Production of Extracellular Hydrogen Peroxide and Peroxidase by Wood-Rotting Fungi

Jerome W. Koenigs

Principal Plant Pathologist, Southeastern Forest Experiment Station, USDA Forest Service, Forestry Sciences Laboratory, Research Triangle Park, North Carolina 27709.

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ABSTRACT

Extracellular H_2O_2 formed by pathogenic and nonpathogenic wood-rotting fungi was detected on two malt extract-heated blood media and by inactivation of a catalase-aminotriazole system. Peroxidase was measured spectrophotometrically. All of 30 species produced some H_2O_2 on blood agar, and 30 of 32 did so from glucose in the catalase-aminotriazole system; brown rot fungi produced more H_2O_2 from glucose than did white rot fungi. No brown rot fungus produced extracellular peroxidase in 3 weeks, but individual isolates of *Lentinus lepideus* and *Coniophora puteana* did so later; 11 of 23 species of white rot fungi secreted peroxidase. This appears to be the first report of the formation of free extracellular H_2O_2 by fungi. This H_2O_2 may be involved in plant pathogenesis and in degradation of plant constituents by wood-destroying fungi.

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Additional key word: pathogenesis.

Hydrogen peroxide is produced intracellularly by several fungal oxidases from appropriate substrates, and is destroyed there by catalase (9). However, the extracellular aspects of its production and the pathological implications of the formation of this chemical by fungi and its activity have been almost totally ignored by plant pathologists. This seems surprising, considering the well-documented role in disease resistance of peroxidase, ascorbic acid oxidase, and catalase.

Hydrogen peroxide itself is a vigorous reagent, and reacts with many host components of interest in pathogenesis. Hydrogen peroxide depolymerizes amylopectin (77) and lowers the ratio of total pectic substances to water-soluble pectic material (24). Even the small amount of naturally occurring H_2O_2 on the surface of cellulose fibers decreases their strength and chemical stability (42); a greater concentration (0.04%) with catalytic amounts of iron completely dissolves and decomposes to CO_2 and H_2O the cellulose in cotton fibers, grass, and sawdust (27). Hydrogen peroxide also oxidizes lignin (see 58, 59, 70), the other major natural polymer. With catalytic amounts of iron and other trace elements, H_2O_2 hydroxylates aromatic compounds (28), oxidizes phenols (37), degrades DNA (82), and oxidizes sugars and the lower fatty acids, hydroxy acids, and dicarboxylic acids (45). Some enzymes such as dehydrogenases (25) and peroxidase and catalase are inactivated by H_2O_2 (63). Hydrogen peroxide lyses bacteria (6, 13), and does so more rapidly with peroxidase (6). The peroxidase system oxidizes phenolic methyl groups (7) such as those found in lignin to form aldehydes and quinones, demethyoxylates lignin compounds (73), and dehydrogenates lignin model compounds (58). Furthermore, the system can split carbon-halogen bonds (29), a feat which may function significantly to aid wood-rotters producing extracellular peroxidase to detoxify chlorinated organic fungicides in wood treated with these preservatives; detoxification of these fungicides by these fungi has been reported (50).

Previous attempts (47, 48, 51) to detect H_2O_2-formation by fungi were unsuccessful; but because of the reactivity of H_2O_2 and its potential for participating in wood decay and pathogenesis directly, or via trace metals or peroxidase catalysis, renewed efforts were made to detect its extracellular presence and to quantify its production by pathogenic and nonpathogenic wood-rotting basidiomycetes. This paper amplifies a previous brief report (43).

MATERIALS AND METHODS.—Extracellular production of H_2O_2 was detected by the growing of several wood-rotting fungi (Table 1) on a heated sheep's blood medium (SBA) similar in its blood component to a chocolate agar used to detect H_2O_2 with anaerobic bacteria; peroxide production was inferred when a green discoloration developed beyond or under colonies growing on SBA and the other blood media (22). The basal medium consisted of 2% malt extract, 2% glucose, 0.1% peptone, and 2% agar (MEA; without agar = MEL) in 950 ml distilled water. The medium was autoclaved for 15 min, and cooled to 80 C, and 50 ml of freshly received, defibrinated sheep's blood (Baltimore Biological Supply) was added, held at 80 C for 10 min and poured, with constant stirring, 25 ml/plate. SBA was adjusted to 4.7 and to 5.4 with concentrated HCl. The plates were inoculated with 28 species of fungi (Table 1) and incubated in the dark to prevent peroxide formation in the medium by light.

Peroxide formation was also detected on similar basal media without sheep's blood but with 11.0 g of bovine hemoglobin powder (type II, Sigma) suspended in 100 ml of cool, sterile distilled water. The suspensions were then added to autoclaved MEA with 900 ml H_2O at 80 C with constant stirring with a magnetic bar to prevent coagulation. The medium (HA) was held at 80 C for 10 min, cooled to 45 C, and adjusted to pH 6.5 with 1 M NaOH, and the plates were poured as described above. The plates were inoculated with 27 species of fungi (Table 1).

