Stable 1,2-Dioxetanes as Labels for Thermochemiluminescent Immunoassay

Hummelen, Jan C.; Luider, Theo M.; Wynberg, Hans

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[39] Stable 1,2-Dioxetanes as Labels for Thermochemiluminescent Immunoassay

By JAN C. HUMMELEN, THEO M. LUIDER, and HANS WYNBERG

Introduction

Since 1976, when Schroeder *et al.* introduced a chemiluminescence immunoassay for biotin,[1] a variety of chemiluminescent techniques has been developed for immunochemical quantification of physiologically important substances. Yet, while fluorescence and enzyme immunoassays have taken a strong position as alternatives for radioimmunoassays, chemiluminescent assays (CIAs) have not grown as a routinely used, commercially available technique. In chemiluminescent techniques light is emitted in a multicomponent reaction. Consequently, reagent purity and mixing on the one hand, and sensitivity toward serum components on the other represent the most important problems in LIA techniques.

A new chemiluminescent system has now been developed in which all of the above-mentioned drawbacks are omitted because inherently chemiluminescent compounds, i.e., stable 1,2-dioxetanes, are used as the label. The emission of light from these compounds can be triggered by thermal activation. This chapter describes the principle of this thermochemiluminescence (TCL), the preparation and properties of TCL labels based on adamantylidene adamantane 1,2-dioxetane, the inclusion of 1,2-dioxetanes in *g*-cyclodextrin, energy transfer TCL, the use of TCL in immunoassay techniques, and the detection of TCL.

Principle of Thermochemiluminescence

1,2-Dioxetanes (Fig. 1, **I**) decompose thermally into two carbonyl fragments. Because of the release of strain energy and because of a favorable reaction path, a fraction of the carbonyl fragments is formed in the first singlet or triplet electronically excited state (II-S_1 and II-T_1). Such electronically excited species can emit light either directly ("direct chemiluminescence," CL) or via energy transfer to a luminescent acceptor molecule A ("indirect chemiluminescence," ICL).

Only very few of the over 200 1,2-dioxetanes known today are stable

[1] H. R. Schroeder, P. O. Vogelhut, R. J. Carrico, R. C. Boguslaski, and R. T. Buckler, *Anal. Chem.* **48,** 1933 (1976).

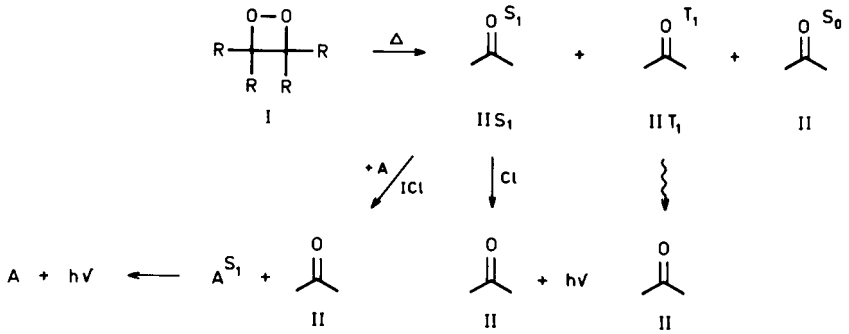

FIG. 1. Chemiluminescent thermal degradation of 1,2-dioxetanes.

(for days) at room temperature.[2] Adamantylidene adamantane 1,2-dioxetane (Fig. 2, **III**) is extremely stable (mp 174-176°)[3] and can be stored for years at room temperature or below without decomposition to adamantanone **IV** (a half-life of 1.2×10^4 years is calculated for **III** at 25°). The exponential relation between the half-life (τ) of **III** and the temperature is shown in Fig. 3. Since the decomposition is a first-order process, the kinetics of the chemiluminescent reaction are independent of the concentration of **III**; the shape of the chemiluminescence curve (I/I_{max} versus time) is a function of the temperature of the sample only. Autocatalytic degradation of **III** has never been observed. The efficiency of direct chemiluminescence (f_{CL}) of **III** is 1×10^{-4}, i.e., 1 mol of **III** emits 6×10^{19} photons when heated.[4] This efficiency is a product of the efficiency of the decomposition reaction ($f_R = 1$), the efficiency of S_1-adamantanone formation ($f_S = 0.02$), and the efficiency of fluorescence of S_1-adamantanone ($f_F = 5.2 \times 10^{-3}$).[4,5] The T_1-adamantanone formed during the decomposition is quenched by collision with other molecules and cannot substantially contribute to the emission of light unless either a fluorescent triplet energy acceptor (e.g., 9,10-dibromoanthracene) is added or collision chances are minimized. The spectrum of direct CL from **III** (Fig. 4) shows a maximum at 425 nm and is identical to the fluorescence spectrum of **IV**.[4,6]

[2] W. Adam and G. Cilento, "Chemical and Biological Generation of Electronically Excited States." Academic Press, New York, 1982.
[3] J. H. Wieringa, J. Strating, H. Wynberg, and W. Adam, *Tetrahedron Lett.* p. 169 (1972).
[4] G. B. Schuster, N. J. Turro, H.-C. Steinmetzer, A. P. Schaap, G. Faler, W. Adam, and J. C. Liu, *J. Am. Chem. Soc.* **97,** 7110 (1975).
[5] A. M. Halpern and R. B. Walter, *Chem. Phys. Lett.* **25,** 393 (1974).
[6] C. A. Emeis and L. J. Oosterhoff, *J. Chem. Phys.* **54,** 4809 (1971).

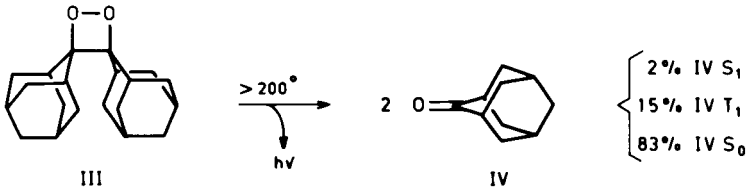

FIG. 2. Reaction of adamantylidene adamantane 1,2-dioxtane.

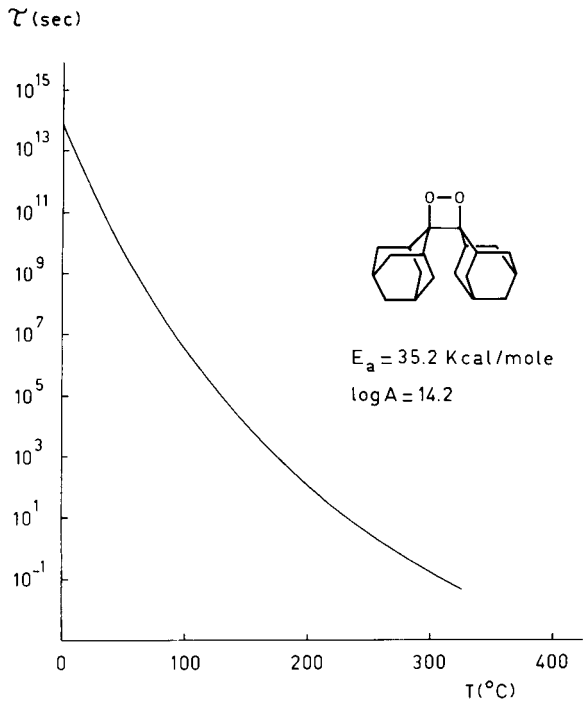

FIG. 3. Half-life of **III** vs temperature.

A detailed analysis of spectra and decay kinetics of TCL from **III** has been reported by Neidl and Stauff.[6a] Coincidentally, the emission spectrum nicely mimics the spectral response of bialkali photomultiplier tubes.

Both **III** and **IV** are colorless compounds [λ_{max}^{abs} (**III**) = 265 nm, e = 21.5; and λ_{max}^{abs} (**IV**) = 280 nm, e = 20].[4] As a consequence, f_{CL} is independent of the concentration of **III**: no self-quenching or absorption occurs,

[6a] C. Neidl and J. Stauff, *Z. Naturforsch. B: Anorg. Chem., Org. Chem.* **33B,** 763 (1978).

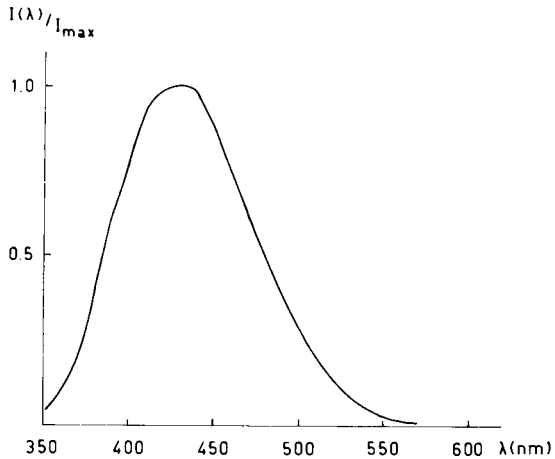

FIG. 4. TCL spectrum of **III** at 200° under an N_2 atmosphere.

even at very high local concentration. Therefore, macromolecular substrates can be heavily labeled with (colorless) derivatives of **III** without relative loss of specific activity. The latter feature of **III** in particular is rather unique for a label, and cannot be found among other chemiluminescent and fluorescent labels.

