The Photochemistry of 5-Fluorouracil*†

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The principal photoproduct formed when 5-fluorouracil is irradiated with ultraviolet light of mainly 253.7-mμ wavelength has been compared with chemically synthesized *dl*-5-fluoro-6-hydroxyhydrouracil. A comparison of a number of physical and chemical properties of the two materials has shown that they are identical. The 5-fluoro-6-hydroxyhydrouracil is unstable in alkali and decomposes to form urea and probably fluoromalonaldehydic acid.

The incorporation of the pyrimidine analog, 5-fluorouracil, into the nucleic acid of tobacco mosaic virus sensitizes the intact virus (Becarevic *et al.*, 1963; Lozeron and Gordon, 1964) and the infectious nucleic acid to ultraviolet light (Lozeron and Gordon, 1964). A study of the photochemistry of 5-fluorouracil has been initiated with the hope that the identification of some of the main photoproducts might be helpful in gaining insight into the molecular mechanism of ultraviolet sensitization of the 5-fluorouracil-containing virus nucleic acid. The present communication describes the isolation and identification of the major photodecomposition product of 5-fluorouracil. A comparison of the properties of this photoproduct with those of the chemically synthesized 5-fluoro-6-hydroxyhydrouracil indicates that the compounds are identical.

EXPERIMENTAL

Irradiation of [2-^{14}C]5-Fluorouracil.—Two-ml samples of aqueous unbuffered solutions of 5-fluorouracil (concn. 10^{-3} M; *p*H 6.0; specific radioactivity 6800 cpm/μmole) were irradiated at room temperature in open petri dishes of 4.5 cm diameter at a layer thickness of 0.125 cm. Irradiations were performed with a Mineralight V-41 low-pressure mercury-resonance lamp equipped with a Corning filter 9863 (Ultraviolet Products Inc., San Gabriel, Calif.). At least 90% of the energy output of this lamp is stated to be at 253.7 mμ. The 2-ml irradiated samples were subsequently concentrated *in vacuo* to 0.10 ml. The concentrated sample (containing approximately 14,000 cpm) was then applied to Whatman No. 1 chromatography paper as a 2-cm band, and the chromatogram was developed by descending chromatography with a 1-butanol-H_2O (86:14) solvent system similar to that used by Wacker *et al.* (1961) for the isolation of the photoproducts of irradiated uracil. Development for 24–30 hours resulted in a solvent front of 52 cm.

In order to determine the number and location of possible radioactive photoproducts on the chromatogram, the developed paper chromatograms were scanned by cutting the chromatograms into 1.0 × 2.0-cm strips which were placed in planchets. The radioactivity of the strips was determined with an open-window gas-flow counter (Nuclear-Chicago Corp., Chicago, Ill.). Counting efficiency by this method was approximately 30% compared to the same amount of radioactive material applied directly to the planchets as an infinitely thin film.

Large-Scale Irradiation of 5-Fluorouracil.—An aqueous solution of 5-fluorouracil, 10^{-3} M, 160 ml, was placed in a 3-quart Pyrex baking dish at a distance of 11 cm from the ultraviolet source. During the course of the irradiation the solution was stirred magnetically and kept at no higher than 4° by cooling with a large tray of ice. The solution was irradiated for 5 hours during which time the optical density decreased to 43% of the original value. Longer periods of irradiation gave rise to larger amounts of additional photochemical products other than the hydrate. A total of 4000 ml of the 10^{-3} M 5-fluorouracil was irradiated by this procedure. In pilot experiments with [2-^{14}C] 5-fluorouracil, 10% of the irradiated material was converted to products other than the photochemical hydrate; accordingly, the maximum yield of 5-fluoro-6-hydroxyhydrouracil that could be expected in the present experiment was 1.88 mmoles [$4000 \times 10^{-3} \times (1.00 - 0.43 - 0.10)$].

Chromatographic Separation of 5-Fluoro-6-hydroxyhydrouracil.—Immediately after irradiation, the solutions were frozen and lyophilized. The residue was dissolved in approximately 25 ml of water and applied to the long edges of 40 sheets of Whatman 3 MM paper of size 46 × 57 cm. The lower edges of the paper sheets were scalloped so that the solvent would drip off the lower edge during chromatography. The paper sheets were developed for 36 hours by descending chromatography using 1-butanol saturated with water. The sheets were then dried, and marker strips were cut from both edges of a given sheet. The marker strips were heated over a hot plate and examined for the appearance of a new band of ultraviolet-absorbing material. The reappearance of ultraviolet absorption upon heating is caused by the dehydration of 5-fluoro-6-hydroxyhydrouracil to 5-fluorouracil. The portion of the main sheet corresponding to 5-fluoro-6-hydroxyhydrouracil was removed. All these portions were then homogenized in a high-speed mixer with the use of about 1000 ml of water. The homogenized paper pulp was placed in a glass column (10 × 100 cm) and eluted with water. The column was monitored by periodically taking 3-ml aliquots of the effluent and adding 0.3 ml of 1 N sodium hydroxide. The elution was discontinued after the OD of an aliquot at 260 mμ had dropped to 0.083 after the addition of alkali. A total of 2125 ml containing 13,455 OD units at 260 mμ was obtained as measured after the addition of alkali to an aliquot. This number of alkaline OD units corresponded to 1.01 mmoles of 5-fluoro-6-hydroxyhydrouracil. An ϵ_{260} of 13,300 was used for the alkaline decomposition product (Duschinsky *et al.*, 1957,

