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A POSSIBLE MECHANISM OF THE GENERATION OF SINGLET MOLECULAR OXYGEN IN NADPH-DEPENDENT MICROSOMAL LIPID PEROXIDATION

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SUMMARY

A simplified system, consisting of NADPH, Fe^{3+}-ADP, EDTA, liposomes, NADPH-cytochrome *c* reductase and Tris · HCl buffer (pH 6.8), has been employed in studies of the generation of singlet oxygen in NADPH-dependent microsomal lipid peroxidation.

The light emitted by the system involves $^1\Delta g$ type molecular oxygen identifiable by its characteristic emission spectrum and its behavior with β-carotene. The generation of another excited species (a compound in the triplet state) could be demonstrated in this system by changes of light intensity and emission spectra which arise from photosensitizer (9, 10-dibromoanthracene sulfonate, eosin, Rose-Bengal)-mediated energy transfers.

Chemiluminescence in the visible region was markedly quenched by various radical trappers and by an inhibitor of NADPH-cytochrome *c* reductase, but not by superoxide dismutase. During the early stage of lipid peroxidation, the intensity of chemiluminescence was proportional to the square of the concentration of lipid peroxide.

These characteristics suggest that singlet oxygen and a compound in the triplet state (probably a carbonyl compound) are generated by a self-reaction of lipid peroxy radicals.

INTRODUCTION

The unsaturated fatty acids of liver microsomal phospholipids readily undergo peroxidation in the presence of NADPH and oxygen [1, 2]. The NADPH-dependent flavoprotein, NADPH-cytochrome *c* reductase, catalyzes the peroxidation of isolated lipids [3] or lipoprotein [4] under similar conditions, provided that EDTA is present in appropriate concentration in the reaction system. EDTA in such reconstructed systems appears to elevate the redox potential of Fe^{3+} and of the iron complex, facilitating thereby the transfer of one electron from NADPH to tri-valent iron through the flavin moiety of the reductase [4]. Reduced iron, free in the solution,

plays an important role in the initiation and propagation of phospholipid peroxidation [4, 5]. NADPH-dependent lipid peroxidation is usually accompanied with an emission of ultra weak light [4, 6, 7]. Recently the emitter in the NADPH-dependent microsomal lipid peroxidation system has been spectrometrically confirmed to be $^1\Delta g$ type singlet molecular oxygen [7].

Little is known, however, of the relationship between lipid peroxidation and $^1O_2$ generation. The present work was undertaken to identify the mechanism of $^1O_2$ generation during peroxidative cleavage of phospholipid in microsomes, using a simplified NADPH-dependent lipid peroxidation system (NADPH-NADPH-cytochrome *c* reductase-Fe^{3+}-ADP-EDTA-liposomes).

MATERIALS AND METHODS

Reagents. ADP and α-tocopherol were obtained from Sigma. NADPH was purchased from Oriental Yeast Co. Ltd. The 9, 10-dibromoanthracene sulfonate (sodium salt) and β-anthracene sulfonate (sodium salt) were kindly supplied by Professor G. Cilento. All other chemicals were of reagent grade. β-Carotene, obtained from Tokyo Kasei Co., was purified by three crystallizations from a benzene/methanol mixture. The molar absorption at 4640 and 4940 Å for crystalline β-carotene in benzene was $11.0 \cdot 10^4$ and $9.4 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$, respectively.

Enzyme preparation. Microsomal NADPH-cytochrome *c* reductase (spec. act., 18–25 μmol ferricytochrome *c* reduced/min per mg protein) was prepared from rat liver microsomes by established method [8]. Superoxide dismutase (spec. act., 2500 units/mg protein) was prepared from bovine red blood cells and assayed in terms of its ability to inhibit (50 %) the reduction of cytochrome *c* by milk xanthine oxidase by the method of McCord and Fridovich [9].

Preparation of liposomes. The lipid was extracted from rat liver microsomes [2] by the method of Folch et al. [10] and the chloroform layer was then stored at −20 °C under anaerobic conditions. Just before the experiment, an aliquot of the chloroform solution was added to a 100 ml round bottomed flask and evaporated under reduced pressure. In some cases β-carotene or α-tocopherol in chloroform was mixed with the chloroform solution of the microsomal lipid, and evaporated to dryness as described above. Distilled water was then added to the flask and the lipids (with or without added chemicals) were thoroughly agitated by means of a Vortex mixer until the lipid film was no longer detectable on the sides of flask.

The amount of lipid was measured as lipid phosphorous by a modification of the method of Bartlett [11].

Preparation of malondialdehyde. Malondialdehyde in free form was prepared by NADPH-dependent microsomal lipid peroxidation [2]. Malondialdehyde-bis-bisulfate (sodium salt) was obtained by treating malondialdehyde-bis(dimethylacetal) with aqueous HCl and mixing it with sodium meta-bisulfite [12]. The free form was then obtained by its hydrolysis in 1 M HCl for 20 min at room temperature. Both the enzymatically and chemically prepared malondialdehyde in free form was then purified by gel filtration on Sephadex G-10 column, using 0.1 M potassium phosphate/NaCl buffer at pH 7.2 [2].

