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Short communication

Re-evaluation of the metabolism of oral doses of racemic carbon-6 isomers of formyltetrahydrofolate in human subjects

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The racemic mixture, [6*RS*]-5-formyltetrahydrofolate, is widely used clinically. In human subjects, orally-administered pure unnatural C-6 isomers, [6*R*]-5-formyltetrahydrofolate and [6*S*]-5,10-methenyltetrahydrofolate, were recently shown to be metabolized to the natural isomer, [6*S*]-5-methyltetrahydrofolate. We re-analysed the data from human studies published during the past four decades in which oral doses (≤10 mg) of racemic mixtures of these folates were used. We re-evaluated the data to determine whether these racemic mixtures are only 50 % bioactive or, as we now predict, more than 50 % bioactive. Our analyses indicate that, in human subjects, oral doses of the racemic mixture of the two formyltetrahydrofolates are 20–84 % more bioactive than would be predicted. These data are consistent with the following pathway: chemical conversion of these folates to 10-formyltetrahydrofolate; oxidation of 10-formyltetrahydrofolate to 10-formyldihydrofolate; subsequent enzymic conversion of 10-formyldihydrofolate to dihydrofolate by 5-amino-4-imidazolecarboxamide ribotide transformylase; and finally the well-established metabolism of dihydrofolate to [6*S*]-5-methyltetrahydrofolate. An additional review of the literature supports the *in vivo* oxidation of 10-formyltetrahydrofolate occurring to a certain extent, as 10-formyl-folic acid is rapidly formed after the administration of folic acid (pteroylglutamic acid) or 5-formyltetrahydrofolate in human subjects. The dogma that an oral dose of the unnatural C-6 isomer of 5-formyltetrahydrofolate is not bioactive in human subjects does not withstand scrutiny, most probably due to the previously unrecognized *in vivo* oxidation of 10-formyltetrahydrofolate. This discovery unveils new folate metabolism in human subjects.

Formyltetrahydrofolates: Unnatural isomer: Oral administration: Bioactivity

Bioactive [6*S*]-5-formyltetrahydrofolate (5-HCO-H_4folate) in bacteria was first isolated by Sauberlich & Baumann (1948). The chemically synthesized racemic mixture, [6*RS*]-5-HCO-H_4folate, has been widely used in combination with methotrexate and 5-fluorouracil in cancer chemotherapy. It was considered to have only 50 % of the bioactivity when compared with the natural isomer (Keresztesy & Silverman, 1951). Baggott &Tamura (1999), however, recently demonstrated that the pure unnatural isomers, [6*R*]-5-HCO-H_4folate and [6*S*]-5,10-methenyltetrahydrofolate (5,10-CH=H_4folates), are metabolized and become bioactive in human subjects, because an increase in microbiologically-active folates in plasma occurs after oral administration. [6*S*]-5-Methyltetrahydrofolate (5-CH_3-H_4folate) accounted for the majority of this increase. They proposed an explanation which included the following steps. First, the well-known chemical formation of 10-formyltetrahydrofolate (10-HCO-H_4folate) from 5-HCO-H_4folate

Abbreviations: 5-CH_3-H_4folate, 5-methyltetrahydrofolate; 5,10-CH = H_4folate, 5,10-methenyltetrahydrofolate; 5,10-CH_2-H_4folate, 5,10-methylenetetrahydrofolate; 10-HCO-folate, 10-formyl-folic acid; 5-HCO-H_4folate, 5-formyltetrahydrofolate; 10-HCO-H_2folate; 10-formyldihydrofolate; 10-HCO-H_4folate, 10-formyltetrahydrofolate.

