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# The Relationship between Chemiluminescence and Lipid Peroxidation in Rat Hepatic Microsomes

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Studies were carried out to determine the relationship between NADPH- and ascorbate-initiated chemiluminescence (CL) and lipid peroxidation (LP) in rat hepatic microsomes. NADPH-initiated CL and LP become maximal 15 min after addition of NADPH to the microsomes and ascorbate-initiated CL and LP become maximal 90 to 120 min following addition of ascorbate. There are four lines of evidence to indicate that both NADPH- and ascorbate-initiated chemiluminescence are related to lipid peroxidation. (i) The time courses for the increases in CL and in LP are identical. (ii) There is a linear relationship between total (integral) or maximal CL and LP. (iii) Drug substrates which inhibit LP also inhibit CL in a quantitatively similar manner. (iv) Inhibitors of lipid peroxidation, such as $Co^{2+}$, $Mn^{2+}$, $Hg^{2+}$, *para*-chloromercuribenzenesulfonic acid, and EDTA, also inhibit chemiluminescence. The results of these experiments indicate that chemiluminescence initiated in hepatic microsomes by either NADPH or ascorbate is directly proportional to lipid peroxidation.

In 1971 Howes and Steele (1) reported that the addition of NADPH and oxygen to rat hepatic microsomes produced chemiluminescence (CL)[1] The mechanism responsible for this chemiluminescent response has yet to be determined. Since CL from a variety of tissues had been attributed to nonenzymatic oxidation of tissue lipids (2), Howes and Steele (1) studied the relationship between CL and lipid peroxidation (LP) in liver microsomes. They concluded from these experiments that CL could not arise solely from lipid peroxidation and that it must have its origin, at least in part, from other sources. They also suggested the involvement of electronically excited molecular oxygen species in the NADPH-initiated chemiluminescent response.

It is well known that lipid peroxidation can be stimulated by the addition of either NADPH or ascorbate to various tissue preparations in the presence of oxygen and either ferrous or ferric iron. Peroxidation of hepatic microsomal lipids induced by NADPH has been reported by several investigators (3–5) and it has been found to be closely associated with the microsomal electron transport chain which catalyzes oxidative drug metabolism. Both the lipid peroxidation and drug-metabolizing systems utilize the enzyme, NADPH-cytochrome *c* reductase (6, 7). Therefore, this NADPH-induced response in hepatic microsomes is sometimes referred to as enzymatic LP. Formation of lipid peroxides can also be initiated by addition of ascorbate to various tissue homogenates (8), to liver mitochondria (9), and to hepatic microsomal suspensions (3, 5). Ascorbate-induced peroxidation of lipids is unaffected by enzyme inactivation and is, therefore, referred to as nonenzymatic lipid peroxidation.

Since the origin of the NADPH-induced chemiluminescent response in hepatic microsomes is still unknown, we decided to further investigate the relationship between NADPH-induced CL and the production of malonaldehyde, a degradation product of lipid peroxides. In this communication,

[1] Abbreviations used: CL, chemiluminescence; LP, lipid peroxidation; TCA trichloroacetic acid; OD, optical density; PCMBS, *para*-chloromercuribenzenesulfonic acid; DABCO, 1,4-diazabicyclo[2,2,2]octane.



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evidence is presented to indicate that NADPH-induced chemiluminescence is directly proportional to the amount of malonaldehyde produced. In addition, we have demonstrated, for the first time, an ascorbate-induced CL in hepatic microsomes and have shown that it, too, is closely related to lipid peroxidation. A preliminary report of these results has appeared previously (10).

## MATERIALS AND METHODS

All experiments were performed with hepatic microsomes obtained from male Sprague–Dawley rats weighing 200 to 250 g (Zivic-Miller Laboratories, Pittsburgh, Pa.). The animals were sacrificed by decapitation and the livers were removed rapidly and homogenized in a solution containing 0.15 M KCl and 0.05 M Tris–HCl (pH = 7.4). Then the microsomal pellets were obtained by differential centrifugation. In some experiments microsomal enzymes were inactivated by heating the microsomal pellet at 100°C for 4 min. For all measurements of chemiluminescence and lipid peroxidation the microsomes were resuspended in 0.1 M phosphate buffer (0.081 M $K_2HPO_4$ and 0.019 M $KH_2PO_4$; pH = 7.4) at a final concentration of 1 to 2 mg microsomal protein/ml. Phosphate buffer is contaminated with sufficient iron to provide the necessary ferrous or ferric iron for LP. Prior to the start of each experiment, oxygen (100%) was bubbled through the microsomal suspension for 1 min.

