Bioactivity of [6*R*]-5-formyltetrahydrofolate, an unnatural isomer, in humans and *Enterococcus hirae*, and cytochrome *c* oxidation of 10-formyltetrahydrofolate to 10-formyldihydrofolate

Joseph E. BAGGOTT¹, Constance B. ROBINSON and Kelly E. JOHNSTON

Department of Nutrition Sciences, 336 Webb Building, 1675 University Blvd., University of Alabama at Birmingham, Birmingham, AL, 35294-3360, U.S.A.

The bio-inactive C-6 isomer, [6*R*]-5-formyl-tetrahydrofolate (5-HCO-H₄F), is not found in Nature. An oral dose of 13.5 μ mol of [6*R*]-5-HCO-H₄F in humans results in the appearance of the naturally occurring [6*S*]-5-methyl-tetrahydrofolate and relatively large amounts of other bioactive folates in plasma. The removal of the asymmetry at C-6 could account for these results. Two oxidized cytochrome *c* [cyt *c* (Fe³⁺)] molecules oxidize one 10-formyl-tetrahydrofolate (10-HCO-H₄F) with second-order kinetics and a rate constant of $1.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. The folate product of this oxidation reaction is 10-formyl-dihydrofolate (10-HCO-H₂F), which has no C-6 asymmetric centre and is therefore bioactive. The folate-requiring bacterium, *Enterococcus hirae*, does not normally biosynthesize cytochromes but does so when given an exogenous source of haem (e.g. haemin). *E. hirae*

grown in haemin-supplemented media for 3 days utilizes both [6*R*]- and [6*S*]-5-HCO-H₄F in contrast to that grown in control medium, which utilizes only the [6*S*] isomer. Since known chemical reactions form 10-HCO-H₄F from 5-HCO-H₄F, the unusually large rate constant for the oxidation of 10-HCO-H₄F by cyt *c* (Fe³⁺) may account for the unexpected bioactivity of [6*R*]-5-HCO-H₄F in humans and in *E. hirae* grown in haemin-containing media. We used an unnatural C-6 folate isomer as a tool to reveal the possible *in vivo* oxidation of 10-HCO-H₄F to 10-HCO-H₂F; however, nothing precludes this oxidation from occurring *in vivo* with the natural C-6 isomer.

Key words: folinic acid, non-enzymic metabolism, haem iron.

INTRODUCTION

The C-6 optical isomer, [6*R*]-5-formyl-5,6,7,8-tetrahydrofolate (5-HCO-H₄F), is a chemically synthesized compound, not found in living organisms [1,2]. [6*R*]-5-HCO-H₄F and its C-6 configurationally related folates are not utilized by folate-requiring bacteria or by enzymes of folate metabolism since these enzymes recognize only the naturally occurring isomer, [6*S*]-5-HCO-H₄F, and its C-6 configurationally related folates [3–13]. Therefore, [6*R*]-5-HCO-H₄F was used here as a tool to uncover possible ‘chemical metabolism’ of this folate and its C-6 configurationally related folates in living organisms.

Oral doses of $\leq 25 \mu$ mol of the natural isomer, [6*S*]-5-HCO-H₄F, (present as such or in the [6*RS*] racemic mixture) are very rapidly metabolized in humans and appear in blood plasma almost exclusively as [6*S*]-5-methyltetrahydrofolate (5-CH₃-H₄F) through pathways shown in Figure 1 [14–18]. Using oral doses of radiolabelled [6*S*]-5-HCO-H₄F, it has been established that within 1–2 h the increase in plasma [6*S*]-5-CH₃-H₄F arises from the metabolism of the former [18]. Our results presented here and those observed previously indicate that oral doses of the unnatural isomer, [6*R*]-5-HCO-H₄F, are also converted rapidly into (i) [6*S*]-5-CH₃-H₄F, (ii) other folates with the natural C-6 configuration and (iii) folates with no asymmetric centre at C-6 [17]. Metabolic alteration of the unnatural isomer, [6*R*]-5-HCO-H₄F, obviously does not involve enzymic metabolism; therefore, it must involve ‘chemical metabolism’ reactions. One likely chemical reaction is rapid oxidation of the unnatural isomer, [6*S*]-10-formyltetrahydrofolate (10-HCO-H₄F), to 10-formyldihydrofolate (10-HCO-H₂F), which now has no asymmetric

centre at C-6, and can be metabolized enzymically (Figure 1) [17,19–21].