In another test, HA was prepared but the pH was adjusted to 5.5, 6.0, 6.5, and 7.0 before autoclaving. The poured plates were inoculated with 10 species (Table 2). Plates and flasks in all tests were inoculated with 6-mm discs cut from the margin of colonies growing on MEA for 1-2 weeks at 25 C. The diameter of the colonies and the green zone were measured and recorded for each isolate at 5 or 7 days, depending on the growth rate of the isolate.

The validity for assuming that the green coloration in media was due to the production of H_2O_2 was tested in two ways: (i) A solution of 0.16 mg hemoglobin (Sigma, H2750)/ml in 0.05 M phosphate buffer at pH 5.5 was treated with H_2O_2 at 2×10^{-4} M final concentration, and the decrease in absorbance at 630 and 405 nm followed with a Beckman DB spectrophotometer and a recorder; (ii) MEL with 5.5 g hemoglobin powder/liter was prepared. The medium (HL) was autoclaved, stirred with a magnetic stirrer for several minutes, then distributed with constant stirring, 30 ml/flask. Flasks were inoculated with 30 species (Table 1), and after 7 days of still-culture incubation at room conditions, culture filtrate was carefully withdrawn from undisturbed flasks, and a spectrum run from 360-720 nm against a blank of autoclaved MEL. The spectrum for each isolate was checked for absence of absorption peaks at 618 nm due to sulfhemoglobin and at 610 nm due to alkaline hematin (52) (both yield a green color that might be confused with that formed by H_2O_2), and for the disappearance of the methemoglobin peak at 630 nm as does occur with authentic H_2O_2 (0.03%). Efforts to detect H_2O_2 production spectrophotometrically at 405 nm in cultural filtrates of MEL with the titanium reagent of Bonet-Maury (63, p. 561) in a ratio of 10 parts of filtrate to 1 of reagent (17) were unsuccessful, apparently because of the low endogenous concentrations.

Production of H_2O_2 was also detected with a system (11) in which peroxide formation is considered proportional to the inactivation of catalase by aminotriazole + microbially generated H_2O_2; inactivation is measured in turn by decomposing 0.006 M H_2O_2 with the noninactivated catalase and titrating the residual H_2O_2 with permanganate (10). These procedures were used as published, except that the catalase-aminotriazole-glucose (C-AT-G) incubation medium was (i) prepared in the 0.05 M phosphate buffer at pH 5.5; and (ii) added to the 0.006 M H_2O_2 solution directly without dilution after incubation; (iii) catalase (Sigma, C-10; 0.25 mg/ml) was added to the buffer 0.5-1.0 hr before adding the other components to destroy any endogenous H_2O_2. Aminotriazole (Sigma) was recrystallized from ethanol and dried in vacuo over oven-dried silica gel for 18-24 hr at room temperature. Cultures for tests with this system were grown in 30 ml of MEL in 125-ml Erlenmeyer flasks for 2 to 3 weeks (Table 1). At the time of harvest, the medium was gently decanted and the cultures were drained well and rinsed with buffer which was decanted and drained. Ten ml of C-AT-G incubation medium was then added to each of three replicate flasks for each isolate. After 30 min, a 0.5-ml sample was withdrawn from each flask and added to the H_2O_2 solution, and the procedure was completed. Controls (three replicates each) consisting of (i) 0.5 ml phosphate buffer pH 5.5 (peroxide control) were run once and (ii) 0.5 ml incubation medium were run approximately every 30 min in each experiment. The production of H_2O_2 by the fungi in the C-AT-G system was calibrated with H_2O_2 generated (10.87 μM/ml per min) by adding 1 mg of glucose oxidase (Sigma, Type II) in 1 ml distilled water/10 ml of C-AT-G medium and withdrawing 0.5-ml samples at 0, 15, 30, and 60 min

TABLE 4. Influence of age of culture on peroxidase activity in culture filtrates of wood-rotting fungi incubated on a malt extract medium

Species[a]	Rot type[b]	Age of culture (days) 3 Absorbance units[c]	3 Dry wt, mg	7 Absorbance units[c]	7 Dry wt, mg	14 Absorbance units[c]	14 Dry wt, mg
Coniophora puteana (Mad. 515)	B	0.000	7.4	0.001	51.1	0.046	
Corticium galactinum	W	0.003	6.7	0.000	41.0	0.005	
Fomes igniarius	W	0.000	7.3	0.000	11.5	0.005	
F. pini	W	0.000	7.8	0.000	17.0	0.036	
F. robustus	W	0.000	5.2	0.042	18.8	0.170	
Merulius tremulosus	W	0.000	29.1	0.016	109.2	0.014	
Pleurotus ostreatus	W			0.001			
Polyporus adustus	W	0.002	29.2	0.029	108.0	0.039	
P. hispidus	W			0.003			
P. radiatus	W	0.000	8.7	0.000	18.8	0.034	
Poria weirii	W	0.005	8.2	0.011	27.5	0.590	

[a] See Table 1 for isolate identification numbers.

[b] W = white rot; B = brown rot.

[c] Absorbance calculated as the rate of increase per min for 2 min at 450 nm of the oxidation of *o*-dianisidine in 0.006 M H_2O_2 at pH 6.0 for 1 ml of pooled culture filtrate from three replicate flasks as explained in the text.

