Cofactor role for 10-formyldihydrofolic acid

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10-Formyl-7,8-dihydrofolic acid (10-HCO-H_2folate) was prepared by controlled air oxidation of 10-formyl-5,6,7,8-tetrahydrofolic acid (10-HCO-H_4folate). The UV spectra of the 10-HCO-H_2folate preparation has $\lambda_{max.}$ 234, 333 nm and $\lambda_{min.}$ 301 nm at pH 7.4, and $\lambda_{max.}$ 257, 328 nm and $\lambda_{min.}$ 229, 307 nm at pH 1. ^{1}H-NMR spectroscopy of 10-HCO-H_2folate (in ^2H_2O; 300 MHz) suggested a pure compound and gave resonances for one formyl group proton, two protons on C-7 and C-9, and no evidence for a C-6 proton, which is consistent with the structure proposed. The spectral properties indicated that the 10-HCO-H_2folate preparation is not appreciably contaminated with 10-HCO-H_4folate, 5,10-methenyltetrahydrofolic acid (5,10-CH=H_4folate) or 10-formylfolic acid (10-HCO-folate). The above data establish that the 10-HCO-H_2folate prepared here is authentic. In contrast, a folate with a UV spectrum having $\lambda_{max.}$ 272 nm and $\lambda_{min.}$ 256 nm at pH 7, which was prepared by 2,6-dichloro-indophenol oxidation of 10-HCO-H_4folate and reported to be 97% pure [Baram, Chabner, Drake, Fitzhugh, Sholar and Allegra (1988) J. Biol. Chem. **263**, 7105–7111], is apparently not 10-HCO-H_2folate. 10-HCO-H_2 folate is utilized by Jurkat-cell (human T-cell leukaemia) and chicken liver aminoimidazolecarboxamide ribonucleotide transformylase (AICAR T'ase; EC 2.1.2.3) in the presence of excess 5-amino-imidazole-4-carboxamide ribotide (AICAR) resulting in the appearance of approximately 1 mol of H_2folate product for each mol of AICAR formylated. The present 10-HCO-H_2folate preparation had a kinetic advantage over 10-HCO-H_4folate resulting from a difference of approx. 5-fold in K_m values when both folates were used as cofactors for Jurkat-cell and rat bone marrow AICAR T'ase. No substantial kinetic advantage was observed using chicken liver AICAR T'ase. 10-HCO-H_2folate had little or no activity with Jurkat-cell or chicken liver glycinamide ribonucleotide transformylase (GAR T'ase, EC 2.1.2.2). The existence *in vivo* of 10-HCO-H_2folate is suggested in mammals by several reports of detectable amounts of radiolabelled 10-HCO-folate in bile and urine after administration of radiolabelled folic acid.

INTRODUCTION

It is an axiom of folate metabolism that the pteridine ring of this cofactor is in the 5,6,7,8-tetrahydro state in order to participate in enzyme-catalysed one-carbon-transfer reactions. This axiom provides the raison d'etre for dihydrofolate reductase, the enzyme that catalyses the reduction of 7,8-dihydrofolic acid (H_2folate) to 5,6,7,8-tetrahydrofolic acid (H_4folate), and for the finding that H_2folate is devoid of activity in the one-carbon-transfer reactions catalysed by serine hydroxymethyltransferase and 10-formyltetrahydrofolate synthetase [1–3]. In contrast with the above, we report here that the folate-dependent purine nucleotide biosynthetic enzyme, aminoimidizolecarboxamide ribonucleotide transformylase (AICAR T'ase) in mammalian cells has a kinetic preference for 10-formyl-7,8-dihydrofolic acid (10-HCO-H_2folate) over 10-formyl-5,6,7,8-tetrahydrofolic acid (10-HCO-H_4folate). The preparation and properties of authentic 10-HCO-H_2folate are described.

EXPERIMENTAL

Preparation of 10-HCO-H_2folate and 10-HCO-H_4folate

10-HCO-H_4folate was prepared by a modified procedure of Rabinowitz [4], and air oxidation of this folate was modified from procedures described by Scott [5], Eto and Krumdieck [6] and Murphy et al. [7]. (6*S*)- or (6*R*,*S*)-5-Formyltetrahydropteroylglutamic acid (Lederle Laboratories) (10 mg) was dissolved in 1 ml of 0.25 M 2-mercaptoethanol, and then 20 μl of 12 M HCl was added. This solution was left at 5 °C for 5 days; the precipitated (6*R*)- or (6*R*,*S*)-5,10-methenyltetrahydropteroylglutamic acid (5,10-CH=H_4folate) was collected by centrifugation and washed with 2 × 0.2 ml of ice-cold 5 mM HCl. The solid was suspended in 2 ml of 5 mM HCl. Tris base (1 M; 25 μl) was added to 1 ml of this suspension and air was bubbled through the solution (room temperature). A 20 μl aliquot of this solution was removed every 30 min, dissolved in 1 ml of 0.1 M H_2SO_4, and the time-dependent increase in A_{356} was recorded ($t_{\frac{1}{2}}$ = 1.4 min for this reaction at room temperature). 10-HCO-H_4folate, not 10-HCO-H_2folate, is converted back into 5,10-CH=H_4folate under acidic conditions with the resultant increase in A_{356}. In 1.5–3 h, all 10-HCO-H_4folate had been oxidized to 10-HCO-H_2folate because no detectable increase in A_{356} was observed. The 10-HCO-H_2folate solution was made 10 mM in 2-mercaptoethanol to prevent further oxidation. Prolonged air oxidation (i.e. 8–18 h) of 10-HCO-H_4folate beyond this point yielded 10-formylfolic acid (10-HCO-folate). 10-HCO-H_2folate was stable in solution for 3 days if stored at −70 °C. The molar absorption coefficients for 10-HCO-H_2folate were based on E_{356} = 2.5×10^4 $M^{-1} \cdot cm^{-1}$ (pH 1) [4] for 5,10-CH=H_4folate (the starting material). 10-HCO-H_2folate was quantified in solution at pH 7.4 using $\epsilon_{234} = 3.4 \times 10^4$ $M^{-1} \cdot cm^{-1}$.

