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LUMINOL LUMINESCENCE INDUCED BY 2,2′-AZO-bis(2-AMIDINOPROPANE) THERMOLYSIS

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2,2′-Azo-bis(2-amidinopropane) thermolysis induces luminol luminescence. The luminescence intensity is quenched by SOD, catalase, Trolox and human blood serum. However, the time course of the light intensity profile is different for the different additives. In particular, the quenching efficiency of Trolox and human blood serum decreases with time after addition. Double quenching experiments show that SOD and Trolox are not competitive quenchers, while a simple competition can be established between Trolox and human blood serum in trapping a common intermediate. From the kinetic analysis of the data it is concluded that, at least at low additive concentrations, Trolox scavenges a luminol derived radical. Higher concentrations of Trolox or human blood serum produce induction times that are proportional to the additives concentrations. The possibility of employing luminol luminescence in the evaluation of TRAP levels and the capacity of biological samples to scavenge free radicals is discussed.

KEY WORDS: 2,2′-Azo-bis(2-amidinopropane), luminol, superoxide dismutase, Trolox, catalase, human blood serum, antioxidant capacity.

INTRODUCTION

The luminescence of luminol can be induced by free radical and/or redox processes. Advantage has been taken of the simplicity and sensitivity of the method to develop analytical procedures to evaluate the role of different active oxygen species and the specificity and efficiency of different oxygen radical scavengers. In particular, the effect of different additives upon the luminol chemiluminescence induced by superoxide,[1–5] hydroxyl, alkoxyl and hydroperoxyl radicals,[3] and singlet oxygen[3] has been studied. These data show that a certain degree of specificity exists in relation to the efficiency of a given additive, depending on the active oxygen species that is primarily produced in the system. However, a kinetic analysis of the data obtained in the HRP-luminol system has indicated that more than one precursor of the luminescence can be trapped, and double quenching experiments were proposed as a tool to determine the steps involved in the luminescence quenching by a given additive.[5]

2,2′-Azo-bis(2-amidinopropane) (ABAP) has been extensively employed as a thermal, clean and controllable source of alkylperoxyl free radicals,[6–9] and quantitative tests have been developed to evaluate the total antioxidant capacity of biological samples based on induction times measured in oxidation processes initiated by its decomposition.[10,11] In the present communication we report data obtained

by measuring the luminol luminescence induced by ABAP under different conditions, and discuss the effect of SOD, catalase and Trolox addition. This study was carried out with the aim of determining the mechanism of the ABAP induced luminescence, and to test if the emitted luminescence could be employed as a simple indicator in the evaluation of TRAP levels in biological samples.

MATERIALS AND METHODS

Luminol, catalase from bovine liver (58,000 Units/mg protein), superoxide dismutase (SOD) from bovine blood (2,800 Units/mg protein) and glycine were from Sigma, St. Louis, MO, USA. 2,2'-Azo-bis(2-amidinopropane) (ABAP) was from Polysciences, Warrington, PA. Trolox was from Aldrich Chem. Co. Catalase and SOD were denatured by heating their solutions at 100°C for 10 minutes. Human blood was obtained from 10 normal volunteers by venipuncture. Serum was obtained by centrifugation and kept frozen until its use. Since serum samples were evaluated in less than a week after preparation, it can be considered that they maintain most of their antioxidant capacity.[12,13]

Chemiluminescence measurements

Room temperature (25°C) determinations were carried out with a LKB Wallac 1250 luminometer coupled to a LKB 2210 two channel recorder. Measurements at 30 ± 0.2°C were performed in thermostatized Pyrex cells of 10 mm diameter, placed in front of an EMI 9502-S photomultiplier (power supply, KEYTLEY model 247).

The experiments were performed in freshly prepared ABAP solutions dissolved in 0.1 M glycine buffer, pH = 8.6, to which luminol at the indicated concentrations was added from a concentrated (10 mM) stock solution.

RESULTS

The time profile of the luminescence emitted when luminol is added to an ABAP solution is shown in Figure 1. Similar light profiles were observed when ABAP was dissolved in 0.1 M phosphate buffer, pH 7.4, but the intensity of the signal was considerably smaller, as expected from the pH dependence of luminol chemiluminescence intensity.[1,2,14] The data given in Figure 1 indicate that the luminescence intensity rapidly reaches a maximum value, remaining thereafter almost constant for several minutes. The dependence of the steady state luminescence intensity with ABAP concentration (luminol 100 μM) and with luminol concentration (ABAP = 10 mM) is given in Figures 2 and 3.

