Terpene Biosynthesis. Part V.[1] Interconversions of Some Monoterpenes in Higher Plants and their Possible Role as Precursors of Carotenoids

By **D. V. Banthorpe,* Hilary J. Doonan,** and **Ann Wirz-Justice,** Christopher Ingold Laboratories, Department of Chemistry, University College, London W.C.1

Feeding of [^{14}C]-labelled *p*-menth-1-en-8-ol (α-terpineol) or *trans*-thujan-3-one (isothujone) to *Tanacetum vulgare* L. or 3,7-dimethylocta-2,6-dien-1-ol (geraniol) to *Artemisia annua* L. led to significant (0·14—6·7%) uptake of tracer into carotenoids, and the labelling pattern suggested incorporation of either undegraded C_{10} units or the biogenetic equivalent of 3,3-dimethylallyl pyrophosphate. Tracer uptake (*ca.* 0·2%) into chlorophyll was consistent with degradation of the precursors to C_2 units.

[^{14}C]*p*-Menth-1-en-8-ol was converted directly into isothujone in *T. vulgare*, but experiments with [^{14}C,^{3}H]isothujone showed that although possible direct conversion into thujols occurred within 40 h of feeding, longer metabolism times led to extensive degradation of the additive and scrambling of its tracer into other monoterpenes.

MONOTERPENES are probably metabolically labile in higher plants, rather than being inert waste products as was once believed; but it is not known, for example, whether interconversions within the C_{10} pool [2] or the transfer of tracer from C_{10} to C_{40} compounds [3] are direct processes or involve degradation and resynthesis from, perhaps, C_2 and C_5 precursors.

Preliminary studies carried out in the course of other experiments [2] showed that tracer was transferred to the pigments of *Tanacetum vulgare* L. (fam. Compositae) in significant amounts (>0·2%) after feeding [^{14}C]-labelled α-terpineol (*p*-menth-1-en-8-ol), isothujone (*trans*-thujan-3-one),† or terpinen-4-ol (*p*-menth-1-en-4-ol) [but not linalool (3,7-dimethylocta-1,6-dien-3-ol)]; and consequently we have carried out a detailed investigation to determine (*a*) the nature of these interconversions, (*b*) the possible role of compartmentation effects in regulating access of precursors to chloroplastic

† *cis*- and *trans*- refer to the disposition of the 1-isopropyl and 4-methyl groups.

[1] Part IV, D. V. Banthorpe, B. V. Charlwood, and M. R. Young, *J.C.S. Perkin I*, 1972, 1532.

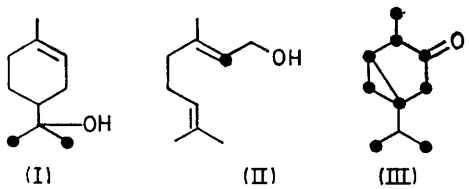
[2] D. V. Banthorpe and A. Wirz-Justice, *J. Chem. Soc.* (*C*), 1969, 541.

[3] T. N. R. Varma and C. O. Chichester, *Arch. Biochem. Biophys.*, 1962, **96**, 419.

sites of synthesis, and (*c*) changes within the monoterpene pool following introduction of exogenous C_{10} compounds. The last effect could be important for the interpretation of labelling patterns obtained after administering C_{10} precursors.

RESULTS

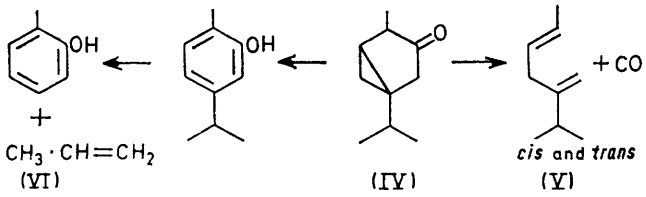
(*a*) *Tracer Incorporation.*—Typically, radioactivity in the 'lipid fraction' (see Experimental section) was at a maximum 5 h after feeding [9,10-$^{14}C_2$]*p*-menth-1-en-8-ol (I) to



(I) (II) (III)

Dot denotes tracer: (III) is generally but not uniformly labelled.

