Decomposing potassium peroxychromate produces hydroxyl radical (·OH) that can peroxidize the unsaturated fatty acids of phospholipid dispersions

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Abstract The unsaturated fatty acyl residues of egg yolk lecithin are selectively removed when bilayer dispersions of the lipid are exposed to decomposing peroxychromate at pH 7.6 or pH 9.0. Mannitol (50 mM or 100 mM) partially prevents the oxidation of the phospholipid due to decomposing peroxychromate at pH 7.6 and the amount of lipid lost is inversely proportional to the concentration of mannitol. N,N-Dimethyl-p-nitrosoaniline, mixed with the lipid in a molar ratio of 1.3:1, completely prevents the oxidation of lipid due to decomposing peroxychromate at pH 9.0, but some linoleic acid is lost if the incubation is done at pH 7.6. If the concentration of this quench reagent is reduced tenfold, oxidation of linoleic acid by decomposing peroxychromate at pH 9.0 is observed. Hydrogen peroxide is capable of oxidizing the unsaturated fatty acids of lecithin dispersions. Catalase or boiled catalase (2 mg/ml) protects the lipid from oxidation due to decomposing peroxychromate at pH 7.6 to approximately the same extent, but their protective effect is believed to be due to the non-specific removal of ·OH. It is concluded that ·OH is the species responsible for the lipid oxidation caused by decomposing peroxychromate. This is consistent with the observed bleaching of N,N-dimethyl-p-nitrosoaniline and the formation of a characteristic paramagnetic ·OH adduct of the spin trap, 5,5-dimethylpyrroline-1-oxide.—**Edwards, J. C., and P. J. Quinn.** Decomposing potassium peroxychromate produces hydroxyl radical (·OH) that can peroxidize the unsaturated fatty acids of phospholipid dispersions. *J. Lipid Res.* 1982. **23:** 994–1000.

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Active species of oxygen, such as ·OH, $O_2^{\cdot -}$ and singlet oxygen are generated in biological systems by a variety of methods (1). These oxygen species have been implicated in the bactericidal action of granulocytes (2, 3), and in lipid peroxidation (4), and they can damage the functional activity of biological membranes (5, 6). The unsaturated lipids of biological membranes are susceptible to oxidative damage and lipid peroxidation has been implicated in photosensitized damage to chloroplasts (7) and to the photoreceptor cells of the retina (8).

Decomposing peroxychromate, K_3CrO_8, has been used as a source of active oxygen species to examine their effects on biochemical functions (9, 10). We have attempted to model these systems by observing the peroxidation of unsaturated lipids in bilayer dispersion (11) and other lipid structures (12). The advantage of this system is that it can produce active species of oxygen capable of causing lipid peroxidation in high amounts, and the effects of lipid peroxidation on membrane structure can be determined by conventional physical methods.

The peroxychromate anion, CrO_8^{3-}, decomposes readily in aqueous systems to release several species capable of causing lipid peroxidation. These include H_2O_2 and ·OH (9), singlet oxygen (13), and possibly $O_2^{\cdot -}$ (14). Although singlet oxygen has been suggested as one of the primary lipid oxidants produced by decomposition of peroxychromate (10), singlet oxygen quenchers such as sodium azide (15) and singlet oxygen traps such as 2,5-dimethylfuran and 2,5-diphenylfuran (7) did not prevent loss of unsaturated fatty acids from lecithin dispersions (11). We have, therefore, studied this oxidation system using other specific oxidant traps and quenchers to identify the oxygen species involved in peroxidation of unsaturated lecithin bilayers in water.

MATERIALS AND METHODS

Suspensions (1.2 mg/ml; approximately 1.5 mM) of pure egg yolk lecithin were prepared by ultrasonication with an M.S.E. ultradisintegrator for 1 min at room temperature in 0.1 M potassium phosphate buffer (pH 7.6). The suspensions were then centrifuged for 10 min at 1000 *g* before use, to remove titanium dislodged from the probe. No steps were taken to remove traces of iron from the phosphate buffer.

Potassium peroxychromate (62 mg) was added to 10.0 ml of phospholipid suspension. The conditions of peroxychromate decomposition were controlled by a Radiometer TTT-1 pH-stat delivering 0.5 N HCl to maintain the system at pH 7.6. Decomposition at a temperature of 30°C was virtually complete within 1 hr under these conditions. This was verified by monitoring the production of chromate by measuring the extinction at 376 nm of samples diluted in 0.1 M potassium phosphate buffer (pH 10.0). High pH provides a more accurate spectral analysis of chromate (16, 17).