The steady state luminescence intensity is decreased by SOD, catalase and/or Trolox addition, both if the compounds are added prior or after the luminol. However the time courses of the luminescence after SOD and Trolox addition are different. In particular, the addition of Trolox produces a transient diminution that can be related to its consumption (Figure 4). If the initial Trolox concentration is high enough to suppress almost totally the emitted luminescence, the light profile allows the evaluation of an induction time, defined as indicated in Figure 4. The dependence of the induction time upon Trolox concentration is shown in Figure 5. These data

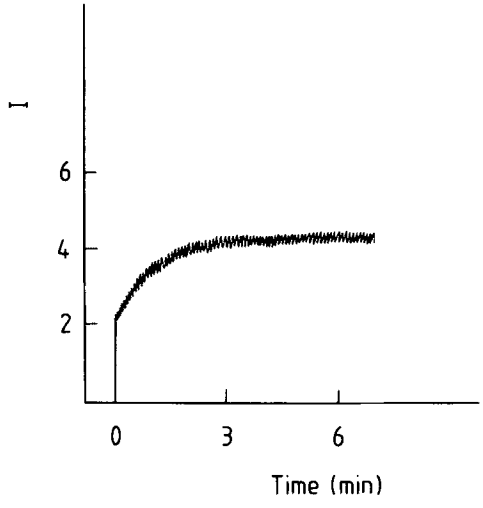


FIGURE 1 Time profile of luminol (100 μM) luminescence induced by ABAP (10 mM) at 25°C. Light intensity measured in relative units.

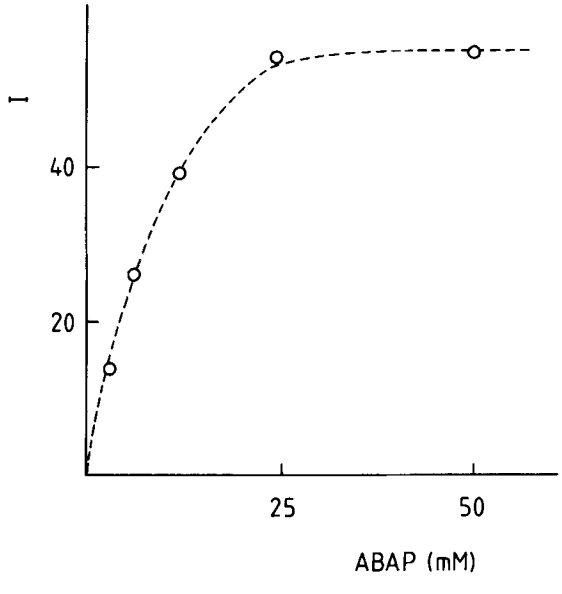


FIGURE 2 Dependence of the steady state luminol luminescence intensity with ABAP concentration. Luminol: 100 μM; temperature: 25°C.

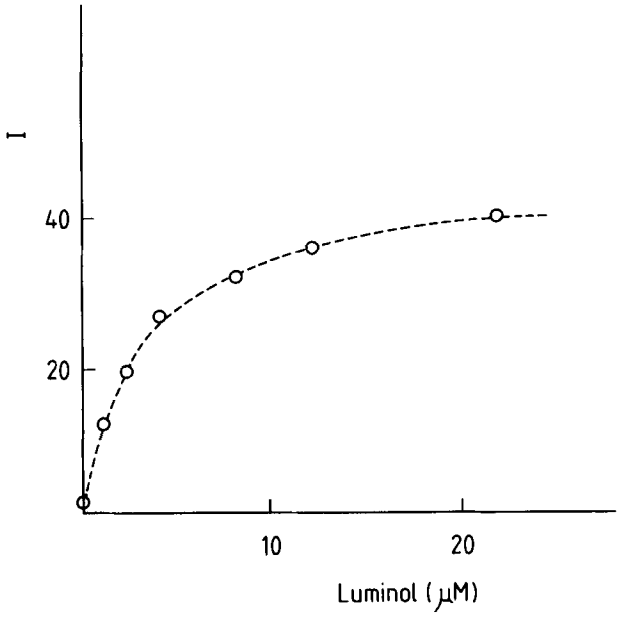


FIGURE 3 Dependence of the steady state luminol luminescence intensity with luminol concentration. ABAP: 10 mM; temperature: 25°C.

