(87%), m.p. 58–59°. Recrystallization from aqueous ethanol gave yellow crystals, m.p. 58–59°; $\lambda_{max.}$ (EtOH): 242, 362 mμ; (pH 13): 242, 370 mμ.

Anal.—Calcd. for $C_{18}H_{18}N_2O_3$: C, 69.7; H, 5.81; N, 9.03. Found: C, 69.5; H, 5.84; N, 8.91.

Similarly, XXX*a* was obtained as an oil that was isolated by ether extraction in 37% yield.

6 - Benzyl - 5 - phenylazo - 2 - thiouracil (XXXIb)—*Method E*—A mixture of 3.10 Gm. (10 mmoles) of XXX*b*, 1.10 Gm. (14 mmoles) of thiourea, and 1.08 Gm. (20 mmoles) of solid sodium methoxide in 20 ml. of methanol was refluxed with magnetic stirring for about 18 hr., then processed as under *Method A*. Recrystallization from ethanol gave 1.9 Gm. (60%) of yellow crystals, m.p. 189–190°. See Table III for additional data.

6 - Benzyl - 5 - (phenylazo)uracil (XIV)—*Method F*—To a stirred solution of 1.61 Gm. (5 mmoles) of XXXI*b* in 20 ml. of 2 *N* aqueous NaOH cooled in an ice bath was added dropwise 20 mmoles of dilute aqueous hydrogen peroxide; the deep red solution became bright yellow during the addition. The solution was then stirred for 2 hr. with the ice bath removed; occasionally the solution was cooled to keep the temperature at 27–30°. The solution was acidified with aqueous HCl, then the product was collected on a filter. After being washed with water and ice cold alcohol, the product was recrystallized from aqueous dioxane; yield, 0.90 Gm. (58%) of red needles. See Table III for additional data.

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Irreversible Enzyme Inhibitors 100

Inhibitors of Thymidine Phosphorylase 8. 6-(*p*-Bromoacetamidobenzyl)uracil, an Active-Site-Directed Irreversible Inhibitor

By B. R. BAKER and MITSUTAKA KAWAZU

Four candidate active-site-directed irreversible inhibitors for thymidine phosphorylase have been synthesized from 6-benzyluracil (I). Of these four, only 6-(*p*-bromoacetamidobenzyl)uracil (III) inactivated the enzyme; since neither iodoacetamide nor the *m*-isomer (VI) of III inactivated the enzyme under the conditions used for III, the inactivation by III most probably proceeds by a neighboring group reaction within the enzyme–inhibitor complex—the so-called active-site-directed mechanism.

PREVIOUS PAPERS in this series have described the mode of uracil binding (1, 2) to thymidine phosphorylase. Hydrophobic bonding to this enzyme by uracil derivatives has also been detected (2–6) and the mode of binding has been summarized (7). An effective inhibitor of this enzyme was 6-benzyluracil (I) (5), which was complexed 4.5-times better than the substrate, 5-fluoro-2′deoxyuridine; increasing the acidity of the uracil system by introduction of a 5-bromo group (II) gave a further ninefold increment in binding (6), II being complexed fortyfold better than the substrate. Therefore, 6-benzyluracil (I) and its 5-bromo derivative (II) were considered logical structures to be further modified for con-

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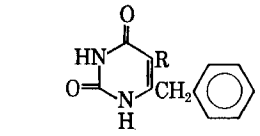
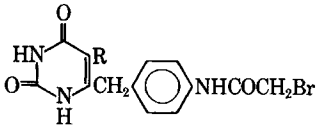
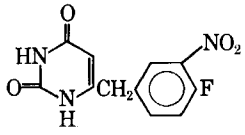
Previous paper: Baker, B. R., Kawazu, M., and McClure, J. D., *J. Pharm. Sci.*, **56**, 1081(1967).
The technical assistance of Pepper Caseria with the assays in Tables I and II is acknowledged.



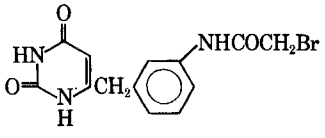
I, R = H
II, R = Br



III, R = H
IV, R = Br



V



VI

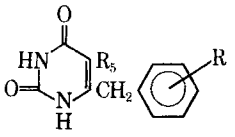
version to potential active-site-directed irreversible inhibitors (8, 9) of thymidine phosphorylase. The *p*-bromoacetamido derivatives (III and IV) of I and II were selected for initial study; also selected were the 3′-nitro-4′-fluoro (V) and the *m*-bromoacetamido (VI) derivatives of 6-benzyluracil (I). The enzyme results with these compounds and their intermediates are the subject of this paper.