A solution of 1 M Tris base and 0.4 M 2-mercaptoethanol (25 μl) was added to the remaining 1 ml of suspension of 5,10-CH=H_4folate. After 1 h at room temperature, it was converted into 10-HCO-H_4folate and used in enzyme assays.

Abbreviations used: 10-HCO-H_2folate, 10-formyl-7,8-dihydrofolic acid; 10-HCO-H_4folate, 10-formyl-5,6,7,8-tetrahydrofolate; 5,10-CH=H_4folate, 5,10-methyltetrahydrofolic acid; 10-HCO-folate, 10-formylfolic acid; AICAR, aminoimidazolecarboxamide ribonucleotide; AICAR T'ase, AICAR transformylase (phosphoribosylaminoimidazolecarboxamide formyltransferase, EC 2.1.2.3); GAR, glycinamide ribonucleotide; GAR T'ase, glycinamide ribonucleotide transformylase (phosphoribosylglycinamide formyltransferase, EC 2.1.2.2.); MTX, methotrexate.

10-HCO-H_4folate was quantified in acid solution (pH 1) as 5,10-CH=H_4folate.

^{1}H-NMR spectroscopy

Approx. 10 μmol of 10-HCO-H_2folate was prepared in 2 ml as described above except that a sodium phosphate buffer (pH 7.0, 1.0 M) replaced the Tris buffer. 10-HCO-H_2folate was separated (all of the following procedures was carried out at 0 °C) by mixing in 3 ml of acetonitrile followed by 1 ml of ethyl ether. After centrifugation (600 *g*; 1 min), the organic layer was removed leaving a yellow oil to which was added 50 μl of ^2H_2O (99.8 %) followed by 3 ml of ethyl ether. The suspension was shaken and centrifuged, and the resulting organic layer was discarded and the yellow oil exposed to a high vacuum (26.7 Pa) for 5 min. The above process was repeated 15 times (in the last cycle the product was exposed to a high vacuum for 20 min), after which the yellow gum was dissolved in 0.8 ml of nitrogen-flushed ^2H_2O.

^{1}H-NMR spectroscopy was performed at 22 °C with a Bruker AMX 300 instrument, using a 5 mm outer-diameter probe (0.6 ml sample) and a spin rate of 21 rev./s. A 20.6 p.p.m. spectral width was measured 32 times requiring 2.65 s of acquisition time per measurement with a 30 s delay (see Figure 2). A preliminary spectrum with a 1 s delay was also measured.

Enzymes

Jurkat cells (ATCC TIB 152) were cultured in a 1:1 (v/v) mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium supplemented with 5 % fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 10^5 units/l penicillin G, 100 mg/ml streptomycin sulphate and 250 mg/l fungizone. Cells were collected by centrifugation, washed twice with PBS, resuspended in 0.1 M sodium phosphate buffer (pH 7.4), frozen (−70 °C) and thawed (37 °C) (×3), and the supernatant (2 min; 5000 *g*) was used as enzyme source.

Young adult male rats were killed and bone marrow expelled from the long bones of hind limbs. After brief vortexing to break up clumps, cells were suspended in minimal essential medium containing 10 % fetal bovine serum, 50 mg/ml ascorbic acid, 10 nM dexamethasone and 1 % of a mixture of penicillin, streptomycin and fungizone. Cells were incubated in a humidified 37 °C atmosphere of 95 % air/5 % CO_2 for 24 h. Non-adherent cells were removed by a medium change and the cells were grown to near-confluence. Cells were subcultured using trypsin/EDTA and plated into 150 cm^2 flasks, grown to confluency, harvested and frozen stocks prepared. Cells were thawed into a 150 cm^2 flask, grown to near-confluence then subcultured into five 150 cm^2 flasks. They were then collected and treated as described above for the Jurkat cells.

