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BIOSYNTHESIS OF SQUALENE AND OTHER TRITERPENES IN *MENTHA PIPERITA* FROM MEVALONATE-2-^{14}C*

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Key Word Index—*Mentha piperita*, Labiatae, peppermint, squalene, triterpenes, biosynthesis from mevalonate-2-^{14}C, degradation of squalene-^{14}C, dimethylallyl pyrophosphate metabolic pools

Abstract—Unlike mono- and sesqui-terpenes, squalene and other triterpenes in peppermint readily incorporate mevalonate-2-^{14}C label (greater than 30% incorporation of *R*-mevalonate in 4 hr) The labelled squalene produced turns over rapidly Squalene derived from mevalonate-2-^{14}C in incorporation times of 1, 4 and 7 hr was degraded chemically and shown to be equivalently labelled, according to theory, in the isopentenyl pyrophosphate-derived and dimethylallyl pyrophosphate-derived portions of the molecule This contrasts with earlier studies on the biosynthesis of mono- and sesqui-terpenes in peppermint from ^{14}C-precursors, in which the isopentenyl pyrophosphate-derived portions of the terpene molecules were found to be preferentially labelled, suggesting the presence of endogenous dimethylallyl pyrophosphate pools The kinetics of squalene biosynthesis, and the labelling pattern of squalene, suggest that sites of triterpene biosynthesis are readily accessible to exogenous mevalonate and that endogenous dimethylallyl pyrophosphate pools do not participate in triterpene biosynthesis to any appreciable extent The triterpene biosynthetic sites in peppermint thus appear to differ significantly from the monoterpene and sesquiterpene biosynthetic sites

INTRODUCTION

EVIDENCE to date suggests that compartmentalization of metabolism is an important feature of terpenoid biosynthesis [1-3] Thus, for example, terpenoid compounds of chloroplasts (except sterols) appear to be synthesized preferentially from endogenous MVA‡ produced from products of photosynthesis, while sterols and certain non-chloroplastidic terpenoids incorporate exogenous MVA readily [1] In peppermint (*Mentha piperita* L) there is evidence suggesting that monoterpenes and sesquiterpenes, both components of the essential oil, are synthesized in separate and distinct compartments [3-5] Monoterpenes incorporate label from $^{14}CO_2$ or ^{14}C-sugars much more readily than from MVA-2-^{14}C Sesquiterpenes incorporate label from MVA-2-^{14}C readily, though still in rather low yield In no case did the incorporation of ^{14}C-precursors into mono- or sesqui-terpenes in peppermint exceed a few per cent

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‡*Abbreviations used* MVA—mevalonic acid, IPP—isopentenyl pyrophosphate, DMAPP—dimethylallyl pyrophosphate

[1] ROGERS, L J , SHAH, S P J and GOODWIN, T W (1968) *Photosynthetica* **2**, 184

[2] LOOMIS, W D (1967) in *Terpenoids in Plants* (PRIDHAM, J B , ed), p 59, Academic Press, London

[3] LOOMIS, W D and CROTEAU, R (1973) in *Recent Advances in Phytochemistry* (RUNECKLES, V C , ed), Vol 6, Academic Press, New York

[4] CROTEAU, R and LOOMIS, W D (1972) *Phytochemistry* **11**, 1055

[5] CROTEAU, R , BURBOTT, A J and LOOMIS, W D (1972) *Phytochemistry* **11**, 2459

The sites of mono- and sesqui-terpene biosynthesis appear to be characterized by largely fermentative metabolism, and by energy deficiency Thus co-administration of sucrose or of CO_2 in the light, enhances the utilization of MVA-2-^{14}C for biosynthesis of both mono- and sesquiterpenes in peppermint[6] Perhaps the most unexpected finding has been the preferential incorporation of exogenous ^{14}C-labeled precursors into the IPP-derived portions of monoterpenes[3,5,7,8] and sesquiterpenes[3,4] This is observed even with $^{14}CO_2$ as substrate[5,8] suggesting the existence of endogenous DMAPP pools at the sites of terpene synthesis As both mono- and sesqui-terpenes are found within peppermint oil glands,[9-12] it is likely that the oil glands function as the primary sites of compartmentalization and that within the glands monoterpene biosynthesis and sesqui-terpene biosynthesis are isolated to different degrees[3] The possible occurrence of mono- and sesqui-terpenes in other cells outside of the oil glands, has been suggested[3,13] but not established