DISCUSSION

Since a number of new intermediates were available from this study, they were investigated for reversible inhibition in order to shed further light on the anomalous binding of 5-bromo-6-(*p*-nitrobenzyl) uracil (VIII) (6). It was previously noted that the 4.5-fold enhancement in binding by 5-bromo group of II and the sevenfold enhancement of the *p*-nitro group of VII were not additive (Table I); in fact, VIII was considerably less effective than II or VII (6).

It was previously proposed (6) that either the nitro group of VII had an electronic effect on the binding of the benzyl moiety or that the nitro group was itself complexed directly to the enzyme. Since the 3-nitro group of IX has even a more beneficial effect on binding than the 4-nitro group of VII, it is clear that the nitro group of VII cannot be binding directly to the enzyme—else it would be position sensitive. Therefore, an electronic effect is much more likely. The evidence previously cited against an electronic effect by the nitro group of VII was the fact that a *p*-sulfonamide group was detrimental to binding (6). This difference can now be attributed to a difference in the hydrophilic character of

TABLE I—REVERSIBLE INHIBITION OF THYMIDINE PHOSPHORYLASE[a] BY



Compd.	R_5	R	μM Concn. for 50% Inhibition	$[I/S]_{0.5}$
I	H	H	90	0.22[c]
II	Br	H	10	0.025[d]
III	H	4-$BrCH_2CONH$-	26	0.065
IV	Br	4-$BrCH_2CONH$-	950[e]	2.4
V	H	3-NO_2-4-F	35	0.088
VI	H	3-$BrCH_2CONH$-	60	0.15
VII	H	4-NO_2	13	0.033[c]
VIII	Br	4-NO_2	67	0.17[d]
IX	H	3-NO_2	3.4	0.0085
X	H	4-CH_3CONH-	670	1.7
XI	H	3-NO_2-4-CH_3CONH-	30	0.075
XII	H	3-NO_2-4-NH_2	2.5	0.0063
XIII	H	F	51	0.13
XIV	Br	3-NO_2-4-CH_3CONH-	420	1.1
XV	Br	4-CH_3CONH-	1200[f]	3.0
XVI	H	4-NH_2	150	0.37[c]
XVII	Br	4-NH_2	11	0.028[d]
XVIII	Br	3-NO_2-4-NH_2	3.2	0.0080

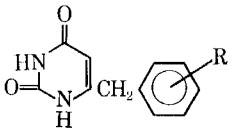
[a] Thymidine phosphorylase was a 45–90% ammonium sulfate fraction prepared from *E. coli* B and assayed with 400 μM FUDR in arsenate–succinate buffer (pH 5.9) in the presence of 10% dimethylsulfoxide as previously described (15). [b] The ratio of the concentration of inhibitor to 400 μM FUDR giving 50% inhibition. [c] Data previously reported (5). [d] Data previously reported (6). [e] Estimated from 30% inhibition at 400 μM, the maximum concentration still allowing light transmission. [f] Estimated from 31% inhibition at 500 μM, the maximum concentration still allowing light transmission.

the nitro group and sulfonamide groups with Hansch π-constants of +0.24 (to −0.28) and −1.82, respectively (10). That a polar group on the benzene is detrimental to binding is indicated by the seven- to eightfold loss in binding when the polar 4-acetamido group is placed on I or IX to give X or XI, respectively. Conversely, if a polar group on the benzyl group is detrimental to binding, then a hydrocarbon group could enhance binding; the latter observation has now been made (11).

That nitration of the benzyl group could enhance binding by 16–60-fold was noted in four out of five cases; compare I and VII, I and IX, XVI and XII, and X and XI where enhancement was good, but in contrast 6-(4-fluorobenzyl)uracil (XIII) showed little change in binding on nitration to V.

