

Effects of Ultrasound on Nucleic Acid Bases[†]

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ABSTRACT: To understand the effects of ultrasound in biological systems at the molecular level, sonolysis of nucleic acid bases at sonic intensities ≤ 5 W/cm² was studied. These sonochemical reactions were followed by UV-spectral decrease and by sonoproduct analysis. The order of reactivity was found to be thymine > uracil > cytosine > guanine > adenine. The extent of sonochemical reactions depends on the exposure time and the rate of the intensity. Aeration was necessary for maintenance of a reasonable reaction rate. "Threshold" intensities for uracil and thymine were observed at ~ 0.5 W/cm². The effectiveness of the dissolved gases in producing sonochemical reactions was Ar > O₂

> air > N₂ > He > N₂O, suggestive of free radicals mediating these reactions since N₂O is an effective radical scavenger. Studies of the effects of substituents have shown that electronic rather than steric effects may have a greater influence. Preliminary identification indicates that *cis*- and *trans*-uracil glycols are the major products of uracil. Thus, a stepwise mechanism of pyrimidine sonolysis is proposed. Sonolysis of a dilute aqueous solution of uracil yielded pseudo-first-order kinetics in terms of [Ura] with a rate constant of 3.9×10^{-2} min⁻¹, implying that the rate-limiting step is the reaction of HO· with the base.

It is generally acknowledged that, at sufficient levels, radiation can produce harmful effects on biological systems. Ultrasonic radiation is no exception. The clinical use of ultrasound has greatly increased in the past few years, principally because the benefit vs. risk judgment appears to be heavily weighted toward the beneficial aspects of this modality (O'Brien, 1977). However, one only needs to examine the history of other forms of radiation to realize that many effects, subtle as they may be, would not have been discovered without comprehensive experimental studies. Thus, a systematic investigation of the fundamental nature of chemical changes in nucleic acids produced by ultrasound was undertaken with an emphasis on the production, isolation, and identification of sonoproducts of nucleic acid components. Such an approach has provided essential breakthroughs resulting in explosive developments in the study of UV¹ radiation of nucleic acids (Wang, 1976). This paper, the first in this series, reports results of a general survey of the reaction parameters thought to be involved in the sonochemical studies of purine and pyrimidine derivatives.

Current activity dealing with the study of the effects of ultrasound on nucleic acids and components is sporadic. The effects of sonication upon nucleic acids in *in vitro* and *in vivo* systems have been briefly reviewed in a related article (Hattman, 1976). Earlier studies of the sonoeffects on purine and pyrimidine bases have been reviewed (El'piner, 1964). Since then, only sonodegradations of cytosine (El'piner and Sokolovskaya, 1963; Sachan et al., 1971) and of thymine (Mead et al., 1975) have been reported. In addition, a phosphate group attached to adenosine producing a stabilizing effect for adenosine has been noted by Braginskaya et al. (1972). They

also observed the relaxation processes induced by sonication which caused a disturbance of the conformational equilibrium existing in aqueous solutions of nucleosides (Braginskaya, 1974).

Experimental Section

Ultrasonic Exposure Systems. Two ultrasonic exposure systems were employed with the majority of experiments performed on the system developed by O'Brien et al. (1974), wherein equipment and dosimetric details are described. Briefly, the sonic irradiations were performed by transferring the solution under study into a cylindrical Pyrex glass vessel, similar to a T tube. The typical dimensions of the vessel are 1 cm inside diameter and 3 cm in length. Access to the vessel was through a stem which was mounted at 90° to the vessel axis and also served to support the vessel in the ultrasonic beam. Acoustically transparent windows (taut Saran Wrap maintained by O rings) sealed both ends of the vessel.

The vessel was positioned in the exposure tank in such a manner that the vessel and the ultrasonic beam axes were coincident and the distance from the transducer surface to the nearest acoustic window was 23 cm. The temperature was controlled at 30 ± 0.2 °C.

