A Chemical Model for the Thymidylate Synthetase Catalysed Methylation of Deoxyuridine Monophosphate [1]

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In a study of a model for the reaction catalysed by the enzyme thymidylate synthetase, a compound previously thought to be the putative intermediate 5-(uracil-5-ylmethyl)tetrahydropteroylglutamic acid (4b) has been prepared and shown to be the 5,10-bis(uracil-5-ylmethyl) adduct (4c). The tetrahydrofolic acid adduct (4d) and its [6-$^2H_1$] analogue have been prepared and have been shown to be thermally converted into thymine (5, R^2 = H). The reaction with deuteriated (4d) shows a high discrimination for the hydrogen at C-6 of the pteridine moiety which rearranges to the methyl group of the resultant thymine (5, R^2 = H). These findings parallel results found for the biochemical process very closely and possible mechanisms for the reaction are discussed. The compounds (4c) and (4d) are neither substrates nor inhibitors of thymidylate synthetase.

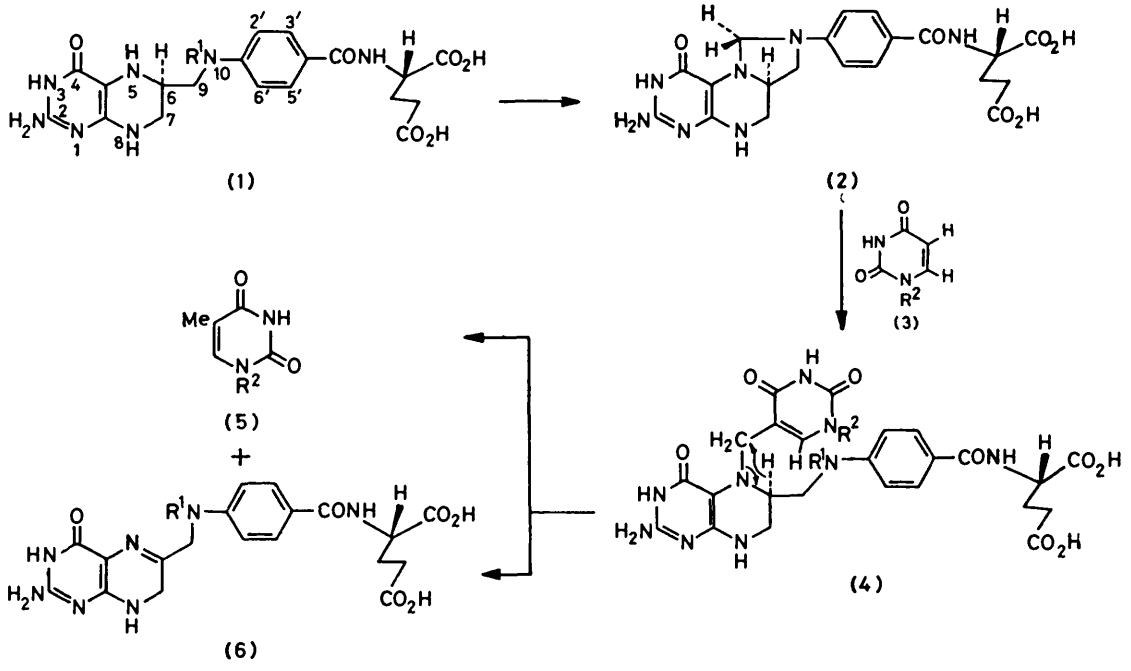
THE RNA base uracil (3; R^2 = H) is converted into the DNA base thymine (5; R^2 = H) by methylation at C-5. This process is vital to the synthesis of DNA and, for this reason, the enzyme which effects the methylation, thymidylate synthetase, is a useful target enzyme in cancer chemotherapy.[2] It has long been recognised [3] that the coenzyme 5,6,7,8-tetrahydrofolic acid (1; R^1 = H) is involved with thymidylate synthetase in the methylation process, both as a one-carbon transfer agent and as a reductant. The one-carbon transfer involves 5,10-methylene-5,6,7,8-tetrahydrofolate (2) rather than 5-methyl-5,6,7,8-tetrahydrofolate which is the more usual substrate for one-carbon transfers at the methanol oxidation level.[4] To achieve the correct oxidation level in the product (5; R^2 = H) the tetrahydrofolate serves as a reductant, being oxidised to 7,8-dihydrofolic acid (6; R^1 = H) and transferring the hydrogen at C-6 of the pteridine ring exclusively to the methyl group of thymine (5; R^2 = H) in the process.[5,6] The methylation is made self-sufficient in the coenzyme 5,6,7,8-tetrahydrofolic acid (1; R^1 = H) by reduction of the 7,8-dihydrofolic acid (6; R^1 = H) formed, by NAD(P)H and the enzyme dihydrofolate reductase. It is the involvement of the dihydrofolate reductase in the methylation process which makes it also a target enzyme in cancer chemotherapy.

