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Title The Biosynthesis of Terpenes in Pelargonium Graveolens

Abstract approved Redacted for Privacy
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Pelargonium graveolens Ait., rose geranium, contains several oxygenated monoterpenes, most of which are related to each other structurally. In this work, the metabolism and interconversions of these terpenes was investigated.

A chromatographic analysis of geranium oil was performed initially for the purposes of identifying the terpenes and determining which chromatographic method was most desirable for this work. It was found that gas-liquid chromatography was superior to thin layer chromatography both in resolution and in affording a convenient and sensitive method of counting radioactivity.

Three types of experiment were used in attacking this problem:

(1) Variations in terpene synthesizing ability with

leaf age were noted in order to determine the site of most active biosynthesis.

(2) Leaves were exposed to $C^{14}O_2$ in light for periodically increasing time intervals, and samples were taken at those intervals. The radioactive terpenes were extracted, and were separated by means of gas-liquid chromatography, and the terpene fractions were counted in a liquid scintillation counter.

(3) Isolated carbon-14 labeled terpenes were used as substrates in interconversion reactions by placing them in contact with sliced or minced geranium leaves in the light.

From the above, it was determined that the interconversions of the terpenes of geranium are very rapid and proceed through the biosynthetic pathway mainly by a series of reductions. It was also shown that the biosynthesis of terpenes is very active only in those leaves that are still expanding.

Besides the above, two types of study were performed that were not directly related to the interconversion problem.

Because it is a widely postulated precursor of terpenes, the incorporation of mevalonic acid into the terpenes of geranium was attempted, and possible incorporation of mevalonic acid-2-C^{14} into geraniol-citronellol,

citral, and a fraction which migrates to the solvent front in thin layer chromatography was observed.

Studies were performed to develop methods of obtaining enzymatically active cell-free extracts from monoterpene producing plants. The enzyme activities extracted were glutamyl transferase (from Mentha piperita, peppermint, Mentha pulegium, pennyroyal, and geranium), mevalonic kinase (from peppermint), and an enzyme activity which possibly produced piperitenone, a monoterpene of peppermint and pennyroyal, using mevalonic acid-2-C^{14} as the substrate and extracts of either peppermint or pennyroyal as the enzyme system.

THE BIOSYNTHESIS OF TERPENES IN PELARGONIUM GRAVEOLENS

by

ROBERT DAVID ALLISON

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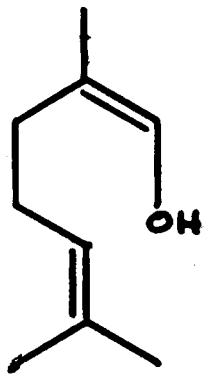
INTRODUCTION

The word "terpene," although it can refer to any compound of five carbons or more possessing an isoprenoid structure (6, 26), will be used here to indicate compounds of that structure possessing between five and 40 carbon atoms. Monoterpenes contain ten carbon atoms, or two "isoprene" groups, and can be cyclic or non-cyclic, oxygenated or hydrocarbons.

Monoterpenes are found in many plants, and among these, the genus Mentha and the genus Pelargonium provide rich sources. Some of the terpenes of Pelargonium graveolens Ait. are shown in Figure 1. (14, Vol. IV, pp. 671-737; 17, 22, 28)

Oil of geranium, which is the commercial name for oil of Pelargonium (14), is a complex mixture of monoterpenes and other organic compounds. Its commercial production is for the perfume industry, and it is commercially obtained by the steam distillation of Pelargonium graveolens plants harvested at a critically selected stage of plant development. The plants can be commercially produced only



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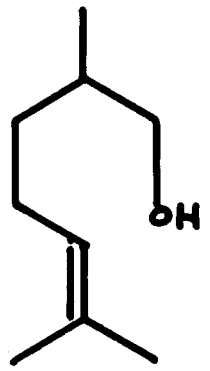

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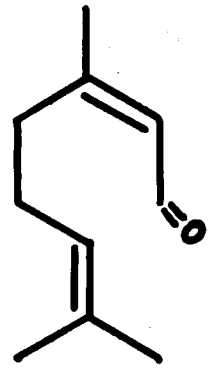

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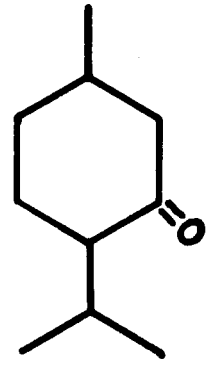
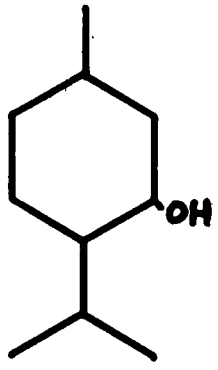


MENTHONE



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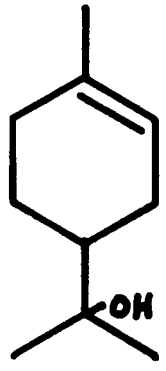
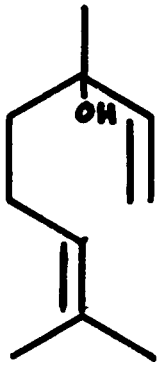
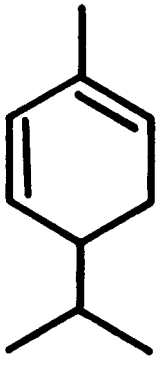


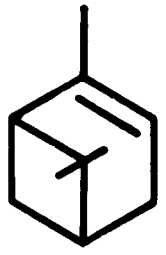
α-TERPINEOL



LINALOOL



α-PHELLANDRENE



α-PINENE

FIGURE 1

SOME TERPENES OF GERANIUM OIL

in warm climates, for they cannot live long at temperatures below 3° C (14). The main constituents of geranium oil are geraniol, the terpene in highest quantity, and citronellol (14, 22, 28). The amounts of these monoterpenes vary from 35 to 50 percent and 25 to 40 percent, for geraniol and citronellol, respectively (14). The only other monoterpene in any quantity in geranium oil is menthone, the others listed in Figure 1 being in very small or trace amounts (14).

Since the structure of monoterpenes shows the "isoprene rule" (26), it has been suggested that their biosynthetic precursors are the same as those found to exist for higher isoprenoids. Of these, one of the most interesting is mevalonic acid. This compound, which has been shown to be incorporated into cholesterol in almost theoretical amounts (29, 33), is considered to be a key precursor of the isoprenoids. Some of the precursors of mevalonic acid have also been used as possible isoprenoid precursors with varying success. Some of these include: isovaleric acid (13), β-methylcrotonic acid (10, 13, 16), and leucine (24).

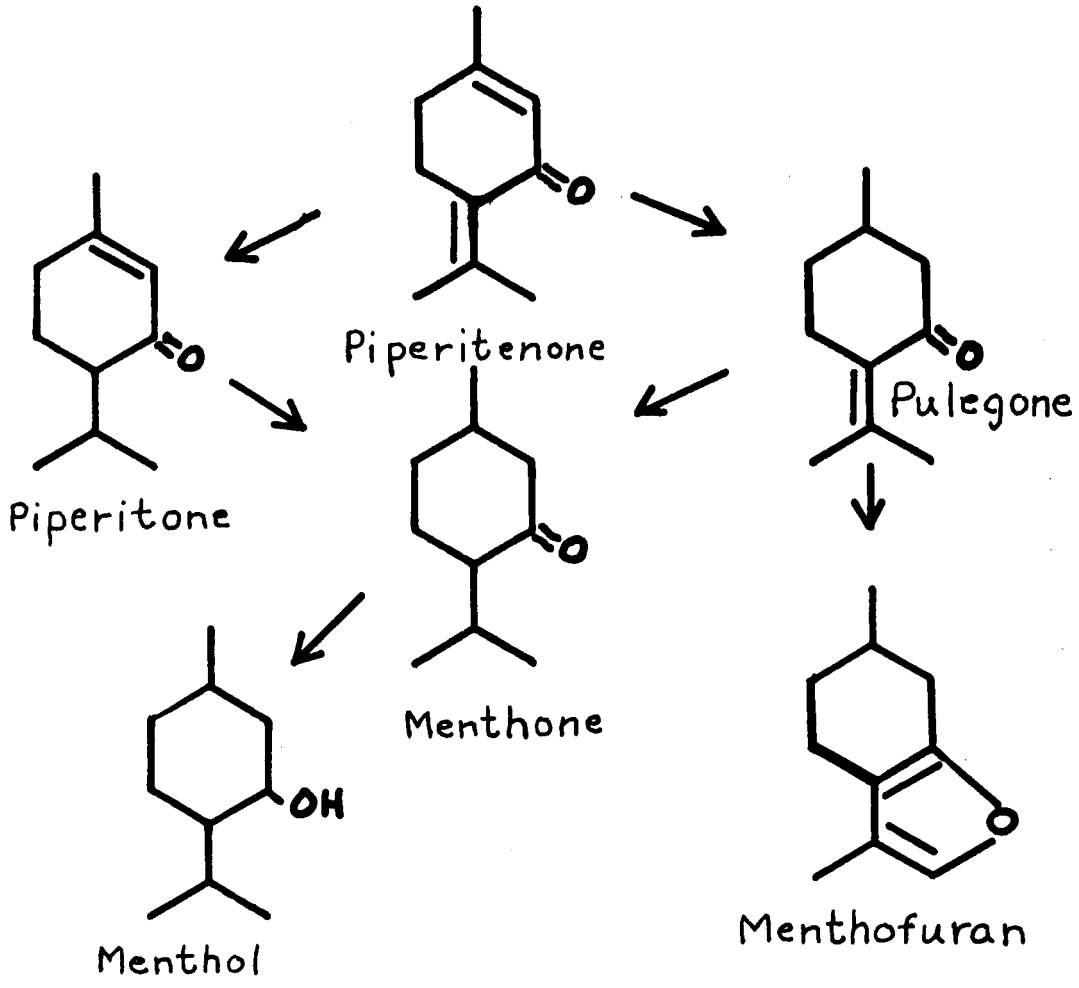
Mevalonic acid has been shown to be a precursor not only of cholesterol, but also of some plant isoprenoid

compounds. Some of these include rubber (23), carotenoids (2, 3), and some terpenes, although much difficulty has been experienced in the case of the monoterpenes (6, 8, 11, 12).

Another approach to the study of the biosynthesis of monoterpenes is to investigate the reactions and interconversions of the terpenes themselves. Most of the work along these lines has been performed on Mentha piperita L., peppermint.

Reitsema (25) was one of the first to do this, and he presented a scheme of interconversions that was later added to and supported by Battaile (6). This scheme indicated a series of reactions starting with piperitenone, which, by a series of reductions, is converted to the most saturated monoterpene in the series, menthol. The scheme is illustrated in Figure 2. It would seem from the above studies that reduction plays the dominant role in the interconversions of monoterpenes in peppermint. Since it has been found that TPNH is formed in plants in the presence of light, there may be a connection between the presence of light and the process of monoterpene interconversion (4, 5).

