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STEREOCHEMISTRY OF PHYTOENE

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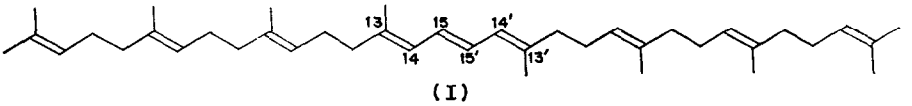
Key Word Index—*Neurospora crassa*; *Phycomyces blakesleeanus*; *Rhodospirillum rubrum*; tomato fruit; carrot oil; phytoene; stereochemistry.

Abstract—Samples of phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene) isolated from higher plant sources, from *Neurospora crassa* and strains of *Phycomyces blakesleeanus* and from diphenylamine-inhibited cultures of *Rhodospirillum rubrum* have been examined by a number of physical methods. All the organisms accumulate predominantly 15-*cis* phytoene while only traces of all-*trans* phytoene are normally present. A comparison with synthetic model compounds has shown that the predominant isomer has a *trans,cis,trans* triene chromophore.

INTRODUCTION

IT IS now generally accepted that phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene, I) is the first conjugated C_{40} compound formed in carotenoid biosynthesis.[1] The central chromophore of three conjugated double bonds offers a number of possibilities for geometrical isomerism. Early studies assigned a *cis* configuration to natural phytoene, mainly on the basis of its inability to form a crystalline thiourea adduct.[2] Later work, in which the IR and NMR spectra of the natural compound were examined and compared with those of unambiguously synthesised all-*trans* phytoene, established that samples of phytoene isolated from tomatoes, from carrot oil and from the G-22 mutant of *Chlorella vulgaris* all had a *cis* configuration at the central 15-double bond.[3,4]



(I)

The 15-*cis* phytoene isolated from carrot oil and from tomato paste was accompanied by traces of another isomer which was apparently 15-*trans*, although the suggestion was made that this could have been formed by isomerisation during processing.[3] The situation is entirely different, however, in *Flavobacterium dehydrogenans*, for the phytoene which accumulates under certain cultural conditions is the 15-*trans* isomer.[5,6]

The stereochemistry of the phytoene molecule is of crucial importance in carotenoid biosynthesis. While the formation of 15-*cis* phytoene is consistent with the $^3H/^{14}C$ ratios

[1] T. W. GOODWIN, in *Carotenoids* (edited by O. ISLER), p. 584, Birkhäuser, Basel (1971).
[2] W. J. RABOURN, F. W. QUACKENBUSH and J. W. PORTER, *Arch. Biochem. Biophys.* **48,** 267 (1954).
[3] F. B. JUNGALWALA and J. W. PORTER, *Arch. Biochem. Biophys.* **110,** 291 (1965).
[4] J. B. DAVIS, L. M. JACKMAN, P. T. SIDDONS and B. C. L. WEEDON, *J. Chem. Soc.* **C,** 2154 (1966).
[5] O. B. WEEKS, in *Aspects of Terpenoid Chemistry and Biochemistry* (edited by T. W. GOODWIN), p. 301, Academic Press, New York (1971).
[6] B. C. L. WEEDON, in *Carotenoids* (edited by O. ISLER), p. 268, Birkhäuser, Basel (1971).

of phytoene formed by higher plant systems from (5*R*)-[2-^{14}C-5-$^{3}H_1$]MVA and [2-^{14}C-5, 5-$^{3}H_2$]MVA,[7,8] it must undergo a subsequent isomerisation to form normal all-*trans* carotenoids. This isomerisation could take place at the phytoene level or at a later stage in the desaturation sequence,[9] for tomato phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene) is probably mainly the 15-*cis* isomer.[10] In some *Chlorella* mutants, even the ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene) occurs as the 15-*cis* isomer,[4] although ζ-carotene and the isomeric 7,8,11,12-tetrahydrolycopene (7,8,11,12-tetrahydro-ψ,ψ-carotene), of higher plants and photosynthetic bacteria respectively, normally occur as all-*trans* isomers.[11]

