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Enhanced Chemiluminescence of Lucigenin with Epinephrine in Cationic Surfactant Micelles Containing Periodate

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Epinephrine (EP) species involved in the lucigenin chemiluminescence (CL) were identified in alkaline solution by comparing the time course of the CL response and the formation of EP oxidation products. EP quinone and adrenolutine (AL) were found to be responsible for the lucigenin–CL reaction. The mechanism of the lucigenin–CL enhancement was investigated using cationic micellar hexadecyltrimethylammonium hydroxide (CTAOH), periodate, and a mixture of micellar CTAOH and periodate. The CL enhancement in the presence of micellar CTAOH and periodate could be explained in terms of increases in the oxidation rate of EP to EP quinone and the intramolecular oxidation rate of adrenochrome to AL.

Keywords: chemiluminescence; lucigenin; epinephrine; surfactant micelle

INTRODUCTION

Catecholamines (CAs) such as epinephrine (EP) play an important role as transmitters in the nervous system. Thus, many analytical methods for CAs have been developed because the determination of CAs in biological fluids is useful for the diagnosis of various diseases and in monitoring their treatments. More attention has recently been given to the application of chemiluminescence (CL) methods for the determination of trace biological compounds, on account of their sensitivity. A few studies have been conducted on CL reaction involving CAs. Slawinska and Slawinski reported on the CL arising from the peroxidation of EP and norepinephrine with hydrogen peroxide (1). An excited carbonyl compound was formed and served as a CL emitter during the peroxidation of CAs (1). The light emission observed from the excited carbonyl compounds in the peroxidation of EP increased in the presence of vesicular media containing manganese as a catalyst. This system was applied for the determination of traces of EP (2).

We have previously reported that 10,10′-dimethyl-9,9′-biacridinium dinitrate (lucigenin) reacts with CAs to emit CL in an aqueous alkaline solution (3). In addition, the CL intensity emitted from the reaction of lucigenin with EP was markedly increased by the use of cationic micellar, hexadecyltrimethylammonium hydroxide (CTAOH) containing periodate. Based on this finding, we have proposed the lucigenin CL method for the determination of EP (4). The sensitivity of the proposed method for EP is comparable to that of the highly sensitive electrochemical detection method. However, the mechanism of the CL enhancement is unknown. The aim of this study was to elucidate the chemical species involved in the CL reaction of lucigenin with epinephrine, and the mechanism of the CL enhancement in the presence of micellar CTAOH and periodate.

MATERIALS AND METHODS

Reagents

10,10′-Dimethyl-9,9′-biacridinium dinitrate (luci-

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genin), hexadecyltrimethylammonium hydroxide and epinephrine were obtained from Tokyo Kasei. Adrenochrome (AC) was purchased from Sigma Chemical Co. All chemicals were used as received with no further purification. EP and AC solutions were prepared daily and were acidified to pH 2 and 4 with HCl, respectively. A 1.0×10^{-3} mol/L CTAOH solution, 1.0×10^{-4} mol/L periodate solution and solution containing both 1.0×10^{-3} mol/L CTAOH and 1.0×10^{-4} mol/L periodate were prepared using 0.1 mol/L sodium hydroxide. All solutions used were prepared with water from a Millipore Milli-Q water purification system.

Apparatus

All CL measurements were made using a fluorometer (Farrand Optical Co., Type Ratio-2) equipped with an automatic injector. The absorption spectra were measured with a Hitachi U-2000-type spectrophotometer equipped with 1-cm quartz cells. The fluorescence spectra were measured with a Hitachi F-2000 type spectrophotometer. Rapid changes in absorbance and fluorescence intensity were monitored with a Ohtsuka Denshi Piras-5000/5500-type photon rapid spectrometer. The light path in the cell of the spectrometer was 1 cm. The ESR spectra of EP semiquinone radical were obtained with a Varian E-9 and E-109 spectrometers operating at 9.5 GHz and employing 100 kHz field modulation.

