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Employment of a Phenoxy-Substituted Acridinium Ester as a Long-Lived Chemiluminescent Indicator of Glucose Oxidase Activity and Its Application in an Alkaline Phosphatase Amplification Cascade Immunoassay

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This paper describes the employment of a novel phenoxy-substituted acridinium ester (di-*ortho*-bromophenyl-AE) as a chemiluminescent endpoint indicator for ligand binding assays. The reactivity of this compound is such that it is capable of generating a high-intensity chemiluminescent signal at neutral pH. Under these conditions, when present in excess, it has been used as an indicator of hydrogen peroxide generation by the action of glucose oxidase (GOx, EC 1.1.3.4) on glucose substrate. The resulting chemiluminescent signal is a long-lived glow. The magnitude of the chemiluminescent signal is directly proportional to the quantity of GOx present and has been used to measure GOx with a sensitivity of 1.8×10^{-16} mol. In addition, this ability to monitor GOx activity has been utilized in an alkaline phosphatase (ALP, EC 3.1.3.1) amplification cascade assay. Here ALP catalyzes the formation of FAD from a prosthetogenic substrate FADP. FAD, a cofactor for a number of oxidase enzymes, then converts inactive apo-GOx to holo-GOx, the activity of which is monitored by the chemiluminescent endpoint and facilitates detection of ALP over the range 10^{-15} to 4.1×10^{-19} mol. The clinical utility of this system has been demonstrated by application to the assay of human thyrotrophin (TSH, sensitivity 0.005 mU/liter).

Chemiluminescence offers a significant combination of advantages in terms of sensitivity, reagent stability and low hazard compared to other isotopic and nonisotopic endpoint detection methods employed in ligand binding assays (1). Consequently, chemiluminescence has had widespread application particularly in the fields of immunoassay and nucleic acid detection. Expressed simply, the chemical entities used to generate the chemiluminescent signal can be divided into two categories: those that result in a relatively short-lived (seconds) but intense signal (flash type) and those which produce a longer-lived signal (minutes or hours), generally of a lesser intensity (glow type). Of the first category (flash type) two classes of compound have had the greatest application, namely acridinium esters (AEs)[2] and luminol (e.g., aminobutylethylisoluminol). Here chemiluminescence is initiated by the addition of oxidant, usually hydrogen peroxide, at high alkaline pH either with (luminol-type compounds) or without (AEs) a catalyst. The resultant signal is short-lived and therefore initiation generally must be performed *in situ*, that is, within the measuring chamber of the luminometric measuring device. Contrastingly, lumi-

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[2] Abbreviations used: AE, acridinium ester; ALP, alkaline phosphatase (EC 3.1.3.1); BγG, bovine γ-globulin; CTAC, cetyltrimethylammonium chloride; GOx, glucose oxidase (EC 1.1.3.4); FADP, flavine adenine dinucleotide-3′-phosphate; NHS, *N*-hydroxysuccinimide ester; PBS, phosphate-buffered saline; RLU, relative light units; TBS, Tris-buffered saline; TSH, human thyrotrophin.

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nometric measurement of the chemiluminescence emanating from compounds of the second category (glow type) does not require *in situ* initiator addition owing to the relatively long-lived nature of the signal. In this category again two main classes have been used: the horseradish peroxidase-enhanced luminol system, and stabilized dioxetanes. With both, ligands are conjugated to enzymes the catalytic actions of which act to initiate reaction pathways culminating in the chemiluminescence. Because of the capacity of enzymes to repeatedly cycle the reaction from substrate to product the signal generation can be long-lived if reactants are present in excess.

We have extensive experience with the application of AEs to ligand binding assays. To date, AEs have been employed exclusively as flash-type labels (2). AE chemiluminescence is triggered by peroxide attack at the C9 ester linkage, characteristically under strong alkaline conditions. It is thus theoretically possible to utilize AE to monitor hydrogen peroxide generated by the action of oxidase enzymes on their respective substrates. However, AEs currently employed cannot easily be coupled to the monitoring of oxidase activity due to the requirement for high ambient pH. This report describes work with a new AE analogue modified such that it is capable of being triggered at neutral pH and therefore can, when present in excess concentration, be capable of reacting with, and therefore reflect the rate of production of, hydrogen peroxide generated by the activity of the oxidase enzyme glucose oxidase (GOx) to produce a relatively long-lived, glow-type, chemiluminescent signal. This ability to monitor an oxidase activity has been used as an endpoint in the previously described alkaline phosphatase (ALP) amplification cascade system (3). Here, the cascade detects ALP via dephosphorylation of flavine adenine dinucleotide -3′-phosphate (FADP) to produce FAD cofactor which combines with apo-GOx to produce holo-GOx. The activity of this enzyme is monitored by AE chemiluminescence. This report describes the development of this system and its application to human thyrotrophin (TSH) immunoassay.

MATERIALS AND METHODS

Chemicals

All chemicals used were analytical grade reagents. D,L-Lysine monohydrochloride, β-D-glucose, FAD, Tween 20, bovine serum albumin, bovine γ-globulin (BγG), and fetal calf serum were obtained from Sigma. Cetyltrimethylammonium chloride (CTAC) was obtained from Aldrich. GOx and ALP were obtained from Biozyme (Blaenavon, Wales). FADP and apo-glucose oxidase (apo-GOx) were obtained from the Department of Biochemistry and Molecular Biology (University College London). Standard human TSH (WHO 80/558) was obtained from NIBSC (England). A matched anti-human TSH antibody pair (mouse monoclonal anti-TSH product code MIT 0406 and affinity-isolated goat polyclonal anti-β-TSH product code PIT 3413) were obtained from Genzyme Diagnostics (UK). Conjugation of anti-β-TSH to ALP was achieved using a kit supplied by Pierce (Rockford, IL). Heterophilic antibody blocker (Heteroblocker) was obtained from Scantibodies Inc. (Santee, CA).

