Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 4, pp. 433–462, 1994
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0731-7085/94 $7.00 + 0.00

Review

Chemiluminescence and immunoassays

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Abstract: The principles of chemiluminescence and the application of chemiluminescent labels and substrates in immunoassays are reviewed. Various immunoassay formats and all known classes of chemiluminescent molecules, including 1,2-dioxetanes, luminol and derivatives, acridinium esters, oxalate esters and firefly luciferins are described as well as the many sensitizers and fluorescent enhancers. Recent promising developments are discussed.

Keywords: *Chemiluminescence; immunoassay; (iso)luminol; dioxetanes; acridinium esters; oxalate esters; bioluminescence.*

Abbreviations: Ab, antibody; ABEI, aminobutylethylisoluminol; ADP, adenosine diphosphate; Ag, antigen; AMP, adenosine monophosphate; $AMPD^-$, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-O^--phenyl)-1,2-dioxetane; AMPGD, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-β-D-galactopyranosyloxy)-phenyl-1,2-dioxetane (disodium salt); AMPPD, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane (disodium salt); AP, alkaline phosphatase; ATP, adenosine triphosphate; DNFB, dinitrofluorobenzene; EDTA, ethylene diamine tetraacetic acid; $FMNH_2$, flavin mononucleotide (reduced form); FMN, flavin mononucleotide (oxidized form); GOD, glucose oxidase; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; HRP, horseradish peroxidase; IgG, immunoglobulin G; LCIA, luminescent cofactor immunoassay; MPO, microperoxidase; NAD^+, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); PIP, *p*-iodophenol; S_1, first singlet excited state; STAV, streptavidin; T_1, first triplet excited state; TCPO, bis(2,4,6-trichlorophenyl)oxalate; XO, xanthine oxidase.

Introduction

Immunoassay methods relying on radioisotopic labels play a major rôle in medicine and related areas [1]. However, many alternatives to radioactive labels have been advocated since Yalow and Berson introduced these powerful methods [2, 3]. The major stimuli to search for alternatives are (1) safety — radioisotopes constitute a health hazard and a waste disposal problem [1, 3]; (2) stability — the relatively short-living nature of radioisotopes limits the shelf-life of labelled antigens and antibodies, and furthermore, radiolysis of conjugates is often observed [1, 3]; (3) speed — a radiolabel is not affected by antigen (Ag)–antibody (Ab) binding and therefore a separation step is always required [3]. An alternative for the radioimmunoassays might be chemiluminescent immunoassays.

Much of the current interest for chemiluminescence arises from the way molecules are being excited. Since there is no need for sample radiation, problems of light scattering, unselective excitation and source instability are absent. Therefore analysis based on chemiluminescence has many advantages [4]. (1) The extreme sensitivity of chemiluminescent analysis, resulting from the low background, is the most important advantage. Further, highly efficient and inexpensive luminometers are commercially available [4–6]. (2) Chemiluminescence has a large linear response. Since it is an emission process, the response is usually linearly proportional to the concentration, from the minimum detectable concentration up to the point where it is no longer possible to maintain excess of reactants relative to the analyte. The linear dynamic range may be up to six orders of magnitude. (3) Most chemiluminescent assays can be performed faster than non-chemiluminescent assays, especially when light is generated in a single ‘flash’. (4) Most chemiluminescent reagents and conjugates are stable. (5) Until now no toxic effects have been reported for chemiluminescent reagents. (6) Assays that use sensitive chemiluminescent markers require

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less reagent than conventional assays and are therefore cheaper. (7) Bioluminescent reactions are highly specific because of the involvement of enzymes.

Chemiluminescence is exploited in many immunoassay formats. These formats may be heterogeneous (I) or homogeneous (II) immunoassays (Fig. 1). Homogeneous assays do not require separation of free and bound fractions, because the activity of the label is altered whenever binding between Ag and Ab occurs. In heterogeneous assays, however, a separation step is always necessary, since there is no change of label activity. In competitive immunoassays (I-A) a labelled and an unlabelled Ag (or Ab) compete for a limited amount of Ab (or Ag). A 'limiting reagent' method is further classified either as an immunoassay (I-AI) when the Ag is labelled or as an immunometric assay (I-A2) when the Ab is labelled [7]. In non-competitive immunoassays (I-B) excess of reagents is used. Such an assay is classified as an indirect immunoassay (I-B2) when a secondary Ab is involved directed against a second Ab, or as a direct immunoassay (I-B1) without such a secondary Ab [7]. Schematic representations of these assay types are given in Fig. 1.

Other ways for classifying the formats of immunoassays are based on the number of antibodies involved and on the incubation time. In a one-site immunoassay the Ag (the analyte) is bound by one Ab. When the Ag is 'sandwiched' between two different antibodies it is a two-site immunoassay. For this latter assay the Ag must be sufficiently large to contain at least two independent epitopes. Two-site immunoassays cannot be used when the Ag is small (hapten).

Measurement of the Ag–Ab interaction is performed preferably at equilibrium which is, usually, very time-consuming. For shorter incubation times, when equilibrium is not attained, large systematic errors might be introduced. However, non-equilibrium assays are frequently used but require very defined incubation times.

Chemiluminescence

Absorption of UV–vis radiation by an organic molecule induces the transition of an electron from the ground state to an excited electronic state and is accompanied by vibrational and rotational changes in the molecule (Fig. 2). The excited molecule is either in a singlet (S_1) or in a triplet (T_1) state (Fig. 2). Return to the ground state (S_0) with emission of radiation is called fluorescence ($S_1 \rightarrow S_0$ transition) or phosphorescence ($T_1 \rightarrow S_0$ transition). The wavelength of the emitted photons is different (i.e. longer) from the radiation originally absorbed. Return of the electron to the ground state by non-radiative processes (Fig. 2) is an alternative pathway but is undesirable from an analytical point of view.

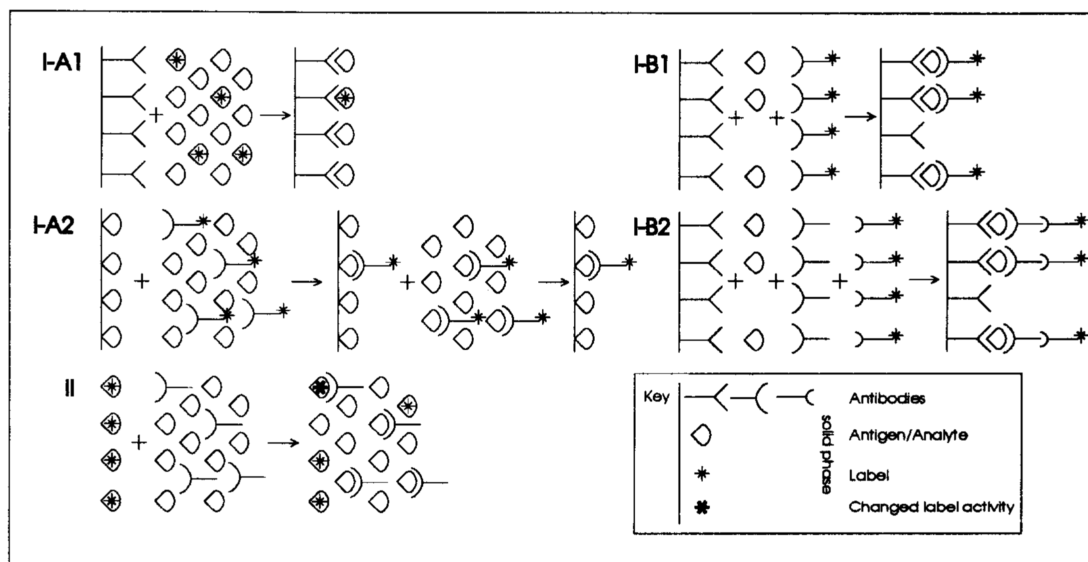


Figure 1
Schematic representations of the immunoassay types as explained in the text. The label is a chemiluminogenic molecule or an enzyme.

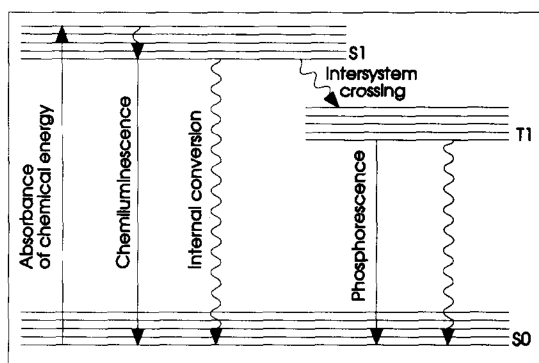


Figure 2
Diagram showing the electronic energy levels and transitions in a chemiluminescent molecule. S_0 is the singlet ground state, S_1 is the first singlet excited state and T_1 is the first triplet excited state. All states are shown with their multiple vibration energy levels.

In chemiluminescence a chemical reaction generates an organic molecule in an electronically excited state without absorption of radiation. However, the processes following excitation hold both for chemiluminescence and fluorescence/phosphorescence. In chemiluminescence, usually, the fluorescent properties of excited molecules are exploited. In most chemiluminescent processes oxidation reactions are involved providing the relatively high energy for excitation.

Bioluminescence or '*in-vivo* chemiluminescence' is found in many living organisms, where the enzyme luciferase catalyses the oxidation of luciferin.

The efficiency of chemiluminescent processes is defined by the quantum yield, ϕ_{cl}, being the fraction of molecules emitting a photon on return to the ground state. The quantum yield 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 yielding an excitable molecule, ϕ_e is the fraction of molecules in an electronically excited state and ϕ_f is the fraction of these molecules emitting a photon. The quantum yield is often very low, typically <1%.

In some instances, when the excited molecule is an ineffective emitter, sensitization or indirect chemiluminescence may be applied. By adding a more efficient fluorophore to the chemiluminescent system, excitation energy is transferred to that fluorophore resulting in a considerable increase in efficiency. The emission characteristics of the chemiluminescent process are then determined by the sensitizer.

Chemiluminescence is observed in the gas and liquid phase, and at solid surfaces [8, 9]. Gas-phase chemiluminescence has found wide applicability in the monitoring of air pollution [4]. Light production at solids has found some application [4]. However, gas-phase and solid-phase chemiluminescence are out of the scope for this review. For immunoassays only liquid-phase chemiluminescence is relevant.

Many liquid-phase chemiluminescent reactions involve peroxide and proceed through the decomposition of reaction intermediates with the simultaneous formation of carbonyl-containing molecules [4]. These classes of chemiluminescent compounds (and a representative example) are: (1) acylhydrazides (luminol); (2) 1,2-dioxetanes (AMPPD); (3) acridinium esters (lucigenin); (4) oxalates (TCPO); and (5) luciferins (firefly luciferin). These classes will be discussed successively.

Acylhydrazides (luminol)

Albrecht reported first on luminol chemiluminescence in 1928 [10–12]. Since that time, the chemiluminescence of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione, Fig. 3a) and isoluminol (Fig. 3b), as well as various derivatives has been studied extensively. All chemiluminescent reactions of (iso)luminol are oxidations being carried out in either protic (water, lower alcohols) or aprotic solvents (dimethylsulphoxide (DMSO), dimethylformamide) [11].

In aprotic media, only oxygen and a strong base are required for chemiluminescence [13]. The reaction in protic solvents usually requires a base, an oxidizing agent and either a peroxide or oxygen [11].

Iron-containing proteins (hematin) catalyse the reaction, and the amount of light emitted is proportional to the amount of protein added (when excess substrate is present). Most frequently the enzymes microperoxidase (MPO), myeloperoxidase, horseradish peroxidase (HRP) and catalase, or the substances cytochrome *c*, haemoglobin and deuterohemin are used.

Other catalysts of the reaction are ozone, halogens, Fe(III) complexes, singlet oxygen, persulphates, Co(II) and Cu(II) ions as well as their complexes [11–13]. Superoxide anion,

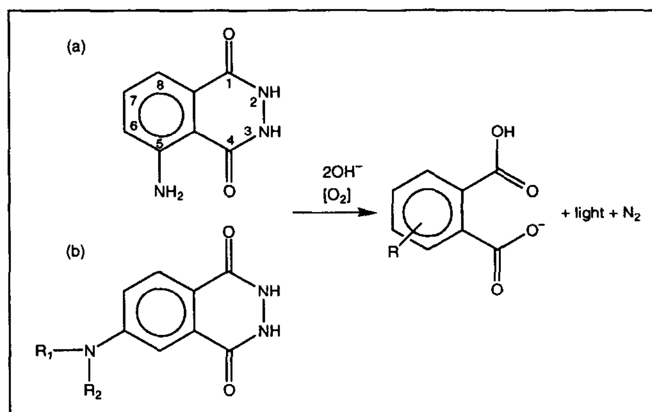


Figure 3
Overall chemiluminescent reaction of (a) luminol and (b) isoluminol (R_1, R_2 = H).

