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ON THE CHARACTERISTICS OF THE VISIBLE CHEMILUMINESCENCE FOLLOWING FREE RADICAL LIPID PEROXIDATION

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The characteristics of the visible luminescence that follows the lipid peroxidative process were investigated either in the autoxidation of rat brain homogenates or in the azo-bis-amidinopropane initiated lipid peroxidation of erythrocyte plasma membranes and liver microsomes. In these systems the luminescence decay observed after total inhibition of the lipid peroxidation is not an iron-catalyzed process, and follows a complex kinetics comprising fast and slow components. The slow component of the decay lasts for several hours at 27°C and amounts to nearly half of the total intensity measured prior to the inhibition of the oxidative process by propyl gallate. The addition of thiols (diethyldithiocarbamate, penicillamine or dithiothreitol) to a lipid peroxidizing system inhibits the chain oxidation and catalyzes the dark decomposition of one (or several) of the luminescence precursors, following first order kinetics. The effect of temperature on the slow luminescence decay corresponds to an activation energy of 18.5 kcal/mol.

KEY WORDS: Visible chemiluminescence, free radical, lipid peroxidation.

INTRODUCTION

Lipid peroxidation in simple[1,2] or complex[2] biological systems produces a significant luminescence in the visible and near UV regions. Detection of this emission has been found useful in monitoring cellular lipid peroxidation[3] and, in most systems studied, the measured luminescence correlates well with other indexes related to the extent of the peroxidative process.[4–6] Nevertheless, differences in the temporal profiles of oxygen uptake, malondialdehyde formation and low-level chemiluminescence have been observed during erythrocyte[5] and liver microsomal[4] lipid peroxidation induced either by t-butyl hydroperoxide[4,5] or by NADPH/ADP-iron.[7] In fact, these studies have pointed out that the luminescent process follows that of lipid peroxidation,[4,5,7] probably due to different pathways for malondialdehyde and electronically excited species formation.[7] In agreement with this view, significant luminescence emission is observed in rat brain homogenates undergoing autoxidation, even under conditions in which the lipid peroxidative process has been completely inhibited.[8] Although the source of the luminescence associated with lipid peroxidation was initially related to free radical processes of the chain-carrying oxy-radicals producing excited carbonyls,[9] it has been suggested that most of the emission could arise from secondary reactions of lipid

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peroxidation products.[8] In this respect, light emission produced after extensive lipid peroxidation was considered to be due to the metal-catalyzed decomposition of lipid peroxides formed at earlier time periods.[10] Furthermore, studies on the decomposition kinetics of the luminescent products produced in the autoxidation of brain homogenates have revealed that more than one intermediate must be produced in the process, with decays exhibiting a rather complex behaviour.[8] This model system comprises a variety of microphases and the luminescence decay observed after the addition of free-radical scavengers (i.e., propyl gallate; PG) or preventive antioxidants (i.e., desferrioxamine; DF) could be related to several parallel processes taking place.[6,8]

The present work extends previous studies by our group, to include the effects of temperature and sulfur compounds upon the luminescence decay produced after lipid peroxidation. Experiments were carried out in rat erythrocyte plasma membranes (EPM) or liver microsomes, model systems in which lipid peroxidation was initiated by azo-bis-amidinopropane (AAP)[11,12] in addition to the brain homogenate autoxidation system.

MATERIALS AND METHODS

Male Wistar rats weighing 250–300 g were kept on a standard pellet diet (Alimentos Balanceados S.A., Santiago) and water *ad libitum*. Blood samples were obtained by cardiac puncture using heparinized syringes on animals anesthetized with nembutal (50 mg/kg, ip), and were centrifuged at 2,300 × g for 10 min at 4°C to remove plasma and buffy coats. The cells were washed three times with cold phosphate-buffered saline (PBS) containing 150 mM NaCl and 5 mM sodium phosphate pH 8.0. Erythrocyte plasma membranes (EPM) prepared by the method of Hanahan and Ekholm[13] and devoided of hemoglobin,[14] were diluted with PBS for protein determinations[15] and peroxidation studies, at a final protein concentration of 2 mg/ml. Brain samples were homogenized (1:4) with cold phosphate-saline buffer containing 10 mM NaCl and 40 mM potassium phosphate pH 7.4, centrifuged at 1000 × g for 15 min at 4°C, and the supernatants were diluted (1:3) with phosphate-saline buffer.[6] Portions of 3 ml of dilute brain homogenate were transferred to 15 ml glass vials for luminescence studies. Liver microsomal fractions were prepared according to conventional procedures.[16] For this purpose, the livers of animals were perfused *in situ* with a solution containing 150 mM KCl and 5 mM Tris pH 7.4 to remove blood. Chopped liver samples were homogenized (1:4) in 150 mM KCl-5mM Tris pH 7.4 and, after two centrifugations at 27,000 × g for 15 min at 4°C, the postmitochondrial supernatant was centrifuged at 105,000 × g for 60 min at 4°C. The microsomal pellet was resuspended in 150 mM KCl- 5 mM Tris buffer pH 7.4 for protein measurement[15] and peroxidation studies, at a final protein concentration of 1 mg/ml.

