

# On the Mechanisms of Folate Cofactors

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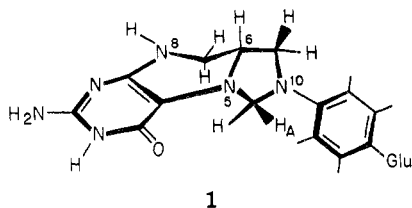
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Through the efforts of numerous investigators<sup>1</sup> over the past 25 years, the requirement for 5,6,7,8-tetrahydrofolic acid ( $H_4$ folate) in the metabolism of single carbon units at varying levels of oxidation has been established. Although its locus in major biosynthetic pathways has been well documented, most studies have been concerned with the purification and the reaction characteristics of the specific enzymes involved and have yielded, in general, only a limited amount of unambiguous and instructive data pertaining to reaction mechanism. There are at this time, however, sufficient data from both the enzymic and nonenzymic reactions, particularly on the oxidation level of formaldehyde and formate, to warrant discussion of some of the more probable catalytic mechanisms.

## Methylene Group Transfer

The structure of  $CH_2$ - $H_4$ folate (1) illustrates the basic



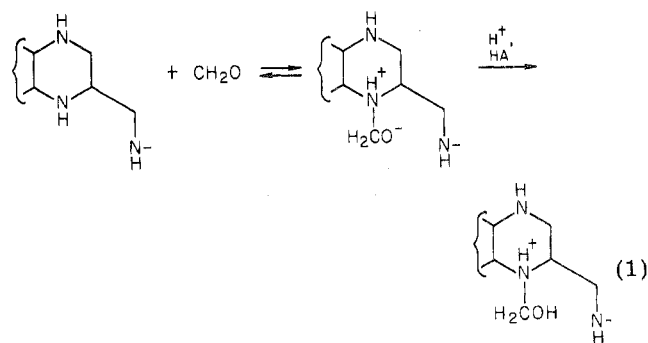
pteroylglutamate structural unit common to the folate cofactors, with the transferable one-carbon unit bridging N-5 and N-10. Poly- $\gamma$ -glutamyl conjugates also occur and are biologically active.<sup>2</sup> There are two asymmetric centers in the parent molecule, one at C-6 in the tetrahydropyrazine ring and the second at the  $\alpha$  carbon of the glutamate residue. The enzyme-catalyzed reactions to be discussed are stereospecific for the (+)-L diastereomer in which the glutamate is in the L configuration, while the absolute configuration at C-6 as yet is unknown.<sup>3,4</sup> Although the cofactor is depicted in one of several possible conformations, all share the common feature that one face of the five-membered imidazolidine ring, the side bearing  $H_A$ , is sterically more accessible than the other.

## Nonenzymic Studies

The formation of  $CH_2$ - $H_4$ folate from formaldehyde and the parent cofactor occurs rapidly in dilute solution in the absence of enzyme.<sup>5</sup> The overall equilibrium lies far on the side of the adduct ( $K_{eq} \approx 10^4$ , pH 7), a fact that originally suggested that the structure of the condensation product involves a methylene bridge between N-5 and N-10 rather than simply an *N*-hydroxymethyl derivative.<sup>5,6</sup> This structural feature has been directly confirmed by direct NMR observation

of the methylene AB doublets<sup>7</sup> centered at  $\delta$  4.99 and 3.78 ( $J = 4$  Hz) characteristic of an AB or AX system in which one proton lies above the plane and the other lies in the plane of an aromatic group.<sup>8</sup> The NMR spectrum furnishes the chief supporting evidence for the conformation depicted above.

The mechanism for the condensation of formaldehyde with tetrahydrofolate is similar to that encountered in a number of carbonyl addition reactions.<sup>9</sup> In the absence of mercaptoethanol,<sup>10</sup> the reaction exhibits a typical bell-shaped pH-rate profile with a maximum at ca. pH 6.0 and inflection points at ca. pH 5.0 and 7.0. Because the latter pH does not correspond to a  $pK_a$  of either reactant, the pH-rate profile must be controlled by the kinetics of the reaction rather than the respective dissociation constants. The profile has been interpreted in terms of rate-determining attack by amine (presumably the more nucleophilic N-5) on formaldehyde at low pH, changing to rate-limiting dehydration of the carbinolamine intermediate at alkaline pH. Both steps are subject to general acid catalysis. The former is probably associated with a stepwise mechanism involving formation of an unstable zwitterionic form of the carbinolamine which reverts rapidly to starting material unless it is trapped by proton transfer from acid or solvent (eq 1).<sup>11-13</sup> The



(1) R. L. Blakley, "The Biochemistry of Folic Acid and Related Pteridines", American Elsevier, New York, N.Y., 1969.

(2) J. K. Coward, K. N. Parameswaran, A. R. Cashmore, and J. R. Bertino, *Biochemistry*, **13**, 3899 (1974).

(3) R. L. Blakley, *Biochem. J.*, **77**, 459 (1960).

(4) A. J. Wahba and M. J. Friedkin, *J. Biol. Chem.*, **236**, PC 11-12 (1961).

(5) R. L. Blakley, *Biochem. J.*, **58**, 448 (1954); M. J. Osborn, P. T. Talbert, and F. M. Huennkens, *J. Am. Chem. Soc.*, **82**, 4921 (1960).

(6) R. G. Kallen and W. P. Jencks, *J. Biol. Chem.*, **241**, 5851 (1966).

(7) C. M. Tatum, Jr., P. A. Benkovic, S. J. Benkovic, R. Potts, E. Schleicher, and H. G. Floss, *Biochemistry*, **16**, 1093 (1977).

(8) L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", 2nd ed., Elmsford, N.Y., Pergamon Press, 1969.

(9) W. P. Jencks, *Prog. Phys. Org. Chem.*, **2**, 63 (1964); J. E. Reimann and W. P. Jencks, *J. Am. Chem. Soc.*, **88**, 3973 (1966).

