The Mechanism of the Condensation of Formaldehyde with Tetrahydrofolic Acid*

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SUMMARY

The bell-shaped pH rate profile for the reaction of tetrahydrofolic acid with formaldehyde to form 5,10-methylene tetrahydrofolic acid has been shown to result from a change in rate-determining step with changing acidity. In alkaline solution, the rate-determining step is the acid-catalyzed dehydration of hydroxymethyl tetrahydrofolic acid and its conjugate base. This step is subject to general acid catalysis with a Bronsted slope, α, of approximately 0.75. In acid solution, attack of tetrahydrofolic acid on formaldehyde is rate-determining. This step is subject to catalysis by general acids, including the solvated proton, with a Bronsted slope of 0.20.

At pH values above 4, the reaction is inhibited by thiols, which combine with formaldehyde in a base-catalyzed reaction to form hemithioacetals. Depending on the reaction conditions, this inhibition can result from a lowering of the equilibrium concentration of formaldehyde or from a trapping by thiol anion of unhydrated formaldehyde, as it is formed from formaldehyde hydrate. The equilibrium constant for hemithioacetal formation from formaldehyde and mercaptoethanol is approximately 620 M⁻¹.

Secondary amines such as morpholine and imidazole catalyze the reaction of tetrahydrofolic acid with formaldehyde by nucleophilic catalysis by a pathway which must involve the intermediate formation of cationic imines:

\[ \text{N}^+\text{H} = \text{CH}_2 \]

The nonenzymic condensation of formaldehyde with tetrahydrofolic acid occurs rapidly in aqueous solution and exhibits a bell-shaped pH rate profile with a maximum at about pH 4.2 and inflection points at pH 3.2 and 5.2 in the presence of 2-mercaptoethanol (1–4). The product of this reaction is 5,10-methylene-THF (I), although methylene-THF is not necessarily the species that is active in enzymic reactions (3–8). A similar reaction occurs with THF and glyoxalate (9). The experiments reported here were undertaken in an attempt to gain a more detailed understanding of the mechanism of this reaction and its relationship to other reactions of carbonyl compounds with amines (10). A preliminary account of this work has appeared (11).

EXPERIMENTAL PROCEDURE

Materials and methods were the same as those described in the preceding paper. The concentration of mercaptoethanol in the THF sample from Nutritional Biochemicals, which was used in some of the early experiments, was not sufficient to interfere with the kinetic results after dilution 1:5000 in the reaction mixtures. THF was diluted daily into deoxygenated glass-distilled water which contained 10⁻³ to 10⁻⁴ M ethylenediaminetetraacetic acid. There was no significant difference in the kinetic results obtained with the different THF preparations.

Reagent grade formaldehyde was obtained from Fisher in 36.6 to 37.2% solutions which contained 10 to 11% methanol. This solution was diluted to a 1.0 M stock solution and was further diluted to 0.025 M every 1 to 2 days. The formaldehyde concentration was determined frequently by iodometric titration (12, 13). Amines were redistilled or converted to the hydrochlorides and recrystallized; imidazole was recrystallized as the free base. Reagent grade formic acid, substituted acetic acids, 2-mercaptoethanol, ethanethiol, mercaptoacetic acid, and inorganic salts were used without further purification.

Reaction mixtures for kinetic measurements (3.0 ml) were equilibrated in a water bath at 25 ± 0.1°C and were deoxygenated by the passage of argon or prepurified nitrogen through the solution for 5 to 10 min before use. It was shown that no detectable loss of formaldehyde occurred from a 0.1 M solution through which argon was passed for 1 hour. The reaction was started by the addition of tetrahydrofolic acid.
by the addition of THF or formaldehyde from a pipette, and the solution was mixed with a stream of argon or by inversion. Spectrophotometric readings were made with a Zeiss PMQ II spectrophotometer equipped with a cell holder maintained at 25° after 5 or 10 sec and every 2½ to 5 sec thereafter for 5 to 10 half-times, usually with about 15 readings. The end points were stable, except at alkaline pH at which a slow decomposition of the product was observed after 5 to 10 half-times. At pH 1 and 7, the spectra of the product agreed with published spectra for enzymatically active N₆,N₁₀-methylene-THF prepared from formaldehyde and THF and by the reduction of N₆,N₁₀-methylene-THF with sodium borohydride (3). The experiments were performed at concentrations of formaldehyde and other reagents that were in great excess over THF and pseudo first order kinetics were observed unless otherwise noted. The ionic strength was maintained at 1.0 M with potassium chloride. Pseudo first order rate constants were obtained from the relation \( k_{\text{obs}} = \frac{0.693/14}{t} \), the half-time of the reaction, was obtained from linear plots of \( \log (A_0 - A_t) \) against time, in which \( A_0 \) and \( A_t \) are the absorbance at 290 m\( \mu \) at the end of the reaction and at a given time, respectively. The observed changes in absorbance varied with pH from about 0.30 at pH 2 to 4 to about 0.15 at pH 7 and above, with a total absorbance of about 1.5 with \( 5 \times 10^{-5} \) M THF. Readings were made against a blank cuvette that contained sufficient p-nitrobenzoic acid to give an absorbance of about 1.0 at 290 m\( \mu \) and required slit widths of 0.5 mm or less. The reaction was shown to proceed to completion by increasing the formaldehyde concentration 4- to 5-fold; the same change in absorbance was observed at all formaldehyde concentrations (by extrapolation of the first order plots to zero time), except in the presence of concentrated formaldehyde at neutral or alkaline pH in which an "initial burst" of absorbance change was observed.

The spectrophotometric measurements of THF in the presence of concentrated formaldehyde shown in Figs. 6 and 7 were made with cuvettes in which the reactants were initially separated by a partition. After the absorbance of the starting material was determined, the reactants were mixed and the absorbances was followed as a function of time against a blank of p-nitrobenzoic acid.

RESULTS

The rate of condensation of THF with a large molar excess of formaldehyde, measured spectrophotometrically at 290 m\( \mu \), is first order in respect to THF in a given experiment and the pseudo first order rate constants were found to increase linearly with increasing formaldehyde concentration in the range 0 to 0.0025 M at pH 2.33, 3.09, and 4.00 and 0 to 0.00167 M at pH 5.60 and 7.00, which indicates that the reaction is also first order in respect to formaldehyde. In the acid region, and to a lesser extent in the alkaline region, catalysis by 0.1 M buffers contributes significantly to the observed rate. The pseudo first order rate constants in the absence of buffer catalysis were obtained by measuring the rates in duplicate at four to five buffer concentrations at each of a series of pH values and extrapolating to zero buffer concentration at each pH. The data obtained with acetate buffers at three pH values are shown in Fig. 1 as an example. In this figure, the observed pseudo first order rate constants are converted to second order rate constants for the free base form of THF by dividing by the formaldehyde concentration and by \( a \), the fraction of THF (pK' 4.82) present as the free base at each pH value.

