Stereochemistry of Reduction of the Vitamin Folic Acid by Dihydrofolate Reductase [1]

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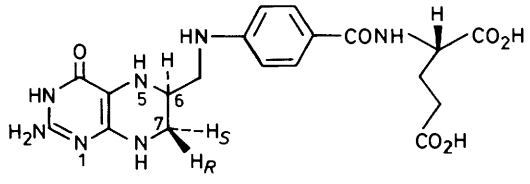
Reduction of the vitamin folic acid (**6**) to the coenzyme 5,6,7,8-tetrahydrofolic acid (**1**) by the enzyme dihydrofolate reductase is shown to involve transfer of the 4-*pro R* hydrogen of NADPH to the same face at both C-6 and C-7 of the pteridine system (the *re* face at C-6 and the *si* face at C-7). The orientations of the pteridine system of folic acid (**6**) and of dihydrofolic acid (**5**) when bound to the enzyme are different from the orientation of the pteridine ring of the anti-cancer drug methotrexate (**11**) when bound to this enzyme.

The coenzyme 5,6,7,8-tetrahydrofolic acid (**1**) is essential in primary metabolism for reactions involving the transfer of a one-carbon unit at the formic acid, the formaldehyde or the methanol oxidation levels.[2] In these reactions the coenzyme first forms a one-carbon derivative at the appropriate oxidation level. This then transfers the one-carbon moiety to a suitable substrate and the coenzyme (**1**) is regenerated so that the process is continuous. An exception to this generalization is the synthesis of thymidine monophosphate (**2**; R^1 = 2′-deoxyribose-5′-monophosphate) from deoxyuridine monophosphate (**3**; R^1 = 2′-deoxyribose-5′-monophosphate) using the enzyme thymidylate synthetase. In this process, although the total transfer is at the methanol oxidation level, the coenzyme-one-carbon derivative used is 5,10-methylenetetrahydrofolic acid (**4**) which is at the formaldehyde oxidation level. The change in oxidation level required in this process is effected by transfer of the hydrogen at C-6 of the coenzyme (**1**) to the carbon which becomes the methyl group of thymine (**2**; R^1 = H).[3–5] The coenzyme is therefore oxidized to 7,8-dihydrofolic acid (**5**) in the process and so a second enzyme, dihydrofolate reductase (E.C.1.5.1.3), is required to reduce this to the coenzyme (**1**) and thus keep thymidine synthesis self-sufficient in coenzyme. Inhibition of thymine synthesis is an important area of cancer chemotherapy and compounds which inhibit the two enzymes involved in this synthesis are used clinically as anti-cancer drugs.[6,7]

Although bacteria can synthesize tetrahydrofolic acid (**1**) *via* dihydrofolic acid (**5**) from purine derivatives, mammals do not have this ability and must take the vitamin folic acid (**6**) in their diet. The enzyme dihydrofolate reductase will reduce the vitamin (**6**), first to dihydrofolic acid (**5**) and then to the coenzyme (**1**). For *Lactobacillus casei* dihydrofolate reductase it has been shown [8] that the first reduction step is 100 times slower than the second. The difference in metabolism between bacteria and mammals has been exploited, and compounds which inhibit steps in the biosynthesis of dihydrofolic acid (**5**) from purines have proved useful antibacterial drugs.[9] Species differences in the enzyme dihydrofolate reductase itself have also been exploited in this way.

In order to understand the catalytic mechanism more fully we



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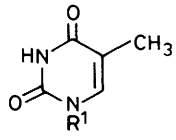

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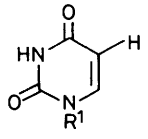

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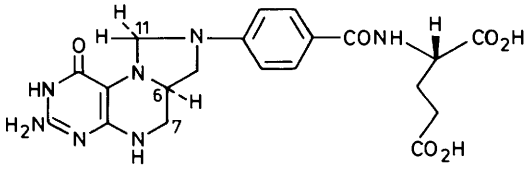

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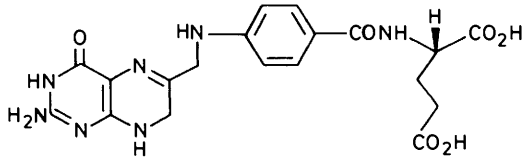