FIGURE 2



INTERCONVERSIONS OF TERPENES IN PEPPERMINT

In his investigation of the biosynthesis of terpenes of P. graveolens, Campbell (12) found that the amounts of citronellol and menthol decreased with age of leaf, but that menthone remained at about the same level. He also found that the uptake of $C^{14}O_2$ was slow at first, increasing to a constant value at about 30 minutes. As with the earlier work on peppermint, however, he was unable to obtain significant incorporation of mevalonic acid-2-C^{14} into the monoterpenes of geranium.

Campbell also did some work on cell-free extracts of peppermint and was able to obtain a small amount of triosephosphate dehydrogenase activity. Although the problem of obtaining enzymatically active cell-free extracts from monoterpene producing plants is important to future studies of terpene biosynthesis, it is made quite difficult for two reasons. (1) The glandular tissue which produces the monoterpenes is a very small part of the total tissue. (2) Apparently there are enzyme inhibitors present in the vacuoles. Techniques which are satisfactory for other plants tend to yield only tyrosinase activity when applied to peppermint. For these reasons, special techniques still awaiting development are needed for this type of work. At this point, the most useful

technique seems to be that of making an acetone powder from the leaf and either extracting the powder and further purifying the extract or simply using the powder as the enzyme system.

In this work, the purpose has been mainly to elucidate the interconversions of the terpenes of Pelargonium graveolens, rose geranium. Although the approach here was concerned for the most part with the relationships between the terpenes, some experiments were also performed using mevalonic acid as a possible terpene precursor. Also, some work was done to develop cell-free systems.

MATERIALS

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

Barium carbonate-C^{14}, specific activity 0.129 millicuries/mg, was obtained from Oak Ridge National Laboratory,

Oak Ridge, Tennessee. This was converted to the sodium salt by adding an excess of perchloric acid to it in an evacuated system in which the released $C^{14}O_2$ was allowed to dissolve in sodium hydroxide. Solutions were four millicuries/ml.

The material used for the chromatoplates was Merck Silica Gel G obtained from Brinkmann Instruments, Great Neck, L. I., New York.

The materials used for the gas chromatography columns were Quadrol (tetrakis (2-hydroxypropyl) ethylenediamine), SAIB (sucrose acetate isobutyrate), and 100/120 mesh fire-brick, all obtained from Wilkens Instrument and Research, Walnut Creek, California.

Geraniol and citral were obtained from Aldrich Chemicals, Milwaukee, Wisconsin.

Citronellol, linalool, citronellal, and isomenthone were obtained from K&K Laboratories, Jamaica, New York.

Menthol was obtained from A. M. Todd Co., Kalamazoo, Michigan.

Menthone and α-phellandrene were obtained from Eastman Co., Rochester, New York.

"Hexane" as used in this work refers to Skellysolve B (essentially n-hexane, boiling range 60-68° C).

Silicone coated anthracene (scintillation grade) glass tubes, and filters were obtained from the Packard Instrument Co., Inc., La Grange, Illinois.

Geranium oil was prepared in this laboratory by the steam distillation of geranium leaves.

Pelargonium graveolens plants were grown in the greenhouse from stock furnished by Dr. F. P. McWhorter.

Mentha piperita plants were the Mitcham variety planted commercially in the Willamette Valley. The plants were grown in the greenhouse from stock furnished by Dr. C. E. Horner.

Mentha pulegium plants were grown in the greenhouse from stock taken from the wild at Kiger Island, south of Corvallis, Oregon. Identification was made by Dr. A. N. Steward of the Oregon State University herbarium.

METHODS

In general, the samples in the experiments to be described were processed as follows: (1) Extraction of the terpenes from the leaves with hexane. (2) Separation of the terpenes in the extracts either on silicic acid chromatoplates or by gas-liquid chromatography.

(3) Determination of the amount of radioactivity in each terpene fraction using either a gas flow counter or a liquid scintillation counter.

Detection and Measurement of Carbon-14

In the cases in which silica gel chromatoplates were used to analyze the extracts in an experiment, the radioactive positions on the plates were determined by radioautography, using Kodak No-screen x-ray film. These positions, masked with plexiglass or polyethylene, were then counted by placing the probe of a Tracerlab TGC-14 gas flow counter about two millimeters above them, the probe being held in place by means of a rack especially constructed for the purpose. On a few occasions the radioactive area was scraped off of the plate, placed into scintillation fluid[1], and counted in a Tracerlab liquid scintillation counter.

When this method first began to be used, it was considered likely that some or all of the radioactive

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[1]Scintillation fluid: 5g 1,4-bis-2(5-phenyloxazolyl)-benzene(POPOP) plus 0.3g 2,5-diphenyl oxazole(PPO), dissolved in one liter toluene.

terpenes on the plates would evaporate as the plates dried after spraying. This was not found to be the case, however, and plates which were counted a few minutes after spraying and then a few days later showed no significant difference in radioactivity. This was also found to be the case with the terpenes of peppermint (6).

Plate counting with the TGC-14 suffered some definite disadvantages as compared to the method later used. Since the separation of the geranium oil terpenes on chromatoplates left something to be desired, it was often very difficult to determine, even with the aid of a radioautogram, exactly what area corresponded to a given component. The counting efficiency of this method was only about five percent, necessitating either very high activities or very long counting times in order to obtain statistically significant results. Often components did not possess high enough activity for significant detection at all. In the case of one experiment to be described later, the results could not be significantly detected until a more sensitive method of carbon-14 detection was used. Although not a serious defect, the fact that chromatoplates are delicate and easily damaged was an additional source of inconvenience.

Fortunately, another, very good method of detecting and measuring radioactivity was found to be applicable to this work. This method, which utilized the gas chromatograph to separate the components of the samples, was developed by the Packard Instrument Company. As each component emerged from the gas chromatograph, it was directed into a glass tube containing silicone coated anthracene. The tubes were fitted into a fraction collector which could be rotated, positioning the next, unused tube, at the press of a button, and the fraction collector was rotated each time a new peak appeared on the recorder trace. Since anthracene is a scintillator to beta radiations, these tubes, with the aid of an adaptor, can be placed in a liquid scintillation counter and counted. The overall counting efficiency of this system was determined elsewhere to be 25 percent (11).

Thin Layer Chromatography

The method of preparing the chromatoplates, although based on earlier work (7), was much simplified by the use of a prepared mixture, Silica Gel G, and the Desaga applicator, obtained from Brinkmann Instruments.

The plates were developed in a 15 liter chromatography jar covered with a glass plate and sealed with rubber cement. The developing solvent mixture used in all of this work was 12 percent (v/v) ethyl acetate in hexane. Other concentrations of ethyl acetate were tried, varying from 5 to 15 percent, but it was found that 12 percent gave optimum separation of the most terpenes. Lower concentrations of ethyl acetate tended to separate terpenes nearest the solvent front better, but adversely affected the separations of the more polar components; higher concentrations tended to drive most of the terpenes together toward the solvent front.

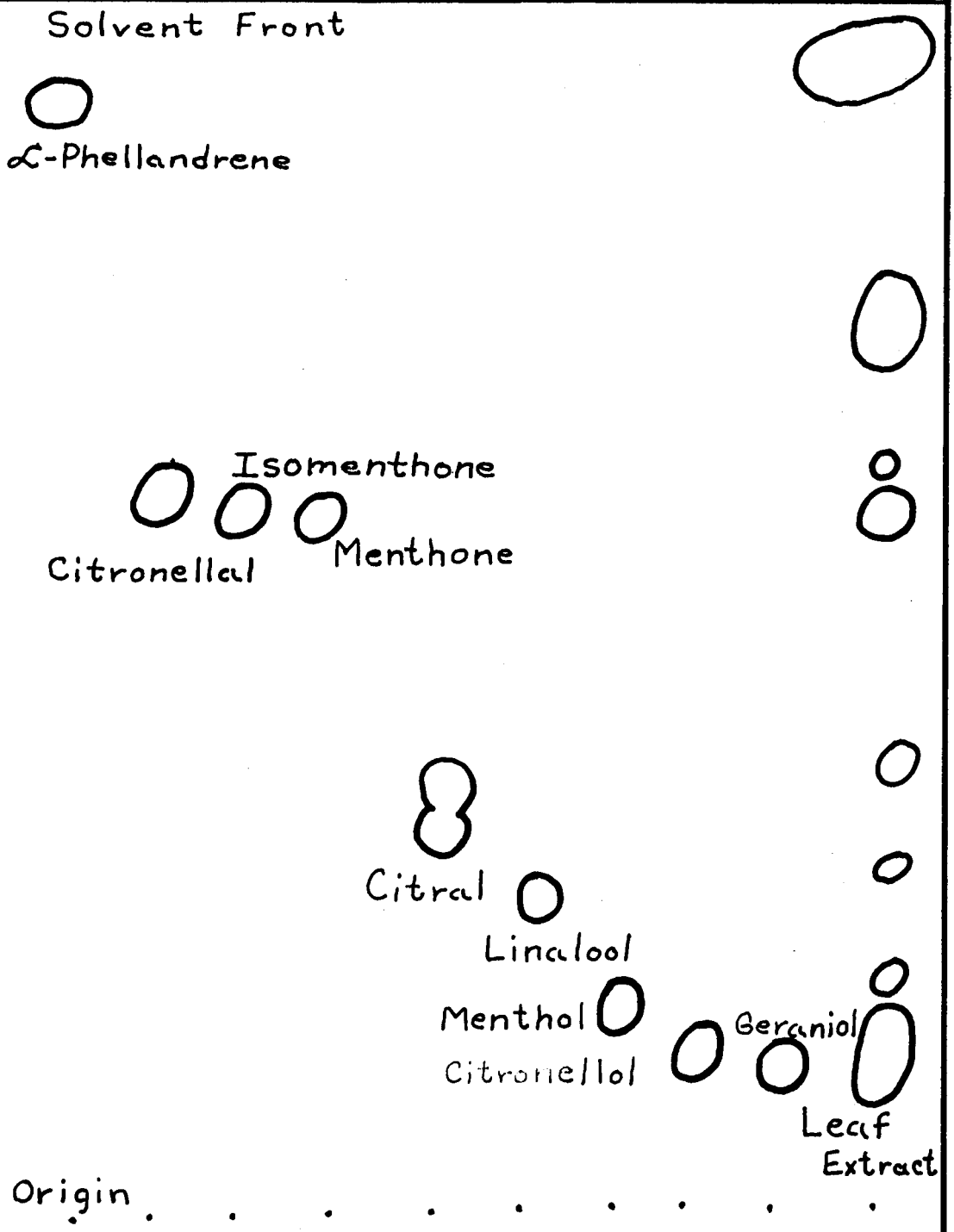
After development, the chromatoplates were routinely examined, first, by permanganate spray, second, by rhodamine B spray (7).