Incubation conditions. The standard reaction mixture consisted of liposomes (0.43 μmol phosphate per ml of incubation mixture), $1 \cdot 10^{-4}$ M $Fe(NO_3)_3$, 1.67 mM

ADP, $5 \cdot 10^{-5}$ M EDTA, 0.16 mM NADPH, NADPH-cytochrome *c* reductase (0.5 unit) and 0.1 M Tris · HCl buffer (pH 6.8) in a total volume of 3 ml.

In some experiments components such as superoxide dismutase, radical scavengers and SH inhibitor were added to the standard incubation mixture to examine their effect on light emission or NADPH consumption. Unless otherwise noted, incubation was carried out at 37 °C.

Other methods. NADPH disappearence and malondialdehyde formation were measured by previously described methods [4]. Lipid peroxide was determined by iodometry [13]. The excitation and fluorescence spectra of dye in 0.1 M Tris · HCl buffer (pH 6.8) and of malondialdehyde in 0.1 M potassium phosphate/NaCl buffer at pH 7.2 were determined by means of a Hitachi Model 203 fluorescence spectrometer. The excitation energy (kcal/mol) of the dye was calculated from its excitation wavelength (λ as Å) according to the equation ($E = 2.8589 \cdot 10^8$ cal $\cdot \lambda^{-1} \cdot$ mol^{-1}). Luminescence was measured by means of a single photoelectron [14] and Packard Model-2311 liquid scintillation counter with the coincidence circuit off [15]. The emission spectrum was determined as previously described [7].

RESULTS

(1) *Factors which promote light emission.* To test the effect of O_2 concentrations on light emission, the reaction mixture containing NADPH-cytochrome *c* reductase, Fe^{3+}-ADP-EDTA, and liposomes was flushed with air or 100 % O_2 for 30 s and chemiluminescence in both systems was compared after the addition of NADPH. In

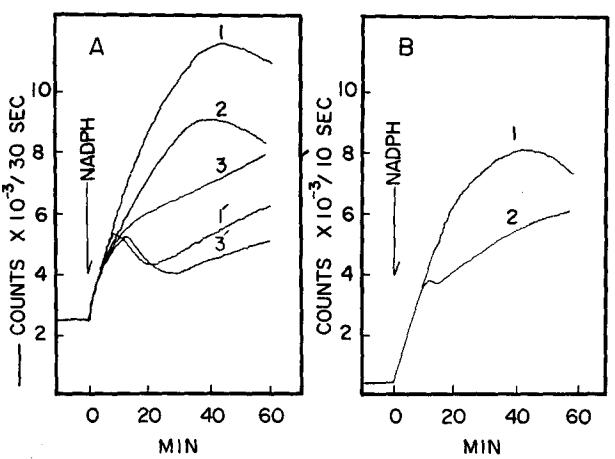


Fig. 1. (A) Effects of O_2 concentration and agitation of the reaction mixture on light emission. The standard reaction mixture (lacking NADPH) in a vial was flushed with air (1 or 1') or 100 % O_2 (2) for 30 s. The system (3 or 3') was sealed from ambient air with 5 ml of paraffin oil (without flushing O_2). After the addition of NADPH, NADPH-dependent light emission was measured by the scintillation counting method described in text. In Expts. 1 and 3 the vessels were shaken whereas in Expts. 1' and 3' they were not but in Expt. 2 were also not shaken during the measurement of light intensity. (B) Effects of the diameter of cell on light emission. The standard incubation mixture was transferred into two circular quartz cells, 6 cm (1) and 3 cm in diameter (2). Light emission was measured without shaking by the single photon counting method. The experiments were carried out at 18 °C.

some experiments the reaction mixture was shielded from air by paraffin oil. Light emission was then measured with or without shaking for a 20-s interval per 1 min at room temperature, using a Packard Model-2311 liquid scintillation counter with its coincidence circuit turned off. As shown in Fig. 1A, the system requires both O_2 and agitation for a continuous increase in light emission. The relation between chemiluminescence and liquid height of the reaction mixture was also studied by our single photon counting method. The reaction mixture with NADPH omitted (2.9 ml) in each of two types of circular quartz cells (3.0 cm in diameter and 6.0 cm in diameter) was placed for 5 min in front of head-on type photomultiplier in a single photon counter.