It has been shown that triterpene synthesis in several plant tissues is readily accessible to exogenous MVA-^{14}C,[1] and previous studies have suggested that MVA-^{14}C is a more efficient precursor of sterols than of monoterpenes in peppermint[14] As farnesyl pyrophosphate is a precursor of triterpenes, and presumably also of sesquiterpenes, it seemed possible that sesquiterpene biosynthesis might not be as closely related to monoterpene biosynthesis as has been supposed but might rather be associated with triterpene synthesis taking place at more accessible sites elsewhere in the plant If sesquiterpene biosyntnesis and triterpene biosynthesis are closely associated in plants, then the incorporation of MVA-2-^{14}C into squalene and sesquiterpenes should show similar kinetics and labelling patterns

Although the biosynthesis of triterpenes in higher plants has received considerable attention, relatively little information is available on the *in vivo* turnover of squalene, or on the *in vivo* labelling pattern of squalene derived from MVA-2-^{14}C Nicholas[15] showed that in cut sections of *Ocimum basilicum* squalene incorporated much more MVA-2-^{14}C label than did unidentified steam volatile substances, and the labelled squalene turned over rapidly (80% turnover in 3 hr) The complete *in vivo* labelling pattern of squalene in plants has not, to our knowledge, been determined, presumably because there was no previous reason to believe that it might differ from the pattern produced by animal systems or by cell-free plant systems For example, Capstack *et al*.,[16] carried out a partial degradation of squalene derived from MVA-2-^{14}C in peas (the carbon atoms that would have become labelled via DMAPP were not examined) and assumed that the labelling pattern of squalene in flowering plants was the same as that in animals (i e equivalent labelling of IPP- and DMAPP-derived moieties) Rees *et al*.[17] degraded β-amyrin produced by pea seedlings from 2-^{14}C-4*R*-4-^{3}H-MVA Six tritium atoms were shown to be incorporated, as predicted by theory,

[6] CROTEAU, R, BURBOTT, A J and LOOMIS, W D (1972) *Phytochemistry* **11**, 2937
[7] BANTHORPE, D V, CHARLWOOD, B V and FRANCIS, M J O (1972) *Chem Rev* **72**, 115
[8] WUU, T and BAISTED, D J (1973) *Phytochemistry* **12**, 1291
[9] HEFENDEHL, F W (1967) *Naturwissenschaften* **54**, 142, (1968) *Riechstoffe Aromen Koerperpflegem* **18**, 523, *Chem Abstr* **71**, 53443
[10] STICHER, O and FLUCK, H (1968) *Pharm Acta Helv* **43**, 411
[11] AMELUNXEN, F, WAHLIG, T and ARBEITER, H (1969) *Z Pflanzenphysiol* **61**, 68
[12] MALINGRE, T M, SMITH, D and BATTERMAN, S (1969) *Pharm Weekblad* **104**, 429, *Chem Abstr* **71**, 53444
[13] AMELUNXEN, F (1967) *Planta Med* **15**, 32
[14] BATTU, R G and YOUNGKEN, H W (1968) *Lloydia* **31**, 30
[15] NICHOLAS, H J (1962) *J Biol Chem* **237**, 1485
[16] CAPSTACK, JR, E, ROSIN, N, BLONDIN, G A and NES, W R (1965) *J Biol Chem* **240**, 3258
[17] REES, H H, BRITTON, G and GOODWIN, T W (1968) *Biochem J* **106**, 659

and five of these were shown to be in the theoretically predicted positions Thus, an endogenous DMAPP pool did not appear to operate in β-amyrin synthesis in peas The indication that endogenous DMAPP pools operate in mono- and sesqui-terpene biosynthesis, however, prompted us to examine the biosynthesis of triterpenes in an essential oil-producing plant The following study was thus undertaken to establish the kinetics of triterpene biosynthesis, to determine the labelling pattern of squalene derived from MVA-2-^{14}C *in vivo*, and to relate triterpene biosynthesis to mono- and sesqui-terpene biosynthesis in peppermint

