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Mechanistic Studies Relevant to Bromouridine-Enhanced Nucleoprotein Photocrosslinking: Possible Involvement of an Excited Tyrosine Residue of the Protein

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ABSTRACT

The results of mechanistic studies on formation of uridine (U) and *N*-acetyl-*m*-(5-uridinyl)tyrosine *N*-ethylamide (2) from irradiation of aqueous, pH 7 solutions of bromouridine (BrU) and *N*-acetyltyrosine *N*-ethylamide (1) are reported. Solutions were irradiated with monochromatic laser emission at 266, 308 and 325 nm. Quantum yield measurements as a function of excitation wavelength suggest that both products result from excitation of the tyrosine derivative followed by electron transfer to BrU, possibly with intermediacy of the hydrated electron. The BrU radical anion ejects bromide to form the uridinyl radical, which then abstracts a hydrogen atom from 1 or adds to the aromatic ring of 1. Formation of adduct 2 is a model for photocrosslinking of nucleic acids bearing the bromouracil chromophore to adjacent tyrosine residues of proteins in nucleoprotein complexes. The value of 325 nm excitation in photocrosslinking, where the tyrosine chromophore is more competitive for photons, was demonstrated with an RNA bound to the MS2 bacteriophage coat protein; more than a 60% increase in the yield of photocrosslinking relative to that obtained with 308 nm excitation was achieved.

INTRODUCTION

Over a period of more than 20 years the 5-bromouracil chromophore has been utilized for photocrosslinking of double-stranded DNA, RNA and single-stranded DNA to associated proteins to establish points of proximity. Studies with small molecule models and with nucleoprotein complexes implicate, primarily, aromatic amino acid residues in the crosslinking. Small molecule model reactions have demonstrated coupling to tryptophan (1,2), tyrosine (2), histidine (2) and cystine (3). Nucleoprotein complexes in which the sites of crosslinking have been established include histidine and tyrosine residues of *lac* repressor protein to operator DNA (4), an alanine residue of GCN4 yeast transcriptional activator to a DNA binding site (5), a tyrosine residue of bacteriophage R17 coat protein to an RNA binding site (6), histidine and tyrosine residues of *Oxytricha* nova telomere binding protein to single-stranded, telomeric DNA (7), histidine and tyrosine residues of the Rel protein NF-κB to a DNA binding site (8), a tyrosine residue of the U1A protein to its RNA binding domain (9) and a phenylalanine residue of the Tyr-Phe mutant of the U1A protein to the same RNA. Although crosslinking to an alanine residue has been observed, the yield was very low.

Many of the mechanistic studies of the 5-bromouracil chromophore have utilized the simple photoreduction to uracil in reducing solvents to define the primary photochemical processes. With excitation at 254 nm, the quantum yield of photoreduction shows significant dependence on reducing solvent concentration in aqueous medium, especially at high reducing solvent concentration (10). The result has been interpreted in terms of a primary carbon–bromine bond homolysis followed by the solvent trapping of the radical pair in competition with radical recombination. The results of deuterium incorporation experiments in variously deuterium-labeled 2-propanol solvent suggested a higher level of complexity (11). Irradiation of 5-bromouracil at 254 nm in 2-deuterio-2-propanol solvent gave uracil with 70% deuterium at the 5-position and in 2-propanol-*d* solvent, 30% deuterium incorporation at the 5-position. Monochromatic irradiation at 308 nm with a XeCl laser in 2-propanol-*d* solvent gave 80% deuterium incorporation (12). Further, deuterium incorporation in 2-propanol-*d* solvent was quenched by *cis*-piperylene and sensitized by acetone. These results have been interpreted in terms of three excited states, two singlet states (a lower energy $^1n,\pi^*$ state and a higher energy $^1\pi,\pi^*$ state) and one triplet state. The $^1\pi,\pi^*$ state undergoes C–Br bond homolysis to form a radical pair and the uracilyl radical abstracts a hydrogen from the 2-position of 2-propanol. The $^1\pi,\pi^*$ state also intersystem crosses to the triplet state, which reacts with 2-propanol by electron transfer. The $^1n,\pi^*$ state does not undergo bond homolysis but intersystem crosses to the triplet state. These processes leading to photoreduction are summarized in Scheme 1. Photoreduction in

