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Model Studies of the Thymidylate Synthetase Reaction. Nucleophilic Displacement of 5-*p*-Nitrophenoxymethyluracils†

Alfonso L. Pogolotti, Jr.,‡ and Daniel V. Santi*

ABSTRACT: Nucleophilic displacement reactions of 5-*p*-nitrophenoxymethyluracil and its N-alkylated derivatives have been examined to provide insight into the mechanism by which thymidylate synthetase catalyzes hydride transfer from 5,10-methylenetetrahydrofolate to the methyl group of thymidylate. All reactions appear to proceed by formation of highly reactive intermediates having an exocyclic methylene group at the 5 position of the heterocycle rather than direct displacement (SN2) of the leaving group. The driving force for the expulsion of the leaving group and formation of such intermediates may be provided by the N-1 anion, where possible, or by attack of a nucleophile at the 6 position of the heterocycle when the 1 position is alkylated. Direct support for the proposed mechanisms was obtained by evaluation of secondary deuterium isotope effects of reactants possessing deuterium at the 5-methylene carbon or the 6 position of the heterocycle. The mechanism involving nucleophilic attack at the 6 position of the heterocycle is analogous to that observed in model studies of other reactions catalyzed by this enzyme, and permits us to propose a unified mechanism for catalysis which is supported by all chemical and biochemical data at hand. Discussion is presented which argues against the existence of a thymidylyl–tetrahydrofolate intermediate in the reaction pathway leading to products.

A minimal mechanism for the thymidylate synthetase catalyzed reductive methylation of dUMP[1] to dTMP must involve at least two steps. This was recognized some time ago when Friedkin and coworkers (Friedkin and Kornberg, 1957; Pastore and Friedkin, 1962) proposed that condensation of CH_2-H_4folate with dUMP results in a 5-thymidylyl-H_4folate intermediate which subsequently undergoes disproportionation *via* a 1,3-hydride shift to give the products dTMP and 7,8-H_2folate (Figure 1). In the first step, an electrophilic substitution reaction occurs in which the methylene carbon of 5,10-CH_2-H_4folate replaces the hydrogen at the 5 position of dUMP without a change in oxidation level. The second step of this mechanism can best be described as a nucleophilic substitution at the incipient methyl group of dTMP by hydride, originating from the 6 position of the cofactor, and resulting in concomitant production of 7,8-H_2folate. Wherever possible, radioisotope tracer experiments have verified salient features of this mechanism (Pastore and Friedkin, 1962; Blakley *et al.*, 1963), but increasing knowledge of the chemistry of the components of this reaction has made it apparent that the enzymic reaction is much more complicated than originally proposed.

Previous reports from this laboratory (Santi and Brewer, 1968, 1973; Santi *et al.*, 1970) dealt with the development of model systems which would help to elucidate the mechanism of the condensation of dUMP and the formaldehyde donor. These studies led to the conclusion that the reaction was initiated by nucleophilic attack at the 6 position of dUMP and resulted in activation of the 5 position toward a species of formaldehyde. Subsequent studies using the quasi-substrate, FdUMP, demonstrated the formation of isolable covalent enzyme–FdUMP complexes (Santi and McHenry, 1972) in which a nucleophile of the enzyme was attached to C-6 of the analog and strongly supported the congruence of the model systems.

A question which remains unanswered is how the second, or oxidation–reduction, stage of the reaction occurs. In view of the poor leaving group potential of amines, it is difficult to envision why the *N*-methylene group of an intermediate such as 5-thymidylyl-H_4folate would be susceptible to nucleophilic attack by hydride; to our knowledge, precedent for direct nucleophilic displacement at the α carbon of a tertiary amine is lacking. In addition, we wondered whether the nucleophilic catalyst required in the initial stage of the reaction might also be involved in the oxidation–reduction step. With these inquiries in mind, we sought to study related reactions in a simpler chem-

† From the Department of Biochemistry and Biophysics and Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143. *Received May 31, 1973.* This work was supported by U. S. Public Health Service Grant No. CA-14394 from the National Cancer Institute.

‡ National Institute of Health Predoctoral Fellow, 1968–1971. Present address: Department of Chemistry, University of Arizona, Tucson, Ariz. 85721.

[1] Abbreviations used are: dUMP, 2′-deoxyuridylic acid; dTMP, deoxythymidylic acid; 5,10-CH_2-H_4folate, 5,10-methylenetetrahydrofolic acid; 7,8-H_2folate, 7,8-dihydrofolic acid; H_4folate, tetrahydrofolic acid; FdUMP, 5-fluoro-2′-deoxyuridylic acid; HMU, 5-hydroxymethyluracil; 1MeHMU, 3MeHMU, and Me_2HMU, 1-methyl-, 3-methyl-, and 1,3-dimethyl-5-hydroxymethyluracil, respectively; NPMU, 5-*p*-nitrophenoxymethyluracil; 1MeNPMU, 3MeNPMU, and Me_2NPMU, 1-methyl-, 3-methyl-, and 1,3-dimethyl-5-*p*-nitrophenoxymethyluracil, respectively; 1APr-3MeNPMU, 1-(3-aminopropyl)-3-methyl-5-*p*-nitrophenoxymethyluracil; 1APr-3MeHMU, 1-(3-aminopropyl)-3-methyl-5-hydroxymethyluracil.

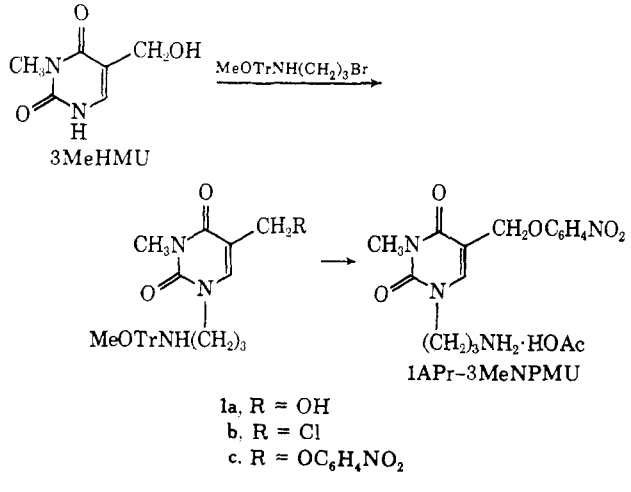
ical system which would mimic that which is catalyzed by the enzyme. We have recently observed that esters and ethers of 5-hydroxymethyluracil are unusually reactive toward nucleophilic displacement (Santi and Pogolotti, 1971), the former undergoing *O*-alkyl rather than *O*-acyl bond cleavage. In addition, when treated with hydride reagents, such compounds rapidly gave rise to corresponding thymine derivatives. In this report, we describe detailed investigations of the mechanisms of nucleophilic displacement reactions for derivatives of 5-*p*-nitrophenoxymethyluracils, and offer these as chemical counterparts of the second step of the thymidylate synthetase reaction. Although the leaving group in the model system greatly differs from H_4folate in structure and reactivity, we feel that the salient features of the reaction reside in the uracil heterocycle, and are retained regardless of the nature of the leaving group. From these studies, we are able to offer a reasonable chemical mechanism for the enzymic reaction which is consistent with all biochemical data reported to date.

Experimental Section

General. All materials were reagent grade unless otherwise specified. Dimethylformamide was dried over Linde 4A Molecular Sieves. Potassium carbonate was dried at 150° prior to use. Melting points were determined on a Mel-Temp apparatus and are corrected. Infrared spectra (KBr) were recorded on a Perkin-Elmer Model 337 spectrophotometer. Deuterium analysis was performed on an AEI-MS902 mass spectrometer equipped with a Mosley 7101B linear streak recorder and a Varian HA-100 nuclear magnetic resonance (nmr) spectrometer in conjunction with a Varian Model C-1024 time averaging computer. Dideuterioparaformaldehyde was purchased from Merck and Co. Methanol-*l-d* (99.7%) and D_2O (99.8%) were purchased from Stohler Chemicals. Sodium deuteroxide solutions were prepared by dissolving metallic sodium in deuterium oxide. Thin-layer chromatography (tlc) was performed on silica gel GF_{254} (Merck) plates and paper chromatography (ascending) was performed on Whatman No. 3MM strips. Spots were visually detected under short-wave ultraviolet (uv) light. Ninhydrin spray was employed to detect primary amines and 10% ethanolic $HClO_4$ was used to detect the monomethoxytrityl blocking group (Schaller *et al.*, 1963). Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

The synthesis of 1APr-3MeHMU (Scheme I) was performed by the following sequence.