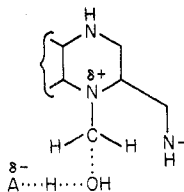
(10) An antioxidant is generally added to minimize oxidation of the parent cofactor.

(11) W. P. Jencks, *Acc. Chem. Res.*, **9**, 425 (1976).

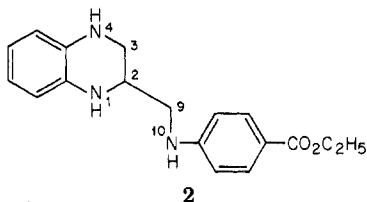
(12) S. Rosenberg, S. M. Silver, J. M. Sayer, and W. P. Jencks, *J. Am. Chem. Soc.*, **96**, 7986 (1974).

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latter is apparently associated with a concerted but imbalanced transition state involving extensive proton transfer and C–O bond breaking with little development of C–N double bond character.<sup>14</sup>

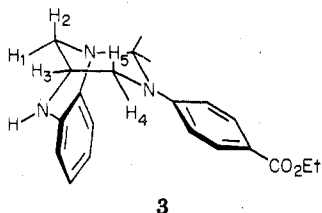


Additional insight into the condensation of formaldehyde with tetrahydrofolate was gained through the substitution of a series of tetrahydroquinoxaline analogues for tetrahydrofolate. The design of these compounds, predicated on the desirability of simplifying experimental problems encountered in studies of reaction kinetics, allowed the removal of several "nonessential" dissociable groups but retained the molecular dissymmetry inherent in the parent cofactor, and in the case of *p*-carboethoxy substitution,  $pK_a$  values for the participating nitrogen atoms closely approaching those for H<sub>4</sub>folate. Two  $pK_a$ 's were measured for **2**, 4.35, assigned to the quinoxaline ring



nitrogens, and -1.10, attributed to the exocyclic amino function.<sup>15</sup> For comparison, the two relevant  $pK_a$ 's of H<sub>4</sub>folate are 4.82 and -1.25 for N-5 and N-10, respectively.<sup>16</sup>

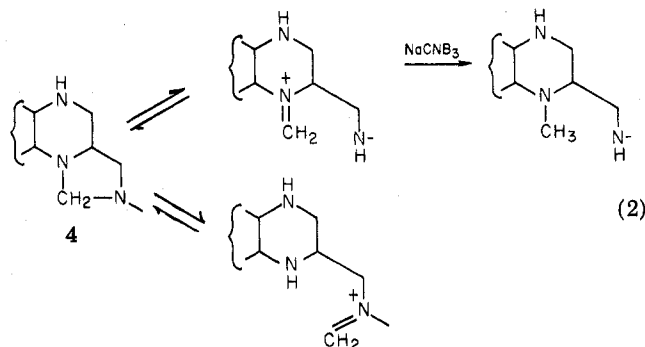
Condensation of formaldehyde with **2** does not form initially the corresponding imidazolidine but proceeds through a kinetically demonstrable addition–dehydration sequence via carbinolamine formation at N-4 to yield the benzotriazocine, **3**.<sup>17,18</sup> The structure of **3**



was characterized via the high-resolution NMR spectrum of the corresponding N-1 methyl derivative and was shown to exist in the depicted chair conformation.<sup>17</sup> Benzotriazocine formation, which has not been observed with the cofactor, can apparently be avoided owing to the lower nucleophilicity of N-8 brought about by its conjugation with the pyrimidine ring. Nucleophilicity

being approximately equal, steric effects on rates and equilibria for formaldehyde condensation at N-1 relative to N-4 are sufficient to direct production of **3**. The second phase of the condensation reaction is the conversion of the benzotriazocine to the 1,10-imidazolidine via the respective parent amine (various para-substituted derivatives) and formaldehyde rather than direct rearrangement of the former to the imidazolidine, which is ca. 20-fold more stable. The most compelling evidence for this conclusion is that rate constants for this conversion do not show the expected sensitivity to alterations in N-10 basicity upon para substitution ( $\Delta pK_a \approx 3$ ) as would be expected for a direct rearrangement of the benzotriazocine to the imidazolidine through the N-10 iminium cation. The pH–rate profile for imidazolidine formation may be readily interpreted in terms of a benzotriazocine  $\rightleftharpoons$  parent amine plus formaldehyde equilibrium. This equilibrium decays through rate-limiting formation of carbinolamine at N-1 catalyzed by solvent and hydronium ion at pH < 7 and, at pH > 7, by rate-limiting dehydration of the carbinolamine with obvious parallels to that described in eq 1. Of considerable interest, however, is the observation that the rate coefficient for hydronium ion catalyzed formation of carbinolamine is sensitive to the  $pK_a$  of the exocyclic nitrogen ( $\beta \approx 1.0$ ). This suggests that an intramolecular proton transfer to the zwitterionic species in eq 1 may be operative.<sup>19</sup> By inference, the cofactor itself may catalyze a one-carbon unit transfer (as a hydroxymethylene unit) in some enzymic reactions.

The investigations of the condensation reaction, however, do not illuminate the mechanistic processes accompanying imidazolidine ring opening, since this step (ring closure in the reverse direction) is not rate limiting. Trapping of the formaldehyde condensation product (**4**), derived from **2** in aqueous solution, by sodium cyanoborohydride yields a single N-1 methyl product over a pH range of 1–7 (eq 2). This was



identified by comparison with the two possible *N*-methyl isomers. The rate of trapping, however, is not independent of the cyanoborohydride concentration, indicating that the iminium cation of thermodynamic control, possibly not identical with that of kinetic control, is being intercepted. Based on our estimated analytic detection limits, the cation at N-1 is present at least in 20-fold higher concentration than that at N-10, and the difference is even greater if one allows for the latter's anticipated higher reactivity toward

(13) J. M. Sayer, B. Pinsky, A. Schonbrunn, and W. Washtien, *J. Am. Chem. Soc.*, **96**, 7998 (1974).

(14) J. M. Sayer and W. P. Jencks, *J. Am. Chem. Soc.*, **99**, 464 (1977).