Preparation of chicken liver AICAR T'ase and glycinamide ribonucleotide transformylase (GAR T'ase) and sources of 5-aminoimidazole-4-carboxamide ribonucleotide and glycinamide ribonucleotide (AICAR and GAR respectively) are described elsewhere [8]. AICAR concentration was estimated at 269 nm (pH 7.4) using $\epsilon_{269} = 1.26 \times 10^4\ M^{-1} \cdot cm^{-1}$ [9].

Enzyme assays

AICAR T'ase from Jurkat cells and rat bone marrow cells was assayed by measuring the increase in absorbance at 312 nm (ΔA_{312}) in a 0.2 cm pathlength cell [10] and by a non-continuous colorimetric assay. Both assay mixtures contained (final concentrations in a total volume of 0.6 ml): 100 mM Tris/HCl (pH 7.4), 150 mM KCl, 10 mM 2-mercapthoethanol, 800 μM

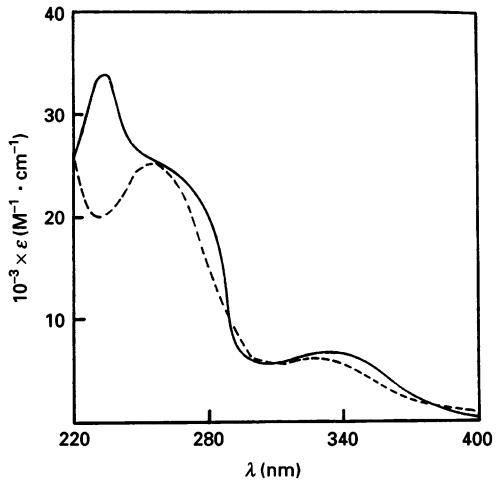

Figure 1 UV spectra of 10-HCO-H_2folate at pH 7.4 (0.1 M Tris/HCl) (——) and at pH 1 (0.1 M HCl) (– – – –).

Spectra were measured with a Varian DMS-200 spectrophometer.

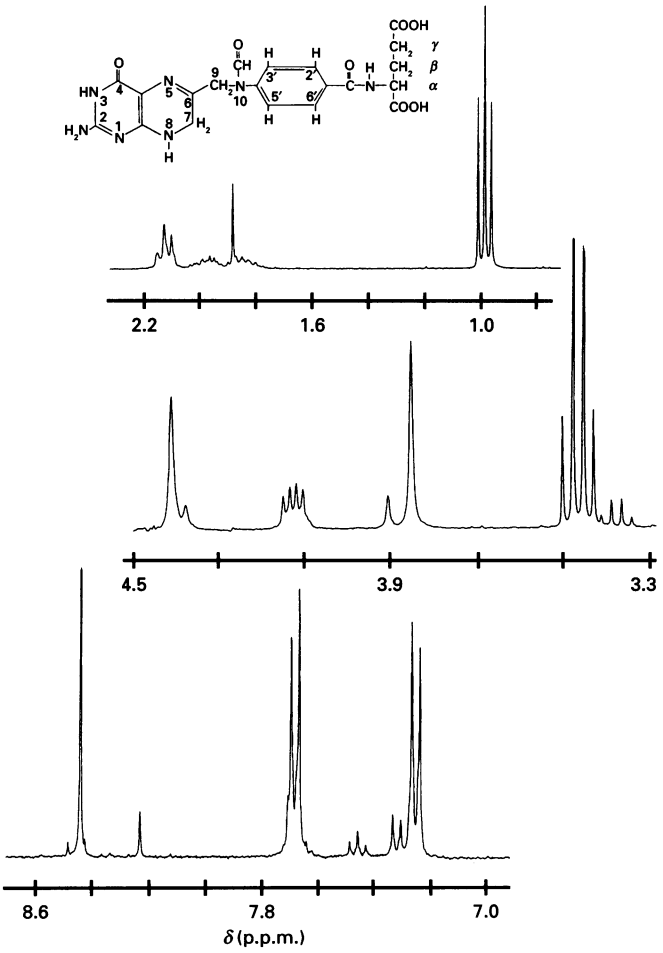

Figure 2 The 300 MHz ^{1}H-NMR spectrum of 10-HCO-H_2folate

10-HCO-H_2folate concentration was 15 mM in ^2H_2O containing 25 mM sodium phosphate buffer with a pH-meter reading of 7.3 at 25 °C. The inset shows 10-HCO-H_2folate structure and numbering. Intensities and abscissae are expanded differently for each region in order to show clearly the details of each resonance and resonances due to small amounts of impurities.

Table 1 NMR chemical shifts and coupling constants for non-exchangeable protons of 10-HCO-H_2folate and for contaminants

Resonance assignments were made on the basis of published chemical-shift data [16–18], multiplicities and coupling constants. The chemical shift of the HO^2H internal standard was 4.67 p.p.m. The integration is the average of C-3′5′ and C-2′6′ resonances set equal to 2.00 [16]; theoretical values are in parentheses. s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets.