For measurements of chemiluminescence, 2.5-ml aliquots of the microsomal suspension were added to dark-adapted plastic liquid scintillation vials. Inhibitors of lipid peroxidation or drug substrates, when used, were added to the vials at this time. Then the vials were placed in a water bath which was maintained at 37°C and incubated for 5 to 10 min prior to the start of the experiment. Chemiluminescence and lipid peroxidation were initiated by adding a small amount of a stock solution of either NADPH or ascorbate to each vial. CL was measured as counts per minute in the tritium channel of a Beckman Model LS-345 liquid scintillation counter (Beckman Instrument Co., Fullerton, Calif.) operated in the out-of-coincidence mode. CL was measured immediately after the addition of NADPH or ascorbate and this measurement was taken to be background. (This level of CL was no different from that obtained before addition of NADPH or ascorbate.) The vials were then returned to the water bath where the temperature was maintained at 37°C and CL was measured at various later times. The CL obtained at each time was expressed as the counts per minute obtained at that time minus the background level. All procedures for determining CL were performed in the dark.

Measurements of lipid peroxidation induced by either NADPH or ascorbate were made on the same samples from which CL was determined. LP was determined by measuring the amount of malonaldehyde formed during the incubation period according to the method of Ottolenghi. After the appropriate incubation period, the samples were placed on ice and an aliquot (0.625 ml) of 40% trichloroacetic acid (TCA) was added to each one. Then 2.5 ml of a thiobarbituric acid solution (0.67%) was added to each sample and the samples were incubated at 90°C for 20 min. After this incubation, another aliquot (0.625 ml) of 40% TCA was added and the samples were centrifuged at 30,000*g* for 15 min. The supernatants were diluted 10-fold with water and the amount of malonaldehyde in each was determined by reading the optical density at 535 nm using a Gilford Model 300-N spectrophotometer (Gilford Instrument Co., Oberlin, Ohio). The amount of malonaldehyde present in each sample was expressed either in optical density (OD) units or as nanomoles of malonaldehyde per milligram of microsomal protein. The molar extinction coefficient used to calculate the amount of malonaldehyde was $1.56 \times 10^5$ $M^{-1}$ $cm^{-1}$ (11).

## RESULTS

The time courses of the chemiluminescence resulting from the addition of three different concentrations of NADPH to rat hepatic microsomes are shown in Fig. 1. The response becomes maximal 15 to 30 min after the addition of NADPH and then gradually diminishes over the next 4 to 5 h. In general, the higher the concentration of NADPH, the longer it takes CL to become maximal and to disappear. The dose–response curve for the effect of NADPH on maximal chemiluminescence is shown in Fig. 2. CL first

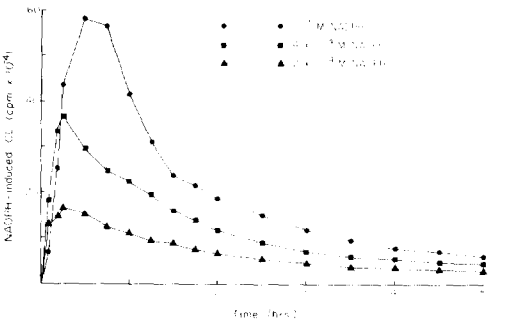

FIG. 1. Plots of NADPH-induced chemiluminescence (CL) vs time for three different NADPH concentrations. Each plot represents the data obtained from one typical experiment. Similar results were obtained from six different experiments.

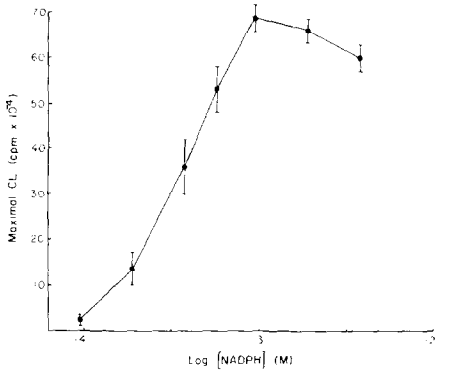


FIG. 2. Maximal, or peak, NADPH-induced chemiluminescence (CL) vs the log of the NADPH concentration. Each point is the mean value from six experiments and the bars represent the standard errors of the means.

