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Biosynthetic Studies on Aromatic Carotenoids

BIOSYNTHESIS OF CHLOROBACTENE

By S. E. MOSHIER* and D. J. CHAPMAN†
Departments of Biochemistry and Biology, University of Chicago,
5630 South Ingleside Avenue, Chicago, Ill. 60637, U.S.A.

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1. The incorporation of [2-^{14}C]mevalonic acid by *Chloropseudomonas ethylica* strain 2K into chlorobactene was studied. 2. Oxidative degradation of chlorobactene of constant specific radioactivity produced labelled benzenecarboxylic acids and indicated that the benzene ring originates from mevalonic acid. 3. Decarboxylation studies demonstrated a stereospecific methyl migration in the formation of the 1,2,5-trimethylphenyl group of chlorobactene. The migrating methyl group was derived from the C-3′ position of mevalonic acid.

Aryl carotenoids were first discovered in the Japanese sea sponge, *Reniera japonica*. In a series of studies, Yamaguchi (1957*a*,*b*, 1958*a*,*b*, 1959, 1960) isolated renieratene ($\phi\chi$-carotene), isorenieratene ($\phi\phi$-carotene) and renierapurpurin ($\chi\chi$-carotene). Subsequently, the new class of carotenoids was identified in photosynthetic (*Chloropseudomonas*, *Chlorobium*, *Pelodyction*, *Chromatium* and *Thiothece*) and non-photosynthetic (*Mycobacterium* and *Streptomyces*) bacteria (Liaaen-Jensen, 1965*a*; Pfennig *et al.*, 1968; Arcamone *et al.*, 1969, 1970; Schmidt & Schiburr, 1970; Andrewes & Liaaen-Jensen, 1972).

Besides the aryl carotenoids there are few plant constituents containing a benzene ring that are formally or demonstrably of solely isoprenoid origin. These include the sesqui- and di-terpenes found in species of the order Cupressales (Erdtman & Norin, 1966; Andersen & Syrdal, 1970) and the naphthalene derivative, gossypol, synthesized by cotton roots (Heinstein *et al.*, 1962). Most plant benzenoid substances originate from the shikimic acid or the polyacetate pathway (Geissman & Crout, 1969). Aryl carotenoids lack the structural characteristics of products of these pathways and are potentially the products of a heretofore unrecognized pathway for the formation of the benzene ring.

The structures of the aryl carotenoids are consistent with an isoprenoid origin. The polyene carbon skeletons of chlorobactene ($\phi\psi$-carotene), hydroxychlorobactene (1′,2′-dihydro-$\phi\psi$-caroten-1′-ol) and okenone (1′-methoxy-1′,2′-dihydro-$\chi\psi$-4-caroten-4′-one) are identical with the carbon skeleton of γ-carotene ($\beta\psi$-carotene), except for the methyl substitution of the benzene ring. Similarly, the carbon skeletons of β-isorenieratene ($\beta\phi$-carotene), isorenieratene, renierapurpurin, 3-hydroxyisorenieratene ($\phi\phi$-caroten-3-ol) and 3,3′-dihydroxyisorenieratene ($\phi\phi$-caroten-3,3′-diol) are identical with the carbon skeleton of β-carotene ($\beta\beta$-carotene) except for the methyl substitution of the benzene ring. The natural occurrence of γ-carotene with chlorobactene and hydroxychlorobactene (Liaaen-Jensen *et al.*, 1964) and of α-carotene ($\beta\epsilon$-carotene) and β-carotene with isorenieratene, renieratene and renierapurpurin (Yamaguchi, 1957*a*, 1958*a*) is consistent with the hypothesis that γ- and β-carotene are the isoprenoid precursors, respectively, of monocyclic and bicyclic aryl carotenoids.

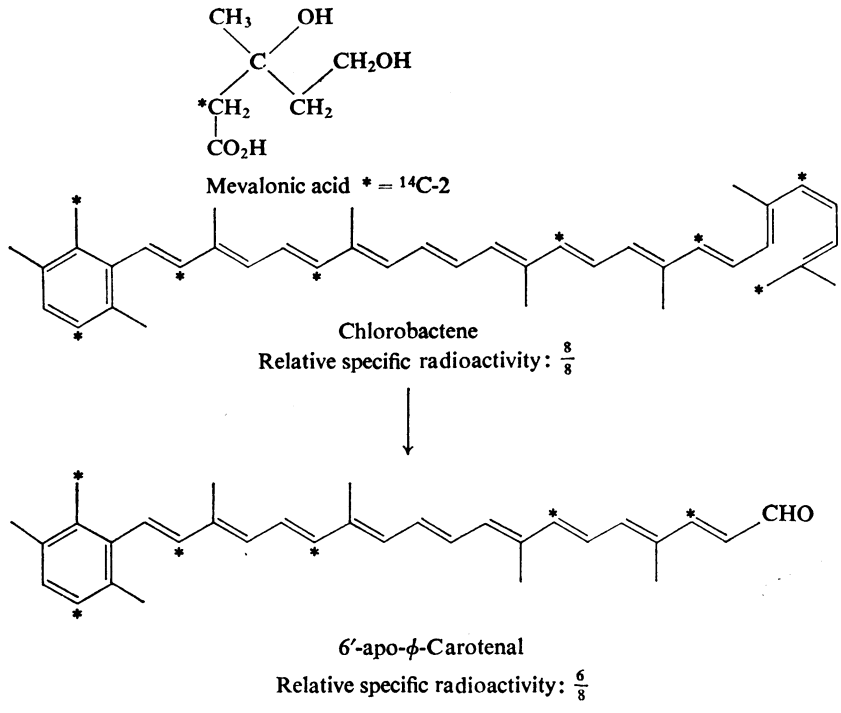
Cooper *et al.* (1963) have suggested that aryl carotenoids are formed by aromatization of a 1,1,5-trimethylcyclohexenyl ring with accompanying methyl migration. This hypothesis suggests that the C-1 and C-2 methyl groups of the 1,2,5-trimethylaryl carotenoids arise from C-2 and C-3′ of mevalonic acid (not necessarily respectively).

