

of the peaks on the chart give the position of the radioactive substances on the chromatogram and an approximate quantitative estimate of the activity of each substance. The counting efficiencies achieved by this method are not great, approximately 10% for ^{14}C and only 1-2% for ^3H . The sensitivity and resolution also depend on such factors as the slit width of the collimator, and the rate of movement of the chromatogram.

C. Radioautography (see Vol. IV, p. 463)

A radioactive sample is chromatographed (usually thin layer) alongside marker samples of authentic compounds, so that any radioactive areas located may be correlated with the positions of known compounds. Generally a plate is spotted with (a) each marked substance individually, (b) a mixture of all markers, (c) analytical sample, and (d) analytical sample plus all markers. After development, the positions of the marker substances are determined in the appropriate way (see Section II, C, 4) and either marked on the plate or recorded by tracing or photography. The portions of the plate with the radioactive samples must not be disturbed. Radioautograms are then prepared, all subsequent operations being carried out in a dark room with an X-ray filtered light source. A piece of X-ray film is placed in direct contact with the adsorbent on the thin-layer chromatogram. The film is cut to the size of the plate and suitably marked by "radioactive ink" or by a series of small cuts by which the corners can be identified, and a clean plate is then positioned to hold the film in place. The plates are then bound firmly together with adhesive tape and the whole is wrapped in aluminum foil and several layers of black paper, and the parcel firmly sealed with adhesive tape and left in a dark place.

After a suitable exposure time (usually 3-4 weeks for detection of a few hundred disintegrations per minute) the film is developed, and the position of radioactive zones on the film is compared with the position of the known marker compounds on the thin-layer plate.

[245] Preparation of ^{14}C - β -Carotene¹

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Biogenesis of β -carotene in plants has been extensively studied using radiotracer techniques. Metabolic studies of β -carotene have been handicapped by the unavailability of labeled carotene with sufficiently high

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specific radioactivities. Most of the systems used for study of carotenogenesis are deficient in either yield of β -carotene or incorporation of label. A survey of various carotenogenic systems indicated that with mated cultures of the mold *Blakeslea trispora*² a greater portion of metabolism is directed to synthesis of β -carotene than with any other known system. Procedures for using this system to produce β -carotene have been developed.³

Materials

Cultures. Mating cultures of *Blakeslea trispora* 2895 and 2896⁴ are carried on potato dextrose agar slants at room temperature. The cultures are kept viable by transplant onto fresh slants every 30 days.

Starter medium, amount per 100 ml: pharmamedia,^{5,6} 5 g; thiamine hydrochloride, 0.2 mg. Sterilized by autoclaving at 121° for 20 minutes

Fermentation medium, amount per 100 ml: pharmamedia, 7 g; lard, 5 g; deodorized kerosene, 5 ml; thiamine hydrochloride, 0.2 mg. Sterilized by autoclaving at 121° for 20 minutes

Labeled compounds: sodium acetate-1,2-¹⁴C. Compounds from several sources are incorporated with equal efficiency. --

Other chemicals: other chemicals used may be purchased from commercial sources as high purity or C.P. grade and used without further purification or treatment.

Methods

Starter Culture. Five-day-old agar slants of each culture are seeded aseptically into separate 500-ml Erlenmeyer flasks containing 150 ml of starter medium. The flasks may be stoppered with cotton or plastic foam plugs and incubated at 28°-30° on a rotary shaker at 200 cycles per minute for 48 hours.

Fermentation. Seven milliliters of each starter culture are transferred aseptically into a single 500-ml flask containing 100 ml of fermentation medium and incubated as the starter cultures. After 48 hours, 0.1 ml of sterile β -ionone is added aseptically. At this time, 0.5 mC of sodium ace-

² R. F. Anderson, M. Arnold, G. E. N. Nelson, and A. Ciegler, *J. Agr. Food Chem.* 6, 543 (1958).

³ A. E. Purcell and W. M. Walter, Jr., *Labelled Compounds* 4, 94 (1968).

⁴ Obtained from C. W. Hesseltine, Northern Utilization Research Development Division, U.S. Department of Agriculture, Peoria, Illinois 61604.

⁵ A defatted cotton seed embryo meal obtained from Traders Protein Division, Traders Oil Mill, Co., P. O. Box 1837, Fort Worth, Texas.

⁶ Use of trade names of specific material does not constitute a recommendation by the U.S. Department of Agriculture to the exclusion of others which may also be available.

tate-1,2- ^{14}C is added by filtering through a sterile membrane filter with 0.1- μ pore size. Fermentation is continued for another 72 hours.

