Hydrolysis of 5,10-Methenyltetrahydrofolate to 5-Formyltetrahydrofolate at pH 2.5 to 4.5

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ABSTRACT: At pH 4.0 to 4.5, 5,10-methenyltetrahydrofolate is hydrolyzed to only 5-formyltetrahydrofolate if reducing agents are present or iron-redox cycling is suppressed. At pH 4.0, the equilibrium position for this hydrolysis is approximately equal concentrations of both folates. If no reducing agents are used or iron-redox cycling is promoted, considerable amounts of 10-formyldihydrofolate are also formed. It is likely that 10-formyldihydrofolate has been misidentified as 5,10-hydroxymethylenetetrahydrofolate, which was reported to accumulate during the hydrolysis of 5,10-methenyltetrahydrofolate to 5-formyltetrahydrofolate [Stover, P. and Schirch, V. (1992) *Biochemistry 31,* 2148−2155 and 2155−2164; (1990) *J. Biol. Chem*. *265*, 14227−14233]. Since 5,10-hydroxymethylenetetrahydrofolate is reported to be the viable in vivo substrate for serine hydroxymethyltransferase-catalyzed formation of 5-formyltetrahydrofolate, and 5,10-hydroxymethylenetetrahydrofolate probably does not accumulate, the above folate metabolism is now doubtful. It is hypothesized that mildly acidic subcellular organelles provide an environment for the hydrolysis of 5,10-methenyltetrahydrofolate to 5-formyltetrahydrofolate in vivo, and there is no requirement for enzyme catalysis. Finally, 10-formyltetrahydrofolate is susceptible to iron-catalyzed oxidation to 10-formyldihydrofolate at pH 4 to 4.5.

During the investigation of the oxidation of 10-formyltetrahydrofolate (10-HCO-H_4F)[1] to 10-formyldihydrofolate (10-HCO-H_2F) at mildly acidic pHs, I became aware that Schrich and co-workers (*1*−*7*) reported the possible formation of a stable folate, 5,10-hydroxymethylenetetrahydrofolate (5,10-HOCH-H_4F). These investigators reported that 5,10-HOCH-H_4F can be chemically prepared from and is in equilibrium with 5,10-methenyltetrahydrofolate (5,10-CH=H_4F) and 5-formyltetrahydrofolate (5-HCO-H_4F) at pH values 2 to 5. If this compound exists, the formation of 5,10-HOCH-H_4F would interfere with an assay for the oxidation of 10-HCO-H_4F to 10-HCO-H_2F at mildly acidic conditions. In addition, 5,10-HOCH-H_4F is reported to be a good substrate for serine hydroxymethyltransferase (SHMT)-catalyzed formation of 5-HCO-H_4F (*1*, *3*).

We recently gave human subjects an oral dose of 5,10-CH=H_4F that had been prepared by incubating a solution of 5-HCO-H_4F at pH 2.5 for 16 h (*8*). According to the reports by Stover and Schrich (*1*−*3*), this procedure would have produced a substantial amount of 5,10-HOCH-H_4F. Therefore, the results reported by us would have reflected the metabolism of the intermediate (i.e., 5,10-HOCH-H_4F) rather than 5,10-CH=H_4F. Furthermore, the formation of 5, 10-HOCH-H_4F from 5,10-CH=H_4F in acidic sites, such as the stomach and the lysosome, would substantially impact our understanding of folate biology. Therefore, in the study presented here, hydrolysis and dehydration reactions of 5, 10-CH=H_4F and 5-HCO-H_4F under mildly acid conditions were reinvestigated to evaluate whether 5,10-HOCH-H_4F is actually formed.

EXPERIMENTAL PROCEDURES

[6*RS*] 5-HCO-H_4F was a gift from Lederle Laboratories (Pearl River, NY). 5,10-CH=H_4F was prepared from 5-HCO-H_4F by dissolving it in 0.1 M H_2SO_4 at 40 °C and allowing the product to precipitate at 5 °C. The precipitated 5,10-CH=H_4F was redissolved in buffers, other chemicals were added, pH was adjusted, and insoluble materials were removed by centrifugation before spectrophotometric measurements were made. 10-Formyl folic acid (10-HCO-F) was prepared by heating folic acid in excess 98% formic acid at 60 °C for 2 h. The reaction solution was placed at 4 °C overnight, and formic acid was removed with ether. The product was recrystallized from hot water to a constant UV spectrum (*9*). Authentic 10-HCO-H_2F was prepared as previously described (*10*). Distilled deionized water was used in all experiments, and all other reagents were commercially available ACS grade.

5-HCO-H_4F and 5,10-CH=H_4F are quantitated at 287 nm ($\epsilon = 3.2 \times 10^4$ M^{-1} cm^{-1}) and 356 nm ($\epsilon = 2.5 \times 10^4$ M^{-1} cm^{-1}), respectively. The decrease in A_{356} of a solution of 5,10-CH=H_4F (pH 2.5 to 4.5) is a measure of the simultaneous formation of both 5-HCO-H_4F ($\Delta\epsilon = -2.4 \times 10^4$ M^{-1} cm^{-1}) and 10-HCO-H_2F ($\Delta\epsilon = -2.14 \times 10^4$ M^{-1} cm^{-1}). All reactions were carried out in 50-mL plastic tubes

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[1] Abbreviations: 10-formyltetrahydrofolate, 10-HCO-H_4F; 10-formyl-7,8-dihydrofolate, 10-HCO-H_2F; 10-formyl-folic acid, 10-HCO-F; 5-formyltetrahydofolate, 5-HCO-H_4F; 5,10-methenyltetrahydrofolate, 5,10-CH=H_4F; 5,10-hydroxymethylenetetrahydrofolate, 5, 10-HOCH-H_4F; desferioxamine, DFX; dithioerythritol, DTE; dimethyl sulfoxide, DMSO; ethylenediaminetetraacetic acid, EDTA; serine hydroxymethyltransferase, SHMT.

