Mechanism of Reduction of Dihydrofolate to Tetrahydrofolate

STUDIES WITH 7-METHYLDIHYDROFOLATE AS A MODEL COMPOUND*

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SUMMARY

7-Methyl-7,8-dihydrofolate was used as a model compound to study the mechanism of reduction of 7,8-dihydrofolate to tetrahydrofolate. 7-Methylfolate is not a substrate but is a competitive inhibitor of folate reductase ($K_I = 1.3 \times 10^{-6}$ M). Since 7-methyl-7,8-dihydrofolate could not be prepared by dithionite reduction of 7-methylfolate, a new method involving reduction with zinc dust in 1.0 M NaOH was used. When this reduction was performed in the presence of tritiated water, tritium was incorporated not only at position 7, but also at carbon 9 and in the 7-methyl group of 7-methyl-7,8-dihydrofolate.

As a result of the study of the mechanism of reduction of 7-methyl-7,8-dihydrofolate to 7-methyltetrahydrofolate by different agents, the following was established. (a) The reduction with dithionite proceeds entirely through an intramolecular rearrangement involving elimination of hydrogen from carbon 7. (b) The catalytic hydrogenation in water proceeds almost entirely by the direct addition of hydrogen to the 5,6-double bond. (c) The reduction with borohydride proceeds mainly through the direct addition of hydrogen as in b, and to the lesser extent through rearrangement as in a. The implications of these results with respect to the mechanism of reduction of dihydrofolate are discussed.

In a previous communication from this laboratory (1), evidence was presented indicating that the enzymatic reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate is preceded by an intramolecular rearrangement (involving expulsion of hydrogen or proton from carbon 7) presumably to 5,8- or 5,6-dihydrofolate (Fig. 1). A similar mechanism has been found to be involved when the reduction of H$_2$-folate was carried out with dithionite. The conclusion that the rearrangement of H$_2$-folate must have taken place prior to the reduction was based on the following observations. (a) Tritium introduced into H$_2$-folate during the reduction of H$_2$-folate is retained in the molecule after H$_2$-folate has been oxidized back to H$_2$-folate. This indicates that tritium must have been incorporated at carbon 7 and not carbon 6 (Fig. 1). (b) When H$_2$-folate in which 1 of the 2 hydrogen atoms at carbon 7 is replaced by deuterium was reduced to H$_2$-folate (in the absence of deuterium), a large portion of deuterium was eliminated.

Recently, Scrimgeour and Vitals presented evidence that the reduction of H$_2$-folate with sodium borohydride proceeds by the direct addition of the hydrogen atoms to the 5,6 double bond of H$_2$-folate (2). This is in agreement with the results obtained earlier by Kaufman who used borohydride for reduction of 2-amino-4-hydroxy-6,7-dimethyl-7,8-dihydropteridine (3).

The present investigation was undertaken in order to clarify these apparent discrepancies. To avoid isotope effects, 7-methyl-H$_2$-folate was chosen as a model compound for the present study. This compound has only 1 hydrogen at carbon 7. Thus, when 7-methyl-H$_2$-folate tritiated at position 7 is reduced to 7-methyl-H$_4$-folate, all tritium should be removed from position 7 if the rearrangement takes place. No tritium, however, should be removed if the reduction proceeds through the direct addition of hydrogen to the 5,6-double bond. It will be shown here that indeed, depending on the conditions used, the reduction may proceed by either or both of the two mechanisms, rearrangement followed by the reduction or direct reduction of 5,6-double bond.