Procedure

The general CL experimental procedure consisted in pipetting 1 mL of a 0.1 mol/L NaOH solution, micellar CTAOH solution, periodate solution or a solution containing both micellar CTAOH and periodate into a 1-cm glass cell in the fluorometer. Next, 0.5 mL of 5.0×10^{-5} mol/L lucigenin solution and 0.5 mL of EP solution were injected simultaneously through Teflon tubing into the cell by using an injector. Thus the CL reaction was initiated and the light emission was detected simultaneously.

The ESR spectra were obtained with a flow system. For these measurements, a 1.0×10^{-2} mol/L solution of EP and a 0.1 mol/L solution of KOH were placed in separate syringes equipped with flow control valves and were mixed at a Y-joint connected to a flat quartz cell mounted in an ESR cavity. The time of attainment to the cell after mixing the solutions was adjusted by the flow control valves.

The oxidation of EP was carried out in the cell in the photon rapid spectrometer (Ohtsuka Denshi Piras-5000/5500-type) under the same conditions.

RESULTS AND DISCUSSION

Effects of micellar CTAOH and periodate on the CL response curve

The CL measurements were performed by adding micellar CTAOH, periodate or a mixture of micellar CTAOH and periodate to the alkaline

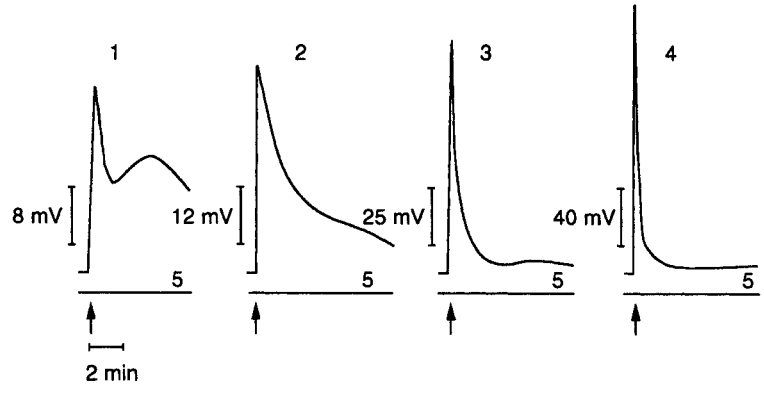
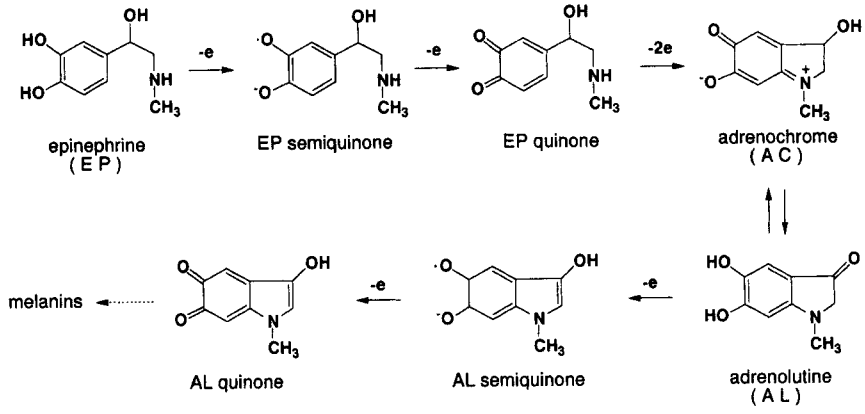


Figure 1. Typical CL response curves for the lucigenin–EP reaction in different reaction media. 1, water; 2, micellar CTAOH; 3, $NaIO_4$; 4, mixture of micellar CTAOH and $NaIO_4$; 5, blank. Concentrations: 1.0×10^{-4} mol/L EP, 5.0×10^{-5} mol/L lucigenin, 1.0×10^{-3} mol/L CTAOH, 1.0×10^{-4} mol/L $NaIO_4$. At the arrow, lucigenin and EP were added



