Free Rad. Res. Comms., Vol. 8, No. 2, pp. 115–121
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DIAZOLUMINOMELANIN: A SYNTHETIC LUMINESCENT BIOPOLYMER

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(Received August 4, 1989)

The purpose of this work was to synthesize a water-soluble derivative of 5-amino-2, 3-dihydro-1, 4-phthalazinedione (luminol) that generated sustained high level luminescence under physiologic conditions without the necessity of a catalyst. The derivative was made by a diazotization reaction with luminol and 3-amino-L-tyrosine. The resulting orange-brown anionic polymer has been given the trivial name of diazoluminomelanin (DALM). It was water soluble above and insoluble at or below pH 5.0. DALM luminesced when treated with hydrogen peroxide without the presence of a catalyst at pHs ranging from 6.5 to 12.0. Microgram quantities produced high levels of chemiluminescence for longer than 52 hr. Dried polymer generated a long-term stable electron spin resonance spectrum. The long-term chemiluminescence of DALM at pH 6.8–7.4 makes it a potentially useful reagent for detecting free radicals and peroxides in cellular and biochemical preparations.

KEY WORDS: Luminol, 3-amino-L-tyrosine, chemiluminescence, derivatized melanin.

INTRODUCTION

Chemiluminescent reactions have found numerous practical applications ranging from immunologic labeling methods to probes for detecting the presence of free radicals and peroxides produced by phagocytes.[1,2,3] These are potentially very sensitive methods, being limited only by background light levels and counting times if photon-counting detectors are used. A number of different chemiluminescent reactions have been characterized,[1,2] but perhaps the most important of these is that of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol).

We have been pursuing the development of a compound with characteristics superior to luminol. These characteristics include: (1) high solubility in physiologic aqueous solutions at pH 7.4; (2) high chemiluminescence yield at pH 7.4 to pH 5.0; (3) sensitivity to hydrogen peroxide, other oxidants, and free radicals without mediation by transition metal ions, hematoporphyrins, or hemoproteins; and (4) long duration of chemiluminescence. This paper describes our first attempts to synthesize a compound with these charaeteristics.

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MATERIALS AND METHODS

Luminol, 3-amino-L-tyrosine hydrochloride (3 AT), horseradish peroxidase (donor: hydrogen-peroxide oxidoreductase; E.C. 1.11.1.7) type VI, dimethyl sulfoxide (DMSO), and phosphate buffer salts were from Sigma Chemical Company (St. Louis, Missouri). Other buffer salts were from Fisher Scientific (Houston, Texas), and 3% hydrogen peroxide was from Hydrox Chemical Company (Elk Grove Village, Illinois). Gel filtration and ion exchange materials were also from Sigma Chemical Company.

Luminometric measurements were made using a Turner Designs (Mountain View, California) 20 e luminometer. In most cases, the samples in the instrument were held at 37°C in an aluminum block by circulating water from a thermostatically controlled water bath. A standard luminol reaction in DMSO activated with sodium hydroxide was used to calibrate the luminometer.[4] One relative light unit was found to be equivalent to 2.23×10^7 photons.

Electron spin resonance spectra were taken of the dry compound at room temperature. A varian E-line spectrometer was used at 9.48 GHz, 10 mW power, and a field set of 3353 G. The scan range was 400 G, with a modulation amplitude of 16 G, a time constant of 3 sec, a receiver gain of 2×10^3, a modulation frequency of 100 kHz, and a scan time of 2 min.

The luminescent compound was prepared by combining solutions of 10 mM luminol in DMSO, 10 mM 3 AT in water, and 100 mM $NaNO_2$ in water. These were mixed in various proportions with the DMSO: water molar ratio kept constant. The orange-brown solution that formed was quantified by its optical absorbance of 500 nm wavelength light measured in polystyrene cuvettes with a Bausch and Lomb Spectronic 2000 spectrophotometer. An orange-brown pigment (the desired product) was precipitated from the reaction solution by adding a 20-fold excess of acetone 5 min after starting the reaction.

