EFFECTS OF INDOLEACETIC ACID AND OTHER OXIDATION REGULATORS ON IN VITRO PEROXIDATION AND EXPERIMENTAL CONVERSION OF EUGENOL TO LIGNIN[1]

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Problems of lignin formation have long occupied the attention of investigators, however, neither the physiological nor chemical mechanisms involved in lignification is known in detail. Nevertheless, a considerable body of knowledge has accumulated through the investigations of Freudenberg, Nord, Brown, Wardrop, and others (4, 5, 6, 10, 13, 15, 23), especially on the biochemistry, histochemistry, and enzymology of lignification.

Investigations using model systems have contributed information on precursors, their transformations, (10, 17, 21) and biophysical factors in formation of lignin polymers (18). Although phenylpropane dehydrogenation polymers formed in model systems are not identical with specific lignins, they belong to this group of natural products (1, 3).

Indoleacetic acid (IAA) may regulate lignin formation. It inhibits lignification and other peroxidations in model systems (18, 19, 21) yet indirectly promotes xylem regeneration (11), presumably by inducing formation of vascular peroxidase (7, 8, 12). Antioxidant activity has been found in organic nitrogen compounds, including IAA, which possess mobile electronic systems (16, 19, 20) and provides a new experimental approach to the study of hormones and regulatory processes.

Our object is presenting evidence for the oxidation-regulating properties of several substances of biochemical interest, and the evaluation of antioxidants as possible factors in lignification.

MATERIAL AND METHODS

BIOASSAY SYSTEM: Vascular bundles of celery (*Apium graveolens*) removed as long strands, virtually free of cortical tissue, are used as a model lignifying system. The composition and properties of the synthetic lignin and its soluble derivative have been reported (18). The product approximates natural lignins (1, 3). The soluble derivative appears to consist of trimeric units containing double linked phenylpropane residues, as in the Freudenberg benzofuran structure. This work is in accord with the proposed conversion of eugenol to lignin via coniferaldehyde (1, 10).

Excised tissue was washed in cold M/15 phosphate buffer (pH 6.0) and stored at 5° C on moist filter paper until used. Excised tissues, pooled and randomized in batches of 10 to 25 gm (wet weight), could be stored and used without change in activity over periods of three to five days.

Experimental samples of 500 mg (±1 %) were shaken at 25±0.1° C for 15 hrs in 25 ml 0.2 M KH_2PO_4 (pH 4.5) containing eugenol (0.5 mM) and H_2O_2 (1.0 mM). Drifts in pH were negligible compared to the spread of the optimum plateau, pH 4 to 6.

Tissues to be analyzed for lignin were first rinsed in running water and 100 ml cold ethanol (redistilled). They were then extracted with boiling ethanol. Two 10 min extractions of tissues with 50 ml portions of alcohol, removed all soluble phloroglucinol-positive interfering substances. No dioxane-soluble product was found; hence ethanol extraction was followed by treatment of the residue with ethanol-3 % HCl which solubilizes lignins by ethanolysis (3). Pre-extracted samples were boiled 15 min in 25 ml acid ethanol, left at room temperature 30 min and the extract made up to 25 ml.

To each alcohol-extracted residue, 1 ml conc. HCl and 130 mg phloroglucinol were added rapidly with agitation, and the phloroglucinol color read at 540 mμ after 1 min (Bausch and Lomb Spectrophotometer). Color develops rapidly, reaches its maximum density in 1 min, and begins to fade after 3 min.

The phloroglucinol color in alcoholic solutions of the isolated trimer (Mol. wt. 667) (18), increased linearly with trimer concentration up to densities of about 0.5. The conversion factor was 34.5 mμ M of trimer per 0.001 density units. The ethanol-HCl extracts contain both endogenous lignin and synthetic products. Each experiment included tissue samples in buffer alone. The phloroglucinol colors in extracts from these contained only solubilized endogenous lignin and were applied as a blank correction to experimental samples incubated with eugenol and H_2O_2.