TABLE 1. Production of H_2O_2 by wood-rotting fungi on sheep's blood (SBA) and bovine hemoglobin agars (HA), in a bovine hemoglobin liquid medium (HL) and during incubation in a catalase-aminotriazole-glucose system (C-AT-G)

Species[a]	SBA, pH 5.4		HA, pH 6.5		HL	C-AT-G
	Growth mm, at 6 days[b]	Reaction ratio[c]	Growth mm, at 6 days[b]	Reaction ratio[c]	Absorbance at 630 nm, % of control[d]	μM H_2O_2/min per mg oven-dry mycelium[e]
Brown rot fungi						
Coniophora puteana (Schum. ex Fr.) Karst. (FP 94042-R)	46	1.15	32	1.78	37	11.64
C. puteana (Schum. ex Fr.) Karst. (Mad. 515)	48	0.00	77	0.51	59	0.08
Fomes pinicola (Swartz ex Fr.) Cke. (A 68)	57	1.11	58	1.05	06	11.84
Gleophyllum trabeum (Pers. ex Fr.) Murr. (Mad. 617)	52	0.98	53	1.08	59	11.28
Lentinus lepideus Fr. (Mad. 534) -S)	44	1.00	51	0.90	74	15.04
L. lepideus Fr. (OKM 2414-S)	24	0.96	26	1.27	89	1.49
Polyporus balsameus Pk. (FP 71191-S)			35	1.17	15	10.16
P. schweinitzii Fr. (FP 14854-S)	22	1.14	46	1.04	57	1.05
Poria incrassata (Berk. & Curt.) Burt (Mad. 563)	52	1.10	62	1.00	28	9.66
P. monticola Murr. (FP 94627-R)	47	1.15	54	1.15	00	13.42
P. vaillantii (Fr.) Cke. (FP90877-R)	37	1.35	39	1.49	22	17.33
						$\bar{X}$ 8.67
White rot fungi						
Armillaria mellea (Fr.) Quel. (A 46)					30	0.04
A. mellea (Fr.) Quel. (OKM 2911-S)					59	0.00
Clitocybe tabescens (Scope.) Gill. (A 139)	16	0.00	11	1.00	76	1.76
C. tabescens (Scope.) Gill. (FP 103448-S)	76	0.89	22	0.86	00	4.54
Corticium galactinum (Fr.) Burt. (FP 105496-S)	14	1.02	42	0.93	13	7.27
Flammulina velutipes (Curt. ex Fr.) Sing. (A 41)			31	0.58	93	0.35
F. velutipes (Curt. ex Fr.) Sing. (OKM 6261-Sp)	54	0.00	81	0.94	57	2.90
Fomes annosus (Fr.) Karst. (C-23)	71	0.77	51	0.88	69	10.72
F. annosus (Fr.) Karst. (C-128)	49	0.86	69	0.90	54	15.28
F. annosus (Fr.) Karst. (C-133)	55	0.75	54	0.68	52	13.07
F. igniarius (L. ex Fr.) Kickx. (L00 17171-S)	26	0.38	40	0.48	20	3.17
F. pini (Fr.) Karst. (FP 53236-S)	16	1.91	20	0.65	83	0.43

Species[a]	SBA, pH 5.4		HA, pH 6.5		HL	C-AT-G
	Growth mm, at 6 days[b]	Reaction ratio[c]	Growth mm, at 6 days[b]	Reaction ratio[c]	Absorbance at 630 nm, % of control[d]	μM H_2O_2/min per mg oven-dry mycelium[e]
F. robustus Karst. (L00 17375-S)	22	0.91	33	0.70	00	0.27
Ganoderma lucidum (Leys. ex Fr.) Karst. (L00 14518-S)	81	0.97				1.18
Lentinus tigrinus (Bull. ex Fr.) (466)	67	0.87	80	0.89	13	3.83
Merulius taxicola (Pers.) Duby (FP 105568-Sp)	29	0.93	30	1.02	34	0.11
M. tremulosus Schrad. ex Fr. (JHG 344-Sp)	52	1.02	70	1.01	09	0.00
Pleurotus ostreatus (Jacq. ex Fr.) Kumm. (OKM 3563-Sp)	28	0.96	37	1.46	20	4.05
Polyporus adustus Willd. ex Fr. (FP 105256-Sp)	81	0.74	83	0.90	24	1.88
P. dichrous Fr. (FP 104268-Sp)	50	0.84			26	0.74
P. hispidus Bull. ex Fr. (FP 48229-S)	81	0.90	13	0.85	13	0.00
P. radiatus Sow. ex Fr. (RLG 5063-Sp)	30	0.60	39	0.44	85	1.08
P. tomentosus var. *circinatus* (B 98)						2.01
P. tomentosus var. *circinatus* (B 236)					59	2.63
P. versicolor L. ex Fr. (A 15)			60	1.04	17	0.10
Poria weirii Murr. (FP 91601-S)	17	0.00	29	0.93	00	0.64
Schizophyllum commune Fr. (A 9)	82	0.90			117	10.14
Stereum frustulatum Pers. ex Fr. (FP 106073-S)	42	0.86	44	0.99	06	0.96
S. sanguinolentum A. & S. (A 150)	42	0.86	42	0.76	59	1.53
						$\bar{X}$ 3.12

[a] Isolates with prefix A from E. B. Cowling, N. C. State University, Raleigh, N. C.; with B, from E. W. Ross, Forestry Sciences Laboratory, Athens, Ga.; with C, from J. W. Koenigs, Forestry Sciences Laboratory, Research Triangle Park, N. C.; with Mad., from Forest Products Laboratory, Madison, Wisc.; other isolates from Forest Disease Laboratory formerly at Laurel, Md., and now located at the Forest Products Laboratory. Numbers following A, B, and C prefixes are the actual numbers from the respective culture collections.

