ASYMMETRIC REDUCTION USING ENZYMES AND CHIRAL CHEMICAL REAGENTS

T H E S I S

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DEGREE OF DOCTOR OF PHILOSOPHY

by

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For my Parents and Irene

A C K N O W L E D G E M E N T S

I would like to express thanks to Professor Hamish C.S. Wood for making much of organic chemistry interesting and intelligible, both as a tutor and as a supervisor.

For advice and helpful discussion during the project I am also indebted to Dr. Colin J. Suckling and Dr. Iain R. Dunkin.

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C O N T E N T S

SUMMARY

This thesis is concerned with the asymmetric reduction of achiral substrates to chiral products on a preparative scale.

In the first case dihydrofolate reductase was used to catalyse the conversion of 7,8-dihydrofolic acid into (6S)-5,6,7,8-tetrahydrofolic acid. The required coenzyme, NADPH, was recycled 1100 fold *in situ* using coupled enzyme techniques. The reaction was quantitative and complete stereospecificity was demonstrated using chiral naphthyl isocyanates and HPLC. (6R)-5,10-Methenyltetrahydrofolate was isolated in 28% yield and hydrolysed to give (6S)-5-formyltetrahydrofolate. The necessary enzymes were immobilised for recovery and re-use. However, despite extensive experimentation, a sufficiently active immobilised dihydrofolate reductase could not be obtained. This was primarily due to the low intrinsic activity of the enzyme.

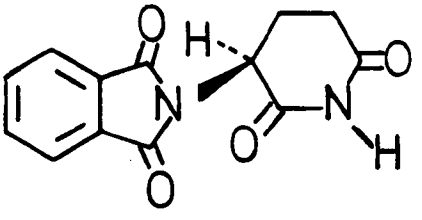
To complement the enzymic studies the chiral chemical reduction of dihydrofolic acid was investigated. The separation of the C6 epimers was thus particularly important for the assessment of the efficiency of the chiral reagents. Two types were investigated a) borate complexes of β-amino alcohols and b) triacycloxyborohydrides prepared from chiral amino acids. The former gave modest chiral induction (up to 18% isomeric excess) whilst the latter were inert. The enzymic route would therefore seem to be the best available.

In the second case tetrahydropyran-3-one was reduced using the chiral catalytic properties of the enzyme horse liver alcohol dehydrogenase. The coenzyme, NADH, was recycled 800 fold using

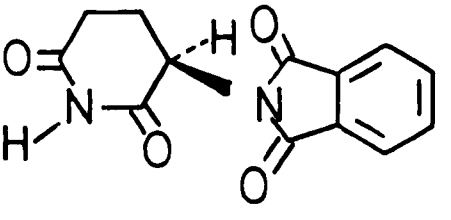
the same methodology as for NADPH. Tetrahydropyran-3-ol was obtained in 49% enantiomeric excess. Acetolysis of the chiral brosylate was undertaken to ascertain whether R_2O_3 anchiomeric assistance was operating during the reaction. This suggestion had previously been surmised from kinetic results. Racemisation occurred during the solvolysis arguing against neighbouring group participation.

CHAPTER 1

The biological properties of organic molecules depend, in large measure, on their stereochemistry. This is true for drugs, insecticides, plant growth regulators, perfumes and flavouring compounds. Thus the biological activity of one enantiomer often differs completely from that of its mirror image. That there is a need for efficient ways of preparing active substances of high stereochemical purity can be demonstrated by example. The thalidomide tragedy was probably caused by use of the racemic drug for it has now been shown, at least in animals, that teratogenic activity comes only from the S-isomer (1.1); the R form (1.2) leads to no deformities.[1]



(S)-Thalidomide (1.1)



(R)-Thalidomide (1.2)

In the broadest sense any preparation of a chiral molecule can be regarded as an asymmetric synthesis. However, this project is concerned with asymmetric synthesis based on the following definition: 'Asymmetric synthesis is a reaction in which an achiral unit in an ensemble of substrate molecules is converted by a reactant into a chiral unit in such a way that the

stereoisomeric products (enantiomeric or diastereomeric) are formed in unequal amounts'.[2] This definition therefore excludes those methods based on resolution or on use of a building block from the chiral pool such as amino acids, sugars, terpenes etc.

We are concerned then,with stoichiometric or catalytic synthesis using chiral reagents. These methods are generally subclassified into microbiological/enzymatic methods and chemical methods. It is pertinent to note that this distinction is not real,in that all methods of preparing optically active compounds are ultimately dependent on materials of natural origin. The requirements for an efficient asymmetric synthesis have been reviewed by Eliel.[3]

(a) The synthesis must lead to the desired enantiomer with high stereoselectivity and high yield.

(b) The chiral product must be readily separable from the chiral reagent.

(c) Unless the chiral reagent is much cheaper than the product it must be capable of being recovered with undiminished optical purity.

We can further expound and state that it would be desirable to use a chiral reagent which is catalytically active because then, in principle, an unlimited amount of chiral product could be produced. While enzymes fit most of the criteria listed above, their use in vitro can be difficult. However, if a suitable system can be formulated, their ability as chiral catalysts cannot be surpassed.

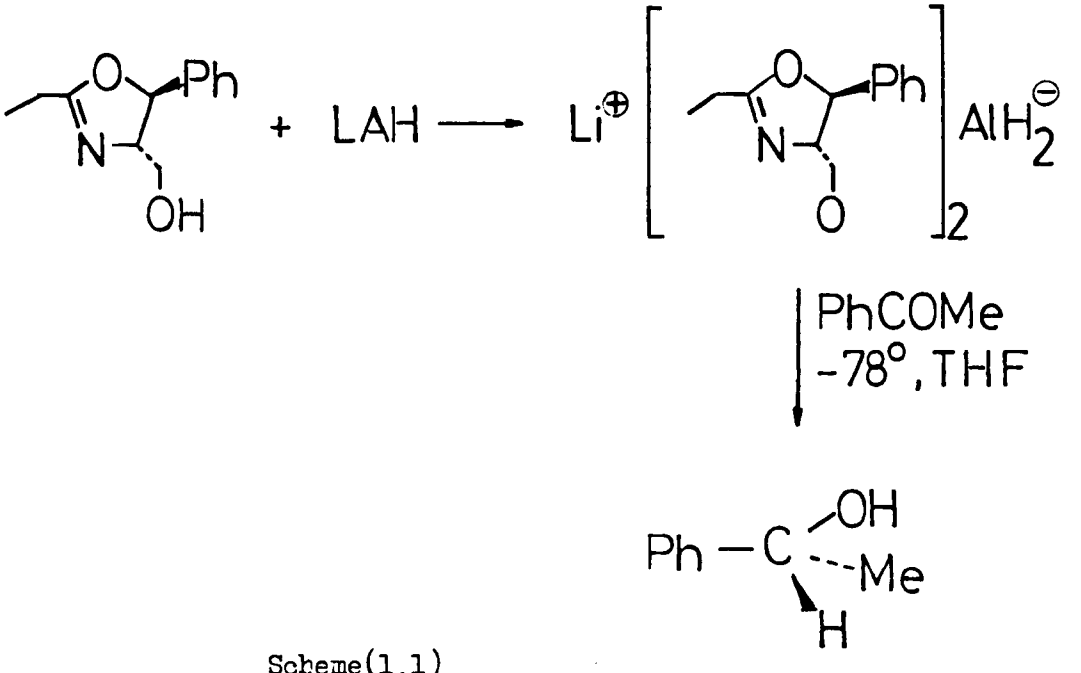
The use of chirally modified chemical reagents to effect stereospecific reactions is also a fast growing research area. High optical yields can be accomplished (greater than 90%) but much work must be done to find general methods. The optimisation of optical yields remains essentially empirical, requiring many experiments.

These matters are discussed in general detail in the following sections of this chapter. The particular topics chosen for extensive review are those most directly related to the principal subject matter of this thesis. This mainly involves a study of the asymmetric reduction of a derivative of folic acid. The section dealing with enzyme mediated synthesis extends beyond the brief of asymmetric reduction. This was done mainly because it was felt that the novelty of the subject required a more complete review.

CHIRAL METAL HYDRIDE COMPLEXES

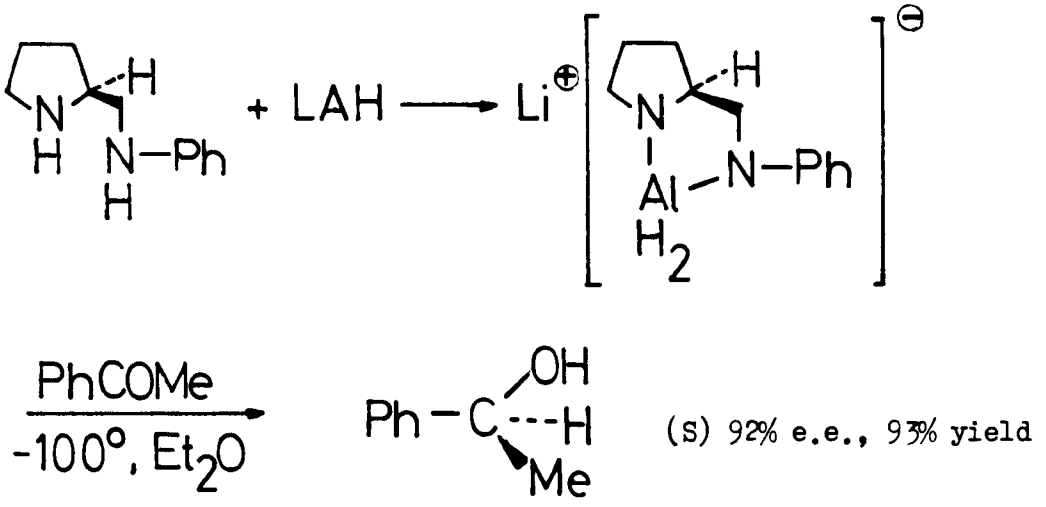
The use of metal hydrides in which chiral organic moieties are ligated to the metal is perhaps the most familiar method of effecting asymmetric reduction. The use of reagents derived from lithium aluminium hydride (LAH) is well documented in reviews of asymmetric synthesis.[4] Borane and borohydride derivatives have received less attention.[5]

Briefly, lithium aluminium hydride has been modified with chiral alcohols[6] (including carbohydrates and diols) 1,2-amino alcohols[7], and amines[8]. The bidentate ligands seem preferable as they show less tendency to dissociate and also form a fairly rigid structure leading to higher stereoselectivity. These reagents are effective in inducing asymmetry in the reduction of the stereoheterotopic faces of the carbonyl group of aldehydes and phenyl alkyl ketones. Some examples are given for the reduction of acetophenone using a reagent derived from a chiral oxazoline (Scheme 1.1)[7b] and a reagent prepared from a chiral di-amine (Scheme 1.2).[8a]



Scheme(1.1)

(R) 65% e.e., 81% yield



Scheme (1.2)

A more recent development has been the use of chiral hydride reagents prepared from lithium aluminium hydride and (S)(-)-2,2'-dihydroxy-1,1'-dinaphthyl and ethanol. Enantiomeric excesses (e.e)* of as high as 100% were achieved in the reduction of acetophenone.[9]

The reactivity and solubility properties of derivatives based on lithium aluminium hydride confines their use to ethereal solvents. These reasons make their use unsuitable for the proposed reaction and they are not discussed further.

Chiral Trialkyl Boranes

Certain B-alkyl-9-borabicyclo[3.3.1]nonanes (B-alkyl-9-BBN) were shown to reduce benzaldehyde under mild conditions.[10] This observation was extended to the reduction of 1-deuteriobenzaldehyde using various chiral alkyl-9-BBN reagents.[11] Optically active terpenes were used to prepare the reagent, (+)-α-pinene(1·3) being the most effective.

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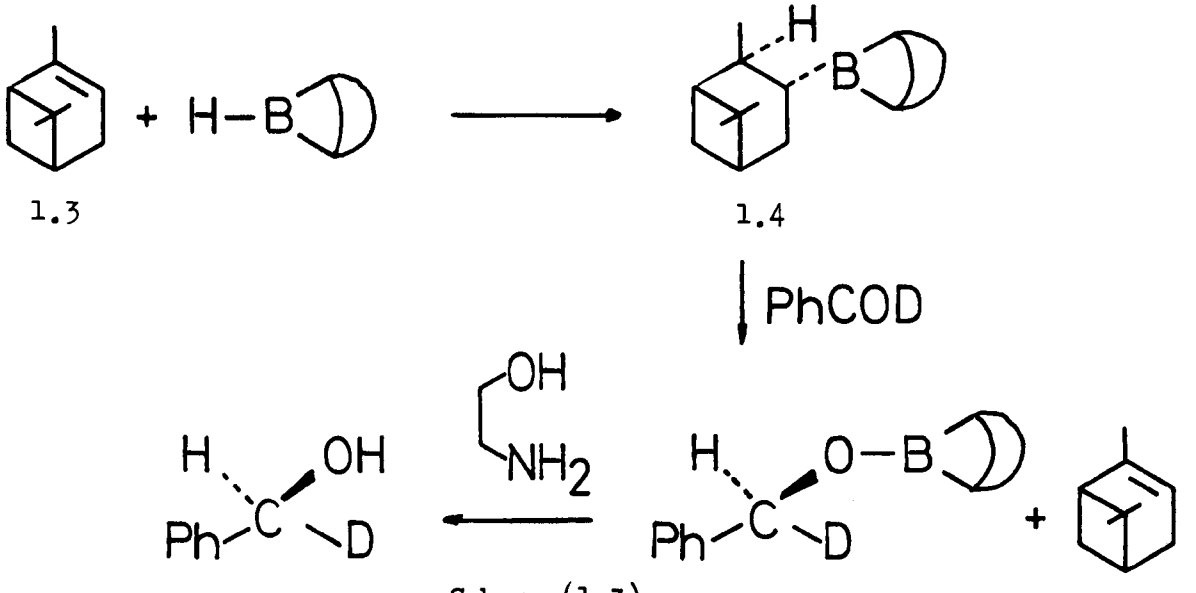
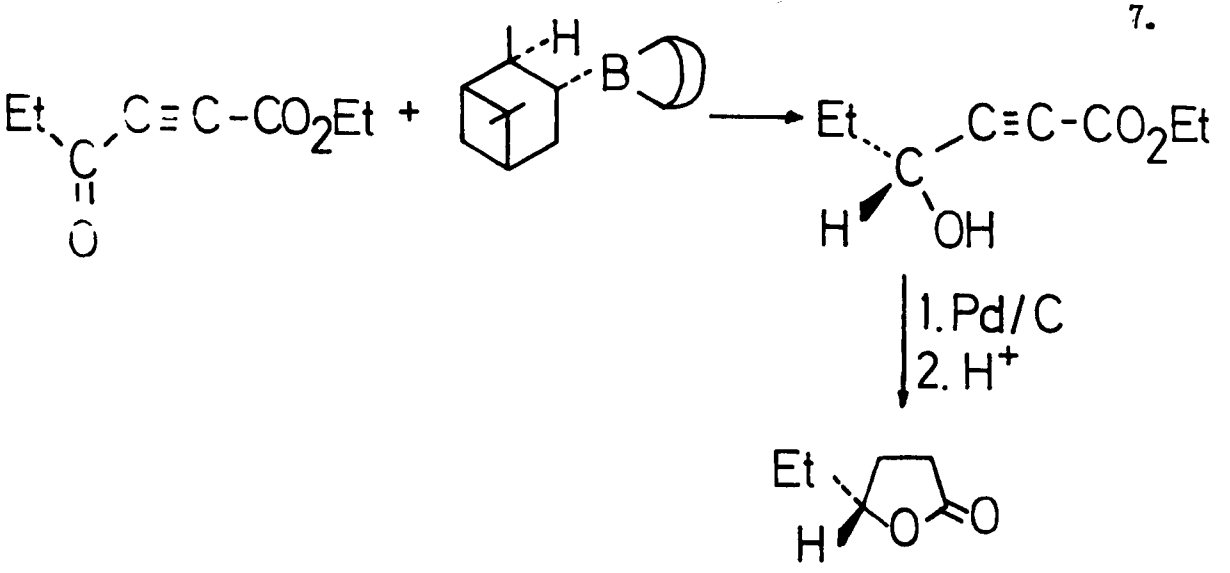
* Enantiomeric excess is defined as the percentage of the major isomer present minus the percentage of the minor isomer. It is usually (but not always) equivalent to optical purity which, expressed as a percentage, is defined as the rotation of the product divided by the rotation of the pure isomer x 100.

In the reduction with B-3-pinanyl-BBN (1.4) the (S)-alcohol was produced in effectively 100% optical yield. The availability of the deuteriated borane reagent also allowed the preparation of a variety of chiral deuterioalcohols from the corresponding aldehydes in e.e.'s ranging from 71-100% (Scheme 1.3). The products are used for mechanistic studies in chemistry and biochemistry.



Scheme (1.3)

B-3-pinanyl-BBN (1.4) will also reduce a variety of α,β-acetylenic ketones to the corresponding propargylic alcohols in 73-100% e.e.[12] These chiral alcohols are useful synthetic intermediates. For example 4-substituted-γ-lactones (often found as pheromonal constituents) can be prepared in this manner. (Scheme 1.4).[13]



Scheme (1.4)

The asymmetric reduction of aliphatic ketones with reagent (1.4) was less satisfactory. Slower reaction times led to dissociation of the reagent and consequently racemic products resulted. Recently Brown[14] circumvented this problem by using the material neat rather than in solution. Ketones of the type R.CO.Me where R = alkyl, aryl, alkenyl, and oxoalkyl were reduced with fair optical yields. For example, 2-butanone and 2-octanone were reduced to the corresponding alcohols in good yields and with 40 and 44% e.e. respectively.

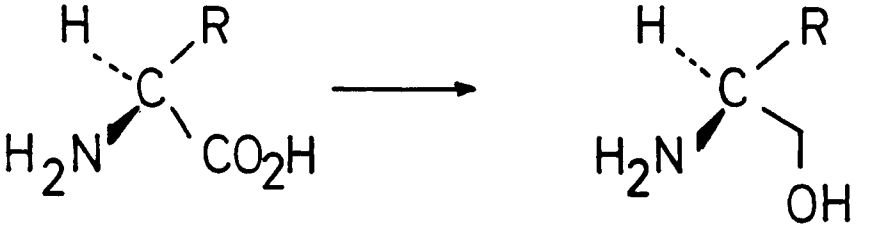
There are no reports of the use of these compounds for the asymmetric reduction of imines. Since reduction of this particular functional group is central to a large part of this research project, these reagents have not been examined further.

Amine and Amino-alcohol Borane Complexes

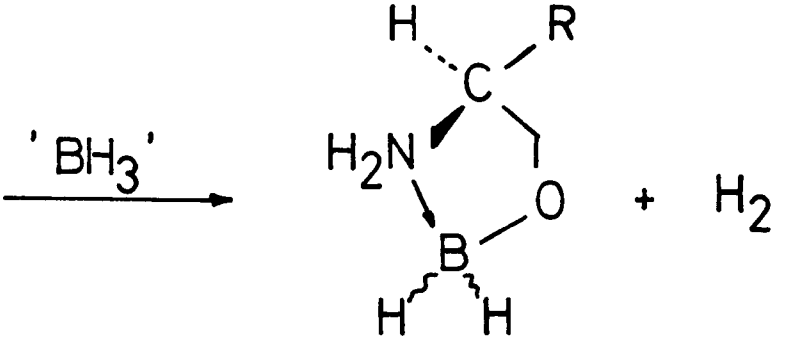
Amine borane complexes[15] have been studied as reducing agents for alkenes, carbonyl groups, and imines. These hydrides demonstrate

good thermal and hydrolytic stability and are soluble in a variety of protic and aprotic solvents. Application of these complexes to the asymmetric reduction of ketones was first reported in 1969.[16] The chiral amine ephedrine was used to prepare the reagent and modest chiral inductions of 5% e.e. were achieved. Similar experiments have been carried out using borane complexes prepared from (R) and (S) - α-methylbenzylamine[17,18] or from α-amino esters in the presence of boron trifluoride etherate.[19] Up to 20% e.e. was achieved in the asymmetric reduction of ketones.

These studies have been extended to the use of alkoxy amine boranes derived from chiral 1,2-amino alcohols (Scheme 1.5).[20,21,22]



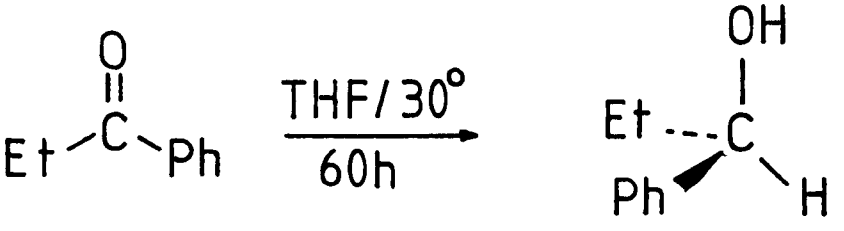
(S)- amino acid



Scheme (1.5)

It is suggested that the reagents consist of a relatively rigid 5-membered ring as shown (Scheme 1.5). Recent work, however, has cast some doubt on the validity of this structure.[23]

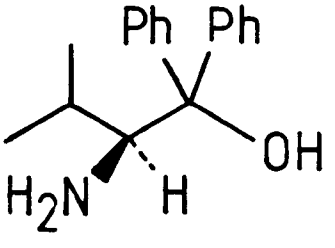
Whatever the structure, reagents derived from (S)-α-amino acids (proline, valine, leucine, and phenylalanine) were successful in reducing aryl ketones stereoselectively (eqn. 1.1).[21]



(Eqn. 1.1)

60% e.e., 100% yield

This work was extended to the use of the reagent prepared from (S)(-)-2-amino-3-methyl-1,1-diphenylbutan-1-ol (1.5) and borane.[22]

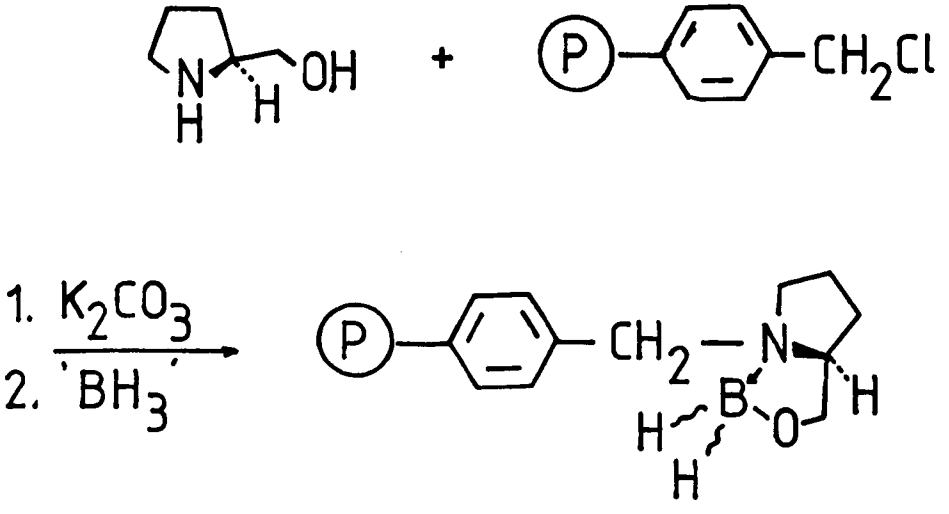


(1.5)

Amino alcohol (1.5) was prepared from (S)-valine methyl ester hydrochloride and an excess of phenyl magnesium bromide. The hydride reduced acetophenone and propiophenone to the corresponding optically active (R)- alcohols in 94% e.e.

The possibility of using these reagents in an aqueous environment extends their versatility, and their reported utility for the reduction of imines suggests that they might well be useful in the proposed research.

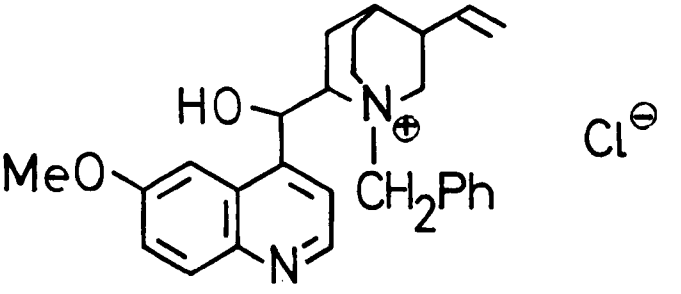
To aid recovery of the chiral moiety polymeric derivatives have been prepared. Chiral boranes attached to a polymeric backbone gave low optical yields[24] but a chiral 1,2-amino alcohol attached to chloromethylated polystyrene gel (Scheme 1.6) gave good optical yields (60% e.e.) in the reduction of propiophenone. The ability to induce asymmetry did not diminish on re-use at least twice.[25]



Scheme (1.6)

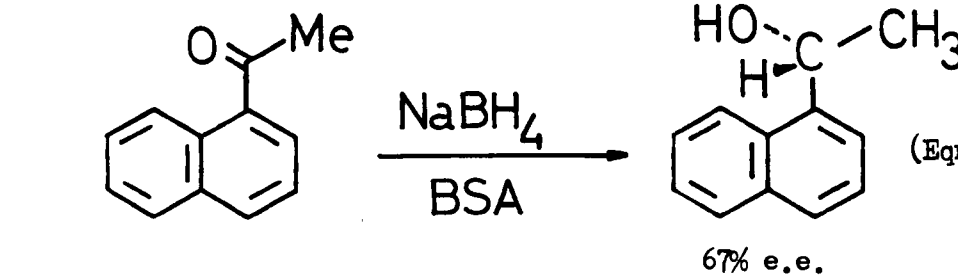
Chiral Borohydrides

Sodium borohydride itself has been used to effect asymmetric reduction of ketones in a phase transfer reaction using an optically active 'onium' salt.[26] For example, using benzyl quininium chloride (1.6) t-butyl phenyl ketone was reduced to the corresponding alcohol in 32% e.e. and 95% yield.



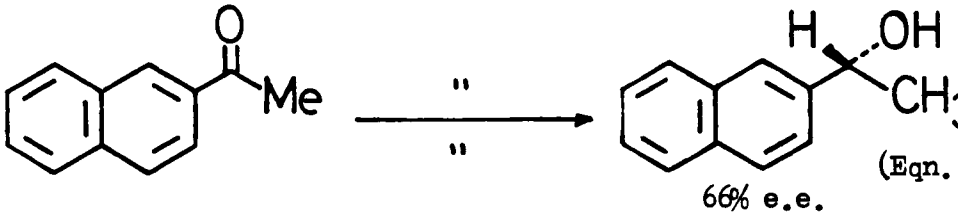
(1.6)

Sodium borohydride in the presence of bovine serum albumin (BSA) also reduced aryl ketones in fair optical yield[27] (eqn 1.2 and 1.3).



(Eqn. 1.2)

67% e.e.



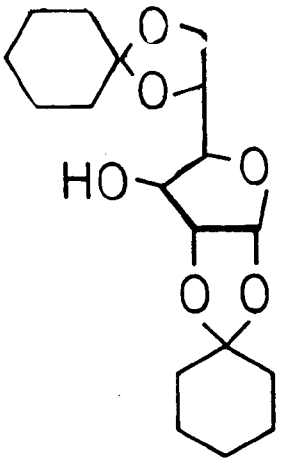
(Eqn. 1.3)

66% e.e.

The high optical induction in these cases is probably due to the fact that naphthalene itself is a good guest compound for bovine serum albumin.

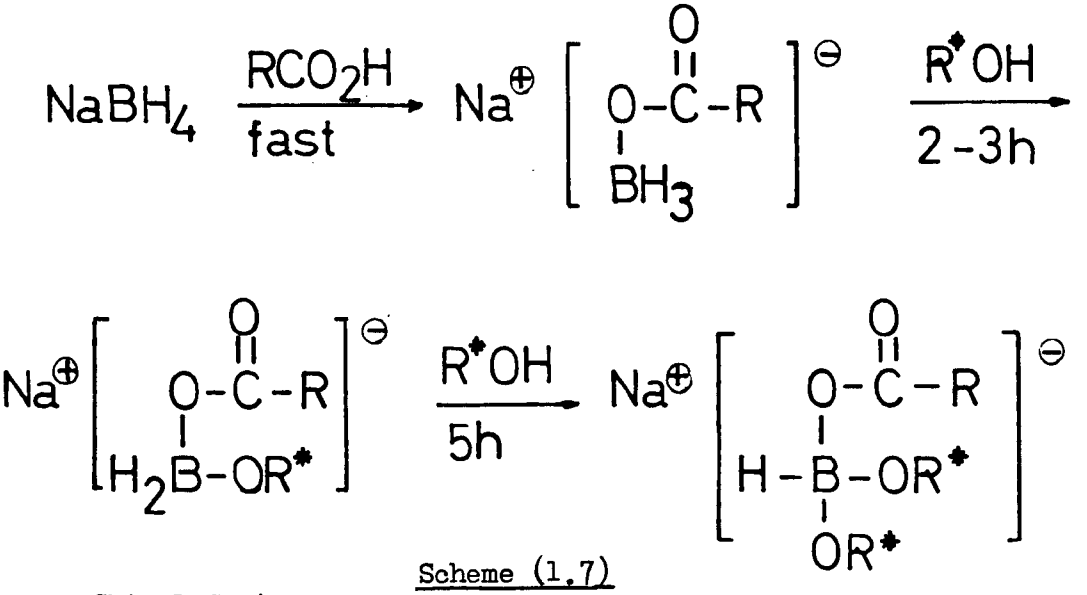
It is generally true that the less reactive a reagent is, the more selective it will be. This is certainly a concept to bear in mind when the synthesis of any chiral reducing agent is contemplated. For example, acyloxy borohydrides, in particular sodium triacetoxy borohydride, will reduce aldehydes but not ketones; this is in contrast with most other hydrides.[28] The reagent is prepared from sodium borohydride and acetic acid in the ratio 1:3 to give $NaBH(OAc)_3$. The mild reducing characteristics may be attributed both to the bulky nature of the reagent and the inductive electron-withdrawing ability of the three acetoxy groups which stabilise the boron hydrogen bond. It would be reasonable to suppose that asymmetric induction might be achieved, using these types of reducing agent, if the carboxylic acid was chiral.

An acyloxy intermediate has indeed been suggested in an asymmetric reducing system comprised of sodium borohydride, a carboxylic acid, and 1,2:5,6-di-O-cyclohexylidene-D-glucofuranose (DCHGF) (1.7).[29]



(1.7)

Aryl ketones are reduced in 35-50% e.e. using this system and the initial formation of an acyloxy borohydride was suspected (Scheme 1.7).



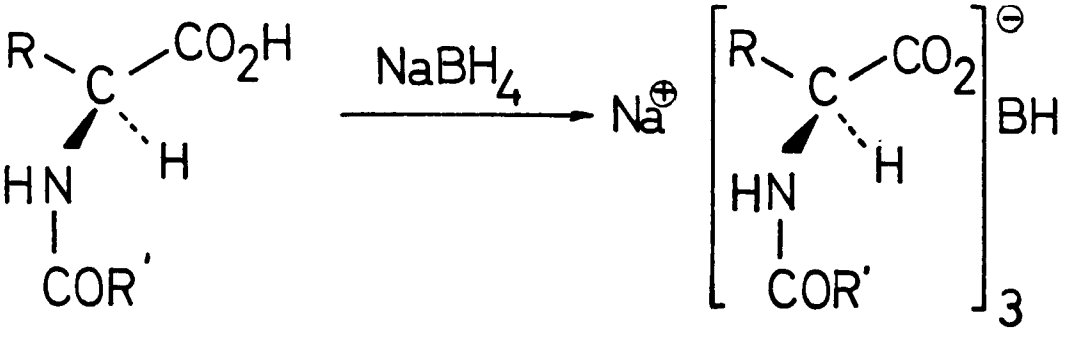
Scheme (1.7)

* = Chiral Centre

Surprisingly it was reported that similar reductions using sodium borohydride and a chiral carboxylic acid gave only a few percent asymmetric product. Perhaps this was because only one

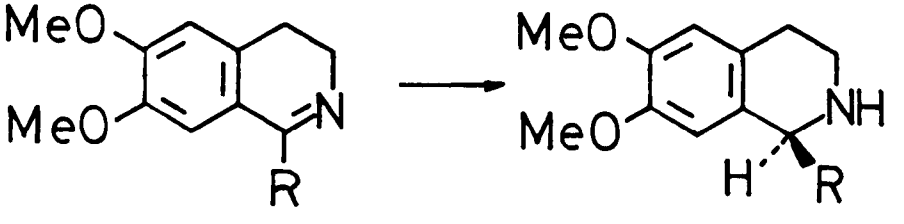
equivalent of the acid was used therefore leaving three 'active hydrides', not increasing the bulk to a great extent and thus the reagent may have been too reactive.

It has been demonstrated more recently in fact that the reaction of N-acyl-α-amino acids with sodium borohydride, in the ratio of 3:1, leads to asymmetric reducing agents (eqn. 1.4).[30]



(Eqn. 1.4)

These derivatives were effective in the reduction of cyclic imines. For example, in the reduction of 3,4-dihydroisoquinoline derivatives (eqn 1.5) 85-90% yields and 70-80% e.e. were achieved.

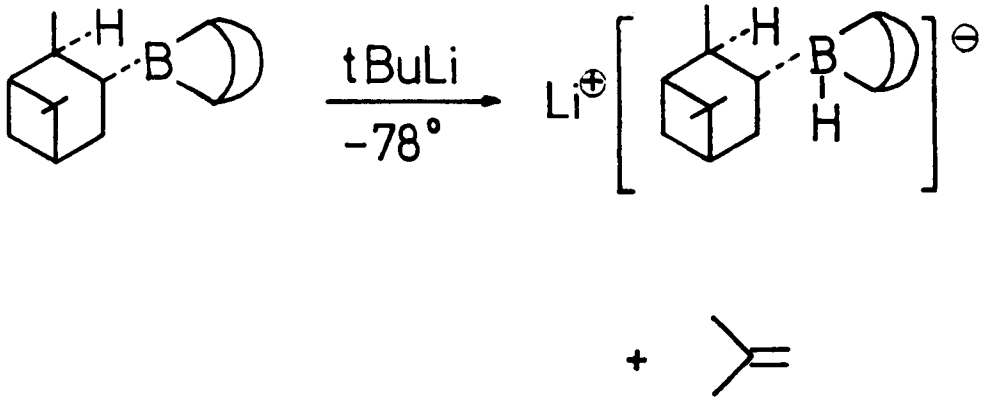


(Eqn. 1.5)

R = methyl,
3,4-methylenedioxyphenyl or
3,4-dimethoxyphenyl

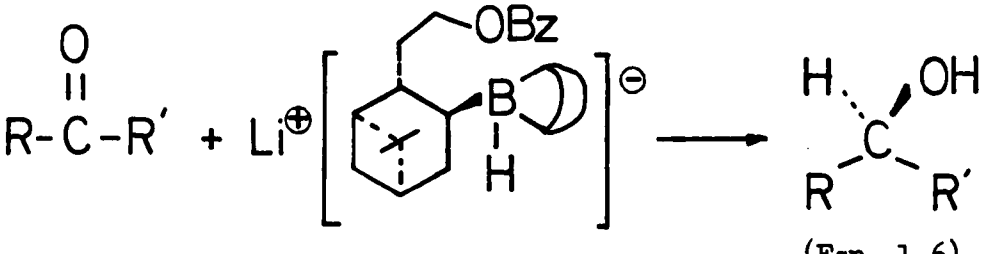
These hydrides are also effective in reducing ketones giving chiral alcohols in 80% yields and 62% e.e.[31]

A further extension of the selectivity/reactivity principle has been the recent development of trialkyl borohydrides (Scheme 1.8).[32]



Scheme (1.8)

The reagent, lithium B-3-pinanyl BBN hydride, reduces dialkyl ketones in 3-37% e.e. A modified reagent using the benzyl ether of 6,6-dimethylbicyclo[3.1.1]hept-2-ene-2-ethanol and 9-BBN has recently been shown to be more effective for aliphatic ketones (eqn 1.6)[33]



(Eqn. 1.6)

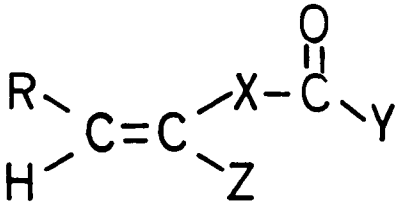
For example, 2-butanone and 2-octanone are reduced to the (S)-alcohols in 76 and 79% e.e. and 70-80% yields.

It is generally true that most of the above reagents are only effective in the asymmetric reduction of aryl ketones. This is presumably because the bulk of the aromatic ring allows better chiral distinction. Newer hydrides have gone some way to being effective in reducing aliphatic ketones and at least one type of derivative is able to induce asymmetry in the reduction of cyclic imines.

CHIRAL HOMOGENEOUS HYDROGENATION CATALYSTS

In 1968 two groups independently reported that the Wilkinson catalyst $Rh(PPh_3)_3Cl$ could be adapted to asymmetric hydrogenation by using chiral phosphines as ligands.[34] The rise of asymmetric homogeneous hydrogenation of prochiral olefins is now well documented.[2,4,35] At first it was expected that it would be necessary to have chirality directly on the phosphorus atom but the majority of systems in use today consist of chelating diphosphines joined by a chiral backbone. They can be looked at, when complexed with rhodium, as a way of arranging four phenyl groups around a metal centre in a chiral conformation. The catalysts themselves are prepared by reacting the phosphine ligand with rhodium 1,5-cyclooctadiene dichloride.

Initial efforts on simple olefins gave modest asymmetric induction[34] and the problem became one of matching substrate to catalyst. The bill was filled by α-acetamidoacrylic acids, the enamide precursors of α-amino acids. It is indeed fortunate that asymmetry could be generated in such an important area by what are, after all, essentially simple catalysts. Enantiomeric excesses of over 90% can usually be obtained if the following structural features are present (1.8):

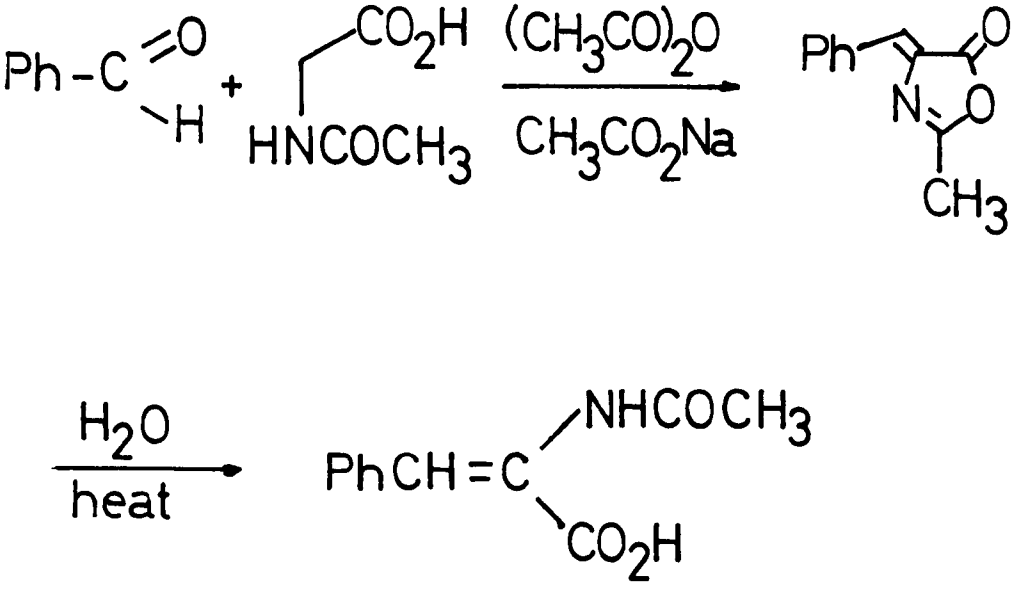


(1.8)

X = NH,O,CH_2
Y = R, OR
Z = Electron-withdrawing group

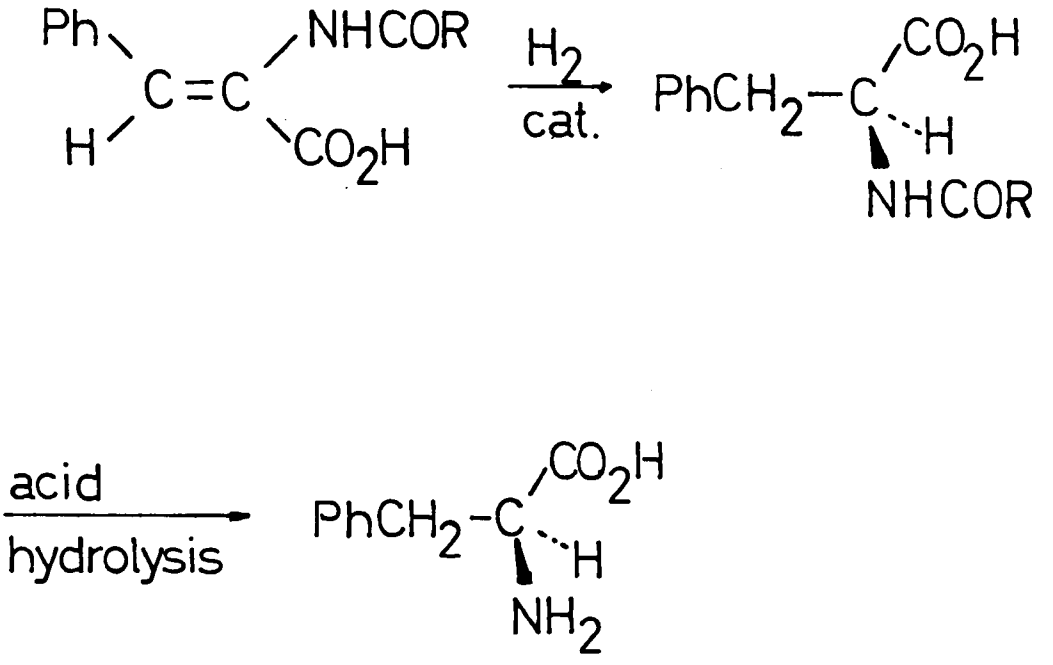
The nature of X and Y do not seem to be crucial but Z should be an electron-withdrawing group and R should be cis to Z. The carbonyl group (or equivalent) is necessary since it probably forms a point of attachment to the metal. It should be borne in mind that the concept of substrate tailoring, as employed here, is acceptable so long as the synthesis of the substrate is efficient.

