BIOSYNTHESIS OF TERPENES IN MINT

by

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BIOSYNTHESIS OF TERPENES IN MINT

INTRODUCTION

The term "terpene" in its most frequent usage indicates a plant product possessing an isoprenoid carbon skeleton containing from five to thirty carbon atoms. Other more comprehensive definitions may be found, all agreeing on the isoprenoid structure, but many extending the molecular size to include the forty-carbon carotenoids and even rubber. Lynen (33) extends the definition even further to include the steroids, which, similarly, are isoprenoid compounds, but are found in animals as well as plants.

Except for several five carbon compounds such as isovaleraldehyde and dimethylacrolein which are occasionally referred to as "hemiterpenes" or "semiterpenes", the simplest terpenes are the monoterpenes, isomers and derivatives of $C_{10}H_{16}$. Sesquiterpenes stem from $C_{15}H_{24}$. Di- and triterpenes contain twenty and thirty carbon atoms.

The various plants of the genus Mentha, the mints, are very rich sources of monoterpenes, especially oxygenated ones, some species producing characteristically linear terpenes and others cyclic compounds oxygenated

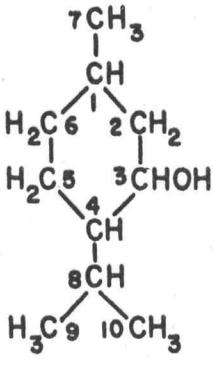
in either the two or the three position of the ring. The principal species used in this study, Mentha piperita or peppermint, produces some small amounts of linear terpenes and of cyclic terpene hydrocarbons, but the chief products are terpenes oxygenated in the three position.

Commercial peppermint oil, obtained by steam distillation of the mint hay, consists primarily of terpenes. The main constituents are menthol, menthone, menthyl acetate, and pulegone. However, numerous other terpenes have been found in the oil: menthofuran, piperitone, piperitenone, menthyl isovalerate, cineole, l-limonene, phellandrene, α- and β-pinene, terpinene, and the sesquiterpene cadinene (23, vol. 3, p. 616-638; 30; and 38, p. 1016). In addition the oil contains a number of simpler compounds which are not terpenes. Formulas for some of the terpenes are given in Figure 1.

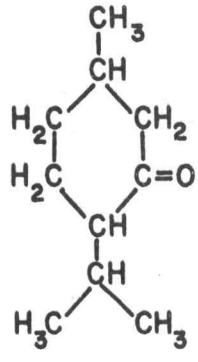
It has already been stressed that terpenes are isoprenoid compounds, a characteristic that they share with carotenoids, rubber, and steroids. Speculation has long held that these four classes of compounds have a common origin, probably in a five carbon compound with the same carbon skeleton as isoprene. Early investigations compared a number of these potential precursors, particularly in systems synthesizing cholesterol, but

FIGURE 1

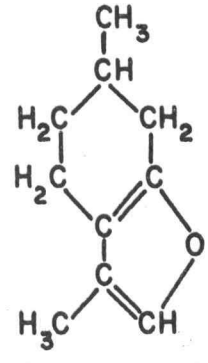
SOME TERPENES OCCURRING IN PLANTS OF THE GENUS MENTHA



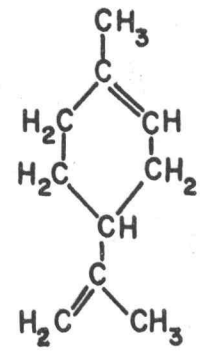
MENTHOL



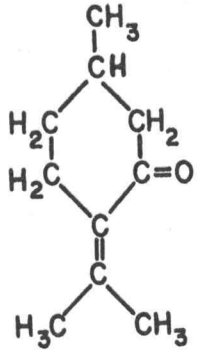
MENTHONE



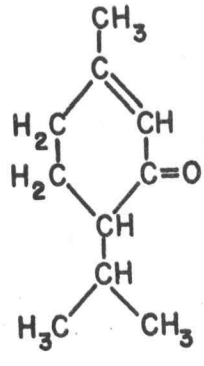
MENTHOFURAN



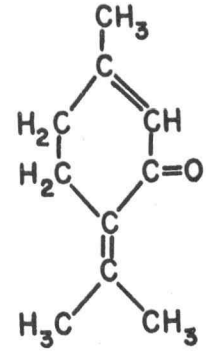
LIMONENE



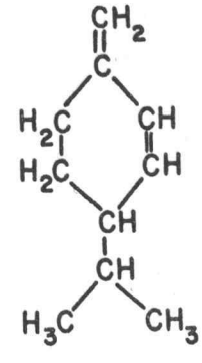
PULEGONE



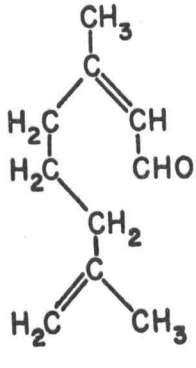
PIPERITONE



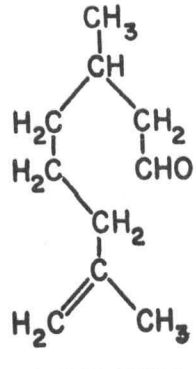
PIPERITENONE



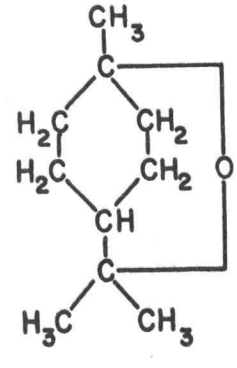
β-PHELLANDRENE



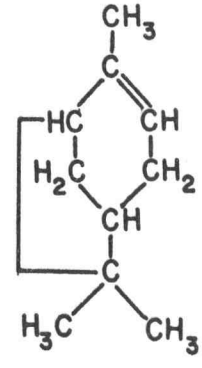
CITRAL



CITRONELLAL



1,8-CINEOLE



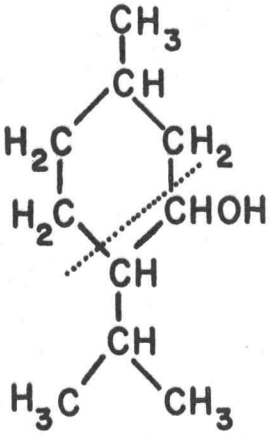
α-PINENE

also in systems producing other isoprenoid compounds. Evidence accumulated that a number of five carbon and also six carbon compounds could act as isoprenoid precursors (13 and 20), none too effectively, however. Among these were isovaleric acid (20), β-hydroxyisovaleric acid (7 and 20), β-methylcrotonic acid (also known as dimethylacrylic or senecioic acid) (14, 20, and 26), cis- and trans-β-methylglutaconic acid (20), β-hydroxy-β-methylglutaric acid (7, 14, 20, 26, 50, and 51), and leucine (40, p. 545-546). Figure 2 illustrates the isoprenoid structures of a typical monoterpene and of some possible precursors.

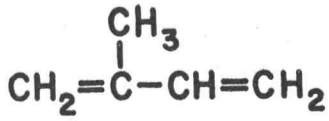
The relative efficacies of these various isoprenoid precursors did not leave a very clear picture of their metabolic connections. A semblance of order began to appear only after the discovery of mevalonic acid and the observation that it was almost completely incorporated into cholesterol (56 and 60). The recent review by Popják (40) and the Ciba Symposium on the Biosynthesis of Terpenes and Sterols (61) are quite helpful in organizing and clarifying results of recent investigations. Figure 3 gives the interrelationships among a number of these compounds according to the experimental information now at hand (19 and 62).

FIGURE 2

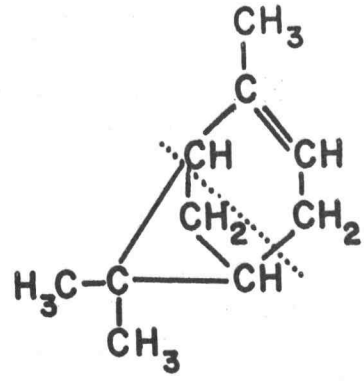
ISOPRENOID STRUCTURE OF MONOTERPENES AND POSSIBLE PRECURSORS



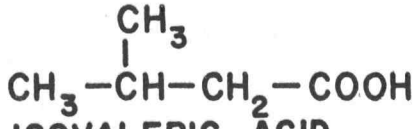
MENTHOL



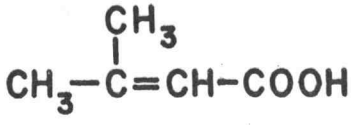
ISOPRENE



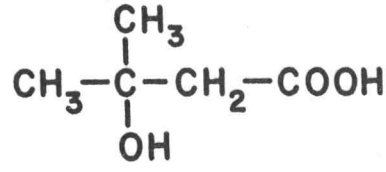
α-PINENE



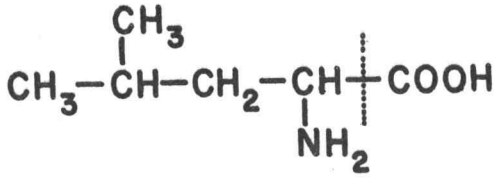
ISOVALERIC ACID



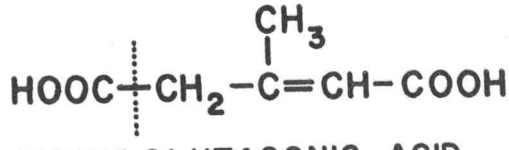
β-METHYLCROTONIC ACID



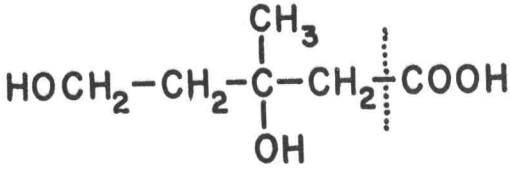
β-HYDROXYISOVALERIC ACID



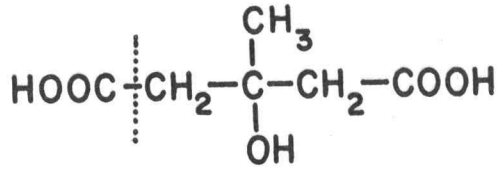
LEUCINE



β-METHYLGLUTACONIC ACID



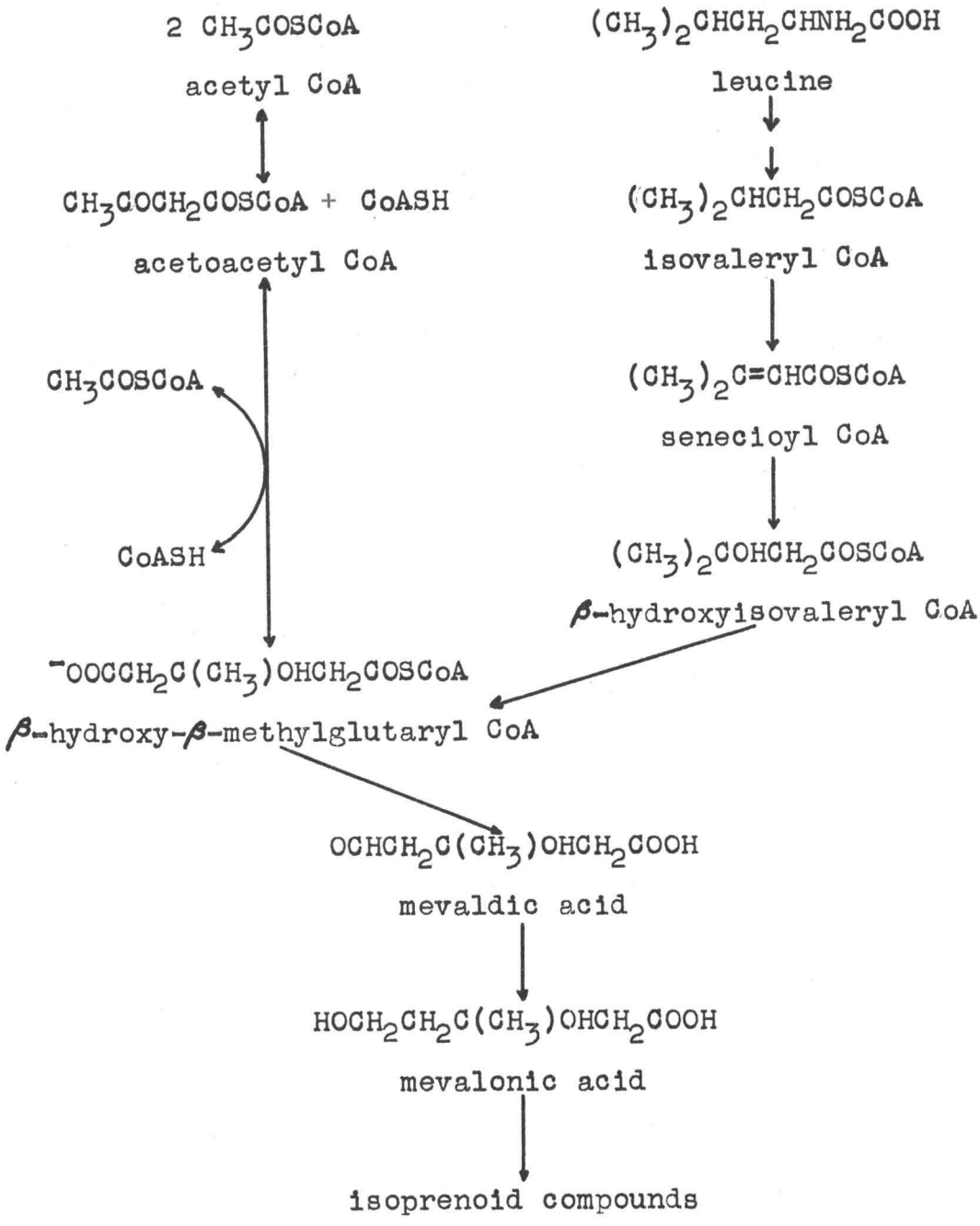
MEVALONIC ACID



β-HYDROXY-β-METHYLGLUTARIC ACID

FIGURE 3

METABOLISM OF ISOPRENOID PRECURSORS



Mevalonic acid (β,δ-dihydroxy-β-methylvaleric acid) has proved to be an excellent precursor not only of cholesterol (2, 27, 48, 49, 57, 58, and 59) but also of rubber (39), carotenoids (15, 16, and 22), and a number of isoprenoid compounds not conveniently classified among the broad groups mentioned: such compounds as felinine (5), mycelianamide (10 and 11), mycophenolic acid (10 and 11), and the olefinic acid recently described by Ogilvie (36 and 37). In addition there have lately appeared in the literature reports of its incorporation into terpenes. In all there are four reports of the incorporation of radioactive precursors into terpenes, and three of them involve mevalonic acid.