After obtaining the background counts, NADPH-dependent luminescence was then measured after the addition of NADPH without shaking during the reaction. Fig. 1B shows that the level of liquid and the surface area are important factors in the continued increase of light emission by this system. The light intensity obtained with a wide quartz cell (6.0 cm in diameter) by the single photon counting method (Method 1) correlated well with those obtained with shaking of vial (2.3 cm in diameter) in a liquid scintillation counter (Method 2). Unless otherwise noted, light intensity was expressed in terms of counts per 10 s (or 30 s) corrected for background. With identical systems, NADPH-induced light intensity measured by Method 1 is approx. three times greater than that measured by Method 2. In the following experiments, either Method 1 or Method 2 was employed for the detection of light intensity. Boiling of the liposomes for 5 min increased the background counts slightly. Omission of either Fe^{3+} or NADPH-cytochrome *c* reductase from the standard mixture did not produce NADPH-dependent light emission. However, omission of EDTA produced detectable light emission which increased linearly in intensity for more than 600 s after the addition of NADPH. This corresponded to about one-fourth of the light intensity of the complete system.

(2) *Effect of enzyme and liposome concentrations on light emission.* When both the initial light intensity (counts at 500 s after the addition of NADPH) and maximal light intensity were measured at a fixed enzyme concentration of 0.17 unit per ml of incubation mixture, both were first order with respect to phospholipid concentration (up to 0.4 μmol phosphate per ml of incubation mixture). With a fixed phospholipid concentration of 0.4 μmol phosphate per ml of the incubation mixture, either NADPH-dependent initial light intensity or maximal light intensity was proportional to the enzyme concentration (up to 0.2 unit per ml of the incubation mixture).

(3) *Effect of SH inhibitor and radical trappers.* NADPH oxidase activity and light emission associated with lipid peroxidation were examined following the addition of *p*-chloromercuribenzoate (inhibitor of the reductase) and 2,5-di-*tert*-butylhydroquinone (radical trapper).

Addition of 1 mM *p*-chloromercuribenzoate 500 s after the introduction of NADPH caused an immediate inhibition of NADPH consumption, but did not inhibit the increase in light intensity for 200 s (Fig. 2). The presence of *p*-chloromercuribenzoate in this concentration prior to the initiation of the NADPH-induced lipoxygenation abolished light emission completely.

Under similar conditions, the addition of $5 \cdot 10^{-5}$ M 2,5-di-*tert*-butylhydroquinone during NADPH-dependent lipid peroxidation accelerated the NADPH consumption (probably by non-enzymatic transfer of electron from NADPH tc

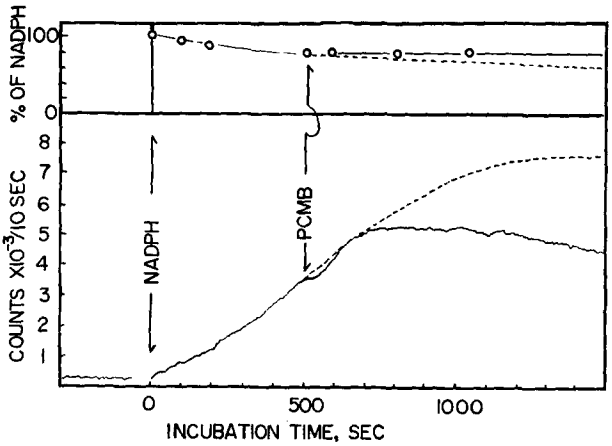


Fig. 2. Effects of *p*-chloromercuribenzoate on light emission and NADPH consumption. The standard reaction mixture was employed in both the experimental and the control incubations; . . . ., control system; —, control system to which *p*-chloromercuribenzoate (1.0 mM) was added after 500 s of incubation. NADPH consumption and light emission were measured by fluorimetry and Method 1, respectively, as described in the text.

di-*tert*-butylphenoxyradical produced in the system), but caused rapid and sustained depression of light intensity (Fig. 3). The addition of this hydroquinone analogue in one-tenth of the above concentration ($5 \cdot 10^{-6}$ M) 500 s after the reaction had been initiated did slightly affect light emission, whereas the presence of the agent in the same concentration prior to the reaction abolished both light emission and malondialdehyde formation completely within more than the 30-min incubation period. This suggests that lipid radicals produced in the beginning of the lipid peroxidation are in small quantities and could be easily scavenged by this agent in the concentration as

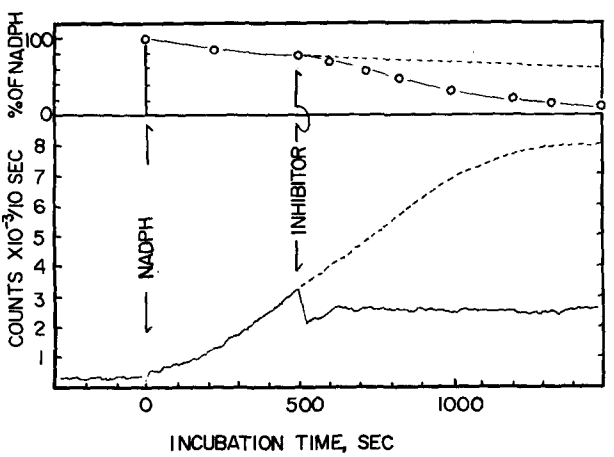


Fig. 3. Effect of 2,5-di-*tert*-butylhydroquinone on light emission and NADPH consumption. The incubation and assay conditions were described in Fig. 2, save that $5 \cdot 10^{-5}$ M di-*tert*-butylhydroquinone (inhibitor), instead of *p*-chloromercuribenzoate, was added at the arrow.