When R is aromatic, for example α-acetamidocinnamic acid, the synthesis of the enamide from the corresponding aldehyde gives good yields (Scheme 1.9).[36]



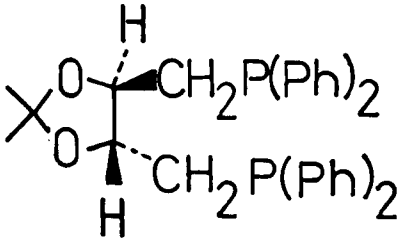
Scheme (1.9)

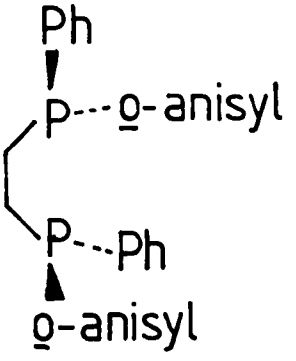
When R is aliphatic condensation yields are so poor that the gain in avoiding resolution is often lost in the synthesis of the substrate. Thus the amino acid route is only practically viable for (Z)-cinnamic acid derivatives (Scheme 1.10).



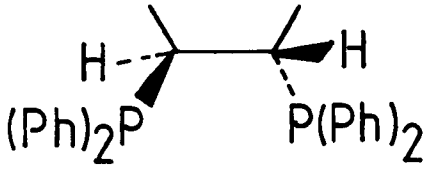
Scheme (1.10)

All of the ligands shown below behave similarly in such systems.

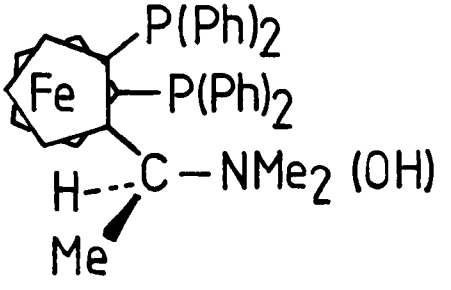
Ligand	% e.e. in reduction of α-acetamidoacrylic acid	ref.
 R,R-DIOP (1.9)	83	37

Ligand	% e.e. in reduction of α-acetamidoacrylic acid	Ref.
	95	38

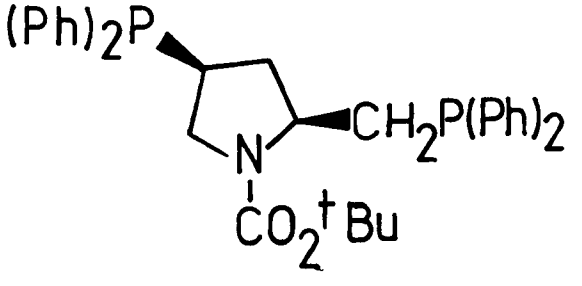
R,R-DIPAMP (1.10)

	95+	39

CHIRAPHOS (1.11)

	93	40

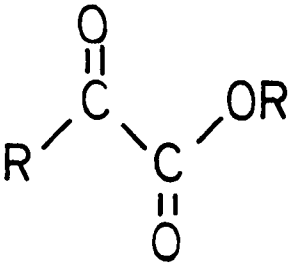
BPPFA (BPPFOH) (1.12)

	91	41

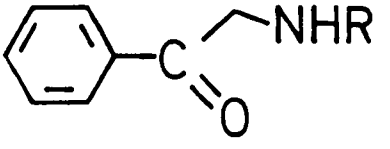
BPPM (1.13)

The above diphosphines represent the major innovations in the field.

Asymmetric hydrogenation of ketones works best for functionalised substrates. One might speculate that the types of substrate that produce high enantiomeric excesses (1.14 and 1.15) do so because they generate a secondary interaction with the rhodium to produce a chelating effect much like that of the functionalised olefins described above.

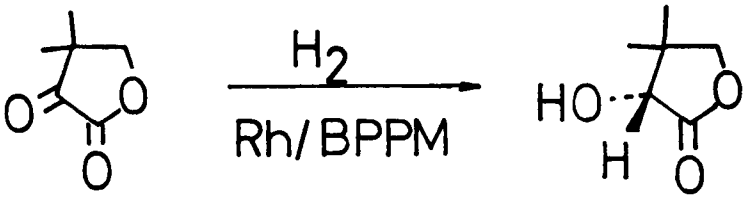


(1.14)



(1.15)

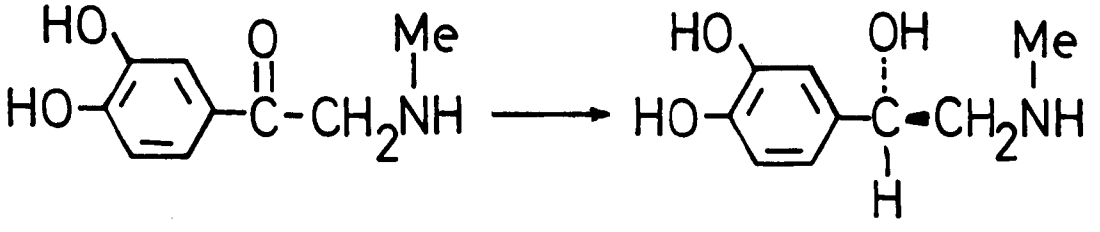
Such structural features are present in α-keto acids and esters and α-amino ketones. Thus pantolactone, the key intermediate in the synthesis of pantheine and coenzyme A, was synthesised using Rh/BPPM (1.13) (eqn. 1.7).[42]



(Eqn. 1.7)

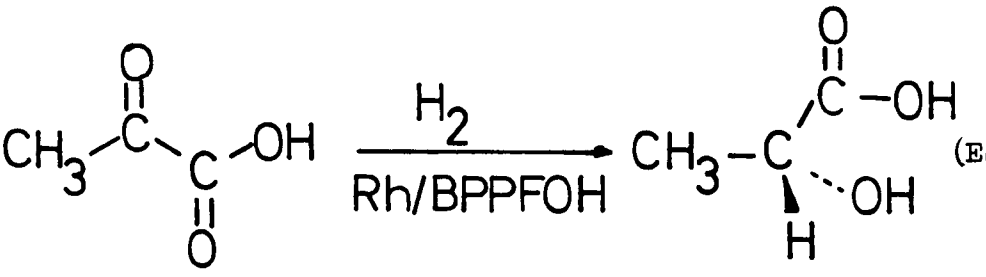
87% e.e.

Epinephrine, an α-amino alcohol, was synthesised by reduction of the corresponding ketone using Rh/BPPFOH (1.12) (eqn 1.8)[43].



100% yield, 95% e.e.
(Eqn. 1.8)

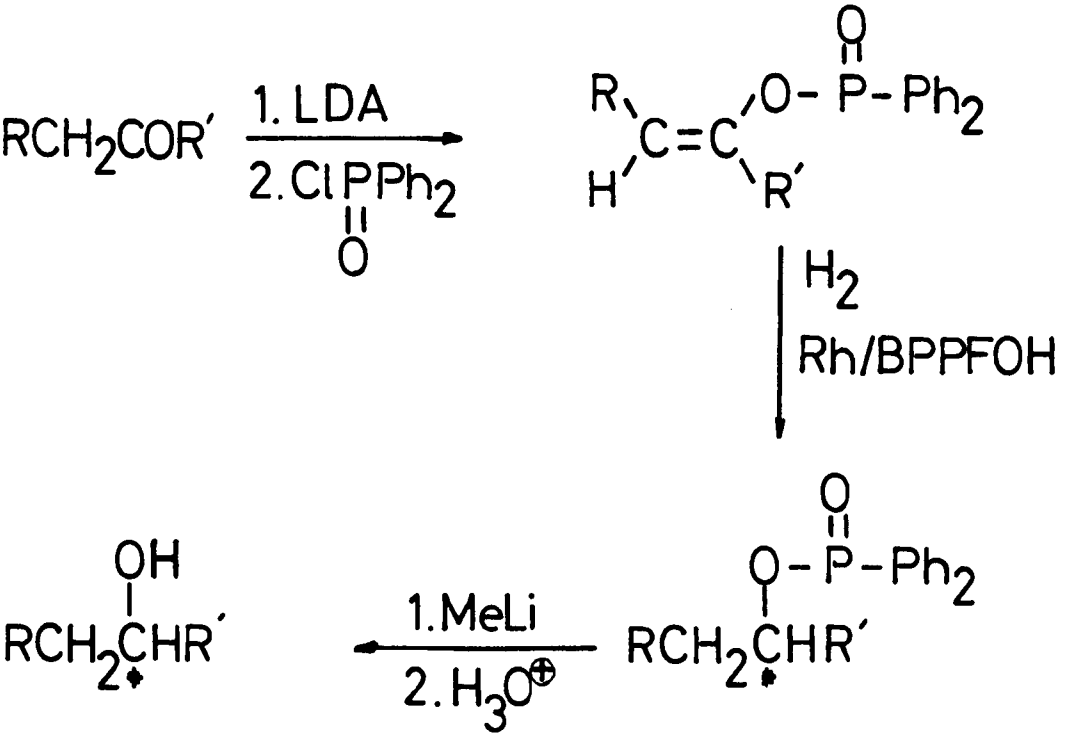
Pyruvic acid, which again contains the necessary structural features, was hydrogenated using Rh/BPPFOH (1.12) to give the corresponding (R)-hydroxy acid in 100% yield and 83% e.e. (eqn. 1.9).[44]



(Eqn. 1.9)

The ability of the BPPFOH (1.12) ligand to cause high asymmetric induction can probably be ascribed to the hydrogen bonding which is possible between the carbonyl group on a substrate and the hydroxy group on BPPFOH (1.12). This may increase conformational rigidity in diastereomeric transition states or intermediates.

Recently Kumada[45] has applied the concept of substrate tailoring to generate secondary alkyl alcohols from ketones. The trick was to generate enol diphenyl phosphinates where P=O replaces C=O in the idealised substrate (Scheme 1.11).



* = Chiral centre

Scheme (1.11)

Enantiomeric excesses of up to 78% were obtained using ketones such as acetophenone, 3-methyl-2-butanone, and 2-octanone. Of course, this represents hydrogenation of an olefin bearing a latent hydroxyl group rather than direct reduction of a carbonyl group and thus depends on being able to make the substrate easily.

The direct reduction of imines has received little study since chiral amines are accessible by reduction of enamides.[46] However the potential is clearly there and it would probably be the case that those chiral catalysts most effective in reducing the carbonyl group would be useful in reducing the imino group.

These catalysts can also be immobilised on a polymer backbone with little loss in optical efficiency, thus facilitating product recovery and catalyst renewal.[47]

In summary these asymmetric reducing agents are catalytically active and can be made insoluble with little loss in efficiency. They do, however, exhibit a narrow substrate specificity but perhaps this is only to be expected. The ability to generate high optical yields and to do so on a wide range of substrates are contradictory requirements. In other words the substrate and catalyst become specific. Other ways of achieving a chiral catalyst, for example resolving a tetrahedral complex with four different groups around the metal, could be investigated.

ENZYMES

The opportunities provided by enzymes (either cell free or in growing microorganisms) for effecting asymmetric synthesis have been recognised and exploited.[48] Enzymes are usually very selective with respect to the type of reaction they catalyse and with respect to the structure and stereochemistry of the substrate and product. This specificity is what distinguishes enzymes from other chiral catalysts or reagents and is a consequence of the detailed three dimensional arrangement of the optically active amino acids which make up the protein. Thus the reactive groupings which constitute the active site (hydrophobic pockets, charged species, nucleophiles etc.) are arranged so as to maximise the energy difference between the diastereomeric enzyme-substrate transition states. This can, for example, lead to complete chiral distinction in the reduction of the stereoheterotopic faces of a trigonal grouping.

Types of Enzyme-Catalysed Reactions[49]

(a) Oxidoreductases. These enzymes catalyse oxygenation such as C-H ⟶ C-OH, or removal or addition of hydrogen atom equivalents, for example CH(OH) ⇌ C=O.

(b) Transferases. The transfer of various groups such as aldehyde, ketone, sugar, phosphoryl and so on, from one molecule to another, are mediated by enzymes of this group.

(c) Hydrolases. The range is broad; esters, amides, peptides, anhydrides, and glycosides for example, are hydrolysed by enzymes.

(d) Lyases. Enzymes of this group catalyse additions to, or formation of, double bonds such as C=C, C=O, and C=N.

(e) Isomerases. Various types of isomerisation, including racemisation, are catalysed.

(f) Ligases. These enzymes mediate the formation of C–O, C–S, and C–N bonds.

The most useful enzymes are those which tolerate broad structural variation within their substrates while retaining the ability to operate stereospecifically or regiospecifically. In general mammalian enzymes fit these criteria best.[48a,50]

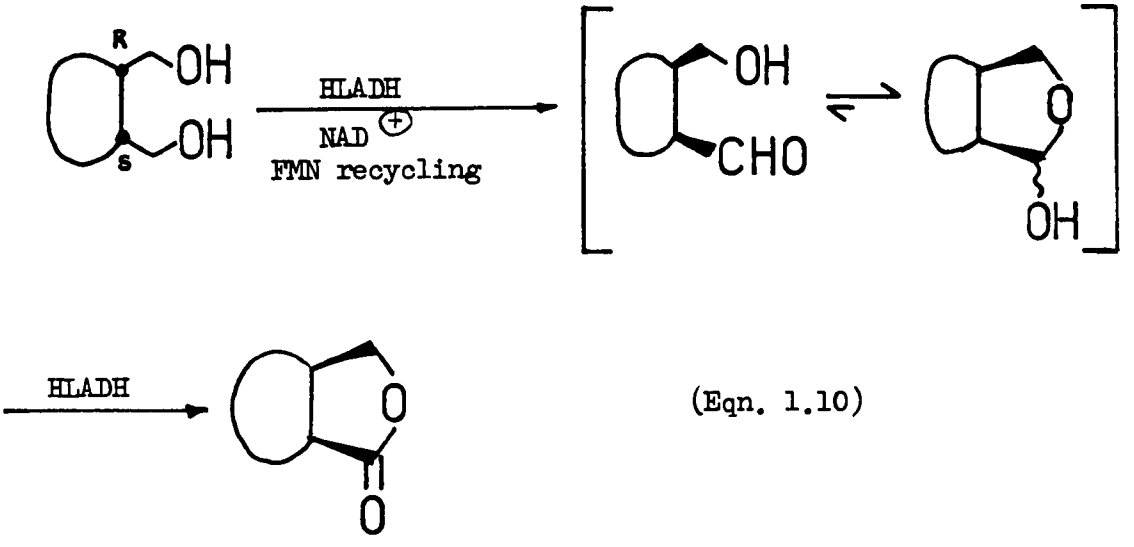
Exploitation of enzyme specificity in organic synthesis

The traditional rôles of enzymes have been (a) in the preparation of stereospecifically labelled materials for use in biosynthetic studies as exemplified by the work of Battersby[51] and Cornforth[52], or (b) in effecting resolutions of racemic mixtures, for example selective hydrolysis of (S)-acyl amino acids in an (R,S) mixture. Recent work however has suggested a growing trend towards what may be the true potential of *in vitro* enzyme mediated synthesis: namely the production of chiral intermediates for further elaboration by conventional synthetic methods. There is nothing new in this ideology of course. Chemists are well versed in plagiarism (nature being the original author) and will happily exploit the chirality present in the naturally occurring building blocks (amino acids, terpenes, carbohydrates) in pursuit of a synthetic goal. The difference in the above approach is that instead of scavenging for a suitable naturally occurring starting material, the chemist may be able

to manufacture an intermediate with chirality and functionality more immediately akin to the problem in hand.

Below are given selected examples of the value of enzymes in the production of chiral templates and their use in an overall synthetic strategy. The examples are all similar in that (a) the enzyme mediated step relies on exploiting the prochiral stereospecificities of enzymes and (b) the chiral product is prepared from a symmetric molecule (therefore all substrate can be converted to product, there is no residual 'wrong' isomer which must often be discarded). The types of reactions that have been successfully used are: enantiotopically selective oxidation, reduction,or hydrolysis. Examples of these are now given.

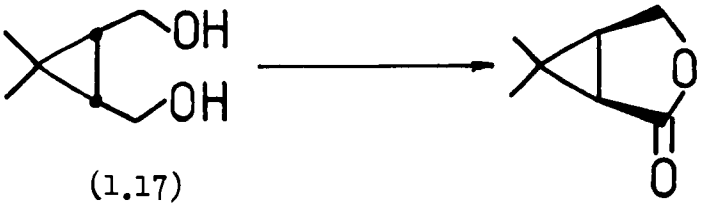
(a) Oxidation of monocyclic meso diols catalysed by horse liver alcohol dehydrogenase (HLADH) provides a direct and convenient one-step route to a broad range of chiral-γ-lactones. (eqn 1.10).[53] Oxidation of the hydroxymethyl group attached to the (S)



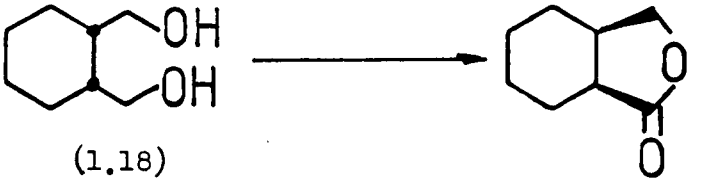
(Eqn. 1.10)

chiral centre occurs exclusively leading to a hemi-acetal which is further oxidised *in situ*. The pure lactones are isolated in high yields with complete optical purity.

(1.16) (+)-1S,2R (1.19) 90% y, 100% e.e.

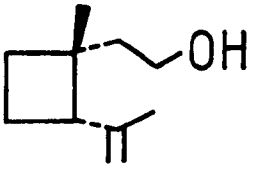


(1.17) (-)-1R,2S (1.20) 71% y, 100% e.e.



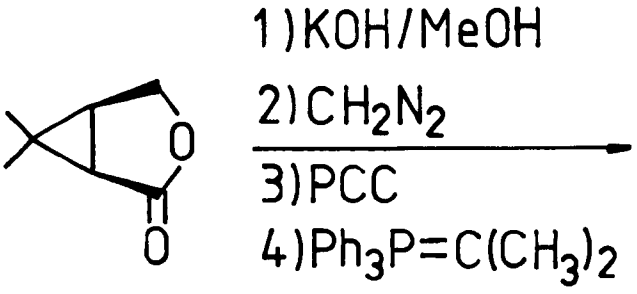
(1.18) (+)-1S,2R (1.21) 79% y, 100% e.e.

These are useful synthetic intermediates. The cyclobutyl lactone (1.19) for example has been converted into enantiomerically pure grandisol (1.22), the boll weevil sex pheromone, in 3% overall yield.[34]



(+)-(1R,2S)-grandisol (1.22)

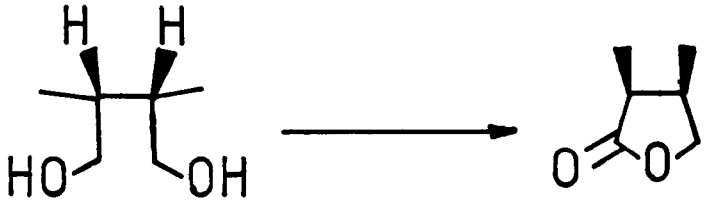
Similarly the dimethylcyclopropyl lactone (1.20), which is readily transformed into (+)-(1R,2S)-cis-methyl chrysanthemate (1.23) (eqn 1.11), leads into the pyrethroids, a group of insecticides of growing commercial importance.[55]



(Eqn. 1.11)

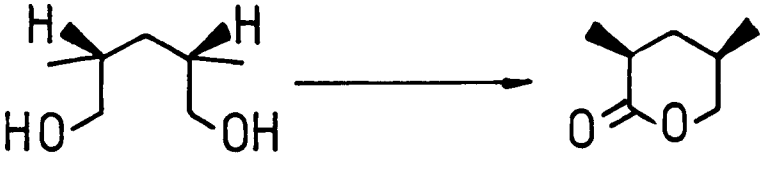
(1.20) (1.23)

Non-cyclic diols are also oxidised to lactones.[56]



64% y, 100% e.e.

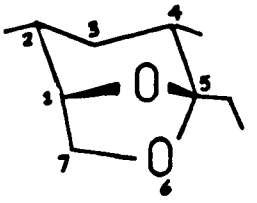
(1.24) (1.26)



65% y, 100% e.e.

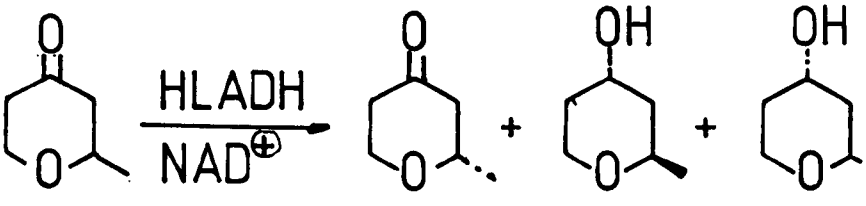
(1.25) (1.27)

Lactone (1.27) for example could be an intermediate for the macrolide antibiotic methynolide.[57] It could also be elaborated into multistriatin (1.28), the elm bark beetle pheromone.[58]



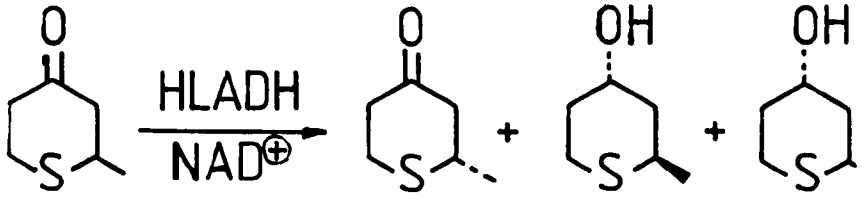
(1S:2R:4S:5R)(-)-α-multistriatin (1.28)

(b) Selective reduction: HLADH has been shown to be an effective catalyst in the reduction of heterocyclic analogues of its carbocyclic substrates. For example, reduction of pyranones (eqn 1.12)[59] or thiopyranones (eqn 1.13)[60] yield the corresponding chiral alcohols in high optical purity.



(Eqn. 1.12)

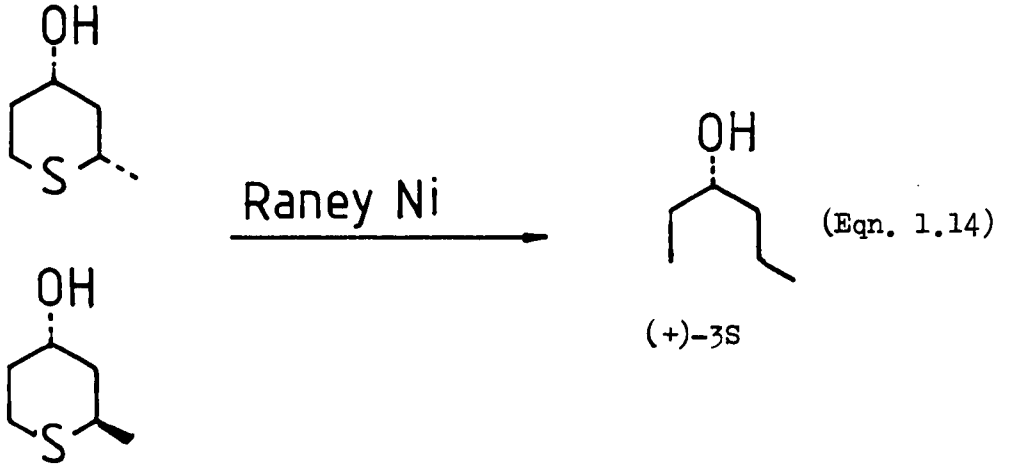
EtOH recycling 2R,4S 33%y,100%e.e. 2S,4S 2%y,36%e.e.



(Eqn. 1.13)

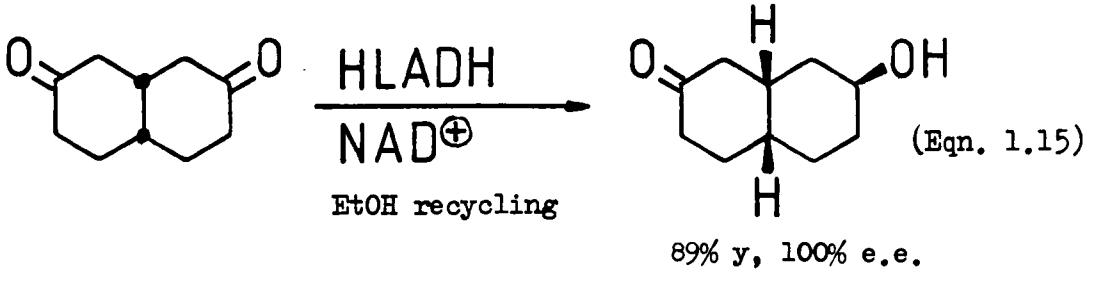
EtOH recycling 2R,4R 29%y,100%e.e. 2S,4S 11%y,100%e.e.

The thioalcohols are particularly useful since they are amenable to modification using sulphur chemistry. The sulphur can be removed using Raney nickel giving acyclic secondary alcohols which cannot be obtained by direct HLADH catalysed reduction of the corresponding ketones (eqn 1.14).



(Eqn. 1.14)

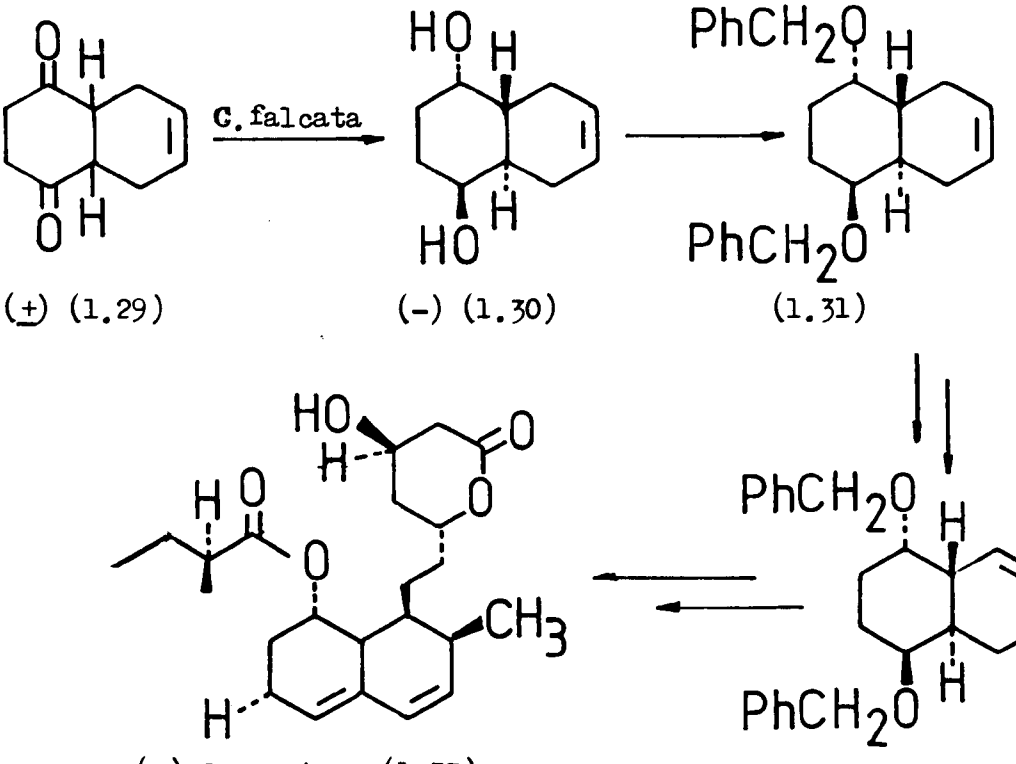
Reduction of cis and trans decalindiones using HLADH gives useful precursors for steroidal or terpenoid structures (eqn 1.15).[61]



(Eqn. 1.15)

89% y, 100% e.e.

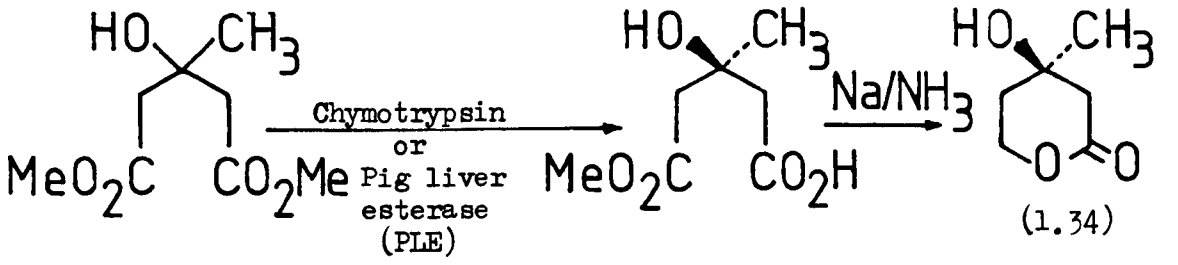
Similarly, reduction of decalindione (1.29) by the microorganism Curvularia falcata gave an intermediate (1.32) used in the total synthesis of the hypocholesterolemic agent (+)-compactin (1.33) (Scheme 1.12).[62]



Scheme (1.12)

Reduction of (±) (1.29) by this method gave (-) (1.30) in 30% yield and 100% e.e. after chromatography on silica gel. Normal reduction on the other hand gave a statistical mixture of the six diastereomeric alcohols.

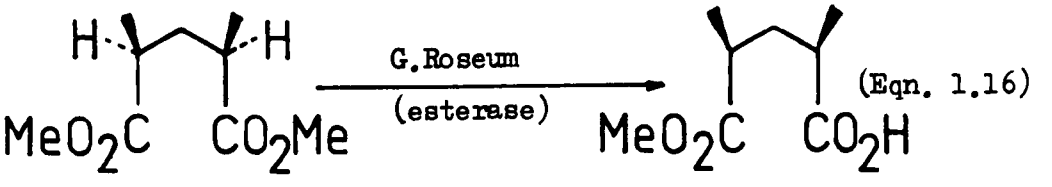
(c) Selective hydrolysis: These reactions involve the enantiotopically specific hydrolysis of one of the prochiral ester groups in a highly symmetrical molecule. (R)-Mevalonolactone (1.34) a key biosynthetic intermediate can be prepared in this manner (eqn 1.15).[63] The pro(S) ester group is hydrolysed.



(1.34)

(Eqn. 1.15)

Partial structural units commonly encountered in macrolide and polyether antibiotics can also be prepared in this fashion (eqn 1.16).[64]

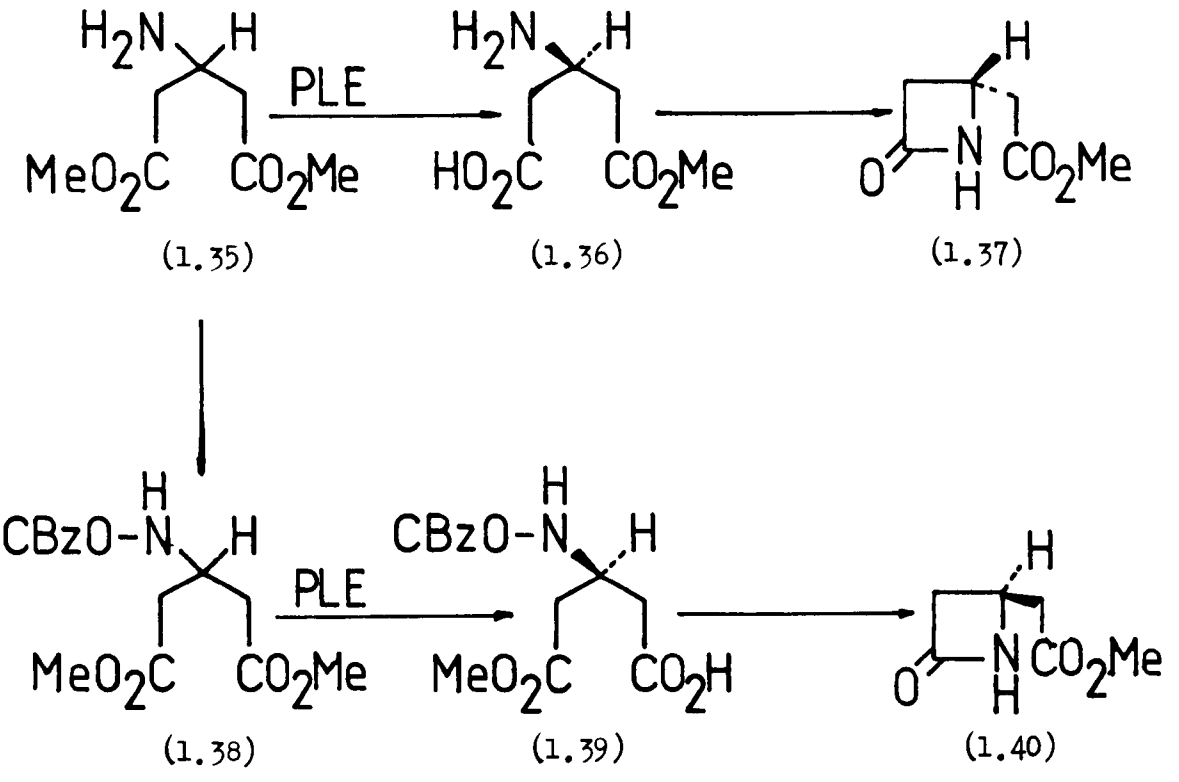


(Eqn. 1.16)

98% e.e.

The pro (R) ester being hydrolysed in this case.

The preparation of a chiral azetidone moiety (1.37) for elaboration into β-lactam antibiotics with a carbapenem nucleus has been achieved by enzymic hydrolysis of dimethyl β-amino glutarate (1.35) Scheme (1.13).[65]



(1.35) (1.36) (1.37)

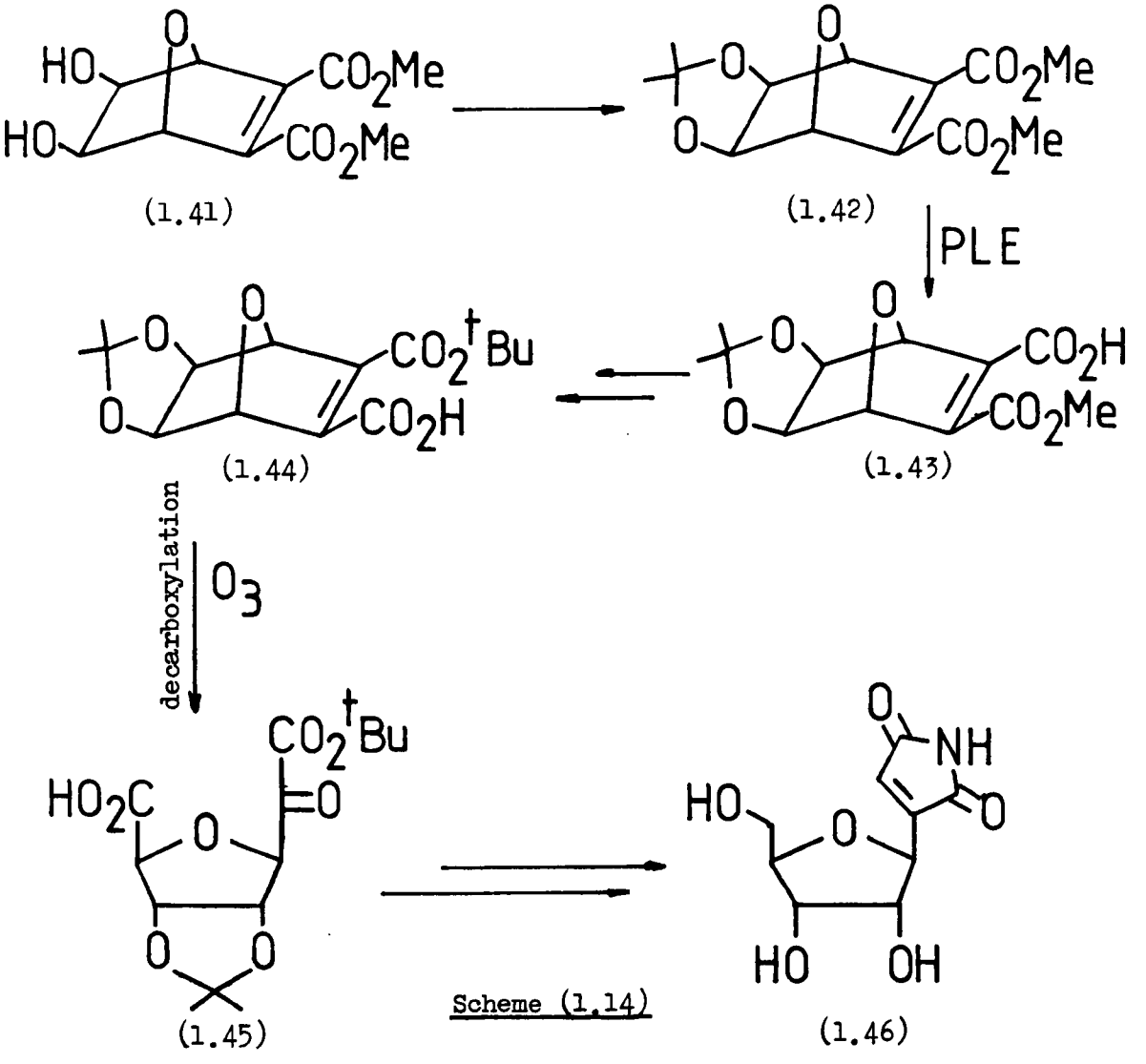
(1.38) (1.39) (1.40)

Scheme (1.13)

It was found that the primary amino group of the diester (1.35) catalysed non-enzymic hydrolysis and this resulted in low optical yields (40% e.e) of the methyl half-ester (1.36). The problem was overcome by masking the amino group with benzyl chloroformate to give the diester (1.38). This was hydrolysed in high optical yield (96% e.e.) to give the methyl half-ester (1.39). Interestingly, the protection of the amino group resulted in a reversal of enzyme specificity (from the pro (S) to the pro (R) ester).

Useful intermediates for elaboration into C-nucleosides (which have antiviral, antibiotic,and anti-tumour activities) have been synthesised from the enzymic hydrolysis product of the

diester (1.41) prepared by a Diels-Alder reaction (Scheme 1.14).[66]



Scheme (1.14)

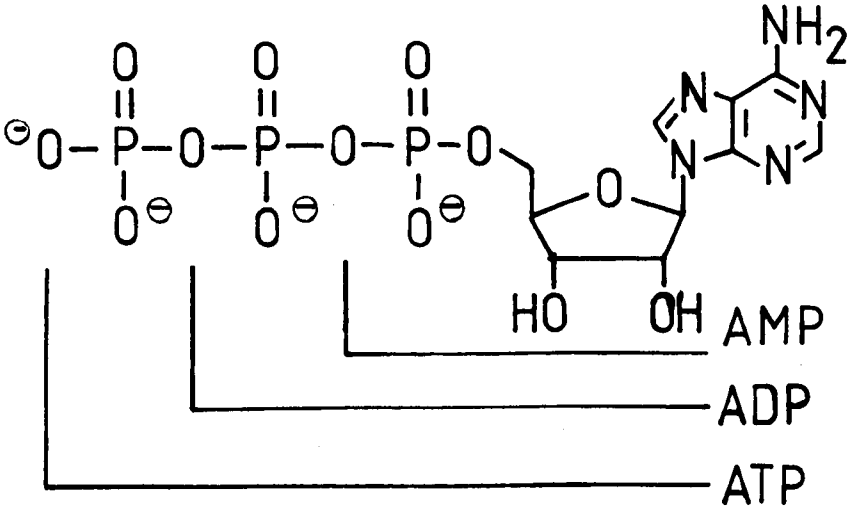
The methyl half-ester (1.43) was prepared in 100% yield. The optical purity was shown to be greater than 98% by conversion to a ribose derivative (which happened to be the L-series). For entry into the D-series the methyl half-ester (1.43) was transformed into the t-butyl half-ester (1.44) of opposite configuration. After decarboxylative ozonolysis and subsequent manipulation the half ester (1.44) was converted into showdomycin (1.46). Recently the carbocyclic nucleosides (-)-Aristeromycin and

(-)-Neplanocin have been prepared by the above approach.[66a]

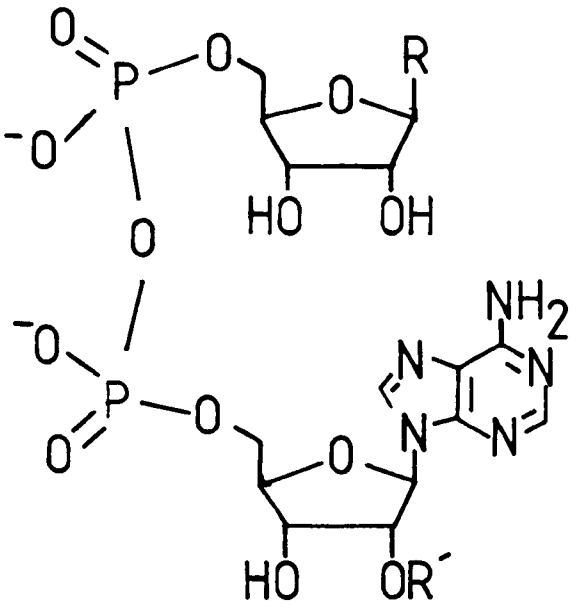
Co-enzymes

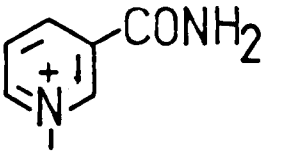
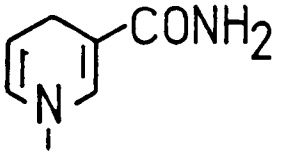
Many enzymes require non-protein co-enzymes (or cofactors) to be catalytically active.[67,68] Co-enzymes are consumed in stoichiometric amounts during the catalytic step and are therefore co-substrates. The cost of those co-enzymes particularly useful in enzyme mediated organic synthesis prohibits their use stoichiometrically as expendable substrates. Some co-enzymes reform automatically under normal conditions (e.g. biotin, oxidised flavins, and pyridoxal phosphate). Others, such as adenosine triphosphate (ATP) (1.47), co-enzyme A, folic acid, and nicotinamide adenine di- and triphosphate in both their oxidised and reduced forms ($NAD(P)^{\oplus}$ and NAD(P)H respectively, (1.48)) must be continuously regenerated *in situ* by an efficient method.

Recycling of ATP (1.47) and the nicotinamide co-enzymes (1.48) represented the most urgent need in the application of enzymic catalysis to organic synthesis. ATP (1.47) costs approximately £500/mole; the cost of the nicotinamide cofactors (1.48) ranges from £1000/mole for $NAD^{\oplus}$ to £170,000/mole for NADPH. Recycling of these co-enzymes is discussed below in some detail.



