Biochem. J. (1970) **117**, 921–927
Printed in Great Britain

Aspects of the Stereochemistry of Torularhodin Biosynthesis

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(*Received* 13 *November* 1969)

1. The incorporation of [2-^{14}C]acetate, [2-^{14}C]mevalonic acid and [2-^{14}C,2-$^{3}H_2$]-mevalonic acid into torulene and torularhodin by *Rhodotorula rubra* and *Rhodotorula glutinis* was studied. 2. A recovery of 14.3% of the label was obtained on decarboxylation of the torularhodin biosynthesized from [2-^{14}C]mevalonic acid. 3. An analysis of the $^{3}H/^{14}C$ ratio in torularhodin gave a value of 9.44:8. 4. These results, obtained by different experimental techniques, show that the reactions in the conversion of the dimethyl group of isopentenyl pyrophosphate into the 16′,17′-position of torularhodin must be free from randomization. A mechanism for the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate is suggested.

MVA‡ has been shown in numerous reports to be an obligatory intermediate in the biosynthesis of the carotenoids (see reviews by Goodwin, 1965; Porter & Anderson, 1967). It has been shown that MVA is converted into MVA 5-phosphate, MVA 5-pyrophosphate, isopentenyl pyrophosphate and dimethylallyl pyrophosphate. Successive condensation reactions of isopentenyl pyrophosphate with dimethylallyl pyrophosphate, geranyl pyrophosphate and farnesyl pyrophosphate yield geranylgeranyl pyrophosphate, which condenses with a similar C_{20} unit to form phytoene. Phytoene is thought to be the precursor of the more unsaturated carotenoids.

MVA labelled in the 2-position with ^{14}C (Steele & Gurin, 1960; Braithwaite & Goodwin, 1960) and ^{3}H (Williams, Britton & Goodwin, 1967) has been shown to label the carotenes at six in-chain positions and in both *gem*-dimethyl positions. It was not possible to determine whether the label in the *gem*-dimethyl groups from [2-^{3}H]- or [2-^{14}C]-MVA is associated with a single carbon atom or is randomized.

The present paper reports the results of the analysis of torulene and torularhodin biosynthesized from MVA labelled in the 2-position with both ^{3}H and ^{14}C. In the conversion of torulene into torularhodin in *Rhodotorula* yeast, one methyl group is oxidized to a carboxyl group. Analysis of the $^{14}CO_2$ from the carboxyl group or of the loss of ^{3}H in the formation of torularhodin should provide information on the nature of the reactions involving this group.

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‡ Abbreviation: MVA, mevalonate.

EXPERIMENTAL

Organism. Rhodotorula glutinis (Fres.) Harrison 48-23T was provided by Dr H. J. Phaff of the University of California, Davis, Calif., U.S.A. *Rhodotorula rubra* var. *longa* was obtained from the Centraal Bureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands. Some of the characteristics of these strains have been reported by Nakayama, Mackinney & Phaff (1954), Simpson, Nakayama & Chichester (1964*a*,*b*) and Scharf & Simpson (1968).

Cultural conditions. The yeast cultures were grown in a medium containing 5% glucose, 0.5% yeast extract and 0.1% $(NH_4)_2SO_4$. On occasion 3% agar was added for semi-solid surface growth. When large volumes of cells were needed, the yeast was cultured in a 14-litre-capacity Microferm laboratory fermenter (model MF14; New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). Ten litres of medium were autoclaved at 120°C for 1h and inoculated with 100ml of a 3-day-old yeast suspension. The culture medium was agitated by means of a mechanical stirrer maintained at 300 rev./min. The temperature was held at 25°C and sterile air (8litres/min) was pumped through the fermenter. Shake cultures were employed when radioactive substrates were part of the culture medium. Portions (50ml) of media in 250ml Erlenmeyer flasks were incubated on a shaker for 11 days.

Disruption of the yeast cells. Disruption of small quantities of cells (less than 40ml packed volume) was accomplished with the use of a French pressure cell (American Instrument Co., Silver Springs, Md., U.S.A.) in conjunction with a hydraulic press (Simpson *et al.* 1964*a*,*b*; Scharf & Simpson 1968).

