AN INVESTIGATION OF ENZYME MECHANISMS USING SUBSTRATE ANALOGUES

A THESIS SUBMITTED TO THE
BOARD OF THE FACULTY OF PHYSICAL SCIENCES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
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UNIVERSITY OF OXFORD

BY

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THE DYSON PERRINS
LABORATORY
DECEMBER 1980

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TO MY PARENTS

'Ohne Phosphor, kein Gedanke'.

Ludwig Büchner (1824-1899)

'Chiral phosphates.....the chemical equivalent of putting a man on the moon'.

Irwin Rose (1926-)

'God is not dead. He is alive and well and working on a much less ambitious project'.

Anonymous

'Progress was all right, it only went on too long'.

James Thurber (1894-1961)

'The habit of analysis has a tendency to wear away the feelings'.

J.S. Mill (1806-1873)

'You cannot have the success without the failures'.

H.G. Hasler (1914-)

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Dr. E.A. Barnard, Dept. Biochemistry, Imperial College, London, for arranging for the gel electrophoresis on Sigma hexokinase to be performed in his laboratory.

NOTE:

(a) Throughout this thesis the Cahn-Ingold-Prelog *RS* nomenclature[412,413] has been employed to describe absolute configuration and where necessary some literature assignments using the *DL* convention have been altered accordingly.

(b) An important section concerning the recently revised absolute isotopic chirality of [^{16}O, ^{17}O, ^{18}O]-phosphate monoesters prepared by our method is dealt with in the APPENDIX at the end of this thesis. All the assignments in the thesis, however, are correct.

Results from this thesis have been published as follows:

G. Lowe, B.V.L. Potter, B.S. Sproat and W.E. Hull, The Effect of ^{17}O and the Magnitude of the ^{18}O Isotope Shift in ^{31}P Nuclear Magnetic Resonance Spectroscopy, *J. Chem. Soc. Chem. Comm.*, (1979), 733.

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G. Lowe and B.V.L. Potter, The Stereochemistry of Yeast Hexokinase Catalysed Phosphoryl Transfer using Adenosine-5'[γ(*S*)-^{16}O, ^{17}O, ^{18}O]-Triphosphate, *Eur. J. Biochem.*, (1981), submitted for publication.

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APPENDIX

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PUBLICATIONS

ABBREVIATIONS

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-phosphate
cAMP	adenosine-3',5'-cyclic phosphate
ATP	adenosine-5'-triphosphate
BuOK	potassium-*t*-butoxide
CD	circular dichroism
DCCI	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
DEAE	diethylaminoethyl
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DPPC	diphenylphosphorochloridate
EDTA	ethylenediamine-N,N'-tetraacetic acid
HMPA	hexamethylphosphoramide
i.r.	infrared
MDCA	4-morpholine-N,N'-dicyclohexylcarboxamidine
NADH	β-nicotinamide adenine dinucleotide (reduced form)
NADP	β-nicotinamide adenine dinucleotide-2'-phosphate
n.m.r.	nuclear magnetic resonance
ORD	optical rotatory dispersion
PEP	phosphoenol pyruvate
p.l.c.	preparative layer chromatography
TEAB	triethylamine bicarbonate
TFA	trifluoroacetic acid
t.l.c.	thin layer chromatography
TMP	trimethyl phosphate
TMS	tetramethylsilane
TSS	sodium-2,2-dimethyl-2-silapentane-5-sulphonate
u.v.	ultraviolet

In diagrams throughout this thesis the following notation has been used for depicting isotopic labelling.

$^{18}O \equiv \bullet$

$^{17}O \equiv \oplus$

$^{16}O \equiv O$

An Investigation of Enzyme Mechanisms using Substrate Analogues

Barry V.L. Potter, Wolfson and Worcester Colleges, Oxford, submitted for the degree of Doctor of Philosophy, Michaelmas term, 1980.

ABSTRACT

The synthesis of several fluorinated analogues of thiamine and precursors has been undertaken, all of which exhibit weak activity against *E.Coli*. A route to 3-fluoro-(*R*)-alanine has been explored.

The first synthesis of the enantiomeric fluorosuccinic acids has been accomplished, and a stereochemical analysis has shown that synthesis *via* esterified malate precursors using diethylamino-sulphurtrifluoride gives *inversion* of configuration, whereas preparation *via* aspartic acid and polyhydrogen fluoride in pyridine gives *retention* of configuration. The circular dichroism spectra of the fluorosuccinic acids are anomalous, being the first example of such behaviour in α-substituted carboxylic acids and derivatives. A previously assigned configuration of a *Pseudomonal* metabolite (+)-fluorosuccinic acid has been corrected. The fluorosuccinic acids are not substrates for malic enzyme, but are potent inhibitors of fumarase. Using (2*S*)-fluorosuccinic acid as a mechanistic probe the fumarase reaction is identified as a bimolecular E2 elimination.

A stereochemical analysis of enzymic phosphoryl transfer reactions using CD spectroscopy has been investigated, and a new synthesis of inorganic pyrophosphate used to prepare P_i-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate whose ^{31}P n.m.r. spectrum is discussed.

The first example of ^{31}P-^{17}O coupling as observed by ^{31}P n.m.r. is demonstrated. The magnitude of the ^{31}P-^{18}O isotope shift is dependent on the nature of the phosphorus to oxygen bond. An elegant method for establishing the isotopic configuration of oxygen chiral phosphates by n.m.r. has been developed, requiring the synthesis of *D*-glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate, adenosine-5'-[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate, and their six membered cyclic phosphate triesters. Chemical cyclisation of these molecules occurs both with *racemisation* and *inversion* of configuration at phosphorus.

The chemical synthesis of adenosine-5'[(*S*)-^{16}O, ^{17}O, ^{18}O]-triphosphate has been achieved and exploited to demonstrate that hexokinase phosphoryl transfer proceeds with *inversion* of configuration at phosphorus. Pyruvate kinase phosphoryl transfer from 2[(*S*)-^{16}O, ^{17}O, ^{18}O]-phospho-(*R*)-glycerate also proceeds with *inversion* of configuration, as does phosphofructokinase phosphoryl transfer from *sn*-glycerol-3[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate.

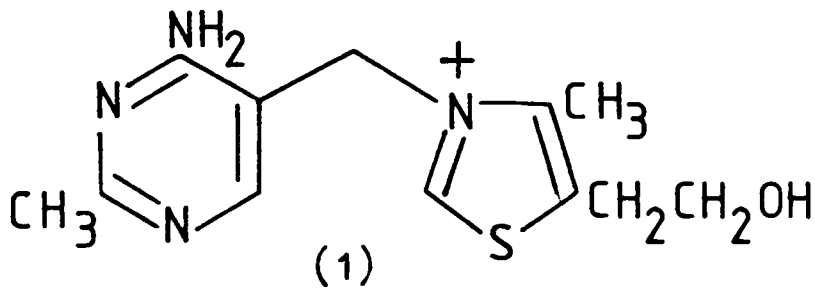
CHAPTER 1

NEW FLUORINATED ANTIMETABOLITES OF THIAMINE AND PRECURSORS

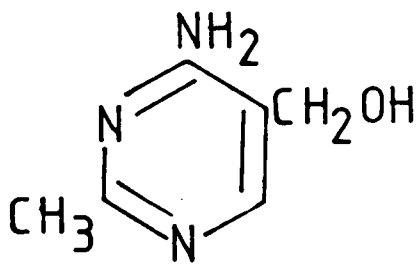
INTRODUCTION

Thiamine

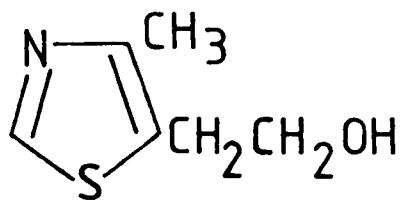
In 1926 Jansen and Donath isolated a crystalline substance from rice polishings having great antineuritic activity.[1] Eijkman, who had previously shown that polished rice was the cause of polyneuritis in birds and beri-beri in man, was subsequently able to confirm the prophylactic and curative activities of this substance against avian polyneuritis[2], although he later confessed that he had originally doubted whether this 'vitamine' [sic] would actually be a single chemical substance. About two years later several workers in the USA, Germany and England improved the method of isolation and thereby obtained sufficient quantities to establish its structural formula (1) and to find methods for its synthesis[3-6] (the isolation of the crystalline vitamin from natural sources, however, never had a chance to compete with the synthesis, first published in 1936 by Williams[3]).



(1)



(2)



(3)

FIGURE 1

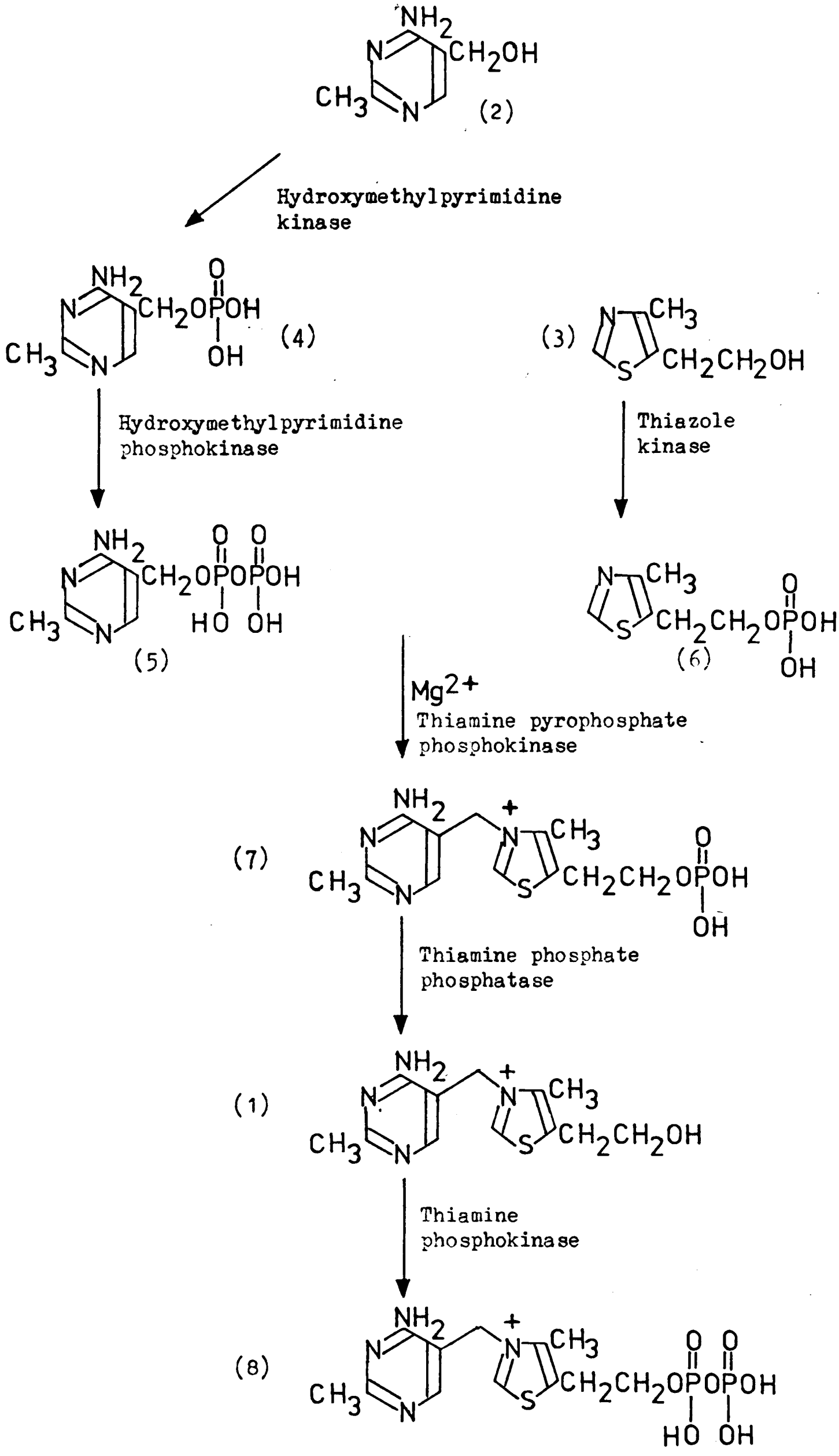
This vitamin was named Thiamine (vitamin B_1), and it was demonstrated that the thiamine molecule consists of a pyrimidine half (2) and a thiazole half (3), the elucidation of this structure being greatly assisted by the observation that thiamine is split quantitatively by sulphite into its pyrimidine and thiazole halves.[7]

The activity of thiamine appears to be very specific, for even small modifications of the molecule give inactive substances, diminish the activity or even produce antagonistic effects. Barton and Rogers[8] give a large number of examples of the influence of alterations to the pyrimidine or thiazole moiety of thiamine to the biological activity of these thiamine analogues and conclude that "...it is evident that the thiamine molecule can undergo very little modification without extensive loss of vitamin B_1 activity."

Thiamine has been found in most forms of life and indeed the ubiquity of thiamine in the plant and animal kingdoms is hardly surprising when one considers the functional role of the vitamin in metabolism. As the coenzyme thiamine pyrophosphate or cocarboxylase (8) (see Scheme 1) it plays an important role in glycolysis and the glycolytic pathway, the citric acid cycle and the hexose monophosphate shunt or pentose pathway. In the glycolytic pathway, widely distributed in animal and insect muscle, yeast, bacteria and plants, thiamine pyrophosphate is essential for the decarboxylation of pyruvic acid leading to the formation of acetyl coenzyme A or ethanol depending on the tissue or organism. In the citric acid cycle which is found in all respiring tissues of animals from protozoa to mammals, in almost all microorganisms including yeast, bacteria and moulds, and in many forms of plant material, thiamine pyrophosphate is again involved in decarboxylation, being necessary for the conversion of α-keto glutaric acid to succinic acid. In the pentose pathway thiamine is essential for the action of transketolase, a carbonyl group transferring enzyme widely distributed throughout the biological kingdom.

The Biosynthesis of Thiamine

Observations concerning the nature of thiamine-requiring microorganisms allow these organisms to be divided into three groups



SCHEME 1 : The Biosynthesis of Thiamine Pyrophosphate

namely (a) those that will utilise thiamine or the pyrimidine portion of the vitamin, (b) those that will utilise thiamine or the thiazole portion of the vitamin and (c) those that require intact thiamine. These observations indicate that the biosynthesis of thiamine proceeds by the independent formation of the pyrimidine and thiazole moieties of the vitamin followed by a final step in which they are joined together to give thiamine. Thus group (a) organisms are deficient in synthesising the pyrimidine portion of thiamine whereas group (b) organisms cannot make the thiazole half, and group (c) organisms are presumably unable to carry out the final stages of thiamine synthesis from the two halves of the molecule.

A great deal of information has now been accumulated about the enzymatic reactions concerned in the formation of thiamine, the initial stimulus being provided by the observation of Harris and Yavit[9] in 1957 that 2-methyl-4-amino-5-hydroxymethyl pyrimidine (2) and 4-methyl-5-(2-hydroxyethyl) thiazole (3) could be converted to thiamine by the action of cell-free extracts of baker's yeast in the presence of ATP. Subsequently it was discovered that the pyrimidine pyrophosphate (5), formed enzymatically from ATP and 2-methyl-4-amino-5-hydroxymethyl pyrimidine is the phosphorylated form of the pyrimidine which reacts directly with the thiazole compound.[10-13] It was also determined that thiamine monophosphate (7) rather than thiamine is the primary product of the yeast enzyme system,[10,12,14] and this suggested that the thiazole monophosphate (6) was the immediate precursor of the quaternised thiamine phosphate, and indeed it was found possible to synthesise thiamine monophosphate enzymatically from the pyrimidine pyrophosphate (5) and the thiazole phosphate (6) in the absence of ATP.[10-14] It was also found that conversion of the pyrimidine (2) to the pyrophosphate was a two stage process involving the

monophosphorylated intermediate (4).[15,16]

Thiamine monophosphate is not phosphorylated directly to cocarboxylase (8) but is first converted to free thiamine which is pyrophosphorylated to the coenzyme in the presence of ATP.[12,14,17,18]

Thus the facts that have accumulated indicate that the conversion of the pyrimidine and thiazole halves of thiamine to thiamine and cocarboxylase occur by the sequence of reactions set out in Scheme 1.[19]

Thiamine Analogues

In the search for selective toxicity one of the best understood mechanisms of antibacterial action is the inhibition of enzyme activity in a vital energy producing or biosynthetic pathway, and consequently the biosynthetic pathways leading to the vitamins, being absent in man, provide a variety of potential targets for antimicrobial agents which are non-toxic to man. Although there are notable examples of this type of therapeutic agent, for example, the sulphonamides as inhibitors of folic acid biosynthesis[20], this approach to new antibiotics remains relatively unexplored. In this respect the biosynthesis and metabolism of thiamine would appear to offer potential although inhibitors in this interesting category have not received much attention. Thus, although numerous analogues of thiamine and its precursors have been synthesised in relation to vitamin activity[8], their potential as antimicrobial agents has been largely ignored. However, there are notable exceptions such as 2-methylthio-4-amino-5-hydroxymethyl pyrimidine (methioprim), toxic to an *E. Coli* mutant requiring the B_1 pyrimidine for growth[21], and the corresponding 2-methoxy derivative (bacimethrin) also bacteriostatic to *E. Coli* and other bacteria and yeasts.[22] 2-Trifluoromethyl-4-amino-5-hydroxymethyl pyrimidine is bacteriostatic towards *B. Subtilis.*, thiamine thiazone

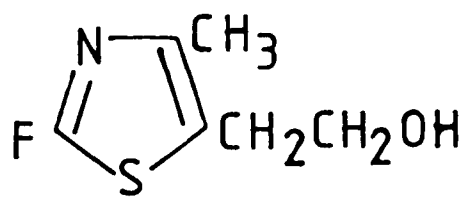
pyrophosphate is a potent inhibitor of pyruvate dehydrogenase from *E. Coli.*[24], and 2-amino-4-methyl-5-(2-hydroxyethyl) thiazole inhibits phosphorylation of the thiazole intermediate in thiamine biosynthesis and is also bacteriostatic to *E. Coli.*[25,26]

Aims of the Present Work

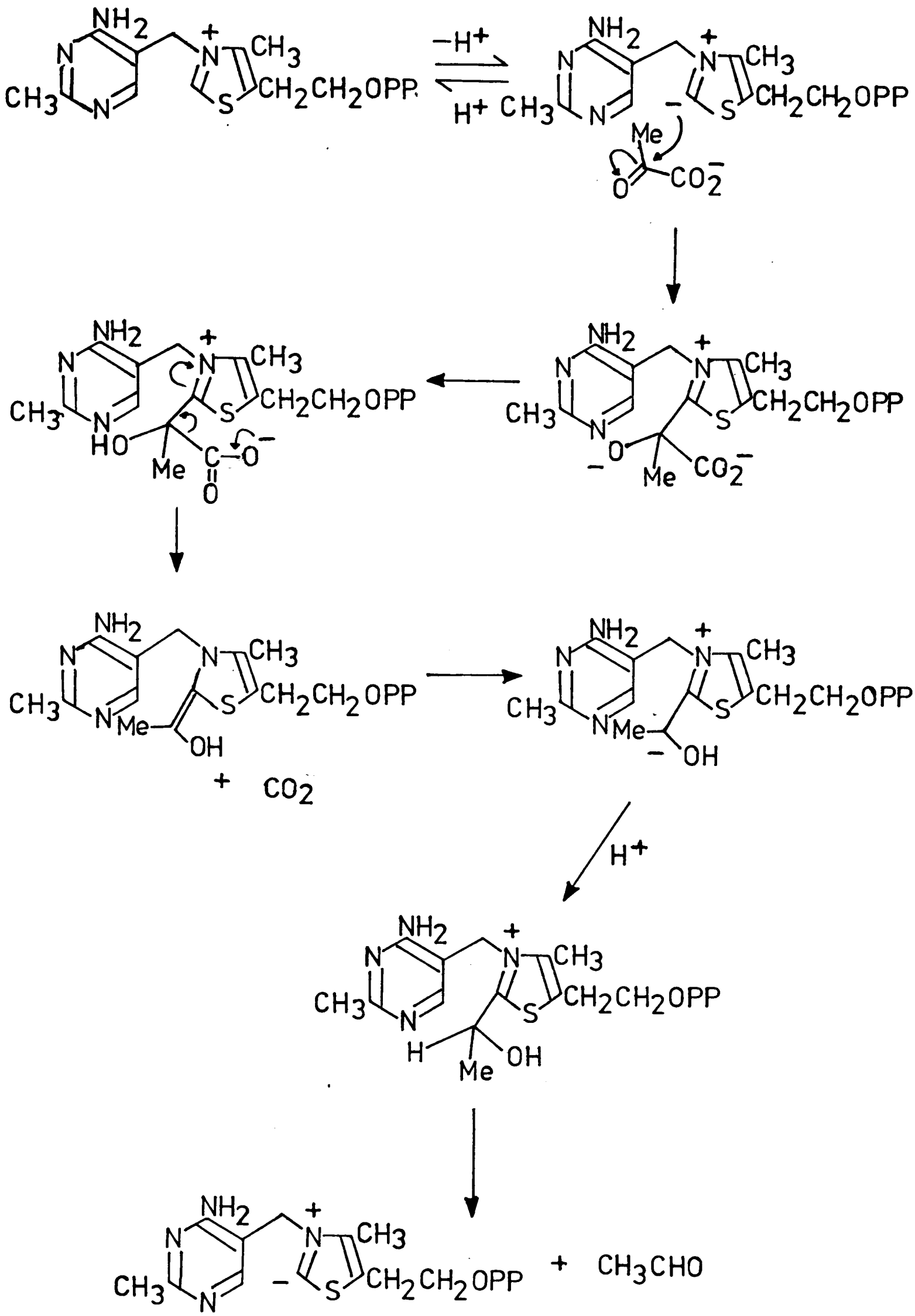
The unique properties of the fluorine atom, namely a high electronegativity and a size sterically considered to be intermediate between the proton and the hydroxyl group, have been considerably exploited in the design of antimetabolites of biologically significant compounds and enzyme inhibitors and substrates.[27-30] A good example is the recently developed 2-fluoro-(*S*)-histidine, an amino acid analogue which inhibits enzyme induction.[31,32,33]

Now thiamine pyrophosphate functions as a coenzyme for enzymes which bring about the decarboxylation of α-keto acids[34], and as such is essential in the primary metabolism of all mammals and parasitic organisms. An essential feature for its activity is the lability of the C-2 proton of the thiazolium ring, the carbanion so formed adding to the keto group of the substrate to initiate decarboxylation.[35] The mechanism of action is illustrated in Scheme 2. This fact prompted the intriguing possibility that if the C-2 position of cocarboxylase could be blocked by a group of similar steric requirements, for example fluorine, the coenzyme would be ineffective and possibly a potent active-site directed inhibitor. Thus 2-fluoro-4-methyl-5-(2-hydroxyethyl) thiazole (9) was considered sterically to be a good structural analogue of the

FIGURE 2



(9)



SCHEME 2 : The Mechanism of Action of Thiamine Pyrophosphate

natural metabolic intermediate (3), and could therefore be a competitive inhibitor or substrate for the thiazole phosphokinase, and moreover, if *in vivo* phosphorylation occurs then the possibility exists that 2-fluorothiamine phosphate and pyrophosphate (10) might be formed, generated by the 'lethal synthesis' concept of Peters.[36]

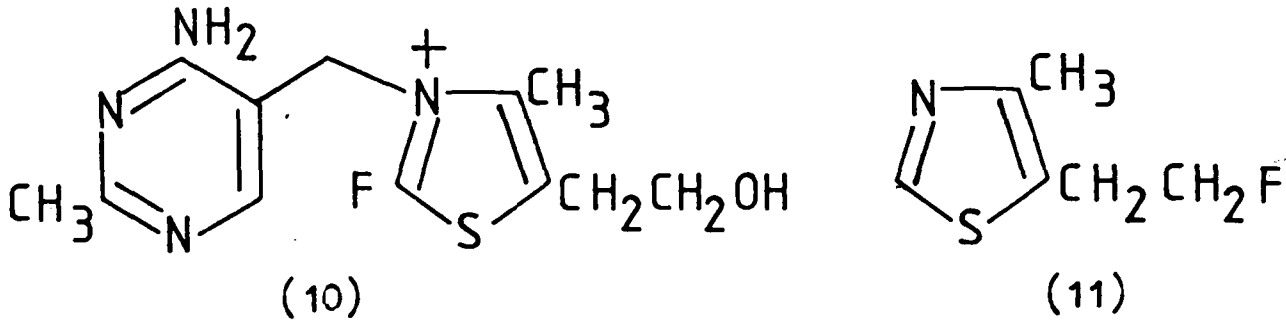


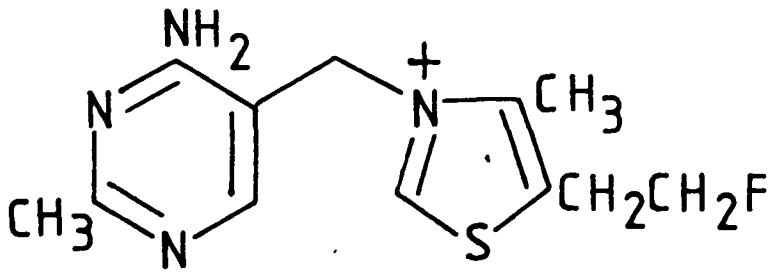
FIGURE 3

Thus the initial synthetic objective was considered to be the preparation of the thiazole (9) in the hope that either it or related derivatives in the biosynthetic pathway might possess anti-bacterial properties.

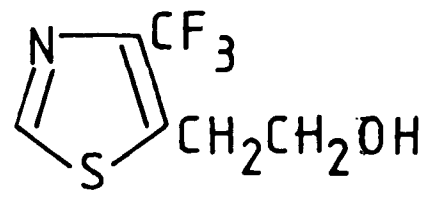
Another approach was also investigated, involving the synthesis of 4-methyl-5-(2-fluoroethyl) thiazole (11) which, since fluorine is a good analogue of the hydroxyl group[28,30], might be expected to be another good analogue of the thiamine thiazole. Although the lack of a hydroxyl group means that this compound could not be phosphorylated there still exists the possibility that it could be a competitive inhibitor of the thiazole phosphokinase and possess bacteriostatic properties. Moreover, it may have interesting pharmacological properties since 4-methyl-5-(2-chloroethyl) thiazole is *inter alia* a widely used and effective sedative and hypnotic.[37]

It was also planned to synthesise the whole thiamine analogue (12) with a view to investigating its interactions with micro-organisms to explore its possible potential as an antimicrobial or anticoccidial agent.

In conjunction with the thiazole (9), and in view of the useful



(12)



(13)

FIGURE 4

reported toxicity of the trifluorinated pyrimidine[23], it was also thought expedient to attempt the preparation of another good steric analogue of the natural thiazole (3) possessing a trifluoromethyl group in the 4-position, 4-trifluoromethyl-5-(2-hydroxyethyl) thiazole (13).

RESULTS AND DISCUSSION

Synthesis of Fluorinated Analogues

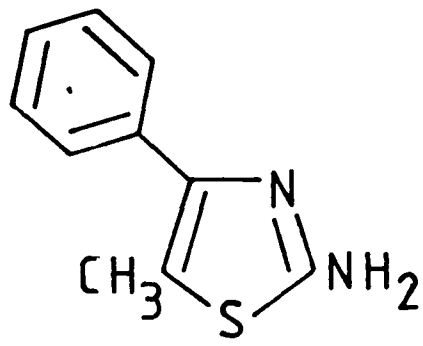
(a) 2-Fluoro-4-methyl-5-(2-hydroxyethyl) thiazole (9)

The synthesis of aryl fluorides is often readily accomplished by the Schiemann reaction, namely the thermal decomposition of diazonium fluoroborates in the solid state[38]. However, when this procedure is attempted with the thiazole nucleus much difficulty has been encountered.[39,40] At the present time there are only three reports in the literature of the successful preparation of 2-fluorothiazoles, two of which utilise the Schiemann reaction[41,42] and the other involves the displacement of a 2-nitro group by fluoride[43], the authors of which cast doubt on the validity of one of the previous syntheses.[41] In the light of our current knowledge of the nature of 2-fluorothiazoles it appears extremely unlikely indeed that the solid product, prepared by the decomposition of the thiazole diazonium fluoroborate prepared *in situ* by Beaty and Musgrave[41], and characterised only by microanalysis, was in fact authentic. Moreover, the apparently successful procedure of

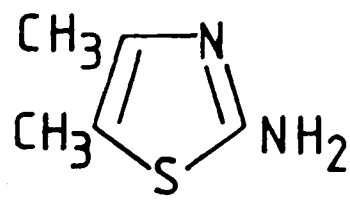
Grünert *et al.*[42] has almost invariably been observed to fail in several hands except in the case of 2-fluoro-5-methyl-4-phenyl-thiazole (14).[39] The synthesis of 2-nitro-4-methyl-5-(2-hydroxy-ethyl) thiazole (18) had also been previously accomplished[40] but displacement of the 2-nitro group by fluoride according to the method of Bartoli[43] was not successful. In view of this rather depressing current situation the synthesis of thiazole (9) was viewed with some pessimism, and it was decided to experiment initially with a model 4,5-disubstituted compound, 2-amino,4,5-dimethyl thiazole (15) in an attempt to perfect the technique.

The diazonium fluoroborate of the thiazole (15)[40] could not be isolated. In many instances although solid products were isolated from Schiemann reaction mixtures they were not diazonium salts as evidenced by their lack of a characteristic $-N\equiv\overset{+}{N}$ stretching band in the infrared spectrum, and they tended to discolour and decompose rapidly on drying. Aryl diazonium hexafluorophosphates, however, have been reported to possess advantages over their tetrafluoro-borate counterparts in the Schiemann reaction[44], and it was indeed found possible to isolate the 2-diazonium-4,5-dimethyl thiazole hexafluorophosphate (16) in good yield. Dry pyrolysis gave a small yield of the desired 2-fluoro-4,5-dimethyl thiazole (17).

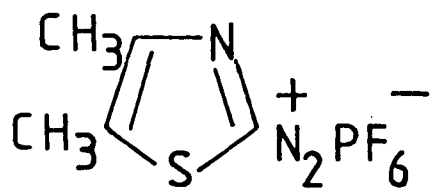
Recently the decomposition of diazonium fluoroborates by a photolytic method has been found to be synthetically useful for imidazoles[31-33] and ring fluorinated tyramines and dopamines.[45] Indeed whilst this work was in progress its use for the preparation of fluorinated pyrazoles and triazoles was also reported.[46] In view of this it was decided to attempt photolysis of the thiazole diazonium hexafluorophosphate (16). Photolytic decomposition through pyrex was found to give a readily observable gas evolution, and after work-up gave 2-fluoro-4,5-dimethyl thiazole (17) in about 25% yield. Photolysis of the tetrafluoroborate *in situ* gave a



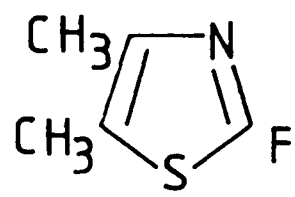
(14)



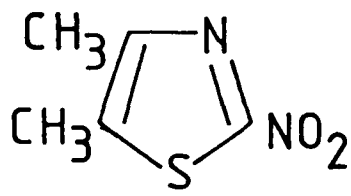
(15)



(16)



(17)



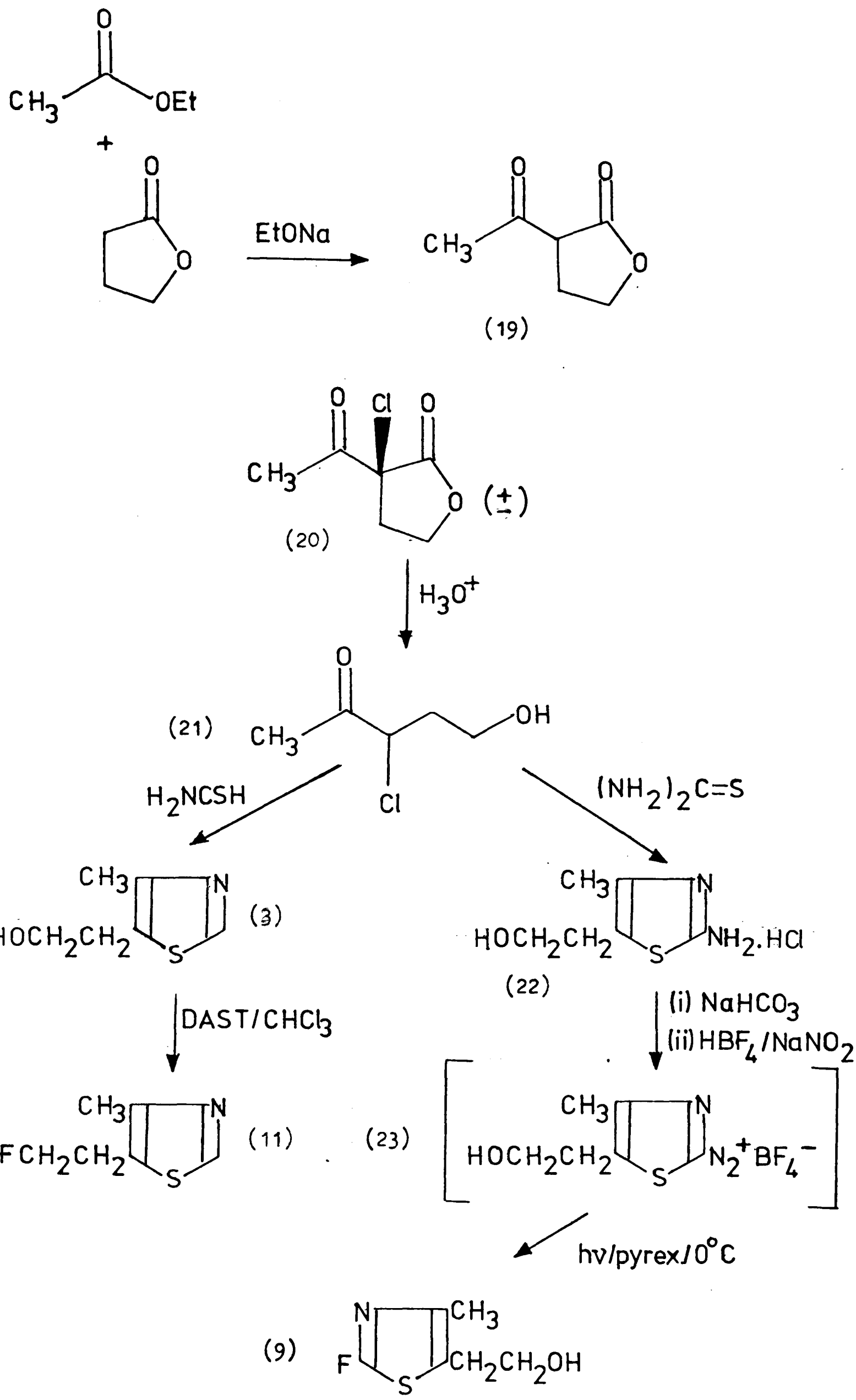
(18)

FIGURE 5

similar yield of the 2-fluorothiazole despite the claim that in general diazonium hexafluorophosphates give higher yields in both the conventional[44] and photolytic[47] Schiemann reactions†. Although the photolytic decomposition did not give exceptionally high yields it was considered to be sufficiently expedient for attempting the préparation of 2-fluoro-4-methyl-5-(2-hydroxyethyl) thiazole (9).

The synthetic scheme to thiazole (9) is illustrated in Scheme 3. 2-Acetylbutyrolactone (19) was prepared by the method of Reppe *et al.*[49] by the condensation of ethyl acetate with γ-butyrolactone. Chlorination using sulphuryl chloride gave 2-acetyl-2-chlorobutyrolactone (20) and acid hydrolysis yielded 3-chloropentan-5-ol-2-one (21) according to the method of Buchmann.[50] Condensation with

† Whilst this work was in progress the stabilisation of diazonium hexafluorophosphates by crown ether complexation in the solid state photolytic Schiemann reaction was reported.[48]



SCHEME 3 : Synthesis of 2-Fluoro-4-methyl-5(2-hydroxyethyl)thiazole and 4-Methyl-5(2-fluoroethyl)thiazole

thiourea by the method of Todd *et al.*[51] yielded 2-amino-4-methyl-5-(2-hydroxyethyl) thiazole (22) as the hydrochloride.

Diazotisation of this aminothiazole (22) gave water-soluble tetrafluoroborate and hexafluorophosphate salts (23) so the protocol for thermal decomposition was not feasible anyway. However, photolytic decomposition in aqueous solution in either case was found to give 2-fluoro-4-methyl-5(2-hydroxyethyl) thiazole (9) in yields of about 28%.

(b) 4-Methyl-5-(2-fluoroethyl) thiazole (11)

The displacement of a good leaving group such as tosylate by fluoride ion in a solvent which permits nucleophilic substitution is a well known method for the preparation of aliphatic fluoro-compounds[52], and thus the synthesis of thiazole (11) was initially attempted according to FIGURE 6.

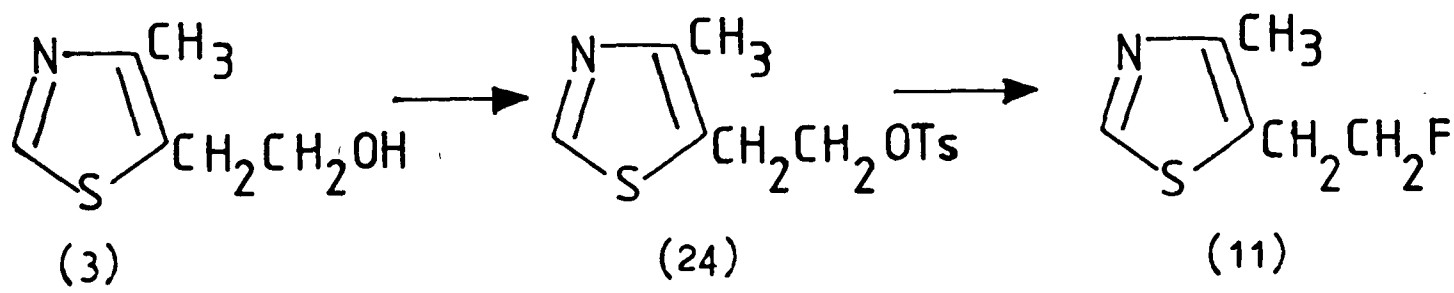
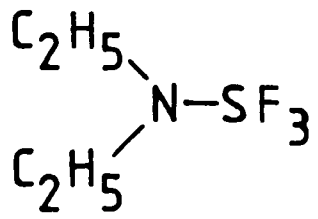


FIGURE 6

4-Methyl-5-(2-hydroxyethyl) thiazole was obtained either by the classic cleavage of thiamine by sulphite according to Williams[7], or from 3-chloropentan-5-ol-2-one (21) by the synthetic method of Buchman.[50] 4-Methyl-5-(2-*p*-toluenesulphonylethyl) thiazole (24) was easily prepared from thiazole (3) with toluene-*p*-sulphonyl chloride in anhydrous pyridine. However, reaction with anhydrous potassium fluoride in dry DMF did not, unfortunately, lead to the desired product.

The availability of diethylaminosulphur trifluoride (DAST) (25) from another investigation (see CHAPTER 3), a reagent which converts



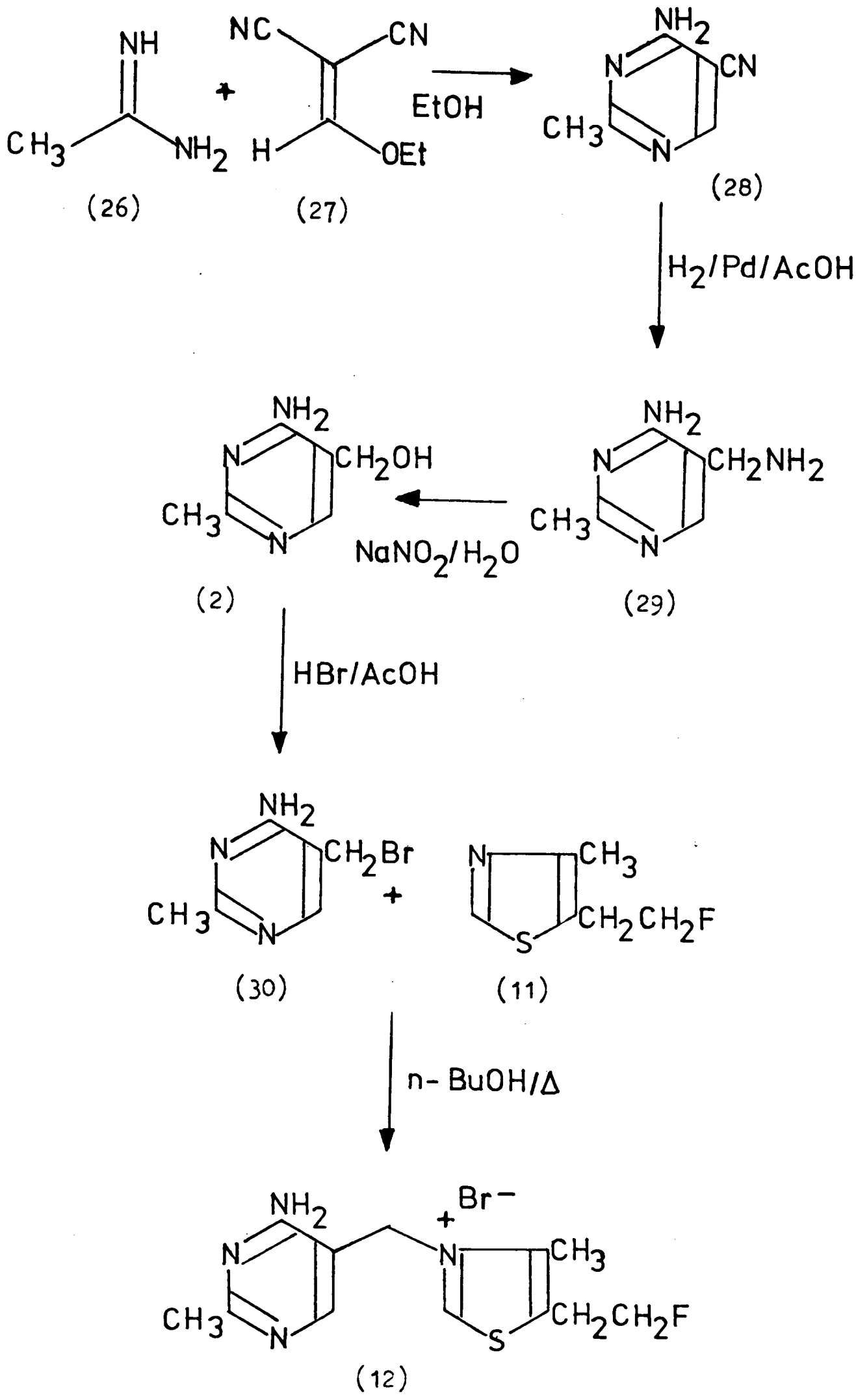
(25)

FIGURE 7

alcohols to fluorocompounds under very mild conditions, and with much less dehydration than is commonly found with other fluorinating reagents[53-55], led to its attempted exploitation in this preparation. Indeed by the simple reaction of DAST with thiazole (3) in dry ethanol-free chloroform solution it was found possible to synthesise the fluorinated thiazole (11) in *ca.* 55% yield. 4-Methyl-5(2-fluoroethyl) thiazole (11) was characterised as its crystalline picrate and methane sulphonate salts.

(c) 3-[(4-Amino-2-methyl-5-pyrimidyl)methyl]-4-methyl-5(2-fluoroethyl) thiazole (12)

The synthetic scheme to fluorothiamine (12) is illustrated in Scheme 4. The substituted pyrimidine (30) required for the quaternisation reaction with thiazole (11) was prepared by literature methods[4,5] as follows. The condensation of acetamidine (26) and ethoxymethylene malononitrile (27) in ethanol gave 2-methyl-4-amino-5-cyanopyrimidine (28) which was hydrogenolysed to 2-methyl-4-amino-5-aminomethyl pyrimidine dihydrochloride (29) using a Pd/C catalyst. Treatment of the pyrimidine (29) with one equivalent of sodium nitrite gave selectively 2-methyl-4-amino-5-hydroxymethyl pyrimidine (2) which was finally converted to 2-methyl-4-amino-5-bromomethyl pyrimidine (30) using hydrogen bromide in acetic acid solution. The final quaternisation step was performed by heating together the pyrimidine (30) and the thiazole (11) in butan-1-ol to yield the thiazolium salt 3-[(4-amino-2-methyl-5-pyrimidyl)methyl]-4-methyl-5-(2-fluoroethyl)



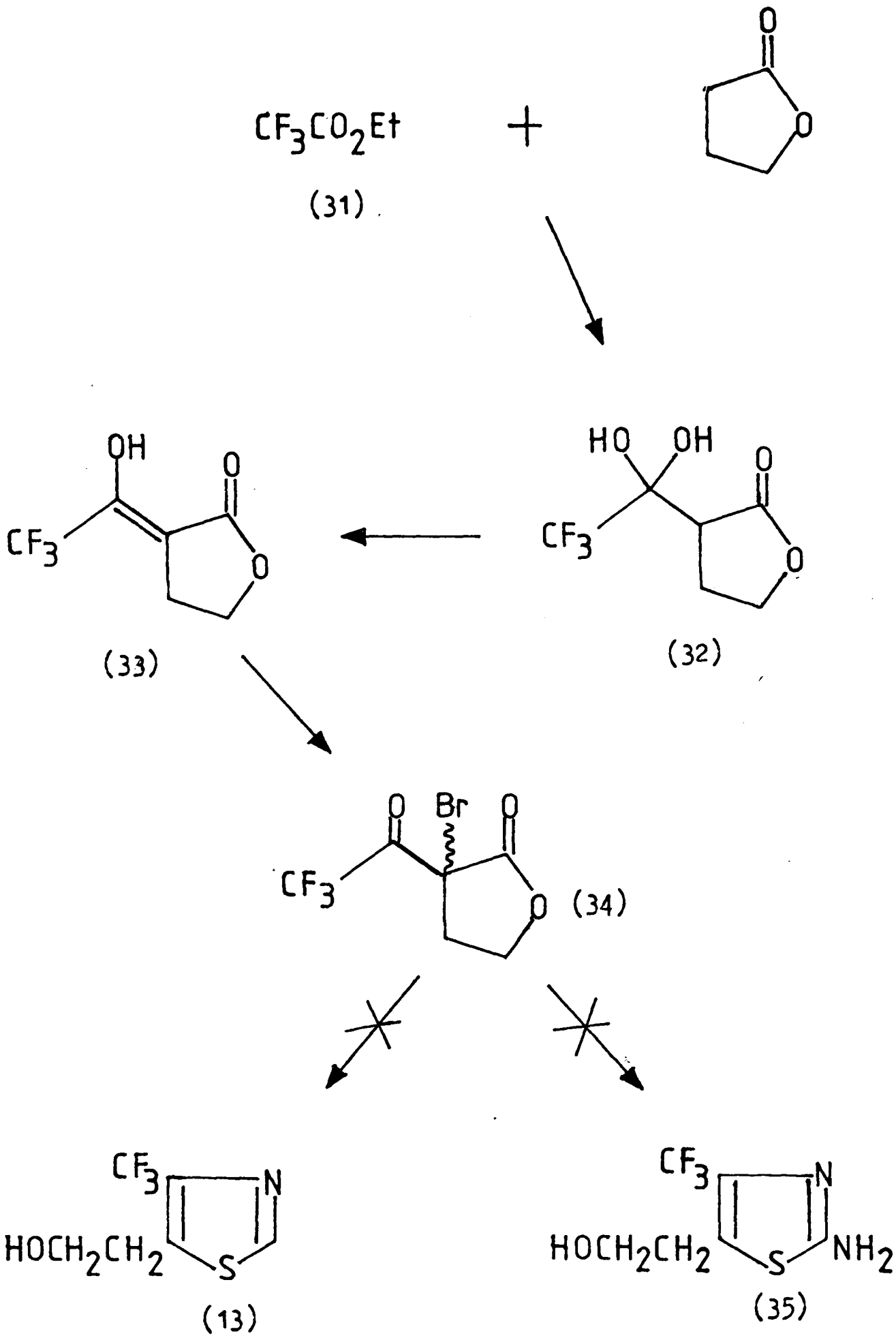
SCHEME 4 : Synthesis of 3[(4-Amino-2-methyl-5-pyrimidinyl)methyl]-4-methyl-5(2-fluoroethyl)thiazole

thiazolium bromide hydrobromide (12).

(d) Attempted Synthesis of 4-Trifluoromethyl-5(2-hydroxyethyl) thiazole (13)

The proposed synthetic route to thiazole (13) is shown in Scheme 5. Ethyl trifluoroacetate (31) was synthesised by the literature method[56] and condensed with γ-butyrolactone. During the work up a solid product crystallised from the reaction mixture. The ^1H n.m.r. spectrum showed, in addition to other complex multiplets, two singlets at 6.89 and 6.72 p.p.m. which disappeared on shaking with D_2O. The highest peak in the mass spectrum was at 182 corresponding to the diketone (32) is its hydrated form). Microanalysis of the multiply recrystallised product indicated the presence of a molecule of water and the product was finally identified as 2-(2,2,2-trifluoro-1,1-dihydroxyethyl) butyrolactone (32) which has a hydrated trifluoroacetyl group. Conventional bromination techniques, such as those described for ethyl-2,2,2-trifluoroacetoacetate[57], failed to yield any of the desired product when this precursor (32) was used. The water of hydration was removed by azeotropic desiccation with benzene to yield 2-trifluoroacetyl-butyrolactone as its enolic form (33). Attempts at halogenation such as the use of molecular bromine, and sulphuryl chloride in dry ether were unsuccessful. However, use of the method of King and Ostrum[58] for difficult brominations, using cupric bromide, gave reasonable yields of 2-bromo-2-trifluoroacetyl butyrolactone (34) (as evidenced by mass spectrometry).

Unfortunately the procedures previously described[50,51] for the preparation of 4-methyl-5-(2-hydroxyethyl) thiazole (3) and its 2-amino counterpart (22) did not appear to be successful with the bromoketone (34), and neither were those described for the direct conversion of 2-disubstituted butyrolactones into thiazoles[59], so attempts at the preparation of thiazoles (13) and (35) were abandoned.



SCHEME 5 : Proposed Synthesis of 4-Trifluoromethyl-5(2-hydroxyethyl)thiazole and 2-Amino-4-trifluoromethyl-5(2-hydroxyethyl)thiazole

The pKa of 2-Fluoro-4-methyl-5(2-hydroxyethyl) thiazole (9)

During the characterisation of the 2-fluorothiazole (9) it was observed that this compound did not form a conventional charge-transfer picrate salt, whereas its non-fluorinated counterpart (3) does so readily.[60] Since it seemed highly likely that this was due to an abnormally low pKa of the ring nitrogen, and that this could be paralleled by a low nucleophilicity, this caused some concern since it might obviously have an adverse effect on the potential of this compound to be enzymatically transformed into the 2-fluoro-thiamine analogue (10). It was thus important to measure the pKa relative to the unfluorinated thiazole (3). There appeared to be no reported value in the literature of the pKa of the naturally occurring thiazole (3) so its value was determined spectrophotometrically and found to be 3.82±0.02 (FIGURES 8 and 9). The pKa† of the fluorinated thiazole (9) was measured spectrophotometrically against the Hammettacidity function[61], using values of Ho obtained by interpolation from the data of Paul and Long[62], and by a method shown to be suitable for thiazoles.[63a,63b] The value was found to be -1.44±0.07 (FIGURES 10 and 11), some 5.26 units below that of thiazole (3) (TABLE 1) and comparable with the recently reported pKa values of 2-chloro- and 2-bromo thiazole (-0.75 and -0.86 respectively).[63b] The HammettM value for this compound is similar to that found for 2-nitrothiazole[63b], possibly for similar reasons. The inductive effect of fluorine thus exerts a very marked influence on the basicity of the ring nitrogen and it now seems likely that the thiazole (9) may not serve as a substrate for the formation of 2-fluorothiamine phosphate, although of course it may still be a substrate or inhibitor of 4-methyl-5(2-hydroxyethyl) thiazole phosphokinase.

† As is customary the pKa of the conjugate acid of the thiazole is used as a measure of basicity.

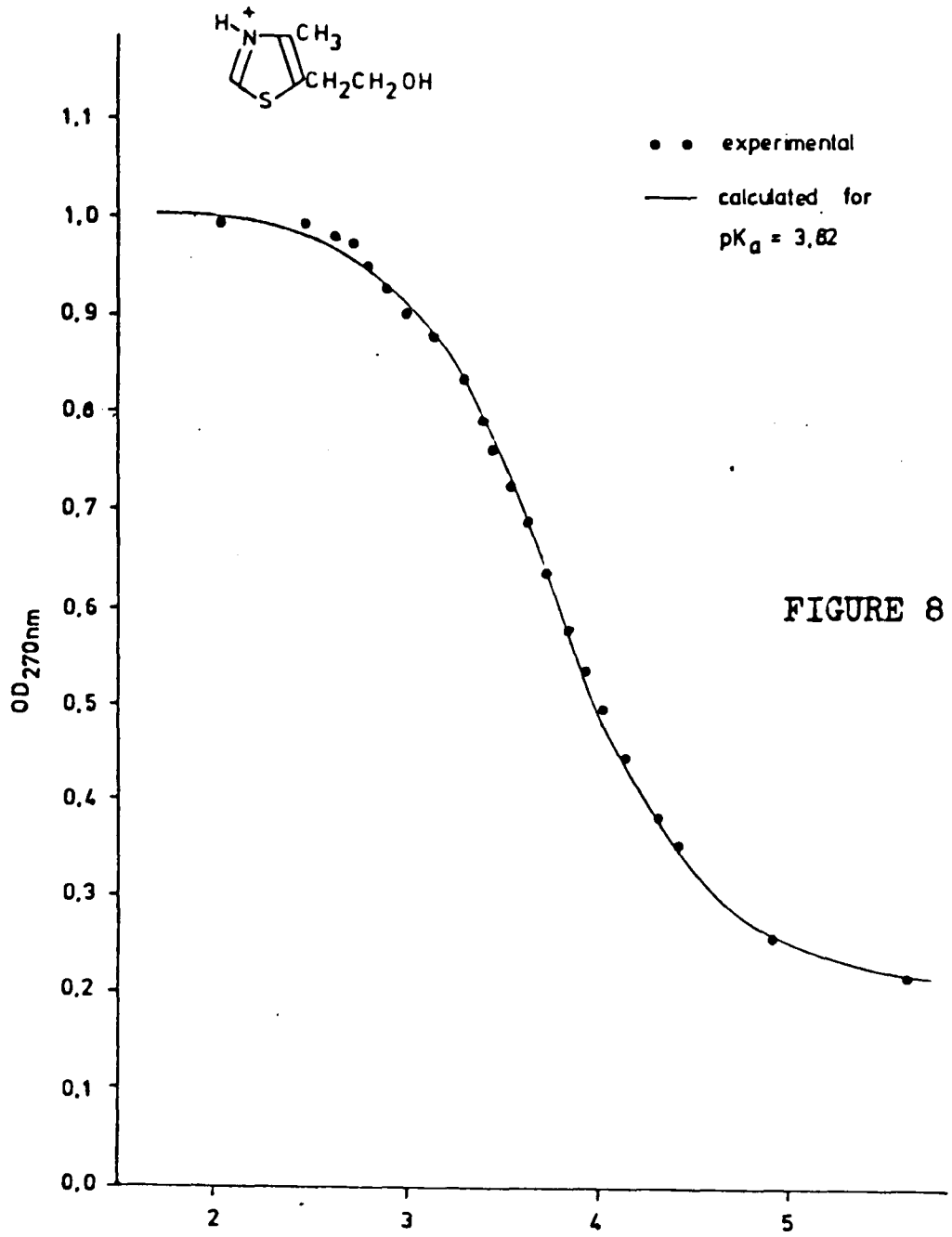


FIGURE 8

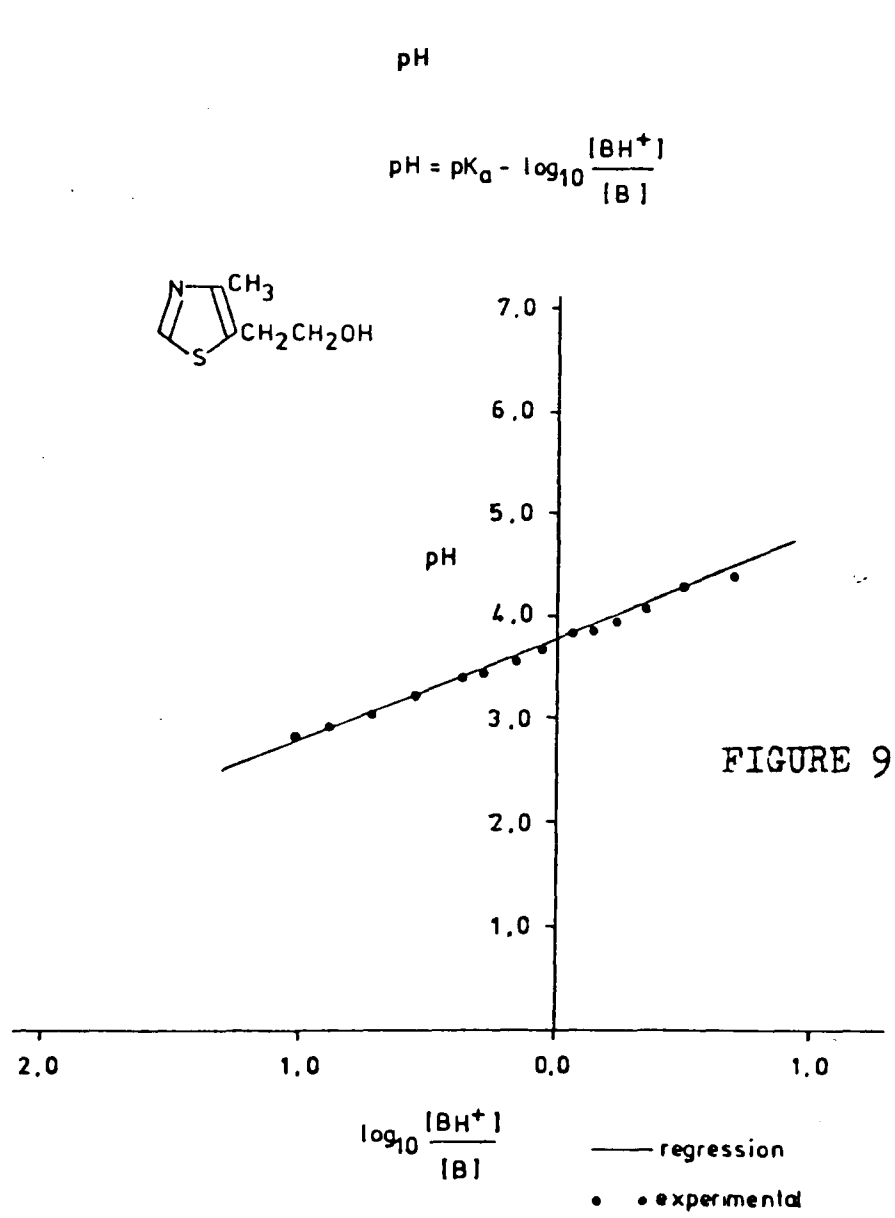


FIGURE 9

pK_a Determination for 4-Methyl-5(2-hydroxyethyl)thiazole

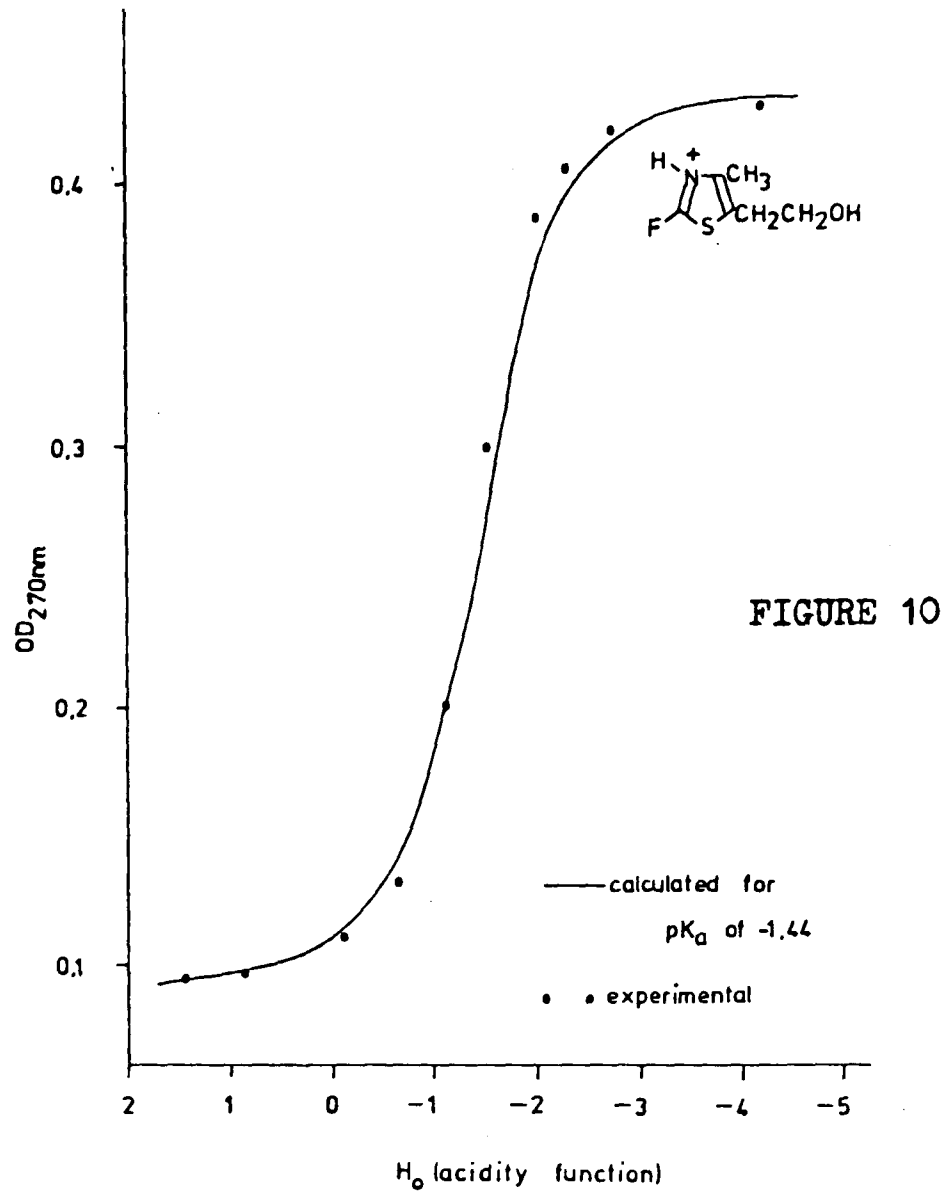


FIGURE 10

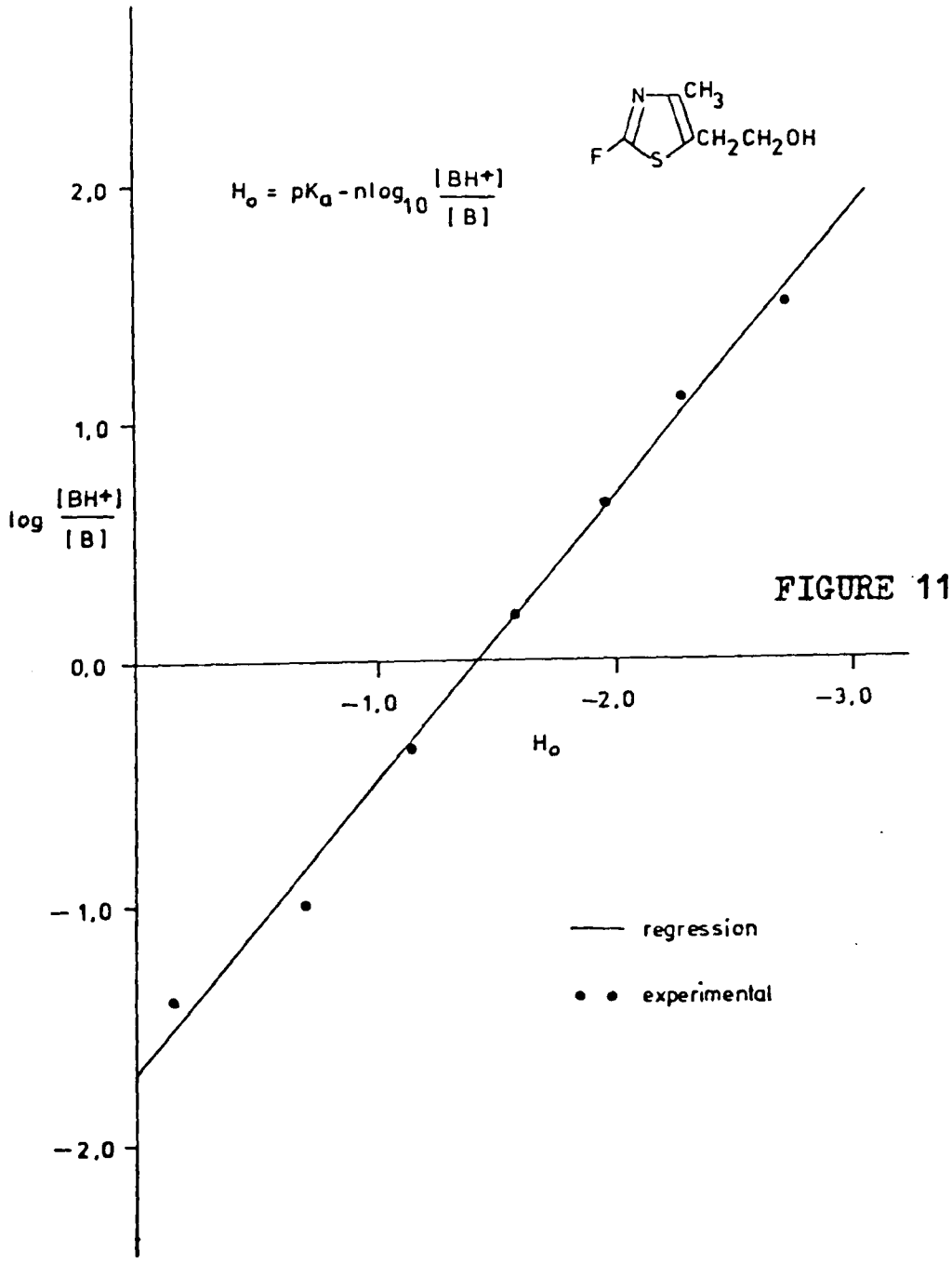


FIGURE 11

pK_a Determination for 2-Fluoro-4-methyl-5(2-hydroxyethyl)thiazole

TABLE 1: pKaS OF THIAZOLES

pKa*	m*	λ^{B}_{max}/nm(ε)+	$\lambda^{BH^+}_{max}$/nm(ε)+	λ_{det}/nm‡
3.82±0.04	1.01±0.07	249 (4190)	258 (4450)	270
-1.44±0.07	0.84±0.03	242 (3969)	257 (3220)	270

* Evaluated from the least squares regression analysis of the $\log_{10}[BH^+]/[B]$ *vs.* pH(Ho) plot, m = slope and pKa is the value of Ph(Ho) for $\log_{10}[BH^+]/[B] = 0$ obtained by extrapolation of pH(Ho) = pKa-m $\log_{10}[BH^+]/[B]$.

\+ B is the unprotonated base.
BH^+ is the protonated base.

‡ Wavelength used in the determination.

Microbiological Testing of Fluorinated Analogues

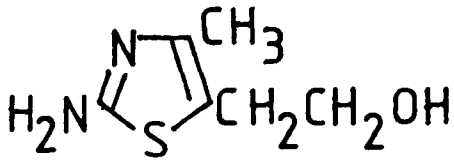
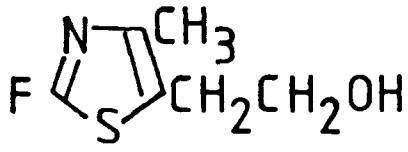
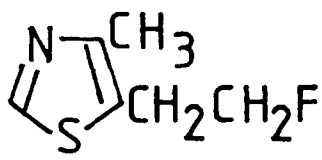
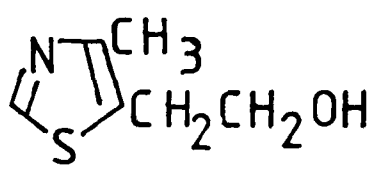
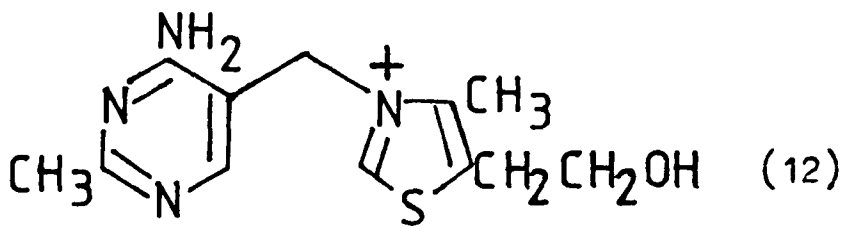
The thiazoles (9) and (11) were tested by standard plate assay techniques using the following four organisms -

E. Coli K12 *Salmonella Typhi* *Alcaligenes Faecalis* *Staphalococcus Aureus*

However, at concentrations of *ca.* 1 mg cm^{-3} no zones of inhibition could be observed after 12 hour incubations. Wider screening of these two thiazoles, however, at ICI Pharmaceuticals revealed that thiazole (9) did possess a weak activity towards gram negative organisms at a concentration of *ca.* 6×10^{-3} *M*.

Since antimetabolite action is often reversed in complex media, and in view of the success of Iwashima and Nose[25] in demonstrating the bacteriostatic activity of 2-amino-4-methyl-5(2-hydroxyethyl) thiazole (22) against *E. Coli* ATCC 9637 *using a minimal incubation medium*, it was decided to test the fluorinated thiazoles in this fashion. Incubation of the aminothiazole (22) with *E. Coli* W3110 in Davis-Mingioli's minimal glucose medium[64] showed effectively no

TABLE 2 : INHIBITION OF THE GROWTH OF E. COLI ATCC 9637 BY THIAMINE ANALOGUES.

COMPOUND		$O.D._{450nm}$ +
Blank		0.63
	(22)	0.15 *
	(9)	0.38
	(11)	0.01
	(3)	0.67
	(12)	0.04
Thiamine		0.69

* Reading taken after 7 h at $37^{o}C$

+ The $O.D._{450nm}$ was recorded after 9 h at 37 C, in a 2mm pathlength cell and is the mean of two samples

growth inhibition. However, when *E. Coli* ATCC 9637 was obtained and used the result of Iwashima and Nose[25] was successfully reproduced, and moreover, incubation of all those fluorinated analogues with this microorganism indicated that they also possess bacteriostatic properties (TABLE 2).

CONCLUSIONS

It is tempting to ascribe these observations of growth inhibition to a particular enzyme in each case, although the present limited experimental data do not really justify this and some caution must be exercised in their interpretation. 4-Methyl-5(2-fluoroethyl) thiazole (11) cannot be phosphorylated and therefore could possibly be expected to inhibit 4-methyl-5(2-hydroxyethyl) thiazole phosphokinase, whilst 2-fluoro-4-methyl-5(2-hydroxyethyl) thiazole (9) might be expected to be either a substrate or inhibitor of this enzyme. If it is a substrate then as 2-fluoro-4-methyl-5(2-phosphoethyl) thiazole it could inhibit the quaternisation step in thiamine biosynthesis. The fluorothiamine analogue (12) may function as an inhibitor of the enzyme responsible for the final stage of cocarboxylase synthesis, thiamine phosphokinase.

All of these speculations, however, require considerable further investigation to place them on a sounder basis, although it is evident that such compounds with structural modifications at or near to a phosphorylation site are interesting candidates for cell free thiazole phosphokinase studies.

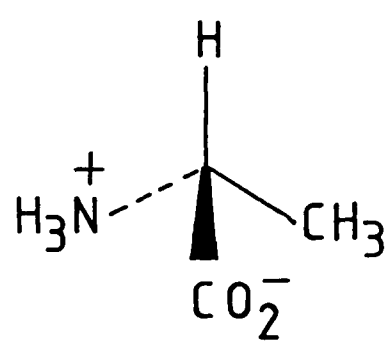
These results have recently been published.[65]

CHAPTER 2

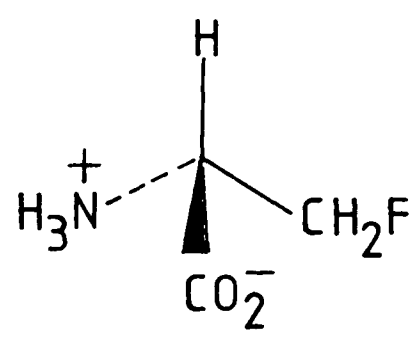
SYNTHETIC ROUTES TO 3-FLUOROALANINE

INTRODUCTION

(*R*)-Alanine (36) is a ubiquitous component of the bacterial cell wall, and is endogenously synthesised by bacteria although it plays no essential role in human metabolism. Recently it has been found that the analogue 3-fluoro-(S)alanine (37) is a potent broad spectrum antibacterial agent[66], its activity being derived from inhibition of the various enzyme systems related to the biosynthesis or utilisation of the (*R*)-alanine component of the peptidoglycan of the bacterial cell wall.



(36)



(37)

FIGURE 12

In the light of the discovery of Diddens *et al.*[67] that peptides such as glycyl-glycyl-(*S*)-alanine, tri-(*S*)alanine and tetra-(*S*)-alanine are actively transported by the oligopeptide transport system into cells of *E. Coli* K12, as are the tripeptide antibiotics (*S*)-Phosphinothioyl-alanyl-alanine and (*S*)-(N^5-phosphono)methionine-S-sulphoximinyl alanyl alanine, which are subsequently hydrolysed inside the cell by a peptidase, suggested that the activity of 3-fluoro-(S)alanine (37) might be enhanced by conversion to such oligopeptide derivatives which would then 'carry' this compound to its target area. Indeed, this type of amino acid antimetabolite would seem to be an ideal choice for this kind of experiment.

The original synthesis of 3-fluoro-(S)alanine (39) was accomplished by the photolysis of (*R*)-alanine (38) in fluoroxytrifluoromethane and liquid hydrogen fluoride.[66] These reagents require specialised handling techniques and possess obvious intrinsic

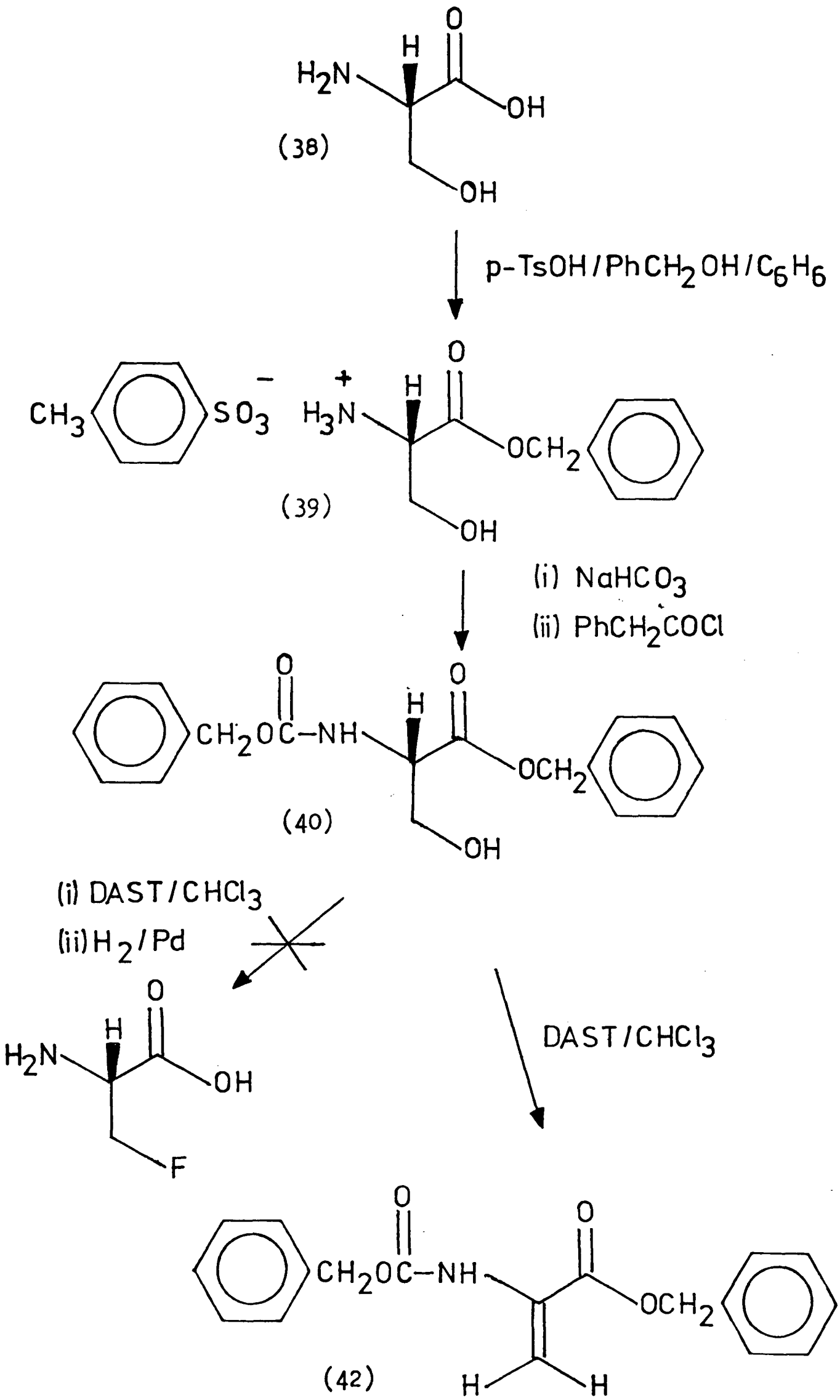
hazards. Another method utilising fluoropyruvic acid gives a racemic product[68] which necessitates a resolution step.[69]

In view of the availability of the mild fluorinating reagent diethylaminosulphur trifluoride (DAST) (25)[53-55] from other investigations (see CHAPTER 3), a reagent whose potential has yet to be fully exploited, it seemed possible that a simple efficient synthesis of both enantiomers of 3-fluoroalanine could be achieved by the action of this reagent on suitably protected serine precursors. Moreover, extension to the generation of oligopeptides could be simply achieved by fluorination of the suitably protected serine peptides followed by deprotection.

RESULTS AND DISCUSSION

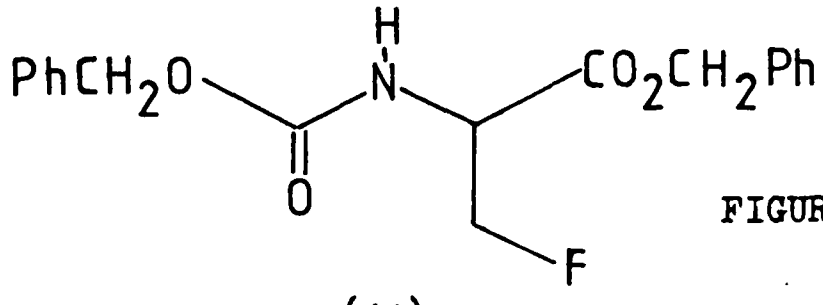
The proposed reaction sequence is illustrated in Scheme 6. Carboxyl groups are readily converted to acyl fluorides by DAST so it was certain at least that the serine would have to be esterified. As a preliminary step the possibility of employing DAST with an unprotected amino function was investigated, and all reactions were carried out using the more readily available and cheaper (*S*)-Serine (38). Thus benzyl-(*S*)-serinate (39) was prepared as its *p*-toluenesulphonate salt by the use of a Dean and Stark apparatus.[70] In the event it was found necessary to protect the amino function, and in order to facilitate the convenience of simultaneous deblocking of the carboxyl and amino group by hydrogenation or with HBr in acetic acid after fluorination, the benzyl-(*S*)serinate (39) was converted to benzyl-N-carbobenzoxy-(*S*)-serinate (40) by reaction with benzyloxy carbonyl chloride.

When this compound was allowed to react with DAST in dry ethanol free chloroform the ^{19}F n.m.r. spectrum showed a triplet resonance at +130 p.p.m. from external trifluoroacetic acid with J_{HF} = 47 Hz (characteristic of *gem* coupling) thus indicating that the desired



SCHEME 6 : Attempted Synthesis of 3-Fluoroalanine using DAST

product (41) had been formed. However, t.l.c. and the ^{1}H n.m.r. spectrum indicated that the major component of the reaction mixture



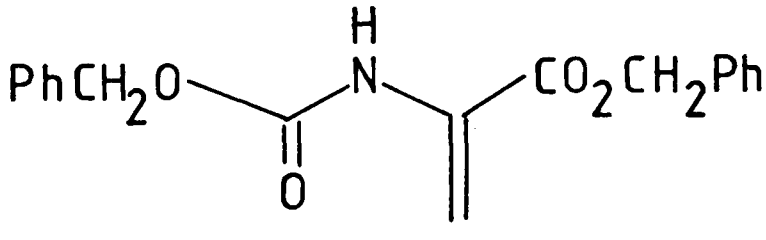
(41)

FIGURE 13

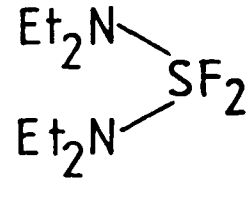
was by far the elimination product benzyl-N-carbobenzoxy-dehydrolanine (42).

The susceptibility of 3-substituted serine derivatives to base-catalysed β-elimination has been previously noted.[71,72] Thus although the occurrence of this type of dehydration reaction has been shown to be much less of a problem when DAST is used in comparison to other fluorinating reagents[55], in this instance it was predominant over fluorination. Repeated attempts at the reaction resulted only in poor yields of the fluorocompound (41) as estimated by t.l.c.

The use of *bis*-dialkylaminosulphur difluorides has been reported to give rise to less of the elimination reaction than when DAST is used with sensitive alcohols.[55] Thus reaction of diethylamine



(42)



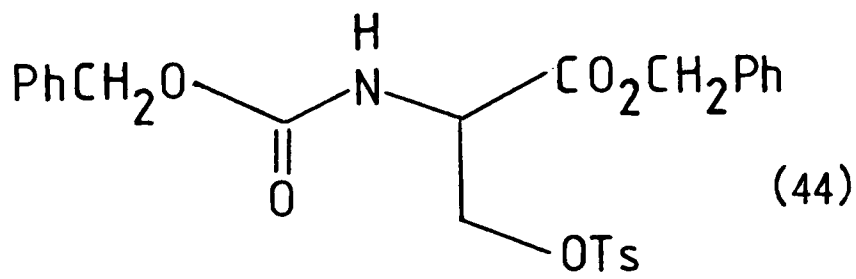
(43)

FIGURE 14

with DAST gave *bis*-diethylaminosulphur difluoride (43) which was allowed to react with the protected amino acid (40). However once again the most prominent product was the dehydroalanine derivative (42).

Nucleophilic displacement of a good leaving group by fluoride ion in a solvent such as DMF is a well known procedure for the synthesis of primary aliphatic fluorides[52], and since good yields of 3-substituted alanines had been obtained by using suitably protected 3-O-tosyl serine derivatives[72,73] it was decided to attempt a similar displacement with fluoride ion.

To this end benzyl-N-carbobenzoxy-3-O-tosyl-(*S*)serinate (44) was prepared by the reaction of the diprotected acid (40) with



(44)

FIGURE 15

p-toluene sulphonyl chloride according to the method of Theodoropoulos *et al.*[73] Unfortunately, however, heating this compound (44) with anhydrous potassium fluoride in dry DMF resulted only in the formation of benzyl-N-carbobenzoxydehydroalanine (42) in almost quantitative yield. The very poor nucleophilicity of the fluoride ion is undoubtedly a major reason for the failure of this reaction which may, however, be possible with the use of a crown ether.

In view of all these difficulties in developing a good new synthesis of 3-fluoroalanine the project was abandoned.

CHAPTER 3

THE SYNTHESIS, ABSOLUTE CONFIGURATION AND CIRCULAR DICHROISM OF THE ENANTIOMERS OF FLUOROSUCCINIC ACID

INTRODUCTION

Fluorosuccinic Acid

Of the monohalosuccinic acids the chloro- and bromo-succinic acids have been well established in the literature since the last century, and iodosuccinic acid since the early part of this century Indeed, the chloro- and bromo-acids appeared in the classical demonstration of the Walden inversion.[75] However, the fluoro-

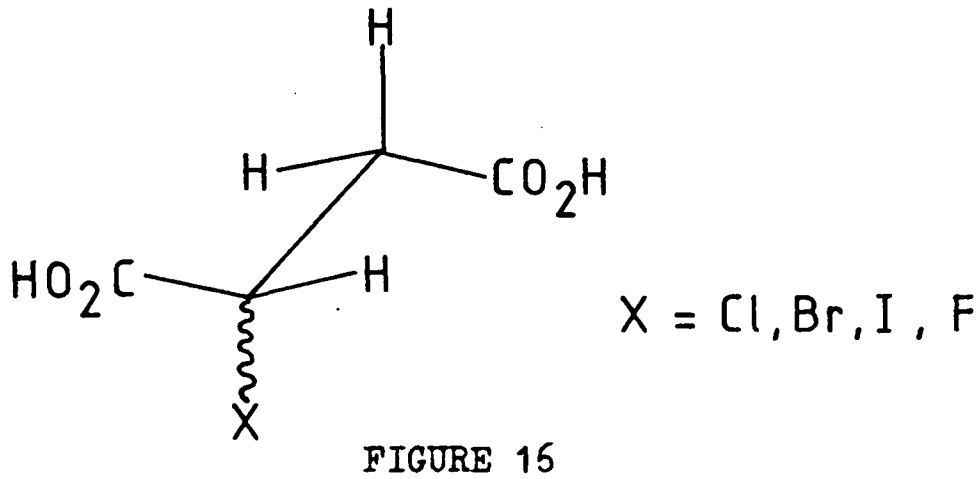


FIGURE 15

analogue did not make its first appearance until 1949 when Martius[76] reported experiments on the acid, and although he expressed thanks for a gift of the acid he gave no indication of its preparation. Peters[77] was later to question the authenticity of Martius' fluorosuccinic acid since his work had yet to be confirmed and "...as several others have not been successful in preparing monofluorosuccinic acid." The investigations of Gitter *et al.*[78] in 1953 demonstrated the non-toxicity of the acid in comparison to the high toxicity of fluorocitrate and fluoroacetate, although once again the origin of their disodium fluorosuccinate was not disclosed. The preparation of impure samples of diethyl-fluorosuccinate (*ca.* 25% yield) and dibenzyl fluorosuccinate by the condensation of the enolate of ethyl fluoroacetate and ethyl bromo-acetate was reported in 1956 by Bergmann and Szinai[79] who noticed the sensitivity of the acid to even mildly alkaline conditions. The much improved synthesis of Dean and Pattison[80] employing diethyl succinate and perchloryl fluoride afforded high yields of the racemic diethyl ester (*ca.* 73%) which could be hydrolysed under

acidic conditions to the free acid with a yield of 67%. Brodie and Nicholls[81] studied the enzymology of (2*RS*)-fluorosuccinic acid prepared by the previous method and were able to rationalise its non-toxicity by showing that it is metabolised to fluorofumarate which in the presence of fumarase is hydrated to the intrinsically unstable 2-fluoromalate (see CHAPTER 4). The spontaneous decomposition of 2-fluoromalate to oxaloacetate and hydrogen fluoride results in a non-enzymic elimination of fluoride. Subsequently Tober *et al.*[82] in their more general study of the interaction of various fluorosuccinic acids with the soluble succinic dehydrogenase system showed that monofluorosuccinic acid is oxidised by the enzyme with a V_{max} of about 45% that of succinate and a K_m about four times as great. Moreover, they claim to have prepared (2*S*)-fluorosuccinate enzymically (although the free acid was not isolated) by the use of racemic 2-methyl-2-fluoro-malonyl coenzyme A and methyl malonyl coenzyme A coupled to coenzyme A transferase in the presence of acetate, and showed that the enzyme oxidises this (2*S*)-acid with a V_{max} approaching its natural substrate. They also reported failed attempts at the resolution of the racemic material due to elimination of hydrogen fluoride from the alkaloid salts. Harper and Blakley[83], in a study of the metabolites of *p*-fluorophenylacetic acid (45) generated by a *Pseudomonal* mutant, were able to isolate *inter alia* an optically active fluorosuccinic acid which exhibited a positive Cotton effect in the optical rotary dispersion (ORD) spectrum. On the basis that (2*S*)-halosuccinates in general had previously been shown to exhibit positive ORD Cotton effects[84] they designated their metabolite as (2*R*)-(+)-fluorosuccinic acid (46) and proposed the following route for its formation (FIGURE 17).

More recently Olah and Welch[85] have demonstrated the potential of the polyhydrogen fluoride - pyridine reagent for the preparation

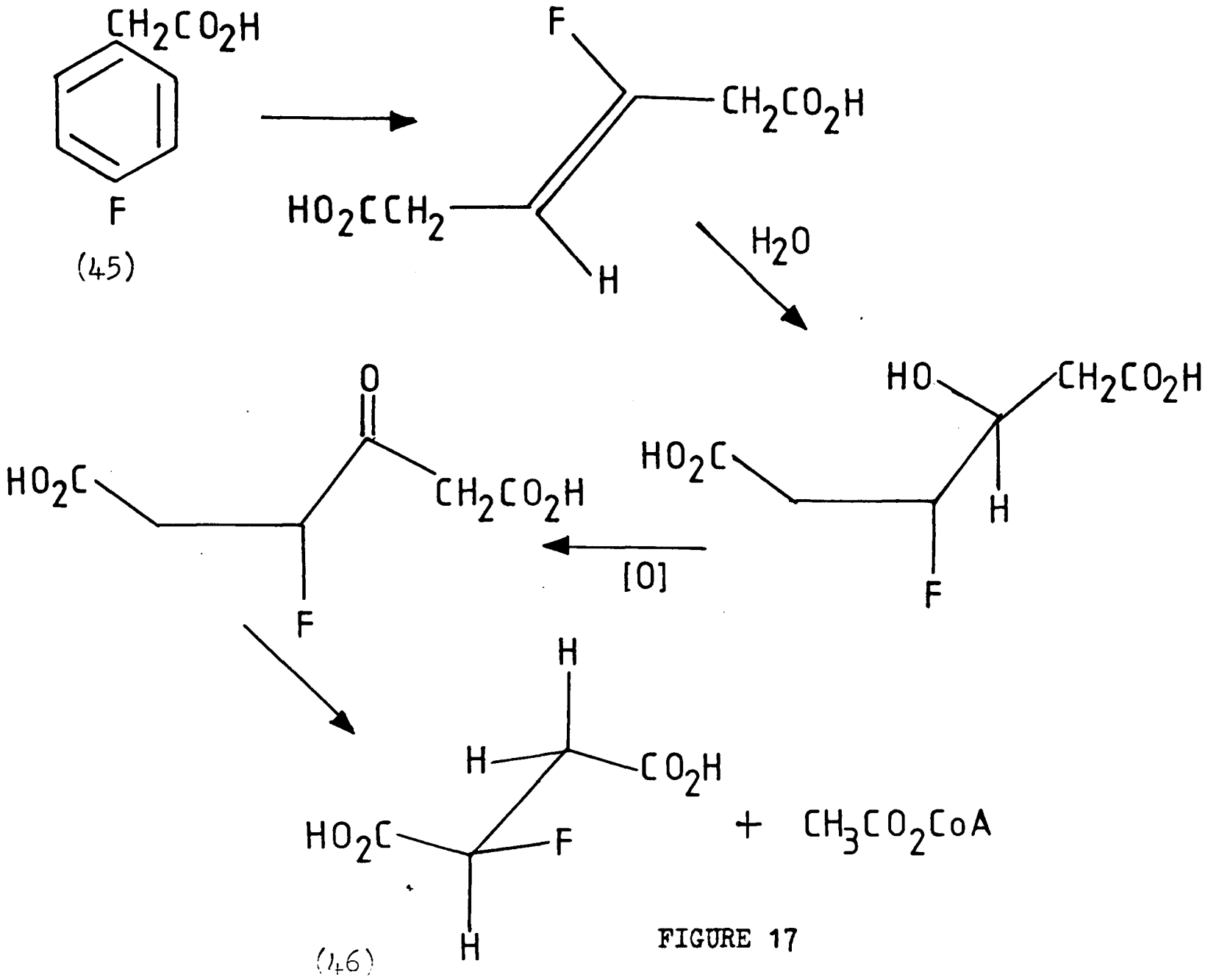


FIGURE 17

of α-fluorocarboxylic acids by the diazotisation of α-amino acids in this medium. Fluorosuccinic acid has been prepared by these workers in 52% yield in this fashion, although no stereochemical consequences of the reaction appear to have been reported. Thus aside from this synthesis, much difficulty has been experienced in the chemical preparation of monofluorosuccinic acid and the only really successful method makes use of the violent reagent perchloryl fluoride which requires special apparatus for its use. Moreover, no chemical synthesis of the *enantiomers* of monofluorosuccinic acid has ever been devised, nor has the tendency to base-catalysed elimination of hydrogen fluoride permitted resolution of the racemic mixture by conventional means.

During our studies on the mechanism of action of the enzyme fumarase, and because of our interest in (2*S*)-fluorosuccinic acid as a valuable analogue of (2*S*)-malic acid (see CHAPTER 4), the natural substrates of fumarase and other enzymes, we undertook an

investigation into the possibility of a stereospecific synthesis of the enantiomers of fluorosuccinic acid, (2*R*)-fluorosuccinic acid (46) and (2*S*)-fluorosuccinic acid, (47) from optically active malate precursors and diethylaminosulphur trifluoride (DAST) (25).

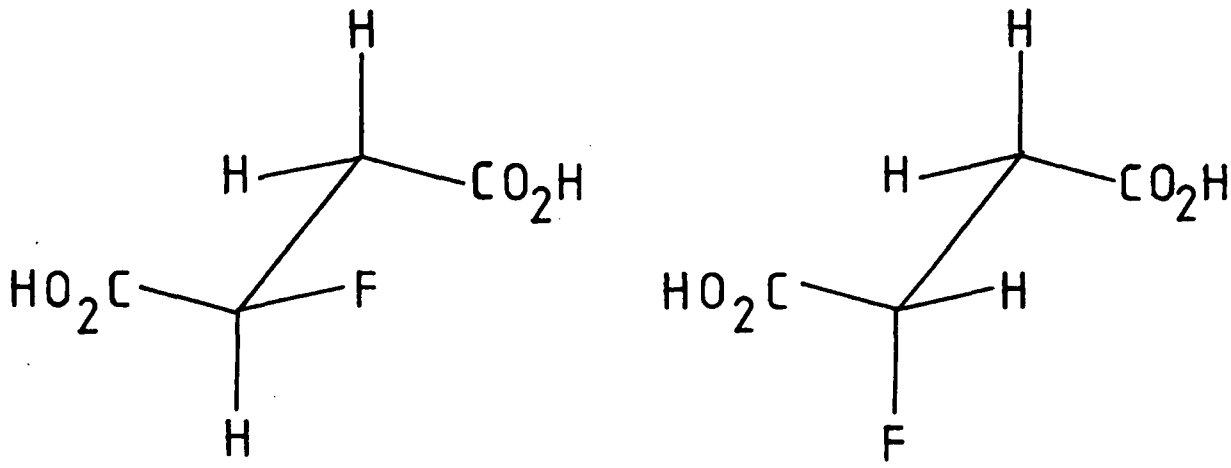


FIGURE 18

Diethylaminosulphur trifluoride (25)

The recently developed mild fluorinating agent diethylaminosulphur trifluoride (25)[53-55] has already found extensive application in the synthesis of monofluoro derivatives of sensitive alcohols and *gem*-difluoro derivatives of ketones using very mild conditions. High yields of fluorinated compounds are obtained as dehydration and elimination side reactions are less of a problem with DAST than with other fluorinating agents. For example in the replacement of a hydroxyl group of an alcohol by fluorine two common side reactions are carbonium ion type rearrangements and dehydration. Middleton[55] reports that crotyl alcohol (48), which is sensitive to both double bond arrangements and dehydration, reacts with

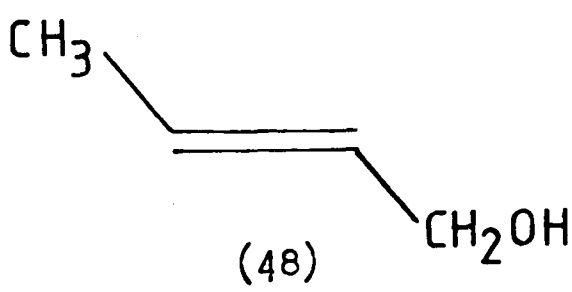
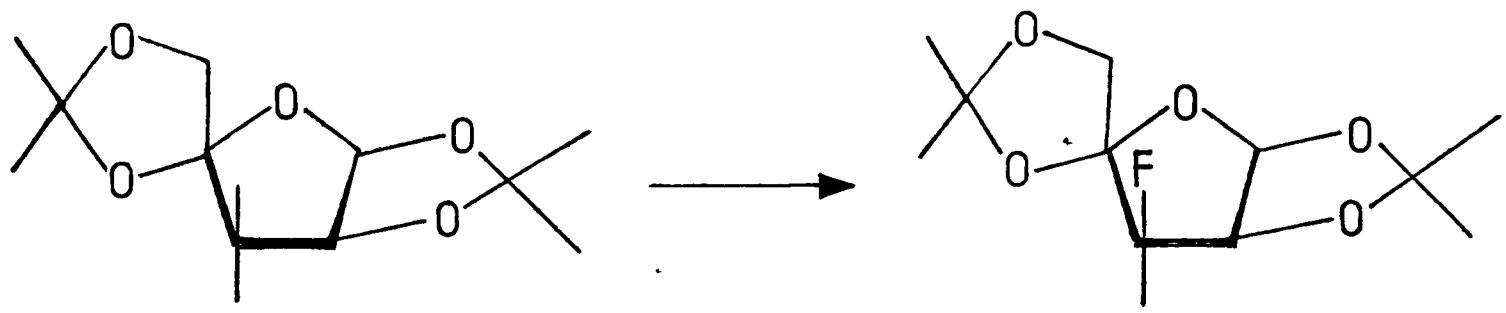


FIGURE 19

sulphur tetrafluoride to give 90% butadiene, 9% 3-fluoro-1-butene

and only a trace of crotyl fluoride. However, reaction with DAST under the same conditions gave virtually no butadiene, 72% of 3-fluoro-1-butene and 28% of crotyl fluoride.

In particular DAST has been used to prepare fluorinated benzodiazepines[86] and chrysenes[87], 6-deoxy-6-fluorohexoses[88], cephalosporins with fluorinated side chains[89], 3-deoxy-3-fluoroglucose[90] and a wide variety of fluorinated steroids[91-93], although only in three cases have stereochemical observations been reported. Tewson and Welch[90] in their synthesis of 3-deoxy-3-fluoroglucose were able to isolate the kind of alkoxydiethylamino sulphur difluoride intermediate (48a) which had been proposed by Middleton[55], and were

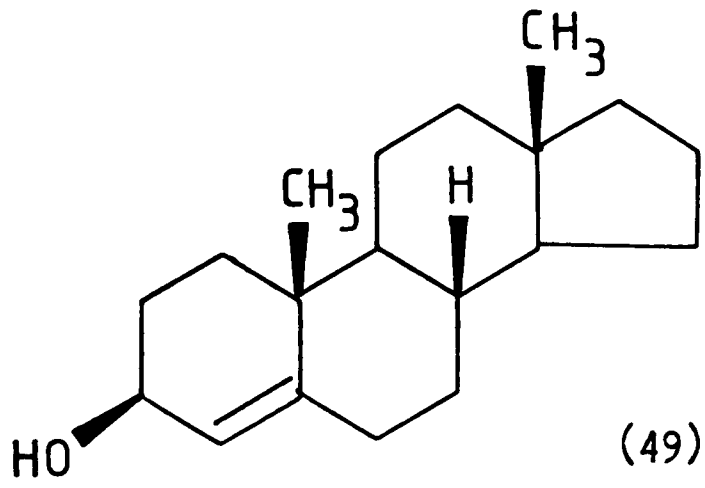


(48a)

FIGURE 20

able to demonstrate the likelihood of a discrete $S_{N}2$ displacement mechanism rather than an ion pair or cyclic transition state, by heating their intermediate (48a) with H ^{18}F in pyridine and observing that 90% of the ^{18}F label was incorporated into the final product. Rather surprisingly, however, they claim that DAST had been introduced as a reagent for effecting mild fluorination with *retention* of configuration. Bird *et al.*[93] have used DAST to prepare a wide variety of fluorinated derivatives of oxygenated 5α-androstanes. Although the most predominant stereochemical consequence was observed to be *inversion* of configuration, cases of retention also occurred, and in instances other than, for example, 3β-OH Δ^5-17-CO androstane (49) where there is potential for neighbouring group participation.

Biollaz and Kalvoda[92] have presented an interesting example of



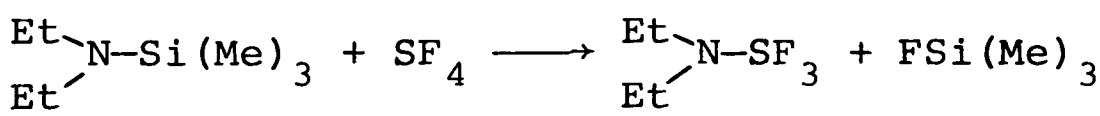
(49)

FIGURE 21

neighbouring group participation by chlorine in a DAST reaction giving overall retention of configuration. They also demonstrated that DAST caused dehydration of 9α-H, 11β-OH and 9α-F, 11β-OH steroids, such dehydrations of highly hindered centres being impossible under other conditions, and that 9α-Cl, 11β-OH steroids gave 9α-Cl, 11β-F compounds.

Preparation of DAST (25)

DAST was prepared by an adaptation of the procedure of Middleton[55] in which diethylaminotrimethylsilane (50) is treated with sulphur tetrafluoride (51) in trichlorofluoromethane at -78 °C *viz.*



(50) (51) (25) (52)

High yields of the product of excellent purity were routinely obtained since the only by-product is the low boiling fluorotrimethylsilane (52) (b.p. 17 °C) and this together with the solvent $CFCl_3$ (b.p. 25 °C) is easily removed by distillation. DAST is a stable, pale yellow liquid b.p. 30-30 °C at 1.3 mm Hg which can be distilled. It reacts explosively with water and fumes in air but

could be stored for months in plastic bottles at 0 °C.

The specially designed apparatus for the preparation of DAST is illustrated in FIGURE 22 and in order to avoid possible hazards in the preparation of this reagent precise experimental conditions are required. For the guidance of future workers a rather detailed description of the method which was developed is given here. The quantities of the reagents are given in the conventional description in the experimental section.

Glassware and rubber tubing withstood exposure to DAST and SF_4 during the short time of the preparation (*ca.* 9 hours). All apparatus was preheated to 110 °C for 3-4 hours and was quickly assembled and allowed to cool whilst being flushed with nitrogen. The complete apparatus was left in a stream of nitrogen overnight. The cold traps and the reaction vessel were cooled to -78 °C with acetone/dry ice and the reaction vessel charged with solvent trichlorofluoromethane. Sulphur tetrafluoride was condensed in the cold trap as follows. With all taps open except T_4, and a slow stream of nitrogen passing through the solution the SF_4 was allowed into the system by opening T_4 and adjusting the needle valve on the cylinder. SF_4 began to condense in the trap at a steady rate and the nitrogen flow was adjusted to obtain a steady passage of gas through the solvent. After *ca.* ½ h sufficient SF_4 (*ca.* 20 cm^3) had collected in the graduated trap and the nitrogen flow was slightly increased and the SF_4 tap closed. With a steady passage of nitrogen and the Dewar removed SF_4 was slowly carried over to the reaction flask and condensed, dissolving in the solvent (complete transfer took *ca.* ½ h). Tap T_1 was then closed and with the reaction vessel isolated a solution of freshly distilled diethylaminotrimethylsilane (50) in trichlorofluoromethane was slowly added over a period of 1-1½ h to give an orange/brown solution. At the end of this period tap T_1 was opened and the flask allowed

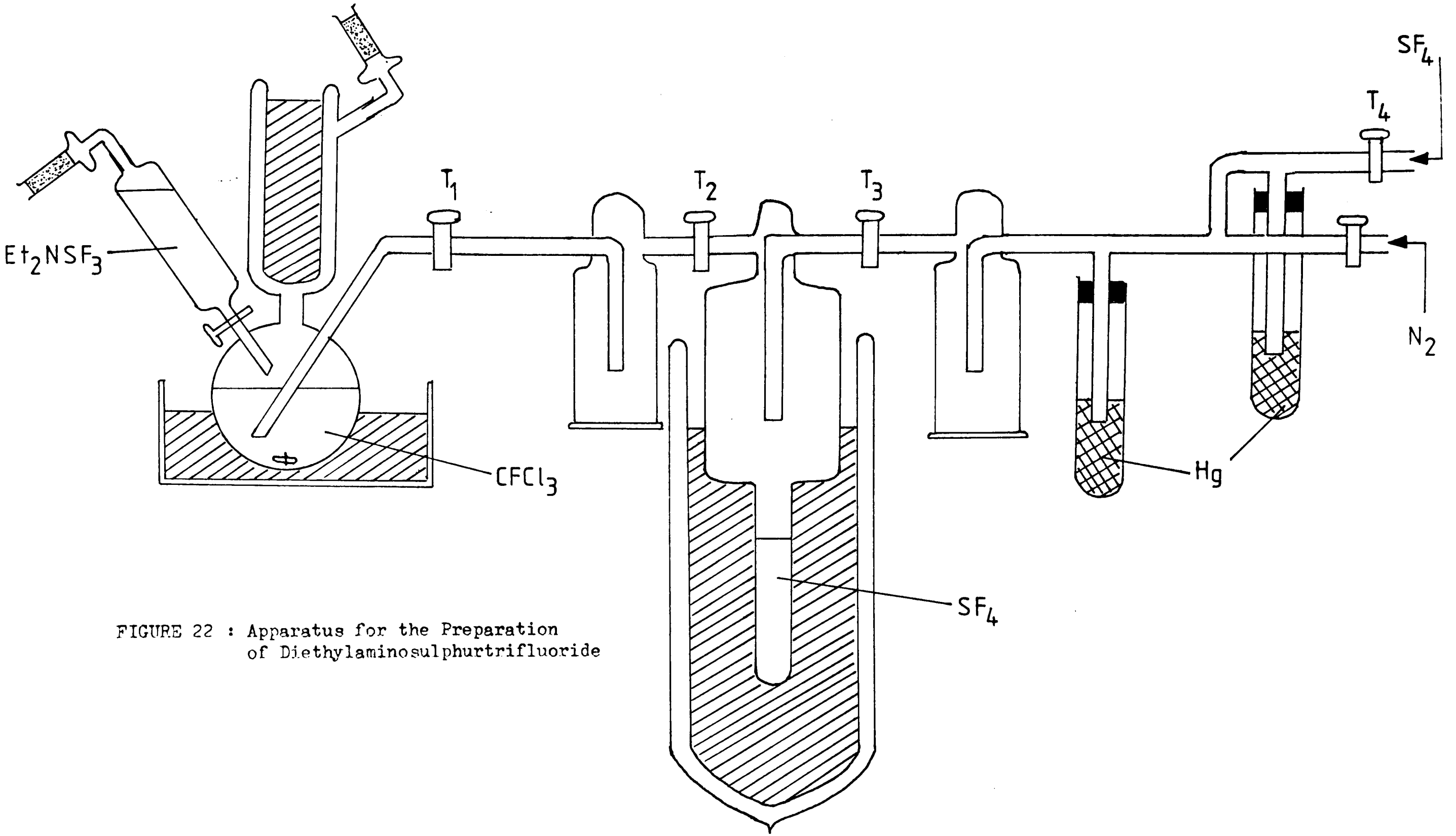
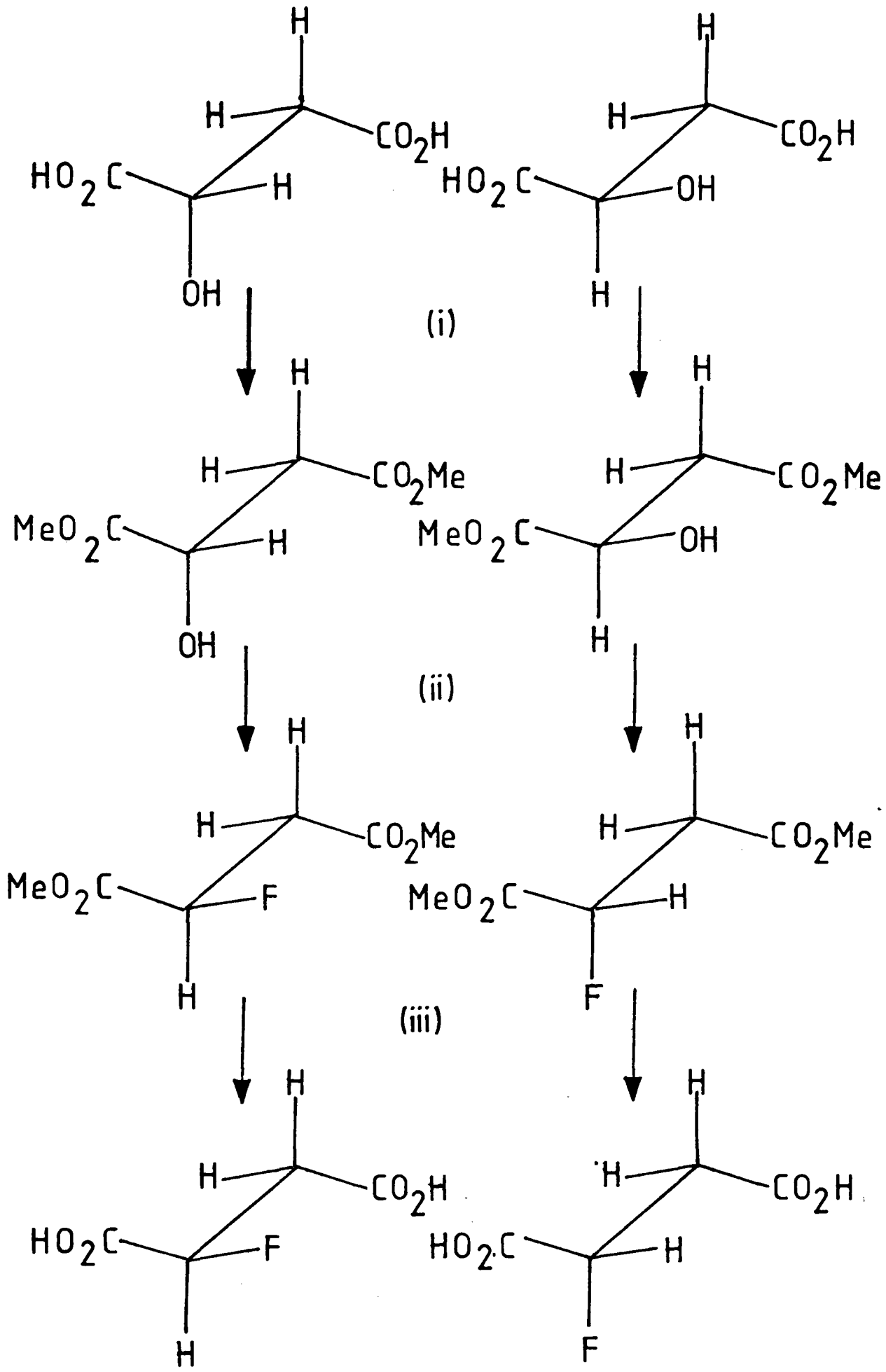


FIGURE 22 : Apparatus for the Preparation of Diethylaminosulphurtrifluoride

to warm to room temperature in a stream of dry nitrogen over 3 h. Excess SF_4 was vented to the atmosphere *via* a silica gel drying tube, and when the evolution of SF_4 had ceased the reaction flask was set up for distillation and the solvent and remaining by-product distilled off at ambient pressure by heating the reaction vessel to *not more than 40* $^{\circ}C$. The dark brown/red reaction mixture was then distilled *in vacuo* with the receiving flask immersed in acetone/dry ice to obtain diethylaminosulphur trifluoride as a pale yellow liquid.

Synthesis of the Enantiomers of Fluorosuccinic Acid

The synthetic route devised for (2*R*)-fluorosuccinic acid (47) and (2*S*)-fluorosuccinic acid (48) is illustrated in Scheme 7. Some protection was required for the carboxyl groups of (2*S*)-malic acid (53) and (2*R*)-malic acid (54), since DAST is known to readily convert carboxyl groups to acyl fluorides.[53-55] Simple ester groups were found to withstand exposure to DAST, and thus the enantiomers of malic acid were protected as their dimethyl esters to give dimethyl (2*S*)-malate (55) and dimethyl (2*R*)-malate (56) using the general methylation procedure of Brenner and Huber.[94] On some occasions, especially in the case of small quantities of material the acids were methylated quantitatively with diazomethane prepared from *p*-tolylsulphonylmethylnitrosamide. Middleton[55] suggests that reactions of hydroxyl groups with DAST be carried out in trichlorofluoromethane solution at -78 $^{\circ}C$, but no adverse effects, however, were encountered when such reactions were performed at 0 $^{\circ}C$ in *dry ethanol-free* chloroform solution. Standard work-up and removal of solvent gave high yields of optically active fluorosuccinic esters which were expected to be dimethyl (2*R*)-fluorosuccinate (57) and dimethyl (2*S*)-fluorosuccinate (58) respectively, the only detectable by-product being *ca.* 6% of



Reagents: (i) $SOCl_2$/MeOH or CH_2N_2/MeOH

(ii) DAST/$CHCl_3$

(iii) H_3O^+

SCHEME 7 : Synthesis of the Fluorosuccinic Acids using DAST

dimethyl fumarate (estimated by u.v. and n.m.r.). The pure ester in each case could be obtained by preparative gas-liquid chromatography (g.l.c.) using a PEGA column operating at 150 °C with an inlet temperature of 190 °C. Interestingly enough, higher temperatures were found to cause complete thermal dehydrofluorination of the ester to give dimethyl fumarate.

Noting the sensitivity of fluorosuccinic acid to even mildly basic conditions as observed by Bergmann and Szinai[79], and the success of Dean and Pattison[80] in their hydrolysis of diethyl (2*RS*)-fluorosuccinate, it was decided to attempt the deprotection under acid conditions. Although some elimination of HF did occur under these conditions, as evidenced by the presence of *ca.* 10% fumaric acid in the final acids, this could be removed by recrystallisation from a variety of solvents to give optically active fluorosuccinic acids, presumably (2*R*)-fluorosuccinic acid (57) and (2*S*)-fluorosuccinic acid (58). The melting points of these crystalline fluorosuccinic acids (130-132 °C) were virtually identical with that of the (+)-fluorosuccinic acid isolated by Harper and Blakley[83], which being the product of an enzymic degradation might be expected to be optically pure. The melting point of the racemic acid is 144 °C.[80] Although it seemed most likely that the fluorination reaction had proceeded with inversion of configuration it was desirable to have positive evidence of this. Moreover, as will be discussed subsequently the circular dichroism spectra of these acids, although exhibiting gratifying mirror-image curves, showed that they were exhibiting Cotton effects *opposite* to what might have been expected on the basis of extrapolation from other halosuccinic acids of known absolute configuration.[84] ((2*R*)-bromo- and (2*R*)-chlorosuccinic acid both show *positive* Cotton effects whereas the present synthetic fluorosuccinic acid obtained by the reaction of DAST with dimethyl (2*S*)-malate showed a *negative*

Cotton effect). Thus in order to make confident configurational assignments a rigorous method of determining the stereochemistry was required.

Determination of the Stereochemical Consequence of the DAST reaction

Initially an attempt was made to distinguish between the two enantiomers of the fluoroester by using the chiral shift reagent *tris*-[3-trifluoroacetyl-*d*-camphorato]Europium(III) [Eu(facam)$_3$][96,97] in order to obtain an estimate of the optical purity. Attention was focussed on the methine proton of the chiral centre. This gave rise to a simple doublet of triplets in the ^{1}H n.m.r. spectrum. Examination of the n.m.r. spectrum of g.l.c.-purified dimethyl fluorosuccinate from dimethyl (2*S*)-malate (55) in the presence of Eu(facam)$_3$ (b) showed a negligible broadening of the methine proton signal compared to that recorded in the absence of shift reagent (a) (see FIGURE 23). However, a 1:1 mixture of dimethyl fluorosuccinate from each dimethyl malate ester precursor in the presence of Eu(facam)$_3$ showed a methine proton signal clearly split by *ca.* 2 Hz. (c), thus demonstrating that the two enantiomers could be distinguished purely on the basis of their pseudo-contact shifts with Eu(facam)$_3$. Unfortunately no greater shift than 2.5 Hz could be induced by this method (and the ^{19}F n.m.r. line widths were too large to be of any use) so it was not possible to obtain the fully resolved situation where accurate enantiomer ratios could be estimated by integration. However, the marked lack of broadening of the g.l.c.-purified enantiomer in the presence of Eu(facam)$_3$ demonstrated that the esters possessed high optical purity.

