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Terpene Biosynthesis. Part I. Preliminary Tracer Studies on Terpenoids and Chlorophyll of *Tanacetum vulgare* L.

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Chlorophyll, β-carotene, and the major monoterpenes of *Tanacetum vulgare* L. were significantly labelled within 1 hour of feeding sodium [1-^{14}C]acetate, but in contrast to results obtained with *Mentha* species, carbon-14 dioxide was poorly incorporated. The changing patterns of tracer in the plant constituents have been measured over several days and the efficiences of carbon dioxide, sodium acetate, and mevalonic acid, all labelled with carbon-14, as precursors have been established under conditions typical of those used in biosynthetic studies. The results are consistent with the sequence of intermediates in the usually accepted hypothetical scheme for monoterpene biosynthesis, and it is suggested that monoterpenes *in vivo* in *T. vulgare* are relatively long-lived and perhaps provide a pool of material for the synthesis of the physiologically important pigments.

Wick-feeding of several ^{14}C-labelled monoterpenes, under conditions that unavoidably must have perturbed the *status quo*, led to rapid and complex interconversions within this class, and more detailed investigations using [^{3}H]geranyl pyroposphate and [^{14}C]terpinen-4-ol demonstrated the passage of tracer, in addition, into pigments, sugars, and amino-acids.

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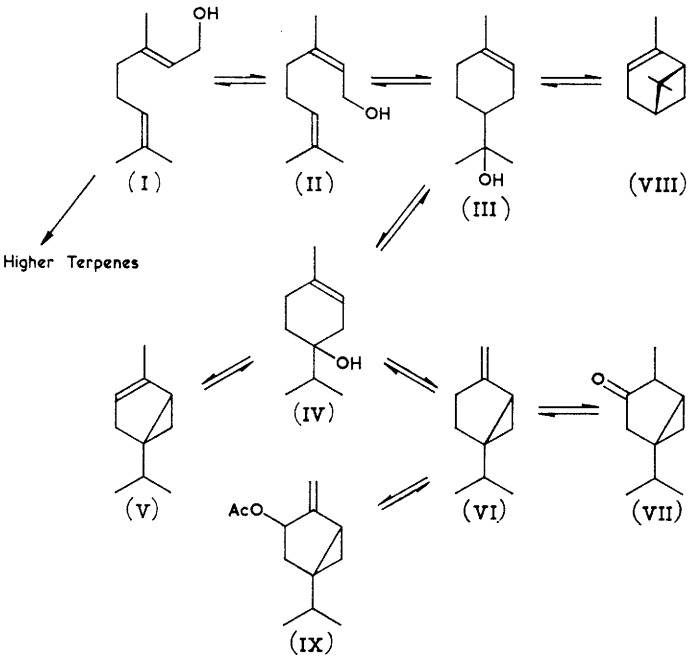
THE pyrophosphates of geraniol (I) and nerol (II) are almost certainly the progenitors of plant terpenoids. They are reasonably assumed to be biosynthesised by a route similar to that established in yeast and mammalian tissue, but details of their further conversions are obscure, although attractive hypotheses have been proposed.[1,2]

Tanacetum vulgare L. (Tansy) yields an oil containing a large quantity of the isomers of thujone (VII) and related compounds. As a preliminary to studies of the biosynthesis of these intriguing compounds we have investigated (a) the time-course of incorporation of sodium [1-^{14}C]acetate into chlorophyll † and certain terpenoids, and (b) the fate of certain individual monoterpenes that were fed into the plant. Most previous studies under heading (a), with notable expections (*cf.* refs. 3—5) either report incorporation of tracer into terpenes of mainly *Mentha* or *Pinus* species over short periods, or record qualitative autoradiography of tracer uptake into a few monoterpenes [6-9] or carotenoids [10,11] at a few intervals after feeding. No investigation has been made on *T. vulgare*. We have used precursor of the highest available specific activity and at the lowest concentration necessary to achieve convenient labelling of the individual monoterpenes of *T. vulgare*; these concentrations were some 100-fold smaller than those typically used in previous biosynthetic studies.[5]

Little is known about the stability *in vivo* or the metabolic significance of monoterpenes.[9,12] Physiological functions have been assigned to carotenoids and to certain sesqui- and di-terpenes, but it is not known whether monoterpenes are stable waste-products of metabolism or whether they play some dynamic role. Limited evidence suggests that the former, traditional, view may be invalid:[2] for example, the administration of [^{14}C]-α-pinene to *Pinus ponderosa* L. led to tracer rapidly appearing in β-pinene and car-3-ene.[13]



SCHEME 1 Biosynthesis of monoterpenes (after Ruzicka). This representation is purely formal. Interconversions probably involve pyrophosphate esters and the structural equivalents of carbonium ions

RESULTS

Composition of Oil.—Table 1 records analyses from 10 week-old plants‡. Specimens from 3 to 30 weeks old gave

† Chlorophyll refers to a mixture of the a- and b-compounds. Pigment will be used as a generic term to include chlorophylls and carotenoids.

‡ Unless specified to the contrary, all experiments were carried out on *T. vulgare*.

[1] L. Ruzicka, *Experientia*, 1953, **9**, 357.
[2] W. D. Loomis, in ' Terpenoids in Plants,' ed. J. B. Pridham, Academic Press, London, 1967, p. 59.
[3] F. W. Hefendehl, E. W. Underhill, and E. von Rudloff, *Phytochemistry*, 1967, **6**, 823.
[4] G. R. Waller, G. M. Frost, D. Burleson, D. Brannon, and L. Zalkow, *Phytochemistry*, 1968, **7**, 213.
[5] R. G. Battu and H. W. Youngken, *Lloydia*, 1966, **29**, 360.
[6] P. Valenzuela, O. Cori, and A. Yudelevich, *Phytochemistry*, 1966, **5**, 1005.
[7] P. Valenzuela, E. Beytia, O. Cori, and A. Yudelevich, *Arch. Biochem. Biophys.*, 1966, **113**, 536.
[8] R. H. Reitsema, F. J. Cramer, N. J. Scully, and W. Chorney, *J. Pharm. Sci.*, 1961, **50**, 18.
[9] J. Battaile and W. D. Loomis, *Biochim. Biophys. Acta*, 1961, **51**, 545.
[10] A. P. Losev and A. A. Shlyk, *Biokhimiya*, 1964, **29**, 457.
[11] T. N. R. Varma and C. O. Chichester, *Arch. Biochem. Biophys.*, 1962, **96**, 419.
[12] D. J. Baisted, *Phytochemistry*, 1967, **6**, 93.
[13] W. Schweers, *Tetrahedron Letters*, 1968, 4425.

