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Review

Chemiluminescence as diagnostic tool. A review

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Abstract

The principles of chemiluminescence and its applications as diagnostic tool are reviewed. After an introduction to the theoretical aspects of luminescence and energy transfer, the different classes of chemiluminogenic labels including luminol, acridinium compounds, coelenterazine and analogues, dioxetanes, systems based on peroxyoxalic acid and their derivatives are described emphasizing the molecules which best fulfil the requirements of today's clinical chemistry. Applications of chemiluminescence and enhanced chemiluminescence to immunoassays, receptor assays, DNA probes, biosensors and oxygen metabolism are discussed as well as the role of enzymes in the selectivity and the sensitivity of these reactions.

Keywords: Enhanced chemiluminescence; Chemiluminogenic labels; Chemiluminescent assays; Oxygen metabolites

1. Introduction

In clinical chemistry, most of the compounds of interest are present in the body fluids at concentrations so low that common analytical methods are not efficient for their determination. In 1959, Yalow and Berson proposed to determine these products using antibody antigen reactions after radiolabeling of one of the partners, the antigen (insulin) in order to discriminate bound and free components [1]. This method is now widely used but known drawbacks of radioisotopes e.g. health hazard, waste disposal problems, short half-life, conjugate radiolysis and legislative bias have induced intensive search for alternative labels [2–4]. Moreover, the widespread opinion that radioactive labels are unsuitable for non-separation protocols increases the need for compounds allowing non-isotopic detection although homogeneous immunoassays have been described, using low-energy as well as high-energy radioisotopes, since the past decade [5,6]. Luminescence and especially chemiluminescence is one of these alternatives.

Luminescence is a term used to describe the emission light, which occurs when a molecule in an excited state relaxes to its ground state. The

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various types of luminescence differ from the source of energy to obtain the excited state. This energy can be supplied by electromagnetic radiation (photoluminescence also termed as fluorescence or phosphorescence), by heat (pyroluminescence), by frictional forces (triboluminescence), by electron impact (cathodoluminescence) or by crystallization (crystalloluminescence). In chemiluminescence, the energy is produced by a chemical reaction [7]. Since excitation is not required for sample radiation, problems frequently encountered in photoluminescence as light scattering or source instability are absent in chemiluminescence. High backgrounds due to unselective photoexcitation are absent too: there is no need for time resolved detection. Consequently, luminometers based on a rough light detection by photomultiplier tubes are among the cheapest devices in the field [8–10].

In an excellent paper, Rongen et al. have reviewed the main advantages of chemiluminescence labeling and detection in immunoassays [11]. They have pointed out the large linear response reaching up to six orders of magnitude, the fast emission of light especially when it is generated in a single flash, the high stability of several reagents and most of the conjugates (increased stability is often observed after conjugation), the low consumption of expensive reagents. They have also noted the short incubation times owing to the high sensitivity generally achieved, the full compatibility with homogeneous or heterogeneous, competitive or not competitive, direct or indirect immunoassays or immunometric assays developed in one step as well as two steps formats and finally the absence of toxicity. These statements valid until 1994, especially the last one, remain true at the present time.

Kricka and his co-workers have also published several review articles dealing with chemiluminescence. Some of these cover the early developments of chemiluminescence [12] or applications to all fields which can benefit from chemiluminescence [13] while others published regularly up to 1997 are devoted to recent advances of this method in clinical chemistry [14–21].

Although chemiluminescence has been widely used as detection method in many fields as flow injection analysis [22], high performance liquid chromatography [23], capillary electrophoresis [24] and thin layer chromatography [25], this paper will focus on applications in the field of diagnostic.

2. Generalities

Chemiluminescence, which is the phenomenon observed when the vibronically excited product of an exoergic reaction relaxes to its ground state with emission of photons, can be defined in simplistic terms: chemical reactions that emit light [26]. The chemical reaction produces energy in sufficient amount (approximately 300 kJ mol^{-1} for blue light emission and 150 kJ mol^{-1} for red light emission) to induce the transition of an electron from its ground state to an excited electronic state. This electronic transition is often accompanied by vibrational and rotational changes in the molecule. In organic molecules, transitions from a π bonding to a π^* anti-bonding orbital ($\pi \rightarrow \pi^*$) or from a non-bonding to an anti-bonding orbital ($n \rightarrow \pi^*$) are most frequently encountered. Return of the electron to the ground state with emission of a photon is thus called chemiluminescence. The excited molecule can also lose energy by undergoing chemical reactions, by collisional deactivation, internal conversion or inter-system crossing. These radiationless processes are undesirable from an analytical point of view when they compete with chemiluminescence (Fig. 1).

The fraction of molecules emitting a photon on return to the ground state is the quantum yield (ϕ_{cl}). It is the product of three ratios:

$$\phi_{cl} = \phi_c \cdot \phi_e \cdot \phi_f$$

where ϕ_c is the fraction of reacting molecules giving an excitable molecule and accounts for the yield of the chemical reaction; ϕ_e is the fraction of such molecules in an electronically excited state and relates to the efficiency of the energy transfer and ϕ_f is the fraction of these excited molecules that return to the ground state by emitting a photon. A lot of organic molecules are chemiluminogenic but the quantum yields are generally very low, typically less or far less than 1%. This inefficiency, in most cases, is due to low yield of the chemical reaction or poor energy transfer but, in some instances, the excited molecule is a poor emitter. The addition to

the system of a more efficient fluorophore results in a non-radiative energy transfer to that fluorophore which emits intense light. The emission characteristics of the chemiluminescent process is then determined by the sensitizer and the efficiency of the transfer complies with the Förster law [27]:

$$E = \frac{(d^{-6})}{(d^{-6} + R_0^{-6})}$$

where E is the efficiency, d represents the distance in Å between the centers of donor and acceptor molecules and R_0 (in Å also) is given by the equation:

$$R_0 = 9700(JK^2\phi_{cl}n^{-4})^{1/6}$$

where J is the spectral overlap integral between donor emission and acceptor absorption spectra, K the orientation factor for dipole–dipole interaction (2/3 for random orientation), ϕ_{cl} the quantum yield of the chemiluminescent energy donor in the absence of the acceptor and n the refractive index of the medium between the donor and the acceptor [28–32]. From these equations, it appears that energy transfer is precisely the most efficient when the donor is a poor emitter provided the distance between donor and acceptor be compatible with the process which requires a minimal concentration of either the donor or the acceptor close to 10 mM.

3. History

Luminous animals are known since the ancient Greek civilization but 'artificial' chemiluminescence was first described in 1877 by Radziszewski who observed the yellow light emission when oxygen was bubbled into an alkaline ethanolic solution of 2,4,5-triphenylimidazole (lophine) [33]. Fifty

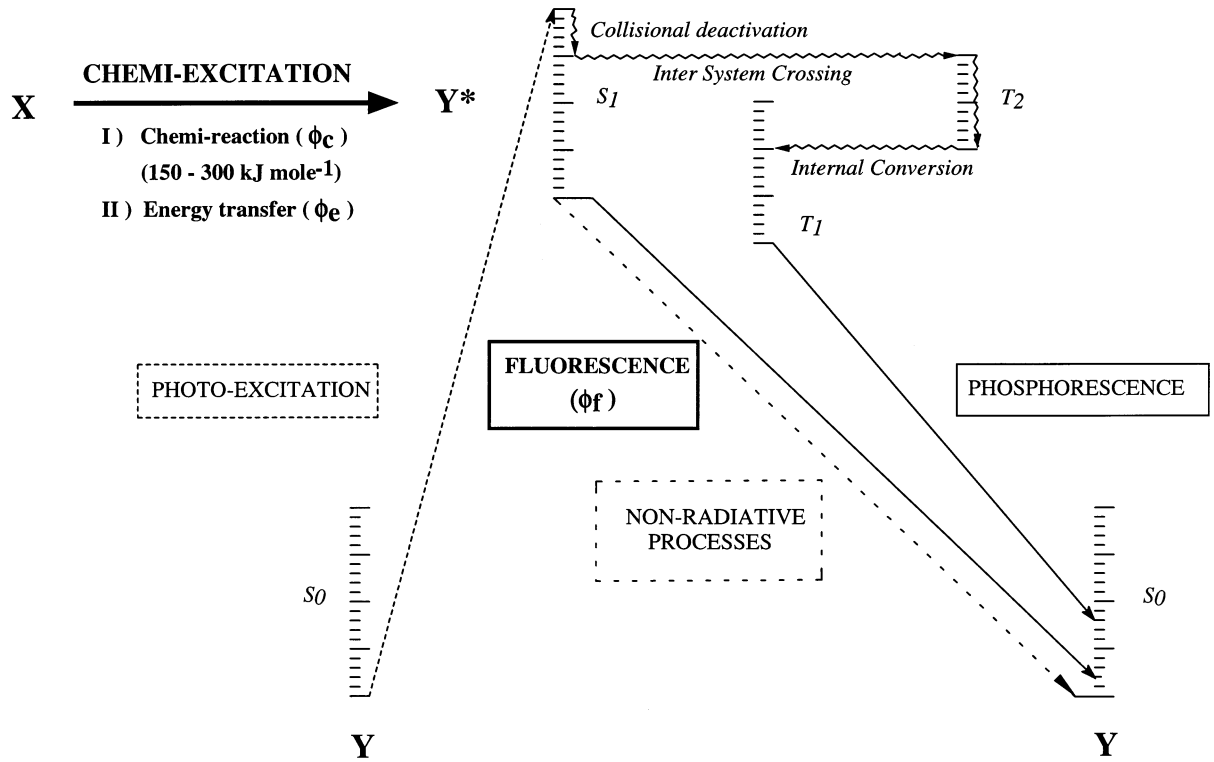


Fig. 1. Diagram placing chemiluminescence among the most typical photophysical processes. The radiationless processes are undesirable from an analytical point of view when they compete with chemiluminescence. The chemiluminescence efficiency is given by $\phi_{cl} = \phi_c \cdot \phi_e \cdot \phi_f$.

