Phytochemistry, 1977, Vol 16, pp. 1665–1667. Pergamon Press. Printed in England.

TURNOVER OF CONIFERIN IN PINE SEEDLINGS

STEFAN MARCINOWSKI and HANS GRISEBACH

Lehrstuhl für Biochemie der Pflanzen, Biologisches Institut II der Universität Freiburg, Schänzlestr. 1, D-7800 Freiburg i.Br., W. Germany

(*Received* 20 *April* 1977)

Key Word Index—*Picea abies*; Coniferae; pine seedlings; coniferin; turnover; lignification.

Abstract—Coniferin (coniferyl alcohol-β-D-glucoside) was not detected in pine seeds (*Picea abies*) but it accumulated in the stems and roots of pine seedlings. Pulse labeling experiments with L-phenylalanine-[U-^{14}C] and 100-day-old pine seedlings in hydroponic solution showed a turnover of coniferin with a half life of about 60 hr. Pulse labeling with $^{14}CO_2$ and seedlings kept in soil gave a half life of about 120 hr for coniferin. The results indicate that coniferin could be an intermediate of lignin biosynthesis in pine seedlings.

INTRODUCTION

Experiments with labeled coniferin (coniferyl alcohol-β-D-glucoside) have shown that it can act as a lignin precursor in a variety of species [1]. However, the role of coniferin in lignification has remained uncertain. Freudenberg *et al.* have postulated that the role of coniferin and the glucosides of other cinnamyl alcohols is to permit transport of the slightly soluble cinnamyl alcohols in a readily soluble form from the site of synthesis to the vicinity of the cambium, where the aglycones are liberated by β-glucosidase action of the cambium and then polymerized [2]. Another hypothesis is that the glucosides act as a reservoir to augment the precursor supply of lignifying cells [3].

The incorporation of phenylalanine-[^{14}C] into 4-coumaryl alcohol glucoside, coniferin, and syringin (sinapyl alcohol-β-D-glucoside) in pine branches has been reported [4]. However, no information on the turnover of these glucosides is available; in this paper we report on pulse-labeling experiments designed to determine the turnover of coniferin in pine seedlings.

RESULTS

The pine seeds (*Picea abies*) did not contain any detectable amount of coniferin. For the pulse-labeling experiments 100-day-old seedlings were used which contained 50–80 μg coniferin/g fr. wt in the experiment with phenylalanine-[^{14}C] and 90–160 μg in the $^{14}CO_2$ experiment. Coniferin was also present in 40-day-old seedlings (70 μg/g fr. wt) and was *ca* equally distributed between stems and roots. The needles did not contain coniferin (for occurrence of phenolics in pine needles see [5]). This finding agrees with that reported by Freudenberg and Torres-Serres for needles from 3–5 year old pines [4]. The coniferin was identified by PC and TLC, by comparison of its UV spectrum with that of an authentic sample and by its enzymatic hydrolysis with emulsin to coniferyl alcohol and glucose.

For pulse-labeling with phenylalanine-[^{14}C] 120 intact seedlings were placed in the phytochamber in a beaker with 200 ml H_2O containing 85 μCi (8.5 μmol) of L-phenylalanine-[U-^{14}C]. After 22 hr, during which 55% of the radioactivity had been taken up by the

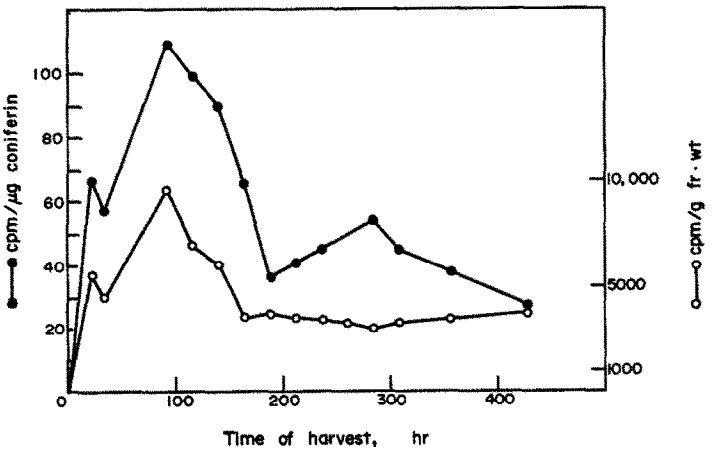

Fig. 1. Pulse-chase experiment with L-phenylalanine-[U-^{14}C]. Pulse from 0–22 hr. Chase from 22–44 hr.