The sonic intensity was determined by two independent radiation force techniques. Beam plots of the ultrasonic field, at 23 cm from the transducer surface, yielded the 3 dB intensity beam width of 1.5 cm with a spatial peak (on-axis) intensity to spatial average intensity (I_{sa}) ratio of 1.4. That is, when the I_{sa} is 1 W/cm², the axial intensity is 1.4 W/cm². All results are reported in terms of the I_{sa} and all irradiations are at a sonic frequency of 1 MHz. The accuracy and precision of the reported I_{sa} are within $\pm 10\%$ and 1%, respectively. It should be pointed out, however, that the field inside the reaction vessel varies and can be several times greater than the free field peak intensity (Christman and Stratmeyer, 1976).

The second system employed was a Macrosonics Multisons 1100-broadband generator. The experiment performed with this sonicator illustrates the use of high pressure liquid chromatography (HPLC) as a method of detecting and assaying sonoproducts. Therefore, no attempt was made to determine the precise intensity or to eliminate standing waves. The details of the system have been reported by Spurlock and Reifsnider

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¹ Abbreviations used are: UV, ultraviolet; HPLC, high pressure liquid chromatography.

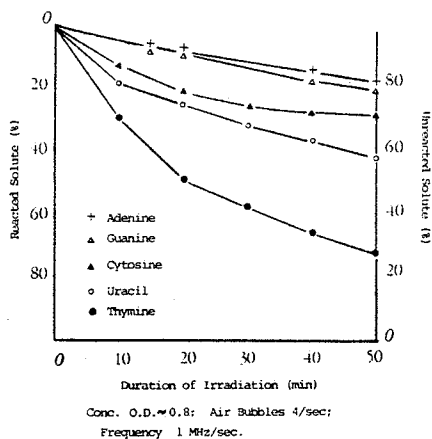


FIGURE 1: Relative sonochemical reactivity of nucleic acid bases at 5 W/cm².

(1970). Briefly, it operates at a frequency of 0.8 MHz with an approximate intensity of at least 10 W/cm², at which streams of air bubbles are clearly visible. Irradiation was carried out with 0.1 mM aqueous solutions for a period of 7 h without aeration. At various intervals during irradiation, aliquots were withdrawn and analyzed by HPLC.

Materials. Thymine (5-methyluracil), uracil, cytosine, adenine, guanine, 6-methyluracil, 5,6-dimethyluracil, and orotic acid were of the highest purity, commercially purchased, and were used without further purification. 1,3-Dimethyluracil (Wang, 1958), 5-bromouracil (Wang, 1959), and *cis*- and *trans*-uracil glycols (Hahn and Wang, 1977) were prepared in this laboratory.

General Sonication Procedures. The sonication vessel was filled with an ~0.1 mM aqueous solution of the compound. Through the stem of the vessel, a 1-mm inside diameter glass tube was inserted for aeration. The solutions were aerated for at least 10 min prior to and during sonication at a rate of 240 bubbles/min. The sonochemical reaction was monitored by UV absorption spectra (Beckman recording spectrophotometer Models DK1 or DB) or by HPLC (Waters Associates). For HPLC, a μ Bondapak-C₁₈ column was used and water served as the eluent. A Schoeffel SF 770 Spectroflow monitor was used at a wavelength of 227 nm for the detection and quantitation of sonoproducts in the eluates.

Results and Discussion

Relative Reactivity of Bases. Aerated aqueous solutions of uracil (Ura), thymine (Thy), cytosine (Cyt), adenine (Ade), and guanine (Gua) were irradiated at a I_{sa} of 5 W/cm² as a function of time. The results are plotted in Figure 1. The apparent order of reactivity as assayed by spectral change is Thy > Ura > Cyt > Gua > Ade. Sonochemical reaction with Thy was found to stop within a few minutes without aeration but was restored with aeration, suggesting that a critical level of dissolved O₂ is required for reactivity. Degassing by ultrasound and consumption of O₂ by oxidative reactions are conceivable causes for O₂ depletion. To test the degassing effect, sonication was carried out with O₂-saturated water. As measured with an oxygen electrode, O₂ was found to decrease from 85% saturation (13.3 ppm) to ~55% (~8.5 ppm) in approximately 3 min. Afterward, continued sonication resulted in no further decrease. The results indicate the O₂ contents could not be reduced below those of atmospheric saturation. Obviously, there is much to be done in order to comprehend the nature of the O₂ and other gas effects. However, the condition of continued aeration has been used throughout this study.