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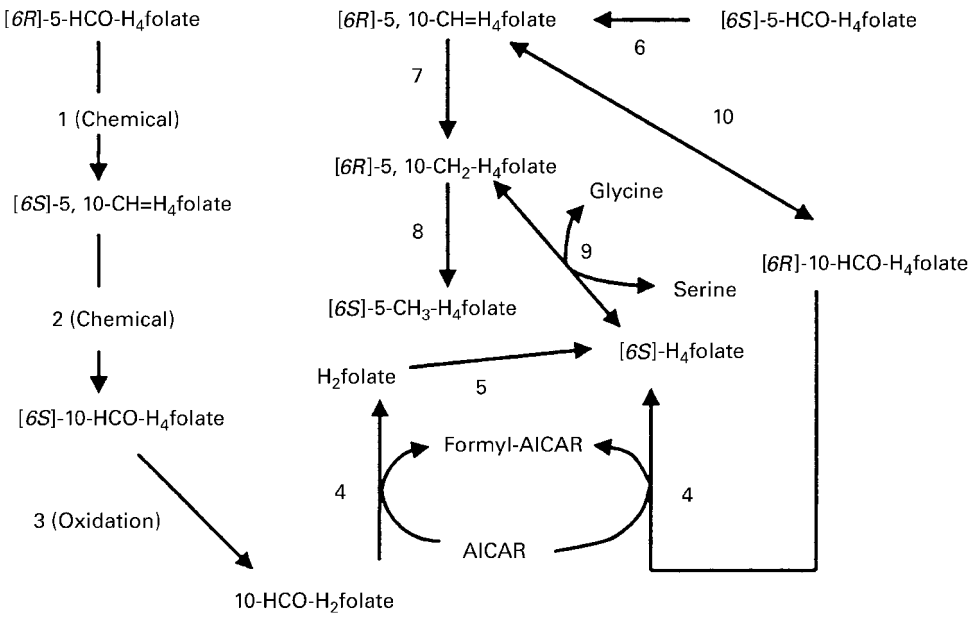

Fig. 1. Metabolism of [6*R*]-5-formyltetrahydrofolate (5-HCO-H_4folate) and the naturally occurring [6*S*]-5-HCO-H_4folate. Reactions 1 and 2 occur at low pH of the stomach and neutral pH of the upper small intestine respectively. Reaction 3 is probably a chemical oxidation, but the possibility of enzyme catalysed oxidation remains. Reactions 4 and 5 are catalysed by 5-amino-4-imidazolecarboximide ribotide (AICAR) transformylase and dihydrofolate (H_2folate) reductase respectively. Reactions 6–8 are catalysed by 5,10-methenyltetrahydrofolate (5,10-CH=H_4folate) synthetase, 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4folate) dehydrogenase and 5,10-CH_2-CH_4folate reductase respectively; this pathway is the shortest route for the conversion of [6*S*]-5-HCO-H_4folate to [6*S*]-5-methyltetrahydrofolate ([6*S*]-5-CH_3-H_4folate). Reaction 9 is carried out by serine hydroxymethyltransferase. Reaction 10 is catalysed by 5,10-CH=H_4folate cyclohydrolase. 10-HCO-H_4folate, 10-formyltetrahydrofolate; 10-HCO-H_2folate, 10-formyldihydrofolate; H_4folate, tetrahydrofolate.

and 5,10-CH=H_4folate takes place due to the changes in pH in the upper gastrointestinal tract (chemical reactions 1 and 2, Fig. 1). This process has been described by Beavon & Blair (1972). Second, enzymic or chemical oxidation of 10-HCO-H_4folate to 10-formyldihydrofolate (10-HCO-H_2folate) subsequently occurs (reaction 3, Fig. 1). Third, the enzymic conversion of 10-HCO-H_2folate to dihydrofolate is catalysed by 5-amino-4-imidazolecarboxamide ribotide transformylase (Baggott *et al.* 1995; Baggott & Johanning, 1999), and dihydrofolate reductase reduces dihydrofolate to tetrahydrofolate (reactions 4 and 5 respectively, Fig. 1). Finally, tetrahydrofolate is converted to [6*S*]-5-CH_3-H_4folate by the well-known enzymic reactions involving serine hydroxymethyltransferase and 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4folate) reductase (reactions 9 and 8 respectively, Fig. 1). Thus, these investigators concluded that the combination of these chemical and enzymic reactions would account for the increase in plasma [6*S*]-5-CH_3-H_4folate in subjects given oral doses of the pure unnatural isomers of 5-HCO-H_4folate and 5,10-CH=H_4folate (Baggott & Tamura, 1999). In contrast, the naturally occurring isomer, [6*S*]-5-HCO-H_4folate, can be metabolized to [6*S*]-5-CH_3-H_4folate through a relatively short enzymic pathway involving 5,10-CH=H_4folate synthetase, 5,10-CH_2-H_4folate dehydrogenase and 5,10-CH_2-H_4folate reductase (reactions 6, 7 and 8 respectively, Fig. 1). Other possible pathways for the conversion of 5-HCO-H_4folate to 5-CH_3-H_4folate include the reaction catalysed by 5,10-CH=H_4folate cyclohydrolase (reaction 10, Fig. 1).