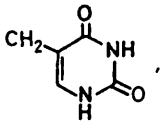
The question of a valid chemical mechanism for this enzymic methylation has excited much speculation, and in 1959 Friedkin revised his earlier [7] theory by suggesting [8] that 2′-deoxyuridine-5′-phosphate (3; R^2 = 2-deoxyribose-5-phosphate) acts as a enamine, so that C-5 of the pyrimidine reacts as a nucleophile with the methylene bridge of the 5,10-methylene-5,6,7,8-tetrahydrofolic acid (2) to give the adduct (4a). This reaction is analogous to the Mannich reaction and uracil is known to undergo Mannich reactions with formaldehyde and secondary amines.[9] The adduct (4a) must now undergo a net 1,3-hydride shift, as shown in Scheme 1, to complete the synthesis of thymidine-5′-phosphate (5; R^2 = 2-deoxyribose-5-phosphate).

The publication of this mechanism stimulated work on chemical models for the process, and two models have been devised using compounds in which dihydroquinoline [10] and tetrahydroquinoxaline [11] replace the tetrahydrofolate moiety in the adduct (4b). This work proved very interesting but we felt that, for a more accurate assessment of the viability of the mechanism as a chemical process, synthesis of the putative intermediate (4b) itself would be required. This would also provide a compound which could be used in feeding experiments to assess the validity of the scheme for the enzymic process itself.

Gupta and Huennekens have reported [12] that the adduct (4b) can be obtained by treating 5,6,7,8-tetrahydrofolic acid (1; R^1 = H) with a two-fold molar excess of 5-chloromethyluracil. The intermediate (4b) therefore seemed to be readily accessible for use in chemical model studies. It was unlikely that pyrolysis of the adduct (4b) would involve a *concerted* thermal 1,3-hydride shift to yield compounds (5) and (6), as in Scheme 1, on the grounds of conservation of orbital symmetry.[13] There was, however, an alternative thermally-allowed pathway from the intermediate (4b) to thymine (5; R^2 = H) and 7,8-dihydrofolic acid (6; R^1 = H) which would result in the hydrogen at C-6 in the intermediate (4b) being delivered entirely to the methyl carbon of thymine (5; R^2 = H). This is outlined in Scheme 2. A thermally-allowed concerted retro-ene reaction [14] would first yield 7,8-dihydrofolic acid (6; R^1 = H) and the intermediate (7). The hydrogen which was at C-6 in the adduct (4b) would now be delivered to C-6 of the pyrimidine (7). A stereospecific enzyme-catalysed prototropic shift is then required to complete the process, finally delivering the hydrogen which had been at C-6 of compound (4b) entirely to the methyl group of thymine (5; R^2 = H). It is evident that this second step would not be stereospecific in a non-enzymic model so that, if the hydrogen at C-6 of the adduct (4b) were labelled by an isotope, this label would appear both at the methyl group and at C-6 of thymine (5; R^2 = H) in a ratio determined by the isotope effect.

To investigate a chemical model for the methylation of deoxyuridine-5′-phosphate by the enzyme thymidylate synthetase, we decided to prepare the intermediate (4b) by the method of Gupta and Huennekens [12] with a view



a; R^1 = H, R^2 = 2-deoxyribose-5-phosphate
b; R^1 = R^2 = H
c; R^1 =  , R^2 = H
d; R^1 = Me, R^2 = H

