Studies in Carotenogenesis

25. THE INCORPORATION OF [1-¹⁴C]ACETATE, [2-¹⁴C]ACETATE AND ¹⁴CO₂ INTO LYCOPENE BY TOMATO SLICES*

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The labelling pattern in β -carotene after the addition of [¹⁴C]acetate to cultures of the fungi *Mucor hiemalis* and *Phycomyces blakesleeana* (Grob, 1956, 1957; Braithwaite, 1958; Goodwin, 1958b, 1959a, b) is now well established; it is the same as that observed in sterols and other terpenoids (see Wolstenholme & O'Connor, 1959; Goodwin, 1960). The methyl carbon atom is found in C-*w*, *w'* and C-*y*, with only slight activity, if any, in C-*x* and C-*z* of the isoprenoid-polymerizing unit (the method of identifying the individual carbon atoms of the isoprenoid residue is

The activity from the carboxyl carbon atom is confined exclusively to C-*x* and C-*z*. Zabin (1957) has examined the distribution of labelling in lycopene (I) synthesized by intact tomato fruit in the

(I)

presence of [¹⁴C]acetate. He degraded the labelled pigment with ozone and isolated and counted the resulting laevulinic acid, formic acid and acetic acid and acetone. From these results, and making some assumptions, Zabin calculated the relative isotope distribution in each carbon atom of the isoprenoid residue. With [1-¹⁴C]acetate the distribution was as indicated in (II), and the distribution observed with [2-¹⁴C]acetate is given in (III). These patterns are radically different from those

observed with other carotenoids and steroids; because of these unexpected results the

incorporation of [1-¹⁴C]- and [2-¹⁴C]-acetate into lycopene by tomatoes has been re-investigated with tomato slices instead of intact fruit. The uptake of ¹⁴CO₂ into lycopene has also been examined. Part of this work has already been briefly reported (Goodwin, 1958c; 1959a).

EXPERIMENTAL

Preparation of tomato slices. Thin slices (1–2 mm.) were cut from the centre of ripe tomatoes and carefully floated on an aqueous solution of the appropriate substrate in Petri

dishes. After being kept at room temperature for 48 hr. the slices were collected, washed with water and dried between sheets of filter paper.

In experiments with ¹⁴CO₂ the slices in Petri dishes were incubated in a large vacuum desiccator with the outlet in the lid sealed with a bung. Sufficient Ba¹⁴CO₃ to provide an atmosphere containing 0.3% (v/v) of CO₂ was weighed into a small tube. This was lowered by a cotton thread through the outlet in the desiccator lid into a small beaker containing a 1:1 (v/v) mixture of lactic acid and water, and the bung immediately replaced. The lactic acid liberates CO₂ slowly.

Extraction, purification and crystallization of lycopene. The tomato pigments were extracted as described by Goodwin (1956). The extract was saponified and the

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unsaponifiable matter obtained by standard procedures (Goodwin, 1955) was dissolved in light petroleum (b.p. 40–60°) and the lycopene separated by column chromatography with methanol-deactivated alumina (equivalent to Brockmann grade 4; P. Spence and Co. Ltd., Widnes, Lancs.) as adsorbent and light petroleum containing 2% (v/v) of acetone as developer (Goodwin, 1955). Unlabelled synthetic lycopene was added to the chromatographically pure lycopene and the mixture was crystallized to constant radioactivity (Goodwin, 1958a).

Determination of lycopene. The amount of lycopene present in various fractions was determined spectrophotometrically with a Unicam SP. 600 photoelectric spectrophotometer (Goodwin, 1956).

Degradation of lycopene. The lycopene was oxidized with alkaline permanganate by the method of Karrer, Helfenstein, Wehrli & Wettstein (1930) adapted to small quantities. Lycopene (10 mg.) in 1.66 ml. of a 1:1 (v/v) mixture of pure toluene and thiophen-free benzene was added to 6.66 ml. of water containing 0.083 g. of KMnO_4 and 0.133 g. of anhydrous Na_2CO_3 in a 50 ml. conical flask. The mixture was shaken for 24 hr. at room temperature, boiled for 2 hr. under a reflux condenser and then distilled for a few minutes to remove benzene and toluene. The aqueous residue was then acidified with phosphoric acid and the excess of KMnO_4 and the residual MnO_2 were reduced by the gradual addition of 30% H_2O_2 . The CO_2 formed during the oxidation was liberated during this process and collected in a $\text{Ba}(\text{OH})_2$ trap. The residual solution was filtered and the acetic acid formed during the oxidation was steam-distilled after first adding 2 ml. of concentrated H_2SO_4 ; the distillate was made just alkaline with NaOH and evaporated to dryness.

