assay is usable for low concentrations of receptor in solutions too dilute to be accurately quantitated by the charcoal method.

Preliminary experiments demonstrate that this assay may also be used for estrogen and aldosterone receptors. Although optimal conditions have not been established for these, it is clear that, at most, only minor modification of the conditions used here will be necessary. Further, utilization of a calibration curve as given in Figure 5, and fluids containing appropriate receptors, would permit a rapid isotope-dilution assay for quantitation of steroids in biological fluids.

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Model Studies of Thymidylate Synthetase. Intramolecular Catalysis of 5-Hydrogen Exchange and 5-Hydroxymethylation of 1-Substituted Uracils†

Daniel V. Santi* and Curtis F. Brewer‡

ABSTRACT: The mechanisms of base-catalyzed 5-hydrogen exchange and 5-hydroxymethylation of 1-substituted uracils have been examined to provide insight into the mechanism of the analogous reactions catalyzed by thymidylate synthetase. Kinetic studies of 5-H exchange demonstrate that the reaction requires attack of a nucleophile at the 6 position of the heterocycle to form carbanionic intermediates, as in classical addition reactions of α,β-unsaturated carbonyl compounds. The intermediate then accepts a proton from water to form the corresponding carbon acid; reversal of these steps results in 5-H exchange. Proton transfers at the 5 position of the saturated pyrimidine intermediates have been found to be susceptible to general acid–base catalysis and may be rate determining. The exchange reaction and 5-hydroxymethylation are greatly facilitated by intramolecular catalysis involving addition of a nucleophile attached to the 1 substituent to the 6 position of the heterocycle; in fact, the rate of intramolecular catalyzed exchange of one of the reactive ionic species of 2′,3′-*O*-isopropylideneuridine is comparable to that of the enzyme-catalyzed reaction. Depending upon the efficacy of the intramolecular catalyst the rate-determining step of the exchange may be either nucleophilic attack at the 6 position of the heterocycle or proton transfer reactions at the 5 position. From these results, a minimal mechanism for the thymidylate synthetase catalyzed 5-H exchange of 2′-deoxyuridylate and its condensation with 5,10-methylenetetrahydrofolic acid is proposed.

Thymidylate synthetase catalyzes the reductive methylation of dUMP[1] to TMP with the concomitant conversion of 5,10-CH_2H_4folate to 7,8-H_2folate (for a recent review, see Blakley, 1969). One of our interests has been the elucidation of the mechanism of catalysis of thymidylate synthetase and, as a prelude to enzymological studies, we sought to develop congruent model systems which would provide a basic understanding of underlying mechanistic features of this reaction (Scheme I).

The complexity of the reaction *in toto* requires that certain simplifying assumptions be made before approaching detailed model studies. Segregation of the overall reaction into two discrete steps permits it to be defined in terms of well-known chemical reaction types: (1) electrophilic substitution

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[1] Abbreviations used are: dUMP, 2′-deoxyuridylic acid; TMP, thymidylic acid; 5,10-CH_2H_4folate, 5,10-methylenetetrahydrofolic acid; 7,8-H_2folate, 7,8-dihydrofolic acid; H_4folate, tetrahydrofolic acid; $(EtOH)_3N$, triethanolamine; Et_3N, triethylamine.

of the 5 position of dUMP by 5,10-CH_2H_4folate, or an equivalent form of formaldehyde, and (2) oxidation–reduction *via* nucleophilic (hydride) attack by the 6-H of H_4folate (Pastore and Friedkin, 1962) at the incipient methyl group of TMP. It is to be noted that the above encompasses the suggestion that a 5-thymidylyl-H_4folate intermediate is formed which undergoes disproportionation *via* a 1,3-hydride shift (Friedkin and Kornberg, 1957).

Lomax and Greenberg (1967) have made the significant observation that thymidylate synthetase also catalyzes exchange of the hydrogen at the 5 position of dUMP for protons of solvent. This reaction represents an electrophilic substitution and, in all likelihood, is more than casually related to the first step of the overall reaction catalyzed by thymidylate synthetase. It is of interest to note that two related enzymes, the dCMP and dUMP hydroxymethylases (Flaks and Cohen, 1959; Yeh and Greenberg, 1967; Roscoe and Tucker, 1964; Dunlap *et al.*, 1971b), also catalyze conversions we associate with the first step of the thymidylate synthetase reaction.

In this paper we describe the kinetics and mechanism of intramolecular catalyzed 5-H exchange and 5-hydroxymethylation of uracil nucleosides and related derivatives. These studies demonstrate common mechanistic features for these reactions and provide the foundation for our proposal of the involvement of nucleophilic catalysis for the thymidylate synthetase and related enzymic reactions.

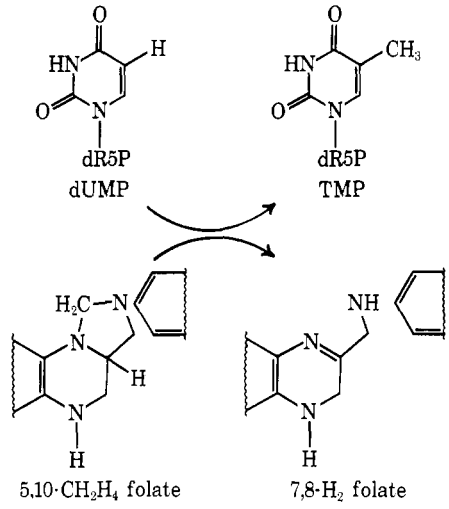
Materials and Methods

General. Proton magnetic resonance (pmr) spectra were recorded with Varian HA-100 or Jeolco C-60H spectrometers and used sodium 3-(trimethylsilyl)-1-propanesulfonate as an internal reference. Ultraviolet spectra were obtained with a Cary 15 spectrophotometer. Spectrophotometric titrations were performed on the apparatus described by Bruice and Maley (1970). pH measurements were determined with a Radiometer Model 26 pH meter using a Metrohm EA-125U combination glass electrode. Hydrolytic rate constants were determined using a Radiometer pH-Stat Model 11 titrator. A Packard Model 3375 Tri-Carb scintillation spectrometer was used for radioisotope measurements. Thin-layer chromatography (tlc) was run on Brinkman silica gel GF_{254} and paper chromatography (ascending) on Whatman No. 1- or 3MM paper. Spots were detected under ultraviolet light (254 nm) or with a Packard Model 7201 chromatogram scanner. Products were quantitatively eluted from paper chromatograms by the centrifugation technique of Edstrom (1968). Corning alkali-resistant glass tubing (No. 7290) was used for kinetic runs above pH 10.

