BACTERICIDAL ACTIVITY OF A SUPEROXIDE ANION-GENERATING SYSTEM

A Model for the Polymorphonuclear Leukocyte*

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Microorganisms ingested by polymorphonuclear leukocytes (PMNs)[1] are exposed to a variety of antimicrobial systems (for review see references 1, 2). In our view, the predominant antimicrobial system for most organisms is one which consists of myeloperoxidase (MPO), H_2O_2, and a halide. After phagocytosis, MPO is released into the phagocytic vacuole from adjacent lysosomal granules, where it interacts with H_2O_2 and a halide, such as chloride or iodide, to form an agent or agents toxic to the ingested organisms. MPO-independent antimicrobial systems also are present in the PMN. PMNs which lack MPO, i.e., from patients with hereditary MPO deficiency, have a major fungicidal and bactericidal defect; however, microbicidal activity is not abolished. Bactericidal activity is observed after a lag period, and this residual activity is dependent in part on oxygen. Oxygen-dependent, but MPO-independent, antimicrobial systems are presumably also present in normal PMNs. The nature of these systems has been the subject of considerable recent interest.

Phagocytosis by PMNs (or, more accurately, perturbation of the plasma membrane) is associated with the reduction of oxygen to water with the formation of highly reactive intermediates. When oxygen accepts a single electron, it is converted to the superoxide anion (O_2^-), a radical which can act both as a reductant or an oxidant. In the human PMN, a pyridine nucleotide oxidase, probably located in the plasma membrane, appears to catalyze this reaction (3, 4). The two electron reduction product of oxygen, H_2O_2, may be formed directly from oxygen by divalent reduction, or by the univalent reduction of O_2^-. A second superoxide radical can serve as the electron donor in the latter reaction in a dismutation reaction as follows:

$$(a)\ O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2.$$
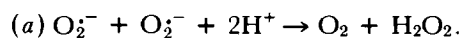
Dismutation can occur spontaneously or be catalyzed by the enzyme superoxide dismutase (SOD) (for review see reference 5). The further reduction of H_2O_2 results in the formation of highly reactive hydroxyl radicals ($OH\cdot$). One mechanism proposed for the production of $OH\cdot$ is the reduction of H_2O_2 by O_2^- (Haber-Weiss reaction [6]) as follows:

$$(b)\ H_2O_2 + O_2^- \rightarrow O_2 + OH^- + OH\cdot.$$
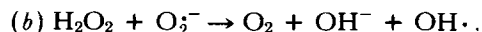
The rate constant for the direct interaction of H_2O_2 and O_2^- is low compared to

competing reactions such as the spontaneous dismutation of O_2^-, making it unlikely that OH$\cdot$ is formed in this way (7-9). However, recently a modification of the Haber-Weiss reaction has been proposed in which a metal ion is first reduced by O_2^- and then oxidized by H_2O_2, with the overall reaction being the generation of OH$\cdot$ from H_2O_2 and O_2^- as shown in (b) (10). When a chemical reaction is induced by a O_2^-- and H_2O_2-generating system, the inhibition of this reaction by SOD, catalase, and OH$\cdot$ scavengers and its stimulation by H_2O_2 has been taken as evidence for the participation of OH$\cdot$ (11).

An additional, potentially toxic, product of oxygen metabolism is singlet molecular oxygen ($^1O_2$), an excited state of oxygen which is formed when one of the valence electrons is shifted to an orbital of higher energy with an inversion of spin (for review see references 12-15). Singlet oxygen may be formed by the interaction of the various products of oxygen reduction. Among the possible mechanisms are: the spontaneous dismutation of O_2^- (16, 17),

$$(c)\ O_2^- + O_2^- + 2H^+ \rightarrow\ ^1O_2 + H_2O_2;$$
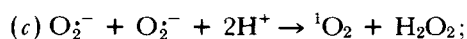
the interaction of O_2^- and OH$\cdot$ (18),

$$(d)\ O_2^- + OH\cdot \rightarrow\ ^1O_2 + OH^-;$$
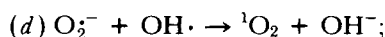
and the interaction of O_2^- and H_2O_2 (19, 20)

$$(e)\ O_2^- + H_2O_2 \rightarrow\ ^1O_2 + OH^- + OH\cdot.$$
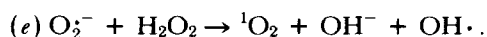
As indicated above, the latter reaction (Haber-Weiss) would require a metal or other catalyst. The excess energy of $^1O_2$ can be dissipated by thermal decay, emission of light, or chemical reaction. The formation of a relatively characteristic $^1O_2$ product in chemical reactions may be employed as evidence for the presence of $^1O_2$. One such reaction which we have employed for this purpose is the conversion of 2,5-diphenyl-furan to cis-dibenzoylethylene (21).