(1.47)



	R	R´
$NAD^{\oplus}$		H
$NADP^{\oplus}$	"	PO_3^{2-}
NADH		H
NADPH	"	PO_3^{2-}

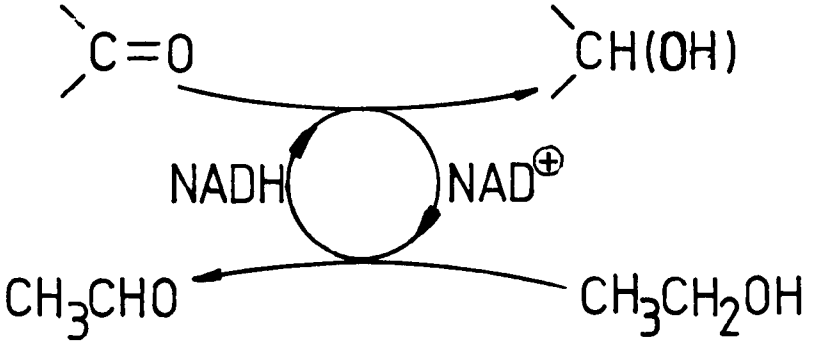
(1.48)

NAD(P)H regeneration[48a]

A large number of systems have been proposed and tested for reduction of the oxidised cofactors. Those based on chemical reduction, using sodium dithionite for example, or electrochemical reduction[69] are not sufficiently selective to achieve the high turnovers necessary. It has been found that enzymatic methods are superior; these may be designated as coupled-substrate or coupled-enzyme techniques.

Coupled substrate

In such a process the cofactor is reformed by adding a second substance that is a good substrate for the enzyme which catalyses the main reaction, but in the opposite direction. This has been applied successfully in horse liver alcohol dehydrogenase (HLADH) catalysed reduction of carbonyl compounds using ethanol to recycle the $NAD^{\oplus}$ (1.48) formed (Scheme 1.15).[48c,d]

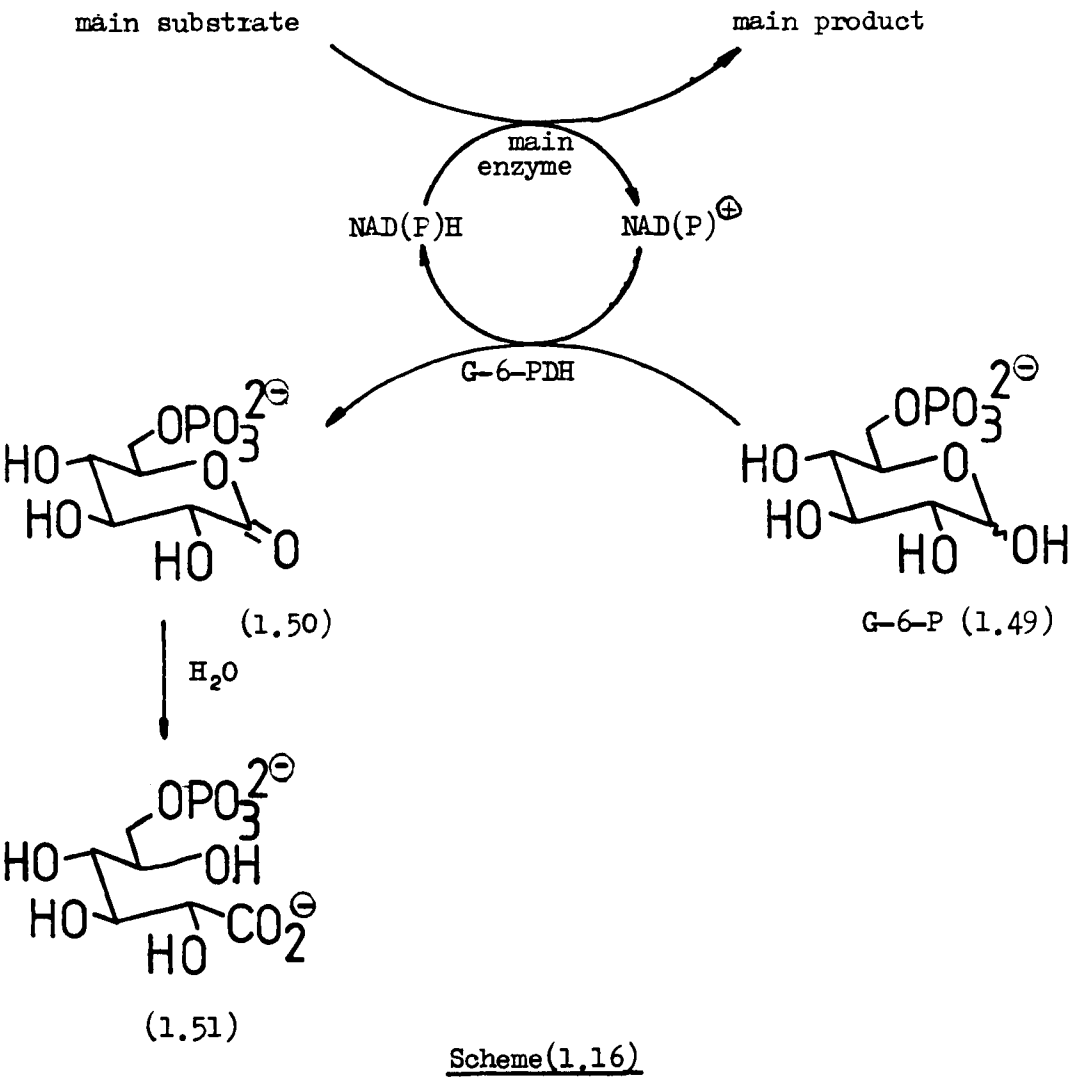


Scheme (1.15)

This is, however, not a generally applicable method because the turnover numbers are fairly low and reaction times fairly long if the carbonyl compound is ketonic. This is due to the redox potential differences between the main and co-substrate.

Coupled enzymes

In this approach the co-enzyme required for the enzyme-catalysed conversion of the main substrate is reformed by a completely separate process catalysed by a different enzyme. As with the scheme outlined above the auxiliary substrate must be cheap. The most generally applicable process is that based on glucose-6-phosphate (G-6-P, 1.49) and glucose-6-phosphate dehydrogenase (G-6-PDH) from Leuconostoc mesenteroides (Scheme 1.16).[70]

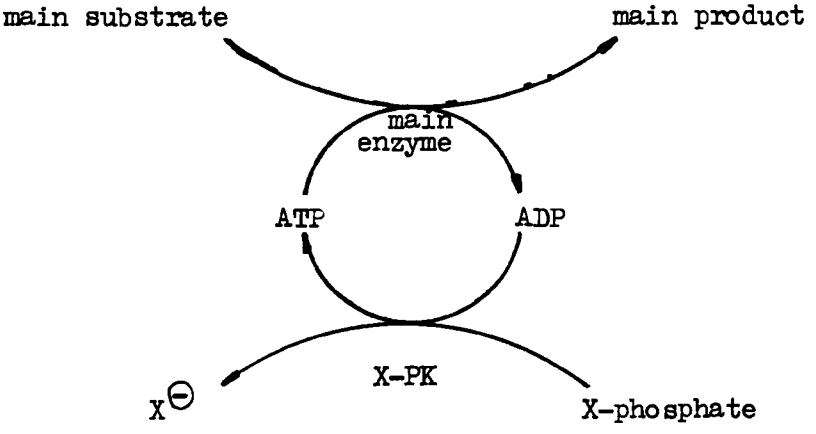


Scheme(1.16)

The advantages of this system are that G-6-PDH from L. mesenteroides is almost equally effective in reducing $NAD^{\oplus}$ and $NADP^{\oplus}$ (1.48), it is stable, inexpensive, and commercially available. The equilibrium lies on the side of products because the reaction is rendered essentially irreversible by the non-enzymic hydrolysis of the initially formed 6-phosphogluconolactone (1.50) to give 6-phosphogluconate (1.51). The scheme requires preparation of G-6-P (1.49) but this can be readily achieved by the hexokinase (HK) catalysed phosphorylation of glucose with ATP (1.47) coupled to an ATP recycling system (see below).

ATP regeneration

Again a coupled enzyme technique for phosphorylation of the ADP (or AMP, 1.47) formed is best (Scheme 1.17). Chemical methods can be rejected; they are incompatible with enzymes and lack the specificity required to give high turnover numbers.



Scheme (1.17)

The requirements of the scheme shown are for an inexpensive phosphate donor (X-phosphate) and the corresponding enzyme X-phosphokinase (X-PK). Some possibilities are listed below in table 1.1.[71]

X-PHOSPHATE	COST	STABILITY	MAX. EQUILIBRIUM CONSTANT
Phosphoenol pyruvate (PEP)	moderate	good	6600
Acetyl phosphate (AcP)	low	fair	400
Creatine phosphate (CrP)	moderate	good	105

Table 1.1

Of these, acetyl phosphate was the most attractive phosphate donor because it is readily prepared in quantity.[72] The enzyme it requires for use in ATP (1.47) regeneration from ADP is acetate kinase (AK) which is fairly cheap and stable. The only serious competitor for approx. mole scale work is phosphoenol pyruvate. A recent synthesis of this phosphate donor which is easier than that of acetyl phosphate has been published.[73] The cost is, however, higher and acetyl phosphate would be preferred for large scale work. Phosphoenol pyruvate is more stable in solution, however, and this makes the recycling simpler in practice. It is also a stronger phosphorylating agent and pyruvate kinase (PK) is less expensive than acetate kinase. Methods based on creatine phosphate offer no advantages over the two given above.

Enzyme Immobilisation

Except for small scale work or where the cost of the product allows, enzymes themselves are too expensive to be treated as expendable reagents. Recovery of protein from dilute aqueous solutions is difficult hence the science of enzyme immobilisation has come into being.[74] An enzyme is said to be immobilised when it is unable to diffuse freely through the reaction mixture. It can be attached physically or chemically to an insoluble support or enclosed within a membrane. While much research has been devoted to this subject there is no one optimal method or support. The approach is therefore largely empirical. There are only isolated cases of success in industrial applications.[74,75]

REFERENCES

REFERENCES

1. V.G. Blashke, H.P. Kraft, K. Fickentscher, and F. Kohler, Arzneimittel - Forsch., 1979, 29, 1640.

2. J.D. Morrison and H.S. Mosher in 'Asymmetric Organic Reactions', Englewood-Cliffs, N.J.:Prentice Hall, 1971.

3. E.L. Eliel in 'Stereochemistry of Carbon Compounds', New York, McGraw-Hill, 1962.

4.(a) D. Valentine and J.W. Scott, Synthesis, 1978, 5, 329.

(b) H. Kagan and J.C. Fiaud in 'Topics in Stereochemistry', N.Y., Wiley-Interscience, 1978, 10, 175.

(c) J.W. Apsimon and R.P. Seguin, Tetrahedron, 1979, 35, 2797.

5. H.C. Brown, P.K. Jadhav, and A.K. Mandal, Tetrahedron, 1981, 37, 3547.

6.(a) S.R. Landor and A.R. Tatchell, J. Chem. Soc. Chem. Comm., 1966, 2280.

(b) R. Andrisano, A.S. Angeloni, and S. Marzocchi, Tetrahedron, 1973, 29, 913.

(c) S.R. Landor, B.J. Miller, and A.R. Tatchell, J. Chem. Soc. Chem. Comm., 1967, 197.

(d) E.D. Lund and P.E. Shaw, J. Org. Chem., 1977, 42, 2073.

7.(a) J.P. Vigneron and I. Jaiquet, Tetrahedron, 1976, 32, 939.

(b) A.I. Meyers, and P.M. Kendall, Tetrahedron Lett., 1974, 1337.

(c) O. Cervinka and O. Belovsky, Coll. Czech. Chem. Comm., 1967, 32, 3897.

8.(a) M. Asami, H. Ohno, S. Kobayashi, and T. Mukaiyama, Bull. Chem. Soc. Jpn., 1978, 51, 1869.

(b) S. Yamaguchi, F. Yashuhara, and K. Kabuto, J. Org. Chem., 1977, 42, 1758.

9. R. Noyori, I. Tomino, and Y. Tanimoto, J. Amer. Chem. Soc., 1979, 101, 3129.

10. M.M. Midland, A. Tramontano, and S.A. Zderic, J. Organometal. Chem., 1977, 134, C17.

11. M.M. Midland, S. Greer, A. Tramontano, and S.A. Zderic J. Amer. Chem. Soc., 1979, 101, 2352.

12. M.M. Midland, D.C. McDowell, R.L. Hatch, and A. Tramontano, ibid., 1980, 102, 86.

13. M.M. Midland and A. Tramontano, Tetrahedron Lett., 1980, 3549.

14. H.C. Brown and G.G. Pai, J. Org. Chem., 1982, 47, 1606.

15. J.A. Snover, U.S.P. 3,317,525., Chem. Abs., 1967, 67, 21921z.

16. J.C. Fiaud and H.B. Kagan, Bull. Soc. Chim. Fr., 1969, 2742.

17. R.F. Borch and S.R. Levitan, J. Org. Chem., 1972, 37, 2347.

18. M.F. Grundon, D.G. McCleery,and J.W. Wilson, J. Chem. Soc. Perkin Trans.I, 1981, 231.

19. M.F. Grundon, D.G. McCleery,and J.W. Wilson, Tetrahedron Lett., 1976, 295.

20. K. Hakko, Jap. P. 57, 146, 786., Chem. Abs., 1983, 98, 107533b.

21. A. Hirao, S.Itsuno, S. Nakahama, and N. Yamazaki, J. Chem. Soc. Chem. Comm., 1981, 315.

22. S.Itsuno, A. Hirao, S. Nakahama, and K. Ito, ibid., 1983, 469.

23. T. Mancilla, F. Santiesteban, R. Contreras, and A. Klaebe, Tetrahedron Lett., 1982, 23, 1561, ibid., 1983, 24, 759.

24. T. Yamashita, H. Mitsui, H. Watanabe, and N. Nakamura, Makromol. Chem., 1980, 181, 2563.

25. S.Itsuno, A. Hirao, S. Nakahama, and N. Yamazaki, Makromol. Chem. Rapid Comm., 1982, 3, 673.

26.(a) S. Colonna, J. Bakells, and R.Fournasier Synthesis, 1976, 266.

(b) S. Colonna and R. Fournasier, J. Chem. Soc. Perkin Trans. I, 1978, 371.

26.(c) J.P. Masse and E.R. Parayre, J. Chem. Soc. Chem. Comm., 1976, 438.

27. T. Sugimoto, Y. Matsumura, S. Tanimoto, and M. Okano, J. Chem. Soc. Chem. Comm., 1978, 926.

28.(a) G.W. Gribble and D.C. Ferguson, J. Chem. Soc. Chem. Comm., 1975, 535.

(b) P. Marchini, G. Liso, A. Reho, F. Liberatore, and F.M. Monacci, J. Org. Chem., 1975, 40, 3453.

29. J.D. Morrison, E.R. Grandbois, and S.I. Howard, J. Org. Chem., 1980, 45, 4229.

30. K. Yamada, M. Takeda and T. Iwakuma, Tetrahedron Lett., 1981, 22, 3869.

31. T. Iwakuma, N. Umino, and N. Ituh, Ventron Alembic, 1981, 21, 3.

32. H.C. Brown, S. Krishnamurthy, and F. Vogel, J. Org. Chem., 1977, 42, 2534.

33. M.M. Midland and A. Kazubski, J. Org. Chem., 1982, 47, 2495.

34. W.S. Knowles and M.J. Sabacky, Chem. Comm., 1968, 1445. L. Horner, H. Siegel, and H. Buthe, Angew. Chem. Int. Ed. Engl., 1968, 7, 941.

35.(a) R.E. Merrill, Chemtech, 1981, 118.

(b) W.S. Knowles, Acc. Chem. Res., 1983, 16, 106.

(c) V. Caplar, G. Comisso, and V. Sunjic, Synthesis, 1981, 85.

(d) B. Bosnich and M.D. Fryzuk, in 'Topics in Stereochemistry', N.Y., Wiley, 1981, 12, 119.

(e) M. Kumada, T. Hayashi, and K. Tamao, Stud. Org. Chem., 1981, 6, 55.

36. R.M. Herbst and D. Shemin, in 'Organic Synthesis', Coll. Vol. 2, N.Y. Wiley, 1943, 1.

37. H.B. Kagan and T.P. Dang, J. Chem. Soc. (C) 1971, 481, J. Amer. Chem. Soc., 1972, 94, 6429.

38. B.D. Vineyard, W.S. Knowles, M.J. Sabacky, G.L. Bachman, and O.J. Weinkauff, J. Amer. Chem. Soc., 1977, 99, 5946.

39. M.B. Fryzuk and B. Bosnich, J. Amer. Chem. Soc., 1977, 99, 6262.

40. T. Hayashi, T. Mise, M. Fukushima, M. Kagotami, N. Nagashima, Y. Hamada, A. Matsumoto, S. Kawakami, M. Konishi, K. Yamamoto, and M. Kumada, Bull. Chem. Soc. Jpn., 1980, 53, 1138 and references cited therein.

41. K. Achiwa, J. Amer. Chem. Soc., 1976, 98, 8265.

42. K. Achiwa, T. Kogure, and I. Ojima, Tetrahedron Lett., 1977, 4431.

43. M. Kumada, T. Nayashi, A. Katsumara, and M. Konishi, Tetrahedron Lett., 1979, 425.

44. T. Hayashi, T. Mise, and M. Kumada, Tetrahedron Lett., 1976, 4351.

45. T. Hayashi, K. Kanehira, and M. Kumada, Tetrahedron Lett., 1981, 4417.

46. A. Levi, G. Modena, and G. Scorrano, J. Chem. Soc., (C), 1975, 6.

M. Kumada, T. Hayashi, Symp. Rhodium Homogeneous Catal., (Proc), 1978, 157.

47. G.L. Baker, S.J. Fritschel, J.R. Stille, and J.K. Stille, J. Org. Chem., 1981, 46, 2954.

48.(a) J.B. Jones and J.F. Beck in: 'Applications of Biochemical Systems in Organic Chemistry', Wiley-Interscience, 1976, Part 1, 107-401.

(b) C.J. Sih and J.P. Rosazza, ibid, 69-106.

(c) J.B. Jones in 'Enzymic and Non-enzymic Catalysis', Ellis Horwood Ltd., 1980, 54-83.

(d) J.B. Jones in 'Enzyme Engineering', Vol. 6, Plenum Press, N.Y., 1982, 107.

49. M. Dixon and E.C. Webb, 'Enzymes', 3rd Ed., Longmans, 1979.

50. C.J. Sih, E. Abushanab, and J.B. Jones, Ann. Repts. in Med. Chem., 1977, 12, 298.

51. A.R. Battersby, Experientia, 1978, 34, 1.

52. J.W. Cornforth, Science, 1976, 193, 121.

53. I.J. Jakovac, H.B. Goodbrand, K.P. Lok, and J.B. Jones, J. Amer. Chem. Soc., 1982, 104, 4659.

54. J.B. Jones, M.A.W. Finch, and I.J. Jakovac, Can. J. Chem., 1982, 60, 2007.

55. M. Elliot, N.F. Janus, Chem. Soc. Revs., 1978, 7, 473.

56. I.J. Jakovac, G. Ng, K.P. Lok, and J.B. Jones, J. Chem. Soc. Chem. Comm., 1980, 515.

57. A. Nakamo et. al. Chem. Lett., 1979, 1019.

58. P.A. Bartlett and J. Myerson, J. Org. Chem., 1979, 44, 1625.

59. J.B. Jones, and J.A. Haslegrave, J. Amer. Chem. Soc., 1982, 104, 4666.

60. J.B. Jones and J. Davies, J. Amer. Chem. Soc., 1979, 101, 5409.
J.B. Jones and H.M. Schwartz, Can. J. Chem., 1981, 59, 1574.

61. D.R. Dodds and J.B. Jones, J. Chem. Soc. Chem. Commun., 1982, 1080.

62. C.T. Hsu, N.Y. Wang, L.H. Latimer, and C.J. Sih, J. Amer. Chem. Soc., 1983, 105, 593.

63. F.C. Huang, L.F.H. Lee, R.S.D. Mittal, P.R. Ravikumar, J.A. Chan, and C.J. Sih, J. Amer. Chem. Soc., 1975, 97, 4144.

64. C.S. Chen, Y. Fujimoto, and C.J. Sih, J. Amer. Chem. Soc., 1981, 103, 5380.

65. M. Ohno, S. Kobayashi, T. Iimori, Y.-F. Wang, and T. Izawa, J. Amer. Chem. Soc., 1981, 103, 2405.

66. Y. Ito, T. Shibata, M. Arita, H. Sawai, and M. Ohno, J. Amer. Chem. Soc., 1981, 103, 6739.

66.(a) M. Arita, K. Adachi, Y. Ho, H. Sawai, and M. Ohno, J. Amer. Chem. Soc., 1983, 105, 4049.

67. J.B. Jones, Tech. Chem., 1976, 10, 1.

68. G.M. Whitesides, C.H. Wong, and A. Pollack, 'Asymmetric Reactions and Processes in Chemistry', A.C.S. Symposium Series, 1982, 205.

69. R.J. Day, S.J. Kinsey, E.T. Seo, N. Weliky and H.P. Silverman, Trans. N.Y. Acad. Sci., 1972, 34, 588.
H. Simons, H. Gunther, J. Bader and W. Tischer, Angew. Chem. Int. Ed.Engl., 1981, 20, 861.
R. DiCosimo, C.H. Wong, L. Daniels and G.M. Whitesides, J. Org. Chem., 1981, 46, 4662.

70. C.H. Wong and G.M. Whitesides, J. Amer. Chem. Soc., 1981, 103, 4890 and references cited therein.

71. C.R. Gardner, C.K. Colton, R.S. Langer, B.K. Hamilton, M.C. Archer, and G.M. Whitesides in 'Enzyme Engineering', Vol. 2, Plenum Press, N.Y., 1974, 209.

72. J.M. Lewis, S.L. Haynie, and G.M. Whitesides, J. Org. Chem., 1979, 44, 864.
G.M. Whitesides, M. Siegel, and P. Garrett, J. Org. Chem., 1975, 40, 2516.

73. B.L. Hirschbein, F.P. Mazenod, and G.M. Whitesides, J. Org. Chem., 1982, 47, 3765.

74. 'Methods in Enzymology', Ed. K. Mosbach, Academic Press, 1976, 44.

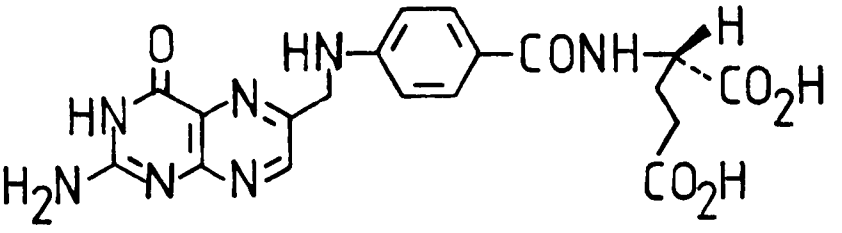
75. I. Chibata, in 'Asymmetric Reactions and Processes in Chemistry', A.C.S. Symposium Series, 1982, 205.
'Immobilized Enzymes', Ed. I. Chibata, Halsted Press, 1978.

CHAPTER 2

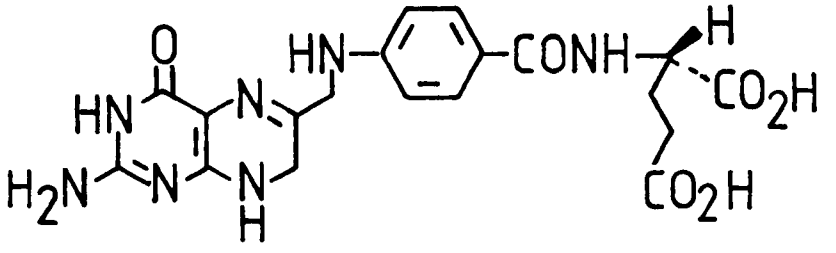
PART I

INTRODUCTION

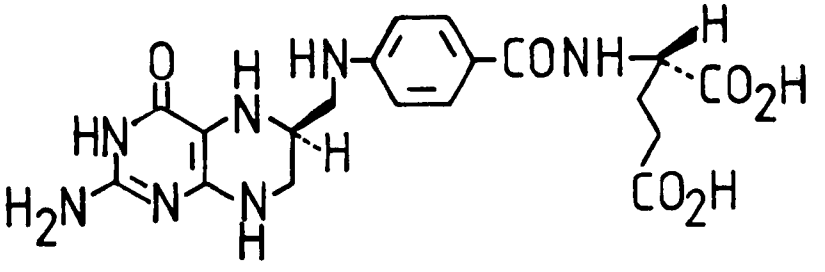
Folic acid (2.1) is a vitamin. Biological activity is expressed, co-enzymically, in the reduced forms 7,8-dihydrofolic acid (2.2) and (6S)-5,6,7,8-tetrahydrofolic acid (2.3).[1]



Folic acid (2.1)



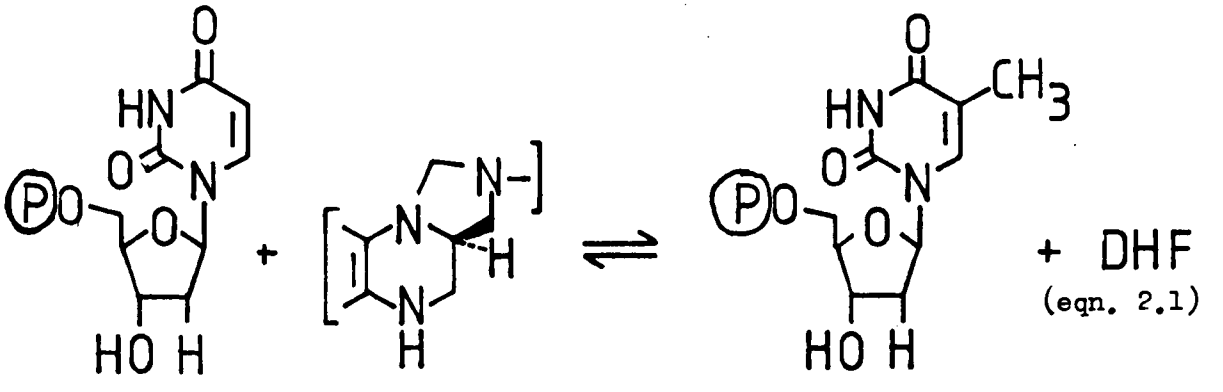
Dihydrofolic acid (2.2)



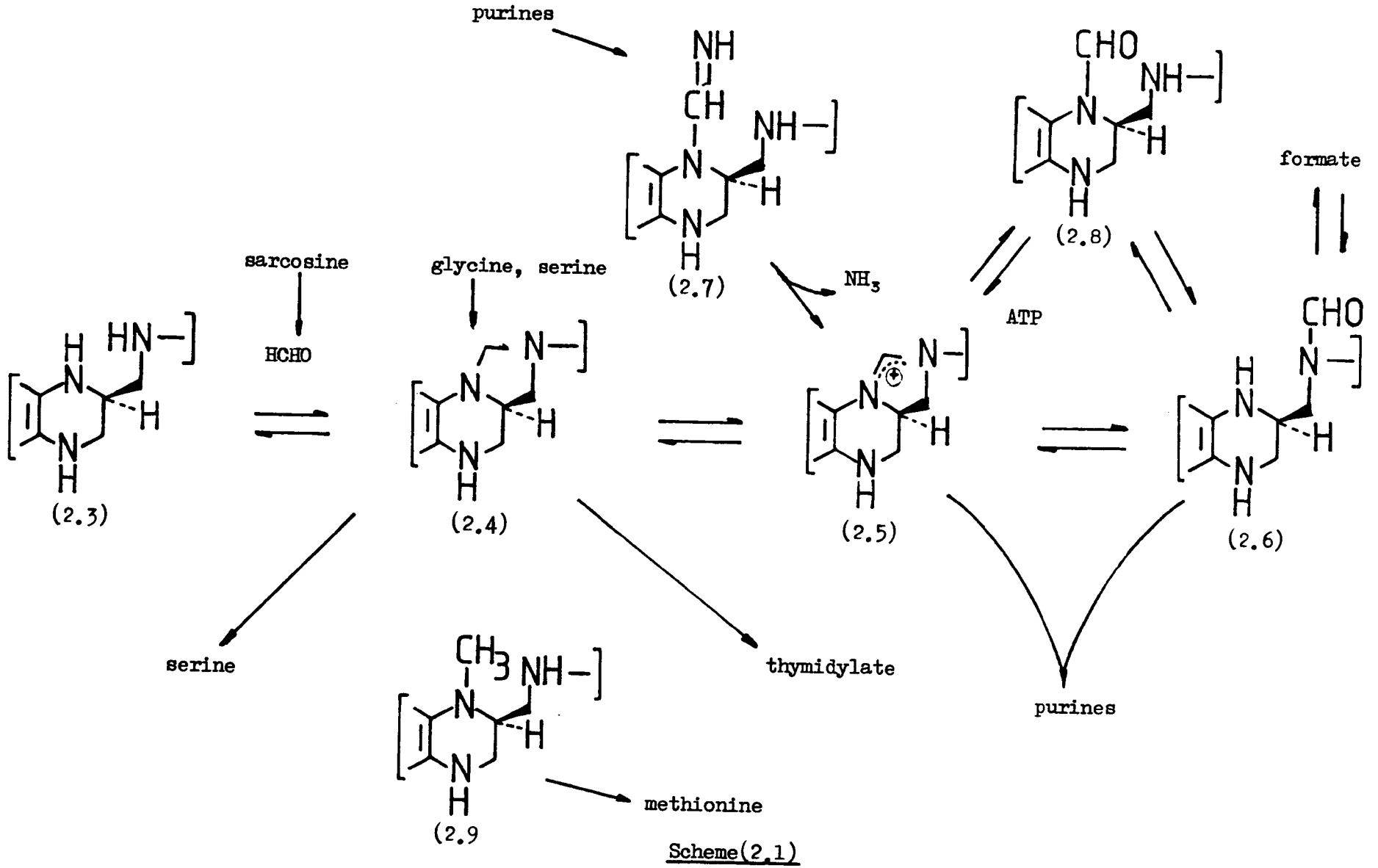
Tetrahydrofolic acid (2.3)

Tetrahydrofolic acid (2.3) is involved in the reversible transfer of one-carbon units at the oxidation levels of formate, formaldehyde, and methanol.[2,3,4] The provision and utilisation of one-carbon fragments, mediated by tetrahydrofolic acid derivatives, is summarised in Scheme 2.1.

These one-carbon tetrahydrofolate derivatives are involved in the synthesis of pyrimidine and purine nucleotides and hence of DNA and RNA. The main interest of the work in this thesis is centred on the fact that a chemotherapeutic treatment of some forms of cancer is based on the inhibition of the biosynthesis of thymine at a step in which a reduced folate is involved. Thus, reductive methylation of deoxyuridine monophosphate, dUMP, (2.10) to give deoxythymidine monophosphate, dTMP, (2.11) is catalysed by the enzyme thymidylate synthase. There is concomitant conversion of (6R)-5,10-methylene tetrahydrofolate (2.4) to 7,8-dihydrofolate (2.2) the cofactor serving as both a one-carbon carrier and a reductant, (eqn. 2.1).[5,6]

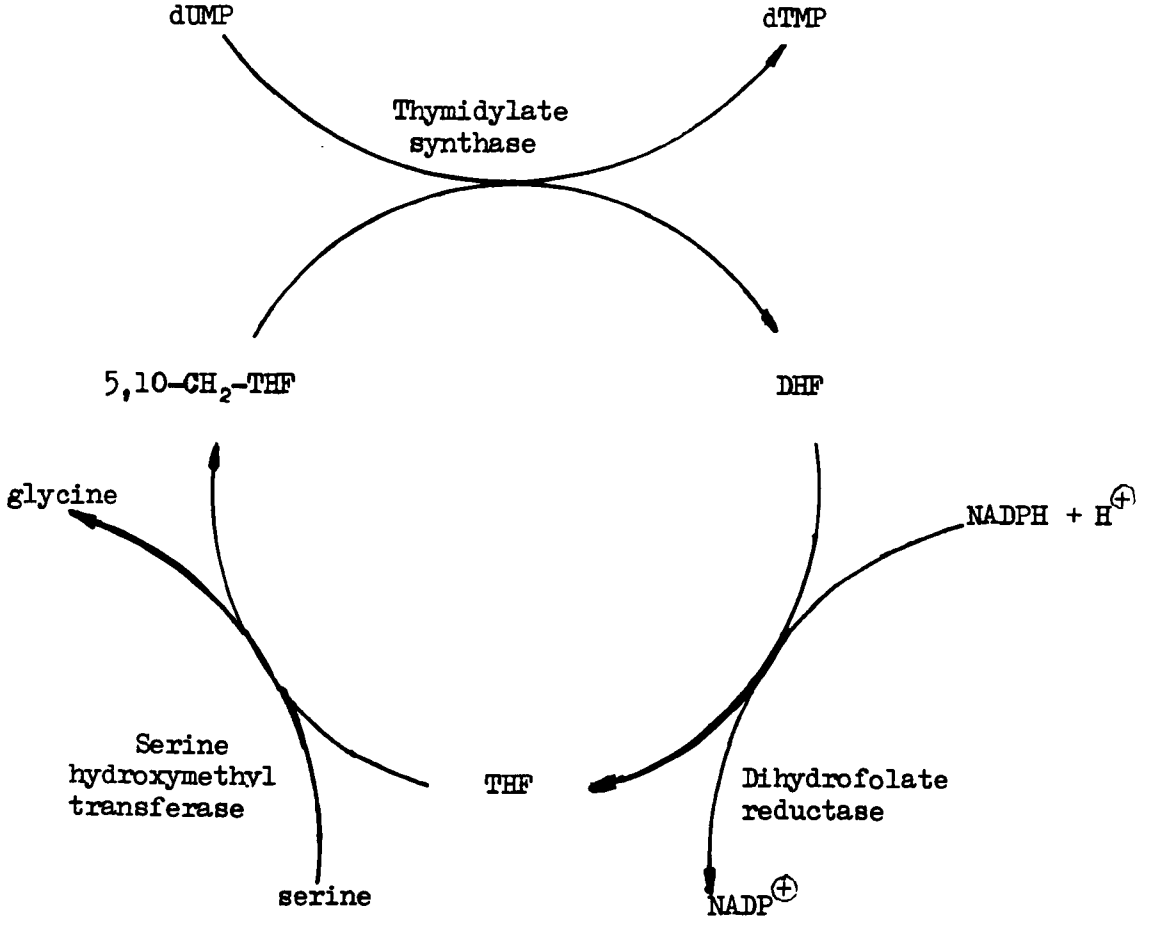


dUMP (2.10) (2.4) dTMP (2.11) (eqn. 2.1)


purines
sarcosine
glycine, serine
formate
HCHO
NH3
ATP
(2.3)
(2.4)
(2.5)
(2.6)
(2.7)
(2.8)
serine
thymidylate
purines
methionine
(2.9

Scheme(2.1)

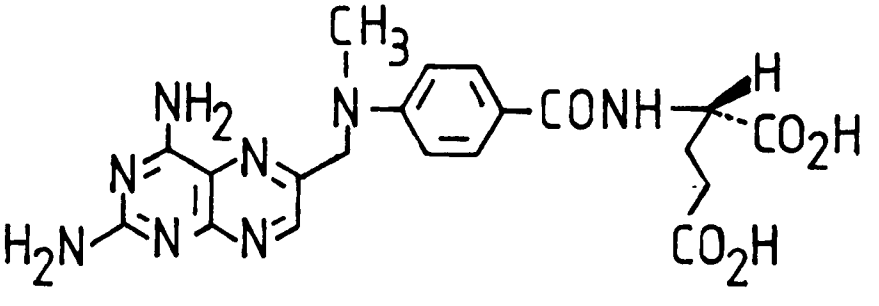
The reaction catalysed by the synthase is highly stereospecific in that 5,10-^{3}H,^{2}H methylene tetrahydrofolate yields dTMP with a chiral methyl group.[7] The reaction is also specific for the (6R)-hydrogen.[8,9] Tetrahydrofolate is regenerated by reduction of dihydrofolate. This reaction is catalysed by the enzyme dihydrofolate reductase (DHFR); NADPH is the required co-enzyme. The biosynthesis of dTMP can thus be represented as shown (Scheme 2.2).



Scheme (2.2)

The cycle is completed by serine hydroxymethyl transferase which catalyses the reversible transfer of the one-carbon unit between serine and glycine. In rat liver, approximately 71% of the β-carbon of serine is catabolised by way of the folate cofactor and thus the reaction catalysed by this enzyme is a major source of the one-carbon units needed for biosynthesis.[10]

As stated above the prevention of the biosynthesis of thymine is central to a large part of cancer chemotherapy. In principle, inhibition of any of the three enzymatic reactions involved would achieve this purpose. In clinical practice however, the inhibition of dihydrofolate reductase is the most effective measure. The most widely used inhibitor of this enzyme is methotrexate, 4-amino-10-methyl-4-deoxyfolic acid (2.12). This analogue binds to the enzyme approximately 10,000 times more tightly than does folic acid (2.1). It inhibits competitively. A degree of selectivity is obtained with the use of this material in that cancer cells have a greater requirement for DNA synthesis.



(2.12)

However, the amount of methotrexate (2.12) needed to be effective means that it is also toxic to normal cells. This problem has led to the use of 5-formyl-5,6,7,8-tetrahydrofolate (2.8) (leucovorin) as a rescue agent.[11,12] This combination therapy is most effective in the treatment of leukaemia. Thus, after administration of a high dose of methotrexate the patient is 'rescued' some 12 to 24 hours later with leucovorin in the form of the calcium salt. This by-passes the block of one-carbon metabolism at the dihydrofolate reductase stage and regenerates the level of reduced folates in the cell. With the development of newer inhibitors of dihydrofolate reductase the use of this combination therapy seems to be increasing. The calcium leucovorin in clinical use is a mixture of diastereomers epimeric at C6. Cosulich *et al*[13] reported that separation by fractional crystallisation of the calcium salts gave one isomer with $[\alpha]_D^{26} = -15.1$ (c. 1.82, H_2O). This material had micro biological activity for *Pediococcus cerevisiae*. Fontecilla-Camps *et al*[11] later prepared 5,10-methenyltetrahydrofolate (2.5) from what appeared to be the pure natural diastereomer of the calcium salt of 5-formyltetrahydrofolate. This calcium leucovorin had $[\alpha]_D^{25} = -25.2 \pm 0.4$ (c.0.91, H_2O) and further recrystallisation did not improve the optical rotation. Compared with a commercial sample of calcium leucovorin this material had a relative biological potency of 1.95 ± 1.16 (SD) using *Pediococcus cerevisiae*. In addition, the material was *at least* twice as effective in the reversal of methotrexate (2.12) toxicity in mice. The natural diastereomer

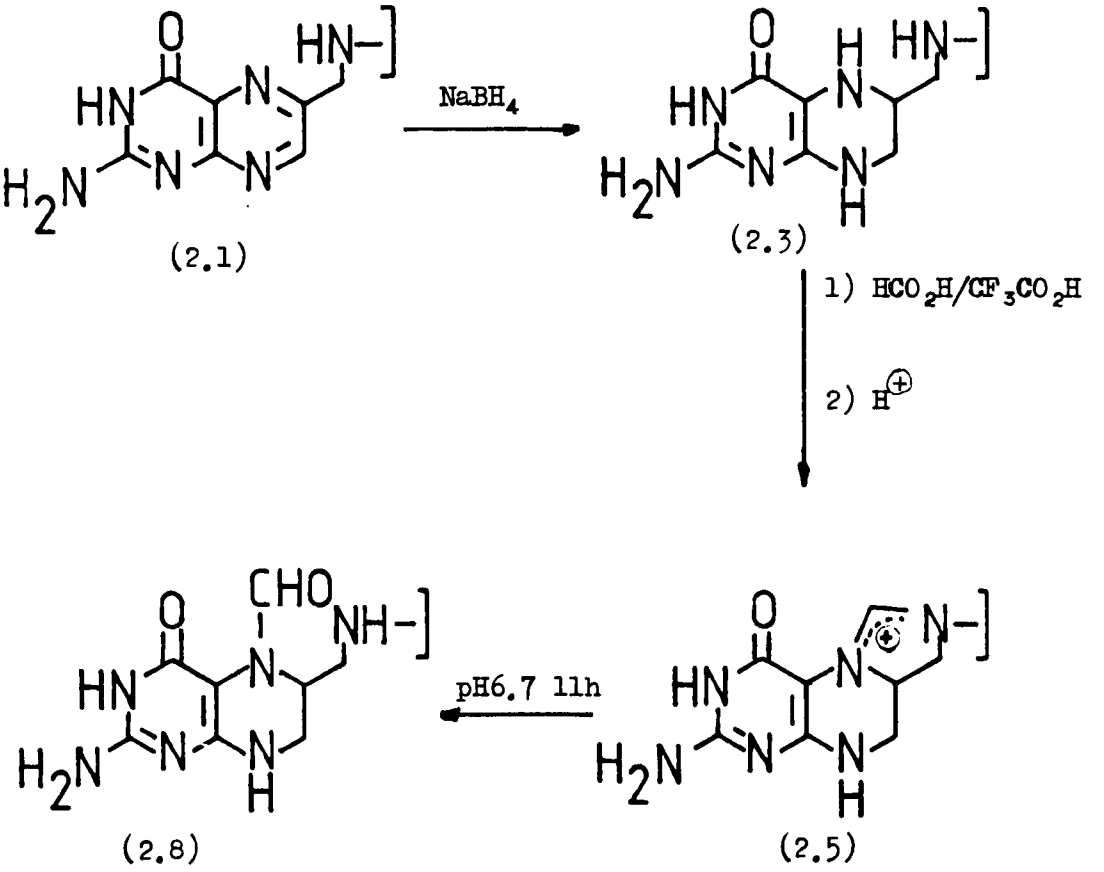
of calcium leucovorin was converted into 5,10-methenyltetrahydrofolate (2.5), $[\alpha]_D^{25} = +11.4 \pm 0.8$ (c.0.95, 12 N HCl). X-ray crystallography of this compound defined the stereochemistry at C-6 as (R). This corresponds to (6S) in natural tetrahydrofolic acid (2.3).

The separation of the diastereomers by fractional crystallisation is not easily achieved and rejection of 50% of the material is not economically sound. This thesis is concerned with the preparation of (6S)-5-formyltetrahydrofolic acid (2.8) by asymmetric reduction of the N5-C6 double bond of a suitable precursor.

The project has potential clinical significance since it would be of interest to monitor the effect of using the biologically active diastereomer in chemotherapy. It can be appreciated that the pure isomer could be twice as effective as the (6-RS) mixture in reversing methotrexate (2.12) toxicity. The situation may be more complicated however. For example, Leary *et al*[14] found that with thymidylate synthase from *L.casei*, the rate of reaction of (6R,S)-5,10-methylene tetrahydrofolic acid (2.4) at 3×10^{-4} M, was 75% of that obtained with 1.5×10^{-4} M of the (6R) isomer. The (6S) isomer was shown to be a competitive inhibitor of the enzyme catalysed reaction. The (6S) isomer was also shown to be a non-competitive inhibitor of the enzyme 5,10-methylene tetrahydrofolate dehydrogenase from *E.coli*[15]. So it seems that, in certain cases at least, the unnatural tetrahydrofolate epimers cannot be regarded as inert components in an enzyme catalysed reaction.

