[CONTRIBUTION FROM THE PHYSIOLOGY DEPARTMENT, TUFTS UNIVERSITY SCHOOL OF MEDICINE]

# Photochemistry of Nucleic Acids and Related Compounds. II. The Ultraviolet Irradiation of the First Product from 1,3-Dimethyluracil[1,2]

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The effects of ultraviolet irradiation on 6-hydroxy-1,3-dimethylhydrouracil resulted in the formation of N,N'-dimethylmalonamide *via* 1,3-dimethylbarbituric acid. The irradiation reaction sequence was investigated. The kinetics were studied, and the mechanisms were discussed.

## Introduction

A systematic study is being carried out in this Laboratory on the photochemical effects of ultraviolet irradiation on nucleic acids and their derivatives. In view of the close relationship between the action spectra of a nucleic acid and the chemical and certain biological effects of ultraviolet irradiation, an attempt is being made to follow the complete reaction sequence to the ultimate irreversible steps. In the case of uridine the first product has been found to be 6-hydroxyhydrouridine. This compound does not support the growth of a uridine-requiring mutant of *Neurospora crassa*,[3] but can be reconstituted to uridine by treatment with heat or acid and will then again support the growth of the mutant. For further study of this and later reactions 1,3-dimethyluracil has been used as a model compound. It is suitable for this purpose because it behaves similarly to the nucleosides and nucleotides toward ultraviolet irradiation. The first product of the irradiation is 6-hydroxy-1,3-dimethylhydrouracil (I) as reported in a previous communication.[4] It is reversible in the same manner as is 6-hydroxyhydrouridine. Compound I has been accumulated, enabling us to study the later reactions resulting from irradiation. The products of these later steps, which do not give reversible reactions, are probably of greater biological significance because of their greater stability.

## Experimental[5]

**Irradiation of 6-Hydroxy-1,3-dimethylhydrouracil (I).**—A $3 \times 10^{-3}$ *M* (380 mg. in 800 cc.) solution of I was prepared in distilled water which had a *p*H 5.7. The $\epsilon_{260}$ readings were $0.22 \times 10^3$ before and $8.30 \times 10^3$ after alkaline treatment, *p*H ~ 12.[4] The solution was irradiated for 25 hr. at 31–33°. After irradiation it had a *p*H 6.1 and the $\epsilon_{260}$ readings were $0.02 \times 10^3$ before and $0.39 \times 10^3$ after alkaline treatment at *p*H ~12. This irradiated solution was lyophilized to dryness. The residue was taken up in absolute methanol, and the filtrate was concentrated to a very small volume. To this, anhydr. ether was added for crystallization and 213 mg. (73% yield) of product melting at 134–136° was obtained. After it had been twice recrystallized from acetone, it gave transparent plate-like crystals, m.p. 137–138°, $\nu_{NH}^{KBr}$ 3251 cm.$^{-1}$.

*Anal.* Calcd. for $C_5H_{10}N_2O_2$: C, 46.14; H, 7.75; N, 21.53. Found: C, 46.07; H, 8.00; N, 21.45.

**Irradiation of 6-Hydroxy-1,3-dimethylhydrouracil (I) under Nitrogen.**—Compound I (157 mg.) was dissolved in 100 cc. of distilled water and was saturated with nitrogen in the irradiator[6] for 0.5 hr. at a rate of about 160 bubbles per minute. The solution then was irradiated for 16.5 hr. under nitrogen with the intensity of lights 42 m.w./sq. ft. and at a temperature between 32–33°. The irradiated solution was worked up as the procedure described above and 115 mg. of the crude product was obtained. The purified material was identical to that obtained above.

**Preparation of N,N'-Dimethylmalonamide (VII).**—According to the procedure of Freund,[7] the product was purified by extracting with chloroform. A crystalline product appeared on the addition of anhydrous ether to the concentrated chloroform extracts. About 1 g. of the product was collected and gave a m.p. at 134–135°. This was recrystallized twice from acetone and melted at 137–138°, m.m.p. 137–138° with the irradiation product, $\nu_{NH}^{KBr}$ 3251 cm.$^{-1}$.

*Anal.* Calcd. for $C_5H_{10}N_2O_2$: C, 46.14; H, 7.75; N, 21.53. Found: C, 46.17; H, 8.05; N, 21.31.