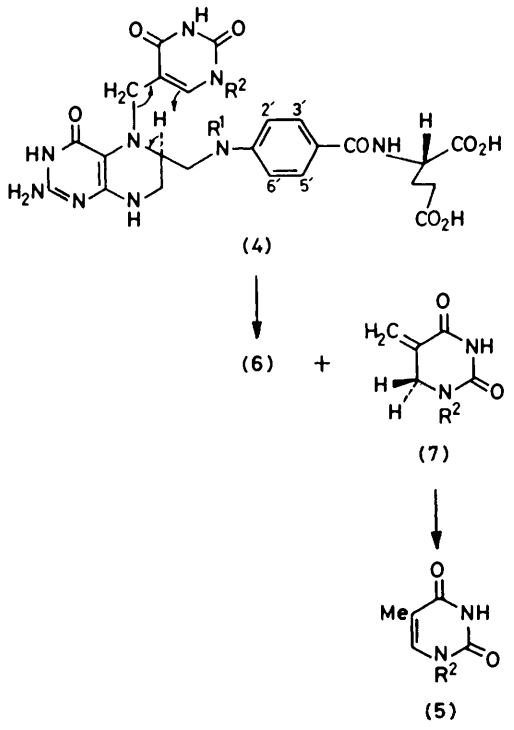
SCHEME 1

to investigating its thermal conversion into thymine. We therefore prepared 5,6,7,8-tetrahydrofolic acid (1; R^1 = H) by reduction of 7,8-dihydrofolic acid (6; R^1 = H) [15] with sodium borohydride. The pH was adjusted to 0—1 to destroy the excess of borohydride and the tetrahydrofolate (1; R^1 = H) was alkylated *in situ* at pH 7.5 with *ca.* two mol equiv. of 5-chloromethyluracil. Although Gupta and Huennekens [12] had purified their product by chromatography on DEAE-cellulose, in our hands preliminary experiments showed this to be unnecessary and indeed led to partial degradation of the product. When the product of the reaction was purified by crystallisation, it had u.v. spectra at acid, neutral, and basic pH which were identical in every way with those reported [12] for the compound (4b) by Gupta and Huennekens. The compound ran as a single ' quenching ' spot on paper chromatography and cellulose t.l.c. Combustion analysis fitted a formula $C_{24}H_{27}N_9O_8 \cdot 4H_2O$ which was in keeping with the structure (4b) suggested for the compound. These figures were, however, also coincidentally in agreement with the formula $C_{29}H_{31}N_{11}O_{10} \cdot 4H_2O$ which would indicate that an adduct had been formed by reaction of *two* molecules of chloromethyluracil with one molecule of 5,6,7,8-tetrahydrofolic acid. ^{1}H N.m.r. spectroscopy and mass spectrometry indicated that in fact the product was such a bis-adduct, in agreement with the structure (4c) for the compound. The ^{1}H n.m.r. spectrum showed, in addition to the typical resonances due to the tetrahydrofolic acid (1; R^1 = H), two resonances for the uracil C-6 protons, one as a singlet at δ 7.05 and the other as a doublet (J 1.8 Hz) at δ 7.79. This latter splitting may be due to long range coupling with one of the bridging CH_2 protons. The bridge CH_2 protons were present in the spectrum as a clear two-proton AB system centred at δ *ca.* 3.6 and a further two protons overlapping with the α-CH of the glutamate moiety at δ *ca.* 4.2. Field desorption mass spectrometry showed a major ion at *m/e* 694 ($C_{29}H_{31}N_{11}O_{10}$ requires *M*, 693.64).

It is necessary therefore to revise the structure (4b), suggested by Gupta and Huennekens [12] for their product, to the structure (4c). They [12] noted, however, that further alkylation of their ' adduct (4b) ' gave a compound to which they assigned the structure (4c). When the compound (4c) was reacted further with chloromethyl uracil, however, a product was obtained with a u.v. spectrum similar to that reported by Gupta and Huennekens.[12] This seemed to be a trialkylated product since the ^{1}H n.m.r. spectrum exhibited three aromatic protons, in addition to signals for 2', 3', 5', and 6'-

H, and there were additional protons in the δ 3.0—4.4 region corresponding to the three methylene bridges.

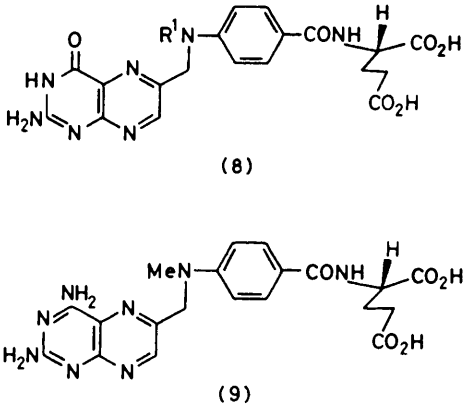
Although the product of alkylation of the tetrahydrofolic acid (1; R^1 = H) had proved to be the *bis*-adduct (4c) rather than the putative enzyme intermediate (4b), it was of interest to study the pyrolysis of this compound since it had the structural features necessary for the proposed mechanism. Although Gupta and Huennekens [12] noted that the compound was stable to heating to 100 °C



SCHEME 2

at pH 7 for 30 min, when we heated the compound as a solid at 250—275 °C for 5 h *in vacuo*, a solid sublimed out of the reaction in 39% yield. This proved on crystallisation to be identical in every respect with an authentic sample of thymine. Preparation of the [6-$^2H_1$]-analogue of the adduct (4c) was then effected by substituting sodium borodeuteride for sodium borohydride in the synthesis. This compound contained *a minimum of* 60% of one deuterium atom at C-6 of the reduced pteridine moiety (by integration of the ^1H n.m.r. spectrum) and when it was pyrolysed at *ca.* 270 °C for 6 h *in vacuo* a 46% yield of thymine was obtained. This was shown to be monodeuteriated to the extent of 29.1% by mass spectrometry, and the ^1H n.m.r. spectrum suggested that most of the deuterium was in the methyl group. The ^2H n.m.r. spectrum was much more revealing, however, showing absorption *only* in the methyl group, with no deuterium residing at C-6 of thymine.

Although encouraging, these results were made difficult to interpret by the presence of the second thyminyl residue at N-10 of compound (4c). Attempts to control alkylation of the tetrahydrofolate to obtain the monoadduct (4b) proved unsuccessful, with either no alkylation or formation of the bis-adduct (4c) occurring. It seemed necessary therefore to protect N-10 of the tetrahydrofolate at either the folic acid (8; R^1 = H) or 7,8-dihydrofolate (6; R^1 = H) oxidation level prior to



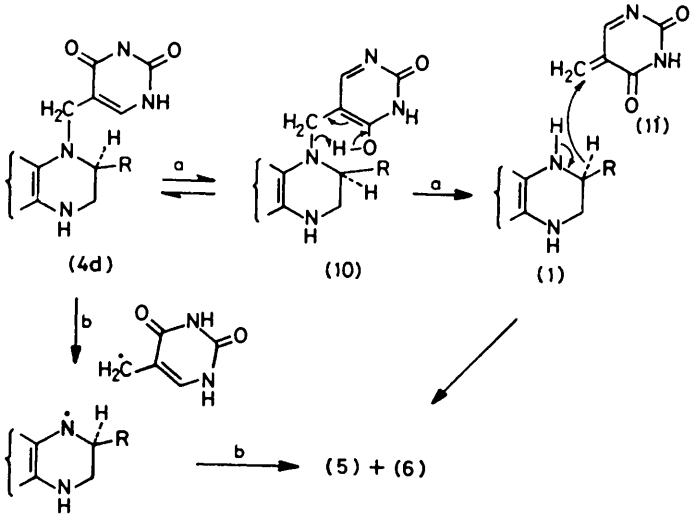
reduction and alkylation. Attempts to achieve this with ' removable ' protecting groups failed and so we settled for the preparation of the methyl analogue (4d) using the cancer drug methotrexate (9) as our starting point.