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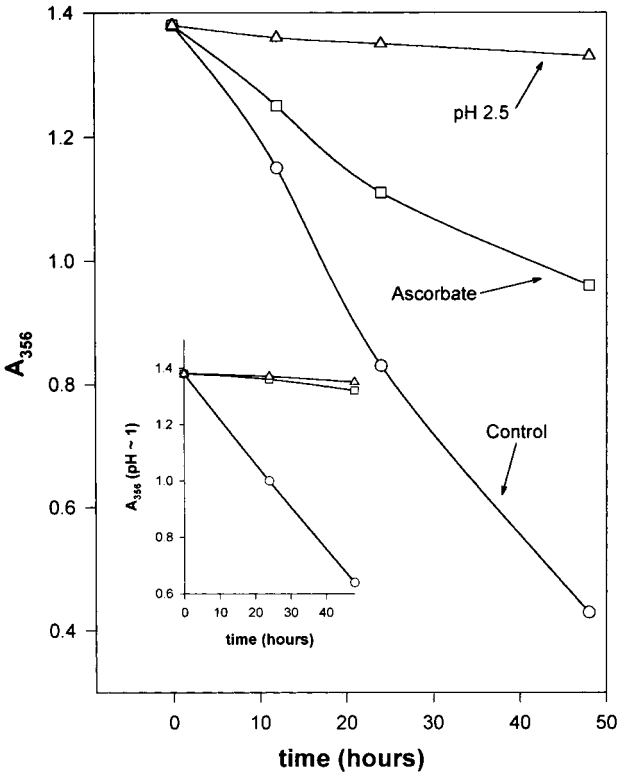

FIGURE 1: Kinetics of the hydrolysis and disappearance of 5,10-methenyltetrahydrofolate. A solution of 5,10-CH=H_4F in 25 mM citrate with an initial A_{356} of 1.38 was divided into three portions. One was made pH 2.5, one was made pH 4.4 (control), and one was made 5 mM ascorbate, pH 4.4 (ascorbate). The A_{356} was monitored at 23 °C. Inset: Aliquots of each reaction were made 0.1 M H_2SO_4, and the final A_{356} is plotted.

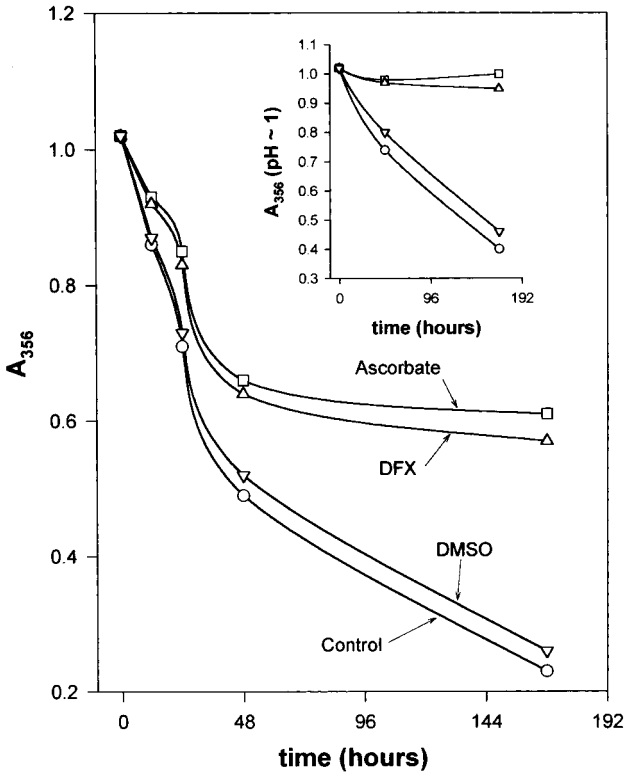

FIGURE 2: Kinetics of the hydrolysis and disappearance of 5,10-methenyltetrahydrofolate. Similar procedures were performed as Figure 1; the initial A_{356} was 1.02, and solutions were made pH 4.0 (control), 5 mM ascorbic acid, pH 4.0 (ascorbate), 1 mM DFX, pH 4.0 (DFX), and 0.1 M DMSO, pH 4.0 (DMSO). Inset shows the same procedures described in Figure 1.

containing 10−25 mL of the reaction solution, and no attempt was made to make the reaction anaerobic. The spectrophotometer was set at zero absorbency using buffers without folates before measurements were made.

The increase in A_{356} is a measure of the formation of 5,10-CH=H_4F ($\Delta\epsilon = 2.4 \times 10^4$ M^{-1} cm^{-1}) in an acidic solution (i.e., 0.1 M H_2SO_4) from 5-HCO-H_4F. The method of Gugenheim (*11*) was used to establish that the rate of formation of 5,10-CH=H_4F from 5-HCO-H_4F was first order for greater than three half-lives and to estimate the final A_{356} after acidification (i.e., 0.1 M H_2SO_4).