Measurements of visible chemiluminescence were carried out in a Beckman LS-315OP liquid scintillation counter at 27°C, operated in the out-of coincidence mode.[17] All chemicals used were obtained from Sigma Chemical Co. (St. Louis, Mo, U.S.A.), except for desferrioxamine (Ciba Geigy, Chile) and 2,2'-azo-bis-(2-amidinopropane) (AAP) (Polysciences, Warrington, PA, U.S.A.).

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RESULTS AND DISCUSSION

The oxidation of EPM by AAP was used in this work to investigate the luminescence behaviour associated with lipid peroxidation, a model system which has been considered suitable to assess the peroxidative damage to biological membranes.[11] AAP thermolysis allows a controlled initiation of membrane lipid peroxidation, which can

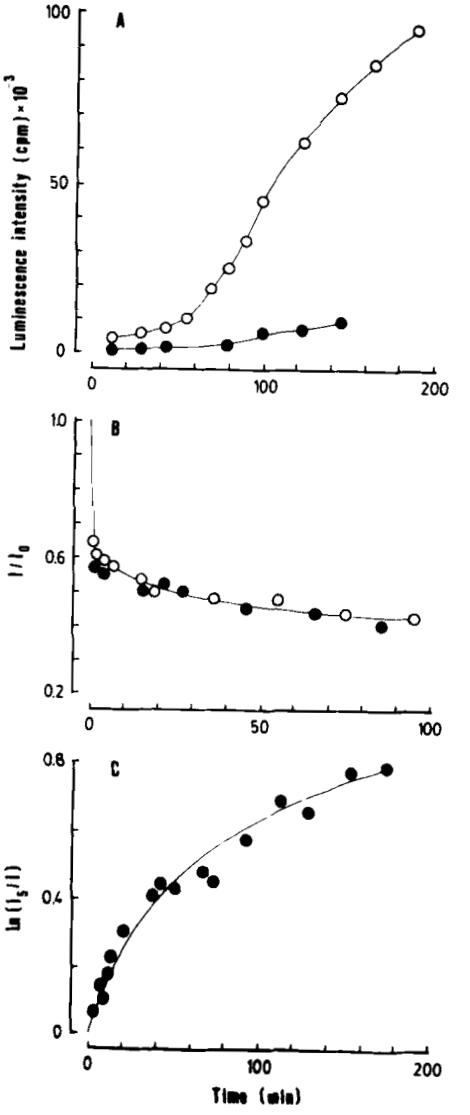

FIGURE 1 *Time course of luminescence intensity of erythrocyte plasma membrane (EPM) suspensions.* (A) following the addition of 5 mM AAP at time zero, in absence (○) and presence (●) of 10 μM propyl gallate (PG); (B) ratio of the intensity (I) at time t after PG addition (30 μM (○) or 100 μM (●) to EPM suspensions preincubated for 60 min. and the intensity prior to PG addition (Io); (C) ln (I_5/I), I_5 being the intensity after 5 min of the addition of 100 μM PG.

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be sustained for prolonged periods of time.[11,17] AAP-induced peroxidation of EPM produced significant visible luminescence that can be almost completely suppressed by the addition of 10 μM PG (Figure 1A). However, the addition of an excess PG (30 and 100 μM) to EPM samples preincubated for 60 min, partially decreased the attained luminescence (Figure 1B). In this case, the luminescence emitted from the system after PG addition showed a fast decay, followed by a considerably slower process (Figure 1B). These results are similar to those previously reported for the autoxidation of brain homogenates,[8] including the nature of the decay of the slow component which did not exhibit a simple monoexponential pattern (Figure 1C). Data summarized in Table I show that the first component of the decay has a half-life of about 10 sec and

TABLE I

Luminescence decay in the 2,2'-azo-bis-(2-amidinopropane) (AAP) initiated lipid peroxidation of erythrocyte plasma membranes (EPM) after the addition of propyl gallate (PG) to preincubated samples.*

Preincubation time (min)	Luminescence+ intensity (cpm × 10^{-3})	PG concentration (μM)	Percentage of fast decay (%)	Lifetime (sec)
95	19.5	100	40	8
100	20.3	100	27	12
115	22.3	100	35	8
121	21.1	100	35	18
134	23.0	30	35	12
138	29.0	300	34	8
188	30.6	100	27	8
194	34.1	30	36	8
204	39.0	100	40	6

* Lipid peroxidation was initiated by the addition of 5 mM AAP to EPM samples (2 mg protein/ml) at time zero and 27°C, and was allowed to proceed for different time periods after which PG was added.

