TERPENE BIOGENESIS IN PINE

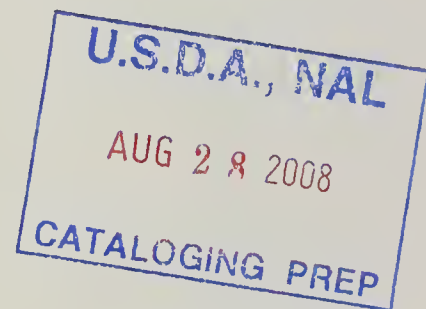by   Robert G. Stanley

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Abstract

Complex turpentine components of pine arise from simple acid molecules. The building blocks and pathway of terpene formation have been demonstrated in pine trees by the use of radioisotopes.

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Understanding the mechanism by which plants form complex products such as terpenes will facilitate the selection, breeding, and management of commercially important sources of these products. Thus, knowledge of the biochemistry of pines may lead to increased production of a desired chemical product, or possibly provide a means of manipulating growing stock to modify a less desired chemical constituent in favor of a more desirable product. A note published in 1958 (25) reported experimental evidence to support the hypothesis that monoterpenes in pine probably arise through enzymatic pathways similar to those recently demonstrated as the route of origin of the more complex animal steroids and plant polyenes. These latter molecules are related to the mono- and di-terpenes of pine through a common isoprene base (21).

The purpose of this paper is to present further experimental details concerning the mechanism by which pines, and probably other plants, form terpenes from acetic acid through mevalonic acid. In addition, some problems which can now be more readily approached by utilizing the techniques and results of these studies will be indicated.

PROPOSED HYPOTHESIS

To permit a clearer understanding of the procedures followed, essential details of the hypothesis used as the basis for these studies have been summarized in Figure 1. According to this 10-step hypothesis, C_{10} monoterpenes are derived from activated C_5 units. These molecules are postulated as arising from the C_6 intermediate, mevalonic acid, which is formed from acetate. Steps 1 to 4 are supported by research concerned with the origin of squalene and cholesterol in animals (7, 19, 23). Other recent hypotheses for the origin of lower plant terpenes are reviewed by Haagen-Smit (12). This summary will therefore be confined to clarifying and integrating aspects of the problem of plant terpene biogenesis which have been published since completion of the aforementioned reviews.

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[1] This article extends a paper entitled "Terpene Formation in Pine" presented at the IV International Congress of Biochemistry, Vienna, Symposium II, pp. 48-55, September 1958.
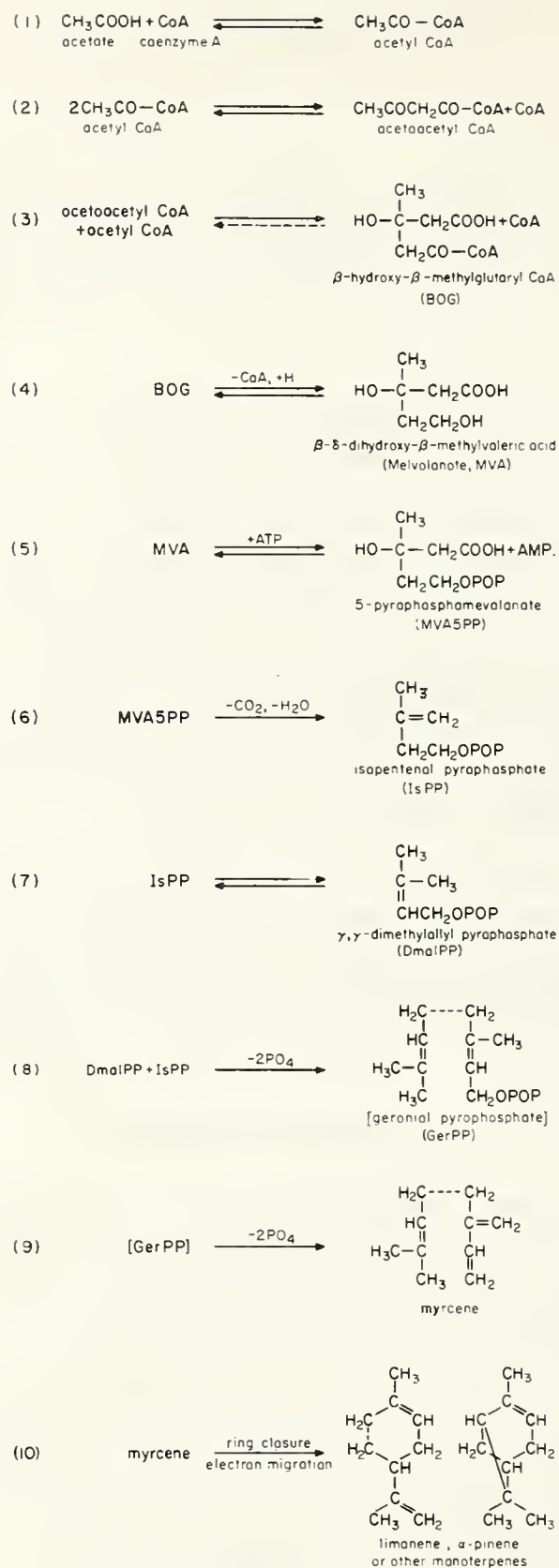
(1) $CH_3COOH + CoA \rightleftharpoons CH_3CO-CoA$
  acetate coenzyme A   acetyl CoA

