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Biomass Photochemistry. V. Modifications of Lignin by Photochemical Treatment and Its Chemiluminescence*

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ABSTRACT

Lignin modifications resulting from different photochemical pretreatments were studied using chemiluminescent methods. In the oxidation of lignin in NaOH solutions at 25°C, the intensity increased with increasing temperature and can be described by an Arrhenius-type exponential equation with an activation energy of 25.8 ± 2.7 kJ/mol. The oxidation of lignin model compounds under these conditions indicated $^1O_2$, $OH^{\cdot}$, and $O_2^{-\cdot}$ generation. Chemiluminescence of the luminol/H_2O_2/Fe^{2+} system was used to study decomposition products of lignin upon irradiation. Unirradiated lignin proved to be an excellent radical trap, an effect initially abolished upon irradiation. At longer irradiation times, however, the radical trapping be-

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havior was restored. The action of the peroxidase/H_2O_2 system on lignin was also investigated using chemiluminescence. Behavior very similar to that using luminol was observed. The intensity increases with increasing time of irradiation up to an optimum value. More prolonged irradiation results in total quenching of the chemiluminescence. This is indicative of depolymerization and posterior aggregation.

Although many rot basidiomycetes are capable of degrading lignin to CO_2, the specific catalyst involved has not been determined [1, 2]. Several studies indicate that the fungal biodegradation of lignin is an oxidative process [3-5]. In addition, Phanerochaete chrysosporium degrades lignin [6, 7] and lignin model compounds [8] approximately 3-fold faster when cultured under 100% oxygen rather than under air.

Some reports indicate that fungi may produce superoxide anion [9], presumably singlet oxygen [10, 11], hydroxyl radicals [12], or unspecified radicals [13].

These preliminary physiological and chemical studies, coupled with the fact that no extracellular enzymes involved in lignin biodegradation have been isolated, led to the postulate that activated oxygen species may be the agents responsible for lignin depolymerization by fungi [13, 14]. The degradation products of lignin model compounds in the presence of activated oxygen species are very similar to those found for degradation by P. chrysosporium [10, 15-17].

Recently, we observed photochemical modification of lignin by light of wavelength longer than 254 nm [18] using both fluorescence and enzymatic methods [19, 20].

In general, it is believed that all of these reactions are oxidative. The oxidation in biological systems can be mimicked by a base-catalyzed oxidation reaction [21, 22]. In order to obtain quantitative information concerning the modifications of the side chain in lignin, the chemiluminescent processes of both lignin itself and model compounds in basic media in the presence of oxygen were studied because it is known that the chemiluminescent method gives information about structural modifications in polymers [23-29]. The chemiluminescent probe luminol, currently used in biological systems [30], is a sensitive method for following any radical formation or trapping. Recently, this method was utilized to study cotton [28]. As it is believed that depolymerization of lignin occurs [31-34] by the peroxidase/H_2O_2 system [35], we have studied the chemiluminescence of this system in the presence of irradiated lignins.

In the present paper we report studies of the chemiluminescence of irradiated lignin and phenolic model compounds under autoxidation conditions in basic media, as well as in the presence of the luminol/

H_2O_2/Fe^{2+} system and the peroxidase/H_2O_2 system. These results are compared to those for unirradiated lignin.

EXPERIMENTAL

Material and Methods

Lignin was obtained from Eucaliptus paniculata (Esc. Engenh. Lorena, M.G., Brazil). The approximate composition is lignin 72.0%, cellulose 19.8%, ash 8.2%. Horseradish peroxidase (Type VI), vanillin, vanillylpyruvic acid, vanillic acid, ferulic acid, and luminol were obtained from Sigma Chemicals. 1,4-Diazobicyclo(2.2.2)octane (DABCO), eosin Y, rose bengal, sodium formate, D_2O (99.8%), and sodium azide were obtained from Merck. 9,10-Dibromoanthracene-2-sulfonate (DBAS) and 9,10-diphenylanthracene-2-sulfonate (DPAS) sodium salts were available from previous work [36]. The lignin irradiation was performed in dioxane/H_2O (1:1) solution, with an Osram HQL 125 W mercury lamp ($\lambda > 254$ nm) at different fluence rates (the fluence of the lamp was 60 $\mu W/cm^2$), which were measured by means of a YSI-Kettering Radiometer (Model 65A). The chemiluminescence studies under basic and enzymatic conditions were carried out in either a Beckman LS-7000 or LS-100c Liquid Scintillation Counter, with a coincidence circuit turned on and off, respectively. The conditions used were the following: To 3 mL of lignin solution (150 mg/L), 40 μL of 5 N NaOH were added and the resultant solution counted each 0.1 min. In the case of model compounds, the final concentration was 10 mM.

