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The Biosynthetic Origin of the Carboxyl Oxygen Atoms of the Carotenoid Pigment, Torularhodin

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The structure of torularhodin was elucidated by Ruegg *et al.* (1958), and it was shown to contain a single carboxyl group at position 1'. The logical precursor to the carboxylated pigment would appear to be γ -carotene (Fig. 1). Kayser & Villoultreix (1960) studied an X-ray mutant of *Rhodotorula mucilaginosa* and found that the amounts of γ - and β -carotene decreased, whereas those of torulin and torularhodin increased, in the mutant. The authors therefore suggested that γ -carotene was the precursor of torulin and torularhodin.

Molecular oxygen is required in the formation of the xanthophylls (van Niel, 1947; Claes, 1959). Sapozhnikov, Maevskaya, Krasovskaya-Antropova, Prialgaukaite & Turchina (1959) and Yamamoto, Chichester & Nakayama (1962*a*) showed that molecular oxygen is incorporated directly into the leaf xanthophylls. In the latter paper it was shown that the biosynthetic origin of the oxygen in the hydroxyl group is the atmosphere, but it was further suggested that water furnished the epoxy oxygen.

Shneour (1962) has shown that molecular oxygen is utilized in the formation of the carbonyl group of the carotenoid spheroidenone in *Rhodopseudomonas spheroides*. The aim of the present investigation was to determine the biosynthetic origin of the oxygen atoms in torularhodin.

EXPERIMENTAL

Organism and growth conditions. A strain of *Rhodotorula rubra* var. *longa* was obtained from the Centraal Bureau voor Schimmelcultures, Yeast Division, Delft, Holland. The culture was grown in shasta flasks (flat whisky bottles of 473 ml. capacity) on the medium described by Nakayama, Mackinney & Phaff (1954), inoculated and harvested as described.

Extraction of pigment from cells. For a volume greater than 500 ml. a colloid mill (Gifford-Wood Co., Hudson, N.Y.; model QV 6) was used for the disruption of the yeast cells before solvent extraction. For volumes of cells of less than 100 ml. a modified French press was used for the disintegration of the yeast cells (Simpson, Wilson, Burton, Nakayama & Chichester, 1963).

Crystalline torularhodin from yeast was obtained by extracting the pigment from the cells harvested from 100 shasta flasks. The washed yeast suspension (1.25 l.) was added to the colloid mill together with 750 ml. of glass beads (average diameter 120 μ) (Minnesota Mining and Manufacturing Co., St Paul, Minn.) and 25 ml. of 0.2M-phosphate buffer at pH 7.0. Usually 1 hr. was required for an 80–90% disintegration of the yeast cells as estimated by microscopic examination.

The modified French press was used according to the procedure of French & Milner (1955) at 25 000 lb./in.². Usually two passages through the press were sufficient for a complete extraction of the pigment.

Fig. 1

The disrupted cells were immersed in an excess of acetone, shaken for 2 min. and centrifuged. Several extractions sufficed to render the residual cell material nearly white. The pigment solution was then mixed with light petroleum (b.p. 40–60°) in a separating funnel and the acetone removed by the addition of water. The light petroleum was dried by passing it through a column of anhydrous sodium sulphate. A considerable amount of colourless impurities was precipitated from the light-petroleum solution by holding it at –10° overnight. On chromatographing the pigment mixture on magnesium oxide-Hyflo Super-Cel (Johns-Manville Co.) (1:2, w/w) torularhodin formed a red band at the top of the column which was moved only very slowly by the developing solvents (light petroleum, diethyl ether-methanol and acetone), which were used successively. The other pigments were eluted completely by these solvents. The band containing torularhodin was cut from the extruded column and eluted from the adsorbent with diethyl ether-acetic acid (10:1, v/v). The acetic acid was washed from the ether with water and the ether was dried over anhydrous sodium sulphate. For the isotopic studies the acetic acid was removed on a rotary high-vacuum flash evaporator and the pigment was removed from the flask with diethyl ether. The pigment was crystallized from diethyl ether-methanol (1:1, v/v) by the slow evaporation of the solvents. The crystals were washed with light petroleum and recrystallized from methanol.

Preparation of synthetic torularhodin. Torularhodin was also prepared from a sample of synthetic torularhodin methyl ester (given by Dr O. Isler, F. Hoffmann-La Roche and Co., Basle, Switzerland). The ester (20 mg.) was dissolved in 200 ml. of diethyl ether, to which an equal volume of a solution in methanol containing KOH (20%, w/v) and water (1%, v/v) was added. Saponification was completed by heating for 2 hr. on a steam bath. The addition of any larger volume of water caused the formation of two phases and greatly reduced the rate of the reaction. The salt of the acid is only slightly soluble in an aqueous alkaline solution and is not soluble in the ether phase. After the addition of water the salt of the pigment was separated from the unsaponified ester by filtration. The pigment was converted into the acid form with dilute HCl and diethyl ether. The ether was washed free of the mineral acid and the pigment was dried and crystallized as described above. A yield of 17.3 mg. of the crystalline pigment was obtained. The natural and the synthetic torularhodin had identical melting points (200–204° with decomposition, uncorrected) and their infrared- and ultraviolet-absorption spectra were superimposable.

The value of 1932 for $E_{1\text{cm}}^{1\%}$ at the characteristic maximum (515 m μ) in CHCl₃ was determined and used for quantitative determinations.

