

Identification of β , β -Caroten-2-ol and β , β -Carotene-2,2'-diol in the Stick Insect, *Carausius morosus* Br.; a Reinvestigation Study

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β , β -Caroten-2-ol, β , β -Carotene-2,2'-diol, Acid Catalyzed Dehydrogenation, *retro* Rearrangement, Insect Carotenoids

Two major carotenoids of the stick insect were reinvestigated and shown to be β , β -caroten-2-ol and β , β -carotene-2,2'-diol and not isocryptoxanthin (β , β -caroten-4-ol) and isozeaxanthin (β , β -carotene-4,4'-diol). Both pigments are esterified with fatty acids. The identification is based on co-chromatography with authentic 2-, 3- and 4-isomers of the mono- and dihydroxy pigment, mass spectra, and chemical behaviour. In acid solution β , β -carotene-2,2'-diol is specifically dehydrogenated and rearranged to ketones with *retro* structures in analogy to the reaction of β , β -caroten-2-ol as recently reported. The final product of the diol is 4,5-dihydro-4,5'-*retro*- β , β -carotene-2,2'-dione.

This is the first demonstration of β , β -carotene-2,2'-diol in an animal.

Introduction

Attention to the carotenoid metabolism of the stick insect, *Carausius morosus*, was drawn from the discovery of an obviously novel keto carotenoid by Willig¹, who also identified the hydroxylated pigments of this phasmid as isocryptoxanthin (β , β -caroten-4-ol) and isozeaxanthin (β , β -carotene-4,4'-diol). However, during own work on the structure of the keto compound, which exhibited oxygen functions at positions C-2 and C-2'^{2,3}, the need arose to reexamine the hydroxy carotenoids.

From a biogenetical viewpoint, based on the metabolic interrelation between both hydroxy and keto compounds, all members of such a pathway should belong to the same substitution type. Indeed, the two hydroxylated carotenoids were found not to be β , β -caroten-4-ol and β , β -carotene-4,4'-diol, but the corresponding isomeric compounds β , β -caroten-2-ol and β , β -carotene-2,2'-diol. This identification is based on chromatographic, chemical and mass spectroscopic evidence. Recently, the 2-ol has been isolated in large quantities from an insect for the first time by Kayser⁴. Now, this is the first report on the occurrence of the diol in an animal after its discovery in a green alga by Kjosen *et al.*⁵.

Material and Methods

Insects

Stick insects (*Carausius morosus* Br., Phasmidae, Orthopteroidea) were obtained from the laboratory

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stock, which is maintained on leaves of *Hedera helix* throughout the year. Insects were killed by vapour of diethyl ether or by freezing. The viscera, but not the ovaries, were removed prior to extraction.

Isolation of carotenoids

Carotenoids were extracted from lyophilized insects and purified by thin-layer chromatography (TLC) first in a partition and subsequently in an adsorption system as reported in preceding papers^{4,6,7}. Saponification was employed on total extracts or on separated zones using either 3% Na-methylate in methanol (w/w) or 5% KOH in methanol (w/w) for at least 24 h at room temperature.

Chemical reactions

Experimental details on acetylation, acid treatment, Oppenauer oxidation, and hydride reduction have been presented otherwise^{4,6,7}.

Spectroscopy

Electronic spectra were recorded on a Zeiss DMR21 spectrometer. The specific extinction coefficients of $E_{1\text{cm}}^{1\%} = 2290$ for β , β -caroten-2-ol and $E_{1\text{cm}}^{1\%} = 2060$ for β , β -carotene-2,2'-diol were adopted from the corresponding algal pigments⁵. — Routine mass spectra were recorded on a Varian MAT CH5 instrument at 70 and 12 eV using the direct inlet system. High precision measurements were done on a AEI MS902S spectrometer with perfluorokerosene as reference. Pigment samples had been purified as described⁶.

Reference carotenoids

Hydroxy compounds: β , β -Caroten-3-ol (cryptoxanthin) and β , β -carotene-3,3'-diol (zeaxanthin)

were isolated from *Capsicum* and *Physalis*, respectively. β,β -Caroten-4-ol (isocryptoxanthin) and β,β -carotene-4,4'-diol (isoeaxanthin) were obtained by NaBH_4 reduction of the corresponding ketones β,β -caroten-4-one (echinenone) and β,β -carotene-4,4'-dione (canthaxanthin), which were synthetic products of Hoffmann-La Roche (Basel). Authentic β,β -caroten-2-ol was isolated from the moth *Cerura vinula*⁴.

