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Inhibition of 5-aminoimidazole-4-carboxamide ribotide transformylase, adenosine deaminase and 5′-adenylate deaminase by polyglutamates of methotrexate and oxidized folates and by 5-aminoimidazole-4-carboxamide riboside and ribotide

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With the use of a continuous spectrophotometric assay and initial rates determined by the method of Waley [Biochem. J. (1981) **193**, 1009–1012] methotrexate was found to be a non-competitive inhibitor, with $K_{i(intercept)} = 72\ \mu M$ and $K_{i(slope)} = 41\ \mu M$, of 5-aminoimidazole-4-carboxamide ribotide transformylase, whereas a polyglutamate of methotrexate containing three γ-linked glutamate residues was a competitive inhibitor, with $K_i = 3.15\ \mu M$. Pentaglutamates of folic acid and 10-formylfolic acid were also competitive inhibitors of the transformylase, with K_i values of 0.088 and 1.37 μM respectively. Unexpectedly, the pentaglutamate of 10-formyldihydrofolic acid was a good substrate for the transformylase, with a K_m of 0.51 μM and a relative $V_{max.}$ of 0.72, which compared favourably with a K_m of 0.23 μM and relative $V_{max.}$ of 1.0 for the tetrahydro analogue. An analysis of the progress curve of the transformylase-catalysed reaction with the above dihydro coenzyme revealed that the pentaglutamate of dihydrofolic acid was a competitive product inhibitor, with $K_i = 0.14\ \mu M$. The continuous spectrophotometric assay for adenosine deaminase based on change in the absorbance at 265 nm was shown to be valid with adenosine concentrations above 100 μM, which contradicts a previous report [Murphy, Baker, Behling & Turner (1982) Anal. Biochem. **122**, 328–337] that this assay was invalid above this concentration. With the spectrophotometric assay, 5-aminoimidazole-4-carboxamide riboside was found to be a competitive inhibitor of adenosine deaminase, with ($K_i = 362\ \mu M$), whereas the ribotide was a competitive inhibitor of 5′-adenylate deaminase, with $K_i = 1.01$ mM. Methotrexate treatment of susceptible cells results in (1) its conversion into polyglutamates, (2) the accumulation of oxidized folate polyglutamates, and (3) the accumulation of 5-aminoimidazole-4-carboxamide riboside and ribotide. The above metabolic events may be integral elements producing the cytotoxic effect of this drug by (1) producing tighter binding of methotrexate to folate-dependent enzymes, (2) producing inhibitors of folate-dependent enzymes from their tetrahydrofolate coenzymes, and (3) trapping toxic amounts of adenine nucleosides and nucleotides as a result of inhibition of adenosine deaminase and 5′-adenylate deaminase respectively

INTRODUCTION

There is considerable experimental evidence indicating that the toxicity of methotrexate (MTX) results from its potent inhibition of dihydrofolate reductase (Jackson & Grindey, 1984). This inhibition diminishes the pool of fully reduced folate coenzymes that are utilized for the biosynthesis of thymidylate and purine nucleotides. In addition, Borsa & Whitmore (1969*a*) and Kisliuk *et al.* (1979) have demonstrated that MTX and polyglutamates of MTX can also directly interfere with thymidylate biosynthesis by inhibiting thymidylate synthase. By contrast, a test for direct inhibition of folate-dependent enzymes of purine-nucleotide biosynthesis *de novo* by MTX indicated that there was no inhibition (Mangum *et al.*, 1979).

The inhibition of dihydrofolate reductase by MTX also results in the accumulation and excretion of folates with 7,8-dihydro or fully oxidized pteridine rings (Nixon *et al.*, 1973; Moran *et al.*, 1975; White & Goldman, 1976; Saleh *et al.*, 1981). Folates with dihydro or oxidized pteridine rings are inhibitors of thymidylate synthetase (Kisliuk *et al.*, 1974; Dolnick & Chen, 1978), glycinamide ribonucleotide transformylase (Chan & Baggott, 1982), methionine synthetase (Whitfield *et al.*, 1970), 5-formino-tetrahydrofolate cyclodeaminase, 5,10-methenyltetrahydrofolate cyclohydrolase (MacKenzie & Baugh, 1980) and methylenetetrahydrofolate reductase (Matthews &

Abbreviations used: MTX, methotrexate (4-amino-10-methylpteroylglutamic acid); $MTXGlu_3$, 4-amino-10-methylpteroyl-γ-glutamyl-γ-glutamyl-γ-glutamylglutamic acid; AICA, 5-aminoimidazole-4-carboxamide; AICA riboside, *N*-1-ribosyl-5-aminoimidazole-4-carboxamide; AICA ribotide, *N*-1-(5′-phosphoribosyl)-5-aminoimidazole-4-carboxamide; formyl-AICA-ribotide, *N*-1-(5′-phosphoribosyl)-5-formamidoimidazole-4-carboxamide; AICAR transformylase, phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3); 10-HCO-$H_4PteGlu_5$, 10-formyl-5,6,7,8-tetrahydropteroylpenta-γ-glutamate; 10-HCO-7,8-$H_2PteGlu_5$, 10-formyl-7,8-dihydropteroylpenta-γ-glutamate; 10-HCO-$PteGlu_5$, 10-formylpteroylpenta-γ-glutamate; $H_4PteGlu_5$, 5,6,7,8-tetrahydropteroylpenta-γ-glutamate; $H_2PteGlu_5$, 7,8-dihydropteroylpenta-γ-glutamate; $PteGlu_5$, pteroylpenta-γ-glutamate.

Haywood, 1979). In all cases, where tested, polyglutamates of dihydro or oxidized folates are better inhibitors than their corresponding monoglutamates.