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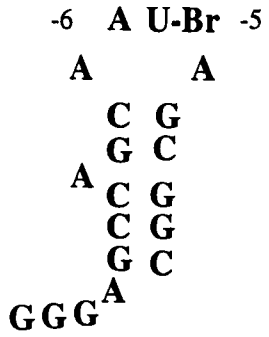
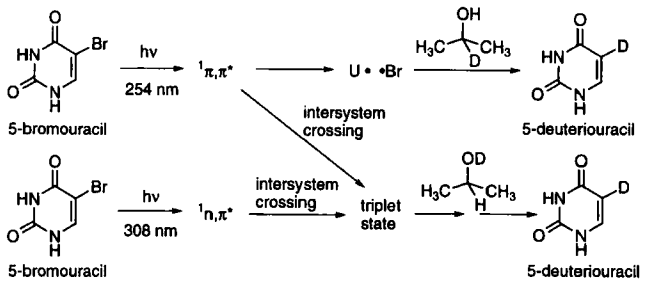


RNA 1

Figure 1. Sequence and secondary structure of an RNA hairpin bearing a BrU at the −5 position that binds to the bacteriophage MS2 and R17 coat proteins. The numbering system relates to the start codon for replicase synthesis in the wild-type sequence. The dissociation constant for the nucleoprotein complex is 3.4 n*M* as determined by a nitrocellulose filter binding assay.

aqueous 2-propanol, especially when sensitized by acetone at high pH, occurs predominantly by a chain mechanism with electron transfer from the 2-hydroxyl-2-propanyl radical or its anion to bromouracil as the chain carrying step (13).



Scheme 1

The coupling of 5-bromouracil to tryptophan, tyrosine, histidine and cystine derivatives is facilitated by 308 nm excitation and has been proposed to occur *via* electron transfer reactivity in the triplet state (1,2). Further, nucleoprotein photocrosslinking with the 5-bromouracil chromophore also appears to be facilitated by irradiation at wavelengths longer than 300 nm, again implicating the bromouracil triplet state (14,15).

The most extensively studied nucleoprotein complex with respect to bromouracil-enhanced photocrosslinking is the R17 bacteriophage coat protein bound to variants of an RNA stem loop structure within the phage genome. Photocrosslinking, illustrated with RNA 1 (Fig. 1), occurs between the −5 position of the RNA and Tyr85 of the coat protein (6,13). With 308 nm excitation of the nucleoprotein complex, the maximum yield of photocrosslinking was 40% based upon RNA. Control experiments suggested that crosslinking stopped at 40% because of photochemical damage to protein leading to dissociation from the RNA. Protein damage was presumed to result from excitation of tryptophan residues. This observation prompted a careful investigation of photoreactivity of the bromouracil chromophore as a function of excitation wavelength with the goal of establishing a reactive wavelength at which tryptophan would be transparent or would absorb minimally. The results of a study of the photoreaction of bromouridine (BrU)† with *N*-acetyltyrosine *N*-ethylamide (**1**) as a function of excitation wavelength and application to photocrosslinking of the R17 nucleoprotein complex are described here.

MATERIALS AND METHODS

Materials. 5-Bromouridine (Sigma Chemical Co., St. Louis, MO), potassium phosphate (Aldrich Chemical Co., Milwaukee, WI) and HPLC-grade acetonitrile (Burdick and Jackson, Muskegon, MI) were used as received. Distilled water was further purified to 18 MOhm cm with a Millipore Q water purification system. *N*-acetyltyrosine *N*-ethylamide and *N*-acetyltryptophan *N*-ethylamide were synthesized from *N*-acetyltyrosine ethyl ester and *N*-acetyltryptophan ethyl ester, respectively, as previously described (2) and recrystallized in ethyl acetate before use. A sample of the photoproduct, *N*-acetyl-*m*-(5-uridinyl)tyrosine *N*-ethylamide, for calibration of the HPLC was prepared by irradiating a mixture of 5-iodouridine (IU) and *N*-acetyltyrosine *N*-ethylamide as described elsewhere (16).

Quantum yield measurements. All quantum yield measurements were carried out using laser light. Light at 266 nm was obtained from a frequency-quadrupled pulsed Nd:YAG laser (Spectra Physics DCR, 10 Hz repetition rate, 4–5 ns/pulse), light at 308 nm from a pulsed XeCl excimer laser (MPB PSX-100, 10–100 Hz repetition rate, 2.5 ns/pulse) and light at 325 nm from a continuous-wave HeCd laser (Omnichrome Series 74, 35–39 mW). For the experiments using pulsed lasers, power per pulse was kept at or below 1 mJ at 10 Hz.