If the chromatographed terpenes were to be recovered from the plates, two methods were used. (1) The terpene solution, in hexane or ether, to be separated and recovered was spotted along with a known standard on either side. After development, the desired portion of the plate was masked with aluminum foil and the markers were sprayed, determining the position of the unsprayed components. Since the solution to be separated and recovered was often

an extract of carbon-14 labeled plant material, this position could usually be further checked by detection of radioactivity peaks corresponding to the component in question. (2) The terpene solution was applied in a band at the origin; so that instead of separate spots, there was one long, horizontal streak. After development, the plate was masked so that only narrow strips on the edges and in the center were exposed, and the plate was sprayed as usual. As above, the positions of the terpenes could also be localized by their radioactivity, detected either by a Geiger-Mueller counter or radioautography.

Figure 3 shows a reproduction of a typical chromatogram of the extract from a single still expanding geranium leaf, weighing about 0.3 gram, compared with known standards chromatographed separately and combined in the figure. The method apparently separates all of the terpenes of geranium oil satisfactorily except for geraniol and citronellol. Instead of separating these terpenes into two distinct spots, one elongated spot is formed by the development. Variations of ethyl acetate concentration only serve to cause more or less elongation but no definite separation. This, of course, creates a serious difficulty in any analytical study of terpene formation, for

FIGURE 3



DISTRIBUTION OF TERPENES ON A CHROMATOPLATE

geraniol and citronellol are not only the major components of geranium oil (14, 22), but are the terpenes that are most highly labeled with carbon-14 when geranium leaves are exposed to $C^{14}O_2$ in the light. Although attempts were made, in some of the experiments to be described, to determine the amounts of radioactivity in the upper half of the spot separately from the lower half, assuming that the bulk of the citronellol was in the upper half, and the bulk of the geraniol in the lower half, it was determined that results obtained in this manner could not be relied upon.

Gas-Liquid Chromatography

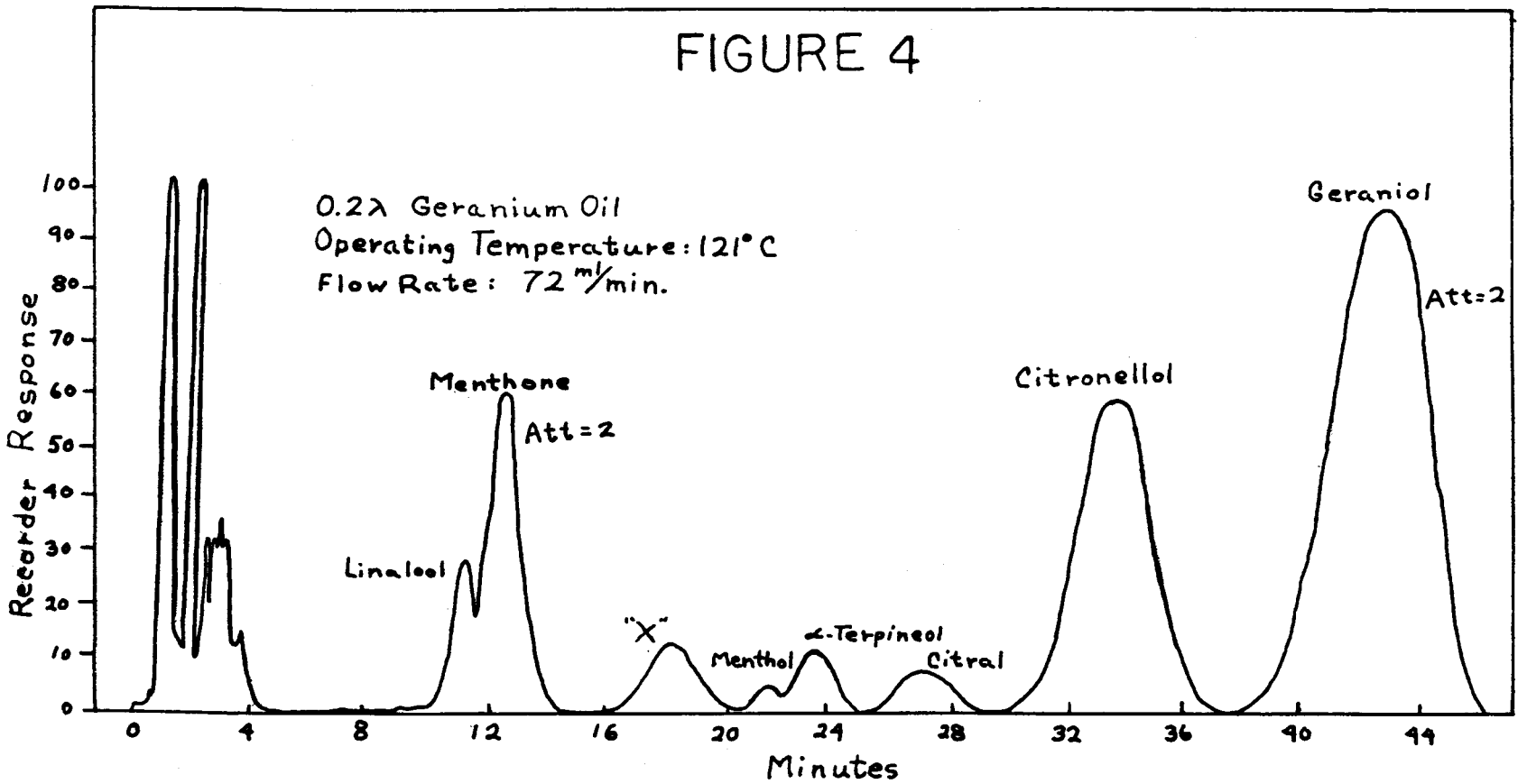
Since thin layer chromatography as a method for the separation of the terpenes of geranium oil possessed some definite disadvantages, the use of gas-liquid chromatography was investigated. This method was immediately found to be superior in many ways for this work. It is more sensitive, in general; with the use of the Disc integrator, it can be easily used for quantitative purposes, such as determining specific activities; and with the use of the Packard fraction collector, very sensitive measurements of the radioactivities of the fractions can be made.

Although many different types of columns have been devised for the gas chromatographic separation of terpenes (1, 9, 30), after some investigation it was decided that a Quadrol-SAIB column which had been developed for peppermint oil (1, 11) would be the most satisfactory for the separation of geranium oil terpenes. This column, after some work on modifications, was found to be a good compromise between very long retention times and poor resolution. It was used in a Beckman GC-2A gas chromatograph with a thermal conductivity detector.

The column used, therefore, in the bulk of this work was constructed of eight feet of one-quarter inch aluminum tubing packed with 100/120 mesh firebrick coated with 4.2 percent Quadrol and 1.6 percent SAIB. The method of column packing used was as described in (30).

Figure 4 shows a typical trace of geranium oil run through this column. As can be seen, the separations of all of the major components is quite complete except for linalool and menthone. These terpenes show little interference with each other in later studies, however; that is, their radioactivities do not seem to "overlap" excessively.

FIGURE 4



SEPARATION OF THE TERPENES OF GERANIUM OIL ON AN SAIB/QUADROL COLUMN

(Att., attenuation)

Although the operating temperature shown here is 121° C, and the gas pressure is 50 psi, these values vary from 120 to 130° C for temperature and 45 to 50 psi for pressure. It was found by the soap bubble method (11) that 45 and 50 psi correspond, respectively, to flow rates of 62 and 72ml/min. with the column connected to an anthracene trapping tube. The variances in operating pressure and temperature are due to slight differences in different columns used and to the effect of age in the same column. Even though each column is prepared in the same manner, individual differences seem to be present in each new one. These are usually slight, resulting most often in slightly longer or shorter retention times but sometimes must be offset by changes in temperature or pressure. Also, it has been found that a column's retention times become shorter, and resolution, as a rule, becomes poorer as the column is used. These effects, caused probably by decomposition or volatilization of Quadrol, its limiting temperature being 140° C (1), can be wholly or partially corrected by a slight (5 psi) decrease in helium pressure, but the maximum operating lifetime of columns of this type does not exceed 250-300 hours.

Although no attempt was made to determine the maximum sensitivity of the instrument used with regard to terpene analysis, very high sensitivity is indicated in Figure 4, which represents 0.2 microliters of geranium oil.

Identification of Terpenes: Thin Layer Chromatography

Identification of the spots on a chromatoplate is based on their positions as compared with known standards, as well as their behavior when treated with the two sprays used. The known compounds used as standards are all terpenes known to occur in geranium oil (14, 17, 22, 28). Thus, although the sprays and R_f values are not, in themselves, completely specific, they do allow reliable identification of components, when compared with the available information on the composition of geranium oil. Since the sprays used do not interfere with each other (7), this method of identification can be of great utility. As can be seen in Figure 3, spots are produced by the geranium oil possessing R_f's corresponding to those of geraniol, citronellol, menthol, linalool, menthone or isomenthone, citral, and citronellal. These spots also produce the same reactions to sprays as the corresponding known standards. The "citral" and "menthol" spots of the geranium oil are

very faint, however, and do not appear in all samples.

Identification of Terpenes: Gas-Liquid Chromatography

The method used for the identification of terpenes with the gas chromatograph utilized the fact that peak area, in general, corresponds to the amount of a particular component present, although the same amount of two different compounds will not produce exactly the same size peak, due to slight differences in thermal conductivity. This is illustrated in Table 1.

Table 1. Calibration values of peak areas.

Terpene	Micromoles	Peak Area*	Micromoles/ Unit Area
Linalool	5.65	2.80×10^{3}	2.02×10^{-3}
Menthone	5.84	2.83×10^{3}	2.06×10^{-3}
α -Terpineol	0.371	0.126×10^{3}	2.95×10^{-3}
Citral	5.85	1.83×10^{3}	3.19×10^{-3}
Citronellol	5.51	2.06×10^{3}	2.68×10^{-3}
Geraniol	5.72	2.75×10^{3}	2.08×10^{-3}

*One sweep of the integrator pen equals one area unit.

In this method, the mixture of interest is injected along with a particular known standard. If a component of the mixture corresponds to the known standard, a peak enlargement is observed; if not, a new peak appears. These observations are compared with the same amount of mixture chromatographed separately. Thus, geraniol, citronellol, linalool, menthone, α-terpineol, menthol, and citral were determined to be probable components of geranium oil by observing successive peak enlargements, but citronellal was not present in detectable amounts. Also, a peak was observed in the trace of geranium oil which did not correspond to any known standard with which it was compared; this is designated in Figure 4 as "x". The known standards used here were: geraniol, citronellol, linalool, menthone, isomenthone, citral, citronellal, menthol, α-terpineol, α -phellandrene, pinene, and l-borneol, all with the exception of citronellal, terpenes that have been found in geranium oil (14, 17, 22). The amounts used were 0.2 microliters of geranium oil plus three microliters of one percent (v/v) solutions in hexane of each of the known standards. Also, a run of 0.2 microliters of geranium oil alone was performed for comparative purposes.

Besides the above, a "combination" technique was used to aid in the identification of the terpenes of geranium oil. This technique is based on the assumption that if an unknown has the same chromatographic characteristics in two different types of chromatography, as compared with a known standard, then the probability is high that the compound is the same as the known standard, especially if the compound is known to exist in the mixture of interest. Two variations of this were used here.