[b] Average diameter of three replicates/test.

[c] Ratio of the average diameter of green zone under colonies for three replicates/test divided by the average diameter of the colonies.

[d] Absorbance calculated as percentage reduction of height of the methemoglobin peak at 630 nm for samples of cultural filtrate from 7-day-old cultures in standard cell and MEL in reference cell for single replicates of each isolate.

[e] Average of three replicates for each fungus for three experiments with 2- to 3-week-old cultures calculated on the basis that 1.0 ml of a glucose oxidase solution generating 10.87 μM H_2O_2/ml per min (2) produced 52.6% inhibition of the C-AT-G system at 15 min.

TABLE 2. Effect of pH on production of H_2O_2 by wood-rotting fungi on bovine hemoglobin agar

Species[a]	Rot type[b]	Age of culture, days	pH 5.5 Growth, mm[c]	pH 5.5 Reaction ratio[d]	pH 6.0 Growth, mm[c]	pH 6.0 Reaction ratio[d]	pH 6.5 Growth, mm[c]	pH 6.5 Reaction ratio[d]	pH 7.0 Growth, mm[c]	pH 7.0 Reaction ratio[d]
Flammulina velutipes (A 41)	W	7	31	0.39	34[e]	0.82	35	0.83		
Fomes annosus	W	7	59	0.70	51	0.88	51	0.80	52	0.77
F. igniarius	W	5	31	0.84	32	0.34	32	0.31	29	0.28
F. pini	W	7	27	1.22	27[e]	1.33	29[e]	1.14	16	1.06
F. robustus	W	7	42	0.57	40	0.63	36	0.75	36	0.72
Gleophyllum trabeum	B	7	62	1.08	62	1.03	59	0.93	58	0.95
Merulius tremulosus	W	5	55	1.04	59	0.97	51	1.04	34	1.12
Polyporus dichrous	W	5	78	0.91	78	0.91	68	0.94	62	0.84
Poria vaillantii	B	7	48	1.33	49	1.31	44	1.30		
P. weirii	W	5	28	0.78	20	0.70	26	0.85	18[f]	0.66

[a] See Table 1 for isolate identification number.
[b] W = white rot; B = brown rot.
[c] Average colony diameter of three replicate plates except where noted.
[d] Average diameter of green reaction zone attributed to H_2O_2 production divided by average colony diameter.
[e] Two replicates.
[f] One plate.

TABLE 3. Influence of age of culture on peroxidase activity in culture filtrates of wood-rotting fungi incubated on a liquid malt medium[a]

Species[b]	Rot type[c]	Time (weeks) 3[d]	4[d]	12[e]	16[d]
		Absorbance, △/min			
Armillaria mellea (A 46)	W	0.000	0.004	0.013	0.014
A. mellea (OKM 2911-S)	W	0.000	0.000	−0.070	0.000
Coniophora puteana (Mad. 515)	B	0.000	0.004		−0.009
Corticium galactinum	W	0.008	0.150	−0.672	0.006
Fomes annosus (C-128)	W	0.000	0.000	0.003	0.000
F. igniarius	W	0.015	0.174	0.003	0.675
F. pini	W	0.050	0.181	0.015	
F. robustus	W	0.439	0.800	0.006	0.220
Ganoderma lucidum	W	−0.002	0.620	0.000	−0.012
Lentinus lepideus (Mad. 534)	B	0.000	0.004	0.000	0.002
L. lepideus (OKM 2414-S)	B	0.000	0.000	0.005	0.000
L. tigrinus	W	−0.004	0.030	0.015	0.012
Merulius tremulosus	W	0.004	0.016	0.019	0.051
Pleurotus ostreatus	W	0.002	0.004		0.003
Polyporus adustus	W	0.028	0.096	0.016	0.004
P. hispidus	W	0.028	0.140		0.003
P. radiatus	W	0.058		0.028	0.521
P. tomentosus (B-236)	W	0.000	0.000	0.454	0.000
P. versicolor	W			0.014	
Poria weirii	W	1.226	5.09	0.405	0.205

[a] The species (from Table 1) not producing peroxidase are not listed in this table.
[b] See Table 1 for isolate identification numbers.
[c] W = white rot; B = brown rot.
[d] Absorbance calculated as the rate of increase per min for 2 min at 450 nm of the oxidation of *o*-dianisidine in 0.006 M H_2O_2 at pH 6.0 for 0.1 ml of pooled culture filtrate from three replicate flasks as explained in the text.
[e] Values based on samples from one flask.

and assaying for inhibition; aliquots of the glucose oxidase solution were assayed simultaneously for gluconic acid production (2), and the rate of H_2O_2 production was calculated. Mycelia were harvested on oven-dried tared Whatman No. 2 filter papers, and the weights were recorded after drying the mycelia for 1-2 days at 60 C.