Baggott & Tamura (1999) used pure C-6 isomers of the folates. However, several research groups have conducted human studies using oral doses of [6*RS*]-5-HCO-H_4folates or [6*RS*]-5,10-CH=H_4folate (Baker *et al.* 1965, 1994; Perry & Chanarin, 1970; Pratt & Cooper, 1971; Brown *et al.* 1973; Ratanasthien *et al.* 1974). These investigators determined the increment of microbiologically active serum/plasma folate concentrations following the oral dose. We have critically re-analysed published data of these human studies and re-evaluated whether these racemic mixtures are only 50 % metabolized and bioactive or, as we now predict, more than 50 % bioactive. We limit our analyses to the studies using oral folate doses of 10 mg (22·7 μmol) or less to minimize artifacts possibly caused by unphysiologically large doses. We calculated the area under the curve using replotted data in figures or tables of the publications cited here. The area consisting of rectangles and triangles was computed after subtracting baseline values.

Baker *et al.* (1965) administered 5·0 mg (11·3 μmol) folic acid (pteroylglutamic acid) and 10 mg (22·7 μmol) [6*RS*]-5-HCO-H_4folate to subjects by the oral and intravenous routes using a paired-study design. The doses of these two folates differed, as it was assumed that only the naturally occurring C-6 isomer of 5-HCO-H_4folate would be bioactive. Serum folate concentrations were measured by microbiological assay with *Lactobacillus casei* for 8 h after each dose. The calculated area under the curve for the orally administered [6*RS*]-5-HCO-H_4folate is larger than that of folic acid (Table 1). In contrast, the area under the curve for the intravenous route is similar for both folates.

Table 1. Area under the curve (AUC) for serum or plasma folate concentrations or maximum increase in total serum folate after independent doses of folic acid, carbon-6 racemic mixtures of 5-formyltetrahydrofolate (5-HCO-H_4folate) or 5,10-methenyltetrahydrofolate (5,10-CH=H_4folate) in human subjects*

Investigators	No. of subjects	Folate	Dose	Route	AUC (nM·h)†	Relative bioactivity‡	
						Found	Expected
Baker *et al.* (1965)	48	Folic acid	5 mg	Oral	5180	1·00	1·00
		[6*RS*]-5-HCO-H_4folate	10 mg	Oral	9530	1·84	1·00
		Folic acid	5 mg	Intravenous	2230	1·00	1·00
		[6*RS*]-5-HCO-H_4folate	10 mg	Intravenous	1860	0·83	1·00
Perry & Chanarin (1970)	15	Folic acid	10 μg/kg	Oral	45	1·00	1·00
	13	[6*RS*]-5-HCO-H_4folate	20 μg/kg	Oral	77	1·71	1·00
Pratt & Cooper (1971)	5	Folic acid	1 mg	Oral	100	1·00	1·00
	2	[6*RS*]-5-HCO-H_4folate	2 mg	Oral	142	1·42	1·00
Ratanasthien *et al.* (1974)	6	Folic acid	5 mg	Oral	920	1·00	1·00
		[6*RS*]-5-HCO-H_4folate	10 mg	Oral	1100	1·20	1·00
		[6*RS*]-5,10-CH=H_4folate	10 mg	Oral	1330	1·45	1·00
Baggott & Tamura (1999)	3	[6*S*]-5-HCO-H_4folate	2·7 μmol	Oral	534	1·00	1·00
		[6*R*]-5-HCO-H_4folate	2·7 μmol	Oral	99	0·19	0
		[6*S*]-5,10-CH=H_4folate	2·7 μmol	Oral	266	0·50	0
Brown *et al.* (1973)	21	Folic acid	0·68 μmol	Oral	15 nM§	1·00	1·00
		[6*RS*]-5-HCO-H_4folate	0·68 μmol	Oral	16 nM§	1·07	0·50
		[6*RS*]-5,10-CH=H_4folate	0·68 μmol	Oral	23 nM§	1·53	0·50

* Folate concentrations were measured by microbiological assay with *Lactobacillus casei*.
† Mean AUC are calculated from time zero until the time of the last blood sampling; this does not include further extrapolation.
‡ The bioactivity for folic acid or pure [6*S*]-5-HCO-H_4folate is set at 1·00 and expected bioactivity of the racemic mixture assumes that only the naturally-occurring C-6 isomer is bioactive. Doses administered in mg or μg are slightly lower on a molar basis for the C_1-substituted tetrahydrofolate when compared with folic acid.
§ Only mean maximum increases in serum folate concentrations are available from this study.

