JOURNAL OF BIOLUMINESCENCE AND CHEMILUMINESCENCE VOL 6 35–43 (1991)

Nucleophilic Addition to the 9 Position of 9-Phenylcarboxylate-10-methylacridinium Protects against Hydrolysis of the Ester

Philip W. Hammond,* Wendy A. Wiese, Alex A. Waldrop III and Norman C. Nelson
Gen-Probe Inc., 9880 Campus Point Dr., San Diego, CA 92121, USA

Lyle J. Arnold Jr
Genta Inc., 10955 John J. Hopkins Dr., San Diego, CA 92121, USA

The chemiluminescent reaction of an acridinium ester (AE) requires addition of peroxide to the 9 position of the acridinium ring. The addition of a hydroxide ion to the 9 position of an acridinium ester to form the carbinol adduct has also been well documented. We have observed a similar addition of other nucleophiles to the acridinium ring to form an acridan adduct. The adduct formed with bisulphite has been particularly well-characterized for rate of formation, rate of reversion, and reaction equilibrium. The formation of an adduct (other than H_2O_2) has been demonstrated to decrease significantly the reactivity of the adjacent ester bond to alkaline hydrolysis. The resulting, more stable adduct is very useful when the acridinium ester is used as a label in DNA probe-based assays. The adduct is highly resistant to hydrolysis under the conditions often desired for DNA probe-based assays (high temperature, elevated pH, extended storage).

Keywords: Acridinium ester; chemiluminescence; DNA probes; ester protection; nucleophilic addition

INTRODUCTION

An acridinium ester has been shown to be a particularly useful label in biological assays and has been used for both antibody-based (Weeks *et al.*, 1983), and DNA probe-based assays (Arnold *et al.*, 1989). It provides an alternative to radioisotopic detection systems with a longer shelf-life, improved sensitivity, and no need for the special handling required by radioisotopes. However, the acridinium ester as a label is not without its problems. Of particular concern is the lability of the ester moiety to alkaline hydrolysis. When this reaction occurs, the label becomes non-chemiluminescent and assay performance is severely curtailed. We have been successful in reducing this problem through the formation of a protective adduct. Reaction at the 9 position of the acridinium ester with an appropriate nucleophile forms a stable yet reversible covalent bond. The resulting adduct has sharply reduced sensitivity to hydrolysis.

The acridinium ester shown in Fig. 1 is used to label DNA probes which contain a primary alkyl amine inserted into the DNA during synthesis. The

*Author for correspondence.

0884-3996/91/010035-09$05.00

Received 10 January 1990
Revised 20 March 1990

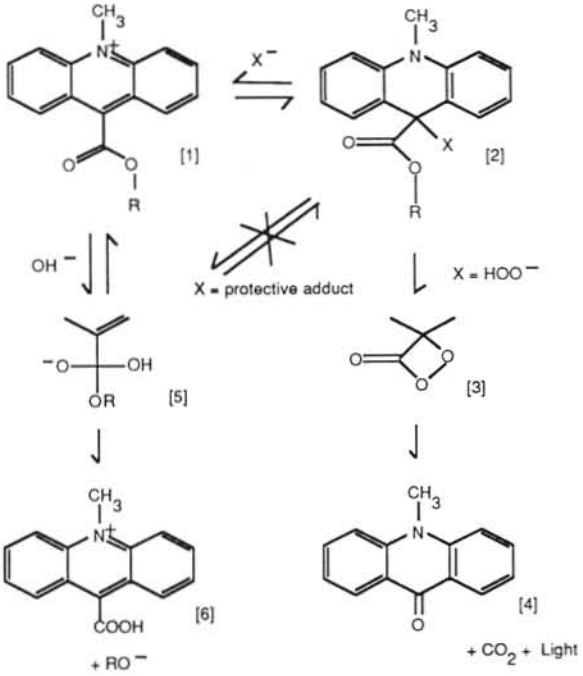

Figure 1. The reaction mechanisms of the acridinium ester. Compound [**1**] is the native acridinium ester. The hydrolysis reaction leading to a loss of chemiluminescence is shown in the pathway through [**5**] to [**6**]. The alternative reaction is nucleophilic attack at the 9 position. When this occurs with H_2O_2, the adduct [**2**] can react with hydroxide to give the dioxetanone intermediate [**3**] which then proceeds through decomposition to give the emitting species *N*-methylacridone [**4**]. If the nucleophilic addition occurs with a 'protective adduct former', the hydrolysis reaction is blocked and a stable adduct is formed.