RESULTS

Determination of Hexane-Soluble Triterpenes in Peppermint Cuttings

Peppermint cuttings (250–350 mg fr wt per cutting) were exhaustively extracted with hexane,* and an aliquot of the extract was analyzed by GLC to determine the approximate monoterpene (3000 nmol per cutting) and sesquiterpene (50 nmol per cutting) content The remaining extract was taken to dryness to remove volatile substances and then separated by TLC into several fractions (hydrocarbon, pentacyclic triterpene alcohol, sterol, origin) The hydrocarbon fraction was analyzed by GLC and shown to contain roughly 10 nmol of squalene per cutting The triterpene alcohol fraction was further purified by TLC and then analyzed by GLC and shown to contain α-amyrin (ursane series) at a level of approximately 1 nmol per cutting Similar analysis of the sterol fraction indicated the presence of sitosterol (about 50 nmol per cutting) with minor amounts of other unidentified sterols The 'origin fraction' from the first TLC separation was rechromatographed using a more polar developing solvent, and a triterpene acid fraction was isolated Methylation followed by GLC analysis indicated the presence of ursolic acid with a minor amount of oleanolic acid (total of 5 nmol per cutting) Other triterpene derivatives (e g sterol esters) were not examined in this study

Mevalonate-2-^{14}C as a Precursor of Triterpenes

Previous studies had shown that, although MVA-2-^{14}C was not incorporated very efficiently into mono- or sesqui-terpenes of peppermint cuttings,[4] it was converted in good yield into other hexane-soluble materials (up to 50% of applied *R*-MVA-^{14}C activity was hexane-soluble after a 2-hr period, and 80% was hexane-soluble after 10 hr[3]) Preliminary studies showed that in periods up to 10 hr virtually none of this hexane-soluble radioactivity could be attributed to saponifiable lipids, suggesting that MVA was not extensively degraded, with resultant randomization of label, but was incorporated directly into hexane-soluble terpenoid compounds. It was presumed that at least part of this radioactive hexane-soluble material was triterpenoid in nature To test this possibility, peppermint cuttings were administered MVA-2-^{14}C through the cut stems in the light, and, after appropriate time intervals, the hexane-soluble material was extracted from each cutting Carrier triterpenes (squalene, sitosterol, α-amyrin and oleanolic acid) were added to each extract, and each extract was then separated into several fractions (hydrocarbon, pentacyclic triterpenol, sterol, origin) by TLC The hydrocarbon fraction was analyzed by liquid scintillation counting and by gas radiochromatography, and the radioactivity of squalene determined. Gas

* Hexane has been used as the extracting solvent in all of our previous studies[3] and was employed again in this instance to provide comparable data on 'hexane-soluble radioactivity' It should be noted, however, that many oxygenated triterpenes are only sparingly soluble in hexane, so quantitative data for the oxygenated triterpenes should be regarded as minimum values

radiochromatography also showed that long-chain paraffins (e g C_{27}, C_{29}) in this fraction were not labelled from MVA-2-^{14}C The pentacyclic triterpenol and sterol fractions were further purified by TLC, and the radioactivity in these fractions was determined by liquid scintillation counting The origin fraction from the first TLC separation was rechromatographed (TLC) using a more polar solvent, and a triterpene-acid fraction was isolated After methylation, the radioactivity in this fraction was determined by liquid scintillation counting Time-courses of incorporation of MVA-2-^{14}C label into squalene, sterols, pentacyclic triterpenols and triterpene acids are shown in Fig 1