SCHEME I



1a, R = OH
b, R = Cl
c. R = $OC_6H_4NO_2$

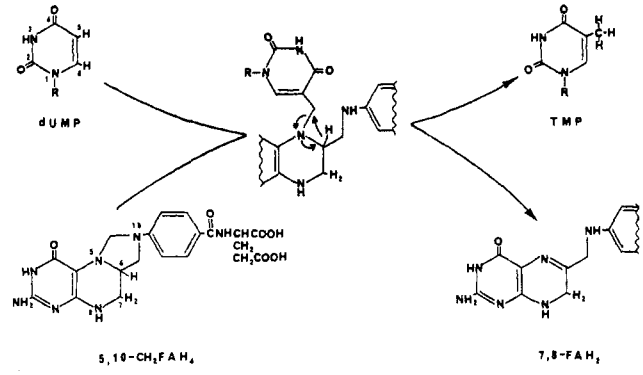


FIGURE 1: Reaction catalyzed by thymidylate synthetase showing the thymidylyl-H_4folate intermediate proposed by Friedkin; R = 5-phospho-2′-deoxyribosyl.

1-[3-(p-Methoxytritylamino)propyl]-3-methyl-5-hydroxymethyluracil (1a). To 50 ml of chilled (5°) $CHCl_3$ containing 5.6 ml (40 mmol) of triethylamine and 2.19 g (10 mmol) of 1-amino-3-bromopropane hydrobromide was added 3.40 g (11 mmol) of *p*-anisylchlorodiphenylmethane over a 30-min period with vigorous stirring. The chilled solution was stirred an additional 30 min and then washed with 3 × 10 ml portions of 10% $KHCO_3$ followed by 3 × 15 ml washings with H_2O. The organic layer was dried ($MgSO_4$) and evaporated *in vacuo* at room temperature to give 3.89 g (97%) of a clear yellow oil which showed only trace impurities on tlc using petroleum ether–EtOAc (10:1). The oil was chromatographed on a silica gel (Woelm) dry column (3.8 × 40 cm) (Loev and Goodman, 1967) using the same solvent system. The product was eluted with 5 × 10 ml portions of $CHCl_3$, dried ($MgSO_4$), filtered, and evaporated *in vacuo* to give 3.60 g (90%) of a colorless oil. Nmr and infrared (ir) spectra were consistent with the assigned structure. The oil gave one uv absorbing spot on tlc using petroleum ether–EtOAc (10:1) which upon spray treatment with 10% ethanolic $HClO_4$ produced an orange color. The product was used immediately in the subsequent reaction.

A suspension of 3.90 g (25 mmol) of 3-methyl-5-hydroxymethyluracil and 3.46 g (25 mmol) of K_2CO_3 in 75 ml of dimethylformamide was magnetically stirred at ambient temperature for 2 hr protected from moisture. After 2 hr, 10.1 g (25 mmol) of the above monomethoxytrityl derivative was added and the suspension was stirred for 5 days at ambient temperature protected from moisture. The dimethylformamide was evaporated *in vacuo* at room temperature. The solid residue was dissolved in 150 ml of $CHCl_3$ and washed with 5 × 20 ml portions of water. The organic layer was dried ($MgSO_4$) and evaporated *in vacuo* to give a white residue. Crystallization from benzene–petroleum ether gave 6.0 g (50.4%) of product; mp 92–93.5°. A portion was recrystallized in similar fashion to give the analytical sample: mp 93–93.5°; λ_{max} (H_2O) 271 nm (pH 7). Tlc using EtOAc–petroleum ether (3:1) showed one spot which gave a positive spray test for the monomethoxytrityl group.

Anal. Calcd for $C_{29}H_{31}N_3O_4 \cdot {}^2/_3C_6H_6$: C, 73.72; H, 6.55 N, 7.82. Found: C, 73.77; H, 6.60; N, 7.88.

1-[3-(p-Methoxytritylamino)propyl]-3-methyl-5-chloromethyluracil (1b). To a magnetically stirred solution (−5°) of 0.970 g (2.0 mmol) of **1a** and 4.0 ml of triethylamine (29 mmol) in 20 ml of $CHCl_3$ was slowly added 0.150 ml (2.1 mmol) of $SOCl_2$. After 30 min at −5° the solution was evaporated *in vacuo* to give a clear glass which was dissolved in a minimal amount of $CHCl_3$ and applied to a silica gel column (2 × 31 cm). Elution with EtOAc–petroleum ether (1:1) gave 0.700 g (71%) of

product pure by tlc in the form of a clear glass. The high reactivity of the 5-chloromethyl group made analysis difficult and structure assignment was based on the absence of OH stretch in the ir spectrum, a positive *p*-nitrobenzylpyridine test for active halogen (Epstein *et al.*, 1955; Baker *et al.*, 1966), a positive spray test for the monomethoxytrityl group, and quantitative conversion to starting material upon treatment with aqueous triethylamine: ν_{max} 3200 (NH), 1710–1640 (C=C, C=O), 1600, 1500, 760, 830 cm^{-1} (phenyl). The product was immediately used in the next reaction.

1-[3-(p-Methoxytritylamino)propyl]-3-methyl-5-p-nitrophenoxymethyluracil (1c). To a well-stirred solution of 2.28 g (14.2 mmol) of anhydrous sodium *p*-nitrophenolate and 0.328 g (2 mmol) of KI in 15 ml of dimethylformamide was added 0.700 g (1.42 mmol) of the blocked chloromethyluracil (**1b**). After stirring for 18 hr at ambient temperature, the solution was evaporated *in vacuo* (<18°) and the residual red solid slurried with 5 × 20 ml portions of cold H_2O. The resulting solid was crystallized from MeCN–petroleum ether to give 0.450 g (52.4%) of product, mp 76–78°. A 0.200-g portion was chromatographed on a silica gel column (3 × 27 cm) using EtOAc–petroleum ether (1:1) to give the analytical sample, 0.140 g, mp 79–81°. Tlc using EtOAc–petroleum ether showed one spot which gave a positive spray test for the monomethoxytrityl carbonium ion. Treatment with 1 N KOH slowly produced a quantitative liberation of *p*-nitrophenoxide: λ_{max} (EtOH) 272, 317 nm; ν_{max} 3170 (NH), 1710–1630 (C=C, C=O), 1600 (phenyl), 1338 (NO_2), 752, 840 cm^{-1} (phenyl).

Anal. Calcd for $C_{35}H_{34}N_4O_6$: C, 69.29; H, 5.65; N, 9.23. Found: C, 69.41; H, 5.78; N, 9.14.

1-(3-Aminopropyl)-3-methyl-5-p-nitrophenoxymethyluracil Acetate (1 APr-3 MeNPMU). To a stirred mixture (10°) of 5 ml of MeCN, 3 ml of H_2O, and 2 ml of glacial HOAc was added 0.126 g (0.21 mmol) of **1c**. After 2 hr at ambient temperature the solution was lyophilized to *ca.* 0.5 ml and taken up in 1.5 ml of 5 mM HOAc. The absorption at 470 nm (log ε 4.67) indicated nearly a quantitative liberation of the water-insoluble monomethoxytritylcarbinol (Deno *et al.*, 1954). The aqueous solution was applied to a phosphocellulose (Bio-Rad; 0.94 mequiv/g) cation exchange column (1.1 × 28 cm) which had been previously equilibrated with 5 mM HOAc. Chromatography was performed at ambient temperature using a linear gradient consisting of 500 ml of 5 mM HOAc in the mixing chamber and 500 ml of 0.875 M HOAc in the reservoir. The product eluted at 0.55 M HOAc to give a 71% yield as determined by quantitative uv. Fractions containing product were pooled, lyophilized until the pH was approximately 4.5, and stored at −20°. Under these conditions, the compound was stable for at least 1 month as determined by uv, chromatography on a phosphocellulose column using the stated solvent system, tlc, and paper chromatography using EtOAc–HCOOH–H_2O (7:2:1) as the solvent system: λ_{max} (H_2O) 270 (ε 9950), 313 nm (ε 10,200) (pH 4.0). Extinction values were obtained by comparing the optical density values at 270 and 313 nm (OD) with the isosbestic point of *p*-nitrophenol (348 nm, ε 4900). The high reactivity of the product precluded its isolation as a solid acetate salt. Lyophilization to dryness at reduced temperature (10°) resulted in degradation of the pyrimidine ring and formation of *p*-nitrophenol. The product gave a quantitative liberation of *p*-nitrophenoxide when treated with base. Tlc and paper chromatography showed one spot which gave a positive ninhydrin test, positive test for *p*-nitrophenol when sprayed with 0.1 N KOH, and a negative test for the monomethoxytrityl blocking group.

1-(3-Aminopropyl)-3-methyl-5-hydroxymethyluracil Acetate (1APr-3MeHMU). The same method described for the preparation of the corresponding *p*-nitrophenyl ether was used. Starting with 0.300 g (0.62 mmol) of N-blocked 1APr-3MeHMU (**1a**), 0.138 g (82%) of a colorless oil was isolated as the acetate salt which showed one spot on tlc using EtOAc–HCOOH–H_2O (7:2:1) as the solvent system. Elution from a phosphocellulose (0.94 mequiv/g) column (3 × 27 cm) occurred at 0.15 M HOAc using the same conditions described for 1APr-3MeNPMU. Unlike 1APr-3MeNPMU, the product was stable at room temperature or in strongly basic solutions (pH >12). The product was characterized by homogeneity on the chromatography system discussed earlier, a positive ninhydrin test, and a negative test for the monomethoxytrityl group: λ_{max}(H_2O) 268 (ε 8120) (pH 2), 269 nm (ε 8190) (pH 10).

