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Photohydrate-Mediated Reactions of Uridine, 2'-Deoxyuridine and 2'-Deoxycytidine with Amines at Near Neutral pH

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Abstract

Photohydrates are formed in high yield when uridine (Urd), 2'-deoxyuridine (dUrd), cytidine (Cyd) and 2'-deoxycytidine (dCyd) are irradiated with UVC in aqueous solution. The thermal reactions of the photohydrates of Urd with amines at pH values near pH 7.5 have been studied using UV spectroscopy, HPLC, mass spectrometry and, in some cases, NMR. It has been found that a number of amines (i.e. ethylenediamine, N,N'-dimethylethylenediamine, glycine, glycinamide, glycylglycine, glycylglycylglycine, putrescine, spermidine and spermine) react thermally with such hydrates to form products with UV spectra characteristic of opened ring uridine-amine adducts. In general, these products display a strong absorption peak with λ_{max} in the range between 288 nm and 310 nm. Mass spectral studies of a number of the products indicate that they contain one molecule of parent nucleoside and one molecule of reactant amine. Upon standing in water these products revert to parent hydrate, while heating produces parent nucleoside. Less comprehensive studies indicate that photohydrates of dUrd and dCyd undergo analogous thermal reactions. Preliminary results suggest that UV-irradiated polyuridylic acid and polycytidylic acid undergo similar reactions. These results may have relevance for obtaining a complete understanding of the biological effects of producing Urd and dCyd photohydrates in a cellular environment.

Introduction

Additions of water to uracil (Ura) and related compounds were among the earliest photoreactions of nucleic acid components to be studied. These reactions involve addition of H₂O across the 5,6-double bond of the parent compound. For example, the product formed by irradiation of uracil in water is 6-hydroxy-5,6-dihydrouracil (1, 2). Photohydration reactions of the pyrimidine nucleobases cytosine (Cyt) and thymine (Thy) also received early attention (1, 2), while the hydrates of 5-methylcytosine (m5Cyt) have been studied more recently (3). The pyrimidine nucleosides of uracil and cytosine are particularly reactive towards photohydrate formation (for general reviews, see (1, 2)).

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Supporting Information The following appendix, figures and table will be found online at DOI: xxxx-xxxxxx.s1 **Appendix S-1** contains sections dealing with the (1) preparation of the photohydrates of Urd, dUrd and dCyd, Ura and 1-MeU, (2) preparation and characterization of reaction products of dUrd and Urd hydrates with various amines, (3) results of exploratory studies of reactions of dCyd photohydrates with various amines, (4) reaction products resulting from incubation of the photohydrates of Urd and dUrd in ammoniacal solution and (5) the results of spectroscopic studies of adducts produced *via* incubation of dUrd hydrate and dCyd hydrates with ethylamine.

Irradiation of uridine (Urd) (4, 5) and 2'-deoxyuridine (6) in aqueous solution induces rapid addition of water. The reaction between uridine (Urd) and water is shown in Scheme 1, in which **1a** is Urd. Because of the asymmetric sugar attached at N1, the photohydrate exists in the two epimeric forms shown as **1b** and **1b'**. Presumably these two forms can interconvert *via* an aldehydic isomer labeled **1b''** in Scheme 1 (5). While thymidine (Thd) hydrates are formed with low quantum yields upon UV irradiation of Thd solutions (Cadet and Wang, unpublished work cited in (1), p. 46), these compounds have been synthesized by an alternative method and their structures determined (7). The formation of two diastereomeric cytidine (Cyd) photohydrates has been demonstrated *via* NMR studies, although these compounds have not been characterized individually (8). Knowledge of the photohydrates of 2'-deoxycytidine (dCyd) is also not very advanced; detailed structural studies of the two diastereomers remain to be accomplished. These hydrates readily undergo deamination processes to form dUrd hydrates (for a detailed discussion, see (9)). The photohydrates of the various pyrimidine nucleobases, as well as those of the nucleosides and other related compounds, are unstable to treatment with heat, acid and base, usually reverting predominately to the parent compound (1, 2)).

In addition to water, other nucleophiles can photoreact with pyrimidine nucleobases, nucleosides and related compounds. For example, the photochemistry of the pyrimidine nucleobases and nucleosides in the presence of amines has received considerable attention in the literature (10, 11). It has been found that the photoproducts formed in such reactions differ significantly in structure from the photohydrates produced in reactions with water. The photoreactions of thymine (Thy) and thymidine (Thd) with a variety of amines (including lysine) were among the first to be studied (12). The predominant reaction undergone by these two compounds at basic pH is a ring-opening reaction in which the reactant amine, say RNH₂, becomes attached to C2 in the parent pyrimidine ring while the N1 nitrogen, along with any substituent (e.g. alkyl group or sugar), is found attached to the C6 of the parent compound in the product. This reaction is shown for Thd in Scheme 2 below.

Interestingly, upon treatment of the opened ring conjugate, shown in the E configuration in Scheme 2, with base or heat causes it to undergo ring closure to form a photoexchange product (PEX) in which the reactant amine is incorporated into the final product and the group originally present at N₁ (e.g. N₁dRib) is expelled. The overall reaction can thus be viewed as a photoexchange reaction. The nature of these reactions was first elucidated by Saito and co-workers; summaries of this early work are available (10, 11). Later work showed that Ura, Cyt, m5Cyt, and 5-bromouracil (5BrU) (13 - 16) undergo similar photoinduced ring-opening and thermal ring closure reactions, as do 5-bromo-2'-deoxyuridine and 2'-deoxycytidine (dCyd) (15, 17).

