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Effect of Surfactants on the Intensity of Chemiluminescence Emission from Acridinium Ester Labelled Proteins

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In order to establish optimum conditions for the chemiluminescent (CL) reaction of two acridinium ester labelled proteins (human albumin and rabbit anti-human albumin IgG), we investigated the effects of the following factors known to influence the CL emission: pH, presence of proteins, relative concentrations of components of CL reaction and presence of surfactants. Under optimal conditions of pH and hydrogen peroxide concentration, hexadecyl trimethyl ammonium chloride (CTAC) increased the intensity of the CL reaction of the acridinium ester labelled albumin by 42-fold. Triton X-100, Tween-20, 23 lauryl ether (Brij 35) and sodium dodecyl sulphate (SDS) exerted a much smaller effect. In the case of the acridinium ester labelled antibody, the greatest increase was obtained with Triton X-100 (15-fold) followed by CTAC, Brij 35 and Tween 20 (SDS decreased the emission intensity).

Keywords: Chemiluminescence; acridinium ester; surfactants; proteins

INTRODUCTION

The synthesis of a stable derivative of an acridinium salt, 4-(2-succinimidyl oxicarbonyl ethyl) phenyl-10-methyl acridinium-9-carboxylate fluorosulphonate, that undergoes a chemiluminescent (CL) reaction in the presence of dilute alkaline hydrogen peroxide has been recently described (Weeks *et al.*, 1983).

In aqueous solution, these compounds reach an equilibrium between the salt and their respective pseudo-bases. High pH usually favours pseudo-base formation, and low pH favours salt formation. The reaction consists of a concerted multiple bond cleavage mechanism via a dioxetanone intermediate to yield a vibronically excited molecule of *N*-methyl acridone (McCapra, 1976). Because the pseudo-base reacts very slowly with

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hydrogen peroxide, the efficiency of the CL reaction is increased when the proportion of acridinium salt is maximum (acidic conditions prior to initiation of the oxidation reaction).

Despite the low counting efficiency of CL labels (15–25% *vs* 60–80% for ^{125}I, depending on the instrument) (Campbell *et al.*, 1985), the use of CL compounds, especially acridinium ester derivatives, as high specific activity tracers has been demonstrated to improve the sensitivity of immunometric assays in comparison to ^{125}I (Weeks *et al.*, 1986; Woodhead and Weeks, 1985).

The efficiency of the CL reaction of acridinium derivatives has been reported to be increased by cyclodextrins as well as surface active agents such as CTAC (Grayeski and Woolf, 1984; Howie and Grayeski, 1987; Paleos *et al.*, 1982). The aim of this work was to investigate the effect of pH, H_2O_2, protein concentrations and a series of surfactants on the CL emission intensity (defined by total photon counts emitted integrated over 10 seconds) of an aryl acridinium ester (AE) and acridinium ester labelled human albumin (Alb*) and rabbit anti-human albumin IgG (Ab*).

MATERIALS AND METHODS

The CL label 4-(2-succinimidyloxicarbonylethyl) phenyl-10-methyl acridinium-9-carboxylate fluorosulphonate (AE) was synthesized as previously described (Weeks *et al.*, 1983).

Human albumin, essentially globulin free (Sigma Chemical Co., St. Louis USA) and rabbit anti-human albumin IgG (Dako Patts, 2600 Glostrup, Denmark), were labelled according to the procedure described elsewhere (Weeks *et al.*, 1983).

The CL compounds were diluted in 0.1 mol/l phosphate buffer pH 6.3 containing 0.15 mol/l NaCl, 0.1 g/l BSA, 0.02 g/l bovine IgG and 0.1 g/l NaN_3 (assay buffer).

The Cl reaction was initiated, unless otherwise stated, as follows: 50 µl of each labelled compound in assay buffer pH 6.3 were dispensed into 11 × 45 mm. polystyrene tubes followed by 100 µ of distilled water. Finally, 100 µl of oxidant reagent (0.2 mol/l NaOH containing 10 mmol/l H_2O_2) were injected automatically in the luminometer.

The chemiluminescence intensity was quantified using a LB9500T manual luminometer (Laboratorium Berthold, Wildbad, FRG) linked to a Apple IIe minicomputer. Light emission was expressed as the total photon counts integrated over 10 seconds following the injection of the oxidant reagent. To study the pH dependence of AE pseudo-base formation, identical amounts of Ab* in HNO_3 (1 mmol/l) were diluted in buffers of various pH. Aliquots (50 µl) were removed at suitable time intervals and measured luminometrically.

