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Formation of Singlet Oxygen by Urocanic Acid by UVA Irradiation and Some Consequences Thereof¶

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ABSTRACT

Singlet oxygen–initiated decomposition of urocanic acid (UCA) (3-(1*H*-imidazol-4(5)-yl)-2-propenoic acid) was used to successfully confirm the report that UCA generates singlet oxygen when irradiated with ultraviolet A light (UVA). The UCA-generated singlet oxygen converts UCA to one or more products that then catalyze the further destruction of the UCA with UVA light by singlet oxygen formation. Some nicking of the ϕX-174 supercoiled plasmid DNA was observed when UCA was irradiated with UVA to complete destruction of the starting material, and the product mixture was then mixed with the plasmid in the dark. More extensive nicking was seen when the photoproduct mixture and the plasmid were irradiated with UVA light. An ''aged'' (4 days) solution of UCA photoproduct no longer caused nicking in the dark but retained the capability to nick the plasmid when irradiated. There is evidence for the presence of hydroperoxides in the UCA photolysis product mixture, and the quenching studies with 2-propanol indicate that free radicals are involved in the plasmid-nicking photochemistry. Singlet oxygen does not appear to play a role in the nicking of the plasmid.

INTRODUCTION

Chronic exposure to ultraviolet A (UVA) light (320–400 nm) can cause premature aging and cancer of the skin (1–4). The mechanisms by which these processes occur have not been completely determined. However, there is strong evidence that reactive oxygen species (ROS) plays a role, presumably generated by an endogenous UVA-absorbing sensitizer (1,5). (*E*)-urocanic acid (UCA) (3-(1*H*-imidazol-4(5)-yl)-2-propenoic acid) is a metabolite of histidine and a substantial constituent of the stratum corneum, making up 0.7% of the dry weight of the epidermis (6). Although there is no significant absorption above 320 nm in the UV absorption spectrum of UCA at neutral pH, there are reports on *E–Z* photoisomerization using broadband light with wavelengths between 340 and 400 nm (7). In addition, it has recently been reported that excitation of UCA with monochromatic light at 351 nm leads to singlet oxygen emission (8). This is the first published report on UCA-generated singlet oxygen formation using UVA light.

Photoacoustic calorimetry (PAC) of UCA does, in fact, indicate the presence of a transition between 320 and 380 nm (8). The maximum for this transition has been estimated to occur at *ca* 340 nm (8). In addition, time-dependent density functional theory calculations have confirmed the existence of transition at *ca* 340 nm (9). It was noted that the PAC spectrum resembles an earlier-published action spectrum (2) for the UVA photoaging (*e.g.* photosagging) of skin (8). Because there is considerable evidence that UVA aging of skin is at least partly attributable to ROS, Hanson and Simon conjecture that the UVA photolysis of UCA may play a role in the photoaging phenomenon (8). In this article we (1) verify that the irradiation of UCA with UVA light produces singlet oxygen; (2) demonstrate that this irradiation also leads to the creation of radicals; (3) show that excitation of UCA in this region can sensitize the nicking of ϕX-174 plasmid DNA; and (4) provide evidence that an intermediate photoproduct may play a major role in these outcomes.

MATERIALS AND METHODS

Chemicals. (*E*)-UCA was obtained from Sigma (St. Louis, MO) and was used as received unless otherwise stated. The (*E*)-UCA was recrystallized as reported previously (10). A photostationary-state mixture of (*E*)- and (*Z*)-UCA was obtained by irradiation of an Ar-saturated (*E*)-UCA solution using 311 nm light. Superoxide dismutase (SOD), Trizma® hydrochloride, Trizma® base, boric acid, catalase and L-histidine monohydrochloride were also from Sigma. (*Z*)-UCA was prepared as reported previously (11). Silica-bound rose bengal was prepared as reported previously (12). NaOD solutions 10% in D_2O were prepared by dissolving 1 g of NaOH in 10 mL of D_2O, evaporating the solvent and reconstituting the solids with 10 mL of D_2O three times. Glycerol and ethidium bromide were from Aldrich (Milwaukee, WI). Bromophenol blue was from Fisher (Fairlawn, NJ). Ethylenediamine tetraacetic acid (EDTA) was from ICN Biomedical Inc. (Aurora, OH). Agarose was from Bethesda Research Lab (Gaithersburg, MD). ϕX-174 plasmid DNA was from New England BioLabs (Beverly, MA). High-performance liquid chromatography (HPLC) water was distilled in glass using a Corning MP-1 water still, whereas other HPLC grade solvents, phosphate salts, NaOH, acetic acid and ammonium acetate were from Mal-

