

# Incorporation of Chlorinated Anilines into Lignin

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Lignin was formed *in vitro* by the peroxidase/hydrogen peroxide mediated polymerization of coniferyl alcohol. In the presence of [<sup>14</sup>C]4-chloroaniline or [<sup>14</sup>C]3,4-dichloroaniline copolymerization occurred with incorporation rates of about 45 mol%. Co-elution of UV-absorbing material (reflecting lignin) and of incorporated radioactivity was observed on a calibrated column of Sephadex LH-60. This method indicated a broad molecular weight distribution of the copolymers with values of between about 20000 and 1000. Most of the copolymer products had apparent molecular weights near 1000.

The <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra of the copolymers were compared with those of *in vitro* lignin. The copolymer spectra showed a relative increase in aromatic peaks and a relative decrease in most of the typical lignin peaks. Three peaks indicative of a new type of phenylpropanoid side-chain structure were detected in the <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra of the copolymers. The corresponding <sup>1</sup>H-NMR chemical shift values were 4.75 ppm ( $\alpha$ -C-H), 4.40 ppm ( $\beta$ -C-H) and 3.70/3.50 ppm ( $\gamma$ -C-H<sub>2</sub>). The <sup>13</sup>C-NMR chemical shift values were 56.9 ppm ( $\alpha$ -C), 84.3 ppm ( $\beta$ -C) and 60.0 ppm ( $\gamma$ -C). These peaks were attributed to a 3-aryl-3-anilino-2-aryloxypropanol-1 structure in the copolymers by analysis of coupling patterns and by comparison with spectral reference data.

The NMR-results and experiments with catalase and inhibitors suggested that a major mechanism of copolymerization consisted of a nucleophilic addition of the anilines to the benzylic  $\alpha$ -carbon of lignol quinone-methide intermediates.

Studies on the plant metabolism of pesticides and other environmental chemicals have frequently led to “insoluble” and “bound” metabolite fractions whose chemical characterization has been difficult (reviewed, [1]). In a few cases evidence for their lignin nature has been obtained (reviewed, [2]). Lignin-like “insoluble” metabolite fractions have in particular been described for chlorinated anilines [3–6].

The present study is concerned with the question whether environmental chemicals can be incorporated into lignin in a biochemically defined system. The pioneering studies of Freudenberg [7] have shown that lignin can be made *in vitro* by the action of peroxidase/hydrogen peroxide on coniferyl alcohol or other substituted cinnamyl alcohols. The polymerization was shown to involve radical and quinone-methide intermediates and to proceed by a spontaneous and essentially random mechanism [7].

A copolymerization of environmental chemicals could be of great importance since lignin is the second most abundant natural polymer which

amounts to some 30% of the dry weight of most woody plants.

Benzo[ $\alpha$ ]pyrene quinones which are primary plant metabolites of the ubiquitous carcinogen, benzo[ $\alpha$ ]pyrene [8], have previously been shown to copolymerize into *in vitro* lignin [9]. The biochemical mechanism of incorporation could, however, not be identified.

Chlorinated anilines which are studied here are released into the environment as components and primary metabolites of a number of widely used pesticides and other chemicals [10]. 4-Chloroaniline and 3,4-dichloroaniline were covalently incorporated into *in vitro* lignin and a biochemical mechanism for incorporation was derived from <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectroscopic data.

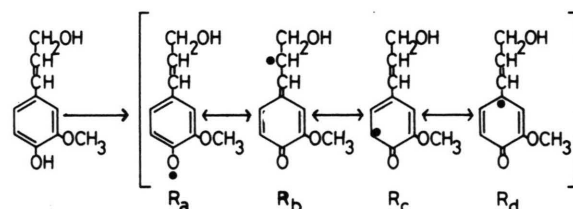


Fig. 1. Mesomeric forms of the radical formed by peroxidase/H<sub>2</sub>O<sub>2</sub> oxidation of coniferyl alcohol. Adopted from [7].

Reprint requests to H. Sandermann.

Enzymes. Catalase, E.C. 1.11.1.6. Peroxidase, E.C. 1.11.1.7.  
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Some of these results have been briefly communicated [11].

