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IDENTIFICATION AND QUANTIFICATION OF CAROTENOIDS AND CAROTENOID ESTERS FROM THE FORAMINIFERAN *ALLOGROMIA LATICOLLARIS* ARNOLD

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ABSTRACT

The ten carotenoids isolated from the cytoplasm of the monothalamous foraminiferan *Allogromia laticollaris* Arnold (Protozoa, Rhizopoda) have been identified as β-carotene (1), lutein (2), anhydrolutein-II (4a), anhydrolutein-II palmitate (4b), zeaxanthin (7a), zeaxanthin ester (7b), astaxanthin dipalmitate (8b) and, after saponification, allogromiarubin (3′,4′-didehydro-3,4-diketo-β-carotene, 10a), β-doradecin (13a) and phoeniconone (17). Most of these pigments represent secondary carotenoids produced in the foraminiferan by metabolism of primary carotenoids, which are derived from the food organism, the green alga *Chlorella*. Anhydrolutein-II (4a), its palmitate 4b and allogromiarubin (10a) have not been found as natural products. The possible biosynthesis of the secondary carotenoids is discussed.

INTRODUCTION

It is well known that among single celled organisms carotenoids are present as cell pigments within the phytoflagellates (Goodwin, 1979) which contain chloroplasts or chromoplasts. Earlier investigations of the two monothalamous foraminifera *Allogromia laticollaris* Arnold (Schwab and Schlobach, 1973, 1979) and *Myxotheca arenilega* Schaudinn (Schwab and Schlobach, 1979) showed that carotenoids and carotenoid esters could also be identified in protozoans lacking chromoatophores. The presence of these pigments in small quantities hindered their identification. Furthermore, it has so far not been clarified, whether the foraminifera are able to synthesize their own cell pigments, or whether the foraminifera only transfer primary carotenoids ingested with the food organism *Chlorella* into secondary carotenoids (cf. Thommen, 1971). Pigment analysis has shown that *Chlorella* possesses numerous primary carotenoids such as α-carotene, β-carotene, rhodoxanthin, sarcinaxanthin, lutein, neoxanthin and zeaxanthin (Karrer and Jucker, 1948; Itawa and others, 1961). Of plant cells it is known, that under special conditions of cultivation (lack of nitrogen, low pH of the incubation medium or strong illumination), primary carotenoids are transferred to oxidized and esterified carotenoids (Tischer, 1941; Goodwin and Jamikorn, 1954; Kleinig, 1967; Czygan, 1968; Kleinig and Czygan, 1969). Secondary carotenoids have also been isolated from *Chlorella* (Dersch, 1960; Kessler and others, 1963; Kessler and Czygan, 1965).

To date, the carotenoids found in animals are only metabolites derived from chemical components ingested with food (Thommen, 1971). In the present investigation 10 pigments have been isolated from the monothalamous foraminiferan *Allogromia laticollaris*. Their structures were established by chemical and spectroscopic evidence. The origin of these pigments is also discussed.

MATERIAL AND METHODS

SPECTROMETERS

IR-spectra: Perkin-Elmer, model 21 (KBr discs); UV-spectra: Beckmann DB-GT; ^{1}H-NMR-spectra: Varian XL 100 (Deuterium as internal standard); mass spectra: Varian MAT 731 (70 eV, high resolution with perfluorkerosene as standard).

CHROMATOGRAPHY

Preparative thin-layer chromatography (prep. TLC): A suspension of 55 g silica gel P/U_{254} (Macherey and Nagel, Düren, FRG) in 120 ml water (yielding 145 ml) was spread on 20 × 40 cm glass plates. The air-dried layers were activated for 3 hr at 130°C.

Thin-layer chromatography (TLC): silica gel plates (Polygram SIL G/UV_{254}, Macherey and Nagel, Düren, FRG). Solvents were benzene/hexane (1:3), chloroform and chloroform/5–10% acetone.

Column chromatography: A homogenized and degassed suspension of precipitated calcium carbonate in benzene/1% methanol was filled in a column (25 × 300 mm, RCT-Multichrom® column, Reichelt-Chemietechnik, Heidelberg, FRG) and compressed to 200 mm at a maximum pressure of 8 bar.

Distribution-chromatography of saponified pigments: cellulose plates (Polygram® CEL 300, Macherey and Nagel, Düren, FRG) which had been impregnated with a solution of 10% triglyceride (plant oil) in hexane. Development solvent methanol/acetone/water (30:10:2) (Kleinig and Czygan, 1969). Polyamide F 1700-micropolyamide thin-layer chromatography plates (Schleicher and Schüll, Dassel, FRG) were used with petroleum ether/methanol/ethyl-methylketone (40:5:5) (Kleinig and Czygan, 1969) as solvent. All zones of the developed chromatograms were numbered from the highest to the lowest R_f-values.

Gas chromatography/mass spectroscopy: Support OV-17 impregnated with 3% SE 30, column 1.5 m × 2 mm, silanized, carrier gas He, injector and detector temperature 270°C, temperature program 130–250°C,

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10°C/min. Gas chromatography: Varian 1700 joined to a mass spectrometer Varian MAT CH 7 by a Biemann-Watson separator.

Reference Substances

Astaxanthin (**8a**), its ester **8b**, β-doradecin (**13a**) and phoeniconone (**17**) were kindly provided by Hoffmann-La Roche and Co., Basel, Switzerland. Astacene (**9a**) (Barton and others, 1963) and isozeaxanthin (**6**) (Petracek and Zechmeister, 1956) have been synthesized from canthaxanthin (**14b**), and zeaxanthin (**7a**) (Karrer and Jucker, 1947) from lutein (**2**). Astacene-dipalmitate (**9b**) was prepared according to Kuhn and Sörensen (1938). Rhodoxanthin (**18**) was isolated from yew tree berries (Karrer and Jucker, 1948).

Anhydrolutein-II (**4a**) [by modification of the method of Zechmeister and Sease (1943)]. Lutein (**2**) (50 mg) was carefully melted together with boric acid hydrate (100 mg) and 2,3-dimethyl-naphthalene (250 mg) in a test tube for about 10 seconds until all **2** was consumed (TLC). Dimethylnaphthalene was separated by prep. TLC (chloroform) and the dehydrolutein mixture was further purified by prep. TLC with chloroform/6.5% ethyl acetate. The anhydrolutein-II (**4a**) from the main zone crystallized from hexane/methanol as irregularly-shaped flat needles; yield 20 mg (41%) **4a**. Under these conditions the anhydroluteins I (**3**) and III (**5**) appeared only in small amounts and were not isolated. UV (hexane): Anhydrolutein-I (**3**): λ_{max} (calc.) = 463 nm, found (Zechmeister and Sease, 1943): 460 nm, Anhydrolutein-II (**4a**): λ_{max} (calc.) = 445 nm, found: 446 nm (own value), 446 nm (Zechmeister and Sease, 1943); Anhydrolutein-III (**5**): λ_{max} (calc.) = 457 nm, found (Zechmeister and Sease): 447 nm.

Cultivation of the Protozoa and Storage of the Cell Material

The foraminifer *A. laticollaris* was cultivated in petri dishes (Ø14 cm) at 20°C in seawater-Erdschreiber medium (seawater,* earth-decoct, 1.1 mM $NaNO_3$ and 0.11 mM Na_2HPO_4) in combination of daylight and artificial light (neon tube Osram L-Interna 40 W/39, spectral composition: small peaks at 400 nm and 420 nm, broad maximum between 500 and 800 nm with peaks at 550 nm and 660 nm; light intensity 500 lux; light/dark cycle: 16/8 h). A marine species of the green alga *Chlorella* served as food organism. The foraminiferan cells were separated from excess water and stored in liquid nitrogen for a period of one year.

Extraction and Separation of the Carotenoids

The collected cell material (123 g = 1.695 g dry weight) was first separated from seawater by filtering through a layer of celite®. The residue was resuspended in acetone (500 ml) together with the filter aid and homogenized in an Ultraturrax. The sediment, after filtration, was washed alternatively with acetone and hexane until the filtrate was colorless. The combined filtrates, after double extraction with water, yielded an oily residue (2.8 g) obstinately retaining some solvent. The pigments from the crude extract were separated into three zone groups (**I–III**, Fig. 1a) by prep. TLC (benzene). The least polar zone group **I** contained three well-separated yellow zones (**Ia–Ic**), which appeared uniform. Group **II** consisted of two incompletely separated reddish-orange zones (**IIa** and **IIb**) and group **III** contained polar substances at the starting region.