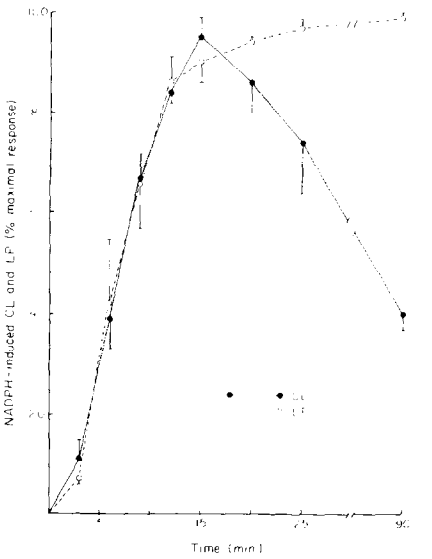


FIG. 3. NADPH (0.4 mM)-induced chemiluminescence (CL) and lipid peroxidation (LP) vs time. CL and LP (measured as malonaldehyde formation) were determined in the same samples. Each point is the mean value from six experiments and the bars represent the standard errors of the means. In these experiments maximal CL was 6.5 (±0.6) × $10^5$ cpm and maximal LP was 16.2 (±0.6) nmol malonaldehyde produced per milligram microsomal protein.

appears at a concentration of $10^{-4}$ M, becomes maximal at $10^{-3}$ M, and then is diminished at higher doses of NADPH. For the remainder of the experiments in which only one concentration of NADPH was used, that concentration was 0.4 mM, a dose which produces a response approximately 50% of maximal.

In order to study the relationship between NADPH-induced chemiluminescence and lipid peroxidation, the two responses were measured at various times in the same samples. The results are shown in Fig. 3. Both CL and LP reach maximal levels in about 15 to 25 min after addition of NADPH and the rates at which both increase appear to be identical. After 15 min, very little additional lipid peroxidation occurs and chemiluminescence diminishes slowly over the next 4 to 5 h (see Fig. 1). These results indicate that the formation of malonaldehyde in the microsomes occurs during the rising phase of CL. Once maximal CL is reached, malonaldehyde formation decreases substantially.

The relationship between either the total amount of light emitted or maximal CL and NADPH-induced lipid peroxidation was studied next. To measure total CL (integral CL), the area under the curve of CL vs time (for 5 h) was cut out and weighed. Total chemiluminescence and lipid peroxidation (measured after 5 h of incubation) were measured in the same samples at five different NADPH concentrations and the relationship between these two parameters is shown in Fig. 4A. The relationship is linear with a correlation coefficient of 0.999, indicating that total NADPH-induced CL is directly proportional to malonaldehyde formation in hepatic microsomes. A graph of maximal, or peak, CL vs LP is shown in Fig. 4B. The correlation coefficient for this plot is 0.994, indicating that maximal CL is also directly proportional to malonaldehyde formation.

The addition of ascorbate to hepatic microsomes is known to initiate lipid peroxidation (3, 5). Therefore, if chemiluminescence is related to lipid peroxidation, the addition of ascorbate to microsomes should produce CL. The CL and LP induced by 0.4 mM ascorbate, a concentration which produces maximal LP (5), are shown as a function of time in Fig. 5. As with the NADPH-induced responses, chemiluminescence and lipid peroxidation increase to