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Abbreviations: EDTA, ethylenediamine tetraacetic acid; Form I, circular supercoiled plasmid DNA; Form II, circular relaxed plasmid DNA; HPLC, high-performance liquid chromatography; PAC, photoacoustic calorimetry; ROS, reactive oxygen species; SOD, superoxide dismutase; UCA, urocanic acid; UVA, ultraviolet A radiation at 320–400 nm.

Table 1. Effects of wavelength, temperature, pH, singlet oxygen quencher and presence of oxygen and solvent on (*E*)-UCA photolysis with UVA light*

Sample description	Light source	Time of irradiation (h)	Solvent	pH or pD	Temperature (°C)	% destruction of UCA ($Z + E$)	% isomerization
(*E*)-UCA	>330	20	D_2O	5.5	8	2.0	3.5
(*E*)-UCA	>330	20	D_2O	7.0	8	15.4	1.8
(*E*)-UCA	>330	20	D_2O	7.8	8	60.2	1.0
(*E*)-UCA	>330	20	D_2O	7.8	0	11.5	1.5
(*E*)-UCA	>330	20	D_2O	7.8	30	80.0	1.0
(*E*)-UCA	>330	20	H_2O	8.0	30	14.0	6.0
(*E*)-UCA	>311	0.5	D_2O	7.8	30	0	50.0
(*E*)-UCA + 15 equivalents of histidine	>330	20	D_2O	7.8	30	3.5	5.0
(*E*)-UCA under Ar	>330	20	D_2O	7.8	30	0	8.0

*In all cases the solution occupies *ca* 25% of the reaction vessel volume, *i.e.* there is a large excess of oxygen present.

linckrodt (Paris, KY). Deuterium oxide was from Cambridge Isotope Laboratories (Andover, MA). EM Quant® peroxide test strips were from EM Science (Gibbstown, NJ).

Instruments. An Orion 290A pH meter with a Triode® pH electrode was used to measure the pH of solutions. The pD values of buffered D_2O solutions were uncorrected pH meter readings. UV absorption spectroscopy was done using a Varian Cary 100 scanning UV–visible spectrophotometer. Samples were analyzed in 1 cm^2 matched quartz cells from Wilmad (Buena, NJ). HPLC analyses were performed using a Varian 5000 series liquid chromatograph fitted with a 7125 Rheodyne injection valve and a 200 μL injection loop. A Varian 2050 variable wavelength detector set at 254 nm monitored the column effluent, and the data were recorded using Packard Radiomatic Flo-One analysis software. An Alltech Econosil C-8 (4.6 mm × 25 cm) reversed-phase stainless steel column was used with the following isocratic condition: 1 mL/min 100% 50 m*M* ammonium acetate buffer (pH 5). The retention times were 5.0 and 9.6 min for the *E*- and *Z*-isomers, respectively. For quantitative work the (*Z*)-isomer area counts were multiplied by a factor of 1.4 to correct for its lower extinction coefficient compared with that of (*E*)-UCA (6).

Irradiations. Photolyses of UCA with λ > 330 nm light used cylindrical Pyrex tubes (12 × 150 mm) in a photolysis box in which samples in a turntable revolved around a water-cooled 450 W Hanovia medium-pressure mercury lamp filtered with a uranium yellow glass filter. The incident light at the tubes was measured as 4.6 mW/cm^2 using a UVX Radiometer with a UVX-36 sensor, from UltraViolet Products, Inc. (San Gabriel, CA). The samples were immersed in a temperature-controlled water bath. Alternatively, monochromatic 311 nm radiation was provided by Philips Ultraviolet B low-pressure 311 nm lamps in a fan-cooled photolysis box with a turntable occupying a central position. Singlet oxygen was generated by the irradiation of silica-bound rose bengal using a slide projector's 500 W tungsten lamp filtered with an LL-500 Corion corp. filter that transmits λ > 500 nm. The presence of peroxide in solution was detected using EM Quant® peroxide test strips.