After decomposition was complete, the lipids were extracted by the method of Bligh and Dyer (18), methylated with 14% (w/v) BF_3 in methanol and analyzed by gas–liquid chromatography as described previously (11). The extent of oxidation was measured by observing changes in the ratio of unsaturated fatty acids relative to the endogenous palmitate of the lecithin. Some of the organic solvent extracts were assayed for lipid hydroperoxide content by the method of Buege and Aust (19).

In the electron paramagnetic resonance (EPR) spin trapping experiment, 9.6 mg of peroxychromate was added to 0.4 ml of 0.1 M phosphate buffer (pH 7.6) containing about 50 μl of 5,5-dimethylpyrroline-1-oxide, but no lipid. An EPR spectrum was recorded immediately at a temperature of about 20°C.

Lecithin was dispersed in buffer (pH 7.6) containing mannitol (50 mM or 100 mM) when required. In experiments with N,N-dimethyl-p-nitrosoaniline, 2 or 20 μmol of the quench reagent in chloroform was mixed with egg yolk lecithin before sonication for 1 min in 0.1 M potassium phosphate buffer (pH 7.6 or 9.0). In another experiment, the lecithin was suspended in buffer (pH 9.0) containing 20 μM N,N-dimethyl-p-nitrosoaniline. Spectra of organic solvent extracts after Bligh and Dyer (18) from samples containing N,N-dimethyl-p-nitrosoaniline were recorded over the range of 200–700 nm.

Catalase was added to lecithin suspensions at pH 7.6 when required to give a final enzyme concentration of 0.2 or 2.0 mg/ml. Catalase solutions were inactivated by heating in a boiling water bath for 15 min. Enzyme activity was assayed by a method based on that of Bergmeyer (20).

Egg yolk phosphatidylcholine was obtained from Lipid Products, Redhill, Surrey. N,N-Dimethyl-p-nitrosoaniline and bovine liver catalase (E.C.1.11.1.6) of nominal specific activity 2100 I.U. were obtained from the Sigma Chemical Co. Ltd., London. 5,5-Dimethylpyrroline-1-oxide was obtained from the Aldrich Chemical Co. Ltd., London. Potassium peroxychromate was prepared as described previously (11). All other reagents were of Analar grade.

RESULTS

Exposure of a dispersion of egg yolk lecithin to decomposing potassium peroxychromate results in a loss of unsaturated fatty acyl residues from the phospholipid. This is illustrated in **Fig. 1**, which shows a significant decrease in the ratio of unsaturated to saturated fatty acids following incubation of phospholipid dispersions with decomposing potassium peroxychromate. This loss of unsaturated fatty acid represents approximately a 12% decrease in the number of moles of oleic acid and approximately a 39% decrease in the number of moles of linoleic acid residues of the phospholipid.

The fatty acid composition of the egg yolk lecithin varied from batch to batch and the following fatty acids were observed in the experiments reported in Fig. 1: palmitic acid (29.8 mol %), stearic acid (14.0 mol %), oleic acid (32.5 mol %), and linoleic acid (23.5 mol

