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FORMATION OF NEOXANTHIN, DIADINOXANTHIN AND PERIDININ FROM [^{14}C]ZEAXANTHIN BY A CELL-FREE SYSTEM FROM *AMPHIDINIUM CARTERAE*

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Key Word Index—*Amphidinium carterae*; Dinophyceae; biosynthesis; zeaxanthin; neoxanthin; peridinin; diadinoxanthin; allene; acetylene.

Abstract—A cell-free system prepared from an axenic culture of the alga *Amphidinium carterae* converted [^{14}C]zeaxanthin into neoxanthin and then into peridinin (62%) and diadinoxanthin (38%). Peridinin, therefore, is made by the excision of three carbon atoms from a C_{40} carotenoid and the acetylene group of diadinoxanthin is formed from the allene of neoxanthin, rather than the reverse.

INTRODUCTION

Little biosynthetic work has been carried out on the many unusual carotenoids found in marine algae. The reasons have to do with the difficulty of growing axenic cultures and the low percentage incorporation of mevalonic acid into the carotenoid fraction. Several biosynthetic sequences have been postulated [1, 2] but these have been based on the occurrence of related carotenoid structures and known chemical and biochemical reactions, rather than on observed interconversions.

The red pigment peridinin is the principal carotenoid of dinoflagellates (50–80%) [2, 3]. Blooms of dinoflagellates are often responsible for 'red tides' where the abundance of peridinin in the cells gives the sea its colour. Peridinin–chlorophyll *a* proteins are the major light-harvesting pigment complexes of dinoflagellates [4, 5]. They absorb maximally in the region 480–520 nm and are efficient harvesters of the predominantly blue-green radiation which penetrates into the sea. Peridinin has two features of considerable biosynthetic interest: it contains an allene group and its carbon skeleton comprises 37 carbon atoms presumably derived by the excision of a three-carbon fragment from a C_{40} polyene chain. Diadinoxanthin, the second most abundant carotenoid in the dinoflagellates (9–30%) [2, 3] contains an acetylenic group.

The presence of these carotenoids in *Amphidinium carterae*, a dinoflagellate that can be grown in axenic culture, suggested that some of the biosynthetic features of the formation of these compounds could be investigated using a cell-free system such as we have used previously [6]. We now report that [^{14}C]zeaxanthin is converted in high yield into neoxanthin and this is then metabolized to peridinin and diadinoxanthin.

RESULTS

Labelling of carotenoids using [^{14}C]*mevalonate*

[^{14}C]Lycopene, β,β-carotene, zeaxanthin (**1**), neoxanthin (**2**), diadinoxanthin (**3**) and peridinin (**4**) (Fig. 1) were isolated from intact cells of *A. carterae* and from the cell-free system to which [^{14}C]mevalonate had been supplied. More efficient incorporation of labelled precursor into the carotenoid fraction was obtained with cell-free systems than intact cells. The yields of labelled carotenoids from cell-free preparations and intact cells were 95 and 85%, respectively, of the values previously published for *A. carterae* [2]. This finding of zeaxanthin and neoxanthin is the first reported occurrence of these carotenoids in the Dinophyceae.

Metabolism of labelled carotenoids

A cell-free system of *A. carterae*, supplied with [^{14}C]zeaxanthin (37 000 dpm, 24 μg) isolated from previous experiments with [^{14}C]mevalonate, converted the carotenoid in very high yield into neoxanthin (**2**). The counts in (**2**) reached a maximum after 30 min when some 70% of the counts added in zeaxanthin were present in neoxanthin (Fig. 2). The amounts of ^{14}C in neoxanthin then fell to very low levels, coincident with the appearance of labelled diadinoxanthin (**3**) and peridinin (**4**) (Fig. 2).

After 3 hr, 98.5% of the zeaxanthin added was accounted for as peridinin (62%) and diadinoxanthin (37%) (Fig. 2). In a separate experiment (Fig. 3), added [^{14}C]neoxanthin (18 000 dpm, 20 μg) was converted into peridinin (66% of the total ^{14}C label) and diadinoxanthin (33%) after 3 hr. Labelled diadinoxanthin (30 000 dpm, 30 μg), as expected, was not converted into peridinin or neoxanthin or metabolized further to any detectable degree (Fig. 4).