Sephadex G-25 (0.9 × 110 cm) column chromatography was used to separate folate derivatives. Fractions (3.6 mL) were collected using 25 mM citrate buffers (pH values 4.0−4.5) as the eluents.

RESULTS

In the reactions of 5,10-CH=H_4F at mildly acidic pH, the decrease in A_{356} is a measure of disappearance of 5,10-CH=H_4F and concerted formation of 5-HCO-H_4F or any other folate with relatively low absorbency at 356 nm. Figure 1 shows that the rate of decrease in A_{356} is much slower at pH 2.5 than at pH 4.4 and that 5 mM ascorbate further slows down the rate at pH 4.4. Figure 2 shows that the rate of decrease in A_{356} at pH 4.0 is slower in 5 mM ascorbate than the control and that these two reactions appear to be approaching two different equilibrium positions at 168 h. Desferioxamine (DFX) reduces the rate of decrease in A_{356}, while DMSO is only slightly effective in this respect. As shown in Figure 3, EDTA increases the rate of decrease in A_{356} at pH 4.5, while dithioerythritol (DTE) reduced this rate as compared to the control. Mannitol only slightly reduced the rate.

The amount of 5-HCO-H_4F formed in the experiments shown in Figures 1−3 can be assessed simply by making aliquots acidic (i.e., 0.1 M H_2SO_4, pH ~1) and by observing the increase in A_{356} as the 5-HCO-H_4F which was formed at pH values 4.0−4.5 is converted back to 5,10-CH=H_4F at pH ~1. If 10-HCO-H_2F is formed at pH values 4.0−4.5, this folate cannot be reconverted to 5,10-CH=H_4F at pH ~1 (*12*); therefore, the original A_{356} cannot be achieved. This assay is also based on the observations of Rabinowitz and others (*13*, *14*) that the rate of formation of 5,10-CH_2=H_4F from 5-HCO-H_4F is slower than the rate of formation of 5,10-CH=H_4F from 10-HCO-H_4F; therefore, if the original A_{356} is achieved, 5-HCO-H_4F must be the only product. Insets in Figures 1−3 show the final A_{356} obtained after the acidification. Figure 1 (inset) shows that close to the original A_{356} was obtained in the reaction at pH 2.5 and in the reaction at pH 4.4 in the presence of ascorbate. In contrast, after 48 h the control reaction achieved less than 50% of the original A_{356} after the acidification. Figure 2 (inset) shows that the reaction in the presence of ascorbate at pH 4.0 produced primarily 5-HCO-H_4F, since the A_{356} was 98% of its original value after acidification. The same was true of the reaction that contained DFX in that A_{356} was returned to 93% of its original value. On the other hand, after 168 h in the control reaction and the reaction in the presence of DMSO, less that 50% of the original A_{356} was recovered after acidification.

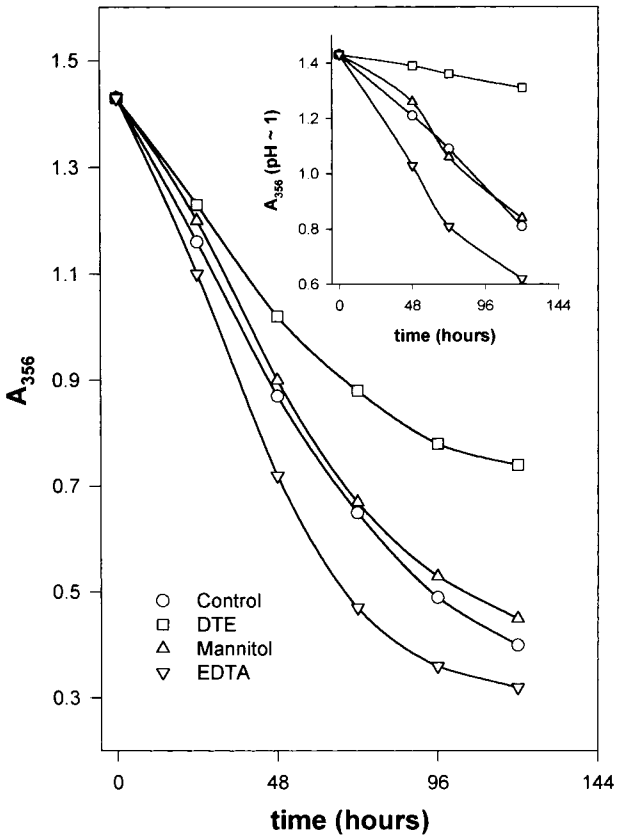

FIGURE 3: Kinetics of the hydrolysis and disappearance of 5,10-methenyltetrahydrofolate. Similar procedures were performed as described in Figure 1; the initial A_{356} was 1.43, and solutions were made pH 4.5 (control); 5 mM DTE, pH 4.5 (DTE), 0.5 M mannitol, pH 4.5 (mannitol); 1mM EDTA, pH 4.5 (EDTA). Inset shows the same procedures described in Figure 1.