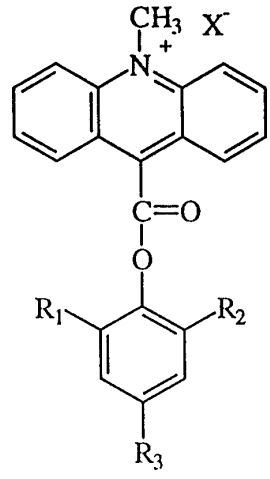
Luminometry

All experiments were carried out using opaque white polystyrene microtiter 96-well plates (Removawell Microlite I or Microlite II strips, Dynatech) which exhibited low cross-talk between adjacent wells. Chemiluminescence was measured in a 96-well microtiter plate luminometer (LB 96P manufactured by EG & G Berthold, Wildbad, Germany). Initial experiments investigating the kinetics of the chemiluminescence were carried out using on-board *in situ* reagent addition of initiating reagents in a single measurement mode. In later experiments, where the chemiluminescent signal was generated enzymatically, reagent addition was performed manually (off board) and measurements were carried out using a continuous, timed repeat measurement cycle. Here, luminescence was measured and integrated for a duration of 1 s every 3 to 5 min over a period of 20 to 40 min. The luminescence data are expressed as relative light units (RLU).

Acridinium Esters

The chemical structures of the AEs used in this study are given in Fig. 1. A description of their synthesis is given by Nelson *et al.* (4). The AE compounds were primarily designed for conjugation through a NHS tail. For the purpose of these investigations the unconjugated AE in free solution was used. It was therefore necessary to first block the NHS moiety by reacting with excess lysine. This was achieved as follows.

One milligram of AE (approximately 1.25 to 1.6 μmol) was taken up to 1 mg/ml in acetonitrile. To this 1.5 ml of a 10 mg/ml solution of D,L-lysine monohydrochloride (approximately 75 μmol) in 0.1 M sodium dihydrogen *ortho*-phosphate 0.15 M sodium chloride, pH 8.0, was added. After 10 min at room temperature 20 μl of concentrated HCl was added with rapid mixing which brought the pH of the solution to approximately 3 (using pH paper). Dilutions from stock solutions of approximately 400 μg/ml of the respective AE was used for all experiments. The solutions appeared to be stable with no apparent loss of luminescent yield when stored at 4°C for 6 months.



	R_1	R_2	R_3	pK_a *
I	CH3	CH3	CO-NHS	10.8
II	Br	Br	(CH2)2CO-NHS	7.13

FIG. 1. Structure and formulae of the two AEs. *Theoretical pK_a of the conjugate acid of the phenoxy moiety, calculated using the algorithms of Perrin *et al.* (6).

The Relationship between AE Structure and pH on Chemiluminescence Initiation

In order to examine the effect of ambient pH on AE chemiluminescence, the kinetics of the initiation reaction were compared over the pH range 14 to 6. The standard initiating conditions used here for the generation of AE luminescence were achieved by automatic *in situ* addition of 100 μl of 0.25 M NaOH containing hydrogen peroxide (0.15 M) and a detergent (CTAC, 0.75% v/v), using a 1-s count duration. In conventional AE flash-type endpoint assays CTAC is used to increase the quantum yield, presumably by increasing the accessibility of the hydrophobic AE molecule to peroxide attack of the phenoxyester. In this experiment, studying the effect of pH, experimental initiating solutions all contained hydrogen peroxide at 0.15 M but no detergent. The following solutions (and their respective pH) were used: 0.2 M NaOH (pH 14), 0.2 M KCl (pH 12), 0.2 M sodium carbonate/bicarbonate (pH 10), 0.2 M Tris/HCl (pH 9, 8, and 7), and 0.2 M sodium acetate (pH 6). Sequential dilutions in 0.01 M HCl of the stock solutions of the two AEs, di-*ortho*-methyl- and di-*ortho*-bromo- (Fig. 1, compounds I and II), were performed to provide concentrations which arbitrarily gave approximately the same integrated luminescence count (5×10^5 RLU per 10 μl aliquot) when using standard initiating conditions. Further 10-μl aliquots of the respective AEs at this dilution were then tested by the priming the luminometer injector with the respective pH-initiating reagent and monitoring the chemiluminescence following addition of 100 μl to the wells. The duration of measurement was 2, 20, or 60 s, except at pH 6 when the reaction was followed for 20, 60, and 120 s.

Studies with GOx

A series of related experiments were carried out to investigate and optimise the use of di-*ortho*-bromo-AE chemiluminescence as an indicator of GOx activity. GOx was dissolved in phosphate-buffered saline (PBS), pH 7.5 (137 mM NaCl, 2.7 mM KCl, 7.7 mM Na_2HPO_4, 1.47 mM KH_2PO_4). Dilutions from the stock solution were made in this buffer (PBS, pH 7.5) or adjusted with HCl to pH 6.5 (PBS pH 6.5). Aliquots (10-μl) of the diluted GOx were added to wells and 100-μl aliquots of PBS at the respective pH containing di-*ortho*-bromo-AE (80 pmol/well) and glucose substrate were added, all at room temperature; luminometry was initiated after 1 min. In some experiments, in order to optimize the conditions, the concentrations of glucose and di-*ortho*-bromo-AE were varied.