The introduction of a 5-bromo group had one of three effects in the seven pairs of compounds that were studied, a loss in binding, a gain in binding, or little change. A 9–14 gain in binding with II and XVII was observed only with the parent benzyluracil (I) or when the phenyl ring was substituted by an electron-donating amino group (XVI). The opposite effect—a loss in binding—was noted when the benzene ring was substituted by electron withdrawing groups; compare the pairs VII and VIII, XI and XIV, and III and IV. Little change in binding was noted with the electronically neutral acetamido group and with a benzene ring containing

TABLE II—IRREVERSIBLE INHIBITION OF THYMIDINE PHOSPHORYLASE[a] BY



Compd.	R	μM Concn. for 50% Reversible Inhibition[b]	μM Incubation Concn.	Time, hr.	% Inactivation
III	4-$BrCH_2CONH$-	26	100	1	24
			100	2	36
			26	2	25
			26	2	25
V	3-NO_2-4-F	35	75	1	0
VI	3-$BrCH_2CONH$-	60	120	2	0
Iodoacetamide		Large	100	2	0

[a] See *Experimental* for inactivation procedure performed at 37°. [b] Data from Table I.

both the electron withdrawing 3-nitro and electron-donating 4-amino substituents; compare the pairs X and XV, and XII and XVIII.

Since 5-bromo-(6-*p*-bromoacetamidobenzyl)uracil (IV) was such a poor reversible inhibitor (Table I), it was not investigated as an irreversible inhibitor. Of the other three candidate irreversible inhibitors, III, V, and VI, only 6-(*p*-bromoacetamidobenzyl)-uracil (III) showed inactivation of the enzyme (Table II). At a concentration of 100 μM, III showed 24% inactivation of the enzyme in 1 hr. and 36% inactivation in 2 hr. In contrast, an equal concentration of iodoacetamide run simultaneously showed no inactivation of the enzyme in 2 hr.; this latter result clearly demonstrates that III does not inactivate the enzyme by a random bimolecular process with no specificity, but supports the suggestion that III inactivates the enzyme within a reversible enzyme–inhibitor complex by the active-site-directed mechanism (12, 13). That the active-site-directed mechanism was operable for irreversible inhibition of thymidine phosphorylase by III was further supported by the observation that the *m*-bromoacetamido isomer (VI) showed no inactivation of the enzyme.

Further studies on inhibition of thymidine phosphorylase by 6-substituted uracils are continuing (11) to determine if additional hydrophobic bonding can be obtained by substitution of hydrocarbon groups on the 6-benzyl group of I or related compounds and to determine if faster active-site-directed irreversible inhibitors can be found before tissue specificity (9) studies are started.

CHEMISTRY

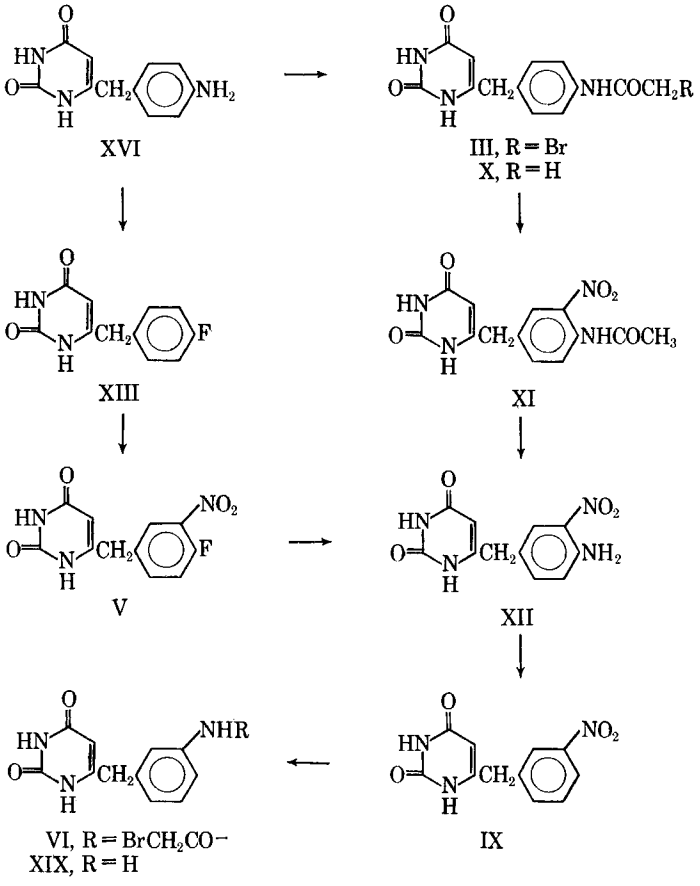
Conversion of 6-benzyluracil (I) to its *p*-amino derivative (XVI) by nitration and hydrogenation has been previously described (5). Reaction of XVI in *N*,*N*-dimethylformamide with bromoacetic anhydride at 0° proceeded smoothly to the bromoacetamide (III) which was uniform on TLC and showed the proper color reactions (14). Similarly, bromoacetylation of 6-(*p*-aminobenzyl)-5-bromouracil (XVII) (6) afforded the 5-bromo bromoacetamide (IV) (Table I).