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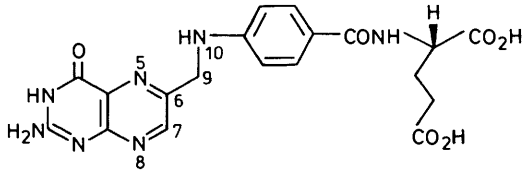

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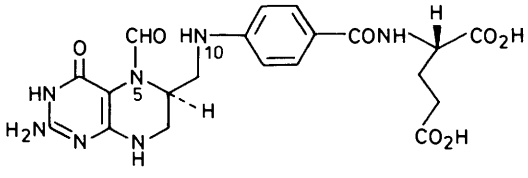
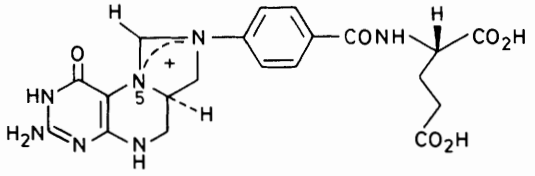


(**7**)

have investigated the stereochemistry of the reduction at C-7 and C-6 of folic acid (**6**) with this enzyme both with respect to the pteridine substrate and to the reducing coenzyme NADPH.

We orginally intended to determine the stereochemistry at C-6 of the coenzyme (**1**) which results from reduction of dihydrofolic acid (**5**) by dihydrofolate reductase using total stereospecific synthesis.[10] In the course of this work, however, the stereochemistry of the *Leuconostoc citrovorum** active diastereoisomer of 5-formyltetrahydrofolic acid, folinic acid (**7**), was shown to be 6*S* by *X*-ray studies[11] on a salt of 5,10-methylidynetetrahydrofolic acid (**8**) which was derived from this compound. The stereochemistry of the active coenzyme would, therefore, be defined formally if tetrahydrofolic acid (**1**) obtained by enzymic reduction of dihydrofolic acid could be converted into one of the C-6 epimers of folinic acid (**7**). We were able to achieve this conversion, first using a sample of (6*RS*)-tetrahydrofolic acid prepared by chemical reduction of folic acid (**6**). Folic acid (**6**) was reduced with aqueous sodium



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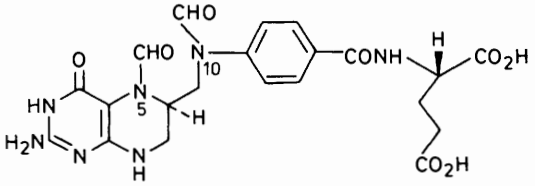
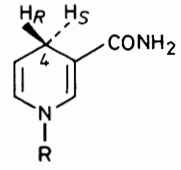


(**9**)

dithionite to yield 7,8-dihydrofolic acid.[12] This was then reduced using sodium borohydride to yield (6*RS*)-tetrahydrofolic acid. The product was scrupulously dried† and treated with freshly prepared[13] formic acetic anhydride to yield a product with u.v. spectrum in keeping with its assignment as 5,10-diformyltetrahydrofolic acid (**9**).[14] Since it was known that, whilst 5-formyltetrahydrofolic acid (**7**) is stable to alkali, 10-formyltetrahydrofolic acid can be deformylated,[15] we were able specifically to deformylate the diformyl derivative (**9**) to obtain folinic acid (**7**) as a mixture of C-6 epimers. Use of ^2H_2O in the dithionite reduction step allowed us to prepare [7-$^2H_1$]folinic acid which we have used in our ^1H n.m.r. spectral studies of the conformations of this compound.[16]

When we applied this synthesis to a sample of tetrahydrofolic acid (**1**) obtained by reduction of dihydrofolic acid (**5**) using NADPH and bovine liver dihydrofolate reductase, we found that it was necessary to remove enzyme and coenzyme NADP(H) before the formylation step. The tetrahydrofolic acid (**1**) was, therefore, purified by chromatography using DEAE-cellulose to give active coenzyme, $[\alpha]_D$ −49.9°. This specific rotation was higher than the value, $[\alpha]_D$ −16.9°, found by Mathews and Huennekens.[17] Diformylation and hydrolysis now gave (−)-folinic acid (**7**) which was purified by chromatography using DEAE-cellulose. The pure compound had a specific rotation, $[\alpha]_D$ −28.5°, expected of the *L. citrovorum* active isomer. Although higher than the value orginally found[18] it was comparable to more recently obtained values.[11,19] The stereochemistry at C-6 of the active coenzyme tetrahydrofolic acid (**1**) can now formally be defined as (*S*) so that reduction must occur from the *re* face at C-6 of 7,8-dihydrofolic acid (**5**). It is known,[3,4] that reduction of dihydrofolic acid at C-6 involves transfer of the 4-*pro R* hydrogen of NADPH (**10**) so that the stereochemistry of this step with respect to both substrate and coenzyme is known.