The *in vitro* chemical oxidation of [6*RS*]-10-HCO-H₄F by oxidized cytochrome *c* [cyt *c* (Fe³⁺)] to produce 10-HCO-H₂F and reduced cytochrome *c* [cyt *c* (Fe²⁺)] is investigated here. Additionally, when the micro-organism, *Enterococcus hirae* (formerly *Streptococcus faecalis*), is given haemin (the haem moiety of cytochromes) in its growth media, both [6*R*]- and [6*S*]-5-HCO-H₄F support the growth of this folate-requiring micro-organism. Using its normal growth media, only [6*S*]-5-HCO-H₄F supports *E. hirae* growth, presumably because there is no biosynthesis of cytochromes needed to oxidize the unnatural isomer, [6*S*]-10-HCO-H₄F, which arises chemically from the unnatural isomers [6*R*]-5-HCO-H₄F and [6*S*]-5,10-methenyltetrahydrofolate (5,10-CH=H₄F) (Figure 1).

EXPERIMENTAL PROCEDURES

Materials

[6*R*]- and [6*S*]-5-HCO-H₄F were obtained from Schircks Laboratories (Jona, Switzerland) and Lederle Laboratories (Pearl River, NY, U.S.A.), respectively. Cyt *c* (Fe³⁺) from bovine heart, [6*RS*]-5-HCO-H₄F, folic acid, methotrexate and haemin were obtained from Sigma (St. Louis, MO, U.S.A.). Media (folic acid casei assay and folic acid assay) for *Lactobacillus casei* and *E. hirae*, respectively, were obtained from Difco (Detroit, MI, U.S.A.). Preparation of 5,10-CH=H₄F, 10-HCO-H₄F, folic acid pentaglutamate and 10-formylfolic acid (10-HCO-F) have been described previously [10,19–22]. All other chemicals were the highest purity commercially available.

Abbreviations used: cyt *c* (Fe³⁺), oxidized cytochrome *c*; cyt *c* (Fe²⁺), reduced cytochrome *c*; 10-HCO-F, 10-formylfolic acid; 5-HCO-H₄F, 5-formyl-5,6,7,8-tetrahydrofolate; 5-CH₃-H₄F, 5-methyltetrahydrofolate; 10-HCO-H₄F, 10-formyltetrahydrofolate; 10-HCO-H₂F, 10-formyl-7,8-dihydrofolate; 5,10-CH=H₄F, 5,10-methenyltetrahydrofolate; AICAR transformylase, aminoimidazole carboxamide ribotide transformylase.

¹ To whom correspondence should be addressed (e-mail matlocks@uab.edu).

Figure 1 Major folate metabolism at the oxidized, dihydro and tetrahydro oxidation states of the pteridine ring

Enzyme-catalysed reactions are: (1) reduction by dihydrofolate reductase of folic acid (F) to 7,8-dihydrofolate (H_2F) and to [6S]-5,6,7,8 tetrahydrofolate (H_4F); (2) thymidylate synthase formation of deoxythymidylate (TMP) from deoxyuridylate (dUMP); (3) aminoimidazole carboxamide ribotide (AICAR) transformylase formation of formyl-AICAR (fAICAR) from AICAR; (4) glycine (GLY) cleavage enzyme; (5) serine (SER) hydroxymethyltransferase; (6) [6R]-10-formyltetrahydrofolate (10-HCO- H_4F) dehydrogenase; (7) [6R]-5,10-methylenetetrahydrofolate (5,10- CH_2 - H_4F) reductase formation of [6S]-5- CH_3 - H_4F ; (8) dimethylglycine (DMG) dehydrogenase; (9) sarcosine (SAR) dehydrogenase; (10) glycylamide ribotide (GAR) transformylase formation of formyl-GAR (fGAR) from GAR; (11) 5,10- CH_2 - H_4F dehydrogenase; (12) methionine (MET) synthase formation of MET from homocysteine (HCY); (13) glutamate formiminotransferase-formiminotetrahydrofolate cyclodeaminase formation of glutamate (GLU) from formiminoglutamate (FIGLU); (14) 10-HCO- H_4F synthetase; (15) [6R]-5,10-methylenetetrahydrofolate (5,10- $CH=H_4F$) cyclohydrolase; (5) serine hydroxymethyltransferase formation of [6S]-5-formyltetrahydrofolate (5-HCO- H_4F) from [6R]-5,10- $CH=H_4F$; and (17) [6R]-5,10- $CH=H_4F$ synthetase. Chemically catalysed reactions are indicated by Chem. and although they are shown with the naturally occurring C-6 isomer, they also occur with the unnatural C-6 isomer.