The aerobic oxidation of its substrate by xanthine oxidase is associated with the formation of O_2^- and H_2O_2 (22) which may subsequently interact to form OH$\cdot$ (11) and possibly $^1O_2$ (19, 20). This has prompted the use of the xanthine oxidase system with xanthine, hypoxanthine, or purine as substrate as a model of the oxygen-dependent antimicrobial systems of the PMN (23-25). With some microorganisms, the antimicrobial effect was inhibited by catalase but not SOD, implicating H_2O_2 (24, 25); in other instances, catalase, SOD, and hydroxyl radical scavengers were inhibitory and OH$\cdot$ generated by the interaction of H_2O_2 and O_2^- was proposed as the microbicidal agent (24). In our earlier study with xanthine as substrate (23), very little antimicrobial activity was observed unless MPO and a halide was added, suggesting that the most potent product of oxygen reduction was H_2O_2 when combined with the other components of the peroxidase system. However, in a recent study in which acetaldehyde was employed as substrate for xanthine oxidase, lysis of erythrocytes was inhibited by xanthine and uric acid (20). This raises the possibility that purines employed as substrate for xanthine oxidase may interfere with microbicidal activity by competing with the microbes for the potentially toxic oxygen products.

In this paper we have reinvestigated the antimicrobial activity of xanthine oxidase in the presence and absence of MPO and a halide, but with acetaldehyde as substrate. The inhibitory effect of purines is described and the role of O_2^-, H_2O_2, OH$\cdot$, and $^1O_2$

in the toxicity considered. The acetaldehyde-xanthine oxidase system in the presence and absence of MPO and a halide appears to be a useful model of the oxygen-dependent antimicrobial systems of the PMN.

Materials and Methods

Special Reagents. Xanthine oxidase (bovine milk, 10 mg/ml, ≅0.4 U/mg suspended in 2.0 M ammonium sulfate and 0.01 M EDTA) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Catalase (bovine liver, 6.1 mg/ml, 60,000 U/mg) obtained from Worthington Biochemical Corp., Freehold, N. J., was dialyzed against water before use. Myeloperoxidase was prepared from canine pyometrial pus by the method of Agner (26) to the end of step 6 and assayed by the ortho-dianisidine method before use (27). 1 U of enzyme is the amount decomposing one μmol of H_2O_2 per minute at 25°C. Superoxide dismutase (bovine erythrocytes, lyophilized powder, 12,300 U/mg) obtained from Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind., was dissolved 5 mg/ml in water and stored at −20°C. Xanthine oxidase, catalase, and myeloperoxidase solutions were heated at 100°C for 15 min and superoxide dismutase was autoclaved at 120°C for 30 min where indicated.

Acetaldehyde (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was distilled and stored in aliquots at −20°C. *Cis*-dibenzoylethylene, *trans*-dibenzoylethylene, and tritiated 2,5-diphenyl-furan (2 μCi/μmol) were obtained as previously described (21). 1,4-Diazabicyclo[2,2,2] octane (DABCO) was obtained from Eastman Organic Chemicals Div., Rochester, N. Y. and xanthine, hypoxanthine, and uric acid were obtained from Sigma Chemical Co., St. Louis, Mo. Trypticase soy broth and agar were from BioQuest, BBL, & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md. Enriched nutrient broth and agar consisted of 12.5 g heart infusion broth, 2.5 g yeast extract, and either 5.4 g nutrient broth or 23 g nutrient agar in 1 l of water. All components were obtained from Difco Laboratories, Detroit, Mi.

Microbicidal Activity. Staphylococcus aureus 502A was maintained on blood agar plates and transferred daily to trypticase soy broth. Overnight cultures were washed twice with 100 mM sodium sulfate and suspended in the same solution to the required absorbance at 540 nm. *Sarcina lutea* (yellow) and a pigmentless mutant, strain 93A (white), were the gift of Dr. Norman Krinsky, Tufts University, Boston (28). They were maintained on trypticase soy agar slants or blood agar plates and were transferred daily to enriched nutrient broth at 30°C (yellow) or trypticase soy broth supplemented with 0.5% yeast extract at 37°C (white). Overnight cultures were washed twice, dispersed for 1 min in a Potter-Elvehjem homogenizer and suspended in 100 mM sodium sulfate to the required optical density at 540 nm.