Luminol experiments were carried out following the method of Pugachevskii and Plesha [28]. The conditions used were the following: To 4 mL of 10 mM NaOH containing 0.25 mM Fe^{2+}, 1.7 mM H_2O_2, and 5 μL of lignin dioxane/H_2O (1:1) solution (150 mg/L), a luminol solution (0.25 mM final concentration) was added and the resulting mixture counted in a Mitchell-Hastings Photometer.

Experiments with the peroxidase/H_2O_2/lignin system were carried out following the method of Young and Steelink [32]. The conditions used were the following: To 2.3 mL of 0.1 phosphate buffer, pH 5.5, containing 0.5 mL of lignin in dioxane/H_2O (1:1) solution and 90 mM H_2O_2, was added a horseradish peroxidase solution (2 μM final concentration) followed by counting each 0.1 min in a Liquid Scintillation Counter.

The spectral distribution was determined on a Hamamatsu TV C-767 Photocounter. The carbonyl analyses were carried out by the known methods (2,4-dinitrophenylhydrazine derivatives). The fluorescence measurements were carried out on a Hitachi Perkin-Elmer MPF-44B Spectrofluorometer.

RESULTS AND DISCUSSION

Upon irradiation at different fluences with $\lambda > 254$ nm, characteristic changes in the fluorescence intensity at λ 486 nm (λ exc. 330 nm) were observed (Fig. 1). This is indicative of initial chromophore formation, followed at longer times by degradation. Figure 2 shows a correlation between carbonyl formation and the increase of this fluorescence intensity for irradiation at low fluences. This correlation implies that carbonyl formation during the irradiation is concomitant with structural modification. As the chemiluminescence is adequate for detecting some differences in the structure of polymers [23-29], the chemiluminescence decay of lignin under basic conditions before and after irradiation is shown in Fig. 3. The pre-irradiated lignin shows higher chemiluminescence, in accord with its higher concentration of carbonyl groups. The cellulose constituents of this lignin did not participate in the chemiluminescence process.

The chemiluminescence from lignin under these conditions was rather complex. Consequently model systems were utilized in the analysis of the species which are present in these processes, employing vanillin, vanillylpyruvic acid, ferulic acid, and vanillic acid as sub-

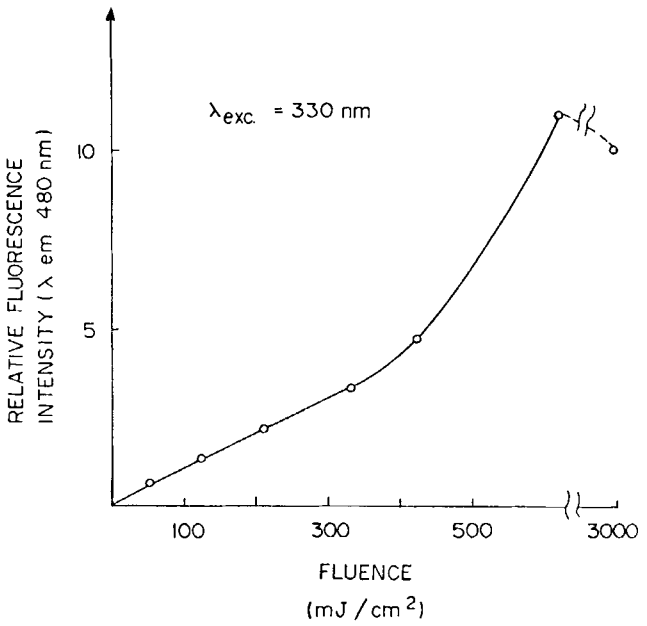


FIG. 1. Fluorescence behavior of lignin (150 mg/L) in dioxane-H_2O (1:1) (λ em 480 nm, λ exc. 330 nm) after treatment at different fluences.