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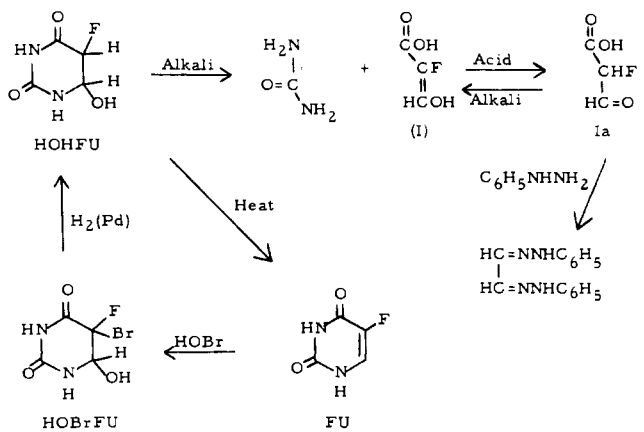
1963) *vide infra*). The solution was lyophilized to dryness to yield 267 mg of a brownish fluffy hygroscopic material which was grossly contaminated with material eluted from the large amount of paper used during chromatography.

Crystallization of 5-Fluoro-6-hydroxyhydrouracil.—A 58.8-mg sample of the crude material from the previous lyophilization was extracted for 30 seconds with 3 ml of boiling absolute ethanol contained in a 25-ml Erlenmeyer flask. The solution was filtered through Whatman No. 1 filter paper into a 10-ml Erlenmeyer flask cooled in ice. The filter paper, which had held back a considerable amount of dark brown material, was returned to the original 25-ml Erlenmeyer flask and reextracted with an additional 3 ml of boiling absolute ethanol for 5 minutes. This second extract was filtered, cooled, and combined with the original extract. The combined pale-yellow filtrates were concentrated to 0.5 ml at room temperature with a jet of nitrogen gas. The residue was warmed, then placed in an ice bath and scratched. A white crystalline material appeared. The flask was stored at −10° for 48 hours. The remainder of the crude 5-fluoro-6-hydroxyhydrouracil (208 mg) was treated similarly using 10 ml ethanol for each hot extraction. The combined extracts in this case were concentrated to 3 ml, cooled, and scratched to give crystalline material. The material from both crystallizations was collected and washed with several portions of cold ethanol totaling 3 ml. The material from this first crystallization was a finely powdered, off-white material.

The combined material from the first crystallization (about 50 mg) was dissolved directly in 10 ml of boiling ethanol, filtered, and rapidly cooled. The volume of the solution was reduced to about 2 ml by means of a jet of nitrogen gas. The solution was cooled in ice, scratched to induce crystallization, and stored overnight at −10°. The following day the material was collected by filtration, washed with several small volumes of cold ethanol, and dried *in vacuo* over phosphorus pentoxide. The yield of the first crop was 29.4 mg. A second small crop weighing 3.5 mg was also obtained.

Chemical Synthesis of 5-Fluoro-6-hydroxyhydrouracil.[1]—The chemical reactions involved are presented as follows:



5-Bromo-5-fluoro-6-hydroxyhydrouracil (HOBrFU).[2]—To a suspension of 25 g (0.192 mole) of 5-fluorouracil (FU) in 250 ml of water, which was cooled in an ice bath, there was added dropwise and with vigorous stirring 31.2 g (0.195 mole) of bromine. Upon continuous stirring and cooling for 20 minutes the mixture first became almost clear and then the colorless semihydrate of HOBrFU crystallized. It was filtered, washed with cold water, and dried at 60° *in vacuo*. The yield was 28.4 g, mp 162–164° (gas evolution). Recrystallization from a mixture of 80 ml of ethyl acetate and 50 ml of petroleum ether yielded 19.26 g of product having the same melting point.

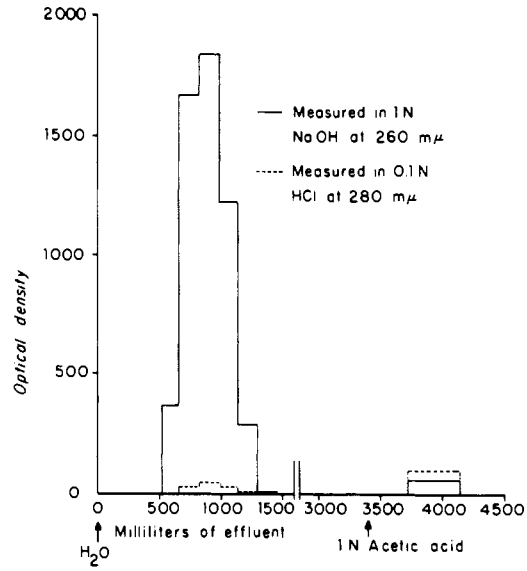


FIG. 1.—Chromatography of hydrogenation mixture on Dowex 1-X4 (acetate).

Anal. Calcd.[3] for $C_4H_4BrFN_2O_3 \cdot {}^1/_2H_2O$: C, 20.36; H, 2.14; Br, 33.86; F, 8.05. Found: C, 20.68; H, 1.97; Br, 33.63; F, 7.75.

Upon drying at 100° *in vacuo* for 2 hours a weight loss of 3.50% (theory for 1/2 H_2O, 3.35%) was observed. The pinkish anhydrous material melted at 181–182.5°. Upon exposure to air it was reconverted into the semihydrate.

Anal. Calcd. for $C_4H_4BrFN_2O_3$: C, 21.16; H, 1.78; Br, 35.20; F, 8.37. Found: C, 21.40; H, 1.86; Br, 35.58; F, 8.22. Ultraviolet (0.1 N HCl): End absorption starting at 280 mμ. Infrared (KBr): 3280, 2070, 2810, 1745, 1695, 1468, 1390, 1282, 1245, 1178, 1150, 1080, 1040, 965, 894, 800, 765, 730, 680 cm^{-1}.

