5,10-Methenyltetrahydrofolate cyclohydrolase, rat liver and chemically catalysed formation of 5-formyltetrahydrofolate

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The 5,10-methenyltetrahydrofolate (5,10-CH=H₄folate) synthetase catalyses the physiologically irreversible formation of 5,10-CH=H₄folate from 5-formyltetrahydrofolate (5-HCO-H₄folate) and ATP. It is not clear how (or if) 5-HCO-H₄folate is formed *in vivo*. Using a spectrophotometric assay for 5-HCO-H₄folate, human recombinant 5,10-CH=H₄folate cyclohydrolase, which catalyses the hydrolysis of 5,10-CH=H₄folate to 10-HCO-H₄folate, was previously shown to catalyse inefficiently the formation of 5-HCO-H₄folate at pH 7.3 [Pelletier and MacKenzie (1996) *Bioorg. Chem.* **24**, 220–228]. In the present study, we report that (i) the human cyclohydrolase enzyme catalyses the conversion of 10-HCO-/5,10-CH=H₄folate into 5-HCO-H₄folate (it is also chemically formed) at pH 4.0–7.0; (ii) rat liver has a very low capacity to catalyse the formation of 5-HCO-H₄folate

when compared with the traditional activity of 5,10-CH=H₄folate cyclohydrolase and the activity of the 5,10-CH=H₄folate synthetase; and (iii) a substantial amount of 5-HCO-H₄folate reported to be present in rat liver is chemically formed during analytical procedures. We conclude that (i) the cyclohydrolase represents some of the capacity of rat liver to catalyse the formation of 5-HCO-H₄folate; (ii) the amount of 5-HCO-H₄folate reported to be present in rat liver is overestimated (liver 5-HCO-H₄folate content may be negligible); and (iii) there is little evidence that 5-HCO-H₄folate inhibits one-carbon metabolism in mammals.

Key words: enzyme-catalysed folinic acid metabolism, folinic acid formation, 5-formyltetrahydrofolate, synthetase.

INTRODUCTION

The 5,10-methenyltetrahydrofolate (5,10-CH=H₄folate) synthetase (EC 6.3.3.2) catalyses the formation of 5,10-CH=H₄folate from 5-formyltetrahydrofolate (5-HCO-H₄folate) and ATP [1]. However, it is not known how 5-HCO-H₄folate is formed *in vivo*. No enzyme has been identified which catalyses the transfer of the 5-formyl group; therefore, the role of 5-HCO-H₄folate in one-carbon metabolism is not clear. Pelletier and MacKenzie [2] developed a spectrophotometric assay for 5-HCO-H₄folate and showed that 5,10-CH=H₄folate cyclohydrolase (EC 3.5.4.9) catalyses the formation of (6*S*)-5-HCO-H₄folate from (6*R*)-10-formyltetrahydrofolate (10-HCO-H₄folate) at pH 7.3. However, the enzyme's catalytic efficiency for 5-HCO-H₄folate formation is very poor when compared with the catalysis of the reversible hydrolysis of 5,10-CH=H₄folate to 10-HCO-H₄folate. Using this assay, we investigated whether the cyclohydrolase also catalyses the formation of 5-HCO-H₄folate under mildly acidic conditions that resemble the environment of subcellular organelles (e.g. lysosomes), and in rat liver homogenate at neutral pH. We hypothesized that acidic subcellular organelles facilitate the formation of 5-HCO-H₄folate by sequestering it from the 5,10-CH=H₄folate synthetase. The assay for 5-HCO-H₄folate was also used to investigate whether the 5-HCO-H₄folate, identified previously in rat liver, was actually formed chemically from 10-HCO-H₄folate and/or 5,10-CH=H₄folate during analytical procedures.

MATERIALS AND METHODS

(6*S*)-5-HCO-H₄folate was a gift from Lederle Laboratories (Pearle River, NY, U.S.A.) and was used to prepare (6*R*)-5,10-

CH=H₄folate solutions as described previously [3]. When stock solutions of 5,10-CH=H₄folate in 0.01 M H₂SO₄ were diluted, the cyclohydrolase and/or the buffer catalyses its conversion into equilibrium concentrations of 10-HCO-H₄folate. Thus these solutions are referred to as 10-HCO-/5,10-CH=H₄folate.