Actinometry was performed using a Scientech 362 power meter, and absorbances were calculated before and after irradiation of samples using either of two Hewlett-Packard UV–visible spectrometers (model 8450 or 8452). The HPLC analyses were performed with a Hewlett-Packard model 1090 HPLC equipped with a diode array detector and a Hewlett-Packard 5 μm C-18 reverse-phase microbore column (2.1 mm × 10 cm) to determine product formation as well as BrU depletion following irradiation. Detection was typically at 280 nm with a 60 nm bandwidth. Mixtures of methanol and 0.001 *M* pH 6.8 phosphate buffer were used as eluents. To determine the number of photons absorbed during an irradiation, the absorbance of BrU was assumed to decay linearly as a function of time, after the absorbance contribution of photoproducts was subtracted. The BrU absorbed power *versus* time curve was then trapezoid-rule integrated to determine the number of photons that BrU absorbed. A similar analysis was carried out to determine the photons absorbed by *N*-acetyltyrosine *N*-ethylamide, except that depletion of this more concentrated compound was never observed by HPLC, and thus its absorbance was assumed constant during all irradiations. An error of 15%, propagated between possible power meter miscalibration and systematic errors arising from the actinometry calculation assumptions, was estimated for the actinometry measurement.

Synthesis and irradiations of RNA 1–MS2 coat protein complex. The RNA 1 was prepared by *in vitro* transcription from synthetic DNA templates by T7 RNA polymerase with [α-^{32}P]ATP and BrUTP substituted for UTP (17). Bromouridine triphosphate was obtained from Sigma Chemical Company. The RNA 1 was renatured by heating to 97°C in water for 5 min and quickly cooling on ice to assure the proper hairpin conformation for binding (18). Prior to irradiation, a 250 μL solution containing 5 n*M* RNA, 200 n*M* MS2 coat protein as a nonaggregating mutant (19), 5 m*M* dithiothreitol, 100 m*M* Tris-HCl (pH 7.5 at 4°C), 80 m*M* KCl, 10 m*M* magnesium acetate and bovine serum albumin (100 μg/mL) was incubated on ice for 30 min. The solution was irradiated at 4°C in a 4 mm wide by 1 cm methacrylate cuvette with an Omnichrome helium cadmium laser emitting 35 mW at 325 nm. The laser beam diameter was 3 mm. Samples were removed after 2, 4, 6, 8, 10 and 12 h, heated to 97°C for 5 min in 80% formamide/10 m*M* EDTA (pH 8.0)/1 mg/mL bromophenol blue/1 mg/mL xylene cyanol and electrophoresed at 400 V through a 20% urea, polyacrylamide, denaturing gel in 89

†*Abbreviations:* BrdU, bromodeoxyuridine; BrU, 5-bromouridine; IU, 5-iodouridine; T, thymidine; U, uridine.

Table 1. Apparent quantum yields calculated with the assumption that photoreaction results from excitation of the respective halouracil*

Entry	XU	Quencher or scavenger	λ (nm)	Apparent quantum yields Φ_{-BrU}	Φ_U	Φ_{XL}
1	BrU	None	266	0.0006 ± 0.0012	0.00011 ± 0.00003	
2	BrU	None	320	0.005 ± 0.005	0 ± 0.0002	
3	BrU	Tyr **1**, 8 m*M*	266	0.078 ± 0.015	0.016 ± 0.003	0.030 ± 0.004
4	BrU	Tyr **1**, 8 m*M*	308	0.0027 ± 0.0007	0.00046 ± 0.00010	0.0013 ± 0.0003
5	BrU	Tyr **1**, 8 m*M*	325	0.15 ± 0.05	0.043 ± 0.010	0.080 ± 0.015
6	BrU	Tyr **1**, 8 m*M* + CH_3CN, 1 *M*	308	0.003 ± 0.003	0.0020 ± 0.0005	0.0017 ± 0.0003
7	IU	Tyr **1**, 8 m*M*	325	0.015 ± 0.003	0.0042 ± 0.0006	0.0081 ± 0.0012
8	IU	Tyr **1**, 8 m*M* + CH_3CN, 1 *M*	325	0.011 ± 0.004	0.0080 ± 0.0012	0.0062 ± 0.0010

*The reactions were conducted with 0.8 m*M* halouridine in 50 m*M* pH 7 phosphate buffer.