The influence of protein concentration in the reaction media was studied by comparing the CL response obtained with identical amounts of labelled compounds (1 ng Ab*/50 µl) dissolved in assay buffer without protein or with increasing amounts of BSA or bovine IgG.

Adsorption of labelled proteins to polyethylene tubes was studied by comparing the CL response obtained with identical amounts of labelled compound (1 ng Ab*/50 µl), freshly prepared (day '0') and after 1 to 5 days storage at 4° C in the same tube and dissolved in the following buffers: (1) PBS 0.1 mol/l pH 6.3 without proteins; (2) as above but with 0.1 g/l BSA; (3) with 0.1% Triton X-100; (4) with 0.1% Triton X-100 and 0.1 g/l BSA; (5) With 0.05% Tween 20; and (6) With 1 g/l gelatin (from swine skin, Sigma).

The influence of micellar solutions produced by different tensoactive agents on the efficiency of CL reaction of AE in aqueous solution, Alb* and Ab*, were studied by comparing the light emission produced using identical amounts of the CL compounds and increasing amounts of the surfactants with the following oxidation procedure: (a) 1 ng of Alb* or Ab*/50 µl of assay buffer or the same volume of a $1/10^7$ dilution in the same buffer of a solution 100 µg/ml (0.172 mol/l) of AE in acetonitrile, were dispensed into polystyrene tubes, followed by (b) 100 µl of HNO_3 0.1 mol/l containing 10 mol/l H_2O_2 and increasing concentrations of each surfactant studied and, finally, (c) NaOH (100 µl, 0.2 mol/l) was injected in the luminometer. The surfactants studied were hexadecyl trimethyl ammonium chloride (CTAC) at 25% in water (Fluka AG, Switzerland), monolaurate sorbitan polyoxyethylene (Tween 20) (Serva, Heidelberg, FRG), 23-lauryl ther (Brij 35) (Sigma Chemical Co., St. Louis, USA), Triton X-100 (Merck, Darmstadt, FRG) and sodium dodecyl sulphate (SDS) (Bio Rad Lab., Richmond, California, USA).

We also studied, under the same conditions, the influence of the relative concentrations of the different compounds of oxidant reagent, the linearity of the dose/response relationship in the luminometer for each labelled compound with the different oxidant reagents and their stability during 15 days storage at 4° C.

Unless otherwise stated, data shown in this paper are corrected for blank values.

RESULTS

The pH dependence of AE pseudo-base formation is shown in Fig. 1. A rapid fall of the light emission was observed within a few seconds after dilution of the CL compound in the corresponding buffer ('0'). This effect was greater at high pH values suggesting a rapid formation of AE pseudo-bases. Stable light intensities (over 120 minutes) were only observed at pH 3. The emission intensity of solutions with pH > 3 reverted to that at pH 3 after addition of 100 µl of HNO_3 0.1 mol to the corresponding aliquot

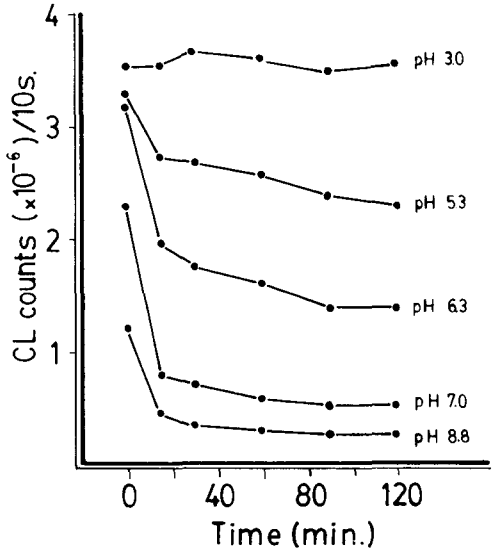

Figure 1. pH dependence of pseudo-base formation. Photon counts over 10 seconds from identical amounts of labelled rabbit anti-human albumin IgG (Ab*) in HNO_3 (1 mmol/l) diluted in buffer (PBS 0.1 mol/l, 0.1 g/l BSA) at different pH values. Aliguots (50 µl) were removed at suitable time intervals and measured luminometrically. Time axis is the time after addition of Ab* to the corresponding buffer. Oxidant reagant:100 µl NaOH 0.2 mol/l containing 10 mmol/l H_2O_2

before luminometry, showing pseudo-base formation to be a reversible process. These results agree with previous findings (Weeks *et al.*, 1983).