Yields of lignin in duplicate or triplicate samples from the same pooled batch of tissue agreed to within ±3 %.

PEROXIDATION OF PYROGALLOL AND EUGENOL IN VITRO: Peroxidation of pyrogallol to purpurogallin was followed photometrically at 425 mμ in a standard system (pH 4.5; 22° C; 0.005 M pyrogallol; 0.025 M H_2O_2; 1.6×10^{-8} M horseradish peroxidase, prepared using purified enzyme obtained from Nutritional Biochemicals Co. or Worthington Biochemical

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Corp.). Measurements were made at 15 sec intervals for 120 sec. For calculating inhibition the reaction rate during the first 60 sec was used. Triplicate measurements usually agreed to ±1 %. The eugenol peroxidation system was similar to that for pyrogallol, but used 0.002 M substrate and 0.005 M H_2O_2. The major products in vitro of eugenol peroxidation are dimeric substances resembling lignins in composition (18). Measurements were made at 10 sec intervals, and for calculating inhibition the overall 30 sec rate was used. (table I). Inhibitors of radical-initiated chain processes increase the induction phase of these reactions, and may be compared on this basis (2, 22). The induction time T_i, was computed graphically as shown in fig. 1.

Although coniferaldehyde forms through slow autoxidation of eugenol (10), stock and vacuum-distilled samples proved to be kinetically similar when used in vitro and in situ (with tissue). Triplicate in vitro determinations generally agreed to within 3 to 4 %.

Precautions included the use of fresh solutions of autoxidizable substances (e.g. pyrogallol, IAA, *p*-benzoquinone); spectrophotometric analysis for impurities (e.g. *p*-benzoquinone, some indoles) and recrystallization of unstable or impure solids (e.g. skatole, IAA, *p*-benzoquinone).

Results

Although the oxidant, H_2O_2, is present in a great molar excess, a number of structurally different compounds are effective inhibitors of the eugenol oxidation (table I). Oxidation in situ is generally more sensitive than in vitro. IAA, isonicotinylhydrazine, and mescaline are most notable as inhibitors, however, the activities of skatole, hydroxytyramine, and the aliphatic amines are also of interest.

The low inhibitory efficiency of indole is associated with its rapid peroxidation to an insoluble violet indigoid compound. The 5-hydroxyindoles, which were unexpectedly inefficient are readily peroxidized to 5,6-dihydroxyindoles, thence to quinones. The enhancement of lignin formation by quinones will be treated below.

Inhibitory activities are also unequal in the two in vitro systems. Eugenol oxidation is generally the more sensitive reaction, and is affected by some compounds, notably isonicotinyl-hydrazine, which are devoid of activity when pyrogallol is used as a substrate.

The two eugenol systems responded to added IAA in a qualitatively similar fashion (fig 2). The sigmoid curve described is commonly associated with inhibitors of radical-initiated reaction chains (2, 22). This comparison emphasized the sensitivity of the in situ lignin-forming system.

The existence of a chain mechanism in the enzymatic oxidation of eugenol was first suggested by its autocatalytic character (fig 1). Preliminary kinetic studies show that neither T_i nor the post-induction rate is first order with respect to peroxidase or peroxide as they should be in a non-chain process (2, 14, 22). The exact values are yet to be determined, but are definitely not integral.

Two substances used for the study of radical-initiated polymerizations (2, 22) were tested as inhibitors of eugenol oxidation-DPPH (the radical of 1,1-diphenyl-2-(2, 4, 6-trinitrophenyl)-hydrazine and iodine. DPPH is a resonance-stabilized, violet radical which can react with a number of other radicals, including those formed from triphenylmethane, H_2O_2 (or H_2O), organic peroxides, olefins, etc. Its decolorization has been applied quantitatively, assuming that one hydrazyl radical is removed for each radical of another kind appearing. Although certain reservations have been pointed out, inhibition by DPPH is strongly indicative of the participation of radicals in the inhibition reaction. Inhibitions found at low concentrations, expressed as increase in T_i, show that