Components indicated in the legends to the tables and figure were added to 10 x 75 mm test tubes and incubated at 37°C in a shaking water bath for 60 min unless otherwise indicated. A 0.1-ml portion of the suspension was serially diluted in 100 mM sodium sulfate and the viable cell count determined by the pour plate method using trypticase soy agar supplemented with 0.25% glucose for *S. aureus* and *S. lutea* (white) and enriched nutrient agar for *S. lutea* (yellow).

Diphenylfuran Conversion. Components of the reaction mixtures indicated in the legends to the tables were incubated in 13 x 100 mm test tubes for 60 min at 37°C in a shaking water bath oscillating 80 times/min. Products of diphenylfuran oxidation were determined as previously described (method B; 21). Briefly, the reaction mixture was extracted with chloroform and the chloroform layer evaporated under nitrogen. The residue was dissolved in 100 μl of ethanol and 10 μl of the solution was separated by thin-layer chromatography with heptane:dioxane (3:1) as the solvent. Unlabeled diphenylfuran, *trans*-dibenzoylethylene, and *cis*-cibenzoylethylene were employed as standards. Spots corresponding to the standards were identified by short wave UV (254 nm) illumination, scraped from the plates, and counted in a Beckman LS-100C liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). 10 μl of the original ethanol solution were employed as a standard and the percent of the added radioactivity in each spot was determined. The amount of dibenzoylethylene (nanomoles) formed was calculated from the initial amount of diphenylfuran employed and was corrected for the amount of dibenzoylethylene found in simultaneous incubations of diphenylfuran in buffer alone.

Data Analysis. For the analysis of the microbicidal data, reaction mixtures yielding sterile pour plates were assigned a value of 10 organisms/ml, the limit of sensitivity of the assay. The number of viable organisms/ml was expressed as the logarithm and arithmetic means, standard

TABLE I

*Comparison of the Bactericidal Activity of the Xanthine Oxidase System in the Presence and Absence of MPO and Chloride**

Acetaldehyde	Viable cell count (organisms/ml $\times$ 10^{-6})		
	Acetaldehyde	Acet. + XO	Acet. + XO + MPO + Cl$^-$
mM			
20	3.9	0.012‡	
10	3.6	0.005‡	
4	4.2	0.1‡	
2	3.6	3.6	
1	3.7	4.2	
0.4	3.7	4.0	
0.2	4.1	4.5	0.0003‡
0.1		4.9	0.01‡
0.04		4.2	0.9§
0.02		3.9	5.1
0.01		3.9	4.7

* The reaction mixture contained 50 mM sodium phosphate buffer pH 7.0, 43 mM sodium sulfate, 4 mM ammonium sulfate, 0.02 mM EDTA, 4.5 × 10⁶/ml *S. aureus*, acetaldehyde (Acet.) at the concentrations indicated and where indicated 10 μg (4 mU) xanthine oxidase (XO), 50 mM sodium chloride and 8 mU MPO in a total volume of 0.5 ml. An isotonically equivalent amount of sodium sulfate (33 mM) was omitted from tubes containing sodium chloride. Incubation-60 min. Results are the geometric mean of three experiments. Significance of difference from initial viable cell count: ‡ $P < 0.005$; § $P < 0.02$; all others NS.

errors, and P values were determined for the logarithmically transformed data (29). The data are expressed as the antilog of the arithmetic mean of the logarithms (the geometric mean of the original untransformed values). Statistical differences were determined using Student's two tailed t test for independent means (not significant, NS, $P > 0.05$).

Results

Bactericidal Activity. The staphylocidal activity of the acetaldehyde-xanthine oxidase system in the presence and absence of MPO and chloride is shown in Table I. Acetaldehyde alone at the concentrations employed was without effect. Bactericidal activity was observed however on the addition of xanthine oxidase at acetaldehyde concentrations ranging from 20 to 4 mM. The further addition of MPO and chloride permitted a 100-fold reduction in the minimal effective acetaldehyde concentration to 0.04 mM. In Fig. 1, the acetaldehyde concentration was maintained at 4 mM and the bactericidal activity of the acetaldehyde-xanthine oxidase system was compared in the presence and absence of MPO and chloride over a 60-min time period. The MPO and chloride-supplemented system had a shorter lag period before the onset of detectable killing (15 vs. 30 min) and a considerably more pronounced bactericidal effect.