+ Luminescence intensity measured prior to PG addition.

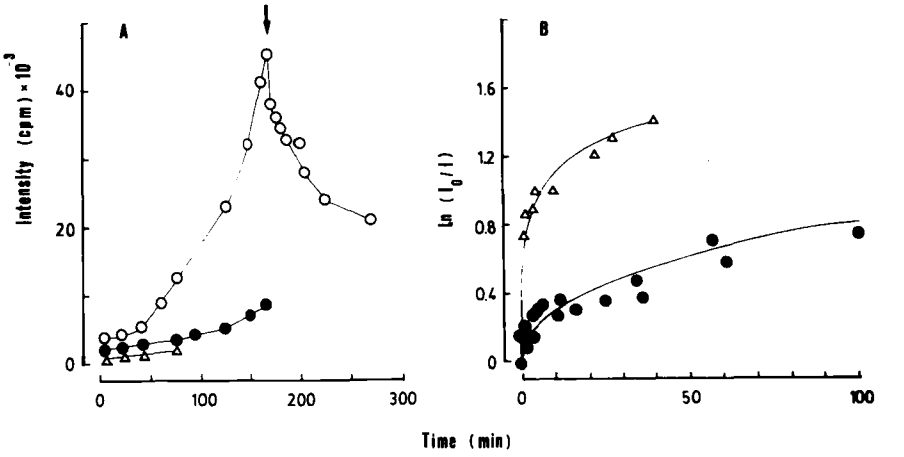

FIGURE 2 *Time course of luminescence intensity of rat liver microsomes.* (A) following the addition of 5 mM AAP at time zero, with propyl gallate (PG) added at time zero (4 μM) (●) or after 170 min preincubation (700 μM) (○) as shown by the arrow; (△) control without AAP; (B) ln (Io/I) after the addition of 700 μM PG to rat liver microsomes (●) or erythrocyte plasma membranes (△), preincubated with 5 mM AAP (Io, luminescence intensity at the time of PG addition; I, luminescence at time t after PG addition).

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that, irrespective of the preincubation time or the PG concentration, it amounts to nearly 35% of the total light intensity measured prior to P6 addition. Furthermore, the half-life of the slow decay, defined as the time required to decrease the luminescence intensity observed 5 min after the PG addition to 50%, was about 100 min, without a clear dependence on the initial intensity.

The luminescent responses induced by AAP in liver microsomes in the absence of added NADPH (Figure 2) show features similar to those observed in EPM (Figure 1). In fact, 4 μM PG added together with AAP at time zero drastically diminished the light intensity found in the absence of the free radical scavenger, while the addition of an excess concentration (700 μM) of PG to samples preincubated for 170 min decreased the luminescence in a complex manner (Figure 2A). Nevertheless, the luminescence decay seen in the microsome-AAP system is considerably slower than that observed in the EPM-AAP system under similar conditions (Figure 2B).

The results obtained both in EPM and liver microsomes presented in this work, together with those previously reported for the autoxidation of brain homogenates,[6,8] suggest that most of the visible luminescence induced seems to be due to a non-radical process that takes place with a complex time-course. This could indicate the contribution of several luminescent intermediates, whose decay is dependent on the characteristics of the peroxidizing system. Also, the light emitted after quenching of peroxidation is not an iron-catalyzed process, as light intensity is not significantly diminished when desferrioxamine (60 μM) is added to an EPM sample previously oxidized with 5 mM AAP for 190 min, or to a sample in which 500 μM PG has been added at 60 min preincubation (data not shown). This is in agreement with the fact that similar decay profiles are observed when PG or desferrioxamine are added to the brain homogenate autoxidation system.[8]