Photocleavage of ϕX-174 phage DNA. Sixty microliters of 50 m*M* phosphate buffer, pD 7.8, was added to 30 μL of commercial ϕX-174 phage DNA to obtain a DNA solution, which was 205 μ*M* in base pairs. Two different solutions were irradiated with DNA: (1) a 3 m*M* solution of 12× recrystallized (*E*)-UCA in 50 m*M* phosphate buffer, pD 7.8; and (2) a 15 mL 3 m*M* solution of recrystallized (*E*)-UCA in 50 m*M* phosphate buffer, pD 7.8, which was saturated with O_2, capped, sealed with parafilm and irradiated with λ > 330 nm for 34 h. These solutions were saturated with O_2 or Ar, and 7 μL was placed into end-tapered tubes containing 3 μL of ϕX-174 phage DNA solution. These were inserted into Corning 10 × 75 mm Pyrex test tubes, the tubes were capped with septa and parafilm sealed, and the atmosphere was saturated with O_2 or Ar. Some experiments included histidine hydrochloride (45 m*M*) to scavenge singlet oxygen, 2-propanol (0.5 *M*) as a free radical scavenger, catalase (25 μg/mL) and SOD (25 μg/mL). Controls also included unirradiated DNA, irradiated DNA free of drug and unirradiated DNA–UCA solutions. After irradiation, 10 μL of 10× stop buffer–loading solution (50% glycerol, 0.2% bromophenol blue, 0.05 *M* EDTA in 100 m*M* Tris buffer, pH 8) was added to each sample, and the samples were loaded onto a 0.8% agarose gel containing ethidium bromide (0.5 μg/mL). The gel was run in 0.5× Tris-borate EDTA buffer containing ethidium bromide (0.5 μg/mL). After electrophoresis at 132 V for approximately 1.5 h, the DNA was visualized by UV light using a NucleoVision gel analyzer. Gel bands were quantified using Gel Expert software, with the intensities corrected for the decreased binding of ethidium bromide to supercoiled DNA (*i.e.* circular supercoiled plasmid DNA [Form I] is 1.4 times less responsive than circular relaxed plasmid [Form II] or linear plasmid DNA) (13,14).

RESULTS AND DISCUSSION

UCA-sensitized formation of singlet oxygen by UVA light

In our initial studies we confirmed that the sensitized formation of singlet oxygen by UVA light could be monitored using the singlet oxygen–initiated decomposition of UCA as our probe (15). A 2 mL solution of 3 m*M* (*E*)-UCA in 50 m*M* phosphate buffer, pH 8, was saturated with O_2 and irradiated at 30°C with λ > 330 nm for 20 h. This is a region where UCA shows negligible light absorption (8,16). HPLC analysis indicated that 6% of the initial (*E*)-UCA had undergone isomerization, whereas 14% of the total UCA (*i.e.* *E*- plus *Z*-isomers) had been destroyed. The reaction depended on the presence of oxygen and was greatly enhanced when D_2O was used as solvent. In the latter case there was as much as 80% destruction of the total UCA after 20 h of irradiation. Also, reaction in D_2O was quenched by histidine, a known singlet oxygen scavenger (17). In addition, this reaction showed temperature and pH dependence, being more reactive at higher temperatures and at alkaline pH. At pH 8, UCA exists predominantly as the carboxylate anion (6). Neither spectroscopy nor theoretical calculations give evidence for any significant electronic perturbations at wavelengths greater than 320 nm across the pH 6–8 region (9). It is interesting that histidine also shows a strong increase in its reactivity with independently generated singlet oxygen on going from pH 6 to 8 in a manner that matches well with the histidine pK curve (18), *i.e.* the protonated imidazole ring is relatively unreactive with singlet oxygen. The salient data are summarized in Table 1.

