

Chemiluminescence from the oxidation of urea and ammonia with hypobromite and *N*-bromosuccinimide

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Abstract

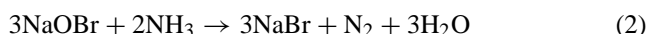
The spectral distribution for the chemiluminescent oxidation of ammonia with hypobromite is significantly different to that for the oxidation of ammonia with *N*-bromosuccinimide. Therefore, in contrast to the assumptions of several authors, the action of *N*-bromosuccinimide is not solely derived from the in situ formation of hypobromite. Neither the oxidation of urea with hypobromite nor the oxidation of urea with *N*-bromosuccinimide involves an initial hydrolysis of urea to ammonia in the alkaline solution. However, these two reactions lead to a common emitter. The addition of xanthene dyes, such as dichlorofluorescein, enhance the chemiluminescence intensity by energy transfer to the efficient fluorophore, but reaction between the sensitiser and hypobromite can result in a significant increase in the background signal. A list of potential interferences has been compiled; particular attention was paid to guanidino compounds, as the chemiluminescence accompanying the oxidation of this functional group has not been previously discussed.

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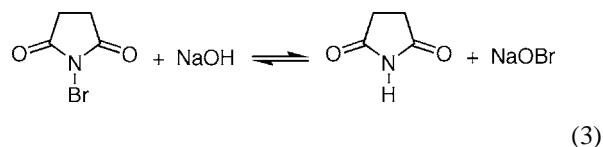
Keywords: *N*-bromosuccinimide; Chemiluminescence; Hypobromite

1. Introduction

Chakrabartty reviewed the chemical properties of hypohalites in 1978 [1]. Hypobromite is conveniently prepared by adding bromine to an alkaline solution, or by chemical or electrochemical oxidation of bromide [2]. Urea and ammonia are both oxidised by hypobromite to molecular nitrogen (Eqs. (1) and (2)). The mechanism of oxidation involves the transfer of halogen cations and loss of halide anions from the *N*-halo intermediates [3], and may proceed via hydrazine and diazine. Unlike ammonia, the oxidation of urea involves an intermolecular rearrangement. Substitution and isotope labelling studies have provided evidence for two reaction pathways; the Hofmann rearrangement and a nitrogen analogue of the Favorskii reaction [4].



A variety of flow analysis techniques have emerged for the determination of urea and ammonia, based on measurement of the chemiluminescence arising from the oxidation of these analytes with either hypobromite or *N*-bromosuccinimide [5–9] (Table 1). The action of *N*-bromosuccinimide as a chemiluminescence reagent in aqueous solution is often assumed to be derived from the in situ formation of hypobromite [10–16] (Eq. (3)), but it has not been established whether the oxidation of either urea or ammonia with hypobromite and *N*-bromosuccinimide produce the same spectral distribution.



In 1962, Stauff and co-workers [17,18] reported that reacting urea, guanidine or gelatine with hypohalites gave chemiluminescence with very similar spectral distributions and they concluded that the same emitting species was generated by the oxidation of the following functional groups; $-\text{CONH}_2$, $-\text{C}(\text{NH})-\text{NH}_2$ and $-\text{CONH}-$. Recently, we [9,19]

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Table 1
Comparison of flow analysis techniques

Analyte	Oxidant	Enhancer	Manifold	Matrix	Detection limit (M)	Proposed emitter	Reference
Urea	Hypobromite		Two-line flow-injection and pulsed flow chemiluminescence analyser	Haemodialysis waste	1×10^{-7}	No emitter suggested	[9]
Urea	Hypobromite		Two-line flow-injection with an on-line column	Urine and goldfish-bowl water	9×10^{-7} 9×10^{-8}	Nitrogen	[7]
Urea	<i>N</i> -bromo-succinimide	Dichlorofluorescein	Three-line flow-injection	Serum	3×10^{-6}	Dichlorofluorescein, energy transfer from nitrogen	[8]
Ammonium ion	Hypobromite		Two-line flow-injection, with a glass filter between the flow-cell and detector	Rainwater and fogwater	6×10^{-6}	Nitrogen or nitrogen dioxide	[5,20]
Ammonium ion	<i>N</i> -bromo-succinimide	Dichlorofluorescein	Two-line continuous flow with auto-sampler	Fertiliser samples	2×10^{-6}	Dichlorofluorescein, energy transfer from nitrogen	[6]

