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PEROXIDASE CATALYZED OXIDATION OF NATURALLY-OCCURRING PHENOLS AND HARDWOOD LIGNINS

MARIA YOUNG and CORNELIUS STEELINK

Department of Chemistry, University of Arizona, Tucson, AR 85721, U.S.A.

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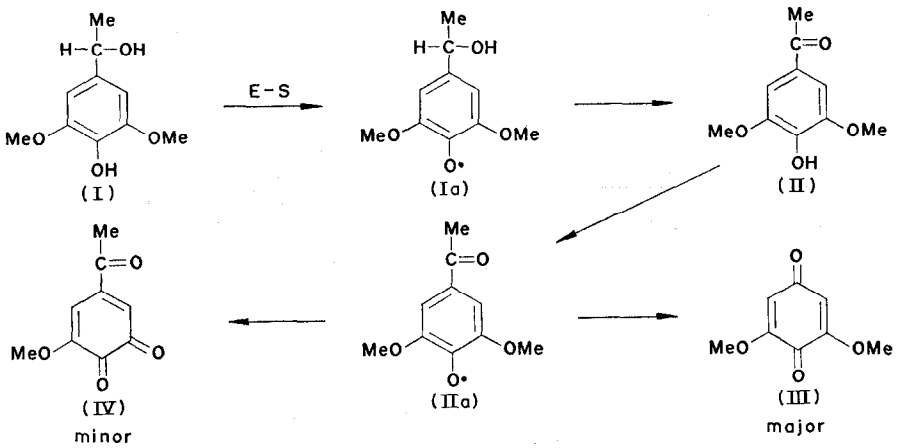
Abstract—Horseradish peroxidase and hydrogen peroxide form phenoxy radicals from 4-substituted-2,6-dimethoxyphenols, milled wood lignin and alkali lignins. A number of factors governing this reaction are examined. Side chain cleavage to quinones is the principal disproportionation reaction of these radicals. Catalysis by UV light and inhibition by quinones is observed. Aerobic oxidation of phenols is catalyzed by small amounts of hydrogen peroxide. Lignin substrates are degraded by the same oxidation mechanism as are the simple phenolic substrates.

INTRODUCTION

RECENTLY[1a] we reported the existence of long-lived phenoxy radical intermediates in the enzymatic oxidation of naturally-occurring phenols. The reaction was shown to proceed in a stepwise fashion. Model compounds such as I→II were chosen because they represent important structural elements in hardwood lignin macromolecules, and because they form stable phenoxy radicals capable of being monitored continuously by ESR spectroscopy. Their behavior with fungal phenoloxidases could represent a number of important patho-



E–S = Peroxidase/hydrogen peroxide or laccase/oxygen or fungal extracts.[1b]

[1] (a) CALDWELL, E. S. and STEELINK, C. (1969) *Biochim. Biophys. Acta* **184**, 420; (b) Aqueous extracts of various *Polyporus* species (unpublished results).

logical processes in plants.[2,3] In addition, the reaction I → III may be important in certain biological transformations of lignin[4,5] and its conversion to humic substances.[6]

We have now examined this reaction in greater detail with the view to elucidating the mechanism of formation and decay of the radical species. Factors such as pH, concentration of reactants, product inhibition, dissolved oxygen, effect of light and dissolved polysaccharides were evaluated. Acetosyringone (II) and α-methylsyringyl alcohol (I) were chosen as substrates for this study. In addition, native and industrial hardwood lignins were also used as substrates, because of the considerable interest in the microbiological decomposition of lignin wastes and pollution control.[7]

RESULTS

pH

The optimum pH for radical (IIa) formation was 5·6; above and below this value, the concentration of the radical species drops off rapidly. The buffer MES [2-(morpholino) ethane sulfonic acid, p*K*, 6·1] was used in all experiments. Other buffer systems gave poorly resolved ESR spectra. At pH, 5·6, the maximum radical concentration was 2×10^{-3} M in a solution which contained 3×10^{-7} M in horseradish peroxidase and 5×10^{-3} M substrate (II).