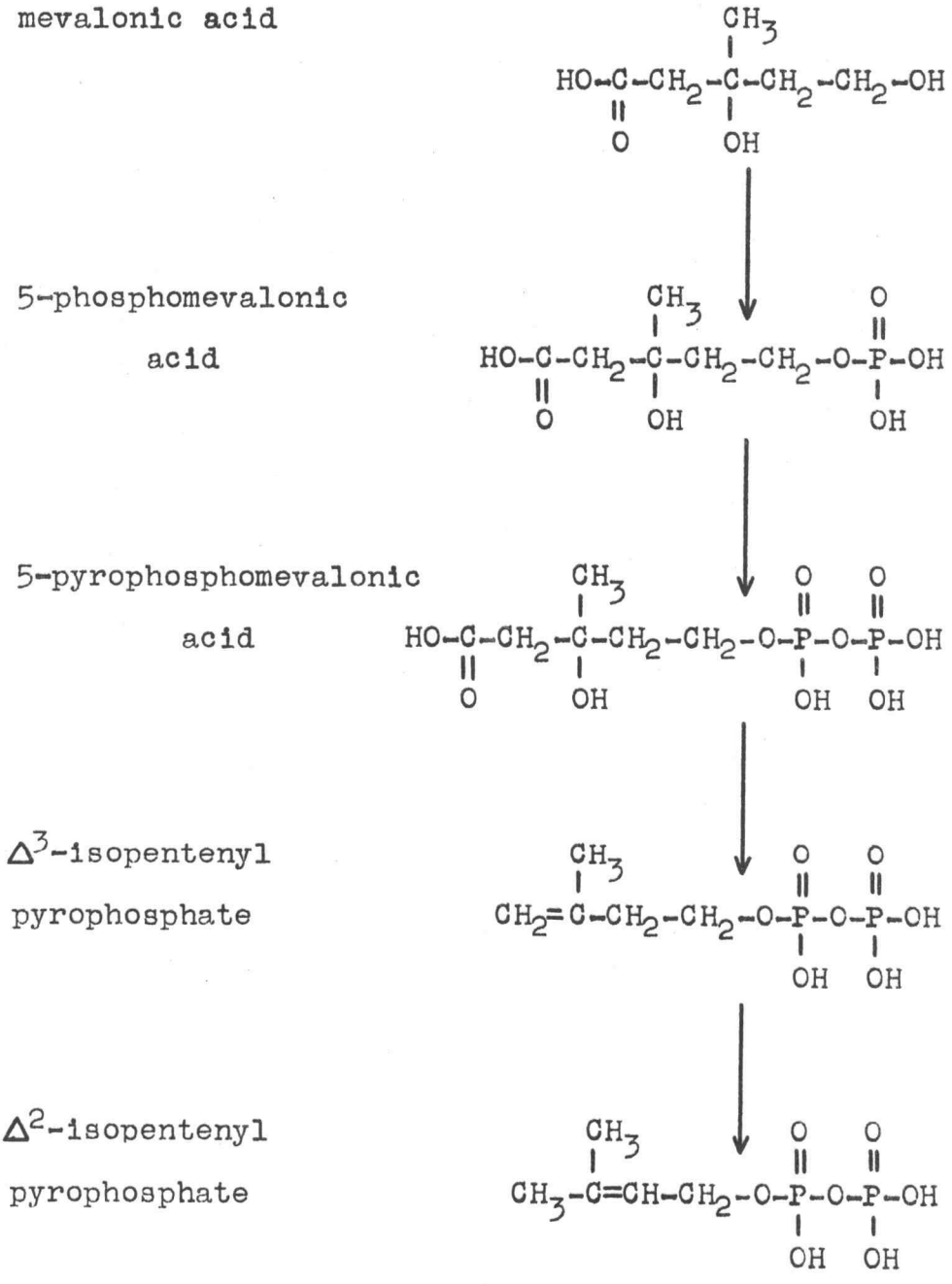
The first labeled terpenes were reported in 1956 by Sandermann and Stockmann (52), who secured labeling of pulegone in Mentha pulegium (pennyroyal) by administration of β-methylcrotonate-2-C^{14}. Partial degradation of the pulegone indicated that the incorporation was of the intact molecule, that no randomization through degradation to acetate had occurred. Arigoni reported in 1958 (3) the incorporation of mevalonic acid-2-C^{14} into a pentacyclic triterpene in soy beans. Degradation of the triterpene in this instance also indicated that no randomization through acetate had occurred. Later the same year Stanley (54) showed the incorporation of

radioactivity from mevalonic acid-2-C^{14} into α-pinene in Pinus attenuata in yields of 0.02 and 0.5 percent. Birch and co-workers (9) have reported quite recently the incorporation of mevalonic acid-2-C^{14} into 1,8-cineole in Eucalyptus globulus. Partial degradation of the cineole was consistent with the expected labeling pattern for incorporation of the intact molecule. A fifth report, by Sukhov (55), is not sufficiently specific to warrant inclusion with the other four.

If mevalonic acid is, as it appears, the precursor of terpenes, it is likely that the initial steps of its utilization are the same as those shown in cholesterol and ergosterol biosynthesis. Chiefly through the efforts of Bloch and his associates (12, 14, 48, 49) it has been shown that mevalonic acid is converted to 5-phosphomevalonate which in turn is converted to 5-pyrophosphomevalonate and then to Δ^3-isopentenyl pyrophosphate. Lynen reports the isomerization of this to Δ^2-isopentenyl pyrophosphate (1), with which he believes it condenses to form the ten-carbon cholesterol precursor (34). It may well be that either or both of these isopentenyl pyrophosphates enter similarly into the formation of terpenes. Figure 4 illustrates the first steps of mevalonate utilization as shown in the synthesis of cholesterol.

FIGURE 4

FIRST STEPS OF MEVALONATE UTILIZATION

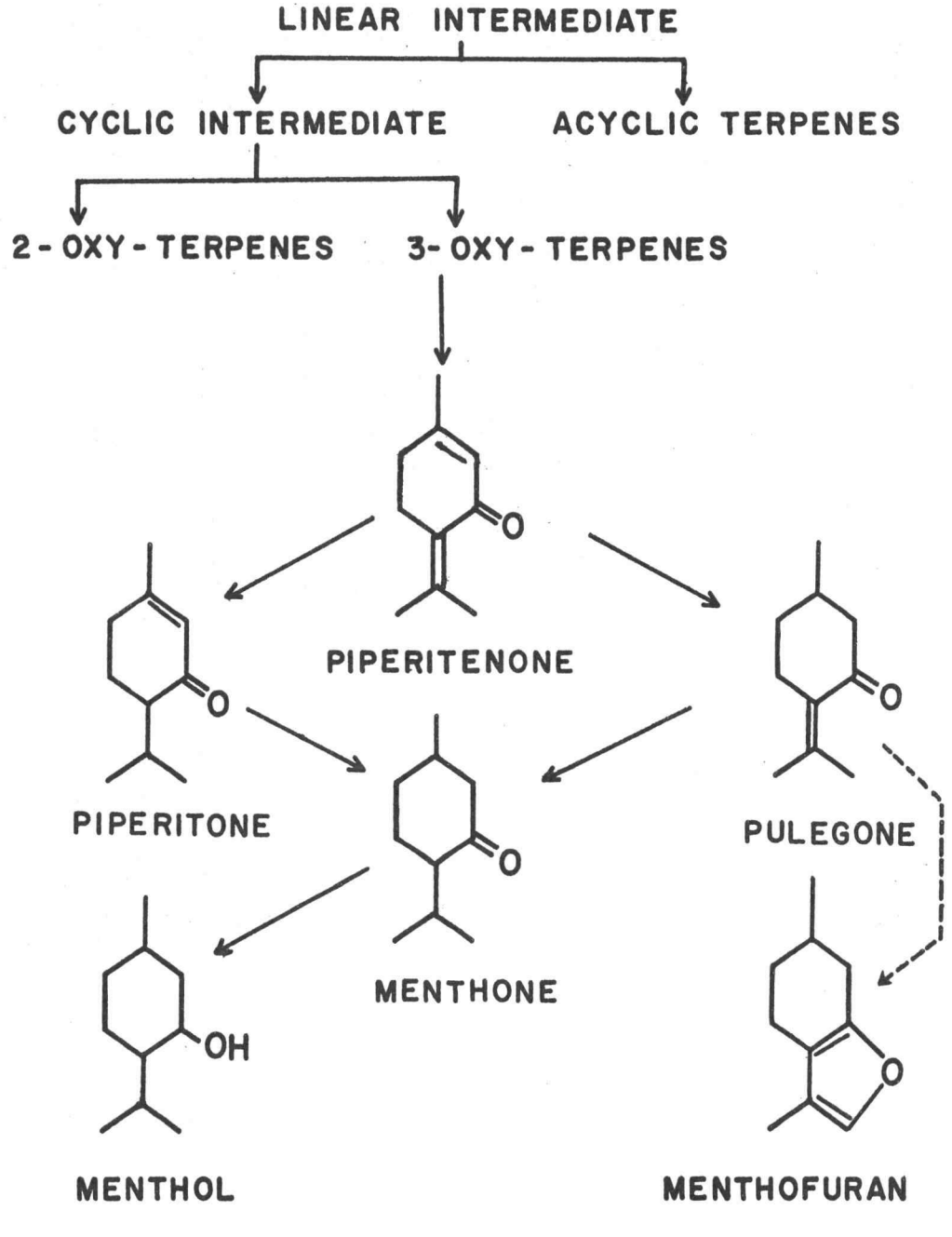


The works thus far discussed represent one approach to the biosynthesis of terpenes: study of the steps from isoprenoid precursor to the first terpene structure. A second approach is the investigation of the interrelationships, interconversions, of the terpenes themselves. Of course, it is possible to postulate a separate synthesis for each terpene so that no interconversions are necessary, but this idea has little to offer in view of the great similarities of terpenes within a species. Many, if not most, of the terpenes of a single species are likely to be related by very simple oxidation, hydrogenation, and hydration steps, which may or may not represent the actual biosynthetic relationships.

The chief investigation directed at this end of terpene biosynthesis and the only scheme of terpene interconversion presented recently is that of Reitsema (42), reproduced in part here in Figure 5. From a study of the terpenes found in various mint species (43, 45) and the differences in oil composition of young, mature, and old foliage (47), he concludes that unsaturated ketones are among the first terpene products. Saturated ketones, alcohols, and esters are produced from the ketones by reduction and esterification reactions. The

FIGURE 5

REITSEMA'S TERPENE INTERCONVERSION SCHEME



unsaturated hydrocarbons, Reitsema believes (42), are not intermediates in the process. No direct experimental evidence has been thus far available to corroborate or refute Reitsema's scheme.

The purpose of this investigation has been a broad one: to gain whatever information possible reflecting on the nature of the process of terpene biosynthesis in mint. The approach has been from both ends, from isoprenoid precursors and from the terpenes themselves, both the de novo synthesis and the interconversions. The methods of investigation have not been deliberately restricted, but because of the extremely small quantities of terpenes present in mint plants, most of the study has involved use of C^{14}-labeled precursors.

LABORATORY INVESTIGATION

Materials

Mevalonic acid-2-C^{14} as the dibenzylethylenediamine salt was obtained from Isotopes Specialties Co., Inc. and from Tracerlab. This was converted to the sodium or potassium salt by adding the alkali to pH 10.5-11.5, extracting the dibenzylethylenediamine with ether, and adjusting the mevalonate solution to approximate neutrality with HCl. Solutions were 100 μc per ml. These were kept frozen when not in use. The Isotopes Specialties Co., Inc. mevalonic acid was found to be quite impure; so its use was discontinued after some early, unsatisfactory experiments.

Sodium β-methylcrotonate-3-C^{14} was purchased from Isotopes Specialties Co., Inc. This was kept frozen in solution of concentration 40 μc per ml.

Sodium acetate-1-C^{14} was obtained from Tracerlab and was used in a solution of 1 mc per ml, which was kept frozen when not in use.

Uniformly labeled leucine-C^{14} was a product of Tracerlab.

Glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, and uniformly labeled glucose were all products of Tracerlab.

Barium carbonate-C^{14} was obtained from Oak Ridge, specific activity 0.127 mc per mg. Additional barium carbonate of low and unknown specific activity was donated by Dr. C. H. Wang of Oregon State College.

A number of terpenes were Eastman products: menthol, citronellal, α-phellandrene and menthone practical grade and 1,8-cineole and d-limonene, both white label.

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

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

Highly purified samples of citral, isopulegol, geraniol, β-phellandrene, and α- and γ-terpinene were kindly supplied by Dr. R. A. Bernhard of the University of California at Davis.

The Mitcham peppermint oil which was used as a standard chromatograph marker throughout this work was supplied by Dr. C. E. Horner at this institution.

Materials used for the chromatoplates were Mallinckrodt 100 mesh analytical reagent silicic acid (catalog no. 2847) and Jean Vivaudou orthopedic plaster of Paris.

Menthyl acetate was prepared in this laboratory by the action of acetic anhydride on menthol.

M. piperita plants were the Mitcham variety planted commerically in the Willamette Valley. All experiments used plants from a single clone grown in the greenhouse from stock furnished by Dr. C. E. Horner.

M. pulegium 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 College herbarium.

Techniques

Growth Chambers

Experiments involving intact mint plants were carried on in growth chambers in the laboratory. During the course of the program, two different chambers were used. The first was a makeshift prototype; the second was designed after the performance of the first had been noted.

Chamber I was made of two cardboard cartons fastened together to form a box sixteen by fifty by eighteen inches high. The interior was painted with several coats of white Tygon paint. Illumination was by

four 40 watt and four 20 watt fluorescent tubes. Light intensity on the floor of the chamber was approximately 800 foot candles. There was no temperature regulation, but while the lights were on (sixteen hours per day), an exhaust fan was used to keep the temperature down.

Chamber II was made of plywood lined with crinkled aluminum foil, dimensions sixteen by fifty by twenty-four inches high. Illumination was with eight 100 watt fluorescent tubes, Westinghouse Super High Output cool white, and two 40 watt incandescents. 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. A blower circulated air through all three of the chambers thus formed. No other temperature control was in use during the course of these experiments.

Preparation of Mint Oil Samples

In the course of these studies three different methods were used for the preparation of mint oil samples. According to the method used, the samples have been arbitrarily called "soaks", "extracts", and "sublimates".

Soaks were prepared by merely soaking the intact leaves at room temperature in sufficient Skellysolve B

to cover them, decanting the liquid, and concentrating it by evaporation in a stream of cool air.

Extracts were prepared by grinding the leaves in a mortar, extracting with Skellysolve B, and concentrating the liquid by evaporation as in the soaks. To facilitate grinding, sodium bicarbonate, calcium carbonate, sand, or silicic acid with a crystal of barium hydroxide was used. The silicic acid-barium hydroxide seemed most effective and easiest; so this was the grinding agent used through all of the late experimental work. Three or more extractions with Skellysolve B were made, the liquid being drawn off each time with a medicine dropper. Extracts were made not only of fresh leaves, but also of leaves from which soaks had already been produced.

Sublimates were prepared by grinding the leaves with silicic acid and barium hydroxide, adding a drop or two of water, and subliming the water and terpenes from a boiling water bath to a dry ice-chloroform cold finger. The condensates were then extracted with Skellysolve B and the resulting solutions concentrated as in the soaks and extracts. Without the extra drop or two of water added before subliming, the terpenes form a feathery growth which falls from the condenser at the slightest disturbance. Extra water changes this into a solid ice cap which may easily be removed to the Skellysolve B.

The sublimations were carried out in a number of simple apparatuses, one of the most effective of which was a 50 ml beaker nested in a 100 ml beaker, the inner beaker containing the dry ice-chloroform, the outer one sitting on a steam bath.

In all three of these procedures, attempts were made to minimize losses of the oil. Solvent quantities in each method were minimum, barely enough to cover the solids being extracted and allow for decantation. Solids used in the grinding processes were just sufficient that the mixtures should not be pasty from the liquid contained in the plant but should rather be dry enough that the grinding compounds could act effectively.