The bifunctional 5,10-CH=H₄folate cyclohydrolase/5,10-methylenetetrahydrofolate dehydrogenase (EC 3.5.4.9/EC 1.5.1.5) was prepared as described previously and stored at –20 °C in a buffer containing 50 mM phosphate (pH 7.3), 35 mM 2-mercaptoethanol, 7 mM NADP, 1 mM benzamidine and 20 % (v/v) glycerol [4]. The protein concentration in the stock solution was 0.5 mg/ml, which contained 15 units of the dehydrogenase activity/ml (1 unit = 1 μmol of product/min). The stock enzyme solution was thawed and diluted into the reaction buffer containing 50 mM acetate (pH 4.0, 4.5 or 5.0) or 50 mM phosphate (pH 6.0, 6.5 or 7.0) and 5.0 mM ascorbic acid. A non-enzymically catalysed reaction was also performed. Fresh, frozen (–70 °C) rat liver was homogenized in 3 vol. (w/v) of 50 mM phosphate (pH 7.0), centrifuged at 3200 *g* for 10 min at 5 °C and a supernatant was used as a source of enzymes.

An assay of 5-HCO-H₄folate developed by Pelletier and MacKenzie [2] was slightly modified. Aliquots of 0.5 ml of the reaction solutions were mixed with 0.5 ml of 1.0 M sodium acetate buffer (pH 3.3) or 0.5 ml of 7 % (w/v) aqueous trichloroacetic acid (TCA). The former solution was kept undisturbed for 10 min. The latter solution was heated at 100 °C for 90 s and cooled to room temperature (23 °C). Both solutions were centrifuged at 3000 *g* for 5 min and the absorbance at 356 nm (*A*₃₅₆) was measured in the supernatant and compared with similarly treated blank (i.e. buffer with or without enzyme). When the rat liver preparation was used, both the acetate and TCA

Abbreviations used: 5-HCO-H₄folate, 5-formyltetrahydrofolate; 5,10-CH=H₄folate, 5,10-methenyltetrahydrofolate; TCA, trichloroacetic acid.

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Table 1 Conversion of 10-HCO-/5,10-CH=H₄folate into 5-HCO-H₄folate during the extraction and work-up procedures for assaying rat liver folates

Amount of 10-HCO-/5,10-CH=H ₄ folate reported (± S.D.)	Estimated % of <i>in vitro</i> conversion into 5-HCO-H ₄ folate	Amount of 5-HCO-H ₄ folate reported (± S.D.)	Newly estimated amount of 5-HCO-H ₄ folate (± S.D.)	Reference
1.42 ± 0.50 (μg/g liver)	17	0.49 ± 0.15	0.20 ± 0.92	[6]*
or		or	or	
22.6 ± 4.2 (% of total folate)	17	7.7 ± 1.9	3.1 ± 7.1	[6]*
20.9 ± 7.0 (% of total folate)	27	12.5 ± 2.6	4.8 ± 12.2	[9]†
10.7 ± 4.4 (% of total folate)	27	4.2 ± 3.2	0.2 ± 8.1	[9]†
16 ± 4 (% of total folate)	27	9 ± 2	3 ± 7	[10]†
29 ± 10 (% of total folate)	27	16 ± 2	5 ± 17	[10]†

* A 1:10 dilution in the pH 7.85 Hepes/Ches buffer followed by heating at 100 °C for 10 min, then γ-glutamyl hydrolase treatment (0.25 vol. in a 0.1 M phosphate buffer, pH 7.0) for 3 h at 37 °C and finally heating at 100 °C for 5 min.

† Same as in the above footnote, except for a 1:2 dilution (versus 1:10) in the buffer and treatment with 0.5 vol. (versus 0.25) γ-glutamyl hydrolase.

solutions were centrifuged at 5000g for 15 min. Using a 1 cm light-path-length cell, the concentration of 5-HCO-H₄folate is directly proportional to the difference between A₃₅₆(TCA) and A₃₅₆(acetate), [A₃₅₆(TCA) – A₃₅₆(acetate)]/2.5 × 10⁴ M⁻¹ (see below). The initial rate of formation of 5-HCO-H₄folate was proportional to the amount of bifunctional enzyme added at pH 4.0 and 7.0 and to the amount of rat liver protein added at pH 7.0.