The observed pseudo first order rate constants for the reaction of THF with 0.00167 M formaldehyde, extrapolated to zero buffer concentration, exhibit a bell-shaped dependence on pH both in the presence (lower curve) and the absence (upper curve) of 0.00267 M mercaptoethanol (Fig. 2). The lower curve is similar to that obtained by Osborn, Talbert, and Huennekens (3) in the presence of approximately the same concentration of mercaptoethanol, and has a maximum at pH 4.6 with inflection points at pH 4.0 and 5.2. These reactions were initiated by the addition of formaldehyde to the reaction mixture. However, if the reaction is carried out in the absence of mercaptoethanol and the THF is protected from oxidation by argon and ethylenediaminetetraacetic acid, the observed rate constants above pH 4 are considerably larger and the maximum and inflection points of the pH rate profile occur at higher pH values (upper curve).

The inhibitory effect of mercaptoethanol on the rate is shown as a function of mercaptoethanol concentration at various pH values in Fig. 3. At pH 3.09, mercaptoethanol has little or no effect, at pH 4.00 it causes 50% inhibition at 0.02 M, and at pH 5.58 the observed rate constants approach zero with increasing mercaptoethanol concentration. This inhibition is attributed to a base-catalyzed reaction of formaldehyde with mercaptoethanol to form the unreactive hemithioacetal (12, 14, 15), which decreases the concentration of formaldehyde available for reaction with THF. Similar inhibition was observed with mercaptosuccinic acid and ethanethiol, which shows that the phenomenon is a general one and does not require a neighboring hydroxyl group.

At pH 6.57, the inhibition is less if the formaldehyde is preincubated with mercaptoethanol than if the reaction is initiated by the addition of formaldehyde (Fig. 4). By varying the time of preincubation, it was shown that at a given concentration of thiol a constant degree of inhibition, i.e. an equilibrium, for the reaction of formaldehyde and thiol is reached in less than 2 min.
FIG. 2. Dependence on pH of the pseudo first order rate constants for the reaction of THF with 0.00167 M formaldehyde, ionic strength 1.0 M and 25°C in the presence (X) and absence (•) of 0.00267 M 2-mercaptoethanol. The rate constants are extrapolated to zero buffer concentration. The pH was maintained with hydrochloric acid, formate, acetate, phosphate, N-methylmorpholine, and triethylenediamine buffers. The lines at low (---) and high (---) pH are the calculated rates of the attack and dehydration steps, respectively, and the upper solid line is the calculated rate from the steady state rate Equation 8 (see text and Table IV).

FIG. 3. Effect of mercaptoethanol on the pseudo first order rate constants for the reaction of THF with 0.00167 M formaldehyde at ionic strength 1.0 M and 25°C. The buffers were 0.033 M formate (▲), 0.033 M acetate (○) and 0.067 M acetate (●). The reactions were initiated by the addition of formaldehyde.

FIG. 4. Inhibition by mercaptoethanol of the reaction of formaldehyde with THF with (○—○) and without (●) a 10-min prior incubation of mercaptoethanol and formaldehyde. The reaction conditions were: 0.00167 M formaldehyde, 5 × 10⁻⁴ M THF, 1.3 × 10⁻⁴ M ethylenediaminetetraacetic acid, and 0.03 M phosphate buffer, pH 6.57, ionic strength 1.0 M, 25°C.

FIG. 5. The effect of mercaptoethanol concentration on the inhibition of the reaction of THF with formaldehyde under conditions in which the formaldehyde and thiol had been allowed to reach equilibrium by a 10-min incubation before the addition of THF. The experimental conditions were the same as in Fig. 4. The theoretical curve for an equilibrium constant for hemithioacetal formation of 620 M⁻¹ is shown as the solid line.

at this pH. By measuring the amount of the rate decrease which is caused by the removal of formaldehyde as the hemithioacetal after a preincubation period of 10 min at this pH at different thiol concentrations (Fig. 5), it was possible to calculate the equilibrium constant for hemithioacetal formation. The value
of $K_H = [\text{H}_2\text{C(SR)OH}]/[\text{H}_2\text{C(OH)}_2] [\text{RSH}] = 620 \text{ M}^{-1}$ so obtained was used to calculate the solid line of Fig. 5, which agrees satisfactorily with the experimental points.

At pH 8.48, there is a rapid initial increase of the absorbance of THF at 290 μm in the presence of 0.55 M formaldehyde, which is too fast to be measured by ordinary techniques, followed by a relatively slow first order formation of product; the rapid increase is labeled "Initial Burst" in Fig. 6. At 290 μm under the same conditions, the initial burst is in the opposite direction, i.e. to an absorbance lower than the initial absorbance, and it is followed by a slower increase to the absorbance of the final product. Similar experiments at a number of wave lengths made possible a comparison of the spectra of the starting material, the product, and the intermediate formed in the initial rapid reaction (Fig. 7).

At low formaldehyde concentrations, the pseudo first order rate constants for the reaction of THF with formaldehyde increase linearly with the formaldehyde concentration, but at high formaldehyde concentrations at alkaline pH, under conditions in which the initial burst is observed, the rates level off and become independent of formaldehyde concentration. At still higher formaldehyde concentrations, an inhibition of the rate is observed. The leveling off is caused by the complete conversion of THF to the intermediate product which is formed in the initial rapid reaction, so that a further increase in formaldehyde concentration cannot cause a further increase in the concentration of the intermediate or the rate of its conversion to the final product. The inhibition at very high formaldehyde concentration suggests that a 2nd molecule of formaldehyde combines with THF to form a compound which does not react further. The results of a typical experiment are shown in the form of a reciprocal plot, analogous to a Lineweaver-Burk plot, in Fig. 8. The intercept of such a
plot at the ordinate gives the reciprocal of the maximum pseudo first order rate constant at high formaldehyde concentrations (i.e. the rate constant for the conversion of the intermediate to product), and the intercept at the abscissa gives the negative of the equilibrium constant for formation of the intermediate. The inhibition of the rate at high formaldehyde concentrations is evident in the upward deviation of the experimental points from the line near the ordinate.

The maximal pseudo first order rate constants at several pH values, obtained from a series of experiments similar to that illustrated in Fig. 8, are plotted logarithmically as a function of pH in Fig. 9. The logarithms of the observed rate constants follow a sigmoid curve, with acid-catalyzed segments at high and low pH values connected by a region in which the rate is almost independent of pH. This behavior suggests that the THF-formaldehyde intermediate undergoes ionization with a $pK_a$ of approximately 10.5 and that both ionic species of the intermediate undergo acid-catalyzed reactions to give the final product.