The economy of the oil contained in a sample was particularly important in those instances in which only a leaf pair or a terminal bud was being sampled, and these comprise most of the experiments in this investigation. It was because of this small sample size that steam distillation was discarded as a method of sample preparation. Reitsema (46) and Howe (24, p. 159-175) have described effective microdistillation apparatuses, but the former requires a minimum of one plant and the latter, although it can handle 10 µl of mint oil, only has a recovery of about 85%. Neither of these devices offers much assurance of recovery of trace terpenes

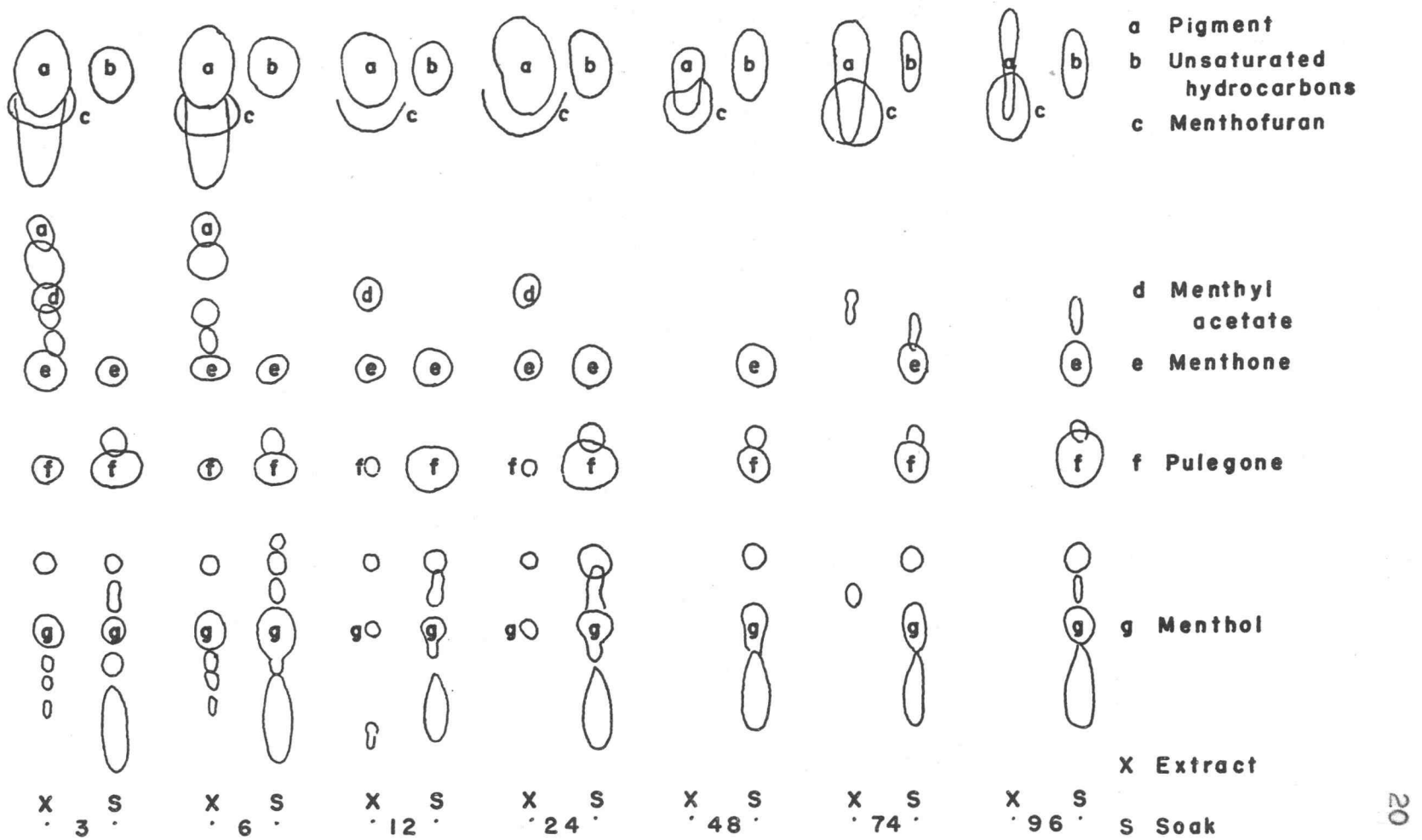
which might, although trace components, nonetheless be important intermediates in the biosynthetic processes.

The three types of oil samples used have distinct differences; each has its own interesting properties, advantages, and disadvantages.

Soaks are of highly variable quality. The length of time of soaking is an obvious source of variation, and this has been studied. Figure 6 shows something of this effect. Another likely source of variation is in the age of the leaves. It is probable that the very young, soft leaves are much more quickly and thoroughly extracted by soaking than are mature leaves. It is disconcerting to note that in some series of peppermint samples, soaks contained all of the menthofuran, while in other series, soaks contained none of the menthofuran of the samples. An interesting possibility is that the soaks may contain chiefly the contents of the oil glands and the waxes of the cuticle, whereas the subsequent extraction may remove lipids from within the cells. In any case, although there was considerable variation in the terpenes present in the soaks, there remained the advantage that the soaks were largely terpene, containing very little of the pigmented materials presumed to be carotenoids. The soaks could be relied upon to be consistently simple mixtures, usually quite similar in composition to the commercial

FIGURE 6

EFFICIENCY OF SOAKING PROCEDURE



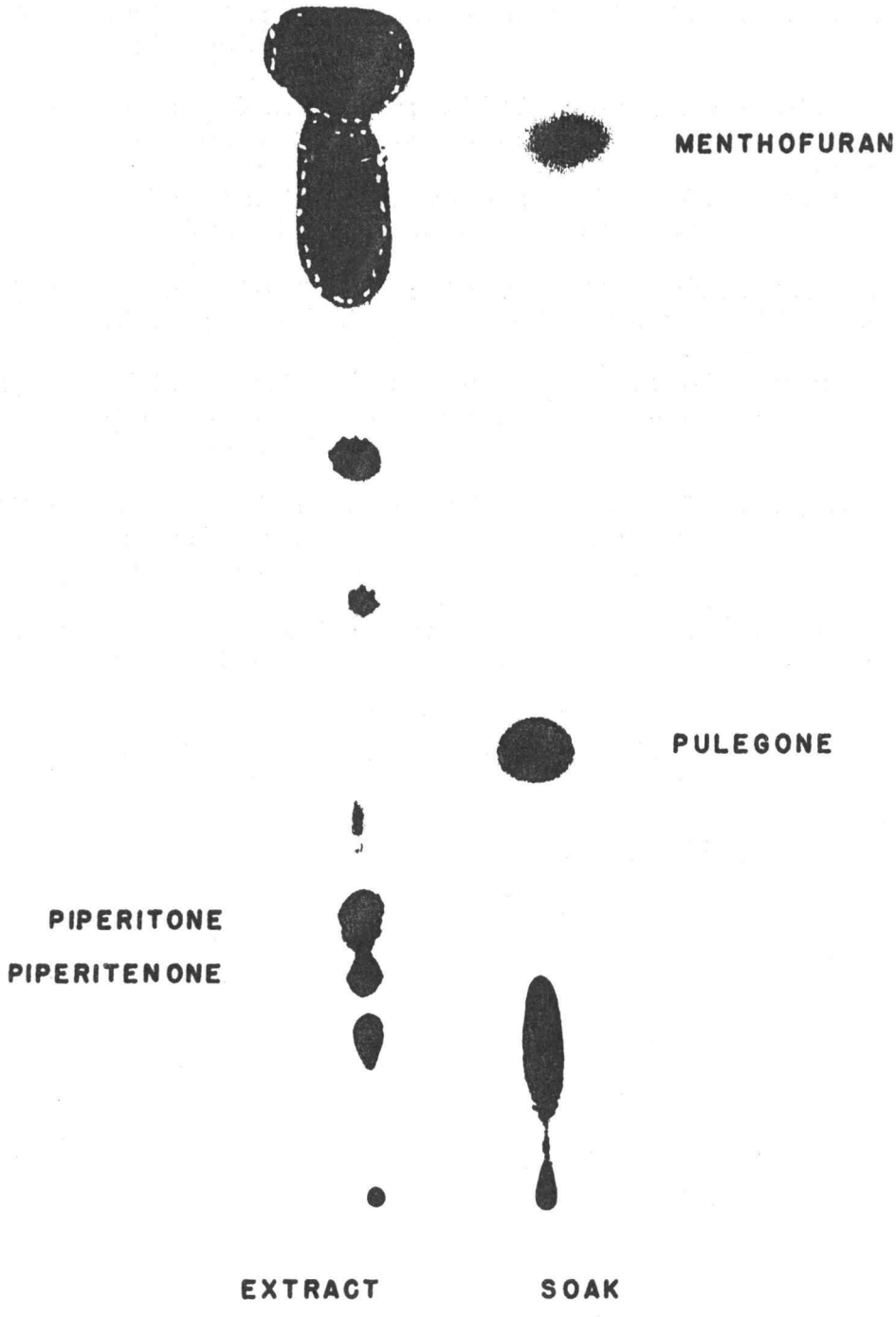
steam distilled oil.

The procedure for preparation of extracts, which was very efficient in recovery of terpenes from the leaves, recovered large quantities of pigments, also. In some cases pigments were present in such quantities that chromatoplates of the oil were too complex to give much information on the terpenes. This was particularly undesirable when radioactive substrates had been used, for it was then difficult to distinguish between labeled terpenes and labeled carotenoids, since they overlapped. The most important use of the extracts was as a check in conjunction with soaks, where the extracts usually showed that the soaks had already removed the terpenes. However, in some experiments, it appeared that while pulegone, menthone, and menthol were removed in the soaks, piperitone and piperitenone were removed primarily in the subsequent extracts. Figure 7 shows the occurrence of different terpenes in a soak and its companion extract.

Sublimates gave an oil sample which was free of the carotenoids and which resembled the peppermint oil of commerce very closely. However, extraction of the residue from sublimation showed that even sublimation prolonged to half an hour was only half effective. A further disadvantage was disclosed when sublimation was tried as a means of recovering terpenes from

FIGURE 7

C-14-LABELING PATTERNS OF SOAK AND EXTRACT OF THE SAME SAMPLE



chromatoplates. As the terpenes were recovered and re-chromatographed, the oxygenated terpenes dwindled while the hydrocarbons increased. It appeared that dehydration reactions were taking place. Regardless of the nature of the changes, however, the fact that changes did occur was sufficient to make the procedure an undesirable one.

Analysis of Mint Oil

It has been necessary or desirable throughout most of this investigation to analyze the oil from one pair of mint leaves or, at most, from a terminal bud with one or two pairs of very small leaves. Gas chromatography, which seemed to offer the greatest promise of good separation and identification, proved to be insufficiently sensitive. No doubt more sensitive equipment is available than that on hand, the Perkin-Elmer Vapor Fractometer Model 154B, but certainly for the analysis of the oil from a single leaf pair this is inadequate. Infra-red spectroscopy likewise proved too insensitive. Indications were that samples ten to one hundred times larger than those available would be necessary for analysis by either method. Chromatography by means of silicic acid chromatoplates as described by Kirchner, Miller, and Keller (28), by Reitsema (44), and by Dunning (18, p. 11-20) gave fair separation and detection but suffered some severe

disadvantages in the delicacy and lack of reproducibility of the plates. For those reasons various reverse phase chromatography systems were tried, but all were without success; chromatoplates had to be used.

The chromatoplates were prepared as follows: A carefully screened mixture of silicic acid and plaster of Paris, 5:1, was quickly stirred with enough water to make a thick slurry. Approximately 20 ml of water was required for 12 g of powder. The slurry was then rapidly spread over a glass plate with a large spatula and briefly shaken to give a smooth surface. Devices for controlling the thickness of the plate seemed desirable, but none was devised to cope satisfactorily with the quickly changing consistency of the plaster of Paris slurry. When the plates were set sufficiently to be undisturbed by moving, they were placed in an oven at approximately 65°C for at least five hours.

After cooling a few minutes, the plates were spotted with samples of the oil to be analyzed and with solutions of known markers. Usually the oil to be analyzed was the total material from one leaf pair reduced to about 10 µl (microliters) of Skellysolve B solution, and markers were 10 µl of 1% solutions of known terpenes. One very useful marker was 10 µl of 2% commercial Mitcham peppermint oil. Piperitone and

piperitenone had to be used in much smaller quantities, 1 µl of 0.1% or thereabouts. After spotting, plates were stood on end in some developing solvent in a carefully sealed chromatography jar. Development is quite rapid with chromatoplates, from one to two hours with an eleven inch long plate, four to six hours with a seventeen inch plate, some variation occurring with temperature and with thickness of the plating material.

The examination of plates was most frequently a five step process. (1) Pigmented materials, mostly yellow and assumed to be carotenoids, are clearly seen by looking through the plates at a fluorescent light source, although in most cases reflected light of any sort is adequate for their detection. (2) Short wave ultraviolet light (mercury vapor, $\lambda = 2537$ Å) reveals unsaturated ketones and aldehydes: piperitenone, piperitone, pulegone, and citral, all of which absorb strongly. Some components of extracts fluoresce under ultraviolet light, but none of these have been identified as terpenes. (3) A 4% solution of trichloroacetic acid in chloroform applied as a spray to the chromatoplate gives a pink to red color with menthofuran after a wait of not more than half an hour (30). Occasionally a blue spot appears after the trichloroacetic acid spray, but this is due to one of the yellow pigments. (4) a very

dilute spray of potassium permanganate in sulfuric acid (approximately 0.2% $KMnO_4$ in 0.04% H_2SO_4) detects the unsaturated terpenes: piperitenone, piperitone, pulegone, citral, citronellal, isopulegol, menthofuran, and the hydrocarbons limonene, phellandrene, terpinene, pinene, and menthene. All of these appear as white or yellow spots against the purple background of unreduced permanganate. (5) Rhodamine B spray in a concentration of 0.05% reveals all of the foregoing materials, although it is rather insensitive to the hydrocarbons. It shows in addition menthol, menthone, menthyl acetate, cineole, and several unidentified compounds. These appear either as dark red areas where the water-soluble dye has not penetrated well into the oily terpenes or, looking at the plate from the back, as white spots against the pink background where Rhodamine B has soaked in. The three sprays just mentioned can be used in succession with very little interference. The menthofuran test does decrease the sensitivity of the Rhodamine B spray for menthone and menthyl acetate but not intolerably. Since the three compounds usually occur well apart on chromatoplates, the interference can be minimized by spraying with trichloroacetic acid only over the area where menthofuran is expected to occur. The permanganate spray fades rapidly over the entire plate, so that a lapse of ten or fifteen

minutes between the two sprays eliminates interference with the Rhodamine B spray. Other useful sprays are glacial acetic acid saturated with o-dianisidine, which detects citral (orange-yellow spot) and citronellal (lemon-yellow spot), and alcoholic or aqueous acidic solutions of 2,4-dinitrophenylhydrazine, which detects a number of carbonyl compounds.

Examination of Rf values of terpenes on these chromatoplates reveals a disconcerting variability. This is partly due to the solvent used for the development. Menthol, for example, can be given an Rf value of from 0.0 to 0.5 by varying the ethyl acetate content of a Skellysolve B-ethyl acetate developer from 0 to 15%. This effect and the effect of temperature on the Rf values of terpenes in this same solvent system are treated in detail by Dunning (18, p. 16-28). However, additional variations can be noted. A decrease in the volume of developing solvent used for a plate decreases the Rf values of some components markedly, particularly those components of low Rf. Particularly bad, however, is a variation of Rf values on a single plate. One compound spotted across the whole plate gives on development a series of spots lying on a parabola, with the lowest Rf values shown by the spots in the center of the plate. In some instances the variation is quite small;

occasionally plates have almost perfectly uniform Rf values; in most, however, the variation is sufficiently great to make it desirable to accompany each unknown material with a spot of known markers on one, if not both sides. A further variation in Rf values is caused by overloading, as would be expected, or by what appears to be something of a mutual solubility effect. That is, the Rf seems to vary with the nature of the other components of the same oil sample. However, none of these effects is so great as to obscure the identity of the components in any normal oil sample. Figure 8 shows the array of spots revealed by this chromatographic separation of a sample of commercial Mitcham peppermint oil and compares these with the spots given by a mixture of compounds known or suspected to be peppermint oil components. Figure 9 reproduces a plate with numerous spots and illustrates the parabolic distribution of a single substance.

