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THE PHENOLIC ACIDS IN WHEAT—III.

INSOLUBLE DERIVATIVES OF PHENOLIC CINNAMIC ACIDS AS NATURAL INTERMEDIATES IN LIGNIN BIOSYNTHESIS*

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Abstract—^{14}C-Labeled compounds were administered to wheat shoots and their incorporation into lignin and into *p*-coumaric, ferulic and sinapic acids was measured. These phenolic cinnamic acids were not present as such but were obtained by alkaline hydrolysis of both the ethanol extract and the insoluble residue. Measurement of the incorporation into lignin was based on alkaline nitrobenzene oxidation of the residue to vanillin, syringaldehyde and *p*-hydroxybenzaldehyde. Labeled carbon dioxide, phenylalanine or tyrosine was incorporated more readily into the phenolic cinnamic acids bound to the insoluble residue than into ethanol soluble derivatives whereas the reverse was true for labeled precursors such as cinnamic, *p*-coumaric, caffeic, ferulic, and sinapic acids. Time-course studies with labeled carbon dioxide suggested the sequence: carbon dioxide →aromatic amino acids→ethanol-insoluble derivatives of the phenolic cinnamic acids→lignin. Soluble derivatives of the phenolic cinnamic acids appear to exchange with the insoluble derivatives and to act as intermediates in lignification especially when the free acids are administered. A scheme is suggested to explain these complex relationships.

INTRODUCTION

IT IS well known that hydroxycinnamic acids, such as *p*-coumaric, caffeic, ferulic and sinapic acids are widely distributed in vascular plants, where they generally are found as esters rather than as free acids.[1] Esterification may occur with the aliphatic hydroxyls of a number of compounds such as glucose, quinic acid or anthocyanins. There are several unidentified esters of these acids in ethanol extracts of wheat plants. In addition, it has been shown that relatively large amounts of hydroxycinnamic acids, particularly *p*-coumaric and ferulic acids, are released on alkaline hydrolysis of the insoluble residue remaining from an ethanol extraction of wheat shoots.[2] Since the hydrolysis can be accomplished by N sodium hydroxide at 30° it may be inferred that these insoluble derivatives are esters rather than phenolic glycosides although other types of linkage (e.g., acid anhydride, thio-ester) are possible. For ease of reference in the ensuing discussion the terms "soluble esters" and "insoluble esters" will be used, respectively, for ethanol-soluble or ethanol-insoluble compounds which release hydroxycinnamic acids on alkaline hydrolysis.

Cinnamic acid and the hydroxycinnamic acids mentioned above have been implicated as intermediates in the biosynthesis of lignin by a number of tracer and enzyme studies, but there are reasons for believing that the free acids are not the natural intermediates.[3] The general occurrence of these acids as a variety of esters suggests that intermediates with activated carboxyl groups must exist. It is also necessary to postulate such intermediates to explain the reduction of a carboxyl group which almost certainly occurs as a step in the biosynthesis of

* N.R.C. No. 8109.

[1] E. C. BATE-SMITH, in *Wood Extractives* (Edited by W. E. HILLIS), p. 136, Academic Press, N.Y. (1962).

[2] S. EL-BASYOUNI and G. H. N. TOWERS, *Can. J. Biochem.* **42**, 203 (1964).

[3] A. C. NEISH, in *Formation of Wood in Forest Trees* (Edited by M. ZIMMERMAN), pp. 219–39, Academic Press, N.Y. (1964).

lignin. By biochemical analogy, e.g. formation of phosphoglyceraldehyde[4] or mevalonolactone,[5,6] these intermediates might be expected to be coenzyme-A esters, enzyme thio-esters or phosphoric acid anhydrides involving the carboxyl group of the cinnamic acid derivative. Levy and Zucker[7] have evidence that the quinates of these acids can act as intermediates but these esters are not ubiquitous in their distribution in vascular plants and may be side-products formed from more active intermediates.

Previous work on the insoluble esters of the hydroxycinnamic acids of wheat showed changes during development which suggest that these esters are not inert storage products but may be active intermediates in phenylpropanoid metabolism.[2] This view has been confirmed by the tracer experiments reported below which indicate that certain insoluble esters may be more directly involved than soluble esters in lignin biosynthesis.