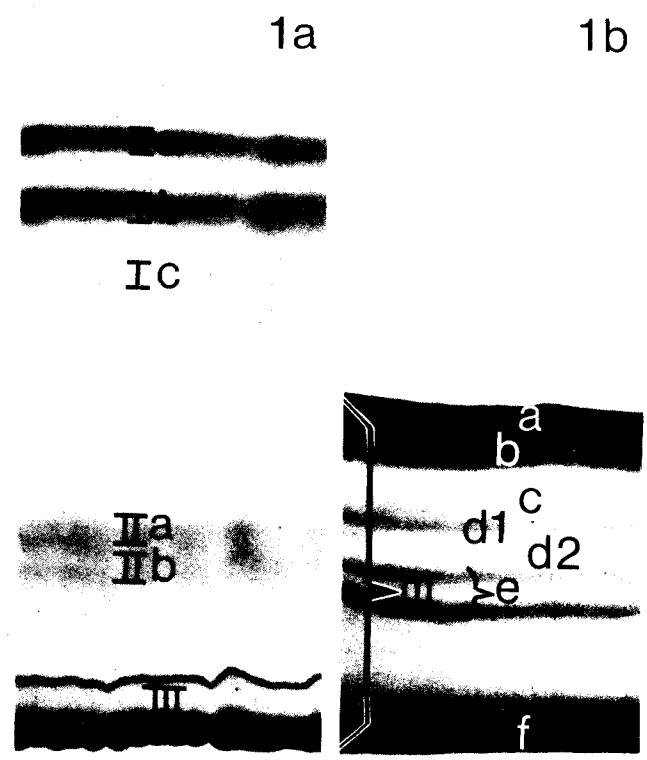


FIGURE 1a. Separation of acetone/hexane pigment extract from *Allogromia laticollaris* by prep. TLC (benzene) into zone groups **I–III**.

FIGURE 1b. Rechromatography (chloroform) of zone group **III** into the zones **IIIa–f**.

The reddish-orange zones **IIa** and **IIb** were separated by further chromatography (prep. TLC) with benzene as solvent. Group **III**, which contained several components, was rechromatographed repeatedly by prep. TLC (chloroform). Thereby the zones **IIIa–e** were separated, whereas zone **IIIf** remained at the starting region (Fig. 1b). The zone **IIId** on rechromatography (prep. TLC, chloroform/5% acetone) separated into two orange zones, $\mathbf{IIId_1}$ and $\mathbf{IIId_2}$. Zone **IIIf** separated into two yellow zones, $\mathbf{IIIf_1}$ and $\mathbf{IIIf_2}$. From zone **IIIe** only the uppermost reddish-orange main component was isolated.

All these pigments appeared to be chromatographically pure on silica gel (benzene/hexane, 3:1, chloroform or chloroform/5–10% acetone) and impregnated cellulose (Kleinig and Czygan, 1969) with regard to the

* The seawater was procured from the Biologische Anstalt Helgoland, List auf Sylt, FRG.

carotenoids, but were contaminated with oily components. Therefore, only the qualitative electron spectra were recorded (Figs. 2–13) and the esters saponified.

Saponification

The different carotenoid esters were dissolved in hexane (20 ml) and warmed to 40°C with 5% ethanolic potassium hydroxide (5 ml) for 3 hr. The solution was diluted with water and the *hypophasic* pigments extracted with chloroform. In order to obtain the *hydrophasic* components the solution was acidified with 1 N HCl (pH 5) and again extracted with chloroform. The free carotenoids obtained by this procedure will be henceforth denoted by an "H" positioned after the zone number.

Pigments

Pigment Ia (IaH, β-carotene, 1)

After filtration of the hexane solution through a layer of Al_2O_3 (activity IV, neutral, Woelm, Eschwege, FRG) the pigment was identified as β-carotene (**1**) by its smell of violets and its R_F-value (TLC, benzene/cyclohexane, 1:1), electron spectrum (Fig. 2) and mass spectrum by comparison with authentic (**1**) (Roth, Heidelberg, FRG).

Pigment Ib (anhydrolutein-II palmitate, 4b)

UV (hexane): λ_{max} = 473, 446, 425 nm sh.

Pigment IbH, IIIcH (anhydrolutein-II, 4a)

The benzene solution, after filtration through a layer of Al_2O_3 (activity IV, neutral, Woelm, Eschwege, FRG) gave on evaporation ochre-colored prisms with clearly hydrophasic behavior in hexane/95% methanol. UV (hexane): λ_{max} (1g ε) = 422 (4.89) 445 (5.03), 473 nm (4.99) (Fig. 3); (benzene): λ_{max} = 433, 457, 486 nm. MS (70eV): m/e = 550(10%, M^+), 532(1%), 430(2%), 428(3%), 427(3%), 426(5%), 415(3%), 414(8%), 413(3%), 412(6%), 411(7%), 400(3%), 399(5%), 398(3%), 397(4%), 396(6%), 314(13%), 91(31%), 85(44%), 83(64%), 81(50%), 71(63%), 57(100%) (Fig. 14). The product did not differ from synthetic anhydrolutein-II (**4a**) in its chromatographic behavior on silica gel, polyamide and impregnated cellulose and in its spectroscopic properties. $C_{40}H_{54}O$, calc. mass: 550.41744, found 550.4173 (MS).

Identification of palmitic acid

Ib was hydrolysed as described above. The alkaline solution acidified after separation of **IbH** (**4a**) and extracted with ether. The extract was evaporated to dryness and the residue methylated with diazomethane. The product was identified as palmitic acid methylester by gas chromatography/mass spectrometry as well as gas chromatographically by coinjection with synthetic methyl palmitate. In the same manner palmitic acid was identified as the only acidic constituent of pigment **IIIa**. Astaxanthin (**8a**), in *A. laticollaris* therefore, is esterified only with palmitic acid.

Pigment Ic (zeaxanthinester, 7b)

UV (hexane): λ_{max} = 475, 447, 425 sh, 337 nm (Fig. 4).

Pigment IcH (zeaxanthin, 7a)

The crude **7a** was separated from several yellow byproducts (TLC, chloroform/5% acetone) yielding the pigment as ochre-colored prisms. UV (hexane): λ_{max} = 471, 447.5, 422 sh, 335 nm (Fig. 4). MS (70eV): m/e = 568(100%, M^+), 550(5%, M-H_2O), 532(1%, M-2 H_2O), 489(2%), 476(32%), 412(2%), 415(2%), 410(5%), 375(2%), 349(3%). $C_{40}H_{56}O_2$, calc. mass: 568.4280, found: 568.4283 (MS). The R_F-value of **7a** on silica gel and polyamide is lower than that of lutein (**2**) or isozeaxanthin (**6**) and does not differ from partially synthetic zeaxanthin (**7a**) (Karrer and Jucker, 1947). Saponification of **7b** carried out for only a short time gave a zeaxanthin monoester with an R_F-value between **Ic** (**7b**) and **IcH** (**7a**).

Pigment IIa (allogromiaxanthin palmitate, 3',4'-didehydro-3-hydroxy-4-keto-β-carotene palmitate, 10b)

UV (benzene): λ_{max} = 485, 470 nm sh (Fig. 5).

Pigment IIaH (allogromiarubin, 3',4'-didehydro-3,4-diketo-β-carotene, 10a)

The pigment was rechromatographed by prep. TLC (chloroform/5% acetone) followed by column chromatography over calcium carbonate (benzene/1% methanol). On evaporation of the hexane solution **10a** crystallized as dark-red needles. The R_F-value of **IIaH** (**10a**) on oxalic acid-silica gel (chloroform) is higher than that of canthaxanthin (**14b**) or rhodoxanthin (**18**).

FIGURE 2–13. UV-spectra of the foraminiferal pigments **Ia–IIIf** and reference carotenoids. For comparison, the qualitative spectra are normed on an extinction of 175,000; wavelength in nm.

FIGURE 2. Isolated (**Ia**, ——) and authentic (·····) β-carotene (**1**) in hexane.

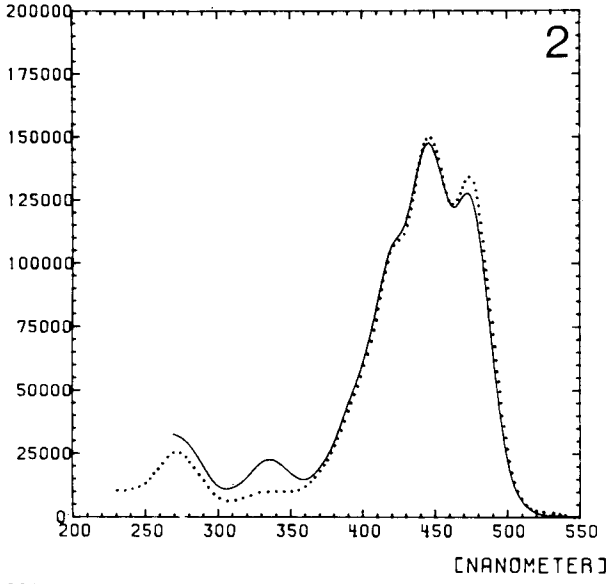
FIGURE 3. Isolated (**IbH, IIIcH**, ——) and synthetic (·····) anhydrolutein-II (**4a**) in hexane.