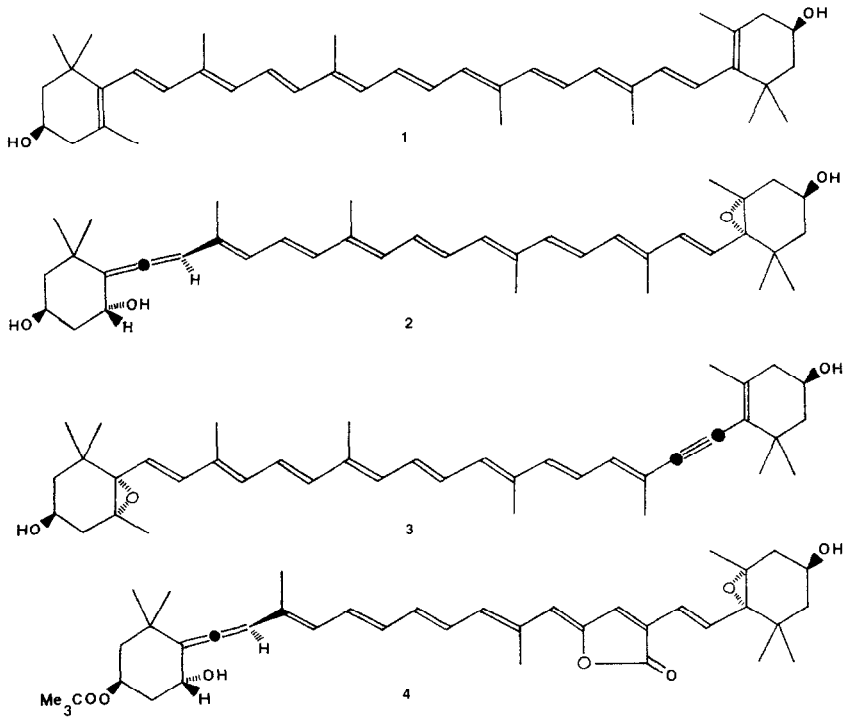

Fig. 1. Structures of carotenoids showing absolute configurations and numbering system. The formation of diadinoxanthin (3) from zeaxanthin (1) and neoxanthin (2) establishes the absolute configuration at C-3, C-5, C-6 and C-3′ of (3) to be as shown. For (1) see ref. [17], (2) [12], (3) [18] and (4) [19].

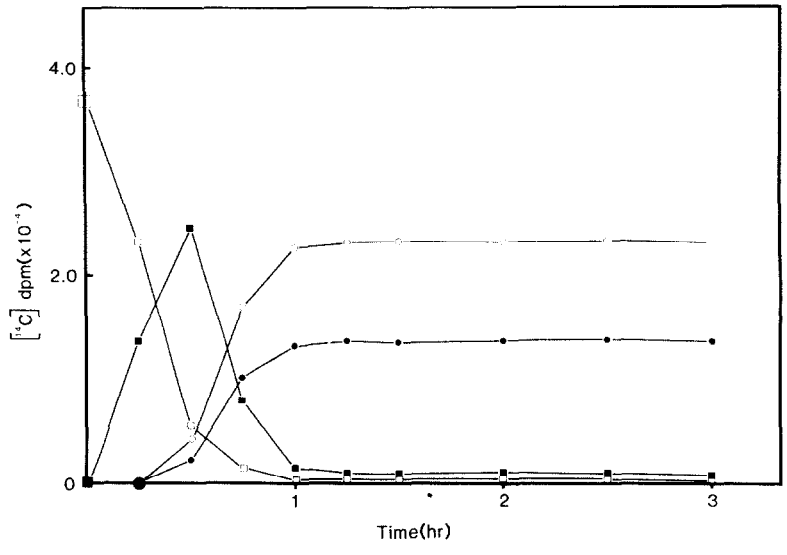

Fig. 2. A whole cell homogenate (5 ml; 1.3 mg carotenoid/ml homogenate) was prepared from a stationary phase culture of *A. carterae* (500 ml) as described in the Experimental. [^{14}C]Zeaxanthin (37 000 dpm, 25 μg) was added in ethanol (100 μl) to the system, which was then incubated under tungsten illumination, at 26° for 3 hr. Aliquots of the homogenate (0.5 ml) were withdrawn during the incubation and zeaxanthin, neoxanthin, diadinoxanthin and peridinin were isolated and their radioactivities counted as described in the Experimental. (□) Zeaxanthin; (■) neoxanthin; (●) diadinoxanthin; (○) peridinin.