Extracellular peroxidase was detected in MEL at 3, 4, 12, and 16 weeks in one test (Table 3) and at 3, 7, and 14 days in another test (Table 4) under cultural conditions and by harvesting as previously described (44); however, peroxidase was assayed with a more sensitive *o*-dianisidine system (1) with and without H_2O_2 (to measure peroxidase plus polyphenol oxidase and polyphenol oxidase activity, respectively), using 0.1 ml of culture filtrate substituted for the enzyme solution in the reference cuvette; peroxidase activity was calculated as the difference in absorbance per minute between the activities of the two systems. Fungi-producing peroxidase after 7 days were tested for polyphenol oxidase in MEA with 0.1% gallic and tannic acids as recommended by Lyr (48) and rated visually (16). Measurement of the production of extracellular catalase in culture filtrates was attempted by following the decrease in absorbance at 240 nm in H_2O_2 solutions with an absorbance of 0.840 (5), and in some instances, 0.255.

RESULTS.—*Detection of H_2O_2 on SBA, HA, and HL media.*—In experiments with SBA at pH 4.7 and 5.4 and in one experiment with HA at 6.5, nearly all brown and white rot fungi produced H_2O_2 after 6 days. Results of typical tests with SBA at pH 5.4 and HA are presented in Table 1. Of fungi not producing H_2O_2 at 6 days, almost all did so by 13 days; e.g., the one negative isolate of *Clitocybe tabescens* and that of *Poria weirii* were 28 and 27 mm in diameter, with reaction ratios of 0.96 and 0.92, respectively. Some fungi showing negative reactions at 6 days overgrew the plates by the end of the 2nd week, and their reactions could not be definitely assessed. The results of the three tests taken as a group, however, confirm that all species can produce H_2O_2. Isolates of *Coniophora puteana, Fomes pini, Lentinus lepideus, Poria vaillantii, Pleurotus ostreatus, Polyporus balsameus,* and *Poria monticola* produced reaction zones clearly beyond their colony margins, and were particularly high producers of H_2O_2. Because reaction zones differed little from the background at low pH levels on both SBA and HA media, it was difficult to conclude whether pH affected production of H_2O_2 or merely the intensity of the reaction.

Spectra from 380-720 nm for buffered hemoglobin showed that H_2O_2 at 2×10^{-4} M final concentration eliminated the methemoglobin peak at 630; it lowered the Soret peak at 405 nm from an absorbance of 0.853 to 0.229, and shifted it to 418 nm. Spectra for isolates grown on HL medium indicated that all isolates except *Schizophyllum commune* caused a variable decrease in absorbance of the methemoglobin peak at 630 nm (Table 1) and of the Soret peak at 405 nm. Neither H_2O_2 nor any fungus caused peaks to appear at 618 nm (sulfhemoglobin) or 610 nm (alkaline hematin); either would have made results difficult to interpret. The curves for isolates *C. tabescens, Fomes robustus, Lentinus tigrinus, P. monticola, P. weirii,* and *Stereum frustulatum* were virtually identical with that for authentic H_2O_2. Those of all fungi other than *S. commune* caused some decrease of the peaks at 630 and 405 nm. Lack of replication makes any further quantitative interpretation unwise now, although the method would appear adaptable to such studies; if this were done, absorption should routinely be measured at 620 nm, an isobestic wavelength (26, 30), to avoid quantitative error among isolates due to pH changes by the fungi.

Effect of pH of HA and concentration of hemoglobin powder on detection of H_2O_2.—All 10 fungi tested gave a positive and a fairly consistent reaction on HA at all pH levels (Table 2). The reaction ratios of *Flammulina velutipes* and of *Fomes igniarius* at pH 5.5 seem to be anomalous and to vary significantly from those at other pH levels. Although most reaction ratios appear quite consistent for each of the other fungi at all pH levels, the medium adjusted to pH 6.5 before autoclaving was more reddish, and reactions were distinctly clearer at this pH and at 7.0.

Generally the usual concentration of bovine hemoglobin powder was more favorable for detecting H_2O_2 production, although in tests not reported here, a lower concentration (5.5 g/liter) resulted in slightly larger ratios of reaction to growth, but zones were not so distinct as with HA. The reaction zone, which even beyond the margin of the colony, extended to the bottom of the plate below the colonies, indicated that the oxidant had diffused from the hyphae.

Detection of H_2O_2 production with the C-AT-G system.—Two- to 3-week-old mycelial mats of nearly all isolates produced H_2O_2 from glucose, as is indicated by inactivation of the catalase-aminotriazole system (Table 1). Some fungi, such as *Polyporus versicolor, Polyporus hispidus,* and individual isolates of *Armillaria mellea* and *C. puteana,* produced so little inactivation in the three experiments that production of H_2O_2 was not confirmed. Detecting H_2O_2 at low levels of production was complicated by rapid inactivation of C-AT-G between 1 and 2.5 hr due to causes intrinsic to the system in the absence of mycelium. Although controls were included to measure the rate of intrinsic inactivation, the test still was ambiguous with a few isolates. Even so, if these same fungi were assayed quickly enough, i.e., before C-AT-G inactivation entered the log phase, they clearly produced some H_2O_2. Results with the assay for these fungi clearly demonstrated extracellular H_2O_2, but are not considered very useful quantitatively. The results reported here are for complete experiments in which results were obtained before intrinsic inactivation reached 20% of its maximal value (volume of 0.01 N $KMnO_4$ = ca. 14% of that required for titrating H_2O_2 controls to a pink end point).