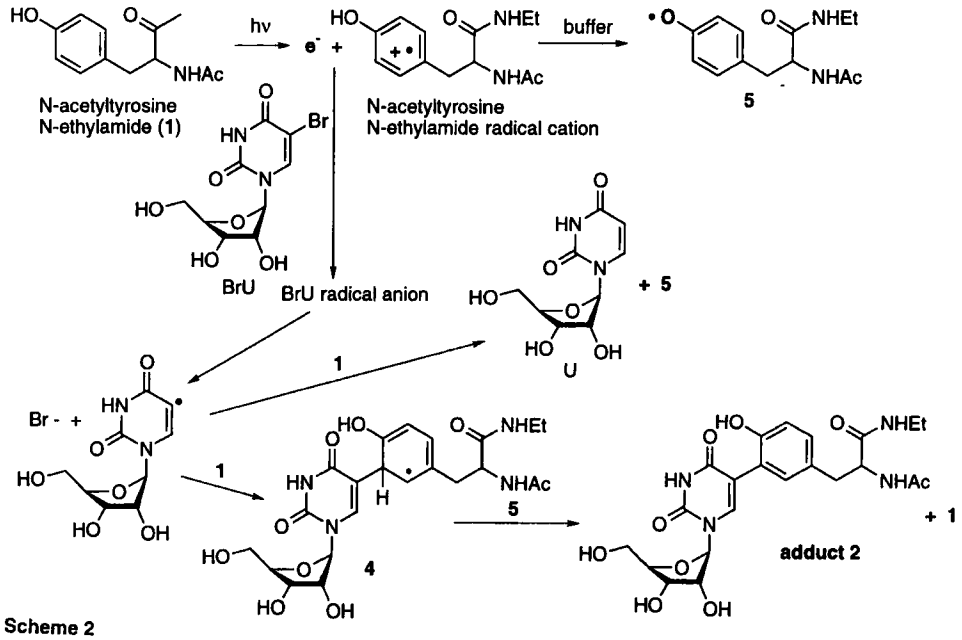
m*M* Tris-borate/2 m*M* EDTA (pH 8.0). The percent crosslinking was quantitated from a phosphor imager scan.

RESULTS AND DISCUSSION

Photoreactions of BrU with a tyrosine derivative

Irradiation of BrU in buffered aqueous medium (pH 7) in the presence of *N*-acetyltyrosine *N*-ethylamide (**1**) yielded two major products, uridine (U) and *N*-acetyl-*m*-(5-uridinyl)tyrosine *N*-ethylamide (**2**), as illustrated in Scheme 2. Adduct **2** was isolated and characterized earlier from spectroscopic data as the product from irradiation of IU in the presence of **1** (16). The HPLC comparison using a diode array detector and coinjection established identity of adduct **2** from the two sources. Earlier work established the structure for the analogous adduct from irradiation of a pH 7 solution of the nucleic acid base, 5-bromouracil, and **1** (2).

Photoreduction of BrU and formation of adduct **2** occur upon excitation at 266 nm with frequency-quadrupled emission from the Nd:YAG laser, at 308 nm with emission from a XeCl excimer laser, and at 325 nm with emission from a HeCd laser. The quantum yields of destruction of BrU and formation of U and adduct **2**, based upon absorption by BrU, appeared to be wavelength dependent with the highest quantum yields from excitation at 325 nm and the lowest at 308 nm. However, repetition of the measurements did not show reproducibility. Two factors contributed to the lack of reproducibility. The overall solution absorbance continued to increase after the irradiation was stopped; although, no similar increase in products was observed. The absorbance rise possibly resulted from bromine or bromide chemistry; the effect was minimized in subsequent experiments by prompt measurement of the absorbance at termination. The second factor was an increase in the quantum yields with increasing BrU conversion, suggesting that the reaction was accelerating as conversion increased. To test for one of the products possibly serving as catalyst, the quantum yields were measured with an initial presence of either uridine (0.04 m*M*), adduct **2** (0.064 m*M*) or sodium bromide (3.8 m*M*). Neither of these reagents increases the quantum yields for photoreduction or adduct formation, and the phenomenon was not explored



Scheme 2

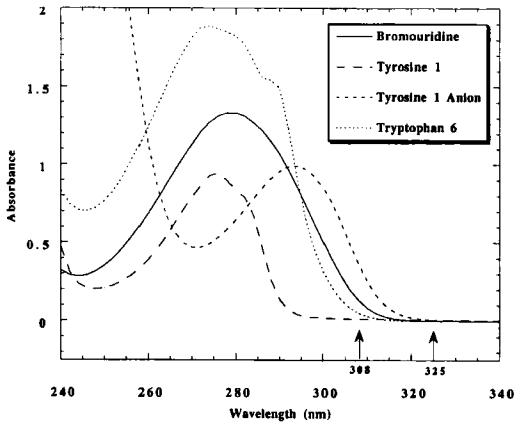


Figure 2. Ultraviolet absorption spectra of 0.18 m*M* BrU (———), 0.73 m*M* tyrosine derivative **1** (— —) and 0.32 m*M* tryptophan derivative **6** (·····) in 50 m*M* pH 7 phosphate buffer and 0.44 m*M* anion of **1** (– – –) in pH 12 aqueous sodium hydroxide.