Degradation of acetate. The acetic acid obtained by degradation of the lycopene was degraded by the Schmidt reaction with a modification of the method of Schuerch & Huntress (1949). Acetic acid (10 m-equiv.) and finely powdered sodium azide (12 m-equiv.) were placed in a filter tube fitted with a bung carrying a tube connected to a source of CO_2 -free N_2 ; the side arm was connected to a $\text{Ba}(\text{OH})_2$ trap. The apparatus was flushed with CO_2 -free N_2 , the bung was partly removed and concentrated H_2SO_4 (analytical grade; 160 m-equiv.) was quickly added and the bung replaced. The reaction vessel was then immersed in a water bath maintained at 60°. The CO_2 evolved was collected in the $\text{Ba}(\text{OH})_2$ trap. After 1 hr. the apparatus was again flushed with CO_2 -free N_2 to recover any remaining CO_2 and the precipitated BaCO_3 was filtered off, washed with CO_2 -free water and methanol successively, and finally dried in a vacuum desiccator.

The reaction mixture was then made alkaline with NaOH and the methylamine distilled into concentrated HCl and collected as the hydrochloride.

Radioactive materials. $[1\text{-}^{14}\text{C}]\text{Acetate}$, $[2\text{-}^{14}\text{C}]\text{acetate}$ and $\text{Ba}^{14}\text{CO}_3$ were purchased from The Radiochemical Centre, Amersham, Bucks.

Assay of ^{14}C . All measurements were made with a proportional gas-flow counter (Nuclear Measurements, Chicago, U.S.A., model PC-3). All counts were corrected for self-absorption and counting was continued to give a standard error of gross count rates of $\pm 5\%$.

Samples of lycopene were plated on to a planchet from chloroform solution, acetic acid samples as sodium acetate from aqueous solution, methylamine samples as the hydro-

chloride from aqueous solution and CO_2 samples as a slurry of BaCO_3 . The planchets used in the proportional counter were flat stainless-steel disks; to obtain samples of constant area, a small disk of lens tissue of slightly smaller diameter than that of the planchet was cut and stuck to the planchet by adding one drop of sucrose solution (1%, w/v) and drying under an infrared lamp. The material to be assayed was then placed on the lens tissue, and dried and assayed. For thick samples, e.g. BaCO_3 , a clamp device was used which allowed samples of equal surface area to be prepared; no lens tissue was used for these. At least two samples of each preparation were assayed.

Specific activity of lycopene. In order to calculate the specific activity of the lycopene originally present in the slices, the amount of chromatographically pure lycopene present was first measured spectrophotometrically, then a known amount of pure lycopene was added and the mixture crystallized. The crystals were dissolved in an appropriate volume of light petroleum and the amount of lycopene present was again measured spectrophotometrically. From these two measurements and the radioactivity of the final solution, the specific activity of the original lycopene could be calculated.

RESULTS

Comparison of the incorporation of $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]\text{-acetate}$ into lycopene in tomato slices. If acetate is incorporated into lycopene by tomatoes via the same mechanism as that demonstrated for sterols (see, for example, Popják, 1958) and carotenoids in other organisms (Grob, 1958; Goodwin, 1958a), then the ratio of the incorporation of $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]\text{-acetate}$ into the pigment should be close to 1.5:1. Zabin (1957) reported a value of 5.5:1. As this is remote from the expected value the experiments of Zabin were repeated with two modifications which it was thought would allow more comparable experimental conditions. Slices from the same fruit were compared instead of different whole fruit, and incubation was carried out for 48 hr. instead of from 10 to 15 days. We are aware that the ideal solution would have been to use doubly labelled acetate but this material was not available to us. In spite of the use of comparable slices it is very difficult to obtain them with the same amount of lycopene present, owing to the differential pigment distribution in tomato fruit. A synthesis of lycopene cannot be demonstrated spectrophotometrically in slices under our conditions, but the most reasonable assumption is that it is proportional to the amount of pigment originally present in the slice. So, in comparing the percentage incorporations into lycopene with $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]\text{-acetate}$, the values must be referred to a unit amount of pigment. Similarly, the ratio of the specific activities of lycopene calculated on the amount of lycopene originally present should closely approximate that for the newly synthesized pigments.

