Carotenoids in the Stick Insect, *Ectatosoma tiaratum* Isolation of β,ε-Caroten-2-ol and β,ε-Caroten-2-one

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2-Oxo-carotenoids, 2-Hydroxy-carotenoids, [^{14}C]β-Carotene, Metabolism, Stick Insects

The carotenoids of the stick insect, *Ectatosoma tiaratum*, were studied by spectroscopic and chemical methods. The β,β-type pigments are β,β-carotene, β,β-caroten-2-one, β,β-caroten-2-ol, β,β-carotene-2,2′-dione, 2′-hydroxy-β,β-caroten-2-one, β,β-carotene-2,2′-diol, 3,4-didehydro-β,β-carotene-2,2′-dione, 2′-hydroxy-3,4-didehydro-β,β-caroten-2-one, and 3,4,3′,4′-tetradehydro-β,β-carotene-2,2′-dione. In addition, the following β,ε-type pigments were identified: β,ε-carotene, β,ε-caroten-2-ol, and β,ε-caroten-2-one. This is the first report on the occurrence of β,ε-caroten-2-ol in an animal and of β,ε-caroten-2-one in nature at all. On treatment with BF_3 in chloroform β,ε-caroten-2-ol is dehydrated to a specific product with the proposed structure of 2,3-didehydro-4,7′-*retro*-β,ε-carotene.

The biogenesis of the β,β-type carotenoids from β,β-carotene is demonstrated by feeding [^{14}C]β-carotene to the insects. Radiolabel was incorporated into all major metabolites of this type. The metabolism of carotenoids in stick insects is discussed applying the "half-molecule substrate" hypothesis to the enzymic transformations of the pigments.

Introduction

In recent studies on insect carotenoids two taxonomically widely different species – the moth, *Cerura vinula* [1, 2], and the stick insect, *Carausius morosus* [3, 4] – were shown to contain β-carotene based pigments with 2-ol and 2-one end rings. Furthermore, from *Carausius* a hitherto unknown type of carotenoids was isolated with 3,4,3′,4′-tetradehydro-β,β-carotene-2,2′-dione as the most outstanding pigment as its chromophore is the longest one of a natural carotenoid so far known. The structure of this red insect carotenoid has been confirmed by comparison with an authentic sample prepared by total synthesis [5]. The other β,β-type carotenoids of *Carausius* display related structures with 2-hydroxy-, 2-oxo-, and 3,4-didehydro-2-oxo-end rings found in nearly all combinations [3, 4]. β,β-Carotene-2,2′-diol is the predominant pigment of this series [6].

A metabolic pathway for the biogenesis of these carotenoids in stick insects has been proposed assuming that the ketones precede the hydroxylated compounds [4]. In this hypothetical scheme β,β-caroten-2-one and β,β-carotene-2,2′-dione are intermediates in the formation of both the hydroxylated pigments and the red 3,4-didehydro-2-oxo-type metabolites. Unfortunately, these two metabolically important carotenoids were found only in trace amounts in *Carausius* and, therefore, their identification is very tentative so far.

In an attempt to find a richer source for the isolation of the two ketones we choose the related stick insect, *Ectatosoma tiaratum*. In this species the females are very big and fat thus facilitating large scale preparation of carotenoids. The present paper deals with the carotenoids in this stick insect. Qualitatively, the pattern of the β,β-type carotenoids is found to be identical with that of *Carausius*; the proportions of the 2-one and the 2,2′-dione are sufficiently high for unequivocal identification. Besides the β,β-type pigments the presence in *Ectatosoma* of β,ε-caroten-2-ol is demonstrated which is the first report in an animal; in addition, the corresponding 2-one could be isolated which has not yet been found in nature before. A brief account on these results has already been given [7].

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Materials and Methods

Insects

Eggs and adults of *Ectatosoma tiaratum* (Phasmatodea, Orthopteroidea) were supplied by Dr. Grimm this university; eggs were also obtained from Dr. Storrer, University of Kaiserslautern. The insects were maintained on bramble leaves (*Rubus fruticosus*) throughout the year and kept under room conditions wih natural illumination. The

insect material was stored in a deep freezer until processing. Heads and viscera were removed prior to pigment extraction from the undried material.

Isolation of carotenoids

The procedures for the isolation of carotenoids were essentially the same as reported in detail in earlier papers [1, 8]. Briefly, the carotenoids were extracted with acetone and acetone/methanol (1:1; v/v) and purified by thin-layer chromatography (TLC) applying a partition system with silica gel-G (Merck; 0.25 mm thick) and an adsorption system with a mixed layer (0.5 mm thick) of $CaCO_3$, MgO, and $Ca(OH)_2$ (30:6:5; w/w/w). In both systems mixtures of petroleum ether (100–140 °C) and propanol-2 were used as solvents. The ratio was varied (100:0.5 to 100:5; v/v) so as to obtain optimal separations. In addition, precoated silica gel layers without gypsum (type Polygram Sil-G; Macherey and Nagel) were used. For mass spectrometry the pigments were finally run on precoated glass plates of ultra pure silica gel type G-25 HR (Macherey and Nagel) after a prewash step with methanol. Development was with *n*-hexane/methanol mixed at a ratio which produced R_f-values between 0.3 and 0.7.

