Occurrence of squalene, di- and tetrahydrosqualenenes, and vitamin MK₈ in an extremely halophilic bacterium, *Halobacterium cutirubrum*

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ABSTRACT The nonpolar (acetone-soluble) lipids of the extremely halophilic bacterium, *Halobacterium cutirubrum*, were found to consist of red carotenoid pigments (43%) and squalenes (48%) with a small amount of a vitamin K-type quinone. The squalenes were shown by n.m.r. and mass spectra to consist of the fully isoprenoid squalene (S; C₃₀H₅₀), dihydrosqualene (S₂; C₂₀H₄₂), and tetrahydrosqualene (S₄; C₄₀H₈₄) in the ratio of 1.0:0.4:0.1. S₂ probably has one reduced internal isoprenoid group, and S₄ has one internal and one terminal reduced isoprenyl group. The vitamin K-type quinone was shown by n.m.r. and mass spectra to have a Cho isoprenoid side chain, and is thus identified as menaquinone-8 (MK₈).

SUPPLEMENTARY KEY WORDS menaquinone-8 . halophilic bacteria

THE LIPIDS of extremely halophilic bacteria, e.g. *Halobacterium cutirubrum*, are remarkable for their unusual molecular structures (1-11). Most of the lipid components are derivatives of a di-O-alkyl glycerol ether (1, 2, 4, 5) identified as 2,3-di-O-(3'R,7'R,11'R,15'-tetramethylhexadecyl)-sn-glycerol (phytanyl glycerol diether) (3, 6-10). The phosphatides so far identified are the phytanyl glycerol diether analogues of phosphatidyl glycerophosphate (3, 6-8, 11) and phosphatidyl glycerol (5, 8, 11). A glycolipid, 1'-O-(glucosyl-mannosyl-galactosyl)-2,3-di-O-phytanyl-sn-glycerol, and its sulfate ester (9) have also been identified.

These polar lipids, which comprise about 92% by weight of the total cellular lipids, are readily separable from the nonpolar lipids by precipitation from cold acetone. The acetone-soluble fraction contains all of the nonpolar lipids, about half of which consists of red carotenoid pigments (12-14), chiefly α-bacterioruberine (13, 14). The remaining nonpolar lipids, however, have not previously been investigated. This communication deals with the isolation and characterization of the nonpolar lipids, apart from the carotenoid pigments, in *H. cutirubrum*, and the identification of squalene, hydro-squalenenes, and vitamin MK₈ as major components of this fraction.

MATERIALS AND METHODS

Culture Conditions

Cells of *H. cutirubrum* were grown aerobically at 37°C in the standard complex medium for halophiles, as described previously (1, 8, 15). In preliminary studies, cells were cultured in a 110-liter fermentor (15), using silicone antifoam agent to suppress foaming and mineral oil to lubricate the stirring propeller. It was subsequently discovered that both the silicone and the mineral oil are entrained by the cells and appear in the lipid extract together with the nonpolar lipid fraction. Cells were
then grown in shake culture with surface aeration in 7.5-liter batches in 15-liter baffled flasks without silicone or mineral oil; they were harvested by centrifugation after 3 days' growth, and resuspended in 25% salt solution to a concentration of about 50 mg of dry cells per ml. 14C-Labeled cells were grown in 1 liter of medium containing 950 μc of acetate-1-14C as described elsewhere (16), harvested by centrifugation after 7 days' growth, and resuspended in 25% salt solution.

**Extraction of Total Lipids**

Cell suspensions were extracted by the method of Bligh and Dyer (17), modified as described previously (8, 15, 16). The total lipids thus obtained were dissolved in a minimum of chloroform and the solution was diluted with 10 volumes of acetone and kept at 0°C overnight. After centrifugation to remove the precipitated polar lipids, the supernatant liquid containing the nonpolar lipids (including the red carotenoid pigments) was brought to dryness in vacuo; the residual deep-red gum was stored in acetone solution at 0°C. The yield of the acetone-soluble fraction was 3.6 ± 1.0 mg per g of dry cells, or per liter of culture; yield of the acetone-soluble fraction was 3.6 ± 1.0 mg per g of dry cells, or per liter of culture (about 10% of the total lipids).