Effect of additives upon the kinetics of the luminescence decay

It has been reported that several additives can promote changes in the luminescence associated with the decomposition of the intermediates produced in the rat brain homogenate autoxidation system.[6] The evaluation of the capacity of a given additive to modify the luminescent behaviour of the intermediates is of relevance in order to relate its effects upon the luminescence to its antioxidant action.[4-6] This can be assessed by the analysis of the profiles of the luminescence decay after the addition of a high antioxidant concentration, in excess to that needed to completely inhibit lipid peroxidation. For typical antioxidants, these profiles must be similar and independent of the concentrations used, as is the case of propyl gallate and desferrioxamine.[8]

This experimental approach was applied to the brain homogenate autoxidation system under the influence of different thiols known to play a significant role in protecting cells against oxidative damage and electrophile attack by reactive chemicals.[8] In these conditions, the Q_2^1 values (i.e. the thiol concentration needed to reduce the integrated emission over a 60 min period to a value equal to 50% of that observed in the absence of additives) were found to be 1.4, 60 and 400 μM for diethyldithiocarbamate (DDC), penicillamine and dithiothreitol, respectively. These results are in agreement with studies performed in a microsomal lipid peroxidation system[19] and point out to DDC as one of the most effective thiol antioxidants. Furthermore, the luminescence profiles found for DDC (Figure 3A), penicillamine and dithiothreitol (data not shown) indicate that the light intensity decay depends on the thiol con-

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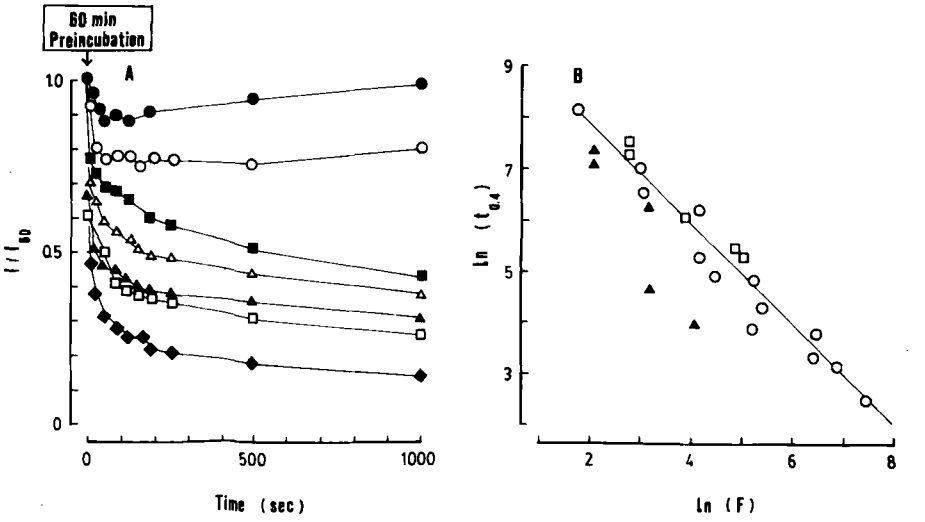

FIGURE 3 *Plots of I/I_{60} versus time after the addition of diethyldithiocarbamate (DDC) to rat brain homogenates* preincubation for 60 min; final concentrations of DDC: 2.7 μM (●). 13 μM (○), 27 μM (■), 90 μM (△), 130 μM (▲). 270 μM (□) and 1.3 mM (◆); I_{60} and I corresponds to light intensities before and after DDC addition, respectively; (B) ln ($t_{0.4}$), i.e., the time required to reach a value of I/Io = 0.4 after the addition of different thiols to rat brain homogenates, as a function of ln (F), defined in the text; concentration ranges used were, 8 μM to 2.8 mM for DDC (○), 0.8 to 10 mM for dithiothreitol (▲) and 3.2 to 25 mM for penicillamine (□).

centrations, even when added in excess to their $Q_{\frac{1}{2}}$ values. The obtained data were expressed as the time ($t_{0.4}$) required to reach a I/Io = 0.4 and were analyzed as a function of the excess thiol concentration defined as $F = Q/Q_{\frac{1}{2}}$ (where Q is the concentration of the added thiol) (Figure 3B). It can be seen that, contrary to PG or desferrioxamine which present a decay profile that is independent of F over two orders of magnitude,[8] the values of $\ln(t_{0.4})$ for the thiols studied were linearly correlated to ln(F), with a slope close to one (Figure 3B). Also, Figure 3B shows that at a given F value, DDC, penicillamine and dithiothreitol exhibited similar values of $t_{0.4}$. Thus, the luminescence decay elicited by thiol addition to a lipid peroxidating system seems to involve (a) a trapping mechanism in which the thiol interacts with the precursors of the luminescence species with first order kinetics that can be represented by dI/dt = kQ, and (b) and interaction mechanism determined by the same factors that control the thiol reactivity towards the chain-carrying radicals,[20] probably comprising an electron transfer from the thiol to the precursor of the emissive species that produces its non-radiative decomposition. This interaction must occur with the slow decaying intermediates, since the addition of DDC to a sample to which an excess PG was previously added produces a significant decrease in the luminescence intensity (Figure 4).