These results are in contrast with what one observes when

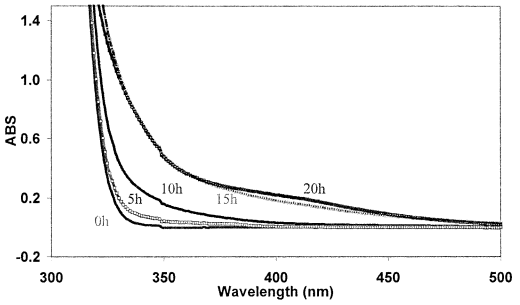


Figure 1. Change in absorption spectra as a function of irradiation time when (*E*)-UCA is photolyzed with light > 330 nm in D_2O, pD 7.8. From left to right in the figure, the time points are 0, 5, 10, 15 and 20 h of irradiation.

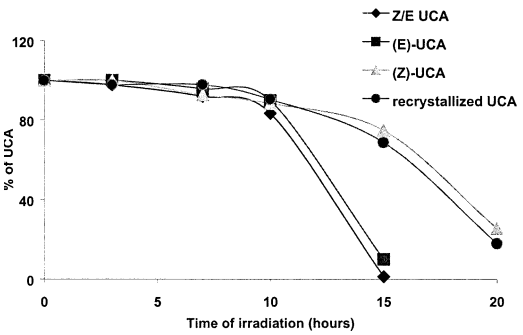


Figure 2. Time study for the photodestruction of various samples of UCA in D_2O, pD 7.8, with light > 300 nm.

UCA is irradiated at 311 nm (Table 1) and are actually more similar to the consequences of irradiation at 254 nm. It has been demonstrated that photolysis of UCA at these wavelengths excites the molecule into two different singlet states that are embodied within one absorption envelope having a λ_{max} at 280 nm at pH 7.2 (19–21). In particular, we have noted in the earlier work (21), and see again here, that 311 nm produces efficient *E–Z* isomerization and little decomposition. Photolysis at 254 nm gives a higher ratio of decomposition to isomerization, at least in part because of the 10-fold less efficient isomerization chemistry at this wavelength (21). These results indicate that excitation beyond 330 nm leads to different chemistry from that seen at 311 nm.

During this work we noted that the self-sensitized oxidation of UCA leads to the development of a yellow color in the photolysis solutions after *ca* 10 h of irradiation. This is evident in the UV–visible absorption spectrum of the reaction mixtures (Fig. 1).

In a more quantitative analysis of the reaction as a function of reaction time, we irradiated four sets of 2 mL solutions of 3 m*M* UCA in oxygen-saturated D_2O containing 50 m*M* phosphate buffer, pD 7.8, at 30°C with $\lambda > 330$ nm. The UCA samples consisted of commercial (*E*)-UCA, a sample of (*E*)-UCA that had been recrystallized five times, synthetic (*Z*)-UCA (12) and a mixture of (*E*)- and (*Z*)-UCA prepared by 311 nm photolysis of the commercial *E* isomer. The reactions were monitored at 5, 10, 15 or 20 h. The total loss of the UCA in each case is presented in Fig. 2.

It is clear from this figure that the photodestruction of the UCA involves an induction period, which we visually observe to be accompanied by the generation of the yellow color. This induction period is present in all the samples. However, the (*Z*)-UCA and the 5× recrystallized sample of (*E*)-UCA show the slowest rate of destruction in the postinduction process. The commercial sample of *E* is faster than either of these, and the (*E*)–(*Z*) mixture is faster yet. The latter observation is consistent with our observation that the reaction of purified (*E*)-UCA, (*Z*)-UCA or an (*E*)–(*Z*) mixture with independently generated singlet oxygen using photosensitization by silica-bound rose bengal indicates that the *Z* isomer is *ca* 1.4 times more reactive toward singlet oxygen than the *E* isomer is (data not shown). There are some striking similarities between our observations and those made with histidine, albeit upon its photolysis at 254 nm (22). Hasselmann and Laustriat (22) noted that a self-sensitized photooxidative degradation of the histidine is accompanied by the formation of a pale yellow polymer. The reaction is faster in basic media, quenched by 2-propanol and facilitated by a histidine photoproduct, with a discernible induction period. This photoproduct is attributed to a histidine hydroperoxide that is formed from His$^{\bullet}$ reacting with ground-state oxygen. The authors suggest that the histidine triplet state initiates the chemistry by generating singlet oxygen (22).