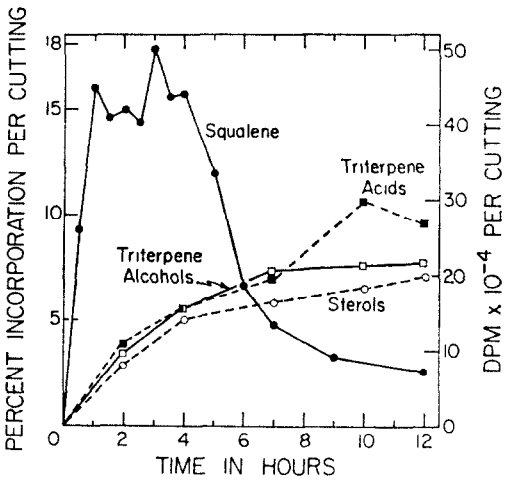


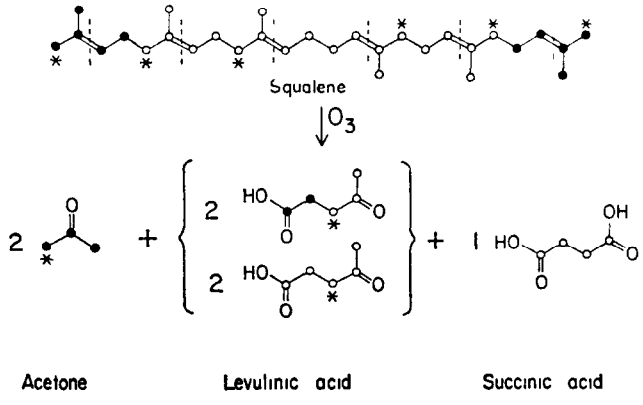
FIG 1 TIME-COURSE OF LABELLING OF TRITERPENES AND STEROLS IN PEPPERMINT FROM MVA-2-^{14}C Data presented for squalene and sterols are from the same experiment, data for triterpene acids and alcohols are from a second, identical, experiment Both experiments used matched cuttings fed 0 4 μmol RS-MVA-2-^{14}C per cutting Squalene was determined by gas radiochromatography, and pentacyclic triterpene alcohols, sterols and triterpene acids by liquid scintillation counting of TLC isolates Time is from start of MVA feeding, and percent incorporation is calculated by assuming that only R-MVA is physiologically active

Chemical Degradation of Squalene Derived from MVA-2-^{14}C

To determine if endogenous DMAPP pools participate in the biosynthesis of triterpenes, squalene biosynthesized from MVA-2-^{14}C was degraded, and the labelling pattern of IPP- and DMAPP-derived units was examined Peppermint cuttings were fed MVA-2-^{14}C through the cut stems, and after 1, 4 and 7 hr from the start of feeding several cuttings were removed and extracted with hexane Carrier squalene was added to each extract, and the hydrocarbon fraction was isolated by TLC Radioactive squalene was isolated from the hydrocarbon fraction by semi-preparative GLC, and, after the further addition of carrier squalene, the specific activity was determined by gas radiochromatography of an aliquot The remaining squalene was then ozonized and oxidatively cleaved to yield acetone, levulinic acid and succinic acid[18] (Scheme 1) Acetone was analyzed as the oxime by gas radiochromatography, while levulinic and succinic acids were converted to the propyl esters before gas radiochromatographic analysis The results of this study are shown in Table 1

[18] RILLING, H C and BLOCH, K (1959) *J Biol Chem* **234**, 1424

and indicate that in all time periods studied, squalene is equivalently labelled (within experimental limits), according to theory, in the IPP-derived and DMAPP-derived units Although the ozonolysis products, acetone, levulinic acid and succinic acid, were not degraded further, the distribution of label among these products is consistent with equivalent labelling, and there is no evidence to suggest either preferential labelling of IPP-derived moieties or extensive randomization of label The small amount of radioactivity in succinic acid probably results from a minor degree of oxidation of levulinic acid to succinic acid during the degradative procedures [18]



SCHEME 1 CHEMICAL DEGRADATION OF SQUALENE THEORETICALLY DERIVED FROM MVA-2-^{14}C Labelled carbon atoms theoretically derived from MVA-2-^{14}C by direct utilization are identified by asterisks Open circles represent carbon atoms derived from IPP Closed circles represent carbon atoms derived from DMAPP Dotted lines indicate ozonolysis cleavage points