Chemical reactions

Chemical modifications of carotenoids such as saponification, acetylation, treatment with BF_3 in chloroform, and reduction with borohydride ($NaBH_4$) were performed according to the standard procedures [1].

Spectroscopy

Electronic spectra were recorded with a Zeiss DMR 21 spectrophotometer. If not stated otherwise, absorption data refer to acetone solutions of the carotenoids. Mass spectra were routinely obtained on a Varian MAT 711 machine. Electron impact (EI) mass spectra were recorded at 70 eV and 8 kV, field desorption (FD) spectra at 8 kV. The EI mass spectra of the acid product of β,ε-caroten-2-ol were run on a AEI MS902S instrument at 70 eV and 20 eV, respectively. Perfluorokerosene was used as reference in high precision measurements. ^{1}H-NMR spectra were recorded on a Bruker HX-90 instrument by the pulsed Fourier transform (FT) technique. Pigments were dissolved in $CDCl_3$ containing tetramethylsilane (TMS) as an internal standard.

Reference carotenoids

The carotenoids of the 2-hydroxy-, 2-oxo-, and 3,4-didehydro-2-oxo-type from *Carausius* [3, 4] served as reference pigments. β,β-Caroten-2-ol was also isolated from the moth, *Cerura* [1]. Authentic β,ε-carotene-2-ol from the green alga, *Trentepohlia* [9], was provided by Prof. Liaaen-Jensen, University of Trondheim. Synthetic β,β-carotene was a product of Merck. Authentic β,ε-carotene from carrots was obtained from Sigma.

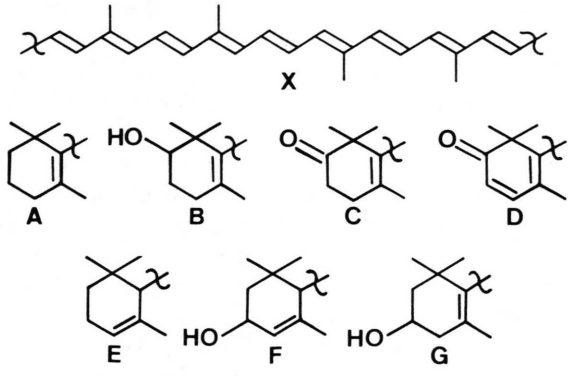
Incorporation of [^{14}C]β-carotene

[15,15′-^{14}C]β,β-carotene with a specific activity of 32 μCi/mg was donated by Hoffmann-La Roche, Basel. The crystalline pigment was repurified by TLC on precoated layers of silica gel and dissolved in olive oil at a concentration of 20 μCi/ml equivalent to 625 μg/ml (*cf.* [10]). The labelled carotene was introduced into the insects by injection of a specified volume of the oil solution through the mouth into the oesophagus without anesthetization. Each insect received 0.2 μCi [^{14}C]β-carotene in 10 μl of oil with the aid of a 50 μl Hamilton microsyringe. The injected insects were starved for two days to avoid rapid loss of the labelled carotene by defaecation. The insects were killed by freezing. The carotenoids were extracted according to the routine procedure. Aliquots were chromatographed on precoated silica gel layers (type Sil G-25 supported on glas plates; 5 × 20 cm; Macherey and Nagel). The chromatograms were developed with a mixture of petroleum ether (100–140 °C) and propanol-2 (96:4; v/v). To record qualitatively the distribution of radioactivity on the chromatograms the plates were scanned with a windowless gas flow counter (scanner system BF 210-23; Berthold and Frieseke) using a 2 × 36 mm slit.

Results

A list of the carotenoids including their structural formulae found in *Ectatosoma* is given in Table I. The identification of these pigments will be presented in the sequence of increasing polarity as shown in the schematic silica gel chromatogram in Fig. 1.

Table I. List of the carotenoids from *Ectatosoma tiaratum*.



No.	Structure	Semisystematic name
I	A-X-A	*β,β*-carotene
Ia	A-X-E	*β,ε*-carotene
II	A-X-C	*β,β*-caroten-2-one
IIa	C-X-E	*β,ε*-carotene-2-one
III	A-X-B	*β,β*-caroten-2-ol
IIIa	B-X-E	*β,ε*-caroten-2-ol
IV	C-X-C	*β,β*-carotene-2,2′-dione
V	C-X-B	2′-hydroxy-*β,β*-caroten-2-one
VI	B-X-B	*β,β*-carotene-2,2′-diol
VII	D-X-C	3,4-didehydro-*β,β*-carotene-2,2′-dione
VIII	D-X-B	2′-hydroxy-3,4-didehydro-*β,β*-carotene-2-one
IX	D-X-D	3,4,3′,4′-tetradehydro-*β,β*-carotene-2,2′-dione
X	G-X-G	*β,β*-carotene-3,3′-diol (zeaxanthin)
Xa	G-X-F	*β,ε*-carotene-3,3′-diol (lutein)

*β,β-Carotene (**I**) and β,ε-carotene (**Ia**)*

The carotenes behave as a single zone on silica gel but separate into two fractions on the adsorption layer. The lower fraction exhibits a *β,β*-type chromophore (*451* and 475 nm; % III/II = 10) and co-chromatographs with synthetic *β,β*-carotene. The upper zone shows a *β,ε*-type electronic spectrum (423, *446*, 474 nm; % III/II = 43) and co-migrates with authentic *β,ε*-carotene. No further work was carried out on these carotenes.