Scheme 1. Radical and molecular intermediates in the oxidation of EP

solution. The critical micelle concentration for CTAOH is 9×10^{-4} mol/L (5). Typical CL response curves are shown in Fig. 1. Two CL peaks were observed in the alkaline solution alone. The first peak reached maximum intensity in 5 s and then began to decay rapidly. The second peak reached maximum intensity in 3 min and then decayed at a much slower rate. The CL intensity of the first peak increased remarkably in the presence of micellar CTAOH. However, the second peak was no longer detectable. The CL intensity of the first peak increased further while that of the second peak became very faint in the presence of periodate. When the mixture of micellar CTAOH and periodate was used, only one peak was observed in the CL response curve and the CL enhancement was maximal. In each run, no light emission was detected in a blank solution which contained no EP, as shown in Fig. 1 (curve 5).

These results may be interpreted as follows. It is known that EP reacts with oxygen in an alkaline solution and is successively converted into EP oxidation products as shown in Scheme 1 (1, 6). The appearance of two peaks is probably due to successive reaction of lucigenin with EP oxidation products. Alternatively, the remarkable change in the CL response curves in the presence of micellar CTAOH and peroxidate may be due to an increased rate of formation of EP oxidation products involved in the lucigenin–CL reaction.

Characterization of EP oxidation product responsible for the first CL peak

In order to determine the time course of EP oxidation products formed during the early stage of

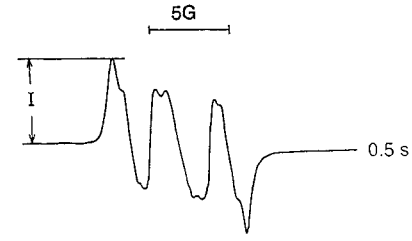


Figure 2. ESR spectrum of EP semiquinone formed during the oxidation of EP. Concentrations: 1.0×10^{-2} mol/L EP, 0.1 mol/L NaOH

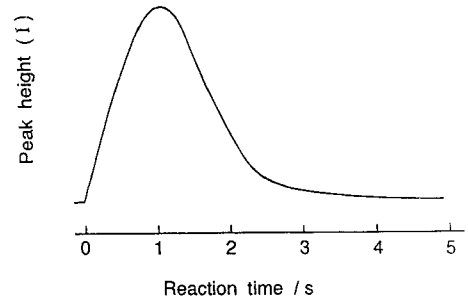


Figure 3. Lifetime of EP semiquinone. Concentrations: 1.0×10^{-2} mol/L EP, 0.1 mol/L NaOH

the oxidation, the reaction of EP was performed in alkaline solution. At first, the variation of the concentration of EP semiquinone was measured by the flow-ESR method. A typical ESR spectrum is shown in Fig. 2. The peak intensity (I) and the time course for I are shown in Figs 2 and 3. The concentration of EP semiquinone reached its maximum at 1 s. No EP semiquinone was detected 3 s after the initiation of the reaction. However, the first CL peak reached its maximum intensity 5 s

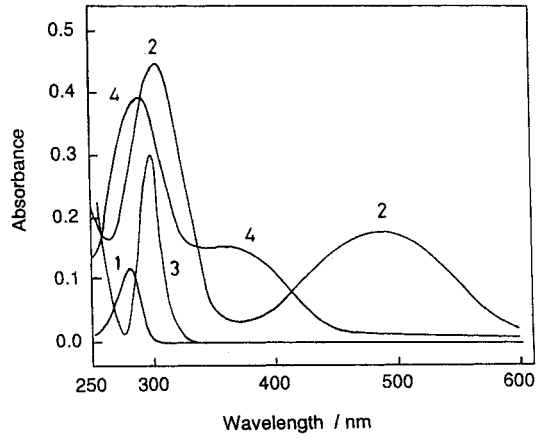


Figure 4. Absorption spectra of EP and EP oxidation products. 1, EP; 2, AC; 3, EP quinone; 4, mixture of AL and AL oxidation products. Concentrations: 5.0×10^{-5} mol/L EP, 5.0×10^{-5} mol/L AC