Enzyme Concentration

At peroxidase concentrations greater than $2{\cdot}5 \times 10^{-7}$ M, the rate of reaction is independent of enzyme concentration. Below 1×10^{-7} M, the rate is proportional to the concentration of enzyme (Fig. 1). In this connection it is interesting to note that Stahmann

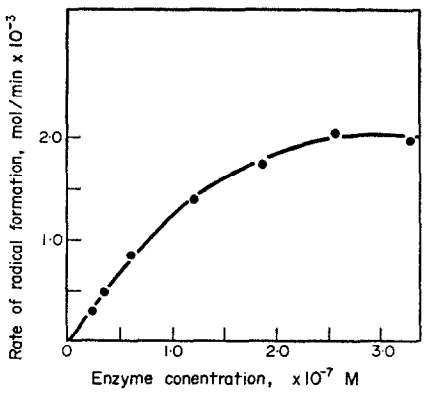


FIG. 1. INITIAL RATE OF RADICAL FORMATION VS. ENZYME CONCENTRATION.
Reactants: Actosyringone, 5×10^{-3} M; hydrogen peroxide, $1{\cdot}3 \times 10^{-2}$ M; buffer, 0·1 M; pH 5·6.

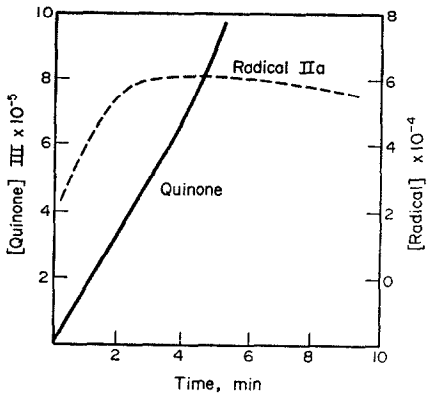


FIG. 2. FORMATION OF RADICAL IIa AND QUINONE III.
Reactants: Acetosyringone, 5×10^{-3} M; hydrogen peroxide, $1{\cdot}3 \times 10^{-2}$ M; horseradish peroxidase, $2{\cdot}8 \times 10^{-8}$ M; buffer, 0·1 M; pH, 5·6.

[2] MACKO, V., WOODBURY, W. and STAHMANN, M. A. (1968) *Phytopathology* **58**, 1250.
[3] STAHMANN, M. A., CLARE, B. and WEBER, D. J. (1966) *Science* **153**, 62.
[4] KENT KIRK, T. (1971) *Ann. Rev. Phytopath.* **9**, 185.
[5] HIGUCHI, T. (1971) *Adv. Enzymology* **34**, 207.
[6] HURST, H. M. and BURGES, N. A. (1967) in *Soil Biochemistry* (MCLAREN, A. D. and PETERSON, G., eds.), pp. 260–86, Dekker, New York.
[7] KLEIN, A., ROCKHILL, R. C., ELDRIGE, J. P. and PARK, J. E. (1970) *TAPPI* **53**, 1469.

et al.[8a,b] reported that peroxidase injected into plants increased disease resistance, but that excess peroxidase had no additional effect. Piette *et al.*[9] reported a linear relationship between rate of radical (benzosemiquinone) formation and peroxidase in the oxidation of hydroquinone. In addition, they noted that the steady state radical concentration was proportional to the square root of the enzyme concentration, a relationship also observed in our work. However, our hindered phenols differ significantly from hydroquinone in the decay reaction: the rate of our reaction is at least 1000 times slower than the hydroquinone reaction and the concentration of our radical species is 10^4 times greater than that reported for the semiquinone.

Most pertinent are the studies of radical formation and quinone formation. Quinone III is the major product of the oxidation of II. It can be monitored in the reaction mixture at 380 nm, at which wavelength the other reactants do not absorb appreciably. At high enzyme concentrations (3×10^{-7} M), radical IIa, and quinone III are formed so rapidly that the formation rates are not measurable. At low enzyme concentrations ($2{\cdot}7 \times 10^{-8}$ M), the rate of radical formation parallels the rate of quinone formation. To a first approximation, the first order rate constants of formation are equal: $k_{rad}[II] = k_{quinone}[IIa]$ (Fig. 2). Thus, the conversion of ketone radical IIa to quinone III must be much more rapid than the dehydrogenation of ketone II.

Hydrogen Peroxide Concentration and the Effect of Dissolved Oxygen

The plot of radical concentration $[RAD]_{max}$ vs $[H_2O_2]$ shows a maximum (Fig. 3). Excess peroxide, therefore, inhibits the reaction as had been noted with other reactions.[10]

When the molar ratio $[H_2O_2]/[II]$ is less than 1·0, anomalous results are obtained. Thus, an 8-fold reduction of peroxide concentration results in only a slight (40%) decrease in free radical concentration. When the experiment is repeated with degassed solutions, the drop in radical concentration is proportional to the drop in peroxide concentration. In other experiments, degassed solutions of the enzyme-substrate-peroxide system always gave lower radical yields than aerated solutions. When the oxidants were only oxygen or only hydrogen peroxide without enzyme present, no radicals were observed.