METHODS

Preparation of 7-Methylfolate 7-Methylfolate was prepared according to the procedure of Boothe et al. (4). The ultraviolet spectrum of this compound at pH 7 was identical with that of folate. In 0.1 N NaOH the spectrum, although very similar to

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Reduction of Folate and 7-Methylfolate—Attempts to reduce 7-methylfolate with dithionite at room temperature as well as at elevated temperatures were unsuccessful. A new method for preparation of H_{2}-folate which involves reduction of folate with zinc in alkaline solution has been worked out. This method after slight modification was also applicable to 7-methylfolate. To reduce folate the following procedure was used. Commercial folate (50 mg) (General Biochemicals) was dissolved in 1 ml of 1 M NaOH, 100 mg of zinc dust were added, and the mixture was stirred at room temperature for 30 min. Zinc was separated by filtration so as to allow the filtrate to flow directly into a solution of 450 mg of sodium ascorbate in 9 ml of water. This diluted solution was chilled in ice and acidified to pH 3 by a slow addition of glacial acetic acid. The partially crystalline precipitate which formed was centrifuged, washed twice with cold 0.1 M acetic acid, twice with acetone, and finally with ether. The yield of the air-dried material was 70 to 80%. This material was identical with H_{2}-folate prepared by the procedure of Blakley (5) with respect to the following properties: ultraviolet spectrum, proton magnetic resonance spectrum, mobility on paper in 0.1 M phosphate buffer pH 7, mobility on DEAE-cellulose column, and substrate activity for dihydrofolate reductase.

To reduce 7-methylfolate the procedure was modified as follows. The reaction mixture was stirred at room temperature for about 18 hours. Then the mixture was diluted with about 10 times its own volume of 0.25 M Na_{2}HPO_{4} containing 1% mercaptoethanol. Zinc was removed by filtration and the clear solution was adjusted to pH 4 with 1.0 M HCl. After centrifugation the material was washed as described for H_{2}-folate. This crude material was suspended in ice and acidified to pH 3 by a slow addition of glacial acetic acid. The partially crystalline precipitate which formed was centrifuged, washed twice with cold 0.1 M acetic acid, twice with acetone, and finally with ether. The yield of the air-dried material was 70 to 80%. This material was identical with H_{2}-folate prepared by the procedure of Blakley (5) with respect to the following properties: ultraviolet spectrum, proton magnetic resonance spectrum, mobility on paper in 0.1 M phosphate buffer pH 7, mobility on DEAE-cellulose column, and substrate activity for dihydrofolate reductase. The residue was redissolved in 1% mercaptoethanol and lyophilized until most of the bicarbonate or acetate was removed. The product thus obtained was the ammonium salt of 7-methyl-H_{2}-folate. Its purity as determined from the ultraviolet spectrum (see below) was about 90 to 95%. In each case the main peak in the effluent was preceded by a smaller peak (about one-tenth of the area of the main peak). This material had a spectrum similar to that of 7-methyl-H_{2}-folate, but the shoulder at 305 nm was much more pronounced (Fig. 2). Small quantities and instability of this minor component did not permit its further investigation. Since it is difficult to remove acetate or bicarbonate quantitatively by lyophilization, an analytical sample of 7 methyl H_{2}-folate was prepared by redissolving an 80 to 90% pure preparation in 10% ascorbate solution and precipitating it with HCl as described for H_{2}-folate by Blakley (5). This compound after drying in vacuum over P_{2}O_{5} gave the following analysis.

\[ C_{14}H_{22}N_{7}O_{6} \cdot 2.5H_{2}O \]

Calculated: C 47.9, H 5.57, N 19.5
Found: C 48.36, H 5.53, N 18.47
Its ultraviolet spectrum was identical with that reported by Blakley for H₂-folate (Fig. 2) (5). When 21.7 mg (4.58 × 10⁻³ mole) of 7-methyl-H₂-folate were hydrogenated in 6 ml of glacial acetic acid over PtO₂ (20 mg), 4.84 × 10⁻³ mole of hydrogen was absorbed after about 80 min. The product of this reaction had an ultraviolet spectrum identical with that of H₂-folate.