Uracil-5,6-d_2 in 98.5% isotopic purity was prepared by catalytic hydrogenolysis of 5-bromouracil-*6-d* by the method of Parkanyi and Sorm (1963) with the exception that D_2 gas and deuterated solvents were employed. In our hands, use of protio solvents and H_2 resulted in a 25% loss of deuterium from the 6 position.

5-Hydroxymethyluracil-6-d (HMU-*6-d*) was prepared in 85% yield by base-catalyzed hydroxymethylation of uracil-*5,6-d_2* according to the method of Cline *et al.* (1959). The product showed identical chromatographic properties and melting point as an authentic sample of the protio compound. Mass spectral analysis showed a parent peak at *m/e* 143 and isotopic purity of greater than 98%. Nmr showed a singlet at (NaOD) 4.18 (7-CH_2).

5-Hydroxymethyluracil-7-d_2 (*HMU-7-d_2*). The base-catalyzed condensation of uracil with dideuterioparaformaldehyde was performed as described for 5-hydroxymethyluracil-*6-d:* yield 75%; mp 280°. Mass spectra showed a parent peak of *m/e* 144 and isotopic purity of 98.4%. Nmr showed a singlet at (NaOD) 7.18 (6-H).

Preparation of Deuterated NPMU Derivatives. All NPMU derivatives deuterated at the 6 position (*6-d*) or 7 position (*7-d_2*) were prepared from the corresponding 5-hydroxymethyluracils by procedures previously reported (Santi and Pogolotti, 1971) for the analogous protio compounds and showed identical melting points, uv spectra, and chromatographic properties. All deuterated derivatives were minimally 98% isotopically pure as determined by mass spectra and nmr.

Product Analysis. With the exceptions of the reactions of 1APr-3MeNPMU and Me_2NPMU at high pH, the sole hydrolytic products of NPMU and its N-alkylated derivatives were shown to be the corresponding HMU derivatives and *p*-nitrophenoxide by uv analysis, chromatographic comparisons to authentic samples, and product isolation in preparative scale reactions. Repetitive scans (235–410 nm) at various pH values showed clearly defined isosbestic points and demonstrated the absence of accumulated intermediates in the conversions; the changes observed corresponded to quantitative conversion to the aforementioned products. This was not the case with 1APr-3MeNPMU or Me_2NPMU at high pH where numerous short-lived intermediates could be detected along with *p*-nitrophenoxide. Upon completion of these reactions, the pyrimidine chromophore was no longer intact and the products were tentatively assigned as N-alkylated urea derivatives resulting from cleavage of the C_4–N_3 amide bond (Kondo *et al.*, 1971; Kondo and Witkop, 1968; Santi and Pogolotti, 1971).

Kinetic Measurements. Unless otherwise stated, kinetic experiments and pK_a determinations were performed in aqueous solution at 25 ± 0.1° with μ = 1.0 (KCl). All solutions were prepared from doubly distilled water which was purged with nitrogen prior to use. Rate measurements were

made on a Beckman DU monochromator combined with a Gilford Model 2000 multiple sample absorbance recorder using 3-ml cuvets. Reactions were monitored under pseudo-first-order conditions for at least 8 half-lives at 402 nm (pH >6) or 308 nm (pH <6). For fast reactions ($t_{1/2}$ < 10 sec) a Durrum-Gibson Model 13001 stopped-flow spectrophotometer was used. A Cary Model 15 spectrophotometer fitted with the titration apparatus described by Bruice and Maley (1970) was used for kinetic experiments performed in the absence of external buffers. A Radiometer Type PHM26 pH meter with a Metrohm EA125U combined electrode was used for pH measurements. Potentiometric pK_a determinations were performed under nitrogen by manual titration using a Radiometer buret syringe.

Reactions having $t_{1/2}$ < 10 hr were initiated by the addition of 5–20 μl of a 1–5 mM stock solution of the reactant in dioxane (MCB, Spectroquality) to the buffer (3-ml cuvets) which was preequilibrated in the thermostated cell compartment of the spectrophotometer. Controls in which the dioxane content of the reaction mixtures was varied from 0 to 4% showed no effect on hydrolytic rates. For longer reactions, 20 ml of a 5 × 10^{-4} M solution of the reactant in the appropriate buffer was placed in a constant temperature bath. At appropriate intervals aliquots were removed and the optical density determined at the appropriate wavelength. For stopped-flow experiments, a freshly prepared stock solution of the substrate in 10^{-4} M HCl was placed in one syringe. The buffer or KOH and KCl concentrations in the other syringe were such that the desired pH and ionic strength were obtained upon mixing. Buffers used were acetate (pH 3.6–5.7), phosphate (pH 6.0–7.9), borate (pH 7.6–9.0), and carbonate (pH 9.2–10.6). Hydrochloric acid and potassium hydroxide were used for the pH ranges of 1.0–2.8 and 11.0–14.0, respectively. The pH values of solutions were measured before and after each kinetic run; solutions which showed a drift greater than 0.03 pH unit over the course of the reaction were discarded. Buffer concentration was generally maintained at 0.1 M since, with the exception of 1APr-3MeNPMU, buffer catalysis was not observed over a 50-fold concentration range. Lyate-catalyzed rates for 1APr-3MeNPMU were obtained in the absence of external buffer using the titration assembly described by Bruice and Maley (1970). For the Brønsted plot with 1APr-3MeNPMU, solutions of the purified amines or amine hydrochlorides were adjusted to the desired pH with HCl or NaOH.

The values of the pseudo-first-order rate constants (k_{obsd}) were calculated from plots of log (OD − OD_t) *vs.* time (t) or from a Culler-Fried on-line computing system served by an IBM 360/75 computer using a standard least-squares program written by Dr. David O. Harris. Rates were obtained in triplicate and agreed within 3%.

Results

Hydrolysis of NPMU and N-Methylated Derivatives. The pH–log k_{obsd} profile for release of *p*-nitrophenol(ate) from Me_2NPMU is shown in Figure 2. In the pH region 0.9–9.8, repetitive scans (235–410 nm) throughout the course of reaction showed clearly defined isosbestic points (*i.e.*, no detectable build-up of intermediates), and the final spectrum together with product isolation demonstrated quantitative conversion to Me_2HMU and *p*-nitrophenol. At high pH (>11), there is a facile liberation of *p*-nitrophenoxide which shows first-order dependence on hydroxide ion (k_{OH} = 0.27 M^{-1} min^{-1}) at 25° which is followed by a slower destruction of the pyrimidine chromophore at 265 nm. The final degradation products, with

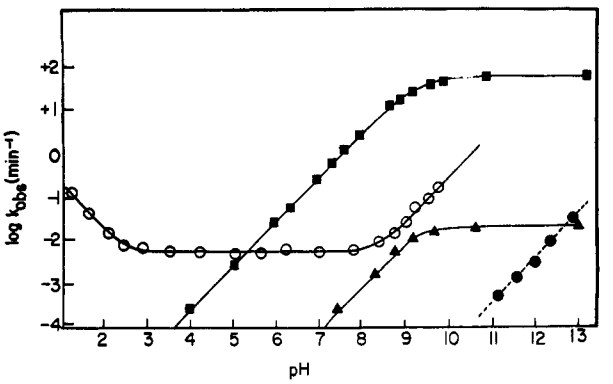


FIGURE 2: The pH–log k_{obsd} profiles for: (a) hydrolysis of Me_2NPMU to Me_2HMU at 90° (○); (b) liberation of *p*-nitrophenoxide in the degradation of Me_2NPMU at 25° (●); hydrolyses of 1MeNPMU (▲) and 3 MeNPMU (■) to 1MeHMU and 3MeHMU, respectively, at 25°. Points are experimental and lines are calculated from equations presented in the text.

the exception of *p*-nitrophenoxide, exhibited no uv absorption above 250 nm and were not characterized. Under identical and more vigorous (pH 13.5) conditions, Me_2HMU was completely stable, showing that it is *not* formed as an intermediate in reactions of Me_2NPMU which occur at high pH. It is to be emphasized that reactions resulting in destruction of the pyrimidine chromophore were only observed with Me_2NPMU at high pH and with 1APr-3MeNPMU (*vide infra*).

Over the pH range 0.9–9.8 the hydrolysis of Me_2NPMU to Me_2HMU can be described by eq 1, where k_0 (5.63 × 10^{-3}

$$k_{obsd} = k_0 + k_{OH}(K_w/a_H) + k_{a_H}a_H \quad (1)$$

min^{-1}) is the catalytic rate constant for spontaneous hydrolysis, k_{OH} (68.0 M^{-1} min^{-1}) is the specific base catalyzed rate constant, k_{a_H} (0.86 M^{-1} min^{-1}) is the specific base catalyzed rate constant, a_H is the hydrogen ion activity as measured by the glass electrode, and K_w (12.54 M) is the autopyrotolysis constant for water at 90° (Wynne-Jones, 1936). Extrapolation of the Arrhenius temperature dependence determined at pH 9.43 for the hydrolysis of Me_2NPMU to Me_2HMU gave k_{obsd} = 1.60 × 10^{-5} min^{-1} and k_{OH} = 0.38 M^{-1} min^{-1} at 30°.