*β,β-Caroten-2-one (**II**) and β,ε-caroten-2-one (**IIa**)*

In the original extract these compounds migrate between the diester fraction of 2,2′-diol (**VI**) and the ester(s) of 2′-hydroxy-2-one (**V**). After saponification the unchanged pigments run between the carotenes (**I**, **Ia**) and 2,2′-dione (**IV**). The two pigments are not separated on silica gel but split into the two structural isomers on the adsorption plate. The lower fraction is predominant, exhibiting the spectrum of *β,β*-carotene (*451* and 476 nm). A molecular weight of 550 is obtained by FD mass spectrometry indicating a mono-oxo carotene ($C_{40}H_{54}O$). This is confirmed by reduction with borohydride. The product exhibits increased polarity and is inseparable from authentic *β,β*-caroten-2-ol on both the partition and the adsorption plate after multiple development (*cf.* [1]). The upper fraction of the native mixture shows maximal absorbance at 423, *446*, and 475 nm (% III/II = 59) indicating the *β,ε*-chromophore. After reduction the product co-chromatographs with authentic *β,ε*-caroten-2-ol in both TLC systems. The electronic spectra do not change on borohydride reduction in either pigment. Conclusively, the two mono-ketones are *β,β*-caroten-2-one and *β,ε*-caroten-2-one. Small amounts moving ahead of the principal zones on the adsorption layer are probably *cis*-isomers as judged from the presence of a *cis*-peak, a hypsochromic shift of 4 nm, and loss of fine structure in the electronic spectra (*e. g.* % III/II = 29 in **IIa**).

*β,β-Carotene-2,2′-dione (**IV**)*

This carotenoid runs ahead of the mono-ol fraction. Two zones are produced on re-chromatography on silica gel the upper of which corresponds to an all-*trans* *β,β*-type pigment (*452* and 478 nm;

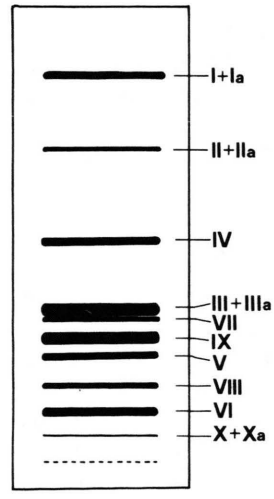


Fig. 1. Schematic silica gel thin-layer chromatogram of a saponified carotenoid extract from *Ectatosoma tiaratum* (females with eggs). For identification of carotenoids see Table I. Solvent: petroleum ether (100–140 °C)/propanol-2 (100:3; v/v), developed twice.

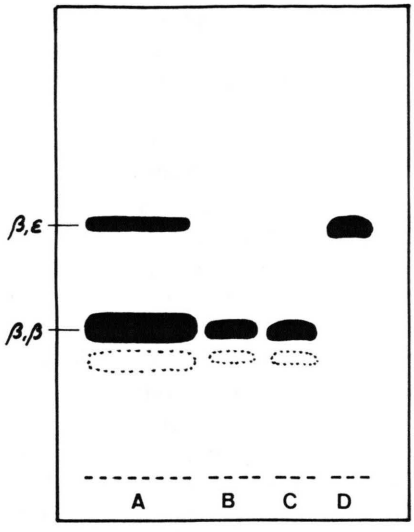


Fig. 2. Identification of β,β-caroten-2-ol (β,β) and β,ε-caroten-2-ol (β,ε) *ex Ectatosoma* (A) by adsorption thin-layer chromatography of the 2-ol fraction from the silica gel chromatogram (**III** + **III a** in Fig. 1). Reference samples: β,β-caroten-2-ol *ex Carausius* (B) and *ex Cerura* (C), β,ε_3-caroten-2-ol *ex Trentepholia* (D). Dotted spots: *cis*-isomers of β,β-caroten-2-ol.

% III/II = 13), the smaller lower zone is probably a *cis*-isomer (*448* and 474 nm; *cis*-peak at 356 nm). No structural isomer of the β,ε-type can be found. Mass spectrometry revealed a molecular weight of 564 corresponding to $C_{40}H_{52}O_2$ according to high precision measurements. Elimination of toluene (M-92), observed at *m/e* 472, is typical for carotenoid mass spectra [11]. Reduction with borohydride proceeds as a two-step reaction yielding a polar final product which behaves identical to β,β-carotene-2,2′-diol in all TLC systems used, thus confirming the structure of β,β-carotene-2,2′-dione for the original pigment.

*β,β-Caroten-2-ol (**III**) and β,ε-caroten-2-ol (**III a**)*

The two mono-ols behave as a single fraction on silica gel but separate into the β,β- and β,ε-type isomers on the adsorption layer (Fig. 2). Maximal light absorbance is found at *452* and 479 nm (% III/II = 20) for the stronger adsorbed pigment, and at 423, *446*, and 475 nm (% III/II = 60) for the weaker adsorbed zone (Fig. 3). The mass spectra of both carotenoids are largely identical to each other with prominent molecular ions at *m/e* 552 (M^+) as expected for mono-hydroxy carotenes. The fragments at *m/e* 460 (M-92) and *m/e* 446 (M-106) correspond to losses of toluene and xylene, respectively [11]. The intensity ratio of M-92 to M-106 is 6.7 in the β,β-type and 10.5 in the β,ε-type pigment, which are both in the range observed for nine conjugated double bonds in the polyene chain [12]. Elimination of water is observed at *m/e* 534 (M-18) confirming the presence of one hydroxyl group in each compound. Exclusively in the spectrum of the β,ε-type pigment (Fig. 4) prominent fragment ions are found at *m/e* 496 (M-56) and *m/e* 404 (M-92-56). A loss of 56 m. u. is reported to be typical for a cleavage of the ε-end ring arising from a *retro*-Diels-Alder rearrangement [12, 13].