We also observed (data not shown) an increase in light emission (10%) when H_2O_2 was included in the nitric acid reagent. Thus, in order to obtain better efficiency and reproducibility, 100 µl of HNO_3 0.1 mol/l containing 10 mmol/l of H_2O_2 was added to the sample to be measured, prior to addition of NaOH, in all subsequent experiments. Using these modifications we obtained 2–4-fold increase in the CL emission intensity in comparison to that obtained with the old oxidant reagent.

We next investigated the effect of increasing amounts of different surfactants in the oxidant reagent on the CL reaction. As shown in Fig. 2(a), the background light emission produced by the reagents was increased by CTAC, Tween 20 and Brij 35 at concentrations higher than 0.3%, and was lower or unchanged with Triton X-100 and SDS up to a concentration of 2.5%.

All surfactants, when added to the oxidant reagent, produced an increase on the light emission intensity of the AE CL reaction (Fig. 2(b)). The greatest increase (3-fold) was obtained with Triton X-100 (2.5% v/v) and Tween 20 (10% v/v). The other surfactants produced a 2-fold increase. With Alb* (Fig. 2(c)) the greatest increase (8-fold) was obtained with CTAC 0.3% w/v. Triton X-100 (2.5% v/v) only produced approximately a 4-fold increase. In contrast, with Ab* (Fig. 2(d)) Triton X-100 produced a maximum 3-fold increase of chemiluminescence emission while CTAC only produced a 2-fold increase. SDS inhibited the CL response at concentrations less than 5% w/v.

Table 1 shows the critical micelle concentration (CMC) for the different surfactants. In our experimental conditions, the concentrations of the surfactants were greater than the coresponding CMC values.

Table 2 shows that the kinetics of the CL light emission differed according to the surfactant used. Thus, CTAC produced rapid reactions while SDS led to very slow reactions. The other surfactants did not produce noticeable changes in the CL reaction kinetics.

We found for labelled antibody (Ab*) (Table 3) that increasing concentrations of NaOH produced faster light emission kinetics, as well as increasing CL counts over 10 seconds in the presence of either nitric acid, CTAC or Triton X-100 (see Fig. 3). Increasing concentrations of

