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THE PENTOSE PHOSPHATE PATHWAY AS A SOURCE OF NADPH FOR LIGNIN SYNTHESIS

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Key Word Index—*Coleus blumei*; *Pisum sativum*; *Helianthus tuberosus*; Labiatae; Leguminosae: Compositae: lignin synthesis; reducing power; pentose phosphate pathway.

Abstract—The aim of this work was to investigate whether the pentose phosphate pathway provides reducing power for lignin synthesis. Explants of the stem of *Coleus blumei* and the storage tissue of *Helianthus tuberosus* were cultured for 4 days on media which caused extensive lignification. [3-^{3}H]-glucose and either [3-^{14}C]- or [U-^{14}C]-glucose were supplied to such 4-day-cultured explants, and also to the roots of 5-day-old seedlings of *Pisum sativum*. Significant amounts of ^{3}H and ^{14}C were recovered in syringaldehyde, vanillin, *p*-hydroxybenzaldehyde, and ligothioglycollic acid from the explants of *Coleus* and *Helianthus*; and in vanillin, *p*-hydroxybenzaldehyde, and milled-wood lignin from pea roots. The ^{3}H/^{14}C ratios in these derivatives and preparations of lignin are held to indicate that much of the reducing power for lignin synthesis comes from the pentose phosphate pathway.

INTRODUCTION

Lignification coincides with appreciable activity of the pentose phosphate pathway in explants of the stem of *Coleus* and the storage tissue of Jerusalem artichoke [1], and in pea roots [2]. This suggests that the pathway provides at least some of the reducing power for lignin synthesis. The aim of the work described in this paper was to investigate this hypothesis by incubating the above tissues in specifically labelled [^{3}H,^{14}C]-glucose, and then determining whether the lignin was labelled in the manner expected from the hypothesis. In view of the difficulties of isolating lignin we determined the labelling pattern in the aromatic aldehydes produced by oxidation of lignin with alkaline nitrobenzene, and in preparations made so as to extract lignin with the minimum of change. Two such preparations were made; one was extraction of lignin as a complex with thioglycollic acid [3], and the other was extraction with dioxan as in the preparation of milled-wood lignin [4].

The major steps in the conversion of glucose-6-phosphate to the precursors of lignin are shown in Scheme 1. Both [1-^{3}H]- and [3-^{3}H]-glucose label NADP in the pentose phosphate pathway. There is considerable evidence of marked discrimination against [1-^{3}H]-glucose in the pathway in plants [5] and animals [6]. As yet there is no such evidence against [3-^{3}H]-glucose, thus we chose this isotope to label the NADP^{3}H, which it does via the reaction catalysed by 6-phosphogluconate dehydrogenase. If [3-^{3}H]-glucose enters glycolysis then the labelled hydrogen is lost completely as water in the isomerization of dihydroxyacetone phosphate to phosphoglyceraldehyde [6]. There is strong evidence that glycolysis and the pentose phosphate pathway are the only routes of carbohydrate oxidation in the tissues which we studied [1, 2]. Consequently, substantial labelling of the lignin of these tissues by [3-^{3}H]-glucose would indicate that the pentose phosphate pathway provided reducing power for lignin synthesis. The extensive transfer of label from [3-^{3}H]-glucose to water during glycolysis could lead to the appearance of label in lignin. However, dilution of tritiated water formed in this way by cellular water is likely to make labelling of lignin by this route small when compared to the flow of carbon to lignin from glucose. Accordingly we assessed this carbon flow by supplying [3-^{14}C]- or [U-^{14}C]-glucose with [3-^{3}H]-glucose.

Our dual labelling experiments allow a comparison between the ^{3}H/^{14}C ratios found in lignin and the aromatic aldehydes derived from lignin, with the values expected from the hypothesis that all of the reducing power for lignin synthesis comes from the pentose phosphate pathway. The following arguments are based on our current understanding of the pathways of carbohydrate oxidation [7] and lignin synthesis [8]. The available evidence [1, 2] strongly suggests that, in the tissues which we used, the activity of the pentose phosphate pathway, though appreciable in absolute terms, is low in relation to that of glycolysis. Thus we suggest that most of the phosphoenolpyruvate used in phenylpropanoid synthesis in these tissues comes from glycolysis. If this is so then the specific activity of the phenylpropanoid units of lignin would be about one and a half times that of the supplied [^{14}C]-glucose regardless of whether it was [3-^{14}C]- or [U-^{14}C]-glucose. Similarly, the specific activity of aromatic aldehydes from lignin would be approximately equal to that of the supplied [3-^{14}C]- or [U-^{14}C]-glucose. The precise values will depend upon the extent to which label from [^{14}C]-glucose reaches the methoxyl groups of the phenylpropanoid units, and on the degree of re-cycling in the pentose phosphate pathway.