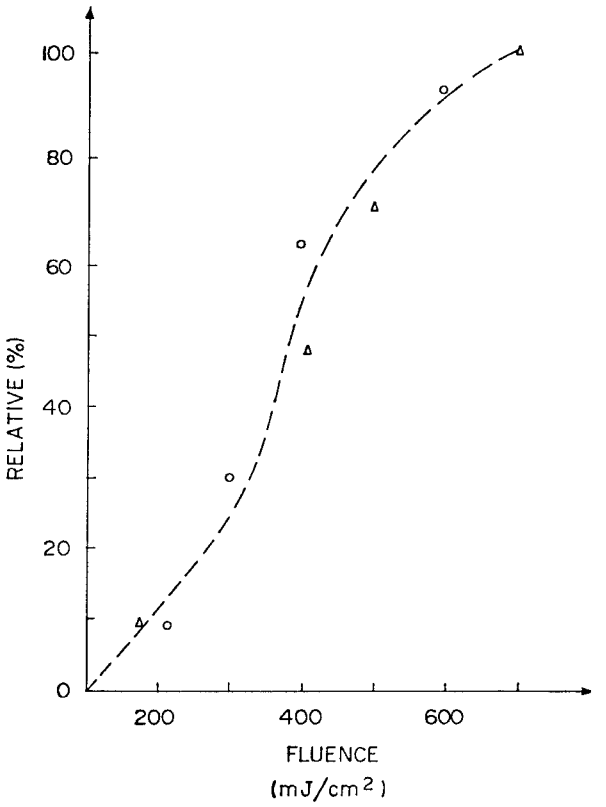


FIG. 2. Correlation of carbonyl compounds appearance (○) and fluorescence intensity (△) of lignin (λ em 480; λ exc. 330 nm) after treatment at different fluences.

strates. Table 1 shows the integrated emission in the presence of the different quenchers and enhancers for different types of excited states and activated oxygen species.

The detection of singlet oxygen as the main emitter in the chemiluminescence of the base-catalyzed autoxidation of vanillin is evident from Table 1. The $OH^{\cdot}$ radical and presumably $O_2^{-\cdot}$ are also important in this emission. The dioxetane intermediate suggested by Nimz and Turzik [16] would appear to be unlikely because no excited carbonyl species were detected. Presumably in this case the hydroperoxide intermediate generates singlet oxygen by the Benson mechanism [29, 37] (Scheme 1) [43]. Table 1 and Scheme 2 show that in the case of vanillylpyruvic acid the main species are electronically ex-