The hydrolysis of 1MeNPMU to 1MeHMU was studied in the pH region 7.4–14.0 at 25 ± 0.1°. The pH–log k_{obsd} profile shown in Figure 2 is described by eq 2 where k_r is the specific

$$k_{obsd} = k_r[K_a/(K_a + a_H)] \quad (2)$$

rate constant for the reaction of the monoanion (1MeNPMU$^-$) with water and K_a is the acid dissociation constant of 1MeNPMU. At high pH ($a_H \ll K_a$) k_{obsd} becomes equal to k_r (2.28 × 10^{-2} min^{-1}); calculation of pK_{app} for 1MeNPMU gives a value of 9.33 which is reasonable agreement with the measured value of 9.65 of the "model" 1-methyl-5-methoxymethyluracil. The kinetically equivalent mechanism involving hydroxide attack on the neutral species may be expressed by eq 3 where k_{OH} (1.0 × 10^3 M^{-1} min^{-1}) is the specific hydroxide

$$k_{obsd} = k_{OH}[K_w/(K_a + a_H)] \quad (3)$$

catalytic constant. Solvent deuterium isotope effects or general acid–base catalysis were not observed over the pH range examined.

In Figure 2 is also shown the pH–log k_{obsd} profile for the hydrolysis of 3MeNPMU to 3MeHMU at 25 ± 0.1° which

may also be described by the kinetically equivalent eq 2 or 3. The profile for 3MeNPMU has the same general shape as 1MeNPMU except that k_{obsd} for 3MeNPMU is at least 10^3-fold greater at any given pH. Assuming spontaneous reaction of the monoanion (3MeNPMU$^-$) with water, calculations using eq 2 give k_r = 66.0 min^{-1} and pK_{app} = 9.30; the measured value for 3-methyl-5-methoxymethyluracil under comparable conditions (25°, μ = 1.0) is 9.67. For the kinetically equivalent hydroxide attack upon the neutral species (3MeNPMU), k_{OH} = 3.4 × 10^6 M^{-1} min^{-1} from eq 3.

The pH–log k_{obsd} profile for conversion of NPMU to HMU and *p*-nitrophenoxide (Figure 3) indicates the involvement of at least two acidic species with apparent pK_a values of 8.89 and 13.54. NPMU possess two ionizable protons which may give rise to four molecular species in solution (Scheme II).

$$k_{obsd} = k_{OH}[OH^-][NPMU] + k_1[NPMU\text{-}1^-] + k_1'[NPMU\text{-}3^-] + k_2[NPMU^{2-}] \quad (4)$$

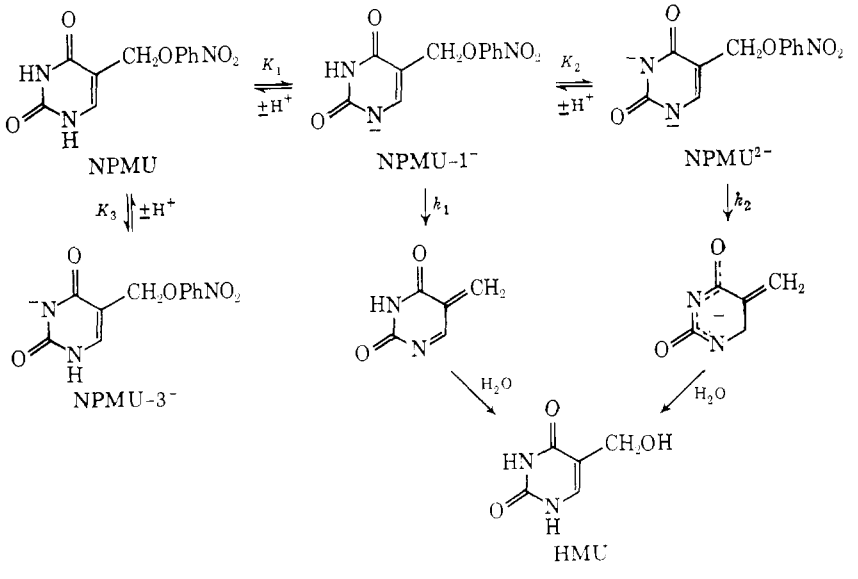
order rate constant associated with hydroxide attack on the uncharged NPMU, k_1 and k_1' are the specific rate constants for spontaneous hydrolysis of the 1-anion (NPMU-1$^-$) and 3-anion (NPMU-3$^-$), respectively, and k_2 is the rate constant for spontaneous reaction of the dianion (NPMU^{2-}) with water. The first three terms of eq 4 are kinetically equivalent and as will be shown later (see Discussion) the reaction of the hydroxide ion with NPMU and the spontaneous reaction of NPMU-3$^-$ are sufficiently slow as to be omitted from consideration. The remaining second and fourth terms of eq 4 may be expanded through material balance, a_H, and the dissociation constants of each acidic species to give eq 5. Fitting the

$$k_{obsd} = \frac{k_1K_1a_H + k_2K_1K_2}{K_1K_2 + (K_1K_3)a_H + a_H^2} \quad (5)$$

data in Figure 3 to eq 5 gives the values k_1 = 56.0 min^{-1}, k_2 = 9.50 × 10^3 min^{-1}, K_1 = 5.03 × 10^{-10} M for dissociation of the N-1 proton, K_2 = 2.24 × 10^{-14} M for the composite dissociation of the second proton of NPMU-1$^-$ and NPMU-3$^-$ to give the dianion NPMU^{2-}, and K_3 = 7.10 × 10^{-10} M for dissociation of the N-3 proton to give the unreactive NPMU-3$^-$. The discrepancy between the pK_{app} value of 8.89 (Figure 3) and the calculated value of 9.30 (eq 5) for ionization of the N-1 proton will be shown to result from a nonproductive preequilibrium dissociation of the N-3 proton in which nearly 50% of the monoanions in solution are present as the unreactive NPMU-3$^-$. The first kinetic dissociation constant observed in the profile is actually the sum of two dissociation constants, K_1 and K_3, in eq 5.

Hydrolysis of 1APr-3MeNPMU. The release of *p*-nitrophenoxide from 1APr-3MeNPMU shows a pK_{app} of 9.70 at 30° (Figure 3) and, as with Me$_2$NPMU at high pH, is accompanied by destruction of the pyrimidine ring as evidenced by the loss of absorbance at *ca.* 265 nm. Since the 5-hydroxymethylpyrimidine (1APr-3MeHMU) was stable between pH 6 and 13, it was concluded that it is not formed as an inter-

SCHEME II



The first pK_{app} agrees with the measured value of 9.18 for the model 5-methoxymethyluracil and the second ionization is in the region expected for formation of the dianion of pyrimidine-2,4-diones (Shugar and Fox, 1952). An empirical expression which describes the possible involvement of the various ionic species of NPMU is given in eq 4 where k_{OH} is the second-

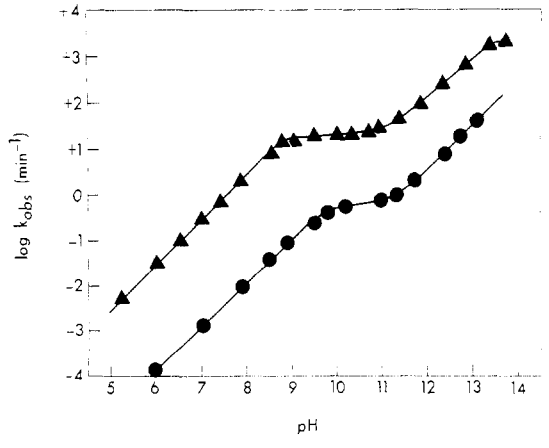


FIGURE 3: The pH–log k_{obsd} profile for the lyate-catalyzed hydrolysis of NPMU (▲) at 25° and 1APr-3MeNPMU (■) at 30°. Points are experimental and lines are theoretical (eq 5 and 6).

