The pH-dependent rearrangements of formyltetrahydrofolates and their nutritional implications

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1. The rates of hydrolysis of 5,10-methenyltetrahydrofolic acid and of cyclization of 10-formyl- and 5-formyltetrahydrofolic acids have been studied in vitro.

2. 10-Formyl- and 5-formyltetrahydrofolate cyclized below pH 4, 10-formyltetrahydrofolate being more rapid; the rates of cyclization for these compounds were not concentration-dependent. 5,10-Methenyltetrahydrofolate was stable below pH 4; above this the rate of hydrolysis depended on pH, nature of buffer and substrate concentration.

3. The relevance of the results to animal and human nutrition is discussed.

The pH-dependent equilibria between 5-formyltetrahydrofolic acid (5 CHO—THF), 10-formyltetrahydrofolic acid (10 CHO—THF), and 5,10-methenyltetrahydrofolic acid (5,10 CH=THF) are shown in Fig. 1. The rates of attainment of these equilibria have not been worked out, but are of importance in animal feeding experiments and in dietary studies, since the folate compound ingested may often not be that which is eventually absorbed.

In the synthesis of 5 CHO—THF (Roth, Hultquist, Fahrenbach, Cosulich, Broquist, Brockman, Smith, Parker, Stokstad & Jukes, 1952) the equilibrium

$$10\ \text{CHO—THF} + H^+ \rightleftharpoons 5,10\ \text{CH=THF} + H_2O$$

was allowed 3 d for completion at pH 0. Graphical results for the cyclization of 5 CHO—THF and 10 CHO—THF in 0·25 M hydrochloric acid have been published (Rabinowitz, 1960), together with studies on the hydrolysis of 5,10 CH=THF, the rate of which is dependent on the nature of the buffer present. The mechanism has been studied (Robinson & Jencks, 1967*a*, *b*) and the equilibrium constants for the cyclization of 5 CHO—THF and 10 CHO—THF have been determined (Kay, Osborn, Hatefi & Huennekens, 1960).

The in vitro work reported here establishes the reaction half-times for the equilibria in Fig. 1 at pH values likely to be met in the alimentary tract.

EXPERIMENTAL

Materials and methods

Chemicals. Calcium leucovorin (providing 5 CHO—THF) was a gift from Lederle Laboratories Inc., Pearl River, New York; 2-mercaptoethanol was obtained from Koch–Light Laboratories Ltd, Colnbrook, Bucks.; 5,10 CH=THF was made by the method of Roth *et al.* (1952); 10 CHO—THF was made by reduction with hydrogen

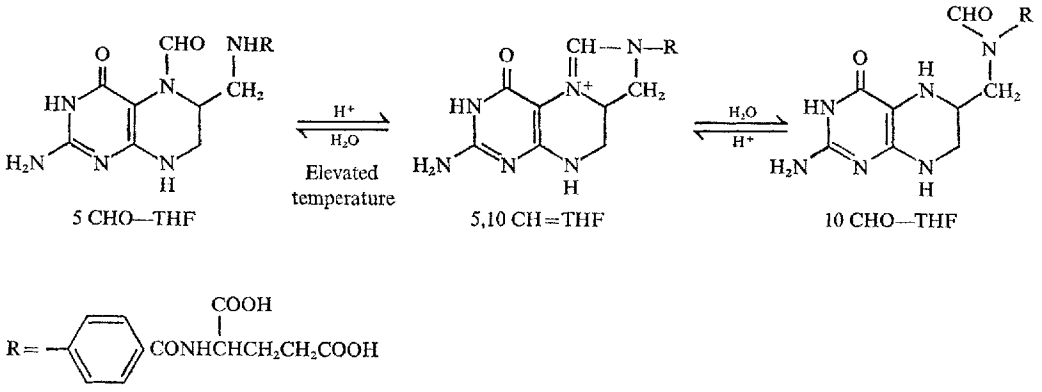

Fig. 1. pH-dependent equilibria between 5-formyltetrahydrofolate (5 CHO—THF), 10-formyltetrahydrofolate (10 CHO—THF) and 5,10-methenyltetrahydrofolate (5,10 CH=THF).

using platinum as catalyst, in glacial acetic acid of 10-formylfolic acid prepared by the method of Blakley (1959).

Spectrophometric measurement. 5,10 CH=THF has a characteristic absorption peak at 355 nm, not possessed by any other tetrahydrofolate. All the reactions were followed by observation of the appearance or disappearance of this peak using a Hilger & Watts Uvispek spectrophotometer at 25°.