The present work is basically a survey of samples of phytoene from a number of natural sources. Thus the stereochemical characteristics of samples of phytoene isolated from the fungi, *Phycomyces blakesleeanus* and *Neurospora crassa*, and from the photosynthetic bacterium, *Rhodospirillum rubrum*, have been compared with those of phytoene from higher plant sources. In addition, the stereochemistry of the entire chromophore of fungal, bacterial and tomato phytoene has been investigated; this has been made possible by the recent synthesis of a series of phytoene analogues.[12] Comparisons of a number of spectroscopic parameters of the natural phytoenes with those of the synthetic analogues, in which four possible combinations of *cis* and *trans* configuration at the 13-, 13′- and 15-double bonds are represented, have led to a complete and stereochemically unambiguous description of the chromophore of the major component of each of the natural phytoenes.

TABLE 1. THE LEVELS OF 15-*cis* AND ALL-*trans* PHYTOENE IN A NUMBER OF NATURAL SOURCES

Source of phytoene	Wet or dry wt of original material	Total phytoene (mg)	Percentages of isomeric phytoenes 15-*cis*	all-*trans*
Tomato fruit	3·6 kg (wet)	12·0	97·2	2·8
Carrot oil	4·8 g	6·6	99·8	0·2
Neurospora crassa	400 g (wet)	16·5	98·0	2·0
Phycomyces blakesleeanus				
Wild type (100 μM DPA)	206 g (wet)	5·8	98·7	1·3
C5-*car*-10(−) mutant	28·6 g (dry)	9·8	96·8	3·2
Rhodospirillum rubrum (65 μM DPA)	24·0 g (dry)	17·8	85·1	14·9

RESULTS

Without exception, the samples of natural phytoene could be separated chromatographically into two distinct components. The proportions of these components, estimated spectrophotometrically, are indicated in Table 1. The less polar isomer, eluted from the alumina column (Brockmann activity grade I) with 2% diethyl ether in light petrol., had its λ_{max} in light petrol. at 286 nm and inflexions at 276 and 297 nm. The second isomer, eluted with 4% ether, had an absorption spectrum in light petrol. which exhibited three

[7] R. J. H. WILLIAMS, G. BRITTON, J. M. CHARLTON and T. W. GOODWIN, *Biochem. J.* **104,** 767 (1967).
[8] M. J. BUGGY, G. BRITTON and T. W. GOODWIN, *Biochem. J.* **114,** 641 (1969).
[9] S. C. KUSHWAHA, G. SUZUE, C. SUBBARAYAN and J. W. PORTER, *J. Biol. Chem.* **245,** 4708 (1970).
[10] B. K. KOE and L. ZECHMEISTER, *Arch. Biochem. Biophys.* **46,** 100 (1953).
[11] B. H. DAVIES, *Biochem. J.* **116,** 93 (1970).
[12] N. KHATOON, D. E. LOEBER, T. P. TOUBE and B. C. L. WEEDON, *Chem. Commun.* (1972). in press.

distinct peaks, at 276, 286 and 298 nm, with absorption minima at 278·5 and 289 nm; the main λ_{max} was that at 286 nm. Comparisons of these spectroscopic and chromatographic properties with those reported in the literature[3,4] indicate that the first and second isomers are 15-*cis* and 15-*trans* phytoene respectively.

These identifications were confirmed by the behaviour of each of the two isomers on isomerisation. On illumination of a light petrol. solution of the 15-*cis* isomer in the presence of iodine, the absorption spectrum underwent an overall increase in extinction and in persistence over a period of 15 min; the inflexions sharpened into distinct absorption maxima at 276 and 297·5 nm. The reverse effects were observed when the 15-*trans* phytoene was photoisomerised and the final equilibrium spectrum had an identical appearance in each case. Chromatography of the mixture resulting from the isomerisation of the 15-*cis* phytoene, after removal of the iodine by treatment with sodium thiosulphate, again yielded the two separate isomers. The isomerisation of the 15-*trans* isomer was on too small a scale to allow a corresponding chromatographic confirmation of its partial conversion to the 15-*cis* isomer.