Methotrexate (9) was hydrolysed to 10-methylfolic acid (8; R^1 = Me) using 1M-sodium hydroxide,[16] and this was reduced to 10-methyl-7,8-dihydrofolic acid (6; R^1 = Me) with sodium dithionite.[16] The u.v. spectra of both compounds were in keeping with the literature [16] and the ^1H n.m.r. spectra were analogous to those of folic acid (8; R^1 = H) and 7,8-dihydrofolic acid (6; R^1 = H) [17,18] respectively, but with additional three proton singlets for the N-10 methyl group. Reduction of 10-methyl-7,8-dihydrofolic acid (6; R^1 = Me) with sodium borohydride, followed by *in situ* alkylation with 5-chloromethyluracil, was carried out in the same way as for the corresponding reaction with tetrahydrofolic acid (1; R^1 = H). The product had the analytical and spectroscopic properties expected for the compound (4d), and substitution of sodium borodeuteride for sodium borohydride in the sequence gave the corresponding [6-$^2H_1$]-analogue. This last compound had a ^1H n.m.r. spectrum which showed a loss of integration of *ca.* 0.7 H in the region assigned to the proton at C-6 of the reduced pteridine moiety but otherwise the spectra were similar to those of the parent compound (4d).

We now had a compound which, having only one uracil moiety, and that at N-5, should give unambiguous results in model studies for the thymidylate synthetase reaction. The compound (4d) was therefore pyrolysed at 255—260 °C for 6 h *in vacuo* and yielded 32% of thymine. Pyrolysis of the corresponding [6-$^2H_1$]-analogue at 255—260 °C for 7 h *in vacuo* gave a 47% yield of thymine which was shown to be 25.3% monodeuteriated by mass spectroscopy. The ^2H n.m.r. spectrum showed absorption only in the region of the

thymine methyl group so that once more the label had been transferred to the methyl group and not to C-6.

Although our results were quite clear, and all the samples of compounds (4c) and (4d) used in the pyrolytic studies were pure (by spectroscopic and chromatographic techniques), it was necessary to be absolutely sure that no more than trace amounts of thymine were present in these samples, since these could have arisen by reduction of 5-chloromethyluracil by residual sodium borohydride and deuteride. This was shown to be the case by checking for the thymine methyl signal in the ^1H n.m.r. spectra run at high gain, with and without added thymine.



SCHEME 3

It would now appear that the labelling results in our chemical model closely parallel the results found in the biochemical reaction. The label at C-6 of the pteridine moiety of the intermediate (4d) is transferred to the methyl group and not to C-6 of thymine. This rules out the concerted retro-ene mechanism of Scheme 2 for the process. The relatively high amount of deuterium transferred in the reaction is remarkable considering the high $^1H : {}^2H$ ratio in the starting compound (4d) and the adverse isotope effect. Thus, although the transfer of hydrogen from C-6 of the pteridine moiety of the adduct (4d) to the methyl group of thymine cannot be concerted, it must still be a favoured reaction. This would suggest that some form of cage process is operating. Decomposition of the enolised form (10) of compound (4d), as in Scheme 3a, would lead to the intermediate (11) and 5,6,7,8-tetrahydrofolic acid (1). Reduction of compound (11) by tetrahydrofolate (1) in a cage might then account for the results. An alternative mechanism, shown in Scheme 3b, would involve homolytic cleavage of the N(5)–CH_2 bond followed by radical transfer of the hydrogen at C-6 within a cage. An Arrhenius calculation originally suggested that this was a feasible process with an analogous homolytic bond cleavage having $t_{\frac{1}{2}}$ *ca.* 2 s at 250 °C.[1] This calculation was based on a heat of formation for *N*-(phenylmethylene)aniline of +60.6 kcal [19,*] but there is an error in the Tables [19] on this point and a revised value was estimated as +45.53 kcal (based on heats of formation of *N*-ethylaniline, ethane, and toluene).[19] A revised Arrhenius calculation using this value, values of +55.0 and +45.1 kcal for the heats of formation of the phenylamino and benzyl radicals, respectively,[20] and assuming [21] $\log_{10}A = 16$, gave a value for $t_{\frac{1}{2}}$ for radical formation of the order of 1 200 h. This does not support a radical process, and suggests that a heterolytic process is more likely.