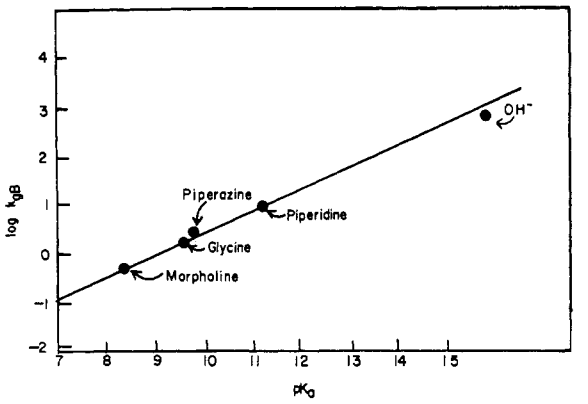


FIGURE 4: Brønsted plot for the second-order rate constants k_{gb} (eq 8) *vs.* the pK_a of the amine buffer in the hydrolysis of 1APr-3MeNPMU at 30 ± 0.1° and μ = 1.0.

mediate in the reaction. The profile in Figure 3 shows a slope of 1.0 from pH 6 to 9, a pK_{app} of 9.70, and a slope of 1.0 at higher pH values (11–13) and may be described by eq 6 where

$$k_{obsd} = k_N \frac{K_a}{K_a + a_H} + k_{OH}[OH^-] \frac{K_a}{K_a + a_H} \quad (6)$$

k_N (0.50 min^{-1}) is the catalytic rate constant for reaction of the free (unprotonated) amine, K_a (2.52 × 10^{-10} M^{-1}) is the dissociation constant of the amine, and k_{OH} (280 M^{-1} min^{-1}) is the specific base catalytic rate associated with the hydrolytic reaction at high pH. At pH 9.43 and 30°, k_{obsd} for hydrolysis of 1APr-3MeNPMU is 2.0 × 10^{-1} min^{-1}, compared to 1.60 × 10^{-5} min^{-1} for Me_2NPMU. From the rate enhancement of 1.25 × 10^4, and the dependence on an ionizable group having pK_{app} in agreement with the expected pK_a of the amino group, it is reasonable to conclude that the amino group directly participates in the reaction. The hydroxide dependence at high pH is more difficult to rationalize. Since the calculated value of k_{OH} at pH 12.0 for hydrolysis of 1APr-3MeNPMU is 10^3 times greater than that observed for Me_2NPMU, the hydrolysis of 1APr-3MeNPMU at higher pH must also be facilitated by the amino group. As will be shown below, the most reasonable interpretation is one in which *hydroxide ion acts as a general base* to facilitate intramolecular nucleophilic attack of the amine to the 6 position of the heterocycle.

Reaction of 1APr-3MeNPMU with Amines. Unlike other derivatives of NPMU, liberation of *p*-nitrophenoxide from 1APr-3MeNPMU is facilitated by general base catalysts. In the pH region of 9.3–10.8 the contribution of the hydroxide-catalyzed rate is negligible and the reaction is described by eq 7. Separation of the constants k_N and k_{gb} can be achieved by

TABLE I: Rate Constants (k_{gb}, k_N) for the Hydrolysis of 1APr-3MeNPMU in the Presence of Added Amine at 30° and μ = 1.0 (Eq 8).

Amine	pK_a	pH	k_{gb} (M^{-1} min^{-1})	k_N (min^{-1})
Morpholine	8.36	9.32	0.52	0.50
Glycine	9.60	9.35	1.67	0.50
Piperazine	9.82	9.80	2.58	0.34
Piperidine	11.22	10.84	9.10	0.79

TABLE II: Activation Parameters for NMPU and Its Methylated Derivatives.[a]

Compd	$\Delta H^{\pm}$ (kcal/mol)	$\Delta F^{\pm}$ (kcal/mol)	$\Delta S^{\pm}$ (eu)
Me_2NPMU	14.7	20.2	−18.4
1MeNPMU	22.0	15.7	+21.0
3MeNPMU	15.4	11.0	+14.8
NPMU	15.6	10.9	+15.8

[a] For compounds having ionizable protons, pK_a values were measured at each temperature using the corresponding 5-methoxymethyluracil derivatives as a model. Calculation of activation parameters took into account the variation of K_w with temperature.

$$k_{obsd} = (k_N + k_{gb}[B])[K_a/(K_a + a_H)] \quad (7)$$

rearranging eq 7 to 8. A secondary plot of $(k_{obsd}/[B])([K_a + a_H]/K_a)$ *vs.* 1/[B] gives k_{gb} as the intercept and k_N as the slope. A Brønsted plot of k_{gb} *vs.* pK_a of the catalyst (Figure 4) gave β = 0.45 and in Table I are recorded the values for k_N and k_{gb}. The satisfactory fit of the point for hydroxide on the Brønsted plot suggests that it behaves as a general base catalyst.

$$\left(\frac{k_{obsd}}{[B]}\right)\left(\frac{K_a + a_H}{K_a}\right) = \frac{k_N}{[B]} + k_{gb} \quad (8)$$

Activation Parameters and Secondary Deuterium Isotope Effects. In Table II are presented activation parameters obtained from plots of log k_{OH} *vs.* $1/T$ for the hydrolysis of Me_2NPMU, 1MeNPMU, 3MeNPMU, and NPMU. Secondary kinetic isotope effects (k_H/k_D) for hydrolysis of the 6-deuterio and 7-dideuterio derivatives of these compounds are recorded in Table III.

Discussion

General Approach. Assignment of mechanisms to the hydrolytic reactions of NPMU and its derivatives is complicated by the numerous kinetically equivalent pathways which may occur. For all compounds except Me_2NPMU, there is at least one ionizable proton and, thus, a multiplicity of mechanisms which cannot be distinguished by pH dependence of rates.

TABLE III: Secondary Deuterium Isotope Effects,[a] k_H/k_D.

Compd	6-*d*	pH	7-d_2	pH
Me_2NPMU[b]	0.89 ± 0.02	8.0–9.5	1.25 ± 0.04	5.0–6.5
	1.01 ± 0.02	4.5–7.1	1.00 ± 0.02	8.2–9.5
1MeNPMU	1.01 ± 0.01	9.4–12.1	1.43 ± 0.02	8.8–13.0
3MeNPMU			1.28 ± 0.01	6.0–7.6
NPMU	1.09 ± 0.02	6.0–7.6	1.27 ± 0.01	5.9–7.1

[a] Reactions were performed at 25 ± 0.05° and μ = 1.0. Reported values of k_H/k_D ± range were obtained from a minimum of 12 runs in which the protio and deuterio compounds were run simultaneously to reduce systematic errors. [b] Rate measurements were made at 90 ± 0.1°.

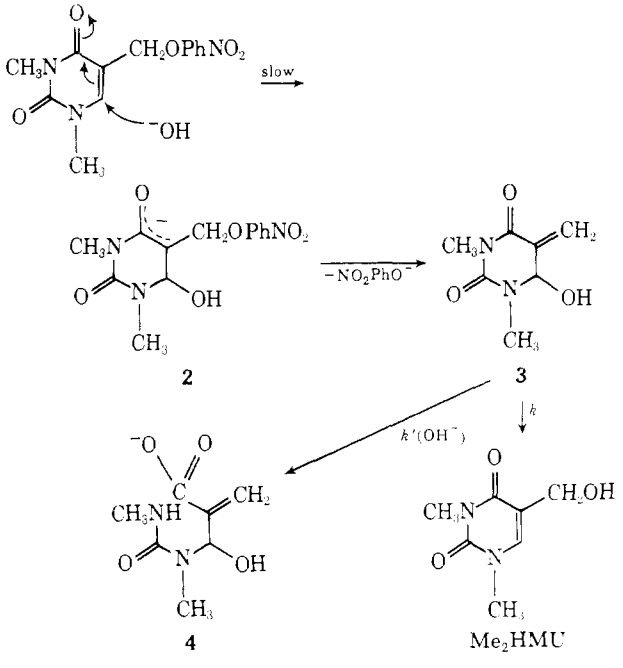
In addition, there are at least two sites of the reactant molecules, C-6 and C-7, which might be susceptible to nucleophilic attack at various stages of the reaction pathway.

To ascertain the reactive ionic species, we have made use of derivatives in which a methyl group has been substituted for the ionizable proton. Since the site of substitution is removed from that of the reactive site(s), it is reasonable to assume that the methylated derivative will resemble the neutral species in its hydrolytic behavior. This approach has been discussed by Jencks (1969) and has been successfully used to distinguish between kinetically equivalent mechanisms in the 5-H exchange of uracils (Santi *et al.*, 1970) and hydrolysis of 5-trifluoromethyluracils (Santi and Sakai, 1971). We also used α-deuterium isotope effects as probes for rehybridization of C-6 and C-7 of the reactants. In conjunction with pH–rate dependences, such studies permitted detailed descriptions of reaction pathways in which we could infer whether liberation of *p*-nitrophenoxide proceeded by SN1 or SN2 type displacements, and whether nucleophilic attack at C-6 was a necessary feature of the reaction. Secondary deuterium isotope effects have been the subject of reviews (Shiner, 1971; Halevi, 1963) and a cursory description of their qualitative use is sufficient for the purpose described here. Substitution of deuterium for hydrogen at a carbon atom undergoing nucleophilic substitution can result in a significant decrease in rate if the carbon atom undergoes sp^3 to sp^2 rehybridization at or before the rate limiting step of the reaction. Although the effect may be as large as 25%, a decrease in rate of 10% per α-deuterium atom may be taken as strong support for the SN1 type mechanism. If rehybridization does not occur, or occurs subsequent to the rate determining step, the effect will not be observed. Concerted displacements of the SN2 type also show α-deuterium isotope effects, but the magnitude of the effect is usually in the order of *ca.* 1%. Analogously, substitution of deuterium for hydrogen at an sp^2-hybridized carbon may result in up to 25% *increase* in rate (*viz.* inverse secondary deuterium isotope effect) if the site of substitution undergoes sp^2 to sp^3 rehybridization at or before the transition state of the rate-limiting step of the reaction.