have refined the structural requirements for an intense emission to $\text{RC}(\text{NH}_2)_2$, where R is an oxo ($\text{O}=\text{}$) or a secondary amine ($-\text{NH}-$) group. Thus, emissions from the oxidation of gelatine [18] and blood serum [9] are likely to be due to the bound arginine content.

In reference to the light-producing pathway, Stauff and Schmidkunz [17] suggested that urea, once halogenated, might decompose to form intermediates that continue to react with the oxidant, to produce OH and OOH radicals. Molecular oxygen and hydrogen peroxide, formed in excited states by radical recombination, were postulated as the emitting species. In contrast, Maeda and co-workers [20] tentatively attributed the luminescence accompanying the oxidation of urea with hypobromite to the second positive band of molecular nitrogen ($^3\Pi_g \leftarrow ^3\Pi_c$). This transition can be generated in the gas phase with N_2 laser excitation [21] but is unlikely to be responsible for the emission from the oxidation of urea as (a) it is a transition between two excited states [22], (b) the upper excited state is around 1000 kJ mol^{-1} above the ground electronic state ($^1\Sigma_g^+$), [22], and (c) the spectral distribution is considerably different to that observed during this reaction [9,20].

Li et al. [8] reported the determination of urea in serum based on the chemiluminescent oxidation of this analyte with *N*-bromosuccinimide in the presence of dichlorofluorescein. Using this oxidant, the large positive interference from protein that hindered the determination of urea with hypobromite [9] was not observed. Li et al. [8] suggested that hydrolysis of urea to form ammonia in the alkaline solution was the first step in the light-producing pathway and then quoted the proposal of Halvatzis and Timotheou-Potamia [6], in which ammonia is oxidised by *N*-bromosuccinimide to form molecular nitrogen in an electronically excited state. Zhou and Chen [15] have suggested

a similar reaction pathway for the chemiluminescent oxidation of four amino acids with *N*-bromosuccinimide. Each of these groups was able to increase the luminescence intensity of their respective reactions by adding xanthene dyes (such as dichlorofluorescein and rhodamine B) [6,8,15].

Maeda and co-workers [5,20] examined the chemiluminescent oxidation of ammonia with hypobromite and found that the corrected emission increased in intensity into the near infrared. Although their knowledge of the wavelength distribution was incomplete, they remarked on the similarity to the luminescence from the $^2\text{B}_2$ excited state of nitrogen dioxide [20], which is formed in the gas-phase reaction of nitrogen oxide with ozone. This luminescence is characterised by a broad emission from 540 to 3000 nm, with a maximum intensity at around 1200 nm [23].

In this paper, we report some chemical and spectroscopic experiments that clarify some of these previous proposals and will assist in the understanding of the chemiluminescent oxidation of urea and ammonia. In addition, we examine the use of alkaline hypobromite for the determination of guanidine compounds, and have assembled a list of potential interferences that should be considered when applying these chemistries to new sample matrices.