**Preparation of 1,3-Dimethylbarbituric Acid (II).**—The procedure was followed exactly as reported by Cope, *et al.*,[8] and the ultraviolet spectra were compared with the data of Fox and Shugar.[9]

**Kinetic Study on Irradiation of 1,3-Dimethylbarbituric Acid (II).**—A $10^{-4}$ *M* solution of II in *p*H 7 phosphate buffer was put in one quartz tube and was carefully warmed to 34°, which was the temperature of the irradiator. The tube with the solution so obtained was placed near the middle of the lights. Samples were taken at time intervals to obtain an optical density reading at 260 mμ. The intensity of the lights was 52 milliwatts/sq. ft.

| Time, min. | 0 | 2 | 4 | 6 | 8 |
|---|---|---|---|---|---|
| $\epsilon_{260} \times 10^{-3}$ | 19.4 | 14.2 | 9.24 | 4.21 | 2.18 |
| $C_t \times 10^4$ | 1.00 | 0.732 | 0.476 | 0.217 | 0.112 |
| Time, min. | 10 | 12 | 15 | 18 | |
| $\epsilon_{260} \times 10^{-3}$ | 1.58 | 1.35 | 1.27 | 1.27 | |
| $C_t \times 10^4$ | 0.082 | 0.070 | 0.065 | 0.065 | |

**Carbon Monoxide Determination of the Irradiation of 6-Hydroxy-1,3-dimethylhydrouracil (I).**—The apparatus setup consists of nitrogen source, 10% sodium hydroxide in gas washing bottle, Oxsorbent trap, the irradiator,[6] a trap (2 × 26 cm.) containing 30 cc. of $CO_2$-free 10% sodium hydroxide solution, Drierite and Ascarite tube in sequence.

Compound I (79 mg.) was dissolved in 100 ml. of water and was irradiated for 16.5 hr. under nitrogen in the same manner as described above. Both the solutions in the irradiation tube and in the 2 × 26 cm. trap were treated in a similar manner for the determination.

| Solutions | Irradiated | Sodium hydroxide |
|---|---|---|
| 10% NaOH added | 10 ml. | .... |
| $BaCl_2$ added | 16 drops | 48 drops |
| $CO_2$ as $BaCO_3$ | None | 15.5 mg. (av.) |
| $Br_2$ added | 0.03 ml. | 0.09 ml. |
| CO as $BaCO_3$ | None | 38.3 mg. (av.) |

(1) This work was done under Contract AT(30-1)-911 with the Atomic Energy Commission.

(2) The author wishes to thank M. Apicella, L. Johnson, M. Salerno and R. Weintraub for their valuable assistance.

(3) D. Rapport, A. Canzanelli and R. Sossen, *Am. J. Physiol.*, **187**, 383 (1956).

(4) S. Y. Wang, M. A. Apicella and B. R. Stone, THIS JOURNAL, **78**, 4180 (1956); **80**, 6196 (1958).

(5) All the melting points are uncorrected and were taken with a Fisher–Johns melting point apparatus. Elementary analyses and infrared spectra (determined with a Baird spectrophotometer upon potassium bromide disks) were carried out by Dr. S. M. Nagy and his associates, Microchemical Laboratory, M.I.T. Ultraviolet spectra were determined with a Beckman spectrophotometer, Model DU.

(6) The irradiator has a quartz tube (1.5 × 10 in.) and is fitted with gas inlet and outlet. It is different from the one used above and is described in a paper by M. J. Kland and L. A. Johnson, THIS JOURNAL, **79**, 6188 (1957).

(7) M. Freund, *Ber.*, **17**, 134 (1884).

(8) A. C. Cope, *et al.*, THIS JOURNAL, **63**, 356 (1941).

(9) J. J. Fox and D. Shugar, *Bull. soc. chim. Belg.*, **61**, 51 (1952).

A solution simulating an irradiation solution was prepared by dissolving 65 mg. of VII and then 42 mg. of $NaHCO_3$ in order to eliminate the possibility of the interference of the irradiated product with the determination. The solution gave 90% of the theoretical amount of $BaCO_3$.