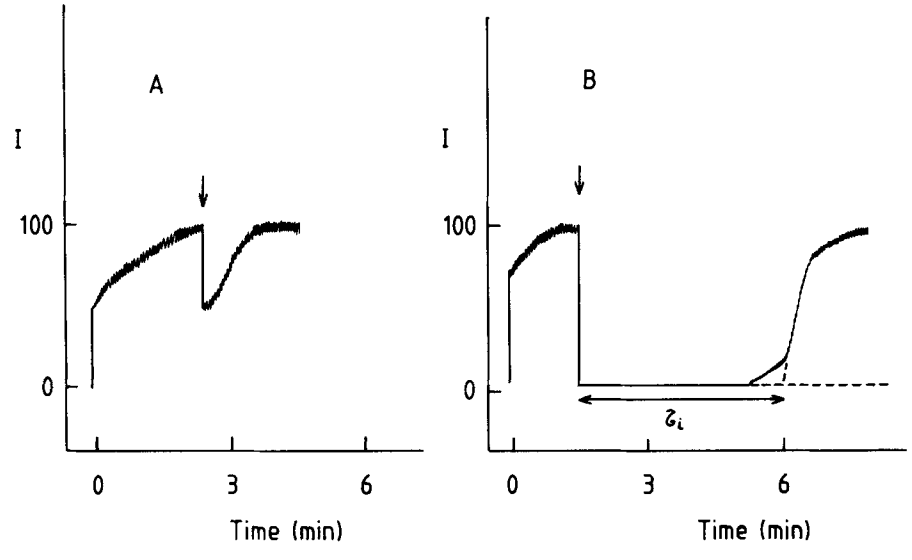


FIGURE 4 Time profile of luminol luminescence intensity after Trolox addition. Luminol: 100 μM; ABAP: 12.5 mM; temperature: 30°C. Trolox addition is indicated by an arrow. Added Trolox concentration: (A) 10 nM; (B) 400 nM. Induction time evaluated by extrapolation to zero light intensity the maximum slope of Trolox consumption.

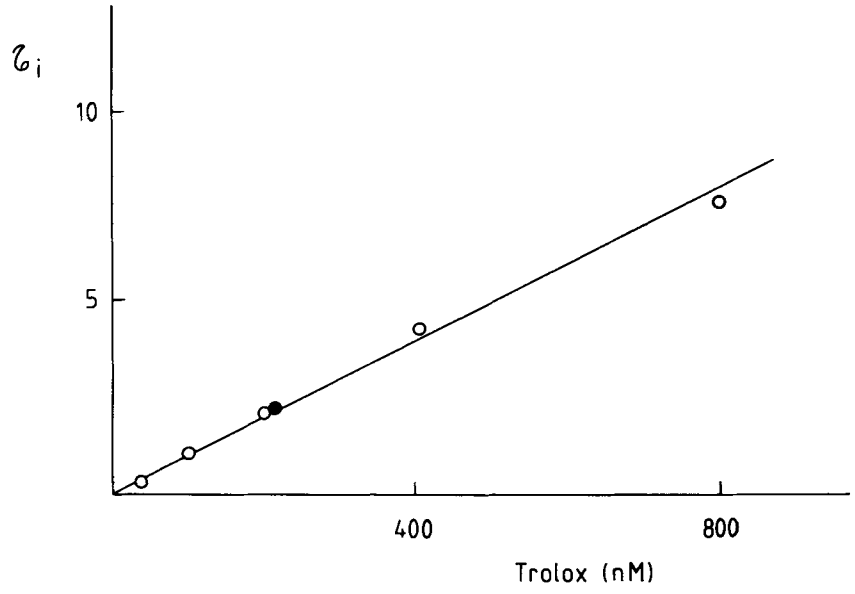


FIGURE 5 Induction time induced by Trolox addition, evaluated as indicated in Figure 4, as a function of added Trolox concentration. Luminol: 100 μM; ABAP: 10 mM; temperature: 30°C. (●) In presence of SOD (0.24μg/ml, 0.67 U/ml).

show a linear relationship between induction time and Trolox concentration, with a slope that yields a Trolox rate of consumption of 3.3 nM/sec (ABAP 12.5 mM, 30°C). This rate of Trolox consumption is in agreement with that reported in other systems in which ABAP has been employed as free radical sources.[6–9]

The extent of luminescence quenching produced by low Trolox concentrations can be determined, particularly at low temperatures and ABAP concentrations (to decrease the rate of Trolox consumption) from the light intensity recorded immediately after the quencher addition. The data obtained, plotted as Io/I against the Trolox concentration, are shown in Figure 6. From these data, a $Q_{\frac{1}{2}}$ value (defined as the quencher concentration needed to reduce to one half the luminescence intensity) of 17 nM can be derived.