Water used for kinetic studies was deionized and distilled in an all-glass apparatus. Deuterium oxide (99.7%) and methanol-*1-d* (99.5%) were obtained from Stohler Isotope Chemicals. Et_3N (bp 89.5°) and $(EtOH)_3N$ (mp 177°, EtOH) were purified prior to use. Other reagents were the highest purity available and used without additional purification. The liquid scintillation solvent consisted of 0.1% of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 4% of 2,5-diphenyloxazole in toluene; nucleosides to be counted were added as a solution in methanol.

Urd, d^2Urd and IpUrd were obtained from Sigma Chemical Co. and used without further purification. The following compounds were prepared by literature procedures and were pure by criteria of constant melting point after repeated recrystallization and thin-layer chromatography (tlc): 1MeUra (Sakai *et al.*, 1968), d^5Urd, and d^5IpUrd (Fox *et al.*, 1960),

SCHEME I



Ara-Ura (Hampton and Nichol, 1966), d^5Ara-Ura (Falco and Fox, 1968), 5-Trt-d^2Lyx-Ura (Horwitz *et al.*, 1964), 2′,3′-Bzl_2Urd (Michelson and Todd, 1956), 1(HOEt)Ura, 1(HOPr)-Ura, 1(HOBu)Ura, and 1(HOPe)Ura (Baker and Schwan, 1966).

[2-^{14}C]Urd (55.2 Ci/mol) and [5-^{3}H]Urd (2.47 Ci/mol) were obtained from New England Nuclear.

[2-^{14}C]- and [5-^{3}H]IpUrd were prepared by modification of the procedure of Hampton (1961). To *ca.* 0.1 mg (or less) of labeled Urd was added 100 μl of a solution of 16.7% 2,2-dimethoxypropane and 0.66% *p*-toluenesulfonic acid in dry acetone. After 28 hr at ambient temperature the reaction was quenched with 100 μl of 1% ammonium hydroxide in methanol and streaked on a strip of Whatman No. 3MM chromatography paper. After development with 1-butanol–water (84:16), the radioactive band corresponding to IpUrd (R_F 0.71) was excised and eluted with 3.5 ml of water. Yields were 96–99% based on radioactivity.

Exchange of 5-H for Deuterium. Prior to each run, reactants were lyophilized three times from D_2O to exchange acidic protons. To a 6.0-ml calibrated alkali-resistant ampoule, continuously flushed with dry N_2, was added 3 ml of CH_3OD and appropriate amounts of $NaOCH_3$ and the pyrimidine. The volume was adjusted to 6.0 ml with CH_3OD to give solutions which were 0.2 M in reactant and 0.47 N in base; the containers were sealed with rubber septa and kept at 60 ± 0.1°. At appropriate intervals (8–12 points/run) 0.6-ml aliquots were removed by syringe, immediately cooled at 0°, and quenched with 25 μl of glacial acetic acid. The CH_3OD was evaporated under a N_2 stream and the residue was dissolved in 0.1 ml of H_2O or H_2O–dimethyl sulfoxide and its paramagnetic resonance (pmr) spectrum was recorded. The extent of 5-H exchange was determined from the relative intensities of the singlet and doublet signals of the 6-H as previously described (Santi *et al.*, 1970). Pseudo-first-order rate constants were obtained by plots of log $(h_H/h_H + h_D)$ *vs.* time, where h_D is the peak height of the C-6 singlet of the pyrimidine-*5-d* present in the mixture, and h_H the combined peak heights of the C-6 doublet of the unlabeled pyrimidine. All such plots were linear for 1 to 2 half-lives; for long runs the reactions were monitored for approximately three-quarters of a half-life. The $NaOCH_3$ titer did not change more than 2% during the course of these reactions, and no degradation products were detected by ultraviolet (uv) or chromatographic analyses. Repeated determinations of k_{obsd} agreed within ±5%.

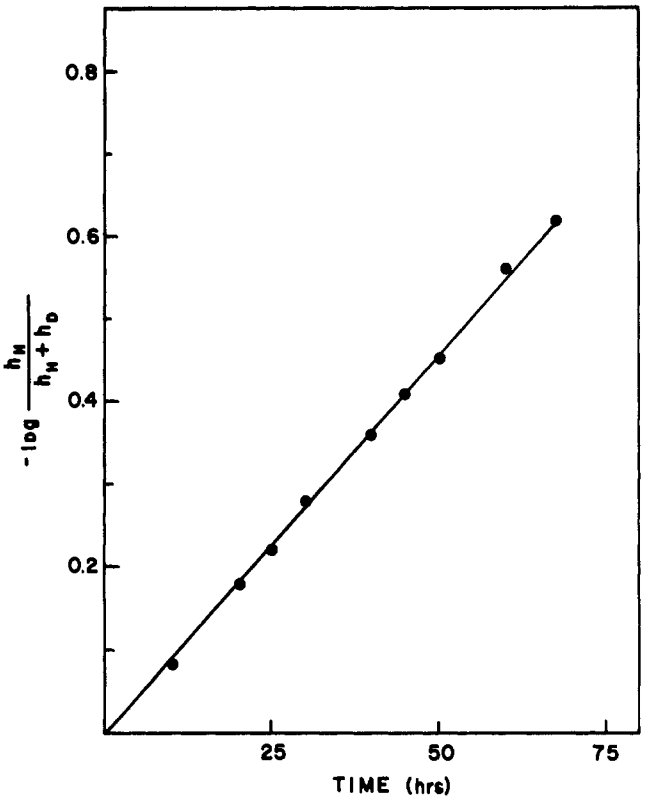


FIGURE 1: Rate of 5-H exchange of 0.2 M 1(HOPr)Ura in 0.47 N $NaOCH_3$–CH_3OD at 60°.

Hydroxymethylation of 1-Substituted Uracils. Reactions were conducted at 60 ± 0.1° in tightly sealed polyethylene containers. Solutions were 0.2 M in pyrimidine, 0.5 N in NaOH, and 3.4 M in formaldehyde. At appropriate intervals, 50-μl aliquots were removed and quenched with 10 μl of acetic acid. For runs with Urd and d^2Urd, the quenched aliquots were applied to Whatman No. 3MM paper strips, and developed [*tert*-butyl alcohol–methyl ethyl ketone–water–formic acid (44:44:11:0.26) for Urd and ethyl acetate–formic acid–water (70:20:10) for d^2Urd]. Zones corresponding to starting materials and products were excised, quantitatively eluted, and quantitated by uv spectra. Separations of 5-hydroxymethylated products from IpUrd, 1(HOPr)Ura, and 1MeUra were performed on tlc using chloroform–ethanol as eluent in the proportions 9:1, 3:1, and 3:1, respectively. Pyrimidines were eluted with 0.01 N NaOH, and the silica gel was removed by centrifugation with the aid of Celite and quantitated by their uv spectra. Corrections for trace uv-absorbing impurities were made by identical treatment of an equivalent amount of silica gel.