(15) S. J. Benkovic, P. A. Benkovic, and D. R. Comfort, *J. Am. Chem. Soc.*, **91**, 5270 (1969).

(16) R. G. Kallen and W. P. Jencks, *J. Biol. Chem.*, **241**, 5845 (1966).

(17) T. H. Barrows, P. R. Farina, R. L. Chrzanowski, P. A. Benkovic, and S. J. Benkovic, *J. Am. Chem. Soc.*, **98**, 3678 (1976).

(18) G. P. Tuszyński, M. Frederick, and R. G. Kallen, *J. Am. Chem. Soc.*, **97**, 7359 (1975).

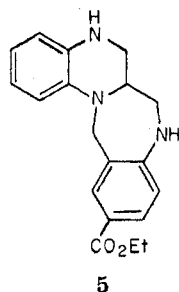
(19) Internal general acid catalysis of carbinolamine dehydration has been observed in the reaction of isobutyraldehyde with diamines of the type  $(CH_3)_2N(CH_2)_nNH_2$ , where  $n = 2$  and  $3$ : J. Hine and F. A. Via, *J. Org. Chem.*, **42**, 1972 (1977), and references therein.

Table I  
Relative Percentage Yields of Product from the Reduction of Methylenediamines with Sodium Borohydride in Dioxane/Water (50% v/v) at pH 6 (Acetate Buffer)<sup>a,b</sup>

System (X, Y)	7	8	9	10
6a (CO <sub>2</sub> CH <sub>3</sub> , H)	9	<0.5	50	41
6b (H, CO <sub>2</sub> C <sub>2</sub> H <sub>5</sub> )	50	45	5	<0.5
6c (F, H)	29	27	23	21

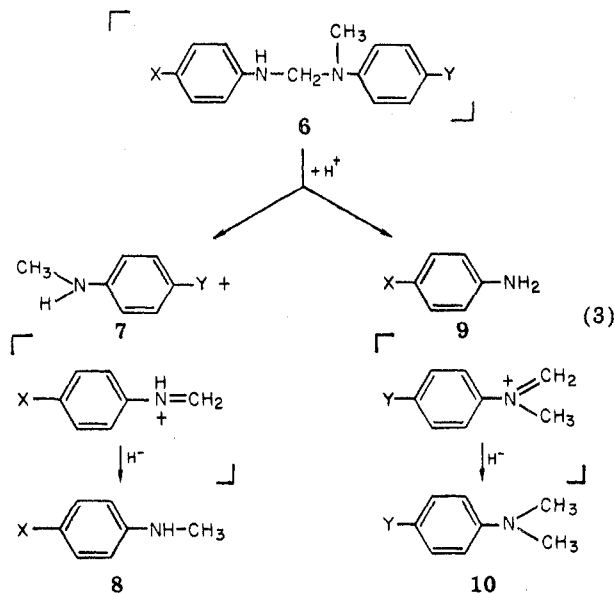
<sup>a</sup> Corrected for hydrolysis of the initial amidine. <sup>b</sup> Average error  $\pm 3\%$ .

hydride. The N-1 cation also is apparently involved in the observed rearrangement in aqueous acid of the imidazolidine to the benzodiazepine, 5, a reaction



possibility not yet investigated for the cofactor. As in the above case of imidazolidine formation, the benzodiazepine does not form directly from 4. Crossover experiments employing deuterated formaldehyde indicate that the methylene bridge of 4 is completely exchanged before yielding the benzodiazepine, implicating a preequilibrium prior to the formation of 5. The total sequence, namely amine plus formaldehyde  $\rightarrow$  benzotriazocine  $\rightarrow$  imidazolidine  $\rightarrow$  benzodiazepine, is an elaborate example of the interplay between thermodynamic and kinetic control encountered with the model analogues.

The question of the direction of imidazolidine ring opening has been approached recently through a series of trapping experiments in systems not complicated by reversibility. The requisite methylenediamines are generated in situ over the pH range 3–11 from the reduction of precursor amidines and are, in turn, trapped by additional borohydride (eq 3).<sup>20</sup> The results

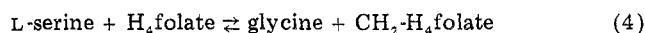


of a series of such experiments are listed in Table I.

For the reduction of 6a and 6b a discrepancy in the 8:7 and 10:9 ratio (expected to be 1:1 on the basis of eq 4 and actually observed in 6c) probably reflects partial hydrolysis of the iminium cation intermediate. Nevertheless, it is clear by simply comparing, for example, the relative concentrations of 7 and 10 in the case of 6a or 8 and 9 in 6b that the reduction proceeds to afford a greater amount of product in which the newly formed methyl group is attached to the more basic nitrogen atom.<sup>21</sup> The partitioning ratio favors expulsion of the less basic amine and formation of the more stable iminium cation by a factor of at least 5:1. The possibility of equilibration of the iminium cations intermediates is rendered unlikely due to the conditions of high dilution and the virtual absence of *N*-methyltoluidine formation (<3%) when the reaction is carried out in the presence of *p*-toluidine. The general acid catalyzed hydrolysis of 2-(substituted-phenyl)-*N,N*-dimethyl-1,3-imidazolidines has been cited as a situation in which stability of the incipient iminium cation contributes to the ease of C–N bond cleavage and facilitates the observation of such catalysis.<sup>22</sup> Collectively, the above results may be interpreted to mean that the  $\Delta pK_a$  between N-5 and N-10 in the parent cofactor would favor iminium cation generation under both kinetic and thermodynamic control at N-5 and that the ring-opening step itself might be subject to concerted acid catalysis rather than preequilibrium protonation.<sup>23</sup>

