Oxidation of Methionine by Human Polymorphonuclear Leukocytes

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ABSTRACT Studies of the photosensitized oxidation have demonstrated that photodynamic oxidation of methionine is mediated by singlet oxygen ($^1O_2$). In this study, we demonstrated that phagocytosing human polymorphonuclear leukocytes (PMN), but not resting PMN, oxidized both intracellular and extracellular methionine to methionine sulfoxide. N-ethylmaleimide, which inhibits phagocytosis and cellular metabolism, inhibited the oxidation of methionine. Neutrophils from patients with chronic granulomatous disease did not oxidize methionine even in the presence of phagocytosis. The oxidation of methionine by phagocytosing normal PMN was inhibited by $^1O_2$ quenchers, (1.4-diazabicyclo-[2,2,2]-octane, tryptophan, NaN_3), myeloperoxidase (MPO) inhibitors (NaN_3, KCN) and catalase. In contrast, superoxide dismutase, ethanol, and mannitol had no effect. Furthermore, $^1O_2$ quenchers did not interfere with the production of superoxide (O_2^-) by phagocytosing PMN. The combination of catalase and SOD did not enhance the inhibition of methionine by phagocytosing PMN. On the other hand, deuterium oxide stimulated the oxidation of methionine by PMN almost 200%.

H_2O_2 at high concentrations oxidized methionine to methionine sulfoxide. However, when similar amounts of H_2O_2 were added to human PMN, they did not oxidize methionine. In contrast, when H_2O_2, at concentrations too low to oxidize methionine by itself, was added to the granular fraction, but not the soluble fraction, they oxidized methionine to methionine sulfoxide. The oxidation of methionine by the combination of H_2O_2 and granular fractions was inhibited by $^1O_2$ quenchers and MPO inhibitors, but it was stimulated by deuterium oxide. Removal of chloride anion also prevented the oxidation of methionine by the granular fractions.

Our results suggest that the oxidation of methionine by phagocytosing PMN is dependent on the MPO-mediated antimicrobial system (MPO-H_2O_2-Cl^-). They also suggest, but do not prove that the oxidation of methionine is mediated by $^1O_2$. Oxidation of methionine may be one of the mechanisms that human PMN damage microorganisms.

INTRODUCTION

Ingestion of particles by polymorphonuclear leukocytes (PMN)[1] is accompanied by the production of several reactive oxygen metabolites that have been implicated in the killing of ingested microorganisms (1). The one electron reduction of molecular oxygen results in the production of superoxide (O_2^-) (2). In human PMN, a pyridine nucleotide oxidase appears to catalyze this reaction (3). Superoxide may then dismutate, either spontaneously or under the enzymatic action of superoxide dismutase (SOD), producing H_2O_2 (1). Recent evidence suggests that O_2^- and H_2O_2, in turn, are precursors of more powerful oxidizing agents such as hydroxyl radical (OH.) (4) or singlet oxygen ($^1O_2$) (5).

Singlet oxygen is an excited state of molecular oxygen in which an electron has been shifted to an orbital of higher energy (6). The excessive energy of $^1O_2$ can be dissipated by the emission of light, by thermal decay or by its participation in characteristic chemical reactions. In 1972, Allen et al. (7) observed the emission of light by PMN during phagocytosis and suggested that this was due to the production of $^1O_2$. However, extensive studies (8) have shown that $^1O_2$ per se contributes little, if anything, to the luminescence of phagocytosing PMN, and that light emission during

[1] *Abbreviations used in this paper*: CGD, chronic granulomatous disease; DABCO, 1.4-diazabicyclo-[2,2,2]-octane; D_2O, deuterium oxide; MPO, myeloperoxide; PMN, polymorphonuclear leukocytes; SOD, superoxide dismutase.

phagocytosis is most likely the result of reactions between certain unspecified constituents of the ingested particles and some or all of the oxidizing agents produced by phagocytosing cells. In 1974, Krinsky (9) observed that human PMN killed a colorless mutant strain of *Sarcina lutea* much more readily than a carotenoid-containing strain and suggested that singlet excited oxygen acted as one of the mediators of PMN bactericidal action. More recently, Rosen and Klebanoff (5) demonstrated the conversion of diphenylfuran to *cis*-dibenzoyl ethylene by the combination of myeloperoxidase (MPO), H_2O_2, and chloride anion, a MPO-mediated bactericidal system present in PMN. They suggested that this chemical conversion was due to the production of $^1O_2$ by the MPO-mediated bactericidal system. However, Harrison et al. (10) showed that the conversion of diphenylfuran to *cis*-dibenzoyl ethylene, the presumed $^1O_2$ reaction, by the MPO-H_2O_2-Cl^- system was due to the generation of Cl_2, not $^1O_2$.

Studies of the photosensitized oxidation have demonstrated that photodynamic oxidation of methionine is mediated by $^1O_2$ (11, 12). At low to neutral pH, two molecules of methionine are oxidized to methionine sulfoxide by one molecule of $^1O_2$ according to the following reaction (11, 12):

$$2CH_3SCH_2CH_2CHCOOH + {}^1O_2 \rightarrow$$
$$\underset{NH_2}{|}$$

$$\overset{O}{\overset{\|}{2CH_3SCH_2CH_2CHCOOH}}$$
$$\underset{NH_2}{|}$$
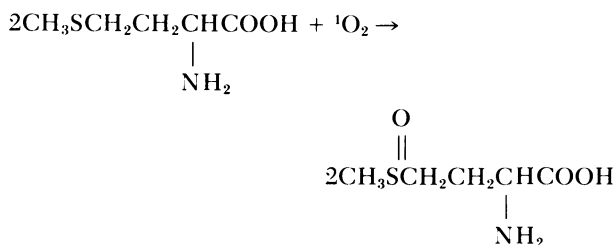
We have studied the oxidation of methionine by human PMN. The results of this study form the basis of this report.

