Production of ω -Haloesters from Alkyl Halides by *Micrococcus cerificans*

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SUMMARY

Micrococcus cerificans grows with *n*-alkyl chlorides and bromides containing 16 to 20 carbon atoms as the sole source of carbon and forms waxy esters. The esters produced from *n*-Hexadecyl chloride were identified to be 16-chlorohexadecyl-16-chlorohexadecanoate (74 %) and 16-chlorohexadecylhexadecanoate (23 %). More than 80 % of the fatty acids of the more polar cellular lipids also contained chlorine at the ω -carbon. These results show that the mechanism of utilization of alkyl halides by this organism is similar to that of alkanes and the initial attack is specifically at the non-halogenated methyl carbon.

INTRODUCTION

Many species of micro-organisms oxidize alkanes (McKenna & Kallio, 1965) and apparently a portion of the hydrocarbons that reach the soil from the cuticular lipids of plants is degraded by micro-organisms (Hankin & Kolattukudy, 1968). *Micrococcus cerificans*, an organism isolated from soil, oxidizes alkanes of medium chain length, C₁₁ to C₂₆ (Finnerty, Hawtrey & Kallio, 1962) as well as very long chains such as *n*-nonacosane (Hankin & Kolattukudy, 1968). Oxidative attack by this organism on the medium-length paraffins starts from the terminal methyl group, giving primary alcohols as the first stable product. Since production of the alcohol is more rapid than its further oxidative degradation, it undergoes esterification with fatty acids and the resulting waxy ester is excreted (Stewart *et al.* 1959). When the hydrocarbon contains a double-bond at one end, the bacterium attacks the other end, thus giving the ω -unsaturated alcohol (Stewart *et al.* 1960). In the present report it is shown that *M. cerificans* can readily grow on alkyl halides by oxidizing the molecule primarily from the non-halogenated end. The bacterium oxidized alkyl halides to ω -halo fatty alcohols and fatty acids, which in turn were esterified to produce the dihalo waxy esters. Since ω -haloacids and alcohols are important intermediates in synthetic organic chemistry, the bacterial production of such intermediates might be of potential use.

METHODS

Organism. *Micrococcus cerificans* (kindly provided by Dr R. E. Kallio, University of Illinois, Urbana), was maintained as a stock culture as previously described (Hankin & Kolattukudy, 1968).

Substrates. *n*-Hexadecylchloride and bromide, *n*-octadecylchloride and bromide, and *n*-eicosylchloride were obtained from Humphrey Chemical Company, North

Haven, Conn., U.S.A. The 1,16-dibromohexadecane was obtained from K and K Laboratories, Plainview, N.J., U.S.A.

Media. The basal mineral medium used was described by Hankin & Kolattukudy (1968). All test substrates were added directly to the mineral medium, which was sterilized by autoclaving. Ultrasonic treatment (Biosonik II, Will Corp., Rochester, N.Y., at 90 % of maximum output for 10 min.) was used to disperse 1,16-dibromohexadecane and *n*-eicosylhexadecane,

Growth studies. Cultures for growth measurements on different substrates were made in 250 ml. Erlenmeyer flasks containing 25 ml. mineral medium + 0.5 % (w/v) alkyl halide. Rate of growth was measured by a plating procedure on Trypticase Soy Agar (Baltimore Biological Laboratories, Baltimore, Maryland). For all tests, the organism was grown in shake culture in a water bath at 30°.

Detection of ester production. At the end of the growth tests the entire culture was mixed with a sufficient amount of 2 + 1 by vol. chloroform + methanol mixture to give a single liquid phase and allowed to stand for at least 18 hr. Total lipids were isolated by the method of Folch, Lees & Sloane-Stanley (1957), and chromatographed on thin-layer plates with benzene as the developing solvent. Lipids were located by a sulphuric acid + dichromate charring method.

