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Complementary Immunoassays
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CHAPTER 14

Thermochemiluminescence immunoassay

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1. INTRODUCTION

The production of light from a process is termed luminescence. A division can be made between physical luminescence and chemiluminescence (CL) according to the source of energy that is needed to induce the luminescent events. Fluorescence and phosphorescence are two types of photoluminescence produced after the absorption of radiative energy, and are therefore physical luminescence phenomena. Chemiluminescence is generally defined as the chemical production of light. Harvey (1957) added the restriction 'at ordinary temperatures'. Later, Seliger and McElroy (1965) stated that the emitted light must be of higher intensity than the expected blackbody radiation of the substance at the same temperature in the same spectral area.

The vast majority of organic chemiluminescent reactions can be classified as oxidative processes. Due to their applicability in analytical procedures a number of examples of this class of reactions have become well known. Thus lophine (I) (2,4,5-triphenylimidazole) and some related derivatives yield green chemiluminescence upon oxidation in basic solution (Radziszewski, 1877; White and Harding, 1964, 1965). The reaction scheme is shown in Fig. 1.

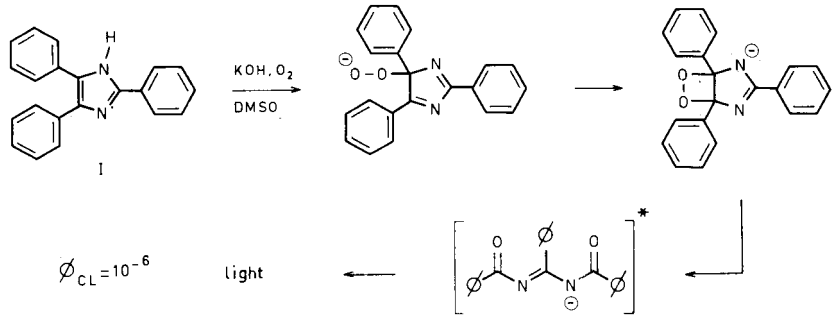


Fig. 1. Chemiluminescent reaction of lophine

Lucigenin (II) (N,N'-bismethylacridinium nitrate; Gleu and Petsch, 1935) emits blue-green light in the presence of hydrogen peroxide (H_2O_2) in a basic solution as illustrated in Fig. 2. Many oxalic acid derivatives react with H_2O_2 to

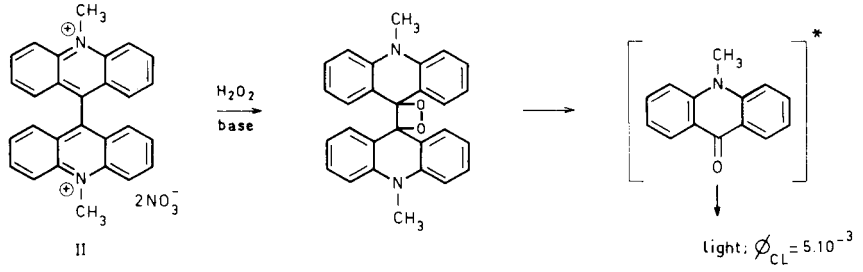


Fig. 2. The oxidation of lucigenin

yield an electronically excited product which can excite a fluorescer in the process mixture as shown in Fig. 3. Under optimal conditions this process is the most efficient non-enzymatic chemiluminescent reaction known to date (Chandross, 1963; Rauhut *et al.*, 1966, 1967; Rauhut, 1969; Roberts and Rauhut, 1972; Zweig and Maulding, 1973). Acridinium esters (McCapra, 1970, 1976) McCapra *et al.*, 1977) undergo a highly efficient oxidative chemiluminescent reaction with H_2O_2 to yield acridone as shown in Fig. 4. This reaction mimics to a certain extent the bioluminescent luciferin/luciferase system in the firefly. Together with the chemiluminescent oxidation of luminol and related phthalhydrazides these examples form the group of systems that have been tested and used extensively in chemiluminescent analytical procedures. All of the above reactions share the same type of intermediate, namely the 1,2-dioxetane four-membered ring moiety, substituted in various ways. This key intermediate yields electronically excited carbonyl products upon degradation. Hence, these chemiluminescent reactions are processes in which a very labile 1,2-dioxetane is formed, that in turn yields a luminescent product.