The structure of the β,β-2-ol is confirmed by its FT-^{1}H-NMR spectrum obtained after application of 71 8000 pulses. This spectrum is basically identical to those of β,β-caroten-2-ol from *Trentepohlia* [9] and from *Cerura* [1] showing a characteristic double doublet at 3.55 ppm (methine proton at C-2) and splitting of the signal of the *gem*-dimethyl group at C-1 into peaks at 1.02 and 1.08 ppm. Other relevant signals were observed at 1.71 ppm (end-of-chain methyls at C-5,5′) and 1.97 ppm (in-chain methyls).

Chemically, the presence of one hydroxyl group is demonstrated by the formation of a mono-acetate in

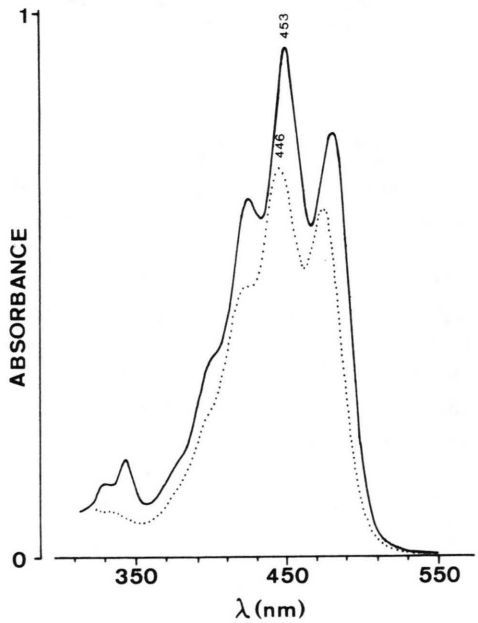


Fig. 3. Electronic spectra of β,ε-carotene-2-ol *ex Ectatosoma* (dotted line: 446 nm; in acetone) and of its product obtained by BF_3-chloroform treatment (full line: 453 nm; in *n*-hexane).