The filtrate of the 28.4 g contained besides 0.001 mole of FU, bromide ions and some electropositive bromine both of which were removed by adsorbing on Dowex 1-X4 acetate and eluting the resin with water. Evaporation of the effluent gave an additional 11 g of crude semihydrate thus raising the total yield to 39.4 g (87%).

5-Fluoro-6-hydroxyhydrouracil (HOHFU).—Palladium charcoal catalyst (3.2 g containing 10% palladium) suspended in a solution of 7.2 g (88 mmoles) of sodium acetate in 300 ml of water was shaken with hydrogen at room temperature and under slight positive pressure until saturation. Then 20 g (84.8 mmoles) of HOBrFU semihydrate was added and the hydrogenation was continued. After 1 hour the reaction had come to a standstill with an uptake of 2180 ml (88 mmoles) of hydrogen. The catalyst was filtered off. The filtrate contained 68,600 OD units measured at 280 mμ in 0.1 N hydrochloric acid ($E_{280}^{0.1N\ HCl}$) and

[1] A preliminary report of this work was presented before the Meeting of American Chemical Society, September, 1963, New York City (Duschinsky *et al.*, 1963).

[2] Abbreviations used in this manuscript are: FU, 5-fluorouracil; HOBrFU, 5-bromo-5-fluoro-6-hydroxyhydrouracil; HOHFU, 5-fluoro-6-hydroxyhydrouracil.

[3] We are indebted to Dr. A. Steyermark of Hoffman-La Roche for microanalyses.

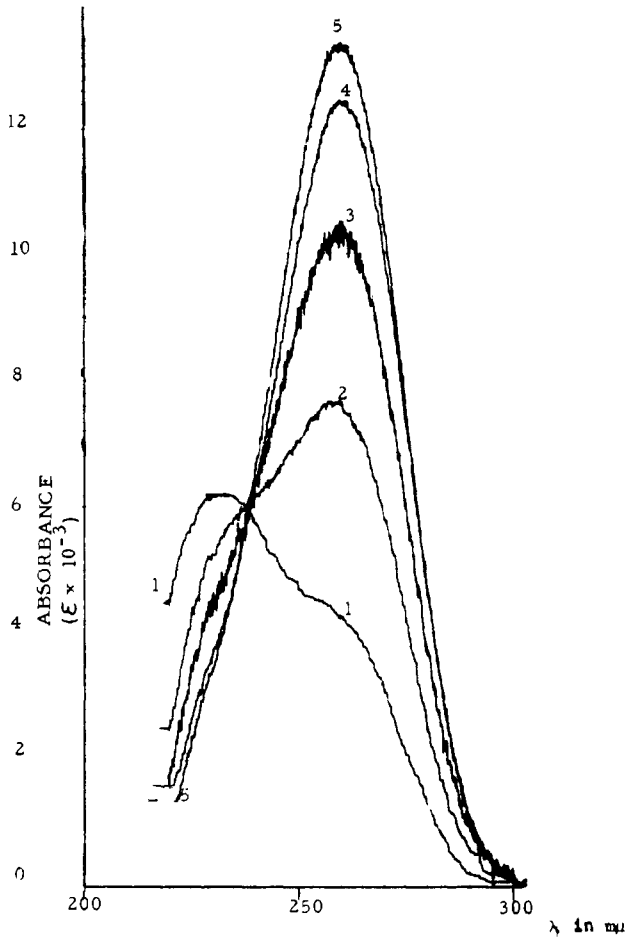


FIG. 2.—Ultraviolet spectrum of HOHFU in 1 N KOH. Time indicated for 260 mμ reading.

Reading:	1	2	3	4	5
Minutes:	6	12	22	36	68

892,000 OD units measured at 260 mμ after letting the sample stand for 30 minutes in 1 N sodium hydroxide ($E_{260}^{1N\ NaOH}$). Based on $\epsilon_{280}^{0.1N\ HCl}$ 4100 and $\epsilon_{260}^{0.1N\ NaOH}$ 3100 for FU and of $\epsilon_{280}^{0.1N\ HCl}$ O and $\epsilon_{260}^{1N\ NaOH}$ 13,300 for HOHFU this would correspond to a content of 16.7 mmoles FU and of 63 mmoles HOHFU.

The solution was neutralized to *p*H 8 by addition of about 100 ml of 1 N sodium hydroxide and put on a column (4 × 50 cm) of Dowex 1-X4 (acetate), 100–200 mesh. Elution was performed first with water which yielded 63 mmoles of HOHFU accompanied by 5.2 mmoles FU, and then with 1 N acetic acid which yielded the balance of 11 mmoles FU (Fig. 1).

The fractions of HOHFU were either evaporated at low temperature *in vacuo* until crystallization or they were lyophilized. Evaporation gave a relatively pure product directly, but in low yield. The lyophilized material was purified by fast recrystallization from 15 to 30 volumes of ethanol. The HOHFU crops thus obtained contained 0.8–2.4% of FU. The total yield was 4.63 g (37%) of crude HOHFU. Repeated recrystallization of the less pure crops gave finally a total of 2.8 g (12.8%) of HOHFU containing not more than 0.8% of FU (melting point, analysis, and spectra, *vide infra*).

When sodium acetate was omitted from the hydrogenation mixture only FU and no HOHFU was obtained. This phenomenon will be discussed later.

