Phytochemistry, 1976, Vol. 15, pp. 1003–1007. Pergamon Press. Printed in England.

CAROTENOIDS OF *PHAFFIA RHODOZYMA*, A RED-PIGMENTED FERMENTING YEAST

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(*Received* 30 *August* 1975)

Key Word Index—*Phaffia rhodozyma*; Deuteromycetes; yeast; pigments; carotenoids; astaxanthin; 3-hydroxy-3′4′-didehydro-β,ψ-caroten-4-one.

Abstract—The red-pigmented fermenting yeast *Phaffia rhodozyma* contains astaxanthin as the principal carotenoid pigment. Echinenone, 3-hydroxyechinenone and phoenicoxanthin were also isolated and identified; isocryptoxanthin and canthaxanthin were absent. Evidence is presented for a new carotenoid, 3-hydroxy-3′4′-didehydro-β,ψ-caroten-4-one. A possible biosynthetic scheme for the formation of astaxanthin in *P. rhodozyma* is suggested.

INTRODUCTION

Recently, Miller and collaborators [1] have described a new genus of yeast, *Phaffia*, with the single species. *Phaffia rhodozyma*. Earlier, this yeast was referred to as *Rhodozyma montanae* [2], but this binomial was not validated by a Latin diagnosis as is required by the International Code of Botanical Nomenclature. Two striking characteristics of *P. rhodozyma* are the intense reddish color associated with mature cultures, coupled with the ability to ferment several sugars. Non-fermenting red-pigmented yeasts are not uncommon, especially in the classes Deuteromycetes and Basidiomycetes as represented by the genera *Cryptococcus*, *Rhodotorula* and *Rhodosporidium*, and the ballistosporogenous genera *Sporidiobolus* and *Sporobolomyces* [3]. Typically, these red yeasts contain β-carotene, γ-carotene, torulene and torularhodin as major carotenoids [3,4]. Plectaniaxanthin [5] and 2-hydroxyplectaniaxanthin [6] have also been isolated from yeasts belonging to the Fungi Imperfecti.

We report here the unusual carotenoid composition of *Phaffia rhodozyma* strain 67-210, investigated as part of the total characterization [1,2] of this new yeast genus.

RESULTS

Phaffia rhodozyma strain 67-210 was cultivated on agar plates and in a large-scale fermentor and the total pigment extracts were not saponified but were chromatographed directly. Non-hydroxylated pigments along with most of the yeast lipids were eluted early in the chromatographic sequence, and this fraction was saponified before rechromatography. Hydroxylated pigments were individually eluted and purified by PLC and TLC.

The total carotenoid mixture comprised 0.003% of the wet cell wt and 0.08% of the dry cell wt (wt after acetone extraction and drying of the cellular residue). All hydroxylated pigments were found unbound, not as esters or other derivatives. Table 1 summarizes the quantitative carotenoid composition in increasing order of adsorption (or polarity), as well as the chromatographic properties and absorption maxima of the carotenoids and pertinent derivatives.

Carotenes, with the exception of β-carotene (**1**; β,β-carotene), were isolated only when *P. rhodozyma* was cultivated on agar plates, but not when it was grown in liquid mass cultures in the fermentor. Neurosporene (**3**; 7′,8′-didehydro-ψ,ψ-carotene) was identified from spectral characteristics and chromatographic behavior. γ-Carotene (**2**; β,ψ-carotene), lycopene (**4**; ψ,ψ-carotene) and β-carotene (**1**) were identified from their visible spectra and co-chromatography with authentic substances. Only β-carotene occurred in any significant amount in the carotene fraction and it was isolated both as the *cis* and *trans* isomers.

Echinenone (**5**; β,β-caroten-4-one) was the only non-hydroxylated keto-carotenoid isolated. Identification followed from direct comparison of physical properties with authentic material. Borohydride reduction [7] yielded a single product (**5a**) with β-carotene chromophore; **5a** gave in turn a monoacetate derivative [8]. Hydride-reduced **5** and its acetate derivative cochromatographed with 4-hydroxy-β,β-carotene and 4-hydroxy-β,β-carotene acetate, respectively.