Table 1
Effects of amino-substitution of isoluminol on chemiluminescence [4]

	R_1	R_2	Relative ϕ	Detection limit (pM)
Isoluminol	H—	—H	5	30
AEI	$H_2N—(CH_2)_2—$	—H	—	—
AEEI	$H_2N—(CH_2)_2—$	$—C_2H_5$	100	1
ABI	$H_2N—(CH_2)_4—$	—H	14	20
ABEI	$H_2N—(CH_2)_4—$	$—C_2H_5$	84	2
APEI	$H_2N—(CH_2)_5—$	$—C_2H_5$	—	—
AHI	$H_2N—(CH_2)_6—$	—H	17	2
AHEI	$H_2N—(CH_2)_6—$	$—C_2H_5$	44	5
AOMI	$H_2N—(CH_2)_8—$	$—CH_3$	—	—
AOEI	$H_2N—(CH_2)_8—$	$—C_2H_5$	—	—

R_1, R_2 — are defined in the structure in Fig. 3b.
Relative ϕ is relative to luminol.
ABEI = aminobutylethylisoluminol, ABI = aminobutylisoluminol, AEEI = aminoethylethylisoluminol, AEI = aminoethylisoluminol, AHEI = aminohexylethylisoluminol, AHI = aminohexylisoluminol, AOEI = aminooctylethylisoluminol, AOMI = aminooctylmethylisoluminol and APEI = aminopentylethylisoluminol.

generated by xanthine oxidase (XO) has also been used to oxidize luminol in aqueous solution [13].

Luminol, isoluminol and derivatives

For luminol, the quantum yield, ϕ_{cl}, is about 5% in DMSO [12] and about 1.0–1.5% in aqueous systems [11, 13]. Isoluminol (Fig. 3b) shows about 5–10% of that efficiency. Several derivatives of (iso)luminol have been tested for their ability to elicit light. The ϕ_{cl} depends both on the structure and the oxidation conditions [12].

Structural modification of the heterocyclic ring of *(iso)luminol* leads to complete loss of the chemiluminescent properties [11, 13]. Analogues in which the amino group is replaced, or with substitution anywhere in the non-heterocyclic ring are generally found to be chemiluminescent [11]. These compounds vary in their light-producing ability (Table 1).

Electron-donating substituents at positions 6 or 7 of the phthalhydrazide moiety decrease ϕ_{cl} more than those at positions 5 or 8. Electron-withdrawing substituents decrease ϕ_{cl} drastically [13].

Alkylation of the amino group in isoluminol leads to a large increase in chemiluminescence capability, whereas similar substitution in luminol leads to a large decrease due to steric hindrance [11, 13]. Especially ethyl derivatives of isoluminol have shown good results. It is observed, that luminescence intensity increases with increasing length of R_2 (Table 1) [14].

Attaching luminol via the aromatic group to, for example, an analyte, is not recommended. However, isoluminol attached via the aromatic amino group is frequently used; mostly through a succinimidic derivative of either Ag or Ab [12].

Diazoluminol (Fig. 4a) has been used as a label in several assays [12]. The ϕ_{cl}, however,

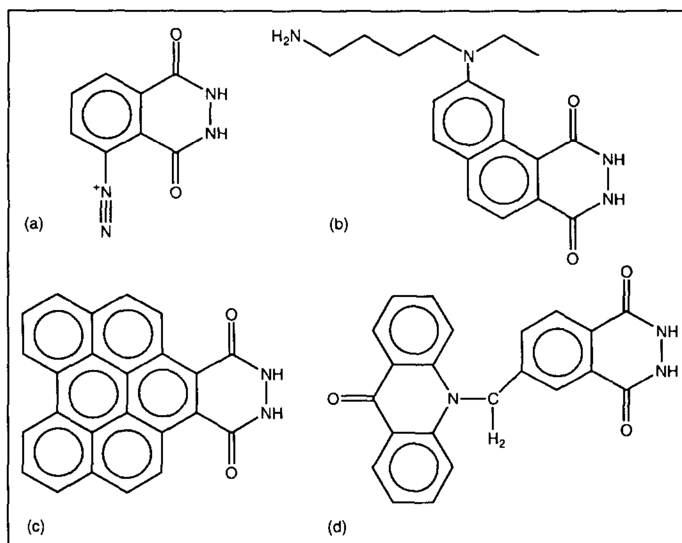


Figure 4
Structures of (iso)luminol derivatives: (a) diazoluminol, (b) aminobutylethyl-naphthalhydrazide (ABENH) [4] (c) benzoperylene derivative and (d) an energy transfer derivative (with the acridone moiety) [11].

largely decreases after coupling to an Ag or Ab. The relative ϕ_{cl} of a diazoluminol conjugate compared with luminol is about 1.3% [12].

Many *aminonaphthalhydrazides* show chemiluminescence, and substitution of the aromatic amino group has a large influence on the ϕ_{cl}. One of the most promising derivatives is aminobutylethyl-naphthalhydrazide (ABENH; Fig. 4b). Its ϕ_{cl} is about 4 times higher than the ϕ_{cl} of luminol [12, 13]. Besides this high ϕ_{cl}, ABENH emits at longer wavelength (515 nm) than luminol and isoluminol (about 420 nm). This may prevent quenching after coupling to an Ag or Ab and interference from other fluorophores [15]. A major disadvantage, however, is that these compounds are easily oxidized by air-oxygen at the surface of the solution [14].

The most efficient chemiluminescent hydrazide yet discovered is a *benzoperylene derivative* (Fig. 4c). Its relative ϕ_{cl} compared with luminol is 700–800% [11, 12]. The fluorescence quantum yield, ϕ_f, of the emitter is only 14% so that the ϕ_e is a remarkable 50%.

Linking of hydrazides to a fluorophore results in an *energy transfer derivative* (Fig. 4d), with a larger λ_{em}, reducing possible interference from other fluorophores [11].

Reaction mechanisms

(1) *The peroxidase-catalysed reaction.* Peroxidases, in particular, catalyse the (iso)-luminol reaction. The mechanism consists of: (1) the pathway leading to a key intermediate, identified as an α-hydroxy-hydroperoxide; and (2) the decomposition pattern of this key intermediate (Fig. 5) [16].

The first part is heavily dependent on the composition of the reacting system (overall concentrations, nature of the oxidant, additives, buffer and pH). In contrast, the decomposition pattern of the key intermediate is unique and depends only on the pH of the system [16].

Catalysis by HRP presumably follows a mechanism as shown in Fig. 6, in which initially an oxidized derivative of the enzyme is formed, HRP-I. Next, HRP-I is reduced to the native enzyme in two one-electron steps via a half-reduced intermediate, HRP-II [17, 18].

Enhancement of peroxidase-catalysed chemiluminescent reactions. The complex reaction mechanism offers several areas for enhancement [19]. For the peroxidase-catalysed reaction the most important enhancers are: 6-hydroxybenzothiazole derivatives ('synthetic luciferins') and *p*-substituted phenols (e.g. *p*-iodophenol (PIP), *p*-hydroxycinnamic acid and *p*-phenylphenol).

The most efficient enhancer is PIP. Enhancement by this compound is markedly pH dependent, with a significant increase in light

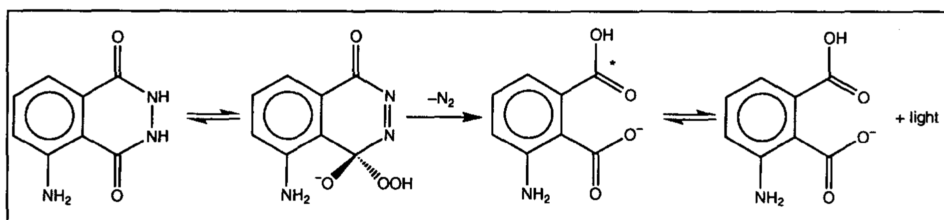


Figure 5
Simplified reaction mechanism of luminol, through the key intermediate (an α-hydroxyperoxide; second structure) into the aminophthalate ion (last structure) and light.

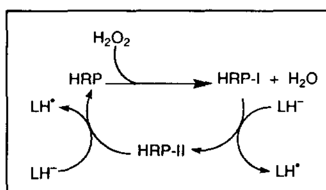


Figure 6
The HRP catalysed reaction mechanism, in which an oxidized derivative of the enzyme is formed (HRP-I), which reacts with the anion of luminol (LH^-) to form a half-reduced HRP-II and the radical LH·. The cycle is completed in a second reaction with a luminol molecule [17].

emission occurring between pH 7.0 and 9.5, and a maximum at approximately pH 8.5 [19, 20].

This enhancement by phenols and 'synthetic luciferins' only occurs when using peroxidase; other iron-containing catalysing proteins, however, show a decrease in light emission [20]. It is suggested, that in the phenol-enhanced reaction, first phenoxy radicals are formed and subsequently luminol radicals. The phenoxy radical (or in general the enhancer radical) acts as an electron-transfer mediator to increase the efficiency of luminol radical formation [17, 21].

(2) *The XO-catalysed reaction.* Xanthine and hypoxanthine are substrates for XO, a molybdenum- and iron-containing flavoprotein [22]. XO catalyses the oxidation of both substrates in the presence of molecular oxygen to yield uric acid, H_2O_2 and superoxide anion radical. A 'ping pong' mechanism is generally accepted, in which the enzyme reduction by the substrate is followed by an electron transfer from the reduced enzyme to molecular oxygen [23, 24]. This transfer may occur by two mechanisms, one giving rise to superoxide anion radical formation, the other to H_2O_2 formation. High pO_2 levels and low substrate levels give rise to the superoxide anion pathway [23]. Superoxide anion radicals induce light emission from luminol derivatives more efficiently than H_2O_2 [25].

It has been proposed that hypoxanthine is oxidized by XO in two successive two-electron transfers, since xanthine is released during the reaction (Fig. 7).

Enhancement of XO-catalysed chemiluminescent reactions. Several approaches to enhance the chemiluminescence have been reported. (1) With sodium dithionite added a sudden increase in light emission occurs. The background light emission in absence of xanthine or hypoxanthine is not enhanced. An explanation for this phenomenon is not yet available [23]. (2) Fe(III)–EDTA and sodium perborate in alkaline buffer form hydroxyl radicals which can oxidize luminol with a high efficiency (Fig. 8) [24, 26, 27]. The light

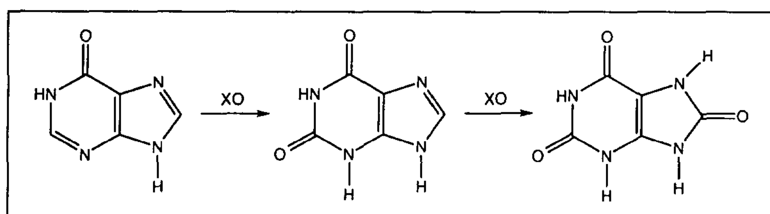


Figure 7
Xanthine oxidase catalysed reaction mechanism of hypoxanthine (and xanthine) to uric acid [22].

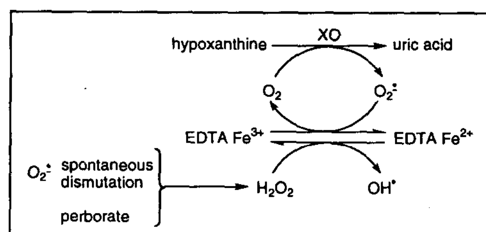


Figure 8
Simplified mechanism of Fe(III)–EDTA enhanced chemiluminescence reaction catalysed by XO [26].

emission is enhanced up to 1000-fold. Free Fe(III) ions do not enhance the reaction. The hydroxyl radicals are formed by a one-electron reduction of peroxide. Without sodium perborate, a slow and progressive increase in the light emission is observed. A low concentration of sodium perborate accelerates the reaction, through the early availability of H_2O_2 [26].

At high pH values the spontaneous dismutation of superoxide anion radical is negligible compared with the reaction with Fe(III)–EDTA, thus leading to a high generation of hydroxyl radicals [26]. A pH optimum, however, is observed (pH 9.8–10.2) because of inactivation of XO at higher pH values [24]. The long-term signal can be monitored for several days without loss of sensitivity [24, 26, 27].