Based on these analyses, we conclude that the orally administered racemic mixture of 5-HCO-H_4folate has a bioactivity almost equal to that of the same amount of folic acid. In contrast, the bioactivity of the intravenous dose of [6*RS*]-5-HCO-H_4folate is approximately half that of folic acid. It is now apparent that the oral route must have allowed the unnatural isomer of [6*RS*]-5-HCO-H_4folate to be converted to bioactive folates as stated earlier. In addition, the results of differential microbiological assay using *L. casei* and *Enterococcus hirae* (formerly known as *Streptococcus faecalis*) indicated that the majority of the serum folate was [6*S*]-5-CH_3-H_4folate following the independent oral dose of both folates. This finding is consistent with the metabolic pathways proposed by Baggott & Tamura (1999) and their results.

Using small oral doses of folic acid (10 μg/kg body weight) and [6*RS*]-5-HCO-H_4folate (20 μg/kg) Perry & Chanarin (1970) conducted a similar study by monitoring serum folate concentrations for 3 h after the dose. The doses differed again because they assumed that only half the racemic mixture was bioactive. As shown in Table 1, the relative bioactivity of [6*RS*]-5-HCO-H_4folate is 1·71, which is higher than the predicted value of 1·0. Differential microbiological assay indicated that the majority of the serum folate was [6*S*]-5-CH_3-H_4folate after the dose of either compound.

Pratt & Cooper (1971) administered 1·0 mg (2·27 μmol) folic acid and 2·0 mg (4·54 μmol) [6*RS*]-5-HCO-H_4folate to subjects, again maintaining a 1:2 ratio to correct for the presumed bioactivity of this racemic mixture. Plasma folate concentrations were measured for 3 h after the dose. Their data indicate a relative bioactivity of 1·42 for the racemic mixture (Table 1). The majority of the plasma folate was also found to be [6*S*]-5-CH_3-H_4folate in this study.

Brown *et al.* (1973) measured the maximum increases in serum folate concentrations at two time points (1 and 2 h) following independent oral doses of only 0·3 mg (0·68 μmol) folic acid, [6*RS*]-5-HCO-H_4folate and [6*RS*]-5,10-CH=H_4folate using a paired-study design. As sequential serum folate values were not reported, only mean maximum increases in total serum folates are presented in Table 1. The oral dose of [6*RS*]-5-HCO-H_4folate produced a slightly greater response than folic acid itself, and an even greater response was observed with [6*RS*]-5,10-CH=H_4folate. These authors noted in the discussion that 5,10-CH=H_4folate was better absorbed when compared with folic acid. This observation is also consistent with a pathway for the conversion of the unnatural isomer, [6*S*]-5,10-CH=H_4folate, to bioactive folates as discussed earlier.

Ratanasthien *et al.* (1974) reported the increase in serum folate concentrations after independent oral doses of 10 mg (22·7 μmol) [6*RS*]-5-HCO-H_4folate and [6*RS*]-5,10-CH=H_4folates and an oral dose of 5·0 mg (11·3 μmol) folic acid. Blood samples were collected for 3 h after each dose. The relative bioactivity of [6*RS*]-5-HCO-H_4folate was 1·20. The greater relative bioactivity of [6*RS*]-5,10-CH=H_4folate of 1·45 occurred presumably because this compound bypassed the first chemical step (reaction 1, Fig. 1). The difference in the relative bioactivities between [6*RS*]-5-HCO-H_4folate and [6*RS*]-5,10-CH=H_4folate are similar to those for the pure unnatural isomers of these two folates reported by Baggott & Tamura (1999).

Finally, Baker *et al.* (1994) measured the increase in plasma total folate and [6*S*]-5-CH_3-H_4folate of portal blood following the oral administration of 10 mg [6*RS*]-5-HCO-H_4folate in one group of subjects and 5·0 mg folic acid in another group. At 30 min after the dose the increases in both total folate and [6*S*]-5-CH_3-H_4folate were two to three times higher in subjects given the [6*RS*]-5-HCO-H_4folate dose when compared with those given the folic acid dose. At the 60 min point the increases were similar in both groups. Their data also suggest that the unnatural isomer, [6*R*]-5-HCO-H_4folate, was converted to bioactive [6*S*]-5-CH_3-H_4folate, although the data allow comparisons only between the 30 and 60 min time points after each dose (data not presented in Table 1).