The addition of SOD instantaneously produces a permanent decrease in luminescence intensity (Figure 7) which can be explained in terms of the SOD catalyzed superoxide dismutation. The dependence of the emitted luminol luminescence with SOD concentration is given in Figure 7B. On the other hand, significant amounts of denatured SOD barely modify the emitted luminescence (Figure 7).

The data obtained in the present work show that Trolox and SOD are not competitive scavengers. This conclusion is based on the lack of effect of SOD addition upon the efficiency of luminescence quenching by Trolox (Figure 5) and the induction time elicited by this additive (Figure 5). In agreement with this conclusion, the rate of Trolox consumption (as evaluated by the luminescence increase after the induction time) is also independent of SOD addition (data not shown).

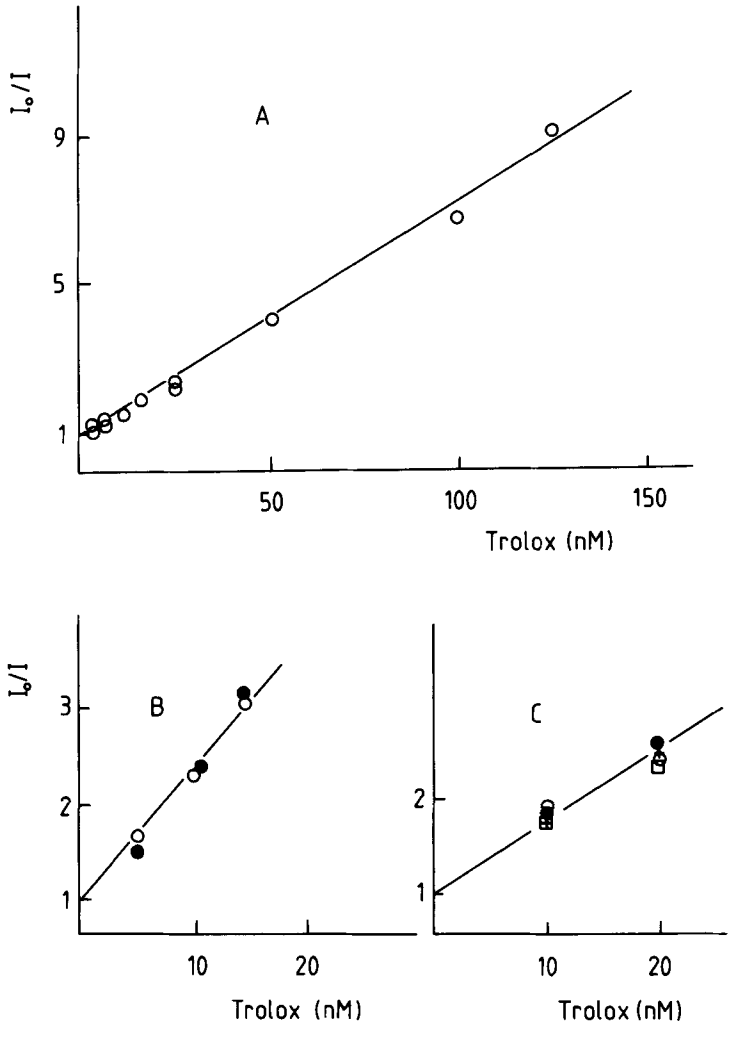


FIGURE 6 Plot of Io/I, where Io stands for the light intensity in the absence of additive, as a function of Trolox concentration. (A) ABAP 10 mM; luminol 12.5 μM; temperature 25°C. Figure 6B shows the effect of added SOD upon the luminescence quenching by Trolox. ABAP: 12.5 mM; luminol: 100 μM; temperature 30°C. (○) Without added SOD; (●) in the presence of an amount of SOD (0.24 μg/ml, 0.67 U/ml) that reduces Io by 38%. Figure 6C shows the effect of luminol concentration upon the luminescence quenching by Trolox. ABAP: 12.5 mM; temperature: 30°C; luminol: (○) 6.25 μM; (●) 12.5 μM; (□) 25 μM.