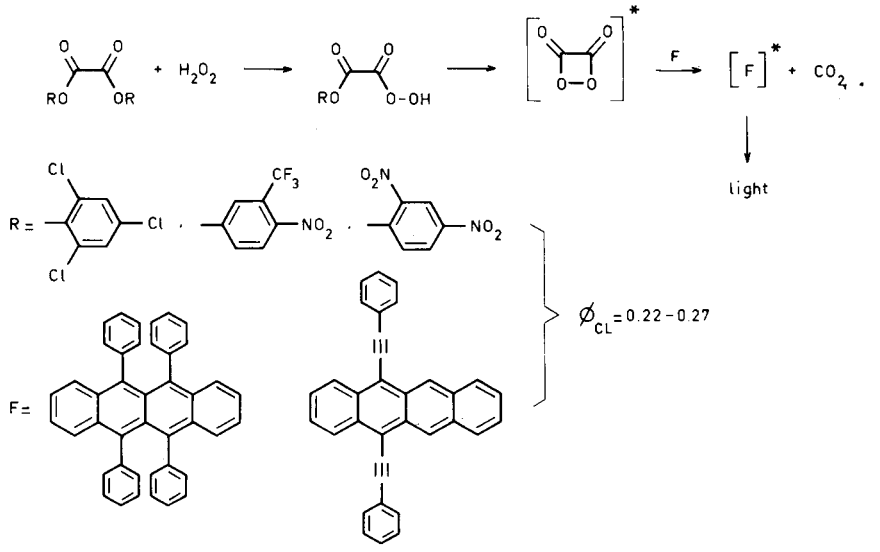


Fig. 3. Indirect chemiluminescence from oxalate ester oxidation

Less well known to workers in the field of immunoassay is the fact that well over 200 different (chemiluminescent) 1,2-dioxetanes have been prepared and isolated since 1968, when trimethyl 1,2-dioxetane was synthesized (Kopecky and Mumford, 1969). Nowadays at least ten different methods are available for the synthesis of 1,2-dioxetanes. Substituents include alkyl, aryl, spiroalkyl, spiroaryl, alkoxy, aryloxy, alkylamino, thioalkyl and thioaryl groups. A number of reviews on 1,2-dioxetanes (and 1,2-dioxetanones as well as 1,2-dioxetanedione) have appeared. These list the scope and limitations of the synthetic methods for most of the compounds that have been prepared, and

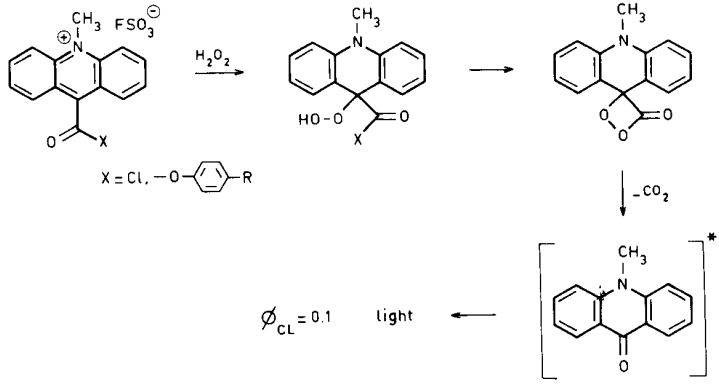


Fig. 4. Acridinium ester chemiluminescence

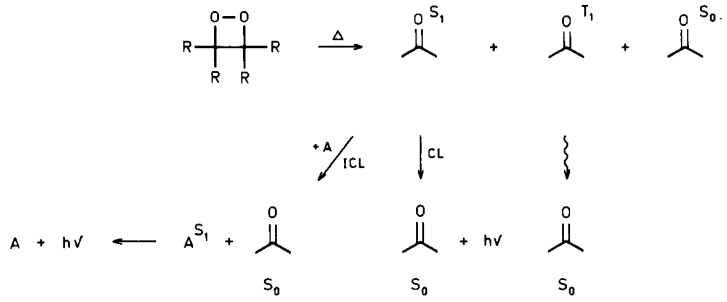


Fig. 5. Pathways for electronic energy in 1,2-dioxetane decomposition

describe their properties (Wilson, 1976; Adam, 1977; Horn *et al.*, 1978; Bartlett and Landis, 1979; Adam, 1980, 1982; Adam and Zinner, 1982; Kopecky, 1982; Bogan, 1982; Adam and Cilento, 1983; Baumstark, 1985; Wilson, 1985).

1,2-Dioxetanes decompose thermally into two carbonyl compounds, of which one can be formed in the first singlet (S_1) or triplet (T_1) electronically excited state. The excited products can emit light through relaxation either directly (direct chemiluminescence, CL) or via energy transfer (ET) to a luminescent acceptor molecule A (indirect chemiluminescence, ICL) as illustrated in Fig. 5.

The direct chemiluminescence from 1,2-dioxetanes is generally very weak compared to compounds such as luminol because most ketones, esters and aldehydes, which are products of the majority of the 1,2-dioxetanes, have a low fluorescence efficiency.

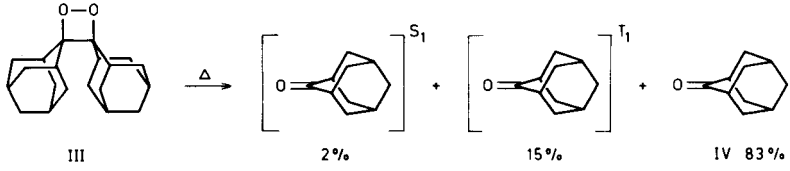


Fig. 6. Thermally induced chemiluminescent decomposition of adamantylideneadamantane 1,2-dioxetane

