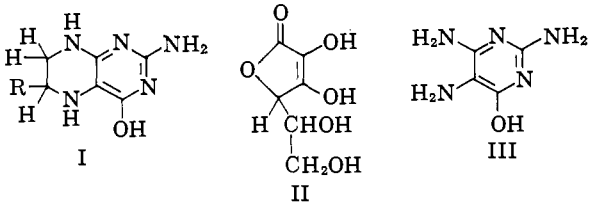
if H_2O_2 (approximately equivalent to the amount which might be formed if ascorbic acid reacted with O_2 to give H_2O_2 and dehydroascorbic acid) is added with no O_2 present then again no products are formed (4). The results of these experiments thus clearly indicate that H_2O_2 is not an intermediate in the oxidation with molecular oxygen. Line 5 of Table I shows the amounts of products formed when the hydroxyl radical (generated by the Fenton reaction)[13] is the oxidizing agent. This reaction did give some of the same products observed with the Udenfriend reaction. However, gas chromatographic analysis revealed at least five other products in the reaction of HO· with cyclohexane and two others with cyclohexene. Under the Udenfriend conditions these products were not observed. Consequently it appears that the Udenfriend reaction on cyclohexane or cyclohexene does not involve the hydroxyl radical as the oxidizing agent. Further evidence for this is obtained if cyclohexene oxide is added at the beginning of the run; it is not significantly reacted under the Udenfriend conditions but under the Fenton conditions it is largely decomposed. If cyclohexanol is added under the Udenfriend conditions then a small amount of it is oxidized to cyclohexanone, and presumably some, if not all, of the cyclohexanone observed under these conditions arises by oxidation of the cyclohexanol formed in the reaction. Cyclohexanone added at the beginning of the run does not appear to be affected under either conditions.

Recently, Kaufman[14] has identified the cofactor for phenylalanine hydroxylase as a tetrahydropteridine of general structure I. Compound I contains a partial structure analogous to the enediol structure of ascorbic acid (II) which is a necessary reagent in the Udenfriend model system. If the enzymatic and model systems react by similar mechanisms then compounds more closely related to I should also react in the model system. Consequently, the hydroxylation of anisole by the model system has been investigated with the ascorbic acid replaced by 2,4,5-triamino-6-hydroxypyrimidine (III). The hydroxylated products obtained are compared in Table II. The similarity of the products formed with II and III is suggestive that the two reactions proceed by related mechanisms. Furthermore, the obvious close similarity of III to the natural cofactor I suggests that the mechanisms of the enzymatic and model hydroxylations are related. A mechanism for the model and enzymatic hydroxylations is considered in the following communication.[15]



I II III

TABLE II

THE HYDROXYLATION OF ANISOLE BY MOLECULAR OXYGEN

Conditions[a]	% yield of methoxyphenols[b]	Isomer distribution *ortho*	*meta*	*para*
Udenfriend system with II	8	43	18	39
Udenfriend system with III	4	49	13	38

[a] The reactions were carried out in 61.5 ml. of water saturated with anisole, buffered with acetate at pH 4.5, and contained 0.04 mmole of $Fe(ClO_4)_2$ and 1 mmole of ascorbate or pyrimidine. The reactions were shaken overnight under an atmosphere of air. [b] Yield based on the initial amount of ascorbate or pyrimidine. The products were analyzed by gas chromatography.

(13) For a review see N. Uri, *Chem. Rev.*, **50**, 375 (1952).

(14) S. Kaufman, *Proc. Natl. Acad. Sci. U. S.*, **50**, 1085 (1963); *J. Biol. Chem.*, **239**, 332 (1964); *ibid.*, **237**, PC2712 (1962).

(15) G. A. Hamilton, *J. Am. Chem. Soc.*, **86**, 3391 (1964).

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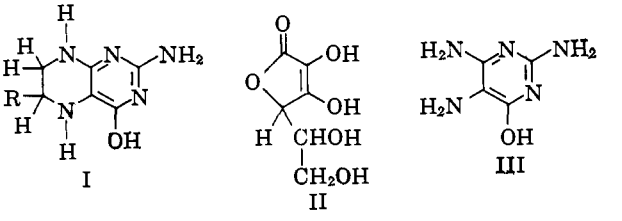
Oxidation by Molecular Oxygen. II. The Oxygen Atom Transfer Mechanism for Mixed-Function Oxidases and the Model for Mixed-Function Oxidases[1,2]

Sir:

The Udenfriend oxidation[3] by molecular oxygen has been considered a model for aromatic hydroxylases and other mixed-function oxidases.[4] It has now been shown that H_2O_2 and the hydroxyl radical are not intermediates in the model oxidation, and that the model system oxidizes aromatic compounds to phenols, saturated aliphatic compounds to alcohols, and olefins to epoxides.[4-6] Such reactions are reminiscent of carbene reactions[7,8] and the implication is that an oxygen species with six electrons and similar to a carbene or carbenoid species is responsible for the oxidations. Other information relevant to a consideration of the mechanism of the model and enzymatic mixed function oxidases is the following: the tetrahydropteridine cofactor (I), required by phenylalanine hydroxylase,[9] has structural features similar to ascorbic acid (II) and 2,4,5-triamino-6-hydroxypyrimidine (III) which



I II III

are necessary for the model oxidations[4]; both the model and enzymatic mixed-function oxidases appear

(1) Presented at the 146th National Meeting of the American Chemical Society, Denver, Colo., Jan., 1964; Abstracts of Papers, Division of Biological Chemistry, p. 13A.

