

Carotenoid Biogenesis in the Stick Insect, *Carausius morosus*, during a Larval Instar

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[^{14}C] β -Carotene was fed to juvenile stick insects, *Carausius morosus*, of the fifth instar. Radioactivity was incorporated into 2-hydroxy-, 2-oxo-, and 3,4-didehydro-2-oxo-carotenoids of the β,β -type. These transformations are due to the insect's own capacity; any contribution by microbial symbionts can be ruled out. A study on the labelling kinetics clearly shows that the biogenesis of hydroxy- and oxo-carotenoids is correlated to a decrease in the carotene precursor, but only up to mid instar. Thereafter, oxidation of the carotene is very low but the transformations of its metabolites continue as before. Predominantly β,β -carotene-2,2'-diol is dehydrogenated to 3,4,3',4'-tetrahydro- β,β -carotene-2,2'-dione via two hydroxyketones. This discontinuous utilization of β -carotene could be due to a stop at mid instar either in the oxidation or in the absorption in the gut of this precursor.

Introduction

The stick insect, *Carausius morosus*, contains a series of carotenoids with 2-hydroxy-, 2-oxo-, 3,4-didehydro-2-oxo-, and non-modified β -ionone rings in nearly all possible combinations [1, 2]. The same carotenoids have been isolated from the related species, *Ectatosoma tiaratum* [3], and also from a number of other stick insects from different genera [4]. Here in addition the 2-hydroxy- and 2-oxo-derivatives of β,ϵ -carotene could be identified [3, 4]. Most carotenoids of this series have not been found in nature before.

Up to date, there is no report on an insect being able to synthesize carotenoids *de novo*, i.e. from smaller precursors. Consequently, the complete carbon skeleton of a carotenoid must be taken up with the food. However, the green leaves on which the insects feed do not contain any of these 2-hydroxy- and 2-oxo-carotenoids. According to the accepted view on the origin of insect carotenoids [5] it must be assumed that a precursor carotene is absorbed in the gut and then structurally modified to produce the whole range of metabolites found in stick insects. In most insect species so far studied this conclusion has been reached indirectly by the demonstration that their food actually lacks the carotenoids in question. Recently, by feeding radiolabelled β -carotene, the biogenesis of β,β -caroten-

2-ol and β,β -caroten-3-ol, respectively, in two moth species [6], and of 2-hydroxy- and 2-oxo-carotenoids in the stick insect, *Ectatosoma* [3], has been directly established.

The present study deals with the carotenoid biogenesis in *Carausius*. Again [^{14}C] β -carotene was used to demonstrate directly the presumed transformation of this carotene, which is readily available in the food, into all β,β -type carotenoids of the 2-hydroxy/2-oxo-series that are present in measurable amounts. In addition, the time-courses of these transformations were followed through a larval instar in order to establish precursor-product relationships among the various carotenoids.

Materials and Methods

Culture and staging of insects

Stick insects (*Carausius morosus* Br., Phasmatodea) were obtained from a laboratory stock which is maintained at 21 °C under a light-dark cycle of 12:12 h. Ivy leaves (*Hedera helix*) were used as food. Juvenile fifth instar insects were used throughout in this study. Those insects which had moulted to the fifth instar within a 24 h interval were collected and starved for two days prior to injection of radiolabelled carotene. This starvation is not expected to produce any non-physiological reaction since, anyhow, the insects do not feed during about two days following a moult. Injections were per-

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formed either on the third day (first experiment), or on the twelfth day (second experiment) after the fourth moult.

Administration of [^{14}C] β -carotene

Crystalline [$15,15\text{'-}^{14}\text{C}$] β -carotene with a specific activity of $32\text{ }\mu\text{Ci/mg}$ was purified by thin-layer chromatography (TLC) on silica gel-G and dissolved in olive oil as described in a previous paper [6]. In preliminary experiments most stick insects usually died after injection of the oil solution. The only successful way to dispense the radioactive solution was to introduce a definite volume of the oil solution through the mouth into the oesophagus without anesthetization [cf. 6]. Each insect received $0.05\text{ }\mu\text{Ci}$ [^{14}C] β -carotene in $2.5\text{ }\mu\text{l}$ olive oil with the aid of a $50\text{ }\mu\text{l}$ Hamilton microsyringe. Only those insects which retained the full quantity of oil were used for the study. The injected insects were kept under standard rearing conditions. However, to avoid rapid loss of radioactivity by defaecation the insects were not fed for one day after the injection. At intervals groups of five to nine insects were selected randomly and killed by freezing.