Reaction of HOHFU *with Alkali.*[4]—The compound is

[4] With the collaboration of Dr. V. Toome (Hoffmann-La Roche, Nutley, N. J.).

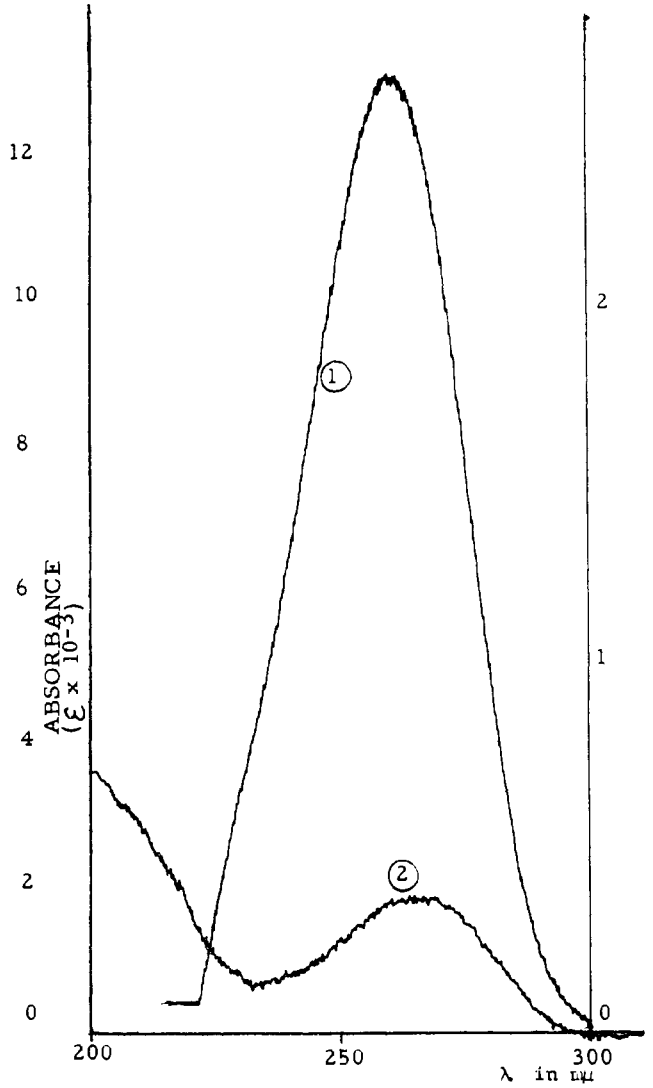


FIG. 3.—Ultraviolet spectra of HOHFU after 20 hours at room temperature in 1 N KOH. Curve 1, spectrum in 1 N KOH (left ordinate). Curve 2, spectrum in 0.05 N HCl (right ordinate).

stable in acid, but undergoes a transformation in 1 N NaOH or KOH. The changes in the ultraviolet spectrum during the transformation are shown in Figure 2. HOHFU has no selective absorption, i.e., is not enolized. In alkali an intermediate with an ultraviolet maximum at 230 mμ was formed very rapidly, presumably by enolization. This reacted to give a compound with an absorption maximum at 260 mμ. The half-time of this reaction was 10.5 minutes at 23° (±1°), the pseudo-first-order constant $K = 0.066\ min^{-1}$.

The final product was examined in acid (Fig. 3) and was shown not to possess any notable absorption down to near 200 mμ, which excluded the presence of conjugation. The two forms of the product, I and Ia, were stable and interconvertible; realkalinization of the acid solution restored the band at 260 mμ. The low band at 265 mμ belongs to a small amount (about 6%) of FU formed.

The cleavage products were identified as urea and fluoromalonaldehydic acid. The well-defined isosbestic point (Fig. 2) indicates that these are essentially the products involved.

Isolation of Urea.—Crude HOHFU (5-mmoles) was dissolved in 75 ml of 1 N sodium hydroxide. After 1 hour the solution contained 66,600 OD units at the maximum of 260 mμ. It was adsorbed on a Dowex

1-X4 acetate column (4 × 30 cm). Elution was performed in the following order: water, acetic acid of increasing concentration from 0.1 to 1.0 N, 0.5 N acetic acid and 0.5 N ammonium acetate, 0.1 N hydrochloric acid, and 0.1 N hydrochloric acid and 1 N ammonium chloride. The effluent was monitored by measuring $E_{280}^{0.1N\ HCl}$ and $E_{260}^{1N\ NaOH}$ and by the urea test according to Fink *et al.* (1956). The water eluate (1500 ml total) contained no ultraviolet-absorbing material, but after passage of 230 ml the Fink test showed the presence of urea in the next 170 ml of effluent. A fraction containing $E_{280}^{0.1N\ HCl}$ 5460 and $E_{260}^{1N\ NaOH}$ 3580 (corresponding to 1.3 mmole of FU) was then eluted with 0.1 N acetic acid and a fraction containing $E_{260}^{1N\ NaOH}$ 2300 was eluted with 1 N acetic acid + 1 N sodium acetate, but the bulk of the $E_{260}^{1N\ NaOH}$ was not found. Evaporation of the urea fraction gave 2.57 g of a solid. (Found: N, 5.45, 5.31%). A solution of 1 g of this material in 10 ml of acetic acid was mixed with a solution of 0.85 g of xanthydrol in 8 ml of acetic acid. The formed crystalline precipitate (0.683 g) was separated by filtration and washed with acetic acid and water. It melted at 261–262° (decomp) and was identified by mixed melting point as dixanthyl urea. This corresponds to a recovery of 3.86 mmoles (77%) of urea from the alkaline hydrolysis.