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results within ±2% (actual values) for the various components. Geraniol, nerol, sabinyl acetate (IX), and α-terpineol (III) could not be detected. The oil comprises *ca.* 0·2% (w/w) of freshly gathered foliage and the yield is *ca.* 20% greater at night than by day; although loss of monoterpenes by evaporation during 24 hr. accounts for only about 1% of the total present. The concentrations of chlorophyll and β-carotene were remarkably constant during the period of these experiments at 4·8 and 1·2 mg. per g. leaf (wet weight) respectively (both ±0·2 mg.). Numerous other species show no diurnal variation of chlorophyll concentration under summer conditions.[14,15]

TABLE 1

Monoterpenes of *T. vulgare* L.

Hydrocarbons	% w/w *	Ketones	% w/w *
Sabinene	3·2	Isothujone	83·1
α-Thujene	0·1	Thujone	0·9
α-Pinene	0·3	Carvone	1·2
β-Pinene	0·1	Camphor	0·7
Camphene	0·3		
α-Terpinene	0·2	Alcohols	
γ-Terpinene	0·1	Isothujol	2·3
Terpinolene	0·3	Neoisothujol	1·0
Limonene	0·1	Terpinen-4-ol	0·9
Myrcene	0·2	Borneol	0·9
Unidentified	0·3		
		Others	
		1:8-Cineole	3·0
		p-Cymene	0·1
		Unidentified	0·7

* Of total plant oil.

Incorporation of Tracer into Pigments and Monoterpenes.— Exposure of *T. vulgare* to carbon-14 dioxide (500 μc) for periods of from 5 min. to 3 days resulted in little accumulation of tracer in terpenoids and chlorophyll. Table 2

TABLE 2

Activity (c.p.m.) of monoterpenes and pigments after feeding of tracer

(1)	*t* (hr.).....	3	4	16	40	64	72
	Activity *	299	293	121	390	125	237
(2)	*t* (hr.).....	3	5	8·5	24	48	
	Activity *	346	1193	1272	2161	430	
(3)	*t* (hr.).....	2	5	7	10	23	29	37
	Activity *	7100	29,010	4125	7820	15,900	12,900	9200

* In c.p.m.

Expt. (1) *T. vulgare*; 500 μc carbon-14 dioxide as tracer source. Expt. (2) *T. vulgare*; 2 μc sodium [^{14}C]acetate, as tracer source. Expt. (3) *P. graveolens*; 15 μc sodium [^{14}C]-acetate, as tracer source.

records a typical experiment using matched leaflets. The incorporation after 3 hr. was distributed: β-carotene, 152 c.p.m.; monoterpenes, 35 c.p.m.; chlorophyll, 22 c.p.m.; the balance being lost in work-up. In contrast, stem-feeding of sodium [1-^{14}C]acetate (2 and 15 μc respectively; 3×10^4 μc mmole^{-1}) to *T. vulgare* and *Pelargonium graveolens* Ait. gave incorporations of *ca.* 0·05 and 0·1% of the administered tracer; *cf.* Table 2. The uptake of carbon dioxide into *T. vulgare* became negligible when the experiment was carried out during 24 hr. in darkness, whereas under the same conditions the uptake of acetate was only reduced by *ca.* 15%. Autoradiography showed that tracer was unevenly distributed over the entire leaf within 1 hr. of the completion of stem feeding, although most accumulated near the level of immersion. After 24 hr., tracer concentrated in discrete spots in the stem and leaves and then passed to the leaf tips, being essentially all thus translocated in 7 days.

Table 2 suggests a periodicity in the pattern of incorporation and more detailed studies on *T. vulgare* are recorded in Figure 1: here the time zero is at the onset of feeding. At time A, an aliquot of 62 mg. chlorophyll had an activity of 172,230 c.p.m. and 15 mg. β-carotene gave 20,900 c.p.m. at time D. Specific activities are in Table 3.

TABLE 3

Incorporation of [^{14}C]acetate into constituents of *T. vulgare* L.

Compound	(1)	(2)	(3)	(4)
Chlorophyll	172,230	2	$2·5 \times 10^6$	2·2
β-Carotene	20,900	4·8	$7·7 \times 10^5$	0·28
Total monoterpenes [a]	10,970	43	$9·1 \times 10^4$	0·14
Terpinen-4-ol	4520	22	$4·2 \times 10^6$	0·06
Thujenes [b]	3510	116	$7·4 \times 10^5$	0·05
α-Pinene	438	116	$1·0 \times 10^6$	0·006
Thujones [c]	638	116	$5·7 \times 10^3$	0·008

(1) C.p.m. for aliquot at maximum incorporation. (2) Time of maximum incorporation (hr.). (3) Specific activity (c.p.m. per mmole) at maximum incorporation. (4) Maximum % incorporation of applied tracer.

[a] Mean molecular wt. ~150 was assumed. [b] Mixture of α-thujene and sabinene. [c] Mixture of thujone and isothujone.

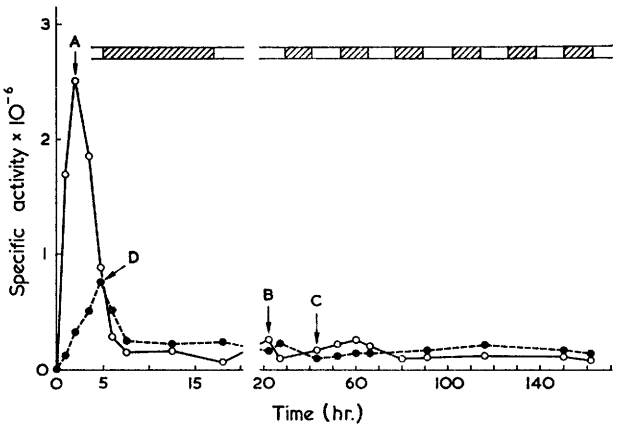

FIGURE 1 Uptake of sodium [1-^{14}C]acetate into pigments; cross-hatching indicates periods of natural darkness —— Chlorophyll; – – – β-carotene

At times A, B, and C, 37, 76, and 59% of tracer in chlorophyll was located in the phytol residue and the balance was in the porphyrin component. In contrast, spinach showed a very similar profile for time-incorporation but with *ca.* 30 and 99% tracer residing in the phytol residue at times corresponding to A and C (observations by Miss Susan Patchin). Figure 2 records the distribution of tracer between monoterpenes and pigments, and Figure 3 shows the specific activities of the major monoterpenes throughout a particular experiment. Duplicate runs gave similar profiles. The existence of geraniol * and α-terpineol was demonstrated by trapping. The activity of samples of

* Mixture of isomers (I) and (II).