In all subsequent studies of bactericidal activity with *S. aureus* as the test organism, the acetaldehyde concentration was 10 mM for the acetaldehyde-xanthine oxidase system and 0.1 mM for the MPO and chloride-supplemented system. Table II demonstrates the requirement for each component of the acetaldehyde-xanthine oxidase and the acetaldehyde-xanthine oxidase-MPO-chloride systems and the loss of
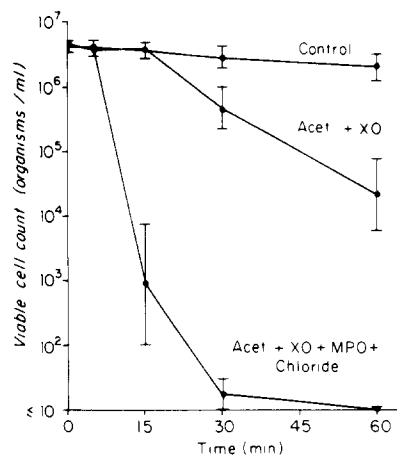
FIG. 1.   Kinetics of Bactericidal Activity. The reaction mixture was as described in Table I except that the acetaldehyde concentration was 4 mM and the incubation period was varied as indicated. The control consisted of *S. aureus* in the phosphate buffer pH 7.0 containing sodium sulfate. Results are the geometric mean ± SE of four experiments.

TABLE II

*Effect of Deletions**

Additions	Viable cell count (organisms/ml $\times 10^{-6}$)	
	Acet. + XO	Acet. + XO + MPO + Cl⁻
Buffer only	3.4	
Complete system	0.006	0.002
Acet. omitted	2.5	4.9
XO omitted	4.5	3.3
XO heated	3.6	4.5
Cl⁻ omitted		3.2
MPO omitted		3.8
MPO heated		3.5

* The reaction mixture was as described in Table I except that 10 mM acetaldehyde was employed in the Acet. + XO system and 0.1 mM in the Acet. + XO + MPO + Cl⁻ system, and components were omitted or heated as indicated. The results are the geometric mean of three experiments.

bactericidal activity on heat inactivation of xanthine oxidase and, where employed, MPO. Catalase strongly inhibited the bactericidal activity of the acetaldehyde-xanthine oxidase system both in the presence and absence of MPO and chloride (Table III), whereas SOD was an effective inhibitor only with the unsupplemented xanthine oxidase system. Heated catalase and SOD had no significant inhibitory effect in either system. This suggests a requirement for both H_2O_2 and O_2^- for the bactericidal activity of the acetaldehyde-xanthine oxidase system, whereas only H_2O_2 is required in the MPO and chloride-supplemented system.

The requirement for both H_2O_2 and O_2^- by the unsupplemented xanthine oxidase system is compatible with a reaction between the two to form some other, more bactericidal, agent. Two candidates have been proposed: hydroxyl radicals and singlet

TABLE III

Effect of Inhibitors on Bactericidal Activity*

Inhibitors	Viable cell count (organisms/ml $\times$ 10^{-6})					
	Acet. + XO			Acet. + XO + MPO + Cl⁻		
None	0.008	(19)		0.001	(19)	
Catalase, 60 μg/ml	4.1	(4)	<0.001	4.9	(4)	<0.001
Catalase heated	0.03	(4)	NS	0.008	(4)	NS
SOD, 1 μg/ml	3.1	(3)	<0.001	0.0005	(3)	NS
SOD heated	0.006	(3)	NS	0.0004	(3)	NS
Mannitol, 100 mM	2.2	(5)	<0.001	0.005	(5)	NS
Mannitol, 10 mM	0.47	(6)	<0.001	0.005	(6)	NS
Mannitol, 1 mM	0.02	(5)	NS	0.005	(5)	NS
Benzoate, 10 mM	4.1	(3)	<0.001	4.7	(2)	<0.001
Benzoate, 1 mM	0.10	(3)	<0.02	0.009	(3)	NS
Benzoate, 0.1 mM	0.007	(3)	NS	0.0005	(3)	NS
Azide, 10 mM	2.9	(6)	<0.001	5.3	(5)	<0.001
Azide, 1 mM	0.20	(5)	<0.01	4.8	(5)	<0.001
Azide, 0.1 mM	0.07	(5)	<0.05	5.5	(5)	<0.001
Azide, 0.01 mM	0.05	(5)	NS	0.20	(5)	<0.001
DABCO, 10 mM	4.1	(3)	<0.001	3.1	(3)	<0.001
DABCO, 1 mM	3.2	(3)	<0.001	0.002	(3)	NS
DABCO, 0.1 mM	0.01	(3)	NS	0.0006	(3)	NS
Histidine, 10 mM	3.7	(3)	<0.001	5.0	(3)	<0.001
Histidine, 1 mM	3.4	(3)	<0.001	4.4	(3)	<0.001
Histidine, 0.1 mM	4.0	(3)	<0.001	0.03	(3)	<0.05
Histidine, 0.01 mM	0.02	(3)	NS	0.001	(3)	NS