Indirect Identification of Fluoromalonaldehydic Acid.—(I and Ia). A freshly prepared solution of 100 mg (0.68 mmole) of HOHFU (containing not more than 0.8% FU) in 5 ml of 1 N NaOH was allowed to stand 1 hour, then acidified with acetic acid and mixed with a solution of 199 mg (1.38 mmole) of phenylhydrazine hydrochloride in a small volume of methanol. After the solution was heated for 10 minutes on the steam bath and cooled to room temperature, 54 mg of red needles, melting at 161–163°, was isolated.

Anal. Calcd for $C_{14}H_{14}N_2$: C, 70.56; H, 5.92; N, 23.51. Found: C, 70.02; H, 5.84; N, 23.59.

The compound was identified as glyoxal diphenylhydrazone by ultraviolet-absorption spectrum and mixed melting point with an authentic sample.

Results and Discussion

In the initial photochemical studies, paper chromatograms of irradiated [2-^{14}C]5-fluorouracil, developed with the 1-butanol-H_2O (86:14) solvent system, were scanned to determine the locations of possible radioactive photoproducts. The first chromatographic analysis was performed on irradiated, aqueous solutions of 5-fluorouracil in which approximately 45% of the analog had been decomposed photochemically as measured by the disappearance of the characteristic absorption peak at 266 mμ. Survey of these paper chromatograms of [2-^{14}C]5-fluorouracil indicated the presence of two principal radioactive peaks (see Fig. 4 for a typical chromatogram). The first peak, having an R_F of 0.42, accounted for 55% of the original starting material and corresponded to unreacted 5-fluorouracil. The second peak, having an R_F of 0.21, accounted for 33% of the starting material and displayed no ultraviolet absorption on examination of the paper chromatogram with a mercury-resonance lamp. Exposure of this area of the chromatogram to heat, however, resulted in the appearance of a new ultraviolet-absorbing spot. A further chromatographic analysis was performed on solutions in which 65% of the analog had been decomposed photochemically. The heat-reversible material accounted for 46% of the starting material. Two additional components were detected. A very minor component, making up 1% of the original starting material, appeared on heating as a band of fluorescent material just tailing the major heat-reversible compound. A second minor component accounted for 7% of the starting material and was found as an ultraviolet-absorbing band slightly ahead of the origin. These two minor components have not been characterized. They possibly are related to fluorobarbituric acid.

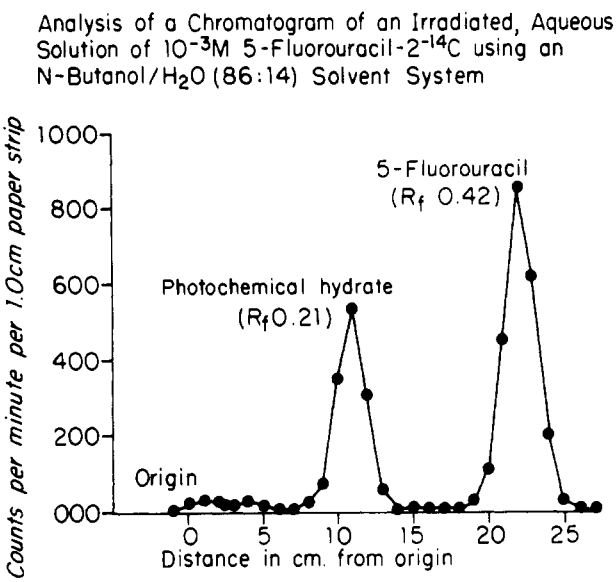


FIG. 4.—Analysis of a paper chromatogram of an irradiated aqueous solution of 10^{-3} M 5-fluorouracil in which 45% of the analog has been photodecomposed. Chromatography was performed on Whatman No. 1 paper using an 1-butanol-H_2O (86:14) solvent system.

We have not been able to detect 5-fluorouracil dimer under the experimental procedures employed. It would be assumed that if 5-fluorouracil dimer did form, its R_F value would be comparable to that of uracil dimer (Wacker *et al.* 1961) and, thus the 5-fluorouracil dimer should remain near the origin. On paper chromatograms of irradiated uracil, uracil dimer is very readily detected slightly ahead of the origin by examination of the chromatogram with a mercury-resonance lamp after a short exposure of this area of the chromatogram to 253.7-mμ irradiation. In the case of uracil, the dimer is converted to uracil by the 253.7-mμ radiation. On chromatograms of irradiated 5-fluorouracil, however, no ultraviolet-absorbing material corresponding to 5-fluorouracil was visibly detected above the background of ultraviolet-absorbing material originally present near the origin when the chromatograms were irradiated further.

The heat-reversible photoproduct (R_F 0.21), therefore, was the major product formed under the experimental conditions employed. On chromatography in the second dimension using the same 1-butanol-H_2O solvent system, the ultraviolet-absorbing material arising upon heating this photoproduct migrated with an R_F value identical to that of 5-fluorouracil. The apparent reversion of the photoproduct of 5-fluorouracil by heat to the parent compound indicated that this photoproduct behaved in a manner similar to that of uracil, uridine, and uridylic acid (Sinsheimer and Hastings, 1949; Sinsheimer, 1954). In these cases the characteristic absorption spectra of these pyrimidines which had disappeared on irradiation could be largely restored by heat at neutral *p*H, and its was postulated that ultraviolet-induced hydration of the C-5,6 double bond of the pyrimidine ring and subsequent dehydration by heat to yield the parent compound could account for the observed spectral changes (Sinsheimer, 1954).