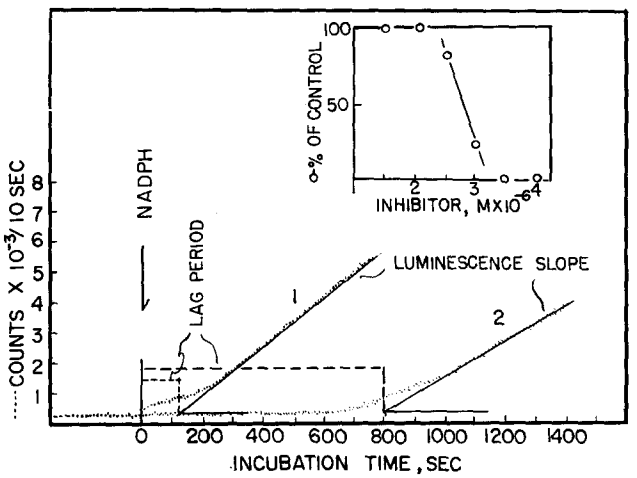


Fig. 4. Measurements of lag time of light emission and chemiluminescence slope. The control (curve 1) and experimental (curve 2) systems were identical with the standard reaction mixture save that the latter contained a radical scavenger. 2,5-di-*tert*-butylhydroquinone (2.5 μM) was added to the reaction mixture (lacking NADPH). NADPH was then added at 0 s to initiate the reaction. The lag time indicated on the figure was measured from zero to the time at which a line tangent to the light intensity (luminescence slope) intersects the "time axis". Prolongation of lag time by this hydroquinone was calculated the lag time in its absence and its presence. The relationship between the concentration of this hydroquinone and the luminescence slope was obtained by plotting each value estimated (as described above) versus concentration of the inhibitor (inset).

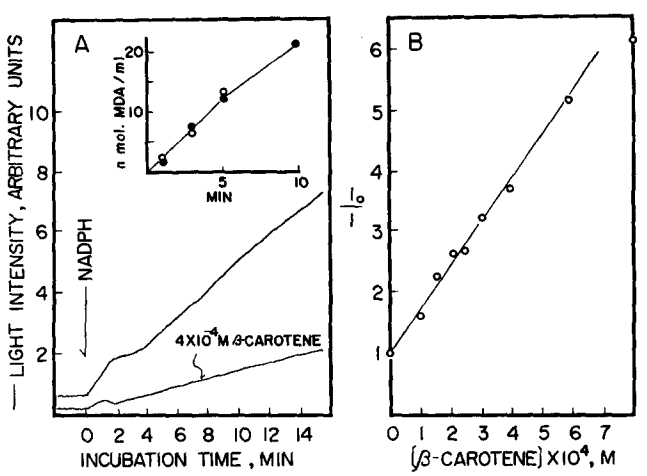


Fig. 5(A). Effects of β-carotene on light emission and malondialdehyde formation. Each reaction mixture was essentially standard, save that the lipid suspension which contained β-carotene (prepared as described under Materials and Methods) was used instead of the neat lipid suspension. Light intensity and malondialdehyde (MDA) were measured at 18 °C by Method 2 and the thiobarbiturate-color test, respectively. The symbols in inset, ● and ○, represent malondialdehyde formation in the systems with and without $4 \cdot 10^{-4}$ M β-carotene, respectively. (B) Effects of the concentration of β-carotene on light emission. Luminescence slopes was measured as described in Fig. 4 in the absence (I_0) and in the presence of β-carotene (I).

low as $5 \cdot 10^{-6}$ M thereby facilitating the inhibition of a propagative formation of the precursor of light species.

(4) *Potency of various antioxidants.* Potencies of agents which inhibit chemiluminescence associated with lipoxygenation can be tested by the addition of these agents prior to the reaction. Their effects can be expressed in terms of the minimum concentration (μM) required to produce a 50 % decrease in the luminescence slope (quenching potency). Fig. 4 shows the effects of 2,5-di-*tert*-butylhydroquinone and indicates the luminescence slope and lag time measured in our experiments. Lag time was prolonged with increasing amount of the hydroquinone analogue. Under our assay conditions, the quenching potencies of 2,5-di-*tert*-butylhydroquinone, 2,6-diiodo-4-hydroxyphenol, 2-butyl-4-methoxyphenol and α-tocopherol were found to be 2.8, 2.8, 5.0 and 600, respectively.

(5) *Effects of $^1O_2$ and $O_2^{-\cdot}$ scavengers on light emission.* Under assay conditions similar to those employed in testing the quenching potency of the antioxidant, β-carotene [16, 17] (a known quencher of $^1\Delta g$ type oxygen) and superoxide dismutase [18] (a known scavenger of $O_2^{-\cdot}$) were tested as possible inhibitors of light emission.