* The reaction mixture was as described for the complete system in Table II except that the inhibitors were added at the concentrations indicated. The results are the geometric mean of (n) experiments. The significance of the difference from no inhibitor is shown.

molecular oxygen. The effect of the hydroxyl radical scavengers, mannitol and benzoate, and the singlet oxygen quenchers, azide, DABCO, and histidine, on the bactericidal activity of the acetaldehyde-xanthine oxidase system is shown in Table III. Significant inhibitions occurred at concentrations at or above 10 mM mannitol, 1 mM benzoate, 0.1 mM azide, 1 mM DABCO, and 0.1 mM histidine. In contrast, the MPO-supplemented xanthine oxidase system is unaffected by mannitol at all the concentrations employed, whereas benzoate was inhibitory at 10 mM (Table III). The $^1O_2$ scavengers, DABCO and histidine, were inhibitory at 10 and 0.1 mM, respectively. Azide, which is both a $^1O_2$ scavenger and an inhibitor of MPO, was a more effective inhibitor of the MPO-supplemented than the unsupplemented xanthine oxidase system.

Xanthine and hypoxanthine (like acetaldehyde) are substrates for xanthine oxidase, and the final product of their oxidation is uric acid. Table IV indicates that 1 mM xanthine and 1 mM hypoxanthine have a small but significant inhibitory effect on the bactericidal activity of the acetaldehyde-xanthine oxidase system. Uric acid was more strongly inhibitory at 1 mM and an effect was also observed at 0.1 mM.

The effect of the acetaldehyde-xanthine oxidase system on two strains of *S. lutea* which differ in their content of carotenoid pigment is shown in Table V. Approximately 40% of the pigmented yellow organisms and 99% of the nonpigmented white

TABLE IV

Effect of Purines on the Acetaldehyde-Xanthine Oxidase Bactericidal System*

Purine	Viable cell count (organisms/ml × 10^{-6})		
None	0.008	(19)	
Xanthine, 1.0 mM	0.4	(7)	<0.001
0.1 mM	0.02	(6)	NS
0.01 mM	0.01	(3)	NS
Hypoxanthine, 1.0 mM	0.06	(7)	<0.02
0.1 mM	0.02	(7)	NS
0.01 mM	0.01	(3)	NS
Uric Acid, 1.0 mM	3.6	(3)	<0.001
0.1 mM	0.2	(3)	<0.01
0.01 mM	0.05	(3)	NS

* The reaction mixture was as described in Table II for the complete acetaldehyde-xanthine oxidase system except that the purines were added at the concentrations indicated. The results are the geometric mean of (n) experiments. The significance of the difference from no purine is shown.

TABLE V

Effect of the Acetaldehyde-Xanthine Oxidase System on S. Lutea*

Supplements	Viable cell count (organisms/ml × 10^{-6})	
	S. lutea (yellow)	S. lutea (white)
Buffer only	5.0 (9)	3.4 (9)
Complete system	2.0‡ (5)	0.03 (5)‡§
Acet. omitted	5.4 (5)	4.1 (4)
XO omitted	4.6 (5)	2.9 (5)

* The complete system was as described for the acetaldehyde-xanthine oxidase system in Table II except that 4 mM acetaldehyde was employed and the test organisms were either wild type S. lutea (yellow) or a pigmentless mutant (white).

‡ $P < 0.005$ compared to buffer only.

§ $P < 0.01$ white compared to yellow (paired analysis).

TABLE VI

Conversion of 2,5-Diphenylfuran to Cis-Dibenzoylethylene by Acetaldehyde Plus Xanthine Oxidase*

Components	cis-Dibenzoylethylene
	nmol
Complete system	25.6 ± 0.7‡ (26)
Xanthine oxidase omitted	0.4 ± 0.1 (4)
Acetaldehyde omitted	0.0 ± 0.1 (4)
SOD added	5.1 ± 0.7 (5)
Heated SOD added	23.4 ± 3.0 (4)
Catalase added	6.0 ± 1.3 (6)
Heated catalase added	22.0 ± 3.0 (5)

* The complete system contained 40 μgm, 16 mU, xanthine oxidase, 40 mM acetaldehyde, 4 mM ammonium sulfate, 0.02 mM EDTA, 44 mM sodium sulfate, 50 nmol diphenylfuran, and 0.25% ethanol in 2.0 ml of 50 mM sodium phosphate buffer, pH 7.0. Xanthine oxidase and acetaldehyde were omitted and 1 μg/ml SOD and 60 μg/ml catalase were added where indicated.

