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Inhibition of Bleach-Induced Luminol Chemiluminescence*

ABSTRACT: The luminol chemiluminescence presumptive test for blood is based on the mild peroxidase activity of hemoglobin in basic peroxide solution. However, this test is subject to interference by strong oxidants, certain transition metal ions, and true peroxidases. This paper reports methods for reducing the interference caused by hypochlorite-containing bleaches. Amines such as 1,2-diaminoethane react rapidly with hypochlorite without interfering significantly with the hemoglobin-catalyzed oxidation. Thus, addition of 0.1 mol/L 1,2-diaminoethane to a standard luminol-peroxide spray lead to almost complete inhibition of hypochlorite-induced chemiluminescence while satisfactory chemiluminescence was still observed from bloodstains. If time allows, an alternative method for reducing interference from hypochlorite bleach is to wait several days until the bloodstains have dried thoroughly, by which time the hypochlorite will have decomposed.

KEYWORDS: forensic science, criminalistics, chemiluminescence, bloodstains, luminol

The luminol-hydrogen-peroxide test for blood can be subject to interferences from peroxidases, metal ions, or oxidants (1). The most likely oxidants to be found at a crime scene are hypochlorite-based bleaches. The presence of these on surfaces being sprayed leads to bright flashes of chemiluminescence, as opposed to the more gradual development of chemiluminescence by blood. Although an experienced forensic scientist can usually differentiate between blood and bleach-induced chemiluminescence this will not always be possible (personal observation, D. Elliott, (2,3)). In addition, the time-averaging implicit in long-exposure photography can lead to the two sources of chemiluminescence appearing identical. A recent paper has reported that the spectra of the bleach-induced and blood-induced chemiluminescence are slightly different (4), but the small shift in emission wavelength combined with the broadness of the emission band mean that spectral filtering is unlikely to be able to separate the two causes.

We have investigated chemical methods for reducing the effect of hypochlorite-containing bleaches on luminol chemiluminescence. Antioxidants such as ascorbic acid or polyphenols (5–7) cannot be used because they also inhibit the blood-catalyzed luminol-peroxide chemiluminescence. Therefore we sought to target the chlorine donor properties of hypochlorous acid (the conjugate acid of hypochlorite), since this is a different mode of oxidation from the mechanism occurring with peroxide. The reaction of amines with hypochlorous acid to form chloramines (Eq 1) is a well-known process (8,9).

$$RR'NH + HOCl \rightarrow RR'NCl + H_2O \qquad R, R' = \text{alkyl or H} \quad (1)$$

Indeed, analytical procedures for amines have been reported based on the inhibition of the chemiluminescence observed when hypochlorite oxidizes luminol (10). Other studies have shown that although primary and secondary amines inhibit oxidative chemiluminescence, tertiary amines actually increase the chemiluminescence, presumably via catalytic formation of the N-chlorotrialkylammonium ions that act as reactive oxidants (11,12). We have investigated whether primary and secondary amines can inhibit the chemiluminescence due to hypochlorite under the conditions typical of forensic luminol sprays, and whether the presence of amines has an effect on the heme-catalyzed luminescence of luminol.

The choice of amines was guided by studies by Margerum (8,9) and Antelo (13,14), who showed that the reaction rate between hypochlorite and amines is pH dependent and depends on the basicity of the amine. The observed pH dependence, Fig. 1, occurs because the reaction involves specifically the amine ($RR'NH$) reacting with hypochlorous acid ($HOCl$) in the reaction, as shown in Eq 1, whereas the protonated amine ($RR'NH_2^+$) and the deprotonated hypochlorite (OCl^-) are much less reactive (9). The reactivity of many with hypochlorous acid amines can be fitted by a curve given by Eq 3 (13),

$$\text{Rate} = k[HOCl][RR'NH^{n+}] \quad (2)$$

$$k = (2.9 \pm 0.5) + (0.48 \pm 0.05)\, pK_a(RR'NH_2^{(n+1)+})\ \text{L mol}^{-1}\ \text{s}^{-1} \quad (3)$$

where $pK_a(RR'NH_2^{(n+1)+})$ is the negative logarithm of the acid dissociation constant of the conjugate acid of the amine. The only amine that was reported to deviate significantly from this relationship was ammonia, which had a rate nine times lower than would be predicted by this equation (9).