Human experiments

The study was reviewed and approved by the Institutional Review Board of the University of Alabama at Birmingham, AL, U.S.A., and each subject signed a consent form. After an overnight fast, three healthy male subjects (27–58 years old) ingested 2.7 μ mol of [6S]- or [6R]-5-HCO- H_4F or 13.5 μ mol of [6R]-5-HCO- H_4F dissolved in 10–50 ml of distilled water. 5-HCO- H_4F was quantified using $\epsilon_{282} = 3.2 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 7.0. A 'washout' period of at least 1 week occurred between each of the three doses. Heparinized blood samples were obtained immediately before (baseline) and 1, 2 and 4 h after the oral bolus and plasma were separated. The procedures of microbiological folate assays were started within 3 h of blood drawing.

Microbiological assays of folates

The *L. casei* and *E. hirae* microbiological assays of plasma folates were performed using a 96-well plate reader as described previously with [6RS]-5-HCO- H_4F as a standard and assuming that only [6S]-5-HCO- H_4F is bioactive [23]. The turbidity at 650 nm (D_{650}) is a measure of the growth of these organisms. An individual plasma concentration was the mean of at least four determinations (two dilutions in duplicate) which fell on the linear portion of the standard curve. The coefficients of variation for the *L. casei* and *E. hirae* assays were $\leq 10\%$ and $\leq 15\%$, respectively [23]. The amount of [6S]-5- CH_3 - H_4F is estimated by the value of the *L. casei* assay minus that of the *E. hirae* assay, since *E. hirae* does not respond to [6S]-5- CH_3 - H_4F . Therefore, the *E. hirae* assay measures 'non-methyl' folates. Baseline plasma concentrations were subtracted from plasma concentrations at 1,

2 and 4 h after the dose. For the experiment evaluating the effect of haemin, *E. hirae* was grown in medium containing 50 μ g/ml haemin and 150 nM [6RS]-5-HCO- H_4F for 3 days (with transfer at the end of each 24 h period) to create 'haemin-conditioned' *E. hirae*.

Quantification of 10-HCO- H_4F

10-HCO- H_4F is unstable and was prepared immediately before its use. The absorbance at 312 nm (A_{312}), an isosbestic point for the hydrolysis of 5,10- $CH=H_4F$ to 10-HCO- H_4F , represents the sum of the concentrations of these two folates [13]. The A_{356} measures primarily the concentration of 5,10- $CH=H_4F$ [19,20]. The following two equations apply:

$$A_{312} = 9.7 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1} [(5,10-CH=H_4F) + (10-HCO-H_4F)]$$

$$A_{356} = 2.5 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1} (5,10-CH=H_4F) + 1.0 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1} (10-HCO-H_4F)$$

If both A_{356} and A_{312} are measured, then the concentration of 10-HCO- H_4F can be shown to be:

$$[10-HCO-H_4F] = 1.04 \times [(A_{312}/9.7 \times 10^3) - (A_{356}/2.5 \times 10^4)]$$

For example, if $A_{356} = 0.5 \times A_{312}$, the hydrolysis of 5,10- $CH=H_4F$ to 10-HCO- H_4F is 84% complete.

Stoichiometry of the reaction of 10-HCO- H_4F with cyt c (Fe^{3+}), a typical experiment

To 0.2 ml of 0.2 M potassium phosphate (pH 8.0) containing 4.0 mM [6RS]-10-HCO- H_4F (90%) + [6RS]-5,10- $CH=H_4F$

Figure 2 Growth of *L. casei* on folates

The turbidity at 650 nm (D_{650} ; ' A_{650} ' on the Figure), which measures cell growth, is plotted against the log (in pM) of the concentration of the folate. Each point is the average of two determinations and lines are spline curves.

(10%) was added 0.2 ml of 4.0 mM cyt *c* (Fe^{3+}). The reaction was allowed to proceed for 5 min at room temperature. At that time, the 500–600 nm spectrum indicated that all of the cyt *c* was in the Fe^{2+} state and no change in this spectrum was observed after the addition of 5 mg of ascorbic acid. A 0.3 ml aliquot of this reaction solution was chromatographed on a Sephadex G-10 column (0.7×42 cm) and eluted with 0.1 M potassium phosphate (pH 6.7) containing 1 mg/ml ascorbic acid [20]. The spectrum of each fraction (0.97 ml) was scanned from 310 to 600 nm in the elution buffer and scanned again 20 min after adding 50 μl of 5.0 M H_2SO_4 to each fraction (see Figure 5, below). The amounts of 10-HCO- H_4F , 5,10-CH= H_4F and 10-HCO- H_2F produced or remaining were estimated from the ϵ_{312} values at neutral pH and ϵ_{356} values at pH 1.0 and the areas under each peak [13,19,20]. In separate experiments, authentic 10-HCO- H_4F , 5,10-CH= H_4F and 10-HCO- H_2F eluted with the same peak fractions as shown in Figure 5. The concentrations of total cyt *c* and cyt *c* (Fe^{2+}) were determined using A_{527} (an isosbestic point; $\epsilon_{527} = 1.1 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and A_{550} ($\epsilon_{550} = 2.8 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$), respectively.