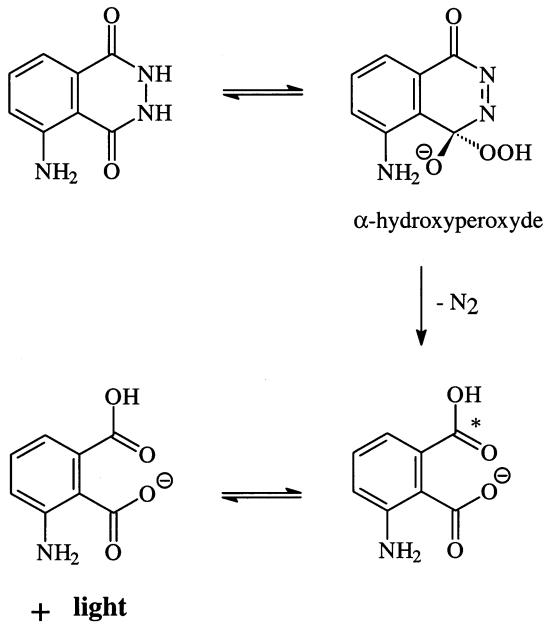


Fig. 2. Simplified reaction mechanism of luminol: the key intermediate is an α-hydroxyperoxide obtained by oxidation of the heterocyclic ring. Its decomposition leads to the aminophthalate ion with light emission.

years later, Albrecht reported the luminescent properties of 5-amino-2,3-dihydrophtalazine-1,4-dione (luminol) [34]. Acridinium derivatives were known as chemiluminogenic molecules since Gleu and Petsch, in 1935, described the blue or green light emission of bis(*N*-methylacridinium) nitrate (lucigenin) [35]. After McCapra, in 1964, has proposed a mechanism based on the formation of a dioxetanone cycle for explaining the chemiluminescence of acridinium salts [36], derivatives of dioxetane and dioxetanedione (peroxyoxalate) have been prepared and experienced [37,38].

4. Luminol, isoluminol and their derivatives

4.1. Reaction mechanism

Luminol derivatives react following a simplified reaction scheme given at the Fig. 2. The key intermediate is an α-hydroxyperoxide obtained by oxidation of the heterocyclic ring. The decomposition pattern of this intermediate leading to the excited state and the light emission is unique and depends only on the pH of the system. In contrast, the first step is strongly dependent of the composition of the medium [39].

In aprotic media (dimethylsulphoxide or dimethylformamide), only oxygen and a strong base are required for chemiluminescence [40]. In protic solvents (water, water solvent mixtures or lower alcohols), various oxygen derivatives (molecular oxygen, peroxides, superoxide anion) can oxidize luminol derivatives but catalysis either by enzymes or by mineral catalysts is required [41]. Since the beginning, many catalysts have been proposed [11,40–42]: enzymes as microperoxidase, myeloperoxidase, horseradish peroxidase, catalase, xanthine oxidase [43–45], metalloproteins as cytochrome *c* [46], haemoglobin especially haptoglobin [47], deuterohemin and mineral catalysts as molecular ozone and halogens or persulphate anion or Fe(III), Co(II) and Cu(II) cations as well as their complexes. More recently, the bacterial peroxidase from *Arthromyces ramosus* characterised by a very high turn-over has been proposed and a hundred times increase in sensitivity is claimed [48,49]. Moreover, many enzymes or enzyme mixtures that produce oxygen derivatives as by-products have been involved in chemiluminescent detection. Alkaline phosphatase, β-D-galactosidase and β-glucosidase in the presence of indoxyl conjugates as substrates [50], lactate oxidase [51], acylCoA synthetase and acylCoA oxidase [52] or diamine oxidase [53] produce hydrogen peroxide; 3-α hydroxysteroid deshydrogenase [54] or glucose-6-phosphate deshydrogenase release NADH which reduces, in the presence of 1-methoxy-5-methylphenazinium methylsulphate, molecular oxygen to hydrogen peroxide which generates light in the luminol microperoxidase system.

Optically pumped chemiluminescence is another technique proposed for diagnostic applications. Pulsed laser light put in an excited state a dye absorbing red light. Luminol can be oxidized by the excited dye with or without the participation of oxygen (Fig. 3) [55].

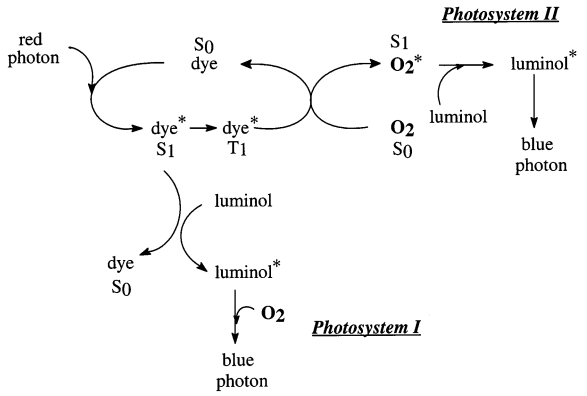


Fig. 3. Optically pumped chemiluminescence of luminol: type I and type II photosensitized oxidation reactions.

4.2. Luminescent properties

The quantum yield of luminol (Fig. 4) does not exceed 5% in dimethyl-sulphoxide [42] and 1–1.5% in aqueous systems [40,41]. Isoluminol (Fig. 4) is far less efficient ($\phi_{cl} = 0.1\%$). Attempts to improve the efficiency have been made in different ways.

It has been shown very early that structural modifications of the heterocycle ring leads to a complete loss of the chemiluminescent properties of both compounds [41,42]. On the contrary, analogues with substitution in the non-heterocyclic ring are luminogenic. Electron-donating substituents are better tolerated in position 5 or 8 than in position 6 or 7 while substitution with electron-withdrawing substituents results in a loss of the chemiluminescence [41,42,56]. Coupling diazoluminol is also very unfavorable. Alkylation of the amino group of luminol decreases drastically the efficiency ($\phi_{cl} = 0.015\%$) but the same modification is so favorable for isoluminol (Fig. 4) that

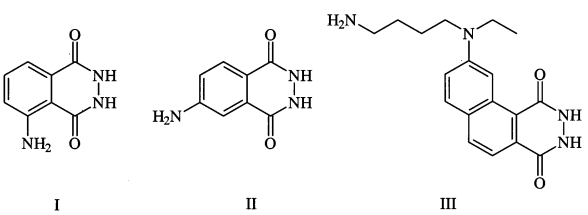


Fig. 4. Structures of luminol (I), isoluminol (II) and aminobutylethylnaphthalhydrazide (III).

the resulting efficiency rises above that of native luminol. Different chain lengths and coupling arms have been investigated (Table 1) [41,42,57,58].

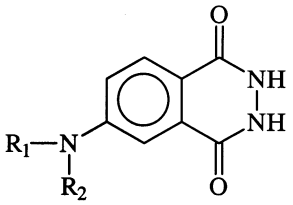
Replacement of the phenyl ring by naphthalene or benzoperylene rings leads to interesting series of molecules. Aminobutylethylnaphthalhydrazide (ABENH; Fig. 4) is about four times more chemiluminogenic than the corresponding derivative of isoluminol and emits at longer wavelengths (515 nm instead of 420 nm) which may prevent quenching or interference from other fluorophores but it is easily oxidized by air oxygen at the surface of the solution [40,57,59]. The benzoperylene derivative is twice as efficient as the former owing to a remarkable value of ϕ_e (50%) [41,42].

Enzyme cycling is another way to increase the light emission [60]. Although the chemiluminescence efficiency of the system and the light intensity are not modified, more light is emitted after a long time because the enzyme substrates are continuously recycled. Malate deshydrogenase and alcohol deshydrogenase are cycling enzymes for NADH while hexokinase and pyruvate kinase have been proposed for ATP.

Nevertheless, the most attractive mode for increasing the sensitivity is certainly the use of chemical enhancers proposed for the improvement of the horseradish peroxidase or the xanthine oxidase as well as the Co(II) catalyzed luminescence.

