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Keyphrases

Pyrrolizidine alkaloids
Tumor inhibition–pyrrolizidine alkaloids
Lipid solubility—antitumor activity
Alkylating ability—antitumor activity

Prediction of Stability in Pharmaceutical Preparations XV

Kinetics of Hydrolysis of 5-Trifluoromethyl-2′-deoxyuridine

By HANS J. NESTLER* and EDWARD R. GARRETT†

The antiviral 5-trifluoromethyl-2′-deoxyuridine, F_3TdR, is hydrolyzed by hydrogen ions and solvent to 5-trifluoromethyluracil, F_3T, and deoxyribose. The F_3T is readily hydrolyzed to 5-carboxyuracil by hydroxyl ion attack on the undissociated and anionic species. At elevated temperatures, 5-carboxyuracil is decarboxylated by hydroxyl ion catalysis to uracil. The F_3TdR is readily hydrolyzed to 5-carboxy-2′-deoxyuridine by hydroxyl ion attack on the undissociated and anion species through an observable kinetic intermediate at high alkalinity, probably 5-hydroxydifluoromethyl-2′-deoxyuridine. The optimum pH range for stabilization is 1–4 where the half-life at 30° is 280 days. The material is readily degraded at pH 7.4 where 1.5 days is the half-life at 30°.

THE KNOWN antitumor and antiviral activity of the 5-halogenated uracils and 5-halogenated pyrimidine nucleosides (1) initiated the synthesis of 5-trifluoromethyl-2′-deoxyuridine (trifluorothymidine, F_3TdR, I) (2). The structure I sterically resembles 5-iodo-2′-deoxyuridine, IDU, which is active against *Herpes simplex* in human keratitis (3). The van der Waals radius of iodine is 2.15 Å. and that of the trifluoromethyl group is 2.44 Å. (4). Since a degradation product of IDU, 5-iodouracil tends to antagonize its activity (5), complete studies of its kinetics of solvolysis were performed in order to determine optimum conditions for pharmaceutical stabilization (6–9).

Preliminary experiments with F_3TdR, I, have

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indicated that it is about 100 times as potent against *Herpes simplex* infections as IDU (10). Intrinsic potency is about 10 times greater but the solubility enhancement is also 10-fold. It also appears that its use is less conducive to the development of resistance (10).

Since the degradation products of nucleosides frequently interfere with the biological activity of its precursors (5, 11, 12), a full study of the kinetics of solvolysis of F_3TdR, I, is necessary to determine the optimum stability conditions for its pharmaceutical formulation and the products of its hydrolysis over the entire pH range. The comparative solvolytic rates of F_3TdR may also provide information on the contributory effects of strongly electronegative 5-substituents to the solvolysis of pyrimidine nucleosides which has been recently discussed in detail (13).

EXPERIMENTAL

Materials—5 - Trifluoromethyl - 2′ - deoxyuridine, F_3TdR, was obtained from P-L Biochemicals, Inc. and 5-trifluorothymine, F_3T, from Merck Sharp and Dohme. All solutions were made up in nitrogen-purged distilled water. Stock solutions of F_3TdR and F_3T were 0.7×10^{-2} *M* and 0.35×10^{-2} *M*, respectively, and were always prepared shortly before using. The pH values of buffer solutions were read at the temperature of the kinetic experiments. The pH values of NaOH and HCl solutions were obtained by calculation and extrapolation from the data of Harned and Owen (14). Ionic strength in all buffer solutions was adjusted to 0.1 with NaCl.

Instruments—A Cary model 15 spectrophotometer equipped with a repetitive scan accessory, automatic sample changer, and a thermostatically controlled cell compartment, and a Beckman DU spectrophotometer, DU-2 were used for ultraviolet absorbance readings. The pH was read on a Radiometer (Copenhagen, Denmark) pH meter equipped with a Beckman high temperature (pH range 1–14) glass electrode with a calomel reference electrode. For potentiometric titrations a Sargent automatic titrator model D was used.

Acid Solvolysis of F_3TdR—An aliquot (2.00 ml.) of F_3TdR stock solution was added to 98 ml. of thermally equilibrated HCl or acetate buffer to produce concentrations of 1.4×10^{-4} *M*. Samples were taken at appropriate time intervals, chilled to room temperature, and an equal volume of 1 *N* NaOH added. After 24 hr. at room temperature the increasing absorbance, *A*, to its asymptotic value A_∞ was read at 300 mμ (Fig. 1). From the slope of the plot $\log/A_\infty - A/$ *versus* time the apparent first-order rate constants were calculated for the temperatures listed in Table I.

Alkaline Solvolysis of F_3TdR—The initial transformation of F_3TdR to a presumed 5-hydroxydifluoromethyl-2′-deoxyuridine intermediate (VIII) was followed at 29.5° by adding 0.5 ml. of F_3TdR stock solution to 10 ml. of NaOH solutions to yield a 3.34×10^{-4} *M* solution. The spectral absorbance increased at 298.4 mμ to a readily obtainable asymptotic value (Fig. 2) prior to further reaction,

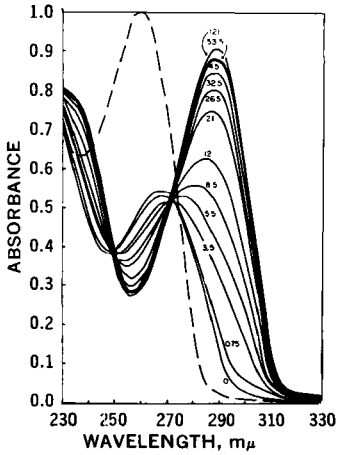


Fig. 1—Typical spectral changes of alkaline-treated (0.5 N NaOH, 24 hr. at room temperature) 5-trifluoromethyl-2′-deoxyuridine, F_3TdR, after the labeled hr. of degradation in 0.002 N HCl at 80.0°. The acid solutions of 1.4×10^{-4} M F_3TdR were diluted 1:1 with 1.0 N NaOH. The spectral end-product, $\lambda_{max.}$ 288 mμ, is 5-carboxyuracil anion. Key: ——, hr.; - - -, spectrum of 5-trifluoromethyluracil in 0.5 N NaOH.

and permitted the first-order rate constants at the cited NaOH concentrations of Table I to be determined. Similar studies were conducted in borate and phosphate buffers but valid asymptotic values for this first transformation were not easily obtained, probably because of the greater relative speed of the subsequent transformation.