The stereochemistry of the reaction of DAST with dimethyl malate, and a better indication of the optical purity of the product was, however, obtained by the following procedure (see SCHEME 8). Fumaric acid (59) was hydrated to 3-deuterio-(*S*)-malate (60) by the

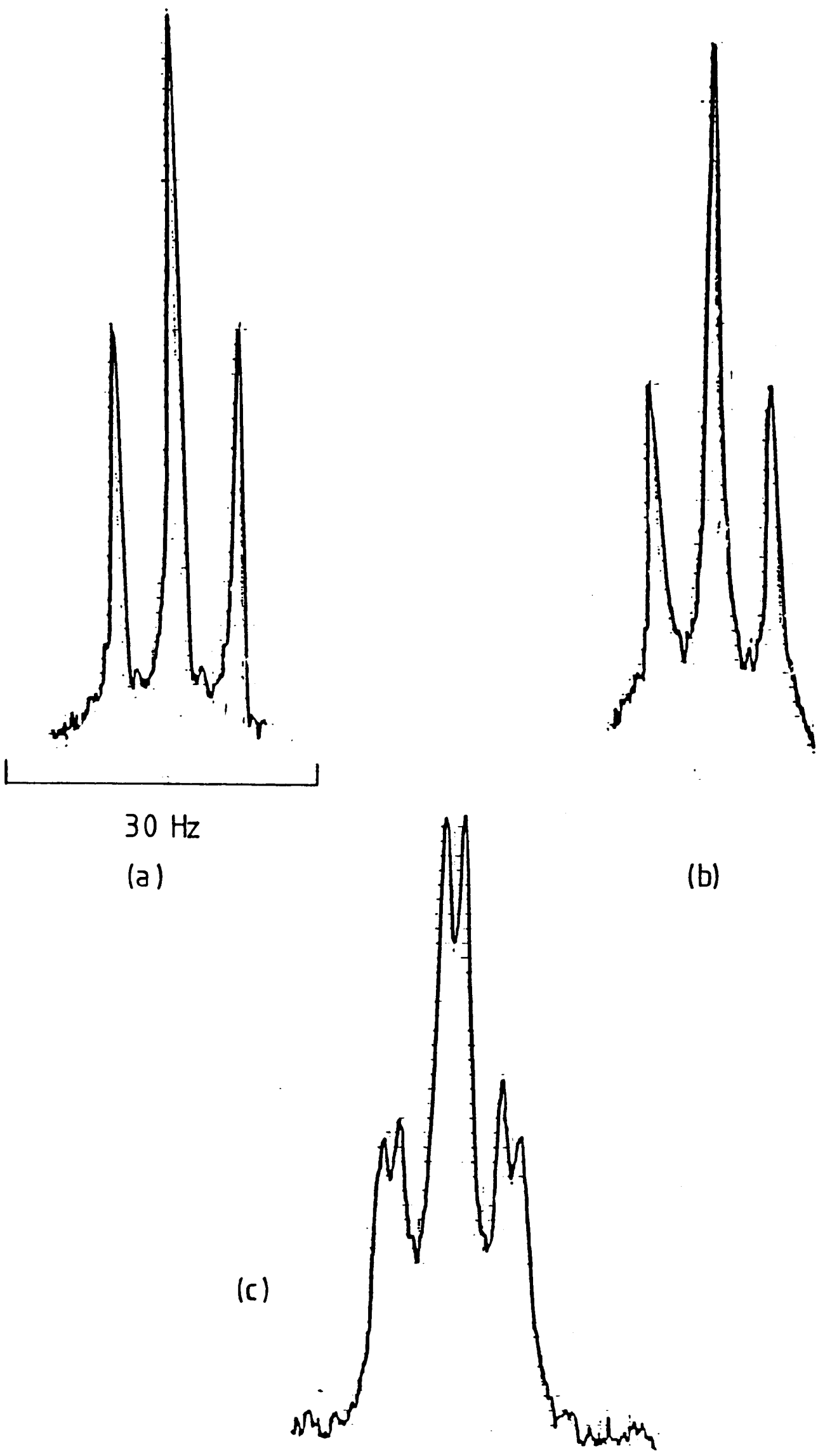
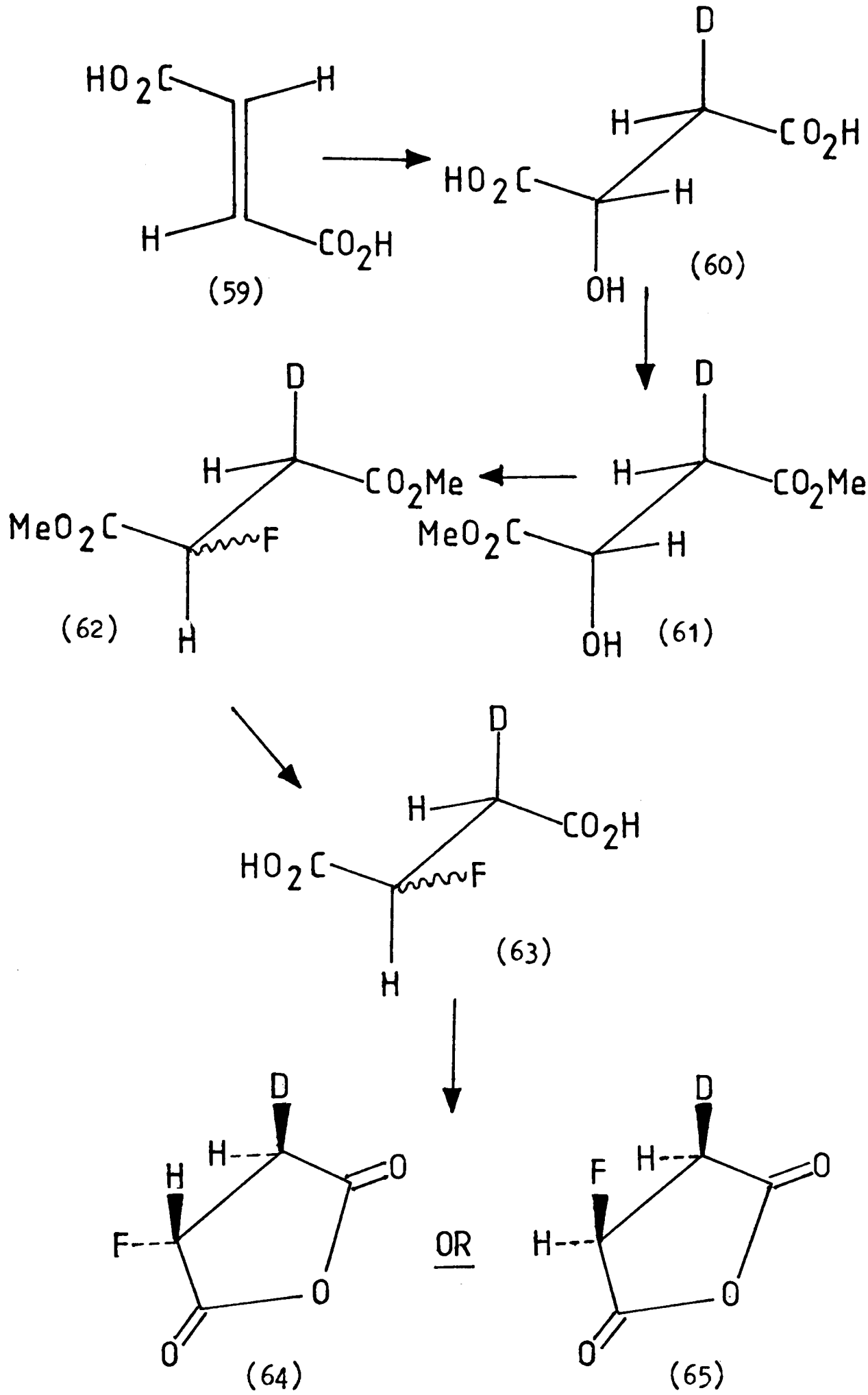


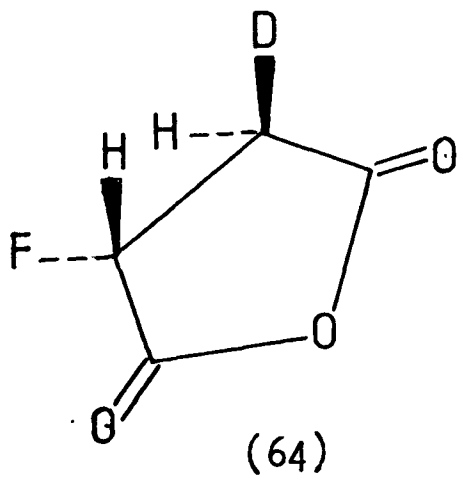
FIGURE 23 : One Half of the 90 MHz ^{1}H n.m.r. Resonance of the Methine Proton for Solutions of Dimethyl Fluorosuccinate in Carbon Tetrachloride. (a) Dimethyl Fluorosuccinate (from Dimethyl (2S)-Malate) (0.37M). (b) As for (a), but in the presence of $Eu(facam)_3$ (0.067M). (c) An equimolar mixture of both optically active fluoroesters in the presence of $Eu(facam)_3$(0.067M).



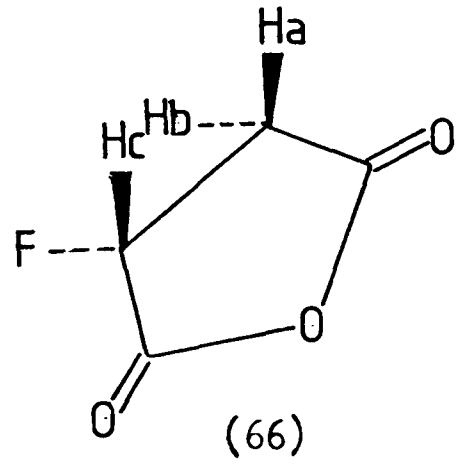
SCHEME 8 : Stereochemical Analysis of the Reaction of DAST with Dimethyl (2S)-Malate.

action of fumarase in deuterium oxide.[98,99] Since it is known that the hydration occurs by a stereospecific *trans* addition the product was designated as (2*S*,3*R*)-[3^2H]-malic acid (60). Esterification with diazomethane yielded dimethyl-(2*S*,3*R*)-[3^2H_1]-malate (61) which was reacted with DAST as described for the unlabelled compounds. Acid catalysed hydrolysis of the resulting ester (62) of unknown configuration at the fluorine centre gave the corresponding deuterio-fluorosuccinic acid (63), also of unknown configuration, which was cyclised using acetyl chloride to the five-membered anhydride (by the same process as for succinic anhydride) which will be either (2*R*,3*S*)-[3^2H_1]-fluorosuccinic acid cyclic anhydride (64) if the DAST reaction has proceeded with *inversion* of configuration, or (2*S*,3*S*)-[3^2H_1]-fluorosuccinic acid cyclic anhydride (65) for *retention* of configuration.

The relative stereochemistry of the two chiral centres could now be examined by n.m.r. It was found easiest to use ^{19}F n.m.r. spectroscopy. An examination of the ^{19}F n.m.r. spectrum of the undeuteriated anhydride (66) derived from (2*S*)-malate demonstrated two $^3J_{HF}$ vicinal coupling constants of 13.5 Hz and 24.9 Hz which were assigned to $^3J_{HaF}$ and $^3J_{HbF}$ respectively (for the stereoisomer shown in FIGURE 24). $^2J_{HcF}$ was always about 50 Hz. (Electron



(64)



(66)

FIGURE 24

diffraction studies of succinic anhydride[100] and tetrafluoro-succinic anhydride[101] have established the essential planarity of the ring system, at least in the crystal and gas phases). Thus it was expected that for a deuteriated anhydride derived from a

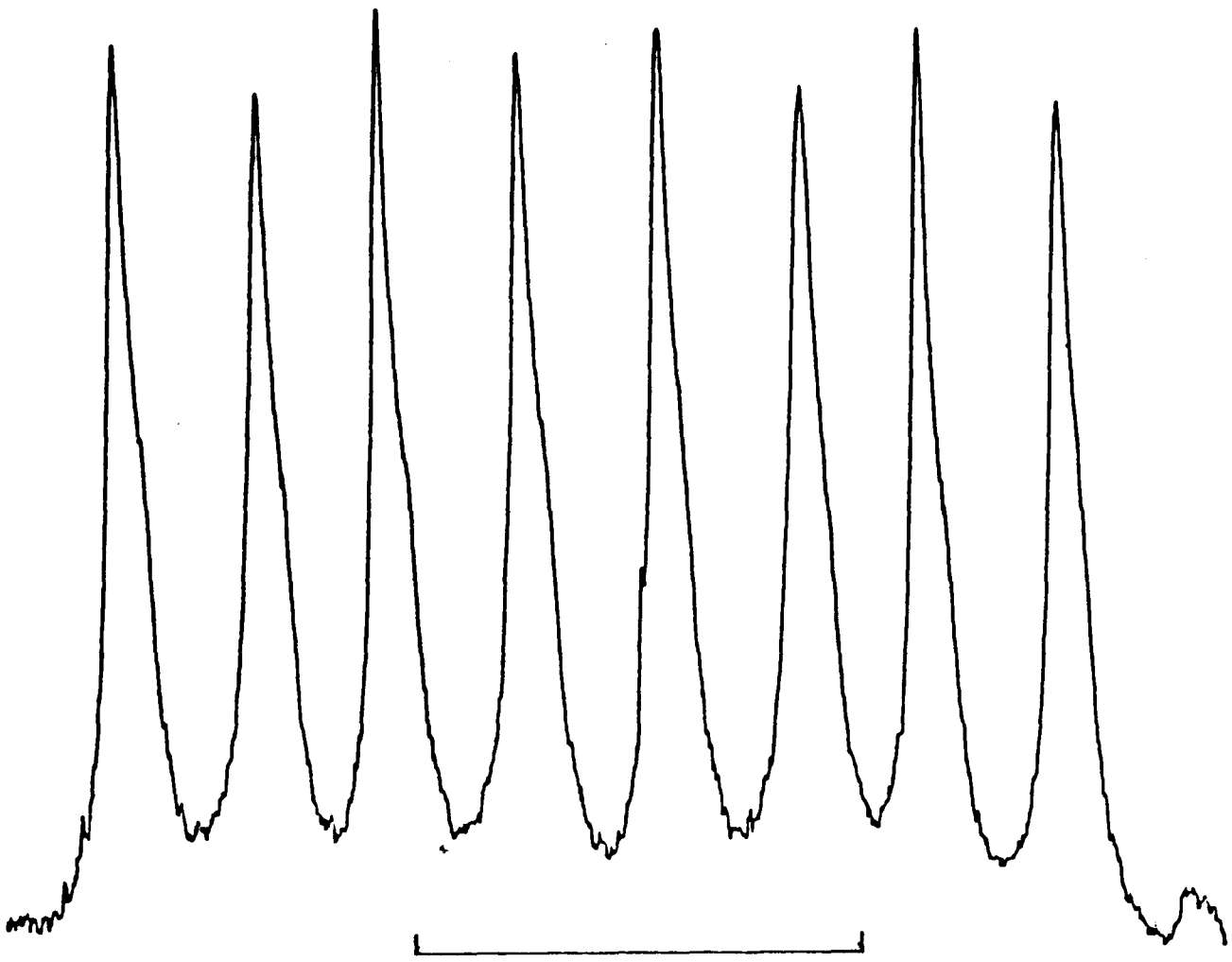
dimethyl malate-DAST reaction proceeding with *inversion* of configuration one would see a $^{3}J_{HF}$ coupling of *ca.* 25 Hz, and for *retention* a $^{3}J_{HF}$ coupling of *ca.* 13.5 Hz. Moreover, should the reaction have occurred with partial racemisation one would expect to see more than four lines in the ^{19}F n.m.r. spectrum, and be able to estimate the enantiomeric purity by integration of the well separated signals.

In the event the ^{19}F n.m.r. spectrum of the product showed only four lines with $^{2}J_{HF}$ = 50 Hz and $^{3}J_{HF}$ = 25 Hz (FIGURE 25), and thus this latter coupling establishes unequivocally the stereochemistry of the anhydride as (64), and the *inversion* of configuration in the fluorination reaction. Whereas a $^{3}J_{DF}$ coupling of *ca.* 4 Hz could be observed in the acyclic species (see FIGURE 26), no finer structure due to this coupling could be seen superimposed on the ^{19}F n.m.r. signals for the anhydride (64), although the lines had $\Delta V_{\frac{1}{2}}$ *ca.* 6 Hz (compare *ca.* 3 Hz for the undeuteriated anhydride (66). It is generally known that fluorine-deuterium coupling constants are approximately $\frac{1}{6}$th of the equivalent fluorine-proton coupling constants (more exactly $J_{HF} = 6.514 \times J_{DF}$ - this represents the ratio of the gyromagnetic constants for hydrogen and deuterium).

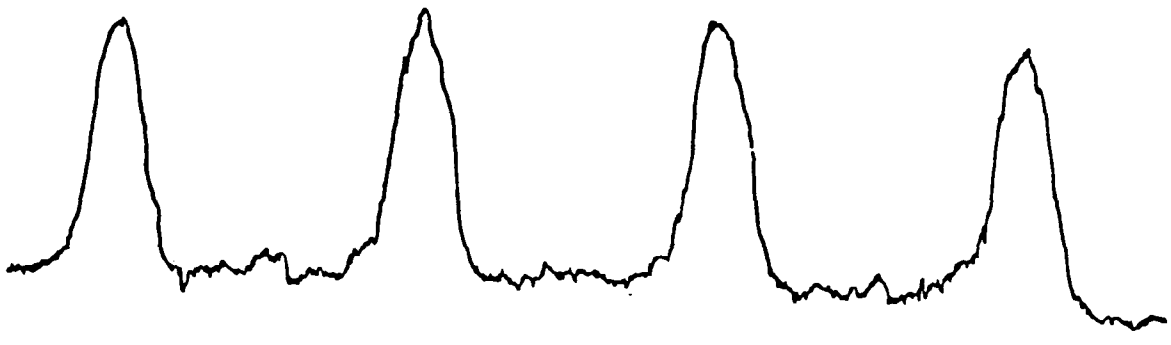
This observation of no apparent $^{3}J_{DF}$ coupling provides additional evidence for structure (64) as does the $^{3}J_{HH}$ coupling constant of 4.5 Hz since the undeuteriated anhydride (66) exhibited $^{3}J_{HaHb}$ = 8.5 Hz and $^{3}J_{HbHc}$ = 6.0 Hz. All these assignments are based on extensive literature precedent, are entirely in accord with expectation[102], and the magnitudes of all three coupling constants are those expected for structure (64).

Thus the following sequence of reactions has been performed. Dimethyl (2*S*,3*R*)-[$3^{2}H_{1}$]-malate (61) is transformed by DAST into dimethyl (2*R*,3*S*)-[$3^{2}H_{1}$]-fluorosuccinate* (62) which is hydrolysed

* The change in configurational assignment at C-3 is a result of the sequence rules governing the *RS* notation.[412,413]



The 84.6 MHz ^{19}F n.m.r. Spectrum of (2R)-Fluorosuccinic Acid Cyclic Anhydride in Deuteriochloroform



The 84.6 MHz ^{19}F n.m.r. Spectrum of (2R, 3S)-[$3^{2}H_{1}$]-Fluorosuccinic Acid Cyclic Anhydride in Deuteriochloroform (64)

FIGURE 25

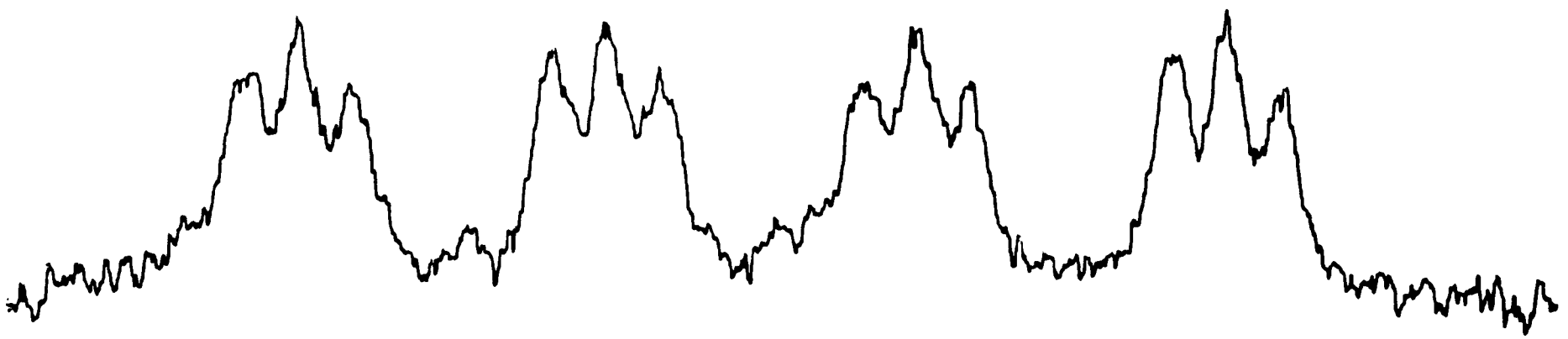


FIGURE 26 : The 84.6 MHz ^{19}F n.m.r. Spectrum of (2R,3S)-[$^{3}H_{1}$]-Fluorosuccinic Acid in D_2O illustrating the Effects of ^{19}F-^{1}H and ^{19}F-^{2}H Spin-Spin Coupling.

to (2*R*,3*S*)-[3^2H]-fluorosuccinic acid (63). Finally treatment with acetyl chloride gives (2*R*,3*S*)-[3^2H_1]-fluorosuccinic acid cyclic anhydride (64). In addition the undeuteriated fluorosuccinic acid cyclic anhydride (66) may now be designated as (2*R*)-fluorosuccinic acid cyclic anhydride (66) with the stereochemistry as shown in FIGURE 24. The presence of only four lines in the spectrum of compound (64) indicates also that the reaction has occurred with a clean stereochemical consequence.

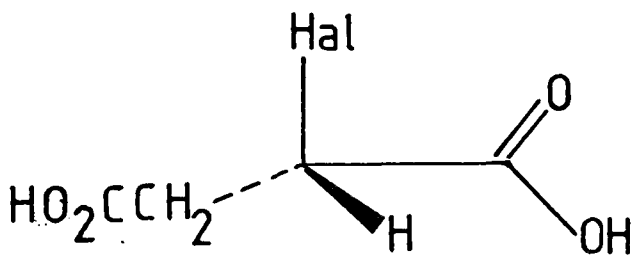
The Circular Dichroism Spectra of α-Halosuccinic Acids

Studies using simple α-hydroxy acids such as lactic, malic and tartaric acid led to some of the early theories of optical activity and to assignments of absolute configuration.[103] Recently a number of investigators[84,104-108] have re-examined the ORD and CD spectra of these compounds, and with the advent of the newer generation of instruments opening up the region below 200 nm for exploration, they have extended measurements into the ultraviolet to include the region of absorption of the carboxyl group (until recently no absorption band had been accessible for the carboxylic acids and their derivatives).

The situation for the carboxyl chromophore, however, is very different from the experimentally well explored saturated carbonyl group where we have a carbonyl group of well established symmetry for which our theoretical knowledge of the transitions involved in the absorption and CD bands is essentially adequate, and whose absorption band was easily within the range of the earlier instruments. Even today with the carboxyl group we are still restricted by the fact that the absorption and CD bands of carboxyl and related groups are rather near the lower limit of penetration of even modern instruments. Moreover, whereas in the classical studies of the saturated ketones the synthesis of such generalisations as the octant rules[109] and the axial haloketone rule[110]

depended upon the measurement of ORD and CD spectra of compounds of fixed conformation which were relatively easy to obtain, carboxyl compounds are particularly difficult examples to study on account of their numerous conformational possibilities due to free rotation about the double band joining the carboxyl group to the rest of the molecule. Since we know relatively little about the preferred conformations attempts to formulate a sector rule[111] analogous to the octant rule have primarily been based upon conformationally rigid lactones.

With a sound theoretical background the potential of CD spectroscopy as a conformational probe is obviously very great, and in this respect Listowsky *et al.*[112] have uncovered some finer points in the CD spectra of certain acyclic carboxylic acids and have showed that the temperature and solvent dependence of CD paramters may be rationalised by the presence of two structural species in equilibrium. Thus, for example, in aqueous solutions of α-hydroxy acids the CD bands at 240 nm (weak) and 210 nm (strong and of opposite sign) are recognised as manifestations of specific rotamers of the acids. They suggested that the rotamer (67) in FIGURE 27 is the preferred conformer and is responsible for the 210 nm band shown by the halosuccinic acids. Gaffield[113] has made careful study of simple aliphatic α-chloro and α-bromo acids. All



(67)

FIGURE 27

acids of the same stereochemical series were found to give curves of the same antipodal type. These results have been rationalised in terms of two conformations (68) and (69) and an *octant* type rule

as shown in FIGURE 28.

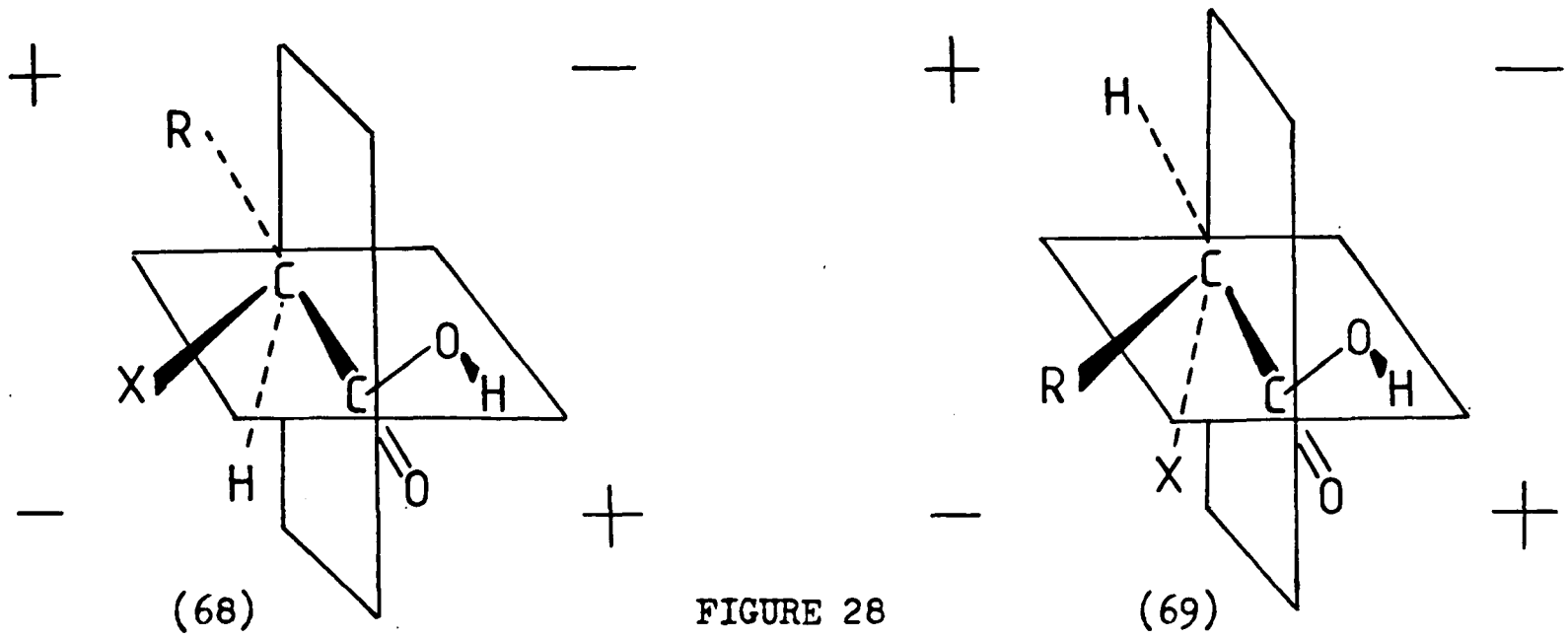


FIGURE 28

In conformation (68) which is responsible for the shorter wavelength band at 200-220 nm the halogen atom X is eclipsed with the carbonyl, and the ethyl group R makes a positive octant contribution. In the alternative conformation (69) which gives rise to the longer wavelength band (usually the smaller band at 240-260 nm) the ethyl group is eclipsed and the halogen atom makes a positive octant contribution.

Craig *et al.*[114] have investigated the effect of conformational preference on the chiroptical properties of α-substituted succinic acids using variable temperature CD spectroscopy, and solvents of different polarity. Their results indicate that α-halo acids and esters exist in the same conformation (70) with the C_α-C_β bond eclipsed by the C = 0 bond. (FIGURE 29).

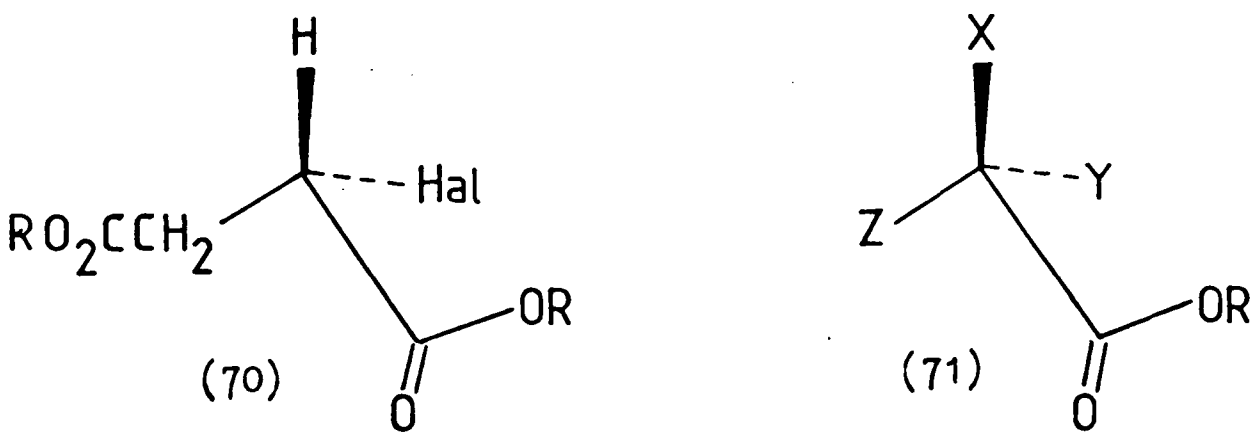


FIGURE 29

3 possible conformers

	X	Y	Z
1d	CH_2CO_2R	H	R'
1e	H	R'	CH_2CO_2R
1f	R'	CH_2CO_2R	H

All their evidence indicates that when R' is alkyl or halogen in (71) the preferred conformation is 1e, and when R' is hydroxy, methoxy or amino the preferred conformation is 1d with the hetero-atom eclipsed. In either case 1f is the least favoured on steric grounds.

These studies, although they support the work of Listowsky in so far as a conformational equilibrium for α-methylsuccinic acid is concerned, do *not* agree with his results for α-bromo- and α-chlorosuccinic acid, since a different behaviour was observed on increasing the temperature. Moreover, Listowsky's 'empirical rule' for prediction is discredited since he incorrectly utilises the sign of the 200 nm CD band as that of the $n \rightarrow \pi^*$ carbonyl transition instead of that at 220 nm. The new investigators rationalise their results in terms of *two* optically active transitions, the lower wavelength CD band being assigned to an $n \rightarrow \sigma^*$ transition for C-Br and the higher wavelength band to an $n \rightarrow \pi^*$ transition.

An octant projection (72) is proposed which predicts successfully the sign of the ellipticity of the $n \rightarrow \pi^*$ transition for any α-substituted succinic acid or ester with appropriate substituent R' and appears also to apply to simple alkanoic acids and esters with the same substituents (FIGURE 30).

In the above octant projection the sign of the Cotton effect will be determined solely by the position of the group X or Y in

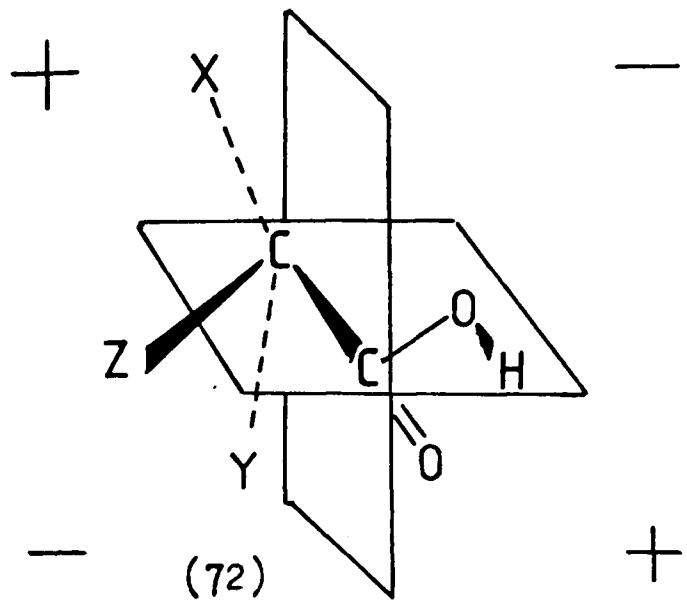


FIGURE 30

the back octants. Since in conformations 1d and 1e one of the two groups is always H, accordingly the sign is always determined by the position of the other group. This octant projection is thus claimed to predict the expected sign of the ellipticity of the $n \rightarrow \pi^*$ transition in the 200-250 nm region for any alkyl, halo, hydroxy, alkoxy or amino succinic acid or ester.

Fluorine-induced Anomalous Circular Dichroism

Perhaps the most well known case of an anomalous CD due to fluorine substitution, as compared with bromine, chlorine and iodine, is in its violation of the axial haloketone rule first proposed by Djerassi[110] in 1957 which states that an adjacent halogen equatorial to a keto group in a cyclohexane (73) does not alter the sign of the Cotton effect with respect to the unhalogenated ketone, whereas axial halogen may do so. Whence, looking

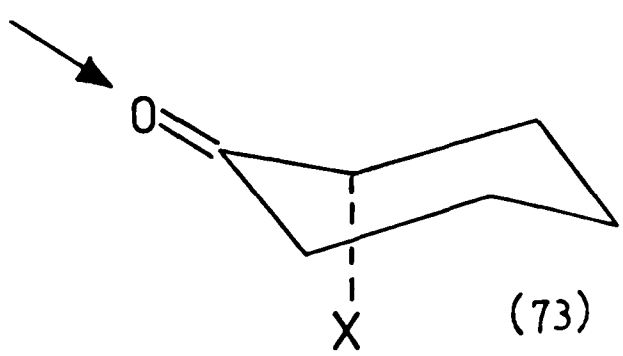
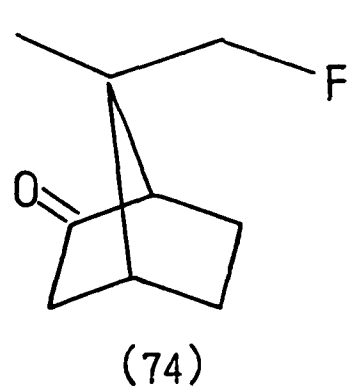


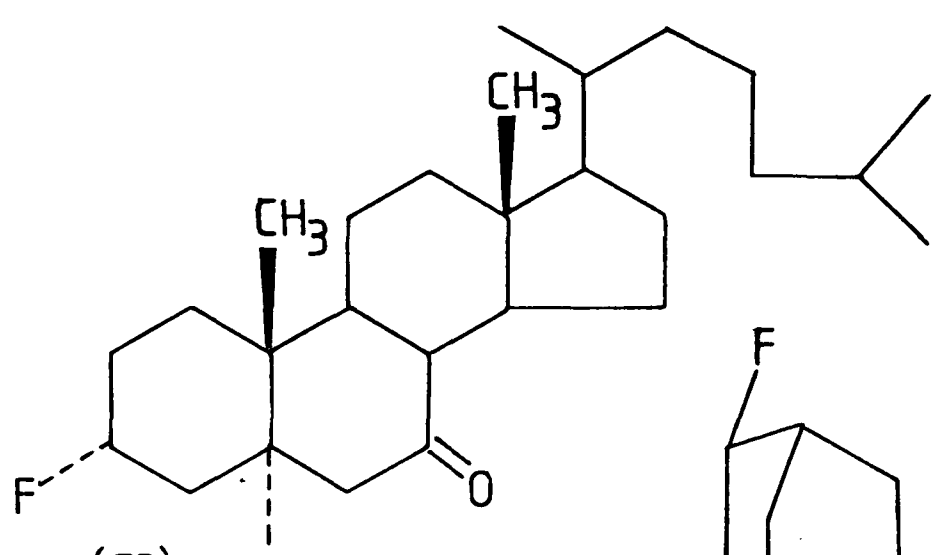
FIGURE 31

down the C = 0 axis of the cyclohexanone if the axial halogen is to

the left as indicated there will be a negative Cotton effect, and *vice versa*. It was, however, realised at a very early stage that fluorine gives rise to effects of sign *opposite* to those from chlorine, bromine and iodine. Extensive studies have been made by Hudec[115,116] in the area of long range interactions of substituents and their effects upon CD spectra. However, the CD spectra of the fluorinated camphor derivative (74) and the δ-fluorinated steroid (75) show no anomalous effects on the carbonyl n → π* transitions as far as the sign of the CD band is concerned, although many have



(74)



(75)

(76)

FIGURE 32

been observed.[117]

Anomalous effects of fluorine substitution, *inter alia*, have also been found by Snatzke and Eckhardt[118] in their studies on substituted adamantanones *e.g.* (76).

Although the techniques of ORD and CD spectroscopy have been applied to a wide range of optically active α-substituted carbonyl compounds, the only halosuccinic acids for which Cotton effects have been reported[84] are the chloro- and bromo-acids. Fredga *et al.*[84] demonstrated that compounds of the (*R*)-configuration gave positive Cotton effects when the substituent is alkyl, aryl or halogen and negative Cotton effects when the substituent is hydroxyl or amino.

Present CD Studies on Fluorosuccinic Acid

In this thesis the CD spectra of the 2-fluorosuccinic acids and their dimethyl esters obtained by the DAST route have been investigated in methanol solution. The spectra obtained in both cases show gratifying mirror image curves indicating that the two esters and acids have antipodal absolute stereochemistries (FIGURES 33 and 34). The single CD band observed at 214 nm in both cases is assigned as usual to the carbonyl $n \rightarrow \pi^*$ transition. The magnitude of the CD maximum $\Delta\varepsilon_{max}$ for dimethyl fluorosuccinate is only slightly smaller than that for the fluorosuccinic acid of the same configuration, as is commonly observed[119-122] (TABLE 3).

TABLE 3: CIRCULAR DICHROIC SPECTRAL DATA FOR α-HALOSUCCINIC ACIDS AND ESTERS

Compound	λ_{max}/nm (MeOH)	$\Delta\varepsilon$	Reference
(2*S*)-Fluorosuccinic acid	214	+1.08	This work[127]
(2*R*)-Fluorosuccinic acid	214	-0.97	This work[127]
(2*R*)-Chlorosuccinic acid	224	+1.17	123
(2*R*)-Bromosuccinic acid	236	+2.01	123
Dimethyl(2*S*)-fluorosuccinate	214	+0.74	This work[127]
Dimethyl(2*R*)-fluorosuccinate	214	-0.74	This work[127]
(2*S*)-Fluorosuccinic acid from (2*S*)-aspartic acid	214	-0.81	This work[127]

Introduction of fluorine into succinic acid results in a λ_{max} at 214 nm, and this continues the trend of a hypsochromic (blue) shift as set by the chloro-compound (λ_{max} 224 nm) and the bromo-compound (λ_{max} 236 nm) (TABLE 3). The signs of the Cotton effects for the free acids and their respective esters are, as expected, the same.

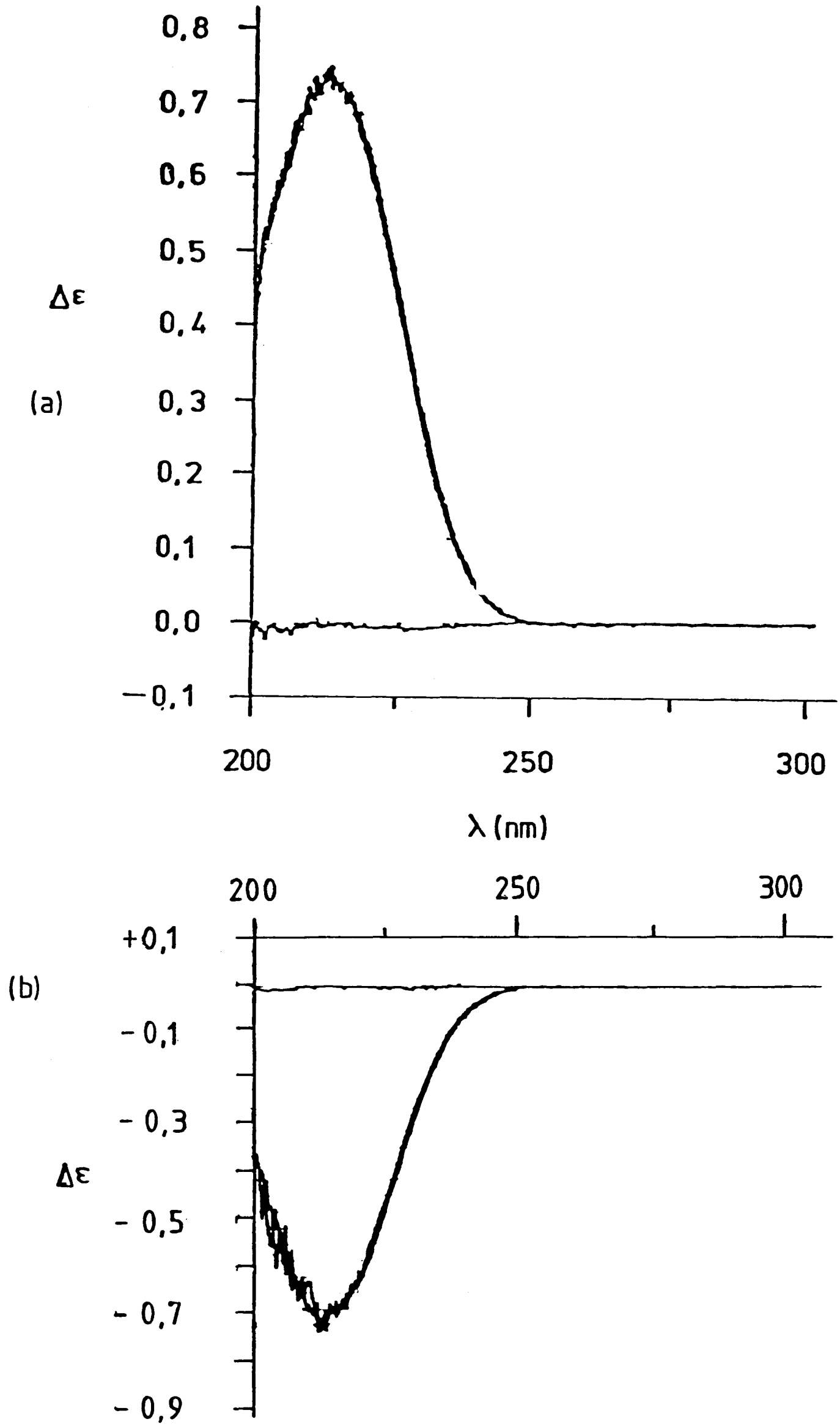


FIGURE 33 : The Circular Dichroism Spectra in Methanol of : (a) Dimethyl-(2S)-(+)-Fluorosuccinate, (b) Dimethyl-(2R)-(-)-Fluorosuccinate

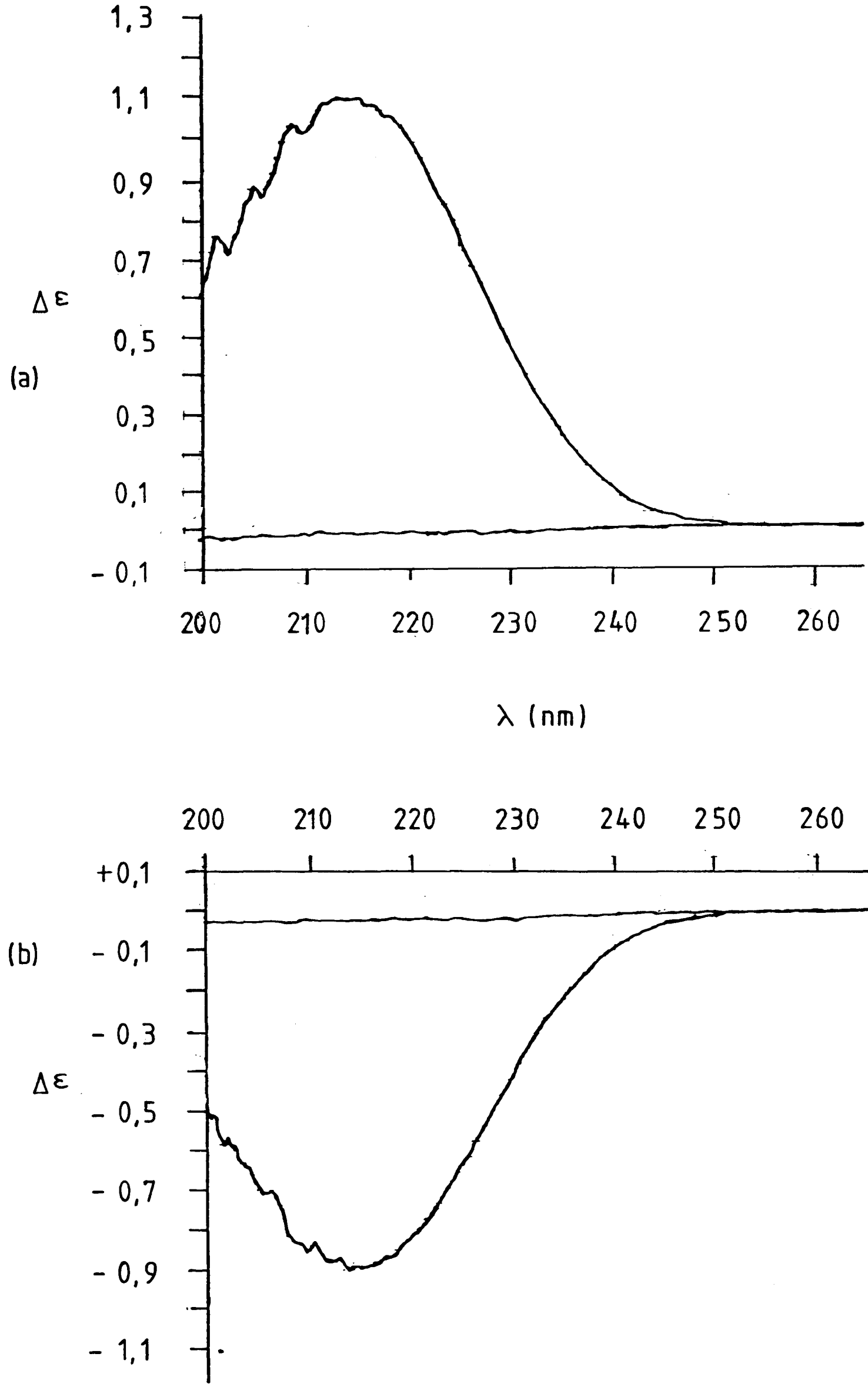


FIGURE 34 : The Circular Dichroism Spectra in Methanol of : (a) (2S)-(+)-Fluorosuccinic Acid, (b) (2R)-(-)-Fluorosuccinic Acid.

However, the overall effect of fluorine is *anomalous*, and thus dimethyl (2*S*)-(-)-malate (55) gives dimethyl (2*R*)-(-)fluorosuccinate (57), but dimethyl (2*R*)-(+)-bromo- and chloro-succinic acids. This is the first example of an anomalous fluorine effect in the CD spectra of carboxylic acids and esters and thus infringes upon the previously mentioned generalisations laid down by Fredga *et al.*[84] and Craig *et al.*[114] The absence of any other bands at longer wavelengths in both the esters and free acids indicates that they exist in predominantly one conformation in the sense previously referred to.

Fluorosuccinic acid was also synthesised by the method of Olah and Welch by the diazotisation of (2*S*)-(+)-aspartic acid (77) in hydrogen fluoride-pyridine reagent. The CD spectrum was examined and at 214 nm this acid exhibited a *positive* Cotton effect. Thus we may conclude that the transformation (2*S*)-(+)-aspartic acid to (2*S*)-(+)-fluorosuccinic acid has occurred, *i.e.* a *retention* of configuration presumably as a result of neighbouring carboxyl participation (there are many examples in the literature of α-carboxyl participation especially with the 'deaminations' of α-amino acids[124]). The $\Delta\varepsilon_{max}$ value, however, was some 25% lower than that of (2*S*)-(+)-fluorosuccinic acid prepared *via* the DAST reaction, and this may reflect a certain degree of racemisation in the dediazotisation reaction.

The ORD spectrum of a fluorosuccinic acid isolated from the metabolism of *p*-fluorophenylacetic acid (45) by a *Pseudomonal* species has been reported and is illustrated in FIGURE 35. This acid showed a *positive* Cotton effect and was therefore assigned the (*R*)-configuration by a rather unwise extrapolation from ORD evidence. This is now shown to be erroneous and we now know that the metabolite was really (2*S*)-(+)-fluorosuccinic acid (in the light of which FIGURE 17 will need to be adjusted). It was thought

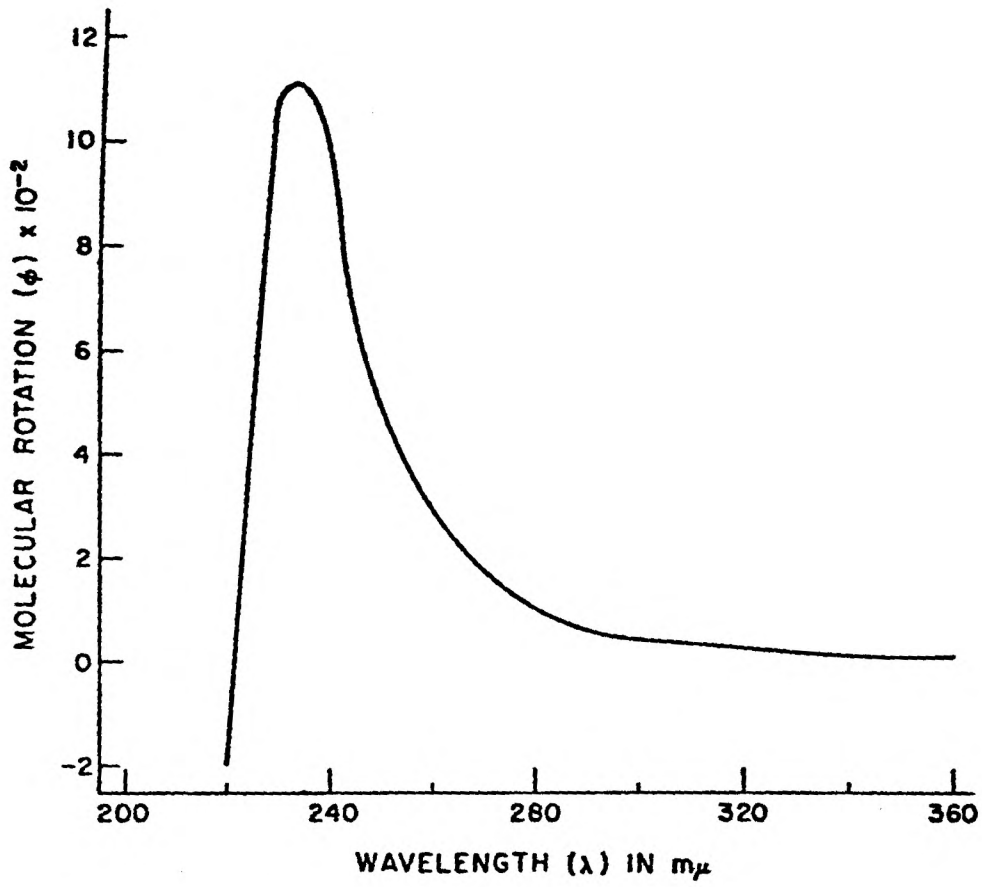


FIGURE 35 : The Optical Rotatory Dispersion Spectrum of (=)-Fluorosuccinic Acid in Methanol at 5°C from the Pseudomonal Degradation of p-Fluorophenylacetic Acid (45) after Harper and Blakley.[83]

expedient that an attempt to compare the published ORD spectrum with the CD results should be made. Unfortunately since the published spectrum was incomplete, in so far as it did not indicate the position of the ORD trough, it was not possible to make a direct comparison between the ORD molecular amplitude a and that estimated from the CD spectrum by the Krönig-Kramers semiquantitative relationship

$$a \sim 40.28\Delta\varepsilon$$

$$\text{where } a = \frac{\Phi_1 - \Phi_2}{100}$$

where Φ_1 and Φ_2 are the molecular rotation extremes. However, by computerised curve fitting an interesting comparison was made possible.

Curve fitting of CD and ORD Spectra

A comparison was made possible by use of the Krönig-Kramers transform.[125] The numerical data are presented in TABLES 4 and 5. The CD spectrum was initially fitted by computer to a single gaussian type curve described by the equation:

$$\varepsilon_K = \varepsilon_K^{\circ} e^{\frac{-(\lambda-\lambda_K)}{\Delta K^2}}$$

A CD band is commonly described by four parameters, the wavelength of the maximum circular dichroism (λ_K), the maximum value of dichroic absorption or of molar ellipticity ($\Delta\varepsilon_K^{\circ}$ or $[\theta]_{max}$ respectively), the halfwidth of the band ΔK which is the wavelength interval in which ε_K falls to $\frac{1}{e}$ ~0.368 of its maximum value, and the rotational strength R which is derived from the area under the CD band and is proportional to the intensity of the transition. The computer was allowed to vary the parameters λ_K, ε_K° and ΔK until

TABLE 4: CURVE FITTING OF CD SPECTRUM OF (2*S*)-(+)-FLUOROSUCCINIC ACID TO A SINGLE GAUSSIAN

Wavelength (nm)	[θ] observed (deg cm^2 $dmol^{-1}$)	[θ] calculated (deg cm^2 $dmol^{-1}$)
205	2739	2700
210	3362	3403
215	3561	3597
220	3287	3187
225	2261	2368
230	1555	1475
235	772	771
240	294	337
245	69	124
250	0	38

Fitted Paramters: λ_K 214 nm, $[\theta]_{max}$ 3608 deg cm^2 $dmol^{-1}$, ΔK 1485 nm.

the least mean square deviation between the observed CD spectrum and the generated gaussian band reached a minimum. Since a reasonably good fit to a single band at 214 nm was obtained it was not necessary to invoke the presence of other bands and we may infer that in methanol the fluorosuccinic acid, and presumably its methyl ester, exist in predominantly one conformation about the carbonyl chromophore, presumably that suggested by Craig *et al.*[114], although of course we must reverse the signs of the octant projection in FIGURE 30.

The procedure adopted for fitting the ORD spectrum was similar but at each iteration the fitted spectrum was generated from the peak parameters by the Krönig-Kramers equation rather than the gaussian equation. This method of curve analysis is not strictly

TABLE 5: CURVE FITTING OF THE ORD SPECTRUM OF (+)-FLUOROSUCCINIC ACID TO TWO GAUSSIANS

Wavelength (nm)	[θ] observed (deg cm^2 $dmol^{-1}$)	[θ] calculated (deg cm^2 $dmol^{-1}$)
220	-200	-220
230	1000	1050
240	1130	1010
250	480	580
260	300	310
270	180	180
280	100	120
290	60	80
300	50	50
310	40	30
320	30	20
330	20	10
340	10	10
350	0	0
360	0	0

Parameters of best fitting gaussians:

λ_K	$[\theta]_{max}$	ΔK
217.3 nm	2830 deg cm^2 $dmol^{-1}$	17.42 nm
152.4 nm	-8518 deg cm^2 $dmol^{-1}$	10.91 nm

a Krönig-Kramers transform in the normal sense, although the computer program does actually carry out several hundred Krönig-Kramers transforms during the curve fitting process. This particular technique has not been employed previously although the principle is of course fairly obvious. In this case, however, one

band was insufficient to describe the spectrum but the fitting to two bands centred at 217 nm and 152 nm appeared to be reasonable. The published spectrum does not really have the form of an ORD curve, probably due to a certain amount of artistic licence exercised by the authors.[126]

In any event in the region of interest the published ORD spectrum was transformed to give a CD maximum at 217 nm with $\Delta\varepsilon$ about 78% of that of (2*S*)-(+)-fluorosuccinic acid prepared by the DAST reaction.

CONCLUSIONS

In the light of the previous results which have been published[127] the conclusions may be summarised as follows:

(a) The fluorination of dimethyl malate by DAST proceeds with *inversion* of configuration. With the isolation of an alkoxyamino-sulphur difluoride adduct by Tewson and Welch[90] we may write a mechanism for the transformation as in FIGURE 36 with reasonable confidence. Thus dimethyl (2*S*)-(-)-malate (55) attacks the DAST molecule (25) to give the intermediate adduct (78) which is attacked by fluoride ion to give dimethyl (2*R*)-(-)-fluorosuccinate (47).

(b) The fluorination of aspartic acid (77) by hydrogen fluoride-pyridine reagent proceeds predominantly with *retention* of configuration probably due to neighbouring group participation from the adjacent carboxyl group as in FIGURE 37. Thus reaction of aspartic acid (77) with sodium nitrite yields the diazonium compound (79), and an intramolecular attack by the neighbouring carboxyl gives the inverted lactone (80) and direct displacement by fluoride ion gives (2*S*)-(+)-fluorosuccinic acid (48).

(c) The effect of α-substituted fluorine on the sign of the $n \rightarrow \pi^*$ transition in the CD spectra of α-halosuccinic acids is

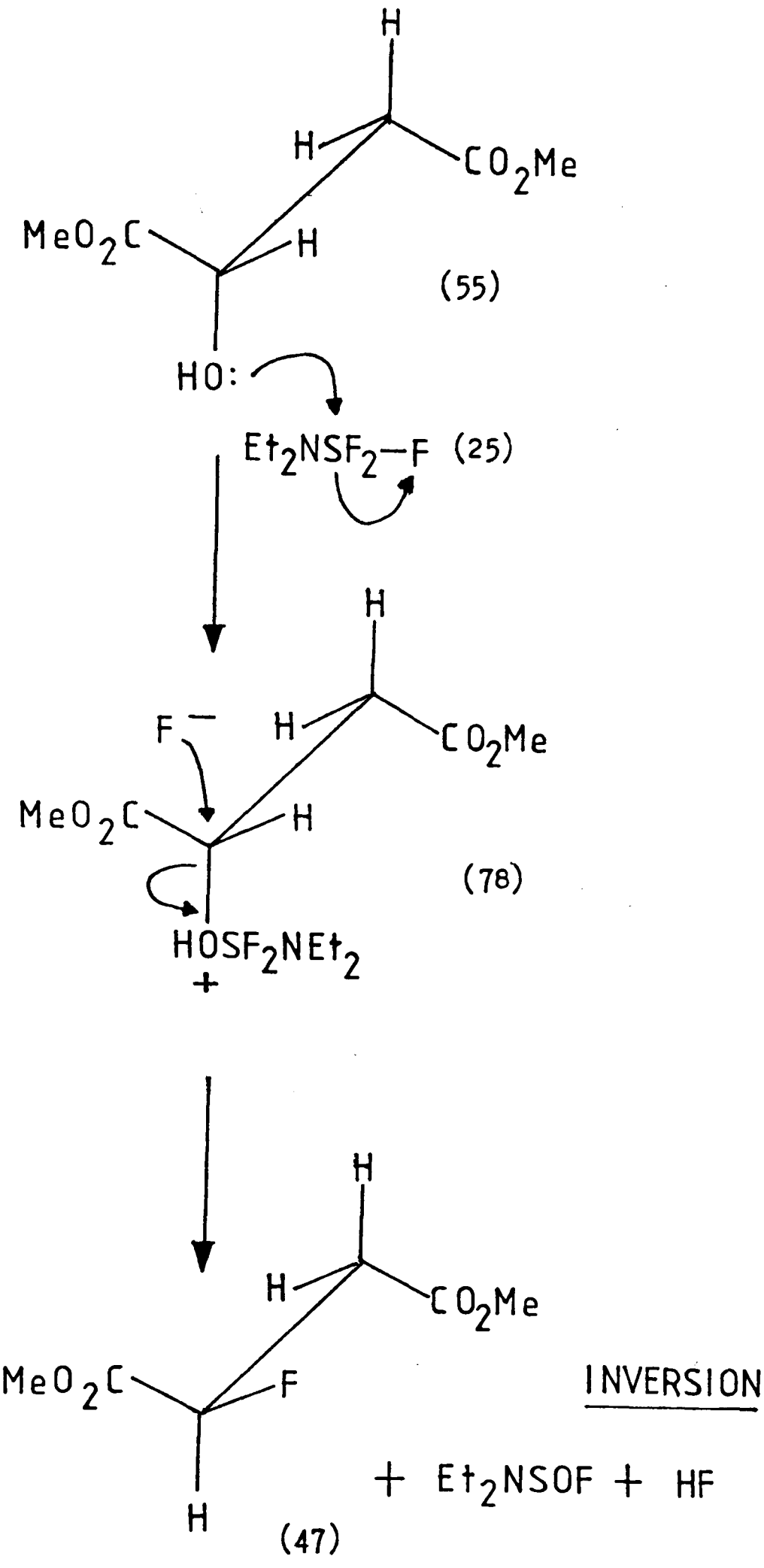


FIGURE 36 : A Proposed Mechanism for the Fluorination of Dimethyl-(2S)-Malate by DAST.

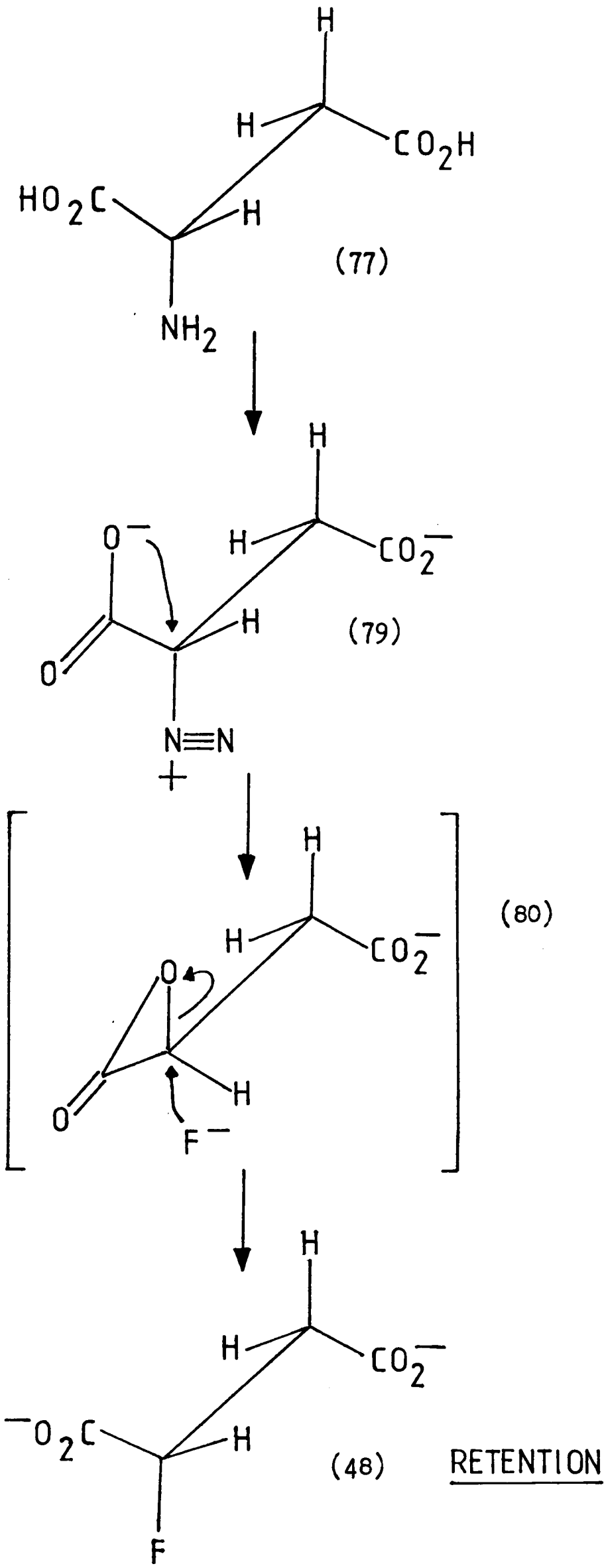


FIGURE 37 :A Proposed Mechanism for the Fluorination of (2S)-Aspartic Acid by Hydrogen Fluoride-Pyridine Reagent

anomalous. Fluorosuccinic acid exists in one conformation about the carbonyl chromophore.

(d) The *Pseudomonal* metabolite isolated by Harper and Blakley[83] was not (2*R*)-(+)-fluorosuccinic acid as reported but (2*S*)-(+)-fluorosuccinic acid.

CHAPTER 4

A REVIEW OF FUMARASE

General Background

The enzyme fumarase (fumarate hydratase E.C. 4.2.1.2) is found in a very wide variety of organisms and is an essential enzyme of the tricarboxylic acid cycle (TCA cycle), a primary metabolic and energy releasing biochemical pathway. The enzyme catalyses the reversible hydration of fumarate (59) to (2*S*)-malate (53) by a process which requires no cofactors.

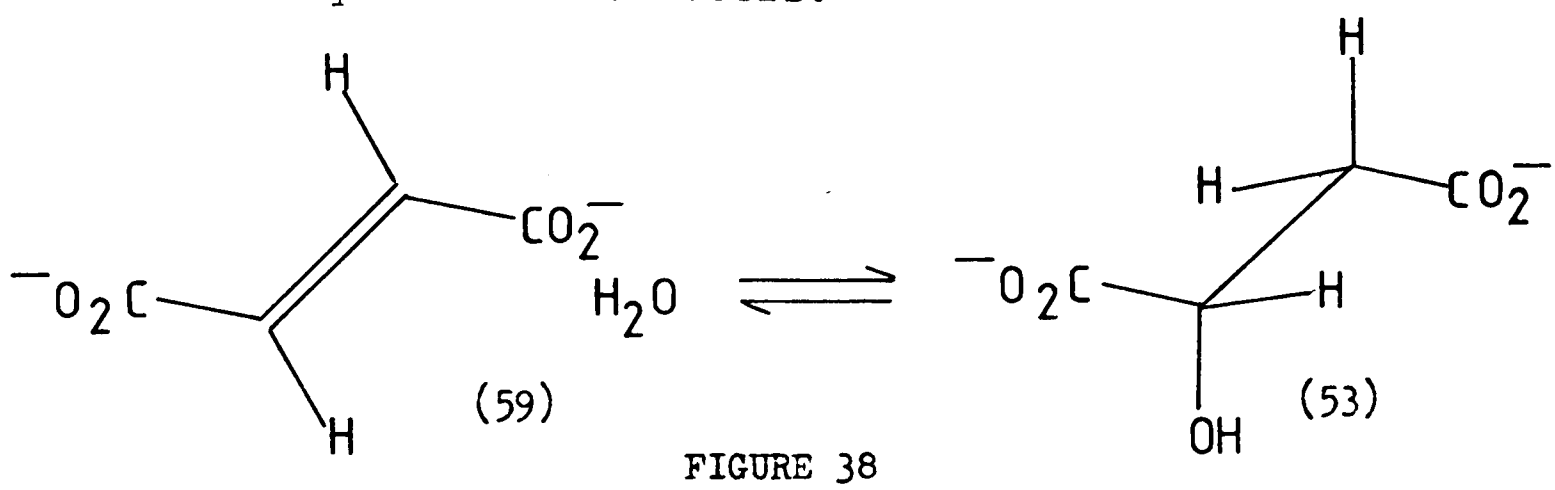


FIGURE 38

Battelli and Stern[128] first described the action of fumarase when they observed the conversion of succinate to malate by beef liver homogenates, although it was later realised that this was really a two step process, the fumarase catalysed hydration being preceded by a prior dehydrogenation of succinate to fumarate. Optically active (2*S*)-malate (53) was shown to be the exclusive hydration product[130], the equilibrium lying heavily to this side.

Difficulties of purification of the enzyme were overcome in 1952 when crystalline fumarase was isolated from swine heart muscle[131] thus facilitating the detailed subsequent investigations of the kinetic, thermodynamic and stereospecific properties of the enzyme. Hopefully a complete understanding of the structure-related mechanism of action will ultimately be achieved.

Physical Properties and Macromolecular Structure

Fumarase absorbs ultraviolet radiation between 250 and 290 nm[128], and since the extinction coefficient depends directly on concentration (for given conditions of pH, temperature and buffer

composition), this can be readily used for estimating enzyme concentrations. Moreover, the absorption of fumarate in the region between 250 and 280 nm is much greater than that of (2*S*)-malate, and this has formed the basis of an assay method for active fumarase.[132,133]

The molecular weight of fumarase has been found, by sedimentation equilibrium, to be 194,000 in 0.5 *M* phosphate buffer[134], whereas it is only 48,500 in 6 *M* guanidine hydrochloride, and this has suggested the existence of a tetrameric native enzyme.

Fumarase contains 1763 amino acid residues in an as yet unknown sequence with an unusually low proportion of tryptophan and half cystine[135], the latter being completely accounted for as cysteine on the basis of the thiol content of the enzyme by titration with thiol reagents such as *p*-mercuribenzoate and iodoacetate), and leading to an estimate of twelve thiol groups per molecule.[134] These are thought to be buried deep in the hydrophobic core of the enzyme since they are less reactive than "free", *i.e.* more accessible, thiol groups, and although they are not believed to be in the active site (but may reside in the subunit contact regions[137]), a loss in enzyme activity proportional to the amount of reagent consumed is observed. The presence of 2-mercaptoethanol has no effect on the molecular weight[134], thus suggesting that the enzyme is devoid of any inter-chain disulphide linkages, and hence the four subunits of the native enzyme must be held together by other than covalent forces. The enzymically active form of fumarase is the tetrameric moiety (equilibrium dialysis studies showing four binding sites, an average of one per subunit), and any conditions which encourage the dissociation of this form lead to a resulting loss of activity. Although fumarase remains undissociated in phosphate buffers even at very low concentrations, the absence of phosphate, substrates,

or competitive inhibitors leads to a rapid inactivation which increases with decreasing enzyme concentration. This has been interpreted as representing a dissociation of the enzyme into its subunits.[137] Urea and guanidine can cause this dissociation as well as an unfolding of the polypeptide chain, although activity can be restored by dialysis.

Nature of the Catalytic Action and the Active Site

The kinetics of the fumarase catalysed hydration have been very thoroughly investigated (see, for example, the extensive review by Alberty[138]), and are of the Michaelis-Menten kind at low substrate concentrations (*i.e.* <5 K_m, where K_m is the equilibrium dissociation constant for the enzyme-substrate complex). However, at higher concentrations substrate activation is observed[139,140,141], and at concentrations greater than 0.1 *M* fumarase is inhibited by its substrate[140,142], this latter fact being explained by a non-productive binding of two substrate molecules at the active site[143], and the former is accommodated, amongst other possibilities, by Conway and Koshland's mechanism of allosteric negative co-operativity.[144]

Fumarase is very sensitive to the nature and concentration of anions[145], which can behave in a similar fashion to the natural substrates. For example, at low concentrations multivalent anions such as phosphate, selenate, borate, arsenate and citrate activate the enzyme, although at high concentrations they may well be competitive inhibitors.[140] Some novel competitive inhibitors are the nucleotides ATP and the less effective GTP, UTP and CTP, whilst ADP and AMP activate the enzyme.[146] The triphosphate chain is thought to be responsible for the inhibitory effects, and such behaviour is believed to be important *in vivo* in the regulation of metabolic pathways.

The bell-shaped pH dependence of the maximum velocity of

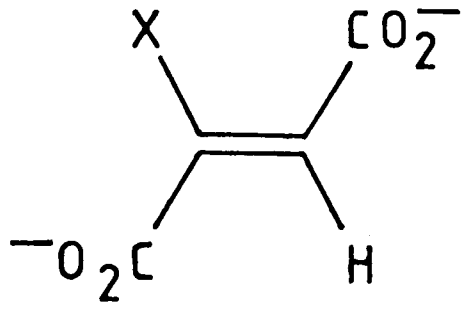
reaction (V_{max}) has shown the maximum activity to be between pH 6 and 8[147,148,149], and this has been interpreted as indicating the presence of protonated and deprotonated functional groups on the enzyme. Indeed, both proton addition and proton loss might be expected to be prerequisites for a hydration reaction. A kinetic analysis has yielded pK_1 and pK_2 values of 6.2 and 6.8 respectively[147,150], and these values closely approximate to those expected for the imidazole side chains of histidine. Hence it may be possible that two of these side chains function as catalytic groups in the active site, although later more thermodynamically orientated studies[138,151] have indicated that while pK_2 may reflect the dissociation of an imidazolium side chain, pK_1 is more likely to reflect that of a carboxyl group perturbed by its environment (compare, for example, the active site carboxyl group responsible for the activity of lysozyme, also of pK 6.2.[152]).

The investigation of structural analogues of fumarate and malate has shown that most dicarboxylic and tricarboxylic acids are competitive inhibitors of the enzyme, whereas monocarboxylic acids, as well as analogues containing derivatized carboxyl groups, are much less effective. From considerations such as these Massey[153] has suggested that the active site contains positively charged regions which interact with at least two negatively charged carboxyl groups in the substrate or inhibitor. Wigler and Alberty[154] have provided evidence for an unsymmetrical active site from a comparative study of the effects on the pH dependence of the initial reaction velocity of the four inhibitors succinate, (2*R*,3*R*)-tartrate, (2*S*,3*S*)-tartrate and (2*S*,3*R*)-tartrate (95). It was found that the dissociation constants K_1E and K_2E were affected differently by the two enantiomers, suggesting that the two catalytic groups of the active site are non-equivalent. The free energies of binding of the tartrates with the enzyme were also measured by

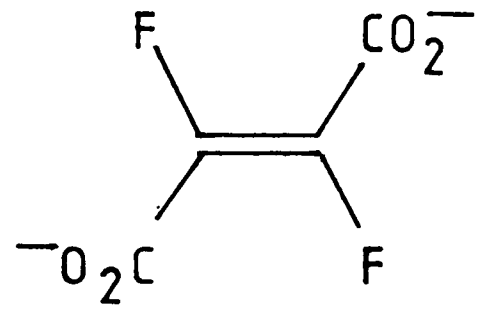
competitive binding with succinate, and it was found that (2*S*,3*R*)-tartrate is bound very tightly, being one of the best competitive inhibitors, whereas the other two enantiomers are bound much less effectively.

Substrate Specificity

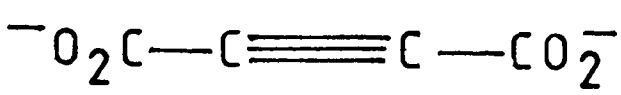
The interconversion of fumarate and malate is stereospecific, (2*S*)- but not (2*R*)-malate being the substrate[130], thus indicating the stereochemical requirements at the carbon bearing the hydroxyl group. Moreover, hydration of fumarate in D_2O introduces only one atom of non-exchangeable deuterium[98], and no deuterium is introduced into fumarate isolated from an equilibrium mixture of malate and fumarate in the same medium. The stereochemical relations of the mono-deuterio-(2*S*)-malate have been deduced by n.m.r., and the molecule has been designated as (2*S*,3*R*)-[3^2H]-malate (60).[99] For many years it was believed that fumarase was specific only for its natural substrates but further insight into the catalytic properties of the enzyme has been provided by the behaviour of some recently discovered unnatural substrates.[155,156] (FIGURE 39). Fumarase has been found to catalyse the hydration of the halofumarates (81-85), acetylene dicarboxylate (87) and mesaconate (86). It also catalyses the dehydration of (2*S*,3*S*)-chloromalate (89), (2*S*,3*S*)-bromomalate (90), (2*S*,3*S*)-tartrate (91) and (2*S*,3*S*) hydroxyaspartate (92). Thus although these unnatural substrates may not be ideally aligned with the active site catalytic functional groups, hydration and dehydration are nevertheless catalysed at an appreciable rate. These observations have revealed two general features necessary for a compound to be a substrate of fumarase. First, two negatively charged carboxyl groups free to adopt an anti-periplanar conformation are required, and secondly the carbon atom bearing the hydroxyl group in all substrates must have the same configuration



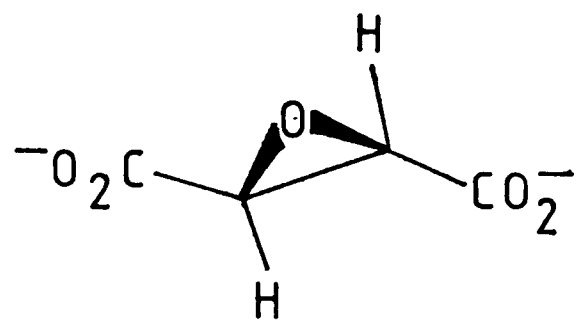
X = F Cl Br I Me
(81) (83) (84) (85) (86)



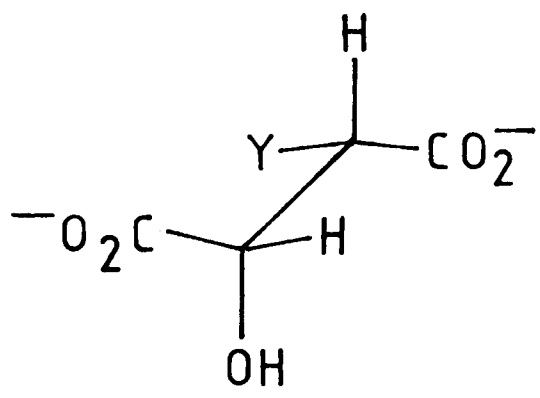
(82)



(87)



(88)



Y = Cl Br OH NH_2
(89) (90) (91) (92)

Fluorofumarate (81)
Difluorofumarate (82)
Chlorofumarate (83)
Bromofumarate (84)
Acetylene Dicarboxylate (87)
(2R, 3R)-Trans-Epoxysuccinate (88)
Iodofumarate (85)
Mesaconate (86)

(2S,3S)-3-Chloromalate (89)
(2S,3S)-3-Bromomalate (90)
(2S,3S)-Tartrate (91)
(2S,3S)-3-Hydroxyaspartate (92)

FIGURE 39 : Unnatural Substrates of Fumarase

as that in (2*S*)-malate. Malate derivatives in which a substituent other than hydrogen is *erythro* to the hydroxyl group are not substrates. No compound has yet been found which is a substrate of fumarase and which does not obey these criteria.

It is of interest to note that fluorofumarate (81) is hydrated three times faster than fumarate itself and is the best substrate known.[156,157,158] However, whereas the other halofumarates give the corresponding stable 3-halomalates, fluorofumarate is hydrated to 2-fluoromalate (93) which then spontaneously decomposes to oxaloacetate (94).

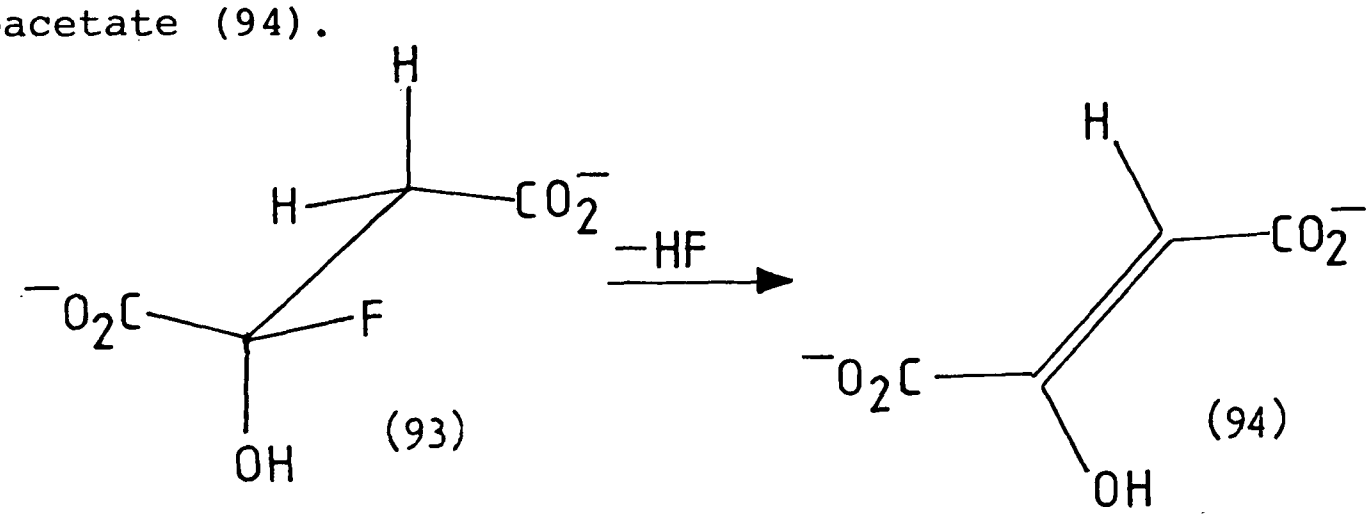
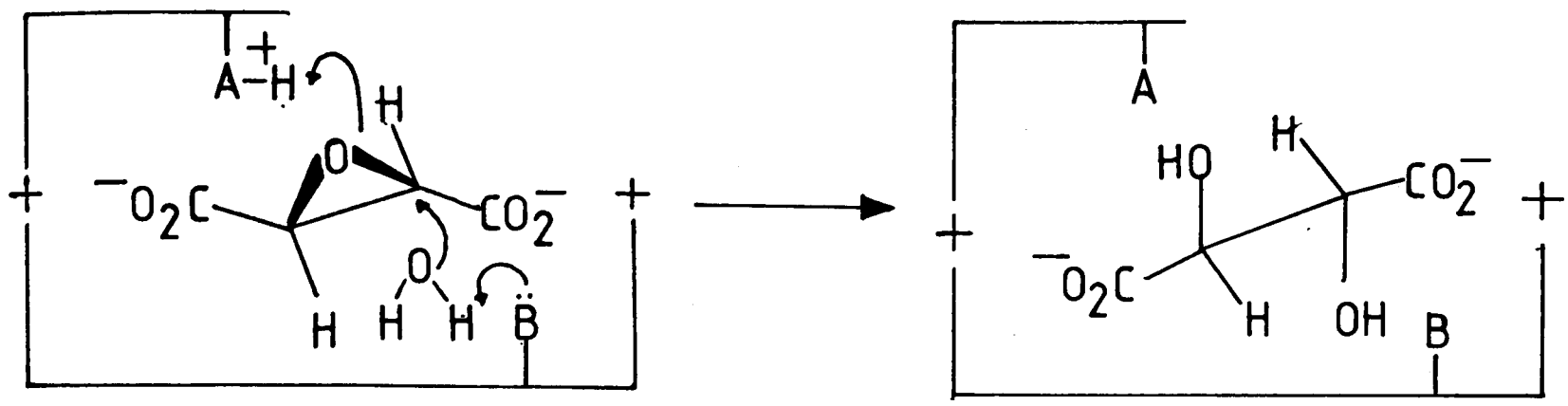


FIGURE 40

These considerations have given an interesting insight into the steric restrictions operative in the active site. Teipel *et al.*[156] have suggested that this contrasting behaviour is due to the fact that all substrates can bind potentially in either of two configurations, although if a substituent is too large only one type of binding is possible. Because of the small size of the fluorine atom fluorofumarate can bind in the mode which will give 2-fluoromalate, whereas the other halo-derivatives, being larger, can only bind in the opposite fashion. Therefore, it has been concluded that the part of the active site catalysing hydroxyl exchange (the "B" site in the schematic diagram below) is more sterically restricted than the region catalysing hydrogen exchange (the "A" site), and this is indeed borne out by consideration of the binding of the novel substrate (2*R*,3*R*)-*trans*-epoxysuccinate (88) which undergoes a fumarase catalysed ring opening to give

(2*S*,3*R*)-tartrate (95).



(88) FIGURE 41 (95)

If it is assumed that enzymic ring opening takes place by the same mechanism as that in free solution, namely a backside attack on the protonated intermediate, then one might expect both the (2*R*,3*R*) and the (2*S*,3*S*) isomers to undergo reaction. However, the fact that only the former does so indicates that the B site is more sterically hindered than the A site, with the consequent restriction on the binding of the (2*S*,3*S*) enantiomer.[155]

Mechanism of Action

The observation that the dehydration of (2*S*,3*R*)-[3^2H]-malate (60) occurs without a primary kinetic isotope effect[159] has suggested that the breaking of the carbon-hydrogen bond is not rate determining. In addition, the exchange rate of the deuteron removed on dehydration has been shown not to exceed the rate of incorporation of hydrogen *via* the back reaction. Thus, since the first step is unlikely to be the removal of a proton from malate to form a carbanion, an enzyme-bound carbonium ion intermediate has been postulated, formed by the initial removal of hydroxide ion (or the addition of a proton in the reverse reaction). See FIGURE 42 for a schematic representation of the mechanism first proposed by Alberty.[98] Support for this mechanism has come from a comparison of the rates of hydration of both mono- and difluorofumarate with that of the natural substrate[156], and this has indicated a

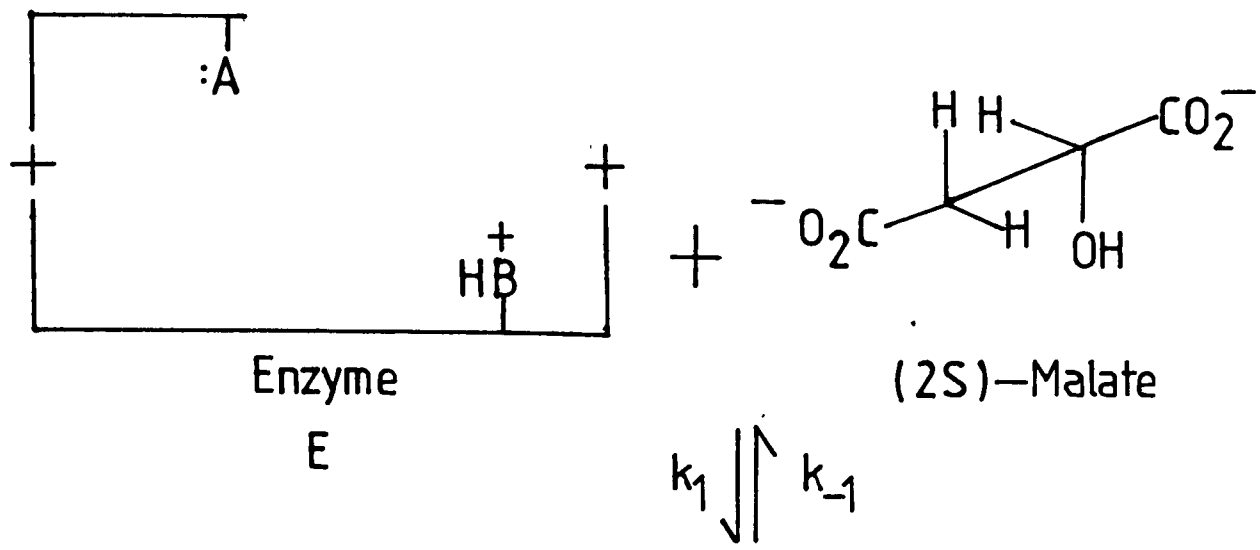


FIGURE 42 : Schematic Representation of the Carbonium Ion Mechanism of Action of Fumarase, after Hill and Teipel (ref. 161).

transition state with carbonium-ion character. This was proposed to explain why the monofluoro-derivative is hydrated faster than the difluoro-compound, for a carbonium ion intermediate is presumably formed more readily in the former compound.[160]

Because of the lack of a deuterium isotope effect in the conversion of the proposed carbonium ion intermediate EX in FIGURE 42 to the enzyme-fumarate complex (EF), and since the dissociation of fumarate (rate k_4) and association of malate (rate k_1) were known to be rapid, the conversion of the enzyme-malate complex (EM) to EX (rate k_2) was thought to be rate limiting. However, a sophisticated use of isotopes by Hansen *et al.*[162] which involved the measurement of both initial and equilibrium exchange rates for ^{18}O, ^{14}C, ^{3}H and ^{2}H labelled species, has shown this not to be the case. Using ^{14}C labelled malate and fumarate the rate of exchange of the carbon skeletons of both substrates was measured and compared with the rate of exchange of the [^{18}O]-labelled hydroxyl group of malate with solvent, as well as the exchange of the *pro-R* methylene proton using ^{2}H and ^{3}H labelling. The following relative rates were obtained indicating that the rate of exchange of the hydroxyl group of (2*S*)-malate with ^{18}O was faster than the rate limiting step, namely the random dissociation of fumarate or the substrate-derived proton from the enzyme.

TABLE 6

	Rel. Rate
^{18}O-malate ⟶ ^{18}O water	4.0
^{14}C-malate ⟶ ^{14}C fumarate	2.5
^{3}H-malate ⟶ ^{3}H water	1.0
^{2}H-malate ⟶ ^{2}H water	1.0

The carbonium ion mechanism attractively accounts for the fact that the rate of exchange of the hydroxyl group of malate was found to be more rapid than that of the *pro-R* proton with solvent. In addition the fact that the exchange of malate with fumarate was found to be faster than that of the *pro-R* proton with solvent required the inclusion in the reaction scheme of an enzyme-proton complex without bound fumarate. The minimal reaction scheme, therefore, was thought to be as follows, with EX as the carbonium ion complex:-

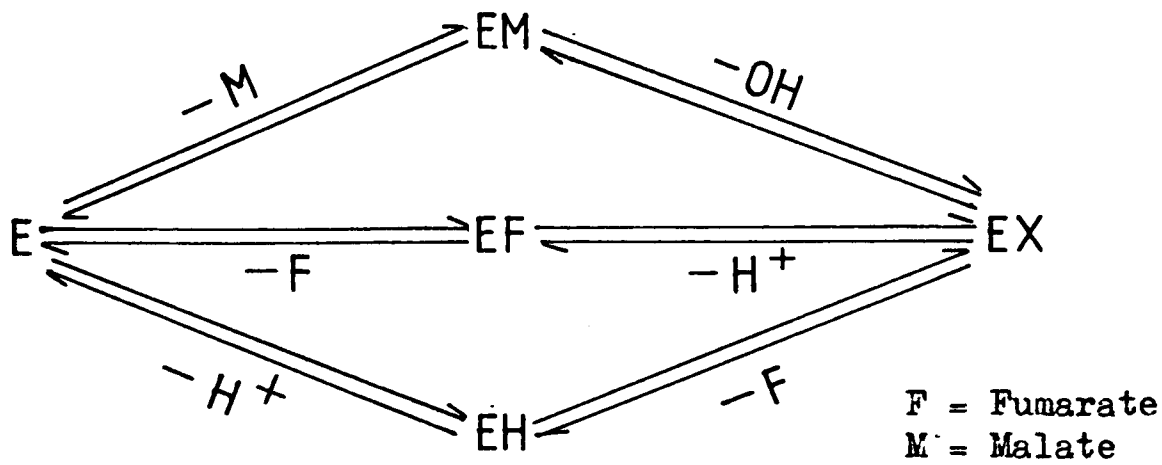


FIGURE 43

It has been calculated that the rates of formation of EX by the three pathways involving EM, EF and EH are virtually the same, and consequently no single step can be regarded as being rate limiting in the catalysis.

Although the climate of opinion at the present time favours the involvement of a carbonium ion intermediate, the slow release of the substrate derived proton makes the evidence also compatible with either a single concerted dehydration, or even the more unlikely mechanism involving a carbanion intermediate. Rose[163] has very concisely summarised the situation as follows "There are no data that focus on the fundamental question of whether the abstraction of a proton from malate precedes, follows, or is concerted with the breaking of the C-OH bond". Alternatively we may say that, using the terminology of Ingold, we do not know whether the mechanism is of the E1, $E1_{cb}$ or E2 type. Recent work in this

laboratory, however, has indicated that a carbonium ion mechanism is much less likely.[164,165]

CHAPTER 5

EVIDENCE SUPPORTING AN E2 MECHANISM FOR THE FUMARASE CATALYSED DEHYDRATION OF (2*S*)-MALATE

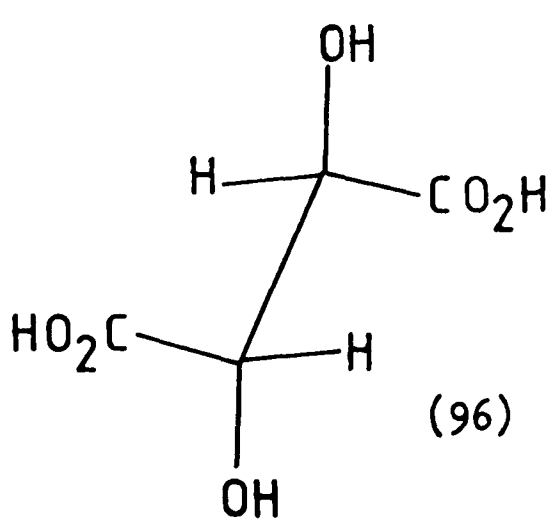
INTRODUCTION

The investigations reported in this thesis form part of a programme initiated in this laboratory using isotopic exchange techniques coupled with competitive inhibitors to examine the intimate mechanism of dehydration of (2*S*)-malate catalysed by fumarase.[164,165]

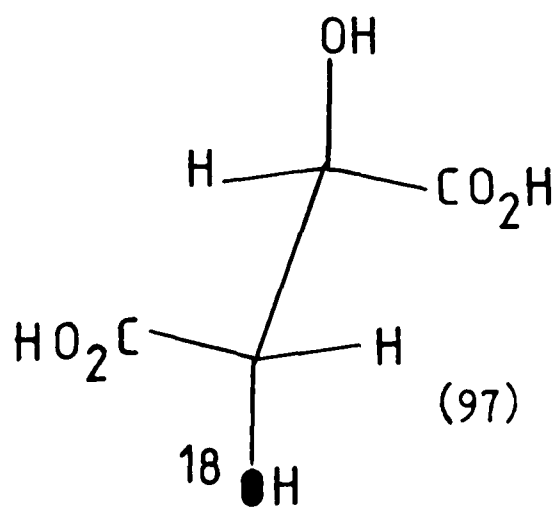
(a) Evidence against an E1 Mechanism

(2*S*,3*R*)-Tartrate (96) is a potent competitive inhibitor of fumarase. Moreover, the pKa values derived from the bell-shaped pH dependence of inhibition[154] are similar to those found for the fumarase-(2*S*)-malate complex.[147,148] (2*S*,3*R*)-tartrate (96) is considered to be a potent inhibitor of fumarase because of its stereochemical equivalence at C-2 with (2*S*)-malate, and the additional bonding capability of the (3*R*)-hydroxyl group. The observed pKa values for the fumarase-(2*S*,3*R*)-tartrate complex are also explained by this model.[154]

Since it is the *pro-R* proton which is abstracted from (2*S*)-malate (53) in the fumarase catalysed dehydration, (2*S*,3*R*)-tartrate (96) is not dehydrated. If, however, a carbonium ion intermediate is involved then (2*S*,3*R*)-tartrate should undergo fumarase-catalysed exchange with $H_2{}^{18}O$. Interest in this area was stimulated by the observation that such ^{18}O exchange was *not* catalysed by fumarase.[164] Since this was contrary to expectation (2*S*,3*R*)-[2-^{18}O]-tartrate (97) was synthesised and equilibrated with fumarase in unlabelled water.[165] No ^{18}O exchange was again



(96)



(97) FIGURE 44

observed and thus these two experiments provide *prima facie* evidence *against* a carbonium ion intermediate. This evidence does not rigorously rule out the E1 mechanism since it depends on the assumption that (2*S*,3*R*)-tartrate (96) binds in the active site in the same mode as (2*S*)-malate (53). The justification for this assumption, however, has already been made.

(b) Investigation of the El_{cb} Mechanism

In order to facilitate an analogous investigation of the El_{cb} mechanism (2*S*)-fluorosuccinic (48) acid was synthesised from (2*R*)-malic acid[127] (54) (see CHAPTER 3) with a view to examining possible fumarase catalysed exchange of the *pro*-*R*-proton with deuteriated solvent. Since there is ample testimony that fluorine can serve as an analogue of hydrogen or the hydroxyl group[28,29], and it is therefore likely that (2*S*)-fluorosuccinate (48) will be a good competitive inhibitor of fumarase, such exchange with deuteriated or tritiated water might be expected to occur if the carbanion mechanism was valid. If no exchange was found then the E2 mechanism would be the only process consistent with all the experimental evidence.

RESULTS AND DISCUSSION

Inhibition of Fumarase by the Fluorosuccinic Acids

With the stereospecific synthesis of the enantiomers of fluorosuccinic acid it was possible to test both of these compounds as substrates and inhibitors of fumarase.

As expected neither (2*R*)-fluorosuccinic acid (47) nor (2*S*)-fluorosuccinic acid (48) was found to be a substrate of fumarase. Thus no increase in u.v. absorbance at 240 nm due to fumarate (59) was observed after extended periods of incubation of these two compounds with the enzyme.

For inhibition studies it was not possible to use the standard assay[132,133] employing the spectrophotometric monitoring of the formation of fumarate (59) from (2*S*)-malate (53) due to the non-linearity of response of the SP1800 spectrophotometer to the small changes in optical density generated. Thus the coupled assay system of Marco *et al.*[166] was used in which fumarase forms part of a three enzyme system. The other enzymes are present in excess so that the overall reaction rate is controlled by the rate of the fumarase reaction (FIGURE 45).

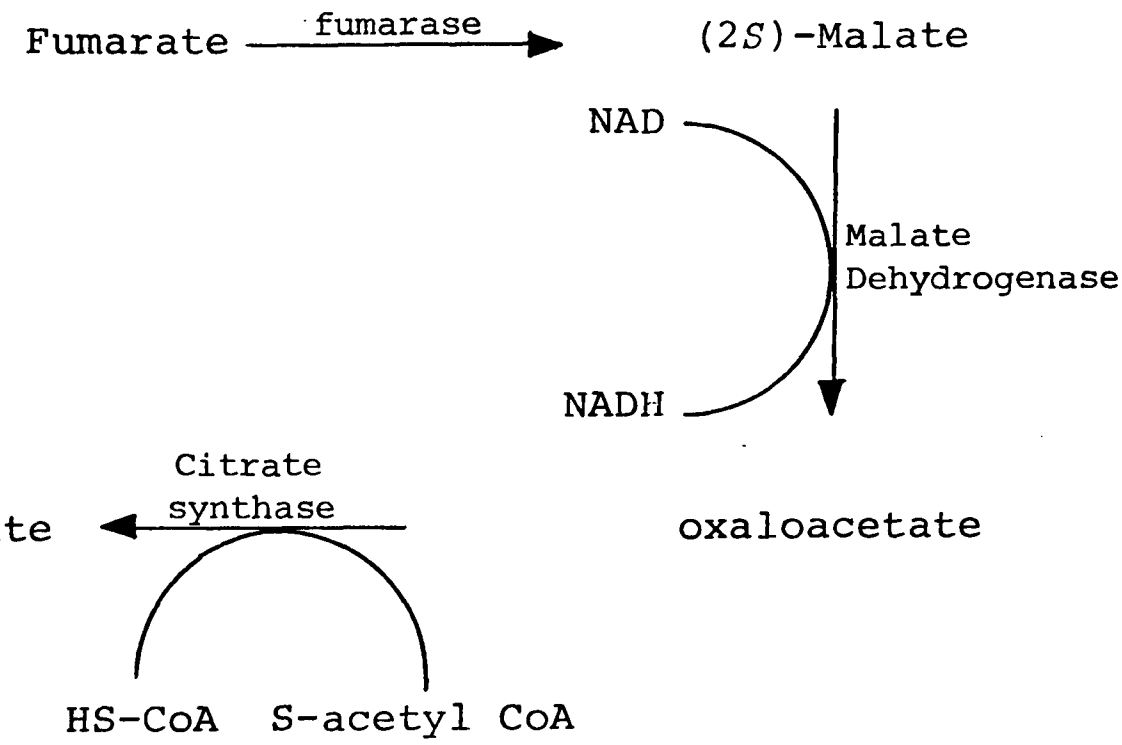


FIGURE 45

Malate dehydrogenase (MDH, E.C. 1.1.1.37) catalyses the oxidation of (2*S*)-malate (53) in conjunction with the cofactor nicotinamide adenine dinucleotide (NAD). The equilibrium position of this reaction lies well to the side of malate and in order to drive the reaction in the required direction two steps are taken. First, an auxiliary enzyme, citrate synthase (E.C. 4.3.3.7), is present to convert oxaloacetate to citrate. This process requires the cofactor S-acetyl coenzyme A. Secondly NAD is replaced by its analogue 3-acetylpyridine adenine dinucleotide which is more readily reduced than NAD itself. Thus when fumarate was mixed

with fumarase in the presence of an excess of the two auxiliary enzymes it was converted to citrate at a rate dependent on its conversion to (2*S*)-malate (53), and which could be monitored by the increase in absorption at 364 nm due to the formation of 3-acetyl pyridine-ADH_2 (reduced form). The assay is sensitive, readily monitoring the turnover of 10^{-5} *M* substrate because ε_{364}(NAD) is much greater than ε_{240} of fumarate.

To investigate the efficiency of the system the k_I value of succinate was initially determined (FIGURE 46). Unfortunately in preliminary experiments it was found that citrate synthase obtained from Sigma London Ltd., was very heavily contaminated with fumarase (*ca.* 100 units/mg protein!). Thus it was not possible to ensure an excess of the coupling enzyme over fumarase. Citrate synthase obtained from Boehringer, however, was only slightly contaminated to the extent of 5 units per mg protein and was thus acceptable. This material was used as a source of both enzymes.

Massey[153] has determined the k_I values of a wide range of inhibitors including succinate. His reported value is 5.2×10^{-2} *M* at pH 6.35 at 25 °C in 0.06 *M* phosphate buffer. Wigler and Alberty[154], however, in their studies of the chirality of the active site of fumarase quote a value of 1.2×10^{-3} *M* for 0.01 *M* tris acetate at 25 °C. Moreover, Massey's value for *D*-tartrate is 2.5×10^{-2} *M* whereas the Wigler and Alberty value is 2.5×10^{-3} *M*. Fumarase is, however, notorious for exhibiting variable kinetic constants under different conditions.

The succinate inhibition data were treated by the method of Dixon.[167] An estimate of k_m (fumarate) [k_m(F)] was required and was obtained by interpolation from the data on the variation of k_m(F) with pH as determined by Frieden and Alberty.[147] A value of 4μ*M* was used. The inhibition data were fitted by a least squares

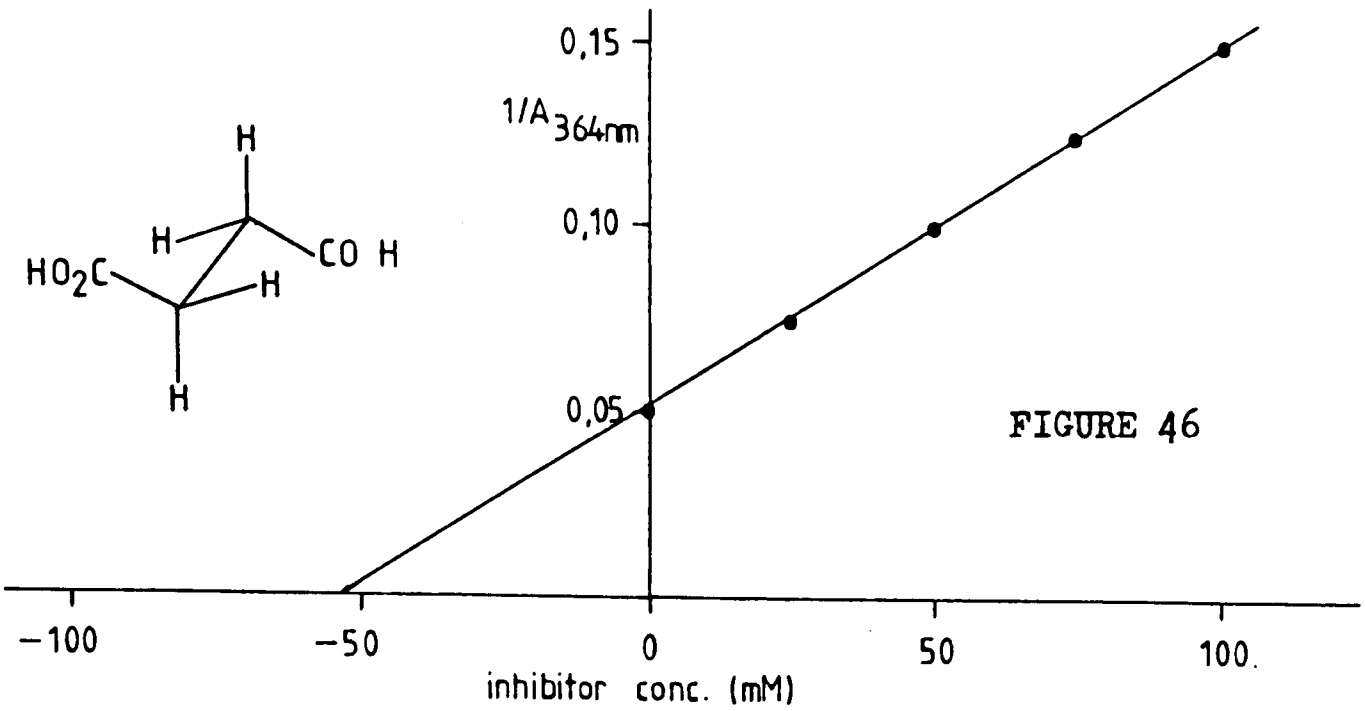


FIGURE 46

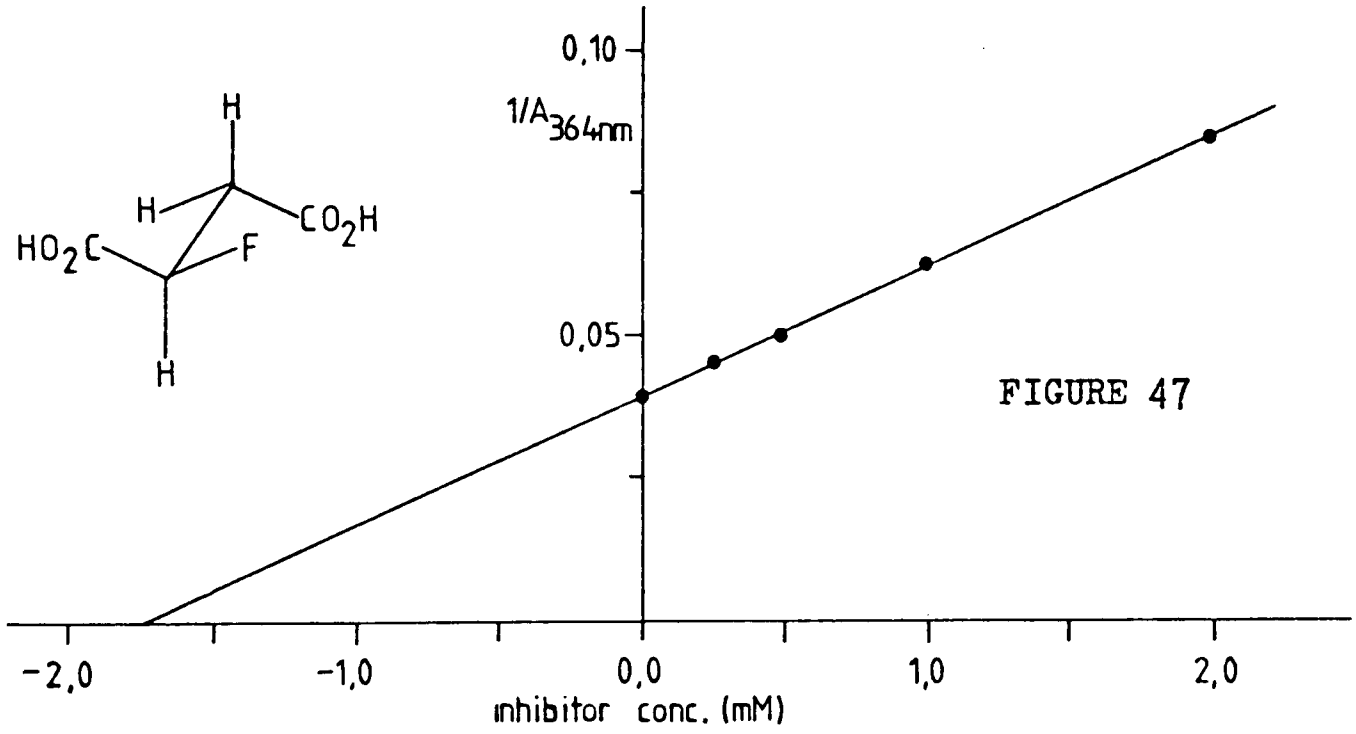


FIGURE 47

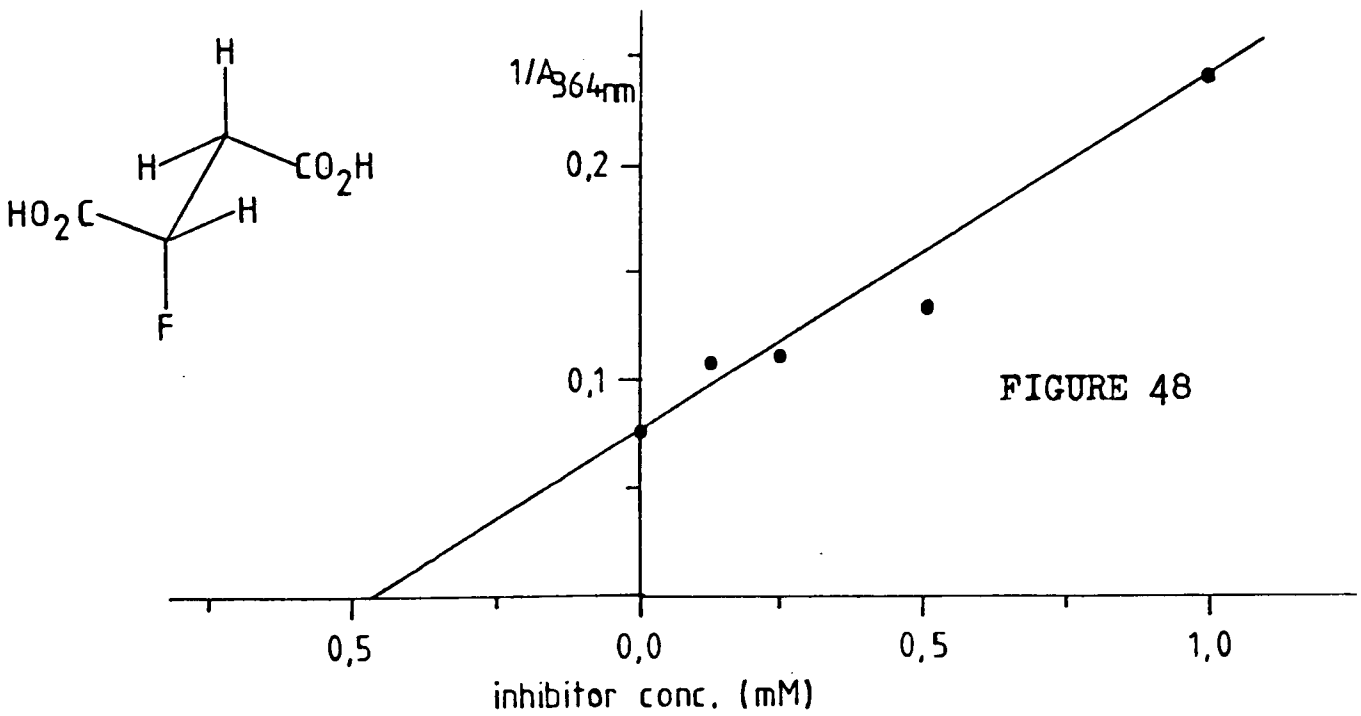


FIGURE 48

FIGURES 46-48 ; Inhibition of Fumarase by Succinate and the Fluoro-succinic Acids.

regression analysis and gave an intercept on the concentration axis (i axis) of $-5.7 \times 10^{-2} \pm 0.4 \times 10^{-2}$ *M*. Now since when $\frac{1}{v} = 0$ $-i = k_I\left(\frac{s}{k_m} + 1\right)$ where i is the inhibitor concentration, k_I the inhibition constant, k_m the Michaelis constant, s the substrate concentration and v the maximum reaction velocity in

$$\frac{1}{v}.V = \frac{k_m}{s}\left(1 + \frac{I}{k_I}\right) + 1$$

and since the fumarate concentration was 6.76×10^{-5} *M* we can obtain a value for k_I of 3.18×10^{-3} 0.22×10^{-3} *M*. In view of the comparison between the data of Wigler and Alberty[154] and those of Massey[153] for *D*-tartrate and succinate this value is quite acceptable.

The effect of (2*R*)-fluorosuccinate (47) and (2*S*)-fluorosuccinate (48) on the rate of the fumarase catalysed hydration of fumarate (6.7×10^{-5} *M*) in 0.01 *M* tris acetate buffer pH 7.3 was now investigated (FIGURES 47 and 48). As k_m(F) is *ca.* 0.4×10^{-5} *M* under these conditions[147] the substrate concentration is of the order of 17 k_m and substrate activation effects should be slight. The results were again analysed by the method of Dixon[167] and k_I values of $9.7 \times 10^{-5} \pm 0.2 \times 10^{-5}$ *M* and $2.4 \times 10^{-5} \pm 0.7 \times 10^{-5}$ *M* at pH 7.3 were found for (2*R*)-fluorosuccinic acid (47) and (2*S*)-fluorosuccinic acid (48) respectively. Thus both acids are potent competitive inhibitors of fumarase and since succinate was found to have a k_I of $3.2 \times 10^{-3} \pm 0.2 \times 10^{-3}$ *M* (Wigler and Alberty[154] give 1.2×10^{-3} *M* at 25 °C in tris acetate at the pH optimum of 7.0) it is clear that the fluorine atom is making a contribution to binding, particularly in the case of (2*S*)-fluorosuccinic acid (48) where it has replaced the hydroxyl group of the natural substrate. It seems reasonable, therefore, that (2*S*)-fluorosuccinate (48) binds in a

conformation in which the fluorine atom occupies the position normally associated with the C-2 hydroxyl group of the natural substrate. Occupancy of this position by fluorine rather than hydrogen must arise from the properties common to the C-F and C-OH bonds and not shared by the C-H bond. For instance the bond length[168] and polarity[169] of the C-F bond resemble those of the C-OH bond more than those of C-H. Moreover, the covalent Van der Waals radii of fluorine and oxygen are similar[170], and covalently bound fluorine has the ability to accept a hydrogen bond.[171] All of these factors may be implicated in the interaction of fluorine with the enzyme which leads to its specific orientation at the active site. (Further information concerning the hydroxyl binding site and its interactions with substrates might be obtained from ^{19}F n.m.r. studies with these inhibitors).

(2*R*)-fluorosuccinate (47), although not quite as effective as the (2*S*)-enantiomer (48) is still expected to be better than succinate on the grounds that (2*R*)-malate (k_I = 6.3 x 10^{-3} *M*[153]) is a better inhibitor than succinate (k_I = 5.2 x 10^{-2} *M*[153]) and this is borne out by the results. Thus, there is obviously an advantage in having a hydroxylic (or analogous group) even in the (2*R*)-position.

Exchange Experiments

Both (2*R*)-fluorosuccinate (47) and (2*S*)-fluorosuccinate (48) were equilibrated with deuteriated water (99.8 atom %) in the presence of fumarase at 25 °C for four days and the solution examined directly by ^1H n.m.r. Attention was focussed on the double doublet of doublets at δ5.35 p.p.m. assigned to the methine proton (fluorosuccinic acid undergoes a conformational change on ionisation and this is reflected in the n.m.r. spectrum[172]). Should deuterium have been incorporated this resonance would collapse to a simple

doublet of doublets.[127] In the event *no* evidence for exchange was obtained from either of the two enzyme experiments. It was estimated that approximately 5% of such [3-^{2}H]-2-fluorosuccinate could have been detected if formed.

In order to be able to define this limit even more closely the experiment was repeated using tritiated water. Once more no significant incorporation of tritium could be detected indicating that the amount of exchange, if any, was less than 0.05%.

CONCLUSIONS

Since (2*S*)-fluorosuccinate (48) has a k_I for fumarase comparable with the k_m of (2*S*)-malate (2.5×10^{-5} *M*)[147,148] it seems very unlikely that the lack of exchange could be due to incorrect binding. Also the possibility that the proton abstracted is always recaptured by the inhibitor and never exchanged with solvent appears improbable. The strong inductive effect of fluorine would be expected to facilitate carbanion formation at C-3 if an El_{cb} mechanism was possible, but it seems likely that if it were formed loss of fluoride ion would occur, and no evidence of fumarate or malate could, however, be found. This, therefore, provides *prima facie* evidence against an El_{cb} mechanism.

Although the evidence from these investigations apparently rules out both step-wise mechanisms and therefore *implies* that the fumarase catalysed dehydration of (2*S*)-malate (53) occurs by a concerted E2 mechanism, positive evidence to support this proposal is tenuous.

The absence of a primary kinetic isotope effect in the fumarase-catalysed dehydration of (2*S*,3*R*)-[3-^{2}H]-malate (60) to fumarate (59) at pH 7.3[159], can be ascribed to the rate limiting release of the substrate-derived proton (deuteron) and fumarate from the enzyme.[162] At pH 5 a kinetic isotope effect (k_H/k_D = 1.31) was observed.[173] In an attempt to detect a kinetic isotope effect at pH 7.3 a comparison was made of

the rate of fumarase-catalysed ^{18}O-exchange from (2*S*,3*R*)-[2-^{18}O,3-^{2}H]-malate and (2*S*,3*R*)-[2-^{18}O]-malate to water at equilibrium. An isotope effect k_H/k_D = 1.15 was reported.[173] These values should not be regarded as true kinetic isotope effects, however, since the elimination step is not rate limiting in the overall reaction nor is it known to be in the ^{18}O reaction. Indeed, if the concept is valid that enzymes have evolved, at least in the central metabolic pathways, to the point where the chemical events occur at rates comparable or faster than the release of products[174], we should not expect the elimination or addition step to be rate-limiting. The isotope effects should therefore be regarded as lower limits to the true kinetic isotope effect of the elimination step. In any event, a deuterium kinetic isotope effect of even 1.3 is consistent with a primary kinetic isotope effect for a reaction with a highly asymmetric transition state.[175,176] Moreover, all the experimental evidence which has been cited as supporting a carbonium ion mechanism is consistent with a concerted elimination in which the transition state has a high degree of carbonium ion character. Indeed, there appears to be no observation which is inconsistent with the conclusion that fumarase catalyses the dehydration of (2*S*)-malate (53) *via* a transition state in which breaking of the C-OH bond is much further advanced than that of the C-H bond.