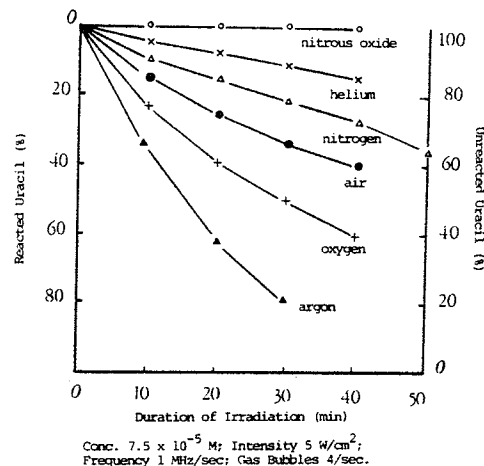


FIGURE 2: Effect of dissolved gases in water on sonication of uracil.

Since the order of reactivity was determined on the basis of absorbance decreases at 270 nm, the actual rates of reaction may be underestimated in cases where sonoproducts also absorb at 270 nm. Preliminary data from HPLC products separation indicate that the Thy reactivity should be augmented by ~10% and Ura requires no correction. Such a revision will not affect the given order of reactivity.

Sonic Intensity and the Rate of Reaction. A 0.1 mM aqueous solution of Ura was sonicated at intensities of 1, 3, and 5 W/cm² as a function of time. The percentage of unreacted Ura seems to be linearly dependent upon exposure time to a first approximation and was 75, 55, and 35% respectively, at 50 min.

"Threshold" Intensity for Thy and Ura. Aqueous solutions of Ura and Thy (0.1 mM) were irradiated for 30 min as a function of intensity from 0.3 to 5 W/cm². Thy exhibited a cutoff of sonolysis around 1 W/cm², and Ura around 0.5 W/cm². Because the increase in structural complexity generally results in a decrease in reactivity (Gupta and Wang, 1976) or an increase in the "threshold" intensity, "threshold" intensities for nucleosides, nucleotides, and nucleic acids remain to be determined.

Effects of Dissolved Gases. A 0.075 mM aqueous solution of Ura was flushed with a test gas for 10 min prior to and during sonication at 5 W/cm² as a function of time. The extent of reaction was monitored by the decrease in UV absorbance at λ_{max} . The results are shown in Figure 2. El'piner (1964) previously reported that the yields of sonoproducts of purine and pyrimidine bases were higher in the presence of dissolved molecular oxygen or argon than in the presence of air, but lowered in nitrogen, helium, or hydrogen, in apparent agreement with current observations. El'piner (1964) suggested that oxidation processes are favored in these sonochemical reactions.

Our finding the absence of sonolysis in N₂O is suggestive of free radicals mediating these sonochemical reactions because N₂O is an effective radical scavenger.

Effects of Electron-Donating Substituents. Aqueous solutions of Ura (0.15 mM), Thy (0.10 mM), Me⁶Ura (0.075 mM), and Me₂^{5,6}Ura (0.075 mM) were sonicated at 5 W/cm² and 25 °C. The order of reactivities, shown in Figure 3, seems to be Me₂^{5,6}Ura > Me⁶Ura > Thy > Ura, indicating that the presence of the electron-donating CH₃ group accelerates the rates of reaction. Thus, the electronic effect may have a greater influence than the steric effect. If the steric effect were operative, the CH₃ groups on the 5,6-double bond, i.e., the site of reaction (discussed below), should retard the rates of reaction.