**Irradiation of 1,3-Dimethyluracil to N,N'-Dimethylmalonamide (VII).**—A $10^{-3}$ *M* solution of 1,3-dimethyluracil was irradiated in the usual manner.[4] The solution had a *p*H of 7 and the intensity of the lights was 52 m.w./sq. ft. The $\epsilon_{260}$ readings of the irradiated solution after reconstitution at *p*H ~12 were

| Time, hr. | 0 | 1 | 3 | 5 | 7 | 9 | 11 | 19 |
|---|---|---|---|---|---|---|---|---|
| $\epsilon_{260} \times 10^{-3}$ | 7.75 | 7.65 | 7.53 | 7.50 | 7.18 | 6.73 | 6.09 | 0.26 |

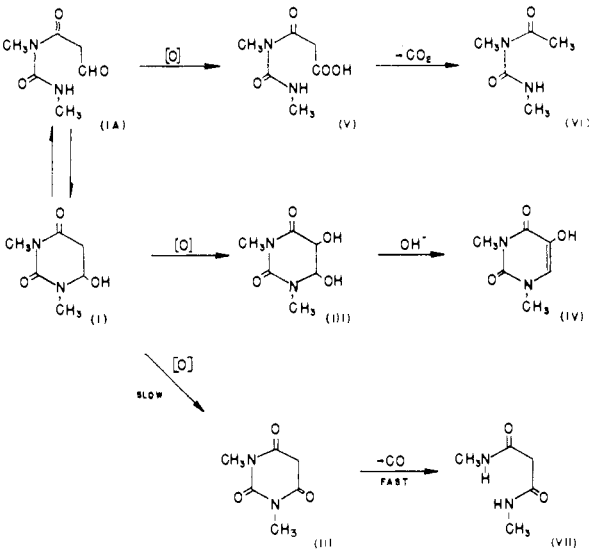
Isolation and identification were done on a solution of 420 mg. of 1,3-dimethyluracil in 300 cc. of water. The solution was irradiated for 40.5 hr. and its product was identified as VII.

**Irradiation of N,N'-Dimethylmalonamide (VII).**—One millimole (130 mg.) of VII was dissolved in 100 cc. of water and was irradiated in the irradiator for 96 hours at 35–37°. The substance was recovered unchanged.

## Results and Discussion

Extensive breakdown of irradiation products from pyrimidines[10] indicated that irradiation of 6-hydroxy-1,3-dimethylhydrouracil (I) in aqueous solution would produce additional reactions; I was thus further irradiated in aqueous solution. Oxidation at position 5 or 6 would be the most probable immediate effect on I. As shown in the figure, if this oxidation occurred at position 6, then 1,3-dimethylbarbituric acid (II, $\lambda_{max}^{H_2O}$ 260 m$\mu$, $\epsilon$ 18.9 $\times 10^3$ at *p*H 7) would be the product. If it occurred at position 5, 5,6-dihydroxy-1,3-dimethylhydrouracil (III) would be the product which on treatment with alkaline solution would lose a molecule of water. The result of this would be 5-hydroxy-1,3-dimethyluracil (IV, $\lambda_{max}^{H_2O}$ 285 m$\mu$, $\epsilon$ 7.35 $\times 10^3$). Samples were removed during the irradiation of I, and spectra were obtained for each sample before and after treatment with sodium hydroxide solution to *p*H~12. However, no absorption peaks occurred at either 260 m$\mu$ before or at 285 m$\mu$ after alkaline treatment. The only peak occurred at 267 m$\mu$ after alkaline treatment. This represents the reconstitution of I to 1,3-dimethyluracil and has been used to determine the extent of the reaction. It therefore appeared unlikely that the reaction occurred *via* II or III. A more probable pathway seemed to be *via* the open chain aldehyde form IA which upon oxidation yields the $\beta$-keto acid V. This in turn would be decarboxylated to produce 1-acetyl-1,3-dimethylurea (VI). Indeed, a compound which gave an analysis of $C_5H_{10}N_2O_2$ was obtained in over 70% yield. The appearance of an NH band in the infrared spectrum of the irradiation product indicated the rupture of C–N bond (bonds) in I. This evidence led us to believe that 1-acetyl-1,3-dimethylurea (VI) was actually the compound sought. However, only a synthetic material could identify this irradiation product beyond doubt. A number of attempted syntheses gave only a very hygroscopic material and analysis of the irradiated solution for $CO_2$ was inconclusive. These observations strongly suggested that, after all, VI was not the irradiation product. Only then the product was found to be identical with authentic N,N'-dimethylmalonamide (VII).