Kinetics of the oxidation of [6RS]-10-HCO- H_4F by cyt *c* (Fe^{3+})

Solid [6RS]-5,10-CH= H_4F was stored in 0.1 M H_2SO_4 containing 10 mM mercaptoethanol. The [6RS]-5,10-CH= H_4F was washed five times with ice-cold 2.0 mM H_2SO_4 (0.02 ml for each mg) to remove the mercaptoethanol. It was then dissolved in ice-cold 2.0 mM H_2SO_4 to make a stock solution of 0.1–0.5 mM (0.2–1.0 mg in 4.0 ml of 2.0 mM H_2SO_4). This solution was diluted 100–1000-fold into a 0.2 M potassium phosphate reaction buffer (pH 6.8, 7.4 or 8.0) and the A_{312} measured. When the

Figure 3 Increase in plasma folates following oral doses of [6R]- and [6S]-5-HCO- H_4F

(A) Increase in plasma [6S]-5- $\text{CH}_3\text{-H}_4\text{F}$ (means $\pm$ S.E.M.) following oral doses of 2.7 μmol of [6R]-5-HCO- H_4F (solid line) and [6S]-5-HCO- H_4F (broken line). (B) Increase in plasma non-methyl folates (means $\pm$ S.E.M.) after oral doses of 2.7 μmol of [6R]-5-HCO- H_4F (solid line) and [6S]-5-HCO- H_4F (broken line).

hydrolysis of [6RS]-5,10-CH= H_4F to 10-HCO- H_4F had achieved at least 84% completion ($A_{356} \leq A_{312}$), a small volume of cyt *c* (Fe^{3+}) stock solution was added, mixed rapidly and the ΔA_{416} recorded. Stock solutions of cyt *c* (Fe^{3+}) were made in the same reaction buffer. The $\Delta \epsilon_{416}$ for cyt *c* (Fe^{3+}) $\rightarrow$ cyt *c* (Fe^{2+}) is $4.0 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [24]. At 416 nm the molar absorption coefficients for 5,10-CH= H_4F , 10-HCO- H_4F and 10-HCO- H_2F are less than $1.0 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and contribute very little to the ΔA_{416} [13,19,20]. Potential inhibitors of 10-HCO- H_4F oxidation were added before the addition of cyt *c* (Fe^{3+}). Initial rates were estimated by the method of Wilkinson [25].

RESULTS

Microbiological assay of folates

The micro-organisms that were used to assay plasma folates effectively only respond to the natural C-6 isomer or to folates with no asymmetric centre at C-6. For example, Figure 2 shows the growth response of *L. casei* to C-6 isomers of folates and to folates with no C-6 asymmetric centres. The C-6 isomers produce approximately half the response (i.e. a 0.3 log unit shift to the right) of the natural isomer and half the response to folates with no C-6 asymmetric centre. The response of the unnatural C-6 isomer, [6R]-5-HCO- H_4F , was relatively small, being shifted about 2 log units to the right of [6S]-5-HCO- H_4F . This small response may be due to contamination of [6R]-5-HCO- H_4F with the natural isomer or some previously unrecognized ability of the micro-organism to metabolize [6R]-5-HCO- H_4F .

Figure 4 Increases in plasma [6S]-5-CH₃-H₄F (□) and plasma non-methyl folates (○) after an oral dose of 13.5 μmol of [6R]-5-HCO-H₄F

Results are shown as means ± S.E.M.

Table 1 Area under the curve (nM × h; mean ± S.E.M., *n* = 3) for plasma folates after independent oral doses of [6S]- and [6R]-5-HCO-H₄F in humans

Values (including percentage of total) with different superscript letters are significantly different (*P* < 0.05) by the paired Student's *t* test. Percentages in parentheses are percentages of the total area under the curve.