T. vulgare. The level had dropped to about four-fifths of this value by 40 h and was then essentially constant up to 150 h after feeding. Very little [^{14}C]carbon dioxide



(VI) (IV) (V)

SCHEME 1

(<0·3% of the applied tracer) was evolved up to 60 h but a burst of radioactivity then occurred over the period 60—100 h, whereafter the level steeply dropped. The distribution of tracer in a typical experiment is shown in Table 1:

* [*g*-^{14}C] Means that the labelling is general but not necessarily uniform.

the balance of tracer was water-soluble (82%) and tissue-bonded (18%).

(*b*) *Tracer Patterns.*—Isothujone (IV), β,β-carotene (VII), and chlorophyll isolated from plant material that had been fed with ^{14}C-precursor and harvested after 102 h were degraded as outlined in Schemes 1—3. In Scheme 2, carbon

TABLE 1

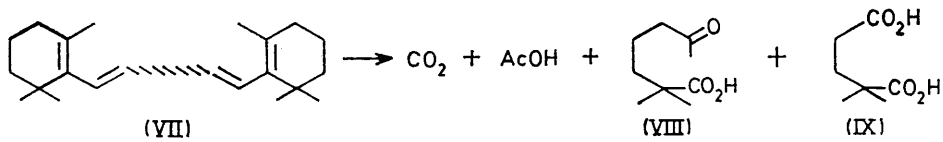
Tracer distribution after feeding [9,10-$^{14}C_2$]*p*-menth-1-en-8-ol[a] (I) [2] (2·1 × 10^6 disintegration min^{-1}) to *T. vulgare* for 102 h

Compound	Activity [b]	Specific activity [c]	% Incorporation
Carbon dioxide [d]	44,100		2·2
Lipid fraction [e]	50,006		2·5
Isothujone [f]	2125	4·11 × 10^3	0·1
β-Carotene	510	3·18 × 10^5	0·02
Xanthophylls [g]	3032	5·05 × 10^6	0·12
Chlorophylls [h]	6103	3·04 × 10^5	0·24
p-Menth-1-en-8-ol	26,120		1·2 [i]
Terpene fraction [j]	20,230		1·0

[a] Initial activity 1·0 × 10^9 disintegration min^{-1} mmol^{-1}. [b] In disintegration min^{-1}. [c] In disintegration min^{-1} mmol^{-1}. [d] Total activity evolved over 102 h. [e] See Experimental section. [f] Isothujone and thujone (99 : 1). [g] Lutein, violaxanthin, and neoxanthin. [h] a and b-compounds. [i] Percentage recovered of that fed. [j] G.l.c. fraction of C_{10} compounds, excluding *p*-menth-1-en-8-ol.

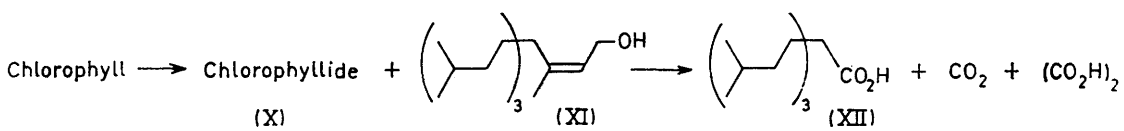
dioxide and acetic acid were mainly derived from oxidation of the conjugated chain linking the β-ionone residues. In one experiment a combined fraction of xanthophylls [lutein, violaxanthin, and neoxanthin, formally represented by (XIII), all oxygenated at C-3 and C-3′ and with the last two carrying oxygen substituents at other positions] were degraded to give the products of Scheme 4.

Incorporations of tracer resulting from feeding [9,10-$^{14}C_2$]*p*-menth-1-en-8-ol to *T. vulgare*, and less detailed data pertaining to feeding of [2-^{14}C]geraniol (II) (3,7-dimethylocta-2,6-dien-1-ol) to *Artemisia annua* L. (fam. Compositae) and [*g*-^{14}C]isothujone * (III) to *T. vulgare* are in Table 2. Results of degradations of certain products obtained in these experiments are in Table 3. Tracers from [2-^{14}C]geraniol and [*g*-^{14}C]isothujone were transferred to the



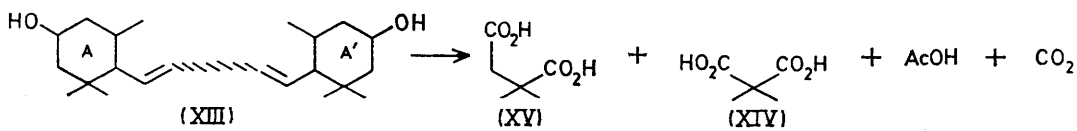
(VII) (VIII) (IX)