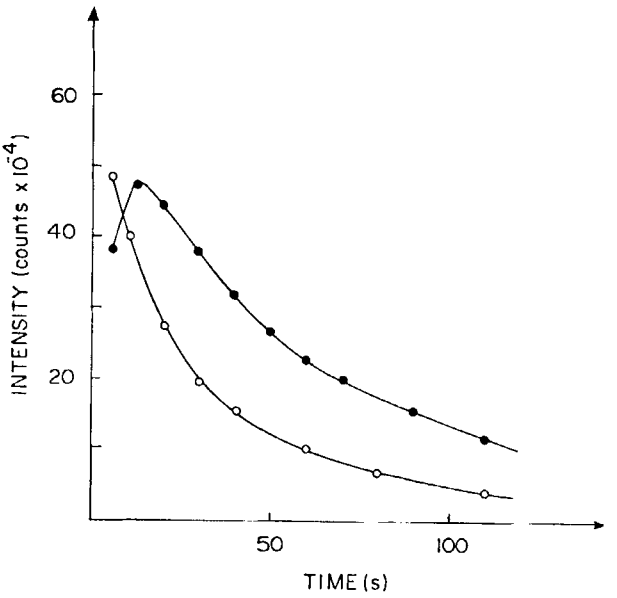


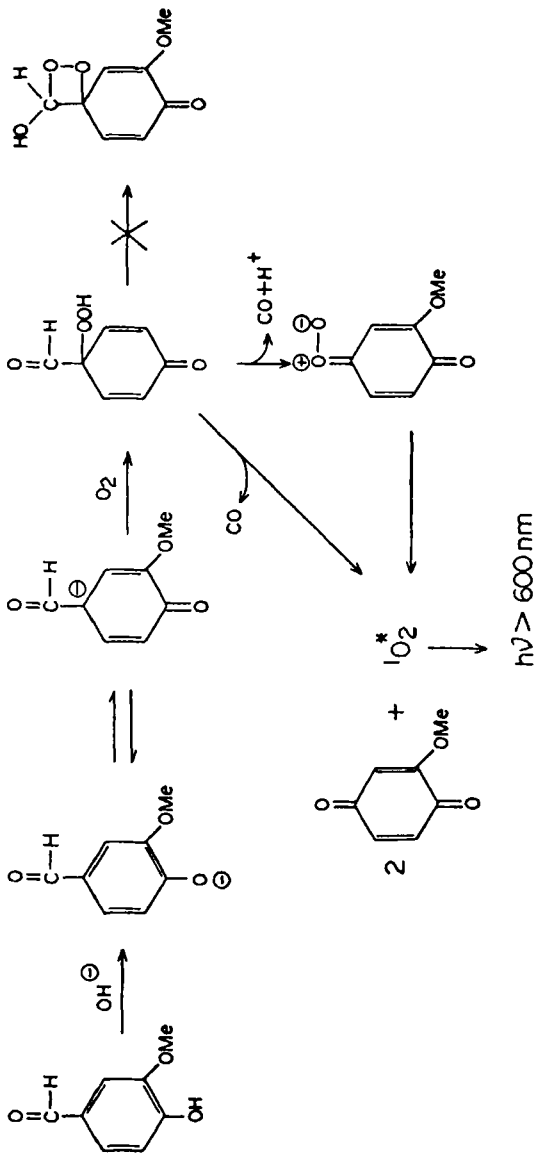
FIG. 3. Temporal behavior of photon emission of unirradiated (○) and irradiated (0.7 J/cm^2) (●) lignin (150 mg/L) in 5 N NaOH solution (LSC-Beckman LS-100c).

cited carbonyl species rather than singlet oxygen. Spectral and TLC analysis showed that one of the emitters is vanillin [44]. No energy transfer from triplet vanillin to oxygen was observed. Similar results were observed with vanillic acid and ferulic acid. The spectral distributions are shown in Fig. 4. From lignin luminescence spectra (unpublished results), it appears that aromatic carbonyls (400-500 nm) and quinones (500-600 nm) are the main emitting species. The chemiluminescence spectrum of lignin in basic media (Fig. 5) shows that the principal emissions are centered around 360-420 nm, 500-600 nm, and above 650 nm. This probably corresponds to ketone, quinone, and singlet oxygen emissions, respectively. The autoxidation of lignin [45] and their models is summarized in Scheme 3.

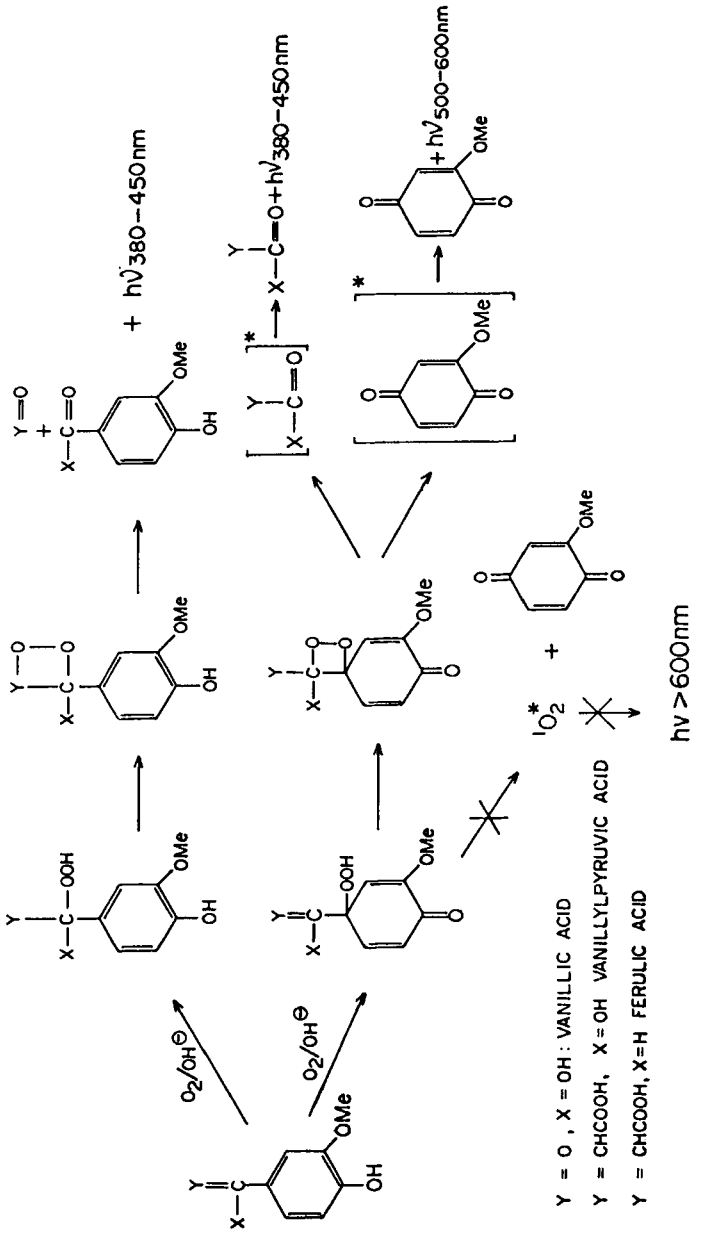
Figure 6 shows the integrated photon emission from unirradiated lignin at three different temperatures. The data can be described by an Arrhenius-type exponential equation with an E_a of 6.18 ± 0.64 kcal/mol (25.8 ± 2.7 kJ/mol). This value of E_a is in agreement with that expected for radical peroxide recombination [46]. The irradiated sample of lignin did not follow the Arrhenius equation, probably due to previous breakage of the macromolecule which can accelerate radical recombination at higher temperatures. Figure 7 shows the