At higher temperatures, *e.g.*, 47°, this first reaction was a fast process so that initial spectra (see 295 mμ in Fig. 3) already represented the complete transformation to the probable 5-hydroxydifluoromethyl-2′-deoxyuridine intermediate. The reaction was studied at a concentration of 1.05×10^{-4} *M* in various NaOH, borate, and phosphate buffer solutions listed in Table I with their pH values at 280 mμ and the rate constants determined. In buffer solutions below pH 8 the initial changes due to the first reaction were not as readily observable, and thus estimates of these rate constants were not as valid as the rate constants for the formation of 5-carboxy-2′-deoxyuridine from degrading F_3TdR.

Decarboxylation of 5-Carboxy-2′-deoxyuridine—The spectrum of 5-carboxy-2′-deoxyuridine was obtained very quickly at 80° in NaOH and borate buffers from F_3TdR. The spectrum then changed slowly to $\lambda_{max.}$ 261 mμ in base and 262 mμ in acid; these spectra are inidcative of 2′-deoxyuridine as the product. The reaction appeared to be pH independent in the alkaline region. 2′-Deoxyuridine was relatively stable in buffer below pH 9, but slowly degraded in strong NaOH with loss of absorbance.

Degradation of Trifluoromethyluracil, F_3T—An aliquot (2.00 ml.) of F_3T stock solution was added to 98 ml. of various NaOH and buffer solutions, preequilibrated at 34°. The reaction to 5-carboxyuracil was followed by monitoring the increasing absorbance at 290 mμ attributed to the 5-carboxyuracil anion. The derived first-order rate constants, solvent conditions, and pH values are given in Table II. This reaction is completed almost instantaneously at 80°. The subsequent

TABLE I—APPARENT FIRST-ORDER RATE CONSTANTS IN SEC.$^{-1}$ FOR THE HYDROLYSIS OF 5-TRIFLUOROMETHYL-2′-DEOXYURIDINE AND A PROBABLE INTERMEDIATE, 5-HYDROXYDIFLUOROMETHYL-2′-DEOXYURIDINE

80.0°				47.0°				29.5°			
Medium		pH[a]	10^5k[b]	Medium		pH[a]	10^5k[c]	Medium		pH[a]	10^5k[d]
[HCl]				[NaOH]	[H_3BO_3]			[NaOH]	[H_3BO_3]		
2.54		−0.34	7.97	0.962	—	13.40	32.2	0.126	—	12.80	205
1.407		−0.04	4.73	0.485	—	12.95	19.4	0.097	—	12.70	183
0.918		0.15	3.69	0.194	—	12.50	11.6	0.068	—	12.55	127
0.608		0.35	3.30	0.097	—	12.20	10.7	0.0485	—	12.40	118
0.217		0.80	2.27	0.0194	—	11.60	7.85	0.0194	—	12.10	46.0
0.100		1.10	2.14	0.0049	—	11.00	7.77	0.0097	—	11.75	22.5
0.010		2.05	2.16	0.050	0.050	9.57	8.29	0.0049	—	11.45	11.8
0.002		2.70	2.10	0.025	0.050	9.00	7.83	0.050	0.050	9.70	1.508
[$HC_2H_3O_2$]	[$C_2H_3O_2^-$]			[Na_2HPO_4]	[NaH_2PO_4]			0.025	0.050	9.00	0.973
1.00	0.100	3.75	1.95	0.033	0.002	8.00	6.10	0.012	0.050	8.30	0.945
0.100	0.100	4.75	2.57	0.025	0.025	6.70	1.18	[Na_2HPO_4]	[NaH_2PO_4]		
				0.068	0.010	5.90	0.255	0.033	0.002	8.00	0.936
				[$HC_2H_3O_2$]	[$C_2H_3O_2^-$]			0.006	0.031	7.40	0.540
				0.025	0.100	5.30	0.0753				
				0.100	0.100	4.60	0.0373				

[a] The pH values were calculated from pH = $-\log a_{HCl}$ [HCl] and pH = $pK_w - \log a_{NaOH}$ [NaOH], respectively, where the activity coefficients for the respective [HCl] and [NaOH] and the pK_w values at the several temperatures (12.63, 80.0°; 13.35, 47.0°; 13.83, 29.5°) were obtained from the literature (14). The pH values in the buffer solutions were measured at the stated temperatures and the ionic strengths of these buffer solutions were adjusted to 0.1 with NaCl. [b] These rate constants at 80.0° were obtained for the acid-catalyzed solvolysis of 1.40×10^{-4} *M* 5-trifluoromethyl-2′-deoxyuridine, I, to 5-trifluoromethyluracil, II, and deoxyribose by monitoring the appearance of 5-carboxyuracil, IV, derived from II after 24 hr. in 0.5 *N* NaOH at room temperature. Additional rate constants obtained for this transformation were: °C., [HCl], 10^5k; 70.0°; 2.54, 2.55; 60.0, 2.54, 0.570; 47.0, 2.54, 0.0994; 75.0°, 0.100, 1.1; 70.0°, 0.100, 0.617; 60.0°, 0.100, 0.253. [c] The rate constants at 47.0° in NaOH solutions were obtained for the hydroxyl ion catalyzed solvolysis of the anion of 5-hydroxydifluoromethyl-2′-deoxyuridine, VIII, to 5-carboxy-2′-deoxyuridine, IX ($\lambda_{max.}$ 272 mμ) after the rapid spectral changes at 295 mμ denoted completion of the formation of VIII from 1.05×10^{-4} *M* 5-trifluoromethyl-2′-deoxyuridine, I. Additional rate constants for the hydrolysis of VIII to IX were °C., [NaOH], pH, 10^5k; 38.0, 0.485, 13.1, 8.70; 26.2, 0.535, 13.6, 1.73. The buffer studies were conducted *in situ* and no significant amount of VIII could be observed indicative that these rate constants in the buffer solutions are for I → VIII with rapid degradation to IX. [d] Additional rate constants obtained at 60.0° for hydrolysis of I to IX through the intermediate VIII were: pH, 10^5k; 5.90 (phosphate), 1.24; 5.30 (acetate), 0.49. The rate constants at 29.5° in NaOH solution were obtained for the formation of the probable anion of 5-hydroxydifluoromethyl-2′-deoxyuridine, VIII, from 3.34×10^{-4} *M* trifluoromethyl-2′-deoxyuridine, I, a rapid reaction which is completed much before the hydrolysis of VIII to IX becomes significant as indicated by the monitoring of spectral absorbance at 295 mμ *in situ*. In the borate and phosphate buffer regions, there is no indication from the 290 and 295 mμ absorbances that VIII has more than a transitory existence and that its transformation to IX is very rapid. Thus, these rate constants in the buffer solutions are for I → VIII with rapid degradation to IX.

slower decarboxylation of 5-carboxyuracil to uracil ($\lambda_{max.}$ 283 mμ) was monitored spectrophotometrically.