RESULTS

In the first experiment labeled precursors were fed to 8-day-old wheat seedlings and incorporation into lignin and the hydroxycinnamic acids was measured after a metabolic

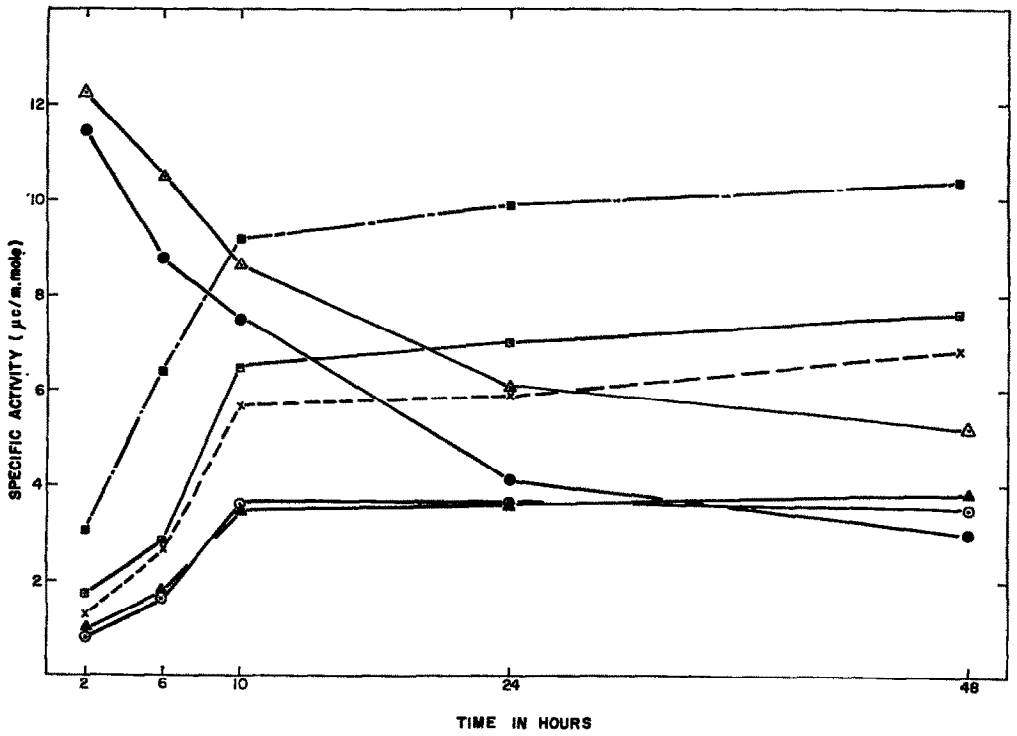

FIG. 1. CHANGES WITH TIME IN SPECIFIC ACTIVITIES OF HYDROXYCINNAMIC ACIDS AND LIGNIN ALDEHYDES AFTER PHOTOSYNTHESIS IN $^{14}CO_2$.

△, ferulic acid (insoluble esters); ⊡, ferulic acid (soluble esters); ●, *p*-coumaric acid (insoluble esters); ⊙, *p*-coumaric acid (soluble esters); ▲, sinapic acid (soluble esters); ×, vanillin; ■, syringaldehyde.

[4] E. RACKER, *Physiol. Rev.* **35**, 1 (1955).
[5] J. J. FERGUSON, I. F. OURR and H. RUDNEY, *Proc. Nat. Acad. Sci. U.S.* **45**, 499 (1959).
[6] F. LYNEN, J. KNAPPE, H. EGGERER, U. HENNING and B. W. AGRANOFF, *Fed. Proc.* **18**, 278 (1959).
[7] C. C. LEVY and M. ZUCKER, *J. Biol. Chem.* **235**, 2418 (1960).

TABLE 1. PERCENT CONVERSION OF $^{14}CO_2$, IN PHOTOSYNTHESIS, AND OF CARBON-14 LABELED PHENYLPROPANOID COMPOUNDS TO HYDROXYCINNAMIC ACIDS AND LIGNIN ALDEHYDES IN WHEAT*

Compound administered Dose = 5 mg of each acid per set of plants)	Percent converted into								
	p-Coumaric acid		Ferulic acid		Sinapic acid		Lignin aldehydes		
	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters	*p*-Hydroxy-benzalde-hyde	Vanillin	Syring-aldehyde
CO_2 (60 μc)	0·016	0·05	0·06	0·24	0·004	0·016	0·17	0·57	0·14
L-Phenylalanine-U-^{14}C (5 μc)	0·125	0·40	0·55	2·40	0·06	0·28	1·90	3·10	1·30
L-Tyrosine-1-^{14}C (5 μc)	0·116	0·54	0·28	2·60	0·03	0·30	ND†	ND	ND
Cinnamic-3-^{14}C (3·1 μc)	5·60	0·46	8·70	2·40	0·49	0·34	5·00	7·00	2·30
p-Coumaric-3-^{14}C (0·96 μc)	4·40	0·39	1·90	1·60	0·18	0·05	8·10	6·10	2·40
Caffeic-3-^{14}C (1·1 μc)	0·13	0·06	0·60	0·82	0·15	0·03	0·80	3·60	1·40
Ferulic-3-^{14}C (2·2 μc)	0·08	0·05	3·80	1·80	0·17	0·06	0·60	12·80	1·50
Sinapic-3-^{14}C (1·4 μc)	0·79	0·15	0·67	0·29	2·87	1·33	2·29	2·20	7·10

