Iodination Catalyzed by the Xanthine Oxidase System: Role of Hydroxyl Radicals†

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ABSTRACT: Three iodinating systems dependent on the acetaldehyde–xanthine oxidase (XO) system are described. In one, optimal activity was dependent on supplementation with myeloperoxidase (MPO) and chloride, and inhibition by catalase but not superoxide dismutase or OH· scavengers suggested that H_2O_2 generated by the xanthine oxidase system is required. The catalysis of iodination by peroxidase and H_2O_2 is well established, and this iodination can be stimulated by chloride. The second iodinating system required supplementation by Fe^{2+} and ethylenediaminetetraacetic acid (EDTA) for optimal activity. Iodination was inhibited by catalase, superoxide dismutase, and the OH· scavengers ethanol and mannitol, suggesting a requirement for OH· generated by the Fe^{2+}–EDTA-catalyzed interaction between $O_2^{-}\cdot$ and H_2O_2 (Haber–Weiss reaction). The Fe^{2+}–EDTA-supplemented system was considerably less sensitive to inhibition by azide than was the MPO–chloride system, and the former but not the latter system was inhibited by tris(hydroxymethyl)-aminomethane buffer. Both systems had a neutral or alkaline pH optimum. The third XO-dependent iodinating system required Fe^{2+} but was inhibited by EDTA. Iodination was optimal in acetate buffer, pH 5.0–5.5, at concentrations of 0.02 M or less and was inhibited by phosphate, lactate, and citrate buffers. The XO system could be replaced by H_2O_2. Iodination was inhibited by catalase, ethanol, mannitol, and azide as in the Fe^{2+}–EDTA-supplemented system; however, superoxide dismutase was not inhibitory unless the Fe^{2+} concentration was lowered. A requirement for OH· formed by the interaction of H_2O_2 and Fe^{2+} (Fenton's reagent) was proposed. All three systems were equally sensitive to inhibition by cyanide, methimazole, and propylthiouracil. These findings are discussed in relation to a possible role for OH· in biological iodination reactions.

The conversion of iodide to organic form (iodination) occurs in a number of biological systems. A covalent bond is formed generally with tyrosine residues in protein to form mono- and diiodotyrosine; however, histidine and sulfhydryl groups and certain other low molecular weight compounds and lipids can be iodinated. The iodination of protein is generally measured by the conversion of iodide to a trichloroacetic acid precipitable form.

Peroxidases, when supplemented with H_2O_2 and iodide, are potent catalysts of the iodination reaction [for review, see Morrison & Schonbaum (1976)]. The predominant site of iodination in the body is the thyroid gland where thyroid hormone synthesis is initiated by the iodination of tyrosine residues in thyroglobulin; a thyroid peroxidase has been implicated in this reaction (Taurog, 1970). Iodination also occurs when certain phagocytes [neutrophils (Klebanoff, 1967), eosinophils (Bujak & Root, 1974; Klebanoff et al., 1977; DeChatelet et al., 1977; Pincus, 1980), monocytes (Baehner & Johnston, 1972; Biggar et al., 1974; Lehrer, 1975)] are stimulated by phagocytosis or by certain soluble stimulants, and a peroxidase is involved here as well. Fertilization of sea urchin eggs is associated with iodination, and an ovoperoxidase, released from cortical granules, and H_2O_2 generated by egg metabolism have been implicated (Klebanoff et al., 1979). Iodination of soluble or cell surface proteins by lactoperoxidase and H_2O_2 is a much used analytical procedure (Morrison & Schonbaum, 1976), and this enzyme may catalyze the iodination reaction in situ in the mammary or salivary glands (Morrison & Steele, 1968).

In this paper, we describe peroxidase-independent iodinating systems of possible biological importance that appear to require hydroxyl radicals (OH·).[1] Fenton (1894) described the strong oxidizing activity of a mixture of Fe^{2+} and H_2O_2, and it was subsequently proposed by Haber & Weiss (1934) that the oxidant was OH· formed as follows:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$$

Fenton's reagent has subsequently been used as a source of OH· (Walling, 1975). Haber & Weiss (1934) also proposed the generation of OH· by the reduction of H_2O_2 by the perhydroxyl radical ($HO_2\cdot$) as follows:

$$HO_2\cdot + H_2O_2 \rightarrow O_2 + H_2O + OH\cdot$$

This reaction with the perhydroxyl radical in its ionized form, the superoxide anion ($O_2^{-}\cdot$), is commonly referred to as the Haber–Weiss reaction:

$$O_2^{-}\cdot + H_2O_2 \rightarrow O_2 + OH^- + OH\cdot$$

The superoxide anion is formed in a number of enzymatic and nonenzymatic systems by the univalent reduction of oxygen, and spontaneous or enzyme (superoxide dismutase)-catalyzed dismutation of these radicals results in the formation of O_2 and H_2O_2 as follows:

$$2O_2^{-}\cdot + 2H^+ \rightarrow O_2 + H_2O_2$$

When a reaction is initiated by an $O_2^{-}\cdot$- and H_2O_2-generating system and this reaction is inhibited by superoxide dismutase, which scavenges $O_2^{-}\cdot$, by catalase, which degrades H_2O_2, and by OH· scavengers such as mannitol or ethanol, this has been taken as presumptive evidence for the involvement of OH· generated by the Haber–Weiss reaction (Beauchamp & Fridovich, 1970; Diguiseppi & Fridovich, 1980).