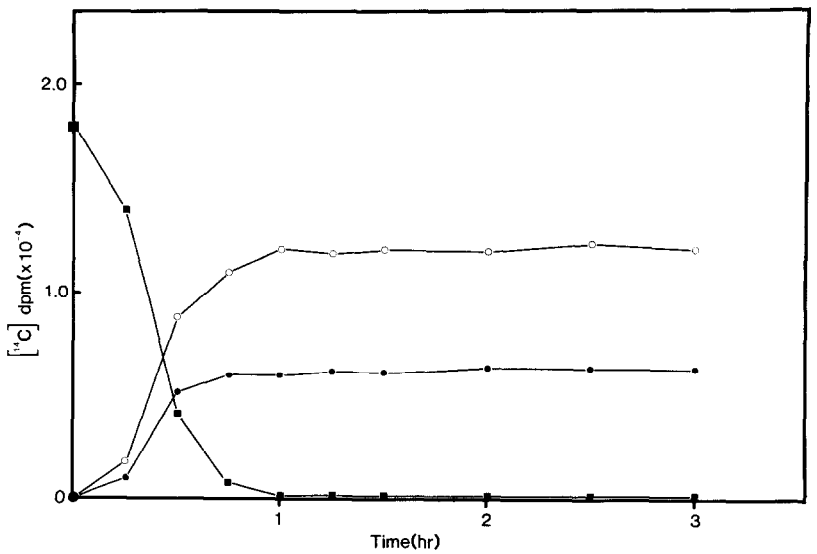

Fig. 3. The same conditions described in Fig. 1 were used, except that [^{14}C]neoxanthin (18 000 dpm, 20 μg) was added in ethanol (100 μl) to the whole cell homogenate. (■) Neoxanthin; (○) peridinin; (●) diadinoxanthin.

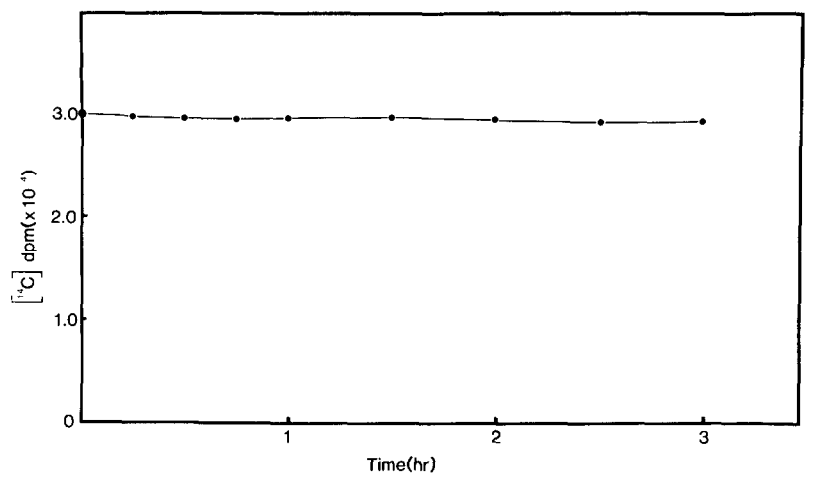

Fig. 4. The same conditions described in Fig. 1 were used, except that [^{14}C]diadinoxanthin (30 000 dpm, 30 μg) was added in ethanol (100 μl) to the whole cell homogenate. (●) Diadinoxanthin.

DISCUSSION

Hitherto zeaxanthin (**1**) and neoxanthin (**2**) have not been identified in members of the Dinophyceae although they could be expected from the occurrence of dinoxanthin (neoxanthin-3-acetate). Neoxanthin is present in relatively minor amounts (1–2% of the total carotenoid fraction) and so can be considered an intermediate in the production of the major carotenoid species [2].

Figure 2 shows that the amount of ^{14}C in peridinin and diadinoxanthin reached and maintained a constant level. It appears, therefore, that both are terminal products and do not turn over rapidly, provided, of course, that the cell-free system gives a true indication of the physiological status of the intact cell over 3 hr. The incorporation of [^{14}C]zeaxanthin by a cell-free system, first into neoxanthin and then into diadinoxanthin or peridinin, establishes the two former as precursors of the two latter compounds (Fig. 2). As expected, diadinoxanthin was not converted into peridinin or any other product. The allene in (**2**) is the precursor of the acetylene in (**3**), rather than the reverse. The order in which the other elaborations of the molecules occur is not yet known.