Early work by Fikus and Shugar (18) showed that solutions of photohydrates of Urd made basic with NH₃ (pH 11.8-12.0) displayed transient appearance of a UV absorption spectrum with λ_{max} at 290 nm. This spectrum disappeared over a period of an hour; during the course of the loss of absorbance at 290 nm, Urd was regenerated in almost quantitative yield. It was suggested that the reversion reaction of hydrate to uridine proceeds by two pathways, one being a direct base catalyzed reaction and the second a ring opening reaction, followed by ring closure and dehydration. Similar results were obtained in solutions containing added NaOH or KOH, except that reversion to Urd was significantly less than quantitative, likely due to a third competing irreversible reaction in which ring opening at the 3,4 bond occurred. It was proposed (18) that the structure of the product displaying $\lambda_{\text{max}} = 290$ nm was **1c** (see Scheme 3), in which a hydroxyl group is attached to C3. It can be noted that **1c** is an isomeric form of **1b''** in Scheme 1.

Can Urd (or dUrd) photohydrates be induced to undergo ring-opening reactions with additives other than hydroxide or ammonia? Do dCyd hydrates react in the same manner? We describe here results of experiments with Urd (dUrd) hydrates and dCyd hydrates that indicate ring-opening reactions do indeed occur with a variety of amines (e.g. ethylamine, ethylenediamine, glycineamide, glycyglycine, spermine) and that a number of these reactions occur readily at pH 7.5. The reaction products are nucleosideamine adducts; a number of these adducts are stable enough to isolate and submit for mass spectrometric study. In addition, utilizing a variety of ^1H and ^{13}C NMR techniques, we have carried out an extensive study of adducts formed upon incubation of Urd hydrates with glycineamide. In less extensive proton NMR studies, we have also characterized the ethylamine and ethylenediamine adducts of dUrd and the ethylamine adduct of dCyd. Finally, returning to the seminal observations of Fikus and Shugar, we present evidence that, at least in part, the absorbance changes observed when uridine hydrates are incubated with ammonia can be accounted for by production of an opened ring Urd-ammonia adduct.

Materials and Methods

General aspects

Urd, dUrd, Ura, 1-methyluracil (1-MeU) and dCyd were purchased from Sigma (St. Louis, MO), as were ethylenediamine.2HCl, glycine, glycineamide.HCl, glycyglycylglycine, putrescine.2HCl, cadaverine.2HCl, spermidine.3HCl and spermine.4HCl. Methylamine, N,N'-dimethylethylenediamine, 1,3-diaminopropane.2HCl and glycyglycine were obtained from Aldrich (Milwaukee, WI). Ethylamine and ethylenediamine (EDA) were from Fluka (Buchs, Switzerland). Polycytidylic acid (K^+ salt) was from P-L Biochemicals (Milwaukee, WI), while polyuridylic acid (K^+ salt) was obtained from Boehringer Mannheim. Ammonium hydroxide and HPLC solvents were from Fisher (Fair Lawn, NJ); NMR solvents were from Aldrich. Preparative separations were accomplished using a Shiseido Capcell UG120 10 $\times$ 250 mm reverse phase column (5 μm particle size (Yokohama, Japan)); hereafter, this column is termed Column A. Analytical HPLC was done on a Capcell UG120 4.6 $\times$ 150 mm reverse phase column (5 μm particle size); in the following we term this Column B. The HPLC system employed was a Rainin binary gradient pumping system (Emeryville, CA) hooked up to a Hewlett-Packard 1040A diode array HPLC detector (Palo Alto, CA). HPLC samples were filtered, using Costar Spin-X micro-centrifuge filter tubes containing a 0.2 μm nylon filter (Corning Incorporated, Corning New York). UV spectra were measured on a Hewlett-Packard 8452A diode array spectrometer or by using the "on the fly" spectral capture capability of the Hewlett-Packard diode array HPLC detector. Rotatory evaporations were carried out using a Büchi R-200 Rotovapor (New Castle, DE) connected to dry ice traps and a mechanical pump.

Electrospray ionization (ESI) mass spectra were run on either a Waters Micromass ZQ4000 instrument (Beverly, MA) or a Sciex API300 triple quadrupole electrospray instrument (Toronto, Canada). Liquid secondary ionization mass spectra (LSIMS) were run on a Kratos MS-50 (Ramsey, NJ). NMR spectra were run at 600 MHz on a Varian INOVA NMR spectrometer (Palo Alto, CA) or at 300 MHz on either a GE QE-300 NMR spectrometer (Fremont, CA) or a Bruker Advance NMR spectrometer with Topspin software (Billerica, MA).

Irradiation methods

Production of the photohydrates of Urd, dUrd, dCyd, Ura and 1-methyluracil (1MeU) were carried out in the cold with 254 nm radiation. This light was provided by unfiltered Spectronics BLE-1T155 15 watt lamps (Spectronics, Westbury, NY) housed in Spectroline XX-15A lamp holders. The parent solutions were placed in 187 mL cylindrical quartz

vessels (47.6 cm length $\times$ 2.6 cm OD) from Southern New England Ultraviolet Company (Branford, CT). These vessels fit snugly between the two lamps in a Spectroline XX-15A lamp holder placed in a “lamps up” orientation and photoreactions were carried out with 165 mL portions of solution with the irradiation vessels placed in this position; one end of the lamp housing was elevated at about a 10 degree angle to the horizontal to avoid leakage of the solution from the tops of the stoppered vessels. During irradiation, the reaction vessels were stoppered with glass taper seal stoppers. Irradiations were done under air in a cold room at about 5°C.

Irradiations in spectrophotometer cuvettes were done in self-masking 0.6 mL volume quartz cells (Precision Cells, Hicksville, NY); these were closed within Teflon stoppers and placed directly against one of the lamps contained in the lamp holder described above. Thermal reactions of the various photohydrates with amines were followed by UV spectroscopy in the same cells.

