Singlet Oxygen Production Associated with Enzyme-catalyzed Lipid Peroxidation in Liver Microsomes*

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Evidence for the formation of singlet oxygen during the oxidation of NADPH by liver microsomes is presented. The evidence is based primarily on the enzyme-dependent formation of dibenzoylethylene from diphenylfuran, a reaction which is specific for singlet oxygen. The apparent formation of singlet oxygen is coupled to the occurrence of peroxidation of microsomal lipid, a phenomenon known to be associated with NADPH oxidation by the particles. Both the peroxidation of lipid and the apparent formation of singlet oxygen are related to the amount of Fe3+ present in the system and the results are consistent with the possibility that the singlet oxygen formed by this system is derived from the breakdown of lipid peroxides. If 'O2 is formed from breakdown of lipid peroxides, it would be dependent on O2 formation because superoxide anion has been shown to undergo reactions in this system which generate extremely reactive free radicals (probably hydroxyl) that initiate lipid peroxidation. These peroxides are quite unstable and their degradation may be the source of 'O2. We have consistently observed that O2 itself is not a reactive radical with respect to lipids or radical scavengers. Hence, O2 cannot be the radical which initiates lipid peroxidation on which 'O2 generation appears to depend. The results may offer at least part of the explanation for the dietary requirement for a-tocopherol which not only scavenges free radicals but quenches singlet oxygen as well.

This report also includes description of studies indicating that another enzyme, xanthine oxidase, which forms superoxide anion during its activity under aerobic conditions, does not form singlet oxygen during its function. This finding is in contrast to reports of others which indicate that xanthine oxidase activity does produce 'O2.

In a previous report we presented evidence that purified microsomal NADPH-cytochrome P-450 reductase activity initiated lipid peroxidation in biological membranes because the enzyme generates superoxide anion radicals (O2) when catalyzing NADPH oxidation. This occurred under conditions which may exist in vivo (1). Our investigations, however, indicated that O2 per se was not the radical species causing lipid peroxidation in microsomes during NADPH oxidation by these particles. Rather, the oxidative attack on membrane lipids was most likely due to hydroxyl free radicals produced by subsequent reactions of O2. Howes and Steele have reported that when liver microsomes oxidize NADPH in the presence of O2, chemiluminescence was observed which they attributed to singlet oxygen (2). Because Kearns has cautioned that the chemiluminescence in such complex systems may not necessarily reflect singlet oxygen formation (3), we examined the problem using compounds which react with singlet oxygen ('O2) to yield specific products. These studies indicated that singlet oxygen does appear to be formed during the enzymic oxidation of NADPH by these particles, but that its formation is Fe3+-dependent. In addition, we have included in this report a description of studies which indicated that xanthine oxidase activity apparently does not produce 'O2, contrasting with the results of other investigators (4) and with the suggestion that the chemiluminescence associated with the activity of this enzyme may involve 'O2 (5). The findings reported in this communication indicate that the activity of the rat liver microsomal NADPH oxidase system not only appears to produce singlet oxygen in addition to the previously reported hydroxyl free radical, but also that the singlet oxygen formed may be a product of the lipid peroxidation process which then contributes to further peroxidative attack on the membrane lipids during microsomal NADPH oxidase activity.

EXPERIMENTAL PROCEDURES

Animals

Adult male albino rats (180 to 250 g) of the Holtzman Sprague-Dawley strain, bred and maintained at this institution, were used in this study. The animals were fed a commercial laboratory ration obtained from Rockland Laboratories, Teckland, Inc., Monmouth, Ill.
Materials

The substances used in these studies were obtained from the following sources: 2,5-diphenylfuran, W. H. Curtin & Co.; trans-1,2-dibenzoylethylene, 1,3-diphenylisobenzofuran, and O-dibenzyldien-
benzoic, Aldrich Chemical Co.; rose bengal, Koch Light Laboratories; sodium ethylenediaminetetraacetate and 2-thiobarbituric acid, Eastman Organic Chemicals; uric acid and xanthine, Calbiochem; p-
chloromercuribenzoic acid, p-glucose-6-phosphate dehydrogenase (from Torula yeast crystalline suspension in 2.6 M (NH₄)₂SO₄ solution) and deuterium oxide (99.8%), Sigma Chemical Co.; Tris-HCl, chloroform, methanol, other organic solvents and inorganic salts, Fisher Scientific; Chelex-100 resin, Bio-Rad; "chromatoquality" heptane and 1,4-dioxane, Matheson, Coleman and Bell. All chemicals and solvents were used as obtained except where otherwise specified.

