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OCCURRENCE OF MONOTERPENES IN *PINUS RADIATA* AND UTILIZATION OF LABELED CO_2 AND MEVALONIC ACID

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Abstract—β-Pinene, α-pinene and limonene are the most abundant monoterpenes of resins, needles, shoot tips and seedlings from *Pinus radiata* grown in Chile. The limonene contents of tissues is much higher than that of resins. More $^{14}CO_2$ is incorporated into β-pinene than into α-pinene in a young tree. In isolated needles, the β-isomer attains higher specific radioactivity than the α-isomer. The properties of mevalonic kinase from *P. radiata* seedlings are similar to those reported for other organisms.

INTRODUCTION

THE distribution pattern of terpenes in turpentines obtained from resins from the genus *Pinus* tends to be characteristic of a given species, within the margins of individual variations and of chemical mutations.[1] There is also a pattern of inheritance which makes the composition of resins from hybrids intermediate between those of the parent species.[2]

We have been interested in the biosynthesis of terpenes, and specifically of β-pinene in *Pinus radiata* D Don, which is the species most abundant in Chile. The reports available[1,2] agree that in this species grown elsewhere β-pinene is the main component of turpentine, α-pinene the next in abundance and that camphene is found only in trace amounts. However, the only report available on *P. radiata* in Chile[3] disagrees with all other evidence[1,2] since it claims the presence of 35 per cent of camphene and only traces of β-pinene. It was thought that the distribution of terpenes in this species should be ascertained prior to any biosynthetic study, and that the composition of turpentines from tissues should also be determined.

The biosynthesis of α-pinene from 2-^{14}C-mevalonic acid (MVA) has been demonstrated in shoot tips or isolated needles from *P. attenuata* and *P. nigra austriaca*.[4,5] Evidence obtained in this Laboratory shows that radioactivity from ^{14}C-MVA, as well as radioactive orthophosphate is incorporated by isolated needles from *P. radiata* or by seedling extracts into the same phosphorylated intermediates (5-phosphomevalonic acid, MVAP; 5-pyrophosphomevalonic acid, MVAPP; isopentenyl pyrophosphate, IPP) described as precursors of steroids,[7] rubber[8] and carotenes.[9] It may also be expected that the same enzymes may be

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TABLE 1. DISTRIBUTION OF MONOTERPENES IN TURPENTINES FROM TISSUES AND RESINS OF *Pinus radiata*

(Experimental Station Rinconada, Chile)

Tree identification no.	Type of material	α-Pinene	β-Pinene	Limonene	β-Phellandrene	Camphene	Δ³-Carene	Sabinene	Myrcene
39	Needles	18·9	47·7	19·0	12·8	0	0	1·6	t
245	Needles	30·9	37·1	19·7	12·3	t	0	0	t
254	Needles	14·5	40·4	11 8	31·9	t	0	1 4	t
255	Needles	18·4	54·9	22·1	2·9	0	0	0	1·7
355	Needles	15 5	43·8	35·5	3 2	0	0	0	2·0
Average		19·4	44·8	21·6					
39	Shoot tips	23·9	36·4	27·3	2 6	t	3·0	5·6	1·2
245	Shoot tips	23·0	30·3	23·0	18·7	0	2·7	0	2·3
254	Shoot tips	18·7	28·4	23·8	13·7	t	0	14·7	0·7
255	Shoot tips	21·8	36·9	21·8	10·6	0	4·9	0	4·0
355	Shoot tips	28·1	41·7	27·5	2·7	0	0	0	t
Average		23·1	34·7	24·7					
39	Fresh resin	31·0	57·8	4·4	0	t	t	6·7	0
245	Fresh resin	45·0	48·4	3·3	3 3	t	t	0	0
254	Fresh resin	25·0	60·5	5·5	5·5	0	0	13·8	0
255	Fresh resin	37·2	46·6	4 3	0	t	t	10·1	1·8
355	Fresh resin	53·0	41·0	4·0	0	0	0	0	2·0
Average		38·2	50·9	4·3					
1	Seedlings	54·2	45 8	0	0	0	0	0	0
2	Seedlings	51·8	48·2	0	0	0	0	0	0
3	Seedlings	55·8	44·2	0	0	0	0	0	0
4	Seedlings	55·5	45·8	0	0	0	0	0	0
5	Seedlings	53·8	46·2	0	0	0	0	0	0
Average		53·9	46 1						