Carbon-14 Detection and Measurement

Estimation of the extent of incorporation of C^{14} labeling into terpenes was accomplished by radioautograms and by counting with a Tracerlab TGC-14 Mylar window gas flow counter. In most instances these were used only to find the distribution of labeling among the various

FIGURE 8

DISTRIBUTION OF TERPENES ON
A CHROMATOPLATE

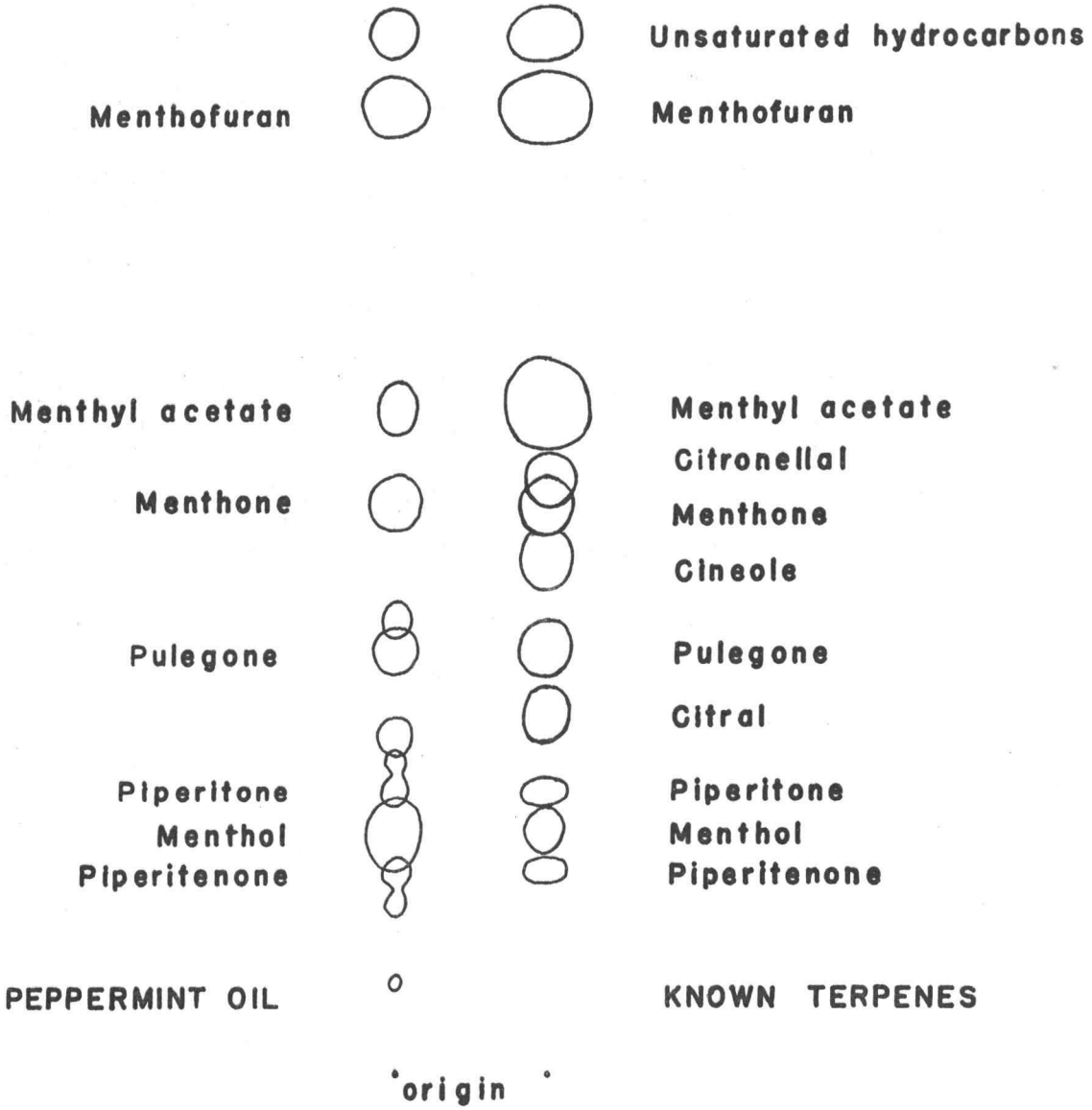
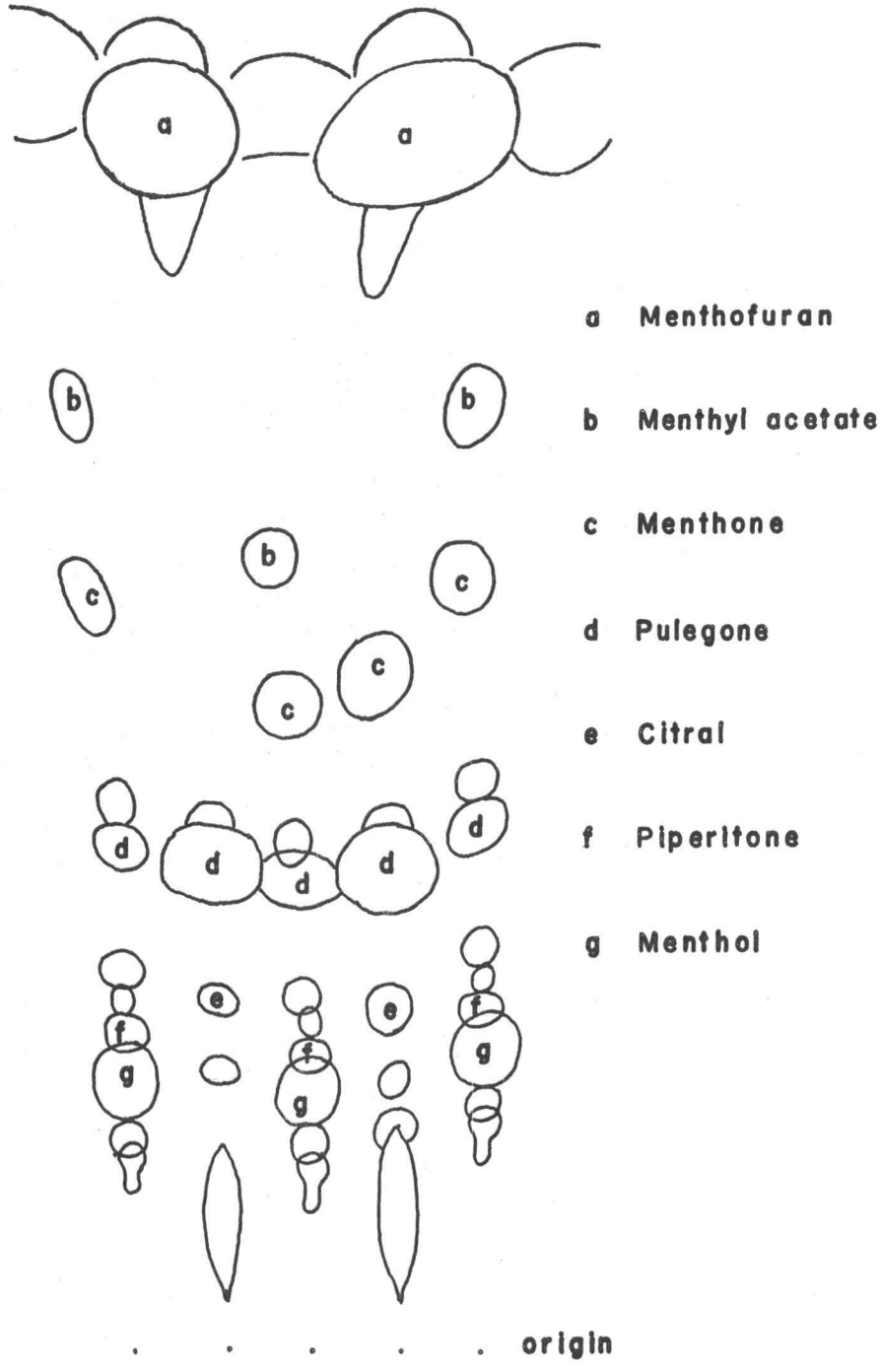


FIGURE 9

PARABOLIC DISTRIBUTION OF Rf VALUES



terpenes, but rough quantitative relationships were also established.

Although it would be expected that terpenes might evaporate from the chromatoplates, early examinations showed that the radioactivity detected on a plate increased slightly for a day or two and then remained constant thereafter. Evidently the initial increase was due to the slow evaporation of the water from permanganate and Rhodamine B sprays. For this reason plates were allowed to wait a day or two before counting or radioautographing.

Radioautograms were made with Kodak no-screen X-ray film. The length of time varied with the activity of the sample, but the usual time was two weeks. For some of the least active samples, for example those which were taken after the mint shoot had had only a five minute exposure to radioactive carbon dioxide, the length of exposure had to be increased to six or eight weeks.

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 upon the shield with the window over the opening in the shield. Counting results were good and quite consistent; background was gratifyingly low. However, the

delicacy of the plates made the method undesirable. By the time plates had been counted one centimeter at a time (the usual slit used was 1 x 2 cm), they were dusting badly and softened over large areas. The method was in the end largely abandoned when it appeared that radioautography gave more information.

A rough correlation and standardization of methods was secured by removing from a plate an area which had been both counted and radioautographed, converting the labeled compound to barium carbonate, and counting this on planchets. The spot used was a sharp spot of pulegone with no other radioactivity on surrounding areas of the plate. A 100 minute count of the spot with the TGC-14 counter gave an activity of 4970 cpm corrected for background. The spot was then radioautographed for periods of 2, 4, 12, and 24 hours.

The spot containing the pulegone was transferred to a wet-combustion apparatus with approximately 3 mg of sucrose as cold carrier. The system was evacuated through a cold toluene trap to see whether the pulegone would evaporate from silicic acid under vacuum. The toluene was examined in a liquid scintillation counter and found to count only background. Oxidation of the pulegone was effected with Van Slyke-Folch mixture: 5 g CrO_3, 1 g KIO_3, 67 ml $H_2S_2O_7$, and 33 ml H_3PO_4

simmered at 150°C until solution was complete (4, p. 44). Due evidently to contamination of the sodium hydroxide with carbonate, the barium carbonate precipitated was much more than expected and had to be collected on two planchets. One planchet, 0.1099 g, transmission 0.25, counted 2628 cpm, hence corrected for self absorption to 10,500 cpm. The second planchet, 0.0666 g, transmission 0.388, counted 2615 cpm, hence corrected to 6,740 cpm. The specific activities agreed moderately well: 95.7 and 101 cpm/mg. Total activity from the two was 17,200 cpm. Since an efficiency of 17.8% had been determined for the TGC-14 counter using standard planchets, this gave an activity of 97,000 disintegrations per minute. The microcurie equivalent for direct counting of plates then became:

$$\frac{2.22 \times 10^6 \times 4970}{9.7 \times 10^4} = 1.14 \times 10^5 \text{ cpm}$$

Considering that the plates were highly irregular in thickness and probably in water content and certainly in surface, it seemed reasonable to use a value of 1×10^5 as a rough millicurie equivalent for the purpose of estimating efficiency of conversion of terpene precursors into terpenes. Through the use of a densitometer this was extended to radioautograms by means of the

standard radioautograms made with the same pulegone spot.

Experiments

It is a common observation that the order in which the experiments of an investigation are carried out is seldom a logical order for their recounting. As an investigation progresses, points of emphasis shift, entirely new problems enter in, and new information arises out of old experiments. Out of one experiment grows a second, which must be discussed first in order to make clear the meaning of the original experiment.

Although experiments to determine the site of terpene biosynthesis in peppermint grew at a late stage out of the investigation of the paths of biosynthesis, the results will be discussed first, because they are important in understanding and interpreting most of the other experiments. The experiments on terpene precursors as a second section seem logically to precede those experiments on terpene interconversion not only physiologically, but also because a C^{14}-labeled terpene precursor yielded labeled terpenes which could serve as substrates to demonstrate terpene interconversion.

Site of Terpene Biosynthesis in Mentha piperita

In some experiments designed to discover the

order of terpene synthesis through time course studies using $C^{14}O_2$ as the labeled substrate, an attempt was made to secure equality of samples by starting only with mature leaves of the same size, apparent age, color, and so forth. The result of these attempts was that practically no labeling of terpenes was observed. In one instance, however, young axillary shoots developing on these stems of mature leaves, even though they became noticeably only after the removal of the labeled carbon dioxide, were found to produce substantial quantities of labeled terpenes; at the same time, the mature leaves had little if any terpene labeling.

Analysis of the oil from each leaf pair of a single mint shoot, an attempt to find composition differences as a function of age, showed not only that such differences did exist, but also that the amount of oil per leaf pair was most constant for the leaves on one shoot, in spite of great differences in leaf size.

Both of the foregoing observations suggested strongly that terpene synthesis is a function of the young leaves. An experiment was accordingly set up to define the site of the synthesis.

In one study four intact shoots of Mentha piperita were transplanted with their soil into a desiccator in growth chamber II. There they were exposed to carbon

dioxide of unknown specific activity. One plant was removed from the desiccator after seventeen hours, at which time the radioactive carbon dioxide was likewise removed. A second plant was removed after three days. The others after this showed extreme rosette formation and looked generally too unhealthy to make reasonable samples, but the shoot tips were sampled after eight days.

The division of the first plant into samples is shown in Table 1, and the pattern of labeling in the terpenes is shown in Figure 10. A corresponding division and analysis was made of the second plant with results so similar that they need not be included here. The shoot tips from the third and fourth plants were analyzed without division; so they gave no evidence on site of terpene synthesis. However, the succession of samples from seventeen hours, three days, and eight days gave some evidence as to rate of terpene biosynthesis and will be further treated in a later section.

A second experiment, in which six shoots were sampled at intervals from 2½ hours to 4 days after initial exposure to $C^{14}O_2$, gave results in complete agreement with those of the first experiment.