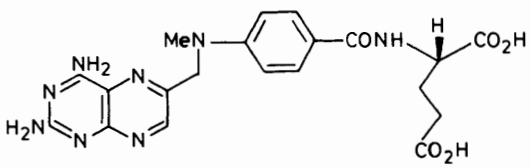


(**10**)

The next objective was to investigate the stereochemistry of reduction of the vitamin folic acid (**6**) by dihydrofolate reductase at C-7. We therefore prepared (4R)-[4-$^2H_1$]-NADPH (**10**; H_R = ^2H) from [4-^2H]-NADP[20] using the 'B-specific' enzyme glucose-6-phosphate dehydrogenase and glucose-6-phosphate.[21] Two n.m.r. tube experiments were now performed at 270 MHz using folic acid and dihydrofolate reductase from *L. casei*. In the first experiment unlabelled NADPH was used as reductant whilst in the second experiment (4*R*)-[4-$^2H_1$]-NADPH (**10**; H_R = ^2H) was used. The ^1H n.m.r. spectra obtained in these experiments are shown in the Figure. The ^1H n.m.r. spectrum of undeuteriated tetrahydrofolic acid (Figure A) exhibited a multiplet for the C-6 proton and separate multiplets for the two C-7 protons (δ 3.49 and δ 3.19 p.p.m.). Each of the multiplets for the C-7 protons featured a large (12 Hz) geminal coupling and a smaller (3.1 and 6.7 Hz respectively) vicinal coupling. The higher field (δ 3.19 p.p.m.) 7-H multiplet had been assigned[22,23] to the proton *trans* to the C-6 proton because it featured the larger vicinal coupling constant and this assignment was confirmed by consideration of the spectra of 6-methyl-5,6,7,8-tetrahydropterin.[24,25] The higher field resonance was therefore due to the 7-*pro R* hydrogen and the lower field resonance to the 7-*pro S* hydrogen. In the spectrum of [6,7-$^2H_2$]tetrahydrofolic acid (Figure B) obtained by reduction of folic acid by (4*R*)-[4-$^2H_1$]-NADPH (**10**; H_R = ^2H) and dihydrofolate reductase, the signals at δ 3.32 and δ 3.49 p.p.m. for 6-H and 7-H_S respectively were no longer present while the 7-H_R resonance was now a broad singlet. Clearly deuterium has replaced the C-7 hydrogen which is *cis* to the C-6 hydrogen, *i.e.* the 7-*pro S* hydrogen.

Reduction of folic acid with dihydrofolate reductase has, therefore, transferred the 4-*pro R* hydrogen of NADPH to the *si* face at C-7 of folic acid. Hydrogen transfer at *both* C-6 and C-7 therefore involves the same face of NADPH (4-H_R) and the same face of the pteridine system in folic acid (C-6 *re*; C-7 *si*). There is thus no major difference in the orientation of folic acid (**6**) and dihydrofolic acid (**5**) on binding to the active site of the enzyme.

It is of interest to note in this context that an *X*-ray structural analysis of a ternary complex of the anti-cancer drug methotrexate (**11**), the coenzyme NADPH and dihydrofolate reductase has shown[26] that whilst 4-H_R of NADPH is close to



(**11**)

* *L. citrovorum* has been renamed *Pediococcus cerevisiae*.

† Traces of water in this reaction were found to cause complications due to formation of 5,10-methylidynetetrahydrofolic acid.