DISCUSSION

The incorporation of MVA-^{14}C into triterpenes of peppermint is much greater than into mono- and sesqui-terpenes (30% of the *R*-isomer for triterpenes vs $< 1\%$ for mono- and sesqui-terpenes), although there is much less triterpene per peppermint cutting than mono- and sesqui-terpene Squalene incorporated label very rapidly for about 1 hr, maintained a nearly constant proportion of label (15% incorporation of R-MVA) for 3 hr, and then lost label rapidly (80% turnover in 8 hr) A similar rapid turnover of squalene was observed by Nicholas in cut sections of *Ocimum basilicum* fed MVA-^{14}C [15] In *Ocimum* cuttings, unidentified ^{14}C-labelled volatile substances (presumably mono- and sesqui-terpenes) also turned over very rapidly In peppermint, the oxygenated triterpenes became labelled more slowly than squalene, each oxygenated fraction acquiring about 5% of administered R-MVA-2-^{14}C label at 4 hr and continuing to acquire label at a slower rate thereafter The kinetics of labelling of squalene and of the other triterpenes are consistent with the precursor-product relationship expected for these compounds However, within the oxygenated triterpene group, the labelling kinetics of the alcohols and the acids show no apparent precursor-product relationships (e g as might be expected for α-amyrin and ursolic acid)

As the various C_5 units of squalene are equivalently labelled, or nearly so, it appears that DMAPP pools do not participate to any great extent in the biosynthesis of triterpenes in peppermint If DMAPP pools do participate in triterpene synthesis, these pools must be small relative to the amounts of triterpene synthesized, and must turn over rapidly The

slight deviation of observed label distribution in squalene from the theoretical distribution (Table 1), if real, is consistent with the latter interpretation The observed deviation from theory is less than the limits of resolution of our methods (less than $\pm 10\%$), but the consistency of the deviation suggests that it may be real In any case, the results described here suggest that triterpene biosynthesis in peppermint is not closely related to either monoterpene or sesquiterpene biosynthesis Certainly the bulk of triterpene synthesis does not appear to be as tightly compartmentalized as the biosynthesis of the lower terpenes That the sites of triterpene and sesquiterpene synthesis (and the farnesyl pyrophosphate precursor pools of these compounds) cannot be the same is apparent from the fact that equivalently labelled squalene has largely turned over at the time that sesquiterpene preferentially labelled in the IPP-derived portion, reaches maximum incorporation of MVA-2-$^{14}C^{4}$ (i e after a 6 hr incorporation period) A similar argument could be made regarding triterpene vs monoterpene biosynthetic sites

TABLE 1 DISTRIBUTION OF LABEL IN SQUALENE BIOSYNTHESIZED FROM MVA-2-^{14}C AFTER VARIOUS INCORPORATION PERIODS

Component isolated (time in hr)	Specific activity (dpm/μmol, 10^{-4})	Observed	Total radioactivity (%)* Theoretical Direct incorporation†	Randomized‡
Squalene			100	100
1 hr	29 4	100		
4 hr	33 1	100		
7 hr	23 5	100		
Acetone (as oxime)			33 3	20
1 hr	4 64	31 6		
4 hr	5 35	32 4		
7 hr	4 16	35 4		
Levulinic acid (as propyl ester)			66 7	66 7
1 hr	5 09	69 2		
4 hr	5 66	68 4		
7 hr	4 02	68 4		
Succinic acid (as dipropyl ester)			0	13 3
1 hr	0 53	1 8		
4 hr	0 70	2 1		
7 hr	0 26	1 1		

* (Specific activity of product isolated $\times$ mol of product per mol of squalene/specific activity of squalene) $\times$ 100 Each mol of squalene yields 2 mol acetone, 4 mol levulinic acid and 1 mol succinic acid

† Assuming direct incorporation of MVA-2-^{14}C, with equivalent labelling of IPP- and DMAPP-derived units