METHODS

Chemicals. L-[U-^{14}C]methionine was obtained from New England Nuclear, Boston, Mass. L-methionine, L-methionine-DL-sulfoxide, guaiacol, catalase[2] (bovine liver, EC 1.11.1.6) and SOD (bovine blood EC 1.15.1.1) were obtained from Sigma Chemical Co., St. Louis, Mo. 1.4-diazabicyclo-[2,2,2]-octane (DABCO) was obtained from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y. Sodium hypochlorite was the product of Mallinckrodt, Inc., St. Louis, Mo. and was standardized by reaction with KI and spectrophotometric measurement of the I_3^- formed using $E_{353} = 2.64 \times 10^4$ M^{-1} cm^{-1} (13). Deuterium oxide (D_2O) was obtained from ICN Pharmaceuticals, Inc., Irvine, Calif. Polystyrene latex (0.79 μm Diam) were obtained from the Dow Corning Corp., Midland, Mich.

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[2] Catalase, 3,400 U/mg, one unit decomposes 1 μmol of H_2O_2/min at pH 7.0 25°C, whereas H_2O_2 concentration falls from 10.3–9.2 μmol/ml of reaction mixture. Superoxide dismutase, 2,810 U/mg. Assayed per method of McCord and Fidovich (*J. Biol. Chem.* **244:** 6049, 1969).

Isolation of human PMN and preparation of cell fractions. These were performed as described previously (14, 15).

Oxidation of methionine by human PMN. To 50 ml conical plastic centrifuge tubes, the following were added in a final vol of 1 ml: 1.5×10^7 PMN and 0.1 μCi [^{14}C]methionine (final concentration, 22 μM unless otherwise indicated). In the tubes designated for phagocytosis, polystyrene latex particles were added to a final concentration of 0.17% (by weight), which provided more than 100 particles per cell. Experimental additions yielded the final concentrations of the chemicals as indicated in the table legends. The tubes then were incubated at 37°C for 30 min in a waterbath with constant shaking. At the end of incubation, the tubes were immersed in ice and immediately centrifuged at 1,000 g for 5 min at 4°C. The supernate was saved for paper chromatography. In some experiments, the pellet was washed once with 5 ml cold modified Hanks' solution, the cells were then extracted with 1 ml distilled water in ice for 1 h. After centrifugation at 1,000 g for 5 min at 4°C, the water extract was chromatographed to determine the intracellular oxidation of methionine.

The ascending paper chromatography with three different solvent systems, which gave adequate separation of methionine from methionine sulfoxide (16) was used. These solvent systems are (*a*) t-butanol:methylethylketone:H_2O:ammonium hydroxide, 40:30:20:10; (*b*) n-butanol:glacial acetic acid:H_2O, 50:25:25; and (*c*) t-butanol:methylethylketone:formic acid:H_2O, 40:30:15:15. Control methionine and methionine sulfoxide were always cochromatographed with the samples. After the development, ninhydrin was used to identify control methionine and methionine sulfoxide, and the R_f values calculated. The chromatograms that contained the samples were cut into 1 × 2 cm strips, which were placed in vials and counted by liquid scintillation in a toluene-based solution. About 20 μl of the samples were chromatographed and the recovery of radioactivity was >95%. The results were plotted on the graph papers and the R_f values compared to those of control methionine and methionine sulfoxide. In the initial experiments, all three solvent systems were used. The R_f values of the oxidative product of [^{14}C]methionine were identical to those of methionine sulfoxide in all three solvent systems. Subsequently, only system I was used routinely.[3] In each experiment, a [^{14}C]methionine standard was also cochromatographed to determine the spontaneous oxidation of methionine during the procedure and the results were corrected accordingly.

Oxidation of methionine by the cell fractions. To 12 × 75 mm plastic test tubes, the following were added in a final vol of 0.5 ml in modified Hanks' solution: soluble or granular fraction (0.15 mg protein) and 0.1 μCi [^{14}C]methionine (22 μM). Experimental additions yielded the final concentrations of the chemicals as indicated in the tables. The tubes then were incubated at 37°C for 30 min in a waterbath with constant shaking. At the end of incubation, the tubes were immersed in ice and immediately centrifuged at 1,000 g for 5 min at 4°C. The supernate was chromatographed to determine the oxidation of methionine as described above.