Isomer	Dose (μmol)	Area under the curve (% of total folates)		
		Total folate	[6S]-5-CH ₃ -H ₄ F	Non-methyl folates
[6S] (natural)	2.7	402 ± 56 ^a	397 ± 55 (99) ^a	5 ± 2 (1) ^a
[6R] (unnatural)	2.7	127 ± 26 ^b	112 ± 23 (88) ^b	15 ± 4 (12) ^b
[6R] (unnatural)	13.5	484 ± 114 ^a	188 ± 31 (42) ^c	296 ± 94 (58) ^c

Plasma folates after oral doses of [6R]- and [6S]-5-HCO-H₄F

The increases in plasma concentrations of [6S]-5-CH₃-H₄F and non-methyl folates following 2.7 μmol oral doses of [6R]- and [6S]-5-HCO-H₄F (a control experiment) are shown in Figure 3. This amount of both isomers produced an increase of predominately [6S]-5-CH₃-H₄F in plasma, and the concentrations peaked at 1 h. In contrast, as shown in Figure 4, the higher dose of [6R]-5-HCO-H₄F (13.5 μmol) produced an increase in primarily non-methyl folates (i.e. folate precursors of [6S]-5-CH₃-H₄F) rather than [6S]-5-CH₃-H₄F in plasma at 1 h after the dose. The kinetics of the appearance of plasma non-methyl folate following the higher dose of [6R]-5-HCO-H₄F (Figure 4) was markedly different from that of the lower dose of [6R]-5-HCO-

Figure 5 Sephadex G-10 chromatography of the reaction of 10-HCO-H₄F with cyt *c* (Fe³⁺)

Procedures are described in the Experimental procedures section. *A*₃₁₂ (○) was measured at pH 6.7. *A*₃₅₆ (□) was measured at ≈ pH 1. The peak at fraction 12 was unreacted 10-HCO-H₄F (pH 6.7) and 5,10-CH=H₄F (≈ pH 1). The peak at fraction 16 was 10-HCO-H₂F. Cyt *c* (Fe³⁺) and cyt *c* (Fe²⁺) eluted at fractions 4–6.

H₄F (Figure 3B) because of a burst of plasma non-methyl folates at 1 h. This burst was about five times the concentration of plasma 5-CH₃-H₄F. Peak plasma concentrations of both 5-CH₃-H₄F and non-methyl folates occurred at 2 h after the oral dose (Figure 4). As shown in Table 1, the area under the curve for non-methyl folates was greater when the higher dose of [6R]-5-HCO-H₄F was used.

Oxidation of 10-HCO-H₄F by cyt *c* (Fe³⁺)

Cyt *c* (Fe³⁺) oxidizes a variety of organic molecules, including ascorbic acid and riboflavins, and appeared to be a good candidate as an *in vivo* oxidant [24,26,27]. Experiments using cyt *c* (Fe³⁺) and 10-HCO-H₄F at millimolar concentrations indicated that a reaction was taking place during the mixing time. These experiments were performed in the absence of any other oxidizable compound (i.e. antioxidants). The stoichiometry of this reaction was determined to be:

An example of this type of experiment is shown in Figure 5, where 0.29 μmol of 10-HCO-H₂F was found after reacting 0.6 μmol of cyt *c* (Fe³⁺) with 0.6 μmol of 10-HCO-H₄F. After the reaction, all of the cyt *c* was in the Fe²⁺ oxidation state. Additional experiments demonstrated that both [6R]-10-HCO-H₄F and [6RS]-10-HCO-H₄F could be oxidized rapidly and completely, indicating that this chemical oxidation was not specific for one of the C-6 isomers. The folate product of this oxidation, 10-HCO-H₂F, is known to have lost the C-6 asymmetric centre [19,20]. Practically, this oxidation is irreversible *in vitro*.

To slow down the rate and better characterize this oxidation, the initial concentrations of the reactants were reduced to the range 0.2–5.0 μM. The log₁₀ initial rates of the reaction [nM cyt *c* (Fe²⁺)/s] were plotted against the log₁₀ concentration of one reactant, keeping the concentration of the other reactant constant

Figure 6 Cyt *c* (Fe³⁺) oxidation of 10-HCO-H₄F

The $\ln$ of the initial rate (in nM/s) is plotted versus the $\ln$ of the concentration of the variable reactant. The constant concentration of the reactant, the slope of the line and the mean rate constant are shown. All rates were measured at pH 7.4 (23 °C).

(Figure 6). For each reactant, the reaction order was estimated to be one, and the overall second-order rate constant was estimated to be $(1.3 \pm 0.1) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (pH 7.4, 0.2 M potassium phosphate, 23 °C). The reaction order and rate constant for cyt *c* (Fe³⁺) were verified using the method of Wilkinson [25] and conditions where [6*RS*]-10-HCO-H₄F concentration was at least in 4-fold excess over the cyt *c* (Fe³⁺) concentration. The reaction order was 1.16 ± 0.07 ($n = 7$) for cyt *c* (Fe³⁺) and the rate constant was $(1.4 \pm 0.3) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. Figure 7 shows two such experiments. As a final check, under more rigorous pseudo first-order conditions, each reactant fit first-order kinetics through several half-lives. Figure 8 shows two of these experiments.