The product designated DALM was centrifuged with a clinical centrifuge, and the supernatant discarded. The material was then dried under vacuum. The compound was redissolved in buffer for pH dependency and chromatographic studies. For long-term chemiluminescence studies, DALM was dissolved in water. In the pH dependency experiments, horseradish peroxidase (HRP) and DALM were dissolved in 0.1 M *Tris* (pH 9.5) or sodium phosphate buffers (pH 6.5 or 7.4), at concentrations indicated in the "Results" section. Each chemiluminescent assay sample was composed of 900 μl of DALM and/or HRP solution activated with 100 μl 0.03% hydrogen peroxide unless otherwise noted. The chemiluminescence was integrated for 10 sec out of every 16, and expressed as relative light units of the Turner Designs 20 e. For the long-term chemiluminescence study, the reaction mixture was composed of 10 μl 3% hydrogen peroxide, 500 μl deionized distilled water, and 50 μl DALM solution (187.5 μg DALM) held at 37°C and counted for 10 sec every 30 min.

RESULTS

No colored products were formed from the luminol and 3 AT reaction mixture if nitrite was omitted. Also, luminol and nitrite did not form a colored product under the conditions of the reaction if 3 AT were omitted. An orange-brown pigment did form when 3 AT and nitrite were reacted, but this pigment was observably darker

when luminol was included. The maximum absorption at 500 nm of the 3 AT, luminol, nitrite solution, when the 3 AT and luminol concentrations were varied, was observed at a 3 AT:luminol molar ratio of 1.67. The total of the 3 AT and luminol concentrations was kept constant at 10 mM, and nitrite concentration was held at 20 mM in order to make polymer with varying molar ratios of 3 AT and luminol.

DALM adsorbed to Sephadex G-25 and G-50 columns, and eluted following a salt fraction (0.1 M) with an ionic strength larger than that normally required for molecular weight separations. The colored product formed smeared bands, even at low flow rates, in the gel filtration columns.

The best column separations were achieved on DEAE Sephadex with 0.1 M pH 5.0 sodium acetate as the eluting buffer. Three components came off the DEAE column: (1) a brown pigment that strongly and visibly chemiluminesced when treated with 0.05 N NaOH and 0.15% hydrogen peroxide, and strongly fluoresced under a long-wavelength ultraviolet lamp (UVL.56, Ultraviolet Products, Inc., San Gabriel, California); (2) an orange-brown pigment that did not chemiluminesce or fluoresce (equivalent to the product of 3 AT and nitrite alone); and (3) an orange-brown anionic component that fluoresced and chemiluminesced. This third component was considered DALM. It precipitated when allowed to stand in pH 5.0 acetate buffer.

Dried acetone-precipitated DALM displayed an electron spin resonance spectrum, thus indicating that this DALM could form stable free radicals (Figure 1). The signal was still present in this sample two months later (only 20% diminished).

Shown in Figure 2 is the chemiluminescent response of DALM at 1 μg/ml in 0.1 M *Tris* HCl buffer (pH 9.5) at room temperature (25°C). The DALM was pre-incubated with the respective concentration of hydrogen peroxide for 5 min, and then the chemiluminescent response was integrated for 10 sec. Each value is a mean of four

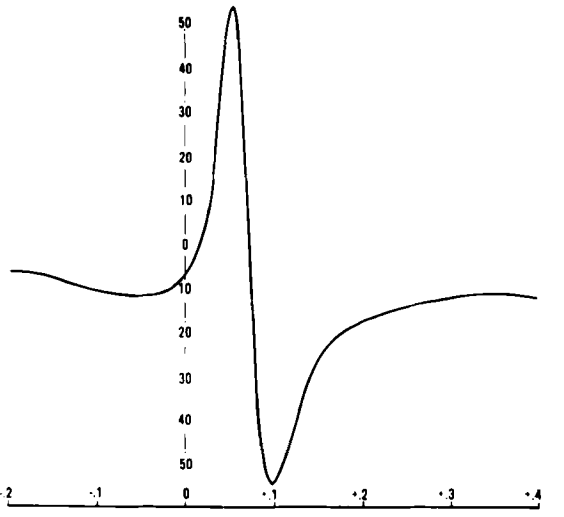

FIGURE 1 The ESR spectrum of stable free radicals in dry DALM at room temperature (25°C). A Varian E-line spectrometer was used at 9.48 GHz, 10 mW power, a field set of 3353 G, scan range of 400 G, modulation amplitude of 16 G, time constant of 3 sec, receiver gain of 2×10^3, modulation frequency of 100 kHz, and a scan time of 2 min.

