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In Vitro Photodecomposition of Uric Acid in Presence of Riboflavin II

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Abstract □ *In vitro* studies on the photodecomposition of uric acid in the presence of the monosodium salt of riboflavin 5′-phosphate in buffers at various pH values, in methanol, and in human plasma are reported. The decomposition rate increased with increasing pH and was independent of solvent or buffer species. The mechanism appears to be an energy transfer process involving triplet riboflavin and singlet oxygen. Riboflavin-enhanced photodecomposition of uric acid occurred *in vitro* in hyperuricemic human plasma.

Keyphrases □ Uric acid—*in vitro* photodecomposition in presence of riboflavin, effect of pH and solvent □ Riboflavin—effect on *in vitro* photodecomposition of uric acid, various pH's and solvents □ Photodecomposition—uric acid in presence of riboflavin, effect of pH and solvent □ Vitamins—riboflavin, effect on *in vitro* photodecomposition of uric acid, various pH's and solvents

The successful phototherapy of psoriasis was reported using methoxsalen and longwave UV light (1), and phototherapy alone was used to treat neonatal hyperbilirubinemia (2–4). Riboflavin was reported to enhance bilirubin photocatabolism in rats (5).

Recently, an *in vitro* photodecomposition of uric acid was observed in the presence of the monosodium salt of riboflavin 5′-phosphate (I) and visible light in aqueous solutions (6). Increasing the I concentration appeared to increase the disappearance rate of uric acid. The addition of potassium iodide (10^{-3} *M*) to the reaction mixture retarded photodecomposition.

Because of the potential clinical importance of this observation (treatment of hyperuricemia), experiments were undertaken to characterize the pH dependence of the reaction and to determine if the reaction would proceed in a nonaqueous solvent, if the reaction was oxygen dependent, and if photodecomposition would occur *in vitro* in human plasma.

EXPERIMENTAL

The irradiation light box used standardized test tubes distributed radially at equal distances (approximately 26 cm) measured from the center of the test tubes to the center of a vertically positioned, unfiltered 15-w fluorescent bulb[1] emitting visible light. The inside of the apparatus was painted flat black to reduce any internally reflected light, and the outside was draped with black cloth to eliminate external light. The light intensity was monitored using a light meter placed underneath a test tube and was held constant using a transformer[2].

The light intensity was approximately 40 foot-candles for all experiments, except the plasma study performed at approximately 50 foot-candles. Test tubes used for the irradiation[3] were standardized (±2%) by measuring[4] the absorbance of a 9.7×10^{-6} *M* aqueous I solution at 450 nm.

All solutions were prepared using deionized water, except for the methanol studies, and stock solutions of uric acid and riboflavin were prepared fresh daily. Irradiation, spectrophotometric analysis[5], and all sample preparation were performed in a darkened laboratory. Plasma samples, analyzed by high-pressure liquid chromatography (HPLC), were kept in light-resistant containers from which samples were withdrawn.

Reagents—Uric acid[6], lithium carbonate[6], and the monosodium salt of I[7] were used as received. All other solvents and reagents were reagent grade.

HPLC System and Conditions—HPLC analysis[8] was performed at a fixed wavelength of 280 nm, using a stainless steel column (0.61 m × 2 mm i.d.) packed with an anion-exchange material[9] (7). A Sorenson phosphate buffer, pH 5.9, was the eluting solution.

Buffers—Sorenson phosphate buffers of approximately pH 5, 6, 7, and 8 and a Delory and King carbonate buffer of approximately pH 9 were

[1] Sylvania F15T8-CW.
[2] Powerstat type 3PN11688, Superior Electric Co., Bristol, Conn.
[3] Pyrex No. 9800.
[4] Coleman Jr. II spectrophotometer.
[5] Beckman model 25 recording spectrophotometer.
[6] J. T. Baker Chemical Co., Phillipsburg, N.J.
[7] Nutritional Biochemical Co., Cleveland, Ohio.
[8] Waters Associates liquid chromatograph model 6000 with Soltec recorder.
[9] VYDAK AM, Catalog No. 301, The Separations Group, Hesperia, Calif.