Synthesis of TCL Labels

A series of TCL labels based on adamantylidene adamantane 1,2-dioxetane has been prepared. Some examples are shown in Fig. 5. Compounds **V** and **VI** react with strong nucleophiles (e.g., free thiol groups of proteins), while the *N*-hydroxysuccinimide esters **VII** and **VIII** react with amino group containing compounds. In this chapter attention will be focused on compound **VIII**.

The synthetic route for compound **VIII** is outlined in Fig. 6, starting from bromoadamantylidene adamantane (**X**). Isomerically pure bromide **X** is obtained upon reaction of *N*-bromosuccinimide with adamantylidene adamantane **IX**, which in turn can be conveniently prepared on large scale (>500 g) from commercially available adamantanone **IV** in a two-step procedure.[7,8] The overall yield of the 7-step synthesis (from **IV** to **VIII**) is 50%.

In the last step of the synthetic route, sensitizer-generated singlet oxygen attacks alkene **XIII** from both the top and bottom side to yield a

[7] E. W. Meijer and H. Wynberg, *J. Chem. Educ.* **59,** 1071 (1982).
[8] P. D. Bartlett and M. S. Ho, *J. Am. Chem. Soc.* **96,** 627 (1974).

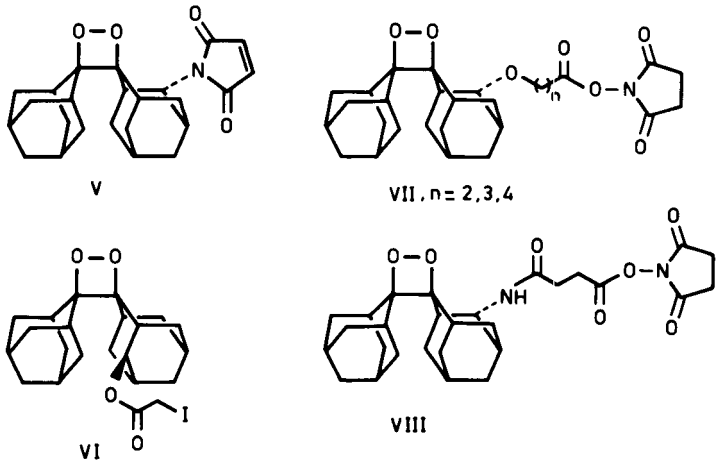

FIG. 5. TCL labels based on **III**.

(~1 : 1) mixture of isomeric 1,2-dioxetanes, *syn* and *anti* **VIII**. This mixture of isomers is used in labeling experiments. *N*-Hydroxysuccinimide ester **VIII** is a crystalline colorless compound which can be kept for months without decomposition if stored dry at or below room temperature.

4-eq-Bromoadamantylideneadamantane **X**

In a slightly modified previously published procedure,[9] 45 g (0.168 mol) **IX** and 55 g (2.1 eq) *N*-bromosuccinimide are dissolved in CH_2Cl_2 (600 ml) and acetic acid (15 ml) in a 1-liter round-bottom flask. The mixture is stirred magnetically and refluxed for 1 hr. The solvents are evaporated at a reduced pressure and the solid residue is dissolved with shaking in 3 liters of a 2 : 1 : 3 mixture of ether, pentane, and 0.5 *N* NaOH. After removal of the aqueous phase, the organic layer is shaken with 1 liter 0.5 *N* NaOH three times. After the addition of $MgSO_4$ (~5 g) and charcoal (~2 g), the suspension is filtered and concentrated to yield crude **X** (55 g) as a yellowish solid. Acetone (200 ml) is added and the suspension is stirred at 0° for 1 hr. The purified bromide is isolated by filtration and dried *in vacuo* (44.5 g, 76.5%); mp 128-130° (lit.[9] 130.5-131.5°).

4-eq-Aminoadamantylideneadamantane **XI**

Bromide **X** (7.0 g, 20 mmol) and dioxane (75 ml) are mixed in a stainless-steel cylinder (400 ml). Then liquid NH_3 (250 ml) is added slowly and

[9] E. W. Meijer, R. M. Kellogg, and H. Wynberg, *J. Org. Chem.* **47,** 2005 (1982).

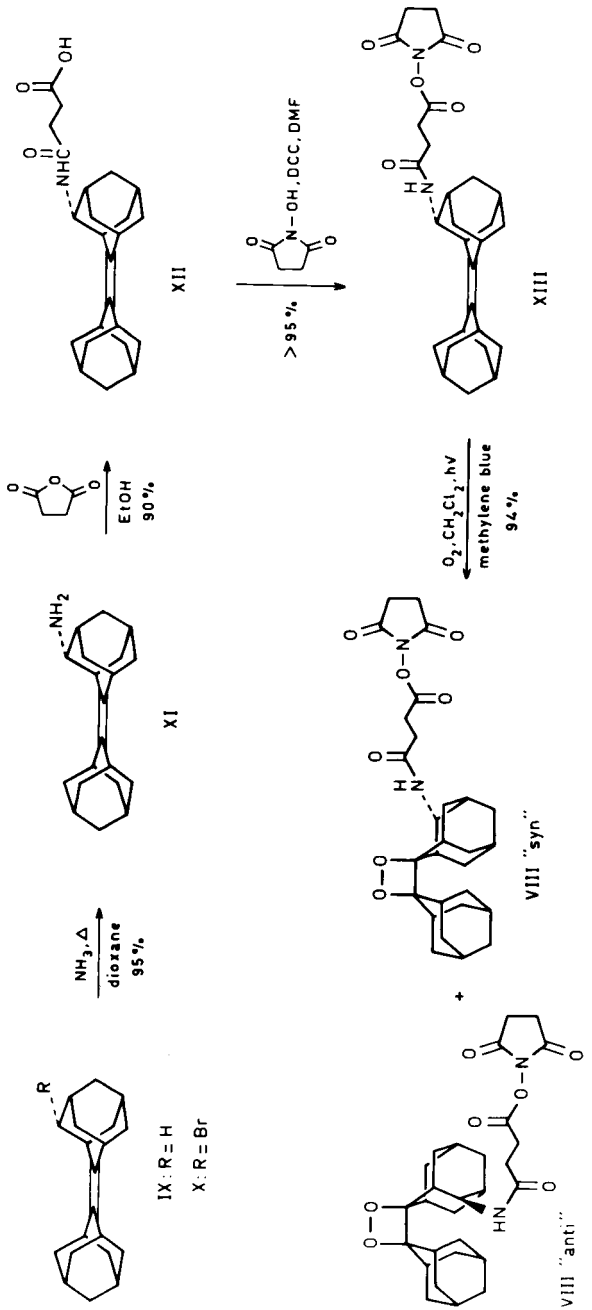

FIG. 6. Synthesis of **VIII**.

the cylinder is closed tightly. The mixture is shaken at 70° for 16 hr in a Carius oven. After cooling to -50° (liquid N_2), opening of the cylinder, and evaporation of unreacted ammonia (this takes 2 hr), the residual solution is poured on 1 *N* NaOH (100 ml). Ether is added (250 ml) to extract the product and the ethereal solution is washed with H_2O (3 × 200 ml), dried over $MgSO_4$, filtered, and evaporated to produce 5.70 g (95%) spectroscopically pure **XI** as a white solid.

A small portion of this material was sublimed (0.001 mm Hg, 150°) to give white crystals; mp 126-160°. IR (KBr): 3350 (br), 2900 (s), 1600 (m), 1450 (s), 1090 (m) cm^{-1}. ^1H NMR ($CDCl_3$, TMS): δ3.15-2.50 (br, 5H); 2.45-0.80 (m, 24H). ^{13}C NMR ($CDCl_3$): δ134.4, 132.0, 56.0, 39.3, 38.7, 38.1, 37.0, 34.7, 32.0, 31.7, 30.5, 30.1, 28.2, 27.7. Mass: M^+ at *m/e* 283 (100%), 266 (20%), 135, 79. Exact mass: calculated for $C_{20}H_{29}N$, 283.230; found 283.228.

N-(Adamantylideneadamant-4-eq-yl)succinic Acid Monoamide **XII**

A solution of amine **XI** (9 g, 32 mmol) in absolute EtOH (75 ml) is added to a solution of succinic anhydride (3.2 g, 32 mmol; Merck) in absolute EtOH (150 ml). From the initially clear solution monoamide **XII** starts separating after a few minutes as an amorphous material. After stirring the mixture overnight, the product is filtered off, washed with a little absolute EtOH, and dried in a desiccator (12.2 g, 90%), mp 253-256°. Monoamide **XII** is insoluble in CCl_4, $CHCl_3$, CH_2Cl_2, ether, and alkanes and is soluble in DMSO and DMF.