* See Experiment I under Experimental.
† Not determined.

TABLE 2. DILUTION VALUES OF HYDROXYCINNAMIC ACIDS AND LIGNIN ALDEHYDES CALCULATED FROM DATA OF EXPERIMENT I*

$$\text{Dilution value} = \frac{\text{Specific activity of compound administered}}{\text{Specific activity of compound isolated}}$$

Compound administered and its specific activity (μc/mmole)	*p*-Coumaric acid		Ferulic acid		Sinapic acid		Lignin aldehydes		
	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters	*p*-Hydroxybenzaldehyde	Vanillin	Syringaldehyde
$^{14}CO_2$ (800)	340	135	120	35·5	222	172	200	141	75·6
L-Phenylalanine-U-^{14}C (165)	135	53	52·7	15·9	86·4	48·8	55·7	84·6	18·4
L-Tyrosine-1-^{14}C (181)	130	45·8	72·4	15·4	165	43·9	ND†	ND	ND
Cinnamic-3-^{14}C (92·2)	5·7	22·5	3·3	8·3	10·5	22·8	17·7	29·6	9·6
p-Coumaric-3-^{14}C (31·5)	7	28	12·8	19·8	30·3	97·2	9·6	38	12·4
Caffeic-3-^{14}C (39·6)	188	440	42·6	46·6	35·4	264	116	66	21·5
Ferulic-3-^{14}C (87·1)	335	622	6	13·4	13·1	158	155	21·4	27
Sinapic-3-^{14}C (63·5)	45·7	235	33·4	117	1·1	9·34	53·8	122	7·3

* See Table 1 and Experimental
† Not determined.

period of 24 hr (Tables 1 and 2). It is noteworthy that $^{14}CO_2$ and the aromatic amino acids were incorporated more readily into the insoluble esters of the hydroxycinnamic acids than into the soluble esters whereas the reverse was true for precursors such as cinnamic, *p*-coumaric, caffeic, ferulic and sinapic acids. The data confirm previous observations that the aromatic amino acids and cinnamic acid derivatives can be readily incorporated into lignin. In this experiment there was an especially active synthesis of "syringyl lignin", judging from the low dilution values for syringaldehyde in Table 2. Tyrosine and phenylalanine were equally good in the production of *p*-coumaric and ferulic acids. Sinapic acid was converted to ferulic acid and even to *p*-coumaric acid with low enough dilution to suggest that this