The final recovery of 7-methyl-H₂-folate varied from experiment to experiment, but the average value was about 25%. The variability of the yield was later found to be due to two factors. First, 7-methyl-H₂-folate is relatively soluble in water and therefore, each washing of the preparation decreased the yield. Second, the rate of reduction is dependent on the speed of mixing of the reaction mixture. Thus, for instance, when very rapid shaking was applied (using Vortex Junior tube mixer) the reduction was practically finished in 1 to 2 hours. If such violent mixing was continued for a longer period of time, the yield of 7-methyl-H₂-folate was lowered.

Oxidation of 7-Methyl-H₂-folate and H₂-Folate—Both of these compounds were oxidized to 7-methyl-H₂-folate and folate, respectively, by the following procedure. About 10 mg of the dihydro compound was dissolved in 1 ml of 0.1 M phosphate buffer of pH 7.7. To this solution 1 ml of an aqueous 10% suspension of manganese dioxide catalyst was added, and air was bubbled through this mixture for 60 min. The reaction mixture was filtered, and the filtrate was applied to a DEAE-cellulose column in acetate form and chromatographed as described elsewhere for folate (1). The final products had the ultraviolet spectra of folate or 7-methylfolate, respectively. The yield was about 90% in the case of folate and about 35% in the case of 7-methylfolate.

Degradation of Folate and 7-Methylfolate—Folate was oxidized to 2-amino-4-hydroxypteridine-6-carboxylate (Fig. 3, Reaction a) in the following way. Potassium permanganate (45 mg) was added to a solution of 15 mg of folate in 1.0 ml of 0.1 M NaOH. The solution was stirred for 90 min. The precipitate of MnO₂ was removed by centrifugation and washed with 1.0 ml of water. The supernatant and wash were combined, and a drop of ethanol was added to decolorize the excess of permanganate. The new precipitate of MnO₂ was again removed by centrifugation and washed with 1 ml of water. The combined wash and supernatant were lyophilized. The residue was redissolved in 1.0 ml of 0.1 M solution of KH₂PO₄ and applied to a DEAE-cellulose acetate column (1 × 14 cm). The elution was carried out with a linear gradient of 150 ml of water and 150 ml of 1 M ammonium acetate solution. Two compounds were eluted at the acetate concentration 0.7 M and 0.82 M, respectively. The first compound had the same mobility as p-aminobenzoylglutamate (see above) but gave negative reaction when tested for aromatic amine. The second one was identified by its mobility on the column and by its ultraviolet spectrum as 2-amino-4-hydroxy-6-formylpteridine-6-carboxylate (7). The recovery of this product was about 8%. Cleavage of folate with bisulfite to 2-amino-4-hydroxy-6-formylpteridine (Fig. 3, Reaction b) was carried out as described elsewhere (8).

7-Methylfolate was oxidized to 2-amino-4-hydroxy-7-methylpteridine-6-carboxylate (Fig. 3, Reaction a) in the following way. 7-Methylfolate (10 mg) was dissolved in 10 ml of 0.05 M phosphate, pH 7. A 10% solution of potassium permanganate was added dropwise until no more was reduced. The excess of permanganate was decolorized with 30% H₂O₂, and the precipitate of MnO₂ was removed by centrifugation and washed with a few milliliters of water. The supernatant and wash were combined and chromatographed on a DEAE-cellulose. The elution was carried out with the linear gradient of 500 ml of water and 300 ml of 1 M ammonium acetate solution. In this case, in addition to 2-amino-4-hydroxy-7-methylpteridine-6-carboxylate (identified by the ultraviolet spectrum (9)), unchanged p-aminobenzoylglutamate (identified by the positive reaction for an aromatic amine and its ultraviolet spectrum) was isolated. The pteridine was eluted immediately before p-aminobenzoylglutamate (concentration of acetate about 0.6 M) and its recovery was about 30 to 40%.