ALP Amplification Cascade

This system was assembled in stages. The ability of apo-GOx to combine with FAD in the presence of di-*ortho*-bromo-AE was examined by adding to 50 μl of a solution of FAD in glycine buffer (0.02 M glycine, 0.8 mM Mg^{2+}, 16 μM Zn^{2+}, pH 9.2) 100 μl of an admixture of apo-GOx, di-*ortho*-bromo-AE, and glucose in 0.1 M

sodium citrate, pH 6.4. The admixture was assembled within 10 min of use. Having optimized the conditions for this reaction the amplification cascade was obtained by a two-stage procedure involving a preincubation of FADP with alkaline phosphatase. To optimize the concentration of FADP, doubling dilutions (40 μl) were added to a 10-μl aliquot containing either zero (control) or 1.53×10^{-16} mol of ALP, both in glycine buffer, and incubated at 37°C for 40 min. The second-stage admixture was then added and repeat luminometric measurements were carried out using room temperature incubation. Having optimized the FADP concentration, the dose response for ALP was obtained using 10-fold dilutions in glycine buffer.

TSH Assay

Microlite II strips were coated with 100 μl of 0.1 M sodium carbonate/bicarbonate buffer (pH 9.6) containing 200 ng of mouse monoclonal anti-TSH for 18 h at 37°C. Following a wash with PBS/0.05% w/v Tween 20, the plates were blocked for 1 h at room temperature in Tris-buffered saline (TBS: 137 mM NaCl, 2.7 mM KCl, 25 mM Tris buffered with HCl to pH 7.5) containing 0.5% BSA and 1% sucrose. Blocked plates were flicked and blotted, dried *in vacuo*, and stored desiccated. The matched affinity-isolated goat polyclonal antibody was conjugated to ALP using mercaptoethylamine. The final preparation was buffer exchanged into TBS, pH 8.2, containing Mg^{2+} and Zn^{2+} (both at 1 mM), 0.02% sodium azide w/v, and glycerol (1 vol) was added and then stored at −25°C. For assay 100 μl of sample, or standard in fetal calf serum, was added to the coated wells followed by 100 μl of anti-TSH conjugate (at an empirically optimized dilution of 1/20,000) in assay buffer (TBS plus 0.05% BSA, 0.005% BγG, 0.05% sodium azide, and 1:100 heteroblocker, which is a commercial preparation used to block possible serum sample endogenous heterophilic antibody activity). After incubation with shaking for 60 min at 40C, the plates were washed in TBS/0.05% Tween 20 and 50 μl of FADP (30 pmol/well) in glycine buffer was added. After a further incubation at room temperature for 40 min, 100 μl of apo-GOx (8 pmol/well) and di-*ortho*-bromo-AE (600 pmol/well) in citrate buffer containing 2% glucose was added. Luminometry commenced after an additional 5 min incubation and repeat measurements were made every 3–5 min. Assay data were processed from the results obtained at 20–25 min using Graph Pad Prism data reduction software. Clinical samples for assay comparison were obtained from the Department of Medical Biochemistry, University Hospital of Wales; the Chiron ACS 180 TSH was used as reference method. A precision profile was constructed from 83 samples and standards measured in three separate assays; the mean response percentage CV of duplicate estimations of 13 bins was plotted against the respective mean interpolated response values.

RESULTS

Effect of pH on AE Chemiluminescence

Classically AE chemiluminescence is initiated by the addition of peroxide in strong alkali (pH 14). In order to examine the feasibility of coupling AE chemiluminescence to oxidase enzyme generation of peroxide from respective substrate, it is necessary to obtain a species of AE capable of reacting at or near neutral pH with sufficient luminescent yield in the absence of detergent (which may interfere in any enzymatic reaction). The effects of initiating pH on the chemiluminescence kinetic profiles of di-*ortho*-bromo-AE at 14, 8, and 6 are depicted in Fig. 2. Here the total measuring time is divided into twentieths by the luminometer software and the total integrated RLU for that interval is plotted against its respective time point. The total integrated RLU for the whole duration of measurement is then obtained by summation of the 20 individual integrals and is given as ΣRLU in the figure legend. The effect of lowering the initiating pH led to a progressive reduction in the rate of measurable photon release; this could be compensated for by increasing the measurement time.

The shape of the kinetic profile altered as the pH of the initiating reagent was lowered, associated with this reduced rate of luminescence. Thus at pH 14 the profile was that of a characteristic symmetrical flash type with a rapid rise to peak height at 0.3 s followed by a rapid decay to background by 0.6 s. At pH 8 the profile was essentially similar to that seen at pH 14 but the reaction proceeded more slowly with peak height at 4 s and a decay to background at 10 s. In contrast, at pH 6 the reaction was a more prolonged glow type with a biphasic kinetic profile consisting of a slow rise to peak height at 36 s followed by an even slower decay such that at the end of the measurement time (120 s) the luminescence was still greater than 50% peak height. This pH-associated pattern of transition from a flash type to the slower biphasic glow type of kinetic profile was observed to occur with both AEs studied. However, the pH at which the kinetic profiles altered from flash to glow and the associated quantum yields was variable and reflected the reactivity of the respective AEs.