Other studies. The fate of methionine in human PMN was analyzed by determining which fractions of radioactivity were acid soluble and acid insoluble as described previously (17). The myeloperoxidase activity of the granular fractions was determined according to Michell et al. (18) and the photodynamic oxidation of methionine by methylene blue was

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[3] Solvent system I can also separate methionine sulfone from methionine and methionine sulfoxide (23). Throughout the entire study, no [^{14}C]methionine sulfone was observed.
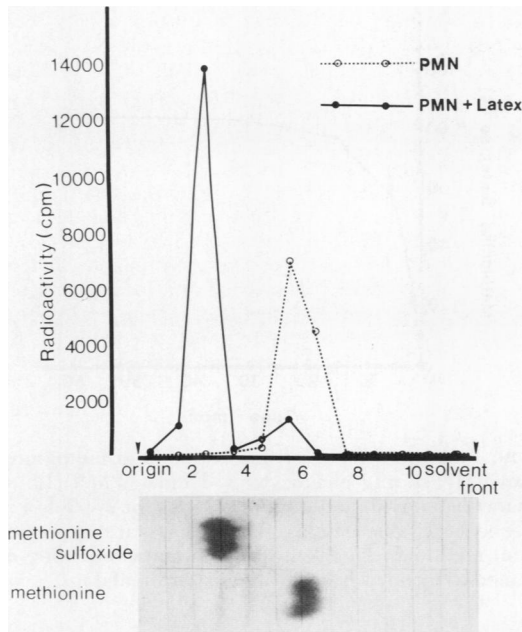
FIGURE 1 Chromatographic separation of methionine and methionine sulfoxide. Cells were incubated with [¹⁴C]methionine in the presence or absence of latex particles for 30 min at 37°C. The final media were then chromatographed by ascending paper chromatography using t-butanol:methylethylketone:H₂O:ammonium hydroxide (40:30:20:10).

performed according to Jori et al. (11). Superoxide production by human PMN was measured by SOD-inhibitable reduction of ferricytochrome c according to Babior et al. (2) as described previously (14). Hydrogen peroxide production

was determined by the disappearance of scopoletin fluorescence in the presence of horseradish peroxidase as described previously (19). The effect of D_2O on the oxidation of methionine by PMN, granular fraction or methylene blue was carried out by substituting H_2O with D_2O to a final concentration of 60% D_2O.

Statistical analysis. Statistical differences were determined using Student's t test for independent means (20).

RESULTS

Oxidation of methionine by human PMN. Using ascending paper chromatography with three different solvent systems, we demonstrated that in the presence of latex particles, methionine was oxidized to methionine sulfoxide by human PMN. Fig. 1 shows one of the typical experiments. After 30 min incubation, 86% of methionine present in the final medium was oxidized to methionine sulfoxide by human PMN in the presence of latex particles; whereas in the absence of latex particles, none of the methionine was oxidized.

Fig. 2A shows the amount of methionine taken up by human PMN at various substrate concentrations (12–62 μM). Resting PMN took up more methionine than phagocytosing PMN. The fractions of methionine taken up by the cells were small; for example, at a concentration of 22 μM, only 5 and 1.5% of methionine was taken up by resting and phagocytosing PMN, respectively. Quantification of the acid soluble and acid insoluble fractions revealed that <10% of the methionine taken up by the cells were incorporated into protein (acid-insoluble fraction).

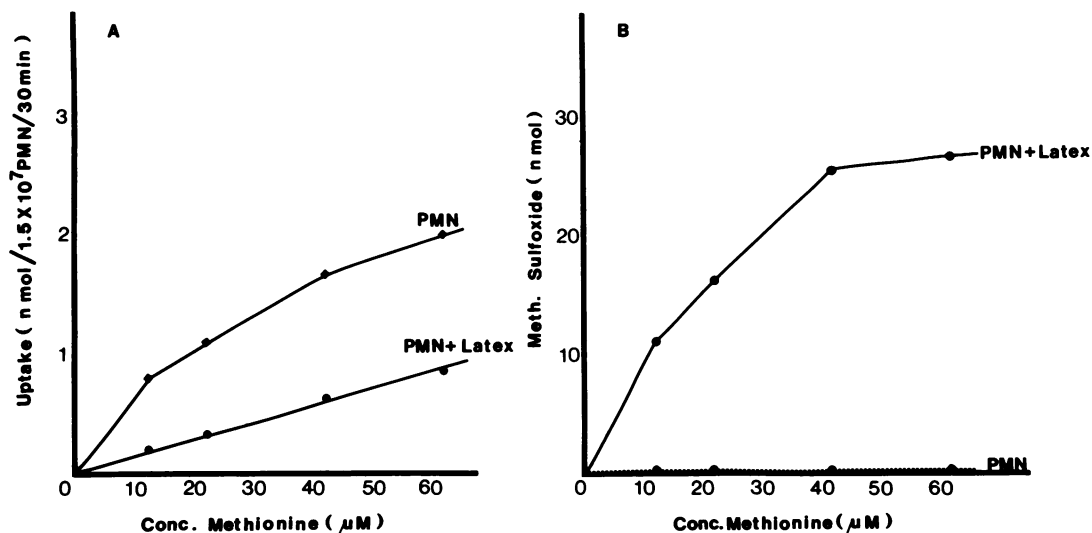Fig. 2B shows the oxidation of methionine by human

FIGURE 2 (A) Methionine uptake and (B) oxidation of methionine by human PMN. Human PMN (1.5×10^7) were incubated with methionine at various concentrations (conc) (12–62 μM) for 30 min in the presence or absence of latex particles. The amount of methionine uptake by the cells or the amount of methionine oxidized to methionine sulfoxide in the final media were then determined. Each point represents the mean of two experiments.

PMN at various concentrations of methionine. In this study, only the oxidation of extracellular methionine was determined. The amounts of methionine sulfoxide produced by phagocytosing PMN increased with increasing concentrations of methionine in the medium; it reached a plateau at a methionine concentration of 42 μM. In contrast, resting PMN did not oxidize methionine to methionine sulfoxide.