3-Hydroxyechinenone (**6**; 3-hydroxy-β,β-caroten-4-one) showed one broad, slightly asymmetrical absorption band in the visible spectrum, the position and shape of which was consistent with literature values [9–11]. On treatment with dilute base in the presence of air, one product (**6a**) was formed which exhibited a small bathochromic shift in the visible spectrum relative to **6**. Both **6** and the oxidized product (**6a**) gave monoacetates and, following borohydride reduction, diacetate derivatives; reduced alcohols and acetates displayed β-carotene chromophores. The product **6a** resulting from basic oxidation was chromatographically similar to starting material on silica gel but distinctly more polar on alumina. This chromatographic difference is quite characteristic of carotenoids with diosphenol end groups produced from oxidation of 3-hydroxy-4-keto-carotenoids [9–13]. Hydride-reduced **6** was chromatographically different from β,β-carotene-4,4′-diol and β,β-carotene-3,3′-diol. Representa-

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Table 1. Carotenoids of *Phaffia rhodozyma* and their properties

Carotenoid (structure number)	% of total carotenoids	λ_{max} (acetone)	R_f on paper*†	Solvent‡ used for paper chromatography
β-Carotene (**1**)	2–2.5	(428), 453, 482	0.40†	1% e/p
γ-Carotene (**2**)	*ca* 0.01	438, 464, 496	0.90†	5% e/p
Neurosporene (**3**)	*ca* 0.01	419, 441, 472	0.85†	5% e/p
Lycopene (**4**)	*ca* 0.01	446, 474, 505	0.77†	5% e/p
Echinenone (**5**)	2–4	460	0.60†	2% a/p
(**5a**)		(428), 452, 481	0.65*	5% a/p
3-Hydroxyechinenone (**6**)	3–4	464	0.52*	8% a/p
(**6a**)		467	0.50†	8% a/p
			0†	20% a/p
3-Hydroxy-3′,4′-didehydro-β-ψ-caroten-4-one (**7**)	0.3–0.5	(465), 492, 522	0.45*	8% a/p
			0.20†	20% a/p
(**7a**)		(466), 493, 523	0.43*	8% a/p
			0†	20% a/p
(**7b**)		(460), 487, 518	0.48†	8% a/p
(**7c**)		(460), 487, 518	0.49*	20% a/p
(**7d**)		(460), 487, 518	0.51*	20% a/p
Phoenicoxanthin (**8**)	5–7	474	0.39*	8% a/p
(**8a**)		(428), 452, 481	0.55*	20% a/p
(**8b**)		478	0.40*	8% a/p
			0†	20% a/p
Astaxanthin (**9**)	83–87	477 (ϵ = 96,500)	0.42*	10% a/p
(**9a**)		(428), 452, 480	0.37*	20% a/p
(**9b**)		481	0†	100% a/p
(**9c**)		(427), 452, 480	0.37*	20% a/p
(**9d**)		358, 468	0.38*	10% a/p

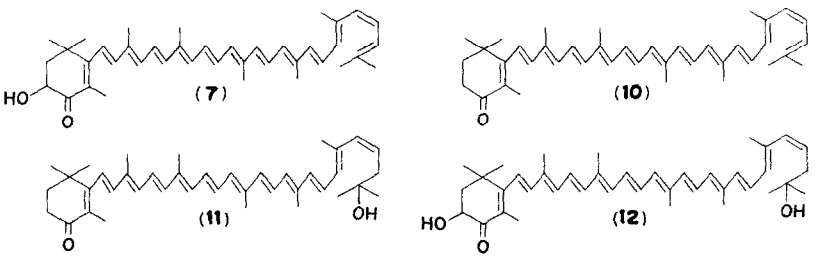
* = S & S 287 paper. † = S & S 288 paper. ‡ = % a/p = % Me_2CO in petrol; % e/p = % Et_2O in petrol.