SCHEME 2



(X) (XI) (XII)

SCHEME 3



(XIII) (XV) (XIV)

SCHEME 4

TABLE 2

Incorporation of tracer from [^{14}C]-labelled precursors after 102 h

Precursor				Product				
Expt.	Compound	Specific activity [a]	Activity [b]	Plant	Compound	Specific activity [c]	Activity [d]	(%) [e]
1	[9,10-$^{14}C_2$]*p*-Menth-1-en-8-ol	101	210	*T. vulgare*	Isothujone	4·03	2150	0·1
2	[9,10-$^{14}C_2$]*p*-Menth-1-en-8-ol	101	210	*T. vulgare*	β,β-Carotene	12·5	201	0·01
3	[9,10-$^{14}C_2$]*p*-Menth-1-en-8-ol	101	210	*T. vulgare*	Xanthophylls [f]	144	3002	0·15
4	[9,10-$^{14}C_2$]*p*-Menth-1-en-8-ol	101	210	*T. vulgare*	Chlorophylls [g]	26·2	4810	0·24
5	[*g*-^{14}C]Isothujone	8·03	2·01	*T. vulgare*	β,β-Carotene	82·0	1342	6·7
6	[2-^{14}C]Geraniol	62·0	59·3	*A. annua*	β,β-Carotene	55·1	896	0·15

[a] In 10^7 disintegration min^{-1} mmol^{-1}. [b] In 10^4 disintegration min^{-1} fed to plant. [c] In 10^3 disintegration min^{-1} mmol^{-1}. [d] In disintegration min^{-1}. [e] % Incorporation of tracer. [f] See *g*, Table 1. [g] See *h*, Table 1.

carotenoid fractions to the extent of 0·15 and 6·7% respectively. These experiments pertain to mature plants. [^{14}C]*p*-Menth-1-en-8-ol was also fed to seedlings and young plants (see Experimental section) of *T. vulgare* and low incorporations (*ca.* 0·1%) of tracer into the carotenoids occurred.

(*c*) *Studies with* [^{14}C,^{3}H]-*Labelled Precursor*.—The distribution of tracer in products after feeding *T. vulgare* with [^{14}C,^{3}H]isothujone is given in Table 4.

TABLE 3

Tracer pattern resulting from feeding of [^{14}C]-labelled precursors, determined by degradation of the products obtained in experiments 1—6 in Table 2

Expt.	Compound	Specific activity [a]	Degradation products and specific activity [a,b]
1	Isothujone	4·03	(V) 3·89; CO 0·11; (VI) 3·93
2	β,β-Carotene	23·5 [d]	CO_2, 0; [c] (VIII) 22·1; [d] (IX) 23·0 [d]
3	Xanthophylls	144	CO_2, 0·23; AcOH, 0·93; (XIV) + (XV) 138
4	Chlorophylls	26·2	(X) 24·6; (XI) 0·66; (XII) 0·39; $(COOH)_2$, 0·23
5	β,β-Carotene	82·0	CO_2, 0; [c] (VIII) 77·5
6	β,β-Carotene	55·1	CO_2, 1·53; (VIII) 53·2

[a] In 10^3 disintegration min^{-1} mmol^{-1}. [b] Key to compounds: (V), 6-methyl-5-methylenehept-2-enes; (VI), propene; (VIII), 2,2-dimethyl-6-oxoheptanoic acid; (IX), αα-dimethylglutaric acid; (XIV), αα-dimethylmalonic acid; (XV), αα-dimethylsuccinic acid; (X), chlorophyllide; (XI), phytol; (XII), 5,9,13-trimethyltetradecanoic acid (see Scheme 3). [c] Count not significantly above background. [d] Results from experiment duplicate to that in Table 2.

TABLE 4

Distribution of tracer after feeding [*g*-^{14}C,^{3}H]isothujone to *T. vulgare*

Compound	*t*/h [a]	% Incorporation [b]	^{14}C : ^{3}H [c]
Isothujone [d]	40		1·0 ± 0·3
Thujanols [e]	40	1 ± 0·5	1·1 ± 0·3
Other terpenoids	40	<0·05	
Isothujone [d]	80		*f*
Thujanols [e]	80	10 ± 5	*f*
β,β-Carotene	80	0·12 ± 0·10	*f*
Other terpenoids	80	30 ± 5	

[a] Time of metabolism. [b] % Incorporation of [^{14}C]-tracer-from additive. [c] Ratio in precursor, 0·66 ± 0·02. [d] Isothujone and thujone (99 : 1). [e] A mixture of all four thujan-3-ols. [f] ^{14}C : ^{3}H > 10; *i.e.*, >90% tracer was ^{14}C.