‡ Mean ± SE of (n) experiments.

TABLE VII

Effect of Inhibitors on 2,5-Diphenylfuran Conversion by Acetaldehyde Plus Xanthine Oxidase*

Inhibitor		Diphenylfuran conversion (% inhibition)				
		Concentration of inhibitor				
		100 mM	10 mM	1 mM	0.1 mM	0.01 mM
Azide	(3)	98 ± 1‡	99 ± 1‡	88 ± 1‡	51 ± 1‡	18 ± 3‡
DABCO	(3)	101 ± 0‡	89 ± 2‡	20 ± 3‡	2 ± 2	
Histidine	(3)	97 ± 1‡	98 ± 1‡	78 ± 1‡	31 ± 2‡	17 ± 7
Mannitol	(4)	33 ± 10‡	2 ± 7			
Benzoate	(3)	49 ± 3‡	5 ± 24			
Xanthine	(4)				96 ± 1‡	74 ± 3‡
Hypoxanthine	(4)				88 ± 4‡	76 ± 7‡
Uric Acid	(3)				97 ± 2‡	81 ± 6‡

* The reaction mixture was as described for the complete system in Table VI except that inhibitors were added at the concentrations indicated. The number of experiments is shown in parentheses. Results are mean ± SE.
‡ $P < 0.05$ as compared to no inhibitor.

organisms were killed by the xanthine oxidase system under the conditions employed. This difference was significant ($P < 0.01$, paired analysis).

Diphenylfuran Conversion. The conversion of diphenylfuran to cis-dibenzoylethylene by acetaldehyde plus xanthine oxidase but not by either alone is shown in Table VI. The formation of trans-dibenzoylethylene was negligible (0.4 nmol). Conversion was strongly inhibited by catalase and SOD (Table VI) and by the singlet oxygen quenchers, azide (≥ 0.01 M), DABCO (≥ 1 mM), and histidine (≥ 0.1 mM) (Table VII). In contrast, the hydroxyl radical scavengers mannitol and benzoate were moderately inhibitory only at the highest concentration employed (100 mM). The purines, xanthine, hypoxanthine, and uric acid, were potent inhibitors of diphenylfuran conversion by the acetaldehyde-xanthine oxidase system at concentrations ≥ 0.01 M.

Discussion

The aerobic oxidation of acetaldehyde by xanthine oxidase in the presence and absence of MPO and chloride is employed here as a model of the oxygen-dependent microbicidal systems of the PMN. The xanthine oxidase system generates O_2^-, H_2O_2, OH·, and possibly $^1O_2$. A portion of the H_2O_2 is formed by the dismutation of O_2^-; the remainder by divalent reduction without an apparent O_2^- intermediate (22). The generation of hydroxyl radicals by the xanthine oxidase system is based on the formation by this system of ethylene from β-methylthiopropionaldehyde (methional). Ethylene formation is inhibited by SOD, catalase, and the OH· scavengers, ethanol and benzoate, and is stimulated by H_2O_2, suggesting the interaction of O_2^- and H_2O_2 to form OH· which initiates ethylene formation (11).

The acetaldehyde-xanthine oxidase system also appears to form $^1O_2$. Kellogg and Fridovich (19) in a study of linolenic acid peroxidation by acetaldehyde plus xanthine oxidase, found an inhibition of peroxidation by SOD, catalase, and scavengers of $^1O_2$, but not by scavengers of OH·. When 2,5-dimethylfuran was employed as a $^1O_2$ scavenger, a product was formed which was identical by thin-layer chromatography to the product formed by a known source of $^1O_2$. It was proposed that $^1O_2$, like OH·,

was generated by the Haber-Weiss reaction (e). Our finding of the conversion of 2,5-diphenylfuran to cis but not $trans$-dibenzoylethylene (Table VI), the strong inhibition of this conversion by SOD, catalase, and the $^1O_2$ quenchers, azide, DABCO and histidine, and the minimal effect of OH· scavengers (Table VI, VII) provides additional support for this mechanism for $^1O_2$ formation by the acetaldehyde-xanthine oxidase system.