The conversion of zeaxanthin and neoxanthin (Figs. 2 and 3) into peridinin has further significance, namely, that peridinin is formed by the excision of a three-carbon fragment from the polyene chain of what had been the C_{40} zeaxanthin rather than by fusion of, for example, a diterpene and a sesquiterpene plus a C_2 moiety. The data do not exclude the possibility that more than three carbon atoms are deleted, however, subsequent experiments [6] have shown that three carbon atoms are indeed removed.

The high percentage of the ^{14}C label recovered in peridinin and diadinoxanthin shows that the added [^{14}C]zeaxanthin is metabolized with considerable efficiency. Obviously, in a cell-free preparation, the

compartmentation normally present in intact cells has been abolished, so carotenoids are readily accessible to enzymes of the biosynthetic pathway.

In order to wash out the label to the observed final specific activity of zeaxanthin, *ca* six times the amount of the zeaxanthin pool (86 μg endogenous + 25 μg added) would have to be formed from unlabelled precursors and converted into diadinoxanthin plus peridinin. This is based on the assumptions that perfect mixing of the labelled and endogenous material occurred and that the material synthesized during the course of the experiment was also mixed completely. These are not unreasonable assumptions for a cell-free system. The amounts of peridinin and diadinoxanthin found at the end of the experiment (220 and 125 μg, respectively, Table 1) are considerably less than the postulated amounts (620 and 310 μg, respectively) which would be observed if no pooling of zeaxanthin occurred. Also, and more importantly, the specific activity of the two terminal products would have to be considerably less (*ca* 37 and 45 dpm/μg, respectively) than the values observed (105 and 112 dpm/μg, respectively).

Clearly, the observations differ so markedly from the values predicted above that it can be concluded that there are two pools of zeaxanthin and the ^{14}C-labelled material added is preferentially metabolized to diadinoxanthin and peridinin. The converse must also be true: that part of the endogenous zeaxanthin (20 μg/g cells) may have a function in the cells other than as an intermediate in the formation of the more elaborate carotenoids.

The [^{14}C]zeaxanthin was metabolized so rapidly in the cell-free system that the equivalent of one-third of the endogenous amount was converted into peridinin and diadinoxanthin during the 3 hr of the experiment, suggesting that the steps beyond zeaxanthin are not regulated (or at least not in the cell-free system). The increase in the amounts of these two carotenoids was in a similar proportion to that in which they occur in intact cells (2.4 mg peridinin and 1.1 mg diadinoxanthin/g cells). It follows, if this is so, that the steps limiting carotenoid formation must be those leading up to zeaxanthin.

If the proportions of peridinin and diadinoxanthin change in response to environmental factors or the physiological state of the cells, then the enzymes operating on products formed from zeaxanthin could be subject to regulation. However, as it appears that a proportion of the zeaxanthin can be removed from the pathway of peridinin biosynthesis in some way, it is possible that the relative amounts of the various carotenoids in the cell could be regulated by differential binding rather than by adjustment of the amount or activity of an enzyme. Such a model has already been proposed for the peridinin–chlorophyll *a* protein complex [7]. The cell-free system we describe now makes these problems susceptible to direct experimentation. However, in order to determine the localization of the various enzymes it will be necessary to fractionate chloroplasts from the rest of the cell and use the cell-free system to study the metabolism of labelled carotenoids in each fraction.

The acetylation of the C-3 hydroxyl to form peridinin may represent an end-of-biosynthesis signal by reducing the polarity of one ring and thereby facilitate positioning of the completed molecule, often in a carotenoprotein complex, in a chloroplast membrane. The reduction in polarity that occurs as neoxanthin is converted into diadinoxanthin may have the same effect. At least in the cell-free system the diadinoxanthin was not acetylated, yet acetylating activity was present as evidenced by the formation of labelled peridinin and isolation of dinoxanthin (neoxanthin-3-acetate) from cell-free systems.

EXPERIMENTAL

Materials. (3*RS*)-[2-^{14}C]Mevalonolactone (22 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks, U.K. [U-^{14}C]Toluene (1.53 mCi/mol) was obtained from Packard Instruments, Downers Grove, IL, U.S.A. Al_2O_3 60 F_{254} (type E) TLC plates were from Merck, Darmstadt. All other reagents and solvents were analytical grade.