The value of the total measurement time-integrated RLU (ΣRLU) was used as a means of comparing the variation in luminescence yield and the reaction kinetics over the range of initiating pH at measuring times of 2, 20, and 60 s (Fig. 3). The ΣRLU data were normalized by expressing the respective value as a percentage of that obtained using the standard luminescence-initiating conditions (i.e., pH 14 plus detergent).

By expressing the data in this way it was possible to discern whether the time course and quantum yield of the released chemiluminescence of the respective AE at each pH was sufficient to provide the degree of sensitivity in terms of signal:noise to enable its employment as an assay endpoint indicator.

The di-*ortho*-methyl-AE demonstrated a large fall off in ΣRLU with a reduction in pH so that by pH 8 the chemiluminescent reaction was not significantly above background even over 60 s total integration. Absence of detergent at pH 14 caused an immediate drop in 2-s integrated RLU for the di-*ortho*-methyl-AE to 35% of that for standard initiating conditions. In contrast, the di-*ortho*-bromo-AE yielded increased RLU when changing from standard to pH 14 initiation, presumably because the luminescence intensity of this more reactive compound under standard conditions, that is in the presence of detergent, overloaded the luminometer dead time. Lowering the pH led to a decrease in the rate of chemiluminescence but even at a pH as low as 9 the di-*ortho*-bromo-substituted compound gave high yields at 2 and 20 s integration. The reactivity of the di-*ortho*-bromo-AE was always greater than the di-*ortho*-methyl-AE such that even at pH 6 a high luminescent yield was obtained over extended measurement time (see Fig. 2).

From these experiments it was concluded that the di-*ortho*-bromo-AE could be a candidate for employment as an endpoint indicator for peroxide-generating oxidase activity, and all subsequent investigations

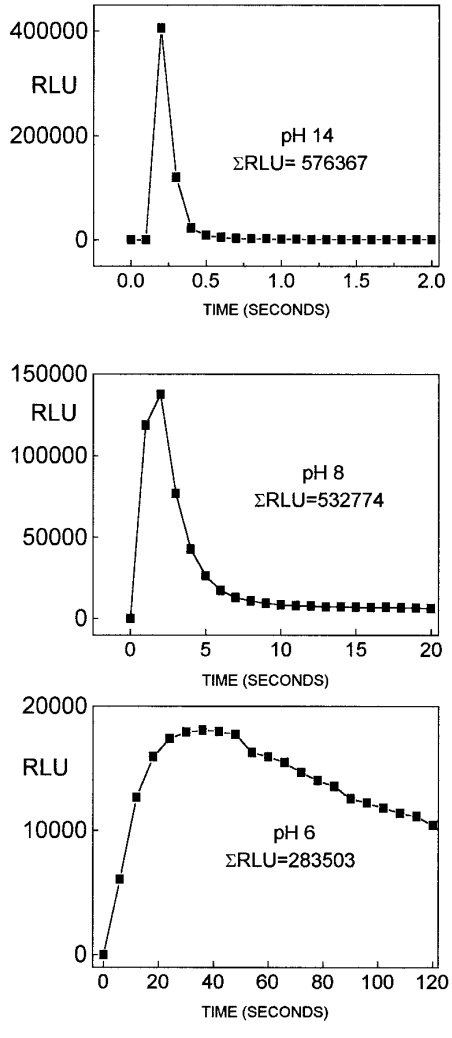


FIG. 2. Time course of the kinetic profile of the chemiluminescence of di-*ortho*-bromo-AE: effect of pH of the initiating reagent. ΣRLU refers to the total integrated RLU over the respective duration of measurement.

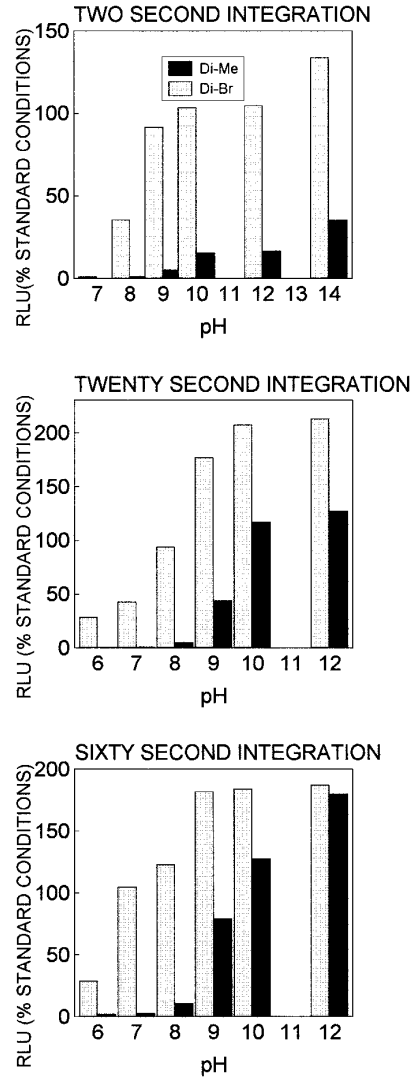


FIG. 3. The relationship between the initiating reagent pH and the chemiluminescent yield of di-*ortho*-methyl-AE (DiMe) and di-*ortho*-bromo-AE (Di-Br). The height of each bar represents the total integrated RLU (ΣRLU), normalized to that obtained under standard initiation conditions (see text for a definition), of each respective AE when measured over 2, 20, and 60 s.