To examine the nature of (*E*)-UCA photodegradation by its photoproducts, we irradiated a 3 m*M* stock solution of 12× recrystallized (*E*)-UCA in 50 m*M* phosphate buffer, pD 7.8, with light >330 nm to complete the destruction of the UCA. Additional (*E*)-UCA was then added to the photolyzed mixture to form a 1 m*M* (*E*)-UCA solution. Irradiation of this solution under O_2 with light > 330 nm for 4 h gave a 92% destruction of the UCA. Irradiation under Ar resulted only in isomerization and not in destruction of the UCA. Photolysis in oxygen in the presence of 15 molar equivalents of histidine or 0.5 *M* 2-propanol led to 9 or 84% destruction of UCA, respectively. These results clearly indicate that the photodegradation of UCA by its photoproducts is oxygen dependent and that the formation of singlet oxygen plays a major role in the sensitized degradation. We believe that it is also more than coincidental that the amount of degradation nonquenchable by histidine (9%) correlates well with the diminution in UCA destruction (8%) in the presence of the hydroxyl radical trap, 2-propanol (see also subsequently). UCA has been shown to be a better hydroxyl radical scavenger than other 4-(5-) substituted imidazole derivatives such as histidine (23). It is noteworthy that a freshly irradiated solution of UCA, taken for the total destruction of UCA, tested strongly positive for the presence of hydrogen peroxide or hydroperoxides (or both) using an EM Quant® peroxide test strip.

In summary, we observe that (1) the UCA forms singlet oxygen with UVA light, with the reaction most efficient in D_2O, on the basic side of a pD range of 5.5–7.8; (2) the UCA reacts with singlet oxygen to form products, one or more of which are colored; (3) the product mixture is responsible for a much more rapid generation of singlet oxygen with UVA light, possibly because of greater light absorption at these wavelengths; (4) the photoproducts include peroxides, and a portion of the UCA degradation involves free radicals; and (5) the commercial (*E*)-UCA contains an impurity that accelerates these processes. We emphasize, however, that the extensively crystallized (*E*)-UCA and the

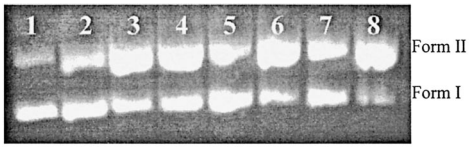
synthetic, purified (*Z*)-UCA show the same ability to generate the yellow intermediate and photosensitize their oxidative self-destruction.

Effects of UVA irradiation of UCA on ϕX-174 supercoiled DNA plasmid

In order to study the effect of UVA irradiation of UCA on biomolecules, we irradiated 12× recrystallized (*E*)-UCA with ϕX-174 supercoiled plasmid DNA at a phosphate–UCA ratio = 0.1. As with the previous experiments, the samples were in 50 m*M* phosphate buffer, pD 7.8. Agarose gel electrophoresis was used to separate Form I from its nicked Form II. We found that irradiation with 12× recrystallized (*E*)-UCA for 4 h produced only a small (*ca* 6%) level of nicking of the plasmid DNA (data not shown). In contrast, the irradiation of prephotolyzed UCA solutions with the plasmid generated extensive nicking. After a number of such experiments, it became clear that aging of the irradiated UCA solutions affected the plasmid results. We, therefore, irradiated a 3 m*M* stock solution of 12× recrystallized (*E*)-UCA in 50 m*M* phosphate buffer, pD 7.8, in D_2O to the complete loss of the UCA (and the concomitant appearance of a yellow color) and divided the solution into two parts. One portion was immediately mixed with the plasmid DNA, and the solution was irradiated for an additional 4 h. The second portion was allowed to stand in the dark for 4 days and then irradiated with the plasmid. The results are shown in Fig. 3.