Attempts to prepare 6-(*m*-aminobenzyl)uracil (XIX) by a primary synthesis starting with *m*-nitro- or *m*-acetamidophenylacetyl chloride were unsuccessful; the condensation of the acid chlorides with ethyl sodioacetoacetate gave black, resinous mixtures. Therefore, attention was turned to the conversion of 6-(*p*-aminobenzyl)uracil (XVI) to XIX by classical benzene chemistry. (Scheme I.)

Acetylation of XVI with acetic anhydride in *N*,*N*-dimethylformamide at 20° gave the *p*-acetamido derivative (X). Nitration of X with a mixture of 70% nitric acid and 96% sulfuric acid at 10–25° proceeded smoothly to the mononitro derivative (XI). That the nitro group had not entered the 5-position of the uracil was shown by nearly identical ultraviolet absorption of X and XI. The *N*-acetyl group of XI was removed with boiling 3 *N* aqueous hydrochloric acid to give a good yield of the nitroaniline derivative (XII). Diazotization of the aryl amino group of XII proceeded with difficulty due to a combination of poor reactivity and poor solubility; diazotization was eventually achieved, although poorly, by using 75% aqueous *N*,*N*-dimethylformamide. The resultant diazonium salt was then reduced *in situ* with hypophosphorus acid. The resultant 6-(*m*-nitrobenzyl)uracil (IX) was contaminated with two other minor products, but could be isolated in 21% yield by preparative TLC. Hydrogenation of IX to the *m*-aminobenzyl uracil (XIX) with a platinum oxide catalyst, followed by reaction with bromoacetic anhydride in *N*,*N*-dimethylformamide afforded the desired 6-(*m*-bromoacetamidobenzyl)uracil (VI).

Attempts to synthesize the 3-nitro-4-fluorobenzyl derivative (V) by diazotization of XII in 50% fluoboric acid with or without *N*,*N*-dimethylformamide as a solvent were unsuccessful due to the difficulty of diazotization. The desired V was then synthesized by an alternate method. Diazotization of 6-(*p*-aminobenzyl)uracil (XVI) in dilute aqueous fluoboric acid gave an insoluble diazonium fluoborate that was converted to XIII in 23% yield by heating at 150°. Nitration of XIII at 40° in a mixture of nitric and sulfuric acids gave V; that the nitro group had entered *ortho* to the fluorine atom was shown by conversion of V to XII with ammonia in *N*,*N*-dimethylformamide at 100°.

The 5-bromouracil derivatives, XIV and XV (Table I), were prepared by bromination of the corresponding uracil derivatives, XI and X, respectively. Hydrolysis of XIV with hot 3 *N* aqueous



Scheme I

hydrochloric acid afforded 6-(4′-amino-3′-nitrobenzyl)-5-bromouracil (XVIII).

EXPERIMENTAL

Melting points were determined in capillary tubes on a Mel-Temp block and those below 230° are corrected. Ultraviolet spectra were determined in 10% ethanol (unless otherwise indicated) with a Perkin-Elmer 202 spectrophotometer. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B or 337 spectrophotometer. All analytical samples gave a single spot on thin-layer chromatography (TLC) on Brinkmann Silica Gel GF when spots were detected by visual examination under ultraviolet light.

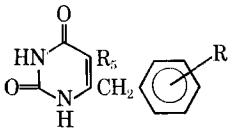
6-(p-Bromoacetamidobenzyl)uracil (III)—*Method A*—To a stirred solution of 540 mg. (2.64 mmoles) of XVI (5) in 5 ml. of *N,N*-dimethylformamide cooled in an ice bath was added 650 mg. (2.7 mmoles) of bromoacetic anhydride. The mixture was stirred at ambient temperature for 4 hr., then diluted with 5 ml. of water. The product was collected on a filter and recrystallized from aqueous *N,N*-dimethylformamide; yield, 420 mg. (50%) of white needles, m.p. 251–252° dec.; $\nu_{max.}$ 3400, 3140, 3000 (NH); 1700, 1680, 1600, 1530 (C=O, NH, C=C); 840 cm.$^{-1}$ (*p*-C_6H_4). See Table III for analytical data. The compound gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen and a negative Bratton-Marshall test for aromatic amine (14).