In order to determine whether the 15-*trans* phytoene was a naturally occurring isomer or had been formed as an artifact during isolation, a sample of pure 15-*cis* phytoene was subjected to the normal isolation procedure. There was no indication of any isomerisation under these conditions. It was noted, however, that after a number of days' handling at laboratory temperatures, there was some isomerisation of 15-*cis* phytoene; this was apparent from increased IR absorption in the 950–990 cm^{-1} region.

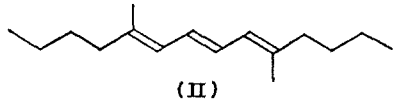
An IR absorption spectrum was determined for each of the samples of natural phytoene from carrot oil, tomatoes, *R. rubrum*, *N. crassa* and the *car*-10 mutant of *P. blakesleeanus*. In all cases, the spectrum indicated a strong absorption at 767 $\pm$ 2 cm^{-1}, with only weak absorption in the 950–990 cm^{-1} region. The strong absorption, corresponding to C–H out-of-plane deformations for the central, disubstituted double bond, indicates a 15-*cis* configuration for the major component of all the samples of natural phytoene. Only in one case, that of *N. crassa* phytoene, was it possible to isolate sufficient of both isomers for examination in this way. The 15-*cis* isomer had no absorption in the 950–990 cm^{-1} region, but had the characteristic strong band at 767 cm^{-1}; in the case of the other isomer, however, the '15-*cis* absorption' at 767 cm^{-1} was absent while a pronounced peak at 990 cm^{-1} and weaker bands at 971 and 962 cm^{-1}, more in keeping with a 15-*trans* configuration, were present.

Since rather larger samples of natural phytoene were required for NMR spectroscopy, not every sample was examined in this way. Such analyses were carried out, however, on a representative selection of samples isolated from tomatoes, from *P. blakesleeanus* (*car*-10 mutant) and from diphenylamine-inhibited cultures of *R. rubrum*. All yielded comparable 100 and 220 MHz spectra. Absorption for the methyl groups occurred at 1·58, 1·61, 1·66 and 1·74 ppm relative to TMS; the bands due to the methyl groups at the end of the central triene chromophore both occurred at 1·74 ppm. Coupling constants were calculated from the 220 MHz spectra for the olefinic protons, and these had the values $J_{14,15} = 12{\cdot}0$ Hz, $J_{14,15'} = -1{\cdot}3$ Hz, $J_{15,15'} = 10{\cdot}6$ Hz and $J_{14,14'} = -0{\cdot}3$ Hz. The two groups of signals for the triene were centred approximately 50 Hz apart ($\nu_o\delta_{AB} = 50$ Hz).

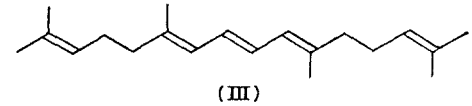
DISCUSSION

Samples of phytoene isolated from a number of higher plants, fungi and bacteria all have the same general properties. All contain two chromatographically separable

components, but in slightly differing proportions (Table 1); the less polar isomer usually comprises at least 95% of the mixture, although its proportion is apparently reduced to 85% in the photosynthetic bacterium, *R. rubrum*. The predominant isomer has been identified, on the basis of its UV spectrum, its spectroscopic and chromatographic behaviour on iodine-catalysed photoisomerisation and its IR spectrum, as 15-*cis* phytoene. The minor component could not, because of its less ready availability, be subjected to such rigorous analysis, but the evidence from its UV spectrum, from the changes occurring on isomerisation and from its IR spectrum, which has no peak at 767 cm^{-1}, all points to a 15-*trans* structure.