Resonance assignment	Chemical shift (p.p.m.)	Multiplicity	Integration	| J | (Hz)
10-HCO-H_2folate				
Formyl	8.45	s	0.83 (1)	–
C-2′6′	7.69	d	2.05 (2)	8.6
C-3′5′	7.26	d	1.95 (2)	8.6
C-9	4.41	s	1.78 (2)	–
αC	4.13	dd	1.08 (1)	4.6, 8.8
C-7	3.86	s	1.91 (2)	–
γC	2.12	t		7.5
βC	1.86	m	5.14 (4)	–
βC	1.99	m		–
Solvents from sample preparation				
Methylene (ethanol)	3.46	q	3.50 (4)	7.1
Methyl (ethanol)	1.00	t	5.64 (6)	7.1
Methylene (ether)	3.38	q	< 0.2	7.1
Acetonitrile	1.89	s	~ 1	–
10-HCO-folate				
C-7	8.24	s	–	–
Unknown	7.46	t	–	–
C-3′5′	7.33	d	–	–
C-9	4.38	s	–	–
αC	3.91	–	–	–

AICAR, 25–1300 μM 10-HCO-H_4folate or 10-HCO-H_2folate and 2.0 μM methotrexate (MTX) (unless indicated otherwise); assays were carried out at 37 °C.

A colorimetric assay used a plastic tube with an airtight cap and is modified from a previously published method [11]: 0.1 ml aliquots were removed at various times (0–4 h), the reaction was stopped by the addition of 0.05 ml of acetic anhydride (with vigorous mixing), and the mixture allowed to stand for 20 min at room temperature. The following ice-cold reagents were added, mixed and incubated at 0 °C for indicated times: 0.05 ml of 5 M H_2SO_4 (1 min), 0.3 ml of 1% (w/v) $NaNO_2$ (5 min), 0.3 ml of 5% (w/v) ammonium sulphamate (3 min) and 0.3 ml of 1% (w/v) *N*-(1-naphthyl)ethylenediamine (20 h at room temperature). After centrifugation (1 min; 600 *g*), the A_{552} in a 1 cm pathlength cell was read against a blank which contained all components of the reaction except AICAR. Only AICAR, not formyl-AICAR, IMP or *p*-aminobenzoylglutamate (an oxidation product of H_4folate), gives a coloured product in the above assay procedure. Changes in A_{552} observed during the reaction (versus the zero time point) were compared with a standard curve of 10–80 nmol of AICAR per tube. As the colorimetric assay is not continuous, corresponding concentrations of the folate cofactors were calculated [12]. The colorimetric assay was also used to follow the progress curve of the AICAR T'ase reaction (see Figure 3).

Chicken liver AICAR T'ase was assayed using the direct spectrophotometric assay (ΔA_{312}) as described above except that AICAR was 200 μM and the assay solution contained 1.7% (v/v) glycerol, 0.7% (v/v) DMSO and no MTX. This assay was also used to follow the progress curve of the AICAR T'ase reaction (see Figure 4).

GAR T'ase from Jurkat cells was assayed using the direct spectrophotometric assay (ΔA_{312} in a 1.0 cm pathlength cell) [13]. The assay mixture contained (final concentrations in a total volume of 1.0 ml): 20 mM Tris/HCl (pH 7.4), 5 mM 2-mercaptoethanol, 50 μM (α,β) GAR, 2.0–100 μM 10-HCO-H_4folate or 10-HCO-H_2folate, 0.5 μM MTX; assays were carried out at 37 °C.

Chicken liver GAR T'ase was assayed as described above except that the assay solution contained 0.25% (v/v) glycerol, 0.1% (v/v) DMSO and no MTX.

Protein concentration (in mg/ml) was estimated to be equal to $1.55\ A_{280} - 0.76\ A_{260}$.

Estimation of kinetic parameters and statistics

Unweighted initial velocities and folate cofactor concentrations (or corresponding concentrations) were fitted to the Michaelis–Menten equation to obtain K_m, $V_{max.}$ and their S.E.M. values using the EZ-fit program (E. I. DuPont de Nemour). Student's *t* test (two-tailed) was used to detect differences in kinetic parameters.

RESULTS AND DISCUSSION

Properties of 10-HCO-H_2folate

10-HCO-H_2folate was prepared by careful monitoring of air oxidation of 10-HCO-H_4folate, and the UV spectra of the former are shown in Figure 1. The UV spectrum of 10-HCO-H_2folate at pH 7.4 ($\lambda_{max.}$ 234 and 333 nm, $\lambda_{min.}$ 301 nm) is different from that reported by Mathews and Huennekens [14] ($\lambda_{max.} \cong 244$ nm); these workers prepared their 10-HCO-H_2folate by dithionite reduction of 10-HCO-folate. The neutral pH spectrum in Figure 1 is very different from that reported by Baram et al. [15] ($\lambda_{max.}$ 272 nm, $\lambda_{min.}$ 256 nm) for their 10-HCO-H_2 folate which was reported to be 97% pure (by HPLC) and which was prepared by 2,6-dichloro-indophenol oxidation of 10-HCO-H_4folate. Therefore the authenticity of the 10-HCO-H_2folate prepared here is established by the following.