2. Experimental

2.1. Instrumentation

A flow-injection analysis manifold was assembled using a peristaltic pump (Gilson Minipuls 3; John Morris Scientific, Balwyn, Vic., Australia) with bridged PVC tubing (Protech Group, Coolum Beach, Qld, Australia) to propel solutions through 0.8 mm i.d. PTFE tubing (Protech). Solution lines

were combined with a Y-piece. Samples were injected into the carrier stream with an automated 6-port valve (Valco Instruments, Schenkon, Switzerland). Detection was achieved using a custom-built flow-through luminometer. The luminometer consisted of a coiled flow cell (PTFE tubing, Protech) mounted flush against the window of a photomultiplier tube (PMT; Thorn-EMI Model 9924SB, Ruislip, Middlesex, UK) which was operated at 900 V by a stable power supply (Model PM28BN, Thorn-EMI) via a voltage divider (C611, Thorn-EMI). The flow cell, PMT and voltage divider were encased in a padded light-tight housing, and the output from the photomultiplier was plotted with a chart recorder (YEW Type 3066, Yokogawa Hokushin Electric, Tokyo, Japan). The development and operation of the pulsed flow chemiluminescence analyser has been previously described in detail [9,24]. In this study, the pulsed flow chemiluminescence analyser is used as a stopped-flow instrument to examine the relationship between chemical structure and chemiluminescence intensity.

Absorbance and fluorescence spectra were measured with a Cary 300 Bio UV-Vis spectrophotometer (Varian Australia, Mulgrave, Vic., Australia) and a Cary Eclipse spectrofluorimeter (Varian) fitted with a R928 photomultiplier tube (Hamamatsu, Iwata-gun, Shizuoka-ken, Japan), respectively. The spectrofluorimeter was also adapted for the measurement of chemiluminescence spectra. A continuous flow manifold incorporating an integrated glass Y-piece and spiral flow cell (0.5 mm i.d., 90 μ l volume, Embell Scientific, Murwillumbah, NSW, Australia) was placed in front of the emission window of the spectrofluorimeter. A peristaltic pump with silicone pump tubing was used to continuously pump reagent and analyte solution through 0.5 mm i.d. PTFE tubing to the flow cell where the chemiluminescence reaction occurred. The excitation source of the spectrofluorimeter was turned off (Bio/Chemiluminescence data mode) and the recorded data was an average of ten scans (1000 ms gate time, 1 nm data interval, 20 nm slit width). Emission spectra were corrected for the wavelength dependence of the detector response and monochromator transmission by multiplication with a correction factor that was established using a quartz–halogen tungsten coiled lamp (45 W) of standard spectral irradiance (OL245M, Optronics Laboratories, Orlando, FL, USA). The lamp was operated at 6.5 A dc, which was supplied by a programmable constant current source (OL65A, Optronics Laboratories).

2.2. Reagents

Deionised water (Millipore, MilliQ Water System, Bedford, MA, USA) and analytical grade reagents were used, unless otherwise stated. Solutions of hypobromite were prepared by disproportionation of bromine (Hopkin & Williams, Chadwell Heath, Essex, UK) in cold aqueous sodium hydroxide (Ajax (APS), Auburn, NSW, Australia) or by adding potassium bromide (Sigma–Aldrich, Castle

Hill, NSW, Australia) to a cold sodium hypochlorite (12.5% (w/v); APS) solution. *N*-bromosuccinimide (99%, Aldrich), urea (AR; Ajax), and ammonium chloride (BDH, Poole, UK) solutions were each prepared by dissolution of the solid in deionised water. The xanthene dyes–dibromofluorescein (Fluka), dichlorofluorescein (disodium salt, Hopkin & Williams), diiodofluorescein (Hopkin & Williams), fluorescein (disodium salt, Ajax), rhodamine B (BDH) and tetrabromofluorescein (EGA-Chemie, Steinheim, Germany) were prepared by dissolving the solid in a 0.1 M sodium hydroxide solution.