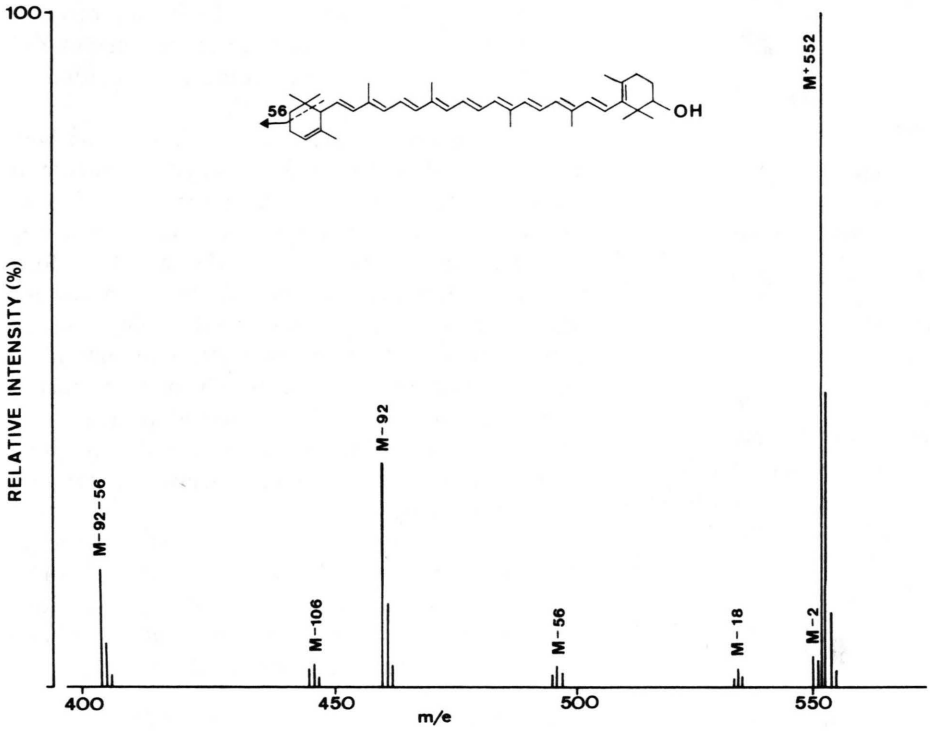


Fig. 4. Mass spectrum (70 eV; 8 kV; 190 °C) of β,ε-caroten-2-ol *ex Ectatosoma.*

both carotenols. This reaction was used to separate quantitatively these mono-ols from the two diketones **IV** and **VII** (*cf.* Fig. 1) in large scale work. Identity of the β,β-type pigment with β,β-caroten-2-ol and of the β,ε-type with β,ε-caroten-2-ol is further confirmed by co-chromatography with authentic pigments from *Carausius, Cerura,* and *Trentepohlia,* respectively (Fig. 2). Some *cis*-isomers run slightly ahead of the main fractions on the adsorption layer and on silica gel HR (*cis* β,β-2-ol: *447* nm; *cis*-peak at 339 nm; *cis* β,ε-2-ol: 442 nm; *cis*-peak at 332 nm). The β,β-2-ol (**III**) is further identified by the dehydrogenation reaction and *retro* rearrangement upon treatment with BF_3 in chloroform found to be specific for this isomer [1]. The product of the *Ectatosoma* pigment shows a *retro*-shaped electronic spectrum with maxima at 350, 366, 432, *456,* and 485 nm (% III/II = 31) and co-chromatographs with the product (4′,5-*retro*-β,β-caroten-2-one) obtained from β,β-2-ol ex *Carausius* by the same treatment (*cf.* [3]).

When β,ε-2-ol (**III a**) from *Ectatosoma* is treated with BF_3 in chloroform a product is obtained which displays a chromatographic behaviour intermediate between the corresponding product of β,β-2-ol (**III**) and the two carotenes (**I, I a**) on both the partition and the adsorption plate (Fig. 5). Authentic β,ε-2-ol from *Trentepohlia* when subjected to the same treatment yields an identical product as judged from chromatographic and spectral properties. The electronic spectrum of this product (Fig. 3) exhibits pronounced fine structure with maxima in acetone at ~ 334, 347, 431, *455,* and 487 nm (% III/II = 49), in *n*-hexane at 333, 346, 428, *453,* 483 nm, and in chloroform at ~ 340, 353, 440, *465,* 498 nm. This spectrum is very similar to that of the product of β,β-2-ol (*cf.* [1]) differences refer mainly to the fine structure in the UV. In the EI mass spectrum of the product (Fig. 6) a prominent molecular ion is found at *m/e* 534.4209 (M^+) with a mass formula of $C_{40}H_{54}$ demonstrating elimination of H_2O on BF_3-treatment. Fragment ions at *m/e* 455 (M-79), 442 (M-92), 428 (M-106), and 376 (M-158) are due to extrusions of a C_6H_7 fragment, toluene, xylene, and a $C_{12}H_{14}$ fragment, respectively, from the polyene chain as commonly observed in carotenoid mass

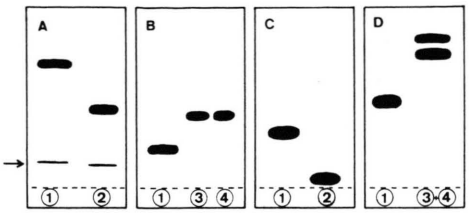


Fig. 5. Chromatograms on silica gel-G (A, B) and on the adsorption layer (C, D) of the BF_3-chloroform product of β,ε-caroten-2-ol (1) and β,β-caroten-2-ol (2), respectively, both from *Ectatosoma.* (3) β,ε-carotene, (4) β,β-carotene. Arrow in (A) points to the unchanged 2-ols.

spectra [11]. The ions at *m/e* 478 (M-56) and *m/e* 386 (M-92-56) arise from a *retro*-Diels-Alder cleavage of the ε-end ring and are of diagnostic value [11, 13]. The fragment at *m/e* 411 (M-123) is attributed to a rupture of the 6′,7′-single bond. No other fragmentations of the polyene chain are observed. The intensity ratio of the eliminations of toluene to xylene (M-92/M-106) is 5.9 which is within the range observed for nine conjugated double bonds in the polyene chain [12]. In the 20 eV spectrum the only prominent ion is the molecular ion at *m/e* 534. To summarize the MS data, the product obtained from β,ε-2-ol by acid treatment is a didehydro-derivative of β,ε-carotene. The elimination of water during the reaction is in contrast to the behaviour of the isomeric β,β-2-ol undergoing an oxidation of the hydroxyl to an oxo-group [1].

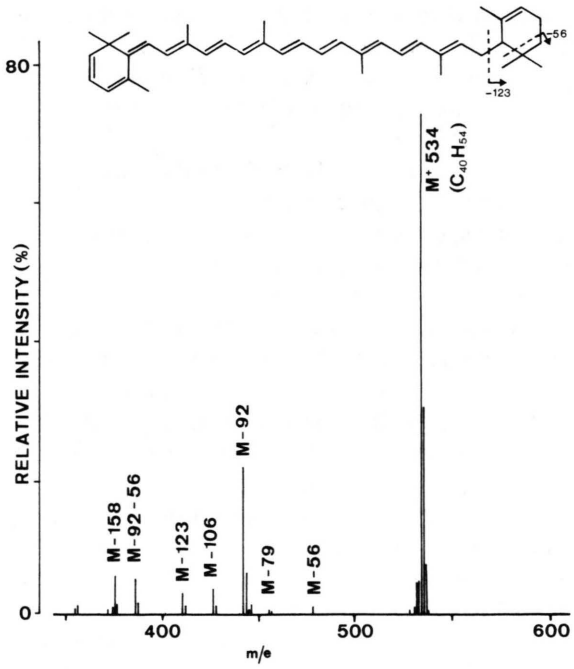


Fig. 6. Mass spectrum (70 eV; 8 kV; 185 °C) of the BF_3-chloroform product of β,ε-caroten-2-ol.