The figures indicate percentages of the total monoterpenes (recovery from the resins was 100%). For experimental data see text. "t" indicates the presence of a peak that is too small to be evaluated quantitatively, whereas 0 indicates absence of deflection of the recorder needle at maximum sensitivity. Calculation of averages is included only for the three main components.

involved. Mevalonate kinase, which catalyzes the phosphorylation of MVA by ATP, could be then the key enzyme in the utilization of MVA for the formation of monoterpenes. Our previous attempts to extract this or other enzymes from needles have been unsuccessful.[10] Since several enzyme activities may be demonstrated in aqueous extracts from *P. radiata* seedlings,[6, 10] this type of preparation was thought to be adequate to explore some of the properties of mevalonate kinase.

RESULTS

The percentage of β-pinene present in the resins of 4-year-old trees is higher than that of α-pinene (Table 1). Only traces of camphene are found. Turpentines obtained by steam distillation of resins from four 10–15-year-old trees of the San Cristobal arboretum, or from stored resins from the same trees contain an average of 40 per cent α-pinene, 59 per cent β-pinene, 1 per cent limonene and traces of camphene. Infrared spectra of the samples of α- and β-pinene and camphene obtained from the resins were found to be identical with those of authentic samples. β-Phellandrene, sabinene and myrcene varied greatly from one specimen to another. The contents of limonene was much higher in needles and shoot tips

TABLE 2. INCORPORATION OF $^{14}CO_2$ INTO α- AND β-PINENE IN A 1-YEAR-OLD *P. radiata*

	Specific radioactivity (counts/min/μmole)		% Incorporation of $^{14}CO_2$	
	α-pinene	β-Pinene	α-Pinene	β-Pinene
Needles	267,920	499,390	0·015	0·035
Stem	136,500	80,400	0·005	0·003
Roots	69,760	114,370	0·003	0·005

than in resins. Seedlings exhibit a fairly constant composition and only two terpenes could be detected.

$^{14}CO_2$ is incorporated into both pinenes in the tissues of a 1-year-old tree or in isolated needles (Table 2 and Fig. 1). In the experiment shown in Table 2, the highest percentage of incorporation of added $^{14}CO_2$ and the highest specific radioactivity of the pinenes is found in needles. β-pinene shows a higher specific radioactivity than α-pinene in needles and roots. The radioactivity incorporated into β-pinene by isolated needles after 45 hr was of the order of 0·03 per cent of the $^{14}CO_2$ fed, and about 0·035 per cent for α-pinene. These values are of the same magnitude as those found for needles in the whole tree (Table 2). The time curve for total radioactivity incorporated by isolated needles roughly parallels that in Fig. 1.

Mevalonic kinase could be demonstrated in cell-free extracts from seedlings of *P. radiata* by the formation of MVAP from 2-^{14}C-MVA. The product was identified by its R_f on paper and by ion exchange chromatography. The enzyme is present in the water-soluble fraction, since centrifugation of the extracts for 2 hr at 34,000 *g* or 170,000 *g* does not affect the activity of the supernatant liquid. The maximum specific activity found in the extracts was from 2 to 5 mμmoles of MVAP formed per minute and per mg protein at 35°. The enzyme may be concentrated by precipitation with 55% saturated ammonium sulphate of saturation; no