For an examination of the relationship between the structure of guanidino and related compounds and chemiluminescence intensity, the following solutions were each prepared by dissolution of the solid in deionised water followed by the appropriate dilutions: agmatine sulfate salt (1-amino-4-guanidinobutane; 99%; Sigma), amiloride hydrochloride hydrate (Sigma), arginine (Ajax), creatine (LR; Ajax), debrisoquin sulfate (Sigma), formamidine sulfinic acid (aminoiminomethane sulfinic acid; 99%; Aldrich), guanethidine monosulfate (Sigma), guanidine hydrochloride (AR; Sigma), 4-guanidinobenzoic acid monohydrochloride (Sigma), 3-guanidinopropionic acid (Sigma), guanidinosuccinic acid (*N*-(aminoiminomethyl-L-aspartic acid; Sigma), methylguanidine hydrochloride (98%; Aldrich), moroxidine hydrochloride (99%; Aldrich), pentanimidamide and streptomycin sulfate (Sigma).

3. Results and discussion

3.1. Chemiluminescence accompanying the oxidation of ammonia

Using the flow manifolds described in the experimental section, the oxidation of ammonia with hypobromite and *N*-bromosuccinimide were performed flush against the emission window of the spectrofluorimeter. After correction, the chemiluminescence accompanying the reaction with hypobromite contained an intense emission leading into the infrared (Fig. 1(i)). This is in agreement with the work of Maeda and co-workers [20]. However, we also observed relatively weak maxima at 575 and 350 nm. The spectrum for the reaction with *N*-bromosuccinimide was significantly different: a peak at 460 nm, with a shoulder around 485 nm (Fig. 1(ii)). Various research groups [10–16,25] that have used *N*-bromosuccinimide as a chemiluminescence reagent have attributed its action to the in situ formation of hypobromite (Eq. (3)). However, a study by Senanayake et al. [26] has shown that, although the equilibrium depicted in Eq. (3) does occur, the overall oxidation process is more complex. Our findings clearly show that the oxidation of ammonia with *N*-bromosuccinimide affords chemiluminescence with a different spectral distribution to that obtained with hypobromite.

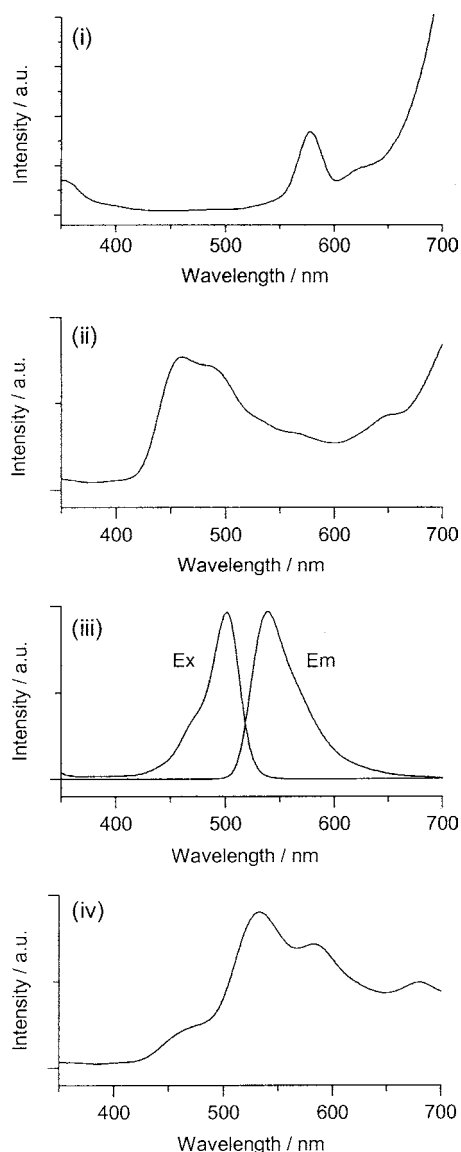


Fig. 1. Chemiluminescence spectra for the reactions of ammonia with (i) hypobromite and (ii) *N*-bromosuccinimide, (iii) the fluorescence (Ex) excitation and (Em) emission spectrum of dichlorofluorescein and (iv) the chemiluminescence spectrum for the reaction of ammonium with *N*-bromosuccinimide in the presence of dichlorofluorescein. Each emission spectrum has been corrected.