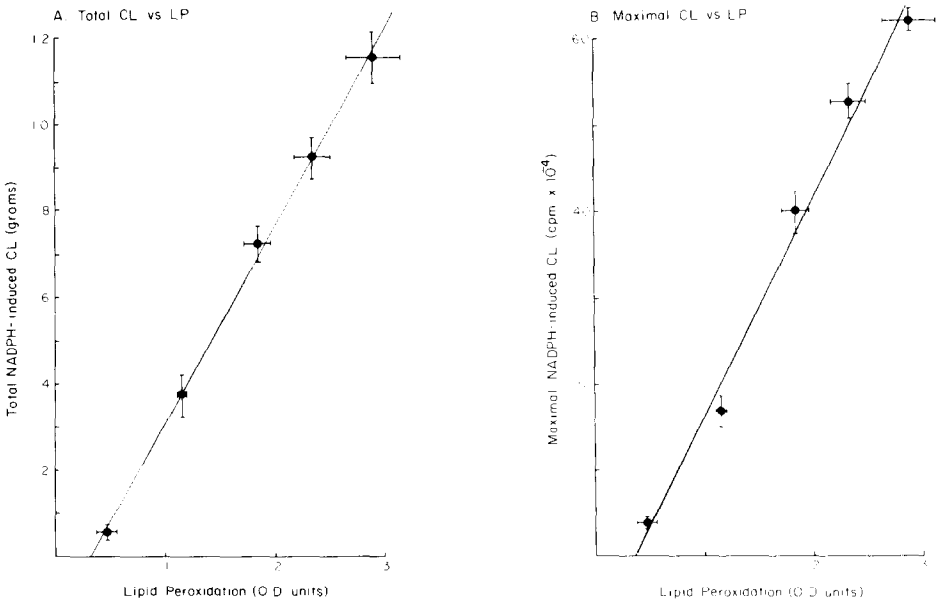


FIG. 4(A). Total NADPH-induced chemiluminescence (CL) vs lipid peroxidation (LP). These data were obtained by measuring CL and LP (as malonaldehyde formation after 5 h of incubation) in the same samples at five different NADPH concentrations: 0.1, 0.2, 0.4, 0.6, and 1 mM. Total CL was measured by plotting CL vs time (over a 5 h period), cutting out the areas under the curves, and weighing them. Each point is the mean value from six experiments and the bars represent the standard errors of the means. The line was drawn by using least-squares analysis and the correlation coefficient is 0.999 (B) Maximal, or peak, NADPH-induced chemiluminescence (CL) vs lipid peroxidation (LP). These data were obtained by measuring peak CL and LP (as malonaldehyde formation after 40 min of incubation) in the same samples at five different NADPH concentrations: 0.1, 0.2, 0.4, 0.6, and 1 mM. Each point is the mean value from six experiments and the bars represent the standard errors of the means. The line was drawn by using least-squares analysis and the correlation coefficient is 0.994.

maximal levels at about the same rates, although the ascorbate-induced responses are much slower. Maximal levels of chemiluminescence and lipid peroxidation are reached in 90 to 120 min. These results indicate that virtually all malonaldehyde production occurs during the rising phase of CL. After peak chemiluminescence is reached, very little additional malonaldehyde is formed.

The relationship between maximal, or peak, ascorbate-induced chemiluminescence (measured after 90 min of incubation) and lipid peroxidation (measured at 120 min), obtained from experiments in which seven different ascorbate concentrations were used, is shown in Fig. 6A. The correlation coefficient is 0.999 which indicates that maximal ascorbate-induced CL is related directly to the formation of malonaldehyde. Experiments were also carried out using microsomal preparations in which the enzymes were inactivated by heating. Inactivation of enzymes eliminates NADPH-induced chemiluminescence and lipid peroxidation but has no effect on ascorbate-induced CL or LP. The relationship between maximal, or peak, ascorbate-induced chemiluminescence and lipid peroxidation is shown in Fig. 6B. This relationship, with a correlation coefficient of 0.999, is very similar to the one obtained using normal microsomes. The results indicate that nonenzymatic, ascorbate-induced chemiluminescence is related directly to malonaldehyde formation in both normal and heat-treated microsomes.

Many substrates for the hepatic microsomal drug-metabolizing system have been shown to inhibit lipid peroxidation (12–15). Recently, we have shown that it is the direct antioxidant properties of the substrate molecules which cause inhibition of both NADPH- and ascorbate-induced lipid peroxidation in liver microsomes (16). Based on these previous findings and the results of our present study, it seemed likely that drug