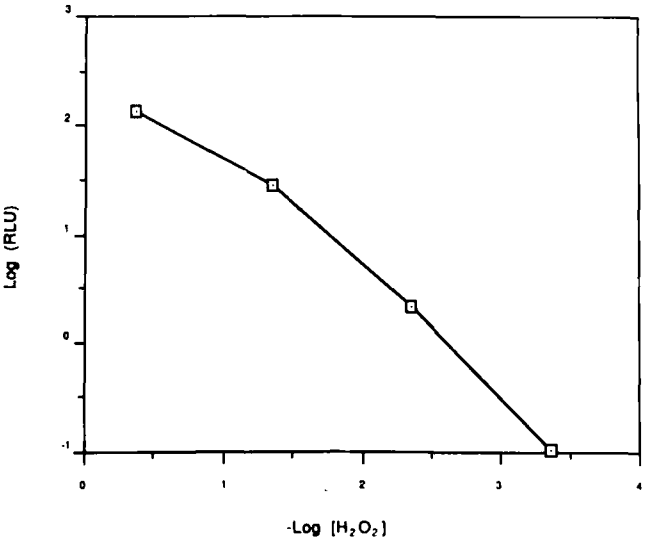

FIGURE 2 The chemiluminescent response in logs of relative light units (RLU) DALM (1 μg DALM/ml) in pH 9.5 (0.1 M) Tris HCl buffer at room temperature (25°C). DALM was pre-incubated with the respective concentration of hydrogen peroxide for 5 min before chemiluminescence was integrated for 10 sec in a Turner Designs 20e luminometer. Each point is a mean of four experiments.

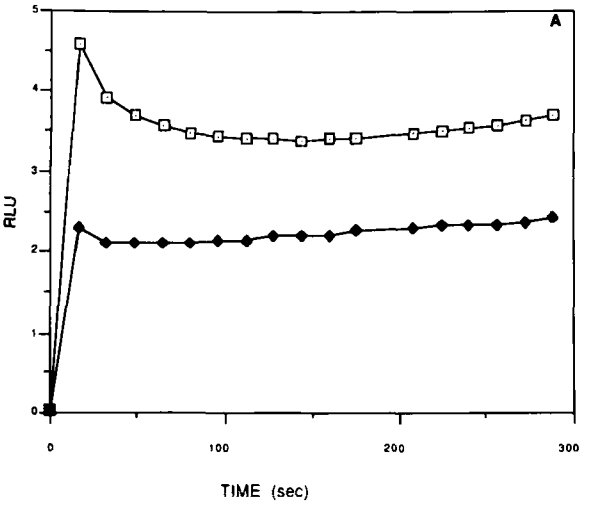

FIGURE 3 Chemiluminescence of DALM (10 μg DALM/ml) at pH 6.5, 7.4, and 9.5 with or without horseradish peroxidase (22.5 nM HRP) at 37°C. The reactions were initiated by adding 100 μl 8.8 mM hydrogen peroxide to 900 μl 0.1 M phosphate (pH 6.5 or 7.4), or Tris HCl (Ph 9.5) buffer containing DALM (closed diamonds), or DALM and HRP (open squares): A, pH 6.5; B, pH 7.4; C, pH 9.5. Counts were for 10 sec out of every 16 sec in a Turner Designs 20e luminometer.