### Stereochemical Probes

The bridging methylene unit is a prochiral center, so that substitution with a heavier isotope, for example, deuterium or tritium, converts this carbon to a third asymmetric center permitting one to follow the steric course of its transfer. Utilizing CH<sub>2</sub>-H<sub>4</sub>folate, chemically synthesized to contain tritium distributed 3:1 at the two prochiral diastereotopic positions of the methylene group, and stereochemically pure (*3R*)- and (*3S*)-[3-<sup>3</sup>H]serines, we have examined the stereochemical course of the serine hydroxymethylase catalyzed reaction<sup>7</sup> (eq 4). The enzyme requires pyridoxal



phosphate (PyP), and considerable spectrophotometric evidence has been amassed implicating the formation of the respective amino acid–pyridoxal phosphate imines as required steps in the above reactions.<sup>24,25</sup> The pyridoxal phosphate–glycine imine anion is a presumed intermediate in the stereospecific replacement of CH<sub>2</sub>OH by H at the *pro-2S* position of glycine.<sup>26,27</sup> The cleavage of serine to glycine was made effectively irreversible by coupling (eq 4) to a NADP<sup>+</sup>-requiring dehydrogenase which oxidizes CH<sub>2</sub>-H<sub>4</sub> folate to 5,10-

(21) There is a probable steric effect by the methyl group on the partitioning of 6, since in 6c the basicities of the respective anilines are equivalent. The mode of decomposition of 6 also appears to be sensitive to substituent effects at the bridging methylene unit: J. L. Hogg, D. A. Jencks, and W. P. Jencks, *J. Am. Chem. Soc.*, **99**, 4772 (1977).

(22) T. H. Fife and J. E. C. Hutchins, *J. Am. Chem. Soc.*, **98**, 2536 (1976).

(23) The  $pK_a$  for protonation of an imidazolidine is ca. 0.5–1.5  $pK_a$  units below that for the parent amine: R. G. Kallen, R. O. Viale, and L. K. Smith, *J. Am. Chem. Soc.*, **94**, 576 (1972).

(24) L. Schirch and M. Mason, *J. Biol. Chem.*, **238**, 1032 (1963).

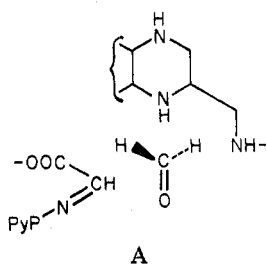
(25) L. Schirch and T. Gross, *J. Biol. Chem.*, **243**, 5651 (1968).

(26) P. M. Jordan and H. Akhtar, *Biochem. J.*, **116**, 277 (1970).

(27) P. Besmer and D. Arigoni, *Chimia*, **23**, 190 (1968).

methenyl- $H_4$ folate ( $CH^+$ - $H_4$ folate). This latter reaction is totally stereospecific, removing a hydride or its equivalent from one face of the imidazolidine ring resulting in either transfer of tritium to the nicotinamide cofactor or retention of tritium in the  $CH^+$ - $H_4$ folate.<sup>7</sup> Starting with (3*S*)-[3-<sup>3</sup>H]serine, we obtained  $CH^+$ - $H_4$ folate containing 76% of the tritium, whereas, with the (3*R*)-[3-<sup>3</sup>H]serine, 24% of the tritium is retained. The remainder in both cases is transferred to the nicotinamide. This curious result is indicative of partial stereospecificity in the transfer process and contrasts with the apparent total stereospecificity usually found in enzyme-catalyzed reactions. This finding, however, is in excellent agreement with an earlier experiment performed with intact rat liver slices.<sup>28</sup> In this experiment, [<sup>3</sup>H]formate was converted to predominantly (3*S*)-[3-<sup>3</sup>H]serine (72% of the tritium) rather than (3*R*)-[3-<sup>3</sup>H]serine through a sequence thought to involve  $CH^+$ - $H_4$ folate reduction to  $CH_2$ - $H_4$ folate. The possibility that the partial racemization arises from nonenzymic racemization of the 5,10-methylene intermediate was eliminated in our case by demonstrating that this process was negligible for the chemically synthesized tritiated  $CH_2$ - $H_4$ folate under the conditions chosen for the coupled enzyme system. Furthermore, the degree of racemization was found to depend on the extent of equilibration attained prior to the addition of the coupling enzyme and independent of the dehydrogenase concentration.

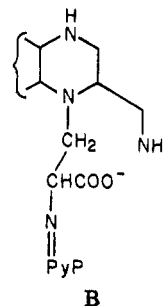
A number of mechanisms constructed in part from the behavior of the model systems have been proposed for the hydroxymethylase reaction, but only two—restricted to the key events involving  $H_4$ folate—need be considered here.<sup>26,29-31</sup> One version (A) features a



A

transient formaldehyde intermediate at the active site, in contrast to a second involving a covalent adduct at N-5. The two mechanisms basically differ in the timing of the species trapping the presumed N-5 iminium cation (water in A and the glycyl anion in B). The major reason for preferring A is based on another interconversion catalyzed by the hydroxymethylase enzyme.

The facile cleavage of  $\alpha$ -methylserine to D-alanine is not explicable if the adduct in B decomposes through a customary elimination-addition rather than a displacement sequence. Our stereochemical studies may be rationalized in terms of A since partial rotation about the torsionally symmetric formaldehyde intermediate



B

would lead to the partial racemization observed. Mechanism A as written, however, does not explain why, in the absence of  $H_4$ folate, the enzyme readily catalyzes the conversion of threonine to glycine and acetaldehyde which is then rapidly released from the enzyme. Moreover, this result implies that tetrahydrofolate is not involved directly in a displacement reaction on the hydroxymethylene unit. It appears plausible that a functional group<sup>32</sup> (possibly SH or OH but not  $NH_2$ )<sup>33</sup> on the enzyme might condense with formaldehyde prior to its transfer to the glycyl anion and that its removal as the imidazolidine of the cofactor is needed for efficient catalysis, a requirement not mandated by the less stable acetaldehyde adducts. Partial rotation occurring at the enzyme-bound formaldehyde intermediate would rationalize the lack of absolute stereospecificity.