Preparation of photohydrates

The protocols for photochemical synthesis and isolation and purification of the various photohydrates are described in Appendix A1 in the Supporting Materials. Using these procedures, we isolated pure samples of the photohydrates of Urd, dUrd, dCyd, Ura and 1-MeU. In those experiments, described below, that used purified nucleoside photohydrates as reactants, we usually used mixtures of the two diastereomers. (Upon standing in the cold, gradual isomerization of each purified diastereomer yields a mixture of two isomers; thus, after purification of the individual diastereomers, we combined them to give the solutions used for thermal incubations.)

Results

Introduction: A puzzling observation

An impetus for the studies reported here was the observation of baffling behavior when dUrd was irradiated at 254 nm in aqueous solution containing ethylenediamine at *near neutral pH*. In addition to the expected disappearance of the parent dUrd, due to formation of photohydrates **Ib** and **Ib'**, there appeared an unanticipated red-shifted band with a λ_{max} at about 296 nm. Indeed, this absorption band steadily increased in intensity, even after irradiation had been terminated. (This behavior is displayed in Fig. S1 in the Supporting Information.) Further investigation showed that other amines induced the same type of behavior. These observations, coupled with those of Fikus and Shugar (18) on the appearance of red-shifted absorption spectra when Urd hydrate is incubated in basic ammoniacal solution (see above), suggested that thermal reactions of photohydrates of Urd (and other pyrimidine nucleoside photohydrates) with amines at near neutral pH values merited further investigation. In particular, these results suggested that Urd and dUrd hydrates reacted thermally with appropriate amines (e.g. ethylenediamine (EDA)) to form either compounds with structures of the type given by **IIa** for Urd adducts (or **IIc** for dUrd adducts) (see Scheme 4) or, alternatively those of the type shown by **IIb** and **IId** in Scheme 4. Below, we present evidence indicating that the latter type of adduct, in which the reactive amine becomes attached to C3, is the more likely product in the observed reactions of hydrate with amine.

Thermal reaction of Urd and dUrd photohydrates with ethylenediamine

Some of our most extensive studies of the thermal reactions of pyrimidine nucleoside photohydrates with amines were done with mixtures of Urd hydrates (**Ib** and **Ib'**) (or dUrd hydrates) and ethylenediamine (EDA). The pK_a values for EDA are 6.85 and 9.93 (19). (Photohydrates were prepared as described in Materials and Methods and in Appendix A1).

Buffers containing a weak acid (e.g. a diprotonated amine) and its conjugate base (a monoprotonated amine) display reasonably high buffer capacities over a pH range between $pK_a \pm 1$ ((19), Table 2.4). Thus, over the pH range 5.85 and 7.85, the equilibrium between diprotonated and monoprotonated EDA provides good buffering action; as a result, the pH in incubation mixtures containing Urd or dUrd hydrates, at the concentrations used in this work (2 mM and smaller), and EDA, present in significant excess at pH values within this range, should be well protected against significant pH changes during reaction. The progress of the ring-opening reaction of mixtures of **Ib** and **Ib'** in solutions containing EDA at pH 7.5 after short reaction times is shown in Fig. S2. As can be seen, absorbance with a λ_{max} at 294 steadily increases over the time interval used. A pH profile for the rate of formation of putative opened ring compounds was constructed by measuring the absorbance at 294 nm produced by incubation for set time intervals at a number of pH values. This profile is shown in Fig. S3. As can be seen, reaction can be detected at a pH value as low as 5.7; at this pH, the percentage of EDA in the form of EDA.2HCl is 93%, while the percentage of total EDA in the form EDA.HCl is 7%. (The percentage of total EDA present as completely unprotonated amine is very small at pH 5.7.) The profile displayed in Fig. S3 confirms that the reaction involves a free amine moiety, as the larger the concentration of unprotonated amine, the greater the amount of hydrate converted to opened ring form in a given reaction time.

The reaction of dUrd hydrate with EDA was also examined to confirm that it behaves similarly to the Urd hydrate. Indeed, spectrometric monitoring of the absorption of a mixture of epimeric dUrd hydrates and EDA (90 mM, pH 7.3) over a 16 min period produced a set of curves very similar to that displayed in Fig. S2. We carried out a considerably more extensive study of purified reaction product in this system, using UV spectroscopy, mass spectrometry and proton NMR as tools. The detailed results of this study are described in Appendix A2a. In short, the product isolated displayed spectroscopic properties consistent with it being a mixture of the E and Z isomers of either **Iic** or **Iid** (see Scheme 4); The UV spectra of the two adducts, as obtained *via* use of the spectral capture technique of the diode array detector, are shown in Fig. S4. These two products, separable by HPLC, were termed PEDA₁ and PEDA₂. (short for ProductEthyleneDiAmine with subscripts 1 and 2 denoting the two isomeric forms).

We were able to obtain a 1D proton NMR spectrum for this mixture of adducts; however, because of the thermal instability of these adducts, we were unable to carry out ¹³C and 2D NMR studies to distinguish between structures of type **Iic** and **Iid**. It can be noted that chemical evidence, discussed in Appendix A2a, favors assignment of the adducts as being E and Z isomers of structure type **Iid**. However, more definitive assignments could be made if various 2D NMR techniques (e.g. heteronuclear multiple bond correlation (HMBC) or correlation spectroscopy (COSY)) were to be applied to adducts less labile than those isolated after incubation of the dUrd hydrate-EDA system. Indeed, we found that adducts arising from the incubation of Urd hydrates with glycineamide (GlyAm) and the dUrd hydrates with ethylamine satisfied this need for increased stability.