Effects of Electron-Withdrawing Group. A similar study was made with Ura (0.09 mM), Br⁵Ura (0.1 mM), orotic acid (Oro, 0.1 mM), and Me₂^{1,3}Ura (0.1 mM), also at 5 W/cm² and 25 °C. As Figure 3 shows, the order of reactivity seems to be Br⁵Ura < Oro < Me₂^{1,3}Ura = Ura. Here, the effects of electron-withdrawing and the steric hindrance of 5-Br- and 6-COOH moieties cannot be distinguished because both result in decreases in the rates of reaction. It is probably the former, however, because steric effects as aforesaid may be ineffective. In contrast, the CH₃ substituents on N(1) and N(3), which are away from the 5,6-double bond, showed little effect on the reaction rate.

Total "Acoustic Dose" Effect on Sonoreactions. Aqueous solutions of Ura (0.15 mM) were sonicated at 1 W/cm² with 2, 10, 20, 40, or 120 "pulses" so that the total exposure time summed to 10 min. As assayed by OD_{260nm} decreases, all irradiated solutions resulted in 93.4% unreacted Ura. The effect seemed to be cumulative and the crucial parameter for this sonoreaction is the total dose; neither the pulse duration nor number of pulses seemed to exert any influence on sonoproduct formation.

Sonochemical Reaction Kinetics of Ura. A 0.075 mM solution of Ura was presaturated with argon for 10 min and then irradiated at an intensity of 5 W/cm² with argon aeration to achieve an increased rate of reaction. The change in optical density readings at 260 nm or in concentrations at time intervals (C_t) was observed. The reaction followed a pseudo-first-order kinetics giving a straight line by plotting log(C₀/C_t) vs. time with a rate constant of 3.9 × 10⁻² min⁻¹.

Tentative Identification of the Major Sonoproducts of Ura. This set of experiments employed the Macrosonics Multisons sonicator using a 0.1 mM solution of Ura. Samples at 0, 1, 2, 4, and 7 h of sonication were analyzed by HPLC. The peaks were identified as Ura and *trans*- and *cis*-Ura glycols by coinjection of each of the respective authentic samples and had retention times of 7.0, 5.25, and 5.0, respectively. These findings indicate that Ura glycols are the major sonoproducts of Ura.

A similar experiment was performed with a more concentrated (80 mM) solution with an 8 h sonication period. HPLC examination of the reaction mixture gave peaks with retention units of 5.0, 5.25, 7.0, 8.0, and 12. Treatment of the crude sonicated solution with either 10% NH₄OH or 10% HCl eliminated the first two peaks. In the case of alkaline treatment, a new peak was produced with a retention time of 18.5 units, identical with that of an authentic sample of isobarbituric acid. Acid treatment yielded no detectable additional peak. These results again identified glycols as sonoproducts (Hahn and Wang, 1977).

Upon lyophilization of the sonicated solution, the reaction mixture was applied on thin layer cellulose plates and the chromatogram was eluted with 1-propanol-water (9:1; v/v). Four spots with R_f values of 0.08, 0.46, 0.57, and 0.65 were revealed either with iodine test (Ekert and Monier, 1959) or by viewing with UV light (254 nm). Both the R_f values and the characteristics correspond well with *cis*- (0.46) and *trans*-glycols (0.57) and Ura (0.65), respectively. Treatment of a second sample with acidic *p*-dimethylaminobenzaldehyde (Fink et al., 1956) developed a faint yellow spot with R_f 0.52 which corresponded to urea.

Remarks

The extent of sonochemical changes may depend on the total dose and rate of the ultrasonic intensity for these pyrimidines and purines. Aeration is necessary for the maintenance of the reactions. "Threshold" intensities observed in the range of 0.5