R group for the labelling reagent is shown in Fig. 2. Fig. 1 shows the reaction schemes of the acridinium ester. Reaction of the native acridinium ester [**1**] with hydroxide ion at the ester moiety results in hydrolysis of the ester bond [**5**] to give the non-chemiluminescent acridinium acid [**6**] and the leaving group. This reaction, which is first order in hydroxide ion concentration over a large pH range, yields no light and results in an irreversible loss of chemiluminescent activity. Reaction at the 9 position of the acridinium ester with a nucleophile X^- yields the compound [**2**]. The nucleophile can be hydroxide ion, peroxide, or an appropriate adduct-forming nucleophile such as those we have characterized. When the nucleophile is peroxide (in alkali) the reaction proceeds through a dioxetanone intermediate [**3**] to give *N*-methylacridone [**4**] and light (McCapra *et al.*, 1965). Alternatively, if the adduct

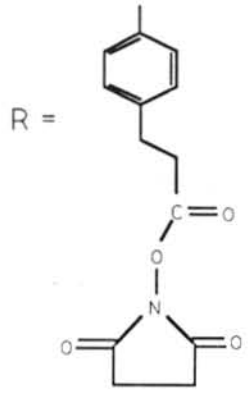

Figure 2. This is the R group in Figure 1 for the acridinium ester labelling reagent. The *N*-hydroxysuccinimide group reacts specifically with a primary amine in the DNA probe.

forming compound X^- is a 'protective adduct former' which does not undergo the cyclization reaction, a 'protected' acridinium ester is generated. In this case, the 9 position is occupied in competition with any other reactive nucleophile, such as peroxide, effectively blocking the light-producing reaction. In addition, the formation of [**2**] changes the electronic structure of the acridinium ring system giving a less strongly electron withdrawing group and increasing steric hindrance around the ester. This makes the carbonyl group less susceptible to nucleophilic attack which leads to a much more stable label. The formation of the adduct is a reversible reaction and full recovery of chemiluminescent activity can be achieved.

MATERIALS AND METHODS

AE-labelled DNA probes were prepared by reacting the *N*-hydroxysuccinimide acridinium ester (Weeks *et al.*, 1983) with a synthetic oligomer containing a primary alkyl amine inserted into the DNA probe during chemical synthesis. The labelled probes were purified by HPLC. The resulting labelled probes have a specific activity that is equal to that of the AE-labelling reagent at 1×10^8 relative light units per picomole (rlu/pmol).

Absorption spectra were obtained using a Varian 9060 POLYCHROM diode array detector. A 20 µmol/l solution of AE-labelling reagent was prepared in 20% (v/v) CH_3CN, 20 mmol/l NaOAc, pH 5.5. Thirty µl of a 0.1 mmol/l aqueous solution of sodium bisulphite was added to 3 ml of AE

solution to give a final concentration of 1 mmol/l. AE solutions with and without bisulphite were injected through the flow cell, using a 1-ml syringe, and scans taken.

Chemiluminescence was measured in a Leader™ I luminometer equipped with two 200-µl pumps. This instrument measures chemiluminescence by photon counting and the readout is in relative light units (rlus). All chemiluminescence measurements were made using an AE-labelled DNA probe.

To determine the rate of bisulphite adduct formation and reversion, AE-labelled probe was diluted in a solution of 0.8 mol/l sodium borate pH 7.65% (v/v) Triton X-100. The solution used for reversion measurements (2 µl per time point) was approximately 600-fold more concentrated than for formation measurements (300 µl per time point). Aliquots were transferred to a 12 × 75 mm tube for each measurement and the sample was placed in the luminometer for assay. The first injection for formation measurements contained sodium bisulphite diluted from a 1 mol/l stock solution in 1% (v/v) Triton X-100. For reversion measurements, the first injection contained the same buffer in which the probe was diluted. For both assays the second injection contained 2 mol/l NaOH, 100 mmol/l H_2O_2. The delay time between automatic injections was varied for incubations less than 25 seconds. For longer incubations the first injection was done manually.