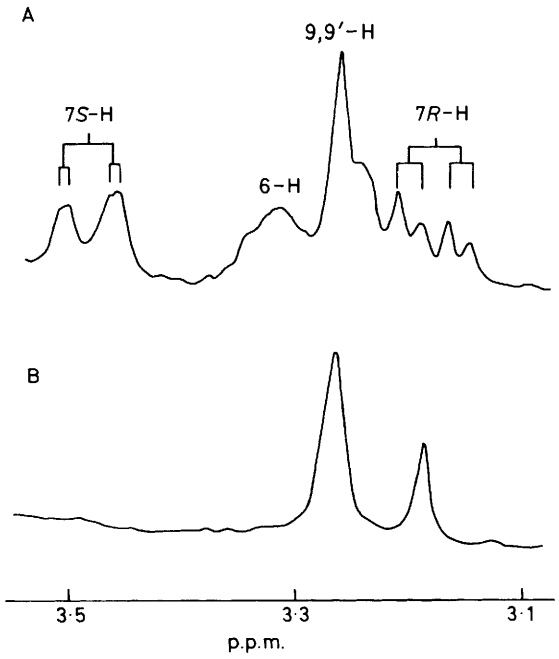


Figure. Part of the 270 MHz ^1H n.m.r. spectrum in ^2H_2O of (A) 5,6,7,8-tetrahydrofolic acid (**1**); (B) [6,7-$^2H_2$]-5,6,7,8-tetrahydrofolic acid from enzymic reduction of folate using (4*R*)-[4-$^2H_1$]-NADPH. The chemical shift scale is referenced to sodium 2,2-dimethyl-2-silapentane-5-sulphonate.

C-6 and C-7 of the pteridine system of methotrexate (**11**), it is in fact *the opposite face of the pteridine system* which is close to this reducing hydrogen from that face of the pteridine system in folic acid (**6**) which is actually reduced. Thus, in spite of the close structural similarity between folate and methotrexate, the pteridine rings of these two compounds bind to the enzyme in quite different orientations. This possibility was recognized by Matthews *et al.*[26] and Hitchings and Roth[27] who pointed out that the results of Fontecilla-Camps *et al.*[11] implied a different orientation of 7,8-dihydrofolate and methotrexate on the enzyme. The present results show that this is not a difference between oxidized and 7,8-dihydropteridine rings, but rather a difference between 2-amino-4-oxo (substrate) and 2,4-diamino (inhibitor) substitution patterns. From the crystal structure[26] and ^{13}C n.m.r. experiments,[28] it is clear that, when bound to the enzyme, methotrexate is protonated at N-1 and forms an ion-pair with the carboxylate of the aspartate residue 26 (Asp-26) of the enzyme. Further, there is a hydrogen bond between the carbonyl oxygen of the amide bond between the amino acids 4 and 5 of the enzyme and the 4-amino group of methotrexate (**11**).[26] Such an interaction is disfavoured with the carbonyl group at C-4 of folic acid (**6**) and dihydrofolic acid (**5**). The pteridine ring of bound folate must differ in orientation by about 180° from bound methotrexate so that N-1 will be remote from Asp-26, and indeed ^{13}C n.m.r. experiments show that bound folate is not protonated at N-1.[28]

The role of Asp-26 in enzymic catalysis has not firmly been established but, if the bound folate is oriented at 180° to the bound methotrexate molecule, then Asp-26 will be in the region of N-5 and the 4-carbonyl group of folate. Thus it is possible that Asp-26 may act either by assisting protonation of N-5 of dihydrofolate so favouring reduction of the 5,6 double bond or by hydrogen bonding with the 4-carbonyl group of the substrates thus holding them in the correct orientation for reduction.

Experimental

^1H N.m.r. spectra were recorded on Perkin-Elmer R12 and R32 instruments and on a Bruker WH270 spectrometer operating in the Fourier-transform mode. ^{13}C N.m.r. spectra were obtained using a Jeol PFT100 spectrometer. U.v. spectra were recorded on Pye Unicam SP800, SP500, and SP1800 spectrophotometers and specific rotations were determined using a Perkin-Elmer 241 polarimeter with a 1 dm path length cell.