TABLE 1. Influence of Various Agents upon the Emission from the Basic Catalyzed Autoxidation of Vanillin and Vanillylpyruvic Acid

Compounds	Intensity integrated at 30 s (counts × 10^{-4})		Remarks
	Vanillin	Vanillylpyruvic acid	
Control	1.5	21.3	
+ DABCO (20 mM)	2780.0	10.0	Inhibition of $^1O_2$ formation [38] and enhancement of $^1O_2$ emission [38]
+ D_2O/dioxane	40.6	21.0	Enhancement of $^1O_2$ emission [39]
+ N_3^- (20 mM)	0.8	21.0	$^1O_2$ trap [40]
+ Benzoate (10 mM)	0.9	21.5	$OH^\cdot$ trap [41]
+ Bicarbonate (20 mM)	23.9	21.0	Enhancement of the emission when the species is $CO_2^{-\cdot}$ [42]
+ Rose bengal (5 μM)	111.0	60.0	Enhancement of $^1O_2$ emission and carbonyl emission
+ Eosin Y (5 μM)	255.0	89.1	
+ DPAS (20 μM)	1.2	20.0	Enhancement of carbonyl emission [36]
+ DBAS (20 μM)	1.3	30.2	
+ Ascorbic acid (10 mM)	0.0	0.0	Radical trap



SCHEME 1.



SCHEME 2.

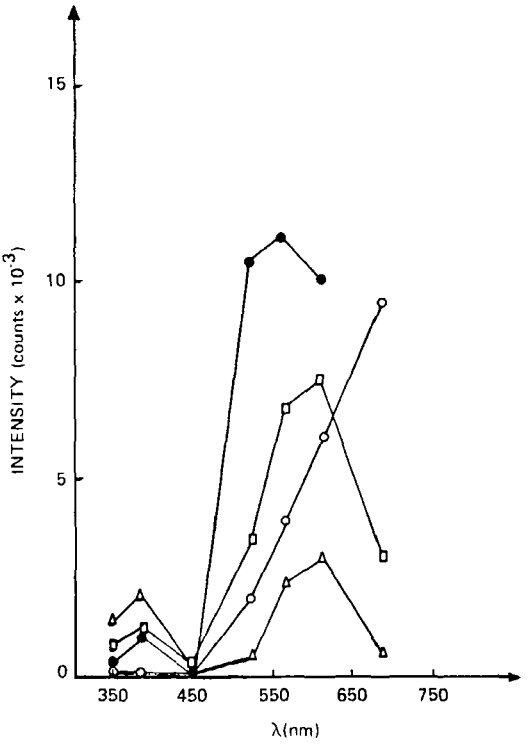


FIG. 4. Spectral analyses of photon emission of basic stimulated chemiluminescence of lignin model compounds (10 mM): (•) vanillyl-pyruvic acid, (□) vanillic acid (intensity ×4), (○) vanillin, and (△) ferulic acid (Hamamatsu TV-C-767 Photocounter).

chemiluminescence of samples irradiated at different fluences. The maximum photon emission occurred at fluences of 1.3 and 2.6 J/cm^2.