**Silicic Acid Column Chromatography**

The acetone-soluble material was fractionated on a column of silicic acid (Unisil, 325 mesh; weight ratio of silica-tetramethylsilane 295); the plates were heat-activated for 2 hr at 120°C. Chromatography was carried out in lined jars by the ascending method, using (A) heptane-benzene 90:10:1 for separation of neutral lipids; and (C) chloroform-acetone-methanol-acetic acid-water 50:43:50:44 ml of total for separation of polar lipids (18). Spots were made visible by exposure to iodine vapor, by charring with H2SO4, or under UV radiation (360 nm). 14C-Labeled spots were detected by radioautography on Kodak X-ray film. The separated components were eluted from the silica gel with chloroform-methanol 9:1, and counted with a thin end-window Geiger-Müller counter.

**Measurement of Spectra**

Visible and UV absorption spectra were recorded with a Cary model 11M spectrophotometer. Infrared spectra were taken on thin films or solutions of the compounds in carbon tetrachloride with an IR-237B Perkin-Elmer spectrophotometer.

The n.m.r. spectra were measured with a model A-60 Varian spectrometer; samples were in carbon tetrachloride solution that contained 1% tetramethylsilane as internal reference standard.

Mass spectra were recorded with a Hitachi RMU-6D mass spectrometer equipped with a direct inlet system set at 370°C, and with an LKB-9000 gas chromatograph—mass spectrometer for monitoring each component emerging from the 2% OV-1 (methyl silicone) column. The ionizing energies used were 70, 20, and 10 ev. Other conditions are described in the Results section. Samples were prepared for mass spectral analyses as described previously (19, 20).

**Gas-Liquid Chromatography**

Samples were analyzed with an F & M 810 gas chromatograph (flame-ionization detector) on a 1.8 m × 3 mm glass column of 2% OV-1 (methyl silicone) on Gas-Chrom P (Applied Science Laboratories, Inc., State College, Pa.); some analyses were performed with a Pye Argon chromatograph on a 1.2 m × 6 mm glass column packed with 10% butanediol succinate polyester on Gas-Chrom A (Applied Science).

The n.m.r. spectra were measured with a model A-60 Varian spectrometer; samples were in carbon tetrachloride solution that contained 1% tetramethylsilane as internal reference standard.

**Measurement of Radioactivity**

14C-Labeled samples were plated on aluminum planchets and counted with a thin end-window Geiger-Müller counter.

**TABLE 1** **Fractionation of Acetone-Soluble Fraction of Lipids from Halobacterium cutirubrum by Column Chromatography**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Solvent</th>
<th>Unlabeled Lipids*</th>
<th>14C-Labeled Lipid †</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Eluate Volume</td>
<td>Weight of Fractions</td>
</tr>
<tr>
<td>I</td>
<td>Heptane ‡</td>
<td>40</td>
<td>3</td>
</tr>
<tr>
<td>II</td>
<td>Benzene</td>
<td>50</td>
<td>45</td>
</tr>
<tr>
<td>III</td>
<td>Chloroform</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>IV</td>
<td>Chloroform-methanol 2:1</td>
<td>50</td>
<td>43</td>
</tr>
<tr>
<td>V</td>
<td>Methanol</td>
<td>60</td>
<td>2</td>
</tr>
</tbody>
</table>

* 202 mg of acetone-soluble lipids were fractionated on a column containing 10 g of silicic acid.
† The acetone-soluble fraction (65.9 × 106 cpm) from 14C-labeled cells grown for 7 days in 1 liter of medium was fractionated on a column containing 2 g of silicic acid.
‡ This fraction was identical in all respects with the heptane fraction of lipid material extracted from the uninoculated culture medium.
A

CULTURE MEDIUM

I II III IV V STANDARDS

B

SQUALENE

VIT K1

CoQ10

STANDARDS

C

SQUALENE

VIT K1

CoQ10

STANDARDS

RESULTS

Fractionation of Acetone-Soluble Lipids

Fractionation of the unlabeled or 14C-labeled acetone-soluble lipids on a column of silicic acid yielded the fractions given in Table 1. Further fractionation of each column fraction by TLC in the neutral lipid solvents A and B and in the polar lipid solvent C gave the results shown in Fig. 1 for the unlabeled lipids and in Fig. 2 for the 14C-labeled lipids. The heptane eluates (fraction I) yielded a colorless oil that amounted to about 3% of the total nonpolar lipids. This...
fraction gave an elongated spot on TLC (Fig. 1), an unresolved multicomponent peak on gas–liquid chromatography (GLC), an infrared spectrum, and a mass spectrum typical of commercial mineral oils (21). It cannot be considered as having been produced by the bacteria, but was probably derived from the culture medium, for the following reasons: (a) the oily material obtained by extraction of the culture medium alone showed the same spot on TLC (Fig. 1) and the same broad unresolved peak on GLC; and (b) the fraction I obtained from 14C-labeled lipids was completely devoid of 14C activity (Table 1; Fig. 2A). Fraction I was therefore not further investigated.

