Studies in Carotenogenesis

27. INCORPORATION OF [2-¹⁴C]ACETATE, DL-[2-¹⁴C]MEVALONATE AND ¹⁴CO₂ INTO CARROT-ROOT PREPARATIONS*

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In the previous paper in this series (Braithwaite & Goodwin, 1960*a*) we have reported on the incorporation of [2-¹⁴C]acetate, DL-[2-¹⁴C]mevalonate and ¹⁴CO₂ into β-carotene by the mould *Phycomyces blakesleeanus*. The present paper deals with the incorporation of these labelled substrates into carrot slices, grated carrots and cell-free extracts of carrots. It is remarkable that although carrot roots are the classical source of β-carotene, this is the first investigation to be reported in which carrots have been used for tracer studies on carotenogenesis.

Part of this work has already been briefly reported (Braithwaite & Goodwin, 1957; Goodwin, 1958*a*, 1959).

EXPERIMENTAL

Carrot-root preparations. Young red carrots bought from a local greengrocer were used in all experiments. Carrot slices were prepared from about the centre of a root, and thin sections cut with a sterile razor blade were floated on a solution of the appropriate substrate in a Petri dish in a manner similar to that described for tomato slices (Braithwaite & Goodwin, 1960*b*). In experiments with ¹⁴CO₂ the slices in Petri dishes were incubated in an atmosphere of 0.3% of CO₂ in a vacuum desiccator as described previously for tomato slices and *P. blakesleeanus* (Braithwaite & Goodwin, 1960*a*, *b*).

Experiments were also carried out with preparations of grated carrot, made by grating carrots, previously chilled to -15°, through a clean wire gauze.

Cell-free preparations of carrot roots were prepared from the grated tissue. The gratings were suspended in ice-cold potassium phosphate buffer (0.1M, pH 7.4) containing nicotinamide (0.03M), MgCl₂ (4 mM) and sucrose (0.125M) and fragmented at 0° in a loose-fitting Teflon-pestle homogenizer for 30 sec. (Bucher, 1953). The cell debris was removed by low-speed centrifuging (350 g) at 0° for 5 min. The supernatant was used for experiments with [¹⁴C]-acetate and DL-[2-¹⁴C]mevalonate.

Extraction, purification, determination and crystallization of β-carotene. The standard techniques developed in our Laboratory have previously been described in detail (Goodwin, 1958*b*; Braithwaite & Goodwin, 1960*a*, *b*).

Degradation of labelled β-carotene. This has previously been described in detail (Braithwaite & Goodwin, 1960*b*).

Radioactive materials. [2-¹⁴C]Acetate and Ba¹⁴CO₃ were purchased from The Radiochemical Centre, Amersham, Bucks. DL-[2-¹⁴C]Mevalonate was kindly synthesized by Dr R. H. Cornforth.

Assay of ¹⁴C. All measurements were made in a proportional gas-flow counter (Nuclear Measurements Corp. Inc., Chicago, Ill., U.S.A.; model PC-3). Full details of the procedures adopted were given by Braithwaite & Goodwin (1960*b*). All counts were corrected for self-absorption and counting was continued to give a standard error of gross count rates of ±5%.

RESULTS AND DISCUSSION

Incorporation of [2-¹⁴C]acetate and DL-[2-¹⁴C]mevalonate into β-carotene in carrot slices

Preliminary experiments showed that both [2-¹⁴C]acetate and DL-[2-¹⁴C]mevalonate were incorporated into β-carotene by isolated carrot slices. Experiments were then carried out to compare the incorporation of these two substrates in slices from the same root. Table 1 shows that both substrates

Table 1. *Incorporation of [2-¹⁴C]acetate and DL-[2-¹⁴C]mevalonate into β-carotene by carrot slices*

Slices (1–2 g.) were floated on a solution (15 ml.) of labelled substrate in Petri dishes for 24 hr. at room temperature. Specific activity of [2-¹⁴C]acetate, 5×10^{12} counts/min./mole; specific activity of DL-[2-¹⁴C]mevalonate, 2.2×10^{10} counts/min./mole.