These conclusions have recently been published.[177]

Malic Enzyme

During the course of the fumarase studies the fluorosuccinic acids were also tested as substrates for 'malic' enzyme (from chicken liver, E.C. 1.1.1.40). Early literature on malic enzyme has been reviewed by Ochoa.[178] Malic enzyme catalyses the following decarboxylation of (2*S*)-malate (53).

$$(2S)\text{-malate} + TPN^{+} \xrightarrow{Me^{2+}} \text{pyruvate} + CO_2 + TPNH$$

The purified enzyme also catalyses the decarboxylation of oxaloacetate to pyruvate, and both of these decarboxylations are stimulated by Mg^{2+} or Mn^{2+} ions.

Gal[179] found that fluoromalate at a concentration of 0.7 m*M* caused 60% inhibition of malate decarboxylation. Dicarboxylic acids[180] (fumarate and mesaconate), and α-hydroxy dicarboxylic acids related to malate (mesoxalic, tartaric) as well as oxalic, aspartic, succinic and citric acids are inhibitory[181], the inhibition being usually partially competitive and partially non-competitive. A mechanism of action has been proposed by Steinberger and Westheimer.[182]

In the presence of all the constituents of the assay mixture and each fluorosuccinic acid, at 25 °C in glycyl glycine buffer malic enzyme effected no change in the optical density at 340 nm after long periods. Fluorosuccinic acid is therefore not a substrate of malic enzyme. However, in the light of the work of Gal[179] it should prove to be an excellent inhibitor and this possibility is being investigated.

CHAPTER 6

A SURVEY OF RECENT PROGRESS IN ENZYMIC PHOSPHORYL TRANSFER

INTRODUCTION

The enzyme catalysed transfer of phosphoryl groups is ubiquitous in intermediary metabolism, such biological reactions being essential to the energy balance and cellular control mechanisms of all organisms at every level. Indeed, essentially every naturally occurring thermodynamically unfavourable metabolic process involves a displacement at the phosphorus atom of a phosphoric monoester or anhydride, such reactions being coupled to the free energy change resulting from the cleavage of the phosphoric anhydride of a nucleoside triphosphate by the phosphokinases and ATPases. The enzymatic transfer of phosphoric esters resulting from displacement at the phosphorus atom of phosphoric diesters is also commonplace.

The classes of enzyme which catalyse displacements at phosphorus are summarised in FIGURE 49. The enzymes which handle phosphoric *monoesters* fall into three categories: the *phosphatases*, where water is the acceptor of the phosphoryl group (these include such enzymes as alkaline phosphatase which is merely hydrolytic, and

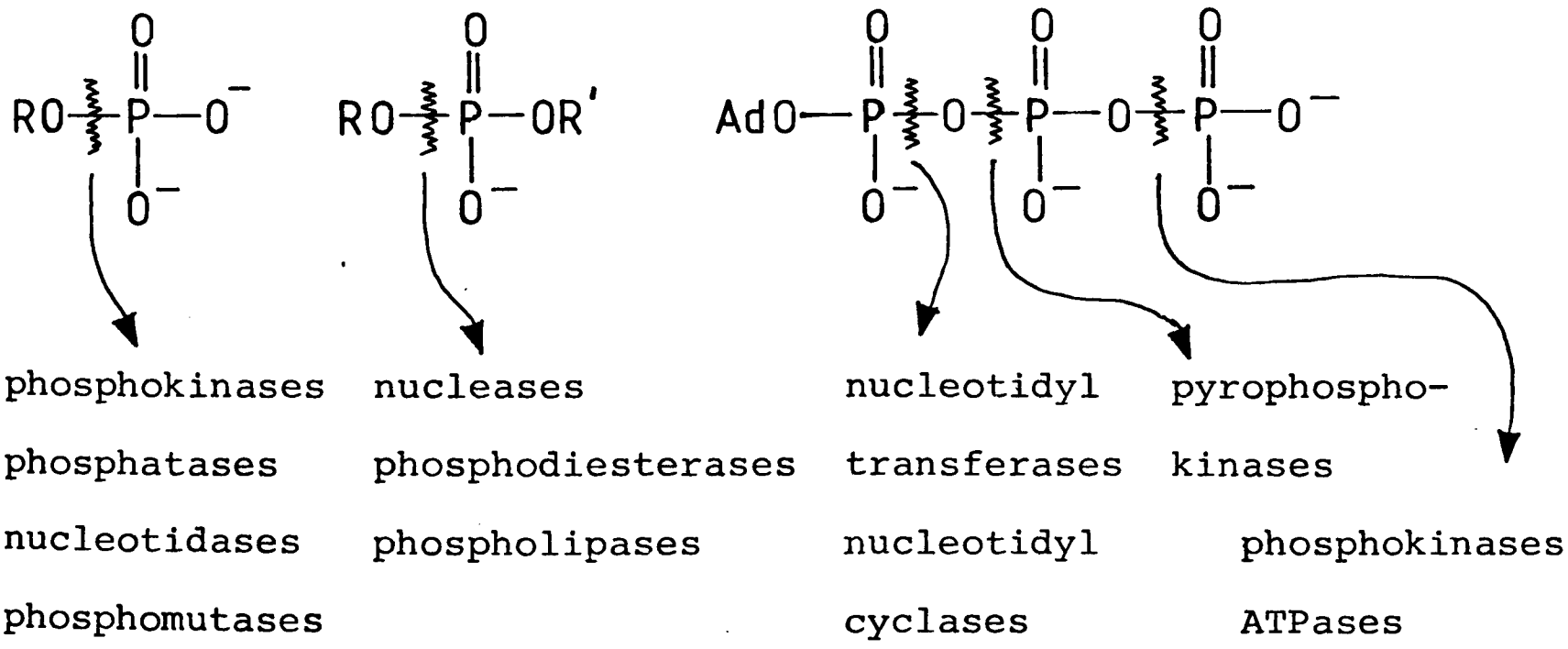


FIGURE 49

enzymes such as the ATPases where the free energy available is coupled to some other metabolic function); the *kinases*, where a

nucleotide triphosphate is the phosphoryl donor, and some molecule other than water is the acceptor (which may be an alcohol, as for hexokinase, a carboxylic acid as for acetate kinase, a nitrogenous compound as for creatine kinase or a phosphorylated compound as for adenylate kinase); and the *mutases* for which the acceptor is another functional group on the donor molecule. The enzymes that handle phosphoric *diesters* are either hydrolytic (*e.g.* the nucleases) or nucleotidyl transfer catalysts.

Until fairly recently the majority of mechanistic information has been based on kinetic techniques, and has been confined to establishing the kinetic mechanism, namely the order in which the substrates bind and dissociate from the enzyme, and very importantly whether a phosphoenzyme intermediate is involved or not. This area has been reviewed by Cleland[183], who has shown how the kinetic mechanism may be deduced.

There are two major classes of kinetic mechanism: *sequential* mechanisms in which all the reactants must combine with the enzyme before any reaction occurs and products are released (such processes are further divisible into *ordered* and *random* processes), and *ping-pong* mechanisms where one or more of the products may be released before all the substrates have been bound to the enzyme, the implication of this being that the enzyme oscillates between two or more stable forms. The ping-pong mechanism as applied to phosphoryl transfer necessitates a phosphoryl-enzyme intermediate, whereas a sequential mechanism has no attendant chemical implications. The existence (or non-existence) of a phosphoenzyme intermediate is central to the understanding of the mechanism of phosphoryl transfer.

In practice the interpretation of kinetic data is often difficult and on a kinetic basis several enzymes, notably hexokinase[184] have had conflicting mechanisms postulated. For acetate kinase, however,

a ping-pong mechanism has been implicated from initial rate experiments[185] and subsequent *isolation* and investigation of the properties of the phosphoenzyme intermediate[186] has confirmed it as a viable entity. There are of course many complications and ambiguities in the kinetic investigation of phosphoryl transfer.

The investigation of the mechanism of enzyme catalysed phosphoryl transfer reactions may be divided into three main categories:

(a) How many reaction intermediates are involved and what are their natures? For example, is there an obligatory phosphoenzyme intermediate[187] and what is the order of substrate binding?
(b) What is the rate limiting transition state and how does a particular intermediate partition?
(c) In the elementary step is the displacement associative or dissociative, and how is each step catalysed by the enzyme?

It is in the last of these categories that this thesis has most relevance, more particularly the stereochemistry of the elementary step, and this area will be discussed more thoroughly.

THE NATURE OF THE ELEMENTARY STEP

Associative *vs*. Dissociative Pathways

We must now ask what is the mechanism of phosphoryl group transfer at the level of the elementary step, and what is the present state of the literature evidence which bears upon how such a single transfer could be catalysed enzymically?

One may extract from the physical organic literature[188,189] four formal mechanistic extremes for reactions at the phosphorus atom of a phosphate ester, there being one *dissociative* pathway and three *associative* reactions (FIGURE 50). The dissociative pathway necessitates the transient involvement of the highly reactive, and

currently controversial monomeric metaphosphate species (at least in the present context) which is subsequently captured by the acceptor group. Now for phosphorus monoester dianions, for example, there is little dependence of reaction rate on the pKa of the entering nucleophile, the value of $\Delta S^{\ddagger}$ is around zero, significantly different from that expected for a bimolecular process, and there is a significant ^{18}O isotope effect for the P-O bond being broken.[188,189,190] This evidence *inter alia*, as well as the generation and trapping of methylmetaphosphate[191] has established the importance of the dissociative pathway.

Protonation or further esterification of the monoester dianion causes the transition state to assume some of the characteristics of an associative process in the case of *mono*-anions, and for triesters the reaction rates are equally sensitive to the pKas of the nucleophile and the leaving group, such reactions seeming to be *associative* in nature and possessing transition states with the nucleophile and leaving group at apical positions.[188,189] However, the pentacoordinate intermediate need not be merely a transition state but may be a viable intermediate (especially for cyclic esters where steric crowding is reduced) and sufficiently long lived to allow *pseudorotation*[192,193], namely the non-dissociative pairwise exchange of equatorial and axial ligands.[111] Thus this possibility provides for the mechanistic extreme in which addition of the nucleophile at phosphorus leads to a pentacoordinate intermediate which may undergo one or more pseudorotations before the leaving group departs. Recent evidence[194] places these pathways on firm ground even for acyclic systems.

The process of pseudorotation has been reviewed by Westheimer[192], and appears to be well established.[195] Several somewhat empirical preference rules[192] have been applied to the nucleophilic attack on P(V) compounds and the stereochemistry of the pentacovalent intermediate.

(i) Groups approach and depart from pentacoordinate species only *via* apical positions.

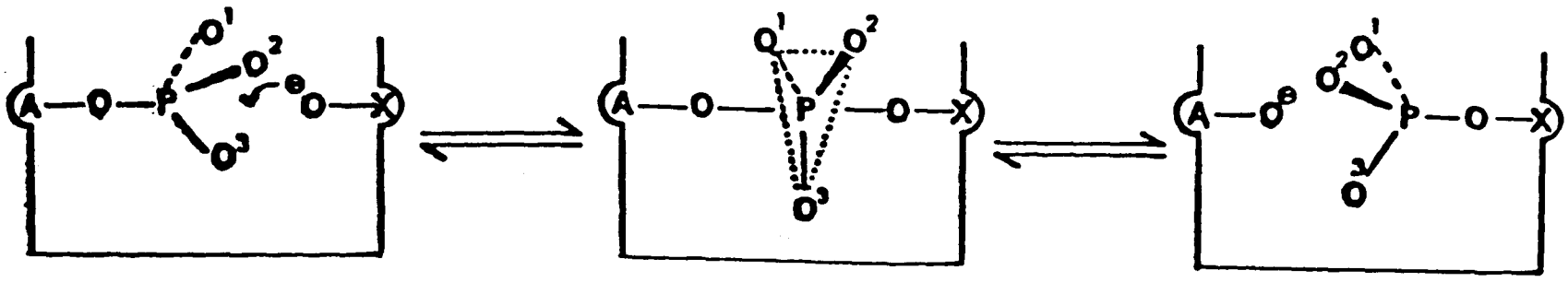
(ii) Electronegative atoms occupy preferentially apical positions.[196]

(iii) Five-membered rings will generally span apical-equatorial positions.

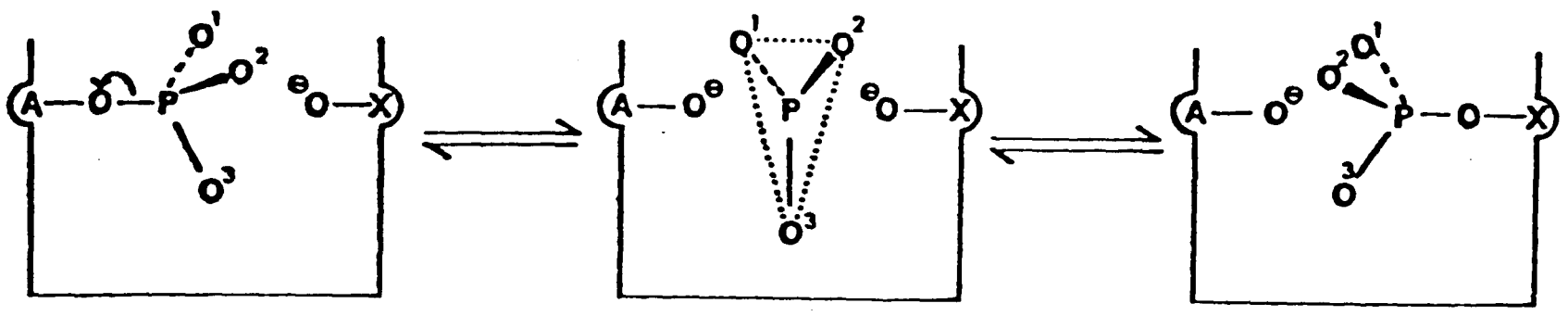
(iv) Whether a particular pseudorotation will take place is determined by the lifetime of the phosphorane and whether the energy barrier to the ligand exchange is surmountable.

What are the consequences of these mechanistic extremes in terms of enzyme catalysed processes? In an enzyme active site, where there is a fixed disposition of attacking, leaving and charge-stabilising groups it may be assumed that the reaction will have a well defined stereochemical consequence. The four most likely possibilities are set out in FIGURE 50 (the oxygen atoms are numbered to show the stereochemical courses). The 'in line' associative $S_N2(P)$ displacement (a) is expected to proceed with inversion of configuration, the nucleophile approaching at an apical position and the leaving group departing from the other apical position. The dissociative $S_N1(P)$ reaction (b) can in principle proceed with inversion or retention although the former is considered to be the most likely since the high energy meta-phosphate is expected to be very tightly bound. The 'adjacent' attack with pseudorotation (c) will proceed with retention of configuration, as will the double displacement (d) involving a phosphoenzyme intermediate which invokes two 'in line' displacements which can be either $S_N1(P)$ or $S_N2(P)$. Two adjacent displacements, however, would also result in retention of configuration.

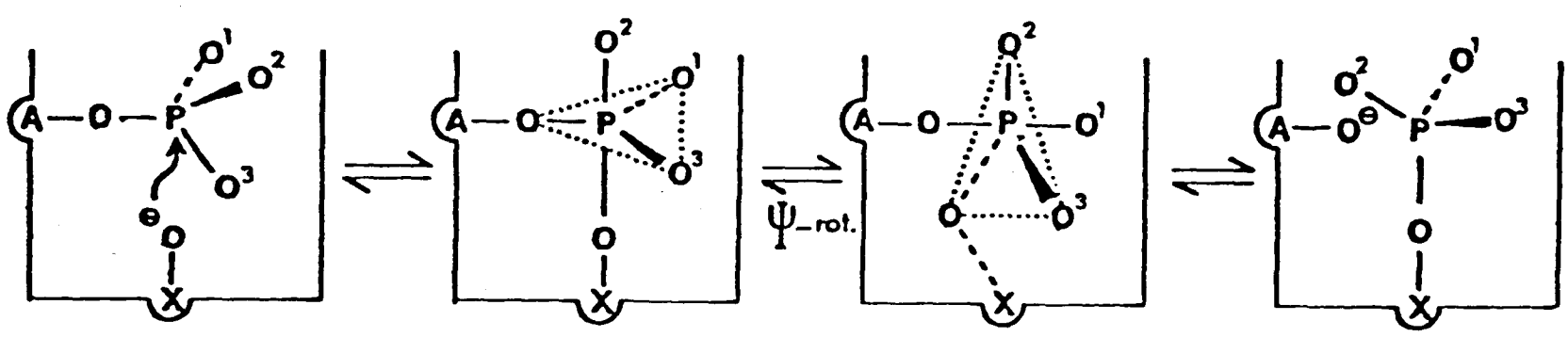
In principle, two kinds of experiment will distinguish between these four mechanisms, namely one to define an associative or



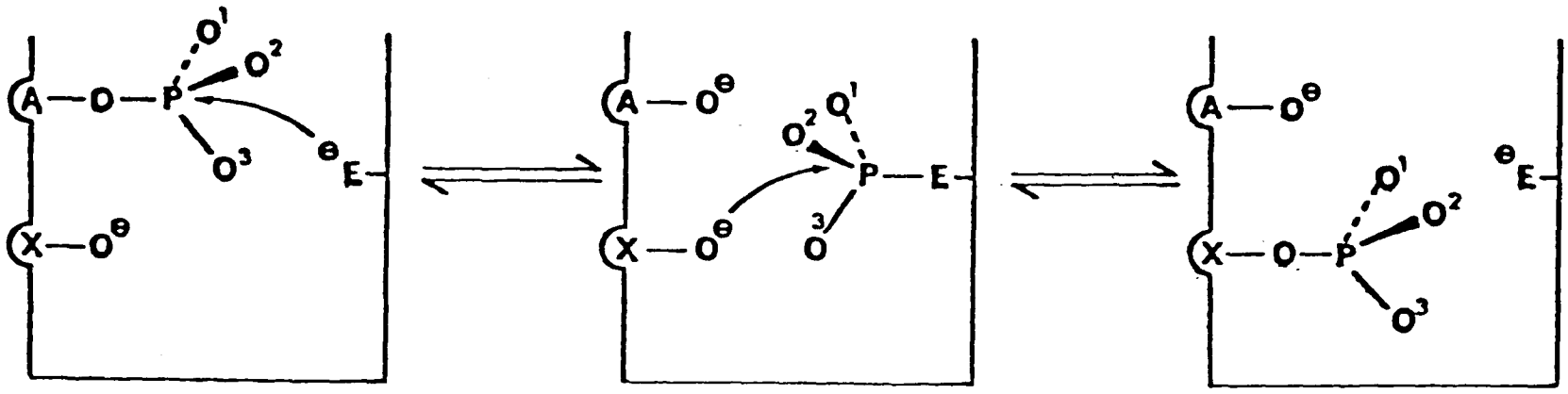
(a)



(b)



(c)



(d)

FIGURE 50 : The Four Possible Mechanisms of Phosphoryl Transfer.

dissociative pathway, and the other to determine the stereochemical course. For the kinases one first requires to know whether in the absence of the other substrate the P_γ-OP_β bond of ATP is broken. This distinction requires the synthesis of a specifically ^{18}O-labelled ATP molecule with ^{18}O either exclusively in the bridging position or completely surrounding the γ residue. The reversible cleavage of the P_γ-OP_β bond will result in scrambling of ^{18}O either out of or into the bridging position provided that the β-phosphoryl residue of the ATP is free to rotate.[197]

In an attempt to distinguish between an associative and dissociative mechanism for pyruvate kinase in this laboratory, Lowe and Sproat[198] have employed such positional isotope exchange experiments to show that pyruvate kinase catalyses the randomisation of ^{18}O between the β,γ bridge and β non-bridging positions of ATP in the absence of cosubstrate. (The rationale behind this type of experiment has recently been reviewed by Rose[199]). This scrambling occurs about 20 times more slowly than when pyruvate is present, and its observation can only be accounted for by an $S_N1(P)$ mechanism or a phosphoenzyme intermediate. The detection of this scrambling has been achieved by mass spectrometry[197] and ^{31}P n.m.r. spectroscopy[198,200] since ^{18}O has been observed to produce a measurable isotope shift on the ^{31}P resonance when directly bonded.[200,201]

However, since there is no evidence for a phosphoenzyme intermediate[202,203] it was suggested that this randomisation indicated a *dissociative* pathway. However, catalysis of positional isotope exchange is not a general phenomenon with phosphokinases, and an alternatively viable rationalisation could be found in the action of an enzyme nucleophile as a poor surrogate for the hydroxyl group of enol pyruvate in an *associative* displacement. Since isotope exchange between the γ-oxygens of ATP and $H_2{}^{18}O$ does not

occur, such an explanation has a prerequisite that P_i at the active site does not tumble and that P-O bond rotations are only possible about the bonds indicated in FIGURE 51.

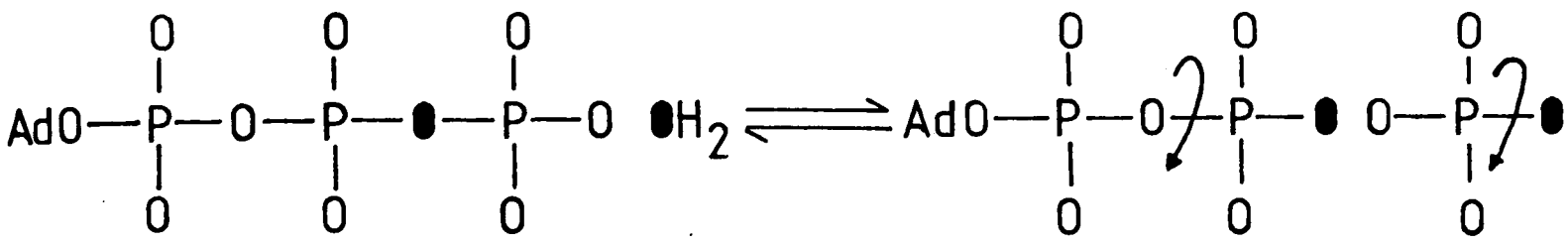


FIGURE 51

Knowles[204] has found that when γ-chiral [^{16}O, ^{17}O, ^{18}O]-ATP is incubated with pyruvate kinase in the absence of cosubstrate the γ-phosphoryl group racemises (if at all) at least 300 times more slowly than the β-phosphoryl group undergoes positional isotope exchange. This is not really surprising since even if monomeric metaphosphate were formed at the active site it would surely be tightly bound and any rotation would be very slow.

One must now examine the stereochemical consequences of such phosphoryl transfers, and indeed it is precisely in this area that so much progress has been made this decade and notably in the past few years, and is the primary subject of this thesis.

Stereochemistry

Now the distinction between a single 'in line' displacement resulting in inversion of configuration and a double 'in line' displacement or a single adjacent displacement, both of which result in retention of configuration, could be made if a *chiral phosphoryl residue was available*. It is this stereochemical investigation which is the concern of this thesis, while the enzyme catalysed ^{18}O scrambling experiments represent a complementary investigation also being carried out within this group. Creatine kinase has also been recently studied in this laboratory

by this latter technique.[205]

Although considerable insight into various aspects of phosphoryl transfer has been gained by kinetic investigations, and the isolation of a phosphoenzyme intermediate has demonstrated that it can be important during catalysis, this isolation will invariably be impossible especially for sequential reactions.

Recognition of the usefulness of creating chirality at phosphorus for studying enzyme mechanism and the pioneering investigation into the mechanism of phosphoryl transfer from a stereochemical viewpoint was made by Eckstein[206,207] in his elegant study of the mechanism of action of ribonuclease A using phosphorothioates. Ribonuclease A catalyses the decomposition of ribonucleic acid by a two stage process, namely transphosphorylation followed by hydrolysis of the resulting cyclic phosphate diester (FIGURE 52).

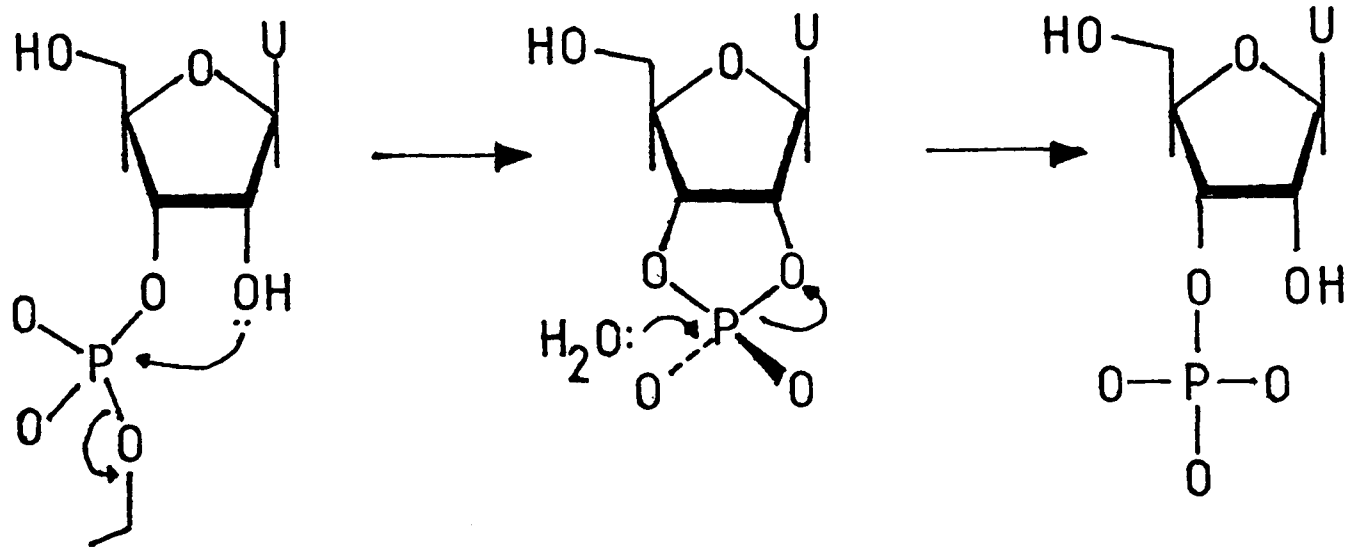
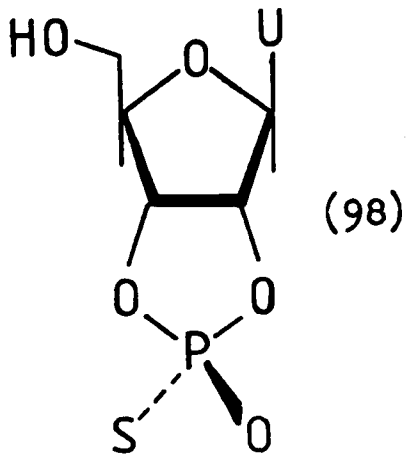


FIGURE 52

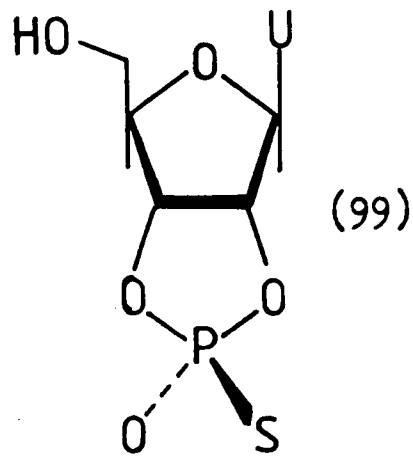
Both steps involve either the formation or hydrolysis of a 5-membered ring phosphate diester which can occur by an 'in line' or adjacent mechanism. The key to the analysis was the isolation of the two diastereoisomers of uridine 2',3'-cyclic phosphorothioate[208] (98 and 99) (FIGURE 53).

The enzyme catalysed ring opening of diastereoisomer (98) was carried out in $H_2{}^{18}O$ to produce uridine 3'-phosphorothioate with ^{18}O incorporated specifically into the thiophosphoryl group (100).



(98)

crystalline
diastereoisomer



(99)

liquid
diastereoisomer

FIGURE 53

Recyclisation to uridine 2',3'-cyclic phosphorothioate using diethyl phosphorochloridate occurred by an 'in line' process.[206] There was thus an equal chance that ^{16}O or ^{18}O was lost on chemical recyclisation but *if* the enzyme catalysed ring opening had been a stereospecific 'in line' process then the loss of ^{18}O would regenerate diastereoisomer (98) while the loss of ^{16}O would produce diastereoisomer (101) (FIGURE 54). A clear demonstration of an

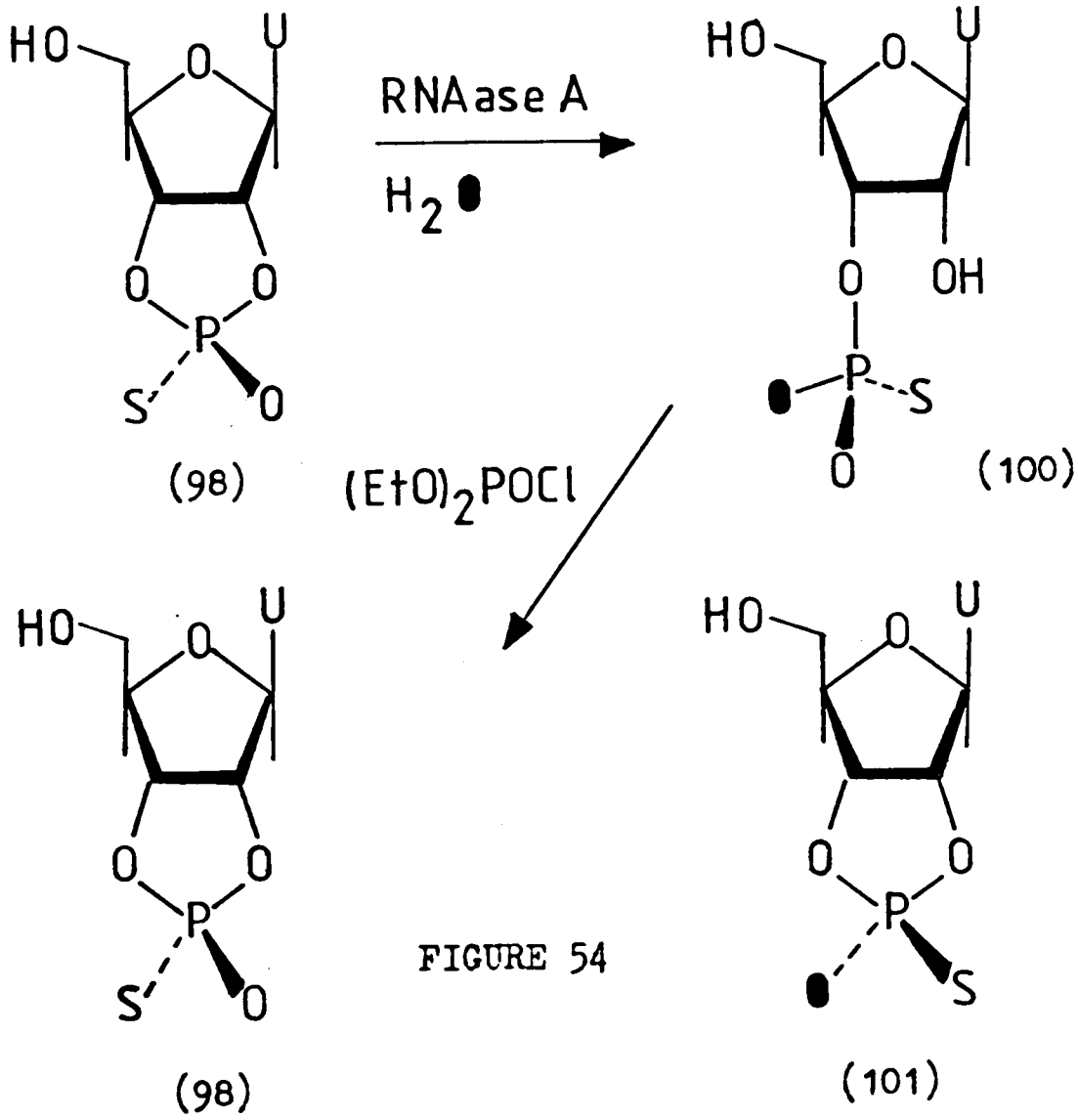


FIGURE 54

'in line' mechanism for the second step of the ribonuclease catalysed reaction resulted from the separation of the diastereoisomers from the chemical ring closure and the estimation of the ^{18}O content by mass spectrometry. The mechanism shown in FIGURE 54 was then established. The first step of the ribonuclease reaction was also shown to be 'in line' by a similar method[207], and Knowles[209] has used similar arguments to allow a comparative study of the stereochemistry of the reactions catalysed by pyruvate kinase, glycerol kinase and hexokinase, all of which have been shown to catalyse reactions with the same stereochemical consequence. However, in spite of Eckstein's work, and although Knowles has shown how this fundamental approach may be extended, it will be appreciated that these methods are not *generally* applicable. The use of nucleoside phosphorothioates to investigate enzyme mechanism has been reviewed by Eckstein.[210]

In order to outline the complexity of this area it will be expedient to consider the whole range of possible displacement reactions. From a stereochemical viewpoint the types of displacement at phosphoric monoesters and diesters may be categorised into three divisions as follows.

(a) Prochiral Substrate → Prochiral Product

In this class the phosphorus at which the displacement occurs is a prochiral centre and the reaction results in the creation of a new prochiral centre at that phosphorus. For example, the enzyme UDP-glucose pyrophosphorylase catalyses the nucleophilic displacement of pyrophosphate from P_α of UTP by the phosphoryl group of glucose-1-phosphate.

$$\text{UTP} + \alpha\text{-}D\text{-glucose-1-phosphate} \rightleftharpoons \text{UDP-glucose} + PP_i$$

The stereochemical course of this reaction has been established

by the experiments of Sheu and Frey[211] who created chirality at the prochiral phosphorus by replacement of a peripheral oxygen by sulphur.

(b) Prochiral Substrate → Pro-Prochiral Product

In this case a prochiral phosphorus is attacked by water to give a phosphate monoester which is *pro*-prochiral at phosphorus. For example the prochiral phosphorus centre of adenosine-3',5'-cyclic phosphate (cAMP) is hydrolysed by cyclic phosphodiesterase to give a *pro*-prochiral product in 5'-AMP. Once again sulphur analogues have proved useful and in this case the stereochemistry was apparently solved by Eckstein *et al.*[212] who hydrolysed the S_P diastereoisomer of cAMP phosphorothioate (cAMPS) in $H_2{}^{18}O$ and then determined the configuration of the resulting chiral $AMP_\alpha S$ ^{18}O. Moreover, recent important work in this laboratory with the oxygen chirally labelled *natural* substrate has shown their result of inversion to be correct.[213]

TABLE 7 summarises all the currently known stereochemical courses of enzyme catalysed displacements at phosphorus for groups (a) and (b).

(c) Pro-prochiral substrate → Pro-prochiral product

This class contains all the enzymes which catalyse phosphoryl group transfer reactions where both substrate and product are phosphoric monoesters. For example, the reaction catalysed by glycerol kinase

$$\text{ATP} + \text{glycerol} \rightleftharpoons \textit{sn}\text{-glycerol-3-phosphate} + \text{ADP}$$

Two approaches have been made to study this class of reaction— the transferred phosphoryl group *has been made chiral* by

TABLE 7: STEREOCHEMICAL COURSE OF DISPLACEMENTS AT PHOSPHORIC DIESTERS

Enzyme	Result	Class[a]	Reference
ribonuclease A	inversion, inversion	a,b	206,207
ribonuclease T_1	inversion	a	240
ribonuclease T_2	inversion	a	241
UDP-glucose pyrophosphorylase	inversion	a	211
galactose-1P-uridylyl-transferase	2 inversions or 2 retentions	a	204
PRPP synthetase	inversion	a	242
RNA polymerase (initiation & exchange)	inversion	a	243
RNA polymerase (elongation)	inversion	a	244,245
DNA polymerase	inversion	a	246
tRNA nucleotidyl transferase	inversion	a	247
polynucleotide phosphorylase (elongation)	inversion[b]	a	248
polynucleotide phosphorylase (exchange)	retention[b]	a	204
acetyl CoA synthetase	inversion	a	249,232
Enterobacter aerogenes phosphatase	inversion	b	250
snake venom phosphodiesterase	overall retention	b	251,252
bovine intestine 5'-phosphodiesterase	overall retention	b	204
aminoacyl-tRNA synthetases	inversion	a	253
cyclic phosphodiesterase	inversion	b	212,213
Brevibacterium liquefaciens adenyl cyclase	inversion	a	204

[a] With the natural unlabelled substrates. [b] This is in the exchange reaction using ADPαS and $^{31}P_i$, which may be a model for the initiation step (as distinct from the elongation steps, which proceed with inversion).

distinguishing the three formally equivalent peripheral oxygen atoms by using either ^{16}O, ^{18}O and sulphur or by using ^{16}O, ^{17}O and ^{18}O. Glycerol kinase has been studied by both methods[214,215], and the fact that both approaches have given the same answer (inversion) has relieved fears that the more slowly reacting phosphorothioates, invariably only poor substrates for the enzyme (although most of the glycolytic enzymes from hexokinase to pyruvate kinase will handle them) may follow a different mechanistic and stereochemical course. These allayed fears, moreover, have also been quelled by the current results for cAMP where phosphodiesterase opens the sulphur analogue with inversion of configuration, and the natural compound with inversion.[213] Indeed phosphoglycerate mutase does *not* appear to tolerate sulphur substitution. Results from this thesis, however, using our new ^{31}P n.m.r. method for analysing the chirality of [^{16}O, ^{17}O, ^{18}O]-labelled phosphates[216] have shown that with their *natural* oxygen chiral substrates the enzymes hexokinase[217], pyruvate kinase[218] and phosphofructokinase[219] all catalyse phosphoryl transfer with *inversion* of configuration.[220] Of these three enzymes hexokinase and pyruvate kinase have previously been shown to catalyse thiophosphoryl transfer with inversion of configuration[209], and also glycerol kinase has been shown to catalyse both transfers with inversion of configuration[214,215] so it would appear that, at least for the kinases, phosphorothioates are reliable analogues for stereochemical studies. All the enzyme catalysed displacements of group (c) whose stereochemistries have been deduced by these methods are summarised in TABLE 8.

Although Eckstein's pioneering stereochemical work[207] was done in 1970 it is only very recently that the data in TABLES 7 and 8 have been obtained. Synthetic approaches to chiral phosphorothioate diesters[221,222], chiral [^{18}O] phosphorothioate

TABLE 8: STEREOCHEMICAL COURSE OF DISPLACEMENTS AT PHOSPHORIC MONOESTERS

Method	^{16}O-^{18}O-S		^{16}O-^{17}O-^{18}O		
Enzyme	Result	Reference	Enzyme	Result	Reference
glycerol kinase	inversion	209,214,215	glycerol kinase	inversion	214
hexokinase	inversion	214,215	acetate kinase	inversion	237
pyruvate kinase	inversion	214,215	alkaline phosphatase	2 inversions or 2 retentions	238
adenylate kinase	inversion	223,224,234			
adenosine kinase	inversion	204	phosphoglycerate mutase		
nucleoside diphosphate kinase	2 inversions or 2 retentions	235	(cofactor dependent and cofactor independent)	2 inversions or 2 retentions	239
nucleoside phospho-transferase	overall retention	236			
			hexokinase	inversion	This work[217]
			pyruvate kinase	inversion	This work[218]
			phosphofructokinase	inversion	This work[219]

monoesters[209,223,224,225], and chiral [^{16}O, ^{17}O, ^{18}O] phosphoric monoesters[214,226,227] have been devised, and essentially any labelling pattern of any of the phosphate groups in ATP can be now achieved. Moreover, several methods for the analysis of the location of isotopic labels in such molecules are now well developed, and one such novel method is reported in this thesis.[216] Mass spectrometry is useful when the amount of material is small[197,228,229,230], but the effect of ^{18}O substitution on the ^{31}P chemical shift[200,201,231] and of ^{17}O substitution on the line width of the ^{31}P n.m.r. signal[231,232,233] (CHAPTER 7) are non-destructive methods that are especially useful when enough product is available.

Thus it appears from the data collected in TABLES 7 and 8 that, provided the phosphorothioates can be considered to be well behaved probes of the stereochemical consequences of phosphoryl transfer reactions in classes (a) and (b), by far the most preferred stereochemical consequence for enzyme catalysed phosphoryl transfer is *inversion* of configuration.

CHAPTER 7

A STEREOCHEMICAL APPROACH TO THE MECHANISM OF ENZYME CATALYSED PHOSPHORYL TRANSFER: A METHOD USING CIRCULAR DICHROISM SPECTROSCOPY

INTRODUCTION

In order to delineate the stereochemical consequences of group (c) phosphoryl transfer reactions (CHAPTER 6) we have developed in this laboratory[226], simultaneously with Knowles *et al.*[214,227] at Harvard, *a general method for establishing whether retention or inversion of configuration of phosphorus occurs in these processes.* This is achieved by the creation of chirality at the required phosphorus centre using the three stable isotopes of oxygen ^{16}O, ^{17}O and ^{18}O. A general phosphorylating reagent has been devised in this laboratory[226], (2*R*, 4*S*, 5*R*)-2-chloro-[2-^{17}O]-oxo-4,5-diphenyl [1-^{18}O]-1,3,2-dioxaphospholane (102b) and has been used to

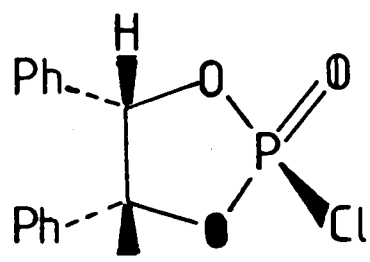
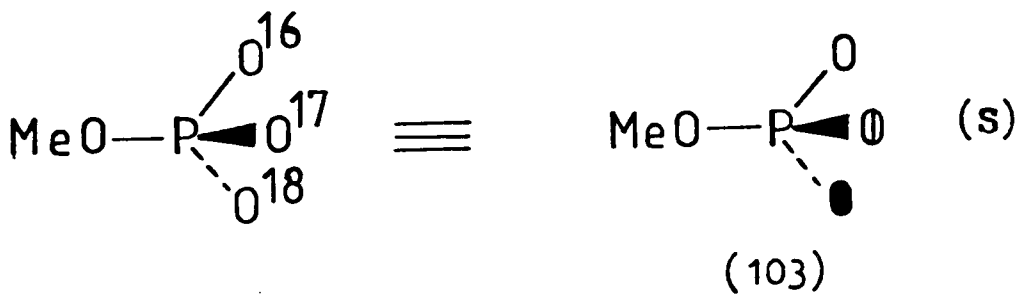


FIGURE 55

(102b)

synthesise methyl [(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate (103). The small isotopic mass chirality has been shown to be sufficient for this molecule to exhibit a small but surprisingly measurable u.v. circular dichroic effect.[226] Thus this effect may in principle be



(103)

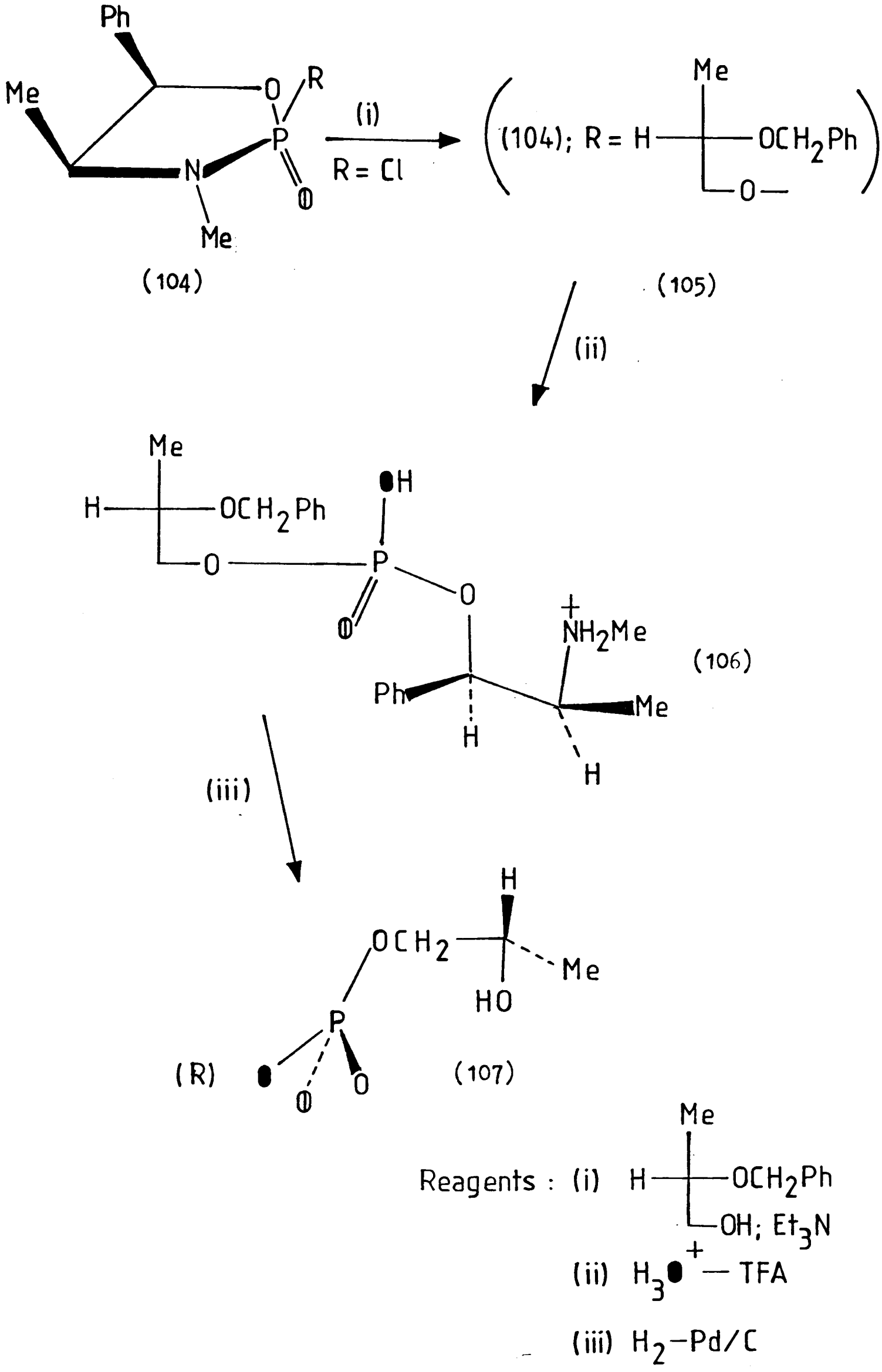
FIGURE 56

used to determine the absolute configuration of a given chiral [^{16}O, ^{17}O, ^{18}O]-phosphate monoester in a conventional fashion since the (*R*)-isotopic enantiomer will of course exhibit a mirror image circular dichroic effect, provided of course that apart from

isotopic substitution the molecule is *achiral*. Moreover, it should be possible to extend such optical activity measurements into the infrared region of vibrational chirality using the recently developed technique of laser Raman circular dichroism spectroscopy (LRCD) as pioneered by Barron[254] who has shown that in contrast to the electronic optical activity of isotopically disymmetric molecules, the vibrational optical activity is expected to be significantly large, and will provide a reliable correlation of absolute configurations. This procedure will, with increasing instrumental sophistication, have great sensitivity advantages over UVCD measurements and may allow such accurate focussing on vibrational modes of interest that other chiral centres may also be present without significant interference.

Knowles *et al.*[214,227] have developed a method of synthesising chiral [^{16}O, ^{17}O, ^{18}O]-phosphates of opposite ((*R*)) configuration as well as those derived from our method[226] (SCHEME 8). 2-Chloro-1,3,2-oxazaphospholidine (104, R = Cl) was allowed to react with (*S*)-propane-1,2-diol monobenzyl ether, a step proceeding with retention of configuration at phosphorus, and the resulting ether ester hydrolysed when ring opening occurred to give compound (106). 1-[(*R*)-^{16}O, ^{17}O, ^{18}O]-Phospho-(*S*)-propane-1,2-diol (107) was finally obtained by hydrogenolysis. Stereochemical analysis of compound (107) presented an intriguing problem which was solved by a lengthy chemical sequence involving cyclisation of (107), methylation, chromatographic separation of diastereoisomers, hydrolysis and metastable ion mass spectrometry.[230] Our proposed analytical procedure is much shorter and involves measurement of spectroscopic properties.

Now most of the natural acceptors of the phosphoryl group from ATP in kinase-mediated reactions are intrinsically chiral and would be poor choices for a subtle spectroscopic analysis. The most



SCHEME 8 : The Synthesis of 1-[(R)-^{16}O, ^{17}O, ^{18}O]-Phospho-(S)-propane-1,2-diol, after Knowles (ref. 214)

desirable and ubiquitous moiety to use as a starting point for the analysis of chirality would of course be ATP. Many enzyme reactions *per se* are reversible, and most phosphoryl transfer reactions should be able to be driven in the direction of ATP synthesis.

Thus providing we can synthesise, by selective protection techniques, a variety of biologically important [^{16}O, ^{17}O, ^{18}O]-phosphate monoesters of known absolute isotopic configuration, and we allow the phosphoryl transfer to ADP to proceed enzymatically then we will always end up with a γ-chiral [^{16}O, ^{17}O, ^{18}O]-ATP molecule of unknown isotopic configuration containing the required stereochemical information for the transformation. The problem is, of course, to determine the configuration, which really means to selectively degrade the ATP to remove the potentially interfering ribose sugar system without harming the γ-phosphoryl residue. The most satisfactory procedure for performing this appears to be the enzymic cleavage of ATP to AMP and inorganic pyrophosphate catalysed by acetyl coenzyme A synthetase. Thus the γ-chiral phosphoryl residue could in principle be split off as pyrophosphate from γ-chiral ATP derived from any enzyme reaction and compared with that of chemically synthesised P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate of known absolute isotopic configuration to deduce the stereochemistry of the phosphoryl transfer. The procedure is illustrated in FIGURE 57 for pyruvate kinase. 2[(*S*)-^{16}O, ^{17}O, ^{18}O]-phospho-(*R*)-glycerate (105) has already been synthesised in this laboratory[255], converted to 2[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphoenol pyruvate (109) by enolase, and the chiral phosphoryl residue transferred to ADP by pyruvate kinase with unknown stereochemical consequence. All that remains is the development of a general method for analysis of the isotopic chirality of the ATP (110) and to this end the preparative degradation of ATP with acetyl coA synthetase was investigated.

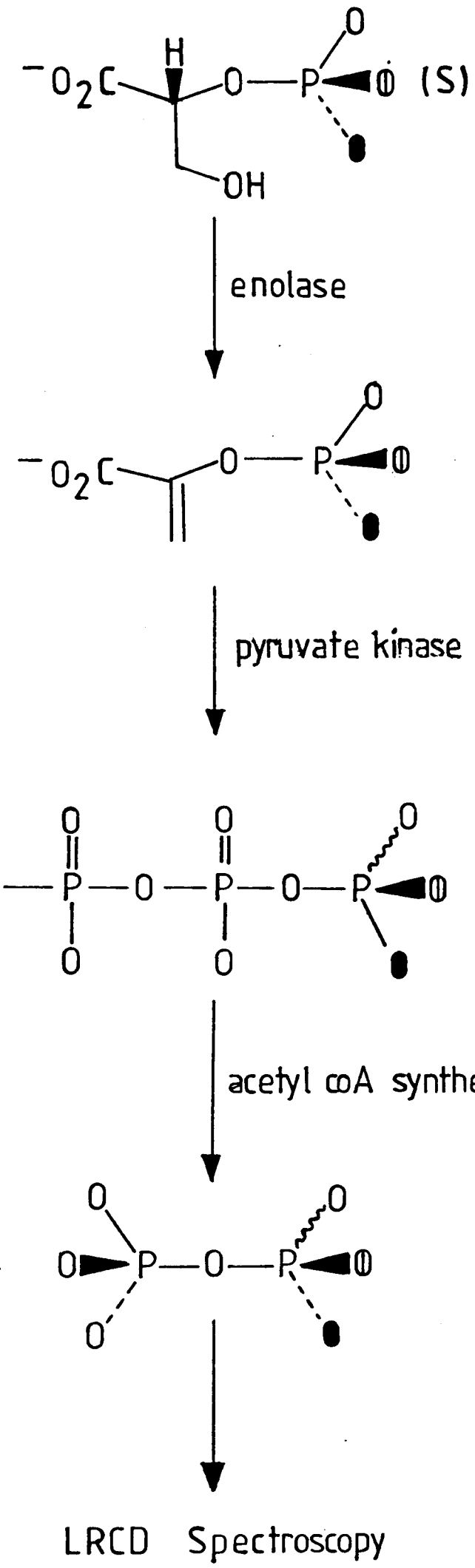


FIGURE 57 : A Proposed Analysis of the Stereochemistry of the Pyruvate Kinase Reaction using LRCD Spectroscopy.

RESULTS AND DISCUSSION

The Acetyl Coenzyme A Synthetase Catalysed Cleavage of ATP

Acetyl coenzyme A synthetase (E.C. 6.2.1.1.) catalyses the reaction:

$$ATP + MeCO_2^{\theta} + CoA \rightleftharpoons AMP + PP_i + AcCoA$$

and in order to drive this reversible reaction in the forward direction and also to avoid the use of large quantities of the expensive cofactor coenzyme A when performed on a preparative scale this reaction was coupled with the essentially irreversible synthesis of citrate from oxaloacetate and acetyl coenzyme A using citrate synthase which thus gives the two reactions:

$$ATP + MeCO_2^{\theta} + CoA \rightleftharpoons AMP + PP_i + AcCoA$$

$$AcCoA + \text{oxaloacetate} + H_2O \longrightarrow CoA + \text{citrate}$$

$$\textit{i.e.}\quad ATP + MeCO_2^{\theta} + \text{oxaloacetate} + H_2O \rightleftharpoons AMP + PP_i + \text{citrate}$$

These reactions were found to couple together quite readily and the overall progress could be monitored on aliquots of reaction mixture by an adaptation of the extremely sensitive citrate assay procedure of Natelson *et al.*[256] The overall reaction was generally found to go to the reasonable extent of *ca.* 75-80% over approximately 2 h which seemed to be quite satisfactory.

An unfortunate problem which manifested itself during experimentation was the contamination of the acetyl coenzyme A synthetase with inorganic pyrophosphatase which catalyses the reaction:

$$PP_i + Mg^{2+} \longrightarrow 2P_i + Mg^{2+}$$

Quite obviously when a preparative scale isolation of inorganic pyrophosphate is desirable the presence of this activity can be very dangerous. A private communication from Midelfort[257], however, demonstrated that this contaminant could be removed by chromatography on a DEAC cellulose column, eluting with phosphate buffers. Indeed when this was performed and the purified enzyme examined by incubation with PP_i and Mg^{2+} in a ^{31}P n.m.r. tube no inorganic pyrophosphatase activity could be detected, although the intrinsic synthetase activity had been much reduced by this treatment. The purification is illustrated in FIGURE 58, the acetyl coA synthetase being the last peak on the elution profile.

After destruction of enzyme activity by agitation with chloroform the products from a typical coupled enzyme run were loaded onto a DEAE Sephadex column run in TEAB pH 10.5. The high pH was necessary to ensure that the pyrophosphate was fully charged so that it would be separated from any citrate present. AMP was eluted first followed by citrate, pyrophosphate and undegraded ATP. Nucleotides were detected by their u.v. absorption at 280 nm and citrate by the Natelson procedure.[256] Thus an enzymic degradation procedure for γ-chiral [^{16}O, ^{17}O, ^{18}O]-ATP was established.

Synthesis of Inorganic Pyrophosphate and P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-Pyrophosphate

In order to establish spectroscopically the isotopic configuration of the P_1-[^{16}O, ^{17}O, ^{18}O]-pyrophosphate split off from the γ-chiral [^{16}O, ^{17}O, ^{18}O]-ATP derived from a kinase reaction we required a stereospecific synthesis of P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate. (The independent synthesis of γ-chiral [(*S*)-^{16}O, ^{17}O, ^{18}O]-ATP should be possible by a modification of the method of Baddiley *et al.*[258] in which P_1P_2-dibenzyl

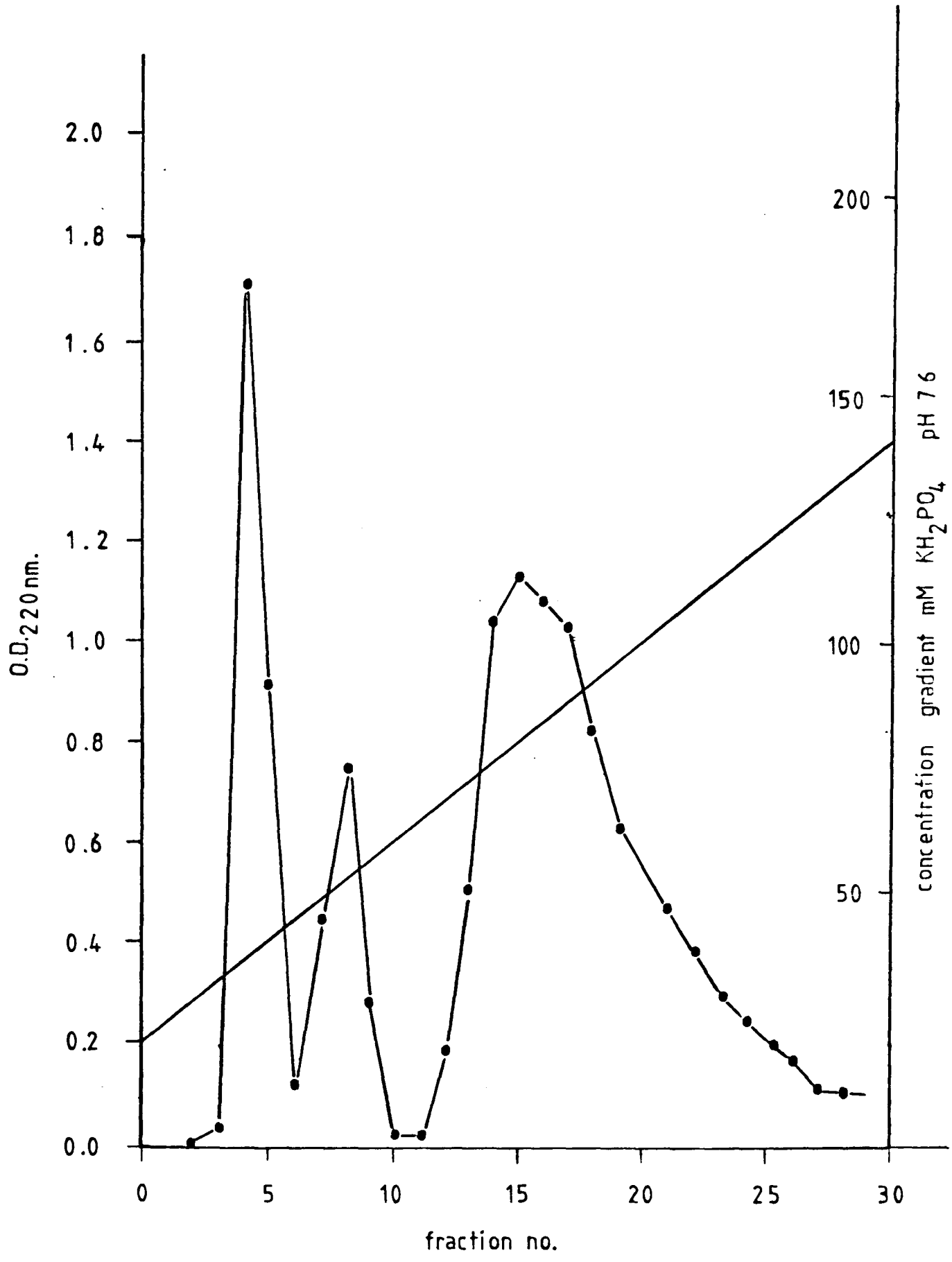
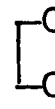


FIGURE 58 : The Purification of Acetyl CoA Synthetase on DEAC 52 Cellulose.

adenosine diphosphate is phosphorylated by dibenzyl phosphorochloridate - see CHAPTER 11 - however, since we ultimately plan to perform a chiroptical analysis on the P_1-[^{16}O, ^{17}O, ^{18}O]-pyrophosphate obtained by the degradation of the labelled ATP, the direct synthesis of this molecule is more appropriate).

A synthetic route developed to inorganic pyrophosphate is illustrated in SCHEME 9. The unlabelled phosphorylating agent (102a) was allowed to react with dibenzyl phosphate (111) to produce *trans*-2-(dibenzylphospho)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (112). A wide variety of conditions was investigated since the usual conditions for phosphorylation, namely generation and *in situ* reaction of the phosphorochloridate (102) were found to produce a number of side reactions. The formation of the asymmetric pyrophosphate tetraester (112) was monitored by ^{31}P nmr spectroscopy and although the spectrum of the reaction mixture in pyridine was complex an appreciable quantity of (112) was produced with the expected doublet signals at -1.5 ppm from TMP ($^2J_{pp}$ = 21 Hz, ) and at -16.2 ppm ($^2J_{pp}$ = 21 Hz, $(BzO)_2P$) as well as an intense singlet at -15.5 ppm which was assigned to tetrabenzyl pyrophosphate (113) produced by the following displacement reaction.

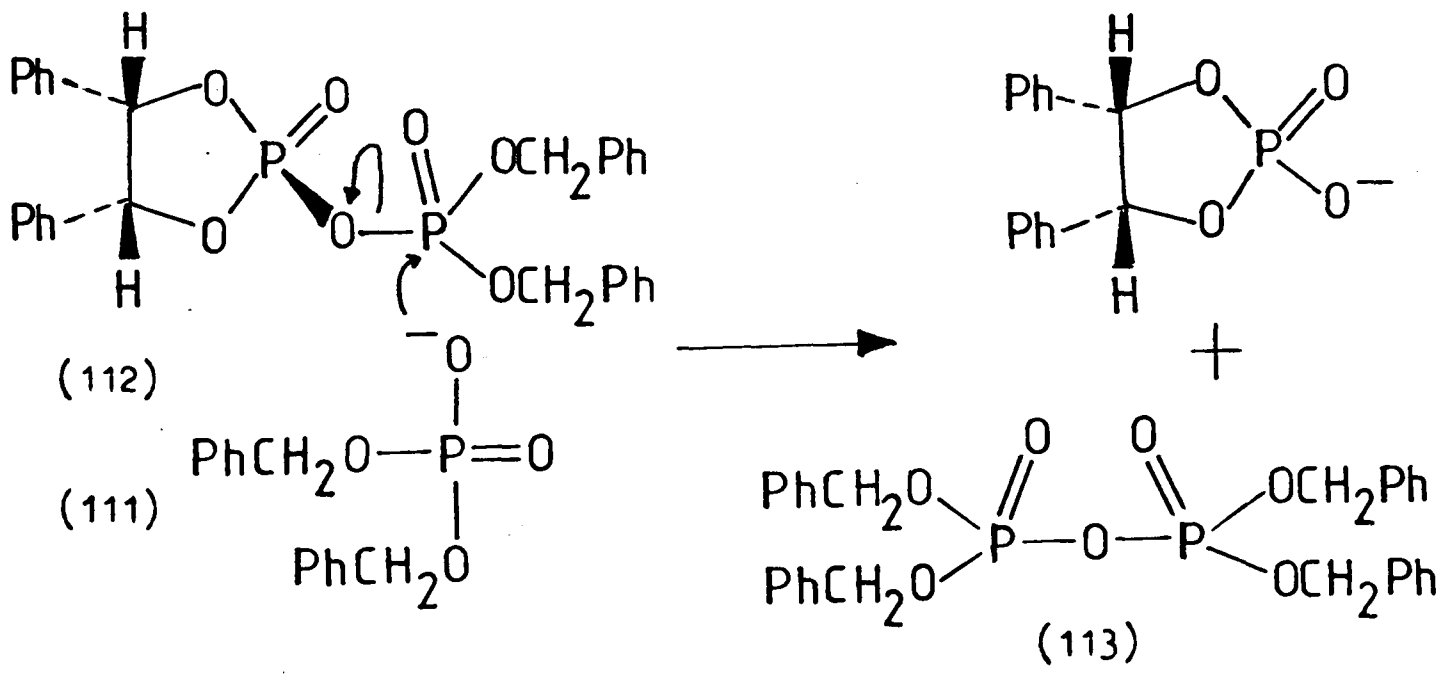
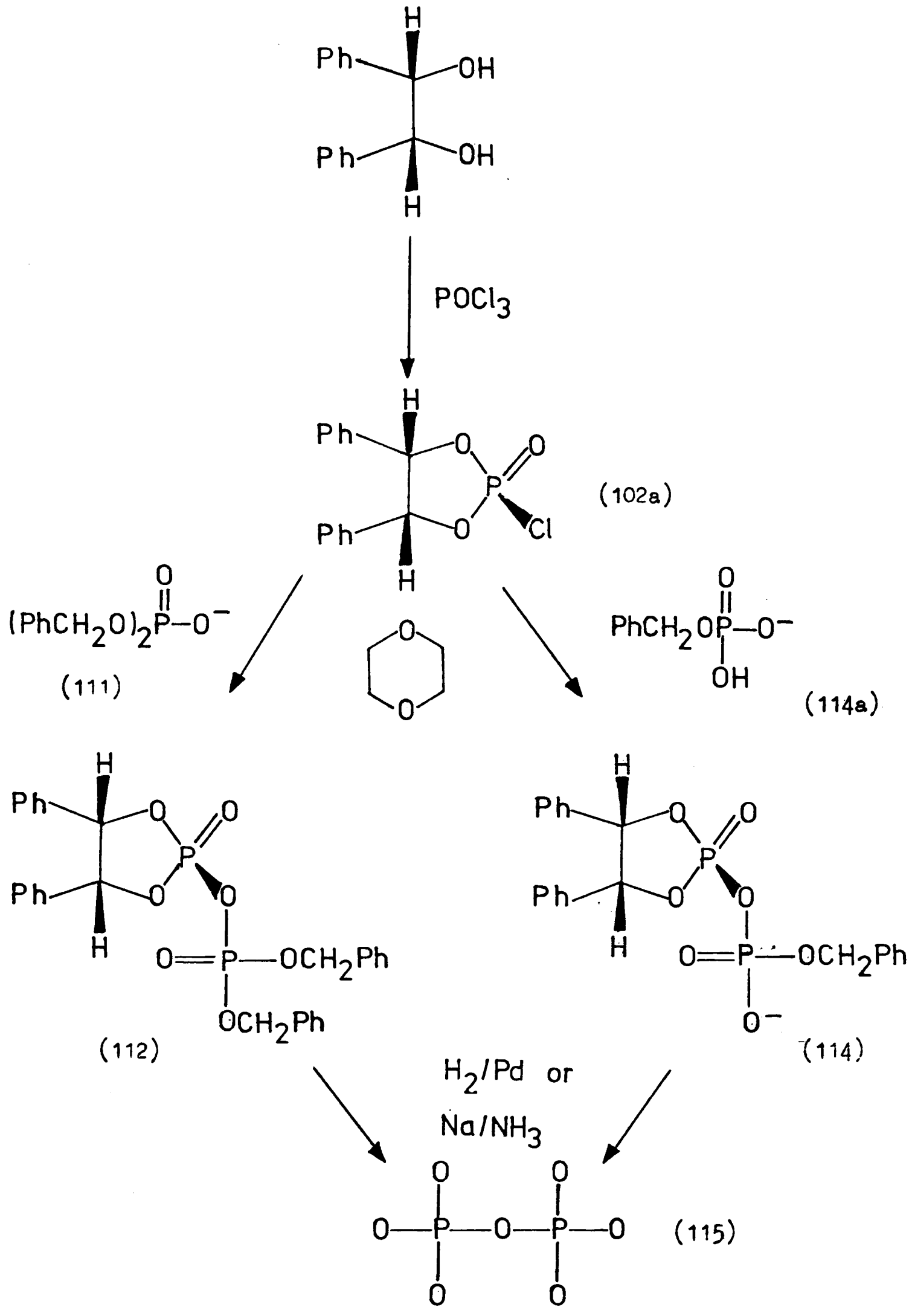


FIGURE 59

This is an analogous reaction to that reported by Corby *et al.*[259] in which P_1-dibenzyl-P_2-diphenyl pyrophosphate was observed to



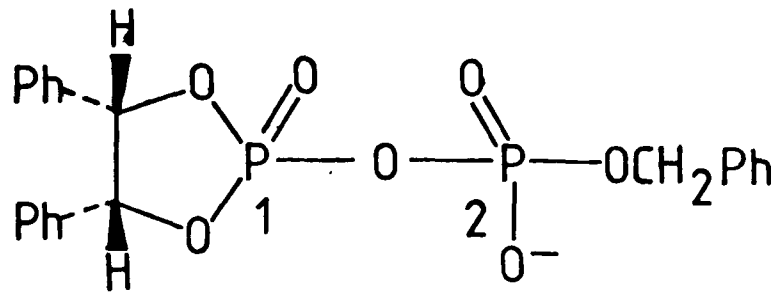
SCHEME 9 : Two Attempted Syntheses of Inorganic Pyrophosphate using the Benzyl Phosphates

react with dibenzyl phosphate to produce diphenyl phosphate and tetrabenzyl pyrophosphate (113). Moreover, it was also found that P_1-di-*p*-methoxyphenyl-P_2-diphenyl pyrophosphate disproportionated to give *p*-methoxyphenyl pyrophosphate and tetraphenyl pyrophosphate which may represent a further explanation of the side reactions observed in pyridine solution.

Reaction in dry THF or toluene gave only small yields of (112). The optimum conditions were realised by reaction in benzene containing one equivalent of pyridine and under these conditions an excellent yield of the unsymmetrical tetraester (112) could be obtained in 1½ hours at room temperature. The undecoupled ^{31}P nmr spectrum of this compound indicated $^{3}J_{PH}$ to be 8.0 Hz and confirmed the structure at the ring phosphorus.

It was decided to investigate the stability of (112) towards water to judge possible deprotection precautions, and so two drops of deuterium oxide were added to a sample and the nmr spectrum recorded. The pyrophosphate tetraester (112) had been completely destroyed in less than 5 min. by this treatment, this increase in reactivity over tetrabenzyl pyrophosphate being associated with the five-membered ring. Westheimer[192] has shown that 2-methoxy-2-oxo-1,3,2-dioxaphospholane hydrolyses *ca.* 10^6 times faster than trimethyl phosphate, and this behaviour has been attributed to release of ring strain in the transition state, and to stereo-electronic effects.[260]

In view of this discovery, conventional hydrogenolysis techniques involving a hydrogen-water interface were obviously unacceptable, and finally it was decided to try and improve the stability of the pyrophosphate precursor before attempting deprotection. The most logical consequence of this was to attempt the preparation of the triester (114) in the hope that the charge would afford some protection against nucleophilic scission of the

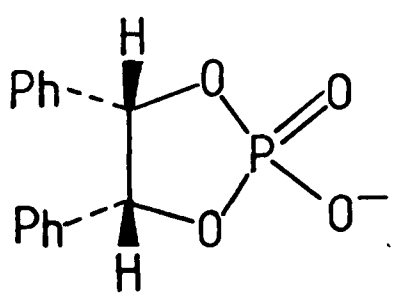


(114)

FIGURE 60

P-O-P system (SCHEME 9).

Thus monobenzyl phosphate (114a) was prepared and allowed to react with the phosphochloridate (102a) as its tributylamine salt. The best solvent was found to be dry dioxan. After 3 hours under anhydrous conditions at room temperature the ^{31}P n.m.r. spectrum showed the formation of *trans*-2(benzylphospho)-2-oxo-4,5 diphenyl-1,3,2-dioxaphospholane (114) in almost quantitative yield with doublets at -15.18 ppm and -3.65 ppm assigned to P_1 and P_2, each with $J_{P_1P_2}$ = 22.06 Hz. When water was added to this compound no destruction of the pyrophosphate linkage could be observed as hoped. The mixture was split into two portions for deprotection; one half was hydrogenolysed in ethyl acetate with a Pd/C catalyst, and the other half submitted to sodium-liquid ammonia reduction. Examination of the ^{31}P n.m.r. spectrum of the hydrogenolysed product showed predominantly a strong resonance at +0.24 ppm which was assigned to inorganic phosphate, although from the less intense peak at -7.57 ppm it was apparent that some inorganic pyrophosphate (115) had been formed. The presence of a peak at +12.88 ppm assigned to the 5-ring diester (116), suggested that the hydrogenolysis had proceeded



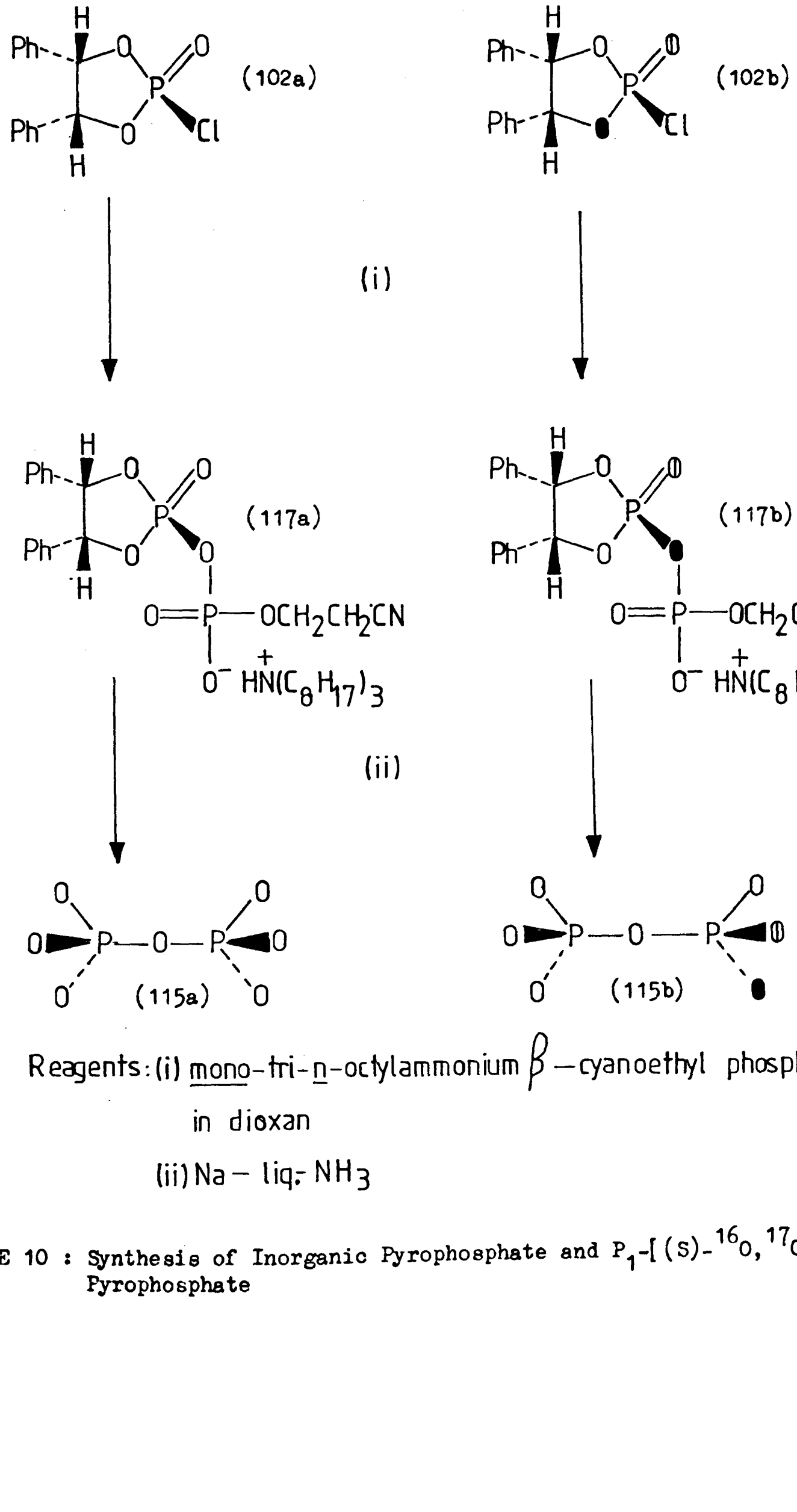
(116)

FIGURE 61

with a rapid removal of the benzyl group to leave the corresponding P_iP_1-pyrophosphate diester which had decomposed. Thus the problem would appear to be the relative rates of hydrogenolysis of the benzyl ester as compared to the 5-ring system.

The alternative deprotection reaction, namely sodium metal in liquid ammonia has the advantage of anhydrous conditions. Arris *et al.*[261] have studied the deprotection of di-, tri- and tetra-benzyl pyrophosphate by this procedure and have found that the di- and triesters give inorganic pyrophosphate, but the tetraester gives mainly orthophosphate and some inorganic pyrophosphate. When the other half of the reaction mixture containing (114) was deprotected by sodium in liquid ammonia only two resonances could be observed in the ^{31}P nmr spectrum, the major one at +3.06 being assigned to inorganic phosphate, and the other at -7.51 to inorganic pyrophosphate (115). It seemed that a similar kind of relative deprotection problem was being encountered in the sodium-liquid ammonia reduction as well, probably due to the more facile formation of benzyl radicals, and therefore what was required was a protecting group on P_2 which would either be completely stable to the deprotection conditions or be removed at a rate much slower than that of the 5-ring system. The choice of the β-cyanoethyl protecting group appeared to be appropriate in this instance for it would be expected that it would be removed by mildly basic conditions and be completely stable to hydrogenolysis. The synthetic scheme is illustrated in SCHEME 10.

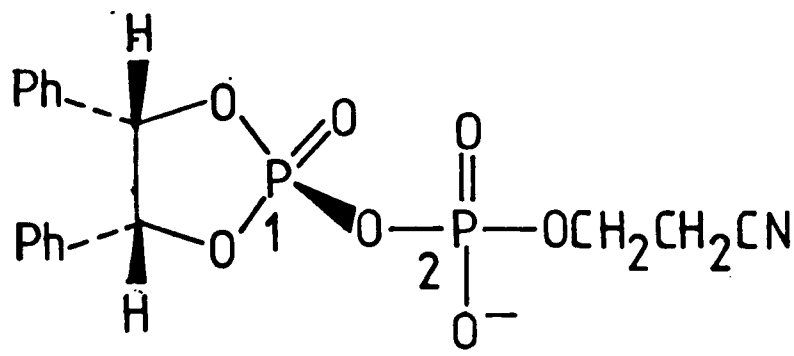
Consequently, exploiting the readily available β-cyanoethyl phosphate, converted to its tri-*n*-octylammonium salt, a reaction was attempted with the phosphorochloridate (102a), as previously. Dry dioxan was used as solvent and after 3 hours at ambient temperature the ^{31}P nmr spectrum indicated an excellent yield of the protected pyrophosphate (117) *trans*-2-(β-cyanoethylphospho)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane. The phosphorus resonances



Reagents: (i) mono-tri-n-octylammonium β-cyanoethyl phosphate in dioxan

(ii) Na – liq. NH_3

SCHEME 10 : Synthesis of Inorganic Pyrophosphate and P_1-[(S)-^{16}O, ^{17}O, ^{18}O]-Pyrophosphate



(117a)

FIGURE 62

were at -15.3 ppm and +0.75 ppm (P_2 and P_1) with $^2J_{pp}$ = 22.4 Hz and $^3J_{pH}$ = 7.5 Hz.

Hydrogenolysis using a dry hydrogen balloon system did not give a satisfactory yield of desired product but reductive cleavage using sodium in liquid ammonia indicated an excellent conversion to inorganic pyrophosphate. Moreover, the β-cyanoethyl group had also been removed, possibly during the aqueous alkaline conditions of the workup.

The reaction was repeated on a large scale and the products after deprotection subjected to ion-exchange chromatography on DEAE Sephadex A-25 using TEAB buffers and detecting the phosphorus products by an adaptaton of the Brigg's phosphate test.[262] Inorganic pyrophosphate (115) could be isolated in a yield of *ca.* 50%.

The way was now clear for the synthesis of chirally labelled P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate which was prepared in the same way as the unlabelled compound except that the chirally labelled phosphorochloridate (102b) was used. Isolation after deprotection gave P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate in 50% yield.

Attempts at Chiroptical Measurements

Although the measurement of both the UVCD and LRCD spectra has been attempted it has not so far been possible to obtain a satisfactory result. However, other LRCD results are awaited and certain experiments related to the application of this technique

in this context have been performed. Difficulties have been encountered in these measurements due to the suspected presence of trace impurities introduced during the synthesis. Chiral polysaccharide impurities from the DEAE columns have probably affected the UVCD measurements and fluorescent impurities the LRCD measurements. Although a UVCD spectrum for P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate (115) has been obtained and is illustrated in FIGURE 63 it must be viewed with some caution since it shows a considerably higher $\Delta\varepsilon$ value than that expected on the basis of the methyl [(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate results[226], and is also to slightly higher wavelength than expected.

In LRCD spectroscopy small traces of fluorescent material can be a source of much annoyance. In view of this, and since examination of the UVCD spectrum of P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate (115) indicated the presence of trace aromatic impurities due to ion exchange resins, it was decided to screen the sample on a simple Laser Raman (LR) spectrometer in Oxford to gauge the results of various purification attempts before sending the sample back to Switzerland (the previous experiment on this machine had shown only the fluorescent background). Suitable bands for optical activity measurements were also required and an idea of the problem could be obtained from a simple LR spectrum. Initial examination of the LR spectrum of unlabelled tetrasodium pyrophosphate (solubility 9 g/100 g water) showed only a weak spectrum because of strong scattering from water. However, when the tetrapotassium salt (solubility 180 g/100 g water) was used a dramatic improvement was obtained and the spectrum is illustrated in FIGURE 64. Four strong bands could be observed. The strongest band at 1020 cm^{-1} (see inset in FIGURE 64 - the large band in the main spectrum between 200 and 300 nm is a step change in background) is assigned to the totally symmetric vibration of the molecule, and is completely

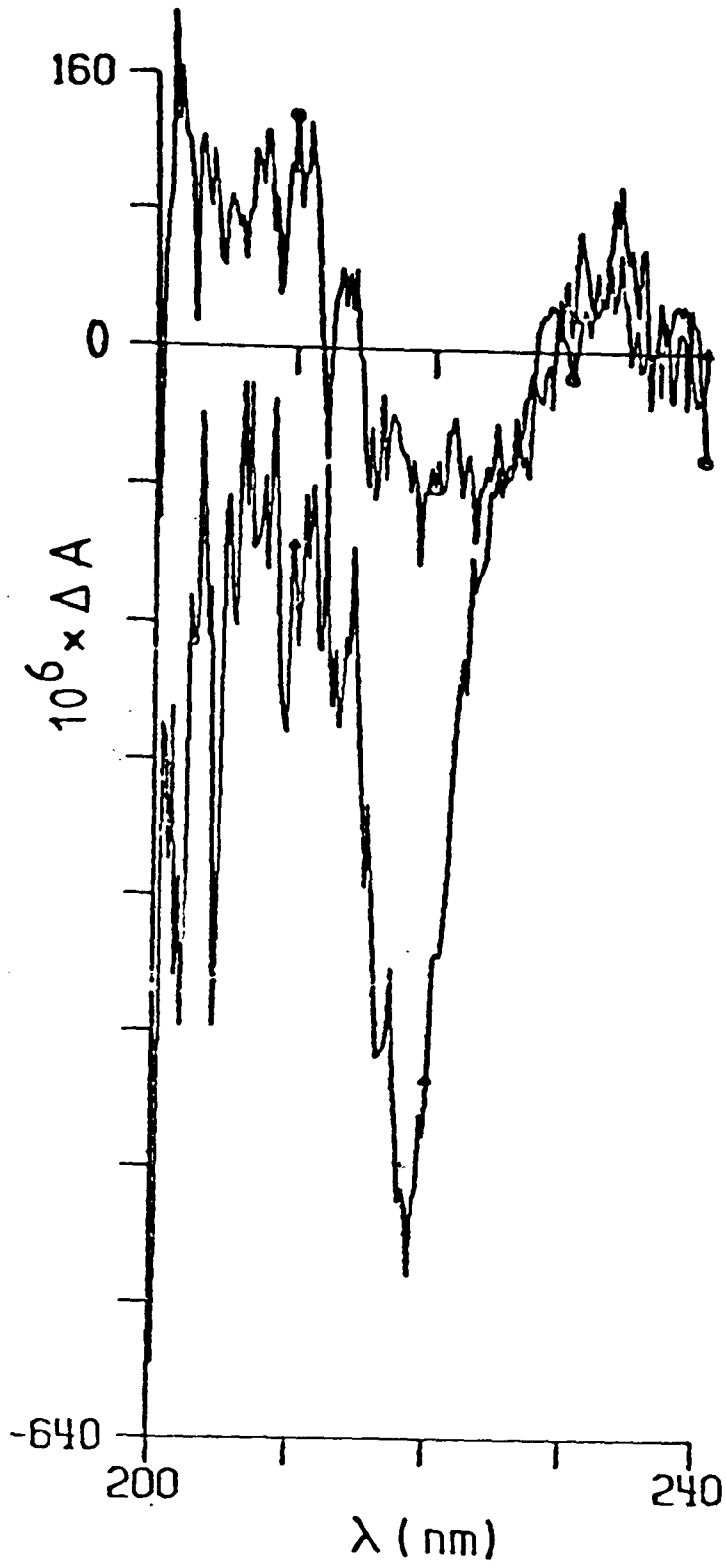
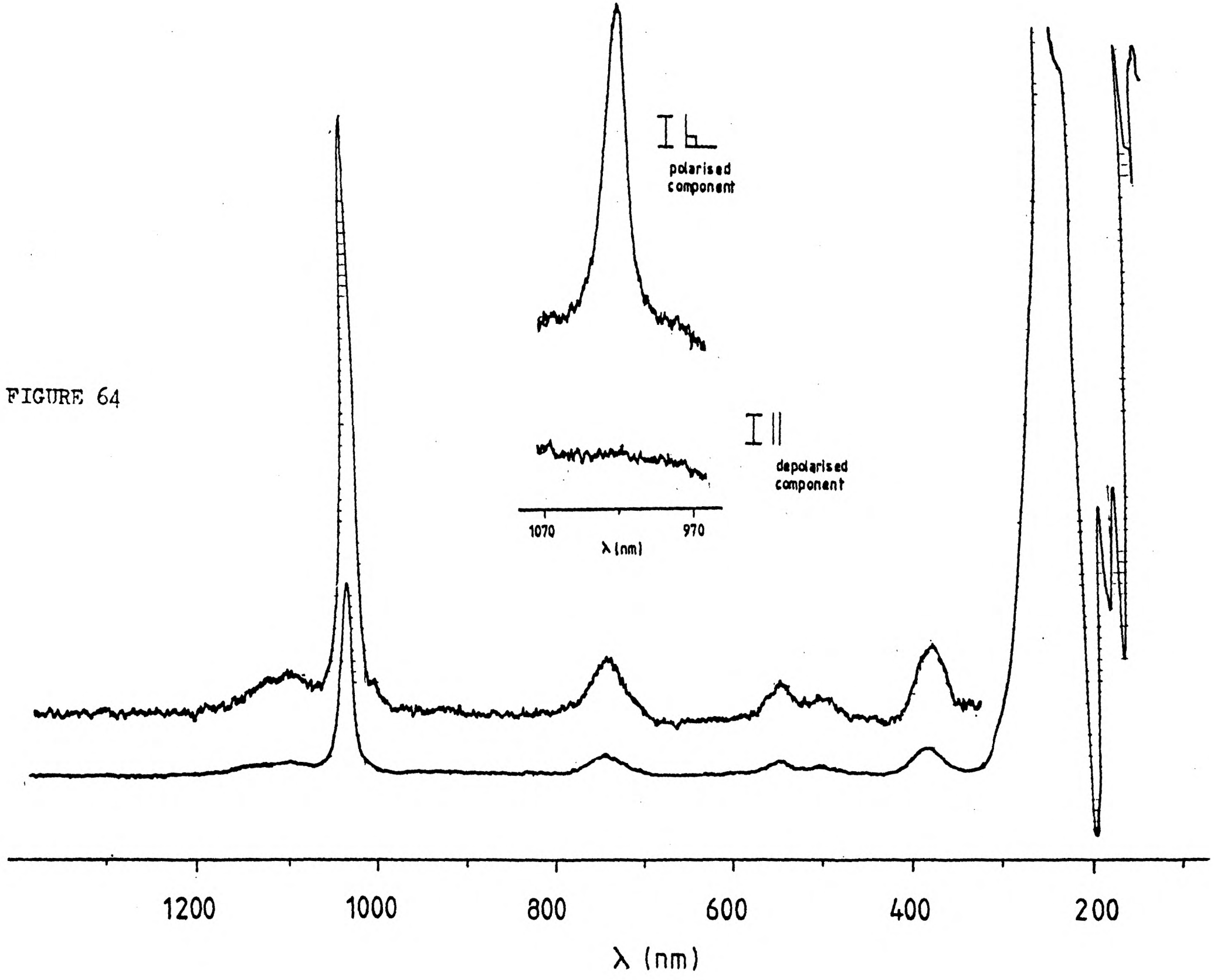


FIGURE 63 : The U.V. Circular Dichroism Spectrum of 5.2mM P_1-[(S)-^{16}O,^{17}O,^{18}O]-Pyrophosphate in water at 20 C; path length 1mm. The uppermost plot is unlabelled inorganic pyrophosphate.

LEGEND TO FIGURE 64(OVER) : The Polarised Argon-Ion Laser Raman Spectrum of Tetra-Potassium Pyrophosphate in Water. The sample was contained in a sealed soda-glass capilliary. Laser parameters were : Exciting wavelength 488nm: Laser power 200mW; Slits 5 cm^{-1}; Period 1sec. Inset : The depolarised and polarised components of the 1020 cm^{-1} band.

FIGURE 64


I⊥
polarised component
I∥
depolarised component
1070
970
λ (nm)
1200
1000
800
600
400
200
λ (nm)

polarised. LRCD optical activity measurements, therefore, would have to be carried out in perpendicular polarisation with the analyser oriented perpendicularly towards the scattering plane, and although this is possible perpendicular measurements are extremely difficult to perform. Moreover, little optical activity has so far been found for polarised bands to date.[263] There are other major bands at 190 cm^{-1}, 163 cm^{-1} and 144 cm^{-1}. The 190 nm band which is probably depolarised is just accessible to the LRCD instrument and could be measured in parallel polarisation which is much easier. This band will be strongest candidate in the search for Raman optical activity.[263]

The LRCD and LR spectra of synthetic P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate (115) were initially examined with the sodium salt and showed a strong fluorescent background. The use of C_{18} Sep. Pak. purification cartridges (Waters Associates Ltd.), however, has proved invaluable in cleaning up such samples and removing trace impurities and has given dramatic effects. When this sample was re-examined after purification although some fluorescence was observable as a background with the 1020 cm^{-1} band superimposed (which could not be seen previously), this was only to high wave-number and the 190 cm^{-1} band was observable.

Thus as the potassium salt there is much hope that this sample will give an interesting LRCD spectrum, and the result is eagerly awaited. Unfortunately, however, technical difficulties have so far precluded this.

CHAPTER 8

THE EFFECT OF ^{17}O AND THE MAGNITUDE OF THE ^{18}O ISOTOPE SHIFT IN ^{31}P NMR SPECTROSCOPY

INTRODUCTION

(a) A coupling constant is a measure of the energy of interaction of two nuclei in a molecule, and all nuclei with non-zero spin quantum numbers (I) can in theory couple to each other. In practice, however, such couplings are often not observed for nuclei with $I \geq 1$ as these nuclei possess quadrupole moments which by interacting with the electric field gradient at the nucleus can decouple the nuclear spin. Thus coupling constants have most often been measured between nuclei with $I = \frac{1}{2}$ such as ^{1}H, ^{13}C, ^{19}F and ^{31}P.

Although the coupling constant $^{1}J_{^{17}O^{31}P}$ has been measured for several phosphorus derivatives by ^{17}O n.m.r. and previously by INDOR techniques[264-268], there is no evidence in the literature of it being determined by ^{31}P n.m.r. spectroscopy and this is presumably due to the quadrupolar line broadening caused by ^{17}O ($I = \frac{5}{2}$) on the ^{31}P resonance. One of the main reasons for this is probably that until recently the maximum isotopic enrichment of ^{17}O in $H_2{}^{17}O$ was below 10%. However, we are now able to obtain enrichments of up to 50% and thus examination by ^{31}P nmr spectroscopy seemed feasible. In order to substantiate our assumptions, and since isotopically labelled [^{17}O]-phosphorus oxychloride (118) (2 atom % ^{16}O, 44 atom % ^{17}O and 54 atom % ^{18}O) was available from other investigations reported in this thesis, we prepared isotopically labelled [^{17}O]-trimethyl phosphate (119) by reaction with methanol and examined the product by ^{31}P nmr.

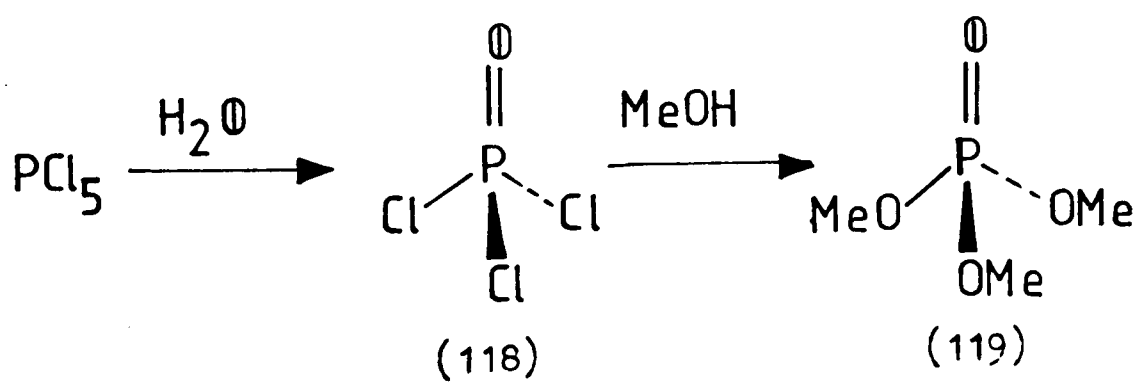


FIGURE 65

(b) The recent demonstration that an isotope shift in ^{31}P nmr spectroscopy is observed when ^{18}O is directly bonded to phosphorus[200,201] has already found extensive application.[198,205,-269-272] In using this technique to determine the absolute configuration at P_α of the diastereoisomers of adenosine-5' (1-thiotriphosphate) the isotope shift on both P_α and P_β caused by ^{18}O in the P_α-O-P bridge in (120) was 0.6 Hz whereas the isotope shift on P_α caused by the non-bridging ^{18}O in (121) was 1.1 Hz at 36.43 MHz.[225] This suggested that the type of bonding of the ^{18}O isotope to phosphorus was important in determining the size of the ^{18}O isotope shift. However, since the effect of sulphur on the electronic distribution in thiophosphates is uncertain the variation in magnitude of the isotope shift reported in this thesis has been investigated in simple methyl phosphate esters and isotopically scrambled ATP species such as (122) and (123) in

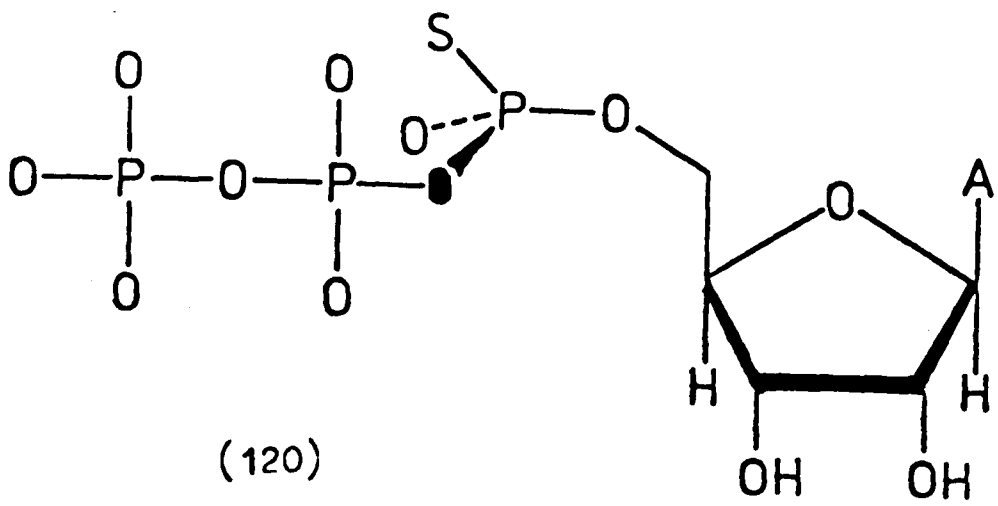
FIGURE 66.

(c) Some interesting points raised by the ^{31}P nmr spectrum of P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate are also discussed.

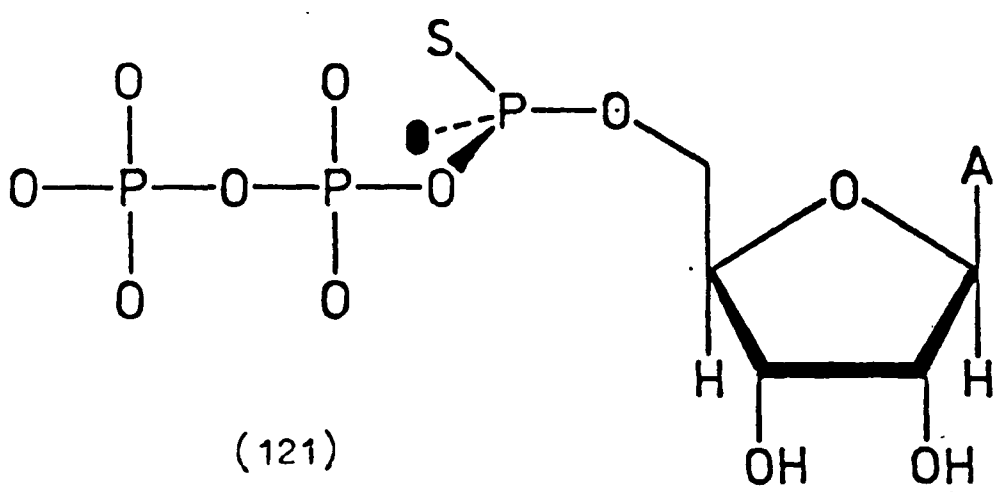
RESULTS AND DISCUSSION

(a) The $^1J_{^{17}O^{31}P}$ Coupling Constant

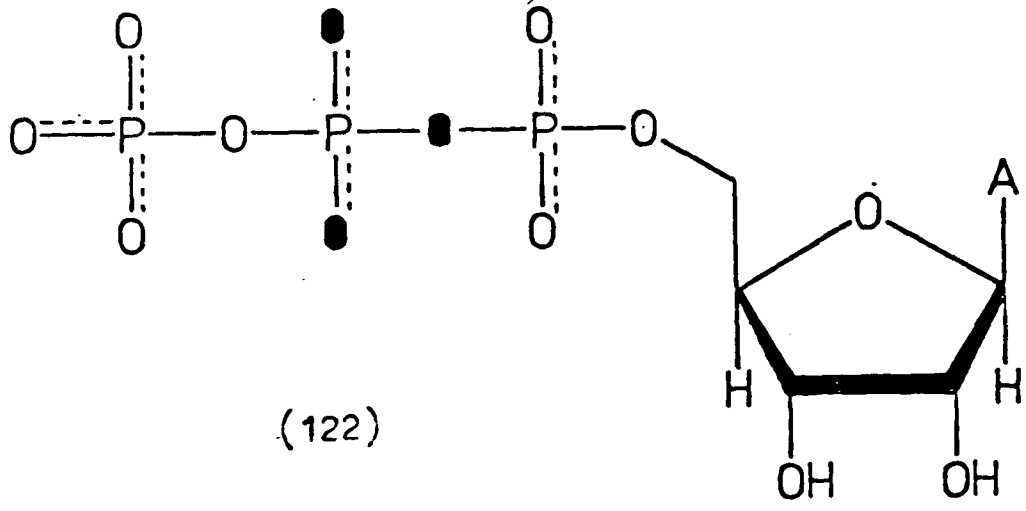
The ^{31}P nmr spectrum of [^{17}O]-trimethyl phosphate (TMP) at 36.43 MHz consisted of a sharp line ($\Delta\nu_{\frac{1}{2}}$ = 0.4 Hz) due to [^{18}O]-TMP (and presumably unresolved [^{16}O]-TMP) together with six very broad ($\Delta\nu_{\frac{1}{2}}$ ~90 Hz) but equally spaced lines due to [^{17}O]-TMP (119), three to the low field and three to the high field side of the single sharp resonance. (FIGURE 67 - in this spectrum the single sharp line of high intensity has been removed by subtraction of an equal resonance of authentic [^{16}O]-TMP leaving behind, at high gain, the rising edges of the two inner lines of the sextet due to



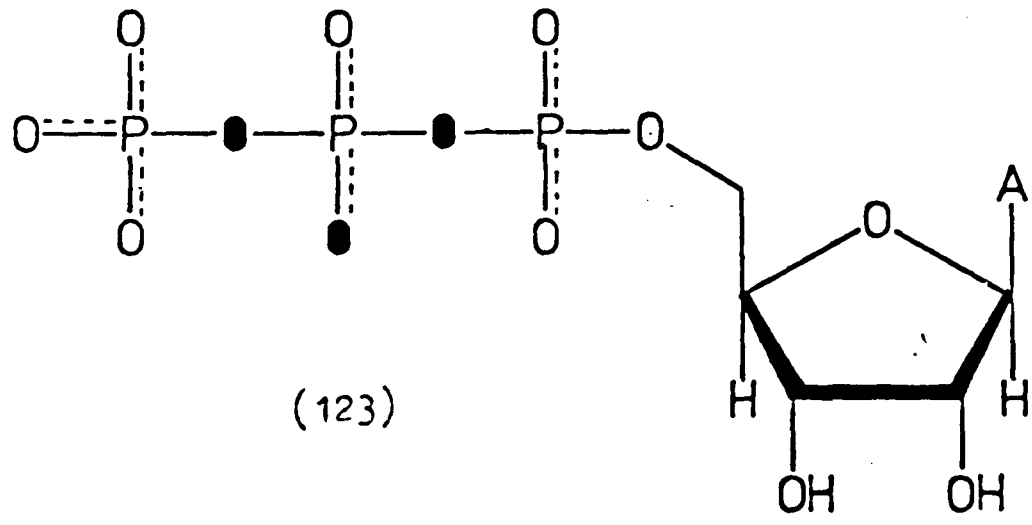
(120)



(121)



(122)



(123)

FIGURE 66

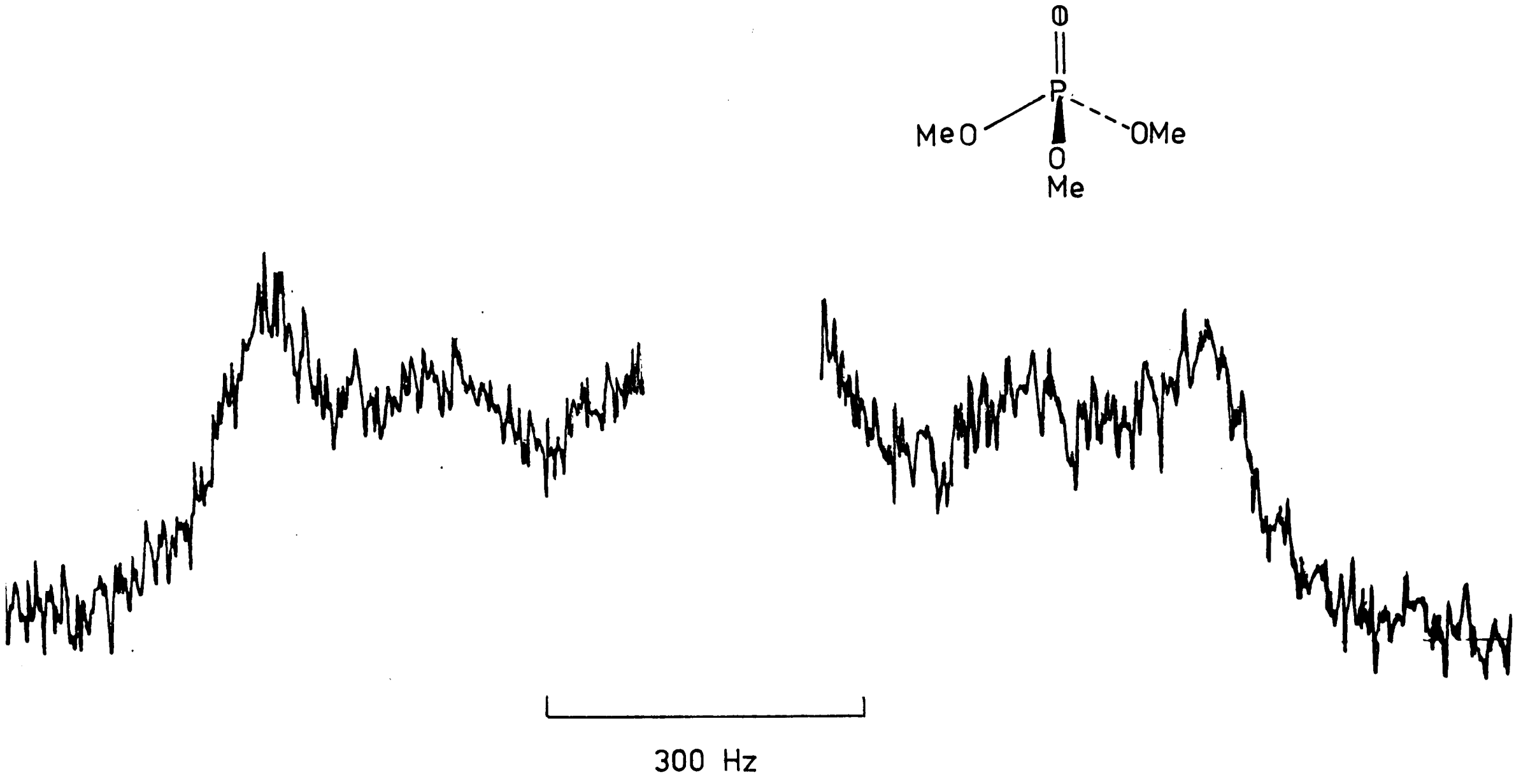


FIGURE 67 : The 36.43 MHz ^{31}P n.m.r. Spectrum of [^{17}O]-Labelled Trimethyl Phosphate. ^{31}P n.m.r. parameters were : offset 3200 Hz; sweep width 3000Hz; pulse width 20 µs; acquisition time 1.3 s; broad band proton decoupling.

^{31}P-^{17}O coupling). A very high sensitivity was required to observe these lines which under normal conditions are buried in baseline noise. The pattern of six lines is in accord with expectation since ^{17}O has a nuclear spin quantum number of $\frac{5}{2}$. Moreover, the spacing of the broad resonances gives the value of $^{1}J_{^{17}O^{31}P}$ which is 156 Hz, in quite good agreement with that determined by ^{17}O INDOR nmr spectroscopy (165 Hz).[264-268] Thus this is the first example of $^{1}J_{^{17}O^{31}P}$ coupling as observed by ^{31}P nmr spectroscopy and has been published.[231] However, although this demonstrates that it is possible to determine $^{1}J_{^{17}O^{31}P}$ by ^{31}P nmr spectroscopy in the favourable case of a highly symmetrical molecule it is expected that ^{17}O in ^{31}P nmr spectroscopy will find its most valuable application in quadrupolar line broadening when directly bonded to phosphorus, since the residual $^{31}P(^{16}O)$ or $^{31}P(^{18}O)$ signal will be at the minimum between two lines of the $^{31}P(^{17}O)$ resonance thus allowing integration of the $^{31}P(^{16}O)$ and $^{31}P(^{18}O)$ signals and hence the ^{17}O content to be estimated from the loss of signal otherwise expected. Indeed, after this work had been completed and submitted for publication[231] just such an example appeared in the literature from Tsai[232], who used the technique to investigate the mechanism of action of acetyl coenzyme A synthetase, and who has recently extended the original concept[233], and demonstrated its wide applicability in the investigation of biological systems.

(b) The Magnitude of the ^{18}O Isotope Shift

The variation in the magnitude of the ^{18}O isotope shift was investigated by preparing the three isotopically labelled methyl phosphates as illustrated in FIGURE 68. [^{18}O]-phosphorus oxychloride (124) was prepared by the reaction of $H_2{}^{18}O$ with phosphorus pentachloride, according to the method used by Laulicht for the unlabelled species[273] and reaction with methanol gave [^{18}O]-TMP (125). Partial hydrolysis of this compound (125) using aqueous base gave a mixture of [^{18}O]-dimethyl phosphate (126) and [^{18}O]-

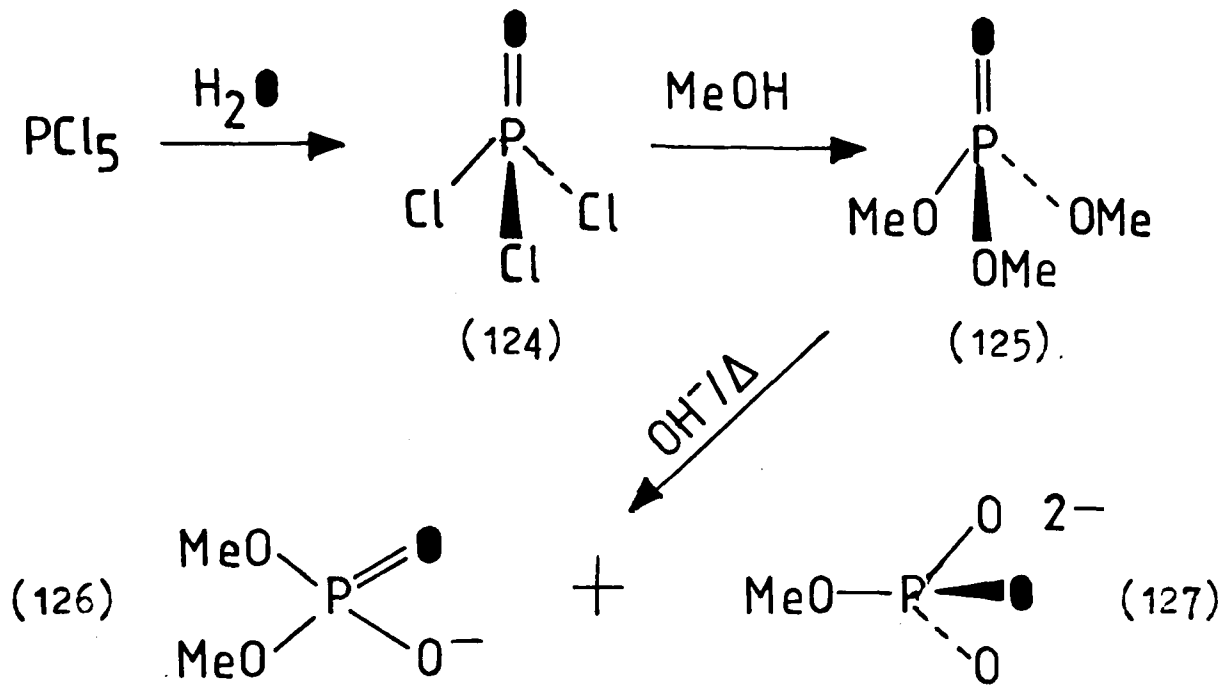


FIGURE 68

methyl phosphate (127). The isotopic enrichment was approximately 50% so that two peaks of almost equal intensity were observed in the ^{31}P nmr spectra of these three species which are illustrated in FIGURES 69 and 70 (in all cases the lines to lower field are assigned to the unlabelled species). For [^{18}O]-TMP (125) this spectrum gave a value for the isotope shift in an ^{18}O-^{31}P pure double bond. In order to investigate systems of lower bond order the [^{18}O]-TMP was partially hydrolysed. TMP is easily hydrolysed to dimethyl phosphate which is then more difficultly hydrolysed to monomethyl phosphate. Thus hydrolysis of [^{18}O]-TMP in dilute base at 120 °C for 40 h in a sealed tube was found to result in a mixture of [^{18}O]-dimethyl phosphate (126) and [^{18}O]-methyl phosphate (127) which were examined by ^{31}P nmr. These spectra which were in all three cases recorded both at 36.43 MHz in Oxford and at 162 MHz in Karlsruhe, Germany illustrate nicely the dramatic effects of high field strength on the resolution of these isotopic splittings.

In order to observe the isotope shift in a phosphorus-oxygen single bond adenosine-5'[$\alpha\beta$-^{18}O, β-$^{18}O_2$]-triphosphate (122) was incubated with pyruvate kinase for a period known to cause partial randomisation of label[198] by Dr. B.S. Sproat. The recovered ATP therefore contained a mixture of starting labelled ATP (122) and randomised ATP (123) enabling the isotope shift on P_α of ^{18}O in

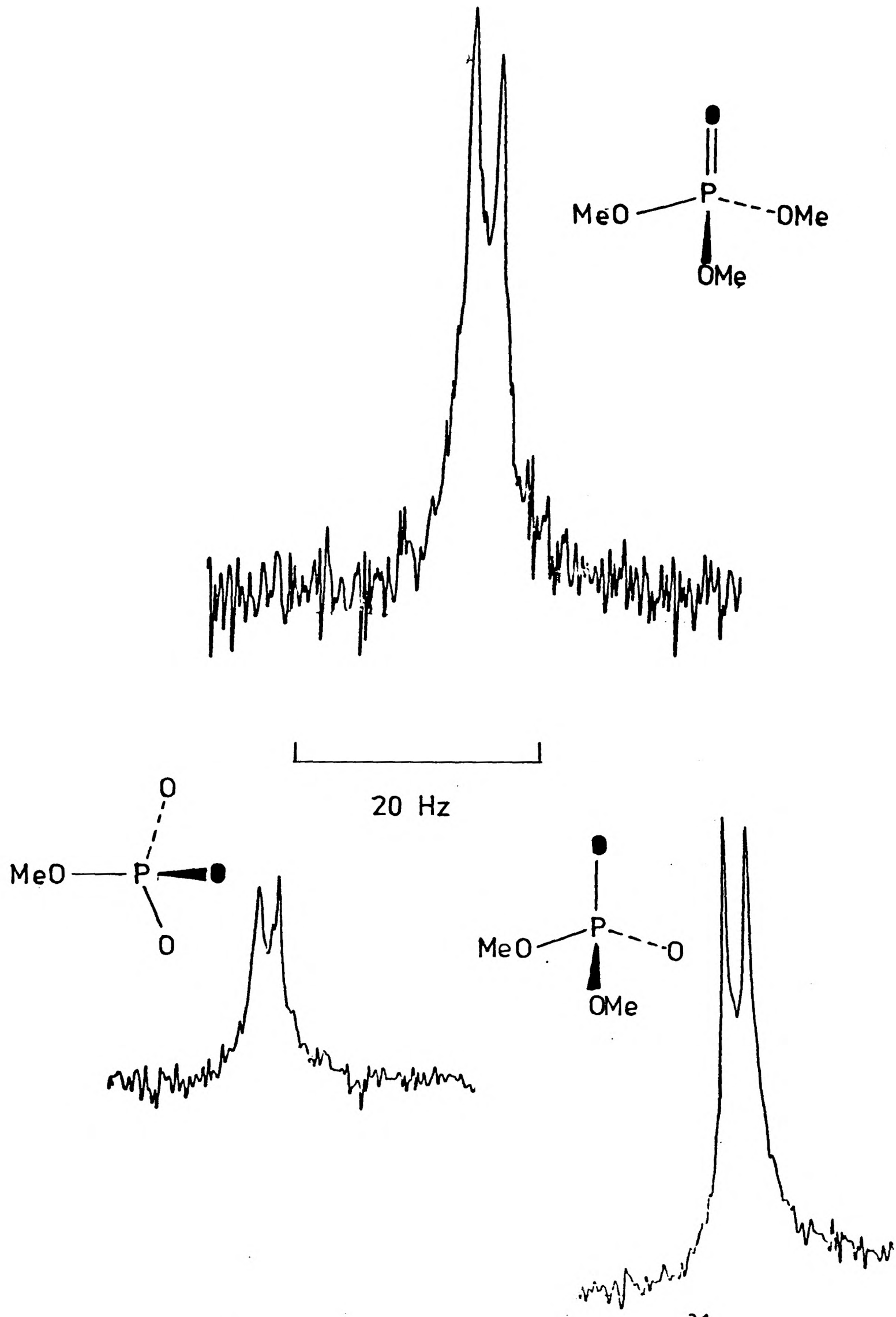
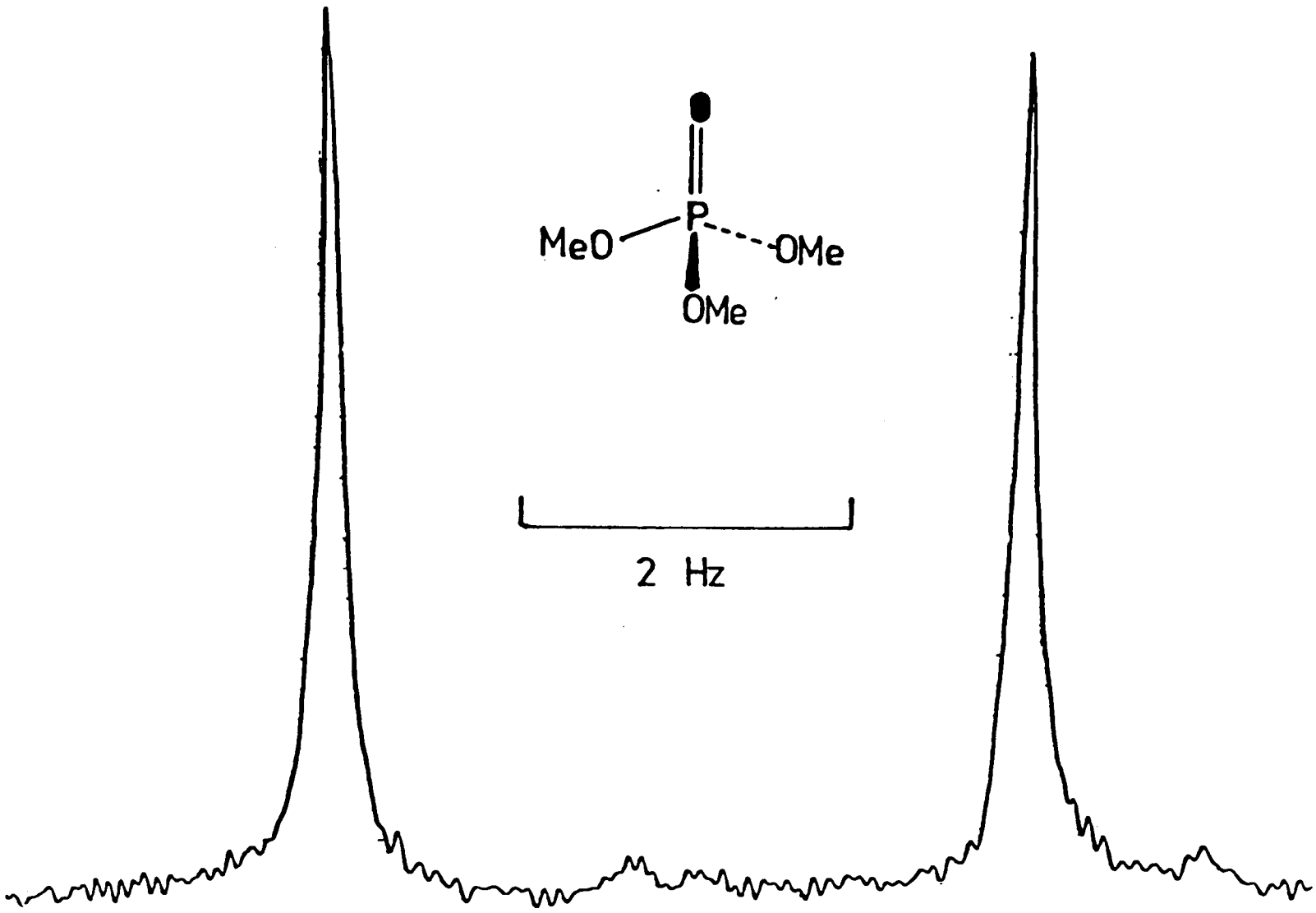


FIGURE 69 : The ^{18}O Isotope Shifts at 36.43 MHz in the ^{31}P n.m.r. Spectra of [^{18}O]-Trimethyl Phosphate, [^{18}O]-Dimethyl Phosphate and [^{18}O]-Methyl Phosphate in D_2O at pH 11. ^{31}P n.m.r. parameters were : offset 3200 Hz; sweep width 3000 Hz; pulse width 15 µs; acquisition time 2.56 s; memory size 4K, but 4K of zeros added before FT; broad band proton decoupling.