Catalysis of the reaction of THF with formaldehyde by acetate buffers at several buffer ratios is shown in Fig. 1, with the rate constants corrected to the non-ionized species of THF ($pK_a$ 4.82; ionization of the carboxyl groups of glutamic acid is neglected). It is evident that the catalysis is proportional to the acidic species of the buffer, and the experimental points are in satisfactory agreement with the lines in the figure, which are calculated from the value 4000 M$^{-2}$ sec$^{-1}$ for the catalytic constant for acetic acid in the rate law

$$v_{cat} = k_{HA}[\text{THF}][F][HA]$$

in which $v_{cat}$ is the rate of the buffer-catalyzed reaction. However, this rate law is kinetically indistinguishable from the rate law

$$v_{cat} = \frac{k_{HA}K_{\text{THF}^+}[\text{THF}^+][F][A^-]}{K_{HA}} = k_{HA}^*[\text{THF}^+][F][A^-]$$

in which $K_{\text{THF}^+}$ and $K_{HA}$ are the ionization constants for THF$^+$ ($pK_a$ 4.82) and the catalyzing acid, respectively, and $F$ is formaldehyde concentration. Since THF is largely in the protonated form under conditions in which catalysis by carboxylic acid buffers is observed, the observed rates of the catalyzed reaction in terms of the total THF may increase with increasing pH, according to this rate law.

The catalytic constants for a number of acids, which were determined in the same manner as that for acetic acid, are summarized in Table I. The values for the solvated proton and for water were calculated from the data shown in Fig. 2 and the rate law

$$v = k_1[\text{THF}][F][\text{H}_2\text{O}] + k_2[\text{THF}][F][\text{H}_2\text{O}^+]$$

TABLE I

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>pH</th>
<th>Concentration range</th>
<th>No. of experiments</th>
<th>Fraction HA</th>
<th>$pK_a$</th>
<th>$k_{HA}$ (M$^{-2}$ sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$^+$</td>
<td>2-4</td>
<td>0.01-0.0001</td>
<td>26</td>
<td>0.1-0.9</td>
<td>2.34</td>
<td>4.8 X 10$^5$</td>
</tr>
<tr>
<td>Cyanocetic acid$^*$</td>
<td>1.6-3.3</td>
<td>0.03-0.40</td>
<td>26</td>
<td>0.1-0.9</td>
<td>2.34</td>
<td>9.1 X 10$^4$</td>
</tr>
<tr>
<td>Chloroacetic acid$^*$</td>
<td>2.34, 3.18</td>
<td>0.03-0.40</td>
<td>26</td>
<td>0.1-0.9</td>
<td>2.34</td>
<td>8.0 X 10$^4$</td>
</tr>
<tr>
<td>Formic acid</td>
<td>3.00-3.65</td>
<td>0.03-0.17</td>
<td>26</td>
<td>0.1-0.9</td>
<td>2.34</td>
<td>5.7 X 10$^5$</td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>3.67</td>
<td>0.03-0.40</td>
<td>26</td>
<td>0.1-0.9</td>
<td>2.34</td>
<td>5.2 X 10$^5$</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>4.20-4.78</td>
<td>0.017-0.10</td>
<td>26</td>
<td>0.1-0.9</td>
<td>2.34</td>
<td>4.0 X 10$^5$</td>
</tr>
<tr>
<td>Phosphate monoanion</td>
<td>5.51-6.38</td>
<td>0.02-0.10</td>
<td>26</td>
<td>0.1-0.9</td>
<td>2.34</td>
<td>2.4 X 10$^5$</td>
</tr>
<tr>
<td>N-Methylmorpholine</td>
<td>3.67-5.71</td>
<td>0.05-0.40</td>
<td>26</td>
<td>0.1-0.9</td>
<td>2.34</td>
<td>1.9 X 10$^5$</td>
</tr>
<tr>
<td>HOH</td>
<td>3.5-6.0</td>
<td>0.05-0.40</td>
<td>26</td>
<td>0.1-0.9</td>
<td>2.34</td>
<td>1.6 X 10$^5$</td>
</tr>
</tbody>
</table>

$^a v = k_{HA}[\text{THF}][F][HA].$

$^b$ Ionic strength maintained at 1.0 M by the addition of potassium chloride.

$^c$ An additional term is present in the rate law for the reaction of monocationic tetrahydrofolic acid (see "Discussion"), $v = k_{HA}^*[\text{THF}^+][F][HA^+]$, in which $k_{HA}^* = 13$ M$^{-2}$ sec$^{-1}$. 

In which $K_{\text{THF}^+}$ and $K_{HA}$ are the ionization constants for THF$^+$ ($pK_a$ 4.82) and the catalyzing acid, respectively, and $F$ is formaldehyde concentration. Since THF is largely in the protonated form under conditions in which catalysis by carboxylic acid buffers is observed, the observed rates of the catalyzed reaction in terms of the total THF may increase with increasing pH, according to this rate law.
which describes the rate of the reaction, exclusive of buffer catalysis, in the acidic pH region and is shown by the dashed line at the left in Fig. 2. In the case of cyanoacetic acid, the strongest carboxylic acid examined, the rate constants at acidic pH are too large to be accounted for entirely by the rate law of Equation 1 and suggest the presence of an additional term

\[ v_{\text{cat}} = k_{\text{HA}}[\text{THF}][F][\text{HA}] + k'_{\text{HA}}[\text{THFH}^+][F][\text{HA}] \]  

(4)

The catalytic constants for cyanoacetic acid were evaluated from a series of experiments carried out at four different buffer ratios between 10 and 90% carboxylic acid. Values of 9100 and 13 M\(^{-2}\) sec\(^{-1}\) were obtained for \(k_{\text{HA}}\) and \(k'_{\text{HA}}\), respectively.

Catalysis by phosphate buffers follows the rate law of Equation 1 at pH 5.5, 6.1, and 6.4 (○), with the rate of the catalyzed reaction proportional to the concentration of phosphate monoanion (Fig. 10). At pH 6.70 to 6.96 (●), the initial rate increase follows the same rate law as at the lower pH values, but as the buffer concentration is increased, the apparent catalytic constant decreases, and the line shows a tendency to level off. The pH of phosphate buffers is not constant with increasing buffer concentration at constant ionic strength in this pH region, but the change in rate caused by this pH variation is negligible compared to the deviation of the points from the upper straight line. It will be shown that this leveling off is another reflection of the mechanism which causes the bell-shaped pH rate profile.