Synthetic Methods

Two general approaches to the chemical synthesis of 5-formyltetrahydrofolic acid (2.8) have been described. In the first, tetrahydrofolic acid was allowed to react with formic acid to give 5,10-methenyltetrahydrofolic acid (2.5). This was then hydrolysed to 5-formyltetrahydrofolic acid (2.8) in hot, neutral or alkaline medium with a reaction time of one hour.[16,17] Alternatively, 10-formyl folic acid (2.6) was catalytically hydrogenated in formic acid. Treatment of the 5,10-methenyltetrahydrofolic acid (2.5) so formed with base *in situ* at elevated temperatures gave 5-formyltetrahydrofolic acid (2.8).[18] A recent synthesis, based on optimisation of the first method has been published.[19] Thus, folic acid (2.1) was reduced with an excess of sodium borohydride to give tetrahydrofolic acid (2.3), and subsequent treatment with formic acid gave 5,10-methenyltetrahydrofolic acid (2.5). High quality 5-formyltetrahydrofolic acid (2.8) resulted from use of isolated (purified) samples of 5,10-methenyltetrahydrofolic acid (2.5) and from conversion of the latter into product under conditions in which the formation of decomposition products was minimised *viz.* pH 6.7 for 11 hours. The yield was approximately 45% from folic acid (2.1) (Scheme 2.3).



(2.1) (2.3) (2.8) (2.5)

Scheme (2.3)

In considering the type of reagent to use to effect the asymmetric reduction, a factor of overriding importance was the need for the reagent to be compatible with an aqueous environment. Only one example of the asymmetric reduction of folic acid (2.1) has been reported[20] and this made use of an optically active rhodium catalyst. $[(py)_2(amide)RhCl(BH_4)]^{\oplus}Cl^{\ominus}$, py-pyridine. A modest enantiomeric excess of the biologically active diastereomer of tetrahydrofolic acid (2.3) was obtained using the optically active amide, (-)N-1-phenylethyl formamide.

While the use of conventional, chemical chiral reducing agents was considered, and indeed reductions were later done using chiral hydride reagents, initial experiments were biased in favour of using the enzyme, dihydrofolate reductase [EC.1.5.1.3]. The reasons for this were:

(a) a supply of the enzyme was available,

(b) the required chirality, (S), at C6 was ensured,

(c) the enzyme is catalytically active, and

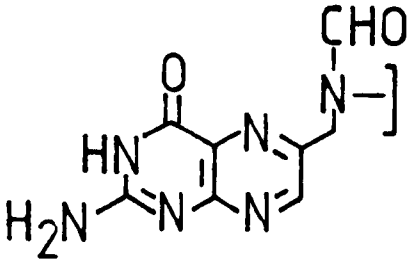
(d) the enzyme functions in an aqueous environment.

The dihydrofolate reductase used was isolated from a trimethoprim resistant strain of E.coli (RT 500).[21] In common with strains isolated from other sources the enzyme exhibits two pH optima, at 7.0 and 5.5, for the reduction of dihydrofolic acid (2.2).[4] The enzyme was available in two separated forms. Under standard assay conditions at pH 7.0 and 25°C, form I, which is more active at pH 7.0, had a specific activity of 8.65 U/mg of solid (27 U/mg of protein); form II had a specific activity of 3.3 U/mg at pH 7.0 and 24.4 U/mg at pH 5.5. Although the enzymatic reductions were carried out at ~ pH 7.0, form II was used initially since we had a greater supply. The reaction catalysed by the enzyme is shown in equation 2.2.

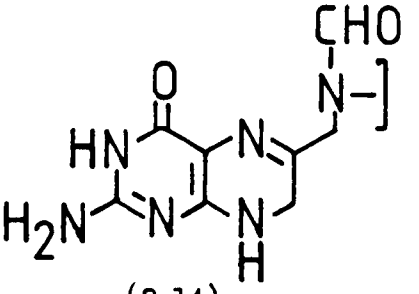
$$\text{Dihydrofolic acid} + \text{NADPH} + H^{\oplus} \rightleftharpoons \text{Tetrahydrofolic acid} + \text{NADP}^{\oplus} \qquad \text{(eqn. 2.2)}$$

Having decided to effect the asymmetric reduction enzymically the final route(s) to 5-formyltetrahydrofolic acid (2.8) would depend on the substrate specificity of the enzyme. The enzyme used will catalyse reduction of folic acid (2.1) at

pH 4.5, requiring 750-fold more enzyme to obtain a reaction rate equal to that with dihydrofolic acid (2.2) as a substrate.[21] The ability of this enzyme to reduce 10-formylfolic acid (2.13) and 10-formyldihydrofolic acid (2.14) under standard assay conditions was examined. A commercial sample of 10-formylfolic acid (2.13) was re-crystallised from water, the u.v. spectrum was as expected,[23] and the combustion analysis was satisfactory.



(2.13)



(2.14)

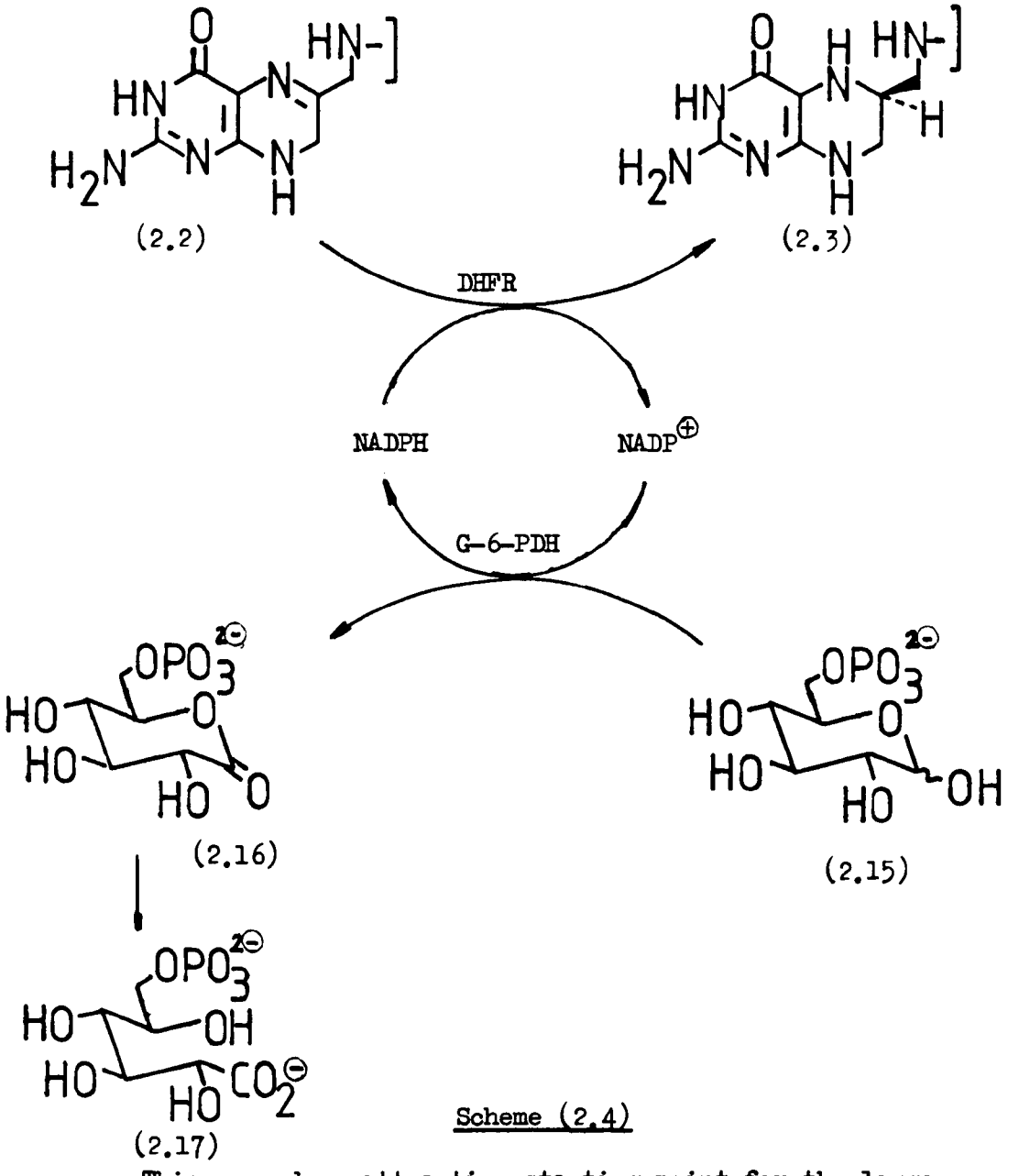
10-Formyldihydrofolic acid (2.14) was prepared by sodium dithionite reduction of this material.[17] The yield was estimated spectrophotometrically by deformylating in base to give dihydrofolic acid (2.2) [(0.1N NaOH)λmax = 283 nm; $\epsilon = 20.4 \times 10^3$]; the yield was $\sim$ 95%. 10-Formyldihydrofolic acid (2.14) was not isolated, the concentration required for the enzymic assay being prepared from an aqueous solution. Neither derivative, at either pH 5.0 or 7.0, was a substrate for dihydrofolic reductase under standard conditions.

The best route to leucovorin, therefore, seemed to involve reduction of folic acid (2.1) to dihydrofolic acid (2.2), enzyme

catalysed reduction of this to (6S)-tetrahydrofolic acid (2.3) and subsequent conversion into 5-formyltetrahydrofolic acid (2.8) *via* 5,10-methenyltetrahydrofolic acid (2.5). Before setting out on this course it was deemed wise to prepare (6R,S)-5-formyl-tetrahydrofolic acid by the method of Scheme 2.3.[19] Thus, 5,10-methenyltetrahydrofolic acid chloride was isolated in 58% yield. This was converted into calcium (6R,S)-5-formyltetrahydrofolate in ~90% yield by careful hydrolysis at pH 6.7. It was discovered that further purification of this material could be effected by careful precipitation from an aqueous solution by the addition of ethanol when the impurities precipitated first. The properties of this material were in keeping with those in the literature.[4,19] The HPLC profile was also comparable to that of a commercial sample. Since this route from tetrahydrofolic acid (2.3) seemed to present no difficulties the next step was to consider the problems involved in the large scale reduction of dihydrofolic acid (2.2) to (6S)-tetrahydrofolic acid (2.3), catalysed by dihydrofolate reductase.

Enzyme Catalysed Reduction

Previously, Mathews and Huennekens[22] prepared (6S)-tetrahydrofolic acid (2.3) by the reduction of dihydrofolic acid (2.2) catalysed by dihydrofolate reductase, isolated from chicken liver. They recycled the coenzyme NADPH approximately 6-fold using glucose-6-phosphate (2.15) and glucose-6-phosphate dehydrogenase (G-6-PDH). The reduction was done on 56 μ mol of dihydrofolate and, after DEAE cellulose chromatography, (6S)-tetrahydrofolic acid (2.3) was isolated in 83% yield. (Scheme 2.4).

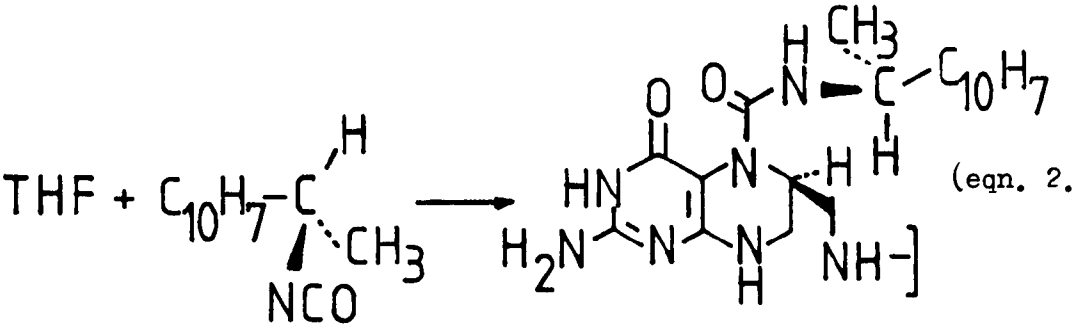


Scheme (2.4)

This seemed an attractive starting point for the large scale enzymic synthesis of (6S)-tetrahydrofolic acid (2.3). As mentioned previously the use of glucose-6-phosphate (2.15) and glucose-6-phosphate dehydrogenase to recycle nicotinamide coenzymes has numerous advantages. Much of the research directed towards developing techniques which would make possible the use of cofactor requiring enzymes in organic synthesis has

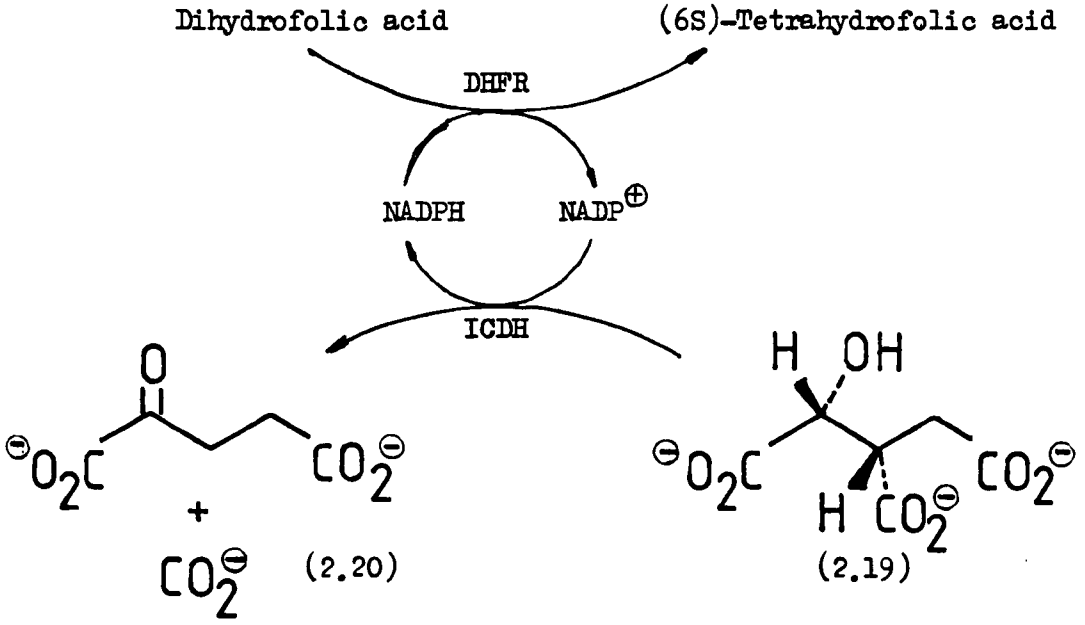
been done by Whitesides and co-workers.[24] They have found in practice that the convenience of this method for recycling NAD(P)H outweighs the disadvantage of having to prepare glucose-6-phosphate (2.15). Indeed, they have developed methods of preparing glucose-6-phosphate (2.15) in situ by the hexokinase catalysed phosphorylation of glucose.

The first requirement for the proposed method was to push the NADPH recycling to an extent that would allow the synthesis of (6S)-tetrahydrofolic acid (2.3) to be economic. Numerous small scale reactions allowed us to reduce dihydrofolic acid (2.2) enzymically with confidence with up to 1,000-fold recycling of NADPH. The required dihydrofolic acid (2.2) was prepared by the method of Futterman, as modified by Blakley.[25] In practice the oxidised form of the co-enzyme, $NADP^{\oplus}$ was used since its solution and storage stability is better than that of NADPH. The tetrahydrofolic acid (2.3) was not isolated but its formation was monitored by reversed phase HPLC. The optical purity of the tetrahydrofolic acid (2.3) so formed was conveniently estimated by reaction with R-(-)-1-(1-naphthyl)-ethyl isocyanate (2.18)[26] which reacts by addition, cf[27], at N_5 of tetrahydrofolate (equation 2.3). Reverse phase HPLC analysis gave good resolution of the C_6 epimers produced by chemical reduction. A single peak was obtained from the material formed in the enzyme catalysed reduction.



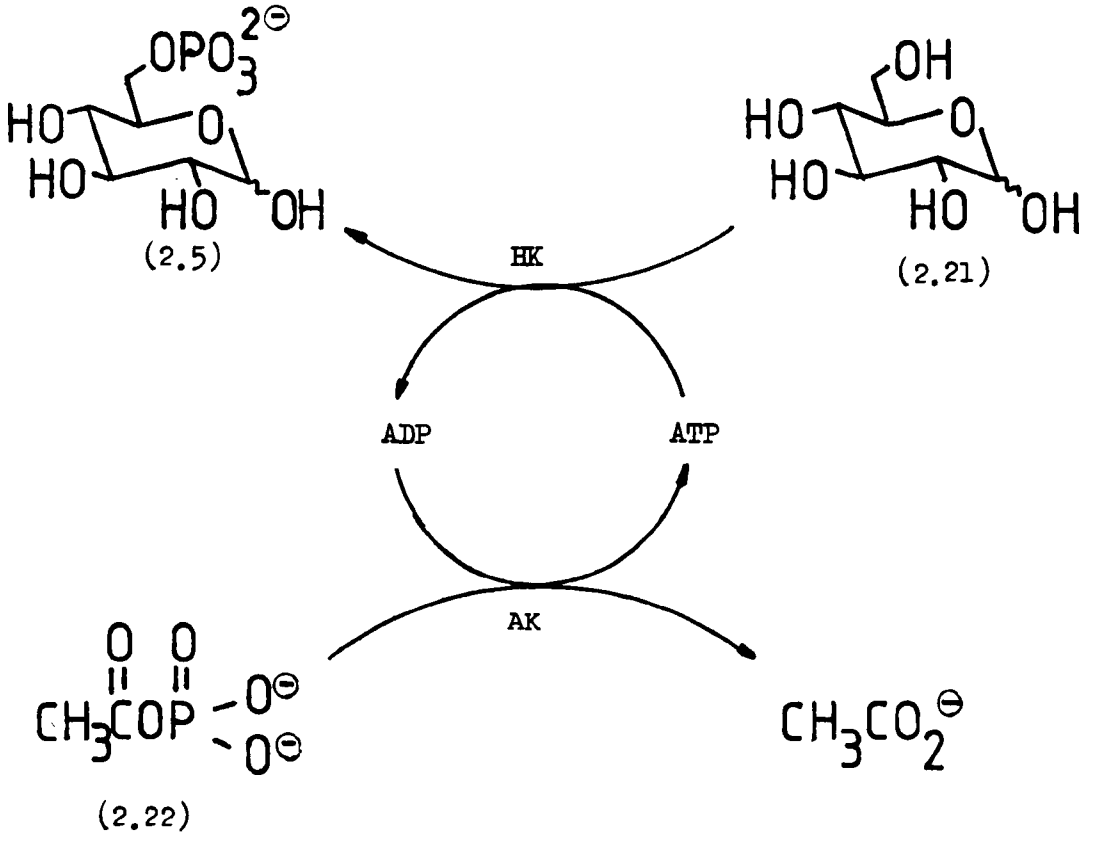
(2.18) (eqn. 2.3)

There are a number of other procedures which are effective for reducing $NADP^{\oplus}$. Many of these methods have been reviewed.[28,29] The use of isocitrate (2.19) and isocitrate dehydrogenase (ICDH) was studied. The enzyme catalyses the oxidation/decarboxylation of D-isocitrate to α-ketoglutarate (2.20) (Scheme 2.5).



Scheme (2.5)

As mentioned the disadvantage in using glucose-6-phosphate /glucose-6-phosphate dehydrogenase as a nicotinamide cofactor regenerating system is the cost of glucose-6-phosphate (2.15). However, glucose-6-phosphate (2.15) can be prepared in quantity by the hexokinase catalysed phosphorylation of glucose (2.21) by ATP, coupled with ATP regeneration.[31] Acetyl phosphate (2.22) phosphorylates the ADP formed in a reaction catalysed by the enzyme acetate kinase (Scheme 2.6).



Scheme (2.6)

In practice DL-isocitrate was used for the sake of economy. In this, and subsequent enzymatic reactions, an aqueous solution of the necessary components was used. The pH was kept at the desired value (around 7.0) by the addition of hydrochloric acid or sodium hydroxide solution, as necessary, using a'pH stat' apparatus. The reactions were performed under nitrogen with the inclusion of dithiothreitol; this fulfilled the function of preventing oxidation of the reduced folates and of essential thiol groups at, or near, the enzyme active site. The reduction was done on 507 μmol of dihydrofolate, using 1.5 units of dihydrofolate reductase and 0.96 units of isocitrate dehydrogenase; 130-fold recycling of NADPH was thus achieved. After seven days the reduction had gone to completion. The reaction mixture was freeze-dried and the yellow lyophilisate was allowed to react with formic acid. The crude (6R)-5,10-methenyltetrahydrofolate (2.5) was purified using DEAE cellulose chromatography, the eluent being 0.1M formic acid - 0.01M β-mercaptoethanol.[30] Effluent fractions exhibiting a value greater than 1.6 for the ratio of absorbance 345:310 nm in 1N-HCl were pooled and lyophilised. The yellow solid obtained was recrystallised from 0.1N HCl - 0.1 M β-mercaptoethanol, to give 46.15 mg, (24% yield based on dihydrofolate) of (6R)-5,10-methenyltetrahydrofolate (2.5), $[\alpha]_D^{24} = +19.2 \pm 2.5$ (c.0.39, 10N HCl). This rotation is in fair agreement with that reported in ref. 1. While this method was eminently suitable for the preparation of research quantities of (6S)-tetrahydrofolic acid (2.3) and derivatives, the procedure based on glucose-6-phosphate (2.15) held most promise,in economic terms,for larger scale syntheses.

An efficient, cheap synthesis of acetyl phosphate (2.22) was therefore required. Acetyl phosphate has been synthesised previously from phosphoric acid by acylation with acetyl chloride[32], ketene[33], isopropenyl acetate[34], and acetic anhydride[35,36] and isolated as the lithium or silver salts.[37] All of these procedures contain difficult work-up and isolation sequences. Two methods involving acylation with ketene or acetic anhydride have recently been published.[38,39] Acetyl phosphate (2.22) was conveniently isolated as its diammonium salt. Initial experiments, using ketene or acetic anhydride to acylate phosphoric acid, failed to produce acetyl phosphate (2.22) of the required purity. The problem was traced to the purity of the phosphoric acid used, 100% phosphoric acid being required. Neither dehydration of 85% syrupy phosphoric acid using phosphorus pentoxide[40] nor drying of the crystalline hemihydrate over magnesium perchlorate *in vacuo* was effective. Eventually a method was developed for dehydration of the hemihydrate by heating to 60°C under reduced pressure (0.1 mm), in the presence of phosphorus pentoxide, for 30 h. It was essential to heat the material above its melting point (30°) but below 100°C to prevent the formation of polyphosphates. On cooling to room temperature the liquid spontaneously crystallised and this material was then used immediately in the preparation of acetyl phosphate (2.22) using acetic anhydride as the acylating agent (equation 2.4).[39] Following the published procedure diammonium acetyl phosphate was obtained with 84% purity, corresponding to a 67% yield based on

$$1{\cdot}8(CH_3CO)_2O + H_3PO_4 \xrightarrow[3{\cdot}7\,h,\,0^\circ C]{EtOAc}$$

$$\xrightarrow[-40 \rightarrow -20^\circ C]{NH_3/CH_3OH} CH_3COP(O^{\ominus}NH_4^{\oplus})_2 \qquad \text{(eqn. 2.4)}$$

phosphoric acid. The purity was estimated by n.m.r. assay using dioxan as an internal standard. The principal impurities being acetamide, ammonium acetate, and inorganic phosphate. Integration of the dioxan peak and of the acetyl protons of acetyl phosphate (2.22), acetamide, and ammonium acetate allows calculation of the percentage composition of the solid. It is important that the acetyl phosphate (2.22) has > 80% purity otherwise complexation of Mg(II) by the phosphate impurities would lower the effectiveness of the enzyme catalysed reactions. It is therefore important to assay the efficiency of the material, prepared in the above manner, in the enzyme catalysed reaction. The usual assay method for the purity of acetyl phosphate (2.22) [or any other phosphate donor and indeed for the activity of the phosphotransferase enzymes] involves linking the reaction in question to another enzymatic reaction, which uses a nicotinamide co-enzyme. This means that the usual continuous spectrophotometric method can be used. Such an assay is shown for acetyl phosphate (2.22) (Scheme 2.7),[39] the change in absorbance at 340 nm being related to the concentration of acetyl phosphate (2.22) used.

$$\text{AcP} + \text{ADP} \xrightleftharpoons{\text{Acetate kinase}} \text{ATP} + CH_3CO_2^{\ominus}$$

$$\text{ATP} + \text{D-glucose} \xrightleftharpoons{\text{hexokinase}} \text{ADP} + \text{D-glucose-6-phosphate}$$

$$\text{D-glucose-6-phosphate} + NADP^{\oplus} \xrightleftharpoons[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-phospho-D-gluconic acid} + NADPH$$

Scheme 2.7

It was felt that this method was tedious and wasteful in that the enzyme and substrate solutions prepared have to be made freshly each day and then discarded. An attempt was thus made to develop a simpler and less expensive assay based on the HPLC separation and quantitation of the ATP formed. HPLC has been used for the assay of several enzymes[41,42,43] and a review has been published.[44] The problem then, was one of separation of the adenosine nucleotides from each other and quantitation of ATP and ADP; this extends the range of phosphotransferase enzymes which could be assayed by this method. The procedure of Danielson and Huth[45] was used and this involved paired ion chromatography using a reverse phase column. A brief description of the terms and materials used in HPLC is therefore necessary.

The columns used are packed with microparticulate, functionalised silica gel. The organic phases are bonded via organochlorosilanes to form stable siloxy bonds (eqn. 2.5).

$$\text{Si-OH} + R_3\text{SiCl} \longrightarrow \text{Si-O-Si-}R_3 \qquad \text{(eqn. 2.5)}$$

In reverse phase chromatography the stationary phase is non-polar and usually consists of an octadecyl hydrocarbon chain. In ion-exchange chromatography an aliphatic or aromatic moiety is converted into a cation ($NR_3^{\oplus}$) or anion ($SO_3^{\ominus}$) exchanger.

Ordinarily, ionic or ionisable species are poorly retained on a reverse phase column, however, good separations can be obtained using paired-ion chromatography.[46] In essence, the technique depends on a phase-transfer reaction. A compound with a hydrophobic tail and a functional group (an alkyl sulphonic acid or a tertiary alkyl ammonium salt) opposite in charge to that of the compound(s) of interest, is added to a water-methanol eluent. The organic counterions ($C^{\oplus}$ or $C^{\ominus}$) form a reversible ion-pair complex with the ionised solute, the complex being electrically neutral. This allows retention on the non-polar stationary phase. The reaction is illustrated for a negatively charged solute, $R^{\ominus}$, (eqn. 2.6).

$$R^{\ominus}(aq) + C^{\oplus}(aq) \rightleftharpoons RC(aq) \rightleftharpoons RC(org) \qquad \text{(eqn. 2.6)}$$

The greatest effect will be obtained at a pH where the solute is maximally ionised.

The pKa value of the primary phosphate group of the adenosine nucleotides are given.[47]

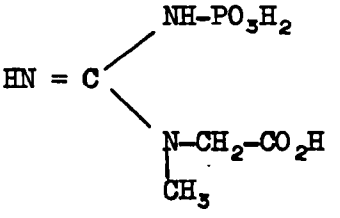
AMP 6.2-6.4)
ADP 6.1-6.7) depending on the cation
ATP 6.0-6.95)

The mobile phase used consisted of an 88% mixture of 50 mM potassium dihydrogen orthophosphate; 0.005 M tetra-n-butyl ammonium phosphate, adjusted to pH 6.8 with 1 N potassium hydroxide, and 12% methanol. This differs slightly from the eluent described in ref. 45 in that the molarity of the buffer, and of the ammonium salt, is lower. For a flowrate of 119 ml/h the retention times of the nucleotides (monitored at a wavelength of 260 nm) were, in

minutes, AMP, 5.2; ADP, 10.2; ATP, 18.4. A linear response was obtained, for a plot of peak area (by triangulation) vs. concentration, for ADP and ATP. Using this method the concentration of acetyl phosphate (2.22) in the sample was assayed by its complete conversion to ATP in the acetate kinase catalysed reaction. Agreement between this method and the n.m.r. assay was good. Hexokinase was also assayed by this method, the activity being related to the concentration of ADP formed with time. For a particular batch of enzyme the specific activity was 9 U/mg; when assayed by a standard spectrophotometric method the activity was 11 U/mg.[48] It was found, however, that the use of this particular ion-pair reagent reduced the working life of the HPLC column to 25% of normal. This effect is attributed to the reaction of the alkyl ammonium salt with un-modified silanol groups on the silica backbone, and this leads to dissolution of the stationary phase. A method was therefore developed for quantitation of ADP and ATP using a strong anion exchange column. The mobile phase used was 0.2 M potassium dihydrogen orthophosphate (pH 4.0 with phosphoric acid solution); 1 M in potassium chloride. The retention times, in minutes, were; AMP, 2.8; ADP, 4.8; ATP, 14.8, for a flowrate of 98.5 ml/h. A linear response was obtained for peak area vs. concentration. This procedure was used to assay acetyl phosphate (2.22), hexokinase, and acetate kinase. Agreement between the two HPLC methods was good. The chromatographic method of assaying enzyme activity is useful for immobilised enzymes in that the enzymatic reaction can be done in any convenient vessel and agitation of the insoluble support by stirring is easy, whereas the u.v. assay involves the use of a

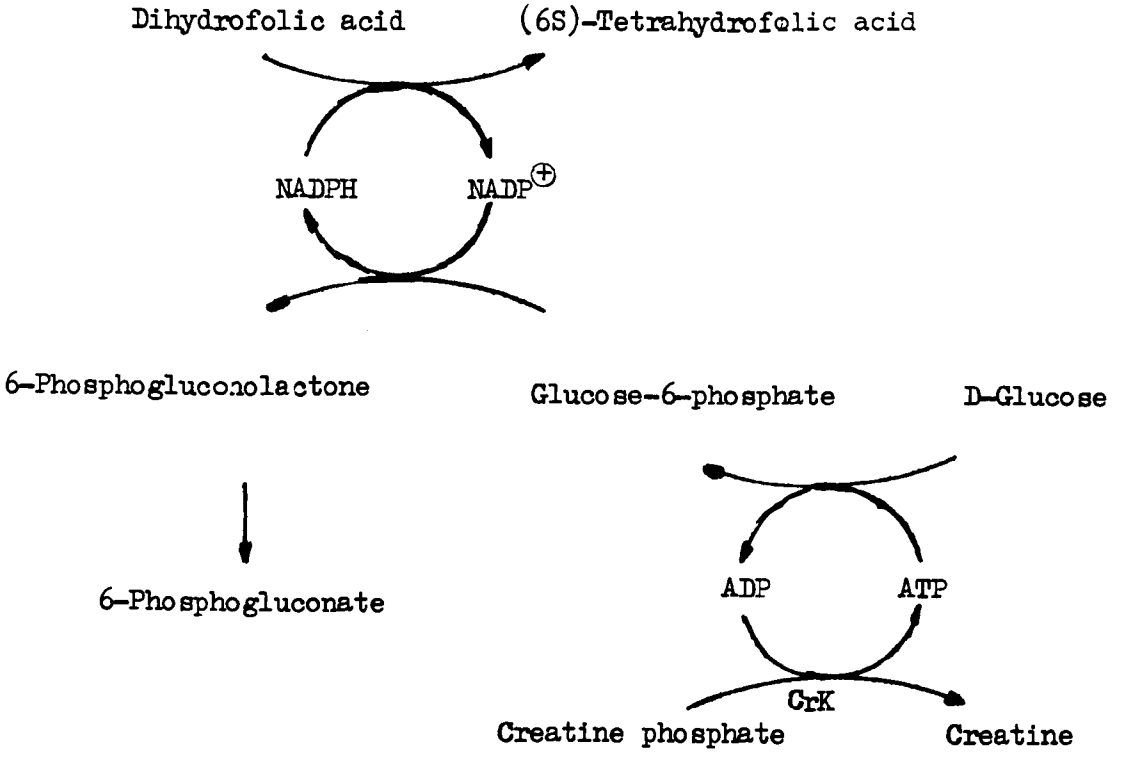
cuvette, with periodic agitation by shaking. Also, if the support is not transparent problems with light scattering can occur in the u.v. assay.

While, in diammonium acetyl phosphate a ready available cheap phosphate donor was thus available, this material was not used in the regeneration of ATP until later, after the enzymes involved had been immobilised.



2.23

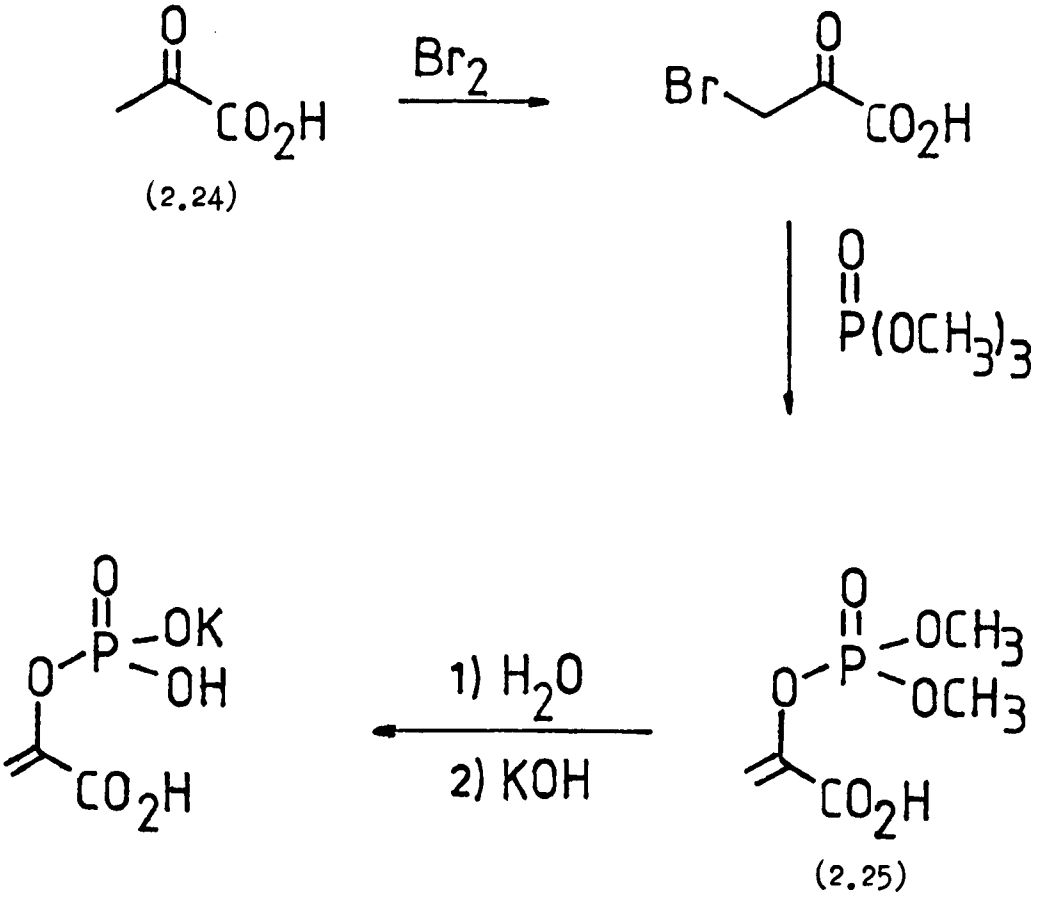
However, the proposed method was shown to be viable since the whole sequence using commercial creatine phosphate (2.23) as phosphate donor and non-immobilised enzymes was carried out. For example, dihydrofolic acid (2.2) 372 μmol was reduced to (6S)-tetrahydrofolic acid (2.3) in 5 days at ambient temperature between pH 7.4 and 7.6 using 4 units each of the enzymes involved; creatine phosphokinase, hexokinase, glucose-6-phosphate dehydrogenase, and dihydrofolate reductase. An equimolar amount of D-glucose was used and a slight molar excess (1.4) of creatine phosphate (2.23). ATP and $NADP^{\oplus}$ were recycled 46 and 93-fold respectively. This was subsequently extended to 70 and 300-fold recycling respectively (Scheme 2.8).



Scheme (2.8)

Creatine phosphate (2.23) has been used previously, with immobilised creatine kinase, to prepare ATP.[49] However existing syntheses of creatine phosphate (2.23) are difficult and not amenable to large scale preparation.[50] Moreover the enzyme, creatine kinase, is isolated from mammalian sources and would probably prove costly in the long term. The one primary advantage that this material has over acetyl phosphate (2.22) is slower hydrolysis in

slightly alkaline solution,[48] which makes enzymatic reactions involving creatine phosphate simpler in practice. In this respect another phosphate donor, phosphoenol pyruvate (2.25), is better than acetyl phosphate. It was not considered during the course of this work but a recently published synthesis makes its use attractive (Scheme 2.9).[51]



(2.24)

(2.25)

Scheme (2.9)

It can be prepared from crude pyruvic acid (2.24) in 50% yield and 95% purity. The synthesis is easier than that of acetyl phosphate (2.22) but the cost of the starting material is higher. Phosphoenol pyruvate (2.25) is a stronger phosphorylating agent than acetyl phosphate (2.22) and the enzyme, pyruvate kinase, is less expensive than acetate kinase. Perhaps the use of this material in an ATP regenerating scheme would be better for work on approximately molar scale while the use of acetyl phosphate (2.22) would be better for larger scale preparations.

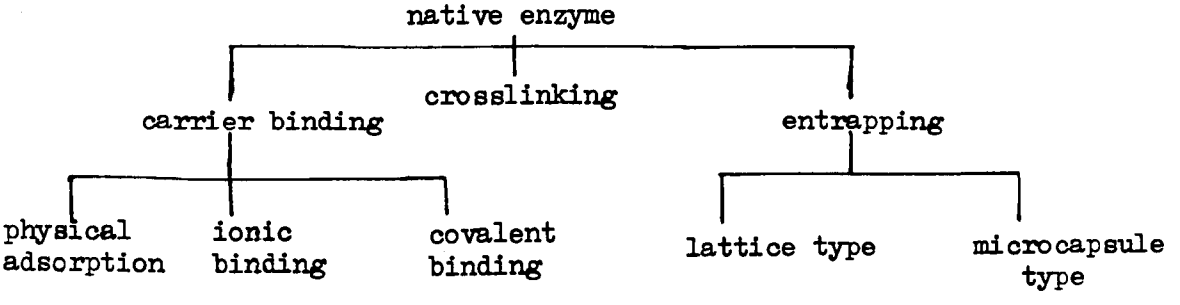
Enzyme Immobilisation

The next stage in our developing enzymatic synthesis of (6S)-tetrahydrofolic acid (2.3) was to look at methods of enzyme immobilisation.

The reasons for immobilising enzymes, when required for organic synthesis, are those of recovery of catalytic activity and of separation from the product. Numerous reviews exist on the subject, varying in depth of coverage and extent of experimental detail.[52,53,54] The catalytic activity, substrate specificity, and stereospecificity of an enzyme are determined by its primary amino acid sequence. The bonds involved in maintaining secondary, tertiary, and quaternary structures are non-covalent (apart from disulphide linkages) and consist of ionic interactions, hydrogen bonds, and hydrophobic interactions. These are individually labile but collectively they lead to a fairly stable, well defined, protein structure. The overall conformation of an enzyme brings the residues comprising the active site into a precise 3-dimensional arrangement of charges

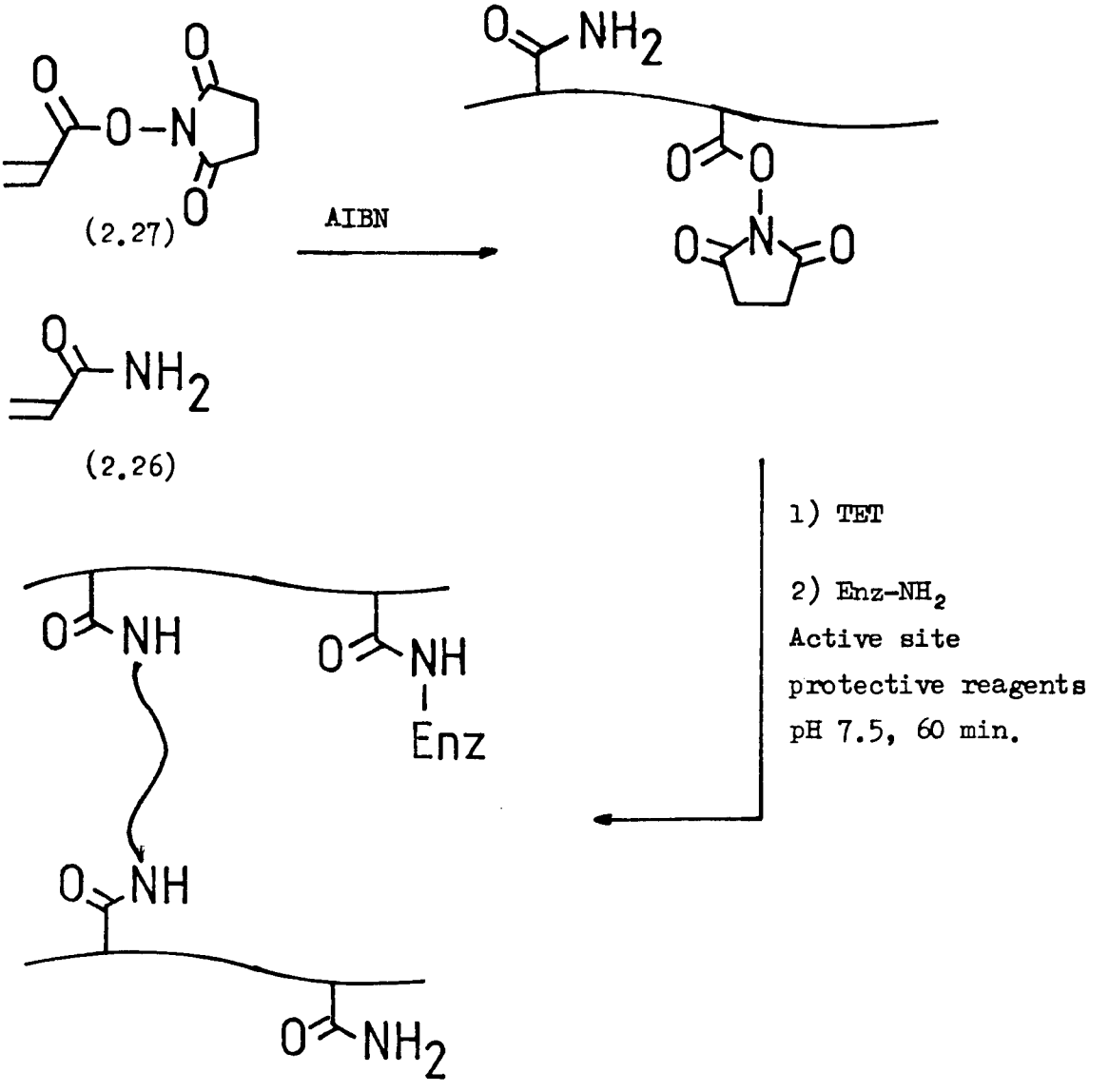
and microenvironment. It follows then, that the ideal immobilisation procedure would incur minimum disruption of the native protein conformation. A lot of research has been devoted to this subject over the last thirty years or so; unfortunately the potential for using immobilising enzymes as specific, recoverable industrial catalysts has been successful only in a few, well-defined areas. Perhaps the solution may lie in genetic modification of an enzyme in such a way that a convenient 'handle' is generated (at a position where minimum disruption of the native conformation results); the 'handle' having sufficiently different chemical reactivity from the rest of the protein making it alone amenable to further reaction under certain conditions, such that it could, for example, be covalently bound to an insoluble support.