Most of our subsequent experiments were done with a methionine concentration of 22 μM. At this concentration, about 80% of methionine was oxidized to methionine sulfoxide by phagocytosing PMN; it gave us excellent sensitivity for testing the effect of various inhibitors. Table I summarizes some of our results. After 30 min incubation, 1.5% of methionine was taken up by the phagocytosing cells (see above). Of this, 77% was oxidized to methionine sulfoxide. Likewise, 80% of the methionine remaining in the medium was oxidized to methionine sulfoxide. In contrast, practically none of the extracellular and intracellular methionine was oxidized to methionine sulfoxide by resting PMN. N-ethylmaleimide, which inhibits phagocytosis and metabolic events associated with the activation of PMN (21), inhibited the oxidation of methionine by PMN in the presence of latex particles. This suggests that the metabolic event associated with phagocytosis is responsible for the oxidation of methionine to methionine sulfoxide. Since the majority of methionine remained in the medium, subsequent studies were performed to determine the oxidation of extracellular methionine only.

Fig. 3 shows a time-course of the oxidation of methionine by phagocytosing PMN. The oxidation of methionine was rapid; it reaches a plateau after incubation for 15 min. This suggests that the agent responsible
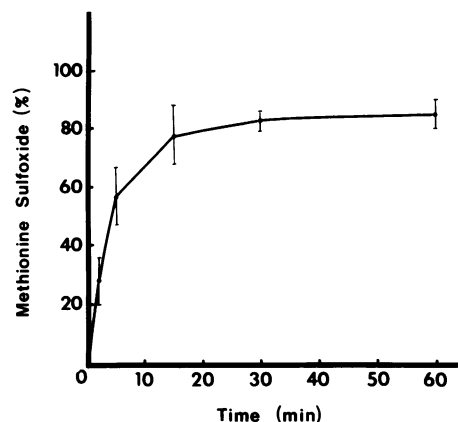

FIGURE 3 A time-course of the oxidation of methionine by human PMN during phagocytosis. Human PMN (1.5×10^7) were incubated with methionine (22 μM) for 2–60 min in the presence of latex particles. The results are expressed as percent methionine oxidized to methionine sulfoxide in the final media (mean$\pm$SEM of three experiments).

for the oxidation of methionine is produced at the early phase of phagocytosis.

Oxidation of methionine by PMN from patients with chronic granulomatous disease (CGD). Neutrophils from patients with CGD ingest particles normally, but fail to produce O_2^-, H_2O_2, and OH$\cdot$normally associated with phagocytosis (1). As a consequence, CGD patients suffer from recurrent, severe infections. Neutrophils from two unrelated patients with X-linked CGD were studied; CGD PMN did not oxidize methionine to methionine sulfoxide even in the presence of latex particles (two experiments, data not shown).

Effect of singlet oxygen quenchers on the oxidation of methionine by human PMN. To determine the agent (or agents) responsible for the oxidation of methionine by phagocytosing PMN, the effect of $^1O_2$ quenchers was first studied. Many of the known $^1O_2$ quenchers such as β-carotene, dimethylfuran (12) are lipid soluble with very limited water solubility. In this study, we only used the water soluble $^1O_2$ quenchers, DABCO (22), tryptophan (12), and NaN$_3$ (23). As shown in Table II, all these $^1O_2$ quenchers prevented the oxidation of methionine by phagocytosing PMN. Other amino acids, lysine, and glycine, had no effect (three experiments, data not shown). At similar concentrations, these $^1O_2$ quenchers did not have any effect on the O_2^- production by human PMN either at rest or during phagocytosis (three experiments, data not shown).

Since NaN$_3$ is known to inhibit heme enzymes such as MPO or catalase; KCN, an inhibitor of heme enzymes but not a $^1O_2$ quencher (5), was also studied. As shown in Fig. 4, both NaN$_3$ and KCN inhibited the oxidation of methionine by human PMN during phagocytosis. However, NaN$_3$ was about 10 times more potent than KCN in inhibiting methionine oxidation, al-

TABLE I
*Oxidation of Methionine by Human PMN**

	Methionine sulfoxide
	%
Resting cells	
Intracellular (5%)‡	1.0±0.6 (3)§
Extracellular (95%)	1.1±0.6 (9)
Phagocytosing cells	
Intracellular (1.5%)	77.0±0.6 (3)
Extracellular (98.5%)	80.0±6.1 (9)
+1 mM n-ethylmaleimide	1.0±0.4 (4)

* The results are expressed as mean±1 SEM of the percentage of methionine oxidized to methionine sulfoxide. PMN (1.5×10^7) were incubated with 0.1 μCi of [^{14}C]methionine (22 μM) in the presence or absence of latex particles or n-ethylmaleimide for 30 min at 37°C.
‡ Percentage of the total radioactivity at the end of incubation.
§ Number of experiments.

TABLE II

Effect of $^1O_2$ Quenchers on the Oxidation of Methionine by Phagocytosing PMN*

	Methionine sulfoxide	P values
	%	
Control	78.2±5.9 (5)	
+ DABCO (5 mM)	7.7±4.9 (5)	<0.001
+ Tryptophan (1 mM)	33.0±6.4 (4)	<0.005
+ NaN$_3$ (0.1 mM)	26.0±2.9 (5)	<0.001

* The results are expressed on the same basis as in Table I.

though KCN is a more potent inhibitor of MPO (24) (also see below).