The effect of catalase addition upon the luminol intensity is shown in Figure 8. Figure 8C shows that the effect of small amounts of catalase increases with time after its addition. The effect observed can then be related to the time required to destroy the accumulated hydrogen peroxide. Figure 8E shows that further addition of catalase does not modify the remaining (ca. 35%) luminescence, indicating that only a fraction of the measured light intensity is mediated by hydrogen peroxide.[15] Furthermore,

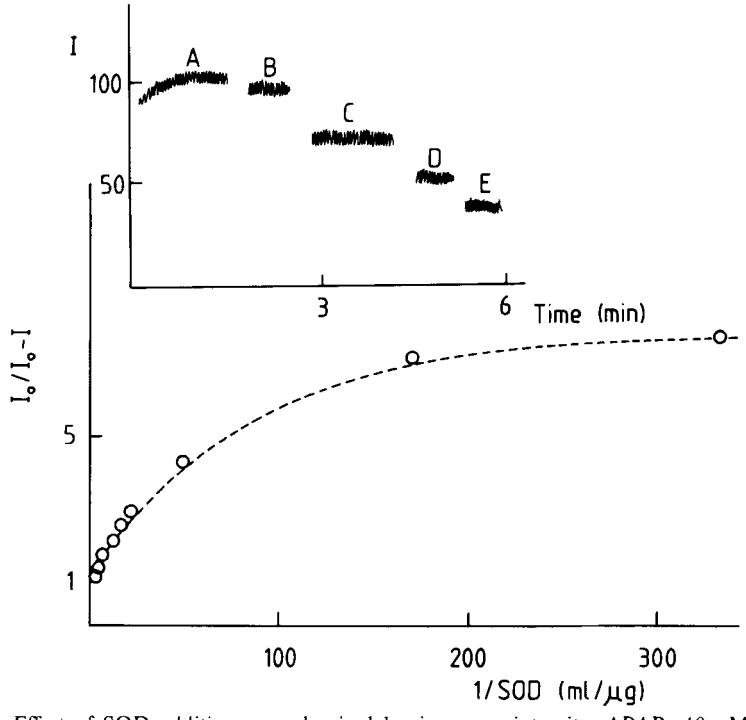


FIGURE 7 Effect of SOD addition upon luminol luminescence intensity. ABAP: 10 mM; luminol: 12.5 μM; temperature: 30°C. Insert shows the light profile after successive SOD additions: (A) in absence of SOD; (B) after addition of 60 ng/ml of heat inactivated SOD; (C) after addition of 60 ng/ml (0.168 U/ml) SOD; (D) after further addition of SOD (180 ng/ml, 0.5 U/ml); (E) after SOD addition (final concentration of active SOD; 540 ng/ml, 1.5 U/ml). In the figure, [Io/(Io − I)] values are plotted as a function of the inverse SOD concentration.

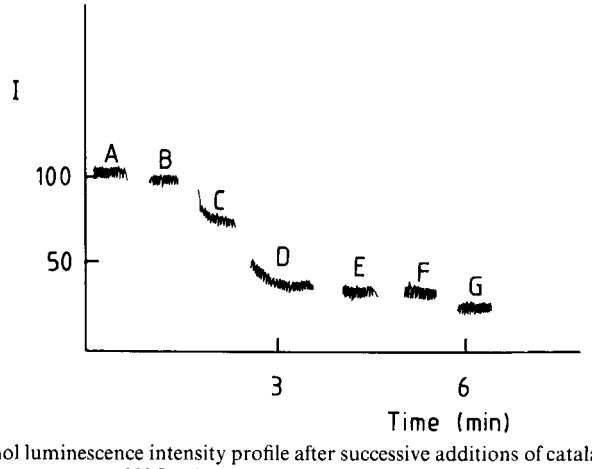


FIGURE 8 Luminol luminescence intensity profile after successive additions of catalase. ABAP: 10 mM; luminol: 12.5 μM; temperature: 30°C. (A) In absence of catalase; (B) after addition of 290 U of heat inactivated catalase; (C) after addition of 15 U of active catalase; (D) after further addition of 43 U of catalase; (E) after addition of 580 U of active catalase; (F) after addition of SOD (0.017 U/ml); (G) after addition of SOD (0.19 U/ml).

the data show that denatured catalase is totally ineffective in quenching the emitted luminescence (Figure 8).

