ASYMMETRIC REDUCTION OF DIHYDROFOLATE USING DIHYDROFOLATE
REDUCTASE AND CHIRAL BORON-CONTAINING COMPOUNDS

by Lilias Rees, Edward Valente, Colin J. Suckling, and Hamish C.S. Wood

Department of Pure and Applied Chemistry, University of Strathclyde,

295, Cathedral Street, Glasgow G1 1XL, Scotland

Abstract: The reduction of dihydrofolic acid to chiral tetrahydrofolic acid has been investigated by enzymic and non-enzymic means. With dihydrofolate reductase from E.coli as catalyst and recycling systems for NADPH, up to 1 g of optically pure stable tetrahydrofolate derivatives was obtained. The technique makes the possibility of synthesising chiral 5-formyltetrahydrofolate (leucovorin) for use in cancer rescue therapy attainable. In contrast, although dihydrofolate was reduced by a number of chiral boranes and borates built from amino acids and amino alcohols, enantiomeric excesses were minimal.

The use of enzymes in the synthesis of chiral organic compounds is now well established[1,2] and their application with current technology is particularly strongly indicated when water-soluble substrates are involved, for example folic acid derivatives. There is also an increasing awareness of the importance of chirality in drugs since it seems likely that the thalidomide tragedy was caused by the teratogenicity of one enantiomer of the drug.[3] In the folic acid field, it has been established[4,5] that the active diastereoisomer[1] C-1 transfer reactions has the S-configuration at position 6. Racemic 5-formyl-(6RS)-tetrahydrofolate (leucovorin) 2, is widely used in 'rescue' therapy for patients undergoing cancer chemotherapy with methotrexate.[6,7] It has, however,

1   2
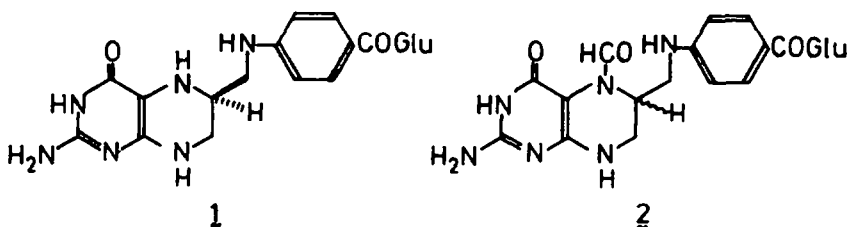
been shown that thymidylate synthase from L. casei is inhibited by the non-natural (6S) diastereoisomer of 5,10-methylene tetrahydrofolate[8] and that 5,10-methylene tetra-hydrofolate dehydrogenase from E. coli is also inhibited by the same diastereoisomer.[9] In these cases at least the non-natural epimers cannot be regarded as biologically inert and there is therefore a potential clinical requirement for the natural (6S) diastereoisomer of leucovorin. Chiral reductions of dihydrofolate have been attempted before by catalytic hydrogenation[10] and by enzymic catalysis.[11] In this paper we report the first substantial scale laboratory synthesis of chiral derivatives of tetrahydrofolate,[12] including leucovorin, together with attempts at asymmetric reduction using chiral boron-containing reducing agents.

Enzyme-catalysed reduction of dihydrofolate

We have studied the preparative scale reduction of dihydrofolic acid catalysed by dihydrofolate reductase from a trimethoprim resistant strain of E. coli kindly provided by Burroughs Wellcome Co. USA.[13]   Dihydrofolate substrate was prepared by reduction of tetrahydrofolate with aqueous sodium dithionite.[14]   The major problem to be solved in developing preparative scale reductions was recycling of the reducing cofactor, NADPH. The work of Whitesides[1,2] has largely solved this problem and several of his techniques were successful with dihydrofolate reductase.   The essentials of each experiment are summarised in Table 1. Assays of the tetrahydrofolates produced by enzymic catalysis and

TABLE 1

Asymmetric reduction of dihydrofolate by E. coli dihydrofolate reductase with NADPH recycling

Recycling method	Reaction time d	NADPH recycling fold	ATP recycling fold	methenyl-tetrahydro F yield mg (%)	leucovorin yield mg (%)
Isocitrate dehydrogenase	7	130	–	46(24)	–
Glucose 6-phosphate dehydrogenase/ creatine kinase	5	300	70	–	–
Glucose 6-phosphate dehydrogenase/ acetate kinase	7	1100	100	2770 (28)	910 (13)
Glucose 6-phosphate dehydrogenase/ acetate kinase Immobilised	8	385	99	–	–

by non-enzymic reagents were carried out by quantitative derivatisation with (R)-1-(1-naphthyl)ethylisocyanate[15] which converts tetrahydrofolate into a substituted urea at N-5 (Figure 1a). Reverse phase hplc cleanly separated the diastereoisomers formed in non-enzymic reduction whereas the product of reduction catalysed by dihydrofolate reductase was a single peak (Figure 1b). Confirmation of the analysis was obtained using n.m.r. at 250 MHz (Figure 2); the striking differences are to be seen in protons near to the naphthyl group (a, b, c, d).

The first enzymic recycling system that we studied used isocitrate dehydrogenase for the direct, one-step reduction of $NADP^+$ (Scheme 1). This reaction was carried out on 500 μmol of dihydrofolate at pH 7, under pH stat control, using 1.5 units of dihydrofolate reductase and 0.96 units of isocitrate dehydrogenase. When reaction was complete as shown by hplc, the reaction mixture was lyophilised and the residue converted into 5,10-methenyltetrahydrofolate with formic acid. The recrystallised product had $[\alpha]_D^{24}$ = + 19.2± 2.5° (c 0.39, 10 M HCl), in fair agreement with previously obtained values.[4] Although this method is practical for small scale experiments, the expense of the recycling enzyme and its substrate recommended the investigation of other methods. We
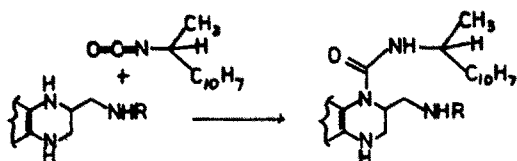
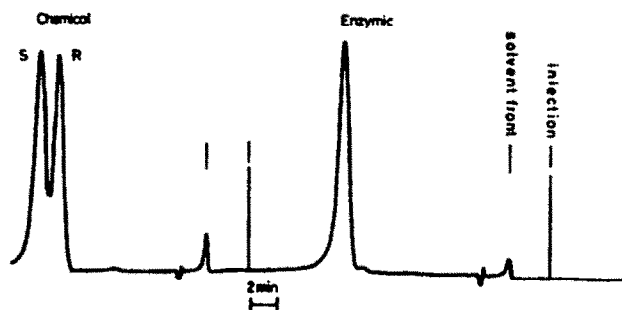Figure 1a   Derivatisation of Tetrahydrofolate

Figure 1b   Hplc analysis of Tetrahydrofolate derivative
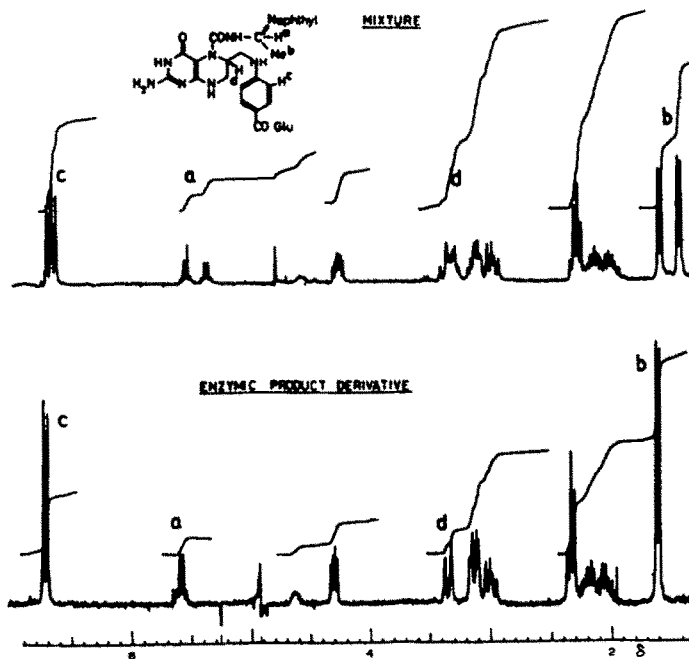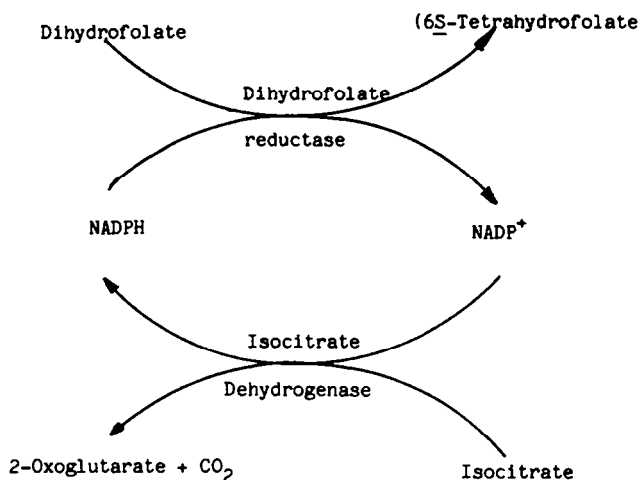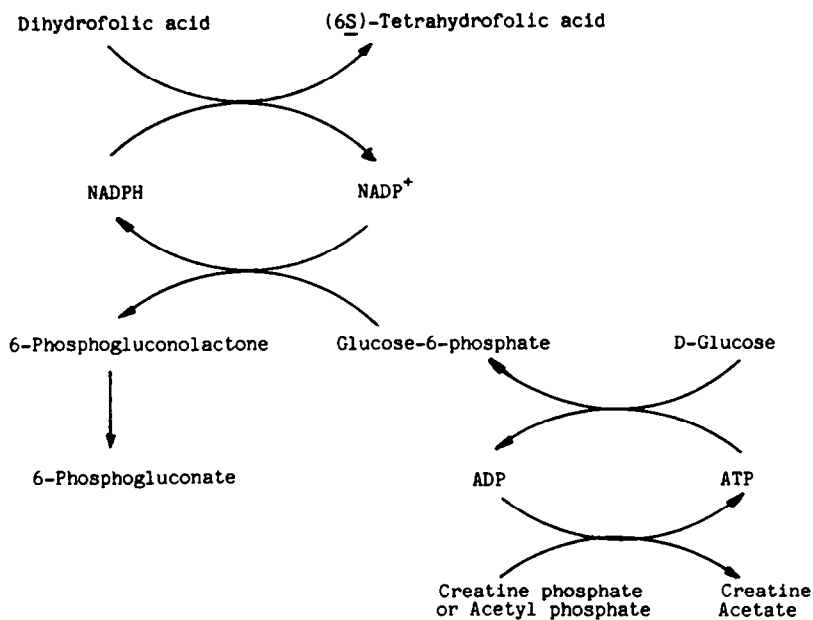   diastereoisomers