Detritiation of [5-^{3}H]Urd and [5-^{3}H]IpUrd. The rates of detritiation were determined in aqueous buffers at 30 ± 0.1° with μ = 1.0 (KCl). Buffers employed were $(EtOH)_3N$-HCl (pH 7.1–8.42), K_2CO_3 (pH 9.42–10.52), and Et_3N-HCl (pH 10.44–11.45). The appropriate buffer solution containing the ^{14}C- and ^{3}H-labeled compounds (^{3}H dpm/^{14}C dpm ≃ 15) was dispensed (Hamilton syringe) in 25-μl aliquots into glass capillary ampoules (alkali-resistant for pH >10) which were fire-sealed and placed in a 30 ± 0.1° bath. At appropriate intervals, the capillaries were broken, and the contents streaked across a Whatman No. 3MM paper strip to which a small amount of unlabeled material had previously been applied across the origin. After development [1-butanol–water (84:16) for IpUrd and 1-butanol–acetic acid–water (2:1:1) for Urd)], the uv-absorbing radioactive band was excised, eluted with 1 ml of methanol, and counted in a liquid scintillation counter. Pseudo-first-order rate constants were calculated from plots of log (^{3}H dpm/^{14}C dpm) *vs.* time. Where buffers were employed, values of k_0 for lyate catalysis were obtained from intercepts of plots of k_{obsd} against buffer concentration; lines from such plots were linear. Duplicate determinations of k_{obsd} generally agreed within ±4%.

TABLE 1: 5-H Exchange Rates for 1-Substituted Uracils at 60° in 0.47 N CH_3ONa–CH_3OD.

Compound	Abbreviation	$k \times 10^5$ (hr^{-1})	k_{rel}
2′,3′-*O*-Isopropylidene-uridine	IpUrd	243	522
5′-Deoxy-2′,3′-*O*-isopropylideneuridine	d^5IpUrd	*a*	*a*
Uridine	Urd	3.62	7.77
2′-Deoxyuridine	d^2Urd	4.03	8.65
5′-Deoxyuridine	d^5Urd	*a*	*a*
2′,3′-Dibenzyluridine	2′,3′-Bzl_2Urd	2.76	5.92
Arabinosyluracil	Ara-Ura	290	623
5′-Deoxyarabinosyluracil	d^5Ara-Ura	547	1174
5′-Trityl-2′-deoxylyxo-furanosyluracil	5Trt-d^2Lyx-Ura	*a*	*a*
1-(2-Hydroxyethyl)uracil	1(HOEt)Ura	0.846	1.82
1-(3-Hydroxypropyl)uracil	1(HOPr)Ura	21.9	47
1-(4-Hydroxybutyl)uracil	1(HOBu)Ura	0.456	0.98
1-(5-Hydroxypentyl)uracil	1(HOPe)Ura	*a*	*a*
1-(3-Phenoxypropyl)uracil	1(PhOPr)Ura	*a*	*a*
1-Methyluracil	1MeUra	0.466	1.0

[a] No exchange observed after as long as 2 weeks.

Results

Base-Catalyzed Labilization of the 5-Hydrogen of 1-Substituted Uracils. Exchange of the 5-H of uracil derivatives for deuterium results in the appearance of a signal in the pmr spectrum midway between the 6-proton doublet, and may be monitored by a peak-height ratio method previously described (Santi *et al.*, 1970). Table I gives the pseudo-first-order rate constants and rates relative to 1MeUra for 5-H exchange of 0.20 M solutions of certain uracil 1-β-D-furanosides and 1-substituted uracils in 0.47 N $NaOCH_3$–CH_3OD at 60°. A typical first-order plot is shown for 1(HOPr)Ura in Figure 1. In the absence of base no exchange is detectable by pmr after as long as 3 weeks. With compounds that underwent very slow base-catalyzed 5-H exchange, proton exchange was also observed at the 6 position of the heterocycle, as indicated by the appearance of a singlet midway between the remaining 5-proton doublet in the pmr spectra. Assuming that the rate of this reaction is independent of the nature of the hydrogen isotope at the 5 position, its occurrence does not affect our rate measurements since the analytical method used depends only on the relative amounts of hydrogen isotopes at the 5 position of the 6-H pyrimidine.

For precise kinetic analysis of 5-H exchange over a wide pH range, and at temperatures where ionization constants could be conveniently determined, a more sensitive analytical method was required. Using [5-^{3}H]IpUrd, low levels of ex-

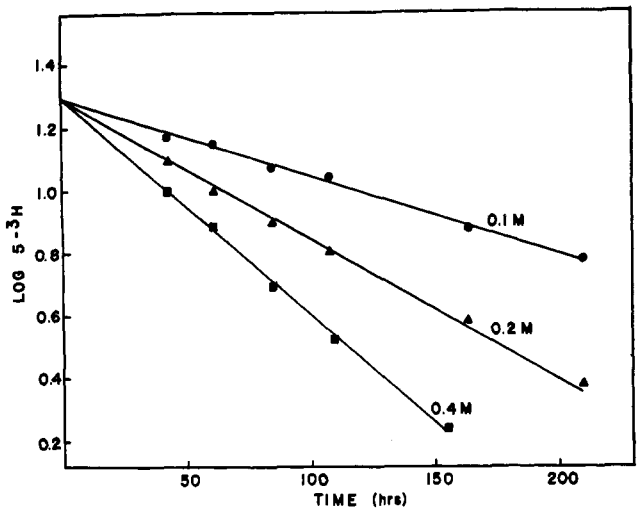


FIGURE 2: Pseudo-first-order plots for tritium release from [5-³H]-IpUrd at 30° and pH 10.72: (●) 0.1 M Et_3N, (▲) 0.2 M Et_3N, (■) 0.4 M Et_3N.

change could be accurately measured at concentrations of the nucleoside which were sufficiently low to negate complications arising from the reactant acting as a general catalyst. Furthermore, by incorporating ^{14}C into the 2 position of the heterocycle, the fraction of 5-³H remaining after a given period of time t can be simply expressed as (^3H dpm/^{14}C dmp)$_t$ × (^{14}C dpm/^3H dpm)$_0$. The method provides a high degree of sensitivity and does not require quantitative recovery of the nucleoside. Typical plots of log (5-³H) *vs.* time obtained by this method are given in Figure 2.