The benzene eluates (fraction II) yielded a yellow-orange oil that represented about one-half by weight of the acetone-soluble lipids, and accounted for about the same proportion of the 14C incorporated into the acetone-soluble lipids (Table 1). TLC of fraction II in solvent A showed the presence of three major components, designated as compounds S, S2, and S4, one of which (compound S) corresponded to authentic squalene; a slower-moving minor component corresponded to authentic vitamin K1. Traces of slow-moving pigments were also detected but none of these corresponded to coenzyme Q (Fig. 1 A and 1 B). Essentially the same pattern was obtained with the 14C-labeled lipids (Fig. 2 A and 2 B). After elution from the plates, compounds S, S2, and S4 accounted for about 95% of the weight of fraction II and about 82% of the 14C in this fraction, whereas the vitamin K-like component accounted for only about 0.5% by weight and contained about 0.5% of the 14C. The yield of squalenes (compounds S, S2, and S4) was calculated to be about 1.5 mg per g of dry cells or per liter of culture, and the yield of vitamin K was about 8 µg per g of dry cells.

The chloroform eluate (fraction III) amounted to only a few percent of the neutral lipids and contained about the same proportion of 14C. This fraction consisted of the same components (S, S2, S4, vitamin K, and pigments) present in fraction II (Figs. 1 and 2), and it was therefore combined with fraction II for further investigation.

The chloroform–methanol eluate (fraction IV) consisted mostly of the red pigments typical of extremely halophilic bacteria (12–14), and accounted for about 43% of the weight and 44% of the 14C in the neutral lipids (Table 1); only traces of phosphatides were present (Figs. 1 and 2). Four discrete red pigments were detected and separated by TLC (Figs. 1 and 2). These pigments were found to have visible spectra similar to those reported (12–14), and were not further investigated.

The methanol eluate (fraction V) amounted to about 2% of the neutral lipids and consisted mostly of polar lipids (the phytanyl diether analogues of phosphatidyl glycerophosphate and phosphatidyl glycerol, and the glycolipid sulfate) and carotenoid pigments (Figs. 1 and 2). The polar components in fraction V appear to be a carryover of acetone-insoluble material from the acetone-precipitation step, and were not further studied.

Identification of Squalene and Hydroxysqualenes

GLC of the benzene fraction II showed three main peaks—S, S2, and S4 (Fig. 3)—corresponding to the three main spots on TLC of this fraction (Fig. 1 A). The retention time of peak S corresponded exactly to that of authentic squalene. From the areas under the peaks
FIG. 3. Gas-liquid chromatographic separation of compounds S, S₂, and S₄ on a column (1.7 m X 3 mm) of 2% methyl silicone on Gas-Chrom P by an F & M gas chromatograph with a flame-ionization detector. Nitrogen inlet pressure, 10 psi; temperature programmed at 8°C per min from 200 to 250°C and held at 250°C.

(Fig. 3), it may be calculated that S, S₂, and S₄ were present in approximatley the same weight ratios, and each component gave a single peak on GLC corresponding to peaks S, S₂, and S₄, respectively, on the chromatogram shown in Fig. 3.

**Mass Spectroscopy.** Mass spectra for compounds S, S₂, and S₄ (Table 2) showed that component S was identical with squalene and that both S₂ and S₄ differed from squalene only in their degree of unsaturation. Both standard squalene (C₃₀H₅₅O) and component S gave parent molecular ions corresponding to M 410. S₂ and S₄, however, had parent molecular ions at M 412 and M 414, respectively. The mass spectra of both compound S and standard squalene (Table 2) showed a base peak at m/e 81, relatively intense doublets at m/e 136 and 137, and fragments at m/e values 341, 367 and 395 corresponding to M-69, M-43, and M-15, respectively. The S₂ component (Table 2) showed a fragmentation pattern similar to that of standard squalene, except that the M-69, M-43, M-15, and parent M ions were two mass units higher. This indicates that compound S₂ has the same structural characteristics as squalene (2,6,10,15,19,23-hexamethyl tetracosa-2,6,10,-

**TABLE 2** **Mass Spectra of Squalenes From H. cutirrorum**

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<th>m/e</th>
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<th>Compound S₄</th>
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* The mass spectra of authentic squalene and compounds S, S₂, and S₄ were obtained with the LKB-9000 mass spectrometer on the components emerging from a 1.7 m X 0.3 cm column packed with OV-1 on Gas-Chrom P. The components were ionized by electron impact at 70 ev. Each peak was scanned in the range of 0-500 mass units.