TABLE I
R_F VALUES FOR URACIL, 5-FLUOROURACIL, AND PHOTOPRODUCTS

	Solvent System		
	1-Butanol-H_2O (86:14)	Ethanol-H_2O (85:15)	Butanol–Acetic Acid-H_2O (50:20:30)
Uracil[a]	0.32		
Uracil hydrate[a]	0.19		
Uracil dimer[a]	0.03		
5-Fluorouracil	0.42	0.66	0.66
5-Fluoro-6-hydroxyhydrouracil (chemicaly synthesized)	0.21	0.58	0.52
5-Flourouracil hydrate (photochemical compound)	0.21	0.56	0.52

[a] Data independently determined by the authors.

Heat reversal of aqueous, unbuffered solutions of 1.35 x 10^{-4} M 5-fluoro-6-hydroxyhydrouracil and photochemical hydrate at 85° C.

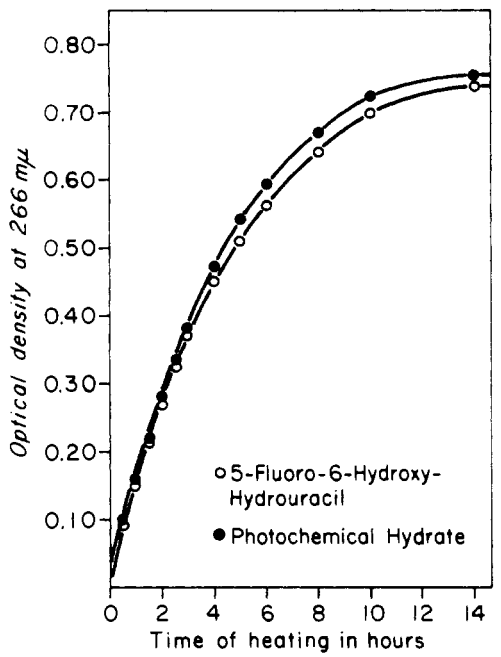


FIG. 5.—Heat reversal of aqueous, unbuffered solutions of 1.35 × 10^{-4} M 5-fluoro-6-hydroxyhydrouracil and photochemical hydrate at 85°.

RATE OF DECOMPOSITION OF HYDRATES IN 1.0N NaOH AS MEASURED BY THE APPEARANCE OF THE 260mμ ABSORPTION PEAK

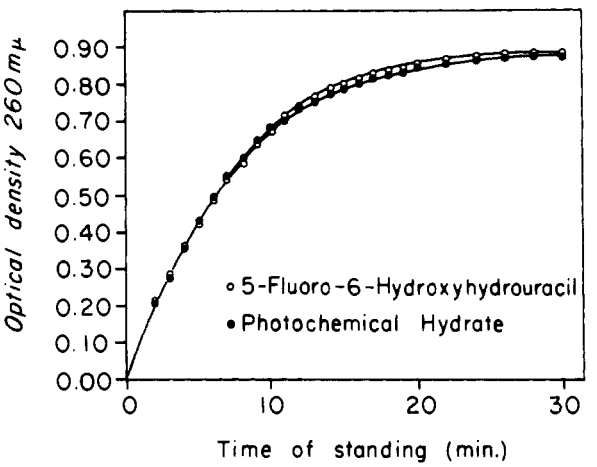


FIG. 6.—Rates of decomposition of 5-fluoro-6-hydroxyhydrouracil and photochemical hydrate in 1.0 N NaOH at 30° as measured by the appearance of the 260-mμ absorption maximum. Aqueous solutions of the respective hydrates were made up in 1.0 N NaOH at a final concentration of 6.75 × 10^{-5} M and the absorption at 260 mμ was immediately followed with the Beckman Model DU spectrophotometer. Rates of decomposition for both compounds were measured simultaneously.

The structure of the irradiation product of the uracil derivative, 1,3-dimethyluracil, was subsequently shown to be that of 1,3-dimethyl-6-hydroxyhydrouracil (Wang *et al.*, 1956; Moore and Thompson, 1957; Wang, 1958). The heat-reversible photoproduct of uracil also has been found to be identical to the chemically synthesized 6-hydroxyhydrouracil obtained by catalytic hydrogenation of 5,5-dibromo-6-hydroxyhydrouracil (Moore, 1958; Gattner and Fahr, 1963). It was reasonable to suspect that our heat-reversible photoproduct would be a hydrate of 5-fluorouracil. A comparison of the chromatographic mobility in the 1-butanol-water solvent system of uracil hydrate (Wacker *et al.* 1961), the heat-reversible photoproduct of 5-fluorouracil, uracil, and 5-fluorouracil (see Table I), indicated that the mobility of the photoproduct of 5-fluorouracil was that expected of a hydrate of 5-fluorouracil.

A large scale irradiation of aqueous 5-fluorouracil followed by paper chromatography yielded a crystalline material. This material was shown unequivocally to be identical with the chemically synthesized 5-fluoro-6-hydroxyhydrouracil by means of the following criteria:

(1) *Anal.* Calcd for $C_4H_5O_3F$: C 32.44; H 3.40; F 12.83. Found for photoproduct: C 32.70; H 3.24. Found for 5-fluoro-6-hydroxyhydrouracil: C 32.29; H 3.42; F 13.15.

(2) *Melting Point.*—Photoproduct: 180–181° with gas evolution, resolidification, remelting at 276°. 5-Fluoro-6-hydroxyhydrouracil: 183–184° with gas evolution, resolidification, remelting at 279°. Mixture of above two: 182–183° with gas evolution, resolidification, remelting at 278°.