Carbonate and bicarbonate containing media are more effective for the detection of low peroxidase concentrations [61]. The horseradish peroxidase catalyzed reaction is also enhanced by several phenols namely 6-hydroxy-benzothiazole derivatives also called 'synthetic luciferins' or *para*-substituted phenols (e.g. *p*-iodophenol, *p*-hydroxycinnamic acid, *p*-phenylphenol, *p*-hydroxybiphenyl) [62–64]. Recently, a new class of enhancers has been proposed: 4-phenylboronic acid which is more effective with the basic isoenzyme of horseradish peroxidase (type VI A) [65]. It will be noted that this last enhancer has been found synergistic with *p*-iodophenol [66]. The mechanism of horseradish peroxidase enhanced chemiluminescence using phenol derivatives has

Table 1
Aminosubstitution of isoluminol by chains of different lengths and coupling arms



		R_1	R_2	Relative ϕ to luminol (%)
Isoluminol	–	–H	–H	5
Aminoethylisoluminol	AEI	$N–(CH_2)_2–$	–H	–
Aminoethylethylisoluminol	AEEI	$H_2N–(CH_2)_2–$	$–C_2H_5$	100
Aminobutylisoluminol	ABI	$H_2N–(CH_2)_4–$	–H	14
Aminobutylethylisoluminol	ABEI	$H_2N–(CH_2)_4–$	$–C_2H_5$	84
Aminopentylethylisoluminol	APEI	$H_2N–(CH_2)_5–$	$–C_2H_5$	–
Aminohexylisoluminol	AHI	$H_2N–(CH_2)_6–$	–H	17
Aminohexylethylisoluminol	AHEI	$H_2N–(CH_2)_6–$	$–C_2H_5$	44
Aminooctylmethylisoluminol	AOMI	$H_2N–(CH_2)_8–$	$–CH_3$	–
Aminooctylethylisoluminol	AOEI	$H_2N–(CH_2)_8–$	$–C_2H_5$	–

been studied by several authors [67–71]. Horseradish peroxidase (HRP) reacts with hydrogen peroxide to form an oxidized HRP (HRP I) that reacts with the anion of luminol to form a half reduced enzyme (HRP II) and a radical of luminol. The enzyme returns to the reduced form (HRP) by reaction with a second molecule of luminol (Fig. 5). It is suggested that catalytic phenols form preferentially phenoxy radicals in contact with horseradish peroxidase and act as electron-transfer mediators to increase the efficiency of luminol radical formation. From chromatographic data, Jansen and van den Berg confirm the increase in rate of reaction with the enzyme but, based on the product formation, they conclude that the mechanism of enhancement is probably different for the various enhancers [72]. Recently, Navas Diaz has given an electrochemical explanation of the phenomenon: only phenoxy radicals having a reduction potential greater than the redox potential of luminol at pH 8.5 (+ 0.8 V) can act as enhancers. They have also correlated the Hammett coefficients of substituents on the phenyl to inhibitory or enhancing effects. Such correlations are of course of predictive value to develop new enhancers [73]. Fluorescein has also been proposed but acts via a completely different mechanism (energy transfer) [74].

The xanthine oxidase reaction is enhanced by mineral or organic compounds. Sodium dithionite increases the emitted light by an unknown mechanism [44]. With complexes of Fe(III) with EDTA or better HEDTA (*N*-(2-hydroxyethyl)ethylenediamine-*N*,*N*′,*N*″-triacetic acid), a tremendous increase in light emission is observed in buffered solutions containing sodium perborate. This increase, due to the formation of hy-

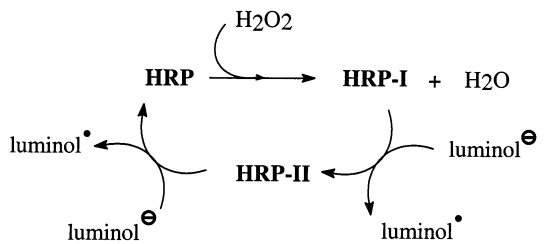


Fig. 5. The horseradish catalysed reaction mechanism: HRP reacts with hydrogen peroxide to form an oxidised HRP (HRP I) which reacts with the luminol anion to form a half-reduced enzyme (HRP II) and a luminol radical. The enzyme returns to the reduced form (HRP) by reaction with a second molecule of luminol.

droxyl radicals, is unfortunately matrix sensitive and consequently of little practical value, especially in homogeneous immunoassays [45,75–77]. Indeed, the signal is completely lost upon addition of urine or serum to a final concentration of 1%.

Penicillins have been reported to enhance the luminol–hydrogen peroxide–Co(II) system. Owing to a complexation mechanism, the β-lactam antibiotic extends the lifetime of the superoxide anion by a few orders of magnitude allowing for more efficient oxidation of luminol [78].

Protein Cu(II) complexes have been recently found more efficient than Cu(II) alone for triggering the luminol peroxide luminescence. The enhancement mechanism is not yet elucidated [79].

Surface-active agents generally impair the light emitted by enzyme mediated luminol reactions although, at selected concentrations, anionic surfactants can increase the rate of horse radish peroxidase reactions [80,81]. Nevertheless, the non-peroxidase luminol-metal porphyrin chemiluminescence reaction is enhanced by non-ionic or negatively charged detergents [82].

4.3. Applications

Luminol, isoluminol and their analogues have been applied in a very broad field including immunoassay or non-immunoassay diagnostic and monitoring techniques as well as biosensors.

4.3.1. Immunoassay applications

The chemiluminescence detection has been mainly applied to immunoassays. Because the oxidation of luminol derivatives has to be catalyzed, antigen or antibody labeling with either the catalyst or the luminogenic substrate has been investigated and heterogeneous immunoassays in various formats (direct or indirect detection in competitive or not competitive mode) as well as homogeneous immunoassays have been proposed. In an excellent review, Rongen et al. have listed more than 60 immunoassays from the different classes [11].

Isoluminol derivatives (AEEI, ABEI and, in a lesser extent, ABENH), that show increased efficiency after coupling, are almost the only tracers to be used in substrate labeled immunoassays. In the early phase of chemiluminescence development, luminol or diazoluminol conjugates have been involved too but the achieved sensitivity was poor [83]. Luminol is more efficient in the free state and is used mostly in enzyme labeled immunoassays. In the last years, enzyme labeled immunoassays using enhanced luminol detection have been proposed for the detection or the determination of prostaglandin E2 (*p*-iodophenol enhancer) [84], vascular endothelial growth factor [85], endothelin-I [86], estrone and testosterone via biotinylated steroid and horseradish peroxidase avidin conjugate [87,88], hepatitis C virus antibody [89], brucellosis and tularaemia [90], human chorionic gonadotrophine β-subunit in high range (up to 5000 IU/L) owing to microsampling and high sensitive detection [91] and free thyroxine [92]. A sensitive non-enhanced chemiluminescent assay based on glucose oxidase for human granulocyte colony stimulating factor (G-CSF) is also described [93].

Enhancers can be formed in situ by enzymatic degradation of precursors (pro-enhancers). In the presence of excess of luminol and peroxidase, the emitted light is proportional to the enzyme concentration. Sub-picomole detection of phosphatase or cholinesterase (*p*-iodophenylphosphate or naphthylacetate respectively as pro-enhancers) is easily achieved using low concentrations of released enhancer but a limited dynamic range is a disadvantage of this method. A similar procedure based on pro-anti-enhancer has also been proposed (*p*-nitrophenol enzymatically derived from *p*-nitrophenylphosphate). In the presence of excess of luminol, peroxidase and the enhancer *p*-iodophenol, a luminescent signal inversely proportional to the enzyme is obtained. One order of magnitude in sensitivity is lost using this last method. Provided conjugates of the involved enzymes to second antibodies are used, these two methods can be of general use in immunoassays. Enzyme immunoassays involving phosphatase as label can be easily turned into chemiluminescent assays by this way [94]. Prosthetogenesis is another method for enhancing luminol chemiluminescence. 0.5 attomole of alkaline phosphatase can be detected. This enzyme removes inorganic phosphate from the coenzyme FADP to give

FAD which combines with an apoenzyme (apo D-aminoacid oxidase). The reconstituted holoenzyme releases hydrogen peroxide that is detected using the luminol peroxidase system eventually itself enhanced by hydroxycinnamic acid [95].

Homogeneous immunoassays are also based on the chemiluminescence of luminol derivatives. Rongen details, in the same review [11], homogeneous immunoassays based on antibody enhancing, energy transfer and quenching of free label. Shortly, the antibody enhanced immunoassay exploits the increased light emission after antibody binding of isoluminol conjugate. This system has been applied to haptens. In the energy transfer immunoassay, a change in the ratio of chemiluminescence at 460 and 525 nm is observed when isoluminol conjugate binds to fluorescein labeled antibody or when isoluminol biotin conjugate binds to fluorescein labeled avidin [96]. Haptens as well as proteins can be determined using this method. Haptens and proteins can also be analyzed by the technique of quenching of free label: analyte and horseradish peroxidase labeled antibody compete for a microtitre plate adsorbed antibody; the chemiluminescence is generated in the presence of a yellow dye which absorbs the light emitted by the free label while the light emitted by the bound label is detected by a photographic plate positioned beneath the microtitre plate. For more specific applications (e.g. complement and antibody measurements), a homogeneous immunoassay based on the release of haemoglobin has been described. In this assay, sheep red blood cells are labeled with hemolysin or antigens for the determination of the complement or antibodies respectively. The extent of complement mediated hemolysis and, consequently, the haemoglobin dependent chemiluminescence is related to the concentration of either the complement or the antibody [97]. Another homogeneous immunoassay exemplified by the determination of anti-insulin antibody has also been proposed [68]. It is based on the fact that a close approach of an effector molecule to the active site of the peroxidase inhibits the enzyme activity. In a recent paper, Handley discusses among other topics the advantages of luminol based homogeneous ligand-binder assays [98].