(2) This investigation was supported by PHS research grant GM-09585 from the Division of General Medical Sciences, Public Health Service.

(3) S. Udenfriend, C. T. Clark, J. Axelrod, and B. B. Brodie, *J. Biol. Chem.*, **208**, 731 (1954).

(4) G. A. Hamilton, R. J. Workman, and L. Woo, *J. Am. Chem. Soc.*, **86**, 3390 (1964), and references therein.

(5) R. O. C. Norman and G. K. Radda, *Proc. Chem. Soc.*, 138 (1962).

(6) G. A. Hamilton and J. P. Friedman, *J. Am. Chem. Soc.*, **85**, 1008 (1963).

(7) (a) J. Hine, "Divalent Carbon," Ronald Press, New York, N. Y., 1964; (b) E. Chinopores, *Chem. Rev.*, **63**, 235 (1963).

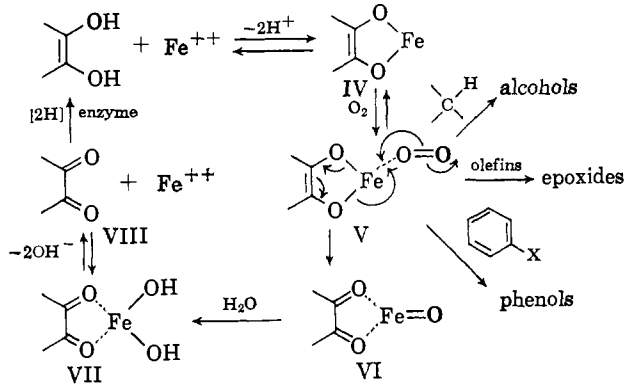
(8) The reaction of a carbene with anisole has been studied by H. Meerwein, H. Disselnkötter, F. Rappen, H. U. Rintelen, and H. Van de Vloed [*Ann.*, **604**, 151 (1957)], and it was observed that the isomer distribution of methylanisoles was *ortho*:*meta*:*para*, 34:35:31, which is not unlike the isomer distribution of phenols formed on hydroxylation of anisole by the model system[4] (*ortho*:*meta*:*para*, 43:18:39). The striking result is that a large amount of *meta* isomer is formed in both cases.

(9) S. Kaufman, *Proc. Natl. Acad. Sci. U. S.*, **50**, 1085 (1963); *J. Biol. Chem.*, **239**, 332 (1964); *ibid.*, **237**, PC2712 (1962).

to require a metal ion, usually Fe^{++} [10]; the stoichiometry of the enzymatic oxidations is

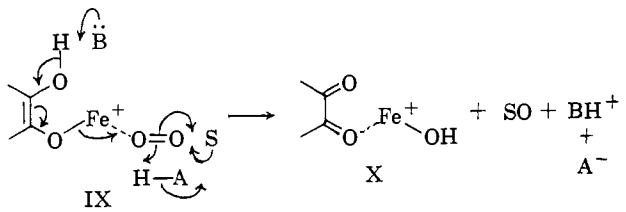
$$O_2 + S + AH_2 \longrightarrow SO + A + H_2O$$

where S, the substrate, is oxidized by two electrons to SO, and a biological reducing agent, AH_2 (eventually reduced diphosphopyridine nucleotide or triphosphopyridine nucleotide in most cases), is oxidized by two electrons to A[10]; for both the enzymatic and model hydroxylations the oxygen in SO comes from molecular oxygen[10]; and the phenylalanine hydroxylase cofactor (I) is oxidized to the level of a dihydropteridine in the same step that phenylalanine is hydroxylated to tyrosine.[9] All this evidence is consistent with the following mechanism for the model and enzymatic reactions.[11]