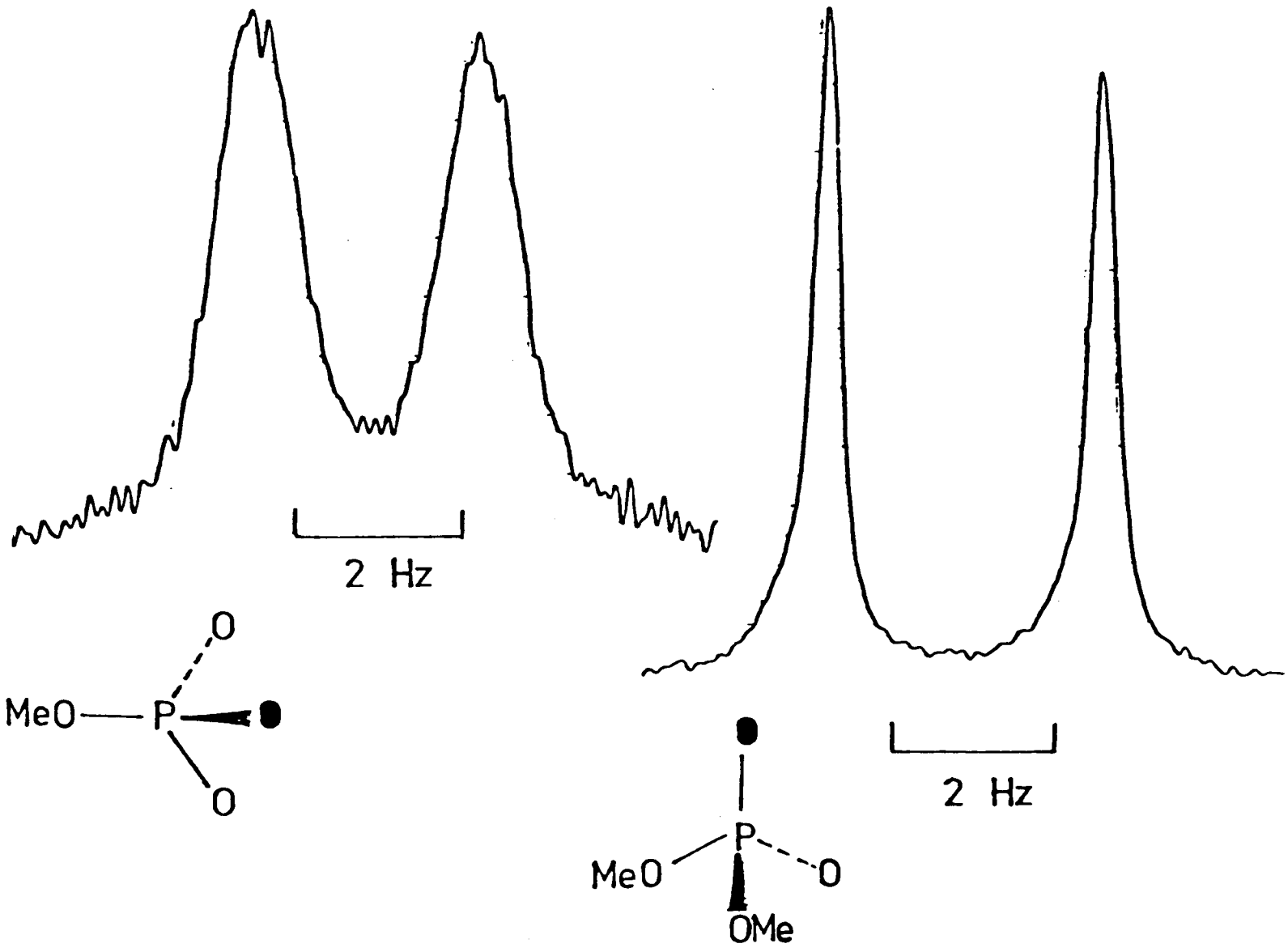


FIGURE 70 : As for FIGURE 69 but recorded at 162 MHz.

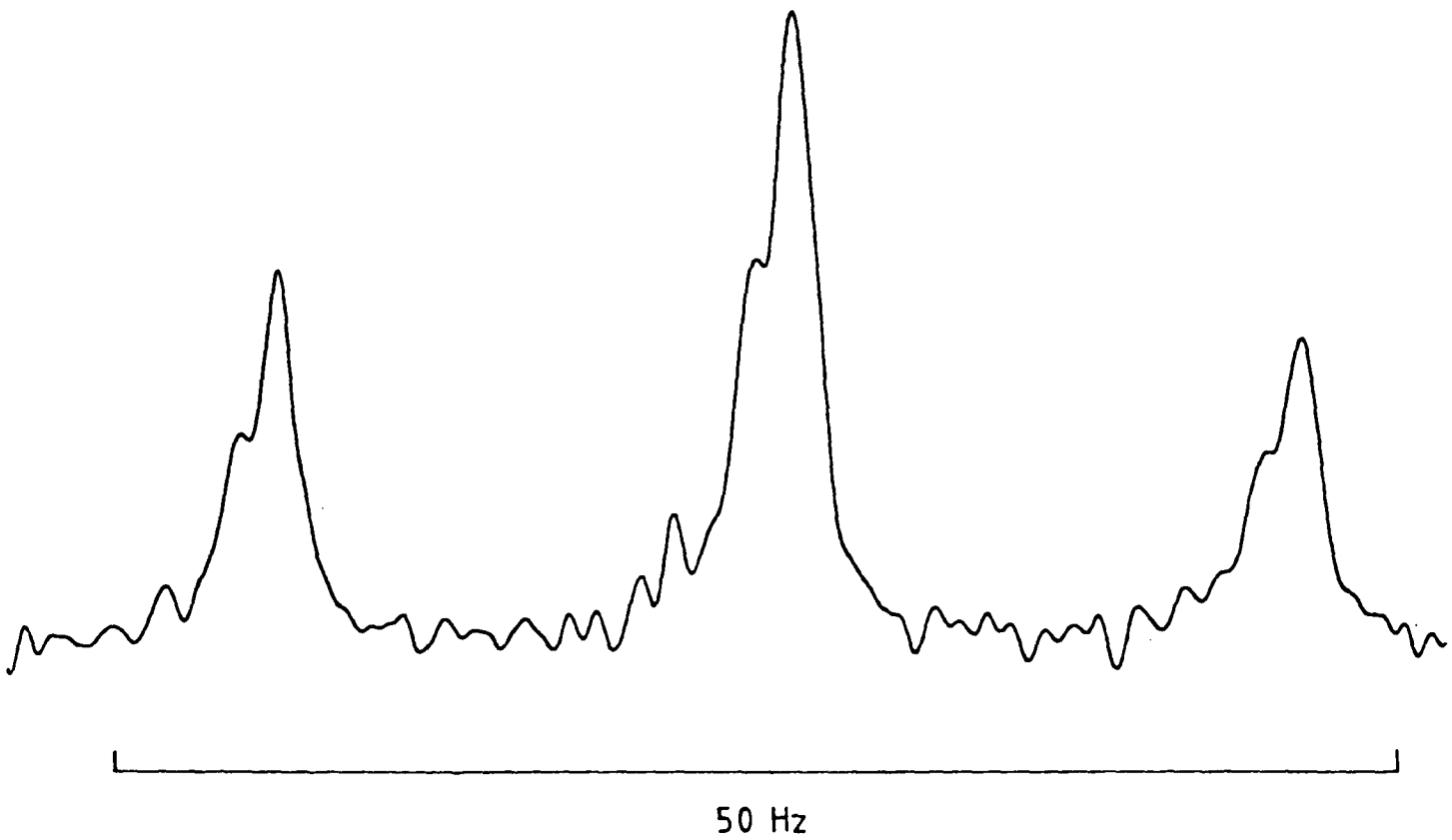
the P_β-O-P_γ bridge to be measured. The values for the various isotope shifts are presented in TABLE 9.

TABLE 9: ^{18}O ISOTOPE SHIFTS IN PHOSPHATE ESTERS (Hz)

	Bond Order	at 36.43 MHz	at 162 MHz
$(MeO)_3P\ ^{18}O$	2.00	1.27	5.83
$(MeO)_2PO\ ^{18}O^{\theta}$	1.50	1.07	4.74
$(MeO)PO_2\ ^{18}O^{2\theta}$	1.33	0.88	3.72
P_γ of (123)	1.00	0.74	3.38

The ^{31}P nmr spectrum of the P_β and P_γ resonances of the mixtures of ATP (122) and (123) is shown in FIGURE 71. It is clear from this that not only are the P_γ resonances clearly resolved at 162 MHz but the P_β resonances are also resolved, the effect being due to ATP (122) possessing one bridging and two non-bridging ^{18}O atoms at P_β whereas ATP (123) possesses two bridging and one non-bridging ^{18}O atom at P_β. Since the isotope shift is always to higher field the high field triplet is assigned to P_β of (123) and the low field triplet to P_β of (124). It seems clear, therefore, that the magnitude of the isotope shift is a parameter which will enhance the range of application of this technique.

From the data in TABLE 9 we can see the general trend of an increase in isotope shift paralleling increasing bond order (see FIGURE 72a). Also a plot of the isotope shift (at 162 MHz) against the square of the totally symmetric A_1-stretching mode of the phosphates[274] shows a good linear relationship (FIGURE 72b) indicating that the isotope shift is related to the force constant of the phosphorus-oxygen bond.



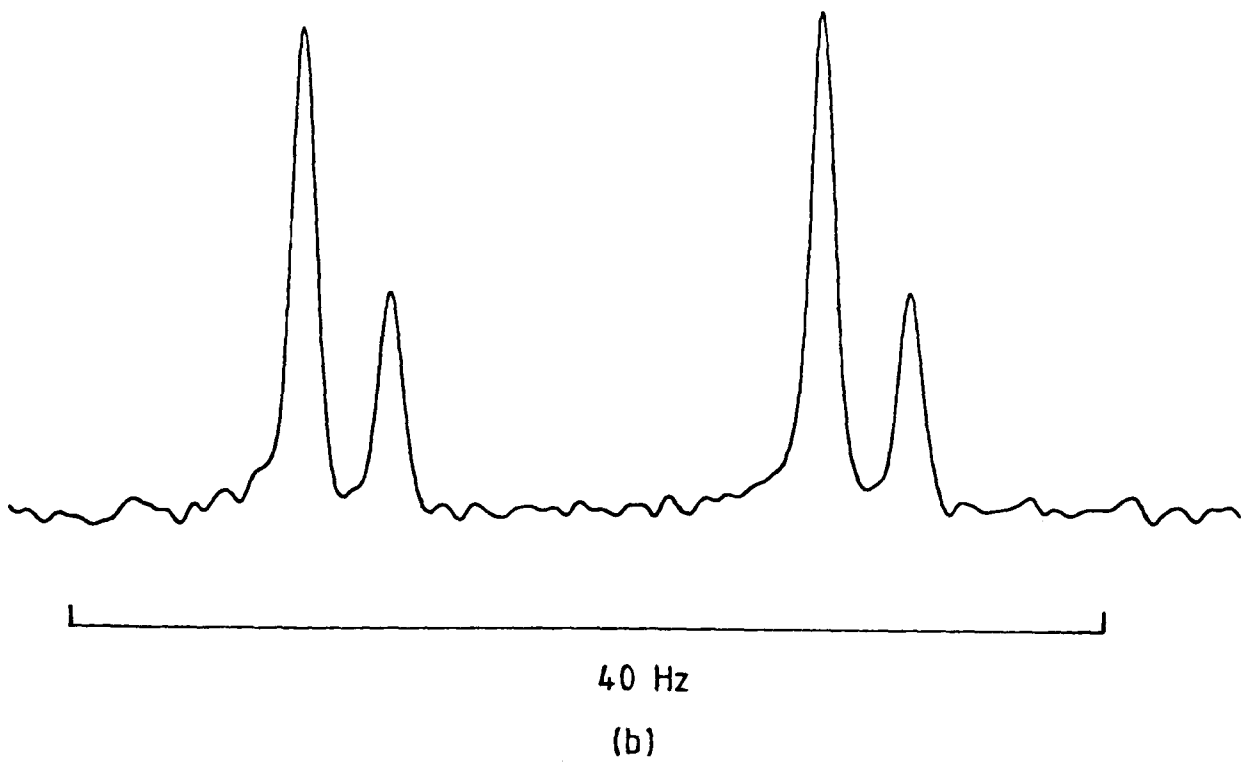
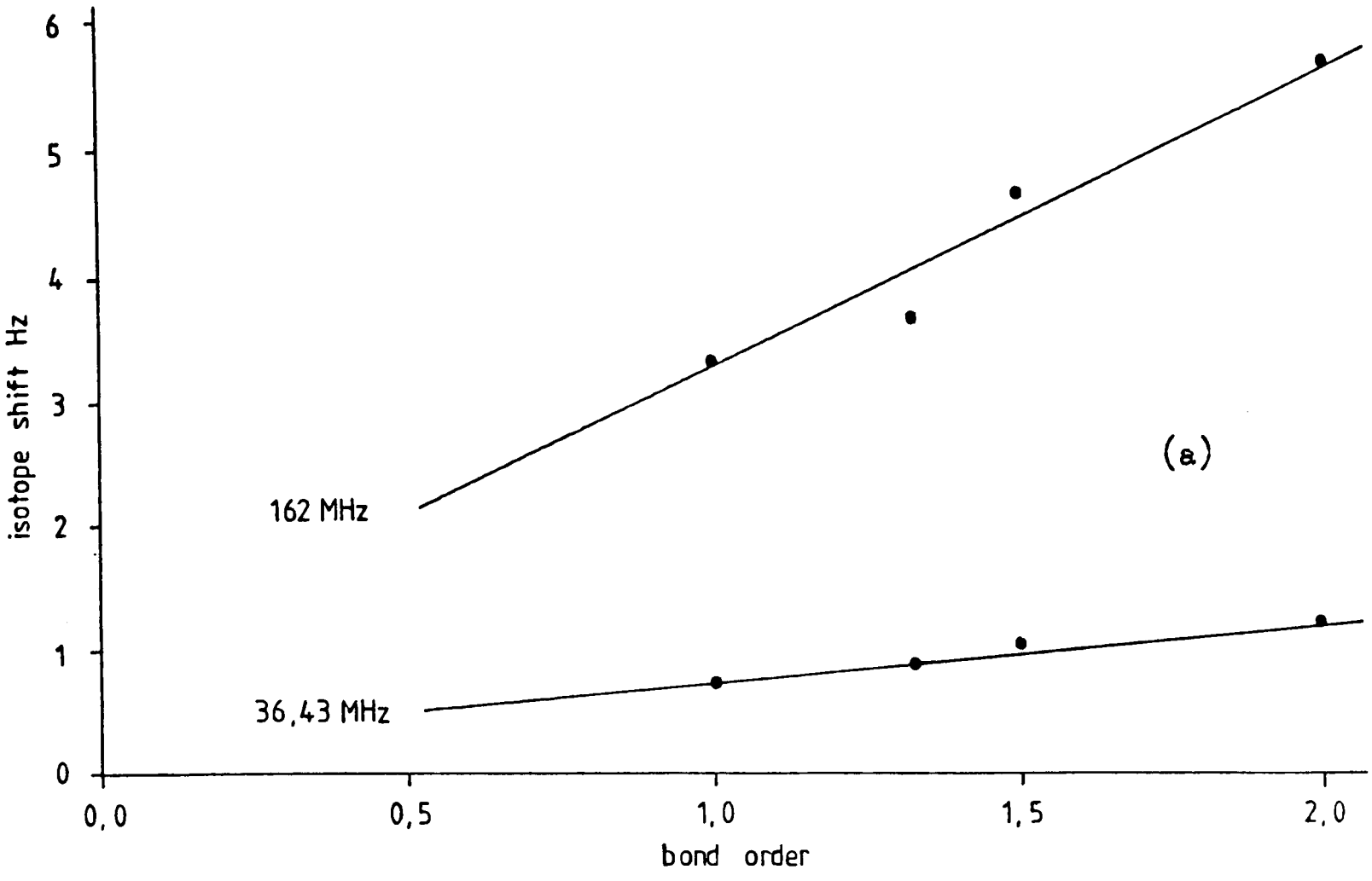


FIGURE 71 : The 162 MHz ^{31}P n.m.r. Spectrum of (a) P_β and (b) P_γ of a Mixture of ATP (122) and (123) Derived by Partial Isotopic Scrambling of ATP (122) by Pyruvate Kinase.



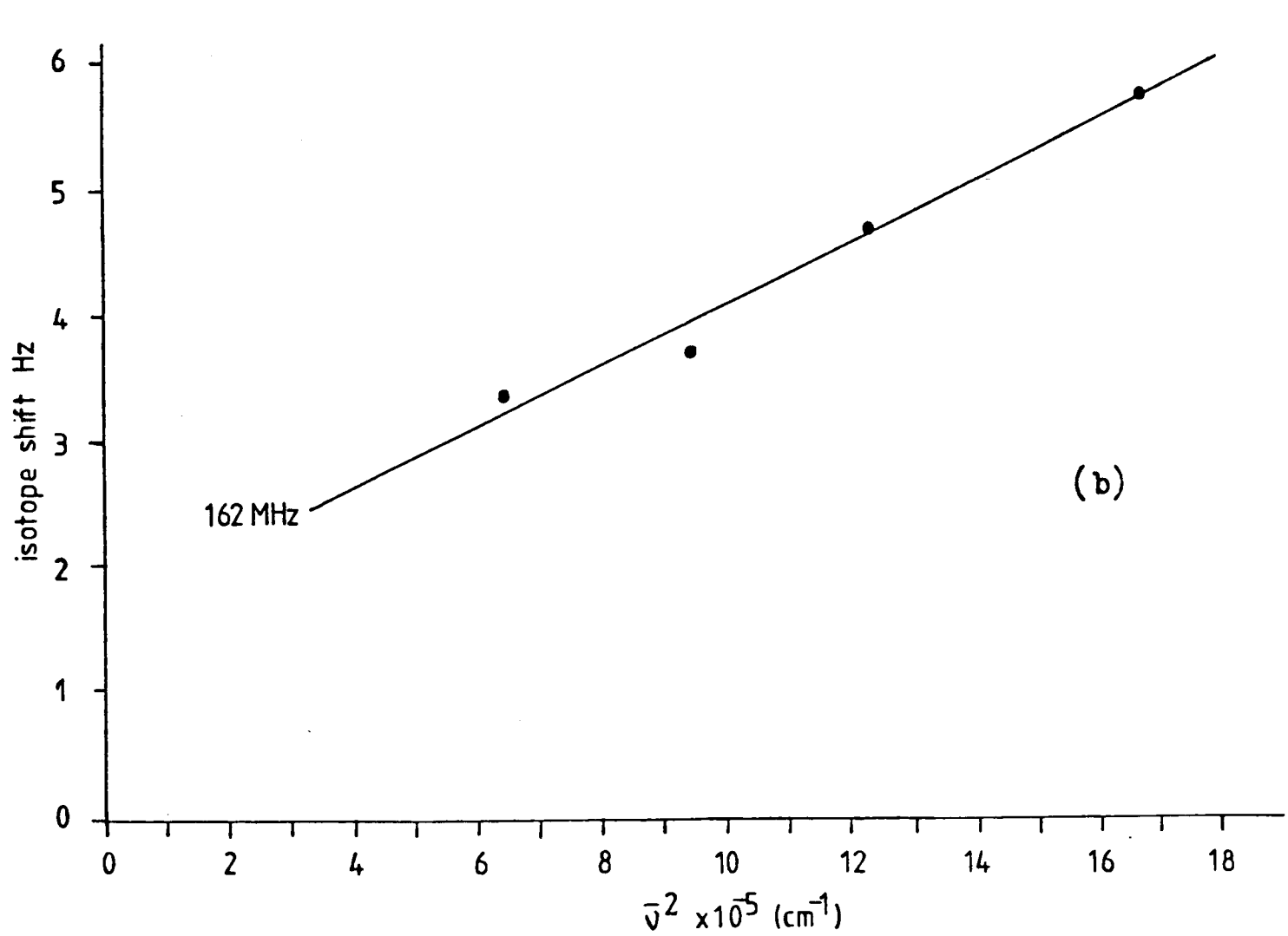


FIGURE 72 (a) : A Plot of the ^{18}O Isotope Shifts for the Phosphate Esters in TABLE 9 against Their Corresponding Bond Orders

FIGURE 72 (b) : A Plot of the ^{18}O Isotope Shifts at 162 MHz for the Phosphate Esters in TABLE 9 against the Square of the Frequency of Their A_1 Stretching Modes. (The best straight line has a correlation coefficient of 0.985).

The development of an elegant method[216] for analysing the isotopic chirality of [^{16}O, ^{17}O, ^{18}O]-phosphate monoesters derived from the observations made here under (a) and (b) will be described in CHAPTER 9.

(c) The ^{31}P n.m.r. Spectrum of P_1-[(S)-^{16}O, ^{17}O, ^{18}O]-Pyrophosphate

The ^{31}P n.m.r. spectrum of P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate (115) (see CHAPTER 7) shows some rather interesting features and is worthy of detailed discussion in the light of the results in this chapter. Since the ^{17}O enrichment of the isotopically chiral centre is only *ca.* 44% (and if we neglect the small amount of ^{16}O in the '^{17}O site') then the spectrum should be a composite of resonances from the following two pyrophosphate species (128 and (115).



FIGURE 73

Initially, when examined at 36.43 MHz the spectrum showed a reasonably sharp singlet at -9.2 ppm from TMP, and also what were apparently two smaller satellites equally spaced at about 11 Hz from the main peak (FIGURE 74). An explanation of this spectrum was not readily forthcoming from the available information at the time so the behaviour of the resonances was studied at higher field strengths. Reference to FIGURE 74 illustrates that the satellite peaks appear to move gradually downfield relative to the main peak as the field strength is increased to 81.01 MHz and then 162 MHz. Moreover, the linewidth of the main peak at 162 MHz is approximately double that of 81.01 MHz. These observations coupled with

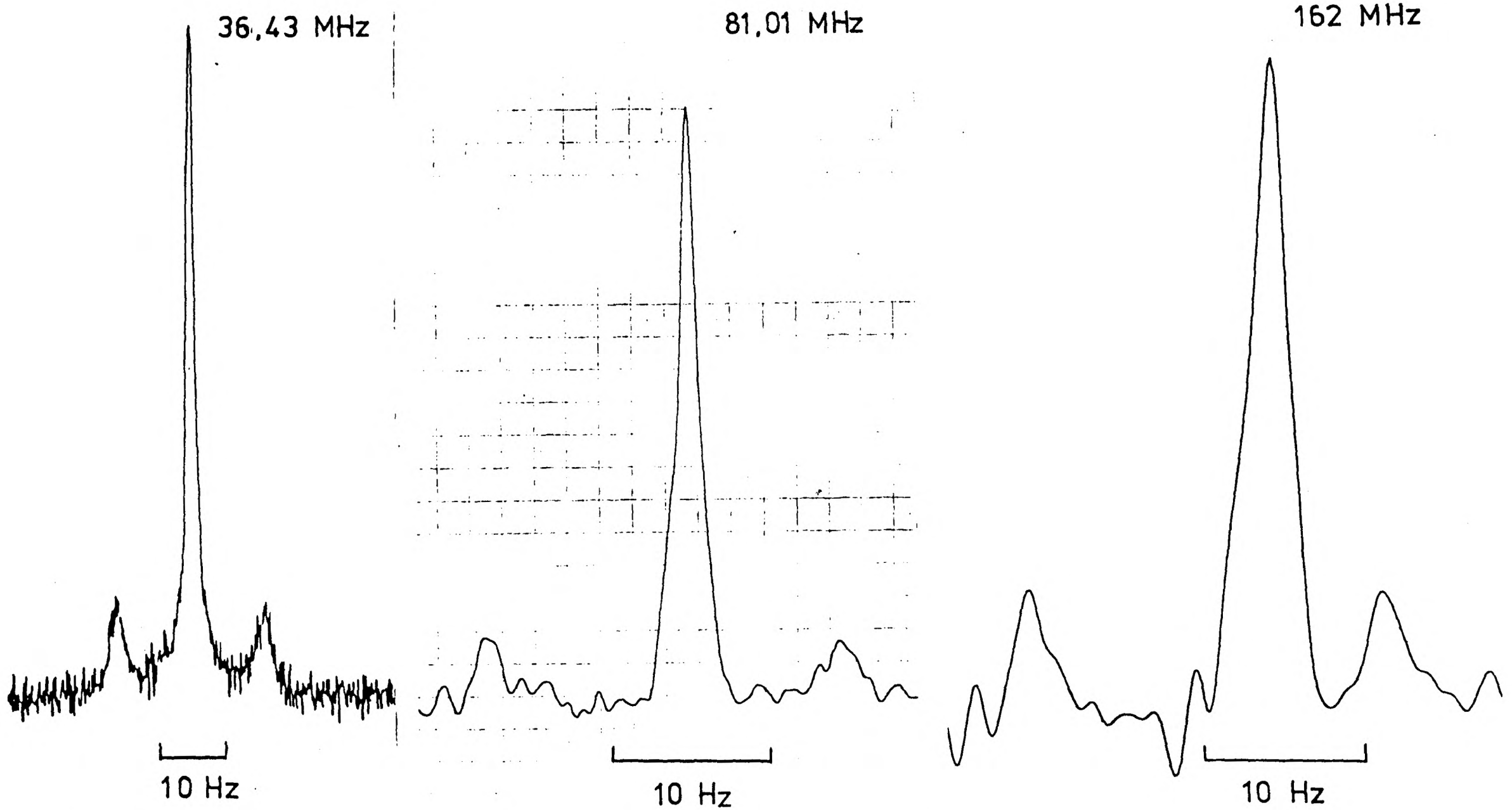


FIGURE 74 : The ^{31}P n.m.r. Spectrum of P_1-[(S)-^{16}O, ^{17}O, ^{18}O]-Pyrophosphate at 36.43 MHz, 81.01 MHz and 162 MHz.

previously observed effects on the isotope shift of phosphorus atoms directly bonded to ^{18}O, and the effect on the linewidth of phosphorus species containing ^{17}O, prompted the following rationalisation of the spectrum (FIGURE 75).

The main peak represents the collapsed most intense lines of an AB quartet due to species (128). The two phosphorus atoms α and β for this molecule are expected to be only slightly different in chemical shift but are sufficiently differentiated by the presence of two ^{18}O isotopes on P_β to couple together, although the resulting collapsed central lines of the AB system are not resolved. The much less intense outer lines are buried in the baseline noise. Such an effect has not previously been observed despite the preparation of a number of isotopically labelled derivatives of, for example, ATP since the phosphorus atoms are already spin coupled due to intrinsically large differences in chemical shift. In this case, however, the only disymmetry is by virtue of the small mass increment due to double isotopic substitution. Evidence that some resolution is being effected is obtained from the observed increase in linewidth with increasing field. Now if we assume that a good approximation for the difference in chemical shifts of P_α and P_β at 162 MHz is twice the isotope shift of 3.72 Hz observed for [^{18}O]-methyl phosphate (127) (the isotope shift is additive[201]), then we would expect the true resonance positions of P_α and P_β to be 3.72 Hz downfield and 3.72 Hz upfield of the centre of the main peak respectively. Furthermore if we assume an approximate value of $^2J_{P_\alpha P_\beta} = 22$ Hz (this will be justified later, but in any event is a good average value for such a coupling constant) we can calculate the separation of the collapsed AB lines, $\Delta_{162\ MHz} = (\nu_3-\nu_2)$, from the following relationships -



$$(\nu_4-\nu_2) = [(\delta_\alpha-\delta_\beta)^2 + J^2_{\alpha\beta}]^{\frac{1}{2}}$$

$$(\nu_4-\nu_3) = 22\ Hz$$

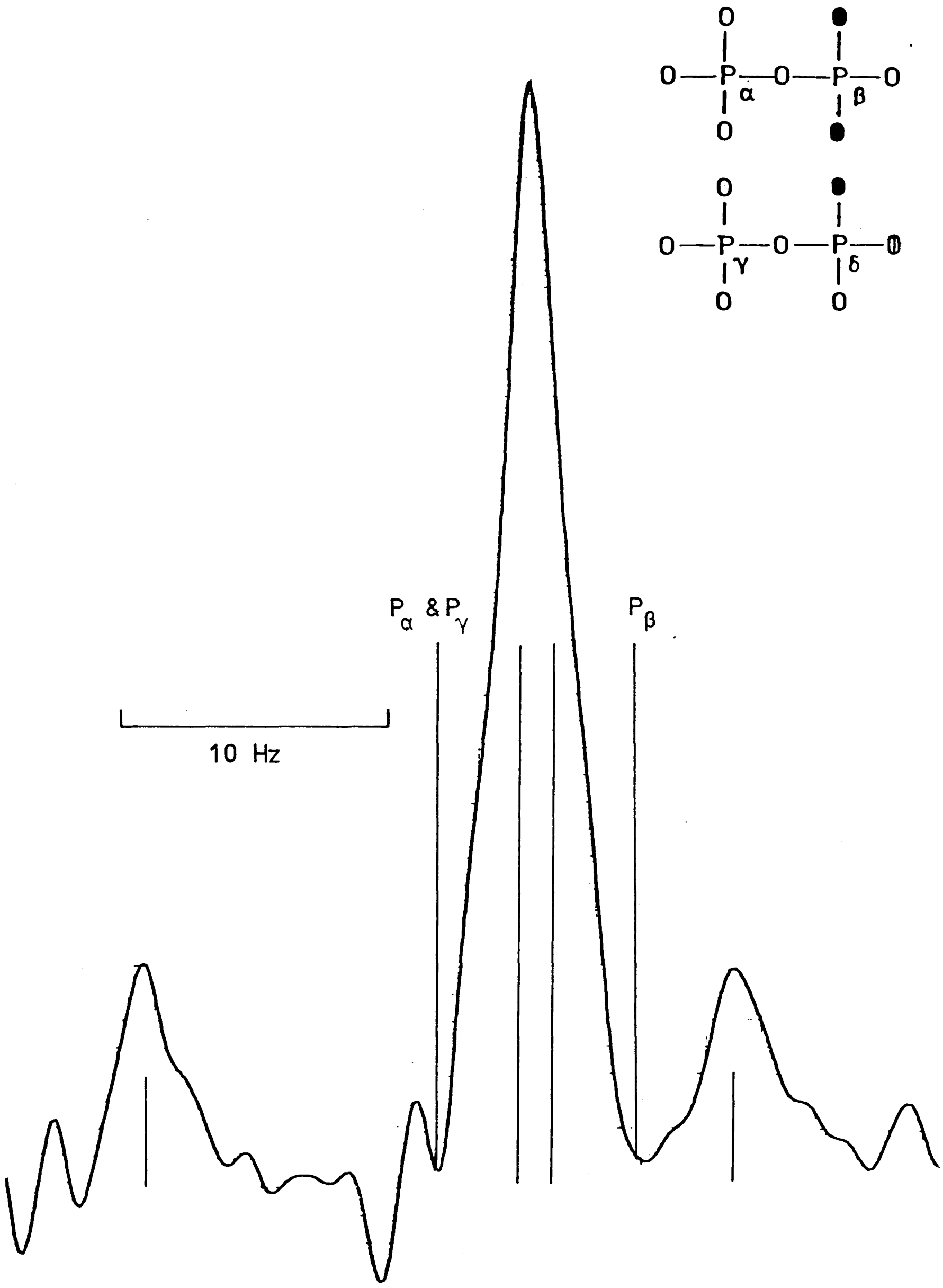


FIGURE 75 : The 162 MHz ^{31}P n.m.r. Spectrum of P_1-[(S)-^{16}O, ^{17}O, ^{18}O]-Pyrophosphate

where ν_n represents the resonance frequency of the nth line of the AB system and δ_α and δ_β are the true chemical shifts of P_α and P_β. Whence we find $\Delta_{162\ MHz} = 1.22$ Hz, and the relative intensity of the collapsed lines to the outermost lines ν_4 and ν_1 is given by

$$I_2/I_4 = \frac{(\nu_4-\nu_1)}{(\nu_3-\nu_2)} = \frac{37}{1}$$

where I_n is the intensity of the nth line in the spectrum. The isotope shift is directly proportional to the field and we may thus calculate $(\delta_\alpha-\delta_\beta)_{81\ MHz}$ to be 3.72 Hz, whence similarly $\Delta_{81\ MHz} =$ 0.31 Hz.

Now for two closely spaced lines of half width $\Delta\nu_{\frac{1}{2}}$ and separated by an amount xHz, the total half width $\Delta\nu_{\frac{1}{2}\ total}$ will be given by

$$\Delta\nu_{\frac{1}{2}\ total} = (\Delta\nu_{\frac{1}{2}} + x)$$

Now if we take a value of say, 0.75 Hz as being a good average representative value for $\Delta\nu_{\frac{1}{2}}$ then we might expect the following values for the total half width at various field strengths.

$$\Delta\nu_{\frac{1}{2}\ total}^{81\ MHz} = (0.75 + 0.31) = 1.06$$

$$\Delta\nu_{\frac{1}{2}\ total}^{162\ MHz} = (0.75 + 1.22) = 1.97$$

whence: $\dfrac{\Delta\nu_{\frac{1}{2}\ total}^{162\ MHz}}{\Delta\nu_{\frac{1}{2}\ total}^{81\ MHz}} = 1.86$ and this is to

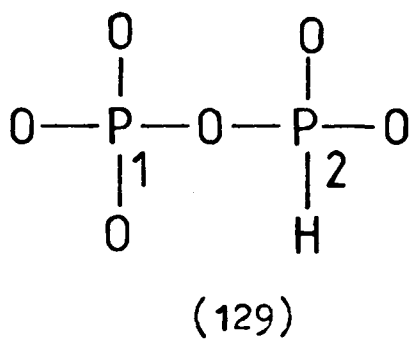
be compared with the observed line width ratio of 1.93 which is

thus in good agreement with prediction.

Now secondary isotope shifts if observable are very small indeed and we can safely ignore their presence when comparing species (128) and (115). Thus *a priori*, we might expect the P_α and P_β resonances to be essentially equivalent. Now the separation between the two satellite peaks does not change from 22 Hz as the field is increased, thus implying that it represents a coupling constant. Moreover, the centre of this pattern at 162 MHz is seen to be almost exactly coincident with the previously estimated true resonance position of P_α. Now for two nuclei in a molecule to be *magnetically equivalent* they must couple equally to all other magnetic nuclei in the molecule. Thus it is clear that the two phosphorus atoms P_γ and P_δ in (115), albeit *chemically equivalent* are *magnetically inequivalent* due to the presence of ^{17}O on P_δ and the resulting non-equivalent $J_{^{31}P^{17}O}$ coupling. In the light of the results from [^{17}O]-enriched trimethyl phosphate (119)[231], a very strong $^1J_{^{17}O^{31}P}$ coupling between P_δ and ^{17}O of the order (but perhaps slightly less) of 150 Hz is to be expected, whereas the P_γ atom is likely to be much less coupled if at all. As a result of this non-equivalence a $^2J_{P_\gamma P_\delta}$ coupling is to be expected, probably of the order of 20-22 Hz as is commonly observed. However, as has been shown earlier with [^{17}O]-TMP the quadrupolar effect of ^{17}O causes marked line broadening of a resonance when directly bonded to a phosphorus nucleus. Thus we would not expect to see the P_δ signal because of the broadening and multiple splitting (six lines for a spin $\frac{5}{2}$ nucleus), although depending on the relaxation rate of P_δ we might still expect to observe a $^2J_{P_\gamma P_\delta}$ coupling on the P_γ resonance. It has been recently found in this laboratory[217] (CHAPTER 10) and in that of Tsai[232,233] that in a polyphosphate chain the neighbouring phosphorus atom to another phosphorus directly bonded to ^{17}O shows no change in resonance intensity, and

remains coupled to the ^{17}O bound phosphorus. Accordingly, therefore, in the spectrum of P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate we might expect to see *a spin coupled resonance for* P_γ *and no resonance for* P_δ. Indeed it is therefore proposed that the two satellites separated by 22 Hz represent this split P_γ resonance. Their behaviour on increasing the field strength is in accord with expectation since as the increasing field begins to resolve the collapsed P_α-P_β AB systems, so the true resonance position of P_α and consequently P_γ will move downfield with respect to the centre of the system.

A reasonably good analogy of our pyrophosphate (115) in the literature is the isohypophosphate ion (129).[275]



(129)

FIGURE 76

The spectrum of this compound together with the spectrum simulated by an NMRCAL program on the Bruker WH90 in this laboratory are illustrated in FIGURE 77. The two phosphorus atoms P_1 and P_2 have a chemical shift difference of only 18 Hz at 12.3 MHz and are mutually coupled with $^2J_{P_1P_2}$ = 17 Hz. However, P_2 suffers a strong $^1J_{P\ H}$ coupling of 620 Hz with the directly bonded proton and thus this molecule bears a semiquantitative resemblance to pyrophosphate (115). However, despite the similarity in chemical shift and thus a δ/J value of 1.06 (a value which might normally be expected to lead to marked second order behaviour in the absence of the other strong coupling) the two main lines of the P_1 resonance in FIGURE 77(a) are of equal intensity. When the NMRCAL program was used with the parameters estimated and obtained for our pyrophosphate

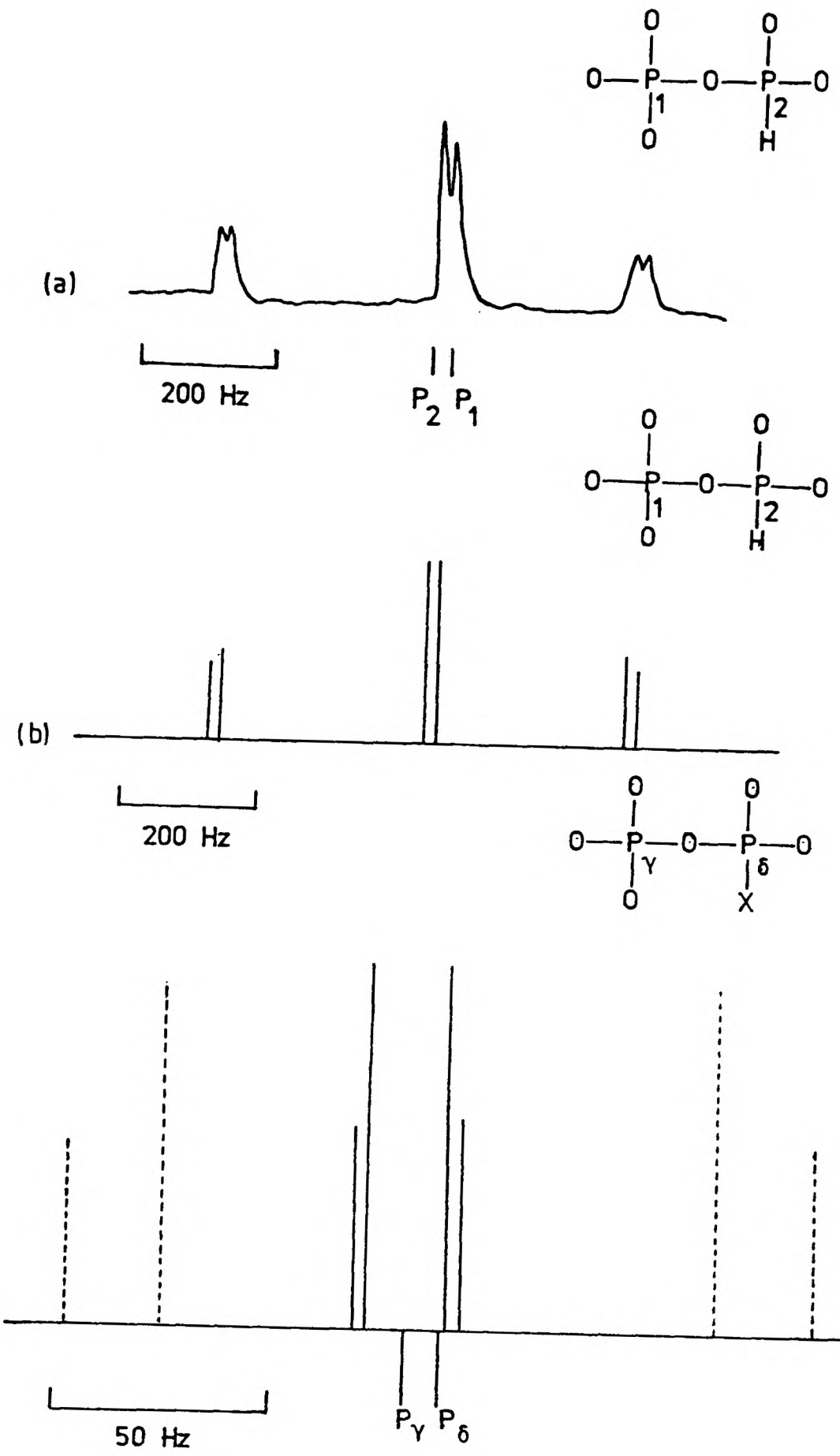


FIGURE 77 : (a) The 12.3 MHz ^{31}P n.m.r. Spectrum of the Isohypophosphate Anion.[275]

(b) The NMRCAL Simulated 12.3 MHz ^{31}P n.m.r. Spectrum as above. Simulation parameters were : $^{2}J_{12}$=17 Hz; $^{3}J_{P_1H}$=0Hz; $^{1}J_{P_2H}$=620 Hz; sweep width, 1000 Hz; δ_1=500 Hz; δ_2=482 Hz; δ_H=50,000 Hz.

(c) The NMRCAL Simulated 162 MHz ^{31}P n.m.r. Spectrum of a 3xspin $\frac{1}{2}$ System. N.m.r. parameters were : δ_α=158 Hz; δ_δ=150 Hz; $^{2}J_{\delta\delta}$=22 Hz; $^{1}J_{\delta x}$=150 Hz; δ_x=50,000 Hz; sweep width 6000 Hz.

(115) the simulated spectrum in FIGURE 77(c) was obtained. (The limitations of the program made it necessary to treat ^{17}O as a spin ½ nucleus only, however the spectrum indicates the qualitative effect. Resonances which would not normally appear due to quadrupolar relaxation are marked with dotted lines). In the case of this molecule (115) δ/J is 0.34 and two extra lines are observed in the central pattern of P_γ which is, however, still symmetrical. The two sets of lines, although of different intensities, show a good positional correspondence with the observed lines in the real spectrum, although no two lines are separated by exactly 22 Hz. The other lines in the spectrum due to P_δ of course are broadened and coupled (impossible to simulate) so as to be unobservable.

Thus this novel example illustrates:

(a) ^{31}P-^{31}P coupling induced by the small effect of isotopic non-equivalence

(b) ^{31}P-^{31}P coupling due to magnetic non-equivalence induced by ^{17}O

(c) Quadrupolar line broadening of a ^{31}P resonance due to directly bonded ^{17}O.

CHAPTER 9

A NOVEL METHOD FOR THE ANALYSIS OF ISOTOPIC CHIRALITY IN [^{16}O, ^{17}O, ^{18}O]-PHOSPHATE MONOESTERS BY ^{31}P NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

INTRODUCTION AND LOGIC OF THE METHOD

Notwithstanding the mass spectrometric analysis procedure of Abbot *et al.*[230] for 1-[^{16}O, ^{17}O, ^{18}O]-phospho-(*S*)-propane diol (107), and the proposed method to be used in this laboratory using CD spectroscopy as outlined in CHAPTER 7, the phenomena reported in CHAPTER 8, namely the quadrupolar broadening of phosphorus n.m.r signals derived from ^{31}P directly bonded to ^{17}O and the variations of the ^{18}O-induced isotope shifts with bond character, suggested a very elegant method for determining isotopic chirality in [^{16}O, ^{17}O, ^{18}O]-phosphate esters by ^{31}P n.m.r. spectroscopy. Such a method would have obvious advantages over the very cumbersome mass spectrometric method[230], and unlike the CD method would require less specialised equipment. Whereas from a purist point of view the comparatively low level of ^{17}O enrichment (*ca.* 40%) at the chiral centre might appear as a slight annoyance it will be shown shortly that it is precisely this incomplete enrichment which will make analysis by n.m.r. feasible (indeed an enrichment of *ca.* 50% would be best of all).

Briefly, displacement of one of the three oxygen isotopes in a [^{16}O, ^{17}O, ^{18}O]-phosphate monoester by a different group would give a mixture of three species, two of which would still contain one ^{17}O atom. Only the other species which would contain one ^{16}O and one ^{18}O atom will give an unquenched ^{31}P n.m.r. signal. Stereochemical information can then be derived by determining whether the ^{18}O isotope is located at the *pro-R* or *pro-S* position. This can be achieved by a stereospecific derivatisation and by distinguishing ^{18}O isotope shifts between the bridge and non-bridge positions.

Initially let us consider a chiral [^{16}O, ^{17}O, ^{18}O]-group of the (*R*) configuration attached to a chiral residue in an imaginary molecule (130) which contains a hydroxyl group suitably disposed

to the formation of a cyclic phosphate *viz.*

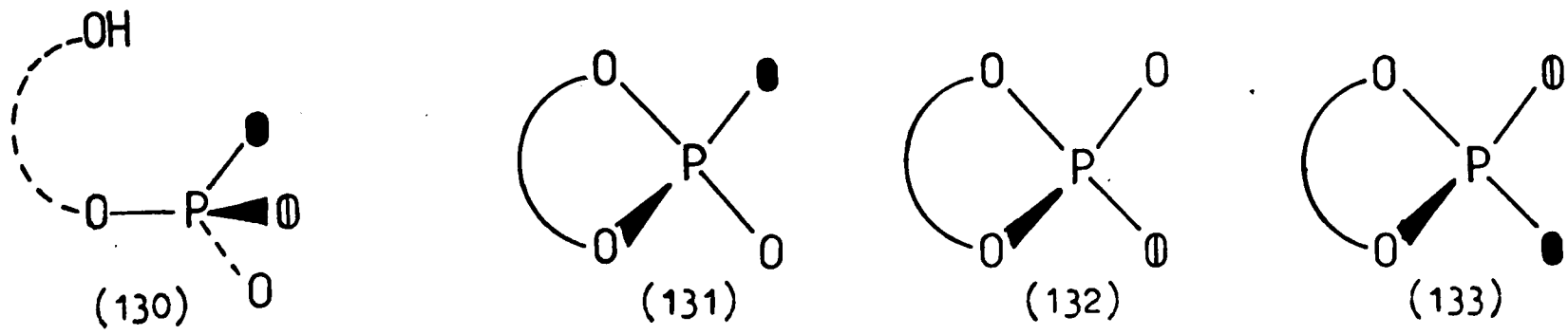
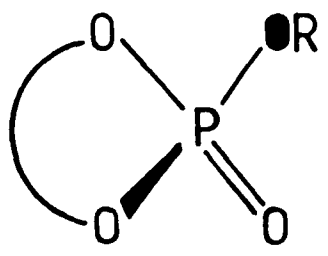
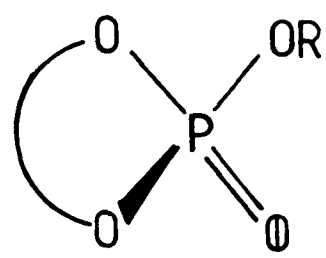


FIGURE 78

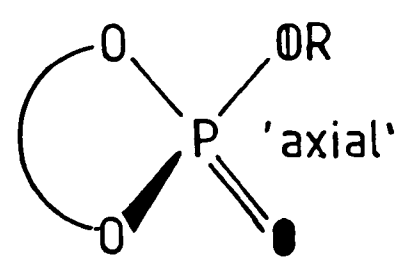
Now cyclisation (consider initially an 'in-line' cyclisation of the type used by Eckstein[206]) will yield the three cyclic phosphates (131-133). These will be diastereoisomeric at phosphorus in an isotopic sense, although it is doubtful whether any direct method could be used to distinguish between them. However, if they were to be further converted to the corresponding triesters then it is likely that a distinction between the absolute stereochemistry at the phosphorus atom (*i.e.* axial or equatorial in the case of, for example, a six-membered ring with respect to the spatial disymmetry of the rest of the molecule) could be defined by n.m.r. spectroscopy since such diastereoisomers are known to possess well separated chemical shifts in their ^{31}P n.m.r. spectra. In general axial triesters appear at higher field than equatorial ones at least for 1,3,2-dioxaphosphorinan-2-ones[276-278], and have phosphoryl infrared stretching frequencies to higher frequency.[279] The resulting six species would thus be produced (134-139) since all three isotopes will have an equal probability of being lost in the cyclisation. Careful consideration of these six species will reveal that they contain all the stereochemical information inherent in the original chiral phosphate. The following features will enable the chirality of the precursor [^{16}O, ^{17}O, ^{18}O]-phosphate to be determined from the ^{31}P n.m.r. spectrum of the above mixture *if we know the stereochemistry of the ring closure reaction.*



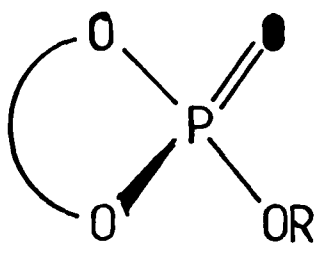
(134)



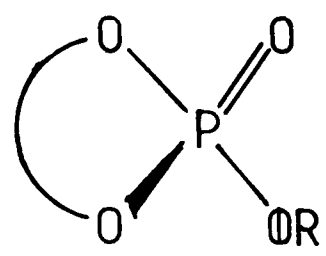
(135)



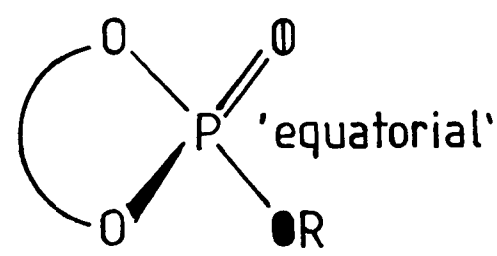
(136)



(137)



(138)



(139)

FIGURE 79

(a) The axial and equatorial series can be distinguished on the basis of their ^{31}P n.m.r. chemical shifts.[276-278] An important advantage of this method is that they do not have to be physically separated.

(b) Triesterification ensures the bridging and doubly bonded natures of the exocyclic oxygens, and thus an ^{18}O isotope in either position *will exhibit a different isotope shift.*[231]

(c) Those triesters containing ^{17}O (*i.e.* (135), (136), (138), (139)) either doubly bonded to phosphorus or in a bridge will not be observed in the spectrum, *but more important since the site is not wholly enriched the residual ^{18}O in that position will be manifest as a peak of decreased intensity as compared to the triesters ((134) and (137)) which have lost ^{17}O in the cyclisation.*

The above considerations, therefore, imply that the ^{31}P n.m.r. spectrum of both the axial and equatorial systems will each appear as three lines, two of which will have decreased intensity due to the obliteration of the species containing ^{17}O. In each set the resonance to lowest field will correspond to that triester which

has only a bridging isotope 'shift' in FIGURE 79 *i.e.* (134) and (138) relative of course *in the ideal case* to the unobserved completely unlabelled species). The next line upfield from this will be from species containing an isotope doubly bound to phosphorus (135) and (137) since this will promote a larger shift. Finally, as a result of the additivity of isotope effects[201] the resonance furthest downfield in each series will be derived from the triesters containing two isotopes (136) and (139) which have lost ^{16}O in the cyclisation. Now it is important to realise here that in, for example, the axial triester series in FIGURE 79 the lowest field base peak will have maximum intensity since it possesses about 99% ^{18}O in the bridging position, in contrast to the lowest field peak in the equatorial series which has some 2% ^{18}O, 44% ^{17}O, and 54% ^{18}O in the bridging position and will therefore show only that proportion of ^{31}P signal derived from phosphorus bound to ^{18}O and ^{16}O, namely 56%. The same kind of arguments hold for the other lines in each series and we find that, ideally, the ^{31}P n.m.r. spectrum of the whole system (134)-(139) should consist of a 'triplet' of lines with intensity ratios 56:100:56 for the downfield equatorial resonances, and an intense peak followed by two equal peaks in the ratios 100:56:56 for the upfield axial triester series. It is vital to realise now that the substitution of a precursor [^{16}O, ^{17}O, ^{18}O]-monophosphate ester of the opposite (*S*) isotopic configuration at phosphorus in the above arguments would lead to an exact reversal of the above intensity ratios, *i.e.* the axial system would appear as the 'triplet' and so on. Thus it may be immediately appreciated that providing we know the stereochemistry of the cyclisation step the above analysis will tell us the configuration of the chiral phosphate and *vice versa*.

Now let us introduce some refinements to this crude model and

deal with real systems. A number of initial requirements deserve consideration.

(a) In order to examine the stereochemical course of biological reactions we wish to determine the isotopic stereochemistry of chiral [^{16}O, ^{17}O, ^{18}O]-ATP. Thus we must effect a suitable *and general* enzymic transfer to an acceptor molecule compatible with the previous arguments.

(b) Asssuming we possess in our acceptor molecule the potential for an 'in line' cyclisation the resulting cyclic phosphate diester should preferably be able to yield a sufficiently robust triester which would not require extensive handling (Knowles' 5-membered ring triesters are *extremely* reactive and susceptible to reaction with atmosphere moisture). Thus in view of the excessive instability of 5-membered cyclic triesters a facile cyclisation to a *conformationally locked six-membered cyclic phosphate system* would be preferred.

(c) We must develop a suitable triesterification procedure (or perhaps some other kind of method) to provide the final distinction).

The following two possibilities concur with our first two requirements.

(i) The transfer of the terminal phosphoryl residue of ATP to adenosine (140) by adenosine kinase yields AMP (141) which may be cyclised to give a six-membered cyclic phosphate system in 3'5'-cyclic AMP (cAMP) (142).

(ii) The transfer of the terminal phosphoryl residue of ATP to *D*-glucose (143) by hexokinase to give *D*-glucose-6-phosphate (144) which may then be cyclised to give *D*-glucose-4,6-cyclic phosphate (145) which does not normally occur in nature.

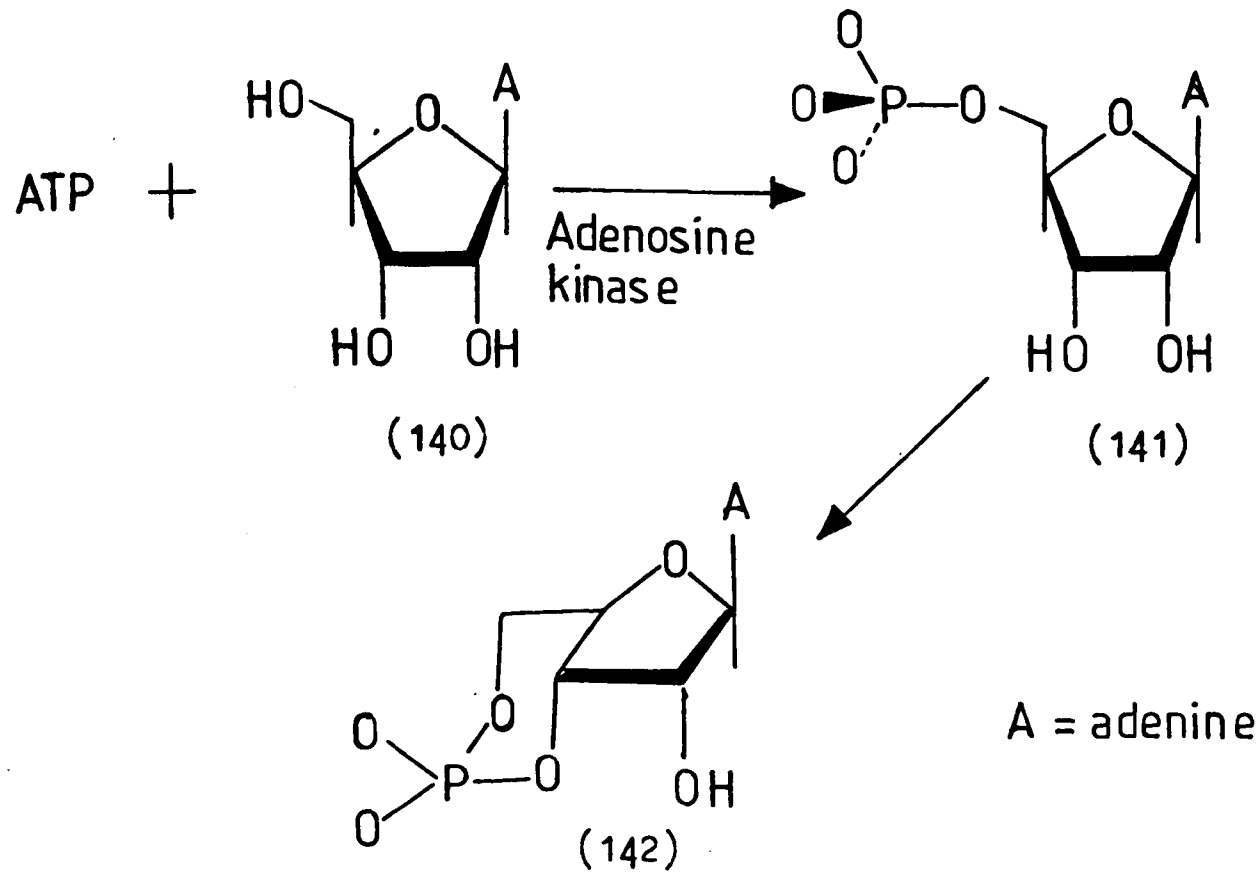


FIGURE 80

For this latter system a methylation procedure had been partially developed[255], and for cAMP a methylation procedure has been developed in this laboratory by Mr. R. Jarvest.[280] Thus with the premise that we can synthesise either adenosine-5'[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate and/or *D*-glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate the first step is to establish the stereochemistry of the ring closure reactions and the second to synthesise γ-chiral [(*S*)-^{16}O, ^{17}O, ^{18}O]-ATP in order to perform the enzymic transfers and to determine their stereochemical consequences. The way will then be clear for an extensive investigation of the wide variety of phosphoryl transfer enzymes which handle ATP.

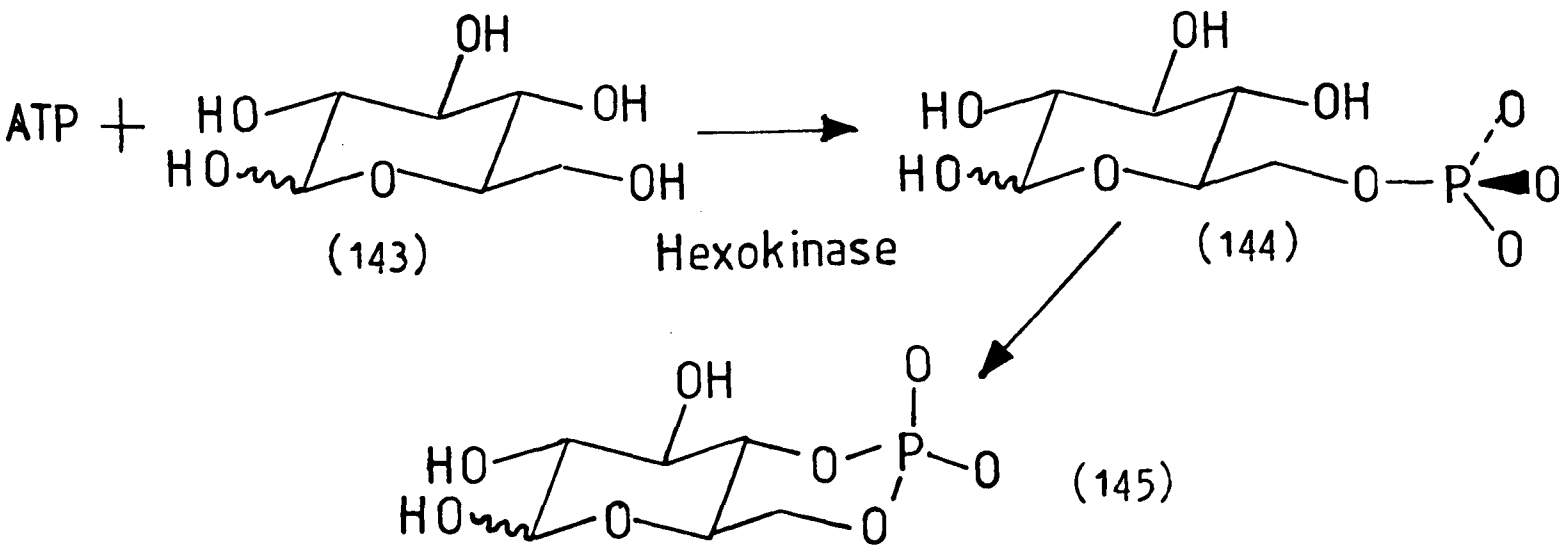


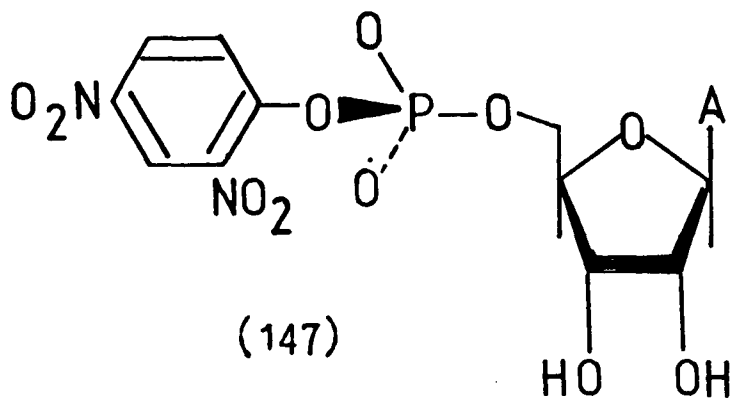
FIGURE 81

Initial Investigations into the Expediency of the Method

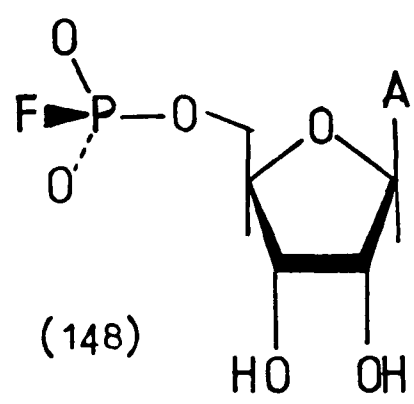
Since, in principle, a cyclisation in the fashion explained above of a phosphate monoester containing only one ^{18}O enriched site should yield qualitatively the kind of isotope shift pattern which we expect for the isotopically chiral phosphate, and since it would also show, per site, what kind of label loss might occur in the procedure the synthesis of adenosine-5'[^{18}O]-phosphate (146) was considered to be a suitable starting point of the investigation. A synthetic route was carried out in collaboration with Mr. R. Jarvest. Chemical methods were rejected in favour of a simple enzymic procedure, the ring opening of adenosine-3',5'-cyclic phosphate (142) by phosphodiesterase in [^{18}O]-enriched water. Using 99% enriched water and repeating the experiment twice gave adenosine-5'[^{18}O] phosphate (146). The isotopic purity of this compound was determined by 162 MHz ^{31}P n.m.r. spectroscopy by relative integration of the peak due to unlabelled AMP and the isotopically shifted major peak which was completely separated (isotope shift 3.72 Hz) and it was found to be 95±5% isotopically pure.

A suitable cyclisation procedure was now required. Attempts at developing an *in situ* cyclisation of AMP-2,4-dinitrophenyl ester (147), prepared by the method of Borden and Smith[281] using 2,4-dinitrofluorobenzene and AMP, with potassium *t*-butoxide as the cyclising agent were not successful. A ^{31}P n.m.r. study of the initial esterification reaction, however, provided a better insight into the reaction than the previous authors' t.l.c. method. Indeed the reaction was found to be essentially complete before the much slower subsequent displacement of the 2,4-dinitrophenyl group by fluoride ion began. Borden and Smith[281] maintain that it is unsafe to leave the esterification reaction longer than 2 h before the competing reaction begins. In our hands, however, the

reaction was found to go 70% to completion in 3 h, as evidenced by the appearance of a sharp singlet at -9.25 ppm assigned to the ester (147), and even after 22 h only trace amounts of the



(147)

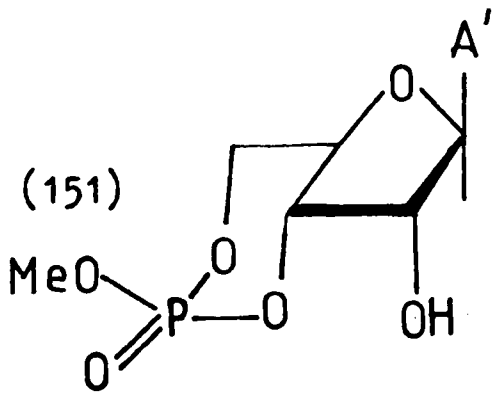


(148)

FIGURE 82

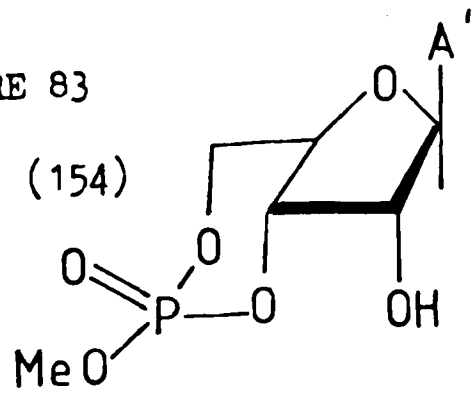
phosphofluoridate (148) could be discerned.

The use of the system triphenylphosphine-2,2'-pyridine disulphide (TPP-PDS) as developed by Mukaiyama and Hashimoto[282] was however, found to give excellent yields of cAMP (142) although the conditions seemed rather drastic (boiling pyridine). Thus the use of this procedure gave adenosine-3',5'[^{18}O]-cyclic phosphate (149) as shown in SCHEME 11 as its 4-morpholino-NN'-dicyclohexyl-carboxamidinium (MDCA) salt. Esterification of cAMP with diazo-methane in HMPA[283] could not be repeated. Attempted methylations using methyl iodide were also unsuccessful probably due to the low nucleophilicity of the cyclic phosphate. However, when the potassium salt of AMP was mixed with one equivalent of 18-crown-6 the resulting complex salt ((15c) for the labelled compound) was found to be clearly methylated overnight to give two peaks in the ^{31}P n.m.r. spectrum at -4.02 and -5.27 ppm in approximately a 1:2 ratio which were assigned to the equatorial and axial (151) cyclic diastereomers respectively. A' = N^1-methyl adenine. The final

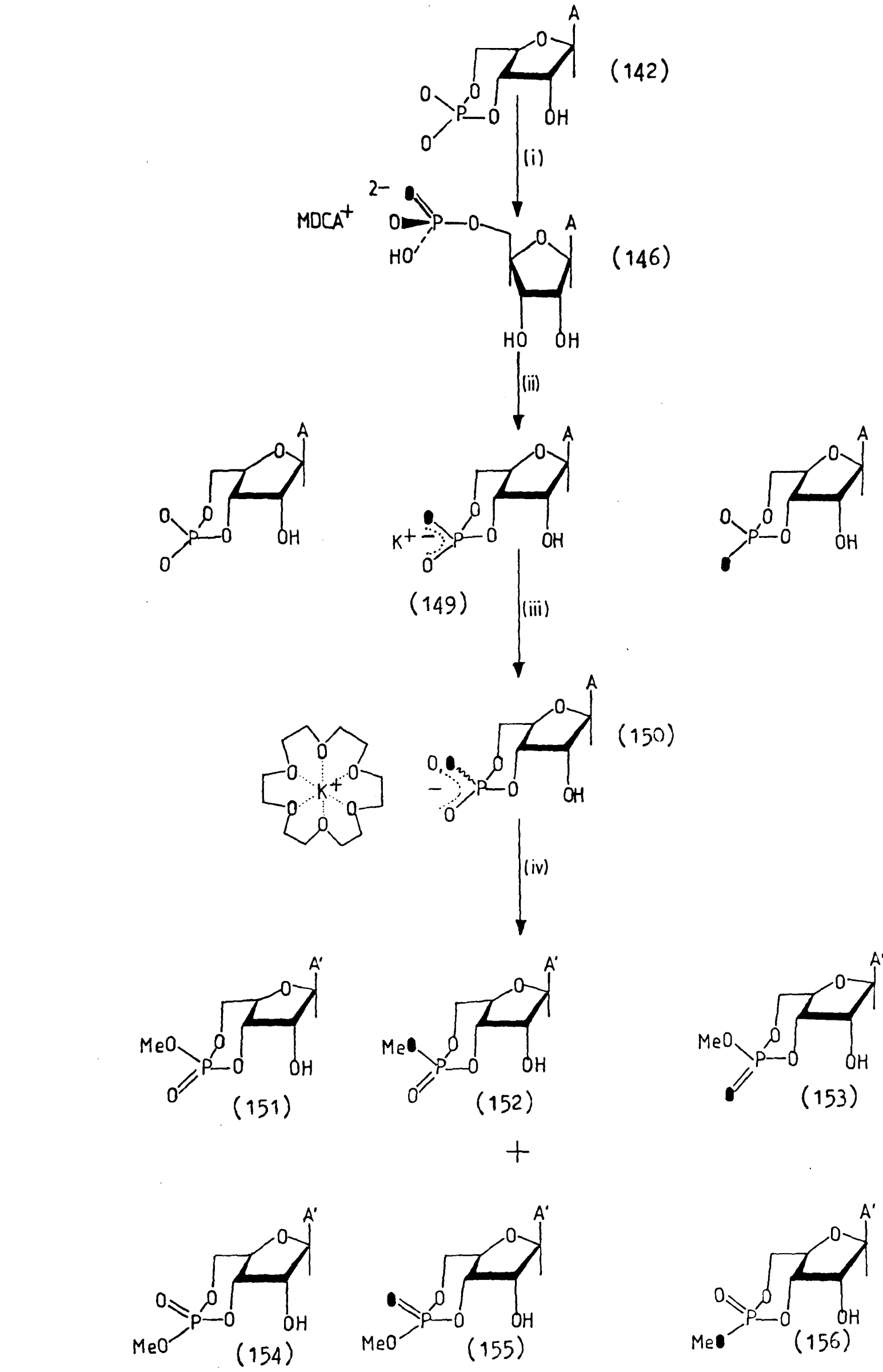


(151)

FIGURE 83



(154)



Reagents: (i) a. cAMP dieserase – H_2O, b. Dowex 50W H^+, 1 equiv. MDCA.

(ii) TPP–PDS (iii) a. Dowex 50W K^+, b. 18–Crown-6

(iv) MeI–DMSO

A' = N^1-methyl adenine

SCHEME 11 : Preparation and Analysis of Adenosine-3',5'-Cyclic[^{18}O]-Phosphate

products were also found to have been methylated at the N^1 position on the adenine ring.[280] Thus methylation in this fashion of the [^{18}O]-cAMP gave the six species (151)-(156) as illustrated in SCHEME 11. Unfortunately, examination of the ^{31}P n.m.r. spectrum at 36.43 MHz gave no useful isotopic shift information so a reexamination of 121.5 MHz was undertaken and the results are presented in FIGURE 84. Isotopic splittings can be clearly seen although it is apparent from the relative intensities that considerable loss of label has occurred (*ca.* 40%). This may have occurred by the intermediacy of P_1P_2-diadenosine-5'-pyrophosphate in the cyclisation procedure (most likely), or possibly by further reaction of the cyclising reagents with cAMP. In any event this kind of cyclisation procedure is clearly unsatisfactory for our purposes.

Nevertheless, in order to examine the isotopic splittings expected in the case of three isotope chirality and to check the feasibility of the method, especially with respect to resolution of the n.m.r. resonances, the synthesis of adenosine-5'[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate (161) was accomplished by our general method[226] in collaboration with Mr. R. Jarvest according to SCHEME 12 and this material cyclised by the TPP-PDS method.

(2*R*, 4*S*, 5*R*)-2-chloro-[2-^{17}O]-oxo-4,5 diphenyl [1-^{18}O]-1,3,2-dioxaphospholane (102) was generated by the usual procedures in pyridine and 2',3'-diacetyladenosine (158) was added over 20 min. in pyridine and the mixture left overnight. ^{31}P n.m.r. indicated a quantitative conversion to (2*R*, 4*S*, 5*R*)-(2',3'-diacetyl-5'-adeninyl)-[2-^{17}O]-oxo-4,5 diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane (159). In a similar preparation Ukita[284] employed 2',3'-isopropylidine adenosine as the attacking nucleophile but was only able to observe the ring opened species (162) and not the corresponding triester similar to (159). In view of the pronounced

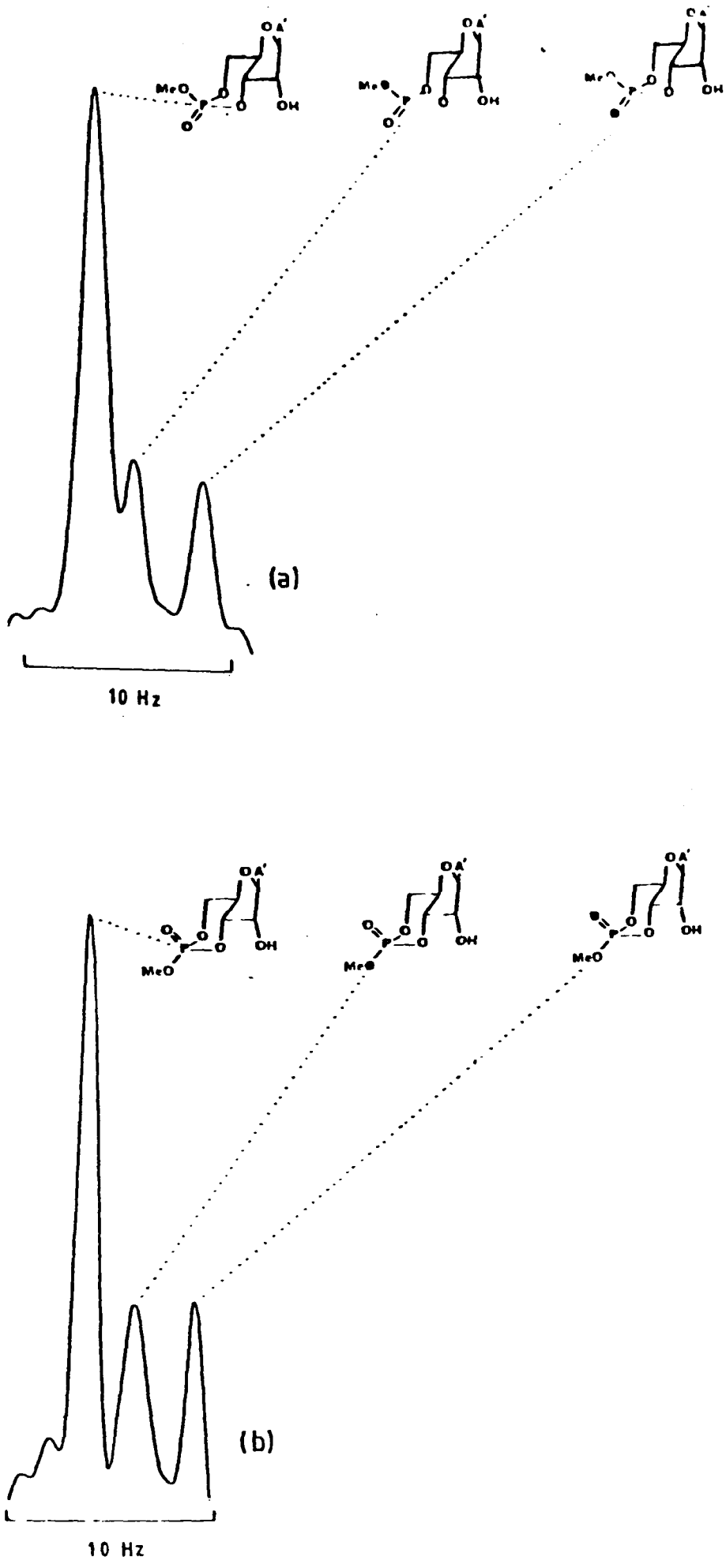
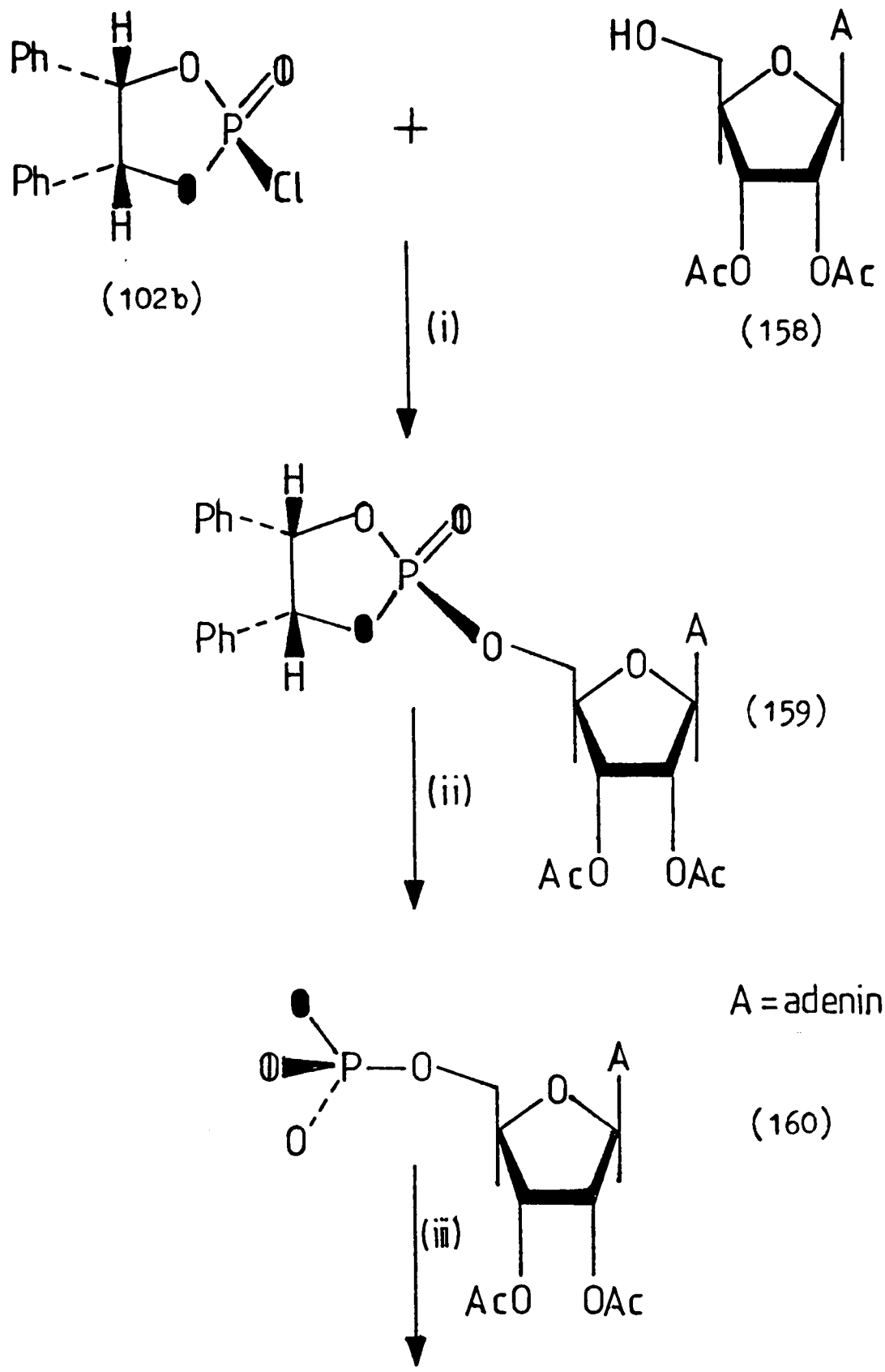


FIGURE 84 : The 121.5 MHz ^{31}P n.m.r. Spectrum in d_6-DMSO of (a) The Axial Diastereoisomer, and (b) The Equatorial Diastereoisomer of N^1-Methyl Adenosine-3',5'-[^{18}O]- Cyclic Phosphate Methyl Ester Derived from the TPP-PDS Cyclisation of Adenosine-5'[^{18}O]-Phosphate. For the axial isomer the isotope shifts were 1.83 Hz ($Me^{18}OP$) and 5.25 Hz ($MeOP{=}O^{18}$), and for the equatorial isomer they were 2.32 Hz and 5.25 Hz respectively. ^{31}P N.m.r. parameters were : 01, 2200 Hz; 02, 6225 Hz: sweep width, 2000Hz; acquisition time, 2.05 s; pulse width, 15 μs; memory size 8K, but processed in32K; zero line broadening: gaussian multiplication (line broadening, -1.2; gaussian broadening, 0.15 Hz); broad band proton decoupling.

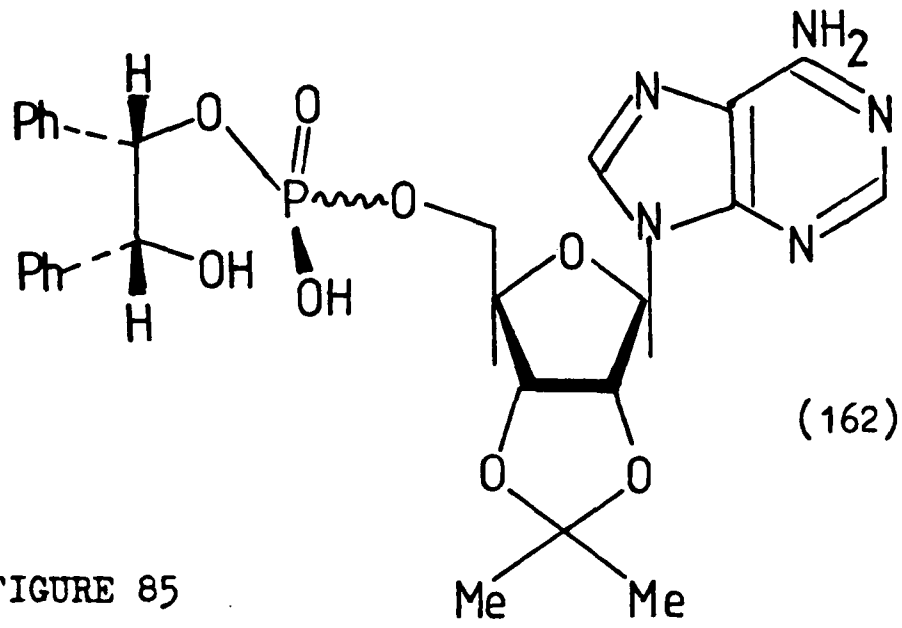


Reagents : (i) C_5H_5N

(ii) H_2-Pd/C-DMF

(iii) NH_3/EtOH

SCHEME 12 : Synthesis of Adenosine -5'[(S)-^{16}O, ^{17}O, ^{18}O]-Phosphate



(162)

FIGURE 85

instability of such cyclic 5-membered ring triesters this product presumably arose from his work-up procedure which involved *aqueous* conditions and gave very poor overall yields. We have found by n.m.r. that *meso*-hydrobenzoin cyclic phosphate triesters can be formed almost quantitatively by Ukita's procedure, and a *careful* work up can give excellent yields of these moieties which are very unstable even to atmospheric moisture. The fact that we are able to isolate the triester (159) whereas Ukita was not, illustrates just how careful one must be.

The triester (159) was hydrogenolysed in DMF to the free acid and deprotected with methanolic ammonia to give adenosine-5'[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate (161) in *ca.* 50% yield. Cyclisation and methylation as previously described gave the two series of triesters which were examined by ^{31}P n.m.r.

The ^{31}P n.m.r. spectrum of the unmethylated adenosine-3',5'-[^{16}O, ^{17}O, ^{18}O]-cyclic phosphate is shown in FIGURE 86. Three peaks can be seen showing that extensive isotopic scrambling has taken place, the peaks being assigned to unlabelled (142), mono-labelled and doubly labelled cAMP on the spectrum in an upfield direction. Methylation, however, splits the middle peak, and the corresponding ^{31}P n.m.r. spectra of the resulting two diastereo-isomers are presented in FIGURE 87. All four peaks expected are visible for the axial isomer with shifts as expected, but the most

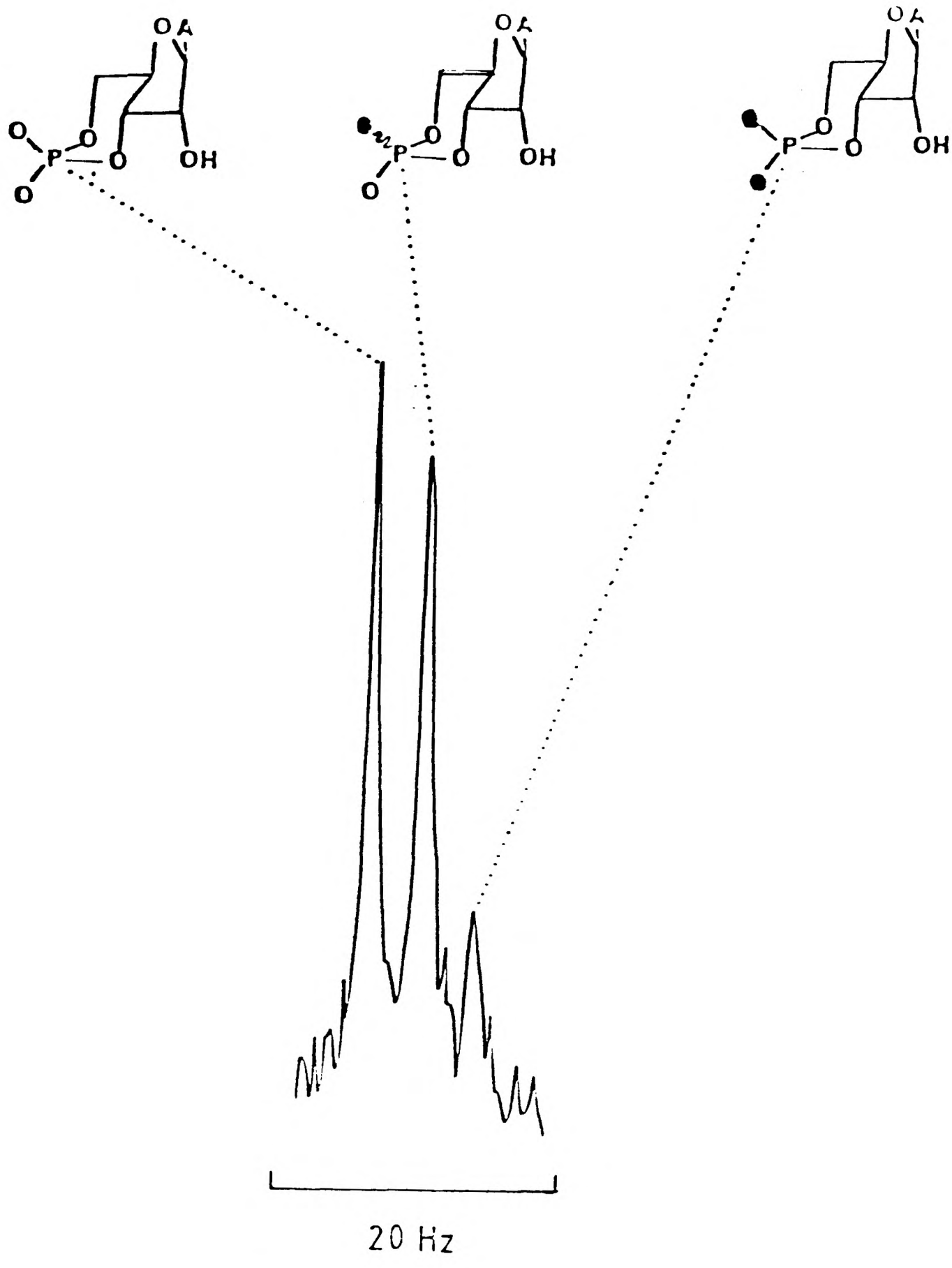


FIGURE 86 : The 121.5 MHz ^{31}P n.m.r. Spectrum in D_2O of Adenosine-3',5'-Cyclic[^{16}O, ^{17}O, ^{18}O]-Phosphate-4-Morpholino-N,N'-Dicyclohexylcarboxamidinium Salt Derived from the TPP-PDS Cyclisation of Adenosine-5'[(S)-^{16}O, ^{17}O, ^{18}O]-Phosphate. The axial and equatorial isotope shifts were both 3.66Hz. ^{31}P N.m.r. parameters were: O1, 2240 Hz; O2, 4365 Hz; sweep width, 2000 Hz;acquisition time, 4.19 s; pulse width, 15 μs; memory size 16 K but processed in 32 K; zero line broadening; broad band proton decoupling.

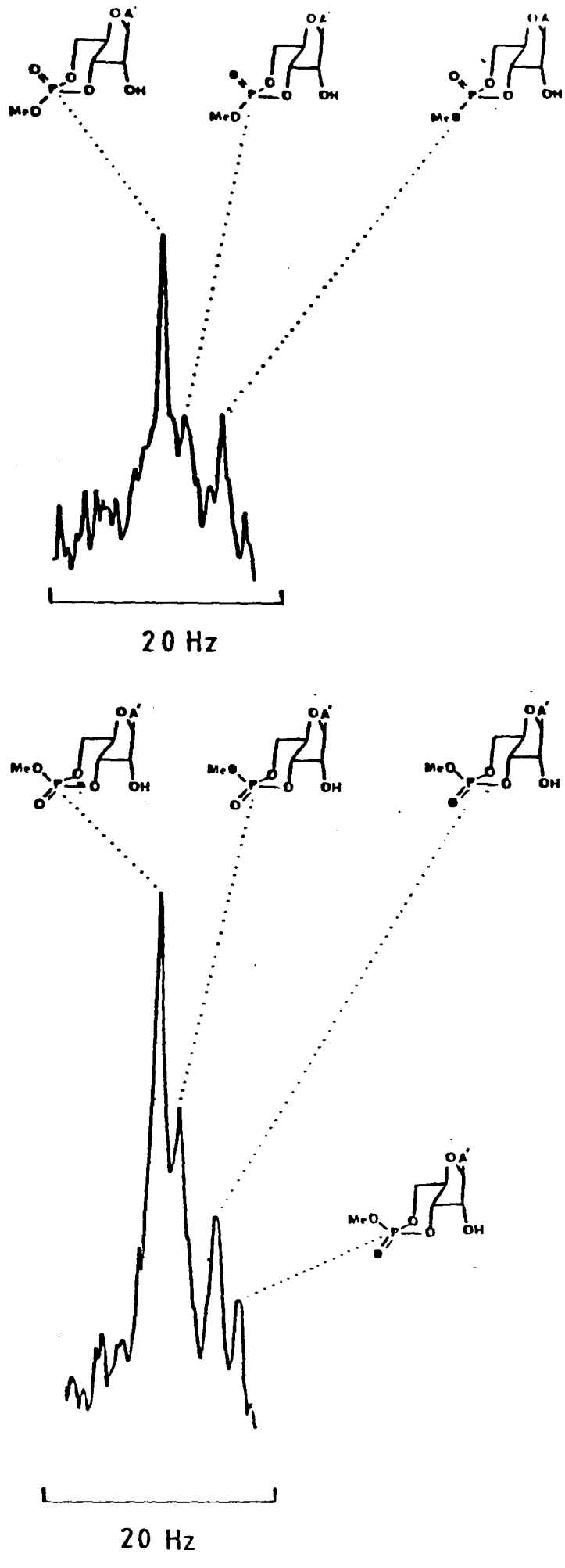


FIGURE 87 : The 121.5 MHz ^{31}P n.m.r. Spectrum of N^1-Methyl-Adenosine-3',5'-[^{16}O,^{17}O,^{18}O]-Cyclic-Phosphate Methyl Ester Derived from the TPP-PDS Cyclisation of Adenosine-5'[^{16}O,^{17}O,^{18}O]-Phosphate. The isotope shifts were for the axial diastereoisomer 1.76 Hz ($Me^{18}OP$), and 4.77 Hz ($MeOP{=}O^{18}$) and for the equatorial diastereoisomer 1.91 Hz and 5.28 Hz respectively. ^{31}P N.m.r. parameters were 01, 2240 Hz; 02, 6200 Hz; sweep width 1201 Hz; acquisition time 3.4 s; memory size 32K; pulse width 16 µs; zero line broadening; broad band proton decoupling.

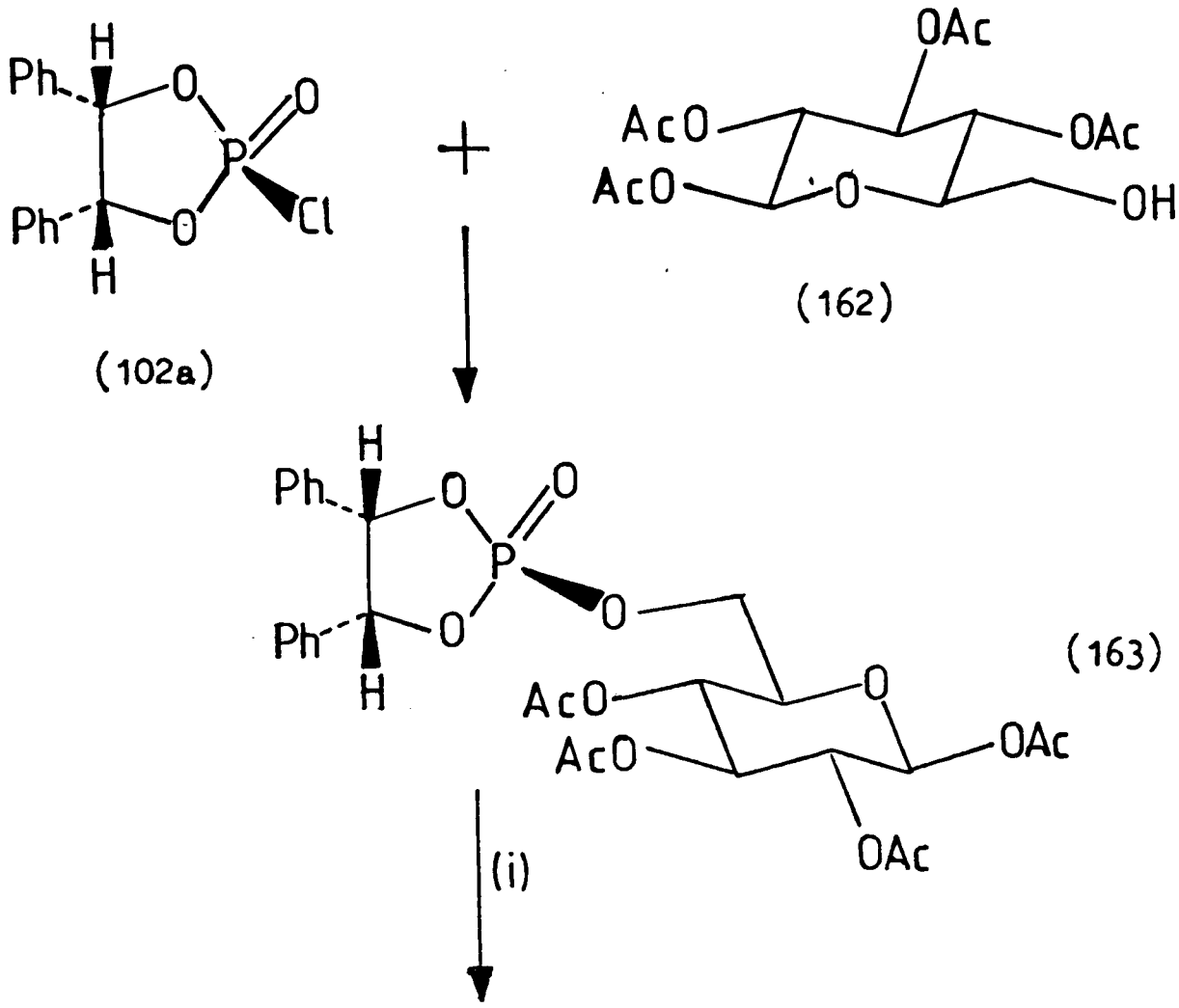
upfield peak on the lower intensity equatorial isomer is buried in the noise. The assignments for these lines are shown on the spectra. Thus despite the isotopic scrambling all four possible lines for this system have been observed and shown to be resolvable.

Better fortune was, however, obtained when these reactions were performed in the *D*-glucose series and this will now be discussed at length. Mr. R. Jarvest has since completed the adenosine series in a similar fashion to the ensuing discussion and the two systems have been published together.[216]

Investigations in the *D*-Glucose Series

A synthesis of *D*-glucose-6-phosphate (144) was required for investigations in this area. The procedure adopted is based on that published by Ukita[284] as well as our general chiral phosphate synthesis[226], however several modifications have been made to Ukita's procedure which greatly improve the overall yield. The same considerations apply to this system as have been previously mentioned for the 2',3'-diacetyl adenosine-5'-*meso*-hydrobenzoin cyclic phosphate (158) as far as instability of the triester is concerned. The synthetic scheme to *D*-glucose-6-phosphate (144) is illustrated in SCHEME 13. Thus β-*D*-glucose-1,2,3,4-tetraacetate (162) was synthesised by the literature procedure[285] and allowed to react with the *meso*-hydrobenzoin cyclic phosphochloridate (102a) as usual in pyridine to give *trans*-2(1,2,3,4-tetraacetyl-6-β-*D*-glucosyl)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (163) which could be isolated in 88% yield.

Ukita[284] deprotected the 1,3,2-dioxaphospholane system by catalytic hydrogenation in methanol. It has been shown conclusively by ^{31}P n.m.r. that such systems are markedly unstable towards methanol and this would therefore seem to be very inappropriate.



SCHEME 13 : Synthesis of D-Glucose-6-Phosphate.

In order to substantiate this theory, freshly prepared triester (163) was allowed to stand in dry methanol for several hours. After 12 h no evidence of the triester could be found by ^{31}P n.m.r. which showed only a complex series of resonances in the area expected for ring opened products. An alternative solvent was thus sought and the triester (163) was shown to be completely stable overnight in dry ethyl acetate which proved to be an excellent solvent in which to perform the hydrogenolysis, which proceeded smoothly using a Pd/C catalyst to give the 1,2,3,4-tetra-acetyl-β-*D*-glucose free acid (164) which was deacetylated without further purification to *D*-glucose-6-phosphate (144) using potassium methoxide in methanol and with an overall yield of 55% (Ukita's overall yield *via the triester* was ~7%).

Notwithstanding the direct synthesis of *D*-glucose-4,6-cyclic phosphate by Baddiley[286] from phenyl-β-*D*-glucose and phenyl-phosphorochloridate, the only literature procedure for the synthesis *via* the cyclisation of *D*-glucose-6-phosphate is that of Khorana[287] who employed dicyclohexylcarbodiimide (DCCI) and the monopyridinium salt of *D*-glucose-6-phosphate in a pyridine-water mixture. This procedure is carried out at room temperature and requires three days for completion. The Polish workers Zmudka and Shugar[288] have reported the effects of solvent variation on the products of this reaction and claim that Khorana's original conditions actually give rise to a mixture of *D*-glucose-4,6-cyclic phosphate and the seven-membered *D*-glucose-3,6-cyclic phosphate (165). However, no evidence in favour of this finding was obtained

(165)

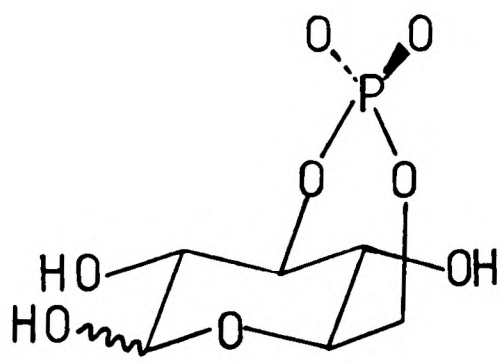
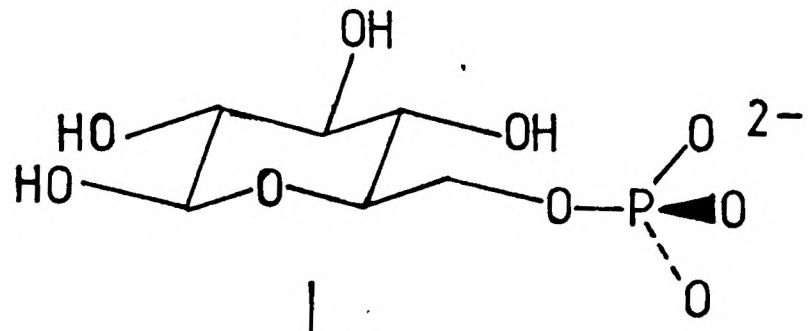


FIGURE 88

during our ^{31}P n.m.r. studies.

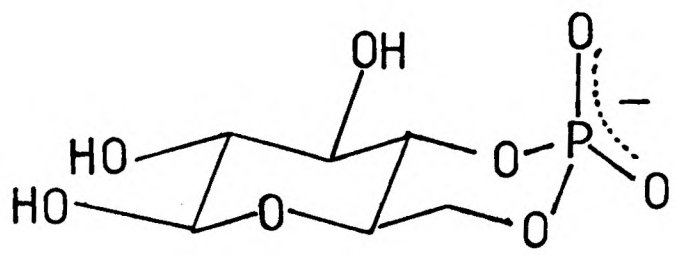
The synthetic scheme to the previously unreported *D*-glucose-4,6-cyclic phosphate methyl triesters (166) and (167) is shown in SCHEME 14. Thus synthetic *D*-glucose-6-phosphate (144) was converted to its *mono*-pyridinium salt by using Dowex 50W (pyridinium form) and this was cyclised in pyridine-water at room temperature to give *D*-glucose-4,6-cyclic phosphate (145) as its pyridinium salt which in D_2O exhibited a ^{31}P n.m.r. spectrum of a singlet at -4.6 ppm characteristic of the six membered cyclic system. Final conversion to the triesters was accomplished using diazomethane on the free acid of *D*-glucose-4,6-cyclic phosphate (145) which gave *D*-glucose-4,6-cyclic phosphate methyl ester as its axial diastereoisomer (166) and equatorial diastereoisomer (167).

The ^{31}P n.m.r. spectrum of this mixture exhibits two resonances at -4.1 and -6.1 ppm separated by *ca.* 1.8-2.0 ppm of approximately equal intensity, and is shown in FIGURE 89. At low sweep widths each line splits into two by an amount which is not wholly independent of the ambient conditions, this splitting being assigned to the α and β anomers of each diastereoisomer. No assignment of these anomeric resonances has been made although it is probably reasonable to assume that the larger peak of each pair (namely the downfield peak for the high field axial diastereoisomer and the upfield peak for the low field equatorial diastereoisomer is derived from the β-anomer on the basis of equatorial preference. The anomeric splitting for the axially substituted diastereoisomer is generally four to five times that of the equatorial and in this case is 15.75 Hz, but this is dependent on solvent to a large extent. Because of the previously mentioned generalisation concerning the chemical shifts of 1,3,2-dioxaphosphorinan-2-ones[276-278] we assign the high field doublet to the anomers of the



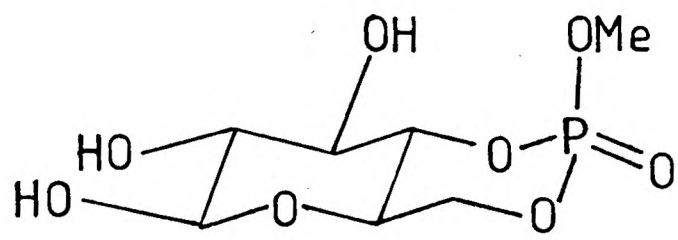
(144)

(i)



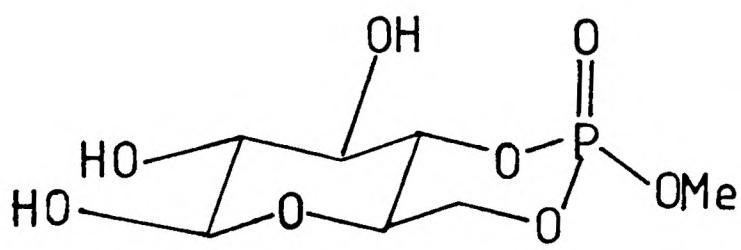
(145)

(ii)



(166)

+



(167)

Reagents : (i) DCCI/pyridine — H_2O

(ii) Dowex − H^+

CH_2N_2 / MeOH

SCHEME 14 : Synthesis of D-Glucose-4,6-Cyclic-Phosphate Methyl Esters

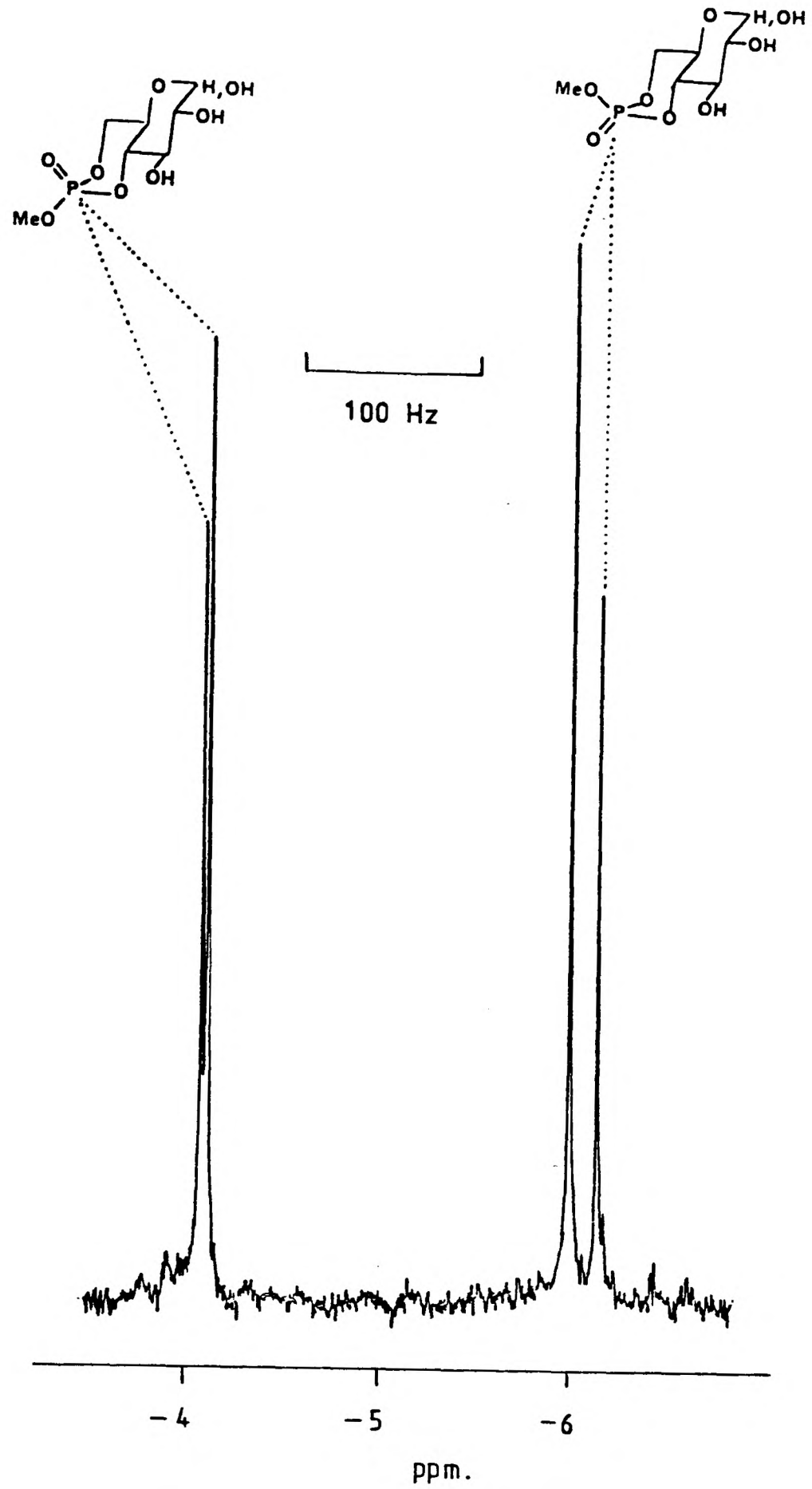
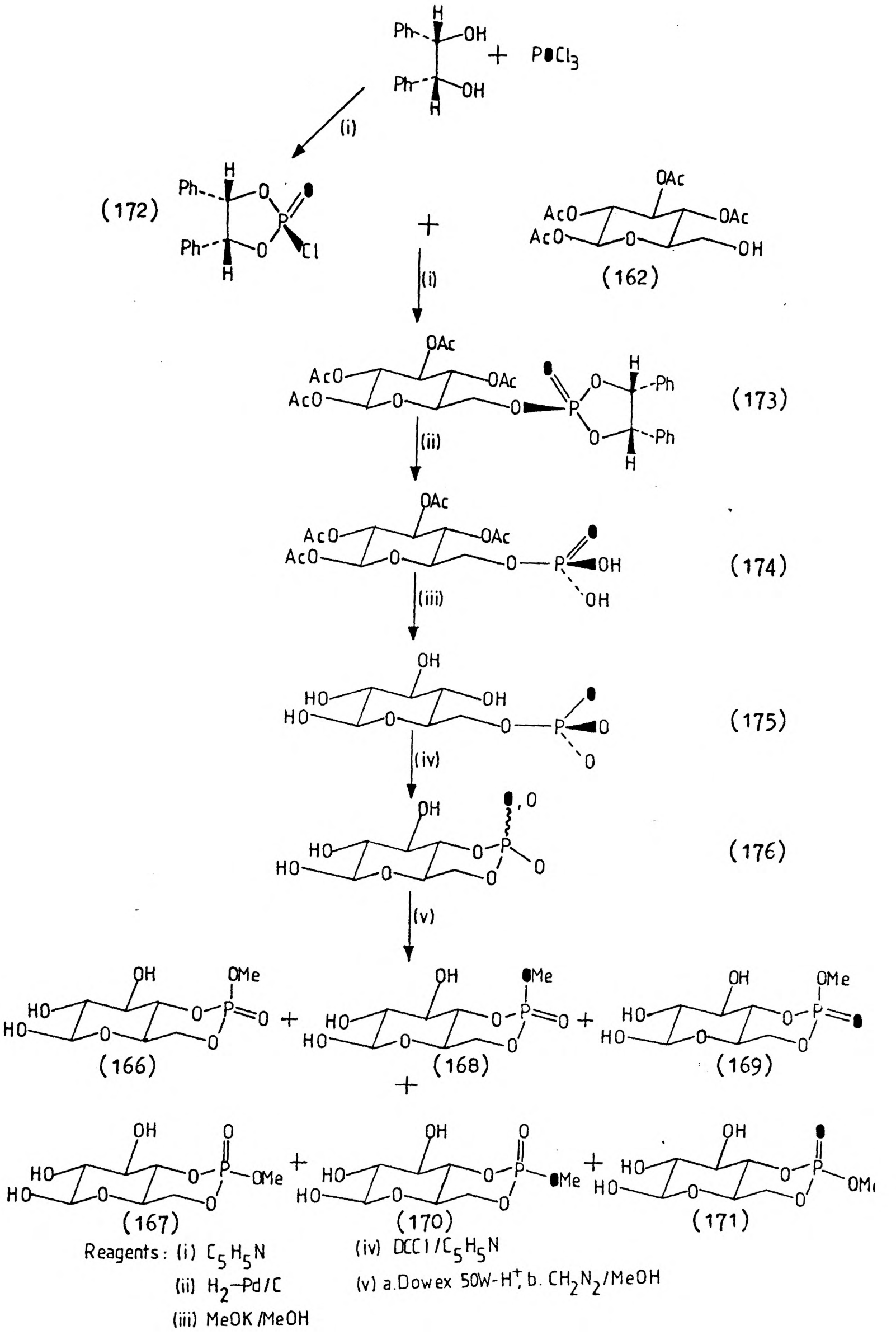


FIGURE 89 : The 121.5 MHz ^{31}P n.m.r. Spectrum of the Axial and Equatorial Diastereoisomers of D-Glucose-4,6-Cyclic-Phosphate-Methyl Ester in 25% d_4-MeOH containing 8-Hydroxyquinoline. ^{31}P n.m.r. parameters were: offset, 2240 Hz; 02, 5980 Hz; sweep width,2000 Hz; acquisition time,2.05 s; memory size,8K, but FT in 32K; pulse width, 16 μs; line broadening, 0.2 Hz; broad band proton decoupling

axial diastereoisomer (166) and the low field doublet to the anomers of the equatorial diastereoisomer (167).

Thus a route was established which should enable a total synthesis of the species required to test the isotopic chirality analysis method. For reasons identical to the logic of the cAMP system the examination of the effects of a single ^{18}O label was planned first. For this system the label was incorporated chemically using *trans*-2-chloro-[2-^{18}O]-oxo-4,6-diphenyl-1,3,2-dioxaphospholane (177) derived from [^{18}O]-phosphoryl chloride (124) and *meso*-hydrobenzoin. The complete scheme is illustrated in SCHEME 15. Thus *trans*-(4*R*,5*S*)-2(1,2,3,4-tetraacetyl-6-β-*D*-glucosyl)-2[^{18}O]-oxo-4,5 diphenyl-1,3,2-dioxaphospholane (173) was hydrogenolysed to give 1,2,3,4-tetraacetyl-β-*D*-glucose 6[^{18}O]-phosphate free acid (174) which gave on deacetylation *D*-glucose-6[^{18}O]-phosphate (175). Cyclisation with DCCI gave *D*-glucose-4,6[^{18}O]-cyclic phosphate (176) and methylation the set of axial diastereoisomers (166), (168) and (169) and equatorial diastereoisomers (167), (170) and (171). Since all the oxygen atoms in the phosphate (175) are torsionally equivalent there is an equal chance of label loss and in the absence of loss of label by other means the final product will consist of an equimolar mixture of the completely unlabelled, bridging labelled and phosphoryl labelled triesters for each diastereoisomeric series.