General acid catalysis of the reaction is also observed in the alkaline pH region. Catalysis by triethylenediamine buffers in the presence of 0.44 M formaldehyde is proportional to the concentration of the conjugate acid of triethylenediamine at pH 8.83 (▲) and 8.54 (●) (Fig. 11). These experiments were carried out at high formaldehyde concentrations in order to avoid a change in the rate caused by the (small) binding of formaldehyde to the buffer (16); at these concentrations of formaldehyde, the rate is independent of formaldehyde concentration and such binding would not affect the rate. The catalytic constants for several acids, obtained from similar experiments, are summarized in Table II.

Secondary amines, such as morpholine and imidazole, are far more effective at low concentrations than are other catalysts of the reaction of THF with formaldehyde. However, as the con-

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**Fig. 10.** General acid catalysis by phosphate monoanion of the reaction of THF with 0.00167 M formaldehyde at pH 5.51, 6.10, 6.38 (○), and 6.70 to 6.96 (●) at ionic strength 1.0 M and 25°C. The curved line is calculated from the steady state rate Equation 9.

**Fig. 11.** Catalysis by triethylenediamine buffers of the reaction of THF with 0.44 M formaldehyde at pH 8.54 (○) and 8.83 (▲) at ionic strength 1.0 M, and 25°C; \(k_0\) is the rate constant extrapolated to zero buffer concentration.

### Table II

Second order rate constants for general acid catalysis of dehydration of hydroxymethyl intermediate of THF at 25°C

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>pH</th>
<th>Concentration range</th>
<th>No. of experiments</th>
<th>Fraction HA</th>
<th>pK(_a)</th>
<th>(k_{\text{HA}}) M(^{-1}) sec(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_3)O(^+)</td>
<td>7.5–8.5</td>
<td></td>
<td></td>
<td></td>
<td>−1.7</td>
<td>2.7 × 10(^7)</td>
</tr>
<tr>
<td>Phosphate monoanion(^a)</td>
<td>6.7–7.0</td>
<td>0.02–0.40</td>
<td>57</td>
<td>0.31–0.05</td>
<td>6.40</td>
<td>42</td>
</tr>
<tr>
<td>(N,N')-Dimethylpiperazine(^c)</td>
<td>7.60–8.57</td>
<td>0.05–0.50</td>
<td>41</td>
<td>0.50–0.90</td>
<td>8.57</td>
<td>0.32</td>
</tr>
<tr>
<td>Triethylenediamine(^c)</td>
<td>8.54–8.83</td>
<td>0.05–0.50</td>
<td>19</td>
<td>0.68–0.78</td>
<td>9.15</td>
<td>0.18</td>
</tr>
</tbody>
</table>

\(^a\) Ionic strength maintained at 1.0 M with potassium chloride.

\(^b\) Obtained from Equation 9 and the data of Fig. 10 (see text).

\(^c\) Measured at 0.44 M total formaldehyde concentration, for which the reaction rate is independent of the formaldehyde concentration.
centration of secondary amine is increased there is a leveling off and then an inhibition of the rate at high amine concentrations, as shown for morpholine in Fig. 12. The catalyzed reaction becomes faster as the pH is decreased. The rate of the secondary amine-catalyzed reaction was found to be directly proportional to the formaldehyde concentration under the following conditions: 0 to 0.0033 M formaldehyde at pH 7.84 for 0.0033 and 0.017 M morpholine; 0 to 0.01 M formaldehyde at pH 8.49 for 0.033 M morpholine; 0 to 0.0063 M formaldehyde at pH 6.29 and 0 to 0.008 M formaldehyde at pH 6.86 for 0.067 M imidazole. The second order rate constants for the maximal velocities, \( k'_{\text{N}} = k_{\text{obs (max)}}/[F] \) in which \( k_{\text{obs (max)}} \) is obtained from the intercept of extrapolations of the linear portions of plots of \( 1/k_{\text{obs}} \) against \( 1/[\text{amine}] \), are plotted logarithmically against pH in the inset of Fig. 12. The data in Fig. 12 suggest that the mechanism of catalysis involves an acid-catalyzed reaction of an intermediate which accumulates at high concentrations of secondary amine.

Catalysis of the reaction by imidazole was studied in less detail than that by morpholine, but it is apparent from the data shown in Table III that the imidazole-catalyzed reaction is also catalyzed by acid and shows saturation with increasing imidazole concentration. Under conditions of saturation in respect to the catalyst and at a given formaldehyde concentration and pH, imidazole is about 10-fold less effective than morpholine as a catalyst. N-Methylpiperazine was also found to be an effective catalyst and to show saturation with respect to amine concentration at pH 5.38.

In the presence of secondary amine catalysts there is an induction period if the reaction is started by the addition of formaldehyde, as shown for the reaction of morpholine at pH 8.02 in

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**Table III**

<table>
<thead>
<tr>
<th>Total imidazole</th>
<th>( k'_{\text{N}} ) (M(^{-1}) sec(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )</td>
<td>pH 5.05</td>
</tr>
<tr>
<td>0</td>
<td>85.0</td>
</tr>
<tr>
<td>0.00167</td>
<td>96.3</td>
</tr>
<tr>
<td>0.0033</td>
<td>112</td>
</tr>
<tr>
<td>0.0067</td>
<td>119</td>
</tr>
<tr>
<td>0.0133</td>
<td></td>
</tr>
<tr>
<td>0.0167</td>
<td>129</td>
</tr>
<tr>
<td>0.033</td>
<td>208</td>
</tr>
<tr>
<td>0.067</td>
<td>306</td>
</tr>
<tr>
<td>0.099</td>
<td>368</td>
</tr>
<tr>
<td>0.130</td>
<td>693</td>
</tr>
<tr>
<td>0.194</td>
<td>822</td>
</tr>
<tr>
<td>0.400</td>
<td>462</td>
</tr>
</tbody>
</table>

* The reaction was started after 10 min of prior incubation of imidazole and formaldehyde; formaldehyde, \( 2.1 \times 10^{-4} \text{ M} \), ionic strength 1.0 M, 25°C.

\( k'_{\text{N}} \) (M\(^{-1}\) sec\(^{-1}\)) = \( k_{\text{abs}}/[F]_{\alpha} \), where \( \alpha \) is the fraction of total THF as the free base.

* Sodium acetate, 0.02 M.
* Potassium phosphate, 0.02 M.
* Potassium phosphate, 0.05 M.
* Extrapolated value, from a plot of \( 1/k'_{\text{N}} \) against \( 1/[\text{imidazole}] \).
Fig. 13 (●). The induction period is abolished by brief preincubation of the amine and formaldehyde before addition of THF (Fig. 13, △). This evidence also suggests that an intermediate is formed from the amine and formaldehyde in this reaction.