Table 1. *Incorporation of [¹⁴C]acetate into lycopene by tomato slices*

Slices were incubated for 48 hr. under conditions described in the Experimental section.

Substrate	Activity of lycopene	
	[¹⁴ C]Acetate*	[2- ¹⁴ C]Acetate*
10 ⁶ × Total radioactivity added (counts/min.) ...	7.8	5.0
Specific activity of lycopene† (counts/min./μmole)		
(a) Chromatographically pure	16 500	4100
(b) Once-recrystallized	3 050	3750
(c) Twice-recrystallized	2 800	3750
(d) Thrice-recrystallized	2 800	—
Total activity in pure lycopene (counts/min.)	2 700	1800
Percentage of total activity added found in lycopene	0.0346	0.0360

* Specific activity (counts/min./mole): 5.0 × 10¹².

† Calculated on the amount of lycopene present originally in the slices.

Table 1 summarizes the results of an experiment carried out under the conditions just described; in both cases the specific activity of the acetate used was 5.0 × 10¹² counts/min./mole. It will be seen that with [¹⁴C]acetate 0.0346% of the total counts added were incorporated into lycopene, whereas with [2-¹⁴C]acetate the figure was 0.0360%; this represents about the same degree of isotope incorporation as observed with the carotenogenic fungus *Phycomyces blakesleeanus* (Braithwaite, 1958). As the amount of lycopene present in the slices exposed to [¹⁴C]acetate was 0.965 μmole and in the [2-¹⁴C] experiment 0.48 μmole, the ratio of percentage incorporation of [2-¹⁴C]acetate to [¹⁴C]acetate per unit (1 μmole) of lycopene is (0.036/0.48):(0.0346/0.965), or 2.1:1. If the specific activity of the lycopene arising from [¹⁴C]acetate is recalculated on the basis of 5.0 × 10⁶ and not 7.8 × 10⁶ counts added [this is justified because such small amounts of acetate (1.0 and 1.56 μmole) were added] then the ratio of the specific activities of the lycopene based on the original amounts present arising from [2-¹⁴C]acetate and [¹⁴C]-acetate is 3750/1796, which is 2.1:1. Both these values are much closer to the expected value and remote from the value reported by Zabin (1957).

Activity of the acetic acid obtained from the oxidation of lycopene. Samples of crystalline lycopene labelled with [¹⁴C]- and [2-¹⁴C]-acetate were oxidized with alkaline permanganate, and the acetic acid arising from C-9, C-9', C-13, C-13' and the methyl groups attached thereto were collected and degraded by the Schmidt reaction. The results obtained (Table 2) indicate that with [¹⁴C]acetate no activity is found in the methyl C atom of the acetic acid, all the activity being present in the carboxyl C atom. When [2-¹⁴C]acetate is used almost all the activity is found in the methyl C atom of the acetic acid, with only trace amounts in the carboxyl C atom. This distribution of activity is characteristic of the pattern observed in β-carotene

Table 2. *Degradation of acetic acid obtained from labelled lycopene oxidized with alkaline permanganate*

Methods of oxidation and degradation are described in the Experimental section. (a) and (b) denote two separate experiments.

Labelled substrate	Activity (counts/min.)		
	[¹⁴ C]Acetate	[2- ¹⁴ C]Acetate	
		(a)	(b)
Isolated acetic acid	160	80	300
C-1	130	10	0
C-2	0	60	250

in *P. blakesleeanus* (Grob, 1956, 1957; Braithwaite, 1958) and other terpenoids, including sterols (Popják, 1958). It is unequivocally different from that reported by Zabin (1957).