If the behavior of this reaction rate is examined as a function of total hypochlorite and total amine species Eq 2 can be rewritten

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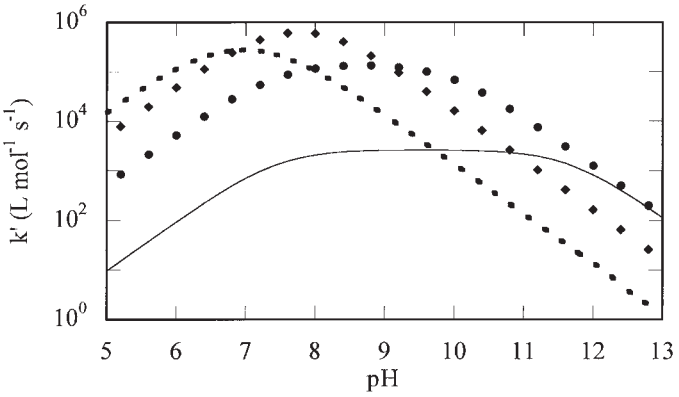

FIG. 1—*Plot showing predicted reactivity of luminol (___), hydrogen peroxide (- - -), TRIS (♦), and 1,2-diaminoethane(●) with hypochlorite as a function of pH. Curves for the amines are based on equation 4 in the text, estimating the rate constants from Eq 3. Curves for luminol and H_2O_2 are derived from analogous equations using literature values.*

in the form

$$\text{Rate} = k'[HOCl]_{total}[RR'NH]_{total} \quad (2a)$$

$$k' = k\left[\left(\frac{[H_3O^+]}{[H_3O^+] + K_a\,(HOCl)}\right) \times \left(\frac{K_a\,(amineH^{(n+1)+})}{[H_3O^+] + K_a\,(amineH^{(n+1)+})}\right)\right] \quad (4)$$

where k′ would be the observed rate constant, $[HOCl]_{total} = [HOCl] + [OCl^-]$, $[RR'NH]_{total} = [RR'NH^{n+}] + [RR'NH_2^{(n+1)+}]$, and $K_a(HOCl)(= 3.6 \times 10^{-8})$ is the acid dissociation constant of hypochlorous acid. A plot of k′ vs. pH, Fig. 1, shows the variation in the rate of this reaction with pH and with the basicity of a given amine. It is seen that amines with low pK_as (e.g., tris, $pK_a(trisH^+) = 8.1$) have a high observed rate constant at their pH optimum, but this rate decreases rapidly (10-fold per pH unit) at higher pH. In contrast, basic amines have a lower maximum observed rate constant but this value is maintained over a greater pH range. At any given pH Eq 4 predicts that the optimum amine will be that for which $pK_a = pH - 0.03$ i.e., $pK_a \approx pH$. Therefore strongly basic amines will be the most effective competitors for hypochlorite under the conditions of common forensic sprays (those reported by Grodsky and Weber both lead to pH > 10). Luminol itself can react with hypochlorous acid, (15,16) and this is indicated in Fig. 1. The observed rate constant for the direct reaction of luminol with HOCl is predicted to be comparatively low at high pH based on the above analysis. This may be incorrect, because luminol has a second deprotonation with pKa ≈ 15 (17), and the basic form may show higher reactivity towards HOCl. Indeed, one literature report shows that the predicted behavior in Fig. 1 is followed until pH > 11.5, where an increase in rate was observed (15). Figure 1 also shows a rate constant-pH curve for hydrogen peroxide (pK_a 11.65), based on literature data. It has the same shape as for amines, but the rate constant for the reaction of HO_2^- and HOCl is 4.4×10^7 L mol/L s^{-1},(18) i.e., seven-fold lower than for an amine of similar basicity.

Methods

Bovine blood obtained from a local abattoir was preserved with 0.2% w/v EDTA and stored at 4°C. Domestic strength sodium hypochlorite bleach was standardized by titration with standardized thiosulfate solution using starch-iodine indicator.

Diethylamine, 1,2-diaminoethane (ethylenediamine), ethylamine, taurine, tris(hydroxymethyl)methylamine (TRIS), and triethylamine were used as received.

The luminol sprays tested were Grodsky: (a solution containing 1 g/L luminol and 50 g/L Na_2CO_3 mixed with an equal volume of 7 g/L sodium perborate solution) and Weber: (25 mL of water containing 0.72 g/L luminol and 2 g/L NaOH was mixed with 25 mL 0.6% H_2O_2 and 175 mL H_2O). In both cases the mixing of solutions was performed as soon as possible before a measurement was to be made.