In order to evaluate the possibility of employing the ABAP-luminol system in the evaluation of TRAP levels and the antioxidant capacity of biological fluids, we have carried out measurements in the presence of human blood serum. Small amounts of serum produce an instantaneous luminescence decrease that is directly related to the serum concentration. A plot of Io/I against the final serum concentration is given in Figure 9. These data show that a simple relationship exists between light intensity and serum concentration, compatible with the trapping of a single free radical intermediate. Furthermore, the data allow an evaluation of $Q_{\frac{1}{2}}$, 0.072 ml/dm^3.

Double quenching experiments were performed in order to test if serum and Trolox are competitive inhibitors. The data obtained after serum addition to a sample without Trolox and to a sample with 15 nM added Trolox are given in Figure 10. These data show that the effect of serum is smaller in the presence of added Trolox. Furthermore, the decrease in slope (a factor 2.3) almost matched the decrease in light intensity elicited by the addition of Trolox. These data indicate that Trolox and serum are competitive inhibitors acting upon the same free radical intermediate.[5]

Higher serum concentrations totally suppress the luminescence. The time course of the luminescence intensity is shown in Figure 11. The behaviour observed is similar to that elicited by Trolox addition (Figures 6 and 11) and the induction times produced are almost additive. A comparison of the induction times produced by Trolox and serum addition indicate that the TRAP of the employed serum corresponds to that of a 400 μM Trolox solution. This value is almost equal to the

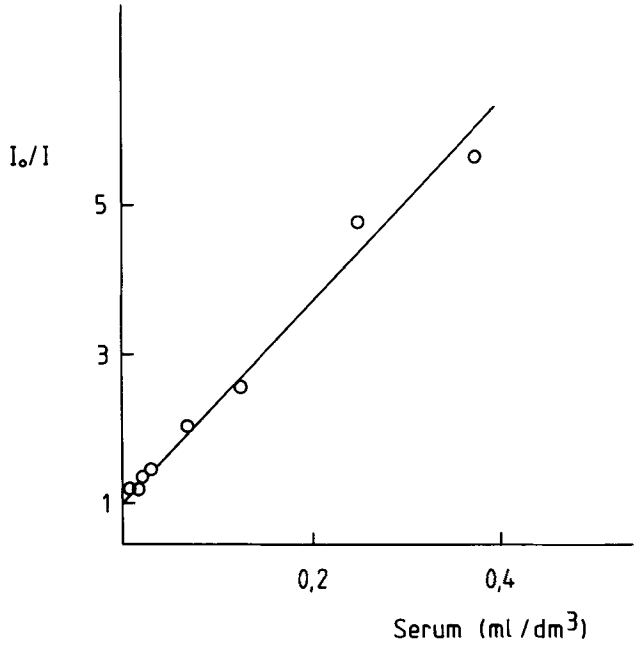


FIGURE 9 Effect of human blood serum upon luminol luminescence intensity. ABAP: 10 mM; luminol: 12.5 μM; temperature: 25°C. Data are plotted as Io/I as a function of amount of serum added.

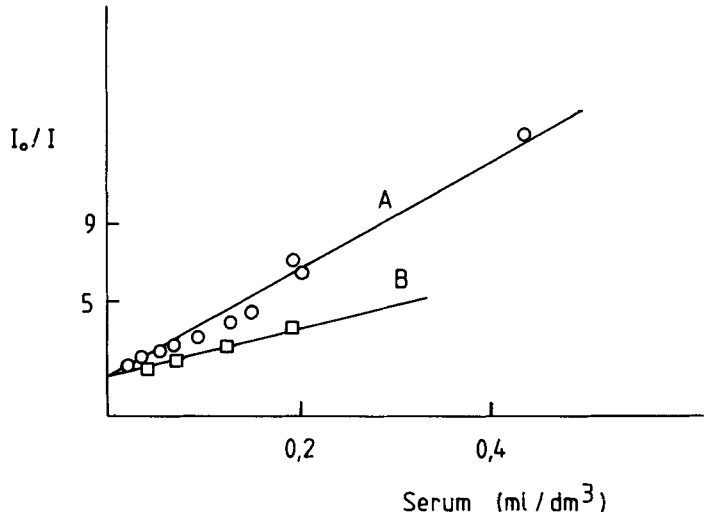


FIGURE 10 Effect of partial quenching by Trolox upon the effect of human blood serum upon luminol luminescence intensity. ABAP: 10 mM; luminol: 12.5 μM; temperature: 25°C. (A) In absence of Trolox; (B) in presence of 15 nM Trolox. This amount of Trolox reduced luminol luminescence to 40% of the initial value.