In order to demonstrate that II might be the transient intermediate, II was synthesized and a $10^{-4}$ *M* solution at *p*H 7 was irradiated and the optical density at 260 m$\mu$ almost completely disappeared in about 20 minutes. On the other hand, the irradiation of I to VII under similar conditions required 6.5 hours for completion. Therefore, the presence of II cannot be detected spectroscopically in the process, most probably because of the rapid formation of VII from II; and the concentration of II, at any one time, is not sufficiently great to form products other than VII. (The spectroscopic studies showed that the irradiation of starting concentrations of II higher than that of a $10^{-4}$ *M* solution did not produce VII alone.)




As for the mechanism of this process, one might consider that the primary process of the oxidation step is the formation of ·OH from water due to the presence of dissolved oxygen, then the sequences occur

$$C_6H_{10}N_2O_3\ (I) + \cdot OH \longrightarrow C_6H_8N_2O_3\ (II) + H_2O + \cdot H$$

$$C_6H_8N_2O_3\ (II) + 2\cdot H \longrightarrow C_5H_{10}N_2O_2\ (VII) + CO$$

Therefore, the water molecules not only serve as an oxidizing agent for the oxidation step but also as a reducing agent for the formation of VII. The decarbonylation of 1,3-dimethylbarbituric acid (II) occurs probably because II is excited by the absorption of ultraviolet photons (4.77 e.v. at 260 m$\mu$) and renders possible an energetic rupture of two C–N bonds (2.11 e.v. per bond).[11] Indeed, this type of decarbonylation is not unprecedented.[12] For further substantiation, semi-quantitative determinations of carbon monoxide were carried out and the data indicated that about 45% of CO and 17–18% of $CO_2$ of the theoretical amount were formed during the reaction (the $CO_2$ may be the artifact resultant from the oxidation of CO during irradiation). This strongly suggests that decarbon-

(10) A. Canzanelli, R. Guild and D. Rapport, *Am. J. Physiol.*, **167**, 364 (1951); W. E. Conrad, *Radiation Res.*, **1**, 523 (1954).

(11) L. Pauling, "The Nature of the Chemical Bond," 2nd ed., Cornell Univ. Press, Ithaca, N. Y., 1945.

(12) G. B. Kistiakowsky and S. W. Benson, U. S. Patent 2,414,880, Jan. 28, 1947.

ylation is a major mechanism if it is not the only one.

The formation of VII also could have been brought about directly by the irradiation of 1,3-dimethyluracil. During this process, the steps leading to the formation of VII occurred only after almost complete conversion of the starting material to I (7 hr.); VII was isolated and identified.

The irradiation on N,N'-dimethylmalonamide (VII) did not bring appreciable change even after 4–5 days at a $10^{-2}$ concentration.

The mechanism of this irradiation process seemed to be a free radical reaction. The rate of the photo-oxidation reaction at $10^{-3}$–$10^{-4}$ $M$ appeared to be a function of its concentration; however, at higher concentrations the rate seemed independent of the concentration of I, about the same reaction time being observed for $3 \times 10^{-3}$ $M$ (21–25 hr.), $6 \times 10^{-3}$ $M$ (21 hr.) and $1.2 \times 10^{-2}$ $M$ (22–28 hr.) with the same reaction conditions. The kinetics of decarbonylation (II to VII) were pseudo zero order at $10^{-4}$ $M$ as shown by a plot of optical density *vs.* time.

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# Concerning the Mechanism of Action of Parathyroid Hormone. II. Metabolic Effects[1,2]

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The effects of varying parathyroid status on arteriovenous differences of pyruvate, lactate and citrate were studied in several tissues in the dog. Bone was shown to be an important source of circulating citrate and this citrate production by bone was dependent upon parathyroid hormone levels. Citrate uptake was shown only by the kidney, but a hormonal effect on this process, if it occurs, was not clearly demonstrated. Interesting trends suggesting a hormonally stimulated utilization of pyruvate and production of lactate and citrate were observed in various tissues in addition to bone and kidney. These findings are discussed in terms of the mechanism of action of parathyroid hormone.

