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THE BIOSYNTHESIS OF THE OXYGENATED MONOTERPENES IN MINT*

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Abstract—Radioactive acetate and mevalonate were found to be incorporated into the volatile oil of *Mentha piperita* L. var. *Mitcham* and *M. arvensis* L. var. *glabrata* Ray. ^{14}C-Labelled acetate, mevalonate and CO_2 were fed under light and dark conditions and the results indicate that acetate and mevalonate are incorporated *per se* and not via prior degradation to CO_2. Acetate-1- and 2-^{14}C and mevalonate-2-^{14}C were incorporated into the oil more slowly than $^{14}CO_2$ in the light; all terpenes studied including menthol and menthofuran showed radioactivity within 5 min of feeding $^{14}CO_2$.

Isolation of individual monoterpenes by GLC and determination of their specific activities upon feeding $^{14}CO_2$ for various periods of metabolism confirmed Reitsema's biosynthetic sequences (piperitenone→piperitone→menthone→menthol; piperitenone→pulegone→menthone and menthofuran) in its major aspects, but it could not be confirmed that piperitenone is the first isolable precursor of these two sequences. Also, isomenthone (which was not considered previously) was found to play a major role, and evidence suggesting the sequence: an unknown precursor→isomenthone→menthone→menthol was obtained.

INTRODUCTION

IT IS now generally accepted that in plants monoterpenes are derived from geranyl pyrophosphate, or a closely related isomer, via the mevalonic acid pathway.[1–4] This precursor, by a number of specific isomerizations, reductions, and oxidations[3,5] is then assumed to give rise to the large number of monoterpenes found in essential oils. The biosynthesis of a number of individual monoterpenes has been studied by means of tracer techniques by several workers.[6–8] Whereas these studies demonstrated the manner in which mevalonic acid or acetate is incorporated (the position of the label was determined by degradation of the terpene), they do not permit conclusions as to the sequence in which closely related terpenes are formed within the plant. A better understanding of such biosynthetic sequences is not only desirable as such, but would be of considerable value in chemo-taxonomic and phylogentic correlations.[9]

A biosynthetic sequence of formation of the oxygenated monoterpenes found in mint species was proposed by Reitsema.[10] In this scheme piperitenone (I) forms a common

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[1] J. H. RICHARDS and J. B. HENDRICKSON, In *Biosynthesis of Terpenes, Steroids and Acetogenins*, p. 113. Benjamin, New York (1964).
[2] H. J. NICHOLAS, In *Biogenesis of Natural Compounds*, p. 641. Macmillan, New York (1963).
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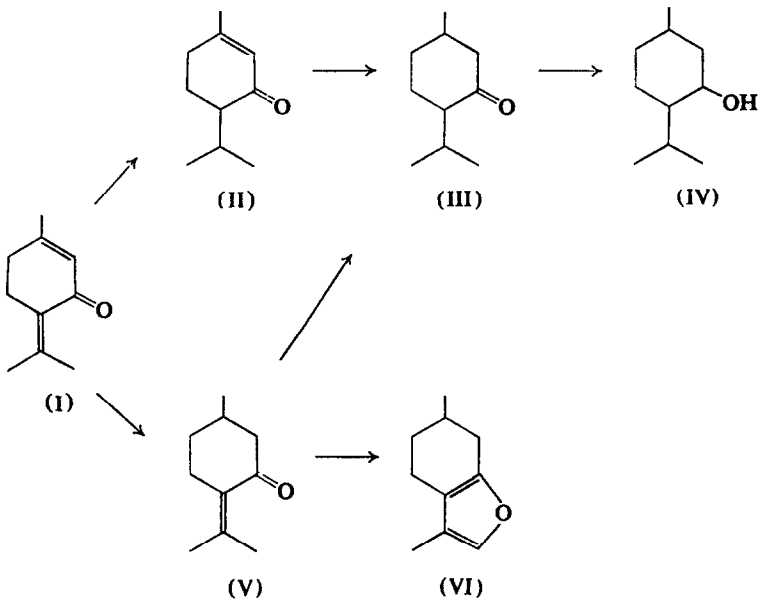
[4] F. LYNEN, B. W. AGRANOFF, H. EGGERER, U. HENNING and E. M. MÖSLEIN, *Angew. Chem.* **71**, 657 (1959).
[5] L. RUZICKA, *Experientia* **9**, 357 (1953).
[6] A. J. BIRCH, D. BOULTER, R. I. TRYER, P. J. THOMSON and J. L. WILLIS, *Tetrahedron Letters* No. 3, 1 (1959).
[7] W. SANDERMANN and W. SCHWEERS, *Tetrahedron Letters* 257, 259 (1962).
[8] R. G. STANLEY, *Nature* **182**, 738 (1958).
[9] E. VON RUDLOFF, *Phytochem.* **5**, 331 (1966).
[10] R. H. REITSEMA, *J. Am. Pharm. Assoc., Sci. Ed.* **47**, 267 (1958).

precursor to two sequences; (*a*) piperitenone→piperitone (II)→menthone (III)→menthol (IV) and (*b*) piperitenone→pulegone (V)→menthofuran (VI). Pulegone (V) can also give rise to menthone (III) and hence to menthol (IV).



Evidence in support of this sequence was put forward by Reitsema *et al.*[11] and also by Battaile and Loomis[12] on the basis of feeding experiments employing radioactive carbon dioxide. The radioactive terpenes formed were separated on thin-layer chromatograms (TLC) and detected by radioautography. This method suffers lack of sensitivity and calculation of specific activities is difficult and subject to large errors. Reitsema *et al.* point out that the method is not quantitative and that the presence of a labelled compound may indicate concentration rather than chronology.