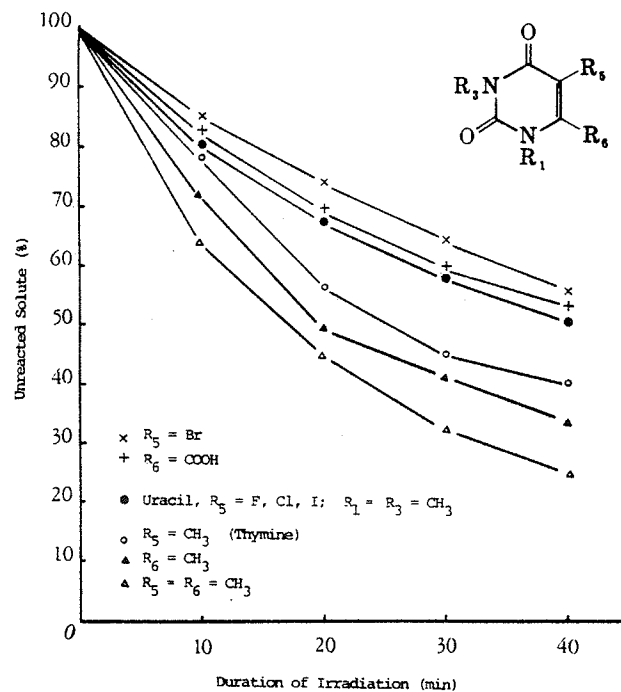
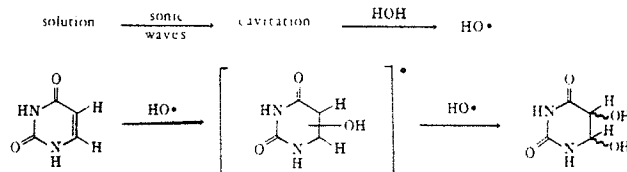


FIGURE 3: Substituent effects on the ultrasonic reactivity of uracil.

to 1 W/cm² suggest the intermediacy of cavitation (step 1) because it is in this intensity region that transient cavitation first appeared in water (Connolly and Fox, 1954). Theoretically, in dilute aqueous solutions such as those examined in this study, a peak ultrasonic pressure of 1 atm would require a sonic intensity of 0.33 W/cm² at 1 MHz. Although various gases seem to affect the rates of reaction, nitrous oxide completely inhibits the reaction. Because nitrous oxide is a known hy-

SCHEME 1: Reaction Scheme for Sonolysis of Uracil in an Aqueous Solution.



dride-radical scavenger, hydroxy radicals are thought to be involved (step 2). The subsequent step (3) must be the reaction of HO• with the bases and the rate-limiting step because the rate expression for this step is pseudo-first-order in terms of [Ura], i.e.,

$$-\frac{d[\text{Ura}]}{dt} = k[\cdot\text{OH}][\text{Ura}] = k'[\text{Ura}]$$

$$\frac{d[\text{Ura}]}{[\text{Ura}]} = k'dt$$

Since step 3 involves the disappearance of UV absorption maxima, the chromophore or the 5,6-double bond is probably the reaction center. This is further corroborated by the finding that only substituents on C(5) and C(6), not on N(1) and N(3), have influence on reaction rates. Intermediates formed in step 3, as shown, would react further to give the final products. The observation that the major products are the *cis*- and *trans*-Ura glycols lends additional support to the reaction scheme.

Along with the formation of HO•, it is likely that H• radicals are formed as water molecules are decomposed. The products possibly generated by H• radicals, such as dihydrouracil, 5-

hydroxy- and 6-hydroxydihydrouracil, were not detectable. It is possible that H \cdot reacts with O $_2$ to yield HOO \cdot which finally produces HOOH. Indeed, HOOH was found to be a major product.

Heating is not believed to be a factor in the results because temperature control of the saline solution in the exposure tank prevented any appreciable temperature fluctuation of the solutions with continuous bubbling. Studies have been made to investigate this and several other variables (Gupta and Wang, 1976).

Another aspect concerns the utilization of rather harsh experimental procedures such as bubbling gas through the media during radiation and subjecting the media at a higher intensity with a Macrosonics Multisons sonicator. Gas bubbling was not for the purpose of creating new sonoreactions but rather for maintaining reasonable rates of reaction. Prolonging a reaction allows a better understanding of the nature of that reaction and the characteristics of its products. Once such characteristics become known, their isolation and identification can be brought about with samples from high intensity radiation experiments which are capable of producing milligram quantities of the desired as well as secondary products.

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