Figure 2   250 MHz nmr of derivatised Tetrahydrofolate
                   diastereoisomers

<u>Scheme 1</u>

Dihydrofolate   (6<u>S</u>-Tetrahydrofolate

Dihydrofolate

reductase

NADPH   NADP$^+$

Isocitrate

Dehydrogenase

2-Oxoglutarate + CO_2 Isocitrate

then turned to glucose 6-phosphate dehydrogenase (Scheme 2); using this enzyme, the driving of the reduction process can be relayed via hexokinase and ATP to a kinase that accepts a chemically synthesised phosphorylating agent.[16] Acetyl phosphate has frequently been used and the diammonium salt was prepared for our studies.[17] Before use, the salt was assayed by an enzymic method followed by hplc using either ion pair chromatography on a reverse phase column[18] or, preferably, using ion exchange on a strong anion exchange column. The first experiments using glucose 6-phosphate dehydrogenase as NADPH recycling enzyme were carried out using creatine phosphate and creatine kinase as recycling agents for ATP. At ambient temperature over five days, 373 μmol dihydrofolic acid was reduced using 4 units each of creatine kinase, hexokinase, glucose 6-phosphate dehydrogenase, and dihydrofolate reductase in the presence of an equimolar quantity of D-glucose and 40% excess of creatine phosphate. This reaction was not further developed because of the greater availability of acetate kinase and acetyl phosphate. Using this system 8.6 g dihydrofolate were reduced anaerobically in the presence of 20-25 units of each of the enzymes. An equimolar quantity of glucose was used and diammonium acetyl phosphate (30% excess) was added in approximately equal proportions over 7 days, after which time reaction was complete. The reaction was worked up by adding ascorbic acid to inhibit oxidation of tetrahydrofolate which was converted initially into 5,10-methenyltetrahydrofolate $[\alpha]_D^{32.7} = + 14.3 \pm 0.8°$(c 0.35, 10M HCl) and then by hydrolysis to 5-formyltetrahydrofolate isolated as the calcium salt. In this way, we obtained nearly 1 g of chiral leucovorin equivalent to 13% yield based upon dihydrofolate.

The possibility of preparing substantial quantitites of chiral tetrahydrofolate derivatives by enzyme-catalysed reduction has thus been demonstrated and we were interested in improving the convenience and economics of the process by using immobilised enzymes. The principal virtues of immobilised enzymes compared with soluble enzymes are that they facilitate extraction of products and maintain catalytic activity. However, the advantages are paid for by the increased expense entailed in immobilisation and the reduction in catalytic activity often encountered. In the case of a high added-value chemical such as leucovorin, the costs of immobilisation can be justified. We therefore investigated a wide range of immobilisation techniques for <u>E. coli</u> dihydrofolate reductase and the other enzymes required; the results are summarised in Table 2. The

Scheme 2

Dihydrofolic acid   (6S)-Tetrahydrofolic acid

NADPH $NADP^+$

6-Phosphogluconolactone   Glucose-6-phosphate   D-Glucose

6-Phosphogluconate   ADP   ATP

Creatine phosphate   Creatine
or Acetyl phosphate   Acetate

TABLE 2

Immobilisation of Enzymes for synthesis of tetrahydrofolate

| Enzyme	Technique	Immobilised activity %	Activity recovered %	Immobilised activity $U\ g^{-1}$
glucose 6-phosphate dehydrogenase	PAN	20	2	140
hexokinase	PAN	42	10	12
creatine kinase	PAN	8.5	0	328
acetate kinase	PAN	42	42	67
dihydrofolate reductase (form II)	PAN	18	70	0.6
dihydrofolate reductase (form I)	PAN	5	69	0.4
dihydrofolate (form I) reductase (form II)	Agarose/CNBr	6 15	0 0	3.6 11.4 unstable to storage
(form II)	AH Sepharose carbodiimide	11.4	70	3
	acrylamide/ acrylic acid/ carbodiimide	11	7	14.5
	AH Sepharose adsorption	-	-	

most extensively studied method used Whitesides polyacrylamide material, PAN.[19] This technique using substrate and cofactor protection for the active sites afforded immobilised preparations of satisfactory catalytic activity per gram of polymer for all enzymes except dihydrofolate reductase. A target activity of at least 50 U g^{-1} support is required for a practical procedure using immobilised enzymes.* We therefore investigated alternative procedures for immobilisation of dihydrofolate reductase (Table 2). Although significant improvements were obtained using the well-established cyanogen bromide technique,[20] the product was still insufficiently active and was unstable to storage. Carbodiimide-mediated coupling to commercial (Pharmacia) AH-Sepharose[21] again was unsuccessful as was a similar coupling to an acrylamide/acrylic acid copolymer.[22] Adsorption on AH-Sepharose at pH 7.0 was complete but, unfortunately, substantial desorption occurred in the presence of 5 mM substrate, dihydrofolate. Dunlap[23] has also experienced difficulties in immobilisation of dihydrofolate reductase and so far, this problem remains unsolved. Nevertheless, we attempted a preparative reduction reaction using enzymes immobilised on PAN and reduction of 372 μmol of dihydrofolate was complete in 8 days using 1 unit of each enzyme at pH 7.4. The availability of a high activity, stable, immobilised form of dihydrofolate reductase would clearly enhance the practical convenience of the enzyme-catalysed method.

Reduction by chiral boron-containing reagents

Unlike many studies of asymmetric reduction, reactions with dihydrofolate must be carried out in aqueous solution. This limits the range of reagents that can be employed but amine-borane complexes[24] have been found to have solubility in protic solvents as have the more recently developed alkoxyamine-borane complexes.[25] We prepared a series of chiral 1,2-aminoalcohols (3-6) by reduction of the parent (S)-amino acid with borane-dimethyl sulphide in the presence of boron trifluoride diethyl etherate,[26] a more convenient procedure than that using lithium aluminium hydride. The oxazoline (8) was prepared from ethyliminoacetate and commercially available (1S,2S)-2-amino-1-phenyl-1,3-propanediol.[27]   (S)-2-Amino-1-phenylethanol (9) was prepared from (S)-mandelic acid amide.[28]   The remaining compounds (7, 10, and 11) were commercial materials. The required borane complexes were prepared by treatment of the amino alcohols with an equimolar quantity of borane-dimethylsulphide complex initially at -78°C. Removal of the solvent afforded viscous oils or solids; the complexes displayed optical activity and were homogeneous by tlc.

Reactions with dihydrofolate were attempted under nitrogen in three solvent systems all containing dithiothreitol to minimise oxidation of tetrahydrofolate. Assays of product chirality were carried out using the chiral naphthylisocyanate as described above. The solvent systems were: A   water/tetrahydrofuran 5:3;   B   20 mM Tris-HCl pH 8.0/tetrahydrofuran 5:3; and C 20 mM Tris-HCl pH 8.0/ethanol 5:3. Table 3 records the results.   It can be seen that, although reduction was complete in many cases, asymmetric induction was poor and, using (S)-amino alcohols, the non-natural (6R)-diastereoisomer of tetrahydrofolate was obtained in excess.