Studies of the co-enzyme specificity of the enzymes responsible for phenylpropanoid synthesis indicate that

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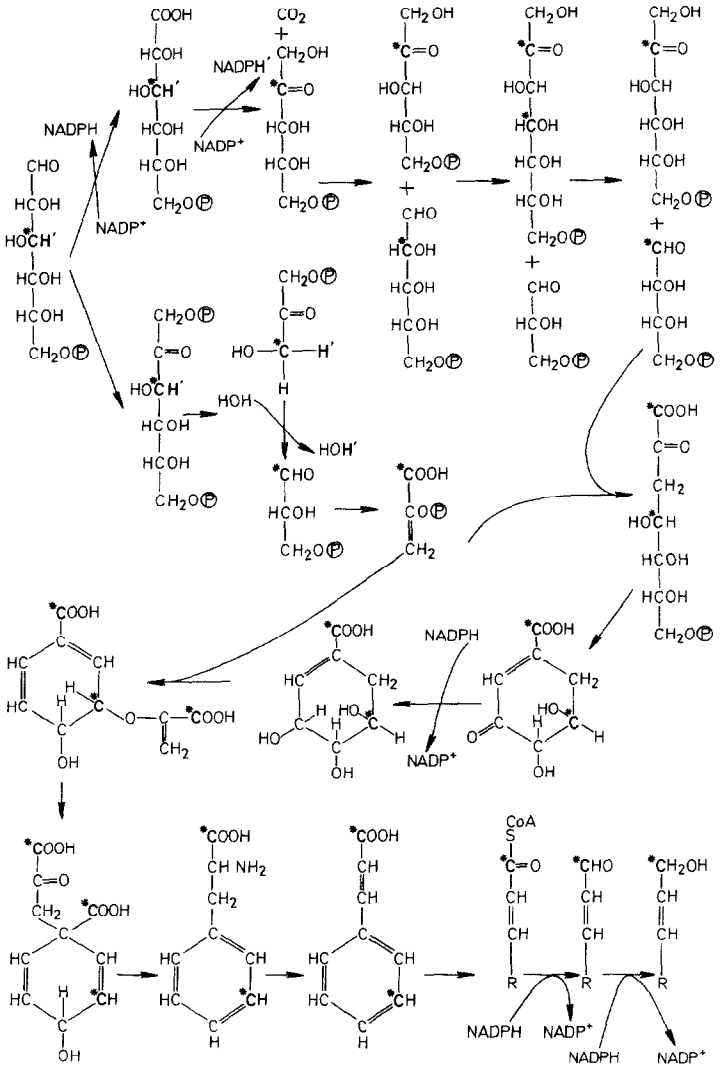

Scheme 1. Principal steps in the conversion of [3-^{3}H]- and [3-^{14}C]-glucose to precursors of lignin.

the following involve direct transfer of hydrogen from NADPH ultimately into lignin: shikimate dehydrogenase [9], cinnamyl-CoA NADPH reductase [10], and cinnamyl alcohol dehydrogenase [11]. It is important to note that most of the hydrogen transferred from NADPH to shikimic acid would be retained on the aromatic ring of the phenylpropanoid unit during the subsequent hydroxylations [12]. The specific activity of NADP^{3}H labelled by [3-^{3}H]-glucose via the pentose phosphate pathway would be half that of the [3-^{3}H]-glucose. From the above we argue that, if all the reducing power for lignin synthesis came from the pentose phosphate pathway, then the specific activity of the substituted cinnamyl alcohols would be about one and a half times that of the [3-^{3}H]-glucose. However, the specific activity of the aromatic aldehydes from lignin would be about half that of the [3-^{3}H]-glucose because they would retain only the ^{3}H transferred to the aromatic ring. Thus it appears, that if all the reducing power for lignin synthesis comes from the pentose phosphate pathway, then feeding [3-^{3}H]-glucose together with either [3-^{14}C]- or [U-^{14}C]-glucose would produce a ^{3}H/^{14}C ratio in lignin of about 1 and, in the aromatic aldehydes derived from lignin, of about 0.5.