Enzyme Sources

Microsomes were prepared from rat liver as described earlier (6). When ready to be used, the frozen microsomal pellets were resuspended with gentle homogenization in 0.05 M Tris-HCl buffer, pH 7.5. The buffer was passed through a column of Chelex-100 resin before use. The volume of buffer was calculated to provide a suspension which contained the microsomes derived from 1.0 g of liver/ml of buffer. Protein content of these preparations was determined by the method of Lowry et al. (7).

Xanthine oxidase (Grade I from buttermilk) was purchased from Sigma Company. Superoxide dismutase was a gift from Dr. Bernard Keefe, University of Alabama and was prepared from bovine erythrocytes by the procedure of McCord and Fridovich (8).

Incubation Systems

Microsomal NADPH Oxidase System—The control system consisted of, per ml of incubation system: 0.1 ml of microsomal suspension (the equivalent of about 1.0 mg of protein); 0.012 mM FeCl₃, which formed a complex with 4 mM ADP and 0.05 mM Chelex-100-treated Tris-HCl buffer, pH 7.5; and 0.3 mM NADPH. The complexing of Fe³⁺ with ADP appears to serve two purposes in this system: (a) maintain-
ing the iron in solution and (b) providing the proper ligand possibilities for the interaction of the iron with microsomes. Other nucleotides are not as effective (9). Control systems either without ADP-Fe³⁺ or without NADPH were always included. Incubations were carried out in 15-ml glass test tubes at 37° in a Dubnoff shaker in air. In some experiments the incubation systems were scaled up to a final volume of 10 ml in order to allow sufficient quantities for certain types of analyses. The larger systems were incubated in 25-ml Erlenmeyer flasks to provide a comparable surface area for gas exchange as in the 1-ml systems. This is important because the rate of oxygen utilization in the reaction is rapid. In most experiments, 0.1 ml of an NADPH-generating system was added per ml of reaction system instead of NADPH. The NADPH-generating system contained, per ml of incubation system: glucose 6-phosphate, 5 mM; NADP, 0.5 mM; and 0.5 Kornberg unit of p-glucose-6-phosphate dehydrogenase activity. Various specific inhibitors, singlet oxygen quenchers and free radical scavengers were added to the basic enzyme system as indicated in the legends accompanying each table or figure. All additions excepting microsomal particles and purified enzymes are expressed as final concentration values in the incubation system.

Xanthine Oxidase System—Xanthine oxidase activity was assayed by measuring the absorption of the uric acid formed at 290 nm. The system contained 0.05 M potassium phosphate buffer, pH 7.8, containing 1.0 x 10⁻¹⁴ M EDTA, 0.1 mM xanthine, and xanthine oxidase (0.015 mg of protein/ml of reaction system). Superoxide formation by this system was assayed by the cytochrome c reduction procedure of McCord and Fridovich (8). Addition of superoxide dismutase (0.008 mg of protein/ml of reaction system) to this reaction system completely inhibited cytochrome c reduction by the enzyme.

Analytical Procedures

Colorimetric Determination of Malondialdehyde—Malondialde-
hyde formed in the reaction systems as a result of lipid peroxidation was determined by the method of Ottolenghi (10). The analyses were carried out on 1.0-ml aliquots of the reaction systems.

Synthesis of cis-Dibenzoylethylene—cis-Dibenzoylethylene was prepared for use as a standard by the method of Lutz and Wilder (11). Purity of the compound was determined by thin layer chromatography and infrared spectra.

