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The Acidic Solvolytic Transformations of an Iodinated Nucleoside, the Antiviral 5-Iodo-2′-deoxyuridine[1]

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The antiviral nucleoside, 5-iodo-2′-deoxyuridine, IDU, is solvolyzed by solvent and hydrogen ions to iodouracil and deoxyribose. The iodouracil is similarly catalyzed in the presence of deoxyribose and its precursors to uracil and iodide ion. The latter mechanism is through a displaced iodouracil–uracil equilibrium where the generated positive iodonium ion is reduced by acid-degraded deoxyribose. Although the rate is independent of the acid-degraded deoxyribose concentration the extent of the reaction is not. The apparent stoichiometry is seven iodouracils to one degraded deoxyribose molecule. The complete kinetics of the solvolytic transformation of IDU → iodouracil → uracil in acid and neutral solutions are given and the Arrhenius parameters are derived. Spectrophotometric, polarographic, and chromatographic assay procedures for the various nucleosides and their products are developed.

The compound 5-iodo-2′-deoxyuridine (IDU) is a biological growth inhibitor[3] and an antitumor agent.[4] IDU is an analog of thymidine which is required in the synthesis of DNA. It has been suggested that the effect of IDU depends upon the blockage of utilization of thymidine and that it may be incorporated into abnormal DNA.[5] Recently, it has been shown that IDU is effective as an antiviral agent in herpes simplex keratitis in rabbits and even human keratitis, a most serious corneal disease.[6]

Sterilization of solutions of IDU in an autoclave results in considerable destruction.[5,7] *In vivo*, IDU is deiodinated and rapidly metabolized. As much as 50–75% of the iodine appears in the urine within about 4 hr. of intravenous administration.[5] This rapid metabolism does not facilitate antiviral action on systemic administration. There are several reports[8–10] discussing the enzymic pathways for degradation of IDU and the dehalogenation of substituted pyrimidines *in vivo*.

The anticipated decomposition products of solution degradation of IDU, the compounds 5-iodouracil (IU) and deoxyuridine (DU), are toxic in cell cultures in concentrations of only 0.001 mg./ml. However, purified IDU showed no apparent toxicity[11] at concentrations as high as 1 mg./ml. IU appears to be irritating or toxic to the human cornea.[11] Moreover, IU inhibits the antiviral activity of IDU as measured by tissue culture assay, even at a concentration of only 0.01% that of IDU.[11] This may be considered as analogous to the observed reversal of the antimicrobial activity of psicofuranine (the adenine nucleoside) by adenine alone.[12–14]

A knowledge of the kinetics and mechanisms of solvolytic transformations could serve as a basis for the understanding of the metabolism of this first significant antiviral. Such studies are of pharmaceutical importance for the estimation of conditions for maximum stability and to inhibit the formation of toxic products of degradation. In addition, the determination of the kinetics of solvolysis of nucleosides is of fundamental interest since, to our knowledge, the numbers of such studies in this class of fundamental biological compounds have been limited[12,15] and in many cases restricted to qualitative comparisons.[16] The elucidation of a mechanism for IDU solvolysis in acidic and neutral solution became more evident when preliminary studies showed that the end products of IDU solvolysis were iodide ion and uracil.

Results

Kinetics of IDU Transformations.—The typical change of spectra for IDU in acid solution is presented in Fig. 1. The absorbance at 288 mμ, λ_{max} of IDU, decreases with time and a new band appears with a maximum at 259 mμ corresponding to uracil. The concomitant formation of iodide ion, but not iodine, has been proven by iodometric tests and by polarography. The retention of an apparent isosbestic point (271 mμ for 80–90% of spectral change) with the loss (at λ_{max} 288 mμ) and gain (at λ_{max} 259 mμ) of absorbance by a first-order process (Fig. 2) is usually strong evidence of a 1:1 transformation. However, polarographic evidence showed no initial loss in the diffusion current assignable to IDU and IU and indicated that IU was accumulating in the system. The iodide formation was not strictly first-order by polarography and an induction period was indicated. The alkaline spectra of acid-degraded solutions of IDU (Fig. 3) provided contributory evidence of an IU intermediate in that the increase in absorbance at >300 mμ in alkaline solution cannot be assigned to U or DU but only to the λ_{max} of IU at 304 mμ. The absorbance at this wave length subsequently decreased as the IU was transformed to uracil.

Conclusive evidence was obtained by following the progress of the reaction by thin layer chromatography where the IDU → IU → U transformations were monitored with time (Fig. 4). For example, degraded IDU in 0.947 *N* HCl at 80.0° definitely showed the disap-

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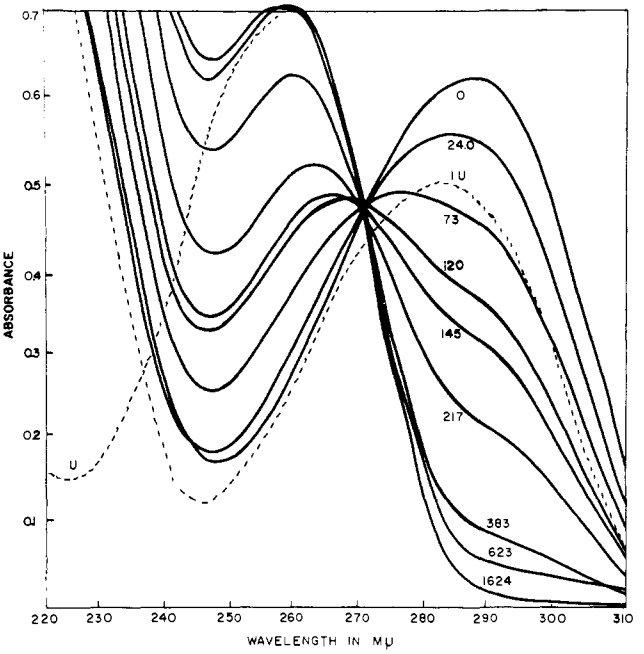


Fig. 1.—Typical curves of the spectral changes of acid-degraded 5-iodo-2′-deoxyuridine. The reacting solution was at 70.0°, 0.458 N HCl with an initial concentration of 5-iodo-2′-deoxyuridine of 0.988×10^{-3} M. The spectra were run after a 2:25 dilution with water. The dashed curves represent the spectra of equimolar 5-iodouracil and uracil under these conditions. Each curve is labeled as to the number of hours after the start of degradation.

pearance of IDU at 24 hr. and the appearance of a chromatographic spot at the same R_f value as IU, which attenuated at 51 hr. and completely disappeared at 119 hr. The spot corresponding to the R_f value of U faintly appeared at 5 hr. and is the only one remaining at 119 hr.

The thin layer chromatograms for the acetic acid-acetate buffer-degraded IDU also showed an IU intermediate which was completely transformed to a uracil product with time.