(II)



(III)

The full relevance of the NMR and other data obtained for the samples of natural phytoene only becomes apparent when these data are compared with those obtained for a number of synthetic models of the phytoene chromophore. Four isomers, *trans,trans-*, *trans-*, *trans,cis,trans-*, *trans,trans,cis-* and *trans,cis,cis-*, of each of a number of trienes, including (II) and (III), have recently been prepared by stereochemically unambiguous syntheses.[12] The 100 MHz NMR spectra of these models indicate that the position of the band due to a methyl group attached to a *trans* double bond at the end of the triene unit is independent of the stereochemistry of the rest of the chromophore. Thus both the *trans-*, *trans,trans* and *trans,cis,trans* isomers of (III) show the bands for both these methyls at 1·74 ppm, that is, at positions corresponding to those for the natural phytoenes. Any possibility that the natural phytoene could have either a *trans,trans,cis* or a *trans,cis,cis* configuration is eliminated because these models of (III) show bands at 1·74 and 1·76 and at 1·74 and 1·81 ppm respectively in their 100 MHz spectra. The band for a methyl group attached to a *cis* double bond moves downfield by 0·02 or 0·07 ppm depending on whether the neighbouring conjugated double bond is *trans* or *cis*.[12]

The IR spectra of the *trans,trans,trans* and *trans,cis,trans* isomers of (II) show a marked difference; while the C–H out-of-plane deformation for the central, disubstituted double bond occurs at 964 cm^{-1} in the case of the *trans,trans,trans* isomer, that for the *trans,cis-*, *trans* model is at 765 cm^{-1}.[12] Thus the natural phytoenes, with their prominent absorption at 767 cm^{-1}, are clearly *trans,cis,trans*.

The unambiguous assignment of this configuration to the natural phytoenes can be confirmed by a comparison of the coupling constants calculated from the 220 MHz spectra for the olefinic protons of the natural and synthetic trienes. The values for the natural phytoenes, $J_{14,15} = 12{\cdot}0$ Hz, $J_{14,15'} = -1{\cdot}3$ Hz, $J_{15,15'} = 10{\cdot}6$ Hz and $J_{14,14'} = -0{\cdot}3$ Hz, agree well with the corresponding values for the *trans,cis,trans* isomer of (II) (11·5, −1·4, 10·8 and −0·2 Hz respectively). The corresponding values for the *trans,trans,trans* isomer of (II) are 11·0, −1·1, 14·6 and 0·1 Hz respectively.[12] The value obtained on phytoene for the chemical shift between the interacting protons ($\nu_O\delta_{AB}$) of 50 Hz agrees with that for the synthetic *trans,cis,trans* compounds; the all-*trans* isomer of (II) had a corresponding frequency separation of 94 Hz.

That the 13- and 13′-double bonds of phytoene are *trans* has been assumed for a number of years, but has awaited unambiguous chemical proof until now. The only real evidence of such a configuration has come from biosynthetic studies. In the biosynthesis of phytoene

from doubly-labelled mevalonic acid by *P. blakesleeanus* and by tomato slices, the loss of all the tritium label from (4*S*)-[2-^{14}C-4-$^{3}H_1$]MVA and the retention of that from (4*R*)-[2-^{14}C-4-$^{3}H_1$]MVA has indicated that the 13- and 13'-double bonds are *trans*.[13]

The present investigation has extended the range of organisms in which the chromophore of phytoene is known to be of the *trans,cis,trans* configuration. Among all the organisms examined in these and other studies, only *Flavobacterium dehydrogenans* has, as a major isomer, a phytoene which shows the characteristics (IR and NMR) of an all-*trans* triene chromophore.[5,12]

The *trans,cis,trans* phytoene is not the only isomer present, however, in higher plants, fungi and photosynthetic bacteria; it is invariably accompanied by small quantities of what is probably all-*trans* phytoene. The relevance of the natural occurrence of both isomers to the stereochemistry of carotenoid formation must await further investigations of a biosynthetic nature, although some evidence for the conversion of 15-*cis* to all-*trans* phytoene has been obtained from studies on *R. rubrum* (P. M. Bramley and B. H. Davies, unpublished work).