The first method consisted of first chromatographing the hexane extracts of two small geranium leaves on a silicic acid chromatoplate, determining the positions of the terpenes by the methods described previously, scraping these areas off the glass plate, and eluting the terpenes from the silica with hexane or ether. The extracts from each position were then concentrated in a stream of air and injected into the gas chromatograph. This method, however, was found to be useful only for geraniol and citronellol, the terpenes of highest concentration in geranium oil, the other spots giving no detection on gas chromatography. The probable reasons for this are two: first, the extraction of the terpenes from the silica gel is not complete; second, the process of concentrating the hexane solution will

usually evaporate some of the terpenes along with the hexane. Of course, higher concentrations of geranium oil could be used on the original thin layer chromatography, but this proved to be inconvenient, especially since another, more readily useful method was available. However, this first method did produce an identification check on geraniol and citronellol.

The second method is simply a reversal of the first. Here, the extract is first run through the gas chromatograph and then applied to a chromatoplate by means of allowing the effluent from the gas chromatograph to flow directly onto the surface of the plate. The plate is then developed and sprayed, and the position of the spot is noted. This method was more convenient than the first and was seemingly more sensitive. The R_f's of all of the peaks shown in Figure 4 corresponded to those of known standards, except for menthol and citral, the positions of which showed no reaction to the sprays on the chromatoplate, even on increasing the geranium oil concentration. It must be assumed that these terpenes are in too low concentration to be detected in this manner; they often cannot be detected even when the extract is applied directly to the plate.

It was also seen here why compounds "x" and α-terpineol were not observed by means of thin layer chromatography. These compounds both possess about the same R_f as geraniol and would be masked by it on a chromatoplate.

Light Chamber

All of the experiments done involving intact or partially intact (such as minced) plant materials were performed in a light chamber built for the purpose. This chamber is described in (6).

Exposure of Plant Materials to $C^{14}O_2$

Experiments using $C^{14}O_2$ as the substrate were performed as follows. The samples were selected, cut, weighed, and measured, and were placed in small beakers containing enough water to cover the tip of the petiole and not too much more, in order to minimize the absorption of $C^{14}O_2$ in the water during the experiment. Usually one leaf was used per sample, but two were sometimes used in order to further reduce variations between samples.

Exposure to $C^{14}O_2$ was carried out in a sealed glass chamber in which the $C^{14}O_2$ was generated by adding perchloric acid to labeled sodium carbonate. The chamber was then placed in the light chamber for illumination. The

$C^{14}O_2$ was removed by flushing the chamber atmosphere through traps filled with 4N sodium hydroxide for about five minutes. After each sample was taken, the chamber was resealed and flushed for five minutes with air from which the carbon dioxide had been removed by being drawn through traps containing 4N sodium hydroxide.

The proportion of $C^{14}O_2$ absorbed by the leaves in the experiments varied from about 50 percent for a one hour exposure to as high as 92 percent for a 16 hour exposure.

Preparation of Samples

Extraction of terpenes from leaves was done routinely by grinding the leaves in a mortar with sodium sulfate, which acts both as an abrasive and as a drying agent. The greenish-yellow pigmented hexane extracts were decolorized with a minimal amount of Norit A charcoal and were concentrated in a stream of air. The extracts were then decanted from the Norit A, which was re-extracted with one to two milliliters of hexane, this extract being added to the first.

EXPERIMENTS

Selection of Plant Materials

When this work was first begun, the plant materials used consisted of terminal buds with very small leaves attached. These materials were chosen in line with the work done by Battaile and Loomis (8) in which it was shown that terpene synthesis in peppermint occurs only in young tissues, and that the synthesis is most active in very young leaves and the growing tips of shoots. However, when materials of this type from geranium were used, the results were somewhat disappointing. Although much care was exercised to obtain materials of the same size and weight, much variation in amount of radioactivity incorporated and amount of terpene synthesizing ability was noted. When larger, but still expanding, leaves were used, it was found that much more uniformity of sampling was possible without sacrificing any terpene synthesizing activity. The geranium leaves used, therefore, in the experiments to be described were matched as closely as possible and varied, from experiment to experiment, from 0.15 to 0.30 grams in weight, from 2.0 to 3.5 centimeters wide at the widest point, and from 1.5 to 3.0 centimeters long, not including

the petiole.

Radioactive Terpene Precursors Used

Although many compounds have been shown to be precursors of terpenoid type compounds (10, 13, 16, 24, 34), only a few have been used successfully in the study of the biosynthesis of monoterpenes. Of these, $C^{14}O_2$ is probably the most widely used. Mevalonic acid-2-C^{14} has also been used, but with much less success, even though it has been widely postulated as a precursor of monoterpenes (6, 12, 35).

In the experiments to be described, only $C^{14}O_2$ and mevalonic acid-2-C^{14} were used as precursors, the former being almost exclusively used, although a few experiments were done using mevalonic acid as the substrate.

Site of Terpene Biosynthesis

This experiment was performed in order to determine which leaf age was most active in the biosynthesis of terpenes and how terpene composition varied with leaf age.

In the experiment, the leaves were exposed to light and 400 microcuries of $C^{14}O_2$ for two hours, after which they were analyzed by the anthracene tube method. Six leaves were used (not including terminal buds), increasing progressively in age and weight and obtained from the same

geranium plant.

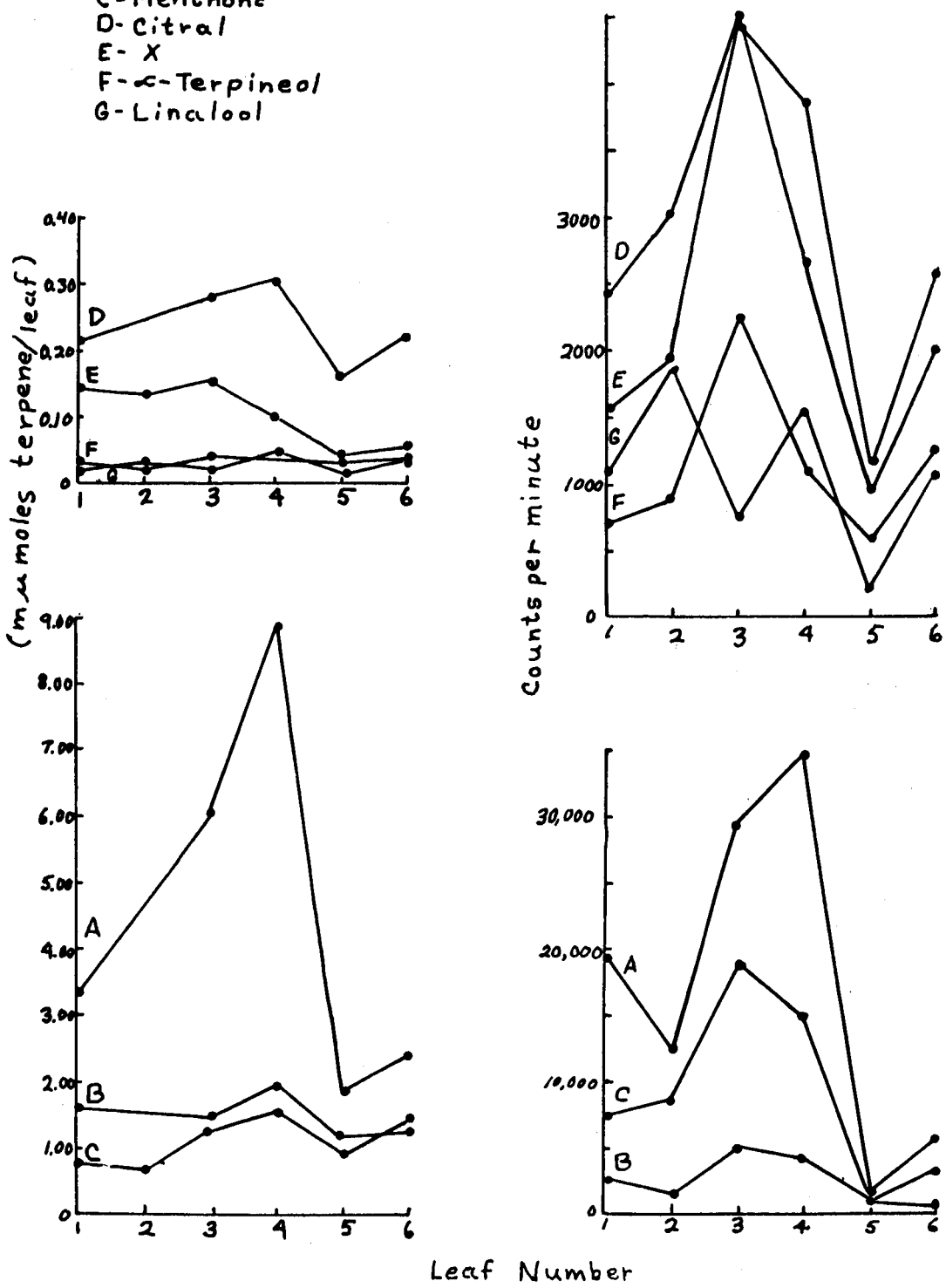
Leaf No.	Weight	Description
1	0.3 g each	Two very small, immature leaves.
2	0.4 g	One small, still expanding leaf.
3	1.3 g	One still expanding leaf.
4	1.6 g	One leaf, just coming to full size.
5	1.2 g	One large, mature leaf.
6	1.8 g	One very large, mature leaf.

Figure 5 shows the results of this experiment. First, it is seen that all of the terpenes except α-terpineol and linalool increase more or less in concentration as the leaves that are still expanding increase in size, but fall to lower levels in the fully expanded leaves, with a very striking rise and subsequent drop seen in the case of geraniol. α-Terpineol and linalool stay at about the same low level in all of the samples. These results differ from the case of peppermint, in which it was found that the most saturated terpenes occurred only in the oldest leaves (6, 8).

Figure 5 also shows a pattern of radioactivity that generally follows the concentration pattern, indicating that the most active site of terpene biosynthesis in

FIGURE 5

A-Geraniol
B-Citronellol
C-Menthone
D-Citral
E- X
F-α-Terpineol
G-Linalool



SITE OF TERPENE BIOSYNTHESIS

geranium is in those leaves that are just coming to full size.