Isolation of 5-Carboxyuracil—F_3T (200 mg.) was dissolved in 10 ml. of 0.5 *N* NaOH and heated for 30 min. at 50°. The cooled solution was adjusted to pH 3.0 with 2.5 *N* HCl. The refrigerated crystals were filtered after 3 days and dried under high vacuum at 60° for 3 hr. The yield was 180.8 mg., m.p. 284–285° dec. [lit. (15) m.p. 278° dec.]. The product was recrystallized from 50% aqueous ethanol; yield 62 mg., m.p. 285° dec. The com-

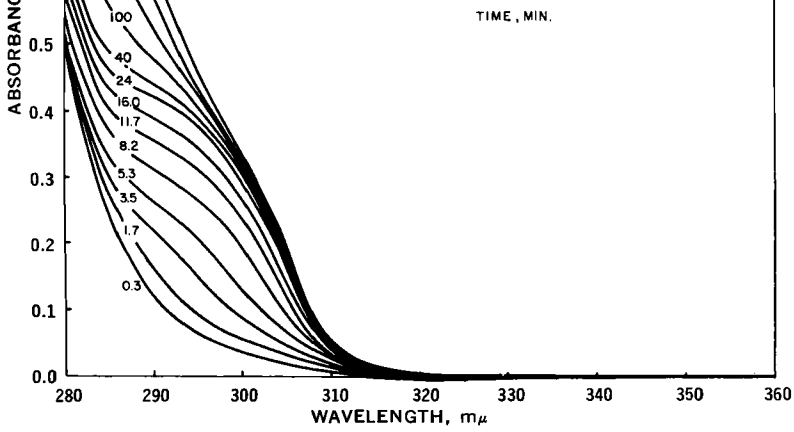


Fig. 2—Typical initial spectral changes for the degradation of 5-trifluoromethyl-2′-deoxyuridine, F_3TdR, in alkaline solution. The conditions were F_3TdR, 3.34×10^{-4} M in 0.126 N NaOH at 29.5°. Key: curves, min. after the start of the reaction; insert, typical plots of the absorbance at 295 mμ against time for the production of the probable 5-difluorohydroxymethyl-2′-deoxyuridine at several NaOH concentrations: ▲, 0.0485 N; ×, 0.097 N; Δ, 0.0049 N.

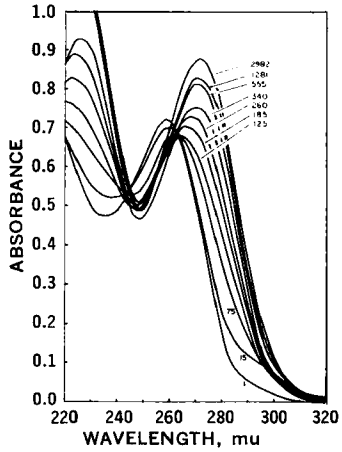


Fig. 3—Typical spectral changes of 5-trifluoromethyl-2′-deoxyuridine, F_3TdR, in alkaline solution. The conditions were F_3TdR, 1.05 × 10^{-4} M, in 0.020 N NaOH at 47.0°. Key: curves, min. after the start of the reaction. The rapid increase in absorbance in the range 290–310 mμ within the first 15 min. may be attributed to the production of a difluoro-monohydroxy intermediate.

pound is claimed to crystallize as the monohydrate (15), mol. wt. 174.1 + 18.0.

Anal.—Calcd. for $C_5H_4N_2O_4 \cdot H_2O$: C, 31.1; H, 3.12; N, 14.6. Calcd. for $C_5H_4N_2O_4$: C, 34.5; H, 3.45; N, 16.1. Found: C, 33.34; H, 3.22; N, 15.76.

Equivalent weight found by titration was 176.5, $pKa_1' = 4.25$, $pKa_2' = 8.90$.

Isolation of 5-Carboxy-2′-deoxyuridine—F_3TdR (200 mg.) was dissolved in 10 ml. of 1 *M* NaOH and heated for 7 hr. at 47°. The pH was adjusted to 6.8 with concentrated HCl. The solvent was removed *in vacuo*. The white residue was triturated with ethanol to which a few drops of concentrated HCl had been added. After filtering off the NaCl, the separated yellow filtrate was evaporated *in vacuo* at 60°. The yield was 115 mg. of yellow crystals, m.p. 165°. Repeated recrystallization from absolute ethanol yielded 31 mg. of a chloride-free product, m.p. 170° dec. The elementary analysis, mol. wt. 272.2, was as follows.

Anal.—Calcd. for $C_{10}H_{12}N_2O_7$: C, 44.12; H, 4.44; *N*, 10.29. Found: C, 44.64; H, 4.48; N, 10.20.

Paper Chromatography—Paper chromatography was used to monitor the basic degradation reactions from F_3TdR to 5-carboxy-2′-deoxyuridine, DU-5-COOH, and from F_3T to 5-carboxyuracil, U-5-COOH. Aliquots (25 μl.) of approximately 10^{-2} *M* aqueous solutions were spotted on Whatman No. 1 paper and developed with a mixture of ethyl acetate, formic acid, and water (21:3:1.5) (16). The substances were visualized by UV light (254 mμ). The acids were detected by spraying the dried chromatogram with alcoholic bromocresol green solution and gave yellow spots against a blue background. F_3TdR and F_3T move with the solvent front while the acids DU-5-COOH and U-5-COOH have an R_f value of ~0.5.

Table III lists the principal UV spectra and pKa′ values determined by potentiometric or spectrophotometric titration.