EXPERIMENTAL

Solvents. All the solvents used were of AR grade. Light petrol. (b.p. 40–60°) and diethyl ether were dried over Na and redistilled from reduced iron powder prior to use. MeOH was distilled from KOH.

Higher plant materials. Carrot oil (5 ml) was saponified by boiling for 10 min in 1 l. MeOH to which 100 ml aq. 60% (w/v) KOH had been added and the unsaponifiable lipid was extracted by a standard procedure.[14] Slightly unripe tomatoes were purchased locally and the lipid was extracted and saponified and the unsaponifiable fraction isolated by standard procedure.[14]

Neurospora crassa. The culture of *Neurospora crassa* Shear and Dodge was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, and was maintained on Czapek Dox Agar (Difco) slopes. Static liquid cultures (100 ml each) were inoculated from these and grown for 7 days in horizontal Roux bottles at 30° under fluorescent light (3400 lx). The liquid medium was prepared using deionized water, and contained, per l.: sucrose, 50 g; KNO_3, 1 g; yeast extract (Difco), 25 g; malt extract, 5 g; NaCl, 2 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 500 mg; $CaCl_2$, 100 mg; $FeCl_3$, 5 mg and $ZnSO_4.7H_2O$, 2 mg. The cultures were harvested by filtering through muslin and the lipid was extracted and saponified by the standard procedure.[14]

Phycomyces blakesleeanus. Two strains of *Phycomyces blakesleeanus* were used. The wild type (22191) was obtained from the Commonwealth Mycological Institute, Kew, Surrey. The C5-*car*-10(−) mutant, which accumulates phytoene,[15] was kindly provided by Dr. K. Bergman, Division of Biology, California Institute of Technology, Pasadena, Calif., U.S.A. The name of this mutant follows the recommendations of the Caltech Group on Phycomyces Genetics Nomenclature[16] and replaces the designation 'Alb 10' which has been used previously.[15] Both strains were grown on a medium prepared using deionised water and which contained, per l.: glucose, 25 g; yeast extract, 500 mg; $MgSO.7H_2O$, 500 mg; thiamin hydrochloride, 0·25 mg; L-asparagine, 1·25 g; L-leucine, 1·25 g and KH_2PO_4, 1·5 g. In the case of the wild type, diphenylamine (DPA) was added at inoculation as an ethanolic solution (2·5 mg/ml) to give a final concentration of 100 μM. Each organism was cultured in 2 l. Erlenmeyer flasks, each containing 1 l. medium inoculated with a suspension of spores from a mature agar slope. Growth was in shake culture (180 rpm) at 24° under fluorescent light (5400 lx) for 72 hr. The cultures were harvested by filtration through muslin and the lipid was extracted and saponified by the usual methods.[14]

Rhodospirillum rubrum. Cultures of *Rhodospirillum rubrum* (N.C.I.B. 8255) were grown anaerobically in the light in the presence of 65 μM DPA under conditions which have been described elsewhere.[11] The bacteria were harvested and extracted and the lipid saponified by methods already described in detail.[11]

Isolation of crude natural phytoene. The preliminary separation of the total natural phytoene from other unsaponifiable components was carried out in each instance on a column of aluminium oxide (Woelm neutral alumina, Brockmann activity grade III) which was developed with light petrol. Up to 100 mg of unsaponifiable material could be accommodated, without overloading, on a 30 × 5 cm column. Fractions

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[14] B. H. DAVIES, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 489, Academic Press, New York (1965).
[15] G. MEISSNER and R. DELBRÜCK, *Plant Physiol.* **43,** 1279 (1968).
[16] K. BERGMAN, personal communication.