IR (KBr): 3400 (m), 3300-2500 (s), 2900 (s), 1720 (s), 1640 (s), and 1550 (s) cm^{-1}. ^1H NMR (DMSO-d_6, TMS): δ7.5 (br, 1H); 3.6 (m, 1H); 2.7 (m, 4H), 2.3 (br, 4H), and 2.3-1.0 (m, 22H). ^{13}C NMR (DMSO-d_6): δ174.0, 170.5, 134.4, 131.5, 53.9, 37.9, 36.7, 35.2, 32.7, 31.7, 31.5, 31.4, 30.6, 30.4, 30.2, 29.4, 27.8, 27.0. Mass: M^+-peak at *m/e* 383 (100%) and 365, 283, 266. Exact mass: calculated for $C_{24}H_{33}NO_3$: 383.246; found: 383.244.

N-(Adamantylideneadamant-4-eq-yl)succinic Acid Monoamide N-Hydroxysuccinimide Ester **XIII**

Monoamide **XII** (1.65 g, 4.3 mmol) is dissolved in warm dry DMF (50 ml). After the solution is cooled to room temperature, dicyclohexylcarbodiimide (900 mg, 4.35 mmol) is added. After stirring the solution for 10 min, dry *N*-hydroxysuccinimide (660 mg, 5.75 mmol) is added and the reaction mixture is stirred under an atmosphere of N_2 for 16 hr. A little precipitated dicyclohexylurea is removed by filtration and the solvent is removed by high vacuum evaporation. The residue is washed with *n*-hexane (2 × 50 ml) and then dissolved in CH_2Cl_2 (50 ml). A little insoluble

material is removed by filtration over a short column of seasand. After removal of the solvent at low pressure the N-hydroxysuccinimide ester remains as a white amorphous powder [mp 191-194° (EtOAc); 2.06 g, 100%].

IR (KBr): 3400 (m, NH), 2900 (s), 1805 (m), 1770 (m), 1720 (s), 1660 (m), 1535 (m), 1200 (m), 1060 (m) cm^{-1}. ^1H NMR ($CDCl_3$, TMS, 200 MHz): δ5.95 (t, *J* = 7 Hz, 1H), 3.9 (m, 1H), 3.02 (t, *J* = 7.5 Hz, 2H), 2.87 (m, 4H), 2.83 (s, 4H), 2.63 (t, *J* = 7.5 Hz, 2H), 2.2-1.0 (m, 22H). ^{13}C NMR ($CDCl_3$): δ168.8, 168.3, 136.0, 130.4, 54.3, 39.4, 39.0, 37.6, 37.1, 35.2, 33.8, 33.2, 32.2, 32.0, 31.6, 31.2, 30.9, 30.6, 28.3, 28.2, 27.3, 26.8, 25.4, 24.7. Mass: M^+ peak at *m/e* = 480, 365 (100%), 267, 266. Exact mass: calculated for $C_{28}H_{36}N_2O_5$: 480.262; found: 480.263.

Syn- and Anti-N-(Adamantylideneadamantane 1,2-Dioxetane-4-eq -yl)-succinic Acid Monoamide N-Hydroxysuccinimide Ester **VIII**

The active ester **XIII** (3.0 g, 6.38 mmol) is photooxygenated for 18 hr in a standard apparatus for photooxygenation of alkenes,[7] using CH_2Cl_2 (700 ml) as the solvent and methylene blue (5 mg) as the sensitizer. The solution is decolorized with charcoal, filtered, and concentrated with a flash evaporator. The yellowish residue is stirred with *n*-pentane (50 ml) and dried in a desiccator to yield the 1,2-dioxetane-active ester (3.0 g, 94%) **VIII** as a mixture of two isomers, spectroscopically pure, in the form of a slightly yellow solid. This material is used as thermochemiluminescent label without further purification (mp 96-110°).

IR (KBr): 3400 (br, NH), 2950 (s), 1810 (m), 1780 (m), 1730 (s), 1650 (m), 1540 (m), 1200 (m), 1080 (m) cm^{-1}. ^1H NMR ($CDCl_3$, TMS; 200 MHz): δ6.20 (d, *J* = 8 Hz, 0.5H), 6.00 (d, *J* = 8 Hz, 0.5H), 4.25 (m, 0.5H), 4.16 (m, 0.5H), 3.00 (t, *J* = 8 Hz, 2H), 2.83 (s, 4H), 2.65 (t, *J* = 8 Hz), 2.78-2.5 (m, 6H), 2.2-1.2 (m, 22H). ^{13}C NMR ($CDCl_3$): δ169.2, 169.1, 168.9, 168.2, 96.0, 95.7, 95.4, 95.3, 49.5, 48.6; signals between δ39.4 and 24.7. Mass: fragments at *m/e* = 362 and 150, 247, 219, 149, 100, 70. No M^+ peak. Exact mass fragment with *m/e* = 362: calculated for $C_{18}H_{22}N_2O_6$: 362.148; found: 362.150.

Application of TCL Labels: Characteristics of Labels and Labeled Compounds

Labeling of Proteins

Proteins that contain free amino groups can be labeled with ester **VIII** in a simple and fast one-step procedure. Because the solubility of **VIII** in aqueous buffers is low, a cosolvent is needed. 1,4-Dioxane is used rou-

tinely, but THF, EtOH 95%, DMF, and acetone suffice as well. When the concentration of cosolvent is kept below 5% (v/v), the immune reactivity of antibodies can be fully preserved. Bovine serum albumin (BSA) can be labeled with a high number of residues of **VIII** if the reaction is performed in dioxane/borate buffer (pH 8.5; 100 m*M*) 1 : 3.

Proteins, labeled with **VIII**, exhibit good solubility in aqueous buffers, e.g., BSA can be labeled with 25 residues **VIII** without significant loss of solubility.

General Procedure. The protein is dissolved in 100 m*M* borate buffer, pH 8.5 (1-20 mg/ml) at room temperature. Into this gently shaken solution is pipetted a fresh solution of **VIII** in dioxane [in such a concentration that a desired labeling ratio is obtained and the final concentration of dioxane is 5-10% (v/v) for antibodies]. The clear (or slightly turbid) solution is kept at 25° for 1 hr. Unreacted label is separated from the conjugate either by dialysis against borate buffer or by column chromatography (Sephadex LH-60/borate buffer). TCL activity of labeled proteins is preserved over a period of several months if stored at -20° in the dark.

Determination of Label Incorporation. Since TCL labels show neither UV absorption (at $\lambda > 280$ nm) nor fluorescence, the number of labels incorporated in proteins can best be determined by TCL measurement or by titration of residual amino groups in the conjugate. Amino group titration of TCL-labeled proteins can be performed using Habeeb's TNBS/SDS method.[10] An example is shown in Fig. 7. This titration indicates that when BSA reacts with 50 equivalents of **VIII**, 18 residues are incorporated, i.e., a labeling efficiency of 36% can be easily attained. Furthermore, the decrease of free amino groups upon conjugation is a good indication for the covalent nature of the label-protein linkage. When a mixture of hydrolyzed **VIII** (i.e., carboxylic acid) and a protein (e.g., BSA, human IgG) is dialyzed, the protein fraction does not exhibit TCL, indicating that strong aspecific (noncovalent) binding does not occur.

Specific Activity of TCL Labels and Labeled Compounds

The thermochemiluminescence spectra of **III**, **VIIa**,**b**,**c**, **VIII**, and TCL-labeled proteins are virtually identical. Additionally, the thermal stability of the 1,2-dioxane moiety is unaltered upon conjugation. Hence, the specific activity under standard conditions can be compared directly for these compounds.

Since the parent 1,2-dioxetane **III** and the free labels **VII** and **VIII** appear to be rather volatile compounds at ~240° (**VII** and **VIII** show

[10] A. F. S. A. Habeeb, *Anal. Biochem.* **14,** 328 (1966).

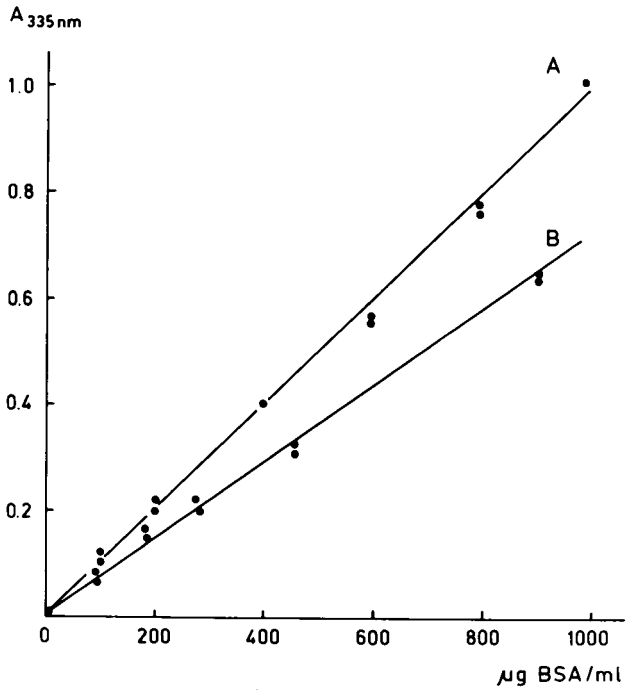

FIG. 7. Amino group titration of BSA and BSA–(**VIII**)$_n$ according to the method of Habeeb.[10] Thus the 100 μl of the protein solution were added 10% SDS (1 ml), 4% Na_2CO_3 (1 ml), and 0.1% (w/v) TNBS (1 ml) in H_2O, successively. After incubation at 37° for 2 hr, the reaction was stopped by addition of 1 ml 1 *N* HCl. UV absorption was measured at 335 nm. Since unlabeled BSA (A) has 61 free amino groups, it can be calculated that this BSA–(**VIII**)$_n$ (B) contains 18 amino group blocking residues of **VIII**.