In Figure 3 (inset), the primary product was 5-HCO-H_4F in the reaction in the presence of DTE. After 120 h in the control reaction and the reactions in the presence of EDTA or mannitol, substantially less than the original A_{356} was recovered after acidification. In all of the above acidification reactions, the half-life for the increase in A_{356} was 4.0−5.5 min at 23 °C, and the reactions were first-order for over three half-lives**.** This rate is consistent with one of the products formed being 5-HCO-H_4F (*13*, *14*).

Similar reactions of 5,10-CH=H_4F were performed using 100 mM phosphate (pH 4.5) at 40 °C to rule out the possibility that the results in Figures 1−3 were artifacts of citrate buffer, ionic strength, or temperature and to repeat the experiments performed by Stover and Schirch (*2*). The reactions were faster at 40 °C as compared to 23 °C. Similar effects of ascorbate, DTE, DFX, and EDTA were observed as shown in Figure 4.

Figure 5 shows repeated UV scans of 5,10-CH=H_4F in citrate buffer or citrate buffer containing DFX. Isosbestic points at 261 and 319 nm were apparent in the reaction containing DFX and were absent in the control reaction. Figure 6 displays repeated scans of 5,10-CH=H_4F in citrate buffer with or without DTE or EDTA. The isosbestic points at 261 and 319 nm were observed in the reaction containing DTE, but absent in the control reaction or in the reaction containing EDTA. The presence of isosbestic points suggests that only one product is formed from 5,10-CH=H_4F.

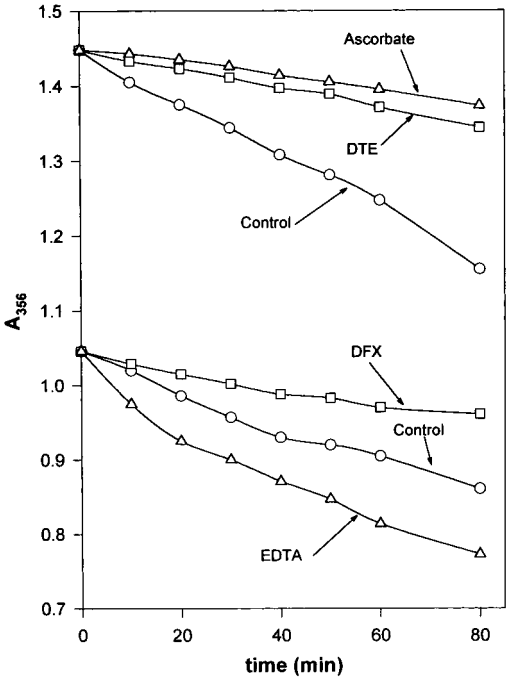

FIGURE 4: Kinetics of the hydrolysis and disappearance of 5,10-methenyltetrahydrofolate. Procedures were the same as described in Figure 1 except that 100 mM potassium phosphate buffer, pH 4.5, at 40 °C was used. Concentrations of ascorbate, DTE, DFX, and EDTA are the same as described in Figures 1−3.

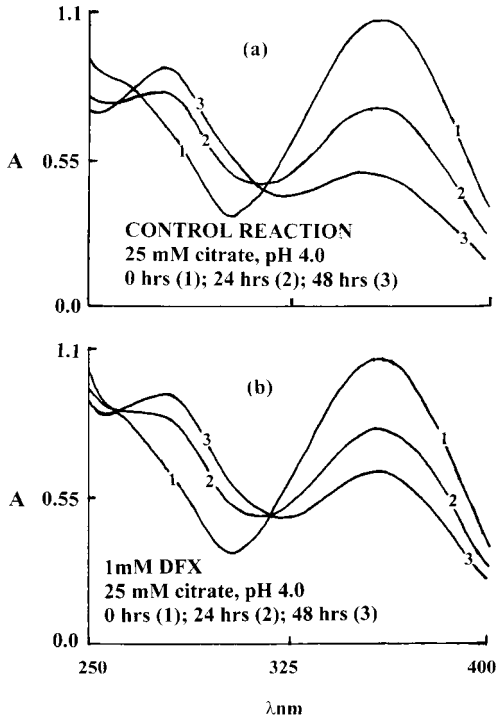

FIGURE 5: Panels a and b. Repeated UV scans of the control reaction a and the DFX containing reaction b described in Figure 2.

Repeated UV scans showing the formation of 5,10-CH=H_4F from 5-CHO-H_4F at pH values 4.4 and 3.3 are shown in Figure 7. Isosbestic points at 261 and 319 nm were observed in the reaction at pH 3.3. The reaction at pH 3.3 apparently reaches equilibrium after 20 h. The equilibrium concentrations calculated from the ΔA_{356} at 20 h in Figure 7, panel a, were 28% as 5-HCO-H_4F and 72% as 5,10-CH=