Table 2. Molar extinction coefficients for BrU, Tyr **1** and the anion of **1** as a function of wavelength and the calculated percent light absorption by each species in a pH 7 phosphate buffer solution containing 0.8 m*M* BrU and 8 m*M* tyrosine derivative*

Reactant	266 nm (% abs) l/mol cm	308 nm (% abs) l/mol cm	325 nm (% abs) l/mol cm
BrU	5390 (39)	747 (96)	3.7 (27)
Tyr **1**	853 (61)	2.1† (3)	1.0 (72)
Anion of Tyr **1**	1280 (0.1)	825 (1)	10.7 (1)
Trp **6**		153	3.4

*The molar extinction coefficients for Trp **6** are also listed. The extinction coefficients for BrU, **1** and **6** were measured in 50 m*M* pH 7 phosphate buffer and for anion of **1** in pH 12 sodium hydroxide. The pK_a of tyrosine derivative **1** was assumed to be 10.

†The measured extinction coefficient was corrected for a contribution from the anion of Tyr **1** with the assumption that the anion is present at about 0.1% at pH 7.

further because it did not appear to be relevant to nucleoprotein photocrosslinking.

To determine if the wavelength dependence on the efficiency of crosslinking were real, quantum yields extrapolated to zero irradiation time were now needed. To accomplish this, small aliquots of the reaction mixture were taken as a function of irradiation time and were analyzed by HPLC. The increase in uridine and adduct **2**, as well as the decrease in BrU starting material *versus* time were each found to fit well to a simple quadratic equation, with the curvature term in each fit representing the degree of acceleration of the reaction. The derivative of each quadratic equation was computed at time t = 0, and this value was used in calculating the quantum yield under starting conditions. Alternatively, the same quantum yields were obtained without the need to extrapolate to t = 0, if BrU destruction was carried to not more than 3–4%.

Reproducible quantum yields of destruction of BrU and formation of U and adduct **2** were measured at pH 7 as a function of reaction conditions and excitation wavelength. Values based upon absorption by BrU are reported in Table 1. Entries 3–5 show the dramatic effect of quantum yield on excitation wavelength with a maximum apparent quantum yield at 325 nm. In fact, the apparent quantum yield of destruction of BrU is more than 50 times higher upon excitation at 325 nm than at 308 where BrU absorbs 96% of the absorbed light. Earlier mechanistic studies demonstrated a wavelength dependency for photoreduction of bromouracil in the region 254–308 nm as mentioned earlier and summarized in Scheme 1. The trend suggested that the quantum yield for reaction with **1** on excitation at 325 nm should be similar to or possibly a little higher than at 308 nm, contrary to the observation.

At all three wavelengths, three species present at time zero actually compete for absorption of photons: BrU, tyrosine derivative **1** and the anion of **1**. The UV absorption spectra of the three species are compared in Fig. 2, and the molar extinction coefficients at 266, 308 and 325 nm are reported in Table 2 as well as the percent light absorption by each based upon the extinction coefficients and respective concentrations. The percent anion of **1** present at pH 7 was assumed to be 0.1% of **1** based upon an estimated pK_a of 10.

Recalculation of the quantum yields based upon excitation of tyrosine **1** and the anion of **1** are reported in Table 3. Entries 3–5 now show little or no dependence upon the excitation wavelength. This suggests that excited **1** and its anion are responsible for reduction to uridine and for formation of adduct **2**.

The role of excited tyrosine derivative in the reduction of BrU to U is also indicated by the relative photostability of BrU in the absence of **1** with excitation above or below 300 nm (Table 1, entries 1 and 2). In principle, U could have been formed in either the presence or absence of **1** *via* excited BrU undergoing C–Br bond homolysis followed by the resulting uridinyl radical abstracting a hydrogen atom from BrU or from **1**, respectively. Relative photostability of BrU in aqueous medium has also been recognized by others (10,11).

A primary photochemical reaction of tyrosine and its derivatives and the respective anions in aqueous medium is electron ejection to form a solvated electron (20). In fact, electron ejection appears to be a general photoreaction of the phenol chromophore. The quantum yield is dependent upon the state of protonation and incident light intensity. At lower incident light intensity the efficiency of electron ejection from phenol is reported to be 0.03 and from phenolyate, 0.2 (21). At high light intensity, achieved with a pulsed laser, the quantum yields for both species are significantly higher (21).