These experiments reveal that H_2O_2 in low concentrations *catalyzes the aerobic oxidation* of II to IIa. Recently, Yamazaki *et al.*[11] reported a similar phenomenon with dihydroxyfumaric acid as substrate for peroxidase. They concluded that hydrogen peroxide initially dehydrogenates hydrogen donors to form organic radicals, which in turn reduce oxygen to perhydroxyl radicals or an activated peroxidase–O_2 complex. The latter are active intermediates which cause further oxidation of substrate before decomposing to hydrogen peroxide. The presence of active perhydroxyl radicals could account for many of the subsequent reactions of IIa in our system, although complicating the mechanism considerably.

Another interesting aspect of this reaction is the nature of the ESR spectra of IIa. In the oxygen-free solutions, the spectra are much better resolved than in the peroxide-oxygen systems. (Coupling from all protons is clearly visible. Thus, two ring protons, 1·75 G;

[8] (a) LOVREKOVICH, L., LOVREKOVICH, H. and STAHMANN, M. A. (1968) *Phytopathology* **58**, 193. (b) MACKO, V., WOODBURY, W. and STAHMANN, M. A. (1968) *Phytopathology* **58**, 1250.

[9] YAMAZAKI, I., MASON, H. S. and PIETTE, L. H. (1960) *J. Biol. Chem.* **235**, 2444.

[10] SAUNDERS, B. C., HOLMES-SIEDLE, A. G. and STORK, B. P. (1964) *Peroxidase*, p. 136, Butterworths, London.

[11] YAMAZAKI, I., YAMAZAKI, H., TAMURA, M., OHNISHI, T., NAKAMURA, S. and IYANAGI, T. (1968) *Adv. Chem. Ser.* **77**, 290.

six methoxyl protons, 1·45 G; three side chain protons, 0·30 G.) This suggests some interaction between oxygen and radical IIa (such as a peroxy-phenoxy radical); a radical enzyme complex seems unlikely since the radical concentration is at least 10^4 times greater than enzyme concentration.

Inhibition by Quinones

Like tannins[12] quinones have been reported[13] to be peroxidase inhibitors. The reported data suggest that the reaction is inhibited at its later stages. To determine whether or not quinone III acts in this capacity, we added it in small amounts to the reaction mixture. The amount of enzyme was kept very low ($2{\cdot}75 \times 10^{-8}$ M) so that a slow rate of radical formation could be maintained and monitored. The results show that the initiation of radical formation is inhibited up to 4 min and that the quinone formation was suppressed for 20 min. The quinone III concentration at the end of the reaction period was lower than the uninhibited reaction (Table 1). Quinone III undergoes no reaction with peroxidase–peroxide.

TABLE 1. INHIBITION OF REACTION BY QUINONE III

[Substrate]	$[H_2O_2]$	[HRP]	Added quinone	Inhibition period	Final quinone
5×10^{-3} M	$1{\cdot}26 \times 10^{-2}$ M	$2{\cdot}75 \times 10^{-8}$ M	$3{\cdot}2 \times 10^{-4}$ M	20 min	$3{\cdot}1 \times 10^{-3}$ M
5×10^{-3} M	$1{\cdot}26 \times 10^{-2}$ M	$2{\cdot}75 \times 10^{-8}$ M	None	None	$4{\cdot}5 \times 10^{-3}$ M

The addition of *p*-benzoquinone had the same effect as III. Addition of small amounts of α-methylsyringyl alcohol (5 %) also suppressed the appearance of radical IIa, as previously reported.[1(a)] The latter phenomenon is due to the lower oxidation potential of α-methylsyringyl alcohol (I) as compared to that of ketone II; both potentials have been recently measured in this laboratory.

Inhibition by Polysaccharides

Reactions of wood-rotting fungi and bacteria take place in biological tissues containing high concentrations of polysaccharides. Conceivably, the presence of these polysaccharides could modify the enzyme-catalyzed reactions which we observe *in vitro*. Therefore, a series of experiments were carried out in concentrated sugar solutions using ketone II as substrate. The rate of radical formation was monitored by ESR spectroscopy and the suspected presence of any substrate-saccharide complex was examined by optical spectroscopy. α- and β-Cyclodextrins were included in the group of polysaccharides and sugars because of their ability to form inclusion compounds which alter the rate of chemical reactions.[14,15] A very high saccharide/substrate molar ratio was used (see Table 2) to maximize the chances of detecting any substrate complexes.