The pH–log rate k_0 profile for lyate-catalyzed tritium release from [5-³H]IpUrd at 30° and μ = 1.0 is given in Figure 3. Throughout the pH range examined, the data may be empirically described by eq 1 where the reactive ionic species are the mono- and dianions (Figure 4) or their kinetic equivalents.

$$\frac{dP}{dt} = k_1(HA^-) + k_2(A^{2-}) \quad (1)$$

Using material balance, a_H, and the acid dissociation constants for acidic species, eq 1 may be expanded to

$$k_0 = \frac{k_1K_{a2}a_H + k_2K_{a1}K_{a3}}{a_H^2 + a_H(K_{a1} + K_{a2}) + K_{a1}K_{a3}} \quad (2)$$

Fitting this expression to the data in Figure 3 (solid line) gives the values: $k_1 = 5.0 \times 10^2$ hr^{-1}, $k_2 = 7.5 \times 10^{-3}$ hr^{-1}, pK_{a1} = 8.7 and pK_{a2} = 13.9. The apparent pK_a values from the experimental data agree with the measured values for the 3-NH (pK_a = 9.0) and the 5′-hydroxyl (pK_a = 13.9) of IpUrd at μ = 1.0. In calculating these values, the assumption is made that the state of ionization of the 3-NH has negligible effect on the pK_a of the 5′-hydroxyl and *vice versa* (*i.e.*, $K_{a1} = K_{a4}$ and $K_{a2} = K_{a3}$).

The exchange reaction was shown to be subject to buffer catalysis in the pH region 7.1–11.5. Typical plots of k_{obsd} *vs.* buffer concentration are given in Figure 5, where the intercept provides the lyate catalyzed rate constant k_0 used in the pH–log k_0 profile and the slope gives the apparent rate constant for the buffer catalyzed reaction, k_{app}, at that pH; all such plots were linear. Plots of k_{app} for three buffers utilized

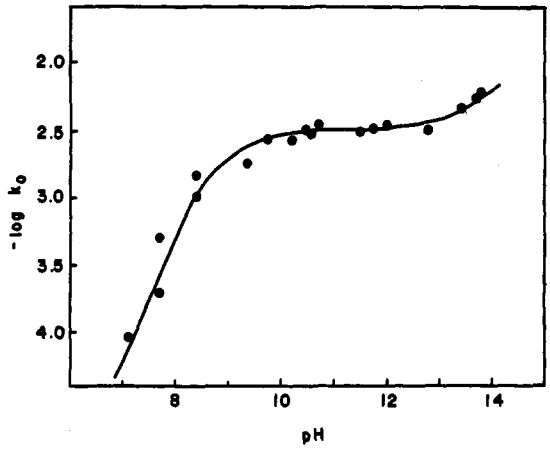


FIGURE 3: The pH–log k_0 profile for exchange of [5-³H]IpUrd at 30°. Points are experimental and the curve is calculated from eq 2.

in the exchange reaction *vs.* the mole fraction of free base are given in Figure 6. The free base of $(EtOH)_3N$ is the only general catalyst, with $k_{(EtOH)_3N} = 13.5 \times 10^{-3}$ M^{-1} hr^{-1} (Figure 6A). Analogous plots (Figure 6B) for carbonate (pH 9.4–10.5) and Et_3N (pH 10.5–11.5) buffers indicate apparent general acid catalysis with values of $k_{HCO_3^-} = 1.6 \times 10^{-2}$ M^{-1} hr^{-1} and $k_{Et_3N\cdot H^+} = 6.6 \times 10^{-3}$ hr^{-1}, respectively.

Release of tritium from [5-³H]Urd was not observed at 30° in carbonate buffer (pH 9.74, μ = 1.0) after as long as 30 days, demonstrating that bimolecular reaction with hydroxide ion is negligible under these conditions.

Approximation of the Acid Dissociation Constant of the 5′-Hydroxyl of Nucleosides. Bruice *et al.* (1962) have shown that the pK_a values for a large number of alcohols are linear functions of the logarithm of the second-order rate constants for alkaline hydrolysis (k_{OH}) of their corresponding acetyl esters. The relationship is based on comparison of the partitioning of tetrahedral intermediates, relating the leaving abilities of

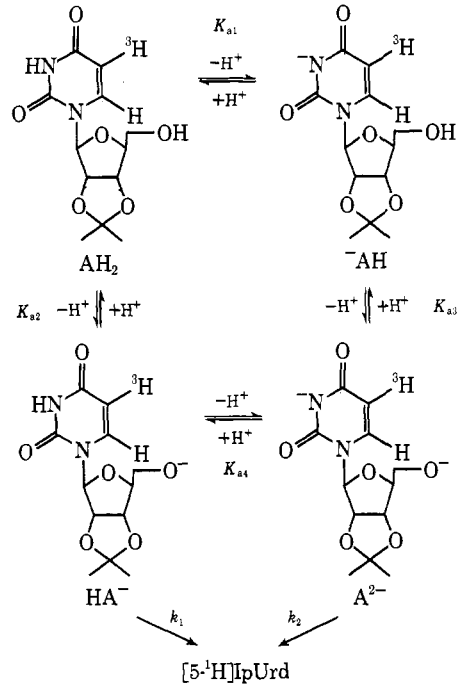


FIGURE 4: Equilibria of ionic species of [5-³H]IpUrd showing the reactive forms leading to 5-³H exchange.

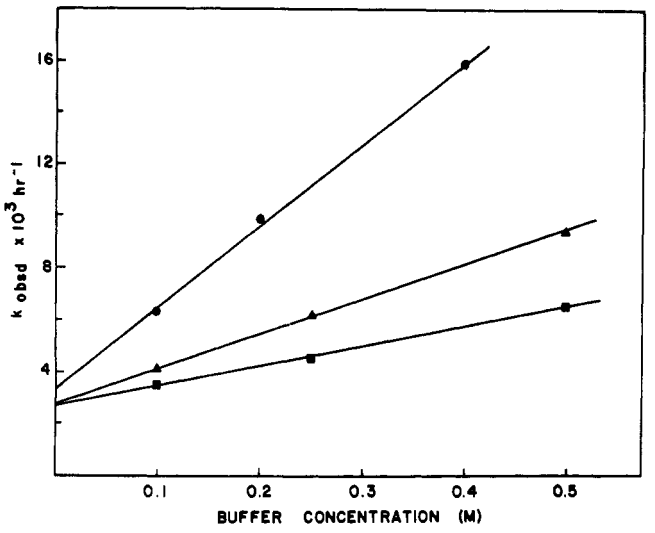


FIGURE 5: Typical plots of k_{obsd} for detritiation of [5-^{3}H]IpUrd *vs.* buffer concentration at 30 ± 0.1° and μ = 1.0: (●) Et_3N, pH 10.72, (▲) carbonate, pH 9.42; (■) carbonate, pH 10.22. Higher buffer concentrations are not shown.

alcohols to their basicities. The k_{OH} for hydrolysis of 5′-*O*-Ac-IpUrd was determined to be 28.2 M^{-1} min^{-1} at 30 ± 0.1°, which when fitted to the data of Bruice corresponds to a pK_a of 13.9 for the 5′-hydroxyl group.