Examination of the ^{31}P n.m.r. spectrum of the above mixture in methanol revealed that the differentiation between the bridging and doubly bonded ^{18}O isotope shifts was indeed discernable at 36.43 MHz on the axial isomer (FIGURE 90) where all three peaks were reasonably resolved for each anomer, although the small anomeric separation on the equatorial system precluded the observation of anything other than overlapping peaks. When, however, the ^{31}P spectrum was recorded at 145.8 MHz in Edinburgh



SCHEME 15 : Synthesis and Analysis of D-Glucose-6[^{18}O]-Phosphate

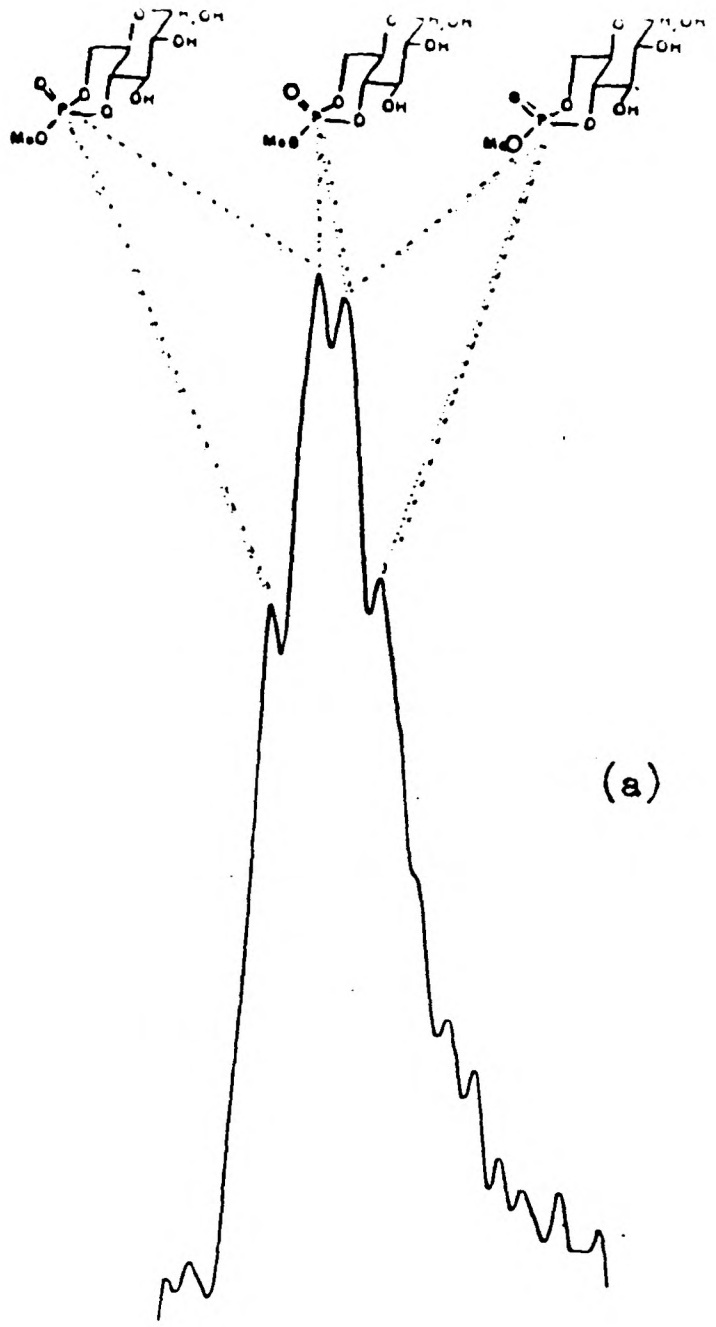


FIGURE 90 :

The 36.43 MHz ^{31}P n.m.r. Spectrum of (a) The Equatorial Isomer, and (b) The Axial Isomer of D-Glucose-4,6-Cyclic [^{18}O]-Phosphate Methyl Ester in d_4-MeOH derived from the Cyclisation of D-Glucose-6[^{18}O]-Phosphate with DCCI in pyridine-water. ^{31}P N.m.r. parameters were: offset, 2200 Hz; sweep width, 400 Hz; pulse width, 17 μs; memory size, 4K; broad band proton decoupling. For the axial diastereoisomer the isotope shifts were : 0.49 Hz ($Me^{18}OP$) and 1.47 Hz ($MeOP{=}O^{18}$), and were not measureable for the equatorial diastereoisomer

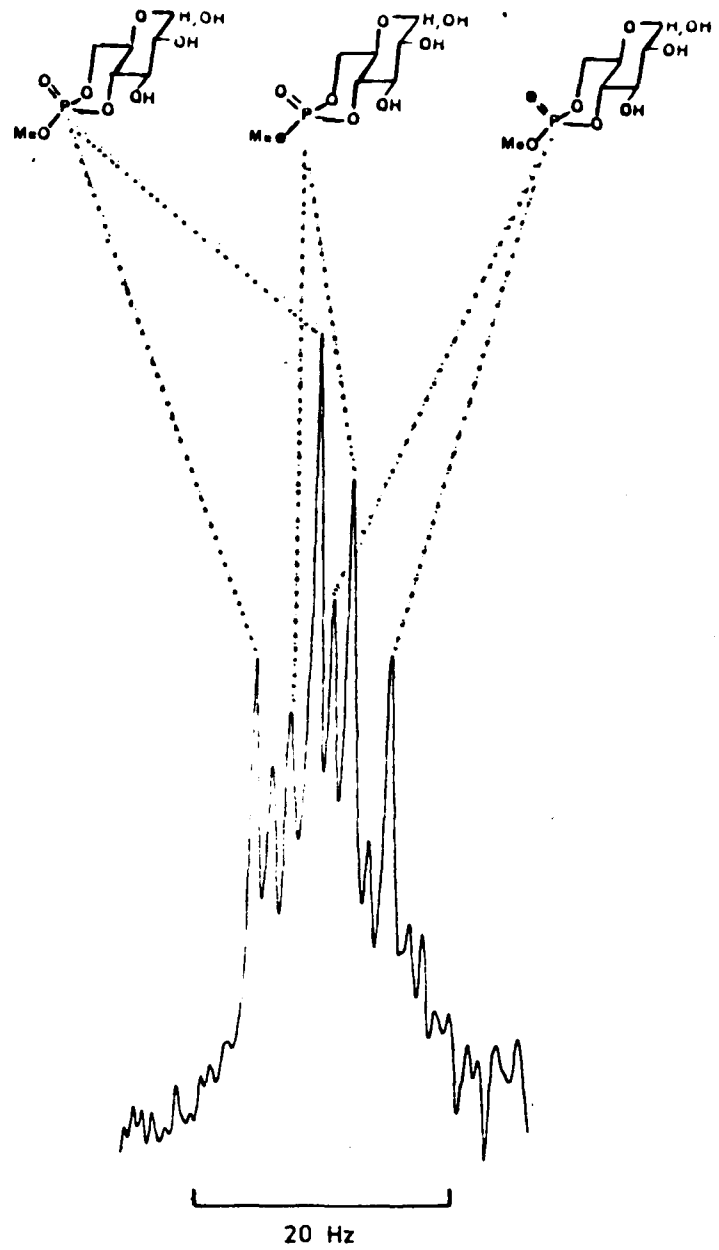
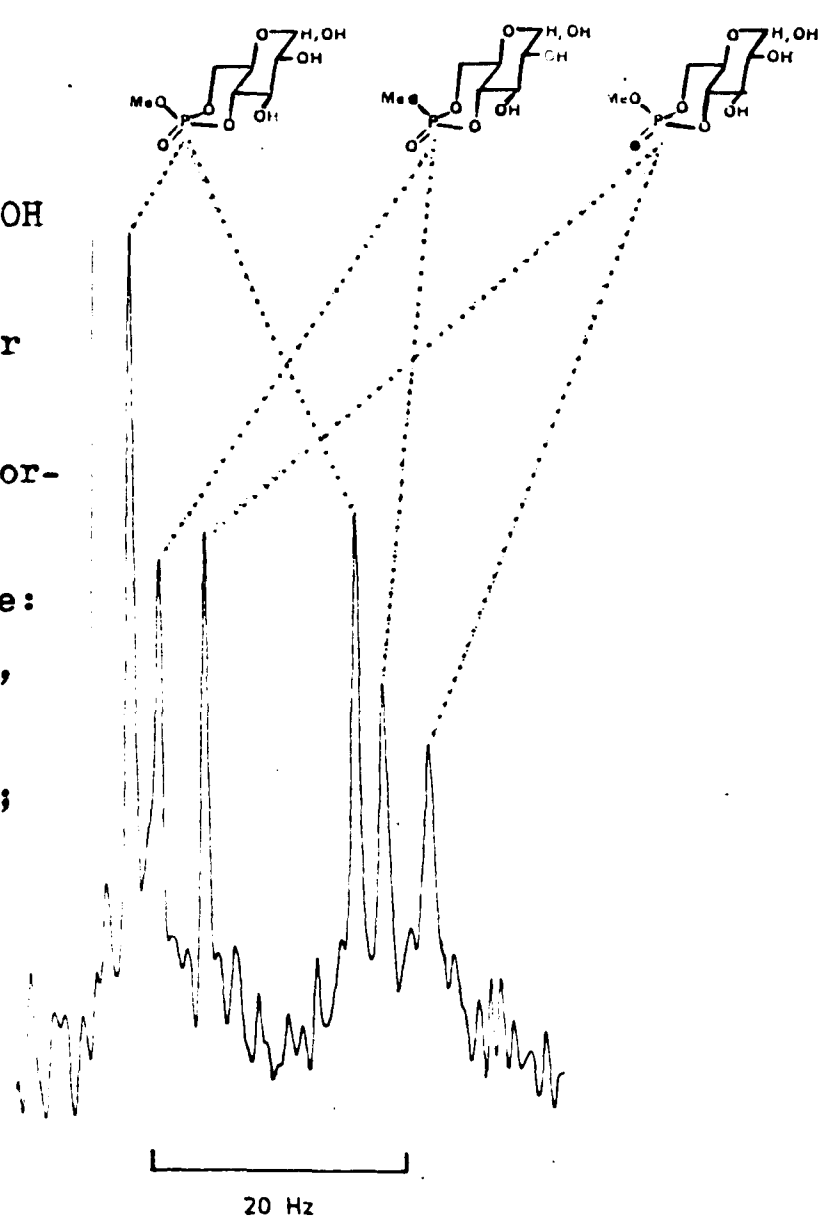


FIGURE 91 :

The 145.8 MHz ^{31}P n.m.r. Spectra in d_4-MeOH of the Axial and Equatorial Isomers from FIGURE 90. For the Axial Diastereoisomer the isotope shifts were 2.14 Hz ($Me^{18}OP$) and 5.72 Hz ($MeOP{=}O^{18}$), and for the equatorial diastereoisomer 2.70 Hz and 5.91 Hz respectively. ^{31}P n.m.r. parameters were: offset, -700 Hz; 02, 6500 Hz; sweep width, 1202 Hz; acquisition time, 3.4 s; memory size, 8K; but FT in 32K; pulse width, 20 µs; broad band proton decoupling



(FIGURE 91) a dramatic change could be seen, allowing measurement of both axial and equatorial isotope shifts, and the spectrum was in excellent agreement with expectation, although the magnitude of the two isotopically shifted peaks was lower than that of the base peak, suggesting some loss of isotope. However, the situation is visually worse than it really is since the relative difference between the labelled and unlabelled peak intensities is actually *twice* the actual amount of isotope lost which is in the region of 15-20%. This loss is serious, however, since it represents the loss from one site only and hence for the [^{16}O, ^{17}O, ^{18}O]-situation the effect would be much greater. This loss may have occurred during the synthesis when the free acid was exposed to aqueous conditions although this is unlikely (see later for a full discussion), and it probably represents a complication in the cyclisation such as for example the intermediacy of a P_1,P_2-diglucose-6-pyrophosphate intermediate.

A much more convenient and efficient synthesis was developed. The dropwise addition of the pyridine-soluble *mono*-4-morpholino-N,N'-dicyclohexylcarboxamidinium (MDCA) salt of *D*-glucose-6-phosphate, prepared *via* the *mono*-pyridinium salt, to a boiling solution of 1 equivalent of DCCI dissolved in anhydrous pyridine led to an almost quantitative conversion to the MDCA salt of *D*-glucose-4,6-cyclic phosphate within 10-15 min (no reaction was observed after 30 min at 60 °C under these conditions), whereas Khorana's original procedure[287] at room temperature takes 3 days for completion. This synthesis has another advantage in that the product of the cyclisation reaction is *D*-glucose-4,6-cyclic phosphate as its MDCA salt which is soluble in methanol. Thus the final exchange reaction to the free acid with Dowex 50W (H^+) may be simply performed in anhydrous methanol prior to the reaction with diazomethane.

When this new procedure was used to cyclise a sample of *D*-glucose-6[^{18}O]-phosphate (175) and the product converted to its methylated triesters, the ^{31}P n.m.r. spectrum was much more encouraging (FIGURES 92,92a,b). The axial diastereoisomer series at -5.65 ppm showed an anomeric separation of *ca*. 9 Hz at 121.5 MHz and allowed complete resolution of the pattern. However, the smaller equatorial diastereoisomer anomeric separation was not resolved (although the equatorial lines were twice as broad as the axial ones, and a simple three line pattern was observed instead of the more complex overlapping pattern which had previously been observed at 145.8 MHz. In other respects the spectrum was entirely in accord with expectation and showed that an acceptably low level of isotope loss had occurred in this case. Indeed a comparison of peak heights for both diastereoisomers showed that the sample was *ca*. 96% labelled, and estimates of the isotopic purity before cyclisation (from the mass spectrum of [^{18}O]-trimethyl phosphate (125) derived from the [^{18}O]-phosphoryl chloride used in the synthesis) did not really exceed this. Thus minimal loss of isotope had occurred.

Convincing proof that the equatorial triplet pattern (FIGURE 92b) was in fact due to two virtually coincident anomers was obtained by running the spectrum again with added DMSO (the final mixture was 5:3 MeOH:DMSO). This different solvent composition resolved the two anomers beautifully into six lines with the expected intensity patterns (FIGURE 93). As will be seen later these sets of resonances are particularly dependent on very precise conditions for resolution and are consequently very unpredictable.

The way was now ready for a synthesis and investigation of the chirally labelled system. Thus we needed to synthesise *D*-glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O] phosphate and put it through the same sequence of reactions. The synthesis of *D*-glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate (178) was achieved according to the general procedure as

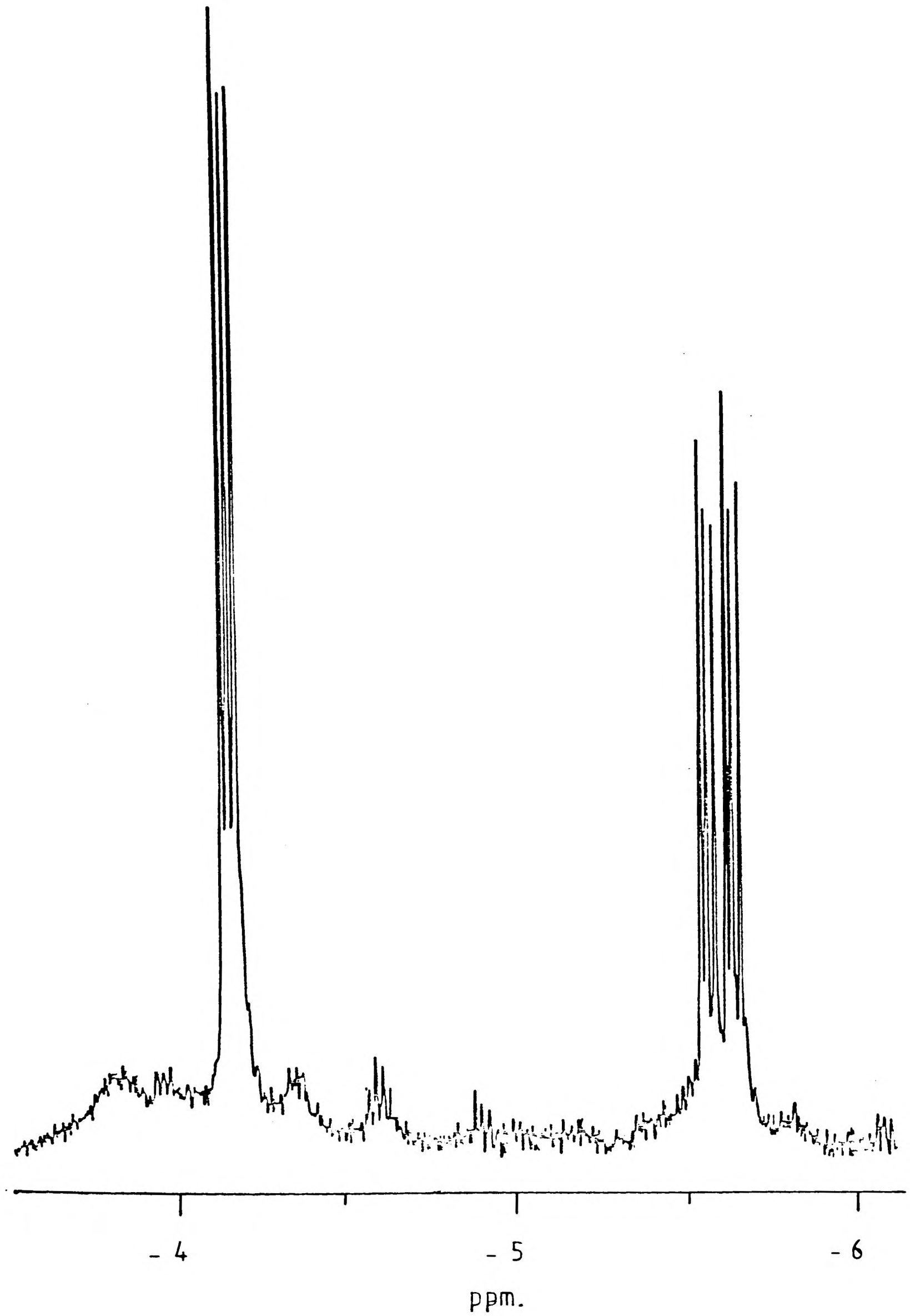


FIGURE 92 : The 121.5 MHz ^{31}P n.m.r. Spectrum in d_4-MeOH of the D-Glucose-4,6-Cyclic-Phosphate Methyl Esters Derived from the Cyclisation of D-Glucose-6[^{18}O]-Phosphate using DCCI

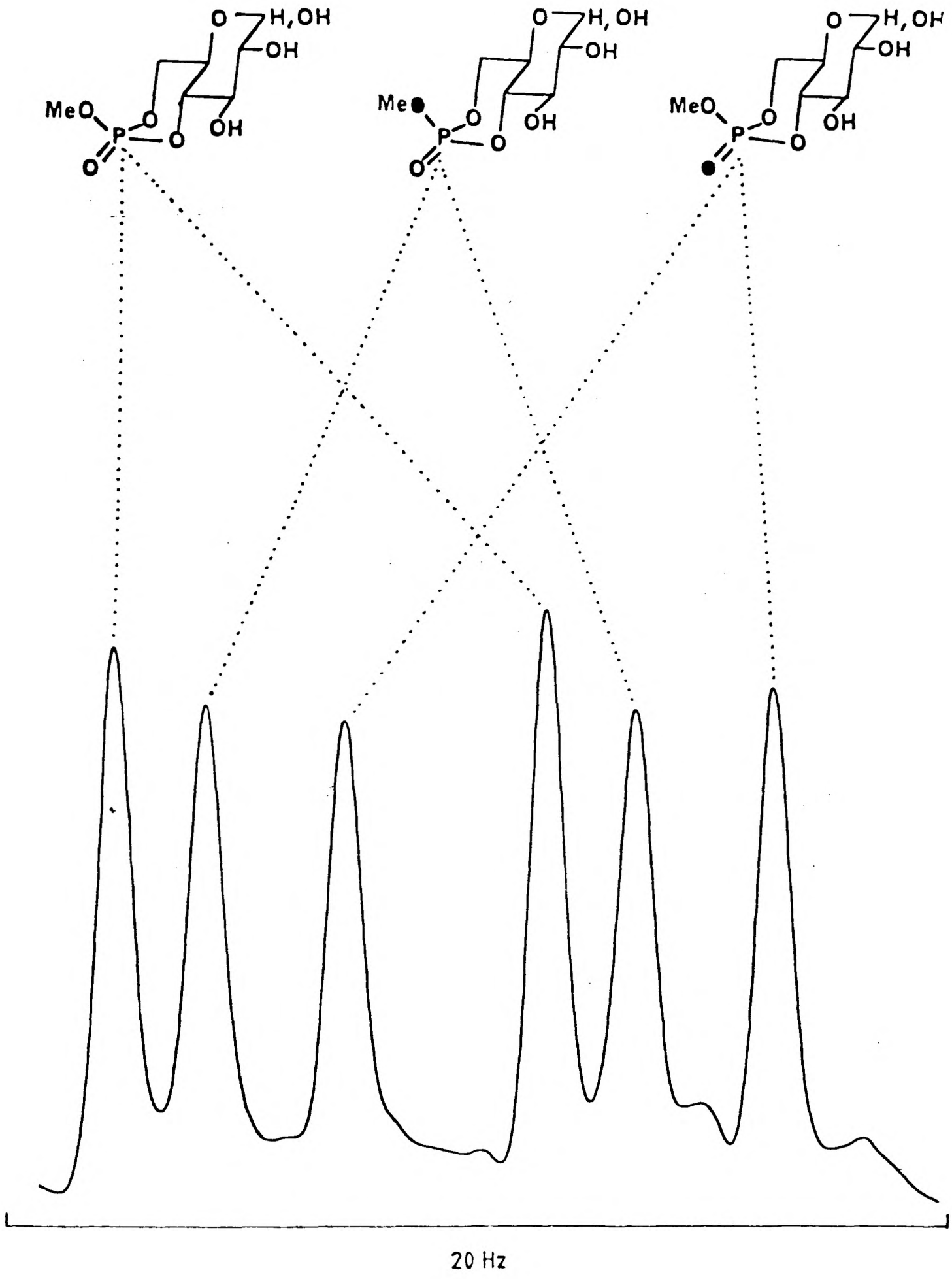


FIGURE 92(a) : An expansion of the axial diastereoisomer as in Figure 90. ^{31}P n.m.r. parameters were : offset, 2649 Hz; 02, 5980 Hz; sweep width, 1202 Hz; acquisition time, 2.05 s; memory size, 4K but FT in 32K; pulse width, 16 μs; broad band proton decoupling; gaussian multiplication (line broadening, -1.0 Hz; gaussian broadening, 0.1 Hz) in 4K.

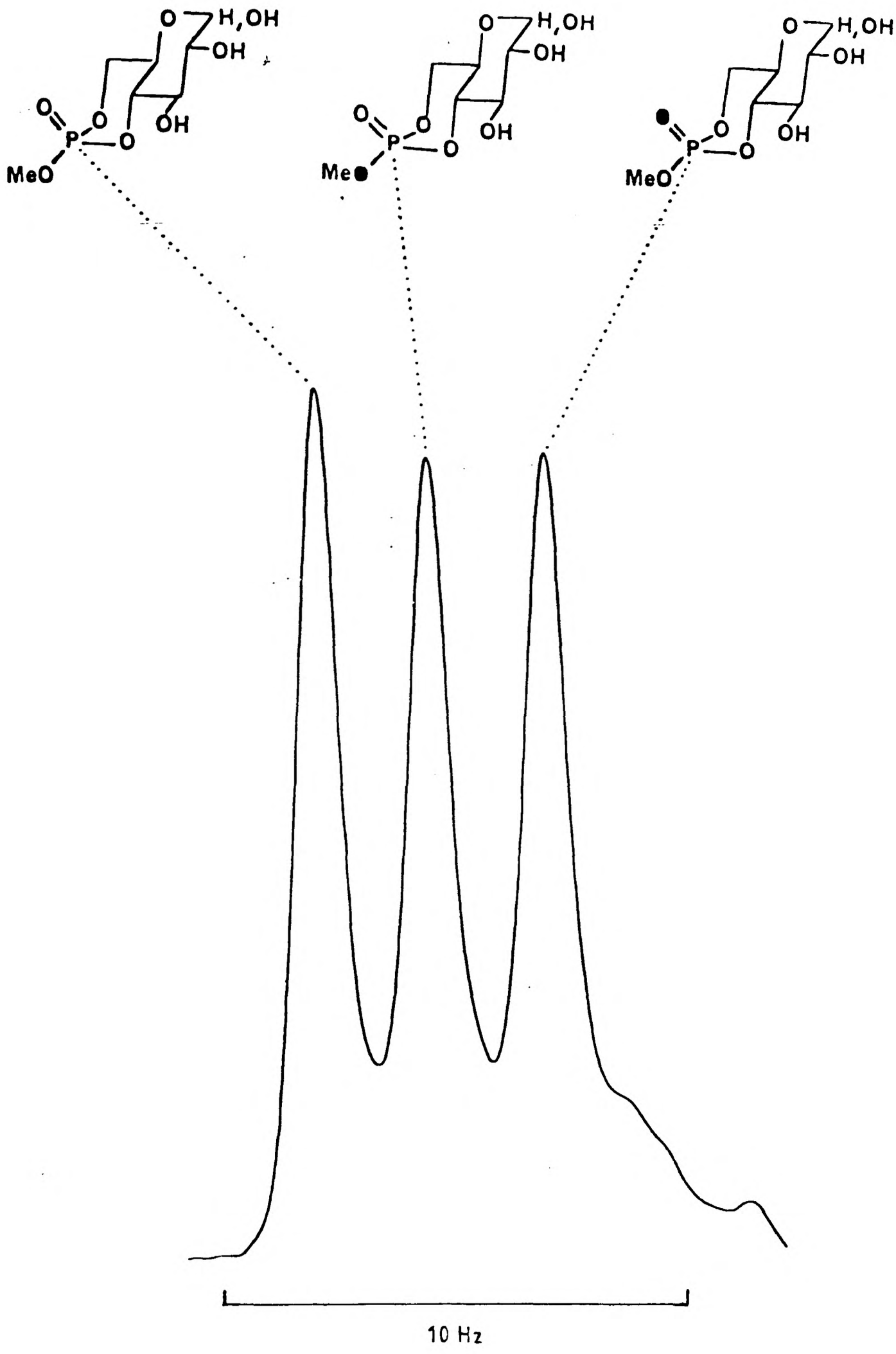


FIGURE 92(b) : An expansion of the equatorial diastereoisomer.

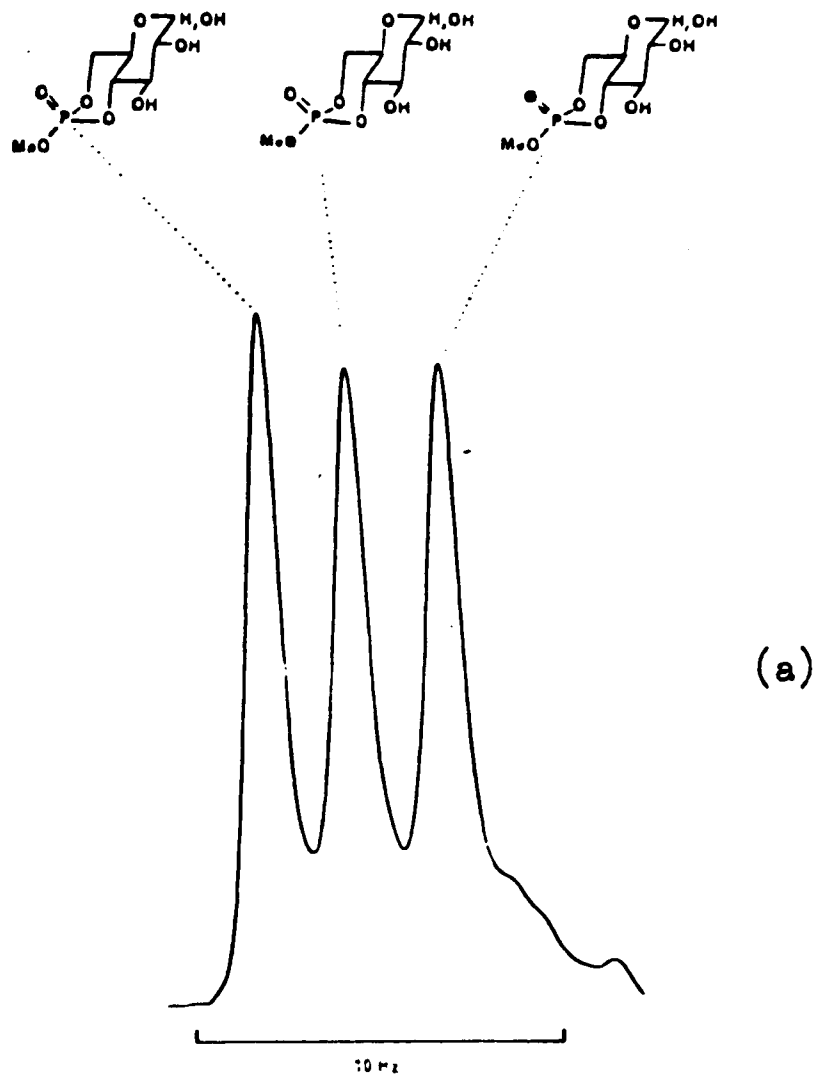


FIGURE 93 : A Comparison of the ^{31}P n.m.r. Spectrum of the Equatorial Diastereoisomer of D-Glucose-4,6[^{18}O]-Cyclic-Phosphate Methyl Ester in (a) d_4-MeOH, and (b) 5:3 d_4-MeOH:DMSO. The n.m.r. parameters were the same as FIGURE 92

illustrated in SCHEME 16. Thus (2*R*,4*S*,5*R*)-2-chloro-[2-^{17}O]-oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane was prepared in the usual fashion and reacted with 1,2,3,4-tetraacetyl-β-*D*-glucose (162) to give (2*R*,4*S*,5*R*)-2(1,2,3,4-tetraacetyl-6-β-*D*-glucosyl)-[2-^{17}O]-oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane (177) which was deprotected by hydrogenolysis and deacetylation to give *D*-glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate (178). This material was cyclised and methylated according to the previously discussed procedures and the ^{31}P n.m.r. spectrum is illustrated in FIGURES 94 and 94a,b. The assignments are on FIGURES a,b, and it must be remembered that *species containing ^{17}O will not be observed.* The downfield equatorial triester exhibits overlapping resonances and will be discussed later, however it can immediately be seen from the axial resonances that each anomer now consists of four lines, the extra most upfield line, in addition to the three we have already discussed from the system starting from a singly labelled species, being derived from the doubly labelled species. One thing should be remembered here, and that is that the two most important lines in any given axial or equatorial diastereoisomeric isotope pattern are the central two lines. These lines are central because in reality we will almost certainly have a small amount of material which will end up completely unlabelled, and of course this will appear in the most downfield position. However, this line and the other most upfield outside line for each anomer of each diastereoisomer act as very useful markers with respect to the overall pattern. However, it is the relative order and ratio of the centra two lines in any anomer which will tell us whether inversion, retention or racemisation has occurred. Bearing this in mind we see immediately from the axial diastereoisomer resonances for each anomer that the heights of the central lines are identical (FIGURE 94a) and we can derive no stereochemical information from them.

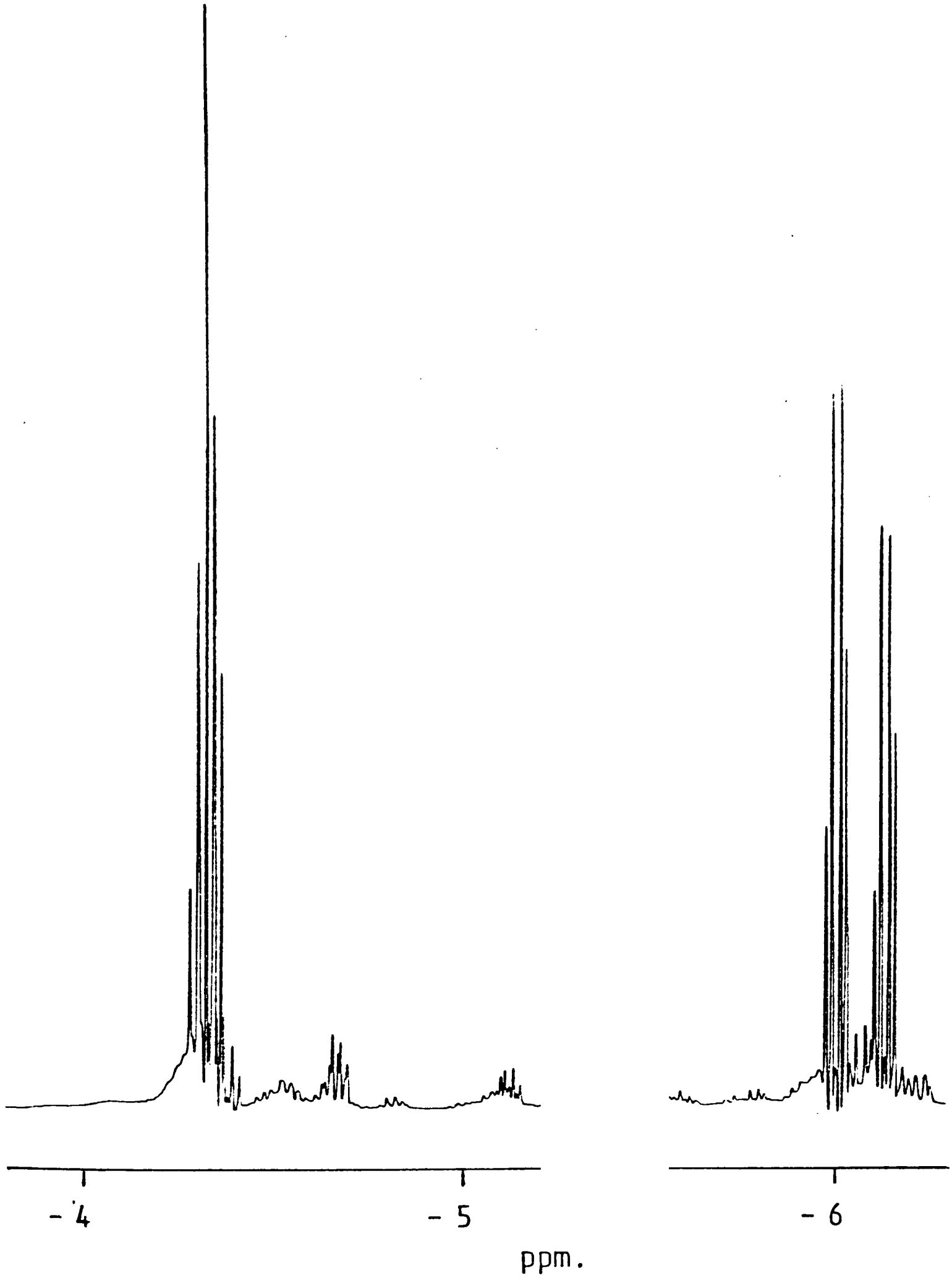


FIGURE 94 : The 121.5 MHz ^{31}P n.m.r. Spectra in 5:3 d_4-MeOH:DMSO of the Axial and Equatorial Diastereoisomers of D-Glucose-4,6-[^{16}O, ^{17}O, ^{18}O]-Cyclic-Phosphate Derived from the DPPC-BuOK Cyclisation of D-Glucose-6[^{16}O, ^{17}O, ^{18}O]-Phosphate and Subsequent Methylation.

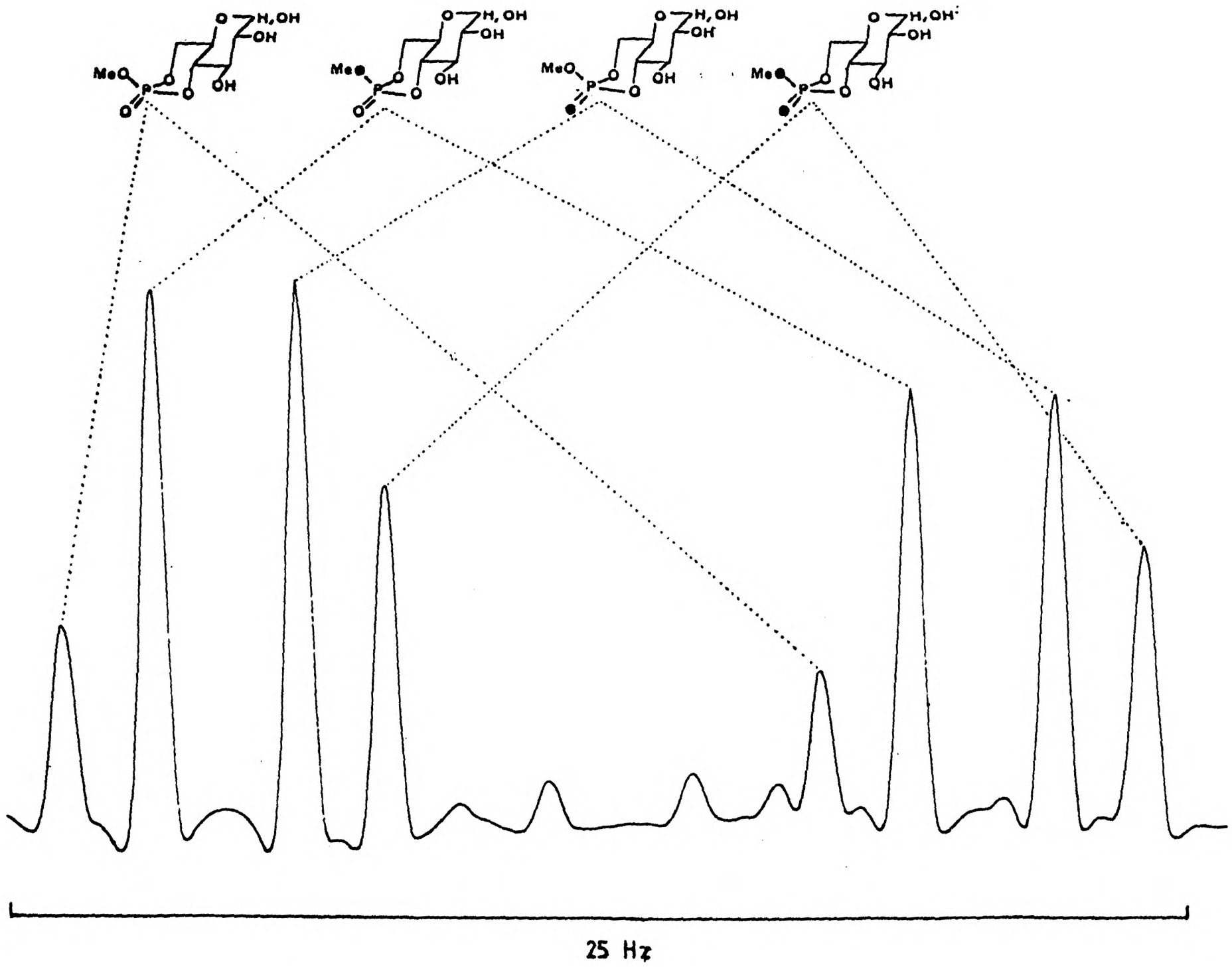


FIGURE 94(a) : An Expansion of the Axial Diastereoisomer

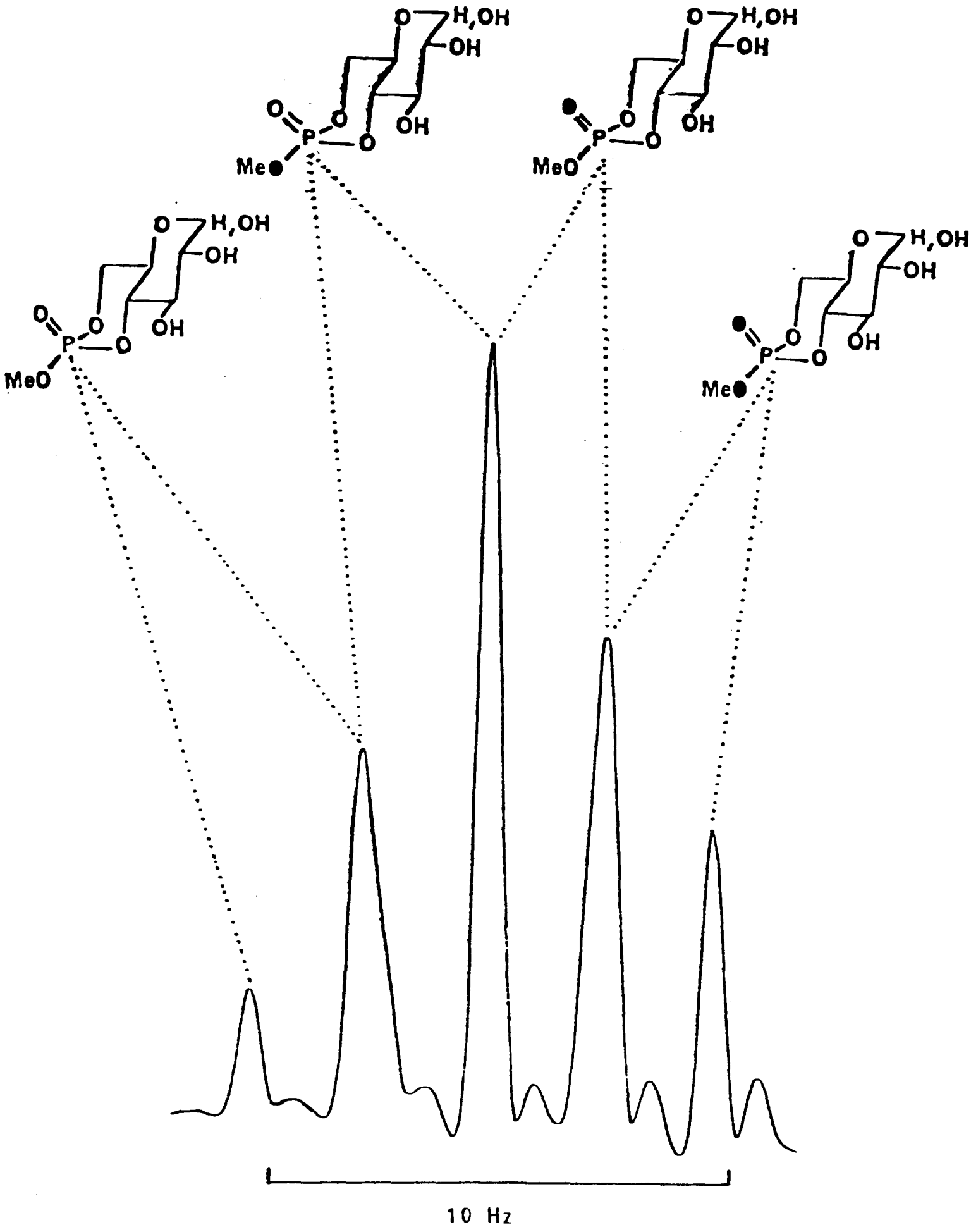


FIGURE 94(b) An Expansion of the Equatorial Diastereoisomer. ^{31}P n.m.r. parameters were : offset, 2240 Hz; 02, 5980 Hz; sweep width, 2000Hz; pulse width, 16 μs; acquisition time, 2.05 s; broad band proton decoupling; gaussian multiplication(line broadening, -0.6 Hz; gaussian broadening, 0.4 Hz) in 8K and FT in 32K.

We know from our stereospecific synthesis that the precursor of the cyclic system had the isotopic (*S*) configuration and therefore the ring closure reaction must have occurred with *racemisation* of the isotopic chirality. The equatorial signals, although more complex, are also in line with this interpretation. Peak heights cannot be used here as a reliable way of assessing the relative contributions of the various species since it can be seen that lines 2 and 4 (numbering in an upfield direction from the smallest peak) are broader than the others due to imperfect anomeric overlap of their constituent lines. However, integration of the whole system enables a good comparison to be made and the pattern is wholly compatible with two anomers of approximately equal size overlapping by one spacing, and with the same relative ratios of all the lines as for the axial series.

In order to account quantitatively for the observed line intensities (and hence determine the stereoselectivity of the cyclisation when we have such a ring closure reaction) a number of factors need to be considered. First, the isotope content of the [^{17}O]-phosphorus oxychloride (118) (determined by mass spectrometry after conversion to trimethyl phosphate) was 33 atom % ^{16}O, 43.5 atom % ^{17}O and 53.2 atom % ^{18}O. Secondly, the (1*R*,2*S*)-[1-^{18}O]-1,2-dihydroxy-1,2-diphenylethane used in the synthesis was derived from the reduction of (2*S*)-[1-^{18}O]-benzoin which was 91.8% (*S*) and 8.2% (*R*), was labelled with 97 atom % ^{18}O and was contaminated with 6% (1*S*,2*S*)-[1-^{18}O]-1,2-dihydroxy-1,2-diphenylethane. Taking these and other factors into consideration the expected relative intensities of all the peaks in the ^{31}P n.m.r. spectrum can be calculated for inversion, retention and of racemisation configuration at phosphorus (this procedure is described in detail in CHAPTER 10), and these are compared with the observed mean peak heights in TABLE 10 (the mean equatorial peak heights are those derived from

TABLE 10: A COMPARISON BETWEEN THE OBSERVED AND CALCULATED NMR INTENSITIES FOR THE DCCI CYCLISATION OF *D*-GLUCOSE-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-PHOSPHATE

	Axial Triester				Equatorial Triester			
	OBS.	Calculated			OBS.	Calculated		
		RETN.	INVN.	RACM.		RETN.	INVN.	RACM.
MeO-P=O	0.20	0.28	0.28	0.33	0.39	0.28	0.28	0.33
Me●-P=O	1.00	1.00	0.74	1.00	1.00	0.74	1.00	1.00
MeO-P=●	1.00	0.74	1.00	1.00	1.00	1.00	0.74	1.00
Me●-P=●	0.65	0.49	0.49	0.56	0.65	0.49	0.49	0.56

RETN. = Retention, INVN. = Inversion, RACM. = Racemisation.

integration of the overlapping system and subsequent 'best fit' calculations). The full details of this calculation will appear later.

We can thus see that since isotopic racemisation has occurred, we cannot use DCCI to facilitate a stereospecific ring closure reaction. However, these results may have interesting implications in the area of the mechanism of action of carbodiimide reactions. A comprehensive review of the carbodiimide area has been published by Khorana[289], and is too detailed to be entered into here. Suffice it to say that it has always been thought likely that carbodiimide reactions resulting in cyclic products may go *via* an intermediate pyrophosphate which is further activated in some way and breaks down to give the cyclic compound and another fragment which is recycled. Because of the poor nucleophilicity of a hydroxyl group relative to another phosphate monoester anion the first step of the reaction is probably the formation of the symmetrical pyrophosphate (180) of the phosphomonoester which must then be

further activated by reaction with carbodiimide. The nature of this activated intermediate is not certain but it may be a monomeric metaphosphate (181) as shown in FIGURE 95 which could torsionally equilibrate and thus readily lose stereochemical information. Extensive scrambling of label, however, cannot take place since there is virtually no loss of label when the synthesis is performed with one label, and there is no evidence from the n.m.r. spectrum of any doubly labelled material. In any event this evidence obviously demonstrates that the straightforward type of ring closure reaction *via* the activated intermediate (179) illustrated in FIGURE 96 does not occur, and implies a pyrophosphate

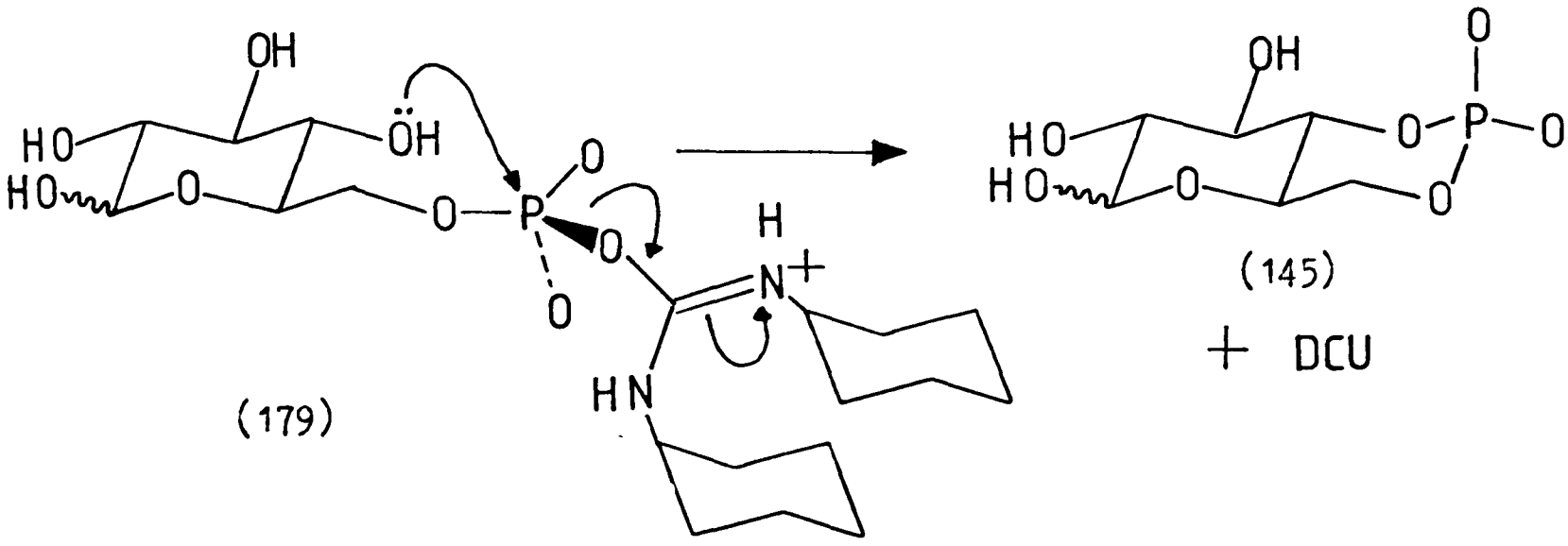


FIGURE 96

intermediate which probably breaks down *via* a metaphosphate. Further work is being carried out in this area - obviously a useful experiment would be to perform the reaction with only half an equivalent of DCCI to try and trap the pyrophosphate which cannot be further activated.

The Cyclisation of *D*-Glucose-6-phosphate using a Michelson-Type Precursor

With the failure of the DCCI method as a *stereospecific* ring closure reaction a better procedure was sought. It seemed prudent to look for a method which would permit quantitative formation of

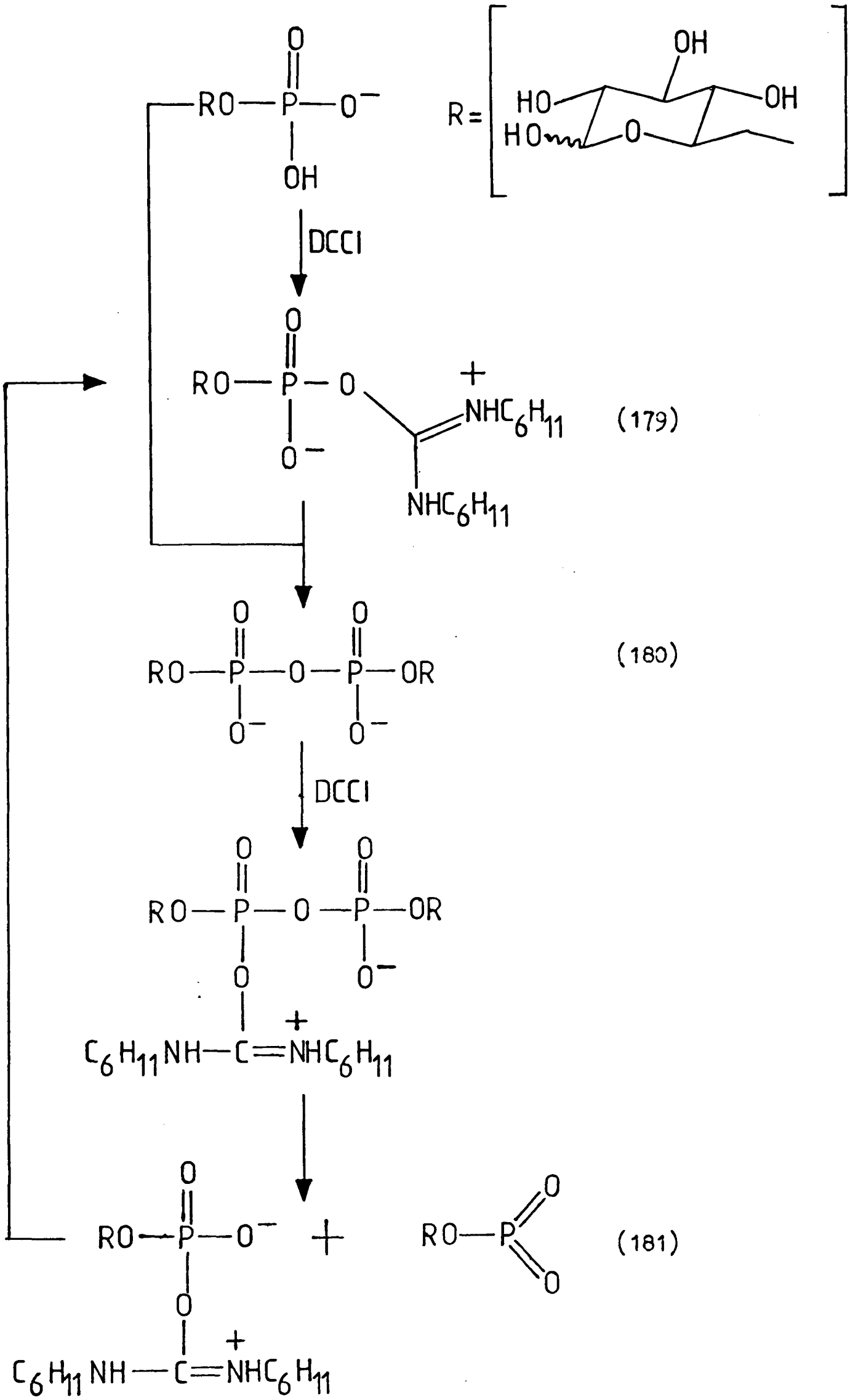


FIGURE 95 : A Proposed Mechanism for the Reaction of a Phosphate Monoester with DCCI (after Khorana [289]).

a cyclisable intermediate, which would be stable to attack from the precursor *D*-glucose-6-phosphate, *before* attempting the cyclisation reaction thus avoiding the difficulties of the DCCI system. The stability of any diglucose-6 pyrophosphate which might be formed would also be desirable under the formation and cyclisation conditions.

From the work of Borden and Smith[281] (a brief mention of applicability to the cyclisation of AMP) a sensible choice of procedure embodying all these aspects seemed to present itself in their adaptation of the classic Michelson method[290] for the activation of nucleoside monophosphate esters using diphenyl phosphorochloridate (182). It had been shown by Michelson that

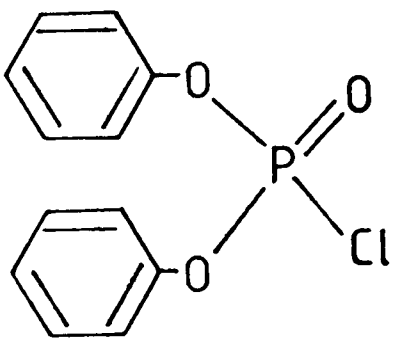


FIGURE 97

(182)

reaction of nucleoside-5'-monophosphates, as their suitable sterically hindered base salts, with diphenylphosphorochloridate (182) in mixtures of dioxan/DMF gives quantitatively the P_1-nucleoside-5'-P_2-diphenyl pyrophosphates (*e.g.* (183) for AMP[290]) which as demonstrated by Borden and Smith, at least for AMP[281], can be cyclised to the 3'5'-cyclic phosphate in reasonable yield by the use of a strong base such as *t*-butoxide in DMSO.

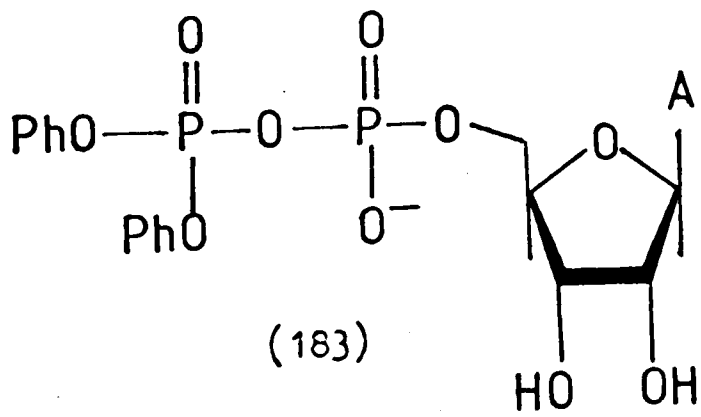
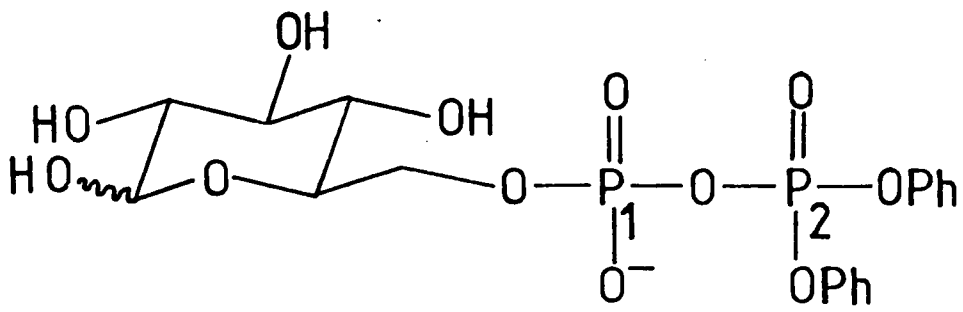


FIGURE 98

(183)

It was thus hoped to extend this procedure to the formation of *D*-glucose-4,6-cyclic-phosphate in a similar, if not easier fashion, since the 4-hydroxyl group of *D*-glucose-6-phosphate is much more favourably oriented towards displacement of a phosphate bound leaving group, being on a six-membered ring. Since it had already been shown that the formation of the mixed pyrophosphate (183) could be accomplished almost quantitatively in dry DMF[291], the formation of the corresponding P_1-(6-*D*-glucosyl)-P_2-diphenyl-pyrophosphate (184) was attempted by treating *D*-glucose-6-phosphate



(184)

FIGURE 99

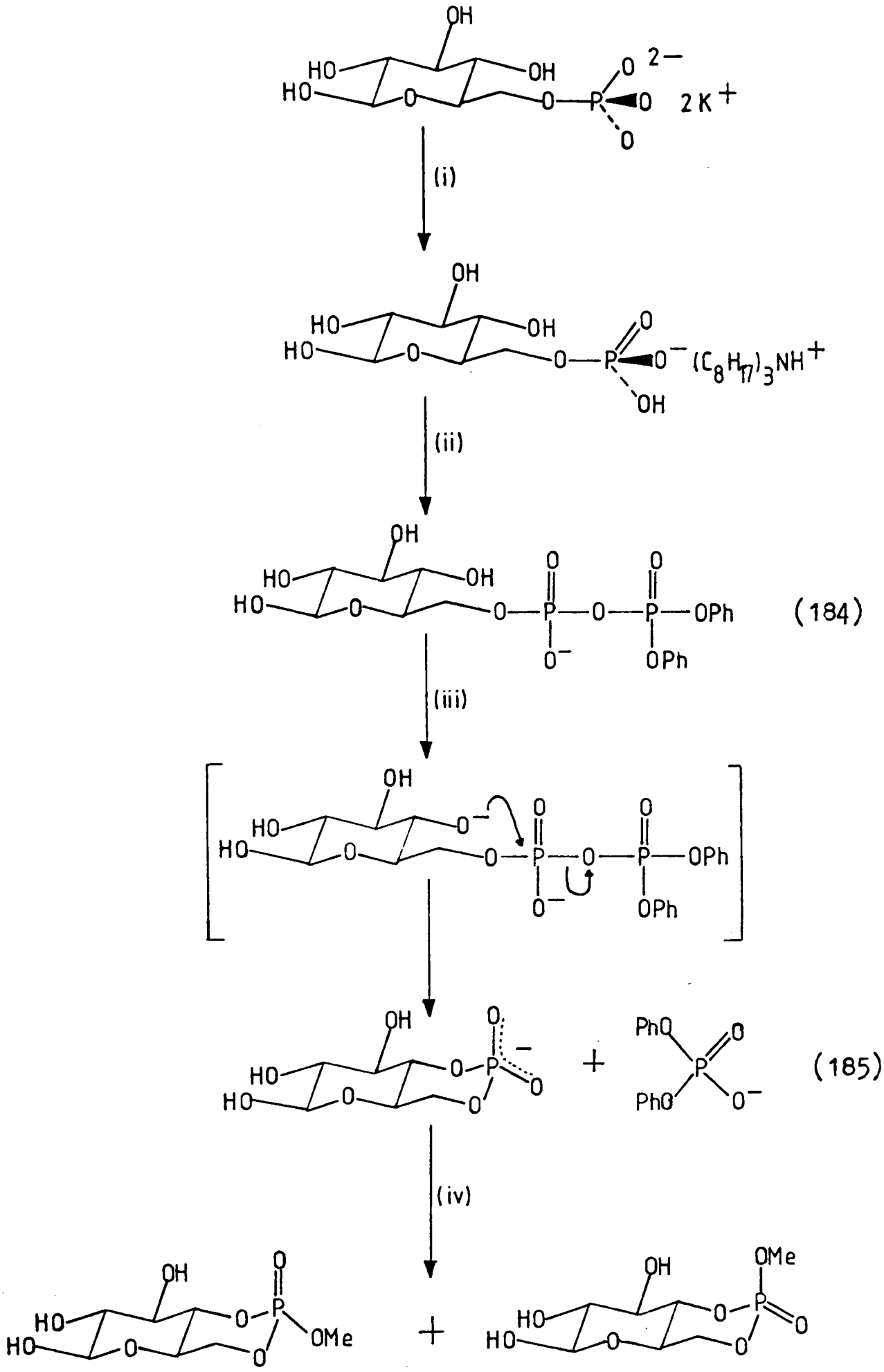
as its *mono*-tributylammonium salt in dry DMF with freshly distilled diphenyl phosphorochloridate dissolved in dry DMF.

Unfortunately when the reaction mixture was monitored by ^{31}P n.m.r. no resonances corresponding to (184) could be observed and the procedure was found to have resulted in an almost complete conversion to the symmetrical P_1P_2-diglucose-6-pyrophosphate which resonated as a sharp singlet at -15.3 ppm. Under these conditions the mixed pyrophosphate was obviously too reactive towards its precursor *mono*-anion. This differing behaviour to adenosine-5'-phosphate is not readily explicable. When the solvent system for the reaction was changed to a mixture of dry dioxan/DMF, however, some of the mixed pyrophosphate was formed as well as the symmetrical pyrophosphate as characterised by its ^{31}P n.m.r. spectrum which consisted of two doublets at -13.43 ppm and -13.55 ppm due to P_1 with $^2J_{P_1P_2}$ (mean) -18.39 Hz (the anomeric effect at C_1 on the glucose ring caused the additional splitting of 0.12 ppm, which was

not shown on P_2), and a doublet at -26.28 ppm with $J_{P_1P_2}$ = 17.65 Hz assigned to P_2 (the P_1 resonance was of relatively higher intensity as the result of a positive nuclear Overhauser enhancement). A considerable amount of symmetrical pyrophosphate was still formed, however, but this decreased as the dioxan:DMF ratio was increased, and was relatively small when the solvent contained 75% dioxan. However, this ratio proved to be the practical limit for this system since the *mono*-tributyl-ammonium *D*-glucose-6-phosphate was insoluble in mixtures containing less than 25% DMF. In an attempt to circumvent the problem of even relatively small quantities of symmetrical pyrophosphate being present in the reaction mixture at the next stage (despite the fact that Borden and Smith[211] had previously demonstrated the stability of P_1P_2-diadenosine-5' pyrophosphate to *t*-butoxide, since it did not give any cyclic products) the solubility of the more hydrophobic *mono*-tri-*n*-octyl-ammonium *D*-glucose-6-phosphate was investigated. Fortuitously this salt proved to be completely soluble (albeit difficultly) in dry dioxan and when the reaction with diphenylphosphorochloridate (182) was performed in this solvent a virtually quantitative yield of the mixed pyrophosphate (182) was obtained. During investigation of this system it was discovered that this pyrophosphate and *D*-glucose-6-phosphate enjoyed peaceful coexistence in dry dioxan.

Having formed the intermediate quantitatively in this solvent it was felt, however, that dioxan would not be a suitable choice in which to effect the desired base-catalysed cyclisation to *D*-glucose -4,6-cylic phosphate (DMSO or DMF are usually the solvents of choice.) Thus the reaction mixture was diluted with dry DMF (to a ratio of approximately 3:1 DMF:dioxan) before treatment with a freshly prepared solution of potassium *t*-butoxide in *t*-butanol, which was prepared by dissolving the required freshly cut weight of potassium metal, under strictly anhydrous conditions, in *t*-butanol

which had previously been freshly dried by distillation from calcium hydride under a nitrogen atmosphere. Dilution of the dioxan reaction mixture with DMF was followed rapidly by addition of the base to minimise possible decomposition of the P_1-*D*-glucose-6-P_2-diphenyl pyrophosphate (184) and resulted in an immediate precipitation of phosphorus-containing material. The supernatant after centrifugation of this mixture showed only diphenyl phosphate (185) which exhibited a characteristic ^{31}P n.m.r. resonance at -14.31 ppm and did not broaden on removing the proton decoupling. The precipitated material was shown by ^{31}P n.m.r. to consist of predominantly *D*-glucose-4,6-cyclic phosphate (145) which had been formed in *ca.* 60% yield and had a ^{31}P n.m.r. resonance at -5.8 ppm. Since this product was formed as the potassium salt it proved to be convenient to straightforwardly esterify the exocyclic phosphate oxygens using methyl iodide on the K^+-18-crown-6 complex salt according to the previously described procedure. The use of neat DMSO for this procedure resulted in the axial and equatorial isomers being formed in the ratio of approx 1:2 as judged by the observation of ^{31}P n.m.r. resonances at -8.56 ppm and -6.43 ppm respectively. The ^{31}P n.m.r. lines in DMSO, however, were rather broad, presumably due to only small differences in the anomeric ^{31}P n.m.r. chemical shifts (0.73 Hz for the anomers of the axial isomer and 1.22 Hz for the anomers of equatorial isomer at 36.43 MHz). This would make the observation of subtle isotope effects extremely difficult to observe, but it was fortunately discovered that the problem could be overcome by adding methanol - the higher the methanol/DMSO ratio the greater the anomeric differentiation. Thus in 50/50 methanol/DMSO the equatorial anomeric separation had become 1.46 Hz and the axial separation 2.8 Hz at 36.43 MHz. Thus the complete conditions for the synthesis of the glucose-4,6-cyclic phosphate methyl esters using this new cyclisation method are set out in SCHEME 17.



Reagents : (i) Dowex 50W-H^+/$(C_8H_{17})_3N$-1 equiv.

(ii) $(PhO)_2POCl$ in dioxan — then Bu_3N

(iii) BuOK in DMF

(iv) MeI-18-Crown-6 in DMSO

SCHEME 17 : The Cyclisation of D-Glucose-6-Phosphate using a Michelson-Type Precursor.

The Stereochemical Course of Cyclisation of *D*-glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate

When this procedure was carried out with *D*-glucose-6-[^{18}O]-phosphate, the synthesis of which has already been described, the ^{31}P n.m.r. spectra illustrated in FIGURES 100a,b were obtained.

Once again we see from the spectra that cyclisation has occurred ostensibly without great label loss (probably about 4%) and the spectra are in complete accord with expectation. The equatorial anomers are nicely resolved and all the expected lines are seen. Thus we were now in a position to repeat the diphenylphosphorochloridate-*t*-butoxide cyclisation with chirally labelled *D*-glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate (the same material was used as for the DCCI cyclisation) and when this was performed the spectra illustrated in FIGURES 101a,b were obtained. This time we immediately see that we can derive stereochemical information from these results since the two triesters containing only one label possess different intensities.

The stereochemical consequences of cyclising *D*-glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate with inversion and retention of isotopic configuration are illustrated in FIGURE 102. It is easy to see on inspection that the most intense resonances are those corresponding to (192) and (195) in the figure and thus the cyclisation has proceeded with the expected *inversion* of configuration. Since the precursor was the same as that used in the DCCI ring closure we can perform the same calculation to compare the observed peak intensities with those expected for either stereochemical outcome. The results are presented in TABLE 11.

In this laboratory Mr. R. Jarvest has performed the same sequence of reactions on adenosine-5'[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate, prepared as described previously. The ^{31}P n.m.r. spectrum is shown for completeness in FIGURE 103, and the assignments are illustrated on

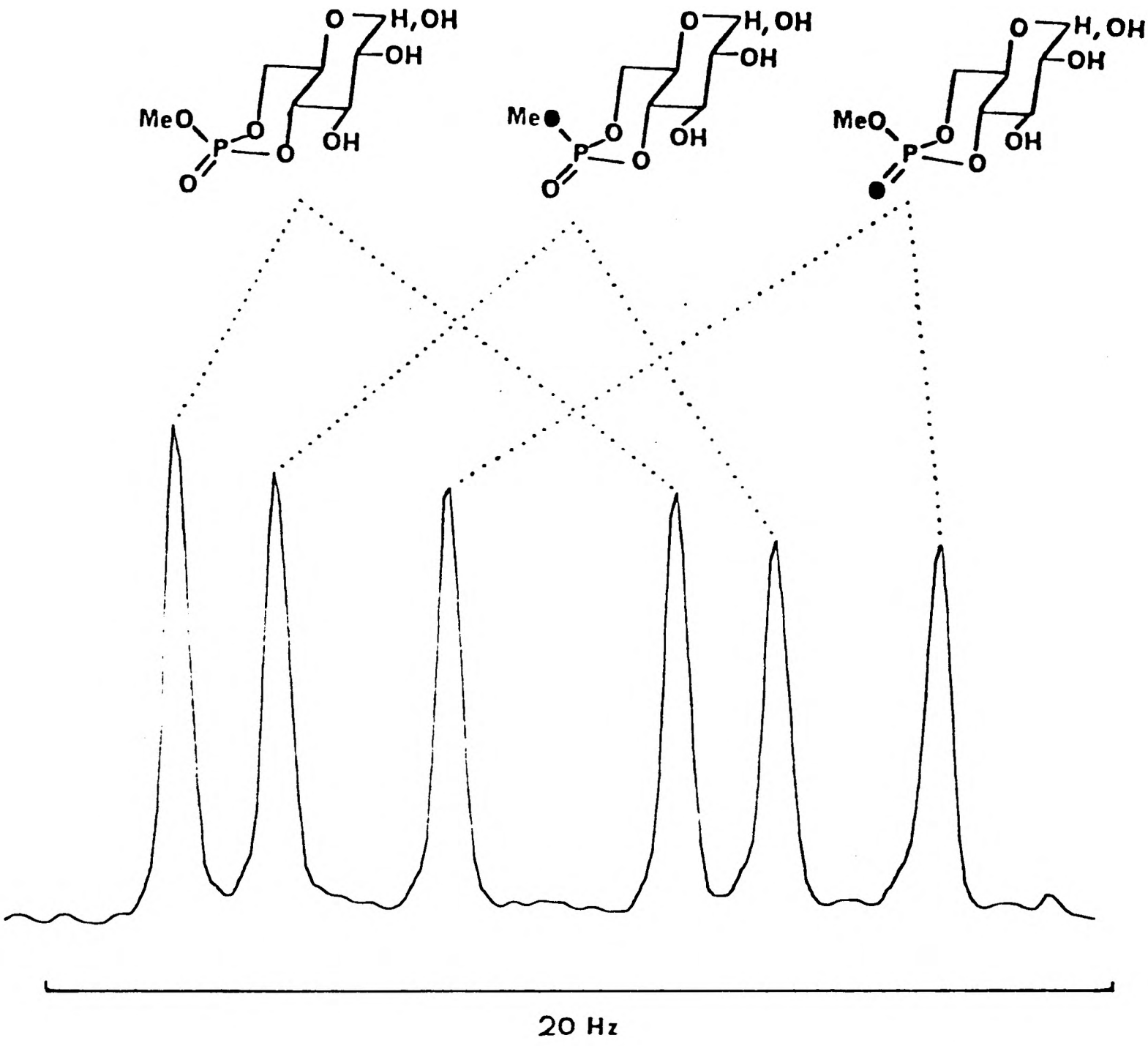


FIGURE 100 (a) : The 121.5 MHz ^{31}P n.m.r. Spectrum of the Axial Diastereoisomer of D-Glucose-4,6[^{18}O]-Cyclic Phosphate Methyl Ester Derived from the Cyclisation of D-Glucose-6[^{18}O]-Phosphate with DPPC-BuOK. The ^{31}P n.m.r. parameters were identical to those in FIGURE 94.

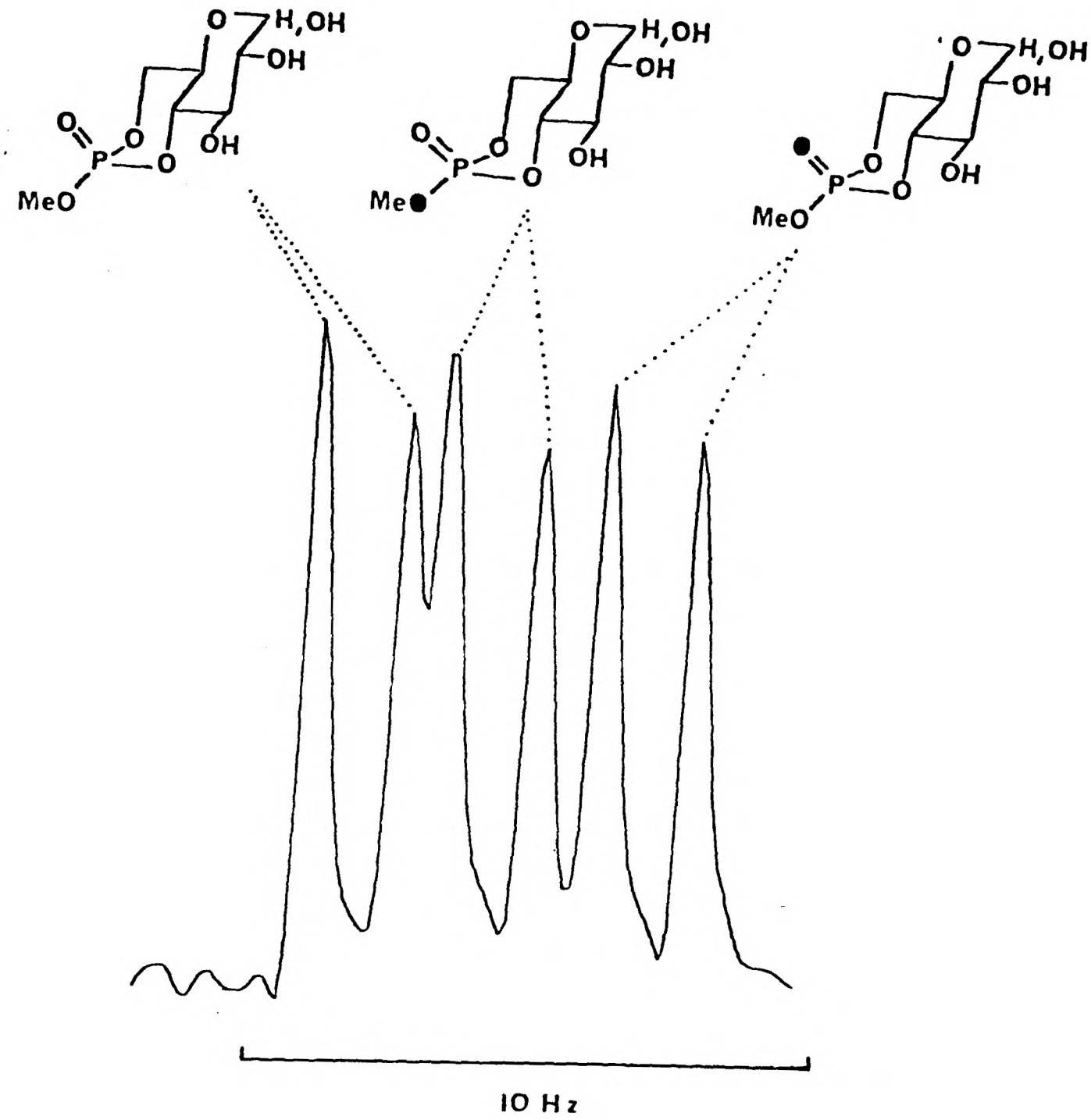


FIGURE 100 (b) : The 121.5 MHz ^{31}P n.m.r. Spectrum of the Equatorial Diastereoisomer as in FIGURE 100 (a), The vertical gain control for this ester is four times that for the axial ester.

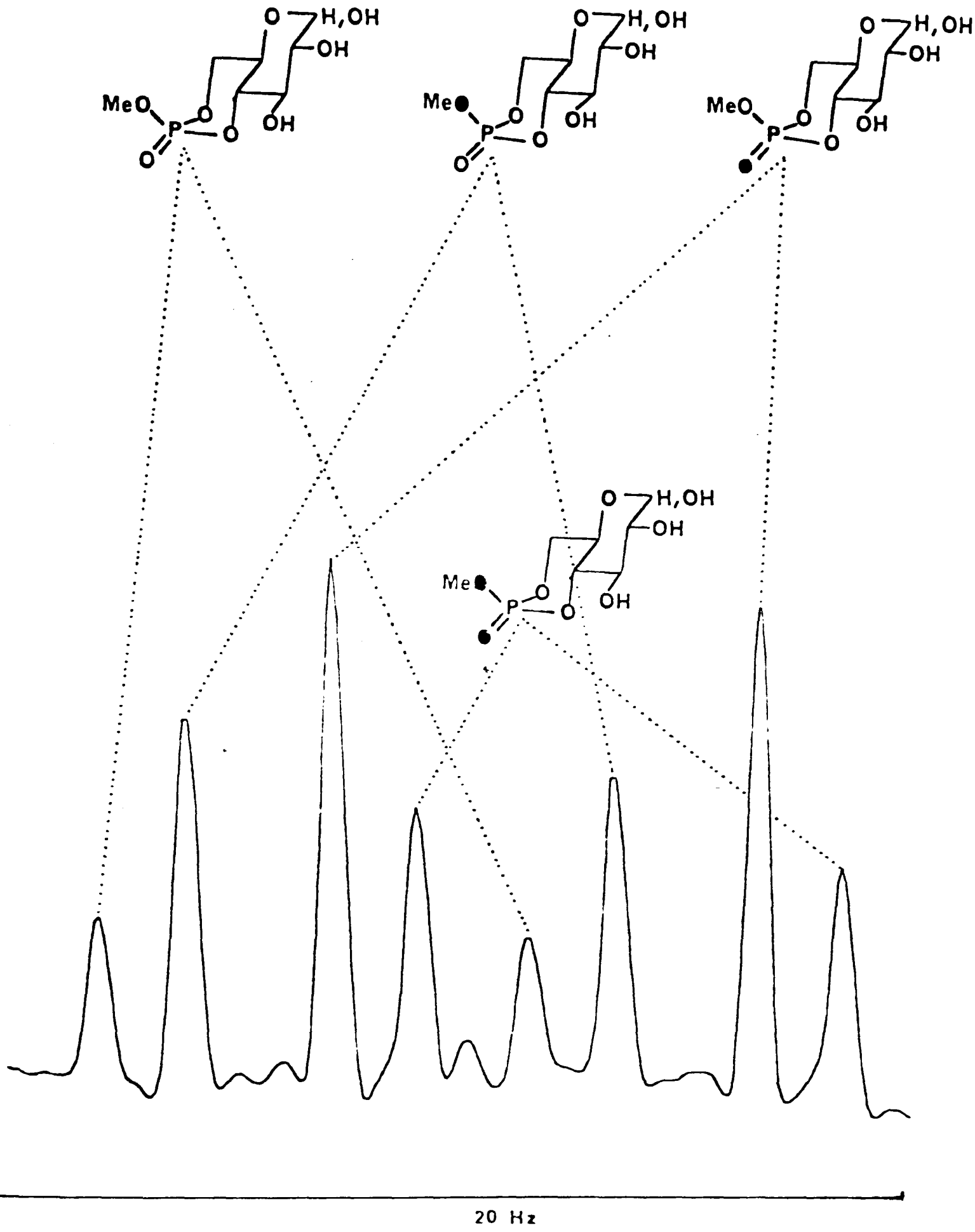


FIGURE 101 (a) : The 121.5 MHz ^{31}P n.m.r. Spectrum of the Axial Diastereoisomer of D-Glucose-4,6[^{16}O, ^{17}O, ^{18}O]-Cyclic-Phosphate Methyl Ester Derived from the DPPC-BuOK Cyclisation of D-Glucose-6[(S)-^{16}O, ^{17}O, ^{18}O]-Phosphate. The isotope shifts were 1.83 Hz ($Me^{18}OP$) and 4.95 Hz ($MeOP{=}O^{18}$), and the ^{31}P n.m.r. parameters were identical to those in FIGURE 94

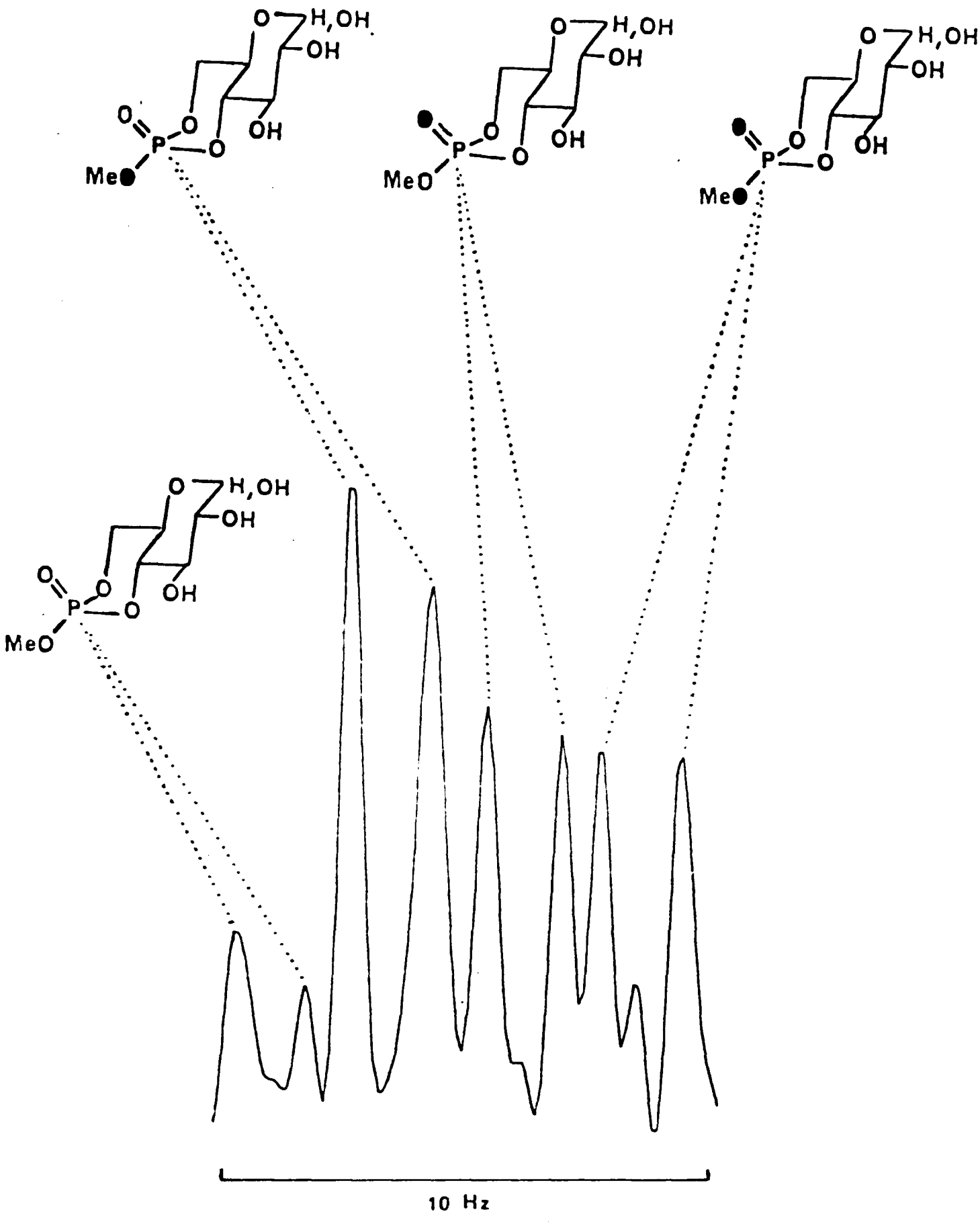


FIGURE 101 (b): The 121.5 MHz ^{31}P n.m.r. Spectrum of the Equatorial Diastereoisomer as in FIGURE 101 (a). The vertical gain control for this ester is sixteen times that of the axial ester. The isotope shifts are 2.26 Hz ($Me^{18}OP$) and 5.00 Hz ($MeOP{=}O^{18}$)

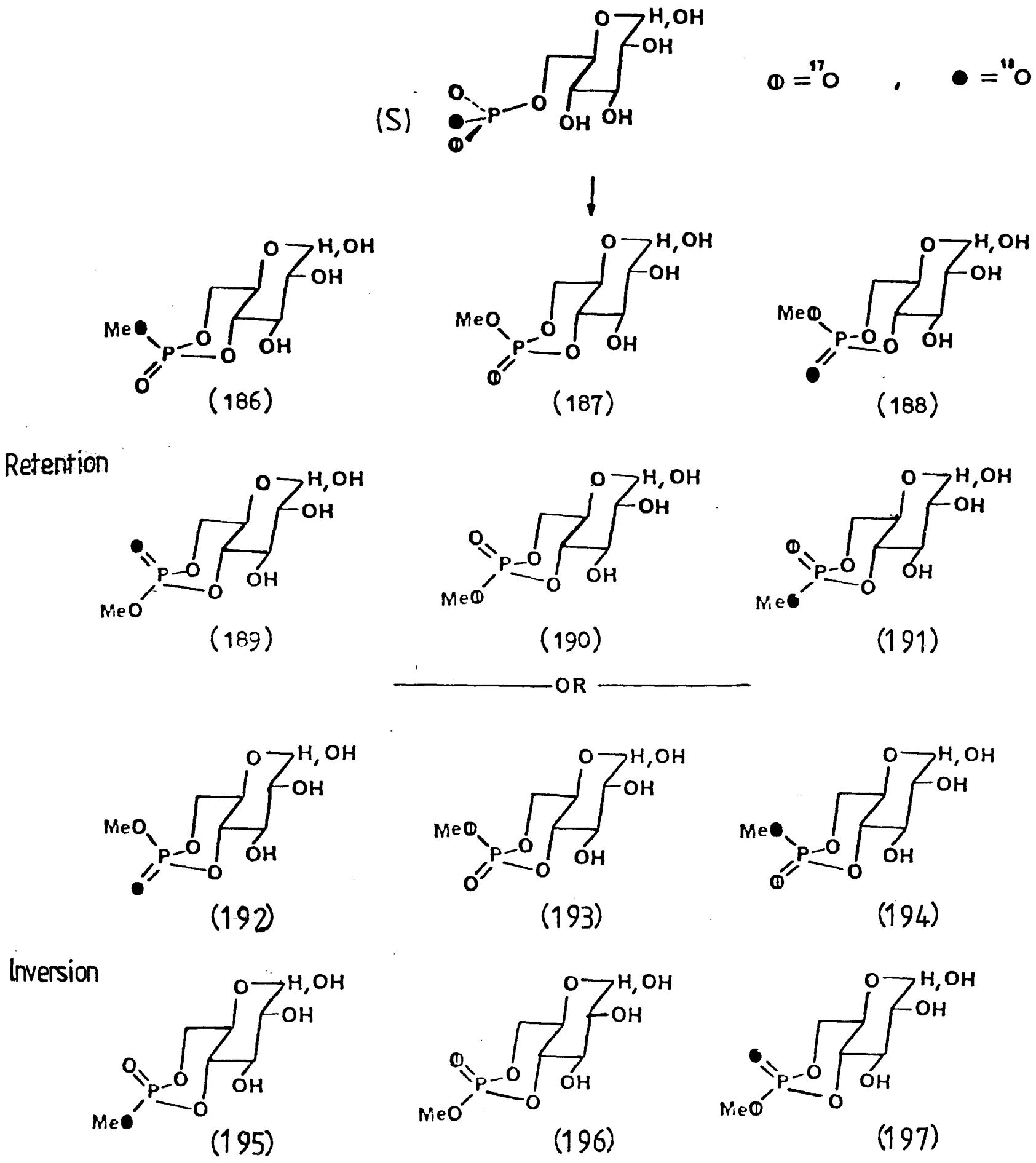


FIGURE 102 : The Stereochemical Consequences of Cyclising D-Glucose-6[(S)-^{16}O, ^{17}O, ^{18}O]-Phosphate with Inversion and Retention of Configuration at Phosphorus.

TABLE 11: A COMPARISON BETWEEN THE OBSERVED AND CALCULATED N.M.R. INTENSITIES FOR THE DPPC-BuOK CYCLISATION OF *D*-GLUCOSE-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-PHOSPHATE

	Axial Triester			Equatorial Triester		
	OBS.	Calculated		OBS.	Calculated	
		RETN.	INVN.		RETN.	INVN.
MeO-P=O	too inaccurate	0.29	0.29	0.41	0.29	0.29
Me●-P=O	1.00	1.00	0.75	0.76	0.75	1.00
MeO-P=●	0.72	0.75	1.00	1.00	1.00	0.75
Me●-P=●	0.65	0.49	0.49	0.62	0.49	0.49

RETN. = Retention, INVN. = Inversion.

the spectrum. The axial ester is assigned to the high field group of signals as before[277,278]; the configuration of the axial ethyl ester of adenosine-3',5'-cyclic phosphate has been confirmed by X-ray crystallography.[291] The absence of anomers of course simplifies the spectrum. Taking into consideration the same factors as for the *D*-glucose system, a similar calculation can be performed and gives almost identical results. Again it is seen that the cyclisation has occurred with *inversion* of configuration. In both these cases a reasonable value for the stereoselectivity of the cyclisation step can be obtained from the ratio of the two *mono*-^{18}O triesters, compared with the calculated value and in both cases this value is probably in excess of 90%.

The cyclisation of *D*-glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate and adenosine-5'[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate with inversion of configuration was anticipated since there is evidence that the cyclisation to five-membered phosphorothioate esters[206] and six-membered phosphoramidates[293] occurs with inversion of

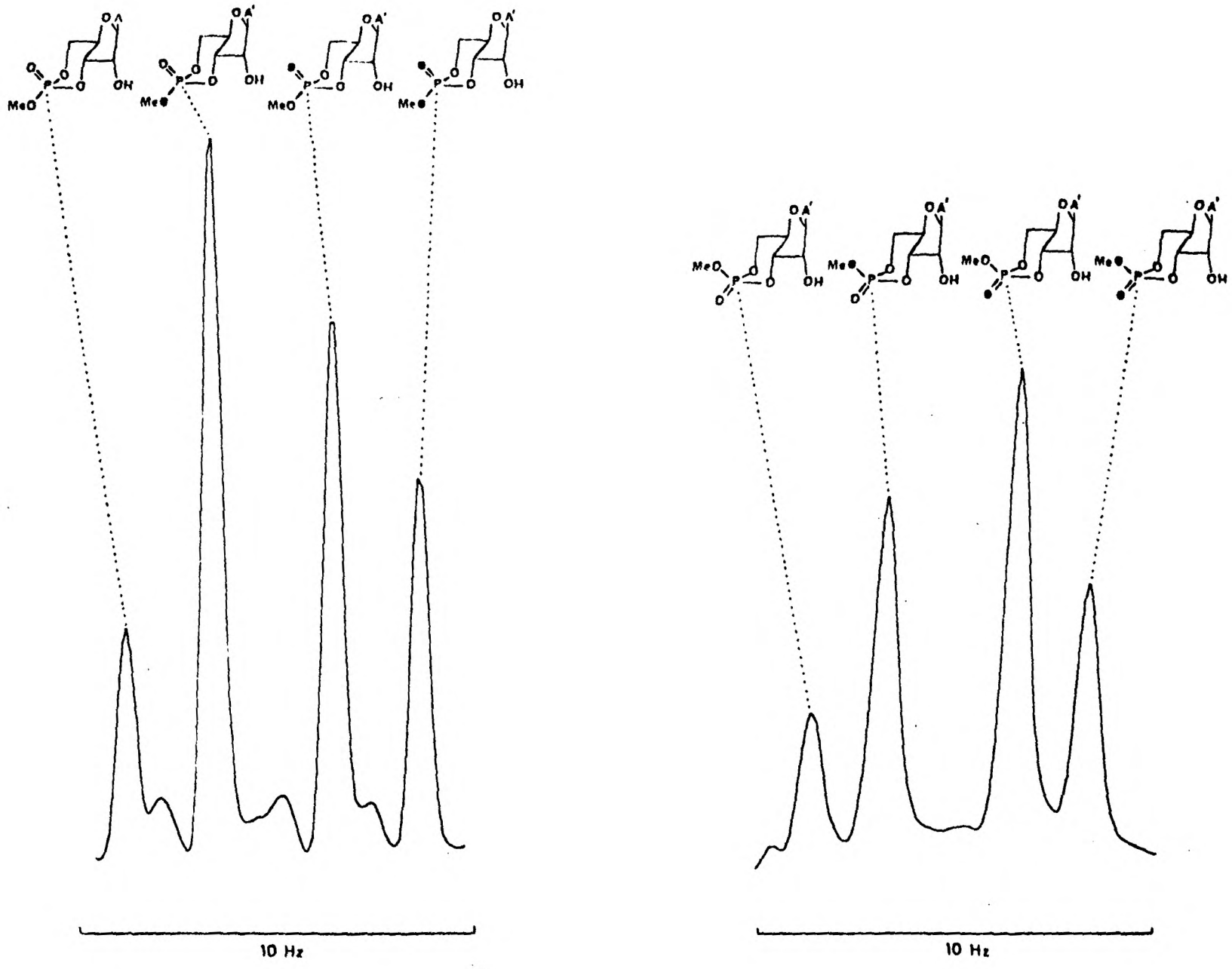


FIGURE 103 : The 121.5 MHz ^{31}P n.m.r. Spectrum in 1:1 DMSO-d_6:DMSO of the Equatorial and Axial Diastereoisomers Derived by the Cyclisation and Methylation of Adenosine-5' (S)-^{16}O, ^{17}O, ^{18}O -Phosphate. For the equatorial triester the isotope shifts are 2.1 Hz ($Me^{18}OP$) and 5.2 Hz ($MeOP{=}O^{18}$), and for the axial triester 1.8Hz and 5.1 Hz respectively. The gain control for the axial triester is half that of the equatorial triester. A'=N^1-methyl adenine. ^{31}P n.m.r. Parameters were similar to FIGURE 94.

configuration at phosphorus. The stereochemical course of the cyclisation is illustrated in FIGURE 104 for the case where an ^{17}O label is lost from an isotopic (*S*) precursor configuration.

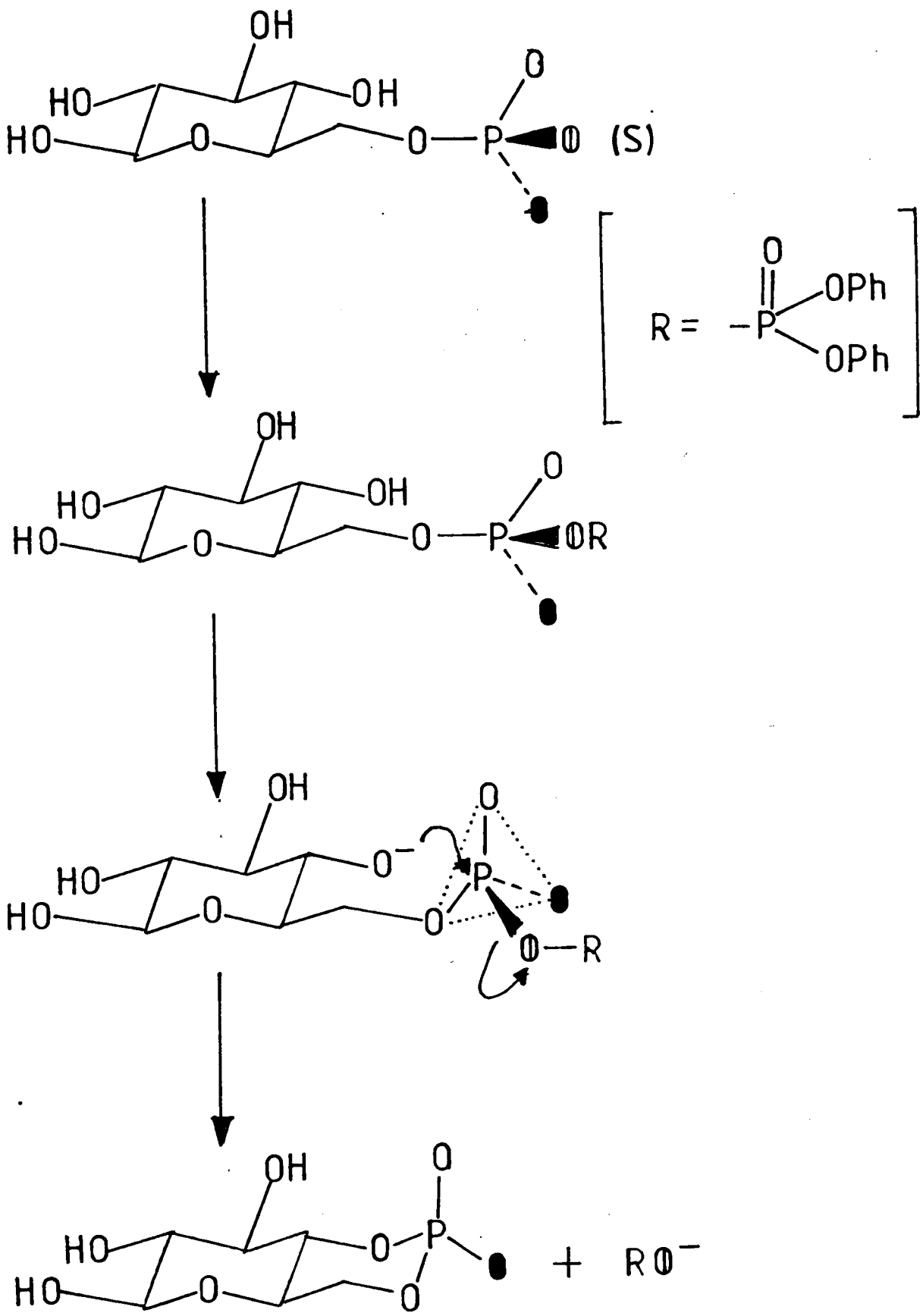


FIGURE 104

With this successful determination of the ring closure stereochemistry we now have a rigorous method for determining the stereochemistry of either *D*-glucose-6[^{16}O, ^{17}O, ^{18}O]-phosphate or

adenosine-5'[^{16}O, ^{17}O, ^{18}O]-phosphate derived from enzyme reactions This method has been published[216], and its application to the determination of the stereochemistry of enzyme reactions will be discussed in later chapters.

CHAPTER 10

SOME OBSERVATIONS AND CALCULATIONS IN THE SYNTHESIS OF ISOTOPICALLY LABELLED PHOSPHATE ESTERS

A Modified Synthesis of (1*R*,2*S*)-[1-^{18}O]-1,2-dihydroxy-1,2-diphenylethane

A synthesis of (1*R*,2*S*)-1,2-dihydroxy-1,2-diphenylethane (*meso*-hydrobenzoin) (198) *via* a new synthesis of (2*S*)-hydroxy 1,2-diphenylethanone (199) has been recently reported by Cullis and Lowe[226,255], and this has been used to synthesise [1-^{18}O]-(2*S*)-1,2-diphenylethanone (200) and (1*R*,2*S*)-[1-^{18}O]-1,2-dihydroxy-1,2 diphenylethane (201). The latter compound is the key to the

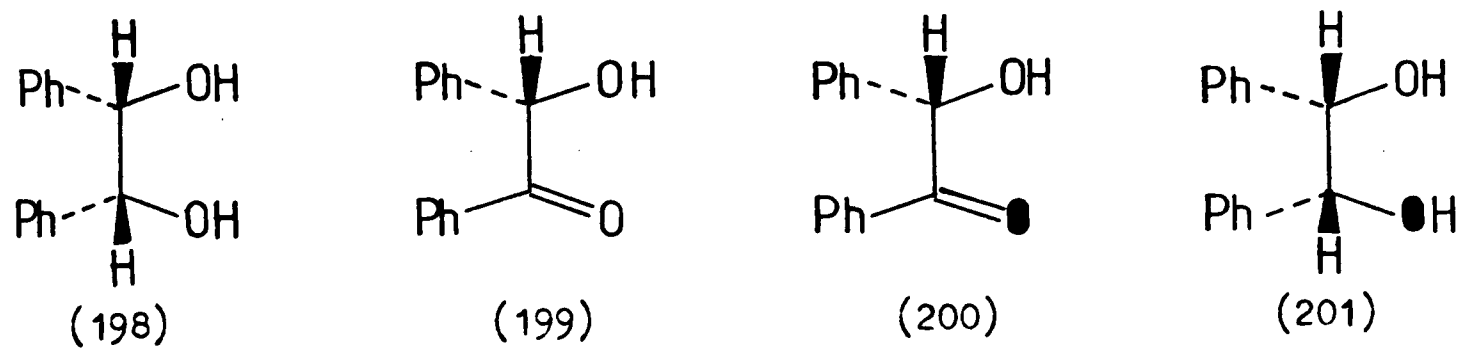
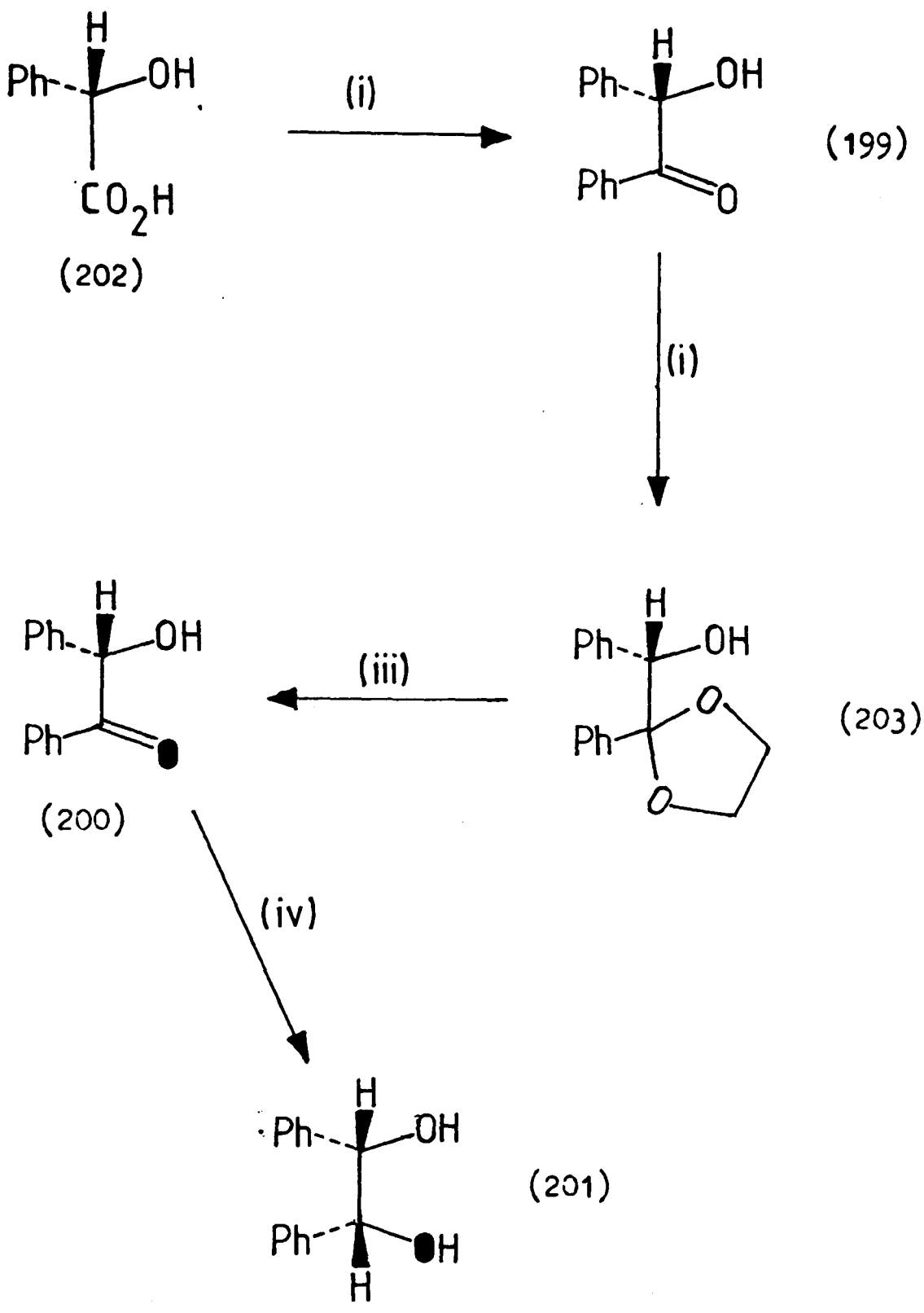


FIGURE 105

synthesis of chirally labelled [^{16}O, ^{17}O, ^{18}O]-phosphate monoesters, indeed its preparation is the only aspect of the whole synthesis[226,255] for which there is no literature precedent. This compound is synthesised according to SCHEME 18. Thus (2*S*)-mandelic acid (202) is converted to (*S*)-benzoin (199) which is ketalised with ethylene glycol to the ketal (203). Acid-catalysed ring opening in H_2 ^{18}O/dioxan gives the labelled benzoin (201).

When this synthesis was repeated using the conditions of Cullis and Lowe and the resulting [^{18}O]-labelled diol (201) used to prepare adenosine-5'[γ-^{16}O, ^{17}O, ^{18}O] triphosphate enzymically to determine the stereochemistry of the pyruvate kinase reaction the product, when examined by ^{31}P n.m.r. spectroscopy at 121.8 MHz (a facility not available to the previous authors), clearly exhibited resonances derived from species containing 0,1,2 and 3 labels on the γ-phosphorus. Whilst a measure of unlabelled and singly labelled species is always to be expected, the triply labelled product is not, and can only have arisen from diol which



Reagents: (i) PhLi

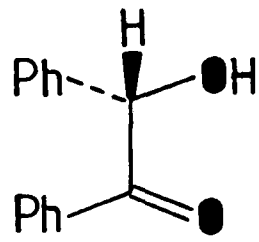
(ii) $HOCH_2CH_2OH$- p-TsOH

(iii) H_2●/dioxan- p-TsOH

(iv) $LiAlH_4$

SCHEME 18 : Synthesis of (1R, 2S)-[^{18}O]-1,2-Dihydroxy-1,2-Diphenylethane.

has both hydroxyl groups labelled, and will have thus arisen from the doubly labelled benzoin (204). Indeed examination of the



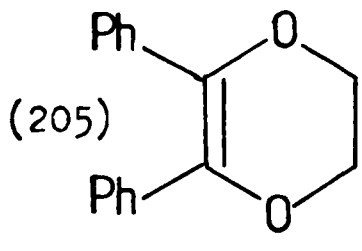
(204)

FIGURE 106

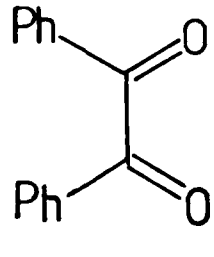
[^{18}O]-labelled benzoin and diol by field desorption mass spectrometry (the previous workers used only conventional mass spectrometry which can give ambiguous results) confirmed the presence of this species both in [^{18}O]-diol prepared in our hands by the previous method, and in samples of diol prepared by the original authors, although some batches appeared to be worse than others. It is obviously imperative to be able to prepare [^{18}O]-diol efficiently, reproducibly and specifically (one batch of this diol was prepared in our hands and used to prepare 'chirally labelled' *D*-glucose-6[^{16}O, ^{17}O, ^{18}O]-phosphate, which in preliminary experiment using the n.m.r. analysis method of CHAPTER 9 was shown to contain very significant levels of the triply labelled product, and be almost totally racemic - all of the isotopically labelled peaks at the triester stage were equal in height!). Thus a detailed investigation and reappraisal of the original conditions was carried out in collaboration with Mr. R. Jarvest using field desorption mass spectrometry and optical rotation studies to assess the quality of the products.

Summerbell[294] has demonstrated that the cyclic ether (205) may be prepared in very good yield from the benzoin ketal (203) by

FIGURE 107



(205)



(206)

refluxing in xylene with *p*-toluene-sulphonic acid and it appeared that this was a very likely candidate to blame for the racemisation problem. Indeed in preliminary studies on improving the system much evidence was gathered demonstrating the presence of compound (205) in the hydrolysis mixtures, as well as benzil (206) which could account for doubly labelled material.

In our improved synthesis of the diol (203) we retained the procedure for introduction of label *via* the ketal (203), although other methods were considered, and a very large number of small scale experimental conditions were tried in an attempt to optimise the specific incorporation of label and minimise racemisation. It would be tiresome to reproduce all these investigations here, so suffice it to say that we have finally found an efficient and *reproducible* synthesis of [^{18}O]-benzoin (200) by variation of the four parameters, solvent composition, quantity of acid catalyst, temperature of incubation - and time of incubation. Thus the original conditions of Cullis and Lowe have been considerably altered as set out in TABLE 12. From a large scale

TABLE 12: HYDROLYSIS CONDITIONS FOR LABELLING (2*S*)-BENZOIN KETAL

after Cullis and Lowe[226,255]		new conditions
$H_2{}^{18}O$	100 µl	100 µl
dioxan	500 µl	200 µl
ketal	130 mg	130 mg
tosic acid	1 mg	10 mg
incubation temp.	100 °C	85-95 °C
incubation time	24 h	3 h

synthesis using (*S*)-benzoin ketal prepared from (*S*)-benzoin with

$[\alpha]_D^{20} = 108^\circ$ (95.6% enantiomer excess) was obtained 97% labelled [^{18}O]-benzoin which exhibited an excellent field desorption mass spectrum and had $[\alpha]_D^{20} = +98.9^\circ$ [83.5% (*S*) enantiomer excess]. Another check on the integrity of this material was obtained from the ^{13}C n.m.r. spectrum, for ^{18}O has recently been demonstrated to also effect on isotope shift in ^{13}C n.m.r. spectroscopy.[295] Thus if we examine the carbon atom in benzoin which has the hydroxyl group attached we should see in the ^{13}C n.m.r. spectrum an upfield isotope shift proportional to the amount of undesired ^{18}O incorporation into this site. The spectrum is illustrated in FIGURE 108, and from the inset, which is an expansion of the required resonance, it can be seen that within the limits of error there is no incorporation of label into the hydroxyl site when our method of synthesis is used.

Our problem, however, was not yet solved since the next stage, the reduction of (*S*)-benzoin to the diol using lithium aluminium hydride by the method of Pohoryles *et al.*[296], was not found to give exclusively the *meso*-hydrobenzoin but rather a mixture of 85% (1*R*,2*S*)-hydrobenzoin and 15% (1*S*,2*S*)-hydrobenzoin as the crude reaction product. Three recystallisations from ethanol-water (as used by Cullis and Lowe[226,255]) reduced the (1*S*,2*S*)-hydrobenzoin content to 6% which was deemed to be acceptable although considerable diol had been lost by this procedure. The final contamination can be estimated by ^{1}H n.m.r. spectroscopy at 300 MHz. FIGURE 109 demonstrates the efficient resolution which can be achieved. In a subsequent preparation, recrystallisation from benzene was found to remove this contaminant much more efficiently. Pohoryles *et al.*[296] used this solvent for their recrystallisations and claim a yield of 90% *meso*-hydrobenzoin from their reduction. Therefore, they may merely not have noticed some 10% of the (1*S*,2*S*)-diol in their product before recrystallisation. When the [^{18}O]-labelled

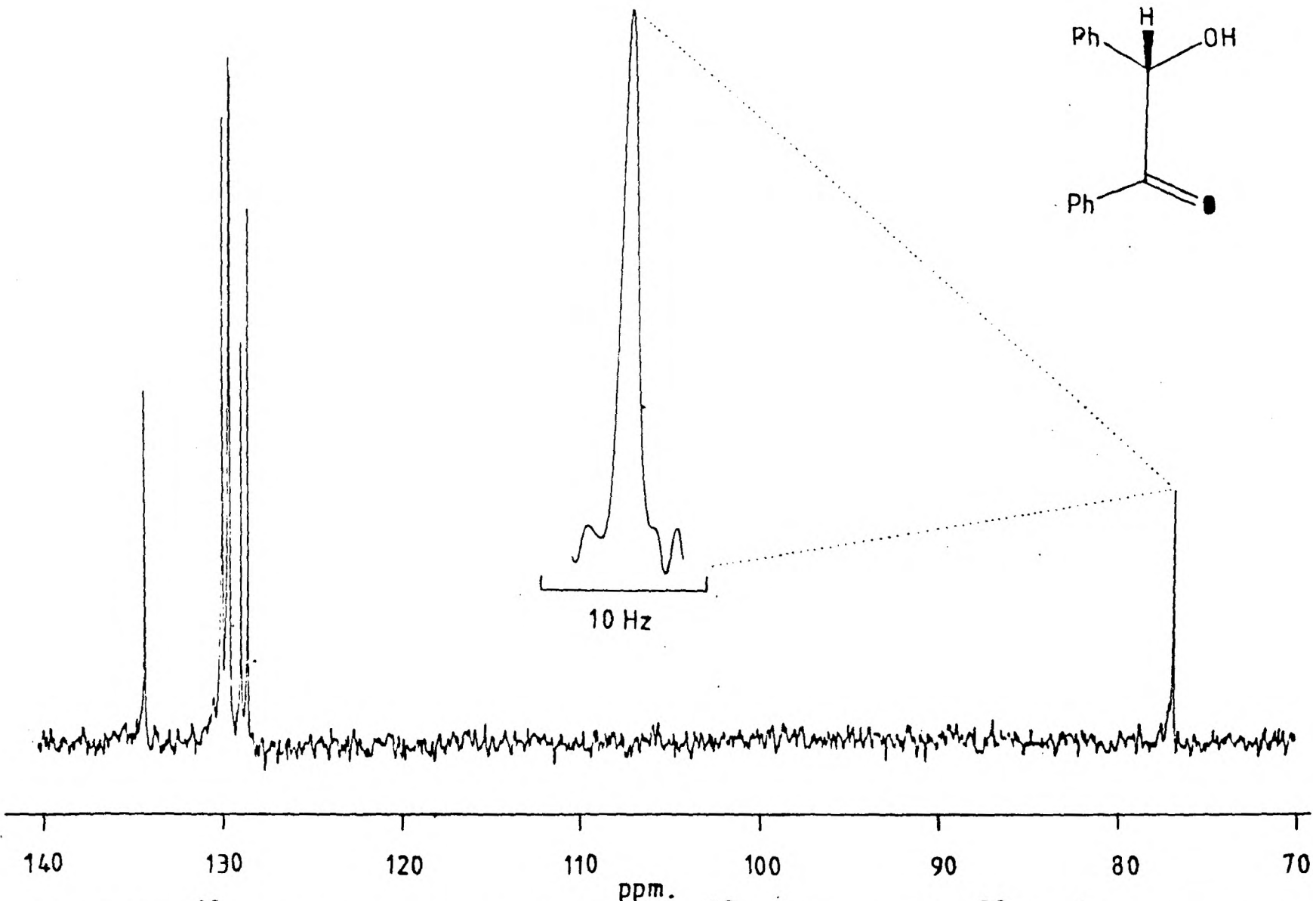


FIGURE 108 : The 75.47 MHz ^{13}C n.m.r. Spectrum of (S) -[1-^{18}O]-Benzoin ($[\alpha]_D^{20}=106^\circ$) in Deuterioacetone (resonance not shown). ^{13}C n.m.r. parameters were: offset, 19900 Hz; 02, 63600 Hz; sweep width 1858 Hz; pulse width, 20 μs; acquisition time, 0.84 s; gaussian multiplication (line broadening, -1.2 Hz; gaussian broadening 0.15 Hz) in 8K, FT in 32K; broad band proton decoupling. Scale referred to TMS

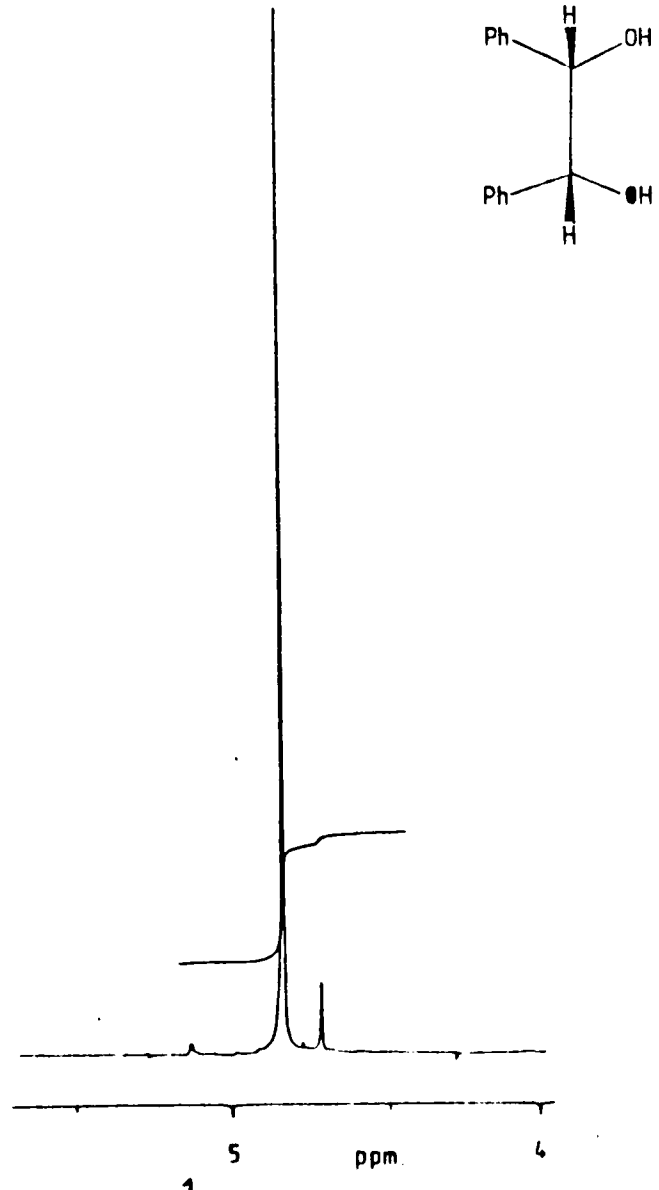


FIGURE 109 : The 300 MHz ^{1}H n.m.r. Spectrum of (201) showing the presence of the (1S, 2S)-isomer.