It should be noted that our work has not ruled out the possibility that photo-induced reaction can also produce products with structures similar to, say, **Iib** or **Iid** when solutions of dUrd or Urd are irradiated in the presence of EDA near neutral pH. Proof that such adducts are formed only *via* thermal pathways will require further research.

Thermal reactions of Urd hydrates with glycineamide

The reaction of Urd hydrates with glycineamide (GlyAm) produced adducts in good yields that are quite stable and that can be readily purified *via* HPLC without using aqueous eluents containing salt (thus eliminating potential product losses during a desalting step). The

protocol for preparation of sufficient amounts of the desired GlyAm adducts for purposes of in-depth NMR study is given in Appendix A2b, along with information concerning their spectral properties. In short, incubation of Urd hydrate with GlyAm produces both a major adduct ($\lambda_{\text{max}} = 291 \text{ nm}$) and a minor adduct ($\lambda_{\text{max}} = 299 \text{ nm}$) that elute quite close to one another; thus, semi-preparative HPLC on Column A resulted in isolation of a mixture of two isomeric forms of the Urd-GlyAm adduct (putatively E and Z in nature). ESI mass spectrometry of the isolated mixture of adducts yielded a single value of 319.1 for the molecular mass, consistent with a structure containing one Urd and one GlyAm.

The characterization of the adduct mixture by NMR proved to be quite informative. The results of these NMR studies show that the adducts formed in the Urd hydrate-GlyAm system correspond in structure to **II**f in Scheme 4, rather than **II**e. Furthermore, 2D NMR studies show that both the E and Z isomers are present in the NMR sample and suggest that the E isomer of the adduct exists as interconvertible conformational forms. The various NMR results, along with their implications, are discussed below; the NMR spectra themselves are displayed in Figures S5, S6, S7, S9 and S10, contained in the Supporting Information.

We first conducted NMR studies of the putative mixture of E and Z adducts in DMSO- d_6 , so as to observe interactions between the anomeric ribose C1'H ($\delta \approx 5.32$) and the NH proton generated by the ring opening reaction. Indeed, we observed this interaction in the ^1H NMR spectrum as a pair of doublets at 9.04 (Z configuration) and 9.25 ppm (E configuration) ($J = 9.4 \text{ Hz}$ in both cases) integrating to 0.14 protons and 0.86 protons, respectively (Fig S5). The structure was further analyzed by heteronuclear multiple bond correlation spectroscopy (HMBC) (see Fig. S6-A), so as to confirm that the NH proton is adjacent to the ribose ring, as manifested by its interaction with the C1' and C2' carbons (Fig S6-B). Little additional useful structural information could be ascertained from the NMR spectra in this solvent, due to spectral broadening and the inherent complexity of the spectrum because of the presence of resonances due to -OH and -NH sites.

To obtain more information, the adduct mixture was analyzed in D_2O , which not only decreased the number of observed protons, but also sharpened the signals for most of the other protons. As expected, the resonances for the NHRib proton (see above) are no longer present. The vinyl protons for the various adduct forms could be readily observed. The C3H protons, next to the attached glycinamide group (Scheme 4), could be observed as a broad doublet at 7.64 ppm ($J = 13 \text{ Hz}$, 0.85 H) and a sharp doublet at 6.79 ppm ($J = 8.1 \text{ Hz}$, 0.15 H) (see Fig. S7). As the coupling constants for hydrogens that are *trans* to one another are generally larger than those that are *cis* with respect to each other, the configuration with $J = 13 \text{ Hz}$ can be assigned to be the E configuration. The C2H proton resonances are present at 4.81 ppm ($J = 13 \text{ Hz}$, 0.85 H, E configuration) and 4.63 ppm ($J = 8.1 \text{ Hz}$, 0.15 H, Z configuration). The observed integrated areas for the protons corresponding to the E and Z isomers imply that the ratio of E to Z in the NMR sample is 85/15. The glycinamide CH_2 protons integrate to only 1.67 protons (84%) at 3.86 ppm with the remaining 16% being found under one of the ribose protons peaks at 3.95 ppm. In the case of the dominant E configuration, the width of the C2H and C3H peaks suggests that interconvertible conformations of this isomer exist, each with somewhat different environments for protons corresponding to these peaks. The sharper corresponding signals for the Z isomer suggest that only one major conformational form is accessible for the C2H and C3H protons in this species. This explanation of the broadness of the C3H and C2H NMR peaks for molecules in the E configuration and the sharpness of the corresponding vinyl protons in the Z configuration draws support from the results of calculations carried out with Spartan 10 for the Macintosh; Fig. S8 displays conformational snapshots of the three lowest energy conformations of the E configuration of the Urd-glycinamide adduct and the single low

energy conformation of the Z configuration, as obtained *via* use of a Hartree-Fock 3-21G basis set for quantum chemical calculations (for further details, see the caption for Fig. S8). While these calculations of relatively low sophistication must be regarded as giving only approximate results, they do indicate that one of low energy conformations (Fig. S8-C), accounting for an estimated 9% of the total population of E isomer, has a different environment for C2H and C3H than that found in the other two low energy conformations (Fig. S8-A and Fig. S8-B) for the same two protons; in particular the adducted glycinamide moiety displays a different conformation, relative to the vinyl group, than the other two conformations. (The main difference in the other two low energy conformations for the E isomer lies primarily in the very different relationship of the ribose ring to the remainder of the molecule.) The same set of calculations suggests that, due to hydrogen bond stabilization of a six-membered ring containing these moieties, the C3H and C2H in the Z isomer see essentially one environment (see Fig. S8-D).