[12] BOUDET, A. (1965) *Compt. Rend.* **261,** 214.
[13] KLAPPER, M. H. and HACKETT, D. P. (1963) *J. Biol. Chem.* **238,** 3736.
[14] CRAMER, F., SAENGER, W. and SPATZ, H. Ch. (1967) *J. Am. Chem. Soc.* **89,** 14.
[15] BRESLOW, R. and CAMPBELL, P. (1969) *J. Am. Chem. Soc.* **91,** 3085.

In these experiments, both of the cyclodextrins inhibited the appearance of radicals, while none of the more common saccharides had any appreciable effect. The existence of an inclusion compound between substrate and cyclodextrin was not apparent, because of the absence of any spectral shifts. Furthermore, calculated interatomic distances for II and those of the cyclodextrins indicated that inclusion compounds are unlikely. Even *p*-hydroxy-acetophenone, a molecule which is smaller than II, underwent no spectral shift in the presence of excess cyclodextrin. Therefore, the inhibition due to these rare saccharides may be due to inclusion of one of the oxidants or to selective binding on an active site of the enzyme. Certainly, none of the common polysaccharides had any effect on the enzyme-catalyzed oxidation of II.

TABLE 2. INHIBITION OF RADICAL FORMATION BY SACCHARIDES*

Saccharide	[Saccharide]/[Substrate]	Inhibition period (min)†	ΔA at 380 nm‡
Maltose	77	1	0·3
Raffinose	77	0	0·3
Dextran	77	2	0·3
Cellulose (insoluble)	—	0	—
α-Cyclodextrin	77	7	0·3
β-Cyclodextrin	38	14	0·22

* The standard experiment is carried out as follows: acetosyringone, 5×10^{-4} M; H_2O_2, $1{\cdot}26 \times 10^{-2}$ M; horseradish peroxidase, $2{\cdot}8 \times 10^{-8}$ M; buffer, 0·1 M; pH, 5·6, are mixed. Five min elapse before an ESR spectrum is observed, in the absence of saccharides.

† Additional time over 5 min standard period.

‡ The change in absorption maximum after reaction is completed. A change of 0·3 in absorption corresponds to $4{\cdot}9 \times 10^{-4}$ M in quinone III.

Oxidation of α-Methylsyringyl Alcohol (I)

Effect of hydrogen peroxide concentration. In the presence of excess hydrogen peroxide, compound I is oxidized to radical Ia, then to ketone II, then to radical IIa and finally to quinones III and IV.[16] Under these conditions, the reaction is too fast to determine at what point in the reaction sequence quinone III formation begins. When ketone II is used as substrate, radical IIa forms immediately in high concentration and decays to quinone III. However, it is conceivable that this quinone may be generated by the decay of radical Ia, as well as by the decay of IIa; in the former case, quinone formation would compete with ketone formation.

To evaluate the proposed reaction routes, we carried out a series of experiments with varying ratios of $[H_2O_2]/[I]$ at reduced reaction rates. The concentrations of I, II and III in solution were determined as follows:

(1) $[I] + [II] + [III] = 5 \times 10^{-3}$ M (initial concentration of I)

Total absorbance $A = E \times c \times 1$

$E_I = 13$; $E_{II} = 40$; $E_{III} = 620$ at 380 nm.

$E_I = 560$; $E_{II} = 10\,400$; $E_{III} = 7000$ at 301 nm.

[16] The identity of quinone IV has recently been established in our laboratory by comparison with a synthetic sample. (Sheldon I. Clare, Ph.D. dissertation, University of Arizona, 1972).

(2) $13 \times [I] + 40 \times [II] + 620 \times [III] = A_{380nm}$ (at length = 1 cm).
(3) $0{\cdot}01 \times 560 \times [I] + 0{\cdot}01 \times 10\,400 \times [II] + 0{\cdot}01 \times 7000 \times [III] = A_{301nm}$ (at length = 0·01 cm).

When the initial molar ratio: $[H_2O_2]/[I]$ was exactly 1·0, radical Ia was generated, but no IIa radicals were formed; i.e. the ketone II was apparently not dehydrogenated. A slight yellow color in the solution was noted before the slow oxidation of I was 50% complete. This indicated that some quinone had already formed. At the completion of the reaction, 4% of I remained, 10% of quinone III had been formed and 84% of ketone II was present.

