Metabolism of a Plant Wax Paraffin (*n*-Nonacosane) by a Soil Bacterium (*Micrococcus cerificans*)

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(Accepted for publication 29 October 1967)

SUMMARY

Long-chain paraffins, such as nonacosane, reach the soil from plants and are degraded by micro-organisms. The ability of one soil organism, *Micrococcus cerificans*, to grow on cabbage paraffin (*n*-nonacosane) was studied. Unlike the pattern seen with hexadecane, waxy esters were not produced when nonacosane was the sole carbon source, but esters were formed when a co-oxidation technique of [¹⁴C] *n*-nonacosane with hexadecane was employed. The alcohol and acid portions of the radioactive esters formed were primarily of chain length C₁₆, C₁₇ and C₁₈; neither nonacosanol nor nonacosanoic acid were detected. Thus the nonacosanol and nonacosanoic acid which might have been produced from *n*-nonacosane must have been oxidized to shorter-chain compounds which in turn were incorporated into waxy esters as well as other lipids.

INTRODUCTION

Large amounts of paraffins reach the soil from plants (Stevenson, 1966) and much of this is degraded by micro-organisms present in the soil. These plant paraffins are generally found to have carbon chain-lengths of C₂₇-C₃₅, with C₂₉ (nonacosane) one of the most abundant (Silva-Fernandes, Baker & Martin, 1964). Little is known about bacterial utilization of these plant paraffins although the bacterial degradation of petroleum paraffins (C₁₂ to C₁₈) has been studied extensively (McKenna & Kallio, 1965). Many species of micro-organisms can grow on paraffins as a sole carbon source and attempts are being made to produce edible microbial proteins from petroleum (Champagnat, 1964). Longer paraffins (C₂₀-C₃₄) are known to be utilized by micro-organisms (ZoBell, 1950) and they can also be utilized by animals (Kolattukudy & Hankin, 1966).

It has been shown (Stevenson, Finnerty & Kallio, 1962; Stewart *et al.* 1959; Stewart & Kallio, 1959) that *Micrococcus cerificans* produces waxy esters from medium chain-length paraffins; the alcohol portion of the ester has the same chain length as the substrate paraffin but the acid portion varies. If this metabolic pattern applies to the very-long-chain plant paraffins then very-long-chain alcohols would be produced. The demonstration that such long-chain alcohols possess androgenic activity (Levin, 1963) raises the possibility that the intestinal flora may produce physiologically active compounds from long-chain paraffins present in the food supply (Kuksis, 1964; Silva-Fernandes *et al.* 1964).

In the present report, the metabolic fate of *n*-nonacosane (from cabbage wax) in *M. cerificans*, a soil organism, is discussed.

METHODS

Organism. *Micrococcus cerificans* was maintained as a stock culture on agar slants prepared with 3.1% of nutrient agar supplemented with 0.5% each of yeast extract and Casamino acids (Difco), and subcultured weekly. The organism was kindly provided by Dr R. E. Kallio, University of Illinois, Urbana.

Media. The basal mineral medium contained per litre of distilled water: $(\text{NH}_4)_2\text{SO}_4$, 2 g.; KH_2PO_4 , 4 g.; Na_2HPO_4 , 6 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg.; MgSO_4 , 200 mg.; CaCl_2 , 1 mg.; H_3BO_3 , 10 μg .; MnSO_4 , 10 μg .; ZnSO_4 , 70 μg .; CuSO_4 , 50 μg .; MoO_3 , 10 μg .; calcium pantothenate, 200 mg. pH 7.5.

Test media (except where indicated) were prepared by adding sterile (autoclaved) paraffins (0.5–1.0% except for radioactive material) to sterile basal mineral medium. To emulsify the hydrocarbon the medium was then subjected to ultrasonic disintegration (Biosonic II, Will Corporation) at 90% of maximum output for 10 min. The ultrasonic disintegration step was omitted when *n*-hexadecane was the substrate.

Growth studies. In comparative growth tests of different substrates, the same amount of inoculum was placed in each flask. At least 27×10^6 bacteria/ml. were used in each test. Growth was measured by a direct microscopic method (American Public Health Assoc., 1948) after staining the bacteria with a solution of crystal violet. For all tests, the organism was grown in shake culture in a water bath at 30°. When radioactive *n*-nonacosane was used, in addition to shaking, sterile air was bubbled through the incubation mixture to remove radioactive CO_2 .

Preparation of substrates and standards. Paraffins other than *n*-nonacosane were a gift from Dr B. J. Humphrey (Humphrey Chemical Co., North Haven, Conn., U.S.A.). Both radioactive and non-radioactive *n*-nonacosane was obtained from the cuticular wax of *Brassica oleracea* as previously described (Kolattukudy, 1965; Kolattukudy & Hankin, 1966). Standard long-chain esters used for gas chromatographic analysis were prepared from stearyl alcohol and acyl chlorides with pyridine as catalyst.