(1) Substantial contamination of 10-HCO-H_4folate is ruled out as no conversion of this compound into 5,10-CH=H_4folate (i.e. ΔA_{356}) in acidic pH is observed in our preparation of 10-

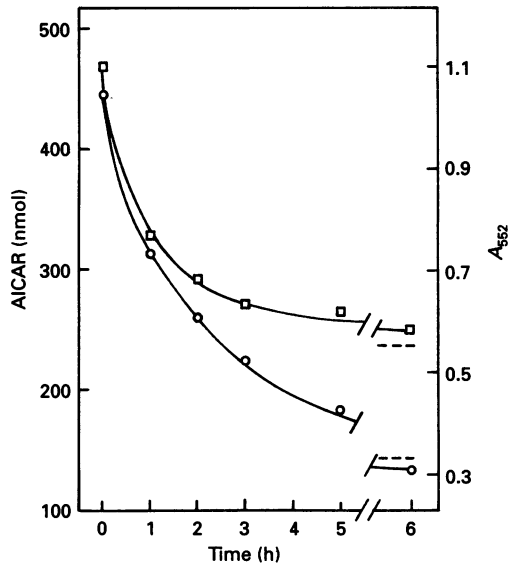

Figure 3 Progress curve (consumption of AICAR) during the Jurkat-cell AICAR T'ase-catalysed reaction using 10-HCO-H_4folate and 10-HCO-H_2folate as cofactors

Assay mixtures contained [final concentrations (and amounts)] 0.15 M KCl, 0.1 M Tris/HCl, pH 7.4, 10 mM 2-mercaptoethanol, 800 μM (480 nmol) AICAR, 2.0 μM MTX, 0.32 mg of protein from Jurkat-cell extract and either 383 μM (230 nmol) 10-HCO-H_2folate (□) or 501 μM (301 nmol) 10-HCO-H_4folate (○) in a total volume of 0.6 ml at 37 °C. At the indicated times, 0.1 ml aliquots were removed and the colorimetric assay for AICAR was performed. The zero time point, taken immediately after the addition of the Jurkat-cell extract, gave an initial value of 468 nmol and 445 nmol of AICAR for the 10-HCO-H_2folate and 10-HCO-H_4folate assay respectively, in agreement with the calculated amount of 480 nmol of AICAR added to each tube. The dashed lines indicate the amount of AICAR which should remain if 230 nmol of 10-HCO-H_2folate and 301 nmol of 10-HCO-H_4folate were completely utilized to convert equimolar amounts of AICAR into IMP.

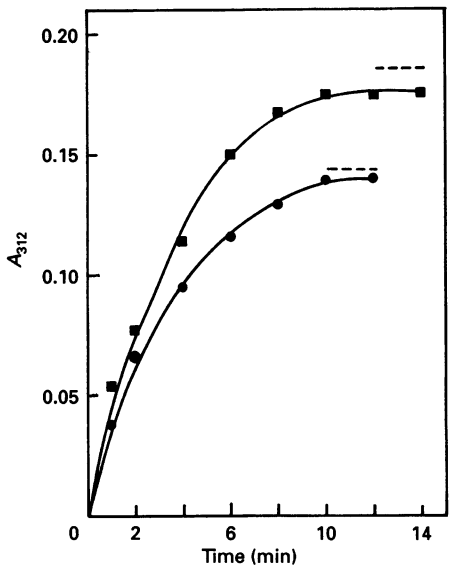

Figure 4 Progress curve (appearance of folate product) during the chicken liver AICAR T'ase-catalysed reaction using (6*R*)-10-HCO-H_4folate and 10-HCO-H_2folate

Assay mixtures contained (final concentrations) 0.15 M KCl, 0.1 M Tris/HCl, pH 7.4, 10 mM 2-mercaptoethanol, 200 μM AICAR, 1.7% (v/v) glycerol, 0.7% (v/v) DMSO, chicken liver enzyme preparation and either 60 μM 10-HCO-H_4folate (●) or 107 μM 10-HCO-H_2folate (■) in a total volume of 0.6 ml at 37 °C. The A_{312} was measured in a 0.2 cm pathlength cuvette. The dashed lines indicate the final A_{312} readings expected if 10-HCO-H_4folate was completely converted into H_4folate and 10-HCO-H_2folate was completely converted into H_2folate using $\Delta\epsilon_{312}$ values of 1.2×10^4 and 8.7×10^3 $M^{-1} \cdot cm^{-1}$ respectively.

HCO-H_2folate. Also the pH 1 spectrum (Figure 1) of 10-HCO-H_2folate ($\lambda_{max.}$ 257 and 328 nm, $\lambda_{min.}$ 229 and 307 nm) is very different from that of 5,10-CH=H_4Folate [4]. Substantial contamination by 10-HCO-folate is ruled out, as this compound has a UV spectrum with $\lambda_{max.}$ 243, 270 and 347 nm and $\lambda_{min.}$ 252 and 305 nm at pH 7.4. 10-HCO-folate with this UV spectrum was, however, produced by prolonged air oxidation of 10-HCO-H_4folate in this laboratory and by others [7] and by permanganate/peroxide oxidation of 5,10-CH=H_4folate [6]. 10-HCO-H_2folate prepared from (6*R*,*S*)-10-HCO-H_4folate has the same spectral properties as the 10-HCO-H_2 folate prepared from (6*R*)-10-HCO-H_4folate.