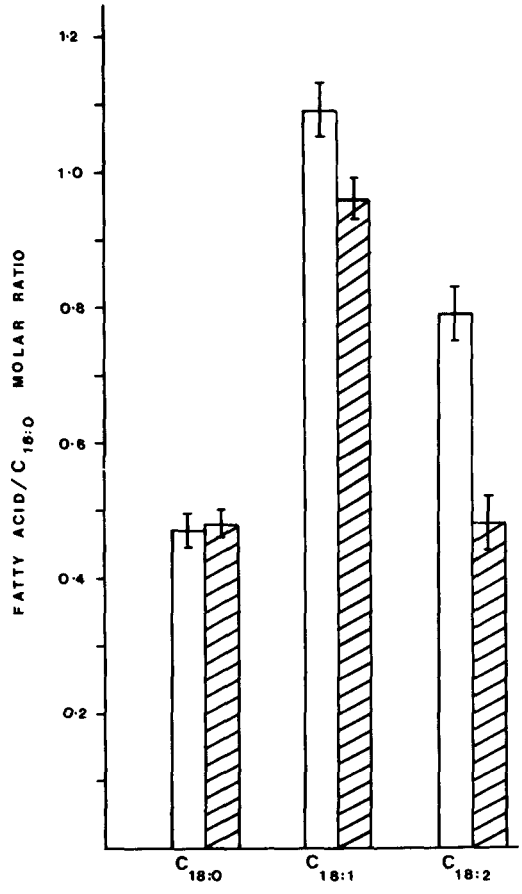

Fig. 1. Histogram of fatty acid molar ratios with respect to palmitate. The means ± SEM of duplicate samples from five experiments are given. Control samples (unshaded areas) consist of egg yolk lecithin dispersions in 0.1 M potassium phosphate buffer, pH 7.6, (1.20 mg/ml) incubated at 30°C for 1 hr. Oxidized samples (hatched areas) were obtained by incubating potassium peroxychromate (62 mg) in 10.0 ml of the phospholipid dispersion under the same conditions as the control, while maintaining the pH at 7.6.

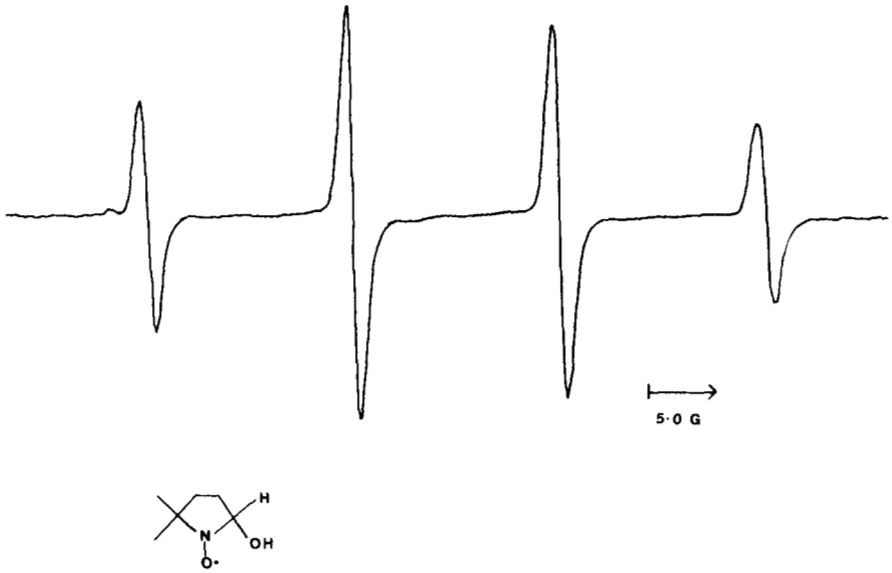

Fig. 2. Electron paramagnetic resonance spectrum of the radical trapped by 5,5-dimethylpyrroline-1-oxide (DMPO) in the presence of decomposing peroxychromate. Peroxychromate was decomposed in 0.1 M phosphate buffer (pH 7.6) containing the spin trap but no lipid. The EPR spectrum observed (a 1:2:2:1 quartet with a splitting constant of 15.22 g) was identical to the hydroxyl adduct of DMPO described by other workers (refs. 29–31, 35) and illustrated in the lower left corner of the figure. No EPR signal was observed in the absence of decomposing peroxychromate. The spectrum was recorded with a modulation amplitude of 0.2 g, a scan rate of 25 g/min, and a time constant of 0.003 S.

%). No other fatty acids were detected in significant amounts although it has recently been reported (21) that this lipid also contains palmitoleic acid (1.4 mol %) and arachidonic acid (1.5 mol %). The extent of oxidation under our standard conditions was different for each batch but the mean oxidation for 39 experiments using six batches of lipid was 18% oleic and 45% linoleic acids oxidized. Solvent extracts (18) from 1:4 dilutions of oxidized lipid dispersions had an absorbance of 0.37 ± 0.01 at 353 nm (4) (corresponding to the production of 0.84 μmol lipid hydroperoxide) when assayed for lipid hydroperoxide. No loss of fatty acid was observed if a lecithin dispersion was mixed with a buffer solution in which potassium preoxychromate had been fully decomposed and then incubated for 1 hr at 30°C, although organic solvent extracts from these dispersions assayed for lipid hydroperoxide had an absorbance of 0.18 ± 0.03 at 353 nm (4) (corresponding to the production of 0.42 μmol lipid hydroperoxide). This suggests that the reactive species that cause losses of unsaturated fatty acids are relatively short-lived compared to those that cause the production of lipid hydroperoxides.

