Isolation and Identification of Echinene from *Micrococcus roseus*

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An orange carotenoid from *Micrococcus roseus* was purified by solvent partitioning followed by column and thin-layer chromatography. Absorption spectra, chromatographic mobility, and partition coefficient suggested that the pigment was echinenone (4-keto-β-carotene). Reduction yielded a pigment with the spectral and polar properties of isocryptoxanthin (4-hydroxy-β-carotene), the expected product. The orange pigment and its reduction product co-chromatographed with the respective authentic pigments, confirming the original pigment as echinenone. To our knowledge echinenone has not been identified previously as a bacterial pigment.

Masses of cells of *Micrococcus roseus* appear orange-pink. The principal colored carotenoid is canthaxanthin (4,4′-diketo-β-carotene) (3), and structures have been suggested for several other pigments (38). The monoketo compound echinenone (4-keto-β-carotene) is generally considered an intermediate between β-carotene and canthaxanthin in animals (14, 17, 20, 25, 29). Therefore, we sought this carotenoid in cells of *M. roseus*.

MATERIALS AND METHODS

In earlier work, *M. roseus* ATCC 516 was cultured on the surface of stock culture agar. In the present work, larger yields of cells were desired so that trace pigments would not be overlooked. Therefore a liquid medium was used which approximated the composition of stock culture agar. Each 11-liter batch contained: beef heart infusion broth, 480 g; protease-peptone, 10 g; gelatin, 10 g; tryptone, 10 g; isoelectric casein, 5 g; sodium chloride, 5 g; disodium phosphate, 4 g; sodium citrate, 2 g; and dextrose, 0.2 g. The pH was adjusted to 6.8. Cultures were incubated in a bench-top fermentor at 28 C aerated at 4,000 cc of air per min. At 72 hr, stationary-phase cells were harvested and washed three times with water.

Cells were extracted repeatedly with methanol under nitrogen in the dark until the pellet remaining after centrifugation was colorless. The combined methanol extracts were partitioned against several portions of petroleum ether (boiling point 30 to 60 C) until the ether phase was colorless. The combined epiphasic petroleum ether phase was concentrated in a rotary evaporator.

The pigment mixture was applied to a column of Silica Gel G: Celite (3:1, w/w). Silica Gel G was obtained from American Optical Corp., Richmond, Calif. Two yellow bands were eluted with petroleum ether and an orange-red band was eluted with 3 to 5% acetone in petroleum ether. The orange-red band was suspected of containing echinenone because of its chromatographic mobility and because its absorption spectrum showed a single broad absorption maximum at 454 to 460 nm.

The crude eluate was concentrated and applied to a thin-layer chromatography (TLC) plate coated with neutral alumina (AG 7, 100 to 200 mesh, BioRad Laboratories, Richmond, Calif.). The plate was developed with 1 to 2% acetone in petroleum ether. Three colored zones were resolved: a yellow pigment, \( R_f \) 0.9; an orange-red pigment, \( R_f \) 0.5; and a red pigment, \( R_f \) 0.1. Preparative TLC was then used to isolate the three pigments.

Spectra were recorded in reagent grade solvents by use of a Bausch & Lomb Spectronic 600 recording spectrophotometer. Partition coefficients were determined in a hexane:95% methanol system as described by Petracek and Zechmeister (32).

Keto groups were reduced to hydroxyl groups with sodium borohydride according to Krinsky and Goldsmith (27). The products were then chromatographed and the products were eluted as before.

Authentic echinenone was a gift of O. Isler, Hoffman-LaRoche, Basel, Switzerland. Isocryptoxanthin (4-hydroxy-β-carotene) was prepared by reducing echinenone with sodium borohydride.

