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The biochemistry of phosphonate analogues of naturally occurring phosphates.

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The Biochemistry of phosphonate analogues of naturally occurring phosphates

submitted by D.J.W. Roach

for the degree of Ph.D.
of the University of Bath
1980

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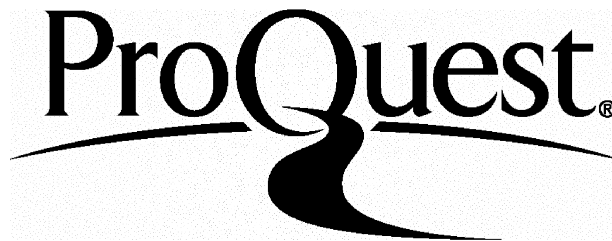

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SUMMARY

6,7 dideoxy-α-D-*gluco*-heptose 7-phosphonic acid, the isosteric phosphonate analogue of naturally-occurring glucose 6-phosphate was synthesised via a novel route, involving the use of an easily-prepared and stable ethyl ester, followed by a facile dealkylation procedure.

6,7 dideoxy α-D-*gluco*-heptose 7-phosphonic acid was shown to behave as a product inhibitor of purified bovine brain hexokinase I, being a competitive inhibitor of $MgATP^{2-}$ and a noncompetitive inhibitor of glucose.

6,7 dideoxy α-D-*gluco*-heptonic 7-phosphonic acid, the isoteric phosphonate analogue of gluconate 6-phosphate was enzymically synthesised from 6,7 dideoxy α-D-*gluco* heptose 7-phosphonic acid and was shown to be a poor substrate for yeast gluconate 6-phosphate dehydrogenase at pH 7.5 and 8.0.

6,7 dideoxy α-D-gluco-heptose 7-phosphonic acid had no effect on cell proliferation of mouse lymphoma cells (at mM concentrations) but was found to be more stable then the natural phosphate in their presence.

α-D-glucose fluorophosphate was tested as an active-site-directed irreversible inhibitor of yeast glucose 6-phosphate dehydrogenase with a view to eventual use of the corresponding phosphonate analogue in similar systems. The fluorophosphate was found to be inactive in this respect.

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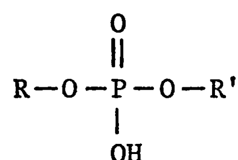
INTRODUCTION

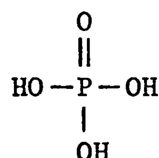
INTRODUCTION

The isolation and characterisation of fructose 1,6-bisphosphate by Harden & Young in 1905 emphasised the importance of phosphorus-containing compounds in living organisms. Organic phosphates have since been found to be widely distributed in nature being present in all living cells and playing an essential role in metabolic processes. Naturally-occurring phosphates are generally of the structural type (I) where R or R' or both are organic groupings



I

(e.g. sugar, lipid or nucleotide) and the compounds can be described as mono (R or R' = H) or diesters of phosphoric acid (II). In view of the importance of such phosphates in metabolism there has been

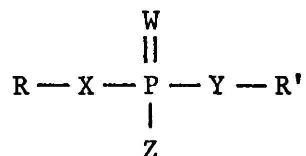


II

considerable interest in the possible use in biological research of structural analogues of these compounds. Analogues of naturally-occurring metabolites in general have potential as metabolic probes. There is always the possibility that an analogue might proceed several steps along a metabolic pathway and then not be metabolised further; such a situation can lead to the build up of intermediates and so give information about the pathway concerned. Possible selective substrate and inhibitory activity of this type can lead to chemotherapeutic

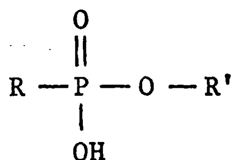
agents capable of blocking pathways in specific pathogenic cells. Substrate analogues also have a place in isolated enzyme studies, e.g. analogues acting as slow substrates can lead to information about enzyme mechanism.

Many kinds of phosphate analogue are possible. The formula (I) of naturally-occurring phosphates can be further generalised to give the structure (III) in which W, X, Y and Z all represent oxygen

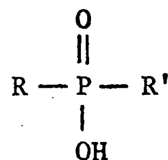


III

atoms. Analogues in which W, X, Y or Z have been substituted with electronegative groups such as -S- or -NH- have been synthesised and studied (as indeed have analogues where the phosphorus atom itself has been substituted). Replacement of X or Y, not with electronegative groups but with substituted carbon atoms leads to phosphonates (IV) or to phosphinates (V) [derivatives of phosphonic acid (VI) or phosphinic acid (VII) respectively].



IV



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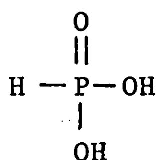
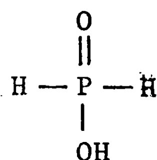
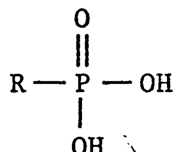


VI



VII

In this thesis we shall be mainly concerned with the occurrence, synthesis and biological activity of phosphonates having the general structure shown in (IV) and more particularly with the dibasic acids (VIII).



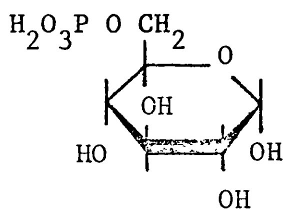
VIII

Over the last twenty years there has been significant interest in the preparation and biological investigation of phosphonate analogues of naturally-occurring phosphates (Engel 1977). The minimal structural alteration of substituting one of the ester oxygen atoms attached to phosphorus (i.e. X or Y in structure III) with a methylene group brings about several physical and chemical changes which could have a profound effect on the biological and chemical activity of the compound.

The replacement of a phosphate ester C-O-P bond with C-C-P in a phosphonate analogue makes such an analogue an unlikely substrate for enzymes catalysing phosphate cleavage, although there is a good chance that it may act as an inhibitor. This situation could lead to a metabolic block and to information about the pathway as discussed earlier (page 1-2). The inability of the phosphonate

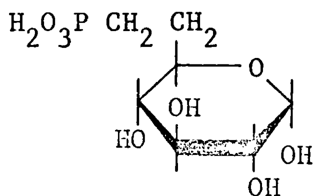
grouping to be hydrolysed by phosphatase enzymes is of potential advantage in protecting an analogue from unwanted degradation e.g. in preserving a metabolic regulator *in vivo* or in stabilising a ligand for affinity chromatographic purification of phosphate cleaving enzymes (M. Landt *et al.*, 1978).

A further result of replacement of C-O-P by C-C-P is the generally-observed weaker acidity of the phosphonate analogue (Freedman & Doak, 1957). This can be seen as resulting from the replacement of an electronegative oxygen atom by an electron-donating alkyl group. For example glucose 6-phosphate (IX) with a



IX

secondary pka value of 6.21 will contain approximately 95% of the dianionic form at both pH 7.5 and pH 8.0. The isosteric phosphonate analogue (X) in contrast has a secondary pka value of 7.46 and will

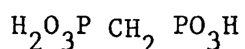


X

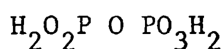
contain 52% and 78% of the dianionic form at pH 7.5 and pH 8.0 respectively. This decrease in acidity could have a profound effect on the activity of phosphonates as substrates of enzymes where the dianionic form of the substrate is necessary for reaction. Such a

situation could be exploited to investigate preferred binding modes in enzyme-substrate interactions. Phosphonates of the type (III) where W = X = Z = O and Y = -C- can of course, only ever exist in the mono anionic form and could be used in similar studies.

Phosphonate analogues can also differ from the natural phosphates in their physical size and shape. Although the phosphonate analogue in which C-O-P of a natural substrate is replaced by C-CH_2-P is designed to mimic the naturally-occurring phosphate in bond lengths and angles, differences can occur. Such differences in 'isosteric' analogues of the type so far discussed, while possibly biochemically significant are usually physically small. However methylene diphosphonic acid (XI) the isosteric phosphonate analogue of pyrophosphoric acid (XII) has differences in the P-X-P bond angles of greater than 10° (Larson _et al._, 1969). Discrepances of this kind can lead to large differences in binding properties for the analogues



XI



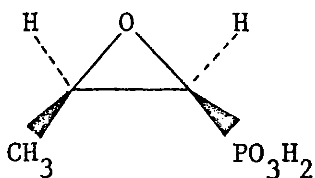
XII

of nucleotide di- and triphosphates where a pyrophosphate linkage has been replaced by a methylene diphosphonate linkage. Many analogues in which the C-O-P of a natural phosphate is replaced simply by C-P have also been studied and in such "non-isosteric" analogues there is an obvious decrease in the overall size. Furthermore the distance

between the phosphoryl oxygens and other possible binding sites or sites of interaction are significantly changed unless strained or unlikely conformations occur. This could result in far greater variation of biochemical activity than that brought about by simple substitution of a methylene group for an oxygen atom. For this reason "isosteric" phosphonate analogues might in principle be considered to be better analogues.

A final difference between phosphonate analogues and the natural phosphates is the loss of a possible binding function of the esterified phosphate oxygen which is absent in the analogue.

The isolation of naturally-occurring phosphonates (Horiguchi & Kandatsu, 1959) from living organisms has stimulated many biochemical investigations. Mutant strains of bacteria have been shown to be capable of using phosphonates as a source of phosphorus, (Zeleznick _et al._, 1963) and a naturally-occurring phosphonate, phosphonomycin (XIII) isolated from _Streptomyces radiae_ (Hendlin _et al._, 1969) has been found to have a broad spectrum of

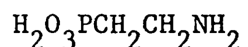
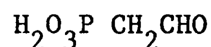


XIII

antibiotic activity caused by its inhibition of bacterial cell wall biosynthesis.

Phosphonates have been shown to occur commonly among lower animals (Kitteridge & Roberts, 1969) chiefly in the form of α-amino

ethyl phosphonate (XIV) and its derivatives and La Nauze *et al*. (1970) have

$H_2O_3PCH_2CH_2NH_2$ $H_2O_3P\ CH_2CHO$

XIV XV

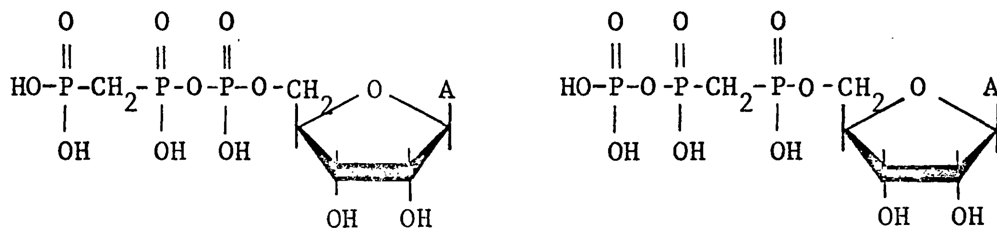
purified (from a strain of *Bacillus cereus*) an enzyme able to cleave the C-P bond of 2 phosphono acetaldehyde (XV) itself a product of 2 amino-ethyl phosphonate.

Thus phosphonates as analogues of naturally-occurring phosphates are of interest and of possible potential use in biochemical investigations as probes of metabolic pathways and of enzyme mechanisms, as chemotherapeutic agents and as stable ligands in affinity chromatographic purification of phosphate-cleaving enzymes. The following introduction will point out the various kinds of phosphonate analogues that have been studied, their uses and biological properties.

(A) NUCLEOTIDES

1. Analogues of ATP

The central role that ATP plays in many biochemical processes has led to an extensive study of the phosphonate analogues XVI and XVII in which the βγ or the αβ oxygens respectively are substituted by a methylene group.



(i) Systems in which phosphonate analogues have been found to be capable of replacing ATP

Analogue XVI was found to be a substrate for RNA polymerase (*Azotobacter vinelandii*) allowing incorporation of labelled UMP into Poly AU 30% to 60% as effectively as when ATP was used (Simon *et al.*, 1965). Wong & Murray (1969) showed that analogue XVI is also a good substrate for phosphoribosyl pyrophosphate synthetase from Ehrlich ascites-tumour cells (Table 1). Both of the above enzymes catalyse

Table 1

compound	K_m	V_{max} (relative to ATP)
ATP	0.06mM	1·0
XVI	0.16mM	0.75

the cleavage of the αβ phosphodiester bond of ATP (unchanged in analogue XVI) and the substrate activity of XVI is perhaps not

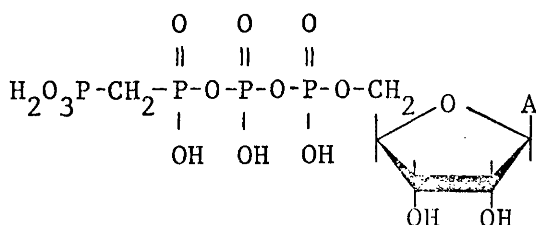
surprising.

Analogue XVI, like ATP, was found to regulate the activity of adenylate deaminase in crude extracts of ascites-tumour cells (Atkinson & Murray, 1967). It should be noted that the similar activities of compound XVI and ATP in this crude extract could result from competing factors. Thus analogue XVI is unlikely to be removed in kinase-catalysed reactions and so any diminished regulatory activity compared to that of ATP could be compensated by its longer lifetime in the system.