All iron complexes with an ethylenediamine structure are able to generate a similar enhancement as the EDTA complex, but HEDTA (*N*-(2-hydroxyethyl)ethylene diamine-*N*,*N'*,*N'*-triacetic acid) shows additionally a lower blank signal, making this chelating agent more suitable than EDTA [24].

Upon adding urine or serum to a final concentration of 1% (v/v), the chemiluminescent light signal completely disappears, probably because of components present in the matrix. This largely limits the use of this system in homogeneous immunoassays [28].

(3) *The glucose-6-phosphate dehydrogenase catalysed reaction.* Glucose-6-phosphate dehydrogenase (G6PDH) was used for the first time in a chemiluminescent immunoassay with bacterial luciferase in 1982 [29]. The enzyme converts glucose-6-phosphate (G6P) into 6-phosphoglucono-8-lactone (6PGL, an intramolecular ester). During this reaction, NAD^+ is converted into NADH, which reduces indirectly, in the presence of the electron mediator 1-methoxy-5-methylphenazinium

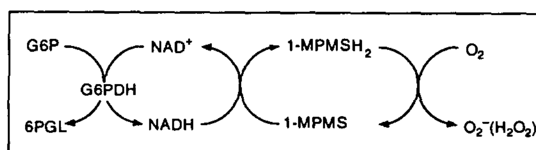


Figure 9
Scheme showing the G6PDH-catalysed chemiluminescence reaction [29].

methylsulphate (1-MPMS), molecular oxygen to superoxide anion radical and/or H_2O_2. These two species generate light in a system containing (iso)luminol (Fig. 9).

No enhancers have been described for this system yet. With this system, 1 amol of enzyme (as a label) can be detected [29].

Applications of cyclic hydrazides in immunoassays

Type I-AI assays. An advantage of substrate-labelled Ag, is the relative ease of coupling it to a wide variety of substances [4, 30, 31]. A disadvantage of most substrate-labelled Ag assays, however, is an additional incubation step with strong base for 30 min at 60°C. By this means, the label is removed from the resulting Ab-label–Ag complex to prevent quenching by the Ab and to adjust the pH for an optimal reaction condition. Another disadvantage is the short time of light production in an assay without enhancer, after addition of an enzyme [30, 31].

Enzyme-labelled Ag assays, however, should be more sensitive, because one enzyme molecule can activate many substrate molecules.

Separation of bound and free Ag is performed in four ways. (1) A wash step in microtitre plates, to which the Ab is adsorbed. (2) The Ag-specific Ab binds a secondary Ab, which is immobilized on polyacrylamide beads. A simple centrifugation step separates the bound and free fractions. (3) The Ag-specific Ab is coupled to a magnetic particle. A magnetic field will immobilize those particles on the bottom of the reaction tube, allowing a simple wash step for separation. (4) A secondary Ab or some gel-forming agent precipitates the Ag–Ab complex, while free Ag does not precipitate.

The conjugates used are all stable and possess no known hazard for health or environment. These techniques are simple, reliable, relatively fast and comparable to radio-

Table 2
Substrate-labelled chemiluminescent immunoassays type I-A1 with (iso)luminol (derivatives)

Analyte	Substrate as label	Catalyst	Enhancer
Aldosteron	ABEI conjugate	MPO–H_2O_2	No
5α-Androstane-3α,17β-diolglucuronide	AEEI conjugate	MPO–H_2O_2	No
Biotin	Isoluminol conjugate	LPO–H_2O_2	No
Cortisol	ABI conjugate	Micelles	No
Cortisol-3-CMO	ABEI conjugate	MPO–H_2O_2	No
Cyclosporin	ABEI conjugate	MPO–H_2O_2	No
Estradiol	ABEI conjugate	MPO–H_2O_2	No
Estradiol	ABEI conjugate	MPO–H_2O_2	No
Estradiol	ABEI conjugate	MPO–H_2O_2	No
Estradiol	ABEI conjugate	MPO–H_2O_2	No
Estradiol	ABEI conjugate	MPO–H_2O_2	No
Estradiol-6-CMO	ABEI conjugate	MPO–H_2O_2	No
Estriol	ABEI conjugate	MPO–H_2O_2	No
Estriol	ABENH conjugate		No
Estriol-16-α-glucuronide	ABEI conjugate	MPO–H_2O_2	No
Estrone-3-glucuronide	AEEI conjugate	MPO–H_2O_2	No
Estrone-3-glucuronide	AEEI conjugate	MPO–H_2O_2	No
17α-Hydroxyprogesterone	Isoluminol conjugate	G6PDH	No
17α-Hydroxyprogesterone	ABEI conjugate	MPO–H_2O_2	No
IgG	Diazoluminol conjugate	Haemin–H_2O_2	No
IgG	Luminol conjugate	Haemin–H_2O_2	No
Methyltestosterone	ABEI conjugate	MPO–H_2O_2	No
Methyltestosterone	ABEI conjugate	MPO–H_2O_2	No
Pregnanediol-3α-glucuronide	AHEI conjugate		No
Pregnanediol-3α-glucuronide	AHEI conjugate	MPO–H_2O_2	No
Pregnanetriol-3α-glucuronide	AEEI conjugate	MPO–H_2O_2	No
Progesterone	ABEI conjugate	MPO–H_2O_2	No
Progesterone	ABEI conjugate	MPO–H_2O_2	No
Progesterone	ABEI conjugate	MPO–H_2O_2	No
Progesterone	ABEI conjugate	MPO–H_2O_2	No
Progesterone-11-hemisuccinate	ABEI conjugate	MPO–H_2O_2	No
Somatomedin C	AEEI conjugate	MPO–H_2O_2	No
Somatomedin C	ABENH conjugate	MPO–H_2O_2	No
Testosterone	Luminol conjugate	Cu(II)	No
Thromboxane B_2	AHEI conjugate	MPO–H_2O_2	No
Thyroxine	ABEI conjugate	MPO–H_2O_2	No
Thyroxine	ABEI conjugate	MPO–H_2O_2	No
Thyroxine	ABEI conjugate	MPO–H_2O_2	No
Thyroxine	ABENH conjugate	MPO–H_2O_2	No
Transferrin	ABENH conjugate	MPO–H_2O_2	No
Triiodothyronine	ABEI conjugate	MPO–H_2O_2	No

Table 3
Enzyme-labelled chemiluminescent immunoassays type I-AI with (iso)luminol (derivatives)

Analyte	Enzyme as label	Catalyst	Enhancer
Aldosterone	HRP conjugate	Luminol–H_2O_2	PIP
Δ^9-THC	HRP conjugate	Luminol–H_2O_2	PIP
Δ^9-THC-11-oic acid	HRP conjugate	Luminol–H_2O_2	PIP
Dehydro-epiandrosterone	HRP conjugate	Luminol–H_2O_2	No
Dehydro-epiandrosterone-sulphate	HRP conjugate	Luminol–H_2O_2	No
Digoxin	HRP conjugate	Luminol–H_2O_2	PIP
Estradiol	XO conjugate	Luminol	Fe(III)–EDTA
Organophosphorous compound, MATP	HRP conjugate	Luminol–H_2O_2	Coumaric acid
Parathyroid hormone	HRP conjugate	Luminol	PIP
Thyroxine	HRP conjugate	Luminol–H_2O_2	PIP
Thyroxine	XO conjugate,	Luminol	Fe(III)–EDTA

ABEI = aminobutylethylisoluminol, ABENH = aminobutylethyl-naphthalhydrazide, ABI = aminobutylisoluminol, AEEI = aminoethylethylisoluminol, AHEI = aminohexylethylisoluminol, B/F = bound/free fraction, CMO = carboxymethyloxime, IU = international units, LPO = lactoperoxidase, MATP = methylphosphonic acid, *p*-aminophenyl-1,2,2,-trimethylpropyl diester and THC = tetrahydrocannabinol.

Table 2
Continued

Sensitivity	Range	Matrix	B/F separation	Reference
0.03 nM	0–2 nM	Serum	Magnetic field	89
13.4 pg μl^{-1}		Urine	Centrifugation	90
50 nM	50–350 nM			91
				92
54 pg $sample^{-1}$	10–1000 nM			4, 93
6.6 $\mu g\ ml^{-1}$	25–1600 $\mu g\ l^{-1}$	Whole blood	Magnetic field	95
0.18 nM		Serum	Microtitre plate	30, 31, 34
0.01 nM	0–20 nM	Saliva	Microtitre plate	30
50 ng l^{-1}		Plasma	Centrifugation	94
0.25 pg $sample^{-1}$	0–50 pg $sample^{-1}$		Microtitre plate	96
3.8 pM		Saliva	Microtitre plate	97
3.25 nM		Plasma	Aspiration	4, 98
4.5 pg $sample^{-1}$	5–750 pg $sample^{-1}$	Saliva		30, 99
<250 pg l^{-1}		Urine		100
3.8 μM		Urine		4, 101
2 pg $sample^{-1}$	2–100 pg $sample^{-1}$	Urine		34
3.25 nM		Urine		100
0.1 pg $sample^{-1}$	0.1–100 pg $sample^{-1}$	Dried blood		29
6 pg $sample^{-1}$		Dried blood	Charcoal	103
10 $\mu g\ sample^{-1}$				4
5 $\mu g\ sample^{-1}$		Serum	Precipitation	4
125 pg g^{-1}	0–800 pg g^{-1}	Muscle tissue		103
3.1 pg $sample^{-1}$	1–400 pg $sample^{-1}$		Microtitre plate	104
2 pg $sample^{-1}$	10–100 pg $sample^{-1}$	Urine		34
30 pg $sample^{-1}$	30–2000 pg $sample^{-1}$	Urine	Aspiration	4, 106
1.6 $\mu g\ l^{-1}$	1.6–3.2 $\mu g\ l^{-1}$	Urine		105
0.54 nM	0.6–127 nM	Serum	Centrifugation	30, 32
0.04 nM	0–10 nM	Saliva	Centrifugation	30, 33
0.32 nM		Saliva	Microtitre plate	30
2 pg $sample^{-1}$	2–100 pg $sample^{-1}$	Plasma	Magnetic field	33
4 pg $sample^{-1}$	15–1000 pg $sample^{-1}$	Plasma	Aspiration	4, 107, 108
16 pg $sample^{-1}$		Serum		109
16 pg $sample^{-1}$		Serum		109
0.1 mg $sample^{-1}$				4, 110
57 pM				4
5.3 nM	33–515 nM	Serum		111
25 nM				4
100 pM				4, 112
2 pM				4, 13
13 amol $sample^{-1}$	13–160 amol $sample^{-1}$	Seminal fluid	Precipitaton	15
0.23 nM	1.4–12.3 nM	Serum		111

Table 3
Continued

Sensitivity	Range	Matrix	B/F separation	Reference
100 fg $sample^{-1}$	140–1180 pM	Saliva/plasma	Microtitre plate	113
0.13 $\mu g\ l^{-1}$	0.1–4 $\mu g\ l^{-1}$	Urine	Microtitre plate	114
0.13 $\mu g\ l^{-1}$		Urine	Microtitre plate	114
25 pg $sample^{-1}$	25–1000 pg $sample^{-1}$	Serum	Microtitre plate	115
100 pg $sample^{-1}$	0.1–10 ng $sample^{-1}$	Serum	Microtitre plate	115
0.4 nM	0.4–6.4 nM	Serum	Microtitre plate	116
20 pg ml^{-1}	20–2000 pg ml^{-1}	Serum	Microtitre plate	26
50 nM		Serum	Microtitre plate	117
1.0 pg $sample^{-1}$	1–115 pg $sample^{-1}$		Microtitre plate	118
10.3 nM	10–323 nM	Serum	Microtitre plate	116
	0–50 $\mu IU\ ml^{-1}$	Serum	Microtitre plate	27

immunoassay results in sensitivity [30–34]. Type I-AI assays using (iso)luminol (derivatives) are presented in Tables 2 and 3.

Type I-A2 assays. Only one type I-A2 assay with a luminol derivative has been reported [35]. In this assay the hapten or Ag is immobilized onto a solid phase by adsorption or by covalent coupling via a carrier protein. The first antibody is biotinylated and a streptavidin (STAV)–ABEI conjugate serves as a universal marker. This assay generates an amplification because of the exceptionally strong affinity between STAV and biotin. Light emission is measured after washing and addition of an enzyme or catalyst and H_2O_2 at high pH value. No loss of immunoreactivity is observed due to the biotinylation [35]. Cortisol and progesterone-11-hemisuccinate have been measured with this assay, using both MPO and catalase in the presence of H_2O_2. Sensitivities have not been reported [35].