(2) $2CH_3CO-CoA \rightleftharpoons CH_3COCH_2CO-CoA + CoA$
  acetyl CoA   acetoacetyl CoA

(3) acetoacetyl CoA
   +acetyl CoA $\rightleftharpoons$
$$HO-\underset{\underset{CH_2CO-CoA}{|}}{\overset{\overset{CH_3}{|}}{C}}-CH_2COOH + CoA$$
   β-hydroxy-β-methylglutaryl CoA
   (BOG)

(4) BOG $\xrightarrow{-CoA, +H}$
$$HO-\underset{\underset{CH_2CH_2OH}{|}}{\overset{\overset{CH_3}{|}}{C}}-CH_2COOH$$
   β-δ-dihydroxy-β-methylvaleric acid
   (Melvalonate, MVA)

(5) MVA $\xrightarrow{+ATP}$
$$HO-\underset{\underset{CH_2CH_2OPOP}{|}}{\overset{\overset{CH_3}{|}}{C}}-CH_2COOH + AMP.$$
   5-pyrophosphomevalonate
   (MVA5PP)

(6) MVA5PP $\xrightarrow{-CO_2, -H_2O}$
$$\underset{\underset{CH_2CH_2OPOP}{|}}{\overset{\overset{CH_3}{|}}{C}}=CH_2$$
   isopentenol pyrophosphate
   (IsPP)

(7) IsPP $\rightleftharpoons$
$$\underset{\underset{CHCH_2OPOP}{||}}{\overset{\overset{CH_3}{|}}{C}}-CH_3$$
   γ,γ-dimethylallyl pyrophosphate
   (DmalPP)

(8) DmalPP + IsPP $\xrightarrow{-2PO_4}$ [geraniol pyrophosphate] (GerPP)

(9) [GerPP] $\xrightarrow{-2PO_4}$ myrcene

(10) myrcene $\xrightarrow[\text{electron migration}]{\text{ring closure}}$ limonene, α-pinene or other monoterpenes

Figure 1.--Probable pathway of terpene formation in plants.

Bonner and associates ($\underline{6}$, $\underline{15}$) demonstrated the incorporation of radioactive acetate into rubber and isolated radioactive β-hydroxy-β-methyl glutaric acid and β-methyl crotonic acid. These two compounds, though structurally related to isoprene, were not readily converted to rubber. A cell-free system from Hevea latex, shown to incorporate acetate into rubber ($\underline{3}$), rapidly formed rubber from mevalonic acid-2-C^{14} [MVA-2-C^{14}] ($\underline{18}$). The C^{14} occurred in the positions expected if condensation of isoprene-like units had occurred according to step 8 in Figure 1.