Gas chromatography. Gas chromatographic analyses were made on a Perkin Elmer 810 gas chromatograph with a flame ionization detector. The columns used have previously been described (Kolattukudy, 1967). Radioactive material emanating from the column was measured with a radioactivity monitor (Barber-Coleman).

Isolation and identification of lipids. Total lipids were extracted from the incubation mixture by the method of Folch, Lees & Sloane-Stanley (1957) with a 2+1 (v/v) mixture of chloroform and methanol. After concentration, the extract was placed on a silica gel column (Kolattukudy, 1967; Kolattukudy & Hankin, 1966). The components were eluted successively with hexane, benzene and methanol (containing 10% of acetic acid). Hexane eluted the paraffins, benzene the esters, and methanol the polar lipids (glycerides, phospholipids). Lipid classes were identified by thin-layer chromatography (Mangold, 1965). Fatty acids from glycerides and phospholipids were isolated after saponification with ethanolic KOH under nitrogen. Methyl esters were prepared with BF_3 as catalyst. The waxy esters were isolated from the benzene eluate of the silica gel columns by thin-layer chromatography on silica gel G with benzene as the developing solvent. The ester fraction was saponified with ethanolic KOH under nitrogen, and the alcohol and acid fractions were isolated by thin-layer chromatography with hexane + diethyl ether + formic acid (40 + 10 + 1, by vol.)

(Kolattukudy, 1967) as the developing solvent. The alcohol fraction was analysed as the acetate, and the acids as the methyl esters by gas chromatography.

RESULTS

Growth of Micrococcus cerificans with long-chain paraffins

Micrococcus cerificans is able to grow on medium-chain-length paraffins (hexadecane, octadecane) as the sole source of carbon (Stevenson *et al.* 1962; Stewart *et al.* 1959; Stewart & Kallio, 1959). We have now observed that this soil organism can also utilize much longer paraffins, such as that from cabbage wax (*n*-nonacosane). Comparable growth was obtained with *n*-docosane (Fig. 1). Since it was noted that with small inocula the organism grew slowly, larger inocula were used in the biochemical studies. In preliminary experiments the paraffin was presented to the organism coated on diatomaceous earth (Celite) since the paraffin is not readily dispersible in aqueous solution. Although it grew with this experimental technique, better growth was obtained when ultrasonic emulsification of the substrate was employed.

Table 1. *Micrococcus cerificans*: co-oxidation of *n*-hexadecane with [^{14}C]*n*-nonacosane (C_{29})

Mineral medium (100 ml.) with 1.0% of *n*-hexadecane and 23.7×10^8 counts/min. [^{14}C]*n*-nonacosane were inoculated with *Micrococcus cerificans* and incubated for 20 hr at 30°. The water layer contained radioactivity but was not analysed further.

Product	% of administered ^{14}C
Respiratory CO_2	8.4
Hydrocarbon fraction	64.0
Ester fraction	0.81
Polar lipids	2.7
Wax acids	0.28
Wax alcohols	0.30
Cell residue	2.5

Ester production on paraffin substrates

The cultures were assayed for waxy ester production by thin-layer chromatography. Ester production was readily detected when hexadecane was the substrate, lesser amounts when docosane was used and none when nonacosane was the sole carbon source. To increase the chances of detecting a metabolite of nonacosane, such as a 1-nonacosanol, [^{14}C]nonacosane was presented to the organism in the presence of relatively large amounts of a readily oxidizable hydrocarbon, namely hexadecane. This co-oxidation technique with non-radioactive hexadecane and radioactive nonacosane resulted in small but significant amounts of radioactive ester being detected and isolated by the column and thin-layer chromatographic methods used (Table 1). Of necessity the growth period was shortened in order to avoid extensive degradation of the presumed intermediate, nonacosanol. Gas chromatography of these esters showed that the major radioactive esters had chain lengths of 31–34 carbon atoms.

*Products formed from [^{14}C]*n*-nonacosane by co-oxidation with hexadecane*

When the ester fraction was hydrolysed, the radioactivity was found to be equally distributed between the alcohol and acid parts (Table 1). The gas-chromatographic analysis of the alcohols (as the acetate) and the acids (as methyl esters) revealed that neither had a chain length comparable to that of the substrate hydrocarbon. The

Fig. 1. Growth of *Micrococcus cerificans* on *n*-nonacosane and *n*-docosane as sole source of carbon; 30°.

Fig. 2

Fig. 3

Fig. 2. Radio gas-liquid chromatogram of alcohols (as acetates) derived from waxy esters produced by *Micrococcus cerificans* during co-oxidation of [^{14}C]*n*-nonacosane with *n*-hexadecane. Essentially all the mass was represented by C_{16} alcohol as measured by the flame ionization detector. Experimental conditions: 183 cm. (0.6 cm. o.d.) coiled copper column, 12 % Apiezon L on 50-60 mesh Anakrome A (Analabs, Hamden, Conn.), temperature of column and injector 230° and 350° respectively. Carrier gas was argon at 75 ml./min. Analysis on a 5 % SE-30 column at 300° did not show significantly radioactive alcohols longer than C_{18} .