The immobilisation methods in use at the present time can be conveniently subclassified as shown (Scheme 2.10).[54]



Scheme 2.10

As far as immobilisation methods go there is no one optimum method and a choice must be made on the basis of the type of enzyme and the final use of the support. The first choice in the present work was a procedure recently developed for use with the relatively delicate and expensive enzymes of interest in enzyme catalysed organic synthesis.[55] Thus a water soluble functionalised prepolymer, poly(acrylamide-co-N-acryloxy-succinimide), PAN, was prepared by the copolymerisation of acrylamide (2.26) and N-acryloyloxysuccinimide (2.27).



Scheme (2.11)

Reaction of this prepolymer with a low molecular weight diamine, triethylenetetramine (TET) crosslinks the polymer chains and forms an insoluble gel connected through amide groups. Reaction of the amino functions of the enzyme with residual active esters covalently links the enzyme to the gel through additional amide linkages (Scheme 2.11). The presence of reagents which bind at the active site affords some protection against modification of that area. Polyacrylamide was chosen as a base for the support because it is hydrophilic and non-denaturing towards many proteins.[56] The N-hydroxysuccinimide active ester group has been used previously in a copolymer with acrylamide as a medium for development of affinity chromatography supports.[57] The novelty of this system is that the entire volume of the buffer solution containing the reactive prepolymer and the enzyme is transformed to gel. The kinetic problems encountered when an enzyme is required to diffuse from solution into a reactive preformed gel (for example, cyanogen bromide activated agarose) are thus avoided and the coupling time is therefore short.

PAN was prepared by free-radical polymerisation of acrylamide and N-acryloxysuccinimide in tetrahydrofuran solution using thermal initiation with azobis (isobutyronitrile) (AIBN).[58] The polymer was assayed for active esters by allowing it to react with aqueous ethylamine solution and measuring the absorbance due to the anion of N-hydroxysuccinimide (ϵ_{259} = 8,600 $M^{-1} cm^{-1}$).[59] The composition of the polymer is specified in terms of the content (in μ equiv./g) of active ester groups. Thus the polymer used in the present work, termed PAN-400, released 400 $\pm$ 25 μmol of N-hydroxysuccinimide per gram of dry polymer on treatment with

excess ethylamine solution. TET was chosen as the cross-linking agent which yielded the gel having the greatest mechanical strength. In the standard procedure a quantity of PAN sufficient to give a 20% w/w solution was weighed rapidly in air and dissolved in 0.3 M Hepes buffer, pH 7.5, containing components intended to protect enzymatic activity. Dithiothreitol (5 mM) was included to inhibit protein oxidation. The cross-linking diamine, TET was then added to this rapidly stirred solution to provide 0.85 equiv.of primary amine groups/equiv. of active ester. This stoichiometry leaves ca. 15% of the active ester groups available for reaction with other nucleophiles. Addition of an enzyme to the reacting solution of PAN and TET before it gelled resulted in its covalent incorporation into the cross-linked polymer network. In a preliminary experiment the appropriate quantity of TET was added with vigorous stirring to the solution containing PAN, and the gel time (typically 2-3 min) for the mixture was measured. The enzyme was subsequently added before (30-60 s) the gel point. The enzyme containing gel was then allowed to stand for 60 min. to complete the coupling reaction, broken up by grinding in a mortar, washed with aqueous buffer containing ammonium sulphate to convert residual active ester groups into amides, and assayed. The yield obtained in the immobilisation is defined by equation 2.7.

$$\text{yield } (\%) = \frac{\text{units (assayed in gel)}}{\text{units (assayed in solution before immobilisation)}} \times 100 \qquad \text{(eqn. 2.7)}$$

In general the yield of immobilised activity was highest using quantities of enzyme which varied between 0.5 and 4 mg/g of PAN. Table 2.1 lists immobilisation yields obtained by applying this procedure to the enzymes required in our synthetic strategy. They are in good agreement with the published results.[55]

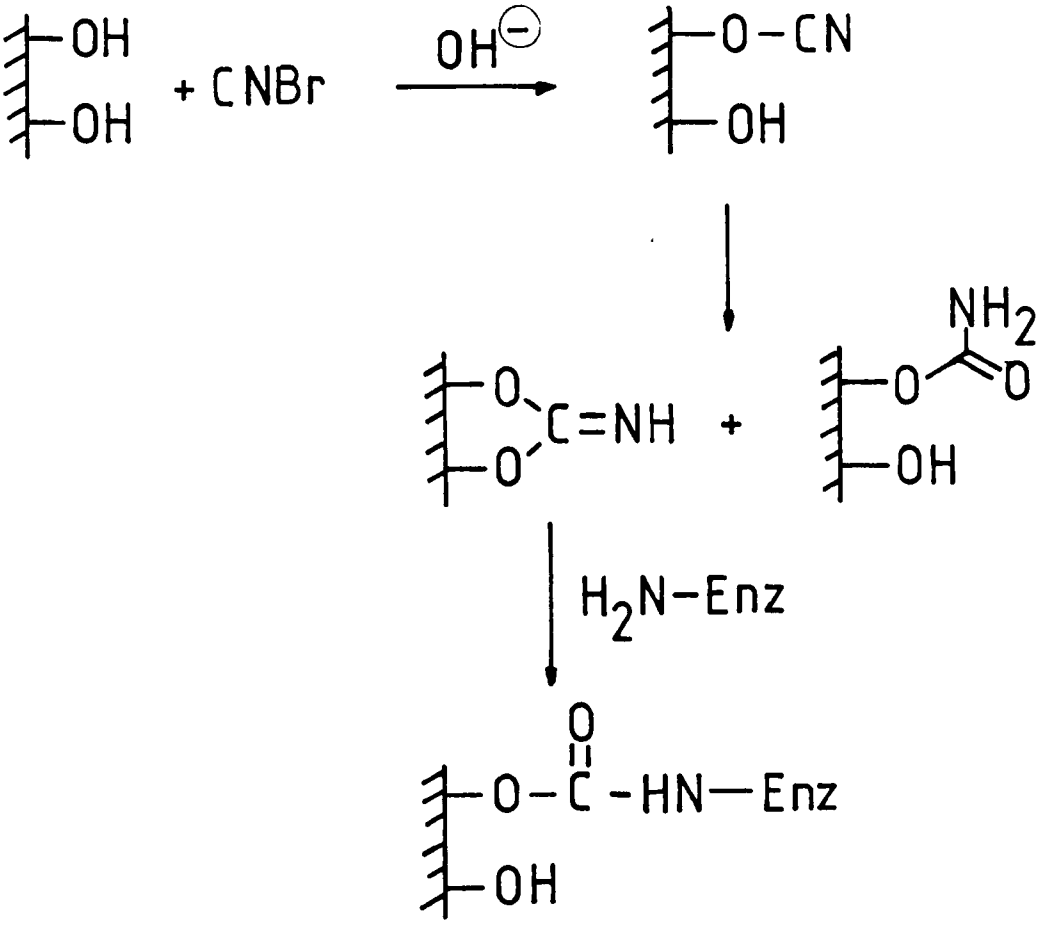
The immobilisation of acetate kinase was done under nitrogen since this enzyme is sensitive to dioxygen. In an effort to improve the immobilisation yield of dihydrofolate reductase (II) the enzyme was allowed to react with PAN before the addition of TET; 60 s before gave a yield of 18% and 78% recovery in washes, while 90 s before gave a yield of 25% with 50% activity in the washes. The lower immobilisation yield of dihydrofolate reductase (I) must reflect the lower purity of this preparation (0.32 mg protein/mg of solid). It is considered that a lower limit of 50 U enzyme activity/g of support is necessary for convenience in organic syntheses. All of the enzymes in Table 2.1 would fulfill this criterion except dihydrofolate reductase (note - the hexokinase used here has a specific activity of $\sim$10 U/mg; hexokinase is readily available however with a specific activity of 150 U/mg which would give a preparation having an activity of 150 U/g on the basis of the results obtained). The low activity obtained with dihydrofolate reductase reflects the low intrinsic activity of the native enzyme. Unfortunately with this, and other immobilisation procedures, higher loading of enzyme per unit weight of support results in a decrease in yield. This was the case for dihydrofolate reductase using this method. If the dihydrofolate reductase (I) was purified to homogeneity its specific activity would be of the order 27 U/mg, which, using this

Enzyme	C mg/g PAN	M conc. mM.	yield %	activity in washes %	activity U/g
glucose-6-phosphate dehydrogenase (yeast)	0.17	G-6-P(6.3)$NADP^{\oplus}$(1.0)	20	2	140
hexokinase	2.6	glucose(25)ADP(10)	42	10	12
creatine kinase	28	CrP(40)ADP(10) KNO_3(100)	8.5	0	328
acetate kinase	1.0	AcP(12.5)ADP(20)	42	42	67.2
dihydrofolate reductase (II)	1.0	DHF(0.32)$NADP^{\oplus}$(0.7)	18	70	0.6
dihydrofolate reductase (I)	1.0	DHF(0.32)$NADP^{\oplus}$(0.7)	5	69	0.4

Table 2.1

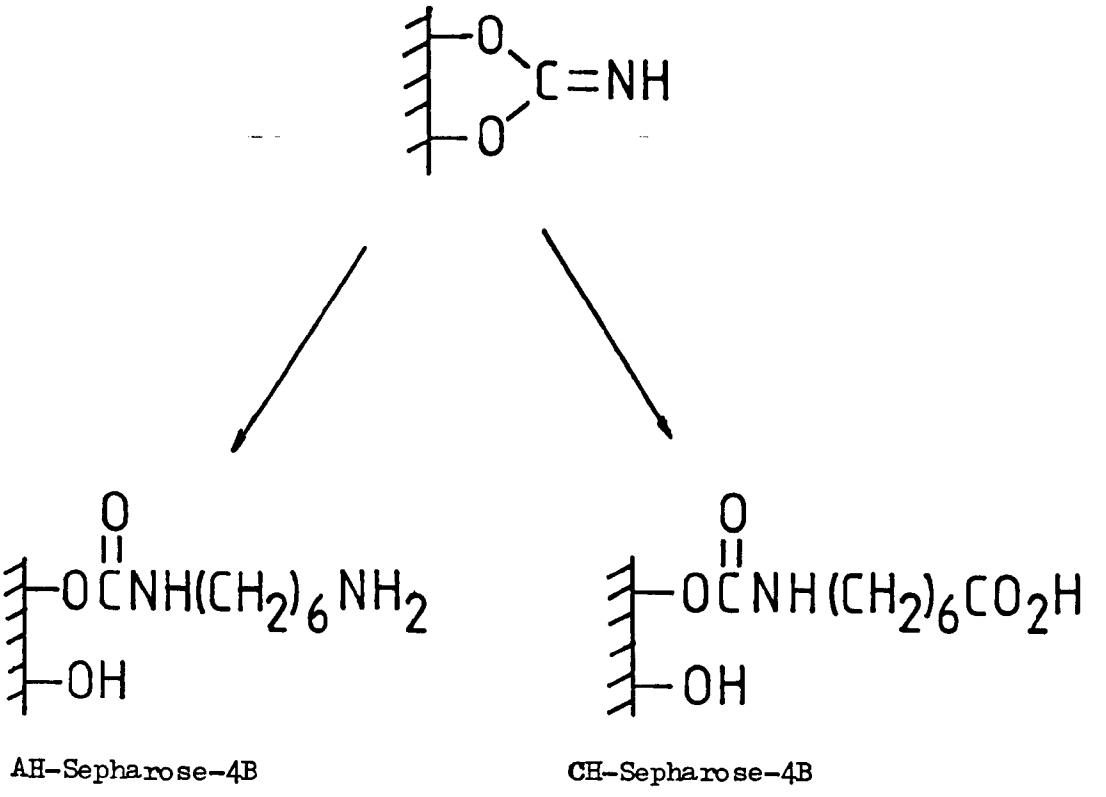
procedure would lead to a support having activity of only 7 U./g.

We therefore decided to look at some more established methods of enzyme immobilisation in an effort to prepare a support having greater activity/unit weight. Covalent attachment of an enzyme to an insoluble support has generally been based on activation of a preformed hydrophilic polymer such as agarose or copolymers of acrylamide. Agarose is available commercially as Sepharose 4B. Undoubtedly the most generally used method for enzyme immobilisation is the cyanogen bromide (CNBr) activation of agarose (Scheme 2.12)[52,60]



Scheme (2.12)

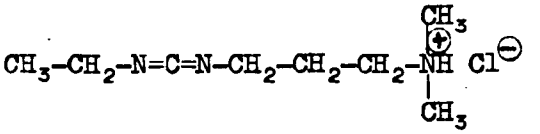
The cyanate formed initially reacts to give unreactive carbamate and reactive imidocarbonate. Reaction of the imidocarbonate with the amino functions of the protein leads to immobilisation. An extension of this technique involves reacting 1,6-diaminohexane or 6-aminohexanoic acid with the activated Sepharose to give supports with free amine groups (AH-Sepharose-4B) or free carboxyl groups (CH-Sepharose-4B). These are then available for coupling with the carboxyl groups or amino groups on enzymes in a carbodiimide mediated reaction (Scheme 2.13).[61] The water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-



AH-Sepharose-4B CH-Sepharose-4B

Scheme (2.13)

imide (EDC, 2.28) is preferred.



(2.28)

Polyacrylamide is also a good basis for supports in enzyme immobilisation. It has good chemical and mechanical stability, it is hydrophilic and is not susceptible to microbial attack. Moreover, due to the number of acrylic monomers available, a variety of copolymers can be prepared for specific purposes. For example, acrylamide can be copolymerised with acrylic acid to give a support containing free carboxyl groups and this can then be used to covalently bind enzymes using the carbodiimide method.[53,62] Dihydrofolate reductase was immobilised on cyanogen bromide activated Sepharose-4B. In some experiments a commercial support was used and in others Sepharose-4B was activated with varying quantities of cyanogen bromide.[53] Thus 2 ml of wet, packed gel was reacted with 500, 200, 55, or 25 mg of cyanogen bromide at pH 12. It has been reported that a loading of approximately 50 mg CNBr/g of gel and a coupling pH of 8.5 gives good coupling efficiency and low multiple point binding for proteins.[63] The commercial preparation has approximately this degree of activation. The enzyme was allowed to react with the support overnight, with gentle stirring, at 4°C. Cofactors (DHF, $NADP^{\oplus}$) were not included as their presence led to no immobilisation. The coupling was done in either 50 mM sodium bicarbonate/sodium carbonate buffer at pH 9.0 (A) or 50 mM potassium phosphate buffer

pH 7.5 (B). The preparation was carefully washed to desorb non-covalently bound protein according to the recommended procedure.[61] The results are given in Table 2.2.

Sepharose	buffer	protein loading (mg/g)	yield (%)	activity in washes (%)	U/g	
Commercial	A	8.6	12.5	29	3.5	
Commercial	B	8.3	27	0	5.6	
Commercial	B	20.5	13	0	6.7	
Commercial	B	30.4	15	0	11.4	Form II
200	A	9.5	17	0	5.3	
200	B	10.6	18	13	6.3	
500	B	6.3	15	0	3.1	
55	B	32	10	14	10.6	
25	B	52	2	—	3.4	
Commercial	A	10.8	4	0	3.6	Form I
Commercial	B	10.2	6	0	5.2	

Table 2.2

From these results pH 7.5 gives better yields and a protein loading of $\sim$10 mg/g is optimal, higher loading gives lower yields. The commercial preparations gave higher immobilisation yields than more active supports, for the same protein loading. The maximum activity per unit weight would appear to be $\sim$10 U/g. As before dihydrofolate reductase form I gives lower activity when immobilised under identical conditions compared with form II. After storing

at 5°C in pH 7.0 potassium phosphate buffer for 5 days about 14% of the immobilised activity was lost. It was also found that stirring of the preparation in 1 mM dihydrofolic acid (2.2) solution resulted in a 20% decrease in activity with the activity lost being present in solution.

Dihydrofolate reductase was also coupled to AH-Sepharose-4B using the water soluble carbodiimide EDC.HCl to form a peptide bond with the carboxyl groups on the protein and the amino group on the support. The reaction was done in water in the presence of dihydrofolic acid (0.01 mM) and $NADP^{\oplus}$ (o.01 mM) at 4°C for 5 h. The protein loading was 8mg/g; 11.4% activity was immobilised and 70% was recovered. This results in a support having an activity of 3U/g.[64] A copolymer of acrylamide and acrylic acid was prepared.[62] Dihydrofolate reductase was bound to this support using the carbodiimide, EDC.HCl, in aqueous solution at 6° for 5 h. The protein loading was 30 mg/g; 11% activity was on the support and 7% was recovered. The preparation gives 14.5 U/g.[64]

Dihydrofolate reductase was entrapped in polyacrylamide by adding the enzyme to a polymerising mixture. The polymer was produced in beaded form by allowing the polymerisation to proceed in an aqueous phase which was dispersed by stirring in an organic solvent mixture.[53,62] Low activity was present in the beads (2.4%). This low immobilisation may have been due to the presence of radicals and organic solvents which could inactivate the enzyme. The fast stirring necessary may also have caused shear of the protein.

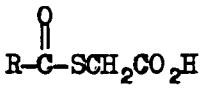
The ionic adsorption of dihydrofolate reductase on AH-Sepharose-4B was also examined. At neutral pH this support will be

positively charged while dihydrofolate reductase is negatively charged.[21] It was found that all of the protein adsorbed onto the support (since washing in buffer at a different pH washed the protein from the support) in water or 10 mM potassium phosphate buffer, pH 7.0. However in the presence of 5 mM dihydrofolic acid 45% activity was eluted from the support in 45 min.

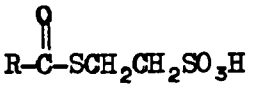
From the above results it would appear that attaining a highly active (50 U/g at pH 7.0) immobilised preparation of dihydrofolate reductase is an intractable problem.

Finally, the preparation of an immobilisation support containing a pendant thiolester group was studied briefly. The reasons for choosing this functionality were; (a) thiolesters display greater reactivity towards amine nucleophiles than do ordinary esters[65] and (b) thiolesters are compatible with an aqueous environment, the rate of acid catalysed hydrolysis being 20-30 fold slower than O-esters while base catalysed hydrolysis proceeds at about the same rate.[66] In terms of acylating strength this functionality parallels the reactivity of an acid chloride. This reactivity is a result of electron-withdrawal from the carbonyl-carbon by the sulphur atom. Thiolesters have been used as intermediates in peptide bond formation after the discovery of the rôle of coenzyme A.[67] Thiolesters can be prepared from a carboxylic acid using an activating agent such as carbonyldiimidazole or a carbodiimide,[68] or from a derivative such as an acid halide, an anhydride, or an ester.[69] The initial attraction of the preparation of a support containing this functionality was based on the demonstration that aqueous solutions of thiolesters of mercaptoacetic acid (2.29) and 2-mercaptoethane sulphonic acid (2.30) were stable to hydrolysis

but would rapidly acylate amino groups on protein (wool).[70] These particular thiols were chosen to confer water solubility on the esters.

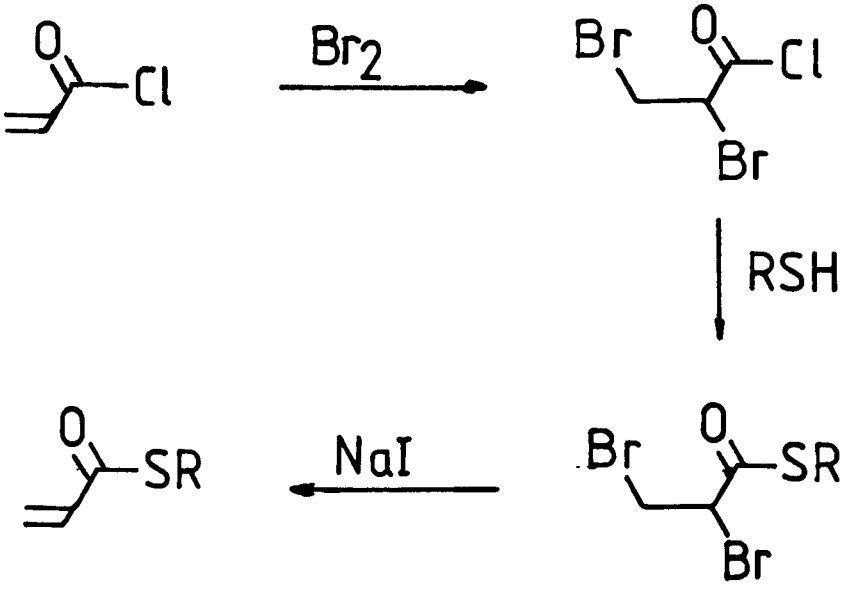


(2.29)



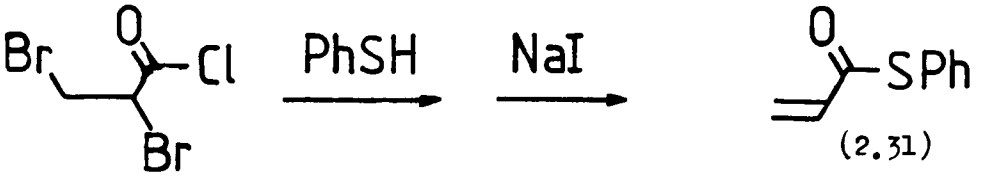
(2.30)

It was hoped to prepare an α,β-unsaturated thiolester from a water soluble thiol and then co-polymerise this monomer with acrylamide to give a water soluble prepolymer as in ref. 55. However, the preparation of a thiolacrylate from acryloyl chloride cannot be done directly since Michael addition is the preferred reaction.[71] Protection of the double bond of acryloyl chloride by bromination was envisaged followed by reaction with a suitable thiol, and then deprotection to yield the α,β-unsaturated thiolester (Scheme 2.14).[72] Acryloyl thiolesters are known to be readily polymerised.[73]



Scheme (2.14)

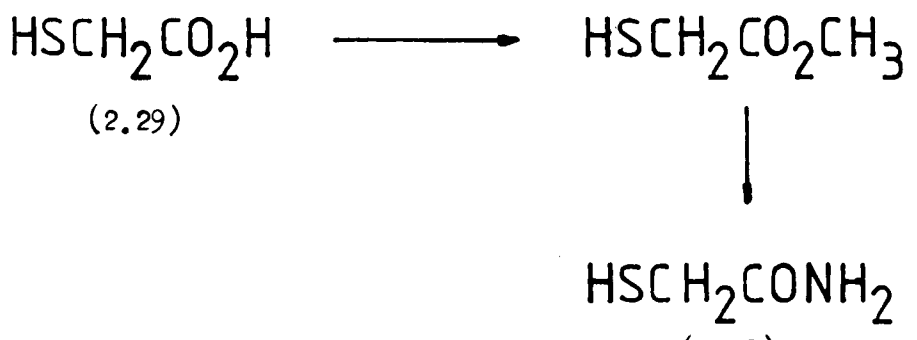
Thus acryloyl chloride was brominated to yield 2,3-dibromopropanoyl chloride as a colourless liquid in 81% yield. This was allowed to react with thiophenol in a model reaction. After work-up t.l.c. indicated a two component mixture which could not be separated by distillation. However, after debromination of this crude material, and then distillation, S-phenylthioacrylate (2.31) was obtained as a yellow viscous liquid in low yield (13%). (Scheme 2.15). The boiling point of this material was not in agreement with the reported value[74] but the spectral characteristics allowed the structure to be assigned with confidence. The low yield may be attributed to side reactions involving displacement of bromine by the thiol. The decision to prepare a monomer using a water soluble thiol was based on the consideration that it would be easily separated from the immobilised enzyme preparation using an aqueous wash. The acidic thiols mentioned earlier were considered to be unsuitable since their release from the support might overload the buffer system used for the enzyme immobilisation. Accordingly the amide of mercaptoacetic acid was chosen. Thus the methyl ester of mercaptoacetic acid was prepared in 68% yield[75]



(2.31)

Scheme (2.15)

and allowed to react with anhydrous ammonia to give the thiolamide (2.32) quantitatively (Scheme 2.16).[76]



Scheme (2.16)

Thioacetamide (2.32) was isolated as a white, amorphous solid, m.p. 45-50°C. No attempts were made to further purify the material since it undergoes oxidation to the disulphide easily, and it was stored desiccated *in-vacuo* at 5°C. The material is readily soluble in water and ethanol. Unfortunately the reaction of this material with 2,3-dibromopropanoyl chloride was complicated by lack of a suitable solvent. A heterogeneous reaction was attempted in dimethoxyethane but this led to a complex mixture of at least four components (t.l.c.). Work-up of this reaction was further complicated by the expected water solubility of any thiolester formed. Some material isolated by extraction into chloroform was tentatively identified as the disulphide (i.r.). This route to an immobilisation support containing a thiolester was therefore abandoned. However, it is expected that a support could be synthesised by reaction of this thiol with a *preformed* polymer (agarose or polyacrylamides) containing a pendant carboxyl group. The reaction could be performed in an *aqueous* medium, using a water soluble carbodiimide to activate the support. There has been one report of the preparation of an insoluble matrix containing a

thiolester (using mercaptoacetic acid). The support was not used to bind enzymes by acylation of the amino functionality but it was reported that 1N hydroxylamine solution cleaved the thiolester.[77]

Large Scale Enzyme Synthesis

Despite this inability to prepare a high activity immobilised preparation of dihydrofolate reductase the reduction of dihydrofolic acid (2.2) to (6S)-tetrahydrofolic acid (2.3) was accomplished with cofactor recycling and with the required enzymes immobilised on PAN. Diammonium acetyl phosphate was used as the ultimate phosphorylating agent. Thus dihydrofolic acid (2.2) (372 μmol) was reduced to (6S)-tetrahydrofolic acid (2.3), the reaction being catalysed using one unit of each enzyme, dihydrofolate reductase, glucose-6-phosphate dehydrogenase, hexokinase, and acetate kinase, immobilised on PAN gel. A slight excess of diammonium acetyl phosphate was used (587 μmol) and this was added in 3 equal portions over 3 days. The reduction was conducted at pH 7.4, using a 'pH stat' apparatus and was reasonably complete after 8 days. ATP and $NADP^{\oplus}$ were recycled 99 and 385-fold respectively. An unexpected difficulty was encountered; it was found that inclusion of PAN gel particles in the reaction medium caused the pH electrode to behave erratically. This may be attributed to clogging of the porous frit on the electrode.

We thus decided to carry out a larger scale reduction using free enzymes. Folic acid (2.1) was reduced using sodium dithionite by a modification of the published procedure.[25] The reaction was followed by HPLC and the yield was 77% giving 8.6 g (19.4 mmol) of dihydrofolic acid (2.2). This was suspended in 1 L of de-aerated

water and brought into solution with 50% sodium hydroxide solution. 20-25 units of the enzymes required were used. An equimolar amount of glucose was used; diammonium acetyl phosphate (25.5 mmol) was added in approximately equal portions over 7 days, after which time the reduction was complete (HPLC). The turnover numbers for ATP and $NADP^{\oplus}$ were 100 and 1100 respectively. Ascorbic acid (5 g) was added to the solution to minimise oxidation of the tetrahydrofolate. The pH of the solution was adjusted to 3.5 with conc. HCl and the yellow precipitate of (6S)-tetrahydrofolic acid (2.3) was collected by filtration under nitrogen and converted into (6R)-5,10-methanyltetrahydrofolic acid chloride as previously described. The yield of the yellow solid was 2.77 g (28% based on dihydrofolic acid). The combustion analysis was satisfactory and the U.V. spectrum and HPLC profile were consistent with those of authentic material; the material had an $[\alpha]_D^{32.7} = +14.3 \pm 0.8$ (c.0.35, 10N HCl) which is in agreement with the reported value $[\alpha]_D^{25} = 11.4 \pm 0.8$ (c.0.95, 12N HCl).[1] This material was hydrolysed to give (6S)-5-formyltetrahydrofolate, isolated as the calcium salt, 0.91 g (46%). Anal. Calcd. for $C_{20}H_{21}N_7O_7\ Ca.\ 3H_2O$: C,42.4; H, 4.81; N, 17.30. Found: C, 42.62; H, 4.63; N, 16.45. The U.V. spectrum was in keeping with that in the literature[4] and HPLC profile was comparable with that of a commercial sample. The $[\alpha]_D^{25} = +2.12$ (c.1.32, H_2O), does not agree well with the reported value of what seems to be the pure biologically active diastereoisomer, $[\alpha]_D^{25} = -25.2 \pm 0.4$ (c. 0.91 H_2O).[1] The material used for the determination of optical rotation was recovered by removal of water <u>in vacuo</u> at 45°. The residue was dissolved in 1 ml of 1 N HCl and left standing for 1 h. The precipitate of 5,10-methenyltetrahydrofolic acid chloride was made up to 10 ml with 10 N HCl.

The optical rotation was determined, the weight of material was calculated as 0.059 g using the determined molecular weights, $[\alpha]_D^{27}$ =+11 (c.0.59, 10N HCl). This rotation agrees with that of the original sample and would suggest that no racemisation had occurred during the hydrolysis. The discrepancy between our value for the rotation of calcium (6S)-5-formyltetrahydrofolate and that reported[1] cannot be explained. A microbiological assay would allow an alternative estimate of the purity of our material.

We have established that it is a straight forward procedure to reduce dihydrofolic acid, in quantity, to (6S)-tetrahydrofolic acid, using the enzyme dihydrofolate reductase as a stereospecific catalyst. The cost of any cofactors needed is easily offset by using enzymic recycling methods <u>in situ</u>. The use of immobilised enzymes is preferable if

(a) activity can be recovered after use,

(b) a preparation of a suitable level of activity can be made,

(c) the cost of the support is not prohibitive, and

(d) the immobilised enzyme can be stored.

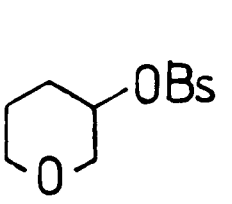
In some cases, especially in the synthesis of pharmaceuticals, the cost of using free enzymes,in conjunction with cofactor recycling, may be acceptable. later experience with enzymes immobilised on PAN suggests that storage, with minimum loss of enzyme activity, would be difficult.

PART II

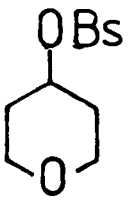
INTRODUCTION

Since a method for recycling of both oxidised nicotinamide cofactors ($NAD^{\oplus}$ and $NADP^{\oplus}$) was now available the use of the enzyme horse liver alcohol dehydrogenase (HLADH) in effecting chiral reduction of a heterocyclic ketone was investigated. The chiral alcohol so formed was required for elucidation of the mechanism of a solvolysis reaction using stereochemical means.

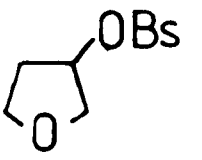
In 1969 Tarbell and Hazen[78] studied the rates of acetolysis of some simple O-heterocyclic sulphonate esters. As expected the rates were slower than the corresponding carbocycles because the dipole associated with the ether oxygen destabilises the incipient positive change. It was found, however, that 3-tetrahydropyranyl brosylate (2.33) solvolysed faster than predicted, the observed rate being approximately that expected in the absence of the dipole effect. For example, the rate was 5-fold faster than that of the 5-membered ring analogue (2.35). This effect was attributed to R_2O_3 anchimeric assistance of the solvolysis reaction by the ether oxygen atom. This type of neighbouring group participation is well documented.[79]



(2.33)



(2.34)

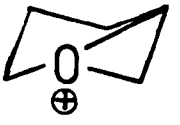


(2.35)

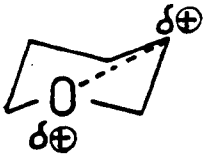
k, x $10^5 s^{-1}$	2.47	1.40	0.55

84.80°C.

The lone pair of electrons on the oxygen atom could conceivably interact with the developing p-orbital to give the oxonium ion (2.36) or a partially bonded intermediate (2.37). This type of participation is not expected to operate in the case of the other

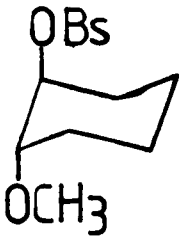


(2.36)



(2.37)

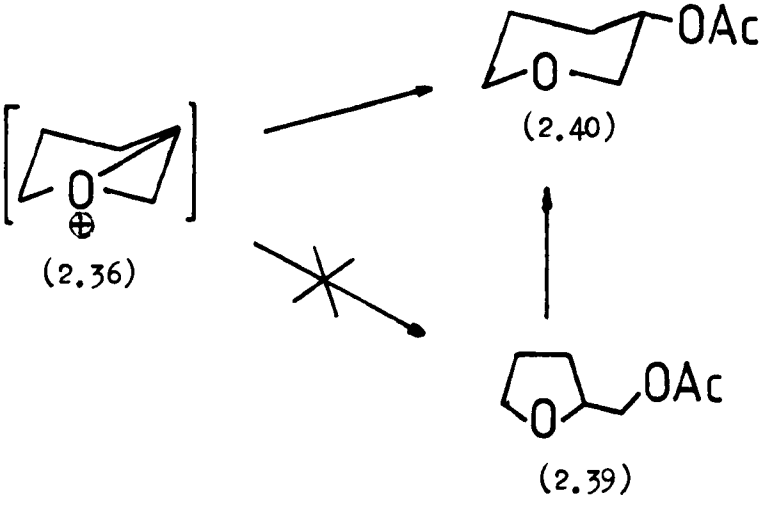
sulphonate esters for reasons of strain. Evidence that the rate enhancement is real is supplied by the fact that the rate is approximately equal to that for the acetolysis of trans-1-methoxy-cyclohexyl brosylate (2.38), in which case there is neighbouring group participation.[80]



$k = 2.44 \times 10^{-5} s^{-1}$

(2.38)

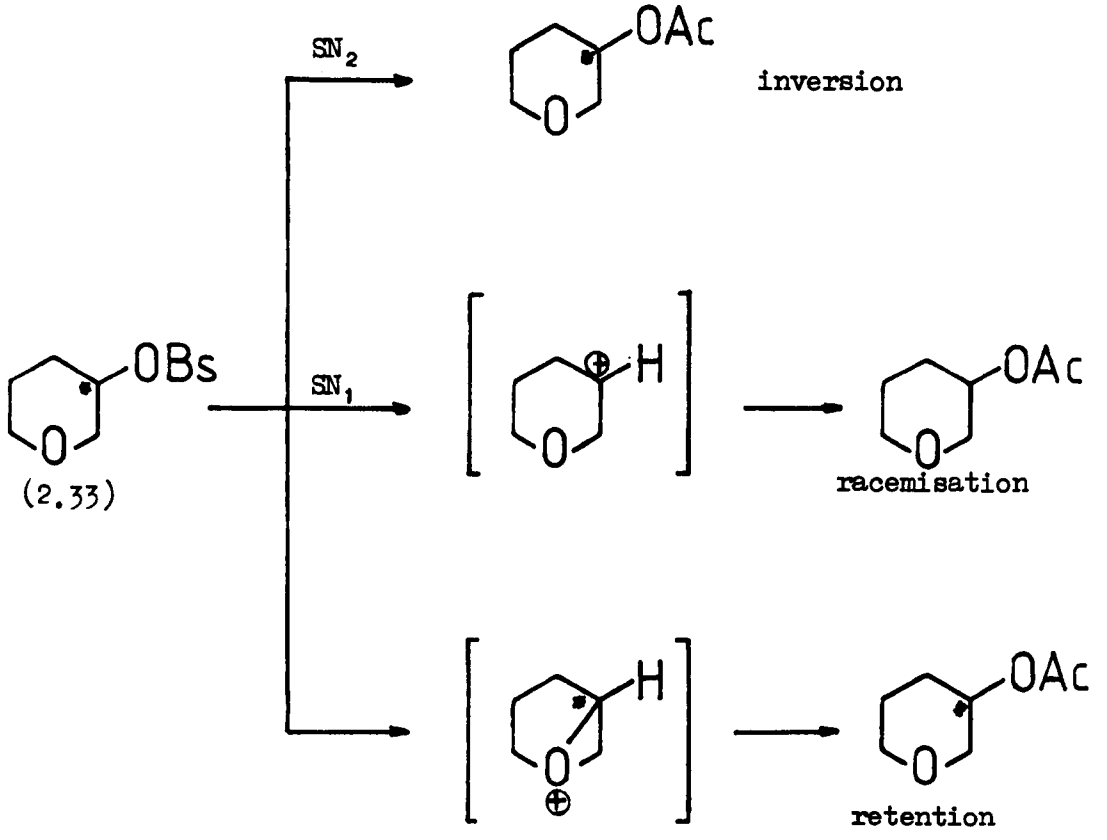
A cyclic intermediate or transition state such as those postulated above (2.36, 2.37) might be expected to lead to rearranged products. In the study by Tarbell and Hazen none were observed. The ring contracted furfuryl acetate (2.39) may have been expected since the ring expansion of this material to the pyranylacetate (2.40) has been reported (Scheme 2.17).[81]



Scheme (2.17)

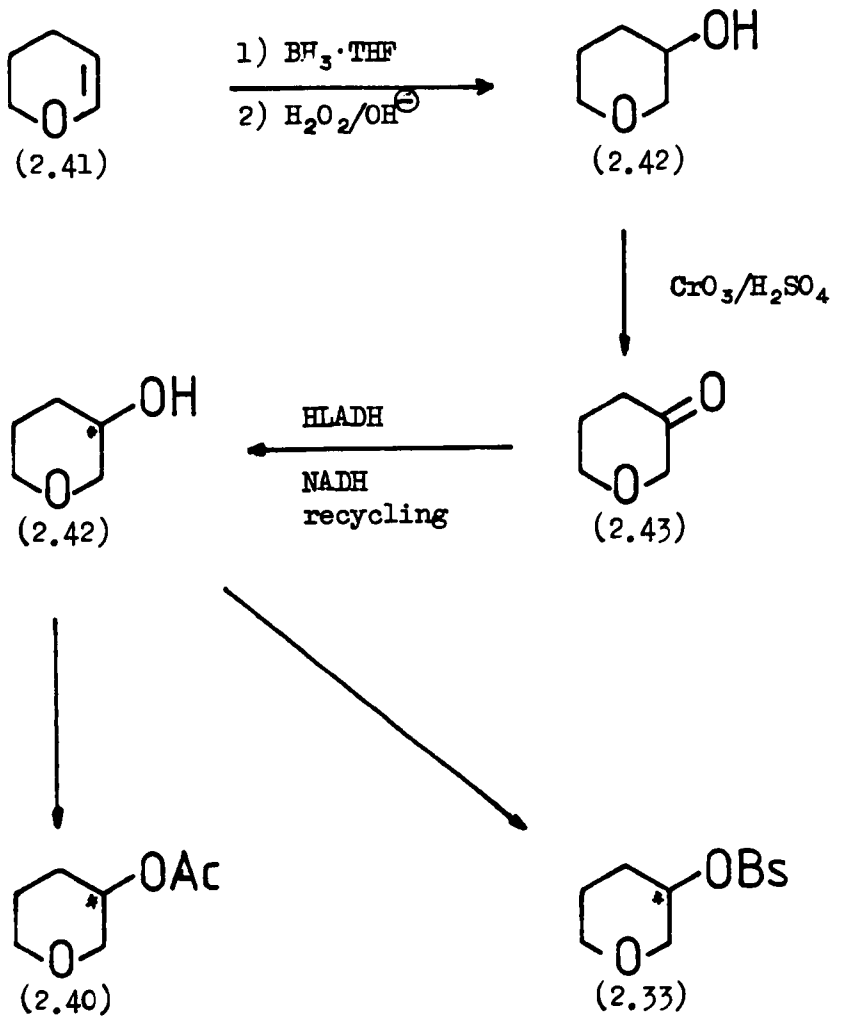
thus the existence of neighbouring group participation is surmised solely from kinetic data; if the brosylate (2.33) was optically active then retention of optical activity in the acetolysis product would be powerful evidence in favour of this mechanism.

Three possible mechanisms may be envisaged for the simple acetolysis of the optically active brosylate (2.33) (Scheme 2.18). A comparison of the optical activity of the acetate formed by acetolysis, with the acetate prepared from the chiral alcohol, would indicate the relative importance of the undernoted mechanisms. Attempts to resolve 3-tetrahydropyranol (2.42) via the half acid phthalate and an optically active amine, by fractional crystallisation of the diastereomeric salts, failed



Scheme (2.18)

because of the inability to induce crystallisation.[82] The synthesis of the optically active alcohol (2.42) by enzyme catalysed reduction of the ketone (2.43) (Scheme 2.19) was thus proposed.