Incorporation of ¹⁴CO₂ into lycopene by tomato slices. The atypical distribution of activity observed by Zabin (1957) in lycopene isolated from tomatoes injected with [¹⁴C]acetate could be explained qualitatively by assuming that part of the labelled acetate was rapidly oxidized to CO₂, which was subsequently fixed into lycopene in a random manner. However, if established routes of CO₂-fixation are functioning even a combination of labelling from CO₂ and from acetate cannot give rise to Zabin's quantitative results. In spite of this it was considered important to find out whether tomato slices can fix ¹⁴CO₂ into β-carotene, because under normal cultural conditions the carotenogenic fungus *P. blakesleeanus* does not; fixation does, however, occur in the presence of the amino acid leucine (Braithwaite, 1958; Goodwin, 1959a). When tomato slices were allowed to take up ¹⁴CO₂ from a gas phase containing 0.3% (v/v) of CO₂, the percentage of total radioactivity incorporated was less than 10% of that incorporated when comparable slices were allowed to incorporate [¹⁴C]-acetate from a solution containing tracer amounts

Table 3. Incorporation of $^{14}\text{CO}_2$ into lycopene by tomato slices

Slices were incubated for 48 hr. in an atmosphere (5 l.) containing 0.3% (v/v) of $^{14}\text{CO}_2$ *; other conditions are described in the Experimental section.

$10^6 \times$ Total radioactivity added (counts/min.) ...	66
Specific activity (counts/min./ μmole) of lycopene†	
(a) Chromatographically pure	2750
(b) Once-recrystallized	2150
(c) Twice-recrystallized	2100
(d) Thrice-recrystallized	2150
Total counts in pure lycopene	1650
Percentage of total counts added found in lycopene	0.0025

* Specific activity (counts/min./mole) = 0.985×10^{11} .

† Calculated on the amount of lycopene present originally in the slices.

of the substrate (Table 3). When, however, the relative amounts of CO_2 and acetate available to the slices are considered, more CO_2 than acetate is fixed into lycopene. The amount of $[2\text{-}^{14}\text{C}]$ acetate incorporated in the experiment recorded in Table 1 is $1.0 \times 0.036/100 = 0.00036 \mu\text{mole}$, whereas the amount of CO_2 fixed is $(5/22.4 \times 0.3/100) (0.0025/100) = 0.01675 \mu\text{mole}$. In this, tomato slices resemble isolated photosynthetic tissues of higher plants in that $^{14}\text{CO}_2$ is a much better substrate for β -carotene formation than is acetate (Goodwin, 1958a); on the other hand, tomato slices differ from the fungus *P. blakesleeanus*.

DISCUSSION

The results reported in Table 2, together with the recent observations that $[^{14}\text{C}]$ mevalonate, a key biosynthetic intermediate in β -carotene and all other terpenoid systems so far examined (see Wolstenholme & O'Connor, 1959), is incorporated into lycopene in tomato systems *in vitro* and *in vivo* (Shneour & Zabin, 1959; Purcell, Thompson & Bonner, 1959), indicate that the biosynthetic pathway from acetate to lycopene is the same as in other terpenoid compounds. The results obtained by comparing the incorporation of $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]$ -acetate into tomato slices (Table 1) also tend to support this conclusion, although unequivocal results could come only from using doubly labelled acetate.

An explanation of the unexpected results of Zabin (1957) must be sought. The passage of acetate through the tricarboxylic acid cycle could account for randomization of C-2 into C-1 and thus the appearance of label in all positions of lycopene when $[2\text{-}^{14}\text{C}]$ acetate was added to the lycopene-synthesizing system of tomatoes; our results with experiments of shorter duration (48 hr. compared

with 10–15 days) show that this randomization does not occur to any significant extent. Furthermore, in experiments with *P. blakesleeanus* lasting 7–14 days no randomization was observed (Goodwin, 1959a); Grob (1956, 1957) also found no significant randomization of C-2 of acetate into β -carotene in *Mucor hiemalis*, and in other terpene systems the same situation exists. Thus the participation of the tricarboxylic acid cycle probably cannot account for the distribution of radioactivity recorded in lycopene (II) by Zabin when $[2\text{-}^{14}\text{C}]$ -acetate was used.