(3) *Reversion to 5-Fluorouracil upon Heating in Aqueous Solution.*—Stock, aqueous solutions of 1.35 × 10^{-4} M photochemical product and 5-fluoro-6-hydroxyhydrouracil were heated in glass-stoppered tubes at 85° in a water bath. The *p*H of these aqueous unbuffered solutions was 6.0. Aliquots were removed at specified time intervals, and the spectra of the heated samples were taken. Aqueous solutions of photochemical product and 5-fluoro-6-hydroxyhydrouracil exhibit only end absorption below 240 mμ. On heating at 100°, an absorption spectrum with a maximum at 266 mμ appeared and was identical with that of 5-fluorouracil. The rates and extent of conversion of the respec-

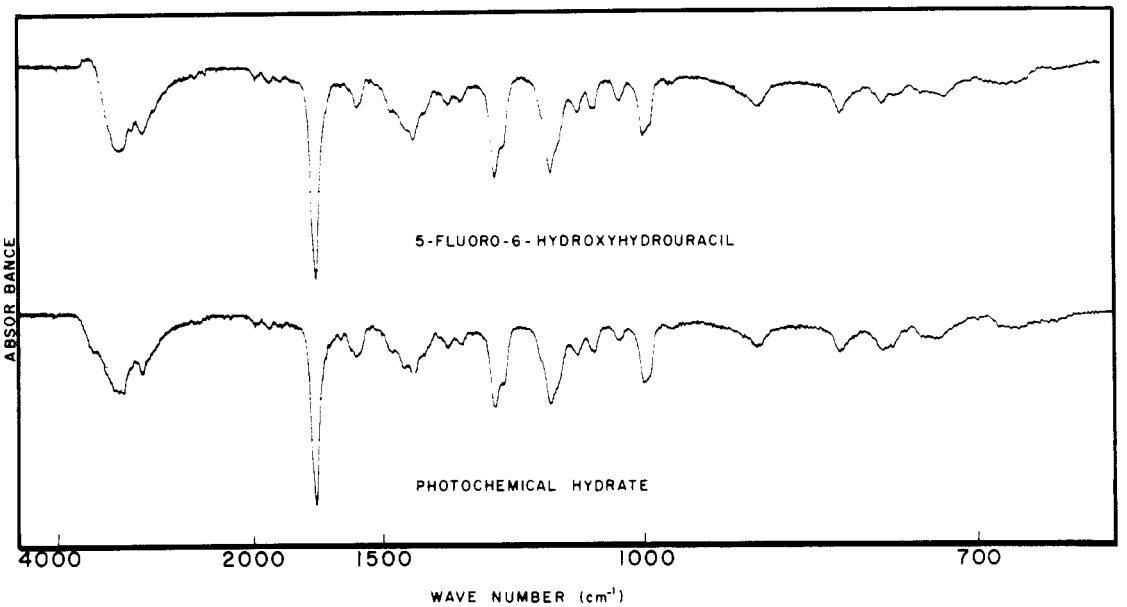


FIG. 7.—Infrared spectra of chemically synthesized material and photoproduct.

tive hydrates, as measured by the appearance of the 266-mμ absorption peak, to 5-fluorouracil were identical (Fig. 5). An approximately 70% conversion was attained on heating the hydrates for 10 hours at 85° (ϵ_{266} mμ for 5-fluorouracil, 7070 [Duschinsky *et al.*, 1957]). Rates of conversion were also identical on heating the hydrates in 0.05 N HCl at 100°.

(4) *Base Decomposition.*—The photochemical compound and 5-fluoro-6-hydroxyhydrouracil behave identically on base degradation. As indicated in Figure 6, both compounds are exceedingly labile in 1.0 N NaOH at approximately 30° as measured by the rate of appearance of a new absorption peak at 260 mμ which rapidly reaches a maximum value. Acidification of these 1.0 N NaOH solutions resulted in the complete disappearance of the 260-mμ absorption peak. On realkalization, this peak reappears immediately.

(5) *Extinction Coefficient of Alkaline-Decomposition Product.*—The extinction coefficient of the alkaline decomposition product calculated from the results under (4) is 13,300 ± 200, in agreement with the value 13,300 obtained for the alkaline-decomposition product of 5-fluoro-6-hydroxyhydrouracil.

(6) *Paper-chromatographic Behavior.*—The photochemical hydrate and the chemically synthesized hydrate were chromatographed on Whatman 3MM paper in the following solvents: 1-butanol–acetic acid–water (5:2:3); ethanol-water (85:15); and 1-butanol-water (86:14). The results are listed in Table I.

(7) *Infrared-Absorption Spectra.*—The spectra of photoproduct and chemically synthesized HOHFU are reproduced in Figure 7. They appear identical in every detail. The details of the spectra are λ_{max} (KBr) 3125, 3050, 2985, 2900, 2780, 1980, 1915, 1860, 1720, 1582, 1480, 1442, 1418, 1333, 1298, 1234, 1215, 1135, 1092, 1066, 1030, 1000, 870, 796, 764, 758 cm^{-1}.

(8) *Nuclear Magnetic Resonance Studies.*[5]—The spectra were obtained with a Varian A-60 NMR spectrometer. Dimethylformamide-D_7 was used as the solvent and tetramethylsilane was the internal standard. A trace of deuterium oxide was added to remove the coupling between H_1 and H_6.

H_6 of HOBrFU appears at 4.63 τ, and H_5 and H_6 of HOHFU are at 4.55 and 4.73 τ, respectively. Verification of the structural assignments of HOBrFU and HOHFU can be obtained from the magnitude of the spin-spin coupling constants. The small coupling (2 cps) between the fluorine and hydrogen in HOBrFU indicates that they are located on different carbons. Jackman (1959) reported coupling constants of 44–81 cps when the fluorine and hydrogen are on the same carbon.

