The Ketonic Carotenoid Canthaxanthin Isolated from a Colour Mutant of *Corynebacterium michiganense*

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During investigation of the carotenoids produced by mutant strains of the bacterial phytopathogen *Corynebacterium michiganense* (E. F. Smith) Jensen (Saperstein, Starr & Filfus, 1954), a pigment was isolated which resembled canthaxanthin, a carotenoid only recently found by Haxo (1950) in the basidiomycete *Cantharellus cinnabarinus*. The present report summarizes the experimental work that leads to the conclusion that the *C. michiganense* pigment is indeed identical with fungal canthaxanthin. In addition, further chemical and physical properties of the pigment are reported and a likely structure is suggested.

METHODS AND RESULTS

Culture. The orange strain (4938) of *C. michiganense*, one of the colour mutants examined by Saperstein *et al.* (1954), was used for this study. This culture had been isolated by Ark (1951) from a naturally occurring yellow strain which had been treated with uranium sulphate. Both the parent and mutant strains were pathogenic for tomato; the mutant was less virulent than the parent, but did cause definite infection with mild symptoms.

Medium. For mass cultivation of the micro-organism, a medium having the following composition per 100 ml. was used: peptone, 1 g.; yeast extract, 0·5 g.; glucose, 1 g.; the pH was 6·8. The medium, less the glucose, was sterilized by autoclaving for 20 min. at 121°; then the glucose, which had been autoclaved separately for 15 min. at 121° as a concentrated, acidified solution, was added aseptically. The methods employed for growing and collecting the cells and for extracting the mixture of carotenoids were similar to those described in other publications of the writers (Starr & Saperstein, 1953; Saperstein *et al.* 1954).

Chromatographic separation of the carotenoids. A light petroleum solution of the extracted carotenoids, previously dried over anhydrous Na_2SO_4, was chromatographed on a column of a 2:1 (w/w) mixture of magnesia (2641 of Westvaco Chemical Co., Newark, California) and Hyflo Super-cel (Johns-Manville Corp., New York). The chromatogram was developed with 5% (v/v) acetone in light petroleum (b.p. 60–70°). A typical column appeared as follows (the figures denote width of zone in mm.): orange (2), cryptoxanthin; purple (8), canthaxanthin; clear (1); dark red (2), *cis*-canthaxanthin; clear (30); orange (2), β-carotene.

The column was cut and each pigment eluted with 1:1 (v/v) benzene:methanol (cryptoxanthin and β-carotene) or 1:1 (v/v) $CHCl_3$:methanol (canthaxanthin). The carotenoids in the two orange zones were identified as cryptoxanthin and β-carotene (Saperstein *et al.* 1954) and will not be considered further here. The pigments eluted from the purple and red zones were rechromatographed to remove traces of the other pigments.

Characterization of canthaxanthin

Crystallization. The purple carotenoid eluted from the column was crystallized from benzene:methanol. The crystals were dark red to purple in colour and formed thin trapezoidal plates and prisms. Following the third crystallization, the melting point of the crystals, determined with a Fisher-Johns apparatus, was 218° (corr., sealed in capillary under N_2). The yield of this pigment was approximately 2·7 mg./10 g. residual dry wt. of cells.

Analysis. The sample purified by chromatography and crystallized from benzene:methanol as described above was analysed. (Found: C, 85·28, 85·34; H, 9·39, 9·41. Calc. for $C_{40}H_{52}O_2$: C, 85·05; H, 9·28. Calc. for $C_{40}H_{54}O_2$: C, 84·75; H, 9·60%.) Catalytic hydrogenation in 1:1 (v/v) *cyclo*hexane:glacial acetic acid using platinum oxide catalyst indicated the presence of 12 carbon-carbon double bonds.

Colour reactions. When a solution of the pigment in ether was layered over conc. H_2SO_4, a blue-purple colour developed. With 85% H_3PO_4 or with conc. HCl, no colour was observed. A blue colour was obtained with the antimony trichloride reagent (Carr & Price, 1926), suggesting the polyene nature of the pigment.

Partition tests. On partition between 95% (v/v) aqueous methanol and light petroleum, the pigment was distributed almost equally in both phases (slightly more in the hypophase). With 85% (v/v) methanol, the pigment was definitely epiphasic.

Spectral data. A single absorption maximum in the visible region, as determined with the Beckman Spectrophotometer model DU, was located as follows: in CS_2, 501 mμ.; in benzene, 480 mμ.; in hexane, 468 mμ.; in ethanol 477 mμ. When the hexane solution was treated with iodine (in light) to catalyse stereoisomerization, the position of the maximum shifted by approximately 4 mμ. towards the shorter wavelengths. The extinction curve for canthaxanthin in benzene is shown in Fig. 1.