Reduction of 7-Methyl-H₂-folate to 7-Methyl-H₂-folate—This reduction was carried out by the following methods: (a) reduction with dithionite as described elsewhere for H₂-folate (1); (b) reduction with borohydride as described elsewhere for H₂-folate (2); (c) catalytic hydrogenation of the ammonium salt of 7-methyl-H₂-folate in water; and (d) catalytic hydrogenation in glacial acetic acid. The weight ratio of the substrate (5 to 15 mg) to the catalyst (platinum dioxide) was in each case approximately 1:1. The ammonium salt of 7-methyl-H₂-folate and the catalyst were suspended in about 5 ml of water or glacial acetic acid, respectively, and hydrogenation was conducted at atmospheric pressure for 90 min when glacial acetic acid was the solvent, and for 4 hours when water was the solvent. The reaction was terminated by the addition of a few drops of mercaptoethanol to the hydrogenation flask. The catalyst was then removed by filtration and the filtrate was lyophilized. All preparations were purified by the procedure described elsewhere (6) for tetrahydrofolate. The ultraviolet spectra of all the 7-methyl-H₂-folate preparations in 0.05 M phosphate, pH 7.3, containing 0.1% mercaptoethanol were identical with that of H₂-folate. After standing in 1 M HCl the product of hydrogenation decomposed with the release of the diazotizable amine at the rate similar to that of H₂-folate (6). Upon heating with 98% formic acid in the presence of mercaptoethanol, a product was formed which had an ultraviolet spectrum identical with that of 5,10-methenyltetrahydrofolate (6).

Measurement of Radioactivity—Radioactivity was measured in a two-channel liquid scintillation spectrometer (Packard model 3002). The size of the samples counted varied between 0.1 and 0.5 µmole. Each sample was dissolved in a small amount of water (0.1 to 0.3 ml) and was added to 20 ml of the scintillation solution described by Bruno and Christian (10). The efficiency of counting (10 to 20%) was determined for each sample.

Fig. 3. Degradation of 7 methylfolate and folate.
from the channel ratio after calibration of the instrument with tritiated toluene of known specific activity. The concentrations of solutions to be counted were determined from their ultraviolet spectra. The following molar extinction coefficients at pH 7 were used: H\textsubscript{2}-folate and 7-methyl-H\textsubscript{2}-folate, 27 X 10\textsuperscript{4} at 297 m\textmu (6); H\textsubscript{2}-folate and 7-methyl-H\textsubscript{2}-folate, 28.5 X 10\textsuperscript{4} at 282 m\textmu (5); and folate and 7-methylfolate, 26.6 X 10\textsuperscript{4} at 280 m\textmu (6).

Proton Magnetic Resonance Spectra—These spectra were obtained at 60 Mcycles by means of a Varian A-60 spectrometer. The compounds were used in the form of their 10\% solutions in 1 n NaOD. The internal standard was 3-(trimethylsilyl)-1-propane sulfonate.

Enzymatic Assays for Folate and Dihydrofolate Reductase—When folate was the substrate, the reaction was carried out at pH 5.2 and the rate was ascertained by the determination of the aromatic amine released spontaneously from H\textsubscript{2}-folate (11). When H\textsubscript{2}-folate or 7-methyl-H\textsubscript{2}-folate were the substrates, the reaction was carried out in the Cary 14 spectrophotometer. The change of absorbance at 340 m\textmu was monitored (12). In each case, a crude cell extract of an amethopterin-resistant strain of cultured Sarcoma 180 was used as the source of the enzyme (13).