The chemiluminescence of lignin in basic media suggest a mechanism in which several activated oxygen species and electronically excited molecules, such as singlet oxygen and carbonyl groups, are involved.

The luminol probe [28] was used to study the structural modifications of pre-irradiated lignin by a more sensitive method. Figure 8 shows that the chemiluminescence from the luminol/$H_2O_2Fe^{2+}$ system, which generates $OH^{\cdot}$ and $O_2^{-\cdot}$, is totally quenched by unirradiated lignin. This is not surprising because it is known that lignin is an excellent antioxidant [47, 48]. Surprisingly, for irradiated samples,

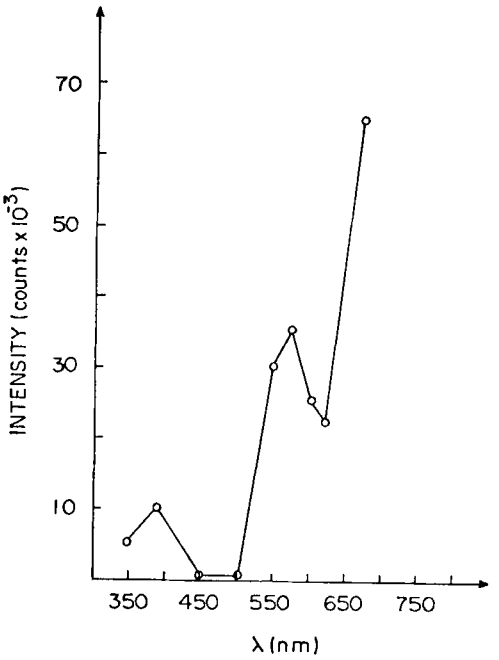
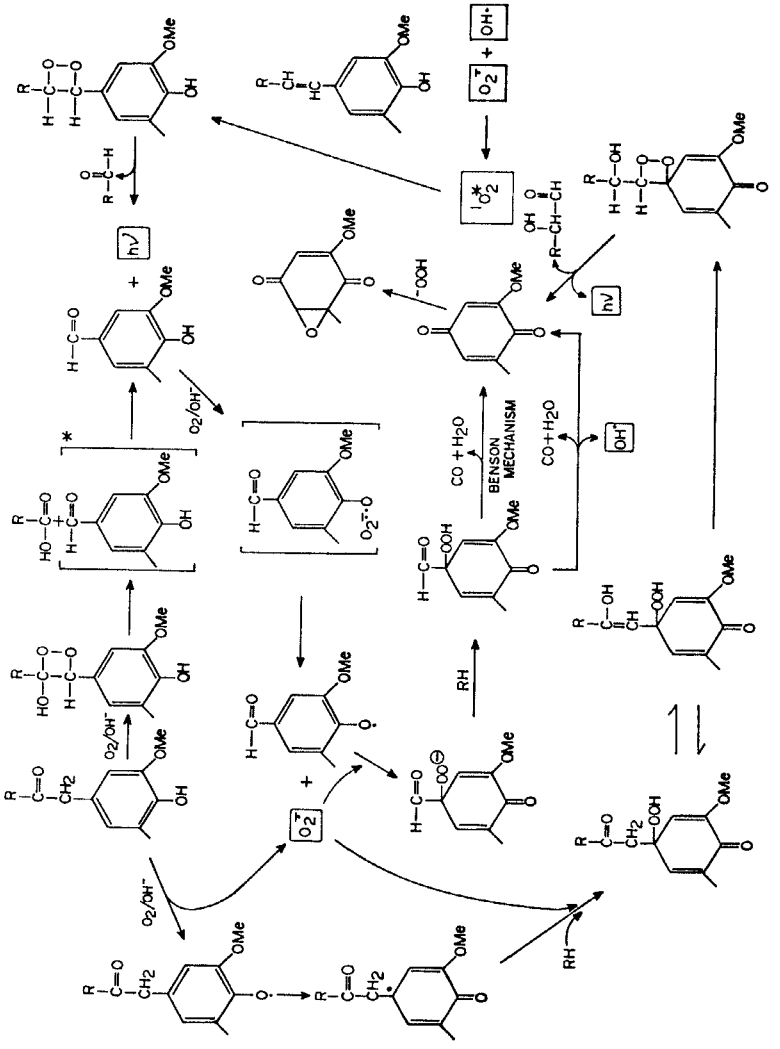