Using linear free energy relationships obtained for the ionization of substituted methanols in water (Ballinger and Long, 1960) or in 2-propanol (Taft, 1953), a high attenuation factor of 0.51/CH_2 (McGowen, 1960), and polar substituent constants obtained for open chain analogs (Ballinger and Long, 1960), we calculate that neutral substituents on the 2′ position, or alkyl groups attached to the 3′-hydroxyl by an ether linkage, should have minimal effects on the pK_a of the 5′-hydroxyl group of nucleosides. Acidities of the 5′-hydroxyl should follow the order IpUrd > Urd > d^{2}Urd, but the range of pK_a values should not exceed 0.3 unit.

Base-Catalyzed Hydroxymethylation at the 5 Position of 1-Substituted Uracils. Figure 7 shows the extent of hydroxymethylation at the 5 position of a number of 1-substituted uracils in the presence of excess base and formaldehyde. The competing Cannizzarro reaction and concomitant base consumption prevented many of the reactions from proceeding to completion and prohibited accurate determination of rate constants. However, since these side reactions expectedly occur to the same extent regardless of the nature of the reactant, comparison of the amounts of hydroxymethylated product at a given time during the linear portions of the reactions provides a valid estimate of the relative rates. It should be noted that the rates of hydroxymethylation depend upon the nature of the 1-substituent and parallel those of 5-H exchange (Table I).

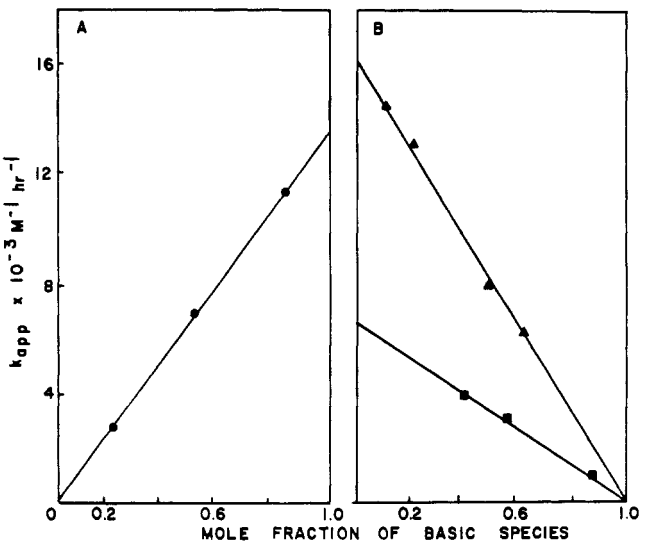


FIGURE 6: Plots of second-order rate constants (k_{app}) for detritiation of [5-^{3}H]IpUrd *vs.* basic species of: (●) $(EtOH)_3N$, (▲) HCO_3^-, (■) Et_3N.

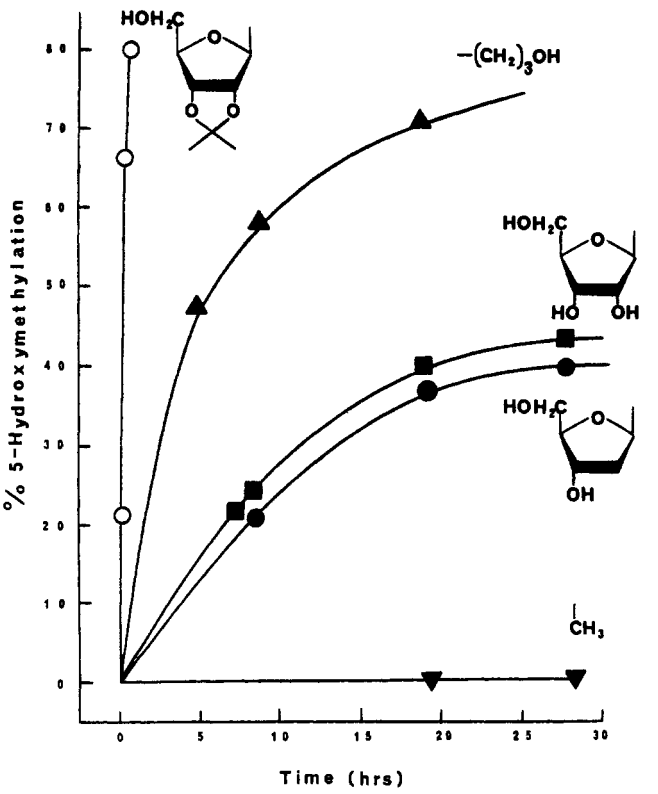


FIGURE 7: Extent of 5-hydroxymethylation of 1-substituted uracils. All reactions were kept at 60° and were 0.2 M in substrate, 3.4 M formaldehyde, and 0.5 N in NaOH.

Discussion

Intramolecular Catalysis of 5-H Exchange and Hydroxymethylation. The base-catalyzed 5-H exchange of uracil nucleosides in methanol-*1-d* may proceed up to 10^3 times faster than 1MeUra or derivatives which do not possess one or more of the free hydroxyl groups. Clearly, a catalytic role for hydroxyl groups attached to the 1 substituent is implicated. In analogy with the bimolecular counterpart (Santi *et al.*, 1970), a general mechanism may be proposed for the exchange which involves anchimeric assistance of an oxyanion attached to the 1 substituent, as depicted in Figure 8.

The only participating nucleophiles in the ribofuranosyl nucleosides are derived from the 5′-hydroxyls, as borne out by the stability of the 5-H of the 5′-deoxynucleosides, d^{5}Urd and d^{5}IpUrd. The 67-fold rate enhancement observed for IpUrd as compared to Urd can be accounted for either by the rigidity imposed upon the furanoside ring by the acetonide group (Hall, 1964; Abraham *et al.*, 1962), or by a higher

acidity of the participating hydroxyl group which would increase the equilibrium concentration of the reactive ionic species ii (Figure 8). Although it has been argued that the pK_a of 5′-hydroxyl groups of nucleosides should not be significantly affected by neutral substituents on the 2′ position, ionization of the 2′-hydroxyl group of Urd (pK_a = 12.5; Izatt *et al.*, 1966) is nearly complete under conditions where the 5′-oxyanion is present in significant concentration. Nevertheless, it is difficult to envision the effect of the 2′-oxyanion of Urd (separated from the 5′-CH_2OH by three carbon atoms) as being sufficiently large to cause the increase of 1.8 units increase in the pK_a of the 5′-hydroxyl necessary to explain the observed rate differences. Furthermore, 2′,3′-Bzl_2-Urd and d^2Urd, neither of which undergo a secondary ionization, exchange at approximately the same rate as does Urd. It may be concluded that the rate enhancement observed with IpUrd is, for the most part, due to stereochemical factors which probably increase the equilibrium concentration of the cyclonucleoside carbanionic intermediate prior to rate determining proton (deuteron) transfer (*vida infra*). A related equilibrium effect has been observed with the 6,5′-cyclic episulfides derived from 5′-S-Urd and 5′-S-IpUrd (Reist *et al.*, 1964; Chambers and Kurkov, 1963). At neutral and acidic pH, 20% of the former exists as its cyclic episulfide whereas the acetonide is completely cyclized. In general agreement with our conclusions, these workers hypothesized that the isopropylidene group forces the sugar ring into a conformation which favors formation of 6,5′-anhydro nucleosides.