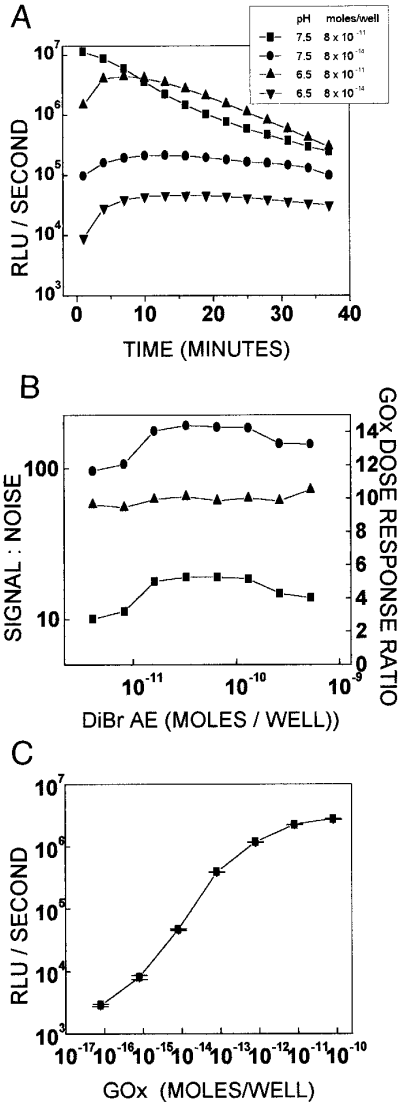


FIG. 4. Glucose oxidase (GOx) activity monitored by di-*ortho*-bromo-AE chemiluminescence endpoint. (A) Time course at two doses of GOx at pH 7.5 and 6.5. (B) Variation in the concentration of di-*ortho*-bromo-AE (DiBr AE): left-hand axis, dose response of di-*ortho*-bromo-AE on signal:noise ratio at two doses of GOx (7.6×10^{-14} (■) and 7.6×10^{-13} (●) mol/well); right-hand axis, ratio of signal between the two doses of GOx at each dose of di-*ortho*-bromo-AE added (◆). (C) Dose response of GOx. Data (means ± SD of triplicates) measured at 20 min following initiation of the reaction.

were carried out using this compound. This was then investigated by examining the response to the action of GOx on glucose in the presence of excess di-*ortho*-bromo-AE. Figure 4A depicts the time course (repeat measurements on the same wells) of the chemiluminescence from approximately 1 min following the addition of two concentrations of GOx to a solution containing AE and glucose in phosphate buffer at pH 7.5 and 6.5. The magnitude of chemiluminescence was GOx dose and pH dependent. At the higher concentration of GOx at pH 7.5 there was an immediate high magnitude chemiluminescent signal in excess of the saturation threshold of the luminometer. This signal decayed slowly to less than 10% of the initial signal over a period of 40 min measurement. Crude integration (cutting and weighing) indicated a recovery of approximately 65% of the available luminescence calculated from the standard conditions flash equivalent RLU added (which was approximately 10^{10} RLU). However, it is difficult to be confident of this calculation since the initial measurement took place 1 min after addition of reagents and at this time the RLU/s was $>10^7$ at which rate the luminometer efficiency is considerably attenuated. At pH 6.5, although the magnitude of the dose-dependent signal was less, the stability in terms of a flatter time course profile was improved.

The incubation conditions were then optimized in terms of pH, glucose, and di-*ortho*-bromo-AE concentration to obtain maximum sensitivity of detection of GOx. Although the chemiluminescence at pH >7.0 was higher because of a higher zero GOx signal at this pH range, both the stability of the signal and the optimal signal:noise were obtained at pH 6.2 to 6.5. At concentrations above 0.5% w/v glucose was found to be nonlimiting. A di-*ortho*-bromo-AE concentration range of 10^{-11} to 10^{-10} mol/well was found to provide the highest signal:noise ratio at low and high doses of GOx (Fig. 4B). Above this concentration the response decreased probably due to self-absorption of the signal by the di-*ortho*-bromo-AE. The signal ratio between the two GOx doses was constant over the range of di-*ortho*-bromo-AE concentrations tested. Using these optimized conditions a dose response for GOx revealed a detection limit (mean plus two standard deviations of zero) of 1.8×10^{-16} mol/well with a response range up to 10^{-12} mol/well (Fig. 4C).

In order to increase the potential sensitivity of this system it was decided to investigate adapting the GOx/AE-linked chemiluminescent endpoint to the alkaline phosphatase/FADP amplification described by Obzansky *et al.* (3). A two-stage incubation protocol was adopted to optimize the dephosphorylation of FADP at alkaline pH and the subsequent development of detectable signal at pH 6.5. In order to assemble this system it was necessary to optimize the amounts of FADP and apo-GOx. The ability of FAD to combine with apo-GOx to form the activated holo-enzyme is depicted in Fig. 5A. The chemiluminescence showed a dose-dependent response over the range 2×10^{-13} to 1.65×10^{-12} mol apo-GOx/well in the presence of 25 pmol/well FAD. A small dose-dependent increase in signal was observed in the absence of FAD, probably indicative of low-level contamination of the apo-GOx by residual holo-GOx.