It is now the prevailing view that the direct interaction of $O_2^{-}\cdot$ and H_2O_2 to form OH· is too slow as compared to com-

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; $HO_2\cdot$, perhydroxyl radical; MIT, monoiodotyrosine; MPO, myeloperoxidase; $O_2^{-}\cdot$, superoxide anion; OH·, hydroxyl radical; PMN, polymorphonuclear leukocyte; Tris, tris(hydroxymethyl)aminomethane.

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peting reactions such as the spontaneous dismutation of $O_2^{-}\cdot$ to be of biological significance (McClune & Fee, 1976; Halliwell, 1976; Rigo et al., 1977; Czapski & Ilan, 1978; Ferradini et al., 1978; Gibian & Ungermann, 1978; Melhuish & Sutton, 1978; Weinstein & Bielski, 1979). Rather it is the current belief that a trace metal such as iron acts as an oxidation–reduction catalyst, being alternately reduced by $O_2^{-}\cdot$ and oxidized by H_2O_2, with the overall reaction being the generation of OH· from $O_2^{-}\cdot$ and H_2O_2 as in the classical Haber–Weiss reaction (McCord & Day, 1978; Halliwell, 1978a,b; Buettner et al., 1978; Czapski & Ilan, 1978; Gutteridge et al., 1979; Diguiseppi & Fridovich, 1980; Rosen & Klebanoff, 1981) as follows:

$$O_2^{-}\cdot + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH\cdot + Fe^{3+}$$

$$O_2^{-}\cdot + H_2O_2 \rightarrow O_2 + OH^- + OH\cdot$$

Some chelating agents, e.g., ethylenediaminetetraacetic acid (EDTA) (McCord & Day, 1978; Halliwell, 1978a,b; Buettner et al., 1978; Gutteridge et al., 1979; Diguiseppi & Fridovich, 1980; Rosen & Klebanoff, 1981) and lactoferrin (Ambruso & Johnston, 1981), have been reported to strongly stimulate OH· formation by the iron-catalyzed Haber–Weiss reaction.

When xanthine oxidase catalyzes the oxidation of its substrate (e.g., xanthine, hypoxanthine, acetaldehyde), oxygen is reduced, in part or totally, to its one electron reduction product $O_2^{-}\cdot$ (Fridovich, 1970). H_2O_2 is formed by the dismutation of $O_2^{-}\cdot$ and, in part, directly from oxygen by divalent reduction. The formation of OH· by the xanthine oxidase system via the metal-catalyzed Haber–Weiss reaction has been proposed based on the formation of ethylene from methional or 2-keto-4-thiomethylbutyric acid (a reaction that can be initiated by OH·) and the inhibition of this reaction by superoxide dismutase, catalase, and OH· scavengers (Beauchamp & Fridovich, 1970; Diguiseppi & Fridovich, 1980). We report here that the xanthine oxidase system can catalyze the iodination reaction and present evidence that suggests that this reaction is dependent on OH· generated either by the metal-catalyzed Haber–Weiss reaction or by Fenton's reagent. The properties of this system are compared to those of peroxidase-catalyzed iodination.

Materials and Methods

Special Reagents. Xanthine oxidase (bovine milk, 10 mg/mL $\simeq$ 0.4 unit/mg suspended in 2.0 M ammonium sulfate and 0.01 M EDTA) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. The preparation was dialyzed against water just prior to use. Acetaldehyde was obtained from Aldrich Chemical Co., Milwaukee, WI. Myeloperoxidase (MPO) was prepared from canine pyometral pus by the method of Agner (1958) to the end of step 6 and assayed by the *o*-dianisidine assay (Worthington Enzyme Manual, 1972). One unit of enzyme is the amount decomposing 1 μmol of H_2O_2/min at 25 °C. Catalase (bovine liver, 6.1 mg/mL, 60 000 units/mg) obtained from Worthington Biochemical Corp., Freehold, NJ, was dialyzed against water before use, and superoxide dismutase (bovine erythrocytes, lyophilized powder, 12 300 units/mg), obtained from Miles Laboratories, Miles Research Products, Elkhart, IN, was dissolved in water (5 mg/mL) and stored at −20 °C. Human serum albumin (essentially fatty acid free), protease (type V from *Streptomyces* griseus), and diethylenetriaminepentaacetic acid (DTPA) were obtained from Sigma Chemical Co., St. Louis, MO, and desferrioxamine (Desferal mesylate) was obtained from Ciba Pharmaceutical Co., Summit, NJ. $Na^{125}I$ (carrier free in 0.1 M NaOH) was obtained from New England Nuclear, Boston, MA.

Iodination of Protein. (A) Cl_3CCOOH Precipitation. The components of the reaction mixture (see legends) were incubated in 15 × 75 mm polystyrene test tubes for 20 min at 37 °C in a water bath oscillating 70 times per minute. The reaction was stopped by the addition of 1.0 mL of cold 10% Cl_3CCOOH. The precipitate was collected by centrifugation at 1400 *g* for 5 min in a refrigerated centrifuge and washed 3 times with 2.0 mL of 10% Cl_3CCOOH. The test tube containing the washed precipitate was placed in a counting tube and the radioactivity of the sample determined in a γ counter. A blank containing iodide, buffer, and albumin was run with each experiment, and the results were subtracted from the experimental values. Less than 0.5% of the total added radioactivity was Cl_3CCOOH precipitable in the blank. A standard containing the total amount of ^{125}I in the reaction mixture was counted, and the percent conversion to a Cl_3CCOOH-precipitable form in the experimental tubes was determined as follows:

$$[(cpm_{exptl} - cpm_{blank})/cpm_{standard}] \times 100$$

Each experimental value was determined in duplicate and the average employed as a single *n* for statistical analysis.

