Superoxide, Hydrogen Peroxide, and Singlet Oxygen in Lipid Peroxidation by a Xanthine Oxidase System*

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There recently has been a surge of interest in the peroxidation of lipids. This interest follows upon the early observations by Hochstein et al. (1–2a) of an enzymatic, NADPH-dependent oxidation of microsomal lipids, and it derives from the obvious importance of unsaturated lipids for the structural and functional integrity of biological membranes. This literature is ample but its impact is unclear. Iron salts and chelating agents, such as ADP or EDTA, have been used routinely by most workers because they have appeared to augment the rate of lipid peroxidation. Pederson and Aust (3, 4) noted that xanthine oxidase, while acting aerobically upon xanthine, could cause lipid peroxidation. Since scavengers of O₂⁻ and of singlet oxygen inhibited, they concluded that O₂⁻, generated by xanthine oxidase, gave rise in turn to singlet oxygen which was then the immediate cause of the lipid peroxidation. The reactivity of singlet oxygen with unsaturated lipids (5–8) makes this a very reasonable supposition. Superoxide dismutase has been used repeatedly to expose the involvement of O₂⁻ in the chain of events leading to lipid peroxidation by the xanthine oxidase system (3, 4, 9, 10), but the presence of chelated iron salts adds a complicating factor which hinders interpretation of these data. In one study (11), the xanthine oxidase system was seen to mount an oxidative attack upon the lipids of lysosomal membranes. In this case, superoxide dismutase augmented lysis rather than inhibiting it, and OH⁻ was implicated as the causative agent. Here too, iron salts were present to cloud the issues.

It seemed necessary to explore the co-oxidation of unsaturated lipids by the xanthine oxidase system, under simple and well defined conditions, so that mechanisms might be discerned more readily. The peroxidation of linolenate has been studied in this way. The results of these studies and the mechanism they suggest form the basis of this report.

MATERIALS AND METHODS

Thiobarbituric acid, mannitol, β-carotene, α-tocopherol, catalase, bovine serum albumin, and hemoglobin were obtained from the Sigma Chemical Co. The catalase was occasionally dialyzed, to free it of a thymol preservative, and it was also freed of a minor contamination with superoxide dismutase by repeated washing over an XM-100 A Diaflo ultrafiltration membrane from the Amicon Corp., without any effect on the results. Starch (Soluble, Lintner) and t-butyl alcohol were purchased from the Fisher Scientific Co. EDTA and trichloroacetic acid were from Matheson, Coleman and Bell, while potassium iodide and ferric chloride were from Baker and Adamson. Linolenic acid was obtained from Mann, and was stored in UCKUO at -20°. Potassium phosphates were from J. T. Baker and, unless otherwise specified, buffers made from these salts were passed through a Chelex 100 column, to remove trace metals. The Chelex 100 was from Bio-Rad Laboratories, Inc. Dimethoxyethane, from Eastman Chemical Co., was stored over metallic sodium and was passed through a column of alumina, to remove peroxides, on the day of use. 2,5-Dimethylfuran, 2,5-diphenylfuran, and 1,4-diazabicyclo[2,2,2]octane were purchased from the Aldrich Chemical Co. Dimethoxyethane was distilled, and the fraction collected between 83–85° was used where indicated. In other cases, it was used as supplied. Diphenylfuran was recrystallized from 95% ethanol, while diazabicyclooctane was recrystallized from acetone. Superoxide dismutase from bovine erythrocytes was purified by Truett Laboratories as previously described (12). It was freed of

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carboxylic anhydrase by affinity chromatography (13). Xanthine oxidase was prepared from cream (14), and was stored frozen under ammonium sulfate.

All glassware was washed twice and was then thrice rinsed in deionized water. In setting up reaction mixtures, dimethoxynaphthyl-soluble components, such as linolenic acid and the singlet oxygen scavengers, were added and mixed before the buffer and the watersoluble components were added. When diene conjugation was to be followed, at 233 nm, all components were mixed and incubated for 1 hour before acetaldehyde was added to start the reaction. This was necessary to avoid artifacts due to light scattering. Increases in absorbance were then followed for 24 min and the linear rate of increase which was achieved after 6 min was recorded. Unless otherwise specified, all incubations and reactions were at 37°C. When products were to be analyzed, by thin layer chromatography, 3.0 ml samples were chilled in an ice-salt bath, were acidified with 0.3 ml of 1.0 M phosphoric acid, and were extracted with 0.3 ml of CHCl₃. After centrifugation, 20 µl of the organic phase were taken for analysis on Silica Gel 6B thin layer sheets from J. T. Baker. The running solvent was petroleum ether/benzene/acetonitrile, 1/1/1. The method of Stahl (15) for organic peroxides was modified for visualization of linolenate products. Thus, 1.0 ml of 4% KI was mixed with 4.0 ml of acetic acid, and this mixture was immediately sprayed onto the thin layer sheet, followed by a spray of a fresh 1% solution of starch. Areas of the sheet bearing peroxides were stained purple. In experiments with dimethylur, the samples were acidified and extracted with CHCl₃ as described above, and the CHCl₃ extracts were applied, as spots, to thin layer sheets. After thoroughly drying these spots under a stream of N₂, the chromatograms were run in benzene/acetone, 10/1, and the spots were visualized by spraying with ethanol/sulfuric acid, 1/1.

