ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 135, 416–418 (1969)

Terpene Biosynthesis: Utilization of Neryl Pyrophosphate by an Enzyme System from *Pinus Radiata* Seedlings[1]

OSVALDO CORI

Laboratory of General Biochemistry (Faculty of Chemistry and Pharmacy) and Department of Chemistry (Faculty of Sciences), University of Chile, Santiago, Chile

Received August 20, 1969; accepted October 4, 1969

α pinene and β pinene (0.6%) are formed from 2-^{14}C neryl pyrophosphate by soluble enzymes from *Pinus radiata* seedlings. Geranyl pyrophosphate is much less active as a precursor of hydrocarbons and the monophosphates are completely inactive.

These data offer further support to the proposed role of the *cis* isomer in the formation of cyclic monoterpenes.

A soluble enzyme system from *Pinus radiata* seedlings forms nerylpyrophosphate and nerol from 2-^{14}C mevalonic acid (1). The *cis* configuration of NPP[2] and its chemical behavior (2) (3) have suggested that it could be a more direct or better precursor of cyclic monoterpenes (1) than its *trans* isomer, GPP. An attempt to demonstrate transformations of NPP or GPP other than hydrolysis is presented in this communication.

EXPERIMENTAL

A 104,000*g* extract, obtained from *P. radiata* seedlings (1) was incubated with radioactive substrates in a total volume of 2 ml containing 70 mM Tris–HCl buffer, pH 7.8, or Tris–succinate, pH 6.0; 1.7 mM $MgSO_4$ and $MnSO_4$; 20 mM 2-mercaptoethanol; 60 mM KF; seedling protein 4 mg and 0.025 mM 2-^{14}C-GPP, NPP, GP, or NP ($1.6 \cdot 10^6$ cpm/μmole). After 4 hr at 37° the reaction mixture was cooled to 0° and extracted with 2 ml of pentane containing carrier limonene, α and β pinene or nerol plus geraniol (0.3 mg of each). The pentane phase was concentrated under gentle vacuum. Thirty per cent of the amount of hydrocarbons was lost during this process, as estimated from the peak area of gas chromatograms. Terpenes were separated in a gas chromatographic column (4) as detailed in Table I. The effluents were collected at 1.5-min intervals for counting (1). Nerol and geraniol were separated on a 2% ethyleneglycoladipate column at 115°. In all columns, the injection port was kept at 130–135° (5). Under these conditions, synthetic ^{14}C nerol or geraniol gave a single radioactivity peak coincident with carrier alcohol. No radioactivity could be detected at retention volumes corresponding to hydrocarbons (below 0.35 × retention volume of nerol).

Thin-layer chromatographic separation of monoterpenes (R_F 0.75-0.85) from C_{10} prenols (R_F 0.17–0.33) was achieved on silica gel G thin-layer plates using ethyl acetate-benzene-hexane (12:25:63, v/v) and iodine vapors to locate the spots. The system does not separate well individual hydrocarbons, but they are very well resolved from alcohols. The silica gel was scraped from the plates in 1-cm portions into scintillation vial sand counted (6). Control experiments with boiled extract were always performed. All values stated are corrected for these nonenzymic controls.

Synthesis of Radioactive GP, GPP, NP and NPP. Radioactive methyl esters of geranic and nerylic acids were synthesized by a modified Wittig reaction (6), (7) from 2-^{14}C methylbromoacetate and 6-methylhept 5(6) en-2-one.

The *cis* was separated from the *trans* ester on a 2% ethyleneglycol adipate column at 115° and collected. The separated esters were reduced to the

[1] This research was partially financed by CONICYT (Chile), by the U. S. Department of Agriculture (FG Ch 101) and by N.I.H. (AM-10714).

[2] *Abbreviations*: GPP, geranyl pyrophosphate; NPP, neryl pyrophosphate; GP, geranyl monophosphate; NP, neryl monophosphate.

corresponding alcohols with $LiAlH_4$, and the alcohols were phosphorylated (6) to a mixture of mono- and pyrophosphates, which were separated on DEAE-Sephadex (8). After this separation each phosphorylated compound had a single radioactivity peak in paper chromatograms (*n*-propanol-NH_3-water 7:2:1 v/v) with an R_F of 0.27 for GPP and NPP and of 0.45 for the monophosphates.

RESULTS AND DISCUSSION

Table I shows that up to 0.6% of the counts added as NPP to a soluble enzyme system from *P. radiata* seedlings could be recovered from a gas chromatographic column associated with carrier α and β pinenes. Little or no radioactivity was associated with limonene. When GPP was the substrate, the radioactivity associated with these terpenes was much less. In parallel experiments, it was found that 96% of the radioactivity of the pentane extract was recovered as nerol when NPP was the substrate, and 84% as geraniol, when GPP was used. It may be assumed that the balance radioactivity from GPP could be found in other isoprenoids (9). At pH 6.0, more radioactivity is released from the substrates; fewer hydrocarbons are formed (Table I) and more radioactivity is found in the corresponding alcohol in parallel experiments.

If NP or GP were used as substrates, more counts were found in the pentane extract than with NPP or GPP. No radioactivity was associated with carrier terpene hydrocarbons, but it could be recovered with nerol or geraniol.

The formation of nerol or geraniol from all phosphorylated substrates used may be attributed to an acid phosphatase activity (1), only partially inhibited by KF. At pH 6.0 this activity is higher; more alcohols and fewer hydrocarbons are formed. Monophosphates appear to be better substrates than pyrophosphates.