3.2. Chemiluminescence accompanying the oxidation of urea and related species

A three-line flow-injection manifold was used to merge urea (0.03 M) and sodium hydroxide (0.6 M) streams, prior to combining with *N*-bromosuccinimide (0.02 M), in front of the emission window of a spectrofluorimeter with the light source switched off. The wavelength distribution of the chemiluminescence was similar to that of the oxidation of urea with hypobromite: a broad peak with a maximum intensity at approximately 700 nm (corrected) [9,20]. This is significantly different to the oxidation of ammonia with either hypobromite or *N*-bromosuccinimide. Therefore, at

Table 2

Chemiluminescence intensities for reactions between hypobromite and mixtures of urea (1×10^{-5} M) and some xanthene derivatives (1×10^{-4} M) using a two-line flow-injection analysis system

Xanthene dye	Relative chemiluminescence intensity	
	Blank	Urea
	0	100
Fluorescein	21	257
Dichlorofluorescein	154	516
Dibromofluorescein	22	394
Diiodofluorescein	17	136
Tetrabromofluorescein	38	367
Rhodamine B	17	521

least at these high concentrations, the light-producing pathway of the reaction between urea and *N*-bromosuccinimide relies on the equilibrium shown in Eq. (3) [26], and not the hydrolysis of urea to form ammonia as suggested by Li et al. [8].

Using the pulsed flow chemiluminescence analyser, a series of guanidino compounds (and some related species) were each oxidised with hypobromite (prepared by adding 200 μ l bromine to 50 ml of a 0.4 M sodium hydroxide solution). Maximum chemiluminescence intensity (peak height) was compared to the emission detected during the oxidation of urea, as shown in Table 2. Although the oxidation of substituted ureas does not result in the intense chemiluminescence observed with the parent compound [9], many mono-substituted guanidino-compounds resulted in greater emission intensities than guanidine. Is not unexpected that streptomycin and amiloride produce an intense emission; Halvatzis et al. [27] have shown that the oxidation of these species with *N*-bromosuccinimide generates chemiluminescence and they utilised this for the analysis of pharmaceutical preparations.

3.3. Sensitised chemiluminescence

Energy transfer from an excited state reaction intermediate to an efficient fluorophore has been widely utilised in analytical chemiluminescence to increase signal intensity and improve detection limits [6,8,28–33]. Xanthene dyes, such as fluorescein [28,29], dichlorofluorescein [6,8,29] and rhodamine B [30,31] have been extensively used for this task. An increased background signal is commonly observed when using xanthene derivatives, owing to reaction between the sensitiser and chemiluminescence reagent [34].

We combined ammonia and a relatively low concentration of dichlorofluorescein with *N*-bromosuccinimide, and were able to observe both the unsensitised peak and a more intense peak, which was similar to the characteristic photoluminescence emission of dichlorofluorescein (Fig. 1(iii) and (iv)). Another peak was also present, which may have resulted from energy transfer to an oxidation product of the

sensitiser, although it is in a similar position to a peak observed in the unsensitised oxidation of ammonia (Fig. 1(i)). Oxidation of the sensitiser without the analyte resulted in a small background emission. The absorbance spectrum of the dichlorofluorescein (Fig. 1(iii)) significantly overlaps with the emission from the oxidation of ammonia with *N*-bromosuccinimide (Fig. 1(i)). This evidence supports the suggestion by Halvatzis and Timotheou-Potamia [6] that dichlorofluorescein enhances this reaction by accepting energy from an excited state intermediate.

