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On the Inhibition of Peroxidase Reactions by Ethers

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The effects of ethers on the peroxidase catalvzed oxidation of iodide and pvrogallol were studied. Anisole, phenyl ether and benzofuran were inhibitory in both systems but inactive in the corresponding non-enzymic oxidations. Diethyl ether inhibited slightly. The pyrogallol system was more sensitive than iodide to all of the ethers. Tyrosine was apparently more active than the ethers in the iodide system but far less active with pyrogallol. Comparisons among the various systems suggest that ethers inhibit reactions unique to enzyme-catalyzed oxidation. The activities of ethers in model biochemical systems may reflect a biologically important function of the aryloxy group.

Some years ago, it was proposed that the mobile electronic system of the thyronine nucleus is involved in the physiological function of thyroactive substances.[1] Recently we have shown that a number of hormones and other bioregulators exhibit antioxidant activity in a variety of test systems.[2-4] Our findings which include the thyronines, agree with the general proposition that antioxidant activity is a function of electron availability.[5] Thus, antioxidant activity may be a measure of the electronic properties which were implicated in the hormone action, as noted above.

In a test system containing peroxidase and H_2O_2, the conversion of I^- to I_2 was inhibited markedly by thyroxine and 3,5,3′-triiodothyronine[6] to a lesser degree by other thyronines and least by tyrosine and 3,5-diiodotyrosine.[2] The presence of the ether linkage contributed more than did iodination to increased activity, using tyrosine as the baseline. These observations suggested that the aryloxy group itself should be considered in seeking out a structural basis for antioxidant properties.

In addition to its occurrence in the thyronines, the aryloxy group appears in many herbicides and growth regulators, the substituted phenoxyacetic acids for example. The aryl ethers have fungistatic and other biological activities.[7] The obvious importance of the aryloxy group prompted us to extend our antioxidant tests to the ethers themselves.

Results and Discussion

Aryl ethers have relatively low water solubilities and antioxidant efficiencies hence 25% inhibition was selected as a reference point for comparisons among these compounds (Table I). The two test systems containing different substrates also differed in their peroxidase, H_2O_2 and buffer concentrations. Therefore inhibitor levels were expressed both as concentration giving 25% inhibition (C_{25}) and as the molar ratio of inhibitor to enzyme required for 25% inhibition (R_{25}).

TABLE I

COMPARATIVE INHIBITORY EFFECTS OF ETHERS ON PEROXIDATION OF IODIDE AND PYROGALLOL

	Peroxidase substrate			
	Iodide		Pyrogallol	
(Compound	C_{25} ($M \times 10^{-4}$)	R_{25}	C_{25} ($M \times 10^{-4}$)	R_{25}
Diethyl ether	>100	$>5 \times 10^6$	10	5×10^4
Anisole	20	1×10^6	0.5	2.5×10^3
Phenyl ether	4	2×10^5	.6	3×10^3
Benzofuran	2	1×10^5	.5	2.5×10^3
Tyrosine	0.5	2.5×10^4	5.0	2.5×10^4

The inhibitory effects of the ethers differ markedly in the two peroxidase systems. To illustrate, the inhibition of iodide oxidation requires 70-fold more phenyl ether or 40-fold more benzofuran per mole of enzyme than does pyrogallol oxidation. In the iodide system, anisole is intermediate to the aliphatic and fully aromatic ethers, whereas all three aryl ethers are equally active inhibitors of pyrogallol oxidation. Diethyl ether is least active in both systems. Tyrosine, which was included as a representative phenolic antioxidant, is a more active inhibitor of iodide oxidation than any of the ethers. In contrast, tyrosine is 10-fold less active than the aromatic ethers as an inhibitor of pyrogallol oxidation. Although its behavior in pyrogallol oxidation suggests that tyrosine may have some potentialities as an antioxidant in other systems, the level of activity encountered in the iodide system was unexpectedly high. It is possible that the figures obtained for tyrosine in present and past studies may be attributed in part to its iodination rather than to its participation in oxidation–reduction phenomena. Our reaction conditions are far from suitable (in the preparative sense) for iodination but may allow sufficient removal of iodine by this route to account for the apparent inhibition. Previously, it was reported that $10^{-5}M$ thyroxine inhibited iodide oxidation 66% but had no effect on the oxidation of pyrogallol.[3] Hence, it is still conceivable that the relative effect of tyrosine on the two systems is correct as observed. Benzene, toluene and chlorobenzene were inactive in both peroxidase systems, and the ethers themselves were inactive at the C_{25} level in the absence of peroxidase.

If the inhibitors exerted non-specific solvent or denaturative effects or acted as metal-poisons, the R_{25} values should be similar in the two test systems. On the other hand, the inhibitors must

(1) H. Lardy in the "Thyroid," *Brookhaven Symposia in Biol.*, **7**, 90 (1955).

(2) S. Siegel, F. Porto and P. Frost, *Arch. Biochem. Biophys.*, **82**, 330 (1959).

(3) S. Siegel and P. Frost, *Proc. Natl. Acad. Sci., U. S.*, **45**, 1379 (1959).

(4) S. Siegel and F. Porto, "Proceedings of the 4th Intl. Conf. on Plant Growth Regulators," R. M. Klein, in press, 1959.

(5) C. Walling, "Free Radicals in Solution," John Wiley and Sons, Inc., New York, N. Y., (1957).

(6) S. Klebanoff, *J. Biol. Chem.*, **234**, 1437 (1959), shows that thyroxine accelerates the peroxidation of epinephrine, other catechol derivatives and ascorbic acid, but not of pyrogallol.