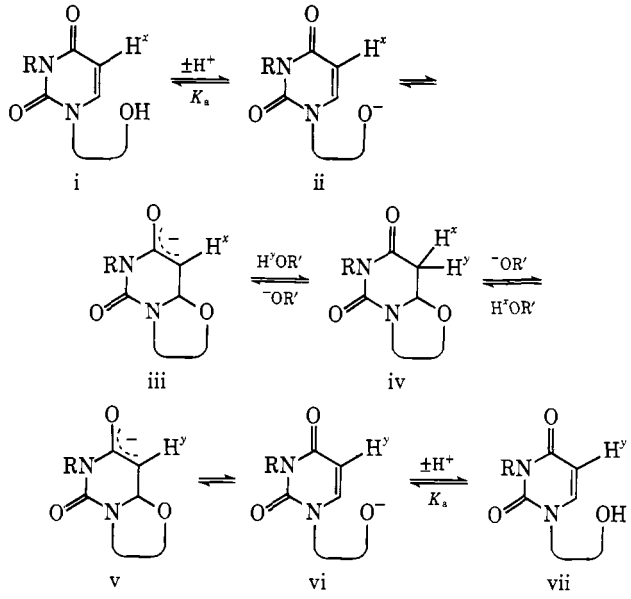


FIGURE 8: General scheme for mechanism of 5-H exchange of 1-substituted uracils involving neighboring group participation by oxyanion where R = H or $^-$, R′ = H or CH_3 (see text). H^{x-} refers to the hydrogen isotope initially present at C-5 and H^{y-} is the isotope present in the solvent; because H^{y-} is present in large excess, reactions may be considered irreversible after loss of H^x from C-5 to form species iv.

Although the 2′-hydroxy of Urd does not participate in catalysis of 5-H exchange, inversion of configuration at this carbon to give Ara-Ura results in a 623-fold enhancement in rate relative to 1MeUra. Assignment of the 2′-"up" oxyanion as the participating group rather than the 5′-oxyanion was confirmed by the observation that the 5-H exchange of d^5-Ara-Ura proceeds over 10^3 times faster than the bimolecular reaction. Intramolecular catalysis involving 6,2′-cyclonucleoside intermediates has also been observed in the base catalyzed hydrolysis of the C-4 amide of 1-β-D-arabinofuranosides of 5-fluorouracil (Otter and Fox, 1967), 5-fluorocytosine (Fox *et al.*, 1966), and 5-bromouracil (Otter *et al.*, 1968), and the deamination of arafuranosylcytosine (Notari *et al.*, 1972). We have also observed (D. V. Santi and D. Farber, unpublished data) that Ara-Ura undergoes cleavage of the C-4 amide bond in 1 *N* sodium hydroxide with $t_{1/2}$ of *ca.* 2.5 hr at 60°. This reaction, which undoubtedly proceeds through a 6,2′-cyclonucleoside intermediate, is not observed under the conditions of exchange because of the reversible closure of the ureido ester formed upon methoxide attack at C-4. Likewise, in contrast to the rapid cleavage in aqueous base, 1-β-D-arabinofuranosyl-5-fluorouracil is reported to be stable in 0.1 N $NaOCH_3$–CH_3OH for as long as 20 hr (Otter *et al.*, 1968). With the 2′-deoxylyxofuranoside, the 3′-"up" hydroxyl does not appear to take part in catalysis of 5-H exchange. Again, this is in accord with the base stability of 1-(2′-deoxy-β-D-lyxofuranosyl)-5-fluorouracil under conditions where the C-4 amide of the corresponding arabinonucleoside is completely hydrolyzed (Fox *et al.*, 1966).

Of the 1-(ω-hydroxyalkyl)uracils examined, only 1(HOPr)-Ura showed a significant enhancement in the rate of 5-H exchange. Formation of a cyclic carbanionic intermediate (iii, Figure 8) is in accord with the ease of formation and stability of six-membered rings. 1(HOBu)Ura and 1(HOPe)Ura, which would have to form unfavorable seven- and eight-membered ring intermediates, show no rate enhancement. It is noted that the hydroxyl group of 1(HOPr)Ura is certainly less acidic than the 5′-hydroxyl of IpUrd, and the relative rate for 5-H exchange is likely a lower estimate of the catalytic effectiveness of the hydroxypropyl group. The observation that the rate of 5-H exchange of 1(HOEt)Ura is comparable to that of the bimolecular process is at first somewhat surprising since formation of five-membered rings usually proceeds with equal or greater facility than formation of six-membered rings (Bruice and Pandit, 1960; Bruice and Benkovic, 1963). However, inspection of molecular models show that in entering the transition state for cyclization, the necessary overlapping of the electrons of the oxyanion with the p orbital at C-6 may only occur with considerable strain and eclipsing of methylene groups, expectedly resulting in an increase in the energy of activation. Since these should not significantly affect the stability of the cyclic intermediate it is likely that ring formation is rate limiting for intramolecular catalysis of 1(HOEt)Ura rather than proton transfer. The five-membered cyclic intermediate formed during 5-H exchange of Ara-Ura is not subject to the same effects since the necessary steric factors are accommodated in the ground state.

5-Hydroxymethylation of 1-substituted uracils represents a chemical counterpart for the initial condensation of dUMP with 5,10-CH_2H_4folate. Rates of the base-catalyzed hydroxymethylation are dependent upon the presence of a hydroxyl group on the 1 substituent, and closely parallel those for the exchange reaction. Thus, as with 5-H exchange, hydroxymethylation apparently requires addition of a nucleophile to the 6 position of the heterocycle and is susceptible to intramolecular catalysis. It is noted that this reaction is directly analogous to that catalyzed by dUMP hydroxymethylase (Flaks and Cohen, 1959), which catalyzes labilization of the 5-H of dUMP in the presence of H_4folate (Dunlap *et al.*, 1971b).