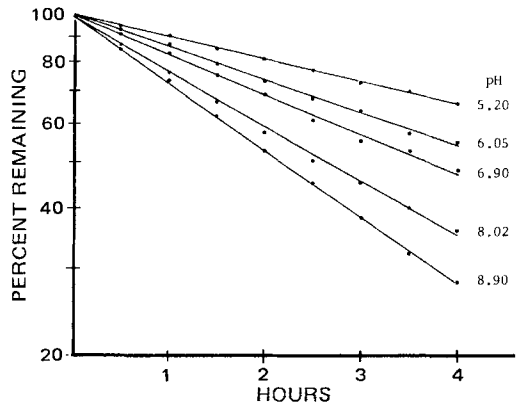

Figure 1—*Apparent first-order photodecomposition of uric acid (1.19 × 10^{-4} M) in the presence of riboflavin (3.88 × 10^{-5} M) in various phosphate buffers. Each point represents the average of four separate experiments performed in triplicate.*

prepared. In addition, a phosphate buffer of approximately pH 9 was prepared by adjusting the pH of 0.067 *M* dibasic sodium phosphate solution with 1 *M* NaOH. All buffers were boiled for 10 min after preparation, cooled, oxygenated for 5 min, and stored in the refrigerator. Before use, the buffers were removed from the refrigerator and allowed to reach room temperature.

Stock Solutions—Stock solutions of I (1.94 × 10^{-3} *M*) and uric acid (5.96 × 10^{-3} *M*) were prepared. The uric acid stock solution was prepared by solubilizing 10.0 mg of uric acid in 2.5 ml of 5.41 × 10^{-2} *M* lithium carbonate solution in a 10-ml volumetric flask and bringing the solution to volume with deionized water.

Test Solutions—Test solutions containing I (3.88 × 10^{-5} *M*) plus uric acid (1.19 × 10^{-4} *M*) and blanks containing I alone (3.88 × 10^{-5} *M*) were prepared from the stock solutions, the various buffers, and methanol. Test solutions of human plasma[10] were prepared containing uric acid (2.98 × 10^{-4} *M*) plus I (3.88 × 10^{-5} *M*) or uric acid alone (2.98 × 10^{-4} *M*) from the stock solutions. The uric acid concentrations in the plasma samples were elevated to simulate the levels in hyperuricemic patients.

pH Studies[11]—The standardized test tubes, each containing 10 ml of test solution or blank, were placed in the irradiation apparatus for light exposure. Other samples were kept in the dark. A stopwatch was used to measure sampling times, and tubes were removed randomly at each time interval. The pH of each solution was recorded before and after irradiation. The decrease in uric acid peak height was recorded using similarly irradiated I solutions as blanks. The wavelength for maximum absorption of the uric acid exhibited a bathochromic shift with increased pH (277 nm at pH 5.2 and 287 nm at pH 8.9).

Methanol Studies—These studies were performed in the same manner as the pH studies, except that methanol was used as the solvent and pH values were not recorded. Spectrophotometric analysis was performed at 293 nm using irradiated I solutions as blanks.

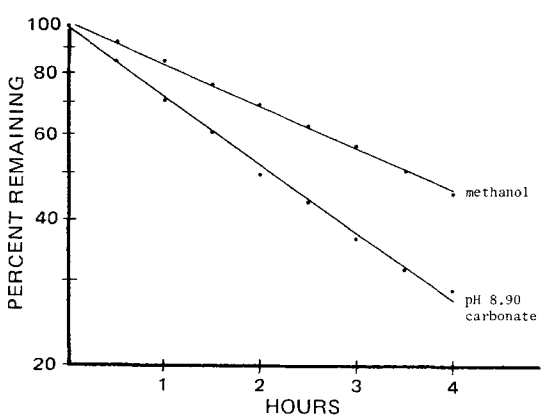

Figure 2—*Apparent first-order photodecomposition of uric acid (1.19 × 10^{-4} M) in the presence of riboflavin (3.88 × 10^{-5} M) in methanol and in a carbonate buffer. Each point is the average of four separate experiments performed in triplicate.*

[10] Outdated plasma, obtained from the Travis County Blood Bank, Austin, Tex.