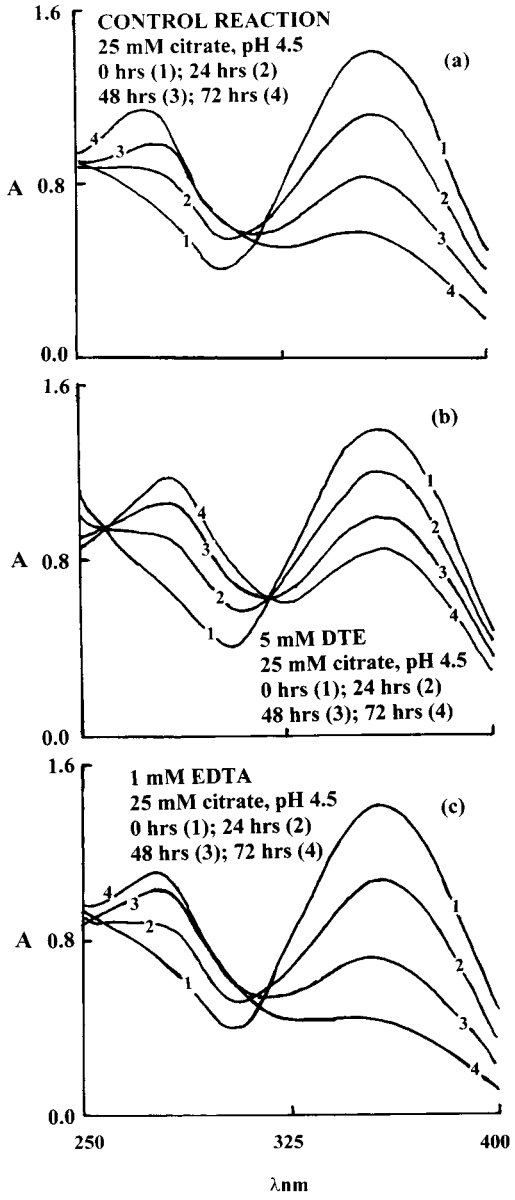

FIGURE 6: Panels a, b, and c. Repeated UV scans of the control reaction a, the DTE containing reaction b, and the EDTA-containing reaction c described in Figure 3.

H_4F of the total folates. However, when it was allowed to proceed for an additional 76 h, both isosbestic points were lost (Figure 7, panel a, broken line). Repeated UV scans of the reaction at pH 4.4 never displayed isosbestic points (Figure 7, panel b), and there was only a small increase in A_{356}. Thus, only a small amount of 5,10-CH=H_4F was formed from 5-CHO-H_4F at pH 4.4.

The reaction solution containing ascorbate shown in Figure 2 was lyophilized, redissolved in water, applied to a Sephadex G-25 column and eluted with 25 mM citrate (pH 4.0). Two peaks appeared when monitored at A_{287}, although the first peak was partially obscured by ascorbate (Figure 8). The first peak (fraction 28) and the second peak (fraction 36) were identified as 5,10-CH=H_4F and 5-HCO-H_4F, respectively. One peak (fraction 28) appeared when monitored at A_{356} and was again identified as 5,10-CH=H_4F (Figure 8). Using peak height measurements and appropriate molar extinction coefficients, this reaction contained 46% as 5-HCO-H_4F and 54% as 5,10-CH=H_4F of the total folate.

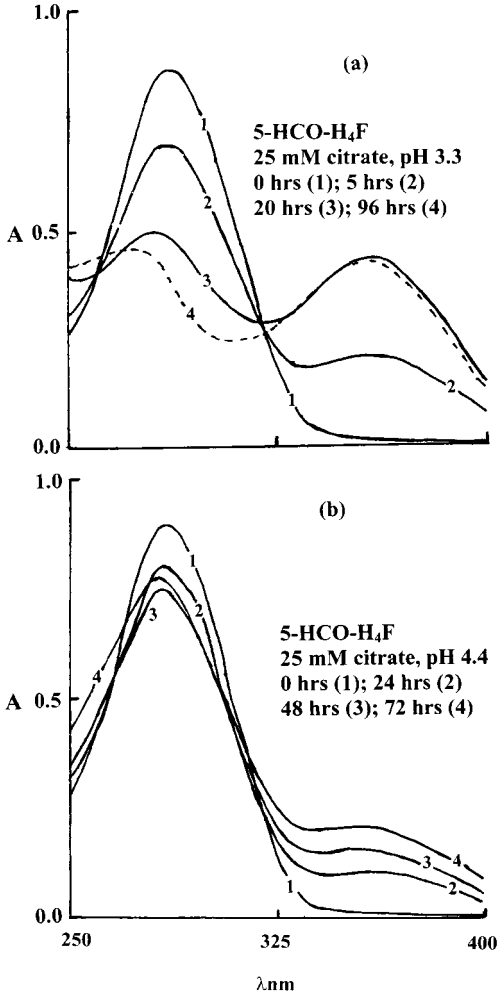

FIGURE 7: Panels a and b. Repeated UV scans of a solution of 5-HCO-H_4F made in 25 mM citrate buffer (23 °C), which was divided and made pH 3.3 (a) or pH 4.4 (b).

Using the ΔA_{356} (Figure 2), this reaction contained 43% as 5-HCO-H_4F and 57% as 5,10-CH=H_4F of the total folate, and these were essentially the same as the values obtained using Sephadex G-25 chromatography.