Procedures with ¹⁸O. For isotopic studies three shasta flasks were inoculated and the yeast was grown for 15 days in an atmosphere of O₂ (5.97 and 5.61 atoms % excess of ¹⁸O) + N₂ (2:8, v/v). The apparatus was similar to the one of Yamamoto *et al.* (1962a). The amount of yeast was estimated from non-isotopic runs to be 2–3 g. dry weight containing at least 500 μ g. of torularhodin. The oxygen uptake was followed manometrically. The torularhodin obtained from the isotopic studies was crystallized and recrystallized with the aid of the previously prepared pigment.

The pigment was pyrolysed according to the method of Rittenberg & Ponticorvo (1956) as modified by Yamamoto,

Chichester & Nakayama (1962b). The ratios for both CO₂ (mass 46–mass 44) and the enriched oxygen atmosphere (mass 34–mass 32) were determined on a Consolidated Electro Dynamics mass spectrometer, through the courtesy of California Research, Standard Oil Co. of California.

RESULTS AND DISCUSSION

Nakayama *et al.* (1954) identified the carotenoids of a number of asporogenous yeasts. One of those described, *Rhodotorula glutinis* (Fres.) Harrison (48–23T), exhibited a marked change in its pigment concentration when grown at 5° rather than 25°. At the lower temperature γ - and β -carotenes were formed, whereas at the higher temperature an increase in the oxygenated compounds, torulin and torularhodin, was observed. When this species was grown at 5° and then transferred to 25° the yeast at the surface became red in 25–48 hr., but the lower layers remained yellow. The red colour was mainly due to torularhodin, as torulin is a minor constituent of this strain of *R. glutinis*.

The strain 48–23T was obtained and it was confirmed that it still showed the same effect of temperature as had been reported. It was hoped that it would be possible to study the incorporation of atmospheric oxygen into torularhodin by aerating washed cells at 25° that had previously been grown at 5°. No change in colour, however, was observed over a 24 hr. period under these conditions. This observation is not clearly understood. Apparently there is not a simple conversion as in the formation of spheroidenone (Shneour, 1962). These results, together with the time required for the formation of red pigment on the slants, indicate that growth is required for the formation of the pigment.

R. rubra, which produces far more torularhodin than the *R. glutinis* species (Nakayama *et al.* 1954), was grown in an enriched atmosphere of ¹⁸O₂. The results of Table 1 show that under these experimental conditions one atom of oxygen is probably contributed by the atmosphere to the carboxyl group.

Oxygen atoms in the carboxylic acid group are subject to both enzymic (Bentley, 1949; Senkus & Brown, 1938) and acid-catalysed (Senkus & Brown, 1938; Cohn & Urey, 1938) exchange with water oxygen atoms. If exchange reactions were to occur during growth or during any of the isolation procedures the effect would be to remove oxygen that had been inserted biosynthetically, and the incorporation of ¹⁸O would be lower than that predicted.

The isolation procedures that were used were such that any unnecessary exposure of the pigment to low pH values was avoided. To assess the magnitude of possible exchange reactions, 3 mg. of crystalline torularhodin was shaken with 20 ml. of diethyl

Table 1. ^{18}O content in torularhodin from *Rhodotorula* grown in an ^{18}O -enriched atmosphere

In the first experiment the atmosphere contained 5.97 ± 0.002 excess ^{18}O , and in the second 5.61 ± 0.002 excess ^{18}O . These percentages were determined by analysis of the gaseous phase in each case. The atoms of ^{18}O contributed were calculated by the use of the expression (atoms % excess $\times$ dilution $\times$ 2)/(atoms excess of $^{18}\text{O}_2$).

Expt.	Atoms % excess	Dilution	Atoms % excess $\times$ dilution	Atoms contributed	Possible atoms contributed
1	0.071	7.32	0.517	0.173	1
2	0.082	7.72	0.631	0.225	1

ether-acetic acid (10:1, v/v) together with 2 ml. of 6 atoms % excess of H_2^{18}O ; 0.3 % of the oxygen of the pigment exchanged with those of the water in 15 min. under these conditions. Although the acetic acid-ether was removed under vacuum in the purification of the yeast pigments, no significant exchange would have occurred had water been used to remove the acid. These results indicate that the exchange with water during the other steps of the isolation would be small.

The pigment obtained from the column usually had a spectrum whose peak was $5\text{ m}\mu$ lower than expected. After crystallization, however, the main absorption band was at $515\text{ m}\mu$. The position of the initial maximum was due to the formation of *cis-trans* isomers from *trans-trans* double bonds during isolation. These isomers were formed in normal handling procedures, particularly in the presence of strong acids.

The use of the crystalline non-isotopic torularhodin (1.5 mg.) as an aid in the crystallization of the ^{18}O -enriched pigment (0.24 and 0.22 mg.) reduced the concentration of the isotopic pigment in the crystalline product below that which was predicted by the extinction values. Since the *trans* pigment is crystallized selectively the net effect was to give a lower value to the atoms contributed than might be expected.

It is reasonable to expect that biochemically the hydroxylation of a methyl group occurs through the direct participation of atmospheric oxygen, whether it is at the γ -carotene stage or earlier. It is also reasonable to assume that subsequent oxidations would probably utilize oxygen from water (Gillette, 1959). The results reported here are thus consistent with those in the biosynthesis of cholesterol from lanosterol. This transformation requires a number of steps, as three methyl groups in the presence of molecular oxygen are lost as CO_2 (Olson, Lindberg & Bloch, 1957).

In *R. rubra* the carboxylic acid pigment torula-

rhodin is not readily further metabolized; thus the origin of the oxygen in the carboxyl group could be determined.

SUMMARY

1. Torularhodin was isolated from *Rhodotorula rubra* grown in an enriched atmosphere of $^{18}\text{O}_2$. Isotopic analysis showed that one atom was incorporated into the carboxyl group.

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