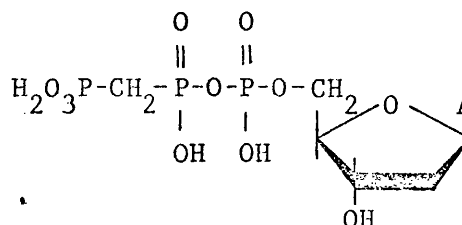
Phosphonate analogues XVI, XVII and XVIII were all found to be good inhibitors of rabbit liver fructose 1, 6 bisphosphatase, but the deoxy analogue XIX had no inhibitory action (Table 2). The

Table 2

addition	[concn.]	relative activity
none	-	100%
XVI	0.005mM	49%
XVII	0.1 mM	35%
XVIII	0.1 mM	66%
XIX	0.1 mM	107%
XXII	0.1 → 0.9mM	104 → 90%



XVIII



XIX

action of analogue XIX is in contrast to that of the natural 2 deoxy-

ATP which is a more effective inactivator than ATP. These results are difficult to interpret and the authors suggested that they were an exaggeration of the random specificity exhibited by the natural nucleotide di and tri phosphates (Taketa et al., 1971).

Analogue XVI was found to be a better inhibitor of bacterial polynucleotide phosphorylase than was ATP (Simon & Myers, 1961).

Flesher et al. (1960) found that analogue XVI was over twenty times more potent than ATP in increasing the vasodepressor activity of 'nembutal' - anaethetized cats. This indicates that the mechanism of action of ATP is the result of binding to a receptor site without cleavage of the phosphate chain. The increase in activity of analogue XVI compared to that of ATP was rationalised as resulting from small deviations in bond angle and lengths around the substituted methylene group which might afford greater affinity for the receptor site.

Issaly et al. (1973) demonstrated that the phosphonate XVI could replace ATP in promoting the release of aspartate transcarbamylase from ribosomes of a mutant strain of *Neurosporo crassa in vitro* after complementation (Table 3). However analogue XVII did not replace ATP in this system and it was concluded that the major

Table 3. Release of aspartate transcarbamylase by nucleotides

addition	ATCase after ribosomal complementation free/bound
none	0.26
ATP 1.0mM	1.15
XVI 1.0mM	0.80
XVII 1.0mM	0.29

mechanism of ATP involved either transfer or liberation of a pyrophosphate group.

Yount *et al*. (1971b) showed that analogue XVI forms one to one complexes with Ca^{2+}, Mg^{2+} and Mn^{2+} cations with affinities equal to, or greater than, those of ATP. This was found to be true at pH 7.4 where protons might be expected to compete effectively with divalent metal ions for the weaker acid group of the phosphonate analogue. This suggests that any failure of XVI in enzymic interactions cannot be attributed to inability to bind divalent metal ions.

The above activities of analogues XVI and XVII as ATP analogues are listed in Table 4 together with some further reported instances.

Table 4. Systems where analogues of ATP are capable of replacing ATP

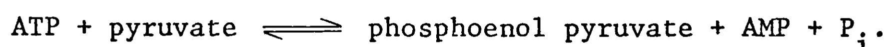
Analogue	System	Effect	Reference
XVI	RNA polymerase	substrate	Simon *et al.* (1965)
XVI	phosphoribosyl pyrophosphate synthetase (from Erlich ascites tumour cells)	substrate	Wong & Murray(1969)
XVI	adenylate deaminase	inhibitor	Atkinson & Murray (1967)
XVI XVII XVIII	fructose 1 6 bisphosphatase (from rabbit liver)	inhibitor inhibitor inhibitor	Taketa *et al.* (1971)
XVI	bacterial polynucleotide phosphorylase	inhibitor	Simon & Myers (1961)
XVI	vasodepressor activity in anaethetized cats	activates	Flesher *et al.* (1960)
XVI	release of ATCase from ribosomes of a mutant strain of N. crassa	releases ATCase from ribosomes	Issaly *et al.* (1973)

Table 4. (contd.)

Analogue	System	Effect	Reference
XVI	binding of divalent cations	similar binding capacity to ATP	Yount *et al.* (1971b)
XVI + XVII	exchange of internal nucleotides from mitochondria	exchanged same as ATP	Duree *et al.* (1968)
XVI	production of relaxed state in insect flight muscle	same as ATP	Goody *et al.* (1975)
XVII	early burst hydrolysis of ATP by Myosin	same as ATP	Koretz & Taylor (1975)

(ii) Systems in which Phosphonate analogues have been found not to be capable of replacing ATP

Phosphoenolpyruvate synthetase from *E. coli* catalyses the overall reaction



$$ATP + pyruvate \rightleftharpoons phosphoenol\ pyruvate + AMP + P_i.$$

Analogue XVI was not a substrate for this enzyme but found to be a competitive inhibitor with respect to ATP (K_i = 2.1μM)(Berman & Cohn, 1970). Preliminary incubation of the synthetase with XVI (in contrast to ATP, Table 5) did not block subsequent phosphorylation of the enzyme with phosphoenolpyruvate nor did it block the [^{14}C] pyruvate - phosphoenolpyruvate exchange reaction. This was interpreted to show that a pyrophosphoryl enzyme intermediate (E-PP) is not formed (for in such a case analogue XVI might be expected to form an inactive E-PCH_2P intermediate which would block both phosphorylation of the enzyme with phosphoenolpyruvate and the exchange reaction of [^{14}C] pyruvate and phosphoenolpyruvate.

Table 5

preincubation compound	moles P_i/mole enzyme
none	1.3
ATP	0.0
XVI	1.1

Lin *et al.* (1975) found analogues XVII and XVIII to be competitive inhibitors of hepatic adenylate cyclase with respect to ATP (Table 6).

Table 6

Compound	K_m (μM)	K_i (μM) at pH 7.5 and 8.5
ATP	55	-
XVII	-	40
XVIII	-	15
XXIV	-	200

The ionic form of the analogues is not likely to be a major factor in these inhibitions as the K_i values obtained were identical at pH 7.5 and 8.0. These results also indicate that the active site of hepatic adenyl cyclase can accommodate an extra phosphate group on the nucleotide (e.g. XVIII) without a reduction in affinity compared to ATP.

Both analogues XVI and XVII were found to behave as competitive inhibitors of ATP with formyl tetrahydrofolate synthetase from Clostridium cylindrosporum (K_i values 10 and 28μM respectively compared to a K_m value for ATP of 200μM)(Curthoys & Rabinowitz, 1971). The authors pointed out that the high affinity of the analogues for the enzyme might be explained in terms of their superior ability to achieve a trigonal-bipyramidal intermediate because of small deviations in bond angles and lengths around the substituted $-CH_2-$ group. Further studies by Buttlaire & Reed (1975) found that, in contrast to the EPR spectra of the ATP-Mn^{2+}- formyltetrahydrofolate synthetase complex, that of the corresponding analogue XVI complex showed considerable broadening of the spectrum of Mn^{2+}. Other differences from the ATP-containing complex were also noted and were attributed to the presence in the analogue of the P-C-P linkage which could affect the

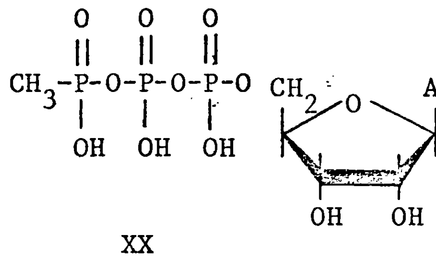
capacity of the synthetase to undergo certain conformational changes.

Microsomal preparations of Na^+, K^+-dependent ATPase from porcine kidney outer medulla and from <u>Electrophorus</u> <u>electroplax</u> catalysed a rapid Mg^{2+} and K^+ dependent exchange of water oxygens ($H_2[^{18}O]$) with inorganic phosphate (Dahms & Boyer, 1973) in the presence of ATP. Analogue XVI was unable to replace ATP in activating exchange of water oxygens from the medium to inorganic phosphate, indicating that the exchange arises by dynamic reversal of a late step in ATP hydrolysis by the Na^+, K^+-dependent ATPase.

In similar studies Holland & Labelle (1974) investigated the effect of replacing ATP by analogue XVI in oxygen-exchange reactions catalysed by rat liver mitochondria. Under conditions where rapid exchange of $H_2[^{18}O]$ with the terminal phosphate oxygens of ATP occurred (unique to oxidative phosphorylation) analogue XVI displayed no incorporation of labelled oxygen from $H_2[^{18}O]$. This suggests that ATP-water exchange can only occur when the terminal phosphate can be cleaved. Alternatively the analogue may not bind at the site where exchange takes place, or the analogue may bind at this site but not form the penta-covalent intermediate. Analogue XVI was found not to inhibit the above exchange between ATP and labelled water ($H_2[^{18}O]$), but did on the other hand inhibit the exchange between inorganic phosphate and $H_2[^{18}O]$ and the exchange between phosphate and ATP. These results suggest the presence of separate sites for ATP in these exchange reactions (Holland & Labelle, 1974).

Moos <u>et al</u>. (1960) first reported investigations on the effects of substituting analogue XVI for ATP in muscle contraction.

Analogue XVI was found not to be able to replace ATP in causing muscle contraction or in lowering the viscosity of actomyosin and was not hydrolysed by a homogenate of glycerinated muscle. It was shown to partially inhibit myofibrillar ATPase but the presence of Mg^{2+} abolished the inhibition. Analogue XX was also shown not to induce contraction of myofibrils nor to be hydrolysed by myosin b (Tonomura et al., 1967) suggesting that ATP must be in the dianionic form for the hydrolysis to occur.



Inhibition of heavy meromyosin ATPase by analogue XVI was found (Yount et al., 1971a) to be mixed in the presence of Mn^{2+} or Mg^{2+} (K_i values 44 and 200μM respectively). This inhibition levelled off at higher concentrations of analogue XVI which may represent interaction of the analogue with a second modifier site.

Werber et al. (1972) found that ATP, but not analogue XVI, induced fluorescence changes in heavy meromyosin, suggesting that the observed changes resulted from local conformational changes of heavy meromyosin induced by cleavage of the terminal phosphate group of ATP. Further studies by Seidal (1975) showed that ATP-induced enhancement of fluorescence and changes in the EPR spectra of myosin and heavy meromyosin, represented firstly, the binding and secondly, the hydrolysis of ATP. Analogue XVII was shown to be capable of binding to myosin but was not subsequently hydrolysed. Koretz & Taylor (1975) demonstrated that myosin gave an 'early burst' of 0.25 → 0.3 protons/

active site with both ATP and analogue XVII. The proton liberation was assumed to be derived from a change in conformation of the enzyme-substrate complex.

The actin monomer of G-actin, binds one molecule of ADP or ATP which is rapidly exchanged with unbound nucleotides and was found to bind analogues XVI and XVII only with an affinity 100 to 200 fold less than ATP, indicating the very specific nature of this binding site (Cook & Murdoch, 1973).

These and similar activities of the phosphonate analogues of ATP are listed in Table 7.

Table 7. Systems where analogues of ATP have been found not to be capable of replacing ATP

Analogue	System	Effect	Reference
XVI	Phosphoenol-pyruvate synthetase (E. coli)	comp. inhibitor of ATP	Berman & Cohn (1970)
XVII + XVIII	hepatic adenylate cyclase	comp. inhibitors of ATP	Lin *et al.* (1975)
XVI	chicken liver formylglycinamide ribonucleotide amido-transferase	comp. inhibitor of ATP (K_i = 0.35mM)	Li & Buchanan (1971)
XVI + XVII	adenylate cyclase (Erlich ascites-tumour cells)	comp. inhibitors of ATP	Murray (1968)
XVII	phosphoribosyl pyro-phosphate synthetase (Erlich ascites-tumour cells)	inhibitor of ATP	Wong & Murray (1969)
XVI + XVII	formyl tetrahydro-folate synthetase (*Clostridial cylindrosporum*)	comp. inhibitors of ATP	Curthoys & Rabinowitz (1971)
XVI	formyl tetrahydrofolate synthetase	no changes in EPR spectrum	Buttlaire & Reed (1975)

Table 7. (contd.)

Analogue	System	Effect	Reference
XVI + XVII	adenylate kinase, pyruvate kinase hexokinase phospho-glycerate kinase	neither sub-strates or inhibitors (XVII reacted slowly c̄ Pi glycerate kinase)	Duree *et al.* (1968)
XVI	ATPase (carrot juice)	comp. inhibitor of ATP	Atkinson & Polya (1967)
XVI	ATPase (rat corpus luteum)	inhibitor of ATP	Stansfield & Francks (1971)
XVI	ATPase (Na^+ K^+ dependent, rat brain)	inhibitor of ATP + ouabain binding	Tobin *et al.* (1973)
XVI	ATPase (Na^+ K^+ dependent)	inhibitor of ATP	Robinson (1975)
XVI	ATPase (Na^+ K^+ dependent, porcine kidney)	does not acti-vate $H_2{}^{18}O$ exchange c̄ inorganic phosphate	Dahms + Boyer (1973)
XVI	ATPase (Na^+ K^+ dependent, Electro-phorus electroplax)		

Table 7. (contd.)