A rapid formation of product(s) from 5,10-CH=H_4F using procedures similar to those described by Stover and Schirch (*2*) (see Figures 1 and 2 of this reference) was performed. A solution of 1.6 mM 5,10-CH=H_4F in 25 mM citrate, pH 4.5, was heated at 50 °C for 75 min. The A_{356} decreased by 28% during this period. The reaction solution was then chromatographed using Sephadex G-25 with the same buffer (pH 4.5) as an eluent. Using A_{261} measurements, the first peak occurred at fraction 28 and a second peak at fraction 36. The first peak was identified as unreacted 5,10-CH=H_4F by its UV spectrum, but the second peak could not be identified by its UV spectrum. Each fraction was then made 0.1 M in H_2SO_4, and the rate of ΔA_{356} was monitored followed by a 60-min incubation. The fractions with measurable rates of ΔA_{356} are presented as a broken line in Figure 9. Any 5-HCO-H_4F that was present will be dehydrated to 5,10-CH=H_4F in this acidic environment resulting in an increase in A_{356}. This procedure and the elution position identified 5-HCO-H_4F as one of the products. Under these acidic conditions, 5,10-CH=H_4F has the same molar extinction coefficient at 305 and 380 nm; therefore, $A_{305} - A_{380}$ will be zero, if only 5,10-CH=H_4F is present. Figure 9 shows

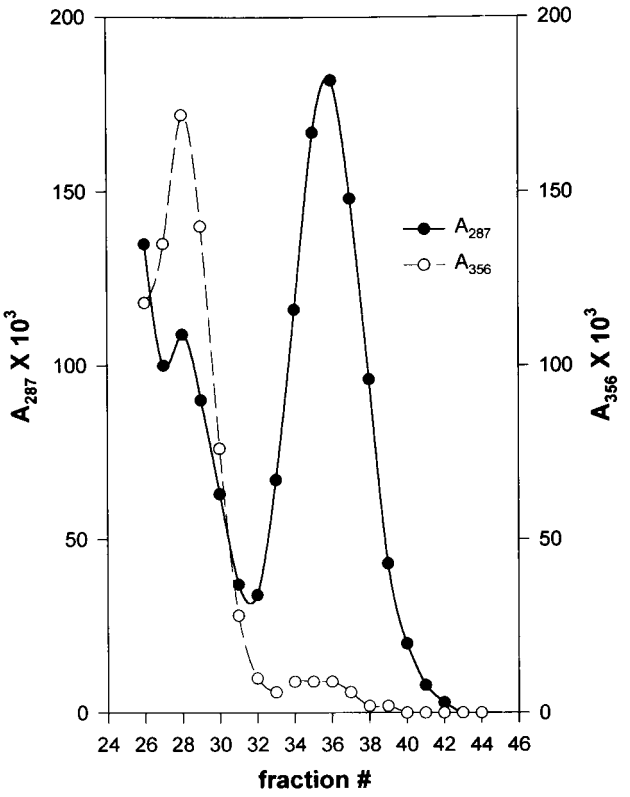

FIGURE 8: Chromatogram of the hydrolysis products of 5,10-methenyltetrahydrofolate in the presence of ascorbate. The ascorbate containing reaction (after 168 h) described in Figure 2 was chromotographed on Sephadex G-25 column using 25 mM citrate pH 4.0 as the eluent. Blue dextran eluted at fractions 10 and 11.

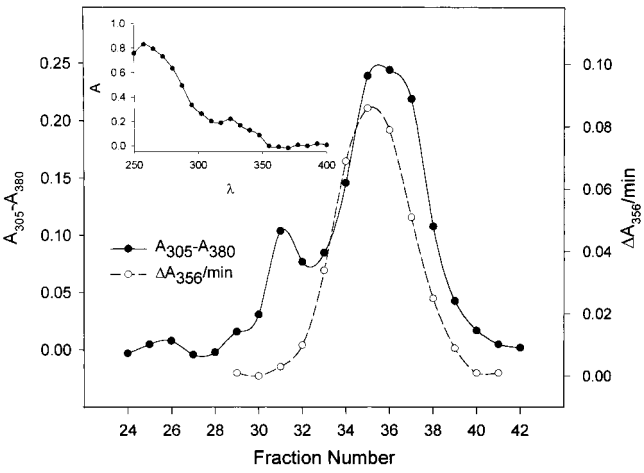

FIGURE 9: Chromatogram of the products of 5,10-methenyltetrahydrofolate at pH 4.5 heated at 50 °C for 75 min. One milliliter of a reaction mixture containing 1.6 mM 5,10-CH=H_4F in 25 mM citrate (pH 4.5) heated at 50 °C for 75 min was chromatographed on Sephadex G-25 column using the same buffer as the eluent. Open circles and dashed lines represent the initial rate of ΔA_{356} after making the fractions 0.1 M H_2SO_4. Closed circles and solid lines represent the $A_{305} - A_{380}$ of the acidified fractions. Inset is the absorbance of acidified fraction 36 minus the absorbance of the 5,10-CH=H_4F, as described in the text.

that a plot of $A_{305} - A_{380}$ (solid line) has a small peak at fraction 31 and a large peak at fraction 36. These two peaks represent two products of this chemical reaction in addition to 5-HCO-H_4F. 10-HCO-H_2F was positively identified in fraction 36 by subtracting the absorbance of 5,10-CH=H_4F formed from 5-HCO-H_4F (in 0.1 M H_2SO_4) from the final absorbance. This difference in absorbance is shown in Figure 9 (inset) and is the spectrum of 10-HCO-H_2F at pH ~1 (λ_{max} 258 and 325−330 nm) (*10*). The small peak of the $A_{305} - A_{380}$ plot was identified as 10-HCO-F (fraction 31), since, in separate experiments, the peaks of pure 10-HCO-H_2F and 10-HCO-F appeared at fractions 36 and 31, respectively.