*Note Although the value for hexokinase obtained was below the target, the initial activity was only 10 U mg^{-1} protein; use of a more active preparation which was commercially available (150 U mg^{-1}) would have led to satisfactory activity in the immobilised product.
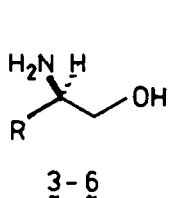
R

(3) $(CH_3)_2CH$
(4) $PhCH_2$
(5) CH_3
(6) $(CH_3)_2CH.CH_2$

3-6

7

8

9

10

11
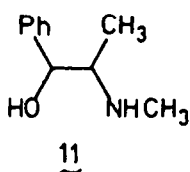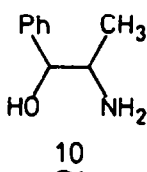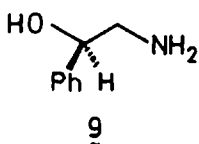
TABLE 3

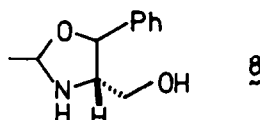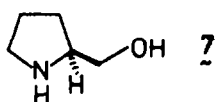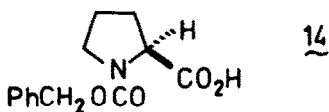| Parent amino alcohol	Solvent	wt. of complex (mg)	extent of reaction (%), time	isomeric excess (%)	Configuration at C6
3	A	55.5	> 98, 5 days	1.8	R
4	A	33.3	> 98, 5 days	9.8	S
5	A	37.8	> 97, 5 days	0.8	R
6	A	26.5	30, 5 days	-	-
7	A	30.6	84, 5 days	9.8	S
8	B	4.8	100, 3 h	11.8	R
8*	B	35.6	100, 1 day	12.6	R
8*	C	9.73	100, 5 days	14.0	R
8*	C	5.22	> 90, 3 days	18.0	R
9	B	23.54	6, 2 days	-	-
10	B	21.97	86, 2 days	5.2	R
(+)11	(B	39.6	- -	-	-
	(C	27.92	- -	-	-
(-)11	(B	42.7	- -	-	-
	(C	34.35	- -	-	-

Table 3   Reduction of dihydrofolate with chiral aminoalcohol borane complexes
* entries were prepared with 2:1 borane : compound 8.   Solvents: A water :
tetrahydrofuran 5:3 v/v containing dithiothreitol (10 mM), B Tris-HCl (20 mM)
pH 8.0 : tetrahydrofuran 5:3 v/v containing dithiothreitol (10 mM), C as B but
with ethanol in place of tetrahydrofuran.

In view of this failure, we examined triacyloxyborohydrides which are known to reduce cyclic imines.[29]   Derivatives of the benzyloxycarbonylamides of (S)-alanine (12), valine (13), proline (14), phenylalanine (15), and serine (16) were prepared by reacting the N-carbobenzoxy amino acid with sodium borohydride in a 3:1 molar ratio in dry tetrahydrofuran.   The complex borohydrides were obtained as white amorphous solids in good yield.

$$
\begin{array}{cc}
 & R \\
(12) & CH_3 \\
(13) & CH(CH_3)_2 \\
(15) & CH_2Ph \\
(16) & CH_2OH \\
\end{array}
$$

PhCH_2OCONH   CO_2H

12,13   15,16

PhCH_2OCO   CO_2H   14
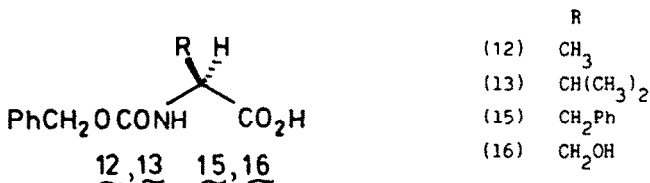
Unfortunately the only one of these reagents to show any significant reduction of dihydrofolic acid was the derivative from (S)-proline (14);   the use of 7 mol. equiv. over 72 h led to 70% reduction.   In this case no asymmetric induction was observed.

The disappointing results with these reagents indicate strongly that for asymmetric reduction of dihydrofolic acid, enzyme catalysed methods are superior to non-enzymic reducing agents.   Further development of the enzyme-catalysed methods, however, will require a more stable and more active form of dihydrofolate reductase.

Acknowledgements

EXPERIMENTAL

Nuclear magnetic resonance (n.m.r.) spectra were recorded on Perkin-Elmer R32 (90 MHz) or Bruker-250 (250 MHz) spectrometers.   Tetramethylsilane was used as an internal standard.   ^{13}C-Nuclear magnetic resonance spectra were recorded on a Jeol PFT 200 instrument.   Ultra-violet (u.v.) spectra were recorded on Pye-Unicam SP8000 and SP800A spectrophotometers.   Infra-red (i.r.) spectra were recorded on Perkin-Elmer 397 or 257 spectrometers.   Specific rotations were determined using a Perkin-Elmer 241 polarimeter with a 1 decimetre path-length, jacketed, cell.   High presssure liquid chromatography (hplc) was carried out using a system comprising a LDC model 396 micropump and a Cecil Instruments CE2012 variable wavelength u.v. monitor.   The prepacked columns were supplied by Watmans or Waters.   All solvents were distilled before use.   pH was automatically maintained using a combi-titrator 3D, Metrohm Herisau, Switzerland.

<u>Hplc analyses of folates</u> were carried out using an octadecylsilyl reverse phase column eluting with 5:95 v/v acetonitrile : 50 mM Tris-HCl pH 7.0 containing 10 mM 2-mercaptoethanol with a flow rate of 40 ml h^{-1} and monitoring the eluates at 254 nm.

<u>Diastereoisomeric derivatives</u> of tetrahydrofolate were prepared by mixing equal volumes of an aqueous solution of tetrahydrofolate with an equal volume of ethanol containing an excess of (<u>R</u>)-1-(1-naphthyl)ethylisocyanate; reaction was complete in ca. 5 min at room temperature. Products were assayed by hplc as above eluting with 20:80 v/v acetonitrile : 50 mM Tris-HCl, pH 7.0 and monitoring the eluates at 290 nm. A typical separation is shown in Figure 1b.

<u>Reference samples of folate deriatives</u> were prepared following published procedures : 7,8-dihydrofolate,[14] 10-formylfolic acid,[30] (6<u>RS</u>)-5,10-methenyltetrahydrofolate chloride,[31] calcium (6<u>RS</u>)-5-formyltetrahydrofolate.[31]

<u>Preparation and assay of diammonium acetyl phosphate</u>[17]

Phosphoric acid hemihydrate was heated for 30 h at 60°C under reduced pressure (0.1 mm Hg) in the presence of phosphorus pentoxide. For the first 12 h the phosphorus pentoxide was replaced every 4 h. On cooling to room temperature the material crystallised after 30 min. The product (100% H_3PO_4) was stored <u>in vacuo</u> over phosphorus pentoxide.

Ethyl acetate (680 ml) and 100% H_3PO_4 (100 g, 1.02 mol) were cooled in a 1-L two neck round bottom flask to 0°C (ice/water). Acetic anhydride (187 g, 1.83 mol) was cooled to 0°C then slowly added to the mixture. The addition took 30 min. and the resulting solution was stirred at 0°C for a further 3.7 h. A 3-L three necked flask was fitted with a thermometer, a gas inlet tube, and an overhead stirrer. The gas outlet was through a side arm adaptor used with the stirrer. Methanol (765 ml) was added and ammonia was bubbled into the solvent, with stirring, at -30 to -40°C (dry ice/acetone/ethylene glycol). After at least 30 mins. the addition of ammonia was stopped and the gas inlet tube was replaced with a 1-L pressure equalising dropping funnel containing the ethyl acetate/acetic anhydride/H_3PO_4 mixture. The addition took 30 min; care was taken to ensure that the temperature did not rise above -20°C during the addition. The fine white solid which filled the flask was collected by suction filtration [after a further portion of methanol (500 ml) was added to ease filtration]. It was washed with methanol (500 ml) and ether (500 ml). Final drying to constant weight was done by placing the solid in a glass dish in a desiccator, and drawing dry air over it by way of an inverted funnel. The air was passed through conc. H_2SO_4 and two calcium chloride drying towers. The desiccator contained calcium chloride. The final weight was 136.7 g. Enzymatic assay (see below) showed that the solid contained 84% acetyl phosphate by weight corresponding to a 67% yield based on H_3PO_4. A n.m.r. assay (see below) gave a composition ratio of 84.4% acetyl phosphate, 0.6% acetamide and 6.3% ammonium acetate. Found: C, 12.25; H, 6.0; N, 15.1. Calc. for $C_2H_{11}N_2O_5P$: C, 13.8; H, 6.35; N, 16.1%. The sample was stored at 5°C in a desiccator.