6-(p-Fluorobenzyl)uracil (XIII)—A solution of 1.08 Gm. (5.26 mmoles) of XVI (5) in 100 ml. of water and 3 ml. of 50% fluoboric acid was cooled in an ice bath with magnetic stirring, then treated dropwise with a solution of 0.40 Gm. (5.8 mmoles) of $NaNO_2$ in 10 ml. of water at such a rate that the temperature was 3–5°. After an additional hour in the ice bath, the mixture was filtered and the diazonium fluoborate was washed with ice water, then dried *in vacuo;* 1.2 Gm. of white solid showing an N≡N band at 2280 cm.$^{-1}$ was obtained.

The diazonium salt was heated in a bath at 150° until gas evolution was essentially complete. The residue was recrystallized from ethanol; yield, 0.25 Gm. (23%) of white crystals, m.p. 274–276° dec.; $\nu_{max.}$ 3350, 3150, 3050 (NH); 1710, 1650, 1610 (NH, C=O, C=C); 1210 (C—F); 830 cm.$^{-1}$ (*p*-C_6H_4). See Table III for additional data.

6-(p-Chlorobenzyl)uracil (XX)—A solution of 1.27 Gm. (6.2 mmoles) of XVI (5) in 150 ml. water and 5 ml. of 12 *N* hydrochloric acid was cooled in an ice bath with stirring, then treated dropwise with a solution of 0.38 Gm. (5.5 mmoles) of $NaNO_2$ in 10 ml. of water at such a rate that the temperature was 3–5°. After being stirred an additional 10 min., the solution was treated with 0.5 Gm. of Cu_2Cl_2. The mixture was stirred an additional 2 hr. in the ice bath, then the product was collected on a filter and washed with water. Recrystallization from ethanol gave 0.38 Gm. (34%), of white plates, m.p. 257–259° dec.; $\nu_{max.}$ 3400, 3100, 3000 (NH);

TABLE III—PHYSICAL CONSTANTS OF



Compd.[a]	R_5	R	Method	Yield,[b] %	M.p. °C., dec.	Anal. Calcd.	Anal. Found	$\lambda_{max.}$ (10% EtOH) pH 6	$\lambda_{max.}$ (10% EtOH) pH 13
III	H	4-$BrCH_2CONH$-	*A*	50[c,d]	251–252	C, 46.2 H, 3.58 N, 12.4	C, 46.3 H, 3.72 N, 12.4	265	270
IV	Br	4-$BrCH_2CONH$-	*A*	43[c,d]	252–253	C, 37.4 H, 2.68 N, 10.1	C, 37.6 H, 2.75 N, 10.2	275	256 (sh) 290 (sh)
V	H	3-NO_2-4-F	*B*[e]	22[d]	280–281	C, 49.8 H, 3.04 N, 15.8	C, 50.0 H, 3.12 N, 15.7	263	283
VI	H	3-$BrCH_2CONH$-	*A*[f]	22[c,d]	269–271	C, 43.8[g] H, 3.96	C, 43.6 H, 3.60	270	285
IX	H	3-NO_2	Exptl.	21	288–291	C, 53.4 H, 3.67 N, 17.0	C, 53.3[h] H, 3.93 N, 16.9	267	283
X	H	4-CH_3CONH-	*A*[i]	49[d]	297–300	C, 60.2 H, 5.02 N, 16.2	C, 60.5 H, 5.21 N, 16.0	264	288
XI	H	3-NO_2-4-CH_3CONH-	*B*	71[j]	311–313	C, 51.3 H, 3.98 N, 18.4	C, 51.2 H, 4.07 N, 18.1	262	285
XII	H	3-NO_2-4-NH_2	*C*	79[k]	298–300	C, 50.4 H, 3.85 N, 21.5	C, 50.3 H, 3.90 N, 21.4	260	286
XIII	H	4-F	Exptl.	23[l]	274–276	C, 60.0 H, 4.13 N, 12.7	C, 60.1 H, 4.28 N, 12.6	265	288
XIV	Br	3-NO_2-4-CH_3CONH-	*D*[m]	68	257–258	C, 40.8 H, 2.89 N, 14.6	C, 40.8 H, 3.07 N, 14.5	280	301
XV	Br	4-CH_3CONH-	*D*	64	>360	C, 46.1 H, 3.58 N, 12.4	C, 46.1 H, 3.56 N, 12.2	250 (sh) 275	245 (sh) 295
XVI	Br	3-NO_2-4-NH_2	*C*	88[k]	>330	C, 38.7 H, 2.66 N, 16.4	C, 38.7 H, 2.84 N, 16.2	260	292
XX	H	4-Cl	Exptl.	34[l]	257–259	C, 55.8 H, 3.80 N, 11.8	C, 55.6 H, 3.94 N, 11.7	263	288