FIGURE 4. Isolated (**IcH, IIIf$_2$**, ——) zeaxanthin (**7a**) and zeaxanthin palmitate (**Ic, 7b**, ·····) in hexane.

FIGURE 5. Allogromiarubin (**IIaH, 10a**, ——), allogromiaxanthin palmitate (**IIa, 10b**, ·····) und allogromiarubin acetate (**10c**, – – –) in benzene.

FIGURE 6. Adonixanthin palmitate (**IIb, 13b**, ——), β-doradecin (**IIbH, 13a**, ·····) and β-doradecin acetate (**13c**, – – –) in benzene.

FIGURE 7. Isolated (**IIbH, 13a**, ——) and authentic (·····) β-doradecin (**13a**) in benzene.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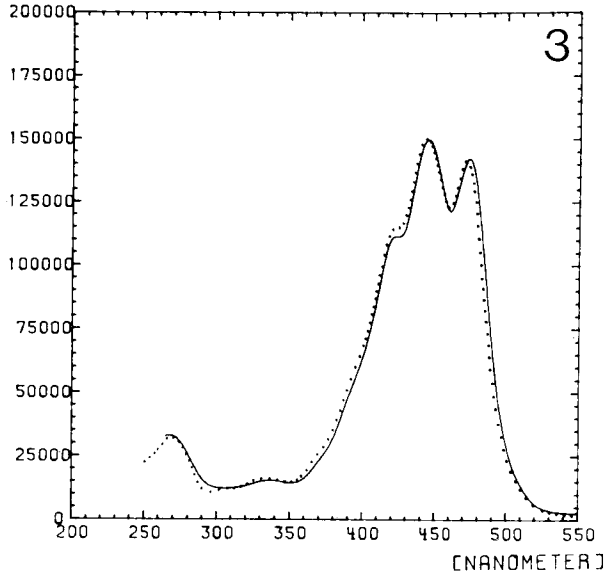
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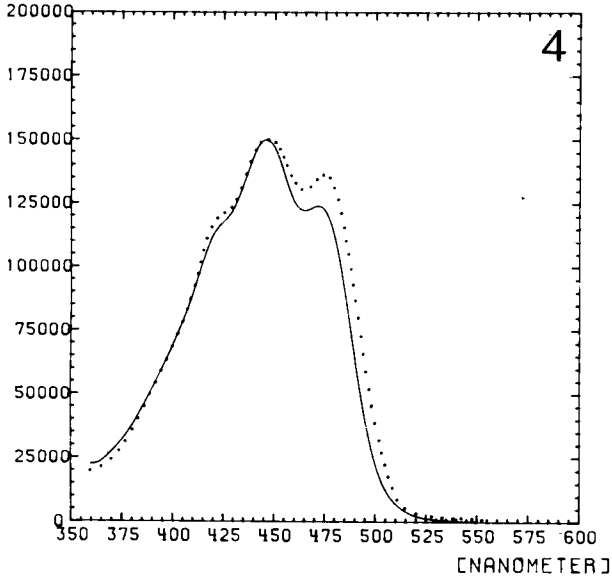
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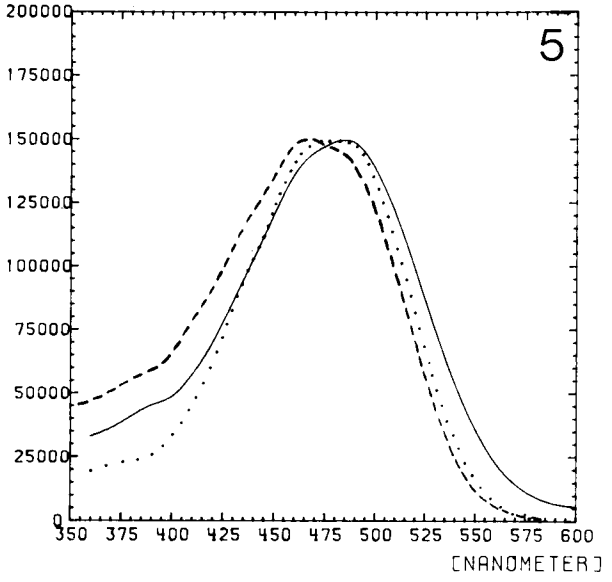
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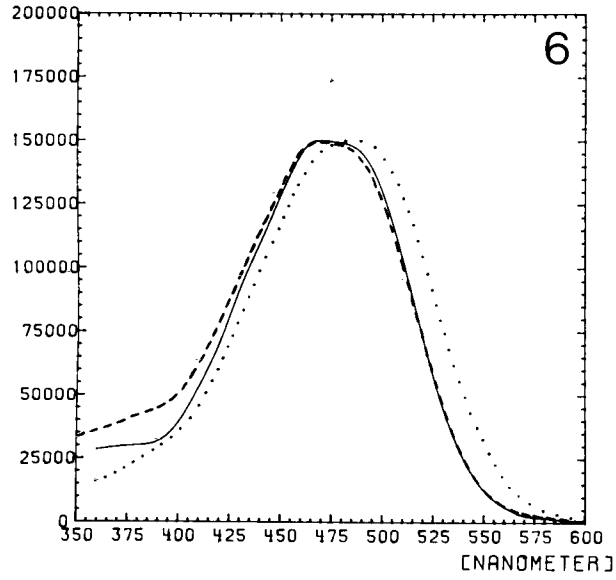
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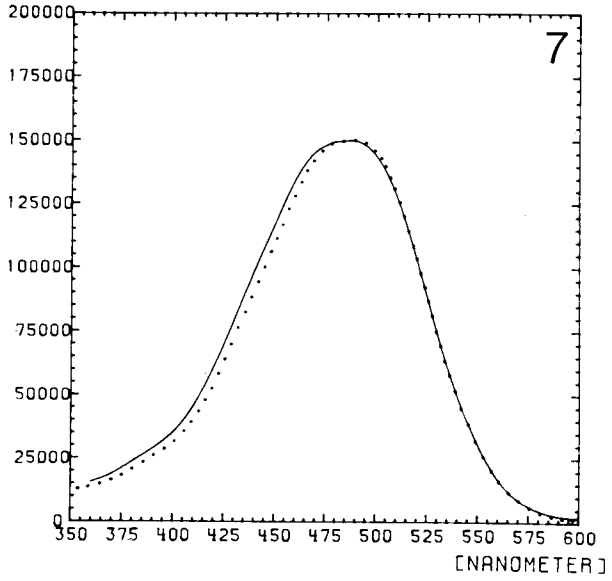
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Pigment **10a** is irreversibly adsorbed on aluminum oxide. UV (benzene): λ_{max} = 486, 475 nm sh (Fig. 5). MS (70eV): m/e = 562(19%, M^+), 494(3%), 410(4%), 269/267(2%), 241(2%), 229(6%), 203(100%), 179(5%), 165(9%), 157(9%), (Fig. 15). $C_{40}H_{50}O_2$, calc. mass: 562.38106, found: 562.3792 (MS).

3',4'-Didehydro-3,4-diketo-β-carotene acetate (10c)

A solution of 3',4'-didehydro-3,4-diketo-β-carotene (**10a**, 0.1 mg) in acetanhydride/pyridine (3:1, 0.3 ml) was kept at room temperature. After 5 hrs the mixture was hydrolyzed and the chloroform extract purified by prep. TLC (chloroform). UV (hexane): λ_{max} = 457, 475 nm sh; (benzene): λ_{max} = 466 nm (Fig. 5). MS (70eV): m/e = 604(5%, M^+), no fragments in the range of m/e = 200–600.

Pigment IIb (adonixanthin ester, 13b)

UV (benzene): λ_{max} = 470, 487 nm sh (Fig. 6).

Pigment IIbH (β-doradecin, 13a)

Rechromatography by prep. TLC (chloroform/5% acetone) followed by column chromatography (calcium carbonate, benzene/1% methanol) gave from the main zone a solid, which crystallized on titrating with hexane as brownish-black needles, identical with an authentic sample. UV (benzene): λ_{max} = 480 nm (Fig. 7); (pyridine): λ_{max} = 478 nm; (hexane): λ_{max} = 460 nm; unstructured bands. MS (70eV): m/e = 580(43%, M^+), 562(9%, M-H_2O), 488(4%), 428(4%), 203(93%), 165(37%), 159(35%), 157(59%), 145(55%), 143(47%), 119(79%), 105(76%), 91(100%). $C_{40}H_{52}O_3$, calc. mass: 580.39162, found: 580.3889 (MS).

β-Doradecin-diacetate (13c)

Preparation as described for pigment **10c**. UV (hexane): λ_{max} = 453, 471 nm sh (Fig. 6); (benzene): λ_{max} = 470 nm. MS (70eV): 664(3%, M^+), 662(4%, M-ketene), 602(3%), 528(8%), 482(20%), 404(5%), 362(21%), 203(25%).