RESULTS AND DISCUSSION

Explants of the third internode of the stem of *Coleus blumei*, and of the storage tissue of Jerusalem artichoke, were placed on media which promoted extensive lignification. For both tissues this lignification reaches its maximum after 4 days [1]. Thus we supplied [^{3}H,^{14}C]-glucose to samples of 4-day-cultured explants for 24 hr under aseptic conditions. We labelled the lignin in pea roots in two ways. In two experiments we supplied the isotopes to the roots of intact 5-day-old seedlings which were arranged in a compartmented box so that the isotopes could be applied locally to the region 6–46 mm from the root apices. In the other experiments we fed the isotopes to samples of steles which we had isolated from the region of the roots 6–46 mm from the apices. The labelling of lignin and its aldehydes was similar in both

Table 1. Labelling of lignin by [^{3}H,^{14}C]-glucose supplied to different plant tissues

Fraction of tissue	Isotope recovered per fraction (dpm × 10^{-3}) from *Coleus* explant ^{3}H	*Coleus* explant ^{14}C	Jerusalem artichoke explant ^{3}H	Jerusalem artichoke explant ^{14}C	Pea root ^{3}H	Pea root ^{14}C
Water-soluble substances	313.40	92.17	200.2	184·3	188.8	44.7
Aldehydes from lignin						
Syringaldehyde	0.64	0·46	0.86	1.50	none detected	
Vanillin	0.80	0.54	4.99	8.23	0.26	1.15
p-Hydroxybenzaldehyde	0.98	0.88	2.98	5.54	0.14	1.10
Lignothioglycollic acid	16.05	8.05	13.79	17.78	—	—
Milled-wood lignin	—	—	—	—	2.82	0.92
Position of label in [^{3}H,^{14}C]-glucose supplied	3	3	3	U	3	3

Labelling of the lignin and the aldehydes from peas, and of the water-soluble substances for all tissues was determined after 6 hr incubations in [^{3}H,^{14}C]-glucose: the labelling of all other fractions was measured after incubation for 24 hr.

types of experiment and the results of all the experiments with peas are treated together. We could not supply the isotopes to pea roots under aseptic conditions. However, the similarity of the data for peas and those for *Coleus* and artichoke, and the fact that lignin is not a microbial product, strongly suggest that the data for peas represent the metabolism of the pea roots.

At the end of the incubations of the plant material in the [^{3}H,^{14}C]-glucose, the tissues were rinsed thoroughly to remove isotope from the free space. Uptake of isotope was estimated by measuring the disappearance of label from the incubation medium. These estimates varied between experiments but were within the ranges 60–80%, 15–20%, and 1–3% of the label supplied for artichoke, *Coleus*, and pea tissue, respectively. In the experiments with explants of *Coleus* and artichoke the labelling of the lignin preparations and the aromatic aldehydes obtained from lignin was appreciable. In pea roots these fractions were much less heavily labelled but the amounts of ^{3}H and ^{14}C present were high enough to be counted accurately. Data from typical experiments for each tissue are given in Table 1 which also shows the extent to which the water-soluble components of the tissues became labelled in rather shorter incubations. The latter data indicate that the labelling of the lignin fractions was significant in relation to the total amount of labelled glucose metabolized. We repeated each feeding experiment three to six times with separately grown batches of plants. The absolute amount of ^{3}H and ^{14}C recovered in the lignin fractions showed some variation with different batches of the same tissue but the ^{3}H/^{14}C ratios were quite similar (Table 2). In fact the ^{3}H/^{14}C ratios in any particular lignin fraction were basically similar in all three tissues. This suggests that the labelling patterns which we found are characteristic of lignifying tissues in general and allows us to discuss our data from all three tissues collectively.