Other characteristics of this oxidation include the following. There was no biphasic nature under conditions where almost all of the [6*RS*]-10-HCO-H₄F is oxidized (Figure 8A). There was no evidence for the reaction occurring preferentially with one of the C-6 isomers. The following pteridine compounds tested at 50–100 μM failed to produce any ΔA_{416} (i.e. failed to react) when added to 2.0 μM cyt *c* (Fe³⁺): 5-HCO-H₄F, 10-HCO-F, folic acid, the pentaglutamate of folic acid and methotrexate. The inhibition of the reaction (pH 7.4, 0.2 M potassium phosphate, 23 °C) of 5.0 μM [6*RS*]-10-HCO-H₄F with 2.0 μM cyt *c* (Fe³⁺) by 50–100 μM concentrations of these pteridines was less than

Figure 7 The method of Wilkinson [25] is used to determine reaction order and rate constants

Time (t) in s divided by the fraction of the reaction completed (f) is plotted versus t . Initial concentrations of [6*RS*]-10-HCO-H₄F and cyt *c* (Fe³⁺) are shown as well as slopes and calculated rate constants.

20%. The above reaction was inhibited less than 40% by increasing the concentration of potassium phosphate from 0.2 to 1.8 M (pH 7.4). Using initial rates, the second-order rate constant was found to be $(1.4 \pm 0.3) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ($n = 9$) at pH 8.0 and $(1.1 \pm 0.1) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ($n = 16$) at pH 6.8. 5,10-CH=H₄F did not react as such, as shown in Figure 9. However, the same initial concentrations of 5,10-CH=H₄F, 10-HCO-H₄F and cyt *c* (Fe³⁺) reacted to reach the same equilibrium position at pH 6.8 with different kinetics. The clear lag phase for the oxidation of 5,10-CH=H₄F is consistent with its hydrolysis to 10-HCO-H₄F before its reaction with cyt *c* (Fe³⁺) (Figure 9).

Bioactivity of [6*R*]-5-HCO-H₄F in haemin-conditioned *E. hirae*

The porphyrin ring binds iron to form haem-moiety-containing proteins such as haemoglobin, myoglobin and cytochromes. The folate-requiring bacteria *E. hirae* and *L. casei* presumably lack the ability to biosynthesize haem and consequently they contain no detectable cytochromes [28,29]. However, others have demonstrated that, given an exogenous supply of haem, *E. hirae* can biosynthesize cytochromes [30]. This organism could therefore be used to test whether cytochromes participate in folate metabolism.

An aqueous solution of 5-HCO-H₄F will approach equilibrium with both 5,10-CH=H₄F and 10-HCO-H₄F [31,32]. If cytochromes (e.g. cyt *c*) play a role in the metabolism of 10-HCO-H₄F, *E. hirae* should also utilize [6*R*]-5-HCO-H₄F after it had been supplied with a source of haem. Figure 10 shows the growth response to [6*RS*]-5-HCO-H₄F of *E. hirae* that had been grown for 3 days in the presence of haemin (a haem compound). This micro-organism was designated as haemin-conditioned. At the lower concentrations of [6*RS*]-5-HCO-H₄F, the growth response of the haemin-conditioned *E. hirae* grown in the haemin medium

Figure 8 Pseudo first-order semi-logarithmic plots of the reaction (ΔA_{416}) of [6RS]-10-HCO-H₄F with cyt *c* (Fe³⁺)

The inserts show the time courses of the reaction. Reactions were performed at pH 7.4 and 23 °C using initial concentrations of cyt *c* (Fe³⁺) and [6RS]-10-HCO-H₄F as shown.

was shifted 0.3–0.5 log units to the left (i.e. 2–3-times greater) compared with either regular *E. hirae* or haemin-conditioned *E. hirae* grown in normal medium without haemin. These results are

Figure 9 The reaction (ΔA_{416}) of cyt *c* (Fe³⁺) with [6RS]-10-HCO-H₄F and [6RS]-5,10-CH₂=H₄F

The reactions were carried out at pH 6.8 and 23 °C with initial concentrations of [6RS]-10-HCO-H₄F, [6RS]-5,10-CH=H₄F and cyt *c* (Fe³⁺) as shown.

consistent with *E. hirae* utilizing both isomers of 5-HCO-H₄F when biosynthesizing cytochromes. The possibility that haemin itself stimulated the growth or reacted with [6RS]-5-HCO-H₄F to produce the greater response is unlikely because *E. hirae* grew poorly after its first day of exposure to haemin (Figure 10).

