AN ABSTRACT OF THE THESIS OF

Alice Joyce Burbott for the M.A. degree in Chemistry

Date thesis is presented May 13, 1963

Title Interconversions of Monoterpenes in Peppermint

Abstract approved [redacted]

The analysis of the distribution of monoterpenes through the leaf pairs of a single shoot of Mentha piperita supports Reitsema's contention that the first terpenes formed are unsaturated ketones and that saturated terpenes are produced from these.

The growth of Mentha piperita under varying conditions of temperature and the leaf-by-leaf analysis of the terpene composition of the oil demonstrate effects of environmental factors on terpene formation and interconversion.

Time-course studies using $C^{14}O_2$ support this portion of Reitsema's scheme: pulegone yields menthone yields menthol yields menthyl acetate.

A seventy-two hour time-course with alternating periods of illumination and darkness demonstrates the interconversion of terpenes in the presence or absence

of light.

The conversion of pulegone to menthone; menthone to menthol and menthyl acetate; and piperitenone to pulegone, menthone and piperitone has been observed in peppermint tissue using labeled terpenes as substrates.

INTERCONVERSIONS OF MONOTERPENES IN PEPPERMINT

by

ALICE JOYCE BURBOTT

A THESIS

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in partial fulfillment of
the requirements for the
degree of

MASTER OF ARTS

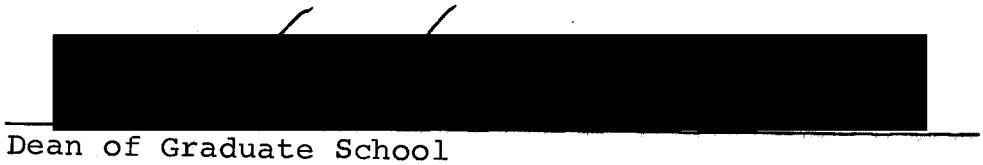
June 1963

APPROVED:

Associate Professor of Chemistry

In Charge of Major

Department Head, Chemistry



Dean of Graduate School

Date thesis is presented May 13, 1963

Typed by Norma Hansen

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INTRODUCTION

The name "terpene" in its most frequent usage designates a plant product possessing an isoprenoid carbon skeleton containing from five to thirty carbon atoms. Some authors extend the molecular size to include the forty-carbon carotenoids, and rubber. Lynen (10) extends the definition even further to include the steroids, which are isoprenoid compounds that are found in animals as well as plants.

The various plants of the genus Mentha, the mints, are very rich sources of monoterpenes, isomers and derivatives of $C_{10}H_{16}$. Some species produce linear terpenes and others, cyclic compounds oxygenated in either the two or the three position of the ring. Mentha piperita or peppermint, the species used in this study, produces some small amounts of cyclic terpene hydrocarbons, but the chief products are terpenes oxygenated in the three position.

The main components of commercial peppermint oil are menthol, menthone, menthyl acetate, and pulegone. The

most recent and complete compilation of terpenes reported to be present in peppermint oil, is given by Cramer.[1] He lists the following terpenes: α - pinene; β - pinene; 1,8-cineole; α -phellandrene; l-limonene; menthofuran; d-isomenthone; l-menthone; d-neomenthol; l-neoisomenthol; d-isomenthol; d-pulegone; menthyl acetate; l-piperitone; l-caryophyllene; piperitenone; cadinene; α -terpinene; menthyl isovalerate; and sabinene hydrate. Formulas for some of these terpenes are given in Figure 1.

The chief purpose of this study has been the investigation of the metabolic interrelationships, and interconversions of the terpenes. Although it is possible to postulate a separate synthesis for each terpene so that no interconversions would be necessary, when the great similarities of terpenes within a species are viewed this seems unlikely. Many, and in some cases, most of the terpenes within a species can be related by very simple oxidation, hydrogenation, and hydration steps, which it is tempting to think, may represent the actual biosynthetic relationships.

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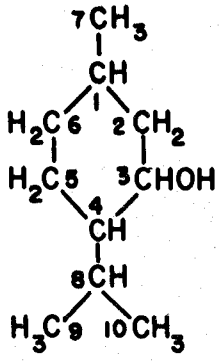
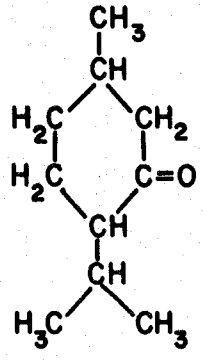
[1] Personal communication from F. J. Cramer, A. M. Todd Co., April 1960.

FIGURE 1

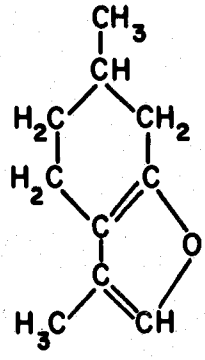
SOME TERPENES OCCURRING IN PLANTS OF THE GENUS MENTHA



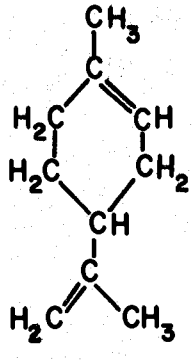
MENTHOL



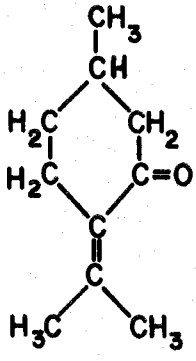
MENTHONE



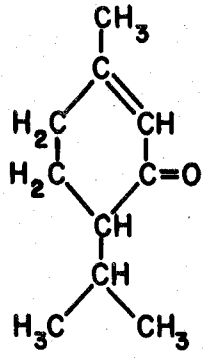
MENTHOFURAN



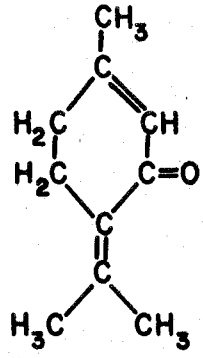
LIMONENE



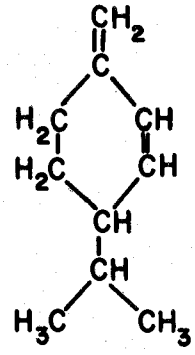
PULEGONE



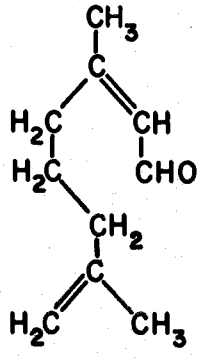
PIPERITONE



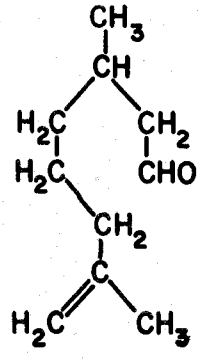
PIPERITENONE



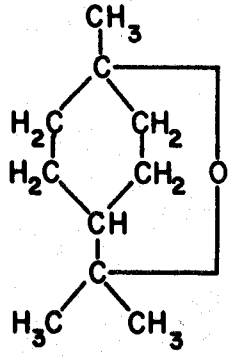
β-PHELLANDRENE



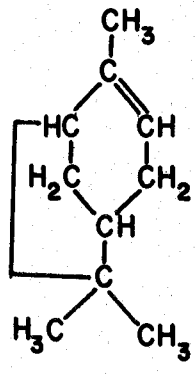
CITRAL



CITRONELLAL



1,8-CINEOLE



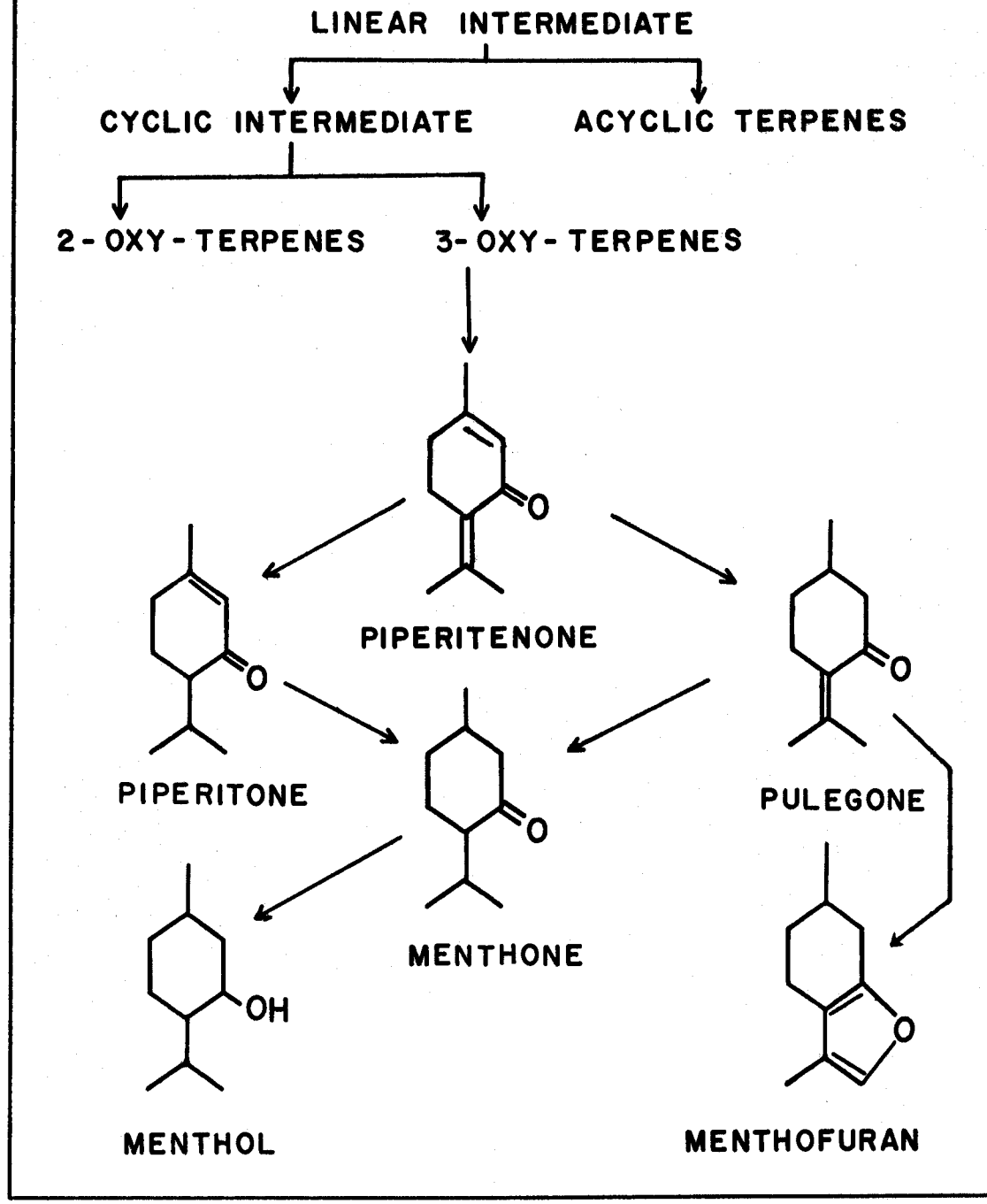
α-PINENE

Two schemes for terpene interconversion in M. piperita have been presented. The first, by Kremers (8), was postulated before many of the components of peppermint oil were known. In this scheme the cyclization of citronellal gives rise to isopulegol which then forms either pulegone or menthol. Menthone is formed by the oxidation of menthol. Neither isopulegol nor citronellal has been shown to be a component of peppermint oil.

The scheme proposed more recently by Reitsema (11) for terpene interconversion in peppermint is presented in part, in Figure 2. From a study of the terpenes found in various mint species (12,13) and the differences in oil composition of young, mature, and old foliage (14), he concluded that unsaturated ketones are among the first terpene products, and that saturated ketones, alcohols, and esters are produced from the ketones by reduction and esterification reactions. Reitsema believes (15) the unsaturated hydrocarbon terpenes are not intermediate in the process. Battaile and Loomis (3), as well as Reitsema and co-workers (15), have presented evidence to support portions of this scheme.

FIGURE 2

REITSEMA'S TERPENE INTERCONVERSION SCHEME



LABORATORY INVESTIGATIONS

Materials

Barium carbonate-C^{14} was obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee, specific activity, 0.129 mc per mg. Solutions of sodium carbonate-C^{14} were produced by decomposing the barium carbonate with acid, in vacuo, in a Van Slyke-Folch wet combustion apparatus and trapping the liberated carbon dioxide in 4.0 N sodium hydroxide. Sodium carbonate solutions used had specific activities of 1.0 mc per ml and 4.0 mc per ml.

Mevalonic acid-2-C^{14}, as the dibenzylethylenediamine salt was obtained from Tracerlab, Inc. Waltham, Massachusetts. This was converted to the sodium salt by adding sodium hydroxide to pH 10.5-11.5, extracting the dibenzylethylenediamine with ether, and adjusting the mevalonate solution to approximate neutrality with hydrochloric acid. Solutions were 100 uc per ml.

Geraniol-C^{14}, prepared by exposing Pelargonium graveolens leaves to labeled carbon dioxide, was furnished by Robert Allison of Oregon State University.

A number of the terpenes were Eastman products:

menthol, citronellal, and menthone, (practical grade), and 1,8-cineole, and d-limonene, both white label.

Piperitone, pulegone, and menthol were donated by the A. M. Todd Company, Kalamazoo, Michigan.

Piperitenone was a donation from the Glidden Company, Jacksonville, Florida.

Geraniol and menthyl acetate were obtained from the K & K Laboratories, Jamaica, New York.

The peppermint oil which was used as a standard chromatographic marker and to evaluate the gas chromatograph columns was supplied by Dr. C. E. Horner of this institution.

Merck Silica Gel G and the Desaga thin-layer chromatography apparatus were obtained from H. Brinkman, Inc., Great Neck, New York.

"Quadrol" (2-hydroxypropyl ethylenediamine), "SAIB" (sucrose-acetate-isobutyrate ester), and firebrick for gas chromatography columns were obtained from Wilkens Instrument and Research, Inc., Walnut Creek, California.