[14] D. W. A. Roberts and H. J. Perkins, *Biochim. Biophys. Acta*, 1962, **58**, 499.

[15] T. N. Godnev and E. F. Shabelskaya, *Vesti. Akad. Nauk. Belorusk. S.S.R.*, 1966, 5; (*Chem. Abs.*, 1966, **65**, 17,365).

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carrier that were added to the plant extract at the various times after feeding, and reisolated, are compared with the activity of terpinen-4-ol (IV) in Table 4. The specific

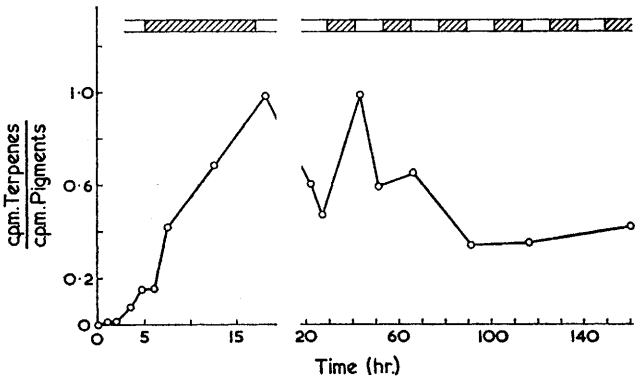

FIGURE 2 Relative uptake of sodium [1-^{14}C]acetate into pigments and terpenes; cross-hatching indicates periods of natural darkness

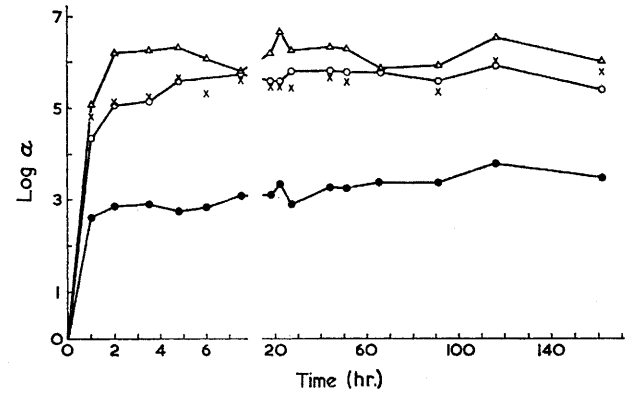

FIGURE 3 Specific activities of monoterpenes; α = specific activity in c.p.m. mmole^{-1}; Δ, terpinen-4-ol; ○, sabinene + α-thujene; ×, α-pinene; ●, thujone

activities of these compounds must exceed 10^9 c.p.m. mmole^{-1}.

TABLE 4

Geraniol and α-terpineol as biosynthetic intermediates

Time (hr.)	1	2	3·5	4·75	6	7·5	18	22
Geraniol *	52	62	92	230	242	103	420	682
α-Terpineol *	25	67	190	350	423	102	518	876
Terpinen-4-ol *	83	103	113	137	74	40	108	283
Time (hr.)	27	43	51	66	91	116	161	
Geraniol *	325	730	308	218	230	142	130	
α-Terpineol *	403	1110	414	137	276	1605	617	
Terpinen-4-ol *	120	131	123	52	58	252	66	

* C.p.m. mg.$^{-1}$ of extracted oil.

Fate of Tracer from Labelled Monoterpenes.—^{14}C-Labelled monoterpenes were concentrated near the point of injection in the stem, but 0·1—9·1% of the applied tracer was recovered in monoterpenes extracted from the leaves, which experiments with defoliated stems showed to be the main sites of terpene interconversions. Formal percentage incorporations are especially meaningless as indices of metabolic lability of these predominaly water-insoluble additives, owing to differences of translocation to the leaves.

The dosages of specific activities of the substrates and the amounts of tracer recovered in the monoterpene fraction were: sabinene (VI), 18 mg., 1800 c.p.m. mg.$^{-1}$, 1·3%; α-thujene (V), 45 mg., 820 c.p.m. mg.$^{-1}$, 8·2%; α-terpineol (III), 0·5 mg., 17,000 c.p.m. mg.$^{-1}$, 9·1%; terpinen-4-ol, 20 mg., 1400 c.p.m. mg.$^{-1}$, 7·9%; sabinyl acetate, 14 mg., 5040 c.p.m. mg.$^{-1}$, 0·2%; thujone, 40 mg., 1000 c.p.m. mg.$^{-1}$, 3·2%; and linalool, 2 mg., 6540 c.p.m. mg.$^{-1}$, <0·1%. Table 5 records the distribution of tracer in monoterpenes extracted from the leaves.

TABLE 5

Interconversions of terpenes *in vivo*

		Distribution of tracer (c.p.m.)							
Substrate	Time [a]	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Sabinene	96	79	39	87	79	—	174	—	—
α-Thujene	40	9	72	2787	21	—	14	—	12
α-Terpineol	24	27	12	275	282	173	—	—	—
α-Terpineol	96	25	3	7	2	—	78	7	—
Terpinen-4-ol	96	346	4	16	291	—	4	—	1544
Sabinyl acetate	40	7	—	26	32	33	19	—	—
Thujone	40	522	—	345	398	—	26	—	—
Linaloöl	40	2	—	5	4	—	4	—	—

[a] Time before harvesting (hr.)

(1) Sabinene. (2) α-Thujene. (3) α-Terpineol. (4) Terpinen-4-ol. (5) Sabinyl acetate. (6) Thujones (n- and iso-). (7) Camphor. (8) Thujols (iso- and neoiso-).

Quantity of tracer used (mμc); sabinene, 15; α-thujene, 17·2; α-terpineol, 3·8; terpinen-4-ol, 12·7; sabinyl acetate, 32·8; thujones, 18·2; linalool, 5·9.