thermochemical breakdown besides 1,2-dioxetane decomposition), TCL quantification of these compounds has to be carried out on strongly absorbing material, e.g., a small piece (~20 mm^2) of aluminum oxide thin-layer chromatography sheet (Neutral, Merck). The TCL of buffered solutions of labeled proteins can be measured by pipetting 1-10 *m*l of the solution on a disk of a thermoresistant polymer (Teflon, Kapton 500H), evaporation of solvent at 100°, and subsequent heating and detection at ~240°. Table I lists the specific activities of some TCL compounds, measured in the apparatus, described at the end of the chapter. This apparatus has a counting efficiency of 0.14%. It can be concluded that (1) the experimental values are in excellent agreement with the expected specific activity (1 mol **III** emits 6×10^{19} photons according to the reported values for f_S and f_F[4,5]); (2) substituted adamantylidene adamantane 1,2-dioxetanes show little variation in specific activity; (3) the specific activity is un-

TABLE I
SPECIFIC ACTIVITY OF SOME TCL COMPOUNDS[a]

Compound	Carrier	Measured specific activity (photon counts/mol)
III[b]	Al_2O_3	1.0×10^{17}
VIIC[b]	Al_2O_3	1.0×10^{17}
VIII[b]	Al_2O_3	5.0×10^{16}
BSA-(**VIII**)$_{18}$[c]	Al_2O_3	8.0×10^{17}
BSA-(**VIII**)$_{18}$[c]	Teflon[d]	8.0×10^{17}
BSA-(**VIII**)$_{18}$[c]	Kapton[e]	1.6×10^{17}

[a] Counting efficiency 0.14%; the sample is heated from 100 to ~240° within 15 sec and kept at that temperature for 45 sec under an atmosphere of nitrogen gas. During this period >90% of the total emission is detected.
[b] As a solution in dioxane.
[c] As a solution in 100 m*M* borate buffer, pH 8.5.
[d] Disk diameter 9 mm; thickness 0.5 mm; background 500 cpm.
[e] Disk diameter 9 mm; thickness 0.125 mm ("Kapton 500H," DuPont); background 170 cpm.

changed after conjugation to proteins; (4) labeling BSA with even 18 residues of **VIII** does not result in a relative decrease of specific activity.

Reproducibility and Linearity of TCL; the Use of g-Cyclodextrin for the Prevention of Evaporation of Free Label

The factors that influence the reproducibility of TCL measurements are quite different from those ruling that of (room temperature) liquid phase bi- or trimolecular chemiluminescent reactions. So are the possible ways to eliminate disturbing factors and the ways to distinguish between true values and artifacts.

At the present state of the art of TCL chemistry and detection, the most important disturbing parameters in reproducibility and linearity are (in order of decreasing importance): (1) thermochemical degradation of the spacer moiety of the TCL labels, resulting in evaporation of the 1,2-dioxetane moiety before TCL occurs, (2) reproducibility of heat contact between the carrier material and the heating element in the TCL detection apparatus, (3) pipet precision (routinely 2-5 *m*l of a solution is pipetted on the carrier material), (4) sample position in the TCL detection apparatus, (5) inherent TCL detection apparatus reproducibility, and (6) counting statistics (only at lower concentrations).

The thermochemical degradation, and subsequent evaporation, is a parameter that operates predominantly at lower concentrations. It can be minimized in several ways: (1) by measuring a sample on a strongly absorbing material [e.g., aluminum oxide thin-layer chromatography material, Whatman glass filter (GS/A)], see Table II, entries 1 and 2; (2) by measuring samples as buffered solutions or as proteinaceous solutions: upon evaporation of the solvent this leaves a (very small) transparent but sticky residue on the carrier surface, thereby preventing evaporation; (3) by covering the sample with a nonvolatile transparent medium (e.g., silicon oil). Thus TCL-labeled proteins cannot be quantified accurately as solutions in pure water (below ~10 *m*g/ml). The disappearance of material during the heating process can be followed by the technique of curve fitting, since the rate observed does not obey the kinetics established for adamantylidene adamantane 1,2-dioxetane decomposition. Both linearity and reproducibility are influenced by this parameter, as can be seen in Table II, entries 3-5 (see also Fig. 8A).

Evaporation of free label **VIII** is completely inhibited upon complexation with *g*-cyclodextrin (*g*-CD). A strong complex is formed by **VIII** and *g*-CD, which can be chromatographed over a Sephadex LH-60 (40-120 *m*m) column. When dissociation of the complex upon dilution is prevented through dilution in 15 m*M* *g*-CD/borate buffer, perfect linearity and reproducibility of TCL are observed (Table II, entry 8; Fig. 8B). Dissociation of the complex can also be prevented by incapsulation of the *g*-CD complexes in liposomes. Solutions of liposomes (cholesterol/phosphatidyl-

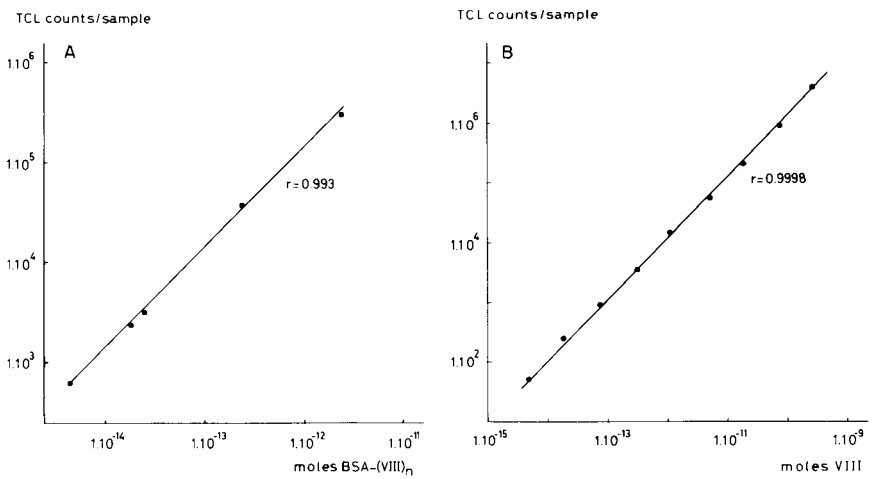

FIG. 8. Linear relationship between TCL output and the amount of BSA–(**VIII**)$_n$ in borate buffer (A), and the amount of free **VIII** in 15 m*M* γ-CD/borate buffer (B).

TABLE II
LINEARITY AND REPRODUCIBILITY OF TCL DETECTION

TCL sample	Solvent	Carrier	Measured range	r^a	CV (%)[a]
1 **III**	Dioxane	Al_2O_3	60 pmol[b]	-	8
2 **VIII**	Dioxane	Al_2O_3	40 pmol[b]	-	6
3 BSA-(**VIII**)$_n$	0.1% aq. BSA	Kapton	250-2.5 fmol[c]	0.996	5-10
4 BSA-(**VIII**)$_n$	B[d]	Kapton	2 pmol-5 fmol[c]	0.993	7-16
5 BSA-(**VIII**)$_n$	H_2O	Kapton	250-2.5 fmol[c]	0.90	30-60
6 BSA-(**VIII**)$_n$	B	Teflon	70 pmol- 70 fmol[c]	0.998	7-28
7 BSA-(**VIIb**)$_n$	B	Teflon	70 pmol- 70 fmol[c]	0.996	6-50
8 **VIII**	15 m*M* *g*-CD in B	Kapton	320 pmol-5 fmol[b]	0.9998	1.4-3.1[e]
9 **VIII**-*g*CD complex in liposomes	B	Kapton	60 pmol-60 fmol[b]	0.996	0.9-2.6[e]
10 **VIII**-*g*CD complex in liposomes	B	Teflon	400 pmol-40 fmol[b]	0.998	4-8

[a] *r*, correlation coefficient of linear regression; *CV* at highest to lowest concentration.
[b] Amount of 1,2-dioxetane.
[c] Amount of protein.
[d] B, 0.1 *M* borate buffer, pH 8.5.
[e] At lowest concentration *CV* corrected for counting statistics.

choline 1 : 1, sonificated to SUVs, centrifuged at 10,000 *g* and chromatographed over a Sephacryl S-300 column) that contain these complexes can be diluted without disturbance of the association equilibrium of **VIII** and *g*-CD, since the local concentration inside the liposomes is kept constant. These TCL liposomes show excellent linearity and reproducibility when measured on Kapton as the carrier (Table II, entry 9). Potentially, these liposomes can be applied as macromolecular TCL labels, containing many *g*-CD complexed 1,2-dioxetane molecules per liposome. A further advantage of liposome incapsulation of the *g*-CD complexes is the creation of a closed and constant surrounding for the 1,2-dioxetanes, protecting them from potential quenchers in analyte solutions.