[11] Model 5 pH meter, Corning Scientific Instruments.

Table I—Observed Rate Constants for Uric Acid Photodecomposition in the Presence of Riboflavin

Solvent	pH	K_{obs}, hr^{-1}
Phosphate buffer	5.20	0.102
Phosphate buffer	6.05	0.152
Phosphate buffer	6.90	0.185
Phosphate buffer	8.02	0.255
Phosphate buffer	8.90	0.317
Carbonate buffer	8.90	0.320
Methanol	—	0.198

Plasma Studies—Samples of 15 ml of plasma containing either uric acid plus I or uric acid alone were irradiated as already described. After irradiation, all samples were transferred to light-resistant containers and frozen until analyzed. The unfrozen samples were subjected to ultrafiltration[12] under darkened laboratory conditions prior to analysis, and the filtrate was collected in light-protected containers. Triplicate 20-μl aliquots of each filtrate were analyzed by HPLC.

A standard curve was prepared using filtered plasma solutions that were first spiked with uric acid plus I or uric acid alone. Analyses also were performed on samples of the original plasma to determine endogenous uric acid levels.

Anaerobic Studies—A 1000-ml volumetric flask containing approximately 900 ml of deionized water and a 500-ml volumetric flask containing 10.0 mg of uric acid dissolved in 2.5 ml of lithium carbonate (5.41 × 10^{-2} *M*) and brought to approximately 400 ml with deionized water were each boiled for 0.5 hr. After the heat was removed, nitrogen was bubbled through both solutions for 15 min. Then both solutions were capped and cooled. A 100-ml flask and 16 test tubes were flushed with nitrogen and placed in a glove bag[13] under a continuous nitrogen flow. A test tube rack and previously weighed packets of I (2.0 and 10.0 mg) were also placed in the bag, which was then sealed with clamps.

The laboratory lights were extinguished, and solutions of uric acid (1.19 × 10^{-4} *M*) plus I (3.88 × 10^{-5} *M*) and I alone (3.88 × 10^{-5} *M*) were prepared in the glove bag. Four blanks and 12 test solutions were placed in the test tubes, and the tubes were capped with waxed paper[14]-covered corks. The test tubes were removed from the bag, wiped clean, and placed in the light box. The glove bag was then placed over the light box to provide a nitrogen blanket during the experimental run. Spectrophotometric analysis was performed as previously described.

RESULTS AND DISCUSSION

Figures 1 and 2 show the pH dependence of the observed first-order disappearance of uric acid in the presence of visible light and riboflavin. There was no measurable decrease in uric acid when uric acid alone was exposed to light or when uric acid plus riboflavin was maintained in the

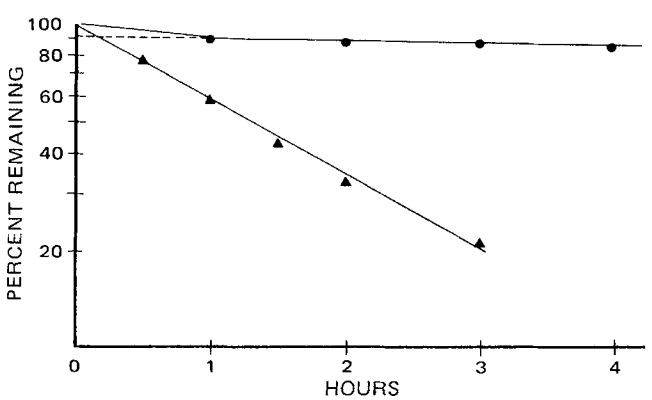

Figure 3—*Effect of oxygen on the photodecomposition of uric acid (1.19 × 10^{-4} M) in the presence of riboflavin (3.88 × 10^{-5} M). Key: ●, deoxygenated solutions; and ▲, nondeoxygenated solutions (6). Each point represents the average of three separate experiments performed in triplicate.*

[12] Millipore 47-mm stirred ultrafiltration cell XX42 047 10 with Pellicon membrane (PT Series, 10K).