(B) Paper Chromatography. The reaction mixture as described in the legend was incubated for 20 min at 37 °C and the reaction stopped by the addition of either 0.05 mL of 0.011 M propylthiouracil (final concentration 0.001 M) or 0.05 mL of 0.011 M propylthiouracil containing 5 mg of protease. The preparation without protease was chromatographed immediately, and the protease-containing preparation was incubated at 37 °C for 24 h. Samples (25 μL) of each were applied to duplicate 1-in. strips of Whatman No. 1 filter paper, and ascending chromatography was performed at room temperature in butanol saturated with 2 N acetic acid for 16–18 h. Autoradiography using Kodak X-Omatic G X-ray film was performed to localize the radioactive products, and the strips were then cut into 1-cm segments and counted in a well-type scintillation counter. Following subtraction of the background radioactivity, the percent of the total activity in each segment of the strip was determined. Standards containing a mixture of iodide, monoiodotyrosine, diiodotyrosine, and thyroxine were run in each experiment and the strips stained with a ceric sulfate–sodium arsenate reagent (Wilkinson & Bowden, 1960).

Iodination of Tyrosine. Following completion of the incubation (see legend), the reaction was stopped by the addition of 0.05 mL of 0.06 M potassium iodide and 0.006 M propylthiouracil, and 25-μL aliquots were removed for paper chromatography as described above.

Results

Iodide is converted to a Cl_3CCOOH-precipitable form by the xanthine oxidase system. In phosphate buffer, pH 7.0, xanthine oxidase in the presence of its substrate acetaldehyde and a protein (human serum albumin) as a source of iodine acceptor groups converted 3.6% of the added iodide to organic form in 20 min ($P < 0.001$ vs. background). Iodination by this unsupplemented xanthine oxidase system could be substantially increased by certain additions. Three systems are described here in which the xanthine oxidase system is supplemented by either (1) MPO and chloride, (2) Fe^{2+} and EDTA, or (3) Fe^{2+} alone.

Iodination by MPO–Chloride-Supplemented Xanthine Oxidase System. Iodination by acetaldehyde and xanthine oxidase (3.6%) was stimulated by the addition of MPO (11.0%; $P < 0.001$) (Table I). Chloride when added alone to the

Table I: Iodination Catalyzed by MPO–Chloride-Supplemented Xanthine Oxidase System[a]

supplements	iodination (%)
acet + XO + HSA	3.6 ± 0.5 (12)[b]
acet + XO + HSA + MPO	11.0 ± 1.5 (4)
acet + XO + HSA + Cl^-	4.1 ± 1.0 (4)
acet + XO + HSA + MPO + Cl^-	48.5 ± 5.0 (4)
acet omitted	1.0 ± 0.5 (3)
XO omitted	−0.03 ± 0.2 (3)
XO heated	1.2 ± 0.1 (3)
MPO heated	3.9 ± 1.0 (3)
HSA omitted	2.6 ± 0.4 (3)

[a] The reaction mixture contained 0.05 M sodium phosphate buffer, pH 7.0, 10^{-5} M sodium iodide (5 nmol; 0.05 μCi of ^{125}I), and, where indicated, 10^{-2} M acetaldehyde (acet), 10 μg of xanthine oxidase (XO), 500 μg of human serum albumin (HSA), 4 milliunits of myeloperoxidase (MPO), and 0.1 M sodium chloride in a total volume of 0.5 mL. XO and MPO were heated at 100 °C for 15 min where indicated. [b] Mean ± SE of *n* experiments.

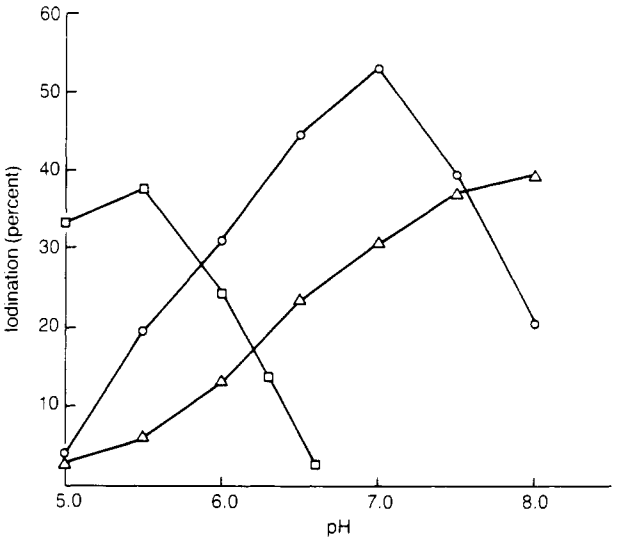

FIGURE 1: Effect of pH. The reaction mixture was as described in Table I for the MPO–chloride-supplemented xanthine oxidase system (○), in Table IV for the Fe^{2+}–EDTA-supplemented system (△), and in Table IV for the Fe^{2+}-supplemented system (□) except that the pH of the buffer was varied as indicated. Phosphate buffer (0.05 M) was employed for the MPO–chloride- and the Fe^{2+}–EDTA-supplemented systems and acetate buffer (0.01 M) for the Fe^{2+}-supplemented system. The results are the mean of three experiments.

xanthine oxidase system did not increase iodination (4.1%; *P* not significant); however, chloride significantly increased iodination when added to the MPO-supplemented system (45.8%; *P* <0.001 vs. MPO alone). Iodination by the acetaldehyde–xanthine oxidase–MPO–chloride system was greatly decreased or abolished by the deletion of acetaldehyde, xanthine oxidase, or albumin or by the heat treatment of either xanthine oxidase or MPO. Iodination with heated MPO was not significantly different from that of the unsupplemented xanthine oxidase system.