When a slight 4% excess of H_2O_2 was initially added, all of the alcohol I was consumed, leaving 28% quinone III and 72% of ketone II. Radical IIa was also observed. If the initial molar ratio was 0·80, then 20% of alcohol I remained at the end of the reaction along with 10% of quinone III and 69% of ketone II. No H_2O_2 was detected at the end of the reaction. These data are summarized in Table 3. Thus, it would appear that alcohol I is stochiometrically oxidized by H_2O_2 to a mixture of ketone II and quinone III. The quinone apparently arises from both radical Ia and IIa (in the presence of excess peroxide)

TABLE 3. CONCENTRATION OF OXIDATION PRODUCTS OF ALCOHOL I*

Initial ratio, $[H_2O_2]/[I]$	Final concentrations (Molar %) Alcohol I	Ketone II	Quinone III
0·8	20	69	10
1·0	4	84	10
1·04	0	72	28

* α-Methylsyringyl alcohol.

Effect of light irradiation. The oxidation of α-methylsyringyl alcohol (I) is catalyzed by light in the range 300–380 nm. If one monitors the ketone concentration [II] at 301 nm continuously in the spectrophotometer (that is, under constant irradiation), an accelerated oxidation of I is observed. A comparison of the dark reaction (monitored intermittently with the 'light' reaction) is shown in Fig. 4.

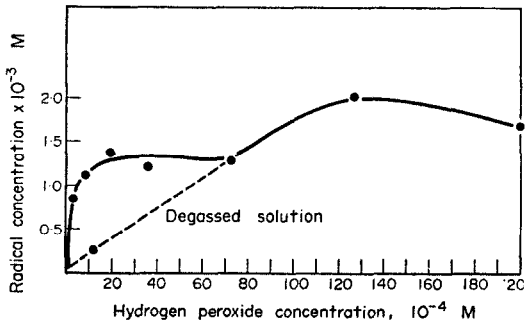


FIG. 3. RADICAL CONCENTRATION VS HYDROGEN PEROXIDE CONCENTRATION.
Reactants: Acetosyringone, 5×10^{-3} M; horseradish peroxidase, $3{\cdot}2 \times 10^{-7}$ M; buffer 0·1 M; pH, 5·6.

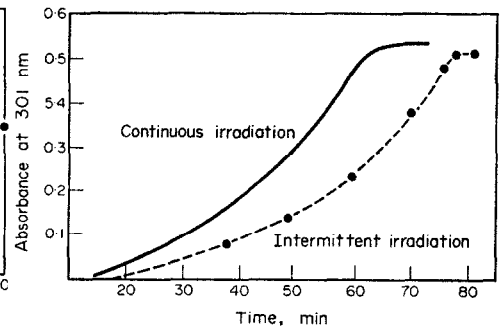


FIG. 4. EFFECT OF LIGHT ON OXIDATION OF α-METHYLSYRINGYL ALCOHOL
Reactants: α-Methylsyringyl alcohol, 5×10^{-3} M; horseradish peroxidase, $3{\cdot}2 \times 10^{-7}$ M hydrogen peroxide, 5×10^{-3} M; buffer, 0·1 M; pH, 5·6.

The oxidation of ketone II does not show this effect; in fact, it is inhibited by quinone formation as previously discussed. Apparently, ketone II can act as a sensitizer for the dehydrogenation of I as it is formed from the latter. Photodegradation of models related to I and II has been observed under non-enzymatic conditions.[17,18]

Enzymatic Oxidation of Macromolecular Phenols

The two model compounds I and II represent important structural units in the hardwood lignin macromolecule. We were interested in determining whether or not the polymeric material would also be an active substrate for the peroxide-peroxidase system. Therefore, we chose as typical substrates: (a) milled wood aspen lignin, (b) hardwood kraft lignin, and (c) Meadol. The latter two substrates are produced in the United States by alkaline delignification of hardwood, and presumably contain a higher percentage of phenolic α-carbonyl groups than does the untreated milled wood lignins.[19]

Lignin solutions (0·5% lignin in dioxane–H_2O, 1:5) were mixed with H_2O_2 and horseradish peroxidase and examined by ESR and optical spectroscopy. All samples immediately gave ESR signals and showed increased absorption at 284 and 380 nm (principal bands of quinone III and to a less extent ketone II). These spectral bands attained their maximum values in 20–40 min. The maximum radical concentration was proportional to the peroxide and enzyme concentrations.

Kraft and Meadol hardwood lignins are polydisperse polymers of different MWs. To obtain an approximate measure of the behavior of the low and high MW fractions, we extracted the samples with methanol. The methanol-soluble and methanol-insoluble fractions were treated separately by the enzyme system (see Table 4). The most active substrate was the methanol-soluble fraction, which yielded 90% of the total radical and quinone species in the polymer.