Effect of SOD and catalase on the oxidation of methionine by human PMN. The effect of SOD and catalase was also studied. As shown in Table III, SOD had no effect on the oxidation of methionine by phagocytosing PMN. In contrast, catalase markedly inhibited the oxidation of methionine. The combination of SOD and catalase did not increase the inhibition suggesting that hydroxyl radicals were not involved. Other hydroxyl radical scavengers such as ethanol and mannitol also had no effect on the oxidation of methionine by phagocytosing PMN. (Table III)

Effect of H_2O_2 on the oxidation of methionine by human PMN. The above results indicate that at least part of the methionine oxidation by phagocytosing PMN is dependant on H_2O_2. Since H_2O_2 is known to oxidize methionine to methionine sulfoxide (25), we studied the effect of exogenous H_2O_2 on the oxidation of methionine by human PMN. As shown in Fig. 5, H_2O_2 oxidized methionine to methionine sulfoxide at relatively high concentrations, e.g., at a concentration of 1 mM, it oxidized 45% of the methionine. However, when the same amounts of H_2O_2 were added to human
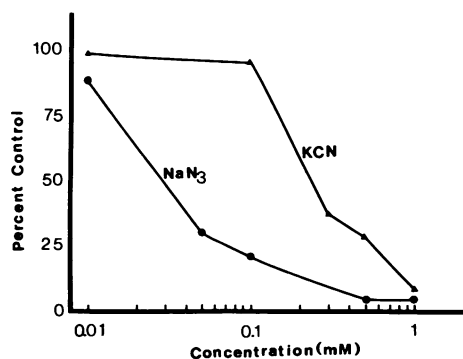
FIGURE 4 The effect of NaN$_3$ and KCN on the oxidation of methionine by human PMN during phagocytosis. Human PMN (1.5 × 10^7) were incubated with methionine (22 μM) and latex particles for 30 min at 37°C in the presence or absence of KCN or NaN$_3$. Each point represents the mean of two to four experiments.

TABLE III

Effect of SOD, Catalase and Hydroxyl Radical Scavengers on the Oxidation of Methionine by Human Phagocytosing PMN*

	Methionine sulfoxide	Percent control
	%	
Control	81.3±6.5 (3)	
+ SOD		
400 U/ml	86.2±8.6 (3)	106
800 U/ml	85.3±8.1 (3)	105
+ Catalase		
100 U/ml	59.3±4.8 (3)‡	73
500 U/ml	51.0±3.2 (3)‡	63
1,000 U/ml	15.4±1.8 (3)‡	19
1,000 U/ml heat inactivated	78.0±5.9 (3)	96
+ (SOD 100 U/ml + catalase 100 U/ml)	61.8±1.4 (2)	76
+ (SOD 400 U/ml + catalase 500 U/ml)	47.9±9.4 (3)‡	59
+ Ethanol 50 mM	85.2±3.1 (3)	104
+ Mannitol 50 mM	79.5±4.2 (3)	98

* The results are expressed on the same basis as in Table I. There was no statistical difference between catalase 100 U/ml and catalase 100 U/ml + SOD 100 U/ml; catalase 500 U/ml and catalase 500 U/ml + SOD 400 U/ml.
‡ P < 0.05 or less (vs. control).

PMN, it did not oxidize either intracellular or extracellular methionine to a great extent. This is most likely due to the removal of H_2O_2 by catalase present in the cells. Direct quantification of H_2O_2 production by phagocytosing PMN in the presence of 2 mM NaN$_3$ to inhibit the cellular destruction of H_2O_2 (19) revealed that 637.5 ± 60.3 nmol (n = 4) of H_2O_2 were produced by 1.5 × 10^7 PMN after 30 min incubation. This resulted in a final H_2O_2 concentration of about 0.6 mM.

Oxidation of methionine by the granular fraction of human PMN. In contrast to the above observation, when H_2O_2 at concentrations (0.01–0.2 mM) too low to oxidize methionine, was added to the granular fractions of human PMN, it oxidized methionine to methionine sulfoxide (Table IV). In the absence of H_2O_2, the granular fraction did not oxidize methionine. When similar amounts of H_2O_2 were added to the soluble fractions, there was no oxidation of methionine. Thus, the granular fraction of human PMN markedly enhanced the ability of H_2O_2 to oxidize methionine.

Effect of $^1O_2$ quenchers and MPO inhibitors on the oxidation of methionine by the granular fraction. The effect of $^1O_2$ quenchers and MPO inhibitors on the oxidation of methionine by the granular fraction was then
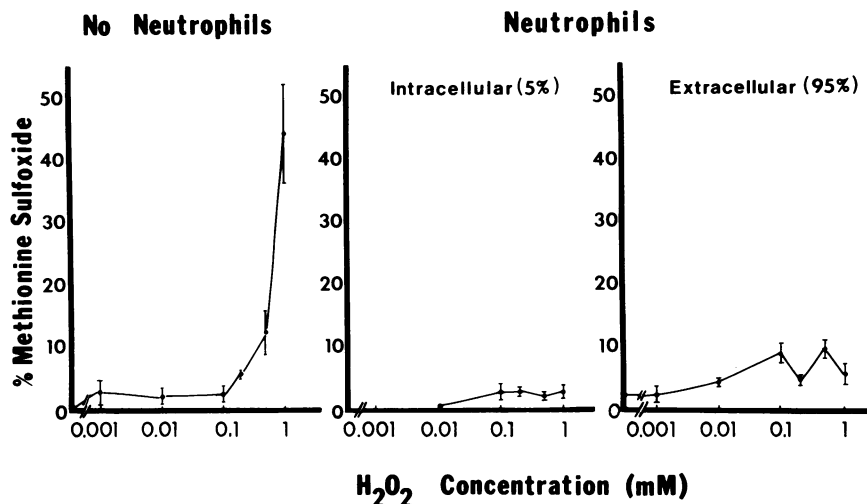
No Neutrophils   ## Neutrophils