Using radioactive substrates, Arigoni ($\underline{1}$) showed that a penta-cyclic sterol in soybean originates from acetate through MVA. Degradation of the molecule showed the labeling to be in accord with the hypothesis as derived through step 8. A diterpenoid rosenonolactone, produced by a mold, was also shown to arise from acetate and MVA in conformity to the squalene-cholesterol concept ($\underline{4}$). Also, the incorporation of acetate and MVA into lycopenes and carotenes of tomatoes ($\underline{20}$, $\underline{24}$) and in carotinoids of Mucor and corn has been demonstrated ($\underline{10}$, $\underline{11}$). The incorporation of MVA into many plant constituents structurally related to isoprene has now been conclusively demonstrated although actual details must still be worked out.

Details of reactions 5 to 7 were recently revealed by laboratories of Lynen and Bloch ($\underline{2}$, $\underline{5}$). In studies with yeast enzyme preparations, which can convert MVA to the C_{30} molecule squalene, these workers have shown that the MVA is phosphorylated (reaction 5), then decarboxylated (reaction 6) followed by an isomerization of the molecule (reaction 7). The C_{15} compound, farnesol pyrophosphate, has been isolated as a condensation product of γ, γ-dimethylallyl pyrophosphate (DmalPP). Lynen indicates the probable mechanism in yeast as being the condensation of a reactive DmalPP and isopentenol pyrophosphate (IsPP) followed by an electron migration to form a reactive terminal allylic pyrophosphate with the DmalPP configuration; this C_{10} further polymerizes to form a C_{15} sesquiterpene, or adds additional IsPP groups to form diterpenes or even larger molecules ($\underline{16}$). If geraniol pyrophosphate, the C_{10} compound formed from the condensation of DmalPP-IsPP, undergoes an electron migration and release of the pyrophosphate unit, it could yield a C_{10} molecule such as myrcene. This may possibly be the case in pine. An aliphatic terpene such as linalööl or myrcene has long been postulated as an intermediate in cyclic terpene formation ($\underline{9}$). In pine, however, myrcene occurs more commonly than linalööl ($\underline{17}$). Enzymatic and non-enzymatic ring closures and group rearrangements are common reactions in sterol terpenoid metabolism.

METHODS AND MATERIALS

In the studies reported here, acetate-2-C^{14} and mevalonate-2-C^{14} were infiltrated into actively metabolizing pine shoots, and the monoterpenes were isolated, identified, and their radioactivity measured. In such experiments it is advantageous to use plant material with turpentine composed of predominantly a single terpene rather than a mixture.

Previous studies of Mirov (17) revealed several pine species in which the turpentine consists primarily of one terpene. Knobcone pine (Pinus attenuata Lemm.), with turpentine composed of 98 percent α-pinene, was selected for the present studies.

Two to three grams of shoot tips from actively growing 2-year-old seedlings were cut and the bases immersed in 2 ml 0.01 M K-PO$_4$ buffer, pH 5.9 in a 4-6 ml conical shaped vial. Radioactive acids of known specific activity were added to the buffer solution. "Parafilm" was used to seal the vial tops to prevent evaporation. The exposed shoot tips were placed approximately 15 inches from a 100-watt incandescent lamp with an electric hair dryer blowing cool air just above the shoots. After 4 hours the radioactive solution had been reduced by uptake into the shoots to about 0.5 ml and additional buffer (1 ml) was added. At the end of 8 hours, little if any solution remained in the vial. The shoot tips were removed, frozen in liquid nitrogen, wrapped in aluminum foil, and stored at -10°C. until analysis, from 1 to 3 days later.

In experiments utilizing tissue slices the shoot tips were sectioned with a razor blade into slices about 2 mm thick under a solution of phosphate buffer at 2°C. Two grams, wet weight, were transferred to Warburg respirometer flasks and shaken aerobically in 2.5 ml buffer plus radioactive substrate for 90 minutes at 30° C. Flask contents were then frozen and stored until analysis 1-3 days later.