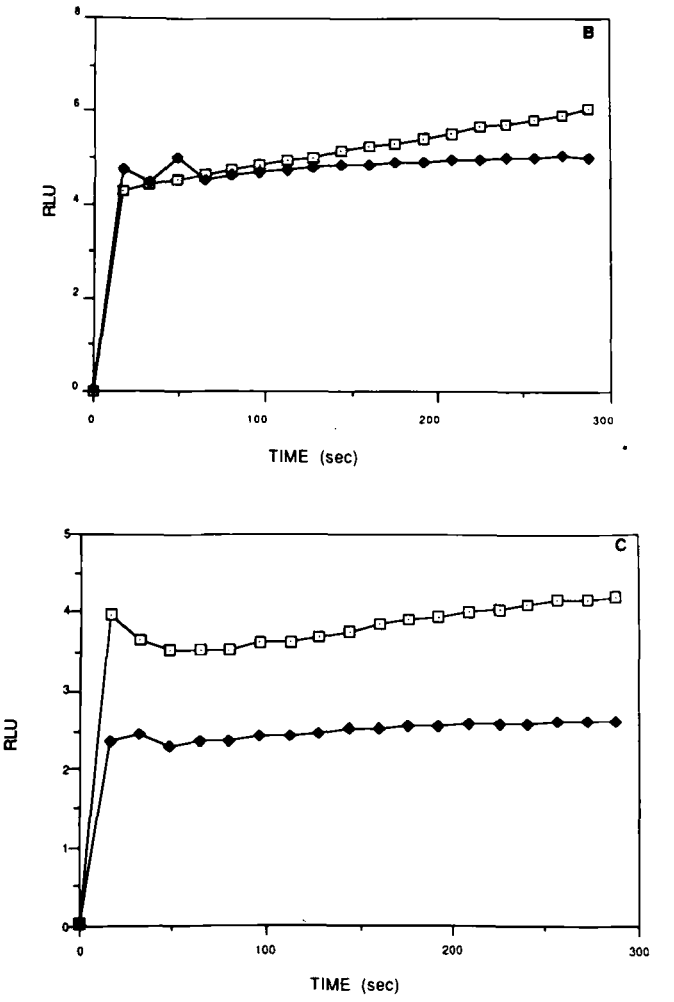

FIGURE 3 (Contd)

experiments. Under these conditions, the lower limit of hydrogen peroxide detection was about 0.88 mM.

This relative insensitivity to hydrogen peroxide was misleading, since Figure 3 (panels A, B, and C) shows that the output of light increased or was near steady-state over 5 min of reaction time (at 37°C) for pH 6.5, 7.4 and 9.5. Therefore, integrating over longer periods than 10 sec would have yielded higher sensitivities to hydrogen peroxide. That HRP, at 22.5 nM, no more than doubled the chemiluminescence is also shown in Figure 3. The reaction ran without the presence of peroxidase, and ran equally well at pH 6.5 and 9.5. Of the three pHs examined, pH 7.4 gave the best results.

When means of triplicate 10-sec integrations after 5-min 20-sec pre-incubations at various concentrations of DALM were examined at pH 6.5, 7.4 and 9.5 and the hydrogen peroxide was at 0.88 mM, the greatest chemiluminescence was seen at 9 μg

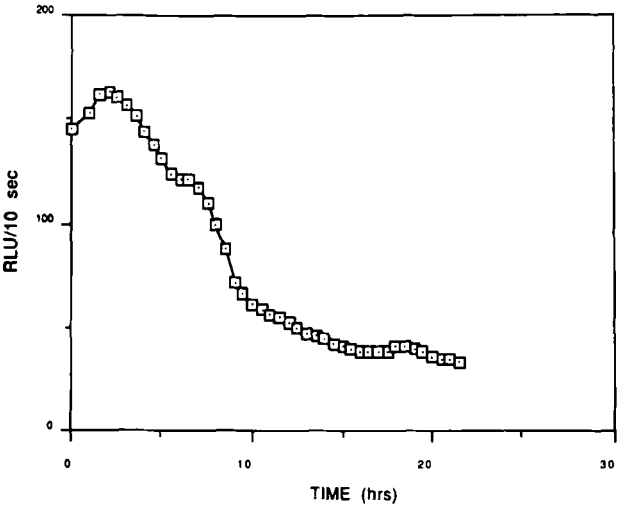

FIGURE 4 Long-term chemiluminescence of DALM. The reaction mixture contained 10 μl 3% hydrogen peroxide, 500 μl distilled deionized water, and 50 μl DALM solution (187.5 μg DALM). The reaction was initiated by addition of hydrogen peroxide, and held at 37°C in the luminometer. Chemiluminescence was recorded for 10 sec out of every 30-min period.