<u>N.m.r. assay for acetyl phosphate</u>

The reaction product (100-150 mg) was dissolved in D_2O (0.6 ml), and to this solution was then added dioxan (10 µl; micropipette). The solution was transferred to a n.m.r. tube and the ^{1}H spectrum recorded. The acetyl protons of acetyl phosphate fall 1.65 ppm upfield from the dioxan protons and are split into a doublet by coupling to phosphorus. Acetyl protons from acetamide are 1.76 ppm upfield while those from ammonium acetate are

found 1.86 ppm upfield. Integration of the dioxan peak and the acetyl proton peaks allow the composition of the mixture to be calculated. The formula used is,

$$M_A \times \frac{N_A}{H_A} = M_B \times \frac{N_B}{H_B}$$

where M = molarity, N = number of protons and H = the height of the integral.

Enzymic assay for acetyl phosphate

The assay is based on the phosphorylation of ADP using acetyl phosphate and acetate kinase to give ATP. The number of moles of ATP formed is directly related to the number of moles of acetyl phosphate used. Care was taken to ensure complete reaction in the stipulated time. The ATP concentration was measured by hplc analysis. The following solutions were made up;

(1)   0.2 M Triethanolamine HCl buffer, pH 7.6, 0.03 M in Mg^{2+} [2.98 g $(HOCH_2CH_2)_3N$ and 0.61 g $MgCl_2.6H_2O$ for 100 ml].

(2)   Water was added to 125.15 mg ADP to give one ml of solution. This was kept cold in an ice bath.

(3)   Approx. 50 mg of the acetyl phosphate sample was brought to 50 ml with H_2O just prior to the assay and chilled immediately. 10 μl of a suspension of acetate kinase in ammonium sulphate solution was used for the assay. This corresponded to approx. 8.5 units.

Procedure: To solution 1 (15 ml) in a sample bottle was added solution 2 (100 μl) and acetate kinase suspension (10 μl). This mixture was incubated at 25°C for 2 mins before addition of H_2O (100 μl) or acetyl phosphate solution 3 (100 μl). The blank was to correct for ATP present as an impurity in the ADP and for any ATP that might be formed during the reaction. The reaction was allowed to proceed for 5 min. (This was shown to be adequate for complete reaction). The solution was then placed in boiling water for 1 min, chilled over 1 min, filtered and injected onto the hplc column in duplicate. Either a reverse phase column or an anion exchange column was used (detector set at 0.05 a.u.f.s. in both cases). Agreement between the two hplc methods and also with the n.m.r. method was good.

Assays of adenine nucleotides and enzymes by hplc

1) Reverse phase column and ion-pair reagent

The mobile phase was made up as follows:

To potassium dihydrogen orthophosphate, KH_2PO_4 (6.804 g) was added approx. 500 ml of water. The ion pair reagent, tetra-n-butyl ammonium phosphate, (TBA) was added [1 vial diluted to one litre gives a concentration of 0.005 M]. The pH was adjusted to 6.8 with 1M potassium hydroxide. The volume was then made up to one litre in a volumetric flask. This gave an aqueous phase of 50 mM KH_2PO_4, pH 6.8, 0.005 M in TBA. Methanol was added to the aqueous phase to give a ratio of buffer : MeOH of 88:12. The volumes were measured separately then mixed. The retention times (minutes) for the nucleotides were: AMP - 5.2, ADP - 10.2, ATP - 18.4 for a flowrate of 119 ml/h.

2) Strong anion exchange column

Optimal conditions for the mobile phase were found to be; 0.2 M KH_2PO_4 brought to pH 4.0 with 0.2M H_3PO_4. The buffer was also 1 M in KCl. The retention times for the nucleotides were (in minutes), AMP - 2.8, ADP - 4.8, ATP - 14.8 for a flowrate of 98.5 ml/h.

Assay of hexokinase by hplc

The assay was based on the measurement of the rate of formation of ADP by hplc analysis.

Assay Mixture	Volume used	conc. in assay
0.1 M Triethanolamine buffer (pH 7.6 with 1 M HCl)	2.4 ml	48 mM
0.5 M glucose solution (1 g/10 ml buffer)	2.0 ml	220 mM
0.1 M $MgCl_2$	0.3 ml	6 mM
14 mM ATP solution (9.5 mg/ml buffer)	0.2 ml	0.56 mM
Hexokinase solution (9.35 mg/100 ml buffer)	0.1 ml	approx. 0.1 unit

All solutions, except that containing the enzyme, were pipetted into a glass sample bottle and incubated at 25°C for 2 min. After addition of enzyme, 1 ml samples were removed at 1, 3, and 5 min. and frozen. The samples were then plunged into boiling water for $1\frac{1}{2}$ min, chilled for 1 min, filtered, and injected onto the column.

Assay of acetate kinase by hplc

The assay was based on the measurement of the rate of formation of ATP by hplc analysis.

Assay mixture	Volume used	conc. in assay
0.1 M Triethanolamine buffer (pH 7.6 with 0.2 M HCl 5 mM in (dithiothreitol)	4.45 ml	89 mM
0.24 M acetyl phosphate solution (50 mg Li/K salt/ml buffer)	0.2 ml	9.6 mM
76 mM ADP solution (40 mg/ml buffer)	0.2 ml	3.0 mM
0.1 M $MgCl_2$ solution	0.05 ml	1.0 mM
Acetate kinase solution (0.2 ml of suspension in 100 ml of buffer)	0.1 ml	approx. 0.1 unit.

The procedure was conducted as above. The assay was done at 25°C and the reverse phase column was used. The detector was set at 0.05 a.u.f.s. Two assays were done and the samples from each at 1, 3, and 5 min. were injected once.

Assay of immobilised enzymes

The immobilised enzymes were assayed by either of the procedures outlined above. For the spectrophotometric assay, aliquots (120-100 μl) of the immobilised enzyme, suspended in the appropriate buffer, were taken and added to a cuvette. The cuvette was stoppered and shaken for 10 s to mix the suspension. The absorption was read at 340 nm for 30 s. This process was repeated so that the response was measured over 9 min. The plot was linear. For the chromatographic assay the solution was stirred with a small overhead air stirrer to keep the suspension agitated. Analysis was done as before.

Enzymatic reduction of dihydrofolate to (6S)-tetrahydrofolate consuming D-isocitrate. Isolation of (6R)-5,10-methenyltetrahydrofolate by ion exchange chromatography

A 250 ml 3 necked flask was equipped with a pH electrode (connected to a 'pH stat' unit) a nitrogen inlet/outlet, and a magnetic stirring bar. The flask was charged with water (150 ml, which had been degassed and then saturated with nitrogen), dithiothreitol (77 mg, 0.5 mmol), and dihydrofolate (225 mg, 507 μmol). The pH was adjusted to 7.4 with saturated ammonium carbonate solution; a solution was obtained after 5 min. To the flask was added $MgCl_2 \cdot 6H_2O$ (101 mg, 0.5 mmol) and DL-isocitrate (322 mg, 510 μmol of D-isomer). The addition of isocitrate caused the pH to rise; this was adjusted automatically to 7.4 using 1 M HCl, by the 'pH stat' apparatus. Dihydrofolate reductase, form II (0.45 mg, 1.5 U), D-isocitrate dehydrogenase (30 μl of a suspension in glycerol, 0.96 U), and $NADP^+$ (3.1 mg, 3.7 μmol) were added. The pH was maintained between 6.8 and 7.7 using 1M HCl. The flask was covered in foil to prevent photo-degradation of the reduced folates and the reaction was left stirring under nitrogen at ambient temperature. After 7 days the reaction was complete (hplc) and the final pH was 6.8. The reaction mixture was freeze-dried and the yellow lyophilisate was dissolved in a mixture of 98:2 formic acid (98%): trifluoroacetic acid (10 ml). After 14 h this mixture was lyophilised and the residue was suspended in 30 ml of 0.5 M HCl containing 2-mercaptoethanol (30 μl). The whole was concentrated in vacuo at 45°C to   20 ml. After standing at room temperature for 18 h a light brown solid was isolated by freeze-drying.

DEAE cellulose was washed extensively with water and packed into a column (4 x 30 cm) to a height of 18 cm. The packing was then washed with 500 ml of the eluent, 0.1 M formic acid - 0.01M 2-mercaptoethanol. The lyophilisate from above was dissolved in 70 ml of the eluent and allowed to percolate onto the column. The flowrate was set at ~2 ml/min. and 10 ml fractions were collected. At the 10th fraction the yellow band started eluting and this and subsequent fractions were collected and frozen. All of the effluent fractions exhibiting a value greater than 1.6 for the ratio of absorbance of 345:310 nm in 1.0M HCl were pooled and lyophilised (tubes 13-22). The yellow solid was dissolved in boiling 0.1M HCl - 0.1 M 2-mercaptoethanol (~15 ml). After standing overnight at 5°C the crystals were collected by centrifugation and washed with ethanol and then with ether. The supernatant was concentrated and a second crop of crystals deposited. After drying in vacuo at 76°C for 1.5 h the yield of (6R)-5,10-methenyltetrahydrofolate was 46.15 mg (24% based on dihydrofolate). U.V. λ_{max}, (1M HCl) 287 and 347 nm. $[\alpha]_D^{24°C}$ = + 19.2 ± 2.5° (c. 0.39, HCl), Lit.[4] $[\alpha]_D^{25}$ = + 11.4 ± 0.8° (c. 0.95, 12 M HCl). The product was compared with an analytically pure racemic sample by hplc (cf Figure 1).

