Hormone Induced Changes in Carotenoid Composition in *Ricinus* Cell Cultures. I. Identification of Rhodoxanthin

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When cell cultures of *Ricinus communis* are grown in light and with kinetin as the sole growth factor red cells are formed. The red pigmentation is due to the accumulation of rhodoxanthin which is the major carotenoid in these cultures. The identification of this *retro*-type carotenoid is based on electronic and mass spectra, on chemical transformation to zeaxanthin, and on comparison with an authentic sample. Rhodoxanthin is not present in any part of the intact plant. The major yellow carotenoid in the red cultures is lutein.

Introduction

Chloroplasts of higher plants contain a fairly constant pattern of carotenoids which function as accessory pigments in photosynthesis and protect the chlorophylls and chloroplast enzymes against photodestruction [1]. In contrast to this type of plastids, chromoplasts contain a great variety of carotenoids, some of which are not found in other types of plastids. These pigments are responsible for the bright red, yellow, and orange colors of many autumn leaves, fruits and flowers [2]. The structural aspects of carotenoid accumulation in chromoplasts [3] and the chemistry and biochemistry of carotenoids [4] are wellknown, however there are only little data on the regulation of carotenoid metabolism.

In our efforts to study plastid differentiation we found a system in which the synthesis of a specific red pigment can be easily induced. The strain A of a cell culture derived from the endosperm of *Ricinus communis* [5] produces red cells when the culture is maintained in light on a medium with kinetin as the sole growth factor [6]. Here we report on the identification of the red pigment as rhodoxanthin, a *retro*-type carotenoid, which is not synthesized by the intact plant.

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Materials and Methods

Plant material

The callus cultures are derived from the endosperm of the castor bean, *Ricinus communis;* only strain A, as characterized elsewhere [5], was used. The cells were cultivated under fluorescent white light (Osram L65W/32, 5 W/m^2) at 20 °C on a solid Gamborg B5 medium [7] supplemented with 2% sucrose and 0.2 mg/l benzylaminopurine (kinetin).

Leaves of the pondweed, *Potamogeton natans*, were collected from a pond near Ulm.

Extraction

Lyophilized *Ricinus* callus cultures were homogenized in a mortar using quartz sand. Carotenoids were repeatedly extracted with acetone. After evaporation of the solvent the pigments were redissolved in chloroform for thin-layer chromatography (TLC). Fresh leaves of *Potamogeton* were homogenized in acetone with an Ultra-Turrax. The pigments were extracted with acetone; the carotenoids were transferred from acetone to petroleum benzine (50–70 °C) after adding of water and a small volume of 10% KOH to render most of the chlorophylls hypophasic. The upper layer was dried with Na_2SO_4 and concentrated under reduced pressure. All operations were performed in dim light.

Chromatography

The carotenoids were isolated by preparative thin-layer chromatography (TLC) according to published procedures [8, 9]. Partition TLC was performed on precoated silica gel layers type Polygram Sil-G (Macherey-Nagel) or type Kieselgel 60 (Merck). Further purification was achieved by adsorption TLC on a mixed layer of $CaCO_3$, MgO and $Ca(OH)_2$ (30:6:5; by wt). In both systems mixtures of petroleum benzine (100–140 °C) and *iso*-propanol were used as solvents; the ratio was varied (100:5 to 100:16; v/v) to obtain optimal separations. For mass spectrometry the carotenoids were finally run on precoated layers of ultrapure silica gel type G-25 HR (Macherey-Nagel) which had been prewashed with methanol.

After TLC the colored bands were scraped off and the pigments eluted with acetone.

Chemical reactions

Standard procedures as detailed elsewhere [8] were employed for saponification (KOH/methanol), acetylation (acetic anhydride/pyridine), etherification (BF_3-etherate/ethanol) and hydride reduction ($NaBH_4$/methanol) of carotenoids. Reduction with zinc powder was performed in acetic acid/pyridine (3:10; v/v) at 50 °C for less than 1 min [10].