Time-Course Experiments with $C^{14}O_2$

Time-course experiments were performed in order to gain information about the sequence of terpene formation in geranium. The purpose of the first of these was to try to find the minimum time which the plant needed in order to form each successive terpene in its synthetic pathway. The anticipated outcome of these experiments was similar to those obtained by Battaile (6) with peppermint, that is, a radioactive labeling pattern which showed successively more terpenes labeled as time progressed. It was found, however, that virtually all of the geranium terpenes became labeled in 50 minutes to one hour. Exposures to $C^{14}O_2$ for less than 20 to 30 minutes generally resulted in too little incorporation into terpenes for effective detection by means of radioautography. Nevertheless, one useful short time experiment was performed. In this experiment, samples were taken at 20, 40, and 60 minutes. A radioautogram made from a thin layer chromatogram of the three samples shows a slight incorporation of carbon-14 into geraniol-citronellol at the 20 minute sample, increasing in the 40 minute sample, but with no other terpenes

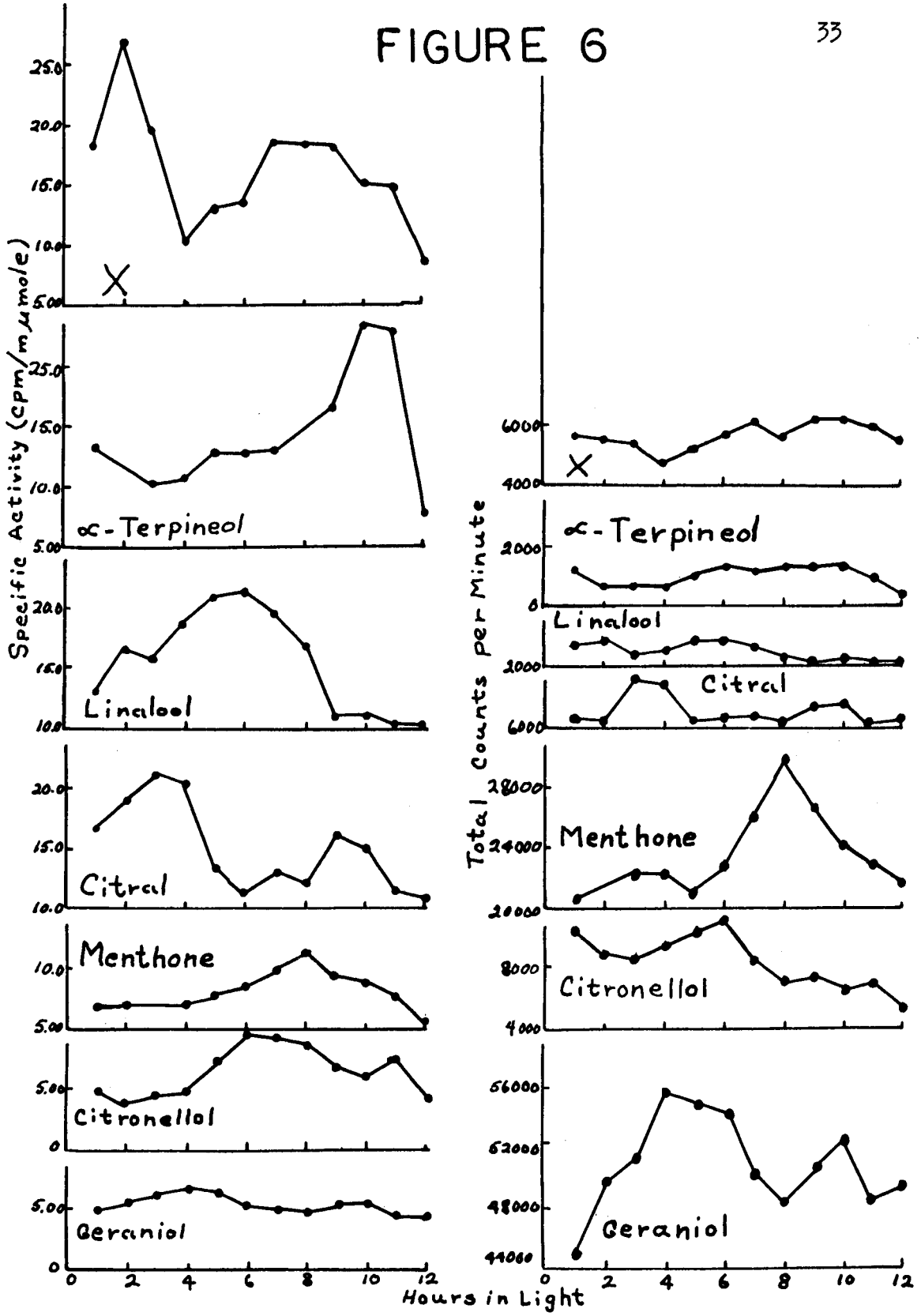
labeled until the 60 minute sample was reached, where labeling was observed for linalool and citral, also.

In all of the experiments to be described in the following, the method of carbon-14 detection and measurement was by the anthracene tube method unless stated otherwise.

When geranium leaves are continuously exposed to $C^{14}O_2$ in light, it has been already shown that the terpenes will become labeled in a fairly short time. If the $C^{14}O_2$ is removed, and samples are taken periodically over a period of a few hours, it might be expected that a pattern of radioactivity and/or specific activity changes of the terpenes would be revealed that would be related to the order of formation of the terpenes. Figure 6 represents the results of such an experiment. In this experiment, each sample was two geranium leaves, each weighing about 0.3 gram, and the leaves were initially exposed to 400 microcuries of $C^{14}O_2$ for one hour, after which, one sample was taken each hour for 12 hours.

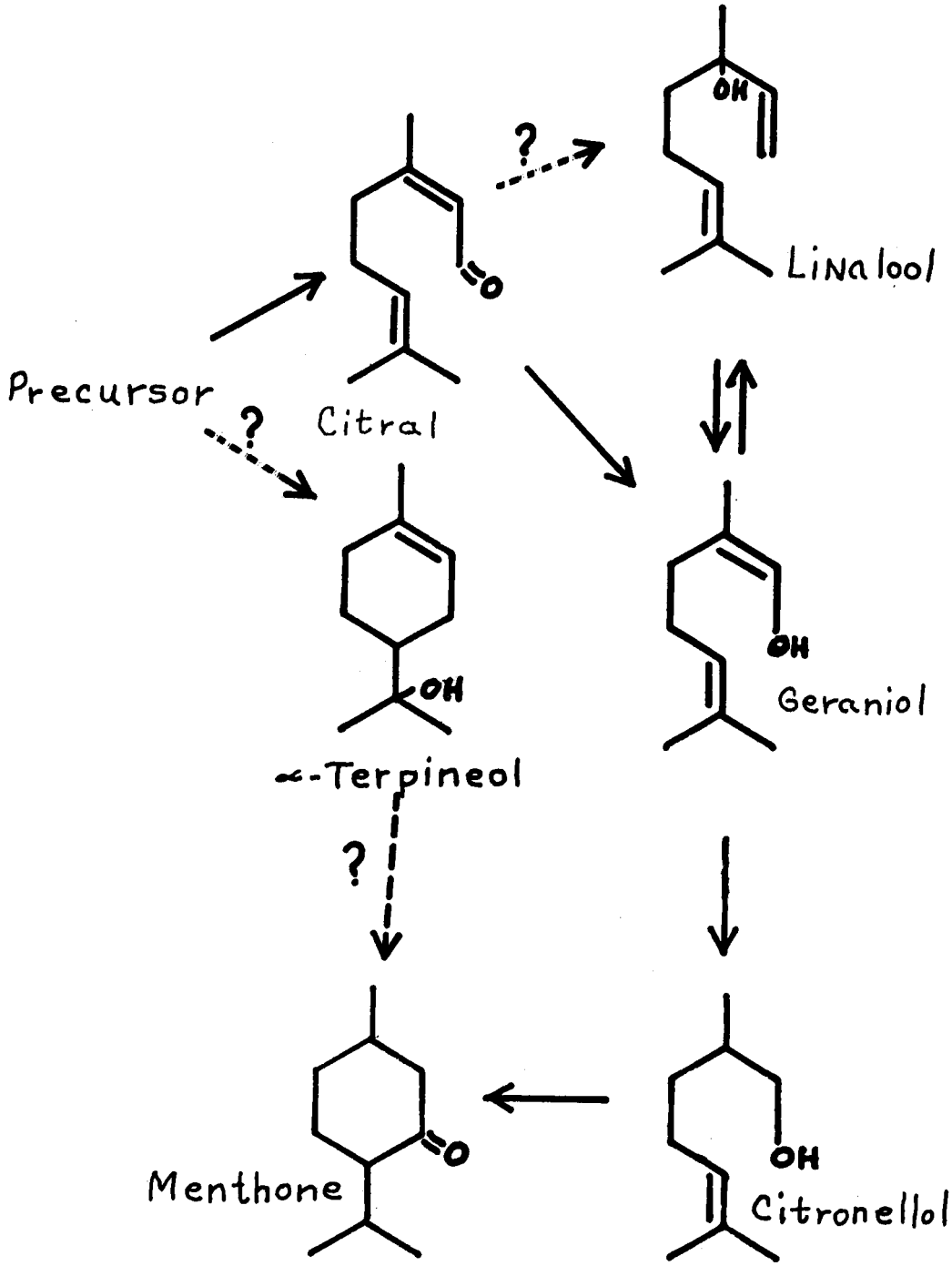
As can be seen in Figure 6, successive specific activity peaks appear, from geraniol and citral to citronellol and linalool to menthone to α-terpineol, indicating a possible biosynthetic sequence of terpene formation as illustrated in Figure 7. This will be discussed later.

FIGURE 6



TWELVE HOUR TIME-COURSE

FIGURE 7



PROPOSED PATHWAY OF TERPENE INTERCONVERSIONS IN P. GRAVEOLENS

The specific activity peaks for α-terpineol are ambiguous, however, because thin layer chromatography of the α-terpineol peak of carbon-14 labeled geranium oil revealed that some of the radioactivity remained in the α-terpineol spot on the plate, but more than half of it migrated to the solvent front, indicating that there is another component in the α-terpineol peak.

A specific activity plot for compound "x" is shown, based on an assumed calibration value, 4.14×10^2 area units/micromole terpene, obtained from an average of the known monoterpenes' values. The plot shows two peaks for "x".

Also in Figure 6 a plot of counts per minute versus time is shown. It is seen that the pattern of total radioactivity change for each terpene is quite similar to that of specific activity.