To ascertain whether displacement of *p*-nitrophenoxide occurred by direct displacement or carbonium ion type mechanisms, the rates of hydrolysis of derivatives of NPMU-*7*-d_2 were compared to their protio analogs. α-Deuterium rate effects (k_H/k_D) of 1.25–1.45 (1.1–1.2 per α-deuterium atom) were taken as evidence for sp^2 rehybridization of the 5-methylene carbon in the transition state of the rate determining step. Since rehybridization would be concomitant with liberation of the leaving group, it is difficult to envision chemical mechanisms in which rehybridization at C-7 could occur in a preequilibrium step. Thus, absence of secondary deuterium isotope effects may be interpreted as evidence that rehybridization of C-7 does not occur (*viz.* displacement is of the SN2 type) or occurs subsequent to the rate determining step. An inverse isotope effect of 10% for derivatives of NPMU-*6*-*d* was interpreted as evidence for sp^2 to sp^3 rehybridization of C-6, resulting from hydroxide attack in the transition state of the rate determining step of the reaction, or at a preequilibrium step. Since the initial steps of all reactions in which isotope effects were examined are either rapid preequilibrium proton transfers from the acidic N-1 or N-3 positions of the heterocycle, or hydroxide attack at C-6, the absence of α-deuterium isotope effects with derivatives of NPMU-*6*-*d* suggests that rehybridization of C-6 does not occur in the hydrolytic pathway.

Hydrolytic Reactions of NPMU and Its Methylated Derivatives. The kinetic study of the hydrolysis of Me_2NPMU to Me_2HMU (Figure 2), a model for reactions of the uncharged heterocycle, indicates the conversion may proceed by specific acid, specific base, and spontaneous hydrolysis. The kinetics in the pH region 8.5–9.8 and the large negative entropy of activation (−18.4 eu) are in accord with (a) SN2 displacement by hydroxide at C-7 or (b) hydroxide attack at C-6 to assist in elimination of *p*-nitrophenoxide. The resistance of *p*-nitrophenyl ethers to hydrolysis in basic media (Tokuyama, 1956; Bunnett and Bernasconi, 1965) and the observation that Me_2NPMU-*6*-*d* exhibits an inverse isotope effect of k_H/k_D = 0.89 provides a strong argument for hydroxide attack at C-6 at or before the rate limiting step. The failure of Me_2NPMU-*7*-d_2 to show an α-deuterium isotope effect further indicates that rehybridization of the 7-CH_2 does not precede or occur in the rate determining step. Taken in concert, these data are in best accord with a mechanism involving rate determining attack of hydroxide ion at C-6 to form the enolate **2**, followed by rapid elimination of *p*-nitrophenoxide to give **3** (Scheme III). The driving force for formation of **2** would be provided

SCHEME III

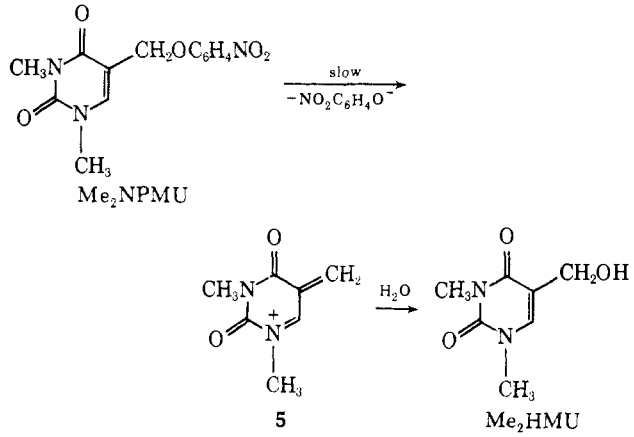


by delocalization of the negative charge and its resultant stabilization. In addition, **2** would be expected to undergo facile elimination of *p*-nitrophenoxide *via* a reverse Michael-type reaction to provide an intermediate **3** possessing a reactive exocyclic methylene group which would subsequently undergo reaction with water.

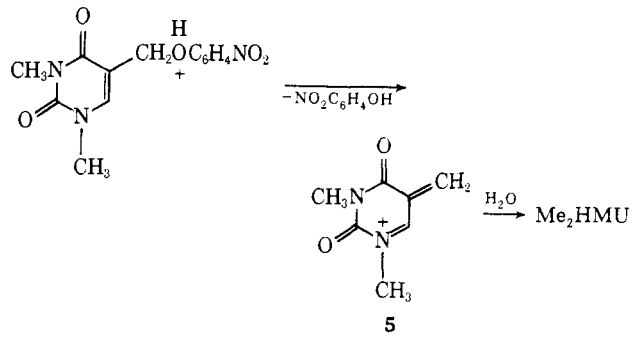
Above pH 11, the liberation of *p*-nitrophenoxide from Me_2-NPMU shares features with the reaction at pH 8.0–9.5 which suggests the occurrence of common pathways in hydrolytic mechanism throughout the entire basic region. Firstly, the liberation of *p*-nitrophenoxide is first order with respect to hydroxide ion and, within experimental error, shows identical second-order rate constants at 25° throughout the pH range of 8.0–13. Secondly, between pH 8.0 and 13, Me_2NPMU-*7*-d_2 shows no deuterium isotope effect whereas Me_2NPMU-*6*-*d* shows an inverse isotope effect of 0.89. However, at higher pH, the pyrimidine chromophore is destroyed in a reaction which is second order in hydroxide; Me_2HMU is not an inter-

mediate since it was shown to be completely stable under identical conditions. It is known that 5,6-dihydrouracils are susceptible to hydrolysis of the C-4 amide bond (Sander, 1969) and that ring opening reactions of most, if not all, uracil derivatives examined to date appear to occur only after initial addition across the 5,6-double bond (Fox *et al.*, 1966; Kondo *et al.*, 1971; Santi *et al.*, 1970). From these, we conclude that the initial stages of the reactions proceed similarly throughout the pH range of 8.0–13 (*viz.* hydroxide attack at C-6 followed by *p*-nitrophenoxide release; Scheme III), but cleavage of the C-4 amide of intermediate **3** by hydroxide proceeds more rapidly at high pH to give **4** than reaction with water to produce Me_2HMU. This proposal is in accord with the observation that treatment of Me_2NPMU with CH_3ONa results in quantitative formation of the 5-methoxymethyl derivative rather than ring cleavage (Santi and Pogolotti, 1971); it has been proposed that alkoxide results in reversible opening of the C-4 amide to form the corresponding ureido ester.

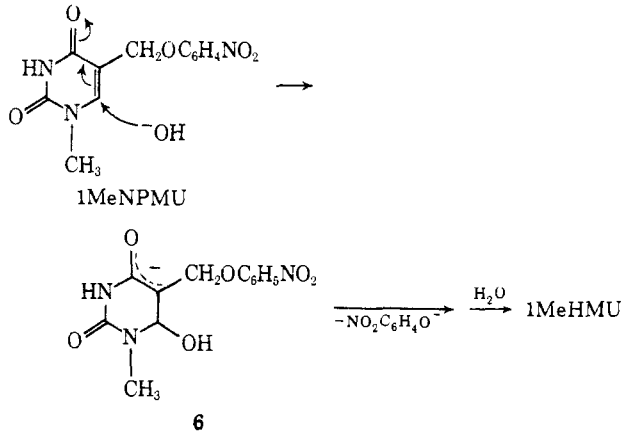
In the pH region 4.5–7.0, the spontaneous hydrolysis of Me_2NPMU-*6-d* exhibited no isotope effect (k_H/k_D = 1.01), while Me_2NPMU-*7-d*$_2$ gave a large α-deuterium isotope effect (k_H/k_D = 1.25), indicating the 5-methylene group to possess considerable sp^2 character in the transition state of the rate determining step. These results argue against the possibility of an SN2 type displacement and a preequilibrium hydration of the 5,6-double bond. The intermediate **5** is in accord with these considerations.



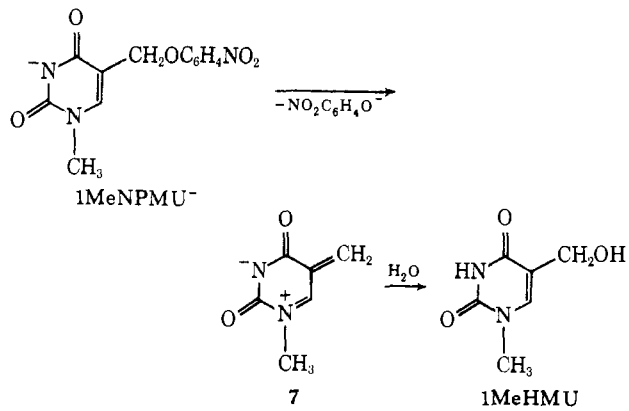
Although the acid-catalyzed hydrolysis of Me_2NPMU was not studied in great detail, a reasonable mechanism in accord with the kinetic data is one in which the ether oxygen is protonated prior to spontaneous release of the leaving group to give **5**.