To obtain more definite data in support of this biosynthetic sequence, both groups of workers employed infiltration techniques wherein a labelled member of the sequence was fed to the mint leaves and the conversion to radioactive products was followed by TLC-radioautograms. In this manner it could be shown that mint leaves can convert menthone to menthol,[11] pulegone to menthofuran and menthone, and piperitenone to piperitone.[12] However, the radioautograms showed that additional radioactive compounds were produced. Hence, it cannot be ruled out that secondary or reversible pathways exist. The problems arising from "feed-back" mechanisms were referred to by Birch.[3] Also, as pointed out by Reitsema *et al.*[11] the reactions brought about by such infiltration techniques need not necessarily be those taking place in the oil gland.

Another approach towards studying the formation of terpenes in mint oils was taken by Hefendehl[13] and by Höltzel.[14] The oil from leaves of different age and at different stages of growth was analysed by gas–liquid chromatography (GLC). Hefendehl could confirm the

[11] R. H. REITSEMA, F. J. CRAMER, N. J. SCULLY and W. CHORNEY, *J. Pharm. Sci.* **50**, 18 (1961).
[12] J. BATTAILE and W. D. LOOMIS, *Biochem. Biophys. Acta* **51**, 545 (1961).
[13] F. W. HEFENDEHL, *Planta Med.* **10**, 241 (1962).
[14] C. HÖLTZEL, Dissertation Univ. of Tübingen (1964).

findings of Reitsema[10] that the primarily secreted terpenes must be more unsaturated relative to the final products. The results of Hefendehl indicate also that a possible sequence of formation need not always proceed in the same manner, but different reactions may occur depending on the physiological state of the mint leaf.

Höltzel obtained data which suggest that two different pathways exist, one of which depends on the length of photoperiod. The sequence piperitenone→piperitone→menthone→menthol is reported to be operative only during long exposures to light (18-hr day; i.e. summer growth) whereas the transformation piperitenone→pulegone→menthofuran is apparently independent of the photoperiod. The reduction of pulegone to menthone as described by Battaile and Loomis could not be confirmed by Höltzel.

Since GLC permits quantitation and isolation of individual components, determination of specific activities may be readily achieved. Also, this technique is considerably more sensitive with regard to detection and separation. Thus, additional information on the biosynthesis of the terpenes in mint oils could be expected by combining this technique with determination of radioactivity by means of a scintillation counter. Therefore, it was decided to re-investigate this aspect of terpene biogenesis in *Mentha piperita* L. var. *Mitcham* and *M. arvensis* L. var. *glabrata* Ray,[15] keeping in mind two major objectives:

(1) to determine whether radioactive acetate and mevalonate are incorporated into mint leaf oils as such or via degradation to $^{14}CO_2$, and
(2) to study the terpene interconversions by feeding $^{14}CO_2$ and evaluating the relationships of the specific activities of the oxygenated monoterpenes.

RESULTS AND DISCUSSION

Feeding Radioactive Acetate and Mevalonate

Single shoots of different age of *Mentha arvensis* and *M. piperita* were fed with 5 μmoles sodium acetate 1-^{14}C per g fresh weight, which was found to be the optimum dose. After allowing the plants to metabolize the precursor for 24 hr, the volatile oil of separate leaf pairs was assayed for ^{14}C content. The specific activity of the oil of *M. arvensis* shoots was found to decrease continuously in going from the youngest to the oldest leaf pair (see Table 1). Noteworthy is the high total activity of the oil of the axillary shoots and the flowering buds. Experiments with *M. piperita* shoots led to similar conclusions, except that maximum total and specific activity appeared in the first insertion rather than the apex. In agreement with the findings of Reitsema *et al.*[11] and Battaile and Loomis,[12] it may be concluded that young shoots with four or five insertions below the apex represent the most suitable material for feeding experiments and shoots of this type (*ca.* 1–2 g fresh weight each) were used in subsequent experiments.

In order to ascertain the metabolic period required for maximum incorporation of the tracer into the volatile oil, sodium acetate-1-^{14}C was fed to shoots of both species and these were allowed to metabolize from 3 to 168 hr. Considerable variation was encountered in duplicate runs and a large number of experiments had to be carried out to arrive at more definite conclusions. Typical results obtained for the two species are shown in Table 2. The specific and total activity increased continuously to about 96 hr and then tended to level off.

Next, sodium acetate-1-^{14}C and -2-^{14}C and mevalonate-2-^{14}C were fed to the shoots and each was allowed to metabolize for 24 hr. Rather consistent results were obtained in this

[15] E. VON RUDLOFF and F. W. HEFENDEHL, *Can. J. Chem.* **44**, 2015 (1966).

TABLE 1. INCORPORATION OF ^{14}C FROM SODIUM ACETATE-1-^{14}C* INTO THE VOLATILE OIL OF DIFFERENT LEAF PAIRS OF *Mentha arvensis* VAR. *glabrata*

Leaf pair†	Young shoot			Shoot with flower buds		
	Weight of oil (mg)	Total ^{14}C (mμc)	Specific activity (mμc/mg)	Weight of oil (mg)	Total ^{14}C (mμc)	Specific activity (mμc/mg)
Top‡	0·23	2·55	11·1	0·21	2·26	10·8
1	0·54	3·13	5·8	0·37	1·65	4·5
2	0·87	2·53	2·9	0·44	1·52	3·5
3	0·61	0·68	1·1	0·53	0·59	1·1
4	0·48	0·17	0·35	0·18	0·31	1·7
5	0·21	0·09	0·42	—	—	—
Axillary shoots	0·72	7·64	10·6	0·78	12·04	15·4
Flower buds	—	—	—	0·66	13·84	20·9

* 26·4 μc of sodium acetate-1-^{14}C was fed each shoot. The plants were allowed to metabolize in continuous light for 24 hr from time of administering the tracer.