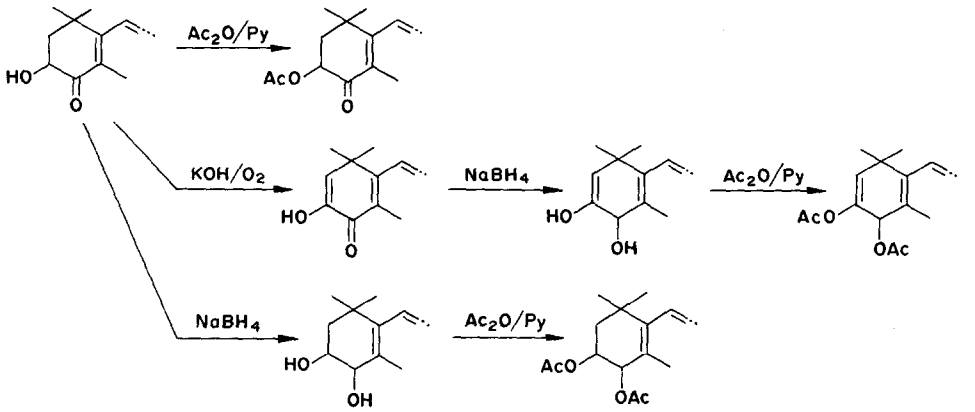
tive reactions used to establish the identity of the 3-hydroxy-4-keto-carotenoid end group are illustrated in Scheme 1.

A minor but persistent component of all *P. rhodozyma* cultures is a new carotenoid, 3-hydroxy-3′4′-didehydro-β,ψ-caroten-4-one (**7**). Structural assignment followed from consideration of the following properties. Chromatographically, **7** was more polar than **6** and less polar than phoenicoxanthin (**8**). The absorption spectrum in acetone (Table 1) was virtually identical to that of anhydro-deoxy-flexixanthin (**10**) in both shape and position of the bands [14]. Structure **7**, however, differs from **10** by having an OH group adjacent to the keto substituent. This structural difference is expected to have little effect on the position and shape of the bands in the spectrum. Deoxy-flexixanthin (**11**) and flexixanthin (**12**) are, for example, nearly indistinguishable when the visible spectra are compared [14]. Treatment of **7** with base in the presence of air gave a single new product (**7a**) with diosphenol properties. On acetylation, **7** gave a monoacetate (**7b**) with identical visible spectrum. Borohydride reduction of **7** and **7a** gave diols **7c** and **7d**, respectively, both of which provided diacetate derivatives. **7c** and **7d** and the acetate derivatives thereof showed visible spectra indistinguishable from torulene (**13**). These data are consistent with the structure (**7**) assigned to this previously unreported carotenoid.

Phoenicoxanthin (**8**; 3-hydroxy-β,β-carotene-4,4′-dione) was the second most abundant carotenoid in *P. rhodozyma*. Identification followed from comparing the visible spectrum with values given in the literature [10,15,16], formation of a monoacetate, borohydride reduction to a trihydroxy compound (**8a**) which displayed the β-carotene chromophore and the formation of a triacetate from the reduced product. Upon air oxidation of **8** in basic solution a single product **8b** was obtained with chromatographic properties of a diosphenol compound, and whose visible electronic spectrum was bathochromically shifted relative to the starting material. The diosphenol product (**8b**) and the borohydride reduction product obtained from **8b** yielded mono- and triacetates, respectively.

Astaxanthin (**9**; mp 213°) was by far the most abundant carotenoid biosynthesized by *P. rhodozyma*; it comprised 83–87% of the total pigment mixture. The absorption spectra in several solvents were consistent with literature values [11,15,17,18]. MS examination gave a M^+ at *m/e* 596 and a fragmentation pattern consistent with an earlier report [19]. PMR signals indicated in structure **9** are comparable with reported values for the dimethyl ether [20] and diacetate [21,22] derivatives and the *gem* dimethyl and C-5 methyl signals correspond well to the values reported by Weedon [22] for the equivalent end group in hydroxyechinenone (**6**). Astaxanthin gave





Scheme 1. Representative chemical reactions on the 3-hydroxy-4-keto carotenoid end group.

a diacetate derivative; on borohydride treatment, it was reduced to a tetrol (**9a**) from which a tetracetate was obtained; both tetrol and acetate derivative showed a β-carotene type of chromophore. Basic oxidation gave the diosphenol product astacene (**9b**) with properties comparable to literature values [10,17,18,23]. Astacene on reduction with borohydride gave a tetrol (**9c**), from which a tetracetate was obtained; both **9c** and acetate derivative had a β-carotene chromophore. Astaxanthin (**9**) from *P. rhodozyma*, although unusual in stereochemistry [32], cochromatographed with authentic material.