Several techniques, currently used in enzyme immunoassays, have also been adapted for the chemiluminescent detection. For liposome immunoassays, entrapping of glucose oxidase allows the detection of 0.3 nM digoxin [99]. Entrapment of the glucose oxidase cofactor FAD in place of the enzyme improves the sensitivity to 10 pM because more smaller molecules of FAD can be entrapped inside the liposomes than the enzyme glucose oxidase [100]. Enzyme immunoassays involving phosphatase, β-D galactosidase or β-glucosidase have been transposed in chemiluminescent assays [101].

Optically pumped chemiluminescence has been applied to the assay of α-fetoprotein detected at 1.5 ng ml^{-1} of plasma [55].

4.3.2. Non-immunoassay applications

Apart from their use in immunoassays, luminol derivatives have been essentially employed for estimating directly a lot of compounds and for monitoring metabolic pathways even if an isolated old paper describes the use of copper-catalyzed luminescence of luminol for dosimetry [102].

A sensitive determination of tyrosine is based on the attenuation of *p*-iodophenol enhanced luminescence by competition for the 4-iodophenoxy radical. The interference of cysteine is removed by incubation with iodoacetic acid [103]. Quantification of lipid hydroperoxides in biological media has been done by direct luminescence measurements. In a first time, separation of interfering substances needs for an extraction or a chromatographic step [104–106] or an enzymatic sample pretreatment [107] but the high sensitivity achieved presently allows direct measurements in plasma using the luminol hemin reaction [108].

Substrates of enzymes releasing hydrogen peroxide are also detected. Total cholesterol determination benefits from enhanced chemiluminescence detection. The reaction proceeds in two steps: esterified cholesterol hydrolysis is performed by cholesterol esterase in the presence of sodium cholate followed by cholesterol oxidation by cholesterol oxidase and assay of the released peroxide in peroxidase reaction with luminol and *p*-iodophenol [109]. A similar reaction has been reported for the determination of free fatty acids

using the enzymes acyl-CoA synthetase and acyl-CoA oxidase [52].

Several thiols such as glutathione, cysteine or cysteamine have been determined using the copper–luminol–microperoxidase system. The time-resolved response curves were suitable for the analysis of binary mixtures of cysteine and glutathione or cysteamine and glutathione [110]. The enhancing effect of proteins on the copper catalysed luminescence of luminol has allowed the determination of different albumines in the 0.1–20 mg l^{-1} range [79].

Associated with flow injection analysis, chemiluminescence detection allows the determination of biological parameters (glucose, choline containing phospholipides, choline and acetylcholine), β-lactam antibiotics, vanilmandelic acid and trace elements (vanadium) [111–116].

Some reports on the use of chemiluminescence for the prediction of the course of infectious diseases and for the study of the antimicrobial activity of the neutrophil peroxidase system are found in the last decade literature [117,118].

When polymorphonuclear leukocytes are activated, oxygen derivatives are released that can be detected by luminol chemiluminescence. Hydrogen peroxide was first thought to be the only species involved in the luminescence [119] but it was very early suspected and then confirmed that superoxide anion and hydroxyl radicals also participate in the luminescence [120,121]. Recently, a specificity of isoluminol derivatives for the release of oxygen metabolites in the extracellular medium has been reported. This specificity has been related to the incapacity of isoluminol to penetrate the cell [122,123]. The possibility to study neutrophil metabolism by chemiluminescence has been widely exploited in the last decade. Several hundred of papers deal with this subject. Intensity of luminescence has been correlated to acute and chronic diseases [124–134]. Drugs have been reported to influence neutrophil metabolism as measured by chemiluminescence [135–139]. It is possible that, in a few cases, alterations of neutrophil metabolism be of clinical significance [136]. Studies of leukocytes chemiluminescence has led to a better understanding of inflammatory processes and oxygen metabolites related diseases [117,118,140,141].

4.3.3. Biosensor applications

Luminol derivatives are very rarely configured for biosensor applications. Aizawa et al. have described an optic fiber modified on the top by depositing an optically transparent platinum film. An auxiliary electrode is constructed around this working electrode and the potential is controlled potentiostatically against a Ag/AgCl reference electrode. At the working electrode bound and free luminol conjugates resulting from a competitive immunoassay are discriminated because only free luminol conjugate is oxidized. Light is measured using a photocounting device [142]. More classical approaches, in which the activity of immobilized enzymes is monitored using luminol luminescence, are more frequently reported [143–147].

5. Acridinium derivatives

5.1. Reaction mechanism

The mechanism has been studied in detail by McCapra [36,148,149]. The most probable mechanism is presented at Fig. 6. All intermediates, except the dioxetanone, have been isolated and characterized [150]. From spectrophotometrical (bright blue chemiluminescence from acridone and bright yellow–green chemiluminescence from its anion) and chemical (effects of base concentration and solvent composition) arguments, White hesitates to recognize the dioxetanone ring as an intermediate in the usual sense of this word but thinks that the dioxetanone does exist more probably as a transition state [151]. McCapra has also proposed other routes (see Fig. 6 for alternative routes) that do not imply the dioxetanone [149]. No catalysts are involved in all these reactions. Only hydrogen peroxide and a strong base are needed for the chemiluminescence of acridinium derivatives. However, the acridinium derivatives are in equilibrium with a non-chemiluminogenic pseudobase formed by hydroxyl substitution at the 9-position of the heterocycle (Fig. 7). This equilibrium is displaced toward the pseudobase in alkaline medium. Kinetic data related to the pseudobase equilibrium are given by Littig: the half-

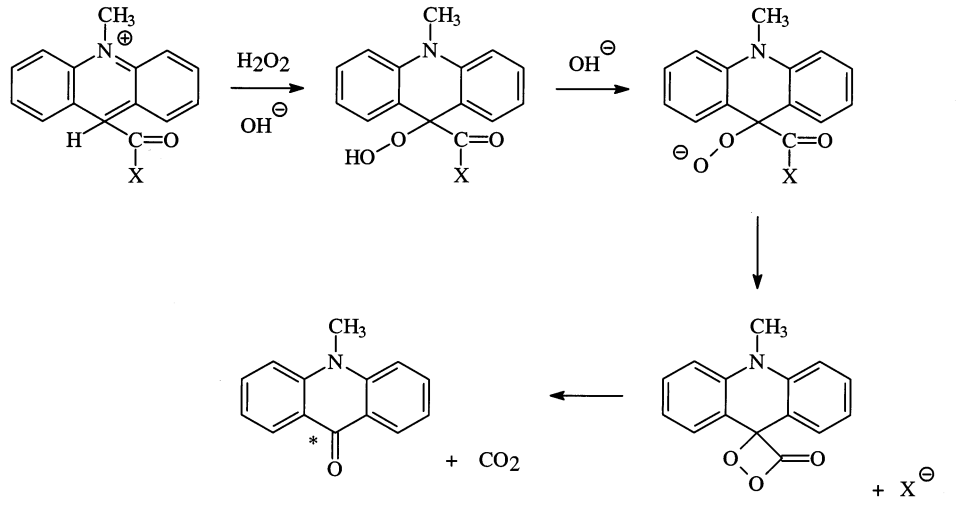


Fig. 6. Most probable mechanism of acridinium derivatives and alternative routes.

life for pseudobase formation is 26 s at pH 9, 8 s at pH 11 but only 1 s at pH 13 [152]. This rapid conversion of acridinium esters to pseudobase requires that care be taken to minimize the reagent mixing time prior to observation of chemiluminescence emission intensity. For this reason, it is of common practice to trigger the acridinium chemiluminescence by sequential addition of a solution of hydrogen peroxide in acidic medium followed by fast alkalinization using injection of a strong base.

Arylmethylene *N*-methyl dihydroacridines have epoxy and open alkylperoxides as intermediates in place of a dioxetanone one [153,154]. Lucigenin reacts with peroxide to form a dioxetane derivative [155].

5.2. Luminescent properties

Except for lucigenin and arylmethylene *N*-methyl dihydroacridines, all the acridinium derivatives are constituted of two parts: the acridinium heterocycle and the leaving group X. Each part plays a major role in the light emission.