Exchange of [5-^{3}H]IpUrd. The pH–rate profile for lyate-catalyzed tritium release from [5-^{3}H]IpUrd at 25° shows a dependence upon an ionizable group having a pK_a of 9.0, and

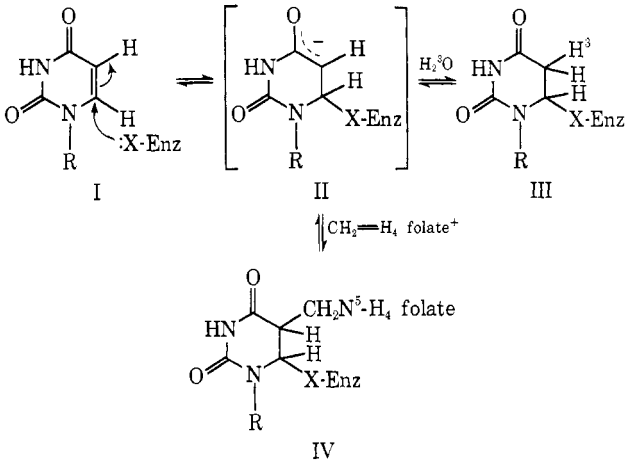


FIGURE 9: A proposed minimal mechanism for the first step of the thymidylate synthetase reaction involving catalysis by a nucleophilic group (:X) of the enzyme.

indicates the involvement of two reactive ionic species. Bimolecular reaction with hydroxide ion cannot be significant since [5-^{3}H]Urd or [5-^{2}H]1MeUra do not undergo observable exchange under comparable conditions. Figure 4 shows the ionic forms of IpUrd which may exist in solution. Between pH 7.1 and 12, the pH–rate profile is in accord with unimolecular exchange of either of the monoanions ($^-$AH or HA$^-$). Although these cannot be distinguished by kinetic means, the negatively charged heterocycle of $^-$AH should be less susceptible to nucleophilic attack at the 6 position than the neutral species (Santi *et al.*, 1970; Santi and Sakai, 1971), and the reactive ionic species in the lower pH range is in all likelihood HA$^-$. It is noted that the bimolecular counterpart also proceeds by hydroxide attack on the neutral heterocycle (Santi *et al.*, 1970), as does the mechanistically related hydrolysis of 1-methyl-5-trifluoromethyluracil (Santi and Sakai, 1971). The rate increase at high base concentrations is consistent with intramolecular attack of the 5′-oxyanion on the ionized heterocycle (A^{2-}). The kinetically equivalent intermolecular attack of hydroxide on the ionized heterocyclic form ($^-$AH) may be ruled out in view of the absence of hydroxide-dependent exchange of 1MeUra at high pH (Santi *et al.*, 1970) and the lack of observable exchange of [5-^{3}H]Urd at 30°. In this regard it is noted that the catalytic facility of the neighboring oxyanion permits a reaction of the ionized heterocycle which is not observed in the bimolecular counterpart.

Although species HA$^-$ is present only in very low concentrations at any given pH, it is expectedly the most reactive of the ionic forms present in solution. Fitting eq 2 to the experimental data, values of $k_1 = 5.0 \times 10^2$ hr^{-1} and $k_2 = 7.5 \times 10^{-3}$ hr^{-1} are obtained for the unimolecular exchange of HA$^-$ and A^{2-}, respectively. The 67,000-fold difference in the rate of 5-H exchange of HA$^-$ and A^{2-} probably reflects the electrostatic repulsion between the anionic reactive groups of the latter species. A similar effect has been observed (Santi and Sakai, 1971) in the analogous hydrolytic reactions of 5-trifluoromethyluracil derivatives and their conjugate bases, and has been explained in a similar manner.

The buffer catalysis which is observed in the 5-H exchange of [5-^{3}H]IpUrd between pH 7.1 and 11.5 indicates proton transfer in the rate-determining step. In Figure 8, the interconversions of iii $\rightleftarrows$ iv and iv $\rightleftarrows$ v differ only in the nature of the hydrogen isotopes transferred, and the rate-determining step at lower pH is best assigned to base-catalyzed tritium abstraction from iv. One consequence of this interpretation is that nucleophilic attack at the 6 position of the heterocycle to give iii occurs in a preequilibrium step and, in this case, the rate dependence on the 1 substituent reflects the stability of the cyclonucleoside rather than a propinquity rate effect. At higher pH, the change to apparent general acid catalysis is best explained in terms of the kinetically equivalent specific acid–general base catalyzed reaction, in which the dianionic species (iv, R = $^-$) is protonated prior to base-catalyzed proton abstraction at C-5, giving an analogous mechanism as previously described for the monoanion.

The recent observation (Wataya and Hayatsu, 1972) that analogous 5,6-dihydrouracil-6-sulfonates undergo amine-catalyzed exchange of the 5-H for protons of solvent support the proposal of general base catalysis abstraction from iv. It should be noted that the rate-determining step of 5-H exchange of uracil derivatives may be dependent upon the nature of the 1 substituents (D. V. Santi and J. Maley, unpublished results), the reactive ionic species of the heterocycle, and the nature of the nucleophilic catalyst. Analogous reactions catalyzed by thiols (Heller, 1968; Kalman, 1971) and other oxyanions described here have not been adequately analyzed to permit distinctions between rate-determining and preequilibrium addition to the 6 position of the heterocycle.

Relationship between Model and Enzymic Reactions. The salient features of the model studies described here which should be considered in mechanisms proposed for thymidylate synthetase are: (a) 5-H exchange and 5-hydroxymethylation of 1-substituted uracils are initiated by attack of a nucleophile at the 6 position of the heterocycle, followed by reaction of the "activated" heterocycle with the appropriate electrophilic species; (b) both the neutral and anionic forms of the heterocycle are susceptible to nucleophilic attack, although the neutral form is *ca.* 7×10^4 times more reactive; (c) the rate determining step may be *either* addition of the nucleophile to the 6 position, or proton transfer at the 5 position of the adduct; the latter step is susceptible to general base catalysis.

From the above, minimal mechanistic features may be suggested for the first step of the thymidylate synthetase reaction (Figure 9). It is proposed that the reaction is initiated by attack of a nucleophilic group of the enzyme (:X) at the 6 position of dUMP to produce an intermediate with high electron density at the 5-carbon of the heterocycle (II or the corresponding enol). The activated intermediate could react with CH_2H_4folate (or an equivalent species of formaldehyde) to give IV, or protons of solvent to provide the unsaturated intermediate III. Intermediate IV would undergo further chemical changes within the central complex to ultimately provide the observed reaction products, whereas reversal of III would account for the observed 5-H exchange. It is noted that intermediate IV differs from that proposed by Friedkin (1959) in that it is *covalently* bound to the enzyme, and the 5,6-double bond of the heterocycle is saturated.