Extraction of carotenoids

The stick insects were minced and homogenized in cold acetone with an Ultra-Turrax (Janke and Kunkel). The carotenoids were extracted by repeated homogenization in acetone followed by acetone/methanol (1:1; v/v). Then, the chlorophylls derived from the gut were removed by extraction of the carotenoids into petroleum ether ($50\text{--}70\text{ }^{\circ}\text{C}$) after addition of water and a few milliliters of 10% KOH to render the chlorophylls hypophasic. The extracts were not saponified since the best separations of the various carotenoids are obtained when the hydroxylated pigments were in their native esterified form.

Chromatography and measurement of radioactivity

The carotenoids were separated by TLC on pre-coated silica gel layers of various types (Macherey and Nagel). Prior to sample application the starting area of the layer was moistened with petroleum ether ($50\text{--}70\text{ }^{\circ}\text{C}$) to minimize possible destruction and isomerization of the carotenoids. In order to record the pattern of incorporation of radioactivity into the various carotenoids about one third of a total extract was applied on two glass sup-

ported layers type Sil G-25 ($5\times 20\text{ cm}$). The plates were developed in mixtures of petroleum ether ($100\text{--}140\text{ }^{\circ}\text{C}$) and 2-propanol (99:1 and 96:4; v/v, respectively) to resolve the less polar fractions on one plate and the more polar ones on the second. The chromatograms were scanned with a windowless gas flow counter (scanner system BF 210-23; Berthold and Frieske) using a $2\times 36\text{ mm}$ slit. For quantitative measurements the remaining part of the extract was applied on two layers type Polygram Sil-G supported on plastic sheets ($20\times 20\text{ cm}$) and developed twice in petroleum ether/2-propanol (97:3; v/v). The carotenoid zones were scraped off and collected in 10 ml tubes. For elution of the carotenoids 2 ml of acetone were added to each tube and, after vigorous mixing and centrifugation (5 min at $5000\times g$), $500\text{ }\mu\text{l}$ aliquots of each supernatant were transferred to scintillation vials using a $500\text{ }\mu\text{l}$ Hamilton microsyringe. After evaporation of the solvent by an air stream the carotenoids were dissolved in 10 ml of a toluene-based scintillation cocktail (Quicksint 501; Zinsser). Generally, samples were prepared in duplicate. Radioactivity was measured by counting for 10 min using a Nuclear Chicago scintillation spectrometer type Isocap/300 with the external standard mode for quench determination. Counting efficiencies of 85% were obtained throughout. Radioactivity in the total extracts was measured in 0.5 ml aliquots of the petroleum ether solution mixed with 10 ml of the scintillation cocktail. Incorporation of radioactivity into a specified carotenoid is expressed as the percentage of total radioactivity of all carotenoid fractions measured. The results thus obtained were qualitatively similar to those related to the radioactivity of the total extracts. Attempts to determine the specific radioactivities of the various carotenoids were unsuccessful due to spectral disturbance by chlorophyll derivatives which were present in many fractions. During initial work incorporation of radioactivity into a specified carotenoid was confirmed by rechromatography first in the same silica gel system and then on an adsorption layer consisting of a mixture of CaCO_3 , MgO , and Ca(OH)_2 [cf. 1–3]. Further confirmation was achieved by derivatizations such as saponification of carotenoid esters, acetylation of hydroxy-carotenoids, and reduction with NaBH_4 of keto-carotenoids [for experimental details see 1–3]. After each procedure the products were subjected to TLC and radioscanning.

Results

Transformation of [^{14}C] β -carotene

The β,β -type carotenoids that have been identified in *Carausius morosus* [1, 2] are listed in Table I. All hydroxy-carotenoids are esterified with fatty acids to a large extent and only a negligible proportion of each is attributed to the free carotenols. This fact facilitates the separation in a single TLC run of nearly all carotenoids of interest.