In recent years, attention has focused upon the importance of citric acid in bone metabolism, yet the true significance of citrate in bone has remained obscure.[3] Parathyroid hormone influences citric acid metabolism[4,5] and, recently, conclusive evidence that this influence is a direct effect of the hormone was provided by the experiments of Freeman and Chang[6] and Elliott and Freeman,[7] who demonstrated that the rise in the level of citric acid in the blood that accompanies nephrectomy is abolished by a prior parathyroidectomy.

In previous publications of the present series,[8,9] this relationship between parathyroid status and citrate metabolism has been confirmed and extended. It was shown that, following the intravenous injection of parathyroid hormone in dogs, a sharp rise in the level of blood citrate occurred reaching a peak one hour following the injection. The rise in citrate was followed closely by a parallel rise in inorganic phosphate, while the calcium level did not increase until 4–6 hours following the injection. The release of citrate by bone and its dependence upon parathyroid hormone, was indicated. Data demonstrating that citrate can exchange for phosphate on the surface of the bone crystals, with a resulting increase in the solubility of the mineral phase, also was presented.

In the present investigations, the uptake and release of pyruvate, lactate and citrate by various tissues, in addition to bone, and the influence of the parathyroid hormone upon such metabolic activity, has been investigated by means of the technique of arteriovenous differences. These studies were carried out in thyroparathyroidectomized, normal and parathyroid-injected dogs.

## Methods

Mongrel dogs were thyroparathyroidectomized and used two days later for blood collection. These animals received 600,000 units of procaine penicillin intramuscularly following the operation. The parathyroid treated animals received one or two subcutaneous injections of parathyroid extract[10] at various intervals (5 to 24 hours) prior to blood collection, at a level of 100 units per kilo. This amount of hormone was selected as one which would elicit a major response in each animal. Effects of a similar nature undoubtedly could be obtained at a lower dosage.[11,12] Three of the control animals were sham operated.

Mixed bone blood was collected from a catheter inserted in a small hole drilled in the spongiosa of the femur of the dog, a technique described earlier.[8] As before, $Sr^{89}$ was given five minutes before blood collection began to permit a comparison of $Sr^{89}$ levels in the catheter blood with arterial levels. Since, in the early time periods, the $Sr^{89}$ is effectively "cleared" in passing through bone and only bone,[9] this provided a rough measure of the proportion of the catheter sample which was truly venous outflow from bone. In many instances, several consecutive samples of catheter blood were obtained to afford a better estimate of venous outflow from bone. A corresponding arterial blood sample was drawn at the midpoint of each bone blood collection, for calculations of A-V differences. In addition to that from

(1) This paper is based on work performed under contract with the United States Atomic Energy Commission at The University of Rochester Atomic Energy Project, Rochester, New York.

(2) Taken in part from a thesis submitted by H. E. Firschein in partial fulfillment of the requirements for the Ph.D. degree in Biochemistry, University of Rochester, 1958.

(3) T. F. Dixon and H. R. Perkins, "The Biochemistry and Physiology of Bone," G. H. Bourne, ed., Academic Press, Inc., New York, N. Y., 1956, Chap. 11.

(4) F. Dickens, *Biochem. J.*, **35**, 1011 (1941).

(5) N. Alwall, *Acta Med. Scand.*, **116**, 337 (1944).

(6) S. Freeman and T. S. Chang, *Am. J. Physiol.*, **160**, 341 (1950).

(7) J. R. Elliott and S. Freeman, *Endocrinology*, **59**, 181 (1956).

(8) W. F. Neuman, H. Firschein, P. S. Chen, Jr., B. J. Mulryan and V. DiStefano, THIS JOURNAL, **78**, 3803 (1956).

(9) H. Firschein, G. Martin, B. J. Mulryan, B. Strates and W. F. Neuman, *ibid.*, **80**, 1619 (1958).

(10) Purchased from the Eli Lilly Company, Indianapolis, Ind.

(11) L. C. Miller, *J. Am. Pharm. Assoc.*, **27**, 90 (1938).

(12) C. I. Bliss and C. L. Rose, *Am. J. Hyg.*, **31**, 79 (1940).