Most 1,2-dioxetanes require an energy of activation for their thermal decomposition of 20-26 kcal mol^{-1}, which means that all such compounds decompose within a few minutes to a few weeks at room temperature. Therefore these compounds are not useful as chemiluminescent labels for *in vitro* analytical procedures. In our laboratory, an extremely stable 1,2-dioxetane, adamantylideneadamantane 1,2-dioxetane (compound III, Fig. 6) was prepared in 1972 (Wieringa *et al.*, 1972). This white and crystalline compound (m.p. 174-176°C) can be stored at room temperature for years without decomposition to adamantanone (compound IV, Fig. 6). A half-life of

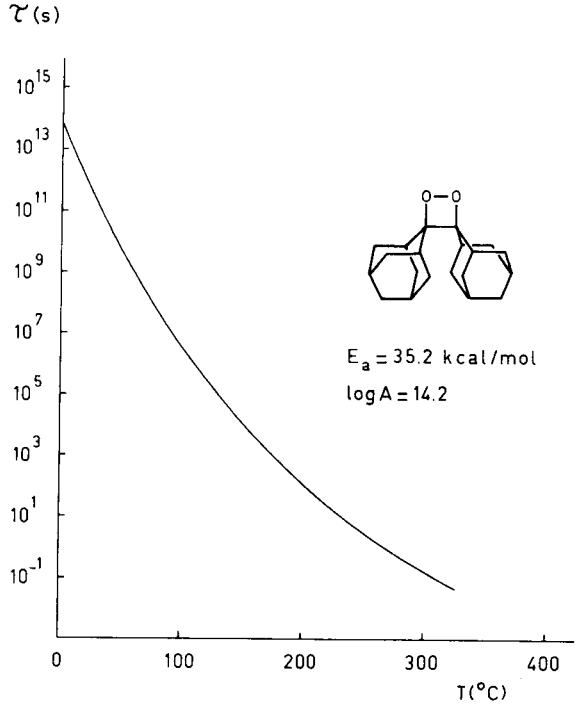


Fig. 7. The half-life of compound III as a function of temperature

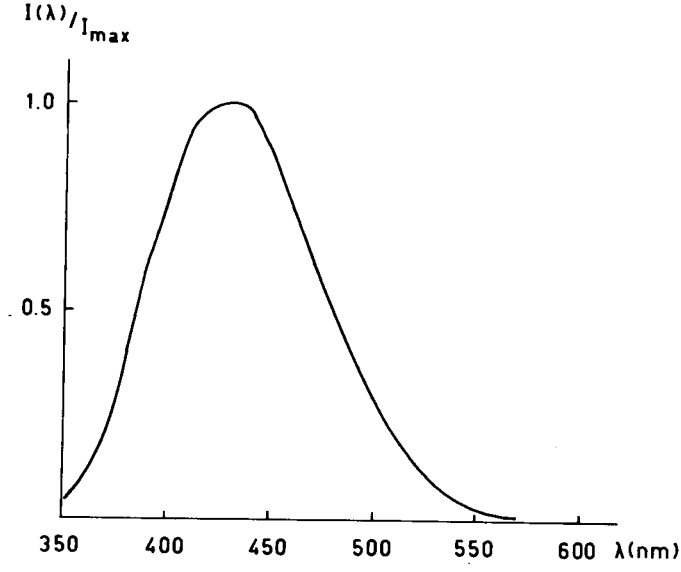


Fig. 8. Spectrum of chemiluminescence from compound III at 200°C under an atmosphere of N_2

10^4 years was calculated for compound III at 25°C. The thermal decomposition is a first order process and the kinetics of the chemiluminescent reaction are independent of the concentration of 1,2-dioxetane. The half-life is shortened exponentially with rising temperature. This relationship for compound III is shown in Fig. 7.

Hence the shape of the chemiluminescence curve (I/I_{max} versus time) is a function of the temperature of the sample. A constant CL curve is observed at all concentrations using a defined temperature programme and a standard detection period. Because this chemiluminescence is initiated and controlled by temperature alone and since no bimolecular reaction is involved, we have called this process thermochemiluminescence (TCL). Both the 1,2-dioxetane and the decomposition product IV are colourless compounds, thus self-quenching does not occur at high (local) concentrations.

The TCL spectrum of compound III is shown in Fig. 8 and is independent of concentration. The TCL maximum is found at 425 nm. The spectrum mimics the fluorescence spectrum of adamanatanone.

2. PREPARATION OF LABELS AND LABELLED COMPOUNDS

We have synthesized TCL labels based on adamantylideneadamantane 1,2-dioxetane III (Hummelen *et al.,* 1986). A few examples are shown in Fig. 9. Because these 1,2-dioxetanes are prepared by photo-oxygenation of 4-equatorially substituted adamantylideneadamantanes, they are formed as (~1:1) mixtures of isomeric (syn and anti) 1,2-dioxetanes. Malemide V is an example of a syn isomer and the iodoacetate VI is an example of an anti isomer. Except for iodoacetate VI, which was prepared as the pure anti isomer, the TCL labels are used in the form of ~1:1 isomeric mixtures.

The N-hydroxysuccinimide esters VII and VIII are the labels that are used routinely since these compounds react readily with the free amino groups of proteins. Compound VIII has the advantage over VII that it is crystalline with superior solubility characteristics in water-miscible solvents. All labels (and their decomposition products) are colourless compounds, which can be kept for a year without decomposition if stored dry at or below room temperature.