FIG. 5. Spectral analysis of photon emission of basic stimulated chemiluminescence of unirradiated lignin (150 mg/L) solution (dioxane-H_2O (1:1)) (Hamamatsu TV-C-767 Photocounter).

this quenching effect was abolished up to a fluence of 2.5 J/cm^2, but at a fluence of 7.2 J/cm^2 the antioxidant properties appeared again. This suggests depolymerization with generation of radicals at low fluences followed by polymerization and cross-linking, giving a polymer similar to native lignin at higher fluences.

Recently, Strel'skii and Chupka [34] studied the action of horseradish peroxidase on pine tree sapwood by the chemiluminescent method. Gel filtration of the reaction product on Sephadex G-100 indicated that the process occurred with degradation and subsequent aggregation of substrates. Lignin was 52.7% degraded by the process, with 13.5% of the product appearing as high-molecular-weight material. This is in line with our observations in the basic and luminol reaction. Having shown that this latter method is very specific for detecting any small modification in biomass [49], we tested this enzymatic system on lignin modified by different times of irradiation. Figure 9 shows again that the fluence range of 1.3 to 2.5 J/cm^2 is the most significant with respect to production of modifications which facilitate attack by oxidative enzymes.



SCHEME 3.

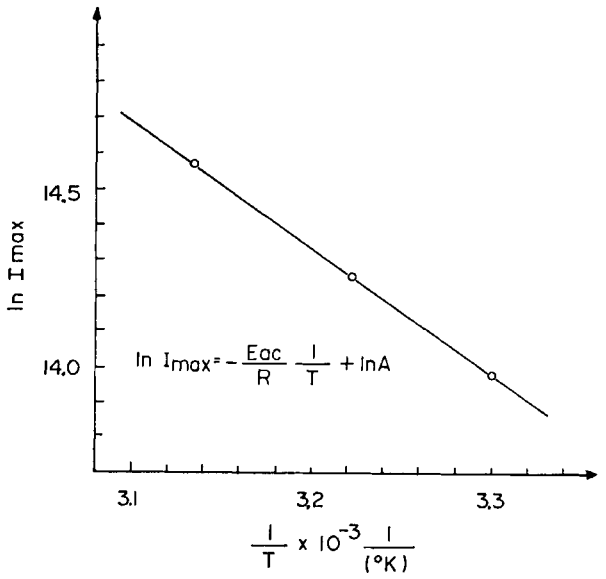


FIG. 6. Arrhenius plot of integrated photon emission of basic stimulated chemiluminescence at different temperatures of unirradiated lignin (LSC-Beckman LS-100c).

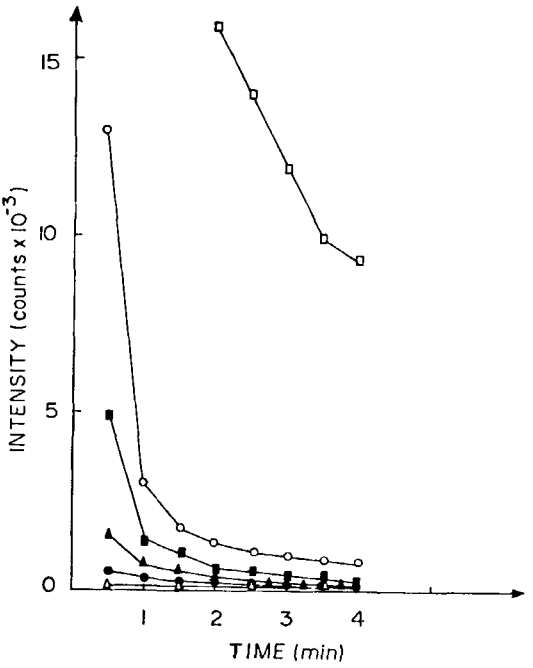


FIG. 7. Temporal behavior of the photon emission of 0.2 mL of lignin (150 mg/L) solution (dioxane-H_2O (1:1)) and 10 μL 5 N NaOH in a total volume of 2 mL of dioxane-H_2O (1:1) at the following fluences: (●) 0.0 J/cm^2, (▲) 0.43 J/cm^2, (■) 0.86 J/cm^2, (□) 1.3 J/cm^2, (○) 2.6 J/cm^2, and (△) 5.2 J/cm^2 (LSC-Beckman LS-7000).