Thymidylate synthetase catalyzes the reductive methylation of 2'-deoxyuridylylate (dUMP) to thymidylate (dTMP) with the concomitant conversion of 5,10-methylenetetrahydrofolate to 7,8-dihydrofolate. The cofactor serves as both a one-carbon carrier and a reductant.<sup>34</sup> An a priori minimal mechanism for this enzyme must involve at least two steps: (a) an electrophilic substitution reaction in which a putative adduct is formed between an enzyme-bound 5,6-dihydrouracil and  $CH_2$ - $H_4$ folate or a 5-hydroxymethyl-5,6-dihydrouracil intermediate is generated, and (b) a disproportionation resulting in a process equivalent to a 1,3-hydride shift.<sup>35</sup> That the cofactor serves as the reductant has been verified by demonstrating transfer of the [6-<sup>3</sup>H] from the cofactor to the methyl group of dTMP. Various model studies also suggest that in step a formation of the reactive dUMP species probably occurs through enzymic nucleophilic addition to the 5,6-double bond of dUMP and (b) the reductive disproportionation step takes place through an exocyclic methylene derivative which is then reduced to dTMP.<sup>36</sup>

Evidence for the presence of an adduct containing both the uracil and  $CH_2$ - $H_4$ folate rather than simply a hydroxymethyl-5,6-dihydrouracil has been derived mainly from studies of the interaction of the enzyme with 5-fluoro-2'-deoxyuridylylate.<sup>37,38</sup> Although a re-

(28) J. F. Biellmann and J. F. Schuber, *Bull. Soc. Chim. Biol.*, **52**, 211 (1970).

(29) S. J. Benkovic and W. P. Bullard, *Prog. Bioorg. Chem.*, **133**-175 (1973).

(30) L. Jaenicke, "The Mechanism of Action of Water Soluble Vitamins", Ciba Foundation Study Groups No. 11, A. V. S. DeRauck and M. O'Connor, ed., Little, Brown & Co., Boston, 1961, p 38.

(31) F. M. Huennekens, H. R. Whitely, and M. J. Osborn, *J. Cell. Comp. Physiol.*, **54**, 109 (1959).

(32) L. Schirch and A. Diller, *J. Biol. Chem.*, **246**, 3961 (1971).

(33) P. M. Jordan, H. A. El-Obeid, D. L. Covina, and M. Akhtar, *J. Chem. Soc., Chem. Commun.*, **73** (1976).

(34) G. K. Humphrey and D. M. Greenberg, *Arch. Biochem. Biophys.*, **78**, 275 (1958).

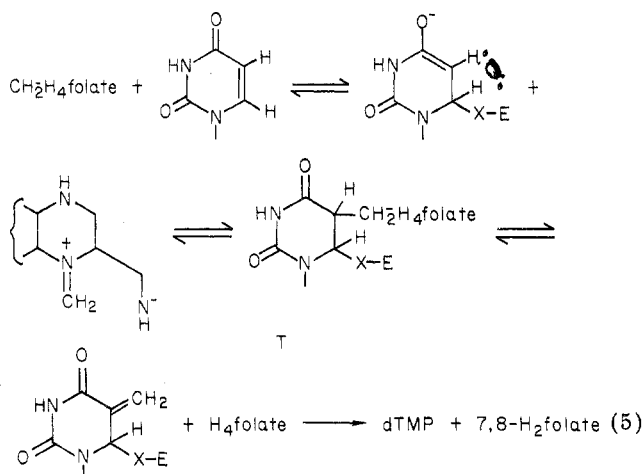
(35) M. Friedkin, "The Kinetics of Cellular Proliferation" F. Stohlmer, Ed., Greene and Stratton, New York, N.Y., 1959, p 59.

(36) For a recent review, see: A. L. Pocolotti and D. V. Santi, *J. Bioorg. Chem.*, in press.

(37) D. V. Santi, C. S. McHenry, and H. Sommer, *Biochemistry*, **13**, 471 (1974); D. V. Santi and C. S. McHenry, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 1855 (1972).

(38) R. J. Langenbach, P. V. Danenberg, and C. Heidelberger, *Biochem. Biophys. Res. Commun.*, **48**, 1565 (1972).

versible binary complex forms between the nucleotide and the synthetase enzyme, in the presence of cofactor a stoichiometric ternary complex is generated. Spectral measurement, as well as the observation of secondary isotope effects, suggest that the 5,6-double bond of the pyrimidine is saturated in the bound complex. This hypothesis is supported by the isolation of a peptide covalently attached to the adduct. The stability of the latter toward loss of formaldehyde by hydrolysis or oxidation of the tetrahydrofolate constitutes primary evidence for favoring substitution at the N-5 position of the cofactor.<sup>39</sup> The above results are combined in the mechanism postulated in eq 5.



We have investigated the steric course of the synthetase reaction by employing the biologically active  $\text{CH}_2\text{-H}_4\text{folate}$  diastereomer produced by serine hydroxymethylase.<sup>40</sup> A chiral methyl group in dTMP was generated from a cofactor containing  $^3\text{H}$  at the C-6 position and a methylene unit formed from (3*R*)-[3- $^3\text{H}$ ,3- $^2\text{H}$ ]- and (3*S*)-[3- $^3\text{H}$ ,3- $^2\text{H}$ ]serine, respectively. The two enzymes were coupled under conditions where nonenzymic racemization would again be negligible. The isolated dTMPs were oxidized to yield the respective chiral acetates which were then assayed for chirality via enzymatic conversion to fumarate through malate. This analysis is not sufficiently sensitive to permit comment on whether the process is partially or totally stereospecific. However, dTMP from (3*S*)-serine gave chiral acetate of *S* configuration (32% tritium retention in fumarate), whereas dTMP derived from (3*R*)-serine yielded chiral acetate of *R* configuration (69% tritium retention in fumarate). When the starting serines were analyzed via an alternate transformation to chiral acetates (with stereospecificity similar to that observed with the hydroxymethylase enzyme), similar tritium retention percentages were obtained. Consequently the reaction catalyzed by thymidylate synthetase is highly stereospecific. This finding is consistent with the mechanism shown in (5) since it shows the absence of a freely rotating methylene group, a condition satisfied by the iminium cation. Since reduction of an exocyclic methylene derivative constitutes an attractive second step, it appears that the additional hydration/dehydration sequence imposed by a transient formaldehyde intermediate is now avoided.