The method of Damm (16) was also used. After carefully washing the sheets in ice-salt, areas of the thin layer sheets, which were identified by spraying with an iodine/potassium iodide solution, were cut out and eluted with 1.0 ml of ethyl acetate. In a second experiment, thin layer sheets were prepared from cream (14), and were stored frozen under ammonium sulfate.

RESULTS

Lipid Peroxidation Measured in Terms of Diene Conjugation—The aerobic action of xanthine oxidase on xanthine produces both O₂⁻ and H₂O₂ (16). Xanthine and its oxidation product, urate, absorb strongly in the ultraviolet. It was, therefore, necessary in studying the effects of O₂⁻ on linolenate to replace xanthine by a substrate whose oxidation does not cause large changes in ultraviolet absorbance. Acetaldehyde served this purpose. When acetaldehyde was added to buffer solutions of xanthine oxidase plus linolenate, there was a linear increase in absorbance with a maximum close to 233 nm. This increase was dependent upon the combined presence of acetaldehyde, xanthine oxidase, and linolenate, and was presumed to reflect the production of conjugated diene hydroperoxides (17). This reaction was studied both in the absence and in the presence of EDTA-Fe³⁺ and the results are summarized in Table I. It is clear that superoxide dismutase at 10 µg/ml was a powerful inhibitor, as was catalase at 8.28 µg/ml. Iron-EDTA was not at all required, but it did somewhat modify the mechanism, since it diminished the inhibition by superoxide dismutase but had no effect on the inhibition by catalase. It follows that in the absence of iron EDTA, the reaction was virtually entirely dependent upon both O₂⁻ and H₂O₂ whereas in the presence of iron-EDTA, the dependence upon O₂⁻ was lessened but not eliminated. A control protein, such as bovine serum albumin at 10 µg/ml, had no effect. Scavengers of hydroxy radical such as mannitol or t-butyl alcohol were also without effect, whereas a scavenger of singlet oxygen such as β-carotene, at 1 x 10⁻⁴ M, inhibited in the absence of iron-EDTA, but not in its presence. It might have been anticipated that mannitol and t-butyl alcohol would be ineffective in this system, since the reaction mixtures contained 10% dimethoxynaphthyl which reacts with OH- with a rate constant of 1.56 x 10⁶ M⁻¹ s⁻¹ (18).

The modest inhibition by 0.01 mM β-carotene, which was seen in the absence of Fe³⁺-EDTA, was probably limited by partial inactivation of this compound during the 60-min incubation which preceded the addition of acetaldehyde. In reaction mixtures containing Fe³⁺-EDTA, this inactivation may well have gone to completion before the period of observation. We cannot, therefore, conclude that singlet oxygen was not involved in the linolenate peroxidation seen in the presence of EDTA-Fe³⁺. It is interesting to note that diene conjugations were not dependent upon the presence of EDTA. In the absence of EDTA, the reaction rate was decreased about 50%, but this is probably due to inactivation of xanthine oxidase by trace metals (19). Superoxide dismutase prevented the absorbance increase at 233 nm due to diene conjugation whether EDTA was present or not.

Superoxide dismutase is an extremely efficient enzyme and acts upon O₂⁻ with a rate constant of approximately 2 x 10⁹ M⁻¹ s⁻¹ (20, 21). It should, therefore, be effective in scavenging O₂⁻ when present at low concentrations. Moreover, there is a danger of artifact when the copper-zinc superoxide dismutase is used at high concentration, since it can catalyze the peroxidation of numerous substances, including unsaturated lipids (22). The effect of varying the concentration of superoxide dismutase was accordingly explored. Fig. 1, which presents these results, demonstrates that superoxide dismutase inhibited perceptibly at 0.01 µg/ml, caused 46% inhibition at 0.1 µg/ml, and 81% inhibition at 1.0 µg/ml. Inhibition was maximal at 10 µg/ml, and appeared to decrease at higher concentrations. This apparent decrease probably relates to the peroxidative activity of this superoxide dismutase (22), and may explain the report by Fong et al. (11) that superoxide dismutase, at 110 µg/ml, augmented peroxidation of the lipids of lysosomal membranes. It is clear that low levels of superoxide dismutase did prevent the action of the xanthine oxidase system upon linolenate, thus implicating O₂⁻ as an essential intermediate.