[a] All compounds were uniform on TLC and had infrared spectra compatible with their assigned structures. [b] Yield of analytically pure material. [c] The compound gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen and a negative Bratton-Marshall test for aromatic amine (14). [d] Recrystallized from aqueous *N,N*-dimethylformamide. [e] Reaction performed at 40°. [f] See *Experimental* also. [g] Monohydrate. [h] Corrected for 2% ash from silica gel. [i] 30% excess of acetic anhydride employed at 20°. [j] Recrystallized from dimethylsulfoxide–ethanol. [k] Recrystallized from *N,N*-dimethylformamide–ethanol. [l] Recrystallized from ethanol. [m] Reaction performed at 80° in suspension. Solution occurred in 20 min. and the product was isolated by cooling.

1720, 1650 (uracil); 845 cm.$^{-1}$ (*p*-C_6H_4). See Table III for additional data.

6-(4′-Acetamido-3′-nitrobenzyl)uracil (XI)—*Method B*—To a stirred mixture of 10 ml. of 70% nitric acid and 10 ml. of 96% sulfuric acid cooled in an ice bath was added portionwise 3.0 Gm. (14.5 mmoles) of X at such a rate that temperature was 7–10°. After being stirred at ambient temperature for 1 hr., the mixture was poured on ice. The product was collected on a filter and washed with water, then ethanol. Recrystallization from hot dimethylsulfoxide by addition of ethanol gave 2.5 Gm. (71%) of yellow prisms, m.p. 311–313° dec.; $\nu_{max.}$ 3350, 3080, 3000 (NH); 1730, 1700, 1640, 1500 (NH, C=O, C=C, NO_2); 1330 cm.$^{-1}$ (NO_2). See Table III for additional data.

6-(4′-Amino-3′-nitrobenzyl)uracil (XII)—*Method C*—A mixture of 2.50 Gm. (10 mmoles) of XI and 30 ml. of 3 *N* aqueous hydrochloric acid was refluxed for about 18 hr., then spin evaporated *in vacuo*. The residue was recrystallized by solution in hot *N,N*-dimethylformamide, then addition of ethanol; yield, 1.5 Gm. (79%) of yellow crystals, m.p. 298–300° dec.; $\nu_{max.}$ 3450, 3300, 3150, 3000 (NH); 1700, 1650, 1570, 1500 (NH, C=C, C=O, NO_2); 1340 cm.$^{-1}$ (NO_2). See Table III for additional data.

This compound was also prepared by treatment of V with *N,N*-dimethylformamide saturated with ammonia at 100° in a Parr bomb for 9 hr. Recrystallization from water gave yellow crystals, m.p. 297–299° dec., that were identical with the preceding preparation.

6-(3′-Nitrobenzyl)uracil (IX)—A warm solution

of 1.7 Gm. (6.8 mmoles) of XII in 75 ml. of *N,N*-dimethylformamide and 1.5 ml. of 12 *N* aqueous hydrochloric acid was cooled in an ice bath with magnetic stirring, then treated with a solution of 0.55 Gm. (8 mmoles) of sodium nitrite in 20 ml. of water at such a rate that the temperature was 3–5°. After being stirred an additional 10 min. in the ice bath, the mixture was treated dropwise with 10 ml. of 10% hypophosphorus acid at such a rate that the temperature was 3–5°. After being stirred for 5 hr. in the ice bath, the mixture was stirred at ambient temperature for about 18 hr. The mixture was filtered, and the filtrate was diluted with 150 ml. of water. The product was collected on a filter and washed with water, then ethanol. The crude product was recrystallized from aqueous *N,N*-dimethylformamide; the resultant solid showed 3 spots on TLC. This product was further purified by preparative TLC on eight 20 × 20-cm. plates by the technique previously described (4). The major product, appearing in the second zone, was eluted with acetone; yield, 0.31 Gm. (21%) of yellow crystals, m.p. 288–291° dec. See Table III for additional data.