It is evident that the conditions used in our model reaction are more drastic than those for the enzyme reaction, but it is of some interest that the chemical reaction occurs and that the labelling results follow the enzymic results so closely. The synthetic problems encountered in our work meant that we had neither compound (4a) nor (4b) to test as possible substrates for the enzymic reaction. It was, however, possible to test the compounds (4c) and (4d) as potential substrates for, or inhibitors of, thymidylate synthetase using the increase in the molar absorption coefficient at $\lambda_{max.} = 340$ nm, due to the formation of 7,8-dihydrofolic acid (6; $R^1 = H$),[22] as a measure of thymidylate synthetase activity. Neither compound (4c) nor (4d) could substitute for the tetrahydrofolate, dUMP, and formaldehyde as substrates for the enzyme. Furthermore, although 10-methyl-5,6,7,8-tetrahydrofolic acid is known to inhibit the enzyme,[23] neither compound (4c) nor (4d) caused any inhibition of the enzyme.

EXPERIMENTAL

^1H N.m.r. spectra were run on Bruker HFX-90, Varian T60, and Perkin-Elmer R32 instruments. For spectra in [$^2H_2$]water solutions, samples were pre-treated by dissolution in [$^2H_2$]water and lyophilisation. ^2H N.m.r spectra were recorded on a Jeol FX 90Q spectrometer. U.v. spectra were recorded on Pye-Unicam SP800, SP500, and SP1800 spectrophotometers, and i.r. spectra on a Perkin-Elmer 577 spectrometer. Electron impact mass spectra were obtained on an AE1-MS30 instrument and field desorption mass spectra were obtained on Varian MAT 731, VG 70/70F, and AEI-MS50 spectrometers. T.l.c. was carried out on cellulose (thickness 0.1 mm; Polygram Cell 300 UV_{254}, supplied by Camlab). Thymidylate synthetase was purchased from The New England Enzyme Center, Boston, Mass., U.S.A.

5,10-*Bis*(*uracil-5-ylmethyl*)*tetrahydropteroylglutamic Acid* (4c).—Sodium borohydride (1 g, 26.4 mmol) was added to a solution of 7,8-dihydropteroylglutamic acid (6; $R^1 = H$) [15] (1 g, 2.25 mmol) in deaerated water (70 ml) and stirred for 15 min at room temperature under nitrogen. The solution was acidified to pH 0—1 with concentrated hydrochloric acid, and after 2 min the pH was readjusted to 7.5 with 1M-aqueous sodium hydroxide. The solution was cooled in an ice-bath and a suspension of 5-chloromethyluracil (0.7 g, 4.36 mmol) in deaerated dioxan (70 ml) was added. The reaction mixture was stirred for 35 min under nitrogen, mercaptoethanol (*ca.* 0.5 ml) was added, and the mixture was lyophilised to dryness. The crude product was dissolved in deaerated water (100 ml) under nitrogen and the

* 1 kcal is 4.184 kJ.

pH was adjusted to *ca.* 9 to effect dissolution. The solution was filtered and the pH readjusted to 3.5 with 2M-aqueous acetic acid. After 90 min in an ice-bath under nitrogen the cream-coloured precipitate was collected by centrifugation at *ca.* 2 °C, washed successively with ice-cold deaerated water (20 ml), ice-cold deaerated water–methanol (1 : 1; 15 ml), and ice-cold methanol (15 ml), and dried *in vacuo* at room temperature. The precipitation/washing sequence was repeated twice to yield the product (4c) (450 mg, 29%), *m/e* (field desorption) 649 (M^+) (Found: C, 45.5; H, 5.1; N, 19.9. $C_{29}H_{31}N_{11}O_{10}\cdot 4H_2O$ requires C, 45.5; H, 5.1; N, 20.1%); $\lambda_{max.}$ (pH 7, 0.1M-phosphate buffer) 275sh and 301 nm (ε 22 600 and 25 000); $\lambda_{max.}$ (pH 1) 267 and 292sh nm; $\lambda_{max.}$ (pH 13) 293 nm [lit.,[12] for the compound assumed to be (4b), $\lambda_{max.}$ (pH 7) 275sh and 302 nm (ε 24 900 and 29 200); $\lambda_{max.}$ (pH 1) 266 and 293sh nm; $\lambda_{max.}$ (pH 13) 293 nm]; δ(1M-NaO^2H in ^2H_2O) *ca.* 2.2 (4 H, br m, β- and γ-CH_2 of glutamate), 3.2—3.4 (5 H, br m, 6-H, 7-, and 9-H_2), *ca.* 3.6 (2 H, AB q, bridge CH_2), 4.0—4.4 (3 H, m, α-CH of glutamate and bridge CH_2), 6.7 (2 H, d, *J* 8.4 Hz, 3′- and 5′-H), 7.65 (2 H, d, *J* 8.4 Hz, 2′- and 6′-H), 7.05 (1 H, s, uracil 6-H), and 7.79 (1 H, d, *J* 1.8 Hz, uracil 6-H). The compound ran as a single quenching spot on cellulose t.l.c. using 0.1M-potassium phosphate buffer (pH 7), 0.05M in mercaptoethanol as eluant.