Precursors of Terpenes

A study of the known metabolic reactions of

TABLE 1

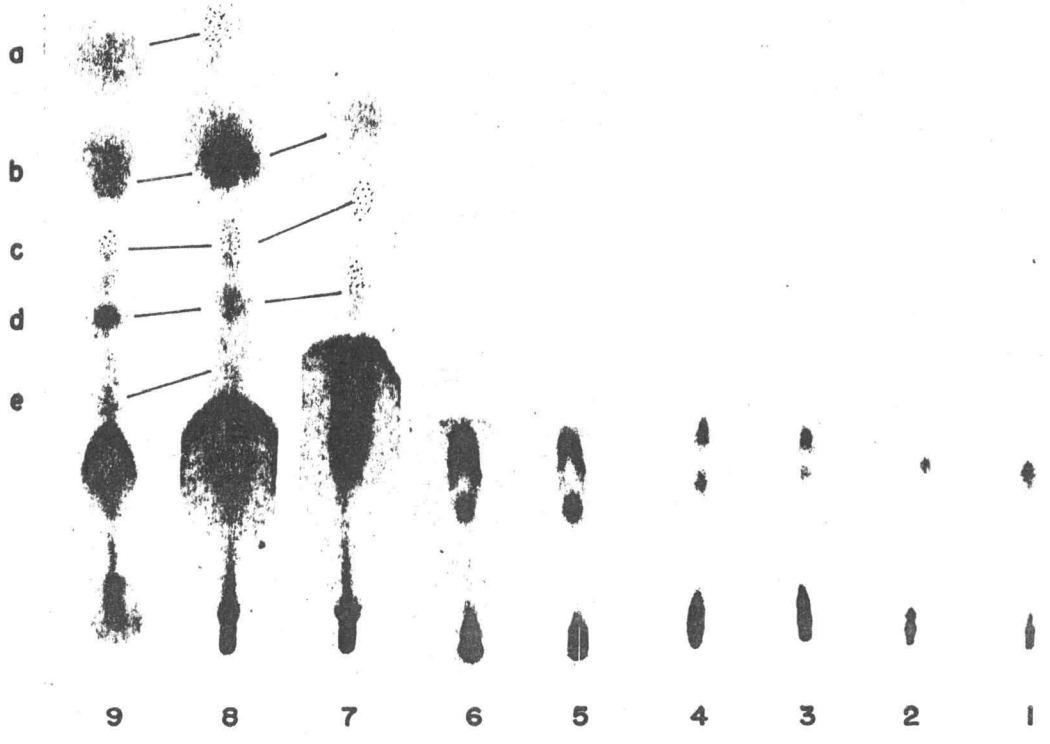
SITE OF TERPENE SYNTHESIS

(Description of the Samples Used in Figure 10)

1.	0.02 g	Oldest and lowest leaf pair; leaves round and pale; oil contained menthol, pulegone, and menthofuran.
2.	0.03 g	Second leaf pair; still round; menthol, pulegone, and menthofuran.
3.	0.05 g	Third leaf pair, round; pulegone, menthol, and two other saturated terpenes.
4.	0.07 g	Fourth leaf pair; leaves of normal, mature shape; pulegone, menthol, saturated terpenes.
5.	0.15 g	Fifth leaf pair; normal shape; oil saturated except for menthofuran and a trace of pulegone.
6.	0.17 g	Sixth pair; youngest of mature leaves; oil largely saturated except for menthofuran and traces of pulegone and piperitone.
7.	0.17 g	Seventh leaf pair; leaves young; oil high in menthone, pulegone, unsaturated terpenes.
8.	0.08 g	Eighth leaf pair, quite juvenile; oil very low in menthol, high in menthone and unsaturated terpenes.
9.	0.03 g	Ninth leaf pair and terminal bud; oil unsaturated except for a little menthone.

FIGURE 10

SITE OF TERPENE SYNTHESIS



The extent of terpene labeling from a 17-hour exposure to $C^{14}O_2$ is shown for each leaf pair of a mint shoot in order from the oldest (1) to the crown (9). Descriptions of the samples are in Table 1.
a-menthone b-pulegone c-citral d-piperitone
e-piperitenone

mevalonic acid and related compounds suggests that mevalonate should be an excellent precursor of terpenes; that β-methylcrotonate should be less effective, although probably more effective than acetate and certainly more than leucine. Acetate should be considerably more effective than glucose. These ideas are based only on numbers of intervening steps between precursors and products, ignoring possible mechanical hindrances to the utilization of exogenous substrates. Considerations of absorption and transport could completely change the pattern. In the experiment detailed below these substrates have been tested under the same conditions so that reasonably valid comparisons could be drawn among them.

Eight young mint shoots about two inches long were selected for comparable size and appearance. All contained vigorous young growth and enough mature foliage to assure detection and recognition of the major terpenes after chromatography. Each shoot was given 10 μc of substrate in about 0.1 ml of solution by uptake through the cut stem. On the average the shoots required about two and one-half hours to take up the substrate.

The substrates used were mevalonate-2-C^{14}, β-methylcrotonate-3-C^{14}, acetate-1-C^{14}, uniformly

labeled leucine, uniformly labeled glucose, glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14}. In the course of the experiment the plant receiving glucose-6-C^{14} wilted but recovered. The plants receiving mevalonate and acetate both showed extensive blackening, but both retained some healthy tissue up until the time of sampling.

A radioautogram of the chromatoplate of the soaks and extracts from the shoots shows that all of the solutions contain numerous radioactive lipids. In the four shoots which received labeled glucoses, pulegone and menthone showed incorporation of the order of 0.01% of the C^{14} administered. Leucine gave less incorporation, something of the order of 0.002%. Mevalonate, β-methylcrotonate, and acetate contributed no labeling to any recognized terpene.

The foregoing is only one of the many experiments in which mevalonate, β-methylcrotonate, and acetate were used as substrates. In no case did labeling from the β-methylcrotonate appear in terpenes, either of Mentha piperita or of M. pulegium. Uptake of the β-methylcrotonate through the cut stems seemed effective in all cases; the tops of the shoots became highly radioactive; however, no labeled terpenes resulted even in M. pulegium, which in a similar experiment by Stockmann (52) converted β-methylcrotonate to pulegone.

Acetate gave rise to small amounts of labeled terpenes in several experiments. These are not unambiguous, for the possibility exists that acetate was degraded to carbon dioxide and the latter was the source of the terpenes. This possibility becomes a likelihood in view of the experiments with $C^{14}O_2$ which will be discussed presently.

Mevalonate, since it is believed to be one of the closest terpene precursors, was the subject of the most intensive investigation. In particular, the method of introduction of the mevalonate into the mint system was widely varied.

In numerous experiments with mevalonic acid as a substrate for Mentha piperita and M. pulegium shoots of various ages, the C^{14}-labeled mevalonic acid was introduced by uptake through the cut stem of the mint shoot. In all of these experiments the top of the shoot was highly radioactive. Carotenoids proved to be labeled, but in no case were the terpenes.

A rooted M. piperita shoot placed in mevalonic acid solution allowed only a very small amount of radioactivity to reach the top of the shoot. This could have been a small amount of mevalonate getting through a surface defect of the root, a degradation product of the mevalonic acid, or just a very small uptake through

intact roots. Whatever the case, no terpene labeling resulted.

Spots of mevalonic acid solution on the leaves of M. pulegium shoots were absorbed, translocated, and used for the synthesis of numerous compounds, none, however, recognizable as terpenes. Spotted on M. piperita leaves, the mevalonate solution showed no translocation and only extremely slow penetration. It may be that the water evaporated rather than being absorbed, but the plant was kept in a high humidity chamber to prevent this. No labeling of terpenes was detected.

Youngest leaves and terminal buds of M. piperita were given mevalonate by vacuum infiltration. As has been true in all other cases of mevalonate introduction, materials at the solvent front on the chromatoplate were labeled, but no materials recognizable as terpenes showed radioactivity.

In separate experiments both stems and young leaves of M. piperita were minced with a sharp blade under water. The tissue slices were then placed in mevalonate solutions in strong light for periods of from four to twelve hours. Extracts of the mint fragments and the solution showed in some instances considerable labeling in the lipids, but in no case could this labeling be attributed to any terpenes.

A small amount of mevalonolactone was produced by treating potassium mevalonate with the acid form of Dowex-50. This was given to a vigorous young M. piperita shoot by uptake through the cut stem. Some radioactivity was detected in the materials at the solvent front on the chromatoplate, but none was clearly associated with a known terpene.

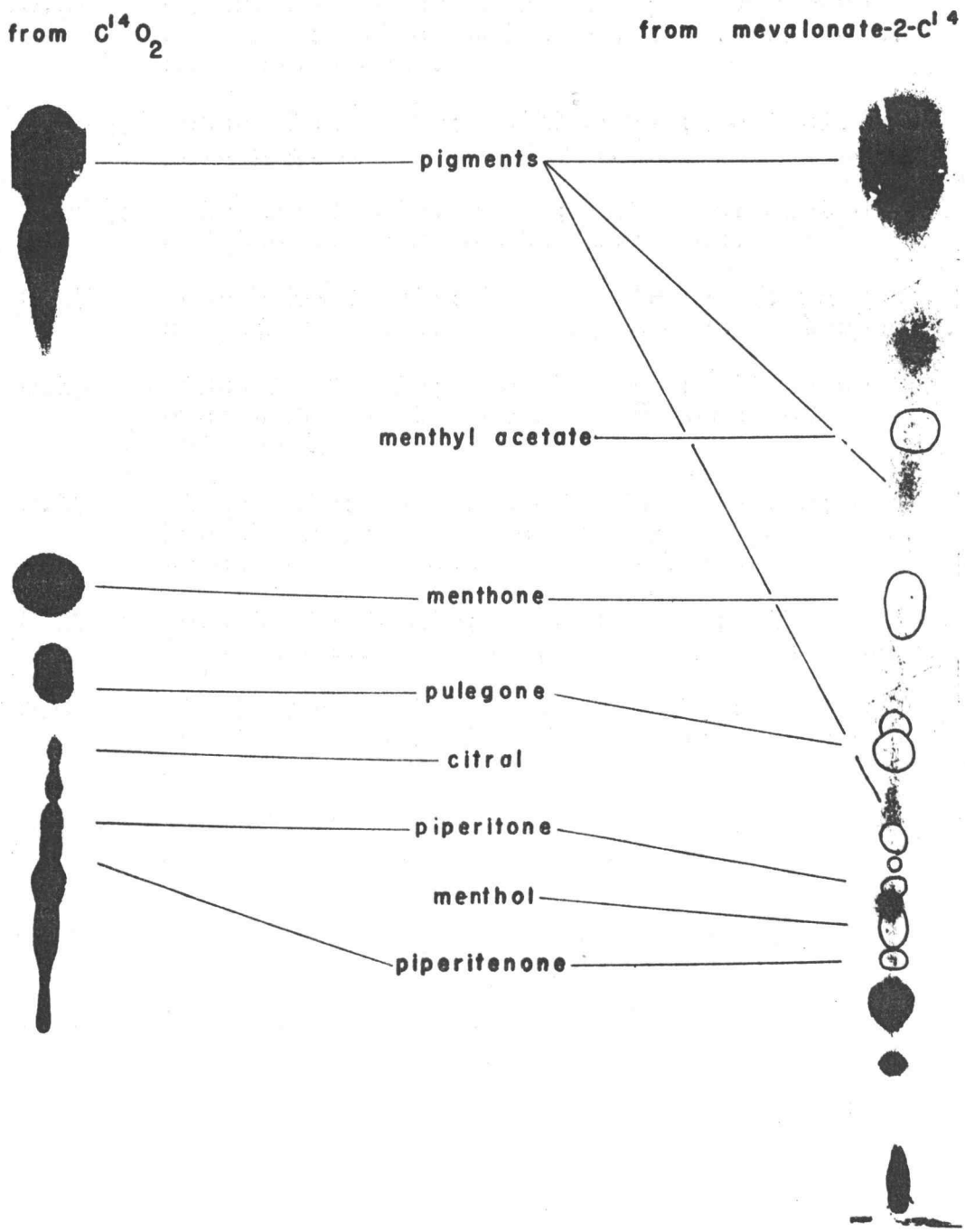
Mevalonate was also tried as a substrate for several cell-free systems. In these no terpene labeling could really be expected, but not even phosphorylation of the mevalonate was observed. The systems tried included: extracts from acetone powders of both peppermint and pennyroyal; peppermint leaves ground in iced mortar with silicic acid and pH 6.6 phosphate buffer; mint juice expressed directly into pH 7.5 phosphate buffer containing sucrose; mint terminal buds and youngest leaves ground in iced mortar with sucrose, sand, pH 7.4 Tris-maleate buffer and glutathione or ascorbate or nicotinamide or all three.

Figure 11 compares the typical distribution of radioactivity in samples labeled from mevalonate with the distribution of labeling resulting from carbon dioxide.

Carbon dioxide as a labeled substrate was first tried merely to establish whether terpene synthesis was

FIGURE II

LABELING DISTRIBUTION IN PEPPERMINT LIPIDS



occurring in plants kept in the laboratory. Consistent failure to obtain labeled terpenes from mevalonate and β-methylcrotonate had made this a question of some doubt. When peppermint and pennyroyal plants were exposed to $C^{14}O_2$, it was discovered that terpenes were, indeed, being synthesized, but more important, that they were highly labeled from the carbon dioxide. It then seemed practical to use labeled carbon dioxide as a precursor for time course studies directed at finding sequences of terpene formation.

The circumstances of these studies have not been such as to make it convenient to calculate percentage yields of labeled terpenes. More than one shoot of the mint under study has always been involved; the different shoots, even though selected for similar size, age, and apparent vigor, can not be expected to synthesize identical quantities of terpenes; the yields and rates of synthesis have often been observed to be quite different. However, rough approximations based on the unlikely premise that the shoots equally divide the available carbon dioxide have been made. Table 2 lists some examples from various experiments showing extent of incorporation of carbon dioxide labeling into terpenes. The examples are selected to illustrate the rapidity of the incorporation as well as its extent.

TABLE 2

EXAMPLES OF TERPENE LABELING FROM $C^{14}O_2$

Plant	Time	Terpene	Counts per minute	% of C^{14} incorporated
peppermint	10 min.	citral?	2.5	0.00002
peppermint	19 hrs.	pulegone	50,000	0.2
peppermint	19 hrs.	menthofuran	50,000	0.2
peppermint	3 days	pulegone	115,000	0.6
peppermint	6 days	menthofuran	140,000	0.7
peppermint	6 days	pulegone	15,000	0.15
pennyroyal	1½ hrs.	pulegone	25	0.0025
pennyroyal	6 hrs.	pulegone	4,000	0.04

The values above are taken from several different experiments to illustrate something of the speed and extent of incorporation of carbon dioxide labeling into mint terpenes.

Interconversion of Terpenes

It was originally hoped that the sequence of formation of terpenes might be deduced from time course studies employing some close terpene precursor such as mevalonic acid or β-methylcrotonic acid. When it was found that labeled terpenes could be effectively produced from neither, three other lines of experimentation were applied to the problem. Time course experiments with labeled carbon dioxide, analysis of terpene distribution through the plant, and use of labeled terpenes as substrates were attempted with some success.

Time course studies are most useful when they employ precursors only a very few steps removed from the reactions of interest and when the periodic samples are equivalent, either samples taken from the same organism or from a large number of organisms so that the variations between individual organisms do not enter in. Neither of these applies to the time course studies of terpene biosynthesis in mint using $C^{14}O_2$ as a labeled precursor. Many steps must intervene between precursor and product of interest. Equivalence of samples is very difficult to secure and cannot be claimed in this investigation. To take successive samples from the same mint shoot is out of the question. In the previous section on site of

terpene biosynthesis it has been shown that only the very young tissue of a shoot is active in terpene biosynthesis, and this is enough only for one sample. It should be possible to use large numbers of shoots, but inconveniences enter in. A large number of shoots in a single sample would give too much oil for analysis as a single sample. If only an aliquot of the total oil were used, then the specific activity of the oil would have to be greatly increased, necessitating use of inconveniently large quantities of $C^{14}O_2$. There is some justification for the use of single shoots as samples when these have been selected for similarity of size, age, and vigor, for all of the mint used in these experiments is from a single clone and hence can be regarded as genetically a single organism.

In spite of the disadvantages attending the $C^{14}O_2$ time course studies, considerable information has resulted from them. One of the most successful experiments in some respects was one of the earliest. In it peppermint shoots kept in a desiccator in growth chamber I were encouraged to develop axillary shoots by clipping the terminal buds and gradually removing mature leaves. When the young growth was fairly advanced and had developed a watery, succulent appearance, it was exposed to approximately 2 mc of carbon dioxide of high specific

activity. Lighting was then continuous until the time of first sampling, 19 hours later. Thereafter the plants were maintained on a sixteen hour day. Sampling was continued for eight days. After that the plants were slowly dying, and no further sampling was done until a final sample was taken at 19 days.