The observed coupling (3.8 cps) between the two protons in HOHFU is consistent with vicinal coupling (2–9 cps) and not with geminal coupling (12–15 cps). In addition, one proton in HOHFU has a typical vicinal-hydrogen-fluorine-coupling constant (2.6 cps) while the other proton has a hydrogen-fluorine-coupling constant (47.5 cps) consistent with a geminal coupling.

The identical chemical shifts and spin-spin-coupling constants observed in the nuclear magnetic resonance spectra of photoproduct and chemically synthesized compound indicate that they are identical. Since vicinal-coupling constants depend on dihedral angles, a difference in the proton-proton and/or the proton-fluorine-coupling constants would be expected if the two compounds were geometric isomers. Recent studies by Shugar and co-workers on the photochemical hydration of 1,3-dimethyl-5-fluorouracil have yielded results which are consistent with the hydration of this material to form, with respect to the hydrogens 5 and 6, a *trans*-1,3-dimethyl-5-fluoro-6-hydroxyhydrouracil (Fikus *et al.*, 1964).

The stereochemistry of HOHFU cannot be considered as firmly established, but the following consideration may shed some light on the question. It is assumed that initial addition of hypobromous acid to FU follows the usual *trans* course (Houben-Weyl, 1960). The hydrogenation of the resulting HOBrFU may take place with or without inversion. Hydrogenation in the very acidic unbuffered medium gives only FU and no HOHFU, while in presence of sodium acetate a mixture of about 20% FU and 80% HOHFU is obtained. It seems reasonable to assume that the source of FU is the more labile of the two possible diastereomers, namely, the *cis* form (with respect to H_5 and H_6) which carries H and OH in *trans* position. Thus it appears that the unbuffered hydrogenation proceeds without inversion and leads to *cis* form, which is readily dehydrated to FU, while the buffered hydrogenation

[5] We are indebted to Dr. E. Billeter and Dr. F. M. Vane of Hoffmann-La Roche for the NMR studies.

proceeds with 80% inversion. Our isolated HOHFU proved to be perfectly stable in the acidic medium and therefore cannot be the source of FU. Consequently it should be the *trans* form with respect to the hydrogens in 5- and 6- position.

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Synthetic Experiments in the Ferrichrome Series*

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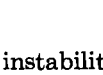
The amino acid sequences within the cyclic hexapeptide moieties of the ferrichrome compounds have been elucidated. The sequence in ferrichrome (cyclo-triglycyltri-δ-*N*-acethydroxamido-L-ornithinyl) was established through chromatographic examination of partial acid hydrolysates and by the synthetic preparation, from three residues each of glycine and δ-*N*-acetyl-L-ornithine, of a cyclic hexapeptide corresponding to a reduction derivative of the natural product. The sequence in ferrichrome A (cyclo-di-L-serylglycyltri-δ-*N*-acylhydroxamido-L-ornithinyl) was determined through chromatographic analysis of partial acid hydrolysates and through the application of the β-elimination reaction characteristic of serylpeptides.

Previous investigations on the structure of the ferrichrome compounds showed these substances to be ferric trihydroxamates in which the metal-binding center is mounted upon a cyclic hexapeptide platform (Fig. 1) (Emery and Neilands, 1961). The peptide moiety was found to contain three residues of neutral amino acids, which may be glycine alone (ferrichrome) or a combination of 1 mole of glycine with 2 moles of serine (ferrichrome A), together with 3 residues of a new amino acid, δ-*N*-hydroxyornithine (Emery and Neilands, 1961; Rogers and Neilands, 1963). The acyl substituents at the hydroxamate bonds in ferrichrome and ferrichrome A are acetic acid and *trans*-β-methylglutaconic acid, respectively (Emery and Neilands, 1960).

In the formula published previously, the precise sequence of the amino acid residues within the cyclic hexapeptides remained as a major unsolved feature of the proposed structure. In the case of ferrichrome four arrangements could exist, while for ferrichrome A many additional sequence isomers are possible. However, deductions based upon the examination of molecular models coupled with the finding of glycylglycine in partial acid hydrolysates of ferrichrome ruled out the possibility of a completely alternating sequence.

Sequence analysis in the ferrichrome peptides is confronted with several obstacles. In the first place these substances do not seem to be attacked by any of the common proteolytic enzymes. Secondly, the cyclic form is relatively stable compared to the open-chain species, and hence partial acid hydrolysis tends to give either the undegraded peptides or the free amino acids (Bamford and Weymouth, 1955). Finally, the hydroxamic acid groups in the side chains would be expected to be cleaved under conditions which open the cyclic-peptide moiety. This would introduce several additional complications, not the least of which is the



instability of the R—N—H group at neutral *p*H. These considerations prompted a search for a more satisfactory and definitive means of determining the amino acid sequences in ferrichrome and ferrichrome A.

It was reasoned that if the hydroxamic acid linkages could be reduced to the amide level then the resulting stable hexapeptide should, in the case of ferrichrome at least, be available by synthesis. Careful comparison of each of the four synthetic model hexapeptides with fully reduced ferrichrome should then reveal the structure of the natural product. While these experiments were in progress, we were greatly assisted by two developments, namely, (a) a personal communication from O. Mikeš and J. Turková[1] in which they announced the amino acid sequence in the peptide core of the antibiotic albomycin to be cyclo-triseryltri-δ-*N*-acethydroxamidoornithinyl and (b) the appearance of

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