Table I

Inhibitor Efficiencies in Peroxidation of Eugenol and Pyrogallol

Catalyst / Substrate / Product	Peroxidase in situ Eugenol Lignin as trimer		Peroxidase in vitro Eugenol Dimer		Pyrogallol Purpurogallin	
Inhibitor (M/L)	5×10^{-5}	5×10^{-4}	5×10^{-5}	5×10^{-4}	5×10^{-4}	5×10^{-3}
H_2O_2/Inhibitor (M/M)	800	80	100	10	40	4
			% Inhibition			
Indole	0	16	0	40	0	21
Skatole	21	55	25	82	0	50
IAA	71	97	53	92	41	97
5 HO-IAA	0	22	17	78	48	86
5 HO-Tryptamine	12	36	15	92	19	50
Isonicotinylhydrazine	70	99	15	97	0	0
Mescaline	70	89	15	38	0	17
HO-Tyramine	22	42	17	68	0	14
Isobutylamine	32	49	0	16	0	0
d-Glucosamine	20	38	0	10	0	0

TABLE II

EFFECTS OF DPPH AND OTHER INHIBITORS ON EUGENOL OXIDATION

INHIBITOR	CONCENTRATION (M)	H_2O_2/INHIBITOR	T_i (SEC)
None	...	...	10 ± 1
DPPH	10^{-6}	5,000	16 ± 2
	10^{-5}	500	21 ± 2
	10^{-4}	50	27 ± 3
IAA	10^{-4}	50	18 ± 1
	5×10^{-4}	10	22 ± 2
Indole	5×10^{-4}	10	14 ± 1
Skatole	5×10^{-4}	10	24 ± 2
Isonicotinyl-	10^{-4}	50	16 ± 2
hydrazine	5×10^{-4}	10	21 ± 2

DPPH is a highly efficient chain breaker (table II). Doubling of T_i is effected by approximately 10^{-5} M DPPH (500 moles H_2O_2 and 125 moles eugenol for each mole of inhibitor). Comparing inhibitor concentrations required for doubling T_i, IAA, skatole, and isonicotinylhydrazine all possess about 1/50 and indole less than 1/500 the activity of DPPH. Both DPPH and I_2 at 5×10^{-5} M inhibited lignin synthesis about 50 %, making them comparable in that system with IAA, isonicotinylhydrazine, and mescaline (table I).

ENHANCEMENT OF LIGNIN SYNTHESES BY QUINONES: Quinones may act as chain-breakers, although their inhibitory activities are sometimes secondary in importance to their chain initiating and chain transfer activities.

After observing that chloranil, tetrachloro-*p*-benzoquinone, effected a rapid multi-step (yellow→violet→red-brown) oxidation of eugenol in bulk and in solution, several quinones were tested as possible promoters of lignin synthesis (table III). When added to the standard in situ test system several quinones enhanced lignin synthesis substantially. Chloranil, although a far more powerful dehydrogenating agent than the others, was comparable with the parent benzoquinone and the indolic adrenochrome. Naphthoquinone was more active than other quinones as a promoter in lignin synthesis.

The behavior of quinones in the in situ assay may in turn account for the low inhibitory efficiency of 5-hydroxyindoles (table I). Autoxidation of these compounds in solution yields highly colored quinonoid intermediates, and ultimately, melanoid polymers.