The radiochromatograms shown in Fig. 1 have been obtained with extracts taken about two weeks after feeding [^{14}C] β -carotene to fifth instar larvae. These radioscans clearly document the incorporation of radiolabel into all β,β -type carotenoids that are present in measurable quantities [cf. 2,4]. The chromatograms show the seven fractions studied, and the negligible proportions of the free diol **VI** and the free hydroxy-ketone **VIII**. The didehydro-dione **VII** which is present in very low amounts [cf. 4], is masked by the diol mono-ester zone **VI'** which also includes small amounts of free 2-ol **III**. No attempts were made to look for radioactivity in the didehydro-dione **VII**. The 2,2'-dione **IV** and the 2-one **II**, which also represent trace compounds, could not be detected on the chromatograms.

The attribution of label to the major carotenoids was confirmed by further purification of these pigments in the silica gel partition system and in an

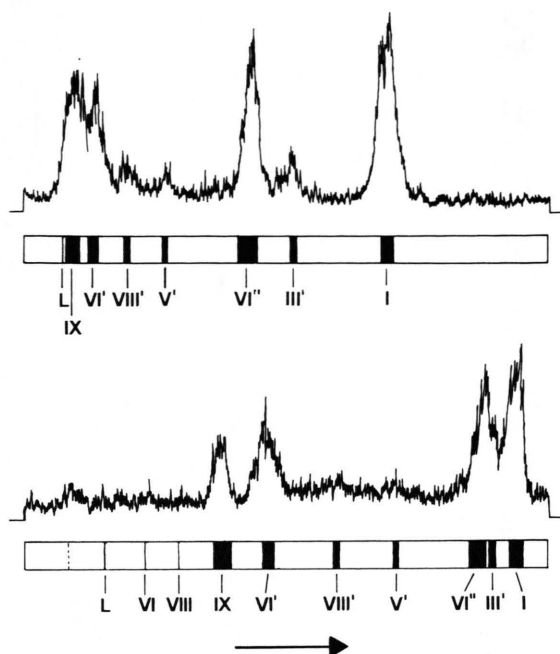
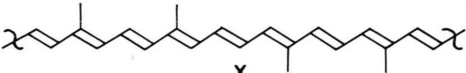
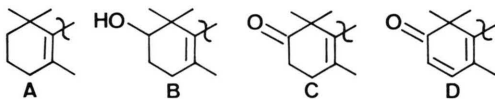


Fig. 1. Radioscans of thin-layer separations of carotenoids from *C. morosus* after feeding [^{14}C] β -carotene **I** to demonstrate its transformation into the carotenoids **III**, **V**, **VI**, **VIII**, and **IX**. Labelled β -carotene was fed to fifth instar juveniles of non-specified age. Incorporation period was about two weeks. For identification of carotenoids see Table I. Primed numbers refer to esters of the corresponding carotenoids. L — lutein (β,ϵ -carotene-3,3'-diol). The chromatograms were developed in petroleum ether (100–140 °C)/2-propanol: 99:1 (v/v) in the upper and 96:4 (v/v) in the lower chromatogram. Arrow indicates direction of solvent flow.

Table I. List of the β,β -type carotenoids from *Carausius morosus* [1, 2].

		
		
No.	Structure	Semisystematic name
I	A-X-A	β,β -carotene
II	A-X-C	β,β -caroten-2-one
III	A-X-B	β,β -caroten-2-ol
IV	C-X-C	β,β -carotene-2,2'-dione
V	B-X-C	2'-hydroxy- β,β -caroten-2-one
VI	B-X-B	β,β -carotene-2,2'-diol
VII	C-X-D	3,4-didehydro- β,β -carotene-2,2'-dione
VIII	B-X-D	2'-hydroxy-3,4-didehydro- β,β -caroten-2-one
IX	D-X-D	3,4,3',4'-tetrahydro- β,β -carotene-2,2'-dione

adsorption system. Furthermore, chemical reactions such as saponification, acetylation, and borohydride reduction were carried out on the purified native fractions and the products were again subjected to TLC. By these procedures it was firmly established that the radioactivity in a silica gel fraction can be unequivocally attributed to the single β,β -type carotenoid which is present in that zone. No other significant radioactivity is detectable in the carotenoid fractions considered. Only in TLC runs of total extracts the starting zone usually contains radioactivity that can not be attributed to any coloured compound. This fraction of radioactivity increases with time as judged from the TLC scans, and probably accounts for the continuous decrease of total radioactivity which is recovered from the specified carotenoid zones under study (Fig. 2).