Retro compounds: 2,3,4',5'-Tetrahydro-4,5'-*retro*- β,β -carotene was synthesized by N-bromosuccinimide (NBS) treatment of synthetic β,β -carotene (Merck)⁸. 3 mol of NBS were used per mol pigment. The purified product exhibited visible absorption maxima at ≈ 404 , 462, 485, and 515 nm in acetone (% III/II = 19), which were in close agreement with those reported in the literature (460, 487, 518 nm in hexane)⁹. — 4',5'-Didehydro-4,5'-*retro*- β,β -carotene was obtained by BF_3 -chloroform treatment⁴ of borohydride reduced 3',4'-didehydro- β,β -caroten-4-one, which had been isolated from the NBS reaction mixture. The electronic spectrum showed maxima at ≈ 380 , 446, 471, and 500 nm in acetone (% III/II = 26), which coincided with published values (382.5, 445, 470, 501 nm in hexane)¹⁰. — 4,5'-Dihydro-4,5'-*retro*- β,β -carotene-4,4'-dione was made from canthaxanthin¹¹ by reduction with zinc powder in a solution of pyridine and acetic acid (10:3; v/v)¹². The reaction was carried out at 50 °C for 5 min. The absorption maxima of the product were at 371.5, 421, 445, and 474 nm in acetone (% III/II = 40). The chromophore of a second product, more polar than the former, was shortened by one double bond as judged from its spectral properties ($\lambda_{\text{max}} = 333$, 404, 427, 454 nm in acetone; % III/II = 66; cf. Fig. 2).

Results and Discussion

Chromatography of the unsaponified carotenoid extract of *Carausius* revealed that both hydroxylated carotenoids, β,β -caroten-2-ol and β,β -carotene-2,2'-diol, are present as mono and diesters, respectively, in the insect. The chromatographic sequence relative to the other carotenoids of *Carausius* will be reported in a following paper³.

β,β -Caroten-2-ol

This pigment from *Carausius* co-chromatographed with authentic β,β -caroten-2-ol from *Cerura* in both the partition and the adsorption system and, consequently, was clearly distinguished from the isomeric 4-ol (cf.⁴). The *Carausius* carotenoid behaved similar also to β,β -caroten-2-ol from the alga *Tren-*

tepholia. In the adsorption system a minor fraction ran just behind the main pigment but was always less polar than isocryptoxanthin. The two fractions were interconvertible by standing in solution for a prolonged period of time, they exhibited small spectral differences and gave identical mass spectra; therefore, the minor fraction was obviously a *cis*-isomer of β,β -caroten-2-ol. It was found in each preparation of *Carausius* but never in *Cerura*.

The electronic spectrum of *all-trans* β,β -caroten-2-ol from *Carausius* exhibited maxima at 452 and 478 nm in acetone (% III/II = 21). The mass spectrum (70 eV) was superimposable with that obtained from the *Cerura* pigment⁴. The molecular ion at *m/e* 552 and the fragment at *m/e* 460 (M-92) were the most prominent ions. Elimination of water (*m/e* 534; M-18) was merely detectable. In the 12 eV spectrum only the molecular ion was found. In case of an allylic hydroxyl group as in β,β -caroten-4-ol the dehydration product would be strongly favoured; this will be shown more detailed in case of the dihydroxy pigment.

The chemical features of β,β -caroten-2-ol from *Carausius* were the same as reported for the *Cerura* pigment (cf.⁴). The time course of acetylation of the *Carausius* pigment was completely identical with that of the *Cerura* 2-ol, thus demonstrating a shielded position of the hydroxyl group (50% acetate after 3.2 h). Oppenauer oxidation and ether formation were both negative. Treatment with 0.1 M BF_3 -etherate in chloroform or ethanol yielded a less polar product which co-chromatographed with the corresponding pigment obtained from the *Cerura* 2-ol (Fig. 1). In the latter case the product has been

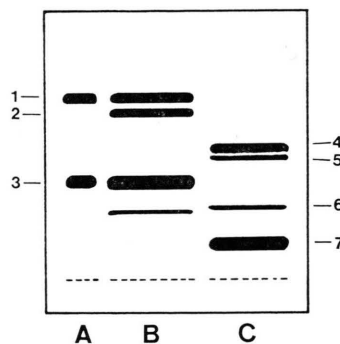


Fig. 1. Silica gel-G chromatogram of the reaction products of BF_3 -chloroform treatment of β,β -caroten-2-ol from *Cerura* (A) and *Carausius* (B), and of β,β -carotene-2,2'-diol from *Carausius* (C). 1, 4 — *all-trans retro* products; 2, 5 — *cis* isomers of 1 and 4, respectively; 3 — β,β -caroten-2-ol; 6 — transient *retro* product; 7 — β,β -carotene-2,2'-diol.