Peroxidase production.—All culture filtrates were tested for extracellular peroxidase production after 3

weeks and most of them after 4, 12, and 16 weeks. None of the nine species of brown rot fungi listed in Table 1 produced extracellular peroxidase after 3 weeks, but individual isolates of *L. lepideus* and *C. puteana* did so slightly by the 4th week (Table 3); activity was variable at 12 and 16 weeks. Eleven of the 23 species of white rot fungi listed in Table 1 produced the enzyme (Table 3); *A. mellea, Ganoderma lucidum,* and *L. tigrinus* did not produce extracellular peroxidase after 3 weeks, but did so by the 4th week. Two white rot fungi, *P. versicolor,* and one isolate of *Polyporus tomentosus* produced peroxidase later. Activity of *P. weirii* and *F. robustus* was exceptionally high.

Fungi producing extracellular peroxidase at 3 weeks were further tested to determine the rapidity of peroxidase formation by harvesting at 3, 7, and 14 days after inoculation and assaying for peroxidase. Slight extracellular peroxidase activity was detected for three fungi after 3 days, for two additional fungi after 7 days, and for all fungi at the end of 2 weeks (Table 4).

Peroxidase-H_2O_2 interference with the Bavendamm reaction.—Bavendamm reactions of the four fungi which produced peroxidase in MEL at 7 days (from Table 3) and their H_2O_2-reaction ratios (from Table 1) are presented in Table 5 along with an estimate of the peroxidase systems's interference with the interpretation of the test for polyphenol oxidase.

DISCUSSION.—The data indicating that at least some isolates of all the fungi produced a characteristic green color on SBA, HA, that some mimicked the spectrum of H_2O_2 in HL, and that nearly all isolates inactivated catalase in the C-AT-G system, suggest that the ability to form extracellular H_2O_2 is a general characteristic of wood-rotting species and not peculiar to either white or brown rot fungi.

The possible role of H_2O_2 and/or the H_2O_2-peroxidase system in the decomposition of wood cellulose is intriguing, as decay has been considered to proceed via cellulase. H_2O_2 (0.04%) and Fe completely decompose cellulose in vitro (27), but apparently this has not been considered a possible reaction in wood decay by microorganisms. H_2O_2 may be a strong oxidizing agent under the acid conditions found in wood (71), and the little H_2O_2 occurring naturally on paper is reported to decrease its strength and chemical stability (42). Because H_2O_2 and Fe decompose cellulose, it might be expected that they would also cause it to swell and decrease its strength. In preliminary experiments, H_2O_2 at 0.03% swelled cotton cellulose 11%, and swelling increased to 39% at 0.5% H_2O_2; 1% H_2O_2 reduced the degree of polymerization 35% (J. Koenigs, *unpulished data*); with 26 μg Fe/ml, H_2O_2 increased swelling, the reducing capacity, and the amount of alkali-soluble material of residual cellulose, whereas strength, toughness, and degree of polymerization decreased rapidly with increasing concentration of H_2O_2. The results with H_2O_2 and Fe on cotton cellulose are virtually identical with those produced by brown rot fungi on cellulose in wood (15) and by acid hydrolysis, and contrast sharply with the effect of white rot fungi (15). Perhaps significant here is that brown rot fungi produced about 3 times as much H_2O_2 from glucose as did white rot fungi (Table 1). Furthermore, the brown rot fungi form H_2O_2 from a greater variety of sugars than do white rot fungi (J. Koenigs, *unpublished data;* glucose, xylose, mannose, galactose, maltose, sorbose, and trehalose were tested).

Lignin decomposition has been viewed almost strictly as an enzymatic process. Polyphenol oxidases of the white rot fungi are the implied agents (41, 70), although the role of phenol oxidase systems apparently is unclear (30, 32, 40, 41). Decomposition of lignin with ground mycelium and culture filtrates have been attempted and oxygen uptake has been reported (23), but the results have been questioned (70), and efforts with purified enzymes have been unsuccessful. If oxidation of dihydric phenols is the primary function of polyphenol oxidase, it is difficult to postulate how this can account for the loss of lignin during decay. However, laccase can split alkyl-phenyl carbon-to-carbon bonds in lignin model compounds; thus, ca. 41% of the phenylpropanoid units in spruce lignin are potentially susceptible to this cleavage (40). The H_2O_2 molecule, because of its small size and the accompanying lack of restricted substrate range inherent to an enzyme system, should be much freer than is polyphenol oxidase, for example, to attack a polymer such as lignin. Formation by H_2O_2 of organic peroxides or free radicals in lignin (58, 76) might lead to its subsequent depolymerization or furnish a "substrate" on which oxidation of lignin can occur (40). The total H_2O_2 available to peroxidase-forming white rot fungi might account for the differences between some of them and the brown rot fungi in their capacity to degrade lignin. Peroxidase does increase the rate of destruction of polymers as complex as bacterial cell walls (6, 13).