† Numbered from apex to base of shoot.

‡ Top refers to the group of three or four very small leaf pairs and apical meristem.

TABLE 2. INCORPORATION OF ^{14}C FROM SODIUM ACETATE-1-^{14}C* INTO THE VOLATILE OIL OF *M. arvensis* AND *M. piperita* AFTER VARYING PERIODS OF METABOLISM

Period of metabolism† (hr)	Fresh weight of plant (g)	Wt. of oil recovered (mg)	Specific activity (mμc/mg)	Total ^{14}C in oil (mμc)
M. arvensis				
Expt. A:				
3	3·2	25·6	0·42	10·7
6	3·2	18·5	0·92	17·1
12	3·0	14·5	2·21	32·0
24	2·7	20·5	3·59	73·6
48	3·2	22·5	4·54	102·2
Expt. B:				
48	2·3	14·9	6·30	93·9
96	2·2	16·7	8·40	140·5
M. piperita				
3	1·6	2·34	1·65	3·9
6	1·8	7·58	0·30	2·2
12	1·8	5·45	0·72	3·9
24	1·8	5·41	1·61	8·7
48	1·7	5·08	4·58	23·3
96	1·7	4·92	6·90	34·0
168	1·7	4·91	7·60	37·3

* 24 μc of sodium acetate-1-^{14}C per shoot were fed to *M. arvensis* L. var. *glabrata*; 22 μc of acetate-1-^{14}C per shoot were fed to *M. piperita* L. var. *Mitcham*. Total acetate fed to each shoot was 5 μmoles/g fresh weight.

† Period of metabolism was the time interval from commencement of tracer feeding until harvesting; metabolic period in continuous light.

series (see Table 3) and the efficiency with which these three compounds were incorporated is in agreement with the postulate that three acetate units combine to give mevalonic acid (with loss of one carbon atom) and this is then incorporated into the monoterpenes. If one allows that only *l*-mevalonic acid is used in terpenes synthesis, then mevalonate is incorporated about ten to twenty times as efficiently as is acetate-2- and 1-^{14}C, respectively. These findings are in good agreement with those reported by Birch *et al.*[6, 16] and Hefendehl,[17] but are in contrast with those of Battaile and Loomis,[12] who did not find mevalonate to be incorporated into the terpenes of mint oils.

TABLE 3. INCORPORATION OF ^{14}C-LABELLED ACETATE AND MEVALONATE INTO THE VOLATILE OIL OF *M. piperita* L. VAR. *Mitcham**

Compound fed	μmole	μc	Fresh wt. of plant (g)	Volatile oil recovered: Weight (mg)	Volatile oil recovered: Specific activity (mμc/mg)	Volatile oil recovered: Total activity (mμc)
Sodium acetate-1-^{14}C	8·0	22·6	1·6	3·74	5·3	19·9
	8·5	22·6	1·7	5·70	4·3	24·7
Sodium acetate-2-^{14}C	8·0	21·0	1·6	4·33	8·2	35·6
	8·0	21·0	1·6	2·90	13·4	38·9
Sodium mevalonate-2-^{14}C	10·9	18·3	1·6	4·50	36·3	163·0
	10·9	18·3	1·6	3·60	43·8	158·0
DBED-Mevalonate-2-^{14}C†	6·64	22·3	1·6	4·03	40·6	164·0

* The plants were allowed to metabolize the tracer in continuous light for 24 hr.
† N,N-Dibenzyl ethylene diamine salt of mevalonic acid.

Battaile and Loomis[12] report that on feeding mint shoots acetate-1-^{14}C radioactivity appeared only very slowly in the terpenes, but rapidly in respiratory carbon dioxide. They thought it likely that acetate was degraded to carbon dioxide and that radioactivity entered the terpenes via this carbon dioxide. To investigate this aspect further, radioactive carbon dioxide and acetate were fed to mint shoots both in light and in the dark (see Table 4).

TABLE 4. INCORPORATION OF ^{14}C-LABELLED SODIUM ACETATE AND CARBON DIOXIDE INTO THE VOLATILE OIL OF *M. piperita* L. VAR. *Mitcham* UNDER LIGHT AND DARK CONDITIONS

Conditions	Compound administered	μc fed	Fresh wt. of plant (g)	Total ^{14}C in volatile oil (mμc)
Light*	Carbon dioxide-^{14}C	93	1·1	1832
	Sodium acetate-1-^{14}C	22·6	1·0	6·1
	Sodium acetate-2-^{14}C	21·0	1·0	35·4
Dark†	Carbon dioxide-^{14}C	93‡	1·1	0·7
	Sodium acetate-1-^{14}C	22·6	1·1	5·1
	Sodium acetate-2-^{14}C	21·0	1·2	29·7

* Plants in continuous light for 24 hr.
† Plants in continuous dark for 24 hr.
‡ The amount of $^{14}CO_2$ recovered after the 24-hr period of metabolism was 51 μc; more than 96 per cent of the administered activity was taken up by the plants in the other feedings.

[16] A. J. BIRCH, R. J. ENGLISH, R. A. MASSY-WESTROPP and H. SMITH, *J. Chem. Soc.* 369 (1958).
[17] F. W. HEFENDEHL, *Planta Med.* **14**, 66 (1966).

Whereas $^{14}CO_2$ was incorporated very extensively into the volatile oil (up to twelve and seventy-five times as much as from acetate-2-^{14}C or -1-^{14}C respectively) in light, this dropped to an insignificant amount in the dark. In contrast, acetate was incorporated almost equally well in the dark as in the light. Hence, it follows that acetate and mevalonic acid are not degraded first to carbon dioxide, but incorporated as such.