This run is especially interesting for the high radioactivity obtained in the terpenes. Yields of single terpenes were estimated up to 0.7% of the $C^{14}O_2$ for menthofuran and nearly the same for pulegone. Although probably little more than half of the mint was sampled, about 2.5% of the radioactivity of the carbon dioxide was recovered in the lipids, and of this scarcely less than a third could be terpene. A second feature which is interesting (especially in the light of later work) is that only in the last sample, the nineteen day sample, does a slight incorporation of radioactivity into menthone occur. In the first few samples, piperitone and piperitenone show labeling. Apart from these, pulegone and menthofuran are the only labeled terpenes, and throughout the experiment these bear the major portion of the labeling. Table 3 summarizes the distribution of radioactivity in the known terpenes of the samples.

For one experiment young peppermint shoots just out of the ground and still possessing the rounded,

TABLE 3

DISTRIBUTION OF LABELING IN PEPPERMINT TERPENES AFTER EXPOSURE TO $C^{14}O_2$

Terpene	Days: 1	2	3	4	5	6	8	19
pulegone	+++	+++	+++	+++	+++	+++	++	-
menthofuran	+++	+++	+++	+++	+++	+++	+++	+++
piperitenone	+++	++	++	+	+	-	-	-
piperitone	+++	++	+++	+	+	-	+	-
menthone	-	-	-	-	-	-	-	+
menthol	-	-	-	-	-	-	-	++

+++ heavy labeling
++ light labeling
+ barely detectable labeling
- no detectable labeling

translucent, juvenile foliage were used. They were accustomed to growth chamber I for several days before being exposed for a half hour to a millicurie of carbon dioxide. Samples were taken at 35 minutes, 1, 2, 4, 8, 15½, 31, 70, 142, and 287 hours. Incorporation of labeling into the terpenes was never very high, reaching a maximum of 0.15% of the initial C^{14} in pulegone in the 142 hour sample. The chief interest in this experiment is that, although menthone was detected by Rhodamine B spray in a few of the samples, it did not become labeled with C^{14} even in the longest periods. Of interest, also, is that pulegone, menthofuran, and materials with Rf's approximating those of piperitenone and piperitone were labeled in the first sample, after only thirty-five minutes.

The distribution through a mint plant of labeled terpenes resulting from exposure of the plant to labeled carbon dioxide has already been discussed in the section on site of terpene biosynthesis in Mentha piperita. The experiment showed quite plainly that more terpene synthesis occurred in young than in mature or old tissue. In addition the study offered some information on sequence of terpene formation: Since one plant was dismembered 17 hours after exposure, one after 3 days, and the remaining two after 8 days, the experiment has

yielded time course results. Furthermore, since old leaves synthesize terpenes more slowly than young leaves, simultaneous sampling of old and young leaves from a plant which has received $C^{14}O_2$ should give results very similar to a time curve. Let us consider the lagniappe information given by these two approaches.

A comparison of the most active tissue (the tops) of the four plants shows that there is a very definite progression from unsaturated to saturated materials. The terminal bud of the 17 hour sample shows labeling of piperitone, piperitenone, pulegone, menthone, and a substance detected by Rhodamine B which partly overlaps the pulegone spot. A spot tentatively identified as citral shows light labeling. The 3 day plant has lost the citral labeling; piperitone and piperitenone have decreased somewhat; and menthone, pulegone, and the material overlapping pulegone have increased in labeling. The two samples taken the eighth day both show their heaviest labeling in the menthone spots. Menthol is very heavily labeled in both. Pulegone is heavily labeled in one, lightly in the other and the spot which overlaps pulegone is labeled in the reverse fashion, though fairly heavily in both. A Rhodamine B-detected material lying between citral and piperitone is labeled in both shoots; what appears to be menthyl acetate is

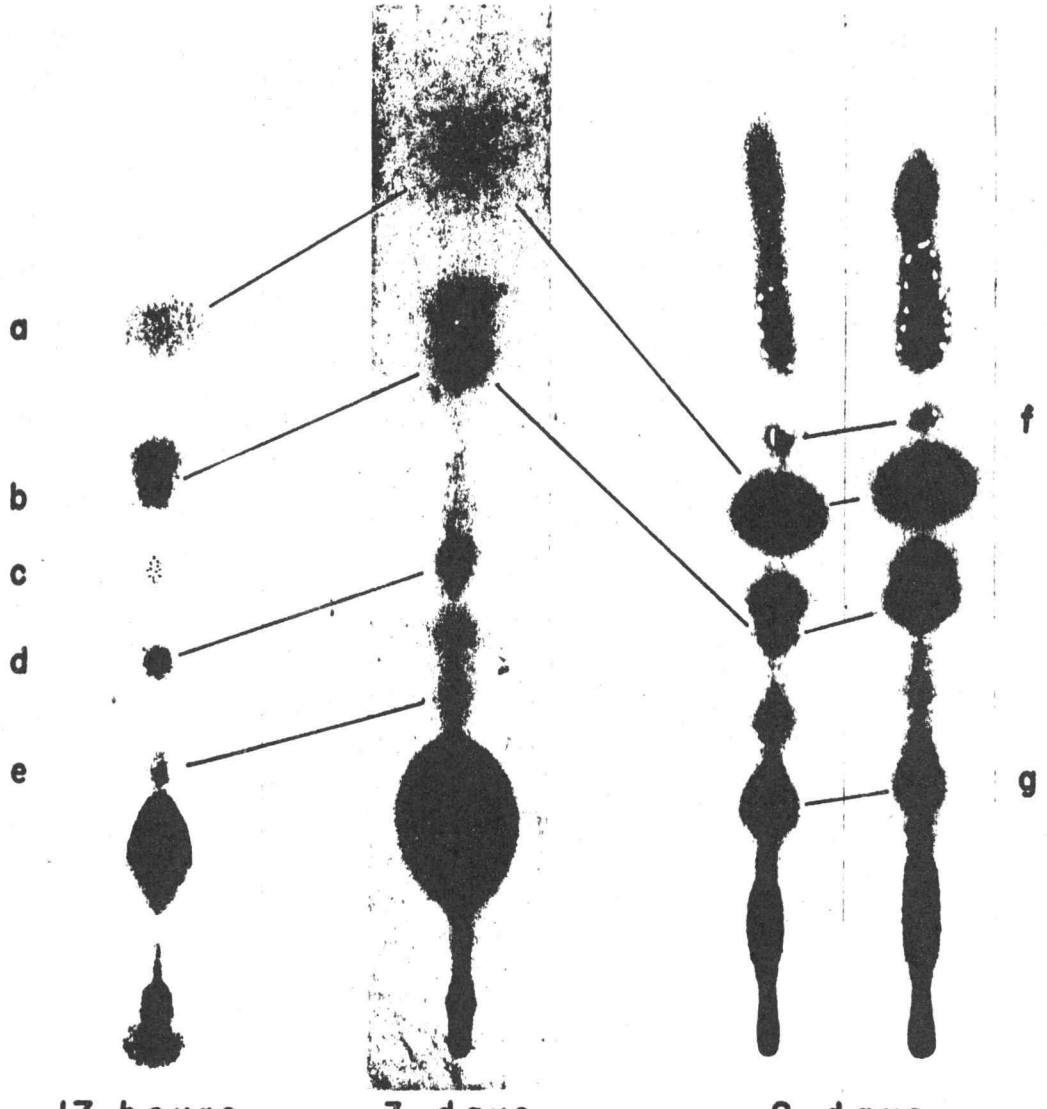
labeled in both; and menthofuran is labeled a trace in both. Piperitone and piperitenone labeling seem to have disappeared entirely and so has the supposed citral labeling. The four samples that give this information are shown in Figure 12.

In Figure 10 given in the section on site of terpene biosynthesis, it can be noticed that the six oldest leaf pairs show no trace of terpene biosynthesis, in spite of the fact that one of the largest leaf pairs of the shoot is included. The seventh leaf pair, the same size as the barren sixth pair, shows slight labeling in pulegone, piperitone, and perhaps very faint labeling in citral. The eighth leaf pair shows heavy labeling of pulegone, moderate labeling of piperitone, and light labeling of piperitenone, menthone, and the spot which overlaps pulegone. The terminal bud shows heavy labeling of menthone, pulegone, the pulegone-overlapping constituent, piperitone, and piperitenone. The results from the second dismembered shoot are in complete agreement with the results of the 17 hour sample, and are therefore not shown.

Mentha pulegium has a rather simple oil, largely pulegone with small amounts of piperitenone, menthone, and piperitone. Sequences of terpene formation should be fairly easily detected. In the one time course

FIGURE 12

CHANGES IN TERPENE LABELING WITH TIME



a menthone
b pulegone
c citral ?
d piperitone
e piperitenone
f menthyl acetate ?
g menthol

experiment in which M. pulegium was exposed to labeled carbon dioxide, results were not very clearly meaningful. Labeling appeared simultaneously and to roughly the same extent in piperitenone and pulegone in the first sample (3/4 hour). In later samples the pulegone, which is present in much greater amounts, was likewise labeled to a greater extent. Piperitone showed faint labeling in the later samples, too. At the last sampling, four days after exposure to the $C^{14}O_2$, some material with an Rf value equal to menthone was heavily labeled, though the material could not be detected chemically.

When it had been noticed that juvenile shoots only an inch or two high did not produce very highly labeled terpenes from carbon dioxide and produced mostly unsaturated terpenes rather than saturated terpenes such as menthone and menthol, a further run was tried using the young tips of older shoots. Fourteen of these M. piperita shoots, each consisting of a terminal bud and the smallest leaf pair, were exposed to a millicurie of carbon dioxide in a small container in growth chamber I. The container consisted of a crystallizing dish inverted over a rubber stopper. The crystallizing dish had a glass side arm covered with a medicine dropper bulb through which sulfuric acid could be added by hypodermic needle to $BaC^{14}O_3$. The rubber stopper contained inlet

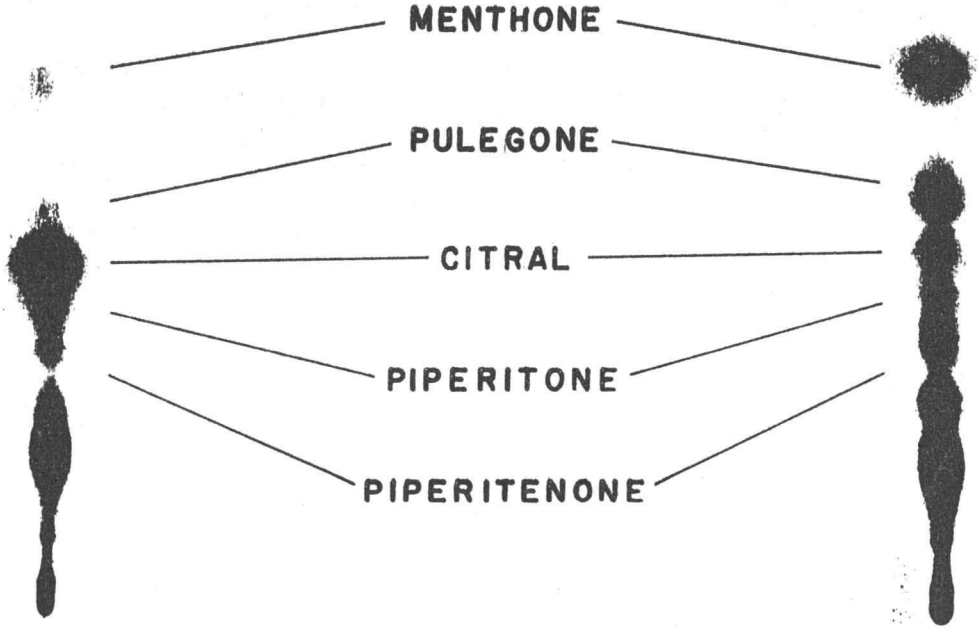
and outlet tubes for sweeping with air. Shoots were placed in wet vermiculite in an aluminum foil dish sitting on the stopper. The chamber was swept with air 31 minutes after introduction of the $C^{14}O_2$; the first sample was taken at 37 minutes; sweeping was continued then until the last sample had been taken.

The chief point of interest in the experiment seems to be in the first two samples, which are reproduced in Figure 13. The sample taken at 37 minutes shows the bulk of the labeling, and very heavy labeling at that, to be in something not detected by sprays but with the same Rf value as citral. Additional faint labeling is in piperitenone, piperitone, pulegone, and menthone. In the sample taken at 65 minutes, the bulk of the labeling is split between the pulegone and menthone, with considerable labeling in piperitenone and piperitone, and still less labeling in the citral location. In succeeding samples, the change in labeling pattern is more subtle; changes seem to be rather minor and representative of no real qualitative differences. It would seem that all but the very slow steps of terpene interconversion are over in the first hour.

To investigate more carefully the early steps of terpene biosynthesis, two experiments were planned. One would expose shoots to carbon dioxide for only five

FIGURE 13

$C^{14}O_2$-INDUCED LABELING PATTERNS IN PEPPERMINT TERPENES



Peppermint shoots were exposed to labeled carbon dioxide for 31 minutes. The samples on left and right above were taken respectively 6 and 34 minutes after removal of the carbon dioxide.

minutes, giving a sharper starting point for the introduction of labeled substrate. The other would have continuous carbon dioxide introduction. Both would have frequent sampling through a period of one hour.

Twelve M. piperita shoots each consisting of terminal bud and one leaf pair were placed in a chamber of approximately 20 ml volume. This was made by inverting a crystallizing dish (with outlet arm in the center of the bottom) over a paraffin block with a shallow cylindrical recess carved into the top. The seal was made with stopcock grease. Shoots stood in water in individual cups punched into the floor of the paraffin chamber. This apparatus was placed in grown chamber II at maximum light intensity. One millicurie of carbon dioxide was released in the chamber, bringing the carbon dioxide content of the air up to approximately 5%, a concentration suitable for rapid fixation.

After five minutes exposure, the container was simultaneously opened and swept by suction from an evacuated desiccator containing sodium hydroxide. The first sample was immediately dropped into Skellysolve B sitting in dry ice to stop enzyme activity as quickly as possible. Thereafter the chamber was left open, and the plants were frozen in Skellysolve B at five minute intervals. Analysis of the samples gave the following

results:

Time	Labeling
6 min. . .	No terpene labeling.
10	Possible faint piperitone.
15	No terpene labeling.
20	No terpene labeling.
25	Pulegone > piperitenone > piperitone > menthone.
30	Pulegone > piperitenone.
35	Equal labeling of pulegone, piperitenone, and menthone.
40	Similar to the 35 minute sample but somewhat fainter.
45	Pulegone > piperitenone >> menthone and piperitone.
50	Pulegone > piperitenone.
55	Pulegone > piperitenone > menthone.
60	Possible, very faint, citral labeling.

The individual variation of these samples is so great that little information is to be gained from them. This is disappointing since the shoots had been carefully matched as usual and since the experiment proceeded smoothly and without difficulty.