7,8-*Dihydrofolic Acid* (**5**).—This compound was prepared by the following modification of the method of Blakley.[12] The pH of a solution of ascorbic acid (20 g, 0.114 mol) in deaerated water (100 ml) was adjusted to pH 6 with 1M-aqueous sodium hydroxide under nitrogen. A solution of folic acid (760 mg, 1.72 mmol) in 0.1M-aqueous sodium hydroxide (32 ml) was added, followed by sodium dithionite (8 g, 45.9 mmol). The solution was stirred for 5 min after dissolution of the reagent. The reaction was cooled to <5 °C and acidified to pH 2.8 by addition of 1M-hydrochloric acid at a rate of *ca.* 1 ml min^{-1}. The precipitate was stirred for a further 5 min and centrifuged at 4 °C. The solid was resuspended under nitrogen in an ice-cold solution of sodium ascorbate (pH 6) prepared as above (same quantities). The pH was adjusted to pH 2.8 by addition of 1M-hydrochloric acid. The precipitate was centrifuged and the solid was washed with ice-cold 0.001M-hydrochloric acid (50 ml). The product was dried *in vacuo* at room temperature (600 mg, 79%); and the u.v. spectrum,[29] the ^1H n.m.r. spectrum,[30] and the ^{13}C n.m.r. spectrum[31] were in keeping with the spectral data reported in the literature.

(6RS)-5-*Formyl*-5,6,7,8-*tetrahydrofolic Acid* (**7**).—7,8-Dihydrofolic acid (250 mg, 0.56 mmol) was suspended in deaerated water (5 ml) in an atmosphere of nitrogen. Sodium borohydride (200 mg, 5.29 mmol) was added and after 10 min the reaction was cooled in an ice-bath. The solution was acidified to pH 0—1 with concentrated hydrochloric acid and neutralized with 1M-sodium hydroxide. Mercaptoethanol (2 drops) was added and the solution was acidifed to pH 3.5 with 2M-aqueous acetic acid and left for 15 min at 0 °C. The precipitate was centrifuged at 0—4 °C and dried *in vacuo* at room temperature. The product (180 mg, 72%) (6*RS*)-5,6,7,8-tetrahydrofolic acid had $\lambda_{max.}$ (pH 7) 297 nm, and a purer sample $\lambda_{max.}$ (pH 13) 297 nm (ε 21.0 × 10^3) [lit.,[32] $\lambda_{max.}$ (pH 7) 297 nm, $\lambda_{max.}$ (pH 13) 290 nm (ε 21.6 × 10^3); lit.,[29] $\lambda_{max.}$ (pH 7) 298 nm, $\lambda_{max.}$ (pH 13) 298 nm]; $\lambda_{max.}$ (pH 13) changed in 1 min to $\lambda_{max.}$ 290 nm without change in ε. The sample was used immediately for the following step.

(6*RS*)-5,6,7,8-Tetrahydrofolic acid (25 mg, 0.56 mmol) was dried thoroughly and added to freshly prepared[13] ice-cold formic acetic anhydride (3 ml). The reaction was stirred in an ice bath under nitrogen for 30 min and the solution was allowed to reach room temperature. Stirring was continued until an aliquot had $\lambda_{max.}$ (pH 13) 265 nm. The time required for complete reaction varied between runs of this experiment, being 20 h in one case. In this experiment excess formic acetic anhydride was removed under reduced pressure after 3 h to yield (6*RS*)-5,10-diformyl-5,6,7,8-tetrahydrofolic acid (**9**) as a yellow solid (33 mg, >100%). The yield was estimated as *ca.* 66% from ε (pH 13, 265 nm) 11.6 × 10^3 [lit.,[14] ε (pH 13, 266 nm) 17.5 × 10^3] and this material was hydrolysed directly to folinic acid.

Crude L-($\pm$)-5,10-diformyl-5,6,7,8-tetrahydrofolic acid (**9**) (5 mg, 0.01 mmol) was heated at 100 °C for 2 h in 0.1M-sodium hydroxide (2 ml). The u.v. spectrum[14] indicated complete *N*-10-deformylation after this time and addition of acid to an aliquot gave, as expected, the u.v. spectrum typical of 5,10-methylidyne-5,6,7,8-tetrahydrofolic acid (**8**)[29] after *ca.* 90 min. The alkaline solution was neutralized with 1M-hydrochloric acid and the

solvent was removed under reduced pressure to give (6*RS*)-5-formyl-5,6,7,8-tetrahydrofolic acid (**7**) identical with an authentic sample and with spectra as reported elsewhere.[16]