Mint extracts were fractionated and trapped by the use of the Beckman GC-2A gas chromatograph in conjunction with the Packard Tri-Carb Fraction Collector. Tri-Carb

scintillation cartridges and silicone-coated anthracene crystals were obtained from Packard Instrument Corporation, La Grange, Illinois.

Plants of Mentha piperita L. were the Mitcham variety planted commercially in the Willamette Valley. All experiments used plants from a single clone grown in the greenhouse from stock furnished by Dr. C. E. Horner.

Mentha pulegium L. plants were grown in the greenhouse from a single original plant taken from the wild at Kiger Island, south of Corvallis, Oregon. Identification was made by Dr. A. N. Steward of the Oregon State University Herbarium.

For experiments lasting more than one week, peppermint cuttings were rooted in nursery grade Perlite, an expanded siliceous rooting medium obtained from the supreme Perlite Company, Portland, Oregon.

Techniques

Growth Chambers. Experiments requiring a steady light source were carried out in a light chamber in the laboratory. The chamber was made of plywood lined with crinkled aluminum foil, dimensions, sixteen by fifty by

twenty-four inches high. Illumination was accomplished with eight 100 watt fluorescent tubes, Westinghouse Super High Output cool white, and two forty watt incandescent bulbs. Light intensity on the floor of the chamber was approximately 1400 foot candles. The lights were separated from the rest of the chamber by two glass plates an inch apart. The large chamber in which the exposure vessels were placed was exhausted, by means of a blower, into the laboratory hood. A second blower circulated air over the lights and between the glass plates. No other temperature control was in use during the course of these experiments.

Fixation of radioactive carbon dioxide was carried on in an exposure vessel consisting of a dessicator top, dimensions, eight inches in diameter at the base by three inches high, sealed to a glass plate by means of plasticine. Sodium carbonate-C^{14} was placed in a container in the center of the chamber and after the chamber was sealed, carbon dioxide was generated by injecting a solution of perchloric acid into the sodium carbonate through the rubber seal. When samples were to be removed from the exposure vessel at fixed intervals, as in

the time-course studies, the chamber was flushed at the end of the exposure period with air which had been washed through two sodium hydroxide traps to minimize the amount of unlabeled carbon dioxide entering the chamber. The chamber was resealed and flushed with sodium hydroxide-washed air after each sample was removed.

Preparation of Mint Oil Samples. In the course of these studies it was found desirable to grind and extract the mint tissue immediately at the conclusion of an experiment or in the case of the time-course studies, directly as the sample was removed from the exposure chamber, since it appeared that some enzyme activity continued even when the tissue was frozen at once in chilled Skellysolve B and then stored in the freezer.

Extracts were prepared by grinding the leaves in a mortar, extracting the ground material with Skellysolve B, and concentrating the extract in a stream of cool air. Sodium sulfate was added to facilitate the grinding. When samples were being prepared for injection into the gas chromatograph a minimal quantity of sodium sulfate was added to the extract to remove traces of water, since water is extremely harmful to the liquid phase of the

column. The ground tissue was routinely extracted four times with Skellysolve B to a total volume of ten ml, the liquid being drawn off each time with a medicine dropper.

It has been observed by both Battaile (1, p. 21) and Campbell (4, p. 28) that when $C^{14}O_2$ or mevalonic acid-C^{14} are used as substrates for peppermint, the label is more actively incorporated into carotenoid materials than into the terpenes and these carotenoids may, in fact, obscure the presence of labeled terpenes on a chromatoplate. This is also a problem when gas chromatography is used to separate the extract into its terpene fractions, as it has been observed that the carotenoid material is not recovered as a single peak or series of peaks, but is eluted more or less continuously from the column. Since it was possible that the terpenes might also be removed by the Norit A and that specific terpenes might be preferentially adsorbed, this was investigated in the following manner. Three peppermint shoots were ground and extracted, and the extracts divided into two five ml aliquots. One aliquot from each extract was decolorized with Norit A and the other

left untreated. Each aliquot was concentrated to fifty microliters and injected into the gas chromatograph. The relative areas of the individual peaks were obtained from the gas chromatograph trace by the use of the Disc integrator.

Relative Peak Areas

	Menthofuran		Menthone		Menthol	
	Treated	Untreated	Treated	Untreated	Treated	Untreated
30 µl	2.0	2.1	15.2	14.8	30.2	33.8
50 µl	1.1	1.1	18.8	20.9	48.4	50.0
50 µl	1.0	1.1	18.5	17.7	41.3	35.8

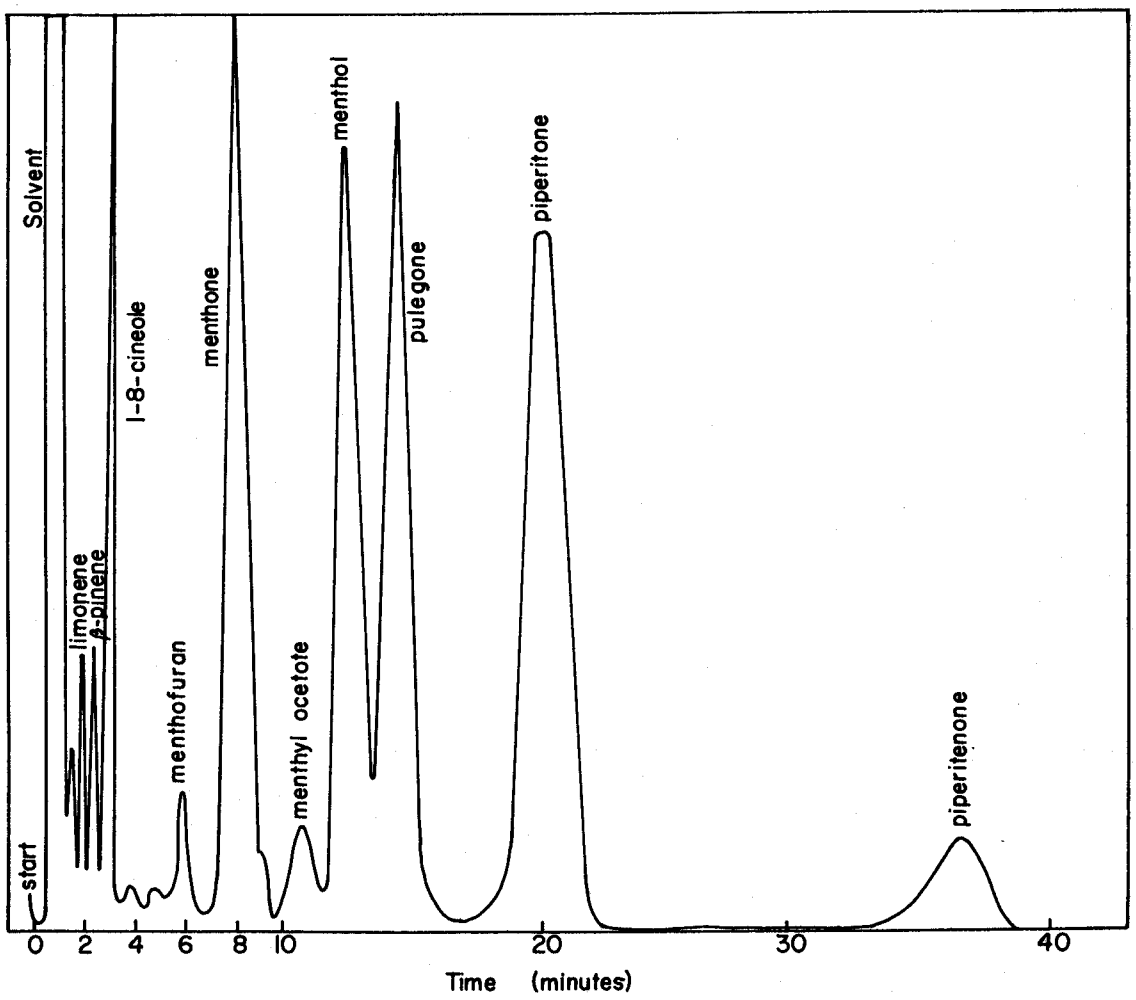
No consistent variation was detected between the relative areas of the extracts treated with Norit A and those which were not decolorized. Removal of pigmented material from the mint extracts by the use of Norit A was carried out routinely throughout these studies.

Analysis of Mint Oil. The mint tissue extracts in these investigations were analyzed by one of two methods: thin-layer chromatography on silicic acid as described by Battaile, Dunning and Loomis (2), using however, Merck Silica Gel G and the Desaga thin-layer apparatus; and gas-liquid chromatography, using the Beckman GC-2A gas chromatograph.

Gas-liquid chromatography was chosen for most of

the analytical work because it made possible more complete separation of the individual terpenes and it facilitated quantitative determination of the terpenes and of carbon-14. Seven foot columns of one-fourth inch aluminum tubing were packed with a seven percent (weight/weight) coating of liquid phase on 100-120 mesh firebrick. The liquid phase consisted of five parts Quadrol and two parts SAIB. The columns were operated at 135° C, at a filament current of 400 ma, and with a helium flow of 65 ml per minute as determined by a bubble flow-meter. This column was a modification of a column originally suggested by Wilkens Instrument and Research, Inc. (16). The liquid phase of the column is extremely sensitive to traces of water and the operating temperature is close to the maximum temperature for the use of Quadrol; therefore the useful life of the column proved rather short and several columns were used in the course of the investigations. The flow rate of the helium was changed slightly with each column to give approximately the same retention time for the individual terpenes on each column. Figure 3 reproduces the standard peppermint oil trace on this column, showing each of the terpenes with

Figure 3


start
Solvent
limonene
β-pinene
1-8-cineole
menthofuran
menthone
menthyl ocetote
menthol
pulegone
piperitone
piperitenone
0
2
4
6
8
10
20
30
40
Time (minutes)

its retention time.

Peaks were identified by comparing retention times with those of known terpenes on this column and on a twenty-foot column of ten percent Carbowax 1000; and by rechromatographing individual components of standard peppermint oil on silicic acid chromatoplates. The components were trapped on the plates as they eluted from the gas chromatograph and were then chromatographed in a solvent system containing eight percent ethyl acetate in Skellysolve B. The Rf values of the trapped materials were then compared with the Rf values of known terpenes.

Although good separation of the individual terpenes of pure mint oil is obtained by gas chromatography, the early hydrocarbon peaks are masked by Skellysolve B in the mint extracts, and only β-pinene can be detected well enough to be trapped separately. Complete separation of menthol and pulegone proved impossible with any of the combinations of columns, coatings, and operating conditions which were tested; however when materials from these two peaks were trapped separately on silicic acid plates and chromatographed in the ethyl acetate-Skellysolve B system, there proved to be very little

pulegone in the menthol peak and only slightly more menthol in the pulegone peak.

The Brown recorder used with the gas chromatograph was fitted with a Disc integrator which made it easy to determine the area under the peaks and thus get a quantitative measurement of the amount of each terpene in the extract. When each full sweep of the integrator pen is assigned a value of one, the numbers of sweeps per 0.1 millimicroliter are as given:

Sweeps per 0.1 millimicromole of terpene

piperitenone	86	menthone	100
pulegone	68	menthol	90
piperitone	79	menthyl acetate	65
menthofuran	82		

Thin-layer chromatography was used, as noted above, to identify the gas chromatograph peaks and check their homogeneity. It also served, in conjunction with radioautography, for analysis of preliminary experiments. The principal use of this method, however, was the separation of carbon-14 labeled terpenes to be used as substrates. When gas-liquid chromatography was tried for this purpose, it was found that the terpene material could not be recovered without considerable decomposition.

Carbon-14 Detection and Measurement. Estimation of

the extent of incorporation of the carbon-14 label into terpenes was accomplished by use of the Packard Tri-Carb liquid scintillation counter, radioautographs, or by counting chromatoplates with a Tracerlab TGC-14 Mylar-window gas flow counter.

By attaching the Packard Tri-Carb Fraction Collector to the sample effluent vent of the gas chromatograph, the effluent gas was passed through Tri-Carb scintillation cartridges and the terpenes were trapped on the silicone-coated anthracene crystals. The cartridges were then counted directly in the liquid scintillation counter. The counting was done at high voltage tap six (900 V) on the Packard 314 Counter, with window settings of 10 to 100 and 100 to infinity. These settings gave a balance point condition for this system. Samples were usually counted for a period of five minutes, though when activity proved to be extremely high, shorter time periods were used. The anthracene cartridges give a low background reading of from twelve to twenty cpm. This system avoids the losses due to evaporation and incomplete elution found in freeze-trapping systems and the low efficiency and tedium of plate counting. It also

proved to be more sensitive than radioautography.

To check the efficiency of the anthracene as a counting system 100 µl aliquots of a Skellysolve B solution of geraniol-C^{14} were injected directly into anthracene cartridges. The radioactivity of this volume of geraniol solution as determined by liquid scintillation counting in toluene-POPOP-PPO[2] counting fluid was 16,000 cpm. The anthracene cartridges had an average activity of 12,000 cpm for an efficiency of seventy-five percent. To check the trapping efficiency, 100 µl aliquots of the same geraniol solution were injected into the gas chromatograph and trapped after passing through the column. The average of the samples was 4,000 cpm or an efficiency of twenty-five percent.

For counting plates with the gas flow counter, a shield of one-tenth inch thick Plexiglass was used. The shield was laid directly on the chromatoplate with an opening over the area to be counted. The counter was then set on the shield with the window over the opening in the shield. Counting results were quite consistent

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[2] POPOP = 1,4 bis-2-(5 phenyloxazole).
PPO = 2,5-diphenyloxazole.

and the background was low. Battaile (1, p. 33) reports a counting efficiency of approximately fifteen percent using this method.

Autoradiograms were made with Kodak single-coated Medical X-ray film. The length of exposure varied with radioactivity levels of the samples, but was usually about two weeks. For some of the less active samples the exposure was increased to six weeks.