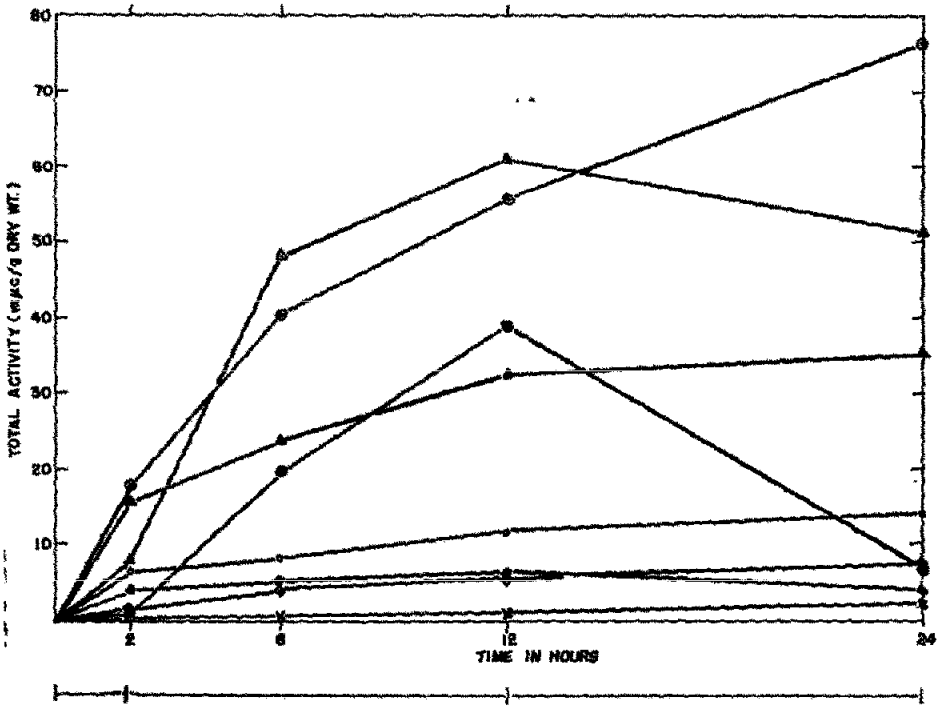

FIG 2. CHANGES WITH TIME IN TOTAL ACTIVITIES OF HYDROXYCINAMIC ACIDS AND LIGNIN ALDEHYDES AFTER ADMINISTRATION OF CINNAMIC ACID-β-^{14}C.

○, ferulic acid (insoluble esters); △, ferulic acid (soluble esters); ◆, *p*-coumaric acid (insoluble esters); ●, *p*-coumaric acid (soluble esters); ×, sinapic acid (soluble esters); ▲, vanillin; ○, *p*-hydroxybenzaldehyde; ◇, syringaldehyde.

might have occurred by removal of one or two methoxyl groups respectively, rather than by conversion through carbohydrate. There have been previous reports that sinapic acid can give rise to guaiacyl derivatives in plants.[8,9] Caffeic acid and ferulic acid were not converted to *p*-coumaric acid as readily as was sinapic acid.

The second experiment (Fig. 1 and Table 3) was carried out with intact wheat seedlings which were allowed to assimilate $^{14}CO_2$ photosynthetically for 2 hr and were then allowed to metabolize under normal physiological conditions for periods up to 48 hr. The amounts of

[8] T. HIGUCHI and S. A. BROWN, *Can. J. Biochem. Physiol.* **41**, 613 (1963).
[9] S. Z. EL-BASYOUNI, D. CHEN, R. K. IBRAHIM, A. C. NEISH and G. H. N. TOWERS, *Phytochem.* **3**, 485 (1964).

TABLE 3. TOTAL AMOUNT (μMOLE/G DRY WT.) AND TOTAL ACTIVITY (mμC/G DRY WT.) OF HYDROXYCINNAMIC ACIDS ISOLATED AFTER ADMINISTRATION OF $^{14}CO_2$*

Metabolic period after administration of $^{14}CO_2$ in photosynthesis (hr)	*p*-Coumaric acid				Ferulic acid				Sinapic acid	
	Soluble esters		Insoluble esters		Soluble esters		Insoluble esters		Soluble esters	
	Amount μm	Total act. mμc	Amount μm	Total act. mμc	Amount μm	Total act. mμc	Amount μm	Total act. mμc	Amount μm	Total act. mμc
2	6	4·86	6·99	80·1	6·49	11·55	20	244·4	1·16	1·18
6	6·56	10·76	6·91	60·8	6·80	19·58	19·79	208·2	1·34	2·45
10	6·11	22·30	7·08	53·2	6·58	42·77	21·44	185·2	1·30	4·56
24	5·69	21·11	6·41	26·7	6·08	42·86	17·99	109·7	1·19	4·32
48	5·27	17·13	6·59	19·8	5·69	43·24	18·35	95·4	1·10	4·22

* See Experiment II under Experimental.

TABLE 4. TOTAL AMOUNT (μMOLE/G DRY WT.) AND SPECIFIC ACTIVITY (μC/MMOLE) OF HYDROXYCINNAMIC ACIDS AND LIGNIN ALDEHYDES ISOLATED FROM WHEAT SHOOTS ADMINISTERED CINNAMIC ACID-β-^{14}C*

Metabolic period after administration of cinnamic acid-3-^{14}C (hr)	*p*-Coumaric acid				Ferulic acid				Sinapic acid		*p*-hydroxybenzaldehyde		Vanillin		Syringaldehyde	
	Soluble esters		Insoluble esters		Soluble esters		Insoluble esters		Soluble esters							
	Amount μm	Specific activity	Amount μm	Specific activity	Amount μmole	Specific activity	Amount μm	Specific activity	Amount μm	Specific activity	Amount μm	Specific activity	Amount μm	Specific activity	Amount μm	Specific activity
2	0·48	<1	8·80	0·45	0·97	7·79	28·41	0·634	Nil	Nil	23·80	0·27	58·95	0·27	10·4	0·15
6	1·18	15·95	8·66	0·63	4·01	12·06	33·91	1·20	traces	<1	24·90	0·33	59·47	0·40	10·5	0·40
12	1·26	31·01	10·76	0·64	4·24	14·66	33·22	1·69	0·75	1·73	25·20	0·46	59·87	0·55	11·3	0·52
24	1·11	6·02	7·49	0·53	5·38	9·31	35·97	2·12	1·55	1·49	26·10	0·53	60·92	0·58	12	0·59

* See Experiment III under Experimental.