The frozen shoots were cut into sections about 1/8 to 1/4 inch long with a razor blade, and ground in successive portions of anhydrous ethyl ether in a mortar with pestle until radioactivity was absent from the ether extract. In tissue-slice experiments the whole flask contents were extracted in this manner. Finally, the tissue residue was shaken in an Erlenmeyer flask on a mechanical shaker for 3 hours with four changes of ether. The remaining ether was removed by pressing the residue by hand between 2 folds of cheese cloth under a large cork. The ether extracts were combined, giving a volume of about 200 ml which was then treated by one of two procedures:

1. In early experiments the 200 ml was first extracted with 20 ml of M KOH and residual water removed by shaking with anhydrous CaCl$_2$. The acid-free ether extract was then concentrated to 20 ml at 38° C. and passed over a "Darco" decolorizing column 30 mm high and 7 mm in diameter. About 50 ml of ethyl either was used to wash the column. The colorless eluant was concentrated to 0.5 ml under an air stream for terpene analysis.

2. In later experiments the 200 ml of extract was initially reduced to 20 ml by distillation at 38°C. This concentrate was passed through a column packed with "Woelm" basic alumina oxide overlaying decolorizing charcoal, 50/50, in a column 70 mm high and 15 mm in diameter. The column was washed with 100 ml of ether, yielding a colorless acid-free eluant, which was concentrated to 0.5 ml by distillation and under an air stream.

Pure α-pinene (0.2 ml) was added to the final concentrate in both procedures.

The α-pinene was separated from the mixture by gas liquid partition chromatography (GLPC). A Wilkens Instrument Co., Model #100 with a Varian 10 ma recorder was used. Helium was the gas phase flowing through a 5-foot column of Carbowax 1540 on diatomaceous earth at about 50 ml per minute. The α-pinene was readily separated from other components at 100° or 120° C. (27). About 93 percent recovery was obtained.

The recovered α-pinene, which presumably contained added non-radioactive "carrier" α-pinene and any which the plant may have formed, was then analyzed for radioactivity. To substantiate the nature of the α-pinene isolated, its infrared spectrum was compared to known pure samples; it was also crystallized as the nitrosochloride, which was compared to pure standards. The specific activity of the isolated α-pinene was determined.

Radioactivity in the α-pinene liquid fractions and nitrosochloride was assayed by dissolving the material in 0.5-1.0 ml toluene. Samples were counted by liquid scintillation in 0.5 percent 2, 5-diphenyloxazole in toluene (8). Counting efficiency was about 90 percent. Samples of the tissue residue were placed in aluminum cups and counted at infinite thickness with a thin end-window Geiger-Müller tube. No correction was made for enantio-morphic (dl) specificity in computing incorporation percentages.

Acetate-2-C^{14} was obtained as the sodium salt from Research Specialties Co., Berkeley, California. dl-mevalonic acid and dl-mevalonic acid-2-C^{14} were obtained as the N, N' - dibenzylethylenediamine salt from the Medical Research Division of Merck, Sharp and Dohme, Rahway, New Jersey.

RESULTS

It was first necessary to establish the validity of the gas-liquid-phase-chromatography system, GLPC, as a method of separating and recovering the terpene, α-pinene, from pine shoots infiltrated with postulated radioactive precursor acids. Experiments were carried out in which samples of pure α-pinene and MVA-2-C^{14} were mixed, extracted with alkali, and separated by GLPC. Three fractions were recovered and assayed for C^{14} activity: the fraction before the α-pinene peak started to emerge, the α-pinene peak, and the post-α-pinene fraction, i.e., components which were eluted when the column temperature was increased to 190° over a 40-minute interval.