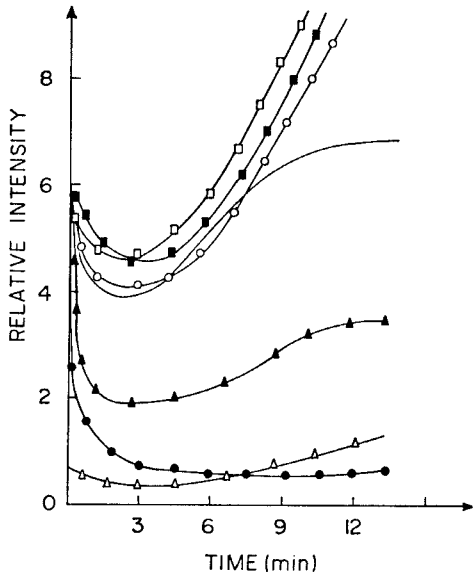


FIG. 8. Temporal behavior of photon emission of the luminol (0.25 mM)/H_2O_2 (1.7 mM)/Fe^{2+} (0.25 mM) system (—) in a total volume of 4 mL of 1.0 mM NaOH, in the presence of 5 μL of lignin (150 mg/L) solution (dioxane-H_2O (1:1)) at the following fluences: (●) 0.0 J/cm^2, (▲) 0.43 J/cm^2, (■) 0.86 J/cm^2, (□) 1.3 J/cm^2, (○) 2.6 J/cm^2, and (△) 5.2 J/cm^2 (Mitchell-Hastings Photometer).

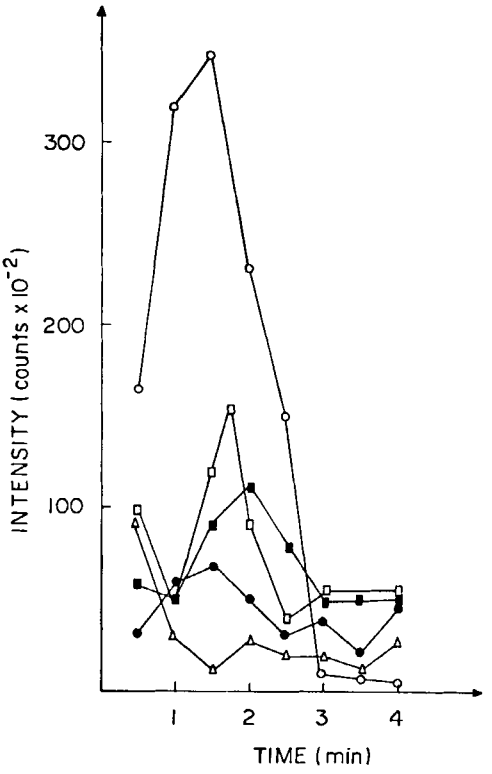


FIG. 9. Temporal behavior of 0.5 mL of lignin (150 mg/L) solution (dioxane-H_2O (1:1)) and horseradish peroxidase (2 μM)/H_2O_2 (90 mM) system in a total volume of 2.3 mL of 0.1 M phosphate buffer pH 5.5, at the following fluences: (●) 0.0 J/cm^2, (■) 0.86 J/cm^2, (□) 1.3 J/cm^2, (○) 2.6 J/cm^2 (intensity × 10), and (△) 5.2 J/cm^2 (LSC-Beckman LS-7000).

Chemiluminescence thus appears to be a very sensitive method for detecting modifications which might increase efficiency of fungal degradation of lignin. Preliminary results for degradation of photochemically pretreated lignolic materials by Aspergillus sp. indeed indicate a nice correlation between the chemiluminescence and the efficiency of fungal degradation [50].

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