Type I-BI assays. Assays with substrate-labelled second Ab are summarized in Table 4. The prolactin and hGH (human growth hormone) assays both use a biotinylated second antibody and a STAV–ABEI conjugate as a label. Theoretically the sensitivity of these reactions is limited only by the Ab avidity for the Ag and the label performance [35, 36].

In the hGH assay, a 10-fold improved sensitivity has been found, due to the use of the STAV–biotin principles, compared with the use of labelled second Ab systems.

Examples of assays with enzyme-labelled second Ab are given in Table 5.

Type I-B2 assays. This assay is a modification of type I-BI. The modification consists of the use of a HRP-conjugated Ab, directed against the second Ab, resulting in higher specificity.

Anti-hepatitis B surface Ag [37], *Listeria monocytogenes* serogroup 4 Ag [38], and *Legionella pneumophila* serogroup 1 urinary Ag [39] are assayed, using fluorescein isothiocyanate (FITC) in a 'FITC–anti-FITC' system. The FITC link is used here, because of the high affinity of the anti-FITC Ab for FITC.

Type II assays. In homogeneous assays, separation is not necessary. However, often a purification step prior to the immunoassay is applied to remove interfering substances from the biological matrix (e.g. an organic solvent extraction for steroid assays). In homogeneous assays a labelled Ag competes with unlabelled Ag for the Ab. The activity of the label changes whenever binding to an Ab occurs. Three types of homogeneous immunoassays have been reported.

(1) *Antibody-enhanced immunoassay.* Steroid-isoluminol derivatives emit light with higher intensity, when the conjugate is bound to an Ab. In most cases, both the peak intensity and the total light intensity are increased whenever binding occurs. In some cases the peak intensity is decreased and delayed in time, but the total light intensity remains of higher value after binding. The reasons for these changes are unknown [40]. Examples of these Ab-enhanced assays are presented in Table 6.

(2) *Energy-transfer immunoassay.* When ABEI-labelled antigens bind to FITC-labelled antibodies, there is a change in the ratio of chemiluminescence at 460 nm (blue) and at 525 nm (green). These energy-transfer assays are at least as sensitive as the conventional RIAs [41] (Table 6).

(3) *Quenching of free label.* In this assay, a photographic plate is positioned beneath a microtitre plate. Analyte and HRP-labelled antigen compete for the microtitre plate-adsorbed Ab. After incubation, a 'yellow dye', luminol, PIP and H_2O_2 are added. Light produced by the HRP conjugate can only reach the photographic plate when this conjugate is very close to this plate, thus only Ab-bound conjugate will be detected. Light emitted by the free conjugate will be absorbed by the 'yellow dye' [42] (Table 6).

Recent developments in luminol-based immunoassays

(1) *Assays using pro-enhancers or pro-anti-enhancers.* These assays are based on the enzymatic generation of enhancers or anti-enhancers from pro-enhancers or pro-anti-enhancers, respectively [43].

Pro-enhancers. An alkaline phosphatase (AP) assay has been described [43]. The substrate is *p*-iodophenylphosphate (PIPP), which is converted by AP to PIP, which is a potent enhancer of the peroxidase-catalysed reaction, whereas PIPP is not. In the presence of a

Table 4
Chemiluminescent immunoassays type I-B1 using isoluminol derivatives

Analyte	Label	Catalyst	Enhancer	Sensitivity	Range	Matrix	Reference
Hepatitis B surface antigen	AHEI conjugate	MPO–H_2O_2	No	1 ng ml^{-1}	2–50 ng ml^{-1}	Serum	119
IgG	AHEI conjugate	MPO–H_2O_2	No	0.5 ng $sample^{-1}$			120
Prolactin	ABEI conjugate	MPO–H_2O_2	No	<5 ng $sample^{-1}$		Plasma	36
TBG	ABEI conjugate			3 μg ml^{-1}	0–50 μg ml^{-1}	Serum	121
hGH	ABEI conjugate	Catalase	No	0.6 pg $sample^{-1}$		Plasma	35
hGH	ABEI conjugate	MPO–H_2O_2	No	0.7 pg $sample^{-1}$		Plasma	35

ABEI = aminobutylethylisoluminol, AHEI = aminohexylethylisoluminol, hGH = human growth hormone and TBG = thyroxine binding globulin (thyrotropin).

Table 5
Chemiluminescent immunoassays type I-B1 using luminol

Analyte	Label	Catalyst	Enhancer	Sensitivity	Range	Reference
Factor VIII related Ag	HRP conjugate	Luminol–H_2O_2	PIP		0–0.5 IU ml^{-1}	20
α-Fetoprotein	HRP conjugate	Luminol–H_2O_2	Luciferin			122
Human choriogonadotropin	HRP conjugate	Luminol–H_2O_2	PIP			20
IgE	XO conjugate	Luminol	Fe(III)–EDTA	0.2 kIU ml^{-1}		26
TSH	XO conjugate	Luminol	Fe(III)–EDTA		0–50 μIU ml^{-1}	27

IgE = immunoglobulin E, IU = International Unit and TSH = thyroid stimulating hormone.

Table 6
Type II chemiluminescent immunoassays using (iso)luminol (derivatives)

Analyte	Label	Catalyst	Enhancer	Sensitivity	Range	Matrix	Reference
Antibody enhanced homogeneous immunoassays							
Cortisol	APEI conjugate	MPO–H_2O_2	No	20 pg sample^{-1}	20–1000 pg sample^{-1}	Plasma	40
Estriol	APEI conjugate	Hematin		10 pg sample^{-1}	10–100 pg sample^{-1}		123
Estriol-16α-glucuronide	ABEI conjugate	MPO–H_2O_2	No	10 pg sample^{-1}	10–100 pg sample^{-1}	Urine	124
Progesterone	ABEI conjugate	Hematin	No				123
Total estrogens	ABEI conjugate	MPO–H_2O_2	No	4 μg l^{-1}	5–500 μg ml^{-1}	Urine	125
Progesterone	AHEI conjugate	MPO–H_2O_2	No	25 pg sample^{-1}	25–400 pg sample^{-1}	Plasma	126
Energy transfer immunoassays							
cAMP	ABEI conjugate	MPO–H_2O_2	No	25 fmol sample^{-1}			41
Complement component C9	ABEI conjugate	MPO–H_2O_2	No	100 pg sample^{-1}			41
IgG	ABEI conjugate	MPO–H_2O_2	No	17 fmol sample^{-1}			41
Progesterone	ABEI conjugate	MPO–H_2O_2	No	0.88 pmol sample^{-1}			41
Quenching of free label							
Testosterone	HRP conjugate	Luminol–H_2O_2	PIP	150 pg sample^{-1}	150–16000 pg sample^{-1}		42

ABEI = aminobutylethylisoluminol, AHEI = aminohexylethylisoluminol, APEI = aminopentylethylisoluminol, cAMP = cyclic adenosine monophosphate, MPO = microperoxidase and PIP = *p*-iodophenol.

limited amount of AP, the light emission is proportional to the enzyme activity [43].

Pro-anti-enhancers. An alternative assay uses *p*-nitrophenylphosphate (PNPP), which after conversion by AP yields PNP (*p*-nitrophenol), an inhibitor of the PIP-enhanced peroxidase catalysed reaction. In the presence of a limiting amount of AP, the light emission is inversely proportional to the enzyme activity [43]. The detection limits for AP, using pro-enhancer or pro-anti-enhancer, are 100 amol and 1 pmol, respectively [43].

At low concentrations of released enhancer or anti-enhancer, large differences in light emission are observed, making a sensitive assay possible. A disadvantage is the limited dynamic range [43].

α-Fetoprotein has been measured in a sandwich-type assay (type I-B1) with an AP-labelled second Ab, using the pro-enhancer PIPP and a luminol–H_2O_2–HRP mixture. The sensitivity obtained was 3.2 $\mu g\ l^{-1}$. The sensitivity for the analogous colorimetric assay is 16.6 $\mu g\ l^{-1}$ [43].

(2) *Liposome chemiluminescent immunoassay.* In 1989 a new, sensitive immunoassay combining a liposome immunoassay with chemiluminescent detection was developed [44]. Lysis of the liposomes with a melittin conjugate releases entrapped glucose oxidase (GOD). GOD plus added substrate produces H_2O_2, which in turn generates light in the presence of isoluminol and MPO.

Two types of assays for digoxin have been developed, both using the liposome lysing capability of free ouabain–melittin conjugate (ouabain resembles digoxin), whereas anti-digoxin antibody-bound conjugate does not. The best assay has a sensitivity of 0.3 nM digoxin.

A modification of this assay has the GOD-cofactor flavin adenine dinucleotide (FAD) incorporated in the liposomes. The sensitivity is improved to 10 pM digoxin because more (smaller) molecules of FAD can be entrapped inside a liposome than GOD [45].

(3) *Flow-injection chemiluminescent immunoassays.* A non-equilibrium competitive flow-injection enzyme immmunoassay for thyroxine (T_4) involving affinity separation of the immunocomplex of HRP-labelled antibodies and Ag from the free labelled antibodies has been described [46, 47]. An enhanced chemiluminescence reaction is used for detection of Ab-conjugated HRP. T_4 and the anti-T_4–HRP conjugate are separated by T_4 immobilized on a column. A T_4-concentration of 10^{-11} M can be detected [46]. The column containing the immobilized T_4 is not regenerable but can be used for more than 100 assays. The total assay time is <5 min.

A flow-injection sandwich enzyme immunoassay, with HRP as a label, for human IgG has also been described [46, 48, 49]. IgG (analyte) and anti-IgG–HRP conjugate are injected. A single polystyrene bead with immobilized anti-IgG Ab is placed in a reaction cell. After incubation and separation, luminol, PIP and H_2O_2 are added to the bead and chemiluminescence is measured. The detection limit for human IgG in this assay is 1 nM; the assay time is <15 min [46, 48].

Another flow-injection immunoassay for mouse anti-bovine IgG uses a transparent thin-layer flow cell as the immunoreactor with antigen immobilized on a membrane. The on-column detection with a photomultiplier tube is with PIP-enhanced luminol chemiluminescence. The detection limit was 1 fmol with an assay time of 10 min [50].

(4) *Two-phase separation of free and bound analyte.* The antigen HRP, which is bound to a monoclonal Ab can be separated from free HRP in an aqueous system, consisting of two phases: 5.4% (w/w) PEG 6000 and 9.0% (w/w) dextran [51]. The partition coefficient of HRP is 1.00–1.05; the partition coefficient for Ab-bound HRP is 0.10–0.15. This system is used to separate Ab-bound Ag and free Ag very quickly and quite efficiently. However, other separation systems are very efficient as well, so the practical importance of this separation procedure has to be proven yet.

(5) *Luminescence biosensors in homogeneous immunoassays.* Optical biosensors are based on molecular recognition by biomolecules, followed by optical changes in absorbance, reflectance, fluorescence or luminescence. Immunosensors with a luminescent label are attractive due to a wide dynamic range and low detection limits. Peroxidase has been utilized as a label with luminol in immunoassays for IgG and β_2-microglobuline [52].

Luminol can be employed as an electroactive

label in an electrochemical luminescence technique (ECL). At the top of an optical fibre an optically transparent electrode (a platinum layer) is constructed. An auxiliary electrode is constructed around the optical fibre. The potential of the working electrode is controlled against a Ag/AgCl reference electrode. The optical fibre is connected to a photon counter. The luminol-labelled Ag emits light after oxidation at the electrode, while Ab-bound conjugate generates no light, probably because of steric hindrance of the structures involved in the electrochemically-induced luminescence process.

This principle has been used in a homogeneous immunoassay in which human serum albumin (HSA, analyte) and HSA–luminol compete for anti-HSA Ab. With this technique, 3 μM HSA can be detected [52].

Conclusions

In conclusion, cyclic acylhydrazide chemiluminescent immunoassays are the most frequently used chemiluminescent immunoassays. Most promising techniques are those using enhancers, such as PIP in combination with a peroxidase and Fe(III)–EDTA–perborate in combination with XO. With these enhancers, not only higher signals are measured, increasing the sensitivity, but the light is emitted over a prolonged period of time, facilitating light measurement.

An advantage of cyclic acylhydrazides is, that they are easily conjugated and can be used for different kinds of assays. A disadvantage is, that they need a catalyst for light emission, leading to higher background signals.