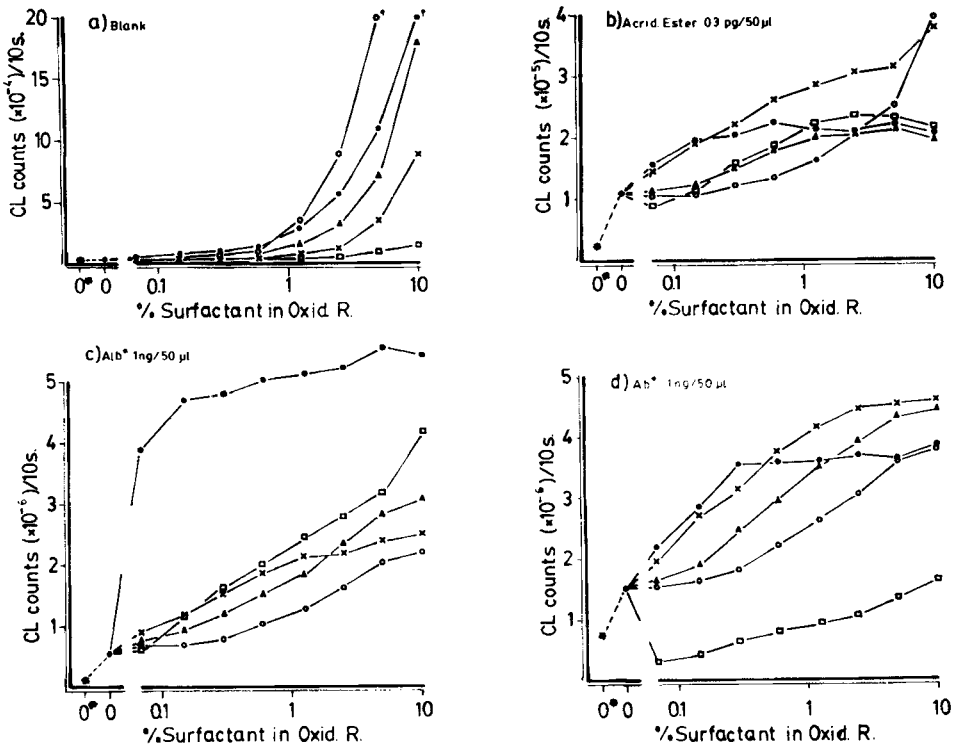

Figure 2. Effect of different concentrations of hexadecyl trimethyl ammonium chloride (CTAC, ●), Triton X-100 (X), Tween 20 (○), 23 lauryl ether (Brij 35, △) and sodium dodecyl sulphate (SDS, □) in the oxidant reagent (100 µl HNO_3 0.1 mol/l containing 10 mmol/l H_2O_2) on the CL reaction emission intensity (total photon counts over 10 seconds) of: (a) blanks (50 µl assay buffer); (b) acridinium ester (AE, 0.3 pg/50 µl); (c) labelled human albumin (Alb*, 1 ng/50 µl); and (d) labelled rabbit anti-human albumin IgG (Ab*, 1 ng/50 µl). The CL reaction was initiated by injection 100 µl of 0.2 mol/l NaOH. 0*: Photon counts over 10 seconds obtained in each case without surfactant and pH 6.3 (old oxidant reagent: 100 µl NaOH 0.2 mol/l containing 10 mmol/l H_2O_2)

H_2O_2 (Fig. 4) in the oxidation reagent also produced a greater chemiluminescence emission intensity from Alb* and Ab*, and this reached a maximum at a concentration of 80 mmol/l independent of the oxidation reagent used, although this increase was more marked when either CTAC or Triton X-100 was also included. However, in the case of AE, the maximal effect in acid without surfactant was reached at a concentration of 10 mmol/l of H_2O_2 and at 80 mmol/l of H_2O_2 in the presence of Triton X-100 or CTAC. At concentrations greater than 80 mmol/l of H_2O_2 we observed (data not shown) slower light emission kinetics. This could explain why in some cases the CL intensity produced was greater using 80 mmol/l of H_2O_2 than with higher H_2O_2 concentrations.

When the optimum concentration of H_2O_2 (80 mol/l) was used, a marked increase in the CL (counts over 10 seconds) of Alb* or Ab* could be obtained with increasing concentrations of either CTAC or Triton X-100. The improvement was more marked in all the cases than that obtained

Table 1. Critical micelle concentrations for the different surfactants

Surfactant	10^3[CMC]M	[CMC]g/100 ml
[a]CTAC	1.29	0.041
[a]Brij 35	0.1	0.012
[a]SDS	8.13	0.234
[b]Tween 20	0.05	0.006
[b]Triton X-100	0.24	0.016

Literature values taken from [a]Reference 9 and [b]Reference 10.

Table 2. Chemiluminescence emission kinetics with different surfactants

Oxidant reagent (100 µl)	AE		Alb*		Ab*	
	tmax.	thmax.	tmax.	thmax.	tmax.	thmax.
HNO_3 0.1 mol/l, 10 mmol/l H_2O_2	0.1	0.5	0.1	0.6	0.1	0.8
HNO_3 0.1 mol/l, 10 mmol/l H_2O_2 with 0.2% CTAC	0.1	0.2	0.1	0.3	0.1	0.3
HNO_3 0.1 mol/l, 10 mmol/l H_2O_2 with 2% Triton X-100	0.2	1.0	0.1	1.1	0.2	1.0
HNO_3 0.1 mol/l, 10 mmol/l H_2O_2 with 2% Tween 20	0.2	0.7	0.2	1.2	0.3	0.8
HNO_3 0.1 mol/l, 10 mmol/l H_2O_2 with 2% Brij 35	0.2	0.7	0.2	1.2	0.3	0.8
HNO_3 0.1 mol/l, 10 mmol/l H_2O_2 with 2% SDS	0.3	1.8	0.4	3.6	0.4	3.5

tmax.: time in seconds at which peak emission occurred.
thmax.: time in seconds of half-maximum emission.
AE: Acridinium ester.
Alb*: human albumin.
Ab*: rabbit anti-human albumin IgG.