Acetaldehyde plus xanthine oxidase is bactericidal at relatively high acetaldehyde concentrations (10^{-2} M). The staphylocidal activity of this system is inhibited by SOD and catalase (Table III) suggesting a requirement for both O_2^- and H_2O_2. Neither O_2^- nor H_2O_2 alone were adequate under the conditions employed because H_2O_2 formation is not decreased by SOD nor is O_2^- formation decreased by catalase. As described above, two possible products of the interaction of O_2^- and H_2O_2 are OH· and $^1O_2$; scavengers of both inhibit the staphylocidal activity of the acetaldehyde-xanthine oxidase system (Table III). Further evidence consistent with the participation of $^1O_2$ comes from studies of the effect of acetaldehyde-xanthine oxidase on two strains of $S.$ $lutea$, one yellow and the other white. The pigmented yellow strain was significantly more resistant to the bactericidal activity of the xanthine oxidase system than was the nonpigmented white strain (Table V), consistent with the protective effect of pigment. The strains are thought to differ only in their ability to synthesize carotenoid pigments which are potent $^1O_2$ quenchers and the differential killing of these strains by PMNs has been taken as evidence for the participation of $^1O_2$ in PMN microbicidal activity (28, 30). Although these findings are consistent with the involvement of both OH· and $^1O_2$ in the microbicidal activity of the acetaldehyde-xanthine oxidase system, it should be emphasized that inhibitor studies must be interpreted with caution, because the specificity of an inhibitor for a particular mechanism is seldom absolute.

In our previous study of the bactericidal activity of the xanthine oxidase system, xanthine was employed as substrate and very little antimicrobial activity was found (23). As shown in Table IV, xanthine, hypoxanthine, and their oxidation product, uric acid, inhibit the bactericidal activity of the acetaldehyde-xanthine oxidase system at concentrations ⅒ (xanthine, hypoxanthine) or ¹⁄₁₀₀ (uric acid) the initial acetaldehyde concentration. Kellogg and Fridovich (20) have observed an inhibition by xanthine and uric acid of erythrocyte lysis by acetaldehyde plus xanthine oxidase and have proposed the quenching effect of purines on $^1O_2$ as a possible mechanism. We found the conversion of diphenylfuran to cis-dibenzoylethylene by acetaldehyde plus xanthine oxidase to be inhibited by low concentrations (<10 μM) of xanthine, hypoxanthine, and uric acid (Table VII), suggesting a high degree of purine reactivity toward the agent responsible for diphenylfuran conversion, presumably $^1O_2$. Purines also have high reactivity towards OH· with rate constants reported to be $>10^9$ M^{-1} s^{-1} (31) and thus would serve as very effective OH· scavengers as well. The inhibition of bactericidal activity by xanthine may in part explain our previous difficulty in demonstrating xanthine oxidase-mediated microbicidal activity with xanthine as substrate.

The microbicidal activity of the xanthine oxidase system was considerably increased by MPO and chloride with either xanthine (23) or acetaldehyde (Table I) as substrate. The supplementation of the acetaldehyde-xanthine oxidase system by MPO and chloride allowed a 100-fold reduction in the minimal effective acetaldehyde concen-

tration, suggesting that the toxic products of the xanthine oxidase system were required in considerably higher concentration in the absence of MPO and chloride than in their presence. When the acetaldehyde-xanthine oxidase system was compared in the presence and absence of MPO and chloride at the same acetaldehyde concentration (4 mM), the microbicidal activity of the MPO and chloride-supplemented system had an earlier onset and killing was more complete (Fig. 1). This difference in the rate and extent of staphylocidal activity was observed even when the acetaldehyde concentration of the xanthine oxidase system was increased to 2.5 times that of the MPO-supplemented system (data not shown).

The staphylocidal activity of the acetaldehyde-xanthine oxidase-MPO-chloride system was inhibited by catalase but not SOD, indicating a requirement for H_2O_2 but not O_2^- (Table III). The MPO-supplemented system was unaffected by mannitol at concentrations up to 100 mM suggesting that OH· are not involved in the toxicity. Benzoate, however, was inhibitory at 10 mM concentration, possibly due to a mechanism other than the scavenging of OH·. The $^1O_2$ scavengers, DABCO and histidine, were inhibitory at 10 and 0.1 mM, respectively. Azide, which both scavenges $^1O_2$ and directly inhibits MPO, inhibited the bactericidal activity of the MPO-supplemented xanthine oxidase system at a considerably lower concentration than was required for a comparable inhibition of the unsupplemented system. The formation of $^1O_2$ by the MPO system and its involvement in microbicidal activity has been proposed (21, 32, 33); however, the data is subject to other interpretations (34), leaving open the question of the role of $^1O_2$ in the MPO system.