Additional information concerning the attachment site of the ribose to the remainder of opened ring was obtained by study of the ^{13}C NMR and HMBC spectra in D_2O . Weak carbon signals are seen for the olefin and glycinamide moieties in the ^{13}C spectrum of the Z isomer, while the corresponding signals are too weak to be seen for the E isomer (Fig. S9-A). The DEPT 135 spectrum (Fig. S9-B) indicates that a weak signal at 50.5 ppm corresponds to the CH_2 of the glycinamide moiety and that a strong resonance at 61 ppm is that of C5'H_2 . (Summaries of the ^1H and ^{13}C NMR assignments for various resonances, seen in the spectra discussed above, are given in Fig. S7 and Fig. S9 respectively.)

Study of the same sample by HMBC (Fig. S10-A) confirms correlation between the glycinamide carbon at 50.5 ppm and the Z isomer C3 proton at 6.79 ppm (Fig. S10-B). In addition, the resonance of the glycinamide methylene protons of the Z configuration is correlated with carbon resonances at 155 (C3) and 176 ppm (CONH_2) (see Fig. S10-C). The glycinamide protons in the E isomer (3.86 ppm) correlate with carbons that should display resonances at 152 and 174 ppm; the absence of these carbon peaks in the ^{13}C spectrum suggests that their signals may be broadened by the existence of readily interconvertible conformations in the E isomer.

Summing up, the data from detailed NMR study of the Urd-GlyAm adducts in D_2O and in DMSO-d_6 shows that these compounds exist as the general structural type shown as **IIIf**, with both the E isomer (major form) and Z configuration (minor form) of this structure being present in the NMR sample; the alternative general structure **IIe** can be ruled out. The NMR data is consistent with the idea that interconvertible conformational states exist for the E configuration of **IIIf** in aqueous solution, but that a single conformational state is dominant for the Z configuration.

Thermal reactions of Urd photohydrates with various amines at near neutral pH

Do other amines, besides EDA and GlyAm, also react with the hydrates of Urd to yield adducts similar in nature to **IIb** and **IIIf**? To answer this question, we examined the reaction products formed when a variety of amines were incubated at ambient temperature with a mixture of the two diastereomeric uridine hydrates at near neutral pH. In addition to EDA and GlyAm, we examined the reactivity of glycine (Gly), glycylglycine (Gly_2), glycylglycylglycine (Gly_3), N,N'-dimethylethylenediamine (DMEDA), propane-1,3-diamine (DAP), putrescine (butane-1,4-diamine) [Put], cadaverine (pentane-1,5-diamine) [Cad], spermidine (N-(3-aminopropyl)butane-1,4-diamine) [Spd] and spermine (N,N'-bis(3-aminopropyl)butane-1,4-diamine) [Sper]. (DMEDA can be regarded as a model of the secondary amine moieties found in Spd and Sper.) Most of these amines have a pK_a (or set of pK_a values) that indicate a significant proportion of the total amine concentration exists in a partially unprotonated state in aqueous solution at pH 7.5 (see Table S1 in the Supporting

Information). Of the various compounds studied, only Cad failed to yield appreciable amounts of product. The products from the various reactions were isolated using HPLC and studied using UV spectrophotometry and ESI mass spectrometry. In all cases (except for Gly), ESI mass spectrometry of the isolated adducts yielded peaks with the predicted molecular mass values, while each of the UV spectra closely resembled those observed for the Urd-GlyAm and dUrd-EDA adducts. These results suggest that Urd hydrates react with these other amines to form the same type of adduct formed by GlyAm and EDA. The protocols used in the preparation and isolation of these compounds, as well as details concerning the UV spectral properties and molecular masses of these compounds are given in Appendix A2c.

It is interesting to note that the purified adducts of the various amines were quite stable in the aqueous frozen state (-20°C) over a 3-month period. However, when incubated in distilled water at room temperature, these compounds reverted to parent hydrates of Urd or dUrd. To obtain some quantitative information about the effect of phosphate buffer on the stability of adducts at physiological temperature and near physiological pH, we studied the rate of loss of the Urd-EDA adduct at 37°C and pH 7.6 at three different phosphate concentrations. For this study, we used HPLC as a tool, examining the peak area corresponding to the Urd-EDA adducts (measured at 291 nm) as a function of incubation time in a temperature controlled water bath. Column A was used for chromatography, using 92% (100 mM NaCl)/8% MeOH flowing at 4 mL/min as eluent. Under these conditions, both the E and Z forms of adduct eluted at 3.8 min. The following pseudo-first order rate constants were measured at various fixed values of [P], the phosphate concentration in moles/L: [P] = 9.5 mM, $k = 0.027 \text{ min}^{-1}$; [P] = 52.5 mM, $k = 0.033 \text{ min}^{-1}$; [P] = 95 mM, $k = 0.039 \text{ min}^{-1}$. When k is plotted versus [P], a slope of $0.117 \text{ L mole}^{-1} \text{ min}^{-1}$ and an intercept of 0.026 min^{-1} ($r^2 = 0.988$) were evaluated. These limited results suggest that phosphate species can participate in a general acid or general base catalyzed reversal mechanism. These results have similarities to those obtained for ring-closure of amine adducts of Thy and Ura to form thymine and uracil hydrates (20).

When a similar incubation of the Urd-EDA adduct was carried out in 0.1% TFA, reaction to form product(s) with an absorption spectrum similar to that of Urd hydrates was complete within 1 min.

Scheme 5 displays a set of reactions that are consistent with our results on the reactions of Urd photohydrates with amines at near neutral pH to give Urd-amine adducts. This scheme assumes that **Ib''**, the aldehydic form of photohydrate **Ib**, reacts reversibly with base (in this case, RNH_2) to form an opened ring compound (**Int**). (As displayed in Scheme 1, **Ib** reversibly converts into its epimer, namely **Ib'** via **Ib''**; both forms would participate in the reaction sequence displayed in Scheme 5, even though **Ib'** is not displayed.) Finally, **Int** undergoes reversible loss of water to form the observed product (**Iip**). This scheme is consistent with the observation that **Iip** reverts to photohydrates upon standing in water in the absence of amine and is also consistent with the data displayed in Fig. S3, in which the rate of reaction to form **Iip** increases with pH (i.e. with the concentration of unprotonated amine groups present in solution).