The conversion of 1MeNPMU to 1MeHMU proceeds either by hydroxide attack on the neutral species or the kinetically equivalent spontaneous reaction of the 3-anion with water. Arguments in support of the latter mechanism may be formulated on the basis that reaction of hydroxide ion with 1MeNPMU to give the intermediate **6** would be expected to

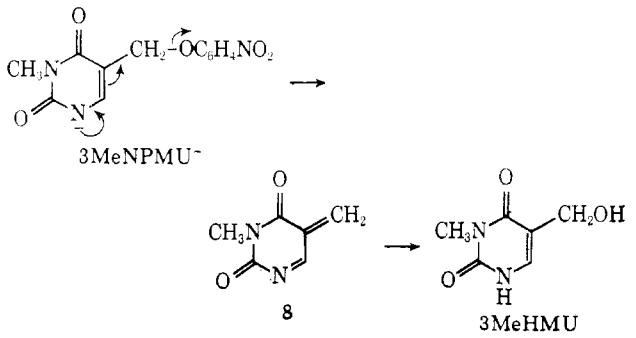


show similar bimolecular rate constants, secondary isotope effects, and activation parameters as obtained with Me_2-NPMU. Firstly, the calculated second-order rate constant for the reaction of 1MeNPMU with hydroxide ion at 25° is 3.7×10^3-fold greater than that obtained for hydroxide-catalyzed hydrolysis of Me_2NPMU at the same temperature. Secondly, the absence of an inverse isotope effect with 1MeNPMU-*6-d* contrasts with the rather large effect observed with Me_2NPMU-*6-d* over the same pH range and suggests that hydroxide attack at C-6 of 1MeNPMU does not occur. It is noted that, in contrast to the base-catalyzed hydrolysis of Me_2NPMU-*7-d*$_2$, a large secondary isotope effect is observed with 1MeNPMU-*7-d*$_2$ (k_H/k_D = 1.43) and indicates that the 5-methylene group possesses considerable sp^2 character in the transition state of the rate determining step. Thirdly, the hydrolysis of 1MeNPMU shows a large *positive* entropy of activation ($\Delta S^{\ddagger}$ = 21 eu), whereas Me_2NPMU shows a corresponding large, but *negative* $\Delta S^{\ddagger}$ (−18.4 eu); bimolecular attack of hydroxide ion with the neutral species of 1MeNPMU would be expected to show a negative $\Delta S^{\ddagger}$, and the large, positive value of $\Delta S^{\ddagger}$ obtained is in best accord with a unimolecular elimination. A mechanism in agreement with these data involves a slow unimolecular release of *p*-nitrophenoxide from the monoanion (1MeNPMU$^-$) to give the reactive intermediate **7**. The subsequent rapid reaction at



the exocyclic methylene group with water would give rise to the product 1MeHMU.

Using similar arguments, the hydrolytic data for 3MeNPMU may best be interpreted as proceeding *via* unimolecular elimination of *p*-nitrophenoxide from the anionic species 3MeNPMU$^-$ to give **8**. Calculation of the bimolecular



rate constant for hydroxide attack on the neutral species 3MeNPMU is some 10^7 times greater than Me_2NPMU. Direct evidence for **8** comes from the α-deuterium isotope effect (k_H/k_D = 1.28) observed in the hydrolysis of 3MeNPMU-*7-d*$_2$ and the large positive entropy of activation (+14.8 eu).

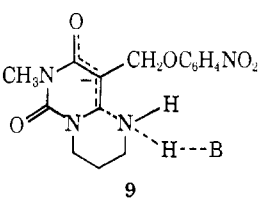
The kinetic data for the hydrolysis of NPMU are somewhat more difficult to interpret since four possible reactive species (Scheme II) involving kinetically indistinguishable pathways may be present; in addition, the microscopic equilibrium constants relating the concentration of each species cannot be determined by direct measurement. However, comparison of kinetic data, secondary deuterium isotope effects, and activation parameters with the previously described methylated derivatives permits conclusions as to which mechanisms are likely.

Since the concentration of the dianion, NPMU^{2-} (pK_{app} = 13.54), is negligible below pH 10, the pathways consistent with the kinetic data are (a) reaction of the neutral species with hydroxide or (b) reaction of either or both monoanions (NPMU-1$^-$, NPMU-3$^-$) with water. Again, the much higher rate of hydrolysis of NPMU compared to Me_2NPMU argues against reaction of the neutral species with hydroxide. Furthermore, an inverse isotope effect would be expected with NPMU-*6-d* if hydroxide reacted with C-6; in fact, NPMU-*6-d* showed k_H/k_D = 1.08. This effect is of the proper magnitude, but in the wrong direction and is in all likelihood the result of an inductive effect of deuterium which causes a slight increase in the pK_a of the 1-NH and consequent decrease in the amount of reactive 1-anion present in solution. In this regard, it is noted that the inductive effect of an α-deuterium atom should be sufficient to produce the decrease in acidity necessary to accommodate the interpretation (*e.g.*, K_{HCO_2H}/K_{DCO_2H} = 1.1; Bell and Miller, 1963). Support that the hydrolysis of NPMU below pH 10 proceeds *via* slow unimolecular solvolysis of the 1-anion (NPMU-1$^-$) is provided by comparison to 3MeNPMU, which has previously been demonstrated to proceed by such a mechanism. The calculated hydrolytic rates for NPMU-1$^-$ and 3MeNPMU$^-$ are almost identical, whereas NPMU-1$^-$ hydrolyzes some 10^3 times faster than 1MeNPMU$^-$. In addition, activation parameters for hydrolysis of NPMU and α-deuterium isotope effects of the 7-dideuterio compound are, within experimental error, identical with those obtained with 3MeNPMU. The large isotope effect observed with NPMU-*7-d*$_2$ again provides support that the slow step of the reaction involves sp^3 to sp^2 rehybridization of C-7 and concomitant release of *p*-nitrophenoxide. The line generated from eq 5 which best fits the hydrolytic data gives values of $K_1 = 5.02 \times 10^{-10}$ and $K_3 = 7.10 \times 10^{-10}$ for dissociation of the 1-NH and 3-NH of NPMU, respectively. Using the equation $K_{NPMU} = K_{NPMU\text{-}3^-} + K_{NPMU\text{-}1^-}$ (Tucker and Irvin, 1951), a pK_a of 9.25 is obtained for the first apparent ionization of NPMU which does not agree well with the kinetically obtained pK_{app} of 8.89. However, significant populations of both monoanions exist in solution, and this discrepancy may be explained to result from ionization of the 3-NH to give the unreactive NPMU-3$^-$ in a nonproductive equilibrium.

The hydrolysis of NPMU at higher pH ($a_H \ll K_1$) can be described by spontaneous elimination of *p*-nitrophenoxide from the dianion or the kinetically equivalent hydroxide reaction with either of the two monoanions (NPMU-3$^-$, NPMU-1$^-$). The latter mechanism can be eliminated since neither 1MeNPMU$^-$ nor 3MeNPMU$^-$ exhibited a rate term associated with hydroxide. The most probable pathway for the hydrolysis of NPMU at high pH is *via* elimination of the dianion NPMU^{2-} (Scheme II). As in the case of 5-H exchange of uracil (Santi *et al.*, 1970) and the hydrolysis of trifluoromethyluracil (Santi and Sakai, 1971), the greater reactivity of NPMU^{2-} ($k_2 = 9.50 \times 10^{-3}$ min^{-1}) as compared to NPMU-1$^-$ (k_1 = 56.0 min^{-1}) may be rationalized in terms of the higher electron density at the 1 position available for assistance.

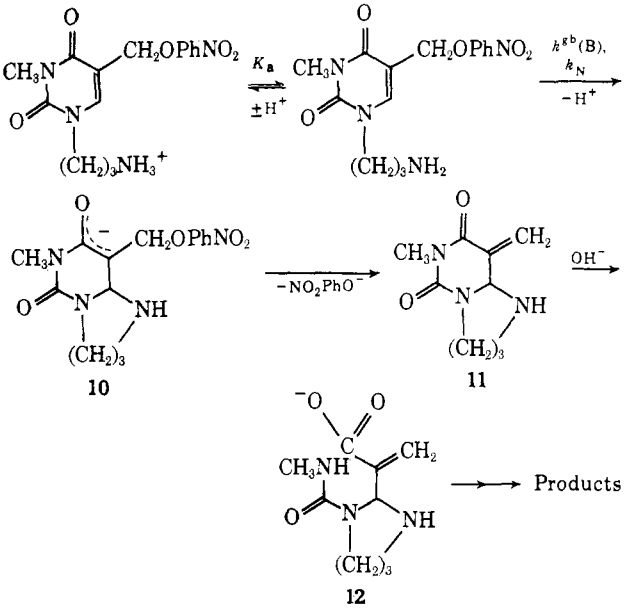
Hydrolysis of 1APr-3MeNPMU. In previous studies (Santi and Brewer, 1968, 1973) it has been demonstrated that 5-H exchange and 5-hydroxymethylation of 1-substituted uracils which possess a hydroxyl group on the 1 substituent are greatly facilitated by intramolecular nucleophilic attack of the oxyanion at the 6 position of the heterocycle. Analogous intramolecular reactions have been observed with 1-(ω-aminoalkyl)-5-trifluoromethyluracils, in which the participating amino nucleophile results in increases of up to 10,000-fold in observed rates of hydrolysis of the 5-trifluoromethyl group (Santi and Sakai, 1971).