	[2- ¹⁴ C]- Acetate	DL-[2- ¹⁴ C]- Mevalonate
10 ⁶ × Total counts added	7.4	2.35*
Percentage of total counts utilized	100	98
Specific activity of β-carotene† (counts/min./μmole)	26 600	15 000
Total counts in β-carotene (counts/min.)	2 700	6 500
Percentage of counts utilized found in β-carotene	0.0365	0.280

* The number of counts added was twice this figure as Wolf *et al.* (1957) showed that DL-mevalonate had only one-half the microbiological activity of the naturally occurring compound.

† Recrystallized three times; specific activity calculated for β-carotene originally present.

* Part 26: Braithwaite & Goodwin (1960*a*).

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are almost completely metabolized by the slices and that, on the basis of total counts incorporated, mevalonate is some seven times more effectively incorporated into β -carotene than is acetate. However, as three acetate methyl-carbon atoms are incorporated into each isoprenoid (C_5) unit for each C-2 of mevalonate incorporated, the overall efficiency of mevalonate compared with acetate is 21:1. This is similar to the figure (16:1) obtained with the mould *P. blakesleanus* (Braithwaite & Goodwin, 1960a).

*Degradation of β -carotene derived from
DL-[2- ^{14}C]mevalonate*

If the incorporation of mevalonate into β -carotene follows the same pathway as that in *P. blakesleanus* (Braithwaite & Goodwin, 1960a), then C-2 should appear in the 'backbone' of the molecule; that is in C-8, -8', -12, -12', but not in the lateral methyl groups attached to C-9, -9', -13, -13' (I). Degradation of β -carotene with alkaline permanganate yields acetic acid derived from C-9, -9', -13, -13' and the adjacent lateral methyl-carbon atoms. When this degradation was carried out on labelled β -carotene formed by isolated carrot slices in the presence of DL-[2- ^{14}C]mevalonate, the resulting acetic acid was unlabelled. Thus mevalonate is incorporated into β -carotene by carrot slices in the same way as by *P. blakesleanus* (Braithwaite & Goodwin, 1960a); the same pattern is also observed in steroids and other terpenes (see Wolstenholme & O'Connor, 1959).

Incorporation of $^{14}CO_2$ into β -carotene by carrot slices

Carrot slices placed on damp filter paper were exposed for 48 hr. to an atmosphere (volume 5 l.) containing 0.3% of $^{14}CO_2$ (60×10^6 counts/min.) in the apparatus described by Braithwaite & Goodwin (1960b). Although some radioactivity was found in the unsaponifiable matter, none was present in the crystallized β -carotene. In this way carrot slices are similar to *P. blakesleanus* grown in absence of leucine (Braithwaite & Goodwin, 1960a), but different from tomato slices, which incorporate significant amounts of $^{14}CO_2$ into β -carotene (Braithwaite & Goodwin, 1960b).

Incorporation of [2- ^{14}C]acetate and DL-[2- ^{14}C]mevalonate into β -carotene by cell-free preparations of carrot root

Many unsuccessful attempts have been made in this Laboratory to prepare cell-free systems from

Table 2. *Incorporation of DL-[2- ^{14}C]mevalonate into β -carotene by carrot gratings*

Gratings were incubated for 24 hr. at room temperature. Specific activity of DL-[2- ^{14}C]mevalonate, 2.2×10^{10} counts/min./mole.

$10^6 \times$ Total counts added*	4.7
Percentage of total counts utilized	20
Specific activity of β -carotene (counts/min./ μ mole)†	5400
Total counts in β -carotene (counts/min.)	1180
Percentage of counts utilized found in β -carotene	0.125

* Twice this number of counts was actually added as DL-[2- ^{14}C]mevalonate.