FIGURE 5 Effect of H_2O_2 on the oxidation of methionine. A reaction mixture containing 0.1 μCi methionine (22 μM) and H_2O_2 (0.001–1 mM) in the presence or absence of human PMN (1.5×10^7) was incubated at 37°C for 30 minutes. The percentage of methionine oxidized to methionine sulfoxide was then determined. In the presence of PMN, 5% of the methionine was taken up by the cells, and 95% remained extracellularly in the medium (see Fig. 2A). Each point represents the mean and SEM of four to six experiments.

studied. Tryptophan, DABCO, NaN_3, and KCN prevented the oxidation of methionine by the granular fractions (three experiments, data not shown). It should be pointed out that NaN_3 again, was more potent that KCN in inhibiting the oxidation of methionine.

The effect of $^1O_2$ quenchers and MPO inhibitors on the MPO activities of granular fraction. To demonstrate that inhibition of methionine oxidation by $^1O_2$ quenchers was not due to their inhibition of MPO, the MPO activities of the granular fractions were measured. There was no difference in MPO activities, whether it was determined in the modified Hanks'

TABLE IV
Oxidation of Methionine by Granular Fraction of Human PMN*

	Methionine sulfoxide	
H_2O_2	Soluble fraction	Granular fraction
mM	*%*	*%*
0	2.5±2.5 (3)	2.0±1.7 (4)
0.01	0±0 (3)	12.9±4.7 (4)
0.02	3.0±3.0 (3)	15.6±4.9 (4)
0.2	0±0 (3)	75.9±11.3 (4)

* [^{14}C]methionine (0.1 uCi, 22 uM) was incubated with 0.15 mg of cell fractions (granular or soluble) in the presence or absence of H_2O_2. The percentage of methionine oxidized to methionine sulfoxide then was determined. The results are expressed as mean±SEM of the percent methionine oxidized to methionine sulfoxide. The number in parentheses indicates the number of experiments.

solution that contained Cl^- or in the phosphate buffer (0.1 M pH 7.0) that did not contain Cl^-. As shown in Table V, DABCO and tryptophan at a concentration of 1 mM, which markedly inhibited the oxidation of methionine (Table II) did not have any effect on the MPO activities of the granular fractions. NaN_3 (1mM), while markedly inhibiting the oxidation of methionine (90% inhibition), inhibited the MPO activities by only 18%. In contrast, KCN at the similar concentration markedly inhibited the oxidation of methionine and the MPO activities.

The effect of Cl^- on the oxidation of methionine by the granular fraction. Our results suggest that the oxidation of methionine by phagocytosing PMN or the granular fraction is dependent on MPO and H_2O_2. To determine whether the MPO-mediated antimicrobial system (MPO-H_2O_2-Cl^-) (1) is responsible for this oxidation, the effect of Cl^- was then studied. When Cl^- was eliminated from the incubation medium by replacing modified Hanks' solution with phosphate buffer (0.1 M, pH 7.0), oxidation of methionine by the granular fraction was markedly reduced, but not completely abolished (32.3±8.4% vs. control 86.9 ±6.5% $n = 5, P < 0.001$). This might be due to a slight contamination of Cl^- in the granular fractions. This reduced oxidation of methionine by the granular fraction in the absence of Cl^- was not due to the inhibition of MPO, since MPO functioned normally in the absence of Cl^- (see above).

Effect of D_2O on the oxidation of methionine by human PMN and the granular fraction. The lifetime of $^1O_2$ is much longer in D_2O than in H_2O (26) and it has

TABLE V

Effect of Singlet Oxygen Quenchers and Myeloperoxidase Inhibitors on the
Peroxidase Activities of the Granular Fraction of Human PMN*

		Peroxidase activity	P value (vs. control)
		μmol tetraguaiacol/min/mg protein	
Control		2.88±0.60 (3)	
+DABCO	1 mM	2.96±0.46 (3)	>0.8
+Tryptophan	1 mM	2.82±0.12 (3)	>0.8
+NaN₃	1 mM	2.38±0.53 (3)	<0.025
+KCN	1 mM	0.01±0.01 (3)	<0.001

* Guaiacol was used as the hydrogen donor. The results are expressed as the
mean±1 SEM (micromoles tetraguaiacol produced/minute/milligram protein).

been suggested that enhancement of $^1O_2$-mediated reaction in D_2O is one way of demonstrating $^1O_2$ intermediacy (12, 26). The effect of D_2O on the oxidation of methionine by human PMN and the granular fraction was then studied. For this purpose, the concentration of methionine was increased to 102 μM (instead of 22 μM used in the above experiments). As shown in Table VI, D_2O had no effect on the methionine oxidation by resting PMN, while it markedly stimulated the methionine oxidation by granular fractions. Since at higher concentrations, H_2O_2 can oxidize methionine, the effect of D_2O was also studied. D_2O had no effect on the oxidation of methionine by H_2O_2 (34.6±8.2% [H_2O] vs. 34.0±2.3% [60% D_2O], H_2O_2 1 mM, methionine 22 μM, mean±SEM of three experi-