For disruption of larger volumes of yeast cells an Eppenbach Colloid Mill (model Q4-6; Gifford Wood Co., Hudson, N.Y., U.S.A.) was used. Glass beads (0.12mm; Minnesota Mining and Manufacturing Co., St Paul, Minn., U.S.A.) (3:5, v/v) were added together with the yeast cells. The mill was cooled by pumping iced water through it. Initially the distance between stator and rotor in the mill was set at 0.072in. This was gradually closed to 0.032in. The yeast cells were disrupted under an atmosphere of N_2 gas for 1h.

Pigment extraction and chromatography. The pigments were extracted from the disrupted yeast with acetone. The extraction procedure was repeated until the extract was nearly colourless. When the colloid mill was used, the yeast cells were decanted from the glass beads and repeatedly extracted with acetone. The pigments were transferred to light petroleum (b.p. 40–60°C) by the addition of water and washed free of acetone. The solution was dried over anhydrous Na_2SO_4 at −10°C for 24h.

Two procedures were employed for separation of pigments. When torularhodin was the only pigment wanted, it was isolated by chromatography on a column (2.6cm internal diam.) of MgO–Hyflo Super-Cel (1:2, w/w). The column was initially developed with acetone–light petroleum (b.p. 40–60°C) (1:99, v/v), then washed with 500ml of acetone–light petroleum mixture followed by 1 litre of acetone. Under these conditions torularhodin remained at the top of the column and the other, less polar, pigments were eluted. The column was dried and extruded, and the torularhodin band was cut from the column. The pigment was eluted from the adsorbent with 0.1M-HCl–diethyl ether (1:9, v/v). The ethereal solution was washed free of HCl with water and dried over anhydrous Na_2SO_4 overnight at −10°C.

A slightly different procedure was followed for recovery of both torulene and torularhodin. The extract was chromatographed on the MgO–Hyflo Super-Cel column. The chromatogram was developed with acetone–light petroleum (1:99, v/v) until the two red bands separated. Torularhodin remained on the top of the column and was eluted from the adsorbent as described above. The torulene band appeared between torularhodin and the yellow carotenoids.

The pigments below the torularhodin band were eluted from the adsorbent with acetone. The light petroleum–acetone extract was washed free of acetone before saponification. An equal volume of 10% (w/v) KOH in methanol was added to the light-petroleum extract of the pigments. The mixture was placed on a steam table in the dark for 30min and a stream of N_2 gas was blown over the mixture during saponification. Portions of the saponified material were slowly poured into a separating funnel containing light petroleum and a small amount of acetone. Addition of water transferred the pigments to the epiphase, which was then washed several times with water and dried over anhydrous Na_2SO_4.

The light-petroleum extract was rechromatographed on a MgO–Hyflo Super-Cel column and developed with acetone–light petroleum (1:99, v/v). The column was extruded and the torulene band was cut from the column plug. Torulene was eluted from the adsorbent with acetone and transferred to light petroleum with water. The light-petroleum solution containing the pigment was dried overnight over anhydrous Na_2SO_4 at −10°C.

The pigment extract was centrifuged at −10°C to remove the precipitate of sterols that had formed. The supernatant was transferred to a centrifuge tube and held at −20°C for 2 days, then centrifuged in a clinical centrifuge at −20°C to remove any precipitate of amorphous material that had formed. The supernatant was decanted into a glass centrifuge tube before crystallization.

Crystallization of labelled pigments. Crystalline torulene and torularhodin, previously prepared, were added as seed crystals to the glass centrifuge tubes containing the corresponding pigment in light petroleum. The centrifuge tubes were placed in a freezer at −10°C until crystallization was complete (2–3 weeks). This material was washed repeatedly with warm light petroleum to remove non-crystalline material. The washed crystalline pigments were dried under a stream of N_2 gas and used within 1 day for the decarboxylation work or scintillation counting.

Identification of carotenoids by their visible-absorption spectra. The pigments were identified by their position on the chromatographic column and by their characteristic absorption spectra (Simpson *et al.* 1964*a*). A Cary 15 recording spectrophotometer was used in determining absorption maxima.