(39) N-5 relative to N-10 or unsubstituted tetrahydrofolate derivatives are more resistant toward aerobic oxidation.<sup>1</sup>

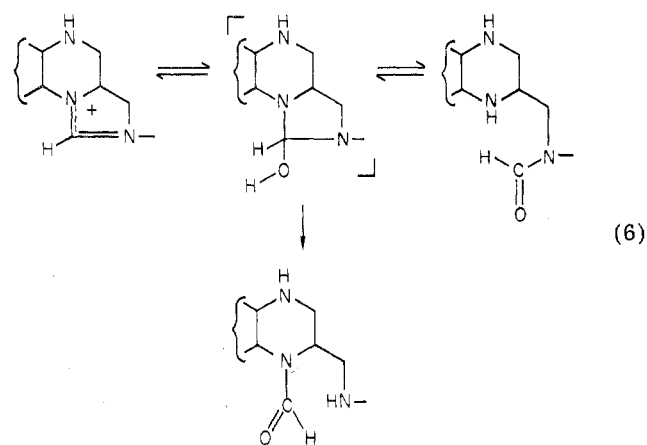
(40) C. Tatum, J. Vederas, E. Schleicher, S. J. Benkovic, and H. Floss, *J. Chem. Soc., Chem. Commun.*, 218 (1977).

Given the elucidation of the absolute stereochemistry at C-6, we would be able to define the overall stereochemical course of this latter step.

### Formyl Group Transfer

Three forms of the cofactor carry carbon at the formate level of oxidation: the N-5 and N-10 formyl derivatives and the 5,10- $\text{CH}^+\text{-H}_4\text{folate}$  formamidineium salt. Only the latter two function in transformylation reactions, as, for example, in the biosynthesis of inosinic acid which requires two transformylation reactions, i.e., the formation of formylglycine amide ribonucleotide and 5-formamido-1-ribosyl-4-imidazolecarboxamide 5'-phosphate.

Kinetic studies of the hydrolysis reaction of the formamidineium salts derived from the tetrahydroquinoxaline analogues revealed the occurrence of kinetic and thermodynamic control in product formation, as outlined in eq 6.<sup>41</sup> Rates measured at  $\text{pH} < 6$  exhibited



nonlinear kinetics due to the subsequent isomerization of the initially formed N-10 formyl product to the more stable N-1 formyl compound; at  $\text{pH} \geq 6$  the N-10 formyl product is formed in 80 to 100% yield depending on the nature of the buffer species present. The latter represents a difference of  $< 1$  kcal for the respective product-determining steps. However, the pH-independent equilibrium for the two N-formyl compounds favors the N-1 formyl species by ca.  $10^2$ . The equilibrium constant for formation of the formamidineium salt from either formyl species is acid dependent and disfavors formation of the N<sup>10</sup>-formyl derivative by ca.  $10^{-5}$  M. The kinetic and thermodynamic dispositions of the formamidineium salt and the related N-formyl derivatives derived from 2 are qualitatively and in most respects quantitatively analogous to those of the corresponding derivatives of tetrahydrofolate. Hydrolysis of 5,10-methenyltetrahydrofolate under basic conditions yields mostly N<sup>10</sup>-formyltetrahydrofolate, with minor amounts of N<sup>5</sup>-formyl arising concomitantly, whereas acidification of the N<sup>10</sup>- or N<sup>5</sup>-formyl derivatives effects reconversion to the starting salt. Upon prolonged incubation, the N<sup>10</sup>-formyl species in weakly basic media undergoes slow isomerization to the N<sup>5</sup>-formyl isomer.<sup>42,43</sup> In broad terms the mechanism for hydrolysis involves preequilibrium addition of hydroxide ion

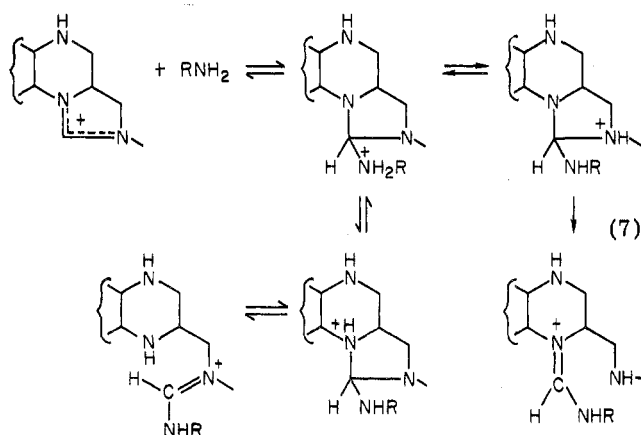
(41) S. J. Benkovic, W. P. Bullard, and P. A. Benkovic, *J. Am. Chem. Soc.*, 94, 7542 (1972).

(42) M. May, T. J. Bardos, F. L. Barger, M. Lansford, J. M. Ravel, G. L. Sutherland, and W. Shive, *J. Am. Chem. Soc.*, 73, 3067 (1951).

(43) D. R. Robinson and W. P. Jencks, *J. Am. Chem. Soc.*, 89, 7098 (1967).