Proteins containing free amino groups can be labelled in a simple one-step procedure using the TCL N-hydroxysuccinimide ester VIII. The label is dissolved routinely in 1,4-dioxane and this solution is added to a buffered (borate 100 mM, pH 8.5) aqueous protein solution (1-20 mg ml^{-1}) with gentle shaking at room temperature. Other water-miscible organic solvents can be used equally well. When antibodies are labelled, the final concentration of 1,4-dioxane is kept below 5% (v/v). Bovine serum albumin (BSA) can be labelled with more than 30 TCL residues, when the reaction is carried out in 33% 1,4-dioxane. The resulting TCL-BSA is still water-soluble.

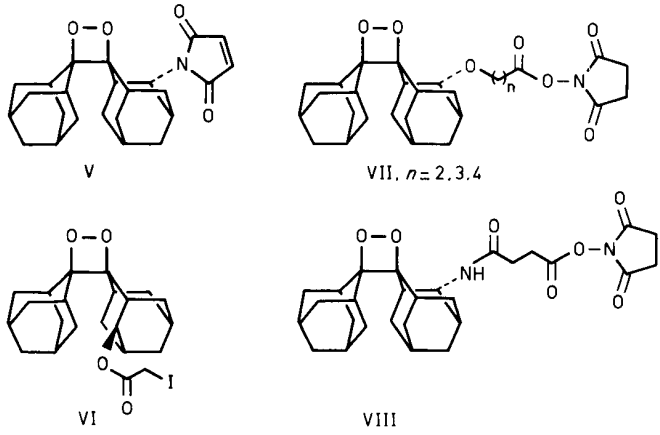


Fig. 9. TCL labels for R-SH (V and VI) and R-NH_2 (VII and VIII)

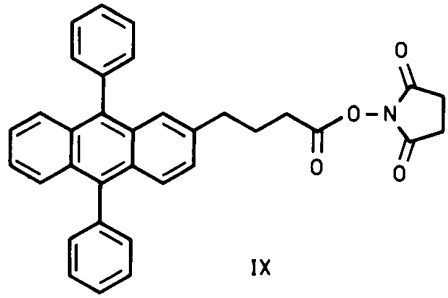
After 1 hour, the reaction is complete and the product is purified by dialysis against borate buffer, or by column chromatography, using Sephadex LH-60, and borate buffer as the eluent. When labelled BSA was lyophilized it showed unaltered TCL specific activity after being stored at -20°C for more than 2 years.

Since the TCL labels do not absorb light of $\lambda > 290$ nm, the number of labels bound to a protein can best be determined by TCL detection or chemical titration. We have found a perfect correlation between the titration of free amino groups (by the method of Habeeb, 1966) of BSA, indicating 18 residues of compound VIII being bound covalently, and the TCL specific activity of this protein.

Hence, the specific activity of label VIII is fully preserved upon conjugation to a protein. Furthermore, the specific activity does not decrease upon heavy labelling of proteins, i.e. BSA labelled with 25 residues of compound VIII shows a specific activity equal to 25 equivalents of compound VIII. During the course of these investigations a great variety of proteins (e.g. antibodies, enzymes and globulins) were labelled. It can be concluded that all proteins can be labelled with compound VIII with conservation of specific activity, unless the protein contains metal or coloured centres.

3. ENERGY TRANSFER THERMOCHEMILUMINESCENCE

As shown in Fig. 5, the electronic energy of the singlet excited ketone product from 1,2-dioxetane decomposition can be transferred in a non-radiative way to a luminescent acceptor molecule. This phenomenon can be used to amplify the TCL signal from 1,2-dioxetane labels and labelled compounds. In the routine procedure, a protein is labelled with both TCL label and the luminescent



IX

Fig. 10. The structure of SCP-DPA (IX)

acceptor molecule. A new fluorescent label, 2-[O-(N-succinimidyl)carboxypropyl]-9, 10-diphenylanthracene (SCP-DPA; IX, Fig. 10), which is stable at 200-250°C under an atmosphere of nitrogen, was developed for this purpose. This compound mimics all fluorescent properties of the well-known acceptor compound 9,10-diphenylanthracene (DPA) to a great extent (Wilson and Schaap, 1971; Turro *et al.*, 1974).

Since the fluorescence efficiency of adamantanone is 5.2×10^{-3} and that of DPA 1.0, a maximal amplification of a factor of 190 could be expected theoretically (at infinite concentration of DPA and at room temperature). The efficiency of ET is concentration dependent. It was predicted by Förster (1948) and confirmed experimentally later (Latt *et al.*, 1965; Stryer and Haugland, 1967; Bücher *et al.*, 1967) that the rate as well as the efficiency of ET through dipole-dipole interaction of a donor and an acceptor depends on the sixth power of the distance between both chromophores. The distance between donor and acceptor at which the efficiency of ET is 0.5 is defined as R_o. Analogously to the work to Stryer (1978) we calculated the value of R_o for the donor-acceptor pair S_1-adamantanone and DPA to be 15.3 Å (in a medium with a refractive index of 1). The relationship between the efficiency of ET and the value of R_o is shown in Fig. 11.