Biological material. Axenic cultures of *Amphidinium carterae* Hulbert (CSIRO Culture Code CS-21 [8]; were grown at 18° in 1.5 l. batches of sterile culture medium [9] modified by replacement of Fe sequestrene with Fe citrate and addition of EDTA (30 g/l.). Aerated cultures received

Table 1. Quantities of carotenoids in a cell-free preparation of *A. carterae* and the metabolism of added [^{14}C]zeaxanthin

	At the commencement of incubation			After 3 hr incubation		
	μg	dpm	dpm/μg	μg	dpm	dpm/μg
Zeaxanthin	85(endogenous) 24(exogenous)	37 000	336	70	400	5.7
Diadinoxanthin	95	—	—	125	14 000	112
Peridinin	170	—	—	220	23 000	105

A white cell homogenate (5 ml; 120 μg/ml homogenate) was prepared from a stationary phase culture of *A. carterae* (500 ml) as described in the Experimental. [^{14}C]Zeaxanthin (37 000 dpm, 25 μg) was added in ethanol (100 μl) to the system, which was then incubated under tungsten illumination at 26° for 3 hr. Aliquots of the homogenate (1.0 ml) were taken before addition of the [^{14}C]zeaxanthin and at the end of the incubation and the carotenoids isolated, quantitatively determined and their radioactivity counted as described in the Experimental.

40 μE/m^2 sec light from 'daylight' fluorescent tubes under 12 hr light–12 hr dark cycles. Cells were harvested after 5 weeks (i.e. used in the stationary phase; 1.2×10^6 cells/ml culture medium).

Preparation of a whole cell homogenate. Cells from a 1.5 l. vol. of culture medium were harvested by centrifugation (20 min, 0°, 9500 *g*). The cell pellet (5 g) was resuspended in the enriched Tris–HCl buffer, pH 7.8 (5 ml) previously described [6] and transferred to a chilled mortar. Celite (500 mg) was added and the mixture ground thoroughly until a homogenous slurry resulted and no intact cells remained. The homogenate was then filtered through four layers of cheesecloth to remove any Celite. The cheesecloth was rinsed with the Tris–HCl buffer, pH 7.8 (10 ml) described above, and the two filtrates were combined. This whole cell homogenate was used to study the metabolic interconversions of labelled carotenoids. It was found that more efficient incorporation of labelled precursor into carotenoids occurred using old intact cells, or whole cell homogenates prepared from cells, than with young cells.

Preparation of labelled zeaxanthin, neoxanthin and diadinoxanthin. The [^{14}C]mevalonic acid was prepared by dissolving the required [^{14}C]mevalonolactone, supplied as a C_6H_6 soln, in 1 M KOH (0.5 ml) and incubating at 22° for 2 hr. The KOH was neutralized with 1 M (0.5 ml) HCl. The C_6H_6 was evaporated under N_2. The aq. mevalonic acid soln was diluted with Tris–HCl buffer (20 mM, pH 7.8, 2.5 ml) before being added to the whole cell homogenate (9.5 ml; 0.9 mg carotenoid/ml whole cell homogenate) or to intact cells (2.4 g) resuspended in culture medium (10 ml; 1.0 mg carotenoid/ml medium). Both systems were shaken under tungsten illumination (24 μE m^2 sec) for 3 hr at 26°. The carotenoids neoxanthin and diadinoxanthin were isolated (see below) after incubation. [^{14}C]Zeaxanthin was prepared by feeding [^{14}C]mevalonate to flowering heads of *Celendula officinalis* and isolating and purifying the zeaxanthin fraction [Swift, I. E. and Milborrow, B. V., unpublished].

Metabolism of labelled carotenoids. A whole cell homogenate (15 ml) was prepared from 1 l. culture and the previously isolated labelled carotenoids added in EtOH (100 μl) to each sample (5 ml). The flasks were shaken under tungsten illumination (24 μE m^2 sec) for 3 hr at 26°. Aliquots (0.5 ml) were withdrawn at intervals and the carotenoids extracted, isolated and counted as described below. This expt was repeated ×4 with similar results; only the data from one set of results are shown here.