Although astaxanthin was isolated principally as the *trans* isomer, every culture of *P. rhodozyma* contained a *cis* isomer (**9d**) of astaxanthin which was identified by iodine-catalyzed stereo-mutation [24]. The *cis* isomer, chromatographically more polar than the *trans* isomer, showed a large *cis* band at 358 nm and the main absorption band was hypsochromically displaced relative to *trans* **9**. It is difficult to say with absolute certainty whether the *cis* isomer is an artifact of workup procedures. However, in light of the fact that no *cis* isomer of astaxanthin having chromatographic properties comparable to that of **9d** has been reported from other biological materials handled by procedures similar to those used here, and considering the mild procedures utilized by us during its isolation from *P. rhodozyma*, the *cis* astaxanthin which was obtained from *P. rhodozyma* probably reflects a genuine biosynthetic product.

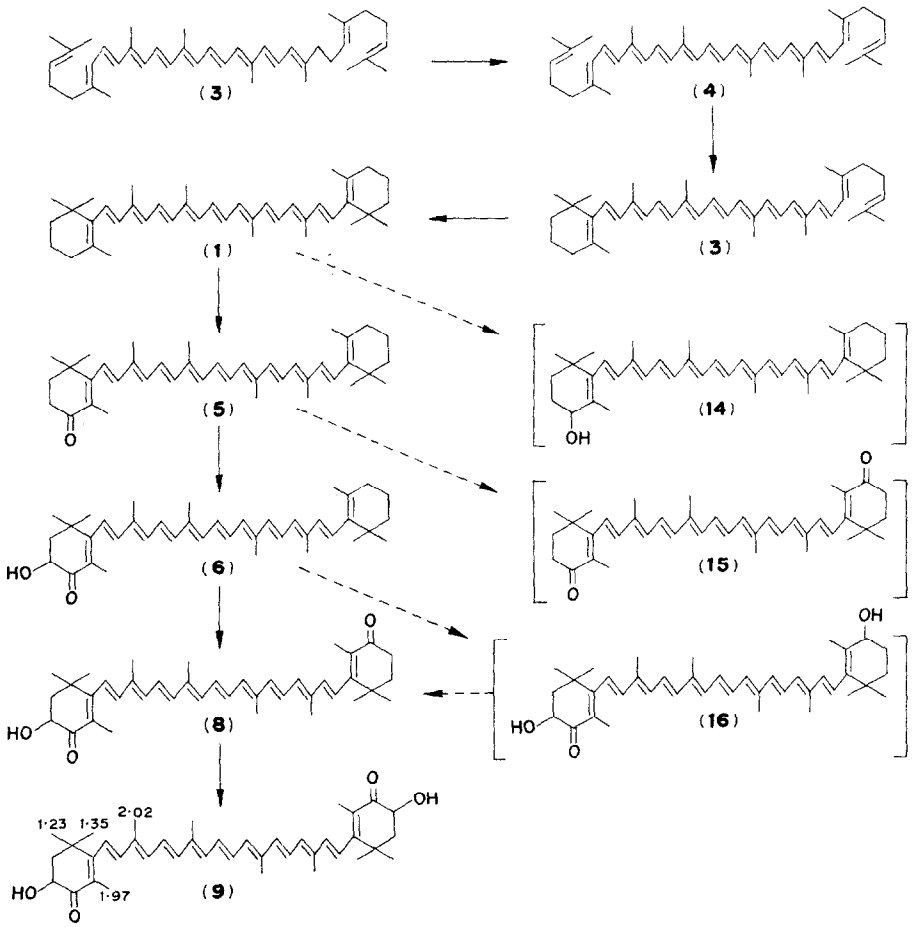
DISCUSSION

Astaxanthin has been isolated from a number of sources including animals, plants and algae [12,18,25]. This is the first instance of the isolation of astaxanthin from a yeast, *Phaffia rhodozyma*, although there have been reports of astaxanthin occurring in basidiomycetous fungi [26,27]. A number of schemes have been proposed for the biosynthesis of astaxanthin, particularly in crustaceans and birds, and these have been summarized in great detail [28]. Chichester and collaborators have advanced a somewhat original proposal for the biosynthesis of astaxanthin in goldfish [29,30].