Coupling the chemiluminescence endpoint to the first-stage reaction was then examined. Figure 5B de-

picts an FADP dose-dependent signal response at a fixed dose of ALP and apo-GOx. The response reached a plateau at 40 pmol/well (assay volume 150 μl) giving a K_m of 10.5 pmol/well (equivalent to 0.21 μM) calculated by double-reciprocal plot (Fig. 5B, inset). In the absence of ALP, a FADP dose-dependent signal was observed which was attributed to residual FAD in the FADP preparation. The response due to this contamination at higher doses of FADP was appreciable and indicated that the optimal sensitivity was to be obtained by using a concentration of FADP which gave the largest signal:noise ratio (5–10 pmol/well). The value of the final signal was directly proportional to the duration of the first-stage incubation, and was optimized in terms of signal magnitude at 30–45 min at room temperature following addition of FADP (data not shown).

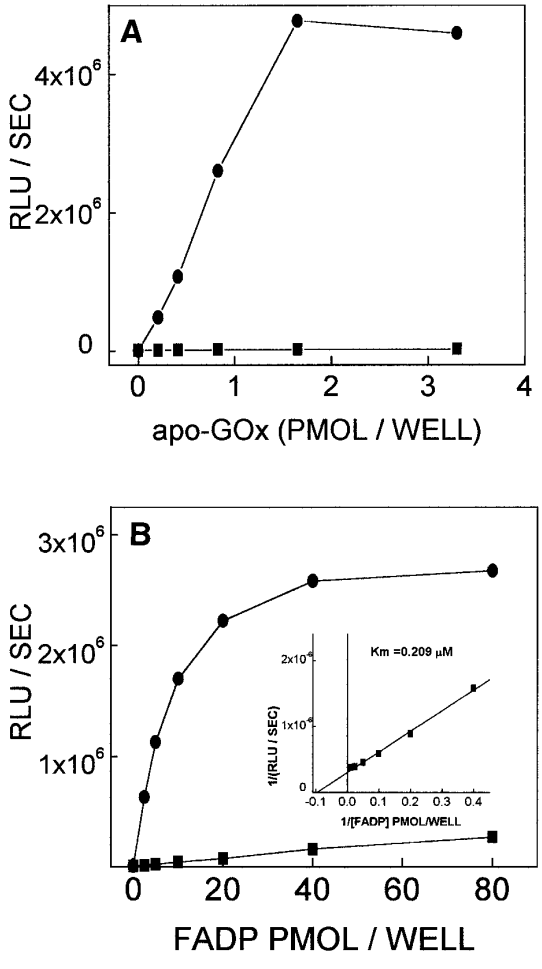


FIG. 5. (A) Conversion of apo- to holo-GOx by FAD; dose response of added apo-GOx in the presence (●) or absence (■) of FAD (25 pmol/well). (B) Generation of GOx activity as a function of conversion of FADP to FAD; dose response of FADP in the presence (●) or absence (■) of 1.5×10^{-16} mol/well ALP.

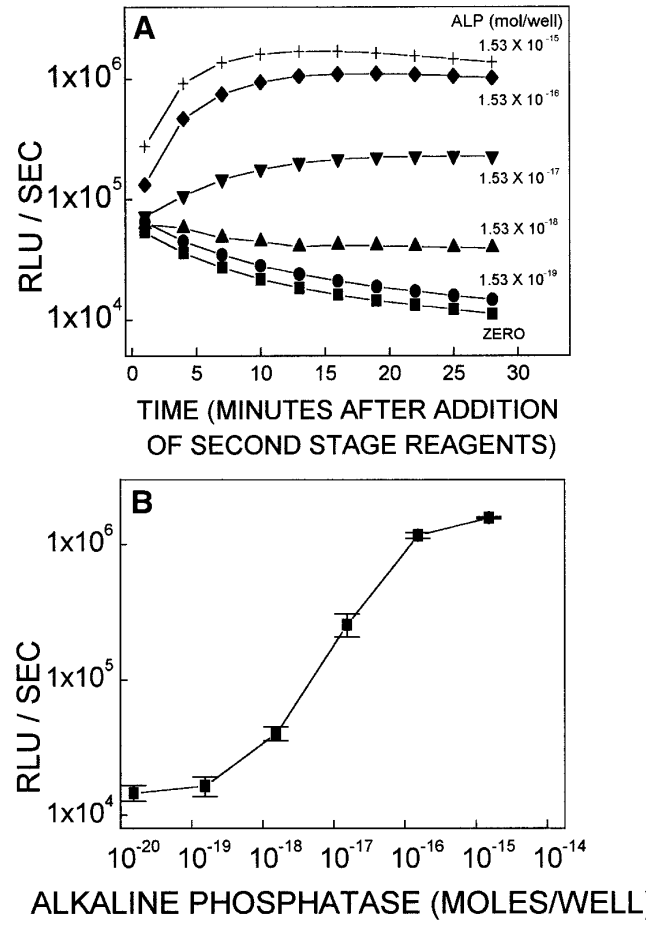


FIG. 6. Alkaline phosphatase activity monitored by the FADP/GOx/AE amplification cascade. (A) Time course of luminescence plotted by dose of ALP (mol/well). (B) Dose response of ALP: Data (means ± SD of triplicates) measured at 22 min following addition of the second stage reagents.