In contrast to the radical trappers, β-carotene was found to produce a decrease in the slope of chemiluminescence without prolongation of the lag period nor depression of malondialdehyde formation (Fig. 5A). The absorption spectra (4000–6000 Å) of benzene extracts obtained from reaction mixtures before and after the incubation were identical to that of β-carotene in benzene, indicating that β-carotene was not altered during lipid peroxidation. As shown in Fig. 5B, kinetically this compound acted as quencher which obeys the Stern - Volmer formula for all types of quenching mechanisms:

$$I_0/I = 1 + k\tau_0[A]$$

Where I_0/I, k, [A] and τ_0 represent the ratio of the unquenched to the quenched intensity of chemiluminescence in terms of the chemiluminescence slope, the quenching rate constant, the quencher concentration and the actual life time of an excited species in the absence of A. From the data shown in Fig. 5B, the $k\,\tau_0$ (slope) was calculated to be $8 \cdot 10^3\ M^{-1}$. Assuming a life time of the light species (as $^1O_2$) in water of $2 \cdot 10^{-6}$ s [19], the rate constant in the above equation is $4 \cdot 10^9\ s^{-1} \cdot M^{-1}$ (i.e. about one-tenth of the k in benzene [20]).

Intact superoxide dismutase at a concentration of 1 μM, known to scavenge more than 70% of $O_2^{-\cdot}$ generated in a system with $3.3 \cdot 10^{-4}$ M xanthine and xanthine oxidase (0.9 unit per ml of the incubation mixture) at pH 6.8 [4], produced 37–40 % inhibition of the chemiluminescence slope and 25 % inhibition of malondialdehyde formation without prolongation of lag time. On the other hand, heat-inactivated superoxide dismutase produced 18–20 % inhibition of the chemiluminescence slope and 8–10 % inhibition of malondialdehyde formation.

Light emission under the same conditions during 20 min in the presence of intact superoxide dismutase was found to be 80 % of that emitted in the presence of heat-inactivated dismutase. This indicates that enzyme-catalyzed inhibition is slight.

(6) *Emission spectrum.* As shown in Fig. 6, the emission spectrum of light species from the present system coincides closely with that from NADPH-dependent microsomal lipid peroxidation [7], viz. both are in 4000–6800 Å region. The locations of the emission peaks, at or near 5200 Å and at or near 6350 Å, and of the shoulder or

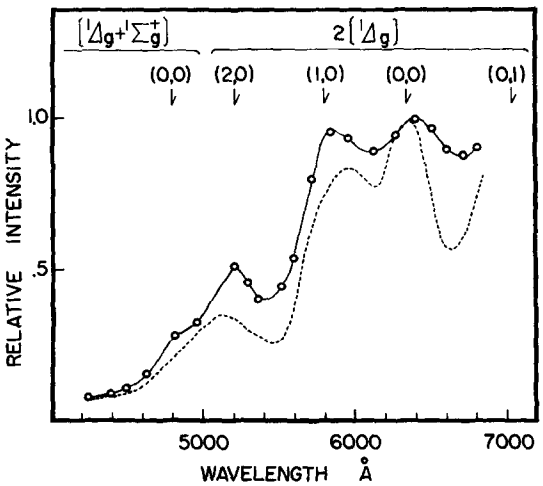


Fig. 6. Emission spectrum of light species in the reconstructed microsomal lipid peroxidation system. Spectral analysis was carried out after the addition of NADPH to the standard reaction mixture (lacking NADPH). Each point (○) is the average of two experiments. Dotted line indicates the spectrum of light emission from the NADPH-dependent microsomal system (data taken from the report of Nakano et al. [7]). Arrows indicate the location of the band labeled according to upper electronic state by energy conversion with vibronic components (after Kahn and Kasha [23]).

distinct peak at near 5800 Å also coincide with those from a $H_2O_2/NaOCl/NH_3$ aqueous system [7]. It seems likely therefore that the simplified lipid peroxidation system produces, at the least, $^1\Delta g$ type-singlet molecular oxygen identical to that observed in the complicated microsomal NADPH-dependent system. It may be postulated that the above three emission peaks (or the two bands and the shoulder) in the observed spectrum represent pairs of metastable $^1\Delta g$ molecules in binary collisions identical with those observed with chemically produced $^1O_2$ in an aqueous solution [21, 22].

(7) *Effects of photosensitizers on chemiluminescence.* Several dyes (each at $2 \cdot 10^{-5}$ M) were examined as possible photosensitizers of electronically excited

TABLE I

SPECTRAL PROPERTIES OF PHOTOSENSITIZERS

Sensitizer*	0-0 band	
	Excitation** (nm)	Emission** (nm)
β-Anthracene sulfonate	377–380	420
9,10-Dibromoanthracene sulfonate	403	435
Eosin	515	537
Rose-Bengal	560	575

* 2.5–5.0 μM in 0.1 M Tris · HCl buffer (pH 6.8).