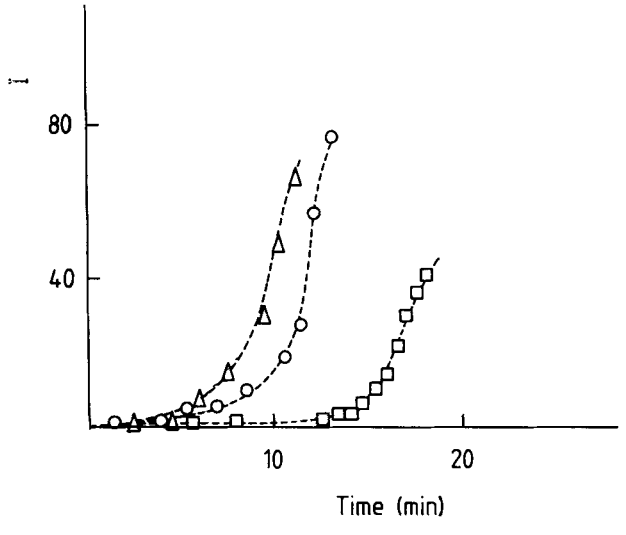


FIGURE 11 Induction time elicited by a sample from a pool of human blood serum. ABAP: 25 mM; luminol: 12.5 μM; temperature: 25°C. The data show the time course of the luminescence intensity in the presence of: (○) 1.25 μM Trolox; (△) 2.5 ml/dm^3 human blood serum; (□) in the presence of Trolox (1.25 μM) and human blood serum (2.5 ml/dm^3).

average value reported for human plasma as evaluated by the classical TRAP procedure.[13,14]

DISCUSSION

ABAP thermolysis in the presence of oxygen can be interpreted in terms of reactions 1 and 2:

$$ABAP \rightarrow 2R\cdot + N_2 \quad (1)$$

$$R\cdot + O_2 \rightarrow ROO\cdot \quad (2)$$

Considering the low concentration of luminol employed in the present work and the high rate generally observed for the addition of oxygen to alkyl radicals,[18,19] it can be considered that reaction (2) takes place quantitatively even in the presence of luminol. The produced peroxyl radicals can be considered the active species that interacts with luminol.

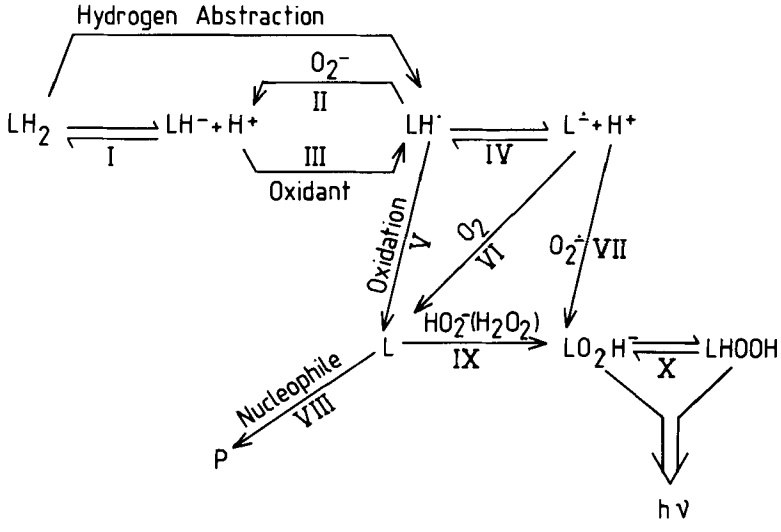
A general mechanism proposed for the luminol (LH_2) luminescence induced by free radical and/or redox processes is given in Scheme 1 [Ref. 15]. The data given in Figures 1 and 2 can be readily explained in terms of the reactions included in Scheme 1. The small time required to reach the maximum intensity could be related to the times required to reach steady state concentrations of superoxide and hydrogen peroxide and/or concentrations high enough to quantitatively trap luminol derived ($LH\cdot$ and $L^{\cdot-}$) radicals, avoiding its back disproportionation to luminol. In this regard it is interesting to point out that the time required to achieve the steady state condition increases notably when the ABAP concentration decreases (data not shown). The lack of dependence of the emitted luminescence upon the luminol concentration observed at high luminol concentrations can be interpreted in terms of the quantitative occurrence of reaction (3)

$$ROO\cdot + LH_2 \rightarrow ROOH + LH\cdot \quad (3)$$

at high luminol concentration. On the other hand, the data of Figure 2 are more difficult to interpret. In the present system, the near independence of the emitted light intensity upon ABAP concentration observed at very high (50 mM) concentration could indicate interference of the azo-compound with the light emitting species and/or its precursors, i.e. by acting as a nucleophile in reaction VIII of Scheme 1. Similar dependences upon the concentration of the additive leading to the production of the active oxygen species have been reported in other systems, but the behaviour was not explained.[3]