It is important to point out that since thiols can interact with the precursors of the light-emitting species from which most of the luminescence associated with lipid peroxidation is produced, caution must be taken in evaluating the effects of these compounds on lipid peroxidation processes assessed by luminescent procedures.

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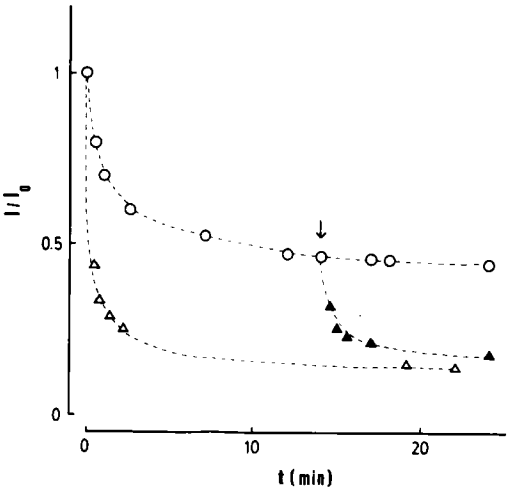

FIGURE 4 *Time course of the luminescence decay of brain homogenate samples.* Preincubation for 60 min, after the addition of 2.5 mM propyl gallate (PG) (○) or 1.3 mM diethyldithiocarbamate (DDC) (△). The arrow indicates the time at which 1.3 mM DDC was added to the system containing PG (▲).

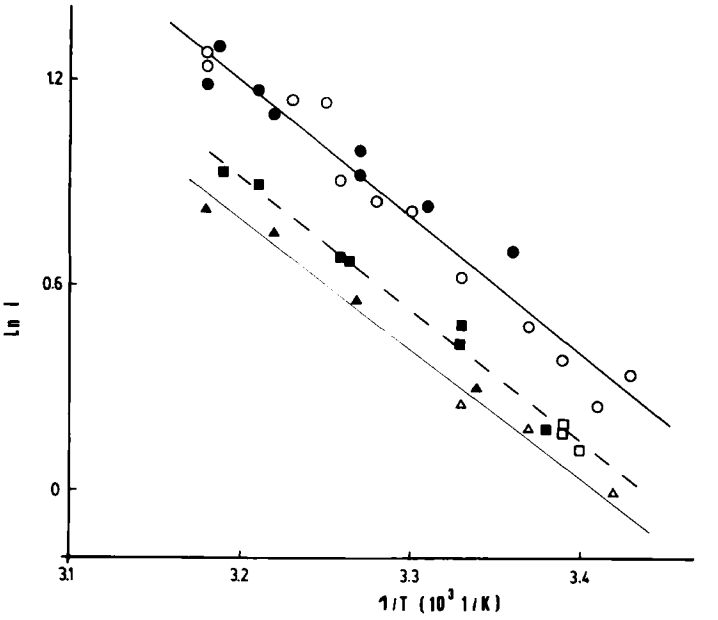

FIGURE 5 *Influence of temperature on the luminescence intensity of preincubated brain homogenate samples.* Samples were preincubated for 140, 70 or 50 min in experiments carried out at 22 (□,■), 27 (△,▲) or 40 (○,●) °C, respectively; then 300 μM propyl gallate was added to completely suppress lipid peroxidation. The open and closed symbols correspond to intensities measured when the samples were cooled or heated, respectively.

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Temperature dependence of the emitted light intensity

The influence of temperature on the light intensity emitted after lipid peroxidation was studied in brain homogenates samples preincubated at 22, 27 and 40°C for different times, to reach comparable light intensities. At these times, an excess PG (300 μM) was added. After 10 min of PG addition, the temperature of the samples was rapidly changed and the light intensity was measured. Heating and cooling cycles were repeated in order to detect any hysteresis, either real or artifactual as a consequence of an incomplete thermal equilibration or sample deterioration. Data presented in Figure 5 show that no significant hysteresis is present and that, irrespective of the preincubation temperature, the luminescence intensity shows an Arrhenius-type behaviour, with an activation energy of 18.5 kcal/mol. The rather high value obtained for the activation energy is not incompatible with that expected for the uni (or pseudo uni-) molecular decomposition of one (or several) dioxetane-like intermediate.[21]

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