Visual observations were conducted in a darkened room after the investigator was dark-acclimatized for at least 5 min. Spraying of luminol solutions was performed using a calibrated spray-gun to ensure uniform delivery of reagent. Substrates were vinyl flooring tiles and unbleached cotton. The tiles were used as received, whereas the cotton was washed with clothes detergent, then rinsed in tap water and finally boiled in Milli-Q deionized water and then dried.

Supporting experiments examining the effect of amines on bleach-induced chemiluminescence were performed using an Ocean Optics S2000 fibre optic diode array spectrophotometer with the reagent feed being controlled by a peristaltic pump. Hypochlorite solution (1:20 dilution) adjusted to pH 12.1 was mixed with luminol-H_2O_2-perborate solution at pH 12.1 at a rate of 10 mL/min (each reagent stream) in a 2 mm diameter T-junction cell with two right-angle turns 1 cm after the initial junction to increase mixing. Light emission in the 2 cm of tubing immediately after the mixing point was monitored under constant flow conditions. The high flow rate was needed to avoid the build up of gas bubbles in the cell. Studies of the effect of amines on the blood-induced chemiluminescence used the same spectrophotometer equipped with a 1 cm cuvette holder.

Safety: All initial experiments involving spraying of luminol solutions were performed in chemical fumehoods. When the sprays were used outside fumehoods appropriate personal protection including goggles, respirator, and gloves were worn. If amines are added to the luminol solution these precautions are especially important, and the room should be thoroughly ventilated after the chemiluminescence has been observed.

Results and Discussion

Inhibition of Hypochlorite Bleach-Induced Chemiluminescence

Preliminary experiments showed that the observed chemiluminescence of luminol upon reaction with either blood or bleach was pH dependent, and that the addition of amines to solutions could change the pH. Therefore the pH was adjusted to pH 12.1 ± 0.1 for the solution studies described in this paper.

The ability of amines to inhibit bleach-induced chemiluminescence from the Grodsky formulation of luminol solution was evaluated using a steady-state flow cell attached via fibre optics to a diode array spectrophotometer. The amines were added at levels of 0, 0.008, 0.02, 0.04, and 0.08 mol/L. The results, Table 1, showed that the inhibition by amines increased with the basicity of the amines and was most marked for 1,2-diaminoethane, with 90% inhibition at 0.08 mol/L. Ammonia caused little or no inhibition, in accord with the reported slower rate of reaction of ammonia with hypochlorous acid (9). Triethylamine caused increases in the observed chemiluminescence, in agreement with literature observa-

tions on tertiary amines (11,12). The metal chelating agent EDTA, which is also a tertiary amine, had no effect on the chemiluminescence at all concentrations tried (0–0.08 mol/L).

Similar results to these were obtained by visual examination of bleach spots sprayed with amine-containing luminol sprays. Depending on the concentration of the bleach used and the length of time it had been on the substrate, concentrations of 0.02 mol/L or 0.1 mol/L 1,2-diaminoethane in Grodsky's luminol spray were sufficient to reduce bleach-induced chemiluminescence to a negligible level. In addition, it was noted that if bleach spots were left for periods of greater than a day under conditions where they could dry that no significant chemiluminescence was observed when they were sprayed with standard forensic luminol-peroxide sprays (Grodsky or Weber formulations).

Effect of Amines on Heme-catalyzed Chemiluminescence

The effect of the amine 1,2-diaminoethane on the blood-catalyzed luminol chemiluminescence was evaluated both spectrophotometrically in solution and visually with bloodstains on substrates. In solution studies the addition of 0.1 mol/L 1,2-diaminoethane at pH 12.1 caused a slight decrease in chemiluminescence over the first 1 min, but was then identical to the chemiluminescence of the control, Fig. 2. This experiment was performed with aged blood, so the initial rapid change may represent activity due to degraded hemoglobin.