The influence on the reproducibility by variation in heat contact/transfer between the carrier material and the heating element of the TCL detector on the total reproducibility is seen in entries 4/6 and 9/10. Teflon disks (0.5 mm thickness) are stiff and slightly bent. In contrast, Kapton disks (0.125 mm thickness) are perfectly flat and thermostable up to >400°. As a result the thermochemiluminescence curve (TCL versus time) of TCL material, heated on Kapton disks is of a very reproducible shape (the same shape of I/I_{max} versus time at all concentrations!); therefore the reproducibility is independent on the TCL integration period of time. When Teflon is used, however, disk to disk variation in heat contact

results in pronounced variation in TCL/time curve shape and thus relatively long integration periods (~2 min) are needed to improve reproducibility of TCL quantification.

At present, pipet precision (*CV* ~0.5%), inherent apparatus reproducibility (*CV* ~0.1%), and counting statistics ($CV = 100\sqrt{N}/N\%$) determine the ultimate reproducibility of TCL detection.

Energy Transfer TCL

Improvement of Specific Activity through Isochromic Energy Transfer

The efficiency of direct TCL of **III** is only ~1 % that of luminol and its analogs at their optimum. The difference in f_{CL} is caused by the relatively low quantum efficiency of fluorescence $f_F = 5.2 \times 10^{-3}$) of adamantanone formed in the TCL reaction. At the theoretical maximum, the TCL intensity of **III** can be amplified by a factor 192 upon the addition of an efficient fluorescer ($f_F \sim 1$), which acts as an acceptor of energy of singlet excited carbonyls (see also Fig. 1).

Since Belyakov and Vassil'ev[11] introduced this technique of excitation energy-transfer CL (ICL) as a way of visualization of poorly luminescent excited carbonyl products in hydrocarbon autoxydation and since Wilson and Schaap[12] showed that 9,10-diphenylanthracene (DPA) acts as a very efficient acceptor in 1,2-dioxetane decomposition, DPA has become a popular acceptor compound. As an acceptor of singlet energy in the decomposition of **III** (at ~200-250°!), DPA is especially very suitable for the following reasons: (l) it is a very efficient fluorescer ($f_F = 0.8\text{-}1$)[13,14]; (2) it is thermally stable; (3) it has a small negative temperature coefficient of f_F; (4) it is an apolar nonbasic and nonacidic molecule, thus a strong influence on f_F and λ_{em} (max) is not to be expected in the presence of proteins and other biological fluid components.[13] Such an influence is seen in the case of fluorescein and its analogs,[15] resulting in a pH optimum of f_F and almost complete loss of fluorescence if bound to an antifluorescein antibody; (5) the absorption and emission spectra of DPA exhibit only a small overlap at 390-410 nm. Thus DPA shows minimal concentration quenching[16]: the concentration at which it has half its maximal f_F:

[11] V. A. Belyakov and R. F. Vassil'ev, *Photochern. Photobiol.* **11,** 179 (1970).
[12] T. Wilson and A. P. Schaap, *J. Am. Chem. Soc.* **93,** 4126 (1971).
[13] A. Schmillen and R. Legler, *in* "Landolt-Börnstein Tables" (K.-H. Hellwege and A. M. Hellwege, eds.), Group II. Vol. 3, pp. 143 and 266. Springer-Verlag, Berlin and New York, 1967.
[14] P. S. Engel and B. M. Monroe, *Adv. Photochem.* **8,** 245 (1971).
[15] T. Förster, "Fluorescenz Organischer Verbindungen," p. 47. Vandenhoeck & Ruprecht, Göttingen, 1951.
[16] W. H. Melhuish, *J. Phys. Chem.* **65,** 229 (1961).

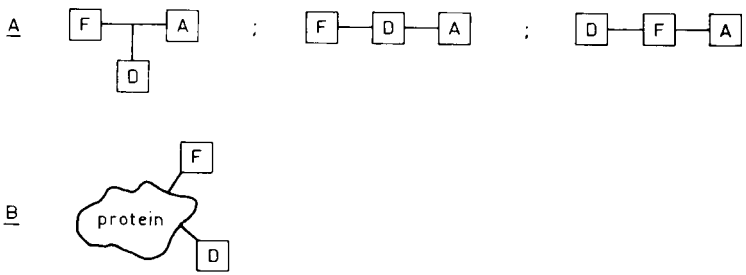

FIG. 9. Fluorescence amplified systems. F, Fluorescer (DPA); D, 1,2-dioxetane; A, reactive group.

C_h ~0.5 M [C_h (fluorescein) = 0.02 M, C_h (acridone) = 0.025 M[17]]; (6) DPA shows no excimer fluorescence.[18]

The end-point quantification step of a TCL immunoassay consists of heating a sample (for example as a precipitate or as a complex, bound to a carrier polymer or as a small volume of an aqueous solution). Sensitive detection requires a high signal-noise ratio rather than simply a strong signal. Upon addition of a constant amount of DPA to all samples before measurement, the background signal and the blank values would be increased significantly. Thus if DPA would be added as such it would not increase the sensitivity of the assays. Therefore DPA has to be linked either to the luminescent label to form a new label (A) or to the substance labeled with the luminescent label as well as a second label (B) (Fig. 9).

In this chapter only examples of system B will be discussed. In order to connect DPA to a protein, it first has to be transformed into a fluorescent label. The new fluorescent label 2-[*O*-(*N*-succinimidyl)carboxypropyl]-9,10-diphenylanthracene (**XIV**, SCP-DPA; Fig. 10) mimics all fluorescent properties of DPA to a great extent. SCP-DPA is prepared from the corresponding carboxylic acid by the standard procedure for such esters (*N*-hydroxysuccinimide, DCC, dioxane). The carboxylic acid, in turn, can be prepared from DPA in ~80% yield via a two-step procedure, described by Douris.[19] Both the emission and the absorption spectrum of SCP-DPA are shown in Fig. 11. Note that the the emission spectrum of SPC-DPA is almost identical to the emission spectrum of **III** and the corresponding TCL label **VIII** (Fig. 4). Thus the spectral distribution of TCL from a mixture of one of these 1,2-dioxetanes and SPC-DPA (or a derivative) is independent on the efficiency of energy transfer. The only visible effect of the addition of SPC-DPA is an amplification of the TCL signal.

[17] A. Schmillen and R. Legler, *in* "Landolt-Börnstein Tables" (K.-H. Hellwege and A. M. Hellwege. eds.), Group 11, Vol. 3, p. 329. Springer, Verlag, Berlin and New York, 1967.
[18] J. B. Birks and L. G. Christophorou, *Proc. R. Soc. London, Ser. A* **277,** 571 (1964).
[19] R. G. Douris, *Ann. Chim.* (*Paris*) [13] **4,** 479 (1959).

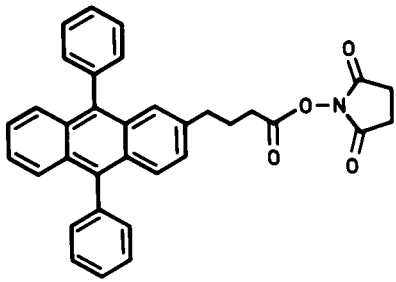

FIG. 10. Structure of SCP–DPA (**XIV**).

The critical distance R_0 for energy transfer from **III** to SPC-DPA (R_0 is the distance between donor and acceptor at which the efficiency of energy transfer is 0.5) is calculated to be 15.3 Å (in a medium with a refractive index of 1), according to the theory of Förster.[20,21] This value indicates that random labeling of smaller proteins with both **VIII** and SPC-DPA can result in a full energy transfer TCL protein, when only a few DPA residues are attached to the protein (in the case of BSA, approximately 6 DPA residues are needed). For efficient energy transfer TCL on IgGs, however, a minimum of approximately 20 residues of SPC-DPA would be needed. This is most likely to eliminate all immunological activity of the antibody.

In practice it appears that the maximal amplification of TCL from a labeled protein using SCP-DPA residues as energy acceptors is a factor 40. Thus BSA, labeled with 17 residues of **VIII**, shows a specific activity of 1×10^7 photon counts/*m*g in a TCL detection apparatus with a counting efficiency of 0.14%, when measured as 1 *m*l of a 1 mg/ml solution in 1% aqueous BSA, evaporated on a disk of Teflon; when this conjugate is labeled with even up to approximately 30 residues of SCP-DPA according to the general procedure for labeling proteins with TCL label **VIII**, the specific activity rises to a maximum of 4×10^8 counts/*m*g (i.e., f_{CL} of this BSA dual conjugate = 4%). This very efficiently luminescent protein, which is readily soluble in aqueous buffers above pH 8.5, can be used as a label by covalently linking it 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 a label, is described in the next section.

[20] T. Förster, *Ann. Phys.* (*Leipzig*) [6] **2,** 55 (1947).
[21] L. Stryer, *Annu. Rev. Biochem.* **47,** 819 (1978).