Fig. 3. Radio gas-liquid chromatogram of fatty acids (as methyl esters) derived from polar lipids produced by *Micrococcus cerificans* during co-oxidation of [^{14}C]*n*-nonacosane with *n*-hexadecane. Experimental conditions same as in Fig. 2 except the column temperature which was 220°. Most of the mass was represented by approximately equal quantities of saturated and unsaturated C_{16} acids.

major alcohol was C_{17} with smaller amounts of C_{16} and C_{18} (Fig. 2). The wax acids were of similar chain lengths. The polar lipid fraction contained radioactivity several times higher than the ester fraction (Table 1). The major radioactive fatty acids were C_{17} , C_{18} and C_{16} , in decreasing order of magnitude (Fig. 3). This distribution pattern is almost identical to that seen with the wax acids and alcohols.

DISCUSSION

A possible explanation for the absence of ester production with *n*-nonacosane is that the rate of oxidation of the very-long-chain paraffins (C_{29}) is much slower than that of the medium-chain-length paraffins. Therefore, the first stable intermediate in very-long-chain oxidation (namely the alcohol) undergoes oxidative degradation as soon as it is formed. Consequently, unlike the case of the medium-length paraffins, the alcohols of the long-chain paraffins do not accumulate, and hence no ester formation occurs. If such be the case, dilution of the alcohol pool might be accomplished by

Fig. 4. Scheme of proposed metabolic fate of *n*-nonacosane in *Micrococcus cerificans*.

providing the organism with the readily oxidizable C_{16} paraffin along with C_{29} paraffin. Under these conditions sufficient nonacosanol (C_{29}) might accumulate for its esterification to occur. However, the absence of C_{29} alcohol from the ester fraction after co-oxidation indicates that in spite of the dilution of the alcohol pool that must have occurred as a result of the co-oxidation, the C_{29} alcohol underwent preferential oxidation. The enzymes that synthesize the waxy esters may prefer the medium-chain-length alcohol (C_{16} alcohol) and thus leave the C_{29} alcohol for oxidative degradation. However, the possibility of an oxidative attack on an internal carbon (such as C_{17}) of the paraffin cannot be ruled out. The radioactive alcohols detected in the ester fraction were probably formed by reduction of fatty acids derived from the oxidation of C_{29} paraffin. This hypothesis is consistent with the fact that the chain lengths of the radioactive acids and alcohols were similar. Stewart *et al.* (1959) did find ester formation when this organism was grown on palmitic acid, although the ester production was very much less than with hexadecanol (C_{16}). This indicates that palmitic acid did undergo reduction to the alcohol.

These results suggest the metabolic fate of C_{29} paraffin with *Micrococcus cerificans* as depicted in Fig. 4. Although no direct evidence was obtained from the present experiments, it is likely that the end carbon of C_{29} paraffin would undergo oxidation

to the corresponding alcohol presumably as described in the case of octane (Gholson, Baptist & Coon, 1963) and hexadecane (Stewart *et al.* 1959; Stewart & Kallio, 1959). This alcohol in turn is presumably oxidized to the corresponding acid which appears to undergo rapid 'chain shortening' to the level of common fatty acids of the organism (C_{16} – C_{18}). Such a conversion could be accomplished by a β -oxidation system; but in that case the β -oxidation would have to cease at the C_{17} stage. Although it is thought that β -oxidation of a fatty acid proceeds to completion, C_{18} acid has been shown to be converted into C_{16} acid in the rat by a 'leakage' in the β -oxidation system (Elovson, 1965). However, a 'chain-shortening' system (counterpart of the chain-lengthening system) similar to, but not identical with the β -oxidation system, could have been responsible for the formation of C_{17} acid from C_{29} acid. If it is a 'leakage', then it must be specific enough to cause 'leakage' primarily at the C_{17} stage. The complete conversion of C_{29} acid into 13 acetate units and a propionate unit, and the resynthesis of C_{17} from such units, cannot be ruled out conclusively. However, the relative magnitude of radioactivity in the various metabolic products makes this unlikely. The medium-chain-length acids, primarily C_{17} , derived from C_{29} paraffin are then incorporated mostly into phospholipids; smaller quantities undergo reduction to the alcohol which in turn is esterified. In the rat, *n*-nonacosane underwent a similar oxidative degradation, resulting in primarily C_{17} acid which was incorporated into phospholipids and glycerides (Kolattukudy & Hankin, 1966).

The organism used in this study, *Micrococcus cerificans*, was isolated by enrichment culture with the use of hexadecane (Finnerty, Hawtrey & Kallio, 1962) from soil. It might be possible to isolate an organism from soil which would more readily utilize, or even prefer, plant wax paraffin, if such paraffins were used in the enrichment procedures.

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