Urinary excretion of 5-aminoimidazole-4-carboxamide (AICA) markedly increases during MTX therapy (Luhby & Cooperman, 1962; McGeer & McGeer, 1963; Donaldson *et al.*, 1969). This compound undoubtedly arises from increased pools of its corresponding riboside and ribotide as a result of blockage at the step in the pathway of purine-nucleotide biosynthesis catalysed by phosphoribosylaminoimidazole-carboxamide formyltransferase (AICAR transformylase) (EC 2.1.2.3). AICA riboside has been reported to be an inhibitor of *Escherichia coli* adenosine deaminase (EC 3.5.4.4) (Kuramitsu *et al.*, 1964). Adenosine deaminase is of critical importance in mammalian lymphocytes because a defiency of this enzyme results in a toxic accumulation of adenine nucleosides and nucleotides (Martin & Gelfand, 1981).

MTX and a typical polyglutamyl metabolite of MTX, 2,4-diamino-10-methylpteroyl-γ-glutamyl-γ-glutamyl-γ-glutamylglutamate ($MTXGlu_3$) (Galivan, 1980), were tested as inhibitors of chicken liver AICAR transformylase assayed with a typical naturally occurring polyglutamyl folate coenzyme, 10-HCO-$H_4PteGlu_5$ (Connor & Blair, 1980). $PteGlu_5$, 10-HCO-$PteGlu_5$, 10-HCO-7,8-$H_2PteGlu_5$ and $H_2PteGlu_5$ were tested as typical oxidized folates that could be inhibitors of the transformylase. Finally, AICA riboside was tested as an inhibitor of adenosine deaminase and AICA ribotide was tested as an inhibitor of a metabolically related enzyme, 5′-adenylic acid (AMP) deaminase (EC 3.5.4.6.)

EXPERIMENTAL

Materials

10-HCO-7,8-$H_2PteGlu_5$ was prepared from 10-HCO-$H_4PteGlu_5$ and quantified as described by Eto & Krumdieck (1980). MTX, AICA ribotide, AICA riboside, adenosine and AMP were obtained from Sigma Chemical Co. $MTXGlu_3$ was synthesized as previously described (Nair & Baugh, 1973) and quantified by using $\epsilon_{307} = 19700\ M \cdot cm^{-1}$ at pH 1 (Blakley, 1969). Other folates and ribotides were synthesized and quantified as previously described (Baggott & Krumdieck, 1979*a*,*b*). Bovine adenosine deaminase (type VIII) and rabbit 5′-adenylic acid deaminase were purchased from Sigma Chemical Co. Affi-Gel 10 was purchased from Bio-Rad Laboratories and [8-^{14}C]IMP was purchased from Schwarz–Mann. All other reagents were the best grade available from commercial sources.

The affinity column for the purification of AICAR transformylase was synthesized as follows. A 100 μmol portion of AICA ribotide was allowed to react with 100 μmol of $NaNO_2$ in 30 ml of 0.06 M-H_2SO_4 and 0.03 M-NaCl at 0 °C for 30 min. To this solution was added 100 μmol of *N*-1-naphthylethylenediamine hydrochloride and after 10 min of stirring the pH was raised to 7 with conc. KOH. A 500 mg portion of Affi-Gel 10 resin was then added and the suspension was slowly stirred for 6 h at 5 °C. The coupling of the azo dye was then terminated by the addition of 500 μmol of glycine and the resin was stirred overnight. The resin was then washed with copious amounts of 10 mM-Tris/HCl, pH 7.4, 50 mM-potassium phosphate, pH 7.4, and distilled water until the wash was colourless. The amount of the *N*-1-naphthylethylenediamine azo dye of AICA ribotide coupled to the resin was determined by subtracting the amount in the wash from the total amount synthesized. The concentration of the azo dye was determined by using an ϵ_{540} value 26400 $M \cdot cm^{-1}$ (pH 1) (Flaks & Lukens, 1963) and the amount coupled to the resin was found to be 51 μmol.

Methods

All spectrophotometric enzyme assays and measurements were performed on a GCA McPherson spectrophotometer.

AICAR transformylase was assayed spectrophotometrically at 37 °C in 0.082 M-potassium phosphate buffer, pH 7.4, by monitoring the conversion of 10-HCO-$H_4PteGlu_5$ into $H_4PteGlu_5$ at 312 nm ($\Delta\epsilon$12000 $M \cdot cm^{-1}$; Smith *et al.*, 1981*a*), by monitoring the conversion of 10-HCO-7,8-$H_2PteGlu_5$ into 7,8-$H_2PteGlu_5$ at 312 nm ($\Delta\epsilon$ 8700 $M \cdot cm^{-1}$; see the Results section) or by monitoring the conversion of 10-HCO-$PteGlu_5$ into $PteGlu_5$ at 300 nm ($\Delta\epsilon = 17000\ M \cdot cm^{-1}$; Smith *et al.*, 1981*b*). In all assays the concentration of AICA ribotide was 200 μM and 2-mercaptoethanol was added to a final concentration of 0.01 M when the tetrahydrofolate coenzyme was utilized. Cuvettes had a 1 cm light path, the reference cuvette contained all components except AICA ribotide and a blank rate was established before the addition of the ribotide. AICA ribotide concentration was estimated by the modified Bratton–Marshall assay (Flaks & Lukens, 1963) and IMP was separated on a Sephadex G-10 column (1 cm × 110 cm) eluted with 10 mM-potassium phosphate buffer, pH 7.4.

IMP cyclohydrolase (EC 3.5.4.10) was assayed spectrophotometrically at 37 °C in 0.082 M potassium phosphate by monitoring the conversion of formyl-AICA ribotide to IMP at 250 nm ($\Delta\epsilon$ 5500 $M \cdot cm^{-1}$; Baggott & Krumdieck, 1979*b*). Cuvettes had a 1 cm light path, the reference cuvette contained all components except the enzyme and a blank rate was established before the addition of the enzyme.

Adenosine deaminase was assayed spectrophotometrically at 25 °C in 50 mM-potassium phosphate buffer, pH 7.4, by monitoring the conversion of adenosine into inosine at 265 mm and by using an experimentally determined $\Delta\epsilon$ value (see the Results section). Cuvettes had 0.2 cm light paths, the reference cuvette contained all components except the enzyme and a blank rate was established before the addition of the enzyme.

