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FLUORESCENCE DETECTION OF CARBON-CENTERED RADICALS IN AQUEOUS SOLUTION

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A simple, highly sensitive method for the simultaneous determination of arrays of carbon-centered radicals in aqueous systems is described. Radicals are efficiently trapped by an amino-nitroxide to form stable products which are then reacted with fluorescamine to produce highly fluorescent adducts. The adducts are easily separated by reversed-phase high performance liquid chromatography. The detection limit for individual radical adducts (0.5 to 2 nM) is two to three orders of magnitude lower than those of current methods employing electron paramagnetic resonance detection. Results on the photolysis of ketones and α-keto acids demonstrate the potential of this technique. This approach should be widely applicable to the study of radical processes in biological and chemical systems.

KEY WORDS: Fluorescence, radicals, nitroxides, high performance liquid chromatography, aqueous photochemistry.

INTRODUCTION

Free radicals play a central role in a variety of chemical,[1,2] biological,[3] and toxicological processes[4]. Because of their high reactivity and ofttimes low rates of formation, steady-state concentrations of these species are usually quite low and are frequently difficult or impossible to detect directly by spectroscopic methods. As an alternative to direct detection, aliphatic aminoxyl radicals (nitroxides) have been employed increasingly to trap and identify transient free radicals.[5-7] Nitroxides react quite rapidly with carbon-centered radicals ($10^8 - 10^9 M^{-1}s^{-1}$), but not with most oxygen-centered radicals, to form stable, diamagnetic alkoxyamine and hydroxylamine products.[5-10] Separation of products by high performance liquid chromatography (HPLC) can allow for the identification of specific free radicals.[5-7] Formation rates of carbon-centered radicals have been estimated by following the loss of nitroxide with electron paramagnetic resonance (EPR) spectroscopy.[11,12]

The sensitivity achieved for the detection of radicals trapped by nitroxides can be substantially improved by employing compounds in which the nitroxide is covalently linked to a fluorophore. We and our colleagues have recently shown that the fluorescence yield of these nitroxide-fluorophores increases substantially upon conversion of the paramagnetic nitroxyl moiety to a diamagnetic form via radical (or redox) reactions.[13,14] The results indicate that this class of compounds should prove broadly applicable as highly sensitive optical probes for radicals.

Our interest in detecting low levels of photochemically generated radicals in aqueous systems prompted us to develop an alternative approach based on the formation

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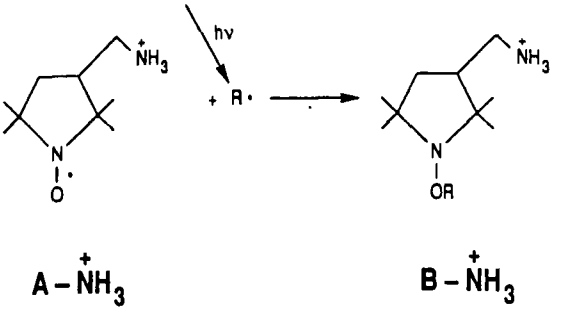
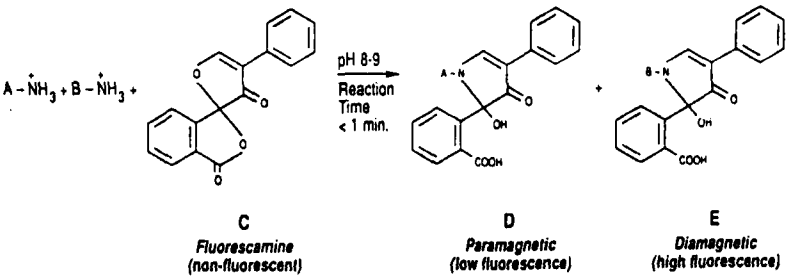
of fluorescent products through a post-irradiation coupling reaction. An amino-nitroxide (3-aminomethyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, 3-AMP) is employed, instead of a nitroxide-fluorophore, to trap photochemically generated radicals (R·):



(1)

$A-\overset{+}{N}H_3$ $B-\overset{+}{N}H_3$

Products and excess 3-AMP are then coupled to fluorescamine:[15]



(2)

C *Fluorescamine (non-fluorescent)* D *Paramagnetic (low fluorescence)* E *Diamagnetic (high fluorescence)*

The 3-AMP-fluorescamine adduct (D) exhibits low fluorescence owing to efficient intramolecular quenching by the nitroxide,[13] while the diamagnetic alkoxyamine- (or hydroxylamine-) fluorescamine adducts (E) are highly fluorescent. This approach is preferable to the direct use of nitroxide-fluorophores since: 1) the latter are only sparingly soluble in water, 2) reaction of highly reactive radicals with the fluorophore is prevented, and 3) photolysis of the nitroxide-fluorophore is precluded.