Feeding of [^{3}H]*Geranyl Pyrophosphate and* [^{14}C]*Terpinen-4-ol.*—Autoradiography of extracts from the previous feeding experiments showed that other compounds, in addition to monoterpenes and pigments, became labelled. A more detailed investigation of the fate of added tracer was therefore made. [^{3}H]Geranyl pyrophosphate (82 mg., $1·4 \times 10^5$ c.p.m. mg.$^{-1}$) was fed and the leaves were harvested after 20 hr. The recovered activity (3·1%, 360,531 c.p.m.) was distributed: geraniol, 299,264 c.p.m.; other monoterpenes, 7466 c.p.m.; β-carotene, 40,373 c.p.m.; other carotenoids, 3994 c.p.m.; and chlorophyll 9434 c.p.m. (65% in phytol). The location of tracer in monoterpenes was: α-pinene (VIII), 804 c.p.m.; camphene, 883 c.p.m.; sabinene 3470 c.p.m.; thujone 290 c.p.m.; terpinen-4-ol 179 c.p.m.; and unidentified, 1840 c.p.m.

[^{14}C]Terpinen-4-ol (4 mg., $6·6 \times 10^4$ c.p.m. mg.$^{-1}$) was fed and the oil was extracted from the entire plant after 44 hr. The bulk of tracer (251,250 c.p.m., 95%) was recovered and was located: terpinen-4-ol, 13,610 c.p.m.; other monoterpenes, 9661 c.p.m.; carotenoids 9500 c.p.m.; chlorophyll 23,500 c.p.m.; amino-acids 10,080 c.p.m.; sugars 18,950 c.p.m.; and tissue-bound (not extracted with solvents) 9370 c.p.m. The distribution in terpenes was: thujol 5320 c.p.m.; thujone 1630 c.p.m.; camphor 1380 c.p.m.; α-terpineol 1226 c.p.m.; unidentified 105 c.p.m. There was negligible incorporation into α-thujene or α-pinene. The large balance of radioactivity that was not located in these particular classes was distributed over the chromatograms at the various stages of separation, and much was water-soluble. Two well defined activities resulted from chromatography of extract A (see Experimental section) on t.l.c. system (*f*). One registered 31,400 c.p.m. and the other 5770 c.p.m.: 11·8 and 2·2% of the administered tracer respectively. Neither was further characterised. The rest of the recovered radioactivity

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could not be assigned to any particular fraction or class of compounds.

Incorporation of Mevalonic Acid.—Incorporation of [2-^{14}C]mevalonic acid is summarised in Table 6 where allowance is made for the utilisation of only the (−)-isomer of the racemate that was fed (observations by B. V. Charlwood). The analyses were made at 73 hr. after feeding, this being the time for maximum incorporation of tracer into thujone. During this period 2·4% of the tracer was evolved as carbon dioxide. After feeding [1-^{14}C]mevalonic acid to *Artemisia annua* L., negligible incorporation of tracer into monoterpenes occurred in 161 hr., but 16·2% was degraded to carbon dioxide. On feeding to *T. vulgare*, 14·5% of tracer from this substrate was released as carbon dioxide in 161 hr., whereas 21·5% sodium [1-^{14}C]acetate was so degraded during the same period.

TABLE 6

Relative efficiencies of tracer incorporation

	% Incorporation [a]		
	Chlorophyll	β-Carotene	Monoterpenes
Sodium [1-^{14}C]acetate	2·2	0·28	0·14
[2-^{14}C]Mevalonic acid	0·46	5·6	0·28
[^{3}H]Geranyl pyrophosphate ...	0·08	0·38	0·05
[^{14}C]Terpinen-4-ol	9·0	3·8	3·8

[a] For details of respective times of metabolism see text.

DISCUSSION

Composition of the Oil.—Our results are in fair agreement with a previous analysis of essential oil from the same genotype,[16] and unusual terpenes such as are produced by mutants [17–19] were not present. Noteworthy is the absence of carvotanacetone and the low yield of camphor. Both are present in considerable (5—15% w/w) quantities in commercial Tansy oil, but they may be formed by thermal and photolytic rearrangements respectively [20] during extraction and storage. The composition of the oil did not appreciably alter before, during, or after flowering, unlike the situation for certain *Mentha* species.[9] It qualitatively resembled the oils from 21 *Artemisia* species that were screened, but was quite unlike those from 6 *Chrysanthemum* species (C. Gatford, unpublished results). This is of interest as *Tanacetum*, *Artemisia*, and *Chrysanthemum* are closely related genera and some authorities have classified *T. vulgare* in the last group.

Incorporation of Tracer from Carbon Dioxide.—The low incorporation of carbon dioxide into terpenes and pigments differs from results obtained from *Mentha* species,[3,9] although, as expected on the basis of other observations,[21,22] such tracer as is incorporated preferentially enters the carotenoids. Under our conditions tracer from acetate is much more readily incorporated than carbon dioxide into both monoterpenes and pigments, whereas opposite conclusions have been reported for the *Mentha* species.[3,9] Our observations are reasonable as carbon dioxide has to be converted into acetate before becoming available for terpenoid synthesis, but the relative efficiencies of the two precursors must be governed by membrane permeabilities and rates of translocation to the synthetic sites, and this may differ between the species.

^{14}C-Labelled acetate and mevalonic acid were incorporated into monoterpenes of *Mentha* species with very low efficiency,[9] and up to 11% tracer was released as carbon dioxide over 336 hours.[5] Consequently, it was suggested that direct uptake of these two compounds was negligible and that carbon dioxide, generated *in situ* from degradation of the additives, was the main precursor of terpenoids. Although acetate and mevalonic acid are considerably broken down to carbon dioxide in *T. vulgare*, and microbial degradation under these conditions is unlikely, we do not favour this route for three reasons. First, [2-^{14}C]mevalonic acid is incorporated into thujone and several other monoterpenes with tracer mainly located at one ring-position, and with little scrambling such as would result from degradation of the substrate to C_1 or C_2 fragments.[23] Secondly, negligible uptake of tracer into monoterpenes was found when [1-^{14}C]mevalonic acid was fed to the closely related species *Artemisia annua* L.: here carbon dioxide must have been generated in large quantities *in situ*. And thirdly, when carbon-14 dioxide, sodium [1-^{14}C]acetate, and [2-^{14}C]mevalonic acid were separately fed in the dark and the treated specimens were so maintained for 24 hours, the incorporation of the first substrate into monoterpenes (an incorporation that was very low in the light) became negligible, whereas uptake of tracer from the other two substrates was practically unaltered. The simplest explanation of these last results, according to a recent discussion of the method as applied to *Mentha piperita* L.,[3] is that degradation *in situ* into, and incorporation of, carbon dioxide, is unimportant.