Chelex Treatment of Buffer—The 0.05 M Tris-HCl buffer solution was passed through a Chelex-100 resin column (4.5 x 45 cm) prior to use and then adjusted to pH 7.5 before use. This reduced traces of iron to 10⁻⁴ M or less. In addition to being the buffer in the NADPH oxidase system, all reagents used in that system were prepared in the same buffer. This procedure was followed so that the amount of iron in the system could be more closely controlled.

Thin Layer Chromatographic Analysis of Diphenylfuran and Pro-
ducts of Its Reaction with O₂—The enzymic systems were extracted with 1 to 2 ml of chloroform/ml of reaction system at the end of each incubation period. The two phase system was vigorously mixed for approximately 15 s, then centrifuged for 10 min at 2000 x g and the chloroform layer was transferred as completely as possible to clean tubes. The chloroform then was removed by a flow of N₂ gas. The chloroform-soluble residue was transferred totally to thin layer plates (Silica Gel G) with small volumes of chloroform. The plates were developed in the dark for 1 hour in a solvent system consisting of 1-heptane/dioxane (3/1), air dried, and then sprayed with 0.5% dimethylphenylhydrazine in 2 N HCl to visualize the diphenylfuran and dibenzoylethylene spots. In some cases the spot corresponding to dibenzoylethylene was eluted from the plate with chloroform/methanol (2/1). The eluate was concentrated and taken up in 1.5 ml of chloroform. The infrared spectrum of the material indicated that it was dibenzoylethylene.

Hydroxylation Analyses—After incubation of aniline in the Fenton system, assays for p-aminophenol formation were performed by the method of Guarino et al. (12), modified for smaller volumes. Acetanilide hydroxylation by the Fenton system was assayed by the method of Krisch and Staudinger (13).

RESULTS

Effect of Singlet Oxygen-quenching Compounds on NADPH Oxidase-dependent Lipid Peroxidation—If singlet oxygen plays a significant role in the process causing lipid peroxidation during enzymic NADPH oxidation by liver microsomes, singlet oxygen quenchers would be expected to inhibit the process. Fig. 1 shows that several compounds known to react with singlet oxygen cause significant inhibition of the peroxidative attack on lipids during incubation with microsomes and NADPH. Lipid peroxidation was followed by determining malondialdehyde formation. We have previously shown that malondialdehyde is produced by the microsomal system in proportion to the extent of polysaturated fatty acid loss (6). All of the substances tested in the experiments described in Fig. 1 have been reported to trap or quench singlet oxygen, and in our studies they caused some degree of inhibition of enzyme-catalyzed lipid peroxidation when added to the reaction system. Diazoxybicyclooctane (14), diphenylisobenzofuran (15), and diphenylfuran (16), are considered to be specific reagents for singlet oxygen. Of the various singlet oxygen reagents tested, diphenylfuran was selected as the most suitable for further study with regard to solubility, stability, and ease of characterization of its singlet oxygen reaction product, cis-dibenzoylethylene. Furthermore, neither diphenylfuran nor dibenzoylethylene act as photosensitizers, nor does diphenylfuran exhibit significant free radical-scavenging activity, as will be demonstrated below.

Since the various singlet oxygen quenchers which were tested (including diphenylfuran) were dissolved in acetone before addition to the reaction system, it was determined that concentrations of acetone of up to 20% had no effect on NADPH-dependent lipid peroxidation in liver microsomes, nor did acetone addition affect the conversion of diphenylfuran to cis-dibenzoylethylene catalyzed by photo-oxidation in the presence of rose bengal.