[1] The synthesis of this compound has been reported recently (Isenberg, N., and Heidelberger, C., *J. Med. Chem.* 10, 970(1967), colorless crystals, m. p. 158–159,° ε = 11,400 ($\lambda_{max.}$ 276 mμ, 0.1 *N* HCl), ε = 6,810 ($\lambda_{max.}$ 271 mμ, 0.1 *N* NaOH).

TABLE II—APPARENT FIRST-ORDER RATE CONSTANTS IN SEC.$^{-1}$ FOR THE HYDROLYSIS OF TRIFLUOROMETHYLURACIL[a] AT 34.0°

[NaOH]	pH[b]	10^4k
0.485	13.3	336
0.194	13.0	285
0.097	12.6	233
0.049	12.35	162
0.0194	11.95	78
0.0049	11.40	44.5

[a] Hydrolysis to 5-carboxyuracil. In borate buffer at pH 9.70 at 34.0°, $k = 5.17 \times 10^{-5}$. [b] Calculated from activity coefficients in the literature (14) where pH = pK_w − log a_{NaOH}[NaOH].

RESULTS AND DISCUSSION

Degradation in Mineral Acid—The glycoside bond of 5-trifluoromethyl-2′-deoxyuridine (F_3TdR), I, is readily cleaved in acid (Scheme I) in a manner common to pyrimidine deoxyribosides (13). The initial products are 5-trifluoromethyluracil (F_3T) (II) and deoxyribose, III. Deoxyribose is not stable under these acidic conditions; it is transformed in part to 5-methyl-3(2*H*)-furanone (17, 18), V, and levulinic acid (VI) (19). The absorbance of the former compound, V, $\lambda_{max.}$ 261 mμ which would interfere with the spectrophotometric monitoring of the F_3TdR, $\lambda_{max.}$ 261 mμ in 0.1 *N* HCl and $\lambda_{max.}$ 259 mμ in 0.1 *N* NaOH, can be removed (13, 17) by degradation of the reaction mixture in 0.5 *N* NaOH at room temperature for 24 hr.

The derived product of acidic solvolysis of I, which is F_3T, II, is completely hydrolyzed under these alkaline conditions to 5-carboxyuracil, IV ($\lambda_{max.}$ 289 mμ in 0.5 *N* NaOH) as shown in Scheme I. The acid-unreacted F_3TdR is also degraded under these alkaline conditions (Fig. 3). The 261 mμ $\lambda_{max.}$ shifts to 271 mμ $\lambda_{max.}$ in NaOH at room temperature in 24 hr., but contributes little to the change in the absorbance of IV at 295 mμ or 300 mμ in alkali. Thus the absorbance of IV at 295 mμ after 24 hr. at room temperature in 0.5 *N* NaOH may be taken as a measure of the extent of hydrolysis of I in acid solution. Typical spectra of the alkaline-treated acid degradations of I are given in Fig. 1. Typical first-order plots for the solvolysis of I in acid are given in Fig. 4 in accordance with the expression

$$\log |A_\infty - A| = -kt/2.303 + \log A_\infty \quad \text{(Eq. 1)}$$

where A is the absorbance with time, t, at 295 mμ after treatment for 24 hr. in 0.5 *N* NaOH at room temperature, A_∞ is the final absorbance, and k is the apparent first-order rate constant for the studies listed in Table I.

A plot of the apparent first-order rate constants (Table I) for the hydrolysis of F_3TdR, I, against [HCl] at 80.0° is linear with a finite intercept. This is indicative of a catalytic attack of hydrogen ion on the undissociated molecule, I, and a pH independent hydrolysis where

$$k = k_0 + k_{HCl}[HCl] \quad \text{(Eq. 2)}$$

where $k_0 = 2.0 \times 10^{-5}$ sec.$^{-1}$ and $k_{HCl} = 2.0 \times 10^{-5}$ L./mole/sec.

Degradation of F_3T—The 5-trifluoromethyluracil II, is known to produce 5-carboxyuracil, IV, in 0.1 *N* sodium bicarbonate and in 1.0 *N* NaOH solutions (2, 20). This can be followed readily by monitoring the absorbance at 290 mμ. Typical spectral changes in alkali with time are given in Fig. 5 and typical first-order rate constants are listed in Table II. Typical first-order plots are given in Fig. 6. At 34.0° the final absorbance was consistent with a stoichiometric yield of 5-carboxyuracil. Paper chromatographic monitoring of the reaction did not indicate any other processes but II → IV.

The rate constants can be well fitted by

$$k = k_{NaOH}'[NaOH]\, f_{u^-} \qquad \text{(Eq. 3)}$$

where $k_{NaOH}' = 4.5 \times 10^{-2}$ L./mole/sec. at 34.0°, where $f_{u^-} = [H^+]/([H^+] + K_a)$ is the fraction of 5-carboxyuracil that exists as the monoanion at the $[H^+]$ determined from the calculated pH (Table II). The K_{a_2}' is taken as 2.5×10^{-13}. This Eq. 3 indicates that the hydrolysis of F_3T, II, is effected by hydroxyl ion attack on the monoanion, or its kinetically equivalent water attack on the dianion at pH values more than one unit above the pK_{a_1}', 7.40 at 25.0°.