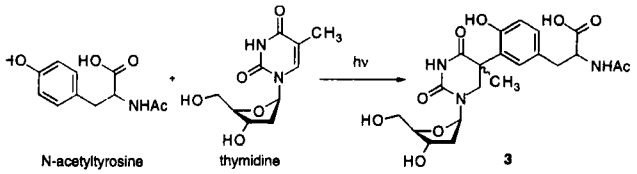
Shetlar and coworkers have characterized the adduct from reaction of *N*-acetyltyrosine with thymidine (T) as *N*-acetyl-*m*-(5-[5,6-dihydrothymidinyl])tyrosine (**3**), a structure analogous to adduct **2** from reaction of tyrosine derivative **1** with BrU (22). They propose excitation of *N*-acetyltyrosine, electron ejection captured by T followed by radical coupling for the reaction mechanism. This reaction is probably the basis of photocrosslinking of wild-type nucleoprotein complexes. *Oxytricha* nova telomeric DNA (T_4G_4) bound to telomere binding protein appears to be an example. The T at the 15-position, numbering from the 3′ end, has been shown to

Table 3. Apparent quantum yields calculated with the assumption that photoreaction results from excitation of *N*-acetyltyrosine *N*-ethylamide (**1**) and its anion*

Entry	XU	Quencher or scavenger	λ (nm)	Apparent quantum yields		
				Φ_{-BrU}	Φ_U	Φ_{XL}
3†	BrU	Tyr **1**, 8 m*M*	266	0.046 ± 0.009	0.010 ± 0.002	0.018 ± 0.003
4	BrU	Tyr **1**, 8 m*M*	308	0.06 ± 0.01	0.011 ± 0.003	0.033 ± 0.008
5	BrU	Tyr **1**, 8 m*M*	325	0.06 ± 0.02	0.018 ± 0.004	0.033 ± 0.007
6	BrU	Tyr **1**, 8 m*M* + CH_3CN, 1 *M*	308	0.06 ± 0.04	0.034 ± 0.009	0.028 ± 0.004

*The reactions were conducted with 0.8 m*M* BrU in 50 m*M* pH 7 phosphate buffer.
†Entry numbers relate to the entry numbers in Table 1.

crosslink to Tyr142 of the α-chain of the telomere binding protein (7). Of relevance to the discussion here, the crosslinking yield is significantly enhanced by bromodeoxyuridine (BrdU) substitution for T at this position.



A mechanism consistent with these observations, shown in Scheme 2, is excitation of **1** and its anion followed by electron ejection to form the hydrated electron, which then reduces BrU to its radical anion. The radical anion then ejects bromide rapidly to form the 5-uridinyl radical. Evidence for rapid elimination of bromide comes from pulsed radiolysis experiments on reduction of BrU (23). The resulting uridinyl radical then abstracts a hydrogen atom from **1** to form U or adds to the aromatic ring of **1** to form transient radical **4**. Radical **4** likely delivers a hydrogen atom to the tyrosine phenoxyl radical **5** resulting from the initial electron ejection followed by loss of a proton to buffer.

The effect of acetonitrile on the quantum yields also suggests the pathway: BrU radical anion to uridinyl radical to U. Acetonitrile at 1 *M* concentration increases the ratio of U to **2** by a factor of three as shown in Table 3 (entries 4 and 6) without changing the overall quantum yield of destruction of BrU. Acetonitrile is a modest hydrogen atom donor and a poor electron donor. The increased ratio results from the uridinyl radical abstracting a hydrogen atom from acetonitrile before it abstracts a hydrogen atom from or adds to **1**.

The quantum yields of BrU destruction and formation of products based upon absorption by tyrosine derivative **1** and its anion in Table 3 are in the range of 0.05–0.06. This is above the literature value of 0.03 for electron ejection by phenol. A contribution from multiphoton excitation was considered as a possible explanation but was rejected because the quantum yields did not decrease with decreasing light intensity. The higher quantum yield may be due to a predicted higher quantum yield of electron ejection from the anion of **1** or a contribution from direct electron transfer from excited tyrosine or its anion to BrU. If the former were the exclusive explanation a more dramatic effect of excitation wavelength should have been observed based upon the absorption properties shown in Table 1. Of the total light absorbed by **1** plus its anion at 308 nm, 25% was by the anion. A further possibility is that the quantum yield of electron ejection from **1** is a little higher than from phenol.