In vitro studies

Cyclization of 5 CHO—THF to 5,10 CH=THF. The substrate solution was aqueous calcium leucovorin (0·4 mg/ml as free acid). Each cuvette (1 cm path length) contained hydrochloric acid (2 ml) at the required pH, toluene (0·2 ml), and either water (100 μl, reference) or substrate solution (100 μl). The effect of dilution was studied using a 4 cm light path cuvette containing hydrochloric acid (8 ml), toluene (2 ml) and either water (100 μl) or substrate solution (100 μl).

Cyclization of 10 CHO—THF to 5,10 CH=THF. The substrate solution was 10 CHO—THF (0·4 mg/ml) in 0·01 % (v/v) aqueous ammonium hydroxide containing 2-mercaptoethanol (20 μl/ml). Each cuvette (1 cm light path) contained hydrochloric acid (2 ml), 2-mercaptoethanol (20 μl), toluene (0·2 ml), and either 0·01 % (v/v) aqueous ammonium hydroxide (100 μl, reference) or substrate solution (100 μl). 2-Merceptoethanol was included to prevent oxidation of 10 CHO—THF to folic acid or 10-formylfolic acid, both of which interfere (λ_{max} at pH 7, 346 and 348 nm respectively (Blakley, 1969)).

Hydrolysis of 5,10 CH=THF to 10 CHO—THF. The substrate solution was 5,10 CH=THF (0·4 mg/ml) dissolved in hydrochloric acid (pH 2). Each cuvette (1 cm light path) contained buffer (acetate or phosphate, 2 ml), 2-mercaptoethanol (20 μl), toluene (0·2 ml), and either hydrochloric acid (pH 2, 100 μl, reference) or substrate solution (100 μl). The pH of the buffers used was unaffected by 100 μl of the acid. Experiments were also done in bicarbonate buffer since this is the predominant buffer ion in the jejunum. Since the buffer capacity is small, it was necessary to use a substrate solution of 5,10 CH=THF (0·04 or 0·4 mg/ml) in pH 4 hydro-

Table 1. *Cyclization of 5-formyltetrahydrofolate to 5,10-methenyl-tetrahydrofolate in hydrochloric acid at 25°* (t *is the reaction half-time in min*)

pH	Substrate* concentration (μg/ml)	t
0·5	19	2·5
1		14
2		81
3		270
4		Stable
1	4·94	14
2		80

* Calcium leucovorin (0·4 mg/ml as free acid).

Table 2. *Cyclization of 10-formyltetrahydrofolate* (10 *CHO—THF*) *to 5,10-methenyl-tetrahydrofolate in hydrochloric acid at 25°. (Substrate* concentration 19 μg/ml;* t *is the reaction half-time in min*)

pH	t
1	1·0
2	8·5
3	35
4	Stable

* 10 CHO-THF (0·4 mg/ml) in 0·01% (v/v) aqueous ammonium hydroxide containing 2-mercaptoethanol (20 μl/ml).

chloric acid, and 4 cm light path cuvettes. Each cuvette contained buffer (8 ml), toluene (2 ml), 2-mercaptoethanol (80 μl), and either hydrochloric acid (pH 4, 100 μl, reference) or substrate solution (100 μl).

RESULTS AND DISCUSSION

The results are given in Tables 1–3.

The fate of each of the three compounds within the alimentary tract will be considered separately. Normal gastric pH is about 4 in the rat (Gordon, 1968) and the gastric evacuation time 1 h (Ely & Ross, 1947). In man, the gastric pH resting value appears to be between 1 and 2, rising to 5 by neutralization with food, after a meal, then returning to between pH 1 and 2 (James & Pickering, 1949). Thirty minutes after a meal of steak the initial gastric pH of 5 in normal subjects has returned to between 2·55 and 3·35; 90 min later to between 1·8 and 2·3 (Fordtran & Lochlear, 1966). These are average values; the antral contents are generally more acid. Thirty minutes after a meal of meat the antral chyme pH lies between 2·5 and 3·4 and 1 h later the pH has reached a steady value of just under 2 (Davenport, 1971). The vigorous movements of a full stomach ensure that all food comes into contact with this fluid at the periphery of the bolus. Freshly secreted gastric juice before being diluted with food has a pH of about 1. Gastric evacuation time varies with the meal composition; a light liquid meal having a half emptying time of about 40 min, a liquid meal of higher osmotic pressure a half emptying time of 60 min and meals containing protein

Table 3. *Hydrolysis of* 5,10-*methenyltetrahydrofolate* (5,10 *CH=THF*) *to* 10-*formyltetrahydrofolate in the three buffers at* 25° (t *is the reaction half-time in min*)

Buffer	pH	Substrate* concentration (μg/ml)	t
Acetate, 0·1 M	4	19	Stable
	5		Stable
Phosphate, 0·1 M	6	19	14
	7		10
Bicarbonate:	6	4·9	116
	7		80
	6	0·49	22
	7		2