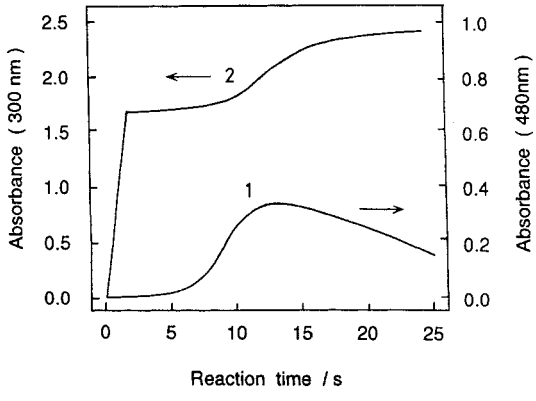


Figure 5. Time course for EP oxidation products during the oxidation of EP. 1, AC; 2, EP quinone. Concentrations: 1.0×10^{-2} mol/L EP, 0.1 mol/L NaOH

after the start of the CL reaction in the alkaline solution. Therefore, these results indicate that EP semiquinone is not responsible for the appearance of the first CL peak.

The reaction of lucigenin with adrenochrome (AC) was performed by adding a 1.0×10^{-4} mol/L solution of AC to the alkaline solution in place of the EP solution. The CL emission appeared immediately after the initiation of the reaction (result not shown). However, the CL intensity observed after addition of AC was ten times lower than that observed by adding EP.

Next, we determined the concentration of adrenochrome during the oxidation by measuring absorbance at 480 nm. Absorption spectra of EP and EP oxidation products are shown in Fig. 4. The wavelength of 480 nm was selected for monitoring AC since no other compounds interfere at that wavelength. The time course for absorbance of AC is shown in Fig. 5 (curve 1). The concentration of AC increased after 5 s and reached a maximum after 12 s from the start of the oxidation. Consequently, these results indicate that AC was also not responsible for the appearance of the first CL peak.

Epinephrine quinone is not available, owing to its instability. In addition, there is no information available on its spectrophotometric properties. Therefore we measured the variation of absorption spectra in the reaction mixture using the photon-rapid spectrometer. The results indicated that the absorbance at 300 nm increased significantly just after the initiation of the reaction, as shown in Fig. 5 (curve 2). Next, the absorbance at various wavelengths was measured 5 s after the start of the oxidation. The absorption spectrum is shown in Fig. 4 (curve 3) and is probably due to the EP quinone, since the concentrations of EP semiquinone and AC were both negligibly small 5 s after the start of the oxidation. Therefore, these results suggest that EP quinone could be responsible for the CL reaction of lucigenin and the appearance of the first CL peak.

Characterization of EP oxidation product responsible for the second CL peak

The second peak is probably due to the reaction of lucigenin with adrenolutine (AL) or AL oxidation products. This conclusion was reached after comparing the CL response curve in Fig. 1 (curve 1) with the time course for AC formation in Fig. 5 (curve 1). Next, the time course of AL formation was determined by measuring the fluorescence intensity due to AL (excitation and emission wavelength, 405 and 515 nm, respectively) (7). No fluorescence spectra of EP and AC were observed under the same conditions. However, the fluorescence spectrum of lucigenin was observed. Thus, the oxidation of EP was performed in water instead of the lucigenin solution. The time course of AL formation is shown in Fig. 6 (curve 1). The fluorescence intensity increased gradually after the start of the oxidation and reached a maximum value at 3 min. The maximal fluorescence intensity of AL peaked at the same time as the CL intensity

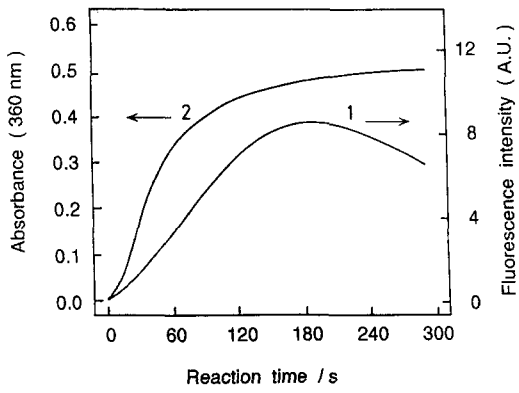


Figure 6. Time course of the formation of AL and AL oxidation products. 1, AL; 2, AL oxidation products. Concentrations: 1.0×10^{-4} mol/L EP, 0.1 mol/L NaOH

of the second peak. Therefore, AL could be responsible for the CL reaction of lucigenin, resulting in the appearance of the second CL peak. In addition, the appearance of the two peaks could be due to an intramolecular redox from AC to AL which occurs late in alkaline solution.