Experiments

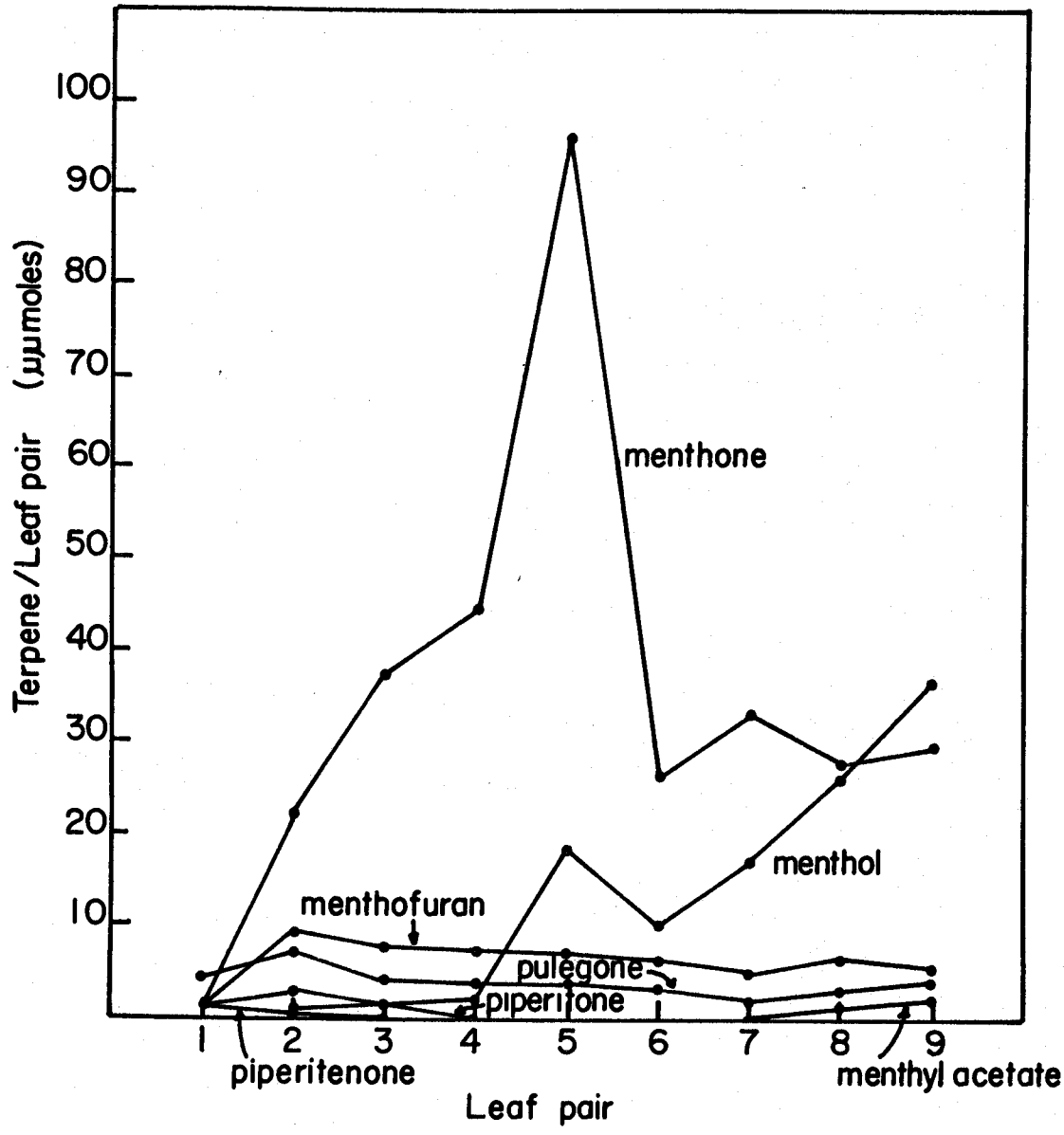
It was desirable as a starting point for these investigations to determine more accurately the terpene composition of peppermint oil as a function of the age and size of the individual leaf, to facilitate proper selection of leaf tissue in the subsequent experiments. Reitsema (12, 14) and Battaile and Loomis (3) had made earlier studies along this line using thin-layer chromatography, but the use of gas-liquid chromatography made possible a more quantitative study.

An actively growing mint shoot, cut in the greenhouse in July, was divided into samples. Each sample consisted of a leaf pair from a single node, except

sample 1, which included the terminal bud and the smallest leaf pair. Extracts were analyzed by gas chromatography on a Carbowax 1000 column which was not calibrated. However, the ratios of terpene to peak area correspond, approximately, to those of the Quadrol-SAIB column, and the calibration values for the latter column were used in plotting the data. These results are shown in Figure 4. Samples 1 through 4 were leaves which were still expanding, while the leaf pairs in samples 5 and 6, though immature in appearance, had reached maximum size. Samples 7 and 8 were mature leaves, and the leaf pair used for sample 9 appeared somewhat senescent. Menthone was the principal component in most of the extracts, while piperitenone and piperitone were found only in immature tissue, and menthol and menthyl acetate in the older leaves. Analysis of shoots carried out in the course of subsequent experiments correspond to this pattern when the mint was growing actively.

When the mint plants were growing slowly the proportions of individual terpenes in the oil changed significantly. Thirty peppermint cuttings were rooted in "Perlite" and grown for twenty-eight days in a constant-

Figure 4


100
90
80
70
60
50
40
30
20
10
Terpene/Leaf pair (µµmoles)
menthone
menthol
menthofuran
pulegone
piperitone
1
2
3
4
5
6
7
8
9
piperitenone
menthyl acetate
Leaf pair

temperature growth chamber. The use of this chamber, a National Appliance product, was made possible by Dr. E. J. Trione of the Science Research Institute. During the course of the experiment, the growth chamber was maintained at a temperature of 1.0° C with a ten minute "defrost" period of five minutes every three hours when the temperature rose to 3.5° C. Humidity in the chamber ranged between eighty and one hundred percent. The chamber was illuminated for a twelve hour period out of each twenty-four hours with Sylvania VHO Gro Lux lamps. Light intensity at shelf level was measured at 700 foot candles with a light meter which has maximum sensitivity to green wavelengths; since the Gro Lux lamps give predominately red and blue light, the actual light intensity should be somewhat higher. At the end of the twenty-eight day period the mint was growing very slowly and had an intense purplish-red coloration in both the leaves and stems. The tips of the lowest leaves were browned but the rest of the leaves appeared undamaged. Four cuttings, consisting of the terminal bud and the upper four leaf pairs were removed from the growth chamber, ground and extracted, and analyzed by gas chromatography. Corresponding leaf pairs from two shoots were included in

each sample. The results are shown in Figure 5.

The total quantity of oil to be found in these samples was much less than in actively growing mint. Menthol and menthyl acetate appear in earlier leaf pairs, while menthone reaches its maximum already in the second leaf pair. The analysis suggests that with the slow growth of tissue the essential oil becomes "mature" while the leaves are of smaller size. Most noticeable is the more or less steady level of menthofuran after the first sample, and the late disappearance of piperitone. Thus the extract from the fourth leaf pair contains principally menthol and menthofuran, a situation not found in rapidly growing mint.

The temperature of the growth chamber was then raised to 15° C., all other conditions remaining unchanged, and the mint was again analyzed after two weeks at this temperature. The mint retained the intense purple color but a new leaf pair developed and it was now possible to obtain five samples from each shoot. As before, corresponding leaf pairs from two shoots were used in each sample. These data are plotted in Figure 6.

Figure 5

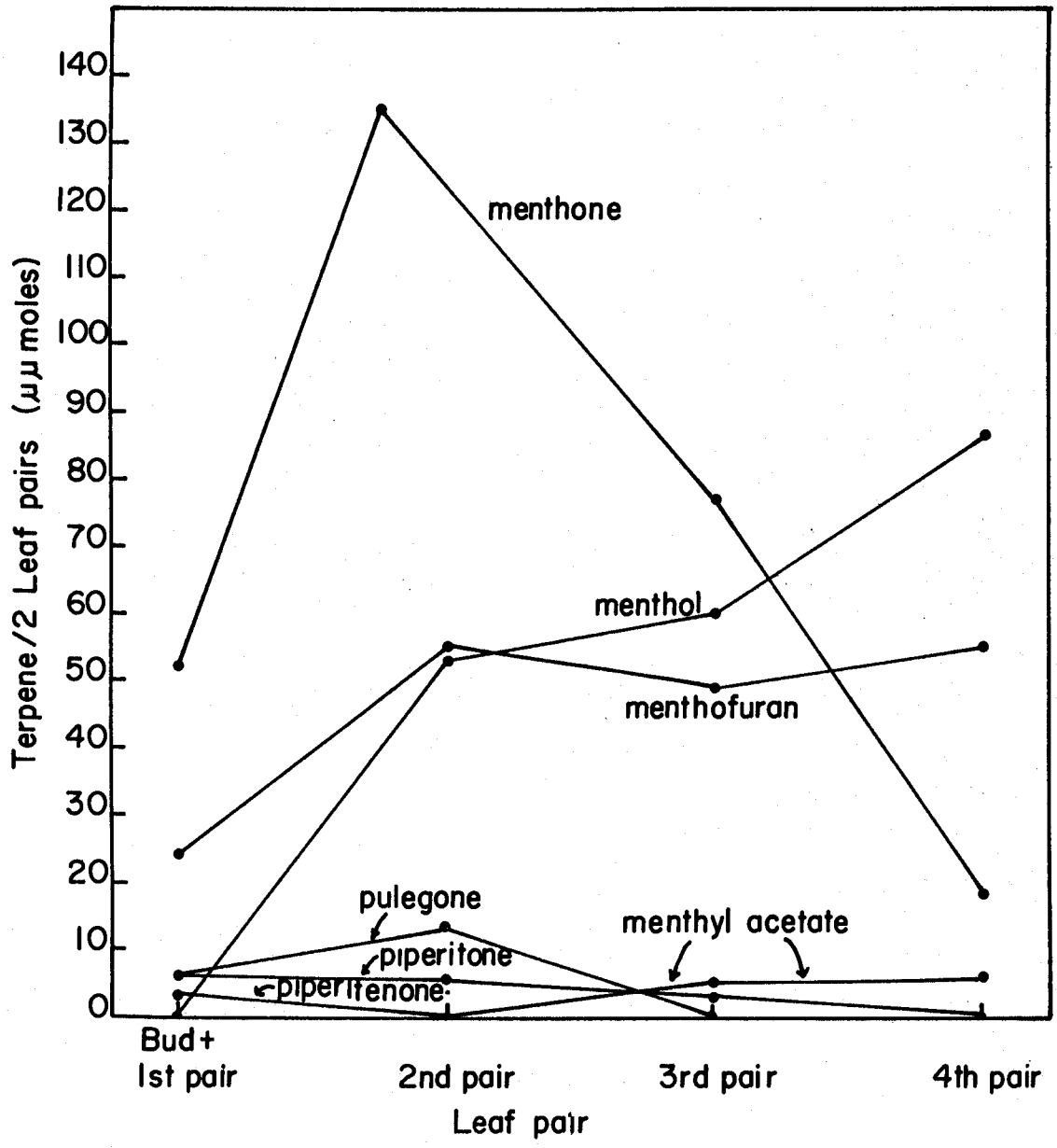
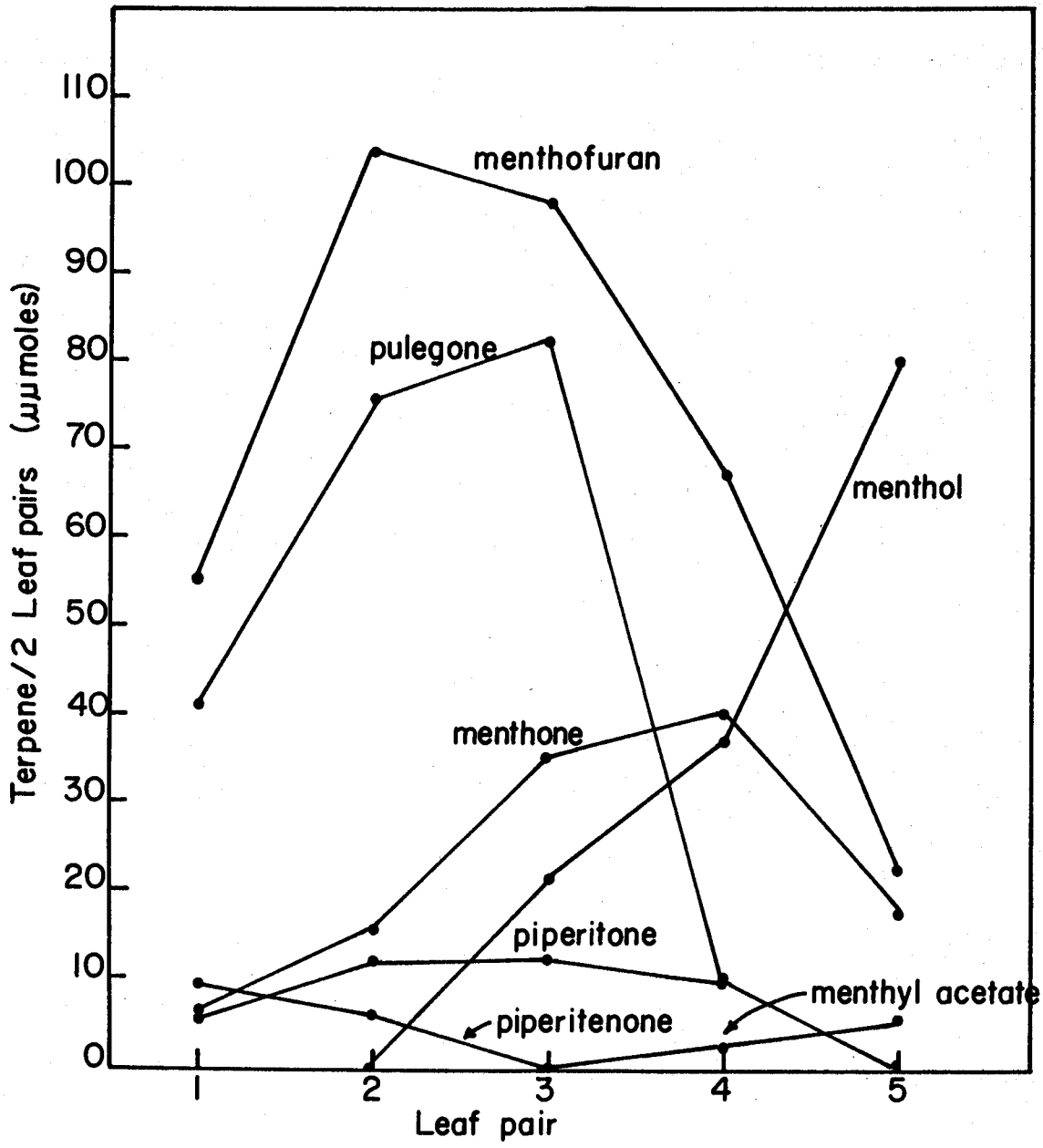

140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Terpene/2 Leaf pairs (μμ moles)
menthone
menthol
menthofuran
pulegone
piperitone
piperitenone
menthyl acetate
Bud+
1st pair
2nd pair
3rd pair
4th pair
Leaf pair

Figure 6


110
100
90
80
70
60
50
40
30
20
10
0
Terpene/2 Leaf pairs (μμmoles)
menthofuran
pulegone
menthol
menthone
piperitone
menthyl acetate
piperitenone
1
2
3
4
5
Leaf pair

The total quantity of oil in each sample has increased. Menthone does not constitute the principal terpene in any of the samples. Menthofuran reaches a peak in the second sample and pulegone in the third. Menthol and menthyl acetate appear one sample later than in the previous analysis.

