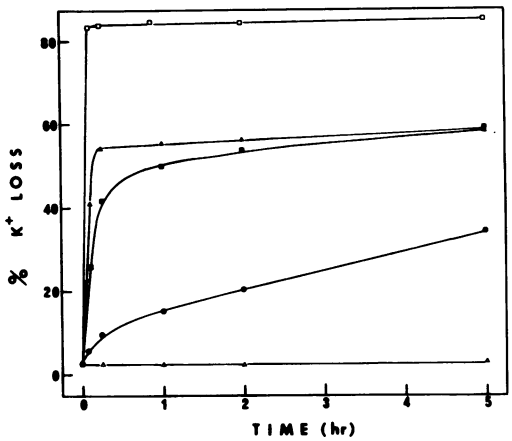


FIG. 1. *Time course of potassium efflux from Cladosporium resinae cells incubated at 30 C in the presence of some typical fatty acids. Symbols:* □ *n-hexanoic acid;* ▲ *n-octanoic acid;* ■ *n-decanoic acid;* ● *n-dodecanoic acid;* △ *n-tetradecanoic acid and control.*

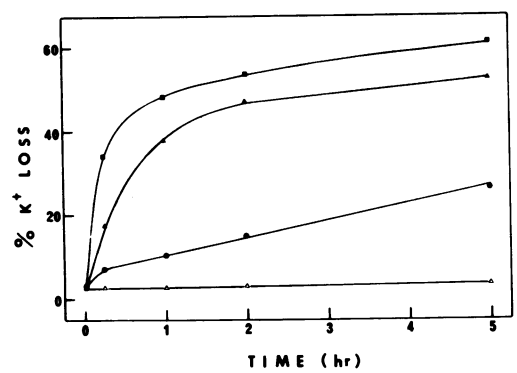


FIG. 2. *Time course of potassium efflux from Cladosporium resinae cells incubated at 30 C in the presence of some typical fatty alcohols. Symbols:* ■ *1-octanol;* ▲ *1-decanol;* ● *1-dodecanol;* △ *1-tetradecanol and control.*

significant proportions of cellular protein, although the latter process was slower and less extensive (Table 4). The dependence of protein leakage on chain length closely paralled that of the potassium efflux, except for the $C_5$ to $C_8$ fatty acids which caused relatively low protein leakage, possibly due to the precipitation of cellular protein by the acidic solutions of these compounds.

From the results of the present investigation, a probable explanation of the toxicity of the shorter fatty acids and alcohols may lie in a direct action of these compounds on the cell membrane. Since short-chain fatty acids (particularly in undissociated form) and alcohols

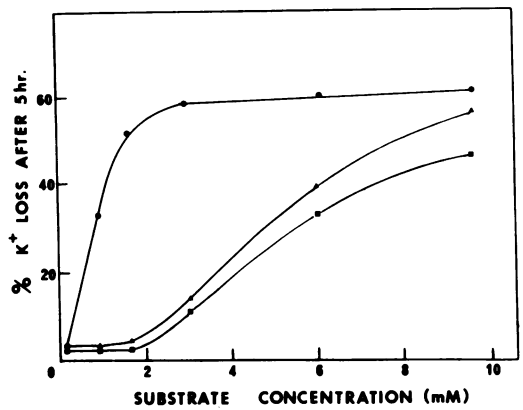


FIG. 3. *Potassium efflux from Cladosporium resinae cells incubated at 30 C with various concentrations of 1-heptanol solution* (▲) *and n-heptanoic acid solution at pH 4.0* (●) *and 6.0* (■).

are more soluble in water than their longer-chain homologues (4), the former might penetrate the cytoplasmic membrane in relatively high concentrations and cause the disorientation of certain essential groups on the membrane. Such disruption of membrane permeability is probably of limited extent, except for the $C_5$ and $C_6$ compounds. Similar effects on the intracellular membrane systems may account for the inhibition of endogenous respiration by the short-chain fatty acids and alcohols. However, other possible mechanisms such as a nonspecific blocking of enzymatic sites (3) or the inhibition of specific reactions of cell metabolism (8) cannot be completely ruled out at present. It is conceivable that all contribute collectively towards the final toxicity of the short-chain compounds.

*C. resinae* is known to utilize *n*-alkanes ranging from *n*-nonane to *n*-octadecane (19). Growth is generally poor on the $C_9$ to $C_{12}$ *n*-alkanes, although these *n*-alkanes have no toxic effect on the cell membrane as observed for *n*-hexane and *n*-heptane (20). In view of the present finding, it is tempting to relate the poor utilization of $C_9$ to $C_{12}$ *n*-alkanes to the toxicity of the corresponding fatty acids and alcohols, assuming that the major pathway for the oxidation of all *n*-alkanes proceeds via the homologous primary alcohol, aldehyde, and monocarboxylic acid, and that the intracellular concentration of these oxidized intermediates are probably not sufficiently high to inhibit completely *n*-alkane utilization and cell growth.

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