Trapping $^{14}\text{CO}_2$. The addition of any metabolizable ^{14}C -labeled substrate releases metabolic $^{14}\text{CO}_2$. A convenient method of trapping this $^{14}\text{CO}_2$ was described by Purcell and Walter.³ A double layer of cheesecloth, 150 by 150 mm, is pushed into the neck of an empty 500-ml Erlenmeyer flask to form a pocket about 40 mm deep. A layer of cotton is loosely packed into the bottom of the pocket to form a layer about 12 mm thick and covered with a double layer of cheesecloth. This stopper is sterilized in an autoclave and transferred to the incubation flask at the time the radioactive compound is added. About 5 g of ascarite is placed in the pocket of the plug and held in place with loosely packed cotton. With low humidity the ascarite may be used up to 48 hours, but with higher humidity it will be necessary to change it more often to prevent the ascarite from liquefying. The ascarite can be changed by removing the upper layer of cheesecloth then replacing it and adding new ascarite and cotton as previously described. The ascarite will be highly radioactive and should be discarded as solid radioactive waste.

In order to avoid the use of ascarite we have made an incubation box of plywood. The box is made to hold four 500-ml Erlenmeyer flasks and is bolted to the rotary shaker. A flanged lid is held on with four springs, and an air tight seal is obtained by cementing thin-walled 4-mm rubber tubing around the inside of the lid. Inlet and outlet connections are installed. A vacuum pump or aspirator is used to draw air out of the box through two traps containing 4 *N* sodium hydroxide. This system appears to trap all the $^{14}\text{CO}_2$.

Extraction and Purification of ^{14}C - β -Carotene. At the end of 120 hours of incubation, 150 ml of absolute methanol is added, stirred into the incubation flask, which is allowed to stand under a hood for 30 minutes. About 10 g of a filter aid such as Hyflo Super Cel or Celite 545 is stirred into the mixture with a glass stirring rod, and the entire contents of the flask are filtered through filter paper in a 125-mm Büchner funnel with vacuum. It is necessary to tamp the mat in order to get it sufficiently dry to facilitate subsequent extraction. The filtrate contains no β -carotene, so it may be discarded as a radioactive waste. The mat, which should be sufficiently dry to crumble is scraped into a beaker, pulverized, stirred in 500 ml of acetone-hexane mixture 1:1, and washed with acetone and hexane until the fresh filtrate becomes pale in color. The mat will still contain about 10% of the total carotene produced. This can be best extracted by blending the mat for several minutes in the hexane-acetone mixture using a Waring blender. Addition of 3-5 ml of water at the end of the blending period facilitates extraction. The mixture is again filtered

and washed. The filtrate becomes colorless after 3 or 4 washings. The mat may be discarded as radioactive waste. Transfer the hexane-acetone extracts to a 2-liter separatory funnel and gently add 100 ml of water with gentle swirling. Two distinct phases form and become clear after standing for a few minutes. The β -carotene is contained in the upper, or hexane, phase. The color in the lower phase is due to water-soluble pigments and traces of hydroxy carotenoids. The lower phase may be discarded as radioactive waste. The upper phase is washed several times by gently swirling it with water to remove traces of acetone. These washings contain a negligible amount of radioactivity.

The hexane phase is saponified by shaking for 2 minutes with one-fourth volume of methanol saturated with potassium hydroxide. If a radioactive balance is being attempted, sodium hydroxide should be used to avoid the radioactivity of potassium-40. Allow sufficient time for two distinct phases to form. Most of the β -carotene will partition into the upper layer. The bottom layer contains a small amount of β -carotene which can be recovered by mixing with an equal volume of ether and adding one volume of water. The upper layer of ether containing the β -carotene is washed with several volumes of water and combined with the hexane extract. The lower phase is discarded as radioactive waste. The combined hexane and ether extract is washed several times with water to remove residual saponified material. Dry the washed extracts by filtration through sodium sulfate and evaporate to dryness under vacuum. Dissolve the residue in 250 ml of ethyl ether, add 100 ml of 5% sodium ethylate, and let stand for 1-2 hours. This strenuous saponification results in sharper chromatographic separation. Add 20 ml of water with a swirling motion and allow the phases to separate. Pass the bottom layer into 150 ml of ether and add 100 ml of water. Combine the ether layers, wash three times with water, and dry by filtration through sodium sulfate. Evaporate to dryness under vacuum and dissolve the residue in 150 ml of hexane.

Chromatography

The β -carotene is isolated by chromatography on a column of magnesium oxide and Hyflo Super Cel 1:1 (w/w). The absorbent is packed into a 3-cm column to a height of 80-90 cm, then packed under vacuum to a height of about 50 cm. Wet the column with hexane, add the hexane solution of nonsaponifiable lipids, and develop this with 5% acetone in hexane until the large orange band of β -carotene is about to be eluted. Let the column run dry for 1-2 minutes. Remove all of the column above the β -carotene band by scraping it out of the tube with a long spatula or a flattened rod and elute the β -carotene into a clean receiver with 50% acetone in hexane.

Purification

We have found that multiple crystallization gives higher yield and greater purity than any other method we have used.