TABLE 4. SPECTRAL ANALYSIS OF ENZYMATIC OXIDATION OF HARDWOOD LIGNINS*

Substrate	Original substrate (%)	$[H_2O_2]$ ($M \times 10^{-2}$)	[Peroxidase] ($M \times 10^{-6}$)	[Radical] ($M \times 10^{-5}$)†	$\Delta A_{380nm}^{0.5\%}$
MWL Aspen	100	8·2	2·5	0·69	ppt
Kraft	100	10·9	3	5·8	2·45
Meadol	100	7·8	2	8·7	2·58
Methanol-soluble‡	58·3	7·8	2	13·0	4·11
Methanol-insoluble‡	41·7	7·8	2	2·8	0·44

* All solutions contain 0·5% substrate.
† Concentration estimated by comparison to a standard sample of diphenylpicrylhydrazyl solution.
‡ Fractions from Meadol lignin.

The rate of formation and decay of the radical species closely parallels that observed for the oxidation of the monomeric model compounds. Despite the heterogeneous nature of the macromolecule, we were able to obtain a reasonably resolved ESR spectrum of the oxidation reaction. (Fig. 5). The spectrum is characteristic of radicals derived from syringyl

[17] BRUNOW, G. and ERIKSSON, B. (1971) *Acta Chem. Scand.* **25**, 2779.
[18] GIERER, J. and LIN, S. Y. (1972) *Svensk. Paperstid.* **75**, 233.
[19] MARTON, J. (1964) *TAPPI* 713.

ketones[1a] of type IIa; it is obtained from the methanol-soluble fractions only. TLC of the methanol-soluble fractions revealed the presence of syringaldehyde and other phenols; the latter appear to be low MW oligomers which yield phenoxy radicals upon enzymatic oxidation. Vanillin was also detected in the extracts; however, it would not give rise to stable phenoxy radicals, since it dimerizes rapidly in aqueous solution.

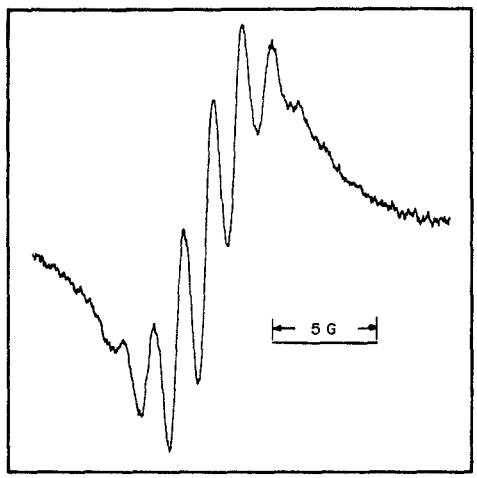


FIG. 5. ESR SPECTRUM GENERATED BY PEROXIDE PEROXIDASE OXIDATION OF METHANOL-SOLUBLE MEADOL FRACTION.

The methanol-insoluble fraction (high MW) was slowly attacked by the peroxide-peroxidase system. It yielded a broad, unresolved ESR spectrum, which overlapped the syringyl spectrum (Fig. 5). A similar ESR spectrum was observed for milled wood lignin, which also yielded a precipitate upon enzymatic oxidation.

In another set of experiments, the lignins were extracted with chloroform until all traces of quinone compounds were removed. The residues were subjected to enzymatic oxidation; the reaction mixtures were extracted with chloroform and the extracts analyzed by TLC. Quinone III was identified as the only prominent oxidation product; the yield was approximately 1–2%. The residual aqueous solutions showed enhanced absorption at 284 nm (Table 5). Subsequent treatment of the residue from the above reaction with fresh peroxide-peroxidase caused a 30% increase in the 284 and 380 nm absorption bands.

TABLE 5. ANALYSIS OF ENZYMATIC OXIDATION OF CHLOROFORM-INSOLUBLE LIGNIN FRACTIONS*

Substrate	Original (%)	$\Delta A_{284nm}^{0.5\%}$	[III]† ($\times 10^{-4}$ M)
MEADOL	58·0	7·6	8·7
KRAFT	56·0	15·3	2·5