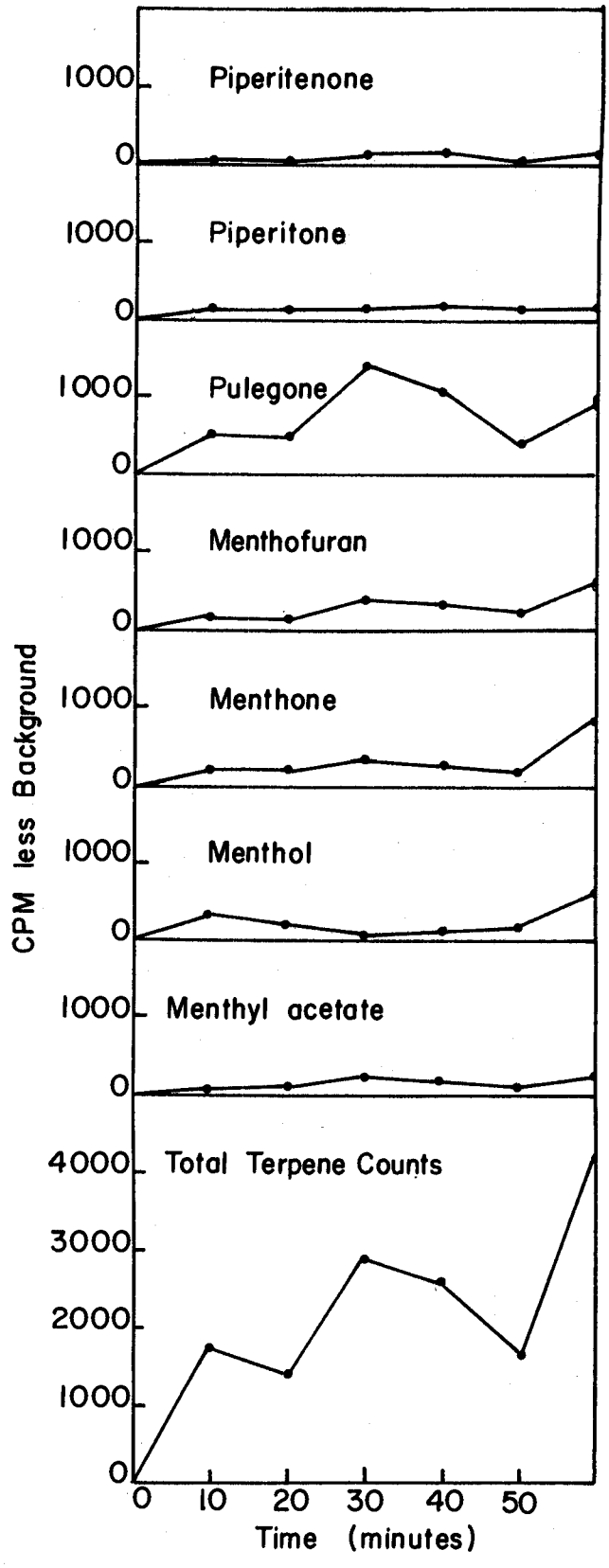
Studies Using Carbon-14. Two differing types of studies were used chiefly in these investigations; time-course studies with $C^{14}O_2$ as the labeled substrate, and experiments in which C^{14}-labeled terpenes were used as substrates.

Battaile and Loomis (3) have shown that de novo synthesis of terpenes in M. piperita occurs only in young, still expanding, leaf tissue, and that terpene formation is a rapid process in these young leaves. Therefore, the portion of peppermint shoots bearing the terminal bud and the first leaf pairs were used routinely in the time-courses. Two shoots were used for each sample in the time-course to increase the quantity of terpenes in the extract. It had been hoped that the combination of very young tissue, high levels of radioactivity, and very short fixation periods would

yield information as to the earliest formed terpenes. The shortest fixation period which proved practicable was one of ten minutes; shorter periods yielded activities too low and inconsistent to be relied upon for accuracy. A fixation period of five minutes, the shortest period tried, gave activities of fifty to one hundred cpm above background in menthol, pulegone, menthofuran, piperitone, and piperitenone.

In one study, sixteen peppermint shoots were cut, the stems placed in water-filled beakers, and exposed, in the light, to 400 μc of $C^{14}O_2$. After ten minutes the chamber was swept with air, and the first sample was removed at twelve minutes after the beginning of the exposure. Samples were taken every ten minutes and the experiment was concluded one hour after fixation began. A curve of the sum of radioactivity in the terpene fraction for each extract is shown in Figure 7. The curve for the sum, although it rises as expected, shows much fluctuation from sample to sample. Pulegone, menthone, and menthofuran, quantitatively the major components of the extracts as indicated by the peak areas on the gas chromatograph trace, show this same

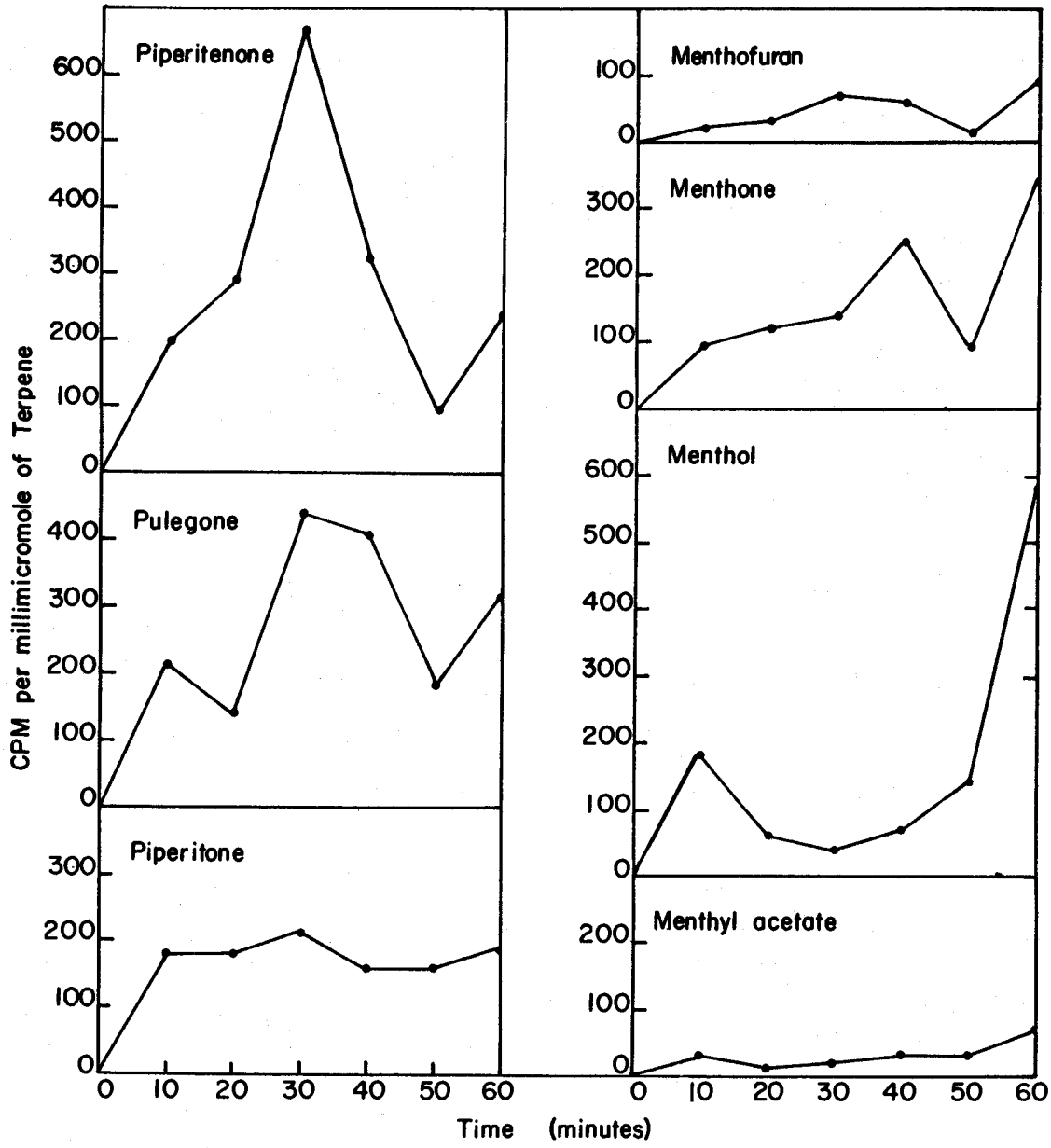
Figure 7


Piperitenone
Piperitone
Pulegone
Menthofuran
Menthone
Menthol
Menthyl acetate
Total Terpene Counts
1000
0
4000
3000
2000
0 10 20 30 40 50
Time (minutes)
CPM less Background

fluctuation. The activities of the individual terpene fractions of each sample are also plotted in Figure 7. To minimize the effect of individual variation between samples, the data from this experiment were also plotted in terms of specific activity, counts per minute per millimicromole of terpene as shown in Figure 8.

When the terpenes are arranged in sequence according to the time period at which maxima occur in the specific activity curves the order is as follows: menthol and menthyl acetate; piperitenone, pulegone, piperitone, and menthofuran; followed by menthone. The early high specific activity of the menthol fraction encountered in both this and a second one hour time-course was unexpected in view of the earlier results of Reitsema (15) and Battaile (1), and suggested further investigation of the material trapped in the menthol fraction. When the menthol peak of young leaf tissue was trapped on a chromatoplate as the effluent emerged from the gas chromatograph, two spots were seen on the resulting thin-layer chromatogram: a spot having the Rf value of menthol in the ethyl acetate-Skellysolve B system and a second spot which had an Rf

Figure 8


Piperitenone
Pulegone
Piperitone
Menthofuran
Menthone
Menthol
Menthyl acetate
CPM per millimicromole of Terpene
Time (minutes)
0
10
20
30
40
50
60

value in the piperitone region. This latter spot did not appear when the menthol peak of standard peppermint oil (derived primarily from mature leaves) was trapped and chromatographed on thin-layer plates.

To investigate this point further, a thirty minute time-course using 400 μc of $C^{14}O_2$ and a fixation period of ten minutes was carried out and samples were taken at ten, twenty, and thirty minutes. In each sample the material from the menthol peak, as it emerged from the gas chromatograph, was trapped and chromatographed on thin-layer plates. This was not an efficient method of trapping and activities of the recovered materials were low. However, in the ten and twenty minute samples the unknown spot had an activity of 100 to 150 cpm above background with the gas-flow counter, while the menthol spot showed no activity until the thirty minute sample. Thus the early appearance of radioactivity in the menthol peak is due to an unidentified contaminant.

Early labeling is often noted in menthyl acetate and may denote incorporation of label into the acetate moiety of the molecule.