RESULTS AND DISCUSSION

Each of the three pigments eluted from alumina TLC plates had a smooth absorption spectrum with a single maximum. Absorption maxima in several solvents are recorded in Table 1. Sufficient quantities of the yellow and red pigments have not been collected to allow adequate analyses, but the yellow pigment does not appear to have keto functions and the red pigment ap-
TABLE 1. Absorption maxima of pigments eluted from alumina TLC plates

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Absorption maxima in nm</th>
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<tbody>
<tr>
<td></td>
<td>Hexane</td>
</tr>
<tr>
<td>Yellow</td>
<td>454</td>
</tr>
<tr>
<td>Orange</td>
<td>458</td>
</tr>
<tr>
<td>Red</td>
<td>464-466</td>
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</table>

**FIG. 1.** Visible absorption spectra of the orange pigment eluted from TLC plates (solid line) and of the pigment after reduction (dashed line).

pears to be a monoketo carotenoid distinct from echinenone.

The absorption spectrum of the orange pigment (Table 1, Fig. 1) was typical of a chromophore containing 12 conjugated double bonds. Lack of fine structure and the slight asymmetry of the spectrum suggested that the chromophore was extended at one end. The pigment was 95% epiphasic in a hexane: 95% methanol system (Table 2), suggesting that it was a monoketo compound. After reduction the pigment was more polar, typical of a monohydroxy carotenoid (Table 2). The absorption spectrum of the product had considerable fine structure with absorption maxima typical of a 3-carotene chromophore (Fig. 1). The change in shape and the 7-nm decrease in the principal absorption maximum indicate that one conjugated keto group was reduced. Similar properties were observed for authentic echinenone and isocryptoxanthin.

Moreover, the orange pigment and echinenone co-chromatographed as one spot on alumina TLC plates developed with 1 to 2% acetone in petroleum ether. After reduction the products also co-chromatographed as one on alumina plates developed with 3 to 5% acetone in petroleum ether.

Therefore, the orange pigment is identified as echinenone, 4-keto-3-carotene (Fig. 2). Keto carotenoids were isolated from other micrococcii, including a radiation-resistant *Micrococcus* sp. (2), from *M. radiodurans* (35) and from *M. lysodeikticus* (33), but none has properties appropriate to echinenone.

Echinenone has been identified in organisms as varied as blue-green algae (22), green algae (10, 12, 16, 21), euglenoids (27, 36), zooflagellates (31), hydra (28), polychaete worms (30), water mites (4, 5, 7), water fleas (11, 20, 37), various shrimps (9, 13, 14, 18, 25, 26), freshwater copepods (6), pill bugs (29), mosquitoes (8), starfish (15), sea urchins (19), and flamingos (17). It is generally believed that animals cannot synthesize carotenoids de novo. They can metabolize carotenoids, and can insert oxygen functions on carotenoids to form xanthophylls. Therefore, echinenone isolated from animals was probably

**FIG. 2.** Structure of echinenone, 4-keto-3-carotene.

**TABLE 2.** Spectral and polar properties of authentic echinenone and orange pigment from *M. roseus* before and after reduction

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Partition coefficient</th>
<th>Absorption maxima in hexane (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange pigment...</td>
<td>95% Epiphasic</td>
<td>458</td>
</tr>
<tr>
<td>Reduced orange pigment</td>
<td>91% Epiphasic</td>
<td>(425)*, 451, 476</td>
</tr>
<tr>
<td>Echinenone......</td>
<td>95% Epiphasic</td>
<td>458</td>
</tr>
<tr>
<td>Isocryptoxanthin.</td>
<td>91% Epiphasic</td>
<td>(425)*, 449, 476</td>
</tr>
</tbody>
</table>

* Figures in parentheses indicate a shoulder rather than a distinct peak.
ingested and stored, or it was formed by oxidation of carotenoids in the diet. Plants and eucaryotes can synthesize carotenoids and convert them to xanthophylls. Thus, animal carotenoids may originate far down the food chain.

Among bacteria, micrococci (2), mycobacteria (23, 24), and flexibacters (1) contain carotenoids with conjugated keto functions on a β-ionone ring. Canthaxanthin, with two such groups, was isolated from *Corynebacterium michagense* (34) and is the principal pigment of *M. roseus* (3, 38). As far as we are aware, this is the first report of echinone as a bacterial pigment. However, it may be fairly widespread among bacteria because it is regarded as an intermediate in synthesis of more oxygenated pigments (14, 17, 20, 25, 29). A pigment with appropriate chromatographic and spectral properties was isolated from *C. michagense* (34).

**LITERATURE CITED**