Iodination by the acetaldehyde–xanthine oxidase–MPO–chloride system increased as the pH of the phosphate buffer increased from 5.0 to 7.0 and then fell as the pH was further increased to 8.0 (Figure 1). Iodination was high with 0.05 M phosphate, cacodylate, acetate, and Tris-HCl buffers, pH 7.0. It was strongly inhibited by catalase, an effect that was abolished by heat inactivation of the enzyme (Table II). In contrast, superoxide dismutase was not inhibitory; indeed, this enzyme had a small but significant stimulatory effect. Iodination by the MPO–chloride-supplemented system also was unaffected by the hydroxyl radical scavengers mannitol and ethanol at 0.1 M concentration but was inhibited by azide, cyanide, methimazole, and propylthiouracil (Table II).

In the studies described above, the iodination of protein (albumin) was detected by the conversion of iodide to a

Table II: Effect of Inhibitors[a]

	iodination by xanthine oxidase system +					
	MPO + Cl^- (pH 7.0)		Fe^{2+} + EDTA (pH 7.0)		Fe^{2+} (pH 5.0)	
inhibitors	% change	*P*	% change	*P*	% change	*P*
catalase (60 μg/mL)	−93.6	<0.001	−92.9	<0.001	−78.2	<0.001
catalase heated	+4.1	NS[b]	−14.7	NS	−13.9	NS
SOD (5 μg/mL)	+18.9	<0.005	−92.6	<0.001	+6.6	NS
SOD heated			−7.5	NS		
mannitol (10^{-1} M)	+6.9	NS	−87.2	<0.001	−93.9	<0.001
mannitol (10^{-2} M)			−32.0	<0.02	−55.1	<0.001
mannitol (10^{-3} M)			+7.2	NS	−4.2	NS
ethanol (10^{-1} M)	+0.6	NS	−86.3	<0.001	−70.8	<0.001
ethanol (10^{-2} M)			−34.8	<0.01	−21.9	<0.05
ethanol (10^{-3} M)			−5.8	NS	−5.4	NS
azide (10^{-3} M)	−96.2	<0.001	−56.4	<0.001	−94.7	<0.001
azide (10^{-4} M)	−91.2	<0.001	−2.2	NS	−30.1	<0.02
azide (10^{-5} M)	−88.3	<0.001	+4.1	NS	−6.7	NS
azide (10^{-6} M)	−49.5	<0.05	+4.0	NS		
azide (10^{-7} M)	+1.1	NS	−2.5	NS		
cyanide (10^{-3} M)	−92.7	<0.001	−87.3	<0.001	−96.3	<0.001
cyanide (10^{-4} M)	−53.8	<0.02	−42.3	<0.05	−82.6	<0.001
cyanide (10^{-5} M)	−9.5	NS	−9.8	NS	−47.9	<0.002
cyanide (10^{-6} M)					−4.6	NS
methimazole (10^{-4} M)	−99.7	<0.001	−99.9	<0.001	−99.9	<0.001
methimazole (10^{-5} M)	−35.5	<0.02	−45.6	<0.002	−88.7	<0.01
methimazole (10^{-6} M)	−10.3	NS	−17.6	<0.05	−11.9	NS
propylthiouracil (10^{-4} M)	−99.9	<0.001	−99.6	<0.001	−99.7	<0.001
propylthiouracil (10^{-5} M)	−68.6	<0.01	−49.1	<0.001	−78.7	<0.01
propylthiouracil (10^{-6} M)	−24.5	<0.002	−18.4	<0.02	−13.8	<0.02

[a] The reaction mixture was as described in Table I for the MPO-chloride-supplemented system, in Table IV for the Fe^{2+}-EDTA-supplemented system, and in Table IV for the Fe^{2+}-supplemented system except that the inhibitors were added in the concentrations indicated. Catalase was heated at 100 °C for 15 min and superoxide dismutase (SOD) was autoclaved at 120 °C for 30 min where indicated. [b] NS, not significant.

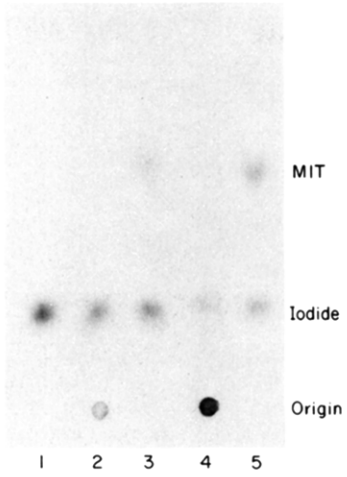

FIGURE 2: Chromatographic separation of reaction products. The components of the reaction mixture were as described in Tables I and IV except that the radioiodide was increased to 0.5 μCi. Tube 1 contained phosphate buffer, pH 7.0, iodide, and albumin; tubes 2 and 3 contained phosphate buffer, pH 7.0, iodide, albumin, acetaldehyde, xanthine oxidase, $FeSO_4$, and EDTA; tubes 4 and 5 contained phosphate buffer, pH 7.0, iodide, albumin, acetaldehyde, xanthine oxidase, MPO, and chloride. The contents of tubes 1, 2, and 4 were chromatographed without further treatment, and the contents of tubes 3 and 5 were treated with protease as described under Materials and Methods prior to chromatography.