The difficulties involved in the isolation of lignin demand caution in the interpretation of the labelling of the lignin preparations. Nevertheless, the similarity of the labelling of these fractions from the three tissues, and the evidence that both lignothioglycollic acid and milled-wood lignin represent uncontaminated lignin material [13], indicate that the labelling of these preparations represents lignin synthesis. In addition, our arguments do not rest on the labelling of the lignin fractions alone but also on that of the aromatic aldehydes which we isolated from the lignin. In all of our experiments there was appreciable labelling of these aldehydes and the lignin preparations by [3-^{3}H]-glucose. This labelling is comparable to that achieved by [^{14}C]-glucose and thus is unlikely to have been due to the transfer of ^{3}H to water in glycolysis. We suggest that the data in this paper, together with our previous evidence of a close association between lignification and the pentose phosphate pathway [1, 2], provide strong evidence that much of the reducing power for lignin synthesis comes from that pathway. Comparison of the ^{3}H/^{14}C ratios found in our

Table 2. ^{3}H/^{14}C ratios in lignin from tissues supplied with [^{3}H,^{14}C]-glucose

Tissue	Position of label in [^{3}H,^{14}C]-glucose supplied ^{3}H	^{14}C	^{3}H/^{14}C ratios in Syringaldehyde	Vanillin	*p*-Hydroxybenzaldehyde	Lignothioglycollic acid	Milled-wood lignin
Coleus explant*	3	U	0.45	0.39	0.35	0.53	—
	3	3	0.34	0.38	0.34	0.52	—
Jerusalem artichoke explant†	3	U	0.35 ± 0.05 (3)	0.32 ± 0.03 (3)	0.27 ± 0.02 (3)	0.45 ± 0.08 (3)	—
Pea roots†	3	3	—	0.28 ± 0.05 (5)	0.25 ± 0.02 (5)	—	0.64 ± 0.07 (6)

* Values are means of data from two experiments.
† Values are means ± S.E. of data from the number of experiments shown in parenthesis.
Pea roots incubated in [^{3}H,^{14}C]-glucose for 6 hr, other tissues for 24 hr.

experiments, with those predicted on the hypothesis that all of the reducing power for lignin synthesis comes from the pentose phosphate pathway, shows that the observed ratios are lower than the predicted ratios. This may be due to the provision of some of the reducing power by a different pathway or to discrimination against NADP^{3}H in lignin synthesis. The complexities of the pathways involved and the difficulties of interpreting labelling patterns obtained with [^{3}H]-substrates [7] preclude quantitative assessment from our data of the contribution of the pentose phosphate pathway to lignin synthesis. However, at a qualitative level, we think that there is enough evidence in this and our previous papers [1, 2] to support the view that one of the functions of the pentose phosphate pathway in plants is to provide a substantial amount of the NADPH used in lignin synthesis.

EXPERIMENTAL

Materials. [3-^{3}H]- and [U-^{14}C]-glucose came from the Radiochemical Centre, Amersham, U.K. and [3-^{14}C]-glucose from N.E.N. Chemicals, GmbH, Dreieichenhain, Germany. The third internodes of stems of 5- to 9-week-old plants of *Coleus blumei* Benth. were excised, sterilised and cut transversely into 1 mm slices. Samples of 5 slices were incubated for 4 days in boiling tubes in the dark at 25° on 2% sucrose, 1% agar and 0.05 mg/l IAA. Disks (8 × 1 mm) were cut aseptically from the storage tissue of Jerusalem artichoke (*Helianthus tuberosus* L.) and samples of 5–10 disks were incubated in the dark at 25° on 3% sucrose and 1% agar supplemented with 5 mg/l IAA, 0.1 mg/l zeatin and 1.0 mg/l gibberellic acid, A_3. Seedlings of *Pisum sativum* L. var Kelvedon Wonder were grown for 5 days and dissected to yield steles as described previously [14].