Measurement of hydrolysis rates was done as follows. To determine the pH dependence 5 µl of AE-labelled probe was added to 100 µl of 0.6 mol/l sodium borate (adjusted to the desired pH with 6 mol/l HCl) in a luminometer tube. To determine protection by bisulphite: AE-labelled probe was added to 0.6 mol/l sodium borate pH 8.0 with or without 5 mmol/l bisulphite and 100 µl aliquots of these solutions were added to luminometer tubes. Aliquots prepared as in 1 and 2 above were incubated 0–24 minutes at 40°C, then removed and placed in an ice bath. The chemiluminescence was assayed as for storage stability testing (see below).

To determine storage stability, an AE-labelled probe was suspended in 0.1 mol/l lithium succinate, pH 5.2, 0.1% lithium lauryl sulphate, with or without 10 mmol/l 3-mercaptopropionic acid. The solution was assayed for chemiluminescence by pipetting 5 µl of a 100-fold dilution into 300 µl of 50% (v/v) formamide, 0.2 mol/l sodium phosphate, pH 6.0, chemiluminescence was measured by automated injection of 200 µl of 0.4 mol/l HNO_3, 0.1% H_2O_2 followed by 200 µl of 1 mol/l NaOH. One-millilitre aliquots of the stock solution were then lyophilized and the resulting residues were incubated 1 or 3 days at 45°C. The lyophils were then reconstituted with water to the original volume and assayed for chemiluminescence as described above.

To determine the hybridization stability of the bisulphite adduct 1×10^6 rlu (0.01 pmol) of AE-labelled probe was suspended in 50 µl of 45 w% diisobutyl sulphosuccinate, 30 mmol/l sodium phosphate, 1 mmol/l ethylenediamine tetraacetic acid (EDTA), 1 mmol/l ethyleneglycol-bis-(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA), 0.7% sodium dodecylsulphate (SDS), pH 6.2 and mixed with 5 µl (0.63 pmol) of it's target *Escherichia coli* rRNA in 3% lithium lauryl sulphate. These solutions contained either no adduct former, 5 mmol/l 4-hydroxythiophenol, or 5 mmol/l 3-mercaptopropionic acid. The hybridization cocktails were incubated at 60°C for 2.5 hours. Each sample was then added to 500 µl of 2% (w/v) hydroxyapatite (HAP), which preferentially binds the hybridized probe, in 0.12 mol/l sodium phosphate, 0.02% SDS, pH 6.8 (wash buffer) and incubated 5 minutes at 60°C, then centrifuged to pellet the HAP, the supernatant was removed as the 'unbound fraction'. The HAP was resuspended in 500 µl of wash buffer, vortexed, then centrifuged, and the washing saved as 'wash'. Finally, the pellet was resuspended in 450 µl of wash buffer. Fifty-microlitre aliquots of these fractions were added to 200 µl of 0.4 mol/l HNO_3, 0.1% H_2O_2 and assayed for chemiluminescence by automatic addition of 200 µl of 1 mol/l NaOH.

RESULTS

Change in absorption spectrum on formation of the bisulphite adduct

Fig. 3 shows the striking change in absorption spectra which occurs when the acridinium ring structure changes to that of an acridan upon adduct formation. Our acridinium ester spectrum correlates well with that of the acridinium cation (Albert, 1966). The acridinium cation has a characteristically strong band near 250 nm (here corresponding to the 260 nm peak in our AE compound). There are also characteristic absorptions at 324 nm, 338 nm, 354 nm, 386 nm, and 402 nm. In the spectrum shown there are small inflections at 334 nm, 350 nm, and the upslope of a

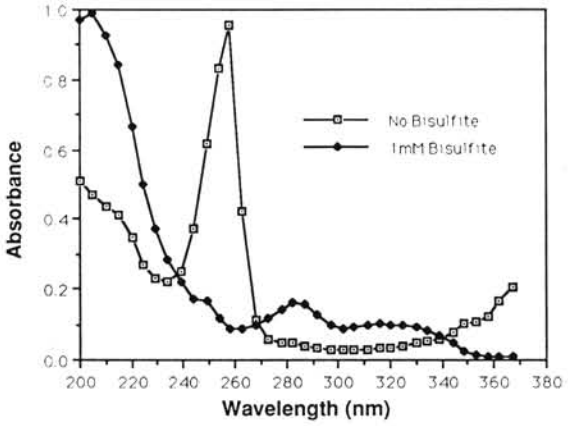

Figure 3. The change in the absorption spectrum of the acridinium ester upon adduct formation with bisulphite is quite marked. The spectrum for the non-adduct is typical of an acridinium ring system. After addition of bisulphite the spectrum changes to that of an acridan.