This assay was based on the fact that at 100 °C in 3.5 % TCA, both 5-HCO-H₄folate and 10-HCO-H₄folate are rapidly and quantitatively converted into 5,10-CH=H₄folate. But at 23 °C in 0.5 M sodium acetate (pH 3.3), only 10-HCO-H₄folate (not 5-HCO-H₄folate) was converted into 5,10-CH=H₄folate within 15 min. In addition, the equilibrium constant at pH 3.3 would prevent the complete formation of 5,10-CH=H₄folate from 5-HCO-H₄folate [3]. Since only 5,10-CH=H₄folate strongly absorbs light at 356 nm, the following equations apply (all folate species shown are before their reactions in TCA or acetate):

$$A_{356}(\text{TCA}) = 2.5 \times 10^4 [5\text{-HCO-H}_4\text{folate} + 10\text{-HCO-H}_4\text{folate} + 5,10\text{-CH=H}_4\text{folate}]$$

$$A_{356}(\text{acetate}) = 2.5 \times 10^4 [10\text{-HCO-H}_4\text{folate} + 5,10\text{-CH=H}_4\text{folate}]$$

Thus

$$A_{356}(\text{TCA}) - A_{356}(\text{acetate}) = 2.5 \times 10^4 [5\text{-HCO-H}_4\text{folate}].$$

The decrease in A₃₅₆ (Δε = 2.5 × 10⁴ M⁻¹ · cm⁻¹) was used as an assay for the cyclohydrolase-catalysed formation of 10-HCO-H₄folate from 5,10-CH=H₄folate. Initial concentration of 5,10-CH=H₄folate was 50 μM in 50 mM potassium phosphate (pH 7.0) and 5 mM ascorbate. The above assay for 5-HCO-H₄folate was used to assay rat liver 5,10-CH=H₄folate synthetase in 50 mM potassium phosphate (pH 7.0), 5 mM ascorbate, 10 mM MgCl₂, 200 μM ATP and 100 μM 5-HCO-H₄folate. Appropriate blanks for these assays contained all components except the enzyme source or the folate. Two protein concentrations (differing by a factor of two) were used to assay each of the above activities.

The pseudo-first-order rate constants for the formation of 5-HCO-H₄folate from 10-HCO-/5,10-CH=H₄folate were estimated by measuring the slope of a plot of ln A₃₅₆ (acetate) versus time. A concentrated solution of 5,10-CH=H₄folate (pH 2.5) was diluted into each buffer (containing 5 mM ascorbate) to obtain a final concentration of approx. 100 μM. Because of the lability of 10-

HCO-H₄folate (i.e. pH 7) at 100 °C, only one or two reaction half-life (t_{1/2}) values could be measured at this temperature before a departure from first-order kinetics was apparent.

The assay for 5-HCO-H₄folate was applied for the extraction of folates from rat liver, in duplicate experiments, using published methods. 10-HCO-/5,10-CH=H₄folate (0.1–1.0 mM) in 50 mM potassium phosphate (pH 7.2) was mixed with the same amount of extraction buffer as for the rat liver. The entire extraction and work-up procedure was then followed. A control sample was treated identically, with the exception that its temperature was maintained at 0 °C for the entire extraction and work-up procedures. In this analysis, the 10-HCO-/5,10-CH=H₄folate reported was assumed to be the source of 5-HCO-H₄folate. The hydrolysis of 10-HCO-/5,10-CH=H₄folate to 5-HCO-H₄folate will be pseudo first order; therefore, the fraction of the reactant converted into the product is independent of the initial concentration of the reactant. If 'F' is the amount (or percentage of total folate) of 10-HCO-/5,10-CH=H₄folate reported and 'fr' is the fraction of 10-HCO-/5,10-CH=H₄folate remaining (i.e. the fraction that has not been converted into 5-HCO-H₄folate) after the extraction and work-up procedures, the amount of 5-HCO-H₄folate formed chemically is given by the following equation:

$$\frac{F}{\text{fr}} - F = \text{amount (or \% of total folate) of 5-HCO-H}_4\text{folate formed chemically}$$

This amount (or percentage of total folate) of 5-HCO-H₄folate formed chemically is subtracted from the reported amount of 5-HCO-H₄folate to give the 'newly estimated amount of 5-HCO-H₄folate' present. The results given in Table 1 are the estimated percentage of *in vitro* conversion of 10-HCO-/5,10-CH=H₄folate into 5-HCO-H₄folate that occurred during the analytical procedures and the newly estimated amount of 5-HCO-H₄folate present. Individual procedures have been briefly described above and the results are presented in Table 1.