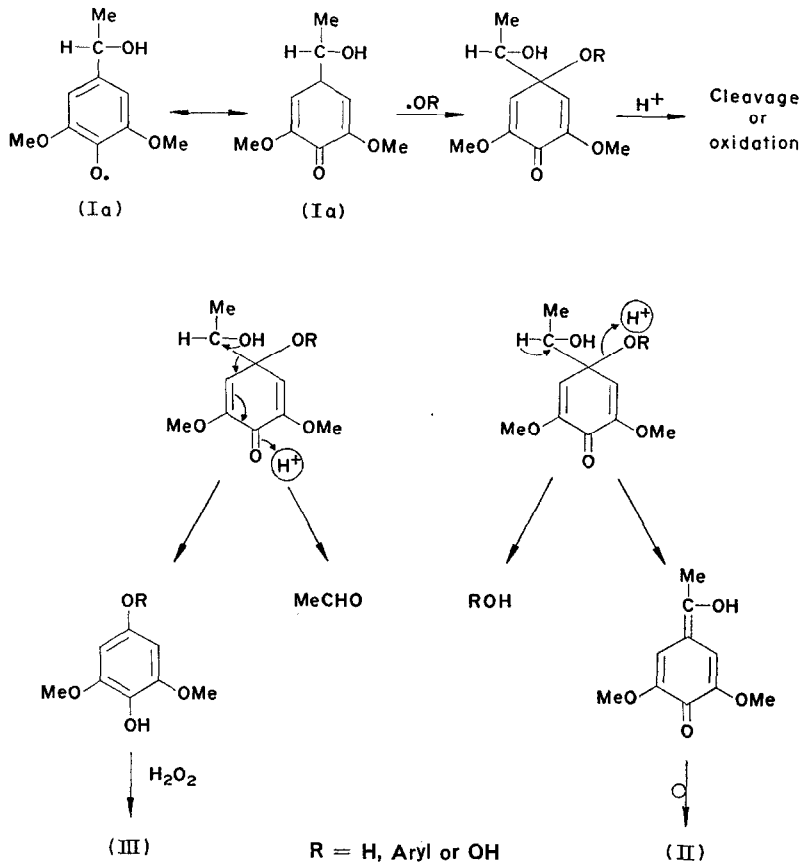
* All solutions contain 0·5% substrate.
† Estimated from TLC.

It is apparent that commercial alkali lignins are much more accessible to enzymatic attack than corresponding untreated lignins. More than 40% of the hardwood lignin (the methanol-soluble fractions) is oxidized rapidly by peroxide-peroxidase via a free radical process. The reaction is remarkably similar to the enzymatic oxidation of monomeric model compounds. Evidently, the low MW fraction contains a higher concentration of phenolic α-carbonyl species such as II than does untreated lignin, since this fraction yields radicals of the type IIa. The high MW fraction mimics the reactions of model compounds related to I. Even though they are much more resistant to enzymatic oxidation, these fractions still yield quinone III in small amounts.

DISCUSSION

The first step in the enzymatic oxidation is a rapid one-electron dehydrogenation of phenolic substrate. Phenoxy radicals are the result of this reaction. The rate of their formation is directly proportional to the enzyme and H_2O_2 concentrations, although saturation of the rate of reaction is easily realized at higher concentrations. The rate is very sensitive to pH changes. Five features of this oxidation reaction deserve comment.

First, aerobic oxidation is catalyzed by peroxidase at very low concentration of H_2O_2. One can observe phenoxy radicals in much greater concentration than predicted from



SCHEME 1.

the peroxide present; when oxygen is removed from the reaction solution, the phenoxy radical content drops. In terms of plant metabolism, this implies that the presence (or release) of minute quantities of H_2O_2 can trigger the reaction of phenols with oxygen to produce significant amounts of radical and quinone products.

Second, side chain cleavage of the radical is extremely rapid and competes with oxidation at the benzyl carbon atom. For 4-substituted α-ketophenols (type II), the rate of side chain cleavage to quinone is so fast that it appears to coincide with radical formation. For compounds with lower oxidation states at the benzyl carbon atom (such at I), side chain cleavage is much slower.

Third, the reaction is markedly inhibited by the end product, 2,6-dimethoxyquinone(III). The nature of this inhibition is not obvious; however, III is not a substrate for the enzyme/peroxide system, as was established by direct experimentation. Other quinones also inhibit the reaction. Inhibition is not observed in the presence of common plant polysaccharides, even when there is a very high (80:1) polysaccharide/substrate ratio.

Fourth, UV light catalyzes the enzymatic oxidation of phenols of type I. This effect is not noticed in the oxidation of ketones of type II, probably because of the rapid formation of quinone III, which inhibits the oxidation. The two effects may well cancel one another under the conditions of the experiment.