## Materials and Methods

### Materials

The [ring- $^{14}\text{C}$ ]-labeled 4-chloroaniline and 3,4-dichloroaniline were purchased from Amersham-Buchler, Braunschweig. Coniferyl alcohol and vanillyl alcohol were obtained from Roth, Karlsruhe, and Fluka, Neu-Ulm, respectively. Other non-radioactive chemicals were of the highest commercially available purity and were purchased from Fluka, Neu-Ulm, Riedel-de-Haen, Seelze, or Aldrich-Europe, Nettetal. Horse radish peroxidase (grade I) and Sephadex LH-60 were obtained from Boehringer, Mannheim, and Pharmacia, Freiburg, respectively.

### Copolymerization of 4-chloroaniline

A published procedure [12] was slightly modified as follows, all steps being performed at room temperature and under red light ( $\lambda \geq 550 \text{ nm}$ ). The aqueous buffer used in all solutions was 10 mM sodium phosphate, pH 7.5, which had been deaerated by vacuum filtration through a  $0.4 \mu\text{m}$  membrane filter and subsequent sparging with  $\text{N}_2$  (1 h). Solutions of a) 1 g (5.55 mmol) coniferyl alcohol and 26.3 mg peroxidase, b) 0.63 ml 30%  $\text{H}_2\text{O}_2$  (5.55 mmol), and c) 708 mg (5.55 mmol, 32  $\mu\text{Ci}$ ) [ $^{14}\text{C}$ ]4-chloroaniline, in a solution volume of 240 ml in each case, were pumped simultaneously and separately over a period of about 1 h into a well-stirred solution of 8.8 mg peroxidase and 17.6 mg (0.11 mmol) vanillyl alcohol in 120 ml buffer (solution d). Solutions a), b) and d) were made up in the above phosphate buffer whereas solution c) was formed from 190 ml buffer plus 50 ml methanol. After an additional stirring and incubation period of 35 min the precipitated product was isolated by low-speed centrifugation, washed four times with deaerated water and lyophilized. The product was then dissolved in dimethylformamide and chromatographed on Sephadex LH-60. Fractions corresponding to molecular weight ranges of 8000 to 22000 (I), 1500 to 8000 (II) and 500 to 1500 (III) were pooled. The total incorporation of 4-chloroaniline into the washed product was 32.5% (w/w), equivalent to 40 mol%, as calculated from the experimentally determined specific radioactivity.

### Copolymerization of 3,4-dichloroaniline

The procedure described for 4-chloroaniline was used employing 899 mg (5.55 mmol, 40.5  $\mu\text{Ci}$ ) [ $^{14}\text{C}$ ]3,4-dichloroaniline in solution c). Total incorporation in this case was 27.4% (w/w) or 30 mol%.

### Two-step copolymerization of 3,4-dichloroaniline

Six ml of solution a) (140  $\mu\text{mol}$  coniferyl alcohol and 0.7 mg peroxidase) and b) (140  $\mu\text{mol}$   $\text{H}_2\text{O}_2$ ) each were pumped simultaneously and separately into 3 ml of the stirred solution d) (2.8  $\mu\text{mol}$  vanillyl alcohol and 0.2 mg peroxidase) over a time interval of 20 min. After a total reaction time of 30 min the remaining  $\text{H}_2\text{O}_2$  was decomposed by addition of catalase (4000 units, Sigma, St. Louis). Subsequently, 6 ml of solution c) containing 14  $\mu\text{mol}$  of 3,4-dichloroaniline (0.23  $\mu\text{Ci}$ ) were added over a period of 20 min. The mixture was stirred for another 100 min and the precipitated product was further processed as described above.

The effect of inhibitors was tested as follows. Six ml of solutions a) and b) each were added to 3 ml of stirred solution d) within 20 min. The inhibitors (280  $\mu\text{mol}$  ascorbate, dihydroxyfumarate or hydroquinone, respectively, or 140  $\mu\text{mol}$  iodine) were then added at once as solutions or suspensions in  $\leq 1 \text{ ml}$  water. Iodine was dissolved in 1 ml ethanol. The reaction was continued for another 20 min, followed by addition of catalase (405 units). After a total of 45 min 6 ml of solution c) (14  $\mu\text{mol}$  3,4-dichloroaniline, 0.23  $\mu\text{Ci}$ ) was pumped dropwise into the mixture over a period of 20 min. After an additional 85 min of stirring the products were collected and further processed as described above.