identified as 4',5-*retro*- β,β -caroten-2-one, demonstrating dehydrogenation of the hydroxyl group and *retro* rearrangement of the polyene system⁴. The *retro*-shaped electronic spectrum confirmed the identity of both acid-products; $\lambda_{\max}$ were at 350, 366, 432, 456, and 485 nm in acetone (% III/II = 36). Eleven double bonds can be calculated from Fig. 2,

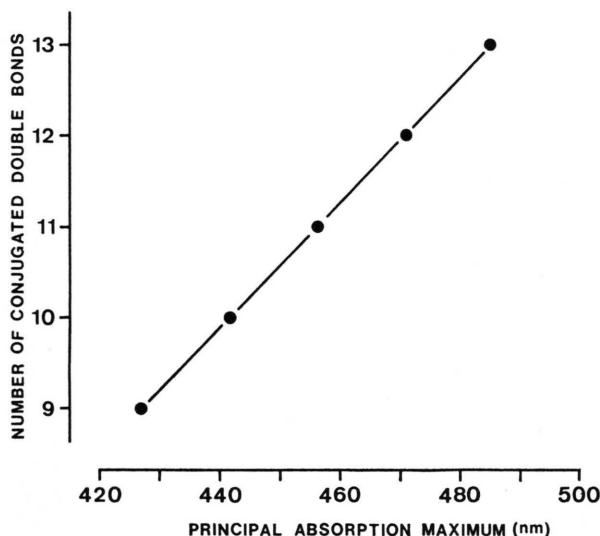


Fig. 2. Positions of principal light absorption maxima of bicyclic *retro* carotenoids as a function of the lengths of their chromophores (acetone solutions).

where the main absorption maxima of authentic *retro* carotenoids are related to the lengths of their chromophores (see Methods).

The *cis*-isomer of β,β -caroten-2-ol exhibited the same specific reaction when treated with BF_3 -etherate though more slowly. This product could be separated from that of the *all-trans* compound on silica gel-G in contrast to the native 2-ols (Fig. 1).

β,β -Carotene-2,2'-diol

In analogy to the monohydroxy carotenoid the dihydroxy pigment from *Carausius* was markedly less polar ($\text{hRf} = 24$ than β,β -carotene-3,3'-diol (zeaxanthin; $\text{hRf} = 15$) and ran slightly ahead of β,β -carotene-4,4'-diol (isoxanthin; $\text{hRf} = 22$) when partitioned on silica gel-G. Furthermore, 3,3'-diol and 4,4'-diol behaved identical in the adsorption system ($\text{hRf} = 27$) where 2,2'-diol was less polar again ($\text{hRf} = 42$). This is in agreement with Kjosen *et al.*⁵. In addition, authentic β,β -carotene-2,2'-diol from the alga *Trentepohlia* and the di-

hydroxy carotenoid from *Carausius* were identical when co-chromatographed. A minor fraction ($\text{hRf} = 37$) was separated by adsorption from the *Carausius* diol and identified as a *cis*-isomer of β,β -carotene-2,2'-diol for the same reasons discussed for the 2-ol.

The electronic spectrum of the *all-trans* β,β -carotene-2,2'-diol showed maxima at 452 and 479 nm in acetone (% III/II = 24). The mass spectroscopic behaviour of β,β -carotene-2,2'-diol agreed with that of the corresponding mono-ol. The molecular ion (m/e 568) was the most prominent ion in the upper mass region (Fig. 3). Dehydrogenation occurred