* 5,10 CH=THF (0·4 mg/ml) dissolved in hydrochloric acid (pH 2).

or fat may persist in the stomach from 4 to 6 h with an extreme of 20 h (Davenport, 1971). The temperature of the empty stomach is about 37° but varies with the meal taken. A 250 ml liquid meal taken at 57° raises the gastric temperature to 44° within 2 min from which it reverts to normal in 18 min (Davenport, 1971). A meal of ice-cream reduces gastric temperature to 22° (Davenport, 1971). The experimental results for cyclization are quoted for a temperature of 25°; the higher gastric temperatures would reduce the half-life for cyclization. Normal jejunal pH in both rat and man is about 6·5 (Gordon, 1968; Benn, Swan, Cooke, Blair, Matty & Smith, 1971).

5 *CHO—THF*. At pH 4, or higher, 5 CHO—THF did not cyclize (Table 1); in the rat it is therefore unlikely that significant amounts of 5,10 CH=THF would be produced. Analysis of faecal radioactivity after administration of [2-^{14}C]-5 CHO—THF (Beavon and Blair, unpublished) showed that 95% of the excreted radioactivity was 5 CHO—THF. In man, gastric pH is low enough for possibly all of the dose to cyclize if given on an empty stomach. If taken in food, the amount of cyclization would vary with the pH and the time it remains in the stomach: where stomach acidity is low, half emptying time short, stomach temperature low and gastric mixing poor, most 5 CHO—THF would be uncyclized and pass to the jejunum unchanged. Where stomach acidity is high, half emptying time long, stomach temperature high and gastric mixing good, a proportion of 5 CHO—THF would be cyclized and a mixture of 5 CHO—THF and 5,10 CH=THF enter the jejunum.

10 *CHO—THF*. At pH 4, 10 CHO—THF did not cyclize (Table 2), thus in the rat stomach, which has a pH of 4, 10 CHO—THF would not cyclize, and would be absorbed as such. In man, in an empty stomach, cyclization would be much quicker than that of 5 CHO—THF, giving 5,10 CH=THF almost entirely, this then being hydrolysed to 10 CHO—THF and possibly some 5 CHO—THF in the jejunum. If taken in food the proportion cyclized would vary with gastric retention time, gastric mixing, stomach temperature and pH but would always be very much greater than that of 5 CHO—THF.

5,10 CH=THF. At pH 5 or lower, 5,10 CH=THF was stable to hydrolysis (Table 3). It will thus pass through the stomach unchanged, but be hydrolysed in the

jejunum. The extent of this is difficult to predict, since reaction rate was dependent both on concentration and on the type of buffer, in agreement with previous studies (Rabinowitz, 1960). Since bicarbonate is probably the chief buffering ion in the jejunum, the discussion refers to the values for that buffer. It is likely that a large proportion will be hydrolysed to 10 CHO—THF; some 5 CHO—THF could also be formed. 5,10 CH=THF is strongly adsorbed on to cellulose powder, and possibly therefore on to fibrous dietary constituents (Luther, Santini, Brewster, Perez-Santiago & Butterworth 1965; Beavon and Blair, unpublished). 5,10 CH=THF adsorbed on to Florisil at pH 1 is eluted by pH 9 ammonium hydroxide partly as 5,10 CH=THF and partly as 5 CHO—THF rather than the expected 10 CHO—THF. Analysis of faecal radioactivity from rats given [2-^{14}C]-5,10 CH=THF showed four components; three were identified as 5,10 CH=THF (*c.* 10%), 5 CHO—THF (*c.* 10%) and 10-formylfolic acid (10 CHO—F; *c.* 70%), the latter probably arising from oxidation of 10 CHO—THF (Beavon and Blair, unpublished). The jejunum is normally anaerobic, so that 10 CHO—THF would escape oxidation.

Conclusion

Formylfolates constitute a significant proportion of folates in food. On microbiological assay with *Pediococcus cerevisiae*, formylfolates accounted for 12% of total folate in asparagus, 44% in chicken liver, and 59% in red kidney beans (Santini, Brewster & Butterworth, 1964). Perry (1971) found 30% of total folate to have *P. cerevisiae* activity in a hospital diet. Distribution of activity between 5,10 CH=THF, 10 CHO—THF and 5 CHO—THF is unclear, partly because of the ease of interconversion, and partly because until the advent of these compounds labelled with ^{14}C (Beavon & Blair, 1971) specific analysis proved difficult. 10 CHO—F has been found in foods (Butterworth, Santini & Frommeyer, 1963; Lewis, 1967), and may be derived from 10 CHO—THF oxidation. The pH-dependent rearrangements have also to be borne in mind when performing administration experiments with formylfolates in metabolic studies.

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