The K_m value of the cyclohydrolase for 10-HCO-/5,10-CH=H₄folate was estimated using the EZ-Fit program (E.I. DuPont de Nemore, Wilmington, DE, U.S.A.). This program uses a non-linear regression fit of the data to the Michaelis–Menten equation.

RESULTS

The assay for 5-HCO-H₄folate at pH 7.0 and 4.0 and in the presence of 10-HCO-/5,10-CH=H₄folate is shown in Figure 1.

Figure 1 Assay of 5-HCO-H₄folate

The $A_{356}(\text{TCA}) - A_{356}(\text{acetate})$ is plotted for 5-HCO-H₄folate in 50 mM potassium phosphate (pH 7.0; ○), in 50 mM sodium acetate (pH 4.0; △) and in 50 mM potassium phosphate (pH 7.0) with 50 μM 10-HCO-/5,10-CH=H₄folate (●). All solutions contained 5.0 mM ascorbic acid. The linear regression line has a y intercept of 0.003 and r^2 of 0.996.

The assay worked well with a 0.5 ml sample when the buffer concentration was 50 mM. For higher concentrations, the sample volume was decreased and water was added to increase the volume up to 0.5 ml before the addition of the TCA or acetate buffer. The $t_{1/2}$ for the dehydration of 10-HCO-H₄folate to 5,10-CH=H₄folate was 2.0–2.5 min in the acetate buffer at 23 °C.

The human cyclohydrolase- and buffer-catalysed formations (23 °C) of 5-HCO-H₄folate from 10-HCO-/5,10-CH=H₄folate at pH 4.0–5.0 and 6.0–7.0 are shown in Figures 2 and 3 respectively. Although the experiments were allowed to proceed for approx. 30 h, there was only a 6 and 2% loss of total formyltetrahydrofolates at pH 4.0–5.0 and 6.0–7.0 respectively (Figures 2 and 3, see the insets). The K_m value of the human cyclohydrolase-catalysed formation of 5-HCO-H₄folate (23 °C) was $9 (\pm 2) \mu\text{M}$ (substrate range 3.4–70 μM) at pH 7.0. At pH 7.0 (23 °C) using a 50 μM initial concentration of 5,10-CH=H₄folate, the ratio of activities for the human enzyme-catalysed formation of 10-HCO-H₄folate to the formation of 5-HCO-H₄folate was 4.9×10^4 . The specific activities (in $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹ $\pm$ S.D.) of rat liver homogenate ($n=3$) for the 5,10-CH=H₄folate cyclohydrolase, 5,10-CH=H₄folate synthetase and the formation of 5-HCO-H₄folate were $1.2 (\pm 0.2) \times 10^{-2}$, $5.5 (\pm 1.2) \times 10^{-4}$ and $6.1 (\pm 2.8) \times 10^{-7}$ respectively at 23 °C and pH 7.0. In rat liver homogenate, the ratio of the specific activity of the cyclohydrolase to that for the formation of 5-HCO-H₄folate was 2.0×10^4 .

The assay for 5-HCO-H₄folate was used to determine the amount of 5-HCO-H₄folate which was formed during the analytical procedures as described in published reports [6–8]. We used a 50 mM phosphate buffer (pH 7.2) containing the 10-HCO-/5,10-CH=H₄folate in an attempt to simulate the intracellular pH and buffering capacity in the liver. This is probably conservative since the intracellular phosphorus and amino acid residue concentrations are approx. 0.1 and 2.1 M respectively in rat liver [5]. Wilson and Horne [6] extracted rat liver by heating Hepes/Ches buffer (containing ascorbate and 2-mercaptoethanol;

Figure 2 Activity of the cyclohydrolase-catalysed formation of 5-HCO-H₄folate at pH 4.0, 4.5 and 5.0

All points represent means for four experiments measuring $A_{356}(\text{TCA}) - A_{256}(\text{acetate})$. ●, pH 4.0; ■, pH 4.5; ▲, pH 5.0. All experiments were performed in 50 mM sodium acetate buffer containing 5.0 mM ascorbate with 70 μg of cyclohydrolase protein/ml of the incubation mixture at 23 °C. The initial concentration of 10-HCO-/5,10-CH=H₄folate was 60 μM. Open symbols represent results from similar experiments without the enzyme. The inset shows means of $A_{356}(\text{TCA})$ for all enzyme-catalysed reactions at three values of pH plotted against incubation time. Error bars represent S.D. and lines were hand drawn to fit the points and error bars.