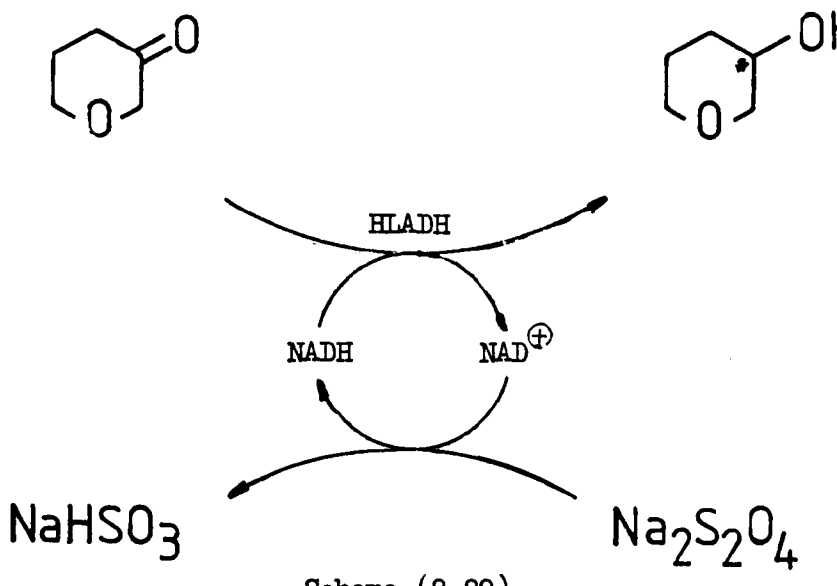
* = optically active centre

Scheme (2.19)

Enzyme Catalysed Reduction

Hydroboration/oxidation of dihydropyran (2.41) by the method of Zweifel and Plamondon[83] gave tetrahydropyran-3-ol in 39% yield. This alcohol was oxidised to tetrahydropyran-3-one (2.42) using Jones reagent, as reported by Gore and Guigues.[84] The yield was 58%. Jones[85] has demonstrated that sulphur analogues of cyclohexanones are substrates for horse liver alcohol dehydrogenase. It was thus expected that this oxygen analogue (2.43) would be reduced without difficulty. Indeed, Jones[86] reported the reduction of O-heterocyclic ketones during the course of this work. To be able to undertake a preparative, enzyme catalysed reduction with confidence the rate of reduction of any new substrate should be at least 1% of that of cyclohexanone. Under standard assay conditions the relative rate of reaction of tetrahydropyran-3-one (2.43) was 7.6% that of cyclohexanone.[85b] The specific activity of horse liver alochol dehydrogenase, for this substrate, was therefore 0.25 U/mg.

For small scale reductions, using horse liver alcohol dehydrogenase, consumption of the coenzyme NADH results in a cost which can be tolerated. However, as part of the overall brief of this work the efficiencies of 3 methods for *in situ* reduction of the oxidised coenzyme, $NAD^{\oplus}$, were investigated.

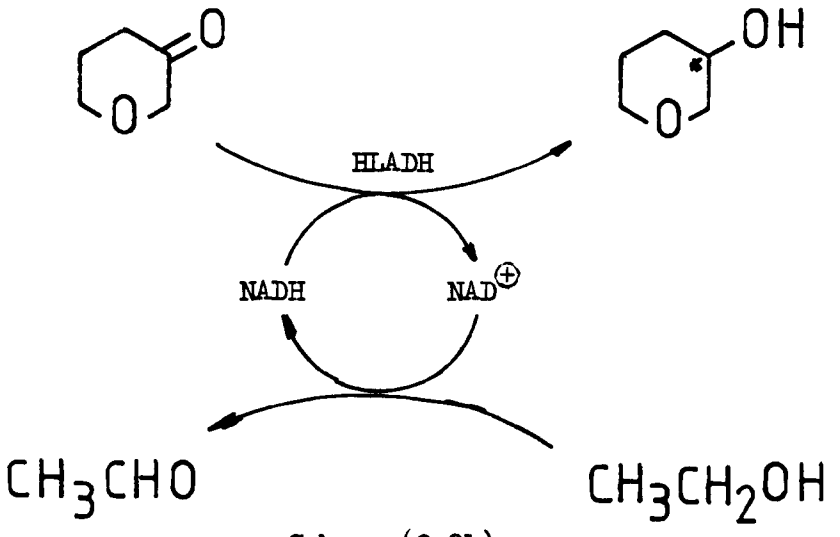


Scheme (2.20)

(1) Sodium dithionite is cheap and the only chemical reducing agent known to give 1,4-reduction of $NAD^{\oplus}$ (Scheme 2.20). However the reduction is not specific enough and turnover numbers of the coenzyme are limited. When this method was used in the reduction of tetrahydropyran-3-one (2.43) in 0.1 M sodium phosphate buffer, 0.1 M in sodium dithionite, at pH 7.0, the formation of an unknown product attended the reduction (gas chromatography).[87] It has been reported that it is necessary to use sodium dithionite under anaerobic conditions otherwise hydrogen peroxide is formed (eqn. 28).[88]

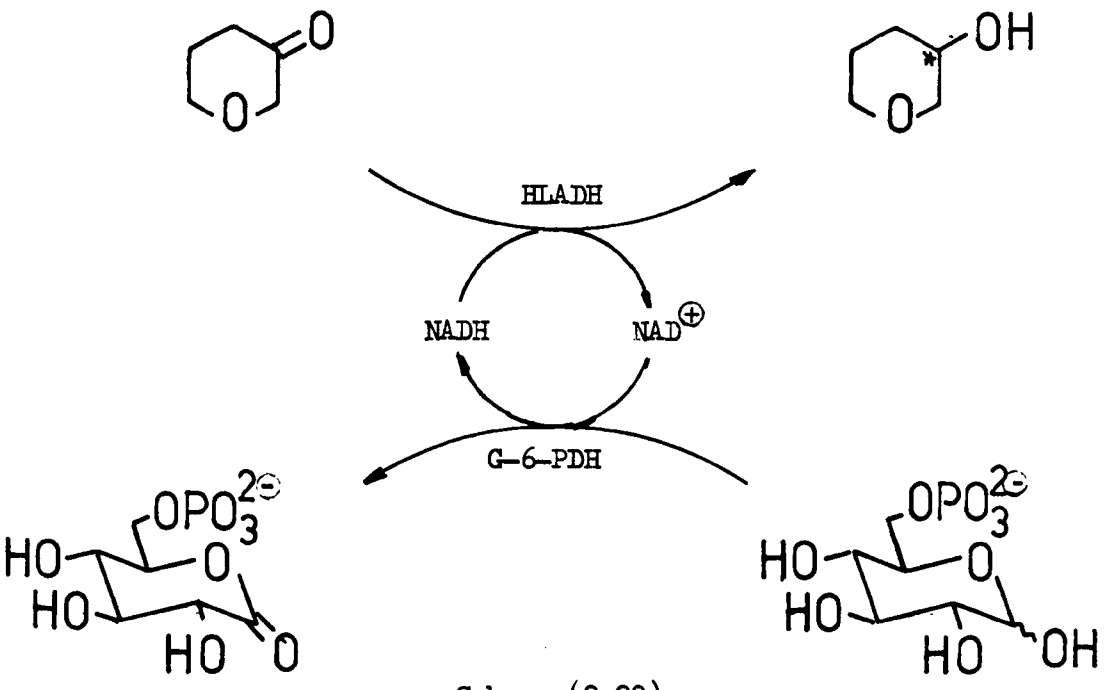
$$Na_2S_2O_4 + O_2 + 2H_2O \longrightarrow 2NaHSO_3 + H_2O_2 \qquad \text{(eqn. 2.8)}$$

This would have a deleterious effect on the enzyme.



Scheme (2.21)

(2) $NAD^{\oplus}$ can be reduced in situ by the addition of ethanol which is a substrate for horse liver alcohol dehydrogenase operating in the opposite direction; the ethanol undergoing oxidation to acetaldehyde (Scheme 2.21).[28] However, for cyclic ketone substrates it is generally found that recycling efficiences are low and that reaction times are long. This is probably because the redox potential difference between the main substrate and the co-substrate is small. This effect was found to operate in this reaction, although the results suggest that the method is feasible.[87]



Scheme (2.22)

(3) The advantages of using glucose-6-phosphate/glucose-6-phosphate dehydrogenase (from L. mesenteroides) to recycle NAD(P)H have been discussed (Scheme 2.22). For this reaction glucose-6-phosphate was synthesised by hexokinase catalysed phosphorylation of glucose, using diammonium acetyl phosphate and acetate kinase to recycle ATP, as described by Whitesides et. al.[31,89] This reaction was attempted using immobilised enzymes (PAN) but problems were encountered with the stability of the preparations. This may have been due to the age of the PAN preparation used. Using ~300 U of each enzyme (free) glucose (193 mmol) was phosphorylated in 60% yield after 4 days at pH 7.4-7.8. Previously prepared diammonium acetyl phosphate (84% purity, 193 mmol) was added in equal portions over this time. The product was isolated as the barium salt of glucose-6-phosphate in 90% purity and 42% yield. Isolation as the barium salt was used because acetate

ion, which is a final component of this reaction scheme, is an inhibitor of horse liver alcohol dehydrogenase.[90] However, barium salts can inactivate enzymes and are toxic. They must be removed before use. Removal of barium is accomplished by dissolving barium glucose-6-phosphate in 0.1-0.2 M sulphuric acid solution, separating the insoluble precipitate of barium sulphate, and neutralising the resulting solution with sodium hydroxide. To a solution of glucose-6-phosphate (20 mmol), prepared by this method, was added 20 mmol of tetrahydropyran-3-one (2.43). This was reduced using glucose-6-phosphate dehydrogenase and horse liver alcohol dehydrogenase, at pH 7.0-7.4. The reduction was followed by g.l.c. and, at times, by enzymatic assay of residual glucose-6-phosphate. The reaction was complete after 4.5 days, NADH being recycled ~ 800-fold. After extraction and distillation 1.4 g (68%) of tetrahydropyran-3-ol (2.42) was obtained. The spectral characteristics were consistent with those of the racemic alcohol and the product showed an $[\alpha]_D^{24.5^\circ} = -8.7$ (c.3.39, $CHCl_3$) and $[\alpha]_{365}^{29.25} = -23.7 \pm 1.4$ (c.3.39, $CHCl_3$). By reaction with (-)-α-methoxy-α-trifluoromethylphenyl acetic acid,[91] and integration of the diastereomeric methoxy proton signals (250 MHz ^1H n.m.r.) in the presence of Eu $(fod)_3$, the enantiomeric excess was found to be 49%. While this e.e. may be too small to be synthetically useful it is enough for the present purpose. Never-the-less, the ability of horse liver alcohol dehydrogenase to make this degree of enantiotopic distinction, on the basis of such a minor structural difference as the position of the oxygen atom in the ring, is remarkable. The results of the above experiment are given in Table 2.3.

Procedure	HLADH (UNITS)	G-6-PDH (UNITS)	pyranone (mmol)	auxiliary reactant (mmol)	reduction (%)	recycling	time (days)	isolated yield (%)
1	10	-	10	50	25	12	1	-
2	12.5	-	9.2	80	60	74	10	-
3	14.2	22.4	20	20	100	820	4.5	68

Table 2.3

Similar investigations by Vandecasteele[92] support the conclusion that an auxiliary enzymic method for the recycling of nicotinamide co-enzymes is best.

The p-bromobenzene sulphonate ester of the chiral alcohol was prepared in 73% yield by standard methods, m.p. = 71-73°, $[\alpha]_{365}^{25.5}$ = -10.3 (c.2.46, $CHCl_3$). The acetate was also prepared $[\alpha]_{365}^{25.5}$ = -24.7 (c.1.64, $CHCl_3$). The rotation of the chiral acetate was unchanged under the conditions of the acetolysis experiment. The acetolysis was done in dry acetic acid containing 0.0384 M sodium acetate and was followed polarimetrically (Hg lamp, 365 nm). The first order rate constants, k, were determined from a plot of ln absolute value of observed rotation vs. time (s), Table 2.4.

Temp (°C)	k (x 10^5 s^{-1})
79.26	1.41
81.43	1.73
82.12	1.73
89.93	7.86

Table 2.4

With the exception of the experiment conducted at 89.93°C the plot was initially curved. G.l.c. analysis of the reaction (heated at 105°C in a sealed glass tube for 22 h to ensure complete reaction) gave two peaks, corresponding to the acetate (2.40) and the alcohol (2.42), confirmed by g.l.c./mass spectrometry. Despite efforts to exclude moisture rigorously this curvature was

always observed. The rate constants were estimated from the linear portion of the curve. A satisfactory Arhennius plot of ln k vs. $\frac{1}{T}$ was obtained, the titrimetrically determined rate constant 2.47×10^{-5} at 84.9°C[78] concurred with these results, Table 2.5.

ln k	$\frac{1}{T} \times 10^3$
- 11.17	12.62
- 10.96	12.28
- 10.96	12.17
- 10.61*	11.78*
- 9.45	11.11

Table 2.5

* from Ref. 78

The products from the acetolysis of the brosylate (2.33) (alcohol and acetate) had no optical activity. Although perhaps not conclusive, this is certainly good evidence against neighbouring group participation of the ether oxygen atom.

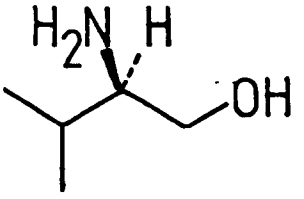
PART III

INTRODUCTION

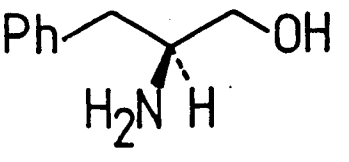
The final investigations in this thesis were concerned with the asymmetric reduction of folic acid (2.1) or dihydrofolic acid (2.2) to tetrahydrofolic acid (2.3) using chiral hydride reducing agents. The selected reagents must be able to perform in an aqueous medium since folates are not soluble in organic solvents. Amine borane complexes have been extensively studied as reducing agents for alkenes, carbonyl groups, and imines.[93] They have good thermal and hydrolytic stability and are soluble in a wide variety of protic and aprotic solvents. Recently developed chiral alkoxy-amine borane complexes have the same properties, and seem to give higher optical induction than chiral amine borane complexes.[94,95] The use of these materials was therefore investigated.

Synthetic Methods

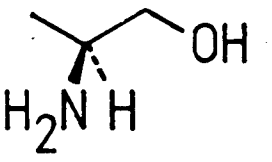
A series of these complexes was prepared from commercially available, or readily synthesised, chiral 1,2-amino alcohols; these are given below. The (S)- amino alcohols (2.44-47) were prepared from the parent (S)-amino acids by reduction with borane dimethyl sulphide, in the presence of boron trifluoride diethyl etherate, in 24-86% yield.[96,97] This method was more convenient than reduction using lithium aluminium hydride.[98,99] The boiling points, spectral characteristics, and optical rotation were in agreement with those in the literature. Trans-(4S,5S)-2-methyl-4-hydroxymethyl-5-phenyl-2-oxazoline (2.49) was prepared in 61%



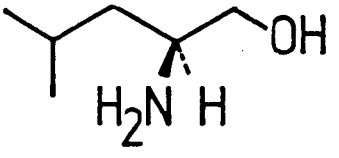
(2.44)
(S)-Valinol



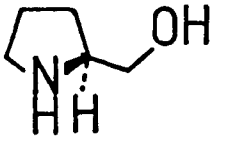
(2.45)
(S)-Phenylalaninol



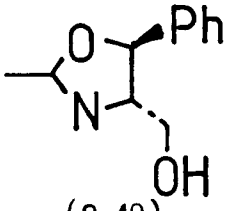
(2.46)
(S)-Alaninol



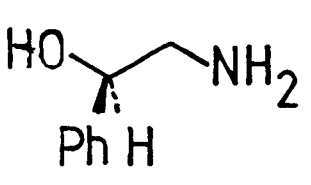
(2.47)
(S)-Leucinol



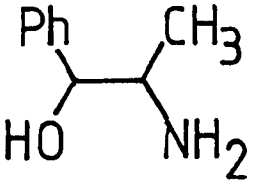
(2.48)
(S)-Prolinol



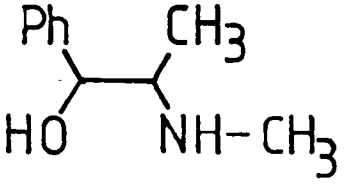
(2.49)



(2.50)

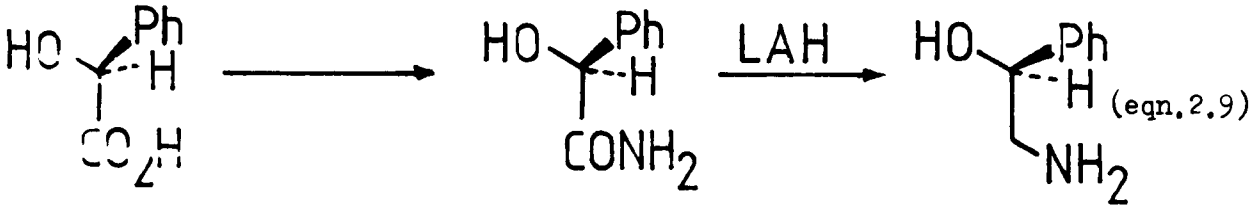


(2.51)



(2.52)

yield by the reaction of ethyliminoacetate hydrochloride with readily available (1S,2S)-2-amino-1-phenyl-1,3-propanediol.[100] (S)-2-Amino-1-phenylethanol (2.50) was prepared from (S)-mandelic acid (2.53), eqn.(2.9).[101]



(2.53) (2.50) (eqn.2.9)

(S)-Prolinol (2.48), (+)-norephedrine (2.51) and (+) and (-)-ephedrine (2.52) were commercial samples. The borane complexes were prepared by reacting the amino alcohols, in equimolar amounts, with borane dimethylsulphide at -78 - -60°, under nitrogen, for 3 h and then stirring at room temperature overnight. They were isolated, by removal of solvent <u>in vacuo</u>, usually as viscous liquids although some complexes were solid. The complexes displayed optical activity, were apparently homogeneous (t.l.c.) and gave a characteristic B-H stretch (between 2,440 and 2,300 cm^{-1}) in the i.r. spectrum. It has been suggested that the complexes contain a relatively rigid five membered ring system (eqn. 2.10).[94a] Further characterisation was not attempted during the present investigation.

(eqn.2.10)

The complexes were allowed to react with dihydrofolic acid (2.2) (5 mg, 11.3 μmol) in 2 ml of the solvent system indicated, at room temperature and under nitrogen. The reaction was followed by HPLC; isomeric excesses were determined as previously described. An excess of the chiral hydride was used in each case because competing hydrolysis was expected; the weight of the complex used is given since the molecular structure is not known with certainty. The solvent systems used were:

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

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

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

(D) 20 mM Tris-Cl, pH 8.0, containing 10 mM DTT

The results are given in Table 2.6.

Parent amino alcohol	Solvent	wt. of complex (mg)	extent of reaction (%), time	isomeric excess (%)	Configuration at C6
2.44	A	55.5	>98, 5 days	1.8	R
2.45	A	33.3	>98, 5 days	9.8	S
2.46	A	37.8	>97, 5 days	0.8	R
2.47	A	26.5	30, 5 days	—	—
2.48	A	30.6	84, 5 days	9.8	S
2.49	B	4.8	100, 3 h	11.8	R
⁎2.49	B	35.6	100, 1 day	12.6	R
⁎2.49	C	9.73	100, 5 days	14.0	R
⁎2.49	C	5.22	>90, 3 days	18.0	R
2.50	B	23.54	6, 2 days	—	—
2.51	B	21.97	86, 2 days	5.2	R
(+)2.52	B	39.6	— —	—	—
	C	27.92	— —	—	—
(-)2.52	B	42.7	— —	—	—
	C	34.35	— —	—	—

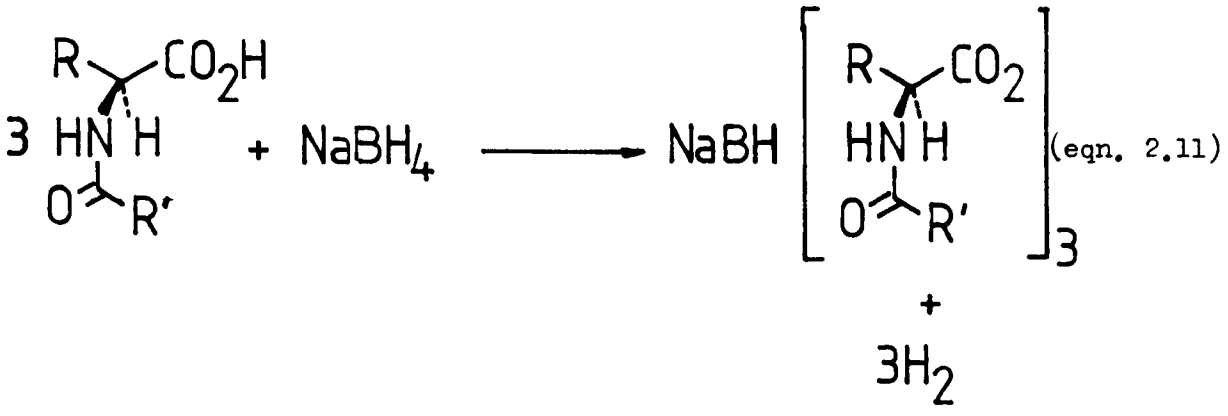
Table 2.6

⁎ - prepared from 2:1 ratio of borane dimethylsulphide : oxazoline.

The isomeric excesses produced are too small to be synthetically useful. For the complexes derived from the amino alcohols of (S)-α-amino acids (2.44-48) the configurations produced in exess at C6 seemed to be related to the sign of the rotation of the complex; (-) gave (S) and (+) gave R. This relationship did not hold with the other complexes. There was a kinetic effect operating during the reductions and so it was necessary to analyse for isomeric excess as close to complete reduction as possible. In the cases where this was not done no allowance was made. The 1:1 complex of oxazoline (2.49) and borane seemed to be more reactive than the 1:2 complex, although the isomeric excesses generated were the same. When the amount of the 1:2 complex used was reduced the isomeric excess increased. The complexes derived from (S)-2-amino-1-phenyl ethanol (2.50) and (+) and (-) ephedrine did not effect any reduction under the conditions stated, this did not seem to be due to low solubility in the medium used.

Another class of chiral hydride reagents, triacyloxyborohydrides, have been shown to be effective in the asymmetric reduction of cyclic imines.[102] These reagents are obtained by reaction of sodium borohydride with N-acyl-(S)-α-amino acids (eqn. 2.11).

The preparation of the benzyloxycarbonyl (CBzO) derivatives (R' = OCH_2Ph) of (S)-alanine, valine, proline, phenylalanine, and serine, was carried out. These derivatives were prepared by standard methods in 31-57% yield.[103] The melting points and optical rotation of these materials were in good agreement with values in the literature, the triacyloxyborohydride derivatives



(eqn. 2.11)

were prepared by reaction of the N-acylamino acid with sodium borohydride, in a 3:1 molar ratio, in dry tetrahydrofuran. Removal of solvent in vacuo gave the hydrides as white, amorphous solids in 82-96% yields. These derivatives had indeterminate melting points and a very weak absorption in the infra-red spectrum (KBr), between 2610 and 2460 cm^{-1} (B-H stretch), was obtained. The results are summarised in Table 2.7.

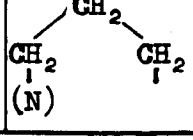
Parent amino acid	R	yield (%)	m.p. (dec) (°C)	$\bar{\nu}$ max (KBr) (cm^{-1})
(S)-alanine	$-CH_3$	91	45-52	2520
(S)-valine	$-CH(CH_3)_2$	82	48-70	2540
(S)-phenylalanine	$-CH_2Ph$	93	58-61	2460
(S)-serine	$-CH_2OH$	96	83-108	2610
(S)-proline		90	56-60	2460

Table 2.7

These chiral hydrides in 3.5-7 fold molar excess, were added to an approximately 1 mM solution of dihydrofolic acid (2.2) at pH 7.0-8.0, under nitrogen. The reaction was followed by HPLC, the results are summarised in Table 2.8.

Parent CBzO amino acid	mol. equiv. used	time (h)	reduction (%)
CBzO-(S)-Phe.	3.7	72	5
CBzO-(S)-Ala.	5.9	48	5
CBzO-(S)-Ser.	4.6	24	0
CBzO(S)-Pro.	7	72	70

Table 2.8

Only that hydride derivative based on (S)-proline gave any significant reduction. HPLC analysis of isomeric excess indicated that no chiral induction had been obtained. The reason for the low reactivity of these derivatives is not obvious. It may have been that the hydrophobic benzyloxycarbonyl group rendered these derivatives too insoluble to react, although these are salts and did appear to dissolve in water. It could be that these derivatives were hydrolysed before they could effect any significant levels of reduction. The apparent reactivity of the proline derivative could have been due to sodium borohydride present.

Finally the asymmetric reduction of dihydrofolic acid (2.2) was attempted in the presence of bovine serum albumin, using sodium borohydride. Bovine serum albumin is a carrier protein in living systems. It will bind aromatic moieties in its

interior, for example naphthalene is a good guest compound.[104] It has been used as a chiral template for the reduction of aromatic ketones using sodium borohydride.[105] Thus bovine serum albumin (M.W. 66,000) was dissolved in 20 mM Tris-Cl, pH 8.0, containing 10 mM DTT to give concentrations of 0.5, 1, 1.5, and 2.0 mM. These solutions (2 ml) were then made 5.63 mM in dihydrofolic acid (2.2), and sodium borohydride (as a solution in water) was added in small portions at five minute intervals until complete reduction was obtained. HPLC analysis of the tetrahydrofolate produced indicated that no chiral induction was achieved. The binding pocket of bovine serum albumin is probably hydrophobic and it seems likely that dihydrofolic acid (2.2)is too highly charged to enter.

REFERENCES

REFERENCES

1. J.C. Fontecilla-Camps, C.E. Bugg, C. Temple, J.D. Rose, J.A. Montgomery, and R.L. Kisluik, 'Chemistry and Biology of Pteridines', eds. R.L. Kisluik and G.M. Brown, Elsevier, North Holland, New York, 1979 p.235 and J. Amer. Chem. Soc., 1978, 101, 6114.
2. S.J. Benkovic and C.M. Tatum, Acc. Chem. Res., 1978, 11, 314.
3. S.J. Benkovic, Ann. Rev. Biochem., 1980, 49, 227.
4. R.L. Blakley, 'The Biochemistry of Folic Acid and Related Pteridines', North-Holland, Amsterdam, 1969.
5. G.K. Humphreys and D.M. Greenberg, Arch. Biochem. Biophys., 1958, 78, 275.
6. M. Friedkin, Fed. Proc., 1959, 18, 230.
7. C. Tatum, J. Vederas, E. Schleicher, S.J. Benkovic,and H. Floss, J. Chem. Soc. Chem. Comm., 1977, 218.
8. A.J. Wahba and M. Friedkin, J. Biol. Chem., 1961, 236, PC11.
9. R.L. Blakley and B.M. McDougall, J. Biol. Chem., 1962, 237, 812.
10. A.L. Kretchman and E.J. Price, Metabolism, 1969, 18, 684.
11. J.F. Bender, W.R. Grove, and C.L. Fortner, Amer. J. Hosp. Pharm., 1977, 34, 961.
12. B.A. Chabner and M. Slavik, Chemother. Rep., 1975, 6, 1. I. Djerrasi, ibid., 3. J.S. Penta, ibid., 6.
13. D.B. Cosulich, J.M. Smith,and H.P. Broquist, J. Amer. Chem. Soc.,1952, 74, 4215.
14. R.P. Leary, Y, Gaumont, and R.L. Kisluik, Biochem. Biophys. Res. Comm., 1973, 56, 484.
15. V.F. Scott and K.O. Donaldson, ibid., 1964, 14, 523.

16. B. Roth, M.E. Hultquist, M.J. Fahrenbach, D.B. Cosulich, H.P. Broquist, J.A. Brockman, J.M. Smith, R.P. Parker, E.L.R. Stokstad, and T.H. Jukes, J. Amer. Chem. Soc., 1952, 74, 3247, and references cited therein.

17. M. May, T.J. Bardos, F.L. Barger, M. Lansford, J.M. Ravel, G.L. Sutherland, and W. Shive, J. Amer. Chem. Soc., 1951, 73, 3067.

18. A. Pohland, E.H. Flynn, R.G. Jones, and W. Shive, J. Amer. Chem. Soc., 1951, 73, 3247, and references cited therein.

19. C. Temple, R.D. Elliot, J.D. Rose, and J.A. Montgomery, J. Med. Chem., 1979, 22, 731.

20. P.H. Boyle and M.T. Keating, J. Chem. Soc. Chem. Comm., 1974, 375.

21. D. Baccanari, A. Phillips, S. Smith, D. Sinski, and J. Burchall, Biochemistry, 1975, 14, 5267.

22. C.K. Mathews and F.M. Huennekens, J. Biol. Chem., 1960, 235, 3304.

23. C.K. Mathews and F.M. Huennekens, J. Biol. Chem., 1963, 238, 3436.

24. G.M. Whitesides, C.H. Wong, and A. Pollack, 'Asymmetric Reactions and Processes in Chemistry', A.C.S. Symposium Series, 1982, 205, and references cited therein, C.H. Wong and G.M. Whitesides, J. Amer. Chem. Soc., 1981, 103, 4890.

25. S. Futterman, J. Biol. Chem., 1957, 228, 1031. R.L. Blakley, Nature , 1960, 188, 231.

26. W.H. Pirkle and M.S. Hoekstra, J. Org. Chem., 1974, 39, 3904.

27. C. Temple, R.D. Elliot, J.D. Rose, and J.A. Montgomery, 'Chemistry and Biology of Pteridines', eds. R.L. Kisluik and G.M. Brown, New York, Elsevier North Holland, 1979, p. 279.

28. J.B. Jones and J.F. Beck, 'Application of Biochemical Systems in Organic Chemistry', eds. J.B. Jones, D. Perlman and C.J. Sih, Wiley Interscience, New York, 1976, p. 107.

29. S.S. Wang and C.K. King, Adv. Biochem. Eng., 1979, 12, 119.

30. F.M. Huennekens, P.P.K. Ho and K.G. Scrimgeour, 'Methods in Enzymology', eds. S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1963, vol. 6, p. 806.

31. A. Pollack, R.L. Baughn, and G.M. Whitesides, J. Amer. Chem. Soc., 1977, 99, 2366.

32. F. Lynnen, Chem. Ber., 1940, 73, 367; F. Lipmann and C. Tuttle, J. Biol. Chem., 1944, 153, 571.

33. R. Bentley, J. Amer. Chem. Soc., 1948, 70, 2183.

34. F. Lipmann and E.R. Stadtman, J. Biol. Chem., 1950, 185, 549; D.E. Koshland, J. Amer. Chem. Soc., 1951, 73, 4103.

35. A.W.D. Avison, J. Chem. Soc., 1955, 732; R.W. Porter, M.O. Modebe, and G.R. Stark, J. Biol. Chem., 1969, 244, 1846.

36. E. Heyde, A. Nagabhushanian, and J.F. Morrison, Biochemistry, 1973, 12, 4718.

37. E. Cherbulliez, 'Organic Phosphorus Compounds', eds., G.M. Kosalopuff and L. Maier, Wiley Interscience, New York, 1973, vol. 6, p. 211.

38. G.M. Whitesides, M. Siegel, and P. Garrett, J. Org. Chem., 1975, 40, 2516.

39. J.M. Lewis, S.L. Haynie, and G.M. Whitesides, J. Org. Chem., 1979, 44, 864.

40. R.N. Bell, Anal. Chem., 1947, 19, 97; L.F. Adrieth and O.F. Hill, J. Chem. Educ., 1948, 25, 80.

41. R. Hartwick, A. Jeffries, A.M. Krstulovic, and P.R. Brown, J. Chromatogr. Sci., 1978, 16, 427.

42. A.M. Krstulovic and C. Matzura, J. Chromatogr., 1979, 176, 217.

43. G.C. Davis and P.T. Kissinger, Anal. Chem., 1979, 51, 1960.

44. J.F. Studebaker, Chromatogr. Sci., 1979, 10, 261.

45. N.D. Danielson and J.A. Huth. J. Chromatogr., 1980, 221, 39.

46. Waters Associates Technical Bulletin D61, Paired Ion Chromatography, 1975.

47. 'Data for Biochemical Research', eds. R.M.C. Dawson, D.C. Elliot, N.H. Elliot, and K.M. Jones, Oxford University Press, 1969, 2nd Ed.

48. H.U. Bergmeyer, 'Methods of Enzymatic Analysis', Academic Press, New York, 1974.

49. N. Ise, H. Kitano, and K. Sakata. Japanese Patent 81 15, 697; Chem. Abs., 1981, 95, 20863y.

50. Ennor and Stocken, Biochem. J., 1948, 43, 190.

51. B.L. Hirschbein, F.P. Mazenod, and G.M. Whitesides, J. Org. Chem., 1982, 47, 3765.

52. M.D. Trevan, 'Immobilised Enzymes', John Wiley and Sons, 1980.

53. 'Methods in Enzymology', ed. K. Mosbach, Academic Press, London, 1976.

54. I. Chibata, 'Immobilised Enzymes', Halsted Press, New York, 1978.

55. A. Pollack, H. Blumenfeld, M. Wax, R.L. Baughn, and G.M. Whitesides, J. Amer. Chem. Soc., 1980, 102, 6324.

56. J.K. Inman and H.M. Dintzis, Biochemistry, 1968, 8, 4074.

57. R.L. Schnaar and Y.G. Lee, Biochemistry, 1975, 14, 1535.

58. Prepared by Mr. J. Haddow, University of Strathclyde.

59. O. Adalsteinsson, A. Lamotte, R.F. Baddour, C.K. Colton, A. Pollack, and G.M. Whitesides, J. Mol. Catal., 1979, 6, 199.

60. R. Axen, J. Porath, and S. Ernback, Nature, 1967, 214, 1302.

61. 'Affinity Chromatography - Principles and Methods', Pharmacia Fine Chemicals, Uppsala, Sweden, 1976.

62. A-C. Johansson and K. Mosbach, Biochem. Biophys. Acta., 1974, 370, 339.

63. G. Kumel, H. Daus, and H. Mauch, J. Chromatogr., 1979, 172, 221.

64. Carried out by Mrs. L. Rees, University of Strathclyde.

65. T.U. Fije and B.R. DeMark, J. Amer. Chem. Soc., 1979, 101, 7379, K.A. Connors and M.C. Bender, J. Org. Chem., 1961, 20, 2498.

66. P.N. Rylander and D.S. Tarbell, J. Amer. Chem. Soc., 1950, 72, 3021.

67. T. Wieland Ann. Chem., 1950, 573, 99. T. Wieland and W. Schafer, Ang. Chem., 1951, 63, 146. R. Schwyzer, Helvetica Chimica Acta., 1953, 36, 414.

68. H.J. Gais, Ang. Chem. Int. Ed.(Engl), 1977, 244. M.V.A. Baig and L.N. Owen, J. Chem. Soc. Chem. Comm., 1966, 540. K. Lloyd and G.T. Young, J. Chem. Soc.(C), 1971, 2890.

69. E. Haslam, Tetrahedron, 1980, 36, 2409 and references cited therein.

70. J.R. Dalton, A. Kirkpatrick, and J.A. Maclaren, Aus . J. Chem., 1981, 34, 759.

71. G. Braude, J. Org. Chem., 1957, 22, 1675. A.A. Schlepprick and F.B. Zienty, J. Org. Chem., 1964, 29, 1910.

72. K. Rufenacht, Helvetica Chimica Acta., 1976, 56, 2186. C.S. Marvel, S.L. Jacobs, W.K. Taft, and B.G. Labbe, J. Pol. Sci., 1956, 19, 59.

73. A.E. Brachman, J. Pol. Sci., 1959, 36, 560. J. Ostsu, K. Tsuda, and F. Fukumizu, Makromol. Chem., 1968, 119, 140.

74. German Patent 856,293, Chem. Abs., 1956, 50, p 1896a.

75. B.R. Baker, M.W. Querry, S.R. Sajir, and S. Bernstein, J. Org. Chem., 1947, 12, 138.

76. H. Sokol and J.J. Ritter, J. Amer. Chem. Soc., 1948, 70, 3517.

77. J.C. Brown and H.R. Horton, Fed. Proc. Fed. Amer. Soc. Exp. Biol., 1973, 32, 496.

78. D.S. Tarbell and J.R. Hazen, J. Amer. Chem. Soc., 1969, 91, 7657.

79. B. Capon and S.P. McManus, 'Neighbouring Group Participation', Vol. 1, Plenum Press, New York, 1976.

80. S. Winstein, J. Amer. Chem. Soc., 1943, 65, 2196; S. Winstein ibid., 1948, 70, 821.

81. D. Gagnaire and A. Butt, Bull. Soc. Chim. Fr., 1961, 312. D. Gagnaire and M. Leclercq, ibid., 1963, 721.

82. I.R. Dunkin and A.J. Paul, University of Strathclyde, unpublished results.

83. G. Zweifel and J. Plamondon, J. Org. Chem., 1970, 35, 898.

84. J. Gore and F. Guigues, Bull. Soc. Chim. Fr., 1970, 10, 359.

85. (a) J.B. Jones and H.M. Schwartz, Can. J. Chem., 1981, 59, 1574.

(b) J. Davies and J.B. Jones, J. Amer. Chem. Soc., 1979, 101, 5405.

86. (a) T. Takemura and J.B. Jones, J. Org. Chem., 1983, 48, 791.

(b) J.A. Haslegrave and J.B. Jones, J. Amer. Chem. Soc., 1982, 104, 4666.

87. E. Valente, Final year research project, University of Strathclyde.

88. J.-P. Vandecasteele and R. Azerad, Inf. Chim., 1978, 176, 225.

89. C.H. Wong and G.M. Whitesides, J. Amer. Chem. Soc., 1981, 103, 4890.

90. H. Sund and H. Theorell, 'The Enzymes', eds. P.D. Boyer, H. Lardy, and K. Myrbach, Academic Press, New York, 1963, Vol. 7.

91. J.A. Dale, D.L. Dull, and H.S. Mosher, J. Org. Chem., 1969, 34, 2943.

92. J.-P. Vandecasteele and J. Lemal, Bull. Soc. Chim. Fr., II, 1980, 46, 101.

93. A. Pelter and K. Smith 'Comprehensive Organic Chemistry', ed. D.N. Jones, Pergamon, Oxford, 1979, Vol. 3.

94. (a) A. Hirao, S. Itsumo, S. Nakahama, and N. Yamazaki, J. Chem. Soc. Chem. Comm., 1981, 315.

(b) K. Hakko, Japanese Patent 57, 146, 786.

95. S. Itsumo, K. Ito, A. Hirao, and S. Nakahama, J. Chem. Soc. Chem. Comm., 1983, 469.

96. C.F. Lane, U.S. Patent 3,935, 280.

97. C.F. Lane, Chem. Revs., 1976, 76, 773.

98. O. Vogel and M. Pohm, Monatsch., 1952, 83, 541.

99. G.S. Poindexter and A.I. Meyers, Tetrahedron Lett., 1977, 3527.

100. A.I. Meyers, G. Knaus, K. Kamata, and M.E. Ford, J. Amer. Chem. Soc., 1976, 98, 567.

101. A.I. Meyers and J. Slade, J. Org. Chem., 1980, 45, 2785.

102. K. Yamada, M. Takeda, and T. Iwakuma, Tetrahedron Lett., 1981, 22, 3869.

103. J.P. Greenstein and M. Winitz, 'Chemistry of the Amino Acids', Vol. 2, John Wiley and Sons, London, 1961 and references cited therein.

104. F. Helner, K. Kilhs, and C. Hansch, Biochemistry, 1968, 7, 2858.

105. T. Sugimoto, Y. Matsumura, S. Tanimoto, and M. Okano, J. Chem. Soc. (C), 1978, 926.

CHAPTER 3

EXPERIMENTAL

Nuclear magnetic resonance (n.m.r.) spectra were recorded on Perkin-Elmer R 32 (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 100 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 resolution mass spectra were recorded on an A.E.I. (Kratos) MS9 spectrometer. Low resolution spectra were recorded on a MS20 instrument.

Gas-liquid chromatography (g.l.c.) was done on Perkin-Elmer F33 and Sigma 3B instruments. The column packing was 5% F.F.A.P. on Chromosorb G.

High pressure liquid chromatography (HPLC) was done using a system comprising a LDC model 396 micropump and a Cecil instruments CE 2012 variable wavelength u.v. monitor. The prepacked columns were supplied by Whatmans or Waters. All solvents were distilled before use.

pH was automatically maintained using a combi-titrator 3D, Metrohm Herisau, Switzerland.

Enzymes and biochemicals were assayed by standard methods.[1]

HPLC Analysis of Folates

The mobile phase used was 5:95, acetonitrile : 50 mM Tris-Cl, pH 7.0, containing 10 mM β-mercaptoethanol. An octadecyl silane reversed phase column was used. The flowrate was approximately 40 ml/h and the effluent was monitored at 254 nm. Relative concentrations were estimated from peak areas.

HPLC Analysis of Diastereomeric purity of Tetrahydrofolic Acid

Tetrahydrofolic acid was reacted with (R)-(-)-1-isocyano-1-naphthylethane.[2] The chiral isocyanate reacts by addition to N5 of tetrahydrofolic acid.[3] Thus an aqueous solution of tetrahydrofolate was mixed with an equal volume of ethanol containing an excess of the isocyanate. The reaction was complete in $\sim$5 min. at room temperature. The eluent used was 20:80 acetonitrile : 50 mM Tris-Cl, pH 7.0. The effluent was monitored at 290 nm. An octadecylsilane reversed phase column was used. The (6R)-isomer eluted first. Diastereomeric excess was estimated from peak height x retention time.

ABBREVIATIONS

ATP	- Adenosine triphosphate
ADP	- Adenosine diphosphate
AMP	- Adenosine monophosphate
AcP	- Acetyl phosphate
NADPH, $NADP^{\oplus}$	- Nicotinamide adenine triphosphopyridine nucleotide (reduced and oxidised forms respectively)
NADH, $NAD^{\oplus}$	- Nicotinamide adenine diphosphopyridine nucleotide (reduced and oxidised forms respectively)
DHF	- Dihydrofolic acid
THF	- Tetrahydrofolic acid
DTT	- Dithiothreitol
EDTA	- Ethylenediamine tetra-acetic acid
TET	- Triethylenetetramine
Tris	- Tris hydroxymethylaminomethane
Hepes	- N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
DEAE - Cellulose	- Diethylaminoethyl cellulose

7,8-Dihydrofolic acid

For small scale work the method of Futterman as modified by Blakely was used.[4] The amounts varied depending on requirements but the procedure is given below.

Ascorbic acid (12 g) was dissolved in distilled water (60 ml). The pH was adjusted to 6.0 with 1N sodium hydroxide. If necessary the final volume was brought to 120 ml with water. Half of this solution was kept ice cold. To the remainder a solution of folic acid (250 mg) in 0.1 N sodium hydroxide (10.5 ml) was added. Sodium dithionite (2.6 g) was then added and the solution was stirred for 5 min. at room temperature. After cooling to 5°C, 1N HCl was added slowly dropwise by burette until the pH fell to 2.8. After stirring for 5 min. the microcrystalline material was recovered by centrifugation at 0°C. The precipitate was then resuspended in the remaining ice cold sodium ascorbate and stirred until dissolution. The pH was re-adjusted to 6.0 if necessary. After maintaining the solution (at times there may be a slight precipitate) at 0°C for 5 min, 1N HCl was again added slowly until pH 2.8 was reached. Crystallisation was complete after 5 min. The crystals were recovered by centrifugation, washed 3 times with 0.005 N HCl (25 ml) and then resuspended in 0.005 N HCl in a 50 ml volumetric flask. The 7,8-dihydrofolic acid was stored in the dark as a suspension at 5°C. It is stable for several months. Estimation of the yield of the reaction was done by U.V. assay.