The addition of xanthene dyes has significantly improved the detectability for a number of *N*-bromosuccinimide induced chemiluminescence reactions [6,8,15,16,27,29]. However, when we evaluated these compounds as sensitisers for the oxidation of urea by hypobromite, the increased blank or background signal (depending on the manifold) negated the advantage of greater emission intensity (Table 3). Townshend and Wheatly [31] arrived at the same conclusion with respect to the addition of enhancers to the chemiluminescent reaction of 2,4-dinitrophenylhydrazine and acidic potassium permanganate.

We also found that the chemiluminescence spectrum for the reaction between urea and alkaline hypobromite in the presence of xanthene dyes was similar to the characteristic fluorescence emission of the dye. Although the fluorescence quantum yields of fluoresceins decrease considerably with greater halogenation [35], in this study some of the halogenated fluoresceins provided greater enhancement of the chemiluminescence intensity than the parent compound. This was also observed by Chen et al. [34] in a study on the oxidation of fluorescein and related compounds with alkaline hydrogen peroxide (catalysed by cobalt(II)). Similarly, Mori et al. [36] found that halogenated fluoresceins (in the 2', 4', 5' and 7' positions) were oxidised faster than the parent compound but substituting chlorine onto the free benzoid moiety of fluorescein increased its resistance to decomposition.

Using a combination of molecular orbital calculations, chemiluminescence spectra and the fluorescence and absorbance spectra of the reaction of products, Chen et al. [34] proposed a degradation mechanism initiated by superoxide attack at the central carbon, leading to polyphenol intermediates known to chemiluminesce when oxidised. Energy transfer to unreacted fluorophores accounted for the dominant emission corresponding to their characteristic fluorescence. However, upon addition of excess hypobromite, we observed a rapid red shift in the visible colour of a fluorescein solution, followed by gradual fading to the characteristic yellow hue of hypobromite. Given that 4',5'-dibromofluorescein can be prepared by bromination in aqueous sodium hydroxide solution [37], it was thought that this may account for the initial red shift. Support for this notion was obtained by comparing the absorbance spectrum of fluorescein with its di- and tetra-bromo derivatives (Fig. 2(i)), and the preliminary intermediate formed during reaction between fluorescein and hypobromite (Fig. 2(ii)). As hypohalites and hydrogen