^{14}C in lignin and soluble and insoluble esters of the hydroxycinnamic acids were measured periodically. It was found that insoluble esters of *p*-coumaric and ferulic acids were labeled quickly and then gradually lost isotopic carbon as it was incorporated into the soluble esters and lignin. In other words, the insoluble esters acted kinetically as if they were precursors of lignin and the soluble esters.

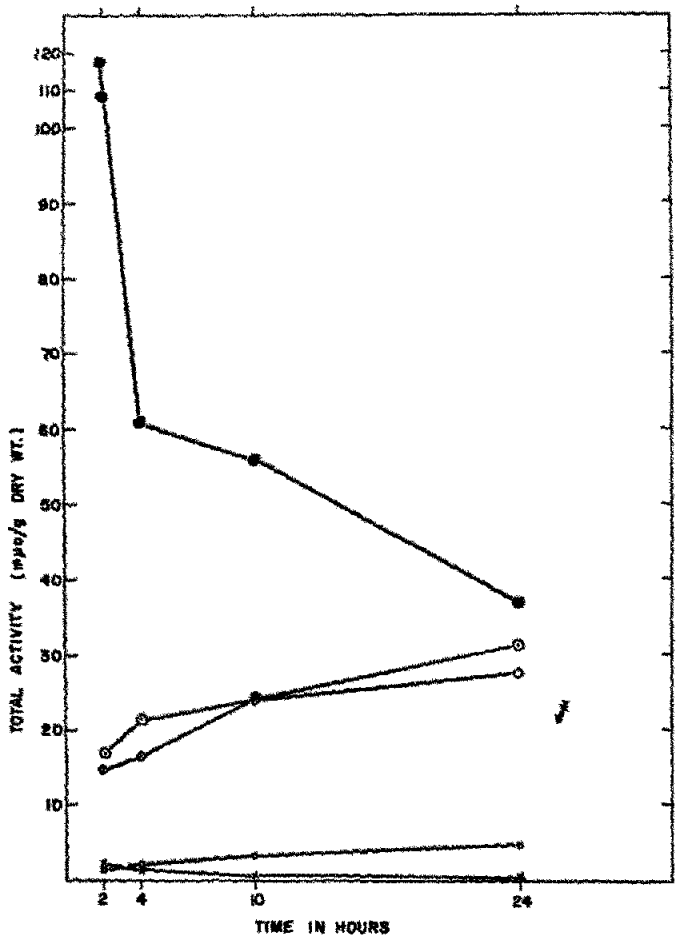

FIG. 3. CHANGES WITH TIME IN TOTAL ACTIVITIES OF HYDROXYCINNAMIC ACIDS AND LIGNIN ALDEHYDES AFTER ADMINISTRATION OF FERULIC ACID-β-^{14}C.

ϕ, ferulic acid (insoluble esters); ●, ferulic acid (soluble esters); ×, sinapic acid (soluble esters); ○, vanillin; ○, syringaldehyde.

A third series of experiments was carried out with shoots cut from 35-day-old wheat plants. Sodium cinnamate-β-^{14}C was fed through the cut ends during a 2 hr period, then the shoots were transferred to a solution of unlabeled sodium cinnamate for 8 hr and then to distilled water. Changes in the total radioactivity (Fig. 2) as well as in the specific activity (Table 4) of various fractions were measured at intervals. There was a gradual incorporation of ^{14}C into lignin but the most striking result was the marked incorporation into insoluble esters of ferulic acid and soluble esters of *p*-coumaric and ferulic acids. The results differed from the experiment with $^{14}CO_2$ in that here it is the soluble esters which appeared to act most like

intermediates. A similar series of experiments was conducted with sodium ferulate-β-^{14}C (Fig. 3 and Table 5) which also showed a rapid initial increase in soluble derivatives of ferulic acid although these results may have included the free acid. The insoluble esters of ferulic acid, however, did not act like intermediates and lignin synthesis must have occurred mainly at the expense of soluble precursors.