Studies on Conversion of Diphenylfuran to Dibenzylo-
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ethylene by Microsomal System—Diphenylfuran was incubated with liver microsomes, NADPH (or an NADPH-generating system), and a low concentration of Fe$^{3+}$ (1.2 x 10$^{-4}$ M). The systems were extracted with chloroform and the extractable material was analyzed by thin layer chromatography as described under “Experimental Procedures.” The resulting separations shown in Fig. 2 indicate that significant amounts of diphenylfuran were converted to dibenzoylethylene in the complete microsomal enzyme system but conversion was nearly undetectable in the control system lacking NADPH. The product of the microsomal reaction had chromatographic behavior, staining characteristics, as well as ultraviolet and infrared absorption spectra, identical with that of authentic samples of dibenzoylethylene and the product formed by photooxidation of diphenylfuran in the presence of a sensitizer (16). Fig. 3 shows that the conversion of diphenylfuran to dibenzoylethylene is apparently dependent on the enzymatic oxidation of NADPH since prior heating of the microsomes at 70° for 2 min before their addition to the system abolished the conversion. In addition, prior treatment of microsomes with p-chloromercuribenzoate prevented dibenzoylethylene formation. Both of these procedures are known to inhibit NADPH oxidation by this microsomal system (17). Mn$^{2+}$ is also an effective inhibitor of lipid peroxidation promoted by this system (17). In order to determine the relationship between the apparent singlet oxygen formation and lipid peroxidation, studies were performed to determine the effect of Mn$^{2+}$ on dibenzoylethylene formation. Fig. 4 shows that when Mn$^{2+}$ is present in the system, dibenzoylethylene is not formed, indicating that lipid peroxidation may be required for $'0_2$ formation since malondialdehyde formation was prevented under those conditions. Mn$^{2+}$ is not acting to quench $'0_2$ since addition of this metal ion in a wide range of concentrations to systems in which $'0_2$ is being generated photochemically to methylene blue had no effect on the conversion of diphenylfuran to dibenzoylethylene.

Because superoxide anion ($O_2^-$) is produced by both the activity of NADPH-cytochrome P-450 reductase and xanthine oxidase, and is probably responsible for the formation of the radicals which initiate lipid peroxidation (1), it is remarkable that superoxide dismutase has no effect on either lipid peroxidation (as indicated by malondialdehyde formation) (Fig. 5) or $'0_2$ production (as indicated by dibenzoylethylene formation).

![Fig. 1](http://www.jbc.org/) Effect of singlet oxygen quenching compounds on malondialdehyde formation during NADPH oxidation by liver microsomes. The basic reaction medium contained 2.0 ml of microsomes (approximately 20 mg of protein), 4 mM ADP, 0.012 mM FeCl$_3$, and an NADPH-generating system (described under “Experimental Procedures”), all in 0.05 M Tris-HCl buffer, pH 7.5. Final volume of all reaction systems was 10 ml. Additions to this system included: O—O, none; △—△, tetrathylpentadiene, 1.67 x 10$^{-4}$ M; □—□, tetraphenylcyclopentadiene, 1.5 x 10$^{-4}$ M; ■—■, diphenylisobenzofuran, 1.0 x 10$^{-3}$ M; ●—●, diphenylfuran, 1.5 x 10$^{-4}$ M; ○—○, diphenylfuran, 1.0 x 10$^{-3}$ M; Mn$^{2+}$, 10$^{-3}$ M. The product of the microsomal reaction had chromatographic behavior, staining characteristics, as well as ultraviolet and infrared absorption spectra, identical with that of authentic samples of dibenzoylethylene and the product formed by photooxidation of diphenylfuran in the presence of a sensitizer (16). Fig. 3 shows that the conversion of diphenylfuran to dibenzoylethylene is apparently dependent on the enzymatic oxidation of NADPH since prior heating of the microsomes at 70° for 2 min before their addition to the system abolished the conversion. In addition, prior treatment of microsomes with p-chloromercuribenzoate prevented dibenzoylethylene formation. Both of these procedures are known to inhibit NADPH oxidation by this microsomal system (17). Mn$^{2+}$ is also an effective inhibitor of lipid peroxidation promoted by this system (17). In order to determine the relationship between the apparent singlet oxygen formation and lipid peroxidation, studies were performed to determine the effect of Mn$^{2+}$ on dibenzoylethylene formation. Fig. 4 shows that when Mn$^{2+}$ is present in the system, dibenzoylethylene is not formed, indicating that lipid peroxidation may be required for $'0_2$ formation since malondialdehyde formation was prevented under those conditions. Mn$^{2+}$ is not acting to quench $'0_2$ since addition of this metal ion in a wide range of concentrations to systems in which $'0_2$ is being generated photochemically to methylene blue had no effect on the conversion of diphenylfuran to dibenzoylethylene.