The other experiment with short sampling periods was beset with numerous difficulties. To sample from a

$C^{14}O_2$-filled chamber without releasing the gas so that exposure could be continuous up to the time of sampling, it was determined that samples should be withdrawn through a liquid lock. It was assumed that shoots might be floated in bicarbonate solution (to retard solution of the $C^{14}O_2$) with threads attached to withdraw them. The shoots floated excellently in water, but sank in a matter of moments in the bicarbonate solution. They were finally placed in a screen from which they could be easily pulled by means of the attached threads. The screen also held a vial of $BaC^{14}O_3$ where it could be reached by a hypodermic needle inserted through the top of the chamber. The chamber was simply a small funnel with a medicine dropper bulb over the stem inverted and lowered over the screen and down into the water until a sufficiently small air space was left. The volume of air in the apparatus was approximately 75 ml, the carbon dioxide 1 mc. The first sample was withdrawn at 5 minutes and plunged immediately into boiling Skellysolve B (70°C), boiled for two minutes, and left there for the preparation of the soak. The first sample was taken without difficulty; others were much less successful. Four of the original twleve shoots were cut in two by the threads, floated back up into the funnel and were lost. Others were recovered in part at the times noted,

but did not represent the whole shoot.

Labeling in all shoots was faint; for that reason reproductions of the radioautograms are not included here. The results, however, are quite clear and are as tabulated below.

Time	Labeling
5 min. . .	A material believed to be citral > piperitenone.
10	Citral > piperitenone.
16	Citral > piperitenone.
23	Pulegone > citral > menthone, piperitenone, and piperitone.
32	Pulegone > menthone > piperitenone, citral and piperitone.
41	Pulegone and citral > piperitenone > menthone.
50	Pulegone > menthone > citral > piperitone.
60	Everything but extremely faint.

If the rate of interconversion of terpenes is not too great, an analysis of their distribution through the plant should give some fair indication of the order in which they are formed. The youngest leaves should contain the first-formed terpenes, and there should be a distinct progression to the oldest leaves, which should contain the end products of the terpene interconversions.

By such a process Reitsema (47) was able to suggest that unsaturated ketones are the first terpenes formed and that menthone and menthol are formed by their reduction. Evidently because of need for a fairly large sample, Reitsema was able to divide his plants for study into only three groups, young, mature, and old leaves. In the experiment detailed here the division was carried further, each leaf pair forming one of the samples analyzed.

A peppermint shoot growing in the greenhouse was taken in mid-April from the ground up and divided into ten samples:

1. First leaf pair, the oldest and lowest leaves, pale yellow-green, one-half maximum size.
2. Second leaf pair, slightly larger and darker than the bottom pair.
3. Third leaf pair, normal adult leaves.
4. Fourth leaf pair, normal adult leaves.
5. Fifth leaf pair, leaves of maximum size, dark green, slightly youthful.
6. Sixth leaf pair, leaves maximum size, youthful, reddened.
7. Seventh leaf pair, not quite full size, reddened.
8. Eighth leaf pair, half grown, pale green.
9. Crown, consisting of two small leaf pairs and the

apical bud.

10. All axillary buds and shoots, ranging from 2 to 10 mm.

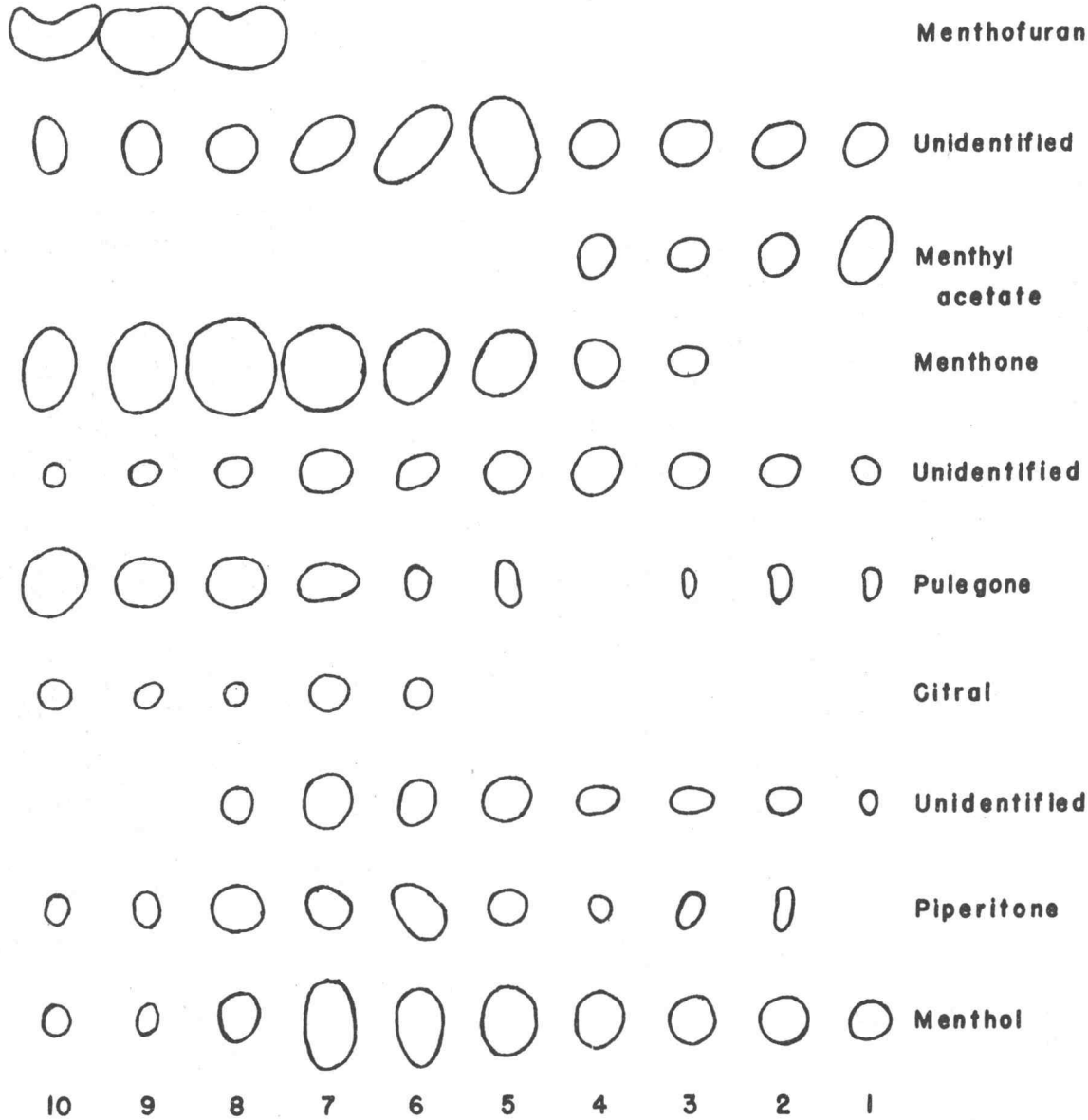
Extracts of these samples gave the chromatograms combined and illustrated in Figure 14. Since the sizes of the spots on chromatoplates are related to the quantities of materials in them, the figure indicates extensive changes in oil composition from one leaf pair to another. Other similar analyses of terpene distribution in the mint shoot were in general agreement with the results of this first experiment, but none were so complete.

The use of labeled terpenes as substrates for mint systems offers several problems. Synthesis of labeled terpenes would be quite difficult, and their isolation from biological systems would probably require addition of cold carrier. Introduction of the labeled terpenes to mint systems then runs into the problem of the slight water solubility of the terpenes. If the terpenes are added as oils to the leaves of mint shoots, even such small quantities as one microliter are sufficient to kill several square centimeters of leaf.

It was observed that sliced mint leaves covered with water are able to utilize mevalonic acid, although apparently not for the production of terpenes. It was

FIGURE 14

DISTRIBUTION OF TERPENES IN A SHOOT



Leaf pairs are numbered from the bottom. Sample 9 is the crown and sample 10 the axillary buds.

also determined that the silicic acid-plaster of Paris mixture from chromatoplates was not harmful to mint plants; so labeled terpenes were obtained by removing them from chromatoplates which had not been sprayed. In this manner samples of the chromatoplate mixture containing radioactive piperitenone, pulegone, and some unidentified materials were obtained. These were covered with small amounts of water and mixed with tissue slices of terminal buds and very young leaves of M. piperita. The systems were then left in one of the growth chambers for 16 hours. Extracts made of the mint leaf fragments and the water in which they were immersed were run on chromatoplates. In most of the experiments only the original labeled substrates were detected, and those were in all cases extremely faint, no doubt due to ineffective extraction. In two instances, however, there were faint suggestions that pulegone had given rise to menthone.

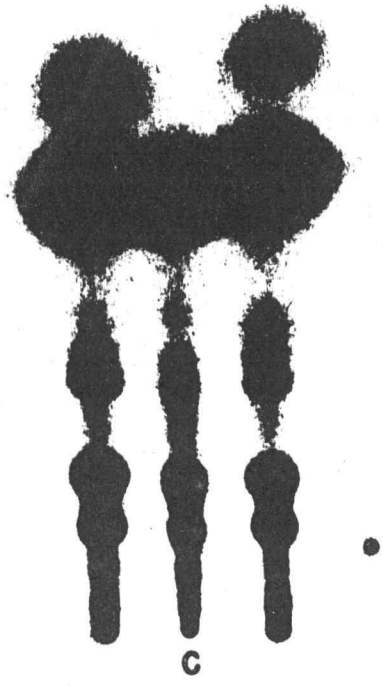
To check the possibility of pulegone conversion to menthone, several young, tender M. pulegium shoots were exposed for 57 hours to one mc of carbon dioxide under the full intensity of light of growth chamber II. The pulegone and piperitenone spots from the chromatogram of the soak of the shoots were counted and radioautographed, showing something of the order of 40,000 cpm of pulegone and 2,000 cpm of piperitenone. These were removed

and slurried with samples of M. piperita sliced under water or with water alone: piperitenone plus tissue slices, piperitenone plus water, pulegone plus tissue slices, pulegone plus water, and pulegone plus tissue slices of blooming mint. (Blooming mint has an especially high menthofuran content; slices of it were included in hope of demonstrating the conversion of pulegone to menthofuran as Reitsema (42) has postulated.) All five slurries contained in addition small amounts of sodium ascorbate. The samples were left in growth chamber II for 15½ hours. Extracts were then made of the mint tissue slices and the solutions in which they had been immersed and also of the control solutions which had contained no mint. The extractions were made with one extra mint leaf (mature) added to provide cold carrier terpenes for identification purposes. The extracts were chromatographed and radioautographed. The resulting radioautograms are shown in Figure 15.

FIGURE 15

INTERCONVERSIONS OF TERPENES

Piperitone (a) is formed from piperitenone (b) by mint tissue slices. Controls (C) contain no tissue slices.



d

e . . . Pulegone is converted to menthone (d) by mint tissue slices.

DISCUSSION

Goodwin tried with cunning, skill, and persistence to show the incorporation of mevalonate into carotenoids of maize seedlings (21) and failed consistently to do so, although in concurrent experiments he was able to show that carotenoids were being synthesized by the incorporation of $C^{14}O_2$ into them. His faith that mevalonate was the true precursor in spite of his failures seems thoroughly justified in view of results with other organisms. The incorporation of mevalonate into carotenoids has been demonstrated in Mucor hiemalis by Grob (22), in Phycomyces blakesleeanus by Braithwaite and Goodwin (15 and 16), and in tomatoes by Purcell (41) and Schneour and Zabin (53). The experiments related in this thesis have repeatedly shown incorporation of mevalonate into yellow pigments soluble in Skellysolve B; so here again should be evidence that mevalonate is a carotenoid precursor. A somewhat similar situation arose in the biosynthesis of rubber: Barlow and Patrick (8) demonstrated incorporation of mevalonate into a number of derivatives by Hevea brasiliensis, but no incorporation into rubber; Park and Bonner found considerable incorporation into rubber by using cell-free systems of the same species (39).

Certainly the experiments related here cannot in any instance suggest that mevalonate is a terpene precursor. Nor, on the other hand, can they be construed as evidence that mevalonate is not a terpene precursor. It does, however, seem strange that glucose and leucine both serve to produce terpenes while mevalonate does not.

Three obvious explanations first come to mind:

(1) Mevalonate is not the terpene precursor.

(2) Mevalonate as administered to the plants has not been in a usable form.

(3) Mevalonate cannot reach the site of terpene biosynthesis.

(1) That mevalonate is not the terpene precursor is at the moment a very difficult idea to accept. The experiments of Birch (9) and of Arigoni (3) are very convincing, as are the numerous experiments on isoprenoid compounds other than terpenes.

(2) That the mevalonate as administered to the plant might not be in a usable form for terpene biosynthesis seems highly unlikely since it is incorporated so readily into carotenoids in the same experiments. It seems unlikely that the initial steps of mevalonate utilization for terpene biosynthesis and for carotenoid biosynthesis should be different. This is especially improbable in view of the results of Dr. W. D. Loomis

(unpublished) showing initial phosphorylation reactions of mevalonate in pumpkin identical to those previously reported in liver and yeast.

(3) The idea that mevalonate for some mechanical reason cannot reach the site of terpene biosynthesis is not a difficult concept to accept at all. Of course, it is true that terpene synthesis has been clearly shown in this investigation to be almost exclusively a function of very young tissue, and numerous of these experiments have shown the introduction of mevalonate into this young growth. However, the structure of the oil gland as elucidated by Howe (25, p. 81-99) is highly suggestive of isolation. It is shaped like a mushroom, with the four to twelve cells of the disc-shaped capitulum connected by a single stalk cell to a single base cell at the leaf surface. There is, it is true, no proof that the terpenes are actually synthesized within the gland itself, but the suggestion is a rather reasonable one. The structure of the oil gland does not offer any suggestion that carbon dioxide should have difficulty entering, at least in the young stages of the gland when the terpenes are being formed. The mature gland seems to have the capitulum almost completely contained within a membranous bag of oil, but at that time the requirement for carbon dioxide for terpene synthesis should be past.