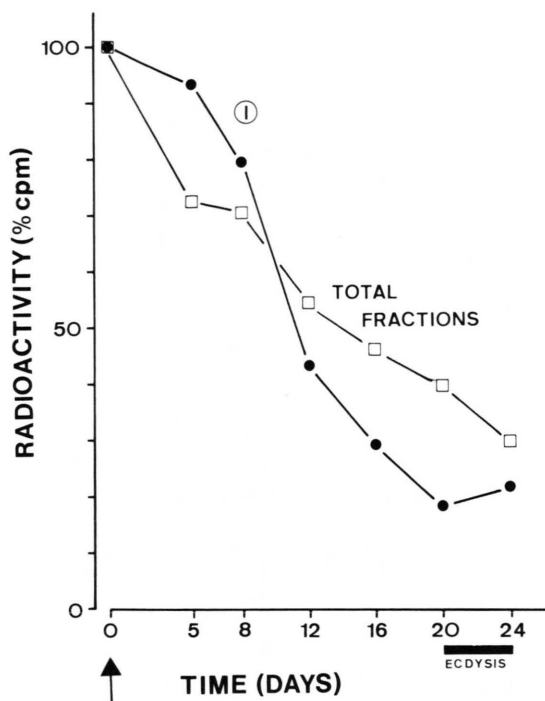


Fig. 2. Time-course of disappearance of [^{14}C] β -carotene **I** during the fifth juvenile instar of *C. morosus*: radioactivity associated with the carotene zone expressed as a percentage of the total activity of all carotenoids (filled symbols). Recovery of radioactivity in the total carotenoid fractions expressed as a percentage of the radioactivity of the total extract (open symbols). Labelled β -carotene was fed (arrow) two days after the fourth ecdysis. Mean-values of duplicate determinations from a single experiment.

Feeding [^{14}C] β -carotene at the beginning of the instar

Labelled β -carotene was given to stick insects at the beginning of the fifth juvenile instar and the carotene transformation was followed up to the next ecdysis. Fig. 2 shows how that proportion of radioactivity which is attributed to the injected β -carotene **I** changes with time. There is a sigmoid decrease indicating a most rapid disappearance (*i. e.* transformation) of β -carotene eight to twelve days after label introduction. It is not known to what extent the kinetic of carotene transformation is influenced by the rate of absorption of the oil solution in the gut.

The labelling kinetics of the various transformation products of the carotene are shown in Fig. 3. In the tetrahydro-dione **IX** and in the esters of the hydroxy-ketones, **V'** and **VIII'**, radioactivity increases throughout the instar. At its end most radio-

activity has accumulated in the red dione **IX** as one of the major carotenoids [*cf.* 4]. Radiolabel accumulates most rapidly in the mono-ester **VI'** and the di-ester **VI''** of the diol which represents the principal carotenoid [4]. Sixteen days after label introduction the radioactivity in the mono-ester **VI'** reaches a maximum and this is followed by a rapid decline. A similar time-course is observed in the diester **VI''**. However, its disappearance starts about four days later. This may indicate that hydrolysis of the mono-ester is preferred to its conversion to the diester. No correlated increase in the free diol **VI** could be observed. Obviously, the diol is rapidly converted to other carotenoids after deacylation during that time. An increase and subsequent decrease