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The amounts of superoxide dismutase added are all capable of completely inhibiting the superoxide anion-dependent reduction of cytochrome c by xanthine oxidase as described by McCord and Fridovich (8).

Inhibition of NADPH Oxidase-catalyzed Lipid Peroxidation by Diphenylfuran—The effect of increasing concentrations of diphenylfuran on the extent of lipid peroxidation by this microsomal system was studied. The results show that increasing concentrations of diphenylfuran in the system resulted in greater inhibition of lipid peroxidation as well as increased dibenzoylethylene production as judged by the size and staining intensity of this substance on thin layer chromatographic analysis (Table I). The data suggests that singlet oxygen may be involved in this enzyme-catalyzed lipid peroxidation. It was found, however, that complete inhibition of lipid peroxidation by this system could not be obtained even at saturating concentrations of diphenylfuran.

Studies Demonstrating that Diphenylfuran Does Not Inhibit Lipid Peroxidation by Scavenging Free Radicals—The experiments with the Fenton system were done to determine whether or not diphenylfuran possesses free radical-scavenging properties. It was considered necessary to determine this possibility since diphenylfuran might have exerted its inhibitory effect on lipid peroxidation by scavenging hydroxyl radicals which are produced during NADPH oxidation by the microsomal system. The Fenton system produces hydroxyl radicals by the reduction of H₂O₂ (18). If diphenylfuran reacts with this electrophilic radical, incubation of the compound in the Fenton system should result in at least part of the compound being oxidized. Chromatographic analyses indicated that diphenylfuran was not reactive in the Fenton system. Neither dibenzoylethylene nor any other product could be detected. This result also agrees with studies performed to determine whether or not diphenylfuran inhibited the hydroxylation of drugs by the Fenton system. Table II shows that the addition of diphenylfuran to Fenton system containing aniline or acetanilide had no influence on the hydroxylation of either of the latter compounds even when the concentration of diphenylfuran was 10-fold that of the drug.

Fe⁺ Requirement for Apparent Singlet Oxygen Production by the NADPH Oxidase System—Previous work in this laboratory had shown that Fe⁺⁺⁺ is required for the enzyme-catalyzed production of HO⁻ radicals which apparently promotes the peroxidative loss of polyunsaturated fatty acids in microsomes (1). In the present study, we have found Fe⁺⁺⁺ was required for the conversion of diphenylfuran to cis-dibenzoylethylene by the microsomal NADPH oxidase system as well. As previously mentioned, the Tris buffer used in preparing all solutions employed in these experiments had been previously analyzed for the presence of superoxide dismutase activity (SOD) with and without diphenylfuran (DPF). The incubation system was as described in Fig. 2. The thin layer analyses shown in Fig. 4 demonstrate that superoxide dismutase had no effect on the conversion of diphenylfuran to dibenzoylethylene under these conditions.

Table I

<table>
<thead>
<tr>
<th>DPF added to system</th>
<th>Malondialdehyde formed/mg microsomal protein</th>
<th>Dibenzoylethylene formation (thin layer plate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>nmol</td>
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<tr>
<td>0</td>
<td>8.00</td>
<td>-</td>
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<tr>
<td>0.05</td>
<td>6.76</td>
<td>+</td>
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<tr>
<td>0.125</td>
<td>5.03</td>
<td>+</td>
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<tr>
<td>0.25</td>
<td>4.78</td>
<td>++</td>
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<tr>
<td>0.375</td>
<td>4.72</td>
<td>++</td>
</tr>
<tr>
<td>0.5</td>
<td>4.38</td>
<td>++</td>
</tr>
<tr>
<td>0.75</td>
<td>4.16</td>
<td>+ +</td>
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<tr>
<td>1.00</td>
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</table>