AMP deaminase was assayed at 25 °C in 0.0 M-sodium citrate (pH 6.5)/0.15 M-KCl/1 mM-2-mercaptoethanol. Stock solutions of AMP deaminase were made by dissolving in excess 66% (v/v) glycerol in 0.33 M-KCl/1 mM-mercaptoethanol. Stock solutions of the enzyme were diluted 50–100-fold into the assay solutions. The formation of IMP, up to a concentration of 300 μM-AMP, was determined spectrophotometrically at 265 nm by using an assay analogous to the adenosine deaminase assay with an experimentally determined $\Delta\epsilon$. Cuvettes had a 0.2 cm light path, the reference cuvette contained all components except the enzymes and a blank rate was established before the addition of the enzyme. At initial AMP concentrations of 0.3–4.0 mM, the enzyme was assayed by using the procedure of Nathans *et al.* (1978).

In all cases where a continuous spectrophotometric assay was used, rates were determined at corresponding

product concentrations and extrapolated to zero product concentration in order to obtain initial rates by the method of Waley (1981). A computer program facilitated calculation of the rates, the corresponding product concentrations, the least-squares extrapolations to zero product concentration and the correlation coefficients.

The test for the denaturation of AICAR transformylase was performed by the method of Selwyn (1965). Kinetic parameters and inhibition constants (and their standard errors) were estimated by using computer programs described by Cleland (1979); however, double-reciprocal plots were used to display the data. When 10-HCO-7,8-H_2PteGlu$_5$ was used as a substrate for AICAR transformylase, the rates at corresponding substrate and product concentrations [which were determined by the method of Waley (1981)] were used to calculate K_i for the product inhibition by H_2PteGlu$_5$. Corresponding product concentrations were arbitrarily chosen so that the data could be displayed as double-reciprocal plots.

AICAR transformylase from chicken liver was partially purified by precipitation with $(NH_4)_2SO_4$, protamine sulphate precipitation and Sephadex G-150 chromatography as described previously (Baggott & Krumdieck, 1979*a*; Mueller & Benkovic, 1981). The enzyme was further purified by affinity chromatography. In a typical purification, 2–4 ml of the enzyme solution was loaded on to the affinity column (1 cm × 6 cm), which had been previously washed with 10 mM-Tris/HCl, pH 7.4. The column was washed with the same buffer until the A_{280} was less than 0.01. The enzyme was then eluted from the column with 20 ml of 50 mM-potassium phosphate, pH 7.4. Stock solutions of the enzyme were stored at −20 °C in this buffer. The protein concentration was determined by the method of Zamenhof (1957), with bovine serum albumin as a standard.

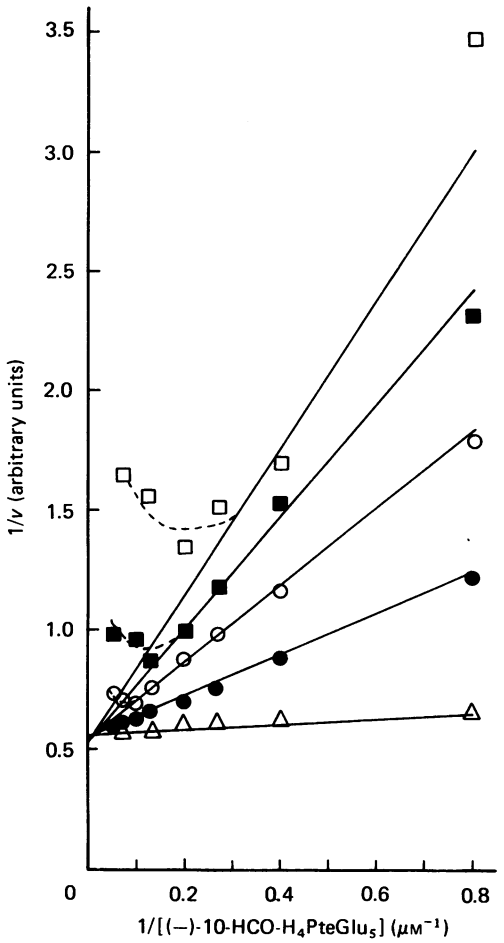

Fig. 1. Inhibition of AICAR transformylase by MTXGlu$_3$

The double-reciprocal plots (continuous lines) are shown in the presence of zero (△), 20 (●), 40 (○), 60 (■) and 80 (□) μM-MTXGlu$_3$. Broken lines show substrate inhibition. Other conditions are described under 'Methods'.

RESULTS AND DISCUSSION

Inhibition of AICAR transformylase by MTX and MTXGlu$_3$

The specific activities of chicken liver AICAR transformylase and IMP cyclohydrolase (which co-purifies with the transformylase) eluted from the affinity column were found to be 4.3 and 11.6 μmol/min per mg of protein respectively when assayed with 40 μM-(±)-10-HCO-H_4PteGlu$_5$ and 25 μM-formyl-AICA ribotide respectively. Thus the ratio of the cyclohydrolase activity to the transformylase activity was 2.7, which is less than the ratio of approx. 6 reported by others (Flaks & Lukens, 1963; Mueller & Benkovic, 1981). The difference is explained by the fact that the assay described above uses saturating concentrations of the folate coenzymes, whereas previous assays did not.

MTX is converted into poly-γ-glutamyl derivatives *in vivo*. Therefore both MTX and a polyglutamyl metabolite, MTXGlu$_3$, were tested as inhibitors of AICAR transformylase (assayed with 10-HCO-H_4PteGlu$_5$). MTX is a non-competitive inhibitor of the transformylase with a $K_{i(intercept)}$ of 72±6 and a $K_{i(slope)}$ of 41±30, whereas MTXGlu$_3$ is a competitive inhibitor (excluding data which displayed substrate inhibition) with a K_i of 3.15±0.69 μM (see Fig. 1). High concentrations of MTXGlu$_3$ also induced substrate inhibition (Fig. 1). Since the reaction catalysed by AICAR transformylase follows an ordered sequential mechanism with the folate coenzyme (mono- or poly-glutamate) binding first, it is likely that both MTX and MTXGlu$_3$ also bind to the free enzyme (Mueller & Benkovic, 1981). The relatively small MTX molecule may bind to a site that is not identical with that of 10-CHO-H_4PteGlu$_5$, producing non-competitive inhibition. By contrast, the larger MTXGlu$_3$ molecule may bind at a site identical with that of 10-CHO-H_4PteGlu$_5$, resulting in the competitive-inhibition pattern. The chicken liver enzyme is known to exist as a dimer (Mueller & Benkovic, 1981), therefore more complex explanations for the inhibition patterns are also possible.