Isolation and fractionation of lipids. To obtain sufficient material for identification purposes, 500 ml. cultures were used. With such large amounts a longer incubation time was necessary to reach peak growth, presumably because aeration was less efficient with the larger volume. After 72 hr of incubation the bacteria were collected by centrifugation. Although the supernatant fluid contained some waxy esters, it was more convenient to isolate them from the bacterial mass. This does not imply that the esters were within the organisms. The wet bacterial mass was stirred with 400 ml. of a 2 + 1 mixture (v/v) of chloroform + methanol at 45° for 4 to 5 hr. Total lipids were isolated from this mixture by the method of Folch *et al.* (1957). The chloroform was evaporated under reduced pressure, and the residue was dissolved in boiling 95 % (v/v) ethanol in water and cooled. The crystalline material was collected and recrystallized twice from 95 % (v/v) ethanol in water. The product at this stage of purification usually melted at 49–50°. Preparative thin-layer chromatography followed by recrystallization from 95 % (v/v) ethanol gave a product melting at 50–51°. This ester fraction was used for the structural analysis.

Since a substantial amount of unbroken bacteria was found after the above treatment, the bacterial residue was freeze-dried and then refluxed with the chloroform + methanol mixture for 6 hr, under nitrogen. The lipids thus extracted were pooled with the mother liquors from the crystallizations described above and were designated 'total lipids'.

Column chromatography. The total lipids were applied to a column (2.5 × 55 cm.) containing SilicAR CC-4, 100–200 mesh (Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.). The unused alkyl halides and the relatively non-polar lipids such as waxy esters, were removed by elution with 500 ml. benzene. The more polar lipids were then eluted with methanol. This fraction is referred to as 'cell lipids'.

Preparation of methyl esters. The cell-lipid fraction isolated by column chromatography was refluxed under nitrogen with 14 % of BF₃ in methanol (Analabs, Hamden, Conn., U.S.A.) for 24 hr. Water was added and the lipids extracted with chloroform. Methyl esters were isolated by thin-layer chromatography.

Reduction of waxy esters with lithium aluminium hydride. To an ethereal solution of waxy ester, several fold excess of lithium aluminium hydride was added and stirred for 30 min. at room temperature. These mild conditions were used to minimize substitution of the chlorine with hydrogen (Johnson, Blizzard & Carhart, 1948). After decomposing the excess of reagent with water, the products were extracted with ether. The alcohols were then purified by thin-layer chromatography.

Thin-layer chromatography. Silica gel G plates 0.5 or 1.0 mm. thick for preparative purposes and 0.3 mm. thick for analytical purposes were used (Kolattukudy, 1965). The waxy esters and methyl esters were chromatographed with benzene as the developing solvent (Kolattukudy, 1965, 1966). The fatty acids and fatty alcohols were chromatographed with a mixture of hexane + diethyl ether + formic acid (40 + 10 + 1, by vol.; Kolattukudy, 1967a).

Gas-liquid chromatography. A Perkin Elmer 810 Gas Chromatograph with flame ionization detector was used. Fatty alcohols, methyl esters of fatty acids and waxy esters were analyzed on a 5 % silicone gum rubber (SE 30) column. Methyl esters were also analyzed on a 15 % stabilized diethylene glycol succinate column. Authentic samples of methyl esters of fatty acids (Analabs, Hamden, Conn.) and fatty alcohols (Humphrey Chemical Co., North Haven, Conn.) were used as standards. The waxy esters used as standards were prepared as previously described (Kolattukudy, 1967b). Conditions for gas-liquid chromatography are given with the legends to the figures.

Infrared and mass spectra. Infrared spectra were obtained on thin films of materia with a Perkin Elmer Model 21 Infrared Spectrophotometer (courtesy of R. A. Botsford of this Station).

Mass spectra was obtained with an MS Mass Spectrometer at 70 eV. with 250° as the source temperature.

RESULTS

Growth of Micrococcus cerificans on alkyl halides

Growth of *Micrococcus cerificans* with various alkyl halides as the sole source of carbon is depicted in Fig. 1. The organism grew readily on C₁₆, C₁₈, and C₂₀ alkyl chlorides, and on C₁₆ and C₁₈ alkyl bromides. In all instances maximum growth was obtained at about 36 hr, and the growth on the alkyl halides was comparable to that observed with the corresponding alkanes. The effect of substituting halogens on both ends of the alkane on the growth of the organism was tested by providing 1,16-dibromohexadecane as the sole carbon source; with this the organism did not grow.