† Recrystallized three times; specific activity was calculated for the β -carotene originally present.

the mould *P. blakesleanus* which would incorporate [^{14}C]acetate or [^{14}C]mevalonate into β -carotene (see Modi, 1955; Braithwaite, 1958). However, it has been possible to prepare such a system with carrot-root tissue. Carrot gratings will effectively incorporate acetate and mevalonate into β -carotene, as the results of an experiment with DL-[2- ^{14}C]mevalonate (Table 2) indicate. Consequently, the gratings were disintegrated in a Teflon-pestle homogenizer for 5 min. The resulting supernatant was incapable of incorporating significant radioactivity from [2- ^{14}C]acetate or DL-[2- ^{14}C]mevalonate into

β -carotene. However, when the gratings were homogenized for 30 sec. (Bucher, 1953) a small but significant incorporation into β -carotene was observed (Table 3). In order to make certain that the incorporation was significant five samples from each experiment were counted and each sample was counted four times. As with whole slices, DL-[2- ^{14}C]mevalonate was more efficiently incorporated than acetate. In an experiment designed to test the effect of the addition of adenosine triphosphate, coenzyme A and di- and tri-phosphopyridine nucleotides (Table 4), the incorporation of [2- ^{14}C]acetate into the control preparation was some three times as great as in the experiment recorded in Table 3. The effect of the added cofactors was ambiguous; on the basis of specific activity of the β -carotene, they approximately doubled the incorporation. However, when total counts incorporated were compared the difference was not significant. This problem was not pursued further.

In all our experiments it was found, in agreement with the original work of Bucher (1953) on cholesterol biosyntheses in liver homogenates, that disintegration of the carrot tissue for longer than 30 sec. rapidly inactivated the preparation. Since this work was first briefly reported (Goodwin, 1958*a*, 1959), similar preparations have been obtained from tomatoes which incorporate [2-¹⁴C]-acetate into lycopene (Shenour & Zabin, 1959). The preparations from carrot roots also incorporate acetate and mevalonate into the other components (mainly sterols) of the unsaponifiable matter of the

Table 3. *Incorporation of [2-¹⁴C]acetate and DL-[2-¹⁴C]mevalonate into β -carotene by cell-free preparations of carrot-root tissue*

Preparation of the cell-free system is described in the Experimental section; extract from 1–2 g. of tissue was suspended in 10–15 ml. of phosphate buffer and, without shaking, incubated for 24 hr. at room temperature. Specific activities of acetate and mevalonate were as given in Table 1.

	[2- ¹⁴ C]- Acetate	DL-[2- ¹⁴ C]- Mevalonate
10 ⁶ × Total counts added (counts/min.)	3.7	0.96*
Specific activity of β -carotene† (counts/min./ μ mole)	1600	550
Total counts in β -carotene (counts/min.)	125	75
Percentage of added counts present in β -carotene	0.0034	0.0078

* Twice this number of counts was actually added as DL-[2-¹⁴C]mevalonate.

† Recrystallized three times; specific activity was calculated for the β -carotene originally present.

Table 4. *Incorporation of [2-¹⁴C]acetate into β -carotene by preparations of carrot-root tissue in the presence and absence of cofactors*

Conditions were as described in Table 2. Cofactors added: adenosine triphosphate (50 μ moles), coenzyme A (1 μ mole), diphosphopyridine nucleotide (1 μ mole), triphosphopyridine nucleotide (1 μ mole).

	No cofactors	Cofactors added
10 ⁶ × Total counts added (counts/min.)	2.44	2.44
Specific activity of β -carotene* (counts/min./ μ mole)	780	1340
Total counts in β -carotene (counts/min.)	235	265
Percentage of added counts present in β -carotene	0.0096	0.0109

* Twice recrystallized; specific activity was calculated for β -carotene originally present.

Table 5. *Comparison of incorporation of [2-¹⁴C]-acetate into the β -carotene and total unsaponifiable matter of cell-free preparations of carrot-root tissue*

The data were obtained from the experiment described in Table 3.