In this report, we demonstrate the potential of this approach through an examination of the aqueous photochemistry of α-keto acids[16,17] and ketones.[18]

MATERIALS AND METHODS

Chemicals

All reagents, of the highest purity available, were purchased from Aldrich and used as received. Solvents were distilled-in-glass grade from Burdick and Jackson. Stock solutions of fluorescamine (Sigma) in acetonitrile [0.08% (w/v)] were stored in the dark at approximately 20 °C. Stock solutions of 3-AMP (10 mM) in sodium borate buffer (pH 8.2, 0.2 M) were stored at −20 °C. Water used in all experiments was from a Millipore Milli Q system.

Irradiation Conditions

Samples in a 1 cm quartz cell were irradiated with a 300 W xenon lamp (Varian Model PS300-1). The light was first filtered through 22 cm of Milli Q water and then passed through a ≥ 275 nm pyrex filter. Unless noted, the light intensity at the cell (determined with a YSI Model 65A radiometer) was 160 mW/cm^2 with the 275 nm cutoff filter. Prior to irradiation, 100 μl of 0.2 M sodium borate buffer (pH 8.2) was added to 3 ml of standards or samples. 3-AMP was then added at concentrations ranging from sub-μM to mM. The concentration of the 3-AMP stock solution was determined as described below. After addition of 3-AMP, samples were deoxygenated for 5 min with N_2 (99.999% purity, Union Carbide) that had been passed through an oxygen trap (Alltech). The cell headspace was continuously purged with N_2 during sample irradiation.

Derivatization Procedure

A 200 μl aliquot of fluorescamine was added to 1 ml of the irradiated sample in an all-Teflon vial. The reaction is complete by 1 min[15] at room temperature. The pH of the reaction solution should be greater than eight for optimal results. The alkoxyamine adducts are quite stable at room temperature ($>$ 1 week), the hydroxylamine aduct is stable if deoxygenated conditions are maintained. Details regarding the optimization of the method will be presented elsewhere.

High Performance Liquid Chromatography

The HPLC consisted of an Eldex Model B-100-S single piston pump followed by a Gilson Model 811B dynamic mixer, a 0–5000 psi pressure gauge, a Valco Model C10W injection valve, and a RCM 8 $\times$ 10 cm Waters radial compression module containing a 0.5 $\times$ 10 cm Nova-PAK column with 4 μm reversed-phase (C_{18}) packing (Millipore); 0.5 μm filters were placed after the pump and injector. A Hitachi Model F1000 fluorescence detector (Tokyo) set at 390 nm (excitation) and 480 nm (emission), was connected to an ELAB PC-based data collection system (OMS). A 0.2 $\times$ 5.5 cm ID enrichment column (Upchurch) with 40 μm C_{18} packing was used in place of a sample loop. Samples were loaded into the enrichment column, then backflushed to inject the sample. The mobile phase composition was controlled by an ELAB low pressure gradient system (OMS). Chromatographic separations were at room temperature and at a flowrate of 1 ml/min. For isocratic separations, the mobile phase was 35% sodium acetate (50 mM, pH 4.0)/ 65% methanol. The precision was 4.2% RSD (n = 8) at 500 nM.

Electron Paramagnetic Resonance

A Bruker/IBM ER 200D EPR spectrometer was employed to obtain spin concentration. Samples were drawn into 50 μl calibrated capillaries, which were then sealed at the top and bottom and placed within standard 3 mm quartz EPR tubes. Concentrations of 3-AMP were determined by comparison of areas obtained by double integration of the EPR signals with a standard curve of area versus concentration determined for 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy. Standard instrument settings

were: 9.77 GHz; power, 10 mW; modulation amplitude, 1.6 G. Nitroxide loss was calculated from the decrease in peak amplitudes, since linewidths were identical for all samples.