The acridinium heterocycle, after oxidation, generates the fluorescer: the excited *N*-methylacridone which is generally released in the medium. Unsubstituted *N*-methyl acridone emits blue light while methoxy substituted *N*-methylacridone emits green light [156]. Ring substitution as well as replacement of the *N*-methyl group by an alkyl chain or a carboxymethyl group has little effect on either the quantum yield or the chemical stability but when the heterocyclic nitrogen is substituted by a carboxymethyl group which is used for protein binding, the light emitting entity will remain attached to the analyte [157,158].

The pK_a of the conjugated acid of the leaving group has a determinant influence on both the chemiluminescence efficiency and the chemical stability. The leaving group must have a pK_a of < 11 (less than the pK_a of H_2O_2) for high yields. Below that critical value, light intensity correlates with the pK_a of the leaving group [148] but very low pK_a predisposes to faster hydrolysis [159]. Phenols, thiols, sulphonamides, fluoroalcohols, heterocyclic endocyclic amines, hydroxamic and sulphohydroxamic acids, thiolamines as well as *O*-esterified oximes and chloroximes can be used as leaving groups [156,160–162] (Fig. 8). From these compounds, those having a sulphohydrox-

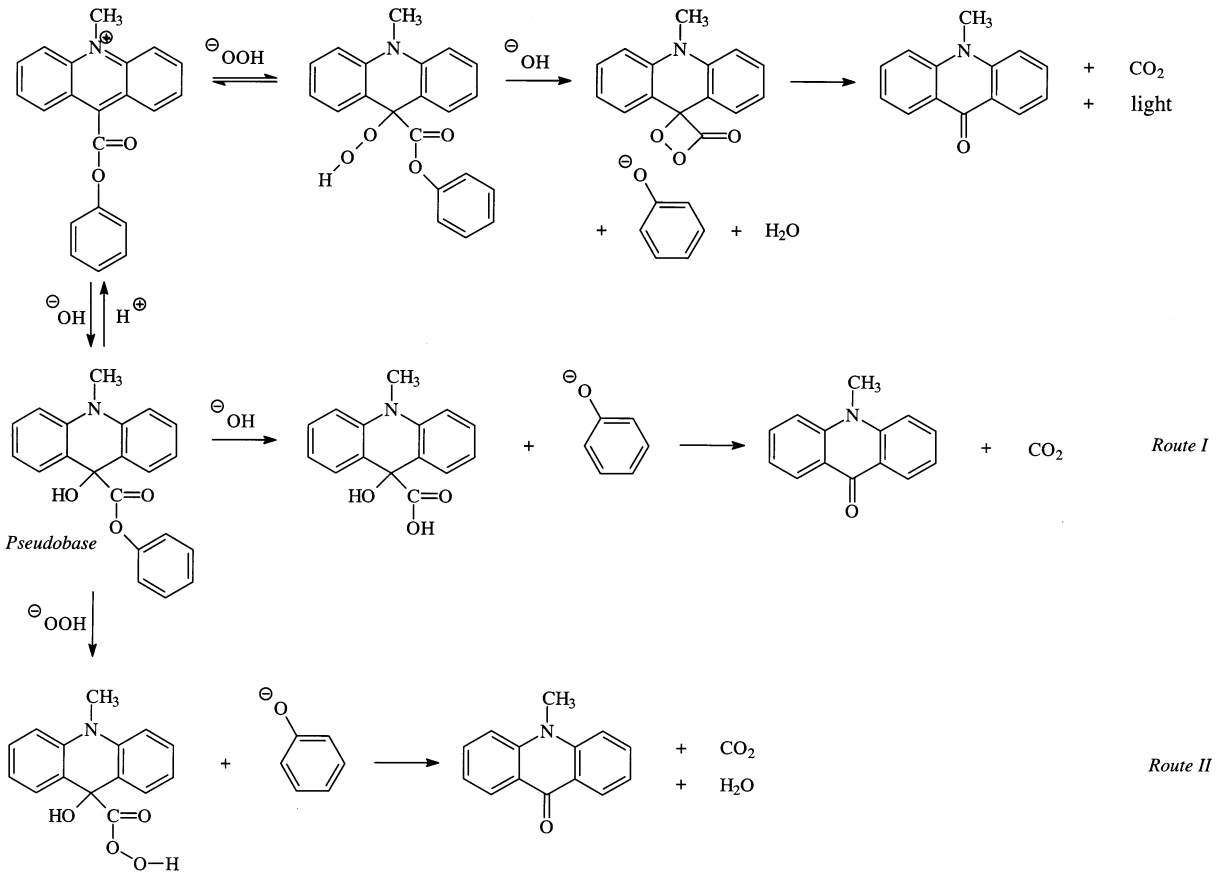


Fig. 7. Non-chemiluminescent pseudobase of acridinium ester and dark reactions via hydroxyl ion attack (I) and via reaction with both hydroxyde and peroxyde (II).

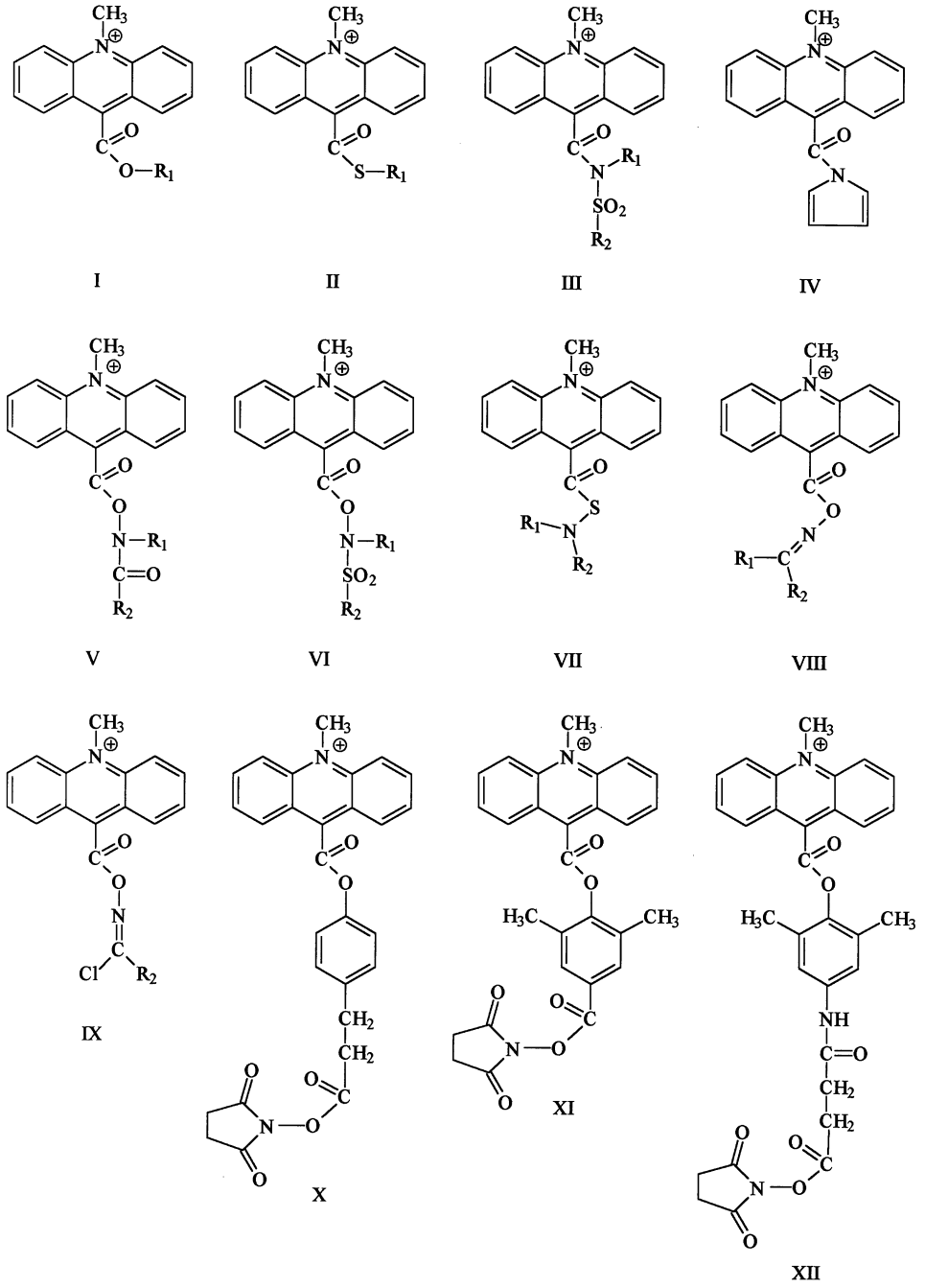


Fig. 8. Structure formulas of acridinium derivatives bearing alcohols or phenols (I), thiols (II), sulphonamides (III), heterocyclic amines (IV), hydroxamic acids (V), sulphohydroxamic acids (VI), thiolamines (VII), oximes (VIII) and chloroximes (IX) as leaving groups. Structure formulas of AE-NHS (X), DMAE-NHS (XI) and a recently developed acridinium derivative giving a long life chemiluminescence (XII).

amic acid as leaving group are probably the most chemiluminogenic acridinium derivatives presently known but the poor synthesis yields (down to 0.01% overall yield) and the complex synthesis and purification processes, especially for those bearing bulky groups, limit their use in practice [160]. Quantum yields up to 7% have been observed in the acridinium series [157]. The first acridinium derivative of practical use was the 4 - (2 - succinimidyl - oxycarbonylethyl) - phenyl - 10-methyl-acridinium-9-carboxylate (AE-NHS) (Fig. 8). AE-NHS does not show the best quantum yield and is not very stable especially at room temperature although its stability is increased after coupling. More efficient compounds are found in the thiol, sulphonamide, hydroxamic, oxime and chloroxime series [160,161,163]. Thiols and sulphonamides derivatives are often five times more luminogenic than AE-NHS [163]. A remarkable characteristic of sulphonamide derivatives is that the improvement in efficiency is not associated with a loss of stability: on the contrary, several compounds are much more stable than AE-NHS. No significant loss of efficiency has been observed even after one year at room temperature [162]. Compounds combining stability and efficiency are also found in the oxime and chloroxime series. Some oxime derivatives have intrinsic stability while, up to now, chloroxime derivatives are only stable after coupling [160,161]. Attempts to improve the stability in the phenol family has made a 2-6-dimethyl phenol analogue of AE-NHS arise (DMAE-NHS) (Fig. 8). This compound met the highest requirements in terms of stability and efficiency.