5,10-*Bis*(*uracil-5-ylmethyl*)[6-$^2H_1$]*tetrahydropteroylglutamic acid* (4c; 6-H = ^2H) was prepared using 7,8-dihydropteroylglutamic acid (6; R^1 = H) [15] (1 g, 2.25 mmol), sodium borodeuteride (600 mg, 14.3 mmol), and [$^2H_2$]water (50 ml) in the initial reduction step, and the above method. The product (170 mg, 11%) had u.v. and ^1H n.m.r. spectra which were identical with the undeuteriated analogue except for a loss of integration of at least 0.6 H in the region δ 3.2—3.4 of the reduced pteridine C-6 proton.

Reaction of the Bis Adduct (4c) *with Chloromethyluracil.*—5-Chloromethyluracil (56 mg, 0.35 mmol) was added to a suspension of 5,10-bis(uracil-5-ylmethyl)tetrahydropteroylglutamic acid (4c) (50 mg, 0.072 mmol) in deaerated 0.5M-aqueous potassium phosphate buffer (pH 7; 4 ml) and dioxan (4 ml) at room temperature under nitrogen. After the mixture had been stirred for 20 min under these conditions, mercaptoethanol (2 drops) was added and the mixture lyophilised to dryness. The crude product was dissolved in deaerated water (20 ml), the pH adjusted to 7 with 2M-aqueous sodium hydroxide, and the solution filtered and acidified to pH 3.5 with 2M-aqueous acetic acid. Mercaptoethanol (2 drops) was added and the solution was left in an ice-bath under nitrogen for 30 min and then filtered. The cream-coloured precipitate was washed with ice-cold water–methanol (1 : 1; 1 ml) and ice-cold methanol (1 ml) and dried *in vacuo* at room temperature (37 mg, 63%); $\lambda_{max.}$ (pH 7; 0.1M-phosphate buffer) 268 and 304 nm; $\lambda_{max.}$ (pH 0—1) 265 and 296sh nm [lit.,[12] for the compound assumed to be (4c), $\lambda_{max.}$ (pH 7) 270 and 303 nm]; δ(1M-NaO^2H in ^2H_2O) *ca.* 2.1 (4 H, br m, β- and γ-glutamyl protons), 3.0—4.4 (*ca.* 12 H, complex m, α-glutamyl CH, 6-H, 7-H_2 and 9-H_2, and 3 × bridge CH_2), 6.5 (2 H, m, 3′ and 5′-H), 6.9 (1 H, s, uracil 6-H), and 7.6 (4 H, br m, 2′ and 6′-H and 2 × uracil 6-H).

Pyrolysis of 5,10-*Bis*(*uracil-5-ylmethyl*)*tetrahydropteroylglutamic Acid* (4c).—5,10-Bis(uracil-5-ylmethyl)tetrahydropteroylglutamic acid (4c) (55 mg, 0.079 mmol) was heated at 250—275 °C *in vacuo* in a Gallenkamp heating block for 5 h. The sublimate was collected (8 mg, 39%) and recrystallised from water. It had an i.r. (KBr) spectrum identical with an authentic sample of thymine, $\lambda_{max.}$ (H_2O) 263 nm; δ[$(CD_3)_2$SO] 1.75 (3 H, s, Me) and 7.2 (1 H, s, 6-H); *m/e* 126 (M^+).

Pyrolysis of 5,10-*Bis*(*uracil-5-ylmethyl*)[6-$^2H_1$]*tetrahydropteroylglutamic Acid* (4c; 6-H = ^2H).—This was performed as above, at 270 °C for 6 h. The product (46%) was recrystallised from water, $\lambda_{max.}$ (H_2O) 263 nm; δ(^2H) (^2H_2O) — 2.93 from the [$^2H_2$] water signal (s, Me); δ(^1H) [$(CD_3)_2$SO] 1.75 (<3 H, s, Me) and 7.1 (1 H, s, 6-H); *m/e* 127 (M^+, indicating 29.1% of a monodeuteriated product).

10-*Methylpteroylglutamic Acid* (8; R^1 = Me).—Methotrexate (9) (500 mg, 1.1 mmol) was dissolved in 1M-aqueous sodium hydroxide (50 ml) under nitrogen and the solution was heated under nitrogen in a water-bath in the dark for 4.5 h. The cooled solution was diluted with deaerated water (550 ml), sodium chloride (2 g, 34.2 mmol) was added, and the pH was adjusted to 3.5 with 5M-hydrochloric acid. The solution was left in an ice-bath under nitrogen in the dark for 5 h and the precipitate was collected by centrifugation at 0 °C, washed with ice-cold 0.001M-aqueous hydrochloric acid (80 ml), and dried *in vacuo* at room temperature (417 mg, 83%). The spectra were identical with those of an authentic specimen; $\lambda_{max.}$ (pH 7; 0.1M-phosphate buffer) 282sh and 302 nm; $\lambda_{max.}$ (pH 0—1) 309 nm [lit.,[16] $\lambda_{max.}$ (pH 7) 282sh and 301 nm; $\lambda_{max.}$ (pH 1) 308 nm]; δ(1M-NaO^2H in ^2H_2O) *ca.* 2.1 (4 H, br m, β- and γ-glutamyl protons), 2.85 (3 H, s, 10-Me), 4.15 (1 H, t, α-glutamyl CH), 4.44 (2 H, br s, 9-H_2), 6.47 (2 H, d, *J* 8 Hz, 3′ and 5′-H), 7.45 (2 H, d, *J* 8 Hz, 2′- and 6′-H), and 8.0 (1 H, s, 7-H).