(2) The ^{1}H-NMR spectrum of 10-HCO-H_2folate in ^2H_2O is shown in Figure 2. Resonance assignments, their multiplicity, coupling constants and integration are given in Table 1. Solvent contaminants are ethyl ether, acetonitrile and ethanol (from the ether) arising from the NMR sample preparation. Coupling constants for the C-2′6′, C-3′5′, αC and γC protons on 10-HCO-H_2folate were within 0.5 Hz of those reported for these protons in 10-HCO-H_4folate [16]. Chemical shifts for the formyl, C-2′6′, C-3′5′, αC, γC and βC protons were within 0.18–0.22 p.p.m. less than these protons in 10-HCO-H_4folate reported by Poe and Benkovic [16]. The above protons are substantially removed from the additional double bond at the 5,6 position which is present in 10-HCO-H_2folate but not present in 10-HCO-H_4folate and their chemical shift should be little altered. In contrast, chemical shifts for C-7 and C-9 protons in 10-HCO-H_2 folate are 0.34–0.51 p.p.m. greater than the corresponding protons in 10-HCO-H_4folate, indicating that both C-7 and C-9 protons are relatively deshielded in 10-HCO-H_2folate by the adjacent 5,6 double bond. A proton on C-6 should have split both C-7 and C-9 proton resonances. The data thus suggest that there is no proton on C-6. No direct evidence for a C-6 proton was observed. Although it may have been buried under the ethanol methylene protons, the correct ratio of methyl to methylene protons was observed for this contaminant, ruling out the contribution of the C-6 proton. The integration values suggest that our preparation is contaminated with approx. 2 mol of ethanol for every mol of HCO-H_2folate. Although the NMR spectrum of 10-HCO-H_2folate was obtained within 2 h of its preparation, detectable amounts of 10-HCO-folate were present (Table 1). This is undoubtedly due to the fact that no antioxidant was added once the 10-HCO-H_2folate had been prepared and the exposure of this compound to air during the sample work-up. The UV spectra of the sample, taken within 1 h of the completion of the NMR spectrum, had $\lambda_{max.}$ and $\lambda_{min.}$ within ± 1 nm of that given above with the exception that, at pH 1, $\lambda_{min.}$ = 311 nm compared with 307 nm for this minimum in the freshly prepared compound. This finding suggests that small differences in the UV spectra of 10-HCO-H_2folate indicate the presence of detectable impurities.

(3) In the presence of excess AICAR and Jurkat-cell AICAR T'ase, 230 nmol of 10-HCO-H_2folate resulted in the utilization of 218 nmol of AICAR (95% of theoretical) at equilibrium (Figure 3). Control experiments (Figure 3) demonstrated that 301 nmol of 10-HCO-H_4folate resulted in the utilization of 312 nmol of AICAR (104% of theoretical). These results demonstrate that essentially all of the 10-HCO-H_2folate preparation can be utilized to convert AICAR into formyl-AICAR or IMP.

(4) Using the $\Delta\epsilon_{312}$ of 1.2×10^4 and 8.7×10^3 $M^{-1} \cdot cm^{-1}$ pre-

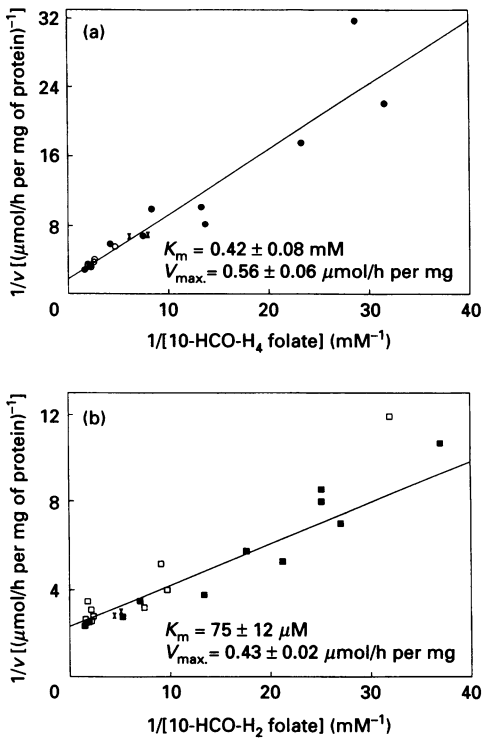

Figure 5 Double-reciprocal plots of the Jurkat-cell AICAR T'ase reaction using (6*R*)-10-HCO-H$_4$folate (a) and 10-HCO-H$_2$folate (b)

Solid symbols represent the direct spectrophotometric assay and open symbols the colorimetric assay. X, Direct spectrophotometric assay without MTX. All assay mixtures contained the same amount of protein from the Jurkat-cell extract.