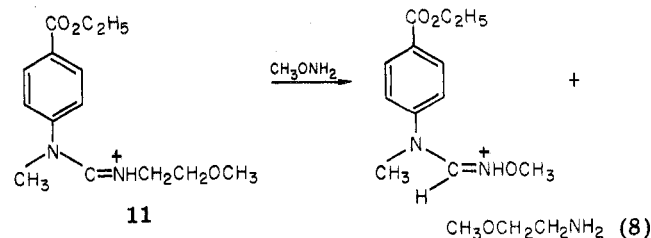
followed either by rate-limiting general acid catalyzed decomposition of the anionic tetrahedral addition compound or by general base catalyzed breakdown of the cationic intermediate.<sup>41,44,45</sup>

In an attempt to more closely mimic the actual one-carbon-unit transfer from the formamidinium cofactor, the condensation of amines with formamidinium analogues was investigated.<sup>46</sup> The reaction in Me<sub>2</sub>SO of  $\beta$ -methoxyethylamine with the formamidinium salt derived from **2**, monitored by NMR, leads to the initial formation of the *N*<sup>10</sup>-formimidoyl isomer followed by its rapid isomerization to the more stable *N*<sup>1</sup> isomer. The initial accumulation of the *N*<sup>10</sup>-amidine follows the pattern of the hydrolytic behavior noted above and is in accord with the expulsion of the more basic leaving group in the initial partitioning of the putative orthoamide intermediate. All of the species in eq 7 decompose through their re-

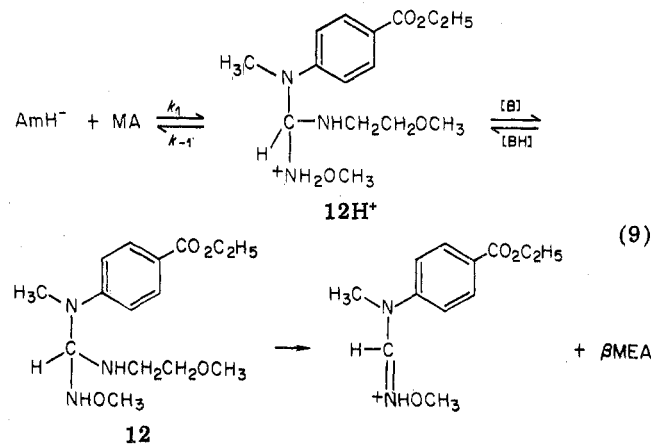


spective conjugate acid form, so that the leaving group is the free amine rather than the unstable amine anion. The net transamidination requires catalyzed tautomerization of the initially formed adduct to that leading to product. Hydrolysis of either of the formimidoyl isomers proceeds through attack of hydroxide ion on the protonated amidine without buffer catalysis and leads to the formation of the *N*<sup>1</sup>- or *N*<sup>10</sup>-formyl isomer but no detectable formyl group transfer. Thus the higher *pK<sub>a</sub>* nitrogen of  $\beta$ -methoxyethylamine is preferentially protonated and expelled from the presumed tetrahedral intermediates, so that basicity rather than leaving group ability determines the locus of bond cleavage.

Before further examining the possible mechanisms necessary to effect the transamidination reaction, let us more closely scrutinize the properties of the proposed orthoamide intermediate. We found that the methoxyaminolysis of the unsymmetrical formamidine **11** (an abbreviated form of the above *N*-10 isomer whose reactions would not be complicated by cyclization) proceeds readily in aqueous solution, furnishing the expected product of transamidination<sup>47</sup> (eq 8). The methoxyaminolysis of **11** exhibits general base catalysis by amines, phosphate, and carboxylates, and gives rise



to a nonlinear Brønsted plot. The catalytic constants are best correlated in the Brønsted relationship by two straight lines of  $\beta \approx 1$  and  $\beta \approx 0$  for  $pK_{BH^+} < 1.5$  and  $pK_{BH^+} > 1.5$ , respectively, suggesting the existence of a short-lived covalent intermediate and kinetically significant transport processes.<sup>11</sup> A mechanism incorporating these observations is given in (9). The



feasibility of a transport process becoming kinetically significant is determined by the lifetime of **12H<sup>+</sup>** which, in turn, is determined by the magnitude of *k*<sub>-1</sub>, the first-order rate constant for reversion of **12H<sup>+</sup>** to starting material. This can be estimated as ca. 10<sup>10</sup> s<sup>-1</sup>. The occurrence of the transition in the Brønsted plot at  $pK_{BH^+} \approx 2$  implicates the methoxyamine nitrogen ( $pK_N = 1.8$  in the orthoamide) as the site of catalysis rather than the  $\beta$ -methoxyethylamine nitrogen in view of the kinetically equivalent general acid catalyzed conversion of **12** to products with proton transfer to the latter nitrogen (eq 9). This does not mean that the  $\beta$ -methoxyethylamine does not undergo protonation prior to its expulsion, but simply that the rate of the second subsequent proton-transfer step is never rate-limiting.<sup>48</sup> It is conceivable but unproved that the required proton transfers for a bifunctional base occur within a one-encounter complex. Thus we can assign transport and proton-transfer processes exclusively the role of determining the mode of decomposition for highly reactive orthoformamides. In brief, within a complex of neutral orthoamide and general acid catalyst, the site of C-N bond cleavage is dictated by the equilibrium or rate of protonation of each of the three possible sites. Returning to our initial problem of attempting to understand overall net formyl transfer

(44) D. R. Robinson and W. P. Jencks, *J. Am. Chem. Soc.*, **89**, 7088 (1967).

(45) Both kinetic terms appear to be associated with diffusion-limited proton transfer steps depending on the *pK<sub>a</sub>* of the buffer species, indicating a stepwise process.

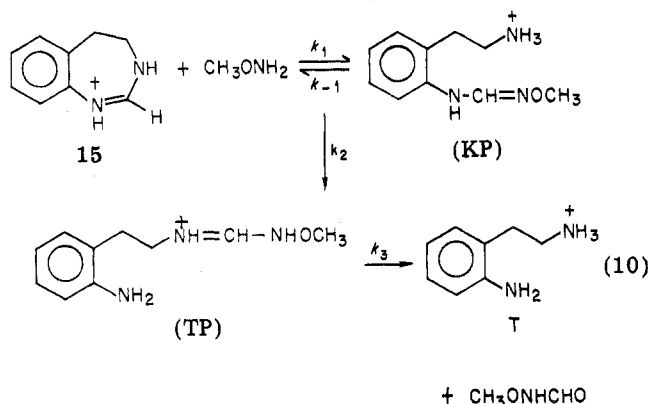
(46) S. J. Benkovic, T. H. Barrows, and P. R. Farina, *J. Am. Chem. Soc.*, **95**, 8414 (1973).