RESULTS AND DISCUSSION

In the near-ultraviolet, Norrish type I photolysis of α-keto acids[16,17] and ketones[18,19] (where the chainlength of $R_1 > R_2$) proceeds through the reaction,

$$R_1C(O)R_2 + h\upsilon \rightarrow R_1\cdot + \cdot C(O)R_2 \quad (3)$$

to produce the primary radicals shown in Table 1. These radicals are trapped to form alkoxyamines, except for CO_2^-, which is expected to reduce 3-AMP to the hydroxylamine.[20,21]

Chromatograms depicting the separation of different radicals as their alkoxyamine- or hydroxylamine-fluorescamine (AF or HF) adducts are shown in Figure 1. Equal concentrations of two products were detected for each ketone or α-keto acid (*vide infra*) in agreement with a Norrish type I cleavage. Adducts were assigned through comparison of the retention times of products formed from different precursors (Table 1). For example, the ethyl radical (peak 7) is formed when solutions of 2-butanone or 3-pentanone are irradiated; acetone, pyruvate, and 2-butanone all show the production of $CH_3\dot{C}O$ (peak 2). Photochemical decomposition of 3-AMP to form fluorescent products was not observed (Figure 1, panels A and F). No AF or HF adducts were produced during the irradiation of aerated samples containing 4 μM 3-AMP, consistent with an efficient scavenging of the primary radicals by oxygen to

TABLE 1

List of primary radicals (R·) expected from the Norrish type I photochemical cleavage of α-keto acids (at pH > pK_a) and ketones in aqueous solution. The CO_2^- radical reduces 3-AMP to the hydroxylamine (H). Also given are relative response factors which relate the loss of 3-AMP as determined by EPR to the integrated fluorescence area of adducts as determined by HPLC (Figure 2). For all adducts, r^2 was ≥0.997 ($n \geq 10$) over the range tested (0.2–3.5 μM); the *y*-intercept ± 95% CI (CI = confidence interval) was not significantly different from zero. A high concentration (310 μM) of α-keto acids and ketones was needed to obtain losses of 3-AMP measurable by EPR. 3-AMP-fluorescamine was titrated with a deaerated aqueous solution of dithionite to form the hydroxylamine (H). Dithionite reduction gave the same product (by HPLC) as ascorbate reduction, which is known to produce the hydroxylamine.[13]

Precursor	R·	Relative Response Factor Slope ± 95%CI (μM/area)	Precursor	R·	Relative Response Factor Slope ± 95%CI (μM/area)
Pyruvate	1) CO_2^- (H)	1.15 ± 0.04	acetone	2) $CH_3\dot{C}O$	0.55 ± 0.03
	2) $CH_3\dot{C}O$	0.60 ± 0.04		6) $\dot{C}H_3$	0.62 ± 0.03
α-ketobutyrate	1) CO_2^- (H)	-	2-butanone	2) $CH_3\dot{C}O$	0.54 ± 0.02
	3) $CH_3CH_2\dot{C}O$	-		7) $CH_3\dot{C}H_2$	0.67 ± 0.02
α-ketovalerate	1) CO_2^- (H)	1.12 ± 0.11	3-pentanone	3) $CH_3CH_2\dot{C}O$	-
	4) $CH_3(CH_2)_2\dot{C}O$	0.56 ± 0.02		7) $CH_3\dot{C}H_2$	-
α-ketocaproate	1) CO_2^- (H)	-	benzoylformate	1) CO_2^- (H)	1.05 ± 0.23
	5) $CH_3(CH_2)_3\dot{C}O$	-		8) $C_6H_5\dot{C}O$	0.54 ± 0.05
3-AMP-fluorescamine	1) H	1.13 ± 0.04			