** Excitation and emission spectra were taken by Hitachi Model-204 fluorescence spectrophotometer.

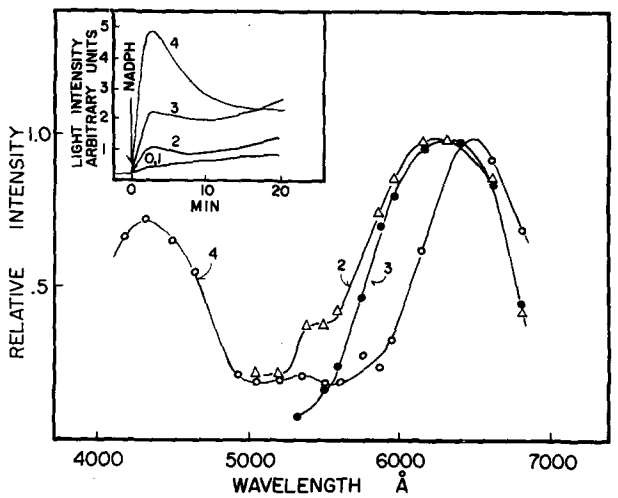


Fig. 7. Chemiluminescence enhanced by several dyes and their spectra. Four dyes (each at $2 \cdot 10^{-5}$ M), β-antharcene sulfonate (1), eosine (2), Rose-Bengal (3) and 9,10-dibromoanthracene sulfonate (4), were added to the standard incubation mixture (○). The reaction was initiated by the addition of NADPH (inset). Light intensity was measured at 18 °C by Method 2. Spectral analysis was carried out as described in Fig. 6.

species in a NADPH-dependent lipid peroxidation system. The spectral properties of the sensitizers are summarized in Table I. Light emission from the NADPH - NADPH-cytochrome *c* reductase-Fe^{3+}-ADP-EDTA-liposome system was enhanced by 9,10-dibromoanthracene sulfonate, eosin and Rose-Bengal, but not by β-anthracene sulfonate (Fig. 7, inset).

Since there is no significant difference between β-anthracene sulfonate and its bromine substituent with respect to lowest excitation energies, the difference between two anthracene analogues on activation of chemiluminescence may be attributable to the effect of heavy atoms in the molecule. As shown in Fig. 7, the emission spectrum of the system containing the dibromoanthracene analogue is quite different from that observed in the absence of this dye (Fig. 6) in that the former has two prominent peaks at 4350 Å and at or neat 6350 Å.

The 4350 and 6350 Å peaks are assigned to the excited singlet (S_1) → singlet (S_0) transition of the dibromoanthracene sulfonate molecule and 2 ($^1\Delta g$) → 2 ($^3\Sigma g^-$) transition of O_2 pairs (O, O), respectively. As shown in the same figure, a very weak emission peak (or shoulder) corresponding to $S_1 \rightarrow S_0$ transition of eosin was observed at or near 5400 Å, while that for $S_1 \rightarrow S_0$ transition of Rose-Bengal was not detectable at or near 5700 Å. In both cases the 6350 Å peak which arises from the molecular pair of $^1\Delta g$ type oxygen (O, O) is only a prominent peak in the region observed.

(8) *Does lipid peroxide involve $^1O_2$ generation?* A typical experiment which shows the time relationship among malondialdehyde concentration, lipid peroxide concentration and light intensity during NADPH oxidase activity is shown in Fig. 8.

The light intensity increased almost linearly with increasing time until lipid peroxide reached its highest concentration; it then reached its maximum while the

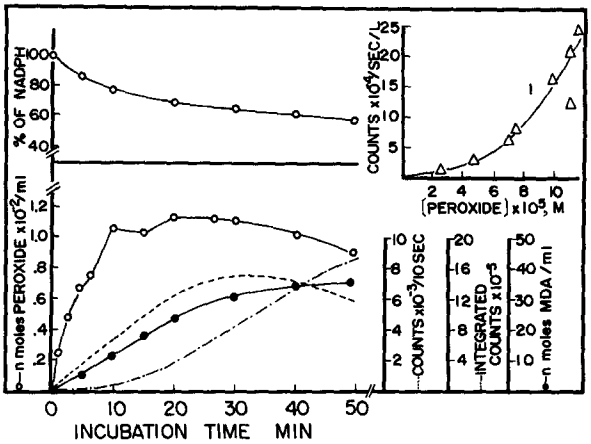


Fig. 8. Time relationship among malondialdehyde concentration, lipid peroxide concentration and light intensity during NADPH oxidase activity. The experimental system consisted of the standard reaction mixture. In the control system NADPH was omitted from the experimental system. Malondialdehyde (MDA) and lipid peroxide (as H_2O_2) in the control and the experimental systems were measured by the methods described in text. Light intensity (counts per 10 s per 3 ml) was measured by Method 1. NADPH consumption in the experimental system was monitored by fluorimetry. Each value, corrected for readings with the control, represents the average of five individual experiments in the case of lipid peroxide concentrations and three individual experiments in the case of the others. The relationship between lipid peroxide concentration and light intensity (inset) was obtained by computation of the data (0–25 min) in Fig. 7. Curve 1 is theoretical, $I = 1.5 \cdot 10^{13}$ counts $\cdot s^{-1} \cdot M^{-1}$ [peroxide]^2.