Others have reported that the chicken liver transformylase is not inhibited by MTX (Mangum *et al.*, 1979); however, their assay used relatively high concentrations of the folate coenzyme (≃ 100 μM) and relatively low concentrations of MTX (10 μM); therefore this MTX concentration would be well below its K_i. In the assay reported here, the MTX concentration is varied from 20 to 80 μM, whereas that of the folate coenzyme is varied from 1.25 to 20 μM, and it should be a more sensitive test for inhibition using more realistic '*in vivo*' concentrations of MTX and the folate coenzyme. Inhibition of the transformylase by MTXGlu$_3$ has not been previously reported. Conversion of MTX into this polyglutamate

increases its potency as an inhibitor of the transformylase and is consistent with analogous results using thymidylate synthetase (Kisliuk *et al.*, 1979).

The apparent K_m of the transformylase for 10-HCO-H_4PteGlu$_5$ is 0.23 ± 0.03 μM [based on the concentration of the (−) optical isomer]. This value is lower than the 2 μM reported previously, where initial rates were estimated by constructing tangents to the progress curve (Baggott & Krumdieck, 1979*a*). The difference may be explained by the fact that the initial rates used here were estimated by extrapolation to zero product concentration as described by Waley (1981). Examples of this method are shown in Fig. 2. Linear regression of the rate versus corresponding product concentration always yielded lines with correlation coefficients greater than 0.95 and frequently greater than 0.975. Analysis of the progress curve by this method also indicated that there was product inhibition. Application of the method of Selwyn (1965) ruled out possible denaturation of the enzyme during the assay. Constructing tangents in order to estimate initial rates is inaccurate, especially when there is product inhibition and a considerable portion of the progress curve must be utilized (Atkins & Nimmo, 1978); this leads to inaccurate estimates of K_m.

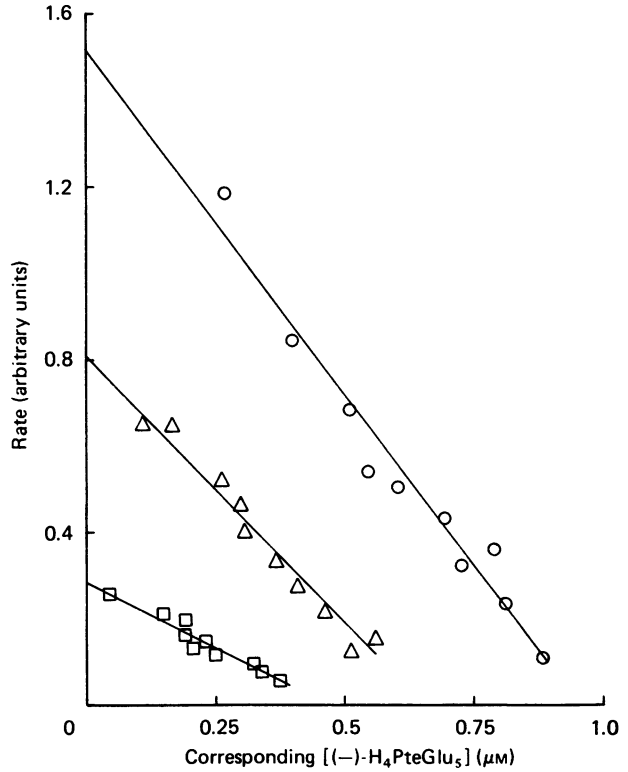

Fig. 2. Determination of initial rates by the method of Waley (1981) of the AICAR transformylase-catalysed reaction

Three examples of this method are given. The rates plotted versus the corresponding product concentration, [(−)-H_4PteGlu$_5$], are shown for initial substrate concentration of 1.25 μM-(−)-10-HCO-H_4PteGlu$_5$ in the presence of zero (○), 20 (△), and 80 (□) μM-MTXGlu$_3$. The lines were calculated by linear regression, extrapolated to zero product concentration to obtain initial rates and have correlation coefficients of 0.980 (○), 0.988 (△) and 0.965 (□).

At a concentration of 80 μM, neither MTX nor MTXGlu$_3$ inhibited IMP cyclohydrolase activity, when assayed with formyl-AICA ribotide at a concentration of 5–25 μM. In addition, MTX and MTXGlu$_3$ at the above concentrations did not affect the $K_{m,app.}$ for AICA ribotide, and at a concentration of 200 μM-AICA, ribotide was saturating. Thus the inhibition of this enzyme by these substances appears to be specific for the folate-dependent site.

Inhibition of AICAR transformylase by 10-CHO-PteGlu$_5$, PteGlu$_5$ and 7,8-H_2PteGlu$_5$: 10-CHO-7,8-H_2PteGlu$_5$ as a substrate for the transformylase

White & Goldman (1976) have demonstrated that Ehrlich ascites-tumour cells incubated with [^{3}H]dihydrofolate accumulated dihydrofolate (and other radiolabelled compounds) when the cells were treated with methotrexate. Saleh *et al.* (1981) found that rats which were given MTX after equilibration with radiolabelled folate excreted greater amounts of folic acid (and other products of the oxidation of both dihydrofolate and tetrahydrofolate) when compared with non-MTX-treated animals. Moran *et al.* (1975) demonstrated that L1210 cells incubated with [^{3}H]folic acid and MTX accumulated polyglutamates of dihydrofolate and possibly folic acid.