(47) W. P. Bullard, L. J. Farina, P. R. Farina, and S. J. Benkovic, *J. Am. Chem. Soc.*, **96**, 7295 (1974).

(48) The ratio of [12·BH<sup>+</sup>] to [12H<sup>+</sup>·B] for bases of  $pK_{BH^+} > pK_{N_1}$  (methoxyamine) is simply  $K_{N_1}/K_{BH^+}$ , whereas the proton-transfer component of [12·BH<sup>+</sup>] conversion to [12H<sup>+</sup>·B] where the  $\beta$ -methoxyamine moiety ( $pK_{N_2}$ ) is protonated in proportion to  $K_{BH^+}/K_{N_2}$  (acids with  $pK_a > 5$ ). Consequently the rate of [12BH<sup>+</sup>] to [12H<sup>+</sup>·B] remains invariant for bases of  $pK_{BH^+} > 5$ , whereas the term  $k_{-1}[12H^+·B]$  decreases. Since diffusion together of 12H<sup>+</sup> and B is rate limiting for bases with limits  $2 < pK_{BH^+} < 5$ , i.e., the partitioning of [12·BH<sup>+</sup>] to starting material is less favorable than conversion to product, proton transfer to the  $\beta$ -methoxyethylamine leaving group is not kinetically important.

as catalyzed by the transformylases, these observations implicate a perturbation at the active site of the relative basicities of the involved amino groups or an equilibration before product release in order to account for net transfer.

Recently our investigation of amidine hydrolysis and methoxylaminolysis has been extended to a series of cyclic amidines in order to establish a model for net formyl transfer, as well as to assess the possibility of directing through stereoelectronic control the breakdown of the ensuing tetrahedral intermediates to the thermodynamic isomers.<sup>49,50</sup> Although the intrusion as well as magnitude of a stereoelectronic effect on product formation could not be unambiguously demonstrated, the examination of the methoxylaminolysis of the 4,5-dihydro-3*H*-1,3-benzodiazepinium (15) amidine reveals a net formyl transfer reaction (eq 10).



Methoxylaminolysis of 15 displays triphasic kinetics interpreted in terms of a rapid preequilibrium formation of the kinetic isomer, followed by its isomerization to give the thermodynamic product and the latter's subsequent hydrolysis to  $\beta$ -(*o*-aminophenyl)-ethylamine and formylmethoxyamine. Both attack by methoxyamine and expulsion of the anilino nitrogen are subject to general acid-base catalysis.<sup>51</sup> The achievement of this transfer is, of course, dependent on the presence of a phenethylamine group intramolecularly disposed to intercept the initial amidine species. However, hydrolysis of the eventual amidine is still

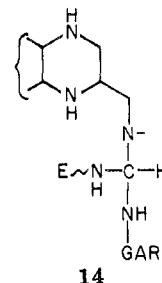
(49) B. A. Burdick, P. A. Benkovic, and S. J. Benkovic, *J. Am. Chem. Soc.*, **99**, 5716 (1977).

(50) According to the principles of stereoelectronic control, the cleavage of a C-O or C-N heteroatom bond is allowed only if lone pairs on the other two heteroatoms (O or N) in the tetrahedral intermediates are oriented antiperiplanar to the bond undergoing fission: J. M. Lehn and G. Wipff, *J. Am. Chem. Soc.*, **96**, 4048 (1974); J. M. Lehn, G. Wipff, and H. B. Burgi, *Helv. Chim. Acta*, **57**, 493 (1974); P. Deslongchamps, P. Atlani, P. Frehel, and A. Malavol, *Can. J. Chem.*, **40**, 3405 (1972); P. Deslongchamps, C. Lebreus, and R. Taillefer, *Can. J. Chem.*, **51**, 1665 (1973).

(51) As noted above the formation/decomposition of the orthoamide intermediate is subject to general acid-base catalysis when the attacking/leaving amine is weakly basic; in the case of strongly basic amines the rate of C-N bond cleavage may become rate determining, so that general acid-base catalysis is not observed: N. Gravitz and W. P. Jencks, *J. Am. Chem. Soc.*, **96**, 499 (1974).

subject to kinetic control and leads to net transfer, owing to the lower  $pK_a$  of the methoxyamine residue in comparison to the phenethylamine.

The implications of the above work suggest the plausibility of a similar equilibration scheme being operative in the amidine-orthoamide equilibria accompanying enzymatic catalysis of formyl transfer. The key orthoamide intermediate (14) features involvement



of the  $N^{10}$ -amino group of the cofactor, a residual amino group of high  $pK_a$  on the enzyme, and the amino moiety of glycine amide ribonucleotide (GAR). According to the conclusions drawn above, the orthoamide species mediates an equilibrium between the three possible enzyme-bound amidines leading to the accumulation of the thermodynamically favored product, the enzyme-glycine amide amidine (with loss of tetrahydrofolate). Selective hydrolysis of the enzyme-linked amidine under kinetic control with expulsion of the more basic amino group results in net formyl transfer to GAR and regeneration of the active amino group of the enzyme. We are presently pursuing studies of the mechanism of action of this transformylase enzyme in order to test the above postulates.

In conclusion, the mechanism of ring opening on the two levels of oxidation differs in several important respects. For the methylene bridge, the stability of the developing iminium cation appears to direct ring opening with C-N-10 bond cleavage, but for the cyclic formamidinium species, ring opening is dictated by the rate or degree of proton transfer. Thus, the leaving amine is generally the more basic one. The change in cleavage mode is apparently derived from the additional driving force for bond breakage available from the exocyclic nucleophile in the tetrahedral intermediate formed with the methenyl derivative. Both ring-opening modes may be subject to general acid catalysis. The nature of the remaining mechanistic steps to achieve one-carbon unit transfer is dependent on the chemical nature of the product, but the fission of the remaining C-N bond, particularly at the formate level of oxidation, presents a unique problem if the product-forming step remains under kinetic control.

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