Stereochemistry of Reduction of Folic Acid using Dihydrofolate Reductase

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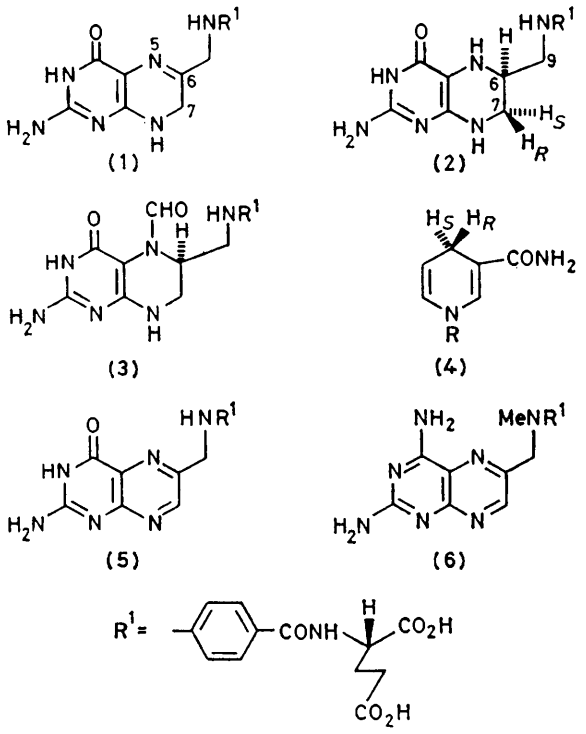
Summary The dihydrofolate reductase catalysed reduction of the vitamin folic acid (**5**) to the coenzyme 5,6,7,8-tetrahydrofolic acid (**2**) involves transfer of the 4-*pro-R*-hydrogen of NADPH to the *si*-face at C-7 of folic acid (**5**); the absolute stereochemistry at C-6 of 5,6,7,8-tetrahydrofolic acid (**2**) from the enzymic reduction has been correlated with that of biologically active folinic acid (**3**), and the reduction at both C-6 and C-7 therefore involves the same side of NADPH and the same face of folic acid.

THE enzyme dihydrofolate reductase catalyses the reduction of 7,8-dihydrofolate (**1**) to 5,6,7,8-tetrahydrofolate (**2**) using NADPH as coenzyme.[1] 5,6,7,8-Tetrahydrofolate (**2**) is the coenzyme responsible for C_1-transfer in primary metabolism and is involved in the methylation of uracil to thymine using the enzyme thymidylate synthetase. In this latter process (**2**) is 'oxidised' to (**1**) and dihydrofolate reductase is required to regenerate the coenzyme (**2**). This enzyme is of considerable pharmacological interest, being the target of anti-folate drugs such as methotrexate and trimethoprim.



The stereochemistry at C-6 of the biologically active diastereoisomer of folinic acid (**3**) has been defined as (*S*) by *X*-ray studies[2] on 5,10-methenyltetrahydrofolate. We have reduced (**1**) to (**2**) using NADPH and dihydrofolate reductase. Purification of the tetrahydrofolate (**2**)† followed by conversion into folinic acid gave the active diastereoisomer (**3**) thus defining the absolute stereochemistry at C-6 of (**2**) from the enzymic reduction as (*S*). Attack of NADPH must therefore occur at the *re*-face of (**1**). In addition, it is known that enzymic reduction of (**1**) involves transfer of the 4-*pro*-*R* hydrogen of NADPH[3] [H_R in (**4**)] so that the stereochemistry of this step is established.

Dihydrofolate reductase from many sources will also reduce folic acid (**5**) to (**2**).[1] With the enzyme from *Lactobacillus casei* this reaction is a hundred times slower than the reduction of (**1**).[4] Nothing is as yet known of the absolute stereochemistry of the enzymic reduction of (**5**) at C-7. We cannot assume that this is the same as for the reduction of (**1**) because of the possibility raised by the recently reported[5] crystal structure of the enzyme–methotrexate (**6**)–NADPH complex that a pteridine ring may bind to the enzyme in a different orientation from that adopted by a dihydropteridine ring. We have now determined the stereochemistry of the enzymic reduction of folic acid.

We prepared [4-^{2}H]NADP by a modification of the method of San Pietro[6] and reduced it using the 'B-specific' enzyme glucose-6-phosphate dehydrogenase[7] to obtain (4*R*)-[4-$^2H_1$]NADPH (**4**, $H_R = {}^2H$). This was used to reduce (**5**) in the presence of dihydrofolate reductase from *L. casei*[4] at pH 6·7. The reduction was followed by the disappearance of the absorption at λ_{max} 340 nm (due to NADPH) and by ^{1}H n.m.r. spectroscopy.