DALM/ml with 22.5 nM HRP, over a concentration range of 0.1 to 10 μg/ml. Under the conditions described, the chemiluminescent assay was sensitive down to 1 μg DALM/ml for pH 6.5, 7.4 and 9.5. At 9 μg DALM/ml, pH 6.5, the maximum enhancement of DALM chemiluminescence by HRP was observed. The catalyzed value was 3.45 times the uncatalyzed value.

At pH 6.5 and 9.5 with 0.88 mM hydrogen peroxide and 9 μg DALM/ml, the chemiluminescent assay was sensitive down to 22.5 nM HRP. At pH 7.4, the limit of sensitivity for HRP was 22.5 pM.

Displayed in Figure 4 is the long-term chemiluminescence of DALM up to 21.5 hr at 37°C and at pH 6.8 in water. At 52 hr after activation with hydrogen peroxide, the chemiluminescence had leveled off at 13.12 relative light units. The long-term chemiluminescence of DALM is unlike that of diazotized luminol. Acidic mixtures of pure luminol and nitrite formed an oxblood-colored product that chemiluminesced with alkaline hydrogen peroxide in the absence of a metal ion catalyst. The brief outburst of light (about 3 s) was visible in an illuminated room. Based on the calibration of the luminometer with a standard luminol and DMSO solution activated with sodium hydroxide and on the luminol content of the polymer (0.37 mole luminol/mole monomeric unit of DALM), 5.96×10^{-3} of the expected emission of luminol occurred in the 52 hr of observation.

DISCUSSION

Diazoluminomelanin (DALM) is an anionic polymer of 3-diazo-L-tyrosine and luminol that has several properties which make it superior to luminol as a chemilumi-

nescent compound. *First*, DALM is water soluble, with an apparent pKa for solubility around pH 5.0. *Second*, its chemiluminescence yield is better at pH 7.4 than 9.5, although it still gives a strong signal at strongly basic pHs. *Third*, DALM yields chemiluminescence at pH 6.5 that is at about the same intensity as that produced at pH 9.5. *Fourth*, DALM does not require a catalyst, and the duration of the reaction is in excess of 52 hr. Luminol, on the other hand, has shown peak luminescence with microperoxidase as the catalyst at 1 sec and half-lives of light emission of 0.5 and 4.5 sec at pH 8.6 and 12.6, respectively.[5]

Whereas the detection limit for luminol when catalyzed by hematin at pH 12.6 is 1 pM (177 femtograms/ml), the detection limit of DALM under the conditions we examined was 900 ng/ml. However, this conclusion is based on 10-sec integration times for DALM; and the time limit for the chemiluminescent reaction has not yet been determined. The uncatalyzed reaction of DALM was running at a near steady-state at 52 hr after activation. Based on the presumed active luminol content of the polymer, the quantum yield of DALM is less than that of luminol for short periods of time; however, the yield may be the same as luminol but extended over a longer period of time or prove to be greater when the entire lifetime is determined.

The lower limit for protohemin detection by luminol has been reported to be 5 pM.[6] For DALM, the catalyzed and uncatalyzed reaction became indistinguishable at pH 7.4, when 22.5 pM HRP was used as the source of the protohemin. Although this is a reasonable sensitivity to protohemin, the uncatalyzed reaction is less than an order of magnitude slower than the catalyzed reaction. On the basis of this property, DALM would not be the reagent of choice for determining the presence of a protohemin source in the reaction mixture.

Because of its strong chemiluminescence at pH 7.4, its autocatalysis, and its long-term luminescence, DALM should be a useful chemiluminescent probe in immunologic assays, in measurement of leukocyte oxidative reactions, and for detection of free radicals.

Acknowledgments

We thank Mr. Kenneth A. Hardy for collecting the electron spin resonance data. This work was supported in part by the United States Air Force Office of Scientific Research.

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Accepted by Dr. M.T. Smith