DISCUSSION

Evidently there is a complex relationship between intermediates of lignin biosynthesis and the soluble and insoluble esters of hydroxycinnamic acids. The experiments with $^{14}CO_2$ (Fig. 2), which represent the closest approach to normal physiological conditions, strongly suggest that the intermediates in lignin biosynthesis are esters of the hydroxycinnamic acids which are not soluble in ethanol. This would appear to eliminate the free acids or quinic acid esters as important intermediates under natural conditions. However, when the free acids are administered they appear to be incorporated into lignin by way of soluble esters. This difference is probably not due to the method of administering the precursors since phenylalanine

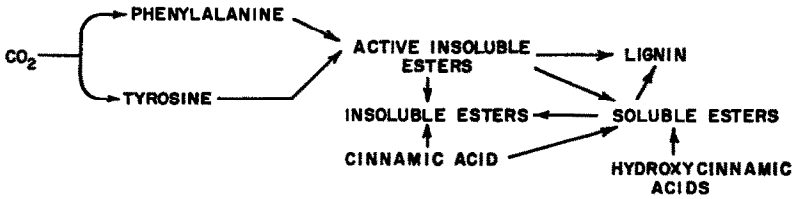

FIG. 4. HYPOTHETICAL OUTLINE OF LIGNIN BIOSYNTHESIS.

and tyrosine, although fed in the same way as the cinnamic acid derivatives, acted like carbon dioxide in labeling the insoluble esters more than the soluble esters (Table 1). This is good evidence that these amino acids are natural precursors of lignin in wheat.

Fig. 4 outlines a working hypothesis which is an attempt to explain these results in the simplest way possible. Under natural conditions plants assimilate carbon dioxide by photosynthesis, and use carbohydrate thus obtained for synthesis of phenylalanine and tyrosine by the shikimic acid pathway. In addition to contributing to protein synthesis these amino acids can undergo loss of nitrogen to form the corresponding cinnamic acid derivatives. It has been demonstrated that plants contain enzymes which can convert L-phenylalanine to cinnamic acid[10] or L-tyrosine to *p*-coumaric acid[11] *in vitro*. It is possible that in the environment of the living cell these acids are not released from the enzymes but undergo further reactions, which eventually lead to formation of lignin, without ever existing as free acids. The formation of the free acids in the *in vitro* systems may be due to high pH ($\sim$ 9) or to the presence of esterases. It can be visualized that an enzyme-ester of a hydroxycinnamic acid would be insoluble in hot 80% ethanol and this may be the nature of the active 'insoluble esters' of Fig. 4. A considerable amount of hydroxycinnamic acids is obtained by alkaline hydrolysis of the ethanol-insoluble fraction and it is not suggested that this is all bound to enzymes. Compounds, such as the ferulic ester of xylan,[12] may be present in greater amounts but need not be regarded as active intermediates. Obviously more information is needed

[10] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).
[11] A. C. NEISH, *Phytochem.* **1**, 1 (1961).
[12] H. FAUSCH, W. KÜNDIG and H. NEUKOM, *Nature* **199**, 287 (1963).

TABLE 5. TOTAL AMOUNT (μMOLE/G DRY WT.) AND SPECIFIC ACTIVITY (μC/MMOLE) OF HYDROXYCINNAMIC ACIDS AND LIGNIN ALDEHYDES ISOLATED FROM WHEAT SHOOTS ADMINISTERED FERULIC ACID-3-^{14}C. (SEE EXP. III UNDER EXPERIMENTAL)

Metabolic period after administration of ferulic acid-3-^{14}C (hr)	Ferulic acid				Sinapic acid		Vanillin		Syringaldehyde	
	Soluble esters		Insoluble esters		Soluble esters					
	Amount μm	Specific activity	Amount μm	Specific activity	Amount μm	Specific activity	Amount μm	Specific activity	Amount μm	Specific activity
2	6·48	16·75	30·13	0·49	1·75	1·34	60·66	0·28	12·1	0·14
4	5·32	11·87	29·43	0·57	1·53	0·90	61·45	0·35	12·7	0·16
10	5·19	10·83	28·87	0·84	1·47	0·50	61·18	0·39	13·1	0·26
24	4·81	9·18	26·94	1·02	1·07	0·49	65·66	0·48	16·5	0·30

concerning the exact nature of the "insoluble esters" of the hydroxycinnamic acids before this data can be interpreted fully.