A number of active oxygen species are believed to be generated in this system, so in order to identify the reactive species responsible for loss of unsaturated fatty acids, specific traps were employed.

The ·OH radical is reputed to be amongst the active oxygen species, and its production was verified by decomposing potassium peroxychromate in phosphate buffer not containing phospholipid in the presence of a spin trap, 5,5-dimethylpyrroline-1-oxide, which forms an identifiable adduct with ·OH. The electron paramagnetic resonance spectrum of the products formed in the reaction is presented in **Fig. 2**. This shows a 1:2:2:1 quartet with a splitting constant of 15.22 G typical of 5,5-dimethylpyrroline-1-oxide-trapped hydroxyl radicals. No attempt was made to quantitate the ·OH generated by the system.

To check if ·OH was involved in unsaturated lipid loss, quench reagents with varying degrees of specificity for ·OH were added to phospholipid dispersions exposed to decomposing peroxychromate. The presence

TABLE 1. Effect of N,N-dimethyl-p-nitrosoaniline on oxidation of egg yolk lecithin by exposure to decomposing peroxychromate

Sample		% Loss C 18:1	% Loss C 18:2
Oxidation at pH 7.6	(5)	11.9 ± 0.4	39.2 ± 2.6
Oxidation at pH 7.6 with ·OH quencher	(4)	0	11.9 ± 0.7
Oxidation at pH 9.0	(2)	4	29
Oxidation at pH 9.0 with ·OH quencher	(2)	0	0

Values are mean percentage losses of oleic and linoleic acid relative to palmitate of egg yolk lecithin dispersions exposed to decomposing peroxychromate at constant pH. The number of experiments is shown in parentheses. The oxidation at pH 7.6 values are calculated from Fig. 1 and the means ± SEM of the values are shown.

of mannitol provided partial protection against lipid loss; 38% of the unsaturated fatty acid loss was prevented when phospholipids were dispersed in 50 mM mannitol and 65% of the unsaturated fatty acid loss was prevented in the presence of 0.1 M mannitol. The color of the reaction mixture, however, became green instead of yellow, indicating that mannitol may interfere with the decomposition of the peroxychromate.

The effect of N,N-dimethyl-p-nitrosoaniline added to the phospholipid dispersion in a molar proportion of 1.3:1 quench reagent to lipid, and cosonicated in buffer of pH 9.0 or 7.6 was examined next. The results are presented in **Table 1** and show that N,N-dimethyl-p-nitrosoaniline provides complete protection against lipid loss at pH 9.0 in the presence of decomposing peroxychromate. At pH 7.6, some loss of linoleic acid was observed, but the loss of the monoenoic fatty acid was completely prevented by the quench reagent. Other experiments performed at pH 9.0, using one-tenth the molar proportion of quench reagent, showed similar effects to those shown in Table 1 for the reaction performed at pH 7.6, namely a complete protection against loss of oleic acid, but only partial protection against loss of linoleic acid. If a phospholipid dispersion prepared at pH 9.0 in the presence of N,N-dimethyl-p-nitrosoaniline (20 μM), was exposed to decomposing peroxychromate (at pH 9.0), no prevention of unsaturated fatty acid loss was observed. The reaction of N,N-dimethyl-p-nitrosoaniline with $^{\bullet}OH$ was checked by recording the absorbance of organic solvent extracts of the reaction mixture in the spectral range 200 to 700 nm. **Fig. 3** shows spectra of lipid extracts from control samples and samples oxidized in the presence of decomposing peroxychromate at pH 9.0. The characteristic absorbance maximum of N,N-dimethyl-p-nitrosoaniline at 425 nm observed in the control samples is removed after exposure to peroxychromate and is replaced by a smaller peak centered at 398 nm.