Pigment IIIa (astaxanthin dipalmitate, 8b)

Rechromatography by prep. TLC (chloroform) or column chromatography (calcium carbonate, benzene) gave brownish-violet prisms on triturating with ethanol. UV (hexane): λ_{max} = 468 nm; (benzene): λ_{max} = 486 nm (Fig. 8). $C_{72}H_{112}O_6$, calc. mass: 1072.9 found: 1072.5 (MS by field desorption, U_A = 2 kV).

Pigment IIIaH (astacene, 9a)

The pigment was purified by chromatography over silica gel (prep. TLC, chloroform/5% acetone) and calcium carbonate (column, benzene/1% methanol). On dilution, the concentrated hexane/methanol solution with water afforded black needles, with a bluish-black sheen. The pigment is hydrophasic in hexane/95% methanol. UV (benzene): λ_{max} (1g ε) = 492.5 nm (4.96) (Fig. 13). MS (70eV): m/e 592(40%, M^+), 577(2%), 500(4%), 486(2%), 203(66%), 91(100%). $C_{40}H_{48}O_4$, calc. mass: 592.35524, found: 592.3550 (MS).

Pigment IIIcH and IIIfH

These pigments were filtered over Al_2O_3 (neutral, activity IV, Wölm, Eschwege, FRG) with chloroform to separate oily impurities, TLC (chloroform/5% acetone) gave the pigments **IIIcH**, **IIIf$_1$H** and **IIIf$_2$H** which were identified as anhydrolutein-II (**4a**, **IIIc** = **IIIcH**), lutein (**2**, **IIIf$_1$** = **IIIf$_1$H**) and zeaxanthin (**7a**, **IIIf$_2$** = **IIIf$_2$H**) by their electron spectra (Figs. 4, 11), mass spectra and R_F-values (silica gel and polyamide).

The following pigments, obtained only in trace amounts, were contaminated with colorless oily impurities and not worked up:

1) Pigment **IIIb**. UV (benzene): λ_{max} = 485, 465 nm sh (Fig. 9).
2) Pigment **IIIbH**, identical with pigment **IIIaH** (astacene, **9a**).
3) Pigment **IIId$_1$**. UV (benzene): λ_{max} = 480 nm (Fig. 10).
4) Pigment **IIId$_2$**. UV (benzene): λ_{max} = 465, 480 nm sh (Fig. 10).
5) Pigment **IIIe$_1$H**, phoeniconone (**17**). UV (benzene): λ_{max} = 487 nm (Fig. 12).

Quantification

For quantitative determination of the carotenoids and carotenoid esters the following specific extinction coefficients were used ($E^{1\%}_{1cm}$ at λ_{max}): β-carotene (**1**) = 2592 (hexane, 453 nm; Davies, 1976); anhydrolutein-II ester (**4b**) calc. as anhydrolutein-II (**4a**) = 1953 (hexane, 445 nm; own data); zeaxanthin ester (**7b**) calc. as zeaxanthin (**7a**) = 2350 (petroleum ether, 452 nm; Davies, 1976); ester **IIa** (**10b**) and ester **IIb** (**13b**) calc. as echinenone (**19**) = 2091 (benzene, 472 nm; Davies 1976); ester **IIIa** (**8b**) calc. as astacene (**9a**) = 1532 (492.5 nm; own data); ester **IIIb**, carotenoids **IIId$_1$**, **IIId$_2$**, **IIIe$_1$** and **IIIe$_2$** calc. as canthaxanthin (**14b**) = 2092 (benzene, 480 nm; Davies, 1976).