In the next experiment, the leaves were exposed to $C^{14}O_2$ in the light for a very short time in order to try to detect any very early terpene interconversions. In this experiment, the samples, which were geranium leaves (one/sample) of 0.3 gram weight, were exposed to 500 microcuries of $C^{14}O_2$ for seven minutes, after which the chamber was flushed and the first sample taken. One sample was then taken every ten minutes for the next 40 minutes. The

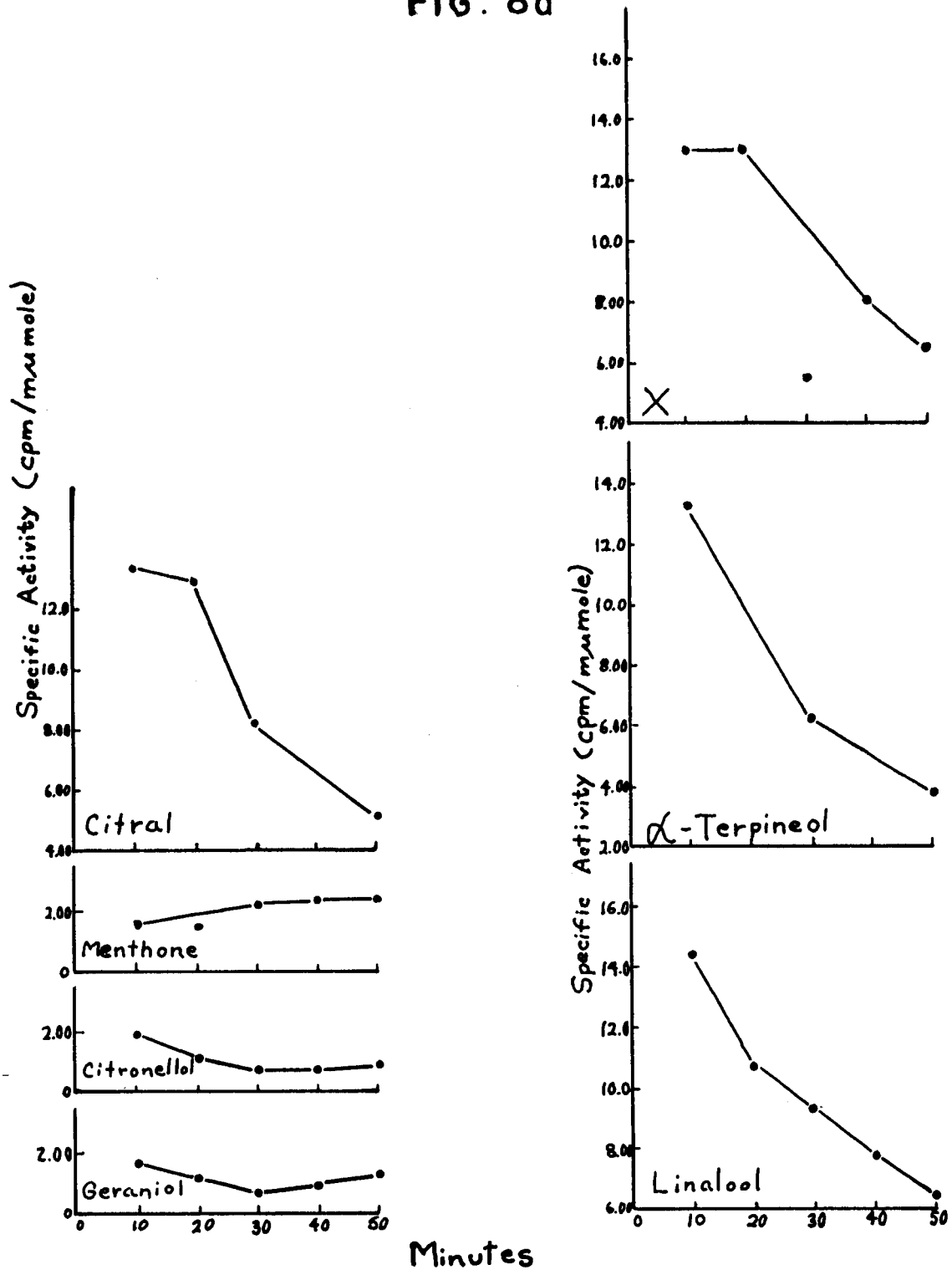
results are illustrated in Figures 8a and 8b.

As is seen in Figures 8a and 8b, the specific activities and total radioactivities of most of the terpenes fall off very rapidly after removal of the $C^{14}O_2$. Only menthone rises in total and specific activity throughout the experiment, possibly indicating that it is formed near the end of the biosynthetic pathway.

Since the metabolism of green plants is known to be affected by the amount and kind of light present (15, 31, 32), an investigation of how the rate of terpene biosynthesis changes from light to dark and vice versa was performed.

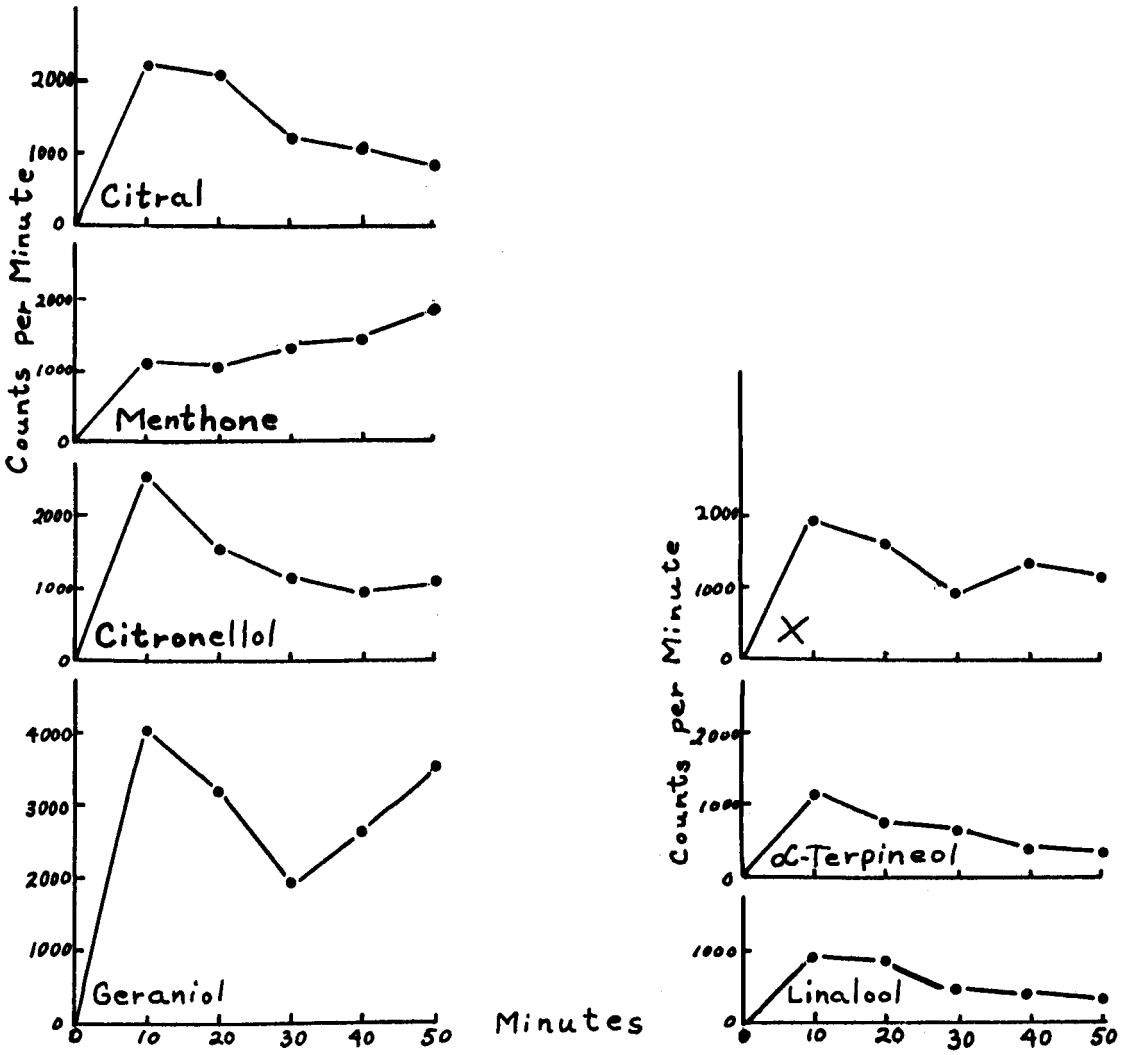
The experiment was set up as previously. The samples were exposed to 400 microcuries of $C^{14}O_2$ for seven hours in the light, after which the chamber was flushed and the first sample taken. The chamber was resealed and exposed to light for seven more hours, when the second sample was taken. All of the leaves were then, with their water-containing beakers, transferred to 35 mm film canisters which were capped, sealing all light out. After five and ten hours, respectively, in the dark, samples were taken; the plants were then again placed in the light chamber for 14 hours. This procedure continued, taking two samples

FIG. 8a



FIFTY MINUTE TIME-COURSE

FIGURE 8B



FIFTY MINUTE TIME-COURSE

per each light and dark period, for 72 hours. The results of this experiment are shown in Figures 9a and 9b.

The light-dark changes seem to have no clear-cut effect on the specific activities of linalool, menthone, or α -terpineol. Although these terpenes vary quite widely in specific activity during the course of the experiment, there is no obvious relationship to the light-dark cycle.

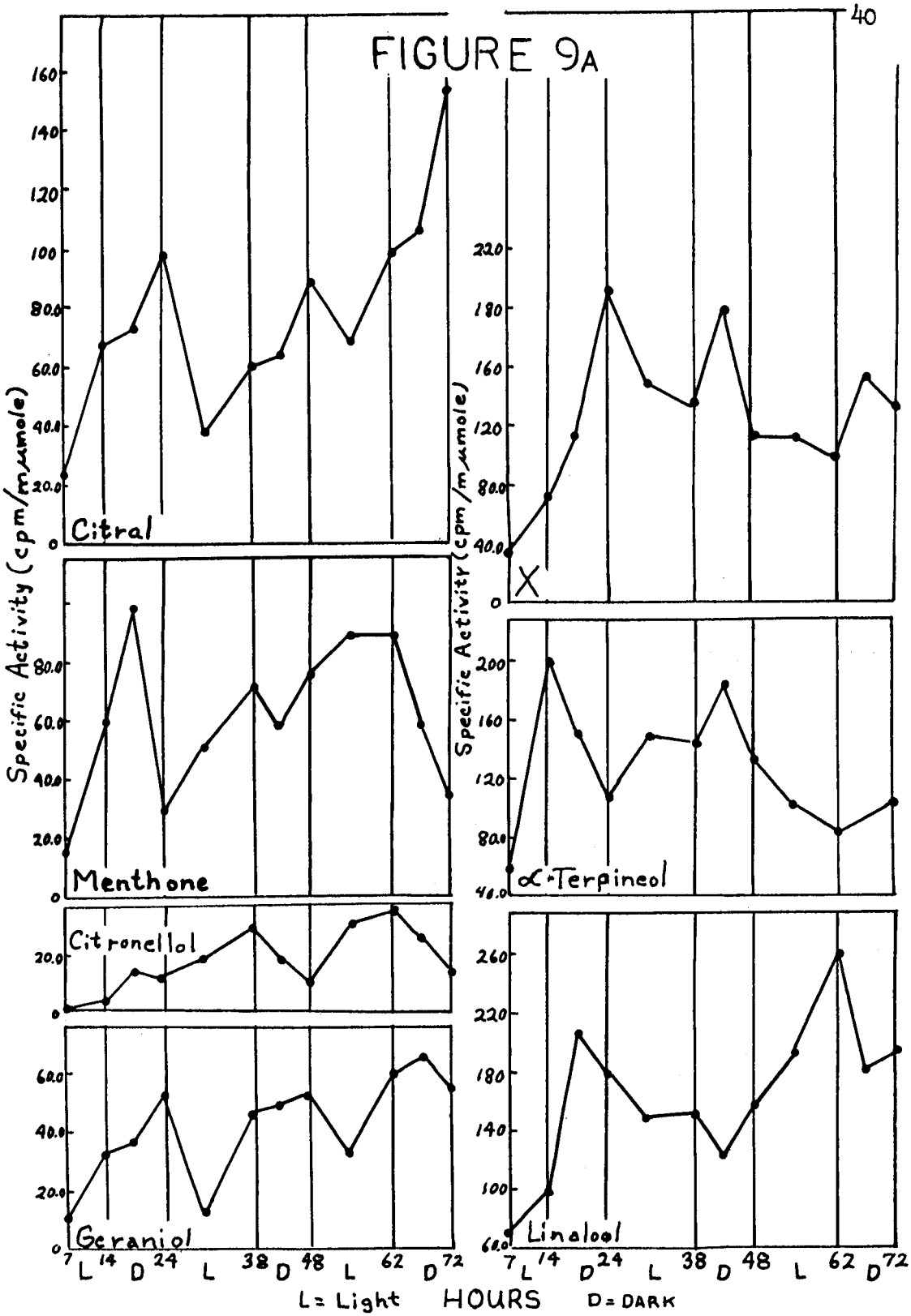
Geraniol, citronellol, and citral, however, seem to follow definite patterns of specific activity change with light and dark periods.