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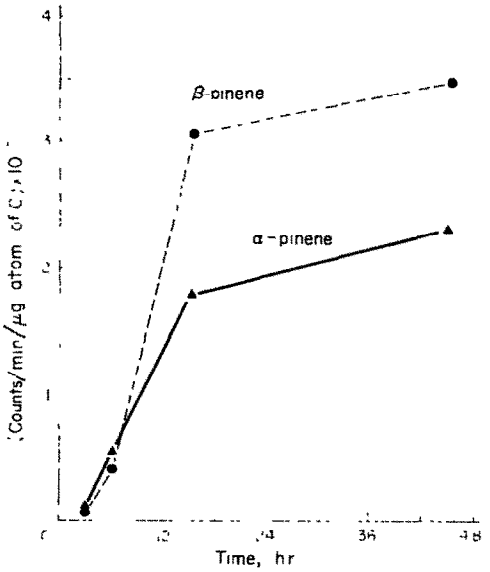

FIG. 1. INCORPORATION OF $^{14}CO_2$ INTO α- AND β-PINENE BY ISOLATED NEEDLES OF *P. radiata*. Bundles of 10 to 20 needles, weighing about 3 g, were illuminated for different lengths of time in the presence of 100 μc of $^{14}CO_2$ with a final specific activity of 0·9 μc/μmole: temperature 18-20°. Reaction was stopped by cooling the tissues to −30° in petroleum ether.

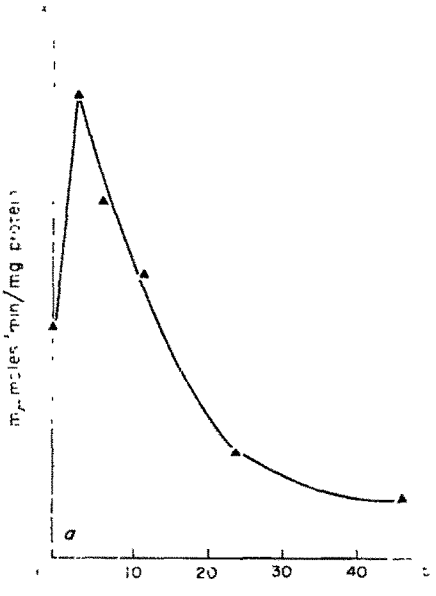

FIG. 2. EFFECT OF Mn^{2+} CONCENTRATION ON MEVALONATE KINASE FROM *P. radiata* SEEDLINGS Total volume: 0·17 ml; incubation time 15 min, temperature 35°. Composition of the medium: 60 mM TRIS-HCl buffer pH 7·5; 23·5 mM ATP; 6 mM MVA (labeled with ^{14}C in C_2, 0·5 mc/mM); ammonium sulphate precipitate (0–55% of saturation) from a seedling extract: 1·6 mg/ml; $MnCl_2$ as indicated. Point *a* contains 20 mM sodium ethylenediaminotetraacetate, pH 7·5. Reaction was stopped by heating 2 min at 100° and MVAP was measured in the supernatant after chromatographic separation.[12]

increase in specific activity is obtained by this procedure. Maximum activity is obtained in Tris-succinate buffer at pH 6·0; addition of 3 mM Mn^{2+} with 23·5 mM ATP produces a maximum of activity, which is decreased by further increase in metal concentration (Fig. 2). Ethylenediaminotetraacetate produces an almost complete inhibition.

The reaction product, MVAP, which was measured on paper chromatograms, was also identified by ion exchange chromatography. In these experiments a radioactive peak appeared in the elution zone corresponding to IPP. Identification of this compound was further confirmed by paper chromatography. The radioactivity present in IPP was about 3 per cent of that recovered in MVAP. No MVAPP could be detected.

When mevalonate kinase in the seedling extracts was assayed for different time periods, it was observed that the amount of remaining DL-2-^{14}C-MVA levelled off at 50 per cent of the added substrate, and that it never did descend below this value, even if incubation was prolonged for 4 hr. Although in short time experiments (15 min), all the radioactivity from MVA could be recovered in MVAP and IPP, this was not the case in long time experiments.