Polyglutamyl folates, which could accumulate in MTX-treated cells, were tested as inhibitors of the transformylase. Both 10-HCO-PteGlu$_5$ and Pte-Glu$_5$ were found to be competitive inhibitors of the enzyme with K_i values of 1.37 ± 0.24 and 0.087 ± 0.011 μM respectively. When 10-HCO-7,8-H_2PteGlu$_5$ was tested as an inhibitor, there was an increase in A_{312} that was completely dependent on both the presence of the enzyme and AICA ribotide. This change in absorbance occurred in the absence of reducing agents and strongly suggested that the transformylase was utilizing 10-HCO-7,8-H_2PteGlu$_5$ as a substrate. In order to confirm these results, the disappearance of AICA ribotide was assayed by using the modified Bratton–Marshall test (Flaks & Lukens, 1963) and IMP was separated from the reaction mixture by Sephadex G-10 chromatography as shown in Fig. 3. The $\Delta\epsilon$ for the conversion of 10-HCO-7,8-H_2PteGlu$_5$ into H_2PteGlu$_5$ was found to be 8700 M·cm^{-1} (Fig. 5 below). The apparent K_m of the transformylase for 10-HCO-7,8-H_2PteGlu$_5$ was found to be 0.51 ± 0.12 μM and the $V_{max.}$ was 3.1 μmol/min per mg of protein or 72% of the $V_{max.}$ when the enzyme was assayed with 10-HCO-H_4PteGlu$_5$. Thus these folate coenzymes bearing a 10-formyl substituent and the pteridine ring at either the tetrahydro or the dihydro state of oxidation are good coenzymes for the transformylase. This result was unexpected, since the tetrahydro state of oxidation of the pteridine ring is widely assumed to be required for folate-dependent enzyme-catalysed one-carbon transfer reactions. However, it is unlikely that this dihydro coenzyme is an important formyl-group donor *in vivo*, since 10-formyltetrahydrofolate synthetase will not utilize dihydrofolate (Himes & Harmony, 1973). In contrast with the above, when it was assayed with 40 μM-10-HCO-PteGlu$_5$ the specific activity of the transformylase was 0.017 μmol/min per mg of protein or only 0.4% of the activity observed when the corresponding tetrahydro coenzyme was used. Thus 10-HCO-PteGlu$_5$, a folate with a fully oxidized pteridine ring, is a relatively poor coenzyme.

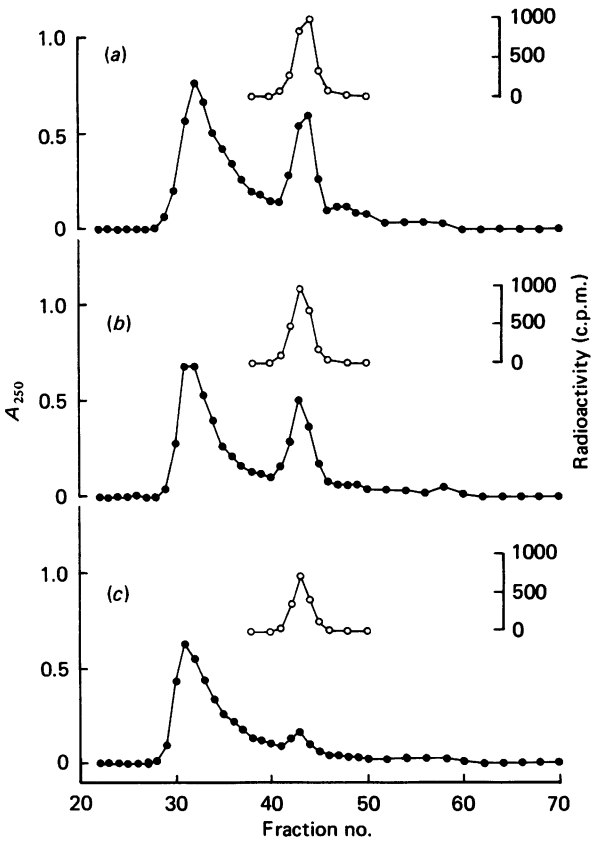

Fig. 3. Separation of IMP from AICAR transformylase-catalysed reaction with 10-HCO-7,8-H_2PteGlu$_5$ 10-HCO-H_4PteGlu$_5$ and 10-HCO-PteGlu$_5$ as formyl donors

The enzyme-catalysed conversion of AICA ribotide into IMP utilizing 10-HCO-7,8-H_2PteGlu$_5$ as the coenzyme was established as follows. The reaction cuvette contained 300 nmol of 10-HCO-7,8-H_2PteGlu$_5$, 600 nmol of AICA ribotide and 0.13 μg of the transformylase in 3 ml of a 0.082 M-potassium phosphate buffer, pH 7.4. The increase in absorbance at 312 nm was monitored and after 100 min the reaction was terminated by the addition of 0.3 ml of 30% (w/v) trichloroacetic acid. The reference cuvette had all components of the assay except for the transformylase, which was added immediately before the addition of the trichloroacetic acid. The modified Bratton–Marshall assay was then performed on the solutions in both the reaction and reference cuvette. After determining the amount of AICA ribotide present, the pH of the Bratton–Marshall-assay reaction cuvette was adjusted pH 7.4, the mixture concentrated to 1 ml and chromatographed on a column (1 cm × 110 cm) of Sephadex G-10 with 10 mM-potassium phosphate, pH 7.4, as the elution buffer. Before loading the sample on to the column, 0.25 nmol (0.01 μCi) of [8-^{14}C]IMP was added as a marker. Fractions (1 ml) were collected, the A_{250} measured and 0.2 ml of each fraction was counted for radioactivity in a scintillation counter (*a* and inset). An identical protocol was carried out with (±)-10-HCO-H_4PteGlu$_5$ as the coenzyme (*b*) and 10-HCO-PteGlu$_5$ as a control (*c*), except that 30 μmol of 2-mercaptoethanol were added to the reaction and reference cuvettes when the tetrahydrofolate coenzyme was employed. With the tetrahydro- and dihydro-folate coenzymes the rate of change in the absorbance at 312 nm at the end of the incubation period was less than 3% of the initial rate. After the incubation period, 144, 114 and 21 nmol of AICA ribotide were consumed when 10-HCO-7,8-H_2PteGlu$_5$, 10-HCO-H_4PteGlu$_5$ and 10-HCO-PteGlu$_5$ were employed respectively, whereas the total change in the absorbance at 312 nm was 0.41 and 0.45 when utilizing the dihydro- and tetrahydro-folate coenzymes respectively. The amount of IMP produced was estimated by measuring the area under the peak and using an ϵ value of 12000 M$\cdot$cm^{-1} at 250 nm and was found to be 140 and 115 nmol when 10-HCO-7,8-H_2PteGlu$_5$ and 10-HCO-H_4PteGlu$_5$ were employed respectively. The average $\Delta\epsilon$ for the transformylase-catalysed reaction was 8700 M$\cdot$cm^{-1} and 12000 M$\cdot$cm^{-1} when utilizing the dihydro- and tetrahydro-folate coenzymes respectively.