A time course, following addition of the second-stage reagents, of the dose response of and a single time point log–log plot of the dose response of ALP is depicted in Figs. 6A and 6B, respectively. Occasionally the signal at zero and low doses of ALP was maximal at zero time and tended to decline as the second-stage incubation proceeded. This was observed when the first incubation took place at 37°C (as depicted) rather than room temperature. From control experiments omitting one of the reaction components an initial low-level spontaneous background AE luminescence was found to be the cause. This AE-dependent signal declined to half its initial value within 5 to 10 min following addition of the second-stage reagents. Subsequent experiments were performed with the first-stage incubation at room temperature where this spontaneous luminescence was considerably lessened. After 10 min the dose response became maximal with a working range from 10^{-15} to $<10^{-18}$ mol/well (limit of detection 4.12 $\times$

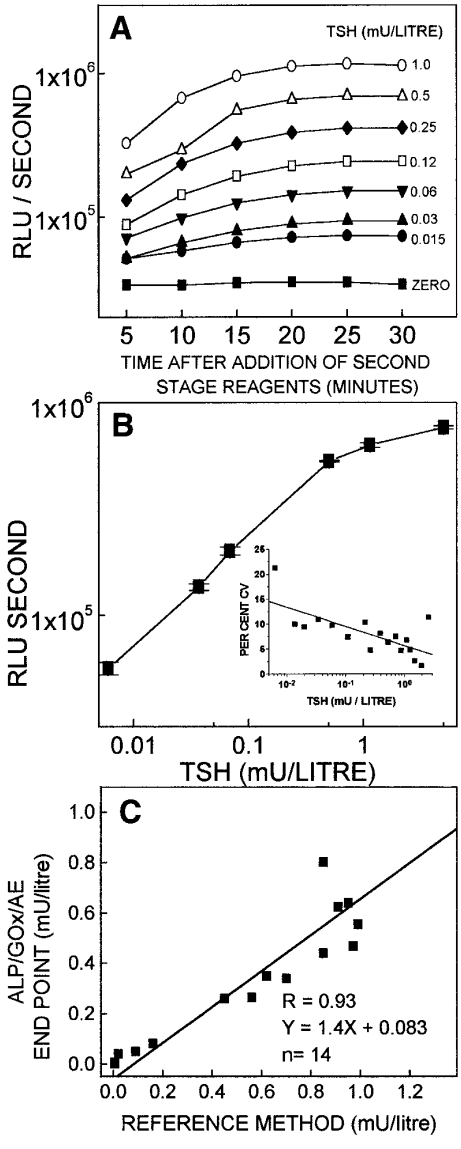


FIG. 7. TSH assay using the amplification cascade/AE chemiluminescence endpoint. (A) Time course of luminescence plotted by dose of TSH (mU/liter). (B) Dose response: data (means ± SD of duplicates) are taken at 20 min following addition of second-stage reagents. Inset: precision profile of 65 samples/standards measured in 3 assays binned into 13 sets, plotted as CV (dose) of duplicates against mean dose. (C) Comparison of clinical sample data with reference TSH assay.

10^{-19} mol/well at 2 standard deviations from mean zero) over a response range of 20-fold above the background at zero ALP (Fig. 6B).

The ability of using this endpoint in a clinical context was examined by its application to a human TSH ELISA (Fig. 7). After the immunochemical reaction between (anti-TSH) capture antibody, TSH, and (anti-TSH) antibody conjugate and subsequent washing, the two-stage reaction described previously was used to measure bound ALP activity. The time course of the luminescence signal TSH dose response is depicted in Fig. 7A. For clinical sample measurement the signal at 20 min following addition of the second stage was used. Figure 7B depicts a dose response for TSH at this time point. The inset to Fig. 7B describes an interassay precision profile of samples and standards (n = 83, binned into sets of 5), obtained from 3 assays using duplicate estimations. From this it can be estimated that the functional working range extends to 0.005 mU/liter at 15% CV (i.e., third-generation sensitivity). A small clinical sample method comparison indicated a high level of correlation between the experimental and reference methods (Fig. 7C).

DISCUSSION

Hitherto, AEs have been exclusively used as flash-type labels where the chemiluminescent moiety is covalently conjugated to the respective ligand. At the assay endpoint, excess hydrogen peroxide is added to initiate AE chemiluminescence. Here our investigations have been directed to the development of a system where exogenous unconjugated AE is present in excess, and the chemiluminescence is generated by analyte dose-dependent production of peroxide. If the latter is continuous, then the chemiluminescent signal is also continuous and thus would exist as a glow rather than a flash. Recently di-*ortho*-bromo-AE was synthesized. It was apparent that it potentially possessed much greater reactivity. The aim of the current study was to investigate this new compound to see if it was reactive at near neutral pH and hence be utilizable in a glow-type system by being coupled to oxidase enzyme peroxide generation.