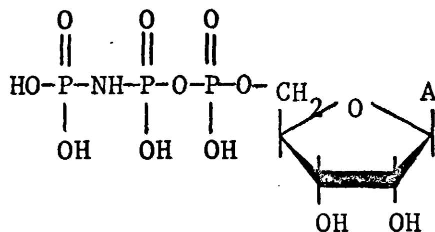
Analogue	System	Effect	Reference
XVI	rat liver mitochondria	Does not exchange $\bar{c}$ $H_2[^{18}O]$	Holland & Labelle (1974)
XVI	yeast hexokinase	(activates pH 6.6)(inhibits pH 8.0)	Kosow & Rose (1971)
XVI	actomyosin	inhibits	Moos *et al.*, (1960)
XX	myosin b	no action	Tonomura *et al.* (1967)
XVI	Heavy meromyosin ATPase	mixed inhibition in presence of Mg^{+} or Mn^{2+}	Yount *et al.* (1971a)
XVI	ATP, heavy meromyosin interaction	did not replace ATP	Werber *et al.* (1972)
XVII	ATP, heavy meromyosin interaction	bound but not hydrolysed	Santi (1975)
XVI	myosin	early burst of protons but not hydrolysed	Koretz & Taylor (1975)
XVI + XVII	G-actin	bound > x100 more weakly	Cook & Murdoch (1973)

(iii) Interaction of amino acyl- tRNA synthetases with analogues of ATP

Papas & Case (1970) observed a broad range of responses of various amino acyl-tRNA synthetases from E. coli to analogue XVI. The analogue showed substrate activity with some synthetases having K_m values similar to or slightly lower than those of ATP, but V_{max} values were all lower than those of the natural substrate (e.g. 50% for lysyl-t RNA, $< 1\%$ for glycyl-tRNA). In the case of synthetases where the analogue was not a substrate but did competitively inhibit ATP, K_i values ranged from 0.02 to 10 times the K_m value of ATP.

Analogue XVII was found (Santi et al., 1971) to be a non-competitive inhibitor of phenyl alanyl-tRNA synthetase from E. coli (indicating the presence of an alternative binding site for adenine nucleotide phosphates in this enzyme) and was a strong competitve inhibitor (K_i = 12μM compared to K_m ATP = 0.5mM) of tyrosyl-tRNA synthetase from E. coli (Santi & Pena, 1973).

Marutzky et al. (1976) found both analogues XVI and XVII to be competitive inhibitors of leucyl-tRNA synthetase from E. coli (K_i values 0.34 and 0.2mM respectively; K_m for ATP 0.3mM). However the equivalent imido analogue XXI was able to replace ATP as a sub-

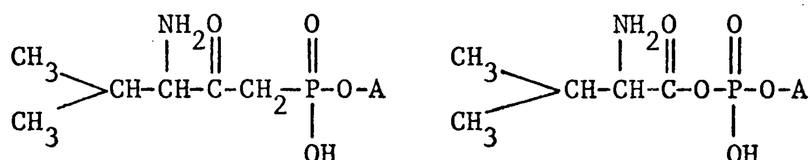


XXI

strate for this reaction (K_m value = 0.05mM, V_{max} 74% of that of ATP). The deviations in bond angle and lengths around the methylene sub-

stituted group in analogue XVI are larger than can be tolerated by the triphosphate binding site of this enzyme.

Southgate & Dixon (1978) found that the isosteric phosphonate analogue (XXII) of valylyl adenylate (XXIII) bound to valyl- tRNA



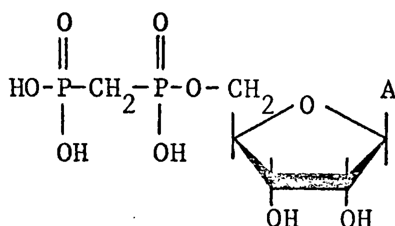
XXII XXIII (A = Adenosine)

synthetase (from E. coli) much less tightly than did the natural compound (dissociation constants, 5×10^{-7}M and 10^{-11} to 10^{-9} respectively). However analogue XXII inhibited both ATP-inorganic pyrophosphate exchange and valylation of tRNA by valyl- tRNA synthetase (K_i, 3μM). The authors suggested that reduced binding of analogue XXII to the synthetase might result because interaction of the enzyme with valylyl adenylate depends upon the anhydride oxygen, missing in the analogue.

2. Analogues of ADP

(i) Systems in which phosphonate analogues have been found to be capable of replacing ADP

Horak & Barton (1974) showed that analogue XXIV could



XXIV

replace ADP in inducing human platelet aggregation and could inhibit ADP induced aggregation. Furthermore the rate of analogue XXIV - induced aggregation was found to increase on raising the pH of the platelet-rich plasma above the pKa of the secondary phosphoryl dissociation of the analogue. These results suggest that analogue XXIV acts at the same site as ADP on platelet membrane and that hydrolysis of ADP is not a prerequisite for the process of aggregation. The observed effect of pH on the rate of analogue XXIV - induced aggregation indicates the importance of the ionization state of the terminal acid group of the nucleotide. Even when fully ionised, however, analogue XXIV is a much less potent aggregating agent than ADP, probably because of conformational differences between the two compounds [Gough *et al*. (1972) working with sheep platelets obtained somewhat different results from the above]. These and some further activities of analogue XXIV are listed in Table 8.

Table 8. Systems where analogues of ADP are capable of replacing ADP

Analogue	System	effect	Reference
XXIV	blood platelet aggregation	induce aggregation	Horak & Barton (1974)
XXIV	exchange of nucleotides from mitochondria	as ADP but slower	Duree *et al.* (1968)
XXIV	exchange of nucleotides from (yeast) mitochondria	as ADP but slower	Lauquin & Vignais (1973)
XXIV	exchange of nucleotides from (rat and mouse liver) mitochondria	as ADP but slower	Jebeleann (1974)
XXIV	5'ribonucleotidase (pig small intestine)	comp. inhibitor better than ADP	Burger & Lowenstein (1970)
XXIV	5'ribonucleotidase (sheep brain)	comp. inhibitor better than ADP	Burger & Lowenstein (1975)
XXIV	5'ribonucleotidase (mouse liver plasma membranes)	non comp. inhibitor	Evans & Gurd (1973)

(ii) Systems in which phosphonate analogues have been found not to be capable of replacing ADP

Le Blanc & Clauser (1974) showed that respiration-dependent Ca^{2+} accumulation in hog heart mitochondria has a specific requirement for ADP. Analogue XXIV was found to be unable to substitute for ADP but did strongly inhibit uptake of Ca^{2+} (Table 9).

Table 9

nucleotide	addition	Ca^{2+} uptake (µatoms/mg protein/10 mins)
none		0.65
ADP	2.4mM	1.2
XXIV	2.4mM	0.16

Binnet & Volfin (1974) demonstrated that internal ADP in rat liver mitochondria is necessary in order that a cytosolic metabolic factor (cmf) can exert its protective effect against Mg^{2+} and Ca^{2+} release from the mitochondria. Analogue XXIV was shown significantly to decrease the effect of cmf and this effect of XXIV was negated by the presence of atractylate. Analogue XXIV probably acts by depleting the endogenous ADP from the mitochondria.

Analogue XXIV inhibited the phosphorolysis of oligo- and polynucleotides (to yield ADP) by polynucleotide phosphorylase from Micrococcus luteus (Chou & Singer, 1970). At concentrations up to ten times those at which deoxy ADP afforded 50% inhibition analogue XXIV was neither a substrate nor an inhibitor of de novo ADP polymerisation catalysed by this enzyme (Chou & Singer, 1971). When the analogue (XXIV) concentration was raised to levels equivalent to that of the

ADP substrate (20mM) significant inhibition of polymerisation did, however, occur. Letendre & Singer (1974) tested the effects of ADP and analogue XXIV on the interaction of N-ethylmaleimide and polynucleotide phosphorylase from Micrococcus luteus (Table 10). In

Table 10

addition	effect of reaction with N-ethylmaleimide mole $[^{14}C]$/ mole enzyme	
	without dithiothreitol	with dithiothreitol
none	0.7	2.5
ADP	0.2	1.7
none	0.3	2.3
XXIV	0.3	1.6

contrast to ADP, analogue XXIV did not effect the reaction of the immediately-available sulphydryl groups with N-ethyl maleimide. Raue & Cashel (1974) demonstrated that E. coli subjected to simultaneous temperature and osmotic shock are capable of carrying out exchange between inorganic phosphate and ribonucleotide 5'polyphosphate. The fact that analogue XXIV inhibited this reaction was interpreted, together with other evidence, as indicating that this reaction is catalysed by polynucleotide phosphorylase.

Interestingly, analogue XXIV did not cause any decrease in rabbit muscle fructose 1,6 bisphosphatase activity, although ADP was found to be the most effective natural nucleotide phosphate inhibitor tested (Table 2) but at high concentrations analogue XXIV did give some protection against ATP inactivation (Taketa et al., 1971).

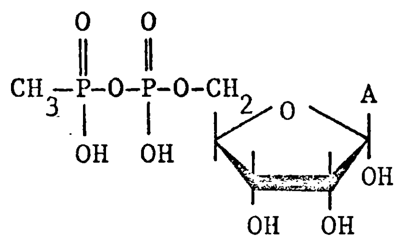
Analogue XXIV did not inhibit ATP binding to formyl tetrahydrofolate synthetase from Clostridium cylindrosporum (Curthoys & Rabinowitz, 1971) nor did it change the EPR spectrum of myosin (during steady state hydrolysis) indicating either that it does not bind or that binding produces no spectral changes (Seidal, 1975).

Although proton relaxation rate measurements showed that analogue XXIV forms a ternary complex with Mn^{2+} and creatine kinase from rabbit muscle it was found to be neither a substrate nor an inhibitor of the reaction catalysed by this enzyme (O'Sullivan et al., 1972).

Gough et al. (1972) reported that analogue XXIV does not initiate aggregation of sheep platelets (or inhibit ADP initiation) which was taken to indicate that hydrolyses of ADP is a necessary, key energy-providing reaction in platelet aggregation (c.f. Horak & Barton, 1974, page 24).

In mitochondrial and chloroplast ATP formation, inorganic phosphate loses an oxygen to water. ADP is the first detected acceptor of the phosphoryl group. Analogue XXIV was found (Jones & Boyer, 1969) not to be detectably phosphorylated; not to stimulate the exchange of oxygen from inorganic phosphate to water in the absence of ADP and not to inhibit this exchange in the presence of ADP. These results are consistent with the theory that exchange of inorganic phosphate oxygen with water results from the dynamic reversal of ATP formation at the catalytic site and that in the phosphorylation reaction the first covalent compound formed is that between inorganic phosphate and ADP.

These and some other activities of the phosphonate analogues of ADP are listed in Table 11.



XXV

Table 11. Systems where ADP analogues have been found not to be capable of replacing ADP

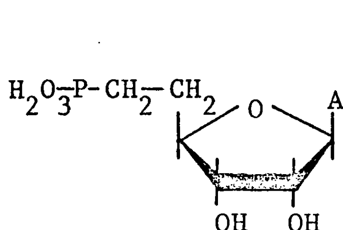
Analogue	System	Effect	Ref.
XXIV	Ca^{2+} accumulation in mitochondria (hog heart)	inhibits ADP dependent Ca^{2+} accumulation	Le Blanc & Clauser (1974)
XXIV	mitochondria (rat liver) cytosolic metabolic factor	inhibited ADP's action on cmf release of Mg^{2+} & Ca^{+}	Binnet & Volfin (1974)
XXV	phenylalanyl tRNA synthetase (E.coli)	non comp. inhibitor	Santi *et al.* (1971)
XXIV	polynucleotide phosphorylase (M. Luteus)	inhibitor	Chou & Singer (1970)
XXIV	polynucleotide phosphorylase (M. Luteus)	not a substrate or inhibitor of polymerisation	Chou & Singer (1971)
XXIV	polynucleotide phosphorylase (M. Luteus)	no effect on action of N-ethyl maleimide	Letendre & Singer (1974)
XXIV	polynucleotide phosphorylase (E. coli)	inhibitor	Raue & Cashel (1974)
XXIV	fructose 1,6 bisphosphatase (rabbit muscle)	not an inhibitor	Taketa *et al.* (1971)
XXIV	formyl tetrahydrofolate synthetase (C. cylindrosporum)	not an inhibitor	Curthoys & Rabinowitz (1971)
XXIV	Myosin	no change of EPR spectrum	Seidal (1975)

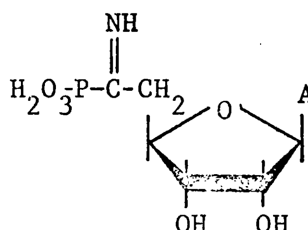
Table 11. (contd.)

Analogue	System	Effect	Ref.
XXIV	creatine kinase (rabbit muscle)	not a substrate or inhibitor	O'Sullivan et al. (1972)
XXIV	aggregation of sheep platelets	does not initiate (or inhibit ADP)	Gough et al. (1972)
XXIV	adenylate kinase, pyruvate kinase, Hexokinase. }	neither substrate nor inhibitor	Duree et al. (1968)
	phosphorylation in yeast mitochondria }	poor P_i acceptor did not inhibit ADP → ATP	
XXIV	chloroplasts and mitochondria $H_2O \rightleftharpoons P_i$ $ADP + H_2O + P_i \rightleftharpoons ATP$	not phosphorylated did not stimulate $H_2O \rightleftharpoons P_i$ exchange	Jones & Boyer (1969)

3. Analogues of AMP

AMP and its isosteric analogue (XXVI) were both found to be competitive inhibitors of adenyl-O-succinate synthetase from E. coli (K_i values 32 and 280μM respectively). The decrease in acidity of the



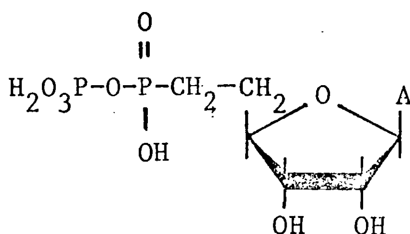


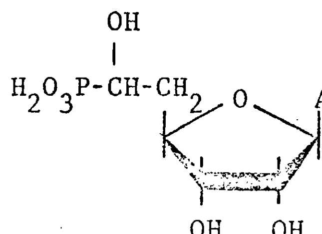
XXVI XXVII

analogue was thought to be the reason for its higher K_i value (Hampton & Chu, 1970).