Fifth, the lignin macromolecule appears to undergo enzymatic oxidation by a mechanism identical to that of the model substrates. This behavior should be of interest to lignin chemists and plant pathologists. As might be expected, the lower MW fractions were attacked much more readily than the methanol-insoluble fractions. However, even these intractable fractions could be re-oxidized to yield quinone products. Our estimate of a 1% yield of quinone from the unfractionated Meadol lignin after 40 min of reaction time compares favorably with a 0·4% yield of quinone III by Ishihara and Miyazaki[20] from the less reactive milled wood maple lignin.

Thus, it would appear that the degradation mechanism proposed for the model compounds is operative with the macromolecules. The industrial lignins, such as kraft and Meadol hardwoods, are much more active substrates than the 'natural' (milled wood lignin). Considerable depolymerization (side chain cleavage) should occur with peroxide/peroxidase, although polymerization may be a competing process[20] as our results with milled wood lignin have indicated. One would anticipate that polymerization would dominate in softwoods,[21] in which guaicyl moieties are the major structural units. For lignin chemists, these results may hold intriguing possibilities for the enzymatic degradation of lignin and lignin waste products. The reaction parameters have been fairly well established and could be optimized. With the use of immobilized enzyme systems[22] the possibility of recycling substrate flow over fixed enzyme bed is not unrealistic, and may render lignin more accessible to microbiological attack and utilization.

With respect to disease resistance in plants, it has been assumed that peroxidase and hydrogen peroxide may act on simple phenolic substrates to produce antifungal metabolites.[3] Quinones such as III have been reported[23] to have antifungal properties. They could arise from the enzymatic oxidation of simple phenols; however, our results indicate that plant lignin, itself, could be the substrate.

[20] ISHIKARA, T. and MIYAZAKI, M. (1972) *Mokuzai Gakkaishi* **18,** 415.
[21] FERM, R., KRINGSTAD, K. P. and COWLING, E. B. (1972) *Svensk Papperstidning*, **75,** 859.
[22] ANON. (1972) *Chem. Engng News* 25 (Jan. 3).
[23] MACE, M. E. and HEBERT, T. T. (1967) *Phytopathology* **53,** 692.

EXPERIMENTAL

Spectra. All ESR spectra were made in a flat quartz solution cell in the cavity of an E-3 Varian spectrometer. The instrument operated at 9·5 gHz with a frequency modulation of 100 kHz. The concentration of free radicals was estimated by comparing the quantity (ht $\times$ width^2), of the first derivative ESR curve with the corresponding value of a standard solution of diphenylpicrylhydrazyl in C_6H_6.

Substrates. Acetosyringone (II) was purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. Quinone III was prepared by the oxidation of 2,6-dimethoxyphenol with lead tetraacetate[24] and purified by sublimation. α-methylsyringyl alcohol (I) was prepared by catalytic reduction of II over Pd–C and recrystallized from C_6H_6, m.p. 96–97°. Meadol hardwood lignin was obtained from the Forest Products Laboratory, Madison, Wisconsin. Kraft hardwood lignin was obtained from the WESTVACO Company, Charleston, South Carolina.

Enzyme. Horseradish peroxidase (Type VI) was purchased from the Sigma Chemical Company, St. Louis, Missouri.

Other materials. α- and β-Cyclodextrins were obtained from Pierce Chemical Company, Rockford, Illinois. The most common buffer used in these experiments was 2-(*N*-morpholino)ethane sulfonic acid, purchased from CalBioChem, San Diego, California.

Methods. Stock solutions of the reactants were maintained at the following concentrations: substrate, $2{\cdot}00 \times 10^{-2}$ M; enzyme, $1{\cdot}83 \times 10^{-6}$ M; H_2O_2, $7{\cdot}6 \times 10^{-2}$ M; buffer, 0·1 M. These were mixed to give a total reaction vol. of 2·00 ml. In a typical experiment, 0·50 ml of substrate, 0·35 ml enzyme, 0·35 ml peroxide and 0·80 ml buffer, were mixed. The pH of these solutions was 5·6. Any variation in concentrations of substrate, enzyme or peroxide were adjusted with an appropriate amount of buffer to give a total vol. of 2·00 ml. Immediately after mixing, the solution was transferred to an ESR cell (or a spectrophotometer cuvette) and scanned over a period of time. In all cases the radical concentration reached a maximum and then decayed. At high enzyme concentrations, the maximum radical concentration (Rad_{max}) was observed almost immediately after the first scan in the E-3 spectrometer. TLC was carried out with silica gel plates; development was normally carried out with C_6H_6–MeOH (4:1). All lignin substrates were dissolved in dioxane–H_2O (1:5) at 0·5% concentration.

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[24] WESSELY, F. and KOTLAN, J. (1953) *Monasch. Chem.* **84,** 296.