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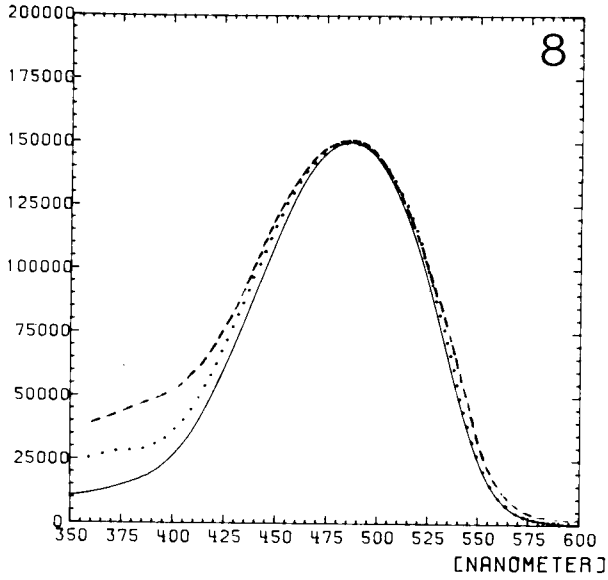
FIGURE 8. Astacene dipalmitate (**9b**, – – –), synthetic (——) and isolated (**IIIa**, · · · · ·) astaxanthin dipalmitate (**8b**) in benzene.
FIGURE 9. Pigment **IIIb** in benzene.
FIGURE 10. Pigment **IIId$_2$** (——) and **IIId$_1$** (· · · · ·) in benzene.
FIGURE 11. Isolated (**IIIf$_1$H**, ——) and authentic (· · · · ·) lutein (**2**) in hexane.
FIGURE 12. Isolated (**IIIe$_1$H**, ——) and authentic (· · · · ·) phoeniconone (**17**) in benzene.
FIGURE 13. Isolated (**IIIaH**) = authentic (——) astacene (**9a**) and astaxanthin (**8a**, · · · · ·) in benzene.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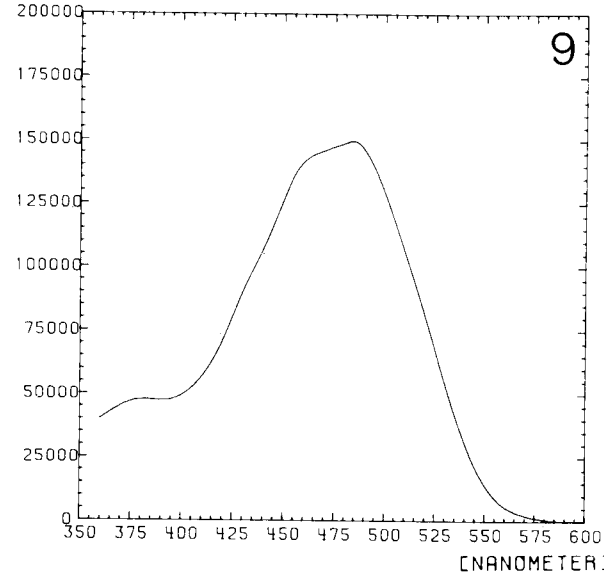
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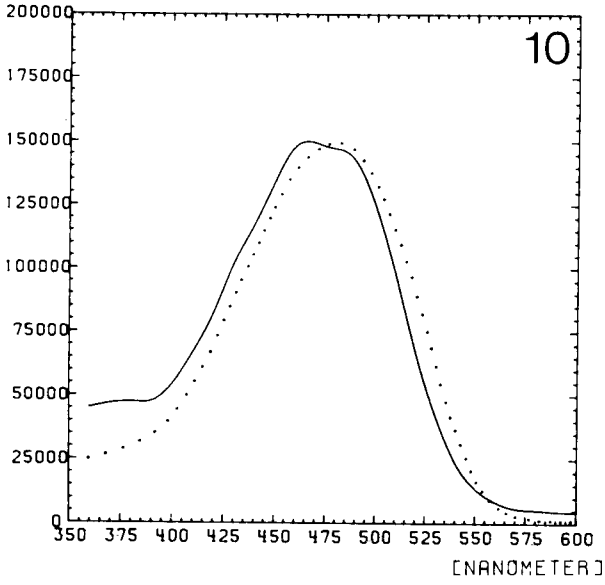
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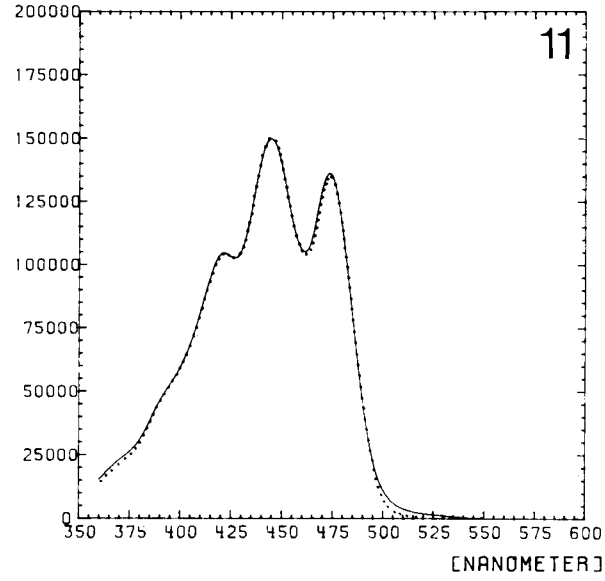
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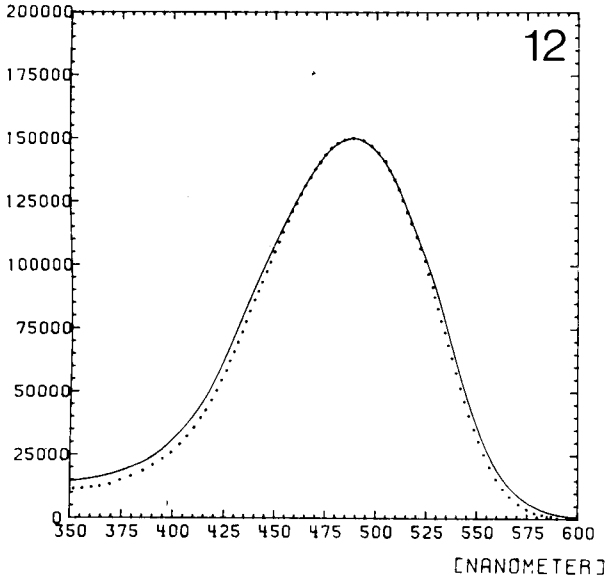
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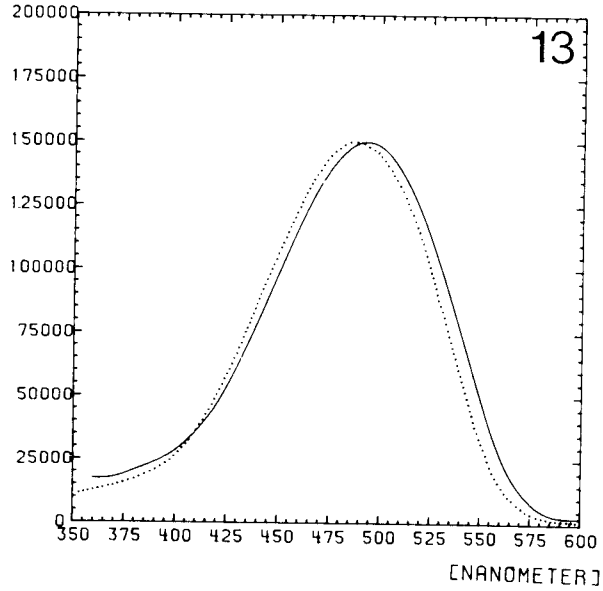
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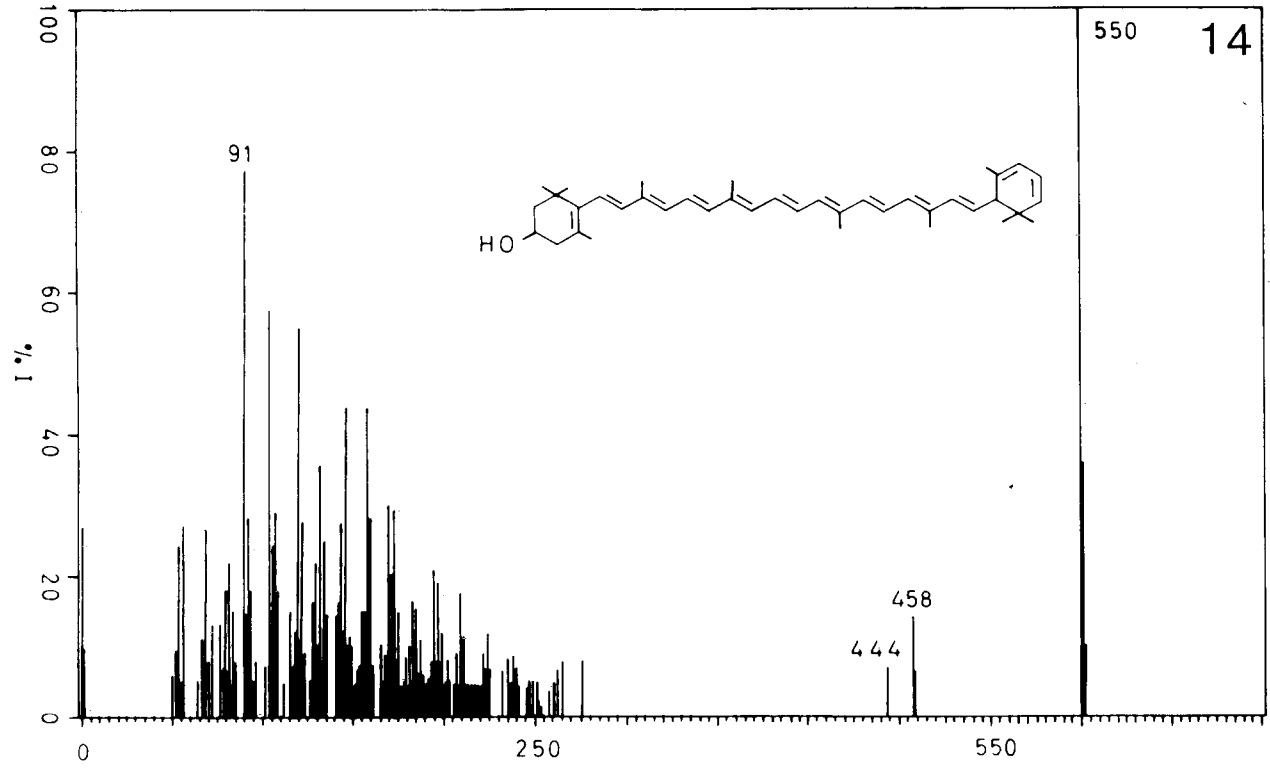


FIGURE 14. Mass spectrum of anhydrolutein-II (**IbH**, **4a**).

RESULTS

Crude acetone/hexane extracts obtained from the deep reddish-orange colored monothalamous foraminiferan *A. laticollaris* were separated into three zones (**I–III**) by repeated chromatography over silica gel. The individual zones after rechromatography over silica gel and calcium carbonate further separated into three yellow pigments (**Ia–Ic**), four orange-to-red components (**IIa**, **IIb**, **IIIa** and **IIIb**) and yellow or red minor constituents (**IIIc–IIIf**) respectively (Fig. 1a/1b).

The main carotenoids showed epiphasic solubility (Kuhn and Brockmann, 1932) in hexane/95% methanol and were homogenous on triglyceride-impregnated cellulose. The electron spectra of the yellow components showed a well-defined band structure whereas the deeper colored components showed only unstructured maxima in benzene and ethanol. For these characteristics the latter were supposed to be keto-carotenoids (Vetter and others, 1971).

Although the components **Ia-Ic** and **IIb** were chromatographically pure, with regard to the pigments, they were found to contain oily substances which made their separation difficult. The crude products therefore saponified in the usual manner, after which the polarity of all the carotenoids increased except that of **Ia**. This establishes the pigments as carotenoid esters. Except pigment **Ic**, the free carotenoids were hydrophasic. By chromatographic purification they were characterized by electron- and mass spectra or by chromatographic and spectroscopic comparison using reference substances. The polar components **IIIc–IIIf** were treated in the same way. They represented free carotenoids as shown by their R_F-values, which remained unchanged by hydrolysis.

The least polar pigment **Ia** was identified as β-carotene (**1**) by comparison with an authentic specimen (smell as β-ionone, R_F-value, UV, MS). The intensity of the band at 335 nm compared to authentic **1** revealed a high content of *cis*-β-carotene (Davies, 1976) (Fig. 2).

The electron spectrum of pigment **Ib** was very similar to that of β-carotene (**1**) (Fig. 2) and showed a characteristic ester-carbonyl band at 1742 cm^{-1} in its IR-spectrum. On saponification only one carotenoid (**IbH**) was formed which was more polar than **Ib**. Its electron spectrum did not differ from that of **Ib** (Fig. 3). The empirical formula was determined by high resolution mass spectroscopy as $C_{40}H_{54}O$. Carotenoids of this composition were obtained by partial synthesis and dehydration from lutein (**2**). Without determining a definite chemical structure, these pigments have been designated as desoxy- or anhydroluteins-I, -II and -III (Zechmeister and Sease, 1943; Budowski and others, 1963; Dutrieu and Hempel, 1964). They may have the chemical formula **3–5**. A comparison of the calculated wavelengths (Vetter and others, 1972) of the main maximum of the isomers **3–5** in hexane showed that only the value predicted for anhydrolutein-II (**4a**) agrees with the spectrum measured from **IbH**. The identities