Feeding Radioactive Carbon Dioxide

Attempts to obtain data relating to the biosynthetic sequence put forward by Reitsema[10] were made by feeding radioactive carbon dioxide. When single shoots of *M. piperita* were fed $^{14}CO_2$ (93 μc each, specific activity 29·5 mc/mmole) and allowed to metabolize for varying periods of time in air ($^{12}CO_2$), maximum incorporation of the tracer was reached in 6–12 hr (see Table 5). Hence, ^{14}C-labelled carbon dioxide entered into the mint oil terpenes much more rapidly than acetate (cf. Table 2). The much slower rate of incorporation of acetate (and perhaps mevalonate) in comparison to that of carbon dioxide in the light remains to be explained. It is possible that carbon dioxide is converted rapidly to a metabolite which is transported readily to the site of terpene synthesis whereas acetate and mevalonate are

TABLE 5. INCORPORATION OF ^{14}C FROM $^{14}CO_2$* INTO THE VOLATILE OIL OF *Mentha piperita* AT VARYING PERIODS OF METABOLISM

Period of metabolism (hr)	Fresh weight of plant (g)	Weight of oil recovered (mg)	Specific activity of oil (mμc/mg)	Total ^{14}C in oil (mμc)
6	1·3	1·73	374	641
12	1·3	2·23	400	888
24	1·3	1·96	370	727
48	1·3	1·85	520	960

* All shoots were treated with 93 μc $^{14}CO_2$ (specific activity 29·5 mc/mmole) in a closed system for 3 hr and then allowed to metabolize in air ($^{12}CO_2$) for the balance of metabolic period.

either transformed only slowly into an active form or the latter is not amenable to rapid transport. Alternatively, transport of precursors via the cut shoot may be slower than assimilation of carbon dioxide by the leaf. The recent report on mevalonate-kinase isoenzymes in plants by Rogers *et al.*[18] indicates the complexity of these aspects and emphasizes the care which may be required in interpreting results from feeding experiments.

By feeding $^{14}CO_2$ for short periods of time it was anticipated that early members of the sequence would be labelled more rapidly than later members. Determination of the specific activities of the individual monoterpenes might thus lend further support to the precursor-product relationships proposed by Reitsema. Although it was realized that specific activity data derived from $^{14}CO_2$ feeding could not offer definite proof for a sequence, such an approach has yielded many fruitful results, particularly in the area of alkaloid biogenesis.[19] Only the main oxygenated monoterpenes (piperitenone, piperitone, isomenthone, menthone, menthol, pulegone and methofuran) of the mint oils were considered in this study. It is noteworthy that whereas the composition of the oil of the mature mint plants agreed with that reported earlier[15, 20] the oil from young shoots contained these oxygenated monoterpenes

[18] L. J. ROGERS, S. P. J. SHAH and T. W. GOODWIN, *Biochem. J.* **100**, 14C (1966).
[19] R. O. MARTIN, M. E. WARREN, JR. and H. RAPOPORT, *J. Am. Chem. Soc.* **86**, 4726 (1964).
[20] K. L. HANDA, D. M. SMITH, J. C. NIGAM and L. LEVI, *J. Pharm. Sci.* **53**, 1407 (1964).

in the amounts shown in Tables 6 and 7. It should also be pointed out that the *M. piperita* plants used in this study, as well as those investigated by Handa *et al.*[20] did not contain detectable amounts of piperitenone. Thus, there may be a subspecies difference with the plants used by Reitsema *et al.*,[11] Battaile and Loomis,[12] and Höltzel.[14]

M. piperita shoots (five shoots for each exposure period) were fed 390 μc of $^{14}CO_2$ (29·5 mc/mmole) for 5, 15, 30 and 60 min respectively, and these were worked up at once. The total radioactivity taken up was 165, 292, 376 and 385 μc, while the total amount of ^{14}C incorporated into the volatile oil was 65, 172, 369 and 907 mμc respectively, which represents an almost linear incorporation with time. The oil was separated into individual components

TABLE 6. INCORPORATION OF ^{14}C FROM $^{14}CO_2$* INTO INDIVIDUAL OIL COMPONENTS DURING 5–60 MIN EXPOSURE

Compound	5-min exposure			15-min exposure		
	μmole of compound†	Disintegrations /min	$10^{-3}\times$ dpm/μmole	μmole of compound†	dpm	$10^{-3}\times$ dpm/μmole
Piperitenone‡	—	102	—	—	453	—
Column background§	—	42	—	—	203	—
Piperitone	0·04	107	2·4	0·06	763	13·8
d-Isomenthone	0·25	792	3·1	0·21	1181	5·7
l-Menthone	6·02	7630	1·3	7·40	34295	4·6
Menthol	1·08	445	0·4	1·55	1783	1·2
Pulegone	0·48	1622	3·4	0·59	6912	11·7
Menthofuran	2·91	426	0·1	1·67	2049	1·2
	30-min exposure			60-min exposure		
Piperitenone‡	—	1393	—	—	5817	—
Column background§	—	513	—	—	3667	—
Piperitone	0·04	723	19·5	0·04	3227	78·7
d-Isomenthone	0·10	1384	13·4	0·10	9920	96·3
l-Menthone	6·92	77952	11·2	9·18	388620	42·3
Menthol	1·05	5086	4·8	1·46	15801	10·8
Pulegone	0·49	13875	28·1	0·85	78716	92·8
Menthofuran	1·60	4514	2·8	2·41	24558	10·2

* Each group of five shoots treated with 390 μc of $^{14}CO_2$ (specific activity 29·5 mc/mmole).
† μmoles of compound collected and counted from 40 per cent of the total oil of each group of five shoots.
‡ Non-labelled piperitenone was added to the isolated oil prior to GLC.
§ Column effluent (at 175°) immediately following emergence of piperitenone was collected for a period corresponding to the total emergence time of piperitenone.

by GLC and the amounts recovered and their specific activities are reported in Table 6. The results obtained show that even after only 5 min exposure of the shoots to $^{14}CO_2$ all components investigated contain appreciable amounts of radioactivity, including menthol and menthofuran which are considered end products in the proposed sequence. This is in contrast to the findings of Reitsema *et al.*[11] and Battaile and Loomis,[12] who could find only a small number of labelled components (the proposed early members of the sequence) in their short-term experiments. The results obtained in our study show not only that all the terpenes are labelled within 5 min on feeding $^{14}CO_2$ but that interconversions between these terpenes occurs much more rapidly than anticipated. Clearly the previous method of ^{14}C-detection (autoradiography) was too insensitive to detect the lower levels of activity.