The sample ran as a single quenching spot, R_F 0.74 on cellulose t.l.c. using 0.1M-potassium phosphate buffer as eluant.

10-*Methyl*-7,8-*dihydropteroylglutamic Acid* (6; R^1 = Me).—1M-Aqueous sodium hydroxide was added to a solution of ascorbic acid (8.75 g, 50 mmol) in deaerated water (44 ml) under nitrogen until pH 6 was reached. 10-Methylpteroylglutamic acid (8; R^1 = Me) (350 mg, 0.77 mmol) in 0.1M-aqueous sodium hydroxide (14 ml) was added to this solution followed by sodium dithionite (3.5 g, 20.1 mmol). The reaction was stirred at room temperature under nitrogen for 5 min and cooled in an ice-bath. The pH was adjusted to 2.8 by addition of 1M-hydrochloric acid at a rate of *ca.* 1 ml min^{-1} and the reaction mixture was left for a further 5 min. The precipitate was collected by centrifugation at 0 °C, washed with ice-cold 0.001M-aqueous hydrochloric acid (40 ml), and dried *in vacuo* at room temperature (206 mg, 59%); $\lambda_{max.}$ (pH 7, 0.1M-phosphate buffer) 282 and 306 nm; $\lambda_{max.}$ (pH 0—1) 252 and 278 nm; $\lambda_{max.}$ (pH 13) 290sh and 305 nm [lit.,[16] $\lambda_{max.}$ (pH 7) 284 and 305 nm; $\lambda_{max.}$ (pH 1) 255 and 284 nm; and $\lambda_{max.}$ (pH 13) 290sh and 305 nm]; δ(1M-NaO^2H in ^2H_2O) *ca.* 2.1 (4 H, br m, β- and γ-glutamyl protons), 2.88 (3 H, s, 10-Me), 3.73—4.0 (4 H, br m, 7- and 9-H_2), 4.22 (1 H, m, α-glutamyl CH), 6.65 (2 H, d, *J* 8 Hz, 3′ and 5′-H), and 7.60(2 H, d, *J* 8 Hz, 2′ and 6′-H).

10-*Methyl*-5-(*uracil-5-ylmethyl*)*tetrahydropteroylglutamic Acid* (4d).—Sodium borohydride (200 mg, 5.3 mmol) was added to a deaerated solution of 10-methyl-7,8-dihydropteroylglutamic acid (6; R^1 = Me) (206 mg, 0.45 mmol) in 0.1M-aqueous potassium phosphate buffer (pH 7; 14 ml). The reaction mixture was stirred under nitrogen for 15 min after which it was quickly acidified to pH *ca.* 1 with concentrated hydrochloric acid. The solution was kept at pH 1 for *ca.* 1 min and then adjusted to pH 7.5 with 2M-aqueous sodium hydroxide. The solution was cooled in an ice-bath and a suspension of 5-chloromethyluracil (130 mg, 0.81 mmol) in deaerated dioxan (15 ml) was added. The reaction

was stirred under nitrogen for 35 min, mercaptoethanol (0.5 ml) was added, and the mixture was lyophilised to dryness. The residue was suspended in deaerated water (15 ml) under nitrogen and the pH was adjusted to 9 with 1M-aqueous sodium hydroxide to effect dissolution. After filtering and adjusting the pH to 3.5 with 2M-aqueous acetic acid, the solution was cooled in an ice-bath under nitrogen for 1 h and the precipitate was collected by centrifugation at *ca.* 2 °C. Further purification was effected by dissolution at pH 8.5 and precipitation at pH 3.5, as above, until a faint quenching spot, R_F *ca.* 0.78, above the major spot, R_F 0.62, had disappeared from a severely overloaded cellulose t.l.c. run in 0.1M-phosphate buffer (pH 7; 0.05M in mercaptoethanol). A pale yellow solid was obtained (61 mg, 23%) (Found: C, 46.3; H, 5.2; N, 19.0. $C_{25}H_{29}N_9O_8 \cdot 3.5H_2O$ requires C, 46.4; H, 5.6; N, 19.5%); *m/e* (field desorption) 584 (M^+); $\lambda_{max.}$ (pH 7, 0.1M-phosphate buffer) 278sh and 304 nm (ε 24 000); $\lambda_{max.}$ (pH 0—1) 269 and 300sh nm; δ(1M-NaO^{2}H in ^2H_2O) *ca.* 2.2 (4 H, br m, β- and γ-glutamyl protons), 2.93 (3 H, s, 10-Me), *ca.* 3.2 (5 H, br m, 6-, 7-, and 9-H), *ca.* 3.7 (2 H, AB q, N(5)-CH_2), *ca.* 4.3 (1 H, m, glutamyl α-CH), 6.69 (2 H, d, *J* 8.4 Hz, 3′- and 5′-H), and 7.7 (3 H, m, 2′- and 6′-H, and uracil 6-H).