† Authentic squalene (Eastman Organic Chemicals).

14,18,22-hexaene), but with one double bond less. Similarly, component S₄ has two double bonds less, since its mass spectrum had the M-69, M-43, M-15, and M ions described above but with values 4 and 2 mass units higher than in S and S₂, respectively (see Table 2).

**Nuclear Magnetic Resonance.** The n.m.r. spectra of compounds S, S₂, and S₄ (Fig. 4) were, in general, similar to that of squalene, with signals at 4.91 τ (olefinic H), 7.96 τ (CH₃—CH==C), 8.0 τ (CH₂—CMe==C), 8.32 τ (cis allylic Me), and 8.37 τ (trans allylic Me).
Compounds $S_3$ and $S_4$ show, in addition, signals at 9.11 and 9.21 $\tau$, corresponding to $\text{CH}_3$ groups in saturated hydrocarbons, which are relatively more intense in $S_4$ than in $S_3$.

A silicone band at 9.91 $\tau$ in the spectrum of compound S (Fig. 4) was traced to the silicone antifoam agent added during the cultivation of large quantities of cells.

By comparison of the areas of the peaks at 8.0, 8.32, and 8.37 $\tau$ (allylic methyl) with the areas of the peaks at 9.11 and 9.21 $\tau$ (alkyl methyl) in the spectra of $S$, $S_2$, and $S_4$, the positions of the saturated prenyl units could be estimated. The ratio of allylic methyls:alkyl methyls for $S_2$ was found to be 7:1. This ratio suggests that an internal double bond was saturated, since saturation of a terminal position would give two methyls, resulting in a ratio of 3:1. On the other hand, the ratio of the areas for $S_4$ was found to be 2:1, which is close to the ratio of 5:3 expected when one internal and one terminal prenyl group is saturated; two saturated terminal groups would give a ratio of 1:1, while two internal
saturated groups give a ratio of 3:1. These findings were confirmed by the IR spectroscopy, which showed the presence of an isopropyl group doublet at 1370–1380 cm⁻¹ in the spectrum of S₄ but not in that of S₂.

On the basis of these results it may be concluded that compounds S, S₃, and S₄ are squalene, dihydrosqualene, and tetrahydroxylsqualene, respectively, and that the S₂ has an internal saturated prenyl unit, while S₄ has one internal and one terminal saturated prenyl group.

Identification of the Vitamin K Component

The vitamin K-like component (Fig. 1 A and 1 B) was isolated by preparative TLC of fraction II in solvent B. The UV spectrum of this yellow component in ethanol solution (Fig. 5), showed maxima at 325, 269, 260, 249, and 243 nm, which are characteristic of a 2,3-disubstituted 1,4-naphthoquinone (22). The quinone structure is supported by the disappearance of the quinone absorption bands at 260 and 269 nm after borohydride reduction (Fig. 5).

Infrared Spectrum. Examination of the IR spectrum of the bacterial naphthoquinone (Fig. 6) also revealed a pattern that was typical of the vitamin K-type quinones (23, 24): an intense band at 1665 cm⁻¹ for C=O stretching of the quinone ring; C=C skeletal in-plane vibration of a conjugated aromatic ring at 1598 cm⁻¹; an intense peak at 720 cm⁻¹ due to CH out-of-plane deformation for the four adjacent ring hydrogens in the naphthoquinone nucleus; and unassigned bands at 1295 and 1330 cm⁻¹ characteristic of the quinone group. The bands at 2962 and 2872 cm⁻¹ are associated with C–H stretching frequencies of CH₃ groups and those at 2924 and 2853 cm⁻¹ are C–H stretching frequencies for CH₂ groups; bands at 1445 and 1379 cm⁻¹ are for C–CH₃ and C–CH₂ groups, respectively. A weak carbonyl band at 1740 cm⁻¹ present in this spectrum (Fig. 6) was probably due to an unidentified contaminant.