Thus in order to obtain an efficient amplification of TCL by SCP-DPA the mean distance between the 1,2-dioxetane and the DPA residue should not exceed 15 Å. This criterion can be met easily when the two components are combined covalently. However, in the present case of labelling a protein with both the TCL label and SCP-DPA in a random way, a statistical distribution of both moieties with a certain mean distance will result. The number of DPA labels per protein molecule, needed for efficient (> 50%) energy transfer, can be estimated roughly if some simplifying assumptions are made. For BSA (with a radius of ~15 Å), we estimate this number to be 6. For IgGs (with a radius of ~25 Å) at least 20 residues of SCP-DPA would have to be bound. From this theoretical estimation it can be concluded that random labelling of IgGs with TCL labels of the type described in this chapter and a functionalized DPA most

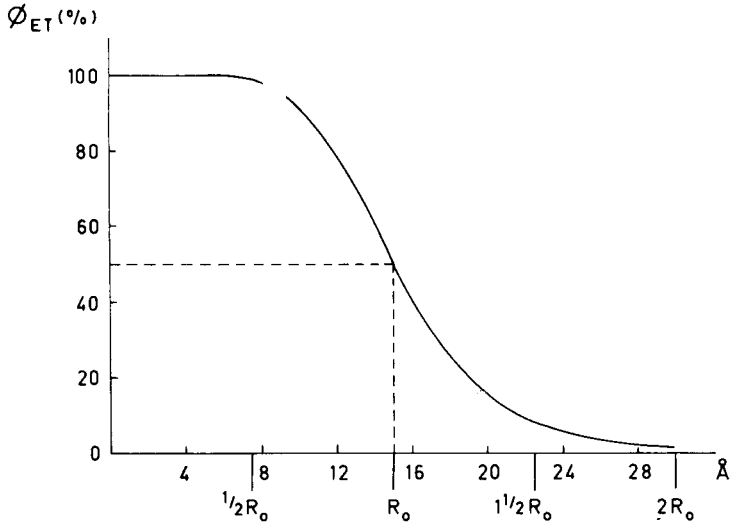


Fig. 11. The efficiency of ET as a function of distance *R* between donor and acceptor chromophore

probably does not result in either optimal amplification of TCL or active antibody conjugates. On the other hand, random labelling of smaller macromolecules with this pair of compounds should give conjugates of high specific activity. In practice it appears that the maximal amplification of TCL by ET is a factor of 40. Thus when BSA was labelled with N-hydroxysuccinimide ester (VIII) first and subsequently with SCP-DPA in large excess under standard conditions for labelling (see Fig. 12), a brightly blue fluorescent and brightly

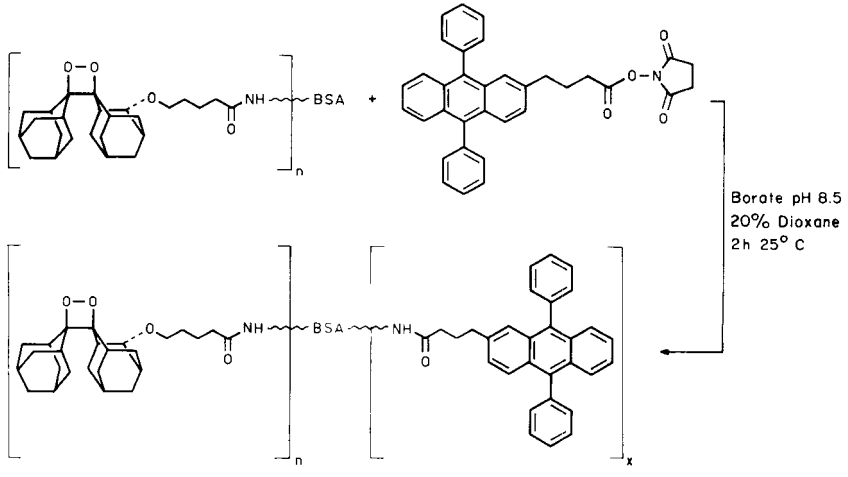


Fig. 12. Preparation of dual labelled BSA

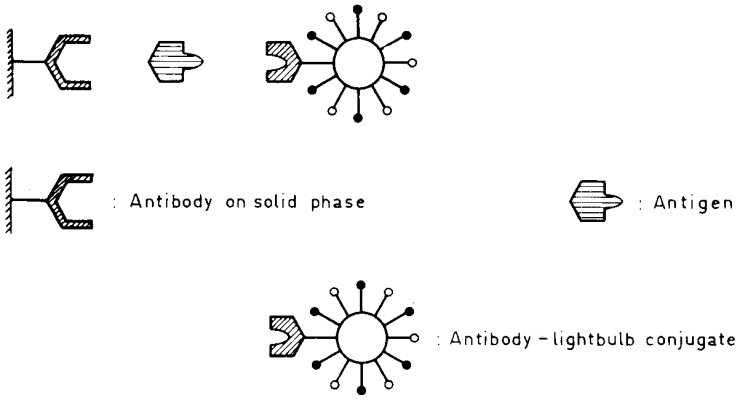


Fig. 13. Principle of FATIMA

blue chemiluminescent dual conjugate was obtained, which was soluble in aqueous buffer solutions.