Metabolism of [^{3}H,^{14}C]*-glucose. Coleus* explants, which had been allowed to lignify for 4 days, were harvested and pooled to give samples of 2.0 g fr. wt which were then incubated aseptically for 24 hr at 25° in the dark in 5.0 ml 0.02 M KH_2PO_4 (pH 5.2) which contained 20 μCi [3-^{3}H]-glucose and either 5 μCi [3-^{14}C]- or 10 μCi [U-^{14}C]-glucose at 0.5 mM. We treated the artichoke explants in the same way. Samples of 10 5-day-old pea seedlings were placed in a compartmented box so that the region of the roots 6–46 mm from the apices lay in a separate compartment to which we added 25 ml 0.02 M KH_2PO_4 (pH 5.2) which contained 15 μCi [3-^{3}H]-glucose and 3 μCi [3-^{14}C]-glucose at 0.5 mM [15]. Each root was aerated throughout the incubation which was for 6–12 hr at 18–22°. Sample of steles, fr. wt 400 mg, from the regions 6–46 mm from the apices of similar roots were incubated for 6 hr in 10 ml 0.02 M KH_2PO_4 (pH 5.2) which contained 17 μCi [3-^{3}H]-glucose and 3 μCi [3-^{14}C]-glucose at 0.5 mM. At the end of the incubations of the *Coleus* and artichoke explants in the labelled glucose solutions samples of these solutions were withdrawn and incubated on nutrient agar at 37° for 7 days. All the data for *Coleus* and artichoke reported in this paper were obtained from samples which showed no sign of microbial contamination after the above test.

Analytical techniques. At the end of the incubation in labelled glucose all samples were rinsed × 3, each time for 2 min, with successive 5 ml portions of water. For measurement of the labelling of the water-soluble substances, tissue, after 6 hr incubation in labelled glucose, was killed and extracted in boiling 80% aq. EtOH. For study of the labelling of the aromatic aldehydes, tissue, after 6–24 hr incubation, was killed in boiling MeOH, and then extracted by refluxing, successively, with 25 ml portions of MeOH (× 2), Et_2O (× 2), and 50% aq. MeOH (× 2), each extraction lasting for 1 hr. The extractive-free material was then dried and samples of 50 mg were added to 0.06 ml nitrobenzene and 1.0 ml 0.02 N NaOH in a 2 ml chamber in a stainless steel bomb which was then shaken at 160 ± 15° in an oil bath for 2.5 hr [16]. The products of the oxidation were treated as described by El-Basyouni *et al.* [17], and the aromatic aldehydes were isolated by PC in the organic phase of *n*-BuOH–NH_3–H_2O (4:1:5), and located with 0.4% (w/v) 2,4-dinitrophenylhydrazine in 2 N HCl. Milled-wood lignin was obtained by complete homogenization of the tissue in 2 volumes of citrate–phosphate buffer (0.165 M Na_2HPO_4–0.018 M citric acid), pH 7.5. The cell-wall material was sedimented by centrifuging at 800 *g* for 30 sec and was then re-suspended in 10 ml H_2O. This cycle of washing and sedimentation was repeated with 15 separate 10 ml lots of H_2O. Protein was removed from the washed cell-wall material by incubation in 10 ml 10 mM glycylglycine buffer, pH 7.5, which contained 200 μg/ml pronase, for 24 hr at 18–22°. The insoluble residue was next washed × 5 with H_2O, as described above, to remove amino acids and once with EtOAc before being extracted with dioxan–water (9:1) for 18–24 hr at 18–22°. Carbohydrate was removed from the dioxan extract by the dropwise addition of C_6H_6 in the presence of Al_2O_3. Finally the extract was filtered and the filtrate was freeze-dried to give what is referred to as milled-wood lignin. We used the method of Freudenberg *et al.* [3] to prepare lignothioglycollic acid. Tissue was thoroughly homogenized in Me_2CO–H_2O (17:3) and the homogenate allowed to stand for 48 hr before centrifugation at 800 *g* for 30 sec. The ppt was then washed × 10, each with 25 ml Me_2CO–H_2O (17:3), dried over $CaCl_2$ *in vacuo*, and then incubated for 3 days at 18–22° with 25 ml thioglycollic acid to which 1.5 ml BF_3 (48% w/v) in Et_2O had been added. The insoluble material was then sedimented at 800 *g*, washed × 3 with H_2O, and incubated for 2 days at 18–22° in 25 ml 2 N NaOH. Next the preparation was centrifuged at 800 *g* and the supernatant and two washings of the pellet with H_2O were collected and amalgamated to give the soln from which the lignothioglycollic acid was pptd by the addition of 4 N HCl. This ppt. was washed with H_2O, dried over $CaCl_2$ and is referred to as lignothioglycollic acid. Analyses of the preparations of lignothioglycollic acid by Kjedahl method failed to reveal the presence of N. Radioactivity was assayed by scintillation spectrometry. The scintillation fluid has been described [2]. The efficiencies for counting ^{3}H and ^{14}C in the same sample were determined according to Price [18] and were 10–25% and 30–50%, respectively

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