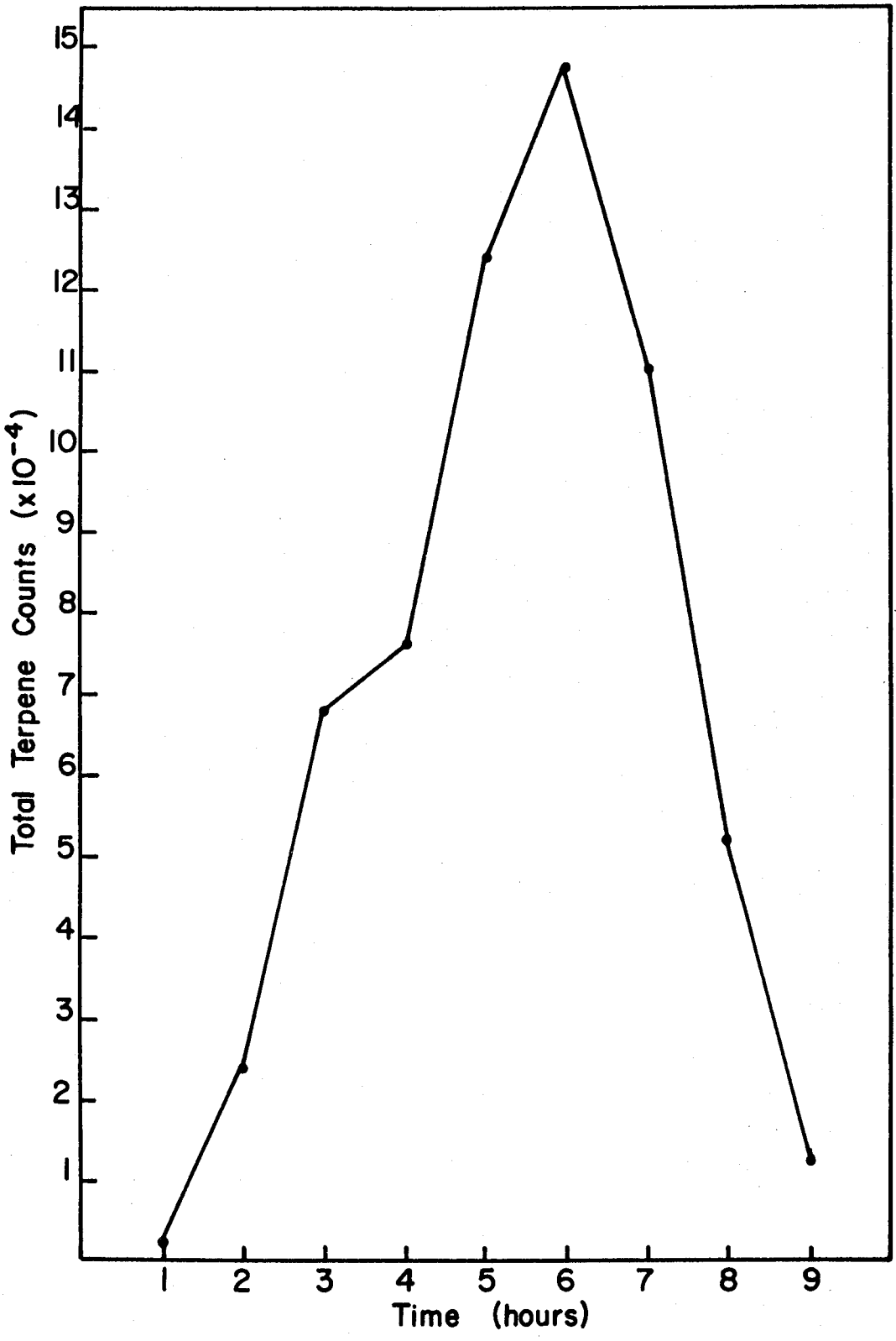
A second time-course was designed to cover a period

of nine hours in continuous light. Twenty peppermint cuttings were exposed to 800 μc $C^{14}O_2$ for a period of one hour. The chamber was then flushed, and samples were taken at intervals of one hour for the subsequent eight hours. The label incoporated into the terpenes was much greater with the longer fixation period and higher substrate level and the variation between sample much less than in the one-hour time-courses. Figure 9 gives the summation curve of activities in the extracts, Figure 10 the activities of the individual terpenes, and Figure 11 the specific activity curves for the individual terpenes in this experiment.

Again, arranging the individual terpenes according to the time at which maxima of specific activity occur the following sequence results: pulegone and piperitenone; piperitone, menthofuran and menthone; menthol and menthyl acetate.

Early investigation of label distribution in terpenes of M. piperita from $C^{14}O_2$ fixation indicated that incorporation of the label into individual terpenes varied considerably with the presence or absence of light. A seventy-two hour time-course experiment, with

Figure 9


Total Terpene Counts (x10⁻⁴)
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
Time (hours)

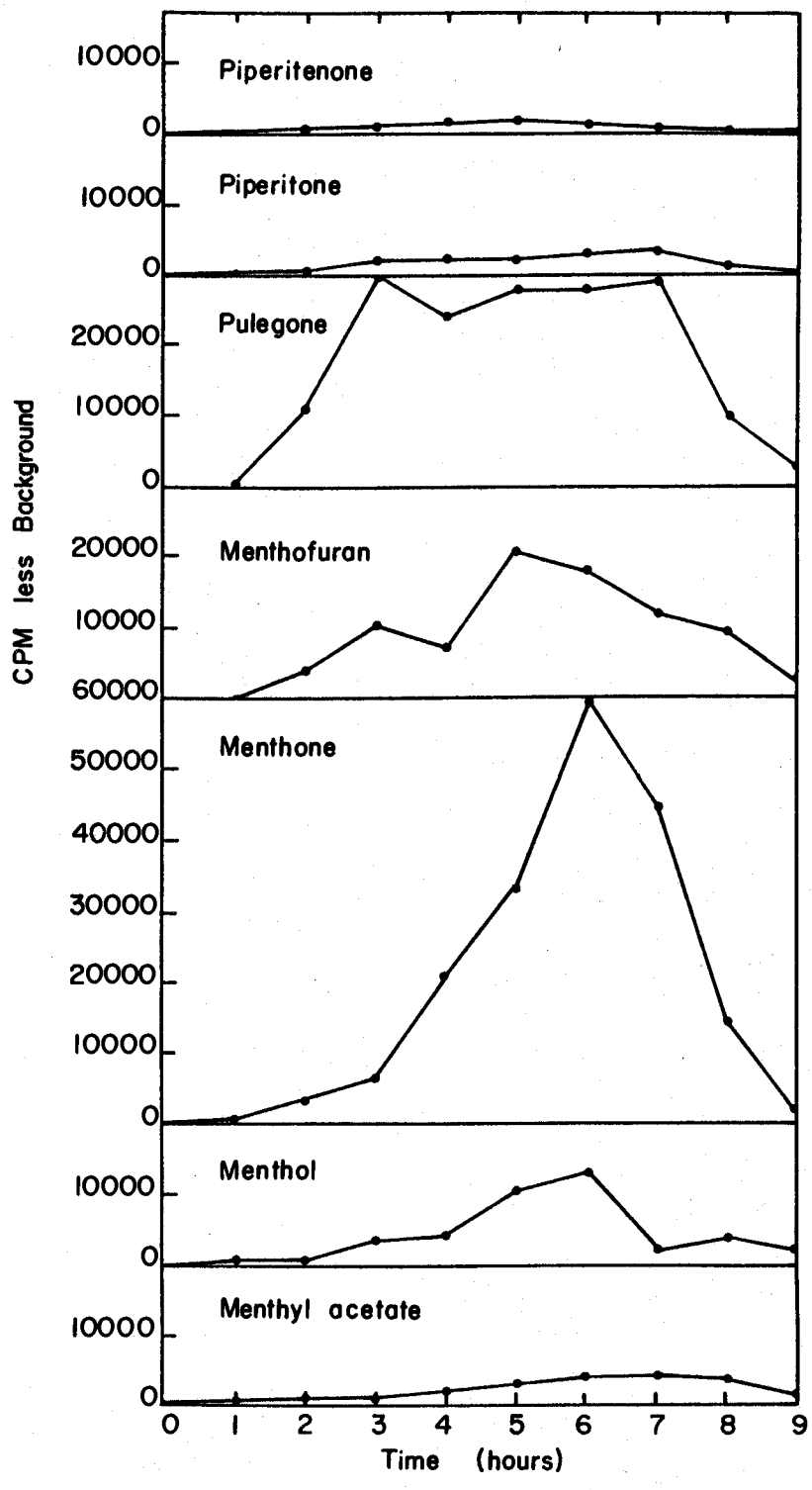
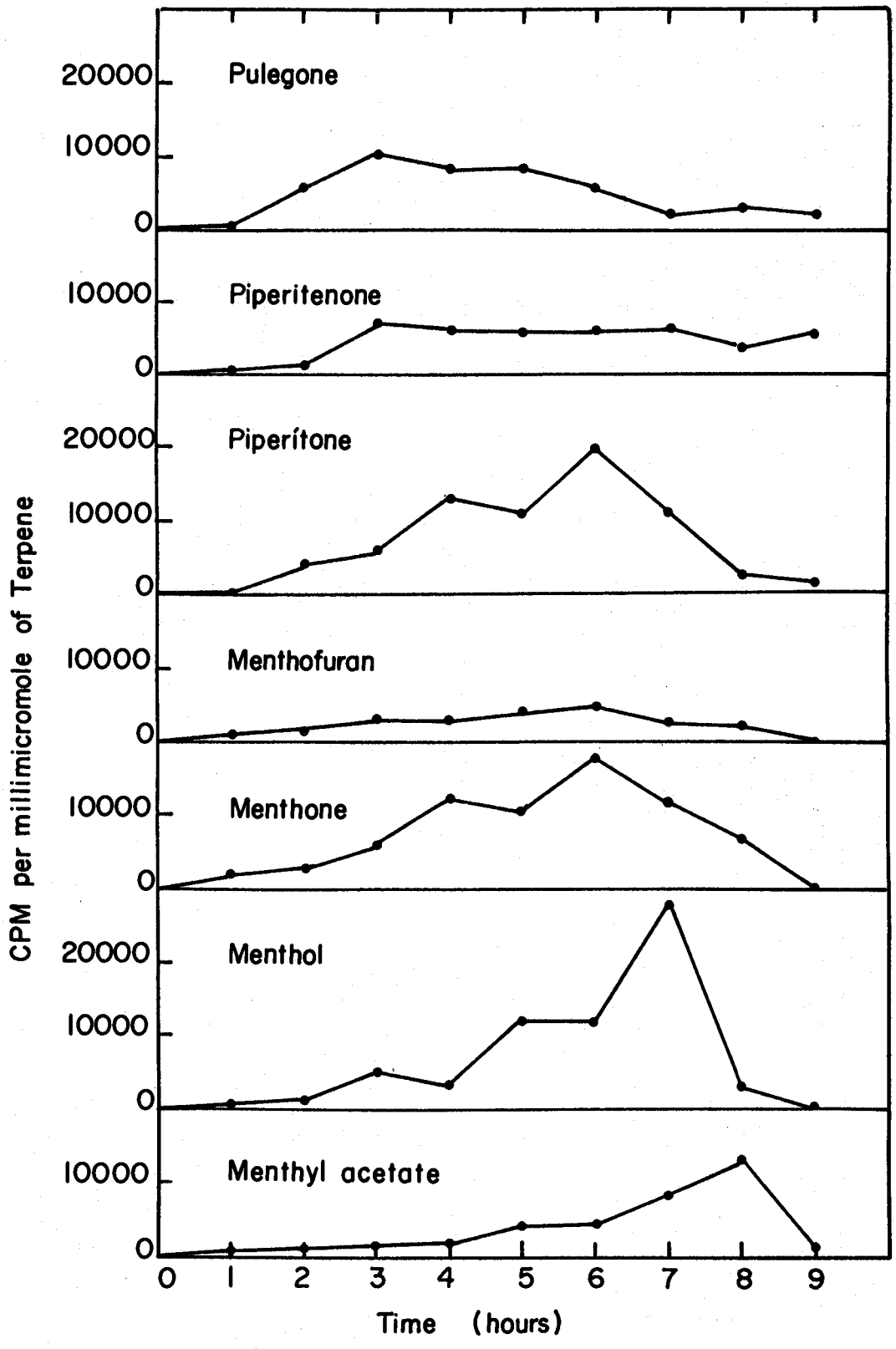

Piperitenone
Piperitone
Pulegone
Menthofuran
Menthone
Menthol
Menthyl acetate
CPM less Background
Time (hours)
10000
0
20000
60000
50000
40000
30000
0 1 2 3 4 5 6 7 8 9

Figure 10

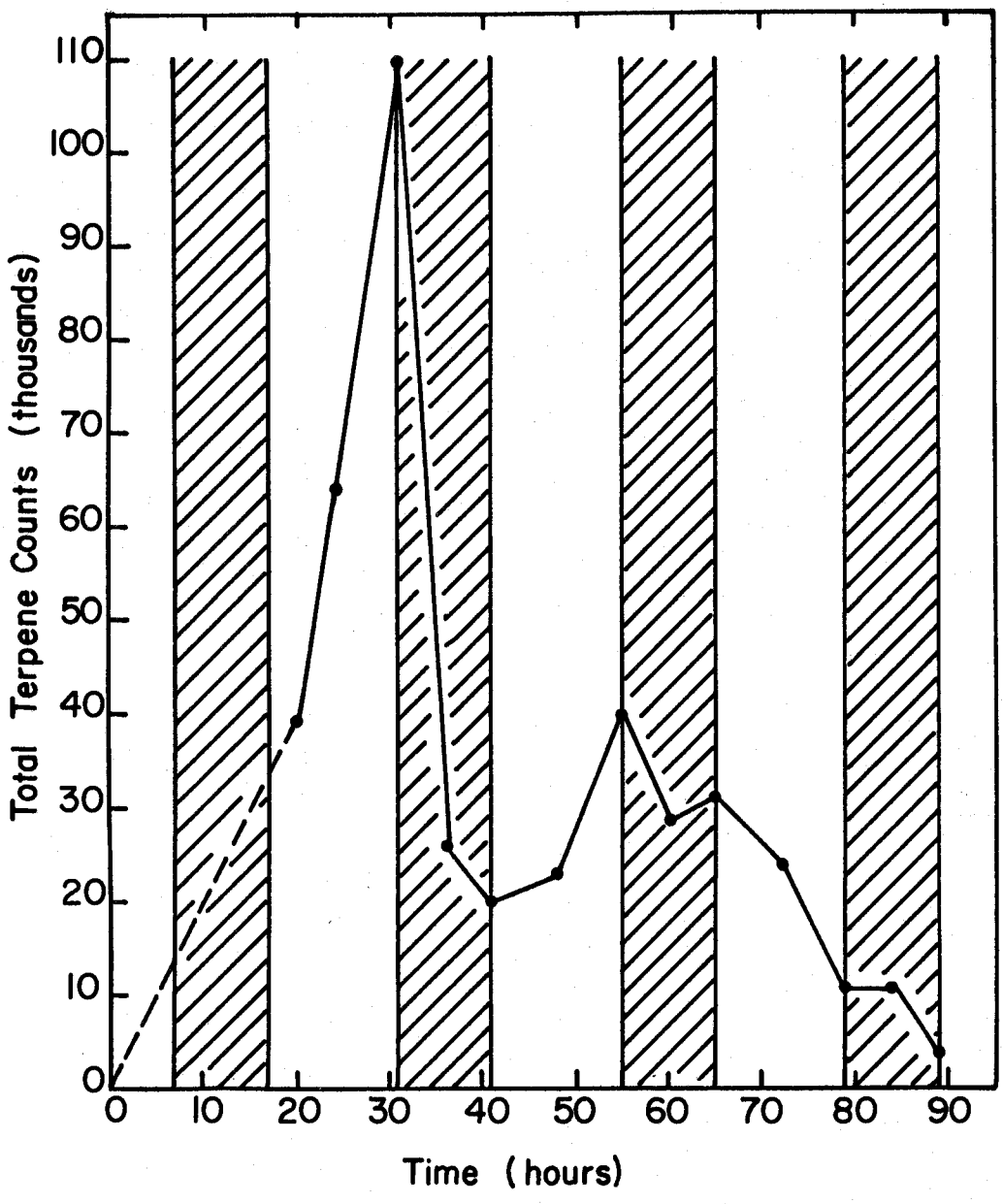
Figure 11


Pulegone
Piperitenone
Piperítone
Menthofuran
Menthone
Menthol
Menthyl acetate
20000
10000
0
CPM per millimicromole of Terpene
0 1 2 3 4 5 6 7 8 9
Time (hours)

a fixed light period of fourteen hours and a dark period of ten hours in each twenty four hour period, was performed to investigate this effect. Twenty-six peppermint cuttings were exposed to 400 μc of $C^{14}O_2$ for a period of twenty hours (seven hours light, ten hours dark, and three hours light). This long fixation period was designed to allow the initial peak of incorporation for each component to pass before sampling began. The exposure vessel was shielded in heavy aluminum foil during the dark periods and as an additional precaution no lights were allowed in the laboratory during the dark period, since it was not known whether low light intensities might influence terpene metabolism as a result of their effect on plant hormones.