Isolation of carotenoids. Both intact cell systems and whole cell homogenates were mixed with MeOH (100 ml) containing the antioxidant 2,6-di-*t*-butyl-4-methylphenol (500 mg) and held in darkness at 22° for 12 and 24 hr for cell-free and intact cells systems, respectively. These and all subsequent manipulations were performed in dull light. The MeOH cell extracts were mixed with an equal vol. Et_2O (100 ml) and sufficient satd aq. NaCl (80 ml) added to achieve phase separation. The Et_2O layer, which contained the carotenoid and chlorophyll fractions, was collected, washed twice with H_2O, dried and evaporated under vacuum at 40°.

The dried Et_2O extracts were redissolved in Et_2O (0.5 ml) and applied to Al_2O_3 TLC plates, which were then developed with toluene–EtOAc (5 : 3). This system gave clear separation of carotenoids and chlorophylls. The carotenoids (three major fractions) were redissolved in C_6H_6 (200 μl) and chromatographed (25 μl injections) on a Waters high-pressure liquid chromatography semi-preparative μPorasil column (Waters Associates, Milford, MA, U.S.A.) in hexane–isopropanol (19 : 1; 4.0 ml/min; 1.4×10^5 kPa) and A_{440nm} was monitored. All solvents were re-distilled, filtered and degassed under vacuum before use.

Identification of carotenoids. Zeaxanthin, neoxanthin, peridinin and diadinoxanthin were identified by their visible absorption spectra and chemical ionization (CH_4) MS. Peridinin was also identified by co-chromatography with an authentic sample provided by Dr. R. Wells. A subsample of the algal culture was analysed to obtain the percent composition of the carotenoids below. Similar, but not identical, percent compositions were found in whole cell homogenates. Lit. λ_{max} values were used to identify the compounds and the absorbances were in the same relationship as published $E^{1\%}_{1cm}$ values. These values were used to determine carotenoid concns. The MS data (chemical ionization) were obtained by Dr. A. M. Duffield and are similar to lit. values obtained by EIMS. The percentages of base peak values from the CI spectra are given below.

Zeaxanthin (1) (20 μg/g cells 0.5% of total carotenoid). UV λ^{EtOH}_{max} nm: 428, 452, 478, $E^{1\%}_{1cm,452}$ (EtOH) 2243 [10]; *m/z* 568 $[M]^+$ (100%), 566 $[M-2]^+$ (5), 550 $[M-18]^+$ (10), 476 $[M-92]^+$ (40), 462 $[M-106]^+$ (1), 455 $[M-153]^+$ (1), 133 (45), 109 (40), 91 (75), 83 (30), 69 (53), 59 (5) and 43 (88) [11].

Neoxanthin (2) (43 μg/g cells, 1% of total carotenoid). UV λ^{EtOH}_{max} nm: 422, 441, 470, $E^{1\%}_{1cm,452}$ (Me_2CO) 2340 [12, 13]; *m/z* 600 $[M]^+$ (30%), 582 $[M-18]^+$ (67), 564 $[M-18-18]^+$ (8), 508 $[M-92]^+$ (10) and 43 (100) [13].

Diadinoxanthin (3) (1.08 mg/g cells, 27% of total carotenoid. UV $\lambda^{Me_2CO}_{max}$ nm: 340, 426, 448, 478, $E^{1\%}_{1cm,448}$ (Me_2CO) 2250 [2]; *m/z* 582 $[M]^+$ (53%), 566 $[M-16]^+$ (3), 564 $[M-18]^+$ (3), 548 $[M-16-18]^+$ (3), 502 $[M-80]^+$ (14) 490 $[M-92]^+$ (6), 352 (2), 221 (80), 181 (12) and 43 (100) [2].

Peridinin (4) (2.4 mg/g cells, 60% of total carotenoid). UV λ^{hexane}_{max} nm: 454, 484, $E^{1\%}_{1cm,454}$ (Me_2CO) 1350 [2, 14]; *m/z* 630 $[M]^+$ (5%), 612 $[M-H_2O]^+$ (41), 594 $[M-2H_2O]^+$ (3), 586 $[M-CO_2]^+$ (5), 570 $[M-AcOH]^+$ (1), 568 $[M-H_2O-CO_2]^+$ (2), 552 $[M-H_2O-AcOH]^+$ (32), 538 $[M-C_7H_8]^+$ (2), 534 $[M-2H_2O-AcOH]^+$ (4), 520 $[M-H_2O-C_7H_8]^+$ (2), 508 $[M-CO_2-AcOH-H_2O]^+$ (2), 478 $[M-C_7H_8-AcOH]^+$ (3), 397 ($C_{28}H_{29}O_2$, 2), 358 ($C_{21}H_{26}O_5$, 3), 234 ($C_{14}H_{18}O_3$, 28), 223 ($C_{17}H_{19}$, 20) 212 ($C_{16}H_{20}$, 37), 197 ($C_{15}H_{17}$, 60), 181 ($C_{11}H_{17}O_2$, 100) and 163 ($C_{11}H_{15}O$, 23) [14, 15].