Incubation of cell-free extracts from seedlings at pH 7·4 with 2-^{14}C-MVA and ATP does not lead to the incorporation of radioactivity into pinenes. However 0·05 per cent of the added radioactivity was recovered with added carrier limonene.

DISCUSSION

Our results agree qualitatively with all the results on the composition of resins of *P. radiata* reported for other geographical locations of this species.[1,2] This further strengthens the point that the composition pattern of resin turpentines is genetically conditioned, and not determined by environment, as might have been concluded if the data of the only discrepant report[3] had proved reproducible.

The contents of limonene found in resins is somewhat higher than reported in the literature[1,2] and it is much higher in needles and shoot tips than in exudated resins. This, and the fact that it is the only compound where some radioactivity may be found in experiments *in vitro*, may suggest a metabolic participation of limonene which should be further explored.

The fact that the needles are more active than the stem or roots in the incorporation of $^{14}CO_2$ into the pinenes, as well as the incorporation observed in the isolated needles points to their importance as sites of formation of terpenes. The difference in the specific activity of terpenes in needles from those found in the stem may be due to isotopic dilution with the larger amounts of resins initially present, and also to differences in photosynthetic activity.

The kinetic experiments performed with groups of isolated needles show that β-pinene is metabolically more active than the α-isomer. No conclusion as to precursor-product relationship can be reached with the present data, but the lack of equilibration of specific radioactivities may suggest some kind of separation between these two components, either as separated pathways or as different compartments. Since probably most of the resin extracted from a needle is in the resin ducts, this observation may also reflect a difference in the equilibration between intracellular and extracellular terpenes.

The properties of mevalonic kinase from seedlings resemble those of the enzyme obtained from yeast,[18] *Cucurbita pepo*[12] and *Hevea brasiliensis*.[19] The activity per mg protein is of the same order of magnitude as that of the yeast extract, higher than the activity that can be estimated from the data reported for the crude pumpkin extract[12] and lower than that of *Hevea* kinase. Our preparations contain a phosphatase which splits ATP and phosphomonoesters at rates from 40 to 100 times the rate of phosphorylation of MVA. This phosphatase

competes with the kinase for ATP, and thus the real values of its activity are probably higher than those obtained.

The effect of Mn^{2+} on mevalonate kinase from *Pinus* seedlings (Fig. 2) is strikingly similar to the curve described for the *Hevea* enzyme.[19] The liver enzyme also attains its maximum activity in the presence of 3 mM Mn^{2+} and is inhibited by higher metal concentrations.[20]

The fact that only up to 50 per cent of the added DL-2-^{14}C-MVA was utilized may most easily be explained by the assumption that the kinase is specific for one of the enantiomorphs. The lack of quantitative recovery of the added ^{14}C in MVAP and IPP could be explained by the formation of volatile compounds. Some direct evidence of this assumption has been obtained in this laboratory.

The formation of IPP by the crude mevalonate kinase preparation, without accumulation of detectable amounts of MVAPP agree with our previous observations in isolated needles,[6] and suggest that the decarboxylation of MVAPP may be a very rapid reaction.

The view that the metabolic steps and enzymes involved in the biosynthesis of terpenes are the same as those established for the formation of other isoprenoid compounds[7-9] finds support in the results reported in this communication. Although up to 17 per cent of the radioactivity added as DL-MVA is incorporated into compounds soluble in petroleum ether by cell-free extracts from seedlings,[6] only 0·05 per cent is found in the terpene fraction of this ether extract. This low incorporation of MVA into terpenes may be due either to the destruction of a required level of organization, to the failure to extract one or more of the enzymes involved in the biosynthetic chain or to the presence of phosphatases which drain phosphorylated compounds from this biosynthetic pathway. The latter assumption is partially supported by the effect of NaF on the accumulation of phosphorylated allylic compounds described elsewhere.[6] Experiments with other labeled precursors are in progress.