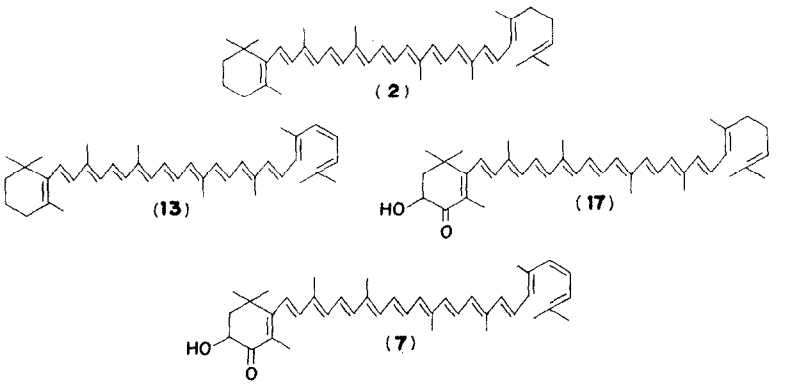
A suggested pathway for the formation of astaxanthin in *P. rhodozyma*, starting from neurosporene (**3**), based on the pigments isolated in this study, is depicted in Scheme 2 and there are deviations from schemes already proposed for astaxanthin biosynthesis. It is proposed that neurosporene (**3**) is converted through well known reactions to β-carotene (**1**), which in turn is converted directly to echinenone (**5**) without passing through the hydroxylated intermediate isocryptoxanthin (**14**; β,β-caroten-4-ol). Despite a careful search, no evidence for isocryptoxanthin (**14**) could be uncovered in any of the *P. rhodozyma* cultures examined. An earlier study [31] with a different organism also concluded that **14** is not necessarily an intermediate in echinenone formation. Scheme 2 shows that echinenone (**5**) is converted to hydroxyechinenone (**6**) in a relatively specific enzymatic reaction. Canthaxanthin (**15**) was not detectable in nutritionally-balanced *P. rhodozyma* cultures. The absence of canthaxanthin implies that the favored pathway starts with the introduction of a keto function at C-4 followed by OH group introduction at C-3 on one end of β-carotene. One might argue that the conversion of canthaxanthin to phoenicoxanthin (**8**) is a very rapid reaction and that the hydroxylation reaction converting **15** to **8** is extremely efficient. This point can only be settled by a thorough study of the intermediate metabolism. The pathway suggested shows that phoenicoxanthin (**8**) results from a direct keto-insertion reaction on hydroxyechinenone (**6**), again without the hydroxylated intermediate, 3,4′-dihydroxy-β,β-caroten-4-one (**16**), which was not detected as a component of the carotenoid mixtures of *P. rhodozyma* cultures. Astaxanthin (**9**), the terminal product of carotenoid biosynthesis in *P. rhodozyma*, is formed from phoenicoxanthin (**8**).

3-Hydroxy-3′,4′-didehydro-β-ψ-caroten-4-one (**7**) cannot be considered as an intermediate in the biosynthesis of astaxanthin (**9**). **7** could be formed (Scheme 3) by dehydrogenation of 3-hydroxy-4-keto-γ-carotene (**17**; 3-hydroxy-β-ψ-caroten-4-one) or alternatively from torulene (**13**). However, neither **13** nor **17** were detected in this study. γ-Carotene (**2**) is formed in small amounts, and could serve as a precursor to **7**.