Interconversion Experiments

One way to gain information on the biosynthetic pathway is to isolate individual carbon-14 labeled terpenes and somehow feed them back to the plants, so that they will be metabolized as if they were endogenous terpenes.

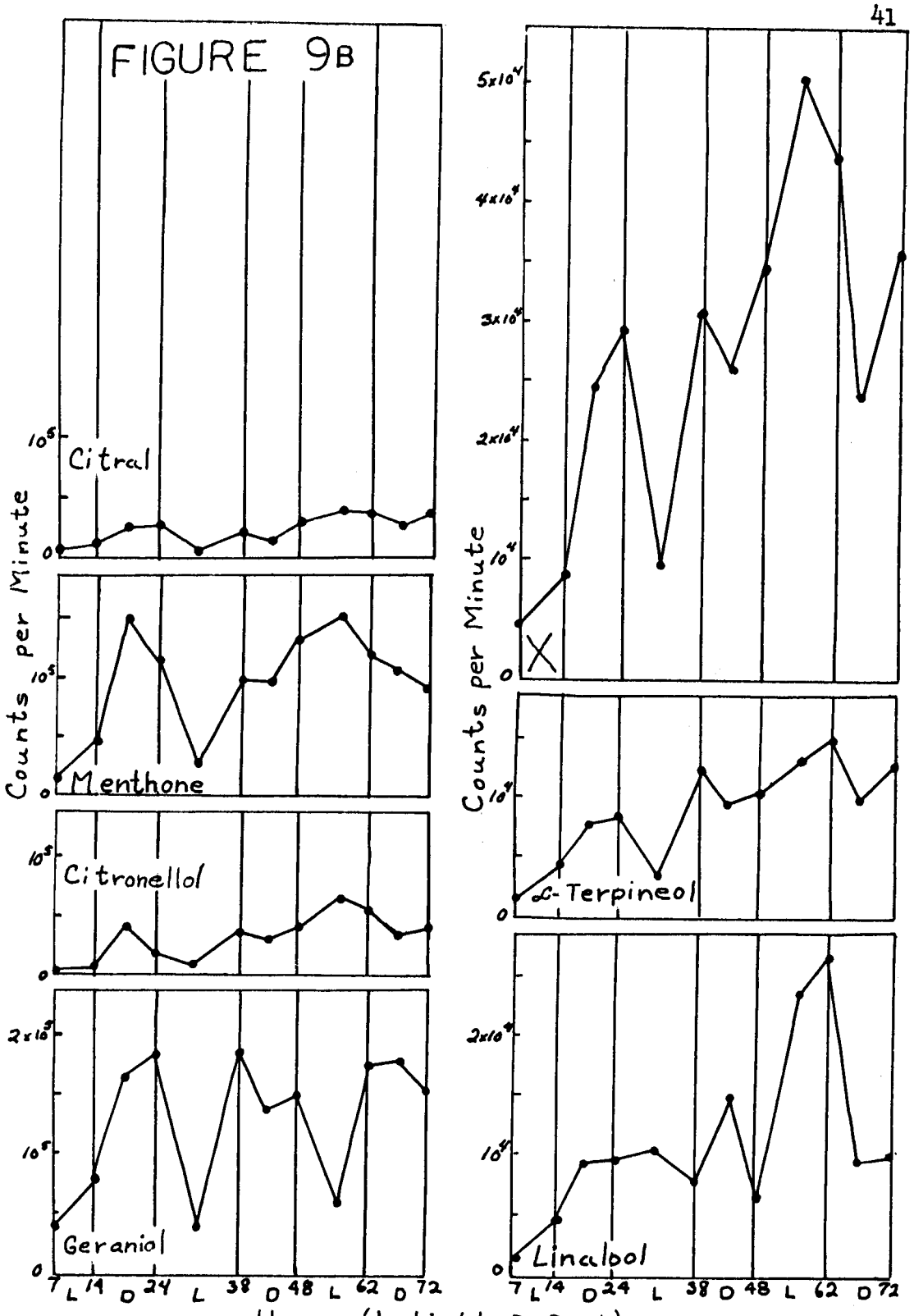
The general method used here was to isolate each carbon-14 terpene from silicic acid chromatoplates as described previously. In this case, however, the geraniol was removed from the extract and isolated by the calcium chloride method (14, Vol. IV, pp. 631-737) before chromatographing. The isolated terpene was then placed, along with the silica scrapings, in a glass vial containing two to

FIGURE 9A



LIGHT DARK TIME COURSE

FIGURE 9B



LIGHT-DARK TIME COURSE

four still expanding leaves. The mixture was covered with water or buffer, shaken, and allowed to react in the light for 12 to 14 hours. Sometimes the leaves were sliced or minced after being immersed in the reaction mixture. After reaction, both the leaves and the liquid were extracted, and the extracts combined.

In the first experiments, the extracts were separated on chromatoplates, but no definite results could be obtained by this method, even though many variations were tried, such as treating the leaves with acetone at dry ice temperature before reaction, or using Tween-20, a wetting agent, in the reaction mixture.

Finally, some results were obtained using gas chromatography with anthracene tubes for counting. The results in Table 2 are from an experiment in which the substrates were added to sliced geranium leaves in water and exposed to light for 14 hours. The conversions shown are not large and explain why the chromatoplate method did not detect them, with its low sensitivity. The blanks were vials which contained no plant material.

The results indicate the following conversions: geraniol to citronellol, citral, linalool, and menthone; citronellol to geraniol and menthone; citral to geraniol

Table 2. Interconversions of Monoterpenes of P. graveolens.

Values given are counts per minute (sample) minus counts per minute (blank) of each fraction collected from the gas chromatograph.

Fraction	Substrate				
	Geraniol	Citronellol	Citral	Linalool	Menthone
Linalool	254	24	0	586*	(148)
Menthone	230	260	0	(122)	2438*
"x"	0	0	166	4	2
Menthol	0	92	274	52	40
α-Terpineol	94	14	30	16	0
Citral	452	42	704*	10	12
Citronellol	488	7524*	386	24	30
Geraniol	9310*	217	512	254	168

*Indicates that this value is the final counts per minute of the substrate.

and citronellol. The numbers in parentheses are considered doubtful because they could have been caused by overlapping of peaks on the gas chromatograph.

Mevalonic Acid-2-C^{14} as a Terpene Precursor

Since monoterpenes are generally considered to be products of the pathway of sterol biosynthesis (6, 12), mevalonic acid should be a precursor of these compounds. But, although much work has been done on this problem (6, 11, 12), very little success in establishing this contention has been experienced. This is thought to be due to difficulties in the polar mevalonic acid molecule getting to the site of terpene biosynthesis (6). Some positive incorporation of meavlonate into monoterpenes has been obtained, however (35).

In this work, also, it was attempted to incorporate mevalonic acid-2-C^{14} into the terpenes of geranium. After experimenting with immersing both cold acetone treated and untreated leaves directly into the solution of mevalonic acid-2-C^{14} and obtaining only incorporation into the fraction which migrates to the solvent front in thin layer chromatography, the "hydrocarbon fraction," some positive results were obtained by immersing the freshly cut petiole

into a solution of three microcuries and allowing the leaf to absorb the solution over a period of 12 hours. The results indicated some incorporation, from plate counting, into geraniol-citronellol (200 cpm), citral (95 cpm), and the hydrocarbon fraction (3300 cpm).

Experiments with Cell-Free Extracts

Definite answers to many of the questions in terpene biosynthesis will require the isolation of enzymatic activity from the plant systems involved. In the work to be described, cell-free extracts from monoterpene producing plants were found to possess two previously well characterized types of enzyme activity, glutamyl transferase (18) and mevalonic kinase (19), as well as possible terpene synthesizing ability. Work of this sort has been attempted by Battaile (6) and Campbell (12) with very little success, although Campbell did isolate some triosephosphate dehydrogenase activity from peppermint.

The glutamyl transferase and mevalonic kinase activities were determined in order to discover ways by which enzymatic activity could be successfully extracted from these plants. These particular enzymes were chosen for assay because they have been well characterized in other

plants (18, 19) and because they are easily assayed.

Glutamyl Transferase. The plants used in these experiments were M. piperita, peppermint, M. pulegium, pennyroyal, and P. graveolens, rose geranium; the preparation of cell free extracts of the first two of these is described as follows.

First, an acetone powder made from fresh, still expanding leaves and terminal buds was prepared as described in (18). Two hundred and fifty mg of the acetone powder was then suspended in five milliliters of 0.10 M, pH 7.4 sodium phosphate buffer containing one mmole of mercaptoethanol. The mixture was allowed to soak in an ice bath under nitrogen atmosphere for about 15 minutes, after which it was squeezed through a few layers of cheesecloth, rewet with buffer and squeezed again. The crude extract, a yellow-green liquid, was then fractionated through a 1 by 14 centimeter Sephadex G-25 column, eluting with buffer. Three fractions of approximately five milliliters each were collected.

All three fractions were tested, and the predicted one (27), fraction one, contained glutamyl transferase activity by the test given in (18).

In the case of geranium, no further refinement of the crude acetone powder was needed to produce glutamyl transferase activity.

Mevalonic Kinase. The plant used here was peppermint, and the method of preparation used was as above.

The method of assay for mevalonic kinase was as in (19), and the results were weak but definitely established the presence of mevalonic kinase in the extract.

Terpene Synthesizing Ability. Both peppermint and pennyroyal were used in these experiments, and the general method used was as follows.