After formation of the key intermediate $LH\cdot$ and/or its deprotonated form ($L^{\cdot-}$), the production of the luminescence precursors LO_2H^- and LHO_2H involves either a superoxide mediated process (Reaction VII) or an hydrogen peroxide dependent process (Reaction IX). The luminescence is accordingly decreased by SOD and or catalase addition (see Figures 7 and 8) independently of the species that is initially interacting with luminol (the $ROO\cdot$ in the present work).

Catalase addition in the 87 U to 1160 U range quenches 65% of the emitted luminescence, suggesting that this fraction of the light arises from intermediates generated through reaction IX of Scheme 1. On the other hand, the effect elicited by SOD appears to be more complex (Figure 7). The addition of small concentrations (20 ng/ml) of SOD produces a moderate (ca. 20%) decrease in luminescence. Further



SCHEME 1.

increase in SOD concentrations leads to a slower decrease in the luminescence intensity, suggesting a dual role for SOD. This behaviour contrasts with that observed in superoxide initiated luminol enhanced luminescence,[5] where Io/I plots are linear with $Q_{\frac{1}{2}}$ values of ca. 4 ng/ml (where $Q_{\frac{1}{2}}$ represents the additive concentration needed to decrease to one half the emitted luminescence). Furthermore, it is noticeable that addition of catalase (final concentration 67 ng/ml, 580 U/ml) and SOD (final concentration 67 ng/ml, 0.2 U/ml) simultaneously does not quench totally the emitted luminescence (Figure 8), suggesting that a reaction path additional to those shown in Scheme 1 might contribute to the luminol luminescence.

In the present system, the effect of a free radical scavenger upon the luminescence can be due to trapping of superoxide, the ROO· radicals and or the luminol derived radicals (LH· and/or $L^\cdot$). When Trolox is employed as a radical scavenger, the first possibility can be disregarded due to its low reactivity towards superoxide anion.[20,21] Furthermore, the data obtained in the present system indicate that Trolox and SOD are not competitive scavengers,[5] and hence both additives are trapping different species. In order to test if Trolox was trapping the azo-derived[22] or luminol-derived radicals, we have analyzed the effect of Trolox at different luminol concentrations. The results obtained (Figure 6) indicate that the effect of Trolox is nearly independent of luminol concentration. Furthermore, Trolox induction times, as well as the rate of Trolox consumption after induction, are independent of luminol concentration in the 12.5 μM to 50 μM range. These results are incompatible with a competition between process (3) and

$$\text{Trolox} + \text{ROO}\cdot \rightarrow \text{inactive radicals} \qquad (4)$$

suggesting that the effect of Trolox must be related to its ability to trap luminol derived radicals. The similarity between $Q_{\frac{1}{2}}$ values obtained in the present work (17 nM) and that obtained at the same temperature when luminol luminescence is

induced by horseradish peroxidase ($Q_{\frac{1}{2}} = 30$ mM),[5] also suggests that in both systems Trolox is trapping the same intermediate. On the other hand, at the rather high Trolox concentrations employed in the evaluation of induction times, trapping of the azo-derived peroxyl radicals cannot be disregarded, and could constitute the main process leading to the observed luminescence quenching.

The low $Q_{\frac{1}{2}}$ values obtained for Trolox, as well as the linearity of the Io/I plots, renders the present system particularly suitable for the evaluation of very low free radical scavenger concentrations. Furthermore, the fact that a similar behaviour is observed employing serum as quencher, as well as its competition with Trolox, would make this system suitable for the evaluation of the capacity of biological fluids to trap free radicals. This information, that is complementary to TRAP values, is of biological significance because it is the parameter that determines, together with the rate of free radical production, the steady state concentration of active radicals.

The data of Figures 4, 5 and 11 indicate that, employing higher additive concentrations, the system allows an estimation of the TRAP capacity of a sample. In this regard it is interesting to note that by small changes in the system, the same experimental technique could provide simultaneously the TRAP value and an estimation of the total reactivity of the sample towards free radicals.

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