Classically AEs which contain a suitable leaving group, such as an aromatic ester, will release potential energy in the form of photonic emission (i.e., chemiluminescence) in the presence of hydrogen peroxide at ambient alkaline pH. A key factor influencing the chemiluminescent reaction is the property of the phenoxide leaving group. McCapra (5) demonstrated that for efficient chemiluminescence the pK_a of the conjugate acid of the leaving group should be $<$12. AEs with such structure, such as I (Fig. 1), have proved to have been extremely successful as labels in ligand binding assays involving flash-type endpoints. This has provoked much interest into modification of the ring structure of the phenolic moiety, in particular the placing of electron-withdrawing groups on the phenolic nucleus. This has the effect of increasing the stability of the phenoate anion upon hydrolysis of the ester bond, and thus rendering the phenoxyester more reactive. It is possible to predict the pK_a of modified phenoxy nuclei using the algorithms described by Perrin *et al.* (6). Thus dibromo substitution results in lowering of the

predicted pK_a value from 10.8 for the di-*ortho*-methyl compound to 7.13 for the di-*ortho*-bromo compound.

From the results of the experiments investigating the effect of lowering the pH of the initiating solution it is apparent that, in comparison to di-*ortho*-methyl-AE, the bromo-substituted AE was much more reactive at near neutral pH. Both AEs displayed a similar pH-related alteration in the reaction kinetics in that the rate became prolonged and the profile flatter with a lower peak height in comparison to the conventional flash seen under standard conditions. This reduced rate of reaction is to be expected since several steps in the chemiluminescent reaction mechanism are pH dependent (for details see Weeks *et al.*, (2)). However, the pH at which these alterations in kinetic profile occurred varied between the two AEs and the trend corresponded to that predicted by the theoretical pK_a data. The di-*ortho*-bromo-AE was reactive at near neutral pH such that at pH 6 over 120 s, the quantum yield was 50% of that seen at pH 14, and at the end of this measurement period the rate was still at 50% of maximum. Thus, under these conditions the chemiluminescent reaction could be subjectively described as a glow rather than a flash. Therefore, the luminescence intensity of this compound at around neutral pH indicated that the di-*ortho*-bromo-AE had potential as an oxidase enzyme endpoint indicator.

Initial experiments, where GOx, glucose, and di-*ortho*-bromo-AE were coincubated, were immediately successful in that a potentially utilizable chemiluminescent yield was obtained. Under these circumstances it was possible to recover a high proportion of added RLU, although it is difficult to achieve accurate reconciliation when comparing RLU obtained by the conventional flash-type and oxidase-dependent reaction. A critical factor in the optimization was the quantity of AE added and the ambient pH (Figs. 4A and 4B). At pH 7.5, although the chemiluminescent yield was higher, the zero GOx background signal was also elevated in comparison to pH 6.5. One further advantage of this lower pH was that the reaction rate is slower so that the signal tends to plateau thus permitting easier and more reproducible luminometry. The quantity of di-*ortho*-bromo-AE added was not critical in terms of signal:background above 200 pmol/well except at higher concentrations where self-absorption occurred. One advantage of this reagent excess situation was that no effect of catalase contamination of differing GOx preparations was observed. It was found necessary to add the di-*ortho*-bromo-AE to the reaction buffer within a short time (10 min) prior to the addition of the GOx, otherwise the resultant yield was reduced. This may have been due to nonchemiluminescent adduct and/or pseudobase formation when the AE was removed from its acidic storage environment (7). Such phenomena may have been occurring during the incubation and may have been partially responsible for the fall in output seen at high GOx concentration in addition to that resulting from peroxide-dependent consumption of AE. However, this did not appear to be of practical importance except that it may have limited the upper measurement limit of GOx. For practical purposes measurements of GOx dose response were obtained from a single time point generally at 10–20 min incubation following reagent addition giving a working range from approximately 10^{-16} to 10^{-11} mol/well GOx. For ligand binding assays where sensitivity is not a prerequisite then direct labeling using, for example, antibody–GOx conjugate, may provide a sufficiently high specific activity tracer.

To capitalize fully on the potential sensitivity of this system it was decided to marry this endpoint to the ALP amplification cascade described by Obzansky *et al.* (3). The resultant sensitivity ($<10^{-18}$ mol/well) compares favorably with other high-sensitivity ALP detection methods (8). The sensitivity appears to be limited by the zero signal which could arise from three sources: spontaneous di-*ortho*-bromo-AE luminescence, contamination of holo-GOx with residual apo-GOx, and FAD contamination of the FADP preparation. Spontaneous di-*ortho*-bromo-AE luminescence decayed with duration of secondary incubation and was practically abolished by performing the primary incubation at room temperature in later experiments. Little contamination of apo-GOx was observed with the supplied material. However, the FAD contamination was significant and necessitated using the FADP at concentrations near the measured K_m for this substrate since the empirically optimal signal:noise was obtained at 10 pmol/well. Thus sensitivity may have been limited by this compromise leading to the use of suboptimal substrate concentrations of FADP. Such a situation was found by Fisher *et al.* (9) using an ALP/FADP amplification cascade employing D-amino acid oxidase, horseradish peroxidase, and enhanced luminol.

The application of the ALP system to the TSH assay was undertaken to demonstrate the applicability of the ALP/GOx/di-*ortho*-bromo-AE cascade in a clinical context. It was by no means an exhaustive investigation and consequently the findings are merely preliminary. However, it was demonstrated that, although the endpoint is somewhat cumbersome with the two-stage incubation, a highly sensitive and precise assay was easily assembled using commercially available reagents.

It may be concluded that the use of the AE chemiluminescence in conjunction with the ALP enzyme cascade produces a highly sensitive ligand binding assay end point. The development of novel AEs which react with high luminescent intensity at neutral pH, and thus are capable of coupling to oxidase-type enzymes,

offers considerable potential as a new class of chemiluminescent indicators.

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