1,2-Dioxetanes

These four-membered peroxides were synthesized in 1969, as a direct result of the prediction that they should be chemiluminescent [4, 53]. Most 1,2-dioxetanes require high temperatures for their decomposition into two ketonic products (Fig. 10).

The mechanism of decomposition is rather simple and well understood. Saturated dioxetans produce remarkable high yields of excited states, unfortunately, often the T_1 states [4]. Since T_1 states are rapidly quenched in aqueous solution, the high yield of excitation is not available for detection [4]. The S_1/T_1 ratio can be altered by variation of substituents [54].

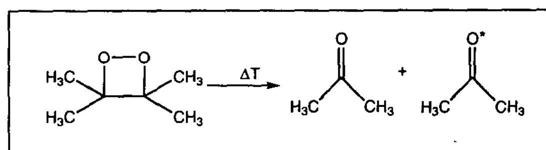


Figure 10
High-temperature decomposition of 1,2-dioxetane into two ketonic products [4].

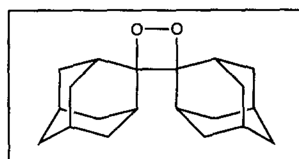


Figure 11
Structure of adamantilydene adamantane-1,2-dioxetane [55].

Substituent effects on the rate of decomposition of 1,2-dioxetanes are substantial and these rates (expressed as $t_{1/2}$) vary from a few seconds to several years [53, 55]. The most stable 1,2-dioxetane discovered yet is adamantylidene adamantane-1,2-dioxetane (Fig. 11), with a $t_{1/2}$ of 21 years at 25°C. This very low rate of decomposition is attributed to a steric effect [53].

Asymmetrically substituted 1,2-dioxetanes, which bear a single adamantyl group, decompose more rapidly than the bisadamantyl derivative, but are sufficiently stable for analytical applications [53]. Other large substituents, such as spirobiaryl groups, destabilize the 1,2-dioxetane, so no conclusive theory about steric effects is available yet [53].

Decomposition of 1,2-dioxetanes

1,2-Dioxetanes decompose thermally into two carbonylic compounds, one of which is in the excited state [53]. Two distinct modes are discerned [53, 56, 57]. (1) The stepwise or diradical mechanism is based on thermodynamic calculations [53, 56–58] (Fig. 12). The first step is rate limiting [53]. This mechanism mainly occurs during thermal decomposition and often low efficiencies of chemiluminescence are obtained, due to a small S_1/T_1 ratio [53]; (2) the chemically initiated electron exchange chemiluminescence (CIEEL) mechanism suggests a concerted, concomitant two-bond breaking process, which may lead to an electronic redistribution and the formation of the two carbonylic products (Fig. 12).

The latter mechanism applies to 1,2-dioxetanes with easily oxidizable substituents [53, 59]. Probably most 1,2-dioxetanes decompose via this CIEEL mechanism [53]. The ϕ_{cl} is usually very high due to large S_1/T_1 ratios [53].

Chemiluminescence activation of 1,2-dioxetanes

The addition of heat to 1,2-dioxetanes causes a decomposition via the diradical mechanism [53]. The removal of a substituent is followed by a CIEEL-like decomposition [53].

In 1982 it was demonstrated, that chemiluminescence from a 1,2-dioxetane bearing a phenolic substituent could be triggered in organic solvents by the addition of a base [60, 61]. Deprotonation generates an unstable phenoxide-substituted dioxetane, which decomposes ca. 4.4×10^6 times faster than the protonated form [60, 61]. Later, other methods were developed for inducing the splitting of thermally stable dioxetanes. Silyloxy-substituted dioxetanes can be triggered by F^- ions [53, 55, 61]. Enzymatic triggering was described in 1987 for the first time [55, 60–62] using arylesterase and AP (Fig. 13).

In all cases, chemiluminescence is derived from the singlet excited aromatic moiety. The rate of enzymatic decomposition is dependent upon the enzyme concentration, while total light emission is not [58, 60].

The basic design of dioxetane based substrates for enzymes should incorporate several important functions in one molecule [53, 55]: (1) a stabilizing moiety (adamantyl nucleus); (2) a group with emissive properties (often a derivatized aromatic substituent, but sometimes a fluorophore; indirect chemiluminescence is observed after an intermolecular electron transfer); and (3) an enzyme-labile function, which causes chemiluminescent activation after removal.

Based on this latter concept, two promising 1,2-dioxetanes have been developed: AMPPD and AMPGD.

AMPPD (3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt) is converted by AP into $AMPD^-$ (Fig. 14). Light emission from AP-activated AMPPD is in the form of a 'glow' [53, 55]. This result is characteristic for a two-step kinetic process. First, in the presence of excess substrate (AMPPD), enzymatic dephosphorylation proceeds at a constant rate proportional to enzyme concentration. Second, the $AMPD^-$ anion produced, decomposes kinetically. The result is a delay preced-

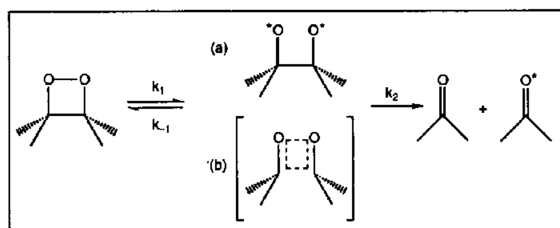


Figure 12
Pathway of (a) diradical, and (b) the chemically initiated electron exchange chemiluminescence (CIEEL) mechanism [53].

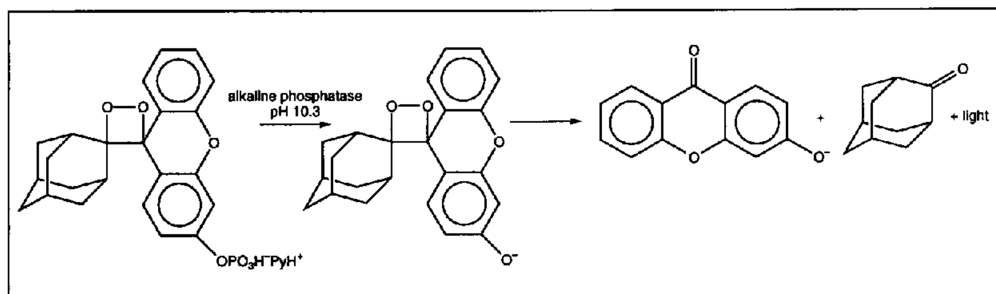


Figure 13
Enzymatic triggering of chemiluminescence from 1,2-dioxetanes by alkaline phosphatase [62].

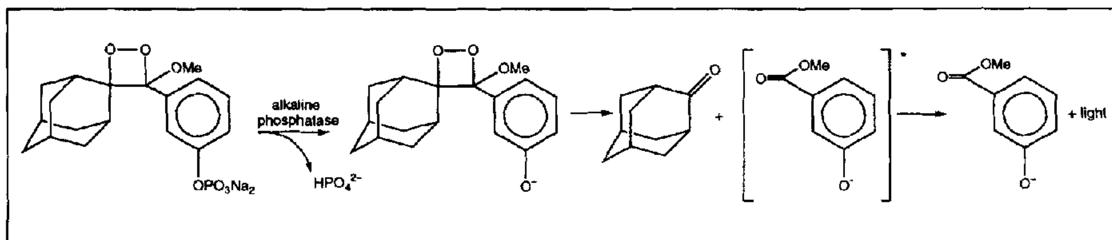


Figure 14
Conversion of AMPPD into AMPD$^-$ (second structure) and light, catalysed by alkaline phosphatase [55].

ing the steady-state chemiluminescence [53, 55]. The plateau which the light intensity reaches is proportional to the AP concentration.

The pH influences the velocity of enzyme-catalysed reactions by affecting the ionization state of functional groups in the active sites of the enzyme. Maximum chemiluminescent light emission (470 nm) from AP-activated decomposition occurs at pH 9.0 [53].

Non-enzymatic hydrolysis of AMPPD (or background) is extremely low at alkaline pH, and becomes only significant below pH 6.0 [53].

The chemiluminescence-emitting moiety is the excited state of the methyl *m*-oxybenzoate anion (Fig. 14). The pK_a of the ground state AMPD$^-$ anion is 9.0. Hence, the intensity of light emission can be dramatically enhanced, when the pH is increased above the pK_a of AMPD$^-$ [53]. Switching the light on and off is possible with this system by simply changing between pH 9.5 and 7.0 [55]. Using AMPPD, 10^{-20} mol of AP can be detected.

AMPGD (3-(2′-spiroadamantane)-4-methoxy-4-(3″-β-D-galactopyranosyloxy-phenyl)-1,2-dioxetane) is a direct chemiluminescent substrate for β-D-galactosidase. AMPGD carries a β-D-galactopyranosyl group that is split off by the enzyme, producing the protonated form of AMPD$^-$ at pH 7.5. When the pH is raised to 12, the anion is formed and chemiluminescent light emission occurs [53]. With AMPGD 10^{-13} mol of β-D-galactosidase can be detected [53].

Enhancement of the AMPPD–AP chemiluminescent reaction

(1) *Addition of macromolecules.* Light emission is enhanced when water-soluble macromolecules, including bovine serum albumin (BSA), or aqueous micelles made with a modified fluorescent molecule and cetyltrimethylammonium bromide are added [53, 55]. The reason for this enhancement (3–400-fold) is probably the interaction of the long-living AMPD$^-$ anion with the hydrophobic microdomains of the enhancer. Since the emitter is an excited-state charge-transfer intermediate, which is easily quenched by proton transfer, the exclusion of water from the immediate vicinity of the emitter increases the relative intensity [53]. The viscosity of BSA and other polymers may also play a rôle in enhancing the emissive pathway. Use of aqueous micelles formed from a fluorescent molecule combines these advantages with the principle of indirect chemiluminescence. With these micelles 1.6×10^{-21} mol AP has been detected [55]. CTAB, plus a fluorescent enhancer, is also used in Lumi-Phos^{TM} (Lumigen Inc., Detroit, MI, USA), a commercially available substrate reagent for AP.

(2) *Energy transfer enhancement.* Another way of signal enhancement has been proposed, utilizing energy transfer to fluorescers (coupled to the methylether, cf. Fig. 14) [55].

Application of 1,2-dioxetanes in immunoassays

Only type I-B1 immunoassays have been described for 1,2-dioxetanes. The results reveal a 4–67-fold improvement in sensitivity, compared with other available methods. All assays use AP as a label and AMPPD as a substrate. The myeloperoxidase assay uses a biotinylated second Ab and an avidin–AP conjugate; the other assays use Ab–AP conjugates. The interferon-γ assay is an indirect chemiluminescent assay; sensitization is performed by use of a fluorophor [63] (Table 7).

Recent developments in dioxetane-based immunoassays

(1) *Use of other enzymes.* The development of modified 1,2-dioxetanes, which can be

Table 7
Immunoassays using the AP–AMPPD system

Analyte	Label	Substrate	Enhancer	Sensitivity	Range	Matrix	Reference
β-Human chorionic gonadotropin	AP conjugate	AMPPD	No	<0.2 mIU sample^{-1}	0.2–200 mIU sample^{-1}		53
α-Fetoprotein	AP conjugate	AMPPD	No	0.03 ng ml^{-1}	0–400 ng ml^{-1}	Serum/amniotic fluid	53
Interferon-γ	AP conjugate	AMPPD (Lumi-Phos™)	Yes (unknown)	10 pg ml^{-1}	10–500 pg ml^{-1}	Plasma	63
hLH	AP conjugate	AMPPD	No	1 ng ml^{-1}	1–10 ng ml^{-1}		53
Myeloperoxidase	AP conjugate	AMPPD	No		0–600 μg l^{-1}		127
TSH	AP conjugate	AMPPD	BSA	4.5 μIU l^{-1}		Serum	128

hLH = human luteinizing hormone, IU = international unit and TSH = thyroid stimulating hormone.

triggered by other enzymes than AP and β-D-galactosidase is being investigated now (sulphatases, aryl esterases and ureases). These may develop into more sensitive assays with 1,2-dioxetanes in the future [55].

(2) *Multi-analyte immunoassays.* The position of the trigger function on the aryl ring relative to the dioxetane ring is of importance: very high singlet chemi-excitation efficiencies are observed, when the triggering function is positioned *meta* on the aryl moiety [55]. Molecular orbital calculations of this system revealed, that the largest amount of charge can be transferred from a donor (e.g. the aryl-O^- anion) to an acceptor (the peroxide O atom) if a *meta* ('odd') rather than *para* ('even') relationship exists [55, 56].