Next, we determined the time course of formation of AL oxidation products. We measured the absorbance at various wavelengths after 3 min from the start of the oxidation. The absorption spectrum is shown in Fig. 4 (curve 4). This spectrum may be composed of absorption spectra of AL and AL oxidation products (no information is available on the absorption and fluorescence spectra of AL oxidation products). Since the mixture had a characteristic absorption at 360 nm, the variation of absorbance at 360 nm in the oxidation of EP was measured. The change of the absorbance is shown in Fig. 6 (curve 2). Although the fluorescence intensity was maximal at 3 min, the absorbance increased gradually with increasing time. Consequently, the variation of absorbance at 360 nm could reflect the time course for the formation of AL and AL oxidation products. These results indicate that AL oxidation products are not responsible for the appearance of the second peak, since the variation of absorbance is not compatible with the time course of the CL intensity of the second peak.

The effects of micellar CTAOH and periodate on the rate of AC formation

The variation of the concentraton of AC was measured by monitoring the absorbance at 480 nm in the presence of micellar CTAOH, periodate or a mixture of micellar CTAOH and periodate. The time profiles for AC formation are shown in Fig. 7. The rate of AC formation in the presence of micellar CTAOH was greater than in the alkaline solution alone. The result may be interpreted as follows. The deprotonated forms of EP are expected to be the main species present in an aqueous alkaline solution, since the acid dissociation constants of EP are $pK_1 = 8.66$ and $pK_2 = 9.95$, respectively (8). The anionic forms of EP interact electrostatically with and bind to a cationic micelle pseudophase such as CTAOH. Consequently, the effective local concentration of EP at the micelle surface is greater than its stoichiometric concentration in bulk water alone. Thus, the oxidation rate of EP is greater in micellar assemblies of CTAOH compared to that in water alone.

The oxidation rate of EP in the presence of periodate was increased significantly compared to that in the alkaline solution alone. This result indicates that periodate is more effective as an oxidizing agent for EP than molecular oxygen. Further, the oxidation rate of EP was greater in the presence of both micellar CTAOH and periodate compared to in peroxidate alone. These results could be explained by peroxidate anion interacting electrostatically with and binding to the cationic micelle pseudophase. Therefore, the effective local concentration of periodate at the micelle surface is greater than its stoichiometric concentration in bulk water alone. Hence, the oxidation rate of EP with

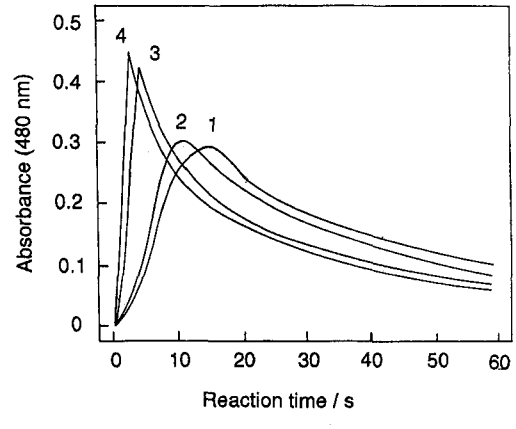


Figure 7. Time course of the formation of AC. 1, water; 2, micellar CTAOH; 3, $NaIO_4$; 4, mixture of micellar CTAOH and $NaIO_4$. Concentrations: 1.0×10^{-4} mol/L EP, 1.0×10^{-3} mol/L CTAOH, 1.0×10^{-4} mol/L $NaIO_4$

periodate is greater in the presence of micellar CTAOH compared to that in water alone.