The release of *p*-nitrophenoxide from 1APr-3MeNPMU is some 13,000 times faster than that of Me_2NPMU at pH values below 9.5 and provides strong evidence for participation of the amino group. In contrast with the hydrolysis of all other compounds studied, the hydrolytic reaction was susceptible to general base catalysis. The Brønsted β value of 0.45 (Figure 4) indicates significant bond formation in the transition state (**9**) of the rate determining step, and development



of positive charge on the general base catalyst. A mechanism consistent with these data is depicted in Scheme IV, where

SCHEME IV

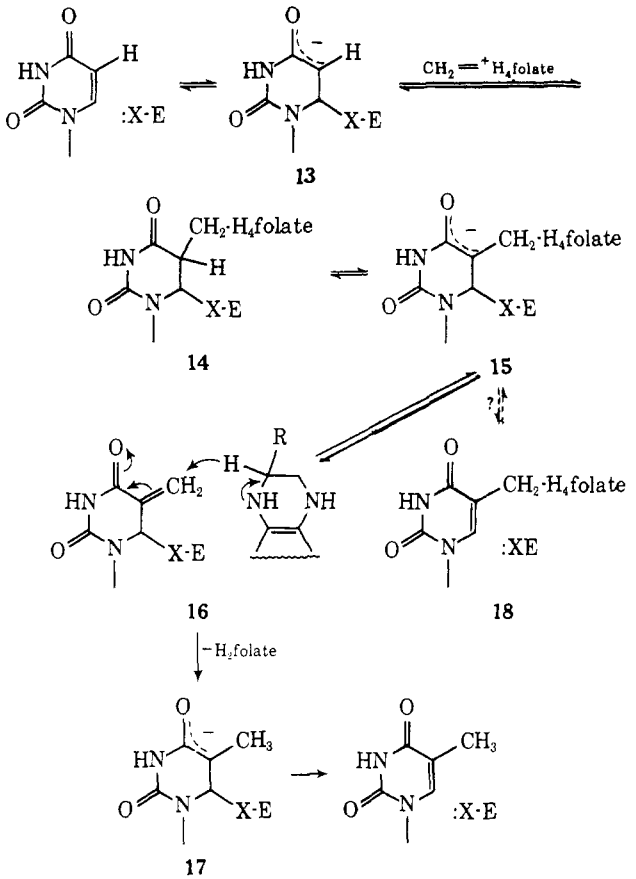


the slow step of the reaction is the intramolecular attack of the 3-amino group at C-6 of the heterocycle. At pH values above the pK_α of the amino group, hydroxide plays a role in the reaction as evidenced by the slope of 1.0 in the pH–rate profile. Since there are no acidic protons on the reactant, our first impulse was to suggest that hydroxide ion participates as a nucleophilic catalyst in the same manner ascribed to Me_2NPMU. However, since the calculated bimolecular rate constant for hydroxide ion reaction with 1APr-3MeNPMU gives a value 10^3-fold greater than that observed with Me_2-NPMU, it is apparent that the reaction at higher pH involves facilitation by *both* hydroxide ion and the neutral species of the 3-amino group. The most reasonable chemical mechanism we can envision for this is one in which hydroxide ion acts as a general base to facilitate proton removal from the amino group in the transition state of the intramolecular reaction. The fact that the intramolecular reaction is facilitated by general bases, and that hydroxide ion satisfactorily fits the Brønsted plot provides good support for this mechanism.

From the same arguments as used for Me_2NPMU, rate limiting attack of the 3-amino group at C-6 should result in a stepwise, rather than concerted, elimination of *p*-nitrophenoxide in which the enolate intermediate **10** would release the leaving group to form the exocyclic methylene intermediate **11**. It is noted that, as with Me_2NPMU at high pH, the liberation of *p*-nitrophenoxide from 1APr-3MeNPMU is accompanied by destruction of the pyrimidine chromophore in a reaction which is second order in hydroxide ion. The observation that 1APr-3MeHMU was stable under comparable conditions demonstrates that, as with Me_2HMU at high pH, the 5-hydroxymethylpyrimidine is not formed in the sequence leading to products. From these analogies with reactions of Me_2NPMU, it is suggested that the intermediate **11** undergoes hydroxide ion catalyzed cleavage at C-4 to give **12** more rapidly than attack at the 5-exocyclic methyl group. A similar ring cleavage has been observed with 1-(3-aminopropyl)uracil (D. V. Santi and C. F. Brewer, unpublished results).

Relationship of Model Studies to the Thymidylate Synthetase Reaction. The mechanism for the thymidylate synthetase reaction which has been favored by most investigators involves formation and concerted disproportionation of a 5-thymidylyl-H_4folate intermediate, as depicted in Figure 1. Although this suggestion encompasses all biochemical data available, the lack of chemical precedent for the two reactions shown led us to believe that essential catalytic features of the reaction were yet unrecognized. From model studies on 5-H exchange and 5-hydroxymethylation (Santi and Brewer, 1968, 1973; Santi *et al.*, 1970), we proposed that the reaction was initiated by attack of a nucleophilic group of the enzyme to the 6 position of dUMP. In this manner, the 5 position of dUMP could be made sufficiently nucleophilic (*viz.* **13**, Scheme V)

SCHEME V[a]



[a] Suggested sequence for the thymidylate synthetase reaction based on model studies. Intermediate **18** is that proposed by Friedkin and Kornberg (1957) which, according to the scheme shown, is not an obligatory intermediate. All pyrimidine structures have a 1-(5-phospho-2′-deoxyribosyl) substituent and R = CH_2-NHC_6H_4COGlu. The pathway shown depicts reactions within the central complex and does not attempt to distinguish the sequence of substrate or product interactions with the enzyme.

to react with CH_2-H_4folate or an equivalent reactive species of formaldehyde. The recent demonstration that 5-fluoro-2′-deoxyuridylate reacts at the 6 position with a nucleophilic group of the enzyme in a similar fashion (Santi and McHenry, 1972; Santi *et al.*, 1974) provided strong support for this proposal. Thus, the initial condensation product between dUMP and CH_2-H_4folate is now generally accepted (see Benkovic and Bullard, 1973; Friedkin, 1973) to be one which is covalently bound to the enzyme and saturated across the 5,6-double bond of dUMP (**14**). The formation of thymidylyl-H_4folate (**18**) would require proton abstraction from **14** to give **15** followed by β elimination of the nucleophilic catalyst.

The question originally posed in this work was why the methylene group of an intermediate such as **18**, if in fact formed, would be susceptible to intramolecular attack by a hydride of C-6 of H_4folate as depicted in Figure 1. The salient features of the studies described here which are relevant to this question are: (a) nucleophilic displacement reactions of models of thymidylyl-H_4folate proceed exclusively *via* SN1 type reactions involving intermediates in which the methylene group is sp^2 hybridized; in no situation is there any indication of direct (SN2) displacement reactions; (b) the driving force for formation of such intermediates may be provided by nucleophilic attack at the 6 position to form a reactive enolate anion analogous to **15**. Based on these results, we can propose a chemically reasonable mechanism for the enzymic disproportionation of thymidylyl-H_4folate (**18**) (Scheme V).[2] The reaction would proceed by addition of a nucleophile to the 6 position of the uracil heterocycle of **18** to form the enolate intermediate **15**. As with the chemical models, **15** should readily undergo a β elimination to produce the highly reactive exocyclic methylene intermediate **16** and H_4folate, bound to the enzyme in close proximity. Intermolecular hydride transfer from H_4folate to **16** would yield dTMP, H_2folate, and the native enzyme. Now, since according to this formulation the enolate intermediate **15** is common to both the formation of a thymidylyl-H_4folate intermediate and in its conversion to reaction products, it follows that, by definition, thymidylyl-H_4folate (**18**) is *not* an obligatory intermediate of the reaction. In fact, since the very existence of 5-thymidylyl-H_4folate is speculatory, we would argue that it is not formed in the thymidylate synthetase reaction.

With minor modification, the mechanism shown in Scheme V would also account for the reactions catalyzed by the dUMP and dCMP hydroxymethylases. In these reactions a hydroxymethyl group is transferred to the **5** position of the heterocycle, but the redox step does not occur. Using similar arguments as described above, we would suggest that these reactions proceed to intermediate **16** in an analogous manner as shown in Scheme V. At this stage, reaction of the heterocycle with water rather than hydride from H_4folate would yield the 5-hydroxymethyl nucleotides and H_4folate.

[2] A similar route for conversion of **18** to dTMP and FAH_2 has been recently considered by Wilson and Mertes (1973) and Benkovic and Bullard (1973).

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