DISCUSSION

The unnatural isomer [6R]-5-HCO-H₄F was used as a tool to uncover a new folate metabolic pathway which involves chemical reactions. If the generally accepted folate metabolic pathways are the only way that folates are metabolized, the unnatural C-6 isomers of tetrahydrofolates should not be bioactive in humans or *E. hirae*, since folate-metabolizing enzymes produce and utilize only the naturally occurring C-6 isomers (Figure 1). Therefore, orally administered doses of the unnatural [6R]-5-HCO-H₄F should not be metabolized and should produce no increase in plasma folates that assay organisms respond to.

The human experiments presented here indicate that an oral dose of the unnatural isomer [6R]-5-HCO-H₄F is metabolized to the natural isomer [6S]-5-CH₃-H₄F and to non-methyl folates that assay organisms respond to. The kinetics of the appearance of plasma [6S]-5-CH₃-H₄F and of non-methyl folates are markedly different when comparing the lower dose (2.7 μmol) with the higher dose (13.5 μmol) of [6R]-5-HCO-H₄F. We observed an initial accumulation or 'burst' of non-methyl folates at the higher dose, which presumably exceeds the enzymic capacity for their conversion into [6S]-5-CH₃-H₄F (Figure 4). These non-methyl folates could be 10-HCO-H₂F, 7,8-dihydrofolate, 5,6,7,8-

Figure 10 Growth (D_{650} ; ' A_{650} ' on the Figure) of wild-type and haemin-conditioned *E. hirae* in normal and haemin media in response to [6RS]-5-HCO-H₄F concentration

Each point is the mean of two determinations and lines are spline curves.

tetrahydrofolate etc. (see Figure 1). It is likely that chemical (not enzyme-catalysed) reactions must be involved in the initial steps of the conversion of [6R]-5-HCO-H₄F into [6S]-5-CH₃-H₄F. Thus, relatively early step(s) of the pathway, which would racemize or remove the asymmetric centre at C-6 of [6R]-5-HCO-H₄F or C-6 configurationally related tetrahydrofolates, are needed to explain the results presented above. The oxidation of 5,6,7,8-tetrahydrofolates to 7,8-dihydrofolates is a reaction that removes the C-6 asymmetric centre. Both 5-HCO-H₄F and 5,10-CH=H₄F, which is formed chemically from the former at acidic pH, are quite stable to oxidation [31–33]. In contrast, 10-HCO-H₄F, which is formed chemically from 5,10-CH=H₄F at neutral pH, can be oxidized readily to the 7,8-dihydro form, i.e. 10-HCO-H₂F, as shown previously [19,20].

This 10-HCO-H₂F can be metabolized enzymically to [6S]-5-CH₃-H₄F, as shown in Figure 1. We have shown that 10-HCO-H₂F is a good substrate for mammalian aminoimidazole carboxamide ribotide (AICAR) transformylase, an enzyme of *de novo* purine nucleotide biosynthesis [19]. The folate product of this AICAR transformylase-catalysed reaction is 7,8-dihydrofolate, which is reduced readily to 5,6,7,8-tetrahydrofolate. This fully reduced folate can then be metabolized to [6S]-5-CH₃-H₄F by several different pathways shown in Figure 1. The fact that 10-HCO-H₂F supports the growth of human leukaemia cells is consistent with this folate being a constituent of the dynamic pool of metabolically interconvertible folates [21].

The chemical conversions of 5-HCO-H₄F into 5,10-CH=H₄F and 5,10-CH=H₄F into 10-HCO-H₄F have been well documented. These reactions should take place in the acidic environment of the stomach and the neutral pH environment of the upper small intestine or the cellular cytoplasm, respectively (Figure 1) [4,31–33]. The rapid *in vivo* oxidation of 10-HCO-H₄F to 10-HCO-H₂F is not so readily explained. Non-protein-bound iron in bile may catalyse this oxidation in the small intestine. For example, iron citrate catalyses the oxygen-mediated oxidation [20]. However, the iron-catalysed reaction may be too slow to explain our results. 10-HCO-H₄F is known to be present in the mitochondria and cyt *c* (Fe³⁺) is known to oxidize a variety of organic bio-molecules [24,26]. In the experiments shown above, cyt *c* (Fe³⁺) oxidized 10-HCO-H₄F to 10-HCO-H₂F with an unexpectedly high rate constant of $1.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, which may be large enough to explain our results in the *in vivo* human study. If the mitochondrial concentrations of cyt *c* (Fe³⁺) and 10-HCO-H₄F are of the order of 100–200 μM and 1–5 μM , respectively, the $t_{1/2}$ for the pseudo first-order formation of 10-HCO-H₂F from 10-HCO-H₄F would be less than 1 s [34,35]. This rate of 10-HCO-H₂F formation may be rapid enough to exceed the subsequent enzyme-catalysed reactions shown in Figure 1. This is also consistent with the burst of non-methyl folates shown in Figure 4.