[13] Model X-37-37, Instruments for Research and Industry, Cheltenham, Pa.

[14] Parafilm.

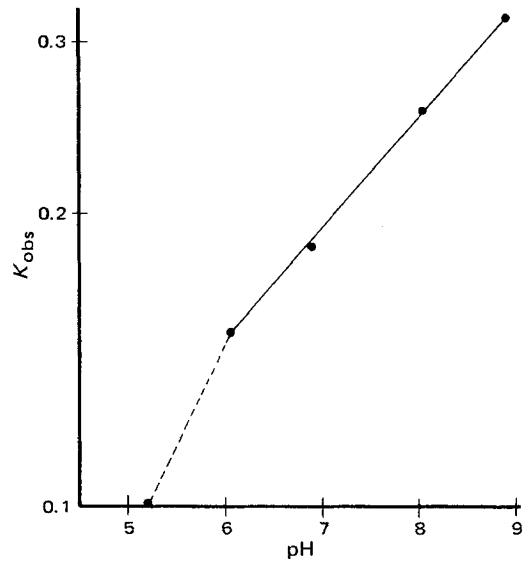

Figure 4—*Semilogarithmic plot of the observed rate constant* versus *pH in phosphate buffer.*

dark. The pH of the buffered solutions showed no significant change during the experiments. Figure 2 also shows that the photodecomposition occurred in methanol, indicating that the reaction is not solvent dependent.

Table I lists the observed first-order rate constants calculated for each buffer solution and methanol. The rate constants at pH 8.9 for both the phosphate and carbonate buffers are in good agreement, indicating that the process is dependent on pH and not the buffer species.

As shown in Fig. 3, the absence of oxygen inhibits the photodecomposition of uric acid in the presence of riboflavin and light. The approximate 10% loss of uric acid during the 1st hr plus the 5% decrease over the next 3 hr is considerably less than the almost 80% decrease previously shown to occur aerobically under the same conditions (6). The occurrence of some photodecomposition may be attributed to residual oxygen not removed during the deoxygenation procedures. Hartley and Kilby (8) showed a similar fast initial hydrolysis of *p*-nitrophenyl acetate in the presence of chymotrypsin followed by a slower steady-state release of product.

Many photooxidation reactions proceed *via* singlet oxygen. It was proposed that the photooxidation of bilirubin alone proceeds by singlet oxygen (9). An increase in singlet oxygen production was found with an increase in pH of aqueous solutions containing a photosensitizer (10). Figure 4 shows that the observed rate constant increased with an increase in pH; this increase seems to involve singlet oxygen in the uric acid photodecomposition.

Flavin triplet also seems to be implicated in the process. Previous work (6) showed that potassium iodide inhibited the reaction. Potassium iodide is known to be an effective quencher of the riboflavin triplet state (11, 12). Digenis *et al.* (13) proposed that riboflavin triplet is involved in the anaerobic photodecomposition of 2-chloro-9-(3-dimethylaminopropyl)acridan phosphate to its acridine derivative through an energy transfer process.

Riboflavin triplet is capable of generating singlet oxygen (14, 15). It was proposed that both riboflavin triplet and singlet oxygen are involved in bilirubin photodecomposition in the presence of riboflavin (16). Therefore, the following mechanism (Scheme I) is proposed to account for uric acid photodecomposition in the presence of riboflavin. Light absorption by ground-state riboflavin, Rb^0, produces riboflavin singlet, Rb^1. A radiationless transition of riboflavin singlet produces riboflavin triplet, Rb^3, which, upon collision with ground-state oxygen, $O_2{}^3$, generates singlet oxygen, $O_2{}^1$, *via* energy transfer. The singlet oxygen reacting with uric acid gives the decomposition products.