Because there is no information on the biosynthesis of the aryl carotenoids (Goodwin, 1971*a*,*b*), we decided to examine the biological origin of these methyl groups to evaluate the hypothesis and to propose possible migration mechanisms.

If incorporation of labelled mevalonic acid into chlorobactene is uniform, the C_{32} aldehyde produced by chromic acid oxidation of labelled chlorobactene should contain 75% of the chlorobactene radioactivity as shown in Scheme 1. Further, if the entire C_{40} skeleton of chlorobactene were derived from mevalonic acid, the dimethylbenzenedicarboxylic acid derivatives obtained from permanganate oxidation would exhibit the labelling pattern shown in Scheme 2. After oxidation in alkaline permanganate 2,4-dimethylbenzene-1,3-dicarboxylic acid and a mix-

* Present address: Asthma–Emphysema Unit, Department of Medicine, Creighton University, Omaha, Neb. 68131, U.S.A.

† Present address: Department of Biological Sciences, University of California, Los Angeles, Calif. 90024, U.S.A.



Scheme 1. *Scheme for oxidation of chlorobactene to indicate whether radioactive label is likely to be uniformly distributed among the sites of label*

Theoretical relative specific radioactivity of 6'-apo-ϕ-carotenal prepared from chlorobactene uniformly labelled from [2-^{14}C]mevalonic acid. 6'-apo-ϕ-Carotenal, obtained by chromic acid oxidation of chlorobactene, was purified by t.l.c., combusted and the radioactivity counted. * marks the labelled positions.

ture of 3,6- and 3,4-dimethylbenzene-1,2-dicarboxylic acid may be isolated, and radioactivity before and after decarboxylation may be compared. The present paper represents the results of analyses of chlorobactene biosynthesized by *Chloropseudomonas ethylica* strain 2K from [2-^{14}C]mevalonic acid. The data establish the isoprenoid origin of the aromatic ring and the origin (in relation to mevalonic acid C-2 and C-3') of ring methyl groups.

Experimental

Organism and culture

C. ethylica strain 2K was kindly provided by Dr. J. M. Olson of Brookhaven National Laboratory, Upton, New York. The bacteria were grown in a modified Olson's medium (Bose, 1963). The following modifications were employed: 0.3g of $Na_2S,9H_2O$/l instead of 0.2g/l and 5.5ml of 0.5% (w/v) $FeSO_4,7H_2O$ in 4M-HCl/l instead of 5.0ml of 0.05% $FeSO_4,7H_2O$ in 0.04M-HCl/l. The initial pH was 6.4–6.6, instead of 7.2–7.4. This modified medium increased cell yield by up to 50%.

Non-radioactive cultures were grown at 30–32°C for 5–7 days and radioactive cultures for 4–5 days in stoppered 12–24 litre carboys filled to the neck with medium. Inocula of 10–25% of the carboy capacity were used and cultures were stirred constantly by means of magnetic stirrers. General Electric Lumiline bulbs providing about 17W/m^2 radiation flux density at the carboy centre were used as light source. Stationary phase was reached in 4–5 days. [2-^{14}C]-Mevalonic acid lactone in ethanol solution was added to the culture medium to give a concentration of 2.63μmol of mevalonic acid/l of culture medium (Expt. 1, specific radioactivity of mevalonic acid 9.5mCi/mmol) or 12.8μmol of mevalonic acid/l of culture medium (Expt. 2, specific radioactivity of mevalonic acid 30.6mCi/mmol). Radioactive uptake was monitored by disappearance from the medium rather than by appearance of label in unsaponifiable matter, since a significant amount of mevalonic acid was expected in the farnesol of the bacterial chlorophylls. Radioactive cultures were harvested at the time of maximum incorporation (1% into unsaponifi-

able matter), just before the stationary phase was reached, because most of the label reappeared in the medium after approximately 8 days.

Chemicals and solvents

Radioactive mevalonic acid was obtained from Schwarz–Mann, Orangeburg, New Jersey, U.S.A. Organic solvents were either reagent grade or scintillation grade as appropriate, unless specified. Organic reagents were obtained from Eastman–Kodak, Rochester, N.Y., U.S.A. or Aldrich Chemical, Milwaukee, Wis., U.S.A.