Table 3. Chemiluminescence emission kinetics of an acridinium ester labelled antibody (Ab*) in response to different NaOH concentrations, used to initiate the chemiluminescent reaction

NaOH (mol/l)	Oxidant reagents					
	HNO_3 0.1 mol/l		with 2% Triton X-100		with 0.2% CTAC	
	tmax.	thmax.	tmax.	thmax.	tmax.	thmax.
0.1	0.2	3.4	0.2	1.0	0.1	0.5
0.2	0.2	0.7	0.2	1.0	0.1	0.4
0.4	0.2	0.5	0.2	0.7	0.1	0.3

tmax.: time in seconds at which peak emission occurred.
thmax.: time in seconds of half-maximum emission.
Ab*: rabbit anti-humna albumin IgG (1 ng/50 µl assay buffer).

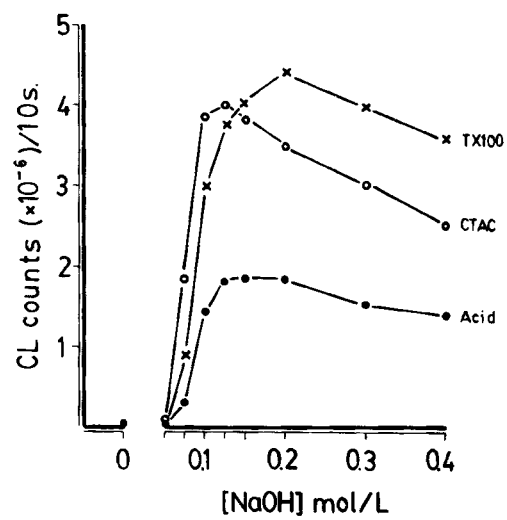

Figure 3. Effect of different concentrations of NaOH on photon counts over 10 seconds produced by 50 µl (1 ng) of labelled rabbit anti-human albumin IgG (Ab*) using three different reagents: (Acid, ●) HNO_3 0.1 mol/l containing 10 mmol/l H_2O_2; (CTAC, ○) as above but with 0.2% w/v CTAC and 2% v/v Triton X-100 (TX100) (X)

when 10 mmol/l of H_2O_2 were used. The maximal effect was observed using 2.5% v/v of Triton X-100 in the case of Ab* (7.5-fold increase *vs* the result obtained with oxidant reagant without surfactant and 10 mmol/l of H_2O_2) and using 0.3% w/v of CATC in the case of Alb* (14-fold increase). Thus, compared with the results obtained with the old oxidant reagent and labelled protein in pH 6.3 buffer, the total increase obtained would be 15-fold for Ab* and Triton X-100 and 42-fold for Alb*and CTAC.

Fig. 5 shows the effect of different concentrations of proteins in the reaction media on the CL emmission intensity of Ab*. It can be seen that concentrations greater than 2 mg/ml (100 µg/tube) of BSA or IgG produced a decrease in the CL. This effect was particularly important when CTAC was present in the oxidation reagent and could only be partially reversed by using high concentrations (80 mmol/l) of H_2O_2.

We also found (Fig. 6) that non-specific adsorption of Ab* to the tube wall, could be best prevented by using a low protein concentration