Figure 5 shows the effect of light on the 450-nm peak of riboflavin in both the absence and presence of uric acid. This peak showed a decrease

$$Rb^0 + h\nu \rightarrow Rb^1 \text{ (riboflavin singlet)}$$
$$Rb^1 \rightarrow Rb^3 \text{ (riboflavin triplet)}$$
$$Rb^3 + O_2{}^3 \rightarrow Rb^0 + O_2{}^1 \text{ (singlet oxygen)}$$
$$O_2{}^1 + \text{uric acid} \rightarrow \text{products}$$

Scheme I

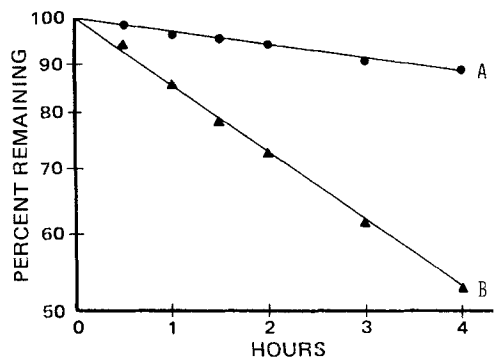

Figure 5—*Effect of light on the 450-nm peak of riboflavin. Key: A, riboflavin alone (3.88 × 10^{-5} M); and B, riboflavin (3.88 × 10^{-5} M) plus uric acid (1.19 × 10^{-4} M). Each point represents the average of three experiments performed in triplicate.*

of 12% in the absence of uric acid and a 47% decrease in the presence of uric acid. On a molar basis, uric acid disappearance was approximately 10 times greater than was the riboflavin loss, as evidenced by the decrease in the 450-nm peak. Thus, uric acid photodecomposition is not a coupled oxidation–reduction involving reduced flavin.

There was no spectral evidence for complex formation between the substrate and riboflavin. The spectra for mixtures of riboflavin plus uric acid exhibited the expected additivity of the spectra for both uric acid and riboflavin alone (Fig. 6).

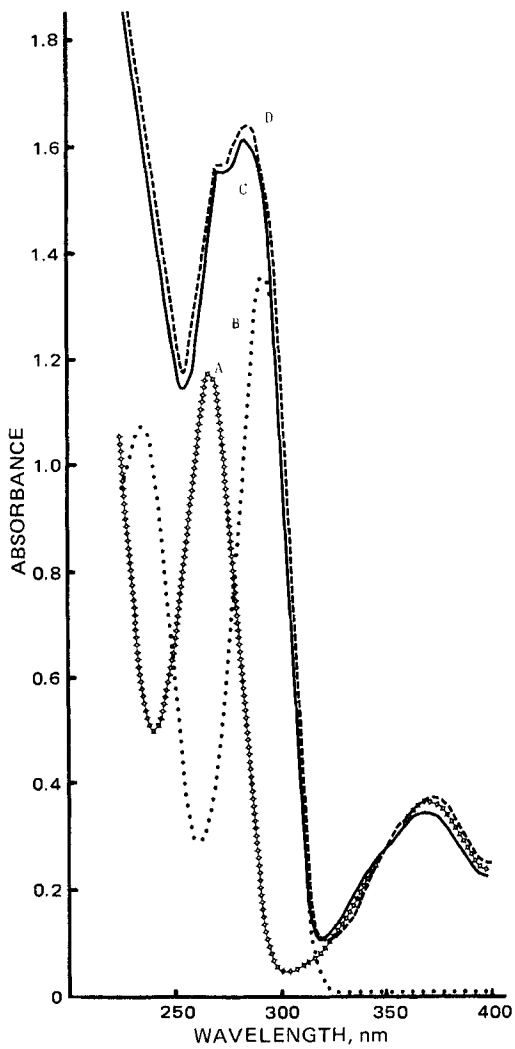

Figure 6—*UV spectra of riboflavin (3.88 × 10^{-5} M) (A), uric acid (1.19 × 10^{-4} M) (B), and a mixture of riboflavin (3.88 × 10^{-5} M) and uric acid (1.19 × 10^{-4} M) (C). The theoretical summation of the mixture is shown by curve D.*