Fig. 5. Formation of malondialdehyde by NADPH oxidase activity in the presence of superoxide dismutase (SOD), with and without diphenylfuran (DPF). The incubation system was as described in Fig. 2. The thin layer analyses shown in Fig. 4 demonstrate that superoxide dismutase had no effect on the conversion of diphenylfuran to dibenzoylethylene under these conditions.
treated with Chelex-100 resin to remove iron. In incubation systems without added iron, there was very little, if any, detectable conversion of diphenylfuran to cis-dibenzylolethylene (Fig. 6). Much lower levels of malondialdehyde formation were also observed when iron was omitted (Fig. 7), but when chelated iron was added to the system (0.012 mM FeCl₃ in 4 mM ADP), malondialdehyde formation was enhanced. Dibenzylolethylene formation then occurred which was detectable by thin layer chromatography (Fig. 6). The appropriate controls showed that even with the addition of iron, it was still necessary to provide NADPH to produce dibenzylolethylene.

**Studies with Xanthine Oxidase System**—It is well documented that xanthine oxidase activity generates superoxide anions (8, 19), although the extent to which it does so may depend upon incubation conditions (20). Using different approaches, two laboratories have reported that xanthine oxidase activity produces singlet oxygen (4, 5). We assayed for singlet oxygen formation during xanthine oxidase activity but obtained negative results. In these studies, we used diphenylfuran as a singlet oxygen reagent, but were unable to show any detectable formation of dibenzylolethylene even when a highly active preparation of xanthine oxidase was used (Fig. 8). This was true either with or without the addition of chelated iron. The activity of the enzyme was sufficient to generate superoxide anion at a reasonably high rate, and was capable of reducing 4 μmol of cytochrome c/min, the reduction being almost totally inhibited when superoxide dismutase was added. In addition, we were not able to show formation of dibenzylolethylene from 1,3-diphenylisobenzofuran (another singlet oxygen trapping agent) which could be specifically attributed to singlet oxygen formation by xanthine oxidase activity, as had been reported by others (4). We observed that this singlet oxygen trapping agent was converted to a product with properties of dibenzylolethylene spontaneously when incubated with xanthine oxidase in the absence of xanthine to about the same extent as when incubated in the complete system. The same situation also occurred when diphenylisobenzofuran was incubated with liver microsomes alone, or in a complete NADPH oxidase system, even in the absence of light. This singlet oxygen reagent appears to be unsatisfactory, therefore, for use in assays involving biological materials.

Chemiluminescence, associated with the oxidation of acetaldehyde by xanthine oxidase, was reported to be considerably greater than that occurring during the oxidation of xanthine by the enzyme (5), suggesting that either xanthine or the product of the enzyme activity, uric acid, may quench singlet oxygen. However, photodynamic oxidation of diphenylfuran by rose

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**Fig. 6. Requirement for ADP-Fe³⁺ for the conversion of diphenylfuran to dibenzylolethylene by microsomal NADPH oxidase activity.** The reaction system, conditions of incubation and analytical procedures are described in Fig. 2 with omissions as indicated. 1, complete incubation system; 2, system without NADPH; 3, system without ADP-Fe³⁺; 4, diphenylfuran standard; 5, cis-dibenzylolethylene standard; and 6, trans-dibenzylolethylene standard.

**Fig. 7. Requirement for ADP-Fe³⁺ in the NADPH oxidase-catalyzed formation of malondialdehyde by rat liver microsomes.** The composition of the reaction system and the analytical procedures are as described in Fig. 2. □—□, complete reaction system; O—O, ADP-Fe³⁺ omitted; △—△, NADPH-generating system omitted.