‡ Assuming label from MVA-2-^{14}C is completely randomized throughout the squalene molecule

Earlier,[19] incidentally to studies of monoterpene biosynthesis, we observed incorporation of label from MVA-2-^{14}C into material that travelled with the solvent front on TLC plates

[19] BATTAILE, J and LOOMIS, W D (1961) *Biochim Biophys Acta* **51**, 545

Since this area was conspicuously yellow-pigmented, we concluded that carotenes had become labelled The present results suggest that most of the label observed in this hydrocarbon fraction was in fact in squalene This interpretation is consistent with the observed compartmentalization of carotenoid biosynthesis within chloroplasts [1]

The results of this and other studies[3-5] suggest rapid synthesis and turnover of terpenoids at the squalene/triterpene sites, with no apparent DMAPP pools, contrasted with slower synthesis and turnover, and DMAPP pools, at the more compartmentalized (but separate) monoterpene and sesquiterpene sites Thus, the picture that emerges from tracer investigations of terpenoid biosynthesis in peppermint is one of multiple compartmentalized sites, each site producing characteristic terpenoid compounds and having its own unique physiology

EXPERIMENTAL

Plant material Peppermint plants were the Black Mitcham cultivar of *Mentha piperita* L , propagated vegetatively from the clone used previously,[19] in a growth chamber maintained at 24° day temp and 10° night temp during a regular 24-hr cycle with 16-hr/day under 10 500–11 000 lx light intensity, as determined with Se photocell [20,21] Illumination was from Sylvania VHO Gro-Lux and Wide-Spectrum Gro-Lux lights in equal numbers Cuttings, consisting of the tuft of youngest leaves at the growing tip plus the next 2 leaf pairs, were taken in the morning 2 5 hr after the beginning of the light period Stems were cut under H_2O and the cuttings were carefully tested before feeding began to insure that they were able to take up H_2O actively Fresh weights of cuttings were between 250 and 350 mg, and cuttings were matched visually as closely as possible In spite of these precautions, some variability is unavoidable, especially when comparing separate experiments that may have been done several months apart

Isolation and fractionation of unlabelled hexane-soluble material Ten cuttings were exhaustively extracted with hexane in the presence of anhyd Na_2SO_4 An aliquot of this extract was decolorized with Norit A activated charcoal and analyzed by GLC[4] to determine the monoterpene (3000 nmol per cutting) and sesquiterpene (50 nmol per cutting) content The remaining extract was taken to dryness under N_2 to remove volatile substances The yellow, waxy residue was taken up in Et_2O and separated by TLC (with standards) on silica gel G (20 cm × 30 cm × 0 3 mm) using C_6H_6–Et_2O (7 3) [22] The developed plate was dried, sprayed with minimum rhodamine 6G (0 05% in EtOH) and the separated bands visualized under UV light The 'hydrocarbon band' (R_f 0 9) was scraped from the plate and extracted with dry Et_2O GLC analysis showed this fraction to contain squalene (10 nmol per cutting) and small quantities of long chain paraffins (e g C_{27}, C_{29}), presumably from the cuticle The 'pentacyclic triterpenol band' (R_f 0 25), 'sterol band' (R_f 0 15) and 'origin band' were removed from the plate and extracted with dry Et_2O Each fraction was then streaked on another silica gel G plate (20 cm × 30 cm × 0 3 mm) which was developed with $CHCl_3$–EtOAc (1 1) [22] The pentacyclic triterpenol band was again located with rhodamine 6G (R_f 0 60), scraped from the plate and eluted with Et_2O GLC analysis indicated the presence of α-amyrin (*ca* 1 nmol per cutting) The sterol band (R_f 0 55) was similarly isolated and analyzed by GLC, and shown to contain primarily sitosterol (50 nmol per cutting) with minor amounts of other unidentified sterols The origin band from the first plate yielded a triterpene acid fraction (R_f 0 2) on rechromatography with $CHCl_3$–EtOAc Methylation with ethereal CH_2N_2 followed by GLC analysis indicated the presence of ursolic acid with a minor amount of oleanolic acid (total of 5 nmol per cutting)