Figure 12, the plot of the total radioactivity in each of the extracts, shows a loss of activity during each dark period, which is only partially recovered during the subsequent light period. The last three samples (at 79, 84, and 89 hours) were badly wilted although the stems were still immersed in water. The failure of the terpenes to recover activity in the third light period (55 to 79 hours) may suggest that the

Figure 12


Total Terpene Counts (thousands)
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
Time (hours)

carbon-14 has by this time been largely converted into metabolically inactive materials, which are not readily converted into terpene precursors. Total activities of the individual terpenes of each extract are plotted in Figure 13, and specific activities of the terpenes are plotted in Figure 14. The data for the pulegone, menthone, and menthofuran fractions are plotted as percentage of the total activity in Figure 15. The bulk of the label was incorporated into these fractions and these terpenes are quantitatively the major components of the extracts.

Of all the oxygenated terpenes of peppermint, piperitenone alone seems unaffected by the presence or absence of light. The piperitone and menthofuran curves display the most periodicity. After the first light-dark cycle, the piperitone specific activity curve shows a maximum in the middle of each dark period, at the same time that pulegone and menthofuran display minima. Menthofuran and pulegone begin to recover activity after the middle of each dark period and the rise displayed by the summation curve toward the end of the second dark period is due to this recovery. The recovery of activity

Figure 13

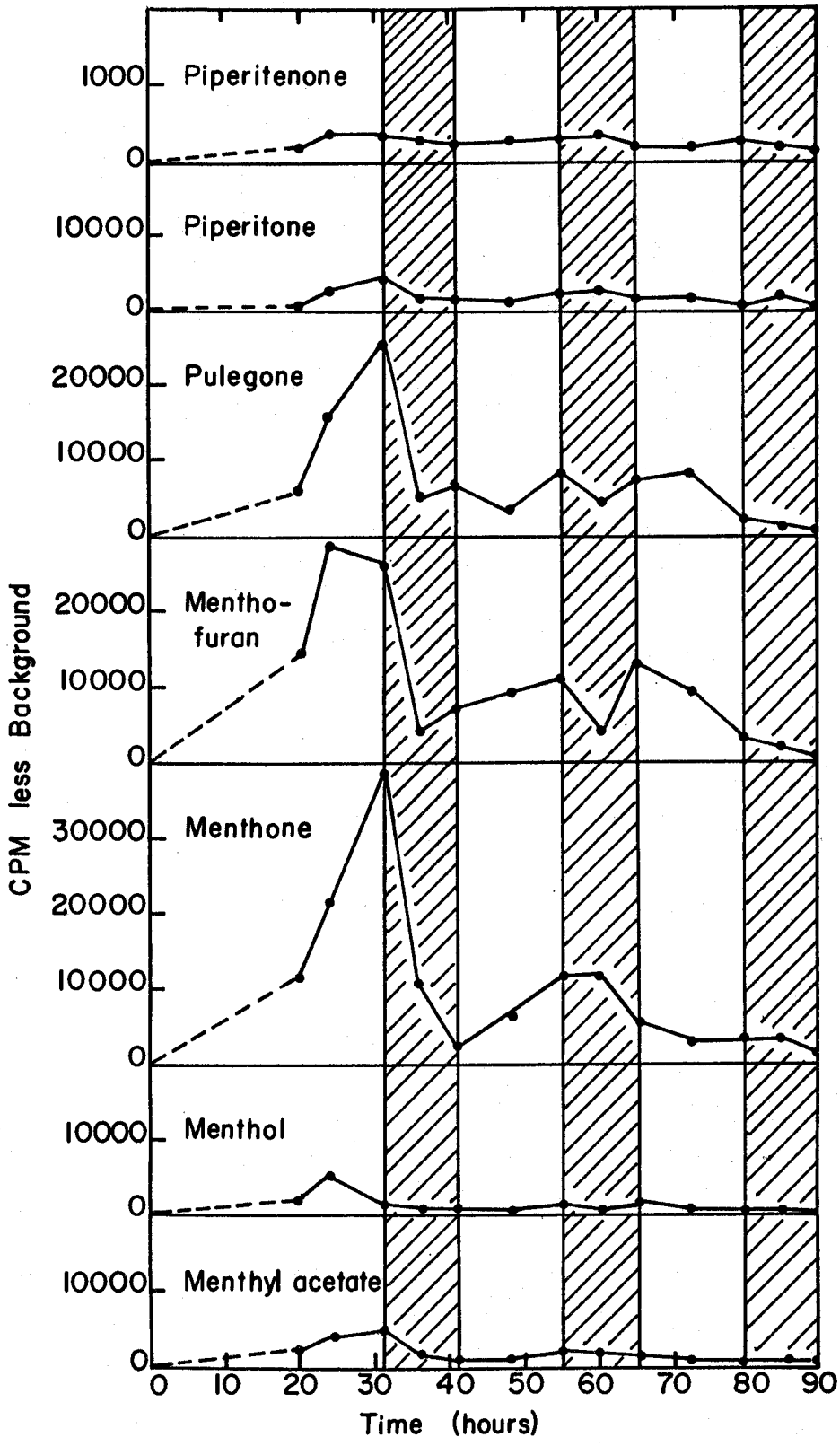

Piperitenone
1000
0
Piperitone
10000
0
Pulegone
20000
10000
0
Mentho-
furan
20000
10000
0
Menthone
30000
20000
10000
0
Menthol
10000
0
Menthyl acetate
10000
0
CPM less Background
0 10 20 30 40 50 60 70 80 90
Time (hours)

Figure 14

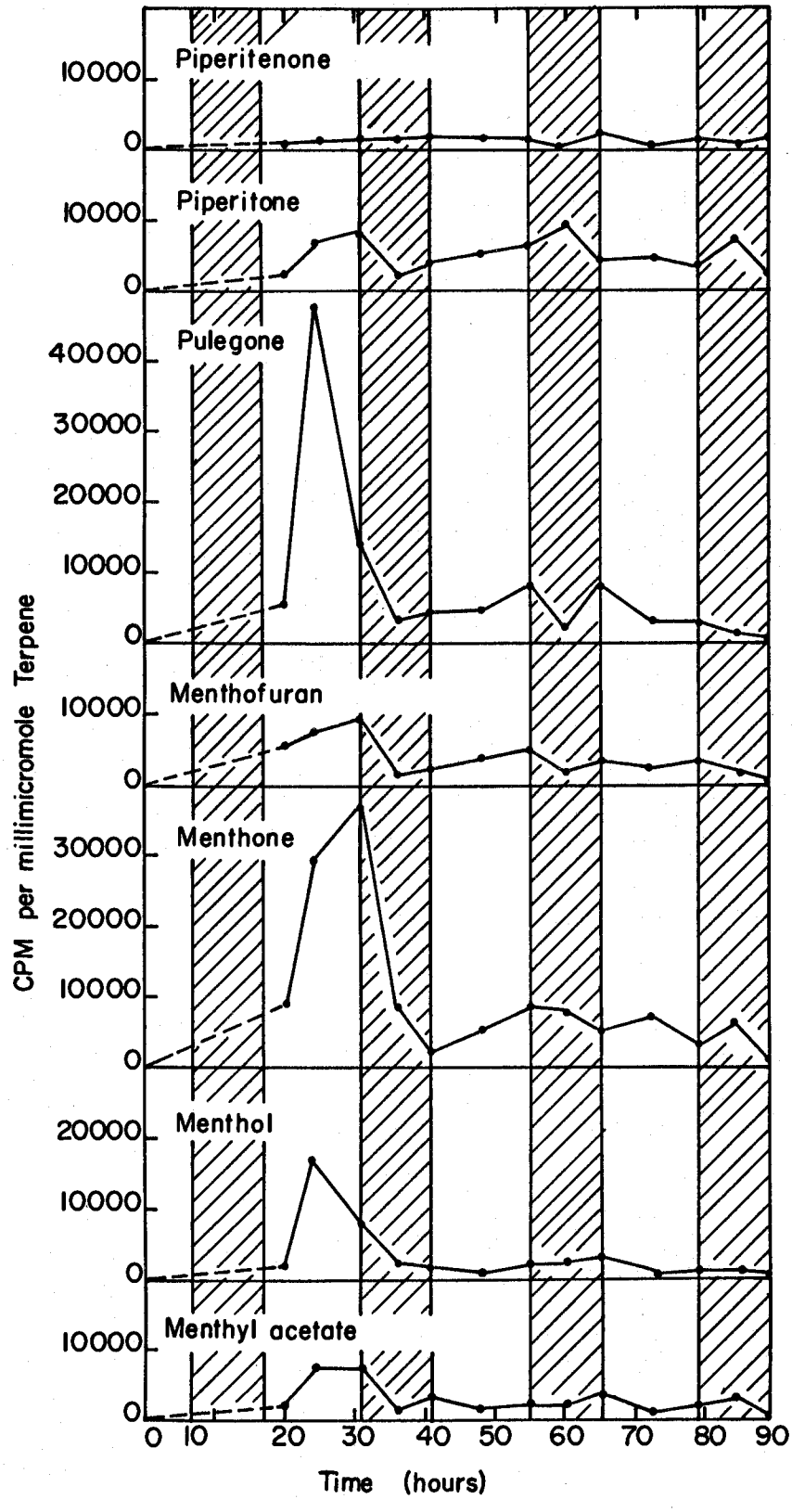
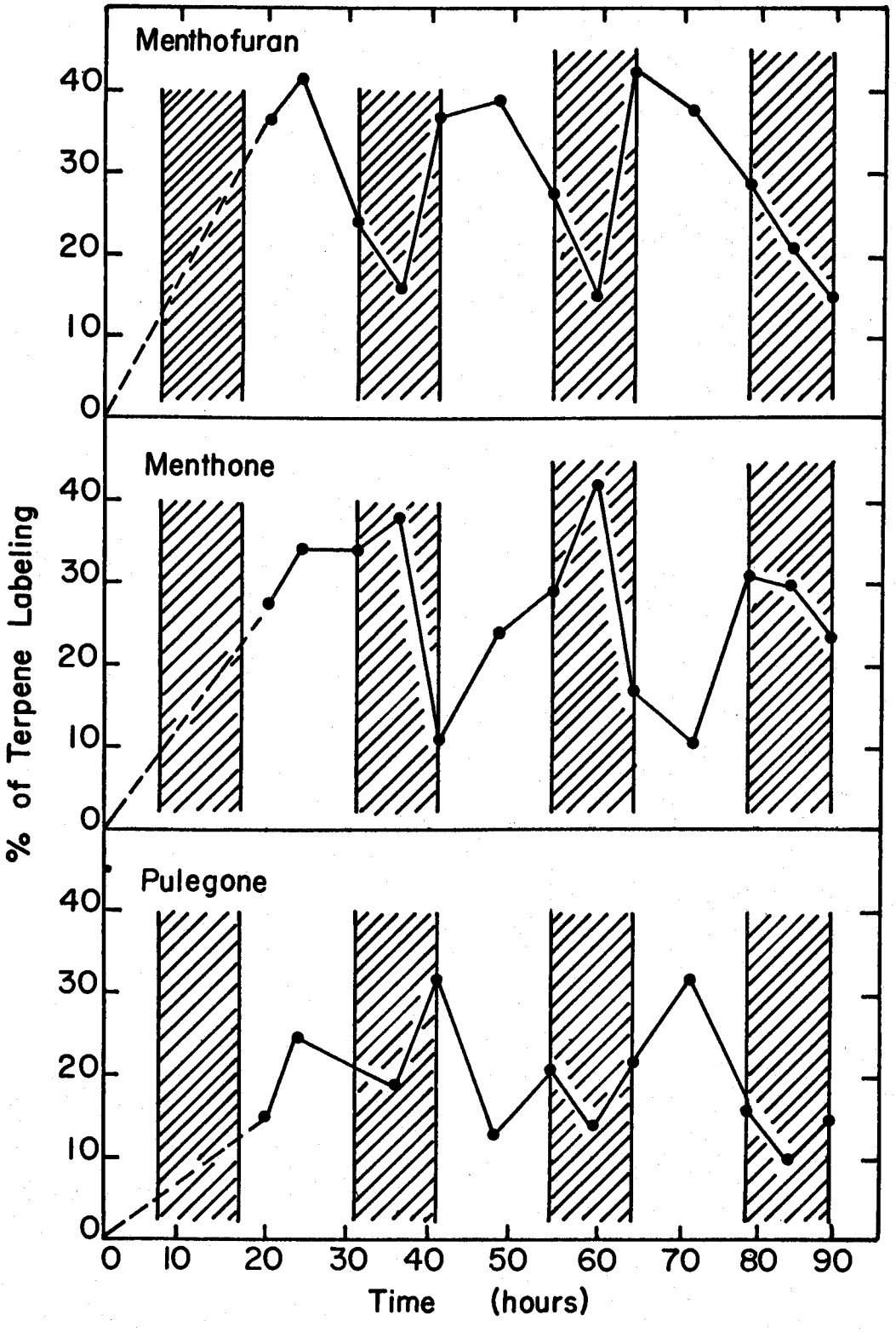

Piperitenone
10000
0
Piperitone
10000
0
Pulegone
40000
30000
20000
10000
0
Menthofuran
10000
0
Menthone
30000
20000
10000
0
Menthol
20000
10000
0
Menthyl acetate
10000
0
CPM per millimicromole Terpene
0 10 20 30 40 50 60 70 80 90
Time (hours)

Figure 15


Menthofuran
Menthone
Pulegone
% of Terpene Labeling
0
10
20
30
40
0 10 20 30 40 50 60 70 80 90
Time (hours)

by menthofuran and pulegone during the dark period was verified in a second similar experiment. The menthol fraction displayed a greater tendency to peak during the dark period in the second experiment than in the experiment shown here.