Visual comparison of 1,2-diaminoethane effects were performed using vinyl and cotton substrates with 1 h, 3 h, and 1-day-old blood stains, and the appearance and duration of the chemiluminescence was noted. On a given day repeated measurements showed good repeatability, and comparisons between different conditions were always made within the same experimental run. It was observed that the strength and duration of chemiluminescence due to blood were decreased by a factor of 2–3 in the presence of high concentrations of 1,2-diaminoethane (0.1 mol/L), Table 2, although the exact level of inhibition was also dependent on the solution pH. However, even though the 1,2-diaminoethane causes slight inhibition in the chemiluminescence due to blood the results obtained are still satisfactory for use at a crime scene. Furthermore, at a crime scene repeated spray applications can be used if the chemiluminescence duration is too short.

TABLE 1—*Effect of amines on chemiluminescence from the reaction of hypochlorite bleach with luminol and hydrogen peroxide. Chemiluminescence is expressed as a ratio compared to that observed when no amine is present.*

Amine	pK_a	Concentration (mol/L) 0	0.008	0.02	0.04	0.08
1,2-diaminoethane	9.93	1	0.71	0.44	0.26	0.12
TRIS	8.08	1	1	0.95	0.88	0.66
Ammonia	9.24	1	1	1	1	1
Taurine	9.06	1	0.77	0.6	0.4	0.28
Triethylamine	10.71	1	3.3	6.3	6.3	6.9

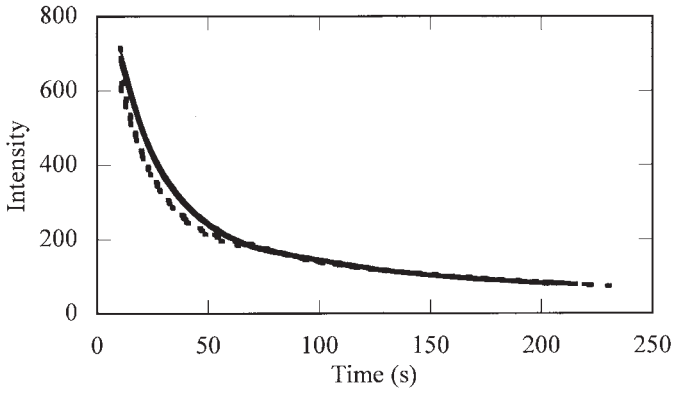

FIG. 2—*Time evolution of the chemiluminescence observed when a dilute blood solution was added to Grodsky's luminol solution in the absence (smooth line) and presence (dashed line) of 0·1 mol/L 1,2-diaminoethane. Each curve is a superposition of three replicate runs.*

TABLE 2—*Duration of chemiluminescence observed when blood on a cotton substrate is sprayed with Grodsky's luminol formulation. The 1,2-diaminoethane was added to the spray solution immediately before use.*

Conditions	Age of Stain (h)	Duration of Observable Chemiluminescence (n = 10; ± s.d.)
Control: Grodsky spray	1	3 min 21 s ± 26 s
	3	3 min 07 s ± 30 s
	72	3 min 38 s ± 29 s
Grodsky spray	1	49 s ± 8 s
+ 0.1 mol/L	3	58 s ± 8 s
1,2-diaminoethane,		
pH 11.77	72	52 s ± 11 s
Grodsky spray	1	1 min 00 s ±14 s
+ 0.1 mol/L	3	1 min 34 s ± 44 s
1,2-diaminoethane,	72	57 s ± 11 s
pH 12.36		
Grodsky spray	1	58 s ± 15 s
+ 0.02 mol/L	3	1 min 27 s ±10 s
1,2-diaminoethane,	72	1 min 20 s ± 13 s
pH 12.01		
Grodsky spray	1	2 min 20 s ± 44 s
+ 0.02 mol/L	3	3 min 55 s ± 27 s
1,2-diaminoethane,	72	3 min 06 s ± 25 s
pH 13		

The addition of 1,2-diaminoethane to Weber's luminol formulation was not as successful at reducing the chemiluminescence due to bleach. Our results suggest that the presence of carbonate in the solution plays a significant role in the observed reactivity.

A simulation of a crime scene where bloodstains on carpet, vinyl, and cloth were washed with bleach at recommended dilutions showed that addition of 0.1 M 1,2-diaminoethane to Grodsky's luminol formulation reduced the bleach interference on all substrates and only slightly affected the blood chemiluminescence. This solution is now included in the ESR protocol for luminol use if there is evidence that bleach may have been used at a crime scene. Appropriate precautions, including the use of safety glasses and respirator masks, are taken when spraying either Grodsky's luminol solution or the solution including 1,2-diaminoethane. Alternatively, if the crime scene is such that it can be left to dry for a few days (e.g., a car interior) then this delay will reduce or remove the bleach interference.