At this stage, furthermore, Howe reports that the cell plate of the capitulum begins to disorganize. It is well to point out in considering the possibility of carbon dioxide fixation by the oil gland that there is no report in the literature concerning the presence of chloroplasts in the gland. Adding to the confusion is the fact that both glucose and leucine introduced by uptake through the cut stem contribute labeling to terpenes. This would suggest that, if terpenes are synthesized in the oil gland, glucose and leucine can be translocated to the gland. If that be true, then it is not necessary to look for chloroplasts in the oil gland. But again, if glucose and leucine are translocated to the gland, what prevents the translocation of mevalonate? Assume that competition for the mevalonate by carotenoid biosynthesis merely leaves insufficient mevalonate to label terpenes noticeably. Why, then, should this be only in the case of introduced mevalonate, why not in the case of mevalonate synthesized from CO_2, glucose, or leucine? Another consideration is the conversion of pulegone and piperitenone to other terpenes by mint tissue slices under conditions that show the conversion of mevalonate to carotenoids. A short consideration of such ideas leads quickly to the notion that there is simply not enough information on hand to support any conclusions as to the

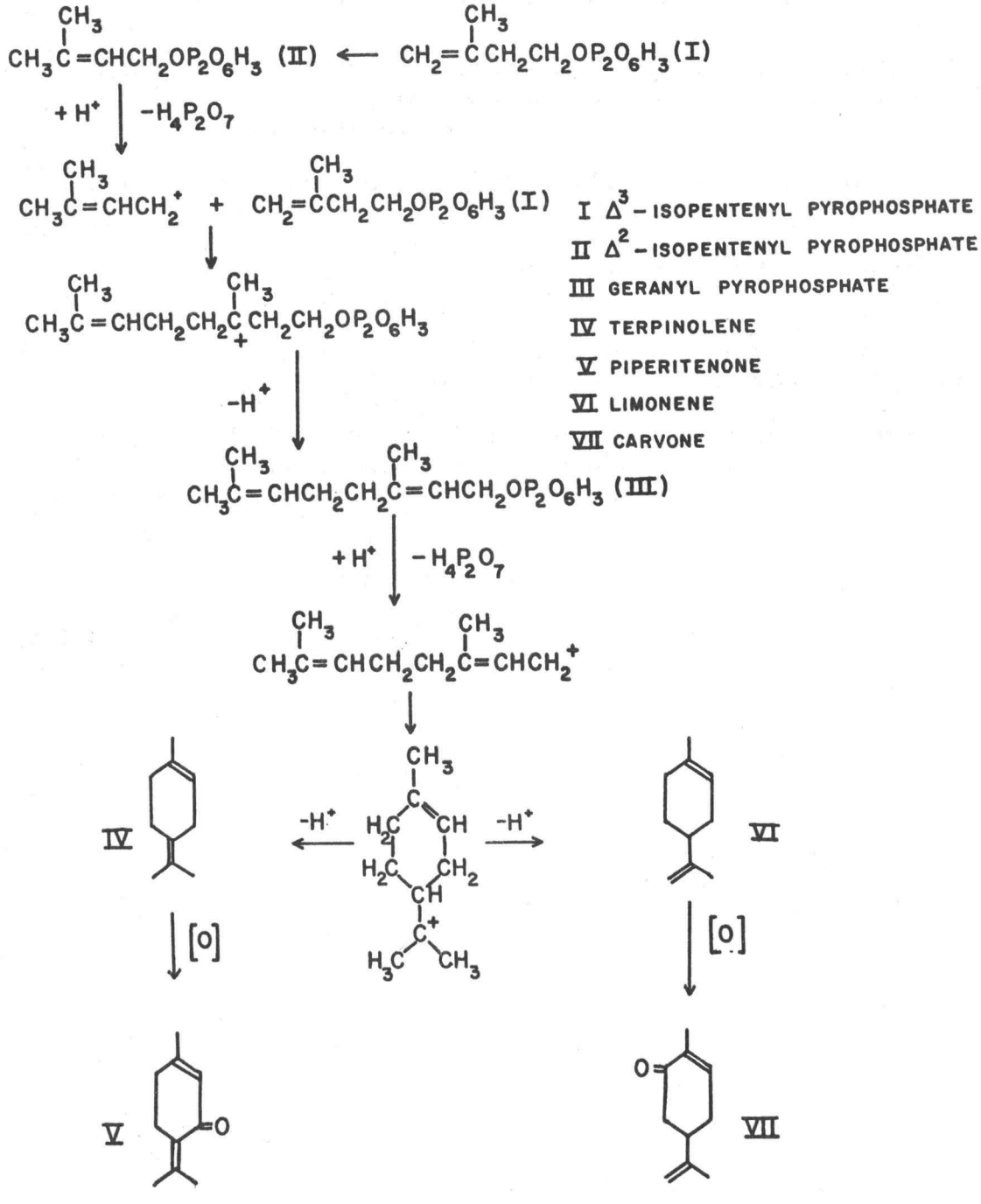
role of mevalonic acid in the biosynthesis of terpenes. Experiments of a much more definitive order are required before a sound explanation can be presented.

It is Lynen's belief (1, 33, and 34) that all isoprenoid compounds stem from mevalonic acid by the steps given in Figure 4, that the Δ^2-isopentenyl pyrophosphate forms a relatively stable carbonium ion for attack upon the Δ^3-isopentenyl pyrophosphate, and that the product rearranges to give the same carbonium ion stabilizing structure with 2,3 unsaturation. From this postulated intermediate of Lynen's, a geranyl ion, one might suggest a ring closure to a monoterpene. These postulated reactions are shown in Figure 16. Two different terpene hydrocarbons could very easily arise from Lynen's geranyl ion: limonene and terpinolene. Limonene is found in a great many terpene-producing plants including M. piperita. Terpinolene, on the other hand, has been reported from very few natural sources. However, it is very unstable to oxidation and polymerization and could conceivably be missed if present in an oil in small quantities.

It is interesting to note that terpinolene, through hyperconjugation, should be extremely easily oxidized in the three position of the ring, which is alpha to both of the double bonds. Limonene, lacking

FIGURE 16

HYPOTHETICAL BIOSYNTHESIS OF CARVONE AND PIPERITENONE



the concerted action of two double bonds, might as easily be oxidized in the six position of the ring, that is, considering the ring double bond to be in the 1,2 position. Upon exposure to air limonene is, indeed, oxidized in the 6 position according to Guenther (23, vol. 2, p. 22-27) with the formation of carvone and carveol as the primary oxidation products.

These suggested oxidations and their products are illustrated in Figure 16. Both piperitenone and carvone occur in mint plants, but, interestingly, they are not known to occur in the same plant. Plants of the peppermint type, Mentha piperita, M. arvensis, M. pulegium, etc., contain piperitenone; plants of the spearmint type, M. spicata and M. niliaca, for example, contain carvone. In M. crispa, according to Reitsema (35), either peppermint type terpenes oxidized in the three position or spearmint type terpenes oxidized in the two position may occur, but not in the same individual. In a genetic study (35) Reitsema found that the inheritance of spearmint or peppermint characteristics was compatible with the idea that one pair of genes controlled the character.

Reitsema's scheme for terpene interconversion (42) given in Figure 5 suggests that piperitenone is the first terpene characteristic of the peppermint type oil, and that the others are derived from it. It has been

possible in the foregoing experiments to show the conversion of piperitenone to piperitone in accordance with Reitsema's scheme. The experiment which showed this, however, casts some doubt on the idea that pulegone is formed from piperitenone, too, but by hydrogenation of the other double bond. In the peppermint sliced for the experiment, and in fact, in the peppermint used for all of these studies to date, piperitone has been a minor terpene while pulegone has been one of the principal ones. There must usually be something of the order of one hundred times as much pulegone as piperitone in the oils examined. Yet in this experiment, piperitenone showed roughly a thirty percent conversion to piperitone without any trace of pulegone appearing. If there could be postulated a difference in mechanism which took into account the fact that the mint tissue slices were in water and might thereby have been deprived of a portion of their oxygen supply, then this lack of pulegone formation might be understandable. The fact is that both pulegone and piperitone production from piperitenone would represent the saturation of double bonds, a similarity which suggests both reactions might well occur under the same conditions. The evidence from this experiment does seem, nevertheless, to suggest strongly that pulegone has another precursor than piperitenone.

The conversion of pulegone to menthone suggested by Reitsema (42) is confirmed by a second experiment with mint tissue slices. While the percent conversion is not so handsome as in the case of the conversion of piperitenone to piperitone, nonetheless the conversion is unmistakeable. A disappointment is the failure of the same experiment to show conversion of pulegone to menthofuran. It has been observed in countless experiments that menthofuran and pulegone are associated, pulegone often observed in the absence of menthofuran, but thus far never vice versa. Both are usually major components of blooming peppermint. Lemli (31) finds menthofuran the main terpene of all extremely active young tissues such as flowers and stolons, and this investigation attests the abundance of pulegone in those. For this reason it was hoped that mint tissue slices from flowering mint incubated with pulegone might yield menthofuran in accordance with Reitsema's scheme.

Information from those experiments employing mint tissue slices and labeled terpenes as substrates is scanty, but it has a tremendous advantage of clarity over the information obtained by other methods. The results of experiments on distribution of terpenes through the shoot and changes of labeling pattern with time are not altogether unambiguous. These can for the most part only

suggest what must be proved by the use of labeled terpene substrates.

The distribution of terpenes in the shoot (Figure 14) seems largely in agreement with the ideas of Reitsema. The youngest leaves contain by far the largest proportion of unsaturated terpenes; the oldest leaves much the greatest proportion of saturated terpenes. Menthofuran occurs only in the youngest leaves in conjunction with large quantities of pulegone, which in itself makes no implication concerning either the origin of the menthofuran or its end: it may or may not be produced from pulegone, and so far as this observation serves, it can disappear equally well with formation of some saturated terpene or the hydroxylactone mentioned by Lemli (31). The abundance of pulegone in the youngest leaves, the early rise and slow fall of menthone content proceeding from youngest to oldest leaves, the somewhat slower increase and decrease of menthol, and the very late appearance of menthyl acetate are all in agreement with Reitsema's postulated conversions: pulegone to menthone to menthol to menthyl acetate. The roles of piperitenone and piperitone do not show so clearly. Piperitone, in fact, seems to behave as an end product rather than an intermediate. In addition several unidentified materials follow the idea of conversion of unsaturated

substances into saturated end products.

Evidence from time course studies is largely in support of Reitsema's scheme, also. Figure 12, for example, shows a progression from labeling of piperitenone, piperitone, and pulegone toward heavier labeling of menthone and, at the last, of menthol. Labeling of pulegone and menthofuran were in many instances observed when no labeling appeared in either menthone or menthol. The reverse was never observed. Menthone labeling was frequently observed when menthol labeling was not, but never vice versa. Menthyl acetate labeling has been definitely observed only long after exposure of the plant to labeled carbon dioxide. This case must be examined with care, however, for labeling of the menthyl acetate can involve the acetate residue just as easily as the menthol moiety. All of these observations can be explained by Reitsema's scheme, but they cannot be construed to be proof of it.

Some information on the interconversions of terpenes ought to be derivable from a study of the differences of composition between soaks and extracts. In most instances soaks remove almost all of the terpenes before the extract is made. However, there are some very interesting exceptions. Menthyl acetate has been very rarely found in soaks even when the succeeding extracts

showed it to be present. Piperitone and piperitenone may be found either in soak or extract, but are more likely to be found in the extract. Menthofuran is a complete puzzle; in some experiments it has been found exclusively in the extracts, in others exclusively in the soaks. Rarely is it found in both. A very strange observation was that in some instances pulegone appeared both in soak and in extract, labeled in the soak and cold in the extract.

The immediate suggestion is that the terpenes are divided into those actively involved in metabolism somewhere within the plant cells and those stored in the oil glands. Loud and Bucher (32) have reported such a division of liver squalene. The oil glands, with their thin envelopes containing the oil outside the main cellular structure of the gland capitulum, do look as if they should readily yield their oil to soaking in Skellysolve B. Menthyl acetate, it might appear, represents the removal of menthol from the gland for some further metabolic use within the plant. The fact that piperitone and piperitenone are mostly in the extract might be indicative of their rapid transformation into other terpenes. This sounds plausible until citral is considered: it is the earliest labeled terpene yet noticed, but it has appeared solely in the soak.

The place of citral in the scheme of terpene interconversion is quite obscure. The first consideration is, of course, the question of identity. In the experiments in which the material tentatively identified as citral appears labeled, it has not been detected by chemical means. The Rf at which the supposed citral appears is one at which no component has been noted in the commercial mint oil sample used throughout this investigation as a standard. It is the Rf at which known citral markers appear. In the chromatography of a number of concentrated soaks of young tissues, a material has been detected at this spot which absorbs ultra violet light, decolorizes permanganate, forms a phenylhydrazone, reacts with o-dianisidine, and catalyzes the oxidation of p-phenylenediamine by peroxide. All of this fits citral perfectly, the Rf, the ultra violet absorption, the oxidation and aldehyde tests. It would seem unlikely that other substances could be found in a terpene oil to match these characteristics. However, although citral is known to occur in some mint species (42, p. 267), it is not known to be a component of peppermint oil. The identification must for the present remain in considerable doubt.

Let us assume for the purpose of discussion that the material in question is citral and summarize the

observations concerning it. (1) Citral becomes labeled in five minutes from carbon dioxide as a precursor. (2) The labeling disappears rapidly from the citral spot concurrently with an increase in labeling of pulegone. (3) Mint samples frozen in Skellysolve B at the moment of sampling do not show citral labeling, whereas those dropped into boiling Skellysolve B show it prominently. In one other sample in which the mint shoot was merely dropped into Skellysolve B at room temperature (the usual treatment) labeled citral was detected. (4) Citral labeling as a prominent feature of mint soaks has been observed only in samples taken short whiles after the commencement of labeled carbon dioxide introduction.

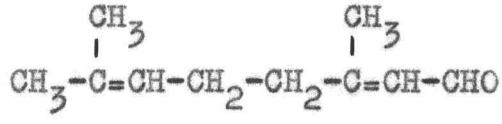
These observations suggest two principal possibilities: First that citral itself is an intermediate in monoterpene biosynthesis, and second, that some very labile intermediate of terpene biosynthesis can be broken down to yield citral.

From the observation of which terpenes appear labeled as citral labeling disappears, it is immediately suggested that citral might be a precursor of pulegone. However, examination of the structures of the two does not encourage the idea. The ring closure would necessitate loss of two hydrogen atoms from what would become positions three and four of the ring and their

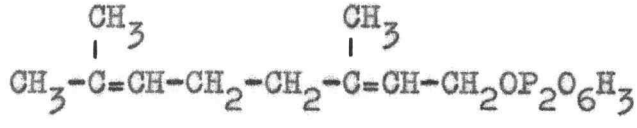
reappearance to saturate the double bond between carbons one and two. A simpler conversion would be to piperitone by transfer of one hydrogen atom from the three position to the eight, but with piperitenone a definite close precursor, it hardly seems reasonable to implicate citral, too. Kremers (29) in 1922 postulated a conversion of citral to citronellal to isopulegol to either pulegone or menthone. Babička, Volf, and Lebeda (6) have patented a process for preparing menthol from citronellal, isopulegol, or pulegol by incubation with Penicillium digitatum for a period of four weeks. A rapid conversion of citral to citronellal seems quite likely, but no labeling in the Rf of citronellal was observed in connection with citral labeling.

The possibility that citral might be a decomposition product of some terpene intermediate is an intriguing one. Compare the structures of citral and the phosphorylated intermediate postulated by Lynen, geranyl pyrophosphate (34).

Citral:



Geranyl pyrophosphate:



While the conversion is not one which might be expected by boiling in Skellysolve B, the similarities are striking.

The circumstances under which citral appears to be labeled are not clearly defined. Samples placed to soak in Skellysolve B at room temperature in most instances appeared to lose citral labeling if they contained any. This is perhaps understandable; enzyme activity may continue for quite a while under those circumstances, for the leaves remain clearly green for at least fifteen minutes before turning brown. In the case of freezing the leaves in Skellysolve B cooled with dry ice, the same explanation may perhaps hold: the leaves remained bright green for a day in the deep freeze, presumably so encased in ice that the Skellysolve B was unable to penetrate the leaf. When they were removed, they had some time to thaw before the leaves were removed from the liquor, time enough, perhaps, for some hardy enzyme to resume function and convert citral to something else. On the other hand, the argument seems equally good that the decomposition of an intermediate to yield citral occurred poorly under all of the conditions tried except at 70°C, the boiling point of Skellysolve B.

SUMMARY

1. Experiments with $C^{14}O_2$ have shown that terpene synthesis in peppermint is a function of very young tissue only, but interconversions occur in older growth.

2. The formation of the first terpenes is a very rapid process, with several terpenes formed from $C^{14}O_2$ in less than a half hour and a material tentatively identified as citral formed in five minutes. Reduction to saturated terpenes occurs much more slowly.

3. Labeling of mint terpenes has been demonstrated using glucose and leucine as substrates, but only labeling of carotenoids has resulted from β-methylcrotonate and mevalonate, although introduction of mevalonate has been attempted by numerous methods.

4. Analysis of the distribution of terpenes through the leaf pairs of a single shoot supports Reitsema's contention that the first-formed terpenes are the unsaturated ketones and that saturated compounds are produced from these. Time studies using $C^{14}O_2$ support the portion of Reitsema's scheme: pulegone yields menthone yields menthol.

5. The conversions of pulegone to methone and piperitenone to piperitone have been observed in peppermint tissue slices using labeled terpenes as substrates.

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