DISCUSSION

(*a*) *Pigments*.—Tracer from [^{14}C]-labelled *p*-menth-1-en-8-ol, geraniol, and isothujone, was incorporated significantly (0·14—6·7%) into carotenoids in various experiments with mature specimens of *T. vulgare* and *A. annua*, and similar results were obtained for [^{14}C]*p*-menth-1-en-8-ol with seedlings and young plants of *T. vulgare*. Dilution factors of 10^3—10^5 were observed (Table 2) based on the specific activities of precursors and products.

Degradations (*cf.* Table 3) showed that essentially all the incorporated tracer was located in the ionone residues: the small amounts found in carbon dioxide and acetic acid could partly or entirely have resulted from oxidation of these residues,[4] or may indicate low incorporation of tracer into the conjugated chain linking them. These results suggest that either the intact C_{10} unit was incorporated or degradation of the additive led to the radioactive biogenetic equivalent of 3,3-dimethylallyl pyrophosphate (DMAPP), and they also indicate that access of either to the biosynthetic sites was not restricted by compartmentation effects.[5-7] The ring-opening or degradation required to effect these routes *in vivo* could have followed a pathway that has been well-characterised in micro-organisms.[8] If the radioactive biogenetic equivalent of isopentenyl pyrophosphate (IPP) was formed, it could not have reached the appropriate biosynthetic sites as tracer was not found in the conjugated chain of the carotenoids.

Tracer from [9,10-$^{14}C_2$]*p*-menth-1-en-8-ol was incorporated into chlorophyll by *T. vulgare* (*ca.* 0·2% incorporation, in dilution of *ca.* 10^4-fold). In contrast to the results for the carotenoids, the tracer appeared to have entered as acetate units. The chlorophyllide portion, where the bulk (*ca.* 96%) of the tracer resided, is known [9-11] to be constructed from a C_2 unit rather than C_5 or C_{10} units and is also believed to be in a rapid state of metabolic turnover. The rest of the tracer was located

[4] W. J. Steele and S. Gurin, *J. Biol. Chem.*, 1960, **235**, 2778.
[5] L. J. Rogers, S. P. J. Shah, and T. W. Goodwin, *Photosynthetica*, 1968, **2**, 184.
[6] K. J. Treharne, E. I. Mercer, and T. W. Goodwin, *Biochem. J.*, 1966, **99**, 239.
[7] T. W. Goodwin in 'Biosynthetic Pathways in Higher Plants,' eds. J. B. Pridham and T. Swain, Academic Press, London, 1965, p. 57.
[8] W. D. Loomis in 'Terpenoids in Plants,' ed. J. D. Pridham, Academic Press, London, 1967, p. 59.
[9] F. G. Fisher, G. Märkel, H. Hönel, and W. Rüdiger, *Annalen*, 1962, **657**, 199.
[10] G. Sironval and M. R. Michel-Wolvertz, *Coll. Internat. Centre Nat. Rech. Sci. Paris*, 1963, **119**, 317 (*Chem. Abs.*, 1964, **60**, 16,222*a*).
[11] E. Coates, *Phytochemistry*, 1966, **5**, 311.

in the phytol and partial degradation showed that the terminal C_5 unit (derived from IPP) contained over one third of the total activity.

One interpretation of these results is that carotenoids can be synthesised at extra-chloroplastic sites, whereas chlorophyll is formed at sites within the organelle, which is only readily accessible to C_2 units. This view is consistent with the observation [12] that xanthophylls are biosynthesised in tissue cultures that do not contain chloroplasts. The xanthophylls were more heavily labelled and were formed with less dilution of tracer than was the β,β-carotene formed in the same experiments. It is possible that the perturbations introduced by administering non-physiological concentrations of the additive may have led to enhanced formation of the oxygenated pigments such as occurs in senescent and necrotic tissue.[13]

Although total incorporations into terpenoids were usually low, only a few percent of the administered radioactivity was released as carbon dioxide (Table 1) and most of the tracer was located in water-soluble non-terpenoids or was tissue-bonded. Similar results were obtained after feeding *p*-menth-1-en-4-ol to *T. vulgare* [2] or geraniol to *Pelargonium* species.[14]