Addition of radioactive substrates. Weighed amounts of the salts of [2-^{14}C]MVA and [2-^{14}C]acetate were added to sterile water. Appropriate portions of the solutions were added to the culture medium 24h after inoculation with yeast cells. All radioactive carbon compounds were obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

The lactone of [2-^{3}H]MVA was received in benzene from Amersham–Searle Corp., Des Plaines, Ill., U.S.A. The biologically inactive lactone was converted into the biologically active free acid by the method of Cornforth,

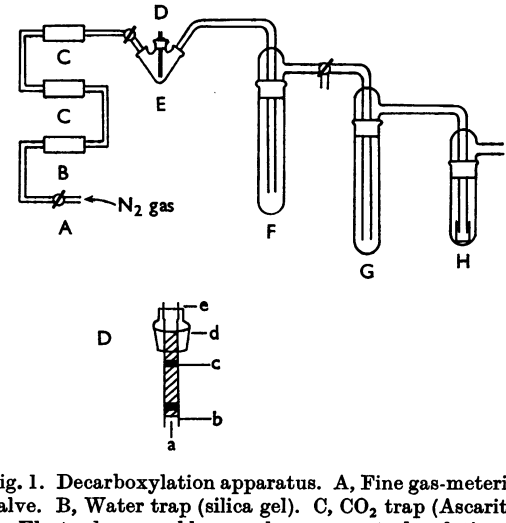


Fig. 1. Decarboxylation apparatus. A, Fine gas-metering valve. B, Water trap (silica gel). C, CO_2 trap (Ascarite). D, Electrode assembly: a, glass support; b, platinum–iridium electrodes; c, Teflon rings; d, standard-taper polyethylene stopper; e, power-supply connectors. E, 15ml three-necked pear-shaped flask. F, Liquid-N_2 gas trap. G, Gas trap (H_2SO_4). H, Trap with glass counting vial containing CO_2 absorbent.

Cornforth, Donninger & Popják (1966). The benzene was evaporated under a stream of N_2 gas and the remaining lactone was dissolved in a slight excess of 1M-KOH. This solution was kept at 37°C for 30min, neutralized with HCl and made up to volume with sterile water. Portions were then added to the culture medium.

The pigments were extracted and crystallized as described. The efficiency of the incorporation of [2-^{14}C]-acetate into crystallized torularhodin was 0.03%. The incorporation of [2-^{14}C]MVA into crystalline torularhodin varied from 0.05 to 0.06%.

Electrolytic decarboxylation. The decarboxylation apparatus, as modified from that described by Cornforth, Gore & Popják (1957), consisted of a gas train containing three N_2 gas scrubbers (two for CO_2, one for water), a three-necked 15ml pear-shaped reaction vessel and three gas traps. Two 24-gauge platinum–iridium wires were sealed through a polyethylene stopper. The wires were supported by a glass rod cemented to the stopper with epoxy cement and the electrodes were maintained 3mm apart and projected to the bottom of the reaction cell (Fig. 1).

The cell was charged with ^{14}C-labelled torularhodin in chloroform (0.2ml). The chloroform was evaporated with a stream of N_2 gas. Pyridine (0.1ml), methanol (0.1ml) and 1–2mg of cinnamic acid were then added to the cell. The addition of cinnamic acid greatly increased the efficiency of the decarboxylation reaction; it may act as a carrier or as an aid in cross-coupling in the Kolbe reaction. The cell was cooled by immersing it in iced water while a 5mA current was passed through the cell. The reaction was assumed complete when the current dropped to zero (3–5h). The cell was then heated in boiling water for 15min.

The CO_2 gas evolved during electrolysis was swept by N_2 gas into a trap immersed in liquid N_2. On warming, the gas was transferred from the liquid-N_2 trap through a gas trap containing 15ml of 1.5M-H_2SO_4 and then to a glass scintillation-counting vial. The counting vial contained 5ml of a solution made up of 27% methanol, 27% phenethylamine and 46% toluene.

Radioactive monitoring of effluent gases during decarboxylation and transfer to the CO_2 absorbent indicated that no labelled gases were lost from the system.

Preparation of samples for liquid scintillation counting and measurement of radioactivity. The crystalline torulene and torularhodin samples were transferred from the glass centrifuge tubes in which they had been crystallized to glass counting vials. Toluene (8ml) was added to the glass counting vial and the pigment was bleached overnight with a sunlamp. Then 8ml of phosphors [0.5g of 2,5-diphenyloxazole (PPO) and 10mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl-POPOP) in 50ml of toluene] was added to the bleached samples for scintillation counting.