Synthesis of 2,4-dimethylbenzene-1,3-dicarboxylic acid and derivatives

2,3,6-Trimethylbenzaldehyde was prepared from pseudocumene (1,2,4-trimethylbenzene) by the procedure of Lowe *et al.* (1958). The aldehyde was oxidized by alkaline permanganate under conditions described by Bentley & Perkin (1897). The 1,3-diacid was purified by t.l.c. (see below) and crystallized from acetone–light petroleum (b.p. 30–60°C) (1:9, v/v). The 1,3-diacid was monitored for the presence of 1,4-diacid by the method of Bryce-Smith (1953). The synthetic 1,3-diacid had an uncorrected melting point of 230°C. Yamaguchi (1958*a*) reported 231°C. The identity of the 2,4-dimethyl-1,3-benzenedicarboxylic acid was confirmed by its mass spectrum. The mass ion appears at *m/e* 194 (74% of base peak intensity). The base peak (*m/e* 148) may arise by typical *ortho*-effect loss of water (Budzikiewicz *et al.*, 1967) to give a fragment at *m/e* 176 (35% of base peak) followed by loss of CO. The fragments at *m/e* 177 (24% of base peak) and *m/e* 149 (15% of base peak) would correspond to a loss of –OH and $-CO_2H$ respectively. The fragments at *m/e* 77 (31% of base peak), 91 (21% of base peak) and 105 (10% of base peak) are typical of alkylbenzenes. The mass spectrum of 2,5-dimethylbenzene-1,4-dicarboxylic acid (D. J. Chapman & S. E. Moshier, unpublished work) is quantitatively distinct from that of 2,4-dimethylbenzene-1,3-dicarboxylic acid, when recorded under similar conditions. Most noticeable is the appearance of the base peak at *m/e* 176 in the former.

The method of Vogel (1957) was used to prepare the di-(4′-phenylazophenacyl)-2,4-dimethyl-1,3-dicarboxylate. The ester was purified by t.l.c. and crystallized from benzene.

Extraction and purification of carotenoids

Enough saturated $CaCl_2$ solution was added with stirring to a culture to cause flocculation and sinking of the cells. The supernatant medium was siphoned off, and the cell mass collected by filtration.

Frozen cells were thawed at room temperature and extracted with methanol–acetone (1:1, v/v) (Liaaen-Jensen, 1962). The extraction process was repeated two or three times with methanol, two or three times with methanol–acetone (1:1, v/v), and finally with acetone until the supernatant was colourless. Saponification was carried out at room temperature in 3–6% (w/v) KOH in methanol for 3–24h. Unsaponifiable matter was extracted into light petroleum (b.p. 30–60°C) (Liaaen-Jensen, 1962), and the light petroleum solution dried over anhydrous Na_2SO_4 before chromatography.

The carotenoids were separated by adsorption chromatography on columns of neutral deactivated alumina, Brockmann grade 2 (M. Woelm, Eschwege, W. Germany) (Liaaen-Jensen *et al.*, 1964). A stepwise gradient of diethyl ether in light petroleum (b.p. 30–60°C) (up to 10%, v/v) followed by acetone in light petroleum (b.p. 30–60°C) (up to 20%, v/v) was used. Chlorobactene isolated by chromatography was crystallized from acetone–light petroleum (b.p. 30–60°C).