Mass Spectroscopy. The mass spectrum of the vitamin K-type quinone (Fig. 7) shows a molecular ion at m/e 716. The base peak of m/e 225 corresponds to the dissubstituted 1,4-naphthoquinone with a double bond in the first isoprene unit of the side chain (25). A peak at m/e 701 (M–15) corresponds to the loss of a methyl group. Peaks at m/e 647, 579, 511, 443, 375, 307, and 239 are due to sequential losses of one terminal (69 mass units) and six internal (68 mass units) isoprene groups, respectively (25–27). The mass spectrum thus provides evidence that the polyprenyl side chain is composed of eight unsaturated isoprene units.

Nuclear Magnetic Resonance. The n.m.r. spectrum of this vitamin K (Fig. 8) was very similar to those of other bacterial vitamin K's (24, 26, 28). Signals were present at 2.20 τ for adjacent aromatic hydrogens; 8.0 τ for side chain methylenes; 8.30 τ for the side chain
methyls that are cis to the olefinic hydrogen; 7.8 for the methyl group on C2 in the quinone ring; 8.2 for the trans methyl on the isoprene unit next to the quinone; signals at 8.72 and 9.12 may be due to the unidentified contaminant. On the assumption that the area of the 6.64-6.75 doublet corresponds to two protons (28), we calculated that the 4.92 signal corresponds to about eight protons or eight unsaturated isoprenoid units in the alkyl side chain.

The spectral data on the bacterial quinone thus establish its identity as a menaquinone with eight isoprenoid units in the side chain, or as a vitamin MK8. According to the rules presented by the IUPAC-IUB commission on biochemical nomenclature (29), the bacterial quinone should be named menaquinone-8 (MK-8).

FIG. 7. Mass spectrum of the vitamin K-type quinone. Spectrum was recorded on a Hitachi RMU-6D mass spectrometer, at 70 ev; sample was introduced by a direct inlet system set at 370°C.

DISCUSSION
The fact that no straight-chain hydrocarbon groups have been identified in the acetone-insoluble phospholipid–glycolipid fraction of H. cutirubrum (8) finds no exception in the acetone-soluble neutral lipid fraction. Only polyisoprenoids and their derivatives have been identified, namely squalenes, carotenoid pigments, and a vitamin K-type quinone. In marked contrast to the polar lipids, which contained no unsaturated hydrocarbon chains, all of the compounds in the neutral lipids were olefins.

The three squalenes isolated—S, S2, and S4—were shown to correspond to the fully isoprenoid compound (C30H50), the dihydroisqualen (C26H42), and the tetrahydroisqualene (C22H34), respectively. Although the exact positions of the reduced double bonds were not established, the n.m.r. data suggest that in S2 an internal isoprenoid group is saturated, and that in S4 one terminal and one internal group is saturated. Determination of the exact position of the reduced double bonds in S2 and S4 would help to establish whether these compounds could have a product–precursor relationship, and
whether an orderly stepwise reduction of squalene to perhydrosqualene takes place in cells of *H. cutirubrum*.

In this connection it may be noted that in 3-day-old cultures S₂ comprised about 33% by weight of the total squalenes (see Fig. 3), but in the 7-day ¹⁴C-labeled culture, S₂ accounted for about 50% of the ¹⁴C in the squalenes (see Fig. 2 A). This observation suggests that an increase in saturation of squalene occurs in older cells, but more detailed studies must be carried out.

Another point that would be worth investigating is whether the halophilic bacteria are also capable of desaturating squalene to dehydrosqualene (C₃₀ phytoene), which has recently been found in *Staphylococcus aureus* (30).

Although the menaquinone-8 was the only quinone detected in *H. cutirubrum*, a more detailed study may reveal traces of other vitamin K-type quinones that have partially saturated side chains. Such compounds have been found in other microorganisms (26, 31–38), and their occurrence in *H. cutirubrum* would not be unexpected since other partially saturated polyolefins, e.g., squalenes, are already known.

The relatively high concentration of squalenes (1.5 mg/g of cells) suggests that they may have a structural role as part of the cell membrane, to which they may impart stability. On the other hand, the low concentration of vitamin MK-8 (8 µg/g of cells) is characteristic of quinones involved in electron-transport systems, and this would be its most likely role in an obligate aerobe such as *H. cutirubrum*.

Manuscript received 13 November 1968; accepted 10 February 1969.

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