Iron and the Thiobarbituric Acid Test—The convenience

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Control rate at 253 nm in presence of 0.1 mM EDTA</th>
<th>% Control rate at 253 nm in presence of 0.1 mM EDTA-Fe³⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100.0 ± 10.0</td>
<td>100.0 ± 3.0</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>5.2 ± 0.9</td>
<td>10.3 ± 2.6</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>10.0 ± 1.6</td>
<td>10.0 ± 3.9</td>
</tr>
<tr>
<td>+10 µg/ml of superoxide dismutase</td>
<td>17.6 ± 3.0</td>
<td>53.0 ± 13.8</td>
</tr>
<tr>
<td>+8.25 µg/ml of catalase</td>
<td>17.4 ± 1.2</td>
<td>17.7 ± 0.6</td>
</tr>
<tr>
<td>+10 µg/ml of bovine serum albumin</td>
<td>107.0 ± 0.2</td>
<td>98.5 ± 1.2</td>
</tr>
<tr>
<td>0.1 M Mannitol</td>
<td>92.0 ± 2.9</td>
<td>86.0 ± 4.0</td>
</tr>
<tr>
<td>0.1 M + t-Butanol</td>
<td>100.0 ± 3.3</td>
<td>107.0 ± 3.0</td>
</tr>
<tr>
<td>10⁻⁴ M β Carotene</td>
<td>98.8 ± 2.6</td>
<td>96.5 ± 4.8</td>
</tr>
<tr>
<td>10⁻³ M β Carotene-linolenic acid</td>
<td>15.1 ± 4.1</td>
<td>—</td>
</tr>
</tbody>
</table>

* Control rate Δ233 min⁻¹ was 3.09 (3.40 - 2.82) x 10⁻³ for the EDTA system; 3.09 (3.01 - 3.16) x 10⁻³ for the EDTA-Fe³⁺ system. Assays were run four times for control and control + superoxide dismutase samples, and in duplicate for all others. The complete reaction system contains 23.4 µg/ml of xanthine oxidase, 1 mM linolenic acid, 48 mM acetaldehyde, and 10% dimethoxynaphtho in 50 mM KPO₄ (pH 8.1). Samples were incubated for 1 hour at 233 nm without acetaldehyde, and after addition of substrate, were monitored at 233 nm for 24 min.
the color yield. Tappel (26) has shown, by several independent studies, that the hydroxyl radical scavengers, butanol or mannitol, had no effect, whereas the singlet scavenger diazabicyclooctane (27) inhibited completely when present at 0.10 M. This effect probably depends upon the Fe3+-catalyzed decomposition of the lipid peroxide to the color-producing malondialdehyde. In view of the widespread use of iron salts in current studies of lipid peroxidation (2-4, 9-11), we re-examined this effect of iron salts. Table II demonstrates that the thiobarbituric acid color, obtained from a fixed amount of peroxidized linolenate, was doubled by 2 x 10^-5 M Fe3+, and was increased nearly 5-fold by 2 x 10^-4 M Fe3+. Iron in the form of hematin was much more effective, and at 8 x 10^-5 M, more than doubled the color yield. Tappel (26) has shown, by several independent techniques, that hematin can catalyze the breakdown of lipid peroxides. It is probable that during the thiobarbituric acid test, the presence of hematin results in a greater yield of thiobarbituric acid-reactive breakdown products. The often repeated observation that iron salts augment the peroxidation of lipids must be re-examined in view of the possibility that the effect was at least partially upon the color yield of the thiobarbituric acid test, rather than upon the actual peroxidation process. Certainly our hands, iron salts did not augment the peroxidation of linolenate induced by the aerobic xanthine oxidase reaction.

Visualization of Linolenate Peroxides on Thin Layer Plates
The results obtained by observing the increases in absorbance at 233 nm, presumably due to the accumulation of conjugated diene hydroperoxides, were verified by thin layer chromatography, which allows visualization of these polar products of lipid oxidation. Fig. 2 demonstrates that lipid peroxides were produced in the xanthine oxidase-acetaldehyde system, that acetaldehyde was essential for their formation, and that superoxide dismutase or catalase, but not bovine serum albumin, inhibited their formation. Fig. 3 demonstrates that the hydroxyl radical scavengers, butanol or mannitol, had no effect, whereas the singlet scavenger diazabicyclooctane (27) inhibited completely when present at 0.10 M. This effect of diazabicyclooctane proved due to its ability to react with and, thus, to eliminate preformed lipid hydroperoxides rather than to its prevention of their formation by scavenging singlet oxygen. Thus, when lipid peroxides were prepared by heating and aerating a solution of linolenate, subsequent incubation with 0.1 M diazabicyclooctane for 60 min at 37°C completely eliminated the preformed peroxide component, as judged by thin layer chromatography.
FIG. 3. Effects of scavengers of OH- and of singlet oxygen. The basic reaction conditions were as described in the legend of Fig. 2. The complete reaction mixture was modified by the following deletions or additions: 1 = no linolenic acid; 2 = complete reaction mixture; 3 = plus 0.10 M t-butyl alcohol; 4 = plus 0.10 M mannitol; 5 = plus 0.10 M diazabicyclooctane.