## Results

### Lignin preparation

The polymerization of coniferyl alcohol with the aid of horse radish peroxidase/ $\text{H}_2\text{O}_2$  was carried out by a slightly modified literature procedure [12] with addition of the  $^{14}\text{C}$ -labeled chloroanilines to the polymerization mixture. The insoluble reaction product was washed, dissolved in a small amount of dimethylformamide and chromatographed on a column of Sephadex LH-60 in the same solvent. The column could be calibrated with polystyrenes of defined molecular weight (Fig. 2). The *in vitro*

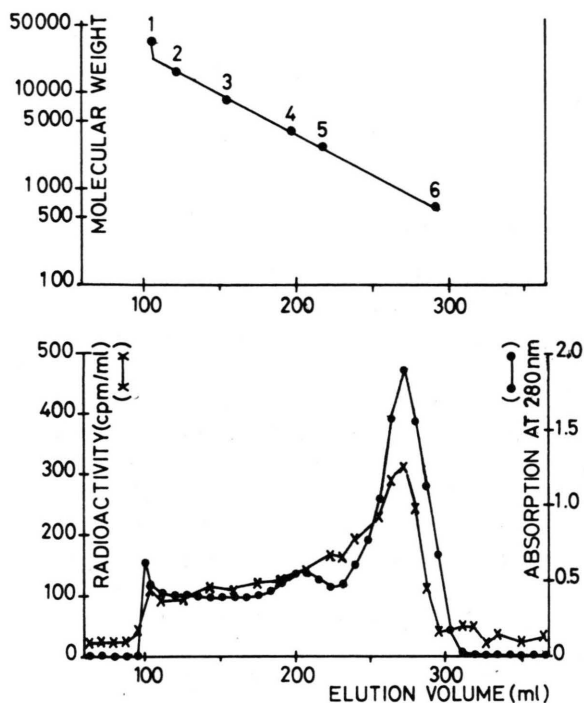


Fig. 2. Gel permeation chromatography on Sephadex LH-60. A column of  $110 \times 2$  cm was used with dimethylformamide as the developing solvent. Upper panel: Calibration curve determined with polystyrenes (Waters Associates) of the following stated molecular weight values, 1. 35000, 2. 17500, 3. 8500, 4. 4000 and 5. 2900. The experimental point no. 6 indicates the position of the detergent, Triton X-100 (molecular weight, about 680). Lower panel: Chromatography of a 3,4-dichloroaniline/lignin copolymer. The absorption at 280 nm ( $\bullet$ — $\bullet$ ) and the radioactivity (cpm/3 ml;  $\times$ — $\times$ ) are plotted against elution volume (ml).

lignin samples were polydisperse but the elution profiles of UV-absorption (reflecting lignin) and of radioactivity (for chloroaniline incorporation) indicated similar molecular weight distributions with maxima near molecular weights of about 1100 in all cases. An elution profile obtained with a 3,4-dichloroaniline copolymer is shown as an example in Fig. 2. Fractions I, II and III were pooled after each column run as described in Materials and Methods. When these fractions were rechromatographed they appeared as defined peaks in the expected molecular weight regions. The further studies were carried out with the low molecular weight fractions III since they contained most of the reaction product and were better soluble in the solvents used for NMR-spectroscopy. The ratios of UV-absorption ( $A_{280}$ ) and of radioactivity indicated that the fractions III contained 50 mol% and 38

mol% of the aniline in the cases of the 4-chloroaniline copolymer and the 3,4-dichloroaniline copolymer, respectively.