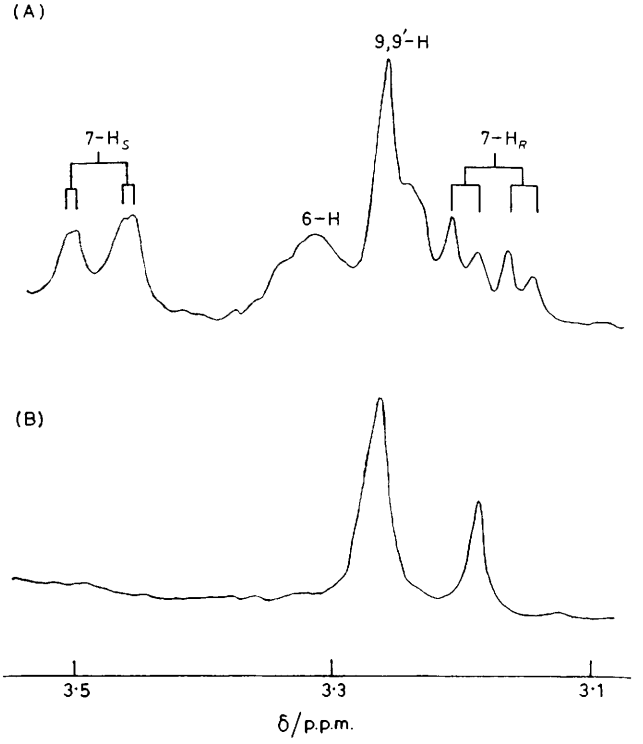


FIGURE. Part of the 270 MHz ^{1}H n.m.r. spectrum in ^2H_2O of (A) 5,6,7,8-tetrahydrofolic acid (**2**); (B) [6,7-$^2H_2$]-5,6,7,8-(terahydrofolic acid from enzymic reduction of folate (**5**) using (4*R*)-[4-$^2H_1$]NADPH. The chemical shift scale is referenced to DSS (sodium 2,2-dimethyl-2-silapentane-5-suphonate).

The ^{1}H n.m.r. spectrum of undeuteriated 5,6,7,8-tetrahydrofolic acid (Figure A) contains a multiplet for the C-6 proton and separate multiplets for the two C-7 protons (δ 3·49 and 3·19). Each of the 7-H multiplets features a large (12 Hz) geminal coupling and a smaller (3·1 and 6·7 Hz) vicinal coupling. The higher field (δ 3·19) 7-H multiplet has been assigned[8,9] to the proton *trans* to the C-6-proton, because it features the larger vicinal coupling constant, and this assignment is confirmed by consideration of the spectra of 6-methyl-5,6,7,8-tetrahydropterin.[10,11] The higher-field resonance is therefore due to the 7-*pro*-*R* hydrogen. In the spectrum of the enzymatically prepared [6,7-$^2H_2$]-5,6,7,8-tetrahydrofolic acid (Figure B), the signals at δ 3·32 (6-H) and 3·49 (7-H_S) are no longer present, while the 7-H_R resonance is now a broad singlet. Clearly deuterium has replaced the 7-H proton which is *cis* to 6-H *i.e.* the 7-*pro*-*S* hydrogen.

Thus, during enzymic reduction of (**5**), hydrogen is transferred from the 4-*pro*-*R* position of NADPH to the *si* face of C-7 of folic acid, so that the hydrogen transfer to

† The tetrahydrofolate (**2**) had $[\alpha]_D^{32}$ −49·9° in 1·5 M Tris containing 0·2 M ethanethiol, compared to a value of $[\alpha]_D^{27}$ −16·9° in the same solvents reported by C. J. Mathews and F. M. Huennekens, *J. Biol. Chem.*, 1960, **235**, 3304. This was converted into folinic acid (**3**), $[\alpha]_D^{32}$ −28·5° (H_2O) in good agreement with the reported[2] rotation for the active isomer.

both C-6 and C-7 involves the same face of NADPH and the same face of folic acid (**5**). There is thus no major difference in the orientation of the oxidised and reduced pteridine rings when bound to the enzyme. We have further provided proof for the suggestion[5,12,13] that this orientation is substantially different from that implied by the crystal structure of the complex of the enzyme with NADPH and the inhibitor, methotrexate (**6**).[5]

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