To the counting vial containing the radioactive CO_2 was added 1ml of phosphor solution [4.0g of 2,5-diphenyloxazole and 80mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 50ml of toluene] and 10ml of toluene. The final volume of all samples was 16ml.

On occasion, labelled pigments in a solvent were transferred to counting vials. The solvent was evaporated under a stream of N_2 gas and then treated in the same manner as crystalline samples.

Measurements of radioactivity were made with a Nuclear–Chicago Mark I liquid-scintillation spectrometer incorporating a ^{133}Ba rod as an external source of radiation. An external standard counting procedure was

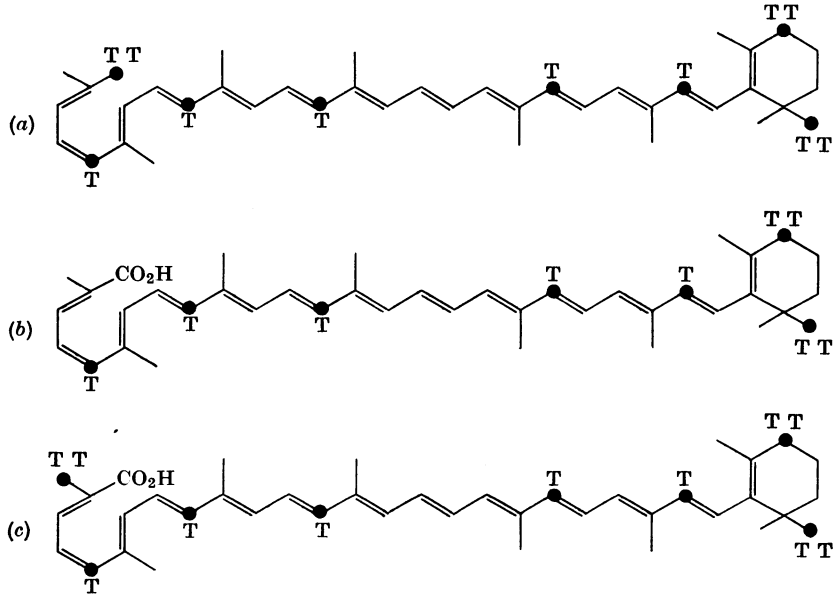


Fig. 2. Structures of torulene and torularhodin. ● and T, Expected label from [2-^{14}C,2-$^{3}H_2$]MVA based on the labelling pattern in β-carotene (Williams *et al.* 1967). (*a*) Torulene, ^{3}H/^{14}C ratio 11:8; (*b*) torularhodin, ^{3}H/^{14}C ratio 9:8; (*c*) torularhodin, ^{3}H/^{14}C ratio 11:8.

followed for measuring the radioactivity of singly-labelled samples. A channels-ratio technique utilizing the ^{133}Ba rod was used to determine counting efficiency.

Purification of solvents. Light petroleum (b.p. 40–60°C) (A.R.), acetone (A.R.), methanol (A.R.) and phenethylamine (A.R.) were all distilled before use. Toluene (A.R.) and spectro quality chloroform and pyridine were used directly. Diethyl ether (A.R.) was distilled and passed through a column of neutral alumina (Woelm, Alupharm Chemicals, New Orleans, La., U.S.A.) to remove peroxides.

RESULTS

It is known that [2-^{14}C]MVA contributes carbon to the end methyl groups of β-carotene and to six other positions in the molecule (Fig. 2). In the formation of torularhodin one or either end methyl group may be utilized in the formation of the carboxyl group. If the enzymes responsible for the conversion show stereospecificity, decarboxylation of ^{14}C-labelled torularhodin should yield an amount of radioactivity equal to 1/8 (12.5%) or none of the total radioactivity in the molecule. Alternatively, if the enzyme does not act in a stereospecific manner, or if randomization occurs in the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate, 1/16 (6.25%) of the total radioactivity should be found in the CO_2 fraction.