The determination of rate of the IDU transformations by spectrophotometric analysis permitted the obtaining of the rate constants listed in Tables I and II. The

TABLE I

CONDITIONS AND OBSERVED FIRST-ORDER RATE CONSTANTS[a] FOR THE SOLVOLYSIS OF 5-IODO-2′-DEOXYURIDINE (10^{-3} M)

		10^6k_1 (sec.$^{-1}$)		
[HCl]		80.0°	70.0°	60.0°
0.948		34.2	10.25	2.47
0.474		20.5	5.19	1.41
0.237		12.7	3.53	0.835
0.119		10.9	2.60	0.683
0.060		8.82	2.20	0.536
[$HC_2H_3O_2$]	[$NaC_2H_3O_2$]			
0.517		...	...	0.375
0.052		...	...	0.396
0.400	0.100	...	...	0.340[b]
0.025	0.025	...	...	0.389[c]
0.050	0.050	...	...	0.369[c]
0.075	0.075	...	...	0.351[c]
0.100	0.100	...	...	0.331[c]

[a] Obtained from the [IDU] calculated from spectrophotometric data in acid and alkaline solutions. [b] pH 4.0. [c] pH 4.6 and at 0.1 ionic strength adjusted with KCl.

kinetic rate constants obtained for the loss of the 283 mμ absorbance when IDU is treated in acid solution was the same as the rate constants for the degradation

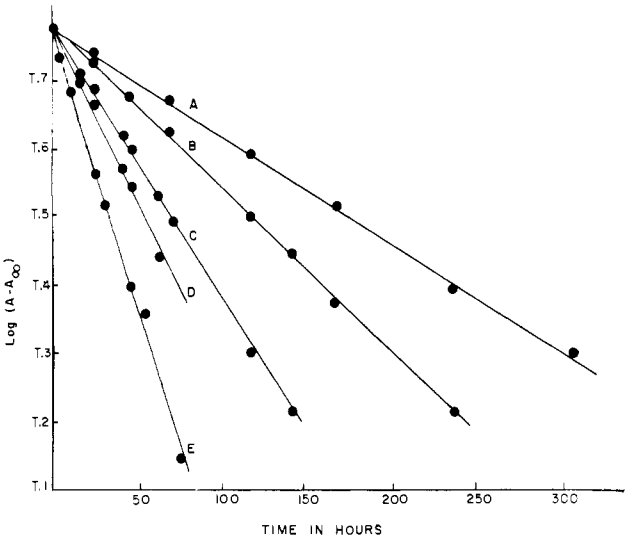


Fig. 2.—Typical apparent first-order plots of the decrease in absorbance (A) at 288 mμ for the 5-iodo-2′-deoxyuridine at 75.0°. The initial concentration of 5-iodo-2′-deoxyuridine was 0.988×10^{-3} M. The solutions were diluted 2:25 before reading. The A_∞ was the value of absorbance at infinite time and usually was 0.020. The [HCl] for the various curves are: 0.114, A; 0.229, B; 0.458, C; 0.612, D; 0.917, E.

of the IU product of IDU as determined from the calculated [IU] values (Table II).

TABLE II

CONDITIONS AND OBSERVED FIRST-ORDER RATE CONSTANTS FOR THE DEGRADATION OF 5-IODOURACIL IN THE PRESENCE OF SOLVOLYTIC PRODUCTS OF PRECURSOR 5-IODO-2′-DEOXYURIDINE (10^{-3} M)

	10^6k_2 (sec.$^{-1}$)			
[HCl]	80.0°	75.0°	70.0°	60.0°
0.948[a]	10.6	..	3.11	1.00
0.917[b]	8.78	4.85[d]	2.62	0.950
0.612[b]	5.39	3.19	1.71	0.600
0.474[a]	4.17	..	1.29	0.459
0.458[b]	4.36[c]	2.54	1.18	0.417
0.237[a]	3.02	..	0.805	0.225
0.229[b]	2.57	1.55	0.844	0.225
0.119[a]	1.65	..	0.556	0.153
0.114[b]	..	1.01	0.517	0.161
0.086[b]	1.97	..	...	...
0.060[a]	1.24	..	0.417	...
0.057[b]	..	..	0.436	...
[$HC_2H_3O_2$]				
0.517[a]	..	..	...	0.125
0.052[a]	..	..	...	0.111

[a] Calculated rate constants from analog computer fitting of [IDU] and [IU] *vs.* time on the premise

$$\text{IDU} \xrightarrow{k_1} \text{IU} \xrightarrow{k_2} \text{U} + \text{I}^-$$

where the concentrations were determined from absorbance data in acid and alkaline solutions. [b] Calculated rate constants from the slope of the apparent first-order plot of log $(A - A_\infty)$ *vs.* time where A is the absorbance in acid solution at 288 mμ and where A_∞ is the asymptotic absorbance at infinite time. [c] The same rate constant was obtained when $[IDU]_0 = 7.91 \times 10^{-5}$. [d] No significant difference in rate constant when the solution was nitrogen purged and shielded from light.

For a wide range of IU concentrations, the rate of 283 mμ absorbance loss on acid degradation of IU in the presence of IDU is first order and apparently independent of the IDU:IU ratio, at least down to 1:3. This is graphically demonstrated by comparing curves B and D of Fig. 5.

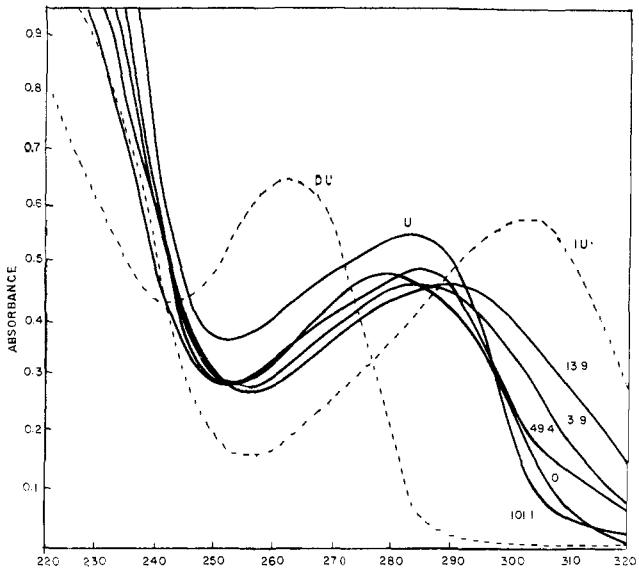
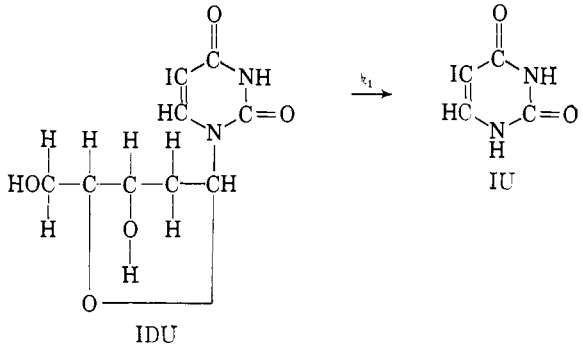
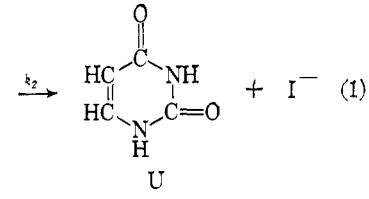


Fig. 3.—Typical curves of the alkaline spectra of acid-degrading IDU. The reacting solution was at 70.0°, 0.947 N HCl with 0.988×10^{-3} M 5-iodo-2′-deoxyuridine. The spectra were run after 2:25 dilution including 3 ml. of 0.795 N NaOH. The dashed curves represent the spectra of equimolar 5-iodouracil and deoxyuridine under these conditions. Each curve of 5-iodouracil is labeled as to the number of hours after the start of degradation.

The sequential reaction





is consistent with the IDU and IU data as clearly shown from the excellent fitting of the analog computer program for all the reactions studied in hydrochloric and acetic acids (Tables I and II). An example of such an excellent fit is given in Fig. 6. If the uracil content is calculated from the absorbance at 259 mμ in acid solution, however, parallel, but higher, values than those generated by the analog computer are obtained.