The TCL specific activity of this thermal lightbulb is unaffected by dilution. The dual conjugate exhibits an almost perfect 1:1 signal/concentration correlation in the range of 0.1 mg ml^{-1}-0.1 ng ml^{-1}. The detection limit of the protein (in 1% aqueous BSA) in our present apparatus with a counting efficiency of 0.14% was 10 attomoles. The material can be stored (preferably in lyophilised form) without decomposition or loss of activity, and can be used as a new label by covalent linkage to other proteins (IgGs) or small analytes, depending on the nature of the assay. A fluorescence amplified thermochemiluminescence immunoassay (FATIMA) using this BSA dual conjugate as the label has been developed in our laboratory according to the format illustrated in Fig. 13.

4. QUANTITATION OF THERMOCHEMILUMINESCENCE

A sample temperature of 200-250°C is needed for a rapid TCL reading. Accordingly, one obvious format for the sample is a dry residue (as a coating, as a bound (immune-) complex, or as a result of an evaporated solution) on a carrier material. Suitable carriers are thermally stable (under an atmosphere of nitrogen), do not emit thermoluminescence in the relevant spectral area of detection between 100 and 250°C, do not quench the TCL signal, and can be used conveniently as carriers in (standard) immunoassay techniques. Kapton 500 H and Teflon (DuPont), among other materials, fulfil these requirements satisfactorily. Components of an immunological reaction can be coated physically on discs (ø 0.9 cm) made of these polymers. ELISAs performed on these solid phase supports are equal to or better than those on commerical (polystyrene) microtitre plates. Kapton 500 H shows a thermal background of ~1 cps at 240°C after a very simple cleaning procedure.

Thus, in the case of a FATIMA, discs of Kapton carrying the TCL two-site immune complex are placed on a small heating element and the TCL output is detected by means of photon counting equipment over a period of time (60 seconds) during which the sample is heated from 100°C to 250°C and kept at that temperature.

The photon counting apparatus consists of a bialkali photomultiplier (PM) tube (EMI 9893 QA/350) in an uncooled housing (Products for Research TE 1004/TS/110) in order to allow the presence of a light conducting glass rod (Scott LST-UV; ø = 1 cm, l = 9 cm), which is coupled to an interference filter (Fairlight Edge Filter, short wave pass; 50% transmittance at 500 nm). The PM signal is preamplified. (EG&G 1121A) and sent to the discriminator (EG&G 1121A)/photon counter (EG&G 1109) unit. A holder with the heating element and sample is connected to the PM housing (to 2 mm from the interference filter) in such away that a small dark chamber results (< 1 cm^3) which is flushed with a gentle stream of nitrogen gas. The equipment is shown in Figs 14, 15 and 16.

In the fully automated detection system a robot (Mitsubishi 'Movemaster') transports the discs carrying sample to the heater and subsequently closes the detection chamber. Thereafter the PM power is turned on and the sample is heated. After a standard heating period (usually 60 seconds) the PM power is turned off, the chamber is opened by the robot and the sample is removed and discarded. The TCL data are collected and analysed by an Apple computer (which controls the whole system), and the dose-response curve is plotted. The system shown can handle four special microtitre-like plates containing a total of 240 samples. The system was chosen for maximal flexibility during the research and development of any type of TCL based assay.

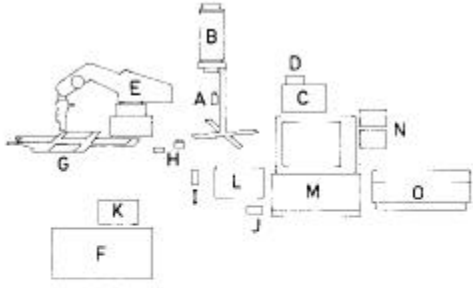


Fig. 14. Automated TCL detection system for TCL based immunoassays. A: heater and sample holder; B: photomultiplier housing and heater adaptor; C: photon counter-discriminator; D: temperature display; E: Movemaster robot with specially designed suction head; F: robot drive unit; G: carousel with 4 microtitre-like plates; H: devices with which the robot can shift a sample disc at its head position in order to deliver with high precision on the sample holder/heater; I: emergency switch; J: teaching box for robot positions; K and L: robot–PC interfacing, drive unit I/O ports and optocoupled relays; M: Apple Personal Computer (PC); N: disk drives; O: printer.