Analogues XXVI and XXVII were found to be substrates or competitive inhibitors (or both) for several enzymes (Table 12) (Hampton et al., 1973a). Action of adenylate kinase on compound XXVI led to analogue XXVIII which was shown to be a substrate for pyruvate kinase.

Hampton et al. (1973b) also investigated the properties of a hydroxyl-substituted analogue XXIX. Analogue XXIX gave a complex





XXVIII XXIX

rate curve in the presence of AMP amino hydrolase. This resulted from different reactivities of the two epimers of analogue XXIX. The V_{max} value for the more active epimer was essentially the same as that for AMP which was in turn five times greater than that for analogue XXVI. The authors suggested that the enhanced substrate activity of the active epimer of analogue XXIX might result from an ability of its C-6' oxygen atom to mimic the oxygen at C-5' of the natural phosphate. Steric hindrance was put forward as a possible reason for the low V_{max} value obtained for the less active epimer of analogue XXIX (eight times lower than that of analogue XXVI). Inhibition of 5' nucleotidase by analogue XXIX but not by analogue XXVII can also be interpreted in terms of the potential ability of the C-6' oxygen atom of analogue XXIX to mimic some function of the C-5' oxygen atom of AMP in the enzyme-AMP interaction. Analogue XXIX was found to be a poor substrate for pig and rabbit adenylate kinases and it was suggested that the hydroxyl group on C-6' will be, in this mixture, close to the site of the catalysed reaction; steric and electronic requirements for substrate activity are likely to be more exacting in this area of the enzyme substrate complex (Hampton et al., 1973b).

Table 12. Effect of AMP analogues with various enzymes

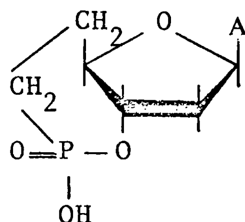
Analogue	System	Effect	Reference
XXVII	5'nucleotidase (snake venom)	competitive inhibitor, K_i= 110μM, (AMP K_m = 27μM).	Hampton *et al.* (1973a)
XXVI	AMP aminohydrolase (rabbit muscle)	substrate K_m = 1.7mM (AMP K_m = 1.0mM)	Hampton *et al.* (1973a)
XXVII	AMP aminohydrolase (rabbit muscle)	substrate K_m = 0.5mM competitive inhibitor K_i = 19μM	Hampton *et al.* (1973a)
XXIX	AMP aminohydrolase (rabbit muscle)	epimer I, substrate K_m = 4.0mM (V_{max} 91%, of V_{max} for AMP), competitive inhibitor, K_i = 210μM	Hampton *et al.* (1973b)
XXIX	AMP aminohydrolase (rabbit muscle)	epimer II substrate K_m = 0.025mM V_{max} 3% of V_{max} value for AMP)	Hampton *et al.* (1973b)

Table 12 (contd.)

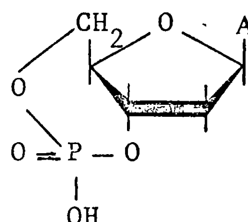
Analogue	System	Effect	Reference
XXVI	Adenylate Kinase (rabbit muscle)	substrate K_m = 0.17mM (AMP K_m = 0.5mM) V_{max} = 2.3% of V_{max} for AMP, competitive inhibitor, K_i = 0.44mM	Hampton *et al.* (1973a)
XXVII	Adenylate Kinase (rabbit muscle)	substrate K_m = 0.027mM (V_{max} 0.4% of V_{max} for AMP), competitive inhibitor, K_i = 0.32mM	Hampton *et al.* (1973a)
XXIX	Adenylate Kinase (rabbit muscle)	substrate K_m = 0.71mM (V_{max} = 0.2% of V_{max} for AMP)	Hampton *et al.* (1973b)

4. Analogues of cyclic nucleotides

In a patent, Jones & Moffatt (1969) reported the synthesis of the isosteric analogue XXX of 3'5' cAMP, with equivalent pharmacological action to that of the natural compound (XXXI) but with a longer half-life *in vivo*. Jones *et al*. (1973) later reported the phosphate ethano

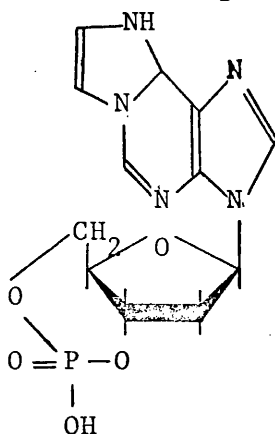


XXX

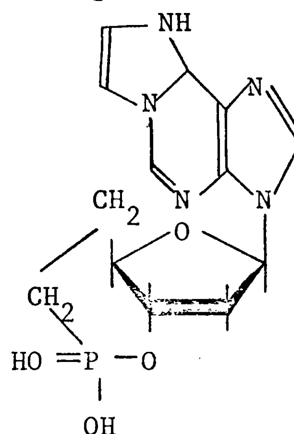


XXXI

derivative (XXXII) to have 54% of the activity of cAMP in stimulating calf brain protein kinase. The isosteric phosphonate ethano derivative (XXXIII), on the other hand had only 0.46% of the activity of cAMP in this system. Kuo & Greengard (1970) investigated the effects of methy-

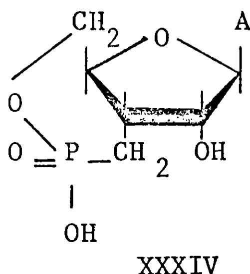


XXXII



XXXIII

lene substitution in the 3' (analogue (XXIV) and 5' (analogue XXX) oxygen position of cAMP on activation of cAMP-dependent and cGMP-dependent protein kinases from various sources. Analogue XXX was found to activate cAMP-dependent protein kinases from bovine brain, bovine heart, rat adipose cells and lobster muscle to the same maximal levels as did cAMP but at concentrations 25 to 500 fold greater than those of cAMP, but was inactive with cGMP-dependent protein kinase from lobster muscle. Analogue XXXIV was found to have no activating effect on either cAMP-

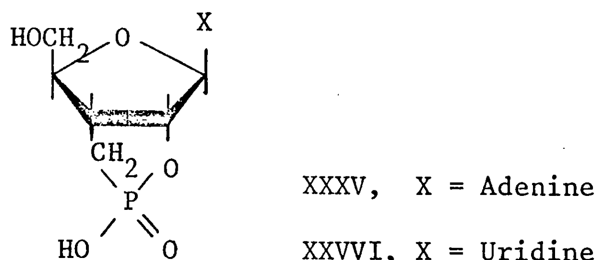


XXXIV

dependent or cGMP-dependent protein kinases. Similar results were obtained for rabbit muscle and bovine brain protein kinase, analogue XXXIV showing no binding affinity (Panitz et al., 1975).

The importance of the position of modification of the methylene analogues is also apparent in the cAMP activity of slime mold chemotactic activity (Malchow et al., 1973). Analogue XXX was found to be as active as cAMP in stimulating chemotaxis in Dictyostelium discordeum, whereas analogue XXXIV had a 10^6 fold-reduced effect. Furthermore the hydrolysis of cAMP by particle bound phosphodiesterase of D. discordeum was inhibited by analogue XXX 100-fold more effectively than by analogue XXXIV. The 3' oxygen of cAMP is obviously more important that the 5' oxygen in interaction with protein kinases, phosphodiesterase and proteins involved in chemotaxis. As well as studying methylene substitution of 3' and 5' oxygen atoms of cAMP, Mato & Konijn (1977) have carried out studies with imido analogues of cAMP. The cAMP chemoreceptor in D. discordeum was shown not to differentiate between, -O-. $-CH_2-$ or -NH- at the 5' position of the phosphate ring. On the other hand the 3' position of the phosphate showed a much higher degree of specificity (-O- > -NH- >> $-CH_2-$) underlining the importance of the lone electron pairs of the C-3' oxygen in interactions of cAMP with proteins.

In another patent Jones & Moffat (1971) reported the synthesis of the isosteric analogue XXXV of 2'3' cyclic AMP which was claimed to be useful for controlling metabolism and producing metabolic deficiencies. The corresponding uridine analogue (XXXVI) was



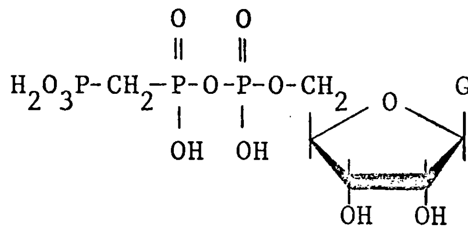
found to bind somewhat more strongly to bovine pancreatic ribonuclease than did the natural substrate 2'3 cUMP (K_m values 0.13 and 2.2mM respectively), however analogue XXXVI was significantly less susceptible to hydrolysis by this enzyme.

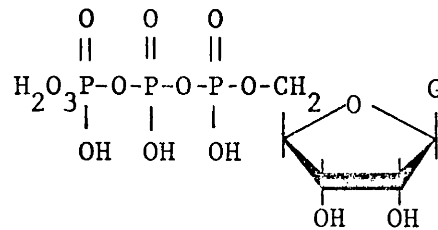
Extensive biochemical investigations have been carried out on many phosphonate analogues apart from those based on adenine nucleotides. The bulk of the literature published in this area has tended to emphasise mechanistic aspects, in attempts to clarify the biochemical role of the natural nucleotide.

5. Analogues of GTP

(i) Studies on protein synthesis

The isosteric analogue XXXVII of GTP (XXXVI) in which the βγ pyrophosphate oxygen is replaced by a methylene group has been thoroughly studied in elucidating the role played by GTP in protein synthesis. Initial investigations showed analogue XXXVII to be an overall inhibitor of peptide synthesis in several systems (Table 13). A full discussion of this area of work is beyond the scope of the present thesis. There have been several reviews on protein synthesis





G = Guanine

XXVII XXVIII

(Lippmann, 1969, Lucas-Lenaro & Lippmann, 1971, Haselkorn & Rothman-Denes, 1973) covering the use of analogue XXXVII in elucidating the role of GTP in protein synthesis. It has been demonstrated that GTP participates in initial binding of an amino acyl tRNA to the ribosomal complex without pyrophosphate cleavage, but that such cleavage does occur during translocation.

Table 13. Analogues used in investigations to determine role of GTP in protein synthesis

Analogue	Effect of analogue	Reference
XXXVII	competitive inhibition of homopolynucleotide synthesis *in vitro*, (*E. coli*) with respect to GTP	Hershey & Monro (1966)
XXXVII	inhibition of formyl methionyl-puromycin *in vitro* (*E. coli*)	Hershey & Tach (1967)
XXXVII	inhibition of valine incorporation into di and oligonucleotides	Tach *et al*. (1967)
XXXVII	stimulated binding of formyl-methionyl-tRNA to *E. coli* ribosomes, but did not replace GTP in reaction of formyl-tRNA-ribosomes with puromycin	Ohta *et al*. (1967)
XXXVII	did not inhibit binding of GTP to form a guanyl nucleotide-phenyl-alanine RNA complex, an intermediate product in enzyme transfer of phenylalanyl tRNA to ribosomes, but did inhibit formation of a dipeptide.	Ravel *et al*. (1968)

(ii) Other studies of phosphonate analogues of GTP

At very low concentrations (0.05μM) GTP stimulates the rate and degree of dissociation of bound, labelled glucagon in plasma membranes of rat liver. Analogue XXXVII shows a similar stimulation but at 100 times higher concentration than that of GTP (Rodbell *et al.*, 1971a). The authors suggested two possible explanations for these results. Either the analogue might exert its effect on glucagon binding by itself binding in a GTP-like manner to the membrane in a process not requiring phosphorylation or the analogue may exert its action by inhibiting the breakdown of endogenous membrane bound GTP.

Further studies by Rodbell *et al.* (1971b) showed that GTP plays an obligatory role in glucagen-induced activation of adenylate cyclase being active at somewhat lower concentrations than those which affect glucagon binding (10nM). In this system analogue XXXVII substituted for GTP with similar effectiveness at only five times the concentration of GTP (i.e. 50nM). The similar activities of analogue XXXVII and GTP indicate that GTP regulates this process through binding and not through phosphorylation. Salomon *et al.* (1975) also showed analogue XXXVII to be capable of substituting for GTP in the activation of the hepatic adenylate cyclase system (again less effectively than GTP).

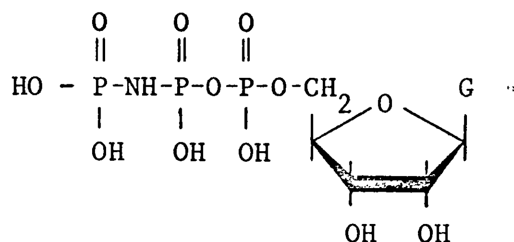
In contrast to the above two reports, Cuatrecasas *et al.* (1975) found that analogue XXXVII stimulated adenylate cyclase in rat fat pad membranes (in the presence or absence of hormones) unlike GTP, which was found to have no effect. The rate of activation was relatively slow and the authors postulated that after forming reversible Michaelis complexes of relatively low affinity, analogue

XXXVII might react irreversibly with the GTP regulatory site of the enzyme, perhaps forming analogue-enzyme covalent intermediates which maintain the activated state of the enzyme.