The apparent first-order rate constants, k_1 and k_2, are plotted against [HCl] for the several temperatures in Fig. 7 and 8, respectively. These plots are linear and do not go through the origin. The respective intercepts $(k_0)_1$ and $(k_0)_2$ can be interpreted as reaction rate constants for the "spontaneous" or solvent reaction. The rate constant dependencies for the apparent first order rate constants are

$$k_1 = (k_{H^+})_1[H^+] + (k_0)_1 \quad (2)$$

$$k_2 = (k_{H^+})_2[H^+] + (k_0)_2 \quad (3)$$

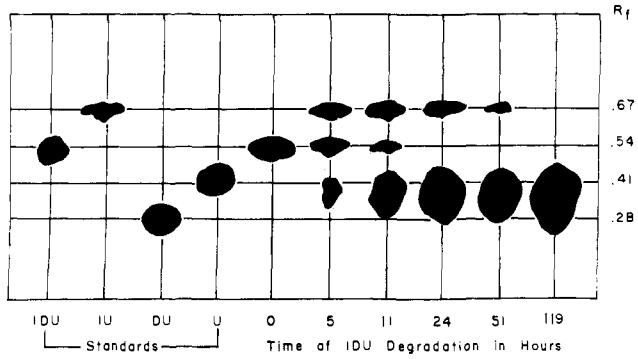


Fig. 4.—Thin layer chromatogram for monitoring 8.47×10^{-3} M IDU decomposition in 0.947 N HCl at 80.0°. The developing solution was 30% isopropyl alcohol–70% chloroform. The standard solutions were developed from 20 λ placed at the origin except for uracil which was 10 λ.

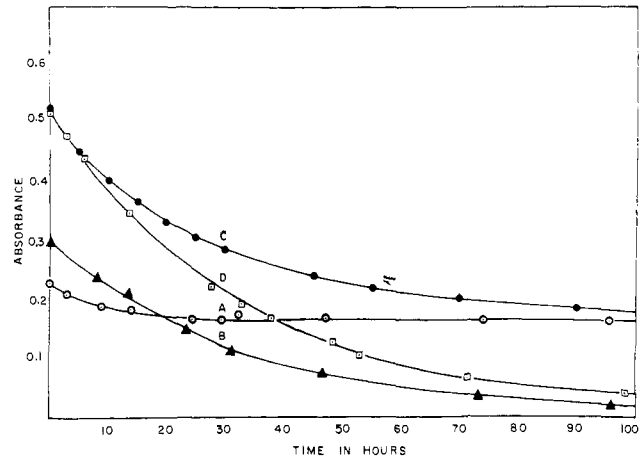


Fig. 5.—Change of absorbance with time of iodouracil (curve A), IDU (curve B), and iodouracil in the presence of equimolar IDU (curve D). The conditions were 0.947 N HCl at 80.0°. The curve C is the sum of curves A and B and represents the expected sum of the absorbances of IU and IDU if there were no interaction.

where $[H^+]$ is defined as equivalent to the stoichiometric [HCl] and the $(k_{H^+})_1$, $(k_{H^+})_2$, $(k_0)_1$, and $(k_0)_2$ values are given in Table III. It is interesting to note that in the solvolysis of IDU to IU, the rate constants observed in acetic acid and in acetic acid–acetate buffer solutions (Table I) are equivalent to the $(k_0)_1$ values. This was also true for the subsequent IU to U transformation in the acetic acids where the apparent first-order rate constants (Table II) were equivalent to the intercept of the k_2 plot against [HCl] (Fig. 8). However, no valid fit of a sequential rate process as given in eq. 1 could account for the [IU] data as a function of time

TABLE III

RATE CONSTANTS (k_{H^+} IN L. MOLE^{-1} SEC.$^{-1}$, k_0 IN SEC.$^{-1}$) AND ARRHENIUS PARAMETERS[a] FOR THE SEQUENTIAL DEGRADATION OF

5-IODO-2′-DEOXYURIDINE $\xrightarrow{k_1}$ 5-IODOURACIL $\xrightarrow{k_2}$ URACIL[b]

T, °C.	$10^6(k_{H^+})_1$	$10^6(k_0)_1$	$10^6(k_{H^+})_2$	$10^6(k_0)_2$
80	28.6	6.90	7.88	1.00
75	...	...	4.40	0.515
70	7.55	1.72	2.26	0.312
60	2.02	0.40	0.75	0.100
ΔH_a (kcal. mole^{-1})	31.1	32.6	27.1	27.2
log P	14.6	15.0	11.6	10.8

[a] Where $\log k_i = -\Delta H_a/2303RT + \log P$. [b] Where $k_1 = (k_0)_1 + (k_{H^+})_1[HCl]$ and $k_2 = (k_0)_2 + (k_{H^+})[HCl]$.

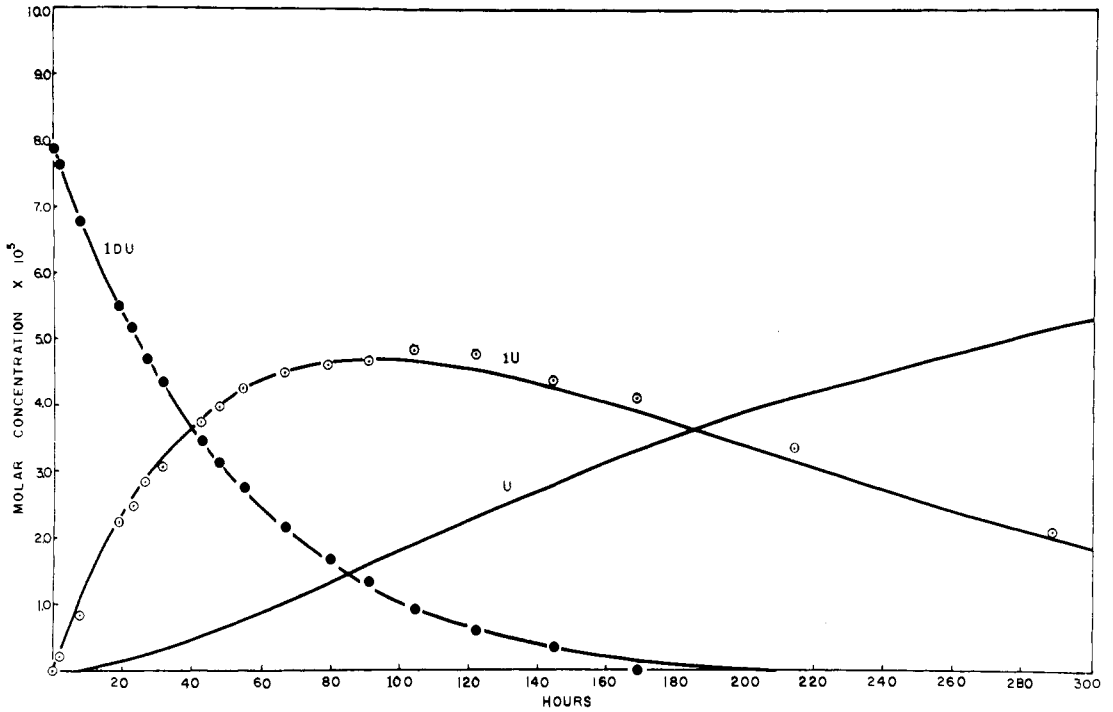


Fig. 6.—Typical analog computer fit of the data for the apparent first-order transformations IDU $\xrightarrow{k_1}$ IU $\xrightarrow{k_2}$ U. The data were obtained from the hydrolysis of 10^{-3} M IDU in 0.474 N HCl at 70.0° after spectrophotometric analysis of aliquots diluted 2:25, $k_1 = 5.19 \times 10^{-6}$ sec.$^{-1}$ and $k_2 = 1.29 \times 10^{-6}$ sec.$^{-1}$.

at the higher pH values extant in the acetic acid–acetate buffers. The apparent first-order rate constant, k_2, for the subsequent transformation of IU to U increased with time. If a k_2 value was chosen to fit the concomitant appearance of IU from IDU and disappearance to U, the rate of IU loss after the complete first-order loss of IDU to zero was faster than expected. Conversely, if the k_2 value was chosen to fit the disappearance of IU to U after the first-order loss of IDU to zero the net rate of appearance of IU from IDU in the initial periods of the reaction was greater than expected.