RESULTS

Biological Properties of 7-Methylfolate and 7-Methyl-H\textsubscript{2}-folate—7-Methylfolate was completely inert as a substrate for folate reductase. In the presence of folate as substrate, 7-methylfolate acted as a competitive inhibitor (Fig. 4) with an inhibition constant of 1.3 X 10\textsuperscript{-6} M. This indicates that 7-methylfolate is bound to the reductase slightly tighter than folate (13). Although 7-methyl-H\textsubscript{2}-folate seemed to be a substrate for dihydrofolate reductase, the rate of reduction of this compound at pH 7 and the enzyme concentration of 1 X 10\textsuperscript{-7} M was only one-tenth of that of H\textsubscript{2}-folate at the enzyme concentration of 1 X 10\textsuperscript{-8} M. At pH 5 and 6 the rate of reduction of 7-methyl-H\textsubscript{2}-folate was about 8 times faster than at pH 7. Although under such conditions the rate of the reduction of 7-methyl-H\textsubscript{2}-folate was comparable to that of H\textsubscript{2}-folate, the extent of the reaction was only one-half or less of that of H\textsubscript{2} folate. The reason for this behavior of 7-methyl-H\textsubscript{2} is not clear. The available evidence indicates that no product inhibition was involved. Since 7-methyl-H\textsubscript{2}-folate possesses an asymmetric carbon (C-7), it might be that only one isomer is active as the substrate for the enzyme.

Degradation of Tritiated 7-Methylfolate—Tritiated 7-methylfolate with a specific activity of 0.012 C per mole (obtained from 7-methyl-H\textsubscript{2}-folate as described above) was oxidized with potassium permanganate at pH 7 (see “Methods” and Fig. 3, Reaction a). Two products were isolated, p aminobenzoylelglutamate, which was free of radioactivity, and 2-amin-4-hydroxy-7-methylpteridine-6-carboxylate with a specific activity of 0.004 C per mole. Thus about 66% of the total radioactivity was removed during this reaction. Since the degradation with permanganate involves removal of both hydrogen atoms from carbon 7, it appears that two-thirds of the tritium of 7-methylfolate was incorporated outside carbon 7.

Degradation of Tritiated Folate—H\textsubscript{2}-folate was prepared by alkaline zinc reduction of folate in the presence of tritiated water.

Incorporation of Tritium into 7-Methyl-H\textsubscript{2}-folate—When zinc reduction of 7-methylfolate was carried out in the presence of tritiated water, highly radioactive 7-methyl-H\textsubscript{2}-folate was isolated. Although this reaction involves hydrogenation of the 6,7-double bond, part of the tritium was incorporated in positions other than at carbon 7. Table I presents the results of experiments in which several preparations of tritiated 7-methyl-H\textsubscript{2}-folate were oxidized to 7-methylfolate. During this reaction, tritium from position 7 should be entirely eliminated. Thus, tritium which was retained in 7-methylfolate must have been incorporated elsewhere. The distribution of tritium between carbon 7 and other positions seems to be, among other factors, dependent on the specific activity of tritiated water used in the reaction. At low specific activity a high percentage of tritium was incorporated outside carbon 7.

Degradation of Tritated Folate—H\textsubscript{2}-folate was prepared by alkaline zinc reduction of folate in the presence of tritiated water.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Sources of tritium & Specific activity & Total tritium & C-atoms & Other positions \\
\hline
H\textsubscript{2}O & 7-Methyl-H\textsubscript{2}-folate & 7-Methyl- & Lost on oxidation & At C-7 & \\
& & folate & oxidation & & \\
\hline
0.9 & 0.234 & 0.104 & 0.130 & 44 & 56 \\
0.9 & 0.010 & 0.033 & 0.097 & 70 & 30 \\
0.9 & 0.017 & 0.006 & 0.011 & 65 & 35 \\
0.225 & 0.074 & 0.054 & 0.020 & 27 & 73 \\
\hline
\end{tabular}
\caption{Incorporation of tritium into 7-methyl H\textsubscript{2}-folate}
\end{table}