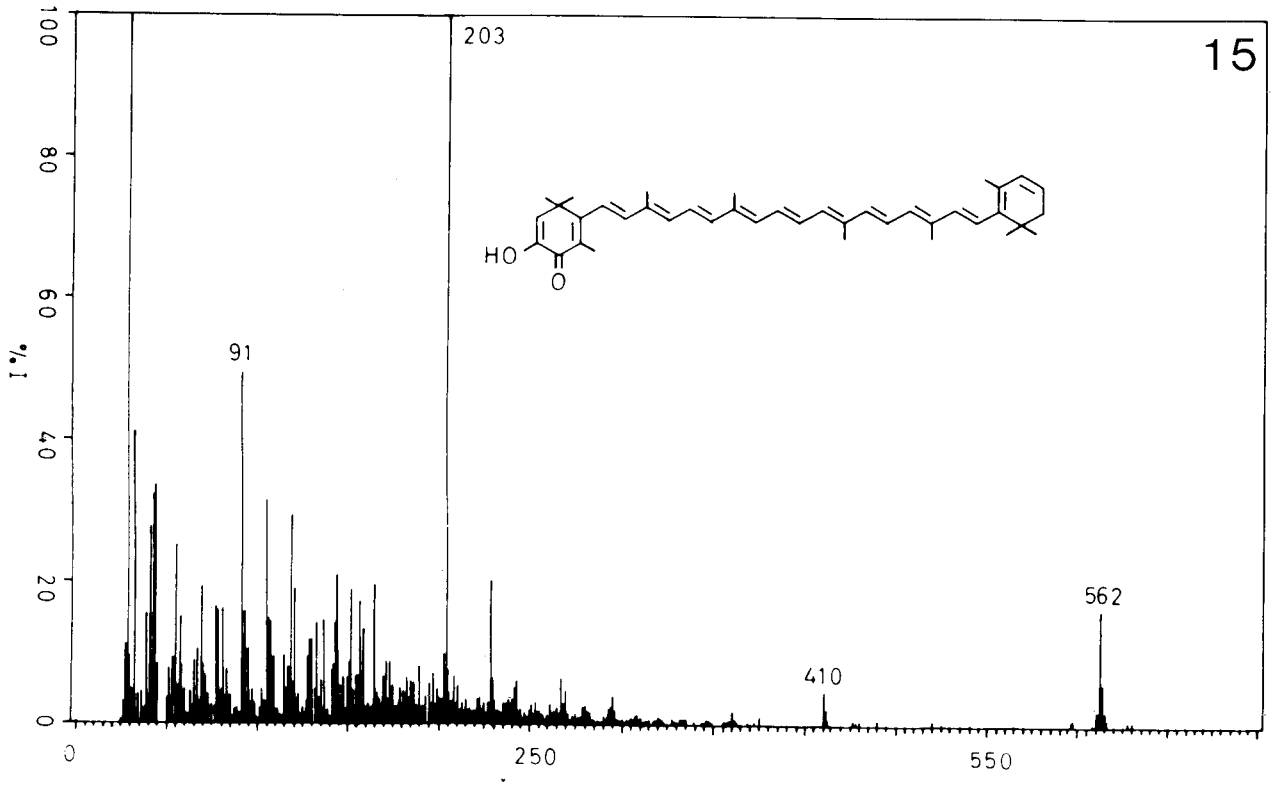


FIGURE 15. Mass spectrum of allogromiarubin (**IIaH**, **10a**).

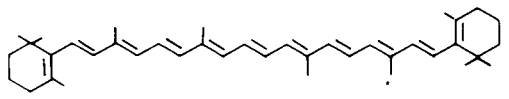
of the pigments **IbH** and anhydrolutein-II (**4a**) were shown by direct comparison (R_F, UV Fig. 3, MS Fig. 14) of the isolated and the partially synthesized material.

The fatty acid obtained after saponification of pigment **Ib** in alkaline solution was methylated with diazomethane and identified as palmitic acid (GC, MS). Anhydrolutein-II (**4a**) must therefore be present in pigment **Ib** as palmitate (**4b**). The chromatographic homogeneity of **Ib** (**4b**) on triglyceride- and paraffinoil-impregnated cellulose (Kleinig and Czygan, 1969), as well as the gas chromatogram of the isolated acid indicated that there are no additional homologous or unsaturated fatty acids present.

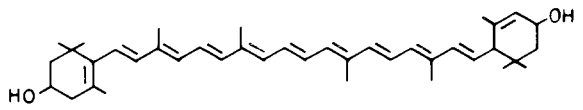
During the saponification of pigment **Ic** (**7b**) a monoester was isolated. The main reaction product **IcH** was a bright yellow solid. This showed the typical α/β-carotenoid electron spectrum with an empirical formula of $C_{40}H_{56}O_2$ (MS). **IcH** was identified as zeaxanthin (**7a**) by the fact that the R_F-value determined on silica gel and polyamide is lower than that of lutein (**2**) and isozeaxanthin (**6**) and by its characteristic mass spectrum (molecule ion base peak; relatively slight release of water compared to lutein (**2**); high M-92 signal; see Enzell and Francis, 1969; Isler, 1971). In the electron spectrum (Fig. 4) an enhanced *cis*-portion was observed. The classification was established by direct comparison with partially synthetic zeaxanthin (**7a**) (Karrer and Jucker, 1947).

The reddish-orange pigments **IIa** and **IIb** showed in hexane, partially, and in ethanol totally unstructured electron spectra (Figs. 5, 6). The saponification of these pigments followed by acetylation yielded acetates with similar spectra. These facts led to the assumption that the pigments are carotenoids, containing conjugated carbonyl groups (Vetter and others, 1971). This was verified by the mass spectra of the hydrolysed products, $C_{40}H_{50}O_2$ (**IIaH**) and $C_{40}H_{52}O_3$ (**IIbH**), showing an intense fragment with a mass of m/e = 203, characteristic for carotenoids containing 3-hydroxy-4-keto- or 3,4-diketo endgroups as in **8a** or **9a** (Isler, 1971). As expected, the reduction of these pigments with sodiumborohydride in 90% ethanol (Hager and Stransky, 1970) was unsuccessful, but succeeded with lithium aluminium hydride as described earlier for astacene (**9a**) (Baldas and Porter, 1969).

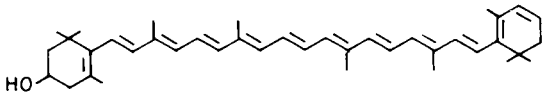
3-Hydroxy-4-keto-carotenoids, such as astaxanthin (**8a**), in alkaline solution on contact with air rapidly dehydrogenated to 3,4-diketo-carotenoids, for example **9a** (drawn as an enol; Kuhn and others, 1939). Since the carotenoid esters were saponified in the presence of air, all compounds containing 3-hydroxy-4-keto-end groups could be excluded as possible structures for pigments **IIaH** and **IIbH** in favor of the end group in **9a**. In agreement with this fact, no release of water could be observed in the mass spectrum of **IIaH** (Fig. 15). Furthermore, no fragments with the mass M-167 and M-233 could be identified, which would have been observed from monoketo-carotenoids with one oxygen per end group (Isler, 1971). The chromatographic be-



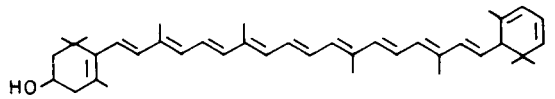
1: ß-Carotene



2: Lutein

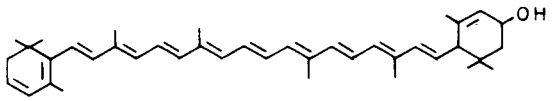


3: Anhydrolutein-I



4a: Anhydrolutein-II

4b: palmitate instead of OH



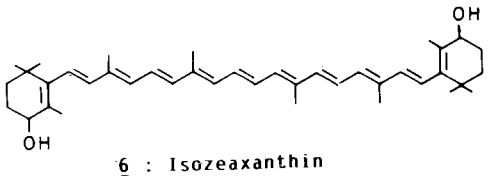
5: Anhydrolutein-III

havior of pigment **IIaH** on triglyceride-impregnated cellulose is different from that of rhodoxanthin (**18**) but corresponds to that of 3,4-diketo-β-carotene (**12a**). Therefore an allene or acetylene configuration is probably absent in the chain (Hager and Stransky, 1970). Pigment **IIaH** could be very easily transferred into a monoacetate (mol mass 604) and then cleaved back into the pigment **IIaH**. This experiment excludes the presence of epoxides or furanoid structures without free hydroxy-groups in the molecule. Considering the biogenetic relationship of the isolated pigments for the pigment **IIaH** with the empirical formula $C_{40}H_{50}O_2$ only two structures, **10a** and **11**, were probable. Since the absorption of pigment **IIaH** is shifted by 6 nm to a longer wavelength, compared to pigment **IIbH** with the structure **13a**, it must be 3',4'-didehydro-3,4-diketo-β-carotene **10a** with an additional conjugated double bond. This is not present in **11**.