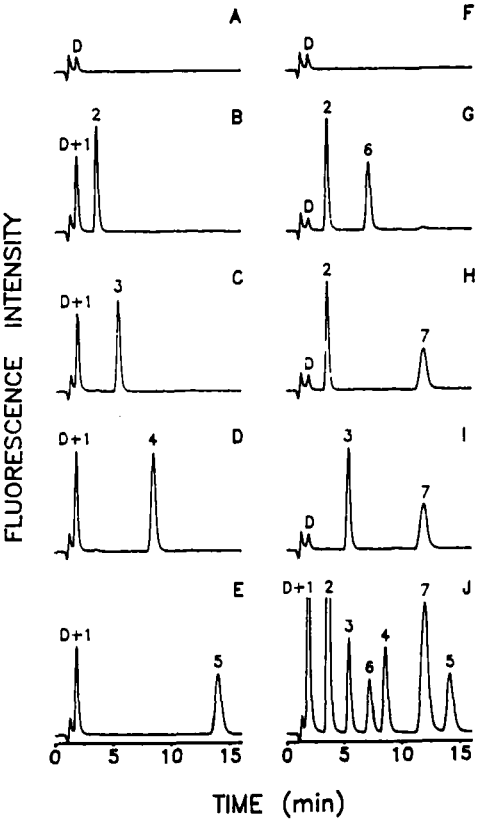


FIGURE 1 Chromatograms showing the separation of the AF or HF adducts of free radicals produced through ketone or α-keto acid photolysis and trapped by 3-AMP. The initial 3-AMP concentration was 7.2 μl. Injection volume was 100 μl. Concentrations of radicals trapped ranged from 100 to 200 nM. Panel A depicts the dark control for the 3-AMP blank (no added ketone or α-keto acid). Panels B-J correspond to samples irradiated for 10.0 min: B) 18 μM pyruvate, C) 18 μM α-ketobutyrate, D) 18 μM α-ketovalerate, E) 18 μM α-ketocaproate, F) 3-AMP alone, G) 60 μM acetone, H) 6 μM 2-butanone, I) 18 μM 3-pentanone, and J) solution of acetone (60 μM), 2-butanone (6 μM), 3-pentanone (18 μM), α-ketovalerate (18 μM), and α-ketocaproate (18 μM). Peak D refers to the paramagnetic 3-AMP-fluorescamine adduct; for all other peaks, refer to Table 1 for indentification of radicals trapped.

form oxygen-centered radicals that are unreactive with 3-AMP. Dark controls (deaerated or aerated with added 3-AMP) and deaerated light controls (no added 3-AMP) showed no production of fluorescent products. Irradiation of ketones at wavelengths longer than their ultraviolet absorption band generated no fluorescent adducts.

The concentration of specific adducts was calibrated by comparing the loss of 3-AMP as monitored by EPR with the integrated fluorescence area of the adducts separated by HPLC. Initially, we assumed that the two products obtained from the photolysis of each ketone or α-keto acid were formed stoichiometrically. Based on this assumption, response factors, defined as the slope of a plot of 3-AMP spin loss versus integrated fluorescence area (Figure 2), are presented in Table 1. That this assumption was indeed correct was verified independently by measuring the response factor for the HF adduct through the reduction of 3-AMP-fluorescamine by dithionite; this

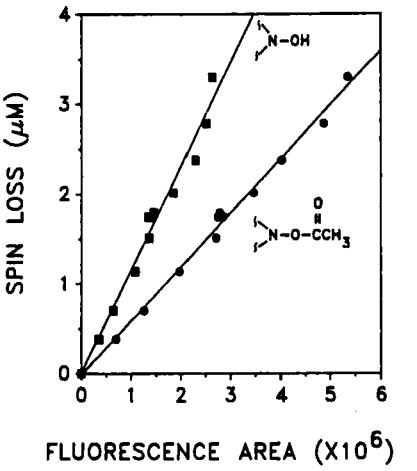


FIGURE 2 Relationship between 3-AMP spin loss as determined by EPR and the integrated fluorescence area of the HF and acyl adducts separated by HPLC. The initial 3-AMP and pyruvate concentrations were 7.3 and 310 μM respectively. Differing levels of 3-AMP loss and product formation were obtained by varying the irradiation time from 0 to 25 min; EPR and HPLC analyses were performed concurrently on identical samples. Response factors obtained from the slopes of the plots are given in Table 1 for the HF and acyl adduct as well as for the adducts of other carbon-centered radicals studied here.

factor was indistinguishable from those of HF adducts formed through photolysis of α-keto acids (Table 1). All AF adducts showed similar response factors (Table 1), suggesting that adduct concentrations of most carbon-centered radicals can be obtained through use of only one or two standards. Based on the analysis of dilute solutions of the hydroxylamine and alkoxyamines, the current detection limit ranges from approximately 0.5 to 2 nM per analyte (5 ml injection) with a signal to noise ratio of four relative to a 7.2 μM 3-AMP fluorescamine blank. The current detection limit is two to three orders of magnitude lower than that of radical trapping methods employing EPR detection[11,22].

To further test the method, we examined the dependence of adduct yields on the concentration of 3-AMP. Results presented in Figure 3A for pyruvate and 3B for acetone show three distinct domains. At low 3-AMP concentrations, adduct yields are reduced owing either to complete consumption of 3-AMP by the primary radicals (Figure 3B), or to incomplete scavenging of the primary radicals (Figure 3A) resulting from competitive radical recombination or disproportionation reactions. At intermediate 3-AMP concentrations, adduct yields reach a maximum and are independent of 3-AMP, indicative of quantitative scavenging of the primary radicals. At high 3-AMP concentrations, adduct yields decrease with increasing 3-AMP. This result cannot be due to light attenuation by 3-AMP ($<$ 5% at 1 mM 3-AMP). The decrease yields are attributed to physical quenching by 3-AMP of the triplet excited state giving rise to the primary radicals. This quenching must be due to the nitroxyl moiety,[23] since at the pH of these experiments, the amine moiety of 3-AMP is protonated and unlikely to participate in quenching (via electron transfer[24,25]). Concentrations necessary for quenching are much higher for pyruvate than for acetone (Figure 3), consistent with the shorter lifetime of triplet pyruvate in water ($\sim$ 0.5 μs[26]) as compared with acetone (5.0 μs[27]). From the slope of a Stern-Volmer plot[18] (Figure 3) and the lifetime