\* These samples were diluted with nonradioactive 7-methyl-H\textsubscript{2}-folate before purification; thus, the yield of the tritium incorporation cannot be ascertained from these two samples.
The incorporation of tritium under these conditions was between 10 and 12%. There was very little tritium removed during the oxidation of H_{2}-folate to folate. This is in agreement with the results reported elsewhere (1) and can be explained by the isotope discrimination effect. Folate thus obtained was then degraded in two ways, (a) by oxidation with permanganate, and (b) by reduction with bisulfite (8) (Fig. 3). The specific activity of folate used in Reaction a was 0.0127 C per mole. 2-Amino-4-hydroxy-6-carboxylate pteridine which was recovered as a product of this reaction had a specific activity of 0.0081 C, per mole. Thus, the decrease of radioactivity was 36.4%. In Reaction b the specific activity of folate was 0.0099 C per mole and the reaction product 2-amino-4-hydroxy-6-formylpteridine had a specific activity of 0.0079 C per mole, which corresponds to the decrease in activity of 20%. Whereas during the permanganate oxidation (Reaction a) both hydrogen atoms from carbon 9 are removed, during the reductive cleavage (Reaction b) only 1 is removed. Thus, there is excellent agreement between the results of both experiments. In addition, these experiments substantiate the previous evidence that during the zinc reduction of 7-methylfolate in alkali, part of the tritium is incorporated into position 9.

Additional evidence that this is indeed the case was based on nuclear magnetic resonance spectroscopy. Fig. 5a shows the proton magnetic resonance spectrum of H_{2}-folate. The assignment of most of the peaks was based on the comparison of this spectrum with those of the fragments of the molecule (e.g. p-aminobenzoic acid, glutamic acid, etc.). The two peaks at -237 cps and -227 cps represent the 7-CH_{3} and 9-CH_{3} groups, respectively. This assignment is based on the following observations. In the spectrum of H_{2}-folate prepared by dithionate reduction of folate in the presence of 99.7% D_{2}O (1), the area of the peak at -237 cps is decreased by one-half. Since it has been previously established (14) that under these conditions an atom of deuterium is incorporated at carbon 7, this peak must correspond to 7-CH_{3}—group. Fig. 5a shows the spectrum of H_{2}-folate prepared by alkaline zinc reduction in the presence of D_{2}O. In this case the area of the peak corresponding to 7-CH_{3}—is considerably decreased, but the peak corresponding to 9-CH_{3}—is almost nonexistent. Thus, under these conditions most of the hydrogen atoms at carbon 9 were replaced by deuterium.

During alkaline zinc reduction of 7-methylfolate, tritium is incorporated at both positions, carbon 9 and 7-methyl group. This indicates that under these conditions, the protons in both positions are labile and exchangeable against the solvent. This exchangeability has not been noticed in the absence of zinc.
Reduction of 7-Methyl-\( \text{H}_2 \)-folate to 7-Methyl-\( \text{H}_4 \)-folate by Different Methods—Table II shows results of experiments in which tritiated 7-methyl-\( \text{H}_2 \)-folate was reduced to 7-methyl-\( \text{H}_4 \)-folate by three methods, (a) reduction with dithionate, (b) catalytic hydrogenation in water, and (c) reduction with borohydride. The second column of Table II shows the specific activity of tritium at carbon 7 of 7-methyl-\( \text{H}_2 \)-folate. The reduction with dithionate led to the loss of tritium equivalent to that present at carbon 7. On the contrary, there was practically no loss of tritium during the catalytic hydrogenation of 7-methyl-\( \text{H}_2 \)-folate; the reduction with borohydride falls between the two extreme cases. Under this condition an equivalent of about 40 to 50% of tritium present at carbon 7 was removed. There is no direct proof in the case of the dithionite reduction that tritium was actually removed from position 7. However, in view of the quantitative agreement between the content of tritium at carbon 7 and the amount of tritium removed during the reduction, as well as in view of the results presented elsewhere (1), it is reasonable to assume that the reduction with dithionite proceeds through an intramolecular rearrangement which leads to the exclusion of tritium from carbon 7. On the other hand, the lack of elimination of tritium during the catalytic hydrogenation indicates that this reaction does not involve rearrangement, but rather it involves direct addition of hydrogen across the 5,6-double bond. Concerning the results obtained with borohydride reduction, the most obvious interpretation would be that this reaction proceeds simultaneously by two mechanisms, to the larger extent by direct addition of hydrogen to 5,6-double bond and to the smaller extent through the rearrangement. Since, however, there is no evidence that the eliminated tritium originates from position 7, other interpretations are also possible. It is of interest that catalytic hydrogenation in glacial acetic acid seems to proceed by a mechanism completely different from that of hydrogenation in water. When 7-methyl-\( \text{H}_2 \)-folate was reduced under this condition, considerably more tritium was removed than could be accounted for by the content of tritium at carbon 7. The interpretation of this result must await further experiments.