The most striking activation of adenylate cyclase by analogue XXXVII was reported by Pfeuffer & Helmreich (1975) who showed the phosphonate to be ten times more potent than GTP in activating pigeon erythrocyte membrane adenylate cyclase. The authors concluded that analogue XXXVII binds non-covalently to the GTP site on the membrane causing an unphysiological, irreversible activation of membrane-bound adenylate cyclase.

Kuwano et al. (1969) isolated a ribonuclease (Ribonuclease V) from cell-free extracts of E. coli and described some of its properties which suggest that ribonuclease V acts as a mRNase in the cell. Analogue XXXVII was not a substrate for but competitively inhibited ribonuclease V activity indicating that hydrolysis of GTP is necessary for RNase V function.

Purified tubulin from bovine brain is made up of a dimer which contains two binding sites for guanyl nucleotides. One contains a tightly-bound GDP molecule which is phosphorylated by GTP bound at an exchangeable site. Analogue XXXVII was not effective in displacing labelled GTP from the exchangeable binding site on tubulin, whereas the imido analogue could do this (Arai et al., 1975). Similar results were obtained for the assembly of bovine neurotubules (Sutherland, 1976). This provides further evidence that mild deviations in bond angles and lengths resulting from the substitution of a methylene group for an oxygen can result in changes in biochemical activity. Macconi & Seeds (1977) found that neither analogues XXXVII nor XXXIX could replace GTP in microtubule formation from lamb brain tubulin.



XXXIX

Analogue XXXVII was found to be a competitive inhibitor of GTP with respect to the action of adenylosuccinate synthetase from E. coli (K_i = 80μM) showing no substrate activity (Rudolph & Fromm, 1969).

Raue & Cashel (1974) found that analogue XXXVII could not replace GTP in stimulating polynucleotide phosphorylase from E. coli, indicating that hydrolysis of the βγ phospho diester bond is required for GTP to be active in the exchange between inorganic phosphate and ribonucleoside 5' polyphosphate.

These effects of the phosphonate analogues of GTP are listed in Table 14.

Table 14. Studies on analogues of GTP

Analogue	System	Effect	Reference
XXXVII	plasma membrane of rat liver	release of glucagon bound to membrane x100 [ ] of XXXVII needed for same effect as GTP	Rodbell *et al.* (1971a)
XXXVII	activation of adenyl cyclase	x5 [ ] of XXXVII needed for same effect as GTP	Rodbell *et al.* (1971b)
XXXVII	activation of adenyl cyclase (hepatic)	x5 [ ] of XXXVII needed for same effect as GTP	Salomon *et al.* (1975)
XXXVII	activation of adenyl cyclase (rat fat pad membranes)	activated (no activation by GTP)	Cuatrecasas *et al.* (1975)
XXXVII	activation of adenyl cyclase (pigeon erythrocyte membrane)	x10 more potent than GTP	Pfeuffer & Helmreich (1975)
XXXVII	adenylate cyclase activation (rat reticulocyte)	activates, and held in activated state	Spiegel *et al.* (1977)
XXXVII	Ribonuclease V (*E. coli*)	inhibits action of GTP	Kuwano *et al.* (1969)
XXXVII	Tubulin (bovine brain)	does not bind XXXVII	Aria *et al.* (1975)

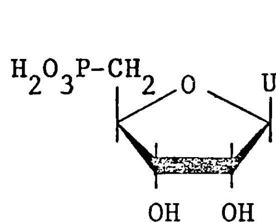
Table 14. (contd.)

Analogue	System	Effect	Reference
XXXVII	bovine neurotubule production	no action	Sutherland (1976)
XXXVII	Adenylosuccinate synthetase (E. coli)	comp. inhibitor	Rudolph & Fromm (1969)
XXXVII	polynucleotide phosphorylase (E.coli)	does not replace GTP as stimulator	Raue & Cashel (1974)
XXXVII	lamb brain tubulin production	no action	Macconi & Seeds (1977)

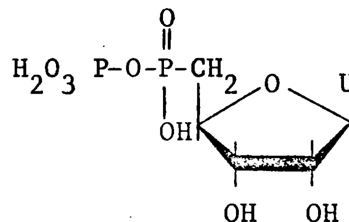
6. Analogues of other nucleotides

(i) Analogues of Uridine nucleotides

As one might expect, the nonisosteric analogue (XL) of UMP was found not to be a substrate for alkaline phosphatase from snake venom, bovine intestine, or E.coli (Holy, 1967). The corresponding nonisosteric analogue (XLI) of UDP was shown to be



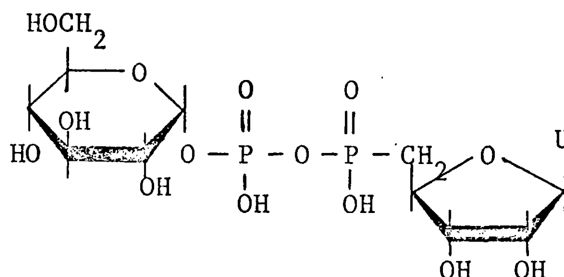
XL



XLI (U = Uridine)

incapable of polymerisation with polynucleotide phosphorylase from Micrococcus lysodeikticus (Yengoyan & Rammler, 1966).

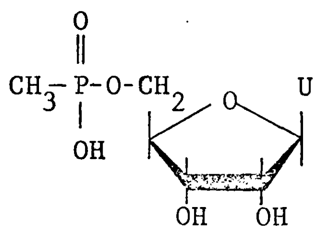
Bax et al. (1970) showed the nonisosteric analogue (XLII)



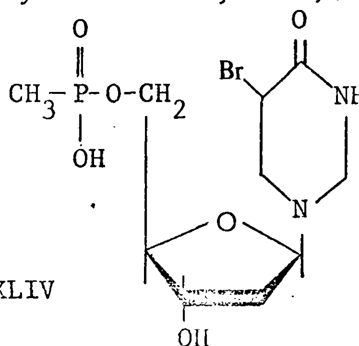
XLII

of UDP-glucose to be a good substrate for bovine UDP-glucose dehydrogenase with a similar K_m value to that of the natural substrate (12μM and 20μM respectively) but with a lower V_{max} value (16% that of the natural substrate). The analogue was, however a poor substrate for UDP-glucose phosphorylase being only 5% as effective as the natural substrate. Analogue XLII was not a substrate for UDP-galactose-4 epimerase.

Analogue XLIII was found to be without effect on thymidylate synthetase from E. coli, indicating a requirement for the dibasic acid for effective interaction (Holy & Vortruba, 1974).



XLIII

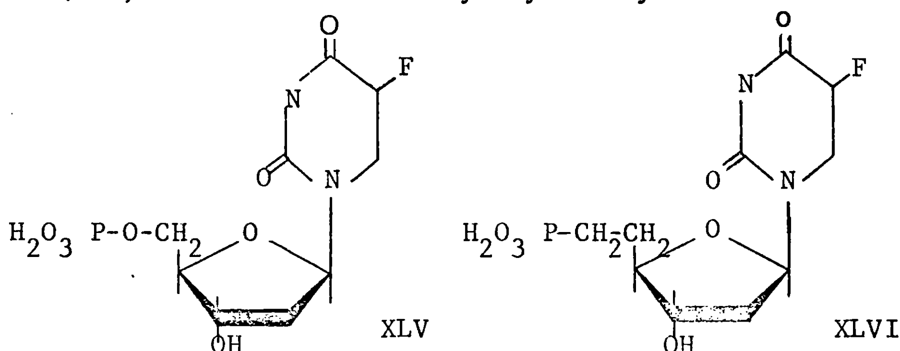


XLIV

Wigler & Lozzio (1972) found that analogue XLIV (5 bromo,2,deoxy-uridine 5' methyl-phosphonate) a structural analogue of 2 deoxy-thymidine caused irreversible cytotoxicity to Chinese hamster cells. Nucleotides in general do not readily penetrate the membranes of living cells but analogue XLIV even when fully ionized

has only one negative charge and might accordingly get into the cell. A delay was in fact observed in the cytotoxic effect caused by analogue XLIV on the hamster cells consistent with time required for sufficient incorporation of the analogue into DNA to cause a lethal effect.

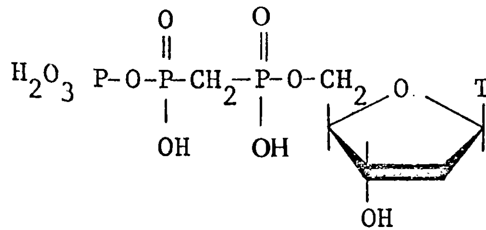
5-Fluorouracil is known to exert its cytotoxic action (and anti cancer effects) after conversion to 2'deoxy 5-fluoro uridylic acid (XLV) which inhibits thymidylate synthetase. Attempts to

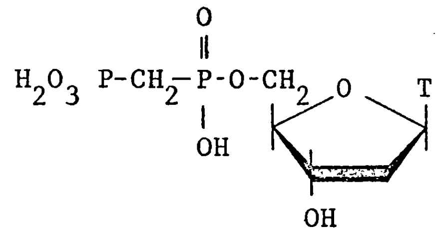


enhance this activity by the direct use of XLV have failed because of its rapid degradation to 5-fluorouracil. Analogue LVI on the other hand should resist dephosphorylation and was accordingly studied by Montgomery et al. (1979). XLVI was found to be a potent inhibitor of thymidylate synthetase from three individual sources, (Lactobacillus casei, E. coli and Coliphage T_2) but nevertheless less potent than compound XLV, requiring preincubation with the enzyme. Analogue XLVI was moderately cytotoxic to H.Ep-2 cells in culture.

(ii) Analogues of deoxy thymidine nucleotides

England et al. (1969) found that analogue XLVII bound to DNA polymerase from E. coli at the same site and with similar affinity to that of deoxy TTP (dissociation constants K = 130 and 80μM respectively). Further investigations (Geider, 1972), showed





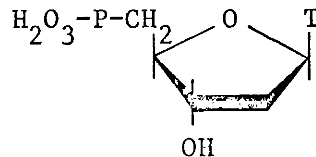
T = Thymidine

XLVII

XLVIII

analogues XLVII and XLVIII to be neither substrates nor inhibitors of DNA polymerase in nucleotide-permeable E. coli cells, showing that although the phosphonate analogues might bind to DNA polymerase, subsequent steps in the polymerisation process cannot proceed.

Rammler et al. (1972) found that analogue XLIX was a weaker inhibitor of micrococcal nuclease than was deoxy TMP (8% and 16% inhibition respectively). The authors suggested that the weaker



XLIX

inhibition observed for the nonisosteric analogue resulted from its smaller size.

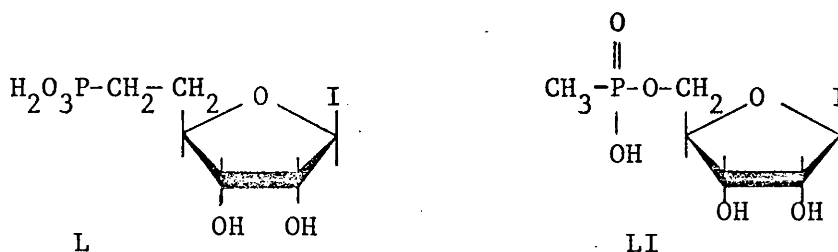
(iii) Analogues of IMP

The isosteric analogue (L) of IMP, like that of AMP (XXIV), was found to be a substrate for adenyl O-succinate synthetase, (Table 15) (Hampton & Chu, 1970) suggesting that the

Table 15

compound	K_m μM	V_{max} (Relative to IMP)
IMP	20	1.0
L	160	0.035

C-5 oxygen atom of IMP does not make a major contribution to the total binding energy of IMP in this system.



Nichol <u>et al</u>. (1967), studying the phosphate-binding sites of inosinic acid dehydrogenase and adenylsuccinate synthetase (from <u>E. coli</u>), investigated the effect of analogue L1, which was found not to be a substrate for either enzyme.

The above sections have outlined studies of mononucleotide analogues in which the structural changes largely involve replacement of CH_2-O-P groupings by CH_2-CH_2-P or CH_2-P moieties. Not only mononucleotides but also dinucleotides (Griffin <u>et al</u>., 1973, and Johnson & Schleich, 1974) have been modified so as to produce phosphonate analogues, the propeṫties of which have been studied. Moreover structural modification has not been confined to the basically simple changes discussed above. For example CH_2-O-P of the naturally-occurring compound has been substituted by $CH_2-\underset{CL}{CH_2}-P$, $CH_2-\underset{NH}{CH_2}-P$ and $CH_2-\overset{CL}{\underset{CL}{CH}}-P$ (Holy & Hong, 1971, 1972 and Gulyaev & Holy, 1972) and many other modifications have been reported.

Other Phosphonate analogues

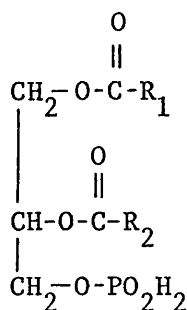
Diphosphonate analogues have been extensively investigated (and used clinically) showing similar properties to inorganic pyrophosphate with regard to calcium metabolism. (Review, Fleish *et al.*, 1977). Analogues of pyridoxal phosphate have also been investigated (Bennett *et al.*, 1959, Hullar, 1967, and Vidgoff *et al.*, 1974) as have phosphonate analogues of intermediates of squalene biosynthesis and other terpenes. (Corey & Volante, 1976, and Sarin *et al.*, 1977).