(*b*) *Monoterpenes.*—[9,10-$^{14}C_2$]*p*-Menth-1-en-8-ol was incorporated with little scrambling of tracer into isothujone, the major monoterpene of *T. vulgare* (Table 3). Although the incorporation of tracer (*ca.* 0·1%) was similar to that into xanthophylls, the dilution factor was considerably larger (*ca.* 10^5). These results imply direct conversion of the additive into isothujone and also suggest a low rate of turnover of the product such as has been inferred from the results of previous [2] tracer studies.

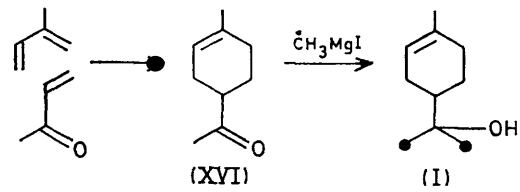
The experiments with [^{14}C,^{3}H]isothujone were performed to determine whether this presumed [2] end-product of monoterpene biosynthesis in *T. vulgare* had a low rate of turnover when its concentration was boosted to non-physiological levels; and also to ascertain whether the interconversions into other monoterpenes that had been demonstrated under these conditions involved modification of the intact C_{10} skeleton or degradation and resynthesis. Isothujone was biosynthesised from either sodium [1-^{14}C]acetate or sodium [^{3}H]acetate by *T. vulgare*. Degradation of similar samples that had been prepared for use in other biosynthetic studies indicated [15] that labelling was general, *cf.* (III), although non-uniform in both cases: specific labelling at alternate positions as expected on the application of simple biogenetic considerations did not occur.

Forty hours after the completion of uptake of doubly-labelled isothujone, only the thujanols were labelled (excluding label in isothujone itself) and the ratio $^{14}C : {}^{3}H$ in thujanols or recovered isothujone was little altered from that in the precursor. However after 80 h, although incorporation into thujanols had increased 10-fold, most of the ^{3}H in these and in recovered isothujone had been lost. Control experiments showed that <25% of the loss could be accounted for by enolisation. These results suggest degradation of the C_{10} compound and resynthesis of monoterpenes from the fragments. This interpretation is consistent with the small evolution of labelled carbon dioxide during the ' induction period ' (up to 50—60 h after feeding) that was followed by increased loss of tracer by this route, presumably when the *status quo* with respect to monoterpene composition was being achieved. Earlier indications [2] of the existence of enzyme systems capable of degrading and interconverting monoterpenes are supported by these present experiments.

EXPERIMENTAL

Many of the techniques used in this work have been described previously.[2]

(*a*) *Preparation of Labelled Substrates.*—[9,10-$^{14}C_2$]*p*-Menth-1-en-8-ol was prepared as shown in Scheme 5.



(XVI) (I)

SCHEME 5

Diels–Alder coupling [16] gave 4-acetyl-1-methylcyclohex-1-ene (XVI), b.p. 82° at 11 mmHg, and a Grignard reaction with [^{14}C]methyl iodide (0·1 mCi) led to the menthenol, which was purified by t.l.c. [silica gel H; ethyl acetate–benzene (12 : 88 v/v)] to give 99·9% chemically (g.l.c.) and radiochemically (radiochromatogram) pure material with specific activity 0·45 mCi mmol^{-1}.

[2-^{14}C]Geraniol (0·3 mCi mmol^{-1}) was prepared using a standard method.[17] [*g*-^{14}C]Isothujone was extracted (see later) after feeding the foliage of *T. vulgare* L. (50 g) with sodium [1-^{14}C]acetate (70 μCi) and maintaining the plant material on nutrient for 4 days under conditions of minimum transpiration. The product was purified to constant specific activity [2,18] to obtain material (20,060 disintegration min^{-1}; $8{\cdot}03 \times 10^7$ disintegration min^{-1} mmol^{-1}). [*g*-^{3}H]Isothujone was similarly prepared from sodium [^{3}H]acetate (300 μCi) and gave a product (15,320 disintegration min^{-1}; $7{\cdot}3 \times 10^6$ disintegration min^{-1} mmol^{-1}). [^{14}C]Labelled 3,7-dimethylocta-1,6-dien-3-ol (linalool) and *p*-menth-1-en-4-ol were available from previous studies [2] (40,230 and 27,325 disintegration min^{-1}; 6·3 and $4{\cdot}3 \times 10^6$ disintegration min^{-1} mmol^{-1}, respectively). All these labelled precursors were obtained with >99·5% chemical and radiochemical purity.