Reitsema (15) showed conversion of menthone to menthol by spotting a hexane solution of the labeled substrate on peppermint leaves. Battaile and Loomis (3) demonstrated the conversion of piperitenone to piperitone, and pulegone to menthone using a technique in which mint leaves were minced under water and floated on aqueous slurries containing the labeled substrate and silicic acid on which the terpenes were separated. Attempts to follow Reitsema's procedure indicated that even such small quantities as one microliter of the pure terpene severely damaged the leaf tissue. Also, since the terpenes to be used as substrate were separated on silicic acid chromatoplates, the use of Battaile's technique avoided the loss of labeled material encountered in removing the terpenes from the silicic acid.

Labeled terpenes to be used as substrates in the interconversion experiments were synthesized by exposing

thirty young peppermint cuttings (including the buds and the first five leaf pairs) and a like number of M. pulegium cuttings to one millicurie of $C^{14}O_2$ for a period of five days. At the end of this time the cuttings were ground and extracted with Skellysolve B. The extract was then concentrated to 100 ml. Aliquots of five or ten ml of this extract were further concentrated and separated on silicic acid chromatoplates just before use. Plate counting with the gas-flow counter of a concentrate of ten ml of this extract gave activities as follows: menthofuran including hydrocarbons, 183,000 cpm; menthone, 92,000 cpm; pulegone; 117,000 cpm; piperitone, 18,000 cpm; menthol, 80,000 cpm; and piperitenone, 86,000 cpm. The extract was spotted across the whole width of the plate at the origin, and after separation and location of the terpenes the plate was divided in half vertically, with one-half of the silicic acid-terpene slurry being used for the experimental sample and the other half for the control.

Mint tissue was chosen with regard to the terpene to be used as substrate in an attempt to obtain leaves at an age when they would normally metabolize the terpene

in question:

Substrate	Mint Tissue				
	Bud	Leaf Pair			
		1	2	3	4
Hydrocarbon	x	x	x		
Piperitenone	x	x	x		
Pulegone		x	x	x	
Piperitone		x	x	x	
Menthofuran				x	x
Menthone				x	x

When available, the fourth through the seventh leaf pairs were used with menthol as substrate; otherwise the oldest leaf pairs on the shoots were used. When the larger leaves were used, the leaf was cut along the midrib and only half of the leaf used in the sample. Although the number of oil glands in the sample tissue was decreased by this practice, it kept the volume of leaf tissue more uniform and manageable and at the same time allowed the use of the same number of shoots in each sample. Five or six leaf pairs were used for each sample.

In each experiment the chosen mint tissue was minced under three ml of distilled water, the labeled terpene along with silica gel from the chromatoplates was added and mixed, and the sample was incubated for sixteen hours in the light chamber. Controls for each experiment consisted of steam-killed mint leaves, three ml of

distilled water and the slurry containing the appropriate terpene. In some cases the steamed mint was omitted from the control. Data from these experiments are given in Tables I through VII.

When extracts were fractioned on the gas chromatograph, not only the terpene peaks but also material eluted from the column between peaks was trapped and counted. This was done to check on trailing of the terpene peaks, to detect any highly labeled material which was not one of the terpenes and to prevent the escape of radioactive material into the laboratory. Terpene peaks were trapped in their entirety. When a fairly long time elapsed between two terpene peaks, the interval was divided into two fractions. Eighteen fractions were thus trapped routinely during the analysis of the mint extracts.

Two events were consistently noticed throughout the interconversion experiments. Firstly, although there was a loss of labeled material in all samples (sometimes as little as ten percent of the label introduced was recovered), the mint tissue in some manner protected the substrate, and substantially greater

amounts of label were recovered in the experimental samples than in the controls. In almost all of the experiments tabulated in the tables, this is well illustrated. Secondly, a varying proportion of the label was found in the fraction eluted from the column just before piperitenone. No peak in this area was ever detected on the gas chromatograph trace and no terpene has been shown to have a retention time on this column which would place it in this area. The activity in this fraction varied from experiment to experiment and with each substrate, but at times it contained the largest activity of any of the fractions. Substantial activity was never found in this area in the $C^{14}O_2$ time-courses. In preliminary experiments in which thin-layer chromatograms were used for separation of the extracts from the interconversion experiments, a material consistently appeared with an Rf value immediately below that of piperitenone. Battaile also noted the appearance of this material and considered it an oxidation product of the labeled terpene. It may be that this spot indicates the same material that eluted just before piperitenone on the gas

chromatograph column.

Pulegone proved to be the most readily utilizable substrate in the conversion experiments. In all five experiments in which pulegone was used as the substrate significant labeling was found in the menthone fraction. In two of the experiments label was found in the menthofuran fraction in small quantities. Experiment 1 of Table I gives the data for one of these experiments. The mint tissue used for this experiment contained about three times as much menthofuran as menthone (1.54 millimicromoles of menthofuran to 0.42 millimicromoles of menthone). The data of the time-courses suggested that menthol is formed most rapidly in the dark, therefore an interconversion experiment was carried out in the dark. Experiment 2 of Table I gives the data for this experiment.

Piperitenone when used as substrate showed, in all cases, incorporation of label into the menthone and pulegone fractions, but, as the data for Experiment 2 of Table II show, label was sometimes encountered in the pulegone fraction of the control. The piperitone fraction had activity in only two out of the five

TABLE I

Substrate: Pulegone

Experiment:		1			2		
Illumination:		Light			Dark		
Fraction	Component	Sample cpm	Control cpm	△ cpm	Sample cpm	Control cpm	△ cpm
1	Solvent	0	0	0	10	0	0
2	Hydrocarbon	0	0	0	10	40	-30
3	Pinene	0	0	0	0	0	0
4	Cineole	20	0	20	40	0	40
5		0	0	0	10	0	10
6		0	0	0	0	0	0
7	Menthofuran	600	0	600	50	60	-10
8	Menthone	5,300	10	5,290	1,400	200	1,200
9		200	40	160	110	90	20
10	Menthyl acetate	700	100	600	350	150	200
11		150	50	100	90	90	0
12	Menthol	850	100	750	12,700	50	12,650
13	Pulegone	7,100	7,500	-400	22,100	4,100	18,000
14		500	600	-100	2,300	800	1,500
15	Piperitone	200	250	-50	1,500	200	1,300
16		100	300	-200	700	200	500
17		100	100	0	250	50	200
18	Piperitenone	100	100	0	200	50	150

TABLE II

Substrate: Piperitenone

Experiment:		1			2		
Illumination:		Light			Light		
Fraction	Component	Sample cpm	Control cpm	△ cpm	Sample cpm	Control cpm	△ cpm
1	Solvent	500	150	350	60	80	-20
2	Hydrocarbon	150	50	100	50	20	30
3	Pinene	0	0	0	0	0	0
4	Cineole	300	80	220	0	20	-20
5		400	100	300	20	10	10
6		250	30	220	50	100	-50
7	Menthofuran	500	300	200	250	50	200
8	Menthone	2,350	600	1,750	300	100	200
9		1,000	400	600	100	60	40
10	Menthyl acetate	600	370	230	130	70	60
11		230	200	30	0	0	0
12	Menthol	600	220	380	60	60	0
13	Pulegone	1,650	300	1,350	160	150	10
14		220	200	20	30	70	-40
15	Piperitone	800	300	500	230	190	40
16		2,300	200	2,100	150	200	-50
17		5,100	800	4,300	300	200	100
18	Piperitenone	4,800	700	4,100	300	300	0

experiments and always in lesser amounts than pulegone. This is contrary to Battaile's experience (1, p. 75). When Battaile exposed mint tissue to C^{14}-labeled piperitenone, about thirty percent of the substrate was converted to piperitone, with no trace of the label in pulegone.

The piperitone fraction of the labeled oil used for substrate had low incorporation of the label. Consequently levels of activity recovered from experiments in which piperitone was used as substrate were too low to be relied upon. The only consistent result was the occurrence of label in the menthone fraction in four out of the five experiments.

Menthone did not give rise to much incorporation of label into the menthol fraction. In four of the five experiments, the greatest incorporation of the label was found in the menthyl acetate peak. It was observed in experiments in which menthol was used as substrate that the control sample often gave evidence of high activity in the menthone fraction and that menthol could apparently be oxidized non-enzymatically to menthone. A small quantity of label was found in

TABLE III

Substrate: Piperitone

Experiment:		1			2		
Illumination:		Light			Light		
		Sample	Control	△	Sample	Control	△
Fraction	Component	cpm	cpm	cpm	cpm	cpm	cpm
1	Solvent	600	200	400	0	0	0
2	Hydrocarbon	300	100	200	0	0	0
3	Pinene	-	-	-	0	0	0
4	Cineole	80	40	40	0	0	0
5		250	50	200	0	0	0
6		300	50	250	50	110	60
7	Menthofuran	200	130	70	30	70	40
8	Menthone	600	200	400	70	40	30
9		220	200	20	0	0	0
10	Menthyl acetate	900	600	300	30	0	30
11		-	-	-	0	0	0
12	Menthol	530	200	330	100	80	20
13	Pulegone	640	280	60	260	80	180
14		200	300	-100	20	70	50
15	Piperitone	700	770	-70	330	40	290
16		1,750	700	1,050	50	60	-10
17		1,650	800	850	70	240	70
18	Piperitenone	2,050	1,000	1,050	1,100	1,400	300

TABLE IV

Substrate: Menthone

Experiment:		1			2		
Illumination:		Light			Light		
Fraction	Component	Sample cpm	Control cpm	△ cpm	Sample cpm	Control cpm	△ cpm
1	Solvent	230	100	130	0	0	0
2	Hydrocarbon	130	140	-10	0	20	-20
3	Pinene	140	0	140	0	0	0
4	Cineole	200	30	170	110	20	90
5		180	10	170	0	0	0
6		-	-	-	60	10	50
7	Menthofuran	250	140	110	100	10	90
8	Menthone	17,700	10,000	7,700	9,150	2,700	6,450
9		1,500	1,200	300	180	90	90
10	Menthyl acetate	2,600	200	2,400	750	90	640
11		-	-	-	170	0	170
12	Menthol	700	340	360	160	40	120
13	Pulegone	800	260	540	300	30	270
14		270	270	0	110	0	110
15	Piperitone	460	400	60	180	0	180
16		1,300	400	900	70	10	60
17		1,900	300	1,600	60	20	40
18	Piperitenone	1,300	600	700	40	0	40

cineole in all experiments in which menthone was used as substrate. Reitsema (15) reported conversion of menthone to menthol using an infiltration method.

Menthofuran is extremely sensitive to auto-oxidation (17) and the most consistent result in experiments with menthofuran as substrate (Table V) was the protection of menthofuran by the plant tissue in the light. Only in Experiment 2, carried out in the dark, was this protective effect absent. Here, the greatest portion of the label is to be found in the unidentified fraction proceeding piperitenone. No consistent incorporation of label into any of the terpene fractions was found in the menthofuran experiments.

Reitsema, et al (15) reported no success in the use of menthol as a substrate for conversion experiments. In the four experiments in which menthol was used as substrate in this investigation (Table VI) the only consistent result was the protective effect also not with menthofuran. This may be due to enzymatic reduction of menthone produced by non-enzymatic oxidation of the substrate. Experiment 1 of Table VI shows the

TABLE V

Substrate: Menthofuran

Experiment:		1			2		
Illumination:		Light			Dark		
Fraction	Component	Sample cpm	Control cpm	△ cpm	Sample cpm	Control cpm	△ cpm
1	Solvent	10	30	-20	120	80	40
2	Hydrocarbon	40	60	-20	100	80	20
3	Pinene	80	30	50	130	110	20
4	Cineole	50	30	20	380	100	280
5		0	0	0	160	170	-10
6		0	0	0	180	100	60
7	Menthofuran	1,400	180	1,220	240	400	-160
8	Menthone	400	130	270	600	300	300
9		20	0	20	400	230	170
10	Menthyl acetate	70	60	10	600	400	200
11		130	110	20	300	200	100
12	Menthol	60	50	10	470	130	340
13	Pulegone	90	70	20	750	300	450
14		100	120	-20	-	-	-
15	Piperitone	100	70	30	950	300	650
16		40	70	30	2,250	300	1,950
17		40	50	-10	1,800	400	1,400
18	Piperitenone	20	80	-60	1,200	500	700

TABLE VI

Substrate: Menthol

Experiment:		1			2		
Illumination:		Light			Dark		
Fraction	Component	Sample cpm	Control cpm	△ cpm	Sample cpm	Control cpm	△ cpm
1	Solvent	50	60	-10	10	70	-60
2	Hydrocarbon	40	60	-20	0	0	0
3	Pinene	0	0	0	40	10	30
4	Cineole	440	70	370	30	0	30
5		-	-	-	10	50	-40
6		170	140	30	0	20	-20
7	Menthofuran	350	70	280	90	30	60
8	Menthone	530	280	250	0	80	-80
9		200	240	-40	20	90	-70
10	Menthyl acetate	410	240	170	180	140	40
11		-	-	-	0	20	-20
12	Menthol	1,880	700	1,180	1,260	480	1,760
13	Pulegone	600	400	200	1,500	50	1,450
14		360	120	240	220	100	120
15	Piperitone	680	500	180	170	80	90
16		-	-	-	90	90	0
17		1,370	1,210	1,160	80	80	0
18	Piperitenone	50	30	20	50	30	20

incorporation of label, from menthol, into cineole. Experiment 2 of the table was carried out in the dark. Considerable labeling of pulegone is seen.