It is interesting to note that the fungi previously reported to contain astaxanthin [26,27] belong to the class Basidiomycetes. While the perfect stage of *P. rhodozyma* has yet to be demonstrated, the available evidence on the morphological and physiological characteristics of *P. rhodozyma* [1,2] suggests that, if sexuality were to be demonstrated, *P. rhodozyma* also would fall into the basidiomycetous fungi. Additional studies concerning the absolute stereochemistry of astaxanthin biosynthesized by *P. rhodozyma* are reported separately [32] as will be the results of our studies on the feasibility of utilizing *P. rhodozyma* as a dietary source of astaxanthin for crustaceans, fish and other animals.



Scheme 2. Biosynthetic pathway for astaxanthin in *Phaffia rhodozyma*; bracketed structures were not isolated; indicate inoperative routes. PMR signals (δ) for astaxanthin are denoted on structure **9**.



Scheme 3. Possible pathway for biosynthesis of 3-hydroxy-3′,4′-didehydro-β-ψ-caroten-4-one.

EXPERIMENTAL

Biological material. The isolation and characterization of *Phaffia rhodozyma* strain 67-210 has been described [1,2]. Cultures were maintained on agar slopes containing 2% malt extract (Fleischmann barley malt which was converted and extracted) and 2% agar. Plate cultures (2% malt extract, 2% agar) were incubated for 5 days at 22°. From 100 plates (harvested with a microscope slide). 90 g of wet cells were obtained. Large-scale cultures (2 × 60 l.; stainless steel fermentor) in a liquid medium containing cerelose (commercial grade glucose; 2%), $(NH_4)_2SO_4$ (0.5%), $CaCl_2 \cdot 2H_2O$ (0.01%), KH_2PO_4 (0.1%), yeast extract (0.05%) and $MgSO_4 \cdot 7H_2O$ (0.05%) were held at 23° for 3 days and aerated at the rate of 65 l./min. pH was monitored at intervals of 10 hr and adjusted at these times to 4.5 by addition of NaOH soln. Cells were harvested with a Sharples continuous flow centrifuge; from each 120 l. batch of culture, 2 kg of wet cells were obtained.

Materials and methods. Column chromatography was carried out on Woelm Si gel and Al_2O_3 (grade I); PLC (1 mm) on Woelm Si gel and TLC on Si gel and Al_2O_3 plates. The mp of **9** was determined in a sealed capillary (uncorr); the PMR at 60 MHz ($CDCl_3$); and the MS at 220° with ionization voltage 70 eV.

Isolation. Cells harvested from plate cultures were ruptured with a French press [33]; cells from large-scale cultures were ruptured with glass beads in a Braun disintegrater [5]. The pigments were extracted with Me_2CO, dried under vacuum and chromatographed on a Si gel column. The non-hydroxylated pigments were eluted with Me_2CO–petrol (1:19) and the more polar pigments by increasing the Me_2CO content of the eluting solvent gradually to 20%. The fraction containing non-hydroxylated pigments was saponified in the usual manner [34], rechromatographed on an Al_2O_3 column and developed with increasing concentrations of Et_2O in petrol. The individual pigments were further purified by TLC on alumina. Hydroxylated pigments were rechromatographed on Si gel PLC or TLC plates developed with Me_2CO–petrol mixtures. R_f values reported in Table 1 are from PC on Schleicher and Schuell 288 and 287 papers.

Acknowledgements—The authors are indebted to Mary Miranda, Department of Food Science and Technology, University of California, Davis, for technical expertise in culturing aspects. This research was supported in parts by grants AI-08426-19 from the U.S. Public Health Service and BMS 75-03189 from the National Science Foundation.