An oxygen-flask method was initially employed to determine the total radioactivity of the sample (Oliverio, Denham & Davidson, 1962). Samples of [7-^{14}C]benzoic acid were found to give 6282 d.p.m./mg on combustion, whereas samples, the radioactivity of which was counted directly, yielded 6311 d.p.m./mg. Because of the similarity of results, the direct counting of the radioactivity of samples was used as the method of determining total radioactivity of radioactive samples.

An electrolytic-decarboxylation apparatus (Fig. 1) was constructed and tested with labelled compounds: *p*-amino[7-^{14}C]benzoic acid yielded approx. 42% of the ^{14}C as recovered $^{14}CO_2$; [2-^{14}C]cinnamic acid yielded less than 1% $^{14}CO_2$ when no ^{14}C was expected.

Torularhodin biosynthesized by *Rhodotorula rubra* cultured on a medium containing [2-^{14}C]-acetate was used as a means of determining the efficiency of the process. Both *gem*-methyl groups plus 20 other positions in the molecule should be labelled. Decarboxylation should yield a theoretical value of 4.17% of the total label. Table 1 shows that 43.2% of the total radioactivity was recovered after decarboxylation.

Torularhodin isolated and crystallized from *R. rubra* cells cultured on a medium containing [2-^{14}C]-MVA was decarboxylated. By utilizing the efficiency of the decarboxylation (Table 1), it was possible to calculate the total label present in the carboxyl group. Table 2 shows that an average of 14.3% of the label was recovered in the CO_2 fraction.

An alternative method of investigation involved the use of double-labelled compounds. Williams *et al.* (1967) showed that phytoene and β-carotene isolated from carrot-root slices incubated with (3*RS*)-[2-^{14}C,2-$^{3}H_2$]MVA yielded mean atomic ratios (^{3}H/^{14}C) of 15.90:8 and 12.25:8 respectively. On this basis torulene would be expected to

Table 1. *Decarboxylation of torularhodin labelled from* [2-^{14}C]*acetate*

Sample no.	Total radioactivity of sample (d.p.m.)	Theoretical yield of CO_2 from $^{14}CO_2H$ (d.p.m.)	Actual yield (d.p.m.)	Percentage decarboxylation
1	4716	197	89	45.2
2	2700	113	45	41.8
3	9320	389	157	40.4
4	4660	194	83	42.8
5	7860	328	153	45.7
			Average	43.2

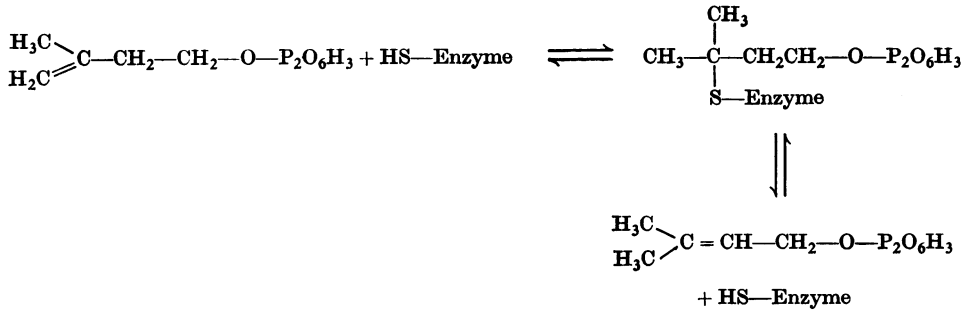
Table 2. *Decarboxylation of torularhodin labelled from* [2-^{14}C]*MVA*

Sample no.	Total radioactivity of sample (d.p.m.)	Radioactivity in recovered $^{14}CO_2$ (d.p.m.)	% of radioactivity in $^{14}CO_2$	% of radioactivity in CO_2H
1	4980	274	5.50	12.7
2	3306	200	6.06	14.0
3	5340	327	6.12	14.2
4	4450	314	7.06	16.3
			Average	14.3

Table 3. *Incorporation of* [2-^{14}C,2-$^{3}H_2$]*MVA into carotenoid pigments by yeast*

Polyene	Radioactivity (d.p.m.) ^{3}H	Radioactivity (d.p.m.) ^{14}C	Mean ^{3}H/^{14}C radioactivity ratio	Mean ^{3}H/^{14}C atomic ratio
Torulene*	176887	25306	6.99	11:8
Torulene†	147231	21870	6.73	11:8
Torularhodin*	15977	2674	5.97	9.40:8
Torularhodin†	14818	2554	5.80	9.48:8
			Average	9.44:8

*, Exp. 1. †, Exp. 2.