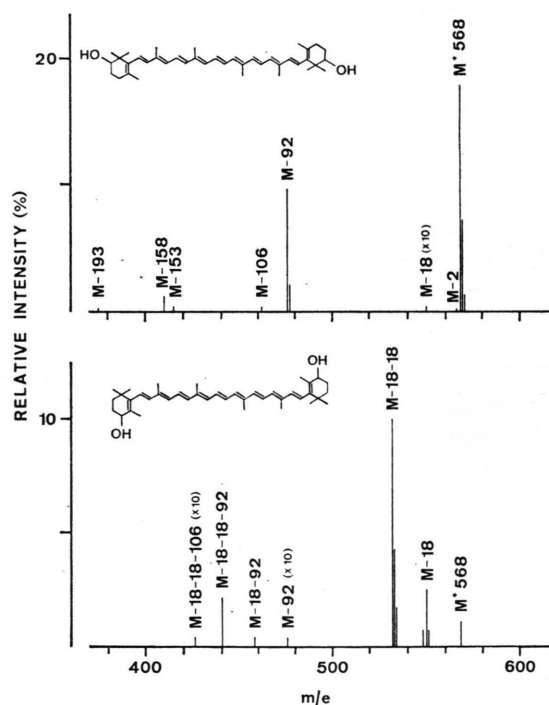


Fig. 3. Mass spectra (70 eV; 3 kV; 190 °C) of β,β -carotene-2,2'-diol from *Carausius* and synthetic β,β -carotene-4,4'-diol. $\times 10$ — peaks are magnified by factor 10.

to a small amount (m/e 566; M-2). Elimination of water was very low and could be definitely stated only for one hydroxyl group (m/e 550; M-18). The ordinary extrusions from the polyene chain¹³ were observed at m/e 476 (M-92), 462 (M-106) and 410 (M-158). Cleavage of the 7,8- and 7',8'-double bond, respectively, with hydrogen transfer to the smaller fragment resulted in the loss of 153 m.u. from the molecular ion (m/e 415). For the rupture

of the 9,10- and 9',10'-bond a corresponding peak at m/e 375 (M-193) was found.

In contrast, the fragmentation pattern of β,β -carotene-4,4'-diol was strikingly different to the C-2 hydroxylated carotenoids (Fig. 3). Due to the allylic nature of the two OH-groups, elimination of water was strongly favoured as demonstrated by the fragments at m/e 550 (M-18) and 532 (M-18-18). The latter ion was the most prominent one in the higher mass region; the molecular ion (m/e 568) was of low intensity. All three ions showed extrusions of toluene at m/e 476 (M-92), 458 (M-18-92) and 440 (M-18-18-92), respectively. But only for the fragment at m/e 532 loss of xylene also was observed (m/e 426; M-18-18-106). In the 12 eV spectrum β,β -carotene-4,4'-diol exhibited the same lability as on high electron impact (70 eV). The tendency to eliminate allylic hydroxyl and ester groups has also been demonstrated for lutein^{14, 15}. To summarize, the mass spectra presented further evidence that the dihydroxy carotenoid of *Carausius* is not isozeaxanthin (β,β -carotene-4,4'-diol).

Regarding the chemistry of β,β -carotene-2,2'-diol the shielded positions of the hydroxyl groups were demonstrated by their slow acetylation. The same effect has been previously shown for β,β -carotene-2-ol from insect⁴ and algal origin¹⁶. As presented in Fig. 4 50% diacetate was formed after 4.5 h in the case of β,β -carotene-2,2'-diol, while the same amount was already found after 1.8 h for lutein. This illustrates the time study of acetylation as an

useful analytical tool in the identification of hydroxylated carotenoids (cf. ⁴).

Attempts to oxidize β,β -carotene-2,2'-diol by Oppenauer oxidation or to etherify the hydroxyls were both negative in contrast to the 4,4'-diol. But under acidic conditions (0.1 M BF_3 -etherate in chloroform or ethanol) a reaction analogous to that of β,β -carotene-2-ol was observed (cf. ^{4, 17}). Two products less polar than the native diol were separated on silica gel-G (Fig. 1). According to time studies, the upper fraction accumulated as the final product, while the lower one behaved as intermediate and was always low in quantity. The electronic spectrum of the main product (Fig. 5) was *retro*-shaped with

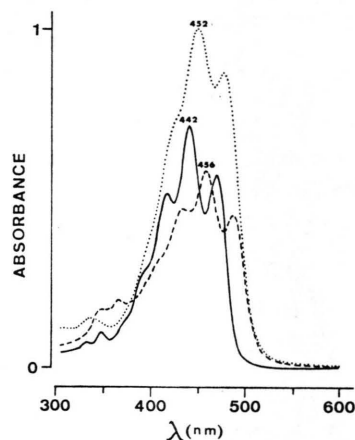


Fig. 5. Electronic spectra (acetone) of β,β -carotene-2,2'-diol (452 nm) and of his *retro* products of BF_3 -chloroform treatment: transient product (456 nm), final product (442 nm).