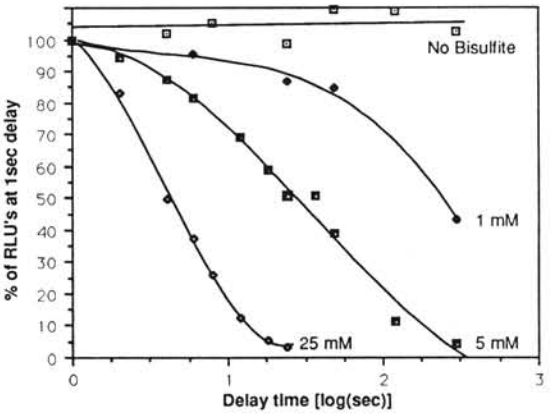

Figure 4. The rate of bisulphite adduct formation was measured at three concentrations of bisulphite. The sample containing an AE-labelled DNA probe was placed in a luminometer tube. A first injection containing the bisulphite was made and then a second made at various intervals containing alkaline peroxide. The light-off reaction with peroxide is over within 2 seconds. The bisulphite competes with hydrogen peroxide for the 9 position of the acridinium ring. The reaction was followed by measuring the loss of chemiluminescence as the adduct forms.

band >370 nm. In contrast, the spectrum of acridan is much simpler. It contains a strong band at 290 nm with inflections at 315 nm and 335 nm and there is no significant absorption at >350 nm in the acridan (Albert, 1966). This agrees well with the spectrum we obtained for the bisulphite adduct and supports the proposed structure. The acridinium ester has a very striking green-yellow colour which fades almost completely upon addition of an adduct-forming compound.

Rate of adduct formation

Fig. 4 shows the rate of formation of the bisulphite adduct as measured by loss of chemiluminescence. The rate of adduct formation varies greatly depending on the nucleophile used. The formation of the bisulphite adduct is relatively slow and therefore readily lends itself to measurement. In contrast, compounds such as 1-propanethiol form adducts so rapidly that it is very difficult to measure the kinetics using methods other than stopped flow techniques. For the bisulphite adduct determinations, an initial injection containing bisulphite was added to the sample. After incubation for the indicated time periods, a second injection was made, which contained the alkaline peroxide required to produce chemiluminescence. The peroxide adds in competition with the bisulphite as described above so that the amount of light obtained is inversely proportional to the extent of reaction with bisulphite. The minimum delay time between injections used was 1 second, the data for longer incubations is reported as the percentage remaining of the rlu obtained at the one-second time point. A significant complication to these measurements is the competition of bisulphite with hydrogen peroxide during the chemiluminescence assay step. This problem was minimized by using a large excess of peroxide and by the large differences in the addition rates of peroxide and bisulphite. The chemiluminescent reaction involving peroxide is essentially complete in less than 2 seconds. The rate of formation of the hydroxide adduct (carbinol or pseudobase) as detailed by Weeks *et al.* (1983), is also sufficiently slow not to compete greatly with measurement of the bisulphite adduct formation (see the data for 'no bisulphite'). The rate of formation is clearly concentration-dependent. As indicated above some of the other adduct formers characterized have very different rates of reaction. Many such as 1-propanethiol (mentioned above) react too quickly to accurately measure while others such as 2-mercaptosuccinic acid and 3-mercaptopropionic acid show intermediate rates of reaction.

Rate of adduct reversion

Reversion of the protective adduct is necessary to restore chemiluminescence. As shown in Fig. 5, the adduct formed with bisulphite is readily reversible by dilution. A 2-µl aliquot of the bisulphite adduct of an AE-labelled probe was diluted by adding 200 µl of the same buffer containing no bisulphite. After the incubation time as indicated, chemiluminescent emission was affected by addition of alkaline peroxide. Reversion is virtually complete in about 25 minutes with 100% of the control sample rlu recovered. Owing to the protection effects of the adduct it is possible to recover greater than 100% of the control value (as seen in Fig. 5). Complete recovery of chemiluminescence activity is a general feature of all of the adducts we have studied. The rate of reversion (like the rate of formation) is very much dependent on the adduct-forming compound. The reversion rate of bisulphite is quite slow (as is the formation rate) and was therefore relatively easy to measure. As with formation, the rate of reversion of 1-propanethiol is too fast to be readily measured using the technique described. While the reversion shown here was accomplished by mechanical dilution, other forms of reversion have been demonstrated. Of particular interest with the thiol compounds has been the use of Aldrithiol™-2(2,2′-dithiodipyridine). This compound reacts via a thiol exchange reaction and effectively drops the concentration of the adduct-forming thiol (a sort of thiol trap). This gives the same effect as dilution, and complete recovery of chemiluminescence has been demonstrated using this technique.