As indicated, no peak corresponding to piperitenone was observed when the oil from

Mp. iperita was assayed by GLC. Hence, a small amount of inactive piperitenone was added to each sample of ^{14}C-labelled oil prior to GLC separation to facilitate isolation. The effluent from the column immediately following the piperitenone peak was also collected (for the same length of time as was required for the collection of piperitenone) and its radioactivity was measured. In each of the four experiments reported in Table 6, a significantly greater amount of activity was present in the piperitenone fraction as compared to the column

TABLE 7. INCORPORATION OF ^{14}C FROM 1 HR EXPOSURE TO $^{14}CO_2$* FOLLOWED BY METABOLISM IN AIR ($^{12}CO_2$)

	Period of metabolism†								
	1 hr			2 hr			4 hr		
Compound	μmole of compound‡	dpm	$10^{-3}\times$ dpm/μmole	μmole of compound‡	dpm	$10^{-3}\times$ dpm/μmole	μmole of compound‡	dpm	$10^{-3}\times$ dpm/μmole
Carrier piperitenone§	—	3165	—	—	4672	—	—	9237	—
Column background¶	—	436	—	—	769	—	—	2050	—
Piperitone	0·03	462	15·9	0·01	483	43·9	0·03	1985	76·3
d-Isomenthone	0·29	6226	21·3	0·19	6776	34·9	0·46	34981	75·8
l-Menthone	14·70	226419	15·4	10·76	342307	31·8	13·68	908476	66·4
Menthol	1·28	12340	9·6	1·27	13936	10·9	1·23	25712	20·8
Pulegone	1·64	44266	27·0	0·86	71272	82·8	0·96	158431	164·6
Menthofuran	5·23	16941	3·2	3·20	28487	8·9	4·72	78260	16·6
	8 hr			12 hr			24 hr		
Carrier piperitenone§	—	7936	—	—	11877	—	—	4569	—
Column background¶	—	5040	—	—	5850	—	—	5020	—
Piperitone	0·02	1953	84·9	0·02	4590	200·0	0·02	3872	215·1
d-Isomenthone	0·34	41057	121·0	0·29	60067	206·0	0·30	55760	183·4
l-Menthone	14·95	1361096	91·0	11·93	2311321	193·7	11·72	1803476	154·8
Menthol	1·60	46657	29·2	1·89	54707	28·9	2·06	33507	16·3
Pulegone	0·77	297225	385·0	0·83	324287	283·0	0·64	110784	173·1
Menthofuran	2·85	154403	54·2	3·51	195968	55·8	3·00	168707	56·2

* Entire group of thirty shoots exposed to 1·9 mc $^{14}CO_2$ (specific activity 29·5 mc/mmole).
† Includes 1 hr exposure to $^{14}CO_2$.
‡ μmoles of compound collected and counted from 40 per cent of the total oil of each group of five shoots.
§ Non-labelled piperitenone was added to the isolated oil prior to GLC.
¶ Column effluent (at 175°) immediately following emergence of piperitenone was collected for a period corresponding to the total emergence time of piperitenone.

background. Since quantitation of the piperitenone as produced by the plants (if any) was impossible, no values for its specific activity could be calculated. Consequently, it was not possible to determine whether the reactions piperitenone→piperitone and piperitenone→pulegone take place. Also, it must be considered that although the fraction collected had the retention time of piperitenone its identity was not established and another unknown trace constituent may account for all or part of the activity recorded (see Experimental).

Most of the calculated values of the specific activities of the monoterpenes reported in Table 6 are consistent with the scheme proposed by Reitsema, but some notable exceptions