Waxy ester production from alkyl halides

Lipids isolated from the cultures at the end of the growth period were analysed by thin-layer chromatography on silica gel G with benzene as the solvent. Three major spots were detected in all cases. The spot nearest to the solvent front (R_F c. 0.85) corresponded to unused alkyl halide. A second spot had an R_F (c. 0.70) identical to that of waxy esters. The more polar lipids remained at the origin. The organism produced less ester from bromides than from the corresponding chlorides. As the chain length increased from C₁₆ to C₂₀, ester production decreased. Thus, more esters were produced from C₁₆ chloride than from the other substrates.

Fig. 1. Growth of *Micrococcus cerificans* on *n*-hexadecylchloride ($C_{16}Cl$) and bromide ($C_{16}Br$), *n*-octadecylchloride ($C_{18}Cl$) and bromide ($C_{18}Br$), *n*-eicosylchloride ($C_{20}Cl$) and 1,16-dibromohexadecane ($BrC_{16}Br$). The arrows on the abscissa indicate times at which growth was measured. The amount of growth at the peak varied, apparently because of the intervals at which growth was measured.

Structure of waxy esters

Since the *n*-hexadecylchloride gave more ester than the other halides it was used to produce esters for structural studies. It is assumed that similar products were formed from the other alkyl halides. The crystalline ester fraction obtained as described under Methods had a melting point of $50-51^{\circ}$. Further preparative thin-layer chromatography and subsequent recrystallization did not alter the melting point. The esters produced by *Micrococcus cerificans* from *n*-hexadecane melted at $46-48^{\circ}$ (Stewart *et al.* 1959). The higher melting point of the ester produced from *n*-hexadecylchloride suggested the presence of chlorine atoms in the ester.

The infrared spectrum (Fig. 2) showed the absorption expected of long alkane chains, absorptions at 1735 cm.^{-1} and 1175 cm.^{-1} characteristic of an ester function as well as at 725 cm.^{-1} and 737 cm.^{-1} indicative of the C-Cl bond.

Gas-liquid chromatography of the wax esters on an SE-30 column showed that there were three esters in the crystalline waxy ester fraction, representing 74, 23 and 3 % of the total mass and marked A, B, C, respectively on Fig. 3. Peak C had a retention time equal to that of cetyl palmitate. Peaks B and A had retention times corresponding to $C_{34.5}$ and C_{37} esters, respectively. Since the mass of one chlorine atom is equivalent to that of 2.5 methylene groups, peak B probably represented C_{32} ester with one chlorine atom, and peak A C_{32} ester containing two chlorine atoms.

Treatment of the ester fraction with lithium aluminium hydride gave a product on thin-layer chromatography with an R_F corresponding to fatty alcohol. This product when isolated from the thin-layer plate and subjected to gas-liquid chromatographic analysis on an SE-30 column gave two major peaks: one peak with a retention time of C_{16} alcohol (about 20 % of the mass); the other peak (about 80 % of the mass) with a retention time corresponding to $C_{18.5}$ which probably represented the C_{16} alcohol containing one chlorine atom.

Fig. 2. Infrared spectrum of waxy esters produced by *Micrococcus cerificans* from *n*-hexadecyl chloride.

Table 1. Measurement of mass of the molecular ions from the dihalo ester

Species	Measured mass	Calculated mass
$C_{32}H_{62}O_2Cl_2$	548.40968	548.4130
$C_{31}C^{13}H_{62}O_2Cl_2$	549.41466	549.4164
$C_{32}H_{62}O_2Cl^{35}Cl^{37}$	550.40877	550.4100
$C_{31}C^{13}H_{62}O_2Cl^{35}Cl^{37}$	551.41375	551.4134