Arrhenius' plots for the specific rate constants of eq. 2 and 3 are given in Fig. 9 in accordance with the expression

$$\log k_i = -(\Delta H_a/2.303R)(1/T) + \log P \quad (4)$$

where the ΔH_a and log P values are given in Table III. The heats of activation for the solvolysis of IDU, both acid catalyzed and acid independent, are 32 kcal. mole^{-1} whereas the heats of activation for deiodination of iodouracil are 27.1 kcal. mole^{-1}. It can be estimated that 1% solvolysis of IDU to IU will occur in 0.5 year at 20° for pH values between 2 and 7.

Effect of Addends on Acid-Catalyzed Iodouracil Degradation.—A typical curve for the change of absorbance in 1 M HCl at 80.0° of IU with time is given as curve A in Fig. 5. When addends such as KI, uracil, I_2–KI, and ribose were added, the changes in absorbance with time for IU were not significantly different.

The complete ultraviolet spectrum of IU in HCl at 80.0° showed a hypsochromic shift of the 283 mμ maximum with time toward 272 mμ. The absorbances at 283 mμ (curve A, Fig. 5) were lessened *ca.* 12% with a hyperchromic effect at less than the 270 mμ which was an apparent isosbestic point. The final asymptotic spectra with time is similar to the 73 hr. curve of Fig. 1. These facts are consistent with an equilibrium established between IU and U in the presence of HCl which favors iodouracil and where uracil has a λ_{max} of 258 mμ.

The presence of deoxyribose, deoxyuridine, and IDU in acidic IU solutions gave spectral shifts with time similar to those observed with IDU alone (see examples in Fig. 1 and 3). The loss of the 288 mμ absorbance of IDU with time in 1 M HCl at 80.0° is represented as curve B in Fig. 5. It may be anticipated that a mixture of the IU and IDU would give the curve C, the sum of the absorbances plotted against time for the compounds reacted separately (curves A and B). However, curve D was experimentally observed.

In the IU plus IDU mixtures studied the absorbance at 283 or 288 mμ decreased with time to approximately zero and the apparent first-order rate constant for this disappearance was the same, $k = 9.2 \times 10^{-6}$ sec.$^{-1}$, and independent of the IDU/IU ratios used (0.25–3.0). The apparent first-order rate constants were the same for the IDU reacted alone under the same conditions of acid and temperature. The results were also similar for deoxyuridine plus IU.

Effect of Varying Deoxyribose Concentration on the Rate and Extent of Iodouracil Loss.—For IU plus deoxyribose (DR) mixtures where the molar ratios, DR:IU, varied from 0.14 to 1.0, the absorbance at 283 mμ decreased to the same asymptote, *ca.* 0.040, with time. The apparent first-order rate constant for the disappearance of the IU chromophore was invariant, $k = 9.1 \times 10^{-6}$ sec.$^{-1}$ and independent of the deoxyribose concentration within the cited range. The results were the same as the IU plus deoxyuridine and IDU cases cited previously (curve D, Fig. 5). However, when the deoxyribose concentrations in

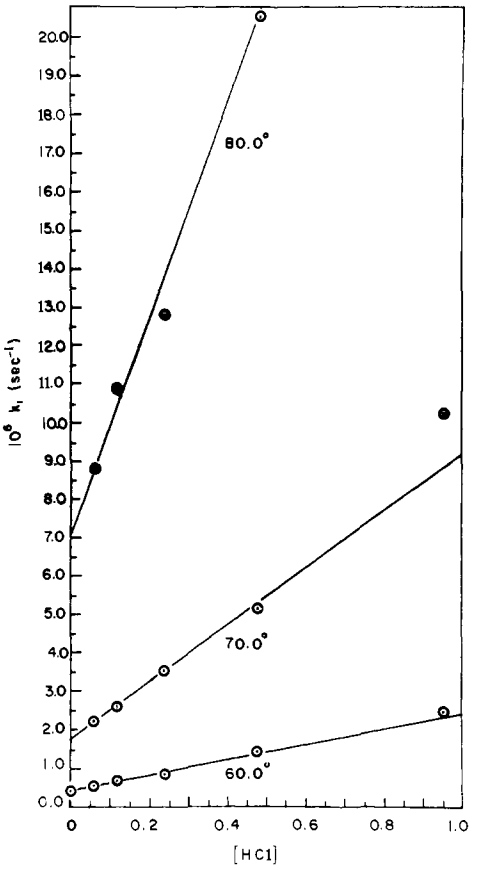


Fig. 7.— Plots of the apparent first-order rate constants at several temperatures for the solvolysis of 5-iodo-2′-deoxyuridine as a function of [HCl].

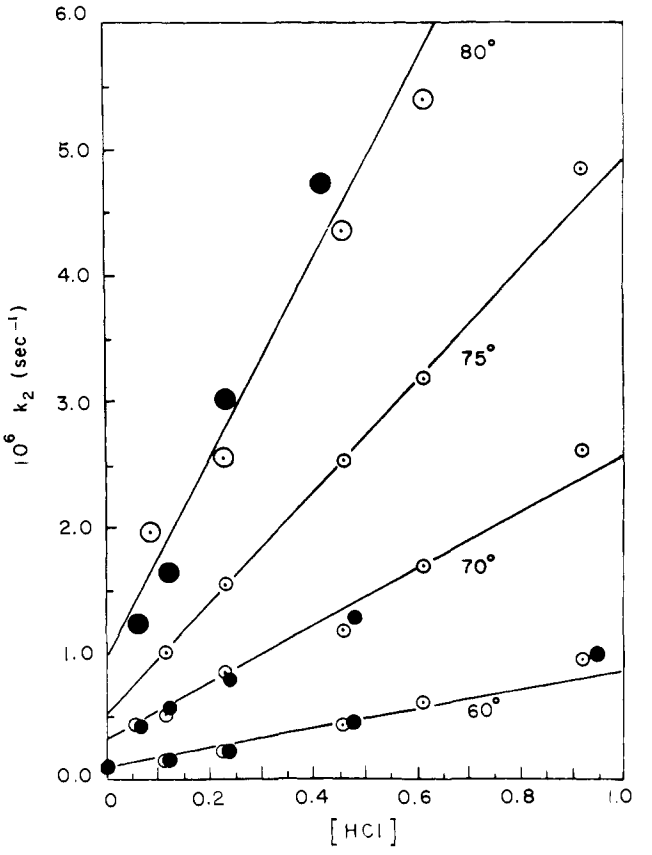


Fig. 8.— Plots of the apparent first-order rate constants at several temperatures for the solvolytic decomposition of iodouracil to uracil as a function of [HCl]. The solid circles are rate constants derived from analog computer fitting of the spectrophotometric data. The open circles are rate constants determined from the rate of change of the absorbance at 288 mμ.

IU–DR mixtures were lessened, the absorbance $A_{283\ m\mu}$ exponentially decreased with time for about 50 hr. to different asymptotic values, A_∞, which values were maintained for at least an additional 100 hr. However, the apparent first-order rate constant, obtained from a plot of log $[A_{283} - A_\infty]$ *vs.* time, $k = 9.1 \times 10^{-6}$ sec.$^{-1}$, was invariant with DR concentration.

The asymptotic absorbances obtained can be corrected to equivalent initial IU concentrations of 5×10^{-4} *M* by the multiplying factor $5 \times 10^{-4}/[IU]_0$ where $[IU]_0$ was the actual initial concentration. After appropriate dilution for spectrophotometric reading at 283 mμ in acid solution, the initial absorbance was 0.8. The [DR]/[IU] concentration ratios used and the asymptotic absorbances at infinite time were, respectively: (a) 0, 0.400; (b) 0.0200, 0.340; (c) 0.0417, 0.298; (d) 0.0634, 0.227; (e) 0.14, 0.020; (f) 0.334, 0.020; (g) 1.00, 0.020. If it is assumed that in 1 *M* HCl at 80.0° $(0.48/0.40)A_\infty$ is proportional to the concentration of IU unaffected by the deoxyribose, then the fraction, *f*, of IU transformed to uracil by the ratio R = [DR]/[IU] can be estimated from the various asymptotic absorbances by $f = [0.48 - (0.48/0.40)A_\infty]/0.48$. The respective *f* and *R* values are: 0.0, 0.0; 0.17, 0.020; 0.25, 0.042; 0.45, 0.063; and 1.0, <0.14 where $f = 1 - 7R$ and whence it appears that one molecule of deoxyribose under these specified conditions is capable of transforming seven molecules of iodouracil to uracil.