TABLE III

ENHANCEMENT OF LIGNIN FORMATION BY QUINONES

QUINONE (10^{-4} M)	LIGNIN YIELD (AS SOLUBLE TRIMER) μM/500	MG TISSUE	% CONTROL
None	2.0, 2.2, 2.4, 2.4	(2.3)	100
p-benzoquinone	3.3, 3.7	(3.5)	152
Chloranil	3.1, 3.6	(3.3)	144
1,4-Naphthoquinone	5.1, 5.3	(5.2)	226
Adrenochrome	3.1, 3.7	(3.4)	148

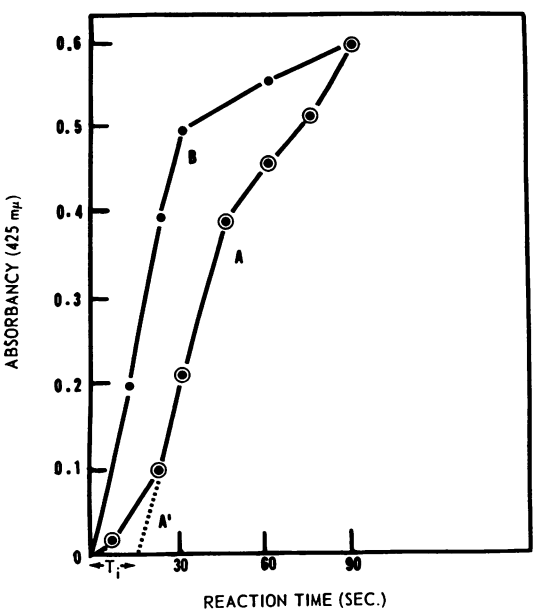

FIG. 1 (*left*). Time course of Enzymic peroxidation with two substrates. Curve A, eugenol; curve B, pyrogallol. Induction time, T_i, is obtained from A', the extrapolated linear segment of A.

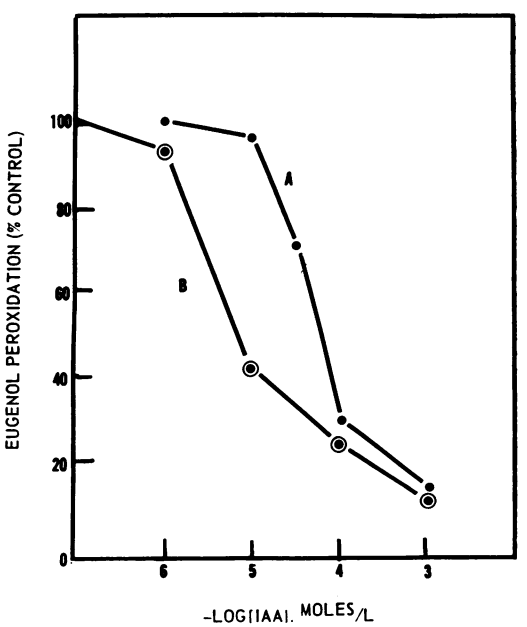

FIG. 2 (*right*). Inhibition by IAA of eugenol peroxidation. Curve A, dimerization in vitro; curve B, polymerization (lignin formation) in situ.

Discussion and Conclusions

The data presented here show that IAA, other indoles, and organic N compounds behave as antioxidants in phenol oxidations of biological interest. Although antioxidant efficiency varies with the system, it is appreciable in both eugenol oxidations studied. Efficiency depends in part upon specific reactants, as is shown by comparison of two peroxidase substrates, eugenol and pyrogallol: I. pyrogallol is oxidized about 500 times faster than eugenol; II. pyrogallol oxidation is initially first order in time whereas eugenol oxidation is autocatalytic (fig 1), and exhibits other features suggesting a chain reaction; III. eugenol and its oxidation products possess far more hydrocarbon character than pyrogallol or its derivatives. Pyrogallol, therefore, should be oxidized to purpurogallin rapidly through reversible formation of short-lived radical intermediates, with the entire reaction sequence localized on the enzyme surface. In contrast, oxidation of weakly bound eugenol should proceed through reversibly formed long-lived, readily desorbed radicals, easily accessible to catalytic reducing agents or chain-terminating scavengers. The appearance in solution of a long-lived intense yellow intermediate during peroxidation of eugenol, and the inhibition of lignin formation by pyrogallol lend support to this picture (17, 18).