lipid peroxide concentration was maintained constant. The decrease in lipid peroxide concentration was accompanied by decreased light intensity. These suggest that the light intensity is dependent upon lipid peroxide concentration, and that lipid peroxide is a precursor of the light species ($^1O_2$). A plot of light intensity against lipid peroxide concentration, at the times cited, yielded a curve which satisfies the following equation, during approx. 30 min following the addition of NADPH (Fig. 8, inset):

$$I = K[\mathrm{ROOX}]^2 \qquad (1)$$

where I and ROOX are counts per s per l of the reaction mixture and the molar concentration of the lipid peroxide (as H_2O_2), respectively, at the times cited. From the data shown in Fig. 7 (inset), K, a constant, was found to be $1.5\ (\pm 0.08) \cdot 10^{13}$ counts $\cdot s^{-1} \cdot M^{-1}$. On the other hand, the oxidation of NADPH parallels the formation of malondialdehyde, but not the integrated light intensity. This suggests that malondialdehyde and light species ($^1O_2$) are formed in different ways and at different times during lipid peroxidation, assuming the absence of any degradation of the formed malondialdehyde.

DISCUSSION

The spectral analyses described in this report indicate that a simplified microsomal lipid peroxidation system produces at least $^1\Delta g$ type molecular oxygen identica

with that formed by a NADPH-dependent intact microsomal system [7]. Kinetic studies of the effect of β-carotene on light emission by the system also support the conclusion that the involved light species is $^1O_2$.

In contrast to the observation that $^1O_2$ (generated by photosensitization or radio frequency discharge) does cause peroxidation of liposomal lipid which is prevented by β-carotene [17, 24], β-carotene had no effect on our NADPH-dependent peroxidation. This discrepancy may be attributable to the reason that in the NADPH-dependent system lipid peroxidation proceeds by means of free radical chain mechanism which is much more dominating than the lipid peroxidation involving $^1O_2$ (produced as a by-product).

In view of the finding that the chemiluminescence in the system was markedly inhibited by radical scavengers but not affected significantly by superoxide dismutase, it seems likely that $^1O_2$ is formed by some reaction involving radical interaction, but not by the reaction: $O_2^- + O_2^- \xrightarrow{2H^+} H_2O_2 + {}^1O_2$.

An excited carbonyl compound is generally believed to be formed in the chain termination process of autoxidation of hydrocarbons, and its singlet excited state [25] or its triplet state [26] appears to emit weak light with its maximum in the range 4200–4500 Å. Even though there is no demonstrable emission band or shoulder which arises from excited carbonyl compounds in the emission spectrum of our system, the experiments with photosensitizers point to the generation of a compound in triplet state by the lipid peroxidation. The 9, 10-dibromoanthracene sulfonate, one of the sensitizers used for this purpose, can theoretically be excited to its singlet state (71 kcal/mol) by an energy level of two $^1\Sigma g^+$ (75 kcal), but this would not be significant because of the very short life of $^1\Sigma g^+$ in water [27]. Therefore, the dibromoanthracene sulfonate-mediated energy transfers are simply explained by following scheme:

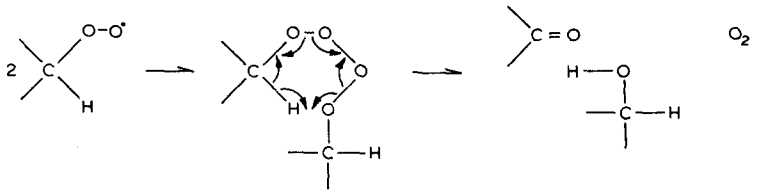
A compound (T_1^δ)+dye $(S_0^\delta) \longrightarrow$ A compound (S_0^δ)+dye (S_1^δ),
Dye $(S_1^\delta) \longrightarrow$ dye $(S_0^\delta)+h\nu$ (4350 Å),
Dye $(S_1^\delta) \longrightarrow$ dye (T_1^δ) (intersystem crossing),
Dye $(T_1^\delta)+O_2^\delta\ (T_0^\delta) \longrightarrow$ dye $(S_0^\delta)+O_2\ (S_1^\delta)$ and
$O_2^\delta(S_1^\delta)+O_2^\delta(S_1^\delta) \longrightarrow 2O_2^\delta\ (T_0^\delta)+h\nu$ (6350 Å).