It can be noted that the results of preliminary studies, described in Appendix A2c, suggest that both Ura photohydrate and 1-MeU photohydrate react with EDA and GlyAm at near neutral pH to form similar types of adducts (albeit in very small amounts).

Exploratory studies of the thermal reactions of dCyd photohydrates with various amines at near neutral pH

The observation of reactivity of Urd (and dUrd) photohydrates with a variety of amines at near neutral pH suggested that dCyd photohydrates might undergo similar reactions. Therefore we prepared and isolated these hydrates (Appendix A1) and incubated them with several of the same amines listed above (EDA, DMEDA, GlyAm, (Gly)₂, Put, Sper). Indeed, *via* monitoring of the reaction by HPLC, we did observe that compounds with the absorption spectra expected for dCyd adducts were formed in these systems (see Fig. S11 for the spectra of two isomers of the putative dCyd adduct with EDA). However, these products were thermally unstable; the corresponding dUrd-amine adducts were among the compounds formed in their decomposition. In three of the incubation mixtures, we found evidence for formation of transamination products of the type produced when dCyd is incubated with amines in the presence of bisulfite (21). Details concerning these experiments and their results are given in Appendix A3.

UV-C irradiated polyuridylic acid (poly rU) and polycytidylic acid (poly rC) appear to form ring-opened adducts when incubated at near neutral pH with DMEDA

It is known that the pyrimidine bases in both poly rU and poly rC undergo photohydration when irradiated in aqueous solution (22-24). We conducted a pair of exploratory studies to determine if rU and rC hydrates, when incorporated into polynucleotides, are also reactive towards adduct formation. The potassium salts of poly rU (14 mg) and poly rC (12 mg) were dissolved with stirring over a three day period in doubly distilled water to produce solutions at a polynucleotide concentration of 1 mg/mL; UV spectroscopic measurements on diluted samples indicated that the absorbances of the resulting solutions were 24.0 and 22.6 respectively. A solution made up of 0.5 mL of poly rU and 9.5 mL of distilled water was prepared in a stoppered quartz tube and irradiated for 30 min as described in Material and Methods. UV spectroscopy indicated that the absorbance at 260 nm had gone from 1.30 to 0.08. Then 1.5 ml of this irradiated mixture was placed in a 3 mL cuvette and 1.5 mL of 100 mM DMEDA (pH = 7.5) was added. (DMEDA is one of the more reactive of the amines utilized in the experiments described above.) Using the H-P diode array spectrometer to monitor the progress of reaction of poly rU at ambient temperature, we observed that the absorbance at 301 nm increased from 0.045 to 0.23 over a 64 min period. After this solution had incubated for 8 days in a refrigerator at 4°C, the absorbance at 301 nm had increased to 0.6. A similar study with poly rC indicated a lesser reactivity, with the absorbance at 297 nm increasing from 0.068 to 0.09 over a 36' incubation period at room temperature; after 8 days incubation in the refrigerator, the absorbance at 297 nm had increased to 0.144. These results suggest that incubation of both irradiated poly rU and poly rC with DMEDA yields ring-opened adducts. The reactivity of irradiated poly rC toward formation of putative adducts is considerably smaller than that of irradiated poly rU. This observation suggests, but does not prove, that uridine hydrate-amine adducts, formed by incubation of RNA containing photohydrates with appropriate amines, could be quantitatively more important products than the corresponding cytidine-amine adducts.

Thermal reaction of dUrd and Urd photohydrates with ammonia at basic pH

As mentioned in the Introduction, Fikus and Shugar (18) found that incubation of the Urd photohydrate in solution, adjusted to pH = 12 with NaOH or KOH, induced formation of a ring-opened product that subsequently reverted to Urd ; a direct reaction of the hydrate to form Urd and an irreversible reaction leading to another product also occurred. They proposed that the reversible product was **1c**, an opened-ring form of the hydrate (Scheme 3). The same reaction appeared to occur in 1 M ammoniacal solutions at pH = 12. They also noted that the same type of reaction and kinetic behavior occurred with dUrd hydrates. We

repeated this work for both Urd hydrates and dUrd hydrates in hydroxylic and ammoniacal solutions and likewise found that very similar UV spectral changes occurred in both systems. Extending this work, using HPLC analysis, we found that two products, both with UV spectral characteristics expected of ring-opened adducts, were formed in ammoniacal solutions of the hydrates of Urd and dUrd. This work is described in Appendix A4. We did not, however, answer the question as to whether these two compounds are ammonia adducts, ring-opened forms of the hydrate or one of each; In principle, questions concerning the nature of these two adducts can be answered *via* mass spectral and proton NMR studies of pure samples of the two adducts; however, for practical reasons, such studies could be quite difficult (see Appendix A4). We instead focused on the question of whether ring-opened amine adducts are formed when photohydrates of dUrd or Urd are incubated with a different amine at basic pH.