(7) W. A. Sexton, "Chemical Constitution and Biological Activity," D. Van Nostrand Company, Inc., New York, N. Y., 1953.

exert an effect on peroxidase itself, as the non-enzymic reactions are not at all retarded at the C_{25} values given. We suggest that the seeming paradox may be resolved if the ethers exert their antioxidant effect on steps of intermediates unique to the enzyme-catalyzed oxidation.

The enzymic oxidation of pyrogallol in acidic solutions yields the trihydroxybenztropolone purpurogallin, whereas autoxidation under otherwise similar conditions, forms instead a variety of quinonoid products, principally of polymeric character.[8] The enzyme, then, restricts the alternative route(s) leading to the formation of quinones and polyquinones. We have observed that the polymerization of aqueous-*p*-benzoquinone in air is not affected by the presence of the ethers at concentrations up to 0.01 *M*. Thus, a model process for the formation of highly colored products during autoxidation is shown to be ether-insensitive. Although the oxidation of iodide to iodine by H_2O_2 is an ionic reaction which proceeds through formation of HOI, radical pathways have been implicated in its photooxidation.[9] Thus the oxidation of each of these substrates may proceed along at least two pathways, one of which may be favored in the presence of peroxidase and particularly ether-sensitive.

Although questions pertaining to mechanism remain to be answered, these data show clearly that simple ethers can act as oxidation inhibitors in a model biochemical system. The effective concentrations are beyond the hormonal range but may reflect a physiologically important property of the aryloxy group. Fungistatic activity and similar biological effects which require relatively high ether concentration may, on the other hand, be more directly explained by the foregoing observations.

(8) S. Siegel, "Sub-Cellular Particles," T. Hayashi, editor, Am. Physiol. Soc., Washington, D. C., 1959.

(9) J. H. Baxendale, *Adv. in Catalysis*, **4**, 31 (1952).

Experimental

Iodine formation (as I_3) was followed photometrically at 360mμ (Bausch and Lomb spectronic 20 Spectrophotometer); purpurogallin formation was followed at 425mμ. The C_{25} and R_{25} values were based upon triplicate measurement at 25° made during the initial, linear phase of the respective reactions—20 min. with iodide and 3 min. with pyrogallol.

The iodide system contained $5 \times 10^{-3}M$, KI $5 \times 10^{-4}M$ H_2O_2, and $2 \times 10^{-9}M$ horseradish peroxidase (Nutritional Biochemicals Corp.) in $M/6$ phosphate buffer, *p*H 5.6. The pyrogallol system contained $5 \times 10^{-3}M$ pyrogallol, $10^{-3}M$ H_2O_2 and $2 \times 10^{-8}M$ peroxidase in $M/30$ phosphate buffer, *p*H 5.0.

The polymerization of *p*-benzoquinone to red-brown products in air was followed at 500 mμ.[8] Reactions were run in the dark for 20 hr. at 25°. Freshly prepared aqueous solutions containing 0.005–0.05 *M* quinone and $M/15$ phosphate buffer, *p*H 5.0, were used.

The only problems of purity were encountered with phenyl ether, which was redistilled, and *p*-benzoquinone, which was recrystallized from ethanol. No phenols were detected in the phenyl ether in tests with $FeCl_3$ and NaOH. Fresh diethyl ether which gave a negative KI test for peroxides was used for each experiment.

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An Enzymatic Examination of the Structure of the Collagen Macromolecule[1]

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The collagenase-catalyzed degradation of soluble ichthyocol has been examined in order to obtain further insight into the structure and configuration of the collagen macromolecule. The kinetics of proteolysis were followed directly by *p*H-stat and colorimetric ninhydrin methods, and it is shown that below 27° (T_c—the temperature at which the collagen → gelatin transition takes place in ichthyocol solutions) the over-all kinetics can be reduced to the sum of two concurrent reactions, both apparently first order in substrate concentration but differing markedly in rate. Since conversion to gelatin reduces the kinetics of proteolysis to a single, apparent first order reaction with a much smaller apparent energy of activation,[25] the complex kinetics observed at temperatures below T_c have been interpreted in terms of local differences in polypeptide chain configuration in the vicinity of the susceptible peptide bonds. Following an examination of the molecular properties of undegraded ichthyocol in neutral salt solution (0.5 *M* $CaCl_2$), the changes produced in the substrate as a consequence of proteolysis were monitored by various physico-chemical techniques. The specific viscosity of ichthyocol solutions falls rapidly (to less than 10% of its initial value) during collagenolysis, also following apparent first order kinetics. Parallel light-scattering experiments revealed that this fall is accompanied by only a slight decrease in molecular weight, but by a marked change in over-all particle shape—the macromolecules becoming more flexible as the reaction proceeds. The non-dialyzable protein concentration and specific rotation of the ichthyocol solution also fall much more slowly than the specific viscosity, indicating that the particle remains relatively unchanged in terms of size and helical content during the early stages of proteolysis. These results are interpreted in terms of a rigid, multi-stranded, inter-chain hydrogen-bonded structure for the collagen macromolecule in solution; enzymatic cleavage of single strands leaves the particle relatively intact but brings about a partial structural collapse by introducing points of increased flexibility.

Introduction

Collagen, the major protein constituent of connective tissue, has been studied for many years. However most examinations have been confined to collagen in the solid state or to degradation products (collectively termed gelatin), chiefly because of the resistance of collagen to solubilization under mild conditions. In 1927, Nageotte reported the first successful preparation of soluble

(1) Presented in part at the 3rd Annual Meeting of The Biophysical Society, February 25, 1959, Pittsburgh, Pennsylvania. The opinions expressed in this article are those of the authors and do not necessarily reflect the opinions of the Navy Department or the naval service at large.

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