The fraction designated as the hydrocarbon fraction is known to contain at least three peaks (one of them, limonene) which are not separated in the extract. However, it was noted in preliminary experiments that this mixed fraction sometimes had high activity early in the time-courses, and therefore it was tested as a substrate in the interconversion experiments. Experiment 1 of Table VII seems to indicate definite incorporation of label into menthone, menthyl acetate, pulegone, and piperitone. The label in piperitenone may indicate incorporation into piperitenone or trailing from the previous high level of activity in the preceding fraction. Experiment 2 of the table would seem to support definite labeling in both piperitone and piperitenone.

During separation of the mint extracts in the preceding experiments, a small but definite peak following piperitone was noticed on the gas chromatograph trace. This peak was largest in the young leaf tissue and was not present in the standard Mitcham peppermint oil used

TABLE VII

Substrate: Hydrocarbon Fraction

Experiment:		1			2		
Illumination:		Light			Light		
Fraction	Component	Sample cpm	Control cpm	Δ cpm	Sample cpm	Control cpm	Δ cpm
1	Solvent	350	180	170	140	20	120
2	Hydrocarbon	300	60	240	50	50	0
3	Pinene	280	90	180	0	0	0
4	Cineole	140	90	50	10	0	10
5		140	90	50	10	30	-20
6		130	70	60	0	30	-30
7	Menthofuran	470	150	320	30	60	-30
8	Menthone	2,350	120	2,130	50	50	0
9	Menthyl acetate	1,200	150	1,050	130	70	60
10		500	170	330	-	-	-
11	Menthol	450	160	290	60	100	-40
12	Pulegone	1,200	300	900	150	70	80
13	Piperitone	1,000	150	850	300	130	170
14		2,000	430	1,570	100	100	0
15		10,000	1,500	8,500	200	100	100
16	Piperitenone	7,500	800	6,700	420	250	170

to calibrate the column. On further investigation this peak proved to have the same retention time as geraniol, and in addition, it gave the same Rf value as geraniol when trapped on silica gel plates and chromatographed in the ethyl acetate-Skellysolve B system. This material gave the reaction as the standard geraniol to the sprays used to detect terpenes on thin-layer chromatograms. Although geraniol has never been reported as a component of peppermint oil, these findings suggested testing geraniol as a substrate in an interconversion experiment. Mint leaves from the terminal bud to the third leaf pair were used for this experiment. The exposure mixture, control sample, and incubation procedure and timing conformed to those previously stated. The data from this experiment are given in Table VIII.

Recovery of label when geraniol was supplied as substrate was never high, but the data indicate that geraniol can be metabolized by M. piperita. Citronellal, an isomer of geraniol, has the same retention time on this column as menthone. Therefore, the label incorporated into the "menthone" fraction could be in

TABLE VIII

Substrate: Geraniol
Illumination: Light

Fraction	Component	Sample cpm	Control cpm	△ cpm
1	Solvent	10	20	-10
2	Hydrocarbon	20	0	20
3	Pinene	0	0	0
4	Cineole	0	0	0
5		10	0	10
6		30	0	0
7	Menthofuran	20	30	-10
8	Menthone	800	50	750
9	Menthyl acetate	200	20	180
10	Menthol	70	20	50
11	Pulegone	160	40	120
12		80	50	30
13	Piperitone	370	170	200
14	Geraniol	2,700	1,200	1,500
15		420	180	240
16		200	120	80
17	Piperitenone	170	10	160

either or in both. Further investigation will be necessary to clarify this point.

If the waxy cuticle covering the peppermint leaves could be softened or removed without damaging the tissue, the mint leaf might become more permeable to the labeled terpene and allow greated incorporation of the substrate. With this in mind, various solvents, chilled with either ice or a dry ice-chloroform mixture, were used to soak the mint leaves prior to incubation.

Benzene, chloroform, and carbon tetrachloride damaged the mint tissue severely and no conversion of labeled substrate occurred with tissue thus treated. Acetone, when chilled to -40° C, froze the mint leaves immediately on contact and when the leaves were thawed under water the tissue appeared undamaged, though somewhat translucent. Unfortunately the tissue thus treated was subject to severe enzymatic browning during the long incubation period required for the interconversion experiments. Addition of adenosine triphosphate ascorbate, buffer systems, sulfhydryl protectors such as 2-mercaptoethanol, and the use of a nitrogen atmosphere did not significantly reduce this browning. Interconversion by the acetone-treated leaves was little if any higher than that resulting with untreated leaves and in later experiments the tissue was not pretreated with solvents.

However, encouraging results were obtained with 2-C^{14}-mevalonate as substrate for these solvent treated leaves. Radioautograms revealed traces of label in the piperitenone, piperitone, and pulegone spots on the chromatograms of the extract. In view of previous

failures to demonstrate incorporation of mevalonate into monoterpenes of peppermint (1, 4), it appears that this technique merits further investigation.

DISCUSSION

Considering the labeling sequences of the two time-courses together, it appears that piperitenone and pulegone are the first oxygenated terpenes to be labeled by $C^{14}O_2$, while the label appears somewhat later in menthone. The early peak of activity which the menthol fraction displays is undoubtedly due to the unidentified material which was discovered when the gas chromatograph peak was trapped and separated by thin-layer chromatography, and as suggested above, the early activity in menthyl acetate may be due to labeling of the acetate moiety. Thus, the true sequence of labeling would appear to be piperitenone and pulegone, menthone, menthol, and menthyl acetate. Piperitone and menthofuran, on the basis of specific activity peaks, can be assigned no definite place in the sequence. The maxima displayed by these two terpenes in the one-hour time-course are slight and it is possible that these

terpenes may not have attained peak incorporation of the label within the duration of this experiment. However data from the time-courses would place them somewhere in the middle of the time sequence, after piperitenone and pulegone, and before menthol and menthyl acetate.

The distribution of labeling in the time-courses and the interconversion experiments strongly supports Reitsema's scheme for the conversion, by hydrogenation, of pulegone to menthone and menthone to menthol in M. piperita. Pulegone has been shown directly to be converted to menthone, and menthone to menthol and menthyl acetate. The time-course data show that activity arises later in menthyl acetate than in menthol, and menthyl acetate may, therefore, be one step further removed from menthone than is menthol. However, in the interconversion experiments, menthyl acetate was formed from menthone but not from menthol. The failure of menthol to be metabolized in these experiments or in Reitsema's experiments may be a result of its relatively high polarity and water solubility, which may prevent it from entering the oil glands.

Reitsema (11) considers piperitenone to be the first terpene characteristic of the peppermint type oil, and the other terpenes to be derived from it. The conversion of piperitenone to either piperitone or pulegone would entail the hydrogenation of one of two double bonds. As previously noted, Battaile showed the conversion of piperitenone to piperitone. The data from interconversion experiments in this study, with piperitenone as substrate, show incorporation of label into both pulegone and menthone, and to a lesser extent, into piperitone. However, the specific activity of the precursor should be equal to if not higher than the specific activity of the product. Only in the one-hour time-course is the specific activity of piperitenone equal to that of pulegone. In the longer time-courses the specific activity of pulegone rises much faster than that of piperitenone and is much higher. In all of the time-courses the specific activity curves of piperitenone and pulegone reach a maximum in the same time period. Thus the time-course data do not support the production of pulegone by the reduction of piperitenone.

Results reported here as well as Battaile's results, indicate that pulegone is a precursor of menthone. The relationship of labeling patterns between menthone and menthofuran observed in the time-courses suggests a close metabolic relationship between the two compounds. Evidence to support the production of menthofuran from pulegone is less clear. The bulk of the label in all the time-courses is to be found in pulegone, menthone and menthofuran; these terpenes are quantitatively the major components of the extracts. The specific activity of the menthofuran fraction is significantly less than those of pulegone and menthone. When pulegone was the substrate in interconversion experiments, label was incorporated into the menthofuran fraction only in leaves which yielded as the major component of the extract. Lemli (9) reports that menthofuran is a substance secreted by very young tissue in which metabolism is most active. Pulegone might, then, form either menthone or menthofuran, depending on the physiological condition of the tissue. The data of the light-dark time-courses and the experiment in which mint was grown at low temperature suggest

that environmental conditions play a role in diverting the conversion of pulegone from menthone to menthofuran.

As in the case of pulegone, the conversion of piperitone to menthone requires the hydrogenation of a double bond. Here again the specific activities of the postulated precursor are lower than those of the product and seem to suggest that little if any menthone is produced by this method. The results of interconversion experiments are inconclusive and neither support nor disprove this contention.

In short term time-courses the earliest peak of activity appeared in the menthol fraction. As noted above, this fraction was found to contain a second component which was not menthol, and the early labeling was shown to be in this contaminant. Battaile (1) reported early labeling in a material he tentatively suggests is citral. The Rf value on thin-layer plates of the material found in the menthol peak is approximately the same as Battaile's material. However commercial citral has been found to have a somewhat longer retention time on the gas chromatograph column used in this study. Battaile found this material only when the

tissue to be extracted was dropped immediately into boiling Skellysolve B at a temperature of 70° C at the conclusion of the experiment. This technique was never used in the present studies. There is not enough evidence to identify the material found in the menthol fraction with Battaile's early-labeled material, however both these materials would seem to warrant additional investigation to identify them and trace their roles, if any, in terpene synthesis.

The one terpene hydrocarbon, β -pinene, trapped separately proved to incorporate label only in the later stages of the time-courses, and the data from these experiments give no hint of a relationship between β -pinene and any of the oxygenated terpenes. The fraction designated "hydrocarbon" is a mixture of components in which only limonene has been identified. The early activity in this fraction and the incorporation of label into oxygenated terpenes when it was used as substrate suggests that this fraction may contain a precursor of the oxygenated terpenes. Reitsema (15) infiltrated C^{14}-labeled pinene and limonene into M. piperita leaves and observed incorporation of the

label into the menthol, menthone, pulegone, and two other areas. However, Reitsema noted that this type of oxidation occurred "in vitro" also.

One of the most striking features of the long term time-courses was the disappearance of label in the later stages of the experiments. The nine hour time-course and a second fourteen hour experiment showed decrease in total label after the peak of label incorporation into menthone, at the sixth hour, had passed. The great bulk of the label incorporated into menthone, did not pass on into menthol and menthyl acetate. The mint tissue used in these time-courses synthesized only minimal quantities of menthol and menthyl acetate and it may be that conditions were such that conversion of only small quantities of the menthone was possible. The volume of individual terpenes in the leaves is almost constant in the samples. The sixth hour sample of the nine-hour time-course contained 0.35 millimicromoles of menthone and the ninth hour sample, 0.38 millimicromoles of menthone, yet the menthone fraction in sample 6 has an activity of 60,000 cpm and the menthone fraction in sample 9,

an activity of 2,200 cpm. Thus it would appear that menthone present in sample 9, has been synthesized in a large part from unlabeled precursors. The menthone synthesized earlier from labeled precursors has been removed in some manner.

Reitsema (15) reports that significant quantities of peppermint oil are lost continuously by evaporation. When he exposed mint cutting to $C^{14}O_2$ for a period of three hours and allowed two-thirds of the cuttings to continue to metabolize in a normal atmosphere for twenty-three hours, one percent of the activity originally in the oil was found in oil which had volatilized into the atmosphere. Chromatography of this oil showed essentially the same composition as the oil extracted from the plants. However, this rate of evaporation could not account for the large loss of label noted in the experiments reported here.

Again, in the light-dark experiment the sharp drop in radioactivity in the terpenes during the dark periods is not reflected by any quantitative loss in terpenes. Both the 31 hour sample (end of the first light period) and the 41 hour sample (end of the first dark period)

contain 0.27 millimicromoles of menthofuran, yet the 31 hour sample has an activity of 26,000 cpm and the 41 hour samples, an activity of 7,600 cpm. This again, suggests a specific loss of the labeled terpenes as distinguished from the unlabeled material. Perhaps the pool of labeled precursors has been largely depleted and the increase of labeling occurring during the second light period merely indicates fixation of labeled carbon dioxide formed by respiration during the night. This does nothing to explain the recovery of the label by the pulegone and menthofuran fraction in the second half of each dark period. Also the long initial period of fixation would be expected to produce a fairly large pool of labeled substrate material. The evidence would seem to point to a more rapid turnover and more active metabolic role for terpenes in M. piperita than was previously postulated.

SUMMARY

1. Analysis of the distribution of terpenes through leaf pairs of a single shoot supports Reitsema's contention that the first terpenes formed are

unsaturated ketones.

2. The growth of Mentha piperita under varying conditions and leaf-by-leaf analysis of the terpene composition of the oil demonstrate the effect of environmental factors on terpene formation.
3. Time-course studies using $C^{14}O_2$ support this portion of Reitsema's scheme: pulegone yields menthone yields menthol yields menthyl acetate.
4. The conversion of pulegone to menthone, menthone to menthol and menthyl acetate, and piperitenone to pulegone, menthone, and piperitone has been observed in peppermint tissue using labeled terpenes as substrates.

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