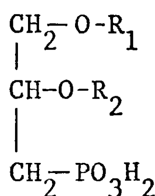
(B) PHOSPHOLIPIDS

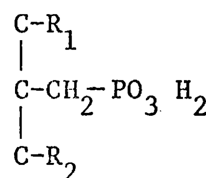
Since phosphonolipids were first demonstrated to occur naturally (Horiguchi & Kandatsu, 1959) there has been a great deal of interest in their organic synthesis and biological activity. They have been synthesised as reference compounds for elucidation of the structure and configuration of naturally-occurring phosphonolipids, for possible use as inhibitors of enzymes utilizing phospholipids and as potential analogues of phospholipids in biochemical investigations generally. Apart from the phosphatidic acids, phospholipids are diesters and their phosphonate analogues may have a C - P linkage on either the base or the glycerol side of the molecule. When both are present the compound is classed as a phosphinate and although such compounds have so far not been found in nature they often show interesting biochemical properties.

1. Analogues of Phosphatidic Acids

Rosenthal & Pousada (1966) demonstrated that the diether analogue (LIII) inhibited the particulate phosphatidate phosphatase from pig kidney. The diether analogue LIII although nonisosteric is a relatively close analogue of phosphatidic acids (LII) and the reported inhibition might in isolation be regarded as the result of





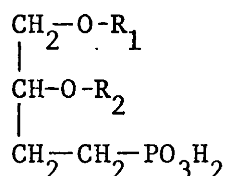


LII LIII LIV

a specific enzyme-analogue interaction. However the sterically

distinct phosphonate analogue LIV containing neither ester nor ether groups was found to be an equally powerful inhibitor of the same enzyme system, suggesting that inhibition in this case might result from relatively non-specific effects unrelated to the detailed structure of the inhibitor. Such indications are reinforced by the observation that the inhibition by phosphonate analogue LIII depended on the length of time of pre-incubation of the enzyme with the analogue and on the degree of dispersion of the latter, suggesting that physicochemical factors might be playing a role in the observed results.

Soluble pig kidney phosphatidate phosphatase was also inhibited by analogue LIII and, surprisingly, to a less extent by the isosteric diether analogue LV (Rosenthal & Han, 1968).



LV

Inhibition of the soluble enzyme was much less influenced by enzyme-analogue pre-incubation than in the case of the particulate system; a fact which further emphasises the possible involvement of physical factors in the latter enzyme and in enzyme-catalysed reactions of phospholipids generally.

As might be expected, phosphonate analogue LIII had no substrate activity for phospholipase C from _Clostridium perfringens_ but was an inhibitor of this enzyme. However, inhibition was not competitive but was again found to result from physical factors.

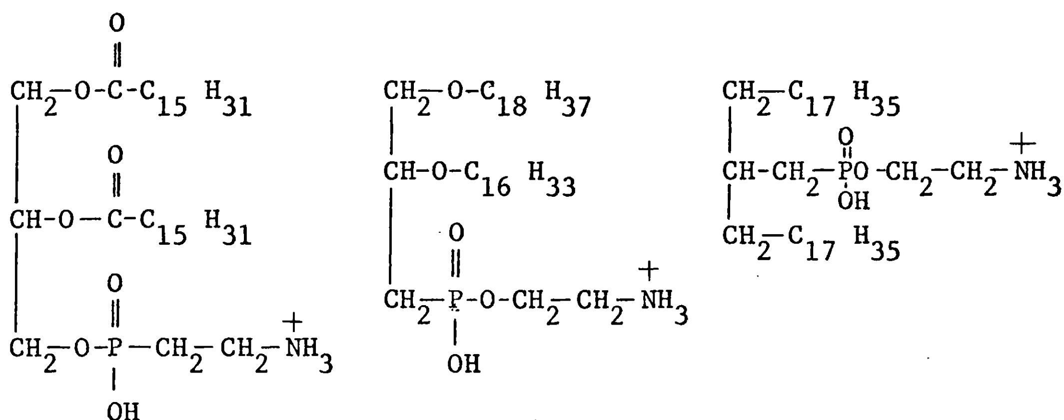
The fact that analogue LIII is negatively-charged means that it could act by altering the electro kinetic properties of the dispersed substrate particles or by binding calcium necessary for activity (Rosenthal & Pousada, 1968).

Rosenthal and his co-workers examined the effects of analogue LIII on _in vivo_ systems. Only two out of thirteen species of bacteria tested were found to be significantly inhibited by the analogue (Rosenthal & Pousada, 1966). Intraperitoneal administration of the phosphonate analogue LIII into rats results in a reversible accumulation of triglycerides; an observation which was not easily related to the demonstrated inhibition of phosphatidate phosphatase (Rosenthal & Han, 1968). A possible explanation could involve the interference of fat removal from the liver to the circulation by complexing of the phosphonate analogue LIII with transport apolipoproteins.

The effect of phosphonate analogue LIII on an _in vitro_ metabolic system was also examined. Addition of rat liver supernatant fraction to microsomal preparations led to stimulation of palmitic acid incorporation into triglycerides and this incorporation was inhibited by phosphonate analogue LIII (Rosenthal & Han, 1968). This inhibitory effect provides supporting evidence that the original stimulation of palmitic acid incorporation is the result of phosphatidate phosphatase present in the supernatant.

2. Analogues of phosphatidylethanolamine

The nonisosteric (relative to the ethanolamine moiety) phosphonate analogue (LVI) of phosphatidylethanolamine was found not to be hydrolysed by phospholipase C from Clostridium welchii (Baer & Stanacev, 1966). However Rosenthal & Pousada (1968) showed that the phosphonate analogue LVI and two other phosphonate analogues of phosphatidylethalolamine were inhibitors of phospholipase C from Clostridium perfringens. The nonisosteric (in relation to the



LVI LVII LVIII

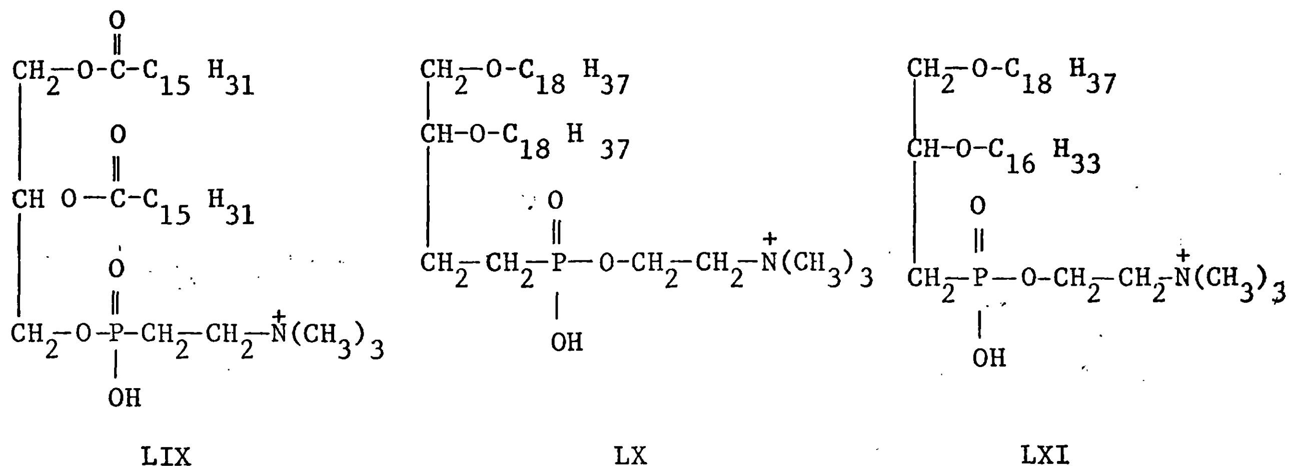
glycerol moiety) diether phosphonate analogue LVII was a better inhibitor than the nonisosteric (in relation to the base moiety) fatty ester phosphonate LVI while the structurally-different branched-chain hydrocarbon phosphonate LVIII was the worst inhibitor. These phosphonate analogues of phosphatidylethanolamine were poorer inhibitors than the corresponding phosphatidylcholine analogues which were also tested in the above system.

The isosteric diether phosphonate analogue LV (R_1=R_2= C_{18}-H_{37}) was found to be an activator of phospholipase A from snake venom, (A. piscivorus) but this activation was demonstrated to be the

result of a physical effect on the substrate lipoprotein (Rosenthal & Han, 1970).

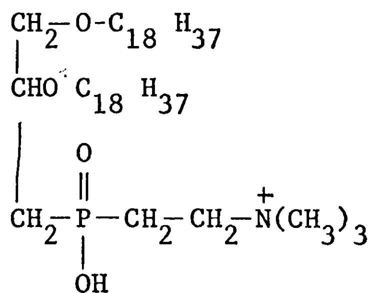
3. Analogues of phosphatidylcholine

Baer & Stanacev (1966) demonstrated that, unlike the phosphatidylethanolamine phosphonate analogue LVI the nonisosteric phosphonate analogue LIX was hydrolysed after several hours in the presence of phospholipase C from Clostridium welchii. Rosenthal & Pousada (1968), on the other hand demonstrated that compound LIX was an inhibitor of phospholipase C from Clostridium perfringens, showing

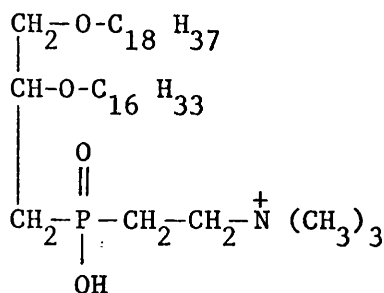


no substrate activity after thirty minutes incubation with the enzyme. The most effective phosphonate analogue inhibitors in this system were found to be the isosteric (LX) and the nonisosteric (LXI) diether analogues in both of which a C - P bond links the phosphorus atom directly to the glycerol moiety. Phosphonate analogues LX and LXI gave relatively simple kinetics, displaying ordinary competitive inhibition, the isostere LX being a better inhibitor than the non-isostere LXI. Further to these studies Rosenthal & Chodsky (1974) investigated the relative inhibitions of phospholipase C by a series of diether phosphinates (LXII → LXV). Simple correlation between

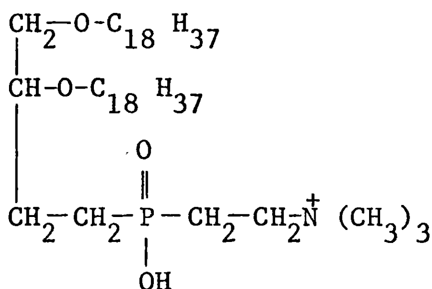
inhibitory activity and structure was not possible. For example none



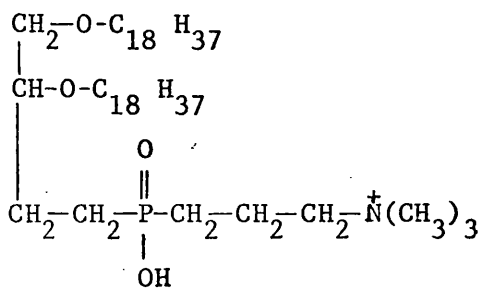
LXII



LXIII



LXIV



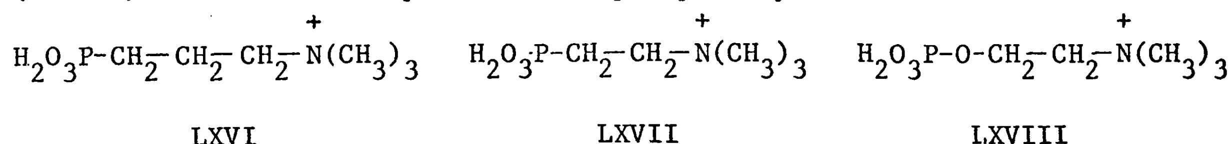
LXV

of the phosphinate analogues LXII → LXV was as strong an inhibitor as was the isosteric phosphonate analogue LX and the corresponding isosteric phosphinate analogue LXV was the worst. This might suggest that the oxygen atom of the choline moiety is important for inhibitory activity. However the nonisosteric phosphinate analogue LXII which lacks such an oxygen atom is a good inhibitor. It is likely that physicochemical properties such as particle size and detergent effect are of particular relevance in this enzyme system.

Studies on phospholipase A from snake venom (A. pescivorus) showed phosphinate analogue LXII to be a stronger inhibitor than either

of the phosphonate analogues LX and LXI. In this system also, however physicochemical effects were found to dominate the interactions of the substrate analogues with the enzyme (Rosenthal & Han, 1970).

Bjerve (1972) demonstrated that the isosteric (LXVI) and the nonisosteric (LXVIII) phosphonate analogues of choline phosphate (LXVIII) were both incorporated into phosphatidylcholines in rat liver



and kidney in vivo. The phosphonate was incorporated as a unit, utilizing the same pathway as that used by the natural substrate. Subsequently excreted compounds were found to be unchanged, indicating that the rat could not break down the C - P bond. Surprisingly, the nonisosteric phosphonate analogue LXVII was a better substrate for phosphocholine cytidyl transferase than was the isosteric phosphonate analogue LXVI. Analogue LXVII was found to be a competitive inhibitor of choline phosphate transfer (and vice versa). (Table 15).