(*b*) *Feeding Methods.*—Seedlings (3 weeks), young (9 weeks), or mature (15 weeks) specimens of *T. vulgare* L. and young (9 weeks) plants of *A. annua* L. were grown from seed

[12] D. V. Banthorpe and A. Wirz-Justice, following paper.
[13] T. W. Goodwin in ' Comparative Phytochemistry,' ed. T. W. Swain, Academic Press, London, 1964, p. 340.
[14] D. J. Baisted, *Phytochemistry*, 1967, **6**, 93.
[15] D. V. Banthorpe and J. Mann, paper in preparation.
[16] K. Alder and W. Vogt, *Annalen*, 1949, **564**, 109.
[17] T. Money, I. G. Wright, F. McCapra, E. S. Hall, and A. I. Scott, *J. Amer. Chem. Soc.*, 1968, **80**, 4144.
[18] D. V. Banthorpe, J. Mann, and K. W. Turnbull, *J. Chem. Soc.* (*C*), 1970, 2689.

obtained from the Royal Botanic Gardens, Kew, and were used over the period April—October. Aliquot portions of the precursors (typically *ca.* 0·1 g) in ethanol–water (10 : 90, v/v; 100 μl) were fed to freshly-cut stems (8—10 cm; 50 g) and after uptake of tracer under forced transpiration,[2] the foliage was maintained on nutrient,[2] whilst enclosed in a large desiccator that was exposed to natural light at 20 ± 5°. The container was periodically flushed and the carbon dioxide in its atmosphere was collected for radiochemical assay. Control experiments showed that the proportion of ethanol in the initial feeding medium had no effect on the vitality of the plant material.

(*c*) *Separation Procedures.*—At predetermined times, the foliage was harvested and was then pulverised in liquid nitrogen with hexane (50 ml) to give a 'lipid fraction'. This was concentrated by passage through a plug (1 × 1 cm) of silica gel H (Merck) whereby essentially all the monoterpenes (with the exception of small amounts of hydrocarbons) and most of the pigments that were extracted by this procedure were absorbed and could be eluted with ether. By this means, the monoterpene fraction could be recovered (*ca.* 90%) in a small (*ca.* 4 ml) volume of solvent and concentration factors of up to 10^2-fold could be achieved in favourable cases.

Monoterpenes were further purified to constant specific activities as previously described [2,18] except that g.l.c. on FFAP (Varian Aerograph) columns at 90—125° was used as a first step, and the eluted fractions were recovered (>70%) by passage into an ethanol trap at −78°. *trans*-Thujan-3-one (isothujone) was purified as the 4-phenylsemicarbazone, m.p. 184° (from ethanol), which could be prepared in solution at pH *ca.* 5·2 under conditions such that no tritium was lost by enolisation (this was shown by the lack of exchange when the preparation was carried out in deuteriated solvents). Solid derivatives of the thujanols that were suitable for scintillation counting could not be prepared in sufficient yields and these compounds were purified by g.l.c. and t.l.c.[2] to the criteria previously outlined for liquids.[18]

Carotenoids and chlorophylls were extracted from the foliage by standard methods [2,19,20] with precautions to prevent photo-oxidation [21] and were chromatographed on a column of magnesium oxide–sucrose (1 : 1 w/w). Hexane eluted β,β-carotene, ether–hexane (50 : 50, v/v) eluted violaxanthin (5,6:5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol), and acetone eluted neoxanthin (5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol). Lutein and chlorophylls were extracted with methanol after extrusion. Fractions from the above were then further purified on at least two of the following t.l.c. systems: (*a*) magnesium oxide–sucrose–calcium sulphate (2 : 1 : 0·3, w/w); hexane–ether or ether–benzene (5 : 95, 50 : 50, and 80 : 20, v/v); (*b*) magnesium oxide; hexane–acetone (90 : 10 and 50 : 50, v/v); (*c*) cellulose (Merck precoated plates); ethanol–water–ammonia (80 : 4 : 16, v/v); (*d*) alumina (activated at 100° for 2 h); ethyl acetate; (*e*) silica gel G; benzene–light petroleum (b.p. 40—60°) (5 : 95, v/v); or (*f*) magnesium oxide–silica gel G (1 : 1, w/w); benzene–light petroleum (b.p. 40—60°) (12 : 88, v/v).