TABLE VI
Effect of D_2O on the Oxidation of Methionine
by Human PMN and Granular Fractions*

		Methionine sulfoxide		Mean stimulation‡
		Experiment 1	Experiment 2	
		%		%
Human PMN				
Resting	H_2O	2.5	0	
	D_2O	1.8	0	—
Phagocytosing	H_2O	22.6	16	
	D_2O	53.1	59.7	192
Granular fraction				
Alone	H_2O	1.4	0	
	D_2O	0	0	—
+H_2O_2 (0.2 mM)	H_2O	18.8	15.9	
	D_2O	46.3	57.9	200

* The experimental conditions were the same as in Table I and Table V except that the concentration of methionine was 102 μM. When D_2O was used, its final concentration was 60%.
‡ Percent stimulation of methionine oxidation in D_2O over H_2O.

ments). In addition, D_2O (60%) had no effect on either the O_2^- production by phagocytosing PMN or the MPO activity of the granular fractions (three experiments, data not shown).

Oxidation of methionine by NaOCl and methylene blue. The formation of hypochlorous acid by MPO, H_2O_2, and Cl^- (13) and the generation of $^1O_2$ by hypochlorite and H_2O_2 (6, 12) have led Rosen and Klebanoff (5) to suggest that this may be the mechanism that $^1O_2$ is produced by PMN during phagocytosis. Since hypochlorite is a potent oxidizing agent, it is possible that the oxidation of methionine by MPO-H_2O_2-Cl^- system is mediated by hypochlorite, rather than $^1O_2$. In fact, it has been shown that the presumed $^1O_2$ reaction described by Rosen and Klebanoff (5) is due to chlorine instead of $^1O_2$ (13). As shown in Fig. 6, hypochlorite oxidized methionine to methionine sulfoxide (hypochlorite at concentrations higher than 0.02 mM oxidized methionine to a new product with a R_f value of 0.82 in the solvent system I). Addition of H_2O_2 did not enhance the oxidation of methionine. Furthermore, D_2O did not have any effect on the oxidation of methionine by hypochlorite either in the presence or absence of H_2O_2. As a control experiment for comparison, photodynamic oxidation of methionine by methylene blue, a known $^1O_2$-mediated reaction (11, 12), was enhanced by D_2O (two experiments, data not shown).

DISCUSSION

In this study, we demonstrated that phagocytosing human PMN, but not resting PMN, oxidized both intracellular and extracellular methionine to methionine sulfoxide. *N*-ethylmaleimide, which inhibits phagocytosis and cellular metabolism, inhibited the oxidation of methionine. Neutrophils from patients with CGD failed to oxidize methionine even in the presence of phagocytosis. Thus, the oxidation of methionine by phagocytosing PMN is dependent upon the oxidative metabolism normally associated with phagocytosis.
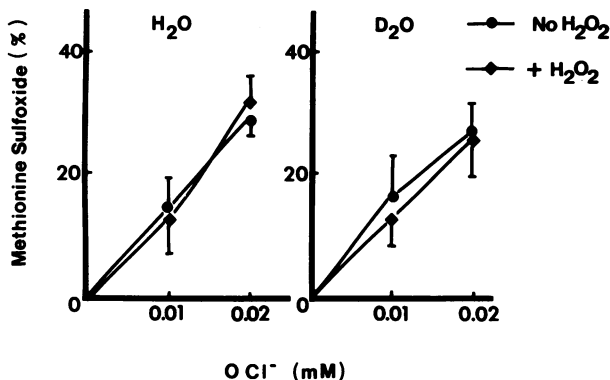
FIGURE 6 Oxidation of methionine by sodium hypochlorite. Methionine (0.1 μCi, 22 μM) and NaOCl were incubated in the presence and absence of H_2O_2 (0.1 mM) in a final vol of 1 ml in phosphate buffer (pH 8.5) for 10 min at 37°C. The percent oxidation of methionine was then determined. When D_2O was used, its final concentration was 60%. Mean±SEM of four experiments. Similar results were obtained when the experiment was performed in modified Hanks' solution (pH 7.4).
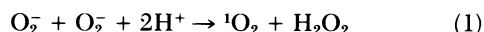
From the data presented, it is clear that the oxidation of methionine by phagocytosing PMN was not due to O_2^- or OH·. On the other hand, the oxidation of methionine by phagocytosing PMN was at least in part dependent on H_2O_2. However, there are several pieces of evidence suggesting that the oxidation of methionine is not directly mediated by H_2O_2. First, KCN, a potent heme enzyme inhibitor, inhibited the oxidation of methionine by phagocytosing PMN. In the presence of KCN, catalase and MPO are inhibited. As a result, more H_2O_2 is released by phagocytosing PMN (27). If the oxidation of methionine by phagocytosing PMN was due to the direct oxidation of methionine by H_2O_2, then KCN-treated PMN would have oxidized more methionine. Second, when H_2O_2, at concentrations sufficiently high to oxidize methionine, was added to PMN it did not significantly oxidize methionine. In contrast, when H_2O_2 (at concentrations too low to oxidize methionine) was added to the granular fractions, it oxidized methionine to methionine sulfoxide. Third, the oxidation of methionine by H_2O_2 was not enhanced by D_2O, whereas the oxidation of methionine by phagocytosing PMN was markedly stimulated by D_2O. Thus, it is more likely that the combination of a KCN-sensitive enzyme (or enzymes) and H_2O_2 is responsible for the oxidation of methionine.