Enzymatic reduction of dihydrofolate to (6S)-tetrahydrofolate consuming creatine phosphate

Dihydrofolate (165 mg, 372 μmol) was suspended in de-aerated water (45 ml) to which was added EDTA (7.5 mg, 20 μmol), $MgCl_2 \cdot 6H_2O$ (101 mg, 500 μmol), and dithiothreitol (74 mg, 480 μmol), dissolved in water to give a final volume of 47.5 ml. Glucose (73.3 mg, 370 μmol) and creatine phosphate (179.5 mg, 520 μmol) were added in 1 ml of water. The reaction was carried out in a 250 ml 3 necked flask equipped as usual. The pH was brought to, and maintained between, 7.4 and 7.6 by a 'pH stat' unit using saturated ammonium carbonate solution. The dihydrofolate was not completely dissolved at the start of the reaction but a solution was eventually obtained. After the pH was reasonably steady at ~ 7.4, hexokinase (0.4 mg, 4 U), glucose 6-phosphate dehydrogenase (0.02 mg, 4 U), creatine phosphokinase (0.03 mg, 4U), and dihydrofolate reductase, form II, (1.34 mg, 4.4 U) were added. The final component, $NADP^+$ (3.32 mg, 4 μmol) was added after the system had stabilised. The reduction was complete after 5 days (hplc).

Large scale reduction of folic acid to dihydrofolate

Folic acid (12 g, 25 mmol) was suspended in water (1250 ml) and dissolved by addition of 50% sodium hydroxide solution. The pH was adjusted to 6, if necessary, and sodium dithionite (15 g, 86 mmol) was added. The pH fell to 5.7 and the solution became dark but reverted to an orange/brown colour. The reaction was conducted under nitrogen at ambient temperature and followed by hplc. After 80 min a further 5 g (29 mmol) of sodium dithionite were added. The reaction was cooled to 5° after 2 h and ascorbic acid (5 g, 28.4 mmol) was added. The pH fell to 5, causing some precipitation, and was adjusted to 6.4. Conc. HCl was added over 23 min. until the pH was 2.8. The reaction was left stirring for a further 10 min. to complete precipitation and the yellow solid was collected by centrifugation at 5°C. The dihydrofolic acid was washed 3 times with 0.005 M HCl (3 x 1 L) and then resuspended in 1 L of 0.005 M HCl for storage before use. The yield of dihydrofolate was 8.6 g (77%) by u.v. assay.

Enzyme-catalysed reduction

Dihydrofolic acid (8.6 g, 19.4 mmol) was suspended in 1 L of de-aerated water and brought into solution with 50% sodium hydroxide solution. This solution was transferred to a 3 neck 3 L flask equipped with a nitrogen inlet/outlet, a magnetic stirring bar and a pH electrode connected to a 'pH stat' unit which maintained the pH between 7.0 and 7.6 by addition of 1 M sodium hydroxide. The pH of the dihydrofolate solution was 13 and it was adjusted manually to 7.0 with conc. HCl. Some precipitation occurred but dissolution was obtained with continued stirring. The flask was then charged with the following components; glucose hydrate (3.85 g, 19.4 mmol), adenosine triphosphate (0.125 g, 0.194 mmol), $NADP^+$ (14.32 mg, 17.5 μmol), $MgCl_2 \cdot 6H_2O$ (1.01 g, 5 mmol), and dithiothreitol (0.57 g, 3.7 mmol). The enzymes were then added; dihydrofolate reductase, form I, (2.88 mg, 25 U), hexokinase, (2.28 mg, 23 U), glucose 6-phosphate dehydrogenase (0.385 mg, 20 U), and acetate kinase (30 μl of suspension in ammonium sulphate, 20 U). The reaction was initiated by the addition of diammonium acetyl phosphate. The total quantity of acetyl phosphate added was 5.68 g (25.5 mmol). It was added in approx. equal portions over 7 days such that the addition was rate limiting (checked by hplc). This minimises non-enzymic hydrolysis where the inorganic phosphate produced would complex with magnesium ions to reduce their effective concentration and so the reaction rate. The reduction was conducted under nitrogen at ambient temperature. The flask was protected from light. After 7 days the reaction had gone to completion. Ascorbic acid (5 g, 28.4 mmol) was added and the pH was brought to 3.5 with conc. HCl (20 min). The yellow precipitate was filtered using a Buchner funnel under an atmosphere of nitrogen and the wet solid was

dissolved in a mixture of formic acid (98%):trifluoroacetic acid (98.2, 70 ml). After standing for 14 h at room temperature the dark red solution was evaporated to dryness in vacuo at a maximum bath temperature of 50°C. The residue was suspended in 0.5 M HCl (200 ml) containing 2-mercaptoethanol (200 μl). The whole was concentrated in vacuo to remove formic and trifluoroacetic acids (45°C). After standing overnight the (6R)-5,10-methenyltetrahydrofolic acid chloride was collected by centrifugation at 5°C and then recrystallised from 0.1 M HCl - 0.1 M 2-mercaptoethanol. After standing at 5°C for 26 h the crystals (2.22 g, 22.5%) were collected, washed with ethanol and ether, and dried in vacuo over phosphorous pentoxide. Concentration of the mother liquor deposited a second crop (0.55 g, 5.5%). The yields are based on dihydrofolate. Found: C, 46.8; H, 4.7; Cl, 6.75; N, 18.9. Calcd. for $(C_{20}H_{22}H_7O_6)^+$ $Cl^-.H_2O$: C, 47.1; H, 4.75; Cl, 6.95; N, 19.2%. $[\alpha]_D^{32.7}$ = + 14.3 $\pm$ 0.8° (c, 0.35, 10 M HCl). (lit.[4] $[\alpha]_D^{25}$ = + 11.4 $\pm$ 0.80°(c. 0.95, 12 M HCl).

Conversion into leucovorin

(6R)-5,10-Methenyltetrahydrofolic acid chloride (1.8 g, 3.54 mmol) was hydrolysed as previously described,[31] in boiling water between pH 6.5 and 6.9. The resulting yellow solution was treated with a clarified solution of calcium chloride (0.8 g in 2 ml). The solution was diluted with 5 ml of ethanol and cooled to 10°C. The brown solid that deposited was removed by filtration. The clear yellow filtrate was diluted with a further 150 ml of ethanol. The resulting slurry of cream coloured precipitate (0.91 g, 48%) of calcium (6S)-5-formyltetrahydrofolate was cooled at 5°C for 18 h, collected by filtration, washed with ethanol, and dried in vacuo over phosphorus pentoxide; Found: C, 42.6; H, 4.65; N, 16.45.   Calcd. for $C_{20}H_{21}N_7O_7Ca.3H_2O$: C, 42.4; H, 4.8; N, 17.3%. λ_{max}, (0.1 M NaOH) 282.5 nm, ϵ 26.63 x 10^3; λ_{max} 282.5: λ_{min} 241.5 = 4.3 $[\alpha]_D^{28}$ = + 2.12°(c. 1.32, H_2O).   Analysis by hplc compared favourably with a commercial sample. The solution for determination of optical rotation (containing 0.06594 g of solid) was evaporated to dryness in vacuo. The residue was dissolved in 1 ml of 1 N HCl, after 1 h the precipitate of 5,10-methyltetrahydrofolate formed was dissolved in 10 M HCl and made up to 10 ml in a volumetric flask, $[\alpha]_D^{27}$ = + 11°(c. 0.59, 10 M HCl).

Enzymic reduction of dihydrofolate to (6S)-tetrahydrofolate using immobilised enzymes (PAN)

Dihydrofolate (165 mg, 372 μmol) was suspended in 40 ml of de-aerated water to which dithiothreitol (79.4 mg, 490 μmol) had been added. The suspension was transferred to a 250 ml 3 necked flask equipped with a nitrogen inlet/outlet and a pH electrode connected to a 'pH stat' unit containing 1 M ammonium carbonate solution in the reservoir. The pH was brought to, and maintained at, 7.4. The dihydrofolate dissolved. The immobilised enzymes were combined to give approx. 1 U of each enzyme in the composite gel mass after centrifugation at 4°C.   The gel was transferred to the flask using 20 ml of water in total.   The enzymes needed were;   dihydrofolate reductase, glucose 6-phosphate dehydrogenase, hexokinase, and acetate kinase. $NADP^+$ (0.72 mg, 0.88 μmol),glucose 76 mg, 383 μmol), ATP (2.18 mg, 3.4 μmol), and $MgCl_2$ $6H_2O$ (49.5 mg, 245 μmol) were added to the flask using another 20 ml portion of water. Acetyl phosphate (108 mg, 387 μmol) was added over 3 days in approx. 3 equal portions to minimise non-enzymic hydrolysis. The pH of the solution was maintained at 7.4-7.6 and the reduction was carried out at ambient temperature, under nitrogen, using a magnetic stirrer to keep the gel suspended. The flask was protected from light. After 8 days the reduction was reasonably constant at 91%.   This corresponds to a recycling ratio of ATP and $NADP^+$ of 99 and 385 times respectively.   The reaction mixture was allowed to settle and the solution was decanted from the gel.   It was intended to use the gel for another reaction; however, the pH

electrode behaved erratically and the reaction was stopped. This alteration of the characteristics of the electrode was found to occur whenever the PAN gel was included as a reaction component. It was therefore difficult to carry out reduction automatically.