Table 1 indicates that a certain amount of acid is not extracted by the alkali treatment. This residual acid is eluted predominantly before the α-pinene peak (experiment 1). Some activity emerges with the α-pinene and finally, a small amount is eluted at the higher temperature. In experiment 2, the α-pinene-MVA-2-C^{14} mixture was not extracted with alkali before being placed on the column. Here, the activity in the α-pinene peak also decreased from that recovered in the pre-α-pinene fraction, but the bulk of the activity was recovered in the high temperature eluting procedure. This might be expected if increased breakdown of MVA and its derivatives occurs as the temperature rises. However, when instead of simply mixing the MVA-2-C^{14} and α-pinene before separation by GLPC, a pine shoot was allowed to metabolize the MVA-2-C^{14} (experiment 3-5), then a different distribution of radioactivity occurred in the eluted

Table 1. RECOVERY OF C^{14}

Experiment	Treatment before separating by GLPC	Added initially	Radioactivity - cpm		
			Pre-α-pinene	Recovered in α-pinene	Post-α-pinene (110-190°C., 40 min.)
1.	mevalonate-2-C^{14} +α-pinene, extracted with KOH	5×10^3	230	145	27
2.	MVA-2-C^{14} + α-pinene	5×10^3	363	150	2,085
3.	P. attenuata shoots infiltrated 8 hrs. with MVA-2-C^{14}, ether extracted with KOH	1×10^4	120	160	75
4.	as 3	1×10^4	150	275	200
5.	As 3 but not extracted with KOH	1×10^5	45	480	2,340

Table 2. INCORPORATION OF C^{14} LABELED ACIDS INTO α-PINENE BY PINUS ATTENUATA

Tissue	Substrate	Hours incubated at 23-25° C.	Percent activity recovered in α-pinene
Whole shoots	MVA-2-C^{14}	8	0.02
Whole shoots	Acetate-2-C^{14}	8	0.01
Shoot slices	MVA-2-C^{14}	2	3.0
Shoot slices	Acetate-2-C^{14}	2	0.5

Table 3. RADIOACTIVITY IN ETHER EXTRACTED TISSUE RESIDUE OF PINUS ATTENUATA SHOOTS

Experiment	Incubation substrate (cpm/gm/8 hrs.)	Treatment of ground ether extracted residue before drying	Percent activity in incubation substrate per gm tissue residue:		mgm loss/gm after drying 10 hrs., 95°C.
			Initial	After drying 10 hrs., 95°C.	
1.	MVA-2-C^{14} 3.5×10^3 cpm	1 hr. at 23° C.	8.9	3.6	74.6
2.	MVA-2-C^{14} 5.8×10^3 cpm	72 hrs. at 50° C.	3.8	2.9	13.7
3.	Acetate-2-C^{14} 8.5×10^4 cpm	72 hrs. at 50° C.	2.2	2.2	10.4

fractions. Again, when the ether extract was not partitioned in KOH before chromatographing (experiment 5) most of the activity was found in the post-α-pinene fraction. But in all experiments the α-pinene peak derived from pine shoots infiltrated with MVA-2-C^{14} showed an increase in radioactivity over that recovered in the pre-α-pinene fraction. This is the reverse of what was observed when a mixture was separated by GLPC as in experiment 1 and 2. It was necessary to elute the column for one hour at 220°C. to recover nearly all the initial activity placed on the column in experiments 1 and 2.

These experiments indicated that: (1) if any non-metabolized or breakdown product of MVA-2-C^{14} is extracted from the plant and placed on the column, it most likely does not have the same retention time as α-pinene; (2) since only the α-pinene from pine shoots which had metabolized MVA-2-C^{14} showed an increase in radioactivity over that occurring in the pre-α-pinene fraction, very probably the plant incorporates MVA-2-C^{14} into α-pinene. However, to be certain this was not an artifact and that the α-pinene was radioactive, it was necessary to separate and measure the specific activity from the rechromatographed and recrystallized α-pinene.

The α-pinene peak (fraction "A" of figure 2) was collected at 120° C. in an experiment similar to number 3 in table 1. The column was then eluted at 190° C. for 30 minutes. Fraction "A" was rechromatographed at 120° C. and "B" was isolated. The dotted lines in figure 2 indicate the peak portions isolated. After again flushing the column, "B" was rechromatographed and the eluant collected in two separate fractions: "C" and "D." Infrared spectra of fractions B, C, and D indicated that the isolated material had the spectra of pure α-pinene. The specific activities of each fraction were found to be constant. These experiments indicated that the radioactive α-pinene apparently had been formed from MVA-2-C^{14}, or one of the derivatives of MVA.