It was, nevertheless, possible to demonstrate that singlet oxygen was essential for the formation of lipid peroxides. Thus, Fig. 4 shows that 2,5-dimethylfuran at 1.0 mM markedly inhibited the formation of lipid peroxide, whereas diazabicyclooctane at the comparable level of 1 mM did not inhibit perceptibly. 2,5-Diphenylfuran was also tested at 0.1 mM, which represents a limit set by solubility. It was not effective at this concentration. It is clear that thin layer chromatography confirms the results obtained by spectrophotometric monitoring at 233 nm, in that O2-, H2O2, and singlet oxygen appear to be essential intermediates for the lipid peroxidation induced by the aerobic action of xanthine oxidase on acetaldehyde.

Peroxidation of Linolenate by a Photochemical Source of Singlet Oxygen—The results in hand suggested that O2- and H2O2 were conspiring to generate singlet oxygen, which was then the immediate cause of linolenate peroxidation. In that case, a photochemical source of singlet oxygen should cause a peroxidation of linolenate which is unaffected by superoxide dismutase or catalase. Fig. 5 presents the affirmation of these consequences. Thus, illumination of linolenate, in the presence of 0.01 mM rose bengal, yielded lipid peroxides whose formation was not prevented by superoxide dismutase or catalase, but was inhibited by the singlet oxygen scavenger 2,5-dimethylfuran.

Production of Singlet Oxygen by the Xanthine Oxidase Reaction—If the aerobic action of xanthine oxidase on acetaldehyde does really generate singlet oxygen as a secondary consequence of the interaction of O2- and H2O2, then this reaction mixture should convert dimethylfuran to the same product as is given by its exposure to a photochemical source of singlet oxygen. Fig. 6 represents a test of this proposal. It is clear that the aerobic action of xanthine oxidase on acetaldehyde did convert dimethylfuran to the same product as did illuminated rose bengal. Furthermore, superoxide dismutase or catalase, but not mannitol, inhibited the formation of this product in the xanthine oxidase system. The production of singlet oxygen, in this system, is thus dependent upon O2- and H2O2, but not upon OH-.

Dimethoxyethane was routinely present as an aid to the solubilization of lipoidal components. In some experiments, it was eliminated, and the linolenate was dispersed into the buffer by sonication under nitrogen. This modification did not introduce any qualitative changes in the behavior of the system. Thus, the xanthine oxidase reaction continued to cause a peroxidation of linolenate, which was prevented by superoxide dismutase or catalase. In this case, however, both 1 mM 2,5-dimethylfuran and 0.1 M mannitol inhibited peroxide formation by about 50%, thus suggesting that both OH- and O2- are involved in the dimethoxyethane-free system. These results are shown in Fig. 7.

DISCUSSION

The peroxidation of linolenate, as induced by the xanthine oxidase reaction, was inhibited by superoxide dismutase or by catalase. It was, therefore, dependent upon both O2- and
peroxidation of linolenate was also inhibited by scavengers of singlet oxygen but not by scavengers of OH. It follows that singlet oxygen is a more discriminating reagent and, therefore, seems unlikely that OH.- would escape indiscriminate scavenging and thus survive to attack critical macromolecules. Singlet oxygen is not the light emitter in this case.

Reaction 1 has profound implications for all respiring cells. Thus, both O$_2^-$ and H$_2$O$_2$ appear to be normal, although perhaps minor, products of the biological reduction of oxygen. It now appears that their interaction can generate both hydroxyl radical and singlet oxygen. Defense, at the primary level, is provided by superoxide dismutases which catalytically scavenge O$_2^-$ and by catalases and peroxidases which similarly scavenge H$_2$O$_2$ (32, 33). Defense, at the secondary level, would necessitate scavengers of OH.- and of singlet oxygen. Since OH.- is a powerful and indiscriminate oxidant (18), it has been proposed (32, 33) as the ultimate agent of oxygen toxicity. However, in view of its nearly universal reactivity and the abundance of potential reactants in living systems, it now seems unlikely that OH.- would escape indiscriminate scavenging and thus survive to attack critical macromolecules. Singlet oxygen is a more discriminating reagent and, therefore, seems likely to be more effective in causing molecular damage at critical sites. If Reaction 1 is of real importance in vivo, the adequacy of the defenses against it may have far reaching implications in the etiology of specific diseases and the senescence of aerobic living systems.

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