EXPERIMENTAL

Samples of fresh resin were collected in spring (November) from five specimens of 3–4-year-old *P. radiata* at the Experimental Station in Rinconada (Central Chile), as described by Bannister.[2] The resin was immediately dissolved in light petrol (b.p. 30–40°). Needles from the preceding spring and 2–3-month-old shoot tips were collected simultaneously from the same specimens. They were extracted with cold light petrol in a mortar with quartz sand. The extracts were bleached by passing them through a 1 × 20 cm column of a mixture of equal parts (w/w) of charcoal (Nuchar C-190, unground, 45–100 μ, acid washed, obtained from West Virginia Pulp and Paper Co., Covington, Va.) and of Whatman cellulose powder. This column did not retain any of the terpenes analyzed, as tested by the recovery of known amounts of standards.

In order to investigate the discrepant report of Bianchi[3] we also took samples of resin from 10–15-year-old trees from the same arboretum where his samples were obtained (San Cristobal, Santiago, Chile). These resins were treated as described above.

Seeds were allowed to germinate in quartz sand without prior stratification, and the seedlings were excised after 45–60 days. The green portions of the seedlings were extracted as above with light petrol and bleached. The petrol extracts of tissues or resins were concentrated under reduced pressure when necessary.

Cell-free aqueous extracts were obtained from seedlings by grinding their green portions in a mortar with one volume of 0·05 M Tris-HCl buffer, pH 7·9, filtering the homogenate through cheese cloth and centrifuging it for 10 min at 30,000 *g* at 0 °.[6]

The experiment of $^{14}CO_2$ incorporation was performed in a closed chromatographic jar with a 1-year-old specimen of *P. radiata* (40 cm height) for 29 hr. Illumination was provided by four 100 W bulbs at 1 m distance. CO_2 was liberated from $NaH^{14}CO_3$ by the slow addition of 4 N H_2SO_4 in a system similar to that described by Slankis.[11] The total radioactivity added was 250 μc. The final specific activity of $^{14}CO_2$ was calculated to be 0·43 μc/μmole. The tissues were extracted as described above. A high-speed homogenator was used to extract the chopped stem.

When isolated needles were used, the experiments were scaled down proportionately and performed in test tubes, with the base of the needles immersed in tap water and illumination provided by two 100 W bulbs at 25 cm. The needles were obtained as described above 1–2 hr prior to use. At the end of the experiments the needles were extracted with light petrol and the extracts bleached as described.

Mevalonic kinase was assayed by measuring the formation of MVAP from DL-2-^{14}C-MVA (Calbiochem, California) as described by Loomis and Bataille.[12]

Gas phase chromatography was performed in a β,β^1-oxidipropionitrile column[13] with He as carrier. This column is very selective for monoterpenes. The instrument used was an F & M Model 720, equipped with a thermal conductivity detector.

Internal standards were frequently used as controls. The amount of terpene in a peak was evaluated either by triangulation or by cutting out and weighing the paper, and compared with a standard curve obtained with known amounts of terpenes.

When radioactive compounds were used, they were collected at the outlet of the gas chromatograph by means of a U-tube containing toluene and cooled in dry ice. Recovery, as controlled by rechromatography of the collected sample, was quantitative. Carrier terpenes were added when the amount of sample to be recovered was expected to be below the sensitivity of the detector. Radioactivity was measured in a liquid scintillation spectrometer using 0·01 per cent 1,4-bis-2-(5-diphenyloxazolyl)benzene (POPOP) plus 0·5 per cent 2-5-diphenyloxazole (PPO) as phosphor in toluene. Radioactivity in paper chromatograms or in effluents of ion exchange columns was measured in a gas flow counter.

Ion exchange chromatography for the identification of phosphorylated products was performed on Dowex-1-formate according to Bloch *et al.*[14] This identification was further confirmed by paper chromatography in three different solvents.[15,16] Proteins were measured by the Biuret method.[17]

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