Although glucose was the principal carbohydrate supplied in all three media in these tests, it should not be inferred that the H_2O_2-producing enzyme is necessarily glucose oxidase. Wood-rotting fungi possess other carbohydrate oxidases capable of oxidizing glucose (49, 80, 81), xylose (35, 49, 60, 80, 81), sorbose (35, 60, 80, 81), galactose (3, 14, 80) and galactosides (3), and other carbohydrates (35, 60). Other of their oxidases act on simple primary (33, 34, 39) and aromatic (20) alcohols. An oxalic acid decarboxylase from white rot fungi (65, 66) functions with oxygen catalytically in other fungi to produce H_2O_2 when reduced phenols are present (19, 22, 46). Oxalic acid oxidase (75) has been isolated from another basidiomycete and from imperfect fungi (18, 19, 46), and a formate oxidase has been recovered from the same basidiomycete (74). Wood-rotters produce oxalic acid from phenyl-propane monomers of lignin (32) and simple carbohydrates (54, 55, 72), and white rot fungi, in contrast to brown rot fungi, further catabolize the oxalic acid (64, 65, 66, 67, 72); as mentioned above,

TABLE 5. Interference of the peroxidase system with the Bavendamm reaction

Species	Peroxidase △A/min[a]	H_2O_2 reaction ratio[b]	Bavendamm reaction[c]		Interference of peroxidase system with polyphenol oxidase	
			Gallic	Tannic	Gallic	Tannic
Fomes robustus	0.042	0 0.70	4	3	–	–
Merulius tremulosus	0.016	1.02	3	3		
Polyporus adustus	0.029	0.90	2	3	+	–
Poria weirii	0.011	0.74	2	3	+	–

[a] From Table 4.
[b] From Table 1.
[c] On 0.1% (48) tannic acid medium (16) rated 1 week after inoculation. 2 = Reaction zone ⟨ mat diameter. 3 = Reaction zone ⟩ mat diameter. 4 = Reaction zone ⟫ mat diameter.

H_2O_2 is produced during oxalic acid catabolism. Although Lyr & Ziegler (51) report that neither glucose oxidase nor oxalic acid-oxidase activity was found in culture filtrates of two white rot fungi, others report that glucose oxidase is extracellular in other fungi (21), and extracellular H_2O_2 was produced from glucose in this study. Oxalic acid oxidase is tightly bound to cell walls of another basidiomycete (75). Thus, several H_2O_2-producing enzymes have been found in wood-rotting fungi; this suggests that these organisms be surveyed for these enzymes, and the relationship between the enzymes and the type of rot which the fungi cause should be explored.

The present study provides experimental evidence that fungi can produce the primary substrate for peroxidase, and thus establishes a tentative but currently absent logic relating this enzyme's association in disease resistance to pathogenicity. In this study, there was no strong relationship between the pathogenicity of the species of wood-rotting fungi and their ability to produce H_2O_2 from glucose. *Fomes annosus* and *Corticium galactinum*, two pathogenic white rot fungi, produced relatively large amounts of H_2O_2 in the C-AT-G system, whereas the pathogens, *A. mellea, C. tabescens, F. pini, Polyporus schweinitzii, Polyporus tomentosus* var. *circinatus*, and *P. weirii* produced little. Peroxide-regulated pathogenicity may be related to other substrates or may be a mechanism for only certain fungi. Live sapwood is resistant to many fungi which readily decay dead sapwood of the same host; dead sapwood, in fact, has little resistance to any wood-rotting fungi (62). In live wood, and probably in recently air-dried lumber with presumably higher levels of residual catalase and/or peroxidase and polyphenol oxidase than dead tissues, the ability of the fungi to parasitize the host or to cause decay could be regulated by the levels of the enzymes and the oxidase substrates in the host or the ability of the fungi to secrete H_2O_2 or inhibitors of the enzymes; e.g., the polyphenol oxidase system which inhibits glucose oxidase (53).

The four fungi producing peroxidase in liquid medium at 7 days were tested for polyphenol oxidase on agar at this same time interval. A comparison of peroxidase activity, peroxide reaction ratios, and Bavendamm reactions, which is manifested by the formation of brown oxidation products, at 1 week indicated that the peroxidase system, which also oxidizes the phenols, could have completely obscured the Bavendamm reaction for *P. adustus* and *P. weirii* on gallic but not tannic acid medium, and possibly for *Merulius tremulosus* on both phenols (Table 5). Less ambiguity in discriminating between peroxidase and polyphenol oxidase is an additional reason for using tannic acid to detect polyphenol oxidase (48). Generally, fungi produced so little peroxidase by 1 week that it appears unlikely to obscure the Bavendamm ratings for most fungi, if the production and diffusion of peroxidase are similar on agar and liquid media, and unless amounts of peroxidase spectrophotometrically undetectable in liquid media can oxidize gallic and tannic acid over a longer period in the solid medium. If polyphenol oxidase reactions are evaluated after 2 weeks, the likelihood increases slightly that the peroxidase system partially interferes with detection of polyphenol oxidase, as nine fungi produced peroxidase in this time interval. Production of H_2O_2 by all fungi on solid media indicates that when polyphenol oxidase and peroxidase are produced, part of the reaction currently attributed to polyphenol oxidase is due to peroxidase, and spectrophotometric methods should be used to assay each separately.