Three pieces of evidence support the possibility that the oxidation of methionine by phagocytosing PMN was due to the production of $^1O_2$. First, $^1O_2$ can oxidize methionine to methionine sulfoxide. The bulk of evidence comes from studies of photosensitized oxidation of methionine (11, 12). Second, the oxidation of methionine by phagocytosing PMN was inhibited by $^1O_2$ quenchers. In this study, we only used water

soluble quenchers, DABCO (22), tryptophan (12), and NaN_3 (23). There are many other $^1O_2$ quenchers such as β-carotene, bilirubin, and dimethylfuran (12). However, these lipid soluble $^1O_2$ quenchers have very limited water solubility. The vehicles used to dissolve sufficient quantities of these quenchers are found to be detrimental to the cells. Third, the oxidation of methionine by phagocytosing PMN was markedly enhanced by D_2O, a known phenomenon of $^1O_2$-mediated reaction (12, 26).

There are several mechanisms that $^1O_2$ may be produced by phagocytosing PMN. These include:

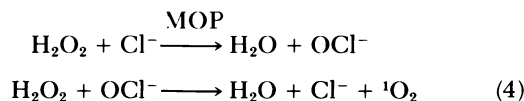The spontaneous dismutation of O_2^- (28)

$$O_2^- + O_2^- + 2H^+ \rightarrow {}^1O_2 + H_2O_2 \qquad (1)$$

The interaction of O_2^- and OH· (29),

$$O_2^- + OH\cdot \rightarrow {}^1O_2 + OH^- \qquad (2)$$
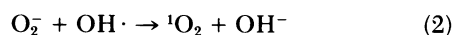
The interaction of O_2^- and H_2O_2 in the presence of a metal ion (Haber-Weiss reaction) (30).

$$O_2^- + H_2O_2 \rightarrow {}^1O_2 + OH^- + OH\cdot \qquad (3)$$

The reaction of H_2O_2 with OCl^- produced by the action of the MPO (5, 6, 13).

$$H_2O_2 + Cl^- \xrightarrow{\text{MOP}} H_2O + OCl^-$$

$$H_2O_2 + OCl^- \longrightarrow H_2O + Cl^- + {}^1O_2 \qquad (4)$$

If the oxidation of methionine by phagocytosing PMN was due to $^1O_2$, then mechanism 1–3 did not produce sufficient $^1O_2$ to oxidize methionine, since SOD did not have any effect on the methionine oxidation. On the other hand, the evidence presented in this paper, suggest that the oxidation of methionine is largely dependent upon the MPO-mediated antimicrobial system ($MPO-H_2O_2-Cl^-$). Although we have not actually used MPO, yet the cellular location (granular fraction) and the response to inhibitor suggest that MPO is most likely the enzyme involved. Thus, one would suggest that mechanism 4 was most likely responsible for the production of $^1O_2$ and the oxidation of methionine. If this were the case, then the combination of OCl^- and H_2O_2 should oxidize methionine to methionine sulfoxide. Unfortunately, OCl^- alone oxidized methionine, the addition of H_2O_2 did not enhance the oxidation of methionine. Furthermore, D_2O did not enhance the oxidation of methionine by either OCl^- or the combination of OCl^- and H_2O_2. Since D_2O did not enhance the oxidation of methionine by OCl^-, it suggests that the oxidation of methionine by phagocytosing PMN or by the combination of granular fraction, H_2O_2 and Cl^- was not due to OCl^-. Failure to show D_2O enhancement of methionine oxidation by OCl^- and H_2O_2 suggests that $^1O_2$ was not produced by OCl^- and H_2O_2 in sufficient quantity to oxidize

methionine. We might not have used the optimal condition for the production of $^1O_2$ by OCl^- and H_2O_2, though we have performed the experiment at pH 8.5, which favors the production of $^1O_2$ from OCl^- and H_2O_2 (26, 31).

Although our results are compatible with the production of $^1O_2$ by phagocytosing PMN, because of the nonspecific nature of the chemical reaction and quenchers used, one really cannot be sure that the oxidation of methionine by phagocytosing PMN or the MPO-mediated antimicrobial system is actually due to $^1O_2$. For example, NaN_3 and tryptophan are shown to be $OH\cdot$ scavengers (32) as well as $^1O_2$ quenchers (11, 12, 23). Recently Held and Hurst (31) have demonstrated that hypochlorous acid preferentially oxidizes DABCO in the presence of H_2O_2 without intermediary formation of $^1O_2$. Furthermore, the effect of D_2O is not specific for $^1O_2$-mediated reaction (33). No matter what is responsible for the oxidation of methionine by phagocytosing PMN, if phagocytosing PMN also oxidizes methionine present at the active site of enzymes, this may be one of the mechanisms by which PMN damage microorganisms. Recently, Matheson et al. (34) have demonstrated that MPO, in the presence of H_2O_2 and halide ion, catalytically inactivates alpha-1-proteinase inhibitor. Amino acid analysis of the inactivated alpha-1-proteinase inhibitor reveals that methionine, which is present at the active site, is oxidized to methionine sulfoxide.

ACKNOWLEDGMENTS

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