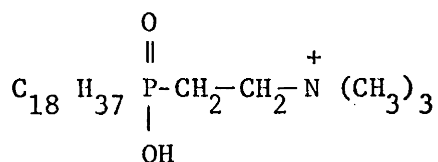
Table 15

substrate	K_m (mM)	K_i mM	V_{max} relative to LXVIII
LXVII	5.2	5.2	1
LXVIII	0.35	0.17	1

The biochemical properties of phosphatidylcholine analogues incorporated into artificial bilayer membranes have been extensively studied. When phosphonate analogue LXI replaced phosphatidylcholine in a phosphatidylcholine - phosphatidylserine-cholesterol (0.7 : 0.3 : 1.0 w/w/w) bilayer membrane there was an inhibition of phospholipase C

action on the membrane (Hendrickson et al., 1974). The degree of inhibition was proportional, over a wide range, to the percentage of analogue LXI that had been substituted for phosphatidylcholine, indicating that the inhibitory effect was non-specific. This is contrary to the inhibition of phospholipase C by the phosphonate LXI in aqueous dispersions (Rosenthal & Pousada, 1968) see page 55. Complete hydrolysis of the phosphatidylcholine in the bilayer membrane must involve reorganisation of the bilayer structure and/or penetration of the enzyme into the inner bilayers and it may be that these processes are inhibited by the presence of the phosphonate LXI.

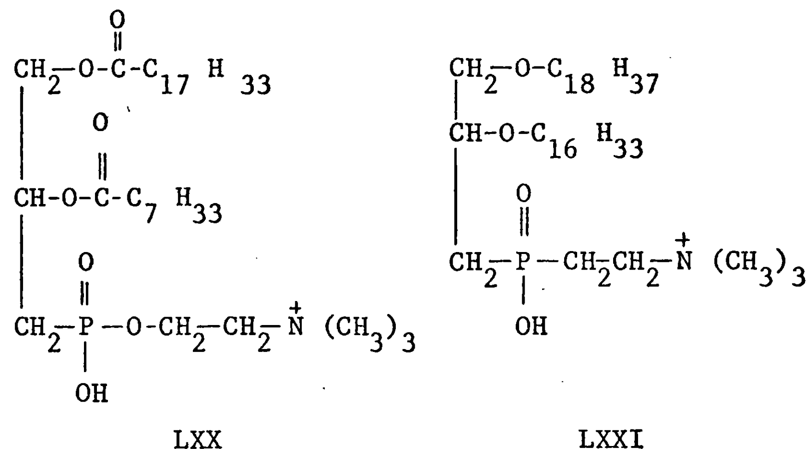
The presence of cholesterol lowers the initial rate of water penetration to the same extent in diester and diether phosphatidylcholine-containing liposomes, suggesting that the carbonyl linkage has a negligible role in the packing of the lipids. Furthermore, liposomes made from the isosteric phosphonate (LX) or phosphinate (LXIV) analogues are affected by the presence of cholesterol similarly to liposomes containing the natural phosphatidylcholine. However liposomes made from the nonisosteric phosphonate(LXI) or phosphinate(LXIII) analogues displayed no interaction with cholesterol (Bittman & Blau, 1972). This indicates that strict steric demands at the polar head must be met in order for a phosphatidylcholine-cholesterol complex to form and that stereoelectronic effects are of secondary importance. In contrast DeKruyff et al.(1973) demonstrated that liposomes made from a nonisosteric straight chain phosphonate LXIX displayed



LXIX

comparable behaviour to that of natural phosphatidylcholine liposomes in the presence of cholesterol.

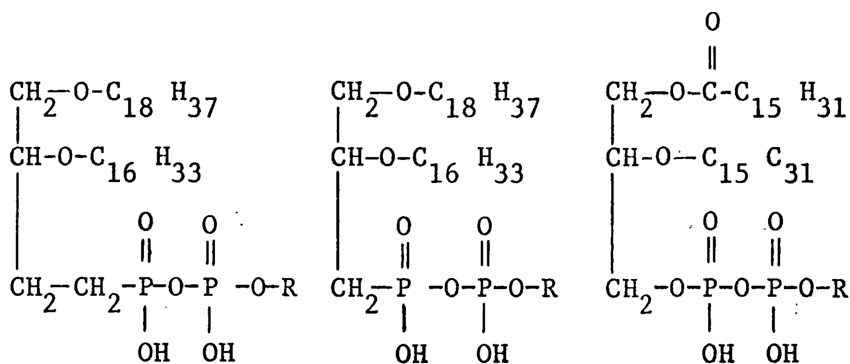
Kinsky et al. (1974) showed that sealed liposomes made from the nonisosteric phosphonate (LXX) and phosphinate (LXXI) analogues do not release labelled glucose in the presence of phospholipase C consistent with the findings (see page 55) that phosphonate and phosphinate analogues of phosphatidylcholine do not function as substrates for phospholipases. Liposomes made from LXX and LXXI, like those made from phosphatidylcholine were however lysed in the

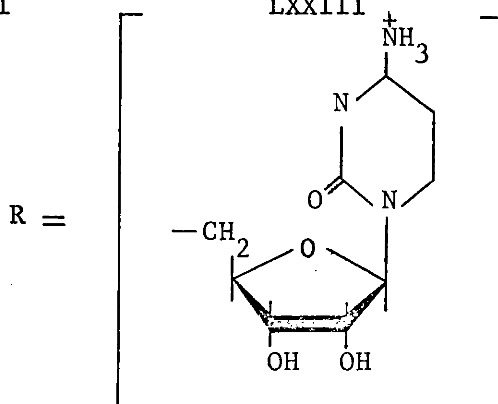


presence of specific antibodies and complement. The authors interpreted these results as demonstrating that the complement-mediated lysis of liposomes (and by implication, of cell membranes) is not the direct result of phospholipase action.

4. Analogues of other phospholipids

The isosteric (LXXII) and the nonisosteric (LXXIII) phosphonate analogues of CDP diglyceride (LXXIV) were found to be substrates for CDP diglyceride: glycerol 3 phosphate phosphatidyl transferase and for CDP diglyceride: L serine phosphatidyl transferase (Tyhach _et al._, 1975). Both phosphonate analogues were





good substrates for the former enzyme having similar kinetic parameters to those of the natural substrate CDP dipalmitin (LXXIV) (Table 16). CDP diglyceride L serine phosphatidyl transferase appeared to have a

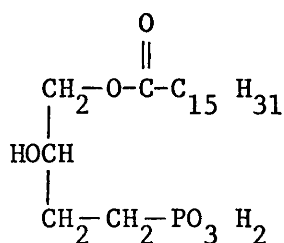
Table 16

Compound	K_m µM	V_{max} (relative to LXXIV)	K_m mM	V_{max} (relative to LXXIV)
	CDP diglyceride glycerol 3-phosphate phosphatidyl transferase		CDP diglyceride:L serine phosphatidyl transferase	
LXXII	80	1	0.8	0.1
LXXIII	60	1	0.4	0.1
LXXIV	44	1	1.4	1.0

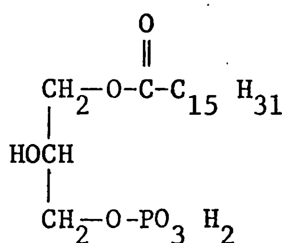
stricter substrate requirement, and the fact that both analogues had the same V_{max} value suggested either that the phosphonate group or the ether groups were responsible for the low activity of these compounds.

Increased hydrophobicity of LXXII and LXXIII may account for the smaller K_m values that were obtained.

The isosteric phosphonate analogue LXXV of lysophosphatidic acid (LXXVI) was a substrate for lysophosphatidic acid acyl



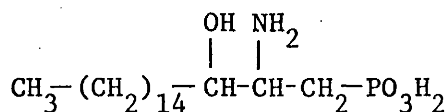
LXXV



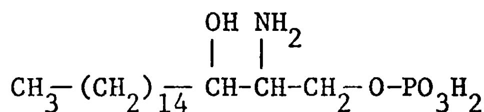
LXXVI

transferase and was found to have a higher K_m value (300μM) compared with that (50μM) for lysophosphatidic acid, indicative of a lower binding affinity of the analogue for the enzyme (Tang *et al*. 1977).

The nonisosteric phosphonate analogue LXXVII of sphinganine phosphate (LXXVIII) has been studied *in vivo* and *in vitro* (Stoffel & Grol, 1974). The phosphonate analogue LXXVII was a substrate for the pyridoxyl phosphate lyase: aldolase having a similar K_m value (16μmoles)



LXXVII



LXXVIII

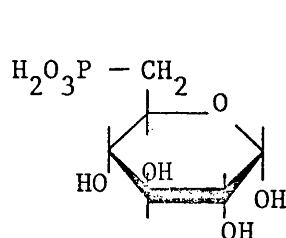
but a smaller V_{max} value (0.1) than those of the natural substrate (LXXVIII), and LXXVII was also found to be a competitive inhibitor of the aldolase (K_i = 5μmoles). When analogue LXXVII was intravenously injected into rats (2μmoles) they died within one minute and 20μmolar concentrations of the phosphonate analogue (LXXVII) were found to haemolyse human red blood cells indicating that the analogue disrupts membrane structure.

(C) CARBOHYDRATES

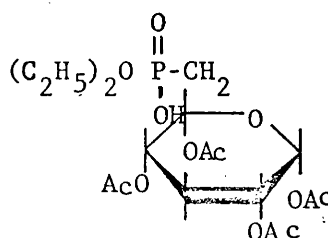
Comparatively little biochemical investigation has been reported to date on phosphonate analogues of carbohydrates.

1. Analogues of Hexoses

Griffin & Burger (1956) found that the nonisosteric phosphonate analogue LXXIX of glucose 6-phosphate did not inhibit choline acetylase, histidine decarboxylase, xanthine oxidase or hyaluronidase. The ester derivative (LXXX) caused production of acetylcholine by choline acetylase in the absence of ATP and increased acetylcholine formation on addition of sub optimal amounts of ATP. The significance of these results is not clear.



LXXIX



LXXX

The isosteric phosphonate analogue X of glucose 6 phosphate was shown to be a good substrate for yeast glucose 6 phosphate dehydrogenase [Table 17] (Adams _et al._, 1976). The K_m value of compound X did not significantly change on raising the pH from 7.5 to 8.0 and it was concluded that the higher K_m values obtained compared with those of glucose 6-phosphate could not be explained in terms of differences in states of ionization. The lower affinity of glucose 6-phosphate dehydrogenase for the analogue X compared to that for the natural substrate was suggested to result from other factors. It could be that the replacement of C-O-P in glucose 6-phosphate by C-CH_2-P in the analogue would lead to loss of interaction with the enzyme, either because the oxygen atom is necessary

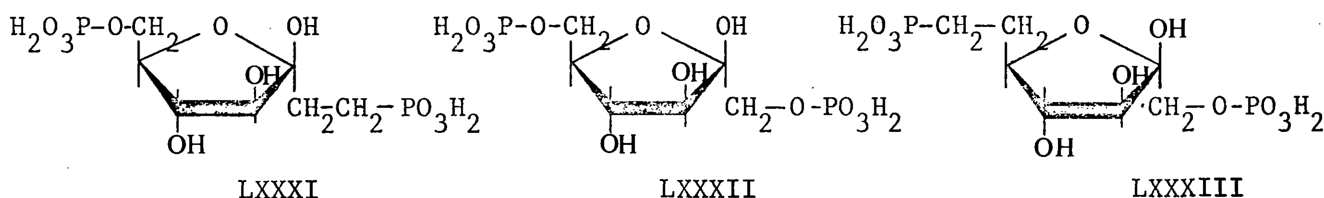
Table 17. Comparison of kinetic parameters for glucose 6-phosphate and its phosphonate analogue (X).

compound	2nd pKa	K_m (μM)		K_m NADP (μM)		V_{max}*	
		pH 7.5	pH 8.0	pH 7.5	pH 8.0	pH 7.5	pH 8.0
glucose 6 -phosphate	6.21	51	46	7.5	6.8	1	1
X	7.50	227	192	6.2	6.5	0.58	0.46

(*Relative to glucose 6-phosphate)

for binding or because of minor geometrical differences between the two structures. The product of enzymic dehydrogenation of analogue X was itself shown to be a substrate for yeast 6 phosphogluconate dehydrogenase (Adams *et al.*, 1976).

A similar pattern of results was obtained (Stribling, 1974) for the enzymically-prepared 1-phosphonomethyl isostere (LXXX1) of fructose 1,6 bisphosphate (LXXXII) in its interaction with aldolase. There was however an even larger decrease in the V_{max} value of LXXXI

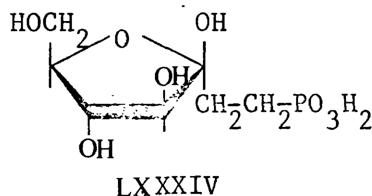


compared to that of the natural substrate LXXXII (i.e. one twentieth that of LXXXII). As one might expect, analogue LXXXI showed no measurable activity with fructose bisphosphatase and was a competitive inhibitor of the natural substrate (K_i = 70μM compared to K_m = 4μM for LXXXII).