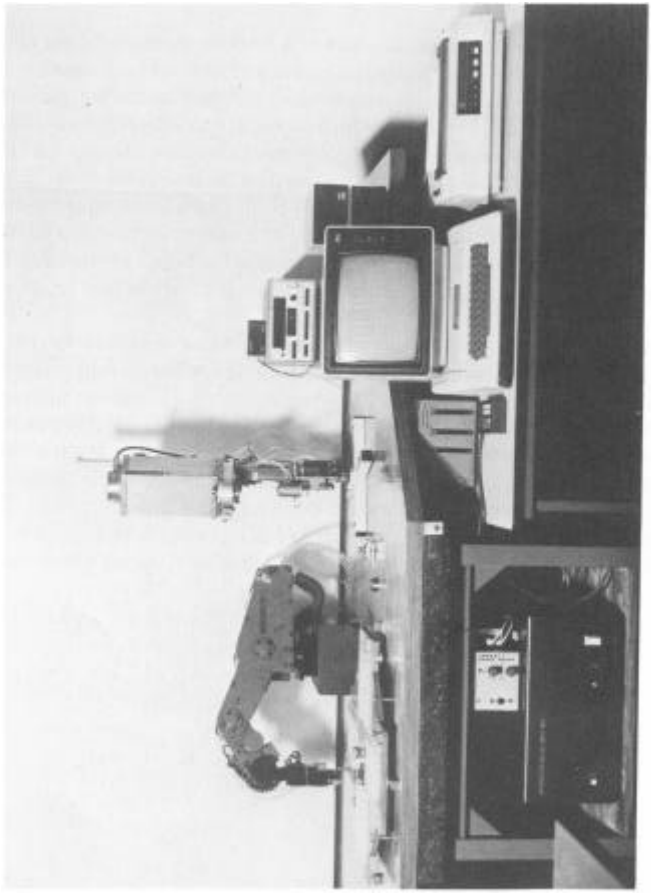


Fig. 15. The automated thermochemiluminescence detection system (see Fig. 14 for details) for TIA

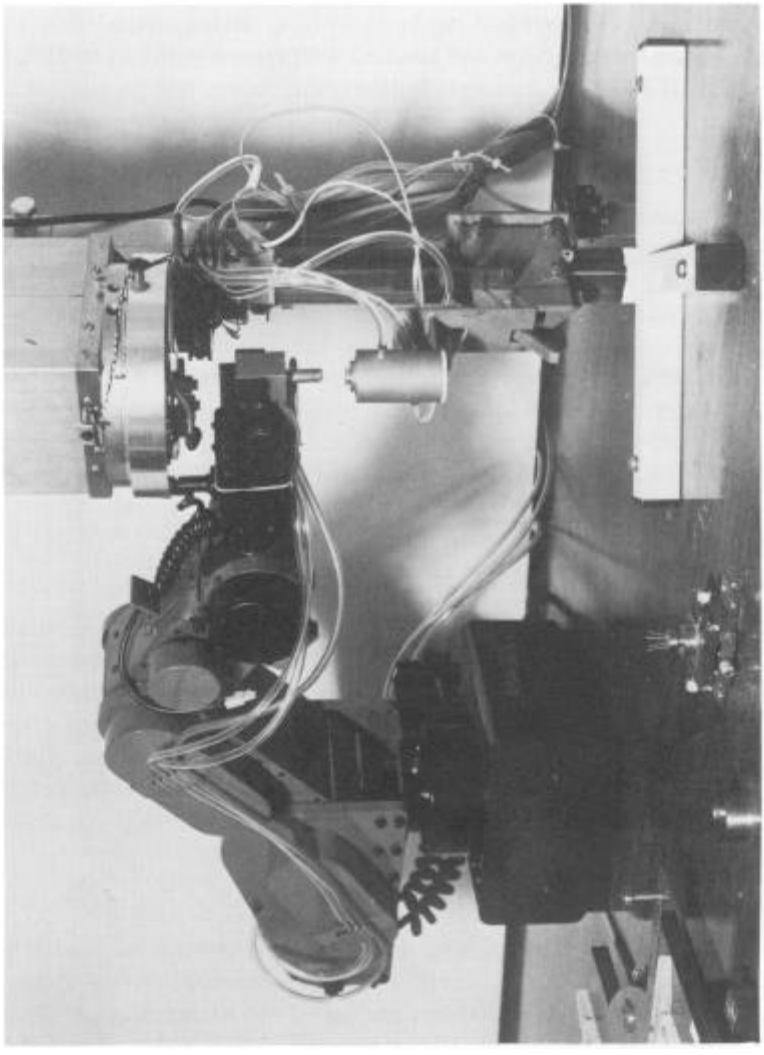


Fig. 16. A close up view of the Movemaster robot, heater and sample holder

5. A THERMOCHEMILUMINESCENCE IMMUNOASSAY FOR hCG

A two-site, solid-phase thermochemiluminescence immunoassay (TIA) for human chorionic gonadotrophin (hCG) was developed using Kapton 500 H discs coated with a monoclonal antibody to hCG and a second monoclonal antibody to a different epitope was labelled with several residues of TCL label (compound VIII). Hence, the amplification principle was not used in this assay.