Conclusion

The false positive response observed when luminol-peroxide solutions are sprayed onto an area that has been cleaned with hypochlorite-containing bleaches can be prevented by adding amines such as 1,2-diaminoethane to the luminol solution. The amines react rapidly with the hypochlorite ions, and the chlor-

amines thus formed do not cause visible chemiluminescence to occur. The amines do slightly reduce the chemiluminescence observed from blood, but the treatment still has sufficient intensity and longevity of light emission that it is useful in a forensic context. During this study it was also noted that the positive interference by bleach is diminished if the area to be sprayed is left for several days. Therefore, where possible a delay before spraying with luminol-peroxide solution can significantly reduce positive interference by bleach. Both of the procedures have been included in the ESR protocols for luminol use.

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References

1. Gaensslen RE. Sourcebook in forensic serology, immunology, and biochemistry. Washington DC: U.S. Department of Justice; 1983.
2. Ballou SM. Moves of Murder. J Forensic Sci 1995;40:675–80.
3. Lytle L, Hedgecock DG. Chemiluminescence in the visualization of bloodstains. J Forensic Sci 1978;23:550–62.
4. Quickenden TI, Cooper PD. Increasing the specificity of the forensic luminol test for blood. Luminescence 2001;16:251–53.
5. Arnhold J, Hammerschmidt S, Arnold K. Role of functional groups of human plasma and luminol in scavenging of NaOCl and neutrophil-derived hypochlorous acid. Biochim Biophys Acta 1991;1097:145–51.
6. Arnhold J, Mueller S, Arnold K, Sonntag K. Mechanisms of inhibition of chemiluminescence in the oxidation of luminol by sodium hypochlorite. J Biolumin Chemilumin 1993;6:307–13.
7. Sakagami H, Sakagami T, Yoshida H, Omata T, Shiota F, Takahashi H, et al. Hypochlorite scavenging activity of polyphenols. Anticancer Res 1995;15:917–22.
8. Gray ET, Margerum DW, Huffman RP. Chloramine equilibria and the kinetics of disproportionation in aqueous solution. In: Brinkman FE, Bellama JM, editors. Organometals and organometalloids: occurrence and fate in the environment. Columbus: American Chemical Society 1978;264–77.
9. Margerum DW, Gray ET, Huffman RP. Chlorination and the formation of N-chloro compounds in water treatment. In Brinkman FE, Bellama JM, editors. Organometals and organometalloids: occurrence and fate in the environment. Columbus: American Chemical Society 1978;278–91.
10. Kraus PR, Crouch SR. Investigation of a chemiluminescent system for the determination of ammonia by flow-injection analysis. Anal Lett 1987;20:183–200.
11. Cobo M, Silva M, Perez-Bendito D. Chemiluminescence measurement of tertiary amines using the continuous addition of reagent technique. Determination of trimethylamine in fish samples. Anal Chim Acta 1997;356:51–59.
12. Lancaster JS, Worsfold PJ, Lynes A. Flow injection procedure for the determination of tertiary amines in water and seawater using chemiluminescence detection. Analyst 1989;114:1659–61.
13. Antelo JM, Arce F, Parajo M. Kinetic study of the formation of N-chloramines. Int J Chem Kin 1995;27:637–47.
14. Antelo JM, Arce F, Castro MC, Crugeiras J, Perez-Moure JC, Rodriguez P. Kinetics of the formation, decomposition, and disproportionation reactions of N-chlorobutylamines. Int J Chem Kin 1995;27:703–17.
15. Seitz WR. Chemiluminescence from the reaction between hypochlorite and luminol. J Phys Chem 1975;79:101–06.
16. Isacson U, Kowalewska J, Wettermark G. Kinetic investigation of reactions between inorganic chlorine compounds and luminol. J Inorg Nucl Chem 1978;40:1653–56.
17. Haapakka KE, Kankare JJ, Linke JA. Determination of the second acidity constant of luminol. Anal Chim Acta 1982;139:379–82.
18. Held AM, Halko DJ, Hurst JK. Mechanisms of chlorine oxidation of hydrogen peroxide. J Am Chem Soc 1978;100:5732–40.

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