	No cofactors	Cofactors added
Activity in total unsaponifiable matter (counts/min.)*	18 300	22 200
Activity in β -carotene (counts/ min.)	235	265
Activity in β -carotene as percentage of activity in un- saponifiable matter	1.29	1.20
Wt. of unsaponifiable matter (mg.)	7.9	3.4
Wt. of β -carotene (mg.)†	0.16	0.106
β -Carotene as percentage of unsaponifiable matter	2.03	3.12

* Mainly sterols.

† Determined spectrophotometrically.

tissue. The results given in Table 5 shows that there is no preferential accumulation of activity in either the sterols or the β -carotene. Thus we are still far from a preparation which will synthesize β -carotene preferentially and attempts to do this will be the aim of future investigations.

SUMMARY

1. Carrot-root slices and gratings will effectively incorporate [2-¹⁴C]acetate and DL-[2-¹⁴C]mevalonate into β -carotene. Mevalonate is more efficiently incorporated than acetate.

2. ¹⁴CO₂ is not incorporated into β -carotene by these preparations.

3. A cell-free system has been obtained from carrot roots which incorporates [2-¹⁴C]acetate and DL-[2-¹⁴C]mevalonate into β -carotene. The other components of the unsaponifiable matter (mainly sterols) and also labelled.

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28. THE EFFECT OF ILLUMINATION ON CAROTENOID SYNTHESIS IN FRENCH-BEAN (*PHASEOLUS VULGARIS*) SEEDLINGS

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It has clearly been established that seeds of many plants, if germinated under the most stringent conditions of light-exclusion, synthesize carotenoids (see Goodwin, 1952). Xanthophylls predominate and, in maize, wheat and barley, are similar to those present in green leaves (Strain, 1938). However, investigators have not been unanimous about the changes undergone on illuminating etiolated seedlings. Xanthophylls were said to develop more quickly than carotenes by Beck (1937), Seybold & Egle (1938) and Nagel (1940), whereas Barrenscheen, Pany & Srb (1942) and Blaauw-Jansen, Komen & Thomas (1950), using wheat seedlings, claimed that carotenes increased more quickly than xanthophylls. More recently it has been stated that illumination of etiolated maize seedlings brought about a specific stimulation of β -carotene (Kay & Phinney, 1956). This observation was followed up by Goodwin (1958), who considered that illuminated etiolated maize seedlings might be a good system for studying the biosynthesis of β -carotene in higher plants. He confirmed that the etiolated seedlings produced mainly xanthophylls, with only a trace of β -carotene, but found that on illumination of the seedlings β -carotene and xanthophylls were synthesized together in the same relative proportions as exist in chloroplasts. Indeed the carotenoids synthesized on illumination are merely a manifestation of the formation of functional chloroplasts. As this is a fundamental change undergone by all etiolated seedlings on illumination, it would appear probable that the type of increase in carotenoids observed by Good-

win (1958) was a general phenomenon, and that the contradictory results previously recorded were due to extraneous factors; in the earlier investigations inadequate analytical methods may well have been the cause.

However, it was decided to carry out similar experiments to those reported for maize on a dicotyledon; the french bean (*Phaseolus vulgaris*) was chosen as suitable material.

EXPERIMENTAL

Seedlings. The variety of French bean (*Phaseolus vulgaris*) used was Canadian Wonder. The seedlings were grown and illuminated under the same conditions as those described for maize by Goodwin (1958).

Extraction, purification, identification and determination of carotenoids. The cotyledons and true leaves were separately examined. The techniques used for the extraction, purification, identification and determination of the constituent carotenoids have been previously described in detail (Goodwin, 1955, 1958).

RESULTS

Carotenoids present in cotyledons of etiolated bean seedlings. The pigments from the etiolated cotyledons were chromatographed on columns of icing sugar and a typical result is given in Table 1. Fraction A, which ran straight through the column when light petroleum was used, was purified on alumina and shown to be β -carotene by chromatographic and spectroscopic comparison with an authentic specimen of synthetic β -carotene.

Fraction B was hypophasic in the light petroleum-aqueous 90% (v/v) methanol test and had an absorption spectrum similar to that of lutein. This

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