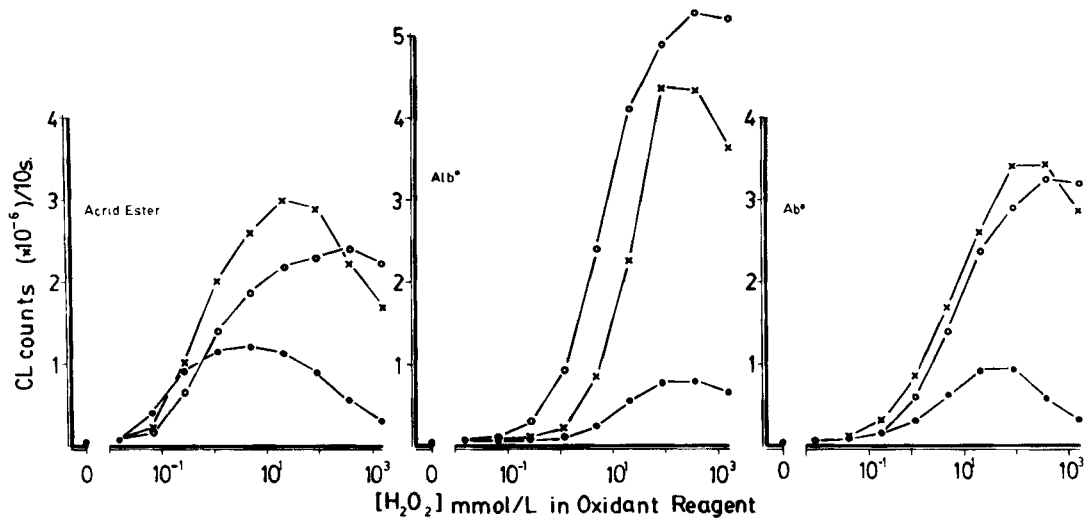

Figure 4. Effect of increasing concentrations of H_2O_2 in the oxidant reagent on total photon counts over 10 seconds produced by CL reaction of acridinium ester (AE, 3 pg/50 µl), labelled human albumin (Alb*, 0.5 ng/50 µl) and labelled rabbit anti-human albumin IgG (Ab*, 0.5 ng/50 µl), using three different oxidant reagents: (●) 100 µl HNO_3 0.1 mol/l; (○) as above with 0.2% w/v CTAC and (X) with 2% v/v Triton X-100. The CL reaction was initiated by injection of 100 µl of 0.2 mol/l NaOH

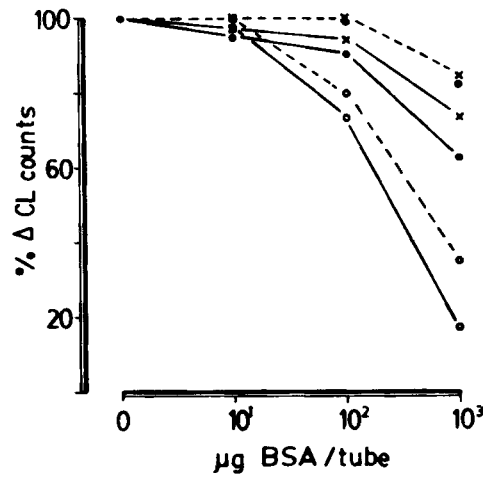

Figure 5. Effect of protein on chemiluminescent reaction. Chemiluminescent response, expressed on percentage of that obtained in a protein free buffer (PBS 0.1 mol/l pH 6.3 with 0.1% v/v Triton X-100), of identical amounts of labelled rabbit anti-human albumin IgG (Ab*, 1 ng) in 50 µl of buffer protein free and with increasing amounts of BSA or bovine IgG (10–1000 µg/tube; 0.2–20 mg/ml), using different oxidant reagents: (●—●) 100 µl HNO_3 0.1 mol/l with 10 mmol/l H_2O_2; (●– –●) as above but with 80mmol/l H_2O_2; (○—○) with 10 mmol/l H_2O_2 and 0.2% CTAC; (○– –○) with 80 mmol/l H_2O_2 and 0.2% CTAC; (X–X) With 10 mmol/l H_2O_2 and 2% Triton X-100; (X_ _X) With 80 mmol/l H_2O_2 and 2% Triton X-100

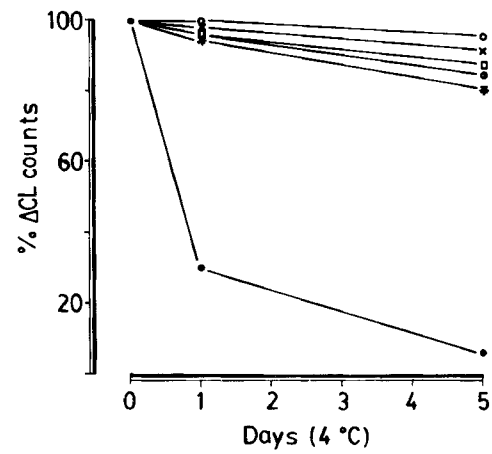

Figure 6. Adsorption of the labelled compound to the walls of polyethylene tubes: chemiluminescent response, expressed as a percentage of that obtained on day '0' of identical amounts of labelled rabbit anti-human albumin IgG (Ab*, 1 ng) in different buffers (50 µl), when stored 5 days at 4°C in the polyethylene tubes; (●) PBS 0.1 mol/l pH 6.3 protein free; (□) as above but with 0.1 g/l BSA; (X) with 0.1% TX100; (○) with 0.1% TX100 and 0.1 g/l BSA; (◉) with 0.05% Tween 20; (* with 1 g/l gelatin. Oxidant reagent: 100 µl HNO_3 0.1 mol/l with 10 mmol/l H_2O_2 and 2% TX 100

buffer (0.1 g/l BSA) containing 0.1% Triton X-100.