DISCUSSION

The spectrophotometric measurements represent four independent assays of reactions of 5,10-CH=H_4F, including the rate of decrease in A_{356}, the rate of increase in A_{356} after acidification, the final A_{356} after acidification, and the presence or absence of isosbestic points. It was concluded that in the presence of reducing agents (ascorbate and DTE) or DFX, 5-HCO-H_4F is the major product of the hydrolysis of 5,10-CH=H_4F under mildly acidic conditions (pH 4.0−4.5). This conclusion was made because alterations in the rates of decrease in A_{356} produced by ascorbate, DTE, DFX, and EDTA (Figures 1−4), the recovery of nearly quantitative amounts of 5,10-CH=H_4F after acidification only in the reactions containing ascorbate, DTE, and DFX (Figures 1−3), the presence of isosbestic points for the formation of 5-HCO-H_4F from 5,10-CH=H_4F only in the DTE and DFX containing reactions (Figures 5 and 6), and the chromatographic separation of 5-HCO-H_4F and 5,10-CH=H_4F (Figure 8). The isosbestic points for the enzyme-catalyzed conversion of 5-HCO-H_4F to 5,10-CH=H_4F were reported to be 258 and 318 nm (pH 6.0), which are similar to the findings reported here (*15*). Beavon and Blair (*14*) reported that 5-HCO-H_4F is dehydrated only to 5,10-CH=H_4F at pH 2−3. The "β-form" of 5,10-CH=H_4F originally proposed by Cosulich et al. (*16*) was later thought to be 5,10-HOCH-H_4F by Stover and Schirch (*2*). However, Kay et al. (*17*) reported in 1960 that the "β-form" of 5,10-CH=H_4F was not observed at pH 3.5 in the presence of mercaptoethanol in the reaction solutions.

In the absence of reducing agents or the presence of iron-redox cycling (EDTA and citrate), both 5-HCO-H_4F and 10-HCO-H_2F are formed from 5,10-CH=H_4F. The formation of 10-HCO-H_2F is strongly suggested because the decrease in A_{356} is greater without the presence of ascorbate or DTE and in the presence of EDTA (Figures 1−4). Only a fraction of the original 5,10-CH=H_4F is obtained after acidification of the reaction mixtures containing EDTA and the control reaction (Figures 1−3), which indicates the formation of 10-HCO-H_2F. Furthermore, the formation of 10-HCO-H_2F is established by Sephadex G-25 chromatography (Figure 9). Even in the presence of ascorbate, Temple et al. (*18*) reported that detectable amounts of 10-HCO-H_2F and 10-HCO-F are formed from equilibrium concentrations of 5,10-CH=H_4F and 10-HCO-H_4F.

The experiments shown in Figure 9 are of particular importance, since they essentially repeat the aerobic incubation and the NMR experiments performed by Stover and Schirch (*2*) (see Figures 1 and 2 of this reference). They concluded that 5,10-CH=H_4F at pH 4.5 heated aerobically for 2.5 h at 50 °C produced no detectable 5-HCO-H_4F; however, when 5,10-CH=H_4F at pH 4.0 was heated for 1 h at 55 °C, substantial hydrolysis occurred (>50% by NMR peak heights) yielding 5-HCO-H_4F as the major product. These contradictory results reported by Stover and Schirch

(*2*) may be explained by the inappropriate detection methods for both 5-HCO-H_4F and 10-HCO-H_2F.

Furthermore, no direct evidence was offered by Stover and Schirch (*1*−*3*) for the accumulation of 5,10-HOCH-H_4F. They did not use reducing agents when 5,10-HOCH-H_4F was prepared from 5,10-CH═H_4F, and it is likely that 10-HCO-H_2F has been misidentified as 5,10-HOCH-H_4F (*2*, *3*). 10-HCO-H_2F shares some of the properties reported for 5,10-HOCH-H_4F. These properties include that it is not an intermediate in the hydrolysis of 5,10-CH═H_4F to 10-HCO-H_4F, it has a UV spectrum that is similar to that reported for 5,10-HOCH-H_4F, and it is not a substrate for the 5,10-CH═H_4F synthetase or 5,10-CH═H_4F cyclohydrolase enzymes (*1*−*3*, *10*, *16*). On the other hand, the chemistry of 10-HCO-H_2F is different from that reported for 5,10-HOCH-H_4F, since it cannot be converted to 5,10-CH═H_4F and 5-HCO-H_4F at pH values below 2 and above 8, respectively, in the absence of a reducing system (*1*−*3*, *10*, *12*). 10-HCO-H_2F cannot be a substrate for SHMT-catalyzed formation of 5-HCO-H_4F (*1*, *3*).

The hydrolysis of 5,10-CH═H_4F to 5-HCO-H_4F is reversible and reaches equilibrium by starting with either folate. At pH 4.0 and 23 °C, the equilibrium concentrations were approximately equal (Figures 2 and 8). Kay et al. (*17*) reported approximately equal concentrations of these folates at pH 3.5. The equilibrium favors the formation of 5-HCO-H_4F at pH 4.5 and formation of 5,10-CH═H_4F at pH 3.3 (Figures 1, 3, and 7). At pH 2.5, 5-HCO-H_4F represents only a few percent of the total folates (Figure 1).