Administration of mevalonate-2-^{14}C For all experiments *RS*-MVA-2-^{14}C (*N,N'*-dibenzylethylene-diamine salt) obtained from New England Nuclear Corp , Boston, Massachusetts, was employed This substance was reported by the manufacturer to possess a specific activity of 6 33 μCi/μmol and a radiochemical purity greater than 99% as determined by PC For time-course studies, cuttings were placed in vials in a small growth chamber under day conditions as described above and given an aq solution of *RS*-MVA-2-^{14}C (2 5 μCi, 0 4 μmol, in 0 05 ml) through the cut stems After the uptake of labelled material (30–50 min) the vials were kept filled with dist H_2O At appropriate time intervals cuttings (in triplicate) were removed, and the hexane-soluble material was immediately extracted For squalene degradation studies at various time periods, incorporation of MVA-2-^{14}C was carried out exactly as described above and cuttings were pooled according to the following schedule 1 hr, 2 cuttings, 4 hr, 2 cuttings, 7 hr, 3 cuttings

Isolation and fractionation of ^{14}*C-labelled hexane-soluble material* Hexane-soluble substances from MVA-2-^{14}C-fed cuttings were analyzed as described above for unlabelled material, except that carrier

[20] BURBOTT, A J and LOOMIS, W D (1967) *Plant Physiol* **42**, 20
[21] BATTAILE, J , BURBOTT, A J and LOOMIS, W D (1968) *Phytochemistry* **7**, 1159
[22] HOLLOWAY, P J and CHALLEN, S B (1966) *J Chromatog* **25**, 336

squalene (40 μg per cutting), sitosterol, α-amyrin and oleanolic acid (50 μg of each per cutting) were added to each hexane extract before fractionation The ^{14}C-labelled hydrocarbon fraction was analyzed by gas radiochromatography, while the ^{14}C-labelled pentacyclic triterpenol, sterol, and triterpene acid fractions were analyzed by liquid scintillation counting

GLC analysis of hexane-soluble components and squalene degradation products Unlabelled hexane-soluble components were analyzed using a Perkin-Elmer 990 GLC equipped with F I D Mono- and sesqui-terpenes were separated on a 6 1 m × 3 175 mm column packed with 1% phenyldiethanolamine succinate—1 5% sucrose acetate isobutyrate (PDEAS-SAIB) on 100–120 mesh Chromosorb G, programmed from 125 to 165° at 1°/min with N_2 flow rate of 30 ml/min The mono- and sesqui-terpene content of peppermint oil and further details of analysis are described elsewhere [4,5] The hydrocarbon fraction was analyzed on a 46 cm 3 175 mm column packed with 3% SE30 (methyl silicone oil) on 70–80 mesh Chromosorb G and operated at 215° with N_2 flow rate of 35 ml/min The pentacyclic triterpenol, sterol and triterpene acid (methyl ester) fractions were also analyzed on this SE30 column, at 250 A 61 cm 3 175 mm column containing 3% SF96 on 100–120 mesh Chromosorb G was also used in analyzing the four triterpene fractions All columns were stainless steel Squalene, α-amyrin, sitosterol, ursolic acid and oleanolic acid (as methyl esters) were identified by co-chromatography with authentic standards on both the SE30 and SF96 columns A standard paraffin mixture[23] was also used in examining the hydrocarbon fraction Peak areas were measured with a Disc integrator, and quantitative analysis was made by comparison with standard curves obtained with pure compounds Several radioactive components were analyzed by GLC using a Beckman Thermotrac temperature programmer fitted with a Carle Micro-Detector (thermal conductivity) Radioactive squalene from the hydrocarbon fraction was analyzed on the SE30 column essentially as described above Acetoxime from the degradation of squalene was analyzed on the PDEAS-SAIB column operated at 100 with He flow rate of 30 ml/min Propyl levulinate and dipropyl succinate from the degradation of squalene were analyzed on the PDEAS-SAIB column at 175° with He flow rate of 30 ml/min Identities of squalene degradation products were confirmed by co-chromatography with authentic standards Peak areas were measured with a Disc integrator and quantitative analysis was made by comparison with standard curves obtained with pure compounds