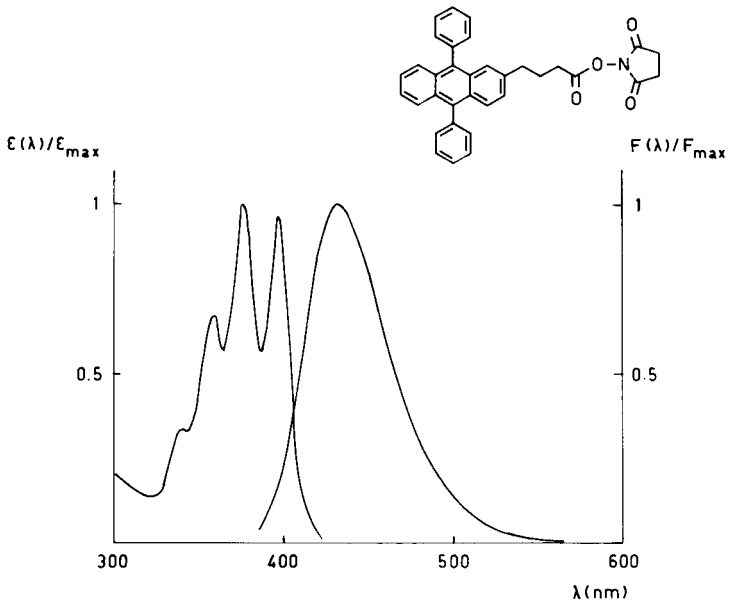

FIG. 11. Absorption and emission spectrum of SCP–DPA.

Red Shifted TCL through Energy Transfer

The many applications of energy transfer fluorescence and chemiluminescence in immunoassay have been based mainly on the color shift of luminescence upon energy transfer. Energy transfer donor-acceptor interactions between suitably labeled immunological counterparts can be used as the basis for homogeneous assays.[22,23] A homogeneous TCL immunoassay, based on energy transfer, has not been developed yet. The main obstacle is the temperature needed (~240°) for rapid TCL detection. Hence, aqueous solutions cannot be measured neat.

It has been found, however, that very efficient energy transfer from **III** and its derivatives to a number of acceptors (e.g., rubrene, perylene, anthracene, 2-(carboxyethylcarbonyl)diphenylanthracene, 2-aminoanthracene, 9,10-dithienylanthracene, fluorescein, carboxyfluorescein, fluorescein isothiocyanate-labeled BSA, rhodamine B) takes place when a sheet of thin-layer chromatography material (aluminum oxide, Merck) is sprayed with a solution of **III** and an acceptor compound (which also may

[22] A. Patel, C. J. Davies, A. K. Campbell, and F. McCapra, *Anal. Biochem.* **129,** 162 (1983).
[23] A. K. Campbell and A. Patel. *Biochem. J.* **216,** 185 (1983).

be chromatographed) and heated to ~200-240°, subsequently. Spots containing fluorescer show bright luminescence of altered color.

Thermostable Solid Phase Materials for Immunoassay

Commonly used solid phase materials (e.g., polystyrene and polyvinylchloride) are not suitable for TCL immunoassay, since these materials degenerate at ~200-300°. Criteria for TCL solid phase materials are (1) thermostability up to ~300° under a nitrogen atmosphere, (2) low inherent thermoluminescence output (background), (3) no quenching effect on TCL emission at the surface, (4) a possibility for coating the surface with proteins, and (5) a convenient format (in the context of washing procedures).

Some materials that meet most or all of these criteria are microcrystalline cellulose, glass, silicon tube, Kapton, and Teflon. For the latter two polymers, that show very low thermoluminescence background and perfect thermostability below 250°, a convenient coating procedure has now been developed. This procedure yields antibody-coated polymer disks that show better immunoreactivity than coated polystyrene microtiter plates. From the two identical solid-phase sandwich immunoassays for hIgG, performed on polystyrene and Kapton 500H, shown in Fig. 12, it can be concluded that antibody-coated Kapton disks show both very little aspecific binding and a high immunoreactivity/cm^2. An identical assay, on Teflon disks, shows a precision and dynamic range identical to the ELISA on Kapton, however superimposed on a constant blank OD value of 0.2. Both ELISAs on Kapton and Teflon result in better precision, sensitivity, and dynamic range than ELISAs on polystyrene. Disks, coated with antibodies, show a TCL background of ~3 counts/sec (Kapton) and ~8 counts/sec (Teflon) at 240°. Antibodies show no thermoluminescence.

First Results of TCL Immunoassay: FATIMA

A solid-phase sandwich immunoassay for carcinoembryonic antigen, CEA, can be performed using the TCL technique described. The standard curve for a CEA-FATIMA in a clinically interesting range of concentrations is shown in Fig. 12.

Procedure. Kapton disks are coated with monoclonal *a*CEA (Roche) as described (Fig. 12). Polyclonal g*a*CEA is labeled with both **VIII** and SCP-DPA **XIV** by reaction with, respectively, 15 and 20 eq of both labels in borate buffer, pH 8.5/5% dioxane to yield an immunologically active, labeled antibody with a specific activity of 1.4×10^6 photon counts/*m*g (measured on Kapton, counting efficiency 0.14%). The *a*CEA-coated

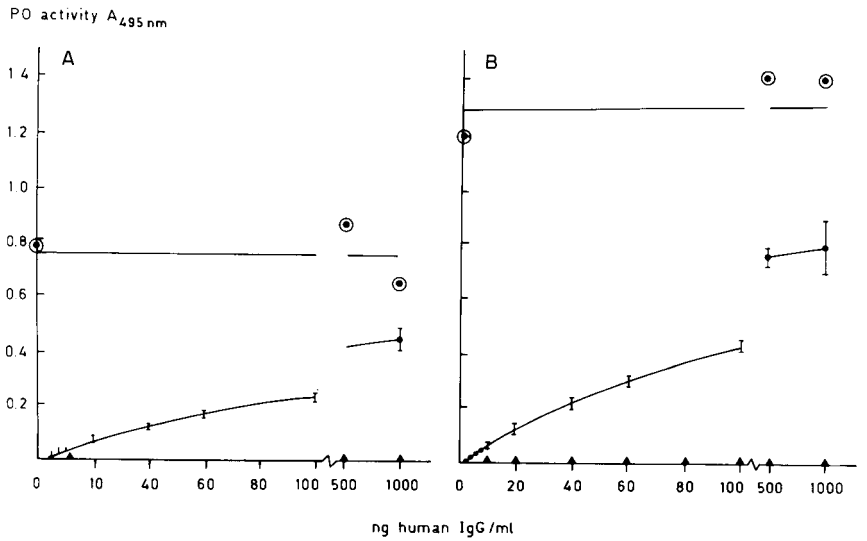

FIG. 12. ELISA for hIgG on a polystyrene microtiter plate (A) and Kapton 500 H (B). Coating procedures. (A) Physical coating with sαhIgG, 0.1 mg/ml in 0.1 *M* carbonate buffer, pH 9.6, for 1 week at 4°. (B) Disks (diameter = 0.9 cm; thickness 0.125 mm) of Kapton were refluxed with several changes of 96% EtOH and several changes of distilled water subsequently, and dried at ~300°. Thereafter these disks were incubated with sαhIgG, 0.1 mg/ml in Tris–HCl buffer, pH 7.6/0.02% NaN_3 at 4° for 2 days. Preincubation: both A and B with 4% BSA in 10 m*M* Tris–HCl (pH 8.0), 0.15 *M* NaCl, 0.05% Tween 20 (Buffer T) at 37° for 1 hr. Incubation: after washing with water, incubation with hIgG in buffer T for 1 hr at 37°. Second incubation: after washing with water, incubation with gαhIgG-peroxidase conjugate in buffer T for 1 hr at 37°. After 1 hr of color development with *o*-phenylenediamine (0.2 ml 2×10^{-3} *M* OPD–HCl in phosphate buffer, pH 5.0/0.0045% H_2O_2), the reaction was stopped with 0.4 ml 1 *N* H_2SO_4. In both assays all standards were measured in triplicate. Triangles, solid phase coated with BSA (blank); double circles, solid phase coated with hIgG (showing maximal capacity and reproducibility of coating).

disks are preincubated with BSA (4% BSA in 0.01 *M* Tris-HCl, pH 8.0, 0.3 *M* NaCl, 0.05% Tween 20) to prevent aspecific binding. The disks are incubated overnight at 37° with 0.2 ml of standard solutions of CEA (Roche) containing, respectively, 0.1, 0.25, 0.5, 2.5, 6.0, and 10 ng CEA/ml in PBS, pH 7.4/20% FCS. The disks are incubated with labeled antibody (0.2 ml of 5 *m*g antibody/ml PBS/20% FCS) for 3 hr at 37°. All washing procedures between and after the incubation are performed using distilled water. The disks are taken out of the assay wells and measured for TCL output directly without a previous drying procedure.