Cl_3CCOOH-precipitable form. In the study shown in Figure 2, the reaction products were separated by paper chromatography with and without prior proteolysis, and the radioactive spots revealed by autoradiography, were identified by comparison to standards. Quantitation was performed by cutting the paper strips into 1-cm segments and counting in a γ scintillation counter. When iodide was incubated with albumin in buffer alone (tube 1), 93.7% of the radioactivity coincided with the inorganic iodide spot, with little radioactivity remaining at the origin. The addition of the xanthine oxidase system supplemented with MPO and chloride (tube 4) resulted in a loss of radioactivity associated with the iodide spot and a corresponding increase in radioactive origin material. Radioactive protein would be expected to remain at the origin with the solvent system employed. Treatment of the radioactive products with protease prior to chromatography resulted in the loss of origin material radioactivity and the appearance of a new radioactive spot that corresponded to the monoiodotyrosine (MIT) standard (tube 5). The percentage of the radioiodine found in each spot is indicated in Table III. Free tyrosine also was iodinated by the MPO–chloride-supplemented xanthine oxidase system with the formation of monoiodotyrosine (Table III).

Iodination by Fe^{2+}–EDTA-Supplemented Xanthine Oxidase System. Iodination of albumin by the acetaldehyde–xanthine oxidase system at pH 7.0 (3.6%) also was markedly increased by the addition of Fe^{2+} plus EDTA (30.1%, $P < 0.001$) (Table IV). Deletion of EDTA or Fe^{2+} from the reaction mixture decreased but did not abolish the increment in iodination whereas iodination was essentially prevented by the omission of albumin, xanthine oxidase, or acetaldehyde. EDTA could not be replaced by the iron-chelating agents DTPA or desferrioxamine under the conditions employed in Table IV; indeed, their addition to the acetaldehyde–xanthine oxidase system inhibited iodination (3.6% vs. 0.9% for DTPA, $P < 0.002$; 3.6% vs. 1.8% for desferrioxamine, $P < 0.05$).

Iodination by the Fe^{2+}–EDTA-supplemented xanthine oxidase system differed in a number of respects from that induced by the MPO–chloride-supplemented system. Iodination increased with pH throughout the pH 5.0–8.00 range employed (Figure 1). Iodination was comparable when phosphate, arsenate, or cacodylate buffer, pH 7.0, was employed but, in sharp contrast to the MPO–chloride-supplemented system, was greatly depressed (<10%) when Tris-HCl buffer, pH 7.0, was used. As with the MPO–chloride-supplemented system, iodination was strongly inhibited by catalase, an effect that was greatly reduced by heat treatment (Table II); however, in sharp contrast, superoxide dismutase (but not the heated enzyme) also was strongly inhibitory. Iodination by the Fe^{2+}–EDTA-supplemented system also was inhibited by mannitol and ethanol at concentrations down to 10^{-2} M but was considerably less sensitive than the MPO–chloride-supplemented system to azide, with an effect observed at a concentration of 10^{-3} M (as compared to 10^{-6} M for the MPO–chloride-supplemented system). The Fe^{2+}–EDTA- and MPO–chloride-supplemented systems were equally sensitive to inhibition by cyanide, methimazole, and propylthiouracil. As with the MPO–chloride-supplemented xanthine oxidase system, the Fe^{2+}–EDTA-supplemented system iodinated the tyrosine residues

Table III: Quantitation of Chromatographically Separated Products[a]

	iodination (%)							
	albumin					tyrosine		
location	1	2	3	4	5	6	7	8
origin	0.3	26.3	0.3	60.7	0.7	1.7	1.7	2.7
iodide	93.7	68.6	68.6	30.7	30.7	95.0	37.7	14.2
MIT	1.2	0.2	26.6	2.4	48.0	0.7	51.6	72.8

[a] For the iodination of albumin, the reaction mixture was as described for tubes 1–5 in Figure 2. For the iodination of tyrosine, the reaction mixture was as described in Figure 2 except that the albumin was replaced by 10^{-3} M tyrosine. Tube 6 contained phosphate buffer, pH 7.0, iodide, and tyrosine; tube 7 contained phosphate buffer, pH 7.0, iodide, tyrosine, acetaldehyde, xanthine oxidase, $FeSO_4$, and EDTA; tube 8 contained phosphate buffer, pH 7.0, iodide, tyrosine, acetaldehyde, xanthine oxidase, MPO, and chloride. Chromatography was performed without prior proteolysis in the tyrosine-containing tubes.

Table IV: Iodination by Xanthine Oxidase System Supplemented with Fe^{2+} and EDTA at pH 7.0 or Fe^{2+} Alone at pH 5.0[a]

	iodination (%)	
supplements	acet + XO + HSA + Fe^{2+} + EDTA	acet + XO + HSA + Fe^{2+}
complete system	30.0 ± 0.8 (12)[b]	32.3 ± 1.4 (3)
EDTA omitted	9.6 ± 1.0 (11)	
Fe^{2+} omitted	11.0 ± 1.2 (10)	1.1 ± 0.08 (3)
HSA omitted	0.8 ± 0.1 (3)	0.4 ± 0.03 (3)
XO omitted	0.3 ± 0.2 (3)	0.1 ± 0.04 (3)
XO heated	0.1 ± 0.003 (3)	0.2 ± 0.04 (3)
EDTA added		1.4 ± 0.4 (3)
phosphate buffer, pH 5.0, added		0.2 ± 0.003 (3)
lactate buffer, pH 5.0, added		0.1 ± 0.01 (3)
citrate buffer, pH 5.0, added		0.4 ± 0.17 (3)