Immobilisation of enzymes on poly(acrylamide-co-N-acryloxy succinimide) (PAN)[19]

The active ester content of PAN was determined as described.[19] It was found to be 385 μ equiv g^{-1}, this was termed PAN 400.

Immobilisation of dihydrofolate reductase (E.C. 1.5.1.3)

PAN-400 (1 g, ca. 400 μmol of active ester groups) was placed in a 25 ml beaker containing a stirring bar and 5 ml of Hepes buffer pH 7.5, containing 0.32 mM dihydrofolate and 0.70 mM NADP$^+$ (approx. 100 times the respective Michaelis constants). To this was also added dithiothreitol (0.5 M, 100 μl). The polymer was dissolved within 90 s by mixing and rubbing against the beaker walls with a glass rod or a syringe plunger. The polymer solution was stirred magnetically for 30 s at room temperature to ensure complete solution and then triethylenetetramine (0.5 M, 340 μl) was added; approx. 60 s later 200 μl of a solution of dihydrofolate reductase form II or form I (1.04 mg, 3.3 units at pH 7.0) were added. In less than 2 min the solution set to a gel (either transparent or with a faint red/brown colour). The gel was allowed to stand for 1 h at room temperature to complete the coupling of enzyme and then transferred to a mortar. The gel was ground with a pestle for 2 min and 25 ml of Hepes buffer (50 mM, pH 7.5, containing 50 mM ammonium sulphate) were added. The grinding was continued for an additional 2 min. The gel suspension was diluted with a further 25 ml of the ammonium sulphate/Hepes buffer and transferred to a centrifuge tube. The suspension was stirred magnetically for 15 min. and separated by gentle centrifugation (~ 3000 rpm). The supernatant (I) was kept. The washing procedure was repeated once with the same volume of the buffer containing no ammonium sulphate and the supernatant (II) was kept. The gel particles were then resuspended in the same volume of Hepes buffer. The gel and the washes (I) and (II) were assayed for enzymatic activity. The activity of the gel was 0.6 U (18%), and 2.4 U (70%) were detected in the combined washes.

Much of the enzymic activity was recovered in the washes suggesting that the amino groups of this enzyme were comparatively unreactive. The immobilisation procedure was therefore modified by allowing the enzyme and the aqueous PAN solutions to react in the absence of crosslinking agent. Triethylenetetramine (TET) was then added and the immobilisation procedure followed the course described above. The results were as follows:

Enzyme added 60 s before TET; gel, 18% activity; washes, 78% activity.

Enzyme added 90 s before TET; gel, 25% activity; washes, 50% activity.

The effect of removing the cofactors was investigated. Without cofactors: gel 3%; washes 4%. With cofactors: gel, 5%; washes 69%.

The stability of dihydrofolate reductase in buffer (Hepes, 50 mM pH 7.5, 10 mM dithiothreitol) at 5°C, free and immobilised, was investigated. After 7 months the immobilised enzyme had retained 78% activity and the soluble enzyme had retained 71% of its activity. This compares favourably with the stability of the soluble enzyme in aqueous solution at 5°C where only 70% activity remained after 2 days.

The immobilisation procedure for the other enzymes was essentially that described above. The immobilisation yields compare favourably with those previously obtained.[19]

Glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49)

Glucose 6-phosphate dehydrogenase (yeast, 0.67 mg, 181 U) was immobilised in PAN-400 (260 mg, ca. 104 μ moles active esters). The Hepes buffer (0.3 M, pH 7.5) contained 15 mM $MgCl_2$, 6.3 mM glucose 6-phosphate and 1.0 mM $NADP^+$. The buffer for washing and storing contained 10 mM Mg^{2+} and 10 mM dithiothreitol. The specific activity of the enzyme was 270 $\pm$ 34 U/mg, at pH 7.6 and 25°C. The washes contained 13.98 U (8%) and the gel 26.5 U (15%) of the enzymic activity. A repeat reaction using 0.26 mg of enzyme (70.5 U) gave gel 14 U (20%) and washes 1.33 U (2%).

Hexokinase (E.C. 2.7.1.1.)

Hexokinase (4.85 mg, 53.4 U) was immobilised on PAN-400 (1.846 g, 738 μmoles of active esters). The buffer contained 15 mM Mg^{2+}, 25 mM glucose, and 10 mM ADP. The buffer for washing and storing contained 10 mM Mg^{2+} and 10 mM dithiothreitol. The specific activity of the enzyme was 11 U/mg. The washes contained 5.4 U (10%) and the gel 22.4 U (42%) of the enzymic activity.

Creatine kinase (E.C. 2.7.3.2.)

Creatine kinase (6.84 mg, 978 U) was immobilisd on PAN-400 (250 mg, 100 μmoles of active esters). The buffer contained 40 mM creatine phosphate, 10 mM ADP, 15 mM $MgCl_2$, and 100 mM KNO_3. The specific activity of the enzyme was 143 U/mg. The gel contained 82.2 U (8.5%); the washes did not contain any detectable activity.

Acetate kinase (E.C. 2.7.2.1.)

Acetate kinase contains disulphide bridges essential for activity. It is very sensitive to dioxygen and so the immobilisation step was done in a beaker under an atmosphere of nitrogen. The grinding and washing were done in air using Hepes buffer containing 10 mM dithiothreitol. The enzyme is supplied as a suspension in ammonium sulphate. It must be separated from this solution otherwise the ammonium sulphate reacts with the polymer forming a weak gel which soon breaks up. The separation can be done by dialysis or by centrifugation; either procedure was satisfactory but centrifugation was preferred because it was quicker.

A suspension of acetate kinase in ammonium sulphate (0.5 ml, 2.5 mg of enzyme, 405 U) was centrifuged at 15,000 rpm and 5°C, for 15 min. The pellet was dissolved in the Hepes/substrate solution (1.5 ml). The enzyme was immobilised on PAN-300 (2.5 g, 750 μmoles of active esters). The buffer contained 15 mM Mg^{2+}, 12.5 mM acetyl phosphate, and 20 mM ADP. The immobilisation up to the gel point was done in a stoppered beaker which had been flushed with nitrogen. After this the beaker was placed in a desiccator and kept under positive pressure of nitrogen. The buffer for washing and storing contained 10 mM Mg^{2+} and 10 mM dithiothreitol. The specific activity of the enzyme was 162 U/mg. The washes contained 170 U (42%) and the gel 168 U (42%) of the enzymic activity.

The preparation of cyanogen bromide activated Sepharose 4B[20]

The activated Sepharose was either purchased from Sigma or prepared in the following manner; Sepharose 4B (2 ml of packed gel) was washed with 25 ml of 1.4 M potassium phosphate buffer (pH 12.1) and sucked dry on a sintered glass filter funnel. The gel was then suspended in 2 ml of cold 5 M potassium phosphate buffer (pH 12.1) and to this was added 4 ml of distilled water. The suspension was cooled (5-10°C) and stirred magnetically. Cyanogen bromide (200 mg in 0.2 ml of acetonitrile) was added over 2 min. and the reaction was allowed to proceed for a further 10 min. The gel was then transferred to a sintered glass filter funnel and washed extensively with distilled water. The gel could now be used immediately for protein coupling; however, usually it was washed with acetone and stored desiccated *in vacuo* at 5°C before use. It was stable for up to 2

days at least. When a different degree of substitution was needed the procedure was the same except that the quantity of cyanogen bromide was varied.

General protein coupling procedure

The method employed was essentially that described in the Pharmacia handbook.[21] Care was taken to ensure that no non-specific protein adsorption occurred. The immobilisation was done at two different pH values. Buffer (A) was 50 mM sodium carbonate/sodium bicarbonate, pH 9.1; buffer (B) was 50 mM potassium phosphate, pH 7.5. The required amount of activated Sepharose was washed and re-swollen with 1 mM HCl (200 ml/g) if necessary. The support was then suspended in the coupling buffer containing the enzyme, and the suspension was stirred gently overnight (18 h) at 5°C. The support was collected and the filtrate assayed for enzyme activity. To block any remaining active sites in the support it was stirred at room temperature for 2 h. in 0.1 M Tris-Cl, pH 8.0 (10 ml/200 mg). Both of these solutions were 0.5 M in NaCl. The beads were then suspended in 50 mM potassium phosphate buffer pH 7.0 for assay and storage. The support and the Tris-Cl wash were assayed for enzyme activity. In some cases the other washes were also assayed.