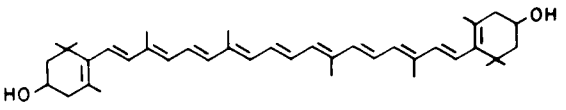
In the electron spectrum (Fig. 7; λ_{max} (hexane) = 460 nm) pigment **IIbH** corresponds well with 3,4-diketo-β-carotene (**12a**, euglenanone; λ_{max} (hexane) = 458) (Foppen, 1971), but differs by an additional hydroxy-group. This was expressed in the empirical formula, by the release of water in the mass spectrum and by the formation of diacetate. The compound was unchanged by hydrogen chloride-saturated benzene (Hager and Stransky, 1970) and was not esterified under standard conditions with butanol/HCl (Hager and Stransky, 1970). On triglyceride-impregnated cellulose it showed similar chromatographic behavior as 3'-hydroxy- and 4'-hydroxy-3,4-diketo-β-carotene (**12b**/**13a**). Adonirubin (**14a**) was excluded by the absence of signals in the mass spectrum at M-138, M-154, M-167, M-203, M-233 (Isler, 1971) and by the stability of **IIbH** against oxygen in alkaline solution. Of the three remaining structures: α-doradecin (**15**), β-doradecin (**13a**) and 4'-hydroxy-3-oxo-echinenone (**12b**), structure **13a** is the most probable one, in accordance with the above-discussed spectroscopic properties. Our electron spectra and those of earlier reports for β-doradecin (**13a**) in hexane (λ_{max} = 465 nm; Foppen, 1971), in pyridine (λ_{max} = 478 nm; Kleinig and Czygan, 1969) and in ethanol (λ_{max} = 465 nm; Davies, 1976) correspond satisfactorily and establish its identity as **13a**. This was further confirmed by direct comparison with an authentic sample.

The chromatographically purified pigment **IIIa** crystallized as brownish-violet, wax-like prisms on trituration with ethanol. The electron spectrum showed no structure in hexane. The carotenoid $C_{40}H_{48}O_4$, obtained by hydrolysis of pigment **IIIa**, corresponds in all properties to partially synthetic astacene (**9a**), (Kuhn and others, 1939). From astaxanthin esters (**8b**) it is well known that they can be very easily transformed into astacene (**9a**) during hydrolysis in the presence of oxygen. Therefore pigment **IIIa** could be an astaxanthin ester or an astacene ester. Pigment **IIIa** is identical in its R_F-value and electron spectrum with partial synthetic astaxanthin dipalmitate (**8b**), (Kuhn and others, 1939), but differs in the shape of the spectrum slightly from **9b**. Its field desorption mass spectrum showed a molecular ion at 1072.5 confirming the structure **8b** for **IIIa**.

As did pigment **IIIa**, pigment **IIIb** also changed to astacene (**9a**) after hydrolysis in the presence of air. The amount of pigment **IIIb** available was not sufficient to repeat the experiment under anaerobic conditions in order to identify the original chromophore in the ester. However, the absorption maximum, which is located at a shorter wavelength than in astacein (**9b**),

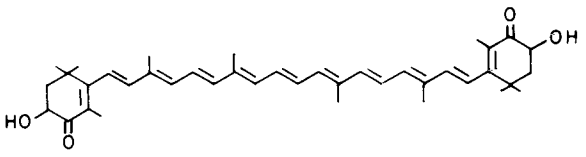


6 : Isozeaxanthin



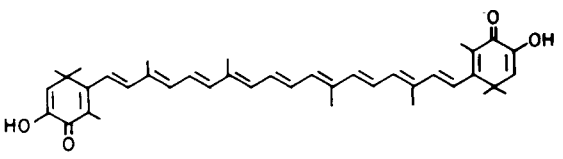
7a : Zeaxanthin

7b : palmitate instead of OH



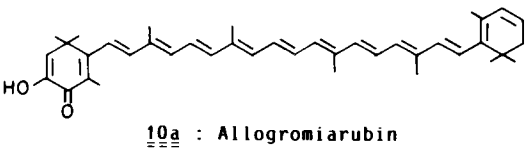
8a : Astaxanthin

8b : palmitate instead of OH



9a : Astacene

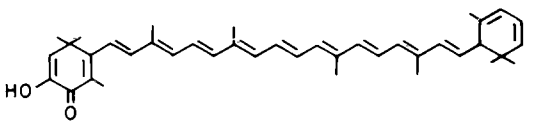
9b : palmitate instead of OH



10a : Allogromiarubin

10b : palmitate instead of OH
double bond C-2,3 hydrogenated

10c : acetate instead of OH·

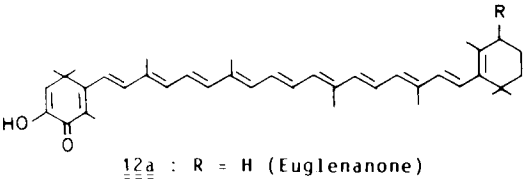


11 : 2',3'-Dehydro-3,4-
diketo-α-carotene

and the distinct band structure points to hydroxyketo-carotenoids like astaxanthin (**8a**) or 2,3-dihydro-astacene (**16**).

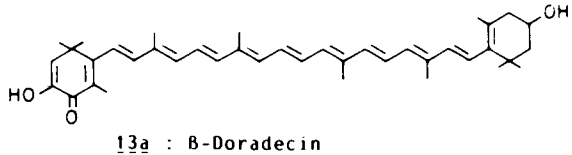
The yellow pigments **IIIc**, **IIIf**$_1$ and **IIIf**$_2$ did not change their chromatographic behavior on saponification. By their R_F-values, IR-, UV- (Figs. 3, 4, 11) and mass spectra they were identified as lutein (**2**), anhydrolutein-II (**4a**) and zeaxanthin (**7a**) respectively.

The reddish-orange pigments **IIId**$_1$, **IIId**$_2$, **IIIe**$_1$ and **IIIe**$_2$ (Figs. 10, 12) were available in very small amounts but precise investigation was carried out on **IIIe**$_1$**H**. Its electron spectrum (Fig. 12) and the R_F-value on triglyceride-impregnated cellulose (Czygan and Kessler, 1967) were the same as obtained from the authentic phoeniconone (**17**). The pigment **IIIe**$_1$**H** in its ester **IIIe**$_1$ is presumably hydrogenated in position 2, 3.



12a : R = H (Euglenanone)

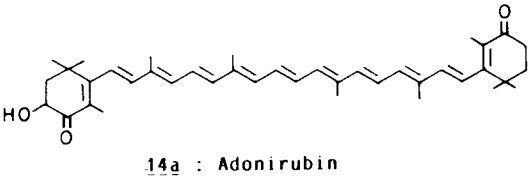
12b : R = OH



13a : β-Doradecin

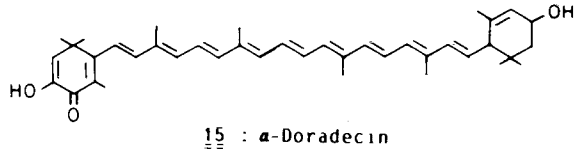
13b : palmitate instead of OH
double bond C-2,3 hydrogenated

13c : acetate instead of OH

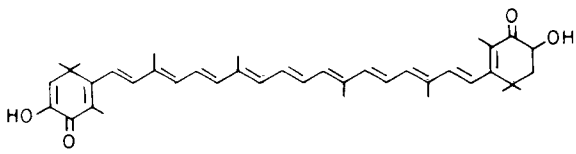


14a : Adonirubin

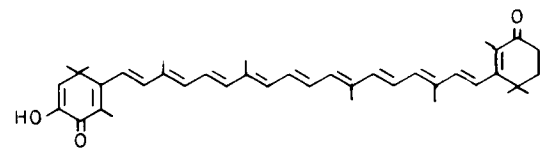
14b : H instead of OH



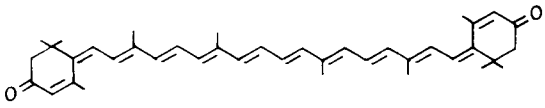
15 : α-Doradecin



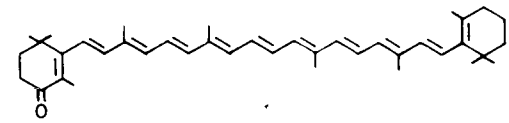
16 : 3-Hydroxy-3',4,4'-triketo-ß-carotene



17 : Phoeniconone



18 : Rhodoxanthin



19 : Echinenone

DISCUSSION

From the cell pigments of the monothalamous foraminiferan *A. laticollaris* ten free or esterified carotenoids have been isolated. They were identified as β-carotene (**1**), lutein (**2**), anhydrolutein-II (**4a**), anhydrolutein-II palmitate (**4b**), zeaxanthin (**7a**), zeaxanthin ester (**7b**), astaxanthin dipalmitate (**8b**), allogromiarubin (**10a**, 3',4'-didehydro-3,4-diketo-β-carotene), β-doradecin (**13a**), and phoeniconone (**17**).

This is the first reported occurrence of anhydrolutein-II (**4a**), its palmitate **4b**, and 3',4'-didehydro-3,4-diketo-β-carotene (**10a**) as natural products. We propose the name allogromiarubin for the latter.