REFERENCES

1. Miller, M. W., Yoneyama, M. and Soneda, M. (1976) *Int. J. Syst. Bacteriol.*, (In press).
2. Phaff, H. J., Miller, M. W., Yoneyama, M. and Soneda, M. (1972) *Fermentation Technology Today: Proc. Int. Ferm. Symp.*, 4th. (Terui, G., ed.), Kyoto, Japan, 759.
3. Goodwin, T. W. (1972) *Prog. Ind. Microbiol.*, vol. 11. (Hockenhull, D. J. D., ed.), Churchill Livingstone, Edinburgh, 29.
4. Simpson, K. L., Chichester, C. O. and Phaff, H. J. (1971) *The Yeasts*, vol. 2, *Physiology and Biochemistry of Yeasts.* (Rose, A. H. and Harrison, J. S., eds.), p. 135. Academic Press, London.
5. Bae, M., Lee, T. H., Yokoyama, H., Boettger, H. G. and Chichester, C. O. (1971) *Phytochemistry* **10**, 625.
6. Liu, I.-S., Lee, T. H., Yokoyama, H., Simpson, K. L. and Chichester, C. O. (1973) *Phytochemistry* **12**, 2953.
7. Warren, C. K. and Weedon, B. C. L. (1958) *J. Chem. Soc.* 3972.
8. Liaaen-Jensen, S., Hegge, E. and Jackman, L. M. (1964) *Acta Chem. Scand.* **18**, 1703.
9. Krinsky, N. I. and Goldsmith, T. H. (1960) *Arch. Biochem. Biophys.* **91**, 271.
10. Egger, K. (1965) *Phytochemistry* **4**, 609.
11. Leftwick, A. P. and Weedon, B. C. L. (1967) *Chem. Commun.* 49.
12. Karrer, P. and Jucker, E. (1950) *Carotenoids*, Elsevier, New York.
13. Liaaen-Jensen, S. (1971) *Carotenoids* (Isler, O., ed.), Birkhäuser, Basel, 61.
14. Aasen, A. J. and Liaaen-Jensen, S. (1966) *Acta Chem. Scand.* **20**, 1970.
15. Fox, D. L. and Hopkins, T. S. (1966) *Comp. Biochem. Physiol.* **17**, 841.
16. Hata, M. and Hata, M. (1971) *Int. J. Biochem.* **2**, 11.
17. Kuhn, R. and Sorensen, N. A. (1938) *Ber. Deut. Chem. Ges.* **71**, 1879.
18. Johansen, J. E., Svec, W. A., Liaaen-Jensen, S. and Haxo, F. T. (1974) *Phytochemistry* **13**, 2261.
19. Enzell, C. R., Francis, G. W. and Liaaen-Jensen, S. (1969) *Acta Chem. Scand.* **23**, 727.
20. Surmatis, J. D. and Thommen, R. (1967) *J. Org. Chem.* **32**, 180.
21. Campbell, S. A., Mallams, A. K., Waight, E. S., Weedon, B. C. L., Barbier, M., Lederer, E. and Salaque, A. (1967) *Chem. Commun.* 941.
22. Weedon, B. C. L. (1969) *Fortschr. Chem. Org. Naturst.* **27**, 81.
23. Katayama, T., Yokoyama, H. and Chichester, C. O. (1970) *Int. J. Biochem.* **1**, 438.
24. Zechmeister, L. (1962) *cis-trans Isomeric Carotenoids, Vitamins A and Arylpolyenes*, Springer, Vienna.
25. Weedon, B. C. L. (1971) *Carotenoids*, (Isler, O., ed.), p. 267. Birkhäuser, Basel.
26. Fiasson, J. L. (1968) Thesis, University of Lyons.
27. Arpin, N., Lebreton, P. and Fiasson, J.-L. (1966) *Bull. Soc. Mycol. Fr.* **82**, 450.
28. Thommen, H. (1971) *Carotenoids*, (Isler, O., ed.), p. 637. Birkhäuser, Basel.
29. Katayama, T., Shintani K., Shimaya, M., Imai, S. and Chichester, C. O. (1972) *Bull. Jpn. Soc. Scient. Fish.* **38**, 1399.
30. Katayama, T., Tsuchiya, H. and Chichester, C. O. (1972) *Proc. Int. Seaweed Symp.*, 7th, 580.
31. Davies, B. H., Hsu, W.-J. and Chichester, C. O. (1970) *Comp. Biochem. Physiol.* **33**, 601.
32. Andrewes, A. G. and Starr, M. P. (1976) *Phytochemistry* **15**, 1007.
33. Scharf, S. S. and Simpson, K. L. (1968) *Biochem. J.* **106**, 311.
34. Davies, B. H. (1965) *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., ed.), p. 489. Academic, New York.