(6RS,7RS)-[7-$^2H_1$]-5-*Formyl*-5,6,7,8-*tetrahydrofolic Acid*.—(7*RS*)-[7-$^2H_1$]-7,8-Dihydrofolic acid was prepared using ^2H_2O in a modification of the method of Zakrzewski[33] in which, after a 5 min reaction, the solution was rapidly quenched with dilute hydrochloric acid to prevent exchange of deuterium at positions other than C-7. The crude product had a ^1H n.m.r. spectrum identical with the undeuteriated compound except that the protons 7-H and 9-H now integrated as 3.5 H. This compound was now taken through the reaction sequence outlined above except that, to prevent exchange, $NaBH_4$ was dissolved in the aqueous solvent *prior* to addition of the dihydrofolic acid. The crude (6*RS*,7*RS*)-[7-$^2H_1$]-5-formyl-5,6,7,8-tetrahydrofolic acid was recrystallized repeatedly from ethanol–water to give the pure product in 16% *overall* yield from [7-$^2H_1$]dihydrofolic acid. The u.v. spectrum was as expected[29] both for the compound itself and for its conversion into 5,10-methylidyne-5,6,7,8-tetrahydrofolic acid (**8**). The pure compound and an authentic sample of 5-formyl-5,6,7,8-tetrahydrofolic acid (**7**) ran as one spot on t.l.c. when mixed together. The ^1H n.m.r. spectrum of this compound is reported elsewhere.[16]

Enzymatic Synthesis of (6S)-5-*Formyl*-5,6,7,8-*tetrahydrofolic Acid* (**7**).—7,8-Dihydrofolic acid (**5**) (75 mg, 0.17 mmol), NADPH (Sigma; 225 mg, 0.25 mmol) and bovine liver dihydrofolate reductase (Sigma; 7.5 units) were incubated in 0.04M-phosphate buffer pH 7.4 (45 ml) at 30 °C for 100 min when the absorbance, $\lambda_{max.}$ 340 nm, due to NADPH had ceased to change. The solution was reduced to a volume of 25 ml under reduced pressure and washed onto a column of Whatman DE23 DEAE-cellulose (3 × 10 cm) which had been equilibrated first with 0.2M-TRIS/HCl pH 7.2 (500 ml) and then with 0.005M-TRIS/HCl pH 7.2 (1 l), both buffers being 0.2M in mercaptoethanol. The column was eluted at 4 °C with a TRIS/HCl pH 7.2 gradient (1 l) which was 0.2M- in mercaptoethanol (1 l, 0.005M to 1 l, 0.2M) followed by 0.2M-TRIS/HCl pH 7.5 buffer, 0.2M in mercaptoethanol. (6*S*)-5,6,7,8-Tetrahydrofolic acid (**1**) eluted in the latter buffer and had $[\alpha]_D$ −49.9° (*c* 0.149, 1.5M TRIS/HCl pH 7.2, 0.2M-mercaptoethanol), based on ε (298 nm) 28 × 10^3.[34] This compared with $[\alpha]_D$ −16.9° found by earlier workers.[17] The (6*S*)-5,6,7,8-tetrahydrofolic acid (**1**) was dried, diformylated, and the crude 5,10-diformyltetrahydrofolic acid was hydrolysed at pH 13 essentially as described above. The crude (6*S*)-5-formyl-5,6,7,8-tetrahydrofolic acid was washed on to a column of Whatman DE23 DEAE-cellulose [3 × 15 cm, equilibrated first with 0.4M-NH_4HCO_3 (500 ml) and then with 4 × 10^{-3}M-NH_4HCO_3 (1 l)] in 4 × 10^{-3}M-ammonium hydrogen carbonate (20 ml). The column was eluted with an ammonium hydrogen carbonate gradient (1 l, 4 × 10^{-3}M to 1 l, 0.4M) to yield pure L-(−)-5-formyl-5,6,7,8-tetrahydrofolic acid (9 mg) with expected spectral properties and $[\alpha]_D$ −28.5° (*c* 0.097, water).