Among the methods used in detecting and studying of radical-initiated chain processes are I. hydroxylation of benzene, II. polymerization of added vinyl monomer, and III. inhibition by one-electron trapping agents. Of these, II can be effected by xanthine oxidase, but neither I nor II can be carried out with hydroperoxidases (14). In spite of the arguments against free-radical mechanisms in hemoprotein catalysis, there are convincing thermodynamic arguments in its favor (9). Leach (14) suggests that radical initiation is obscured by competitive OH-consuming reactions. Further, the body of chemical evidence which may be applied to the study of phenol oxidation (14, 22) demonstrates the importance of radical intermediates in these reactions.

The generation of anions from phenols undoubtedly represents an important ionic process in these reactions, but the oxidation itself almost certainly proceeds via one-electron steps (22). Thus, the controversy noted above, seems to apply to the behavior of H_2O_2-peroxidase only in the absence of phenolic substrates.

The relative efficiencies of antioxidants or scavengers in autoxidations and polymerizations vary widely (2, 22). In autoxidation of cumene, the efficiencies of substituted phenols and anilines span a nearly 500 fold range. In benzoyl peroxide-initiated vinyl acetate polymerization a series of chain-breaking nitrobenzenes and quinones of 2:1 stoichiometry (2 chains stopped inhibitor molecule) varied in efficiency by a factor of about 500. In the latter system, DPPH and I_2 were so efficient, that they could not be analyzed kinetically on a common scale with the simple aromatic inhibitors (2). Our data show that IAA, isonicotinylhydrazine, mescaline, and radical scavengers tested are of more or less comparable efficiency as inhibitors of eugenol oxidation in situ.

The H_2O_2/inhibitor ratios of 10 to 500 which were required for the doubled induction period are comparable with those observed in chemical systems using instead of H_2O_2, initiators such as benzoyl peroxide or azobisisobutyronitrile (22).

The foregoing discussion of experimental findings has necessarily placed emphasis upon chemical problems, rather than biological implications. Nevertheless, the physiological role of anti-oxidant activity warrants a brief comment. It is common knowledge that peroxidases exhibit little specificity toward their electron (or H-) donor substrates. Accordingly, the elevation of peroxidase activity under hormonal influence could in itself lead to the loss of essential thiol groups, formation of toxic quinones, hence to premature cessation of growth. In the light of existing knowledge (7, 8, 10, 11, 12), our experiments suggest that although IAA as a peroxidase-inducer is essential to vascular differentiation, its activity as an antioxidant temporarily constrains peroxidase from indiscriminate attack upon essential structures and metabolites. Such a delaying action would prolong favorable conditions for growth.

Summary

I. Indoleacetic acid and other organic nitrogen compounds inhibit oxidations involving peroxidase (in vitro) and celery vascular tissue (in situ).

II. The conversion of eugenol to lignin-like polymers in situ is especially sensitive to the antioxidants IAA, isonicotinylhydrazine, and mescaline.

III. Oxidation in vitro of eugenol is more inhibited by some compounds than oxidation of pyrogallol. This difference may reside in the differential accessibility to inhibitors of intermediate radicals formed during the oxidation of these substrates.

IV. Preliminary kinetic evidence is consistent with the chemical picture of phenolic oxidations as radical-mediated reactions. Eugenol oxidation displays some features of a radical-initiated chain reaction, whereas pyrogallol does not.

V. Free radical scavengers (chain breakers) are efficient inhibitors of eugenol oxidation in both systems. Using the H_2O_2/inhibitor ratio as an index, the inhibitory activity of the scavengers, IAA, and other compounds compares well with the activities of chain breakers and antioxidants in established oxidation and addition polymerization reactions.

VI. Quinones accelerate oxidation of eugenol in situ to lignin-like polymers, an effect which may account for the failure of autoxidizable hydroxyindoles as inhibitors in this system.

VII. The antioxidant activity of IAA may constrain the peroxidase which it induces from a premature attack upon metabolites essential to growth.

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