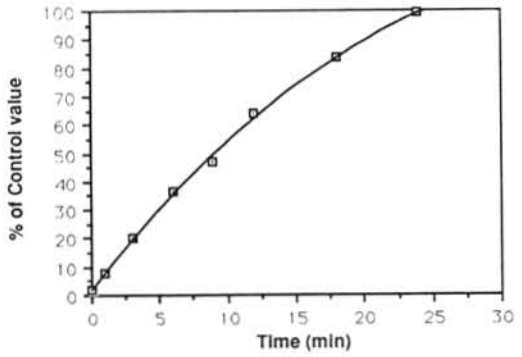

Figure 5. The acridinium ester adduct formed with bisulphite is completely reversible upon dilution. Two microlitres of a solution containing AE-labelled DNA probe and 5 mmol/l bisulphite was added to a luminometer tube. A first injection of 200 µl gave a 100 × dilution of the bisulphite adduct. After a varied time interval a second injection of alkaline peroxide was made to assay for chemiluminescence. The rate of return of chemiluminescence corresponds to the rate of reversion of the bisulphite adduct.

The adduct formed with hydroxide (carbinol adduct) has shown the interesting property of rapid acid reversion. The carbinol adduct forms and reverts very slowly. The reversion reaction was monitored by measuring chemiluminescence from a sample of carbinol adduct in the presence of alkaline hydrogen peroxide over time. As the adduct reverts, it is rapidly attacked by peroxide to give light. When the pH is reduced to less than pH 2.0 using HNO_3, the reversion is complete in a matter of seconds. We believe that protonation of the hydroxide makes it a better leaving group. This results in relatively rapid reversion when compared to the back reaction at elevated pH.

pH-dependence of AE hydrolysis

The ester hydrolysis of AE-labelled probe in aqueous solution is first order in hydroxide ion. AE-labelled probe was incubated for various times at pHs from 6.0 to 8.5. The log of the rlu remaining at each time point was plotted against the time of incubation as shown in Fig. 6. The rate of hydrolysis was determined from the slope of these plots. It is important to note that only the linear range of hydrolysis was used for the slope calculation. At high pH (most notably 8.5), the hydrolysis reaction goes essentially to completion within 12 minutes. Experiments (data not presented) have shown that the amount of carbinol formed under these conditions is less than 1% of total AE and so does not markedly affect these results. The log of the rate of hydrolysis was then plotted against the pH. The resulting plot is very linear throughout the pH range tested, indicating the hydrolysis reaction is first order with respect to hydroxide. This agrees with the mechanism of hydrolysis (hydroxide cleavage of the phenyl ester) described above.

Reduced rate of hydrolysis for the bisulphite adduct

Formation of an adduct of the acridinium ester causes large changes in the electronic structure of the acridinium ring system. As a result, the 9 position of the ring is no longer conjugated to the

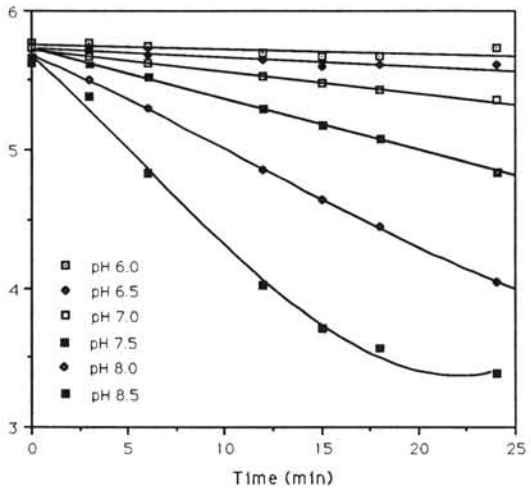

Figure 6. The rate of hydrolysis of the acridinium ester when attached to a DNA probe was measured at six different pHs. Identical samples were prepared for each time point by addition of 5 µl of AE-labelled probe to 100 µl of sodium borate adjusted to the desired pH with 6 mol/l HCL. The samples were incubated for the indicated time at 37°C. The remaining chemiluminescence was then assayed. The Log of rlus is plotted here versus time of incubation. The rate of hydrolysis is taken from the slope of the linear range of these plots.