Mass spectral analysis. The molecular ion at 548 corresponded to the structure $ClC_{15}H_{30}COOCH_2C_{15}H_{30}Cl$. Mass measurements of the various species of the molecular ion by a peak-matching technique with perfluorokerosene (Table 1), agreed with the proposed structure. The relative abundance of ions at 548, 550, and 552 confirmed the presence of two chlorine atoms in this ion. As expected from such an ester ($RCOOR'$) there were dominant ion intensities at 291 corresponding to the $RCOOH_2^+$, and at 258 corresponding to $(R'-H)^+$. The other characteristic ions expected from the proposed structure were also present as shown in Table 2. The presence of the chlorine atoms in the fragment ions was confirmed by the characteristic isotopic ratios due to chlorine. As indicated by the gas-liquid chromatographic analysis, the ester contained a monohalo ester giving rise to the ion at 514. The chlorine atom contained in this ion was expected to be on the ω carbon atom of the alcohol. Confirming this prediction there were fragment ions derived from the non-halo acyl moiety, but ions indicating a non-halo alcohol moiety were hardly detectable. In accordance with the assignment of the minor component (3 %) of the ester as a non-halo ester there was

an ion at 480 which was most likely the molecular ion from etyl palmitate. Thus the mass spectral analysis confirmed the conclusions drawn about the structure of the esters from the other analyses described above.

Fig. 3

Fig. 4

Fig. 3. Gas-liquid chromatogram of waxy esters produced by *Micrococcus cerificans* from *n*-hexadecyl chloride. Column: 5 % SE 30 on Anakrom SD, 4 ft., 0.25 in o.d. at 305° with carrier-gas argon 75 ml./min. A, ω -chlorohexadecyl ω -chlorohexadecanoate; B, ω -chlorohexadecyl hexadecanoate; C, hexadecyl hexadecanoate.

Fig. 4. Gas-liquid chromatogram of methyl esters of fatty acids of cell lipids isolated from *Micrococcus cerificans* grown on *n*-hexadecyl chloride. Column same as in Fig. 3 at 212° with carrier-gas argon at 80 ml./min. The fatty acids are identified by the number of carbon atoms; u represents unsaturation; Y, ω -chlorohexadecanoate; X, unsaturated ω -chlorohexadecanoate; Z, ω -chlorotetradecanoate. A, before bromination; B, after bromination.

Table 2. The major ions in the mass spectrum of the waxy ester

Mass/charge	Probable structure of the ion
548	$[\text{ClC}_{15}\text{H}_{30}\text{COOCH}_2\text{C}_{15}\text{H}_{30}\text{Cl}]^+$
514	$[\text{C}_{15}\text{H}_{31}\text{COOCH}_2\text{C}_{15}\text{H}_{30}\text{Cl}]^+$
480	$[\text{C}_{15}\text{H}_{31}\text{COOCH}_2\text{C}_{15}\text{H}_{31}]^+$
331	$\text{CH}_2:\text{CHC}(\cdot\text{OH})\text{OCH}_2\text{C}_{15}\text{H}_{30}\text{Cl}$
318	$\text{CH}_2:\text{C}(\text{OH})\text{CH}_2\text{C}_{15}\text{H}_{30}\text{Cl}$
303	$\text{ClC}_{15}\text{H}_{30}\text{CH}_2\text{OCO}^+$
291	$\text{ClC}_{15}\text{H}_{30}\text{COOH}_2^+$
290	$\text{ClC}_{15}\text{H}_{30}\text{COOH}^+$
273	$\text{ClC}_{15}\text{H}_{30}\text{CO}^+$
258	$\text{ClC}_{16}\text{H}_{31}^+$
257	$\text{ClC}_{16}\text{H}_{30}^+$

Cellular lipids

The gas-liquid chromatographic pattern of the methyl esters prepared by transesterifying the cell lipids with methanol containing 14 % BF_3 is shown in Fig. 4, tracing A. About 80 % of the mass was represented by two peaks of approximately equal size, marked X and Y. Peak Y had a retention time equivalent to $\text{C}_{18.5}$ and peak X corresponded to unsaturated $\text{C}_{18.5}$. The other major peak (13.6 %) corresponded to C_{16} and the usual fatty acids C_{12} (1 %), C_{14} (0.6 %), C_{18} (1.7 %) and unsaturated C_{18} (2.3 %) were also found in smaller quantities. Small quantities (3.4 %) of an acid corresponding to $\text{C}_{16.5}$ was also detected. To confirm these assignments, the methyl