Chemical degradation

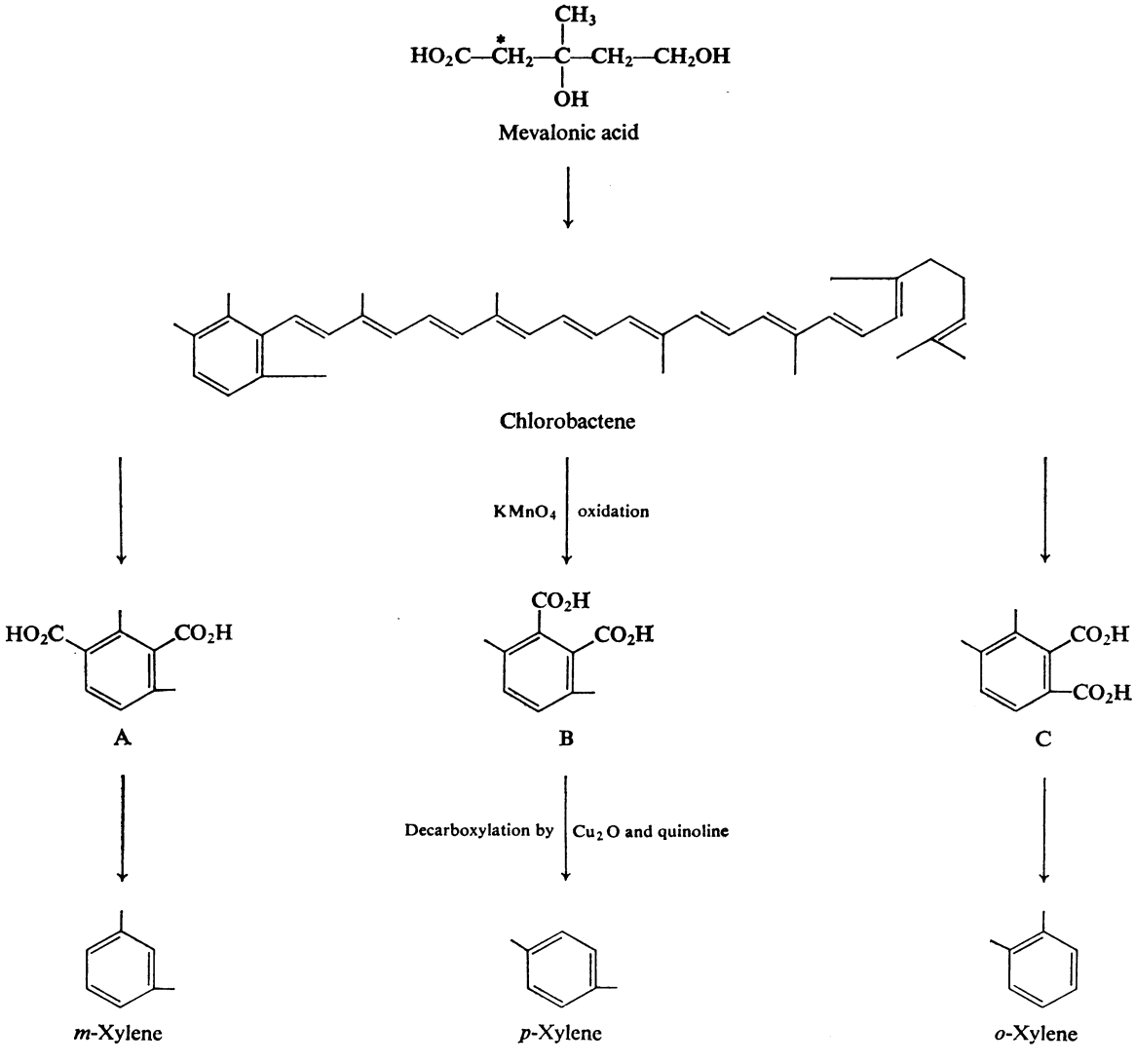
(*i*) *Alkaline permanganate oxidation.* The control of the oxidative degradation of chlorobactene is critical to the formation of the dimethylbenzenedicarboxylic acids. These methylbenzene diacids are soluble in the aqueous phase, so the efficiency of further oxidation of the dimethylbenzenedicarboxylic acids will be high. It is necessary, therefore, to select an amount of permanganate that is sufficient to oxidize the benzenoid substances to the dicarboxylic acids (permanganate is also being consumed concurrently in the oxidation of the polyene chain) without further oxidation to the tri- or tetra-carboxylic acids or degradation of the aromatic ring (Lee, 1969).

Two methods of oxidizing chlorobactene were satisfactory. The first was a modification of the method of Armitage *et al.* (1964). In a typical oxidation 20mg of crystalline chlorobactene was stirred for 15h at room temperature with 2ml of water, 0.8ml of 2-methylpropan-2-ol, 2ml of benzene and 160mg of potassium permanganate. Benzene and 2-methylpropan-2-ol were removed by rotary evaporation. Sodium bisulphite and H_2SO_4 were added to reduce the brown MnO_2 precipitate. The remaining aqueous solution was extracted repeatedly with benzene. The reaction vessel was washed with benzene and the combined benzene solutions were extracted repeatedly with hot water. The combined aqueous fractions were decreased in volume by rotary evaporation, and extracted several times with diethyl ether. The combined diethyl ether extracts were dried over anhydrous Na_2SO_4 before chromatography.

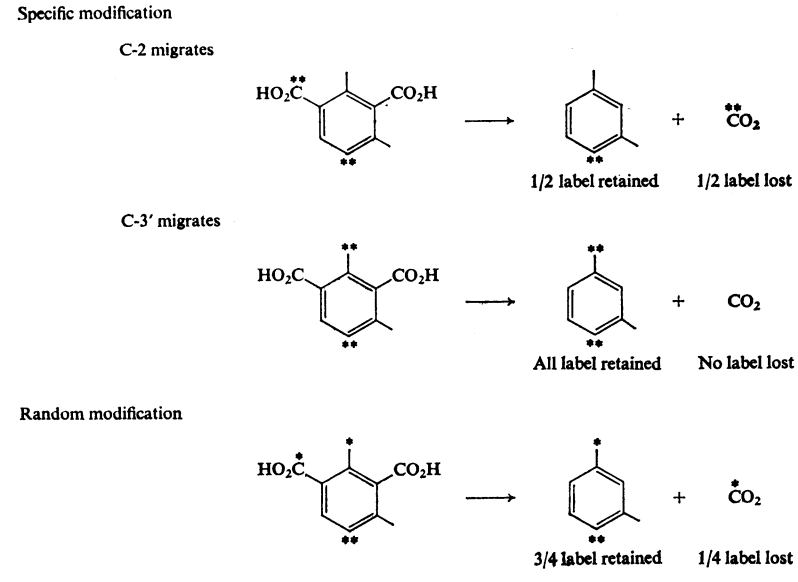
The second method was modified from Yamaguchi (1958*a*). In a typical oxidation 20mg of crystalline chlorobactene were stirred at room temperature in a

mixture of 3ml of water, 3ml of benzene, 260mg of potassium permanganate and 260mg of sodium carbonate for 15h. The reaction products were worked up in a fashion identical with that described for the Armitage *et al.* (1964) permanganate oxidation.