Spectroscopic methods

Electronic spectra were recorded with a Zeiss DMR 21 spectrophotometer; the pigments were dissolved in acetone if not stated otherwise. Electron impact mass spectra were obtained with a Varian MAT 711 machine using the direct inlet system. The conditions were: ionizing voltage 70 eV, acceleration voltage 8 kV, temperature range 190–240 °C.

Quantitative measurements of carotenoids were made by using $E^{1\%}_{1\,cm} = 2500$ for their principal absorption maximum in acetone.

Reference carotenoids

Authentic rhodoxanthin was isolated from leaves of *Potamogeton natans* [11], as described above, and characterized by its electronic and mass spectra. A synthetic sample of racemic zeaxanthin (*β,β*-carotene-3,3′-diol) was kindly provided by F. Hoffmann-La Roche (Basel). Lutein was from the insect *Cerura vinula* [8].

Results and Discussion

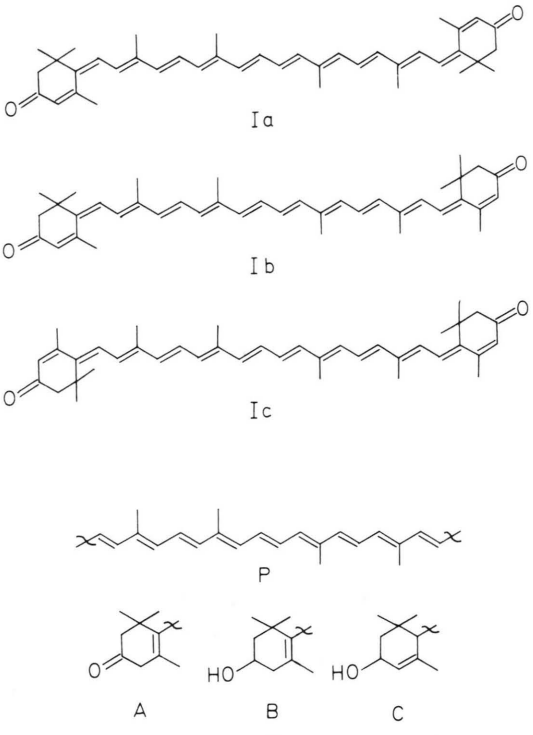
A representative silica gel chromatogram of the carotenoids from red cultures of *Ricinus* is shown in Fig. 1. Besides two minor yellow zones (1 and 2) one prominent red (3) and one yellow (4) zone were observed. Below zone 4 three additional yellow zones with very low concentrations were observed but neglected in further work. Extracts from green or dark cultivated pale cultures displayed only the yellow carotenoid zones [6]. In a typical extract from red cultures the pigment zones 1 to 4 comprised approx. 2%, 8%, 70% and 20% respectively of the total carotenoids which amounted to 250 µg per g of dry weight.

Zone 1 was tentatively identified as *β,β*-carotene on the basis of its spectrum (*450*, 478 nm) and chromatographic behavior. Zone 2 exhibited a spectrum with three maxima (λ_{max} *442* nm) and was labile to alkali: saponification produced a number of orange, red, and yellow zones which behaved more polar than zone 3. It remained unclear whether zone 2 represented a carotenoid ester; no further studies were possible. The prominent zones 3 (red) and 4 (yellow) were shown to represent rhodoxanthin (4′,5′-didehydro-4,5′-*retro*-*β,β*-carotene-3,3′-dione) and lutein (*β,ε*-carotene-3,3′-diol) respectively as detailed below (for structures see Scheme 1).

Rhodoxanthin (**I**)

The red pigment (zone 3) from *Ricinus* exhibited a single light absorption maximum at 486 nm in

Fig. 1. Schematic representation of a silica gel thin-layer chromatogram of the carotenoids from red callus cultures of *Ricinus communis*. Numbers refer to the major pigment zones (see text).