#### UV-spectra

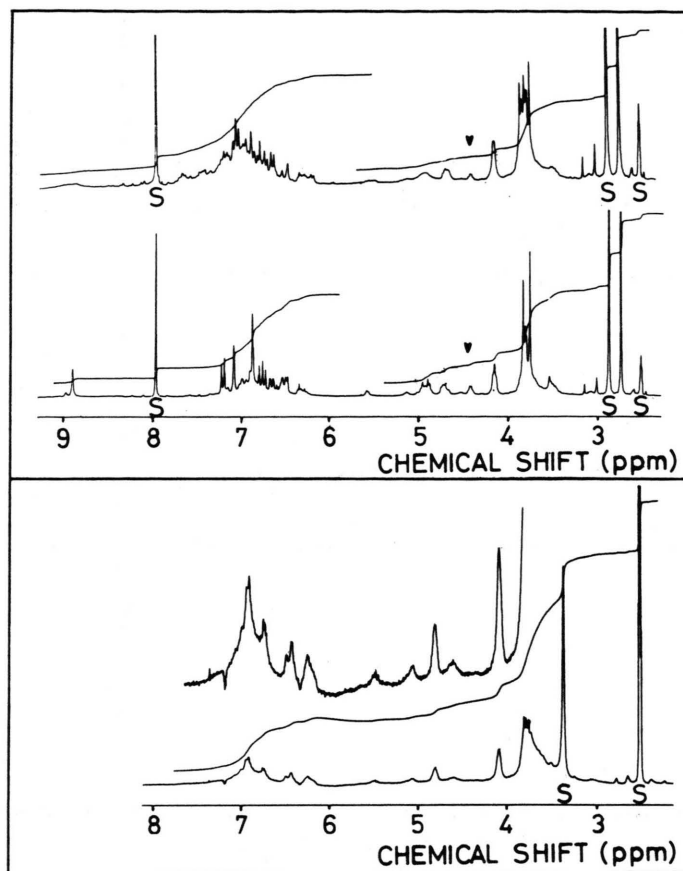
The UV-spectra of fractions III of the 4-chloroaniline and 3,4-dichloroaniline copolymers resembled the previous UV-spectra of lignin and of the benzo[ $\alpha$ ]pyrene quinone copolymer [9] except for an additional absorption shoulder extending up to 500 nm.

#### $^1\text{H}$ -NMR spectra

The  $^1\text{H}$ -NMR spectra of *in vitro* lignin and of the copolymers with 4-chloroaniline and 3,4-dichloroaniline are shown in Fig. 3. The ratios of the integrated peak areas corresponding to aromatic and olefinic hydrogen atoms (7.7 to 6 ppm) and aliphatic hydrogen atoms (mainly  $-\text{OCH}_3$ ; 5.5 to 3.1 ppm) were as follows, 0.46 for unmodified lignin, 1.17 for the 4-chloroaniline copolymer and 0.77 for the 3,4-dichloroaniline copolymer. From the increases in aromatic hydrogen contents incorporation rates of 60 mol% and 40 mol% are calculated for 4-chloroaniline and 3,4-dichloroaniline, respectively. These rough estimates were of the same order as those determined independently from the specific radioactivity values (see above). In addition to the quantitative increase in contents of aromatic hydrogen atoms there was also a considerable spectral complexity in the aromatic region of the copolymer spectra. For example, simple superposition of the lignin and 4-chloroaniline spectra should have led to the known sharp signals at 6.57 and 7.02 ppm due to protons 2/6 and 3/5 of the 4-chloroaniline. Peaks could be discerned near these ppm-values but the general complexity in the aromatic region indicated either non-equivalence of the formerly identical AA'- and BB'-type protons or formation of new aromatic linkages. In the case of 3,4-dichloroaniline, protons 2 (6.76 ppm) and 6 (6.52 ppm) were shifted in the copolymer to 6.89 ppm and 6.72 ppm, respectively, whereas proton 5 remained unchanged at 7.18 ppm. These assignments were supported by decoupling experiments.

A common feature of the  $^1\text{H}$ -NMR-spectra of Fig. 3 was the presence of olefinic propenyl side-chains with typical signals near 6.60 ppm ( $\alpha$ -C-H, d,

Fig. 3.  $^1\text{H}$ -NMR spectra. The upper panel shows the spectra of the 4-chloroaniline copolymer (top) and the 3,4-dichloroaniline copolymer. The spectrum of the reference *in vitro* lignin is shown in the lower panel. The spectra were taken in deuterated dimethylsulfoxide using a Bruker WM 250 instrument at 23 °C with tetramethylsilane as the internal standard. Peaks due to contaminating residual solvents are labelled "S". The copolymer quartet structures discussed in the Results section are marked by arrows.



$J = 16$  Hz), 6.44 ppm ( $\beta$ -C-H, dt,  $J = 16$  and 5 Hz) and 4.15 ppm ( $\gamma$ -C-H<sub>2</sub>, d,  $J = 5$  Hz). These signals were decreased in the copolymer spectra.