Further evidence for the incorporation of MVA-2-C^{14} into α-pinene was obtained by adding "carrier" non-radioactive α-pinene directly to the ethyl ether extract from P. attenuata which had metabolized MVA-2-C^{14} in an experiment similar to number 3 in table 1. A crystalline nitrosochloride derivative was then prepared. In typical experiments of this type, the α-pinene nitrosochloride, m.p. 105-108° C., after three and four recrystallizations contained 75 and 79 cpm per mg. respectively.

These experiments, showing constancy of specific activity through many purification steps, indicate that the C^{14} is incorporated in the α-pinene molecule. Thus, we may conclude that one of the products metabolized from MVA-2-C^{14} by pine shoots is α-pinene-C^{14}.

In experiments of this type which utilize whole plant organs, one seldom recovers high percentages of initially added substrate in the final product. The low recovery may result from failure of the substrate to penetrate to the site of active conversion, or it may result from formation of alternate products from the substrate. Insight was sought into these problems, and into the capacity of pine to form monoterpenes from a simpler acid precursor substrate than MVA.

Figure 2.--Curves indicate the
   relationship of α-pinene
   fractions recovered by GLPC
   from shoots of Pinus atten-
   uata after infiltration and
   metabolism of MVA-2-C[14]. The
   specific activities (cpm/μl)
   were determined on samples
   recovered during the inter-
   vals between the dashed lines.
   Sample B was obtained by re-
   injecting A; C and D were
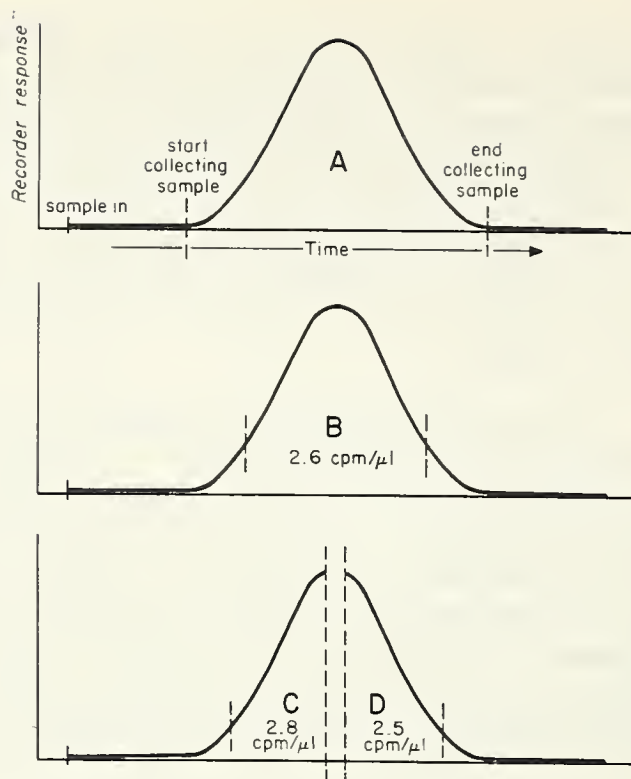   portions of the emerging peak
   of reinjected sample B.

Table 2 shows that substrate incorporation increased when greater
amounts of tissue surface were exposed by slicing the shoots. Studies of
animal cholesterol biogenesis using tissue extracts have shown as high as
45 percent of the added MVA-2-C[14] going into the sterol whereas only a
few percent went in from acetate. However, table 2 shows no such marked
difference between MVA and acetate in monoterpene formation by whole pine
shoots, although there was some increase in the slices. It has long been
recognized that acetate is metabolized by many other pathways in pine
(26) and Hall and Gisvold (13) demonstrated that carotenoids and sterols
are also synthesized in pine shoots. The formation of these alternate
products could markedly change the ratios of MVA to acetate incorporated
into α-pinene in pine. The possible synthesis of products other than
α-pinene during the above experiments was therefore investigated.