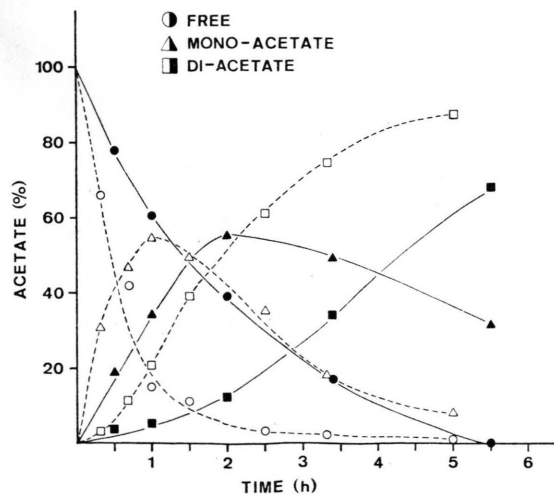


Fig. 4. Time courses of acetylations. Filled symbols: β,β -carotene-2,2'-diol; open symbols: lutein (β,ϵ -carotene-3,3'-diol).

maxima at 337, 349, 418, 442, and 471 nm in acetone (% III/II = 43). This was consistent with a shortening of the chromophore from eleven to ten double bonds (Fig. 1) as shown by spectral identity to the *retro* compound obtained from canthaxanthin by zinc reduction (371.5, 421, 445, and 474 nm in acetone; % III/II = 40). The mass spectrum (Fig. 6) exhibited a strong molecular ion at m/e 566.4131 corresponding to $\text{C}_{40}\text{H}_{54}\text{O}_2$ (calcd. 566.4122), which demonstrated a dehydrogenation of β,β -carotene-2,2'-diol. Ions at m/e 474 (M-92), 460 (M-106), 408 (M-158) and 368 (M-92-106) were due to the common fragmentation pattern of the polyene chain (cf. ¹³). The *retro* structure was confirmed by the ratio of toluene to xylene elimination (M-92/M-106 = 0.64), which corresponded well with ten in-chain double bonds¹⁸. This means, that

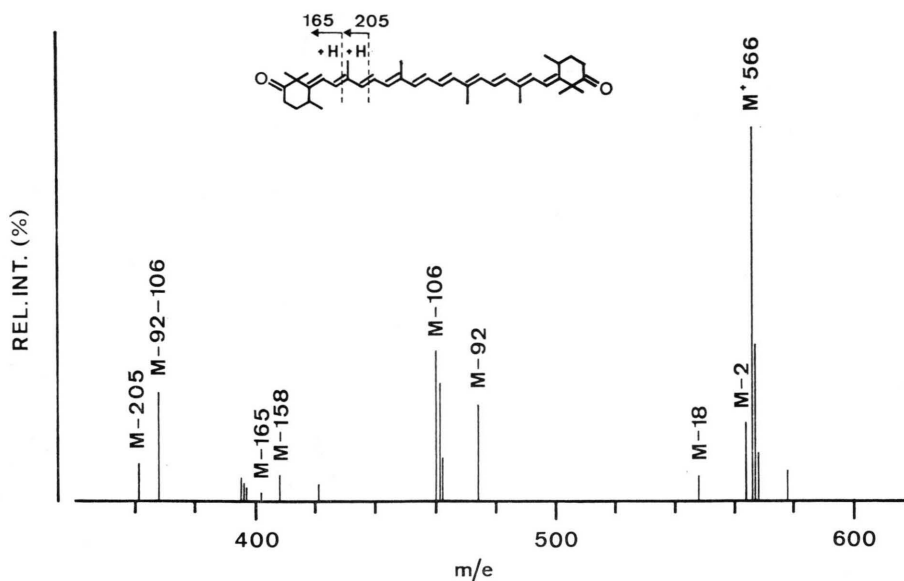


Fig. 6.
Mass spectrum (70 eV;
8 kV; 180 °C) of the
final *retro* product
obtained by BF_3 -
chloroform treatment of
 β,β -carotene-2,2'-diol.

the entire chromophore system is limited to the polyene chain. The fragment ion at m/e 401 (M-165) was attributed to rupture of the 8,9- and 8',9'-double bond, respectively, with hydrogen transfer to the smaller uncharged fragment. The analogous rupture of the 10,11- and 10',11'-bonds resulted in a peak at m/e 361 (M-205). No attack of the 6,7 (6',7')-double bonds was observed in the high mass region (*cf.* ⁴). The fragmentation pattern, generally, confirmed the molecular symmetry of the acid-product.