Decoupling experiments which are not shown in detail furthermore indicated that the following sequence of coupling protons was present only in the copolymers. The broad quartet structure near 4.40 ppm (marked by arrows in Fig. 3;  $J = 5$ –6 Hz) was due to a  $\beta$ -C-H species which was coupled to an  $\alpha$ -C-H (located at 4.75 ppm; d,  $J = 4.5$  Hz) and two  $\gamma$ -C-H species of AB-type at 3.70 ppm (dd,  $J = 11.5$  and 6.0 Hz) and at 3.50 ppm (dd,  $J = 11.5$  and 5.0 Hz). These various chemical shift values and coupling constants indicated that a 3-aryl-3-anilino-2-aryloxy-propanol-1 structure was present in the copolymers (see below). More detailed interpretations were hampered by the strong solvent dependence of the  $^1\text{H}$ -NMR spectra.

#### $^{13}\text{C}$ -NMR spectra

The  $^{13}\text{C}$ -NMR spectra of the same samples that had been used to obtain the spectra of Fig. 3 are shown in Fig. 4. The comparison of the three spectra showed that the basic features of the spectrum of the *in vitro* lignin were also present in the copolymer spectra. Difference spectra of the copolymer spectra minus the lignin spectrum (normalized to the  $-\text{OCH}_3$  carbon) showed that extra-absorption peaks were present near the signals known for the aniline carbons (not shown). Unassigned additional difference peaks were also present in the aromatic region (105–165 ppm) of the copolymer spectra.

The assignment of the lignin spectral peaks was readily achieved by reference to the extensive  $^{13}\text{C}$ -NMR spectral studies of Nimz *et al.* [13–15].



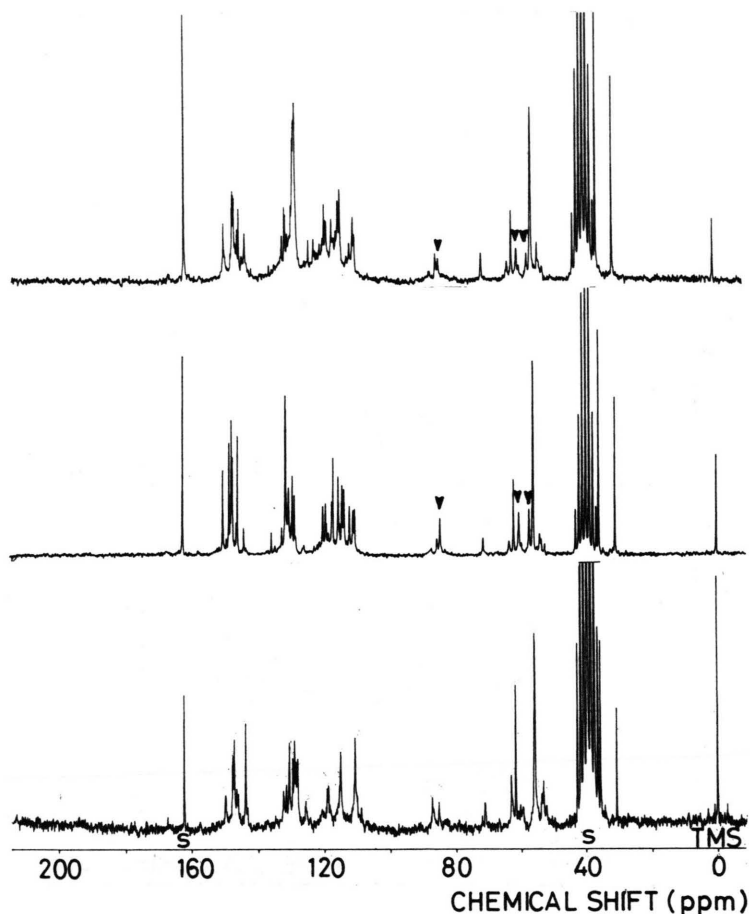


Fig. 4.  $^{13}\text{C}$ -NMR spectra. From top to bottom the spectra were obtained with the 4-chloroaniline and 3,4-dichloroaniline copolymers, and reference *in vitro* lignin, respectively. The spectra were taken on a Bruker WP-80 instrument. Deuterated dimethylsulfoxide was used as solvent, some residual dimethylformamide also being present. Solvent peaks are labeled "S". The copolymer peaks discussed in the Results section are marked by arrows. Tetramethylsilane served as the internal standard.

The present report is, however, only concerned with spectral features related to the incorporation of the anilines.