**DISCUSSION**

**Hemithioacetal Formation from Formaldehyde and Thiols**—The inhibition by thiols of the condensation of THF with formaldehyde at neutral and alkaline pH is undoubtedly caused by the well-known reaction of thiols with formaldehyde and other carbonyl compounds (12, 14, 15) to form the hemithioacetal (Equation 5).

The fact that inhibition of the reaction of formaldehyde with THF is not observed in acid solution indicates that hemithioacetal formation is base-catalyzed; i.e., the thiol anion is the reactive species. It was shown by Ratner and Clarke that the reaction of N-acetylcysteine with formaldehyde is subject to base catalysis (15).

Inhibition by thiols of the reaction of THF with formaldehyde occurs by two different mechanisms, depending on the experimental conditions. If the thiol and formaldehyde are allowed to react with each other to reach equilibrium before THF is added to the reaction mixture, the inhibition is simply the result of the decreased concentration of formaldehyde at equilibrium which is caused by hemithioacetal formation. However, if the reaction is initiated by the addition of formaldehyde, the inhibition is greater, at a given thiol concentration (Fig. 4), and a different mechanism of inhibition must occur. This type of inhibition is attributed to a competition between THF and thiol for the limited amount of free, unhydrated formaldehyde which is made available by the dehydration of formaldehyde hydrate.

The equilibrium constant for formaldehyde hydration, $K_H = [\text{H}_2\text{C(OH)}_2]/([\text{HCHO}][\text{HOH}])$, is $10^8$ to $10^9$ and indicates that formaldehyde is more than 99.9% hydrated in aqueous solution (17, 18). The rate of dehydration of formaldehyde hydrate to free formaldehyde has been determined (19) and is more than adequate to supply unhydrated formaldehyde for the reaction with THF at concentrations of these compounds which were used in these experiments in the absence of competing nucleophilic reagents. That the dehydration of formaldehyde hydrate is ordinarily not rate-determining is also shown by the fact that the reactions are first order in respect to THF.

However, if formaldehyde is added to a solution which contains an excess of thiol at a sufficiently high pH, the unhydrated formaldehyde will react with thiol to form hemithioacetal as fast as it is formed by the relatively slow dehydration of formaldehyde hydrate, thereby lowering the concentration of unhydrated formaldehyde available for the reaction with THF. Under these conditions, the THF competes with thiol for the limited amount of unhydrated formaldehyde and, since the reactive thiol is present in much larger concentrations than that of THF, the THF reaction is inhibited. Under equilibrium conditions, the smaller inhibition is caused only by the decrease in the equilibrium concentration of unhydrated formaldehyde, because at equilibrium the rate of formation of hemithioacetal is equal to its rate of breakdown to unhydrated formaldehyde, so that the formation of unhydrated formaldehyde is no longer rate-determining in the THF reaction.

The fact that inhibition of the THF-formaldehyde reaction by thiols can occur by the trapping of free formaldehyde as it is formed demonstrates that it is free formaldehyde and not formaldehyde hydrate that is the reactive species in the reaction with THF.

The equilibrium constant of 620 M$^{-1}$ for hemithioacetal formation from formaldehyde and mercaptoethanol at pH 6.57, estimated from the inhibition of the THF-formaldehyde reaction under conditions in which it was shown that equilibrium had been reached in the hemithioacetal reaction, is similar to that of approximately 500 M$^{-1}$ for the addition of glutathione to methylglyoxal, which is also hydrated in water (20), and is somewhat larger than the values of 18 to 136 M$^{-1}$ for the addition of various thiols to pyridoxal phosphate (21, 22).

The fact that the inhibition by mercaptoethanol is dependent on pH accounts for the difference in the positions of the ascending and descending limbs of the pH rate profile in the presence and absence of mercaptoethanol (Fig. 2) and means that the inflection points of these curves in the presence of mercaptoethanol cannot be assigned to dissociation constants of THF (cf. Reference 3). Preliminary evidence has been reported for the existence of an enzyme which catalyzes the reaction of THF with formaldehyde in the presence of 0.01 M mercaptoethanol at pH 7.5 (23). This concentration of mercaptoethanol would be expected to reduce the rate of reaction of formaldehyde with THF to a small fraction of the uninhibited value and raises the possibility, among several others, that this enzyme activity involved a facilitation of the liberation of free formaldehyde from the hemithioacetal, rather than of the condensation of formaldehyde with THF.

**Significance of Bell-shaped pH Rate Profile for Reaction of THF and Formaldehyde**—The midpoints of the ascending and descending limbs of the pH rate profile for the reaction of THF with formaldehyde do not correspond to dissociation constants of THF (24), either in the presence or in the absence of mercaptoethanol. Although the midpoint of the acidic limb of the curve in the absence of mercaptoethanol is close to the pK$_a$ value for ionization of the N$_3$ position of THF, there is no ionization in either THF or (hydrated) formaldehyde near the midpoint of the alkaline limb, at about pH 7. The bell-shaped pH
rate profile must be, therefore, an expression of the kinetics of the reaction rather than of the ionization constants of the reactants. Such behavior has been observed in a number of carbonyl group reactions (10, 25) and at least one enzymic reaction (26) and may be most simply described as a change in the rate-determining step of the reaction with changing pH, such that attack of the nucleophilic reagent on the carbonyl group is rate-determining in one pH region, while dehydration of the resulting addition product is rate-determining in another pH region. These steps are shown for the reaction of THF with formaldehyde in Equation 6. It is probable that the intramolecular attack of an amine on the cationic imine in the last step of the reaction is very fast, so that the pH rate profile may be interpreted in terms of rate-determining attack of the basic N5 nitrogen atom of THF in one pH region (Step 1) and rate-determining dehydration of the carbinolamine intermediate (II) to a cationic imine (III) in another pH region (Step 2).

The dependence on pH of the rates provides evidence for a change in rate-determining step, but does not show which step is rate-determining in each pH region. Analogy with other condensations of carbonyl groups with nitrogen compounds suggests that attack of amine on the carbonyl group is rate-determining at acidic pH, while dehydration of the carbinolamine intermediate is rate-determining at alkaline pH (10). The dehydration step is acid-catalyzed, which accounts for the increasing rate with decreasing pH in this region, but as this rate becomes very fast with increasing acidity the rate of amine attack can no longer keep up with dehydration and amine attack becomes rate-determining. The rate of the attack step increases with increasing pH in the region in which the amine is protonated, because the free base form of the amine is the reactive species. It was possible to obtain direct evidence that this assignment of the rate-determining steps is correct also for the reaction of THF with formaldehyde. At alkaline pH values there is a very rapid reaction of THF with formaldehyde, so that the rate law in this region is based on this equation and the rate constants summarized in Table IV.