Crystallisation of the resulting purified carotenoids on the small scale proved as difficult as has been generally found.[22] Attempts were made by dissolving β,β-carotene in the minimum amount of benzene, adding methanol (3—4 vol) and leaving for several h at −20°, and by dissolving the xanthophylls in the minimum amount of methanol, cautiously adding a few drops of water, and cooling. Except for a few experiments, which gave small recoveries, these were unsuccessful. The carotenoids purified by t.l.c. were subjected to a strong evacuation to remove any traces of volatile impurities and used as such.

Identification was by (*a*) u.v. spectra,[22] (*b*) co-chromatography with authentic standards [12] on at least 3 of the t.l.c. systems, and (*c*) observation of characteristic acid-induced shifts [23] of $\lambda_{max.}$ of xanthophylls. Violaxanthin was also identified and assayed by paper chromatography.[24] M.p.s of these carotenoids vary with the rate of heating and with the experimental procedure used in the determination and are not good criteria for the compound or for purity.[25]

Spectrophotometric assays using compiled data [$\lambda_{max.}$/nm ($E^{1\%}_{1\,cm.}$); solvent] [22] were made for β,β-carotene [451 (2505); light petroleum, (b.p. 40—60°)], lutein [4·75 (2160); carbon disulphide], neoxanthin [438 (2270); ethanol], and violaxanthin [456 (2216); benzene], and enabled specific activities to be measured. Compounds were assigned radiochemically pure when specific activities (disintegration min^{-1}/optical density measured at two or three wavelengths) were unchanged after t.l.c. on 2 or 3 systems. This has been a general procedure in tracer studies on carotenoids (*cf.* refs. 19 and 20). In a few cases, the specific activities in segments taken across the t.l.c. spot were shown to be constant.

Chlorophylls were purified by t.l.c. on systems (*a*) and (*d*) and were assayed by their absorption at 562 nm under defined [26] conditions.

(*d*) *Degradation Procedures.*—Thujan-3-one (IV) was photolytically cleaved to carbon monoxide and *cis*- and *trans*-6-methyl-5-methylenehept-2-enes (V). The latter were purified to constant specific activity [18] and the carbon monoxide was scrubbed with acid and then oxidised over copper oxide at 800—900° to form carbon dioxide which was collected in barium hydroxide. Another sample was converted into 5-isopropyl-2-methylphenol, which was degraded to propene (VI) and *o*-cresol by treatment with phosphorus pentoxide.[15] The olefin was converted into the dibromide which was purified to constant specific activity by t.l.c.

β,β-Carotene or the combined xanthophylls (Expt. 3, Table 3) were augmented with carrier (25—50 mg) and were oxidised with chromic acid.[4] This well-established procedure causes much less degradation of the ionone residues (with the formation of carbon dioxide and acetic acid) than does use of potassium permanganate. The carbon dioxide was swept out in a stream of nitrogen, passed through a trap containing concentrated sulphuric acid, and after cooling by passing through another trap at −78° was collected in barium hydroxide solution. The residue was adjusted to pH 2 and acetic acid and traces of the other acids were extracted with ether. This extract and the residual acids were purified by t.l.c. using systems that had

[19] R. J. H. Williams, G. Britton, J. M. Charlton, and T. W. Goodwin, *Biochem. J.*, 1967, **104**, 767.
[20] S. Wieckowski and T. W. Goodwin, *Biochem. J.*, 1967, **105**, 89.
[21] J. S. Friend and T. O. M. Nakayama, *Nature*, 1959, **184**, 66.
[22] B. H. Davies in 'Chemistry and Biochemistry of Plant Pigments,' ed. T. W. Goodwin, Academic Press, London, 1965, p. 489.
[23] N. I. Krinsky and T. H. Goldsmith, *Arch. Biochem. Biophys.*, 1960, **91**, 271.
[24] V. H. Booth, *Analyst*, 1963, **88**, 627.
[25] B. C. L. Weedon in ref. 22, p. 75.
[26] J. Bruinsma, *Biochim. Biophys. Acta*, 1961, **52**, 576.

been developed for the column separation of this mixture [27] and also by using silica gel H (Merck) with ethyl acetate–benzene (15 : 85, v/v).