Dinoxanthin (neoxanthin-3-acetate) (240 μg/g cells, 6% of total carotenoid). UV $\lambda^{Me_2CO}_{max}$ nm: 418, 442, 470, $E^{1\%}_{1cm,442}$ (Me_2CO) 2100 [2]; *m/z* (as dinoxanthin acetate) 684 $[M]^+$ (65%), 666 $[M-18]^+$ (12), 624 $[M-60]^+$ (15), 604 $[M-80]^+$ (7), 592 $[M-92]^+$ (8), 586 (52), 544 (6), 263 (65), 223 (38), 221 (35) and 43 (100%) [2].

Scintillation spectrometry and mass spectrometry. The method described previously [16] was used.

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REFERENCES

1. Strain, H. H., Svec, W. A., Aitzetmüller, K., Grandolfo, M. C., Katz, J. J., Kjosen, H., Norgard, S., Liaaen-Jensen, S., Haxo, F. T., Wegfahrt, P. and Rapoport, H. (1971) *J. Am. Chem. Soc.* **93**, 1823.

2. Johansen, J. E., Svec, W. A. and Liaaen-Jensen, S. (1974) *Phytochemistry* **13**, 2261.
3. Jeffrey, S. W., Sielicki, M. and Haxo, F. T. (1975) *J. Phycol.* **11**, 374.
4. Haxo, F. T., Kycia, J. H., Somers, G. F., Bennett, A. and Siegelman, H. W. (1976) *Plant Physiol.* **57**, 297.
5. Prézelin, B. B. and Haxo, F. T. (1976) *Planta* **128**, 133.
6. Swift, I. E. and Milborrow, B. V. (1981) *Biochem. J.* **199**, 69.
7. Song, P. S., Koka, P., Prézelin, B. B. and Haxo, F. T. (1976) *Biochemistry* **15**, 4422.
8. Jeffrey, S. W. (1979) *CSIRO Div. Fish Oceanogr. Annu. Rep.* (1977–1979) 22.
9. Guillard, R. R. L. and Ryther, J. H. (1962) *Can. J. Microbiol.* **8**, 229.
10. Cholonky, L., Gyorgyfy, K., Szabolcs, J., Weedon, B. C. L. and Waight, E. S. (1966) *Chem. Commun.* 404.
11. Enzell, C. R., Francis, G. W. and Liaaen-Jensen, S. (1969) *Acta. Chem. Scand. Ser. B.* **23**, 727.
12. Aasen, A. J. and Liaaen-Jensen, S. (1966) *Acta. Chem. Scand. Ser. B.* **20**, 2322.
13. Cholnoky, L., Gyorgyfy, K., Ronái, A., Szabolcs, J., Tóth, G., Galasko, G., Mallams, A. K., Waight, E. S. and Weedon, B. C. L. (1969) *J. Chem. Soc. C.* 1256.
14. Strain, H. H., Svec, W. A., Wegfahrt, P., Rapoport, H., Haxo, F. T., Norgard, S., Kjosen, H. and Liaaen-Jensen, S. (1976) *Acta. Chem. Scand. Ser. B.* **30**, 109.
15. Kjosen, H., Norgard, S., Liaaen-Jensen, S., Svec, W. A., Strain, H. H., Wegfahrt, P., Rapoport, H. and Haxo, F. T. (1976) *Acta. Chem. Scand. Ser. B.* **30**, 157.
16. Swift, I. E. and Milborrow, B. V. (1980) *Biochem. J.* **187**, 261.
17. Bartlett, L., Klyne, W., Mose, W. P., Scopes, P. M., Galasko, G., Mallams, A. K., Weedon, B. C. L., Szaboks, J. and Tóth, G. (1969) *J. Chem. Soc. C.* 2527.
18. Buchecker, R. and Liaaen-Jensen, S. (1977) *Phytochemistry* **16**, 729.
19. Johansen, J. E., Borch, G. and Liaaen-Jensen, S. (1980) *Phytochemistry* **19**, 441.