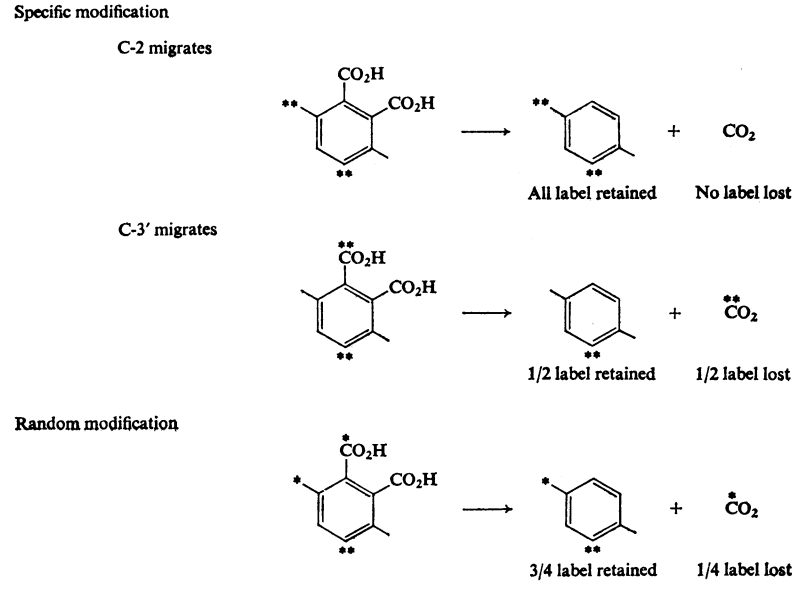
The ether solution was applied to thin layers of silica gel G (250–500μm, E. Merck, Darmstadt, Germany) which were first developed with light petroleum (b.p. 30–60°C) to separate the oil present. The plates were then chromatographed with propan-2-ol–aq. NH_3 (3:1, v/v) with 2,4-, 2,5- and 3,4-dimethylbenzenecarboxylic acid, trimellitic acid, 2,2-dimethylsuccinic acid, phthalic acid, isophthalic acid, terephthalic acid and oxalic acid as reference substances. The chromatographed acids were detected by spraying the plate with alkaline Bromcresol Green solution (Table 1). The acids were eluted from a thoroughly dry plate with methanol, then benzene and diethyl ether, and crystallized from acetone–light petroleum (b.p. 30–60°C) (1:9, v/v) as small needles. In this chromatographic system aromatic carboxylic acids migrate according to the number and position of the carboxylic groups, and acids, such as 2,2-dimethylsuccinic acid, produced by permanganate



A: 2,4-Dimethylbenzene-1,3-dicarboxylic acid



B: 3,6-Dimethylbenzene-1,2-dicarboxylic acid [the 3,4-dimethyl isomer, C, has no carboxyl group at a position of interest (see the text)]



Scheme 2. *Scheme for analysis of the* 1,2- *and* 1,3-*dicarboxylic acid derivatives of labelled chlorobactene derived from C. ethylica grown in the presence of* [2-^{14}*C*]*mevalonic acid*

The 1,3-diacid was separated from the 1,2-diacids by t.l.c., eluted and the radioactivity counted. The 1,2-diacids could not be separated further. They were eluted and the radioactivity was counted. CO_2 from decarboxylation was trapped in 2-aminoethanol–ethylene glycol monomethyl ether (1:2, v/v) and the radioactivity counted. 3,4-Dimethylbenzene-1,2-dicarboxylic acid is omitted from the decarboxylation scheme since neither carboxyl group will appear labelled from chlorobactene biosynthesized from [2-^{14}C]mevalonic acid. * marks the labelled positions.

Table 1. *R_F values of benzene mono- and di-carboxylic acids and aliphatic dicarboxylic acids*

The carboxylic acids were separated by t.l.c. on silica gel G in a solvent system of propan-2-ol–aq. NH_3 (3:1, v/v).

Acid	R_F value
2,4-Dimethylbenzoic acid	0.64
2,5-Dimethylbenzoic acid	0.64
3,4-Dimethylbenzoic acid	0.64
Isophthalic acid	0.35
2,4-Dimethylbenzene-1,3-dicarboxylic acid	0.35
Phthalic acid	0.25
Terephthalic acid	0.15
Trimellitic acid	0.09
2,2-Dimethylsuccinic acid	0.04
Oxalic acid	0.00

oxidation of cyclohexylidene rings in β-carotene and γ-carotene remain at, or near, the origin.