The stimulus for studying the bactericidal activity of the xanthine oxidase system was the similarity between its oxygen-derived products and those of phagocytosing PMNs. Like the xanthine oxidase system, phagocytosis by PMNs is associated with the reduction of oxygen to O_2^- (35) and H_2O_2 (36) and their interaction to form OH· (37) and possibly $^1O_2$ (32) have been proposed. These oxygen-derived products are formed at the cell surface and presumably within the phagocytic vacuole where they can interact with MPO and other released granule proteins. These agents combine to rapidly kill most ingested organisms. Microbicidal activity is impaired in the absence of MPO, i.e., in PMNs from patients with hereditary MPO deficiency. Fungicidal activity against *Candida albicans* is almost abolished and bactericidal activity is greatly decreased (38, 39) despite the increase in phagocytosis-induced oxygen metabolism in these cells (40-42). The difference in the kinetics of the bactericidal activity of normal and MPO-deficient PMNs is similar to that of the acetaldehyde-xanthine oxidase system in the presence and absence of MPO and chloride (Fig. 1). In both, bactericidal activity, in the absence of MPO, is characterized by a lag period after which death of the organisms is observed (41). The xanthine oxidase system is effective at considerably lower acetaldehyde concentrations in the presence of MPO than in its absence suggesting a more efficient usage of the products of oxygen metabolism for microbicidal activity.

These findings support an important role for the MPO system in the microbicidal activity of the PMN. It is the predominant antimicrobial system during the early postphagocytic period and its broad specificity and high potency make it unlikely that many organisms will survive its action. In the absence of MPO, the respiratory burst is increased (40-42), possibly due to an MPO requirement for its termination (43), with a corresponding increase in the products of oxygen reduction and excitation. Our studies with the unsupplemented acetaldehyde-xanthine oxidase system suggest

that under these conditions bactericidal activity may be mediated in part by the interaction of O_2^- and H_2O_2 to form other more potent bactericidal agents, specifically OH$\cdot$ and $^1O_2$.

Summary

The acetaldehyde-xanthine oxidase system in the presence and absence of myelo-peroxidase (MPO) and chloride has been employed as a model of the oxygen-dependent antimicrobial systems of the PMN. The unsupplemented xanthine oxidase system was bactericidal at relatively high acetaldehyde concentrations. The bactericidal activity was inhibited by superoxide dismutase (SOD), catalase, the hydroxyl radical (OH$\cdot$) scavengers, mannitol and benzoate, the singlet oxygen ($^1O_2$) quenchers, azide, histidine, and 1,4-diazabicyclo[2,2,2]octane (DABCO) and by the purines, xanthine, hypoxanthine, and uric acid. The latter effect may account for the relatively weak bactericidal activity of the xanthine oxidase system when purines are employed as substrate. A white, carotenoid-negative mutant strain of *Sarcina lutea* was more susceptible to the acetaldehyde-xanthine oxidase system than was the yellow, carotenoid-positive parent strain. Carotenoid pigments are potent $^1O_2$ quenchers. The xanthine oxidase system catalyzes the conversion of 2,5-diphenylfuran to *cis*-dibenzoylethylene, a reaction which can occur by a $^1O_2$ mechanism. This conversion is inhibited by SOD, catalase, azide, histidine, DABCO, xanthine, hypoxanthine, and uric acid but is only slightly inhibited by mannitol and benzoate. The addition of MPO and chloride to the acetaldehyde-xanthine oxidase system greatly increases bactericidal activity; the minimal effective acetaldehyde concentration is decreased 100-fold and the rate and extent of bacterial killing is increased. The bactericidal activity of the MPO-supplemented system is inhibited by catalase, benzoate, azide, DABCO, and histidine but not by SOD or mannitol. Thus, the acetaldehyde-xanthine oxidase system which like phagocytosing PMNs generates superoxide (O_2^-) and hydrogen peroxide, is bactericidal both in the presence and absence of MPO and chloride. The MPO-supplemented system is considerably more potent; however, when MPO is absent, bactericidal activity is observed which may be mediated by the interaction of H_2O_2 and O_2^- to form OH$\cdot$ and $^1O_2$.

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