Iodide, Iodine and Iodine Monochloride Interaction with and Formation from Iodinated Nucleosides and Their Possible Degradation Products.—The compounds IDU and IU in aqueous solution do not form iodine when subjected to normal visible light as observed over a day. However, 5-iodouridine is light sensitive.

Solutions of iodine at 80.0° in 0.6 *M* HCl are transformed by deoxyribose (DR) and IDU in that order of reactivity. Ribose has no significant effect. Thiosulfate titration of ICl in 0.115 *M* HCl maintained for a day at 60.0° showed no apparent loss of halogen. Although the addition of deoxyribose to an acid solution of ICl did not change the halogen concentration in a day's time, subjection to 60.0° did cause a significant decrease in the total halogen.

Iodouracil in 1 *M* sulfuric or hydrochloric acids at 80.0° did not release iodine except after the addition of KI, indicative of the formation of an iodo compound which can generate iodine with facility on the addition of iodide ion.

When IDU (or IU in the presence of acid degraded IDU) is subjected to 60.0–80.0° in varying acetic and hydrochloric acid solutions, no such iodo compound is observed with either starch, starch–iodide, or by chloroform extraction. Only when oxidizing agents such as H_2O_2 or KIO_3 are added to the acid reacted IDU is the iodine test positive by both starch, starch–iodide, and by chloroform extraction. This is indicative of iodide ion.

The addition of ICl to uracil in acid solutions within a short interval of time (within 20 min. at 80.0° and at 1.15 *M* in HCl within 1.4 hr. at 70.0° and at 0.058

M HCl) transformed uracil to iodouracil. If ICl was in excess, a stoichiometric transformation was manifested. If ICl was insufficient the resultant products were a mixture of uracil, U, and iodouracil, IU, with minimal amounts of ICl remaining.

The above facts can be summarized in the following equations. For an acid HX

$$IU + HX \rightleftharpoons IX + U \quad (5)$$

where the equilibrium greatly favors iodouracil and where X ≠ I since

$$IX + I^- \longrightarrow I_2 + X^- \quad (6)$$

and the moiety X may be hydroxyl or other radical. When X = Cl

$$IU + HCl \rightleftharpoons U + ICl \quad (7)$$

and

$$ICl + H_2O_2 \longrightarrow \text{no reaction} \quad (8)$$

but

$$ICl + I^- \longrightarrow I_2 + Cl^- \quad (9)$$

It has also been shown that

$$I_2 + DR \xrightarrow[\Delta]{H^+} 2I^- + \text{products} \quad (10)$$

$$I_2 + IDU \xrightarrow[\Delta]{H^+} 2I^- + \text{products} \quad (11)$$

The reaction of eq. 10 occurs more readily than that of eq. 11. The latter expression is most likely due to the degradation products of IDU and can be restated as

$$I_2 + IU + DR \xrightarrow[\Delta]{HCl} 3I^- + U + \text{products} \quad (12)$$

It has also been shown that over a day

$$ICl + DR \xrightarrow[\text{cold}]{H^+} \text{no reaction} \quad (13)$$

$$ICl + DR \xrightarrow[60^\circ]{H^+} I^- + Cl^- + \text{products} \quad (14)$$

where in all cases

$$2I^- + H_2O_2 + 2H^+ \longrightarrow I_2 + 2H_2O \quad (15)$$

Discussion

The anticipated pathways for the solvolysis of IDU in acid solution could be through an IU intermediate or a DU intermediate. However, DU was rejected as a major intermediate because the alkaline spectra of acid-degraded IDU (Fig. 3) did not show the characteristic band of DU (λ_{max}^{alk} 262 mμ) and negligible DU was obtained from monitoring the reaction by thin layer chromatography. Some DU may be expected on the basis of a possible equilibrium[17] between ICl, IDU, and DU but the excellent fit of the [IDU] and [IU] data with time by the analog computer (Fig. 6) permits the conclusion that the transformation sequence of IDU is as given in eq. 1.

IU alone in acid solution is not stoichiometrically transformed to U (Fig. 5) but is in equilibrium with U and an iodo compound (eq. 5 and 6) which is not iodine, not oxidizable to iodine, but can form I_2 on the addition of iodide ion (eq. 6, 8, and 9).

The observed IU transformations to U and iodide ion occur only in the presence of deoxyribose or deoxyribose precursors, *e.g.*, IDU or deoxyuridine, and are not affected by iodine, iodide ion, ribose, or a ribose precursor such as uridine. The U can be transformed stoichiometrically to IU by ICl which can be reduced by heating with deoxyribose in acid solution. Iodine can be reduced similarly by deoxyribose and deoxyribose precursors.

(17) A. D. Brownstone, *Nature*, **199**, 1285 (1963).

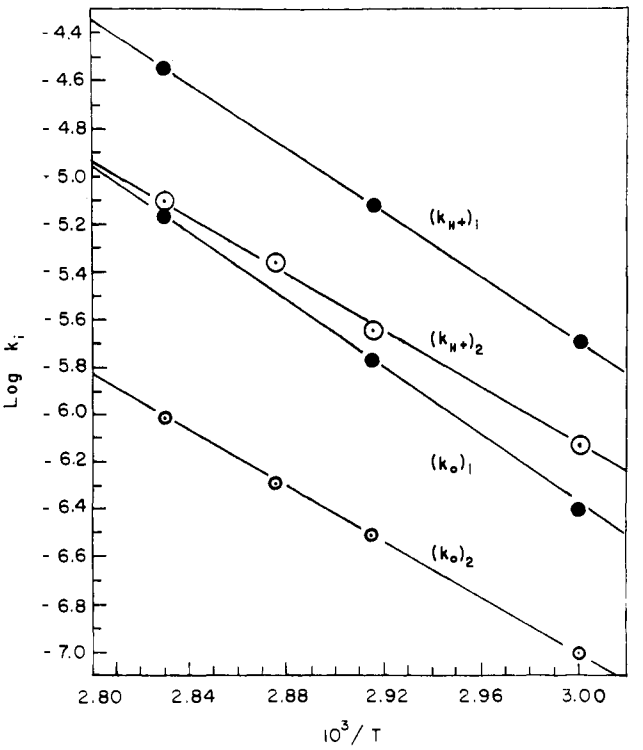


Fig. 9.—Arrhenius plots for the apparent rate constants, k_i, for the solvolytic transformations of 5-iodo-2′-deoxyuridine, $IDU \xrightarrow{k_1} IU \xrightarrow{k_2} U$ where $k_1 = (k_{H^+})_1[HCl] + (k_0)_1$ and $k_2 = (k_{H^+})_2[HCl] + (k_0)_2$.

The extent of the transformation of IU to U and iodide ion is dependent on deoxyribose concentration where seven molecules of IU can be transformed per molecule of deoxyribose in the acid solution studied. However, the apparent first-order rate constant for the achievement of the final uracil concentration is independent of deoxyribose (DR) concentration. Similarly, a deoxyribose precursor such as IDU has no effect on the rate of IU reaction for as low an [IDU]/[IU] ratio as 1:3.