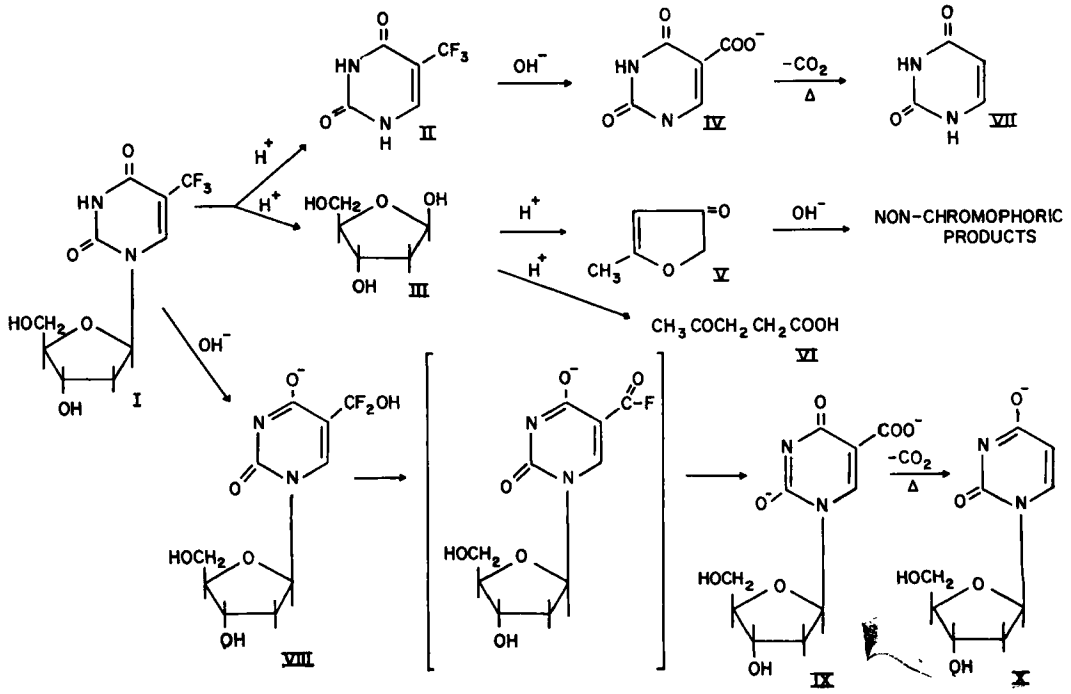
At elevated temperatures the anion of 5-carboxyuracil, IV, decarboxylates (19) to uracil, VII, but much more slowly than the production of IV from II. This is spectrally observable from the loss of absorbance at the 290 mμ $\lambda_{max.}$ in alkali of IV and the appearance of a 283 mμ $\lambda_{max.}$ with the lessened absorbance of the spectrum of VII.

At 80.0° in 0.02 *N* alkali the spectrum of 5-carboxyuracil, IV, is produced almost within 0.5 hr. from F_3T, II. The apparent first-order rate constants for the subsequent decarboxylation of IV were obtained from the slopes of typical first-order plots in accordance with Eq. 1. They were 8.17×10^{-7} and 2.07×10^{-7} sec.$^{-1}$ in 0.485 *N* (pH

TABLE III—SPECTRAL AND pKa′ VALUES

		M.p., °C.	pK_{a_1}'	pK_{a_2}'	0.1 *N* HCl $\lambda_{max.}$	0.1 *N* HCl ε	0.1 *N* NaOH $\lambda_{max.}$	0.1 *N* NaOH ε
5-Trifluoromethyl-2′-deoxyuridine, I (5-trifluorothymidine)	F_3TdR	186–189	7.85[a]	—	261	10,350	259	7,150
5-Hydroxydifluoromethyl-2′-deoxyuridine (5-hydroxydifluorothymidine)	HOF_2TdR	—	—	—	—	—	260[d]	7,000[d]
5-Carboxy-2′-deoxyuridine	DU-5-COOH	170	4.0[a]	9.8[a]	277	12,300	272	7,800
2′-Deoxyuridine	DU	161–163	9.3[a]	—	262	9,700	262	7,300
5-Trifluoromethyluracil (5-trifluorothymine)	F_3T	245–248	7.4[a]	12.6[c]	256	8,700	270	8,000[e]
5-Carboxyuracil	U-5-COOH	285	4.20[a] 4.25[b]	9.10[a] 8.90[b]	276	11,400	290	14,000
Uracil	U	335	9.0[a]	>13[a]	259	8,100	283	6,200

[a] Determined by spectrophotometric titration at 25.0°. [b] Determined by potentiometric titration at 25.0°. [c] Estimated from kinetic data at 34.0°. [d] Estimated. [e] Obtained by extrapolation to zero time.



Scheme I

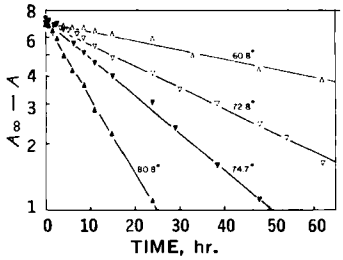


Fig. 4—Typical first-order plots for the degradation of 1.4 × 10⁻⁴ M 5-trifluoromethyl-2′-deoxyuridine in 0.1 N HCl at various temperatures. The absorbance values, A, were measured at 297.5 mμ for aliquots diluted 1:1 with 1.0 N NaOH and held for 24 hr. at room temperature where A_∞ is the asymptotic absorbance.

12.15) and 0.0218 *N* (pH 10.85) NaOH, respectively, at 80.0°. At 80.0° in pH 8.7 borate buffer, k = 2.0 × 10⁻⁷ sec.⁻¹ and in pH 6.7 phosphate buffer, k = 5.8 × 10⁻⁷ sec.⁻¹. At 80.0° in acetate buffers, k = 10.1 × 10⁻⁷ and 1.63 × 10⁻⁷ at pH values 4.75 and 3.75, respectively. It has been demonstrated that the major kinetic dependency for decarboxylation is by the attack of hydroxyl ion on the anion (21). Thus, it is probable from these rate constants that the specific catalytic rate constant for the attack of hydroxyl ion on the monoanion (or its kinetically equivalent water attack on the dianion) below the pKa_2' 9.0 (25.0°) but above the pKa_1' 4.2 (25.0°) of the carboxyl is of greater magnitude than the specific catalytic rate constant for the attack of hydroxyl ion on the dianion above the pKa_2'. The rate of decarboxylation most probably plateaus in the vicinity of this value and then decreases with solutions of decreasing pH values until values below the pKa_1' are reached. The values obtained in phosphate and acetate buffer solutions may be indicative of general base catalysis by phosphate and acetate ions where such catalysis has been reported for the decarboxylation of salicylic acids (21).

An expression consistent with the rate data excluding the values in acetate and phosphate buffers is

$$k = (k_0'' + k_{OH}''a_{NaOH}[NaOH])f_{u-} \quad \text{(Eq. 4)}$$

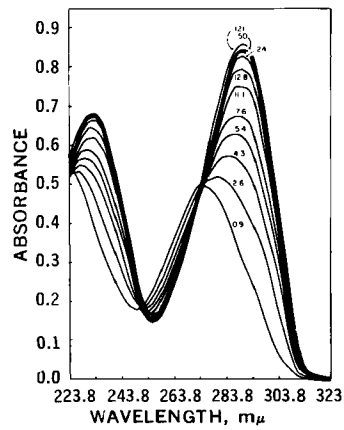


Fig. 5—Typical spectral changes for the degradation of 7.0 × 10⁻⁵ M 5-trifluoromethyluracil, in alkaline solution at low temperatures. The conditions were 0.200 N NaOH at 34.0°. Key: curves, min. after the start of the reaction.