The products were characterised (m.p. in agreement with literature values; i.r. and n.m.r. spectra consistent with proposed structures) as 2,2-dimethyl-6-oxoheptanoic (geronic) acid (VIII), an oil [2,4-dinitrophenylhydrazone, m.p. 137° (from ethanol)], αα-dimethylglutaric acid (IX), m.p. 85° (from benzene–light petroleum), αα-dimethylmalonic acid (XIV), m.p. 192° (from benzene–ethanol), αα-dimethylsuccinic acid (XV), m.p. 141° (from benzene–ethanol), and *S*-benzylisothiouronium acetate, m.p. 135° (from aqueous ethanol). The glutaric acid (IX) and the derivatives of the heptanoic (VIII) and acetic acids were recrystallised to constant specific activity in the radiochemical experiments: the others were purified to constant specific activity on the two t.l.c. systems.

Previous reports [4,28] were confirmed that oxidation of β,β-carotene by this procedure gave good yields of the heptanoic acid (VIII) (*ca.* 60% based on the ionone rings) together with the glutaric acid (IX) (*ca.* 30%) but little (*ca.* 1%) of the acids (XIV) and (XV). Xanthophylls gave moderate (*ca.* 40%) yields of (XIV) and (XV). Yields of carbon dioxide and acetic acid formed by cleavage of the conjugated chain of both β,β-carotene and xanthophylls were *ca.* 90 and 35% respectively.

Chlorophylls were cleaved by treatment with sodium hydroxide (0·1N; 24 h at 20°), and phytol and the chlorophyllide residue were purified on t.l.c. system (*d*) or on silica gel G (Merck) with benzene–ethyl acetate (85 : 15, v/v) or chloroform. The phytol [phenylurethane, m.p. 29° (from ethanol)] was cleaved with potassium permanganate [28] to form oxalic acid, m.p. 189° (from water), and 5,9,13-trimethyltetradecanoic acid (XII), m.p. 153° (from benzene): these were purified on the same t.l.c. systems and subsequently recrystallised.

(*e*) *Counting Methods.*—Quantities of material for radioactive counting were determined by spectrophotometry, g.l.c., or weighing with a Cahn micro-balance.

Samples purified to constant specific activity by recrystallisation or by chromatographic techniques were assayed in aliquot portions (10 ml) of 2,5-diphenyloxazole (3% w/w) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0·3 w/w) in toluene as scintillation medium or (preferably) in similar aliquot portions of Butyl-PBD (Ciba) (0·8% w/w) in toluene. Counting efficiencies were *ca.* 97 and 75% for ^{14}C and ^{3}H in the latter system. Pigments were bleached (to eliminate their quenching properties) by exposure of the vial to a Siemens MB/D 125 W mercury vapour lamp (with the glass envelope removed) for 1 h. Quenching by other solutes was negligible when the second scintillation system was used.

Each sample was counted to accumulate 4×10^4 disintegrations above background to give $2\sigma \pm 1\%$, unless very low levels of radioactivity were present. However errors in weighing or spectrophotometric estimations caused the specific activities recorded in the Tables to have standard deviations of *ca.* ±3%.

Carbon dioxide was purified by scrubbing with concentrated acid and then passing through traps at −78° into barium hydroxide solution or into a mixture of 2-aminoethanol (2 ml) and 2-methoxyethanol (4 ml). The barium carbonate formed in the former method was either counted in a thixotropic gel after Carb-O-Sil had been added to the scintillation medium, or carbon dioxide was regenerated and passed onto 2-aminoethanol–2-methoxyethanol and the resulting solution (2 ml) was added to the counting medium (8 ml); $^{14}C : ^{3}H$ ratios were measured with a Beckman scintillation system (counting efficiency determined by a ^{137}Cs external standard; channels-ratio method).

We thank Unilever Ltd (London) and the S.R.C. for studentships (to A. W-J. and H. J. D., respectively). We are also grateful to Drs. J. Mann and B. V. Charlwood and Mr. C. Gatford and Miss E. Jackson for providing samples and carrying out certain experiments.

[1/2502 *Received*, 31*st December*, 1971]

[27] D. Braun and H. Geenen, *J. Chromatog.*, 1962, **7**, 56.
[28] L. Zechmeister and A. Polger, *J. Amer. Chem. Soc.*, 1944, **66**, 137.