Incorporation of Tracer from [^{14}C]*Acetate.*—The maximum uptake of tracer into chlorophyll and β-carotene occurred at about 2 and 5 hours respectively after the onset of feeding. Both the phytol residue and the pyrrole moiety of the chlorophyll were labelled, the former becoming almost exclusively so in spinach. Both parts of the chlorophyll molecule are known to be rapidly degraded and to be built up readily from acetate units in other plant species.[21,24,25] Most of the initial uptake of tracer was lost by 8 hours after feeding, and the specific activities of both β-carotene and chlorophyll then became essentially constant.

[16] E. von Rudloff and E. W. Underhill, *Phytochemistry*, 1965, **4**, 11.
[17] E. Stahl, *Arch. Pharm.*, 1964, **297**, 385.
[18] E. Stahl and W. Scheu, *Naturwiss.*, 1965, **52**, 394.
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[25] C. Costes, *Phytochemistry*, 1966, **5**, 311.

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The fluctuations in the ratio of tracer in monoterpenes and pigments (Figure 2) may be diurnal in origin as similar profiles were obtained for *Pelargonium graveolens* Ait. and *Juniperus sabina* L. (K. W. Turnbull, unpublished results). Tracer may pass from terpenes to pigments when growth is more rapid but revert to the terpene pool at night. After 3 days, the tracer becomes distributed between the classes in fairly constant proportions. A build-up of tracer into the terpenes, followed by a fall-off of activity without a corresponding reduction in concentration of the terpenes, has been observed for other species,[2,26] although, in contrast, tracer from [^{14}C]acetate is steadily incorporated into monoterpenes in one species for up to 168 hours after feeding.[27] Such differences in the profiles of time-incorporation could be controlled by many factors, in particular by varying dosages of tracer-containing substrates and differences in translocation.

It is difficult to assess the significance of specific radioactivities of compounds whose individual rates of synthesis have not been determined and to interpret unambiguously feeding experiments of presumed intermediates.[28] However, we have calculated specific activities on the basis of the total concentrations of the terpenoids (which are constant over the course of the experiments), and venture tentative conclusions.

Scheme 1 for monoterpene biosynthesis is based on Ruzicka's speculations.[1] The order of specific activities throughout the course of the experiments recorded in Figure 3, together with those estimated by trapping procedures (Table 4), *viz.*,

$$(I) + (II) > (III) > (IV) > (V) + (VI) \sim (VIII) > (VII)$$

is consistent with this scheme. It is inconsistent with a proposal, based on theoretical considerations, of concerted bicyclisation of C_5 units or of an acylic C_{10} compound followed by ring-fission to form monocyclic terpenes,[29] and also make doubtful a suggestion[30] that hydrocarbons are not intermediates in the biosynthesis of oxygenated monoterpenes.

Interconversions of Monoterpenes.—The presumed end-products of terpene synthesis (*e.g.*, thujone) and also the hypothetical intermediates (α-terpineol, sabinene, α-thujene, etc.) readily transfer tracer to the terpene pool on feeding to *T. vulgare* (*viz.*, Table 5). Three tentative conclusions can be drawn. (*a*) Sabinyl acetate and linalool, which do not occur in the natural oil, play no key, if any, role in the above scheme. Their low efficiency as precursors (in the general sense of being capable of transferring their tracer) suggests that they are either not translocated to the synthetic sites or are not substrates for the enzyme systems catalysing the interconversions. (*b*) α-Terpineol and terpinen-4-ol appear to be efficient precursors of bicyclics. (*c*) α-Thujene is not an efficient precursor for bicyclics. If this compound was translocated to the reaction sites, attractive routes to thujone and thujol are either by anti-Markownikoff addition of water to the double-bond, an orientation favoured by the formation of a non-classical ion following the initial step of protonation,[20] or by photoxidation accompanied by rearrangement of the double bond to form sabinol followed by reduction. The last route has been demonstrated in a model system.[31] Our results suggest that α-thujene occupies a side branch on the route to thujone, as shown in Scheme 1, rather than being on the direct pathway, as has been implied.[32] Sabinene, an effective precursor of thujone, is a more likely candidate for a position on the direct route, as γ-oxidation without bond migration followed by redox steps could lead to thujone.

Tracer from water-soluble geranyl pyrophosphate is not incorporated into the terpene pool more efficiently than that from the other substrates. The ester group may hinder the penetration of cell membranes or, more probably, the substrate is hydrolysed by phosphatases known to be widespread in higher plants. The percentage incorporation of geranyl pyrophosphate and terpinen-4-ol are in Table 6: the values have no quantitative significance as they were obtained at arbitrary intervals (20 and 44 hours) after feeding but they are minimum values. Good incorporation of tracer is found especially from the latter compound. Noteworthy are the uptakes of 4·0 and 7·2% of the applied tracer into amino-acids and sugars, presumably *via* degradation of the substrate and assimilation of the fragments. In *Pelargonium graveolens* Ait. oxidative degradation of added [^{14}C]geraniol has been found to be extensive, although 9·4% of the tracer is incorporated into triterpenes.[12] However, only 0·22% of this precursor is incorporated into cineol in *Rosmarinus officinalis* L.[33]

Under our conditions, mevalonic acid, geranyl pyrophosphate, and monoterpenes all transfer tracer to pigments as well as to the monoterpene pool. In contrast, mevalonic acid has been found to be efficiently incorporated into extra-chloroplastic terpenoids in germinating pea and pine seedlings,[22,34] but much less so into pigments and phytols which are believed to be synthesised within the chloroplast. This has been attributed to the impermeability of the chloroplast membrane to this substrate, and to the inter- or intra-chloroplastic location of the relevant enzyme systems. Our results may be due to the use of a different plant species at a different stage of development, or may result

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from degradation to acetate that can penetrate into the chloroplast. We hope to gain further information by use of doubly labelled precursors.