The magnitude of the rate constant for cyt *c* (Fe³⁺) oxidation of 10-HCO-H₄F is intermediate between that for the cyt *c* (Fe³⁺) oxidation of ascorbate (10^2) and riboflavin radicals (10^8) [26,27]. The latter rate is thought to be the diffusion-controlled upper limit. Clearly, two cyt *c* (Fe³⁺) molecules were required for the oxidation of one 10-HCO-H₄F to produce one 10-HCO-H₂F; yet the overall reaction was second, not third, order. One explanation for the reaction order would be the formation of a 10-HCO-H₄F free radical at C-6 as a first and rate-limiting step followed by a diffusion-controlled rate of reaction of this free radical with another cyt *c* (Fe³⁺). Since C-6 is a tertiary carbon, its free radical should be relatively stable. A difference of 10^4 (i.e. $10^8/10^4$) in the rate constants would prevent the C-6 free radical of 10-HCO-H₄F from accumulating.

The possibility that cytochromes oxidize 10-HCO-H₄F was tested *in vivo*. *E. hirae* contains no cytochromes. When given an exogenous source of haem, *E. hirae* has been shown to biosynthesize cytochromes and this bacterium also uses cyt *c* (Fe³⁺) as an electron acceptor [29,30]. Both isomers of 5-HCO-H₄F support the growth of this haemin-conditioned *E. hirae* in contrast to a small response to the unnatural isomer when this bacterium is not supplied with haem. During the 18 h of the *E. hirae* assay, the formation of both [6RS]-5,10-CH=H₄F and [6RS]-10-HCO-H₄F from [6RS]-5-HCO-H₄F would occur. While the natural [6S]-5-HCO-H₄F, [6R]-5,10-CH=H₄F and [6R]-10-HCO-H₄F are utilized enzymically, the unnatural [6R]-5-HCO-H₄F, [6S]-5,10-CH=H₄F and [6S]-10-HCO-H₄F, which approach equilibrium with each other, must either utilize a pathway to bioactive folates or remain metabolically inert. The formation of cytochromes by *E. hirae* creates such a pathway; namely, the oxidation of the unnatural [6S]-10-HCO-H₄F to 10-HCO-H₂F.

Taken together, the *in vitro* human and *E. hirae* data are consistent with cytochromes participating in a novel oxidative step for 10-HCO-H₄F metabolism. The use of unnatural isomer [6R]-5-HCO-H₄F is a tool to reveal this oxidative pathway. In addition, the oxidative pathway certainly uses the naturally occurring isomer [6R]-10-HCO-H₄F. For example, a number of researchers have identified radiolabelled 10-HCO-F, which appears rapidly in various fluids obtained from humans and animals after the administration of radiolabelled folic acid

[36–40]. How was 10-HCO-F formed *in vivo*? Folic acid must first be reduced to the tetrahydro oxidation state before it can accept a one-carbon fragment in enzyme-catalysed reactions (Figure 1). Therefore, radiolabelled 10-HCO-F must have arisen *in vivo* from the oxidation of radiolabelled [6*R*]-10-HCO-H₄F (the naturally occurring isomer) with the obligatory intermediate, 10-HCO-H₂F (Figure 1). 10-HCO-H₂F may not accumulate *in vivo* to a great extent, because it is either utilized by AICAR transformylase or oxidized further to 10-HCO-F (Figure 1).

Finally, it is unlikely that humans or animals would need an enzyme to catalyse oxidation of 10-HCO-H₄F. Assuming mitochondrial concentrations of 2.0 and 200 μM for 10-HCO-H₄F and cyt *c* (Fe³⁺), respectively, the initial rate of 10-HCO-H₂F formation would be 5.2 $\mu\text{M} \cdot \text{s}^{-1}$. Since rate constants for the formation of enzyme–substrate complex are about 10⁸ M⁻¹ s⁻¹, the initial rate of formation of an enzyme–10-HCO-H₄F complex with a typical 1.0 μM enzyme concentration and a 2.0 μM 10-HCO-H₄F concentration would be 200 $\mu\text{M} \cdot \text{s}^{-1}$ [41,42]. If we assume that the rate of formation of enzyme–substrate complex is equal to the rate of reaction, at most, this enzyme would produce a trivial 40-fold increase in the rate (i.e. 200/5.2). It is also unlikely that an enzyme would utilize both C-6 isomers of 10-HCO-H₄F; however, this possibility cannot be ruled out. The oxidation of cyt *c* (Fe²⁺) back to the (Fe³⁺) state would require the enzyme cyt *c* oxidase and this cycle would represent a coenzyme-like role for cyt *c* (Fe³⁺) in this metabolism.

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