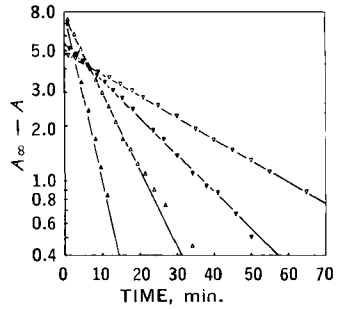


Fig. 6—Typical first-order plots for the degradation of 7.0 × 10⁻⁵ M 5-trifluoromethyluracil in various NaOH concentrations at 34.0°. Key: A, absorbance values were measured at 290 mμ where A_∞ is the asymptotic absorbance; curves and their respective N NaOH: ▽, 0.0049; ▼, 0.1094; Δ, 0.0485; ▲, 0.485.

where k_0'' = 2.0 × 10⁻⁷ sec.⁻¹, k_{OH}'' = 2.40 × 10⁻⁷ L./mole/sec., a_{NaOH} is the activity coefficient (14) for hydroxyl ion in [NaOH] solutions, and f_u = is the fraction of IV as the dianion at 80.0°.

The acid-catalyzed solvolysis of I in mineral acid at 80.0° produced the F_3T, II, or its hydrolytic product, IV, without any apparent decarboxylation. The alkaline-treated samples (room temperature, 24 hr.) from these studies produced a stoichiometric yield of the expected 5-carboxyuracil, IV. However, samples from the hydrolysis of I in acetate buffers at 80.0° at pH values of 3.7 and 4.7 when treated in alkali for 24 hr. did not produce a stoichiometric yield of desired 5-carboxyuracil. The presence of increasing amounts of uracil with time of acetate buffer degradation is consistent with the fact that 5-carboxyuracil is formed in these buffers at 80.0° and decarboxylated in the pH region of its pKa_1' 4.2 where it may exist in part as the carboxylate anion.

Since hydrolysis of II to IV and its subsequent decarboxylation to VII is a slow process with relation to the formation of II from I, an A_∞ corresponding to the theoretical yield of IV on alkaline treatment could be assumed. The initial points of the plot in accordance with Eq. 1 gave good estimates of the rate constants for hydrolysis of I in the acetate buffers at 80.0°.

Degradation of F_3TdR in Alkali—The facile degradation of F_3TdR, I, ($\lambda_{max.}$ 259 mμ) in alkaline solution is similar to that of F_3T in that the trifluoromethyl group is hydrolyzed to 5-carboxy-2′-deoxyuridine (DU-5-COOH), IX (272 mμ $\lambda_{max.}$ in 0.01 *N* NaOH), probably through alcohol, VIII, and acyl fluoride intermediates (Scheme I). The latter is most likely a highly reactive and transitory intermediate. This sequence is readily observable on changes in alkaline solution (Fig. 3). The spectra of alkaline-degrading I demonstrate a very fast initial increase in absorbance in the range 290–310 mμ (Fig. 3) which can be monitored with time at lower temperatures, *e.g.*, 30°. The apparent $\lambda_{max.}$ (260 mμ) of this intermediate is probably that of the curve for the 15-min. sample in 0.02 *M* NaOH at 47° and appears to be not very much different from the precursor, I (Fig. 3). This indicates that only minor changes in the electron patterns of the molecule are occurring, consistent with the structure of the 5-hydroxydifluoromethyl-2′-deoxyuridine, VIII, as the probable intermediate. The sub-

sequent production of a $\lambda_{max.}$, 272 mμ, is a slower process at these lower temperatures and the final absorbance and spectrum are consistent with the stoichiometric production of the anion of DU-5-COOH, IX, which was isolated from the alkaline-reacted solution and identified by elemental analysis and ultraviolet spectrum.

When the rapid increase of absorbance of higher concentrations of I in alkaline solutions is monitored at about 295 mμ at lower temperatures, *e.g.*, 30° (Fig. 2), plots of this absorbance against time permit the estimate of asymptotic values (Fig. 2 insert) before the further solvolysis to the carboxyl significantly affects the absorbance at this wavelength (Fig. 3). First-order plots of these data (Fig. 2 insert) in accordance with Eq. 1 permit estimates of the apparent first-order rate constants for the hydrolysis of I to VIII. These rate constants are listed in Table I and are consistent with the fact that the only significant process above pH 11 is the attack of hydroxyl ion on the monoanion where

$$k = k_{OH}' a_{NaOH}[NaOH] f_{u-} \quad \text{(Eq. 5)}$$

where k_{OH}' at 29.5° is 2.70 × 10^{-2} L./mole/sec., a_{NaOH} is the activity coefficient (14) for the specified [NaOH]. Above pH 11, the F_3TdR, I, exists as the monoanion since $pKa_1' = 7.85$ at 25°.

In the borate and phosphate buffer regions, first-order plots in accordance with Eq. 1 at both 280 and 290 mμ for data obtained at 47° were linear and of similar slopes for the complete duration of the reaction. No sequence of reactions could be observed at 30° and at these wavelengths in these buffer solutions, only the first-order process to IX. These facts are consistent with the premise that the intermediate, VIII, is rapidly hydrolyzed to IX in these lower pH regions by hydroxyl ion attack on the undissociated molecule (or its kinetically equivalent water attack on the monoanion) and that the rate-determining step is the hydrolysis to VIII.

At 80.0° in NaOH solutions, the hydrolysis of I to IX is too rapid to follow. However, it was spectrally observed that deoxyuridine (X) is rapidly produced by decarboxylation and slowly degrades subsequently.