[4-^{2}H]-*NADP*.—The compound was prepared by the following modification of the method of San Pietro.[20] NADP (Sigma; 150 mg, 0.19 mmol) and potassium cyanide (0.75 g, 11.5 mmol) were dissolved in ^2H_2O (9 ml) containing 1M-KO^2H (0.9 ml). The pH was adjusted to *ca.* pH 12 with 1M-$HCOO^2H$ (98—100% HCOOH diluted with ^2H_2O) and the reaction was left at room temperature for 90 min. The solution was acidified with 1M-$HCOO^2H$ to pH 3.5 and the HCN was removed by bubbling nitrogen through the solution into a sodium hypochlorite trap for 1 h. Cold acetone (6.5 volumes) was added and the solution was left at *ca.* 4 °C overnight. The acetone was decanted off and the remaining yellow oil was dissolved in water and lyophilized to dryness. No further purification was attempted. The ^1H n.m.r. spectrum (60 MHz, ^2H_2O) was in agreement with a spectrum of authentic NADP except for the loss of the resonance assigned to the proton at C-4 (*ca.* δ 8.65).

(4R)-[4-$^2H_1$]-*NADPH*.—D-Glucose-6-phosphate (100 mg, 0.35 mmol) was dissolved in 0.02M-aqueous magnesium chloride (3 ml) and the solution was adjusted to pH 7.6 by the addition of 1M-aqueous sodium hydroxide. The crude [4-^{2}H]-NADP prepared above was added to this solution together with a catalytic amount of glucose-6-phosphate dehydrogenase (Sigma) and the pH was readjusted to pH 7.6 with 1M-aqueous sodium hydroxide. The reaction was incubated at 25 °C and the pH was continually readjusted to pH 7.6. The course of the reaction was monitored by consideration of the ratio of absorbance in the u.v. spectrum at 260 and at 340 nm. The product, NADPH, should display a ratio 260:340 nm of 2.4:1.[35] No improvement was found after a ratio of 2.9:1 had been reached, and so the reaction was stopped and diluted with water to a total volume of 100 ml.

The solution (100 ml) was added to a DEAE-cellulose column (Whatman DE 52, 1.7 × 12 cm) which had previously been equilibrated with water (150 ml, containing 2 drops 0.88M-NH_4OH) at 4 °C. The rate of elution was set at *ca.* 1 ml min^{-1}. The u.v. absorbance of the effluent was monitored at 250 nm to ensure that the (4*R*)-[4-$^2H_1$]-NADPH was retained on the support. Once all the (4*R*)-[4-$^2H_1$]-NADPH had been adsorbed, the column was eluted with a gradient of aqueous sodium bromide (500 ml water in the mixing flask and 500 ml 0.1M-sodium bromide in the reservoir; both solutions contained 2 drops 0.88M-NH_4OH). The (4*R*)-[4-$^2H_1$]-NADPH was expected to elute at *ca.* 50 mM sodium bromide. Fractions of *ca.* 5 ml were collected and the u.v. absorbance of the effluent was monitored at 260 and at 340 nm. The tubes displaying approximately the correct absorbance ratio at these values were combined (total 310 ml). The pooled sample displayed an absorbance ratio 260:340 nm of 2.8:1. The sample was lyophilized to dryness to give inorganic salt and (4*R*)-[4-$^2H_1$]-NADPH (total *ca.* 900 mg). The salt was extracted by suspending the lyophilized sample in ethanol (60 ml) and, after stirring at room temperature for 30 min, the suspension was centrifuged and the supernatant liquid was discarded. The pellet was extracted again with ethanol (50 ml) as above and after centrifugation the pellet was dissolved in ^2H_2O (2 ml) and lyophilized. The yield, at this stage, was calculated to be 72 mg based on the weight and on an expected extinction coefficient ε = 6.2 × 10^3 ($\lambda_{max.}$ 340 nm, pH 7.5),[35] $\lambda_{max.}$ (pH 7.5) 260 and 340 nm (ε ratio 2.8:1).

Enzymic Reduction of Folic Acid (**6**).—Two experiments were conducted in an n.m.r. tube, one using NADPH and the other using (4*R*)-[4-$^2H_1$]-NADPH. The solution (0.38 ml) was 530 mM in KCl, 50 mM K_3PO_4 buffer in ^2H_2O (pH *ca.* 6.8), 10.5 mM in NADPH or [^{2}H]-NADPH, 11.4 mM in folate and 0.08 mM in *L. casei* dihydrofolate reductase. The pH was continuously adjusted to 6.8 using ^{2}HCl. The signals due to tetrahydrofolate appeared after 30 min at 25 °C and are shown in Figure A for the run using NADPH and Figure B for the run using (4*R*)-[4-$^2H_1$]-NADPH.

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