Lability of Terpenes in the Unperturbed Plant.—Two approaches are available for estimating the turnover of the terpene pool *in vivo*: the activity of the components of the pool can be measured at various times after feeding a labelled precursor, or labelled terpenes can be fed and their fate determined. Both methods usually perturb the system as large dosages are necessary to obtain sufficient radioactivity in the members of the pool, but an exception is for studies of the uptake of carbon-14 dioxide which can be introduced at its natural abundance (0·04% v/v in air). Our method of feeding probably resulted in a slow translocation of tracer to the active metabolic sites which would minimise these perturbations; and the percentage incorporation was not appreciably changed when the weight of substrate was varied by 160-fold, nor was the development of new synthetic capabilities or the accumulation of atypical metabolites revealed by chromatographic screening. Nevertheless our feeding experiments undoubtedly led to overloading and extensive degradation, probably by routes that have been characterised in micro-organisms and other high plants,[2,12] in order to revert to the *status quo*.

The low uptake of carbon dioxide into the pigments and terpenoids of the ' unperturbed ' plant, the approximately constant specific activities of chlorophyll, β-carotene, and the major monoterpenes after the initial perturbation caused by feeding acetate had subsided, and the location of tracer from [2-^{14}C]mevalonic acid in one ring-position of thujyl compounds many days after feeding,[23] suggest a low rate of turn-over of these compounds in *T. vulgare*. Qualitative measurements have shown a similarly low rate of turnover of the terpenes of *Pinus* species.[35] However, preliminary studies indicate that if the concentrations of pigments and terpenoids are depleted by etiolation, carbon dioxide is incorporated with an efficiency comparable with that of acetate into both classes for the first 12 hours that the plant is exposed to light, when these pools are being refurbished.

The initial maximum specific activities of chlorophyll and β-carotene may be due to overloading with acetate which enters the chloroplast and temporarily causes an imbalance of synthesis that is rapidly corrected by the operation of oxidative degradation. Passage of acetate into monoterpenes lags behind, and degradative and other pathways account for the majority of tracer. The little which does enter the terpene pool, however, is mainly retained. Many other workers have noted the low incorporation of acetate and mevalonic acid into monoterpenes;[2] undoubtedly overloading of the system and consequent massive degradation has commonly occurred. In a recent study,[5] feeding of [^{14}C]acetate or mevalonic acid to *Mentha piperita* L. led to the incorporation of tracer into each presumed intermediate in turn *en route* to menthone and the expected precursor-relationships were demonstrated. However, massive dosages of acetate or mevalonic acid (about 100-fold those used in our experiment) were applied here and a relatively ' steady state ' may not have been attained over the course of the experiments. In our hands the variability of the plant material, inevitable in work with whole plants, precluded such a detailed approach, and we prefer to record the logarithmic profile of Figure 3 to indicate the trend obtained, rather than attempt analyses of the small individual fluctuations in specific activity.

Labelled terpenes were unavoidably fed in quantities which again perturbed the *status quo*. The excess of additive is probably again degraded, and the tracer passes into other constituents such as sugars and amino-acids. Some becomes incorporated into the terpene pool, although further experiments are required to determine if direct incorporation of the additive or breakdown and resynthesis occurs. The former route may apply: incorporation of acetate is usually inefficient and tracer from the carbonyl carbon of thujone and other thujyl compounds does not scramble to other positions.[23]

The traditional view is that terpenes are irreversibly formed side- or end-products of metabolism:[36,37] ' indifferent ballast ' that accumulates with age.[38] This has been questioned[2,39] and recently, certain alkaloids, members of another class of secondary metabolites of obscure function, have been found to be rapidly turned-over in plants.[40-42] Our results show that pathways of synthesis, degradation, and interconversion of terpenoids are available, although the half-life of the compounds is probably many days, at least in the unperturbed plant. An attractive hypothesis is that the monoterpenes provide a pool of material for synthesis of the physiologically important pigments, and perhaps other compounds.

EXPERIMENTAL

Materials and Separation Methods.—Specimens of *T. vulgare* were grown from the seed of a flower-head obtained from the Royal Botanic Gardens, Kew. Pairs of leaflets from the same or corresponding nodes of young plants (6—9 in.; 10 weeks), grown outdoors from March to October, were used for acetate-feeding experiments, and shoots (3 in.; 4 weeks) with 4 or 5 leaf pairs plus the top leaflets and the apical meristem, were used for the terpene feeding. Other species were obtained from Kew or from

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the Cambridge University Botanical Garden, except spinach which was obtained locally.

Reference compounds were obtained by preparative gas-liquid chromatography (g.l.c.) or thin-layer chormatography (t.l.c.) of commercial oils of Tansy, Savin, and *Thuja occidentalis* L. (Fritzsche Bros., New York), and characterised by comparison of i.r. spectra with those of authentic samples.

A Wilkens Aerograph A700 gas chromatograph equipped with helical copper columns (25 ft. × ¼ in. o.d. and 25 ft. × ⅜ in. o.d. for analytical and preparative operation respectively), with helium carrier gas (inlet 50 lb./in.^{2}; flow rate 3—7 l. hr.$^{-1}$), or a Pye series 104 instrument with helical steel columns (5 ft. × ¼ in. o.d.) and argon carrier gas (inlet 40 lb./in.^{2}; flow rate *ca.* 2·5 l. hr.$^{-1}$) were used Acid–alkali washed kieselguhr (80—100 mesh; J. J. Ltd., Kings Lynn) coated with 15 or 30% w/w Carbowax 20M were stationary phases for analytical and preparative operation, and 2,4-dinitrophenyl 2-naphthyl ether (15% w/w on kieselguhr) was useful for the separation of hydrocarbons. The latter were chromatographed at *ca.* 90° and then temperature-programmed to 150—160° to elute ketones and alcohols. Sample sizes were 0·5 μl. to 100 μl. and the collection efficiency was 65—90% depending on the nature and size of the sample. Care was taken to prevent radioactive contamination of the columns by purging the apparatus between separations at the maximum operating temperature with a rapid flow of nitrogen.

Preparative and analytical t.l.c. plates (1·0 and 0·25 mm. layer thickness, prepared with a Shandon apparatus) were developed until the solvent front had travelled 14 mm. Several solid supports and solid systems were used.