II: A–P–A III: B–P–B IV: B–P–C

Scheme 1. Structures of 6,6′-di-*cis* (**Ia**), 6-mono-*cis* (**Ib**) and all-*trans* (**Ic**) rhodoxanthin, β,β-carotene-3,3′-dione or "dihydrorhodoxanthin" (**II**), zeaxanthin (**III**), and lutein (**IV**).

acetone; some spectral fine structure was observed in *n*-hexane (~ 455, *480*, ~ 510 nm). Typically, on analytical silica gel plates (see Fig. 2) the red pigment split into three red respectively orange-red fractions; the middle one predominated. Authentic rhodoxanthin from *Potamogeton* also provided three fractions which co-chromatographed with those of the *Ricinus* pigment and exhibited also identical spectra: the single peak was at 490 nm in the lower fraction, at 486 nm in the middle fraction and at 483 nm in the upper one.

Rhodoxanthin is known to easily isomerize at the 6(6′)-exocyclic double bond(s) and thus occurs as a mixture of three geometric isomers (all-*trans*, 6-mono-*cis* and 6,6′-di-*cis*; see Scheme 1) which have been characterized by their NMR spectra after HPLC separation [12]. Though the electronic spectra of the isomers have not been reported, the three rhodoxanthin fractions obtained by TLC from both *Ricinus* and *Potamogeton* most probably represented these isomers: the lower fraction with the maximum at 490 nm was the all-*trans* isomer and the upper fraction absorbing at shortest wavelengths (483 nm) the 6,6′-di-*cis*-form; the principal middle fraction (486 nm) then represented the 6-mono-*cis* isomer which is the most stable molecular species [12].

The mass spectra of rhodoxanthin from *Ricinus* and *Potamogeton* were superimposable. The molecular ion was observed at *m/e* 562 (M^+, base peak) as expected. Fragment ions characteristic for carotenoids were at *m/e* 470 (M-92) and *m/e* 456 (M-106) which originated from extrusion of toluene and xylene respectively from the polyene chain [13]. The intensity ratio of these ions (M-92/M-106) ranged from 0.5 to 1.0 in the *Potamogeton* pigment (only the mono-*cis* isomer was used) and from 1.0 to 1.36 in that from *Ricinus* (mixture of all isomers); values between 0.44 and 1.0 have been reported as typical for the presence of ten double bonds in the polyene chain as in rhodoxanthin [14].

Reduction of rhodoxanthin with borohydride should furnish eschscholtzxanthin (4′,5′-didehydro-4,5′-*retro*-β,β-carotene-3,3′-diol). Attempts to isolate this product for spectral studies were unsuccessful, however, since it was easily re-oxidized to the diketone. When rhodoxanthin from both *Ricinus* and *Potamogeton* was reduced with Zn (Fig. 2) a yellow less polar product with the chromophore of β,β-carotene was obtained (~ 428, *451*, 479 nm; %**III/II** = 40) representing β,β-carotene-3,3′-dione or "dihydrorhodoxanthin" (**II**) [10]. However, mass spectrometry provided a molecular weight of 562 instead of 564 indicating that re-oxidation to rhodoxanthin [*cf.* 10] took place during insertion and heating of the probe.

Treatment of the yellow diketone **II** with borohydride yielded a product with a similar electronic spectrum (~ 428, *452*, 479 nm; %**III/II** = 25) and a polarity higher than that of rhodoxanthin (Fig. 2). This second yellow product should represent zeaxanthin (β,β-carotene-3,3′-diol) (**III**) [15] which was confirmed by co-chromatography with a synthetic sample both on silica gel and the adsorption layer. Moreover, a correct molecular weight of 568 was determined by mass spectrometry.

In conclusion, these data prove that the red *Ricinus* pigment is identical with rhodoxanthin.