Table 3 indicates that radioactive precursor acids are also
incorporated into volatile and non-volatile constituents of pine. In
these studies the shoot tissue residues, after ether extraction, were
subjected to several drying treatments in an effort to determine the
nature of residual radioactivity. In experiment 1, the tissue standing
at room temperature--after being pressed dry of ether and spread out in
a thin layer on a watch glass to dry further--still contained considerable
radioactivity. A large amount of volatile material was removed after
drying 10 hours at 95°C. If the MVA-2-C[14] incubated material was pre-
dried for 72 hours at 50°C., as in experiment 2, then lesser amounts of
volatile components were present, and a smaller decrease in C[14] activity
occurred on drying at 95°C. With tissue residue of acetate-2-C[14]
incubated shoots, experiment 3, about the same amount of volatile com-
ponents were lost as in experiment 2, but the removable residue was not
radicactive.

It is apparent that radioactivity of a volatile nature remained in the ether extracted residue. This indicated that not all the ether (or water) held by the particles, or in the cells, was removed by the extraction and pressure drying procedure used. Also, there appeared to be a certain residual C^{14} activity incorporated into fixed-non-volatile components of pine shoots. Sandermann and Stockmann (22) found radio-active sterols and phytol in pine seedlings incubated for six weeks with radioactive β, β-methyl acrylic acid, an "active isoprene" like moiety. Whether sterols were formed in shoots used in these studies is not known, but sterols are derivable from MVA-2-C^{14} and acetate-2-C^{14}. The charcoal which was used to extract the pigment from the ether extract was also highly radioactive. These results indicate that pines synthesize many cell constituents from MVA and acetate besides the α-pinene which we assayed.

DISCUSSION

These experiments and related studies by other workers indicate that the chemical pathway by which pines synthesize simple monoterpenes such as α-pinene is similar to that by which animals and plants form related compounds of the isoprenoid series, that is, from acetate through mevalonate. However, not all the steps have been demonstrated. For example, if MVA is a key intermediate in pine terpene formation, it should theoretically be possible to isolate radioactive MVA after the shoots have metabolized acetate-C^{14}. Preliminary experiments using the paper chromatographic procedure of Hong and Wright (14) failed to detect MVA in shoots infiltrated with acetate-2-C^{14}. Demonstrating inter-mediates such as MVA usually requires purer, that is, cell-free, enzyme systems. Obtaining such a cell preparation from pine is the essential next step for the elaboration of the reaction involved in pine terpene snythesis.

It is also possible, via such in-vivo and in-vitro reaction systems used and suggested in these studies, to determine if terpenes are metabolized as indicated by the work of Sukhov (28) or are merely terminal metabolic end-products. The method of translocation and cellu-lar origin of terpenes may also possibly be demonstrated through the use of radioactive molecules produced by these techniques.

SUMMARY

1. An hypothesis relating the pathway of monoterpene formation in pine to acetic acid and mevalonic acid is outlined. The basis of this con-cept lies in recent studies by several workers who investigated the mechanism by which terpenoid units such as sterols, carotenes, lycopenes, and rubber, arise in animals and plants.

2. In the present experiments, mevalonic acid-2-C^{14} and acetic acid-2-C^{14} were both shown to give rise to radioactive α-pinene in shoots of Pinus attenuata. Radioactivity per mg, both liquid α-pinene isolated by gas liquid partition chromatography and crystalline α-pinene nitroso-chloride derived from mevalonic acid-2-C^{14}, was found to be constant through 3 and 4 purification steps.

3. In pine shoots mevalonic acid and acetate appear to be incorporated into other constituents than monoterpenes and terpenoid pigments.

4. The logical next step in testing the hypothesis would be to isolate from pine an active cell-free enzyme system capable of forming terpenes.

ABBREVIATIONS USED

GLPC: gas liquid partition chromatography

MVA-2-C^{14}:   mevalonic acid labeled with radioactive
carbon 14 at the number two carbon

cpm: counts per minute

ATP: Adenosine triphosphate

AMP: Adenosine monophosphate

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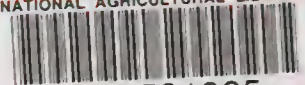   UNESCO, Paris.   1-11.