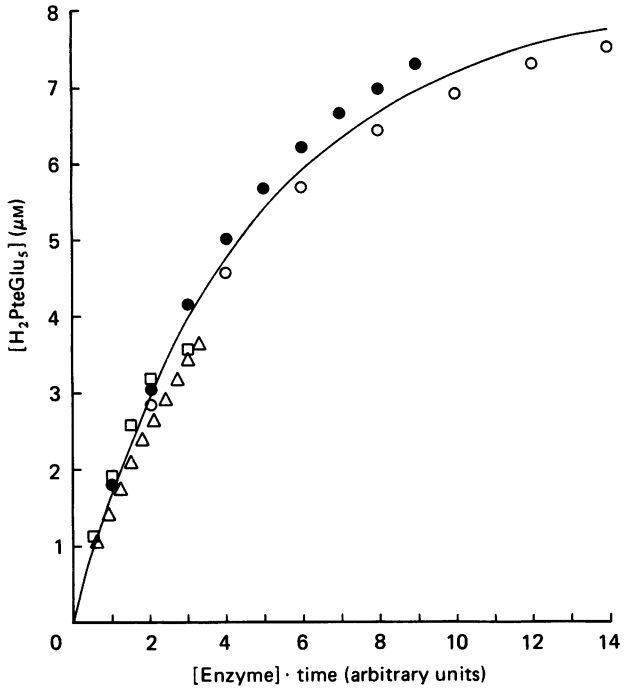

Fig. 4. Test for inactivation of AICAR transformylase by the method of Selwyn (1965)

The initial substrate concentrations were 12 μM-10-HCO-7,8-H_2PteGlu$_5$ and 200 μM-AICA ribotide in 0.082 M-potassium phosphate, pH 7.4 at 37 °C. The A_{312} was monitored and converted into concentrations of H_2PteGlu$_5$ by using a $\Delta\epsilon$ of 8700 M$\cdot$cm^{-1} which is plotted versus the enzyme concentration multiplied by the time. Relative amounts of enzyme used are 1 (△), 1.67 (□), 3.33 (●) and 6.67 (○). The reactions were monitored from 5 to 11 min.

An analysis of the progress curve utilizing 10-HCO-7,8-H_2PteGlu$_5$ in the transformylase-catalysed reaction suggested that there was product inhibition. In order to test for denaturation of the enzyme during the assay period, the method of Selwyn (1965) was employed and indicated that denaturation could not account for the fall in the rate (Fig. 4). Denaturation of the enzyme would be indicated by non-overlapping curves in Fig. 4. When IMP (one of the products) was added at a concentration of 100 μM, no inhibition was detected when the enzyme was assayed with 10 μM-10-HCO-7,8-H_2PteGlu$_5$. Thus the inhibition of the transformylase was due to the production of H_2PteGlu$_5$ (the other product) and an analysis of the progress curve was utilized to estimate the K_i of H_2PteGlu$_5$, since this substance would be difficult

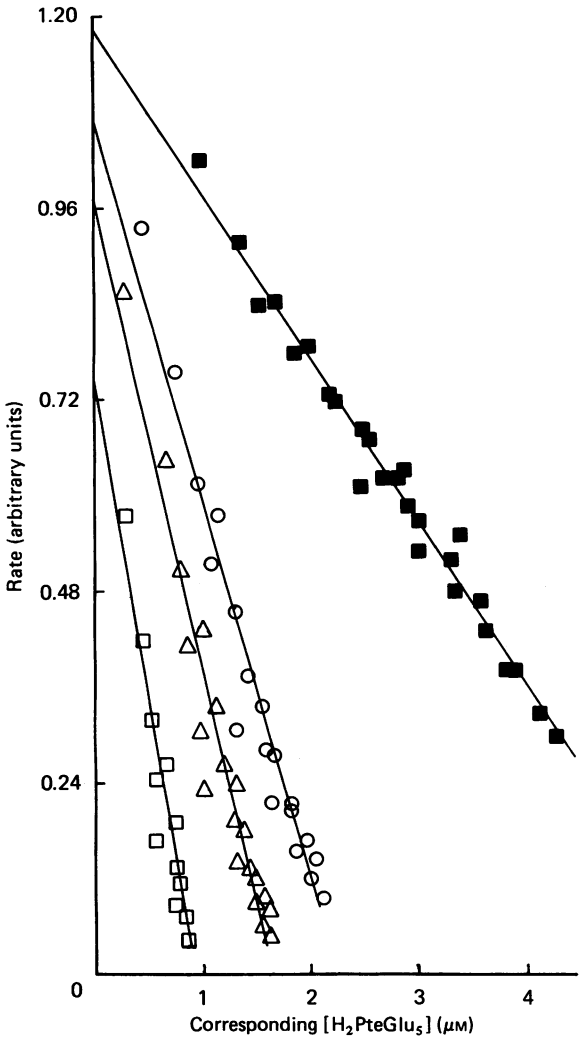

Fig. 5. Application of the method of Waley (1981) to determine initial rates of the AICAR transformylase-catalysed reaction utilizing 10-HCO-7,8-H_2PteGlu$_5$ as a formyl donor

Examples of this method are shown. The rate is plotted versus the corresponding product concentration, [H_2PteGlu$_5$], for initial substrate concentrations of 1.4 (□), 3 (△), 4 (○) and 10 (■) μM-10-HCO-7,8-H_2PteGlu$_5$. Lines were drawn using linear regression, and correlation coefficients were 0.952 (□), 0.959 (△), 0.977 (○) and 0.990 (■).