Further evidence for the formation of such an adduct is the leveling off of the rate with increasing formaldehyde concentration at alkaline pH (Fig. 8). When THF is completely converted to the hydroxymethyl (carbinolamine) addition product, a further addition of formaldehyde cannot result in a further rate increase and the rate of dehydration of the intermediate may be observed directly. The equilibrium constant for the formation of this adduct, obtained from a series of reciprocal plots of these data similar to that shown in Fig. 8, is 32 ± 6 \( \times 10^{-4} \), which is in the range of the values of 18 to 42 \( \times 10^{-4} \) reported for the addition of formaldehyde to 2-amino-6-methyl-5,6,7,8-tetrahydro-4-pteridinone, a model for the tetrahydropteridinone.

Evidence that supports this assumption is given in the last section.

rate-determining attack of the basic N₅ nitrogen atom of THF in one pH region (Step 1) and rate-determining dehydration of the carbinolamine intermediate (II) to a cationic imine (III) in another pH region (Step 2).

The calculated line for this rate law, based on the measured equilibrium constant for addition product formation, and the rate constant for its dehydration, which are summarized in Table IV, is shown as the dot-dashed line on the right side of Fig. 2. The fact that the observed rates do not drop to zero with increasing acidity at pH values below the maximum means that there is an acid-catalyzed as well as an uncatalyzed addition of the free base form of THF to formaldehyde, so that the rate law in this region is that of Equation 3. The dashed line on the left side of Fig. 2 is based on this equation and the rate constants summarized in Table IV and accounts satisfactorily for the observed rates in the acidic pH region.

At intermediate regions of pH, both steps are partially rate-determining and the rate can be described only by the use of a steady state rate equation with the form of Equation 8, which is based on the mechanism of Equation 6.

The calculated rate from this equation and the rate constants given in Table IV is shown as the solid line in Fig. 2. This line shows satisfactory agreement with the observed rate constants over the entire range of pH, within the experimental error of up to 20% for reactions with half-times of 3 to 5 sec.

Further evidence for a change in rate-determining step comes from the dependence of the rate on buffer concentration at a constant pH. At pH 5.51, 6.10, and 6.38, the reaction is catalyzed by phosphate buffers and the rate is directly proportional to the concentration of the phosphate monoanion. However, at
TABLE IV

<table>
<thead>
<tr>
<th>Apparent rate and equilibrium constants for reaction of tetrahydrofolic acid and formaldehyde at 25°, ionic strength 1.0 M, water activity 1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{\text{app.~act}} = \frac{[\text{THF}] [F]}{[\text{A}] [\text{H}]}$</td>
</tr>
<tr>
<td>$K_1 = \frac{[\text{THF}] [\text{CH}_2\text{OH}]}{[\text{A}] [\text{H}]}$</td>
</tr>
<tr>
<td>$K_2/K_1 = \frac{[\text{THF}] [\text{CH}_2\text{OH}]}{[\text{A}] [\text{H}]}$</td>
</tr>
<tr>
<td>$k_1 = \frac{[\text{THF}] [\text{F}]}{[\text{A}] [\text{H}]}$</td>
</tr>
<tr>
<td>$k_1' = \frac{[\text{THF}] [\text{F}][\text{H}^+]}{[\text{A}] [\text{H}]}$</td>
</tr>
<tr>
<td>$k_1'' = \frac{[\text{THF}] [\text{F}][\text{H}_2\text{PO}_4^-]}{[\text{A}] [\text{H}]}$</td>
</tr>
<tr>
<td>$k_{-1} = \frac{[\text{N}_2\text{CH}_2\text{OH}]}{[\text{H}^+] [\text{A}] [\text{H}]}$</td>
</tr>
<tr>
<td>$k_{-1}' = \frac{[\text{N}_2\text{CH}_2\text{OH}][\text{H}^+]}{[\text{A}] [\text{H}]}$</td>
</tr>
<tr>
<td>$k_{-1}'' = \frac{[\text{N}_2\text{CH}_2\text{OH}][\text{H}_2\text{PO}_4^-]}{[\text{A}] [\text{H}]}$</td>
</tr>
<tr>
<td>$k_2 = \frac{[\text{N}_2\text{CH}_2\text{OH}][\text{H}^+]}{[\text{A}] [\text{H}]}$</td>
</tr>
<tr>
<td>$k_2' = \frac{[\text{N}_2\text{CH}_2\text{OH}][\text{H}_2\text{PO}_4^-]}{[\text{A}] [\text{H}]}$</td>
</tr>
<tr>
<td>$k_3 = \frac{[\text{A}] [\text{H}^+]}{[\text{H}_2\text{PO}_4^-]}$</td>
</tr>
<tr>
<td>$k_3' = \frac{[\text{A}] [\text{H}^+]}{[\text{H}_2\text{PO}_4^-]}$</td>
</tr>
<tr>
<td>$k_{3a} = \frac{[\text{A}] [\text{H}^+]}{[\text{H}_2\text{PO}_4^-]}$</td>
</tr>
</tbody>
</table>

The steady state rate Equation 9 may be derived from the scheme of Equation 6 by including the rate constants for buffer catalysis $k_{3a}[\text{HA}], k_{3a}[\text{HA}]$ and $k_{3a}[\text{HA}]$ for the first and second steps. The lower solid line of Fig. 10 was calculated from Equation 9 and the rate constants shown in Table IV and shows satisfactory agreement with the experimental data.

General Acid Catalysis of Attack Step—The Brønsted slope, $\alpha$, is approximately 0.20, for a logarithmic plot of the catalytic constants of a series of general acid catalysts against $pK_a$ for catalysis of the attack of the $N_4$ position of THF on formaldehyde (Fig. 14). The solvated proton and water show deviations of about one order of magnitude from the line drawn through the points for carboxylic acids. This value of $\alpha$ for the attack of the $N_4$ position of THF ($pK_a 4.8$) is similar to the values of 0.25 for general acid catalysis of the attack of semicarbazide ($pK_a 3.7$) and aniline ($pK_a 4.6$) on aldehydes and suggests that the catalysis occurs by a similar mechanism in these reactions (28, 29). There is evidence which supports the mechanism shown in transition state V for these reactions (10, 28, 30), but Mechanism VI, which has the same stoichiometric composition and is kinetically indistinguishable, must also be considered. In addition to the analogy to other similar reactions, the following evidence provides support for Mechanism V for the reaction of THF with formaldehyde.

$$H-\text{N} \cdots \text{C} \cdots \text{O} \cdots \text{H} \cdots \text{A} \cdots \text{H} \cdots \text{N} \cdots \text{C} \cdots \text{OH}$$