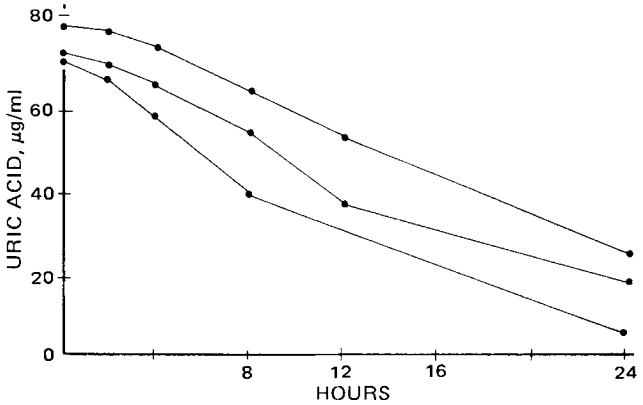

Figure 7—*Photodecomposition of uric acid in the presence of riboflavin in human plasma. Each line represents a different human plasma. Each point represents the average of triplicate determinations.*

The plasma curves (Fig. 7) indicate that the photodecomposition occurred in plasma *in vitro*. Uric acid levels at the termination of each experiment were lower than endogenous levels. Plasma containing uric acid in the absence of riboflavin showed no significant loss of uric acid after exposure to light for 24 hr. During HPLC analysis, the uric acid peak appeared 2.75 min after sample injection. A minor peak, which was not present in the aqueous solutions, was observed 1.75 min after sample injection and may be attributed to a lower molecular weight compound which was not retained by the membrane filter. The nonlinearity of the plasma curves may be due to the settling out of less translucent plasma components during irradiation. Also, a more complex reaction may occur in plasma than in the aqueous solutions.

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ac Polarography for Tetracycline Analysis

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Abstract □ The electrode processes for the reduction of several tetracyclines by ac polarography were examined. In pH 4.0 Walpole acetic acid–acetate buffer, two main waves occurred; the first was quasireversible and the second was reversible. Results showed that the first wave can readily be used for quantitative work. The second wave would also be suitable provided that there was no interference from other electroreducible substances.

Keyphrases □ Tetracyclines, various—ac polarographic analysis, electrode processes identified □ Polarography, ac—analysis, various tetracyclines, electrode processes identified □ Antibacterials—various tetracyclines, ac polarographic analysis, electrode processes identified

Many methods have been reported for the quantitative analysis of the tetracycline antibacterials (1), including a range of electroanalytical methods (2). One method utilized ac polarography for the assay of tetracycline, chlortetracycline, and oxytetracycline with a mercury pool reference electrode (3). This method also was used to analyze doxycycline capsules (4). Studies on minocycline, lymecycline, and demeclocycline have not been reported. Therefore, it was of interest to see whether these antibiotics behaved similarly and to extend the examination, using phase-sensitive ac polarography, to some pharmaceutical preparations on the British market.

Previous workers (3, 4) who investigated the application of ac polarography to tetracycline analysis made no attempt to determine whether the wave was the result of a reversible, a quasireversible, or an irreversible electrode process. Bond (5) stated that a knowledge of the reversibility of the electrode reaction involved is very important when using ac polarography for quantitative analysis but that the analytical use of quasireversible ac electrode processes can be extremely unreliable. Moreover, irreversible electrode processes may be a source of interference (5).

EXPERIMENTAL

Apparatus—All polarograms were obtained on a polarograph[1] equipped with a saturated calomel electrode (SCE), a dropping mercury electrode (DME), a mercury pool counter electrode, and a drop timer.

[1] Cambridge polarographic analyzer model 82P.