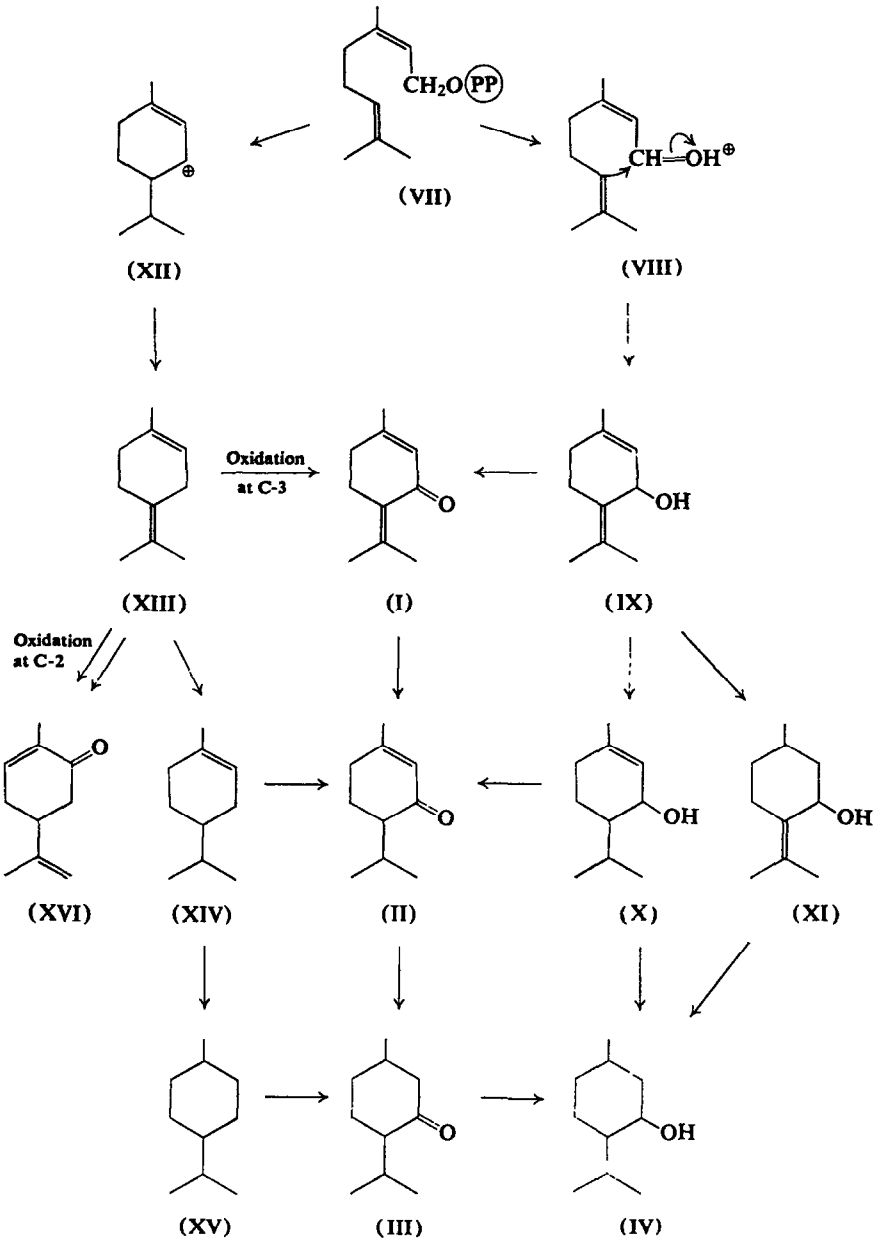
were observed. Thus, the data from all four experiments are consistent with the postulated transformation of piperitone→*l*-menthone→*l*-menthol. Similarly, the specific activities of pulegone and menthofuran are such as to confirm conversion of the former to the latter. The specific activity of *l*-menthone was in all cases lower than both piperitone and pulgeone and whereas it is possible that it is derived from both of these two precursors, the data obtained do not provide a definite verification. Surprisingly high activities were recorded for *d*-isomenthone, a component not considered by either Reitsema *et al.*[11] or Battaile and Loomis.[12] Its specific activity is similar to that of piperitone and surpasses this precursor's activity in the 5- and 60-min experiments. The data obtained suggest two possibilities pertaining to the formation and metabolism of *d*-isomenthone, namely

(1) piperitone→*d*-isomenthone→*l*-menthone
(2) precursor other than piperitone (e.g. pulegone and/or an unknown)→*d*-isomenthone→*l*-menthone.

However, as the mole ratio of isomenthone to piperitone is approximately 4:1 and their specific activities are very similar, it is unlikely that piperitone is the main precursor of *d*-isomenthone. Both on chemical grounds and on the data shown in Table 6, pulegone is a possible precursor of *d*-isomenthone. However, in some preliminary short-term (3–6 min) feeding experiments using $^{14}CO_2$ the specific activity of *d*-isomenthone was considerably greater than that of pulegone. Hence, it appears that *d*-isomenthone is mainly derived from a hitherto unsuspected precursor.

Since the data from the above experiments were somewhat inconclusive, another approach was tried. A number of shoots were kept in the dark for 24 hr to minimize the carbohydrate pool present in the shoots. They were then fed in the light with $^{14}CO_2$ for 1 hr and allowed to metabolize in air ($^{12}CO_2$) for varying periods of time. If a sequence in the formation of the various oxygenated monoterpenes exists then the early members of this sequence should show a more rapid decline in radioactivity than any of the later members or end products. Surprisingly, all compounds investigated showed an increase in their specific activities even up to 6–8 hr of metabolism (see Table 7). Hence, it appears that a very large pool of highly labelled precursors (carbohydrate pool) is maintained for such a period of time. Therefore, the data obtained up to 8 hr metabolism had to be interpreted on the same basis as the data shown in Table 6, and similar conclusions could be drawn. Thus, menthofuran is clearly an end product and pulegone can be its precursor. On the basis of comparing specific activities alone, piperitone, isomenthone or menthone could equally well be precursors of menthofuran, but this would require a number of chemical steps which are less likely to occur than the conversion of pulegone to menthofuran. This is in complete agreement with Reitsema's proposals and the findings of Battaile and Loomis[12] as well as Höltzel.[14] However, taking into account the significant drop in specific activity values of pulegone after 8 hr, pulegone must be transformed to other products besides menthofuran. Comparison of ratios of specific activity values suggests isomenthone and possibily menthone as the most likely products. This is in agreement with the finding of Battaile and Loomis,[12] but is in contrast with the conclusions of Höltzel.[14] However, the ratios of specific activities shown in Table 6 indicate that isomenthone cannot be derived from pulegone and piperitone alone and it is therefore most likely that a third, as yet unknown precursor is involved. The data obtained are also fully compatible with the view that menthone is converted to menthol. Since isomenthol or neoisomenthol have not been found in significant amounts in the oil of *M. piperita*[20] it follows that *d*-isomenthone must be converted mainly to *l*-menthone. This is supported by

the ratios of specific activities of these two compounds. No such direct relationships are discernible for piperitone and the menthones. This is presumably due to the larger errors