U.V. λ_{max} (0.1 N NaOH) 285 nm ($\epsilon = 20.4 \times 10^3$). The yield varied in the range 70-80%.

Assay of Dihydrofolate Reductase, Forms I and II

Assay mixture	volume used	conc. in assay
1) 50 mM KH_2PO_4 (pH 7.0 with KOH)	2.6 ml	46.5 mM
2) 1.0 mM DHF [0.44 mg/ml 50 mM potassium phosphate + 0.12 mM β-mercapto-ethanol (10 µl/ml]	100 µl	0.035 mM
3) 1.2 mM NADPH (1.0 mg/ml of 50 mM potassium phosphate)	100 µl	0.042 mM
4) DHFR solution (approx. 1 mg/ 100 ml H_2O)	50 µl	

These solutions apply to form I assays; for form II (1) is substituted by 50 mM succinate (pH 5.0 with KOH). Solution (1) is kept at 25°C. Solutions (2) (3) and (4) are kept cold.

Procedure: Solutions (1) to (3) were incubated at 25°C for 10 mins in a cuvette. The enzyme sample was then added and the change in absorbance noted. In this case the change in absorbance at 340 nm is due to DHF reduction as well as NADPH oxidation. ϵ NADPH = 6.22 $cm^2/\mu mol$ ϵ DHF = 5.05 $cm^2/\mu mol$. The contribution of NADPH oxidation to the change in absorbance with time is therefore 0.55 x ΔA. This is the ΔA value used when calculating the specific activity.

Form II, pH 7.0, 3.3 U/mg

pH 5.0, 24.4 U/mg

Form I, pH 7.0 27 U/mg of protein
8.65 U/mg of solid.

It was found that the activity of DHFR (form II) decreased over the period of this work to a value of 2-2.5 U at pH 7.0.

10-Formyldihydrofolic acid[5]

10-Formylfolic acid was recrystallised from water in the presence of decolourising charcoal. Anal. calcd. for $C_{20}H_{19}N_7O_7 \cdot H_2O$: C, 49.48; H, 4.42; N, 20.03. Found: C, 49.90; H, 4.74; N, 21.83. U.V. λ_{max} (pH 1) 321 and 252 nm (lit.,[6] 321 and 252 nm).

10-Formylfolic acid (20 mg, 41 μmol) was dissolved in 10 ml of 0.1 M potassium phosphate buffer (pH 2.5). Sodium dithionite (200 mg, 1.15 mmol) was then added to the stirred solution. The reaction was followed by HPLC. After 65 min. at room temperature, cold ethanol (5 ml) was added to precipitate excess sodium dithionite. The solid was removed by centrifugation. Ethanol was removed on a rotary evaporator to give an aqueous solution of the product. The yield was estimated by deformylation of the 10-formyldihydrofolic acid to dihydrofolic acid in basic solution. The U.V. spectrum smoothly changed over 1 h to give λ_{max} 283 nm. Using 20.4×10^3 as the extinction coefficient for dihydrofolate the yield of 10-formyldihydrofolic acid was 18.4 mg (95%). An estimate from the HPLC trace gave 90%.

Investigation of 10-formylfolic acid and 10-formyldihydrofolic acid as substrates for dihydrofolate reductase (form II) at pH 5.0 and 7.0.

Assay mixture	Volume used	Conc. in assay
1 (a) 50 mM KH_2PO_4, pH 7.0 (b) 50 mM succinate, pH 5.0	2.75 ml	46 mM
2 (a) 0.5 mM 10-formyl DHF in buffer 1(a) (b) 0.5 mM 10-formylfolic acid in buffer 1(a)	100 μl	0.016 mM
3 0.7 mM NADPH [0.86 mg in 1.5 ml of buffer 1(a)]	100 μl	0.023 mM
4 Enzyme solution (0.79 mg/100 ml H_2O)	50 μl	

Solutions 1 to 3 (as appropriate) were incubated at 30°C for 10 min. in a cuvette. The enzyme sample was then added and the change in absorbance noted. Allowance was made for the decrease in absorbance at pH 5.0 for a blank reduction. Neither derivative, at either pH, was a substrate.

(6R,S)-5,10-Methenyltetrahydrofolic acid chloride.[7]

To a suspension of folic acid·$2H_2O$ (60 g, 0.126 mol) in water (1.25 L), which was under an atmosphere of N_2 and cooled in an ice bath (5°C), was added slowly with stirring 50% sodium hydroxide (25 ml). The resulting clear yellow/brown solution (pH 7.85, meter) was treated over a 40 min. period with a solution of sodium borohydride (56.2 g) in water (200 ml). The temperature was maintained at 5°C. The solution was stirred for an additional 60 min. followed by decomposition of the excess sodium borohydride with conc. HCl (93 ml). Cooling was required to keep the temperature below 24°C, the addition took 75 min. The resulting solution (pH 7.7) was adjusted to pH 6.6 over a period of 30 min. with conc. HCl (33 ml). β-Mercaptoethanol was then added (3 g, 38 mmol) and the pH was brought to 2.65 by further addition of conc. HCl. A yellow suspension was obtained which turned brown on standing overnight under N_2. The suspension was filtered under an atmosphere of N_2. The wet solid was then dissolved in a mixture of 98:2 formic acid (98%): trifluoroacetic acid (400 ml). After standing at room temperature for 20 h the dark red solution was evaporated to dryness *in vacuo* at a maximum water bath temperature of 45°C. The superficially dried solid was suspended in 0.5 N HCl (1050 ml) containing β-mercaptoethanol (1 ml). The whole was concentrated

in vacuo at 45°C to remove formic and trifluoroacetic acids. After standing at room temperature for 18 h the (6R,S)-5,10-methenyltetrahydrofolic acid chloride was collected by filtration, washed with 0.01 N HCl, ethanol, and ether and dried in vacuo over phosphorus pentoxide: yield 28.65 g (44%). Concentration of the filtrate deposited a second crop, yield 9 g (14%). Anal. calcd. for $(C_{20}H_{22}N_7O_6)^{\oplus}Cl^{\ominus}\cdot H_2O$: C, 47.11; H, 4.74; Cl, 6.95; N, 19.23. Found: C, 46.26; H, 4.72; N, 19.13. A sample was recrystallised from 0.1 N HCl - 0.1 M β-mercaptoethanol in the presence of decolourising charcoal.[8] Found: C, 47.27; H, 4.94; Cl, 8.17; N, 19.63. U.V. λ_{max} (1.0 N HCl) 287 and 347 nm. ϵ = 13.32 x 10^3 and 27.10 x 10^3. λ_{max} 347: λ_{min} 303 = 2.4 (lit.,[8] 290 (12.4 x 10^3) 348 (26.5 x 10^3); 2.46).

Calcium (6R,S)-5-formyltetrahydrofolate[7]

Solid (6R,S)-5,10-methenyltetrahydrofolic acid chloride (10 g, 0.0196 mol) was added with stirring under nitrogen to boiling water (200 ml). During the addition and thereafter for one hour, hot, de-aerated 3.7 N sodium hydroxide (25 ml) was added at a rate to maintain the pH between 6.5 and 7.0 (meter). The resulting solution was refluxed for 12 h; it was necessary to add 1 N HCl (6 ml in total) to maintain the pH between 6.6 and 7.1. After standing for an additional 12 h without heat the mixture was treated with a clarified solution calcium chloride (4.75 g in 11.5 ml). The solution was cooled in an ice/salt bath and a yellow solid was formed on addition of ethanol (20 ml). After standing overnight at 5°C the yellow solid was collected and dried in vacuo over phosphorus pentoxide, yield 5.84 g (54%).

The clear filtrate was further diluted with ethanol (600 ml) and after standing for 2.5 days at 5°C a further crop was obtained, yield 3.89 g (36%). Ascending paper chromatography (Whatmans No. 1, 0.1 M phosphate, pH 7.0) showed some baseline impurities when examined under long wave U.V. light. The material was purified by dissolving in boiling water and adding ethanol until turbidity just remained. Re-dissolving and cooling deposited a solid. This process was repeated twice to give a further 2 fractions. All the fractions were dried in vacuo over phosphorus pentoxide. Fractions 2 and 3 which constituted the bulk of the solid showed no baseline impurities, only an absorbing spot, r.f. 0.61, on paper chromatography. Anal. calcd. for $C_{20}H_{21}N_7O_7Ca \cdot 2H_2O$: C, 43.87; H, 4.60; N, 17.90; Ca. 7.32. Found: C, 43.80; H, 4.54; N, 17.90; Ca. 7.15 (determined as ash CaO, ~10%) U.V. λ_{max} (0.1 N NaOH) 282 nm ϵ = 27.5 x 10^3. λ_{max} 282: λ_{min} 242 = 3.65 (lit.,[7] 282 nm (28.8 x 10^3) max:min = 3.6) $[\alpha]_D^{21}$ = + 11.27° (c. 0.222 g, water). T.l.c. (silica with fluorescent indicator) r.f. 0.62 (0.1 M phosphate pH 7.0).

Further experiments using triethylamine as the base in essentially the same procedure offered no obvious advantages over the method above.

<u>Enzymatic reduction of dihydrofolate to (6S)-tetrahydrofolate; consuming glucose-6-phosphate and recycling $NADP^{\oplus}$ 540X</u>

Dihydrofolate (250 mg, 564 μmol) was suspended in 50 ml of 50 mM Tris-Cl (pH 7.0); 5 mM in DTT, in a 250 ml 3 necked flask. The flask was equipped with a pH electrode, a N_2 inlet/outlet, and a serum stopper. A solution was obtained by adding saturated Tris and stirring magnetically over 10 min. Glucose-6-

phosphate (564 mg, 2,000 μ mol) was dissolved in 5 ml of the buffer and introduced into the flask. If necessary the pH was corrected to 7.0 with Tris. Glucose-6-phosphate dehydrogenase (0.1 mg ~ 25 U), dihydrofolate reductase, form II (5 mg, ~ 16.5 U) and $NADP^{\oplus}$ (0.086 mg, 105 μmol) were dissolved in 1 ml of buffer and added to the flask. The reaction was stirred gently under N_2, the temperature was maintained at 25°C using a thermostated water bath. Complete reduction to (6S)-tetrahydrofolate was accomplished in 6 h (HPLC).

Enzymatic reduction of dihydrofolate to (6S)-tetrahydrofolate; consuming D-isocitrate and recycling $NADP^{\oplus}$ 137X. 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 N_2 inlet/outlet, and a magnetic stirring bar. The flask was charged with water (150 ml, which had been degassed and nitrogenated) 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 N 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 $NADP^{\oplus}$ (3.1 mg, 3.7 μmol) were added. The pH was maintained between 6.8 and 7.4 using 1 N HCl. The flask was covered in foil to prevent photo-degradation of the reduced folates and the reaction was left

stirring under N_2 at ambient temperature. After 7 days the reaction was complete (HPLC) 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 N HCl containing β-mercaptoethanol (30 µl). The whole was concentrated *in vacuo* at 45°C to ~ 10 ml. After standing at room temperature for 18 h a light brown solid was isolated by freeze-drying once more.

DEAE cellulose was washed extensively with water and then 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 - 0.01 M β-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 collections were frozen. All of the effluent fractions exhibiting a value greater than 1.6 for the ratio of absorbance of 345:310 nm in 1.0 N HCl were pooled and lyophilised (tubes 13-22). The yellow solid was dissolved in boiling 0.1 N HCl - 0.1 M β-mercaptoethanol (~ 15 ml). After standing overnight at 5°C the crystals were collected by centrifugation and washed with ethanol and then 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 (1 N HCl) 287 and 347 nm. $[\alpha]_D^{24°C} = +19.2 \pm 2.5°$ (c. 0.39, conc. HCl, sp. gravity 1.16, approx. 10 N). Lit.[24],

$[\alpha]_D^{25°} = +11.4 \pm 0.8°$ (c. 0.95, 12 N HCl). The product was compared with an analytical racemic sample by HPLC.

Ketene

Ketene was produced by the pyrolysis of acetone vapour in a quartz tube. The apparatus was similar to that described by Rice et. al.[9] Acetone was continuously distilled from a round bottom flask, under a stream of dry N_2, and passed through a quartz tube contained in an oven. The solvent level in the flask was maintained by continous addition of acetone. The acetone and N_2 flows were the same for all experiments. After the oven temperature had equilibrated at 700-725°C the rate of ketene generation was estimated by diverting the output of the generator through 50 ml of a cooled, stirred solution of ethanolamine (1.329 N), in 2-propanol, for 30 min. The excess amine was then titrated with standardised 1.0 N HCl.[10] Using methyl red the colour change was yellow to red, the titration proceeded until the first persistent red tinge. The output was 0.55 ± 0.16 mol/h, the relative standard deviation was 23% (from five determinations).

Attempted preparation of diammonium acetyl phosphate

Phosphoric acid (88%) was dehydrated by adding 19.15 g of phosphorus pentoxide with stirring at room temperature. The resulting straw coloured liquid was allowed to stand over magnesium perchlorate in a desiccator for 1 h.

Ethyl acetate (200 ml) and phosphoric acid (29.4 g, 0.3 mol) were cooled to -10°C in a 500 ml 3 neck flask. The output of the ketene generator was passed through this solution for 70 min. Neutralisation with a saturated solution of ammonia in methanol

and isolation of the solid were as described (see below). A n.m.r. assay indicated that the solid did not contain acetyl phosphate.

Diammonium acetyl phosphate.[11]

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 in vacuo 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 futher 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% AcP, 0.6% acetamide and 6.3% ammonium acetate. Anal. calcd for $C_2H_{11}N_2O_5P$: C, 13.80; H, 6.37; N, 16.09: Found: C,12.25; H, 5.98; N, 15.08. The sample was stored at 5°C in a desiccator.

NMR Assay for AcP

100-150 mg of the reaction product was dissolved in 0.6 ml of D_2O, and to this solution was added dioxan (10 µl; micropipette). The solution was transferred to a n.m.r. tube and the spectrum recorded. The acetyl protons of AcP 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 allows 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.

Enzymatic Assay for AcP

The assay is based on the phosphorylation of ADP using AcP and acetate kinase to give ATP. The number of moles of ATP formed are directly related to the number of moles of AcP 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 $Mg^{2\oplus}$ (2.98 g $(HOCH_2CH_2)_3N$ and 0.61 g $MgCl_2 \cdot 6H_2O$ 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 5 ml of solution (1) in a sample bottle was added 100 µl of (2) and 10 µl of acetate kinase suspension. This mixture was incubated at 25°C for 2 mins before addition of 100 µl of H_2O or 100 µl of AcP solution. 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). The ATP

concentration was calculated as outlined below. Agreement between the two HPLC methods and also with the n.m.r. method was good.

Separation and quantitation of adenosine diphosphate and adenosine triphosphate by HPLC[12]

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 H_2O. 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 1 N 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 reaction times (minutes) for the nucleotides were:

AMP - 5.2

ADP - 10.2

ATP - 18.4

for a flowrate of 119 ml/h.

Quantitation of ATP at 0.05 a.u.f.s.

ATP (202.735 mg) was made to 1-L with H_2O. A molecular weight of 643.8 was calculated on the basis of $\epsilon = 15.4 \times 10^3$ for λ_{max} 259 nm in 0.1 M potassium phosphate buffer pH 7.0[13]. Aliquots of this standard solution were withdrawn and diluted with H_2O to give the required concentrations of ATP for injection.

ATP conc, x 10^4 M	Peak area, cm^2
0.063	0.310
0.158	0.950
0.315	1.888
0.630	3.617
0.944	4.804
1.253	7.493
1.566	9.262

The instrument scale was set at 0.05 a.u.f.s. and the injections were duplicated.

The areas quoted were the average. The slope was 5.858×10^4 and the intercept was -7.8×10^{-2}.

Quantitation of ATP at 0.2 a.u.f.s:

ATP conc, x 10^4 M	Peak area, cm^2
1.253	1.562
1.880	2.770
2.507	3.588
3.134	4.606

The injections were duplicated. The slope was 1.587×10^4 and the intercept was -0.35.

Quantitation of ADP at 0.05 a.u.f.s.

ADP (152.905 mg) was made to 1 L with water. A molecular weight of 523.27 was calculated on the basis of $\epsilon = 15.4 \times 10^3$ for λ_{max} 260 nm in 0.1 M potassium phosphate buffer pH 7.0.[13] Aliquots of this standard solution were diluted for injection in duplicate.

ADP conc, x 10^4 M	Peak area, cm^2
0.0585	0.412
0.1460	0.826
0.2922	1.545
0.5844	3.258
0.8766	4.664
1.1168	6.559

The slope was 5.682 x 10^4 and the intercept was -3.4 x 10^{-2}.

Separation and quantitation of adenosine diphosphate and adenosine triphosphate by HPLC

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 0.2 M H_3PO_4. The buffer also being 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.

Quantitation of ATP at 0.02 a.u.f.s.

Samples prepared as before. Injections done in duplicate.

ATP conc, x 10^4 M	Peak area, cm^2
0.062	0.825
0.154	1.838
0.308	3.640
0.616	7.472
0.924	11.636

The slope was 12.548 x 10^4 and the intercept was - 9.79 x 10^{-2}.

0.05 a.u.f.s.

ATP conc, x 10^4 M	Peak area, cm^2
0.308	1.685
0.924	4.520
1.540	7.942
2.156	11.130

The slope was 5.155 x 10^4 and the intercept was -3.22 x 10^{-2}.

Quantitation of ADP at 0.1 a.u.f.s.

Samples prepared as before, injections done in duplicate.

ADP conc, x 10^4 M	Peak area, cm^2
0.120	0.389
0.240	0.630
0.480	1.252
0.721	1.864
0.961	2.494
1.201	3.115

The slope was 2.545 x 10^4 and the intercept was 4.44 x 10^{-2}.

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 N 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 1½ min, chilled for 1 min, filtered, and injected onto the column. Typical results for a determination of enzyme activity using the reverse phase column are given. Three assays were done, the samples from each were injected once.

Time (min)	Assay (1) Peak Area (cm^2)	Assay (2) Peak Area (cm^2)	Assay (3) Peak Area (cm^2)
1	2.188	2.100	2.223
3	4.489	4.113	4.140
5	6.333	5.303	6.138

The average change in area was 0.938 ± 0.137 cm^2/min.

The relative standard deviation was 10.6%.

The detector was set at 0.05 a.u.f.s: the equation for this range was:

$$\text{Area } (cm^2) = 5.682 \times 10^4 \times \text{conc}(M) - 3.4 \times 10^{-2}$$

$$\therefore \quad \Delta[ADP]/min = \frac{0.938 + 3.4 \times 10^{-2}}{5.682 \times 10^{4}}$$

$$\Delta \text{ moles/min} = \frac{0.938 \times 3.4 \times 10^{-2}}{5.682 \times 10^{4}} \times \frac{5}{1000}$$

1 μmol/min = 1 unit

$\therefore$ 100 ml stock solution contained 86.0 units.

The specific activity of hexokinase was $\frac{86.0}{9.53}$ = 9.0 units/mg.

The activity of this batch of enzyme when assayed spectrophotometrically was 11 U/mg.

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 N HCl 5mM in DDT)	4.45 ml	89 mM
0.24 M AcP 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.

The average change in area was 2.074 $\pm$ 0.14 cm^2 /min.

The equation used was:

$$\text{Area } (cm^2) = 5.858 \times 10^4 \times \text{conc.}(M) - 7.83 \times 10^{-2}$$

$$\therefore \Delta\text{moles/min} = \frac{2.074 \times 7.83 \times 10^{-2}}{5.858 \times 10^{-4}} \times \frac{5}{1000}$$

$$= 0.184 \times 10^{-6}$$

The acetate kinase suspension contains 5 mg/ml. The specific activity of the acetate kinase was 184 U/mg. A linked U.V. assay which actually measures the reaction in the opposite direction gave 161 U/mg.

An assay of a different batch of enzyme using the strong anion exchange column gave a specific activity of 156 U/mg, spectrophotometric assay of this enzyme gave 136 U/mg.

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 ATP and recycling $NADP^{\oplus}$

50 ml of 50 mM Tris-Cl (pH 7.2) 10 mM in $Mg^{2\oplus}$ and 5 mM in DTT, which was de-aerated by bubbling N_2 through for 30 min.,

was placed in a 100 ml 2 necked flask. The flask had a N_2 inlet/outlet and a serum stopper. To this was added glucose. H_2O (27 mg, 136 μmol), dihydrofolate (25 mg, 56.4 μmol), dihydrofolate reductase, form II, (1.8 mg, 6 U), glucose-6-phosphate dehydrogenase (0.1 mg, 25 U), and hexokinase (1.68 mg, 18.5 U). The solution was incubated at 25°C in a water bath for 5 mins after which time adenosine triphosphate (70.5 mg, 109 μmol) and $NADP^{\oplus}$ (0.64 mg, 0.78 μmol) were added. The addition of ATP caused some precipitation. A further 10 ml of buffer were added but complete dissolution was not obtained. The suspension was stirred magnetically at 25°C under N_2. It was hoped that the solubility properties of tetrahydrofolate would result in dissolution as the reaction proceeded. After 2 days the reaction was allowed to settle and HPLC analysis indicated 50% reduction of the material in solution. At this point glucose · H_2O (18 mg, 91 μmol) and ATP (23 mg, 36 μmol) were added. After 15 days the reaction in solution had proceeded to > 94% although there was still unreacted dihydrofolate suspended in the reaction. It is clear that the recycling procedure would be viable provided complete solubility is obtained.

<u>Enzymatic reduction of dihydrofolate to (6S)-tetrahydrofolate; consuming creatine phosphate and recycling ATP and $NADP^{\oplus}$ 46 and 93 X respectively</u>

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 DTT (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 done 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, 4 U) and dihydrofolate reductase, form II, (1.34 mg, 4.4 U) were added. The final component, $NADP^{\oplus}$ (3.32 mg, 4 μmol) was then added after the system had stabilised. The reduction was complete after 5 days (HPLC). This system was subsequently extended to recycling ATP and $NADP^{\oplus}$ 300 and 70 X respectively.

Immobilisation of enzymes on poly(acrylamide-co-N-acryloxy succinimide) (PAN)[14]

The active ester content of PAN was determined as described.[14] 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)[DHFR]

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 DHF and 0.70 mM $NADP^{\oplus}$ (approx. 100 x the respective Michaelis constants, km). To this was also added 100 μl of a 0.5 M solution of DTT. 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 340 µl of a 0.5 M aqueous solution of TET were added; approx. 60 s later 200 µl of a solution of DHFR form II (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 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.

Since a fair amount of the enzymic activity was recovered in the washes the inference was that the amino groups of this enzyme were comparatively unreactive. This postulate was tested by modifying the immobilisation procedure. The enzyme and the aqueous PAN solutions were allowed to react in the absence of crosslinking agent. TET was then added and the immobilisation procedure followed the course described above.

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

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

DHFR form I was immobilised using the standard procedure. The effect of removing the cofactors was investigated. Without cofactors: gel 3%; washes 4%,with cofactors: gel, 5%; washes 69%.

The stability of DHFR in buffer (Hepes, 50 mM pH 7.5, 10 mM DTT) at 5°C, free and immobilised, was investigated. After 7 months immobilised enzyme had retained 78% activity : 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, 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.[14]

Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49; G-6-PDH)

G-6-PDH (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 G-6-P and 1.0 mM $NADP^{\oplus}$. The buffer for washing and storing contained 10 mM $Mg^{2\oplus}$ and 10 mM DTT. The specific activity of the enzyme was 270 ± 34 U/mg, at pH 7.6 and 25°C. The washes contained 13.98 U (8%) and the gel 26.5 U (15%). A repeat of the reaction using 0.26 mg of enzyme (70.5 U) gave; gel 14 U (20%) washes 1.33 U (2%).

Hexokinase (E.C. 2.7.1.1., Hk)

Hk (4.85 mg, 53.4 U) was immobilised on PAN-400 (1.846 g, 738 μmoles of active esters). The buffer contained 15 mM $Mg^{2\oplus}$,

25 mM glucose, and 10 mM ADP. The buffer for washing and storing contained 10 mM $Mg^{2\oplus}$ and 10 mM DTT. The specific activity of the enzyme was 11 U/mg. The washes contained 5.4 U (10%) and the gel 22.4 U(42%).

Creatine kinase (E.C. 2.7.3.2., Ck)

Ck (6.84 mg, 978 U) was immobilised 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, Ak)

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 N_2. The grinding and washing were done in air using Hepes buffer containing 10 mM DTT. The enzyme is supplied as a suspension in ammonium sulphate. It must be separated from this solution otherwise the ammonium sulphate crosslinks the polymer forming a weak gel which soon breaks up. The separation can be done by ultrafiltration 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, 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 $Mg^{2\oplus}$, 12.5 mM AcP, and 20 mM ADP. The immobilisation up to the gel point was done

in a stoppered beaker which had been flushed with N_2. After this the beaker was placed in a desiccator and kept under positive N_2 pressure. The buffer for washing and storing contained 10 mM $Mg^{2\oplus}$ and 10 mM DDT. The specific activity of the enzyme was 162 U/mg. The washes contained 170 U (42%) and the gel 168 U (42%).

The preparation of cyanogen bromide activated Sepharose 4B[15]

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 CH_3CN) 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 the quantity of cyanogen bromide was varied. 500, 200, 55, and 25 mg were used hence the Sepharose preparations were designated Sigma, 500, 200, 55, and 25 accordingly.

General protein coupling procedure

The method employed was essentially that described in the Pharmacia handbook.[16] 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). The gel was then washed for 1 h alternately with 0.1 M potassium phosphate buffer, pH 5.5, and the coupling buffer (25 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.

The stability of dihydrofolate reductase to the coupling procedure

Dihydrofolate reductase (2-3 mg) was stirred overnight at 5°C in 2 ml of either buffer (A) or (B). The effect of including the cofactors $NADP^{\oplus}$ and DHF at a concentration of 2.7 mM and 1 mM respectively and also the effect of DTT, at a concentration of approx. 10 mM, were investigated. Inclusion of all the above compounds resulted in 90% recovery of activity. Without the cofactors 55-60% activity remained while omission of only the DTT resulted in 50% recovery. Without any additives the enzyme retained 55-60% of its activity. It was found that successful immobilisation occurred only when there were no additives.

The Immobilisation of Dihydrofolate Reductase (DHFR), on Cyanogen Bromide Activated Sepharose 4B Form II

(a) The enzyme (1.16 mg, 2.4 U.) was added to 135 mg of activated Sepharose (Sigma). The mixture was stirred overnight (18 h) at 5°C in 2 ml of buffer (A). The filtrate contained 0.7 U (29%), the Tris-Cl wash contained no activity, and 0.3 U (12.5%) was detected in the support. After 5 days storage at 5°C the activity had fallen to 0.26 U (86%).

(b) 0.88 mg (2.08 U) of DHFR was immobilised on 100 mg of activated Sepharose (Sigma) in 1 ml of buffer (B). There was no activity in the filtrate, the support contained 0.56 U (27%).

(c) 2.05 mg (5.12 U) of DHFR was immobilised on 100 mg of activated Sepharose (Sigma) in 1 ml of buffer (B). There was no activity in the filtrate, the support contained 0.67 U (13%).

(d) 3.04 mg (7.6 U) of DHFR was immobilised on 100 mg of activated Sepharose (Sigma). There was no activity in the filtrate, the support contained 1.14 U (15%).

(e) 1.43 mg (3 U) of DHFR was immobilised on 150 mg of Sepharose (200) in 2 ml of buffer (A). There was no activity in the filtrate or the Tris-Cl wash, the support contained 0.5 U (17%). After 5 days storage at 5°C that activity had fallen to 0.37 U (74%).

(f) 1.06 mg (2.3 U) of the enzyme was immobilised on 100 mg of Sepharose (200) in 2 ml of buffer (B). The filtrate contained 0.3 U (13%), there was no activity in any other wash and the

support contained 0.42 U(18%). After 4 days storage at 5°C the activity had fallen to 0.36 U(86%). The support was stirred for 1 h in 10 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 1.2 mM dihydrofolate. This treatment decreased the activity further to 0.28 U, 0.05 U was detected in the filtrate.

(g) 0.95 mg (2 U) of DHFR was immobilised in 0.4 g of wet Sepharose (500) [dry weight ~150 mg] in 2 ml of buffer (B). The filtrate and the washes contained no activity, the support contained 0.29 U (15%).

(h) 0.84 mg (1.76 U) of the enzyme was immobilised on 26 mg Sepharose (55) in 2 ml of buffer (B). The filtrate contained 0.24 U (14%) and the support had an activity of 0.17 U (10%). There was no activity in any other wash.

(i) 1.15 mg (2.5 U) of DHFR was immobilised in 22 mg of Sepharose (25). The support had an activity of 0.05 U (2%).

Form I

(a) 1.09 mg (9.2 U) of DHFR was immobilised on 100 mg of Sepharose (Sigma) in 1 ml of buffer (A). There was no activity in the filtrate or the washes, the activity on the support was 0.36 U (4%).

(b) 1.01 mg (8.5 U) of DHFR was immobilised on 100 mg of Sepharose (Sigma) in 1 ml of buffer (B). There was no activity in the filtrate or the washes, the support contained 0.5 U (6%).

Entrapment of Dihydrofolate reductase in polyacrylamide beads.[17]

Monomer mix: Acrylamide (1.9 g, 0.027 mol), N,N'-methylene diacrylamide (0.1 g, 0.65 mmol), and tetramethylethylenediamine (300 µl, 2 mmol) were dissolved in 9.8 ml of 0.1 M Tris-Cl buffer pH 6.8. DHFR (4.56 mg, 15 U) was added to this solution, 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-sided 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 N_2 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 N_2 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 β-mercapto-ethanol. 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%).

Immobilisation of Dihydrofolate reductase on AH-Sepharose-4B by physical adsorption.[16]

AH-Sepharose 4B (250 mg) was washed at room temperature with 2 x 25 ml portions of 0.5 M NaCl, 5 min. each; 3 x 25 ml of 50 mM KH_2PO_4, pH 7.0 containing 5 mM β-mercapto-ethanol, (5 min. each). DHFR form II (1.87 mg, 6.2 U) was added to the support in 50 mM KH_2PO_4 pH 7.0 containing 0.32 mM DHF. 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%). To ascertain the level of desorption in buffer solutions the beads were again filtered and washed in 50 mM KH_2PO_4 buffer x2, 50 min. and then 10 mM KH_2PO_4 x1, 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

DHFR (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 DHF and 10 mM β-mercapto-ethanol. It was adjusted to pH 7.0 with ammonium carbonate. Washes 0 U, beads 0.7 U (11.6%). The effect of removing DHF was investigated; thus DHFR (2.12 mg, 7 U) was immobilised on the Sepharose (250 mg) in water. There was no activity in the washes, the beads

contained 0.7 U (10%). The beads were then stirred in a 5 mM DHF solution at RT for 45 min. to simulate reaction conditions. The activity in the DHF 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.

2,3-Dibromopropanoyl Chloride[18]

To a solution of acryloyl chloride (13.2 ml, 0.128 mol) in 15 ml of carbon tetrachloride at - 10°C, which was protected from light and under N_2, was added bromine (6.56 ml, 0.128 mol). The addition was as rapid as possible while maintaining the temperature at -10°C, approx. 20 min. The solution was allowed to warm to room temperature and left standing for 22 h. After this time the flask was warmed to 40°C under a positive pressure of N_2. The colour due to bromine had disappeared after 8 h. Distillation gave the acid chloride as a colourless liquid (26 g, 81%), b.p. 89-94°/33 mm (lit.,[18] 73-74°/12 mm). $\bar{\nu}_{max}$ (CCl_4) 1775 cm^{-1}. δ (CCl_4) ABC system, 3.55-4.15 (2H, m), 4.6-4.9 (1H, m). ^{13}C, ppm ($CDCl_3$) 28.7 (t), 49.3 (d), 164.3 (s). The sample gave a single peak on g.l.c. (210°C).

S-Phenylthioacrylate

The compound was prepared by a modification of the literature procedure.[19] 2,3-Dibromopropanoyl chloride (4 g, 0.016 mol) and thiophenol (1.57 g, 0.016 mol) were dissolved in 10 ml of dimethoxy ethane. The mixture was cooled to 0°C. Under N_2, and with

stirring, pyridine (1.29 ml, 0.016 mol) dissolved in 5 ml of dimethoxy ethane, was added dropwise. After stirring overnight at room temperature the mixture was decanted and the solvent removed at atmospheric pressure. The residue was dissolved in ether (50 ml) and washed with 5% sodium bicarbonate (50 ml); water (50 ml) and saturated brine (50 ml). After drying (Na_2SO_4) the solvent was removed *in vacuo*. Carbon tetrachloride (25 ml) was added to assist drying by azeotropic removal of any water. Chromatography (t.l.c. and g.l.c.) indicated a two component mixture. The residue could not be purified by distillation and so the debromination was carried out on the crude material. The residue (1.98 g) was dissolved in 10 ml of acetone and added dropwise to sodium iodide (2.11 g, 0.014 mol) in 20 ml of acetone. The mixture was left stirring overnight at room temperature under N_2. After concentration *in vacuo* to a third of the volume the mixture was filtered to remove sodium bromide. The filtrate was dissolved in ether (50 ml) and washed with 10% sodium thiosulphate solution (3 x 10 ml), water (30 ml) and saturated brine (30 ml). After drying (Na_2SO_4) and solvent removal *in vacuo* the residue was distilled to give the thiolester as a yellow viscous liquid (0.3 g, 13%), b.p. 115-128°C/16 mm (lit.,[20] 150-180°/10 mm). Anal. calcd. for C_9H_8OS: C, 65.82; H, 4.91. Found: C, 64.77; H, 5.05. High resolution mass measurement of the parent ion in the mass spectrum gave M 164.0298, calcd. 164.0296. The M + 2 ion gave 166.0258, calcd. 166.0254. $\bar{\nu}_{max}$ (CCl_4) 3060, 1682, 1610, 1473, 1436, and 1388 cm^{-1}. δ (CCl_4) 7.36 (5H, s, Ar H), 6.3-6.45 (2H, m) and 5.69 (1H, m). ABC system, J_{AC} = 5.5 Hz, J_{BC} = 4.2 Hz. The sample was considered to have > 98% purity by g.l.c. (210°C).

Thiomethylacetate

Thioacetic acid (30 ml, 0.44 mol) was added to 200 ml of chloroform containing methanol (47 ml, 1.16 mol) and conc. sulphuric acid (5 ml). The mixture was refluxed overnight under N_2. After cooling the mixture was washed with water and then with 5% sodium bicarbonate. The solvent was removed at atmos. pressure and the residue distilled to give the ester as a colourless liquid (31.6 g, 68%), b.p. 54-56°C/25 mm (lit.,[21] 49-51°/16 mm) $\bar{\nu}_{max}$ (CCl_4) 3000, 2950, 2840, 2580, 1738 and 1435 cm^{-1}.

Thioacetamide[22]

Anhydrous ammonia was bubbled slowly through cooled (ice/water) thiomethylacetate (25 ml, 0.28 mol) for 24 h. After removal of methanol the solid was dried *in vacuo* over phosphorus pentoxide to give the amide as a white solid (24.2 g, 95%), m.p. 45-50°C (lit.,[23] 52°C). $\bar{\nu}_{max}$ (KBr) 3380, 3180, 2910, 2545, 1640 and 1415 cm^{-1}. The amide oxidises easily to the disulphide, no attempts were made to purify the material; it was stored *in vacuo* at 5°C. The thiolamide is soluble in water, ethanol and methanol, it is insoluble in dichloromethane, chloroform, and acetonitrile.

Attempted preparation of S-Acetamidethioacrylate

The reaction of thioacetamide with 2,3-dibromopropanoyl chloride, in the presence of pyridine, was done in dimethoxyethane at 40°C. The thiol seemed to be partly soluble in this solvent. After stirring overnight under N_2 and removal of the solvent *in vacuo* the yellow oil obtained was examined by t.l.c. At least four spots were apparent. Various modifications of the procedure outlined above, for example using calcium carbonate as base or

omitting the base altogether, were not successful. The only material isolated on workup (selective extraction into chloroform) was tentatively assigned as being the disulphide by I.R. This route to a polymer containing a pendant thiolester group was abandoned. Some possibilities for the solution of this problem are presented in the discussion.

Enzymatic reduction of Dihydrofolate to (6S)-Tetrahydrofolate using immobilised enzymes (PAN) and recycling ATP and $NADP^{\oplus}$ 99 and 385 X respectively.

Dihydrofolate (165 mg, 372 μmol) was suspended in 40 ml of de-aerated water to which DTT (79.4 mg, 490 μmol) had been added. The suspension was transferred to a 280 ml 3 necked flask equipped with a N_2 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. $NADP^{\oplus}$ (0.72 mg, 0.88 μmol) glucose (76 mg, 383 μmol) ATP (2.18 mg, 3.4 μmol) and $MgCl_2 \cdot 6H_2O$ (49.5 mg, 245 μmol) were added to the flask using another 20 ml portion of water. Acetyl phosphate (108 mg, 587 μmol) was added over 3 days in approx. 3 equal portions (solid form) 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 N_2, 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 $NADP^{\oplus}$ of 99 and 385 X 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.

Large scale reduction of Folic acid to Dihydrofolate

Folic acid (12 g, 25 mmol) was suspended in 1250 ml of water and dissolved with 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 N_2 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 then the yellow solid was collected by centrifugation at 5°C. The dihydrofolic acid was washed 3 times with 0.005 N HCl (3 x 1 L) and then resuspended

in 1 L of 0.005 N HCl for storage before use. The yield of dihydrofolate was 8.6 g (77%) by U.V. assay.

(6R)-5,10-Methenyltetrahydrofolic acid chloride

Dihydrofolic acid (8.6 g, 19.4 mmol) was suspended in 1 L of de-areated water and brought into solution with 50% sodium hydroxide solution. This solution was transferred to a 3 neck 3 L flask equipped with a N_2 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 with 1 N sodium hydroxide. The pH of the dihydrofolate solution was 13, 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·H_2O (3.85 g, 19.4 mmol), adenosine triphosphate (0.125 g, 0.194 mmol), $NADP^{\oplus}$ (14.32 mg, 17.5 μmol) $MgCl_2 \cdot 6H_2O$ (1.01 g, 5 mmol) and DTT (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, 20U) 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, the inorganic phosphate produced would complex with magnesium ions to reduce their effective concentration and so the reaction rate. The reduction was conducted under N_2 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 in a Buchner funnel under an atmosphere of N_2 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 0.5N HCl (200 ml) containing β-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 0.1N HCl - 0.1 M β-mercaptoethanol. After standing at 5°C for 26 h the crystals were collected, washed with ethanol and ether, and dried in vacuo over phosphorus pentoxide: yield 2.22 g (22.5%). Concentration of the mother liquor deposited a second crop, yield 0.55 g(5.5%). The yields are based on dihydrofolate. Anal. calcd. for $(C_{20}H_{22}N_7O_6)^{\oplus}Cl^{\ominus} \cdot H_2O$: C, 47.11; H, 4.74; Cl, 6.95; N, 19.23. Found: C, 46.78; H, 4.70; Cl, 6.74; N, 18.91. $[\alpha]_D^{32.7^\circ} = +14.3 \pm 0.8^\circ$ (c, 0.35, 10 N HCl). (lit., [24] $[\alpha]_D^{25} = +11.4 \pm 0.80$ (c.0.95, 12 N HCl).

Calcium (6S)-5-formyltetrahydrofolate

(6R)-5,10-Methenyltetrahydrofolic acid chloride (1.8 g, 3.54 mmol) was hydrolysed as previously described, 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 of calcium(6S)-5-formyltetrahydrofolate was cooled at 5° for 18 h, collected by filtration, washed with ethanol and dried in vacuo over phosphorus pentoxide; yield 0.91 g (46%). Anal. calcd. for $C_{20}H_{21}N_7O_7Ca.3H_2O$: C, 42.4; H, 4.81; N, 17.30. Found: C, 42.62; H, 4.63; N, 16.45. U.V. λ_{max} (0.1 N 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). HPLC compared favourably with commercial sample. The solution used 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-methenyltetrahydrofolate formed was dissolved in 10 N HCl and made to 10 ml in a volumetric flask, $[\alpha]_D^{27}$ = + 11 (c. 0.59, 10 N HCl).

Tetrahydropyran-3-ol[25]

To Δ^2-dihydropyran, 41 g (0.488 mol) in freshly distilled tetrahydrofuran (140 ml) was added a 1 M solution of borane in tetrahydrofuran (250 ml: 0.5 equiv. of hydride) at 0°C under N_2. The addition took 75 min. After the reaction had been stirred at 0°C for a further 2 h the temperature was allowed to rise to 20°C and the mixture was stirred at this temperature for an additional 2 h. The oxidation was done by adding sodium hydroxide (89 ml, 3 N) over 30 min. then 50% hydrogen peroxide (35.1 ml) (15 min.). The temperature did not rise above 40°C. After further stirring at room temperature for 1 h sodium chloride was

added and the upper phase separated. Anhydrous potassium carbonate was added to the aqueous phase which was then extracted with 2 x 50 ml portions of tetrahydrofuran. The organic phases were combined and dried ($MgSO_4$) in the presence of ferrous sulphate. The solvent was removed in vacuo. Distillation of the residue gave the alcohol as a colourless liquid (19.21 g, 39%), b.p. 80.5-85°/15 mm. (lit.,[25] 90°/21 mm). Anal. calcd. for $C_5H_{10}O_2$: C, 58.80; H, 9.87). Found: C, 59.88; H, 10.09. $\bar{\nu}_{max}$ (liquid film) 3420-3360, 2945, 2855, and 1095 cm^{-1}. δ ($CDCl_3$) 1.13-2.33 (2H, m), 3.14-4.0 (5H, m). A single peak was obtained by g.l.c. (100°C).

Tetrahydropyran-3-one[26]

Jones reagent was prepared by adding water (42 ml) to chromium trioxide (22 g) and then adding conc. H_2SO_4 (19 ml). Any precipitated salts were dissolved with the minimum amount of water.

Tetrahydropyran-3-ol (9 g, 88.2 mmol) was dissolved in 400 ml of acetone. Jones' reagent was added dropwise to the vigorously stirred solution (mechanical overhead stirrer), so that the temperature did not rise above 35°C. The addition was continued until the characteristic brown colour of the reagent remained for $\sim$ 20 min. ($\sim$30 ml). The solution was decanted and the residual green solid was washed with acetone. The rinsings were added to the main solution. Anhydrous potassium carbonate was added to neutralise and dry the solution. After standing overnight the mixture was filtered and the cake washed with acetone. The solvent was removed by distillation and the residue was dissolved in ether

and dried (Na_2SO_4). The ether was removed by distillation and the residue was distilled to give the ketone as a colourless liquid, (5.15 g, 58%) b.p. 56-58°/13 mm (lit.,[26] 61°/15 mm). Anal. calcd. for $C_5H_8O_2$: C, 59.98; H, 8.05. Found: C, 58.87; H, 8.05. $\bar{\nu}_{max}$ (liquid film) 2955, 2855, 1720, and 1097 cm^{-1}. U.V. λ_{max} (MeOH) 298 nm. δ (CCl_4) 1.9-2.2 (2H, m), 2.35-2.6 (2H, m), 3.7-3.86 (2H, t), 3.88 (2H, s).