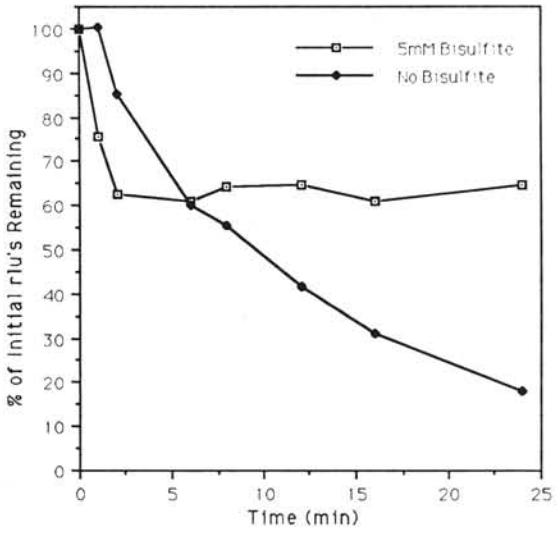

Figure 7. The hydrolysis kinetics of the bisulphite adduct differs greatly from that of the native acridinium ester. Upon formation of the adduct the hydrolysis reaction slows markedly. A stock solution containing AE-labelled DNA probe in sodium borate pH 8.0 with and without 5 mmol/l bisulphite was aliquoted to luminometer tubes. These were then incubated for the indicated time at 37°C and assayed for remaining chemiluminescence. The initial drop in chemiluminescence in the bisulphite containing sample corresponds to additional adduct formation at the elevated temperature.

rest of the π system. Upon adduct formation, the electron-withdrawing nature of the acridinium ring system is reduced, resulting in a decrease in the partial positive charge on the ester carbonyl carbon and consequently in the electrophilic nature of this carbonyl. In Fig. 7, the hydrolysis kinetics of the bisulphite adduct are compared to those of the native ester. The native ester shows first order hydrolysis reaction kinetics as measured by the loss of chemiluminescent activity. In marked contrast, the bisulphite adduct shows virtually no loss of chemiluminescence from 2 to 24 minutes of incubation. The initial rapid decrease in chemiluminescence is due to the formation of additional adduct at the higher temperature of 37°C, as all of the original chemiluminescence can be recovered by sufficient reversion. A similar protection effect has been seen for all of the adduct-forming nucleophiles (except H_2O_2). Indeed, the departure from first order hydrolysis kinetics has been used as an indicator of adduct formation. The magnitude of the change is dependent on the rate of adduct formation and reversion of each individual compound. Those adducts which have a very fast rate of exchange show a greater loss of rlus as the hydroxide is allowed significant opportunity for attack. The result is a kinetic curve somewhere between the two shown in Fig. 7. This reduction in hydrolysis rate has been of primary importance in using acridinium esters as labels in DNA probe-based assays.

Other compounds which form adducts

The formation of adducts with acridinium molecules has been observed since the late 1800s, but the structure of the adducts were not clarified until 1899. Over the following 10 years, acridinium adducts were reported with ammonium ion, cyanide, sulphide, thiophenol, sulphite, and hydroxylamine. In 1957, adducts were observed with Michael reagents. For a review of these findings see Albert (1966). As noted above, acridinium ester adducts have long been known to form with peroxide as part of the light-off reaction. In addition, Weeks and co-workers reported the formation of the hydroxide adduct. We have tested a range of compounds for their ability to form adducts with this acridinium ester. We used as our criterion the

ability to protect the ester from hydrolysis. Using this method, we have observed strong adduct formation with a variety of thiols ranging from simple primary thiols such as 1-propanethiol, 2-aminoethanethiol, cysteine, dithiothreitol to simple and complex secondary (2-propanethiol, and 2-mercaptosuccinic acid) and tertiary thiols (4-hydroxythiophenol, 8-mercaptomenthone, and 2-methyl-2-propanethiol). We have tested several amines (i.e. ethylene diamine, aniline, hydroxylamine) and cyanide without observing adduct formation at 5 mmol/l concentrations although some amines have been observed to form adducts at concentrations of 50 mmol/l or greater (i.e. ethylene diamine). In addition, some miscellaneous compounds such as hypochlorite and phosphate (at high concentrations above pH 6.5) have been observed to form adducts. Several of Good's buffers such as HEPES and MOPS also form adducts at high concentration which is useful for working with the acridinium ester in buffered solutions at elevated pH.