involved in quantitation of this minor component. From the results shown in Table 6 it seems most likely that the conversion piperitone→menthone takes place. Taking into account the anomalous data obtained in this study with piperitenone (see above) the question arises whether precursors other than piperitenone, piperitone or pulegone may be involved in the

formation of the menthones and menthol. Birch[21] suggested that piperitone (II) may be formed from geranyl pyrophosphate (VII) via the ion VIII, piperitenol (IX) and piperitol (X). A single-step isomerization of piperitol (X) to the menthones (III) could be postulated as a pathway in which piperitone is not the primary precursor. However, neither piperitol nor piperitenol have as yet been found to be mint oil components. The above pathway could even lead to menthol (IV) (via piperitol (X) or pulegol (XI)) without the menthones (III) as intermediates. However, this is not in agreement with present or previous findings. Another pathway which merits consideration is initial cyclization to a hydrocarbon (e.g. terpinolene XIII) via the carbonium ion XII.[5] Oxidation at C-3 prior or after reduction of double bonds (e.g. to XIV and XV) could then lead to piperitone, pulegone or the menthones by several different pathways. Loomis[22] points out that a pathway via a hydrocarbon would explain more readily the formation of C-2 oxygenated monoterpenes, such as carvone (XVI) in *M. spicata*[10] and in *Anethum graveolens*.[23] These views are in no way contradictory to Reitsema's original proposals; they expand the possibilities and show that several parallel sequences, or even reversible transformations, may be part of the biosynthetic sequence which could complicate interpretation of results. More accurate experimental methods, especially with regard to quantitation of minor and trace constituents, are required to clarify these aspects.

CONCLUSIONS

1. In agreement with the results of Reitsema *et al.*[11] and of Battaile and Loomis[12] young mint leaves and shoots were found to be most suitable for a study of the formation of the oxygenated monoterpenes by means of feeding radioactive precursors. Noteworthy is the high incorporation of radioactivity into the volatile oil of the axillary shoots and buds.

2. Contrary to the views of Battaile and Loomis[12] labelled acetate and mevalonate were incorporated into the monoterpenes without apparent degradation to carbon dioxide. However, radioactivity from these two precursors entered the volatile oil much more slowly than from carbon dioxide. This difference is remarkable since the accepted sequence $CO_2 \rightarrow$ carbohydrates $\rightarrow$ acetate $\rightarrow$ mevalonate $\rightarrow$ isopentenyl pyrophosphate $\rightarrow$ geranyl pyrophosphate $\rightarrow$ monoterpenes would lead to the opposite expectation. Possibly, acetate and mevalonate are only slowly converted to a form which allows transport to the site of terpene synthesis.

3. Acetate and mevalonate were incorporated into the mint oils almost equally as well in the dark as in the light, whereas carbon dioxide was incorporated only to a negligible extent in the dark. These precursors are presumably drawn from a large carbohydrate pool.

4. All monoterpenes studied (including the end products menthol and menthofuran) were found to contain radioactivity within 5 min of metabolizing the precursors. Thus formation and transformation of the mint oil terpenes takes place much more rapidly than anticipated.

5. The results obtained give, in general, good support to the biosynthetic sequence put forward by Reitsema, but some notable exceptions and additions were encountered.

(*a*) The position of piperitenone as the initial isolable member of the sequence could not be confirmed. A study of this compound in an oil wherein it is present in relatively large proportions may be necessary to establish whether this is the precursor of piperitone and

[21] A. J. BIRCH, In *Chemical Plant Taxonomy* (Edited by T. SWAIN), p. 157. Academic Press, New York (1963).
[22] W. D. LOOMIS, Personal communication.
[23] W. SANDERMANN and K. BRUNS, *Planta Med.* **13**, 364 (1965).

pulegone (as proposed by Reitsema) or whether other pathways and hence some other precursor(s) are involved.

(*b*) Pulegone is not only the precursor of menthofuran, but may play a role in the formation of menthone as suggested by Battaile and Loomis.

(*c*) Isomenthone is an important terpene not previously considered in any sequence. It is possible that its conversion to menthone is reversible, but the data obtained suggest that it is formed prior to menthone and presumably from a precursor other than piperitone and pulegone.

(*d*) Other reversible reactions may take place which complicate interpretation of resuits. Hence, more refined analytical techniques or plants which are more amenable to studies of this nature are required to obtain further details.

MATERIALS AND METHODS

Cultivation of Plants

Mentha piperita L. var. *Mitcham** and *M. arvensis* L. var. *glabrata* Ray[15] were cultivated in growth chambers in vermiculite using a modified Hoagland solution. Fluorescent and tungsten light (13,000 lx at the plant surface) was applied for 18 hr daily, which corresponds to the "long day" photoperiod of *M. piperita*.[24] Under these conditions the stolons of *M. arvensis* failed to produce healthy shoots during the latter part of the winter, whereas the vigorous growth of *M. piperita* continued unimpaired.

Administration of ^{14}C-labelled Compounds

Mint shoots bearing an appropriate number of leaf pairs (generally five leaf pairs plus the top three or four very small leaf pairs and apical meristem) were cut under water with a razor blade and placed individually into 6 × 30 mm glass vials containing labelled acetate or mevalonate in 0·1 ml of aqueous solution. The solution was almost completely absorbed in 15 min and this was followed by two 0·1 ml rinses of water before adding enough water to suffice for the total time of metabolism. In this manner more than 99 per cent of the radioactive precursor was absorbed by the shoots, except in the experiments carried out in the dark where more than 96 per cent was taken up.

To determine optimum dose of radioactive precursor, 0·5–45 μmoles of acetate-1-^{14}C per g fresh weight were fed to shoots of each plant species. Optimum incorporation of ^{14}C was obtained at 5 μmoles per g fresh weight in both plants and this dose was used in all subsequent experiments. Amounts above 30 μmoles/g were found to be toxic in *M. arvensis*.