When salts of cinnamic acid or the hydroxycinnamic acids are fed there is apparently an activation of the carboxyl group leading to formation of soluble esters from which lignin can be synthesized. There is also a slow exchange between the soluble and insoluble esters but the data suggest (Figs. 2, 3) that the insoluble esters formed here are not active intermediates in lignin biosynthesis. Apparently wheat plants are able to incorporate hydroxycinnamic acids into lignin by at least two routes but it has not been established whether the lignins formed by these routes are identical. Under natural conditions the route involving ethanol-insoluble intermediates seems to be the more important.

EXPERIMENTAL

Plant Material

Kharkov wheat (*Triticum aestivum* L. emend Thell. spp. *vulgare* var. Kharkov 22 M.C.) was used exclusively and plants were germinated as described previously.[2,11]

Radioactive Compounds

L-Phenylalanine-U-^{14}C, L-tyrosine-carboxyl-^{14}C and $Na_2^{14}CO_3$ were purchased from Merck, Sharpe and Dohme, Montreal. The preparation of cinnamic, *p*-coumaric, caffeic, ferulic and sinapic acids, all labeled in the β-position, has been described.[13,14]

Experiment I

Wheat seedlings, 8 days old, were obtained from 14 trays germinated at the same time. Uniform plants with an average height of 6 in. were selected. Shoots were cut under water and randomly grouped into 8 sets, each consisting of 200 shoots. One set was allowed to photosynthesize in the presence of $^{14}CO_2$, while to each of the other sets a ^{14}C-labeled phenylpropanoid compound was administered.

Photosynthesis with $^{14}CO_2$. Shoots were held in a vial with their cut ends immersed in water and placed in a photosynthetic chamber (Fig. 5). A partial vacuum was created in the chamber and $^{14}CO_2$ was generated by the action of 2 N HCl on a measured amount of $Na_2^{14}CO_3$ solution. The internal pressure was equalized with CO_2-free air through the $^{14}CO_2$ generator. Plants were allowed to metabolize, in the presence of $^{14}CO_2$, for 5 hr after which the remaining gas was flushed out with air and further metabolism in $^{12}CO_2$ was allowed for 17 hr, which brought the total metabolic period to 24 hr. Continuous light was provided by means of a bank of incandescent lamps giving an average light intensity of 1200 ft-candles at the outer surface of the chamber.

Administration of Phenylpropanoid Compounds

L-Phenylalanine-U-^{14}C and L-tyrosine-carboxyl-^{14}C were administered as their aqueous solutions. Members of the cinnamic acid series were administered as their sodium salts in aqueous solutions of neutral pH. Shoots were held in small vials containing 5 mg of the radioactive compound in 10 ml water. Amounts of radioactivity are given in Tables 1 and 2. They were allowed to metabolize in light (1200 ft-candles) for 24 hr during which time the solutions were completely absorbed. Small volumes of water (3 ml) were added subsequently to prevent desiccation of the shoots. At the end of the metabolic period, the plants were washed with distilled water and analysed as described below.

[13] S. A. Brown and A. C. Neish, *Can. J. Biochem. Physiol.* 33, 948 (1955).
[14] S. A. Brown and A. C. Neish, *Can. J. Biochem. Physiol.* 34, 769 (1956).

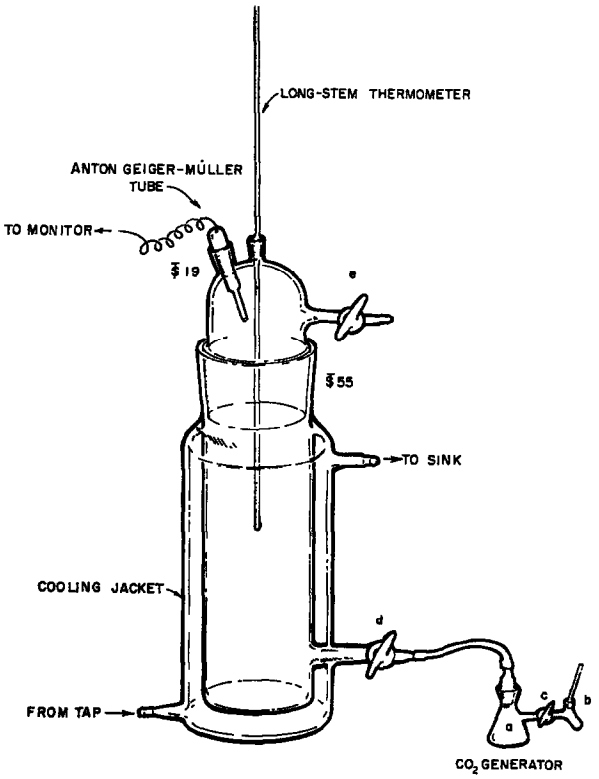

FIG. 5. PHOTOSYNTHETIC CHAMBER.