Storage stability of AE-labelled probe as an adduct

One of the most severe problems associated with the use of radioisotopes as detectable labels is their relatively short half-lives (^{32}P about 14 days and ^{125}I about 60 days). A DNA probe labelled with one of these molecules will lose sensitivity in a relatively short time. This requires that experiments performed over several days or weeks must always be corrected for decay. By using an acridinium ester adduct with 2-mercaptosuccinic acid, we have been able to demonstrate storage times of several months when lyophilized as described above. In a short time course including a non-adduct control we achieved excellent stability. After one day of storage of the lyophilized probe at 45°C the control value dropped to 61% of the starting value, on day three it was down to 23%. The corresponding values for the adduct protected samples were 101% and 105% of the starting value. Longer time courses have been performed but as indicated by the data it is impossible to carry along a non-adduct control for very long. Determining stability using short incubations at elevated temperatures is a common way of estimating stability at lower temperatures over a long time period. Long-term storage at or above room temperature is very desirable for commercialization of this non-isotopic label technology. A large batch of probe can be labelled and lyophilized and aliquots reconstituted as required. Results over a period of months can be easily compared.

Table 1. Signal recovery from an AE-labelled probe after a 2.5 hour hybridization with adduct protection

	Relative light units		
Protective adduct former	Support	Non-bound	Wash
None	6450	5027	671
4-Hydroxythiophenol	21677	43692	3111
3-Mercaptopropionic acid	23476	11160	1041

Hybridization stability of AE-labelled probe as an adduct

DNA probe hybridizations are often done at elevated temperatures and for extended periods of time. It is often necessary to denature a double-stranded target by heating prior to hybridization. At elevated temperatures the AE-label is very susceptible to hydrolysis. As a result the assay loses sensitivity. By using an AE-adduct for these hybridizations, hydrolysis can be greatly reduced, and the signal obtained greatly increased. As shown in Table 1, the addition of 5 mmol/l 4-hydroxythiophenol or 5 mmol/l 3-mercaptopropionic acid increases the recovery of signal in this assay about four-fold. In a system where the background is substantial such an increase in signal is very valuable. When interpreting this data, it is important to remember that the adduct-former is still present to some extent in the unbound fraction and competes with peroxide during the measurement of chemiluminescence. This effect is particularly evident in the 3-mercaptopropionic acid unbound fraction. Residual adduct former is not a problem with the sample bound to the support as the wash step effectively dilutes the adduct-forming compound enough to give complete reversion.

DISCUSSION

The 9-phenylcarboxylate-10-methylacridinium ester studied here rapidly forms a reversible adduct with any of several different nucleophiles. Adduct formation is accompanied by a shift from the

acridinium ring structure to an acridan, which is accompanied by marked changes in the absorption spectra. In addition, adduct formation with compounds other than H_2O_2 prevents the chemiluminescent reaction by competing with H_2O_2 for the 9 position of the acridinium ring. These adduct-forming compounds vary greatly in their rates of addition and reversion. The more ionic compound bisulphite reacts relatively slowly, perhaps because of charge–charge repulsion with the phosphate backbone. More neutral adduct molecules such as 1-propanethiol are in rapid equilibrium. The adduct formed with mercaptosuccinic acid forms at an intermediate rate, approximately half as fast as 3-mercaptopropionic acid. This observation supports the theory of charge–charge repulsion. All of these adducts have been observed to be fully reversible upon dilution.