Most of the typical lignin peaks were decreased in the copolymer spectra indicating a relative decrease or loss of lignin substructures. This was, for example, true for the signals of phenylcumaran units and olefinic side-chains. There was apparently no meta-addition of nitrogen to  $\text{C}_5$  of the guaiacyl-rings, *e.g.* via radical form  $R_c$  of Fig. 1. In that case, a signal for  $\text{C}_2$  of the guaiacyl-ring should appear at 105–109 ppm [16] where, however, no peak could be seen in the copolymer spectra. By a similar argument substitution of the anilines by O-aryl groups in *meta*-position to the amino-group could be excluded since in that case a signal of the substituted carbon is expected at 158–161 ppm. *Ortho*-substitution of the anilines could, however, not be excluded.

The most striking new spectral features of the copolymer spectra concerned the side-chain signals. The intensities of the aliphatic peaks at 87.0 ppm ( $\alpha/\beta\text{-C}$ ) and at 62.8 and 61.7 ppm ( $\gamma\text{-C}$ ) were clearly decreased in the copolymer spectra, with the concomitant appearance of new peaks at 84.3, 60.0 and 56.9 ppm (see arrows in Fig. 4). These new peaks appeared as a doublet, a triplet and a doublet, respectively, under of-resonance conditions and they were of about equal intensities.  $^{13}\text{C}$ -NMR chemical shift values of a number of model compounds were collected in order to facilitate the assignment of the observed new spectral peaks (Table I). These values and the observed coupling pattern suggested that the peak at 56.9 ppm had to be assigned to  $\alpha\text{-C}$ , the peak at 84.3 ppm to  $\beta\text{-C}$  and the peak at 60.0 ppm to  $\gamma\text{-C}$  of a 3-aryl-3-anilino-2-aryloxy-propanol-1 side-chain. This assignment was further supported

Table I.  $^{13}\text{C}$ -NMR chemical shift values (ppm) of selected phenylpropanoid and phenylethyl-compounds. The chemical shift values of the 4-hydroxy-3-methoxyphenyl compounds were taken from the literature [13, 15]. The remaining substances were studied in deuterated dimethylsulfoxide on a Bruker WP-80 instrument.

Aromatic substituent	Side chain	Chemical shift values		
		$\alpha$ -C	$\beta$ -C	$\gamma$ -C
4-Hydroxy-3-methoxyphenyl	2,3-di-O-arylpropanol-1	80.7	84.1	61.7
4-Hydroxy-3-methoxyphenyl	2-O-aryl-1,3-propanediol	73.5	86.5	61.7
Phenyl	2-amino-1,3-propanediol	73.1	58.9	63.0
Phenyl	1,2-ethanediol	73.8	67.5	—
Phenyl	2-amino-ethanol-1	57.4	67.9	—
Phenyl	1-amino-ethanol-2	74.6	50.2	—

by selective proton decoupling experiments (not shown) which correlated the above  $\alpha$ -,  $\beta$ - and  $\gamma$ -carbon signals with the aforementioned new proton signals at 4.75 ppm ( $\alpha$ -C-H), 4.40 ppm ( $\beta$ -C-H) and 3.70/3.50 ppm ( $\gamma$ -C-H<sub>2</sub>).

#### Effect of catalase and inhibitors

In order to examine whether the incorporation of 3,4-dichloroaniline had an absolute requirement for the action of peroxidase/H<sub>2</sub>O<sub>2</sub> the usual copolymerization experiment was carried out as a two-step procedure. First, coniferyl alcohol was polymerized with the aid of peroxidase/H<sub>2</sub>O<sub>2</sub>. Then a large excess of catalase was added in order to decompose

H<sub>2</sub>O<sub>2</sub> before the addition of 3,4-dichloroaniline (see Materials and Methods). The addition of catalase led only to a modest decrease of 3,4-dichloroaniline incorporation from 2.9 to 2.4 mol%. The molecular weight distribution of the copolymer was significantly shifted to lower molecular weight (Fig. 5). When the two-step procedure was carried out with addition of a two-fold molar excess of ascorbate, dihydroxyfumarate or hydroquinone, or of an equimolar amount of iodine before the addition of catalase the total incorporation of 3,4-dichloroaniline was decreased by 85%, 74%, 54% and 57%, respectively.