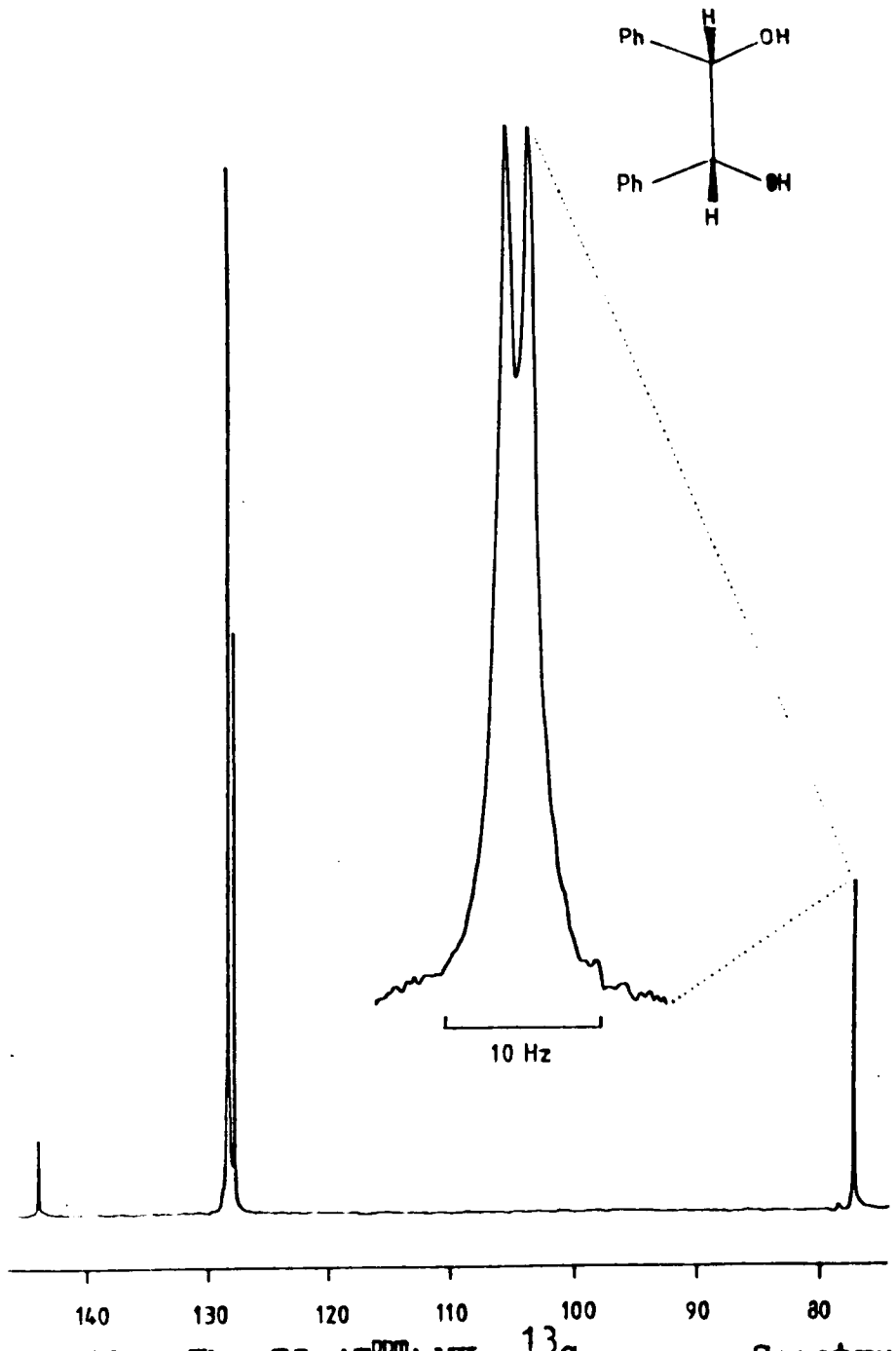
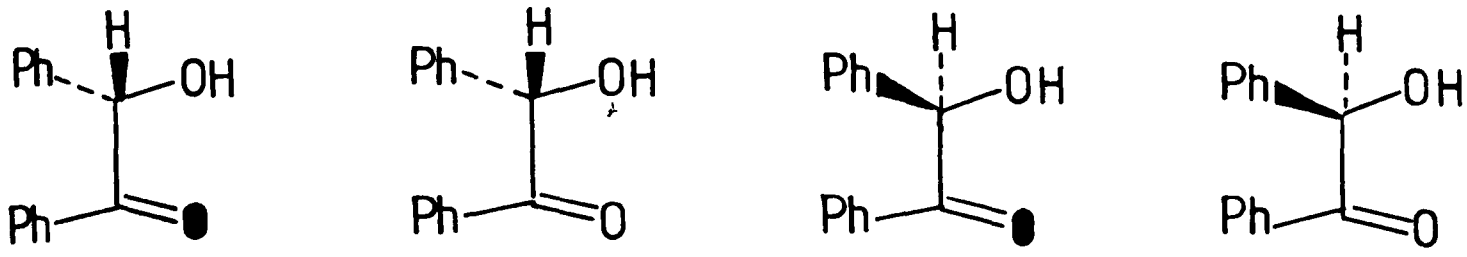


FIGURE 110 : The 75.47 ppm. MHz ^{13}C n.m.r. Spectrum of (201) in deuterioacetone. ^{13}C n.m.r. parameters were identical to FIGURE 108. Isotope shift 1.42 Hz

product of this reduction was examined by ^{13}C n.m.r. spectroscopy a beautiful spectrum was obtained (FIGURE 110) which illustrated nicely an ^{18}O isotope shift on carbon of 1.42 Hz thus confirming the labelling which was estimated to be better than 97% at the required centre. Thus the new isotope incorporation procedure coupled with recrystallisation of the diol from benzene results in a labelled product whose composition may be closely defined. The complicated mixture of labelled species resulting from all these procedures (including the subsequent use of the multiply labelled [^{17}O]-phosphorus oxychloride in the chiral phosphate synthesis) necessitates a detailed calculation in order to be able to define accurately the final composition of the resulting [^{16}O, ^{17}O, ^{18}O]-phosphate, and make some numerical estimations on the stereoselectivity of the cyclisation reaction and subsequent enzymic transfers.

Calculation of the Isotopic Composition of [^{16}O, ^{17}O, ^{18}O]-Phosphates, and the Resulting NMR Analysis Patterns

By mass spectrometry we find that the [^{17}O]-phosphorus oxychloride was labelled as follows 3.3% ^{16}O, 43.5% ^{17}O and 53.2% ^{18}O. From an independent experiment we estimate that at the triester stage in the synthesis there is *ca.* 5% exchange at the phosphoryl centre. Thus we may describe the phosphoryl centre in the final chiral phosphate as 8.14% ^{16}O, 41.32% ^{17}O and 50.54% ^{18}O The [^{18}O]-(2*S*)-benzoin had $[\alpha]_D^{20} = +96.3^\circ$ (c = 1, acetone). Since the rotation for 100% enantiomeric purity is 118.40[297] the enantiomer excess of the (*S*) isomer is therefore 81.3%, and therefore the actual composition of the benzoin is 90.65% (*S*) and 9.35% (*R*). We assume the benzoin to be 97% labelled, whence -

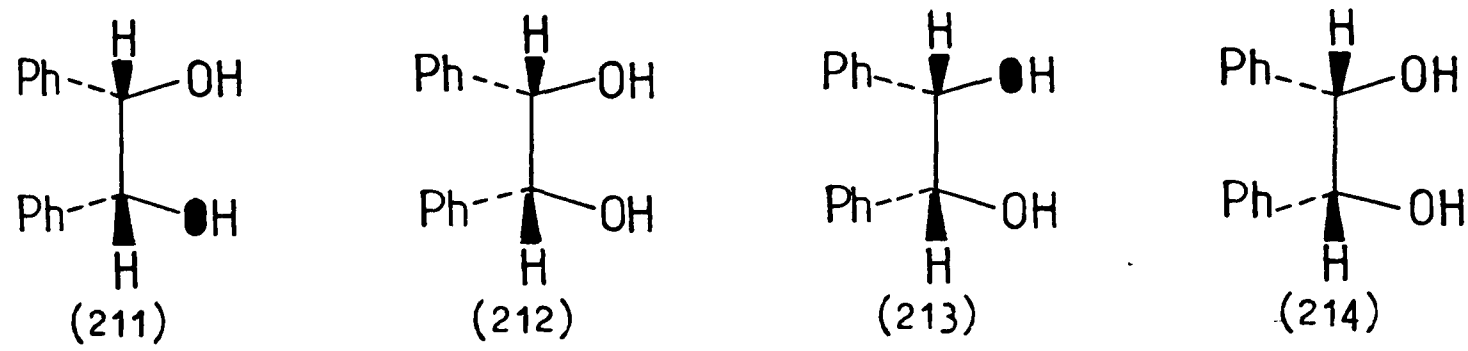


(207) (208) (209) (210)

(*R*) 90.65% (*S*) 9.35%

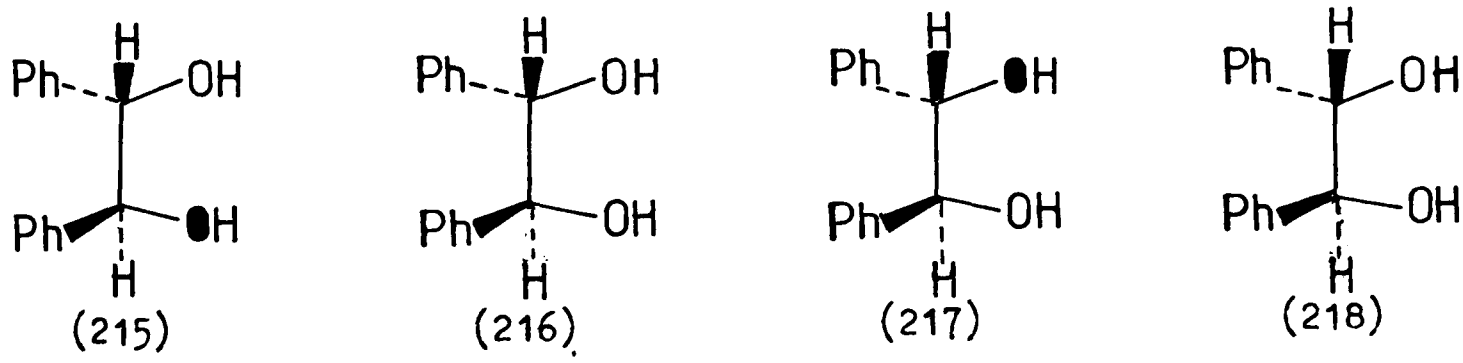
87.93% 2.72% 9.07% 0.28%

(All subsequent figures are % contributions). From the 300 MHz ^{1}H n.m.r. spectrum we know that there is *ca.* 6% (1*S*,2*S*)-hydrobenzoin, thus we have the following 8 diols after reduction (211)-(218).



(211) (212) (213) (214)

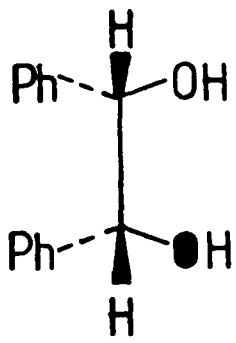
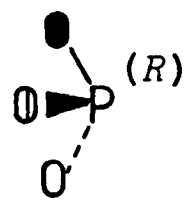
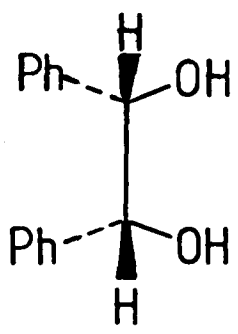
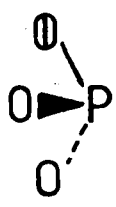
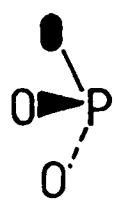
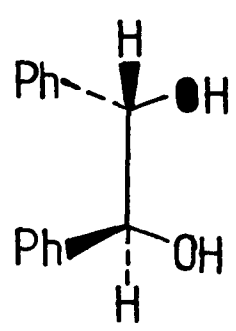
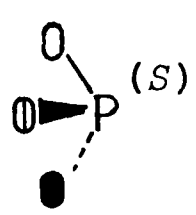
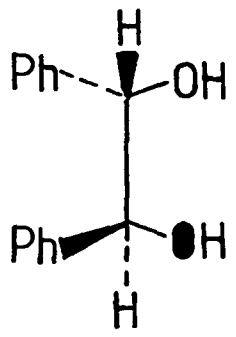
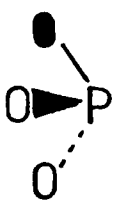
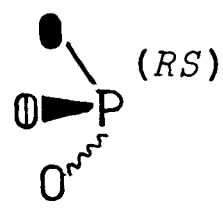
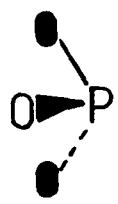
82.65 2.56 8.53 0.51



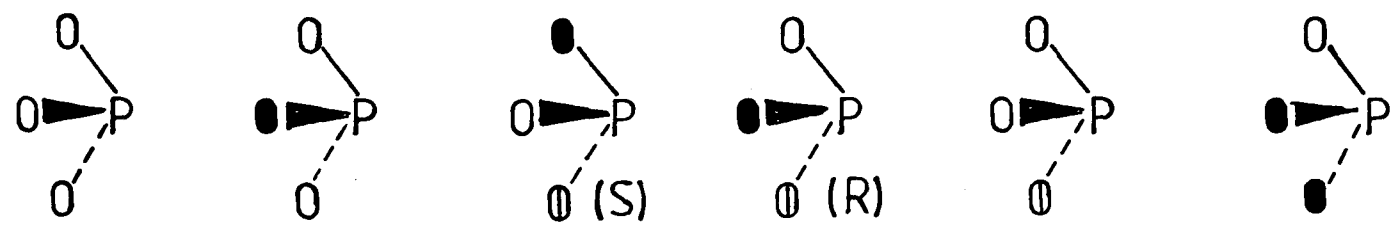
(215) (216) (217) (218)

5.28 0.16 0.54 0.02

Diols (212), (214), (216) and (218) will all contribute no labels. Whence, bringing in the phosphoryl centre we generate the following labelled phosphates.

	8.14% ^{16}O	41.32% ^{17}O	50.54% ^{18}O
			
	6.72	34.15	41.77
			
	0.27	1.34	1.64
			
	0.70	3.52	4.31
			
	0.43	2.18	2.67

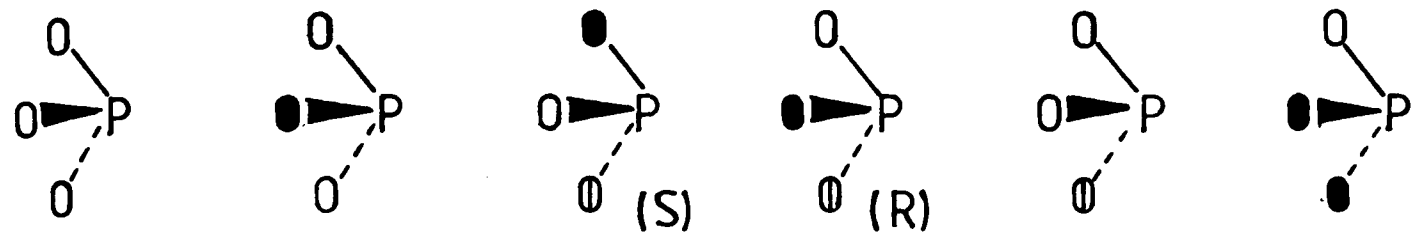
Whence we can summarise the species as follows:



0.27	9.49	35.23	4.60	1.34	48.75

Now an independent experiment indicated *ca.* 10% ring opening during hydrogenolysis, thus we lose 5% of label from the ring system to

give an achiral product, and also get 5% inversion of configuration at the chiral centres (assuming this ring opening to occur with inversion of configuration). Whence the distribution becomes:

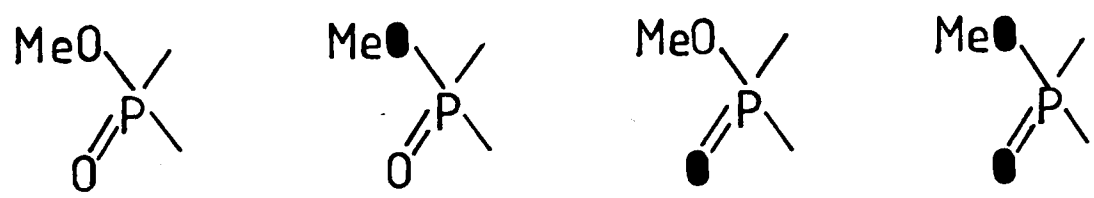


0.75 11.45 31.94 5.90 3.33 46.31

In the cyclisation reaction we estimate a 4% loss of label per site, thus the final distribution becomes

1.34 16.21 28.35 6.47 4.72 42.61

Now the final distribution of the cyclic triesters (for example the axial triester) can be calculated for a ring closure to the 4,6-cyclic phosphate proceeding with *e.g.* inversion of configuration, *ignoring those triesters containing* ^{17}O.



O_3POG	1.34			
$\bullet O_2POG$	5.40	5.40	5.40	
$O\bullet\Phi$ POG(R)		2.16		
$O\Phi\bullet$ POG(S)			9.45	
$O_2\Phi$ POG	1.57			
$O\bullet_2POG$		14.2	14.2	14.2
whence:	8.31	21.76	29.05	14.2

and normalising we have:

0.29	0.75	1.00	0.49

Thus for the axial triester (the central heights will of course be reversed for the equatorial triester) we predict the following line intensity pattern for the cyclisation with various stereochemical consequences.

0.29	:	1.00	:	0.75	:	0.49	RETENTION
0.29	:	0.75	:	1.00	:	0.49	INVERSION
0.33	:	1.00	:	1.00	:	0.56	RACEMISATION

Is There Oxygen Exchange at Ambient pH in Aqueous Solutions of *D*-Glucose-6-Phosphate Free Acid?

The possibility of acid-catalysed oxygen exchange from phosphate groups has always been a source of worry in the preparation of isotopically labelled phosphates. Since brief exposure to acidic conditions may occur during such syntheses and subsequent cyclisation procedures, it was thought expedient to try a simple test of whether this exchange occurs by seeing if we can exchange ^{18}O label *into* a phosphate group under such conditions. To this end *D*-glucose-6-phosphate monosodium salt was converted to the free acid by treatment with Dowex 50W (H^+-form). The gummy free acid was dissolved in 50/50 [^{18}O]-labelled water and incubated at ambient temperature overnight. The labelled water was removed by lyophilisation and the residual free acid dissolved in water and neutralized. Examination of the ^{31}P n.m.r. spectrum was not very informative at this stage and indicated two broad overlapping lines in the ratio of *ca.* 60:40. These lines were assigned to the α and β anomeric forms of the *D*-glucose-6-phosphate. The assignment of

the more intense downfield line to the β-anomer and the upfield line to the α-anomer has been reported by Battersby and Radda.[298] It would have been impossible to observe isotopic splittings under these conditions. The salt was converted to the *mono*-pyridinium salt and then to the MDCA salt which was cyclised with DCCI according to the procedure in CHAPTER 9. Methylation with diazomethane gave the axial and equatorial diastereoisomers which appeared as sharp lines in the 121.5 MHz ^{31}P n.m.r. spectrum and were clearly devoid of any isotopic splittings.

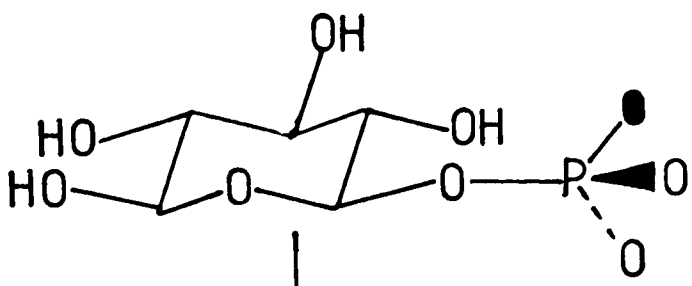
It can therefore be concluded that essentially no exchange of isotopic labels occurs under these conditions and it is safe to expose labelled phosphates to these kind of acidic conditions (this may not apply in the presence of *added* acid).

The Detection of the Extent of [^{18}O]-Labelling in *D*-Glucose-6-phosphates by n.m.r.

After the synthesis of an [^{18}O]-labelled monophosphate ester it is highly desirable to be able to determine the isotopic purity of the product especially if there is concern as to whether label may be lost in subsequent reactions (*e.g.* cyclisations). In the synthesis of these labelled phosphates the isotopic purity of the [^{18}O]-labelled phosphorus oxychloride may be simply and accurately determined by conversion to [^{18}O]-TMP followed by mass spectrometry. Of course with [^{18}O]-monophosphate esters it should be possible to achieve a direct estimation of isotopic purity by high field ^{31}P n.m.r. spectroscopy, performing a relative integration on any unlabelled material which will appear downfield of the main labelled peak. This has been previously described for cAMP (CHAPTER 9), but unfortunately is not possible for the glucose system because of the overlapping anomeric splittings (the α and β anomers have been reported to be separated by 2.45 Hz of 36.43 MHz and pH 7.0[298] but this is very pH dependent and in our hands has varied between

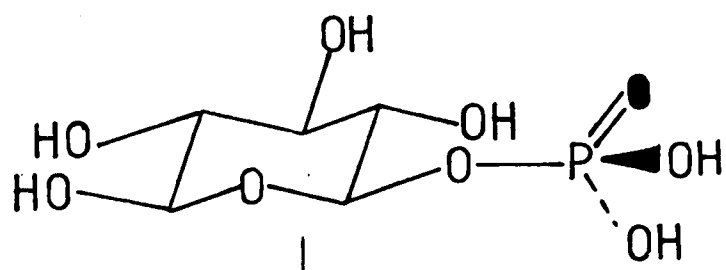
0.6 and 1.46 Hz - in any case the lines are too close together). Esterification of the phosphate oxygens, however, results in sharp well separated lines being observable for each anomer and any unlabelled material should therefore show up clearly. Thus *D*-glucose-6-phosphate *mono*-sodium salt was converted to the free acid by treatment with Dowex 50W (H^+ form), dissolved in dry methanol and heated with an ethereal solution of diazomethane to yield *D*-glucose-6-phosphate dimethyl ester, which showed two sharp resonances at -0.21 and -0.30 ppm in the ^{31}P n.m.r. spectrum. Despite the assignment of Battersby and Radda[298] of the α and β anomeric ^{31}P n.m.r. signals for the salt, this does not necessarily apply to the ester. However, as will be demonstrated, assignments were possible for the labelled compound where advantage was taken of the differing intensities of the two peaks, and ^{1}H n.m.r. data.

Thus in the same way *D*-glucose-6[^{18}O]-phosphate (175) was converted to the free acid and methylated to give the esters (219a-c) as shown in SCHEME 19. The 121.8 MHz ^{31}P n.m.r. spectrum is shown in FIGURE 111 with the assignments on the spectrum. This spectrum is explainable on the following basis. Since the peripheral oxygen atoms are all torsionally equivalent there is a 2 in 3 chance of an ^{18}O isotope ending up in a bridging position in the triester ((219a) and (219b)) and a 1 in 3 chance that it will be in a phosphoryl group (219c). Of course since bridging and phosphoryl labels can be distinguished by high field ^{31}P n.m.r. spectroscopy a 100% labelled sample should thus appear as a doublet of intensity ratio 2:1 for each anomer, the downfield line in each case being the most intense. Any unlabelled material would of course appear furthest downfield and integration of this peak relative to the other two will give the amount of unlabelled material present and thus enable any loss of label during the synthesis to be quantified. This spectrum was obtained from



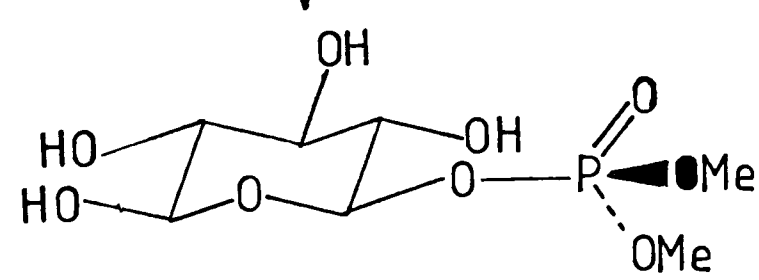
(175)

Dowex 50W (H^+)



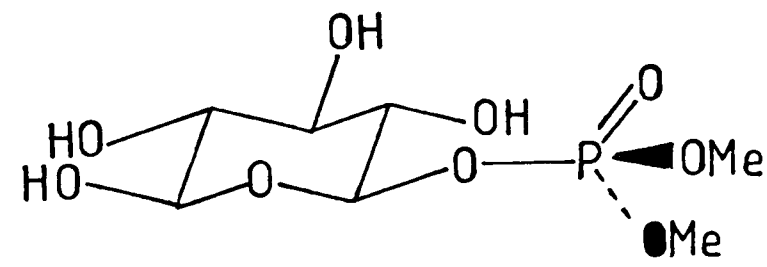
etc.

CH_2N_2/MeOH



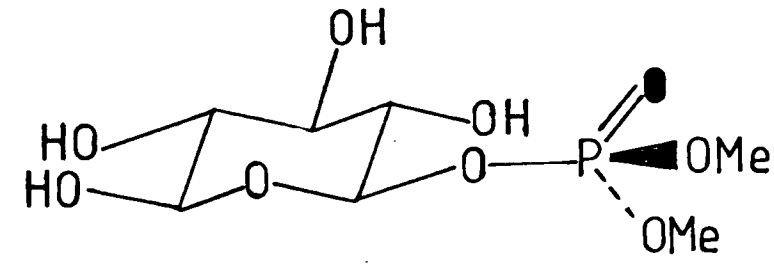
(219a)

+



(219b)

+



(219c)

SCHEME 19 : Synthesis of D-Glucose-6-[^{18}O]-Phosphate Dimethyl Ester.

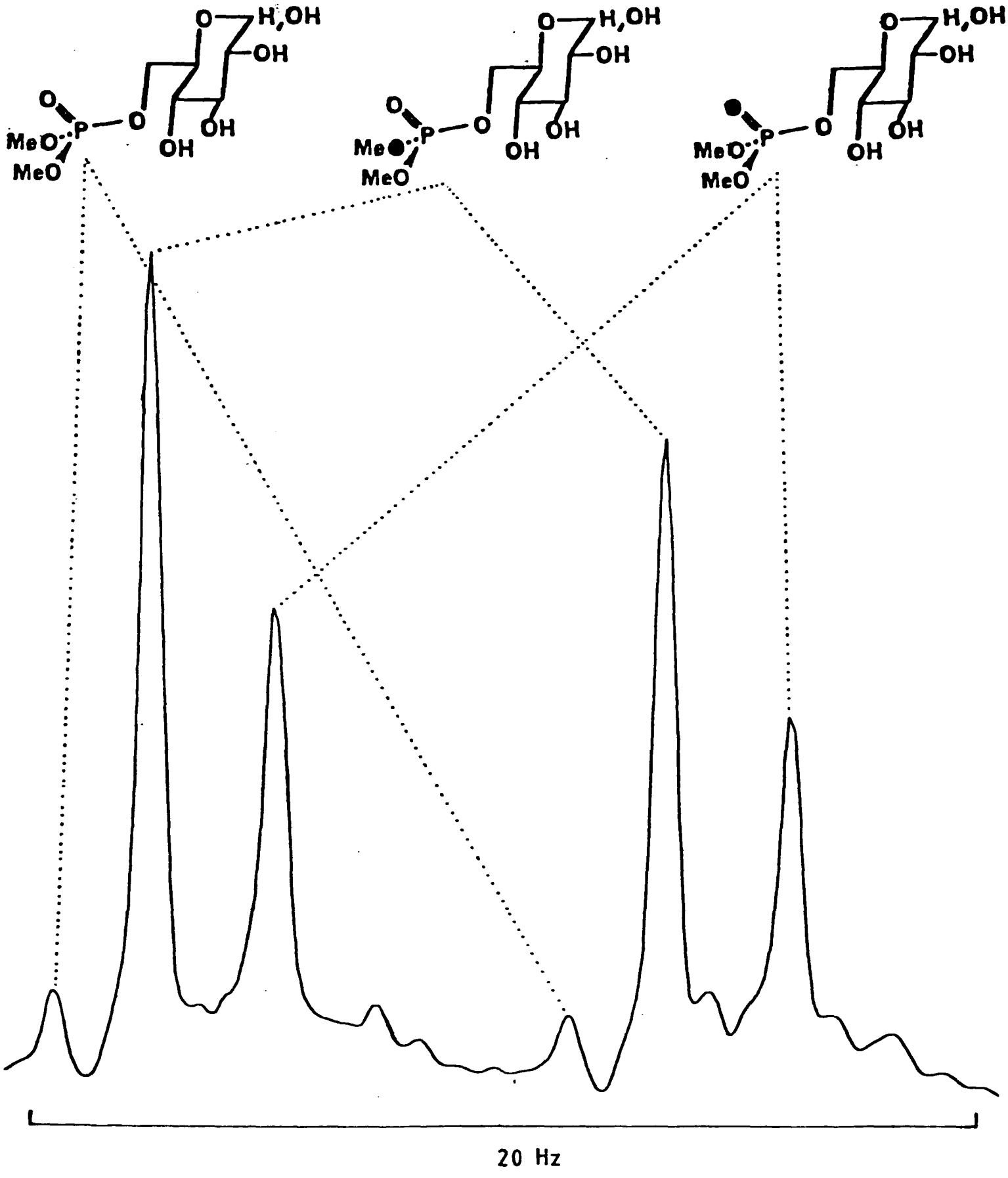


FIGURE 111 : The 121.5MHz ^{31}P n.m.r. Spectrum of D-Glucose-6[^{18}O]-Phosphate Dimethyl Ester in d_4-MeOH. ^{31}P n.m.r. parameters were : O1, 2500 Hz; O2, 5980 Hz; sweep width, 2000Hz; acquisition time 2.05 s; pulse width, 16 μs; gaussian multiplication(line broadening, -0.6 Hz; gaussian broadening, o.4 Hz;) on 8K, FT in 32K; broad band proton decoupling.

material which had been shown to be at least 99% isotopically pure by mass spectrometry on the [^{18}O]-phosphoryl chloride used and indicates that the material is better than 95% isotopically pure. Furthermore it provides additional evidence that it is safe to convert such labelled phosphates to their corresponding free acids under aqueous conditions for reasonable time periods without the danger of excessive loss of label. Of course, it is not inconceivable that the *ca.* 4% loss of label occurred during exposure to such conditions, although in the light of the previous reverse experiment carried out under more serious conditions this seems to be unlikely and the label was probably lost during the synthesis.

Assignment of Anomeric Resonances

This assignment was achieved by recourse to the ^{1}H 300 MHz n.m.r. spectrum of the [^{18}O]-labelled ester. In all these anomeric systems the C_1 (α) and (β) protons exhibit well separated ^{1}H n.m.r. resonances.[299] Moreover, in view of the *trans* nature of H_{c_1} and H_{c_2} in the β (equatorial) form it is apparent that $^{3}J_{H_1H_2}$ (β) > $^{3}J_{H_1H_2}$ (α) which provides another distinction. The integration of these resonances gives the relative ratio of the α and β forms. In the case of *D*-glucose-6[^{18}O]-phosphate dimethylester the downfield (α) form was the more intense in the ^{1}H n.m.r. spectrum and thus the higher intensity (downfield) peak in the ^{31}P n.m.r. spectrum (FIGURE 111) is assigned to the α-anomer. This is *opposite* to the assignment reported for *D*-glucose-6-phosphate in water.[298]

CHAPTER 11

A REVIEW OF HEXOKINASE

A Review of Hexokinase

Of the innumerable phosphokinase enzymes by far the most thoroughly investigated mechemistically is yeast hexokinase (E.C. 2.7.1.1) and it seems likely that many of the features displayed by hexokinase will prove to be applicable to phosphokinases in general. Hexokinase is a key enzyme in the glycolytic pathway whereby glucose is converted to two molecules of pyruvate, a pathway common to all organisms, this sequence of essentially reversible reactions being named (after some of the prominent early workers) the Embden-Meyerhof-Parnas pathway and is illustrated in FIGURE 112. Hexokinase is a dimer of molecular weight 99,000 (although the monomer is active) and catalyses the transfer of a phosphoryl group from adenosine-5'-triphosphate to the 6-position of *D*-glucose as previously described in FIGURE 81. Interest in hexokinase has long been established and there are two recent reviews available.[300,301] A number of important publications, however, are subsequent to these reviews including a detailed X-ray structure.[302] Some of the early work may need to be re-evaluated in the light of the observation that the native enzyme is readily partially degraded by proteases during the extraction procedures, and yet still retains its activity.[303] Methods are now available for the isolation and separation of the native isoenzymes[304,305] in apparently good yield.

Steady state kinetic studies[306,307] on hexokinase show intersecting double reciprocal plots which indicate a sequential rather than a ping-pong pathway, *i.e.* phosphoryl transfer occurs within ternary complexes of the enzyme and its substrates. However, it needs emphasing that such classical experiments may be misleading, for parallel double reciprocal plots may in fact be just slightly converging (indeed this error was made for mammalian hexokinase, which was originally thought to show a parallel initial velocity

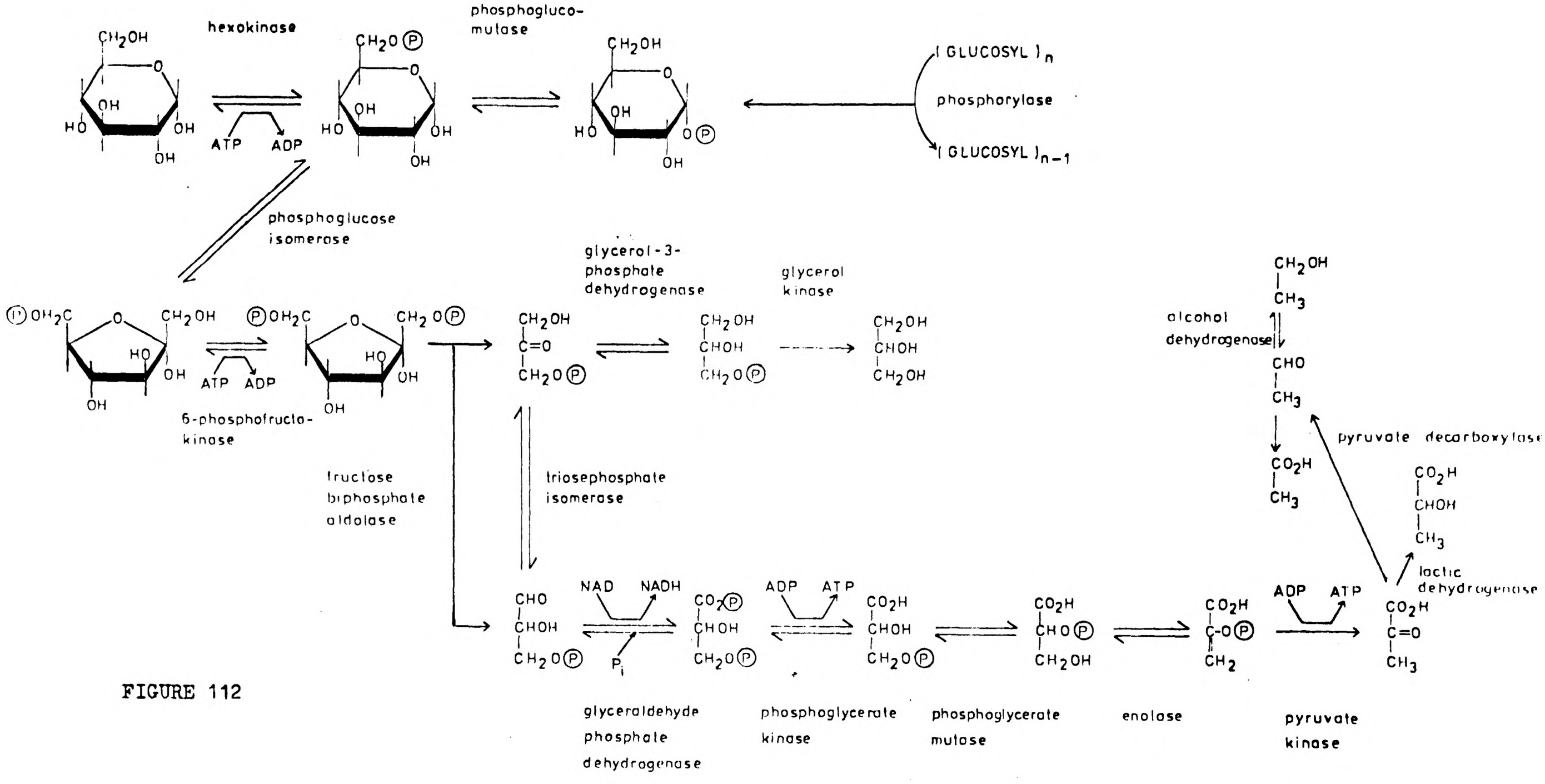


FIGURE 112

THE EMBDEN MEYERHOF PARNAS PATHWAY.

pattern) and these are also circumstances in which false intersections can be observed (*e.g.* if one substrate is contaminated with its coproduct, or if the intermediate in a ping pong pathway is chemically labile).[183] The study of kinase reactions is further complicated by such problems as what to do about the essential divalent metal ion (*e.g.* should one simply keep the metal ion concentration constant, or should one buffer the metal ion to maintain a constant level of free cation?), and how to treat the questions of multiple binding sites (*e.g.*[308]), substrate inhibition or activation, and salt effects (*e.g.*[309]). These elements of ill behaviour often result in non-linear double reciprocal plots (*e.g.*[310]), the interpretation of which becomes more a matter of whim than judgement. In the light of the above, inhibition studies using products, product analogues, and substrate analogues are essential before reliable deductions can be made from steady-state kinetics.[183]

While it is clear that hexokinase follows a sequential pathway the degree of order in substrate addition is less ambiguous. The consensus now is that the pathway is a largely ordered one, with glucose leading, but the alternative order (ATP binding before glucose) can become dominant under conditions of high nucleotide concentration.[311,312] This explains the observation of effectively random substrate addition at certain substrate concentrations. The 'mainly ordered' sequence of FIGURE 109 is supported by the equilibrium flux measurements of Britton and Clarke[313] who found that the ratio of the two fluxes: glucose-6-phosphate $\rightleftharpoons$ ATP and glucose-6-phosphate $\rightleftharpoons$ glucose, is unaffected by the glucose concentration but rises as the ATP concentration increases. This result is expected for an ordered pathway with glucose leading, however, it had previously been shown that each of the equilibrium exchange reactions G-6-P $\rightleftharpoons$ glucose *and* ADP $\rightleftharpoons$ ATP, rises to a

plateau level when the concentration of the other substrate pair is raised.[314] If the *only* possible way of loading the enzyme were by the binding of glucose followed by ATP one would have predicted a suppression of the glucose-6-phosphate $\rightleftharpoons$ glucose exchange reaction by very high ADP and ATP levels. The fact that such suppression was not observed suggests that the alternate order of binding may become preferred at high nucleotide levels.[315] Further embellishments on the scheme in FIGURE 113 have come from recent work from Rose's group using the isotope trapping method.[316]

The above discussion brings us to the point where the nature of the interconversion of the two ternary complexes, E. Glucose. ATP $\rightleftharpoons$ E. Glucose 6-phosphate.ADP has to be considered: is the phosphoryl group transferred directly between the bound substrate or is it transferred *via* a phosphoenzyme complex E~P. Glucose.ADP? Three results have been cited in support of the latter (falsely as will be seen). First, in common with many phosphokinases, hexokinase shows an intrinsic hydrolytic activity towards ATP[317,319], and while this reaction is 4×10^4 times slower than that of glucose phosphorylation, unsubstantiated analogy with the phosphatases has led to suggestions of the intermediacy of a phosphoenzyme for the ATPase reaction. However, the striking promotion of the ATPase activity of hexokinase by glucose analogues such as lyxose[318] indicates that the ATPase reaction may only be a trivial diversion from the normal phosphorylation of glucose within the ternary complex. In the absence of glucose the active site will not, after all, be empty and solvent water could act as a poor surrogate for the 6-hydroxyl group of glucose. Secondly, hexokinase catalyses the slow isotopic exchange of ATP and ADP in the absence of glucose.[317,319] Such half reactions (eqn) have

$$\text{Enzyme} + \text{ATP} \rightleftharpoons \text{phosphoenzyme} + \text{ADP}$$

$$\text{phosphoenzyme} + \text{acceptor} \rightleftharpoons \text{phospho-acceptor} + \text{enzyme}$$

been widely cited[320] as evidence for phosphoenzyme intermediates in phosphokinase reactions, and although in the case of hexokinase the exchange occurs at a rate about 10^4 times slower than the normal reaction and is not accelerated by lyxose[321], substrate synergism[322] could always be invoked. (The term substrate synergism describes the need to bind the cosubstrate in order to achieve the maximal rate of the half reaction.[322]) Unfortunately the phosphokinase field is peppered with erroneous conclusions that arise from the presence of substrate or enzyme contaminants and for many such studies, efforts have not been reported to ensure the absence of cosubstrate or cosubstrate analogues (the presence of which naturally allows ATP-ADP exchange by reversal of the overall reaction) or to ensure the absence of contaminating enzymes (such as adenylate kinase) which would catalyse the observed exchange.[323,324] Thirdly, hexokinase is phosphorylated on incubation with ATP and *D*-xylose[325], and while this phosphoenzyme is catalytically inactive, the phosphorylation can be reversed with ADP + *D*-xylose.[325]

All these results have led to suggestions that the phosphoryl group transfer occurs *via* the enzyme (an 'active' phosphoenzyme that in the presence of *D*-xylose rearranges to an inactive form). The alternative is that the slow reactions referred to are merely side reactions irrelevant to the normal pathway. Certainly the transient phosphorylation of a substrate surrogate (whether it be water[317,318], an amino acid side chain[325], or even a peptide link[326]) could give rise to these observations. Nearly every phosphokinase that has been scrutinized has its complement of slow partial reactions; the problem is to decide whether they are misleading artifacts or useful clues pointing to a phosphoenzyme.

One important approach to the detection of cryptic phospho-enzymes, namely those which cannot be isolated or are ill-behaved

either chemically or kinetically, was devised by Midelfort and Rose[197] who recognised that even if a kinase reaction involved the formation of a phosphoenzyme from ATP, the ADP might not freely dissociate and the expected ATP-ADP exchange reaction would not be observed. Accordingly, on the reasonable basis that even tightly bound ADP could still suffer rotation of its β-phosphoryl group, Midelfort and Rose[197] developed the 'β,γ-bridge to non-bridge' positional isotope exchange experiment previously discussed (CHAPTER 6). Using this method, the transient formation of a phosphorylated intermediate in the glutamine synthetase reaction was established. When, however, this method is applied to hexokinase (using highly purified enzyme in the complete absence of glucose), it fails, even in the presence of lyxose.[204] The *absence* of positional isotope exchange leaves one uncertain as to whether there is no phosphoenzyme intermediate or whether the β-phosphoryl group of ADP cannot rotate during the lifetime of the intermediate. Such an ambiguity exists for starch phosphorylase, for example, where positional exchange of the C-O-P bridge and non-bridge phosphoryl oxygens of glucose-1-phosphate is seen in the presence of maltotriose as primer but *not* during starch elongation.[327] In this case the rate of formation of the putative intermediate may be slower than the rate of its collapse. An analogous case is glucose-1,6-bisphosphate synthetase[328], which proceeds *via* a phosphoenzyme intermediate[329] but does not catalyse the non-bridge to bridge randomisation of a carbonyl ^{18}O-label in the substrate 1,3-bisphosphoglycerate.[329]

The clearest evidence on the nature of the phosphoryl group transfer in the interconversion of the ternary complexes E. Glucose. ATP and E. Glucose-6 phosphate.ADP comes from stereochemical studies. The transfer of a *thiophosphoryl* group from ATP to glucose has been shown to proceed with *inversion* of configuration

at phosphorus[209,214] by the Knowles group at Harvard. This strongly suggests that transfer occurs directly between the substrate molecules within the ternary complex without the covalent intervention of the enzyme. However, it must be emphasised that since this transfer involves the use of a substrate analogue in the form of the thiophosphoryl group some reservation about this result must be made. This is especially so in view of the fact that there has been only one convincing demonstration of the comparability of phosphoryl and thiophosphoryl transfers in glycerol kinase.[214]

A totally convincing answer has been provided by the outcome of a study using adenosine-5'[$\gamma(S)$-^{16}O, ^{17}O, ^{18}O]-triphosphate, which is the concern of this thesis and which is soon to be published.[217]

CHAPTER 12

A STEREOCHEMICAL INVESTIGATION OF YEAST HEXOKINASE CATALYSED PHOSPHORYL TRANSFER USING ADENOSINE-5'[$\gamma(S)$-^{16}O, ^{17}O, ^{18}O]-TRIPHOSPHATE

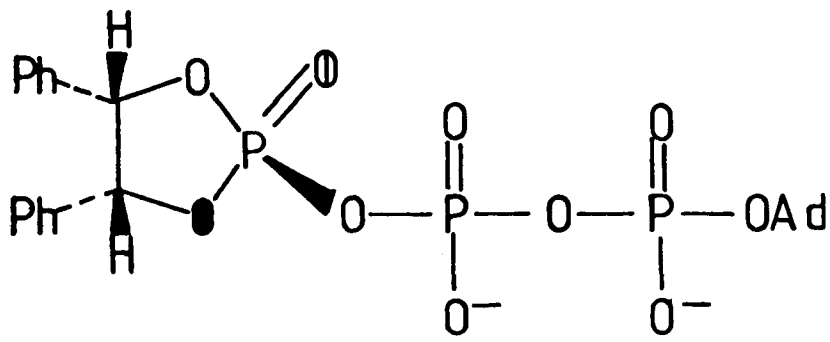
INTRODUCTION

We now have á method for analysing the isotopic chirality of phosphorus of *D*-glucose-6[^{16}O, ^{17}O, ^{18}O] phosphate (CHAPTER 9), the product of the hexokinase catalysed phosphoryl transfer reaction, and would like to extend our original concept to the study of specific enzymic phosphoryl transfers. To make our procedure general for all kinase enzymes handling ATP we would like to have a procedure for analysing the isotopic chirality of adenosine-5'[γ-^{16}O, ^{17}O, ^{18}O]-triphosphate and this is provided by the hexokinase reaction. We must first, of course, synthesise adenosine-5'[γ-^{16}O, ^{17}O, ^{18}O]-triphosphate of known absolute isotopic configuration and put this through the hexokinase reaction to generate a *D*-glucose-6[^{16}O, ^{17}O, ^{18}O] phosphate whose configuration can be analysed by the methods which have already been described.

The Synthesis of Adenosine-5'[$\gamma(S)$-^{16}O, ^{17}O, ^{18}O]-Triphosphate

ATP was initially synthesised by Baddiley *et al.*[258] from dibenzyl phosphorochloridate and P_1,P_2-dibenzyl ADP. Subsequent chemical syntheses (from ADP) have been the morpholidate method of Wehrli *et al.*[330] and the imidazolide method of Hecht and Kozarich.[331] The chemical synthesis of adenosine-5'[$\gamma(S)$-^{16}O, ^{17}O, ^{18}O] triphosphate has been reported by Knowles *et al.*[214] Obviously an enzymic method presents itself using pyruvate kinase, phosphoenol pyruvate and ADP and this will be described in CHAPTER 14.

The synthesis of adenosine-5'[$\gamma(S)$-^{16}O, ^{17}O, ^{18}O] triphosphate should be possible by the reaction of a suitable salt of ADP with our phosphorylating agent (2*R*,4*S*,5*R*)-2-chloro-[2-^{17}O]-oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane (102b) to generate (2*R*,4*S*,5*R*)-2(adenosine-5'-diphospho)-[2-^{17}O]-oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane which could probably be deprotected



(221)

FIGURE 114

by catalytic hydrogenation. The phosphorylation reaction should be possible without protection of the ribose ring hydroxyl groups or an oxygen of the α-phosphoryl group of ADP because of the much greater nucleophilicity of the terminal oxygens of the β-phosphoryl group, and this was initially investigated using unlabelled compounds.

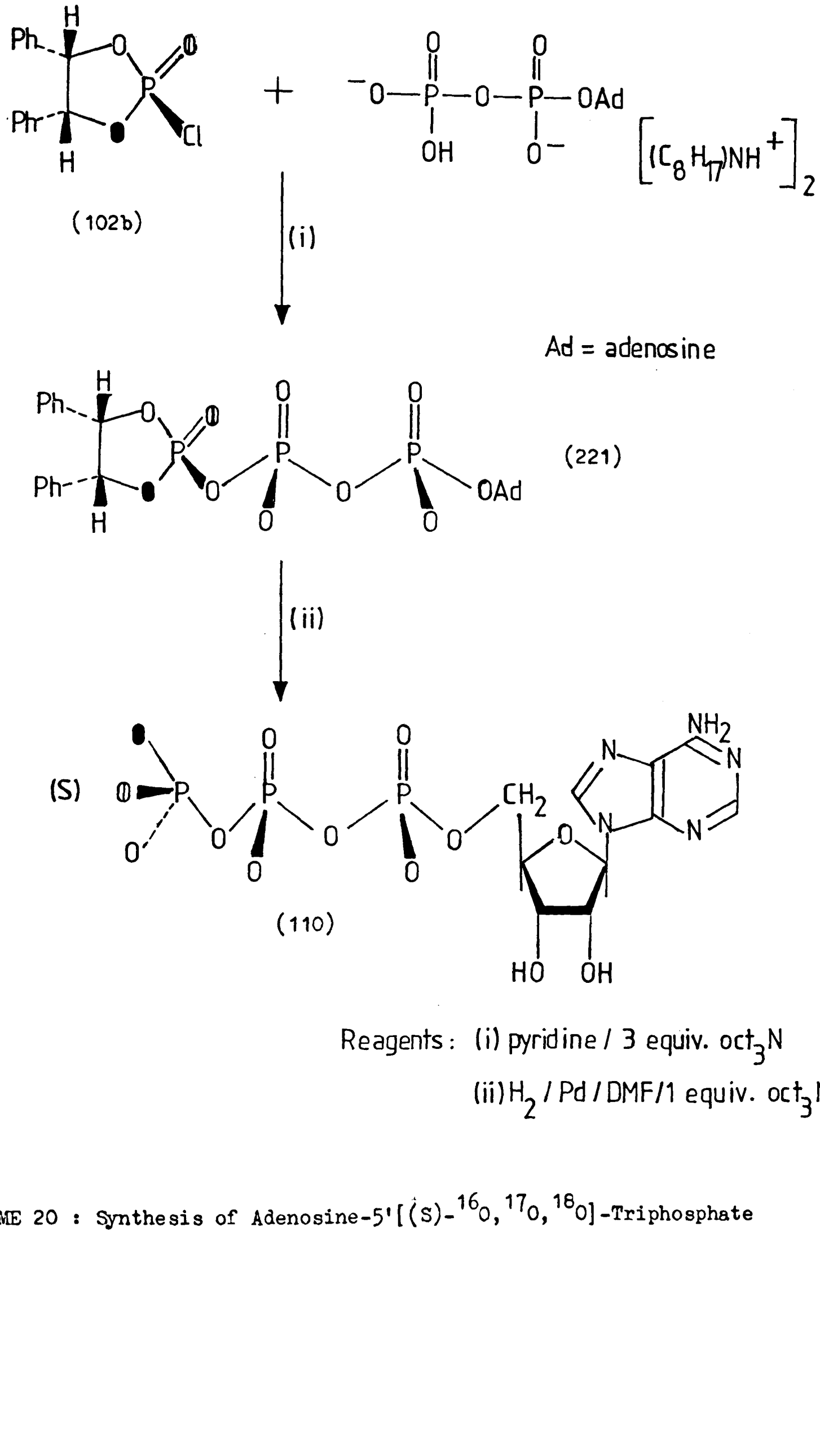
RESULTS AND DISCUSSION

The choice of solvent was an important problem in this reaction. The usual solvent for this type of reaction is pyridine, but because of the known tendency for ATP type molecules to undergo dismutation reactions in this solvent this was initially disfavoured. However, solvents such as DMSO and DMF were clearly incompatible with the generation of the phosphorylating reagent from phosphorus oxychloride with which they both react (although it might be possible to generate the reagent in pyridine and then use a solvent such as DMF in a similar fashion to the synthesis of P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-pyrophosphate). Knowles' synthesis[214] was performed in DMSO but employed their considerably more stable ephedrine-derived phosphorylating reagent (104, R = Cl).

The use of pyridine, therefore, seemed mandatory as no other solvents were compatible, and a wide variety of salts of ADP were investigated. The tributylammonium salts were insoluble in pyridine as was the *tris*-tri-*n*-octylammonium salt. However, the

bis-tri-*n*-octylammonium salt was soluble and this was therefore employed. Unfortunately, when this salt was added to a solution of the phosphorylating reagent (102a) in pyridine an immediate precipitation of *bis*-pyridinium ADP occurred. This was, however, overcome by the addition of three equivalents of anhydrous tri-*n*-octylamine (two equivalents to keep the nucleotide in solution and one more to trap the HCl formed during the displacement reaction) to the phosphorylating reagent prior to the addition of the ADP. This time a clear solution resulted and examination of the ^{31}P n.m.r. spectrum only 5 min after the addition indicated that the reaction was complete. A main point of interest was that the γ-phosphoryl group of the product (222) resonated as a doublet at -0.65 ppm which is approximately 8 ppm further downfield than in ATP, and exhibited a nuclear Overhauser enhancement comparable with P_α. This is indicative of the five-membered 1,3,2-dioxaphospholane system, and moreover, on removing the decoupler the signal became a doublet of triplets with $^3J_{PH}$ = 7.35 Hz confirming the intact nature of the ring system. The desired product (222) had been formed in *ca.* 60% yield. The ^{31}P n.m.r. spectrum also indicated a singlet at +11.8 ppm which split into a triplet with $^3J_{PH}$ = 7.0 Hz and was assigned to the cyclic phosphate (116), and a corresponding amount of ADP. This was seen as evidence of the instability of the triester (222), and indeed when the ^{31}P n.m.r. spectrum was monitored over a period of time the compound was seen to gradually decompose to give such complex products as, for example, P_1,P_4-diadenosine-5'-tetraphosphate (223) as follows.

The product (221) was removed from the pyridine solution as soon as possible after addition of the reagents, and the pyridine removed quickly *in vacuo*. The triester was then hydrogenolysed in DMF over Pd/C in the presence of one equivalent of tri-*n*-octylamine. Crude *tetra*-ammonium ATP was obtained after evaporation of the



SCHEME 20 : Synthesis of Adenosine-5'[(S)-^{16}O, ^{17}O, ^{18}O]-Triphosphate

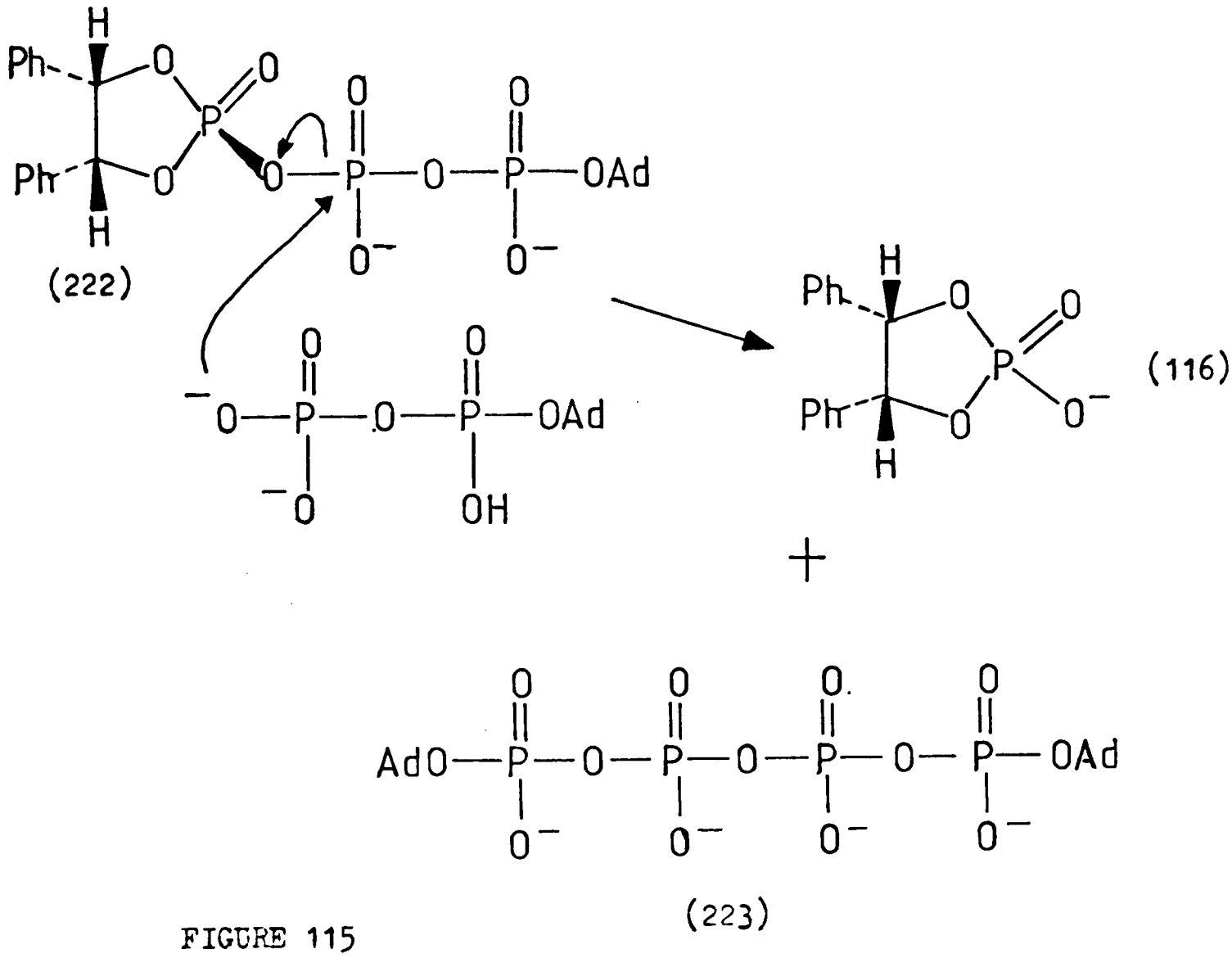


FIGURE 115

filtrate from the hydrogenation and the washings from the catalyst, which was treated with 300 m*M* 50/50 aqueous/ethanolic ammonia.[332] The ATP was purified on a column of DEAE Sephadex A-25 and was obtained as the *tetra*-triethylammonium salt in a yield of 22%; this compound was shown to be a substrate for pyruvate kinase.[332]

The synthesis was repeated using labelled compounds according to SCHEME 20 to give adenosine-5'[γ(*S*)-^{16}O, ^{17}O, ^{18}O]-triphosphate (110) as its *tetra*-triethylammonium salt which was used in this form for the hexokinase catalysed phosphoryl transfer.

Hexokinase Catalysed Phosphoryl Transfer

This was performed first for unlabelled ATP using hexokinase obtained from Sigma, London (type 301 - essentially the fraction 1 of Kaji *et al.*[303] - this was examined for us by Dr. E.A. Barnard at Imperial College London and found to be essentially the SI isoenzyme by gel electrophoresis). The *D*-glucose-6-phosphate formed in the transfer was monitored by ^{31}P n.m.r. as shown in FIGURE 116

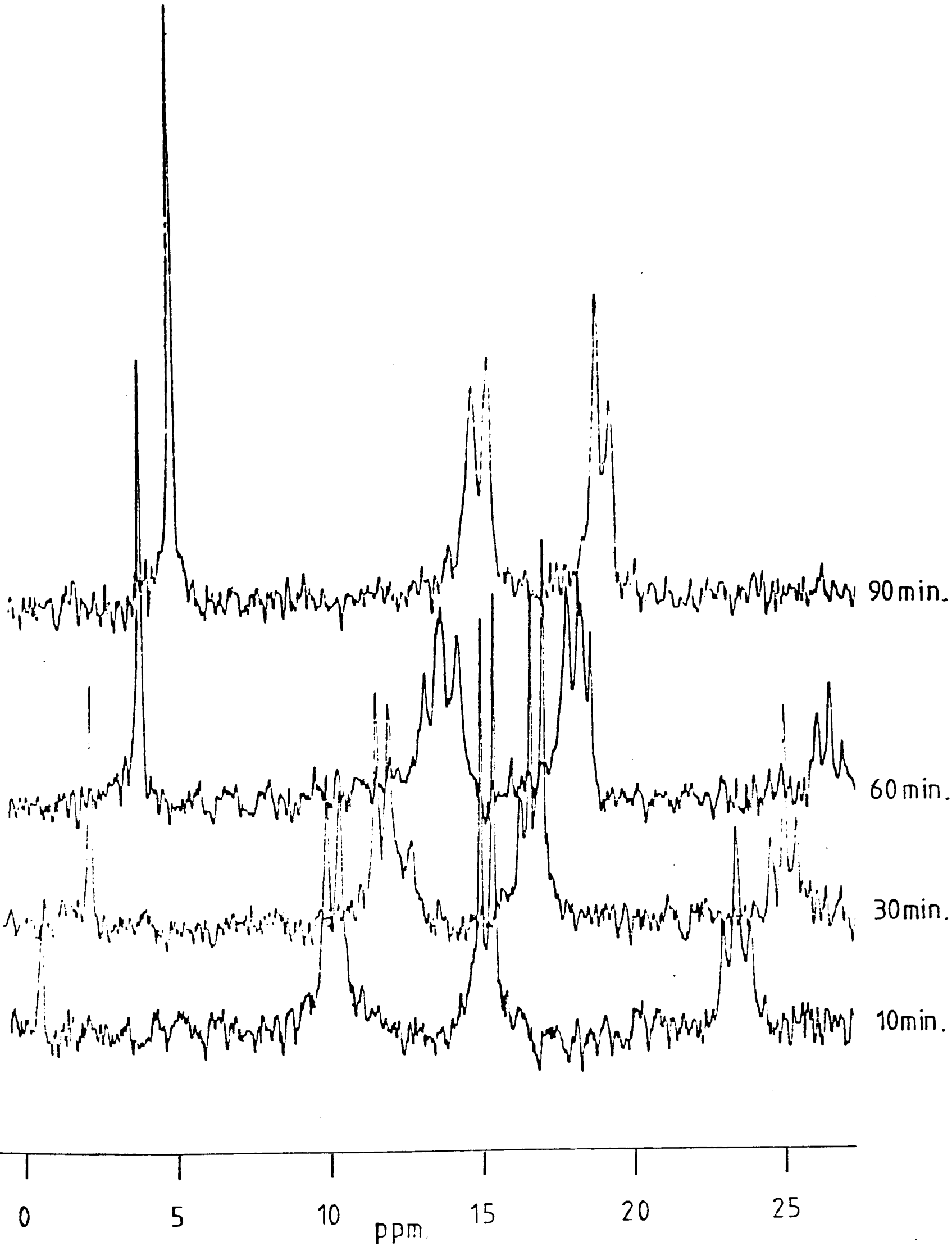


FIGURE 116 : 36.43MHz ^{31}P n.m.r. Spectra Illustrating the Hexokinase Catalysed Phosphoryl Transfer from ATP to D-Glucose. The solution was made up in 0.05M triethanolamine buffer pH 8.0 and was 20mM in ATP, 100mM in D-Glucose and 6.6mM in magnesium chloride at 25°C. The mixture contained 2 units of hexokinase per ml. N.m.r. parameters were: offset, 3200 Hz; sweep width, 3000 Hz; pulse width, 17 μs; broad band proton decoupling; 100 transients in each case. The p.p.m. scale applies to the 10 min. spectrum only, and the D-glucose-6-phosphate is always the most downfield peak in the spectrum.

and when reaction was judged to be complete (*ca.* 90 min) the enzyme was denatured and the *D*-glucose-6-phosphate isolated by column chromatography on DEAE Sephadex A-25. It was detected in the column fractions and subsequently assayed by means of the *D*-glucose-6-phosphate dehydrogenase-NADP system (E.C. 1.1.1.49).[333] Although 100% recovery of ADP was obtained from the ion-exchange column, as judged by the $OD_{260\ nm}$ of the fractions, only 80-90% of *D*-glucose-6-phosphate could be routinely detected in the fractions. This presumably reflects the presence of contaminating enzymes in the commercial product which destroy either ATP or *D*-glucose-6-phosphate. The *D*-glucose-6-phosphate was isolated from the column as its *bis*-triethylammonium salt which was then converted to the *mono*-tri-*n*-octylammonium salt and cyclised and methylated according to previous procedures (CHAPTER 9).

The experiment was repeated with adenosine-5'[$\gamma(S)$-^{16}O, ^{17}O, ^{18}O]-triphosphate and the chirality of the *D*-glucose-6[^{16}O, ^{17}O, ^{18}O -phosphate of unknown isotopic configuration analysed as follows using the previously described methods.

<u>The ^{31}P n.m.r. Spectrum of Adenosine-5'[$\gamma(S)$-^{16}O, ^{17}O, ^{18}O]-triphosphate and the Analysis of the Chirality of *D*-Glucose-6[^{16}O, ^{17}O, ^{18}O]-phosphate from the Yeast Hexokinase Phosphoryl Transfer</u>

On the basis of the published work concerning the effect of ^{17}O in ^{31}P n.m.r. spectroscopy[231-233], it is to be expected that in the ^{31}P n.m.r. spectrum of adenosine-5'[$\gamma(S)$-^{16}O, ^{17}O, ^{18}O]-triphosphate the resonance due to the γ-phosphorus will have its intensity decreased by an amount depending on the isotopic enrichment of ^{17}O at that site. Moreover, ATP contains a convenient internal reference in the β-phosphoryl group (the α-phosphoryl resonance is nuclear Overhauser enhanced) and the isotopic enrichment of P_γ should be obtainable by relative integration of P_γ and P_β, provided of course that P_β is unaffected by the isotopic

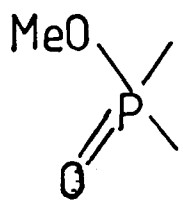
substitution as has been shown to be the case.[232]

The ^{31}P n.m.r. spectrum of adenosine-5'[$\gamma(S)$-^{16}O, ^{17}O, ^{18}O]-triphosphate is shown in FIGURE 117. The P_γ doublet resonance is furthest downfield and shows that there has been some loss of label during the synthesis, as doublet resonances assignable to ATP containing no labels and one label are observable downfield from the major peak representing two labels. As described above it is possible from this spectrum to calculate the relative contributions of all the labelled ATP species in this sample, and this is illustrated in TABLE 13.

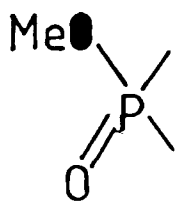
TABLE 13: ISOTOPIC COMPOSITION OF ADENOSINE-5'[$\gamma(S)$-^{16}O, ^{17}O, ^{18}O]-TRIPHOSPHATE

No. of labels	0	1	2 [^{16}O, ^{17}O, ^{18}O]	2 2 x ^{18}O
% contribution	20	15	28	37

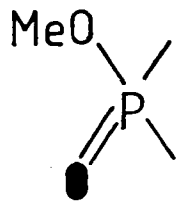
On cyclisation and methylation this leads to the following intensities for *e.g.* the axial triester* (if *inversion* of configuration has taken place).



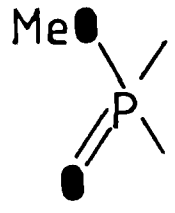
25.0



25.4



18.5



12.3

* From the diol calculation the composition of the [^{16}O, ^{17}O, ^{18}O] material was 81% (*R*) and 13% (*S*).

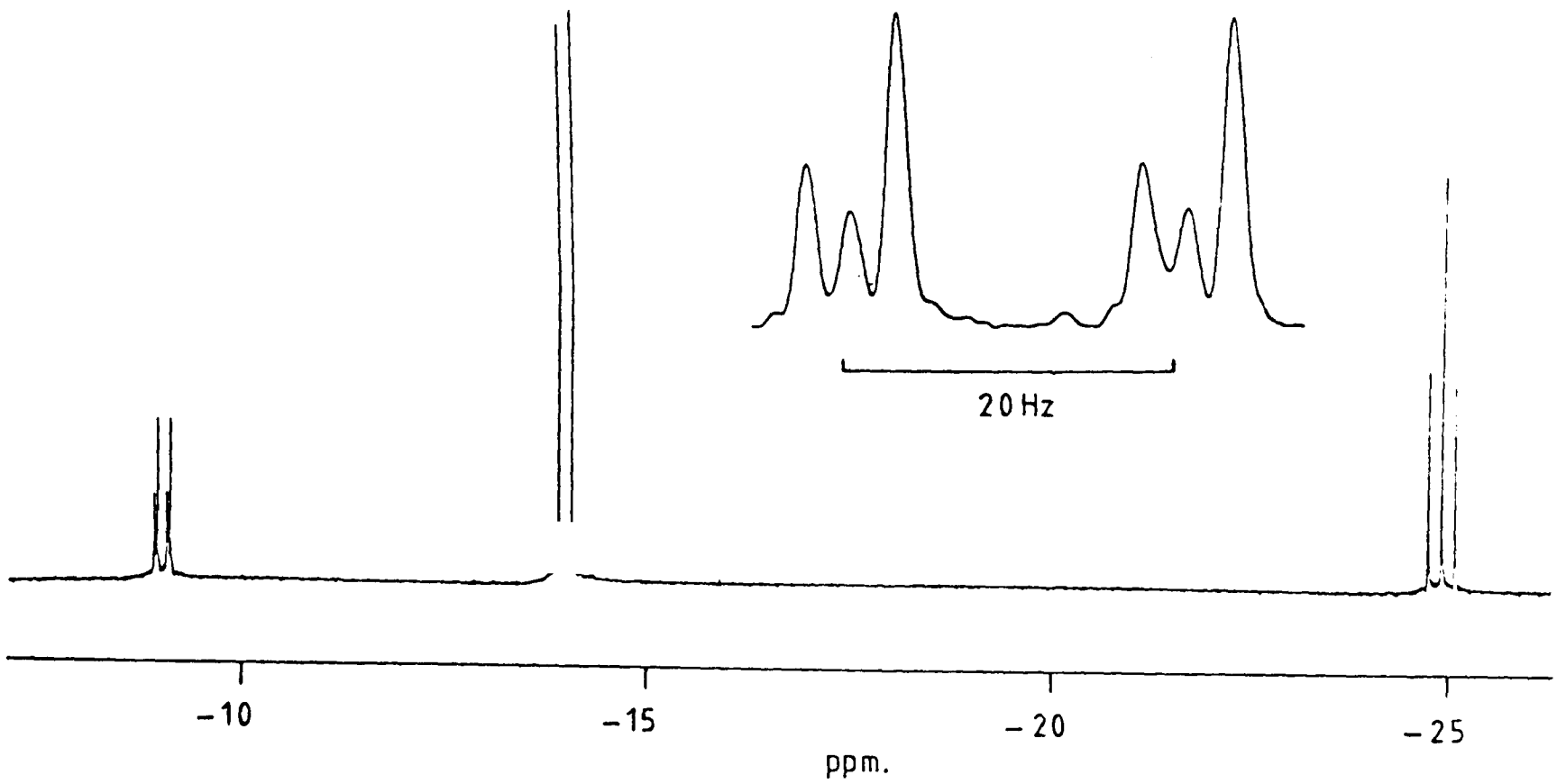


FIGURE 117 : The 121.5 MHz ^{31}P n.m.r. Spectrum of Adenosine-5'[(S)-^{16}O, ^{17}O, ^{18}O]-Triphosphate. The sample was made up in 100mM 2-amino-2-methyl-1,3-propanediol hydrochloride at pH 9.0 containing 10mM EDTA in 25% deuterium oxide. ^{31}P n.m.r. parameters were : offset, 900 Hz; 02, 5580 Hz; sweep width, 3000 Hz; pulse width, 15 μs; acquisition time, 1.36 s; memory size, 8K; broad band proton decoupling; gaussian multiplication (line broadening, -1.2 Hz; gaussian broadening 0.3 Hz) in 8K and fourier transform in 32K. The inset is the expanded P_γ resonance.

and normalising we have:

0.99 1.00 0.73 0.49

The ^{31}P n.m.r. spectra of the labelled triesters derieved from the yeast hexokinase phosphoryl transfer are shown and FIGURE 118 and their relative intensities compared with the above calculated values in TABLE 14 from which it can be seen that the phosphoryl

TABLE 14: A COMPARISON OF OBSERVED AND CALCULATED N.M.R. INTENSITIES FOR THE DPPC-BuOK CYCLISATION OF *D*-GLUCOSE-6[^{16}O, ^{17}O, ^{18}O]-PHOSPHATE DERIVED FROM THE HEXOKINASE CATALYSED PHOSPHORYL TRANSFER

	Equatorial Triester			Axial Triester		
	Observed	Calculated		Observed	Calculated	
		RETN.	INVN.		RETN.	INVN.
MeO-P=O	1.04	0.99	0.99	0.99	0.99	0.99
Me●-P=O	0.75	1.00	0.73	1.00	0.73	1.00
MeO-P=●	1.00	0.73	1.00	0.73	1.00	0.73
Me●-P=●	0.54	0.49	0.49	0.54*	0.49	0.49

* In calculating the average observed value the Me●-P=● peak in the upfield anomer of the axial triester was discounted since it probably does not reflect the true height.

RETN. = Retention, INVN. = Inversion.

transfer reaction has occurred with *inversion* of isotopic configuration at phosphorus, this follows because it has been shown previously (CHAPTER 9) that *D*-glucose-6-[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate is cyclised with *inversion* of configuration at phosphorus.[216] Since the relative intensities of the triesters in FIGURE 118 are reversed from those discussed in CHAPTER 9 (the

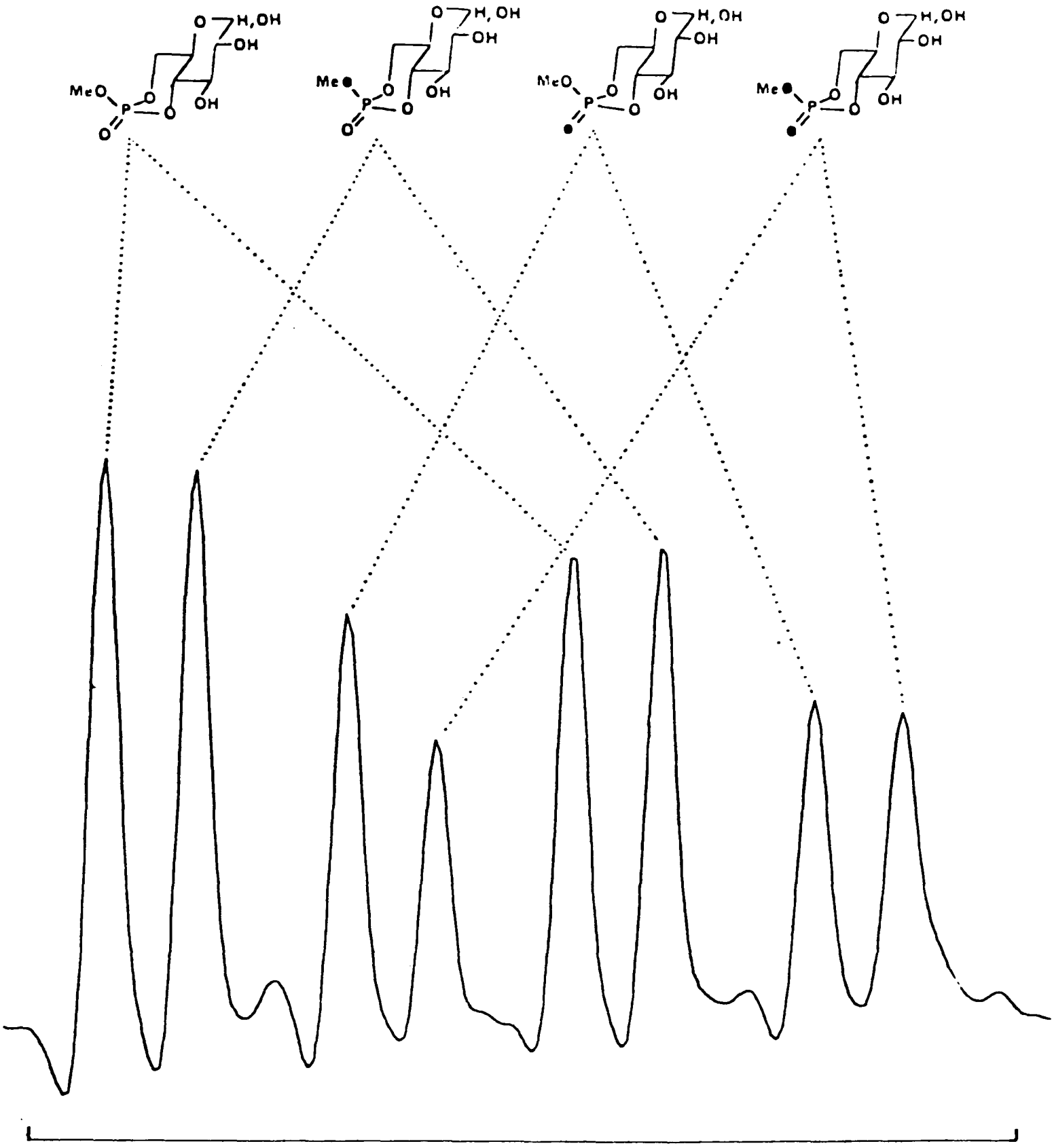


FIGURE 118 (a) : The 121.5 MHz ^{31}P n.m.r. spectrum in 33% CD_3OD, 33% MeOH and 33% DMSO of the axial diastereoisomer of D-glucose-4,6-[^{16}O,^{17}O,^{18}O]-cyclic-phosphate methyl ester, derived by the cyclisation and methylation of D-glucose-6- [^{16}O,^{17}O,^{18}O]-phosphate of unknown isotopic chirality obtained from the hexokinase catalysed phosphoryl transfer from adenosine-5' [γ(S)-^{16}O,^{17}O,^{18}O]-triphosphate to D-glucose. ^{31}P N.m.r. parameters were : offset, 2240 Hz; sweep width, 2000 Hz; acquisition time, 2.05 s; pulse width, 16 μs; memory size, 8K; gaussian multiplication (line broadening, -1.0 Hz; gaussian broadening, 0.3Hz) in 8K and FT in 32K; broad band proton decoupling. The vertical gain control for the equatorial triester is twice that for the axial triester

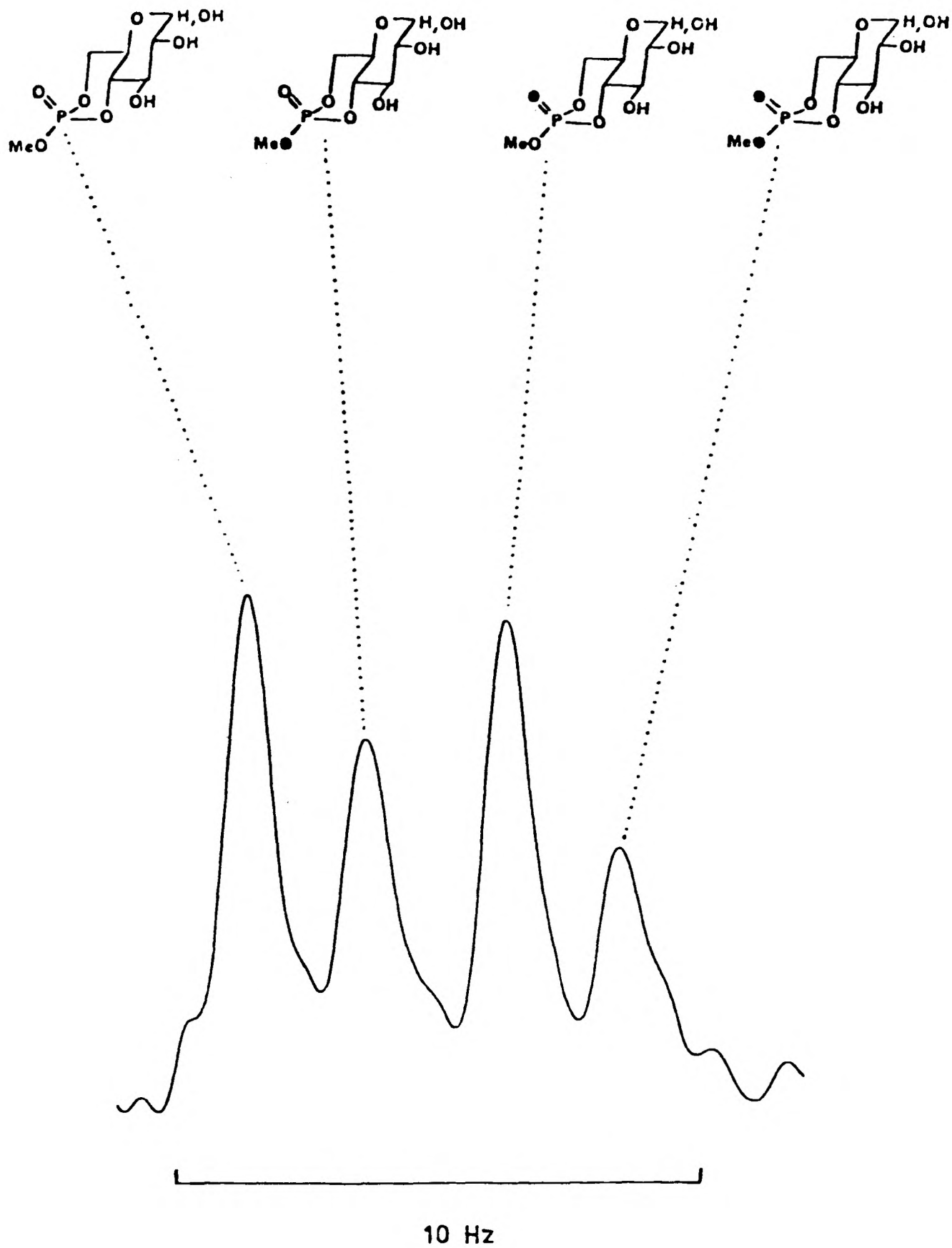


FIGURE 118 (b) : The 121.5 MHz ^{31}P n.m.r. spectrum of the equatorial diastereoisomer as in FIGURE 118 (a).

equatorial system is broader due to the unresolved anomers here) the *D*-glucose-6[^{16}O, ^{17}O, ^{18}O]-phosphate obtained by the hexokinase reaction must have the (*R*)-configuration at phosphorus and thus phosphoryl transfer has occurred with *inversion* of configuration at phosphorus, with a stereoselectivity which is probably in excess of 90%. This process is illustrated in FIGURE 119.

This result eliminates the adjacent mechanism followed by pseudorotation and the double displacement mechanism with a transient phosphoryl enzyme intermediate (see CHAPTER 6, FIGURE 50). According to our original postulate the mechanism must be either an associative or dissociative 'in line' mechanism, that is, the phosphoryl group must be transferred directly between substrates in the ternary complex, or by way of a metaphosphate intermediate. The latter mechanism is, of course, simply an extreme version of the associative mechanism in which bond cleavage is complete before bond formation takes place. Since, in the absence of *D*-glucose, hexokinase does not catalyse isotopic randomisation in the βγ-bridge to non-bridge type of experiment (see CHAPTER 6) we may probably rule out the dissociative mechanism and thus propose an associative $S_N2(P)$ mechanism of phosphoryl transfer for hexokinase (we are, however, not *rigorously* certain of the elimination of the $S_N1(P)$ mechanism by the nature of the experiment). The observation that the Λ isomer of the β,γ-bidentate $Co(NH_3)_4ATP$ is a substrate for hexokinase suggests that Mg^{2+} in the hexokinase-*D*-glucose-MgATP complex is bound to the β,γ residues of ATP.[334] This mode of binding would be expected to favour the associative mechanism.[189]

All the phosphokinases which have been investigated using chiral [^{18}O]-thiophosphate analogues of the natural substrates have transferred the thiophosphoryl group with inversion of configuration.[204,335] The rate at which hexokinase catalyses the transfer of the thiophosphoryl group from adenosine-5'([γ-^{18}O]γ-thio)-triphosphate was not recorded.[269] It had been reported

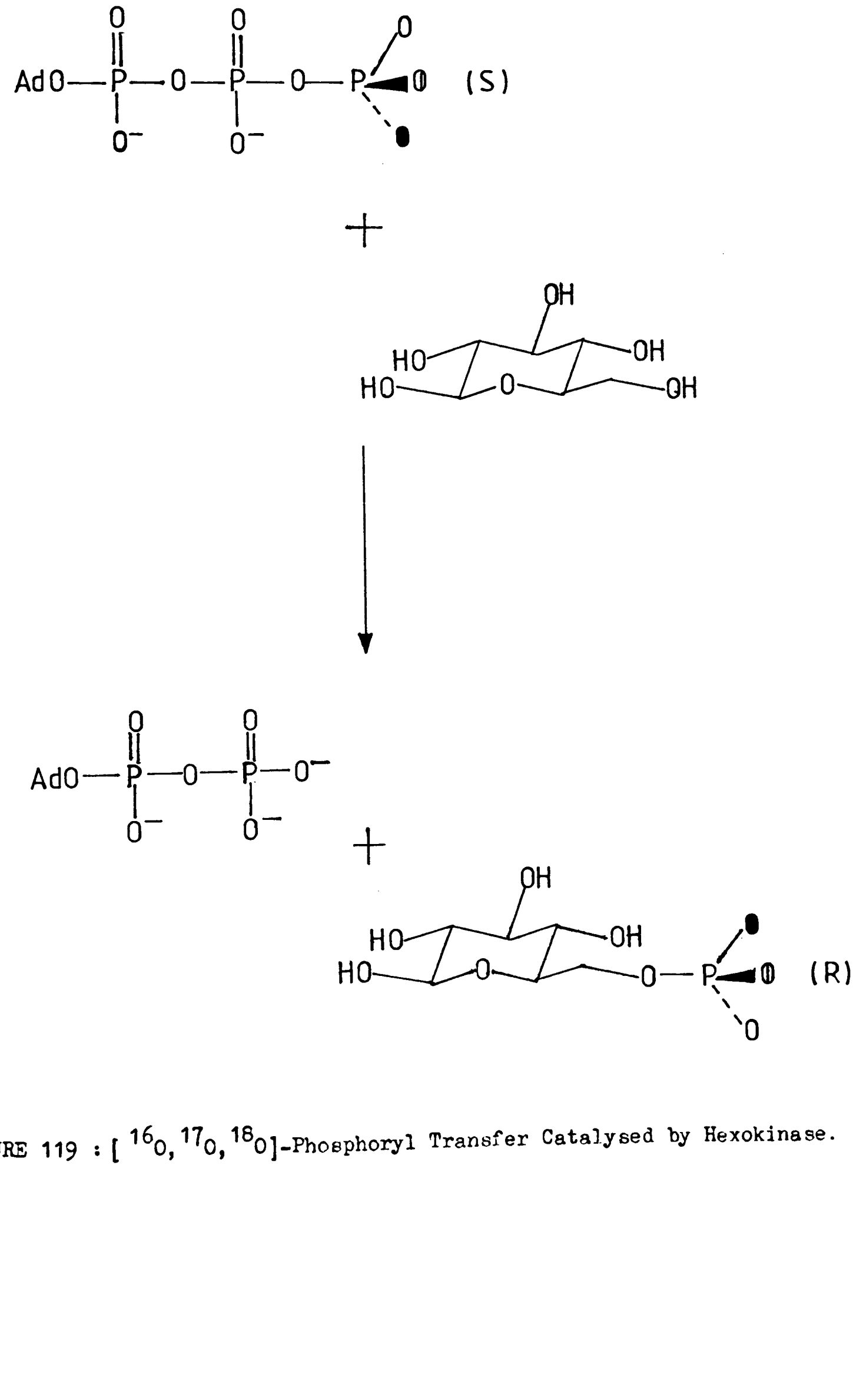


FIGURE 119 : [$^{16}O, ^{17}O, ^{18}O$]-Phosphoryl Transfer Catalysed by Hexokinase.

previously that this compound was not a substrate for hexokinase[336,337], so presumably the transfer is exceedingly slow. The possibility that the mechanism followed for phosphoryl transfer was energetically unfavourable for thiophosphoryl transfer allowing an alternative mechanistic pathway to become more favourable had to be considered.

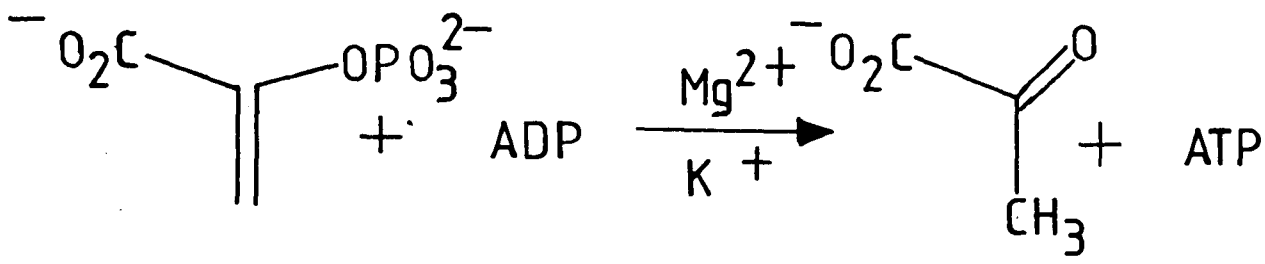
The X-ray structural analysis of the yeast hexokinase isoenzyme A-*D*-glucose complex has shown that the protein conformation is dramatically different[336] from the B isoenzyme which crystallises in the absence of *D*-glucose.[339] Evidence has been presented that this conformational charge is induced on binding *D*-glucose, causing a 12° rotation of the two domains of the subunit, and is not due to the two isoenzymes having a fundamentally different folding of the polypeptide chain.[340] In the hexokinase A-*D*-glucose complex the *D*-glucose is almost engulfed by the protein, only the 6-hydroxyl group being accessible, and even this is hydrogen bonded to ASP-195.[341] The site to which MgATP binds has been deduced from binding studies with adenosine.[342] Model building of the triphosphate chain of ATP on to the adenosine in an extended conformation still leaves the γ-PO_3 group of ATP (the site is actually occupied by sulphate) about 0.6 nm from the 6-hydroxyl group of *D*-glucose. The suggestion that a further conformational change occurs when MgATP binds to the hexokinase-*D*-glucose complex seems almost certainly correct in view of the evidence presented here for an associative 'in line' transfer of the phosphoryl group.

CHAPTER 13

A REVIEW OF PYRUVATE KINASE

Pyruvate kinase review

Pyruvate kinase (ATP: pyruvate-0-2-phospho-transferase, E.C. 2.7.1.40 catalyses the transfer of a phosphoryl group from phospho-enol pyruvate (PEP) to adenosine-5'-diphosphate as follows.



and is an important enzyme in the control of glycolysis and gluconeogenesis[343-345], the equilibrium being in favour of pyruvate and ATP. The above reaction actually involves two processes, namely phosphoryl transfer from PEP to ADP and proton transfer from water to the C-3 position of the resulting enol pyruvate to give pyruvate. The implication of the tightly bound enolate ion of pyruvate[346,347] is substantiated by the observation that oxalate, an analogue of enol pyruvate, is a very potent inhibitor of PK[348], and elicits the same structural change on binding as does PEP.[349] Much effort has been put into the study of the proton transfer reactions catalysed by pyruvate kinase[350,351] and it has been shown that for the pyruvate kinase catalysed proton exchange of pyruvate K^+ and MgATP or an analogue of it are required.[352] When inorganic phosphate and other analogues are used it is found that the pH dependence of the enolisation follows the pKa of the activator.[352]

Although the primary physiological reaction of pyruvate kinase is to perform phosphoryl transfer, it is unique amongst the kinases in that it shows secondary kinase reactions[353] such as 'fluorokinase' activity[354], hydroxylamine kinase' activity[354,355] and 'glycollate kinase' activity.[356] The enzyme also functions as a PEP hydrolase[357] and an oxalacetate decarboxylase.[358,359] The 'fluorokinase' and 'hydroxylamine kinase' activities are stimulated

by bicarbonate ions and it is thought that the bicarbonate ion may occupy the same site as the pyruvate carboxyl group and the acceptor fluoride atom or hydroxylamine molecule the site of the pyruvate carboxyl group.[354] In both cases the specific activities are approximately an order of magnitude lower than the normal activity.

Those enzymes isolated from mammalian tissues[354,360,361] require Mg^{2+} or Mn^{2+}, and K^{+} for activity.[362] Enzymes isolated from various sources can be characterised by their different kinetics. Initial velocity studies have been consistent with a rapid equilibrium random mechanism for the muscle enzyme[363,364], an ordered addition of substrates - and dissociation of products for the yeast enzyme[365], and a ping-pong mechanism involving a phospho-enzyme intermediate for the liver enzyme[366], and such differences in mechanism must reflect considerable structural differences at the respective active sites. An elegant investigation of the kinetics of rabbit muscle pyruvate kinase has recently been reported by Dann and Britton[367], whose results indicate that product dissociation must be rapid and random, and that there is a high degree of cooperativity in binding whatever the order of addition of substrates.

Since the early reviews of pyruvate kinase[346,347] the bulk of the studies, particularly n.m.r., e.p.r., X-ray work and detailed kinetics have been carried out on the muscle enzyme so attention will now be focussed on this.

Rabbit skeletal muscle pyruvate kinase is generally prepared by the method of Tietz and Ochoa.[354] An amino acid analysis has been reported[368] but as yet no sequence has been published, although the amino terminus has been identified as N-acetyl serine and the the carboxy terminus appears to be blocked.[369] The enzyme is a tetramer of molecular weight 237,000[370] composed of four chemically

similar, if not identical subunits[368], and a preliminary X-ray study suggests that the molecule is composed of active dimers formed from conformationally non-identical monomers[371] which are not active.[372] An X-ray study of the cat muscle enzyme stands well advanced at 2.6 Å.[373]

An area of keen interest is, of course, the active site, located at the carboxyl end of a β-sheet[374] and positioned in the cleft between the two unequally sized domains in the subunit.[375] In the active site region chemical modification studies have demonstrated the presence of four essential lysyl ε-amino group groups[376] and four essential sulphydryl groups[377] subsequently identified as cysteine residues[378], per tetramer. Location of these residues in the active site has been demonstrated by proton relaxation experiments[379], and a lysyl ε-NH_2 has been implicated in ADP binding.[376]

The enzyme requires metal ion activation *in vivo* by Mg^{2+} and K^+, however the divalent metal ion requirement *in vitro* can be satisfied by Mn^{2+}[363,350], Co^{2+}[380,381] or Ni^{2+}[380], and the monovalent ion requirement *in vitro* can be satisfied very well by NH_4^+, Rb^+ or Tl^+, the enzyme thus activated being *ca.* 60-80% as active as in the K^+ system.[347] The tetrameric enzyme contains four binding sites for univalent[382] and divalent cations[383,384] as well as for PEP.[382] The effects of these metal ion activators have been extensively investigated by magnetic resonance methods *e.g.* ^{205}Tl[385] and ^7Li n.m.r.[386] and there are a wealth of data concerning the active site geometry of the various complexes.

It has recently been shown that in addition to the tightly bound divalent cation muscle pyruvate kinase requires a second, more weakly bound, divalent cation for activity[387,388], presumably coordinated to the nucleotide as for other kinases.

The role of the metal ions has been the subject of much

investigation. The monovalent cation is located close to the tightly bound divalent cation and the distance between these when monitored by ^{205}Tl n.m.r., has been shown to decrease on adding PEP.[385] The conformational change implicated by this observation has been thoroughly investigated by Nowak.[389,390]

The extensive investigations of the active site using n.m.r. techniques and paramagnetic probes are too detailed to review here but have led to the construction of the total substrate geometry at the active site[381,391,392,393] (FIGURE 120).

The results show partial overlap between the γ-phosphoryl group of ATP and the phosphoryl group of PEP which is in accord with the competition detected by other techniques, and the enzyme bound divalent cation forms second sphere complexes between the phosphoryl groups of PEP and ATP.

Although we have a very detailed picture of the active site of pyruvate kinase (FIGURE 120) there is, however, still room for speculation concerning the mechanism of action.[394] There is evidence against a phosphoryl enzyme intermediate, for no ^{14}C exchange into PEP from [^{14}C]-pyruvate was observed except in the presence of all the substrates.[395] Also the enzyme was not labelled with ^{32}P on incubation with [γ-^{32}P]-ATP and no inorganic phosphate was formed.

A direct phosphoryl transfer must be strongly favoured on the basis of the active site geometry, although it would be unwise to propose an $S_N2(P)$ mechanism purely on the basis of the proximity of the phosphoryl donor and acceptor. Indeed recent isotopic scrambling evidence from this laboratory has suggested that a dissociative $S_N1(P)$ mechanism may be involved[198], although this would be expected to proceed with an 'in line' geometry. Knowles *et al.*[209] have demonstrated that thiophosphoryl transfer takes place with inversion of configuration although, of course, as for

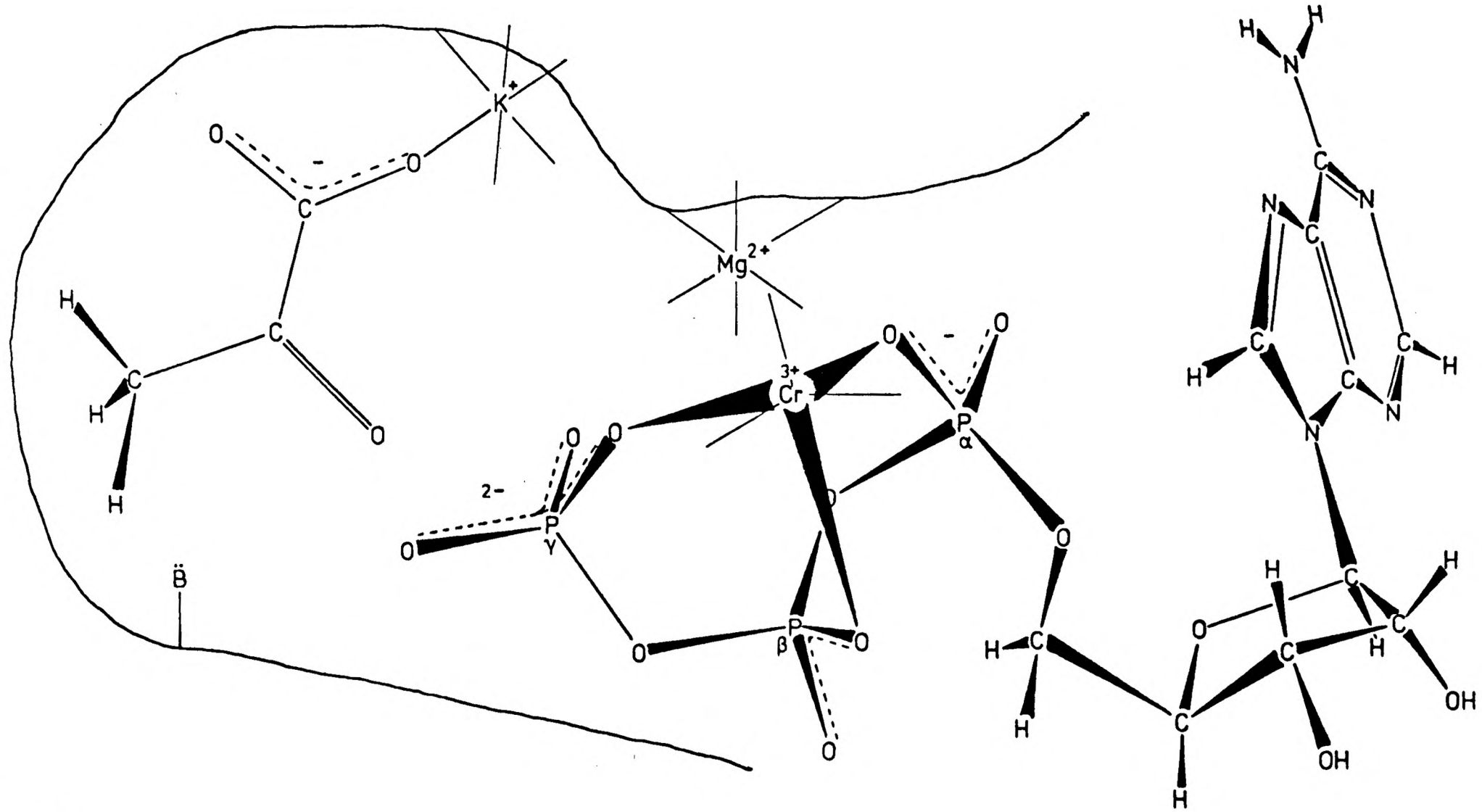


FIGURE 120 Active complex of pyruvate kinase, monovalent and divalent activators, CrATP, and pyruvate. The group labelled B is involved in the deprotonation of pyruvate.

hexokinase this result cannot be regarded as definitive in view of the doubt over the validity of thiophosphoryl analogues as accurate mechanistic probes. The unequivocal answer must come with the use of the [^{16}O, ^{17}O, ^{18}O]-labelled natural substrate which is the concern of this thesis.

CHAPTER 14

A STEREOCHEMICAL INVESTIGATION OF PYRUVATE KINASE CATALYSED PHOSPHORYL TRANSFER

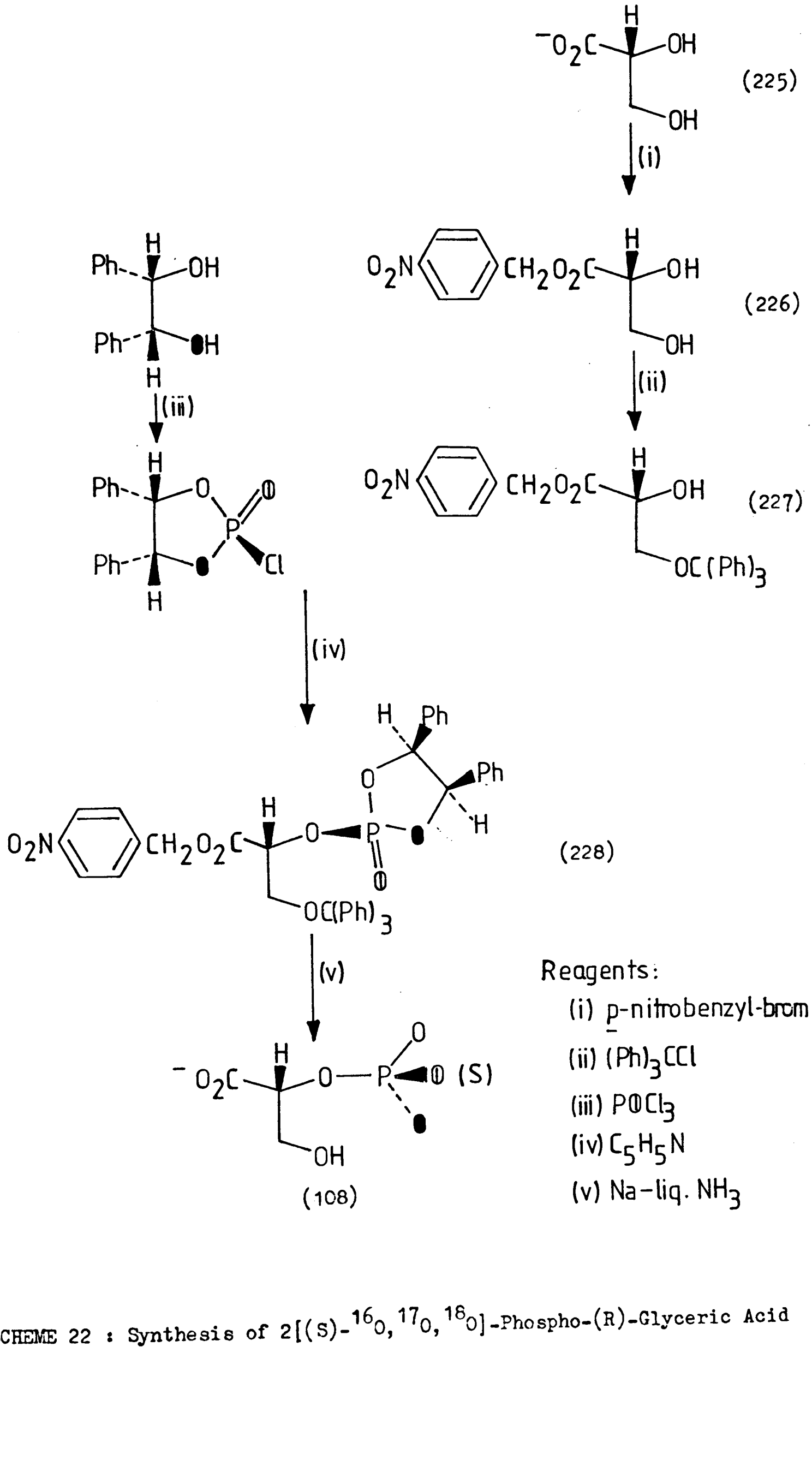
INTRODUCTION

In view of the vast range of kinase reactions it is perhaps necessary to explain why pyruvate kinase was the first enzyme to be chosen for investigation after hexokinase. Pyruvate kinase appeared particularly attractive for a pioneering study since the equilibrium constant for the conversion of PEP to ATP is 6.45×10^3 at pH 7.4[397], and thus adenosine-[γ-^{16}O, ^{17}O, ^{18}O]-triphosphate (111) can be generated in almost quantitative yield from chirally labelled phosphoenol pyruvate.

It was decided that 2-[(*S*)-^{16}O, ^{17}O, ^{18}O]-phospho-(*R*)-glyceric acid (108) would be an appropriate synthetic objective since this could be converted to 2[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphoenol pyruvate (109) enzymically with enolase and the phosphoryl transfer effected with pyruvate kinase. This sequence is illustrated in SCHEME 21.

Synthesis of 2[(*S*)-^{16}O, ^{17}O, ^{18}O]-phospho-(*R*)-glyceric acid

The synthetic scheme developed by Dr. P.M. Cullis in this laboratory is illustrated in SCHEME 22. (*R*)-Glyceric acid (225) is not commercially available and for this reason all preliminary investigations were performed on unlabelled racemic material. Since pyruvate kinase only handles the (*R*) form of 2-phospho-glycerate it was desirable to find a source of (*R*)-glyceric acid. Although a chemical synthesis from optically active starting material has been reported[398] the most direct way of obtaining this material was established to be the hydrolysis of commercially available 3-phospho-(*R*)-glyceric acid by alkaline phosphatase. The (*R*)-Glyceric acid prepared in this way was converted directly to the *p*-nitrobenzyl ester (226) by reaction with *p*-nitrobenzyl bromide (224) by Mr. D.C. Bailey. Reaction of this compound with triphenylmethyl chloride gave *p*-nitrobenzyl-3 0-triphenylmethyl-(*R*)-glycerate (227), and the ^1H n.m.r. spectrum in DMSO showed that



SCHEME 22 : Synthesis of 2[(S)-^{16}O, ^{17}O, ^{18}O]-Phospho-(R)-Glyceric Acid

the glycerate methine proton was coupled to the secondary hydroxyl group thus demonstrating the selective protection of the 3-position.[399] Next, reaction of the protected glycerate (227) with the chirally labelled phosphorylating reagent (102b) in pyridine under the usual conditions gave (2*R*,4*S*,5*R*)-2-(*p*-nitrobenzyl 3'-O-triphenylmethyl-(*R*)-glycero-)-[2-^{17}O]-oxo-4,5 diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane (228) which showed a single sharp ^{31}P resonance at +11.8 ppm which split into a quartet (two overlapping triplets) when coupled to ^{1}H thus demonstrating that the five-membered ring was intact. The coupling constant of $^{3}J_{PH}$ = 8.0 Hz for the methine protons of the 4,5-positions of the 1,3,2-dioxaphospholane ring was obtained from this spectrum. This triester was hydrogenolysed in ethyl acetate using a Pd/C catalyst but this attempt at deprotection was apparently unsuccessful as an enzyme assay with the coupled system enolase/pyruvate kinase/lactate dehydrogenase did not reveal any 2-phosphoglyceric acid. However, deprotection with sodium in liquid ammonia under rather precise conditions did result in the required compound. Thus the triester (228) in dry ether was added to a solution of excess sodium in liquid ammonia at -33 °C under dry nitrogen and the solution stirred for 10 min before being quenched by addition of solid ammonium chloride. Enzyme assay confirmed the production of 2[(*S*)-^{16}O, ^{17}O, ^{18}O]-phospho-(*R*)-glycerate (108) in *ca.* 35% yield showed a single sharp ^{31}P n.m.r. resonance at +1.27 ppm which split into a doublet with $^{3}J_{pH}$ = 8.8 Hz when coupled to ^{1}H.

Pyruvate kinase catalysed transfer from 2[(*S*)-^{16}O, ^{17}O, ^{18}O]-phospho-(*R*)-glycerate

The enzyme substrate (108) was not isolated but was incubated with enolase, pyruvate kinase and a twofold excess of ADP and allowed to come to equilibrium at 37 °C. After *ca.* 1½ h the enzymes were denatured and the labelled ATP formed by the

phosphoryl transfer was isolated and purified by ion-exchange chromatography on DEAE Sephadex A-25 to give adenosine-5'[γ-^{16}O, ^{17}O, ^{18}O]-triphosphate, finally isolated as the tetrasodium salt in 30% overall yield based on the starting diol.

Stereochemical Analysis of the Pyruvate Kinase Reaction

The chirally labelled ATP from the pyruvate kinase catalysed phosphoryl transfer was subjected to the same stereochemical analysis as described for hexokinase in CHAPTER 12. Thus the hexokinase catalysed phosphoryl transfer gave *D*-glucose-6-[^{16}O, ^{17}O, ^{18}O]-phosphate of unknown isotopic chirality at phosphorus, but by a step which was known to proceed with inversion of configuration. Cyclisation and methylation gave the usual triesters which were examined by ^{31}P n.m.r. as previously described The spectra are presented in FIGURE 121 and since the central line intensities for each diastereoisomer are reversed from that found for hexokinase this demonstrates that the pyruvate kinase catalysed phosphoryl transfer has also proceeded with *inversion* of configuration at phosphorus. This process is illustrated in FIGURE 122, and the calculation is as follows.

The chirally labelled ATP used in this experiment was of slightly inferior quality to that used for hexokinase and was in fact slightly racemised. This reflected the same quality in the precursor 2[(*S*)-^{16}O, ^{17}O, ^{18}O]-phospho-(*R*)-glyceric acid, the source of this complication of course, being traced back to slightly inferior [^{18}O]-diol. Although detailed numerical data on the diol were not available in this case, in the light of later work with this system we can make very reasonable estimations, and moreover the ^{31}P n.m.r. spectrum of the actual ATP provides a firm basis for calculation. From the relative intensities of the P_γ and P_β lines in this spectrum we can draw up TABLE 15.

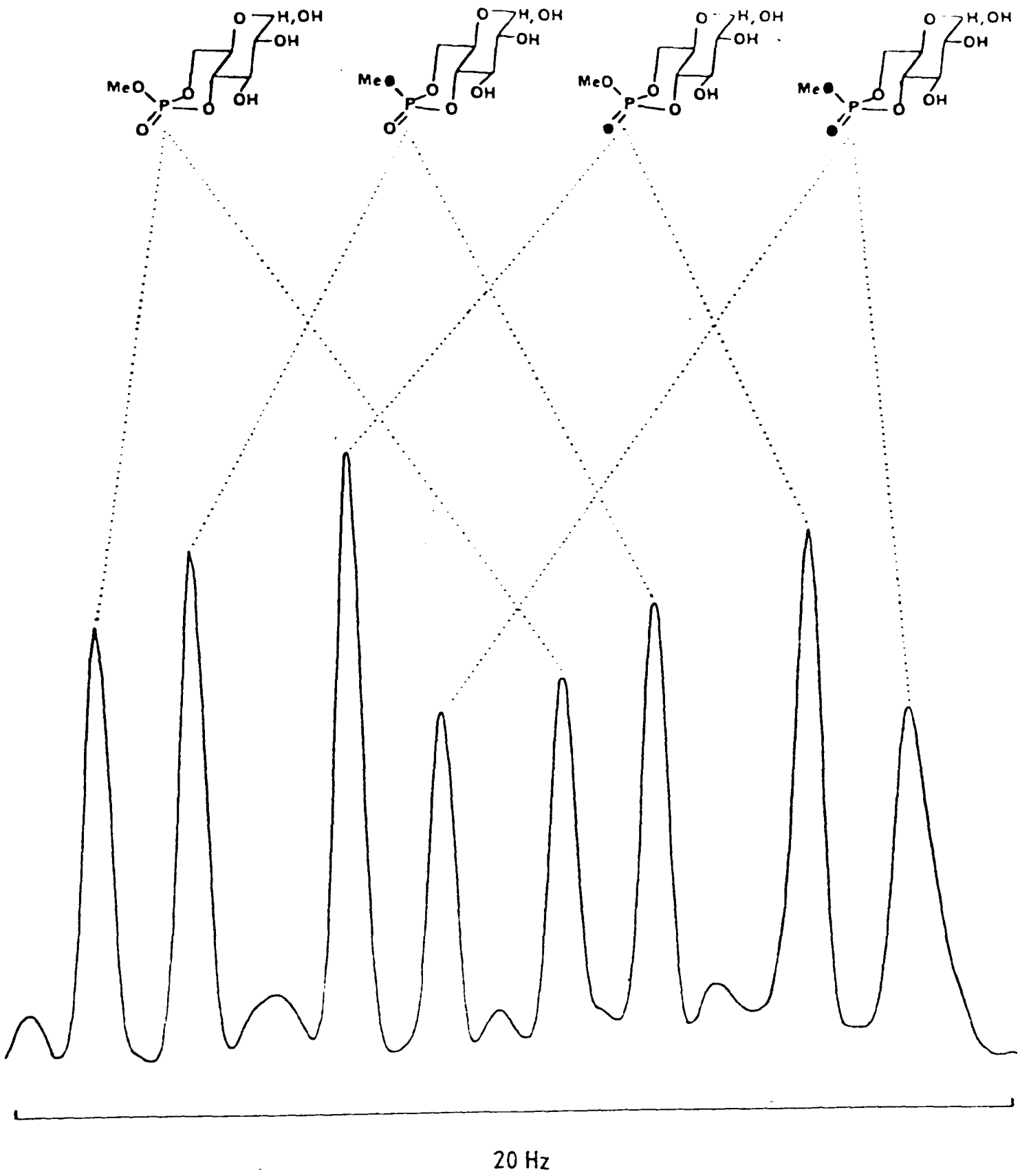


FIGURE 121 (a) : The 121.5 MHz ^{31}P n.m.r. spectrum in 67% CD_3OD and 33% DMSO of the axial diastereoisomer derived by cyclisation followed by methylation of D-glucose-6[^{16}O, ^{17}O, ^{18}O]-phosphate of unknown isotopic chirality obtained by the pyruvate kinase catalysed transfer of the phosphoryl group from 2[(S)-^{16}O, ^{17}O, ^{18}O]-phospho-(R)-glycerate to ADP and the hexokinase catalysed transfer of the phosphoryl group from the resulting adenosine-5'[γ-^{16}O, ^{17}O, ^{18}O]-triphosphate to D-glucose.