The quantum yield of formation of products from irradiation of BrU in the presence of 8 m*M* tyrosine derivative **1** is about 40 times higher than the quantum yield of formation of products from the irradiation of *N*-acetyltyrosine in the presence of thymidine at pH 7 reported by Shetlar and coworkers (21). The relative quantum yields presumably parallel the electron affinities of the acceptors, BrU and T. Coupling to BrU is probably also enhanced by rapid ejection of bromide in competition with back electron transfer. The relative quantum yields for the model reactions are also consistent with the relative crosslinking yields observed with telomeric DNA bound to *Oxytricha* nova telomere binding protein (7).

Although the reactions presented here appear to occur predominantly *via* excitation of the tyrosine component, excited bromouracil and BrU are indeed photoreactive. Reduction to uracil and U, respectively, occurs upon excitation in a reducing solvent such as 2-propanol by both C–Br bond homolysis (70%) and electron transfer (30%). The overall quantum yield is 0.02, much higher than the quantum yield of photoreduction in aqueous medium. Although reduction by electron transfer from water is not expected, C–Br bond homolysis followed by hydrogen atom abstraction from another molecule of BrU should still be a viable pathway. Why is bond homolysis so inefficient in water? The literature suggests radical pair recombination in competition with cage escape (10).

An example of a less efficient reaction in pH 7 aqueous medium is the coupling of bromouracil to *N*-acetylhistidine *N*-ethylamide with excitation at 308 nm where *N*-acetylhistidine *N*-ethylamide is completely transparent. The quantum yield of coupling to 3 m*M* histidine derivative was estimated at 1×10^{-3}; U was a byproduct formed with a quantum yield of 1×10^{-4} (2). The overall quantum yield of reaction of bromouracil with the histidine derivative is high relative to the quantum yield of photoreduction of BrU in aqueous medium (Table 1, entries 1 and 2). This suggests that the reaction with the His derivative is probably initiated by collisional electron transfer rather than by C–Br bond homolysis. The mechanism of coupling of the bromouracil chromophore to tyrosine and possibly tryptophan chromophores may be distinct because of the facility with which these amino acid residues undergo photoelectron ejection (20).

The results of a recent study of diarylmethylchloride pho-

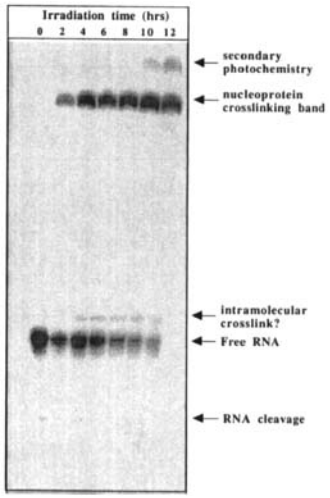


Figure 3. A phosphor imager scan of a 20% urea, denaturing, polyacrylamide electrophoretic gel showing the time course (0, 2, 4, 6, 8, 10 and 12 h) of irradiation at 325 nm of RNA 1–MS2 bacteriophage coat protein complex. The free RNA and crosslinked nucleoprotein are labeled on the right side. The percent crosslinking determined from the phosphor imager scan as a function of irradiation time (time in hours, percent crosslinking) is as follows: 0, 0%; 2, 27%; 4, 41%; 6, 47%; 8, 59%; 10, 66% and 12, 62%.

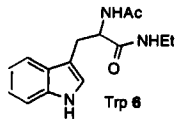
tochemistry by Peters and co-workers suggest a rationale for the inefficiency for C–Br bond homolysis in aqueous medium *versus* 2-propanol medium (24). They observed decay of an initially formed diarylmethyl–chlorine geminate radical pair to the ground-state surface by electron transfer in competition with radical cage escape. Partitioning between reformation of starting material and a contact ion pair then occurred. Possibly, aqueous medium facilitates electron transfer decay within the uridinyl–bromine geminate radical pair to the ground-state surface in competition with radical cage escape. On the ground-state surface, reformation of starting material must then be favored over formation of a contact ion pair because products from an ionic intermediate are not apparent.

5-Iodouridine reacts with tyrosine derivative **1** to give the same products as does BrU. However, the mechanism for initiation of the reaction is distinctly different. The quantum yields of reaction of IU based upon absorption by IU show no wavelength dependence; yet, acetonitrile affects the ratio of U to adduct **2** in an analogous manner (Table 3, entries 7 and 8). These and other observations led to the proposal of initial C–I bond homolysis of excited IU to form the uridinyl radical and an iodine atom followed by reaction of the uridinyl radical with tyrosine **1** (15). The different reactivity of the two chromophores with **1** may result in part from a weaker bond to the halogen in IU.