ester fraction was treated with bromine and rechromatographed (Fig. 4, tracing B). The peaks assigned to the unsaturated fatty acid, $C_{18\alpha}$ and X, were virtually eliminated by the bromine treatment in accordance with the identification described above. Peak Y, corresponding to $C_{18\beta}$, must be due to the methyl ester of 16-chloropalmitic acid, because the mass of one chlorine atom is equivalent to that of 2.5 methylene groups. Therefore X represented unsaturated 16-chloropalmitic acid. By the same reasoning peak Z was identified as the methyl ester of ω -chlorotetradecanoic acid. When the same samples were analysed on a diethyleneglycol succinate column, the two major peaks of approximately equal size, represented by saturated and unsaturated chloropalmitic acid, had retention times 5.5 and 6.5 times that of C_{16} acid respectively as expected from the added mass and polarity introduced by the chlorine atom. Bromine treatment and rechromatography on this column confirmed the presence of unsaturation.

DISCUSSION

Since the organism grew just as readily on the alkyl halides as on the corresponding alkanes, the organism may be able to attack the terminal carbon irrespective of the atoms attached to it. Alternatively, it may attack specifically the non-halogenated end or the alkyl halides are metabolized by an altogether different mechanism. However, 1,16-dibromohexadecane failed to support growth of the organism indicating its inability to attack carbon atoms which bear a halogen. Thus the specificity of the initial oxidation is for a methyl group.

Fig. 5. Proposed metabolic fate of *n*-hexadecyl chloride in *Micrococcus cerificans*.

The formation of waxy esters is very similar to that observed with alkanes (Stewart *et al.* 1959; Stewart & Kallio, 1959), and suggests that the alkyl halide is oxidized in a manner similar to that described for paraffins (Stewart *et al.* 1959; Baptist, Gholson & Coon, 1963; Gholson, Baptist & Coon, 1963). From the structure of the esters produced it is clear that the bacterium attacked specifically the non-halogenated end and produced the ω -haloalcohol which in turn must have undergone further oxidation to the ω -haloacid. The acid and alcohol were subsequently esterified to give the major dihalo-waxy ester (Fig. 5).

From the structure of the esters produced by the organism from various alkanes (Stewart & Kallio, 1959) it appears that the saturated fatty acid pool of the organism takes part in esterifying the excess fatty alcohol produced by the alkane oxidation. Thus the fatty alcohol of the ester usually contains the intact carbon chain of the alkane, but the acids always reflect the composition of the general saturated fatty-acid

pool. While growing on the alkyl halides, *Micrococcus cerificans* probably produced nonhalogenated C₁₆ acid by *de novo* synthesis although the major source of fatty acids would be the direct oxidation on the non-halogenated end of the alkyl halides. Thus, the monohalo ester is probably derived from the halo alcohol and the non-halo fatty acids produced by the organism by *de novo* synthesis. Oxidative attack on some of the halogenated carbons could also produce the non-halogenated fatty acids. However lack of growth on 1,16-dibromohexadecane makes this an unlikely possibility.

The ratio of halo acids to nonhalo acids in the waxy esters was 3:1. The same ratio was found for saturated halo acids to saturated non-halo acids of the cell lipids. This observation strongly supports the contention that the saturated fatty acid pool of the organism takes part in the esterification process.

The organism can apparently perform all its biochemical and structural functions with the ω -halo acids instead of the usual fatty acids. It is obvious that even the unsaturated fatty acids could have a halogen on the ω -end without impairing their usual functions in the cell because the major unsaturated fatty acid in the cell was halo C₁₆ acid.

Under the conditions of growth used in this study about 80 % of all the fatty chains produced by the organism from hexadecyl chloride contained chlorine atoms on the ω -carbon atoms. Thus, total lipids could be a convenient source for ω -halo acids and these acids are extremely useful as intermediates in many syntheses. It may be possible to alter growth conditions of the organism so as to obtain a profitable yield of the halo acids. For example, the organism may readily oxidize alkyl halides while growing on inexpensive and readily obtainable media.

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