Iron-redox cycling is involved in the production of 10-HCO-H_2F. Considerable evidence indicates that the chelators, EDTA, citrate, and DFX, are good, moderate, and poor, respectively, in supporting iron-redox cycling (*19*−*24*). The decrease in A_{356} of a 5,10-CH═H_4F solution at pH 4.0 to 4.5 containing these substances followed this trend (Figures 2−4). It is remarkable that trace amounts of iron in ACS grade reagents at pH 4.0 to 4.5 readily catalyzed the oxidation of smaller concentrations of 10-HCO-H_4F in equilibrium with a larger concentration of 5,10-CH═H_4F. The use of these chelators can reveal the presence of trace iron in reagents. For example, it has been reported that in purified reagents and no added iron, the rate of oxidation of ascorbate is seven times faster in an EDTA-containing buffer than one containing DFX (*25*). Unexpectedly, the hydroxy-free-radical scavengers such as DMSO and mannitol provided only a small amount of protection for the oxidation of 10-HCO-H_4F in the experiments presented here.

The assertion that 5,10-CH═H_4F is a substrate for SHMT-catalyzed formation of 5-HCO-H_4F and that this is the in vivo source of 5-HCO-H_4F made by Stover and Schirch (*1*, *3*) deserves further comment. Stoichiometric concentrations of SHMT were used to demonstrate this reaction (*1*, *3*). Frequently, Stover and Schrich (*1*, *3*) performed an indirect assay for the formation of 5-HCO-H_4F catalyzed by SHMT. Furthermore, they stated that at cytosolic pH, enzyme kinetic data suggest that only 5,10-HOCH-H_4F (not 5,10-CH═H_4F) would be a viable substrate for SHMT-catalyzed reaction (*3*). On the basis of the data presented here, it is unlikely that these authors used 5,10-HOCH-H_4F as a substrate for SHMT. Increasing SHMT activity in *E. coli* and the subsequent increase in 5-HCO-H_4F does not provide proof that this enzyme is forming this folate (*1*). An increase in SHMT activity simply could provide more folate precursors of 5,10-CH═H_4F; therefore, more 5,10-CH═H_4F and thus form more 5-HCO-H_4F prior to analysis. An additional concern is the citation by Stover and Schirch (*1*) of the data published by Cossins et al. (*26*). Stover and Schrich (*1*) suggested that there was a positive correlation between SHMT activity and 5-HCO-H_4F content in wild-type and two mutant strains of *Neurospora crassa*. However, Cossins et al. (*26*) reported that SHMT activities and 5-HCO-H_4F contents were, in paired order, 0.98, 0.76, 0.74, 0.48, 0.27, and 0.09 [nmol product min^{-1} (mg of protein)$^{-1}$] and 1.9, 9.0, 6.5, 2.0, 9.9, and 2.4 (ng/mg of dry weight), respectively. Therefore, no apparent positive correlation existed.

Stover and Schirch (*1*, *3*) speculated that SHMT-catalyzed reaction is the sole in vivo source of 5-HCO-H_4F. If SHMT does not catalyze the formation of 5-HCO-H_4F, how could it be formed in vivo? Cellular proton pumps maintain relatively acidic microenvironments (*27*). Folates are found in and enter and exit the mildly acidic (pH 4 to 5) endosomes, mitochondria, and lysosomes of eukaryotic cells (*28*−*34*). About 10% of the folates in rat liver cells is located in lysosomes (*29*). Folic acid and reduced metabolites are found in isolated rat liver lysosomes 2 h after a dose of radiolabeled folic acid (*29*). Furthermore, in cultured methotrexate-resistant hepatoma cells with increased activity of lysosomal pteroyl-γ-glutamyl hydrolase, the polyglutamyl chain lengths of folates are shorter as compared to wild-type cells (*33*). There appears to be a facilitative system for transporting both polyglutamyl folates and methotrexate into lysosomes (*35*). Thus, folates must enter and exit the acidic environment of lysosomes. Any 10-HCO-H_4F or 5-formiminotetrahydrofolate which enters the lysosome will form 5,10-CH═H_4F nonenzymatically (*13*), and 5,10-CH═H_4F could be converted to 5-HCO-H_4F as demonstrated in this study. Initial rates for the formation of 5-HCO-H_4F from 5,10-CH═H_4F in Figure 4 (i.e., reactions in the presence of ascorbate, DFX, and DTE) suggest a half-life of ∼10 h for this first-order reaction at pH 4.5 (40 °C). Although this is a relatively slow reaction, one-carbon metabolism does not take place in lysosomes, and 5,10-CH═H_4F hydrolysis to 5-HCO-H_4F would occur without any competing reactions. The 5-HCO-H_4F that is formed could be released relentlessly to the cytoplasm, the intercellular space, and other neutral pH sites. Thus, it is still possible that chemically catalyzed reactions are indeed one in vivo source of 5-HCO-H_4F as suggested by Benkovic et al. (*36*).

In conclusion, there is no compelling evidence for the existence of 5,10-HOCH-H_4F that is reported by Stover and Schirch (*1*, *3*) to be the viable in vivo substrate for SHMT-catalyzed formation of 5-HCO-H_4F. It is likely that 10-HCO-H_2F has been misidentified as 5,10-HOCH-H_4F by these investigators. Chemical reactions in mildly acidic subcellular organelles could explain the formation of 5-HCO-H_4F from 5,10-CH═H_4F. Finally, iron-redox cycling is efficient in catalyzing the oxidation of 10-HCO-H_4F to 10-HCO-H_2F at pH 4.0 to 4.5.

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