Generally, acridinium derivatives emit light as a short flash within a 5-s period or less after triggering the chemical reaction. Nevertheless, slower or faster emission rates have been observed after modification of the acridinium ring as well as after substitution of the leaving group. In the phenol series, methylation of the acridinium ring slows the kinetics of light emission; monosubstitution slows the reaction slightly but disubstitution slows the reaction even more. Electron withdrawing groups introduced in the phenyl ring increase both efficiencies and reaction rates while electron donating groups has the opposite effect. Hydrolysis rates are also affected by substituents of both the phenyl and acridinium rings. A parallelism is often observed between the effects on hydrolysis and chemiluminescent reactions because both involve nucleophilic attack, the sites of which are only one carbon apart. Nevertheless, the relative magnitude of the effects can differ greatly from one compound to another and, in some cases, increased stability can be associated to fast emission properties. Steric hindrance could explain these characteristics since most of the compounds exhibiting this stability are substituted in the *ortho* position of the phenol by bulky groups (methyl) or atoms (Br) while the same substitution in another position is not efficient from this point of view [164]. Recently, substitution of the phenyl ring by alkylcarboxamido groups has led to unexpected results: the luminescence efficiency is unaffected but the kinetics of the emission and the rate of hydrolysis is strongly dependent on the position of the substituent. At our knowledge, the *para* substituted derivatives are the slowest emitters described up to now in the acridinium series (duration ≈ 60 s) [165].

Large excess of various nucleophilic reagents (hydroxyl, thiolate and sulfite ions) attack acridinium derivatives at the carbon bearing the carboxy. The adducts formed with thiols or sulfite are more stable than the corresponding native compound or conjugate but the chemiluminescent properties are lost. The chemiluminescence is slowly recovered after water dilution (sulfite) or after reagent removal by 2-2′-pyridinedisulfide (thiols). Formation of adducts has been proposed for long term storage of unstable acridinium conjugates [166]. By reaction with hydroxyls, a non-luminogenic pseudobase is reversibly obtained. Before triggering the chemiluminescence, the equilibrium has to be displaced to the acridinium by reaction with acids (HNO_3, HCl) [166,167].

No chemiluminescence enhancers are currently used in the acridinium series although epinephrine in cationic surfactant micelles containing periodate has been reported to increase the luminescent signal of lucigenin [168]. Nevertheless, depending on the labeled protein and the matrix characteristics, the luminescent signal of acridinium esters is

increased by surfactants used at concentrations greater than the critical micellar concentration. Triton X-100 and hexadecyl trimethyl ammonium chloride (CTAC) are the most efficient ones [169].

5.3. Applications

Following the chemiluminescent reaction, the emissive species is released from the molecule and, consequently, the emission characteristics are relatively independent of the conjugate microenvironment. Moreover, high quantum yields and low background signals (no need for catalysts) allow very sensitive detection. These reasons combined with the easy coupling to protein using activated esters or imidates [167,170] explain why the chemiluminogenic acridinium derivatives have been early proposed for ultrasensitive immunoassays of thyroid stimulating hormone (TSH) [171–173], tumor markers (α-fetoprotein) [174,175], immunoglobulins and related compounds [167]. In the last decade, some competitive and many non-competitive immunoassays have allowed the determination of various analytes at the sub-picomolar level: human growth hormone [176,177], interleukins, interferons and related peptides [178–181], antibodies [182], proinsulin [183], parathyroid hormone related peptide [184], apolipoprotein B [185] and haptens [186–188]. A chemiluminesence receptor assay has been described for vitamin B12 [189]. This assay need for a full calibration curve for avoiding a bias at low concentration.

Acridinium compounds reveal their best capabilities of labeling DNA strands to do chemiluminogenic DNA probes. Indeed, after inclusion in DNA helix, acridinium labels show increased stability toward hydrolysis and no more reactivity with nucleophilic thiols and sulfites. Various non-separative determinations are based on these properties [164,190] which have also been exploited for the very accurate determination of hybridization rate constants and thermodynamic affinities of oligonucleotide probes binding to simple synthetic targets as well as to complex biological targets [191]. Multianalyte determinations based on different decay kinetics of several acridinium derivatives have been made using DNA probes although these methods could probably be applied for labeled antibody based assays [189, 190].

In the acridinium series, only lucigenin has been used for non-immunoassay applications namely for specific quantification of cellular superoxide anion response after oxidative stress [192–195].

Although chemiluminogenic isomers of acridinium and related heterocycles (phenanthridinium and isoquinoleinium) have been claimed more than 10 years ago, no applications have been found in the medical literature [196].

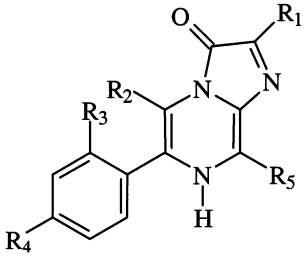
6. Coelenterazine and synthetic derivatives

The structure of coelenterazine is given at Table 2. Coelenterazine, which is the prosthetic group of a coelenterate protein [197], has been synthesized by different methods [198–201].

The chemiluminescence of coelenterazine is triggered by the superoxide anion. In contrast with luminol, the reaction is very specific and there is no need for catalytic removal of hydrogen peroxide before its determination. Consequently, coelenterazine has been proposed as a sensitive and selective chemiluminogenic probe for the study of reactive oxygen metabolite release by neutrophils [202].

Several synthetic coelenterazine analogues have been prepared up to twenty years ago [203,204]. CLA (2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one) and especially the more efficient MCLA probe (2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one) have been used up to now for monitoring superoxide [205–208]. Some other analogues have been obtained recently (Table 2) [209]. The old and new ones have been screened in a comparative study for their superoxide dependent chemiluminescent intensity [209]. The alkyl substitution at the position 5 of the imidazopyrazinone ring results in a decrease of the luminescence intensity whereas the addition of a dimethylene bridge between the position 5 and the phenyl ring bound to the position 6 dramatically increases the light emission indicating the potential usefulness of this type of compound as a probe for superoxide

Table 2
Structures of native and synthetic coelenterazines



R_1	R_2	R_3	R_4	R_5
$CH_2C_6H_4OH$ (*p*)	H	H	OH	H[a]
CH_3	H	H	H	H[b]
CH_3	H	H	OCH_3	H[c]
$CH_2C_6H_4OH$ (*p*)	H	H	OH	$CH_2C_6H_5$
CH_3	CH_2CH_2		OCH_3	$CH_2C_6H_5$
CH_3	H	H	OCH_3	$CH_2C_6H_5$
$CH_2CH(CH_3)_2$	H	H	OCH_3	$CH_2C_6H_5$
$C(CH_3)_3$	H	H	OCH_3	$CH_2C_6H_5$
CH_2CH_2COOH	H	H	OCH_3	$CH_2C_6H_5$
$CH_2CH_2CON(CH_3)_2$	H	H	OCH_3	$CH_2C_6H_5$
$CH_2C_6H_4OH$ (*p*)	H	H	OCH_3	$CH_2C_6H_5$
$CH_2CH_2COOC_6H_3Cl_2$ (*o*, *p*)	H	H	OCH_3	$CH_2C_6H_5$
CH_3	CH_3	H	OCH_3	$CH_2C_6H_5$
CH_3	$CH(CH_3)_2$	H	OCH_3	$CH_2C_6H_5$
$C(CH_3)_3$	CH_3	H	OCH_3	$CH_2C_6H_5$
$C(CH_3)_3$	H	H	OCH_3	H
CH_2CH_2CONH-α-CD	H	H	OCH_3	H
CH_2CH_2CONH-β-CD	H	H	OCH_3	H

[a] Coelenterazine (native).
[b] CLA.
[c] MCLA.

anion. The modification of the substituent in position 2 has little effect on luminescence. Particularly, the introduction of a propionyl or a propanamido group do not affect significantly the chemiluminescence intensity but hinders deleterious interactions with bovine serum albumin (BSA) and allows the covalent binding of the compound. Conjugates to α- or β-cyclodextrins are water-soluble and the latter is rather insensitive to matrix effects. In contrast with the other analogues, its luminescence is not increased by hexadecyltrimethyl ammonium bromide (CTAB), probably owing to the inclusion of the chromophore in the hydrophobic cage of the cyclodextrin.