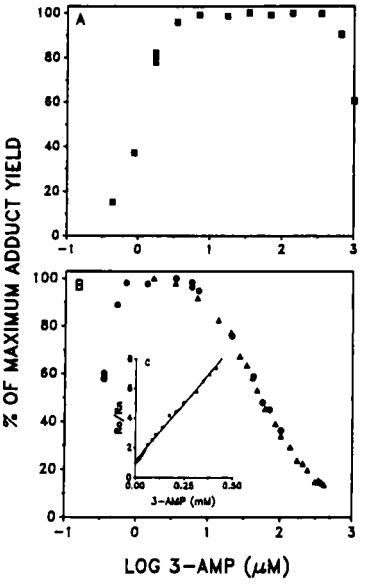


FIGURE 3 The dependence of total adduct yield (normalized to the maximum) on the initial 3-AMP concentration for photolysis of: A) pyruvate (■) and B) acetone (● 3/8/89, ▲ 3/15/89). The initial pyruvate or acetone concentration was 330 μM; the fluram solution was 0.16% (w/v) in acetonitrile. Irradiations were for 5.0 min; other conditions are in text. The maximum yield of total adducts (Table 1) was 0.56 μM for acetone and 1.05 μM for pyruvate. At low concentrations of 3-AMP, the total adduct yield is a substantial percentage of the initial 3-AMP concentration; therefore, in this range, an assumption of initial rates is not valid. Inset C: Stern-Volmer plot for the quenching of triplet acetone by 3-AMP. R_0 is the average initial rate of $CH_3\dot{C}O$ adduct formation in the absence of quenching, while R_q is the initial rate of formation in the region of quenching. The regression line shown is the best fit of the acetone data (r^2 = 0.994; y-intercept ± SE = 1.08 ± 0.15; slope ± SE = 1.57 ± 0.03 × $10^4 M^{-1}$).

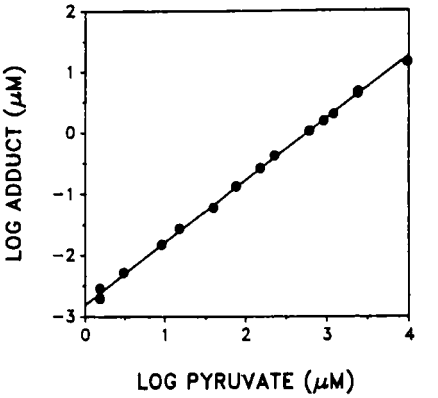


FIGURE 4 Dependence of acyl adduct formation on the pyruvate concentration; r^2 = 0.999; y-intercept ± SE = −2.81 ± 0.05; slope ± SE = 1.02 ± 0.01. The initial concentration of 3-AMP was 10.1 μM, except for the two highest pyruvate concentrations where 3-AMP was increased to 110 μM. Irradiations were for 5.0 min and employed a ≥ 315 nm pyrex filter (intensity = 145 mW/cm²). All other conditions are given in the text.

of triplet acetone,[27] the rate constant for 3-AMP quenching was calculated as $3.1 \times 10^9\ M^{-1}s^{-1}$, which is comparable to values reported for nitroxide quenching of aromatic ketone triplets.[23] Based on two points (Figure 3A), the constant for pyruvate is crudely estimated as $2\text{–}3 \times 10^9\ M^{-1}s^{-1}$. Thus, information on the lifetimes of excited states producing radicals can also be obtained by this method.

Davidson and co-workers[26] suggested recently that pyruvic acid photolysis in aqueous solution occurs through a bimolecular electron transfer reaction between triplet and ground state pyruvic acid. Because of the wide dynamic range of the method, we were able to critically test this possibility for the pyruvate anion (pH 8.2) by measuring the acyl adduct yield as a function of the initial pyruvate concentration. This yield varied linearly with pyruvate concentration over four orders of magnitude (Figure 4). This result is consistent with a unimolecular scission[16,17] of the triplet pyruvate anion and is inconsistent with a bimolecular electron transfer reaction[26].

This method is simpler and far more sensitive than current techniques[11,12,16,17,22] for the detection and identification of carbon-centered radicals in aqueous solution. In future work, microbore HPLC combined with mass spectrometry (LC/MS) should provide a means for positive identification of unknowns.

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