Entrapment of dihydrofolate reductase in polyacrylamide beads[22]

Monomer mix: Acrylamide (1.9 g, 0.027 mol), N,N'-methylene diacrylamide (0.1 g, 0.65 mol), and tetramethylethylenediamine (300 µl, 2 mmol) were dissolved in 9.8 ml of 0.1 M Tris-Cl buffer pH 6.8. Dihydrofolate reductase (4.56 mg 15 U) was added to this solution and just prior to the addition of this aqueous phase to the hydrophobic phase an ammonium persulphate solution (100 µl, 0.04 mg/ml of buffer) was added.

Hydrophobic phase: To a 150 ml round bottomed flanged-topped flask was added 48 ml of toluene, 18 ml of chloroform and 0.8 ml of an emulsion stabilising agent (a 2:1 mixture of SPAN 80 : SPAN 85). This phase was stirred at a temperature of 4°C under nitrogen for at least 30 min. The stirrer shaft and blade were composed of steel. The shaft fitted into an indent in the bottom of the flask. The stirring rate was set at 500 rpm. After addition of the aqueous phase the mixture was stirred under nitrogen for 35 min. to complete polymerisation. The small, regular beads were filtered on a porous sintered funnel. They were then washed in ice cold toluene (50 ml, 10 min), 0.1 M NaHCO$_3$ (50 ml, 45 min), 0.5 M NaCl (50 ml, 45 min) and 50 mM Tris-Cl pH 7.0 containing 0.1 mM EDTA and 1 mM 2-mercaptoethanol. The washed beads were then suspended in 50 mM Hepes pH 7.5 in a 100 ml volumetric flask. The Tris buffer wash contained 0.1 U (0.6%) and beads contained 0.36 U (2.4%) of the enzymic activity.

Immobilisation of dihydrofolate reductase on AH-Sepharose-4B by physical adsorption[21]

AH-Sepharose 4B (250 mg) was washed at room temperature with 2 x 25 ml portions of 0.5 M NaCl (5 min. each) and   3 x 25 ml of 50 mM KH$_2$PO$_4$, pH 7.0 containing 5 mM 2-mercaptoethanol, (5 min. each). Dihydrofolate reductase form II (1.87 mg, 6.2 U) was added to the support in 50 mM KH$_2$PO$_4$ pH 7.0 containing 0.32 mM dihydrofolate. The suspension was stirred gently at 5-10°C for 2 h. The beads were filtered on a sintered glass funnel and the filtrate retained. After washing the beads in 5 ml of buffer they were then suspended in a further 5 ml of buffer and assayed. The beads contained 0.5 U (8%) and the combined washes 0.06 U (1%) of the enzymic activity. To ascertain the level of desorption in buffer solutions the beads were again filtered and washed twice in 50 mM KH$_2$PO$_4$ buffer for 50 min. and then with 10 mM KH$_2$PO$_4$ for 50 min. The activity in these washes (3, 4, and 5), on the beads, and in the original assay buffer was determined.

Original assay buffer	0.023 U,
wash 3	0.0372 U
wash 4	0.0372 U
wash 5	0 U
beads	0. 34 U

Dihydrofolate reductase (1.84 mg, 6 U) was also immobilised on 250 mg of AH-sepharose-4B using water instead of buffer. The aqueous solution contained 0.32 mM dihydrofolate and 10 mM 2-mercaptoethanol. It was adjusted to pH 7.0 with ammonium carbonate. The washes contained no activity and the beads 0.7 U (11.6%). The effect of removing dihydrofolate was investigated; thus dihydrofolate reductase (2.12 mg, 7 U) was immobilised on the Sepharose (250 mg) in water. There was no activity in the washes while the beads contained 0.7 U (10%). The beads were then stirred in a 5 mM dihydrofolate solution at room temperature for 45 min. to simulate the reaction conditions. The activity in the dihydrofolate solution was 0.4 U and on the beads, 0.46 U. This is a reduction of 34% of the activity of the beads. When a weak buffer solution (10 mM) or an aqueous medium is used all of the enzyme is adsorbed onto the beads. However, only about 10% of the activity is expressed. This means that the enzyme is probably immobilised in a non-active conformation.

Preparation of alkoxyaminoboranes

(S)-Phenylalaninol-borane   A 25 ml 2 neck flask equipped with a magnetic stirring bar, a nitrogen inlet/outlet and a pressure equalising dropping funnel was charged with (S)-phenylalaninol (4) (1.02 g, 6.8 mmol) and tetrahydrofuran (10 ml). The amino alcohol was not completely soluble. The mixture was cooled to -60°C and borane-dimethyl sulphide (0.7 ml, 7 mmol) in 5 ml of tetrahydrofuran was added over 20 min. After stirring at -60°C for 3 h (dissolution obtained) the solution was allowed to warm to room temperature and was left stirring overnight. The solvent was removed in vacuo and a gummy white residue was obtained. ν_{max} (CHCl$_3$) 3620, 3300, 3260, 2940, 2440, 1580 and 1160 cm^{-1}. $[\alpha]_D$ = - 14.6° (c 0.7 CHCl$_3$). T.l.c.; Watmans silica gel SG41, n-propanol: NH$_4$OH: acetone; 7:3:1, one spot rf. 0.51.

The following alkoxyamine-borane complexes were prepared by the above method.

(S)-Valinol-borane   1 g (9.69 mmol) of (S)-valinol (3) gave 1.346 g of a viscous liquid. ν_{max} (CHCl$_3$) 3620, 3320, 3260, 2960, 2870, 2310, 1375, 1460, and 1160 c^{-1}. $[\alpha]_D$ = + 10.5° (c 2.39, CHCl$_3$). T.l.c., one spot, rf. 0.34.

(S)-Leucinol-borane   0.848 g (6.75 mmol) of (S)-leucinol (6) yielded 0.895 g of a colourless viscous liquid. ν_{max} (CHCl$_3$) 3620, 3315, 3260, 2950, 2870, 2340, 1580, 1460, 1365, and 1160 cm^{-1}. $[\alpha]_D$ = + 15.4° (c 1.99, CHCl$_3$). T.l.c., one spot, rf. 0.48.

(S)-Alaninol-borane   0.789 g (10.52 mmol) of (S)-alaninol (5) yielded 1.05 g of a viscous liquid. ν_{max} (liquid film) 3450, 3230, 3140, 2970, 2935, 2870, 2320, 1590, and 1165 cm^{-1}. $[\alpha]_D$ = + 25.8° (c 2.23, tetrahydrofuran). T.l.c., one spot, rf. 0.39.

(S)-Prolinol-borane   1.025 g (10.13 mmol) of (S)-prolinol (7) gave 1.17 g of a viscous liquid. ν_{max} (CHCl$_3$) 3620, 3245, 2955, 2880, 2300, 1455, 1395, 1153, and 1066 cm^{-1}. $[\alpha]$ = -12.9° (c 1.9, CHCl$_3$). T.l.c., one spot, rf. 0.66.

Trans-(4S,5S)-4-hydroxymethyl-2-methyl-5-phenyl-2-oxazoline-borane   1 g (5.22 mmol) of the oxazoline (8) gave 1.128 g of a solid after trituration with petrol (40-60°). ν_{max}, (liquid film) 2320, and 1660 cm^{-1} $[\alpha]_D$ = -144.1° (c 2.25, tetrahydrofuran). Reaction of 1 g (5.22 mmol) of the oxazoline with 2 mol. equivalents of borane yielded a white amorphous solid, 1.11 g. ν_{max} (KBr) 2320 and 1655 cm^{-1} $[\alpha]_D$ = -132.1° (c 2.28, tetrahydrofuran).

(S)-2-Amino-1-phenylethanol-borane   0.5 g (3.65 mmol) of (S)-2-amino-1-phenylethanol (9)
gave 0.69 g of a white solid. ν_{max} (CHCl$_3$), 3590, 3505, 3390, 3310, 3260, 3220, 2980,
2960, 2870, 2310, 1587, 1395, 1160, and 1050 cm^{-1}. $[\alpha]_D$ = +75.6°(c 1.90, CHCl$_3$).

(+)-Norephedrine-borane   1 g (6.6 mmol) of (+)-norephedrine (10) gave 0.89 g of a white
solid. ν_{max} (CHCl$_3$) 3590, 3305, 3235, 2965, 2930, 2860, 2335, 1580 and 1325 cm^{-1}.
$[\alpha]_D$ = +37.9 (c. 0.86, CHCl$_3$).

(+)-Ephedrine-borane   1 g (6.05 mmol) of (+)-ephedrine (11) yielded 1.34 g of a viscous
liquid. ν_{max} (CHCl$_3$) 3595, 3480, 3250, 2970, 2940, 2870, 2330, 1450, 1380, 1155, and
1050 cm^{-1}. $[\alpha]_D$ = +24.0°(c 2.92 CHCl$_3$).