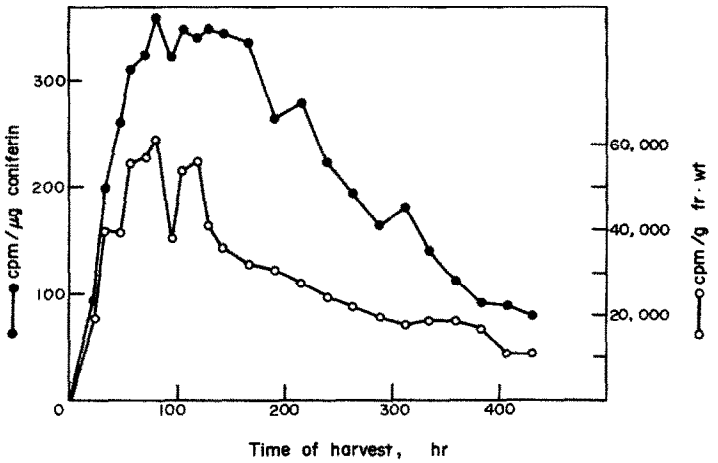

Fig. 2. Pulse-chase experiment with $^{14}CO_2$. Pulse from 0–50 min. For the rest of the time the seedlings were kept in air.

seedlings, the plants were rinsed with water and then placed in 250 ml of a solution of unlabeled phenylalanine (62 μmol) for another 22 hr. The seedlings were then planted into quartz sand which was kept moist with a hydroponic solution. At definite intervals samples of 7 plants each were worked up for coniferin as described under Experimental and the total radioactivity and sp. act. of coniferin were determined. The results shown in Fig. 1 show a turnover of coniferin in the seedlings with a half life of *ca* 60 hr.

Another pulse-labeling experiment was carried out with $^{14}CO_2$. For this purpose 250 seedlings in soil were placed inside a plexiglas chamber. After 50 min photosynthesis in the presence of 1 mCi $^{14}CO_2$ the atmosphere was replaced by normal air. The seedlings were then kept in the phytochamber for the rest of the experiment. Eight seedlings were worked up for coniferin for each value shown in Fig. 2. The half life for coniferin calculated from the sp. act. is *ca* 120 hr.

Samples of [^{14}C]coniferin from both experiments were analyzed for distribution of radioactivity between coniferyl alcohol and glucose. For this purpose coniferin was hydrolyzed with emulsin and the hydrolysis products separated by PC (Table 1). With phenylalanine-[^{14}C] as precursor the bulk of radioactivity is, as expected, localized in the aglycone. With $^{14}CO_2$ an almost equal distribution of activity between aglycone and glucose was found with time with only a slight increase in the relative portion in the aglycone.

Table 1. Distribution of radioactivity in coniferin from pulse-labeling experiments

hr	Glucose (cpm)	Coniferyl alcohol (cpm)	Activity ratio glucose/coniferyl alcohol
I L-phenylalanine-[-^{14}C]			
22–188*	274	10600	1:38
212–284*	359	3650	1:10
II $^{14}CO_2$			
23	3840	3370	1:0.88
95	8660	8670	1:1
143	12100	12800	1:1.06
239	5910	6810	1:1.15
283	2390	3260	1:1.36

* Samples from this time interval were collected.

DISCUSSION

Both pulse-labeling experiments prove a rapid synthesis of coniferin in the pine seedlings and a slower turnover of this compound with a half life in the order of 60–120 hr. The longer half life calculated from the plot of sp. act. from the $^{14}CO_2$ experiment may be due in part to the slight decrease of total coniferin content of the seedlings during the time of the experiment. Whereas in the phenylalanine-[^{14}C] experiment the seedlings had to be taken out of the soil, put into water and then kept under hydroponic conditions, the plantlets in the $^{14}CO_2$ experiment could be kept in soil all the time. It follows from these results that a turnover of coniferin occurs under normal physiological conditions. This finding would be in agreement with the assumption that a part of or all lignin biosynthesis occurs via coniferin in pine seedlings but of course does not prove it. A quantitative correlation between coniferin turnover and lignin formation was not possible under our conditions. The average fr. wt of the seedlings did not increase significantly during the time of the experiments. An increase in lignin content, if measurable at all, would certainly be too small to allow any quantitative correlations. However, the small pool size of coniferin of about 50–70 nmol coniferin per seedling (average wt 190–250 mg) and its relatively slow turnover suggest that only a part of lignin synthesis occurs via coniferin.

In our experiments no differentiation was made between coniferin from stems and roots. It should be considered that the turnover in these two organs could be different.

EXPERIMENTAL

Seeds of *P. abies* were obtained from the institute for Waldbau, Freiburg. Plants were raised in soil in a phytochamber at 20° (60% rel. humidity) under long-day conditions (15 hr, 12000 lx).