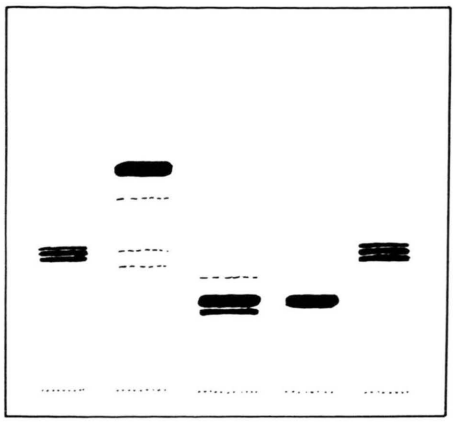


Fig. 2. Products of chemical reactions with rhodoxanthin. (A) Rhodoxanthin from *Ricinus communis;* (B) Zn reduction of A; (C) borohydride reduction of B; (D) synthetic zeaxanthin; (E) rhodoxanthin from *Potamogeton natans.* Note the three zones of geometric isomers in A and E (see Scheme 1).

Lutein (**IV**)

Fraction 4 was more polar than rhodoxanthin on silica gel (Fig. 1) and co-migrated with lutein from *Cerura.* The electronic spectrum exhibited three maxima at 424, *446* and 474 nm (%**III/II** = 56) identical to those of lutein. In the mass spectrum a prominent molecular ion was found at *m/e* 568 (M^+, base peak). The presence of two non-equivalent hydroxyl groups was demonstrated by fragment ions at *m/e* 550 (M-18) and *m/e* 532 (M-18-18) with intensities of 80% and 15% respectively of the base peak. Other typical ions originated from dehydrogenation (*m/e* 566, M-2; *m/e* 548, M-18-2) and from losses of toluene (*m/e* 476, M-92; *m/e* 458, M-18-92), xylene (*m/e* 444, M-18-106) and a $C_{12}H_{14}$ fragment (*m/e* 392, M-18-158) as known from lutein mass spectra [13].

Further evidence for the identity of fraction 4 with lutein was obtained from chemical reactions (Fig. 3). Upon treatment with BF_3-etherate in ethanol the *Ricinus* pigment yielded a less polar product supposed to be the 3′-ether from a parallel experiment with authentic lutein; the 3′-ether could be further acetylated. Thus, pigment 4 has two hydroxyl groups one of which is in allylic position (C-3′) as in lutein.

Taxonomic distribution of rhodoxanthin in higher plants

Thus far, rhodoxanthin has been identified in members of most gymnosperm families including the wellknown examples *Taxus baccata* and *Thuja occidentalis*, and has also been established in some species of pteridophytes [for references see 16]. Concerning the angiosperm plants, rhodoxanthin was found in the red-brown leaves of *Potamogeton natans* [11] and in the red berries of two *Lonicera* species [17]. The identification of rhodoxanthin in callus cultures of *Ricinus communis*, another angiosperm, is remarkable in that this carotenoid is not detectable in any part of the intact plant as revealed so far (the red pigment of the stigma of the *Ricinus* flower is watersoluble and may therefore be an anthocyanidin). It is assumed that the novel biosynthetic capability resulting in the accumulation of rhodoxanthin reflects the differentiation of the chromoplast switched on by specific hormone levels. This feature will be the subject of a following paper [6].

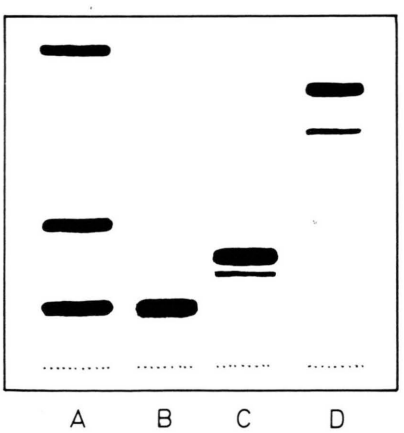


Fig. 3. Products of chemical reactions with lutein. (A) Reference carotenoids from the insect *Cerura vinula:* β,β-carotene, β,β-caroten-2-ol and lutein (from top); (B) lutein from *Ricinus communis;* (C) etherification of B with BF_3/ethanol; (D) acetylation of C.

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