**Fig. 8. Thin layer chromatographic analyses: 1 to 3, xanthine oxidase activity with xanthine or acetaldehyde in the presence of diphenylfuran; 4 to 7, photo-oxidation of diphenylfuran in the presence of xanthine or uric acid.** The enzymatic incubation system (Numbers 1 to 3) consisted of xanthine oxidase as specified, 10 mM acetaldehyde or 1 × 10⁻⁴ M xanthine, in 0.05 M potassium phosphate buffer containing 10⁻⁴ M EDTA, pH 7.8. The photodynamic system (Numbers 4 to 7) consisted of 3 μmol rose bengal, 1 mM xanthine or 1 mM uric acid, in D₂O/H₂O (1/1). The light source was a 500 watt incandescent bulb which was 9.5 cm from the reaction vessels. Diphenylfuran, 1.0 mM, was added to all systems in 0.1 ml acetone/0.9 ml of reaction system. The final volume of all systems was 2.0 ml. Incubation period was 30 min at 37°. All systems were extracted and analyzed by thin layer chromatography as described under "Experimental Procedures". 1, xanthine oxidase (6 μg of protein) with acetaldehyde as substrate; 2, xanthine oxidase (90 μg of protein) with acetaldehyde as substrate; 3, xanthine oxidase (6 μg of protein) with xanthine as substrate; 4, photodynamic system plus xanthine; 5, photodynamic system plus uric acid; 6, photodynamic system; 7, photodynamic system minus rose bengal; 8, diphenylfuran, trans-dibenzylolethylene, and cis-dibenzylolethylene standards (top to bottom, respectively). One unit of xanthine oxidase used was the amount of enzyme required to oxidize 1.0 μmol of xanthine/min at 25°. The enzyme preparation contained 0.82 unit/mg of protein.
DISCUSSION

The possibility that singlet oxygen may be involved in some biological oxidations has been indicated by at least two types of studies on enzyme systems; those involving measurement of chemiluminescence due to singlet oxygen decay (2, 21), and those based on the effect of singlet oxygen quenching or trapping compounds in reaction systems in which O$_2^-$ is believed to be a participant (15). Although it has been claimed that singlet oxygen can arise from the chemical dismutation of superoxide anion (22), Nilsson and Kears have provided convincing evidence that this is not the case (23). The results described in this report demonstrate that singlet oxygen appears to be produced by microsomal electron transport associated with NADPH oxidation and that it may promote part, but not all, of the lipid peroxidation which accompanies enzymic activity. These conclusions are based on the observations that diphenylfuran is converted to dibenzoylethylene during the enzymic reaction and that the peroxidation of lipids in the microsomal membrane is inhibited by only 50 to 60% at saturating concentrations of diphenylfuran. It should be noted that the solubility of diphenylfuran in water is only $5 \times 10^{-7}$ M (16), and addition in acetone of diphenylfuran in the amounts employed in these studies results in a finely dispersed suspension of the compound which, as the concentration increases, causes greater inhibition of peroxidation and qualitatively larger production of dibenzoylethylene (Table I). This effect is probably due to enhanced availability of diphenylfuran to the lipophilic portion of the microsomal membrane due to the fine dispersion. Singlet oxygen apparently can initiate peroxidation of unsaturated fatty acids (24, 25) and form hydroperoxides which, being unstable, degrade and initiate free radical-mediated chain reactions, yielding numerous lipid degradation products, including malondialdehyde. Our studies had already shown that lipid peroxidation catalyzed by at least two purified oxido-reductase enzyme systems is the result of free radical (probably hydroxyl) formation from the interaction of superoxide anion (generated by the enzyme activity) with inorganic iron (1). The formation of O$_2^-$ by the microsomal NADPH oxidase system may occur during degradation of lipid peroxides rather than by a reaction of O$_2^-$ with the microsomal electron transport system during NADPH oxidation. If that were the case, it may explain why O$_2^-$ was not observed in the xanthine oxidase system in which lipid peroxidation was not occurring. It would also explain why Mn$^{2+}$ inhibited apparent $O_2^-$ formation by the microsomal system, because Mn$^{2+}$ completely inhibits lipid peroxidation on which $O_2^-$ formation may depend in this system. Mn$^{2+}$ did not affect $O_2^-$ reactions in nonenzymic photodynamic system. It also would explain the requirement of Fe$^{3+}$ for $O_2^-$ formation since it is already known that Fe$^{3+}$ promotes NADPH-dependent lipid peroxidation in microsomes, apparently by augmenting HO$^-$ formation (1). It may also be relevant that the concentrations of ADP and inorganic iron required for efficient promotion of lipid peroxidation in these systems are similar to those present in the soluble fraction of liver cells (0.032 mmol of inorganic iron/kg wet liver tissue) and 3.2 mmol of ADP/kg wet liver tissue (26).