(*ii*) *Chromic acid oxidation.* Milder oxidation of crystalline chlorobactene was performed by the method of Yamaguchi (1958*a*). The light petroleum (b.p. 30–60°C) phase was evaporated to dryness, redissolved in benzene and purified by t.l.c. The main zone, 6′-apo-φ-carotenal, was eluted with acetone and crystallized from light petroleum (b.p. 30–60°C).

(*iii*) *Decarboxylation of the aromatic dicarboxylic acids.* The decarboxylation was accomplished by a method similar to that of Birch *et al.* (1955). Quinoline containing a trace of Cu_2O powder and the dicarboxylic acid was heated to 180°C under a reflux condenser to retain the resulting xylene. Evolved CO_2 was carried in a stream of N_2 first to a trapping solution of 2-aminoethanol–ethylene glycol monomethyl ether (1:2, v/v; Jeffay & Alvarez, 1961) and then to a saturated solution of $Ba(OH)_2$ to detect any CO_2 which was not absorbed by the first solution.

Sample combustion

A semi-automatic sample combustion system (model 3151, Nuclear Chicago Corp., Des Plaines, Ill., U.S.A.) was used to convert a labelled sample into CO_2 and water. After combustion and cooling, 15–20ml of the absorbing solution, 2-aminoethanol (Matheson, Coleman and Bell, Elk Grove Village, Ill., U.S.A.)–ethylene glycol monomethyl ether (Fisher Scientific Co., Fairlawn, New Jersey, U.S.A.) (1:2, v/v; Jeffay & Alvarez, 1961) was added to the combustion flask through the serum cap and the contents were stirred for 30min.

Liquid-scintillation spectrometry

A Unilux III liquid-scintillation system (Nuclear Chicago Corp.), utilizing low-potassium counting vials, was used for all counting of radioactivity. To 15ml of a scintillation medium containing 5.5g of 2,5-diphenyloxazole (Packard Instruments, Downers Grove, Ill., U.S.A.)/l in scintillation-grade toluene–ethylene glycol monomethyl ether (2:1, v/v; Jeffay & Alvarez, 1961), was added 3ml of the CO_2-trapping solution described above.

Quenching was corrected for by the method of Peng (1960). Counting efficiency was determined by assay of standards under the same conditions.

Chromatography and radioautography

T.l.c. of chlorobactene was carried out on plates of microcrystalline cellulose (Cellex MX; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) containing 40% by weight of alumina (alumina for t.l.c.; M. Woelm). The air-dried plates were activated for 20min at 100°C and developed with ligroin (b.p. 60–90°C). The 6′-apo-φ-carotenal and di-(4′-phenylazophenacyl)-2,4-dimethyl-1,3-dicarboxylate were chromatographed in benzene on thin layers of neutral alumina (M. Woelm).

cis isomers from stereomutation studies were separated by chromatography on paper discs in ligroin (b.p. 60–90°C) on Schleicher and Schüll no. 288 (aluminum oxide-impregnated) paper (Jensen & Liaaen-Jensen, 1959). The paper was activated for 15min at 150°C (Jensen, 1960) before use.

For radioautography an X-ray film and a glass plate were placed over a thin-layer chromatogram. The assembly was clamped, taped around the edges, and kept dark and cold for 2 weeks. The X-ray film was developed and compared with the chromatogram.

Physical methods

Mass spectrometry was performed by Dr. Josef Fried (Departments of Chemistry and Biochemistry, The University of Chicago) at an ionizing voltage of 70eV on a 1015 Finnigan mass spectrometer equipped with a direct inlet system and a Systems 150 computer. Absorption spectra were recorded on a Cary model 11 recording spectrophotometer.

Results

Identity of chlorobactene

The major carotenoid recovered from the column chromatography was monitored for homogeneity by t.l.c. Column chromatography was repeated until the product was pure. The chromatographically pure chlorobactene (15mg/10 litres of culture) was crystallized three times as rosettes of needles. The uncorrected melting point was 146–147°C (lit. 147–148°C). The light-absorption maxima (494, 463, 437nm) in light petroleum (b.p. 30–60°) corresponded

to those published by Liaaen-Jensen *et al.* (1964). Absolute identity was established by comparison of the results of iodine-catalysed isomerization (Liaaen-Jensen *et al.*, 1964) and of the mass spectrum (Enzell *et al.*, 1969; Enzell, 1969) with the published values.

The identity of the 2,4-dimethylbenzene-1,3-dicarboxylic acid arising from an alkaline permanganate oxidation of chlorobactene was established by co-chromatography with the authentic synthetic sample, by co-chromatography of the 4′-phenylazophenacyl ester (both chromatographic identifications were also used to confirm the radioactive acid), by melting point of the ester [167–169°C uncorr.; cf. 169–172°C (Yamaguchi, 1958*b*)] and by a mass spectrum similar to that of the authentic sample.

The 6′-apo-ϕ-carotenal was identified by its mass spectrum (Fig. 1).