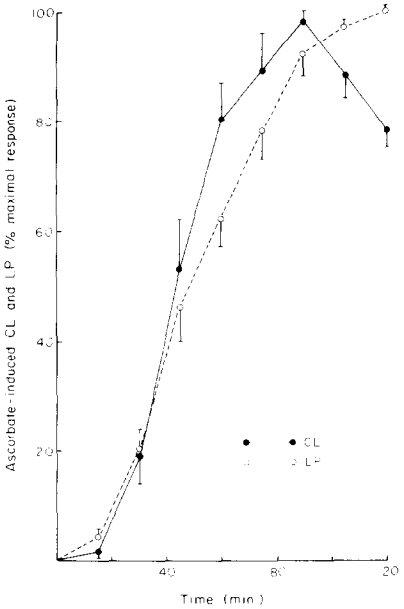


FIG. 5. Ascorbate (0.4 mM)-induced chemiluminescence (CL) and lipid peroxidation (LP) vs time. CL and LP (measured as malonaldehyde formation) were determined in the same samples. Each point is the mean value from six experiments and the bars represent the standard errors of the means. In these experiments maximal CL was 5.4(±0.2) × $10^5$ cpm and maximal LP was 15.3(±0.3) nmol malonaldehyde produced per milligram microsomal protein.

substrates should also inhibit chemiluminescence. The effect of one of these substrates, aniline, on NADPH-induced CL and LP is shown in Fig. 7. Both CL and LP appear to be affected in the same manner by aniline. Similar results were obtained with other drug substrates for both the NADPH- and ascorbate-induced responses. The data from these experiments were replotted in the form of double-reciprocal plots and the concentration of each substrate which produces one-half maximal inhibition ($K_{1/2}$) was determined. The results for four substrates, benzo(*a*)pyrene, SKF 525A, aniline, and ethylmorphine, are shown in Table I. The $K_{1/2}$ values for all substrates tested are the same for both CL and LP. Thus, these data provide further evidence that the chemiluminescent response is related to malonaldehyde formation.

Lipid peroxidation in hepatic microsomes can also be inhibited by various heavy metal ions including $Co^{2+}$, $Mn^{2+}$, and $Hg^{2+}$, by the sulfhydryl modifier, *para*-chloromercuribenzenesulfonic acid (PCMBS) (5), and by the heavy metal ion chelating agent, EDTA (17, 18). Therefore, we tested the effects of these substances on both chemiluminescence and lipid peroxidation. The results are

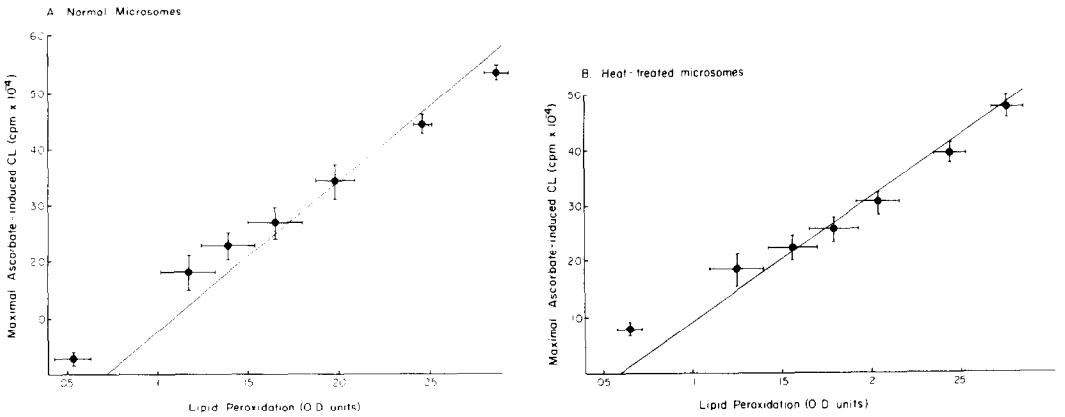


FIG. 6(A). Maximal, or peak, ascorbate-induced chemiluminescence (CL) vs lipid peroxidation (LP) in normal microsomes. These data were obtained by measuring peak CL and LP (as malonaldehyde formation after 2 h of incubation) in the same samples at seven different ascorbate concentrations: 0.01, 0.05, 0.075, 0.1, 0.15, 0.2, and 0.4 mM. Each point is the mean value from six experiments and the bars represent the standard errors of the means. The line was drawn by using least-squares analysis and the correlation coefficient is 0.999. (B) Maximal, or peak, ascorbate-induced chemiluminescence (CL) vs lipid peroxidation (LP) in heat-treated microsomes (i.e., microsomal enzymes were inactivated). Peak CL and LP (measured as malonaldehyde formation after 2 h of incubation) were measured in the same samples at seven different ascorbate concentrations: 0.01, 0.05, 0.075, 0.1, 0.15, 0.2, and 0.4 mM. Each point is the mean value from six experiments and the bars represent the standard errors of the means. The line was drawn by using least-squares analysis and the correlation coefficient is 0.993.