Experiment II

Five sets, each consisting of 160 8-day-old seedlings, were placed, with their roots completely immersed in Hoagland's nutrient solution in polyethylene containers, in a large vacuum desiccator. $^{14}CO_2$ (1 mc) was generated and introduced into the vacuum desiccator. The plants were allowed to photosynthesize for 2 hr (1200 ft-candles) after which the remaining $^{14}CO_2$ was trapped as $Ba^{14}CO_3$ by repeated cycles of suction-flushing with CO_2-free air and one set of plants was removed for analysis. The remaining sets were allowed to metabolize for further periods in cycles of 18 hr light and 6 hr dark. Sets were removed, one at a time, for analysis, at intervals shown in Fig. 1.

Experiment III

Thirty-five-day-old plants with an average height of 18 in. were used. The shoots were cut under water and divided into 2 equal sets of 48 plants each. Sodium cinnamate-β-^{14}C was administered to one set and sodium ferulate-β-^{14}C to the other through the cut ends of the shoots in the light. The radioactive solutions were completely absorbed within the first 2 hr after which 12 plants from each set were removed for analysis. Subsequent treatment was as follows: The remaining plants of each set, after washing of their immersed ends, were transferred to a non-radioactive solution of the administered compound at the same concentration. Twelve plants from each set were removed for analysis after 2–4 hr and another 12 after 8–10

hr. The remaining 12 plants from each set were transferred to distilled water and analysed after a further 12–14 hr. Plants were maintained in light for the duration of the experiment.

Analyses of Phenolic Acids and Lignin Aldehydes

The plant material was homogenized with hot ethanol in a Waring Blendor and thoroughly extracted with 80% ethanol under reflux on the steam bath. The combined filtered extracts were evaporated to dryness in a rotary evaporator. The residue, thus obtained, was treated with boiling water and filtered hot through a bed of Celite. To the cooled filtrate was added sufficient 10 N NaOH to make a 2 N solution, and, after standing for 14 hr in the refrigerator, the solution was acidified with HCl to pH 4 and continuously extracted with ether for 20 hr. The ether extract was dried *in vacuo* and the residue taken up in a measured volume of ethanol and quantitatively chromatographed for the ethanol soluble phenolic acids. The bound phenolic acids in the ethanol-insoluble residue were obtained by suspending 300 mg of the air-dried ethanol-insoluble cell wall residue in a test tube, in 20 ml of N NaOH at 30° for 4 hr. The suspension was subsequently acidified with conc. HCl and continuously extracted with ether for 20 hr.

Alkaline nitrobenzene oxidations were carried out using the micro method of Stone and Blundell[15] with some modifications. To 300 mg of air-dried ethanol-insoluble residue were added 0·7 ml nitrobenzene and 10 ml 2 N NaOH in a stainless steel bomb. After the mixture was heated at 160° for 2½ hr in a constant temperature paraffin-oil bath, the bomb was cooled in crushed ice and its contents were transferred to a separatory funnel and extracted by shaking with four 500-ml portions of ether. The extracts were discarded and the aqueous layer was acidified and continuously extracted with ether for 20 hr. This ether extract contained the phenolic aldehydes resulting from oxidation of lignin.

Chromatographic separation of phenolic acids was carried out using Whatman No. 1 chromatography grade paper and benzene:acetic acid:water (10:7:3) for the first direction and 2% aqueous formic acid for the second. Phenolic aldehydes were separated using the benzene–acetic acid solvent for the first direction and n-butanol:conc. NH_4OH:water (4:1:5) as the second solvent.

Quantitative determination of the phenolic acids has been described previously.[2] Phenolic aldehydes were eluted from paper chromatograms with absolute ethanol. The filtered eluates were dried and made to 100 ml in absolute ethanol containing 4 ml of an 0·2% ethanolic KOH solution. Optical densities were measured at 354 mμ for vanillin, 370 mμ for syringaldehyde and 336 mμ for *p*-hydroxybenzaldehyde using a Beckman Model DU spectrophotometer. A complete u.v. absorption spectrum was obtained for each sample of phenolic acid or aldehyde eluted from chromatograms and those that deviated from the spectrum of the standard were rejected. For this purpose a Bausch and Lomb Spectronic 505 recording spectrophotometer was used.

Radioactivity measurements were made with a Packard Tri-Carb Series 314A Liquid Scintillation Spectrometer.

[15] J. E. Stone and M. J. Blundell, *Analyt. Chem.* **23**, 771 (1951).