All carotenoid esters were homogenous with respect to their fatty acid residues. Two of them were identified as palmitic acid in the pigments **Ib** (**4b**) and **IIIa** (**8b**). Therefore the acid component in the remaining pigments **Ic** (**7b**), **IIa** (**13b**) and **IIb** (**10b**) were presumed to be palmitic acid, also.

It is well known that 3-hydroxy-4-keto-carotenoids are easily dehydrogenated at position C-2,3 in alkaline solution in the presence of air, as shown for the formation of **9a** from **8b**. For that reason, generally, carotenoids containing 3,4-diketo-end groups are believed to be artefacts (Isler, 1971). The esters isolated from the foraminiferan had been saponified in the presence of air. Therefore it is most likely that three further foraminiferal carotenoids containing diketo-end groups (**10a**, **13a** and **17**) present in the cell in esterified form, arose during the isolation procedure from the esters of allogromiaxanthin (**10b**), adonixanthin (**13b**) and adonirubin (**14a**) also.

Astaxanthin dipalmitate (**8b**) is the main pigment in *A. laticollaris* and is present in a concentration of 0.75% (calc. as **8a**) of the cell dry weight (Table 1). This and the esters of allogromiarubin (**10a**) and β-doradecin (**13a**) are responsible for the deep reddish-orange coloration of the foraminiferan cell.

Feeding experiments using carotenoid-free bacteria yielded a considerable decrease in pigmentation of the foraminifera (Arnold, 1956). Although these results do not completely rule out the *de novo* synthesis of the C_{40}-skeleton in this protozoan, the formation of the foraminiferal pigments could be better explained by an appropriate metabolism of incorporated primary carotenoids of the food organism.

It is well known that under physiological conditions the food organism of *A. laticollaris,* the green alga *Chlorella,* contains, besides other carotenoids, β-carotene (**1**) and lutein (**2**), but no 3,4-diketo-carotenoids or 3-hydroxy-4-ketocarotenoids (Iwata and others, 1961). In accordance with their oxygen content, all the

TABLE 1. Quantitative determination of carotenoids isolated from the foraminiferan *A. laticollaris.*

Pigment	Yield [mg] from 1.695 g dry cell material	Percent (of dry weight)
β-Carotene (**Ia**, **1**)	3.6	0.21
Anhydrolutein-II palmitate (**Ib**, **4b**)	2.3*	0.55
Zeaxanthin dipalmitate (**Ic**, **7b**)	1.6*	0.09
Allogromiaxanthin palmitate (3',4'-didehydro-3-hydroxy-4-keto-β-carotene palmitate, (**IIa**, **10b**)	7.1*	0.42
Adonixanthin palmitate (**IIb**, **13b**)	6.4*	0.38
Astaxanthin dipalmitate (**IIIa**, **8b**)	12.7*	0.75
Astaxanthin monopalmitate ? (**IIIb**)	3.1*	0.18
Anhydrolutein-II (**IIIc**, **4a**)	1.5	0.09
not identified (**$IIId_1$**)	0.3	0.02
not identified (**$IIId_2$**)	0.2	0.01
Phoeniconone or the ester (**$IIIe_1H$**, **17/14a**)	trace	—
not identified (**$IIIe_2$**)	trace	—
Lutein (**$IIIf_1$**, **2**)	trace	—
Zeaxanthin (**$IIIf_2$**, **7a**)	trace	—
	Total	2.7%

* Weight calculated as for the free carotenoid.

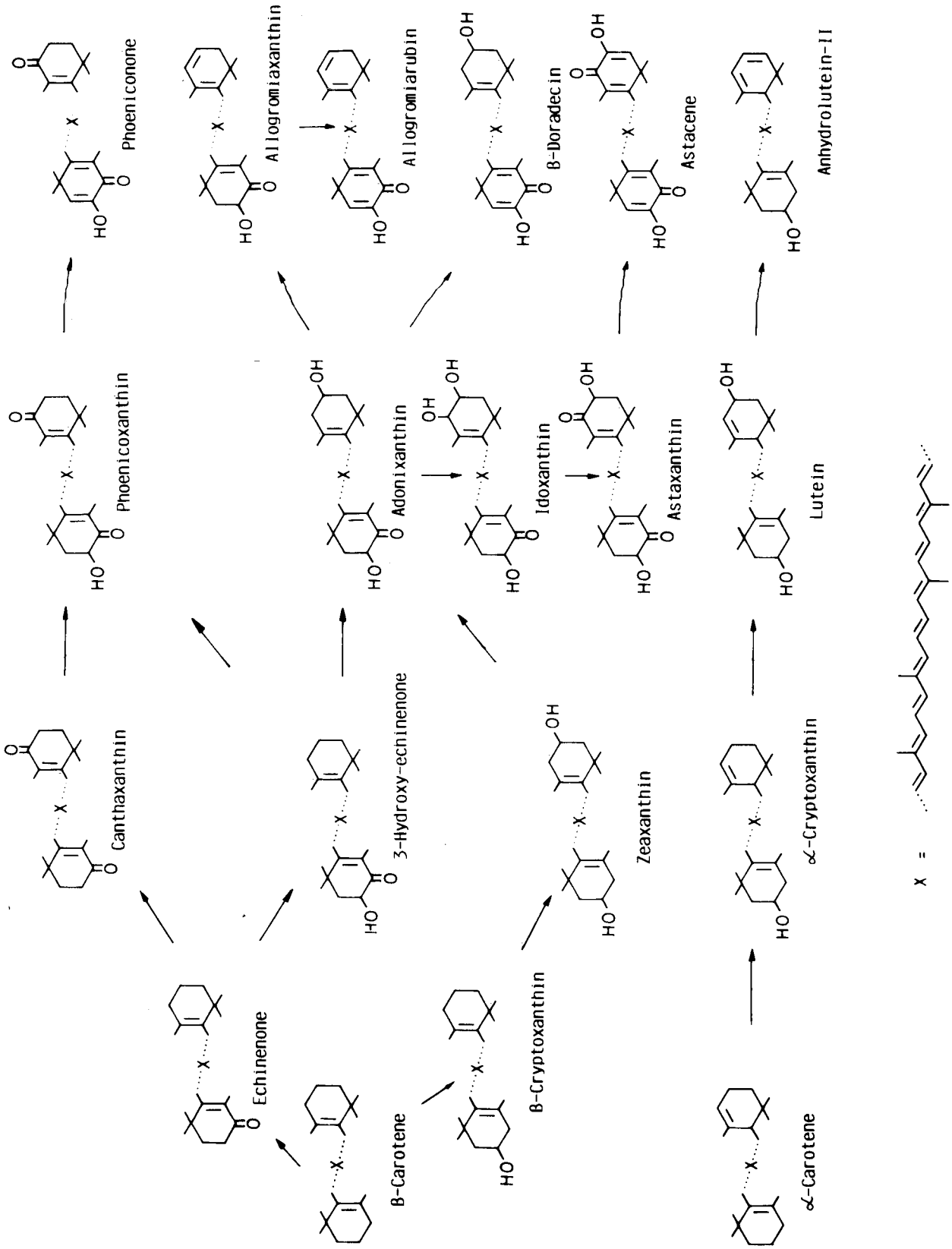


FIGURE 16. Proposed pathway of pigment biosynthesis in the foraminiferan *Allogromia laticollaris*.

isolated pigments may be derived from β-carotene (**1**) by one- to fourfold hydroxylation and/or dehydrogenation.

It has not been clarified whether secondary carotenoids formed within *Chlorella* after incorporation in food vacuoles of *A. laticollaris,* are important for the pigmentation of the foraminiferan. Primary and secondary *Chlorella* pigments could be accumulated in the cytoplasm and not metabolized further. However, the fact that not all carotenoids which are present in *Chlorella* can be identified as foraminiferal pigments indicates metabolism of *Chlorella* pigments in *A. laticollaris.* In addition, the absence of dehydrocarotenoids **10a** and **4a** in *Chlorella* or other natural sources suggested that the foraminiferan cell possesses special abilities for pigment synthesis.

The biogenesis of foraminiferal pigments from β-carotene or other primary *Chlorella* pigments (**2** or **7a**) may be explained as shown in Fig. 16.

Our suggested pathway of pigment synthesis in *A. laticollaris* agrees with the proposal of Thommen (1971) and seems very likely because the metabolism of zeaxanthin into astaxanthin has already been demonstrated in several animals and similar modes of synthesis have also been proposed for plants (see Isler, 1971).

Depending on the stage of development of the cell, the foraminiferan pigments themselves are subjected to metabolism forming uncolored compounds. Apparently another foraminiferan, *Myxotheca arenilega* Schaudinn, easily and quickly metabolizes its own pigment during gametogenesis. Its pigment disappears within 24–36 hours and the cell color changes from reddish-orange to light cream or white. *Allogromia laticollaris* faded slightly during the corresponding stage of development.

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DEDICATION

This paper is dedicated to Professor Dr. V. Leonhardt with best wishes on the occasion of his 65th birthday.

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