(*a*) Sucrose–heavy magnesium oxide–calcium sulphate (1 : 3 : 0·4 w/w) were slurried with methanol and the plates activated at 90° for 1 hr. Elution with ether–benzene (5 : 95 v/v) separated the pigments into several bands and the monoterpenes into one broad band.

(*b*) Silica gel G (Merck) was slurried with water and the plates activated at 110° for 1 hr. Elution with benzene–ethyl acetate (85 : 15 v/v) or chloroform gave good separation of monoterpenes.

(*c*) Silica gel G and 12·5% aqueous silver nitrate gave good separations of terpene hydrocarbons [43] on elution with hexane–ethyl acetate (85 : 15 v/v).

(*d*) Silica gel G–anthracene was prepared as previously described,[44] and the plates were developed with the previous solvents for autoradiography of ^{3}H-labelled compounds.

(*e*) Merck precoated cellulose plates (0·1 mm.) were eluted with n-butanol–acetic acid–water (5 : 1 : 2 v/v), then at right angles with phenol–water (4 : 1 v/v), to give good separations of sugars, phosphates, and amino-acids.

(*f*) Alumina was slurried with water and the plates activated at 100° for 2 hr. Elution with ethyl acetate gave good resolution of carotenes and chlorophylls.

Terpenes were located with iodine vapour or by spraying with 15% ethanolic phosphomolybdic acid and heating at 100° for 2 min. Pigments were similarly treated, or located by their fluorescence in u.v. light. Amino-acids were detected with ninhydrin, and carbohydrates, phosphates, and organic acids with specific sprays.[45–48] Recoveries of greater than 95% could be achieved on the preparative scale. Columns (8 × ⅝ in. o.d.) packed with sucrose (top layer) and heavy magnesium oxide (1 : 3 w/w) were used for larger-scale preparations. Monoterpenes and β-carotene were eluted with hexane, when chlorophyll was absorbed on the sucrose and carotenoids were retained on the magnesium oxide. T.l.c. system (*b*) could be scaled up for column chromatography provided that ' t.l.c.' grade silica gel was used.

Incorporation of Tracer.—Carbon-14 dioxide was generated in a large bell jar by treatment of barium [^{14}C]carbonate (500 μc, 0·15 mmole) with lactic acid to give an atmosphere 0·04% v/v in the gas. However, stem-feeding of leaves (*ca.* 12 g.) with aqueous sodium [1-^{14}C]acetate (4 μc, 3·5 × 10^{-5} mmole, 0·01 ml.) under illumination (natural daylight + 60 w Mazda lamp with heat filter, at 2 ft.) and mild forced transpiration, led to better incorporation into the compounds of interest. The stem was cut under water, and after the tracer and 2 aliquots (each 0·1 ml.) of water had been absorbed (time required *ca.* 1 hr.), the leaves were maintained under outdoor conditions (March—October) either on Pfeffer's solution or on a medium containing ATP (0·1 mg. ml.$^{-1}$) and glucose (1 mg. ml.$^{-1}$). The latter is an adaption of a medium that stimulates uptake and incorporation of tracer into rubber in *Hevea brasiliensis*.[49] Media were sterilised by filtration through Millepore membranes (pore size 0·5 μ) and care was taken to minimise microbial contamination. Microscopic examination of sections from several stems, roots and leaves revealed no sign of fungal or protozoal symbionts.

At predetermined intervals, leaves were ground with liquid nitrogen and the residue was extracted with hexane (3 ml.) at −10° to remove monoterpenes and β-carotene: in some experiments commercial Tansy oil (20 μl.) or individual terpenes (2 μl.) were added as carriers. Extraction with acetone removed terpenes and all the pigments. The hexane solution was separated on t.l.c. system (*a*) when monoterpenes and β-carotene had R_F from 0·0 to 0·5. The fast-moving bands were eluted from the stationary phase with acetone, and rerun on systems (*a*), (*b*), or (*c*) with authentic compounds as markers. In some cases the eluted fractions were submitted to preparative g.l.c. The final products were homogeneous on analytical g.l.c. and t.l.c. and corrections were made for losses during the purification procedures. No carbohydrates or organic acids could be detected on the chromatograms of the initial hexane extract, and ninhydrin-positive material formed only a faint trace at the base-line. No general spray for lipids is available but, except for the presence of β-carotene, the leaf extract showed an identical pattern on t.l.c. as that of the steam-volatile oil which is known [16] to contain only mono- and sesqui-terpenes.

Less conveniently, chlorophyll and carotenoids were qualitatively extracted from the debris after pulverisation by stirring for 2 hr. with petroleum (b.p. 40—60°–benzene–methanol (0·9 : 0·1 : 0·3 v/v; 1·3 ml.), and resolved by t.l.c. system (*b*) or by column chromatography.[50] Chlorophyll and β-carotene were estimated by their absorption at

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652 and 418 mμ.[51] Phytol was cleaved from the chlorophyll with methanolic lithium hydroxide and purified by t.l.c. systems (*b*) and (*e*).

^{14}C-Incorporation was qualitatively measured by autoradiography of developed t.l.c. plates using Kodirex *X*-ray film (Kodak), with an exposure time of up to 10 weeks. Uptake of ^{3}H was revealed by similar autoradiography of plates from t.l.c. system (*d*) at −78° with an exposure time of 1 day.[44] Routine investigations were made with a Tracerlab radiochromatography scanner, model 5C-525B, which had counting efficiencies of *ca.* 15% for ^{14}C and 4% for ^{3}H, and with a Pye Radiochromatograph. A Packard Tricarb liquid scintillation spectrometer, model 3315, was used for quantitative assays with aliquots (10 ml.) of 2,5-diphenyloxazole (3% w/w) and 1,4-di-[2-(5-phenyloxazolyl)]benzene (0·3% w/w) in toluene as a scintillator; and sometimes a Beckman instrument with Butyl-PBD (Ciba), 0·8% w/w, in toluene was also employed. Counting efficiencies were about 97 and 75% for ^{14}C and ^{3}H in the latter system. Bray's solution [52] was used as a scintillator for aqueous solutions, when the presence of water (10% v/v) resulted in counting efficiences of 66 and 12% for ^{14}C and ^{3}H respectively. Corrections were made to allow for the heavy quenching caused by ketones and pigments, or the former types of compound were reduced with lithium aluminium hydride before counting and the latter bleached in the counting vial by exposure to a Siemens MB/D 125 w u.v. lamp with the envelope removed. Each vial was assayed for a total of 10^{4} counts, unless very low radioactivity was present. Individual and specific activities are recorded in the Tables as formally measured and calculated, but the accuracy of the counting techniques is probably ±1% of the values recorded.