Nucleic acids bearing BrU and IU nucleotides are also photolabile with respect to formation of alkali-labile bonds (25). The mechanisms of reaction appear to parallel those proposed for reaction with tyrosine **1**. In a small self-complementary duplex DNA bearing a single BrdU in each strand, depurination to form an alkali labile strand occurs most efficiently with a deoxyadenosine in the position 5′ to a BrdU; a mechanism involving initial excitation of the adenine base followed by electron transfer to the bromouracil base is proposed (26). In an analogous duplex DNA bearing a single iododeoxyuridine, formation of an alkali-labile strand appears to result from initial C–I bond homolysis (27).

Photocrosslinking of a BrU–RNA nucleoprotein complex

A nucleoprotein complex that has been utilized to define and improve photocrosslinking with the 5-bromouracil chromophore is RNA 1 bearing a BrU at the −5 position (Fig. 1) bound to the bacteriophage R17 coat protein. Degradation and sequencing experiments established that crosslinking occurred between the −5 position of the RNA and Tyr85 of the coat protein. The yield of crosslinking based upon RNA with excitation at 308 nm appeared to be limited to about 40% by competitive damage to coat protein, presumably because of excitation of tryptophan residues. The UV spectrum of *N*-acetyltryptophan *N*-ethylamide (Trp **6**) is compared with that of Tyr **1** in Fig. 2. Of particular significance are the extinction coefficients for absorption at 308 and 325 nm shown in Table 2 relative to those for Tyr **1**. The extinction coefficient for **6** at 325 nm is 3 times larger than the extinction coefficient for **1**, but 50 times larger at 308 nm.



Trp **6**

The observations of formation of **2** upon irradiation at 325 nm and higher relative absorption by **1** at 325 nm prompted exploration of photocrosslinking with excitation at 325 nm. Because of availability, the protein was changed to the almost identical bacteriophage MS2 coat protein prepared as a nonaggregating variant. Previous experience suggests that the two proteins behave almost identically in binding and crosslinking to RNA 1. Photocrosslinking of the nucleoprotein complex at 325 nm was studied as a function of irradiation time. The extent of reaction was established by denaturing polyacrylamide gel electrophoresis as shown in Fig. 3. The maximum crosslinking yield was now 66% and occurred after 10 h of irradiation. As observed earlier (13) with irradiation at 308 nm, three additional, radiation-dependent bands appear in the gel. The one slightly above RNA 1 and the ones below RNA 1 appear to be primary photochemical products based upon the timing of their appearance and likely represent the products of RNA intramolecular crosslinking and RNA cleavage, respectively. The high molecular weight band above the primary nucleoprotein crosslinking band appears to be a result of secondary photochemistry because it only appears after 10 h of irradiation.

Although the long irradiation time needed to achieve 66% crosslinking may not be practical in some cases, the dividend of high yield can be particularly helpful if degradation and sequencing are an ultimate goal. A qualitative comparison of nucleoprotein crosslinking experiments employing the bromouracil chromophore suggests that the RNA 1–MS2 system is inefficient. Hence, other systems may not require 10 h of irradiation time to achieve maximum yield. Further, the irradiation time could be significantly reduced with a higher power HeCd laser; lasers emitting almost three times as many photons per second are now commercially available.

The work reported here suggests that photocrosslinking of RNA 1–MS2 coat protein complex occurs *via* excitation of Tyr85 of the coat protein. An alternate possibility within the nucleoprotein complex is excitation of a BrU tyrosine π-complex. The co-crystal structure of an analogous nucleoprotein complex locates the pyrimidine at the −5 position in a π-stacking arrangement with Tyr85 of the coat protein (28). Excitation of a π-complex might then result in direct electron transfer from the tyrosine residue to the bromouracil of the RNA. No evidence was observed for this pathway in the model reaction studies; in particular, the UV absorption spectra of mixtures of **1** and BrU showed no ground-state complex formation.

CONCLUSIONS

Irradiation, with monochromatic light at 266, 308 or 325 nm, of a mixture of BrU and *N*-acetyltyrosine *N*-ethylamide (**1**) in pH 7 aqueous medium yields U as a minor product and *N*-acetyl-*m*-(5-uridinyl)tyrosine *N*-ethylamide (**2**) as a major product. The ratio U:**2** is not affected by wavelength but is increased by the presence of 1 *M* acetonitrile. Quantum yield measurements together with UV absorption measurements suggest that the reactions result from excitation of the tyrosine derivative and its anion rather than excitation of BrU. Irradiation of the nucleoprotein complex consisting of RNA 1, bearing a single BrU, bound to bacteriophage MS2 coat protein at 325 nm, where the tyrosine chromophore absorbs more competitively, gave a record 66% yield of crosslinking to a tyrosine residue.

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