DISCUSSION

Although 7-methylfolate and its dihydro derivative differ structurally from folate and \( \text{H}_2 \)-folate, respectively, by the presence of one methyl group only, considerable differences in the biological and chemical behavior of these compounds were encountered. 7-Methylfolate is much more resistant to reduction than folate. Dithionite which reduces folate in 10 min at room temperature (15) does not attack 7-methylfolate even at 75°C. The use of zinc in alkaline solution proved to be satisfactory for reduction of 7-methylfolate, but here also, profound differences in reactivity between this compound and folate were noted (see "Methods"). No difference between reducibility of 7-methyl-\( \text{H}_2 \)-folate and \( \text{H}_2 \)-folate was noticed when dithionite or borohydride was used. However, the rate of catalytic hydrogenation in water of 7-methyl-\( \text{H}_2 \)-folate was much slower than that of \( \text{H}_2 \)-folate. In view of these differences, the results of the present study must be applied to \( \text{H}_2 \)-folate with caution. Thus for instance, the conclusion may be drawn that there is more than one possible mechanism by which \( \text{H}_2 \)-folate can be reduced to \( \text{H}_4 \)-folate and that this mechanism of reduction depends on the conditions and the nature of the reducing agent. However, there is no justification to suggest that the contribution of either of the mechanisms of reduction must be quantitatively the same for 7-methyl-\( \text{H}_2 \)-folate and for \( \text{H}_4 \)-folate. Scrimgeour and Vitols (2) presented evidence that reduction of \( \text{H}_2 \)-folate with borohydride does not involve rearrangement. This is in apparent disagreement with the results reported here where the reduction of 7-methyl-\( \text{H}_2 \)-folate with borohydride proceeds to the extent of about 40% through the rearrangement. It is obvious that these differences may be due exclusively to the fact that the properties of 7-methyl-\( \text{H}_2 \)-folate are not identical with those of \( \text{H}_2 \)-folate.

It is peculiar that the apparently very similar reactions such as the catalytic hydrogenation of 7-methyl-\( \text{H}_2 \)-folate in water and in acetic acid proceed through different mechanisms. Similarly, enzymatic (1) and borohydride (2) reduction of \( \text{H}_2 \)-folate, both involving hydride ion transfer, also proceed through the different mechanisms. On the other hand, reactions as different as dithionite reduction and enzymatic reduction of folate seem to have a similar mechanism (1). Since tritium (as well as deuterium) on carbon 7 of 7,8-dihydrofolate (and 7-methyl-7,8-dihydrofolate) is perfectly stable and cannot be removed by repeated recrystallization or chromatography, it is unlikely that in alkaline or neutral solution 7,8-dihydrofolate should exist in equilibrium with its 5,8- or 5,6-dihydro isomers. Thus it must be assumed that the reducing agent affects partial rearrangement of 7,8-dihydrofolate. In addition other factors such as the nature of the solvent, salt concentration, pH, and temperature may be influential in determining the course of the reaction. These external conditions, by affecting parameters like conformation and solvation of the molecule, may increase the rate of one pathway of the reduction more than the other. Although final clarification of this problem must await further experimental evidence, the results presented above substantiate the previously reported observations which indicate that the enzymatic as well as dithionite reduction of \( \text{H}_2 \)-folate proceeds through the intramolecular rearrangement (1).

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REFERENCES

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