Mevalonic acid origin of chlorobactene

Chlorobactene extracted from 90 litres of *C. ethylica* strain 2K (129g dry wt. of cells) grown in the presence of [2-^{14}C]mevalonic acid (specific radioactivity 30.6mCi/mmol) was purified to constant specific radioactivity by three crystallizations following two purifications by column chromatography. A final specific radioactivity of 34100d.p.m./mmol of chlorobactene was obtained, providing direct evidence for the mevalonic acid origin of chlorobactene.

Origin of the benzene ring and of the C-1 and C-2 methyl groups

Labelled chlorobactene (53.4mg, specific radioactivity 34100d.p.m./mmol) and 38.9mg of unlabelled crystalline carrier chlorobactene were oxidized by alkaline permanganate. Both the 1,2- and 1,3-dicarboxylic acid bands were collected by t.l.c. purification. Radiochemical purity of the purified 2,4-dimethylbenzene-1,3-dicarboxyic acid was monitored radioautographically by preparing di-(4′-phenylazophenacyl)-2,4-dimethylbenzene-1,3-dicarboxylate and demonstrating radioactivity only in the ester. One-half of the recovered diacids was combusted, and one-half was decarboxylated. The results of this experiment are presented in Table 2, which shows that the counts in the diacids derived from incorporation (Expts. 1 and 2) were of the same magnitude, even though different amounts of chlorobactene derived from mevalonic acid of differing specific radioactivities were used. The low specific radioactivity of recovered chlorobactene, compared with a theoretical maximum calculated from the specific radioactivity of the initial mevalonic acid, may be attributed perhaps to variations in pool size of pre-existing metabolic intermediates and chlorobactene at the time of radioactive inoculation. Additionally, Expt. 1 was performed with cells grown in the original Olson's medium. We have some evidence that the differences between the two media affect the uptake of mevalonic acid.

The data also provided evidence that the C-3′ of mevalonic acid migrates in the process of aromatization. It was expected that the CO_2 from the 1,3-diacid should contain one-half of the label if C-2 migrated, none if C-3′ migrated, and one-fourth for random migration (Scheme 2). The data supported C-3′ migration. In the case of the 1,2-diacid mixtures radioactivity would be expected to be present in the evolved CO_2 for C-3′ and random migration and

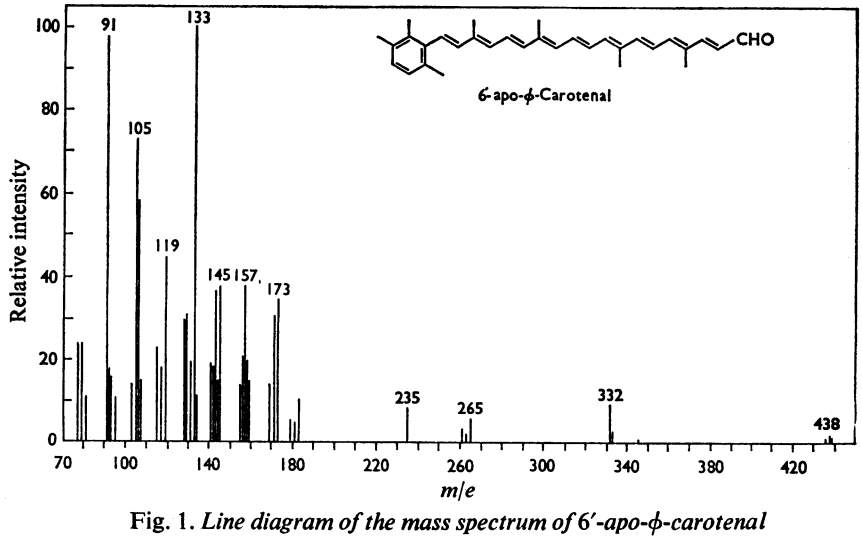


Fig. 1. *Line diagram of the mass spectrum of 6′-apo-ϕ-carotenal*

Table 2. *Radioactivity of* 2,4-*dimethylbenzene*-1,3-*dicarboxylic acid,* 3,6- *and* 3,4-*dimethylbenzene*-1,2-*dicarboxylic acids, and* CO_2 *from decarboxylation of the diacids prepared by permanganate oxidation of* [^{14}C]*chlorobactene grown in the presence of* [2-^{14}C]*mevalonic acid*

The 1,3-diacid was separated from a mixture of 1,2-diacids by t.l.c. The 1,2-diacids could not be separated further. The values are given as means ± S.E.M.

	Radioactivity (c.p.m.)	
	Expt. 1	Expt. 2
[^{14}C]Chlorobactene oxidized (mg) ...	4	53.4
2,4-Dimethylbenzene-1,3-dicarboxylic acid	100 ± 12	88 ± 9
CO_2 from 1,3-diacid	0	0
3,6- and 3,4-Dimethylbenzene-1,2-dicarboxylic acids	180 ± 15	275 ± 10
CO_2 from 1,2-diacids	10 ± 4	45 ± 5

Table 3. *Relative specific radioactivities of chlorobactene and* 6′-*apo*-ϕ-*carotenal*

The 6′-apo-ϕ-[^{14}C]carotenal was prepared by CrO_3 oxidation of the [^{14}C]chlorobactene, purified by t.l.c., combusted and the radioactivity counted. The theoretical value was based on uniform incorporation of [2-^{14}C]mevalonic acid into chlorobactene.