Chromatography. On a column of $Ca(OH)_2$:Hyflo Super-cel (Shell Brand lime, chemical hydrate; 98% through 325 mesh), canthaxanthin is adsorbed from light petroleum below lycopene, but above γ-carotene. Chromatographic homogeneity with an authentic sample of canthaxanthin from *Cantharellus* was shown by the following: after each had first been chromatographed separately on columns of $Ca(OH)_2$, the *C. michiganense* pigment was mixed with the fungal canthaxanthin. The benzene solution of the two carotenoids was percolated through a column of $Ca(OH)_2$:

Hyflo Super-cel (2:1, w/w) and the resulting red band was developed with light petroleum, followed by 8% (v/v) acetone in light petroleum. A single major red band moved down the column preceded by a faint, and much narrower, orange band (see below). A similar mixed chromatogram test was performed by Dr F. T. Haxo at his laboratory; using MgO:Celite columns, he was not able to separate the bacterial carotenoid from the *Cantharellus* pigment.

Provitamin A activity. Canthaxanthin from *C. michiganense* was assayed for provitamin A activity by the 28-day bio-assay of the U.S. Pharmacopoeia XIII. No activity was observed when the carotenoid was given to three groups of fifteen rats each at levels of 1, 2, or 4 μg./day. If one-half of the molecule had the configuration corresponding to vitamin A, detectable activity would have been found at the 1 μg. level. Since the group receiving 4 μg./day was also negative, canthaxanthin can be considered as devoid of provitamin A activity; hence, the compound probably lacks an unsubstituted β-ionone ring.

Examination of the red pigment

The red pigment falling just below canthaxanthin on the MgO chromatogram and appearing orange on a $Ca(OH)_2$ column, gave chemical and physical tests similar to canthaxanthin. The position of its absorption maximum, however, was displaced several mμ. towards the shorter wavelengths compared with canthaxanthin. When a hexane solution of this red pigment was treated with iodine in the light, the position of the absorption maximum shifted towards the longer wavelengths (observed visually with a Zeiss hand spectroscope). Thus, this pigment is probably a *cis* isomer of canthaxanthin. The faint orange band noted on the $Ca(OH)_2$ column when fungal canthaxanthin and the bacterial pigment were chromatographed together, consisted of a pigment which behaved like the red pigment described in this section and undoubtedly is, also, a *cis* isomer of canthaxanthin.

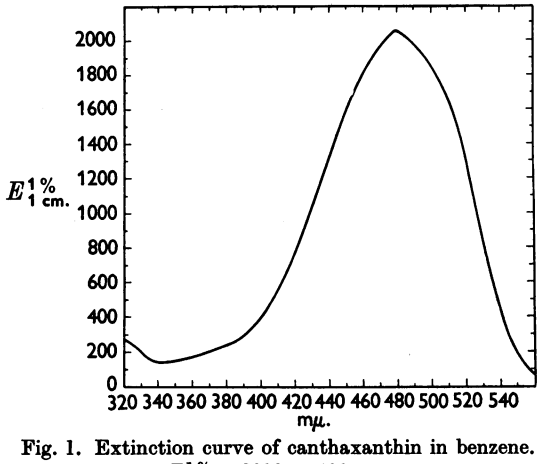


Fig. 1. Extinction curve of canthaxanthin in benzene. $E^{1\%}_{1cm.} = 2090$ at 480 mμ.

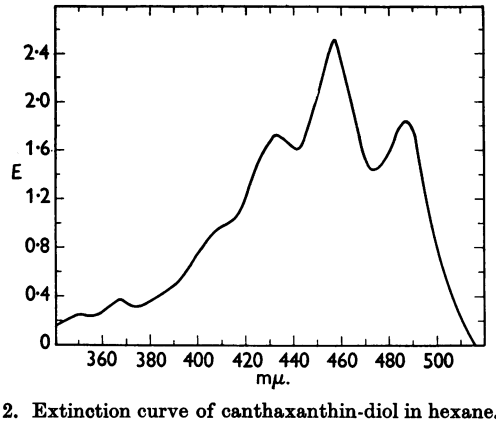


Fig. 2. Extinction curve of canthaxanthin-diol in hexane.