An extract obtained from a seedling acetone powder forms only nerol or geraniol from all four substrates; no radioactivity could be found associated with pinenes or limonene. This could be interpreted as being due to the stability of the phosphatase in spite of the acetone treatment, which inactivates the enzyme or enzymes responsible for the formation of terpenes. Furthermore, this as well as the negative result obtained with NP speaks against the possibility that the allylic alcohols present in the pentane extract could have been dehydrated in the gas chromatograph to bicyclic terpenes. Dehydrations of terpene alcohols to hydrocarbons have been reported (5) although at somewhat more drastic conditions.

Experiments similar to those described in Table I were performed with twice the

TABLE I

INCORPORATION OF RADIOACTIVITY FROM NPP AND GPP INTO MONOTERPENES BY AN ENZYME SYSTEM FROM *P. radiata*

	Carrier peak	Retention volume[b]	Radioactivity collected (cpm/tube)			
			pH 6.0, Substrate		pH 7.8, Substrate	
			NPP	GPP	NPP	GPP
Total cpm in pentane extract	—	—	21,500	25,700	9,800	11,700
Hydrocarbons:	None	0–0.32	60	7	105	7
	σ pinene	0.42	86	33	210	49
	β pinene	0.61	179	29	239	31
	None	0.8	173	41	132	9
	Limonene	1.0	0	52	0	18

[a] For medium composition see under Experimental; 80,000 cpm were used as substrate in all tubes. Controls with boiled enzyme were provided, and all results are corrected for these controls. Gas chromatographic column: 250 × 0.62 cm of β,β'-oxidipropionitrile 15% on Chromosorb W 60/80 mesh at 85°. Helium flow: 70 ml/min; injection port: 130°; thermal conductivity detector, 180°.

[b] Referred to limonene = 1.0. Relative retention volumes below 0.3 have been reported only for santene; 0.8 has been reported for α phellandrene and myrcene (4).

amount of substrate, and terpene alcohols were separated as a group from hydrocarbons by thin-layer chromatography; 360 cpm per tube were recovered from the hydrocarbon spot (R_F 0.75–0.85) and 16,000 cpm from the prenol spot (R_F 0.17–0.33) when NPP was the substrate. The hydrocarbons contained 45 cpm and the alcohols 27,000 when GPP was used. If GP or NP were used, no radioactivity could be recovered from the hydrocarbon spot.

The results reported show that as discussed in previous reports (1, 2), NPP is a better precursor of terpene hydrocarbons than GPP, and that the monophosphorylated substrates are not utilized.

The fact that some radioactivity from GPP is recovered with carrier cyclic hydrocarbons could be explained in terms of a *trans–cis* isomerization. This, however, is not a very common biochemical reaction (10), and there is no evidence for it in the *P. radiata* enzyme system, whereas stereospecific processes are the rule in isoprenoid biosynthesis (11).

The experiment shown in Table I also suggests that the hydrocarbons formed may be bicyclic. Negative results obtained with the monophosphates exclude a possible artifact under the conditions employed in gas chromatography.

The amount of radioactivity recovered with hydrocarbons is very low, and it does not permit more rigorous identification procedures, but a consistent pattern is observed in all experiments: More radioactivity is recovered with terpene hydrocarbons from NPP than from GPP, and the monophosphates are inactive.

The phosphatase activity, which hydrolyzes most of the pyrophosphates added, is probably responsible for the low amount of radioactivity recovered with hydrocarbons. The aqueous medium is another obstacle for the formation of hydrocarbons in this system. Experiments are in progress attempting to eliminate the phosphatase activity and thus to accumulate amounts of hydrocarbons suitable for further characterization.

ACKNOWLEDGMENTS

The author is very indebted to Mr. Emilio Cardemil for his participation in some of the experiments.

Part of the work was performed at the Department of Chemistry of U.C.L.A. while the author participated in the "Chile-California" exchange program.

REFERENCES

1. Beytia, E., Valenzuela, P., and Cori, O., *Arch. Biochem. Biophys.* **129,** 346 (1969).
2. Valenzuela, P., and Cori, O., *Tetrahedron Letters* **1967,** 3015.
3. Cramer, F., and Rittersdorf, W., *Tetrahedron* **23,** 3015 (1967).
4. Klouwen, M. H., and TerHeyde, R., *in* 3*rd Intern. Symp. Gas Chromatography,* 1961 (N. Brenner, E. Callen, and M. D. Weiss, eds.), p. 485. Academic Press, New York (1962).
5. Naves, Y. R., and Bondavalli, I., *Helv. Chim. Acta* **48,** 563 (1965).
6. Upper, C. D., and West, C. A., *J. Biol. Chem.* **242,** 3285 (1967).
7. Wadsworth, W. S. Jr., and Emmons, W. D., *J. Amer. Chem. Soc.* **83,** 1377 (1961).
8. Oster, M. O., and West, C. A., *Arch. Biochem. Biophys.* **127,** 112 (1968).
9. Porter, J. W., and Anderson, D. G., *Ann. Rev. Plant, Biochem.* **18,** 197 (1967).
10. Topper, Y. J., *in* "The Enzymes" (P. D. Boyer, H. Lardy and K. Myrbäck, eds.), 2nd edition, Vol. 5 p. 416, Academic Press, New York (1961).
11. Popjak, G., and Cornforth, J. W., *Biochem J.* **101,** 553 (1966).