Thermal reaction of dUrd photohydrates with ethylamine at basic pH

We next examined the HPLC of reaction mixtures in which dUrd hydrate was incubated with ethylamine. A solution of dUrd (167 mL, 1 mM) was irradiated in the cold at 254 nm for 60 min. UV spectroscopy of the irradiated solution that was diluted 10 fold indicated that little dUrd remained. We then added sufficient 1 M ethylamine to make the solution 100 mM in this compound (pH = 10.7). We made 10-fold dilutions of appropriate volumes of the samples taken after a number of incubation times and measured the UV spectrum. The observed time course of the overlaid spectral curves was very similar to the time course displayed for the Urd hydrate-ammonia system (see above). Portions of the samples were rotatory evaporated to dryness, redissolved in distilled water and chromatographed on Column B using 80/20 H₂O/MeOH flowing at 2 mL/min as eluent. After 4 min incubation, two product peaks with UV spectral properties expected of ring-opened adducts appeared with elution times of 2.78 min ($\lambda_{\text{max}} = 297 \text{ nm}$) and 5.33 min ($\lambda_{\text{max}} = 307 \text{ nm}$). The ratio of the peak areas, calculated using values measured at λ_{max} , was $A_{2.78}/A_{5.33} = 21$. With continuing time of incubation, the peak areas for the two putative adducts decreased and the amount of dUrd in solution increased; this behavior is similar to that seen in incubations of the photohydrates of Urd and dUrd with ammonia at basic pH.

An adduct formed after irradiation of dUrd in the presence of ethylamine is identical to the thermal reaction product of dUrd hydrate with ethylamine

In previously unpublished work, Hom (17) showed that after dUrd (1 mM) was irradiated in 100 mM ethylamine solution at pH 10.4, a mixture of ring-opened adducts were present as the main products. The general structure of the photoadducts was that displayed in Scheme 4 as **IIIh** (the E-isomer of the adduct); this structure was established by use of UV spectroscopy, mass spectrometry and proton NMR spectroscopy (see details in Appendix A5). We repeated this photochemical reaction, analyzing the reaction mixture using the same HPLC conditions described above; we observed two peaks corresponding to putative adducts. The retention times of the two adduct peaks seen after incubation of dUrd hydrate with ethylamine and those of the two adduct peaks produced when dUrd is irradiated in the presence of ethylamine are almost identical. In addition, the UV spectra of the material eluting in each of the corresponding adduct peaks in the two experimental systems, were superimposable, as shown by using the spectral capture feature of the diode array detector. Thus, we can identify adducts formed by incubation of the dUrd hydrate as being of type **IIIh** (in E and Z forms). It appears likely that at least a portion (if not all) of the **IIIh** formed, upon irradiation of dUrd in the presence of ethylamine at pH 10.4, is due to thermal reaction of the 2'-deoxyribose analogs of **Ib** and **Ib'** with ethylamine.

Analysis of the NMR spectrum of **IIIh** in d₆-DMSO (one-dimensional and correlated spectroscopy (COSY)) has been done and the results are presented in Appendix A5. It has

been found that multiple forms of **IIIh** are present in the NMR sample; indeed, the NMR data for the non-exchangeable protons have similarities to the NMR data obtained for the EDA adduct PEDA₂ (see above and Appendix A2a). Thermal reaction of dCyd photohydrate with ethylamine at basic pH leads to formation of an adduct analogous to **IIIh**. After incubation of dCyd photohydrates with ethylamine at basic pH (e.g. 10.5), compounds with HPLC characteristics and absorption spectral profiles similar to those expected for opened ring adducts appeared. As shown in Appendix A6, the predominant product formed in this incubation is (N-(N'-2'-deoxyribose-1'-yl)carbamoyl)-3-ethylaminoacrylamidine, which is analogous in structure to **IIIh**; this compound is also found as one of the products when solutions containing dCyd and ethylamine are irradiated at basic pH at 254 nm. More extensive information, including UV spectra, the molecular mass and proton NMR spectroscopic data, will be found in Appendix A6. The analogous results for the corresponding dCyd-ethylamine adduct, found after dCyd is irradiated in the presence of ethylamine at basic pH, are given in Appendix A6. The adduct N-(N'-ethylcarbamoyl)-3-(2'-deoxyribose-1'-yl) aminoacrylamidine, which has a structure analogous to **IIc**, is also produced in this irradiated system (17).

Discussion

In the preceding Results section, we have described the production of ring-opened amine adducts of Urd, dUrd and dCyd that occurs when the photohydrates of these compounds are incubated in the presence of the parent amine at near neutral pH. It can be conjectured that similar reactions to form adducts occur in UV-irradiated RNA or DNA in its cellular environment. In such putative reactions, Spd, Sper and, possibly, Put would react with RNA or DNA pyrimidine nucleosides that had been previously converted to hydrates by absorption of UV light. Proving that such reactions actually occur at the cellular level could present a worthy challenge for a resourceful photobiologist. One obstacle to successful investigation could be the potential instability of adducts towards formation of parent hydrate (in the case of Urd adducts) or deaminated adduct (in the case of dCyd or Cyd adducts) after transferal from amine-containing medium (the cellular milieu) to water, phosphate buffer or other media used for *in vitro* investigations (see Scheme 5, Scheme A3-3 and the accompanying discussions). If such reactions do occur *in vivo*, delineation of the biological ramifications of such lesions could provide interesting opportunities for investigation.

Are photohydrates formed upon UV irradiation of cellular structures containing DNA and RNA? In the case of DNA, mass spectrometric studies indicated that dCyd hydrates are, in fact, formed upon irradiation of cellular DNA with UVC (9). However, the yield was at least 100-fold less than the total yield of dimeric type compounds (i.e. cyclobutane dimers and (6-4) adducts). (It should be noted that the amounts of dCyd hydrate formed were monitored by assessing the amounts of dUrd hydrate present in digests of the irradiated DNA; thus, the amount of dCyd hydrate that, instead, underwent reversion to parent dCyd was not measured.) Gorelic (25) has shown that Urd hydrates are formed when *E. Coli* 30 S ribosomes are irradiated with UV light and that this reaction appears to be competitive with UV light-induced RNA-protein cross-linking reactions. Early studies, summarized in (26), suggest that Urd photohydrates and, in some cases Cyd hydrates, are formed in tRNA, mRNA and RNA viruses when irradiated with UVC light. As one example (27), it was shown that irradiation of single stranded genomic RNA, isolated from the R17 virus, with monochromatic 280 nm light induced both Urd and Cyd hydrate formation; it was determined that, under the irradiation conditions used, the photoproducts consisted of about 63% pyrimidine cyclobutane dimer, 42% Urd hydrate and 5% Cyd hydrate. Based on the results referenced above, it is plausible that UVC irradiation can produce photohydrates in single stranded regions of irradiated cellular DNA, as well as in various cellular RNA