As mentioned above, autoxidative peroxidation of extracted unsaturated lipids is believed to be initiated by singlet oxygen (24, 25). Our results indicate that this may also be true for part of the lipid peroxidation catalyzed by the activity of some oxido-reductases. These results do not agree with those of Smith and Teng (27), and Sternson and Wiley (28), who found no evidence for singlet oxygen formation during NADPH oxidation by microsomes. However, our studies support and extend those of Howes and Steele who detected chemiluminescence in a similar system which they attributed to singlet oxygen formation (2). The discrepancy may be related to the amount of Fe$^{3+}$ present in the different systems.

Results of the studies on the xanthine oxidase system also present a dilemma. They indicate that O$_2^-$ is not formed in this system in any amount detectable by the method used. These results contrast with those of Arneson et al. (5) and Pederson and Aust (4) who reported that the activity of this system produces singlet oxygen using a similar method. As mentioned above, however, we observed that the singlet oxygen detecting agent (diphenylisobenzofuran) used in one of these studies (4) can convert spontaneously to its singlet oxygen reaction product (dibenzoylbenezene) in the presence of xanthine oxidase alone, even in the dark. The substrate, xanthine, was not required, hence the conversion does not appear to be due to xanthine oxidase activity per se. This observation is in agreement with those of Mayeda and Bard who demonstrated that diphenylisobenzofuran can react spontaneously with ground state oxygen in the dark to form dibenzoylbenzene (29). Diphenylfuran, the trapping agent used in the studies presented in this paper, does not convert spontaneously to dibenzoylethylene under the conditions employed, nor did it produce any singlet oxygen reaction product during xanthine oxidase activity with or without iron.

If singlet oxygen is not produced during xanthine oxidase activity, how does chemiluminescence associated with xanthine oxidase activity arise? It has been suggested that the chemiluminescence in systems involving singlet oxygen formation may be due to direct energy transfer from singlet oxygen to some acceptor organic molecule with subsequent emission of the energy as light (30). Arneson et al. concluded that O$_2^-$ was produced during xanthine oxidase activity from their studies on the chemiluminescence of that system. But chemiluminescence in reactions involving oxygen could be derived in different ways so that light emission per se may not be indicative of a particular mechanism (3). Indeed, the validity of the results of all of the studies described in this report depends on the specificity claimed for the reaction:

\[
\text{diphenylfuran} \xrightarrow{\text{O}_2^=} \text{dibenzoylethylene}
\]

which is reasonably well documented (16).

It is possible that the dibenzoylethylene formed by the microsomal system might be due to a reaction of diphenylfuran with lipid peroxide per se (31), but this does not seem likely in view of the unreactivity of diphenylfuran with hydrogen peroxide in the Fenton system.

Although the studies with superoxide dismutase presented in this report indicate that O$_2^-$ may not be involved, previous studies in our laboratory with a soluble NADPH-cytochrome
P-450 reductase system indicated that O$_2^-$ is involved in the lipid peroxidation phenomenon (1). Studies by Pederson and Aust provided similar results (32). We interpret this finding to mean that the site of O$_2^-$ formation in the intact microsomal membrane may not be accessible to the dismutase, or at least not sufficiently so to interfere with the events which lead to HO· formation and lipid peroxidation.

Since the number of enzymes believed to form superoxide anion during their activity is growing, the studies described in this report imply that lipid peroxidation might be an ongoing process in normal tissue in which membrane damage is offset by homeostatic mechanisms. The latter may include a function for α-tocopherol in biological systems since this compound is known to inhibit lipid peroxidation in biological membranes, and is capable of terminating free radical reactions as well as reacting with singlet oxygen (33). Studies recently completed in this laboratory with isolated, intact liver cells, appear to confirm this conclusion.2

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Singlet oxygen production associated with enzyme-catalyzed lipid peroxidation in liver microsomes.
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