10-Methyl-5-(uracil-5-ylmethyl)[6-$^2H_1$]tetrahydropteroylglutamic Acid (4d; 6-H = ^{2}H).—This compound was prepared from 10-methyl-7,8-dihydropteroylglutamic acid (6; R^1 = Me) (215 mg, 0.47 mmol) and sodium borodeuteride (200 mg, 4.8 mmol) using the above method. The product (80 mg, 29%) (Found: C, 47.15; H, 5.7; N, 19.2. $C_{25}H_{28}{}^2HN_9O_8 \cdot 3H_2O$ requires C, 47.0; H, 5.7; N, 19.7%) had spectra identical with the undeuteriated compound except for a loss in integration of *ca.* 0.7 H in the region assigned to the C-6 proton (δ *ca.* 3.3).

Pyrolysis of 10-Methyl-5-(uracil-5-ylmethyl)tetrahydropteroylglutamic Acid (4d).—The adduct (4d) (36 mg, 0.062 mmol) was heated at 255—260 °C *in vacuo* in a Gallenkamp heating block for 6 h. The sublimate was collected (2.6 mg, 32%) and recrystallised from water. It had the spectral characteristics of authentic thymine and ran as a single spot with authentic thymine on a cellulose t.l.c. run in 0.1M-potassium phosphate buffer.

Pyrolysis of 10-Methyl-5-(uracil-5-ylmethyl)[6-$^2H_1$]tetrahydropteroylglutamic Acid (4d; 6-H = ^{2}H).—This was conducted as above, heating the adduct (42 mg, 0.072 mmol) at 255—260 °C *in vacuo* for 7 h to obtain a 47% yield of thymine which recrystallised from water, *m/e* 127 (M^+ showing 25.3% of monodeuteriation), δ(^{2}H) (^2H_2O) — 2.93 from [$^2H_2$] water (s, Me).

Investigation of the Interaction of Compounds (4c) *and* (4d) *with the Enzyme Thymidylate Synthetase.*—(a) *Assay for enzyme activity.* A buffer solution (A) was made up to 0.05M in TRIS buffer, 0.01M in mercaptoethanol and 0.001M in EDTA adjusted to pH 7.4 with 1M-hydrochloric acid. This buffer (1 ml) was used to dilute a commericial solution of thymidylate synthetase (0.01 ml) to a 1% enzyme solution. The enzyme was then assayed by mixing the 1% enzyme solution (0.1 ml) in buffer (A) (1.7 ml) with 0.6 ml of a solution (B) made up from tetrahydropteroylglutamic acid (1; R^1 = H) (1.8 ml of a solution containing 5 mg ml^{-1} in 1M-aqueous mercaptoethanol, adjusted to pH 7.4 with 1M-NaOH), 1M-aqueous mercaptoethanol (adjusted to pH 7.4 with 1M-NaOH; 6 ml), 0.3M-aqueous formaldehyde (3 ml), 0.5M-aqueous magnesium chloride (3 ml), 0.001M-aqueous dUMP (3 ml), and water (1.2 ml). This solution was put in the test cuvette of the u.v. spectrophotometer whilst a similar solution from which the dUMP had been omitted was used in the reference cuvette. The assay was run at *ca.* 35 °C and the signal $\lambda_{max.}$ = 340 nm was recorded at 2-min intervals. During 10 min the change in absorbance was 0.28 absorbance units. The standard of activity of the enzyme preparation was therefore 0.028 absorbance units per min.

(b) *Assay for compounds* (4c) *and* (4d) *as substrates.* A 1% enzyme solution (0.1 ml) in buffer (A) (1.7 ml) was mixed with 0.6 ml of a solution made up from the test substance (4c) or (4d) (0.3 ml of a solution containing 1.5 mg in 0.3 ml of 1M-aqueous mercaptoethanol, adjusted to pH 7.4 with 1M-NaOH), 1M-aqueous mercaptoethanol (adjusted to pH 7.4 with 1M-NaOH; 1 ml), 0.5M-aqueous magnesium chloride (0.5 ml), and water (1.2 ml). This solution was placed in the test cuvette whilst the reference cuvette contained a similar solution from which the enzyme had been omitted. In neither case was any change in absorbance at $\lambda_{max.}$ 340 nm observed on incubation at 35 °C. After 16 min the solution (B) (0.4 ml) was added to both cuvettes; the increase in absorbance at $\lambda_{max.}$ 340 nm during 10 min was 0.14 absorbance units in the test of compound (4c) and 0.13 absorbance units in the test of compound (4d).

(c) *Assay for compounds* (4c) *and* (4d) *as inhibitors.* A 1% enzyme solution (0.1 ml) in buffer (A) (1.7 ml) and 0.6 ml of a solution made up from 1M-aqueous mercaptoethanol (adjusted to pH 7.4 with 1M-NaOH; 1.3 ml), 0.5M-aqueous magnesium chloride (0.5 ml), and water (1.2 ml) were placed in the test cuvette. The same mixture but without enzyme was placed in the reference cuvette. After incubation at *ca.* 35 °C for 16 min, the enzyme reaction was activated by addition of solution (B) (0.4 ml) to both cuvettes. The increase in absorbance at $\lambda_{max.}$ 340 nm over 10 min was 0.15 absorbance units, *i.e.* almost identical with the increase observed in the presence of the compounds (4c) and (4d) outlined in assay (b) above. Neither compound therefore appeared to inhibit the activity of the enzyme.

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