A similar assay for hIgG can be performed by using a second antibody coupled covalently to heavily labeled BSA [BSA(**VIII**)$_{16}$-(**XIV**)$_{\sim 10}$], using *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide-HCl (EDC) in a one-

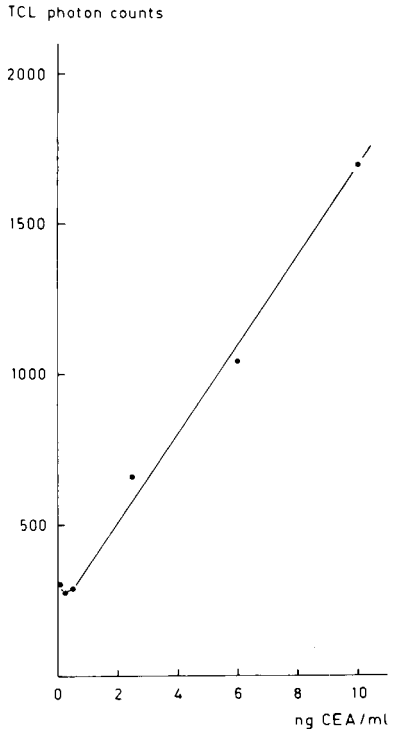

FIG. 13. Standard curve for a CEA–FATIMA using a dually labeled antibody conjugate. The number of photon counts shown is the total integration over a time interval of 14 sec, including the TCL peak intensity, corrected for a standard background by substraction of 145 counts.

step procedure.[24,25] This coupling procedure yields a high-molecular-weight conjugate according to SDS-gel electrophoresis on polyacrylamide. The procedure for the preparation of the protein-protein conjugate is as follows: to 2 ml of a mixture of 1.3 mg BSA(**VIII**)$_{16}$(**XIV**)$_{\sim 10}$ and 0.8 mg g*a*hIgG/ml 10 m*M* borate buffer, pH 8.5, is added 13 *m*l of a 100 mg/ml solution of EDC in distilled water. After stirring for 3 hr in the dark at room temperature, the reaction mixture is thoroughly dialyzed against PBS/0.02% NaN_3 at 4°. After dilution, this crude antibody conjugate solution is used in the assay without further purification.

The protocol for the standard curve for a hIgG-FATIMA shown in Fig. 14 is identical to the one described for the CEA-FATIMA, with the

[24] S. Bauminger and M. Wilchek, this series, Vol. 70, p. 151.
[25] T. L. Goodfriend, L. Levine, and G. D. Fasman, *Science* **144,** 1344 (1964).

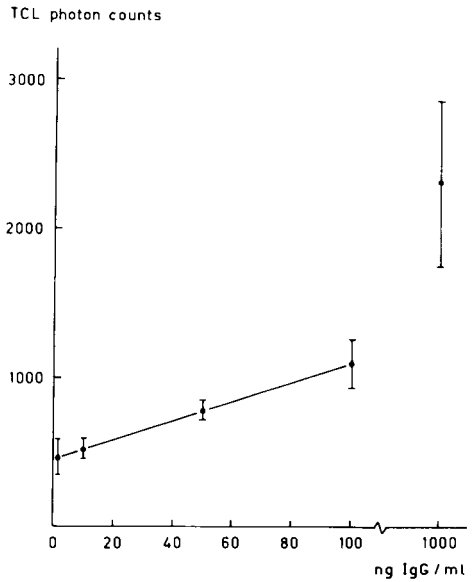

FIG. 14. Standard curve for hIgG–FATIMA using gαhIgG coupled covalently to BSA(**VIII**)$_{16}$–(**XIV**)$_{10}$. The number of counts taken is the total integration of TCL output over a 32 sec time interval without background substraction.

exception that the period of both the first and the second incubation is 1 hr.

TCL Detection Apparatus

The stable 1,2-dioxetanes differ greatly from other chemiluminescent compounds with respect to the way in which the emission is stimulated: the thermochemiluminescent compounds have to be heated to elevated temperatures. For practical reasons as well as to obtain a high peak signal-to-noise ratio, a short detection period (a sharp luminescence peak) is advantageous. In order to decompose 99% of any amount of **III** (or a derivative of **III**) within 30 sec, heating of the sample to a temperature as high as ~250° is necessary (see Fig. 3). Thus the detection of TCL labels based on **III** cannot be carried out in aqueous solutions (at 1 atm). Therefore, all commercial luminometers for chemiluminescence and bioluminescence, designed for the detection of low level luminescence in (aqueous) solutions at ambient temperatures, are of no use for TCL detection.

This section describes a TCL detector, which is designed to meet the following criteria: (1) high sensitivity, (2) low (thermal) background,

(3) variable detection temperature (100-270°), (4) short heating period, and (5) high reproducibility and stability.

The technique of photon counting was chosen, since it offers the best possibilities for combination of high gain with the exclusion of electronic noise. Samples to be quantified consist of minute amounts of dry residues on small and thin disks (f < 1 cm, thickness 0.1-1 mm) of thermoresistant materials. Consequently, the sample compartment can be made extremely small in comparison with sample chambers of liquid phase CL detectors. Thermal insulation is the limiting factor in design of a TCL detector with optimal optical dimensions.

Four types of sources of background luminescence during TCL measurements can be distinguished: (1) CL from oxidative degradation of materials (in the sample) at ~250°, due to reaction with O_2, present in the air, (2) thermoluminescence, (3) blackbody radiation, and (4) X-ray radiation from the environment and from traces of radioactive isotopes. The oxidative degradation can be inhibited effectively by flushing the sample compartment with N_2 gas. The thermoluminescence of a series of carrier materials was investigated. Teflon, Kapton 500H, glass slides, and Whatman glass filter were found to be optimal with respect to thermostability and low thermoluminescence emission. The blackbody radiation of a perfect blackbody, in the spectral area of interest (~350-550 nm), was calculated to be only 0.7 photons sec^{-1} cm^{-2} at 250°. When the hot object is an aluminum heater or a disk of one of the above mentioned carrier materials, the expected blackbody radiation is even much lower, since these materials reflect (or transmit) light of this wavelength to a high extent. Any X-ray originated background is minimized by the use of a specially shielded photomultiplier housing and a sample compartment made of aluminum and brass. Figure 15 shows the block diagram of the TCL detector.

When the heater and sample are connected to the adaptor on the photomultiplier housing (Products for Research Te 1004/TS/110; cooling not used), the microswitch (Honeywell BZ-2RW822) gives the Apple II computer control over the multiplier power study (Öltronix A 2.5K10HR, switched on negative output) via the protection unit. The signal from the photomutiplier tube (EMI 9893 QA/350, selected on low darkcount, i.e., 0.5 cps!) is preamplified (EG&G 1121A) and transported to the discriminator (EG&G 1121A)/photon counter (EG&G 1109) unit. The data enter the Apple II computer thereafter. These data are shown on the monitor after the measurement and printed subsequently. The computer switches four variables via an optocoupler during the measurement: (1) the oven controller can be set at 100° (on/off), (2) the power supply

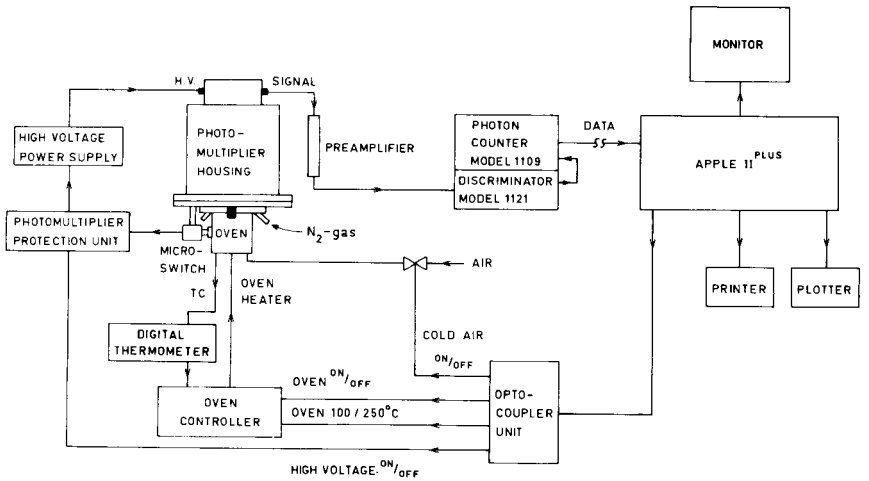

FIG. 15. Block diagram of TCL detection apparatus.

(on/off), (3) the oven controller at 240° [on = 240°, off = 100°; when (1) is "on"], and (4) an electric valve for cold air, which cools the oven at the end of a measurement. The temperature of the oven is shown continuously on a digital display (Analog Wakefield AN 2572).