Figure 3 Activity of the cyclohydrolase-catalysed formation of 5-HCO-H₄folate at pH 6.0, 6.5 and 7.0

All points represent means for three experiments. ●, pH 6.0; ■, pH 6.5; ▲, pH 7.0. Experiments were performed in 50 mM potassium phosphate buffer as described in the caption to Figure 1. Open symbols and inset imply the same as for Figure 2. Error bars and lines are described in Figure 2.

pH 7.85) at 100 °C for 10 min, then treated with γ -glutamyl hydrolase in phosphate buffer (pH 7.0) for 3 h at 37 °C followed by another heating for 5 min at 100 °C. As shown in Table 1, most of the reported 5-HCO-H₄folate would be formed chemically from 10-HCO-/5,10-CH=H₄folate. The analytical procedures used by Tamura and co-workers [7,8] differed slightly from that described

Table 2 Pseudo-first-order rate constants k and $t_{1/2}$ for the formation of 5-HCO-H₄folate from 10-HCO-/5,10-CH=H₄folate

Buffer	pH	37 °C		100 °C	
		k (h ⁻¹)*	$t_{1/2}$ (h)†	k (min ⁻¹)*	$t_{1/2}$ (min)†
0.1 M Tris‡	8.0	0.022	32	0.008	87
	7.5	0.033	21	0.019	36
0.1 M Phosphate‡	8.0	0.015	46	0.016	43
	7.0	0.017	41	0.020	35
Hepes/Ches§	7.85	0.009	77	0.004	170

* The rate constant k is derived from the equation $d[5\text{-HCO-H}_4\text{folate}]/dt = k[10\text{-HCO-/5,10-CH=H}_4\text{folate}]$.

† $t_{1/2}$ is the time required for one half of the initial concentration of 10-HCO-/5,10-CH=H₄folate to be converted into 5-HCO-H₄folate, $t_{1/2} = 0.693 k^{-1}$.

‡ These buffers contained 5 mM ascorbate.

§ Based on Wilson and Horne [6].

by Wilson and Horne [6]. The liver folates were extracted with 2 vol. (instead of 10 vol.) of the same Hepes-Ches buffer and the extract was treated with 0.5 vol. (instead of 0.25 vol.) of rat serum γ -glutamyl hydrolase (T. Tamura, personal communication). Both of these alterations favoured more formation of 5-HCO-H₄folate; thus, most of the 5-HCO-H₄folate detected by Tamura and co-workers [7,8] was probably formed chemically during the analytical procedures (Table 1). The newly calculated values for 5-HCO-H₄folate in Table 1 led to an increase in the error (i.e. propagation of error) such that none of them was significantly greater than zero ($P > 0.05$, t test).

In the procedures described above, control experiments were performed involving the incubations at 0 °C (i.e. elimination of any heat treatment). This resulted in 2 % conversion of 10-HCO-/5,10-CH=H₄folate into 5-HCO-H₄folate. Because of the use of antioxidants, a loss of only 2 % of the total 10-HCO-/5,10-CH=H₄folate plus 5-HCO-H₄folate occurred during the brief treatments at 100 °C.

Since heating at 37 and 100 °C are frequently used in the extraction and work-up of cellular folates for analysis, the pseudo-first-order rate constants and $t_{1/2}$ values were estimated for the formation of 5-HCO-H₄folate from 10-HCO-/5,10-CH=H₄folate and are shown in Table 2. In general, the reaction is faster at lower pH and is slower in the Hepes/Ches buffer used by Wilson and Horne [6] when compared with the Tris and phosphate buffers.

DISCUSSION

The assay for 5-HCO-H₄folate used in the present study was based on the immutable chemical and physical properties, permitting its detection even in the presence of 10-HCO-/5,10-CH=H₄folate. The slow rate of the buffer-catalysed formation of 5-HCO-H₄folate (Figures 2 and 3) is undoubtedly the reason why others [9,10] believed that 5,10-CH=H₄folate is exclusively hydrolysed to 10-HCO-H₄folate at pH 6.5 at 25 °C.