We evaluated studies where the changes in serum or plasma folate concentrations were compared after various doses of folic acid or either pure unnatural isomers or racemic mixtures of formyltetrahydrofolate. The results of all studies uniformly indicate that a significant portion of the orally administered unnatural isomers of formyltetrahydrofolate is bioactive in human subjects. Among the studies reviewed here, the combination of independent oral and intravenous doses, the 8 h follow-up and a paired-study design by Baker *et al.* (1965) may be the strongest to support the hypothesis proposed by Baggott & Tamura (1999). The similarity of the relative bioactivities of the intravenous dose of folic acid and [6*RS*]-5-HCO-H_4folate virtually rules out the possibility that unnatural isomer, [6*R*]-5-HCO-H_4folate, in high concentrations in the circulation, would enter tissues and displace pre-existing cellular folates back into the circulation. Furthermore, their paired-study design corrects for any genetic differences in physiology and folate metabolism.

In the proposed metabolic pathway for the unnatural C-6 optical isomers of formyltetrahydrofolate, the oxidation of 10-HCO-H_4folate to 10-HCO-H_2folate is a critical step (reaction 3, Fig. 1), as it removes the asymmetric centre at C-6, allowing further metabolism of the compound to [6*S*]-5-CH_3-H_4folate through well-established enzymic reactions. To our knowledge, the first evidence for the *in vivo* oxidation of 10-HCO-H_4folate in human subjects was reported by McLean & Chanarin (1966). They detected substantial amounts of radiolabelled 10-formyl-folic acid (10-HCO-folate) in a 4 h urine sample following an intravenous dose of radiolabelled folic acid (15 μg/kg). Their data suggest that the intravenously administered radiolabelled folic acid was first metabolized to 10-HCO-H_4folate, and subsequently oxidized to 10-HCO-folate, with 10-HCO-H_2folate as an obligate intermediate in this oxidation process. Pratt & Cooper (1971) used an oral dose of radiolabelled folic acid (1·0 mg) and detected radiolabelled 10-HCO-H_4folate, 10-HCO-H_2folate and 10-HCO-folate in 2 h bile samples. The detection of all three oxidation states of 10-formyl-substituted folates strongly suggests the existence of the *in vivo* oxidation process in human subjects. Whitehead *et al.* (1972) identified 10-HCO-folate in portal plasma collected 20 min after an oral dose of [6*RS*]-5-HCO-H_4folate (2·0 mg). Their data suggest that the formation of 10-HCO-H_4folate from 5-HCO-H_4folate and the subsequent oxidation of 10-HCO-H_4folate to 10-HCO-folate had occurred.

In a study of a patient with scurvy Stokes *et al.* (1975) suggested that the oxidation of 10-HCO-H_4folate was sensitive to vitamin C nutriture. Before the patient was treated with ascorbic acid (150 mg/d) 10-HCO-folate was the major form of folate in a 24 h urine sample following an oral dose of [6*RS*]-5-HCO-H_4folate (5·0 mg). However, the presence of [6*S*]-5-CH_3-H_4folate was not mentioned. On the other hand, [6*S*]-5-CH_3-H_4folate was the major form and no 10-HCO-folate was detected in a 24 h urine sample following oral [6*RS*]-5-HCO-H_4folate (10 mg) after 6 d of ascorbic acid therapy. The authors did not mention the use of reducing agents, such as ascorbic acid, during the urinary collection. If we accept the data, one possible logical explanation for these findings is that the *in vivo* oxidation rate of 10-HCO-H_4folate derived from the dose of 5-HCO-H_4folate was much greater when the subject was vitamin C deficient. Again 10-HCO-H_2folate is an obligate intermediate in the oxidation of 10-HCO-H_4folate to 10-HCO-folate. One should not forget that the data demonstrating the presence of 10-HCO-folate in the studies noted earlier may be the result of oxidation during the analytical procedures. It remains to be tested, therefore, whether 10-HCO-folate detected in biological samples is exclusively formed by oxidation during the analyses.

In conclusion, a recent finding by Baggott & Tamura (1999) indicating that independent oral doses of pure unnatural C-6 isomers of formyltetrahydrofolate are bioactive in human subjects is consistent with results using racemic mixtures reported by other investigators. Additionally, there is evidence suggesting the occurrence of the *in vivo* oxidation of 10-HCO-H_4folate in human subjects. This oxidation and the reaction catalysed by 5-amino-4-imidazolecarboxamide ribotide transformylase (reactions 3 and 4 in Fig. 1) are previously unrecognized folate metabolic steps in human subjects. The long-held belief that an oral dose of the unnatural isomer of 5-HCO-H_4folate is inactive in human subjects appears to be wrong.

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