For the didehydro-derivative of β,ε-carotene both a 2,3-didehydro- and a 3,4-didehydro-structure is ruled out from the electronic spectrum (*cf.* Fig. 3). A *retro* shift of the chromophore towards the ε-ring would be inconsistent with the MS data. Therefore, a *retro* rearrangement of the polyene chain towards the β-end ring must be presumed joining the 2,3-double bond (introduced by water elimination) to the chromophoric system which now comprises eleven double bonds. The observed principal absorbance peak (455 nm in acetone) is in full agreement with a *retro* carotenoid possessing a chromophore of this length [2].

Conclusively, the structure of the BF_3-product of β,ε-2-ol is tentatively assigned to 2,3-didehydro-4,7′-*retro*-β,ε-carotene. Preliminary PMR data at least confirm the presence of a conserved ε-ring and hence are in favour of the presumed structure.

*3,4-Didehydro-β,β-carotene-2,2′-dione (**VII**)*

This is a red pigment with a broad absorption maximum at 474–476 nm. In the mass spectrum the molecular ion is found at *m/e* 562 with a composition of $C_{40}H_{50}O_2$. Besides the ion at *m/e* 470 (M-92) no other significant fragments are found. The properties of the compound are not affected by saponification or acetylation, however, on reduction with $NaBH_4$ a more polar compound is produced co-migrating with β,β-carotene-2,2′-diol on silica gel. On the adsorption plate this product zone is split into two fractions the upper of which is identical with β,β-2,2′-diol on the basis of co-chromatography and electronic spectrum. The lower predominant fraction exhibits an asymmetrical absorbance peak at 461 nm demonstrating the chromophoric system of 3,4-didehydro-β,β-carotene [4]. The hypsochromic shift of ca. 14 nm indicates that one of the keto groups is conjugated to the polyene chain. Furthermore, the main reduction product is not separable from 3,4-didehydro-β,β-carotene-2,2′-diol obtained as a side reduction product of the tetradehydro-dione (**IX**) from *Carausius* [4] thus confirming the didehydro-dione structure for **VII** from *Ectatosoma.* Moreover, the native pigment from *Ectatosoma* co-chromatographs with the corresponding *Carausius* carotenoid.

3,4,3′,4′-Tetradehydro-β,β-carotene-2,2′-dione (**IX**)

This principal red pigment co-migrates with the corresponding carotenoid from *Carausius* in both the partition and the adsorption system. Its electronic spectrum with a single peak at 491 nm is also identical with that of the *Carausius* pigment (*cf.* [4]). The mass spectrum shows a prominent molecular ion at *m/e* 560 (M^+) corresponding to $C_{40}H_{48}O_2$. In the higher mass region fragments are observed at *m/e* 545 (M-15) and *m/e* 468 (M-92). The pigment is readily reduced with borohydride to a product with a polarity very similar to that of 2,2′-diol on silica gel. On the adsorption plate this product zone is split into three fractions which co-chromatograph with those obtained from the *Carausius* pigment. In the sequence of increasing R_f-values these products are (*cf.* [4]): 1) 3,4,3′,4′-tetradehydro-β,β-carotene-2,2′-diol (symmetrical absorption peak at 470 nm), 2) 3,4-didehydro-β,β-carotene-2,2′-diol (asymmetrical peak at 461 nm), and 3) β,β-carotene-2,2′-diol (β,β-type spectrum: *451* nm). Compounds 1 and 2 are identical with the reduction products of the didehydro-dione **VII** from *Ectatosoma.*

*2′-Hydroxy-β,β-caroten-2-one (**V**)*

This carotenoid displays a β,β-type electronic spectrum (*451* nm; % III/II = 20) and can be completely separated from the red pigment **IX** either by adsorption TLC or by acetylation. For the peracetylated compound a prominent molecular ion is found at *m/e* 608 corresponding to $C_{42}H_{56}O_3$. The fragment at *m/e* 548 (M-60) is due to loss of one molecule of acetic acid from the molecular ion which consequently is assigned to be a mono-acetate. On this basis for the parent carotenoid a molecular weight of 566 and a mass formula of $C_{40}H_{54}O_2$ can be calculated. The presence of one keto group is demonstrated by reduction with borohydride yielding a product identical with β,β-carotene-2,2′-diol as judged from its behaviour on co-chromatography and acid treatment (see below for **VI**).

*2′-Hydroxy-3,4-didehydro-β,β-caroten-2-one (**VIII**)*

The chromophore of this red pigment is identical with that of the didehydro-dione **VII** (474–476 nm). The presence of one hydroxyl group is established by the formation of a mono-acetate. For the native pigment the molecular ion is found at *m/e* 564 and a mass formula of $C_{40}H_{52}O_2$ is calculated for it. On reduction with borohydride two products can be separated by adsorption TLC which are identical with those of the fully reduced didehydro-dione **VII** on the basis of chromatography and electronic spectra. Thus, one conjugated keto group is present in addition to the hydroxyl. Furthermore, identity of **VIII** from *Ectatosoma* with that from *Carausius is* also established by co-chromatography of the native pigments.