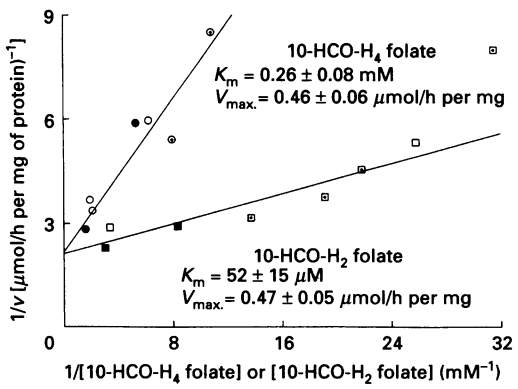

Figure 6 Double-reciprocal plot of the rat bone marrow cell AICAT T'ase-catalysed reaction using (6*R*)-10-HCO-H$_4$folate (circles) or 10-HCO-H$_2$folate (squares)

Solid symbols indicate direct spectrophotometric assay, open symbols colorimetric assay and open symbols with crosses direct spectrophotometric assay without MTX. All assay mixtures contained the same amount of protein from the rat bone marrow cell extract.

viously established for the AICAR T'ase reaction utilizing 10-HCO-H$_4$folate and 10-HCO-H$_2$folate respectively [10,18], and in the presence of excess AICAR, chicken liver AICAR T'ase converted 64 nmol of 10-HCO-H$_2$folate into 60 nmol of H$_2$folate (93% of theoretical) and 36 nmol of 10-HCO-H$_4$folate into 35 nmol of H$_4$folate (97% of theoretical) at equilibrium (Figure 4). These results again confirm that essentially all of the 10-HCO-H$_2$folate preparation can be utilized by the enzyme. 10-HCO-folate is utilized at a very low rate by chicken liver AICAR T'ase [10], and no increase in A_{312} was observed when 10-HCO-folate replaced 10-HCO-H$_2$folate in the experiments shown in Figure 4. 10-HCO-H$_2$folate prepared from (6*R*,*S*)-10-HCO-H$_4$folate was utilized by chicken liver AICAR T'ase to the same extent as 10-HCO-H$_2$folate prepared from (6*R*)-10-HCO-H$_4$folate. Thus the chicken liver enzyme, which is known to be inactive with (6*S*)-10-HCO-H$_4$folate, does not distinguish 10-HCO-H$_2$folate prepared from the two stereoisomers. This suggests that the chiral carbon 6 is indeed missing in 10-HCO-H$_2$folate supporting the assignment of the 7,8-dihydro structure.

(5) 10-HCO-H$_2$folate was identified as one of two products of the permanganate/peroxide oxidation of 5,10-CH=H$_4$folate [6]. 10-HCO-H$_2$folate was separated by HPLC and had a UV spectrum at pH 7.0 with $\lambda_{max.}$ and $\lambda_{min.}$ within 2 nm of those of the pH 7.4 UV spectrum in Figure 1. Thus 10-HCO-H$_2$folate is also a product of this method of oxidation and can be isolated by HPLC.

(6) 10-HCO-H$_2$folate has been prepared by air oxidation of 10-HCO-H$_4$folate and migrated as a single UV-detectable spot on TLC systems [5].

Taken together, the above results and data indicate that the method described here produces authentic 10-HCO-H$_2$folate and establishes the stoicheiometry of its utilization by AICAR T'ase. The chemical nature of the essentially pure folate product (i.e. the product with $\lambda_{max.}$ 272 nm) produced by Baram et al. [15] by the 2,6-dichloro-indophenol oxidation of 10-HCO-H$_4$folate is not known.

Biological properties of 10-HCO-H$_2$folate

Both 10-HCO-H$_4$folate and 10-HCO-H$_2$folate were utilized by the Jurket-cell AICAR T'ase enzyme (Figure 5). The continuous direct spectrophotometric assay (ΔA_{312}) and the non-continuous colorimetric assay are independent assays and gave comparable results (Figure 5). 10-HCO-H$_2$folate had a kinetic advantage over 10-HCO-H$_4$folate, the dihydro cofactor having a lower K_m (75 μM compared with 0.42 mM) ($P < 0.0005$). Values for $V_{max.}$ were statistically ($P < 0.05$) but not substantially different.

Only 10-HCO-H$_4$folate served as a one-carbon donor in the Jurkat-cell GAR T'ase-catalysed reaction ($K_m = 4.9 \pm 1.0$ mM, $V_{max.} = 1.10 \pm 0.07$ μmol/h per mg of protein). GAR T'ase activity with 10-HCO-H$_2$folate (50 μM; 10 min) was not detectable; under identical conditions 12% of 10-HCO-H$_4$folate was converted into H$_4$folate.

The possibility that the Jurkat-cell extract reduces 10-HCO-H$_2$folate to 10-HCO-H$_4$folate, which is then utilized by AICAR T'ase, is ruled out by the following considerations.

(1) MTX (at concentrations that do not inhibit either GAR T'ase or AICAR T'ase) is included in the assays to inhibit dihydrofolate reductase or dismutase activities [19].