Carotenoid	Sp. radioactivity (d.p.m./mmol)	Ratio (%, relative to chlorobactene)
Chlorobactene	34100	100
6′-apo-ϕ-Carotenal	25000	73.3
6′-apo-ϕ-Carotenal (theoretical)		75

absent for C-2 migration (Scheme 2). The radioactivity observed indicated either a C-3′ or random migration. Without separation and quantification of the individual 1,2-dicarboxylic acids, this choice could not be resolved. Because the radioactive 3,4-dimethylbenzene-1,2-dicarboxylic acid would yield unlabelled CO_2, whereas the other diacid would yield labelled CO_2, the ratio of the radioactivity in the mixture before carboxylation to the radioactivity in the evolved CO_2 would depend upon the relative proportions of the two diacids in the original mixture. Only one of the two expected phthalic acids (3,6-dimethylbenzene-1,2-dicarboxylic acid) has a carboxyl group at a position of interest. That acid is likely to be present as a minor constituent of the mixture because of the steric hindrance of its formation. Unequivocal separation of the two 1,2-diacids has not been achieved.

Chromic acid oxidation of chlorobactene

Chlorobactene behaved like lycopene rather than like β-carotene to chromate oxidation and was cleaved at the 5′,6′-double bond instead of the 7,8-double bond to give 6′-apo-ϕ-carotenal (Scheme 1). This cleavage product was useful for determining the distribution of radioactivity throughout the eight sites of label.

Labelled crystalline chlorobactene (21 mg) was oxidized with chromic acid. The crystalline 6′-apo-ϕ-carotenal (2.1 mg) recovered from the oxidation had an m.p. of 166–167°C (uncorr.).

The mass spectrum of 6′-apo-ϕ-carotenal (Fig. 1) had a parent ion of *m/e* 438. The base peak at *m/e* 133 was characteristic of aryl carotenoids. Other prominent peaks at *m/e* 91, 105 and 119 were characteristic of alkylbenzenes and probably represented tropylium and methyl- and dimethyl-tropylium ions. In addition, peaks too small to appear in Fig. 1 were located at *M*−15 and *M*−29. These are peaks found also in the mass spectrum of renieral (Enzell *et al.*, 1969). Other peaks common to both 6′-apo-ϕ-carotenal and renieral were *M*−106, *M*−144 and *M*−106. The carotenal and chlorobactene had, in common, peaks at *m/e* 77, 91, 105, 119, 133, 145, 157 and 173.

Analysis of the labelled chlorobactene and carotenal revealed that 73.3% of the label of chlorobactene remained in the carotenal (Table 3). This value corresponded closely to the expected theoretical value of 75% if the eight carbon atoms of chlorobactene arising from C-2 of mevalonic acid (cf. Scheme 1) were assumed to be uniformly labelled. It is probably

safe to assume therefore that the four labelled carbon atoms remaining in the polyene chain portion of the carotenal molecule are likewise uniformly distributed and that chlorobactene itself is therefore uniformly labelled throughout both the aromatic and olefin parts of the molecule.

Discussion

Products expected from the permanganate oxidation were determined on the basis of preferential initial attack at the benzylic carbon–hydrogen bond (Lee, 1969; Cullis & Ladbury, 1955*a*,*b*,*c*). Attack was always at the α-carbon and the order of reactivity from greatest to least was isopropylbenzene, *n*-propylbenzene, ethylbenzene and toluene. One expects, therefore, that C-7 will be oxidized first. Then only one 1,3-benzenedicarboxylic acid would be encountered, and only two 1,2-diacids (3,6- and 3,4-dimethylbenzene-1,2-dicarboxylic acid). Improvement is still needed especially in the separations of the various diacids.

These results established the mevalonic origin of the aromatic carotenoid chlorobactene (including the aromatic ring portion) and, by extension, of the other aryl carotenoids. Aromatization involved the migration of a methyl group derived from the C-3′ of mevalonic acid. Presumably a similar specific methyl migration occurs during the formation of the other 1,2,5-trimethylphenyl carotenoids.

Cooper *et al.* (1963) suggested that aryl carotenoids may be formed by aromatization of a 1,1,5-trimethylcyclohexenyl ring with accompanying methyl migration. The idea of a cyclic rather than an acyclic immediate precursor to the aromatic carotenoids may be supported by the occurrence of γ-carotene in organisms containing monocyclic aromatic carotenoids and of β-carotene in organisms containing bicyclic aromatic carotenoids (Yamaguchi, 1957*a*; Liaaen-Jensen *et al.*, 1964; Liaaen-Jensen, 1965*b*).

Among naturally occurring carotenoids there are a number of structural types that could be precursors to aromatic carotenoids. They include carotenoids with a β-ionone ring as mentioned above, for they occur with aryl carotenoids, are cyclized, and need only be dehydrogenated with methyl migration. Carotenoids with γ-ionone rings (Arpin *et al.*, 1971) and 3,4-dehydro-β-ionone rings are also possibilities.

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