Independently of the CL compound (AE, Alb* or Ab*) and of the oxidation reagent used, a widely linear dose/response relationship was always obtained. With AE in aqueous solution, we obtained a linear dose/response relationship oxidation reagent: 100 µl HNO_3 0.1 mol/l containing 80 mmol/l H_2O_2 and 2% v/v Triton X-100 from 1.5 to 15 000 fg and a detection limit of 1.2 fg (2 attomoles) when calculated as a function of the precision of 12 blank replicates (mean + 3SD). The respective detection limits obtained for Alb* and Ab* in the optimal conditions for each case were 50 and 200 fg.

Finally, we studied the stability of different oxidant reagents during storage at 4° C for 15 days. They were stable for at least a week with losses not higher than 10% in any case. The oxidant reagents with 80 mmol/l of H_2O_2 showed a lower stability in the presence of CTAC.

DISCUSSION

Our data demonstrate that surfactants greatly increase the emission itensity of the acridinium ester CL reaction in aqueous solution and this is in agreement with previous reports (Grayeski and Woolf, 1984; Hinze *et al.*, 1984; Howie and Grayeski, 1987; McCapra, 1976; Paleos *et al.*, 1982).

Clear differences were found in the effect, qualitative as well as quantitative, of these compounds on different labelled proteins. Thus, SDS increased the CL reaction emission intensity of Alb* while decreasing that of Ab*. Also, while TX100 produced the greatest increase of the CL emission intensity of both AE and Ab*, CTAC produced the greatest enhancement in the case of Alb*. The increase in the CL reaction emission intensity of acridinium ester derivatives by surfactants has been variously suggested to be due to association of a non-polar intermediate in the oxidation reaction or the acridinium ester with the hydrophobic environment of a micelle (Grayeski and Woolf, 1984; Paleos *et al.*, 1982), and owing to an increase in the rate of oxidation within the micelle at the expense of 'dark' reaction pathways (McCapra, 1976).

In studies with lucigenin it has been suggested that the effect of a surfactant is due to the altered hydroxide concentration in the micellar microenvironment (Hinze *et al.*, 1984). However, the reasons for the differences in the effects of these surfactants on the AE labelled albumin and IgG are not completely understood at present, although they may be related to the differing physicochemical properties of the proteins and surfactants, (e.g. CTAC is a cationic surfactant, Triton X-100 is mainly non-ionic and SDS is anionic, albumin is an acidic protein IgG is basic protein).

Apparent differences could also arise from the fact that CTAC produced fast oxidation reactions, Triton X-100 did not affect kinetics and SDS led to very slow oxidation reactions. Rapid reactions may yield 'multiphoton' events at the PMT of the luminometer and hence yield apparently lower photon counts. Although the mechanism for the enhancement is unknown, it is clear from our data that marked increases in the specific activity of CL labelled proteins can be obtained by establishing optimum conditions of pH, H_2O_2 and protein concentrations, in addition to the inclusion of a suitable surfactants in the reaction system. Using this approach we obtained a 42-fold increase in the light emission intensity from AE labelled albumin (pH 3, 80 mmol/l H_2O_2 and 0.3% w/v CTAC) and a 15-fold increase from AE labelled rabbit anti-human albumin IgG (pH 3, 80 mmol/l H_2O_2 and 2.5% v/v Triton X-100).

Also, under optimal conditions a linear response from 1.5 to 15 000 fg and a detection limit of 2 attomoles (1.2 fg) was obtained with acridinium ester in assay buffer (the detection limits for Alb* and Ab* were 50 and 200 fg, respectively). Although buffers with high protein concentration are frequently used to avoid non-specific adsorption of analyte to the tube wall, our data show that they may exert a marked inhibitory effect on the CL reaction emission intensity. This problem can be overcome by using a low concentration of BSA (0.1 g/l) together with Triton X-100 (0.1% v/v).

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