Evaporate the chromatographic fraction to dryness in vacuum and redissolve in a minimum volume of boiling hexane, about 1 ml of hexane per milligram of carotene. The minimum volume can be determined by removing hexane vapors with a stream of nitrogen while warming the flask in a water or steam bath until an insoluble ring of carotene begins to form. Add several drops of hexane to dissolve the ring, then add about 2 volumes of methanol, and warm to boiling. Allow this mixture to sit in the dark at room temperature for several hours. Remove the crystals by filtration and wash them with small volume of methanol. Redissolve the crystals in a minimum volume of boiling hexane and allow crystals to form in the hexane without addition of methanol. After crystals have formed, chill the flasks to -10° for at least 1 hour to increase the yield. Repeat crystallization from hexane once more. Further attempts at purification do not change the specific radioactivity of the ^{14}C - β -carotene.

Specific activity can be conveniently determined by measuring the amount of β -carotene spectrophotometrically and counting a known amount in suitable ^{14}C counting systems. Spectrophotometric determination in hexane at 450 nm using an absorption coefficient of 0.250 mg per liter gives excellent agreement with gravimetric determination. If radioactivity is to be determined by scintillation spectrometry, best results will be obtained if the carotene is decolorized by adding about 1% benzoyl peroxide to a toluene solution of carotene and exposing the mixture to light.⁷

By these procedures, yields up to 15 mg of purified β -carotene containing over 5 million disintegrations per milligram, i.e., 1.2 mCi per millimole, have been obtained.

Discussion

In the production of β -carotene by *Blakeslea trispora*, aeration and rate of stirring appear to be more critical than nutrition. Growth in fermentors would provide an ideal way of regulating growth and controlling metabolic $^{14}\text{CO}_2$, but optimum conditions of aeration and stirring must be developed for each fermentor. If stirring is too slow, a mycelial mat will form and limit growth; if too fast the mycelia are torn apart and growth and carotene production are low. If aeration is too low, growth is limited; if too high, the mycelia are torn by the turbulence and carotene production

⁷ W. M. Walter, Jr. and A. E. Purcell, *Anal. Biochem.* 16, 466 (1966).

is low. Shake flasks have given the most consistent yield of carotene and incorporation of label.

Blakeslea trispora will apparently produce β -carotene on a wide variety of media. Anderson *et al.*² used the following media:

Starter culture medium, amount per 100 ml: acid-hydrolyzed soybean meal, 4.7 g; thiamine hydrochloride, 0.2 mg; acid-hydrolyzed corn, 2.3 g

Fermentation medium: The fermentation medium is prepared by adding 5 g of animal fat, 5 ml of deodorized kerosene, and 0.12 ml of nonionic detergent to each 100 ml of starter culture

Ciegler *et al.*⁸ produced β -carotene with *Blakeslea trispora* grown in laboratory fermentors. These workers used the following media:

Starter culture medium, amount per 100 ml: cotton seed embryo meal, 5.0 g; ground whole corn, 2.5 g; thiamine hydrochloride, 0.2 mg

Fermentation medium, amount per 100 ml: cotton seed embryo meal, 5.0 g; ground whole corn, 5.0 g; citrus molasses, 5.0 g; Triton X-100, 0.12 g; thiamine hydrochloride, 0.2 mg; pH adjusted to 6.7; then vegetable oil, 5 ml; deodorized kerosene, 5 ml

It is believed that with either of these media, incorporation of label will parallel the amount of carotene produced.

The β -carotene produced by *Blakeslea trispora* in the presence of sodium acetate-1,2-¹⁴C has not been degraded to establish the position of labeling. On the basis of other work⁸⁻¹¹ there is no reason to believe that labeling would not be uniform. The β -ionone added to the fermentation media is not a precursor of β -carotene but functions in some other manner,¹²⁻¹⁴ so the presence of β -ionone need not be considered in connection with labeling patterns.

Since glucose and mevalonic acid are incorporated into the carotenoids of *Blakeslea trispora*,³ there is a possibility that selective labeling may be achieved by using these precursors labeled in the various positions.

² A. Ciegler, A. A. Lagoda, V. E. Sohns, H. H. Hall, and R. W. Jackson, *Biotechnol. Bioeng.* **5**, 109 (1963).

³ E. C. Grob and R. Butler, *Helv. Chim. Acta* **37**, 1908 (1954).

¹⁰ E. C. Grob and R. Butler, *Helv. Chim. Acta* **38**, 1313 (1955).

¹¹ E. C. Grob and R. Butler, *Helv. Chim. Acta* **39**, 1975 (1956).

¹² T. W. Goodwin, *Advan. Enzymol.* **21**, 295 (1959).

¹³ G. B. Engel, J. W. Wisch, and M. Zimmerman, *Helv. Chim. Acta* **36**, 1771 (1953).

¹⁴ P. Reyes, C. O. Chichester, and T. O. M. Nakayama, *Biochim. Biophys. Acta* **90**, 578 (1964).