[a] The acet + XO + HSA + Fe^{2+} + EDTA system contained 0.05 M sodium phosphate buffer, pH 7.0, 10^{-5} sodium iodide (5 nmol; 0.05 μCi of ^{125}I), and, where indicated, 10^{-2} M acetaldehyde (acet), 20 μg/mL xanthine oxidase (XO), 500 μg of human serum albumin (HSA), 5×10^{-5} M ferrous sulfate (Fe^{2+}), and 10^{-4} M EDTA in a total volume of 0.5 mL. The acet + XO + HSA + Fe^{2+} system was as described above except that the phosphate buffer, pH 7.0, was replaced by 0.05 M acetate buffer, pH 5.0, and 0.05 M sodium phosphate, lactate, or citrate buffer, pH 5.0, was added where indicated. XO was heated at 100 °C for 15 min where indicated. [b] Mean ± SE of n experiments.

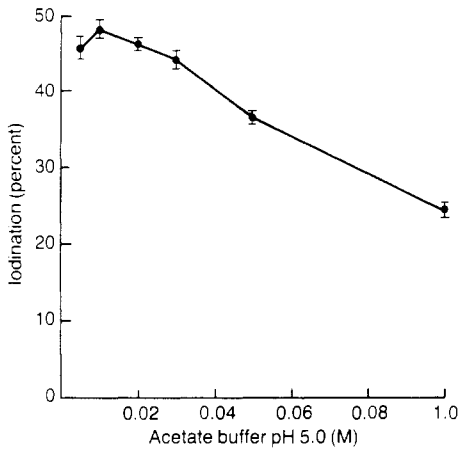

FIGURE 3: Effect of acetate buffer concentration on iodination by Fe^{2+}-supplemented xanthine oxidase system. The reaction mixture was as described for the acet + XO + HSA + Fe^{2+} system in Table IV except that the acetate buffer concentration was varied as indicated. Results are the mean ± SE of three experiments.

of albumin (Figure 2, tubes 2 and 3; Table III) and free tyrosine (Table III) to form monoiodotyrosine.

Iodination by Fe^{2+}-Supplemented, EDTA-Inhibitable Xanthine Oxidase System. As shown in Figure 1, there was little iodination by the acetaldehyde–xanthine oxidase–Fe^{2+}–EDTA system in 0.05 M phosphate buffer, pH 5.0. When the phosphate buffer was replaced by an equimolar concentration of a number of other buffers at pH 5.0 (lactate, citrate, acetate), iodination remained low; however, when EDTA was omitted from the reaction mixture, a difference between the buffers was observed. Iodination remained low in 0.05 M phosphate, lactate, or citrate buffers but was high when acetate buffer was employed or when no buffer was added (pH of the reaction mixture 5.3). Iodination by the acetaldehyde–xanthine oxidase–Fe^{2+} system in 0.05 M acetate buffer, pH 5.0 (Table IV), was greatly decreased or abolished by the deletion of each of the components of the system, by the addition of EDTA, or by the addition 0.05 M phosphate, lactate, or citrate buffer, pH 5.0, presumably accounting for the absence of iodination when the latter buffers were used. Iodination also was inhibited by acetate buffer at concentrations greater than 0.03 M (Figure 3). The pH optimum of the Fe^{2+}-supplemented xanthine oxidase system in 0.01 M acetate buffer was optimal at pH 5.0–5.5 and fell sharply as the pH was increased to 6.5 (Figure 1).

The effect of inhibitors and scavengers of oxygen products on this system (Table II) was comparable to their effect on the Fe^{2+}–EDTA-supplemented xanthine oxidase system, pH 7.0, with one notable exception: the absence of inhibition by superoxide dismutase. Thus iodination was inhibited by catalase, but not heated catalase, by the hydroxyl radical scavengers mannitol and ethanol, by azide at high concentrations relative to those that inhibited the MPO–Cl^--supplemented system, and by cyanide, methimazole, and propylthiouracil. However, as with the MPO–Cl^--supplemented system, superoxide dismutase was not inhibitory. This lack of inhibition by superoxide dismutase was dependent on the iron concentration, which was 10^{-4} M in Table II. As shown in Table V, iodination decreased when the iron concentration was lowered, and when the iron level was 2×10^{-5} M or below, inhibition by superoxide dismutase was observed.

The lack of inhibition by superoxide dismutase at high iron concentrations can best be explained by a Fenton-type reaction in which H_2O_2 generated by the xanthine oxidase system reacts with iron to form hydroxyl radicals, and indeed acetaldehyde

Table V: Effect of Superoxide Dismutase at Various Iron Concentrations[a]

$FeSO_4$ (M)	iodination (%)		
	complete system	+SOD	+heated SOD
10^{-4}	40.6 ± 4.0[b]	38.2 ± 4.6 (NS)	34.9 ± 5.1 (NS)
5×10^{-5}	25.0 ± 4.1	27.9 ± 4.1 (NS)	22.4 ± 2.3 (NS)
2×10^{-5}	11.1 ± 1.1	4.9 ± 0.9 (<0.01)	10.6 ± 0.5 (NS)
10^{-5}	7.6 ± 1.5	1.4 ± 0.7 (<0.01)	7.8 ± 0.8 (NS)
5×10^{-6}	4.6 ± 0.4	0.3 ± 0.1 (<0.001)	4.6 ± 0.4 (NS)
2×10^{-6}	3.2 ± 0.5	0.1 ± 0.1 (<0.002)	3.1 ± 0.5 (NS)
	0.4 ± 0.1	0.1 ± 0.1 (NS)	0.5 ± 0.1 (NS)