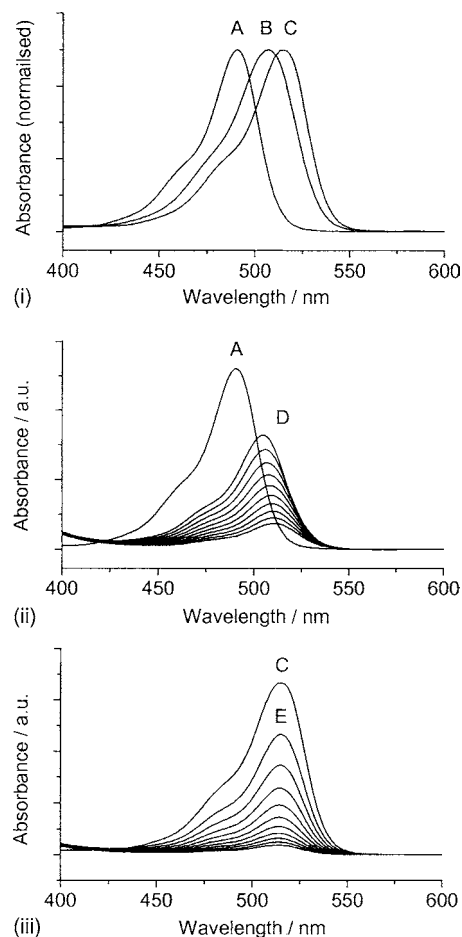


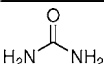
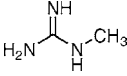
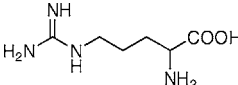
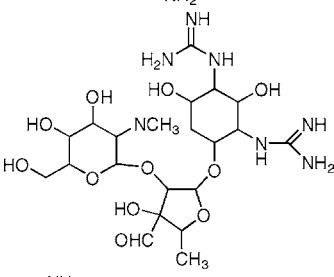
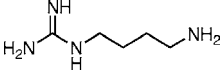
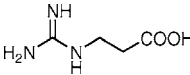
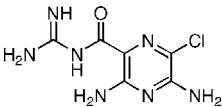
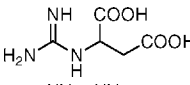
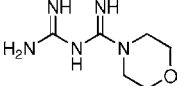
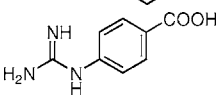
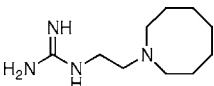
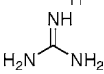
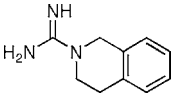
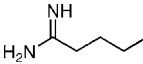
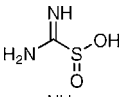
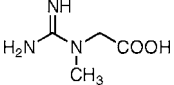
Fig. 2. Absorbance spectrum distribution for (A) fluorescein, (B) dibromofluorescein and (C) tetrabromofluorescein, and (D, E) changes due to the addition of hypobromite.

peroxide are commonly used in chemiluminescence reactions [38], a greater understanding of the emission mechanism for the oxidation of xanthene dyes and the mode of energy transfer will facilitate a more systematic approach to sensitiser selection.

3.4. Interfering species

A survey of the literature has enabled us to assemble a list of potential interferences that should be considered when applying this chemistry to new sample matrices. Table 4 shows the compounds that have been identified as analytes or interferents in chemiluminescence reactions involving either hypobromite or *N*-bromosuccinimide. Although this list appears extensive, the reagents are quite selective against a variety of compounds that are related to urea and ammonia. Substituted ureas and alkyl amines, carboxylic acids, and most amino acids, common inorganic compounds and metal ions produce little or no interference [5,7,9]. Interestingly, there is a difference in the reactivity of hypobromite and *N*-bromosuccinimide towards urea and ammonia. In the oxidation of ammonia with *N*-bromosuccinimide, urea was

Table 3
Oxidation of guanidino-compounds and related species with hypobromite

Compound	Structure	Relative Intensity
Urea		100
Methylguanidine		~100 ^a
Arginine		90
Streptomycin		72
Agmatine		66
3-Guanidinopropionic acid		48
Amiloride		17
Guanidinosuccinic acid		11
Moroxidine		10
4-Guanidinobenzoic acid		4
Guanethidine		2
Guanidine		2
Debrisoquin		0.6
Pentanimidamide		0.6
Ormamidinesulfinic acid		0.4
Creatine		0.3

^a Approximate value, chemical formula (C₂H₇N₃·nHCl).

Table 4
Potential interferents for chemiluminescence reactions involving hypobromite or *N*-bromosuccinimide

Species	Reference
Ammonium	[5,7,9,39]
Arginine	[9,15]
Bilirubin ^a	[40]
Glycine, serine, threonine ^a	[15]
Guanidino compounds	[9,27]
Humic acid	[5,7,41]
Hydrazine and linear hydrazides	[12,25,42]
Hydrogen peroxide	[43]
Pharmaceuticals ^a	[16,25,44,45]
Protein	[9,40]
Pyrogallol	[5,7]
Selected complexing agents ^a	[6]
Selected metal cations ^a	[6,15]
Strong reducing agents (sulfite, nitrite, ascorbic acid)	[5,6,8]
Sulfide	[6,29,33]
Urea	[5,7,9]

^a These species have only been observed as interferents using *N*-bromosuccinimide.

noted as a severe interferent only at concentration ratios of greater than one [6]. In contrast, the oxidation of ammonia with hypobromite produces a far lower emission intensity that the oxidation of urea (using a conventional photomultiplier tube) [7].

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