Extraction of coniferin. After removal of the needles, the seedlings (7–8) were cut into small pieces and then ground in a

mortar with quartz sand and 20 ml of 25% EtOH. The homogenate was heated to boiling for 10 min. After low speed centrifugation, the sediment was washed with 25% EtOH and the combined supernatants were extracted ×3 with 10 ml of Et_2O. The aq. phase was concd *in vacuo* to a small vol. and applied to a TLC plate (0.8 mm, Si gel, F 254). The starting zone was concd by developing the plate twice for a short distance in MeOH. The plate was then developed with Me_2CO–EtOAc–H_2O (10:10:1). The zone of coniferin (R_f 0.38) was detected under UV, scraped off and eluted with 30% MeOH. Coniferin was rechromatographed by PC on prewashed Whatman 3 MM paper with BuOH–2% NH_3. The coniferin zone (R_f 0.3) was eluted with 30% MeOH, the solvent evapd and the residue dissolved in a known vol. of 30% MeOH. The concn of coniferin was determined at 295 nm. For determination of radioactivity 0.5 ml of the soln was counted in 10 ml Triton scintillation fluid (12.5 g PPO, 800 ml Triton X-100, 1600 ml toluene) plus 0.5 ml H_2O in a liquid scintillation counter. Part of the coniferin was rechromatographed by PC with BuOH–HOAc–H_2O (4:1:2,2) (R_f 0.66) and eluted from the paper, and the sp. act. was determined as described above.

Experiment with L-*phenylalanine*-[*U*-^{14}C]. 120 pine seedlings (100 days old) were placed in a beaker containing 200 ml H_2O with 85 μCi L-phenylalanine-[U-^{14}C] (Radiochemical Centre Amersham, 10 Ci/mol). During the time of the expt the seedlings were kept in the phytochamber under conditions described above. After 22 hr the plants were rinsed with H_2O and transferred to a beaker with 250 ml 0.25 mM 'cold' L-phenylalanine. After a further 22 hr the seedlings were planted into sea-sand which was kept moist with a nutrient soln of the following composition (in g/l. H_2O): 0.58 $Ca(NO_3)_2 \cdot 4H_2O$, 0.25. KNO_3, 0.3 NH_4NO_3, 0.52 $MgSO_4 \cdot 7H_2O$, 0.25 $(NH_4)_2SO_4$, 0.25 KH_2PO_4, 0.2 $(NH_4)_2HPO_4$, 0.1 Na_2SiO_3, 0.065 Na–Fe–EDTA and 1 ml trace elements (AZ-soln [6]). The pH was 5–5.5.

Experiment with $^{14}CO_2$. A tray with 250 pine seedlings (100 days old) in soil was placed in a plexiglas chamber of 3.5 l. vol. The chamber was illuminated with two 300 W tungsten lamps with a heat absorbing filter. $^{14}CO_2$ was generated from 1 mCi $Na_2{}^{14}CO_3$ (60.1 Ci/mol) with 2 ml conc H_2SO_4 and the air inside the chamber was circulated by a small ventilator. After 35 min 0.5 ml 33 mM 'cold' Na_2CO_3 soln was added to the H_2SO_4 to expel the $^{14}CO_2$ completely. After 50 min the remaining CO_2 was trapped in KOH soln. The tray with the seedlings was then placed in the phytochamber under the conditions described above.

Enzymatic hydrolysis of coniferin. Coniferin was dissolved in 0.03 M McIlvaine buffer, pH 5, and incubated for 20 hr at 37° with emulsin (Serva). The reaction vial was then heated for 10 min at 100° and the supernatant applied to Whatman 3 MM paper. PC was performed with BuOH–HOAc–H_2O (4:1:2,2). The radioactive zones detected by a chromatogram scanner corresponded to coniferyl alcohol (R_f 0.95, detected under UV) and glucose (R_f 0.3, detected by aniline phthalate). The radioactive zones were cut out and counted in a toluene scintillation fluid.

Acknowledgements—This work was supported by Deutsche Forschungsgemeinschaft (SFB 46) and by Fonds der Chemischen Industrie. S.M. thanks the Fonds der Chemischen Industrie for a Doktorandenstipendium. We thank Prof. Weinges, Heidelberg, for a sample of coniferin.

REFERENCES

1. Brown, S. A. (1966) *Ann. Rev. Plant Physiol.* **17**, 236.
2. Freudenberg, K., Reznik, H., Boesenberg, H. and Rasenack, D. (1952) *Chem. Ber.* **85**, 641.
3. Sarkanen, K. V. and Ludwig, C. H. (1971) *Lignings: Occurrence, Formation, Structure and Reactions* p. 110. Wiley–Interscience, New York.
4. Freudenberg, K. and Torres-Serres, J. (1967) *Ann. Chem.* **703**, 225.
5. Dittrich, P. and Kandler, O. (1971) *Ber. Dtsch. Botan. Ges.* **84**, 465.
6. Strasburger, E. (1962) *Lehrbuch der Botanik* p. 207. G. Fischer Verlag, Stuttgart.