[a] The complete system was as described for the acet + XO + HSA + Fe^{2+} system in Table IV except that the ferrous sulfate concentration was varied as indicated and SOD (5 μg/mL) or SOD autoclaved at 120 °C for 30 min was added where indicated. [b] Mean ± SE of four experiments. The *P* value for the difference from the complete system is shown in parentheses.

plus xanthine oxidase could be replaced by 10^{-4} M reagent H_2O_2 in this iodinating system (55.2 ± 1.8% iodide incorporation, $n = 3$). The properties of the H_2O_2–Fe^{2+} iodinating system were precisely those of the acetaldehyde–xanthine oxidase–Fe^{2+} system; iodination was optimal at pH 5.0–5.5 in acetate buffer at concentrations of 0.02 M or below, was unaffected by superoxide dismutase, and was inhibited by EDTA, phosphate, lactate, or citrate buffer and by catalase, mannitol, ethanol, azide, cyanide, methimazole, and propylthiouracil at the same concentrations that inhibited the Fe^{2+}-supplemented acetaldehyde–xanthine oxidase system (data not shown).

Discussion

Three iodinating systems are described here that are dependent on the aerobic oxidation of acetaldehyde by xanthine oxidase: one requires supplementation by MPO and chloride, the second requires supplementation by Fe^{2+} and EDTA, and the third requires supplementation by Fe^{2+} and is inhibited by EDTA.

The conversion of iodide to organic form by peroxidase and H_2O_2 is well established (Morrison & Schonbaum, 1976). It is not surprising therefore that the addition of MPO to a H_2O_2-generating system such as acetaldehyde–xanthine oxidase stimulates iodination. Iodination by the MPO-supplemented xanthine oxidase system was further increased by the addition of chloride. Chloride stimulation of iodination by MPO, H_2O_2, and iodide, particularly when the iodide to H_2O_2 ratio is low, has previously been reported (Klebanoff, 1970). Iodination by the acetaldehyde–xanthine oxidase–MPO–chloride system was inhibited by catalase, emphasizing the need for H_2O_2; superoxide dismutase, in contrast, produced a small but significant stimulation of iodination, an effect that would be consistent with increasing H_2O_2 production through enzyme-catalyzed dismutation. The hydroxyl radical scavengers mannitol and ethanol were without effect at 0.1 M. Our findings suggest that, in this iodinating system, iodide is oxidized by MPO and H_2O_2 generated by the xanthine oxidase system to form an iodinating species that binds in covalent linkage to tyrosine or tyrosine residues of protein to form monoiodotyrosine. Oxidation of iodide by the peroxidase–H_2O_2 enzyme–substrate complex may be direct or be mediated by the prior oxidation of chloride.

The second xanthine oxidase dependent iodinating system was effective in the absence of peroxidase but required Fe^{2+} and EDTA for optimal activity. Its properties were considerably different from the peroxidase-dependent system. The

acetaldehyde–xanthine oxidase–Fe^{2+}–EDTA iodinating system was strongly inhibited by catalase, superoxide dismutase, and the OH·scavengers mannitol and ethanol, suggesting a requirement for $O_2^{-}\cdot$, H_2O_2, and OH·. This is compatible with the following sequence of reactions. Oxygen is reduced by the xanthine oxidase system to $O_2^{-}\cdot$. H_2O_2 is formed either by the dismutation of $O_2^{-}\cdot$ or directly from oxygen, and the two reduction products, $O_2^{-}\cdot$ and H_2O_2, interact to form OH· by the iron–EDTA-catalyzed Haber–Weiss reaction. Oxidation of iodide by OH· [or an "OH· analagous" species (Bors et al., 1979)] may then form an iodine product capable of covalent linkage to tyrosine residues. The initial reaction is presumably an electron transfer as follows:

$$OH\cdot + I^- \rightarrow OH^- + I\cdot$$

Secondary reactions would be expected to rapidly follow with the possible formation of other iodine species. A number of measurements of the rate constant of the interaction of iodide and OH· have been made; most values have been in the region $k = 10^{10}$ M^{-1} s^{-1} (Dorfman & Adams, 1973).

Azide inhibited iodination by the acetaldehyde–xanthine oxidase–Fe^{2+}–EDTA system but only at a concentration (10^{-3} M) considerably higher than that required to inhibit the MPO-catalyzed system (10^{-6} M). Azide reacts readily with OH· with a rate constant at pH 9.0 of $k = 1.1 \times 10^{10}$ M^{-1} s^{-1} (Dorfman & Adams, 1973) and may inhibit iodination in this way. Cyanide also inhibited the Fe^{2+}–EDTA-supplemented xanthine oxidase system; indeed, it appeared to be a more effective inhibitor than azide under our experimental conditions. Cyanide reacts with OH· with a rate constant at pH 9 of $k = 4.5 \times 10^9$ M^{-1} s^{-1} (Dorfman & Adams, 1973). It is also an efficient chelating agent, and both actions may contribute to its inhibitory activity. Of note was the very low level of iodination observed with Tris buffer at pH 7.0, an effect that suggests scavenging of OH· by Tris (Paschen & Weser, 1975). Iodination by both the MPO–chloride- and the Fe^{2+}–EDTA-supplemented xanthine oxidase systems occurred best at neutral or alkaline pH.