In contrast to untreated β,β -carotene-2,2'-diol the final product of acid treatment yielded no acetate. Consequently, both hydroxyl groups should have been converted to carbonyl groups which was confirmed by the chromatographic behaviour of the product (*cf.* Fig. 1). The carbonyl groups, however, were not reduced by NaBH_4 , which is in agreement with the acid-product of β,β -caroten-2-ol (*cf.* ⁴).

The investigation of the transient product of BF_3 -treatment was illuminating for the understanding of the reaction. Its polarity was between the end product and the starting diol (Fig. 1). Thus, the presence of one hydroxyl group was suggested, which could be confirmed by the formation of a mono-acetate running similar to the end product on silica gel-G. The electronic spectrum (Fig. 5) with maxima at 432, 455 and 484 nm in acetone (% III/II = 28) coincided with that of the *retro* product of β,β -caroten-2-ol, demonstrating identical

chromophores with eleven double bonds. The isolated compound under investigation was transferred to the less polar end product on incubation with BF_3 -chloroform, thus supporting its intermediate nature. In the mass spectrum of the final reaction product (Fig. 6) elimination of water was observed to a small amount from the molecular ion (m/e 548; M-18). This may be explained by its isomerization to the intermediate compound due to thermal effects or electron impact.

Conclusively, the following reaction scheme is proposed for the transformation of β,β -carotene-2,2'-diol in acid solutions (Fig. 7): In analogy to

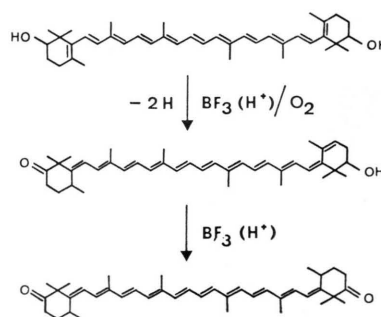


Fig. 7. Proposed scheme for the reaction of β,β -carotene-2,2'-diol in acid solution.

the 2-ol (*cf.* ^{4,17}) one alcohol group is dehydrogenated to the ketone and the conjugated chain is shifted by one position to form 2'-hydroxy-4',5-

retro- β,β -caroten-2-one. This intermediate product is subsequently rearranged by migration of the endocyclic double bond to form the second carbonyl group resulting in the more stable 4,5-dihydro-4,5'-*retro*- β,β -carotene-2,2'-dione.

The oxidation of the mono-ol and the diol of *Carausius* required molecular oxygen as an acceptor for the hydrogen eliminated from the hydroxyls, since under nitrogen the reaction proceeded markedly slower as already shown for 2-ol from *Cerura*⁴.

Conclusive Remarks

This paper presents clear evidence that the main hydroxy carotenoids of the stick insect are not isocryptoxanthin and isozeaxanthin as formerly reported by Willig¹, but β,β -caroten-2-ol and β,β -carotene-2,2'-diol. This identification is based on the following features: a. co-chromatography with authentic samples, b. slow acetylation kinetics, c. stability of the molecular ions on high electron impact, and d. specific dehydrogenation and *retro* rearrangement in acidic solutions.

β,β -Caroten-2-ol has been previously isolated from the moth *Cerura vinula* and identified by spectroscopic methods including NMR⁴. This was the first finding in an insect. Now, β,β -carotene-2,2'-diol has been found in an animal for the first time. This compound was recently discovered in the green alga

Trentepohlia together with β,β -caroten-2-ol and β,ϵ -caroten-2-ol⁵.

No doubt, the natural occurrence of 2-hydroxylated carotenoids is not restricted to only few "curious" species of the plant and animal kingdoms. Due to analytical insufficiencies the identification of hydroxylated carotenoids has often been equivocal. These problems are now overcome by the discovery of the dehydrogenation of 2-hydroxylated β,β -carotenes and their rearrangement to *retro* chromophores, which are easily identified by their typical electronic spectra (cf. 4, 17).

The 2-hydroxy carotenoids of *Carausius* are obviously biosynthesized — along with structurally related keto carotenoids^{2,3} — from diet derived β,β -carotene by the insect since its food is lacking these pigments at all. This assumption is confirmed by results of labelling experiments¹⁹ now in progress to study the metabolic pathway of carotene oxidation in the stick insect.

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