(2) 10-HCO-H$_2$folate has a lower K_m than 10-HCO-H$_4$folate for AICAR T'ase and, using the same enzyme source, has no activity with GAR T'ase. Thus 10-HCO-H$_2$folate is not reduced to 10-HCO-H$_4$folate which would have resulted in GAR T'ase activity.

As Jurkat cells are a neoplastic T-lymphocyte cell line and may not be representative of normal cells, the activity of the dihydro- and tetrahydro-folate cofactors was tested with rat bone marrow cell AICAR T'ase. As shown in Figure 6, 10-HCO-H$_2$folate again had a kinetic advantage over 10-HCO-H$_4$folate, the dihydro cofactor having a substantially lower K_m (52 μM com-

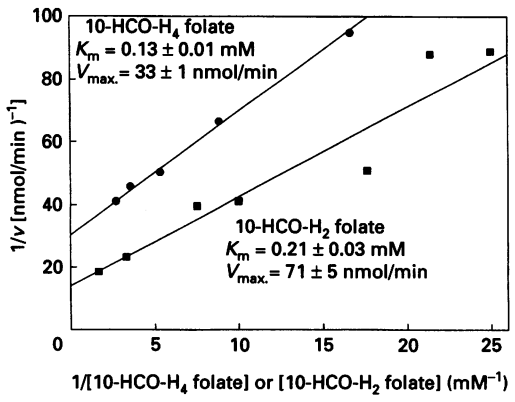

Figure 7 Double-reciprocal plot of the chicken liver AICAR T'ase-catalysed reaction using (6*R*)-10-HCO-H_4folate (●) or 10-HCO-H_2folate (■)

All assay mixtures contained the same amount of chicken liver enzyme.

pared with 0.26 mM) ($P < 0.025$). Values for $V_{max.}$ were essentially the same for both folate cofactors. Thus the rat bone marrow cell enzyme gave quantitatively similar results to these of the Jurkat-cell enzyme.

It had been previously shown that 10-formyl-7,8-dihydropteroylpentaglutamate was a substrate for chicken liver AICAR T'ase albeit a relatively poor one compared with the corresponding 5,6,7,8-tetrahydrofolate cofactor [10]. Therefore both 10-HCO-H_4folate and 10-HCO-H_2folate were tested using chicken liver as a source of AICAR T'ase (Figure 7). 10-HCO-H_2folate did not have a substantial kinetic advantage over 10-HCO-H_4folate because the higher $V_{max.}$ (71 compared with 33 nmol/min; $P < 0.005$) of the dihydro cofactor was offset by the lower K_m (0.13 compared with 0.21 mM; $P < 0.05$) of tetrahydro cofactor. This indicates that AICAR T'ase from mammalian and avian sources have different substrate specificities. Thioinosinic acid inhibition of AICAR T'ase from chicken liver and mouse blood cells were found to be quantitatively different, also suggesting that avian and mammalian enzymes are not identical [20].

When tested with 100 μM 10-HCO-H_2folate and 10-HCO-H_4folate ($K_m \cong 5$ μM) in the reaction catalysed by chicken liver GAR T'ase, the rate of reaction with the dihydro cofactor was 0.6% that with the tetrahydro cofactor. Thus 10-HCO-H_2folate is not utilized efficiently by avian GAR T'ase. In this respect, the avian and mammalian GAR T'ase enzymes are similar, in contrast with AICAR T'ase from these sources.

Conclusions

The ease with which 10-HCO-H_4folate is oxidized to 10-HCO-H_2folate (using only air) virtually confirms the existence *in vivo* of the latter. For example, 10-HCO-folate has been isolated from horse liver [21]. Other have reported 10-HCO-folate in human and rat urine [7,22,23] and human bile [24] as a metabolite of radiolabelled folic acid. As folic acid cannot be enzymically formylated by a direct process, these findings strongly suggest that folic acid was first reduced to the tetrahydro oxidation state, the 10-formyl group introduced enzymically, and then 10-HCO-H_4folate was oxidized to 10-HCO-folate with 10-HCO-H_2folate as an intermediate in the process.

The biological reason why AICAR T'ase, but not GAR T'ase, has two folate cofactors (i.e. the dihydro and tetrahydro) which can serve as one-carbon donors is not known. One may speculate that, as the K_m of 10-HCO-H_4folate is approx. 5 μM (Jurkat cell and chicken liver) in the GAR T'ase reaction and is orders of magnitude below the K_m of this cofactor in the AICAR T'ase reaction (i.e. 0.13 and 0.42 mM), the AICAR T'ase enzyme needs to have both dihydro and tetrahydro cofactors at its disposal to keep pace with the activity of GAR T'ase. This speculation is reasonable given that, in Jurkat cells, the specific activity of GAR T'ase is 2-fold higher than the specific activity of AICAR T'ase. Finally, the data suggest that mammalian and avian AICAR T'ases are not similar and that folate-dependent purine biosynthesis in cells targeted by antifolates (e.g. lymphoid and bone marrow cells) may be a complex process.

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