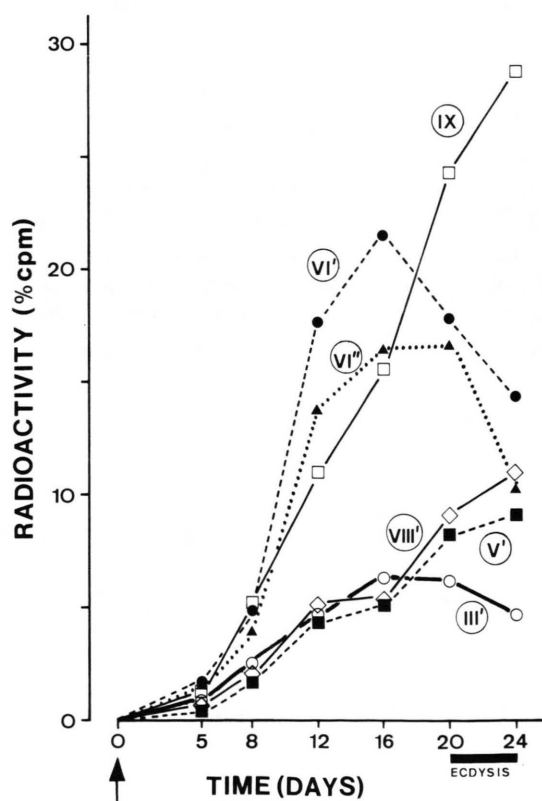


Fig. 3. Time-course of label incorporation from [^{14}C] β -carotene into the carotenoids **III**, **V**, **VI**, **VIII**, and **IX** (*cf.* Table I) in *C. morosus* during the fifth juvenile instar. Labelled β -carotene was fed (arrow) two days after the fourth ecdysis. Radioactivity in a single carotenoid is expressed as a percentage of the total activity of all carotenoids of an extract. Mean-values of duplicate determinations from a single experiment. Primed numbers refer to esters of the corresponding carotenoids.

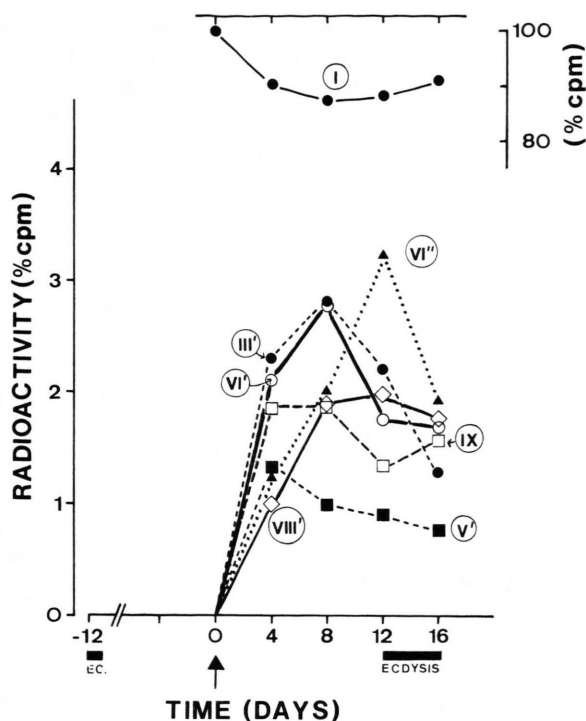


Fig. 4. Time-course of transformation of [^{14}C] β -carotene I (upper diagram) and label incorporation into the carotenoids III, V, VI, VIII, and IX (lower diagram) in *C. morosus*. Labelled β -carotene was fed at day twelve of the fifth instar (arrow). For further explanation see legend of Fig. 3.

in radioactivity is also found in the 2-ol ester III' which parallels the behaviour of the diol esters.

At least up to mid instar radioactivity is clearly incorporated from β -carotene into the other β,β -type carotenoids. It appears that after mid instar the oxidation of β -carotene is greatly reduced but the diol, which has accumulated up to that time, is further transformed to the tetrahydro-dione IX via V' and VIII'.

Feeding [^{14}C] β -carotene at mid instar

As outlined before β -carotene may be utilized only during the first half of the instar. Consequently, the transformation of this carotene should be rather low if it is supplied at mid instar or later. This hypothesis was tested in a second experiment. Radiolabelled β -carotene was injected at the twelfth day after the fourth moult, i.e. shortly before mid instar, and incorporation of radioactivity into the

various carotenoids was again followed up to the next ecdysis. The results of this experiment are shown in Fig. 4. As expected incorporation of radio-label is very low throughout this period and never exceeds 3.3% in any fraction. At the end of the period about 90% of radioactivity is still present in the carotene zone representing the unchanged precursor. This shows that the observed restriction of carotene oxidation to the first half of the instar, as deduced from the first experiment, is not due to vanishing amounts of the labelled precursor. Incorporation of radioactivity into the esters of the diol (VI' and VI'') and of the 2-ol (III') first increases and then decreases again as also observed in the first experiment.