Although, coelenterazine and its analogues have been widely used for superoxide monitoring, the main application of these compounds is undoubtedly their use as prosthetic groups of different photoproteins as aequorin, obelin, mnemiopsin, beroverin and phialidin sensitive to calcium and several other inorganic ions (lanthanides, baryum and strontium but not magnesium) [210,211]. Among these photoproteins, aequorin is the best known for immunoassay applications and intracellular calcium measurements [212,213] but these uses are beyond the scope of this review (bioluminescence).

7. Dioxetanes

From the chemiluminescence mechanism of previously developed chemiluminogenic tracers, it can be predicted that substituted 1,2-dioxetanes be luminogenic emitters [8]. 3-3,4-trimethyl-1,2-dioxetane has been synthesized since 1969. More than 200 different molecules have been prepared up to now [214].

7.1. Reaction mechanism

The dioxetanes decompose thermally, chemically or enzymatically into two carbonylic compounds, one of which is in the excited sate [214–216]. Two distinct modes are discerned (Fig. 9). The diradical mechanism mainly occurs during thermal decomposition. Very high yields of excited states are obtained but, unfortunately, often the T1 state which is rapidly quenched in aqueous solutions and, therefore, of poor utility in diagnostic applications. Enzymatic or chemical decomposition is achieved through a chemically initiated electron exchange chemiluminescence (CIEEL) mechanism: a concerted concomitant two-bond breaking process leads to an electronic redistribution and the formation of the two carbonylic products. Large S1 versus T1 ratios are generally obtained which makes this reaction much more efficient in aqueous solutions. Although these two mechanisms seem rather simple, several aspects are still discussed especially those

dealing with the rate determining step [173,216–219].

7.2. Luminescent properties

1,2-Dioxetanes, in their earliest developments (1969), were characterized by several unpleasant properties: their thermal instability, the quenching of the luminescence in aqueous solutions and the difficulty to control the luminescence process make them rather unsuitable for diagnostic applications but the great dependence of the S1 versus T1 ratios and the half-life on the molecule substituents prompted researchers to look for more stable dioxetanes [214,216,220,221]. In 1972, Wynberg describes the synthesis of the very stable adamantylidene adamantyl 1,2-dioxetane characterized by a decomposition temperature higher than 160°C and a half-life of 21 years at 25°C [222]. It has been suggested that steric effects could explain the exceptional stability of this derivative. Nevertheless, other large substituents, such as spirobiaryl groups destabilizing the dioxetane, no conclusive theory on the role of steric effects is available yet [216]. A few years later, in 1977, McCapra prepares the first asymmetrical and sufficiently stable dioxetane: the 9-(2-adamantylidene)-*N*-methylacridan-1,2-dioxetane still emits light — through the excited singlet state of *N*-methylacridone — after thermal decomposition but opens the way to a new class of asymmetrical dioxetanes whose luminescence is triggered by chemical or enzymatic removal of a protecting group of the stable state of the dioxetane [217]. In 1982, it has already been observed that chemiluminescence from a 1,2-dioxetane bearing a phenolic substituent can be triggered in aprotic solvents by the addition of a base because the deprotonated dioxetane decomposes ca. 4.4×10^6 times faster than the protonated form [223]. Unfortunately, this method does not run in a protic medium as water, the phenol–phenoxy equilibrium resulting in sufficient amounts of the deprotonated form to decrease the overall stability of the molecule. Five years later, Schaap protects the phenol substituting this function by a silylated or an ester group and triggers the luminescence by fluoride anions or arylesterase respectively [224–226]. One of the most widely used dioxetane, (3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt also named 3-4-methoxyspiro(1,2-dioxetane-3,2′-tricyclo[3.3.1.1.3,7]-decan)-4-yl phenylphosphate disodium salt (AMPPD) is based on the same concept and was prepared by Bronstein who also prepared (3-(2′-spiroadamantane)-4-methoxy-4 - (3″ - β - D - galactopyranosyloxyphenyl) - 1,2 - dioxetane (AMPGD). These compounds are the substrate of high turn-over enzymes currently used in immunoassays, alkaline phosphatase and β-D-galactosidase respectively [216]. A recent advance is the 3-4-methoxyspiro(1,2-dioxetane-3,2′-(5′-chloro)tricyclo-[3.3.1.1.3,7]decan)-4-yl phenylphosphate disodium salt (CSPD), a derivative of AMPPD [227–229].

AMPPD is stable in water solutions: its half-life is one year in slightly alkaline medium at room temperature. The light emission is simultaneously

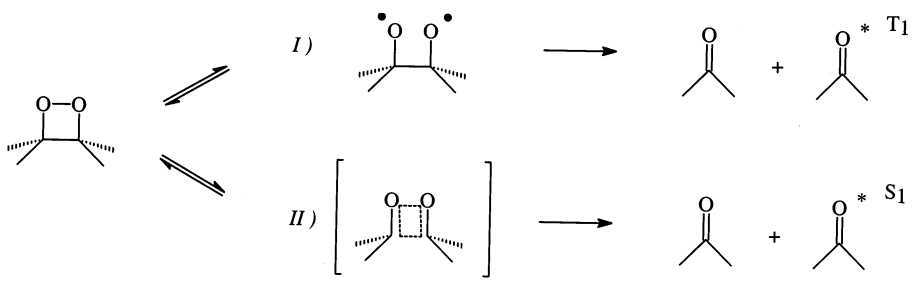


Fig. 9. The two modes of decomposition of 1,2-dioxetanes: (I) the diradical mechanism and (II) the chemically initiated electron exchange chemiluminescence (CIEEL). The diradical mechanism most often generates triplet excited states (T1) while CIEEL generally results in singlet states (S1).

controlled by the kinetics of the enzymatic deprotection and the destabilized dioxetane decomposition with a finite half-life. The result of this two-step process is a delay preceding the steady-state chemiluminescence which is proportional to the alkaline phosphatase concentration. The pH influences the velocity of both reactions and the maximum light emission occurs at pH 9. The chemiluminescence-emitting moiety is the excited state of the methyl 3-hydroxybenzoate anion that emits a glow at 470 nm [216]. CSPD and AMPPD react similarly but higher light intensities and shorter delay to reach the steady-state are claimed using CSPD. The chlorination of the adamantane moiety of AMPPD which minimizes the aggregation of CSPD and its dephosphorylated anion by altering their amphiphilic nature could explain the best properties of this new molecule [228].

AMPGD has the same behavior as AMPPD excepted for the deprotection that is carried out at lower pHs (7,5). At this pH, the phenol is in the protonated form (pK_a of the phenol = 9) and, therefore, is a slow emitter: raising the pH above 10 produces light. It will be noted that it is possible to switch the light on and off by shifting the pH in the range 7–12 [216,230].

As for the other chemiluminescent emitters, dioxetanes and especially emitters through the (3-(2′-spiroadamantane)-4-methoxy-4-(3″-hydroxyphenyl)-1,2-dioxetane anion (AMPPD and AMPGD) and its chlorinated derivative (CSPD) are more efficient (3–400 ×) when they are protected from proton quenching in solutions containing large proteins (BSA) or surfactants (CTAB) [216,230].

Indirect chemiluminescence is also possible with the dioxetanes. A very hydrophobic derivative of a fluorescer (5*N*-tetradecanoyl-aminofluoresceine) can be included in the micelles of the surfactant CTAB (Lumi-Phos™ from Lumigen, Detroit, MI, USA) or the fluorescer itself can be conjugated to the AMPPD analogues by substituting the methoxy in 4-position on the dioxetane ring [230].

The decomposition of adamantylidene adamantyl 1,2-dioxetane has been induced by a photo-excited rare earth metal [231].

7.3. Applications

For various reasons listed here above, thermally unstable dioxetanes are not suitable for diagnostic applications. Chemically triggered dioxetanes have no more attracted attention from end-users. On the contrary, probably because β-D galactosidase and phosphatase enzymes were used for a long time as labels in immunoassays and currently used enzymatic immunoassays can be adapted to chemiluminescent detection [232,233], a great interest is arisen very early in the luminogenic substrates AMPGD and especially AMPPD and CSPD, the two last ones allowing a more sensitive detection [216]. AMPGD has therefore been used for detecting trace amounts of β-D galactosidase in bacteria and transfected cells [234,235] while AMPPD and CSPD have been applied in clinical chemistry and related fields.

As seen above, the enzyme cleavable dioxetanes AMPPD and CSPD need for a rather long period of time before to reach a constant light emission. This feature represents an unwelcome added incubation time in immunoassays and explain why AMPPD and CSPD have been more frequently used in various procedures involving visualization on photographic films. An appropriate photoexcitable storage phosphor screen has been developed recently for dioxetane detection [236].