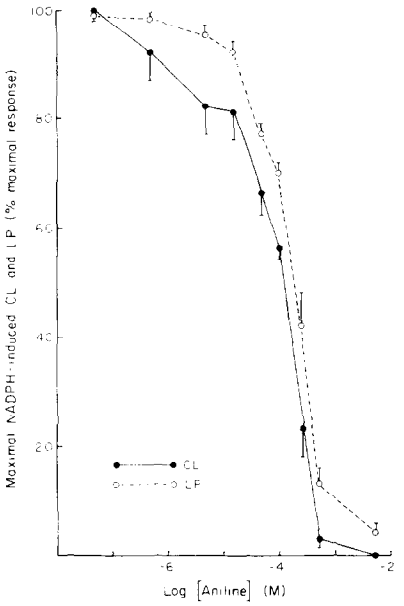


FIG. 7. The effect of various concentrations of aniline on maximal, or peak, NADPH (0.4 mM)-induced chemiluminescence (CL) and lipid peroxidation (LP) (measured as malonaldehyde formation). CL and LP were determined in the same samples. Each point is the mean value from six experiments and the bars represent the standard errors of the means. In these experiments maximal CL was 3.4(±0.6) × $10^5$ cpm and maximal LP was 7.7(±0.8) nmol malonaldehyde produced per milligram microsomal protein.

shown in Table II. All inhibitors of lipid peroxidation cause almost complete inhibiton of both NADPH-induced LP and CL. In addition, $Co^{2+}$, $Mn^{2+}$, and EDTA produce almost complete inhibition of the ascorbate-induced responses, while $Hg^{2+}$ produces partial inhibition and PCMBS has no effect. Wills (5) previously reported that PCMBS does not affect ascorbate-induced lipid peroxidation. The similarity of the effects of these inhibitors on both chemiluminescence and lipid peroxidation, whether induced by NADPH or ascorbate, provides further evidence that chemiluminescence is related to malonaldehyde formation in liver microsomes.

TABLE II

EFFECTS OF INHIBITORS OF LIPID PEROXIDATION ON CHEMILUMINESCENCE AND LIPID PEROXIDATION[a]

| Inhibitor (mM) | Inhibition (%) | | | |
|---|---|---|---|---|
| | NADPH-Induced | | Ascorbate-Induced | |
| | CL | LP | CL | LP |
| EDTA (0.2) | 100 | 97(±1) | 100 | 99(±1) |
| $Hg^{2+}$ (0.2) | 100 | 98(±1) | 74(±3) | 58(±3) |
| $Co^{2+}$ (0.2) | 100 | 97(±1) | 100 | 97 |
| $Mn^{2+}$ (0.2) | 100 | 98(±1) | 100 | 97 |
| PCMBS (0.1) | 100 | 97(±1) | 0 | 0 |

[a] All experiments were carried out using normal microsomes. Each of the values shown for percentage inhibition of chemiluminescence (CL) and lipid peroxidation (LP) is the mean value obtained from five experiments. The numbers in parentheses represent the standard errors of the means. The concentration of both NADPH and ascorbate used to initiate CL and LP was 0.4 mM.

TABLE I

$K_{1/2}$ VALUES FOR DRUG SUBSTRATE EFFECTS ON CHEMILUMINESCENCE AND LIPID PEROXIDATION[a]

| Substrate | NADPH-induced responses | | Ascorbate-induced responses | |
|---|---|---|---|---|
| | LP | CL | LP | CL |
| Aniline | 2.2(±0.2) × $10^{-4}$ | 2.0(±0.1) × $10^{-4}$ | 2.3(±0.4) × $10^{-4}$ | 1.7(±0.4) × $10^{-4}$ |
| Ethylmorphine | 1.1(±0.2) × $10^{-3}$ | 1.2(±0.1) × $10^{-3}$ | 3.4(±0.5) × $10^{-3}$ | 3.0(±0.5) × $10^{-3}$ |
| SKF 525 A | 7.7(±0.2) × $10^{-5}$ | 9.5(±1.7) × $10^{-5}$ | 8.8(±1.5) × $10^{-5}$ | 9.8(±1.0) × $10^{-5}$ |
| Benzo(*a*)pyrene | 6.4(±0.4) × $10^{-6}$ | 4.2(±0.3) × $10^{-6}$ | 14.4(±3.0) × $10^{-6}$ | 8.8(±0.6) × $10^{-6}$ |