Investigation of H_2O_2 and peroxidase reactions may apply to other problems in wood deterioration, such as enzymatic and nonenzymatic discolorations which develop during milling operations. With catalytic amounts of H_2O_2 and Mn, the peroxidase-H_2O_2 complex acts as a phenol oxidase (61), and Mn accumulates in sugar maple wood discolored by fungi (68). Other trace metals and H_2O_2 may oxidize phenols nonenzymatically (61) to yield colored products. Furthermore, in the presence of Mn and oxidogenic phenol donors, the peroxidase complex can oxidize oxalic acid to H_2O_2 (38). The possibility that H_2O_2 could have been formed from oxalic acid and involved in pathogenic or degradative effects in addition to its direct effect on polygalacturonase (4) should be entertained.

In preliminary trials, steady-state H_2O_2 levels were low in liquid cultures of wood-rotting fungi, or H_2O_2 may have been present for only a short time (36). Thus, it seemed desirable to use methods which

would physically separate H_2O_2 and catalase or incorporate a biochemical H_2O_2 trap. In solid, heated blood media, H_2O_2 apparently diffused more rapidly than any extracellular catalase or pseudocatalase (79) or the blood pigments slowed its decomposition (36). Similar relative diffusion rates might be expected in wood; and thus, the concentration of H_2O_2 could become relatively high, and the chemical would be free to react chemically before it was destroyed; e.g., in kiln-dried lumber. The principle of the C-AT-G system involves a biochemical trap, and it too yielded positive indications of H_2O_2 production.

Although heated blood media have been used for many years to detect production of H_2O_2 by anaerobic bacteria (see 22), little information is available about the chemistry of the reactions and the rationale of the preparative steps. The medium is heated, apparently to destroy catalase and to convert oxyhemoglobin to methemoglobin; there were indications during these tests that temperature and duration of heating should be carefully regulated. If the medium as finally prepared were to contain residual catalase activity, sensitivity in detecting H_2O_2 secretion by the microorganisms would be less; if all catalase activity were destroyed, H_2O_2 of atmospheric origin might affect the contrast between inoculated and noninoculated areas of the medium.

Authentic H_2O_2 produced a green discoloration on heated blood agar, and did so in both SBA and HA media. Although 47 compounds form colored complexes with methemoglobin (52), only sulfhemoglobin (absorbance maximal at 618 nm), alkaline hematin (absorbance maximal at 610 nm), and the discoloration due to H_2O_2 are green. Lack of peaks at 618 and 610 nm, the disappearance of the methemoglobin peak at 630 nm upon treatment of HL with H_2O_2 (8), and similar spectra from 380 to 720 nm for culture filtrates of some of the fungi plus the production of the green discoloration by them and authentic H_2O_2 on solid blood media are evidence that the oxidant was H_2O_2. Hydrogen peroxide reactions were most distinct at the two higher pH levels, perhaps because methemoglobin is a pH indicator which is brown at pH 5.5 and red nearer neutral (69). It is useful for methodological reasons to know that hemoglobin powder can be substituted for fresh blood after autoclaving, and that the pH can be adjusted before autoclaving without significantly affecting results (Table 3). Twenty-four per cent of the species produced a clear brown zone attributed to a protease (22) on HA, but 93% did on SBA. Substituting the powder requires that it be suspended in H_2O and added slowly to the basal medium, and that pH be adjusted slowly. The medium, after autoclaving, should be vigorously and constantly stirred to insure a uniform, fine suspension in each plate.

Although the aminotriazole-H_2O_2 complex inactivates catalase, ethanol inhibits inactivation (10), and wood-rotting fungi produce ethanol (56, 78). Furthermore, the oxidases (21) and H_2O_2 (36) may be produced only for a short time, and added H_2O_2 disappeared from inoculated MEL more rapidly for most of the fungi than from noninoculated controls [measured with Bonét-Maury reagent (61, p. 563, J. Koenigs, *unpublished data*)], even though extracellular catalase was not detected in the culture filtrates of nine fungi tested. On the other hand, the concentration of glucose and other H_2O_2-yielding substrates may not be present in host tissue at concentrations used in these tests. Thus, data in Table 1 may not indicate actual rates of production of H_2O_2 from glucose in vivo.

The intrinsic inactivation of C-AT-G noted in the controls could have been due to contamination of the catalase with an H_2O_2-producing oxidase, e.g., glucose oxidase (57), or to decomposition of the components for unreported and unrecognized reasons such as entrapment of ethanol during recrystallization of aminotriazole. In retrospect, it appears that the concentration of catalase may have been too low, and this resulted in a system more delicately poised than was the original (10). The onset of inactivation varied at times with pH of the buffer and with concentration, purification, and samples of aminotriazole within the same lot and between commercial sources. After these studies were completed, it was learned that lots of catalase from another source differ in stability in the presence of aminotriazole (12). It is important to include appropriate controls at frequent intervals, and to avoid using data obtained after rapid inactivation begins, as H_2O_2 produced during this time is not necessarily additive to that which would be inferred by reference to the control curve.

The results indicate that it may be fruitful to investigate H_2O_2 as a factor in wood decay, and perhaps in pathogenesis.

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