Temperature Dependence—Rate constants for the several temperatures are given in Table I and its footnotes. The Arrhenius parameters were calculated from the plots of the logarithms of the rate constants in $sec.^{-1}$ against the reciprocal of the absolute temperature, T, in accordance with

$$\log k = \log P - (\Delta H_a/2.303R)(1/T) \quad \text{(Eq. 6)}$$

where R = 1.987 cal./degree/mole. The heats of activation, ΔH_a, in Kcal./mole and the log P values for the several conditions were: 2.54 N HCl, 29.7, 14.4; 0.100 N HCl, 26.9, 12.0; 0.485 N NaOH, 19.8, 9.78. The values in HCl are for hydrolysis of the nucleoside, I, to trifluoromethyluracil, II, and deoxyribose, III. The values in NaOH are for the hydrolysis of 5-hydroxydifluoromethyl-2′-deoxyuridine, VIII, to 5-carboxyl-2′-deoxyuridine, IX.

Log *k*-pH Profile—The log k-pH profiles for the degradation of F_3TdR, I, are given in Fig. 7 (solid lines) and are based on the data of Table I. They demonstrate maximum stability in the pH region 1–4 which may be assigned to solvent attack, k_0, on the undissociated molecule. The rising branch in

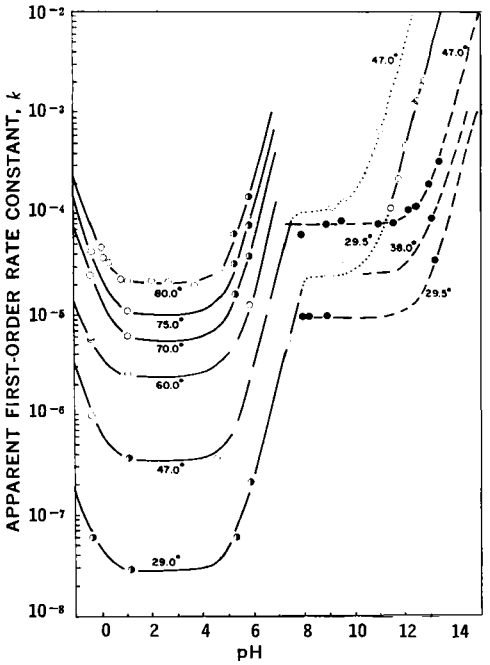


Fig. 7—Key: ——, *log k-pH profiles for the hydrolysis of 5-trifluoromethyl-2′-deoxyuridine and probable intermediate;* - - -, *5′-hydroxymethyl-2′-deoxyuridine where the lines are calculated;* ○, *represent data obtained from the loss of 5-trifluoromethyl-2′-deoxyuridine;* ◌, *estimated values with a degree of uncertainty;* ◑, *predicted from the Arrhenius relations; the dashed lines were calculated and fit the solid circles that represent data obtained for the formation of 5-carboxy-2′-deoxyuridine from the probable intermediate;* . . ., *estimated curves for the initial solvolysis of 5-trifluoromethyl-2′-deoxyuridine.*

the lower pH regions is consistent with hydrogen ion attack, $k_H[H^+]$, on the undissociated molecule. The products of these kinetic dependencies are F_3T, II, and deoxyribose, III (Scheme I).

The increasing rate constants with pH in the pH region 4–7.5 may be assigned to hydroxyl ion attack, $k_{OH}[OH^-]$ on the undissociated molecule and result in the 5-carboxyuracil-2′-deoxyribose, IX. Subsequent slow decarboxylation to X occurs at higher temperatures. No aberrations were observed in the spectra monitored in acetate or phosphate buffers (pH 4–7.5) as a function of time that could be assigned to a possible precursor, VIII, of the final product, IX. This can be explained on the basis of two possible premises. One explanation is that in the acetate and phosphate buffer regions, the HOF_2TdR, VIII, is more rapidly hydrolyzed than F_3TdR, I. Thus, the dashed lines (Fig. 7) for the presumed hydrolysis of HOF_2TdR, VIII to DU-5-COOH, IX by solvent attack on the dissociated molecule, k_0'' (or its kinetically equivalent hydroxyl ion attack on the undissociated molecule) may extend horizontally to lower pH values than the pKa′ of F_3TdR, I, *i.e.*, 7.9. This would imply that HOF_2TdR, VIII, has a significantly lower pKa_1' and that, even when it is completely undissociated, hydroxyl ion attack on this species is of much greater magnitude than hydroxyl ion attack, $k_{OH}[OH^-]$, on the undissociated F_3TdR, I.

TABLE IV—CATALYTIC RATE CONSTANTS[a] AND THERMODYNAMIC PARAMETERS[b] FOR THE CONSTRUCTION OF LOG k-pH PROFILES

	°C.							
	80.0	75.0	70.0	60.0	47.0	29.5	ΔH_a	log P
$10^6 k_0$	2.1	1.00	0.55	0.24	0.035	0.0028	27.1	16.2
$10^5 k_H$	2.2	1.45	0.80	0.13	0.029	0.00135	30.6	16.4
k_{OH}	430	460	250	130	48	14	17.0	13.3
k_{OH}'	—	—	—	—	0.13	0.026	18.2	11.6
$10^6 k_0''$	—	—	—	—	10.4[c]	2.5[c]	—	—
$10^5 k_0''$	—	—	—	—	7.85	0.97	22.3	11.1
$10^6 k_{OH}''$	—	—	—	—	25	10	10.3	3.5

[a] These catalytic rate constants may be used to predict the overall first-order rate constant for the hydrolysis of F_3TdR ($k = k_H[H^+] + k_0 + k_{OH}[OH^-]$ in the pH range -1 to 7.5 and $k = k_{OH}'[OH^-] + k_0'$ in the pH region >8) and of HOF_2TdR ($k = k_0'' + k_{OH}''[OH^-]$ in the pH region >8). [b] These values may predict the catalytic rate constant at other temperatures where $\log k = -(\Delta H_a/2.303R)(1/T) + \log P$. [c] These are estimated values based on approximations.

An alternative explanation is that in acetate and phosphate buffers, the spectrum of undissociated HOF_2TdR, VIII, is not significantly different than undissociated F_3TdR, I, and that the hydrolysis of the precursor I to VIII is much faster than the hydrolysis of VIII to IX so that the good first-order plots would still be obtained. This would lead to the conclusion that F_3TdR is more rapidly degraded than indicated in Fig. 7 for the pH range 5–7. However, in buffers about pH 10, there were no other spots observed on paper chromatographic monitoring that could be possibly assigned to a HOF_2TdR, VIII.