Reduction product

The canthaxanthin obtained from *C. michiganense* was reduced with aluminium *iso*propoxide according to the method of Karrer & Solmssen (1935). The major pigment obtained from this treatment was purified chromatographically on $Ca(OH)_2$ columns. The absorption maxima (black type) and minima of this pigment in hexane, determined with the spectrophotometer, were as follows: **487**, **474**, **457**, **442**, **432**, **374**, **368**, 362 mμ. (Fig. 2). A light petroleum solution of the reduced canthaxanthin, when partitioned over 95% (v/v) aqueous methanol, showed an equal distribution of the pigment between the two phases. When partitioned over 90% (v/v) methanol, the reduced pigment was epiphasic. The new pigment gave a purple colour with conc. H_2SO_4, and a blue colour with 85% H_3PO_4. No colour was observed with conc. HCl. In accordance with recommended nomenclature (Amer. chem. Soc., 1946), this reduction product, presumably a carotenoid alcohol, is named canthaxanthin-diol.

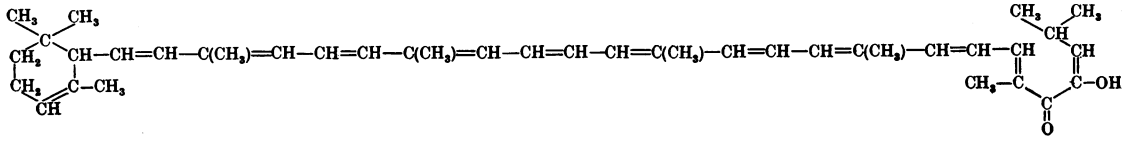
DISCUSSION

The *Corynebacterium* pigment is almost certainly the same as *Cantharellus* canthaxanthin, as indicated by a comparison of our data with those of Haxo (1950), with respect to the following characteristics: melting point, colour reactions with acids, partition behaviour, spectral absorption properties, position on chromatographic columns relative to known carotenoids and properties of *cis* isomers. Furthermore, Dr Haxo and we ourselves have independently been able to demonstrate chromatographic homogeniety of the two pigments.

This study provides a few additional clues to the structure of canthaxanthin. Carotenoids possessing a ketone group attached terminally to a system of conjugated double bonds, such as capsanthin or rhodoxanthin, exhibit an absorption spectrum which generally shows two or more maxima or one maximum and a definite point of inflexion (Karrer & Jucker, 1950). Cross-conjugation of the ketone group results in an absorption spectrum with only one maximum. Reduction of the ketone in the latter instance is thought to yield an alcohol derivative which exhibits an absorption curve having more than one spectral maximum. Of the two other carotenoids known to possess cross-

conjugated ketone groups, astacene and myxoxanthin, alteration of the absorption curve upon reduction of the ketone group has been shown for myxoxanthin only. The results obtained by different experimenters for astacene appear to be contradictory (Goodwin & Srisukh, 1949; Karrer & Würgler, 1943; Kuhn & Sörensen, 1938); however, one might assume that the later studies, employing more accurate instruments, are the more reliable ones.

By analogy with these ketonic carotenoids, it is likely that canthaxanthin is a carotenoid having a carbonyl group cross-conjugated in the system of double bonds. This idea is substantiated by the shape of the absorption curve of reduced canthaxanthin, the relative absorption maxima of the pigment and its reduction product, and the number of carbon-carbon double bonds found by catalytic hydrogenation. Our assumption that the oxygen



atoms are n the 3′ and 4′ positions, rather than distributed one to each end-group, is based on the tenuous evidence that the reduced pigment is not hypophasic in the usual partition tests as are all known polyhydroxy carotenoids having at least one hydroxyl group in each end-group.

Canthaxanthin appears to have the formula $C_{40}H_{54}O_2$ ($\pm$ 2H). Based on the foregoing arguments, the above structure is tentatively assigned to this compound.

Further experimental study is clearly required for more complete elucidation of the constitution of this carotenoid.

SUMMARY

1. The major carotenoid pigment obtained from an orange colour mutant of *Corynebacterium michiganense* is identical with canthaxanthin from the fungus *Cantharellus cinnabarinus*.

2. The compound appears to be a ketonic carotenoid, showing a single absorption maximum in the visible, with a probable formula $C_{40}H_{54}O_2$ ($\pm$ 2H) and a cross-conjugated carbonyl group. A tentative structure is suggested.

3. Reduction of canthaxanthin yields canthaxanthin-diol, a new carotenoid alcohol.

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Uridine Compounds in Glucuronic Acid Metabolism

1. THE FORMATION OF GLUCURONIDES IN LIVER SUSPENSIONS

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Although much interest has been shown in recent years in the biochemistry of glucuronic acid, very few facts can be regarded as firmly established and virtually nothing is known of the mechanisms by which it is synthesized or incorporated into those compounds which contain it. We have accordingly extended earlier work on the synthesis of glucuronides (glucosiduronic acids) by liver slices (Storey, 1950) to broken-cell suspensions, in the hope that a study of this relatively simple process might yield some insight into such problems. In the course of this work we observed that glucuronide formation

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