V

VI

For the reaction catalyzed by the solvated proton, the most probable pathway for the formation of transition state VI involves the protonation of free formaldehyde in a prior equilibrium step, followed by the removal of a proton by water, acting as a general base catalyst, from the attacking THF. From the lowest

pH 6.70 to 6.96, in the region in which the change in rate-determining step occurs, the rate shows the same proportionality to phosphate monoanion concentration at low phosphate concentration, but shows a tendency to level off at higher buffer concentrations (Fig. 10). The step which is subject to catalysis by phosphate monoanion at low pH is the attack step and this is the step which shows catalysis at low buffer concentrations at neutral pH; however, as the buffer concentration is increased in this pH region the attack step becomes faster and the dehydration step, which is less subject to buffer catalysis, becomes partly rate-determining. Similar behavior is observed in semicarbazone formation, in which a change in rate-determining step
estimated value for the equilibrium constant for formaldehyde hydration, \( K_H = [H_2C(OH)\text{H}]_0/[H_2C(O)(OH)] = 10^6 \) (17) and the assumption that the basicity of free formaldehyde is as large as that which has been estimated for acetone by spectrophotometric titration (pK\( \alpha \) for protonated acetone = -7.2 (32)) a value of \( 7.4 \times 10^7 \ m^{-1} \sec^{-1} \) may be calculated for the rate constant of the solvated proton catalyzed reaction by this mechanism. This is more than four orders of magnitude larger than the value of \( 1.4 \times 10^2 \ m^{-1} \sec^{-1} \) for the fastest known diffusion-controlled reaction, the reaction of hydroxide ion with the solvated proton (32), which effectively rules out this mechanism. Reactions which involve other general acids, HA, would require involvement of their conjugate bases A\(-\) according to Mechanism VI. These bases would be expected to be more effective catalysts than water, so that the rates for such reactions would have to be even faster than that for the solvated proton if Mechanism VI held. These considerations favor Mechanism V for all general catalysts.

Cyanoacetic acid, a relatively strong carboxylic acid, catalyzes the attack on formaldehyde of THFH\(^+\) as well as THF (Equation 4). The reactive species of THFH\(^+\) in this reaction is not established, but it is probable that it is one of the forms of THFH\(^1\) formed in a microscopic ionization, with the N\(_3\) position free and the proton on the N\(_1\) or N\(_2\) positions; rough estimates of the expected microscopic ionization constants suggest that this is a reasonable assumption. In any case, the ratio of the rate constants for the cyanoacetic acid-catalyzed reactions of THF and THFH\(^+\), \( k_H/k_nA = 700 \). A similar ratio is expected for the water-catalyzed reaction of THF and THFH\(^+\) with formaldehyde. Such reactivity ratios show only a very small sensitivity to the strength of the catalyzing acid; for example, the reactivity ratio for p-nitrobenzaldehyde to p-hydroxybenzaldehyde with semicarbazide changes less than a-fold with a change of 10\(^17\) in the strength of the catalyzing acid (28). This gives an expected value of 0.13 m\(^-1\) sec\(^-1\) for the water-catalyzed reaction of THFH\(^+\). This is much smaller than the value of 7.3 m\(^-1\) sec\(^-1\) based upon the observed rate of the proton-catalyzed reaction, but expressed as the kinetically indistinguishable water-catalyzed reaction of THFH\(^+\). Thus, the latter mechanism appears to make only a small contribution to the observed rate at low pH and the observed reaction may be interpreted as true general acid catalysis by the solvated proton of the attack of the free base form of THF.

Dehydration Step—The rate of the dehydration step can be measured directly under conditions in which THF has been largely converted to the hydroxymethyl adduct in an initial fast reaction; the specific rate of the dehydration step is obtained from extrapolation to infinite formaldehyde concentration, at which all of the THF is hydroxymethylated, by the use of reciprocal plots (Fig. 8). The dependence on pH of the dehydration step indicates that it is acid-catalyzed and that both hydroxymethyl-THF and the conjugate base of hydroxymethyl-THF react at significant rates (Figs. 2 and 9). The pK\( \alpha \) of hydroxymethyl-THF, which may be estimated from Fig. 9, is 10.5, the same as the pK\( \alpha \) of the THF itself; the hydroxymethyl group would not be expected to have a large effect on the loss of a proton from the N\(_3\) position (24). The solid line in the figure is a calculated line based on this pK value.

The dehydration step is subject to general acid catalysis, but this can be measured only between pH 7.5 and 8.5, because the change in rate-determining step occurs at lower pH and the reaction of the hydroxymethyl-THF anion becomes significant at higher pH. The limited data which could be obtained for this catalysis (Table II) indicate that it has a Brønsted coefficient, \( \alpha \), of approximately 0.75 and the absence of detectable catalysis of the dehydration step by water is in accord with such a high value of \( \alpha \). This large value of \( \alpha \) also reflects the experimental fact that catalysis by the solvated proton is dominant, so that catalysis by weaker acids is of comparatively little importance. The difference between this value and the value \( \alpha = 0.20 \) for general acid catalysis at low pH values is further evidence for a difference in the nature of the rate-determining step at alkaline and acid pH.

The probable mechanism of this step, shown in transition state, VII, is similar to that for related reactions (10).

\[
\begin{array}{cccc}
\delta^+ & H \delta^+ & \delta^- \\
N \overset{\text{CH}_2 \cdots O \cdots H \cdots A} {\text{VII}}
\end{array}
\]

In this case there is no kinetic ambiguity, because the alternative mechanism, which involves pre-equilibrium protonation of the leaving hydroxyl group and general base catalysis by removal of a proton from the nitrogen atom, concomitantly with the expulsion of water, is not possible for THF, because THF has no proton on the nitrogen atom of the hydroxymethylated secondary amine. The suggestion by Swain et al. that catalysis may involve solvation of the partial positive charge on the nitrogen atom by a catalyzing anionic base in the transition state (33) does not appear to be tenable in this case, because catalysis is observed with both neutral and anionic catalysts; this point is considered in greater detail elsewhere (34, 35).

Nucleophilic Catalysis by Secondary Amines—The evidence that catalysis of the reaction of THF with formaldehyde by secondary amines, such as morpholine and imidazole, is nucleophilic catalysis is as follows. (a) Secondary amines are far more effective catalysts than general acid-base catalysts and show effective catalysis in the 0.01 m concentration range. (b) As the concentration of amine catalysts is increased, the rate levels off and finally decreases at high concentrations of amine. This suggests the formation and accumulation of an intermediate at high concentration. (c) An induction period in the amine-catalyzed reaction is eliminated if the amine and formaldehyde are allowed to react with each other before the addition of THF.