In the alkaline region the sequential steps could be readily analyzed kinetically. The data at 29.5° in the pH region > 11 (open circles, solid line, Fig. 7) is for the faster production of an asymptotic absorbance at 295–300 mμ (Fig. 2) than for the production of the spectrum assigned to DU-5-COOH, IX (Fig. 3) represented by the solid circles and dashed lines of Fig. 7. The intermediate is most probably HOF_2TdR, VIII, and is produced by hydroxyl ion attack, $k_{OH}'[OH^-]$, on the anion of F_3TdR, I. The dotted circles at 47.0° and 29.5° on the dotted lines are estimates of the rate constants in the borate buffer region, pH 8–11, and are not too accurate due to the small spectral difference at 295 mμ that had to be used. However, they do serve as a basis for estimating the stability of I in this intermediate pH region (dotted line, Fig. 7) where the rate constants for I and VIII are of the same order of magnitude.

The subsequent hydrolysis of the intermediate, the presumed VIII to IX, is represented by the dashed lines and the observed rate constants by the solid circles (Fig. 7). This obviously represents a solvent attack, k_0'', on the anion of VIII (or its kinetically equivalent hydroxyl ion attack on the undissociated molecule) and a hydroxyl ion attack, $k_{OH}''[OH^-]$, on the anion of VIII at higher pH values.

The basic expression used in the construction of the log k – pH profiles for the hydrolysis of F_3TdR (solid lines, Fig. 7) were

$$k = k_H[H^+] + k_0 + k_{OH}[OH^-] \qquad \text{(Eq. 7)}$$

in the pH region -1 to 7.5 (solid lines, Fig. 7), and

$$k = k_{OH}'[OH^-] + k_0' \qquad \text{(Eq. 8)}$$

in the pH region >8 (dotted and solid lines, Fig. 7). The expression used in the construction of the log k-pH profile for the appearance of DU-5-COOH, IX probably from the hydrolysis of HOF_2TdR, VIII was

$$k = k_0'' + k_{OH}''[OH^-] \qquad \text{(Eq. 9)}$$

in the pH region >8 (dashed lines, Fig. 7). The profiles of Fig. 7 were constructed from the $[H^+]$ and $[OH^-]$ in terms of their activities and the listed constants of Table IV. The drawn lines are calculated on the basis of the Eqs. 7–9 and are consistent with the experimental values of Fig. 7.

The half-life at 30° in the most stable pH range 1 to 4 is 280 days (580 days at 25°) and at pH 7.4 is 1.5 days (16 hr. at 37°). Thus solutions of F_3TR should be kept refrigerated. The products of degradation under the former conditions would be trifluorothymine and deoxyribose, and under the latter conditions would be fluoride ion, 5-carboxymethyl-2′-deoxyuridine and 5-hydroxydifluoromethyl-2′-deoxyuridine.

The obvious prediction is that F_3TdR incorporated into RNA or DNA will probably not remain as such. It should be readily transformed to the 5-carboxyl derivative at physiological pH values and temperatures. This would readily explain the fact that the use of F_3TdR is less conducive to the development of resistance than are the other nucleosidic antivirals (10).

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Keyphrases

Stability prediction—pharmaceuticals
5-Trifluoromethyl-2′-deoxyuridine—hydrolysis
pH effect—stability
Temperature effect—stability
Kinetic equations—degradation rates
Paper chromatography—degradation monitoring
UV spectrophotometry—degradation monitoring

Metabolism of β-Phenethylbiguanide

By PATRICK J. MURPHY and ARNE N. WICK

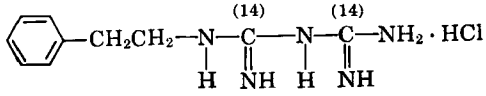
Carbon-14 labeled β-phenethylbiguanide (phenformin) was administered orally to male Sprague-Dawley rats. The radioactive material excreted in the urine was isolated and analyzed. The metabolic products were divided into "weak" and "strong" base components by their behavior on acidic ion-exchange resins. Chromatographic and spectroscopic evidence confirms the structure of the strong base fraction to be ρ-hydroxy-β-phenethylbiguanide. This hitherto unreported biguanide has been prepared synthetically for comparative purposes. The weak base metabolite has been shown to be the glucuronic acid conjugate of the phenolic biguanide. This fact was determined by acid hydrolysis to ρ-hydroxy-β-phenethylbiguanide and glucuronic acid. The more specific hydrolysis by β-glucuronidase confirmed the conjugate structure. The hydroxylated β-phenethylbiguanide along with its glucuronide conjugate were found to be present in the urine in approximately equal amounts. These two compounds seem to be the major metabolic products of β-phenethylbiguanide.

In diabetic humans, β-phenethylbiguanide hydrochloride has a hypoglycemic effect. Although this effect has been extensively studied, there is no unified concept which will explain the hypoglycemic action of this drug. Since the physiological activity of a chemotherapeutic agent is dependent in many cases (1) upon the mode of metabolism of the drug, an understanding of the metabolism of β-phenethylbiguanide may be helpful in elucidating the site and mode of action of the drug.

Preliminary studies in these laboratories have shown that the radioactivity of the labeled drug after administration is initially concentrated in the liver and gastric juice and is almost entirely eliminated in a 24-hr. period (2). Subsequent studies showed that the excreted metabolic products were chemically different from the ingested compound (3). The purpose of this study was the elucidation of the structure of the metabolic products excreted after the administration of β-phenethylbiguanide hydrochloride.

EXPERIMENTAL METHODS

In order to facilitate the metabolic studies, radioactive β-phenethylbiguanide hydrochloride synthesized by Walton (4), was used. The carbon-14 label was in the biguanide nucleus as depicted in the following:



Radioactivity was determined by the following means: (*a*) when the specific activity was to be determined the sample was oxidized to carbon dioxide by the "wet oxidation" method of Van Slyke and Folch (5) and precipitated as barium carbonate. The barium carbonate samples were mounted on planchets and counted for radioactivity in a Nuclear-Chicago gas-flow counter; (*b*) when relative radioactivity was desired, as in the case of column chromatography, aliquots were placed directly on the planchets, evaporated, and counted for radioactivity.

All paper chromatography was done by ascending technique on Whatman No. 1 paper. The spray

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