These facts can be reconciled with the postulation of the following mechanisms in acid solution.

$$IDU \xrightarrow{k_1} DR + IU \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} U + I^+ \quad (16)$$

$$I^+ + \text{degraded deoxyribose} \longrightarrow I^- + \text{products} \quad (17)$$

$$I^- + I^+ \longrightarrow I_2 \quad (18)$$

$$I_2 + \text{degraded deoxyribose} \longrightarrow 2I^- \quad (19)$$

The IDU hydrolysis can be catalyzed by hydrogen ion and solvent to IU and deoxyribose. The IU tends to lose a positive iodonium ion and an equilibrium is established which favors the IU. In the presence of HCl, the formation of ICl occurs although the formation of IOH and other iodonium compounds is also feasible in the rate-determining step. The formation of these latter iodonium compounds can account for

the HCl independent solvolysis where water attack on IU discharges the positive iodonium ion. The deoxyribose has the peculiar property of being degraded in the solutions studied[18] and acting as a reducing agent for both ICl and I_2. The product formed is iodide ion which in turn produces iodine from ICl and may again be reduced by degraded deoxyribose. It follows that if the reactions of eq. 17–19 are fast the rate-determining step is the formation of uracil by the acid- and water-catalyzed dissociation of IU into a positive iodonium ion and uracil. The amount of reducing degraded deoxyribose determines the extent of the reaction but not the rate of achievement of the new equilibrium since k_2 and k_{-2} are constants under constant solvent conditions.

The Anomalous Pseudo-First-Order Rate of the Over-all Reaction IDU → IU → U.—The phenomenon of a pseudo-first-order loss of IDU absorbance at 288 mμ (Fig. 1 and 2) with the retention of an apparent isosbestic point (Fig. 1) is usually almost sufficient proof of a 1:1 molecular transformation with only one rate-determining step. This anomaly can only be observed if a unique set of relations exists among the absorptivities of IU and IDU and the respective rate constants k_1 and k_2.

The experimental evidence implies that the rate of decrease of the absorbance, A, at 288 mμ is a first-order function of the magnitude of the absorbance which must be the sum of the contributions of IDU and IU so that

$$-dA/dt = -d(\epsilon_{IDU}[IDU] + \epsilon_{IU}[IU])/dt$$

$$= -\epsilon_{IDU}d[IDU]/dt - \epsilon_{IU}d[IU]/dt$$

$$= kA = k(\epsilon_{IDU}[IDU] + \epsilon_{IU}[IU]) \quad (20)$$

However, from the fact that $k = k_2$ after the complete transformation of IDU to IU, it follows that

$$k_1(\epsilon_{IDU} - \epsilon_{IU})[IDU] + k_2\epsilon_{IU}[IU] \equiv$$

$$k_2\epsilon_{IDU}[IDU] + k_2\epsilon_{IU}[IU] \quad (21)$$

which can only be true if

$$k_1/(k_1 - k_2) = \epsilon_{IDU}/\epsilon_{IU} \quad (22)$$

which at 288 mμ has the numerical value of 1.25. When the $k_1/(k_1 - k_2)$ values are calculated from the corresponding values of k_1 and k_2 given in Tables I and II they average 1.3 for all HCl solutions below 0.900 N excluding one outlying value at 0.237 N HCl at 60.0°. The 0.95 N HCl studies give a ratio of 1.45.

Comparisons of Nucleoside Solvolysis.—The linearity of the apparent first-order rate constant for the solvolysis of IDU with [HCl] (Fig. 7) does not indicate any ability of the IDU molecule and its pyrimidine ring to act as a formal base with an assignable pK_a' for its conjugate acid. A kinetic assignment of such a pK_a' is possible during the acid hydrolysis of amides[19] and for nucleosides capable of being protonated.[15] In accordance with the concepts of Kenner[20] and Dekker[21] where the acid-catalyzed solvolysis of nucleosides is assisted by the transfer of a proton from a nitrogen atom of a heterocyclic base to the ring oxygen of a sugar, the solvolysis of IDU will be a slow process since the pyrimidine ring is not readily protonated. The comparably fast hydrolysis and the smaller heat of activation (26 kcal. mole^{-1} as against 32 kcal. mole^{-1} for IDU) of the adenine nucleoside, psicofuranine,[15] which is capable of protonation is consistent with this premise. The rates of hydrolysis of IDU ($k = 6 \times 10^{-4}$ min.$^{-1}$ at 80.0° in 0.1 N HCl) are of the same order of magnitude as that reported by Levene and Sobotka[22] for aldonucleoside solvolysis (7×10^{-4} in 0.1 N HCl at 100°, k presumably in min.$^{-1}$). An interesting point is that our preliminary studies have shown similar solvolytic rates for deoxyuridine but very insignificant rates for the acid solvolysis of uridine. Minor changes in the sugar structure appear to have an enormous effect on the acid-catalyzed solvolysis of nucleosides.

The apparent hydrolysis by solvent of IDU is of interest since variation of acetic acid concentrations and acetic acid–acetate buffer concentrations at constant pH and ionic strength (Table I) had no systematic effect on the rates of IDU solvolysis. Thus, general acid–base catalysis was not decidedly implicated. In the detailed study of psicofuranine[15] no solvent effects or general acid–base catalysis was observed. The cause of the solvent action on IDU is not apparent. The solvolyses of systematically varied nucleosidic structures must be studied to determine whether the nature of the heterocyclic, the halogen substituents, the keto or aldehydo linkage of the glycoside, or the peculiarities of the sugar structure are responsible and to what degree.

The dissociation of the iodouracil may be promoted by protonation of one of the nitrogens of the pyrimidine ring to permit the formation of an activated complex which is stabilized by a dipole. Facile rearrangement would give uracil.

One possible representation of this sequence is

(18) Preliminary studies on the degradation of deoxyribose in neutral and acidic solutions indicate that the sugar quickly develops a chromophore with a maximum at 261 mμ which is shifted to 291 mμ at pH 11–12 with negligible absorbance at 310 mμ. The chromophore of acid-degraded deoxyribose appears to be readily destroyed at pH 11–12 with an apparent half-life of *ca.* 20 min. at room temperature. Thus degraded deoxyribose would give minimal interference in the spectrophotometric assay of IU in the alkaline solutions of acid-degraded IDU. The dilutions used in our studies for determination of IU and IDU absorbances at 283 and 288 mμ in acid solution did not show significant interference of the degraded deoxyribose chromophore at these wave lengths. This was checked by degrading equimolar deoxyribose and reading at the same dilutions. However, the chromophore of the degraded sugar does interfere with the uracil maximum at 259 mμ. When ICl was added to degraded deoxyribose the absorbance at the 261 mμ maximum was lessened although the end absorption below 250 mμ, an apparent characteristic of the product ICl reacted with degraded deoxyribose, was accentuated. This is clearly shown by the comparison of the uracil spectrum and the spectrum of the final products of IDU degradation in Fig. 1. The absorbances at 259 mμ of degraded IDU cannot be used directly for determining uracil concentration. These phenomena are reported in detail in a manuscript by J. Seydel and E. R. Garrett, submitted to *Anal. Chem.*

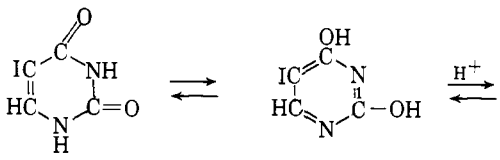
The additional fact that at elevated temperatures and for IDU in acetic acid–acetate buffers (pH >4) the rate of IU loss to U is not truly first order implies that the degradation of deoxyribose to a reducing substance becomes rate limiting. Initially, the deoxyribose is in insufficient quantity to reduce the generated ICl. As the reaction proceeds the deoxyribose concentration increases. The over-all result is an acceleration of the rate of uracil appearance with time from degrading IDU.

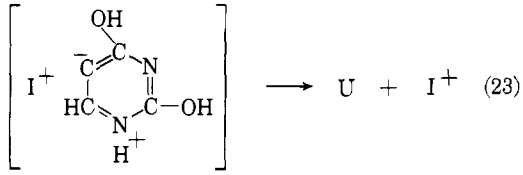
(19) E. R. Garrett, *J. Pharm. Sci.*, **51**, 811 (1962).