The reaction mixture contained rat liver preparation (34, p. 150), 0.5 ml; sodium mevalonate-2-C^{14}, 250 millicuries; ATP,[2] 2.5 micromoles; $MgSO_4$, 5 micromoles; $MnSO_4$, 5 micromoles; BAL,[3] 15 micromoles; TPNH,[4] 0.5 micromoles; and extract, 0.5 ml. Also, 0.5 ml of 0.20 M, pH 6.4 sodium maleate buffer was added to each tube and was used to bring the final volumes of the tubes to 2.0 ml. The rat liver preparation was used to convert mevalonate into isoprenoid

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[2]ATP, adenosine triphosphate.
[3]BAL, 2,3-dimercapto-1-propanol.
[4]TPNH, reduced triphosphopyridine nucleotide.

intermediates which are presumed precursors of monoterpenes (2; 34, p. 150). The reaction was carried out in a nitrogen atmosphere for two to four hours depending on the specific experiment. The reaction mixtures were then extracted with hexane, after adding five microliters of carrier peppermint oil to them, and were chromatographed on chromatoplates.

In the cases where pennyroyal was used as the extract source, the plates were sprayed, and the spots were scraped into scintillation fluid and counted in a Tracerlab liquid scintillation counter. The results of this experiment indicated possible incorporation of mevalonate-2-C^{14} into piperitenone.

Where peppermint was used as the extract source, radioautograms were made from the plates. The results showed a faint but definite radioactive spot corresponding to the position of piperitenone. In one experiment, the amount of ATP was varied in the four reaction tubes, and it was found that the "piperitenone" spot was extracted only from the tube with the highest ATP concentration (5.0 micromoles/2.0 ml.).

In experiments in which attempts were made to concentrate the "terpene synthetase" enzyme activities by the

methods of Anderson and Porter (2), no success was experienced.

DISCUSSION

The first time-course experiment, using chromatoplates and radioautography, indicated an initial formation of geraniol-citronellol followed by linalool and citral. The initial formation of geraniol agrees with the hypothesis that geranyl pyrophosphate is the direct precursor of the monoterpenes (6, 21), and it would be expected that citronellol formation might closely follow that of geraniol by a reduction of the geraniol, similar to the situation found in peppermint (6, 11). Linalool and citral are isomer and oxidation product, respectively, of geraniol, and, again, might be expected to closely follow its formation. However, the more detailed experiments following this made the situation much more complicated, and, based on the results of these experiments, the biosynthetic pathway illustrated in Figure 7 is proposed.

Support in general for this pathway is based, first, on evidence found in the 12 hour time-course experiment. Since the precursors in a biosynthetic pathway normally

possess higher specific activities than their products, it can be seen in Figure 6 that by consideration of both specific activities and position of specific activity peaks, one can arrive at a pathway such as illustrated in Figure 7. This scheme is recommended also by the fact that the major conversions here are reductions (except for the citronellol to menthone step), and it was found in the studies of terpene interconversions in peppermint (6, 11) that the process of reduction was the predominant one.

By consideration of only specific activities, one would conclude that geraniol cannot be a precursor of linalool, since its specific activity is much lower than that of linalool, but another interpretation can be made which changes the picture somewhat, that of the possible existence of metabolically inactive "pools" of terpenes.

Since 80-90 percent of geranium oil consists of geraniol, citronellol, and menthone (14, 22, 28), it would not seem unreasonable to assume that the bulk of these terpenes are metabolically inactive; that is, most of the oil is stored in the oil gland either removed from the site(s) of interconversion or in an inactive form. If, for instance, interconversion only takes place in one part of the cell, only the geraniol in direct or close contact

with that site could be considered metabolically active. This would, of course, apply to the other terpenes, also, but a terpene of low concentration might more likely be in close contact with the site of its formation and interconversion at all times and not be stored in a "pool" at all.

If this were the case, the reason why the specific activities of geraniol, citronellol, and menthone, the major components of geranium oil, are lower than those of linalool, citral, and α-terpineol, minor components, would not be that there is less carbon-14 labeling in the former than the latter, but that linalool, citral, and α-terpineol are in such low quantity that comparatively small changes in radioactivity produce large ones in specific activity, since virtually all that is present in the plant of each of these compounds would be turned over in a reaction. The opposite applies to geraniol, citronellol, and menthone, and the specific activities of the metabolically active portions of these terpenes would be much higher than the data indicate.

The above indicates how geraniol could be the precursor of linalool, even though the apparent specific activity of geraniol is lower than that of linalool. Citral is still placed first in the scheme for two reasons. (1) The

biosynthetic scheme is primarily one of reductions; so the conversion of citral to geraniol is more likely chemically than the reverse reaction. (2) Citral possesses the earliest peaks of specific activity and total radioactivity, both of which drop off after the first few hours.

More direct support for the contention that linalool is formed from geraniol rather than from citral is provided by the results of the interconversion experiment shown in Table 2. It was observed here that geraniol was converted to linalool but citral was not.

In this experiment, however, many difficulties were experienced, and the incorporations observed were low. There were two probable causes of this. First, it can be easily imagined how difficult it would be for the fairly large, non-water soluble terpene molecule to penetrate through the hairy surface of the geranium leaf and enter the oil gland for reaction. Second, when the leaves were minced in order to provide better contact between the labeled terpene and the sites of reaction, part of the leaf was killed. Therefore, the results could only be significantly detected when a very sensitive method of radioactivity detection was used. These observations do not exclude the possibility that citral actually was converted

first to linalool, but they also do not support it. On the other hand, geraniol was definitely observed to be converted into linalool, as well as citronellol, citral, and menthone, further supporting the proposed scheme of Figure 7.

If one radioactive compound is formed from another, it is reasonable to assume that a drop in radioactivity of the first should result in a comparable rise in the radioactivity of the second. In Figure 6, two examples of this can be observed. First, it is seen that as the radioactivity of geraniol drops by 2000 cpm between four and six hours, the radioactivity of citronellol increases by approximately the same amount, consistent with a possible conversion from geraniol to citronellol. Second, as the radioactivity of menthone increases by 9000 cpm between five and eight hours, that of geraniol decreases by 7000 cpm and citronellol by 3000 cpm, consistent with a conversion of geraniol and citronellol to menthone.

The assertion that menthone is formed from the earlier synthesized terpenes, such as geraniol and citronellol, is supported by two other observations. First, it is seen in the 12 hour time-course that menthone is formed late. Second, in the 50 minute time-course experiment, the

specific activities and total radioactivities of all of the terpenes except menthone drop rapidly after the removal of the $C^{14}O_2$. Since the specific activity and total radioactivity of menthone begins to rise at this point, one is brought to the possible conclusion that menthone is an end product of the biosynthetic pathway and that it is formed from the other terpenes.

The light-dark experiment yielded some interesting results and some difficult problems of interpretation.

The patterns of citral and geraniol indicate that they are accumulating in the dark and being converted to their products in the biosynthetic pathway in the light, since the trends of specific activity from the middle of the light period to the end of the dark period are upward and those in the first half of the light period are downward. Even though the depletion of a compound should not change its specific activity, this can be seen to be possible if the "pool" model as described above is considered. That is, if the radioactive geraniol formed is assumed to be localized close to its site of formation, the most highly radioactive geraniol would be preferentially used in a conversion reaction. In this case, when radioactive precursors form geraniol, the overall specific activity

rises, and when geraniol is converted to other terpenes, the overall specific activity falls. Even so, the specific activity trends throughout the experiment as a whole are upward. The reaccumulation of label in geraniol and citral in the second half of the light periods might be due to a refixation of respiratory $C^{14}O_2$. An effect such as this, but not so marked, occurred in the 12 hour time-course in continuous light, in which second peaks were observed at the nine and ten hour points for citral and geraniol, respectively.

The patterns of citronellol and menthone indicate that they are accumulating in the light and possibly being converted to other compounds in the dark. However, if a specific activity drop actually indicates conversion to another compound, it is difficult to see to what citronellol and menthone are converted.

The similarities of the patterns of citral and geraniol suggests that they are closely related in the metabolic scheme. Possibly both citral and geraniol are produced from the same precursor, and citral and/or geraniol is converted to citronellol only when light produces the reducing agent. Or, more consistent with Figure 7, citral might be formed first and be converted in a fast reaction

to geraniol, which, in turn, is converted to citronellol in the light.

In all of the above discussion, the roles of compounds "x" and α-terpineol remain hazy. Since no structure is known for "x", very little can be said about it except that it is an early formed compound. Since α-terpineol is cyclic, and since two sites of cyclization might be considered unlikely, it might be postulated that it is a precursor of menthone. However, no evidence was found to support this other than the fact that α-terpineol is formed early, and menthone is formed late. It is also difficult to see how the structure of α-terpineol correlates to that of menthone.

It was shown by Battaile and Loomis (8) that the interconversions of the terpenes of peppermint are rather slow, no radioactivity appearing in menthol, the last terpene in the biosynthetic pathway, until between three and eight days after exposure to $C^{14}O_2$. On the other hand, it was found that the synthesis of the earliest formed terpene of peppermint, piperitenone, is very rapid, coming to a steady level of specific activity in about one hour (11).

In the case of geranium, however, the picture is somewhat different. Figures 8a and 8b show that the

interconversions of the terpenes of geranium are very rapid, all of the terpenes becoming labeled in about ten minutes. But it is seen in Figure 6 that the probable first formed terpene of geranium, citral, takes three hours to reach its peak of specific activity, after which it drops off to attain a more or less steady value.

Peppermint and geranium differ in another way, also. It has been shown that the amount of terpene biosynthesis in peppermint decreases with expanding leaf size (8) and is at its highest with youngest leaves. With geranium, the results (Figure 5) indicate that the biosynthesis of terpenes proceeds at a high rate only in those leaves that are still expanding, but in contrast to peppermint, the rate increases as the leaves become larger and is at its highest close to the time when the leaves' expansion is completed.

SUMMARY

1. Time-course studies on geranium using $C^{14}O_2$ indicated a series of interconversions of monoterpenes which paralleled the earlier findings with peppermint that the most unsaturated terpenes are formed first and

react by means of a series of reductions to form the more saturated terpenes of the system. Unlike peppermint, however, it was found with geranium that the interconversions to the more saturated terpenes were very rapid.

2. The conversions of geraniol to citronellol, citral, linalool, and menthone; citronellol to geraniol and menthone; and citral to geraniol and citronellol were observed in sliced geranium leaves using labeled terpenes as substrates.

3. Possible incorporation of mevalonic acid-2-C^{14} into geraniol-citronellol, citral, and the "hydrocarbon fraction" was observed.

4. Variations in terpene synthesizing ability with geranium leaf age were noted, and it was found that only those leaves that were still expanding synthesized terpenes at a high rate.

5. A chromatographic analysis of geranium oil was performed, using both thin layer chromatography and gas-liquid chromatography. The latter was found to be superior both in resolution and in affording a convenient and sensitive method of counting radioactivity.

6. Studies were performed to develop methods of obtaining enzymatically active cell-free extracts from

monoterpene producing plants. The enzyme activities extracted were glutamyl transferase (from peppermint, pennyroyal, and geranium), mevalonic kinase (from peppermint), and an enzyme activity which possibly produced piperitenone, using mevalonic acid-2-C^{14} as the substrate and extracts of either peppermint or pennyroyal as the enzyme system.

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