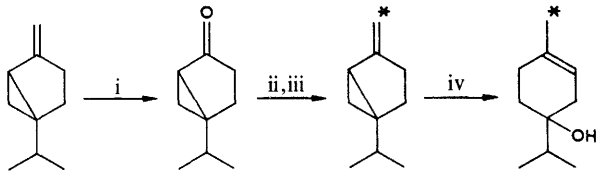
The loss of labelled carbon dioxide and terpenes from the leaves was measured using a previously described apparatus: [8] the former was trapped in ethanolamine and the latter were condensed at −78°. [2-^{14}C]Mevalonic acid was fed as previously described [23] and the labelled products were separated by t.l.c. and assayed by the use of the radiochromatograph scanner, or by the g.l.c. radiochromatograph.

Composition of the Oil.—Extracts were made from batches of 15 plants, each batch being of ages 3, 5, 10, 15, 20, 25, and 30 weeks old, and all being harvested in September. After pulverisation in liquid nitrogen and extraction, the residue of the leaves was steam-distilled and the ethereal extract of the distillate was pooled with the hexane extract. Components were characterised by their relative retention times on g.l.c. columns (25 ft. × ¼ in. o.d.) of Carbowax 20M, silicone oil SE30, dinonyl phthalate, and ββ′-oxydipropionitrile. Major components were isolated from the pooled batches by preparative g.l.c. and identified by i.r. spectroscopy. The response of the chromatograph detector was calibrated for each compound and the quoted figures have a standard deviation of about ±0·5% (actual value).

Synthesis and Feeding of Labelled Monoterpenes.—[^{3}H]Geranyl pyrophosphate (2·4 × 10^{3} c.p.m. mg.$^{-1}$, unspecifically labelled) was kindly donated by Dr. D. Barnard (Natural Rubber Producers' Research Assocn., Welwyn Garden City). ^{14}C-Labelled compounds (10—40 mg.) with specific activities in the range 820—8500 c.p.m. mg.$^{-1}$ after dilution with carrier, were isolated from the oils of appropriate species that had been fed on sodium [1-^{14}C]-acetate and maintained on nutrient medium for 8 days. *Juniperus sabina* L. gave sabinene (0·008% tracer incorporation) and sabinyl acetate (0·018% incorporation); terpinen-4-ol was synthesised from the former.[53] *Thuja plicata* L. gave thujone (0·007%) which was partly reduced to a mixture of thujols, that gave, in turn, α-thujene on dehydration.[20] *Pinus attenuata* L. gave α-pinene (0·004%) which was converted into α-terpineol by a standard route.[54] *Pelargonium graveolens* Ait. gave geraniol (0·02%) which was isomerised to linalool.[53] *Tanacetum vulgare* L. gave isothujone containing a few percent of thujone (0·002%). Solutions of the labelled terpenes (1—5 mg.) and ATP (1·0 μg.) in 90% aqueous dimethyl sulphoxide (0·1 ml.) were wick-fed into the stem of potted or cut specimens of *T. vulgare*, of total weight *ca.* 12 g. The plants were then maintained in nutrient solution for 48—100 hr. and the oil was harvested [sometimes with the addition of commercial oil (20 μg.) or individual terpenes (2 μg.) as carriers] and pure samples of representative monoterpenes and pigments were isolated and assayed for tracer. To complete the isolation of terpene alcohols, the small quantities of terpenyl pyrophosphates were extracted with water (5 × 0·5 ml.) from the pulversied debris after hexane extraction, hydrolysed,[55] and added to the hexane extract.

A series of exploratory feedings using [^{14}C]geraniol showed that omission or increase of ATP, the use of other solvents, the use of emulsions with sapogenins, sodium lauryl sulphate, or bile salts, or application by injection, irrigation, spraying, or wounding techniques such as have been reported for the introduction of tracer, either substantially reduced the healthy life of the plant or resulted in poorer incorporation into the terpenoids.

Synthesis and Feeding of [^{14}]*Terpin-4-ol of High Specific Activity.*—Sabinene (500 mg.) isolated from oil of Savin [20] was converted into [^{14}C]terpinen-4-ol by the sequence in Scheme 2 using known reactions.[20,53] The product (4 mg.)



SCHEME 2 Synthesis of [^{14}C]terpinen-4-ol; * denotes ^{14}C. i, $KMnO_4$, 25°; ii, $^{14}CH_3I$, Mg, 0°; iii, 0·1N-H_2SO_4, 20°; iv, 1·0N-H_2SO_4, 30°.

was homogeneous on analytical g.l.c. and t.l.c. and had 6·6 × 10^{4} c.p.m. mg.$^{-1}$. A modified procedure was applied to isolate labelled products 44 hr. after feeding. The pulverised leaves were extracted with cold (−10°) pentane (5 × 5·0 ml.) to give fraction A, containing monoterpenes and β-carotene. The residual tissue was then refluxed for 3 hr. with acetone to give a green solution containing the remainder of the pigments and a trace of monoterpenes; after removal of most solvent the extract was partitioned between pentane (fraction B) and water (fraction C). The remaining solid tissue was treated with 15% alcoholic potassium hydroxide at 25° for 3 hr. to give a brown liquid, fraction D, and a residue, fraction E. Fraction A contained no sugars or amino-acids, and after isotopic dilution was chromatographed on t.l.c. systems (*a*) and (*b*),

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and on sucrose–magnesium columns. Fraction B contained all the pigments except β-carotene and was separated on t.l.c. system (*f*); the chromatogram was eluted in bands after u.v. mapping of the various pigments. Fraction C contained amino-acids, carbohydrates, and minor amounts of pigments, and was chromatographed on t.l.c. system (*e*): the chromatogram was divided into 5 cm. squares and each was eluted and assayed for tracer. Fraction D also contained amino-acids and carbohydrates and was similarly assayed after chromatography on t.l.c. systems (*e*) and (*f*). Fraction E was dried at 100° and was counted as a suspension in Packard thixotropic gel.

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