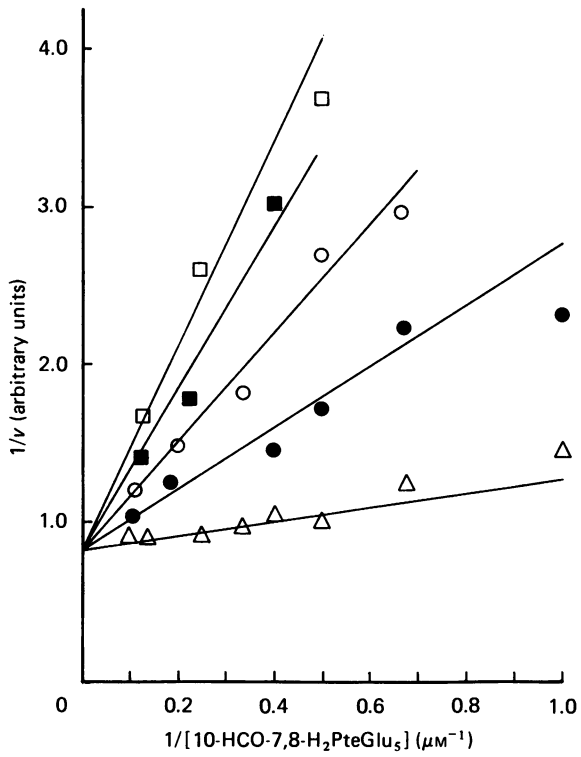

Fig 6. Inhibition of AICAR transformylase by H_2PteGlu$_5$

Double-reciprocal plots are shown. The rates were estimated in the presence of corresponding H_2PteGlu$_5$ concentrations of zero (△), 0.5 (●), 1.0 (○), 1.5 (■) and 2.0 (□) μM by the method of Waley (1981).

to prepare in pure form. Rates and corresponding substrate and product (inhibitor) concentrations were estimated by using the method of Waley (1981) (examples are shown in Fig. 5). These data sets (one set from each enzyme-catalysed reaction) were then used to estimate the K_i by Cleland's (1979) program (Fig. 6). H_2PteGlu$_5$ was found to be a competitive inhibitor with a K_i of 0.14 μM. Waley's (1981) method is only strictly applicable to a one-substrate one-product enzyme-catalysed reaction. However, under the assay conditions, the AICA ribotide concentration is saturating, is much higher than the concentration of the folate coenzymes, and essentially remains constant during the reaction. Therefore, the experimental conditions were chosen to closely approximate a one-substrate one-product reaction where this type of analysis is valid (Orsi & Tipton, 1979). Finally, Atkins & Nimmo (1973) have obtained reliable kinetic parameters using a linear fit to the integrated Michaelis–Menten equation provided that the initial substrate concentration is greater than its K_m. Waley's (1981) method also utilizes an integrated form of this equation, and experimental conditions were chosen so that the initial concentrations of 10-HCO-7,8-H_2PteGlu$_5$ were greater than its apparent K_m.

The K_i values for these oxidized and dihydrofolate folates for the transformylase are of the same order of magnitude as the K_m for the folate coenzymes bearing the same number of γ-linked glutamic acid residues. Thus a relatively small conversion of polyglutamyltetrahydrofolate coenzymes to polyglutamyl dihydro or oxidized folates could have an anti-purine effect greater than that predicted by the depletion of fully reduced coenzymes alone. In support of this hypothesis, Moran (1982) observed that deoxyuridine incorporated into DNA is inhibited by 90%, whereas the pool of the tetrahydrofolate coenzymes decreased only 5% in MTX-treated Lewis lung cells. Jackson & Grindey (1984) suggested that inhibition of thymidylate synthase by increased levels of polyglutamates of dihydrofolate would be one mechanism that could account for these results. The relatively low K_i values for H_2PteGlu$_5$ and the oxidized polyglutamyl folates would make it feasible for an analogous mechanism to inhibit the transformylase.

Inhibition of adenosine deaminase and AMP deaminase by AICA riboside and ribotide

We hypothesized that AICA riboside was an inhibitor of mammalian adenosine deaminase, and in order to test this hypothesis, we intended to use the continuous spectrophotometric assay developed by Kalckar & Shafran (1947). However, Murphy *et al.* (1982) reported large negative deviations from Beer's law when this assay was employed and when initial adenosine concentrations above 100 μM were used and concluded that this assay was invalid above this concentration. If correct, these results would have precluded the use of this assay to determine the K_i for AICA riboside. Therefore the $\Delta\epsilon_{265}$ for the conversion of adenosine into inosine was measured as a function of the adenosine concentration and in the presence of AICA riboside in order to verify the results of Murphy *et al.* (1982). As shown in Fig. 7, $\Delta\epsilon$ decreased from 8700 M·cm^{-1} to 8300 M·cm^{-1} (a 4.6% decrease) when the initial adenosine concentration was raised from 10 to 500 μM, whereas Murphy *et al.* (1982) reported a decrease from about 8000 M·cm^{-1} to about 500 M·cm^{-1} over this concentration range and attributed these results to aggregation of adenosine in solution. The modest 4.6% decrease in $\Delta\epsilon$ that we report here is only an apparent negative deviation from Beer's law, since a decrease in the bandwidth of the incident beam on the spectrophotometer lessened this deviation but resulted in increased noise. As a compromise, a bandwidth of 0.6–1.4 nm was chosen to minimize the apparent deviation from Beer's law with tolerable noise levels. Thus, in the presence of 800 μM-AICAR riboside, which required a relatively high incident-beam bandwidth, the apparent negative deviation from Beer's law is more pronounced (see Fig. 7). The dramatic negative deviations from Beer's law reported by Murphy *et al.* (1982) are apparent, are not a result of adenosine aggregation and are artefacts probably resulting from relatively high incident-beam bandwidths. This spectrophotometric assay is valid provided that small negative deviations in Beer's law can be ignored or corrected for.