Radioactive carbon dioxide was released from $Na_2{}^{14}CO_3$ (29·5 mc/mmole) by addition of dilute sulphuric acid. The shoots were allowed to metabolize $^{14}CO_2$ for a predetermined length of time and the residual atmosphere was pumped through 1 N sodium hydroxide solution by means of a diaphragm pump to determine the amount of $^{14}CO_2$ not used by the plants.

In all feeding experiments about 10,000 lx of fluorescent light was applied at the plant surface at an ambient temperature varying from 23° to 28°.

Steam-distillation of the Volatile Oil

The mint shoots were steam-distilled at once in a circulatory distillation apparatus.[13,25] Steam-volatile acids were removed by addition of Na_2CO_3. The volatile oil was recovered as an ethereal solution by rinsing the condenser and side arm with a minimum of ether. The amount of oil recovered was determined gas-chromatographically (flame-ionization detector), using fenchone as internal standard. The different detector response for menthyl acetate (12 carbon atoms) and sesquiterpenes (15 carbon atoms) was taken into account.

Gas–Liquid Chromatography

For the resolution and isolation of individual components of the mint oils an F & M Scientific Corp. Model 500 gas chromatograph equipped with a thermal conductivity cell was used. For quantitation (see below) an Aerograph Model 600 D (Wilkens Instrument Corp.) with a flame ionization detector was employed. This instrument permitted the use of sample sizes of less than 10 γ, but the sample could not be recovered as it is lost in the combustion.

* We wish to thank Dr. J. A. Murray, A. M. Todd Co., Kalamazoo, Mich., U.S.A., for the gift of stolons of this species.

[24] H. A. ALLARD, *J. Agr. Res.* **63**, 55 (1941).
[25] H. KAISER and W. LANG, *Deut. Apotheker-Ztg.* **91**, 163 (1951).

The mint oil samples were resolved into individual components on a 300 × 0·6 cm o.d. column containing sucrose diacetate hexaisobutyrate (15%; SAIB) on HMDS treated Chromosorb W (60–80 mesh).[13, 20] Optimum separation was obtained by temperature-programming from 110° to 165° at 1·5° per min. Under these conditions isomenthone and piperitone were obtained impure and these were purified further on a 150 × 0·4 cm o.d. column containing fluorinated silicone polymer (15%; QF-1) on Anakrom ABS (60–70 mesh) at 110° (isothermal operation).

Using aliquots of up to 5 μl of neat oil and operating at maximum sensitivity the mint oils obtained in this study showed no peak with the retention time corresponding to piperitenone. The detector sensitivity was such as to show 0·01 per cent as a distinct peak. Hence, piperitenone (isolated from the oil of *M. pulegium*[20]) was added as carrier to facilitate isolation and counting of trace amounts of this ketone if formed in the plant.

All individual components were collected in small glass tubes containing ether (2·0 ml). Of this solution 90 per cent was used for determination of radioactivity. To the residual 10 per cent a specific amount of fenchone was added as internal standard and the quantity of each component collected, as well as its purity, was determined by GLC. The area of the peaks relative to that of fenchone as recorded with the flame-ionization detector on 300 × 0·3 cm o.d. SAIB or silicone rubber (15%, SE-30) columns was calculated by the triangulation method. Since the response of detector is virtually the same for all unsubstituted monoterpenes, no correction factors were employed. The percentage error was ±3–5 per cent for the major components, and increased to about 20 per cent for the minor ones. The high dilution and high detector sensitivity required for determining the very small amounts of piperitone resulted in a larger error for this component. The percentage recovery for 0·5–5 μl aliquots of limonene, 1:8-cineole, menthone, pulegone, and menthyl acetate was found to be 80–90 per cent, which decreased slightly when using larger or smaller aliquots. When the mint oil was injected and collected in one fraction, re-injection showed all components to be present in approximately the same proportions. Thus, no unusual loss due to aerosol formation or decomposition of any one component of the oil was encountered. This conclusion was confirmed with ^{14}C-labelled oil wherein the radioactivity was used as a measure of recovery.

When radioactive fractions were collected it was found that radioactive material was eluted even when no peak was recorded. This was especially noticeable after elution of a strongly labelled component and suggests that tailing may be part of the cause. Since this activity increased with continued use of the column, continuous elution of less volatile material may also be involved. By maintaining the GLC columns at maximum operating temperature between experiments this background radioactivity could be kept within reasonable values.

Measurement of Carbon-14

In the initial experiments where the incorporation of radioactive precursors into the total oil was studied (Tables 1–5), the radioactivity present in the precursor and volatile oil was determined by oxidation of the samples to carbon dioxide with the van Slyke reagent[26] and measurement by means of a Nuclear Chicago electrometer (Dynacon Model 600). In experiments where the amount of carbon-14 was determined in the individual components of the oil (Tables 6 and 7) all measurements were made using a Nuclear Chicago Mark I liquid scintillation counter. Lipophilic substances were counted in a PPO-POPOP-toluene solution (4·0 g 2,5-diphenyloxazole and 0·05 g 1,4-bis(2-5-[phenyloxazolyl)-benzene] respectively per litre). Hydrophilic compounds were counted in Bray's solution.[27] The error in counting was less than 2 per cent in all experiments.

Acknowledgements—The technical assistance by Messrs. J. Dyck, M. Granat and D. F. Kirkland is gratefully acknowledged.

[26] D. D. VAN SLYKE, J. FOLCH and J. PLAZIN, *J. Biol. Chem.* **136**, 509 (1940).
[27] C. A. BRAY, *Anal. Biochem.* **1**, 279 (1960).