Proton exchange reactions at the 5 position of 1-substituted uracils were susceptible to both general acid (D. V. Santi and J. Maley, unpublished results) and general base catalysis. The mechanisms elucidated closely follow those classically observed for hydration–dehydration of α,β-unsaturated carbonyl compounds, and enolization of ketones (see Bruice and Benkovic, 1966; Fedor and Glave, 1971; Bell, 1959). In the mechanism proposed in Figure 9, a general base could aid in proton abstraction from intermediates III and/or IV. General acid catalysis might facilitate nucleophilic catalysis *via* formation of an enol intermediate, and the resultant conjugate base

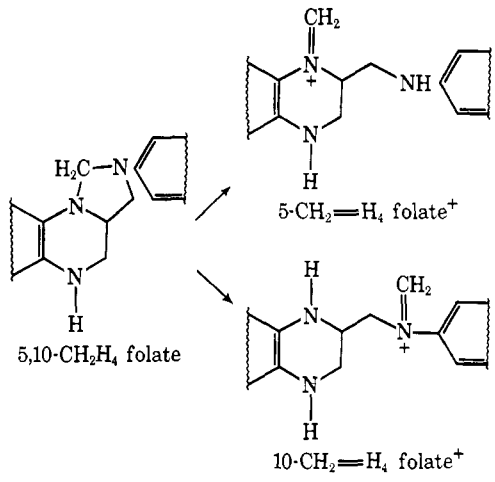
could aid the subsequent proton-transfer reactions. In this regard it is of interest to note that affinity-labeling experiments have provided evidence for the existence of a primary amino group which is positioned in the vicinity of the enzyme proximating the 5 position of the heterocycle (Santi and Sakai, 1972).

The 5-H exchange of dUMP could proceed *via* reversal of intermediate III or an intermediate further along the reaction pathway than IV in which the 5-H has equilibrated with solvent. We favor the former since a number of mechanistically similar enzymes (*viz.*, dUMP and dCMP hydroxymethylases) catalyze an analogous exchange reaction which requires H_4folate, but not CH_2H_4folate (Dunlap *et al.*, 1971b; Yeh and Greenberg, 1967). The apparent requirement for CH_2H_4-folate in the thymidylate synthetase catalyzed 5-H exchange of dUMP may be accommodated by a mechanism in which the cofactor must add to the enzyme before the nucleotide, or is required to initiate the catalytic sequence (Reyes and Heidelberger, 1965; Santi and McHenry, 1972). Direct evidence for this has been obtained (D. V. Santi and Cheryl L. Weill, unpublished results) by demonstrating that certain analogs of H_4folate which cannot undergo condensation with dUMP will serve as cofactors for 5-H exchange of the nucleotide substrate. This demonstrates an interesting situation in which the nucleophilic catalyst of the enzyme attacks from only one face of the heterocycle, but C-5 reacts with solvent from both sides, albeit not necessarily to the same degree.

Although there is little doubt that the thermodynamically stable adduct formed between formaldehyde and H_4folate is 5,10-CH_2H_4folate, it can be stated with a fair degree of certainty that 5,10-CH_2H_4folate is *not* the immediate donor of the one carbon unit. sp^3 hybridized adducts of aldehydes are unreactive towards nucleophilic displacements of the SN2 type; rather, such reactions occur by elimination–addition pathways in which the aldehyde carbon undergoes sp^2 rehybridization before reaction with nucleophiles (Jencks, 1964). Kinetic evidence has been obtained which demonstrates that the formation and, through microscopic reversibility, the hydrolysis of 5,10-CH_2H_4folate (Kallen and Jencks, 1966) and related tetrahydroquinazoline models (Benkovic *et al.*, 1969) proceed *via* the sp^2 hybridized iminium cation (5-CH_2=H_4-folate$^+$). These workers pointed out that such iminium ions are more likely to be the reactive electrophilic species than 5,10-CH_2H_4folate toward acceptor substrates (Scheme II). Three general possibilities exist for the electrophilic species of formaldehyde which undergoes reaction with the nucleophilic acceptor: (a) it may be associated with H_4folate as in 5- or 10-CH_2=H_4-folate$^+$; (b) free formaldehyde may be released from the cofactor before reaction with the substrate; (c) the one carbon unit could be transferred from 5,10-CH_2H_4folate to a nucleophilic group of the enzyme prior to transfer to the final acceptor.

The conclusions pertinent to the thymidylate synthetase reaction which may be derived from the above considerations are that 5,10-CH_2H_4folate is converted on the enzyme to the 5- or 10-iminium cation prior to attack by any nucleophile, and that the immediate formaldehyde donor to dUMP is sp^2 hybridized. At this time it is not possible to ascertain whether other nucleophiles (*viz.*, H_2O or enzyme) mediate the transfer of formaldehyde from 5,10-CH_2H_4folate to dUMP. We have recently observed (D. V. Santi and A. L. Pogolotti, unpublished results) that use of 5,10-CD_2H_4folate in the thymidylate synthetase reaction results in a sizeable secondary deuterium isotope effect (k_H/k_D = 1.21). Although the conversions responsible for this effect can only be speculated on,

SCHEME II



the result does provide direct evidence that the one carbon unit of 5,10-CH_2H_4folate undergoes sp^3 to sp^2 rehybridization-(s) at or before the rate-determining step of the reaction.

We have recently obtained direct evidence supporting the likelihood of nucleophilic catalysis in the thymidylate synthetase reaction by demonstrating that the 6 position of 5-fluoro-2′-deoxyuridylate forms a covalent bond with a nucleophilic group of the enzyme (Santi and McHenry, 1972). Because of the sensitivity of the enzyme toward sulfhydryl reagents (Dunlap *et al.*, 1971a), the suggestion has been made (Kalman, 1971) that the nucleophilic catalyst is a cysteine residue of the enzyme. Although this is a reasonable candidate, a number of nucleophilic groups (*viz.*, mercaptide, oxyanion, amino, hydroxide) have been demonstrated to participate in nonenzymic counterparts and we would remain noncommittal on this point until supporting evidence is obtained. We do note that the enzyme probably does not act as a general base to catalyze the addition of hydroxide to the 6 position of dUMP. 5,6-Dihydro-6-hydroxy-dUMP, the intermediate which would result from this reaction, is not a substrate for the *Lactobacillus casei* enzyme but rather a competitive inhibitor (D. V. Santi and A. L. Pogolotti, unpublished results).

As a final point, we would like to suggest that analogous mechanisms involving nucleophilic catalysis are a general feature of other enzymic reactions which involve electrophilic substitution at the 5 position of uracil and cytosine heterocycles. These would include the dUMP and dCMP hydroxymethylases, the pyrimidine methylases of DNA and RNA, pseudouridylate synthetase and a number of others. Experiments aimed at obtaining direct evidence for this proposal are in progress.

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