6-(3′-Bromoacetamidobenzyl)uracil (VI)—A suspension of 120 mg. (0.51 mmole) of IX in 50 ml. of glacial acetic acid was shaken with hydrogen at 2–3 Atm. in the presence of 50 mg. of platinum oxide catalyst; reduction was complete in 30 min. The filtered solution was spin evaporated *in vacuo*. The residue was dissolved in 5 ml. of 3 *N* aqueous hydrochloric acid. Spin evaporation *in vacuo* gave a residue of XIX hydrochloride that was recrystallized from 90% ethanol; yield, 70 mg. (54%) of XIX hydrochloride as white prisms, m.p. 279–281° dec.; $\nu_{max.}$ 3400 (NH); 2570, 2350, 2200 (NH^+); 1700, 1660, 1645, 1600–1500 (NH, C=O, C=C); 718 $cm.^{-1}$ (*m*-C_6H_4); $\lambda^{H_2O}_{max.}$ (pH 6): 279 mμ; (pH 13): 293 mμ.

Treatment of IX with bromoacetic anhydride by method *A* gave VI as off-white prisms, m.p. 269–271° dec., that was uniform on TLC, gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen, and a negative Bratton-Marshall test for aromatic amine (14). See Table III for additional data.

6-(p-Acetamidobenzyl)-5-bromouracil (XV)—*Method D*—To a solution of 300 mg. (1.2 mmoles) of X in 30 ml. of glacial acetic acid at 60° was added 200 mg. (1.25 mmoles) of bromine. Orange crystals rapidly separated. The mixture was cooled, then the product was collected on a filter and washed with ethanol. Recrystallization from aqueous *N,N*-dimethylformamide gave 250 mg. (54%) of white needles, m.p. above 360°; $\nu_{max.}$ 3400, 3250, 3000 (NH); 1700, 1650, 1620, 1560 (NH, C=C, C=O); 830 $cm.^{-1}$ (*p*-C_6H_4). See Table III for additional data.

ENZYME ASSAYS

Thymidine Phosphorylase—A 45–90% ammonium sulfate fraction from *E. coli* B was prepared as previously described (15). The final volume from 40 Gm. of frozen cells was 47 ml. in 0.05 *M* Tris buffer, pH 7.4.

Reversible Inhibition—Reversible inhibition of thymidine phosphorylase was performed as previously described with 0.4 m*M* 5-fluoro-2′-deoxyuridine (FUDR) as substrate in succinate–arsenate buffer (pH 5.9) containing 10% dimethylsulfoxide.

Irreversible Inhibition—The rate o cleavage of FUDR was previously observed to be proportional to the enzyme concentration when 0.4 m*M* FUDR was employed in the usual manner (15) as long as the total reaction did not exceed 0.5 absorbance units. The enzyme in pH 7.4 Tris buffer in the presence of 10% dimethylsulfoxide was stable over the 2-hr. incubation period at 37°. The inactivation experiments were performed as follows.

Incubation—In two tubes were placed 125 μl. of enzyme solution in 0.05 *M* Tris buffer (pH 7.4) and 0.55 ml. of deionized water in a 37° bath. After 5 min., 75 μl. of dimethylsulfoxide was added to tube 1 and 75 μl. of dimethylsulfoxide containing the inhibitor was added to tube 2. The contents were mixed, the time noted, then a 0.38-ml. aliquot was withdrawn from each tube as rapidly as possible and stored in an ice bath until ready for assay. The aliquot from the enzyme control was labeled C_1; the aliquot from the inhibitor tube was labeled I_1. The remainder in the two tubes (labeled C_2 and I_2) were then kept at 37° for the specified incubation time, then cooled in an ice bath until ready for assay.

Assay—An assay mix was prepared from 1.50 ml. of 4 m*M* FUDR, 7.50 ml. of 0.2 *M* succinate–arsenate buffer (pH 5.9), and 4.50 ml. of water; a larger amount can be made up if many assays are to be done, since the solution is stable at room temperature.