The tricarboxylic acid cycle certainly cannot be implicated to explain Zabin's observed randomization with $[1\text{-}^{14}\text{C}]$ acetate (III) for the label should either remain in C-1 or be lost as carbon dioxide. The only possible way of obtaining overall labelling with $[1\text{-}^{14}\text{C}]$ acetate would be as a result of the operation of an active carbon dioxide-fixing mechanism. Our results (Table 3) show that $^{14}\text{CO}_2$ can be more actively incorporated than acetate (the amount being some 50 times as great in our experiments) into lycopene in tomato slices. However, in spite of this observation, a number of arguments against the view that Zabin's experiments are due to carbon dioxide-fixation can be put forward: (i) no known mechanism of carbon dioxide-fixation can give rise to two-carbon-atom units which would quantitatively label the isoprenoid residue of a terpene as indicated in (II) or (III); (ii) if acetate were being actively metabolized to carbon dioxide then, even in 48 hr. experiments such as ours, randomization of label should have occurred, because of the effective carbon dioxide-fixing mechanism which is present; (iii) similarly, if acetate were being actively metabolized, an effective carbon dioxide-fixing mechanism would, over 1–15 days (the period of Zabin's experiments), lead to a general labelling which would tend to be the same irrespective of whether the initial source of ^{14}C was $[1\text{-}^{14}\text{C}]$ - or $[2\text{-}^{14}\text{C}]$ -acetate.

The only alternative explanation of Zabin's results is that in the ozonolytic degradation which he used, the resulting formate did not arise exclusively from the expected positions. This type of error has previously been reported (see Popják, 1958) and is probably the correct explanation in this case.

SUMMARY

1. Experiments on the incorporation of $[^{14}\text{C}]$ -acetate and $^{14}\text{CO}_2$ into tomato slices are reported.
2. The patterns of incorporation of $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]$ -acetate into lycopene are the same as those observed in β -carotene in *Phycomyces blakesleeanus* and in all other terpenoid compounds examined.
3. There is a significant incorporation of $^{14}\text{CO}_2$ into lycopene, the amount incorporated being some

50 times as great as when [^{14}C]acetate is the substrate.

4. The previously reported atypical labelling pattern in lycopene synthesized by intact tomatoes (Zabin, 1957) was probably the result of the use of an inadequate method of degradation of the labelled pigment.

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26. THE INCORPORATION OF [^{14}C]ACETATE, [^{14}C]MEVALONATE AND $^{14}\text{CO}_2$ INTO β -CAROTENE BY THE FUNGUS *PHYCOMYCES BLAKESLEEANUS**

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When mevalonic acid ($\beta\beta$ -dihydroxy- β -methylvaleric acid) was found to be an extremely effective precursor of cholesterol in liver preparations (see Popják, 1958; Wolstenholme & O'Connor, 1959), it was important to ascertain whether it was also an effective carotenoid precursor. The major part of this paper is concerned with experiments which have been carried out on the incorporation of DL-[2- ^{14}C]mevalonate into β -carotene by the fungus *Phycomyces blakesleeanus*, and to compare it with [2- ^{14}C]acetate.

The incorporation of $^{14}\text{CO}_2$ into β -carotene by *P. blakesleeanus* in the presence and absence of leucine has also been investigated, in an attempt to explain the results of Mackinney and his colleagues on the incorporation of [^{14}C]leucine into β -carotene in the same organism (Wuhrmann, Yokoyama & Chichester, 1957; Yokoyama, Chichester, Nakayama, Lukton & Mackinney, 1957; Mackinney,

Chandler & Lukton, 1958; Chichester, Yokoyama, Nakayama, Lukton & Mackinney, 1959).

The results of part of this investigation have been briefly reported previously (Braithwaite & Goodwin, 1957a, b; Goodwin, 1959a, b).

EXPERIMENTAL

Cultural conditions. The strain of *P. blakesleeanus* used in this investigation was that originally used by Garton, Goodwin & Lijinsky (1951). It was maintained on slopes of Sabouraud agar (Oxoid). The methods used for sterilizing and inoculating liquid media have been described in detail by Garton *et al.* (1951). Experimental cultures were generally carried out in 250 ml. Erlenmeyer flasks containing 30 ml. of the standard medium of Garton *et al.* (1951). The flasks were continuously shaken in a rotary shaker and incubated at 28° in an illuminated cabinet (Garton *et al.* 1951). For the experiments with $^{14}\text{CO}_2$, mats of *P. blakesleeanus* were cultured in Petri dishes.

Extraction, purification and crystallization of β -carotene. The extraction and purification of β -carotene from the harvested mycelia have been described (Garton *et al.* 1951; Goodwin, 1952). Unlabelled synthetic β -carotene was

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