The enediol structure, Fe^{++}, and O_2 could be in equilibrium with a complex such as V. This complex is proposed as the actual oxidizing agent. By a shift of electrons as shown an oxygen atom with six electrons could be transferred from V to an organic substrate if at the same time the enediol complex is oxidized to the dicarbonyl complex VI. It is not proposed that complex V would generate a free oxygen atom but in the presence of a substrate molecule complex V could react as an "oxenoid" species (named in analogy to the carbenoid species which can transfer a divalent carbon during reaction). The formation of phenols, alcohols, and epoxides by the reaction of V with appropriate substrates should occur in a way analogous to the formation of methyl compounds and cyclopropanes by the reaction of carbenes or carbenoid species with similar substrates. The transformation of V to VI is an overall four-electron oxidation and requires that the enediol be oxidized at the same time as the substrate is oxidized. As mentioned earlier, this is observed for phenylalanine hydroxylase.[9] Complex VI would be expected to add water to give VII, which should be in equilibrium with Fe^{++}, OH^-, and the dicarbonyl compound VIII. In the model system the Fe^{++} could recycle but during the oxidation the ascorbic acid would be oxidized to dehydroascorbic acid and could not recycle.[12] However, in the enzymatic reaction the oxidized cofactor would have to be reduced back to the tetrahydropteridine level so that it too can recycle. This would explain the requirement for a reducing agent in oxidations by mixed-function oxidases.

Several variations on the proposed mechanism can be envisaged. One good possibility is that the enediol, Fe^{++}, and oxygen form a complex such as IX which,



by a shift of electrons and a proton as shown, could presumably transfer an oxygen atom to an organic substrate (S) and form complex X. Such a reaction would be subject to general acid and general base catalysis which could partially explain why the enzyme reaction proceeds more rapidly than the model reactions. Also, if such a mechanism were true, then it would not be necessary for both groups to complex with the metal ion and vinylogous enediols might also be reactants.

The main features of the proposed mechanism are that a metal ion must complex with molecular oxygen and with some system capable of oxidation by two electrons. The latter is satisfied by the cofactor for phenylalanine hydroxylase and the reactants, II and III, in the model system. The mechanism predicts that cofactors for other mixed-function oxidases should be capable of similar oxidation. The function of the metal ion in the proposed scheme is presumably to form an electronic link between the enediol and the oxygen.[6] In addition, it probably allows conversion of the triplet oxygen molecule to a singlet species so that radical or diradical intermediates would not be necessary in the reaction of V with the substrate.[14]

Another reaction of carbenoid species with aromatic compounds is the expansion of a benzene ring to a cycloheptatriene ring.[7,8] An analogous oxygen species would be expected to react with a benzene ring to give an oxepine and since such systems are known to cleave readily to open-chain compounds in aqueous solution[15] then a similar mechanism may be involved in the cleavage of aromatic rings in biological systems. Some such model reactions are being investigated.[16]

(10) (a) H. S. Mason, *Advan. Enzymol.*, **19**, 128 (1957); (b) for a recent review see "Oxygenases," O. Hayaishi, Ed., Academic Press, Inc., New York, N. Y., 1962.

(11) The mechanism is depicted for the ascorbate reaction but one of the oxygens in the enediol of ascorbate could be replaced by an N–R as in the phenylalanine hydroxylase cofactor (I) or the pyrimidine (III) with little change in mechanism expected.

(12) Dehydroascorbic acid is formed under the conditions of the oxidation but this is not good evidence for the proposed mechanism since ascorbic acid is oxidized by O_2 to dehydroascorbic acid even in the absence of other substrates. When 2,4,5-triamino-6-hydroxypyrimidine (III) was used instead of ascorbic acid, the ultraviolet spectrum of the products indicated that the same pyrimidopteridines were formed as when the triaminohydroxypyrimidine is oxidized in air in the absence of other substrates.[13] This dimer is presumably formed from the dehydro-2,4,5-triamino-6-hydroxypyrimidine which the mechanism requires as the product in the hydroxylation mechanism.

(13) E. C. Taylor, H. M. Loux, E. A. Falco, and G. H. Hutchings, *J. Am. Chem. Soc.*, **77**, 2243 (1955); H. M. Loux, Ph.D. Thesis, University of Illinois, 1956, p. 92.

(14) To describe the mechanism proposed here the author originally suggested the term "oxene mechanism" in order to emphasize the analogy to carbene reactions, but a referee has objected on the grounds that a free "oxene," or atomic oxygen, is not formed. This is a valid objection and the term "oxenoid," or "direct oxygen atom transfer" is a better description.

(15) M. J. Jorgenson, *J. Org. Chem.*, **27**, 3234 (1962).

(16) The concept of a direct oxygen atom insertion may be helpful in understanding other oxidation reactions. For example, in the oxidation of hydrocarbons to alcohols by chromate and permanganate, retention of configuration is often observed.[17] Direct carbon–hydrogen insertion would be expected to give retention.

(17) K. B. Wiberg and G. Foster, *J. Am. Chem. Soc.*, **83**, 423 (1961); R. H. Eastman and R. H. Quinn, *ibid.*, **82**, 4249 (1960); K. B. Wiberg and A. S. Fox, *ibid.*, **85**, 3487 (1963).

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