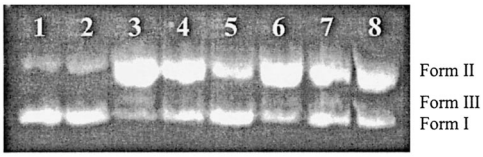
There are several noteworthy observations. (1) UVA light has no effect on the plasmid; the results in Lane 1 are typical of what we see for the plasmid when left in solution in the dark. (2) There is a modest, but apparently real, nicking of the plasmid by the fresh solution in the dark that is not evident for the ''aged'' solution (Lane 2 *vs* Lane 1). (3) Irradiation of either solution produces extensive nicking (*cf* Lane 4). To determine the effect of potential quenchers, we use the data from Lanes 3 to 8 *vs* Lane 2, relative to the results for Lane 4 *vs* Lane 2. (4) The histidine provides no protection against nicking (Lane 6), *i.e.* there is no evidence for singlet oxygen as a source of the nicking. Likewise, Ar degassing provides no protection (Lane 8). The contrasting effects of histidine and Ar here, relative to those observed for the sensitized self-destruction (see previously), may be a consequence of the very high sensitivity of UCA toward singlet oxygen (24). Note that Ar degassing is by necessity incomplete because the 3 μL solution of DNA was not degassed to avoid mechanical nicking of the plasmid. However, we would have expected at least partial protection by degassing the UCA solution. (5) The addition of 2-propanol provides significant protection (Lane 5), evidence for radical intermediates as part of the nicking process. (6) There is evidence for protection by SOD (Lane 7), especially in the fresh solutions. Though UCA has been shown to generate superoxide (25), it is hard to reconcile this result with the Ar studies at this point. Similarly, though the observation is reproducible, we have no explanation as yet for the catalase result in the aged solution (Lane 3).

The combination of a lack of protection by Ar degassing, quenching of nicking by a free radical scavenger and susceptibility to further UVA irradiation is consistent with the



Digitized Data

Lanes	1	2	3	4	5	6	7	8
% Form I	77.8	66.2	47.2	49.5	64.3	41.1	59.8	33.9



Digitized Data

Lanes	1	2	3*	4	5	6	7	8
% Form I	83.9	80.1	20.6	41.5	64.8	36.0	49.1	39.9

*6.9% Form III

Figure 3. Irradiation of UCA photodecomposition products with plasmid DNA using light > 330 nm for 4 h (see Materials and Methods for details). A fresh photoproduct solution was used for the experiment shown in the top gel; a 4 day aged solution was used for the bottom gel. Lane 1, DNA + light; Lane 2, DNA + photoproducts of UCA in the dark; Lane 3, DNA + photoproducts of UCA + catalase (25 μg/mL) + light; Lane 4, DNA + photoproducts of UCA + light; Lane 5, same as lane 4 + 2-propanol (0.5 *M*); Lane 6, same as lane 4 + histidine (45 m*M*); Lane 7, same as lane 4 + SOD (25 μg/mL); Lane 8, same as lane 4 under Ar.

presence of hydroperoxides in the UCA photoproduct mixture (see previously). Organic peroxides and hydroperoxides absorb in the UVA region and can photolytically generate free radicals in an oxygen-independent mechanism (26). UCA solutions irradiated to complete destruction with light > 330 nm and tested immediately after irradiation gave a much more (*ca* 10-fold) positive hydroperoxide test than aged solutions did. Such hydroperoxides could also be responsible for the dark nicking seen for freshly irradiated UCA solutions.

CONCLUSIONS

We have successfully confirmed the report that UCA generates singlet oxygen when irradiated with UVA light. The singlet oxygen converts UCA to one or more products that are more effective in causing the destruction of UCA with UVA light than UCA is. The photodestruction of UCA by its photoproduct(s) is caused by the generation of singlet oxygen. A freshly generated UCA photoproduct solution causes some nicking of Form I plasmid DNA. This dark nicking is not evident for an aged (4 days) photolysis solution. UVA irradiation of the UCA product mixture with the plasmid causes extensive nicking that is inhibited by 2-propanol but not by histidine or by Ar degassing. Thus, the formation of singlet oxygen does not appear to play a role

in the nicking of the plasmid. The UCA photoproduct solutions test positive for the presence of hydroperoxides, and these are likely to be major contributors to the plasmid-nicking chemistry. The studies designed to elucidate the structure of the UCA photoproduct(s) and the mechanisms of the dark and photoinitiated nicking of plasmid DNA are in progress. We are also further elaborating the potential photobiological consequences of UCA–UVA photochemistry.

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