(20) G. W. Kenner in "Ciba Foundation Symposium on the Chemistry and Biology of Purines," Little, Brown and Co., Boston, Mass., 1957, p. 312.

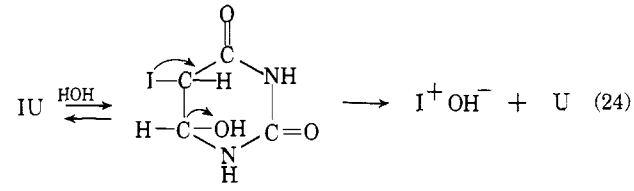
(21) C. A. Dekker, *Ann. Rev. Biochem.*, **29**, 453 (1960).

(22) P. A. Levene and H. Sobotka, *J. Biol. Chem.*, **65**, 463 (1925).





Dissociation without prior protonation could account for the noncatalyzed deiodination. Of course addition can occur across the 4-5 bond[16] with subsequent regeneration of uracil after the elimination of the positive iodonium ion.



Experimental

Kinetics of IDU Transformations by Spectrophotometry.—The 5-iodo-2′-deoxyuridine, IDU, and the iodouracil, IU, were obtained from the California Corporation for Biochemical Research, Los Angeles.

Appropriate quantities of IDU (generally 17.5 mg.) were weighed into 50 ml. volumetric flasks and made up to volume with solvent of the appropriate acid concentration so that the molar concentration in IDU was generally 0.988×10^{-4} M. The solvent was previously equilibrated at the temperature of the kinetic study. In several instances kinetic studies were conducted with and without shielding from light and at other substrate concentrations. No significant effect on rate constants was observed with these variations under the conditions studied.

The solutions were maintained in constant temperature baths at 60.0, 70.0, 75.0, and 80.0° at the acid and buffer concentrations specified in Tables I and II. Spectrophotometric readings were obtained in acid solution after diluting 2:25 with distilled water with a resultant pH in the range 1.3 to 2.5, and in alkaline solution after similar dilutions with water and 0.8 N NaOH so that the resultant solution was in the pH range 11.5 to 12.00. Typical spectra as recorded on the Beckman DB for the acid solutions and alkaline solutions with time are given in Fig. 1 and 3, respectively. The dashed lines in Fig. 1 are the spectra of equimolar IU and U in acid solution whereas the spectra at the labeled 0 and 1624 hr. are consistent with IDU and U, respectively. The dashed lines in Fig. 3 are the spectra of equimolar IU and DU in alkaline solution whereas the spectra at labeled 0 hr. is consistent with IDU.

The data superscripted *b* in Table II were obtained from the apparent first-order rates of change of the 238 mμ spectra of the specified solutions diluted 2:25 with distilled water with a resultant pH in the range 1.3 to 2.5. The apparent first-order rate constants for the increase of absorbance at 260 mμ were the same. Typical first-order plots are given in Fig. 2. All other apparent first-order rate constants were calculated from the concentrations of IU and IDU calculated from spectral readings at 292 mμ in acid on similar dilution and at 310 mμ in alkaline solution by dilution 2:25 with requisite amounts of water and 0.8 N NaOH so that the resultant solutions were in the pH range 11.5 to 12.0. The spectral reading for these calculations were made on the Beckman Model DU ultraviolet spectrophotometer.

The molar absorptivities in acid solution at 292 mμ for the cited compounds are: IDU, 7523; IU, 5524; DU, 160; and U, 100. The molar absorptivities in alkaline solution at 310 mμ for the cited compounds are: IDU, 722; IU, 6386; DU, 50; and U, 150. Since the absorptivities of U and DU are relatively insignificant at these wave lengths, the concentrations of IDU and IU at any time can be given by the following expressions.

$$[\mathrm{IU}] = 1.7077 \times 10^{-4} A_{310}^{\mathrm{alk}} - 1.6395 \times 10^{-5} A_{292}^{\mathrm{acid}} \quad (25)$$

$$[\mathrm{IDU}] = 1.3292 \times 10^{-4} A_{292}^{\mathrm{acid}} - 0.7342[\mathrm{IU}] \quad (26)$$

The values for k_1 were calculated from the slopes of plots of the log [IDU] against time as expressed in the equation

$$\log [\mathrm{IDU}] = -kt/2.303 + \log [\mathrm{IDU}]_0 \quad (27)$$

These are given in Table I.

The first-order sequence of eq. 1 and these k_1 values were programmed on the Pace TR-10 analog computer, Electronic Associates Inc., Long Branch, N. J. The k_2 values which best fit the [IU] *vs.* time plots were obtained and are reported in Table II and superscripted *a*. A typical analog computer fit of [IDU] and [IU] with time in acid solution is given in Fig. 6.

Polarographic Investigations.—The generation of iodide ion in the acid-catalyzed transformations of IDU was followed polarographically. The literature value for the polarographic wave of the iodide ion is *ca.* −0.1 v.[23] Chloride ion interfered with the polarographic assay of iodide ion. An apparent $E_{1/2} = -0.16$ v. was established in 0.417 N Na_2SO_4 with the Sargent Model XXI recording polarograph. The diffusion current at −0.6 v. was determined to be proportional to the iodide ion concentration as measured against a reference cell of saturated calomel in a 2 ml. capacity H cell with an agar potassium nitrate bridge.

It was also shown that under these same conditions that both IDU and IU had half-wave potentials of *ca.* −1.2 v. The diffusion current at −1.7 v. was shown to be proportional to the IDU concentration. In all cases, the contribution from the supporting electrolyte was subtracted by the classical procedure.[24] The apparent ratio of the diffusion currents for equimolar IDU and IU solutions was 0.72.

The compound IDU (1.976×10^{-3} M) was treated in 0.835 N H_2SO_4 at 80.0°. Polarographic readings were recorded as a function of time on aliquots diluted 1:1 with sufficient NaOH solution to adjust the pH to 2.5. The sample solution was purged with nitrogn for 8 min. before the polarographic assay.

Polarographic analysis of such samples showed an initial diffusion current, i_D of 0.67 μa. at −1.7 v. which was relatively invariant for 30 min., subsequently decreased with time, and then further decreased by a first-order rate equivalent to the apparent first-order rate loss of the 288 mμ absorbance of the solution. The total i_D was the sum of contributions from IDU and the formed intermediate IU and eventually could be attributed to IU alone. The appearance of iodide ion as calculated from the i_D at −0.6 v. gave an S-shaped curve against time since sufficient IU had to be produced prior to iodide ion release.

Effect of Addends on Acid-Catalyzed Iodouracil Degradation.—Solutions were prepared that contained 1 to 10×10^{-4} M IU in 1.00 M HCl alone and with uracil, KI, I_2-KI, uridine, or ribose in equivalent concentrations. These solutions were reacted at 80.0°, diluted 2:25 with distilled water, and the absorbances read as a function of time. There were no observed differences. Similarly, solutions of IU were prepared with IDU, deoxyuridine, and deoxyribose and similarly studied. The results have been given previously. The specific compositions of the IU-deoxyribose solutions which were used to evaluate the molecularity of the iodouracil transformation to uracil by deoxyribose (DR) under the 80.0°, 1 M HCl conditions were (a) 0.494×10^{-3} M IU, (b) 0.475×10^{-3} M IU and 0.97×10^{-5} M DR, (c) 0.455×10^{-3} M IU and 1.90×10^{-5} M DR, (d) 0.435×10^{-3} M IU and 2.76×10^{-3} M DR, (e) 0.865×10^{-3} M IU and 0.123×10^{-3} M DR, (f) 0.741×10^{-3} M IU and 0.247×10^{-3} M DR, and (g) 0.494×10^{-3} M IU and 0.494×10^{-3} M DR.