The first process is forbidden by the spin conservation rule but is allowed because of the heavy bromine atoms [28, 29].

Eosine (or Rose-Bengal)-mediated energy transfers, would be essentially the same as those of dibromoanthracene sulfonate, save that $S_1 \rightarrow T_1$ transition of the dyes may occur very easily.

The $T_1 \rightarrow S_0$ transition in these dyes (phosphorescence), located at about 6750–7000 Å [30, 31], could not be detected in the present experiments because of the wavelength limitation of the colored glass filters in the filter spectral analyzer. It may be inferred therefore that the microsomal system produces both singlet oxygen and a compound in triplet state. One possible route for the generation of such electronically excited products is shown in the following scheme on the next page.

From the hypothetical cyclic intermediate either the ketone (a carbonyl compound) will be generated in a triplet state or oxygen in a singlet state (Russell's mechanism) [32, 33].



Scheme I

Linoleic acid and other unsaturated fatty acids in microsomal phospholipid should be convertible to their hydroperoxides through their peroxy radicals by a NADPH-dependent peroxidation system.

If the self-reaction of such peroxy radicals produces a cyclic transition state, the subsequent decomposition of this transition state would be expected to be exothermic by over 100 kcal/mol, judging by calculations of the heat of reaction for the decomposition of a secondary-butylperoxide dimer [33]. This should provide enough energy to form $^1\Delta g$ (22 kcal/mol over grand state oxygen) and $^1\Sigma g^+$ (37.5 kcal/mol over grand state oxygen). Alternatively, a excited species in triplet state mentioned above, probably a carbonyl compound, would also be generated by this mechanism. One of the carbonyl compounds produced during microsomal phospholipid peroxidation is the easily detected malondialdehyde. In our studies chemically and enzymatically prepared malondialdehyde in buffer absorbed light at 2650 Å, whereas it did not emit radiation in the region 3000–7000 Å at room temperature. Malondialdehyde can therefore be ruled out as a detectable emitter in the region observed during microsomal phospholipid peroxidation.

The fact that the light intensity is proportional to the square of the lipid peroxide concentration is also an important clue to solve the mechanism of $^1O_2$ generation in the lipid peroxidation system, i.e. $I = K[\mathrm{ROOX}]^2$.

Assuming that light emission observed is only produced by binary collisions of $^1O_2$ pairs, the following expression can be derived from this equation:

$$I = K' [^1O_2]^2 = K[\mathrm{ROOX}]^2$$

where K' is a constant, including fluorescent quantum yield. Judging by our recent findings that linoleic acid - 13-hydroperoxide in aqueous solution did not produce chemiluminescence unless this system contained Ce^{4+} (known to abstract hydrogen from the hydroperoxy group [34]) or Fe^{2+} (known to split to O-O conjugation in peroxides and promote their corresponding peroxy radicals by a chain reaction [35]), lipid peroxide should be convertible to its corresponding peroxy radical to produce chemiluminescence in our present system. Hence, one plausible mechanism of phospholipid peroxidation is that phospholipid radicals formed in the first stage of the reaction react with O_2 to produce lipid peroxy radicals in a myelinic structure which easily abstract hydrogen atoms from adjacent lipid molecules. In the process of phospholipid cleavage the segmented lipid hydroperoxide (ROOH) is converted under the influence of reduced iron to the "active lipid peroxy radical" (ROO ·).

Assuming that phospholipid cleavage occurs rapidly and $^1O_2$ generation follows Russell's mechanism, the relationship between ROOH (or ROO ·) and O_2 can be explained by the following reactions and equations:

$$2\,\mathrm{ROOH} \xrightarrow{k_a} \mathrm{ROO}\cdot + \mathrm{ROH} + {}^{\cdot}\mathrm{OH}$$

$$2\,\mathrm{ROO}\cdot \xrightarrow{k_b} {}^1\mathrm{O}_2 + \text{other compounds}$$

$${}^1\mathrm{O}_2 + {}^1\mathrm{O}_2 \xrightarrow{k_c} 2\,{}^3\mathrm{O}_2 + h\nu$$

$$\frac{\mathrm{d}[\mathrm{ROO}\cdot]}{\mathrm{d}t} = k_a[\mathrm{ROOH}]^2 - 2k_b[\mathrm{ROO}\cdot]^2$$

$$\frac{\mathrm{d}[{}^1\mathrm{O}_2]}{\mathrm{d}t} = 2k_b[\mathrm{ROO}\cdot]^2 - 2k_c[{}^1\mathrm{O}_2]^2$$

where k_a—k_c are rate constants. If a stationary state is obtained in an early stage of the reaction, the left hand portions of the two equations equal zero and the resulting equation ($k_a[\mathrm{ROOH}]^2 = 2k_c\,[{}^1\mathrm{O}_2]^2$) are in good agreement with that derived from the experimental results ($K[\mathrm{ROOX}]^2 = K'[{}^1\mathrm{O}_2]^2$).

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