A similar adduct chemistry has been carefully studied in the reactions of pyridine nucleotides with cyanide and bisulphite (Fig. 8). The formation of nicotinamide adenine dinucleotide (NAD) adducts with dihydroxyacetone and other Michael reagents has also been demonstrated, but these adducts undergo an intramolecular cyclization reaction which is very different from the acridinium ester chemistry (Arnold *et al.*, 1979). The formation of cyanide adducts with NAD has been carefully studied (San Pietro, 1955; Burton and Kaplan, 1954) and has some striking similarities to the acridinium ester system described here. Because of the close similarities between these two systems, some inferences about the acridinium ester adduct chemistry can be made. Nucleophilic attack on the NAD ring occurs primarily at the N-4 position, and in the acridinium ester at the 9 position, in both cases *para* to the quaternary nitrogen. The unsubstituted nicotinic acid was used as a control in this reaction, and the quaternary ring nitrogen was found to be essential for the adduct formation. Formation of the adduct results in a loss of ring aromaticity and is accompanied by large changes in the absorption spectra of the NAD molecule. This nucleophilic addition is readily followed by the change in absorption spectra and is reversible by dilution. The log of the initial rate of formation of the NAD adduct with dihydroxyacetone displays a linear relationship to the reciprocal of the dielectric constant (Burton and Kaplan, 1954). This indicates that the reaction is ionic in nature, i.e. the positively charged quaternary nitrogen ring system interacts with the negatively charged nucleophile to form a neutral adduct. This has not been tested for this acridinium ester but similarities between the systems would indicate a like mechanism. The double bond in the ring systems reacts similarly to a carbonyl group; however, in the NAD system several carbonyl reactive compounds which were tested did not form an adduct (hydroxylamine, hydrazine, sulphydryl, phosphate) (Colowick *et al.*, 1951). It has been reported that an adduct may be forming with cysteine thiols at high protein concentrations (Racker and Krimsky, 1952).

Differences in rates of ester hydrolysis with different substituents have been well characterized, and variation over several orders of magnitude is possible (Bamford and Tipper, 1972). In general, strongly electron-withdrawing substituents will activate an ester. Replacement with an electron-donating group will deactivate the ester. In the case of AE, we are able to change the nature of the substituent through reversible adduct formation. This protection effect is not just chemically interesting: it also has practical applications. Using an acridinium ester for biological assays can require subjecting it to elevated temperature and pH. In order to be useful in DNA probe-based assays, a label must survive the reaction conditions. Using the acridinium ester as an adduct provides this stability.

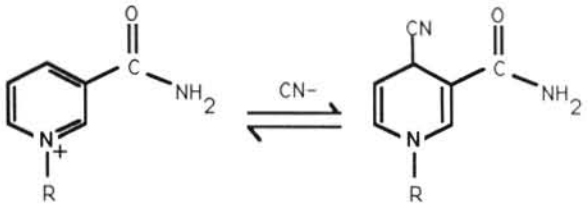

Figure 8. The reaction of pyridine nucleotide with cyanide is very similar to adduct formation with the acridinium ester.

Acknowledgement

We thank Gen-Probe Inc. and San Diego State University for allowing this work to comprise the body of Philip Hammond's Masters Degree project.

REFERENCES

Albert, A. (1966). *The Acridines*, Edward Arnold, London.

Arnold, L. J. Oppenheimer, N. J., Lee, C.-Y. and Kaplan, N. O. (1979). Proton nuclear magnetic resonance study of the

conformation and configuration of the cyclized pyridine nucleotides adducts. *Biochemistry*, **18**, 2787–2973.

Arnold, L. J., Hammond, P. W., Wiese, W. A. and Nelson, N. C. (1989). Assay formats involving acridinium-ester-labelled DNA probes. *Clin. Chem.*, **35**, 1588–1594.

Bamford, C. H. and Tipper C. F. H. (1972). Ester formation and hydrolysis and related reactions. In *Comprehensive Chemical Kinetics*. Bamford, C. H. and Tipper, C. F. H. (Eds), Elsevier, New York.

Burton, R. M. and Kaplan, N. O., (1954). The reaction of pyridine nucleotides with carbonyl compounds. *J. Biol. Chem.*, **206**, 283–297.

Colowick, S. P., Kaplan, N. O. and Ciotti, M. M. (1951). The reaction of pyridine nucleotide with cyanide and its analytical use. *J. Biol. Chem.*, **191**, 447–459.

McCapra, F., Richardson, D. G. and Chang, Y. C. (1965). Chemiluminescence involving peroxide decompositions. *Photochem. Photobio.*, **4**, 1111–1121.

Racker, E. and Krimsky, I. (1952). The mechanism of oxidation of aldehydes by glyceraldehyde-3-phosphate dehydrogenase. *J. Biol. Chem.*, **198**, 731–743.

San Pietro, A. (1955). On the structure of the diphosphopyridine nucleotide–cyanide complex. *J. Biol. Chem.*, **217**, 579–587.

Weeks, I., Beheshti, I., McCapra, F., Campbell, A. K. and Woodhead, J. S. (1983). Acridinium esters as high-specific-activity labels in immunoassay. *Clin. Chem.*, **29**, 1474–1479.