Scheme 1. Proposed mechanism of isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate (Lynen *et al.* 1959).

have atomic ratio 11:8 and torularhodin could have atomic ratios 9:8, 10:8 or 11:8, depending on the conditions of its synthesis.

Rhodotorula glutinis was cultured on a substrate containing (3*RS*)-[2-^{14}C,2-$^{3}H_2$]MVA. Table 3 shows that the average ^{3}H/^{14}C ratio found in torularhodin was 9.44:8.

DISCUSSION

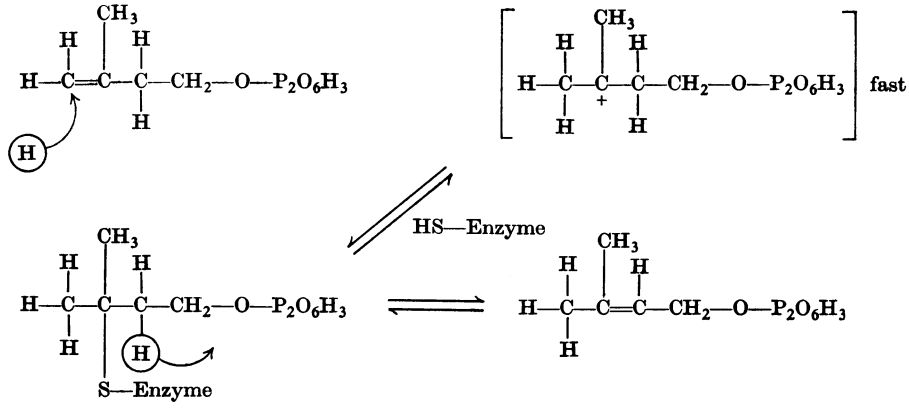
Decarboxylation of torularhodin formed from [2-^{14}C]MVA gave an average value of 14.3% of the total label in the molecule. These results are closer to the theoretical value of 12.5% than either of the alternative values (6.25 or 0%). These results show that the label is lost on decarboxylation. The evidence from studies with double-labelled substrates also supports the stereospecific conversion of torulene into torularhodin. For the conversion to be stereospecific, the ^{3}H/^{14}C ratio should be 11:8 or 9:8. A 9:8 ratio would be required for agreement with the decarboxylation studies. A 9.44:8 ratio was obtained.

These results require that all previous reactions involving these atoms be free from randomization. One possible point of randomization could occur in the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate.

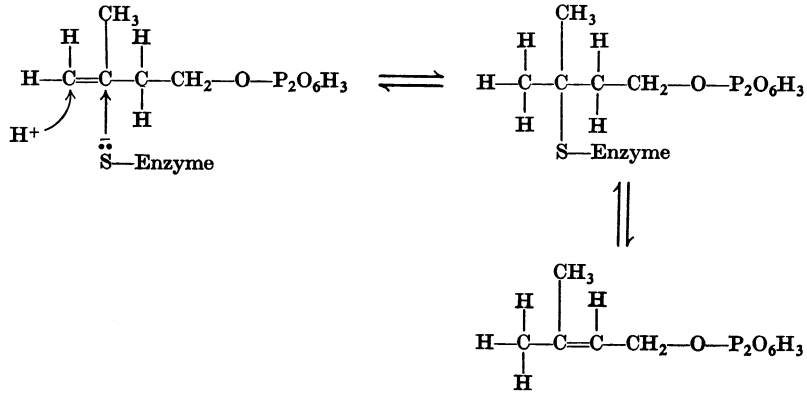
Lynen, Agronoff, Eggerer, Henning & Moslein (1959) showed that the isomerase catalysing the reaction contains an active thiol group. Lynen *et al.* (1959) proposed the mechanism shown in Scheme 1.