Not only the ϕ_{cl} varies with substitution, but also the emission λ_{max} [56]. The fact that different trigger functions generate light of different wavelengths could be used to design 'multi-analyte' assays, to measure different antigens simultaneously in a single sample [55, 56]. Currently, investigations have been initiated with dual channel prototypes, using naphthyl and phenyl substituents with enzyme activation by galactosidase and AP [56].

(3) *New 1,2-dioxetanes.* Recently, a new, more sensitive substrate for AP, a chlorinated 1,2-dioxetane (CSPD) has been developed by Tropix Inc. (Bedford, MA, USA). The structure of this new compound is unknown. CSPD emits light at higher intensity and the maximum in the light output is achieved earlier in time.

It is obvious, that in the future more 1,2-dioxetanes will be developed for use in immunoassays.

Conclusions

In summary, this ultrasensitive detection system using 1,2 dioxetanes, and especially, AMPPD with AP offers several advantages [55]: (1) the sensitivity is similar or greater than radioactive detection; (2) elimination of complex chemiluminescent systems, involving extensive optimization of the different additives (such as for luminol); (3) stable substrates that can be activated directly; (4) simple detection systems; (5) low background and wide linear range; (6) no radiation hazard; and (7) relatively inexpensive reagents and equipment compared with radioimmunoassays.

The advantages over other analytical systems, combined with the prolonged light emission using AMPPD and AP make this system a promising tool for chemiluminescent (immuno)assays.

Acridinium Compounds

Acridinium compounds were designed by McCapra in 1964 [64] in search for a model to help to elucidate the mechanism of firefly bioluminescence [65]. It turned out, that the acridinium compounds prepared are very efficient chemiluminogens. The reduced form of acridinium compounds (acridan) provides an excellent model for luciferin chemiluminescence [64], also because it is among the best understood examples of chemiluminescence [4]. Figure 15 presents the reaction scheme [4, 55].

All intermediates, except the dioxetanone,

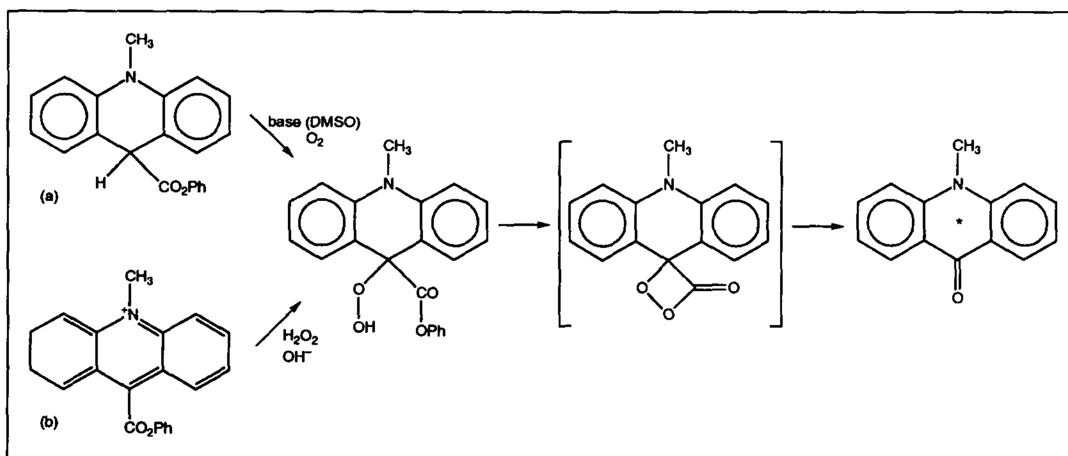


Figure 15
Conversion of acridinium compounds [acridan (a) and acridinium (b)] into the activated *N*-methylacridone [9].

have been isolated and characterized [9]. The peroxide intermediate can be derived either from oxidation of the acridan or the acridinium compound with H_2O_2 [64].

The quantum yield can be over 7% [65] and substitution on the N or of the phenyl moiety, may lead to significant improvements [4]. The yield of the product, *N*-methylacridone (Fig. 15), is quantitative under most conditions and the acridinium salts react well in aqueous alkaline H_2O_2 without further need for a catalyst.

Acridinium esters

The acridinium compounds mostly used are the esters, which in aqueous solutions are in equilibrium with their corresponding pseudobases (Fig. 16). The reaction involves attack by hydroxyl ions on the acridinium species, and formation of the electronically excited *N*-methylacridone (Fig. 15). A pH of 5 shifts the reaction towards the quaternary nitrogen species, and so favours attack by peroxide. The formation of the pseudo-base is decreased [66].

The efficiency of the chemiluminescent reaction correlates with the pK_a of the conjugated acid of the leaving group (the phenolic moiety in Fig. 16) [65]. The leaving group must have a pK_a of <11 for high light yields [64]. A very low pK_a predisposes faster reactions, including hydrolysis (in the absence of H_2O_2). In an assay kit this is prevented by means of bulky groups [64].

Phenols, thiols, sulphonamides and fluoroalcohols can be used as a leaving group [65]. In immunoassays, acridinium phenyl carboxylates are used as a label [67].

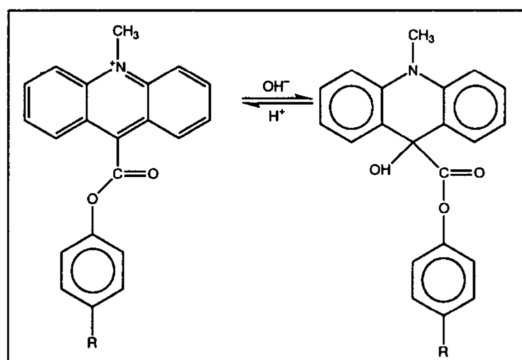


Figure 16
Structure of acridinium esters and the equilibrium with its corresponding pseudobases [66].

Application of acridinium esters in immunoassays

In all applications only one acridinium ester is used: 4-(2-succinimidyloxycarbonylethyl)-phenyl-10-methyl-acridinium-9-carboxylate fluorosulphonate. This acridinium ester reacts spontaneously with proteins to yield stable, immunoreactive derivatives of high specific activity [65, 66, 68, 69]. Following the chemiluminescent reaction, the emissive species is released from the molecule. Thus the emission characteristics (ϕ_{cl}, λ_{max}) are relatively independent of structural changes of the phenolic moiety used for coupling to an immunoreactant [70].

In general, acridinium conjugates have the advantage of high quantum yield, low background signal (no catalyst is required) and ease of coupling to other molecules [71]. A disadvantage is the fast light flash after addition of H_2O_2, complicating the light measurement [65].

The problem with labelled-Ag assays (type I-A1) is the low solubility, so in most assays labelled Ab is used [72]. One type I-A1 has been reported, in which the Ag (albumin) labelled with the acridinium ester competes with unlabelled analyte (albumin) for Ag-specific Ab [73]. In a type I-A2 assay described, the Ab is coupled to the acridinium ester and the analyte thyroxine (T_4) and T_4–IgG conjugate compete for this labelled Ag. Separation takes place by anti-IgG–Ab coupled to magnetic particles [74].

One of the type I-B1 assays, the IgG assay, uses the STAV–biotin system: the second Ab is biotinylated and STAV-acridinium ester conjugate is added in an incubation step (Table 8).

Recent developments in acridinium immunoassays

(1) *Multi-analyte assays. N*-functionalized acridinium compounds are a new class of chemiluminogenic labels (Fig. 17) [65]. The chemiluminescence-reaction kinetics can easily be adjusted, by modifying the leaving group. The λ_{max} of the emitted light can be selected by substituting the acridine nucleus with suitable groups. The analyte is attached via a spacer to the acridine N-atom. As a consequence, the light-emitting entity will remain attached to the analyte.

These new labels will make new multi-analyte assays possible in the future, by label-

Table 8
Immunoassays using acridinium esters

Analyte	Label	Assay type	Sensitivity	Range	Matrix	Reference
Albumin	Acridinium ester	I-A1	0.016 mg l^{-1}	0.1–5 mg l^{-1}	Urine	73
Thyroxine	Acridinium ester	I-A2	<20 nM	20–190 nM	Serum	74
Complement component C9	Acridinium ester	I-B1	10 ng ml^{-1}	10–2500 μg l^{-1}	Plasma	129
α-Fetoprotein	Acridinium ester	I-B1	5 ng ml^{-1}	5–200 ng ml^{-1}		66
IgG	Acridinium ester	I-B1	200 amol $sample^{-1}$			71
TSH	Acridinium ester	I-B1	0.04 mIU l^{-1}		Serum	56
TSH	Acridinium ester	I-B1	0.028 mIU l^{-1}	0.23–113 μIU ml^{-1}		130
TSH	Acridinium ester	I-B1	0.07 mIU l^{-1}	0.5–60 mIU l^{-1}	Serum	131

IU = international unit and TSH = thyroid stimulating hormone.

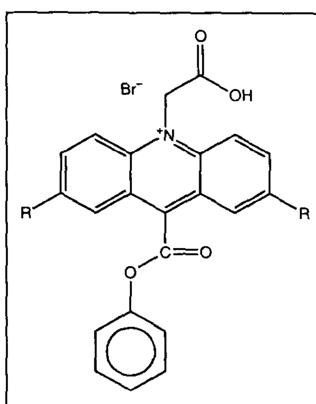


Figure 17
Structure of an *N*-functionalized acridinium compound, to be used in future multi-analyte assays [65].

ling different analytes with different acridinium labels [65].

(2) *Flow-injection analysis.* Common solid-phase immunoassays are usually relatively slow, complex and cannot easily be automated [75]. A flow-injection method for IgG that is at the same time sensitive, rapid in performance and easily automated, makes use of a transparent immunoaffinity column, where an anti-IgG Ab is covalently coupled to a rigid support [75]. A second anti-IgG Ab is labelled with an acridinium ester. Subsequently, the sample, an anti-IgG–acridinium ester conjugate and H_2O_2 in OH^- are injected. The chemiluminescence generated in the transparent affinity column is measured. By changing the pH to 1.8, the column is regenerated and within 12 min, a new assay can be performed [75].

A similar set-up has been developed by Hage and Koa [76] for the detection of parathyroid hormone, except that the acridinium-ester-labelled antibody is eluted from the column and chemiluminescence is generated post-column by adding peroxide. The lower limit of detection is 0.24 pM.

Conclusions

Acridinium esters can successfully be used in chemiluminescent immunoassays. Advantages are a high quantum yield, a low background signal and a simple coupling procedure to proteins. They have one substantial disadvantage: light is emitted instantaneously.

Oxalate Esters

Oxalate esters are the most efficient chemiluminogens of all the synthetic compounds, with readily obtainable quantum yields of over 20% [4]. Oxalate esters are probably unique in that these compounds, which react with the oxidant H_2O_2, are themselves not capable of light emission, but transfer the energy of the reaction to an added fluorescent molecule. This offers flexibility because the oxalate ester can be chosen for maximum reactivity, independent of the different requirements for maximum yield of fluorescence [4].

In aqueous systems oxalate esters are only moderately effective, since hydrolysis of the very reactive oxalate derivatives competes with the attack by H_2O_2. However, intensity and lifetime can be improved by the addition of weak acids, bases and certain salts [8]. Good leaving groups facilitate ring closure to the dioxetanedione intermediate and thus increase chemiluminescent efficiency.

The first developed oxalate esters used electronegatively substituted phenols and although the reaction mechanism is complex, the outline shown in Fig. 18 probably reflects the essential features [4, 8, 72].

The best known ester of oxalic acid is TCPO (bis(2,4,6-trichlorophenyl)oxalate, Fig. 18), which reacts with peroxide in the presence of a suitable fluorescer (rubrene) in some of the most efficient non-enzymatic chemiluminescent reactions known [8]. The ϕ_{cl} may be as high as 27%. This efficiency is due to: (1) the dioxetanedione–fluorescer complex generating the singlet excited state of the fluorescer in high yield; (2) the high efficiency of the fluorescers used; (3) the key intermediate being stable toward unimolecular decomposition; and (4) unwanted, non-luminescent side reactions being reduced by the suitable selection of reaction conditions [8].

Efficient chemiluminescent oxalate derivatives included electronegatively substituted aliphatic and aromatic esters, amides, sulphonamides, oxalylchloride, *O*-oxalyl hydroxylamine derivatives and mixed oxalic–carboxylic anhydrides [8].