The apparent K_m of the cyclohydrolase for 10-HCO-/5,10-CH=H₄folate of 9.0 μ M at pH 7.0 may be physiologically relevant. The curve depicting the progress of the reaction at pH 7.0 is not characteristic of a K_m of 9.0 μ M and suggests that the product, 5-HCO-H₄folate, inhibits the cyclohydrolase (Figure 3). The ratios of the specific activity of cyclohydrolase to that for the formation of 5-HCO-H₄folate for the human enzyme and for rat liver homogenate are of similar orders of magnitude. This suggests

that some of the capacity of rat liver to catalyse the formation of 5-HCO-H₄folate is due to the activity of the cyclohydrolase. It is important to note that only the catalytic capacity of rat liver is measured and not the 5-HCO-H₄folate formation *in vivo*.

Since low recoveries of 10-HCO-H₄folate have been reported, it is not surprising for us to find that 10-HCO-/5,10-CH=H₄folate was converted into 5-HCO-H₄folate during the tissue extraction procedures. Recoveries of a 10-HCO-/5,10-CH=H₄folate standard were reported to be only 69 % by Wilson and Horne [6] and even as low as 26 % by Belz and Nau [11]. Very substantial losses of 10-HCO-H₄folate at pH 7.4 have been reported after only 5 min heating at 100 °C [12]. It is probable that the low recoveries of 10-HCO-/5,10-CH=H₄folate reflect its conversion into 5-HCO-H₄folate. Rebello [13] reported that 8.7 % of the total rat liver folates was 5-HCO-H₄folate; however, the recovery of the folate standards added together was 55 % for 10-HCO-H₄folate and 123 % for 5-HCO-H₄folate, strongly suggesting that the chemical conversion of 10-HCO-/5,10-CH=H₄folate into 5-HCO-H₄folate occurred during the assay. Heating a solution of 10-HCO-H₄folate at 100 °C for 10 min at pH 7.4 in the presence of ascorbate followed by HPLC and microbiological assay was reported to produce an 11 % conversion of it into 5-HCO-H₄folate [14]. An unheated 10-HCO-H₄folate stock solution even contained 7 % 5-HCO-H₄folate, which was probably generated during storage [14]. The results in Figures 2 and 3 and Table 1 demonstrate that the formation of 5-HCO-H₄folate from 10-HCO-/5,10-CH=H₄folate in neutral or mildly acidic buffers can never be stopped, it can only be slowed down. The formation of 5-HCO-H₄folate in procedures used to detect it, including tissue extractions, work-ups, sample storage on HPLC autosamplers etc., have led to its over-estimation.

Our results raise the question of whether 5-HCO-H₄folate is actually present in mammalian tissues. It should be seriously considered that Rosenblatt et al. [15] and McMartin et al. [16] concluded that all the 5-HCO-H₄folates they reported were formed chemically during the extraction of folates in cultured human fibroblasts and rat liver and that Duch et al. [17] did not detect 5-HCO-H₄folate in rodent liver and kidney. These results are in agreement with our results in Table 2. Others have reported that 10 % of the total rat and mouse liver folates is in the form of 5-HCO-H₄folate [18,19]. However, in a study of a single rat liver, the tissue extract (at pH 7.6) was heated for 20 min at 100 °C [18]. Furthermore, these studies [18,19] reported that the liver pools of tetrahydrofolate and 5-methyltetrahydrofolate were 53–64 % and 17–24 % of the total folate respectively. These percentages are outside the values generally reported for these folates in rat and mouse liver [6–8,12,13,16,17,20]. Thus there may be reasons to question the accuracy of these results. In any case, the spectrophotometric assay for 5-HCO-H₄folate could be applied to extraction, work-up and analytical procedures to back correct for 5-HCO-H₄folate formation from 10-HCO-/5,10-CH=H₄folate. This approach may help clarify the results reported in the literature.

Is 5-HCO-H₄folate efficiently biosynthesized and does it accumulate in mammalian liver after consumption of a large dose? Previous reports of active biosynthesis of 5-HCO-H₄folate in rat liver *ex vivo* involved 100 °C treatments at pH 7.4 and 6.5, no separation of folate species followed by long-term microbiological assays [21]. These procedures would chemically convert 10-HCO-/5,10-CH=H₄folate into 5-HCO-H₄folate. Recent results are consistent with very little net accumulation of 5-HCO-H₄folate following a dose. A dose of 5-HCO-H₄folate (90 mg/kg) was given to mice. Only 6 h later, the dose started to accumulate or reach steady-state levels, and represented only approx. 1 % of the total liver folates [20]. This research group