Hydrogen peroxide is another possible oxidant that could be responsible for loss of unsaturated fatty acids. This may be formed directly as a product of peroxychromate decomposition or by the reaction of two $^{\bullet}OH$ radicals. The results presented in **Table 2** show that addition of catalase (0.2 mg/ml or 2.0 mg/ml) or of boiled catalase (2.0 mg/ml) gave partial protection against lipid loss. However, there was no significant difference between the effects of the native and boiled catalase (both 2.0 mg/ml) on lipid loss. Measurement of the native catalase activity after exposure to decomposing peroxychromate showed it to be completely inactivated during peroxychromate decomposition. In other experiments it was found that incubating dispersions of egg yolk phosphatidylcholine with potassium chromate (206 mM) and H_2O_2 (95 mM), in approximate concentrations expected if peroxychromate completely decomposed to give only peroxide and chromate, caused a marked loss of unsaturated fatty acids: 11% of the oleoyl residues and 53% of the linoleoyl residues were removed.

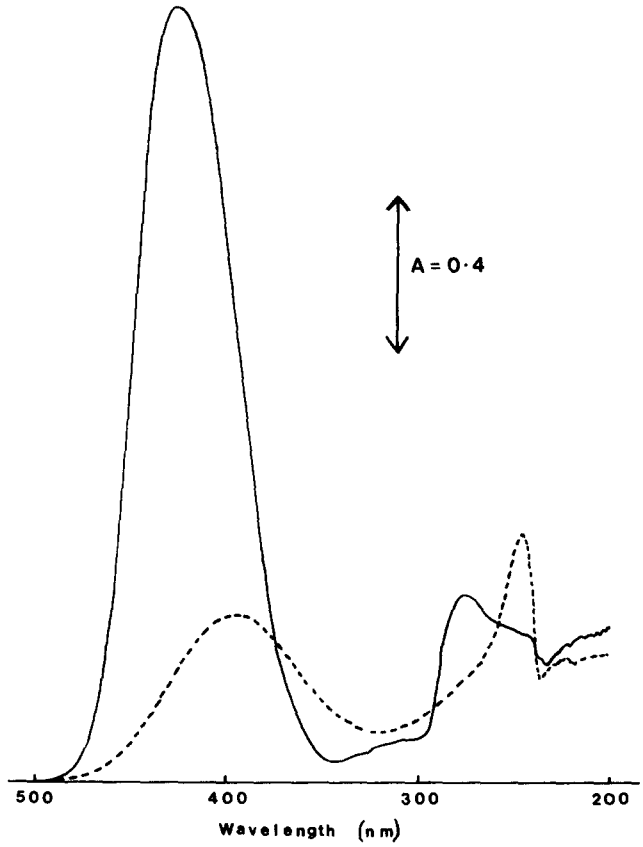

Fig. 3. Typical UV-visible spectra of organic solvent extracts from phospholipid dispersions containing N,N-dimethyl-p-nitrosoaniline at pH 9.0. A spectrum of an extract of a control sample diluted tenfold in chloroform (——): spectrum of an undiluted extract from a sample in which peroxychromate has been decomposed (- - -).

DISCUSSION

The peroxychromate anion, CrO_8^{3-}, consisting of a central chromium atom in the oxidation state +5 surrounded by four peroxy anions (O_2^{2-}) in a dodecahedral arrangement (22), decomposes spontaneously in neutral or alkaline aqueous solutions according to the reaction:

$$4\ CrO_8^{3-} + 2\ H_2O \rightarrow 4\ CrO_4^{2-} + 4\ OH^- + 7\ O_2 \quad 1)$$

Some of the oxygen released in the reaction is known to be in the singlet form (13), and it has been estimated that the yield is 6 ± 2% based on peroxychromate (23). It was assumed in earlier studies that singlet oxygen was

TABLE 2. Effect of catalase and boiled catalase on the oxidation of egg yolk lecithin by exposure to decomposing peroxychromate

Sample		% Loss C 18:1[a]	% Loss C 18:2[a]
K_3CrO_8 − catalase	(4)	17 ± 2	53 ± 3
K_3CrO_8 + catalase (0.2 mg/ml)	(2)	13	38
K_3CrO_8 + catalase (2.0 mg/ml)	(2)	2	31
K_3CrO_8 + boiled catalase (2.0 mg/ml)	(4)	12 ± 3[b]	36 ± 8[c]

[a] Means ± SEM are shown.
[b,c] Significantly different from K_3CrO_8 − catalase: [b], $P < 0.05$; [c], $P < 0.01$.