A second mechanism proposed by Popják & Cornforth (1960) and extended by Shah, Cleland & Porter (1965) suggests the formation of a free carbonium ion intermediate in the isomerization reaction. Tritiated water was used by Shah *et al.* (1965) in an attempt to establish the mechanism of the reaction. It was found that the addition and removal of a proton at C-2 of isopentenyl pyrophosphate and dimethylallyl pyrophosphate is stereospecific. These authors favoured a non-concerted mechanism for the reaction (Scheme 2). Shah *et al.* (1965) further state that 'In this mechanism, the methylene carbon atom of isopentenyl pyrophosphate is first protonated, resulting in the formation of a carbonium ion. This ion is then neutralized, possibly by covalent bond formation with sulfhydryl group of the protein'. They indicated that the step involving stabilization of the carbonium ion occurs very rapidly and that only those hydrogen atoms on the methylene carbon are exchangeable.

It is equally probable that the reaction involving the proposed carbonium ion could not proceed at a rate that would prevent randomization. Our



Scheme 2. Proposed mechanism of isomerization of isopentenyl pyrophosphate and dimethylallyl pyrophosphate (Shah *et al.* 1965).



Scheme 3. Proposed mechanism for the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate.

results, which show no randomization of the label in torularhodin, favour a concerted attack by the proton and the enzyme as shown in Scheme 3.

This mechanism would be consistent with our findings that the label is not randomized in the dimethyl groups of torularhodin. It would follow that all of the acyclic carotene precursors of torularhodin (Simpson *et al.* 1964*a*) would have to be of the same configuration. It would not necessarily follow that the cyclic carotene dimethyl groups would be of the same configuration.

Williams *et al.* (1967) proposed a mechanism for the cyclization of carotenes to form α- or β-carotene. One could envisage an expanded two-stage mechanism that would involve a carbonium ion on C-1. The mechanism could allow the dimethyl groups to be randomized through the carbonium ion. On the basis of the results reported here, one would have to suggest that the concerted mechanism proposed by Williams *et al.* (1967) would be a more likely mechanism.

We thank Dr H. J. Phaff of the University of California for the cultures used in this work and Dr O. Isler of F. Hoffman–La Roche, Basle, Switzerland, for providing synthetic torularhodin methyl ester. This work was supported in part by National Science Foundation Grant GB6213 and the U.S. Public Health Service Grant no. 1 RO1 NB 08516-01 of the National Institute of Neurological Diseases and Stroke. This paper is contribution no. 1332 of the University of Rhode Island Agricultural Experiment Station.

REFERENCES

Braithwaite, G. D. & Goodwin, T. W. (1960). *Biochem. J.* **76**, 5.

Cornforth, J. W., Cornforth, R. H., Donninger, C. & Popják, G. (1966). *Proc. R. Soc.* B, **163**, 492.

Cornforth, J. W., Gore, I. Y. & Popják, G. (1957). *Biochem. J.* **65**, 94.

Goodwin, T. W. (1965). In *Chemistry and Biochemistry of Plant Pigments*, p. 143. Ed. by Goodwin, T. W. New York: Academic Press Inc.

Lynen, F., Agranoff, B. W., Eggerer, H., Henning, U. & Moslein, E. M. (1959). *Angew. Chem.* **71**, 657.

Nakayama, T., Mackinney, G. & Phaff, H. J. (1954). *Antonie Van Leeuwenhoek*, **20**, 217.

Oliverio, V. T., Denham, C. & Davidson, J. D. (1962). *Analyt. Biochem.* **4**, 188.

Popják, G. & Cornforth, J. W. (1960). *Adv. Enzymol.* **22**, 281.

Porter, J. W. & Anderson, D. G. (1967). *A. Rev. Pl. Physiol.* **18**, 197.

Scharf, S. S. & Simpson, K. L. (1968). *Biochem. J.* **106**, 311.

Shah, D. H., Cleland, W. W. & Porter, J. W. (1965). *J. biol. Chem.* **240**, 1946.

Simpson, K. L., Nakayama, T. O. M. & Chichester, C. O. (1964*a*). *J. Bact.* **88**, 1688.

Simpson, K. L., Nakayama, T. O. M. & Chichester, C. O. (1964*b*). *Biochem. J.* **92**, 508.

Steele, W. J. & Gurin, S. (1960). *J. biol. Chem.* **235**, 2778.

Williams, R. J. H., Britton, G. & Goodwin, T. W. (1967). *Biochem. J.* **105**, 99.