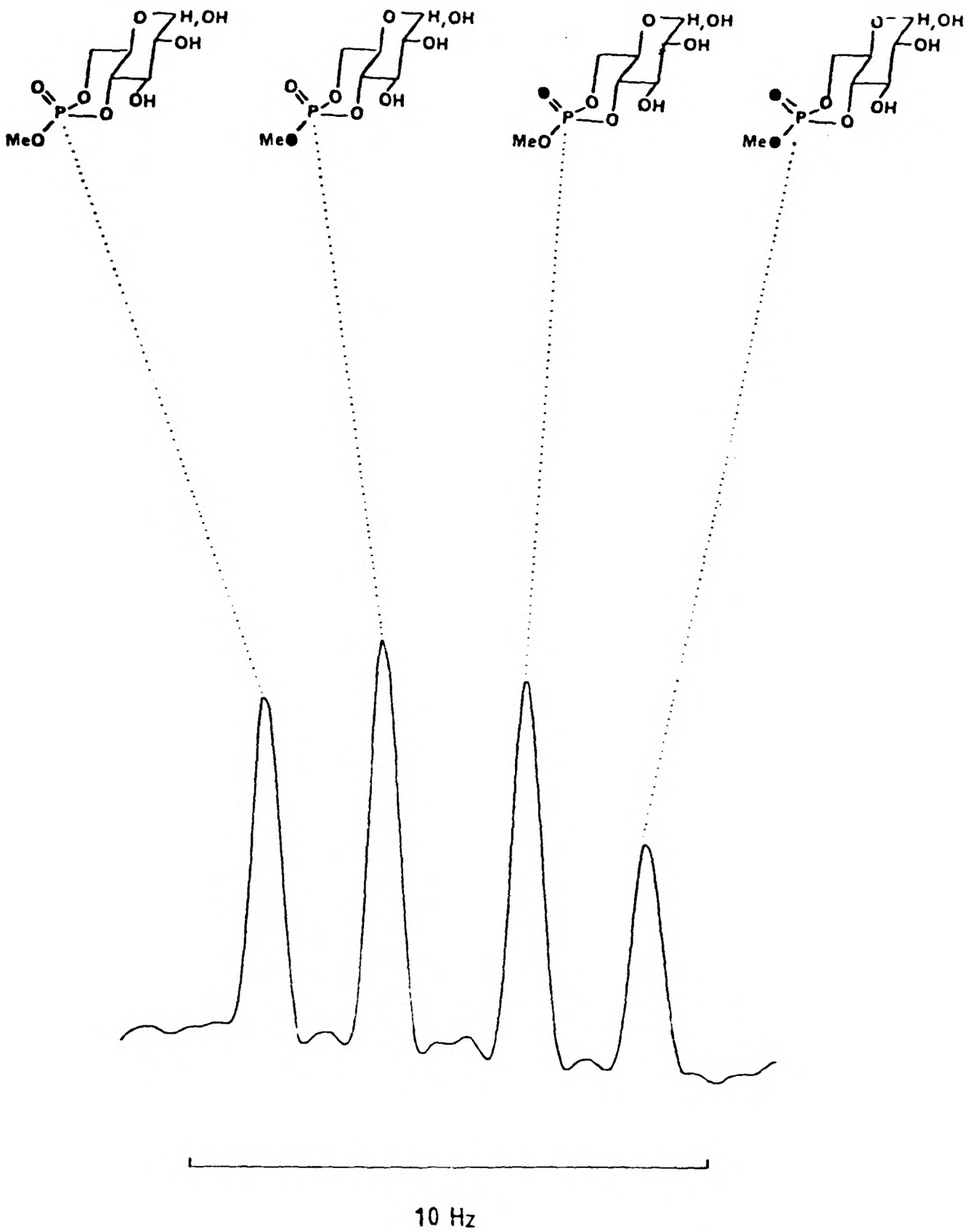
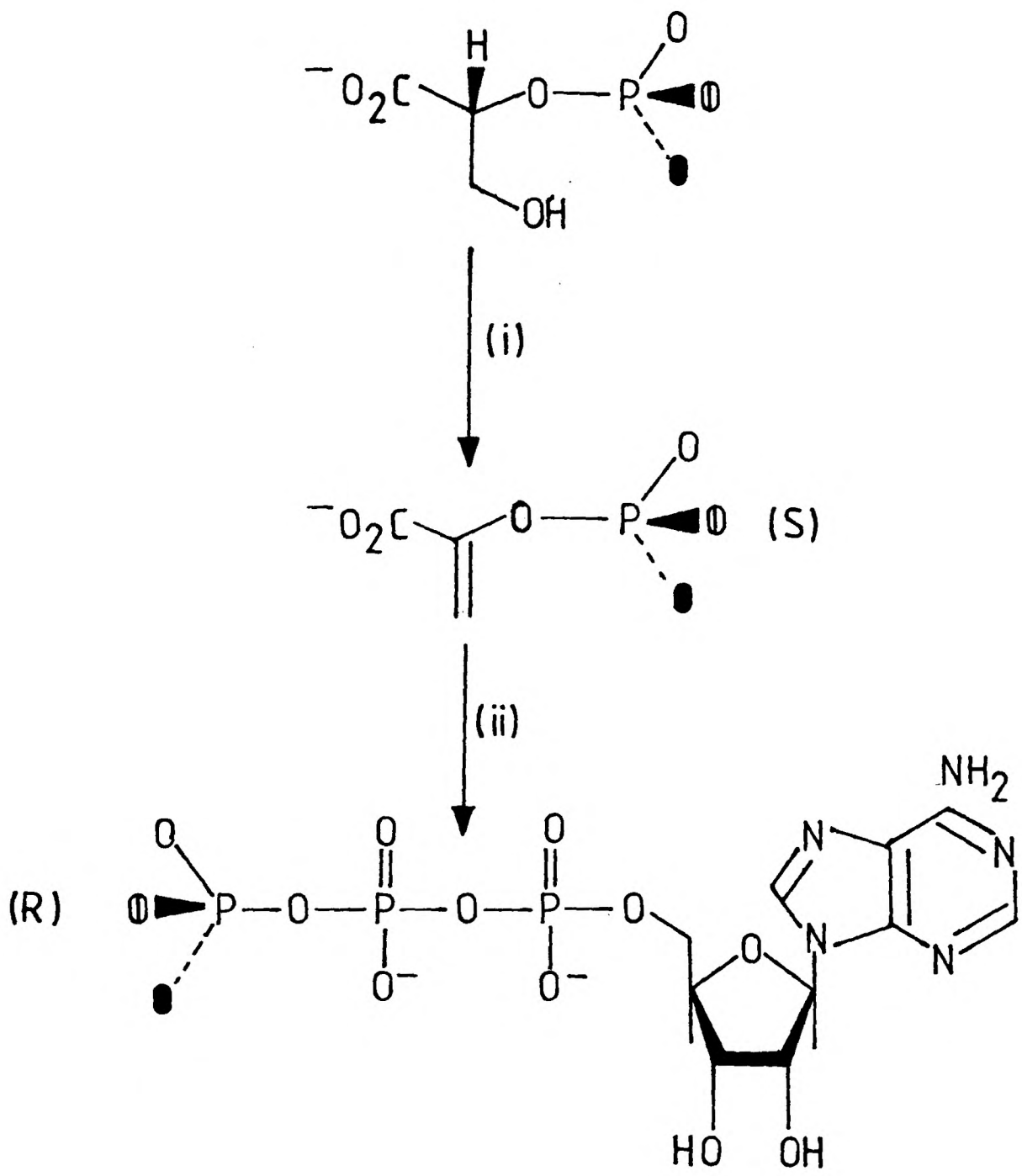


FIGURE 121 (b) : The 121.5 MHz ^{31}P n.m.r. spectrum of the equatorial diastereoisomer as in FIGURE 121 (a). The ^{31}P n.m.r. parameters were: offset, 2240 Hz; O2, 5980 Hz; sweep width, 2000 Hz; pulse width 16 μs; acquisition time, 2.05 s; gaussian multiplication (line broadening, -0.9 Hz; gaussian broadening, 0.35 Hz) in 8K and FT in 32K; broad band proton decoupling. The vertical gain control for both axial and equatorial diastereoisomers is the same. The anomers of the equatorial triester are not resolved in this spectrum.



Enzymes: (i) enolase

(ii) pyruvate kinase

FIGURE 122 : Pyruvate Kinase Catalysed Phosphoryl Transfer from 2[(S)-^{16}O, ^{17}O, ^{18}O]-Phospho-(R)-Glycerate.

TABLE 15: ISOTOPIC COMPOSITION OF ADENOSINE-5'[γ-^{16}O, ^{17}O, ^{18}O]-TRIPHOSPHATE

No. of Labels	0	1x^{17}O	1x^{18}O	2x^{18}O	[^{16}O, ^{17}O, ^{18}O]	[^{17}O, $^{18}O_2$]	3x^{18}O
% Contribution	7.2	10.3	17.3	35.1	21.0	3.4	5.7

From this data we can calculate the expected normalised isotope pattern for initially 100% *inversion*, assuming the [^{16}O, ^{17}O, ^{18}O]-centre to be, as for hexokinase, 13% (*R*) and 87% of the (*S*) configuration. The comparison between observed and expected values is made in TABLE 16.

TABLE 16: A COMPARISON OF OBSERVED AND CALCULATED NMR INTENSITIES FOR THE DPPC-BuOK CYCLISATION OF *D*-GLUCOSE-6[^{16}O, ^{17}O, ^{18}O]-PHOSPHATE DERIVED FROM THE PYRUVATE KINASE PHOSPHORYL TRANSFER

	Equatorial Triester			Axial Triester		
	Observed	Calculated		Observed	Calculated	
		RETN.	INVN.		RETN.	INVN.
MeO-P=O	0.86	0.80	0.80	0.71	0.80	0.80
Me●-P=O	1.00	0.81	1.00	0.85	1.00	0.81
MeO-P=●	0.90	1.00	0.81	1.00	0.81	1.00
Me●-P=●	0.52	0.73	0.73	0.62	0.73	0.73

RETN. = Retention, INVN. = Inversion.

An examination of these figures shows that agreement is good. Agreement would be maximal if the actual configuration of the chiral phosphate had been 20% (*R*) and 80% (*S*), and since it could

not be better than 13% (*R*) and 87% (*S*) this leaves us with 7% extra (*R*) to account for which is probably actually not far from the range of experimental error. In any case we know from the ATP spectrum in this instance that the diol used was slightly worse than previously - using these figures means it turns out to be *ca.* 7% worse, which is quite reasonable. In any case there is no doubt that the phosphoryl transfer has proceeded with *inversion* of configuration for this is clear from inspection of the spectra in FIGURE 121, and in this case it can be said again that the stereoselectivity of this reaction is probably better than 90%. If, as has been demonstrated for pyruvate kinase[198], there is some evidence for a $S_N1(P)$ pathway (however, see CHAPTER 6) there is o course a faint chance that some racemisation may occur in the enzyme reaction by rotation of the metaphosphate, although this is considered to be highly unlikely, and indeed, would probably not be detected in a single turnover experiment like the one performed here.

Although we cannot rule out the possibility of a small degree of enzyme catalysed isotopic racemisation in view of these figures, we regard this occurrence as unlikely and we can be sure that the bulk of the racemisation observed was derived solely from the synthetic procedures.

Pyruvate kinase catalyses phosphoryl transfer with *inversion* of configuration. This observation rules out the adjacent attack with pseudorotation and the double displacement mechanism *via* a phosphoenzyme intermediate leaving the two 'in line' processes as most likely. A proper choice between the $S_N1(P)$ and $S_N2(P)$ mechanisms cannot be made at present in view of the doubts expressed in CHAPTER 6 concerning the $S_N1(P)$ mechanism, so we must be content with merely describing the phosphoryl transfer as an 'in line' process.

Thus this kinase as well as hexokinase catalyses both phosphoryl transfer and thiophosphoryl transfer with inversion of configuration.

Addendum to CHAPTER 14: The Stereochemical Course of Rabbit Muscle Phosphofructokinase

Very recent results in this laboratory carried our in collaboration with Mr. R. Jarvest, have led to an insight on the stereochemical course of the vital glycolytic allosteric control enzyme phosphofructokinase, which has not been previously investigated even using thiophosphate analogues. The key to this investigation was the synthesis of *sn*-glycerol 3[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate (229) which was accomplished by the general method of synthesis as outlined in FIGURE 123 by phosphorylation of 1,2-dipalmitoyl-*sn*-glycerol and subsequent deprotection.

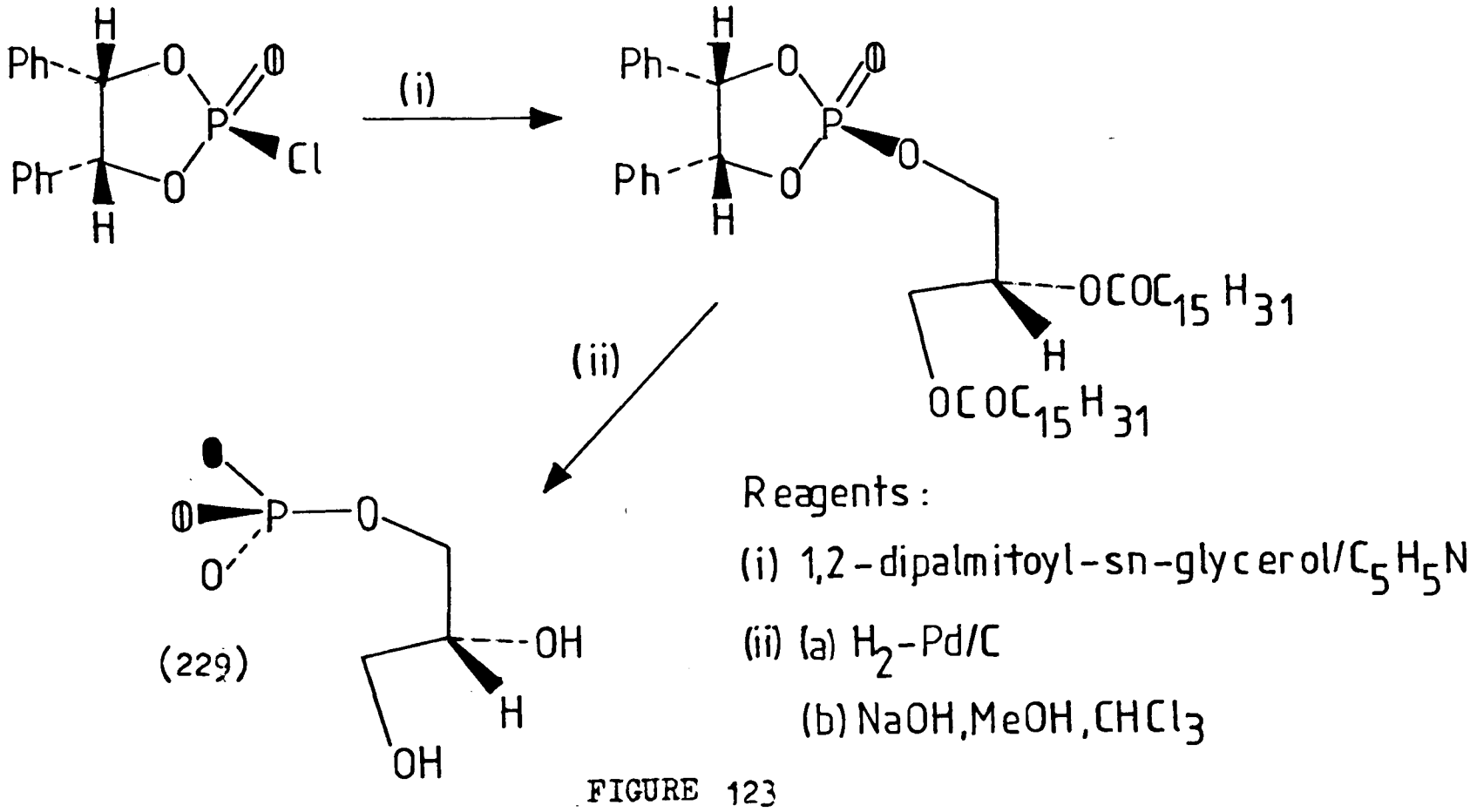
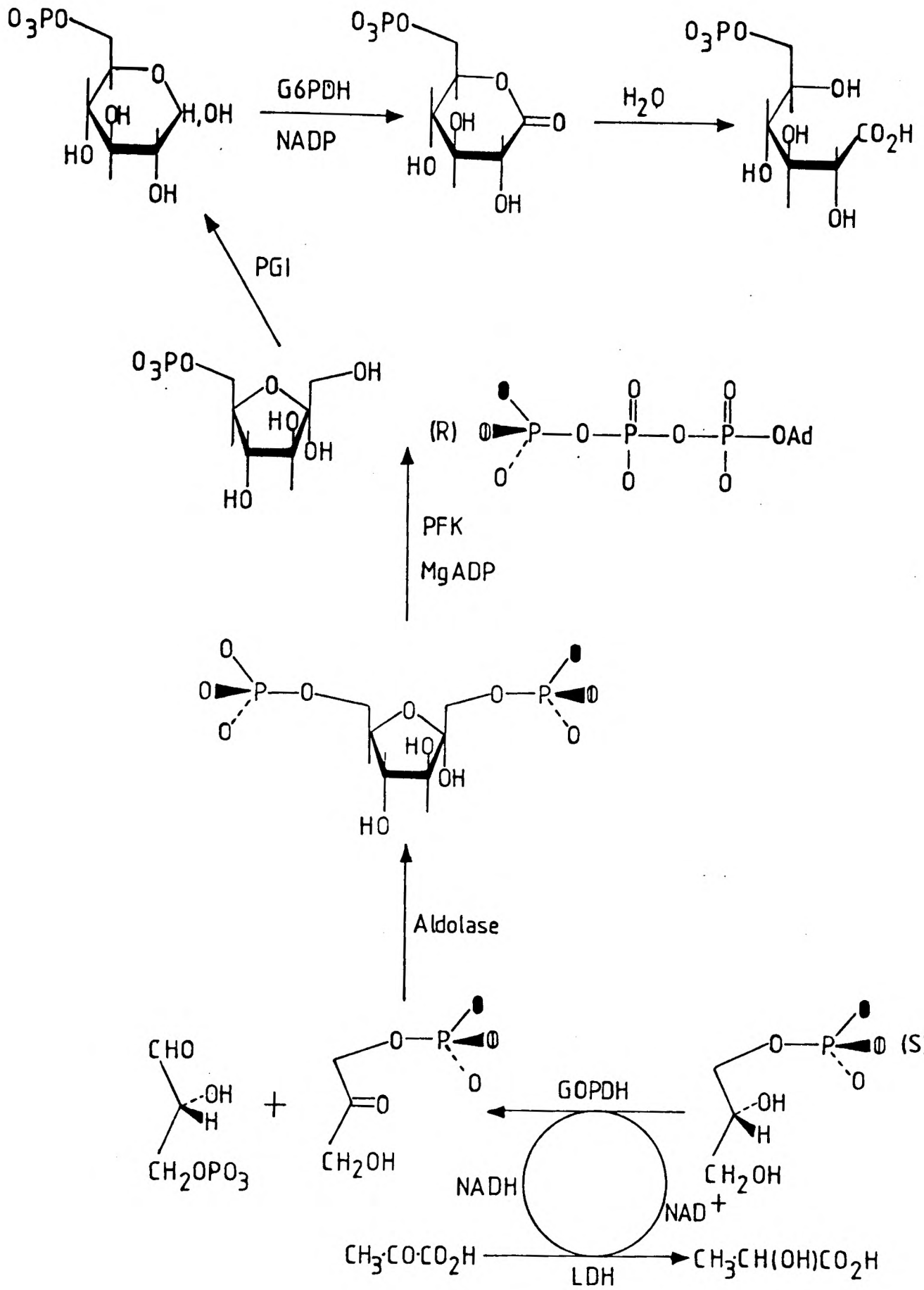


FIGURE 123

The *sn*-glycerol 3[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate was incubated with the enzymes and cosubstrates as indicated in FIGURE 124. 3-Chloro-acetol phosphate was added to the reaction mixture to inhibit possible traces of triose phosphate isomerase[400] (see FIGURE 112) which would catalyse the interconversion of



Enzymes : G6PDH—glucose-6-phosphate dehydrogenase
PGI—phosphoglucose isomerase
PFK—phosphofructokinase
GOPDH-glycerol-3-phosphate dehydrogenase
LDH lactate dehydrogenase
aldolase

FIGURE 124 : [$^{16}O, ^{17}O, ^{18}O$]-Phosphoryl Transfer catalysed by Phosphofructokinase

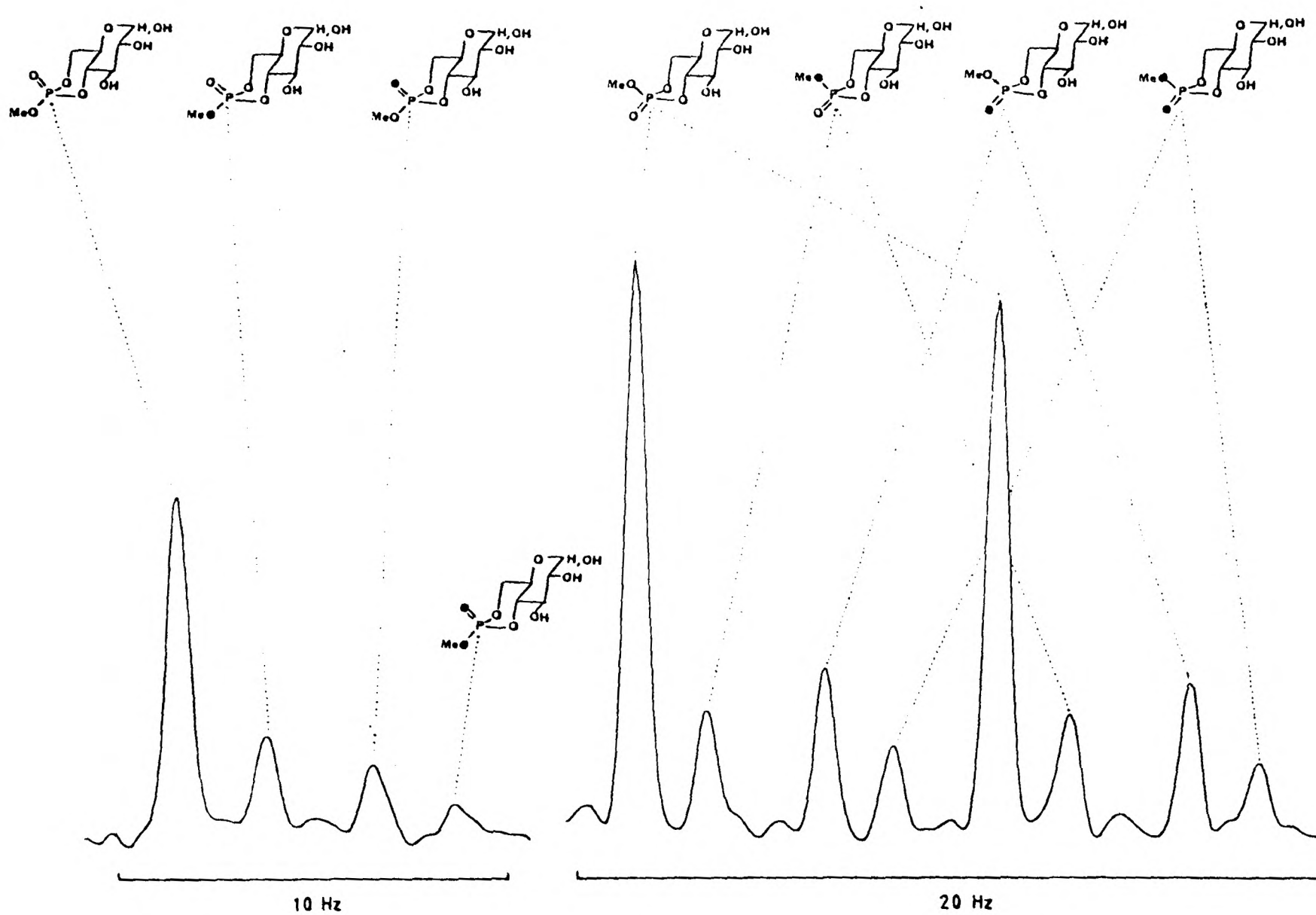


FIGURE 125 : The 121.5 MHz ^{31}P n.m.r. spectra of the axial and equatorial diastereoisomers of D-glucose-4,6-[^{16}O,^{17}O,^{18}O]-cyclic-phosphate derived from the phosphofructokinase catalysed phosphoryl transfer from sn-glycerol-3[(S)-^{16}O,^{17}O,^{18}O]-phosphate

dihydroxyacetone phosphate and *D*-glyceraldehyde-3-phosphate. In the absence of triose phosphate isomerase the [^{16}O, ^{17}O, ^{18}O] group should be exclusively transferred to ADP in a reaction sequence involving only one phosphoryl transferase namely phosphofructokinase. The reaction was driven by converting the *D*-fructose-6-phosphate to *D*-glucose-6-phosphate and then to *D*-gluconolactone-6-phosphate which spontaneously hydrolyses. The adenosine-5'-[^{16}O, ^{17}O, ^{18}O]-triphosphate was isolated and then incubated with *D*-glucose and yeast hexokinase and the *D*-glucose-6[^{16}O, ^{17}O, ^{18}O]-phosphate isolated and analysed as before. The results, which will be fully presented and discussed elsewhere[401,402], indicate that rabbit muscle phosphofructokinase catalyses phosphoryl transfer between *D*-fructose-1,6-bisphosphate and ADP with *inversion* of configuration at phosphorus. The stereochemistry of phosphofructokinase from *B. Stearothermophilus* is also being investigated in this laboratory, and has also been found to proceed with inversion (see FIGURE 125).

General Stereochemical Conclusions for the Glycolytic Enzymes

Yeast hexokinase[217], rabbit muscle pyruvate kinase[218], rabbit muscle phosphofructokinase[402] and glycerol kinase[214] all catalyse phosphoryl transfer with *inversion* of configuration, which is most simply interpreted in terms of an 'in line' transfer of the phosphoryl group between substrates in the ternary complex. To determine whether this transfer occurs by an associative or dissociative mechanism requires independent evidence which can be provided by a positional isotope exchange experiment.

Hexokinase, pyruvate kinase and glycerol kinase have previously been shown to catalyse thiophosphoryl transfer with inversion of configuration at phosphorus[209] so that the stereochemical courses of *three* glycolytic kinases have now been shown to occur with inversion of configuration for both phosphoryl and thiophosphoryl

transfer. It appears, therefore, that when the natural substrate adopts an 'in line' mechanism the thiophosphate analogue will follow the same stereochemical course. It will, however, be necessary to examine the stereochemical courses of many enzymes using isotopically labelled natural substrates before any firm generalisations can be considered secure.

Phosphoglyceromutase (both from wheat germ and rabbit muscle) is the only other glycolytic transferase which has been investigated using isotopically labelled natural substrates and here retention of configuration was observed.[239] This is in accord with an adjacent mechanism with pseudorotation or a double displacement mechanism. Since this enzyme is known to form a kinetically competent phosphorylenzyme intermediate, the double displacement mechanism provides the simplest interpretation of all the experimental evidence. Since phosphoglucomutase also possesses such an intermediate it is likely that this also follows a double displacement mechanism, and indeed, this is the next enzyme to be investigated in this laboratory.

The question naturally arises as to why the phosphokinases have evolved a catalytic mechanism with direct phosphoryl transfer between substrates in the ternary complex, whereas the phosphomutases have evolved a double displacement mechanism involving a phosphoenzyme intermediate. A possible explanation is provided by the fact that the two substrates for a phosphokinase are grossly different so that the enzyme must provide a distinct binding site for each substrate. By requiring both substrates to be present simultaneously at the active site the 'in line' mechanism can be utilised. By contrast the two substrates (and cosubstrate) for the mutases possess the same carbon oxygen skeleton, so that a single binding site could suffice to bind both substrates, but not simultaneously. The phosphoryl group to be transferred must

therefore become temporarily bound to the enzyme while substrate exchange takes place. Since the phosphoryl transfer potential of the substrates which the mutases transform is low, the intervention of a covalent phosphoenzyme intermediate could also have a catalytic advantage. So by providing a refuge for the phosphoryl group on the enzyme, the overall activation energy for phosphoryl transfer should be lowered, and the need to evolve more than one substrate binding site unnecessary.

Is it reasonable that 'in line' displacements should be preferred over 'adjacent' displacements? It does seem more likely that 'in line' S_N2 type reactions could be more readily catalysed than 'adjacent' displacements from what we know of the physical organic chemistry of reaction at phosphate esters.[188,403] To catalyse a displacement at phosphorus the enzyme presumably needs a general base (B) to deprotonate the attacking group (ROH), a general acid (HA) to assist the departure of the leaving group (R'OH) and some disposition of functional groups to stabilise the transition state for the displacement. For an 'in line' displacement the transition state will have the trigonal bipyramidal structure shown in FIGURE 126, in which there are partial negative charges on each of the equatorial oxygen atoms. Provided that the enzyme can provide these electron deficient sites, such as positive charges or hydrogen

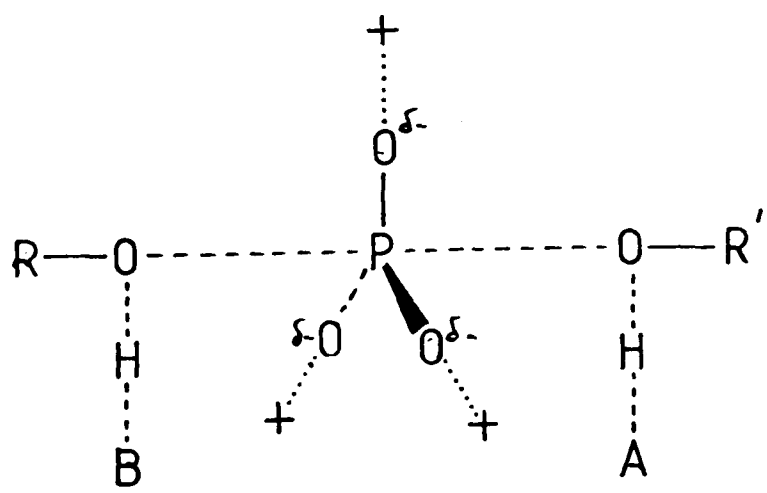


FIGURE 126

bond donors, positioned so as to stabilise the trigonal bipyramidal arrangement rather than the tetrahedral disposition of oxygen atoms in either the starting material or product, then catalysis will result. In contrast the positioning of those groups required to catalyse an adjacent displacement, necessarily involving at least one pseudorotation, is clearly much more demanding. Thus mechanistic economy, in the absence of any facts that suggest otherwise, favours the 'in line' path. With the accumulation of evidence cited in this thesis and elsewhere, the phosphoenzyme intermediate is an unnattractive alternative to the simple elegance of direct 'in line' transfer between the two participating substrates. The stereochemical conclusions reported herein, combined with the large number of high resolution crystal structures of the phosphokinases that are now emerging, will provide the more precise mechanistic definition of these important enzymes.

CHAPTER 15

EXPERIMENTAL

MATERIALS AND METHODS

Oxygen-18 enriched water (99.5 atom %) was obtained from Prochem. Ltd. Oxygen-17 enriched water (44.0 atom %) was obtained from the Monsanto Research Corportion, Miamisburg, Ohio. Deuteriated water was obtained from the Ryvan Chemical Company, Southampton, and from Fluorochem. Ltd., Glossop, Derbys. Tritiated water was obtained from the Radiochemical Centre, Amersham, Bucks. Deuteriochloroform containing 1% TMS was obtained from Merck Sharpe and Dohme, Canada Ltd., and Nuclear Magnetic Resonance Ltd., High Wycombe, Bucks. Deuteriomethanol and dimethylsulphoxide-d_6 were obtained from Service des Molécules Marquées, France. DEAE Cellulose DE52 was purchased from Whatman Ltd., and DEAE Sephadex A-25 from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Dowex 50W 50X4-200R was purchased from Sigma Chemical Co., London, Ltd. AMP, disodium ADP and disodium ATP were purchased from Boehringer Mannheim GmbH, Germany. Disodium NADH, NADP, 3',5'-cAMP, 5-acetyl coenzyme A, 3-acetyl pyridine adenine dinucleotide, monosodium fumarate and thiamine were purchased from Sigma, London, Ltd. (2*R*) and (2*S*)-Malic acids and 2-amino-4,5-dimethyl thiazole were obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset. Hexafluoro-phosphoric acid was obtained from Cambrian Chemicals Ltd., Croydon, Surrey, and tetrafluoroboric acid from BDH Chemicals Ltd., Poole, Dorset. Methyl iodide was distilled before use and stored over Hg in a dark bottle. Hydrogenation catalysts were obtained from Johnson Matthey Chemicals Ltd., London.

All other chemicals were Analar grade or of the highest purity commercially available and were purchased from BDH Chemicals Ltd., Koch Light Laboratories Ltd., Colnbrook, Bucks., Fisons Scientific Apparatus Ltd., Loughborough, or Aldrich Chemical Co. Ltd.

Fumarase, hexokinase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, citrate synthase and malic enzyme were purchased

from Sigma London Ltd., as crystalline suspensions in 3.2 *M* ammonium sulphate. Acetyl coenzyme A synthetase was purchased from Sigma London Ltd., and from Boehringer Mannheim GmbH.

E. Coli ATCC 9637 was obtained from the American Type Culture Collection, and other microorganisms were from cultures kept by Mr. John Keeping who also provided assistance with the microbiological work.

Solvents were purifed as follows[414]: Chloroform for use in DAST reactions was freed from ethanol by passage through an alumina column and distillation from magnesium sulphate. Anhydrous methanol and ethanol were obtained by distillation from magnesium and iodine. Carbon tetrachloride, chloroform and dichloromethane were purified and dried by distillation from phosphorus pentoxide and stored under nitrogen in dark bottles. Benzene and toluene were distilled from phosphorus pentoxide and stored over sodium wire. AR acetone was always used. Commercial peroxide-free ether was dried with sodium wire. Petrol (40-60°) was fractionally distilled and dried with sodium wire when required. Ethyl acetate was fractionally distilled from anhydrous potassium carbonate and stored over Linde type 4A molecular sieves. *t*-Butanol was distilled from calcium hydride under nitrogen immediately before use. Reference to potassium *t*-butoxide refers to freshly prepared reagent made by dissolving potassium metal in fresh *t*-butanol under nitrogen Acetonitrile was distilled from calcium hydride and then from phosphorus pentoxide and was stored over Linde type 4A molecular sieves. Pyridine was refluxed with potassium hydroxide, distilled and stored over calcium hydride or potassium hydroxide pellets. Dimethylsulphoxide was stirred with calcium hydride, distilled under reduced pressure and stored under dry nitrogen over Linde type 4A molecular sieves. N,N-dimethyl formamide was stirred overnight with anhydrous potassium carbonate, refluxed with phthalic

acid under reduced pressure of dry nitrogen, distilled and stored under dry nitrogen in a dark bottle. Dioxan and tetrahydrofuran were passed down an alumina column, refluxed with sodium and benzophenone until a permanent deep blue colour appeared, distilled and stored over sodium wire under dry nitrogen.

High grade deionised water used for preparing all buffers was obtained from a Fisons Fi-streem distillation system and a MilliQ3 water purifying system supplied by the Millipore Corp., Bedford, Mass.

Melting points were determined on a Kofler block and are uncorrected. Optical densities, enzyme assays and turbidimetric measurements were performed on Pye Unicam SP1800 and SP8-100 spectrophotometers. A Radiometer titrator type TTT1c, standardised against BDH buffers was used to determine pH values. Thin layer and preparative layer chromatography plates were prepared by Mr. P. Prior, and t.l.c. on silica gel H plates refers to the use of unbaked plates impregnated with a fluor and visualised under u.v. light at 254 nm and/or 366 nm. Preparative g.l.c. was performed on a PEGA column obtained from Pye Unicam Ltd., and fitted to a Pye Unicam series 108 chromatograph. Microanalyses were performed by Dr. F.B. Strauss and his staff. Mass spectrometry was performed by Dr. R.T. Aplin and his staff on AE1 MS9 and Varian CH7 instruments. High resolution mass spectrometry was performed by Mr. D. Perrin of the Dept. of Pharmacology on a V.G. Micromass 7070F mass spectrometer. Infrared spectra were performed on a Unicam SP1000 i.r. spectrometer. Optical rotations were performed by Mrs. A. Rivers on a PE241 photoelectric polarimeter. Scintillation counting was performed on an LKB liquid scintillation counter in a mixture comprising: naphthalene (120 g), ethanol (640 ml), *p*-bis[2-(5-phenyloxazolyl)benzene] (0.24 g) and 2,5-diphenyl oxazole (6.0 g) made up to 2.5 ℓ with Analar toluene.

Routine proton nuclear magnetic resonance (^{1}H n.m.r.) was performed on a Hitachi Perkin-Elmer R24A spectrometer at 60 MHz. Service ^{1}H n.m.r. spectra were recorded by Lady E.E. Richards and her staff on a Perkin Elmer R32 spectrometer at 90 MHz or on a Perkin Elmer R14 spectrometer at 100 MHz. Pulsed Fourier Transform spectra were recorded on a Bruker WH300 spectrometer at 300 MHz. ^{19}F n.m.r. spectra were recorded at 84.67 MHz on either the Perkin Elmer R32 spectrometer (continuous wave) or the Bruker WH90 spectrometer in the Fourier Transform mode. FT ^{13}C n.m.r. spectra were recorded on a Bruker WH300 spectrometer at 75.47 MHz. ^{31}P n.m.r. spectra were obtained at 36.43 MHz on a Bruker WH90 spectrometer in the FT mode with broad band proton noise decoupling. Signal averaging was performed by a Bruker data system BNC12 computer interfaced with the spectrometer. Sample temperature was controlled by a Bruker variable frequency temperature controller. Aqueous samples containing at least 10% D_2O (providing a field frequency lock on the deuterium resonance) were contained in 10 mm outside diameter precision n.m.r. tubes (Wilmad Glass Co., Buena, New Jersey) fitted with teflon vortex supressors. Samples in other solvents were contained in 8 mm outside diameter tubes which were mounted coaxially inside 10 mm tubes containing D_2O to provide the lock signal. In all cases the chemical shifts (denoted by the symbol δ) are reported as positive when the resonances are downfield from the reference signal at 0 p.p.m. Chemical shifts are referred to internal tetramethylsilane (TMS) or sodium 2,2-dimethyl-2-silapentane-5-sulphonate (TSS) for ^{1}H n.m.r. spectra, external tetramethylsilane for ^{13}C spectra, external trimethylphosphate (TMP) in D_2O for ^{31}P n.m.r. spectra and external trifluoroacetic acid (TFA) for ^{19}F spectra.

Ion-exchange chromatography used DEAE cellulose or DEAE Sephadex A-25 resins in the bicarbonate form with a linear concentration

gradient of aqueous TEAB applied by an LKB 1130 Ultrograd gradient mixer and an LKB Perpex peristaltic pump. The column effluent was monitored by an LKB Uvichord II continuous flow detector at 254 nm, coupled to a chart recorder, before passing to an LKB 7000 Ultrorac fraction collector which was linked to the chart recorder through an event marker. Concentration of dilute aqueous acetyl coenzyme A synthetase solutions was achieved in a ultrafiltration cell model 12 (10 ml capacity) or model 52 (60 ml capacity) manufactured by the Amicon Corp., Lexington, Mass., using a diaflo ultrafiltration membrane type PM10. An Amicon macrosolute concentrator was used for dialysis of fumarase solutions.

SYNTHETIC

2-Diazonium-4,5-dimethyl thiazole hexafluorophosphate (16)

2-Amino-4,5-dimethyl thiazole (0.5 g, 3.9 mmole) from the hydrobromide was suspended in water (4 ml) and concentrated hydrochloric acid (2 ml) added, followed by sodium nitrite (0.5 g, 7.2 mmole). To the clear green solution was added hexafluorophosphoric acid (2 ml) and the immediate yellow precipitate which formed was collected at the pump and washed with water to give 2-diazonium-4,5-dimethyl thiazole hexafluorophosphate (0.5 g, 44%) which was dried *in vacuo* over phosphorus pentoxide. m.p. 105 °C (dec.); ^{1}H n.m.r. δ(DMSO-d_6) 2.0 (s,2*Me*); ^{19}F n.m.r. δ_F(DMSO-d_6)-5.5 (d, $^{1}J_{PF}$ = 723 Hz); i.r. (nujol) ν_{max} 2240 cm^{-1} ($-N_2^+$).

2-Fluoro-4,5-dimethyl thiazole (17)

(a) Pyrolysis of (16)

To a round-bottomed flask preheated to 120 °C were added small portions of the dry diazonium salt (16) which decomposed vigorously to give predominantly a red/brown tar and a small quantity of liquid. Extraction with ether and p.l.c. using 10% MeOH/$CHCl_3$ as

eluant gave 2-fluoro-4,5-dimethyl thiazole (50 mg, 22%). ^{1}H n.m.r. $\delta(CDCl_3)$ 2.00 (s,*Me*-, $^{5}J_{HF}$ = 0.6 Hz) 2.16 (s,*Me*-); ^{19}F n.m.r. $\delta_F(CDCl_3)$ 0.6 s

(b) Photolysis of (16)

The thiazole diazonium hexafluorophosphate (16) (or the tetrafluoroborate salt prepared *in situ*) was photolysed through pyrex as a suspension in water, agitation being provided by a stream of nitrogen. Work up after 4h gave 2-fluoro-4,5-dimethyl thiazole (57 mg, 27%).

2-Acetylbutyrolactone (19)

The title compound was prepared by the method of Reppe *et al.*[49] To γ-butyrolacetone (50 g, 0.58 mmole - which had been dried with magnesium sulphate and freshly fractionally distilled) warmed to 80 °C in a three necked flask fitted with a condenser, thermometer and dropping funnel were added over the course of 3h ethyl acetate (51 g, 0.58 mmole) and sodium metal (13.6 g, 0.59 mmole) in small portions. A further 15.5 g of ethyl acetate was added and after 4h the clear dark red solution was acidified with acetic acid (50%, 81 ml) and shaken with saturated brine (155 ml). The red oil which separated was neutralised with sodium bicarbonate, taken up in ether which was dried ($MgSO_4$) and evaporated to give the crude product (40 g) which was distilled under reduced pressure to give pure 2-acetylbutyrolacetone (32 g, 43%) b.p. 120.5-130 °C at 0.8 mm (lit.[49] 130-132/18 mm); ^{1}H n.m.r. $\delta(CDCl_3)$ 2.08 (s,3H,*Me*-), 2.25 (m,2H,-CH-C*H*$_2$-CH$_2$O-), 3.62 (t,1H,-C*H*-,J_{HH} = 8.0 Hz), 4.02 (t,2H,-CH$_2$C*H*$_2$O,J_{HH} = 7.5 Hz).

2-Acetyl-2-chlorobutyrolacetone (20)

The title compound was prepared by the method of Buchman.[50]

Freshly fractionally distilled sulphuryl chloride (34 g, 0.25 mmole) was added dropwise over 2.5h to 2-acetylbutyrolacetone (32 g, 0.25 mmole) stirred in a two-necked flask fitted with a dropping funnel and a rubber outlet tube. Copious fumes of sulphur dioxide and hydrogen chloride were evolved. The pale yellow mixture was washed with water and dissolved in ether which was dried ($MgSO_4$) and evaporated to give crude product. Distillation under reduced pressure gave 2-acetyl-2-chlorobutyrolactone as a colourless oil (28 g, 69%) b.p. 98 °C at 1 mm (lit.[50] 84-86 °C at 3 mm); ^1H n.m.r. $\delta(CDCl_3)$ 2.64 (s,3H,*Me*-), 3.18 (dt,2H,-C*H*$_2$CH$_2$O, J_{HH} = 8.0 Hz), 4.43 (t,2H,-CH$_2$C*H*$_2$O, J_{HH} = 6.5 Hz).

3-Chloropentan-5-ol-2-one (21)

The title compound was prepared by the method of Buchman.[50] 2-Acetyl-2-chlorobutyrolacetone (28 g, 0.17 mmole) was heated on a steam bath with water (28 ml) and concentrated hydrochloric acid (5 ml) for 1.5h. Decarboxylation occurred, as was evidenced by a vigorous reaction, and two layers formed on cooling. Both layers were extracted with ether which was dried ($MgSO_4$) and evaporated to give crude 3-chloropentan-5-ol-2-one as a pale yellow oil (21 g, 90%) which was not further purified. ^1H n.m.r. $\delta[(CD_3)_2CO]$ 2.31 (s,3H,*Me*-), 4.70-5.60 (m,5H,-C*H*CH$_2$C*H*$_2$OH).

2-Amino-4-methyl-5(2-hydroxyethyl)thiazole (22)

The title compound was prepared by the method of Todd *et al.*[51] Crude 3-chloropentan-5-ol-2-one (10 g, 73 mmole) was mixed with powdered thiourea (6 g, 73 mmol) and heated on a steam bath. A vigorous reaction occurred which rapidly subsided. After further heating for 10 min the mixture was cooled to give an oil which crystallised overnight. The solid was broken up, washed with ether and chloroform and recrystallised from ethanol-ethyl acetate to giv

2-amino-4-methyl-5(2-hydroxyethyl)thiazole hydrochloride as a pale pink solid. (7.74 g, 65%) m.p. 152-153 °C (lit.[51]-not recorded); ^{1}H n.m.r. δ(D_2O) 2.12 (s,3H,-*Me*), 2.80 (t,2H,-C*H*$_2$CH$_2$OH, $^3J_{HH}$ = 6 Hz), 3.7 (t,2H,-CH$_2$C*H*$_2$OH, $^2J_{HH}$ = 6.0 Hz); Found C, 37.22; H, 5.82; N, 14.31; S, 16.57; $C_6H_{11}N_2FOSCl$ gives C, 37.02; H, 5.66; N, 14.40; S, 16.57%.

2-Fluoro-4-methyl-5(2-hydroxyethyl)thiazole (9)

(a) Via the diazonium fluoroborate

2-Amino-4-methyl-5(2-hydroxyethyl)thiazole hydrochloride was converted to the free base by stirring with saturated sodium bicarbonate solution (50 ml) and extracting the solution with ethyl acetate (4 x 25 ml). Evaporation of the solvent *in vacuo* gave a red liquid which was dissolved in fluoroboric acid solution (20 ml) and cooled in an ice salt mixture to below 0 °C. The cold fluoroborate salt mixture was diazotised *in situ* by the addition of solid sodium nitrite (1.2 g, 17.4 mmole) added carefully over 20 min to the vigorously stirred solution. The resulting pale green cold solution of the diazonium fluoroborate was transferred to a 1.5 x 12.5 cm pyrex test tube contained in a pyrex Dewar containing ice-salt mixture, and photolysed through pyrex with strict temperature control (below 0 °C) using a 450 W medium pressure Hg vapour Hanovia lamp. Gas evolution became apparent after about 5 min and was virtually complete after *ca.* 4h when the now pale orange mixture was neutralised to pH 8.0 by the addition of first aqueous, then solid sodium bicarbonate, and the dark red mixture exhaustively extracted with ether for 6h. Evaporation of the ether gave the crude 2-fluoro-thiazole as an orange liquid which was purified on a column of silica gel (35 g) developed in 25/75 light petroleum (40-60 °C)/ethyl acetate to give on evaporation of fractions identified by t.l.c. 2-fluoro-4-methyl-5(2-hydroxyethyl)-thiazole as a pale yellow liquid (350 mg, 28%). ^{1}H n.m.r. δ($CDCl_3$)

1.18 (s,3H,*Me*-), 2.84 (dt,2H,-C*H*$_2$CH$_2$OH,J_{HH} = 6.2 Hz,J_{HF} = 3.0 Hz), 3.35 (broad s,1H,-CH$_2$O*H*), 3.77 (t,2H,-CH$_2$C*H*$_2$OH,J_{HH} = 6.2 Hz); ^{19}F n.m.r. $\delta_F(CDCl_3)$ 6.00 (t,J_{HF} = 3.0 Hz); $\delta_F[(CD_3)_2CO]$ 10.3; High resolution mass spectrum M^+ Calculated for C_6H_8NOFS 161.0310; Found 161.0298.

(b) Via the diazonium hexafluorophosphate

2-Amino-4-methyl-5(2-hydroxyethyl)thiazole hydrochloride (1.5 g, 7.7 mmole) was converted to the free base as above and dissolved in water (2 ml). Hexafluorophosphoric acid solution (5 ml) was added and the mixture cooled to below 0 °C in an ice-salt bath. Solid sodium nitrite (1.2 g, 17.4 mmole) was carefully added over 20 min during which time stirring became more difficult due to gel formation, so more hexafluorophosphoric acid (2 ml) was added. The resulting mixture was left at 0 °C for 1h and then photolysed and worked up as for (a) above to give the fluorothiazole (9) (330 mg, 24%).

4-methyl-5(2-hydroxyethyl)thiazole (3)

(a) From thiamine

The procedure of Williams[7] was used. Thiamine hydrochloride (5 g, 14.8 mmole) was dissolved in water (30 ml) and sodium meta-bisulphite added (13 g, 125 mmole). The pH was adjusted to 4.6 with sodium hydroxide and the mixture left to stand. After only a few minutes white crystals of the sparingly soluble pyrimidine sulphonic acid began to separate and the mixture was left overnight. The pyrimidine cleavage product was filtered off, and the pale yellow solution made pH 10 with sodium hydroxide and extracted with seven equal volumes of chloroform (25 ml). The extracts were evaporated to give crude thiazole (1.0 g, 50%) which was distilled *in vacuo* to give pure product b.p. 119 °C at 1 mm (lit.[7] 93-95 °C at 2 mm).

^{1}H n.m.r. δ($CDCl_3$) 2.33 (s,3H,*Me*-), 2.95 (t,2H,-C*H*$_2$CH$_2$OH, $^3J_{HH}$ = 6.0 Hz), 3.77 (t,2H,-CH$_2$C*H*$_2$OH, $^3J_{HH}$ = 6.0 Hz), 5.04 (broad s,1H, -CH$_2$CH$_2$O*H* disappears in D_2O), 8.47 (s,1H,*thiazole H*).

(b) From 3-chloropentan-5-ol-2-one

The method of Buchman[50] was used. 3-Chloropentan-5-ol-2-one (10 g, 73.3 mmole) and crude thioformamide (7.05 g, 116 mmole) were mixed with ethanol and allowed to stand in a stoppered vessel for 3 days at room temperature, during which time an additional 3.0 g of thioformamide was added. The product was isolated in a similar fashion to (a).

pKa Determinations for (3) and (9)

The pKa of 4-methyl-5(2-hydroxyethyl)thiazole was measured spectrophotometrically using a 0.01% solution of the thiazole in water containing potassium chloride (100 m*M*) to minimise changes in ionic strength during the titration. A good sigmoidal curve was obtained which was converted to linear form and analysed by a least squares procedure to determine the pKa. The pKa of 2-fluoro-4-methyl-5(2-hydroxyethyl)thiazole was measured essentially by the method of Katrizky.[61] A stock solution of AR sulphuric acid was standardised by titration against standard 0.1 *M* sodium hydroxide. To sulphuric acid of known molarity (3 ml) in a quartz cuvette (1 cm path length) was added a solution of the 2-fluorothiazole in water (100 µl, 1 mgml^{-1}), the solution was thoroughly mixed by inversion and the absorbance at 270 nm recorded against an appropriate solvent blank. The H_0 value of the resulting solution was obtained by interpolation from the data of Paul and Long[62] and the sigmoidal curve analysed as for the unfluorinated compound.

4-Methyl-5(2-*p*-toluenesulphonyl)thiazole (24)

4-Methyl-5(2-hydroxyethyl)thiazole (1.1 g, 7.7 mmole) was

dissolved in dry pyridine (10 ml) and *p*-toluene sulphonyl chloride (2.5 g, 13.1 mmole) added. The yellow mixture was left at 0 °C for two days and after filtration the pyridine solution was poured into water (75 ml) to give a brown oil. The supernatant was decanted off and the oil taken up in ether, which was dried ($MgSO_4$) and evaporated to give a pale red oil which crystallised overnight. The tosylate was preabsorbed onto silica (5 g) and purified on a column of silica eluting with ether to give the pure product (760 mg, 33%) m.p. 68-69.5 °C. ^1H n.m.r. $\delta(CDCl_3)$ 2.46,2.47 (s,3H each,2*Me*), 3.14 (t,2H,-C*H*$_2$CH$_2$OH,J_{HH} = 6.0 Hz), 4.20 (t,2H, -CH$_2$C*H*$_2$OH,J_{HH} = 6.0 Hz), 7.5 (dd,4H,tosyl Ar), 8.57 (s,1H,*thiazole H*); Found: C, 52.46; H, 5.01; N, 4.84; $C_{13}H_{15}O_3NS_2$ requires C, 52.50; H, 5.08; N, 4.71%.

Attempted preparation of 4-methyl-5(2-fluoroethyl)thiazole *via* (24)

4-Methyl-5(2-*p*-toluenesulphonylethyl)thiazole (500 mg, 1.68 mmole) was stirred and heated at 100 °C in dry DMF for 3h. T.l.c. in ether indicated a complex series of products and ^{19}F n.m.r. showed no organic fluorine compounds so the reaction was abandoned in favour of a more straightforward procedure using DAST.

4-Methyl-5(2-fluoroethyl)thiazole (11)

To a solution of DAST (1.45 g, 9.0 mmole) in dry ethanol-free chloroform (5 ml) cooled in ice was added dropwise a solution of 4-methyl-5(2-hydroxyethyl)thiazole (1.0 g, 7 mmole) dissolved in dry chloroform (5 ml). After the addition the mixture was allowed to warm up to ambient temperature over a period of 30 min, and water (10 ml) was carefully added dropwise with vigorous stirring. The chloroform layer was washed with saturated sodium bicarbonate solution and saturated brine, then dried ($MgSO_4$) and evaporated *in vacuo* to yield 4-methyl-5-(2-fluoroethyl)thiazole as a pale

yellow liquid which was purified on a column of silica gel (100 g) eluting with ethyl acetate. Yield 550 mg (55%). ^{1}H n.m.r. δ($CDCl_3$) 3.14 (t,2H,FCH_2CH_2-,$^{3}J_{HH}$ = 6 Hz,$^{3}J_{HF}$ = 24.0 Hz), 2.40 (s,3H,*Me*-); 4.55 (t,2H,FCH_2CH_2,$^{3}J_{HH}$ = 6.0 Hz,$^{2}J_{HF}$ = 47.5 Hz) 8.57 (s,1H, *thiazole H*); ^{19}F n.m.r. δ_F($CDCl_3$) 140 (tt,$^{3}J_{HF}$ = 47.5 Hz,$^{3}J_{HF}$ = 24.0 Hz); Picrate m.p. 107-110 °C; Found C, 38.49; H, 2.84; N, 14.79; $C_{12}H_{11}N_4SFO_7$ requires C, 38.51; H, 2.96; N, 14.97%.
The methane sulphonate salt was prepared by adding methane sulphonic acid (150 mg, 1.56 mmole) in a little acetone to (11) (226 mg, 1.56 mmole) dissolved a small volume of acetone. The salt crystallised rapidly, rosettes m.p. 90-92°.

2-Methyl-5-cyano-6-amino-pyrimidine (28)

The title compound was prepared by the method of Grewe.[4] Sodium ethoxide (100 mmole) was prepared by dissolving sodium metal (2.3 g, 100 mmole) in dry ethanol (100 ml) under nitrogen. This was then added to a stirred suspension of acetamidine hydrochloride (9.45 g, 100 mmole) in ethanol. The precipitated sodium chloride was filtered off and the supernatant added to a solution of ethoxy-methylene malononitrile (122 g, 100 mmole) in ethanol (100 ml). An immediate yellow precipitate of 2-methyl-5-cyano-6-aminopyrimidine formed which was filtered off at the pump and crystallised from methanol (400-500 ml required). ^{1}H n.m.r. δ(DMSO-d_6) 2.40 (s,3H, *Me*-) 7.71 (broad s,2H,-NH_2) 8.48 (2,1H,*pyrimidine H*).

2-Methyl-5-aminomethyl-6-amino pyrimidine (29)

The title compound was prepared by the method of Grewe.[4] 2-Methyl-5-cyano-6-amino-pyrimidine (544 mg, 4 mmole) was dissolved in glacial acetic acid (20 ml) and dry HCl gas passed in for 5 min. 10% Pd/C catalyst was added and the mixture was shaken in an atmosphere of hydrogen for 3h until the calculated amount of

hydrogen had been absorbed. The mixture was then mixed with water (20 ml) to dissolve precipitated product, filtered from the catalyst through glass fibre paper and evaporated to dryness *in vacuo* to give a pale yellow gum which solidified to a foam. This was taken up in warm MeOH and ether added to precipitate 2-methyl-4-aminomethyl-6-amino pyrimidine as the dihydrochloride (500 mg, 74%). ^{1}H n.m.r. $\delta(D_2O)$ 2.93 (s,3H,*Me*-) 4.54 (s,2H,-C*H*$_2$-) 8.58 (s,1H,*pyrimidine H*).

2-Methyl-5-hydroxymethyl-6-amino pyrimidine (2)

The title compound was prepared by the method of Andersag and Westphal.[5] 2-Methyl-5-aminomethyl-6-amino pyrimidine dihydrochloride (420 mg, 2.13 mmole) was dissolved in water (8 ml) and sodium nitrite added (120 mg, 1.74 mmole) in a little water. The solution immediately turned yellow and bubbles of nitrogen were evolved. The mixture was warmed to 60 °C over 1h, the pH adjusted to *ca.* 11, and the mixture evaporated down to give a yellow gum which was triturated with methanol, filtered and the solution evaporated to give a sticky solid which was purified on a column of silica gel (35 g) by preabsorption and elution with 20/80 methanol/ether to give 2-methyl-5-hydroxymethyl-6-amino pyrimidine as a pale yellow solid (90 mg, 30%). ^{1}H n.m.r. $\delta(D_2O)$ 2.73 (s,3H, *Me*-) 4.86 (s,2H,-C*H*$_2$OH) 8.30 (s,1H,*pyrimidine H*).

2-Methyl-5-bromomethyl-6-amino pyrimidine (30)

The title compound was prepared by the method of Andersag and Westphal.[5] 2-Methyl-5-hydroxymethyl-6-amino pyrimidine (90 mg, 0.65 mmole) was dissolved in warm glacial acetic acid (3 ml) and an equal volume of 40% hydrogen bromide in acetic acid added. The yellow mixture was transferred to a hydrolysis tube, sealed and incubated at 60 °C overnight. Initially an off-white precipitate of the hydrobromide salt of (2) appeared but this soon dissolved

and crystals of the product appeared slowly. After 12 hours at 60 °C the crystals were filtered off, washed with acetic acid and ether and dried over phosphorus pentoxide to give 2-methyl-5-bromomethyl-6-amino pyrimidine (120 mg, 63%). ^{1}H n.m.r. δ(D_2O) 3.28 (s,3H,*Me*-) 5.19 (s,2H,-C*H*$_2$Br) 8.90 (s,1H,*pyrimidine H*).

3[(4-amino-2-methyl-5 pyrimidinyl)methyl]-4-methyl-5(2-fluoroethyl)-thiazolium bromide hydrobromide (12)

To 4-methyl-5(2-fluoroethyl)thiazole (50 mg, 0.35 mmole) dissolved in butan-1-ol (0.3 ml) was added 4-amino-2-methyl-5-bromomethyl pyrimidine hydrobromide (63 mg, 0.22 mmole) and the mixture heated to 100-120 °C for 15 min. The pyrimidine dissolved and shortly afterwards crystals of the product (12) appeared, which were filtered off, washed with cold ethanol and recrystallised from 95% ethanol to give the thiazolium salt (12) (22 mg, 23%) m.p. 253 °C (dec.). ^{1}H n.m.r. δ(D_2O) 3.07 (s,3H,*Me*-) 3.15 (s,3H, *Me*-) 3.93 (dt,2H,-C*H*$_2$CH$_2$F, $^3J_{HH}$ = 6.0 Hz, $^3J_{HF}$ = 24.0 Hz) 5.26 (dt, 2H,-CH$_2$C*H*$_2$F, $^3J_{HH}$ = 6.0 Hz, $^2J_{HF}$ = 47.5 Hz) 6.08 (s,2H,-C*H*$_2$-) 8.54 (s,1H,*pyrimidinyl H*) 11.14 (s,1H,*thiazolium H*, disappears in D_2O); ^{19}F n.m.r. δ_F(D_2O) 142 (tt, $^3J_{HF}$ = 24.0 Hz, $^2J_{HF}$ = 47.5 Hz); Found C, 31.94; H, 4.37; N, 11.71; $C_{12}H_{19}N_4Br_2FS.H_2O$ requires C, 32.28; H, 4.29; N, 12.34%.

Ethyl-2,2,2-trifluoroacetate (31)

The title compound was prepared by the method of Henne *et al.*[56] To trifluoroacetic acid (100 g, 0.88 mmole) in a round-bottomed 250 ml flask were added dry ethanol (75 g) and concentrated sulphuric acid (10 g). The mixture was refluxed for 10 min until turbid and then distilled through a Vigreaux column. The azeotrope of water/ethanol and ethyl trifluoroacetate boiling at 54-56 °C was collected. On standing overnight this separated into two layers. The mixture was poured into cold water (200 ml) and the

organic layer separated, washed with brine and dried with magnesium sulphate to yield crude ester (98 g). Redistillation through a column packed with Fenske helices gave pure ethyl trifluoroacetate as a colourless liquid (92.4 g, 74%) b.p. 60.5 °C (lit.[56] 61-62 °C). ^{1}H n.m.r. δ(neat) 3.98 (q,2H,CH_3CH_2-,$^{4}J_{HH}$ = 8.0 Hz) 0.98 (t,3H, CH_3CH_2-,$^{5}J_{HH}$ = 8.0 Hz); ^{19}F n.m.r. δ_F(neat) 2.7 (s,CF_3-).

2-(1,1-dihydroxy-2,2,2 trifluoroethyl)butyrolactone (32)

To freshly distilled γ-butyrolactone (40 g, 0.47 mmole) warmed to 80 °C in a three necked flask fitted with a thermometer, condenser and dropping funnel were added over the course of 3h ethyl trifluoroacetate (66 g, 0.47 mmole) and sodium metal (11 g, 0.47 mmole). A further 19 g of ethyl trifluoroacetate was added and after four hours the deep dark red solution was acidified with 50% acetic acid (70 ml) and shaken with saturated brine (120 ml). On salting out the product came out as a dark red oil which was neutralised with sodium bicarbonate, dissolved in ether which was dried ($MgSO_4$) and evaporated to yield a red oil which began to crystallise immediately. The crystals were washed with chloroform and recrystallised from chloroform-petrol to give 2(1,1-dihydroxy-2,2,2-trifluoroethyl)butyrolactone (25 g, 27%) m.p. 90-92 °C. ^{1}H n.m.r. δ[$(CD_3)_2CO$] 2.78 (dt,2H,-CHCH_2-,$^{3}J_{HH}$ = 9 Hz,$^{3}J_{HH}$ = 4.5 Hz) 3.42 (t,1H,CH-,$^{3}J_{HH}$ = 9 Hz) 4.70 (m,2H,-CH_2O-) 6.72,6.89 (s,1H each, $CF_3C(OH)$-,disappears on D_2O shake); ^{19}F n.m.r. δ_F[$(CD_3)_2CO$] 9.6 (s,CF_3-,$^{4}J_{HF}$ = 1 Hz); Found C, 36.17; H, 3.32; $C_6H_8F_3O_4$ requires C, 36.01; H, 3.51%.

2(2,2,2-trifluoroacetyl)butyrolactone enol (33)

The enolic form was prepared by azeotropic desiccation of the hydrate (32) (550 mg, 2.76 mmole) which was refluxed with sodium dried benzene for 2h and the benzene/water azeotrope distilled off

to yield the enol as a pale yellow oil (400 mg, 80%), u.v. λ_{max} ($CHCl_3$) = 250 nm (5.5 x 10^3); ^{1}H n.m.r. $\delta(CDCl_3)$ 3.09 (dt,2H, $-CH_2CH_2O-$, $^3J_{HH}$ = 7.5 Hz, $^3J_{HH}$ = 3.0 Hz), 4.50 (t,2H,$-CH_2CH_2O-$, $^3J_{HH}$ = 8.0 Hz), 11.05 (broad s,1H,*enolic-OH*); ^{19}F n.m.r. $\delta_F(CDCl_3)$ -4.05 (s,enol CF_3), +2.4 (s,ketone CF_3).

2(2,2,2-trifluoroacetyl)bromobutyrolactone (34)

The halogenation method of King and Ostrum for difficult enols was adopted.[58] To cupric bromide (1.25 g, 4.35 mmole) which had been thoroughly dried over phosphorus pentoxide *in vacuo*, suspended in dry refluxing ethyl acetate (10 ml) was added freshly prepared enol (33) (500 mg, 2.7 mmole) in a few ml of dry chloroform and the mixture left to reflux with the exclusion of moisture. After 55h t.l.c. using 25/75 ether/petrol showed a slightly higher running spot and *ca*. 66% of the initially black cupric bromide had turned white. The pale green mixture was filtered and evaporated down to yield a brown oil which was purified by p.l.c. using 25/75 ether/petrol. The main band was extracted with Analar acetone (3 x 25 ml) and evaporated down to give a pale red oil (204 mg, 29% 29%). ^{1}H n.m.r. $\delta(CDCl_3)$ 3.01,3.20 (2t,2Hoverall,$-CH_2CH_2O-$, $^3J_{HH}$ = 9 Hz), 4.4,4.5 (2m,2Hoverall,$-CH_2CH_2O-$); ^{19}F n.m.r. $\delta_F(CDCl_3)$ 3.7 (s,CF_3-); m.s. M^+ at m/e 260 and 262.

Benzyl-(*S*)-serinate-*p*-toluene sulphonate (39)

The title compound was prepared by the general method outlined in Greenstein and Winitz.[70] In a 200 ml round-bottomed flask was placed (*S*)-serine (3.6 g, 34 mmole), *p*-toluenesulphonic acid (7.0 g, 40.7 mmole), benzyl alcohol (30 ml) and benzene (50 ml) and this mixture was refluxed for 10h on a Dean and Stark apparatus. The opalescent yellow mixture was filtered and an excess of ether added giving an emulsion which was left at 0 °C overnight. The supernatant was decanted from the oil which was dissolved in acetone,

filtered and evaporated *in vacuo* to give crude (*S*)-serine benzyl ester *p*-toluenesulphonate as a pale yellow oil which was not further purified. ^{1}H n.m.r. δ(DMSO-d_6) 2.29 (s,3H,ArC*H*$_3$), 5.22 (s,2H, ArC*H*$_3$-), 3.0-4.0 (m,3H,*serine H*), 7.38 (s,5H,*Ar*CH$_2$-), 7.35 (dd,4H, *tosyl Ar*), 8.46 (broad s,5H,-N$\overset{+}{H}_3$).

Benzyl-N-carbobenzoxy-(*S*)-serinate (40)

Benzyl-(*S*)-serinate *p*-toluene sulphonate (5 g, 12.89 mmole) was vigorously stirred with saturated sodium bicarbonate solution (100 ml). Benzyl chloroformate (2.5 g, 14.6 mmole) was added and after 10 min solid product began to appear. Stirring was continued for 1h and the crude product (4.2 g, 99%) collected at the pump and recrystallised from ether-petrol to give pure benzyl-N-carbobenzoxy-(*S*)-serinate as needles, m.p. 80-82 °C (lit.[404] 83-84 °C); ^{1}H n.m.r δ($CDCl_3$) 2.11 (s,*Me*Ar-), 4.0-6.0 (m,3H,*serine protons*), 5.07,5.15 (2s,2H each,ArC*H*$_2$-), 7.30 (s,10H,*Ar*); Found C, 66.36; H, 5.99; N, 4.19; $C_{18}H_{19}NO_5$ requires C, 66.64; H, 5.82; N, 4.25%.

Attempted preparation of benzyl-N-carbobenzoxy-3-fluoro-(*S*)-alaninate (41) using DAST

To benzyl-N-carbobenzoxy-(*S*)-serine (300 mg, 0.91 mmole) in dry ethanol free chloroform (2 ml) at -78 °C was added DAST (0.15 cm^3, 1.2 mmole) in dry ethanol-free chloroform. The mixture was warmed to ambient temperature over 1h when water (2 ml) was cautiously added and the chloroform layer washed with sodium bicarbonate and brine and dried ($MgSO_4$). Evaporation gave a pale yellow oil (273 mg) which had a ^{19}F n.m.r. spectrum consistent with the required product (41) δ($CDCl_3$), 130 (t,-CH$_2$*F*,$^{2}J_{HF}$ = 47 Hz). The major product, however, was separated by p.l.c. using 20/80 ethyl acetate-chloroform and was identified as benzyl-N-carbobenzoxy-dehydroalaninate. ^{1}H n.m.r. δ($CDCl_3$) 5.14,5.23 (2s,2H each,ArC*H*$_2$-) 5.83 (d,1H,C*H*=C-NH-,*trans*,$^{4}J_{HH}$ = 1.5 Hz), 6.26 (s,1H,C*H*=C-NH-,*cis*,)

7.35 (s,10H,*Ar*-).

Benzyl-N-carbobenzoxy-3-0-*p*-toluenesulphonyl-(*S*) serinate (44)

The title compound was prepared by the method of Theodoropoulos *et al.*[73] Benzyl-N-carbobenzoxy-(*S*)-serinate (1.5 g, 4.6 mmole) was dissolved in dry pyridine (5 ml) and tosyl chloride added (0.83 g, 46 mmole) to give a bright yellow solution which was left at 0 °C for two days. After removal of pyridinium hydrochloride by filtration the orange solution was poured into an excess of water and the precipitated solid collected and recrystallised from ethanol to give benzyl-N-carbobenzoxy-3-0-*p*-toluenesulphonyl-(*S*)-serinate m.p. 72-75 °C (lit.[73] 75-77 °C); ^{1}H n.m.r. δ($CDCl_3$) 2.34 (s,3H, ArCH_3), 5.04,5.10 (s,2H each,ArCH_2-), 3.0-4.0 (m,3H,*serine H*), 7.28 (s,10H,*Ar*CH_2-), 7.41 (del,4H,ArCH_2). When (44) (250 mg, 0.53 mmole) was heated at 55 °C with anhydrous potassium fluoride (300 mg, 5.2 mmole) in both AR acetone and DMF for 6h, t.l.c. and n.m.r. of the worked-up product indicated a quantitative conversion to benzyl-N-carbobenzoxy dehydroalaninate. The same result was obtained at room temperature.

Diethylaminotrimethyl silane (50)

The title compound was prepared by the method of Pike and Schank.[405] Trimethylsilylchloride was freshly distilled under nitrogen (b.p. 57 °C), and diethylamine was dried and distilled from KOH (b.p. 55.5 °C) before use. Trimethylchlorosilane (108 g, 1 mmole) was added to anhydrous ether (350 ml) in a 2ℓ three-necked flask fitted with a dropping funnel, reflux condenser and mechanical stirrer fitted with a mercury seal. The whole apparatus was kept under a slow stream of nitrogen, for without the exclusion of moisture it was impossible to get a good yield of product. Diethylamine (201 g, 2.75 mmole) was added to the vigorously stirred

solution over *ca.* 2h and immediately a thick precipitate of diethylamine hydrochloride became apparent. The mixture was stirred for a further two hours and the diethylamine hydrochloride removed by filtration through a sintered glass funnel and washed with ether (300 ml). The combined filtrates were distilled through a Vigreaux column to give diethylaminotrimethylsilane as a colourless liquid (85 g, 59%) b.p. 125-126 °C (lit.[405] 126 °C); ^{1}H n.m.r. δ(neat) 0.06 (s,9H,(*Me*)$_3$Si-), 0.99 (t,6H,C*H*$_3$CH$_2$,$^{3}J_{HH}$ = 8.0 Hz), 2.81 (q,4H,CH$_3$C*H*$_2$-,$^{3}J_{HH}$ = 8.0 Hz); Found C, 57.79; H, 13.54; N, 9.32; $C_7H_{19}NSi$ requires C, 57.86; H, 13.18; N, 9.64%.

Diethylaminosulphur trifluoride (25)

The title compound was prepared by an adaptation of the method of Middleton.[55] The special apparatus required has been previously described. Sulphur tetrafluoride (*ca.* 20 ml) was condensed in a cold trap at -78 °C and passed with a flow of dry nitrogen into a reaction vessel containing $CFCl_3$ (100 ml) at -78 °C. A solution of freshly distilled diethylaminotrimethylsilane (48 g) in $CFCl_3$ (50 ml) was added dropwise to the stirred solution of SF_4 over 1.5h to give a pale yellow reaction mixture. On warming to ambient temperature the mixture turned a deeper yellow. The solvent was removed by distillation at 40 °C and the residue distilled under reduced pressure to give DAST as a pale yellow liquid (43.4 g, 82%) b.p. 30-35 °C at 2.2 mm (lit.[55] 46-47 °C/10 mm). The liquid was stored in a plastic bottle at 0 °C. ^{1}H n.m.r. δ(neat) 1.13 (t,6H,C*H*$_3$CH$_2$-,$^{3}J_{HH}$ = 6.5 Hz) 3.35 (q,4H,CH$_3$C*H*$_2$-,$^{3}J_{HH}$ = 6.5 Hz); ^{19}F n.m.r. δ(neat) -115 (s,-S*F*$_3$).

Dimethyl(2*S*)-malate (55)

The general method of Brenner and Huber was used for methylation.[94] Thionyl chloride (9 ml, 139 mmole) was cooled to

-20 °C and added dropwise to well stirred dry methanol (50 ml) cooled to -20 °C. The temperature was not allowed to rise above -15 °C. (2*S*)-malic acid (7.2 g, 54 mmole) was added dropwise to the stirred mixture which was then allowed to warm to room temperature and refluxed for 2h, filtered and evaporated to yield crude product. Distillation *in vacuo* afforded the pure dimethyl (2*S*)-malate (5.6 g, 64%), b.p. 132-134 °C at 1 mmHg (lit.[112] 240-245 °C); ^{1}H n.m.r. δ($CDCl_3$) 2.78 (d,2H,-C*H*$_2$CO$_2$Me,$^3J_{HH}$ = 6.0 Hz), 3.67,3.75 (2s,3 Head,-CO$_2$*Me*), 4.13 (s,1H,-CHO*H*- disappears with D_2O), 4.52 (t,1H,-C*H*OHCO$_2$Me,$^3J_{HH}$ = 6.0 Hz); Found C, 44.75; H, 6.35; $C_6H_{10}O_5$ requires C, 44.45; H, 6.22%.

Dimethyl(2*R*)-fluorosuccinate (57)

To a stirred solution of DAST (2.0 g, 12.4 mmole in dry ethanol-free chloroform (10 ml) cooled to 0 °C in ice, was added dropwise over 15 min a solution of dimethyl(2*S*)-malate (2.0 g, 12.4 mmole) in dry ethanol-free chloroform (10 ml). The mixture was allowed to warm up to ambient temperature over 30 min and an equal volume of water cautiously added. The organic layer was separated, washed with sodium bicarbonate and saturated brine, dried ($MgSO_4$) and evaporated to give dimethyl(2*R*)-fluorosuccinate as a pale yellow mobile liquid (1.73 g, 85%) contaminated with *ca*. 6% dimethyl fumarate. The pure ester was obtained by preparative g.l.c. using a PEGA column at 150 °C. ^{1}H n.m.r. δ_F[$(CD_3)_2CO$] 2.78,3.08 (2m,2H, -C*H*$_2$-), 3.67,3.75 (s,3H each,-CO$_2$*Me*), 5.26 (dt,1H,-C*H*F,$^2J_{HF}$ = 45 Hz, $^3J_{HF}$ = 6.0 Hz); ^{19}F n.m.r. δ[$(CD_3)_2CO$] 114.8 (dt,$^2J_{HF}$ = 49.5, $^3J_{HF}$ = 25.0 Hz); c.d. λ_{max} 214 nm, Δε-0.74.

(2*R*)-Fluorosuccinic acid (47)

Dimethyl(2*R*)-fluorosuccinate was refluxed with 5% sulphuric acid (10 ml) for 1.25h. The solution was made strongly acidic with

concentrated sulphuric acid and extracted with ether, the extract dried ($MgSO_4$) and the solvent removed *in vacuo* to give (2*R*)-fluorosuccinic acid as a crystalline solid (1.02 g, 71%). Recrystallisation from ethyl acetate-chloroform gave the pure acid m.p. 130°-132 °C which could be vacuum sublimed (*ca.* 115 °C at 0.5 mmHg), ^1H n.m.r. δ(DMSO-d_6) 2.67,2.95 (2m,2H,-CH_2-), 5.2 (ddd,1H,-C*H*F, $^2J_{HF}$ = 47.5 Hz, $^3J_{HH}$ = 5 and 7 Hz), ^{19}F n.m.r. δ_F[DMSO-d_6] 110.1 (dt, $^3J_{HF}$= 25.0, $^2J_{HF}$ = 47.8 Hz); c.d. λ_{max} 214 nm, Δε-0.97; Found C, 35.5; H, 3.9; $C_4H_5FO_4$ requires C, 35.3; H,3.7%.

Dimethyl(2*R*)-malate (56)

This compound was prepared from (2*R*)-malic acid as the procedure for (2*S*)-malic acid.

Dimethyl(2*S*)-fluorosuccinate (58)

This compound was prepared for dimethyl(2*R*)-malate as for dimethyl(2*R*)-fluorosuccinate, c.d. λ_{max} 214 nm, Δε+0.74.

(2*S*)-Fluorosuccinic acid (48)

This compound was prepared from dimethyl(2*R*)-malate as for (2*R*)-fluorosuccinic acid, m.p. 130-132.5 °C; c.d. λ_{max} 214 nm, Δε+1.08.

Succinic anhydride

The title compound was prepared by the method of Fieser and Martin.[406] To succinic acid (1.18 g, 10 mmole) in a 10 ml flask was added acetyl chloride (5 ml) and the mixture heated under reflux for 1.5h until all the acid had dissolved. The mixture was allowed to cool and shortly afterwards crystals appeared. After further cooling the crystals were collected, washed with ether and dried *in vacuo* to yield succinic anhydride (750 mg, 75%) m.p. 123-124.5 °C (lit.[406] 118-119 °C). ^1H n.m.r. δ($CDCl_3$) 3.0

(s,-CH_2-); ν_{max} ($CHCl_3$) 1795 cm^{-1}(s), 1875 cm^{-1}(w) (saturated 5-ring anhydride).

(2*R*)-Fluorosuccinic acid anhydride (66)

The title compound was prepared as for succinic anhydride, but from (2*R*)-fluorosuccinic acid (250 mg, 18.4 mmole). Yield 200 mg (92%). ^{1}H n.m.r. δ($CDCl_3$) 2.7-3.6 (m,2H,-CH_2-), 5.54 (td,1H,-C*H*F, $^2J_{HF}$ = 50.0 Hz, $^3J_{HH}$ = 8.5 Hz and 6.0 Hz); ^{19}F n.m.r. δ_F($CDCl_3$) 117.3 (td,-CH*F*, $^2J_{HF}$ = 52.5 Hz, $^3J_{HF}$ = 13.5 Hz and 24.9 Hz); ν_{max} ($CDCl_3$) 1810 cm^{-1}(s) and 1895 cm^{-1}(w) (saturated 5-ring anhydride).

(2*S*,3*R*)-[3-$^2H_1$]-Malic Acid (60)

The title compound was prepared essentially by the method of Fisher *et al.*[98] Monosodium fumarate (410 mg, 2.97 mmole) was dissolved in water (10 ml); made up to pH 7.6 with sodium hydroxide, lyophilised, dissolved in D_2O and lyophilised again. The solid was dissolved in D_2O, fumarase was added (0.3 cm^3, 300 units) and the solution adjusted to pH 7.6 with sodium deuteroxide. The turnover of fumarate was monitored by measuring the decrease in optical density at 240 nm of aliquots of reaction mixture (0.01 ml) diluted to 1 ml with water and read in a 2 mm quartz cuvette. Most of the reaction was complete in 4h but the solution was allowed to stand overnight in a sealed vessel and then quenched with concentrated hydrochloric acid (3 ml) which brought the pH to below 1. The solution was extracted with ether (4 x 25 ml) to remove fumaric acid and then exhaustively extracted with ethyl acetate (10 x 25 ml) the combined extracts dried ($MgSO_4$) and evaporated *in vacuo* to yield (2*S*,3*R*)-[3-$^2H_1$]-malic acid as a white crystalline hygroscopic solid which was not further purified (210 mg, 52%), m.p. 98-100 °C (lit.[98] 100 °C); ^{1}H n.m.r. δ(D_2O) 3.32 (dt,1H,-C*H*D-, $^2J_{HD}$ = 2.0 Hz, $^3J_{HH}$ = 6.5 Hz), 5.07 (d,1H,-C*H*OH-, $^3J_{HH}$ = 6.5 Hz).

Dimethyl(2*S*,3*R*)-[3^2H_1]-malate (61)

(2*S*,3*R*)-[3^2H_1]-Malic acid (210 mg, 1.56 mmole) was dissolved in dry methanol and a freshly distilled ethereal solution of diazomethane added to the vigorously stirred solution until the pale yellow colour of the diazomethane persisted, whereupon excess of methylating agent was purged in a stream of dry nitrogen and solvent evaporated to yield dimethyl (2*S*,3*R*)-[3^2H_1]-malate as a colourless liquid (215 mg, 85%). The isotopic purity of the deuteriated centre was found by mass spectrometry to be 94%±0.7% (Fisher *et al.*[98] report 97%). ^1H n.m.r. $\delta(CDCl_3)$ 2.77 (dt,1H,-C*H*D-,$^2J_{HD}$ = 2.0 Hz,$^3J_{HH}$ = 6.2 Hz), 3.69,3.78 (2s,3H each,-CO_2*Me*), 4.48 (d,1H, -C*H*OH,$^3J_{HH}$ = 6.2 Hz).

Dimethyl(2*R*,3*S*)-[3^2H_1]-fluorosuccinate (62)

The title compound was prepared from dimethyl(2*R*,3*S*)-[3^2H_1]-malate (210 mg, 1.29 mmole) as described for dimethyl(2*R*)-fluorosuccinate. Yield 169 mg (80%). ^1H n.m.r. $\delta(CDCl_3)$ 2.95 (ddt,1H, -C*H*D-,$^2J_{HD}$ = 2.0 Hz,$^3J_{HH}$ = 4.0 Hz,$^3J_{HF}$ = 26.5 Hz), 3.68,3.80 (2s, 3H each,-CO_2*Me*), 5.26 (dd,1H,-C*H*F, $^2J_{HF}$= 47.5 Hz, $^3J_{HH}$ = 4.0 Hz); ^{19}F n.m.r. $\delta_F(CDCl_3)$ 115.9 ppm (qt,-CH*F*,$^2J_{HF}$ = 27.0 Hz,$^3J_{HF}$ = 49.0 Hz,$^3J_{DF}$ = 3.2 Hz).

(2*R*,3*S*)-[3^2H_1]-Fluorosuccinic acid (63)

The title compound was prepared from dimethyl (2*R*,3*S*)-[3^2H_1]-fluorosuccinate (169 mg, 1.02 mmole) as described for (2*R*)-fluorosuccinic acid. Yield 97 mg (69%). ^1H n.m.r. $\delta(D_2O)$ 3.09 (ddt,1H, -C*H*D-,$^2J_{HD}$ = 2.0 Hz,$^3J_{HF}$ = 24.0 Hz,$^3J_{HH}$ = 4.0 Hz), 5.35 (dd,1H, -C*H*F,$^2J_{HF}$ = 48.0 Hz,$^3J_{HH}$ = 4.0 Hz); ^{19}F n.m.r. $\delta_F(CDCl_3)$ 115.1 (ddt,-CH*F*,$^2J_{HF}$ = 50.0 Hz,$^3J_{HF}$ = 25.0 Hz,$^3J_{DF}$ = 4.0 Hz).

(2*R*,3*S*)-[3^2H_1]-Fluorosuccinic acid anhydride (64)

The title compound was prepared as described for (2*R*)-fluoro-

succinic anhydride but using (2*R*,3*S*)-[3$^{2}H_{1}$]-fluorosuccinic acid (90 mg, 0.67 mmole). Yield 65 mg (81%). ^{1}H n.m.r. $\delta(CDCl_3)$ 3.16 (ddt,1H,-C*H*D,$^{2}J_{HD}$ = 3.0 Hz,$^{3}J_{HH}$ = 4.5 Hz,$^{3}J_{HF}$ = 25.0 Hz), 5.60 (dd,1H,-C*H*F,$^{2}J_{HF}$ = 51.0 Hz,$^{3}J_{HH}$ = 4.5 Hz); ^{19}F n.m.r. $\delta_F(CDCl_3)$ 116.5 (dd,-CH*F*-,$^{2}J_{HF}$ = 55.0 Hz,$^{3}J_{HF}$ = 25.0 Hz).

Purification of (2*S*)-Fluorosuccinic acid from (2*S*)-aspartic acid

Fluorosuccinic acid was prepared[164] from (2*S*)-aspartic acid by the method of Olah.[85] This compound was purified for CD studies by p.l.c. using a double elution with chloroform/ethyl acetate/formic acid 50/10/2.5. After extraction from the plate with ethyl acetate (4 x 25 ml) evaporation of solvent gave (2*S*)-fluorosuccinic acid which was recrystallised from ethyl acetate-chloroform; m.p. 130-132 °C; c.d. λ_{max} 214 nm, $\Delta\varepsilon$+0.81.

[^{17}O]-Phosphorus oxychloride (118)

The title compound by the method of Laulicht as for the unlabelled compound.[273] [^{17}O]-Water (*ca.* 44 atom % ^{17}O, 91 µl) was added dropwise by microsyringe to solid phosphorus pentachloride (1.07 g, 5.1 mmole) cooled in an ice bath. The flask was allowed to warm up to room temperature with stirring after the last addition and the clear solution heated under reflux for 30 min and subsequently distilled to give [^{17}O]-phosphorus oxychloride as a colourless liquid (0.57 g, 73%) b.p. 105-106 °C (lit.[273] 105-106 °C).

[^{17}O]-Oxytrimethylphosphate (119)

[^{17}O]-Phosphorus oxychloride was added dropwise to rapidly stirred excess methanol (5 ml) cooled in ice. After 30 min the flask was warmed to room temperature and the solvent removed *in vacuo* to give a liquid which was dissolved in benzene and evaporated down several times to remove hydrogen chloride.

^{31}P n.m.r. $\delta_P(CDCl_3)$ 0.0 (s, $^{18}OP(OMe)_3$), (sextet, $^{17}OP(OMe)_3$, $\Delta\nu$~90 Hz, $^1J_{^{17}O,^{31}P}$~156 Hz) typical mass spectrum M^+ 140(6) [$^{16}O(POMe)_3^+$], 141(79), [$^{17}OP(OMe)_3^+$], 142(100, [$^{18}OP(OMe)_3^+$].

[^{18}O]-Phosphorus oxychloride (124)

This was prepared as for [^{17}O]-phosphorus oxychloride but using [^{18}O]-water (99 atom % ^{18}O).

[^{18}O]-Oxytrimethylphosphate (125)

This was prepared as for [^{17}O]-oxytrimethylphosphate but using [^{18}O]-phosphorus oxychloride.

Partial hydrolysis of [^{18}O]-Oxytrimethylphosphate

Initially the partial hydrolysis of [^{16}O]-trimethylphosphate was investigated. [^{16}O]-Oxytrimethylphosphate (40 mg, 0.28 mmole) was dissolved in D_2O (1.0 ml) and NaOD was added (200 µl, 40% solution in D_2O). This mixture was sealed in a hydrolysis tube and incubated at 125 °C for 43h. At the end of this period the ^1H and ^{31}P n.m.r. spectra were recorded. ^1H n.m.r. $\delta(D_2O)$ 3.56 (s,*Me*OH), 3.67 (d,3H, $MeOPO_3^{2-}$, $^3J_{PH}$ = 9.5 Hz), 3.80 (d,6H,$(MeO)_2PO_2^-$, $^3J_{PH}$ = 10.0 Hz); ^{31}P n.m.r. $\delta_P(D_2O)$ 2.13 (s,$MeOPO_3^{2-}$), 0.11, (s,$(MeO)_2PO_2^-$).

This procedure was repeated with [^{18}O]-oxytrimethylphosphate prepared from 50 atom % ^{18}O water (the rest being ^{16}O) to obtain the ^{31}P n.m.r. isotope shifts reported in TABLE 5.

Dibenzyl hydrogen phosphate (111)

The title compound was prepared by the method of Coulson.[407] Bromine (2.87 ml, 56 mmole) in CCl_4 (12 ml) was added dropwise over 3h to a rapidly stirred mixture of dibenzyl phosphite (18 g, 68 mmole), CCl_4 (20 ml), pyridine (16 ml) and water (48 ml) cooled in an ice bath. The mixture was stirred for a further 1h at ambient

temperature and the product isolated. Dibenzyl hydrogen phosphate was obtained as small colourless needles from CCl_4-hexane, m.p. 78-79 °C (lit.[407] 78-79 °C); ^{1}H n.m.r. δ(CCl_4) 4.96 (d,4H,-C*H*$_2$- $^3J_{PH}$ = 7 Hz) 7.22 (s,10H,*Ar*), 12.24 (s,1H,P-O*H*); ^{31}P n.m.r. δ_P(THF) -3.15 (s,(PhCH$_2$O)*P*-, ^{1}H decoupled, q, $^3J_{PH}$ = 4 Hz).

Trans-(4*R*,5*S*)-2-chloro-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (102a)

The title compound was prepared by the method of Ukita.[284] (1*R*,2*S*)-1,2-dihydroxy-1,2-diphenyl-ethane (214 mg, 1 mmole) dissolved in dry pyridine (3 ml) was added dropwise to a cooled solution of freshly distilled phosphoryl chloride (94 µl, 1.05 mmole) in dry pyridine (1 ml) over 2h with vigorous stirring and with exclusion of moisture. The mixture was stirred for a further 30 min at 0 °C and for 30 min at ambient temperature to give *trans*-(4*R*,5*S*)-2-chloro-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane which was usually used *in situ*; ^{31}P n.m.r. δ_P(C_6H_6)D_2O lock 15.1,s.

Trans-(4*R*,5*S*)-2(dibenzylphospho)-2-oxo-4,5 diphenyl-1,3,2-dioxaphospholane (112)

The phosphorylating agent (102a) (1 mmole) was generated as previously described, the pyridine carefully removed *in vacuo*, excluding moisture from the system and releasing the vacuum under nitrogen and the residue was suspended in dry benzene. Dibenzyl phosphate (280 mg, 1 mmole) which had been thoroughly dried *in vacuo* over P_2O_5 was dissolved in dry benzene (10 ml) and this volume was evaporated down four times to ensure dryness. It was then dissolved in dry benzene (3 ml) and added to the cooled stirred solution of (102a) together with an equivalent of pyridine (45 µl). After 1½h at ambient temperature the suspension of pyridinium hydrochloride was allowed to settle and a sample removed for ^{31}P n.m.r. ^{31}P n.m.r. δ_P(C_6H_6)D_2O lock, -16.2 (d,(PhCH$_2$O)$_2$*P*O-

$^{2}J_{PP}$ = 21 Hz), -15.5 (s,[$(PhCH_2O)_2PO]_2O$), -4.2, (s,$(PhCH_2O)_2P$-), -1.5, (d,*ring P*,$^{2}J_{PP}$ = 21 Hz,^{1}H undecoupled signal,$^{3}J_{PH}$ = 8.0 Hz).

Benzyl dihydrogen phosphate (115)

The title compound was prepared according to Cramer and Weimann.[408] Crystalline phosphoric acid (0.196 g, 2 mmole), benzyl alcohol (10 g), triethylamine (0.404 g, 4 mmole) and trichloroacetonitrile (1.44 g, 1 mmole) were added together and after 4h at 75 °C cyclohexylamine (2 g, 20 mmole) was added, the excess benzyl alcohol distilled off and the product isolated as its *bis*-cyclohexylammonium salt from acetone-water. Yield 77%, m.p. 233 °C (lit.[408] 233 °C); ^{31}P n.m.r. δ_P(dioxan-*bis*-Bu_3N salt)D_2O lock; -3.05,(s).

Trans-(4*R*,5*S*)-2(benzylphospho)-2-oxo-4,5-diphenyl-1,3,2 dioxaphospholane (114)

The phosphorylating reagent (102a) was prepared as previously described and the pyridine removed *in vacuo*. The residue was dissolved in dry dioxan (4 ml) - the pyridinium hydrochloride did not dissolve. Benzyl phosphate free acid (189 mg, 1 mmole - from the cyclohexylammonium salt and amberlite IR120 (H^+) resin) was dissolved in dry dioxan and tributylamine added (185 mg, 1 mmole), the salt dried by coevaporation of dry dioxan and the mixture quickly added in one portion to the phosphorochloridate together with further tributylamine (1 mmole). The mixture was left stirring for 3h and the ^{31}P n.m.r. spectrum recorded. ^{31}P n.m.r. δ_P(dioxan) D_2O lock -15.8, (d,$PhCH_2OP$-, J_{PP} = 22.06 Hz), -3.65, (s,$PhCH_2OPO_3^-$), 0.00 (d,*5-ring P*,$^{2}J_{PP}$ = 22.06 Hz,^{1}H undecoupled $^{3}J_{PH}$ = 8.0 Hz).

Attempted deprotection of (114)

The reaction mixture was divided into two portions. One half was transferred to a hydrogenation flask containing ethyl acetate

(10 ml) and 10% Pd/C (180 mg) and hydrogenolysed overnight. After filtration the mixture was extracted with sodium bicarbonate solution which was evaporated and the residue dissolved in D_2O ^{31}P n.m.r. $\delta_P(D_2O)$ -7.57 (s,*PP*$_i$), 0.24 (s,*P*$_i$), 12.88 (s,*5-ring phosphate*).

The other half was deprotected in sodium/liquid ammonia as will be described subsequently. ^{31}P n.m.r. $\delta_P(D_2O)$ -7.51 (s,*PP*$_i$), 3.06 (s,*P*$_i$).

Trans-(4*R*,5*S*)-2(β-cyanoethylphospho)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (117a)

β-Cyanoethyl-phosphate dihydrate (322 mg, 1 mmole) was converted to the pyridinium form with Dowex 50W (pyridinium form) and suspended in methanol (5 ml). Tri-*n*-octylamine (353 mg, 1 mmole) was added and the mixture stirred until solution was obtained. This salt was dried by repeated evaporation of dry pyridine and was dissolved in dry dioxan (3 ml) with tri-*n*-butylamine (186 mg, 1 mmole) and this solution was added all at once to a solution of the phosphochloridate (102a) prepared *in situ* in pyridine, which had been evaporated and replaced by dry dioxan (the amine hydrochloride doesn't dissolve). The mixture was left to stir for 3h when reaction was complete. ^{31}P n.m.r. δ_P(dioxan)D_2O lock -15.50 (d, -P-O-*P*OCH$_2$-, $^2J_{PP}$ = 22.8 Hz), -1.00 (s,*free β-cyanoethyl phosphate*), -0.75 (d,5-ring-*P*-O-P,J_{PP} = 22.06 Hz,1H undecoupled, $^3J_{PH}$ = 7.5 Hz).

Inorganic pyrophosphate (115a) - deprotection of (117a)

(a) Hydrogenation

Half of the reaction mixture of (115a) was hydrogenolysed overnight in ethyl acetate (10 ml) with Pd/C (80 mg). After work-up the ^{31}P n.m.r. spectrum showed many lines.

(b) Sodium/liquid ammonia

Liquid ammonia (*ca.* 60 ml) was distilled from sodium metal, and the required amount of sodium metal for the reaction (15 mmole, 40 cm wire) added under anhydrous conditions in dry nitrogen. The dioxan reaction mixture containing (177a) was added rapidly by syringe to the mechanically stirred blue solution, and after waiting exactly 10 min the mixture was quenched by addition of solid ammonium chloride until the blue colour was discharged. The ammonia was allowed to evaporate in a stream of dry nitrogen to leave a white solid which was partitioned between chloroform and water. The aqueous layer was evaporated down to leave a solid which was examined by ^{31}P n.m.r. ^{31}P n.m.r. $\delta_P(D_2O)$ -8.70 (s,PP_i), -1.50 (s,P_i).

This reduction was repeated on a 1 mmole scale and after work-up the aqueous sample was diluted to 300 ml and the pH adjusted to 10.5, when the sample was applied at a flow rate of 80 mlh^{-1} to an equilibrated column of Sephadex A-25. A 48h gradient was selected from 100-600 m*M* TEAB pH 10.5 and fractions were collected at 15 min intervals. Samples (100 µl) were withdrawn and examined by an adaptation of the Briggs phosphate test[262] (see later). Pyrophosphate was located in fractions 26-38 which were evaporated down and repeatedly coevaporated with dry methanol to yield a gum which was stirred with Dowex 50W (Na^+ form) and the solution lyophilised to give anhydrous *tetra*-sodium pyrophosphate in 50% yield. ^{31}P n.m.r. $\delta_P(D_2O)$ -9.2 (s,PP_i).

The Briggs phosphate test

The Briggs phosphate test[262] was adapted for the detection of pyrophosphate as follows. Fractions (250 µl) from the ion exchange column were collected and transferred to individual vials and evaporated to dryness *in vacuo* overnight over concentrated sulphuric

acid. *M* Sulphuric acid was added (500 µl) and the mixture incubated at 90 °C overnight. Water (250 µl) was added followed by ammonium molybdate solution (500 µl, 2.5 g in 20 ml water +7.5 ml conc. sulphuric acid), hydroquinone solution (250 µl, 0.5 g in 100 ml water +1 drop sulphuric acid) and sodium sulphite solution (250 µl, 4 g in 20 ml water) and the vials sealed and left for several days. Generally after *ca*. 48h the blue colouration of phosphate could be discerned.

(2*R*,4*S*,5*R*)-2-chloro-[2-^{17}O]-oxo-4,5 diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane (102b)

The title compound was prepared by the methods of Ukita[284] and Cullis and Lowe[226,255], and were the same as for the unlabelled compound (102a) except that [^{17}O]-phosphoryl chloride and (1*R*,2*S*)-[1-^{18}O]-1,2-dihydroxy-1,2-diphenylethane were used.

(2*R*,4*S*,5*R*)-2-(β-cyanoethylphospho)-[2-^{17}O]-oxo-4,5 diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane (117b)

The title compound was prepared in the same fashion as the unlabelled compound (117a) except that the labelled phosphorochloridate (102b) was used.

P_1-[(*S*)-^{16}O, ^{17}O, ^{18}O]-Pyrophosphate (115b)

The deprotection was performed on the precursor (117b) in the same fashion as for the unlabelled compound, followed by ion exchange chromatography. ^{31}P n.m.r. $\delta_P(D_2O)$ -9.2 (see special discussion).

Adenosine-5'-*p*-nitrophenylphosphate (147)

The compound was prepared *in situ* by the method of Borden and Smith.[281] AMP *bis*-triethylammonium salt (0.5 mmole) was dissolved in dry DMSO (2.5 ml), tributylamine was added (372 mg, 2 mmole)

followed by 2,4-dinitrofluorobenzene (186 mg, 1 mmole). The mixture was stirred and monitored by ^{31}P n.m.r. After 22h the major product was by far the *p*-nitrophenylphosphate ester with δ_P -9.25 (s). There were, however, traces of the AMP phosphofluoridate which had δ_P -12.82 (d, $^1J_{pH}$ = 914 Hz). After 3h the reaction was 66% complete with no traces of any other product.

Trans-(4*R*,5*S*)-2(2',3'-diacetyl adenosine-5'-yl)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane

The labelled phosphorochloridate (102b) was prepared as previously described (2 mmole) and to the cooled solution in pyridine was added 2',3'-diacetyl adenosine (703 mg, 2 mmole) in pyridine (5 ml) and the solution stirred overnight to give *cis*-(4*R*,5*S*)-2(2',3'-diacetyl adenosine-5'-yl)-2-oxo-4,5-diphenyl 1,3,2-dioxaphospholane in quantitative yield by ^{31}P n.m.r. ^{31}P n.m.r. δ_P(pyridine)D_2O lock +11.8 (s,*5-ring P*).

Adenosine-5'-monophosphate (141)

The pyridine solution from above was evaporated down *in vacuo*, dissolved in dry DMF and evaporated down twice (2 x 20 ml). The residue was dissolved in dry DMF (10 ml), catalyst added (10% Pd/C, 1 g, Pd black, 90 mg and the mixture stirred in an atmosphere of dry hydrogen. An uptake of *ca*. 400 ml was observed over 3h. The mixture was filtered and the catalyst washed thoroughly with 300 m*M* 50% ethanolic ammonia (*ca*. 800 ml, monitored by the optical density of the filtrate at 280 nm) and the resulting solution evaporated to dryness. The residue was dissolved in methanol and added to methanol which had been saturated with ammonia at 0 °C. Ammonia was passed through for a further few minutes and the resulting suspension stirred at room temperature for 0.5h. The solvent was removed and the residue dissolved in water, brought to pH 8.0 and conductivity of 50 m*M* TEAB, and this solution applied to an ion-

exchange resin, Sephadex A-25, developed over 24h in 50-200 m*M* TEAB run at 82 $ml h^{-1}$. Fractions were collected every 15 min and AMP eluted in fractions 37-57 between *ca.* 105-135 m*M*. After evaporatior *in vacuo* adenosine-5'-phosphate *bis*-triethylammonium salt was obtained in 52% yield on starting materials, 77% on recovered nucleotide. ^{31}P n.m.r. $\delta_P(D_2O)$ -1.13 (s,-CH_2OP).

Adenosine-3',5'-cyclic phosphate (149)

AMP was cyclised according to the procedure of Mukaiyama and Hashimoto.[282] Adenosine-5'-monophosphate (55 mg, 0.1 mmole) was converted to the free acid using Dowex 50W (H^+) and dissolved in pyridine (5 ml) and water (1 ml). 4-Morpholine-N,N'-dicyclohexyl-carboxamidine (30 mg, 0.1 mmole) was added with slight warming and the solvents removed *in vacuo* and the salt dried by repeated evaporation of dry pyridine. The resulting gum was dissolved in pyridine (15 ml) and heated to boiling point under reflux. To the boiling solution were added in one portion triphenylphosphine (131 mg, 0.5 mmole) and 2,2'-dipyridyl disulphide (110 mg, 0.5 mmole) and the mixture boiled for 3h, when water (25 ml) was added and the mixture left for a further 2h. The mixture was evaporated *in vacuo* and the residue partitioned between water (20 ml) and dichloromethane (20 ml). The aqueous layer was exhaustively extracted with dichloromethane (5 x 50 ml) and evaporated to yield adenosine-3',5'-cyclic phosphate MDCA salt. ^{31}P n.m.r. $\delta_P(D_2O)$ -4.67 (s,*6-ring P*).

N^1-Methyl-adenosine-3',5'-cyclic phosphate methyl esters (151) and (154)

The MDCA salt of cAMP was dissolved in water and stirred with Dowex 50W (K^+ form). The filtrate was evaporated to give the potassium salt of cyclic AMP which was dissolved in a little water and 18-crown-6 added (26 mg, 0.1 mmole) and the mixture evaporated

thoroughly *in vacuo* to give a gum which was dissolved in dry DMSO-d_6 (1 ml) and methyl iodide added (100 µl, 1.5 mmole). The mixture was stirred overnight for 20h, the methyl iodide thoroughly removed *in vacuo* to give a solution containing N^1-methyl-adenosine-3',5'-cyclic phosphate methyl ester. ^{31}P n.m.r. δ_P(DMSO-d_6) -4.02 (s, *equatorial ester*), -5.27 (s,*axial ester*).

Adenosine-5'[^{18}O]-phosphate (146)

Glycylglycine (2.8 mg, 21.5 mmole), magnesium acetate tetrahydrate (1.0 mg, 4.7 µmole) and cAMP hemihydrate (40 mg, 120 µmole) were dissolved in water (0.4 ml) and sodium hydroxide added (*M*, 0.1 ml). The solution was lyophilised and the residue dissolved in [^{18}O]-water (99 atom %, 0.5 ml). cAMP phosphodiesterase (from beef heart, 4 mg, 0.81 l) was added at 4 °C and the mixture was incubated at 37 °C, the pH of the solution being adjusted by the addition of solid tris as follows:

time (min)	25	55	85	115	145	205
tris (mg)	2.9	2.9	2.8	2.7	2.6	final pH = 7.7

After 3.5h the solution was frozen and the [^{18}O]-water recovered by lyophilisation on a vacuum line. The same procedure was repeated and the two residues combined, dissolved in water and vigorously shaken with chloroform to denature the protein prior to application to a column of DEAE Sephadex A-25. A gradient of TEAB pH 8.0 was run from 20 m*M*-200 m*M* over 16h at a rate of 22 mlh^{-1}, collecting fractions every 30 min. [^{18}O]-AMP was identified in fractions 14-22 which were evaporated *in vacuo* to give adenosine-5'[^{18}O]-phosphate *bis*-triethylammonium salt as a glassy solid (106 mg, 82%). ^{31}P n.m.r. δ_P(D_2O) -1.13 (s,-CH_2OP).

Adenosine-3',5'[^{18}O]-cyclic phosphate (149)

This compound was cyclised as for the unlabelled compound.

N^1-methyl-adenosine-3',5'-[^{18}O]-cyclic phosphate methyl esters (151-156)

This material was prepared from adenosine-5'[^{18}O]-phosphate as described for the unlabelled compound.

(2*R*,3*S*,5*R*)-2(2',3'-diacetyl adenosine-5'-yl)-[2-^{17}O]-oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane (159)

The title compound was prepared from the labelled phosphorochloridate (102b) and 2',3'-diacetyl adenosine as described for the unlabelled compound.

Adenosine-5'[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate (161)

The title compound was prepared by deprotection of (159) as described for the unlabelled compound.

Adenosine-3',5'[^{16}O, ^{17}O, ^{18}O]-cyclic phosphate

The title compound was prepared by the TPP-PDS cyclisation of (159) as described for the unlabelled compound.

N^1-methyl-adenosine-3',5'[^{16}O, ^{17}O, ^{18}O]-cyclic phosphate methyl esters

The title compound was prepared by the methylation of adenosine-3',5'[^{16}O, ^{17}O, ^{18}O]-cyclic phosphate as described for the unlabelled compound.

Trityl chloride

The title compound was prepared by the method of Bachmann.[409] Freshly recrystallised trityl alcohol (*ex.* CCl_4, m.p. 161.5-162.5 °C 140 g, 0.54 mmole) was suspended in benzene (44 ml) and warmed on a steam bath. Acetyl chloride (28 ml) was added rapidly at first then dropwise over 10 min, and the resulting clear solution refluxed for 30 min. The flask was quickly cooled and fine cubic

crystals of the trityl chloride began to appear. The addition of petrol and further cooling in ice over 1.5h crystallised most of the product which was rapidly filtered, washed with a small amount of dry petrol and dried *in vacuo* over potassium hydroxide and concentrated sulphuric acid m.p. 109-111 °C (lit.[409] 111-112 °C).

6-Trityl-β-*D*-glucose-1,2,3,4-tetraacetate

The method of Bachmann[409] was used. A mixture containing anhydrous *D*-glucose, dried over P_2O_5 (40 g, 0.22 mmole), trityl chloride (73 g, 0.28 mmole) and anhydrous pyridine (200 ml) was warmed until solution was complete, strictly anhdrous conditions being maintained to avoid hydrolysis of the trityl chloride. Without cooling, for higher temperatures favour the β-anomer, acetic anhydride (120 ml) was added in one portion and after standing for 12h the dark red reaction mixture was poured in a very fine stream into iced water (3 ℓ) to which acetic acid had been added (150 ml), and the resulting mixture was vigorously stirred mechanically for 2h. The granular precipitate was filtered and immediately stirred for a short time with ice water (3 ℓ), filtered washed well with water and air dried. The dried solid was digested with ether (200 ml) - the α-anomer is soluble and the β-anomer insoluble - and allowed to stand. The insoluble portion was dissolved in hot 95% ethanol (1ℓ) and filtered, and the filtrate on cooling deposited fine white needles of 6-trityl-β-*D*-glucose-1,2,3,4-tetraacetate of sufficient purity for further use, which were recrystallised from ethanol (45 g, 35%), m.p. 164-166 °C (lit.[409] 166-166.5 °C); ^{1}H n.m.r. δ($CDCl_3$) 1.61,1.97,2.00,2.10 (4s, 3H each,*Me*-), 3.2 (m,2H,H_{C_6}), 3.7 (m,1H,H_{C_5}), 5.72 (d,1H,H_{C_1}, $^3J_{HH}$ ~8 Hz), 5.20 (m,3H,$H_{C_{2,3,4}}$) 7.10-7.50 (m,15H,*Ar*).

β-*D*-Glucose-1,2,3,4-tetraacetate (162)

The method of Reynolds and Lloyd-Evans was used.[285] A solution

of 6-trityl-β-*D*-glucose-1,2,3,4-tetraacetate (23 g, 0.039 mmole) in acetic acid (100 ml) was prepared by warming and the solution cooled to *ca.* 10 °C and a saturated solution of dry hydrogen bromide in acetic acid was added (9 ml). The mixture was shaken vigorously for 45 seconds, the precipitated trityl bromide removed *immediately* by filtration and the filtrate poured immediately into cold water (500 ml). The tetraacetate was extracted with chloroform (2 x 75 ml) and the extract washed four times with iced water and dried ($MgSO_4$). After filtration the chloroform was evaporated *in vacuo* and the remaining syrup covered with anhydrous ether (50 ml) and rubbed with a glass rod. Crystallisation took place immediately and the product was filtered and recrystallised from chloroform-ether using the *minimum* amount of chloroform to give pure β-*D*-glucose-1,2,3,4-tetraacetate (6 g, 44%) m.p. 124-126 °C (lit.[285] 128-129 °C); ^{1}H n.m.r. δ($CDCl_3$) 2.01,2.04,2.09 (3s,12H overall,*Me*-), 3.67 (m,3H,$H_{C_{5,6}}$), 5.2 (m,3H,$H_{C_{2,3,4}}$), 5.72 (d,1H, H_{C_1}, $^3J_{HH}$ ~8 Hz).

<u>*Trans*-(4*R*,5*S*)-2(1,2,3,4-tetraacetyl-6-β-*D*-glucosyl)-2-oxo-4,5-diphenyl-1,3,2 dioxaphospholane</u> (163)

The phosphorochloridate (102a) was generated in the usual fashion *in situ*, and a solution of β-*D*-glucose-1,2,3,4-tetraacetate (384 mg, 1 mmole) in pyridine (3 ml) was added dropwise over 20 min to the stirred solution of (102a) cooled in ice. The mixture was left stirring overnight and the pyridine evaporated to give a foam to which was added dry ethyl acetate (25 ml). The pyridinium hydrochloride was filtered off under nitrogen and the filtrate evaporated to give (163) as a white crystalline solid which was pumped exhaustively for 2h (530 mg, 88%), ^{31}P n.m.r. δ_P(EtOAc)D_2O lock 11.42 (s,*5-ring P*); ^{1}H n.m.r. δ($CDCl_3$) 2.0 (broad s,12H,*Me*-), 3.95 (m,1H,H_{C_5}), 4.38 (m,2H,H_{C_6}), 5.2 (m,3H,$H_{C_{2,3,4}}$), 5.78 (d,1H,

H_C, $^3J_{HH}$ = 8 Hz) 5.82 (d,2H,-C*H*-in 5-ring, $^3J_{pH}$ = 7.5 Hz), 7.06 (broad s,10H,*Ar*).

D-Glucose-6-phosphate (144)-deprotection of (163)

This compound was deprotected essentially by the method of Ukita.[284] The triester (163) was dissolved in anhydrous ethyl acetate (25 ml) and hydrogenolysed using 10% Pd/C catalyst (500 mg) which had been thoroughly dried *in vacuo* over phosphorus pentoxide. When uptake of hydrogen ceased (*ca.* 1h) the mixture was carefully filtered through glass fibre paper and the catalyst washed thoroughly with dry ethyl acetate and dry methanol. The filtrate and washings were evaporated *in vacuo* to give a mixture of 1,2,3,4-tetraacetyl-β-*D*-glucose-6-phosphate free acid and diphenylethane as a gummy solid which was dissolved in dry methanol (6-10 ml) and a freshly prepared solution of potassium methoxide in methanol added (*M*, 2 ml) to precipitate within a few minutes the *bis*-potassium salt of *D*-glucose-6-phosphate which was left at -20 °C overnight. The solid was centrifuged down, washed thoroughly with cold dry methanol, dissolved in water, filtered and lyophilised to give *bis*-potassium-*D*-glucose-6-phosphate as a white crystalline solid (185 mg, 55%). ^{31}P n.m.r. $\delta_P(D_2O)$ 2.2 (s,G-6-*P*).

D-Glucose-4,6-cyclic phosphate (145)

The title compound was prepared in two ways:

(a) Using a modification of the method of Khorana.[287] The potassium salt of (144) (100 mg, 0.36 mmole) was converted to the *mono*-pyridinium salt using Dowex 50W (pyridinium form) and the gum after evaporation of water was dissolved in water (0.5 ml). To the solution was added excess DCCI (0.42 g) in pyridine (5 ml) and the resulting mixture stirred for three days at room temperature, when water (15 ml) was added and the crystalline DCU filtered off. The

aqueous filtrate was extracted three times with ether and evaporated down *in vacuo* to yield pyridinium *D*-glucose-4,6-cyclic phosphate as a gum. ^{31}P n.m.r. $\delta_P(D_2O)$ -5.4 (s,*6-ring P*).

(b) *D*-Glucose-6-phosphate dipotassium salt (100 mg, 0.36 mmole) was converted to the *mono*-pyridinium salt as above. Addition of 4-morpholine-N,N'-dicyclohexylcarboxamidine (106 mg, 0.36 mmole) to a solution of this salt in 50/50 pyridine/water followed by evaporation *in vacuo* and successive evaporation of several volumes of dry pyridine gave the *D*-glucose-6-phosphate-*mono*-MDCA salt as a glassy solid. This salt was dissolved in dry pyridine and was added dropwise to a boiling solution of DCCI (61.9 mg, 0.3 mmole) in dry pyridine (20 ml) under anhydrous conditions. After 30 min the mixture was cooled and evaporated to small bulk *in vacuo*, water was added to precipitate the DCU which was filtered off and washed with water. The filtrate and washings were evaporated to give *D*-glucose-4,6-cyclic phosphate *mono*-MDCA salt as a gummy solid.

D-Glucose-4,6-cyclic phosphate methyl esters (166) and (167)

To the above product (145) from (a) was added water and Dowex 50W (H^+-form) and the solution stirred for 15 min. After evaporation *in vacuo* the gummy free acid was dissolved in methanol and a freshly distilled ethereal solution of diazomethane added until the yellow colour persisted. Excess diazomethane was purged under N_2 and the solution evaporated to give the methyl esters (166) and (167) in quantitative yield.

The product from (b) above was soluble in methanol and the ion-exchange procedure was performed directly in this solvent, the rest of the procedure being identical. ^{31}P n.m.r. δ_P(25% CD_3OD in MeOH) -6.5 (anomeric doublet-*axial* diastereoisomer), -4.5 (anomeric doublet-*equatorial* diastereoisomer).

Trans-(4*R*,5*S*)-2-chloro-2[^{18}O]-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (172)

The title compound was prepared in the same way as for the unlabelled phosphorochloridate (102a) except that [^{18}O]-water (99 atom %) was used.

Trans-(4*R*,5*S*)-2(1,2,3,4-tetraacetyl-6-β-*D*-glucosyl)-[2-^{18}O]-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (178)

The title compound was prepared in the same fashion as for the unlabelled compound (163) except that the [^{18}O]-labelled phosphorochloridate (172) was used.

D-Glucose-6[^{18}O]-phosphate (175)

The title compound was prepared in the same way as for the deprotection of the unlabelled triester (163) except that (178) was used.

D-Glucose-4,6[^{18}O]-cyclic phosphate (176)

This compound was prepared by both procedures reported for the cyclisation of the unlabelled compound (144).

D-Glucose-4,6[^{18}O]-cyclic phosphate methyl esters (166-171)

The title compound was prepared from (144) by both methods reported for the methylation of the unlabelled compound (145).

(2*R*,3*S*,5*R*)-2(1,2,3,4-tetraacetyl-6-β-*D*-glucosyl)-[2-^{17}O]-oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane (177)

The title compound was prepared in the same way as for the unlabelled triester (163) except that the chirally labelled phosphorochloridate (102b) was used.

D-Glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate (178)

The title compound was prepared by the deprotection of the

labelled triester (177) in the same way as described for the unlabelled compound (144).

D-Glucose-4,6[^{16}O, ^{17}O, ^{18}O]-cyclic phosphate

This compound was prepared from (178) by both methods reported for the preparation of the unlabelled compound (145).

D-Glucose-4,6[^{16}O, ^{17}O, ^{18}O]-cyclic phosphate methyl esters (192)-(197)

The title compound was prepared in the same fashion as the unlabelled compound (145).

P_1-(6-*D*-glucosyl)-P_2-diphenyl pyrophosphate (184)

D-Glucose-6-phosphate *bis*-potassium salt (121 mg, 0.36 mmole) was converted to the *mono*-tri-*n*-octylammonium salt as follows. The salt was stirred with Dowex 50W (H^+ form) and the filtrate and washings evaporated to dryness. Several volumes of dry dioxan (in which the free acid is insoluble) were evaporated off and a solution of tri-*n*-octylamine (126 mg, 0.36 mmole) in dry dioxan (2 ml) added with vigorous shaking. Methanol was also added to assist formation of the salt. After evaporation of solvent and the evaporation of several volumes of dry methanol followed by dry dioxan, the gum was dissolved in dry dioxan (2 ml) and to this stirred solution under anhydrous conditions were added freshly distilled diphenylphosphorochloridate (97 mg, 0.36 mmole) in dry dioxan (0.5 ml) followed immediately by dry tri-*n*-butylamine (67 mg, 0.36 mmole) in dry dioxan (0.5 ml). A precipitate of amine hydrochloride appeared immediately and the solution was stirred for a further 5 min to facilitate complete conversion to P_1-(6-*D*-glucosyl)-P_2-diphenyl pyrophosphate. ^{31}P n.m.r. δ_P(dioxan)D_2O lock -13.43,-13.55 (2d, $-CH_2O-P-$, α and β anomers, $^2J_{PP}$ = 18.39 Hz), -26.28 (d, $(PhO)_2P-O$, $^2J_{PP}$ = 17.65 Hz).

D-Glucose-4,6-cyclic phosphate: *t*-butoxide-catalysed cyclisation of (184)

To the solution of (184) in dry dioxan as above was added with vigorous stirring dry DMF (15 ml) followed immediately by a freshly prepared solution of potassium-*t*-butoxide (*M*, 2 ml). The immediately precipitated material was centrifuged down, washed four times with dry DMF, dissolved in water and the pH adjusted to 8.0 with hydrochloric acid to yield the potassium salt of *D*-glucose-4,6-cyclic phosphate in *ca*. 60% yield. ^{31}P n.m.r. $\delta(D_2O)$ -5.8 (s, *6-ring P*); H n.m.r. $\delta(D_2O)$ 3.2-4.2 (m,all protons except at C_1) 4.55 (d,$H_{C_1}(\beta)$,$^3J_{HH}$ = 8.0 Hz), 5.07 (d,$H_{C_1}(\alpha)$,$^3J_{HH}$ = 4.00 Hz).

Methylation of *D*-glucose-4,6-cylic phosphate prepared *via* (184)

The cyclisation yields the potassium salt which was converted to the 18-crown-6 complex as previously described. Several volumes of dry DMF were removed to ensure the absence of water and the material dissolved in dry DMSO (0.75 ml) - some potassium chloride does not dissolve. Methyl iodide was added (100 µl) and the mixture stirred overnight and a 50/50 mixture of dry methanol/d_4-methanol added (1.35 ml). The material was carefully filtered and was then ready to be examined by n.m.r.

BuOK-DPPC-Cyclisation of *D*-glucose-6[^{18}O]-phosphate (175)

D-Glucose-6[^{18}O]-phosphate was cyclised and methylated by the above procedures for the unlabelled compound.

BuOK-DPPC-Cyclisation of *D*-glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate (178)

D-Glucose-6[(*S*)-^{16}O, ^{17}O, ^{18}O]-phosphate was cyclised and methylated by the above procedures for the unlabelled compound.