Most applications are based on DNA probes and AMPPD. HIV-1 [237], parvovirus [238,239], cytomegalovirus even in patients with AIDS [238,240], parvovirus after in situ hybridization [56] and, generally, nucleic acids [241,242] are detected. These determinations are carried out using digoxin labeled DNA probes and phosphatase labeled antidigoxin antibodies or antibody fragments. In the last case, CSPD is the used substrate. Biotinylated DNA and phosphatase labeled avidin is an alternative applied for detecting HIV-1 using AMPPD [243] or cystic fibrosis transmembrane conductance regulator gene using CSPD [229]. Direct phosphatase labeling of oligonucleotides is another possibility that has allowed the detection of human transferrin [244], hepatitis B virus [245], chlamydia trachomatis [246] and DNA in forensic analysis [247]. CSPD has been evaluated in the determination of human transferrin.

Dioxetanes have been occasionally used in enzyme immunoassays. Tumour markers as α-fetoprotein, carcinoembryonic antigen, CA-19-9 and CA125 [232,248], β-HCG, LH, FSH [216] and TSH [233] are AMPPD based examples.

After having compared different luminescent and non-luminescent detection methods, different authors have concluded that dioxetanes are among the most sensitive ones [249–251].

8. Peroxyoxalic derivatives

8.1. Reaction mechanism

Several oxalate derivatives are oxidized by hydrogen peroxide giving high-energy intermediates. A gaseous intermediate has been isolated from the reaction mixture of oxalate and hydrogen peroxide and used subsequently to produce emission in the presence of a fluorescent acceptor molecule. The proposed intermediate is dioxetanedione. In contrast with the chemiluminogenic compounds cited above, the high-energy intermediate produced in this reaction is not fluorescent and, therefore, cannot emit light by itself. Light emission undergoes through energy transfer to a fluorescer, which is excited in a S1 state. As for several dioxetanes, a CIEEL mechanism is involved in the luminescent process [252,253].

8.2. Luminescent properties

From the reaction mechanism, it is evident that the oxalate and the fluorescent sensitizer can be chosen independently. This offers flexibility because each partner of the chemiluminescent reaction can be selected either to maximize ϕ_c (oxalate derivatives) or to increase ϕ_e and ϕ_f (fluorescer) or to meet the requirements of the assay (aqueous or non-aqueous medium, buffer composition, pH, wavelength of the emitted light, conjugate synthesis). Energy can also be transferred to near infrared fluorescent acceptors [254]. In Table 3, oxalate derivatives and fluorescers are listed. Their main characteristics are also given [255,256].

By coupling the more efficient partners, overall efficiencies as high as 34% have been reported. Unfortunately, these results are obtained in non-aqueous solvents while the efficiency falls in water or water solvent mixtures to values typical for acridinium or enhanced luminol chemiluminescence.

The reaction can be carried out even in acidic media but the optimum pH is close to neutrality. Organic acids impair the reaction in contrast with weak bases especially imidazole, which is reported to have a catalytic effect through the formation of 1,1′-oxalyldiimidazole [257]. γ-Cyclodextrins are proposed to preserve a minimal efficiency in water by encageing the reagents in a low polar microenvironment [258].

High background is frequently observed in peroxyoxalate chemiluminescence. The formation and decomposition of chemiluminescence intermediates seems to be responsible for this background. This luminescence can be distinguished kinetically from fluorophor-induced chemiluminescence and is reduced at high hydrogen peroxide–oxalate ratios. Continuous reagent addition has been proposed for suppressing background emission [259].

8.3. Applications

Oxalate derivatives are not stable in water or in moist solvents. After partial water hydrolysis, the obtained monosubstituted derivative is further decomposed by decarboxylation and decarbonylation [260]. That instability strongly limits their applications in the diagnostic field. Another reason is the low water solubility of both partners of the chemiluminescent reaction: this characteristic makes them unsuitable for protein coupling.

Nevertheless, proteins labeled with 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF) have been detected using TCPO [261] and prostate specific antigen (PSA) has been indirectly determined after release by an alkaline phosphatase antibody conjugate of an indoxyl dye from the corresponding indoxyl phosphate [262].

Peroxyoxalate chemiluminescence has also been used for the detection of hydrogen peroxide forming enzymes such as uricase, choline oxidase, cholesterol oxidase, xanthine oxidase and glucose oxidase [256,258]. The determination of hydrogen

Table 3
Main characteristics of some oxalate derivatives and examples of fluorescers used

Chemiluminogenic reagent		Total CL[a]	Emitting time (s)
Bis(2,4-dinitrophenyl)oxalate	DNPO	7500	11
Bis(2,4,6-trichlorophenyl)oxalate	TCPO	8990	23
Bis[2-(3,6,9-trioxadecyloxycarbonyl)-4-nitrophenyl]oxalate	TDPO	9370	13
Bis(2,6-difluorophenyl)oxalate	DFPO	5650	97
Bis[2-(3-oxabutyloxycarbonyl)-4-bromophenyl]oxalate	MBO-1	4360	630
Bis[2-(3,6-dioxaheptyloxycarbonyl)-4-bromophenyl]oxalate	MBO-2	5000	750
4,4′-Oxalyl-bis[(trifluoromethylsulphonyl)-imino]trimethylene-bis(4-methyl-morpholinium)trifluoromethanesulfonate	MPTQ	5710	1090
Fluorescent compound			
Perylene	PE		
9,10-Diphenylanthracene	DPA		
Dipyridamole	DP		
2,4,6,8-Tetramorpholinopyrimido[5,4-d]-pyrimidine	MP		
2,4,6,8-Tetrathiomorpholinopyrimido[5,4-d]-pyrimidine	TMP		
Bis(*p*-diethylaminostyryl)benzene	DSB		
9,10-Bis(*p*-diethylaminophenylethynyl)-anthracene	DPEA		
9,10-Bis(phenylethenyl)anthracene	BPEA		
9,10-Bis(phenylethenyl)naphtacene	BPEN		
Rubrene			
3,7-Bis(dimethylamino)phenothiazin-5-ium (1+) chloride	Methylene blue[b]		
3-[4-(4-Dimethylaminophenyl)-1-3-butadien-1-yl]-1-ethylpyridinium tetrafluoroborate	Pyridine 1[b]		
[7-(Diethylamino)-3H-phenoxazin-3-ylidene]diethylammonium	Oxazine 1[b]		
3,3′-Diethylthiadicarbocyanine iodide	DTDCI[b]		

[a] Total chemiluminescence: area under the emission decay curve.
[b] Near-infrared fluorescent acceptors.

peroxide in biological fluids or homogenates is another target of this system [263,264].

Low molecular weight drugs, hormones, oligonucleotides and chemicals have been quantified after solvent extraction or column chromatography using peroxyoxalate detection. A derivatization step is often needed depending on the compound itself is fluorescent or not [259,265–268].

Any multianalyte assays are proposed until now although many fluorescers emitting in a wide range of wavelengths are known.

9. Conclusions

This review deals with the application of various chemiluminescent detection methods in the fields of diagnostic and biomedical research. Compounds belonging to five chemical classes: acylhydrazides, acridinium derivatives, dioxetanes, coelenterazines and peroxyoxalic derivatives are currently used. Each of them has advantages well balanced by some drawbacks with the result that none can be definitively preferred to the others.

Acylhydrazides like (iso)luminol are still the most frequently used chemiluminogenic compounds in immunoassays and in oxygen metabolism studies partly because they can be used for different kinds of assays but they need a catalyst for light emission and an enhancer to be competitive in terms of sensitivity. This can result in higher background signals.

Acridinium derivatives have high quantum yields even after easy coupling to proteins. As

they do not need catalysts, background signals are low and high sensitivities are frequently obtained. The instantaneous light emission, which has been considered in the past as a disadvantage (measuring problems), allows high rates in automated analyzers.

The dioxetanes used for diagnostic applications are enzyme triggered dioxetanes. As for acridinium derivatives, low background signals are observed. Moreover, dioxetanes exhibit a prolonged light emission but they need for a rather long period of time before to reach a constant signal. This last feature represents an unwelcome added incubation time in immunoassays.

Coelenterazine and its analogues are essentially used in association with catalytic proteins as apoaequorin. Used alone, it is a specific luminogenic reagent for superoxide anion.

In the presence of a fluorescer, oxalate derivatives are the most efficient non-biological emitters. Fluorescers and oxalates are chosen independently. Efficiency and flexibility are therefore the main advantages of this system. Non-resolved problems of water solubility and stability added to a loss of efficiency in water certainly explain the little success of peroxyoxalte chemiluminescence in immunoassays and biomedical applications.

In the last years, several papers dealing with new chemiluminogenic compounds and more than 1500 per year dealing with applications in immunoassays and biomedical research have been published. Although significant improvements of noise and sensitivity, new developments in multianalyte analysis and homogeneous immunoassays, advances in selectivity of coupling and triggers are expected in a near future, the chemiluminescence has already become an essential tool in medical research as well as in routine analysis.

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