The rate of EP quinone formation may also increase in proportion to that of AC formation. Consequently, the relative effectiveness of enhancing the CL intensity of the first peak, as shown in Fig. 1, parallels the order of increasing the rate of EP quinone formation. In the micellar media, excitation efficiency effects (9) could also underlie the CL enhancement apart from the effect of the oxidation rate of EP on the CL intensity. That is, lucigenin probably partitions and binds to a micelle pseudophase, to which EP oxidation products also partition and bind. Consequently, the excited state *N*-methylacridone as an emitter could be efficiently formed and be stabilized by the less polar micro-environment of micellar media, thus resulting the increase of the CL intensity (9).

The effects of micellar CTAOH and periodate on the rate of AL formation

We measured the rate of AL formation by monitoring the fluorescence intensity in the presence of micellar CTAOH, periodate or the mixture of CTAOH and periodate. The time course for the formation of AL is shown in Fig. 8. These results indicate that the rate of intramolecular redox from AC to AL increases in the following order: mixture of micellar CTAOH and periodate > periodate > micellar CTAOH > water alone. The concentration of AL reached its maximal value at 10 s from the start of the oxidation in the mixture of micellar CTAOH and periodate. The time of the appearance of the second CL peak may be much shorter as a result of an increase in the rate of AL formation. Therefore, only one peak appears in the CL response curve and its intensity increase parallels the increase in the rate of AL formation (Fig. 8).

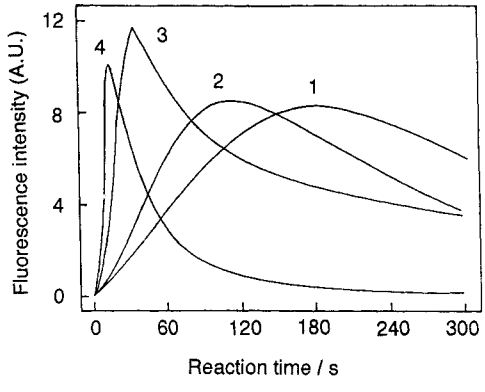


Figure 8. Time course of the formation of AL. 1, water; 2, micellar CTAOH; 3, $NaIO_4$; 4, mixture of CTAOH and $NaIO_4$. Concentrations: 1.0×10^{-4} mol/L EP, 1.0×1.0^{-3} mol/L CTAOH, 1.0×10^{-4} mol/L $NaIO_4$

The maximal concentration of AL formed in the presence of both micellar CTAOH and periodate was less than that in periodate (Fig. 8; curves 3, 4). This is because the oxidation of AL proceeds faster in the mixture, since the absorbance at 360 nm increased rapidly compared to that in the presence of periodate alone (results not shown). However, the CL intensity of the first peak in the presence of both micellar CTAOH and periodate was greater than that in periodate alone (Fig. 1), suggesting that the increased rate of EP quinone, as opposed to AL formation, was important in the increase of the CL intensity of the first peak.

In conclusion, among the epinephrine oxidation products shown in Scheme 1, EP quinone was found to be responsible for the CL reaction of lucigenin to produce the first CL peak. The formation of EP semiquinone was confirmed by the flow-ESR method. However, the CL reaction of lucigenin with EP semiquinone could not be characterized because the lifetime of EP semiquinone is so short. On the other hand, AC reacted with lucigenin to emit CL. However, the CL intensity in the presence of AC was lower than that of the first peak. Consequently, EP semiquinone and AC was not responsible for the appearance of the first CL peak. Adrenolutine underwent a CL reaction with lucigenin, resulting in the appearance of the second CL peak. However, there was no CL reaction of lucigenin with AL oxidation products. EP quinone and AL react successively with lucigenin to emit light. When intramolecular oxidation of AC to AL occurs, two peaks appear in the CL response curve. Meanwhile, the addition of micellar CTAOH, periodate or mixture of micellar CTAOH and periodate to the alkaline solution increased the oxidation rate from EP to EP quinone and from AC to AL, resulting in the appearance of one peak and in the enhancement of the CL intensity. The elucidation of the mechanism of the present CL enhancement will be useful for the development of a highly sensitive method for other catecholamines.

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