Isotope analysis The pentacyclic triterpenol, sterol, and triterpene acid (methyl ester) fractions isolated by TLC were counted directly in a Packard Tri-Carb liquid scintillation spectrometer using a scintillation solvent composed of 0 4% 2,5-diphenyloxazole (PPO) and 0 003% *p*-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in toluene The system had an efficiency of 86% with a background of 23 dpm and a counting error of 3%. Labelled compounds exhibited no quenching at the concentrations employed, and well over 50 000 counts were accumulated for each sample Components separated by GLC (squalene, acetoxime, propyl levulinate and dipropyl succinate) were assayed for radioactivity with a Nuclear-Chicago Biospan 4998 continuous gas flow counter attached directly to the outlet of the GLC thermal conductivity detector The instrument was calibrated with toluene-^{14}C, and peak areas were determined with a Disc integrator Specific activities were determined on aliquots containing 5000–30 000 dpm and 0 1–0 5 μmol of sample Using this system specific activities could be determined routinely to within ±10% of true value

Purification of labelled squalene Hydrocarbon fractions were prepared from ^{14}C-labelled hexane extracts as described above About 0 2 mg of carrier squalene was added to each extract, and radioactive squalene was isolated by semipreparative GLC on the SE30 column under conditions described above [squalene was trapped off the exit port of the thermal conductivity detector in capillary tubes filled with glass wool wetted with hexane (70% yield, 100% radiopurity)] Another 0 7 mg of carrier was added and the specific activity of an aliquot (representing about 25% of the sample) was determined by gas radiochromatography

Ozonolysis of squalene The procedures followed were essentially those of Rilling and Bloch,[18] which minimize secondary oxidation of levulinic acid to succinic acid Squalene (< 1 mg) in hexane was ozonized for 3–5 min at 0° with an ozone–oxygen stream Acetic acid (0 1 ml) was added and ozonization continued for another 3 min A solution of 10% H_2O_2 (0 1 ml) was added to the reaction mixture which was shaken for 30 min at room temp Another portion of 10% H_2O_2 was then added with further shaking Finally, 0 5 ml H_2O_2 and 0 5 ml H_2O were added, and the reaction mixture was warmed to 70 for 30 min under a water-cooled reflux condenser The solution was then cooled, 0 5 ml H_2O was added, and the pH was adjusted to 5 with NaOH Acetone was distilled in an N_2 stream into hydroxylamine solution in a screw-top vial (5 ml H_2O, 5 ml EtOH, 1 5 g NH_2OH HCl and 3 g KOH) The vial was sealed, warmed, shaken, and held overnight This mixture was then diluted with 20 ml H_2O, and the acetoxime was extracted with Et_2O The extract was then dried over Na_2SO_4 and concentrated under N_2 for gas radiochromatography After the distillation of acetone, the original reaction mixture was made strongly acidic with 1 ml of 2 5 N H_2SO_4, and the excess H_2O_2 destroyed by adding $FeSO_4$ in the cold Succinic and levulinic acids were removed by exhaustive extraction with Et_2O The extract was dried over Na_2SO_4, filtered into a small flask, and brought to dryness under a stream of N_2 To the mixture of succinic and levulinic acids was added 1 ml BF_3 in *n*-propyl alcohol (14%, w/v) which was then refluxed for 15 min [24] The boiled mixture was cooled, 5 ml sat

[23] GASKIN, P, MACMILLAN, J, FIRN, R D and PRYCE, R J (1971) *Phytochemistry* **10**, 1155
[24] SALWIN, H and BOND, J F (1969) *J Ass Offic Anal Chem* **52**, 41

$(NH_4)_2SO_4$ solution added, and the propyl esters extracted with Et_2O The Et_2O solution was then dried and concentrated for gas radiochromatographic analysis The propyl esters, rather than the methyl esters, were employed in this instance because methyl levulinate (b p 196°) and dimethyl succinate (b p 195 9°) could not be adequately resolved by GLC

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