At the end of the incorporation period some of the injected insects had already moulted and spent four days in the next juvenile instar. In these specimens incorporation of radioactivity into the carotene metabolites is about twice as high (data not shown) as compared to the non-moulted insects after the same time span. This may be due to an increased oxidation of β -carotene after the moult which would correspond to the situation in the first experiment. No further work has been done on this instar.

Discussion

All animals studied so far proved to be unable to synthesize carotenoids from smaller precursors and therefore completely depend on the dietary supply of the carotene skeleton. This is also valid for insects the majority of which is able to absorb carotenoids from their food. In some insects the carbon skeleton of the absorbed carotenoid(s) may also be modified mostly by introduction of hydroxy- and/or keto-groups as originally outlined by Goodwin [5] and recently reviewed by Kayser [8].

The present study demonstrates that in *C. morosus* dietary β -carotene is metabolized to a series of carotenoids with oxygen functions at C-2 of the β -ionone rings [1, 2]. The same set of β,β -type carotenoids has also been identified in some other stick insects from different genera [3, 4]. In one of these species, *Ectatosoma tiaratum*, transformation of radiolabelled β -carotene into this series of metabolites has been demonstrated, too [3]. Another direct proof for the oxidative modification by insects of the carotene skeleton is the transformation of [^{14}C] β -

carotene to β,β -caroten-2-ol and β,β -caroten-3-ol by the moths, *Cerura vinula* and *Phalera bucephala*, respectively [6]. All these studies have not been performed under aseptic conditions, hence an eventual contribution by any symbiotic microorganisms to carotenoid biogenesis in the insects can not be excluded. However, there is a well documented relation between the diet of insects and their possession of symbionts [9]. Generally, microbial symbionts are found in those insects which eat a restricted diet deficient in certain essential nutrients. For example, wood-eaters, plant-sap feeders, and permanent blood-suckers are typical symbiont bearers, whereas insects feeding on leaves or vegetables do not have any type of symbionts. This "law" is also well established in Lepidoptera and Phasmids. Therefore, any foreign contribution to carotenoid biogenesis in the insects studied so far can be ruled out. Furthermore, the feeding experiments with radiolabelled β -carotene exclude the other possibility that the metabolites in question might have been accumulated by the insects from an unknown food source as recently discussed for the presence of fungal carotenoids in ladybird beetles [10]. In *Carausius* the well characterized [2] carotene metabolites are principally found in the integument and fat body of the insects [11] and also in the eggs [4]. The incorporation of radioactivity into these carotenoids clearly demonstrates that the label, which was initially localized to the gut lumen, has actually entered the body either in the precursor carotene or in metabolic product(s) formed in the gut cells. In conclusion, it is beyond all doubt that in all direct demonstrations of carotenoid transformations in insects as discussed before the radiolabelled β -carotene has in fact been metabolized to the specified oxo- and/or hydroxy-carotenoids by the insects themselves.

In *Carausius* it is obvious from the results presented that the carotenoid transformations proceed

in a discontinuous mode at least during that juvenile instar under investigation. The oxidation of β -carotene representing the initial precursor to the series of 2-hydroxy- and 2-oxo-carotenoids is confined to the first half of the instar. This interpretation is confirmed by the second experiment in which the carotene was administered at mid instar. Presently, the cause of the stop of carotene transformation at mid instar is only subject to speculations. Possibly, the oxidation of the carotene itself ceases or is drastically attenuated wherever this initial attack of the precursor takes place. Another possibility is that the absorption of β -carotene is largely confined to the first half of the instar. Then, the availability of the initial precursor could well become a limiting factor after mid instar. From that time the 2,2'-diol **VI**, representing the predominant carotenoid, and the 2-ol **III**, both of which have been accumulated as esters, are further metabolized (after deacylation) to the apparent end product tetrahydro-dione **IX** which is continuously produced during this instar. The hydroxy-ketones **V** and **VIII** behave as intermediates in this pathway.

The demonstrated transformation of the diol **VI** to the tetrahydro-dione **IX** is in apparent contradiction to the proposed carotenoid pathway in *Carausius* [2] according to which the diol should be an end product. This situation has already been discussed in a recent review [8] and will be considered in detail in following papers.

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