Values indicate the percentage losses of oleic and linoleic acid relative to palmitate of egg yolk lecithin dispersions exposed to decomposing peroxychromate at constant pH (7.6). The catalase was added after sonication of the lipid. The number of experiments is shown in parentheses.

responsible for the oxidation of unsaturated lipids (10, 12), but the lack of protection by singlet oxygen quenchers (11) suggests that active oxygen species other than singlet oxygen are involved. This observation, however, does not exclude the possibility that singlet oxygen is produced during peroxychromate decomposition.

The present experiments suggest that $^{\bullet}OH$ is responsible for the loss of unsaturated fatty acids from phospholipid dispersions exposed to decomposing peroxychromate. The evidence supporting this contention is first, that the presence of N,N-dimethyl-p-nitrosoaniline provides complete protection at pH 9.0 against selective loss of unsaturated fatty acids. This reagent is not known to react with any other form of oxygen at pH 9.0 other than $^{\bullet}OH$ (24), its use as a specific $^{\bullet}OH$ trap has been validated (25, 26), and its reaction with species generated during peroxychromate decomposition is clearly demonstrated (Fig. 3). It is noteworthy that relatively high concentrations of the trap are required for complete protection against lipid loss and this implies that the number of potential oxidizing species is high in this particular oxidizing system. Some loss of linoleic, but not oleic acid, at lower concentrations of the trap (or higher concentrations at pH 7.6) indicates that $^{\bullet}OH$ may be required to oxidize monoenoic fatty acids, whereas dienoic fatty acids may react with other active oxygen species that are derived initially from $^{\bullet}OH$. One example is the formation of H_2O_2 in the reaction:

$$^{\bullet}OH + {}^{\bullet}OH \rightarrow H_2O_2 \qquad 2)$$

We have attempted to examine this point by observing the effect of catalase in the system, but similar protection is obtained with both the native and denatured enzyme. Since H_2O_2 in the presence of chromate can cause lipid oxidation, the failure of native catalase to provide better protection than the boiled enzyme suggests that removal of H_2O_2 is not the factor involved in preventing oxidation. Inactivation of catalase during peroxychromate decomposition suggests reaction with $^{\bullet}OH$ and is consistent with ability of a variety of organic molecules to quench $^{\bullet}OH$ nonspecifically (27). Nevertheless, the possibility that other active species generated initially from $^{\bullet}OH$ cause lipid loss cannot be excluded. Another indication of the primary role of $^{\bullet}OH$ is that mannitol, an $^{\bullet}OH$ quencher (28), inhibits lipid loss although it could be argued that the sugar interferes with the mechanism of peroxychromate decomposition.

The production of $^{\bullet}OH$ was clearly shown by formation of a radical species trapped by 5,5-dimethylpyrroline-1-oxide, with spectral characteristics identical to those described for 5,5-dimethylpyrroline-1-oxide-trapped hydroxyl radicals (29–31), when the reagent was present in buffer in which peroxychromate was decomposing. No evidence for production of hydroperoxyl radical (protonated superoxide anion radical) was obtained, although it can be detected by this spin trap (32). Although $O_2^{\bullet -}$ has been reported as a product of peroxychromate decomposition (14) this has been challenged (9).

The possibility that contaminating iron salts were responsible for $^{\bullet}OH$ production (33) was excluded by failure to detect spin-trapped radicals when 5,5-dimethylpyrroline-1-oxide was incubated with buffer; this may be because of rapid autoxidation of the Fe^{2+}, which is an efficient generator of $^{\bullet}OH$ (34) into the less efficient Fe^{3+} by phosphate buffer (35).

We conclude that $^{\bullet}OH$ radical is produced during peroxychromate decomposition and is probably the primary species responsible for loss of unsaturated fatty acyl residues of phospholipids in aqueous dispersions. The $^{\bullet}OH$ radical has previously been shown to react with aromatic compounds incorporated into bilayers of saturated lecithins (36), to initiate oxidative damage to model membrane systems (37), to be the cause of alloxan-induced diabetes (38), and to be involved in radiation-induced damage to alveolar macrophages (39). The bactericidal action of xanthine oxidase is also believed to be due to the formation of $^{\bullet}OH$ from $O_2^{\bullet -}$ and H_2O_2 (40) but the involvement of $^{\bullet}OH$ in this and the liver microsomal NADPH-oxidase systems in lipid peroxidation has been disputed (41, 42). Peroxidation of membrane lipids has been shown to result in increased membrane rigidity (43) and increased bilayer permeability to solutes (44, 45). The extent to which cellular function is perturbed by such changes remains to be assessed.

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