This evidence and the fact that the rate of the amine-catalyzed reaction increases with increasing acidity support the mechanism shown in Equation 10 for catalysis by secondary amines. The secondary amine catalyst (with the circled nitrogen atom) and formaldehyde react to form a hydroxymethylamine, \( \text{NCH}_2\text{OH} \), which undergoes dehydration to give the reactive cationic imine intermediate, \( \overset{\text{N=CH}} {\text{I}} \). This intermediate reacts with the N\(_3\) nitrogen atom of THF to give the methylenediamine intermediate, VIII, which undergoes acid-catalyzed loss of the catalyzing amine to give the same cationic imine as in the uncatalyzed pathway, III, which rapidly undergoes ring closure to give 5,10-methylene-THF (I). Inhibition of the reaction at high concen-
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trations of amine is caused by the formation of the unreactive methylenediamine, \(\text{NCH}_2\text{N}\), from formaldehyde and 2 molecules of secondary amine (Step G).

The determination of the equilibrium constants for the formation of the hydroxymethylamine and methylenediamine adducts of morpholine (16) permits a comparison of the rate of the catalyzed reaction with the concentrations of these species, which provides further support for this scheme. In Fig. 15 are plotted the rate constants for the morpholine-catalyzed reaction as a function of total morpholine concentration and the concentrations of hydroxymethylmorpholine and methylenedimorpholine, calculated from the equilibrium constants for the formation of these species. It is evident that, within experimental error, the rate of the catalyzed reaction follows the concentration of hydroxymethylmorpholine and not that of methylenedimorpholine. In particular, the decrease in the rate at higher morpholine concentrations occurs as the concentration of hydroxymethylmorpholine begins to decrease, because of methylenedimorpholine formation, and as the concentration of methylenedimorpholine continues to increase. Hydroxymethylamines and methylenediamines have both been suggested as intermediates in a number of other reactions which involve formaldehyde and amines (36-39).

The induction period which is abolished by prior incubation of formaldehyde and amine is attributed to the time-dependent formation of hydroxymethylamine (Equation 10, Steps A and B). The rate constant of 90 M\(^{-1}\) sec\(^{-1}\) for the reaction of the \(\text{NC}\) nitrogen atom of THF with formaldehyde suggests that the reaction of relatively concentrated solutions of the considerably more basic morpholine would be even faster, so that the rate-determining step for hydroxymethylamine formation would be the dehydration of formaldehyde hydrate to the reactive free formaldehyde. The rate constant for this dehydration has been reported to be 0.27 min\(^{-1}\) (19), which is of the correct magnitude to account for the observed induction period. As in the case of thiols, morpholine will compete with the much more dilute and less reactive THF for the free formaldehyde as it is formed, so that the reaction with THF will be inhibited until enough of the reactive formaldehyde-amine intermediate is formed to allow the catalyzed reaction pathway to become significant.

The above evidence, the fact that the rate of the catalyzed reaction is proportional to the proton concentration under conditions in which all of the formaldehyde is converted to hydroxymethylmorpholine, and the kinetic data for the reaction of hydroxymethylmorpholine and formaldehyde with tetrahydrofolic acid (15) are all consistent with the mechanism shown below.

\[
\begin{align*}
\text{HCOH} + \text{N} &\rightarrow \text{NCH}_2\text{N} + \text{H}_2\text{O} & \text{(10)} \\
\end{align*}
\]

Fig. 15. Dependence on total morpholine concentration of the pseudo first order rate constants for morpholine catalysis of the reaction of THF with 0.00167 M formaldehyde in 0.05 M triethylenediamine buffers, pH 8.49, ionic strength 1.0 M, and 25°C. The solid line shows the calculated concentration of hydroxymethylmorpholine, \(N_x\), and the dashed line that of methylenedimorpholine, \(N_y\) (ordinates on right). The value of \(k_4\), for the small contribution of the noncatalyzed reaction, was calculated from \(k_{\text{obs}}\) at zero morpholine concentration multiplied by the calculated fraction of formaldehyde in the free, unbound form. The morpholine and formaldehyde were allowed to react for 10 min prior to the addition of THF.
methylmorpholine (Fig. 12, inset), and the fact that the rate is first order in respect to THF permit the conclusion that the transition state for the catalyzed reaction contains the elements of formaldehyde, THF, 1 mole of morpholine and a proton, under conditions in which the induction period has been abolished by prior incubation of amine with formaldehyde. The rate determining step in the scheme of Equation 10, therefore, can be either D, the reaction of the cationic imine with THF, or E, the acid-catalyzed expulsion of morpholine from the intermediate VIII. It has not yet been possible to distinguish between these two possibilities.

The rapid rate of the catalyzed reaction, incidentally, shows that the ring closure in the final step of the reaction (Step F) is not rate-determining in either the catalyzed or uncatalyzed reaction. It is not rate-determining in the uncatalyzed reaction because the catalyzed reaction proceeds at a much faster rate and involves the same intermediate. It is not rate-determining in the catalyzed reaction because this step does not involve the catalyst and the kinetic evidence shows that a molecule of catalyst is present in the transition state.

Mechanisms can also be written for both the catalyzed and uncatalyzed reaction pathways in which an S_N2 displacement occurs on a saturated carbon atom (IX and X). Similar mechanisms have been proposed for the Mannich reaction and related reactions (37, 38, 40, 41). We reject such mechanisms on the ground that almost all reactions of carbonyl compounds or their derivatives that have been studied in detail occur by an elimination-addition pathway rather than by a direct S_N2 displacement (10). In particular, the condensation of acetone, cyanide, and a secondary amine (42) and the amine-catalyzed reaction of aldehydes with hydroxylamine and semicarbazide (43) occur at rates that are independent of the concentration of the final carbonyl group acceptor. This shows that the formation of an unsaturated imine intermediate, rather than an S_N2 displacement on a saturated intermediate, must be the rate-determining step in these reactions.

The foregoing supports the hypothesis (7, 8) that in some enzymic reactions of formaldehyde and THF, cationic imine intermediates (e.g. III), rather than methylene-THF itself, are the reactive species. The fact that the N_3 position of THF is a secondary amine ensures the formation of such an intermediate rather than the less reactive, neutral imine which would be formed from a primary amine. Secondary amine catalysts such as imidazole, in the active sites of enzymes may also facilitate such reactions.

Imidazole, although less efficient than morpholine, is an effective nucleophile catalyst. The aromatic ring of imidazole would be expected to destabilize a cationic imine formed from this compound and imidazole was found to be ineffective as a nucleophilic catalyst for the formation of pyridoxal phosphate semicarbazone (44).

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