used specific enzyme-catalysed reactions to convert 5-HCO-H₄folate into 5,10-CH=H₄folate, which could then be detected by sensitive radiochemical methods [22]. A larger dose of 5-HCO-H₄folate (400 mg/kg) has been administered to tumour-bearing mice, and again 5-HCO-H₄folate levels in liver essentially returned to baseline levels only after 6 h [23]. Heat treatments and work-up procedures probably created most of the baseline levels of 5-HCO-H₄folate reported in this study [23]. The tumour, which contained barely detectable levels of the 5,10-CH=H₄folate synthetase, did accumulate 5-HCO-H₄folate in amounts approx. five times that of the normal liver at 6 h, and these results are likely to be real and emphasize the metabolic role of this synthetase in keeping 5-HCO-H₄folate levels low [23].

Our results indicate that the specific activity of the 5,10-CH=H₄folate synthetase in rat liver is 900 times that of the catalytic capacity for the formation of 5-HCO-H₄folate. Thus it is probable that any accumulation of 5-HCO-H₄folate *in vivo* must occur at a cellular site sequestered from the 5,10-CH=H₄folate synthetase, a cytosolic enzyme. Stokstad and co-workers [24] reported that the lysosomes of rat liver contain folate. This subcellular compartment would sequester folates from the 5,10-CH=H₄folate synthetase, where cyclohydrolase-catalysed (or chemically catalysed) formation of 5-HCO-H₄folate (albeit very small) could occur. We suggest that very small amounts of 5-HCO-H₄folate could be formed and stored in lysosomes where it would be sequestered from (and therefore not affect) almost all enzymes of one-carbon metabolism. In support of this hypothesis, the capacity of lysosomes to internalize (and release) folates and to hydrolyse folyl polyglutamates has been shown to be one important feature for determining cellular folate polyglutamate chain length ([25] and references therein). This metabolism is probably one reason why lysosomes contain folates [24].

An alternative hypothesis to ours states that (i) serine hydroxymethyltransferase efficiently catalyses the formation of 5-HCO-H₄folate *in vivo* from the putative substrate, 5,10-hydroxymethylenetetrahydrofolate, and (ii) a metabolic pool of 5-HCO-H₄folate can substantially inhibit or bind to enzymes involved in one-carbon metabolism including folate-dependent enzymes of purine, pyrimidine and serine metabolism as well as 5,10-methylenetetrahydrofolate dehydrogenase and dihydrofolate reductase [26,27]. We find that this hypothesis is not supported in mammalian liver because (i) the putative substrate, 5,10-hydroxymethylenetetrahydrofolate, does not exist or is not formed in significant amounts [3]; (ii) the cyclohydrolase also contributes to the capacity of rat liver to catalyse 5-HCO-H₄folate formation; (iii) the amount of 5-HCO-H₄folate actually present in rat liver is overestimated (Table 1); and (iv) the activity of the 5,10-CH=H₄folate synthetase greatly exceeds the activity required for the formation of 5-HCO-H₄folate in rat liver and should prevent 5-HCO-H₄folate accumulation in the cytoplasm [22,23]. Furthermore, the absence of reported toxicity of high-dose 5-HCO-H₄folate in cancer patients and healthy subjects [28–30] is inconsistent with its postulated strong inhibition of one-carbon metabolism [26,27].

Thermodynamic equilibrium at pH 4.5–8 favours the formation of 5-HCO-H₄folate from 10-HCO-/5,10-CH=H₄folate, and it is difficult to imagine why there is any need for an efficient enzyme-catalysed formation. Since 5-HCO-H₄folate must be formed continuously *in vivo* and without the activity of the 5,10-CH=H₄folate synthetase, a majority of cytosolic tetrahydrofolates would eventually end up as a pool of metabolically inactive 5-HCO-H₄folates. Thus there is a reason for the existence of the 5,10-CH=H₄folate synthetase, as a salvage enzyme, keeping steady-state amounts of 5-HCO-H₄folate in mammalian liver at

negligible levels. The 5,10-CH=H₄folate synthetase would also metabolize 5-HCO-H₄folate formed during cooking and storage of food. Finally, 5-HCO-H₄folate may actually accumulate and get stored in substantial amounts in non-mammalian cells, e.g. in the large acidic turgor vacuoles of plant cells.

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