Tests on Iodide, Iodine and Iodine Monochloride Formation and Interaction with Iodinated Nucleosides and Their Possible Degradation Products.—The IDU and IU solutions reacted at the temperatures and in the HCl concentrations stated in Table I and II did not liberate free iodine during the reaction as checked by the starch-iodide test. Only after the addition of periodate or hydrogen peroxide was iodine observed on the addition of starch. This was true even in the case where starch solution was added without accompanying iodide ion.

(23) I. M. Kolthoff and C. S. Miller, *J. Am. Chem. Soc.*, **63**, 1405 (1941).

(24) I. M. Kolthoff and J. J. Lingane, "Polarography," Vol. 1, Interscience Publishers, Inc., New York, N. Y., 1952.

The following solutions were prepared in 0.65 M HCl and heated at 80.0°: (h) 6.70×10^{-4} M I_2, (i) 6.70×10^{-4} M I_2 and 6.60×10^{-4} M IDU, (j) 6.70×10^{-4} M I_2 and 6.60×10^{-4} M ribose, (k) 6.70×10^{-4} M I_2 and 6.60×10^{-4} M deoxyribose. The starch–iodide test was used to test for the presence of iodine and was positive at zero time in all cases. In 1.5 hr. no iodine was observed in solution k with deoxyribose whereas all others were equally positive and equivalent to the control solution (h). In 18 hr., no iodine was observed in solution i with IDU whereas the positive test of solution j with ribose was equivalent to the control solution h at that time. The extent of the starch–iodide test for the control solution appeared to diminish but was definitely positive throughout the study.

Solutions of 10^{-3} M IU were prepared in 0.970 N H_2SO_4 and in 0.947 N HCl and heated at 80.0°. When these colorless solutions were tested with starch after 5 hr. no positive test was noted. On the addition of KI, a positive iodine test was observed in both cases. Only a very slightly pink coloration was observed on chloroform extraction. Potassium iodide was added to the HCl solution and the subsequent chloroform extract was strongly pink. Addition of hydrogen peroxide did not give a positive starch–iodide test for iodine. However, a 10^{-4} M IDU solution in 0.947 M HCl at 80.0° when tested at 16 hr. gave a negative starch–iodide test for iodine until one drop of hydrogen peroxide solution was added when the addition of starch alone gave a positive iodine test.

An amount of 0.1 ml. ICl (Eastman) was dissolved in 200 ml. of 0.115 M HCl. Three milliliters of 10% KI were added to 3 ml. of this solution and the released iodine was titrated by 5.82 ml. of 0.01 N $Na_2S_2O_3$ using a starch indicator. Addition of 1.00 ml. of 0.01 M deoxyribose to 3 ml. of the ICl solution in the cold with subsequent KI addition also gave a titer of 5.82 ml. of 0.01 N $Na_2S_2O_3$ with a starch indicator. Similar amounts of ICl solution with and without the added deoxyribose were maintained at 60.0° for 15 hr. The 3 ml. of ICl solution consumed 5.77 ml. of 0.01 N $Na_2S_2O_3$ whereas the 3 ml. of ICl + 1 ml. of deoxyribose consumed only 4.66 ml. of the $Na_2S_2O_3$.

An amount of 0.1 ml. of ICl in 200 ml. of 1.15 N HCl was made 0.010 M in uracil and heated at 80.0° for 20 min. A 3 ml. aliquot of the solution without uracil consumed 5.82 ml. of 0.010 N $Na_2S_2O_3$ after the addition of 3 ml. of 10% KI to the starch end point. After heating, 3 ml. of the acid solution of ICl and uracil consumed only 3.56 ml. of the $Na_2S_2O_3$ by a similar titration. One milliliter of the reacted uracil–ICl solution was diluted to 100 ml. with water and a complete spectrum in acid solution was obtained. Two milliliters of 0.800 N NaOH was added to 1 ml. of reacted ICl–uracil solution and a complete spectrum in alkaline solution was obtained. The 259 mμ maximum of uracil in acid solution had disappeared whereas the 283 mμ maximum of iodouracil had appeared. The alkaline spectra was that of iodouracil with a flat maximum *ca.* 304 mμ and not of uracil. The transformation of uracil to iodouracil was apparently stoichiometric under the conditions studied.

A solution of 0.1 ml. of ICl in 200 ml. of 1.15 M HCl was mixed 1:1 with an 0.020 M uracil solution and subjected to 70.0°. Each solution had been equilibrated at this temperature before mixing. One ml. of the final solution was diluted to 100 ml. with water and the 283 mμ absorbance values obtained; the first one labeled at 0 hr. was assayed as quickly as possible. The time in hours and the observed absorbances were, respectively, 0.0, 0.129; 0.6, 0.399; 1.4, 0.402; 18, 0.408; 46, 0.408. Addition of KI and titration with $Na_2S_2O_3$ against starch indicator showed almost complete consumption of ICl by the 1.4 hr. sample. The complete spectrum demonstrated that the reaction resulted in a mixture of IU and uracil under these conditions. When the relative absorptivities of the product spectra in acid and alkaline solution were considered, the solution at complete reaction had a ratio of IU:U of 2:1. Whereas IDU in aqueous solution showed no significant photolytic degradation, an aqueous solution of 5-iodouridine became yellow (I_2 by starch–iodide test) on subjection to laboratory light. However, no iodine release was observed in solutions protected from light for 25 hr.

Solvolysis of IDU as Monitored by Thin-Layer Chromatography.—A typically monitored solvolysis of IDU in HCl solution follows. A 0.3% solution (8.47×10^{-3} M) of light shielded IDU in 0.947 N NCl was reacted at 80.0°. At intervals 4 ml. of this solution were neutralized with 2 N NaOH to *ca.* 4.0 pH and evaporated to dryness at 35° by the Arinco with use of the water aspirator. This residue was redissolved with 3 ml. of H_2O and 20 λ of this 11.3×10^{-3} M solution was spotted at the origin of the plate. The plates were prepared with a 400 μ layer of Silica Gel G with phosphor indicators (catalog no. 8071, Research Specialties Co.). The developing solvent of 30% isopropyl alcohol–70% chloroform was used for 10 cm. travel. The resulting spots were observed under ultraviolet light. Standards (20 λ of 0.1% solutions of IDU, IU, DU, and U) were spotted simultaneously before the development of the chromatogram. All compounds were viewed under short-wave ultraviolet, 2537 Å. A typical thin layer chromatogram is given in Fig. 4.

Similarly an 0.2% solution (5.65×10^{-3} M) of light-shielded IDU in 0.2 M sodium acetate–0.05 M acetic acid, pH 5.83) was heated at 80.0°. After cooling, 40 λ aliquots were spotted at various time intervals and developed with 30% isopropyl alcohol–70% chloroform for 8.2 cm. travel. A standard solution in water of a mixture of IDU, DU, IU, U, and isobarbituric acid (each 0.1%) was also spotted. Except for the isobarbituric acid which was viewed under long-wave ultraviolet 3660 Å., the other materials were observed under 2537 Å. ultraviolet light. The thin layer chromatograms of the IDU solutions showed only IDU at zero time, lessened amounts of IDU at 30 and 73 hr. with none at 94 hr. IU was observed at 53 and 73 hr. but not at zero and 94 hr. Uracil started to appear at 53 hr. and was the only identifiable product at 94 hr. No spot assignable to DU or isobarbituric acid was observed at any time.

The silica gel corresponding to each R_f value was scraped off the glass and extracted in 6 ml. of water. Spectra of these extractions were run at acid and alkaline pH values. The expected spectral shifts with pH were evident and proved the identity of IDU, IU, and U spotted from the degraded solution. The chromatograph (Fig. 4) of the HCl-treated IDU indicated the possibility of some overlap of DU and U. However, the spectra of the extracted spots could be assigned largely to U. The maximum amount of DU possible at any time was 10% of the total IDU.

Approximately 80% of the original concentration of the IDU could be accounted for by spectral analysis after extraction of the chromatographed degradation products. In the acetate buffer studies, no DU was observed as an intermediate.