*β,β-Carotene-2,2′-diol (**VI**)*

Chromatographic identity of this *Ectatosoma* pigment with the corresponding one from *Carausius* is shown in both TLC systems. The electronic spectrum is of the β,β-type (*452* and 478 nm; % III/II = 22). A minor fraction, more strongly adsorbed, is a *cis*-isomer (*448* and 474 nm; % III/II = 10; *cis*-peak at 340 nm). A molecular weight of 568 is found by mass spectrometry corresponding to $C_{40}H_{56}O_2$. The mass spectrum is superimposable to that obtained with the 2,2′-diol from *Carausius* (*cf.* [3]). The fragments at *m/e* 476 (M-92), *m/e* 462 (M-106), and *m/e* 410 (M-158) are due to the common losses (*cf.* [11]). The ratio of M-92/M-106 is 11. Elimination of one molecule of water is observed at *m/e* 550 (M-18) with an intensity of only 5% of that of the parent ion. The presence of two hydroxyl groups is however clearly confirmed by the formation of a diacetate. Final evidence on the structure of β,β-carotene-2,2′-diol is obtained from the FT-^{1}H NMR spectrum (77 300 pulses). The two singlets at 1.04 and 1.08 ppm of equal intensity (*gem*-dimethyls) and the (badly resolved broad) peak at ~ 3.5 ppm (H-2,2′) are of diagnostic value (*cf.* [9]). Other prominent singlets are at 1.71 ppm (end-of-chain methyls), 1.96 ppm (in-chain methyls), and 6.11 ppm (H-7,8,7′,8′) [9]. Treatment of the native diol with BF_3 in chloroform resulted in a product of lower polarity exhibiting a *retro*-shaped electronic spectrum (335, 351, 418, *442*, and 471 nm; % III/II = 44). This product is identical with that of 2,2′-diol from *Carausius* for which the structure of 4,5-dihydro-4,5′-*retro*-β,β-carotene-2,2′-dione has been assigned [3].

*β,β-Carotene-3,3′-diol (zeaxanthin, **X**) and β,ε-carotene-3,3′-diol (lutein, **Xa**)*

On the silica gel layer these two pigments represent the most polar zone which separates into the

two isomeric diols on the adsorption plate. The upper zone co-migrates with authentic lutein and also displays its chromophore (*445* nm; % III/II = 50). The lower zone is tentatively identified with zeaxanthin (*450* nm). These diols could not be further studied due to the very limited amounts available.

Carotenoid biogenesis in Ectatosoma

The most probable precursor of the oxidized β,β-type carotenoids found in the stick insects is β-carotene which must be sequestered from the food (*cf.* [4]). To confirm the hypothesis that dietary β-carotene is converted to this series of metabolites within these insects [^{14}C]β-carotene was fed to *Ectatosoma* larvae of the last instar as described in Methods. After ten days the carotenoids were extracted and the chromatograms subjected to radioscanning. According to the scans of the plates with the original extract, as shown in Fig. 7 A, the bulk of radioactivity is associated with the fast moving fraction consisting of a non-resolved mixture of the carotenes **I** and **Ia**, the diester(s) of 2,2′-diol **VI** and the ester(s) of the 2-ols **III** and **IIIa**. After saponification of the total extract, however, only little radioactivity remains with the carotene zone (Fig. 7 B); most of the label is clearly correlated with the 2-ol fraction and with 2,2′-diol. No attempt was made to separate the β,β-2-ol from the isomeric β,ε-2-ol since the β,β-type predominates [6]. Radioactivity is also correlated with tetradehydro-dione (**IX**) and 2′-hydroxy-2-one (**V**) (Fig. 7 B) which can not be sufficiently separated on silica gel. Therefore, the combined zones were scraped off from the plate and, after acetylation, the acetate **V′** of the hydroxy-ketone **V** is separated from the unchanged red diketone **IX** on silica gel. It is clear from the radioscan, shown in Fig. 7 C, that radioactivity is incorporated into both pigments the tetradehydro-dione **IX** and the hydroxy-ketone **V**.

It is remarkable that nearly all of the labelled carotene has disappeared after the ten days period (*cf.* Fig. 7 B). This demonstrates both a rapid absorption in the gut of the supplied precursor and its subsequent transformation into the other β,β-type carotenoids as demonstrated at least for the major pigments of *Ectatosoma.* The carotenoids of the β,ε-type are supposed not to be derived from β,β-carotene **I** but from diet-derived β,ε-carotene **Ia** the presence of which in this species is shown in this paper. When the labelled carotene was given to egg-laying females no transfer of radioactivity from the adult insect to the deposited eggs could be observed during a period of five weeks. However, incorporation of radioactivity into the β,β-type carotenoids of the adult insect is clearly established in these specimens demonstrating that the labelled carotene had been absorbed in the gut.