Most efficient oxalates, based on sulphonamides result in a ϕ_{cl} of 34%. Suitable fluorescent partners are bis-phenethynyl anthracenes [4]. Water-soluble oxalates and fluorescers can be used, but the best ϕ_{cl} observed is only about 8%. The fluorescer must be stable to H_2O_2. Rhodamine B and sulphonic acid derivatives of 9,10-diphenylanthracene are effective fluorescers [4].

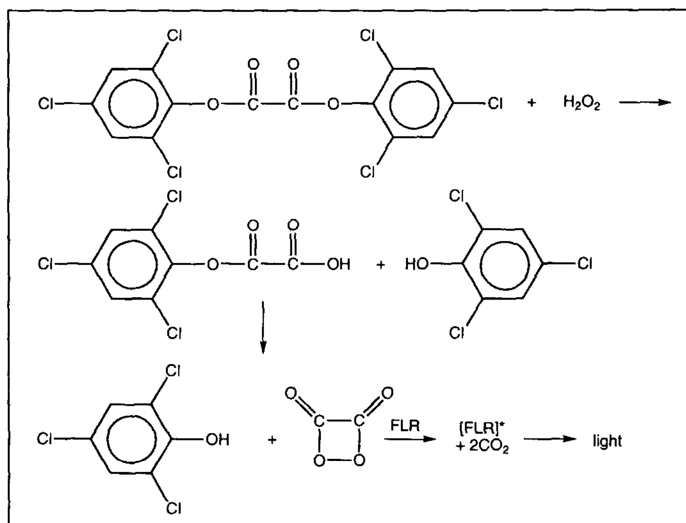


Figure 18
Chemiluminescent reaction of the ester of oxalic acid: bis(2,4,6-trichlorophenyl)oxalate (TCPO). FLR represents a fluorescer like rubrene [8].

Application of oxalate esters in immunoassays
Almost all analytical applications deal with the determination of analytes which act as sensitizers [9]. In such assays, the dioxetanedione excites the fluorophore (analyte). The advantage of chemical excitation is the low background signal.

Only a few immunoassays using oxalate esters have been published, probably due to the low water solubility of most reagents and the susceptibility to hydrolysis [9, 77].

Type I-AI assays. Immunoassays using TCPO and GOD have been published. The principle involves the detection of H_2O_2 released during the oxidation of glucose by GOD. The oxalate is not used as a label, but detects H_2O_2 produced by Ag–GOD conjugates (Table 9) [4].

When β-D-galactosidase is used as a label, lactose is converted via α-D-glucose (and β-D-galactose) into β-D-glucose, which is the substrate for GOD. TCPO and 8-anilino-1-naphthalene-sulphonic acid (ANS) are used for detection (Tables 9 and 10) [78].

Type I-BI assays. In the patent literature a method has been described in which a fluorescent compound is used as a label in a sandwich assay, and a large amount of H_2O_2 and an oxalate ester are used to activate this label [4]. An assay has been developed for γ-globulin, but no further details are given [4].

Conclusion
Oxalate esters can be used very efficiently in an immunoassay, when a fluorescer is added. The advantage is the flexibility of the system. The disadvantage is the low water solubility of most reagents and the susceptibility to hydrolysis, which decreases the ϕ_{cl}.

Bioluminescent Systems

Interest in bioluminescence predates the study of chemiluminescence. Detailed reports of light from living organisms have been made since antiquity [4]. It was not until 1952, that it became fully understood, that the reaction involved was that of an oxidation of a small molecule (a luciferin) catalysed by an enzyme (a luciferase) [79].

Although terrestrial luminescent organisms such as the firefly and glow-worm are the best known bioluminescent organisms, most of the other examples are sea-living organisms, from bacteria to fish. Two-thirds of the organisms in the upper 200 m of oceanic water are bioluminescent. To date, less than 1% of the known luminous organisms have been studied [67].

Only bioluminescent systems relevant for immunoassays will be discussed. Four categories can be discerned, based on a mechanistic classification [67]: (1) pyridine-nucleotide linked systems (especially marine bacteria); (2) adenine-nucleotide linked

Table 9
Immunoassays, type I-A1, using oxalate esters and GOD

Analyte	Enzyme	Substrate	Sensitivity	Range	Matrix	Reference
17-α Hydroxyprogesterone	GOD	TCPO–ANS	0.5 pg sample^{-1}	0.5–100 pg sample^{-1}	Plasma	4, 132
Thyroxine	GOD	TCPO–ANS	2.5 μg l^{-1}	2.5–200 μg l^{-1}		78

ANS = 8-anilino-1-naphthalene-sulphonic acid and TCPO = bis(2,4,6-trichlorophenyl)oxalate.

Table 10
Immunoassays, type I-A1, using oxalate esters and galactosidase

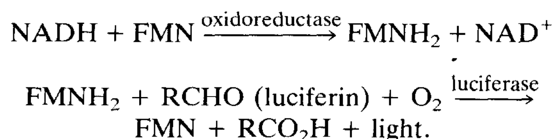
Analyte	Enzyme	Substrate	Sensitivity	Range	Matrix	Reference
Digitoxin	Galactosidase	TCPO–ANS	3.75 ng sample^{-1}		Serum	133
α-Fetoprotein	Galactosidase	TCPO–ANS	1.0 ng ml^{-1}		Serum	133
Phenytoin	Galactosidase	TCPO–ANS	10 ng ml^{-1}	0.1–40 μg ml^{-1}	Serum	133
TSH	Galactosidase	TCPO–ANS			Serum	133

ANS = 8-anilino-1-naphthalene-sulphonic acid, TCPO = bis(2,4,6-trichlorophenyl)oxalate and TSH = thyroid stimulating hormone.

systems (firefly); (3) enzyme–substrate systems without cofactors; and (4) 'precharged' systems (*Aequorea*).

Marine luminous bacteria (the pyridine-nucleotide linked system)

Many luminous bacteria exist, both free living and in symbiotic relationship with several fish [4]. The bioluminescent system involves the reaction between flavin mononucleotide ($FMNH_2$), the luciferin (a long chain aldehyde), O_2 and the luciferase [4]. *In vitro* reactions can be carried out by reduction of FMN either by NADH and an associated oxidoreductase or by various chemical means [4]. A reaction scheme is:



$$NADH + FMN \xrightarrow{\text{oxidoreductase}} FMNH_2 + NAD^+$$

$$FMNH_2 + RCHO\ (\text{luciferin}) + O_2 \xrightarrow{\text{luciferase}} FMN + RCO_2H + \text{light}.$$

The ϕ_{cl} is in the range 10–20%. Generally, the emission spectra are characterized by a broad spectrum, with a λ_{max} near 478–505 nm [67].

A key intermediate, the enzyme-bound flavin peroxide has been isolated (Fig. 19). In this system, the aldehyde is oxidized to the corresponding carboxylic acid. Only aliphatic aldehydes (luciferins) with a chain length of 8 or more C-atoms are effective [67]. The enzyme acts only once in the cycle.

All types of luciferases consist of two different subunits, without metals or cofactors [67]. One subunit catalyses the reaction; the rôle of the other subunit remains to be elucidated, although it is essential for bioluminescent activity [67]. The enzyme activity is highly specific to $FMNH_2$, but shows weak activity towards other flavins and flavin analogues [67].

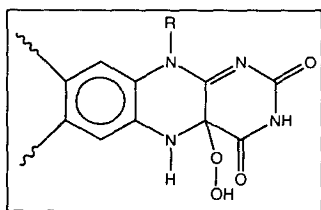


Figure 19
Partial structure of the key intermediate for the chemiluminescence reaction by the pyridine-nucleotide linked system [67].

Application of marine luminous bacteria bioluminescence in immunoassays

Type I-AI assays. Luciferases, especially bacterial luciferases, are deactivated when used as a label, as a result of a binding to a thiol group in the active centre of the enzyme. Some assays exploit the reversible modulation in enzyme activity in an active centre-based enzyme immunoassay.

The assay design involves labelling of an Ag with bacterial luciferase via the thiol group. This deactivated conjugate is then allowed to compete with Ag for a limited number of binding sites on an Ab and then the bound and free fractions are separated using a secondary Ab. Luciferase activity is restored by the addition of a reducing agent. This assay has been applied to insulin and estriol, but no detailed evaluation using clinical samples is available yet [79, 80].

Type II assays.

(1) *Co-immobilization assay.* Homogeneous assays are possible with bacterial luciferases using an enzyme channelling assay design. In a homogeneous assay a bacterial luciferase, NADH–FMN-oxidoreductase and an Ag are co-immobilized on a bead. The immobilized Ag competes with Ag (analyte) for an Ab labelled with G6PDH. Addition of substrate mixture (G6P, NAD and FMN) leads to production of NADH by the G6PDH label.

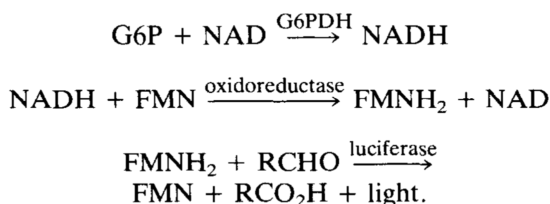
On the bead, NADH reacts with the co-immobilized reductase (yielding $FMNH_2$) and $FMNH_2$ reacts with luciferase (yielding excited luciferin), so light is emitted from the surface of the bead [79]. In solution, NADH is also produced by the unbound G6PDH conjugate and this is destroyed by lactate dehydrogenase, which oxidizes NADH to NAD^+. This scavenging reaction is only effective in solution, so light emission is solely attributed to bound G6PDH activity, which is inversely proportional to the Ag concentration in the sample. This assay has been used to measure progesterone in extracted plasma, with a detection limit of 96 pg per sample [79].

Other assays use an Ab co-immobilized with the oxidoreductase and the luciferase. These assays have been used to determine α-fetoprotein, prolactin and luteinizing hormone [79].

(2) *EMIT-assay (enzyme multiplied immunoassay technique).* This is a homogeneous

immunoassay, which relies on the alteration of enzyme activity, when an enzyme-labelled Ag binds to an Ag-specific Ab [3]. As a label, often G6PDH is used. Coupled to an Ag, this enzyme remains active, but when bound to an Ab, the enzyme is deactivated [3, 67].

High analyte concentrations result in a relative high amount of unbound Ag–G6PDH conjugate. After addition of G6P, NAD, FMN, luciferase, oxidoreductase and long-chain aldehyde, the next reactions take place:



$$\text{G6P} + \text{NAD} \xrightarrow{\text{G6PDH}} \text{NADH}$$

$$\text{NADH} + \text{FMN} \xrightarrow{\text{oxidoreductase}} \text{FMNH}_2 + \text{NAD}$$

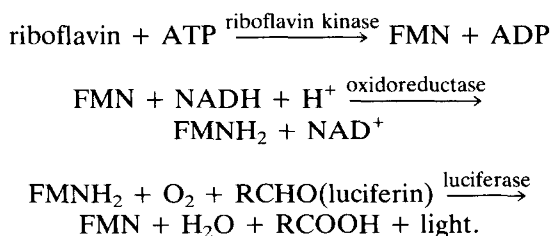
$$\text{FMNH}_2 + \text{RCHO} \xrightarrow{\text{luciferase}} \text{FMN} + \text{RCO}_2\text{H} + \text{light.}$$

This assay has been used to determine phenytoin [3].

(3) *Luminescent cofactor immunoassay (LCIA).* In these assays, NAD is coupled to an Ag [3, 81]. The Ag-bound NAD is normally available for the reaction, while Ab-bound conjugate is not. Often Ag (analyte) competes with NAD-Ag conjugate for Ab binding sites. After incubation, ethanol and alcohol dehydrogenase are added. Free NAD-Ag is converted into NADH–Ag and this reacts with NADH–FMN-oxidoreductase to yield $FMNH_2$ (and light). Light output is proportional to the concentration of Ag [3, 77]. With this assay, biotin and 2,4-DNFB (dinitrofluorobenzene) have been measured [3, 67].

Recent developments in 'marine bacterial' immunoassays

An amplified luminescence assay has been described measuring AP [82]. It relies on the hydrolysis of riboflavin-5′-phosphate (5′-FMN) and riboflavin 4′-phosphate (4′-FMN) yielding riboflavin:



$$\text{riboflavin} + \text{ATP} \xrightarrow{\text{riboflavin kinase}} \text{FMN} + \text{ADP}$$

$$\text{FMN} + \text{NADH} + \text{H}^+ \xrightarrow{\text{oxidoreductase}} \text{FMNH}_2 + \text{NAD}^+$$

$$\text{FMNH}_2 + \text{O}_2 + \text{RCHO(luciferin)} \xrightarrow{\text{luciferase}} \text{FMN} + \text{H}_2\text{O} + \text{RCOOH} + \text{light.}$$

With this system, 16 amol AP can be detected, making this system a valuable detection system for AP labels [82], although it is not as sensitive as the AMPPD system. The light output continues, until the NADH is depleted. FMN is recycled in this scheme.