Three pairs of tubes are necessary to assay each time point for each solution; the C_1 and I_1 sets can be made up for assay during the 1–2-hr. incubation period. The back tube of each set serves as a zero time tube. In each back tube were placed 0.45 ml. of assay mix and 300 μl. of 3 *M* aqueous potassium hydroxide; the contents were mixed, then 50 μl. of C_1 incubation was added and the contents mixed again. In the three front tubes were placed 0.45 ml. of assay mix and 50 μl. of C_1 incubation mixture; the contents were mixed and the time noted. After 10–20 min., the time depending upon the activity of the enzyme (15), 300 μl. of 3 *M* aqueous potassium hydroxide was added and the contents mixed. The absorbance of each pair of tubes was read using the same 1-ml. cell for a pair of tubes. The C_2, I_1, and I_2 aliquots were assayed similarly. The average absorbance change between a front and back pair of tubes, which is proportional to the enzyme concentration, was plotted on a log scale against the incubation time on a linear scale (13). This procedure is satisfactory for a routine screen for a plus or minus answer on inactivation.

When a compound showed inactivation, a larger amount of inhibitor–enzyme mixture could be set up and a number of aliquots removed at varying times in order to obtain the half-life of inactivation.

The results of the inactivation experiments are shown in Table II.

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Method to Estimate Small Amounts of L-Triiodothyronine in D-Triiodothyronine

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One of the requirements for the use of D-triiodothyronine as a serum cholesterol-lowering agent is that it be essentially free of its optical isomer, L-triiodothyronine. A method for estimating small amounts of L-triiodothyronine in D-triiodothyronine is described. After removing any free triiodothyroacetic acid that may be present, the sample is reacted with stereospecific L-amino oxidase (from snake venom) to oxidize the L-triiodothyronine to triiodothyropyruvic acid. The triiodothyropyruvic acid is degraded to triiodothyroacetic acid, extracted, and chromatographed on paper to separate it from the unoxidized D-triiodothyronine. The final quantitative estimation is made by comparing the size and intensity of the colored spot, as visualized by an appropriate spray reagent, with those obtained from known mixtures of L- and D-triiodothyronine carried through the entire procedure. As little as 0.05 per cent L-triiodothyronine could be estimated in the various samples evaluated.

L-TRIIODOTHYRONINE (L-T3), the most potent of the naturally occurring thyroid hormones, is both a powerful calorigenic agent and an active serum cholesterol-lowering agent. Its use for the latter purpose is rather limited, however, since it cannot be used safely in patients suffering from angina pectoris and related diseases. In contrast, its optical antipode, D-triiodothyronine (D-T3), is potentially a very valuable agent for this purpose since it retains much of the serum cholesterol-lowering properties of the natural isomer without the liability of the latter's hypermetabolic activity.

Since L-T3 is such a potent calorigenic agent, its presence in any appreciable amount in lots of the D-T3 destined for clinical trials as a serum cholesterol-lowering agent would badly confound the results. For this reason, heroic efforts were made to limit the amount of L-T3 present in test lots of D-T3 to very small values (of the order of less than 0.2%). In this connection a procedure was required that would be capable of estimating the L-T3 content of samples of D-T3 in the 0–1% range. Since the analytical procedure was designed primarily for acceptance or rejection purposes, sensitivity was deemed more important than great accuracy, and values accurate to one or at most two significant figures were considered satisfactory.

Direct measurement of optical rotation as a means of determining the amount of L-T3 in samples of D-T3 proved to be rather too insensitive for this purpose. With a specific rotation of $-24.5°$ for D-T3, it was not found possible to reliably distinguish samples that contained 0.5% L-T3 from those that contained less than 0.1%. In any case, it was not possible by this procedure to detect 0.2% of L-T3 in samples of D-T3.

The most widely used procedure for determining small amounts of an L-amino acid in the presence of a large amount of its enantiomorph is by stereospecific enzymatic degradation with micromanometric measurement of gas taken up or released (1–5). While this procedure is reported to be capable of estimating as little as 0.1% of an L-amino acid in the presence of its D-isomer, the authors were not able to reliably detect less than 2% of L-T3 in samples of D-T3 manometrically. This may be due to the poor aqueous solubility of the triiodothyronines which prevented the use of an adequate size sample.

Although micromanometric procedures for determining L-amino acids in the presence of the D-isomers were not sensitive enough for the purposes of this work, stereospecific oxidation with an enzyme such as L-amino acid oxidase appeared to be a promising approach to the solution of this problem, provided a sensitive procedure could be

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