The third xanthine oxidase dependent iodinating reaction occurred in an unbuffered reaction mixture (pH 5.3) or in acetate buffer at an acid pH and required the addition of Fe^{2+}. In contrast to the Fe^{2+}–EDTA-dependent system described above, EDTA was inhibitory. Our findings are compatible with the initiation of iodination in this system by OH· formed by a Fenton-type reaction. The evidence is as follows. (1) Fe^{2+} was required. (2) Iodination was inhibited by catalase, indicating a requirement for H_2O_2. (3) Superoxide dismutase was without effect, indicating that $O_2^{-}\cdot$ was not required. (4) Iodination was inhibited by the hydroxyl radical scavengers mannitol and ethanol. (5) Acetaldehyde and xanthine oxidase could be replaced by reagent H_2O_2. Iodination by this system was inhibited by 0.05 M phosphate, lactate, and citrate buffers and by high concentrations of acetate buffer, pH 5.0, which may be due to the scavenging of OH· and/or the chelation of iron. The properties described above were observed when iron was employed at relatively high concentration. When the iron concentration was decreased to 2×10^{-5} M or below, inhibition by superoxide dismutase occurred. Fe^{2+} is initially oxidized by H_2O_2 generated by the xanthine oxidase system. Our studies suggest that at low iron concentration, its reduction by $O_2^{-}\cdot$ and reutilization are required for optimum iodination. Under these conditions, iron acts catalytically, and the Fenton-type reaction becomes a Haber–Weiss reaction and is inhibited by superoxide dismutase.

Our findings suggest that iodination can be initiated by hydroxyl radicals and provide evidence for two mechanisms for their generation for this purpose: the metal-catalyzed Haber–Weiss reaction and Fenton's reagent. It is the generally held view that iodination in situ, e.g., in the thyroid gland and leukocytes, is dependent largely on catalysis by peroxidase. Is there a contribution by OH·? Xanthine oxidase has been detected in bovine thyroid gland and has been proposed as a source of H_2O_2 required for thyroidal iodination (Lee & Fischer, 1978). A contribution by OH· formed by this enzyme in the thyroid thus is possible. Iodination by appropriately stimulated polymorphonuclear leukocytes (PMNs) and monocytes that lack peroxidase, i.e., from patients with hereditary MPO deficiency, is generally decreased but not abolished (Lehrer, 1972; Klebanoff & Clark, 1977; Nauseef et al., 1981). Since OH· also appears to be generated by stimulated phagocytes (Weiss et al., 1977, 1978; Tauber & Babior, 1977; Klebanoff & Rosen, 1978; Green et al., 1979; Rosen & Klebanoff, 1979; Repine et al., 1979, Drath et al., 1979; Sagone et al., 1980), iodination by this radical may account, in part, for iodination by MPO-deficient (and presumably normal) leukocytes.

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Interaction of Benzene with Bilayers. Thermal and Structural Studies†

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ABSTRACT: The thermal and structural properties of saturated phosphatidylcholine liposomes are significantly altered by benzene. Upon the addition of benzene, the liposomes first swell and then disperse into small multilamellar vesicles. At 20 °C these vesicles contain striations or ripples in the plane of the bilayer. Major changes in the thermal behavior of DSPC–benzene liposomes occur near mole ratios of 2:1 and 1:1. At a 2:1 mole ratio, the area under the main endothermic peak, ΔH_m, essentially disappears; however, the total heat absorbed, ΔH_f, remains approximately equal to that of the control. This occurs because for benzene mole fractions $0.12 < x < 0.50$, benzene increases the apparent molar heat capacity, C_p, of the gel phase to about 1.2 kcal/(mol·deg). We interpret this increase in heat capacity to be due to an increase in the concentration of defects (or disorder) in the gel phase. At mole fractions of benzene between 0.5 and 0.9, the transition temperature decreases by 20–30 °C, and broad, multiple transitions are observed. From $0.5 \leq x \leq 0.9$, the apparent molar heat capacity of the liquid-crystal phase increases to that of the defected rippled gel phase. The value of ΔH_f approaches the heat of fusion for 2 mol of *n*-octadecane, suggesting that benzene uncouples the liquid-crystalline acyl chains. The lipids affected by benzene or "boundary lipids" have higher heat capacity than nonperturbed lipids. The apparent molar specific heat, C_p, of 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine (and 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine) multilamellar vesicles is 0.20 ± 0.05 kcal/(mol·deg) in the Lβ', Pβ, and Lα phases. C_p fluctuates about this value in all three phases upon repeated phase transitions in the same sample. However, the value of C_p in the Pβ (rippled) phase exhibits much greater fluctuations in C_p than that in the Lα phase. We attribute these fluctuations to crystal packing defects.

The study of benzene by itself and mixed with other solvents has led to a better understanding of solvent–solute interactions (Hildebrand et al., 1970). The fact that benzene is the parent molecule for many drugs and biological molecules such as phenylethylamines, phenols, aromatic amino acids, cholesterol, bile salts, and fluorescent probes (Radda, 1975), coupled with its implication as a carcinogen (Tough et al., 1970), has stirred numerous investigations on how benzene interacts with biological materials.

In particular, the interaction of benzene at high mole fractions (x)[1] with phosphatidylcholines (lecithins) has been

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[1] Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphorylcholine; x, mole fraction in the organic phase; DSC, differential scanning calorimetry; BZ, benzene; OD, optical density; T%, percent transmittance; %, percent by weight; C_p, constant pressure heat capacity.

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