Firefly (adenine-nucleotide linked systems)

Firefly luciferin is a 6-hydroxy-benzothiazole [79]. With luciferin itself ($R_2 = R_3 = H$ and R_1 = AMP; Fig. 20) the first formed excited state loses one of the protons on the thiazoline ring, forming the excited dianion, which emits light with a λ_{max} of 562 nm (yellow; Fig. 20) [4, 67].

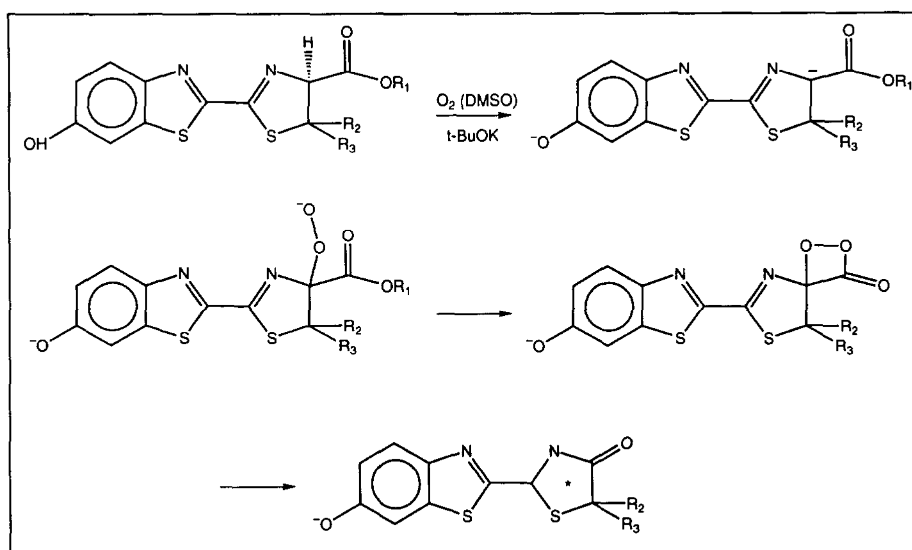


Figure 20
Reaction mechanism of 'luciferin derivatives' catalysed by firefly luciferase [4].

If both R_2 and R_3 are alkyl groups, λ_{max} shifts to 605 nm. The initial reaction is the rapid conversion of luciferin in the presence of Mg^{2+} and ATP to luciferyl-adenylate which, in the presence of luciferase combines with molecular oxygen to give an oxyluciferyl-adenylate–enzyme complex in an excited state. After light emission, the ground state complex dissociates to form enzyme, AMP, CO_2 and oxyluciferin.

For high ϕ_{cl} it is necessary to make esters of the luciferin and to perform the reaction in dipolar aprotic media [4]. Optimally, quantum yields are up to 88% [83], but the system cannot be used in aqueous environments, because hydrolysis of the ester competes with ionization and oxidation on the adjacent C-atom [4].

Intensity and λ_{max} are altered by different luciferases, change in pH, ionic strength, temperature and the addition of $ZnCl_2$ or $CdCl_2$ [67]. Luciferin is very stable in neutral solutions, whereas in acid solutions (pH <2) a rapid degradation occurs. In alkaline solutions, luciferin is oxidized to dehydroluciferin, which is a potent inhibitor of firefly luciferase [84].

Firefly luciferase is a dimer, but only one of the subunits exhibits enzymatic activity in the bioluminescent reaction. It is one of the most hydrophobic enzymes known [67]. The reaction is not specific for ATP. Other nucleotides are also claimed to stimulate light emission [67]. Mg^{2+} ions may be replaced by Mn^{2+} ions [72]. Inhibitors of the bioluminescent reaction are SCN^-, I^-, NO_3^-, Br^- and Cl^-. Anaesthetics such as procaine and lidocaine are inhibitors as well and this fact has been the basis of some assays [67].

A series of firefly luciferins derivatized at the 6-position with sulphate, phosphate, galactoside, arginine, L-phenylalanine and ester groups is commercially available [79, 84]. These luciferins do not react with the enzyme, but by splitting the substituted derivative with an appropriate enzyme, the original luciferin is reactivated and will subsequently react with firefly luciferase yielding light emission [79].

Application of firefly bioluminescence in immunoassays

Type I-A1 assays. An assay for methotrexate (MTX) has been published [3], using conjugates of MTX and firefly luciferase (enzyme activity after coupling is 60% of the original activity). This conjugate competes with Ag for binding sites on an Ab directed against MTX and after incubation and separation steps, light emission of the bound fraction is measured. The light emission is inversely proportional to the Ag concentration [3].

Type II assays. As with the bacterial luciferase system, a LCIA for firefly luciferase has been developed [3]. ATP is bound to an Ag, which competes with unlabelled Ag for Ab binding sites. The Ab-bound ATP–Ag conjugate is no longer available for a reaction, while free Ag–ATP conjugate is [3, 67]. After the incubation, firefly luciferase, luciferin and Mg^{2+} are added.

High Ag concentrations result in high light emission. With this assay 2,4-DNFB-β-alanine has been measured with a detection limit of 0.5 nM [3, 85].

Miscellaneous assays. AP as label can be detected with firefly luciferase, when D-luciferin-*O*-phosphate is used as substrate [68, 79]. AP converts the substrate into D-luciferin, which reacts with firefly luciferase to yield light (unlike the phosphate derivative). Several substances have been measured (Table 11).

Recent developments in 'firefly' immunoassays

Recently several firefly luciferases have been discovered, varying in a few amino acids and producing light with different wavelengths with the same substrate, yielding green, yellow-green, yellow or orange light [86]. Eleven clones were isolated and expressed in *Escherichia coli.*

Each luciferase has a characteristic spectral distribution. Since the substrates within these groups do not differ, the colour variation must be due to differences in enzyme structure [86].

Table 11
Various enzyme (AP) immunoassays using D-luciferin-*O*-phosphate

Analyte	Enzyme	Substrate	Assay type	Sensitivity	Range	Matrix	Reference
Kallikrein	AP conjugate	D-Luciferin-*O*-phosphate	I-A1	5 pg l^{-1}	5–5000 pg l^{-1}	Urine	134
Kallikrein	AP conjugate	D-Luciferin-*O*-phosphate	I-B1		5–5000 pg l^{-1}	Urine	134
Bradykinin	AP conjugate	D-Luciferin-*O*-phosphate	I-A1	5 pg l^{-1}	5–5000 pg l^{-1}		134
Killikrein	AP conjugate	D-Luciferin-*O*-phosphate	I-B1		5–5000 pg l^{-1}	Urine	134

Investigations suggest, that the potential for colour variation is much greater than the four 'colours' isolated. With these different luciferases, it should be possible to measure different analytes simultaneously: multi-analyte systems [86].

Other bioluminescent systems

(1) *Enzyme substrate systems without cofactor.* These systems do not use any cofactor. They are among the simplest bioluminescent systems discovered. No immunoassays relying on this system have been described [4].

(2) *Precharged systems* (Aequorea). The bioluminescent system of the jellyfish *Aequorea* consists of a protein in close association with a chromophore (luciferin), and is called a photoprotein. The luciferin is oxidized by the protein in the presence of Ca^{2+} to produce a bluish luminescence at 469 nm, independent of dissolved O_2. Sr^{2+} and Ba^{2+} also catalyse the reaction [67].

The gene for apoaequorin has been fused with the IgG heavy chain gene, and the fused gene expresses a protein, that retains the properties of both the apoaequorin and the IgG heavy chain [80]. No applications have been described yet.

Conclusions

Bioluminescent immunoassays have been described using various formats and several reagents. In the future, manipulation of luciferase genes may lead to luciferases with improved stability and improved (e.g. constant) quality, since luciferases are not very stable. Disadvantages are that the enzymes are very expensive and there exists a large batch-to-batch variation in quality.

A further prospect is the development of hybrid molecules, produced by the fusion of a luciferase gene and the gene for an Ab or a protein Ag [65] as with aequorin.

Currently, the assays most likely to make the transition from research to routine laboratory are the bioluminescent immunoassays using AP and D-luciferin-*O*-phosphate and the co-immobilized bacterial luciferase assays [79].

General Conclusions

This review deals with the application of chemiluminescence in immunoassays. The most sensitive immunoassays possible are non-competitive immunoassays which use labels with a high specific activity (enzymes). Two-site immunoassays are more specific than other non-competitive assays. All heterogeneous assays have as an advantage over homogeneous assays the removal of potential quenching factors in the biological sample. Homogeneous assays often rely on an extraction step to eliminate these interferences. Low-background signals can be achieved by using a chemiluminescent substrate for the enzymes (labels).

In the case of chemiluminescent immunoassays, the label used is a reactant in the chemiluminescent reaction; often a substrate for an enzyme, a cofactor or the enzyme itself is used.

Chemiluminescence and immunoassays are combined in many ways, using various chemiluminescent systems and different formats of immunoassays, resulting in a complex picture, complicated by the abundance of information available.

Cyclic acylhydrazides like (iso)luminol are the most frequently used chemiluminescent compounds in immunoassays. The most promising techniques are those using enhancers, such as PIP in combination with peroxidase enzymes and Fe(III)–EDTA–perborate in combination with XO. The advantage of cyclic acylhydrazides is, that they can be used for different kinds of assays. A disadvantage is, that they need a catalyst for light emission, leading to higher background signals.

1,2-Dioxetanes are relatively new chemiluminescent substrates for the enzyme AP. The most important advantages of this system are: a prolonged light emission, a similar or greater sensitivity than radioimmunoassays, stable substrates which can be activated directly, a low background and a wide linear range. This system needs no extensive optimization of the different additives as for luminol.

Acridinium esters can successfully be used in chemiluminescent immunoassays. Advantages of acridinium esters are that they have a high quantum yield, a low background signal and a simple coupling procedure to proteins. They have one major disadvantage: light is emitted instantaneously.

Oxalate esters can very efficiently be used in an immunoassay, when a fluorescer is added. An advantage is the flexibility of the system. A disadvantage is the low water solubility of most

reagents and the susceptibility to hydrolysis, decreasing ϕ_{cl}.

Various bioluminescent immunoassay formats have been described using several reagents. In the future, manipulation of the genes for luciferases may lead to enzymes with improved stability and improved (constant) quality. Disadvantages are that luciferases are very unstable, very expensive and that the batch-to-batch quality varies largely. A future prospect is the development of hybrid molecules, produced by the fusion of a luciferase gene and the gene for an Ab or a protein Ag [65] as with aequorin. The bioluminescent immunoassays most likely to make the transition from research to routine laboratory are using AP and D-luciferin-*O*-phosphate and the co-immobilized bacterial luciferase [79].

It can be concluded that the use of chemiluminescence as a detection system provides the advantages of high sensitivity, a large linear response, relative high speed of analysis, high specificity, low cost, and high stability and no toxicity of the reagents involved.

In general, chemiluminescent immunoassays are superior (or at least comparable) to the most commonly used radioimmunoassays with respect to sensitivity, speed of analysis, cost and stability. A big advantage over the use of isotopes is the safety of the chemiluminescent labels.

Besides these advantages of the chemiluminescent labels, immunoassays give high specificity to the analytical method, creating a powerful analytical tool.

Most promising chemiluminescent labels in immunoassays are labels, of which the light output can be enhanced, resulting in a lasting light signal, making more accurate measurements possible and simplifying the measurement procedure. Such combinations are the luminol–peroxidase–H_2O_2 system, enhanced by *p*-substituted phenols, the luminol–XO system, enhanced by Fe(III)–EDTA and sodium perborate in alkaline buffer and the AMPPD–AP system.

Recent developments in chemiluminescent immunoassays are multi-analyte systems, with 1,2-dioxetanes, acridinium esters or firefly luciferin. Also flow-injection analysis with luminol or acridinium esters, is utilized more often, resulting in fast detection systems.

Routine clinical applications of chemiluminescence immunoassays are also stimulated by the availability of luminometers and luminescence readers [5, 6], and kits containing both Ab and chemiluminescent or bioluminescent substrate reagents [87], and last but not least an entire journal is devoted to chemiluminescence [88] demonstrating its vitality.

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[Received for review 9 July 1993; revised manuscript received 11 October 1993]