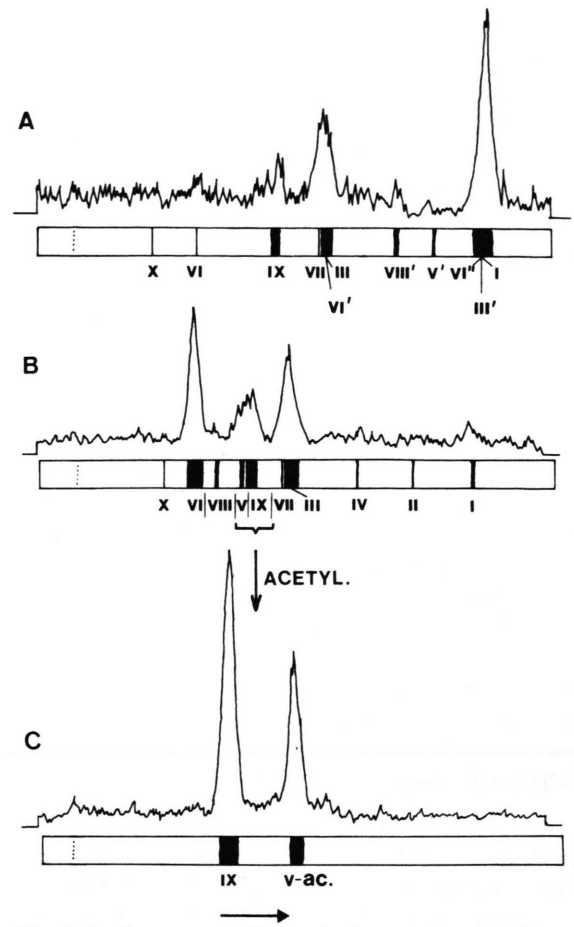


Fig. 7. Radioscans of silica gel chromatograms of carotenoids from *Ectatosoma* extracted ten days after feeding [^{14}C]β-carotene in olive oil. Total extract before (A) and after (B) saponification. C – mixed zone of **V**+**IX** from B after acetylation. For identification of carotenoids see Table I. Primed numbers refer to esters of the corresponding carotenoids. Arrow indicates direction of solvent flow.

Discussion

As demonstrated in this study the β,β-type carotenoids of *Ectatosoma tiaratum* are identical to those of the related species, *Carausius morosus* (*cf.* [3, 4]).

On the other hand, no carotenoid of the β,ε-type (except of lutein) is present in *Carausius* [4], whereas in *Ectatosoma* β,ε-carotene and its 2-hydroxy- and 2-oxo-derivatives could be unequivocally identified. Up to now, β,ε-caroten-2-ol has been isolated only from the green alga, *Trentepohlia iolithus* [9], so the demonstration of its presence in *Ectatosoma* is the first report in an animal at all. The natural occurrence of the oxo-derivative, β,ε-caroten-2-one, has not been reported before.

In previous studies on the chemical behaviour of β,β-caroten-2-ol and β,β-carotene-2,2′-diol these carotenoids have been shown to undergo a specific oxidation on treatment with BF_3 in chloroform yielding *retro*-oxo-compounds which are easily recognized by their electronic spectra [1, 3, 14]. It is interesting to see that in the case of the isomeric β,ε-caroten-2-ol the hydroxyl is not oxidized but eliminated resulting in a product with a chromophore very similar to that of the β,β-2-ol derivative but with a markedly lower polarity on chromatography. So, the identification of the two mono-ols with isomeric end rings does not require their prior separation, thus providing a specific method for their discrimination on micro-scale when NMR spectrometry is not possible and authentic reference samples are not available.

The present study has unequivocally demonstrated the occurrence in *Ectatosoma* of β,β-caroten-2-one (**II**) and β,β-carotene-2,2′-dione (**IV**) by mass spectrometry and chemical reactions. These carotenoids could only tentatively been identified during previous work on *Carausius* [4] due to their low proportions. The relative importance of these two ketones is based on a suggested pathway of carotenoid biogenesis in the stick insects [4] according to which the apparent end product 3,4,3′,4′-tetradehydro-β,β-carotene-2,2′-dione is directly synthesized *via* the 2-one **II**, 2,2′-dione **IV** and 3,4-didehydro-2,2′-dione **VII**; the hydroxylated carotenoids are presumed to be reduction products of the corresponding keto compounds.

Dietary β,β-carotene has been suggested to be the precursor for the various 2-hydroxy- and 2-oxo-carotenoids with two β-end rings in *Carausius* [4]. This has been confirmed in the present study for the related stick insect, *Ectatosoma*, by demonstrating the incorporation of radiolabelled β,β-carotene into at least the major carotenoids in this species. β,ε-Caroten-2-ol and the corresponding 2-one are supposedly not derived from β,β-carotene, which would require an isomerization, but most probably from dietary β,ε-carotene which is in fact present in *Ectatosoma* as shown here. This oxidation process is thought to follow the same pattern as presumed for the β,β-type carotenoids [4]. The transformation of radiolabelled β,β-carotene into 2-hydroxy- and 2-oxo-carotenoids has now also been firmly established in *Carausius* and will be reported in a following paper [15].

It is not essential to postulate different enzymes for the attack of β,β-carotene and β,ε-carotene, respectively, if one applies the concept of "half-side" reactivity for the carotenoid biosynthesis in plants, as outlined by Britton [16], to carotenoid transformations in insects. This means that the substrate of the enzyme is a carotenoid half-molecule (comprising one β-end ring) rather than the entire compound including possibly an ε-ring to which the enzymes of *Ectatosoma* obviously do not exhibit any reactivity. This "half-molecule substrate" hypothesis greatly simplifies the enzyme set expected to be necessary for the formation of the numerous carotenoids in the stick insects and rationalizes the network of their suggested transformations.

Note added in proof: The *retro*-products of the 2-hydroxy β,β-type carotenoids have now been shown not to possess 2-oxo groups as initially proposed [14] but 2,5-oxygen bridges [K. Aareskjold, H. Kayser, and S. Liaaen-Jensen, Tetrahedron Letters, in press].

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