The rate of Horse liver alcohol dehydrogenase catalysed reduction of Tetrahydropyran-3-one compared to Cyclohexanone[27]

Assay mixture	Vol. used	Conc. in assay
1) 0.1 M KH_2PO_4 (pH 7.0 with NaOH)	2.5 ml	86 mM
2) a) 96 mM Cyclohexanone (94.14 mg in 10 ml H_2O)	0.1 ml	3.31 mM
b) 97 mM Tetrahydro pyran-3-one (96.9 mg in 10 ml H_2O)	0.1 ml	3.34 mM
3) 5 mM NADH (3.9 mg/ml H_2O)	0.1 ml	0.17 mM
4) HLADH (3.78 mg in 10 ml of 50 mM Tris-Cl pH 7.0)	0.1 ml	

Solutions 1, 2(a) or (b), and 3 were incubated at 25°C for 5 min. in a cuvette. The reaction was started by addition of solution 4. The rate of change of absorbance at 340 nm was noted. The rate of reduction of tetrahydropyran-3-one was 7.6% of that of cyclohexanone. There were, therefore, 0.25 U/mg of enzyme for this substrate.

Attempted Enzymatic Synthesis of Glucose-6-phosphate using Immobilised Enzymes (PAN) and recycling ATP[28]

A 1 L 3 neck flask equipped as before was charged with 400 ml of a solution containing glucose·H_2O (38.3 g, 193 mmol), adenosine triphosphate (0.8 g, 1.24 mmol), $MgCl_2.6H_2O$ (2.7 g, 13.5 mmol), and DTT (0.371 g, 2.4 mmol). After de-aerating the solution by bubbling N_2 through, PAN gel particles containing acetate kinase (160 U) and hexokinase (160 U) were added. The pH was automatically maintained at 7.4 using 4 M KOH. Diammonium acetyl phosphate (84% purity, 40.02 g, 193 mmol) was added in 9 equal portions over 3 days. The solution was analysed enzymatically for glucose-6-phosphate concentration, it was found to be 0.036 mol. The reaction was left stirring for a further 24 h. The glucose-6-phosphate concentration was 0.0395 mol and the glucose concentration was 0.138 mol (enzymatic assay). These concentrations accounted for 92% of the glucose added. It was suspected that the enzyme activities were lower than stated. The gel was separated from the solution by centrifugation and resuspended in 150 ml of 50 mM Hepes pH 7.5, 10 mM in $MgCl_2$ and 5 mM DTT. The activity of hexokinase was 39.3 u (23.6%) and of acetate kinase (26.1 u, 15.7%). From these results it was clear that the low conversion was not due to non-enzymic hydrolysis of glucose-6-phosphate, but to the low activity of the immobilised enzymes. The reaction should have gone to completion in $\sim$3 days using these conditions. The deterioration of the immobilised enzymes had taken place over 2 weeks under the optimum storage conditions.[14] A similar reaction was initiated using enzymes immobilised immediately beforehand.

After 4 days 50% of the acetyl phosphate had been added, the conversion to glucose-6-phosphate was 23%. This again was lower than expected and the reaction was terminated. These results have cast some doubt on the general usefulness of immobilised enzymes.

Enzymatic Synthesis of Glucose-6-phosphate using free Enzymes and recycling ATP

A 400 ml solution containing glucose·H_2O (38.3 g, 193 mmol) adenosine triphosphate (0.7 g, 1.08 mmol), and DTT (0.371 g, 2.4 mmol) was de-aerated and maintained under N_2. Hexokinase (10.26 mg, 320 U) and acetate kinase (0.4 ml of suspension in ammonium sulphate, 308 U) were added to this solution. Diammonium acetyl phosphate (84% purity, 40.02 g, 193 mmol) was added to the stirred reaction mix portionwise as outlined below. The pH was maintained at 7.4-7.8 by automatic addition of 4 M KOH. The reaction was performed at ambient temperature. The acetyl phosphate was added in approx. 4 g portions. By assaying glucose-6-phosphate formation it was estimated that this is used up in $\sim$3 h. The acetyl phosphate was thus added at 3-hourly intervals over a working day. After 4 days the conversion to glucose-6-phosphate was 60 $\pm$ 5%. The solution was separated from the gel by centrifugation. $BaCl_2.2H_2O$ (21.2 g, 0.086 mol) was added to precipitate inorganic phosphate (the difference between G-6-P formed and AcP added); this material was removed by centrifugation. To the supernatant was added $BaCl_2.2H_2O$ (24.4 g, 0.1 mol) followed by slow addition of ethanol (400 ml). The precipitated solid (42.1 g) contained 90% Ba (G-6-P).$7H_2O$ (0.0807 mol, 42% yield). This salt was stored desiccated at 5°C.

Reduction of Tetrahydropyran-3-one to tetrahydropyran-3-ol catalysed by HLADH; consuming Glucose-6-phosphate and recycling $NAD^{\oplus}$ 826 X

The barium salt of glucose-6-phosphate (11.6 g, 20 mmol) was stirred vigorously in 0.18 M sulphuric acid(111 ml)for 45 min. The bulk of the precipitate ($BaSO_4$) was removed by centrifugation, however, the solution was clarified by adding some filter aid and filtering. The filtrate was neutralised with sodium hydroxide. To this glucose-6-phosphate solution (185 ml), which had been de-aerated by passing N_2 through, was added tetrahydropyran-3-one (2 g, 20 mmol), $MgSO_4$ (76.8 mg, 0.4 mmol), $NAD^{\oplus}$ (12.6 mg, 18.3 μmol), horse liver alcohol dehydrogenase (56.6 mg, 14.15 U), and glucose-6-phosphate dehydrogenase (from leuconostoc mesenteroides, 0.36 mg, 22.4 U). The pH was maintained at 7.0-7.4 automatically with 4 M KOH. The reaction was conducted under N_2 with magnetic stirring in a 250 ml 3 neck flask covered in foil. The reduction was followed by g.l.c. and by enzymatic assay of glucose-6-phosphate concentration.

TIME (h)	EXTENT OF REDUCTION (%)	
	g.l.c.	assay
2	11.3	
4	17.8	
*24	39.4	30
48	69.0	61.4
72	86.3	79.6

* A further portion of $NAD^{\oplus}$ (4.04 mg, 5.9 μmol) and $MgSO_4$ (17.6 mg, 19 μmol) were added at this point. The reduction was complete

after 4.5 days. The mixture was centrifuged at 5°C to remove a light precipitate. The supernatant was saturated with sodium chloride and continuously extracted with ether (250 ml) for 3.5 days. After drying ($MgSO_4$) the solvent was removed in vacuo. Kugelrohr distillation of the residue gave the alcohol as a clear liquid (1.4 g, 68%). Anal. calcd. for $C_5H_{10}O_2$ C, 58.80; H, 9.87. Found: C, 60.42; H, 10.02. The spectral characteristics were consistent with the alcohol prepared previously. $[\alpha]_D^{24.5}$ = - 8.7 ± 1.4° (c. 3.39, $CHCl_3$). $[\alpha]_{365}^{29.25}$ = - 23.7 ± 1.4° (c. 3.39, $CHCl_3$).

Determination of Enantiomeric Excess

(-)-α-Methoxy-α-trifluoromethylphenylacetic acid chloride (MTPA-Cl) was prepared from the acid by the method of Mosher.[29] The colourless liquid was obtained in 56% yield. b.p. 45°C/0.5 mm (lit.,[29] 54-56/1 mm. $[\alpha]_D^{18.5}$ = - 129.2 (c. 4.76, CCl_4) [lit.,[29] $[\alpha]_D^{24}$ = - 129.0 ± 0.12 (c. 5.17, CCl_4)].

Chiral tetrahydropyran-3-ol (20.79 mg, 0.2 mmol) and MTPA-Cl (51.88 mg, 0.2 mmol) were mixed in 5 drops of carbon tetrachloride. Pyridine (5 drops) was added to the solution, a white precipitate formed immediately. The reaction was left shaking at room temperature overnight. 1 ml of water was added and the mixture was transferred to a separating funnel with ether. The organic phase was washed with 0.1 N HCl (5 ml), saturated sodium bicarbonate (2 ml), and then water (1 ml). After drying ($MgSO_4$) the solvent was removed in vacuo to give the ester which was not further purified. The racemic alcohol was treated in this manner to give the racemic ester for comparison. The enantiometric excess was

determined by 250 MHz ^1H n.m.r. ($CDCl_3$) examination of the diastereomeric methoxy protons in the presence of $Eu(fod)_3$ shift reagent. By integration of the signal the e.e. was found to be 49%.

Chiral 3-Tetrahydropyranyl p-bromobenzenesulphonate

Chiral tetrahydropyran-3-ol (319.5 mg, 3.13 mmol) and p-bromobenzenesulphonylchloride (879 mg, 3.44 mmol) were stirred together in 5 ml of dry pyridine at 5°C for 2 days. Ice water was added to hydrolyse the excess acid chloride; the whole was taken up in sufficient water and the aqueous phase was extracted with ether after adding sodium chloride. The organic phase was then washed with 1N HCl 5% sodium bicarbonate, and saturated brine. After drying ($MgSO_4$) the solvent was removed in vacuo to give an oily residue which spontaneously crystallised to a white solid. Recrystallisation from ether/petrol (40-60°) gave the brosylate (735 mg, 73%) m.p. 71-73°C. Anal. calcd. for $C_{11}H_{13}BrO_4S$: C, 41.13; H, 4.08. Found: C, 40.81; H, 4.05. $\bar{\nu}_{max}$ ($CHCl_3$) 3080, 3030, 2945, 2850, 1575, 1365 and 1088 cm^{-1}. δ ($CDCl_3$) 1.4-2.05 (4H, m), 3.59 (4H, m), 4.55 (1H, m), and 7.75 (4H, m, AA'BB'). $[\alpha]_{365}^{25.5} = -10.3°$ (c. 2.46, $CHCl_3$).

Chiral 3-Tetrahydropyranyl acetate

Chiral tetrahydropyran-3-ol (308.5 mg, 3.02 mmol) and acetic anhydride (339.4 mg, 3.3 mmol) were combined in 1 ml of dry pyridine and stirred at 5°C for 2 days. Work up as usual gave the acetate as a colourless liquid after Kugelrhor distillation (366.8 mg, 84%). Anal. Calcd. for $C_7H_{12}O_3$: C, 58.32; H, 8.39. Found: C, 57.13;

H, 8.32. $\bar{\nu}_{max}$ ($CHCl_3$) 2945, 2848, 1723, 1245-1200, and 1090 cm^{-1}. δ ($CDCl_3$) 1.45-2.05 (4H, m), 2.09 (3H, s), 3.63 (4H, m), and 4.8 (1H, m). $[\alpha]_{365}^{25.5^\circ} = -24.7^\circ$ (c. 1.64, $CHCl_3$).

Acetolysis of Chiral 3-Tetrahydropyranyl p-bromobenzenesulphonate

Acetic acid was dried by refluxing overnight with 5% (by volume) acetic anhydride and 1% (by weight) chromium trioxide. It was then distilled in a dry atmosphere.

Sodium acetate (A.R.) was dried in an oven at ~ 200°C for 2 h and allowed to cool in a desiccator. A quantity of sodium acetate was dissolved in acetic acid to give a 0.04 M solution. The solution was standardised by titrating with a perchloric acid solution to the methyl violet end point. The perchloric acid solution had been standardised using a potassium hydrogen phthalate solution. The molarity of the sodium acetate solution was 0.0384 M.

Polarimetric procedure: The chiral brosylate (~100 mg) was weighed into a 10 ml volumetric flask and brought to volume with the sodium acetate solution. This solution was then transferred to a 1 ml jacketed polarimeter tube (Hg lamp, 365 nm). An initial reading was made after 5 min to allow thermal equilibration. Subsequent readings were taken at suitable intervals. The remaining 9 ml of solution were transferred into a glass tube which was sealed in vacuo. After heating at 105°C for 22 h the product was extracted. It was shown to be racemic. The acetolysis was followed polarimetrically at the following temperatures, 79.26, 81.43, 82.12 and 89.93°C. The results are given below.

	Temperature	Time(s)	obs. Rotation	ln. absolute value of observed rotation
1.	79.26°C	0	- 0.123	- 2.096
		5,390	- 0.107	- 2.235
		10,890	- 0.096	- 2.743
		14,250	- 0.090	- 2.408
		20,430	- 0.081	- 2.513
		25,910	- 0.77	- 2.564
		32,070	- 0.70	- 2.660
		42,690	- 0.061	- 2.797
		47,220	- 0.052	- 2.865
2.	81.43°C	0	- 0.116	- 2.156
		5,340	- 0.091	- 2,397
		11,310	- 0.074	- 2.604
		16,200	- 0.063	- 2.765
		21,060	- 0.062	- 2.781
		27,300	- 0.052	- 2.956
		33, 480	- 0.047	- 3.058
		48, 960	- 0.036	- 3.324
3.	87.12°C	0	- 0.124	- 2.088
		5,340	- 0.101	- 2.293
		10,980	- 0.085	- 2.465
		16,860	- 0.073	- 2.617
		21,900	- 0.068	- 2.688
		25,580	- 0.066	- 2.718
		32,160	- 0.059	- 2.830
		37,320	- 0.053	- 2.938
4.	89.93°C	0	- 0.133	- 2.017
		2,220	- 0.110	- 2.207
		4,080	- 0.094	- 2.364
		6,900	- 0.076	- 2.577
		8,700	- 0.075	- 2.696
		11,400	- 0.055	- 2.900
		14,940	- 0.042	- 3.170
		20, 340	- 0.027	- 3.612
		21,180	- 0.024	- 3.709

With the exception of the experiment conducted at 89.93°C a plot of ln. observed value of rotation vs. time was initially curved (over approx. 3 h), after which time the plot was approximately linear. It was suspected that this behaviour was being caused by the presence of some water. Despite efforts to rigorously exclude moisture this effect was always observed. Examination of

the products extracted from the sealed tube experiments by g.l.c. (100°C) gave peaks with retention times corresponding to the alcohol and the acetate, the ratio of each being 1:2.5 respectively. The identity of these peaks was confirmed by linked g.c./mass spectrometry. The fragmentation patterns were as expected.[30] The 1st order rate constants (k) were estimated from the linear portion of the curve. An Arhennius plot of ln k vs. $\frac{1}{T}$ was satisfactory.

RUN	$k \times 10^5$	$\frac{1}{T} \times 10^3$	ln k
1	1.41	12.62	- 11.17
2	1.73	12.28	- 10.96
3	1.73	12.17	- 10.96
4	7.86	11.11	- 9.45
ref. 30	2.47	11.78	- 10.61

The results concur with a titrimetrically determined rate constant, 84.9°C, $k = 2.47 \times 10^{-5}$.[31]

The chiral acetate was shown to be stable to heating at 105°C for 18 h in a sealed tube. The observed rotations before and after were - 0.275 and-0.264 (Hg, 365 nm, in sodium acetate/acetic acid solution).

(S)-Valinol[32]

A dry, 100 ml 3 neck flask equipped with a condenser, N_2 inlet/outlet, mechanical stirrer, and a pressure equalising dropping funnel was charged with 10 g (85.4 mmol) of (S)-valine and 30 ml of dry tetrahydrofuran. Boron trifluoride diethyl etherate (11.66 ml, 94 mmol), freshly distilled from calcium hydride, was then added dropwise with stirring. When the addition was complete the reaction mixture was refluxed until dissolution

obtained. Gentle heating was then continued as 12.8 ml (128 mmol) of borane dimethylsulphide was added at a rate sufficient to maintain gentle reflux. The reaction mixture was then refluxed gently overnight after which a mixture of tetrahydrofuran and water (30 ml and 5 ml respectively) was added followed by 45 ml of 6 N sodium hydroxide. After refluxing for a further 5 h the reaction was cooled and the clear upper organic layer was separated. The aqueous phase was extracted with tetrahydrofuran (3 x 30 ml). The organic phases were combined and dried over anhydrous potassium carbonate. The solvent was removed in vacuo to give the amino alcohol as a colourless liquid (7.54 g, 86%) b.p. 76-78°/7 mm (lit.,[33] 75°/8 mm). $\bar{\nu}_{max}$ ($CHCl_3$) 3650, 3480, 2930, 2870, 1585, 1460, and 997 cm^{-1}. δ ($CDCl_3$) 0.93 [6H, d, $(CH_3)_2$ J = 6.6Hz], 1.3-1.8 [1H, m, $(CH_3)_2$ CH], 2.5 (3H, s, NH_2, OH), 2.55-2.70 (1H, m, NCH), and an ABX system 3.16-3.75 (2H, dq, CH_2OH). J_{BX} = 3. 6 Hz, J_{AX} = 8 Hz and J_{AB} = 9 Hz. $[\alpha]_D^{24.5}$ = - 27.6 (c. 9.38, EtOH) [lit.,[33] $[\alpha]_D^{20}$ = - 15.0 (c. 6.1, EtOH)]. Purity by t.l.c.; Whatmans silica gel SG 41, n-propanol : NH_4OH : acetone; 7:3:1, one spot r.f. 0.603.

The following amino-alcohols were prepared by the above method.

(S)-Phenylalaninol

(S)-phenylalanine (10 g, 60.5 mmol) gave a white solid on removal of solvent in vacuo. Recrystallisation from ethanol/petrol (60-80°) gave the amino-alcohol as white needles (6.69 g, 72%) m.p. 92.5-94.5°. (lit.,[34] 85-86°). $\bar{\nu}_{max}$ ($CHCl_3$) 3630, 3370, 2990, 2930, 2850, 1580, 1490, 1452, and 1025 cm^{-1}. δ ($CDCl_3$) 2.05 (1H, s, NH_2, OH), ABX system 2.39-2.95 (2H, dq, Ar-CH_2) J_{BX} = 4.8 Hz, J_{AX} = 8.4 Hz, and

J_{AB} = 3.2 Hz, J_{AX} = 6 Hz, and J_{AB} = 10 Hz, 7.25 (5H, m, ArH). $[\alpha]_D^{23.5}$ = - 22.7 (c. 1.58, EtOH) [lit.,[35] $[\alpha]_D$ =-25.3 (c. 1.4, EtOH)]. T.l.c.; one spot, r.f. 0.598.

(S)-Leucinol

(S)-Leucine (10 g, 76.2 mmol) gave 2.8 g (31.3%) of S-leucinol. b.p. 57-59°/0.5 mm (lit.,[34] 95-100°/10 mm). $\bar{\nu}_{max}$ ($CHCl_3$) 3630, 3370, 2950, 2930, 2870, 1580, 1460, 1365 and 1035 cm^{-1}. δ ($CDCl_3$) 0.93 (6H, 2d, $(CH_3)_2$), 1.1-1.4 (2H, m, CH_2), 1.5-1.93 (1H, m, $(CH_3)_2CH$), 2.38 (3H, s, NH_2, OH), 2.75-3.05 (1H, m, NCH) and an ABX system 3.1-3.75 (2H, 2q, CH_2O) J_{BX} = 3.2 Hz, J_{AX} = 7.4 Hz, and J_{AB} = 10.2 Hz. $[\alpha]_D^{24.0}$ = + 63.25 (c.5.22 EtOH) [lit.,[34] $[\alpha]_D^{18}$ = + 4.2 (c. 9 .0, EtOH)]. T.l.c.; one spot, r.f. 0.565.

(S)-Alaninol

(S)-Alanine (10 g, 112.3 mmol) gave 2 g (24%) S-alaninol. b.p. 64-66°/7 mm (lit.,[34] 78-80°/12 mm). $\bar{\nu}_{max}$ ($CHCl_3$) 3640, 3365, 2920, 2860, 1580, 1453 and 1030 cm^{-1}. δ ($CDCl_3$) 1.04 (3H, d, CH_3, J = 6 Hz) 2.83 (3H, s, NH_2, OH) 2.86-3.08 (1H, m, NCH), and an ABX system 3.1-3.64 (2H, 2q, CH_2O) J_{BX} = 3.6 Hz, J_{AX} = 7.2 Hz, and J_{AB} = 10 Hz. $[\alpha]_D^{24.5}$ = + 22.3 (c. 3.9 EtOH) [lit.,[34] $[\alpha]_D^{17}$ = + 20.1 (EtOH)].

Ethyliminoacetate hydrochloride[36]

Dry hydrogen chloride was passed through a solution of acetonitrile (100 g, 2.44 mol) in ethanol (113 g, 2.5 mol) which was cooled in an ice/salt bath. The hydrogen chloride was generated by adding conc. HCl (400 ml), via a capillary tube, into conc. H_2SO_4 (400 ml). This theoretically generates 120 g of hydrogen chloride.[37] After the acids had been added to each other the reaction mixture had set to a white solid. It was left standing at room temperature

for 3 days. Recrystallisation from dichloromethane/ether gave the imino ether (170 g, 56%) m.p. 107-108°. (lit.,[38] 107-108°). δ ($CDCl_3$) 1.63-1.39 (3H, t), 2.51 (3H, s), and 4.53-4.80 (2H, q).

Trans-(4S,5S)-4-hydroxymethyl-2-methyl-5-phenyl-2-oxazoline[38]

(1S,2S)-1-phenyl-2-amino-1,3-propanediol (25 g, 149.5 mmol) was added in one portion to a solution of ethylimino acetate hydrochloride (20.76 g, 168.8 mmol) in dry dichloromethane (120 ml) at 0°C. After stirring for 4 h at 0°C the mixture was left overnight at room temperature and then poured into ice water (150 ml). The organic layer was separated and the aqueous layer was extracted with 2 x 80 ml of dichloromethane. After drying over magnesium sulphate the solvent was removed in vacuo to give an oil which solidified on standing. Recrystallisation from ether (150 ml) by cooling to - 78°C gave the oxazoline (17.56 g, 61%). m.p. 62-65° (lit.,[38] 64-65°). $\bar{\nu}_{max}$ ($CHCl_3$) 1665 cm^{-1}. δ ($CDCl_3$) 2.07 (3H, d, CH_3), 3.5-4.1 (2H, m), 4.45-5.0 (1H, s, OH), 5.35 (1H, d), and 7.3 (5H, s, ArH). $[\alpha]_D^{20} = -148.7$ (c. 4.0, $CHCl_3$) [lit.,[38] $[\alpha]_D^{24} = -174.6$ (c. 10.5, $CHCl_3$)].

(S)-1-Hydroxy-1-phenyl ethanamide[39]

(S)-Mandelic acid (16.7 g, 100 mmol) was dissolved in 125 ml of methanol and cooled to 0°C. Acetyl chloride (6 ml) was added and the mixture was stirred overnight at room temperature. The solvent was removed in vacuo and the residue was redissolved in 100 ml of methanol and cooled to 0°C. Dry ammonia was passed through the solution which was then left for 2 days at 5°C. The solvent was removed in vacuo and the residue was recrystallised from ethanol to give the amide (12.22 g, 96%) m.p. 125-126.5° (lit.,[39] 121°.

$[\alpha]_D^{20}$ + 72.3 (c. 1.55, acetone) [lit.,[39] $[\alpha]_D^{24}$ = + 76.4 (c. 1.7, acetone)].

(S)-2-Amino-1-phenylethanol[39]

Lithium aluminium hydride (8 g) was suspended in tetrahydrofuran (180 ml) in a 500 ml 3 neck flask equipped with a condenser and a mechanical stirrer. (S)-1-Hydroxy-1-phenylethanamide (12.22 g, 81 mmol) dissolved in 150 ml of tetrahydrofuran was added slowly. After being refluxed for 6 h the mixture was allowed to stir overnight at room temperature after which water (8 ml), 40% sodium hydroxide (3.5 ml), and water (31 ml) were added. After stirring for 1 h the mixture was filtered through Celite. The cake was extracted with hot tetrahydrofuran, and after combining with the filtrate and drying over anhydrous potassium carbonate the solvent was removed *in vacuo*. The residue was recrystallised from ether to give the amide (2.46 g, 22%) m.p. 60.5-62.5° (lit.,[39] 55-57°) δ ($CDCl_3$) 2.65-3.1 (5H, m), 4.58 (1H, t), and 7.30 (5H, s). $[\alpha]_D^{22}$ = + 45.1 (c. 2.6, EtOH) [lit.,[39] $[\alpha]_D^{23}$ = + 47.9 (c. 2.4, EtOH)].

(+)-Norephedrine

(+)-Norephedrine hydrochloride (3 g, 16 mmol) was suspended in chloroform (50 ml). The mixture was cooled in an ice salt bath and a stream of dry ammonia was bubbled through. After filtration the solvent was removed *in vacuo* to give the amine (2.37 g, 98%). $\bar{\nu}_{max}$ ($CHCl_3$) 3590, 3360, 2950, 2920, 2865, 1595, 1580, 1445 and 1370 cm^{-1}. δ ($CDCl_3$) 0.95 (3H, d), 1.96 (3H, s), 3.13 (1H, m), 4.46 (2H, d), and 7.3 (5H, s). $[\alpha]_D^{22.5}$ = + 26.1 (c. 2.89, $CHCl_3$).

(S)-Phenylalaninol-borane

A 25 ml 2 neck flask equipped with a magnetic stirring bar, a N_2 inlet/outlet and a pressure equalising dropping funnel was charged with (S)-phenylalaninol (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. Yield not recorded. $\tilde{\nu}_{max}$ ($CHCl_3$) 3620, 3300, 3260, 2940, 2440, 1580 and 1160 cm^{-1}. $[\alpha]_D^{18}$ = - 14.6 (c. 0.7, $CHCl_3$). T.l.c.; Whatmans silica gel SG41 , n-propanol: NH_4OH: acetone; 7:3:1, one spot, r.f. 0.507.

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

(S)-Valinol-borane

1 g (9.69 mmol) of (S)-valinol gave 1.346 g of a viscous liquid. $\tilde{\nu}_{max}$ ($CHCl_3$) 3620, 3320, 3260, 2960, 2870, 2310, 1575, 1460, and 1160 cm^{-1}. $[\alpha]_D^{18}$ = + 10.5 (c. 2.39, $CHCl_3$). T.l.c., one spot, r.f. 0.34.

(S)-Leucinol-borane

0.848 g (6.75 mmol) of (S)-leucinol yielded 0.895 g of a colourless viscous liquid. $\tilde{\nu}_{max}$ ($CHCl_3$) 3620, 3315, 3260, 2950, 2870, 2340, 1580, 1460, 1365, and 1160 cm^{-1}. $[\alpha]_D^{18}$ = + 15.4 (c. 1.99, $CHCl_3$). T.l.c., one spot, r.f. 0.48.

(S)-Alaninol-borane

0.789 g (10.52 mmol) of (S)-alaninol yielded 1.05 g of a viscous liquid. $\bar{\nu}_{max}$ (liquid film) 3450, 3230, 3140, 2970, 2935, 2870, 2320, 1590, and 1165 cm^{-1}. $[\alpha]_D^{18}$ = + 25.8 (c. 2.23, tetrahydrofuran). T.l.c., one spot, r.f. 0.39.

(S)-Prolinol-borane

1.025 g (10.13 mmol) of (S)-prolinol gave 1.17 g of a viscous liquid. $\bar{\nu}_{max}$ ($CHCl_3$) 3620, 3245, 2955, 2880, 2300, 1455, 1395, 1153, and 1066 cm^{-1}. $[\alpha]_D^{18}$ = - 12.9 (c. 1.9, $CHCl_3$). T.l.c., one spot, r.f. 0.66.

Trans-(4S,5S)-4-hydroxymethyl-2-methyl-5-phenyl-2-oxazoline-borane

1 g (5.22 mmol) of the oxazoline gave 1.128 g of a solid after trituration with petrol (40-60°). $\bar{\nu}_{max}$ (liquid film) 2320, and 1660 cm^{-1} $[\alpha]_D^{20}$ = - 144.1 (c. 2.25, tetrahydrofuran). Reaction of 1g (5.22 mmol) of the oxazoline with 2 mol.equivalents of borane yielded a white amorphous solid, 1.11 g. $\bar{\nu}_{max}$ (KBr) 2320 and 1655 cm^{-1}. $[\alpha]_D^{20}$ = - 132.1 (c. 2.28, tetrahydrofuran).

(S)-2-Amino-1-phenyl ethanol-borane

0.5 g (3.65 mmol) of (S)-2-amino-1-phenyl ethanol gave 0.69 g of a white solid. $\bar{\nu}_{max}$ ($CHCl_3$), 3590, 3505, 3390, 3310, 3260, 3220, 2980, 2960, 2870, 2310, 1587, 1395, 1160, and 1050 cm^{-1}. $[\alpha]_D^{22}$ = + 75.6 (c. 1.90, $CHCl_3$).

(+)-Norephedrine-borane

1 g (6.6 mmol) of (+)-norephedrine gave 0.89 g of a white solid. $\bar{\nu}_{max}$ ($CHCl_3$) 3590, 3305, 3235, 2965, 2930, 2860, 2335, 1580 and 1325 cm^{-1}. $[\alpha]_D^{22}$ = + 37.9 (c. 0.86, $CHCl_3$).

(+)-Ephedrine-borane

1 g (6.05 mmol) of (+)-ephedrine yielded 1.34 g of a viscous liquid. $\bar{\nu}_{max}(CHCl_3)$ 3595, 3480, 3250, 2970, 2940, 2870, 2330, 1450, 1380, 1155, and 1050 cm^{-1}. $[\alpha]_D^{20}$ = + 24.0 (c. 2.92, $CHCl_3$).

(-)-Ephedrine-borane

1 g (6.05 mmol) of (-)-ephedrine gave 1.19 g of a viscous liquid. $\bar{\nu}_{max}$ ($CHCl_3$) 3595, 3480, 3250, 2970, 2940, 2870, 2330, 1450, 1380, 1155, and 1050 cm^{-1}. $[\alpha]_D^{20}$ = - 22.9 (c. 2.86, $CHCl_3$).

Reduction of Dihydrofolate using Alkoxy-amine-borane complexes

5 mg of dihydrofolic acid was dissolved in the minimum volume of 0.1 N NaOH, the pH was adjusted to 7.0 (paper) 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 N_2 until completion (HPLC). An excess of the hydride reagent was used since competing solvolysis was expected. The amount of the reagent which gave the best induction was lowered to ascertain whether any improvement could be obtained. The isomeric excess was determined as described earlier.

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

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

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

(D) 20 mM Tris-Cl, pH 8.0, containing 10 mM DTT.

(S)-Valinol-borane (55.5 mg) gave > 98% reduction after 5 days using system (A) and an isomeric excess of 1.8% (6R).

(S)-Phenylalaninol-borane (33.3 mg) gave > 98% reduction after 5 days using system (A) and an isomeric excess of 9.8% (6S).

(S)-Alaninol-borane (37.8 mg) gave > 97% reduction after 5 days in system (A) and an isomeric excess of 0.8% (R).

(S)-Prolinol-borane (30.6 mg) gave 84% reduction after 5 days in system (A) and an isomeric excess of 9.8% (6S).

(S)-Leucinol-borane (26.5 mg) gave ~ 30% reduction after 5 days in system (A). The isomeric excess was not determined.

Chiral oxazoline-borane (1:1, 4.8 mg) gave 100% reduction after 3 h in system (B) and an isomeric excess of 11.8% (6R).

Chiral oxazoline borane (1:2, 35.6 mg) gave 100% reduction after 23 h in system (B) and an isomeric excess of 12.6% (6R). 9.73 mg of this reagent in system (C) gave 100% reduction after 5 days and an isomeric excess of 14% (6R). 5.22 mg of this reagent in system (C) gave > 90% reduction after 3 days and an isomeric excess of 18% (6R).

(S)-2-Amino-1-phenylethanol-borane (23.54 mg) in system (B) gave 6% reduction.

(+)-Norephedrine-borane (21.97 mg) in system (B) gave 86% reduction after 47 h and an isomeric excess of 5.2% (6R).

(+)-Ephedrine-borane (39.6 mg) in system (B) and 27.92 mg in system (C) did not effect any reduction.

(-)- Ephedrine-borane (42.7 mg) in system (B) and 34.35 mg in system (C) did not effect any reduction.

N-Benzyloxycarbonyl-(S)-Amino Acids[40,41]

(S)-Alanine (3 g, 33.7 mmol) was dissolved in 2 N sodium hydroxide (16.85 ml) in a 100 ml 3 neck flask equipped with a mechanical stirrer. The solution was cooled to 5°C and 2N sodium hydroxide (20.22 ml) and benzylchloroformate (5.73 g, 33.7 mmol) were added simultaneously in 5 equal portions over 35 min. with vigorous stirring. The mixture was stirred for a further 1.5 h at room temperature. After extraction with ether (20 ml) the aqueous solution was cooled in an ice bath and acidified carefully with 5N HCl to congo red. The resulting oily white precipitate was extracted with ethyl acetate (3 x 20 ml). After drying over magnesium sulphate the solvent was removed *in vacuo*. The residue was recrystallised from ether/petrol (40-60°) to give the N-acyl amino acid, 2.36 g, 31% m.p. 85-87° (lit.,[42] 84°). $[\alpha]_D^{27}$ = - 14.1 (c. 1.56, acetic acid) [lit.,[42] $[\alpha]_D$ = - 14.3 (acetic acid)].

The following N-benzyloxycarbonyl-(S)-amino acids were prepared. Any deviations from the above procedure are noted.

(S)-Serine (2 g, 19 mmol) gave, after recrystallisation from ethyl acetate, 1.68 g, 37% of the N-acyl derivative m.p. 120.5-121° (lit.,[42] 117-119°). $[\alpha]_D^{27}$ = + 5.5 (c. 4.32, acetic acid) [lit.,[42] $[\alpha]_D$ = + 5.8 (acetic acid)].

(S)-Valine (3 g, 25.6 mmol) was reacted with benzylchloroformate in the above manner. However, after acidifying and extracting with ethyl acetate, this organic solution was extracted with ice cold 7% sodium bicarbonate solution. After acidifying, with 5 N HCl to congo red the oil obtained crystallised on standing overnight in the cold. The crystals were washed with water and dried *in vacuo* over

phosphorus pentoxide. Recrystallisation from benzene/petrol (60-80°) gave the N-acyl derivative 3.68 g, 57% m.p. 60-62.5° (lit.,[43] 60-62°). $[\alpha]_D^{25}$ = zero, (c. 5.0, ethanol) [lit.,[43] $[\alpha]_D^{25}$ = + 1.5 (c.5, ethanol)].

(S)-Phenylalanine (3.3 g, 20 mmol) gave, after recrystallisation from ethyl acetate/60-80° petrol, 2.83 g (47.3%) of the N-acyl derivative, m.p. 85-87° (lit.,[44] 88-89°). $[\alpha]_D^{23}$ = + 6.3 (c. 2.0, ethanol) [lit.,[44], $[\alpha]_D^{20}$ = + 5.1 (c. 2.0, ethanol)].

(S)-Proline (2.3 g, 20 mmol) gave an oil on removal of ethyl acetate in vacuo. This was induced to crystallise by triturating with 40-60° petrol and standing at - 20° overnight. The white solid obtained was recrystallised from ether/40-60° petrol to give the N-acyl derivative, 2.6 g (52%), m.p. 73-75° (lit.,[45] 76-77°). $[\alpha]_D^{23}$ = - 79.7 (c. 4.3, acetic acid), [lit.,[45] $[\alpha]_D^{20}$ = - 61.7 (c. 5.3, acetic acid)].

Preparation of Chiral Sodium triacyloxyborohydrides by reaction of sodium borohydride with N-benzyloxycarbonyl-(S)-amino acids.[46]

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). $\bar{\nu}_{max}$ (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). $\bar{\nu}_{max}$ 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). $\bar{\nu}_{max}$ 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). $\bar{\nu}_{max}$ 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). $\bar{\nu}_{max}$ 2540 cm^{-1}.

Attempted Reduction of Dihydrofolate using Chiral Triacyloxyborohydride

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, after 3 days. HCLP analysis of isomeric excess indicated no chiral induction was achieved.

Sodium borohydride reduction of Dihydrofolate in the presence of Bovine Serum Albumin

To 2 ml of a solution containing dihydrofolate (5.63 mM) and DTT (10 mM) in 20 mM Tris-Cl, pH 8.0, was added bovine serum albumin to give a concentration of 0.5, 1, 1.5, or 2.0 mM (66, 132, 198, and 264 mg). Sodium borohydride (11.28 g) was dissolved in 4 ml of buffer and 100 µl was added to each reaction at 5 min. intervals. Extra hydride (0.43 mg) was added to each to achieve complete reduction. Excess hydride was destroyed with 0.1 N HCl and the reactions were analysed for asymmetric induction. None had been achieved.

REFERENCES

REFERENCES

1. H.U. Bergmeyer 'Methods of Enzymatic Analysis', Academic Press, New York, 1974.
2. W.H. Pirkle and M.S. Hoekstra, J. Org. Chem., 1974, 39, 3404.
3. C. Temple, R.D. Elliot, J.D. Rose, and J.A. Montgomery, 'Chemistry and Biology of Pteridines', eds. R.L. Kisluik and G.M. Brown, Elsevier North Holland, New York, 1979.
4. S. Futterman, J. Biol. Chem., 1957, 228, 1031. R.L. Blakley, Nature, 1960, 188, 231.
5. C.K. Mathews and F.M. Huennekens, J. Biol. Chem., 1963, 238, 3436.
6. C.K. Mathews and F.M. Huennekens, J. Biol. Chem., 1960, 235, 3304.
7. C. Temple, R.D. Elliot, J.D. Rose, and J.A. Montgomery, J. Med. Chem., 1979, 22, 731.
8. F.M. Huennekens, P.P.K. Ho, and K.G. Scrimgeour 'Methods in Enzymology', eds. S.P. Colowick and N.O. Kaplan, Academic Press, New York, Vol. 6, 1963.
9. F.O. Rice, J. Greenberg, C.E. Waters, and R.E. Vollrath, J. Amer. Chem. Soc., 1934, 56, 1760.
10. 'Standard Methods of Chemical Analysis', eds. C.R.N. Strouts, H.N. Wilson, and R.T. Parry-Jones Oxford University Press, 1962, Vol. 1.
11. J.M. Lewis, S.L. Haynie, and G.M. Whitesides, J. Org. Chem., 1979, 44, 864. G.M. Whitesides, M. Siegel, and P. Garrett, J. Org. Chem., 1975, 40, 2516.
12. N.D. Danielson and J.A. Huth, J. Chromatogr., 1980, 221, 39.
13. 'Data for Biochemical Research' eds., R.M.C. Dawson, D.C. Elliot, W.H. Elliot, and K.M. Jones, Oxford University Press, 1969, 2nd Ed.

14. A. Pollack, H. Blumenfeld, M. Wax, R.L. Baughn, and G.M. Whitesides, J. Amer. Chem. Soc., 1980, 102, 6324.

15. J. Porath and R. Axen, 'Methods in Enzymology', ed., K. Mosbach, Academic Press, 1976, Vol. 44.

16. 'Affinity Chromatography - Principles and Methods', Pharmacia Fine Chemicals, Uppsala, Sweden, 1976.

17. A.C. Johansson and K. Mosbach, Biochem. Biophys. Acta, 1974, 370, 339.

18. K. Rufenacht, Helvetica Chimica Acta, 1976, 56, 2186.

19. C.S. Marvel, S.L. Jacobs, W.K. Taft, and B.G. Labbe, J. Polymer Sci., 1956, 19, 59.

20. German Patent 856, 293: Chem. Abs., 1956, 50, p 1896a.

21. B.R. Baker, M.W. Querry, S.J. Sajir, and S. Bernstein, J. Org. Chem., 1947, 12, 138.

22. H. Sokol and J.J. Ritter, J. Amer. Chem. Soc., 1948, 70, 3517.

23. P. Klason and T. Carlson, Chem. Ber., 1906, 39, 736.

24. J.C. Fontecilla-Camps, C.E. Bugg, C. Temple, J.D. Rose, J.A. Montgomery, and R.L. Kisluik, 'Chemistry and Biology of Pteridines', eds., R.L. Kisluik and G.M. Brown, Elsevier North Holland, New York, 1979.

25. G. Zweifel and J. Plamondon, J. Org. Chem., 1970, 35, 898.

26. J. Gore and F. Guigues, Bull. Soc. Chim. Fr., 1970, 3521.

27. J.B. Jones and J. Davies, J. Amer. Chem. Soc., 1979, 101, 5405.

28. A. Pollack, R.L. Baughn, and G.M. Whitesides, J. Amer. Chem. Soc., 1977, 99, 2366.

29. J.A. Dale, D.L. Dull, and H.S. Mosher, J. Org. Chem., 1969, 34, 2543.

30. H. Budzikiewicz and L. Grotjahn, Tetrahedron, 1972, 28, 1881.

31. D.S. Tarbell and J.R. Hazen, J. Amer. Chem. Soc., 1969, 91, 7657.

32. C.F. Lane, U.S. Patent, 3,935, 280; Chem. Abs., 1976, 84, p 135101p.

33. Y. Kawakami, T. Sugiura, Y. Mizutani, and Y. Yamashita, J. Polymer Sci. Pol. Chem. Ed., 1980, 18, 3009.

34. P. Karrer, P. Portmann, and M. Suter, Helvetica Chimica Acta, 1948, 31, 1617.

35. A.I. Meyers, D.R.Williams, and M. Drudinger, J. Amer. Chem. Soc., 1976, 98, 3032.

36. A.W. Dox, 'Org. Syn'., Coll Vol. 1, ed., H. Gilman, Wiley, London, 1932.

37. O.R. Sweeney, J. Amer. Chem. Soc., 1917, 2187.

38. A.I. Meyers, G. Knaus, K. Kamata, and M.S. Ford, J. Amer. Chem. Soc., 1976, 98, 567.

39. A.I. Meyers and J. Slade, J. Org. Chem., 1980, 45, 2785.

40. H.E. Carter, R.L. Frank, and H.W. Johnston, 'Org. Syn'., Coll. Vol. 3, Wiley, London.

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42. M. Bergman and C. Zervas, Chem. Ber., 1932, 65, 1192.

43. H. Schwarz, F. Merlin Bumpus, and I.H. Page, J. Amer. Chem. Soc., 1957, 79, 5697.

44. W. Grassman and E. Wünsch, Chem. Ber., 1956, 91, 449, 462. W. Grassman, E. Wunsch, and A. Reidel, ibid., 1958, 93, 455.

45. A. Berger, J. Kurtz, and E. Katchalski, J. Amer. Chem. Soc., 1954, 76, 5552.

46. K. Yamada, M. Takeda, and T. Iwakuma, Tetrahedron Lett., 1981, 22, 3869.