(20 ml) were collected and monitored spectrophotometrically; those which did not contain phytoene were discarded. This procedure resulted in the recovery of natural phytoene contaminated with a little phytofluene (detected by its green fluorescence in 360 nm light) and, in some cases, with squalene.

Separation of isomeric phytoenes. The crude phytoene from the preliminary separation was resolved into its isomers by a procedure which was a modification of an earlier method.[3] The phytoene was applied as a concentrated solution in light petroleum to a column (20 × 1·8 cm, 30 g) of alumina (Woelm neutral, Brockmann activity grade I). Development with 150 ml 0·5% diethyl ether in light petrol. (E/P) eluted any contaminating squalene; this was detected by TLC.[17] The column was then developed in turn with 300 ml 2% E/P and 250 ml 4% E/P; 10 ml fractions of the eluate were collected. Quantitative and qualitative spectrophotometric monitoring of these fractions showed approximately Gaussian elution profiles for two phytoene isomers. The maximal concentration of the less polar component in the eluate was reached after 150 ml 2% E/P had passed through the column, while the more polar component was eluted at maximal concentration after 120 ml 4% E/P had percolated through the alumina. The differences between the properties of these two isomers are described above.

UV absorption spectra. UV absorption spectra of the samples of phytoene were recorded from solutions in light petrol. contained in 1 cm quartz cells in a Unicam S.P. 800 recording spectrophotometer. The calibration of the instrument in the appropriate region was checked by using the 279·4 nm band of a standard holmium oxide filter. Quantitative determinations of phytoene were carried out on solutions of known volume by using the MW of 544·5 in conjunction with the molar absorption coefficients in this solvent at 286 nm; these are $41{\cdot}2 \times 10^3$ and $49{\cdot}8 \times 10^3$ l. mol^{-1} cm^{-1} for natural or 15-*cis* phytoene and all-*trans* phytoene respectively.[4]

Iodine-catalysed photoisomerisation. Determinations of whether samples of phytoene had a *cis* or a *trans* configuration were carried out by following the changes in their absorption spectra on iodine-catalysed photoisomerisation.[3,18] Isomerisations were carried out on light petrol. solutions of phytoene contained in 1 cm stoppered quartz spectrophotometer cuvettes. Iodine (1–2% of the quantity of phytoene) was added as a solution in light petrol. and the spectra were recorded at intervals during periods of up to 30 min illumination with two parallel fluorescent lamps (Philips MCFE, 65 W, 4400°, warm white) at a distance of 40 cm. Larger scale preparative photoisomerisations were carried out by dissolving the phytoene in light petrol. and dividing the solution into a number of 200 ml aliquots (2·5 mg phytoene per 200 ml). These samples, after the addition to each of 50 μg iodine as a concentrated solution in light petrol., were illuminated simultaneously in separate flasks and spectrophotometric monitoring was carried out on samples taken from one flask. When stereoisomeric equilibrium, indicated by the stabilisation of the absorption at 286 nm, had been reached, the contents of all the flasks were bulked and washed with aq. 5% sodium thiosulphate to remove the iodine. The iodine-free solution was dried over anh. Na_2SO_4 and the mixture of isomeric phytoenes recovered after filtration and distillation of the solvent.

Protection of samples from isomerisation. Unless undergoing deliberate photoisomerisation, all samples were protected from the effects of light at every stage of manipulation. Chromatography columns and all items of glassware were covered by wrapping in aluminium foil.

IR spectra. IR spectra were recorded from liquid films of phytoene. The Perkin–Elmer Infracord spectrophotometer was calibrated using the 907 and 1029 cm^{-1} absorption bands of a standard polystyrene film.

NMR spectra. The NMR spectra were recorded at 100 MHz on CCl_4 solutions with a Varian HA-100 instrument at the Department of Chemistry, Queen Mary College, London, from where the samples were sent to the Science Research Council Physico-Chemical Measurements Unit (Runcorn) for analysis at 220 MHz.

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