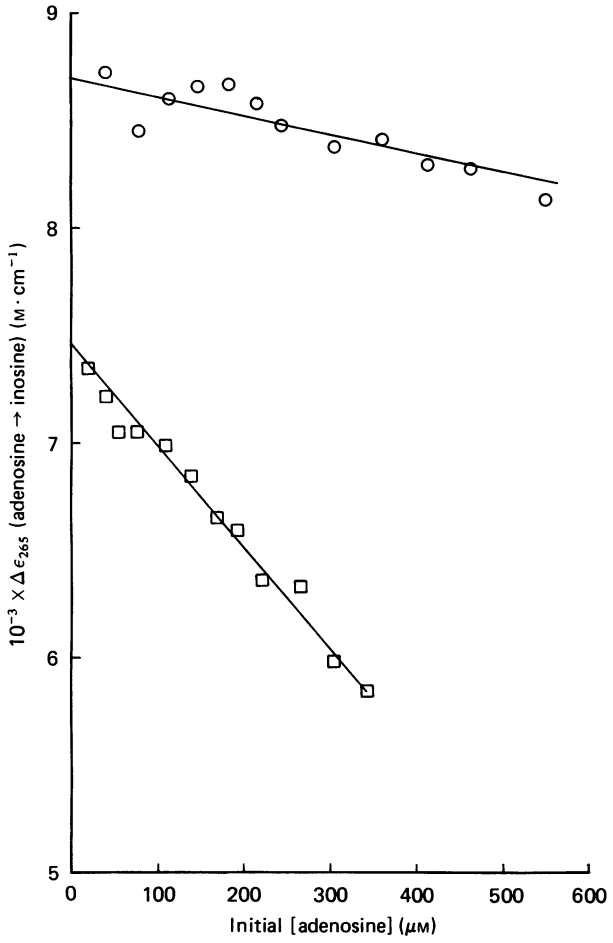

Fig. 7. $\Delta\epsilon$ at 265 nm for the conversion of adenosine into inosine as a function of initial adenosine concentration

The molar absorption coefficients for adenosine and inosine were measured in 50 mM-potassium phosphate buffer, pH 7.4 (25 °C) in the presence (□) or absence (○) of 800 μM-AICA riboside. The $\Delta\epsilon$ values were obtained by subtraction. The bandwidth of the incident beam of the spectrophotometer was either 0.6 nm (○) or 1.4 nm (□). The conversion of adenosine into inosine was also determined as a function of initial adenosine concentration in the presence of 200, 400 and 600 μM-AICA riboside.

AICA riboside is a competitive inhibitor of adenosine deaminase. AICA ribotide did not inhibit this enzyme. The K_i for AICA riboside was 362 ± 54 μM, which is higher than the K_m of 17.4 ± 1.7 μM for adenosine. However, Tomisek *et al.* (1985*a*,*b*) demonstrated that AICA ribotide and riboside concentrations increase 50–100-fold in MTX-treated intestinal cells, L1210 tumour cells and *E. coli*. Thus this relatively high K_i/K_m ratio may still have physiological significance. Kuramitsu *et al.* (1964) estimated a K_i of 90 μM for AICA riboside with the deaminase from a purine-requiring mutant of *E. coli* and reported that AICA riboside added to the media inhibited the growth of this bacterium.

AMP deaminase is an enzyme of purine interconversion that is metabolically related to adenosine deaminase. AICA ribotide is a competitive inhibitor of this enzyme, with a K_i of 1.01 ± 0.19 μM, whereas the K_m for AMP was 0.343 ± 0.025 mM, in general agreement with a previously reported K_m value (Smiley *et al.*, 1967). Thus the K_i is only 3-fold higher than the K_m and is likely to be physiologically important. Again, the inhibition was specific; AICA riboside did not inhibit AMP deaminase.

Borsa & Whitmore (1969*b*) demonstrated that adenosine and deoxyadenosine potentiated the cytotoxicity of MTX in L-cells, whereas guanosine and deoxyguanosine were much less effective potentiators. Taylor *et al.* (1982) and Tattersall *et al.* (1974) found that dATP concentrations in a variety of tumours increased after MTX treatment. Deoxyadenosine in the absence of MTX was not toxic to tumour cells; however, when an inhibitor of adenosine deaminase was also added, deoxyadenosine was toxic (Borsa & Whitmore, 1969*b*; Taylor *et al.*, 1982). Finally, Hryniuk (1972) reported that L5178Y cells were substantially protected from MTX toxicity when hypoxanthine was added to the media, whereas Tattersall *et al.* (1974) demonstrated that deoxyadenosine by itself did not protect this cell line from MTX toxicity.

The above evidence suggests that MTX treatment blocks the conversion of adenosine, deoxyadenosine and their nucleotides into their corresponding inosine compounds. The adenine nucleotides and nucleosides are relatively toxic because dATP is an inhibitor of ribonucleotide reductase, whereas the nucleosides are inhibitors of *S*-adenosylhomocysteine hydrolase, an enzyme required for the efficient utilization of *S*-

adenosylmethionine (Martin & Gelfand, 1981). The accumulation of AICA riboside and ribotide, with the resulting inhibition of adenosine deaminase and AMP deaminase, would be one mechanism allowing MTX treatment to trap purines as adenine nucleosides and nucleotides.

Conclusion

The primary mechanism of action of MTX is inhibition of dihydrofolate reductase. However, MTX treatment of cells sensitive to the drug unavoidably results in (1) the metabolic conversion of MTX into polyglutamates, (2) the accumulation of dihydro and oxidized folate polyglutamates and (3) the accumulation of AICA ribotide and riboside. The above metabolic events may be integral elements in the production of the cytotoxic effect of this drug by (1) producing tighter binding of MTX to folate-dependent enzymes other than dihydrofolate reductase, (2) producing inhibitors of folate-dependent enzymes from their tetrahydrofolate coenzymes and (3) trapping toxic levels of adenine nucleosides and nucleotides as a result of inhibition of adenosine deaminase and AMP deaminase respectively.

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