(-)-Ephedrine-borane   1 g (6.05 mmol) of (-)-ephedrine (11) gave 1.19 g of a viscous
liquid. ν_{max} (CHCl$_3$) 3595, 3480, 3250, 2970, 2940, 2870, 2330, 1450, 1380, 1155, and
1050 cm^{-1}. $[\alpha]_D$ = -22.9°(c 2.86, CHCl$_3$).

Reduction of dihydrofolate using alkoxyamino-borane complexes

Dihydrofolic (5 mg) acid was dissolved in the minimum volume of 0.1 M NaOH and the pH
was adjusted to 7.0 with HCl if necessary.   To this solution was added 2 ml of one of the
solvent systems given below and then the alkoxy-amine-borane was added in one portion.
The reaction was stirred under nitrogen until completion (hplc).   An excess of the hydride
reagent was used since competing solvolysis was expected.   The isomeric excess was
determined as described earlier and results are listed in Table 3.

(A) Water containing 10 mM dithiothreitol : tetrahydrofuran, 5:3.

(B) 20 mM Tris-Cl, pH 8.0, containing 10 mM dithiothreitol :   tetrahydrofuran, 5:3.

(C) 20 mM Tris-Cl, pH 8.0, containing 10 mM dithiothreitol : ethanol, 5:3.

Benzyloxycarbonylamino acids were prepared by published procedures[32-35]

Preparation of chiral sodium triacyloxyborohydrides by reaction of sodium borohydride with N-benzyloxycarbonyl-(S)-amino acids[29]

N-Benzyloxycarbonyl-(S)-alanine (1 g, 4.48 mmol) was added to a stirred suspension of
sodium borohydride (57.6 mg, 1.493 mmol) in dry tetrahydrofuran (10 ml) at 5-10°.   After
hydrogen evolution the mixture was stirred at room temperature for 3 h and then
concentrated in vacuo.   The residue was digested with petrol (60-80°) and filtered to give
the triacyloxyborohydride as a white powder 0.955 g, 91%, m.p. 44-52° (dec). ν_{max} (KBr)
2520 cm^{-1}.

The N-benzyloxycarbonyl derivatives of the following amino acids were reacted by the above
procedure.   The derivatives were obtained as white amorphous powders.

CBzO-(S)-Phenylalanine (1.0 g, 3.3 mmol) and sodium borohydride (42 mg, 1.1 mmol) yielded
0.97 g (93.4%), m.p. 58-61° (dec). ν_{max} 2460 cm^{-1}.

CBzO-(S)-Proline (1.0 g, 4.01 mmol) and sodium borohydride (51 mg, 1.67 mmol) yielded 0.94
g (90%), m.p. 56-60° (dec). ν_{max} 2460 cm^{-1}.

CBzO-(S)-Serine (1.0 g, 4.19 mmol) and sodium borohydride (53.8 mg, 1.39 mmol) yielded
1.006 g (96%), m.p. 83-108° (dec). ν_{max} 2610 cm^{-1}.

CBzO-(S)-Valine (1.0 g, 3.98 mmol) and sodium borohydride (51 mg, 1.32 mmol) yielded 0.853
g (82%), m.p. 48-70° (dec). ν_{max} 2540 cm^{-1}.

Attempted reduction of dihydrofolate using chiral triacyloxyborohydrides

The hydride derivatives were added to an approximately 1 mM solution of dihydrofolate
(5-10 ml) in 3.5-7 fold molar excess.   The reaction was conducted at pH 7.0-8.0, under
nitrogen, and at room temperature.   The reactions were allowed to proceed for 1 to 3 days

and were followed by hplc.   Less than 5% reduction was achieved in all cases except that
of the proline derivative.   This gave 70% reaction, using a 7-fold molar excess of complex
hydride, after 3 days.   Hplc analysis of isomeric excess indicated that no chiral
induction was achieved.

REFERENCES

1.   'Enzymes in Organic Synthesis', Ciba Foundation Symposium 111, R. Porter and S. Clark (eds.), Pitman, London 1985.
2.   G.M. Whitesides, and C-H. Wong, Aldrichimica Acta, 1983, 16, 27.
3.   V.G. Blaschke, H.P. Kraft, K. Fickentscher, and F. Kohler, Arzneimittel Forsch., 1979, 29, 1640.
4.   J.C. Fontecilla-Camps, C.E. Bugg, C. Temple, J.D. Rose, J.A. Montgomery, and R.L. Kisliuk, J. Am. Chem. Soc., 1978, 101, 6114.
5.   P.A. Charlton, D.W. Young, B. Birdsall, J. Feeney, and G.C.K. Roberts, J. Chem. Soc., Chem. Commun., 1979, 922.
6.   J.F. Bender, W.R. Grove, and C.L. Fortner, Amer. J. Hosp. Pharm., 1977, 34, 961.
7.   B.A. Chabner and M. Slavik, Chemother. Rep., 1975, 6, 1; I. Djerassi, ibid., 3; J.S. Penta, ibid., 6.
8.   R.P. Leary, Y. Gaumont, and R.L. Kisliuk, Biochem. Biophys. Res. Commun., 1973, 56, 484.
9.   V.F. Scott and K.O. Donaldson, Biochem. Biophys. Res. Commun., 1964, 14, 523.
10. P.H. Boyle and M.T. Keating, J. Chem. Soc., Chem. Commun., 1974, 375.
11. C.K. Mathews and F.M. Heunnekens, J. Biol. Chem., 1969, 235, 3304.
12. L. Rees, E. Valente, C.J. Suckling, and H.C.S. Wood, in 'Chemistry and Biology of Pteridines', J. Blair (ed.), de Gruyter, Berlin, 1973, p. 533; C.J. Suckling in 'Enzymes in Organic Synthesis', Ciba Foundation Symposium 111, R. Porter and S. Clark (Eds.), Pitman, London, 1985, p. 161.
13. D. Baccanari, A.W. Phillips, S. Smith, D. Sinski, and J.J. Burchall, Biochemistry, 1975, 14, 5267.
14. S. Futterman, J. Biol. Chem., 1957, 228, 1031; R.L. Blakley, Nature, 1960, 188, 231.
15. W.H. Pirkle and M.S. Hoekstra, J. Org. Chem., 1974, 39, 3904.
16. C.H. Wong and G.M. Whitesides, J. Am. Chem. Soc., 1981, 103, 4890.
17. J.M. Lewis, S.L. Haynie, and G.M. Whitesides, J. Org. Chem., 1979, 44, 864; G.M. Whitesides, M. Siegel, and P. Garrett, ibid, 1975, 40, 2516.
18. N.D. Danieson and J.A. Huth, J. Chromatog., 1980, 221, 39.
19. A. Pollak, H. Blumenfeld, M. Wax, R.L. Baughn, and G.M. Whitesides, J. Am. Chem. Soc., 1980, 102, 6324.
20. R. Axen, J. Porath, and S. Ernback, Nature, 1967, 214, 1302; J. Porath and R. Axen in 'Methods in Enzymology', Ed. K. Mosbach, Academic Press, 1976, vol. 44, p.19.
21. 'Affinity Chromatography - Principles and Methods', Pharmacia Fine Chemicals, Uppsala, Sweden, 1976.
22. A-C. Johansson and K. Mosbach, Biochem. Biophys. Acta., 1974, 370, 339.
23. F. Ahmed and R.B. Dunlap in 'Chemistry and Biology of Pteridines, Ed. J. Blair, de Gruyter, Berlin, 1983, p. 539.
24. A. Pelter and K. Smith in 'Comprehensive Organic Chemistry', Ed. D.N. Jones, Pergamon, Oxford, 1979, vol. 3, p 925.
25. A. Hirao, S. Itsomo, S. Nakahama, and N. Yamazaki, J. Chem. Soc., Chem. Commun., 1981, 315; S. Itsomo, K. Ito, A. Hirao and S. Nakahama, J. Chem. Soc., Chem. Commun., 1983, 469.
26. C.F. Lane, Chem. Rev., 1976, 76, 773.
27. A.I. Meyers, G. Kraus, K. Kamata, and M.E. Ford, J. Am. Chem. Soc., 1976, 98, 567.
28. A.I. Meyers and J. Slade, J. Org. Chem, 1980, 45, 2785.
29. K. Yamada, M. Takeda, and T.Iwakuma, Tetrahedron Lett.,1981, 22, 3869.
30. C.K. Mathews and F.M. Huennekens, J. Biol. Chem., 1963, 238, 3436.
31. C. Temple, R.D. Elliot, J.D. Rose, and J.A. Montgomery, J. Med. Chem., 1979, 22, 731.
32. M. Bergman and C. Zervas, Chem. Ber., 1932, 65, 1192.
33. H. Schwarz, F. Merlin Bumpus, and I.H. Page, J. Am. Chem. Soc., 1957, 79, 5697.
34. W. Grassman and E. Wünsch, Chem. Ber., 1956, 91, 449, 462; W. Grassman, E. Wünsch, and A. Reidel, ibid., 1958, 93, 455.
35. A. Berger, J. Kurtz, and E. Katchalski, J. Am. Chem. Soc., 1954, 76, 5552.