[a] All experiments were carried out using normal microsomes. The $K_{1/2}$ values (in moles per liter) were obtained from double-reciprocal plots. Each of the values is the mean value for five or six experiments and the numbers in parentheses represent the standard errors of the means. The values obtained for LP and CL in the presence of each drug are not significantly different at the 0.05 level. The concentration of both NADPH and ascorbate used to initiate CL and LP was 0.4 mM.

## DISCUSSION

The results of these experiments indicate that the chemiluminescence initiated in hepatic microsomes by either NADPH or ascorbate is directly proportional to the formation of malonaldehyde, a degradation product of lipid peroxides. There are four lines of evidence to support this conclusion. (i) A linear relationship exists between total or maximal chemiluminescence and the amount of malonaldehyde produced. (ii) The time courses for the increases in chemiluminescence and malonaldehyde formation are identical. (iii) Drug substrates which inhibit lipid peroxidation also inhibit chemiluminescence in a quantitatively similar manner. (iv) Inhibitors of lipid peroxidation, such as $Co^{2+}$, $Mn^{2+}$, $Hg^{2+}$, PCMBS, and EDTA, also cause inhibition of the chemiluminescent response.

The results of our experiments differ somewhat from those reported previously by other investigators. Howes and Steele (1) were unable to establish a correlation between NADPH-induced chemiluminescence and malonaldehyde formation in hepatic microsomes and, therefore, concluded that CL does not arise solely from lipid peroxidation. Their conclusions were based primarily on two lines of investigation. Howes and Steele (1) studied the relationship between total NADPH-induced CL (integral CL) and LP and found that they did not correlate well. However, their experiments were carried out at 21°C and measurements appear to have been made before the CL response was maximal. Thus, they probably measured less than half the area under the total curve for CL which would not necessarily correlate with LP. In our experiments the entire chemiluminescent response which occurred over a 5-h period was measured. In addition, Howes and Steele (1) found that with increasing concentrations of microsomal protein, malonaldehyde formation increased while CL actually decreased. It is possible that the large amounts of tissue employed in their studies produced a color quenching of the chemiluminescent response. Thus, the apparent discrepancies between our results and those of Howes and Steele may be attributed largely to technical differences in experimental approach.

Although it has been established that chemiluminescence is related to the formation of malonaldehyde, the identity of the light-emitting reaction remains unknown. It is well known that various reactive forms of oxygen, such as superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl radical, can participate in light-emitting reactions in other systems, e.g., in phagocytizing polymorphonuclear leukocytes (19, 20) and alveolar macrophages (21). Furthermore, there is some evidence for the involvement of singlet oxygen in NADPH-induced microsomal lipid peroxidation (22, 23). In preliminary experiments we have studied the effects of various inhibitors of and quenchers for these reactive forms of oxygen on chemiluminescence and lipid peroxidation in hepatic microsomes. Superoxide dismutase and catalase which reduce the levels of superoxide anion and hydrogen peroxide, respectively, and the hydroxyl radical scavengers, benzoate and ethanol, have no effect on either chemiluminescence or lipid peroxidation in hepatic microsomes. However, the singlet oxygen quenchers, diphenylamine and 1,4-diazabicyclo[2,2,2] octane (DABCO), cause inhibition of both NADPH- and ascorbate-induced chemiluminescence and malonaldehyde production. Diphenylamine (1 mM) totally inhibits both reactions but DABCO (20 mM) causes only about 60% inhibition. These data support the findings of Nakano and Noguchi (23) that singlet oxygen is involved in NADPH-induced lipid peroxidation in hepatic microsomes. In addition, the data also suggest the involvement of singlet oxygen in the NADPH- and ascorbate-induced chemiluminescent responses. This latter observation is consistent with the hypothesis of Howes and Steele (1) concerning the role of an active oxygen species in NADPH-induced chemiluminescence. Thus, the close association between chemiluminescence and lipid peroxidation established by our studies can probably be attributed to the role of singlet oxygen in both processes.

## ACKNOWLEDGMENTS

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