5.1 Preparation of labelled antibody

Monoclonal mouse anti-hCG (Organon Technika) was dissolved in 1ml of 0.9% NaCl (3.1 mg ml^{-1}). One millilitre of 100 mM borate buffer (pH 8.3) was added followed by 200 μl of a 1.25 mg ml^{-1} solution of compound VIII in 1,4-dioxane. The solution was shaken gently for 2 hours at room temperature. The reaction was stopped by dialysis against borate buffer at 4°C. A labelling efficiency of 40% was calculated from the TCL activity of this conjugate (6×10^5 counts μg^{-1}). The immune reactivity of monoclonal anti-hCG $(VIII)_{10}$ was confirmed through competition with peroxidase labelled monoclonal anti-hCG in an ELISA. The TCL specific activity was fully preserved upon the addition of bovine serum and hCG standards.

5.2 Assay protocol

Kapton 500 H discs were coated with α-chain specific mouse monoclonal anti-hCG, washed twice with borate buffer (100 mM, pH 8.3), and incubated overnight with 160 μl of hCG standards containing 40% bovine serum, 50-60% borate buffer and 19 μg ml^{-1} of the labelled conjugate. The dose-response curve shown in Fig. 17 is the result of a quintuplicate experiment. The TCL data were obtained using the automated equipment described in the preceding section.

6. CONCLUDING REMARKS

Inherently chemiluminescent compounds can be prepared in such a form that they can be coupled to components of an immunoassay. When stable 1,2-dioxetanes are used as the luminescent factor, the quantification of such a labelled component only requires the addition of heat. 1,2-Dioxetanes derived from functionalized adamantylideneadamantanes are very suitable for these purposes for a number of reasons.

(i) The thermal as well as the chemical stability allow long storage periods of the labels and conjugates, and the use of a great variety of buffers over a large range of pH, serum components and organic solvents without noticable decomposition.

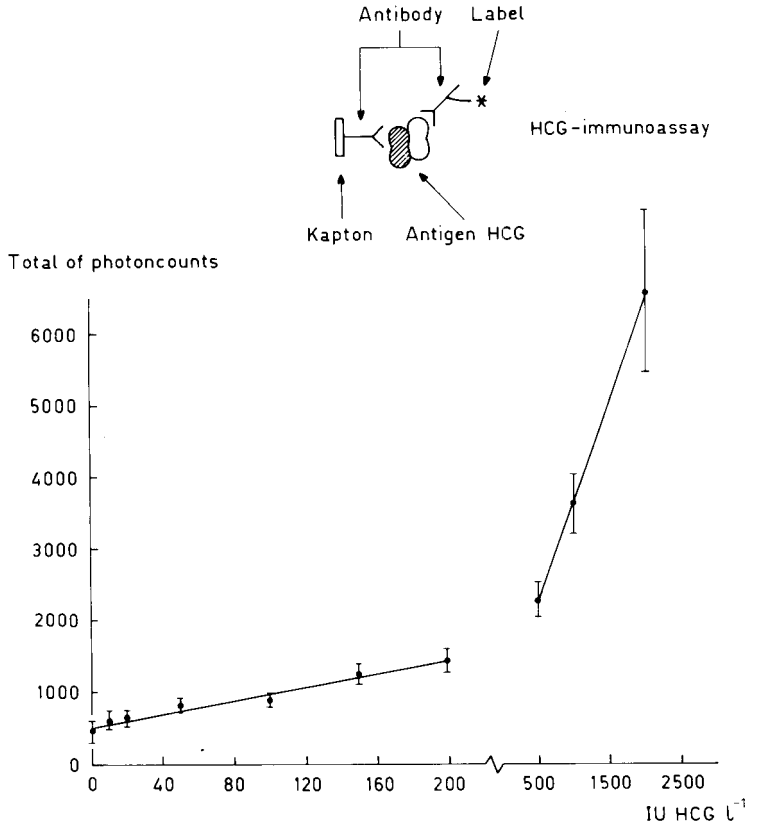


Fig. 17. Thermochemiluminescence immunoassay (TIA) for hCG

(ii) The thermochemiluminescent reaction is a first order process. Hence the kinetics of the reaction are independent of the amount of label present in the sample. False readings can be rejected through the use of curve fitting algorithms.

(iii) No additive chemicals are needed for TCL detection.

(iv) TCL labels can by synthesized conveniently, yielding products of constant high purity and specific activity.

(v) Because these labels are colourless, no self-quenching is observed and proteins can be labelled with high numbers of residues without relative loss of specific acitivity.

(vi) Although the specific activity of adamantylideneadamantane 1,2-dioxetanes is moderate (6×10^{19} photons mol^{-1}), the CL can be intensified maximally 40-fold through energy transfer to thermally stable fluorescers such as SCP-9,10-diphenylanthracene. Red shifted TCL is observed when acceptor compounds like fluorescein or rubrene are added.

(vii) Small proteins (e.g. BSA) can be labelled with a high number of TCL labels (~25) and (~10) DPA residues to yield a very efficiently thermochemiluminescent protein (~1-6 × 10^{22} photons mol^{-1}), which can be used as a water soluble label.

At the present stage the format of the TCL samples is restricted to dry residues that can be heated to 250°C; aqueous solutions (> 10 μl) cannot be assayed. While most of the elements of this new technique are subject to further investigation, a major improvement is expected to be found in the development of a heating system which combines high speed with focused and accurate heating. The assay for hCG clearly shows that the principles of TIA and FATIMA are feasible.

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