macromolecules and RNA macromolecular complexes containing single stranded RNA, Such photohydrates presumably would be available for reaction with polyamines to form adducts. One important unanswered question for the photobiologist would be “Can treatment of cells with UVB light induce such reactions (i.e. formation of Urd, rCyd and/or dCyd hydrates, followed by reaction with polyamines)?”. Of relevance to this question, dCyd displays a UV spectrum with significant absorbance between 290 and 300 nm (see Fig. S12 in the Supporting Information).

Budowsky and co-workers (28) have indicated that cytosine hydrates, formed by UVC irradiation of the RNA bacteriophage MS2, are responsible for the observed cross-linking of the coat protein to the genomic RNA of this virus. These hydrates evidently react thermally with lysines in the coat protein of this phage, resulting in RNA-protein cross-linking. The proposed nature of this type of cross-linking is different than the general type of cross-link that would be produced by the reactions described in this paper. Acid digestion of the irradiated MS2 phage yields a modified cytosine, namely ϵ -N-(2-oxopyrimidyl-4)-lysine (29) as one of the hydrolysis products. It was therefore concluded that this conjugate (a cytosine transamination product) is responsible for the observed UV-induced RNA-protein cross-linking in MS2 phage, as well as that observed when this same phage was treated with sodium bisulfite.

What kind of processes might be affected by formation of adducts? Many *in vitro* studies have been made of the interactions of polyamines with RNA of various types (e. g. ribosomal and transfer RNAs) (30), as well as DNA (30, 31). It is thought that many of the same types of interactions occur *in vivo* as well and are involved in the regulation of cellular processes, including, for example, chromatin remodeling and transcription (32). Formation of dCyd adducts with Spd and Sper, followed by their conversion to more stable dUrd adducts, could have effects on transactions involving DNA, perhaps leading to undesirable effects on cellular function. Similarly, formation of Urd-Spd (Cyd-Spd) or Urd-Sper (Cyd-Sper) adducts in nuclear or cytoplasmic RNAs (e.g. transfer RNAs, ribosomal RNA, messenger RNA) could have adverse consequence in processes involving these macromolecules. Presumably such biological effects would be mediated by alterations in local structure and/or changes in accessibility of the modified base toward proteins or other molecules involved in biochemical processing activities. It should be reemphasized that a particular adduct at a given site may have a transient existence. Adducts appear to form in the presence of amine and revert to hydrate when amine is removed (see above). This suggests that a dynamic equilibrium exists between photohydrate and adduct (see Scheme 5). Consider, for example, a RNA population containing several RNAs with hydrate lesions or a DNA containing several hydrates. Unless equilibrium constant considerations strongly favor adduct formation at the concentration of polyamine in the RNA or DNA environment (i.e. nearly all hydrates are converted to adducts), then an adduct decomposition-formation cycle would be set up. As a result, it is possible that the existence of amine adducts might be transient at a particular site and that sites of adduct formation could move around.

It is interesting to note that the level of two of the polyamines studied here are present in the millimolar range in rat liver nuclei, with measured values of 5.3 mmole/kg of tissue for Spd and 4.5 mmole/kg for Sper (33); the level for Put was determined to be 58 μ mole/kg. (The concentration of Sper used in our incubations was about 22.5 mM, while that of Spd was 45 mM.) These three polyamines are also present external to the nucleus, with 90% of total Spd and Sper being in extranuclear compartments and 80% of total Put being there (33).) The effective concentrations of the various polyamines in the neighborhood of DNA and RNA molecules in the cell would be expected to be higher because of attractive interactions between the negative charges on the phosphate backbone of the nucleic acids and the multiple positive charges carried by polyamines at physiological pH.

Another possible reaction that could occur at physiological pH is adduct formation *via* reaction of N-terminal amino groups of proteins complexed with photohydrate-containing DNA or RNA. The peptides (Gly)₂ (pK_a = 8.25) and (Gly)₃ (pK_a = 7.91) can be viewed as models for N-terminal amino acid regions in proteins. The observed reactivities of the amino groups of these peptides with nucleoside photohydrates suggest that, under the right circumstances, thermal adduct formation could occur in UV irradiated nucleic acid-protein complexes. If so, this would lead to formation of transient DNA-protein cross-links with unknown biological consequences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Scheme 2.

Scheme 3.

- IIa: $R = \text{NH}(\text{CH}_2)_2\text{NH}_2$, $R' = \text{Rib}$
IIb: $R = \text{Rib}$, $R' = \text{NH}(\text{CH}_2)_2\text{NH}_2$
IIc: $R = \text{NH}(\text{CH}_2)_2\text{NH}_2$, $R' = \text{dRib}$
IId: $R = \text{dRib}$, $R' = \text{NH}(\text{CH}_2)_2\text{NH}_2$
IIe: $R = \text{NHCH}_2\text{CONH}_2$, $R' = \text{Rib}$
IIf: $R = \text{Rib}$, $R' = \text{NHCH}_2\text{CONH}_2$
IIg: $R = R' = \text{Ethyl}$
IIh: $R = \text{dRib}$, $R' = \text{Ethyl}$

Scheme 4.

Scheme 5.