## Discussion

### Evidence for true copolymerization

An apparent incorporation of chloroanilines into lignin could be due to the formation of non-covalent inclusion compounds or of two separate homopolymers consisting of lignin and of chloroaniline, respectively. These possible artifacts were excluded, i) by repeated gel permeation chromatography of defined copolymer fractions and ii) by  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra with distinct new peaks which could not be explained by superposition of pure lignin and chloroaniline spectra.

### A mechanism for copolymerization

The side-chain substitution of cinnamyl alcohols or lignols by a radical mechanism is only possible in  $\beta$ -position and involves cinnamyl radicals of the type  $R_b$  of Fig. 1. However, the  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR results clearly indicated that much of the chloroanilines was bound to the benzylic  $\alpha$ -carbon so that a non-radical mechanism became likely. A

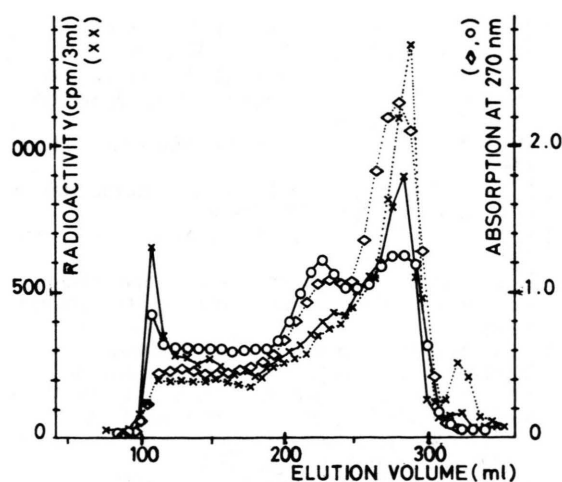


Fig. 5. Effect of catalase treatment on the molecular weight distribution of 3,4-dichloroaniline copolymers. The two-step incubation procedure of Materials and Methods was performed with (....) or without (—) addition of catalase, followed by chromatography on Sephadex LH-60 under the conditions of Fig. 2. The profiles of UV-absorption at 280 nm (○, ◇) and of radioactivity (cpm/3 ml; × ×) are plotted against elution volume (ml).

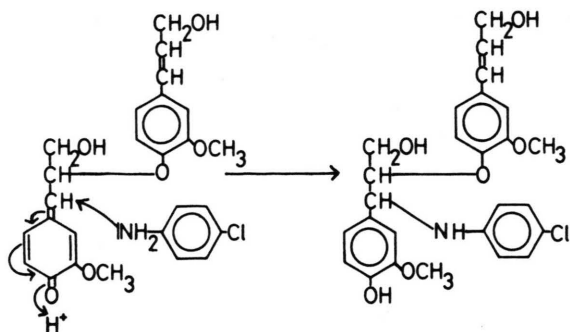


Fig. 6. Proposed mechanism for the incorporation of anilines into lignin. The dilignol quinone-methide intermediate shown as a typical acceptor molecule is thought to arise through addition of the radical  $R_a$  of Fig. 1 to radical  $R_b$  [7].

non-radical mechanism was also indicated by the catalase and, less conclusively, by the inhibitor experiments. Evidence for a benzylamine-structure in chloroaniline/lignin copolymers has previously been obtained by a pyrolysis/mass spectrometry procedure [6, 17].

The  $\alpha$ -substitution of cinnamyl alcohols and lignols requires the intermediacy of quinone-methides [7] so that the mechanism of copolymerization shown in Fig. 6 results. Since the incorporation appears to occur by a simple nucleophilic addition

reaction a wide variety of chemicals with *e.g.*,  $-\text{NH}_2$ ,  $-\text{OH}$ ,  $-\text{SH}$  or  $-\text{COOH}$  substituents should be amenable to incorporation into lignin.

This study appears to be the first where the covalent incorporation of a foreign chemical into lignin and a molecular mechanism for incorporation have been conclusively demonstrated. The incorporation of benzo[*a*]pyrene quinones into *in vitro* lignin has been achieved by similar methods [9]. While most "insoluble" pesticide metabolites isolated from plants are as yet ill-defined [1] it has recently been demonstrated that an insoluble metabolite fraction containing the herbicide 2,4-dichlorophenoxyacetic acid and its 4-hydroxy derivative consisted of lignin [18]. Evidence for the lignin-nature of "insoluble" metabolite fractions of chloroanilines has previously been reported [3–6]. The huge biomass of lignin may more generally enable plants to act as an important but so far not recognized sink for environmental chemicals.

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