[1-^{18}O]-(2*S*)-Hydroxy-1,2-diphenylethanone (200)

The title compound was prepared by the modified procedure of

Cullis and Lowe.[226,225] 2-Phenyl-2[α-(*S*)-hydroxybenzyl]-1,3-dioxolane (203) was prepared by the method of Summerbell from (2*S*)-1,2-diphenylethanone synthesised in 52% yield by the method of Cullis and Lowe[226,225] with $[\alpha]_D^{20} = 108^\circ$ after two recrystallisations for ethanol. This ketal (2.6 g, 10 mmole) was sealed in a hydrolysis tube under nitrogen with dry dioxan (4 ml), [^{18}O]-water (2 ml) and *p*-toluenesulphonic acid (200 mg) and was kept at a temperature of 85-95 °C for 3h. The mixture became homogeneous within the first 0.5h. After 3h the tube was opened and the contents transferred to a flask attached to a vacuum line on which the [^{18}O]-water/dioxan mixture was recovered for further use. The solid residue was dissolved in chloroform and washed with sodium bicarbonate solution then water, dried and evaporated down to give [1-^{18}O]-(2*S*)-hydroxy-1,2-diphenylethanone as a crystalline solid (1.66 g, 99%). Recrystallisation from ethanol gave needles, m.p. 132-133° (lit.[297] m.p. 132-133 °C); $[\alpha]_D^{20} = +98.9^\circ$ (c = 2.5, (lit.[297] unlabelled compound +118.4°).

(1*R*,2*S*)-[1-^{18}O]-1,2-Dihydroxy-1,2-diphenylethane (201)

The reduction of (200) to the title compound was achieved by the method of Pohoryles *et al.*[296] Solid lithium aluminium hydride (350 mg, 9.1 mmole) was cautiously added to a suspension of [1-^{18}O]-(2*S*)-hydroxy-1,2-diphenylethanone (1.70 g, 7.94 mmole) in dry ether (50 ml) cooled to 0 °C and the mixture was stirred for 1½h. Excess reducing agent was destroyed by the addition of ether saturated with water. The precipitated salts were removed by filtration and the filtrate washed with water, dried ($MgSO_4$) and evaporated to give [1-^{18}O]-1,2-dihydroxy-1,2-diphenylethane as a white crystalline solid (1.5 g, 87%). Recrystallisation from ethanol-water gave plates m.p. 132-133 °C (lit.[296] 134 °C).

D-Glucose-6-phosphate dimethyl ester

D-Glucose-6-phosphate was converted to the free acid by treatment with Dowex 50W (H^+ form) and the filtrate and washings evaporated to dryness *in vacuo*. To a stirred solution of the free acid in dry methanol was added freshly distilled ethereal diazomethane until the yellow colour persisted. After removal of excess diazomethane in a stream of nitrogen the solution was evaporated to dryness to yield *D*-glucose-6-phosphate dimethyl ester. ^{31}P n.m.r. $\delta_P(CD_3OD)$ -0.21 (s,α-*anomer*), -0.30 (s,β-*anomer*); ^{1}H n.m.r. $\delta(CD_3OD)$ 3.50-4.30 (m,ring protons except H_{C_1}), 3.62,3.67 (2d,*Me*OP,$^{3}J_{PH}$ = 11.25 Hz, α and β anomers respectively), 3.69,3.75 (2d,*Me*OP,$^{3}J_{PH}$ = 11.25 Hz, α and β anomers respectively), 4.50 (d,H_{C_1}(β),$^{3}J_{HH}$ = 7.80 Hz), 5.13 (d,H_{C_1}(α),$^{3}J_{HH}$ = 3.75 Hz).

D-Glucose-6[^{18}O]-phosphate dimethyl ester (219a,b,c)

The title compound was prepared from *D*-glucose-6[^{18}O]-phosphate as described above for the unlabelled compound. ^{31}P n.m.r. $\delta(CD_3OD)$ -0.18,-0.20,-0.22 (3s,MeOP=O,Me^{18}OP=O,MeOP=^{18}O, α-anomers respectively, -0.27,-0.29,-0.31 (3s,MeOP=O,Me^{18}OP=O,MeOP=^{18}O, β-anomers respectively.

Trans-(4*R*,5*S*)-2(adenosine-5'-diphospho)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (222)

Bis-tri-*n*-octylammonium adenosine diphosphate was prepared as follows: treatment of the *bis*-sodium salt of adenosine diphosphate (471 mg, 1 mmole) with Dowex 50W (H^+ form) gave ADP free acid. After evaporation *in vacuo* of water and several volumes of dry methanol, the free acid was an insoluble gummy solid. Methanol was added (25 ml) and tri-*n*-octylamine (706 mg, 2 mmole) and the mixture shaken vigorously for 10 min whereupon the nucleotide went into solution. Evaporation of solvent followed by coevaporation of four volumes of dry pyridine (4 x 50 ml) gave the required salt

as a gummy solid.

The phosphorylating agent (102a) was generated in the usual fashion (1 mmole) *in situ* and to this solution was added carefully with exclusion of moisture, tri-*n*-octylamine (1.06 g, 3 mmole) which had previously been dried by coevaporation of dry pyridine. To this stirred solution was added over 5 min the nucleotide salt from above in dry pyridine and after 5 min the pyridine was rapidly removed *in vacuo* and traces of this solvent removed by coevaporation of dry DMF to yield *trans*-(4*R*,5*S*)-2-(adenosine-5'-diphospho)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane as a mobile oil. ^{31}P n.m.r. δ(pyridine)D_2O lock +11.9 (s,*5-ring phosphate*, ^{1}H undecoupled,t, $^{3}J_{PH}$ = 7.0 Hz), -0.65 (d,*5-ring γ-phosphate triester*,$^{2}J_{PP}$ = 22.8 Hz, ^{1}H undecoupled,dt,$^{3}J_{PH}$ = 7.35 Hz), -15.24 (d,P_{α},$^{2}J_{PP}$ = 19.9 Hz, ^{1}H undecoupled,broad d), -26.5 (t,P_{β},$^{2}J_{PP}$ = 21.4 Hz, ^{1}H undecoupled, no change).

Adenosine-5'-triphosphate-deprotection of (222)

To a solution of (222) in dry DMF was added dry tri-*n*-octylamine (353 mg, 1 mmole) and 10% Pd/C catalyst (0.6 g) which had been thoroughly dried over P_2O_5, and the mixture was hydrogenolysed over 1.5h when absorption of hydrogen (*ca.* 60 ml) was complete. The resulting mixture was filtered through glass fibre filter paper supported on a sinter funnel and the residue was washed first with DMF and then *ca.* 500 ml of 300 m*M* ammonia solution in 50/50 aqueous ethanol to remove adsorbed nucleotide. The washings were concentrated *in vacuo* and then partitioned between ether and water to remove diphenylethane and the precipitated oily tri-*n*-octylamine. Evaporation of the aqueous layer gave crude *tetra*-ammonium ATP and this mixture was adjusted to pH 8.5, diluted to the conductivity of starting buffer and applied to a column of DEAE Sephadex A-25 (*ca.* 100 ml) which was run with a gradient of 200-600 m*M* TEAB over

24 h at a rate of 82 mlh^{-1}, collecting fractions every 15 min. Fractions containing ATP were identified and measured by their absorbance at 260 nm and by enzyme assay using the pyruvate kinase system[332] and these fractions evaporated down to give *tetra*-triethylammonium ATP in *ca.* 22% yield. ^{31}P n.m.r. $\delta_P(D_2O,$ pH 9.0) -8.73 (d,P_γ,$^2J_{PP}$ = 19.12 Hz), -13.94 (d,P_α,$^2J_{PP}$ = 19.90 Hz), -24.62 (t,P_β,$^2J_{PP}$ = 19.49 Hz).

(2*R*,4*S*,5*R*)-2(adenosine-5'-diphospho)-[2-^{17}O]-oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane (221)

This compound was prepared as from the unlabelled compound except that the chirally labelled phosphorochloridate (102b) was used.

Adenosine-5'[(*S*)-^{16}O, ^{17}O, ^{18}O]-triphosphate (110)

This compound was prepared by the deprotection of (221) as described for the unlabelled compound.

ENZYME AND MICROBIOLOGICAL

Initial testing of thiazole analogues

The microorganisms *Staphalococcus Aureus*, *Escherichia Coli K12*, *Alcaligenes Faecalis* and *Salmonella Typhi* were streaked onto petri dishes containing nutrient agar and Lab.-Lemco, brain heart infusion, peptone-water and peptone-water respectively, and punched holes filled with a small quantity of a solution of the thiazoles being tested. After incubation of 37 °C overnight by which time the organism had covered the plate no zones of inhibition were observed.

Testing of thiamine analogues with *Escherichia Coli* ATCC 9637

E.Coli ATCC 9637 was transferred from nutrient agar to Davis-Mingioli's minimal glucose medium[64] (10 ml) and was grown aerobically

overnight on a shaker (static solution did not grow) at 37 °C. To the medium (10 ml) containing the appropriate concentration of fluoro-compound was added an aliquot of the above resting cells (0.1 ml) and the solution shaken overnight at 37 °C. The growth of the microorganism was monitored turbidimetrically at 450 nm in 2 mm quartz cuvettes.

Coupled assay of fumarase

This assay was performed essentially according to Marco and Marco.[166] To a quartz cuvette (1 cm path length) were added the following (final concentrations in parentheses).

Malic dehydrogenase (2 units), citrate synthase (1 unit, intrinisic fumarase activity (0.05 units), monosodium fumarate (6.76×10^{-5} *M*), S-Acetyl Coenzyme A (0.62 m*M*), 3-acetyl pyridine adenine dinucleotide (0.78 m*M*), 0.01 *M* tris acetate buffer pH 7.3 (2.4 ml). All the above solutions were in buffer. In practice all the above constituents were added to the cuvette and equilibrated at 25 °C except fumarate which was added last to initiate the reaction. The increase in absorbance was monitored at 364 nm.

Inhibition of Fumarase

The above assay mixture was used except that the volume of buffer was varied as follows so as to give the following succinate and fluorosuccinate concentrations.

Succinate

Buffer added (ml)	Succinate soln. added (0.77 *M*)	Succinate conc. (m*M*)	$\frac{1}{A_{364\ nm}}$
2.4	0.0	0.0	0.051
2.3	0.1	25.9	0.077
2.2	0.2	51.8	0.100
2.1	0.3	77.7	0.125
2.0	0.4	103.6	0.143

(2*S*)-Fluorosuccinate

Buffer added (ml)	Fluorosuccinate added (15.7 m*M*) (ml)	Fluorosuccinate conc. (μ*M*)	$\frac{1}{A_{364\ nm}}$
2.40	0.000	0.0	0.075
2.38	0.025	131.0	0.107
2.35	0.050	262.0	0.109
2.30	0.100	524.0	0.129
2.20	0.200	1048.0	0.242

(2*R*)-Fluorosuccinate

Buffer added (ml)	Fluorosuccinate added (15 m*M*) (ml)	Fluorosuccinate conc. (m*M*)	$\frac{1}{A_{364\ nm}}$
2.40	0.00	0.00	0.40
2.35	0.05	0.25	0.45
2.30	0.10	0.50	0.50
2.20	0.20	1.00	0.63
2.00	0.40	2.00	0.85

The inhibition constants K_I for all these inhibitors were calculated as described in CHAPTER 5. (The fluorosuccinic acids were observed to have no effect on the malate dehydrogenase -NAD^+ system used in this coupled assay (for which K_I(succinate) is *ca.* 20 m*M*) at these concentrations).

Exchange experiments with fumarase

(a) D_2O

(2*S*)-Fluorosuccinic acid (6 mgs, 44 μmole, and the other enantiomer in a separate experiment) was dissolved in 0.05 *M* phosphate buffer pH 9.6 (2 ml), lyophilised, dissolved in ^2H_2O

(99.8 atom % ^{2}H) and lyophilised again. $^{2}H_2O$ (2 ml) was added and the solution divided into two portions. One was kept as a blank and to the other was added fumarase (12 units). The solution was incubated in a sealed container at 20 °C for 4 days and then examined by ^{1}H n.m.r. spectroscopy. The methine resonance of fluorosuccinate appeared at 5.35 ppm as a double doublet of doublets. If deuterium had been incorporated this resonance would have appeared as a doublet of doublets.[127] It is estimated that 5% of (2*S*)-fluoro-[3^{2}H]-succinate in the presence of (2*S*)-fluoro-succinate could be detected.

(b) $^{3}H_2O$

(2*S*)-Fluorosuccinic acid (10 mg, 73.5 μmole, and the other enantiomer in a separate experiment) was dissolved in 0.05 *M* phosphate buffer pH 7.6 and the solution lyophilised. To the residue was added [^{3}H]-water (2 ml, 255 mCi) and the solution was divided into two portions, one was kept as a control and to the other was added fumarase (12 units). The solutions were kept at 20 °C for 4 days and the enzyme was then denatured by the addition of concentrated hydrochloric acid to bring the pH to below one. The [^{3}H]-water was removed on a small scale vacuum line by lyophilisation and the residue repeatedly lyophilised with unlabelled water (six times). To each residue was added water (0.1 ml) and the solutions added to a scintillation cocktail (12 ml) and counted for radioactivity. No incorporation of isotope (<0.015%) could be detected.

Test of the fluorosuccinic acids as substrates of malic enzyme

The enzyme was assayed as follows according to the method of Stickland.[181] To a quartz cuvette, (3 ml volume, 1 cm path length) were added the following constituents.

Glycyl-glycine buffer pH 7.5 (2.6 ml, 50 µmole), $NADP^+$ (0.1 ml, 0.1 µmole), manganese acetate tetrahydrate (0.1 ml, 5 µmole), (2*S*)-malate (0.1 ml, 2 µmole). This mixture was equilibrated at 25 °C and neat enzyme solution added (50 µl, 0.5 units). The increase in absorbance at 340 nm due to the formation of NADPH was monitored.

In all the above ingredients except (2*S*)-malate was added (in two separate experiments) either (2*R*) or (2*S*)-fluorosuccinic acid in buffer (10 µmole). The optical density at 340 nm the wavelength of NADPH absorption was monitored for long periods but no increase was observed in either case.

Purification of acetyl coenzyme A synthetase

Acetyl coenzyme A synthetase was assayed by a modification[410] of the method of Berg.[411] To a test tube was added the following:

	blank (ml)	sample (ml)
Buffer-0.5 *M* phosphate pH 7.5	0.25	0.20
Magnesium chloride (0.1 *M*)	0.05	0.05
ATP (100 m*M*)	0.10	0.10
Potassium fluoride (0.5 *M*)	0.10	0.10
Potassium acetate (0.05 *M*)	0.20	0.20
Glutathione (100 m*M*)	0.10	0.10
Coenzyme A (6 mg/ml)	-	0.05
Hydroxylamine pH 7.5	0.20	0.20

The above constituents were mixed, incubated at 37 °C and enzyme solution added 0.10 0.10

The mixture was incubated at 37 °C for 20 min and then quenched with ferric chloride solution (0.37 *M*) in 3.3% w/v trichloroacetic

acid and 6.6% v/v conc. hydrochloric acid in water

2.00 2.00

whereupon the solutions were read at 546 nm. If $\Delta\varepsilon = \varepsilon_s - \varepsilon_b$ where ε_s and ε_b are the optical densities of sample and blank solutions respectively, then the activity U of the enzyme is given by:

$$U = \frac{\Delta\varepsilon \times 3.10}{0.98 \times 20 \times 0.1} \text{ units ml}^{-1}$$

The enzyme was purified by a modification of the method of Midelfort.[257] Commercial acetyl coenzyme A synthetase (Sigma, 5 mg protein, 5.6 units mg^{-1}) was dissolved in water (15 ml) and applied at 22 mlh^{-1} to a DEAE 52 Cellulose column (28 x 0.7 cm) equilibrated in 0.02 *M* K_2HPO_4 buffer pH 7.5 at 4 °C. A gradient of 0.02 *M* to 0.15 *M* K_2HPO_4 pH 7.5 was run over 8h and fractions (5 ml) of effluent collected and examined at 220 nm which revealed three peaks, the last of which was identified as acetyl coenzyme A synthetase. Fractions 12-26 were combined and concentrated to 4 ml under a pressure of nitrogen in a Diaflo apparatus. *M* K_2HPO_4 solution (pH 7.5, 2 ml) was added to bring the phosphate concentration up to 0.05 *M* under which condition the enzyme is stable, and the solution was frozen, stored at -20 °C overnight and lyophilised to give pyrophosphatase-free enzyme as a white powder (330 mg).

Cleavage of ATP with acetyl CoA synthetase

The reaction mixture contained the following constituents: ATP (330 µmole), potassium acetate (400 µmole), magnesium chloride (150 µmole), dithiothreitol (150 µmole), tris (4 mmole), oxaloacetate (400 µmole), coenzyme A (5 mg), citrate synthase (120 units and acetyl CoA synthetase (56 units). The final pH was adjusted to 8.60 and the solution (10 ml) kept at 37 °C. Aliquots (0.05 ml)

were removed and assayed for citrate. The reaction was essentially complete after 20 min when the enzymes were denatured and the mixture diluted to the conductivity of 100 m*M* TEAB pH 10 and applied to a DEAE A-25 Sephadex column run at 80 $ml h^{-1}$ from 100-600 m*M* TEAB over 2 days, the pyrophosphate detected by the Briggs phosphate test and the required fractions evaporated down to give *tetra*-triethylammonium pyrophosphate. ^{31}P n.m.r. $\delta_P(D_2O)$ -9.2.

General Procedure for the hexokinase-catalysed transfer from ATP to glucose

Tetra-triethylammonium adenosine-6'-triphosphate (0.20 mmole) direct from the DEAE Sephadex column was dissolved in 0.05 *M* triethanolamine buffer pH 8.0 (10 ml). Magnesium chloride hexahydrate was added and *D*-glucose to give the following concentrations:

Adenosine-6'-triphosphate	20 m*M*	in 0.08
D-Glucose	100 m*M*	triethanolamine
Magnesium chloride	6.6 m*M*	pH 8.0

This solution was equilibrated at ambient temperature and hexokinase added (Sigma type C-301, 100 µl of the commercial preparation @ 400 units ml^{-1}). The mixture was left at room temperature for 1.5h and then shaken with chloroform to denature the enzyme. After separation of the aqueous layer and washing of the chloroform layer the solution was degassed, made up to pH 8.0 and adjusted to the ionic strength of 25 m*M* triethylammonium bicarbonate buffer pH 8.0 and applied to a 100 ml column of DEAE Sephadex A-25 which was then run from 25-400 m*M* TEAB pH 8.0 over 24h at a flow rate of 82 $ml h^{-1}$ and collecting fractions every 15 min. Chloride ion from the buffer was detected directly in the fractions by means of silver nitrate solution and the fractions

between chloride and ADP were examined enzymically with the glucose-6-phosphate dehydrogenase/NADP system[333] to detect *D*-glucose-6-phosphate, and the required fractions were evaporated down.

Detection of *D*-glucose-6-phosphate by the G6PDH-NADP system

To a cuvette (1 cm path length, 3 ml volume) was added:

0.05 *M* triethanolamine buffer 6.6 m*M* in Mg	2.8 ml
NADP solution in buffer (10 mg ml^{-1})	0.1 ml
Column fraction sample	0.1 ml
Glucose-6-phosphate dehydrogenase soln. in buffer (Sigma: 10 µl commercial prep. diluted to 200 µl with buffer)	0.01 ml

The desired fractions were evaporated down and several volumes of dry methanol removed *in vacuo* to leave *D*-glucose-6-phosphate *bis*-triethylammonium salt as an opaque gum (0.18 mmole, 90% yield).

The transfer from adenosine-5'[γ(*S*)-^{16}O, ^{17}O, ^{18}O]-triphosphate was accomplished in the same fashion.

APPENDIX

APPENDIX

The Stereochemistry of 2-Substituted-2-Oxo-4,5-Diphenyl-1,3,2-Dioxaphospholanes: Assignment of Absolute Configuration to the Related Oxygen Chiral [^{16}O, ^{17}O, ^{18}O]-Phosphate Monoesters

Whilst the manuscript of this thesis was being prepared new experimental information came to light concerning the stereochemistry of the oxygen chiral phosphates prepared by our method.[226] We have now recently shown that they possess the (*S*)-absolute configuration and not the (*R*) as was previously assigned.[206] This has important implications for this thesis and the conclusions drawn in two recent associated publications[213,216], and the assignment criteria are present briefly in this appendix as follows.

Ukita[284,415] has shown that *meso*-hydrobenzoin and phosphorus oxychloride react in pyridine to give a single diastereoisomer of 2-chloro-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane, which readily reacts with an alcohol to give a single diastereoisomer of the cyclic phosphate triester. 2-Methoxy-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (Ukita's triester) prepared in this way has previously been assigned the *cis* configuration (a).[226] since it differs in melting point and ^{1}H n.m.r. parameters from the *trans*-diastereoisomer (b) (Table 1), whose structure has been established by X-ray crystallography.[416] For a number of diastereoisomers of cyclic five membered phosphate esters and amides, it has been shown that the configuration can be assigned with some confidence on the basis of the deshielding effect of the P=O group, the H-4 and H-5 protons of a *cis*-diastereoisomer resonating 0.1 to 0.4 ppm to lower field than the related *trans*-diastereoisomer.[417] The data in Table 1 appear to fulfil this expectation.

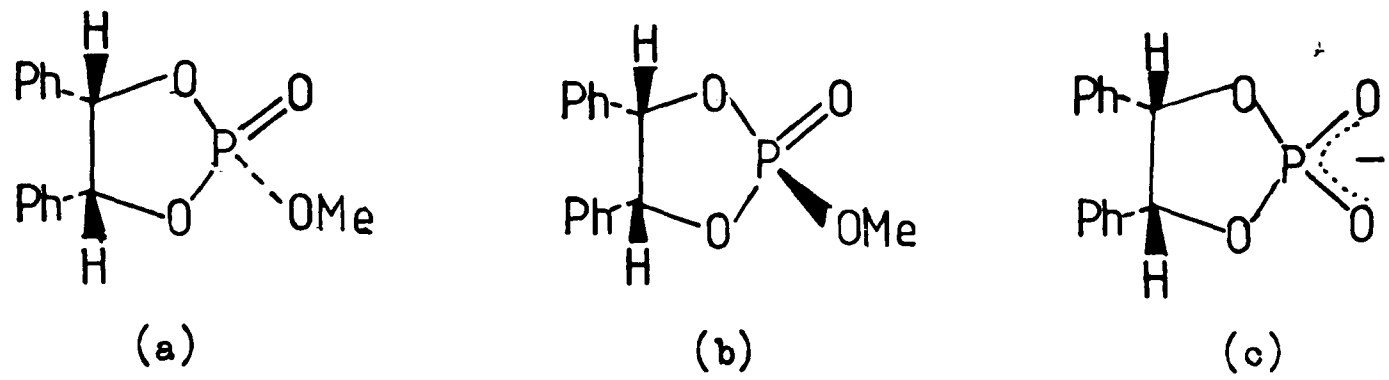
The synthetic route to Ukita's triester has been developed into a general method of synthesis of chiral [^{16}O, ^{17}O, ^{18}O]-phosphate esters.[226] A method of analysis of chiral [^{16}O, ^{17}O, ^{18}O]-phosphate

Table 1. Comparison of the properties of Ukita's triester[415], with those of authentic *trans*-2-methoxy-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (b).[416] The ^{1}H n.m.r. data of Ukita's triester are from our own work.[226]

Newton-Campbell's Triester (b)	Ukita's Triester
m.p. 74-75 °C	m.p. 101-102 °C
δ_H ($CDCl_3$)	δ_H ($CDCl_3$)
3.76 (d, J_{PH} 11.4 Hz, CH_3)	3.96 (d, J_{PH} 11.5 Hz, CH_3)
5.45 (d, J_{PH} 9.0 Hz, 2CH)	5.76 (d, J_{PH} 7.9 Hz, 2CH)

esters based on ^{31}P n.m.r. spectroscopy has also been developed which led to the unexpected conclusion that the cyclisation of phosphate monoesters to cyclic six-membered phosphate diesters occurs with retention of configuration at phosphorus.[216] Although the factors which control the stereochemical course of substitution at phosphorus in phosphate esters are not well understood[418], this conclusion together with the finding that the stereochemical course of the enzymic hydrolysis of isotopically labelled adenosine 3',5'-phosphate occurs with retention of configuration[213], whereas adenosine 3',5'-(S_P)phosphorothioate is hydrolysed by the same enzyme with inversion of configuration[212], led us to reconsider the stereochemistry of Ukita's triester.

Treatment of the pyridinium salt of the cyclic phosphate diester (c)[419] in acetonitrile, with diazomethane gave a mixture of the diastereoisomers (a) and (b) in the approximate ratio of 1:2.



FIGURE

The ^{1}H and ^{31}P n.m.r. data for the two diastereoisomers derived in this way are shown in Table 2. The assignments were made by adding to this mixture, authentic *trans*-diasteroisomer (b) prepared by the method of Newton and Campbell[416], which enhanced the intensity of one set of resonances. Addition of Ukita's triester to the mixture enhanced the intensity of the same set of resonances in both the ^{1}H and ^{31}P n.m.r. spectra. Moreover a mixture of Ukita's triester and the authentic *trans*-diastereoisomer (b) in approximately equal amounts showed resonances only of the *trans*-diastereoisomer in both the ^{1}H and ^{31}P n.m.r. spectra. Ukita's triester is therefore *trans*-2-methoxy-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (b) and it is evident that the ^{1}H n.m.r. data of Newton and Campbell (Table 1) are inaccurate. Having unequivocally assigned the *cis*- and *trans*-diastereoisomers to the n.m.r. data, it is worth noting that the *correct* ^{1}H n.m.r. data shown in Table 2, are in accord with the expectation[417] that the ring protons (H-4 and H-5) in the *cis*-diastereoisomer resonate at lower field (0.14 ppm) than those in the *trans*-diastereoisomer.

Newton and Campbell purified the *trans*-diastereoisomer by sublimation[416], whereas Ukita's triester was purified by crystallisation.[415] It seemed possible therefore that the substantive difference in melting point may be due to polymorphism. However when authentic *trans*-diastereoisomer (b) prepared by the method of Newton and Campbell was purified by crystallisation, its melting

Table 2. ^{1}H and ^{31}P n.m.r. data for the *cis*- and *trans*-diastereoisomers of 2-methoxy-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane prepared from the cyclic phosphate diester (c) by treatment with diazomethane.

trans	*cis*
δ_H ($CDCl_3$)	
3.96 (d, J_{PH} 11.6 Hz, CH_3) 5.76 (d, J_{PH} 7.9 Hz, 2CH)	4.05 (d, J_{PH} 11.6 Hz, CH_3) 5.90 (d, J_{PH} 7.9 Hz, 2CH)
δ_P ($CHCl_3$)	
+13.36 ppm	+14.23 ppm

point remained unchanged (m.p. 73-74.5 °C). We are unable to explain the higher melting point observed by Ukita (Table 1).[415]

When 2',3'-diacetyladenosine is phosphorylated by 2-chloro-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (prepared *in situ* from *meso*-hydrobenzoin and phosphorus oxychloride) in pyridine, only one diastereoisomer is obtained. If the cyclic phosphorochloridate is prepared in tetrahydrofuran with only two equivalents of pyridine, both diastereoisomers are formed which react with 2',3'-diacetyladenosine in the presence of a further equivalent of base, to give both diastereoisomers of the cyclic phosphate triester. This is analogous to the observations made during the preparation of the diastereoisomers of 2-methoxy-4,5-diphenyl-1,3,2-dioxaphospholane-2-thione.[225] The P n.m.r. chemical shifts [δ_P +11.4 ppm (*trans*) and δ_P +12.1 ppm (*cis*) in tetrahydrofuran; the assignments were made by comparison with the relative ^{31}P chemical shifts

in Table 2] indicate as expected, that the single diastereoisomer formed in pyridine (δ_p +11.4 ppm in tetrahydrofuran) has the *trans*-stereochemistry, *i.e.* it has the same stereochemistry as Ukita's triester.

The recognition that Ukita's triester is *trans*-2-methoxy-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (b) means that our general method of synthesis[226], gives chiral [^{16}O, ^{17}O, ^{18}O]-phosphate esters with the (*S*)-configuration. It also means that the cyclisation of *D*-glucose-6[^{16}O, ^{17}O, ^{18}O]-phosphate and adenosine-5'[^{16}O, ^{17}O, ^{18}O]-phosphate occurs with *inversion of configuration* at phosphorus, contrary to our earlier published conclusion.[216] Finally it follows that isotopically labelled adenosine-3',5'-phosphate is hydrolysed by beef heart cyclic AMP phosphodiesterase with *inversion of configuration* at phosphorus.[213] This is in agreement with the observed stereochemical course of hydrolysis of adenosine-3',5'-(S_p)-phosphorothioate[212], and 2'-deoxyadenosine-3',5'-phosphate[420], catalysed by the same enzyme.

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The Effect of ^{17}O and the Magnitude of the ^{18}O-Isotope Shift in ^{31}P Nuclear Magnetic Resonance Spectroscopy

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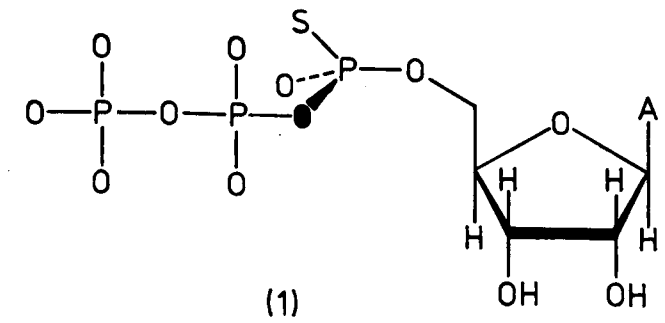
The Effect of ^{17}O and the Magnitude of the ^{18}O-Isotope Shift in ^{31}P Nuclear Magnetic Resonance Spectroscopy

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Summary The effect of ^{17}O directly bonded to ^{31}P causes marked line broadening of the ^{31}P resonance, but the coupling constant can be detected in favourable systems; the magnitude of the isotope shift caused by ^{18}O directly bonded to ^{31}P depends on the nature of the phosphorus to oxygen bond.

ALTHOUGH the coupling constant $^1J(^{17}O^{31}P)$, has been measured for several phosphorus derivatives by ^{17}O n.m.r. spectroscopy,[1] there appears to be no report of it being determined by ^{31}P n.m.r. spectroscopy, presumably due to the quadrupolar line broadening caused by ^{17}O on the ^{31}P resonance. In order to substantiate this assumption, phosphorus oxychloride containing 2 atom % ^{16}O, 44 atom % ^{17}O, and 54 atom % ^{18}O,[2] was converted into isotopically labelled trimethyl phosphate with methanol. The ^{31}P n.m.r. spectrum at 36·43 MHz consisted of a sharp line ($\Delta\nu_{\frac{1}{2}}$ 0·4 Hz) due to trimethyl [^{18}O]phosphate (and presumably the unresolved trimethyl [^{16}O]phosphate) together with six very broad ($\Delta\nu_{\frac{1}{2}}$ 90 Hz) but equally spaced lines due to trimethyl [^{17}O]phosphate, three to the low field and three to the high field side of the single sharp resonance. This is in accord with expectation since ^{17}O has a nuclear spin quantum number of 5/2; the observed coupling constant $^1J(^{17}O^{31}P)$ 156 Hz is in reasonable agreement with the value of 165 Hz determined by ^{17}O n.m.r. spectroscopy.[1] Although it is possible therefore to determine $^1J(^{17}O^{31}P)$ by ^{31}P n.m.r. spectroscopy in favourable cases, we expect ^{17}O in ^{31}P n.m.r. spectroscopy will find its most valuable application in quadrupolar line broadening when directly bonded to phosphorus, since the residual $^{31}P(^{16}O)$ or $^{31}P(^{18}O)$ signals will be at the minimum between two lines of the $^{31}P(^{17}O)$ spectrum, allowing integration of the $^{31}P(^{16}O)$ and $^{31}P(^{18}O)$ signals and hence the ^{17}O content to be estimated from the loss of signal otherwise expected.†

† *Added in proof:* Just such an application has been reported recently: M.-D. Tsai, *Biochemistry*, 1979, **18**, 1468.


S
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is assigned to P_β of (**4**). It seems clear that the magnitude of the isotope shift is a parameter which will enhance the range of applications of this technique.

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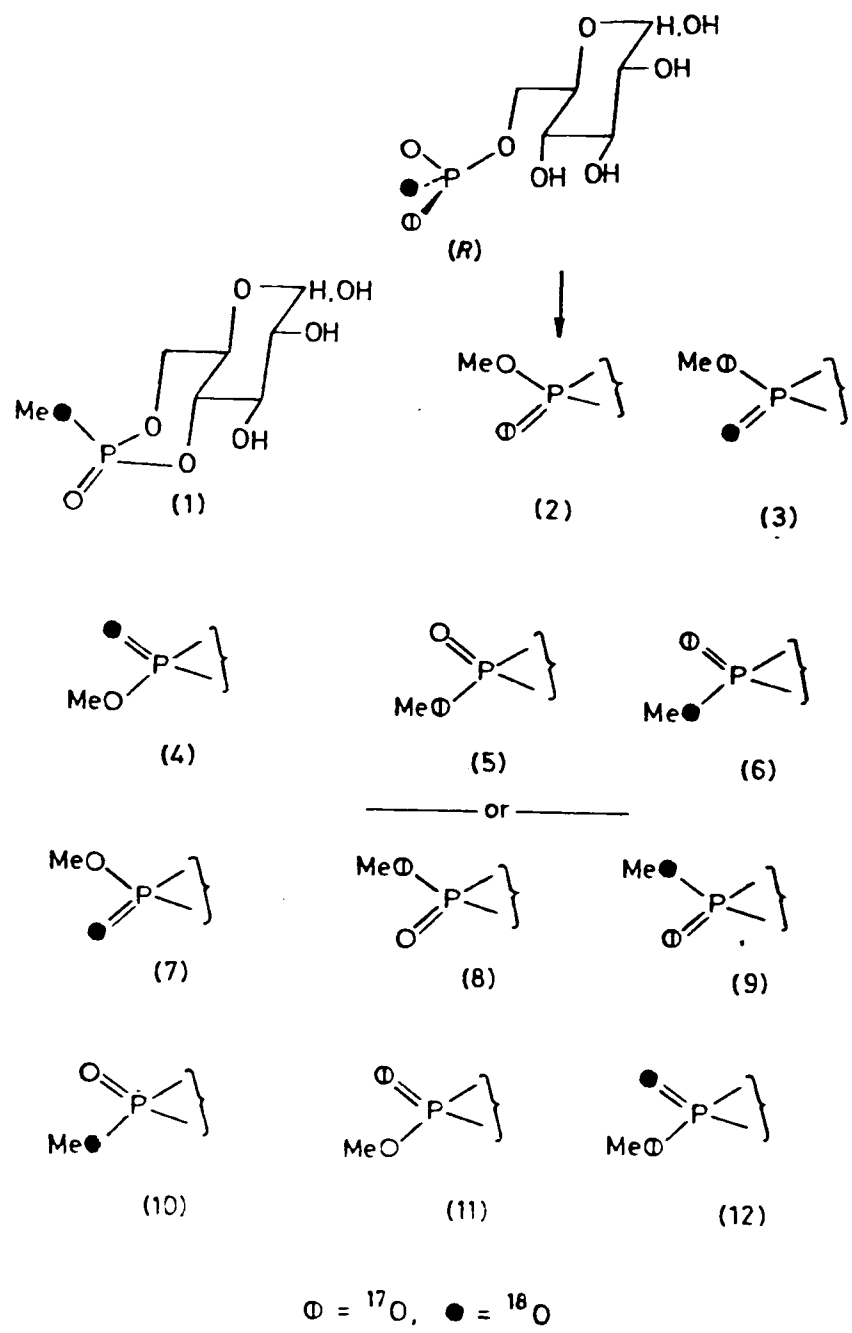
A Stereochemical Investigation of the Cyclisation of D-Glucose-6[(*R*)-^{16}O,^{17}O,^{18}O]-phosphate and Adenosine-5′[(*R*)-^{16}O,^{17}O,^{18}O]phosphate

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Summary D-Glucose-6[(*R*)-^{16}O,^{17}O,^{18}O]phosphate and adenosine-5′[(*R*)-^{16}O,^{17}O,^{18}O]phosphate were synthesised and converted (by treatment with diphenyl phosphorochloridate, followed by potassium t-butoxide) into the cyclic 4,6-phosphate and 3′5′-phosphate diesters respectively with retention of configuration at phosphorus; the absolute configuration of D-glucose-6[^{16}O,^{17}O,^{18}O]-phosphate and adenosine-5′[^{16}O,^{17}O,^{18}O]phosphate of unknown chirality at phosphorus can now be determined by ^{31}P n.m.r. spectroscopy.

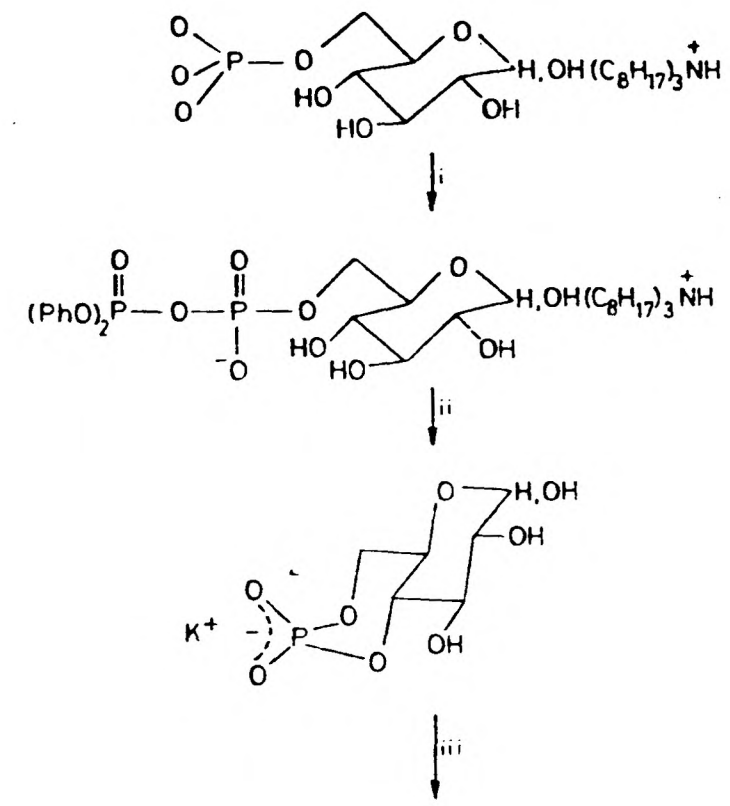
Two routes for the synthesis of chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters have been reported, one giving esters of known absolute configuration,[1] the other giving esters whose configurational assignment required independent analysis.[2] Two analytical methods have also been reported for assigning the absolute configuration of chiral [^{16}O,^{17}O,-^{18}O]phosphate monoesters. The most direct method involves determining the sign of the circular dichroism of the chiral phosphate,[1] but this requires the phosphate ester to be in a molecule which is otherwise achiral and to be free of chiral impurity. The second method depends on the enzymic transfer of the chiral phosphate to (*S*)-propane-1,2-diol, followed by ring closure to give the cyclic phosphate diester, methylation, separation of the *syn*- and *anti*-isomers (which are extremely susceptible to hydrolysis), reaction with methanol, and finally analysis by linked-scan metastable-ion mass spectrometry.[2] The former method is likely to be unreliable for quantitative analysis, while the latter is technically difficult to perform. We now report analytical procedures based on ^{31}P n.m.r. spectroscopy that are experimentally simple, stereochemically rigorous, and well suited for the stereochemical investigation of reactions of phosphate mono- and di-esters.

The analysis depends on converting a chiral [^{16}O,^{17}O,^{18}O]-phosphate mono-ester into the two diastereoisomeric, conformationally locked, six-membered cyclic phosphate triesters. In the cyclisation step, any one of the peripheral oxygen isotopes will be lost with equal probability (the kinetic isotope effect being negligible) and the residual oxygen isotopes will take up axial or equatorial positions. Alkylation of the isotopically labelled cyclic phosphate diester should give the diastereoisomeric axial and equatorial triesters. Suitable phosphate esters for this analysis would be D-glucose-6[^{16}O,^{17}O,^{18}O]phosphate and adenosine-5′[^{16}O,^{17}O,^{18}O]phosphate. In Scheme 1, species (**1**)—(**6**) would be formed by cyclising D-glucose-6[(*R*)-^{16}O,^{17}O,^{18}O]-phosphate with inversion of configuration at phosphorus, followed by methylation, whereas species (**7**)—(**12**) would be formed if cyclisation occurred with retention of configuration at phosphorus. Now ^{17}O bonded to phosphorus virtually obliterates the ^{31}P n.m.r. signal owing to its nuclear electric quadrupole moment,[3,4] so that only those species containing ^{16}O and ^{18}O bonded to phosphorus will be observed, namely (**1**) and (**4**), or (**7**) and (**10**) if the ^{17}O is fully enriched. Furthermore, ^{18}O when singly bonded to phosphorus causes a smaller isotope shift than when doubly bonded to phosphorus,[3] so that the isotope shift on the ^{31}P resonance in the axial ester (**1**) should be smaller than for the axial ester (**7**). Similarly, the isotope shift on the ^{31}P resonance of the equatorial ester (**10**) should be smaller than that of the equatorial ester (**4**). Although the six-membered cyclic phosphate triesters are quite stable, an important advantage of the n.m.r. method is that the diastereoisomers do not have to be separated since their ^{31}P chemical shifts are significantly different. Although the ^{18}O enrichment currently available is in excess of 99 atom%, the highest available enrichment of ^{17}O is about 50 atom%. However, the presence of ^{16}O and ^{18}O in the '^{17}O' site has a distinct advantage since the [$^{16}O_2$]-, the additional [^{18}O]-, and the [$^{18}O_2$]-triesters which will be formed, will serve as internal n.m.r. references.



SCHEME 1.

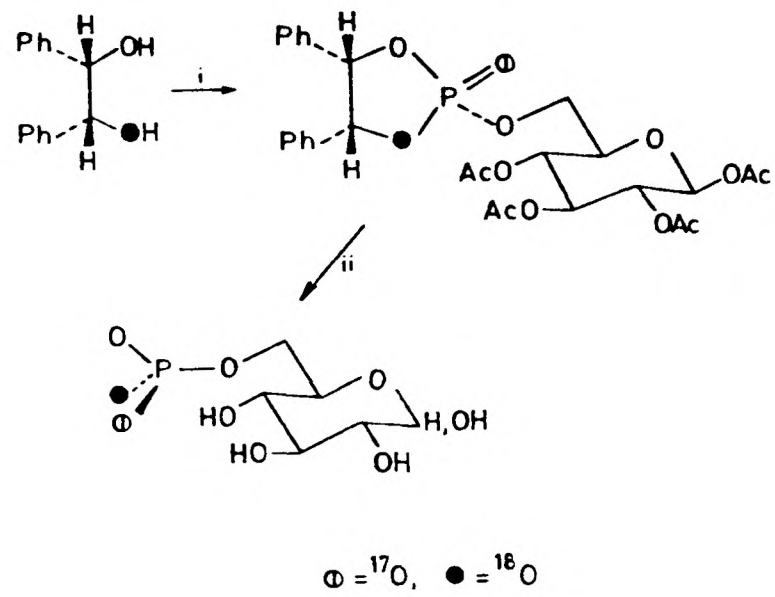
The experimental conditions necessary to bring about the transformation in Scheme 1 were first established with D-glucose-6-phosphate and are outlined in Scheme 2. The ^{31}P n.m.r. spectrum showed the axial and equatorial ester resonances to be separated by about 2 p.p.m. The axial ester was assigned to the high-field resonance as is invariably found for 1,3,2-dioxaphosphorinan-2-ones.[5,6] Both esters exist as α- and β-anomers whose ^{31}P chemical shift differences in dimethyl sulphoxide are comparable to the expected isotope shifts. However, by adding methanol to the solution, the chemical shift difference between the anomers can be progressively increased until a convenient separation is achieved.



SCHEME 2. Reagents: i, (a) $(PhO)_2POCl$ in dioxan; (b) Bu^n_3N in dioxan; ii, Bu^tOK in Me_2NCHO; iii, 18-crown-6–MeI in Me_2SO.

D-Glucose-6[^{18}O]phosphate (estimated by ^{31}P n.m.r. spectroscopy to contain 95 atom% ^{18}O) was cyclised and methylated as in Scheme 2; the ^{31}P n.m.r. spectrum of the product is shown in Figure 1a. As expected the axial and equatorial esters each appear as two sets (due to the α- and β-anomers) of three resonances; the assignments are shown on the spectrum. The integration shows that loss of isotope in the course of this chemical transformation is about 4%.

D-Glucose-6[(*R*)-^{16}O,^{17}O,^{18}O]phosphate was prepared by the method developed for the synthesis of chiral [^{16}O,^{17}O,^{18}O]-phosphate esters of known absolute configuration,[1] as outlined in Scheme 3. After cyclisation and methylation



SCHEME 3. Reagents: i, (a) $P^{17}OCl_3$, C_5H_5N (b) 1,2,3,4-tetra-acetyl β-D-glucose, C_5H_5N; ii, (a) H_2, Pd/C (b) KOMe, MeOH.

as in Scheme 2, the ^{31}P n.m.r. spectrum of the axial and equatorial esters shown in Figure 1b was obtained. All the resonances observed in Figure 1a are present, together with those of the anomers of the [$^{18}O_2$]axial and equatorial triesters. It is easy to see that the most intense resonances correspond to (**7**) and (**10**) in Scheme 1. In order to account quantitatively for the spectrum and hence determine the stereoselectivity of the cyclisation, a number of factors need to be considered. First, the isotope content of phosphorus [^{17}O]oxide trichloride (determined by mass spectrometry after conversion into trimethyl phosphate) was 3·3 atom% ^{16}O, 43·5 atom% ^{17}O, and 53·2 atom% ^{18}O. Secondly, the (1*R*,2*S*)-[1-^{18}O]-1,2-dihydroxy-1,2-diphenylethane used in

TABLE. The observed relative peak intensities of the ^{31}P resonances (from Figure 1b, average of both anomers, and Figure 2) of the ^{18}O-labelled diastereoisomeric triesters derived by cyclisation followed by methylation of D-glucose-6[(*R*)-^{16}O,^{17}O,^{18}O]phosphate (G-6P) and adenosine-5′[(*R*)-^{16}O,^{17}O,^{18}O]phosphate (A-5′P), are compared with the expected values for cyclisation with retention and inversion of configuration at phosphorus. The stereoselectivity of the cyclisation is determined by the ratio of the intensities of the two mono-^{18}O triesters, compared with the calculated value. ● = ^{18}O.

	Equatorial triester				Axial triester			
	Observed		Calculated		Observed		Calculated	
	G-6P	A-5′P	Retention	Inversion	G-6P	A-5′P	Retention	Inversion
MeO–P=O	Too inaccurate	0·35	0·28	0·28	0·41	0·34	0·28	0·28
Me●–P=O	1·00	1·00	1·00	0·74	0·76	0·75	0·74	1·00
MeO–P=●	0·72	0·75	0·74	1·00	1·00	1·00	1·00	0·74
Me●–P=●	0·65	0·55	0·49	0·49	0·62	0·59	0·49	0·49

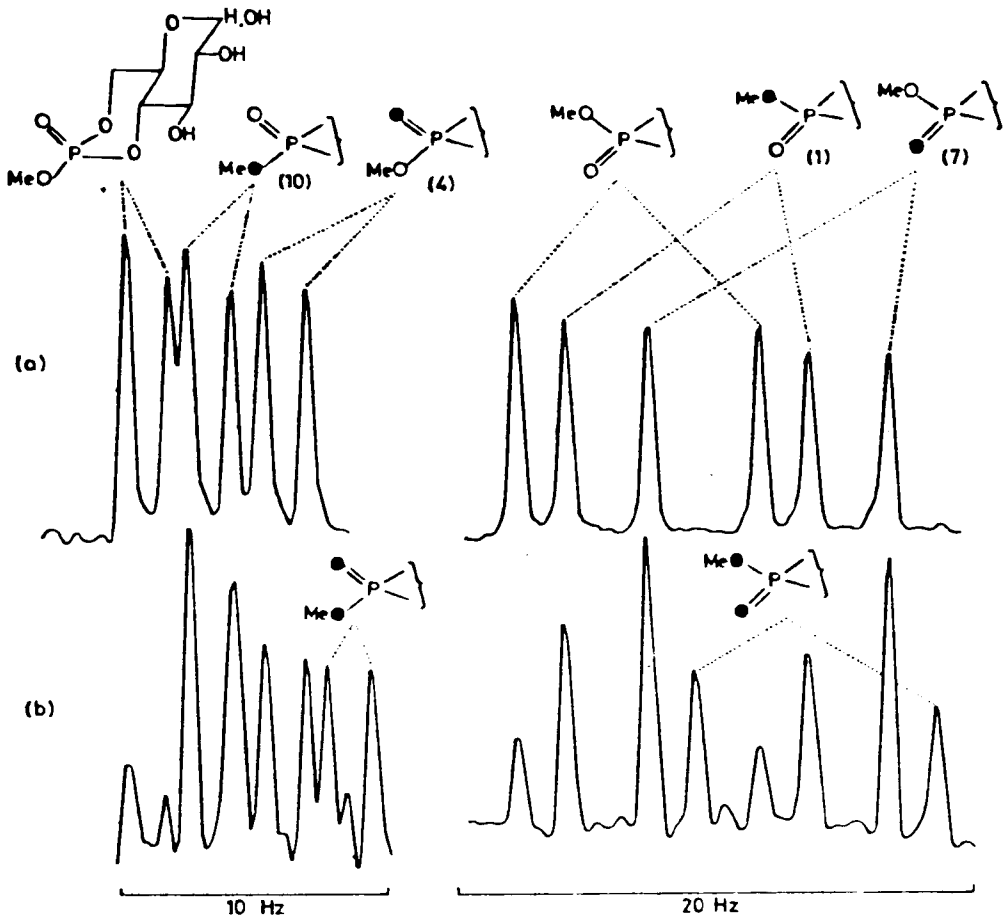


FIGURE 1. The ^{31}P n.m.r. spectra (121·5 MHz, on a Bruker WH 300 spectrometer with quadrature detection, in 25% CD_3OD, 25% MeOH, 50% Me_2SO) of the equatorial and axial triesters derived by cyclisation, followed by methylation, of (a) D-glucose-6[^{18}O]-phosphate and (b) D-glucose-6[(*R*)-^{16}O,^{17}O,^{18}O]phosphate. For the equatorial ester [$\delta(^{31}P)$ — 4·22 p.p.m., *i.e.* upfield from external $(MeO)_3PO$] the isotope shifts are 2·3 Hz ($Me^{18}OP$) and 5·0 Hz ($^{18}O{=}P$). For the axial ester [$\delta(^{31}P)$ — 6·29 p.p.m.] the isotope shifts are 1·8 Hz ($Me^{18}OP$) and 5·0 Hz ($^{18}O{=}P$). The assignments are shown on the spectra.[3] The ^{31}P n.m.r. parameters are: offset 2240 Hz, sweep width 2000 Hz, acquisition time 2·05 s, pulse width 16 μs, broadband proton decoupling, gaussian multiplication (line broadening —0·6 Hz, gaussian broadening 0·4) in 8K, and fourier transform in 32K. The gain control for the equatorial ester is four times that for the axial ester in (a) and sixteen times that for the axial ester in (b).

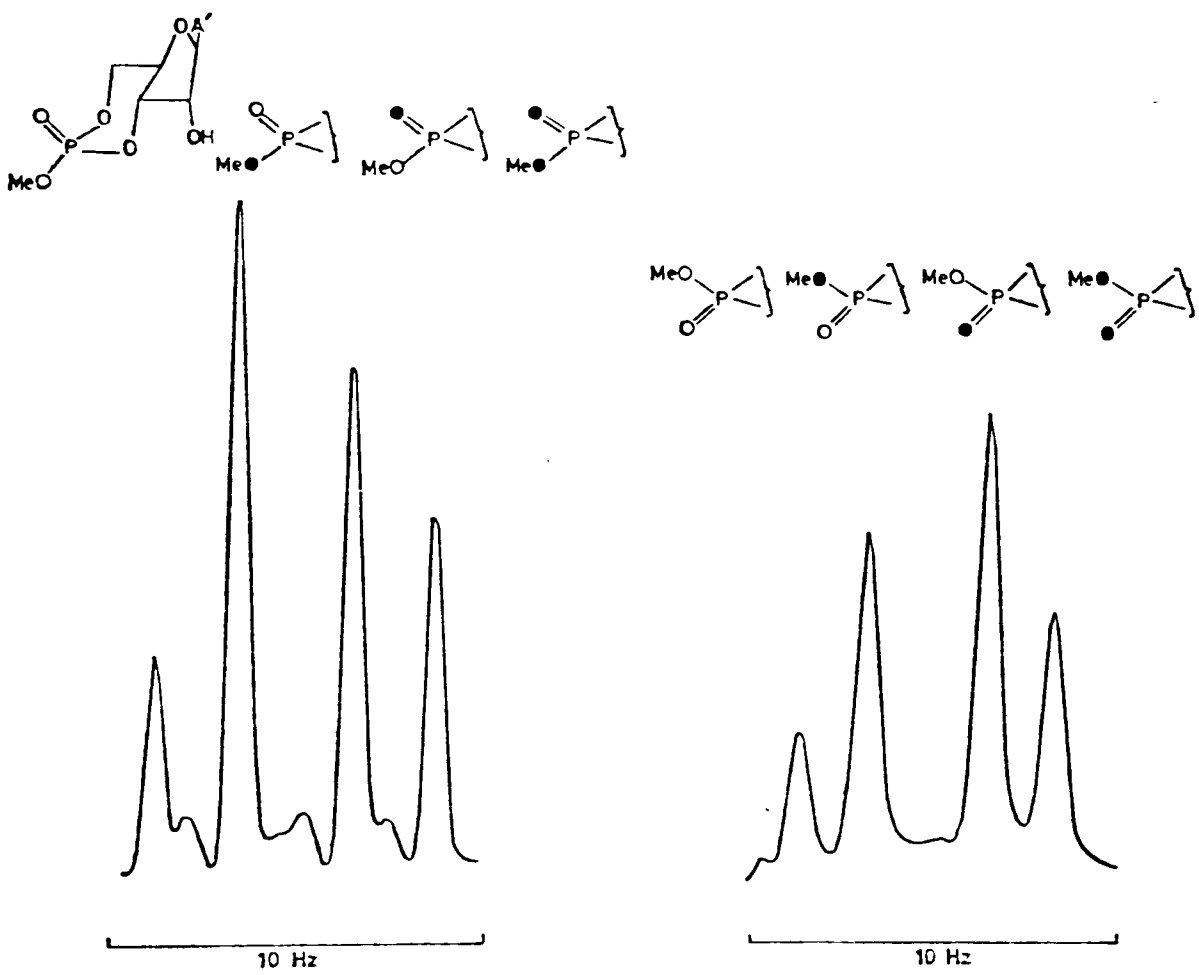


FIGURE 2. The ^{31}P n.m.r. spectrum (121·5 MHz, in 1 : 1 Me_2SO, [$^2H_6$]Me_2SO) of the equatorial and axial triesters derived by cyclisation, followed by methylation, of adenosine-5′[(*R*)-^{16}O,^{17}O,^{18}O]phosphate. For the equatorial triester [$\delta(^{31}P)$ — 4·02 p.p.m.] the isotope shifts are 2·1 Hz ($Me^{18}OP$) and 5·2 Hz ($^{18}O{=}P$). For the axial triester [$\delta(^{31}P)$ — 5·27 p.p.m.] the isotope shifts are 1·8 Hz ($Me^{18}OP$) and 5·1 Hz ($^{18}O{=}P$). The assignments are shown on the spectrum. The ^{31}P n.m.r. parameters are similar to those in Figure 1. The gain control for the equatorial triester is twice that of the axial triester. A′ = N^1-methyladenine.

the synthesis was derived by reduction of (2*S*)-[1-^{16}O]-benzoin which was 91·8% (*S*) and 8·2% (*R*), was labelled with 97 atom% ^{18}O, and was contaminated with 6% of (1*S*,2*S*)-[1-^{18}O]-1,2-dihydroxy-1,2-diphenylethane. A small loss of isotope was also detected during the hydrogenolysis of the triester (in Scheme 3) even though the catalyst was extensively dried (over P_2O_5). From an independent experiment this was estimated to be about 5% exchange of the phosphoryl oxygen and about 10% ring opening. Finally, the previously estimated 4% loss of label during the cyclisation step was allowed for. Taking all these factors into consideration the expected relative intensities of the peaks in the ^{31}P n.m.r. spectrum were calculated for inversion and retention of configuration at phosphorus and are compared in the Table with the observed intensities from Figure 1b. Clearly, the cyclisation has occurred with retention of configuration at phosphorus, and with a stereoselectivity which is in excess of 94%.

Adenosine-5′[(*R*)-^{16}O,^{17}O,^{18}O]phosphate was prepared,[1] and then cyclised and methylated as for D-glucose-6-phosphate. The ^{31}P n.m.r. spectrum is shown in Figure 2; the absence of anomers of course simplifies the spectrum. From the calculated intensities (see Table) it is seen that the cyclisation has occurred with retention of configuration at phosphorus with a stereoselectivity in excess of 94%.

The cyclisation of D-glucose-6[(*R*)-^{16}O,^{17}O,^{18}O]phosphate and adenosine-5′[(*R*)-^{16}O,^{17}O,^{18}O]phosphate with retention of configuration was not anticipated since there is evidence that the cyclisation to five-membered phosphorothioate esters,[7] and six-membered phosphoramidates occurs with inversion of configuration at phosphorus.[8] Retention of configuration implies that the intramolecular alkoxide ion attacks the activated phosphate ester adjacent to the leaving group, so requiring a pseudorotation to occur before the product can be formed. It seems likely that the adjacent attack with pseudorotation is preferred because the 'in line' approach of the alkoxide ion is stereoelectronically and electrostatically disfavoured.

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Evidence against a Step-wise Mechanism for the Fumarase-Catalysed Dehydration of (2*S*)-Malate

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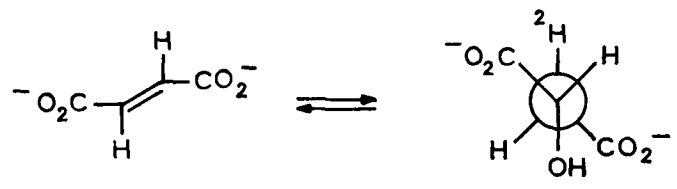
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(Received February 1, 1980)

Fumarase does not catalyse ^{18}O exchange between (2*S*,3*R*)-tartrate and solvent water, nor does it catalyse ^{2}H or ^{3}H exchange into (2*S*)-fluorosuccinate from isotopically labelled water. Both of these substrate analogues are good competitive inhibitors of fumarase. This lack of isotopic exchange provides *prima facie* evidence against the stepwise carbenium ion and carbanion mechanisms. This, together with evidence from the literature, suggests that the fumarase-catalysed dehydration of (2*S*)-malate occurs by a concerted mechanism in which breaking of the C–OH bond is much further advanced than that of the C–H bond in the transition state.

Fumarase catalyses the stereospecific hydration of fumarate to (2*S*)-malate by *trans* addition of the elements of water [1]. When the hydration is performed in $^{2}H_2O$, (2*S*,3*R*)-[3-^{2}H]malate is obtained and the reverse dehydration (in H_2O) occurs without a primary kinetic isotope effect [2] (Scheme 1). This observation led to the suggestion that as carbenium ion intermediate might be involved. More recently the comparative rates of the fumarase-catalysed hydration of the natural substrate with monofluorofumarate and difluorofumarate [3], as well as the observed secondary kinetic isotope effects, have indicated a transition state with carbenium ion character [4]. The most sophisticated use of isotopes to probe the mechanism of action of fumarase involved measurement of both the initial and equilibrium exchange rates for ^{18}O, ^{14}C, ^{3}H and ^{2}H [5]. From this investigation it was clear that the rate of exchange of the hydroxyl group of (2*S*)-malate with $H_2{}^{18}O$ was faster than the rate-limiting step, namely the random dissociation of fumarate or the substrate-derived proton from the enzyme. This evidence is attractively accounted for by the carbenium ion mechanism, but the slow release of the substrate-derived proton from the enzyme makes it also compatible with either a single concerted dehydration step or even a mechanism occurring by way of a carbanion intermediate.

Although the climate of opinion at the present time favours the involvement of a carbenium ion intermediate in the fumarase reaction pathway, Rose has very succinctly stated that 'there are no data that focus on the fundamental question of whether abstraction of a proton from malate precedes, follows or is concerted with breaking of the C–OH bond' [6]. In the terminology of Ingold [7], we do not know whether the mechanism of fumarase action is of the E1, $E1_{cb}$ or E2 type.



Scheme 1. *The stereochemical course of the fumarase-catalysed hydration of fumarate in deuterium oxide*

Fumarase catalyses the hydration of the halofumarates, acetylene-dicarboxylic acid and mesaconate. It also catalyses the dehydration of (2*S*,3*S*)-chloromalate, (2*S*,3*S*)-bromomalate, (2*S*,3*S*)-tartrate and (2*S*,3*S*)-hydroxyaspartate [8]. Thus although these unnatural substrates may not be ideally aligned with the catalytic functional groups in the active site of the enzyme, hydration and dehydration are nevertheless catalysed at an appreciable rate.

(2*S*,3*R*)-Tartrate is a potent inhibitor of fumarase. Moreover the pK_a values derived from the bell-shaped pH-dependence of inhibition [9] are similar to those found for the fumarase–(2*S*)-malate complex [10]. (2*S*,3*R*)-Tartrate is considered to be a potent inhibitor of fumarase because of its stereochemical identity at C-2 with (2*S*)-malate and the additional bonding capability of the (3*R*)-hydroxyl group; the observed

Enzyme. Fumarase (EC 4.2.1.2).

pK_a values for the fumarase–(2*S*,3*R*)-tartrate complex are also explained by this model [9].

Since it is the *pro-R* proton which is abstracted from (2*S*)-malate in the fumarase-catalysed dehydration, (2*S*,3*R*)-tartrate is not dehydrated. If however a carbenium ion intermediate is involved in the fumarase-catalysed reaction, then (2*S*,3*R*)-tartrate should undergo fumarase-catalysed exchange with $H_2{}^{18}O$. This and other isotope exchange experiments designed to elucidate the mechanism of fumarase-catalysed dehydration of (2*S*)-malate are now reported.

MATERIALS AND METHODS

Materials

Pig heart fumarase was obtained from Sigma London Chemical Co. Ltd as a crystalline suspension in 3.2 M ammonium sulphate at pH 7.5. The unit of activity is defined as the quantity of enzyme required to turnover 1.0 μmol (2*S*)-malate/min at pH 7.4 and 25 °C in the standard assay [11]. Malate dehydrogenase, (2*S*)-malic acid, 3-acetyl-pyridine-adenine dinucleotide, *S*-acetyl-coenzyme A and monosodium fumarate were obtained from Sigma London also. Deuterium oxide was obtained from Ryvan Chemical Co. Ltd; isotopically enriched water (containing 98.5 atom % ^{18}O, 51.5 atom % ^{2}H) or 90–99 atom % ^{18}O was obtained from Prochem, British Oxygen Co. Ltd. [^{3}H]Water (≈ 5 Ci/ml) was obtained from the Radiochemical Centre, Amersham. Scintillation counting was performed using an LKB liquid scintillation counter in a mixture comprised of naphthalene (120 g), ethanol (640 ml), p-bis[2-(5-phenyloxazolyl)benzene] (0.24 g) and 2,5-diphenyl-oxazole (6.0 g) made up to 2.5 l with analytical grade toluene.

Methods

Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Enzyme assays and ultraviolet measurements were performed on a Unicam SP 1800 spectrophotometer. Optical rotations were determined on a Perkin Elmer model 241 polarimeter. ^{1}H NMR spectra were recorded on a Perkin Elmer R32 spectrometer and Bruker WH 90 FT spectrometer at 90 MHz. Mass spectra were recorded on AEI MS9 and Varian CH7 spectrometers. pH measurements were made on a Radiometer type TTT1c pH meter standardised with BDH buffers.

Incubation of (2S,3R)-Tartrate with Fumarase in [^{18}O]Water

Fumarase (0.04 ml of the ammonium sulphate suspension) was diluted with 0.5 ml 0.1 M potassium phosphate buffer pH 7.4, concentrated in a Minicon A25 dialyser (to 0.078 ml) and lyophilised. The residue was dissolved in deionised water (0.5 ml) and an aliquot (0.01 ml) added to 0.63 ml 0.1 M phosphate buffer pH 7.4. The solution was assayed [11] and gave an absorbance difference, ΔA_{240}, of 0.09 min^{-1}, corresponding to an activity of 222 units/mg protein originally present.

(2*S*,3*R*)-Tartaric acid (1 ml 10 mg ml^{-1}, pH 7.4) was added to the enzyme solution and the combined solution lyophilised. To the residue was added 0.25 ml $H_2{}^{18}O$ (96.9 atom % ^{18}O) and 0.75 ml deionised water, giving 24 atom % ^{18}O-enriched solution. The solution was sealed and allowed to equilibrate at 20 °C for 15 h. An aliquot was removed, diluted with 0.63 ml 0.1 M phosphate buffer pH 7.4 and assayed. An absorbance difference, ΔA_{240}, of 0.044 min^{-1} was observed, that is 34% of the activity before tartrate addition.

The enzyme was irreversibly inhibited by adding iodoacetic acid (10 mg = 50 μmol, i.e. 30-fold excess over enzyme) and heating the solution in a water bath at 100 °C for 5 min. The solution was lyophilised. The residue was dissolved in 0.1 ml $H_2{}^{18}O$ (96.9 atom % ^{18}O) and 0.03 ml 1 M sodium hydroxide solution added; the solution was applied immediately to a column (0.9 × 15 cm) of Dowex 1 (1X8-400, $^{-}$OH form) and a linear gradient of 200 ml 6 M formic acid and 200 ml water [12] was pumped on to the column at 25 ml/h. The fractions (5 ml each) containing tartrate (fractions 16–25) were detected enzymically (inhibition of fumarase), combined, evaporated, dissolved in dry methanol and esterified with diazomethane. Dimethyl (2*S*,3*R*)-tartrate was purified by vacuum sublimation (0.1 mm Hg = 13 Pa, 65–75 °C) to give a crystalline product, m.p. 112–113 °C (cf. m.p. 114 °C [12]). The mass spectrum of dimethyl tartrate gives a fragment at *m/e* 119 due to $HO^{+}{=}CH \cdot CH(OH) \cdot CO_2CH_3$. The results of this experiment are shown in Table 1.

Synthesis of (2S,3R)-[2-^{18}O]Tartaric Acid

Hydroquinone was converted into barium *cis*-epoxysuccinate dihydrate [13,14] which on treatment with hot hydrochloric acid gave racemic 3-chloromalic acid [13]. This was resolved as its morphine salt $[\alpha]_D^{20}$ −71.0 °C [15]. (2*S*,3*R*)-3-Chloromalic acid was obtained, after treatment of the morphine salt with Dowex 50X2-400 (H^{+} form), as colourless crystals, m.p. 158–164 °C, $[\alpha]_D^{20}$ + 6.75° [15].

(2*S*,3*R*)-3-Chloromalic acid (50 mg) was dissolved in [^{18}O]water (approx. 50 atom % ^{18}O, 0.3 ml) and heated in a Carius tube at 110 °C for 45 h. The solution was lyophilised to give (2*S*,3*R*)-[2-^{18}O]tartrate. Treatment of a portion of the product with diazomethane gave dimethyl (2*S*,3*R*)-[2-^{18}O]tartrate, which was shown by mass spectrometry to contain 48 atom % ^{18}O.

Table 1. *The ratio of the mass spectral absolute ion intensities of (2S,2R)-tartrate (as the dimethyl ester), before and after fumarase-catalysed equilibration with [^{18}O]water (24 atom-% ^{18}O)*
The ratios are the mean of seven mass spectra on each sample

(2*S*,3*R*)-Tartrate	$m/e = 121 : m/e = 119$	$m/e = 120 : m/e = 119$
Unequilibrated	0.033	0.049
Equilibrated: 1st exp	0.053	0.051
Equilibrated: 2nd exp	0.049	0.058

Table 2. *The ratio of the mass spectral absolute ion intensities of (2S,3R)-[2-^{18}O]tartrate (as the dimethyl ester) before and after fumarase-catalysed equilibration with normal water*
The ratios are the mean of seven mass spectra on each sample

(2*S*,3*R*)-[2-^{18}O]Tartrate	$m/e = 121 : m/e = 119$
Unequilibrated	0.92
Equilibrated	0.95

Incubation of (2S,3R)-[2-^{18}O]Tartrate with Fumarase in Water

Fumarase (0.04 ml of the ammonium sulphate suspension) was diluted with 0.5 ml 0.1 M potassium phosphate buffer pH 7.4 and was dialysed and concentrated as before. The resulting enzyme solution was made up to 0.5 ml, in 0.1 M phosphate buffer, pH 7.4 and the solution assayed [11]. The activity was 160 units/mg protein originally present.

(2*S*,3*R*)-[2-^{18}O]Tartrate (10 mg) in 1.0 ml 0.1 M potassium phosphate buffer pH 7.4 was added to the fumarase solution and the solution incubated for 18 h at 25 °C. The enzyme was still active after this period.

The enzyme was irreversibly denatured by the addition of iodoacetic acid (10 mg) and the solution heated to 80 °C for 10 min. The mixture was separated by ion-exchange chromatography as before and the tartaric acid esterified with diazomethane. The dimethyl ester was purified by vacuum sublimation. The mass spectral results are shown in Table 2.

(2S)-Fluorosuccinic Acid and (2R)-Fluorosuccinic Acid

These were prepared as described by Lowe and Potter [16].

Inhibition Experiments with Fumarase

The more sensitive coupled enzyme assay [17] was used to determine the competitive inhibition constants of succinate, (2*S*)-fluorosuccinate and (2*R*)-fluorosuccinate in 0.01 M Tris acetate, pH 7.3 at 25 °C. In this assay (2*S*)-malate formed from fumarate is converted into oxaloacetate with malate dehydrogenase and 3-acetyl-pyridine-adenine dinucleotide, and then by citrate synthetase and acetyl-CoA to citrate. Citrate synthetase from Sigma (London) was too heavily contaminated with fumarase to be able to achieve a satisfactory excess of coupling enzyme; however, the Boehringer enzyme (110 units mg^{-1}) contained only 5 units fumarase/mg protein and was thus acceptable.

Incubation of (2S)-Fluorosuccinate with Fumarase in ^2H_2O

(2*S*)-Fluorosuccinic acid (44 μmol) was dissolved in 2 ml 0.05 M potassium phosphate buffer pH 7.6, lyophilised, dissolved in ^2H_2O (99.8 atom % ^{2}H) and lyophilised again. 2 ml ^2H_2O (99.8 atom % ^{2}H) was added and the solution divided into two portions. One was kept as the blank and to the other was added fumarase (12 units). The solution was incubated in a sealed container at 20 °C for 4 days and then examined by ^{1}H NMR spectroscopy. The methine resonance of fluorosuccinate appeared at 5.35 ppm as a double doublet of doublets. If deuterium had been incorporated this resonance would have appeared as a doublet of doublets [16]. It is estimated that 5% of (2*S*)-fluoro-[3-^{2}H]succinate in the presence of (2*S*)-fluorosuccinate could be detected.

Incubation of (2S)-Fluorosuccinate with Fumarase in [^3H]Water

(2*S*)-Fluorosuccinic acid (10 mg) was dissolved in 2 ml 0.05 M phosphate buffer pH 7.6 and the solution was lyophilised. To the residue was added 2 ml [^{3}H]water (255 mCi) and the solution was divided into two portions: one was kept as a control and to the other was added fumarase (12 units). The solutions were kept at 20 °C for 4 days and the enzyme was denatured by addition of concentrated hydrochloric acid which brought the pH to below one. The [^{3}H]-water was removed on a small-scale vacuum line by lyophilisation and recovered, and the residues repeatedly lyophilised with unlabelled water (six times). To each residue was added water (100 μl) and the solution added to a scintillation cocktail (12 ml) and counted for radioactivity. No incorporation of isotope (< 0.015%) could be detected.

RESULTS AND DISCUSSION

(2*S*,3*R*)-Tartrate was equilibrated with [^{18}O]-water (24 atom % ^{18}O) in the presence of fumarase at pH 7.4 and 20 °C for at least 15 h in two separate experiments. The enzyme was still active at the end of this period. The enzyme was irreversibly denatured by heating the solution in the presence of an excess of iodoacetate.

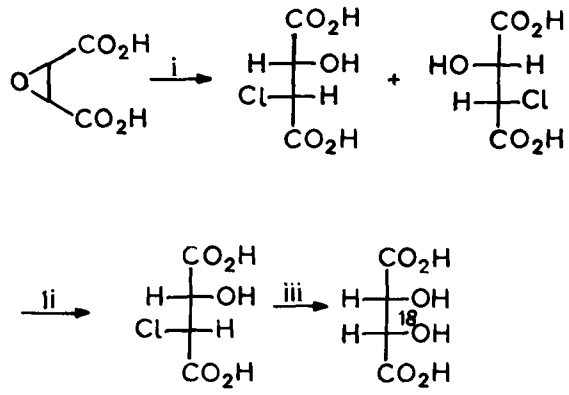
Since competitive inhibitors stabilise the enzyme against denaturation [18], great care was taken to ensure that irreversible denaturation was complete. The (2*S*,3*R*)-tartaric acid was isolated by anion-exchange chromatography and converted into its dimethyl ester. The two most intense peaks in the mass spectrum of dimethyl (2*S*,3*R*)-tartrate are at *m/e* 119 and 90 which were assigned to the α-cleavage ion $[HO^+=CH \cdot CH(OH) \cdot CO_2CH_3]$ and the McLafferty rearrangement ion radial $[CH_3O(HO)C=CH(OH)]^+$ respectively.

The diffusion-controlled association rate constant (k_{on}) for molecules the size of tartrate and fumarase is approximately $10^8\ M^{-1}\ s^{-1}$ and the K_i value for (2*S*,3*R*)-tartrate at pH 7.4 is 3×10^{-6} M [9]. The rate constant for dissociation (k_{off}) should therefore be approximately $3\times10^2\ s^{-1}$. At the concentrations used, each (2*S*,3*R*)-tartrate molecule should bind and dissociate from the enzyme about 1000 times during the period of equilibration. However no significant incorporation of ^{18}O into (2*S*,3*R*)-tartrate could be detected by mass spectrometry of its dimethyl ester (Table 1). The small apparent variations in the *m/e* = 121 : *m/e* = 119 ratio are within the reproducibility of the spectrometer; the *m/e* = 120 : *m/e* = 119 ratio is shown for comparison. Furthermore the *m/e* = 92 : *m/e* = 90 ratio does not differ significantly from that of the unequilibrated sample.

Since the lack of fumarase-catalysed exchange between (2*S*,3*R*)-tartrate and $H_2{}^{18}O$ was contrary to expectation if the carbenium ion mechanism was correct, (2*S*,3*R*)-[2-^{18}O]tartrate was prepared as outlined in Scheme 2, and the sample was equilibrated with normal water in the presence of fumarase. No loss of ^{18}O from (2*S*,3*R*)-[2-^{18}O]tartrate could be detected by mass spectrometry of its dimethyl ester (Table 2).

Lack of fumarase-catalysed ^{18}O exchange between (2*S*,2*R*)-tartrate and water provides *prima facie* evidence against a reaction mechanism involving a carbenium ion intermediate. The evidence does not rigorously rule out the E1 mechanism since it depends on the assumption that (2*S*,3*R*)-tartrate binds to the active site of fumarase essentially in the same mode as (2*S*)-malate. The justification for this assumption, however, has already been made (see introduction).

In order to investigate the $E1_{cb}$ mechanism, (2*S*)-fluorosuccinate was prepared from (2*R*)-malic acid [16]. (2*S*)-Fluorosuccinate was found to be a potent competitive inhibitor of fumarase ($K_i = 2.4 \pm 0.7 \times 10^{-5}$ M at pH 7.3) and (2*R*)-fluorosuccinate was also a good competitive inhibitor ($K_i = 9.7 \pm 0.2 \times 10^{-5}$ M at pH 7.3). There is ample testimony that fluorine can serve as an analogue of H or the OH group (see symposia [19,20]), but since succinate has a $K_i = 3.2 \pm 0.2 \times 10^{-3}$ M for fumarase at pH 7.3 in Tris-acetate buffer (others [9] give 1.2×10^{-3} M at



Scheme 2. *Preparation of (2S,3R)-[2-^{18}O]tartrate.* Reagents: i, HCl; ii (a) morphine, (b) ion exchange; iii, $H_2{}^{18}O$

25 °C in Tris-acetate buffer at the pH optimum of 7.0), it is clear that fluorine is making a contribution to binding, particularly in (2*S*)-fluorosuccinate where fluorine has replaced the hydroxyl group of the natural substrate.

(2*S*)-Fluorosuccinate was equilibrated with ^2H_2O (99.8 atom % ^{2}H) in the presence of fumarase at 35 °C for 4 days. The enzyme was still active after this period, but the ^{1}H NMR spectrum of the equilibrated (2*S*)-fluorosuccinate was identical with that of the unequilibrated sample. When the experiment was repeated in [^{3}H]water, no significant incorporation of tritium was detected, indicating that the amount of exchange, if any, was less than 0.015 %.

Since (2*S*)-fluorosuccinate has a K_i for fumarase comparable with the K_m for (2*S*)-malate (2.5×10^{-5} M) [10], it seems very unlikely that the lack of exchange could be due to incorrect binding. The possibility that the proton abstracted is always recaptured by the (2*S*)-fluorosuccinate and is never exchanged with solvent also appears improbable. The strong inductive effect of fluorine would be expected to facilitate carbanion formation at C-3 if an $E1_{cb}$ mechanism was possible, but it seems likely that if it were formed, loss of fluoride ion would occur. No evidence of fumarate or malate could however be found. This then provides *prima facie* evidence against an $E1_{cb}$ mechanism.

Although the evidence presented apparently rules out both of the step-wise mechanisms, and therefore implies that the fumarase-catalysed dehydration of (2*S*)-malate occurs by the concerted mechanism, positive evidence to support this proposal is tenuous.

The absence of a primary kinetic isotope effect in the fumarase-catalysed dehydration of (2*S*,3*R*)-[3-^{2}H]-malate to fumarate [2] at pH 7.3, can be ascribed to the rate-limiting release of the substrate-derived proton (deuteron) and fumarate from the enzyme [5]. At pH 5 a kinetic isotope effect ($k_H/k_{2_H} = 1.31$) was observed [21]. In an attempt to detect a kinetic isotope effect at pH 7.3, a comparison was made of the rate of fumarase-catalysed ^{18}O exchange from (2*S*,3*R*)-[3-^{2}H,2-^{18}O]malate and (2*S*,3*R*)-[2-^{18}O]malate to water at equilibrium. An isotope effect, $k_H/k_{2_H} = 1.15$

was reported [21]. These values should not be regarded as true kinetic isotope effects however, since the elimination step is not rate-limiting in the overall reaction nor is it known to be in the ^{18}O exchange reaction. Indeed if the concept is valid that enzymes have evolved, at least in the central metabolic pathways, to the point where the chemical events occur at rates comparable or faster than the release of products [22] we should not expect the elimination or addition step to be rate-limiting. The isotope effects should therefore be regarded as lower limits to the true kinetic isotope effect of the elimination step. In any event a deuterium kinetic isotope effect even of 1.3 is consistent with a primary kinetic isotope effect for a reaction with a highly asymmetric transition state [23, 24]. Moreover, all the experimental evidence which has been cited as supporting a carbenium ion mechanism is consistent with a concerted elimination in which the transition state has a high degree of carbenium ion character. Indeed we know of no observation which is inconsistent with the conclusion that fumarase catalyses the dehydration of (2*S*)-malate by a concerted mechanism via a transition state in which breaking of the C–OH bond is much further advanced than that of the C–H bond.

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Synthesis, Absolute Configuration, and Circular Dichroism of the Enantiomers of Fluorosuccinic Acid

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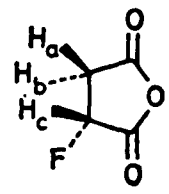
Diethylaminosulphur trifluoride (DAST) converts (2*S*)- and (2*R*)-malate esters into the enantiomeric fluorosuccinate esters. (2*S*,3*R*)-[3-$^2H_1$]Malate, obtained from fumarate by fumarase-catalysed hydration in deuterium oxide, was used to show that the reaction with DAST occurs stereospecifically with *inversion* of configuration. Conversion of (2*S*)-aspartic acid into fluorosuccinate with sodium nitrite in polyhydrogen fluoride–pyridine occurs predominantly with *retention* of configuration. The circular dichroic spectra of (2*S*)- and (2*R*)-fluorosuccinic acids and their methyl esters are ' anomalous ' and consequently the absolute configuration previously assigned to a *Pseudomonal* metabolite, (+)-fluorosuccinic acid, is shown to be erroneous.

During an investigation of the mechanism of action of the enzyme fumarase,[1] the need arose for (2*S*)-fluorosuccinate as an analogue of the natural substrate (2*S*)-malate. (2*RS*)-Fluorosuccinic acid has been synthesised,[2] and is a substrate for succinate dehydrogenase.[3] However no resolution of the racemate has been reported, although attempts have been made.[3] (+)-Fluorosuccinic acid was obtained as a *Pseudomonal* metabolite of *p*-fluorophenylacetic acid, but the D-configuration, assigned to it by comparison of its optical rotatory dispersion curve with those of other D-(+)-halogenosuccinic acids, cannot be regarded as secure.[4]

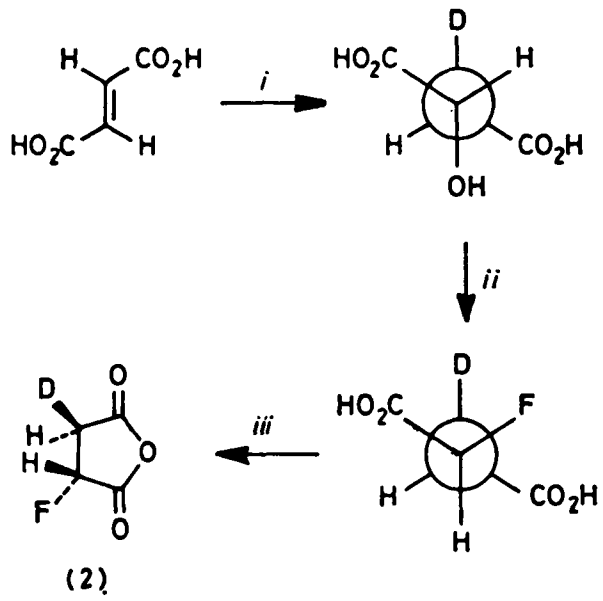
Diethylaminosulphur trifluoride (DAST) [5,6] converts alcohols into fluoro-compounds under mild conditions and with much less dehydration than is commonly encountered with other fluorinating reagents.[7] Moreover, good evidence has been provided that the reagent first forms an alkoxydiethylaminosulphur difluoride intermediate which is attacked by fluoride ion with inversion of configuration.[8] However, in an extensive investigation of the reaction of DAST with oxygenated 5α-androstanes, several examples of substitution of hydroxy-groups with fluorine occurred with retention of configuration, not all of which could be explained by neighbouring-group participation.[9]

Synthesis of (2S)- *and* (2R)-*Fluorosuccinates.*—(2*R*)- and (2*S*)-Malic acids were esterified and converted with DAST at 0 °C into the enantiomeric fluorosuccinic dimethyl esters in high yield, the only by-product being *ca.* 6% of dimethyl fumarate. The esters, after purification by preparative g.l.c., were shown to be pure enantiomers by n.m.r. spectroscopy using the chiral shift reagent tris-[3-(trifluoroacetyl)-(+)-camphorate]-europium(III).[10] The esters were hydrolysed under acidic conditions,[2] in view of the known propensity of fluorosuccinic esters to give fumarate under even mildly basic conditions.[11] The recrystallised acids had melting points virtually identical with that reported for (+)-fluorosuccinic acid.[4]

Assignment of the Absolute Configuration of the 2-*Fluorosuccinic Acids.*—In order to establish rigorously the absolute configurations of the enantiomeric fluorosuccinic acids and the stereochemical course of the DAST reaction, the synthesis was repeated with (2*S*,3*R*)-[3-$^2H_1$]malic acid which was obtained by the fumarase-catalysed hydration of fumarate in deuterium oxide.[12,13] Purification of the 2-fluoro-[3-$^2H_1$]succinic ester followed by acid-catalysed hydrolysis gave a 2-fluoro[3-$^2H_1$]-succinic acid (Scheme) 2-Fluorosuccinic acid derived from (2*S*)-malic acid was converted into its anhydride (1) by refluxing with acetyl chloride.[14] The ^{19}F n.m.r. spectrum is shown in Figure 1(a) from which the coupling constants $^3J_{H_aF}$ 13.5, $^3J_{H_bF}$ 24.9, and $^2J_{H_cF}$ 52.5 Hz can be obtained. These assignments are based on extensive literature precedent and are entirely in accord with expectation.[15] The ^{19}F n.m.r. spectrum of the 2-fluoro-[3-$^2H_1$]succinic anhydride derived from 2-fluoro-[3-$^2H_1$]succinic acid consisted of four lines [Figure 1(b), broader than those in 1(a) owing to unresolved deuterium coupling] with $^3J_{HF}$ 25 and $^2J_{HF}$ 55 Hz. The



(1)



(2)

Scheme *Reagents: i*, fumarase, D_2O; *ii* (a) CH_2N_2, (b) DAST, (c) H_3O^+; *iii*, CH_3COCl

$^3J_{HF}$ coupling constant establishes the structure unequivocally as (2*R*,3*S*)-2-fluoro-[3-$^2H_1$]succinic anhydride (2).*

Circular Dichroism of (2S)- *and* (2R)-*Fluorosuccinic Acids and Esters.*—The anomalous behaviour of fluoroketones, when compared with chloro-, bromo-, and iodoketones is frequently but not invariably observed in circular dichroism, the phenomenon first being encountered as a violation of the axial halogeno-ketone rule.[16] The c.d. spectra of (2*S*)- and (2*R*)-fluorosuccinic acids and their dimethyl esters demonstrate their antipodal stereochemistries. The single Gaussian band with maximum at 214 nm in both acids and esters is assigned to the carbonyl $n \longrightarrow \pi^*$ transition (Figure 2; only dimethyl (2*S*)-fluorosuccinate is shown) and shows the hypsochromic shift expected by comparison with (2*R*)-chlorosuccinic acid ($\lambda_{max.}$ 224 nm) and (2*R*)-bromosuccinic acid ($\lambda_{max.}$ 236 nm).[17] The magnitude of $\Delta\varepsilon_{max.}$ for the

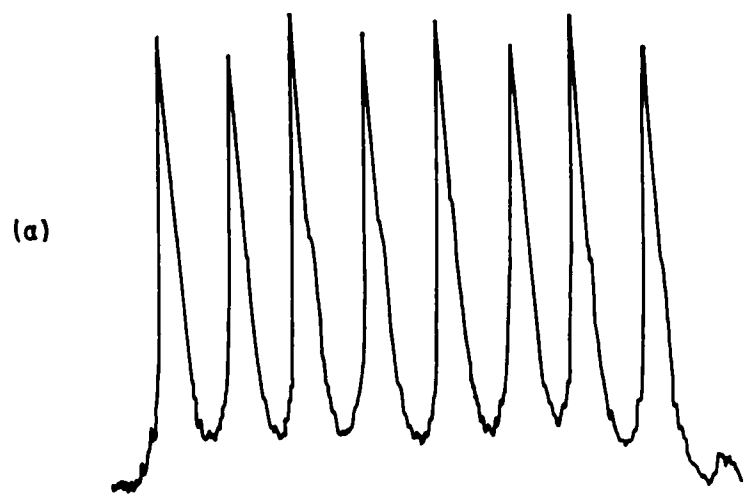


FIGURE 1 ^{19}F N.m.r. spectra at 84.6 MHz of (a) fluorosuccinic anhydride (1) and (b) (2*R*,3*S*)-2-fluoro-[3-$^2H_1$]succinic anhydride (2)

esters is similar to that of the acids, as is commonly observed (Table).[18] However the effect of fluorine on the signs of the c.d. spectra is 'anomalous,' (2*R*)-fluorosuccinic acid and its diester exhibiting negative circular dichroism, whereas (2*R*)-chloro- and (2*R*)-bromo-succinic acids have positive c.d. spectra.[17,19] This is the first example of an 'anomalous' c.d. spectrum for a fluorocarboxylic acid or ester and infringes upon the generalisations proposed for carboxylic acids.[20]

Fluorosuccinic acid has also been prepared by the diazotisation of aspartic acid in polyhydrogen fluoride–pyridine, but no comment about the stereochemistry was made and its chiroptical properties were not measured.[21] Fluorosuccinic acid made by this method from (2S)-aspartic acid showed a positive c.d. curve with $\lambda_{max.}$ 214 nm $\Delta\varepsilon$ + 0.81. Thus the transformation occurs with *retention* of configuration [(2*S*)-aspartic acid to (2*S*)-fluorosuccinic acid] presumably as a result of neighbouring carboxy-group participation. The $\Delta\varepsilon$ however is 25% lower than that of (2*S*)-fluorosuccinic

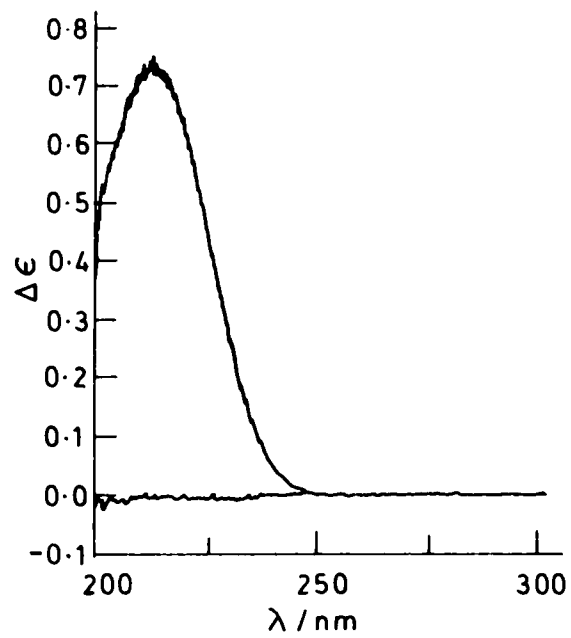


FIGURE 2 C.d. spectrum in methanol of dimethyl (2*S*)-fluorosuccinate

acid prepared by the DAST reaction, reflecting some racemisation.

The optical rotatory dispersion spectrum of a fluoro-succinic acid isolated after metabolism of *p*-fluorophenylacetic acid by a *Pseudomonas* species showed a positive Cotton effect which was interpreted, by analogy, as arising from an acid possessing the D-configuration.[4] This is now seen to be erroneous and the metabolite is actually (2*S*)-fluorosuccinic acid. A transform of the optical rotatory dispersion curve showed it to possess a c.d. maximum at 217 nm with $\Delta\varepsilon$ approximately 78% of that of (2*S*)-fluorosuccinic acid obtained by the DAST reaction.

Circular Dichroic Spectral Data for Halogenosuccinic Acids and Esters

Compound	$\lambda_{max.}$(MeOH)/ nm	$\Delta\varepsilon$	Reference
(2*S*)-Fluorosuccinic acid	214	+1.08	This work
(2*R*)-Fluorosuccinic acid	214	−0.97	This work
(2*R*)-Chlorosuccinic acid	224	+1.17	17
(2*R*)-Bromosuccinic acid	236	+2.01	17
Dimethyl (2*S*)-fluorosuccinate	214	+0.74	This work
Dimethyl (2*R*)-fluorosuccinate	214	−0.74	This work

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage apparatus. ^{1}H and ^{19}F N.m.r. spectra were recorded on a Perkin-Elmer R32 spectrometer at 90 and 84.6 MHz respectively. Chemical shifts are reported in p.p.m. from tetramethylsilane and external trifluoroacetic acid respectively, ^{19}F chemical

* The change in configurational assignment at C-3 follows from the sequence rules governing the *R*,*S* notation.

shifts being quoted as positive if upfield from the reference. Mass spectra were recorded on a VG micromass high-resolution 16F spectrometer and i.r. spectra on a Unicam SP1000 spectrophotometer. Deuterium oxide was obtained from Fluorochem Ltd., fumaric acid (monosodium salt) and fumarase were obtained from Sigma Chemical Co. Ltd., and polyhydrogen fluoride–pyridine reagent and (2*R*)- and (2*S*)-malic acid were obtained from Aldrich Chemical Co. Ltd. Preparative g.l.c. was performed on a PEGA column obtained from Pye Unicam Ltd. and fitted to a Pye Unicam Series 105 Chromatograph.

Dimethyl (2S)-*Malate*.—This diester was prepared from (2*S*)-malic acid ($[\alpha]_D^{20}$ −28.3, *c* 5 in pyridine) in 64% yield by the method of Brenner and Huber.[22]

Dimethyl (2R)-*Fluorosuccinate*.—To a stirred solution of diethylaminosulphur trifluoride[7] (2.0 g, 12.4 mmol) in dry ethanol-free chloroform (10 cm³) cooled to 0 °C was added dropwise over 15 min a solution of dimethyl (2*S*)-malate (2.0 g, 12.4 mmol) in dry ethanol-free chloroform (10 cm³). The mixture was allowed to reach ambient temperature (30 min) and an equal volume of water added cautiously to the vigorously stirred solution. The organic layer was separated, washed with saturated sodium hydrogencarbonate solution and saturated brine, dried ($MgSO_4$), and evaporated under reduced pressure to give dimethyl (2*R*)-fluorosuccinate (1.73 g, 85%) contaminated with *ca.* 6% dimethyl fumarate (estimated from the ^1H n.m.r. spectrum). The pure ester was obtained by preparative g.l.c. using a PEGA column at 150 °C; τ[$(CD_3)_2CO$] 4.74 (dt, $^3J_{HH}$ 6.0, $^2J_{HF}$ 45.0 Hz, 1 H, CHF) 6.25 and 6.33 (s, 3 H each, CO_2Me), and 6.92 and 7.22 (m, 2 H, CH_2); δ_F +114.8 (dt, $^2J_{HF}$ 49.5, $^3J_{HF}$ 25.0 Hz), c.d. $\lambda_{max.}$ 214 nm, Δε −0.74.

(2R)-*Fluorosuccinic Acid*.—Dimethyl (2*R*)-fluorosuccinate (1.73 g) was refluxed with 5% sulphuric acid (10 cm³) for 1.25 h. The solution was made strongly acidic with concentrated sulphuric acid and extracted with ether, and the extract was dried ($MgSO_4$) and the solvent removed to give (2*R*)-fluorosuccinic acid as a crystalline solid (1.02 g, 71%). Recrystallisation from ethyl acetate, or ethyl acetate–chloroform gave the pure *acid*, m.p. 130—132 °C, which could be vacuum-sublimed (*ca.* 115 °C and 0.5 mmHg); τ([$^2H_6$]DMSO) 4.8 (ddd, $^2J_{HF}$ 47.5, $^3J_{HH}$ 5 and 7 Hz, 1 H, CHF), and 7.05 and 7.33 (m, 2 H, CH_2). δ_F ([$^2H_6$]DMSO) + 110.1 (dt, $^3J_{HF}$ 25.0, $^2J_{HF}$ 47.8 Hz), c.d. $\lambda_{max.}$ 214 nm, Δε −0.97 (Found: C, 35.5; H, 3.9. $C_4H_5FO_4$ requires C, 35.3; H, 3.7%).

(2R)-*Fluorosuccinic Anhydride* (1).—(2*R*)-Fluorosuccinic acid (250 mg) was suspended in acetyl chloride (10 cm³) and refluxed for 1.5 h. The resulting solution was evaporated under reduced pressure to give (2R)-fluorosuccinic anhydride (200 mg, 92%), $\nu_{max.}$ ($CHCl_3$) 1 810 and 1 895 cm^{-1} (CO·O·CO); τ($CDCl_3$) 4.46 (td, $^3J_{H_aH_c}$ 8.5, $^3J_{H_bH_c}$ 6.0, $^2J_{H_cF}$ 50.0 Hz, 1 H, CHF), and 6.4—7.3 (m, 2 H, CH_2); δ_F ($CDCl_3$) +117.3 (ddd, $^2J_{H_cF}$ 52.5, $^3J_{H_aF}$ 13.5, $^3J_{H_bF}$ 24.9 Hz, CHF).

(2S)-*Fluorosuccinic Acid*.—(2*R*)-Malic acid ($[\alpha]_D^{20}$ +27.0, *c* 5 in pyridine) was esterified, converted into dimethyl (2*S*)-fluorosuccinate (c.d. $\lambda_{max.}$ 214 nm Δε +0.74), and hydrolysed as above to give (2*S*)-fluorosuccinic acid, m.p. 130—132.5 °C, c.d. $\lambda_{max.}$ 214 nm, Δε +1.08.

Dimethyl (2S,3R)-[3-$^2H_1$]*Malate*.—Monosodium fumarate (410 mg) was converted into (2*S*,3*R*)-[3-$^2H_1$]malate by fumarase in deuterium oxide as described by Fisher *et al.*[12] The acid in dry methanol was treated with ethereal diazomethane. Removal of the excess of reagent and solvent gave dimethyl (2*S*,3*R*)-[3-$^2H_1$]malate (85%) with a deuterium content of 94 ± 0.7% (by mass spectrometry).

Dimethyl (2R,3S)-*Fluoro*[3-$^2H_1$]*succinate*.—Dimethyl (2*S*,3*R*)-[3-$^2H_1$]malate was converted into the dimethyl [3-$^2H_1$]-fluorosuccinate with DAST as described for dimethyl (2*R*)-fluorosuccinate, τ($CDCl_3$) 4.74 (dd, $^3J_{HH}$ 4.0, $^2J_{HF}$ 47.5 Hz), 6.20 and 6.32 (2 s, 3 H each, CO_2Me), and 7.05 (ddt, $^2J_{HD}$ 2.0, $^3J_{HH}$ 4.0, and $^3J_{HF}$ 26.5 Hz); δ_F ($CDCl_3$) +115.9 (qt, $^2J_{HF}$ 27.0, $^3J_{HF}$ 49.0, $^3J_{FD}$ 3.2 Hz).

(2R,3S)-*Fluoro*[3-$^2H_1$]*succinic Acid*.—The dimethyl ester was hydrolysed with 5% sulphuric acid as for (2*R*)-fluorosuccinic acid to give (2*R*,3*S*)-fluoro[3-$^2H_1$]succinic acid (69%), τ(D_2O) 4.65 (dd, $^3J_{HH}$ 4.0, $^2J_{HF}$ 48.0 Hz, 1 H, CHF) and 6.91 (ddt, $^2J_{HD}$ 2.0, $^3J_{HF}$ 24.0, $^3J_{HH}$ 4.0 Hz, 1 H, CHD); δ_F($CDCl_3$) +115.1 (ddt, $^2J_{HF}$ 50.0, $^3J_{HF}$ 25.0, $^3J_{FD}$ 4.0 Hz).

(2R,3S)-*Fluoro*[3-$^2H_1$]*succinic Anhydride* (2).—(2*R*,3*S*)-Fluoro[3-$^2H_1$]succinic acid (90 mg) was suspended in acetyl chloride (5 cm³) and refluxed for 1.5 h. Evaporation under reduced pressure gave (2*R*,3*S*)-fluoro[3-$^2H_1$]succinic anhydride (65 mg, 81%); $\nu_{max.}$ ($CHCl_3$) 1 810 and 1 885 cm^{-1} (CO·O·CO); τ($CDCl_3$) 4.40 (dd, $^3J_{HH}$ 4.5, $^2J_{HF}$ 51.0 Hz, 1 H, CHF), 6.84 (ddt, $^2J_{HD}$ 3.0, $^3J_{HH}$ 4.5, $^3J_{HF}$ 25.0 Hz); δ_F ($CDCl_3$) +116.5 (dd, $^2J_{HF}$ 55, $^3J_{HF}$ 25 Hz).

(2S)-*Fluorosuccinic Acid from* (2S)-*Aspartic Acid* (*with* V. T. Jones).—Fluorosuccinic acid was prepared from (2*S*)-aspartic acid by the method of Olah and Welch.[21] The acid was purified by preparative t.l.c. on silica gel with chloroform–ethyl acetate–formic acid (50 : 10 : 2.5 v/v/v) as eluant and recrystallised from ethyl acetate–chloroform, m.p. 130—132 °C, c.d. $\lambda_{max.}$ 214 nm, Δε +0.81.

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Bacteriostatic Activity of Fluoro-analogues of 5-(2-Hydroxyethyl)-4-methylthiazole, a Metabolic Intermediate in the Biosynthesis of Thiamine

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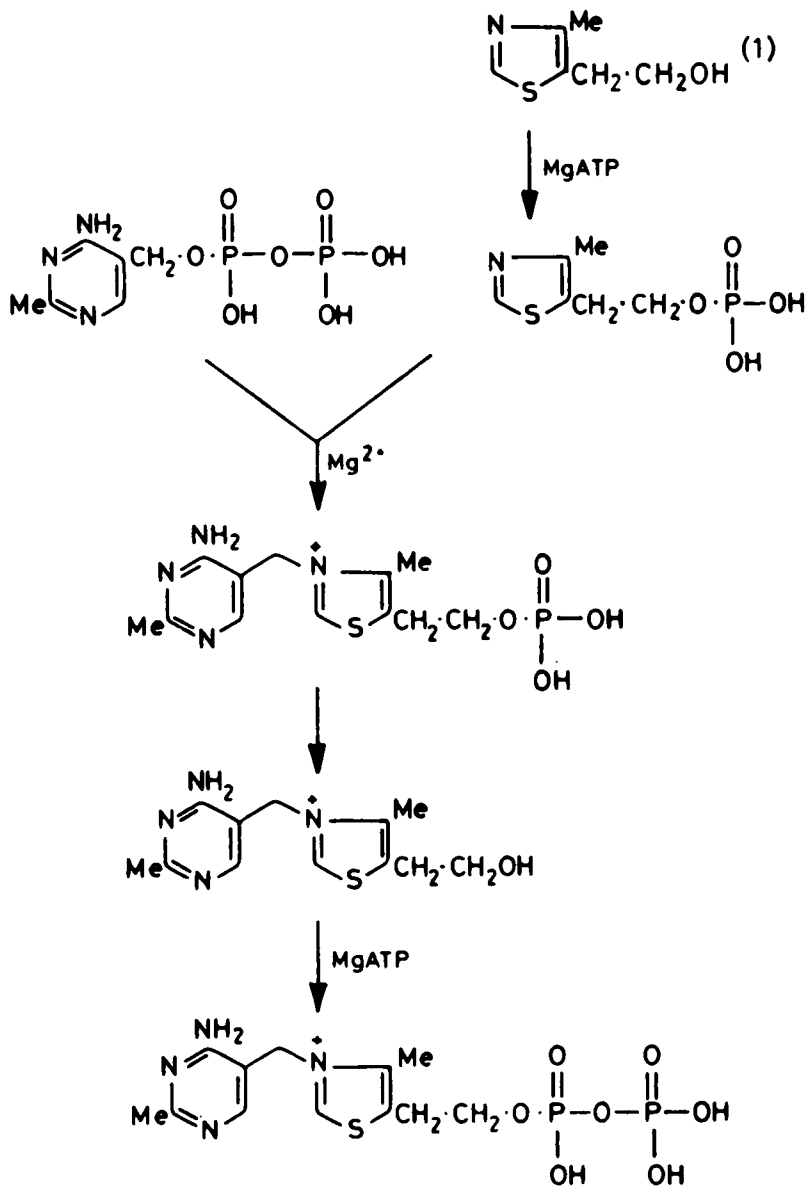
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Bacteriostatic Activity of Fluoro-analogues of 5-(2-Hydroxyethyl)-4-methylthiazole, a Metabolic Intermediate in the Biosynthesis of Thiamine

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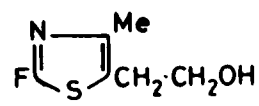
2-Fluoro-5-(2-hydroxyethyl)-4-methylthiazole and 5-(2-fluoroethyl)-4-methylthiazole have been prepared and the latter converted into a fluoro-analogue of thiamine. All three compounds show bacteriostatic activity against *E. coli*.

THE biosynthetic pathways leading to the vitamins provide a variety of potential targets for antimicrobial agents which are non-toxic to man. Although there are notable examples of this type of therapeutic agent, for example, the sulphonamides as inhibitors of folic acid biosynthesis,[1] this approach to new antibiotics remains relatively unexplored. Thus, although numerous analogues of thiamine and its precursors have been synthesised in relation to vitamin activity,[2] their potential as

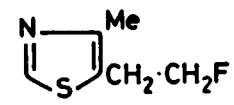


SCHEME The later stages in the biosynthesis of thiamine pyrophosphate

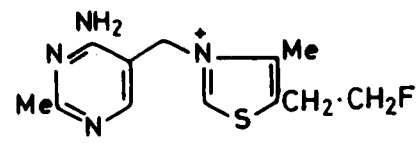
antimicrobial agents has been largely ignored. Exceptions are 4-amino-5-hydroxymethyl-2-methylthiopyrimidine, methioprim,[3] and the corresponding 2-methoxy-derivative, bacimethrin.[4] Thiamine thiazone pyrophosphate is a potent inhibitor of pyruvate dehydrogenase from *E. coli*,[5] while 2-amino-5-(2-hydroxyethyl)-4-methylthiazole inhibits phosphorylation of the thiazole intermediate in thiamine biosynthesis.[6,7] The



(2)



(3)



(4)

later stages in the biosynthesis of thiamine pyrophosphate are outlined in the Scheme.[8]

Thiamine pyrophosphate is a co-factor for enzymes which decarboxylate α-keto-acids.[9] An essential feature for its activity is the lability of the C-2 proton of the thiazolium ring, the carbanion so formed adding to the ketone of the substrate to initiate decarboxylation.[10] If the C-2 position is blocked by a group of similar steric requirement, for example fluorine,[11,12] the co-enzyme would be ineffective and possibly a potent active-site-directed inhibitor.

2-Fluoro-5-(2-hydroxyethyl)-4-methylthiazole (2) was considered to be a good structural analogue of the natural metabolic intermediate 5-(2-hydroxyethyl)-4-methylthiazole (1), and could therefore be a competitive inhibitor or substrate for the phosphokinase. If *in vivo* phosphorylation occurs then the possibility exists that 2-fluorothiamine phosphate and pyrophosphate might be formed.

Aryl fluorides are conveniently synthesised by the thermal decomposition of diazonium fluoroborates,[13] but for fluoroazoles,[14-17] photochemical decomposition is often a more satisfactory procedure. A preliminary study with 4,5-dimethylthiazole-2-diazonium hexafluorophosphate (the tetrafluoroborate salt [18] could not be isolated) showed that both thermal and photochemical decomposition gave 2-fluoro-4,5-dimethylthiazole in similar yield.

2-Amino-5-(2-hydroxyethyl)-4-methylthiazole [19,20]

gave on diazotisation water-soluble diazonium tetrafluoroborate and hexafluorophosphate salts, so that the protocol for thermal decomposition was not possible. Photochemical decomposition in aqueous solution however gave 2-fluoro-5-(2-hydroxyethyl)-4-methylthiazole (2).

The pK_a of 2-fluoro-5-(2-hydroxyethyl)-4-methylthiazole (2) was measured spectrophotometrically against the Hammett acidity function, H_0,[21-23] and was −1.44, some 5.26 units below that of 5-(2-hydroxyethyl)-4-methylthiazole (1) and comparable with the recently reported pK_a values of 2-chloro- and 2-bromo-thiazoles (−0.75 and −0.86 respectively [23]). The low pK_a was reflected in its inability to form a picrate salt, whereas 5-(2-hydroxyethyl)-4-methylthiazole (1) does.[24] Since it seems probable that the low pK_a will be paralleled by low nucleophilicity, 2-fluoro-5-(2-hydroxyethyl)-4-methylthiazole phosphate may not serve as a substrate for the formation of 2-fluorothiamine phosphate. It could however be a substrate or inhibitor of 5-(2-hydroxyethyl)-4-methylthiazole phosphokinase and indeed showed activity against *E. coli* ATCC 9637 at a concentration of 6 mM.

5-(2-Fluoroethyl)-4-methylthiazole (3) has also been prepared from the natural metabolic intermediate 5-(2-hydroxyethyl)-4-methylthiazole (1) by reaction with diethylaminosulphur trifluoride (DAST).[25] Moreover it was converted into the fluorothiamine (4) by reaction with 4-amino-5-bromomethyl-2-methylpyrimidine.[26,27] Both showed bacteriostatic activity against *E. coli* ATCC 9637 at concentrations of 6 and 4.4 mM respectively. 5-(2-Fluoroethyl)-4-methylthiazole is also being tested for pharmacological properties, since 5-(2-chloroethyl)-4-methylthiazole is a versatile pharmacological agent.[28]

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage apparatus. Preparative t.l.c. was performed on 20 cm × 20 cm silica gel H plates containing Fluor, bands being located with u.v. light at 254 or 366 nm. ^{1}H and ^{19}F N.m.r. spectra were recorded on a Perkin-Elmer R32 spectrometer. ^{19}F Chemical shifts are in p.p.m. from external trifluoroacetic acid, downfield resonances being assigned a negative value. High-resolution mass spectra were measured on a V.G. Micromass 7070F mass spectrometer. U.v. and turbidimetric measurements were performed on a Unicam SP 1800 spectrophotometer and i.r. spectra measured on a Unicam SP 1000 spectrophotometer. pH Measurements were made on a Radiometer type TTT1c pH meter. *E. coli* ATCC 9637 was obtained from the American Type Culture Collection, and inhibition of growth was tested as described by Iwashima and Nose.[6]

The following compounds were synthesised by literature methods: 2-amino-5-(2-hydroxyethyl)-4-methylthiazole,[19] 5-(2-hydroxyethyl)-4-methylthiazole,[20] 4-amino-5-bromomethyl-2-methylpyrimidine hydrobromide,[26,27] and diethylaminosulphur trifluoride (DAST).[25] Chloroform used for reactions involving DAST was freed from ethanol and distilled. 2-Amino-4,5-dimethylthiazole hydrobromide was obtained from Aldrich Chemical Co., hexafluorophosphoric acid (65% solution in water) from Cambrian Chemicals Ltd., and tetrafluoroboric acid (40% solution in water) from British Drug Houses Ltd.

2-Fluoro-5-(2-hydroxyethyl)-4-methylthiazole (2).—(a) Via *the diazonium fluoroborate*. 2-Amino-5-(2-hydroxyethyl)-4-methylthiazole (1.20 g, from 1.5 g, of its crystalline hydrochloride) was dissolved in fluoroboric acid solution (20 cm^3) and cooled to −5 °C. Solid sodium nitrite (1.2 g) was added in portions over 20 min to the vigorously stirred solution. The cold green solution was transferred to a Pyrex tube and kept at −5 °C with an ice–salt mixture, whilst being photolysed with a 450-W medium-pressure mercury-vapour lamp (Hanovia). Gas evolution was complete after 4 h. The solution was brought to pH 8.0 with solid sodium hydrogencarbonate and then extracted with ether continuously for 6 h. The residue, after removal of the ether from the extract, was chromatographed on silica gel (35 g) with light petroleum (b.p. 40—60 °C)–ethyl acetate (1 : 3 v/v) to give 2-fluoro-5-(2-hydroxyethyl)-4-methylthiazole (2) as a pale yellow liquid (0.35 g, 28%), δ_H ($CDCl_3$) 3.77 (t, *J* 6.2 Hz, 2 H, CH_2CH_2OH), 3.35 (br s, 1 H, CH_2OH), 2.84 (dt, *J* 6.2 and 3.0 Hz, 2 H, CH_2CH_2OH), and 2.18 (s, 3 H, Me); δ_F ($CDCl_3$) + 6.00 (t, *J* 3.0 Hz) (Found: *m/e* 161.029 8. Calc. for C_6H_8FNOS: *M*, 161.031 0).

(b) Via *the diazonium hexafluorophosphate*. The same amounts of material were used as in (a) except that the fluoroboric acid solution was replaced by hexafluorophosphoric acid solution (7 cm^3). The yield of 2-fluoro-5-(2-hydroxyethyl)-4-methylthiazole (2) was 0.30 g (24%).

2-Fluoro-4,5-dimethylthiazole.[18]— 2-Amino-4,5-dimethylthiazole (0.5 g, from its hydrobromide) was suspended in water (4 cm^3) and 10M-HCl added (2 cm^3) followed by sodium nitrite (0.5 g). Addition of hexafluorophosphoric acid (2 cm^3) to the clear green solution gave immediately a yellow precipitate, which was filtered off, washed, and dried. The 4,5-dimethylthiazole-2-diazonium hexafluorophosphate (0.5 g, 44%), m.p. 105 °C (decomp.) had δ_H ([$^2H_6$]-DMSO) 2.0 (s, 2 Me); δ_F ([$^2H_6$]DMSO) −5.5 (d, J_{PF} 723 Hz); $\nu_{max.}$ (Nujol) 2 240 cm^{-1} ($-N_2^+$).

Pyrolysis. The dry diazonium salt was added in portions to a preheated (120 °C) round-bottomed flask. Extraction of the residue with ether and preparative t.l.c. using MeOH–$CHCl_3$ (1 : 9 v/v) as eluant gave 2-fluoro-4,5-dimethylthiazole (50 mg, 22%).

Photolysis. A suspension of the 4,5-dimethylthiazole-2-diazonium salt in water was photolysed by a 450-W medium-pressure mercury-vapour Hanovia lamp, a stream of nitrogen being used to agitate the suspension. After evolution of nitrogen ceased, the product was extracted and purified by preparative t.l.c. to give 2-fluoro-4,5-dimethylthiazole (57 mg, 25%) δ_H ($CDCl_3$) 2.00 and 2.16 (s, Me); δ_F ($CDCl_3$) + 6.0 (s).

5-(2-Fluoroethyl)-4-methylthiazole (3).—To a solution of DAST (1.45 g) in chloroform (5 cm^3) at 0 °C was added dropwise a solution 5-(2-hydroxyethyl)-4-methylthiazole (1.0 g) in chloroform (5 cm^3). After addition was complete the mixture was allowed to warm up to room temperature (30 min) and water (10 cm^3) was added dropwise with vigorous stirring. The chloroform layer was washed with saturated sodium hydrogencarbonate solution, followed by saturated NaCl solution, dried ($MgSO_4$), and the solvent removed to give a pale yellow liquid which was chromatographed on silica gel (100 g), eluting with ethyl acetate. 5-(2-*Fluoroethyl*)-4-*methylthiazole* (3) (0.55 g, 55%) had

δ_H ($CDCl_3$) 4.55 (dt, J_{HH} 6.0, J_{HF} 47.5 Hz, 2 H, FCH_2CH_2), 3.14 (dt, J_{HH} 6.0, J_{HF} 24 Hz, 2 H, FCH_2CH_2), 2.40 (s, 3 H, Me), and 8.57 (s, 1 H, N=CH); δ_F ($CDCl_3$) + 140 (tt, J_{HF} 24.0 and 47.5 Hz); and formed a picrate, m.p. 107—110 °C (Found: C, 38.5; H, 2.9; N, 14.8. $C_{12}H_{11}FN_4O_7S$ requires C, 38.5; H, 3.0; N, 15.0%), and a methanesulphonate salt, m.p. 90—92 °C.

3-[(4-*Amino*-2-*methylpyrimidin*-5-*yl*)*methyl*]-5-(2-*fluoroethyl*)-4-*methylthiazolium Bromide Hydrobromide* (4).—To a solution of 5-(2-fluoroethyl)-4-methylthiazole (3) (50 mg) in butan-1-ol (0.3 cm^3) was added 4-amino-5-bromomethyl-2-methylpyrimidine hydrobromide (63 mg) and the mixture heated at 100—120 °C for 15 min. The pyrimidine dissolved and shortly afterwards crystals appeared. Hot ethanol (1 cm^3) was added and the solution allowed to cool. The product was filtered off and recrystallised from ethanol to give the *thiazolium salt* (4) (22 mg, 23%), m.p. 253 °C (decomp.), δ_H (D_2O) 3.07 (s, 3 H, Me), 3.15 (s, 3 H, Me), 3.93 (dt, J_{HH} 6.0, J_{HF} 24.0 Hz, 2 H, FCH_2CH_2), 5.26 (dt, J_{HH} 6.0 and J_{HF} 47.5 Hz, 2 H, FCH_2CH_2), 6.08 (s, 2 H, $\overset{+}{N}$=CHS, CH_2), 8.54 (s, 1 H, N=CH), and 11.14 (s, 1 H, N=CHS, disappears in D_2O), δ_F (D_2O) + 142 (tt, J_{HF} 24.0 and 47.5 Hz) (Found: C, 32.0; H, 4.4; N, 11.7. $C_{12}H_{19}Br_2FNS{\cdot}H_2O$ requires C, 32.3; H, 4.3; N, 12.3%).

pK_a *Determinations*.—The pK_a values of the thiazoles (1) and (2) were measured spectrophotometrically.[21-23] To a stock solution of AnalaR sulphuric acid (2 cm^3) of known molarity in a quartz cuvette was added a solution of 2-fluorothiazole in water (100 μl, 1 mg cm^{-3}) containing potassium chloride (100 mM). After mixing, the absorbance was recorded against the appropriate blank solvent. The H_0 value of the solution was obtained by interpolation from the data of Paul and Long.[29] For the thiazole (1): free base $\lambda_{max.}$ 249 nm (ε 4 190), conjugate acid $\lambda_{max.}$ 258 nm (ε 4 450), $\lambda_{determination}$ 270 nm; for thiazole (2): free base $\lambda_{max.}$ 242 nm (ε 3 969), conjugate acid 257 nm (ε 3 220), $\lambda_{determination}$ 270 nm. The pK_a values were evaluated by least-squares regression analysis of plots of $\log_{10}$ $[BH^+]/[B]$ against H_0 or pH.

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