The characteristic part of the TCL detector is shown in Fig. 16. This part is connected to the photomultiplier housing. A soldering iron element (50 W, 24 V; Weller) is used as the heating element. On top of this hollow element an aluminum "oven" is clamped. On this oven a piece of carrier material can be placed. A 1-mm rim on the oven prevents the sample from slipping away. A thermocouple (copper/constantan) is placed into a small hole in the bottom of the aluminum oven. A 3-mm-diameter hollow pipe enters the hollow soldering element to 0.5 cm distance from the aluminum oven. Through this pipe cold air is introduced after completion of each measurement in order to cool the oven to 100°. The soldering element is strengthened by a brass cylinder. On this brass cylinder a progressively protruding piece of brass switches the microswitch on upon connecting the heating device to the adaptor on the photomultiplier housing. The connection is a bayonet-type without a locking position: the heating device is pressed against the adaptor and subsequently rotated 60°. Because of the light-trap construction of the heater-adaptor combination, a perfectly dark sample compartment is obtained.

A horizontal flow of nitrogen gas (±0.4 liters/min) enters the sample compartment from two opposite positions. The nitrogen flows slowly around the oven through the light trap.

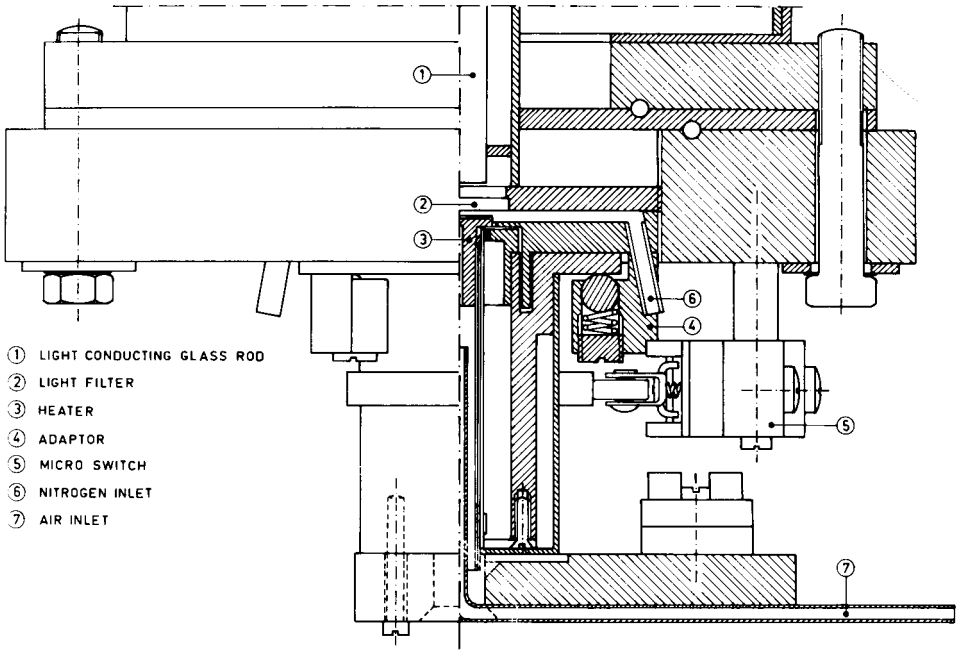

FIG. 16. Heating device and adaptor, filter, and light-conducting glass rod of TCL detector.

The heating device is coupled to the adaptor in such a way that the aluminum oven is positioned only 2 mm from the interference filter (Fairlight Edge filter, short wave pass; 50% transmittance at 500 nm) above the sample. Thus the sample compartment consists of the aluminum oven, brass "walls," and a glass topside.

A light-conducting glass rod (Scott LST-UV, 10 mm diameter, 90 mm 1ength) transports the light, transmitted by the filter, to the photomultiplier cathode surface.

Apparatus Calibration

The apparatus was calibrated, using a disk (10 mm diameter and 1 mm thick) of scintillating plastic (Nuclear Enterprises Ltd. NE 134-^{14}C, activity: 0.0095 *m*Ci). The plastic disk emits 1.7×10^5 photons/sec with a spectral distribution that mimics the thermochemiluminescence spectrum of adamantylidene adamantane 1,2-dioxetane **III** to a high extent. For this scintillator a counting efficiency of 0.14% was observed. Since the disks of Kapton and Teflon, used as solid phase supports, have the same diame-

ter as the scintillator, we estimate the counting efficiency for labeled immune complexes on these polymers to be 0.14% as well.

TCL Measurement

A typical TCL plot for BSA, labeled with **VIII**, is shown in Fig. 17 A. As a standard procedure for determination of TCL of solution, 1-5 *m*l of the solution is pipetted on a disk of Kapton 500H, which is placed on the heater (at 100°). Within a few seconds the solvent evaporates and thereafter the heater (+ sample) is connected to the adaptor on the PM housing. The sample is heated to ~250° and TCL is recorded over a ~0.5-min period.

The blank measurement (i.e., a clean disk of Kapton 500H) shows a very small increase of background signal with temperature: as a mean value a thermoluminescence noise of only 2.5 cps is observed for Kapton at 240° (Fig. 17B). Thus for a 32 sec measurement of TCL a total background of 57 ± 8 counts is observed. Kapton disks, coated with proteins (e.g., BSA, g*a*hIgG, *a*CEA) show a total blank value of 130 ± 15 counts for 32 sec. The latter value is the blank value for a solid phase sandwich immunoassay on Kapton 500H, if no aspecific binding of labeled second antibody occurs.

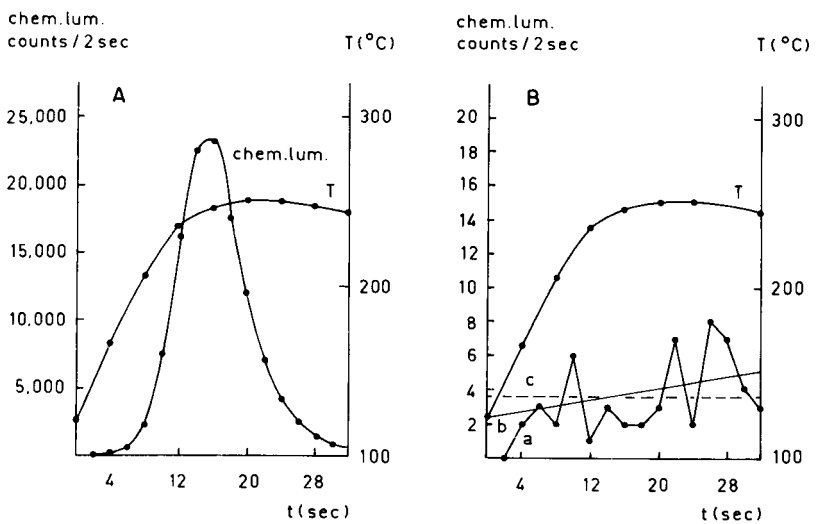

FIG. 17. (A) TCL intensity plotted versus time for BSA(**VIII**)$_{n}$, measured on Kapton 500H as 1 μl of a solution of the protein in phosphate buffer. (B) Typical blank measurements. (a) A single measurement; (b) mean value in time for clean Kapton disks; (c) mean value of background for 32 sec.

Conclusion

Stable 1,2-dioxetanes, derived from functionalized adamantylidene adamantanes, can be prepared and these compounds can be used as labels or tracers in analytical procedures since they are effective inherently chemiluminescent compounds.

These thermochemiluminescent labels exhibit some special features. (1) The light emission is the result of a unimolecular first order decomposition reaction. Thus, both the integration time for TCL detection is independent on the amount of label present in the sample, and the shape of the TCL curve (I/I_{max} versus time) is a function of the temperature of the sample only. Therefore, for a number of potentially disturbing factors, true and false TCL values can be distinguished by curve fitting. (2) Part of the background of a TCL measurement can be rejected because resolution in time is observed for background and 1,2-dioxetane TCL due to differences in activation energies for these processes. (3) Neither the 1,2-dioxetanes nor their degradation products absorp light in the spectral area of TCL emission. Hence, self-quenching does not occur and high local concentrations of these compounds can be used as is shown in the cases of heavily labeled proteins and liposomes without relative loss of specific activity. Although the TCL labels show a moderate specific activity ($\sim 6 \times 10^{19}$ photons emitted per mol), enhancement can be achieved through energy transfer to a variety of fluorescent compounds. The new fluorescent label SCP-DPA has been specially developed for enhancement of 1,2-dioxetane TCL, but it can also be used as fluorescent label or as a label that enhances peroxyoxalate ester luminescence.

Both the TCL labels and TCL-labeled compounds show good long-term stability at ambient temperature.

Thermochemical side reactions, leading to a deficiency in specific activity, take place under certain conditions. These reactions can be inhibited by complexation of the TCL labels with *g*-cyclodextrin. The requirement of a sample temperature of ~240° for rapid detection limits the sample format to that of a (dry) residue on a thermostable carrier. An advantageous aspect is that the size of the samples is minimal.

Linearity and reproducibility of TCL detection are dependent on a number of factors. Under optimal conditions, linearity is perfect over a range of five orders of magnitude and CV values of ~1-3% can be reached.

The FATIMAs for CEA and hIgG presented here are to be regarded as the first results of a new technique that required specific development of several components: a thermostable solid phase, a coating procedure for these specific solid-phase materials, and a TCL detection apparatus. All

elements of the analytical procedures using 1,2-dioxetanes as tracers are subject to further improvement.

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