More recently the 6-phosphonomethyl isostere LXXXIII of fructose, 1,6-bisphosphate has been enzymically-prepared and found to

be a substrate for aldolase (Webster et al., 1976). Surprisingly LXXXIII was, however, not a substrate for 1,6 bisphosphatase but was a competitive inhibitor, with a K_i value (150μM) nearly two orders of magnitude greater than the K_m value of the natural substrate (LXXXII). The authors put forward two possible explanations for this:- that interaction between the oxygen normally present on C-6 and some part of the enzyme is necessary before catalysis can occur or that analogue LXXXIII may bind the wrong way round so precluding catalysis. However analogue LXXXIII was found to be a substrate for the sequential actions of glucose 6-phosphate isomerase, and glucose 6-phosphate dehydrogenase, (the product of the isomerase reaction appeared to be a poor substrate for the dehydrogenase) confirming the work of Adams et al. (1976).

Tang et al. (1978) recently reported the synthesis of the isosteric phosphonate analogue LXXXIV of fructose 1-phosphate. The analogue was shown to inhibit the growth of mutant strains of E. coli



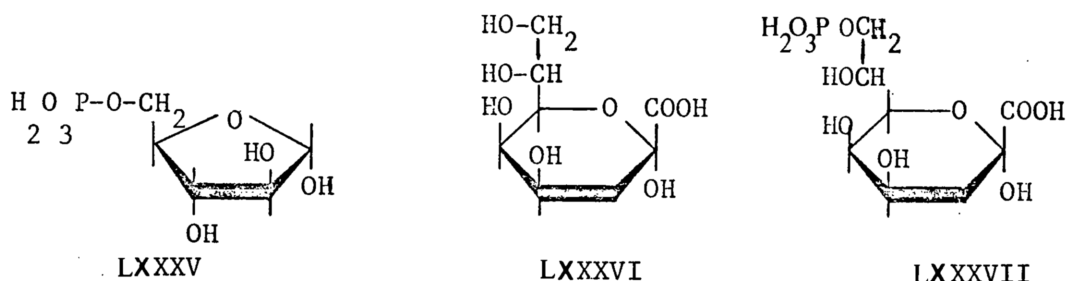
LXXXIV

that are capable of transporting hexose phosphates.

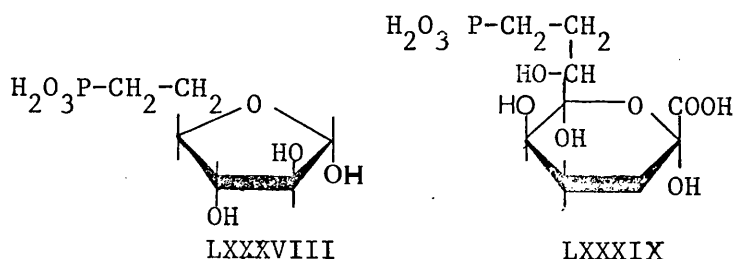
2. Analogues of Pentoses

Arabinose 5-phosphate (LXXXV) is a precursor in the biosynthesis of 3 deoxyoctulosonate (LXXXVI), a component present in lipopolysaccharide structures of all Gram-negative organisms. The first step involves condensation of the pentose LXXXV with phosphoenolpyruvate catalysed by 3-deoxyoctulosonate 8-phosphate synthetase to give 3 deoxyoctulosonate 8-phosphate (LXXXVII) which in turn is

dephosphorylated by a specific phosphatase to give LXXXVI . The isosteric analogue of arabinose 5-phosphate was found to be a

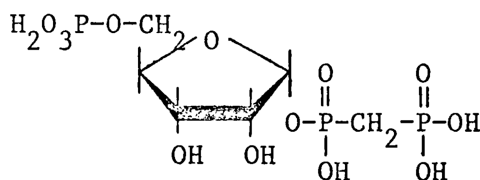


substrate for 3-deoxyoctulosonate 8-phosphate synthetase (Unger et al., 1978). The crude enzyme preparation used in these studies contained both the synthetase and the specific phosphatase but only limited formation of phosphonate LXXXIX was detected as concentrations of LXXXVIII were increased. It was suggested that the phosphonate inter-



mediate LXXXIX accumulated because it was not a substrate for the phosphatase and that product inhibition of the synthetase resulted. Such inhibition might provide a basis for the rational design of an antibacterial agent.

The methylene isostere (XC) of 5-phosphoribosyl pyrophosphatate (PRPP) was isolated after the reaction of ribose 5-phosphate and O-adenyl methylene diphosphate (XVI) in the presence of phosphoribosyl pyrophosphate synthetase (from Erhlich ascites tumour cells) (Murray et al., 1969). This reaction clearly involves a transfer of the pyrophosphate analogue as a unit, and the transfer in the reverse direction (i.e. from XC to AMP giving XVI) has also been demonstrated.



XC

Analogue XC was shown to be a substrate for three phosphoribosyl transferase enzymes (Table 18). The lower V_{max} values, (compared to those of the natural substrate) obtained for XC with adenine and hypoxanthine transferases are thought to result partially from the lower electronegativity of the phosphonate analogue, as the reaction between the nucleotide and the sugar involves nucleophilic displacement at the C-1 position of the sugar. Analogue XC was not recognised by 5-phosphoribosyl 1 pyrophosphate amido transferase or oratate phosphoribosyl transferase (Murray et al., 1969).

Table 18

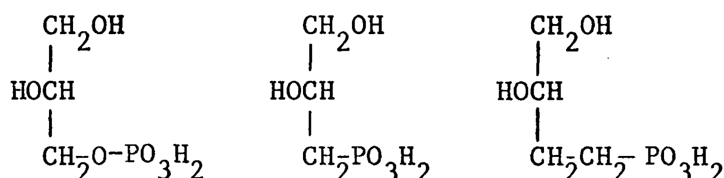
enzyme	K_m (μM)	K_m (μM)	V_{max}
	PRPP	XC	relative to PRPP
adenine phosphoribosyl transferase	13.2	2.2	0.011
hypoxanthine " "	23.0	4.3	0.26
nicotinamide " "	19.8	125.0	1.1

3. Analogues of otherglycolytic intermediates

(i) Analogues of glycerol 3-phosphate

Phosphonate analogues of sn-glycerol 3-phosphate (XCI) have been extensively studied. Initial work with the nonisosteric analogue (XCII) was not promising, showing neither substrate nor inhibitory activity with rabbit muscle glycerol 3-phosphate dehydro-

genase (Baer et al., 1969). However Cheng et al. (1974) found that the isosteric analogue XCIII had similar kinetic parameters to that of glycerol 3-phosphate with rabbit muscle glycerol 3-phosphate dehydrogenase (Table 19). Comparable results were obtained by



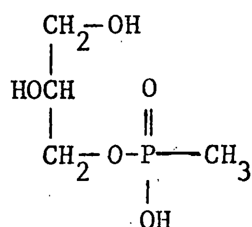
XCI XCII XCIII

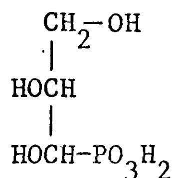
Adams et al. (1974) who also demonstrated that the mono anionic methylphosphonate analogue XCIV and the nonisosteric hydroxymethyl phosphonate analogue XCV were totally inert with glycerol 3-phosphate dehydrogenase. These results indicate that although the esterified

Table 19

compound	K_m (mM)	K_m NAD^+ (mM)	V_{max} (relative to glycerol 3-phosphate)
glycerol 3-phosphate	0.24	0.16	0.066
XCIII	0.19	0.20	0.059

C-3 oxygen of sn-glycerol 3-phosphate has no major direct interaction with the enzyme, the spatial relationship of the carbon-3 atom to the phosphorus atom must approximate to that in the sn-glycerol 3-phosphate





XCIV XCV

molecule for efficient binding to the enzyme and that substrates bind to the enzyme in their dianionic forms.

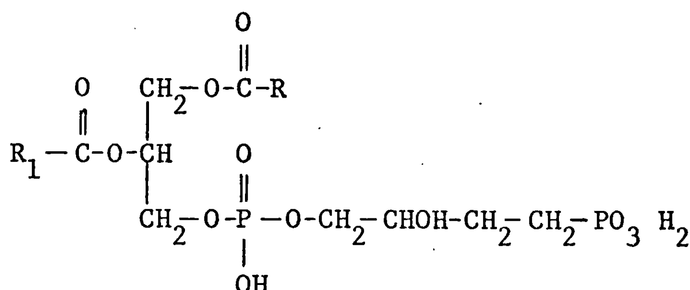
Cheng et al. (1975) studied the ability of the E. coli enzymes involved in glycerol 3-phosphate metabolism to recognise the phosphonate analogues XCII and XCIII . sn-Glycerol 3-phosphate and the isosteric analogue XCIII were found to inhibit competitively the reduction of dihydroxyacetone phosphate by soluble glycerol 3-phosphate dehydrogenase (K_i values 19μM and 42μM respectively) whereas the nonisosteric analogue XCII (as could be predicted from the results with rabbit muscle glycerol 3-phosphate dehydrogenase) showed no inhibitory activity. Neither compound XCII nor XCIII showed substrate activity with membrane-bound glycerol 3-phosphate dehydrogenase or with acyl glycerol phosphate acyltransferase. The isosteric analogue XCIII did not show any inhibitory activity with either of these enzymes. XCI was also without inhibitory effect upon the membrane-bound dehydrogenase but did appear slightly to inhibit the acyltransferase. Most importantly, XCIII was found to be a substrate (K_m = 450μM compared to sn-glycerol 3-phosphate K_m value = 140μM) for glycerophosphate phosphatidyl transferase. Analogue XCIII did not effect phosphatidylserine synthase.

Since Kabak et al. (1972) demonstrated that the phosphonate XCIII is capable of inhibiting the growth of certain strains of E. coli, the mechanism of this inhibition has been thoroughly studied. It was demonstrated that XCIII is a bacteriostatic agent and that it differs in its action from that of the natural phosphate (which also inhibits the particular strains of E. coli studied)(Shopsis et al., 1972). Incorporation studies showed that analogue XCIII not only

inhibits the uptake of labelled acetate into the lipid fraction of *E. coli* but also brings about a marked change in the distribution of the labelled acetate in the phospholipids (Shopsis *et al*., 1973). This altered composition of the phospholipids was quantified by Shopsis *et al*. (1974) who used low concentrations of XCIII (30μmolar) in order to strongly inhibit phospholipid synthesis, while only mildly affecting cell growth. There was found to be a marked decrease in the cellular content of phosphatidyl glycerol which was shown to result from the inhibition of its synthesis (the rate of catabolism of phosphatidyl glycerol was unchanged). Phosphatidylethanolamine and diphosphatidyl glycerol (cardiolipin) synthesis were both inhibited to a lesser extent. The fact that inhibition of phosphatidylethanolamine synthesis was less marked and occurred later than phosphatidyl glycerol synthesis suggested that this effect of compound XCIII is a secondary one. The observed lesser inhibition of synthesis of diphosphatidyl glycerol compared to that of phosphatidyl glycerol itself may result from the preferential conversion of phosphatidyl glycerol into diphosphatidyl glycerol.

Cheng *et al*. (1975) postulated that the observed inhibition of phosphatidyl glycerol synthesis in cells treated with XCIII is the result of a competition (observable *in vitro*) of XCIII and glycerol 3-phosphate for glycerophosphate phosphatidyl transferase. [3-^{3}H]-labelled XCIII has been incorporated into a polar lipid material both by mutant strains of *E. coli* and *in vitro* by glycerophosphate phosphatidyl transferase (Tyhach *et al*., 1976). The product was identified as the isosteric phosphonate analogue of phosphatidyl glycerol 3-phosphoric acid (XCVI) which would not be expected to

be enzymically converted to phosphatidyl glycerol. The appearance of such a phosphonate might have an effect upon membrane structure and



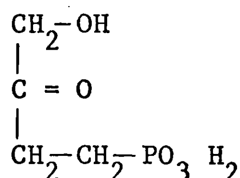
XCVI

function (as might also the decrease in cellular content of phosphatidylglycerol).

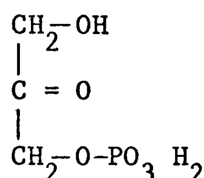
Analogue XCIII has been shown to be actively transported (reversibly) by the glycerol transport system of mutant strains of E. coli (Z. Leifer et al., 1977), with a K_m value (200μMolar) 12 times larger (and a V_{max} three times larger) than that of glycerol 3-phosphate. The addition of cold glycerol 3-phosphate to E. coli cells incubated in the presence of [3-^{3}H] labelled XCIII resulted in a decrease in the intracellular pool of labelled XCIII. Analogue XCIII also caused the release of intracellular glycerol 3-phosphate, and this exchangeability of XCIII and glycerol 3-phosphate could cause depletion of intracellular glycerol 3-phosphate in cells cultured in the presence of XCIII. The resultant glycerol 3-phosphate starvation might explain observed effects of XCIII *in vivo*, and the degradation of XCIII *in vivo* may be the result of abnormal enzyme-substrate interactions resulting from elevated concentrations of XCIII.

(ii) Analogues of Dihydroxyacetone Phosphate

The isosteric analogue XCVII of dihydroxyacetone phosphate XCVIII (like the isosteric analogue of glycerol 3-phosphate) has



XCVII



XCVIII

been shown to be a substrate for glycerol 3-phosphate dehydrogenase, with similar K_m values to those of the natural substrate. Cheng et al. (1974) studied the enzyme from rabbit muscle and found that XCVII although having a similar K_m value, was reduced 25 times more slowly than the natural substrate (Table 20). Surprisingly the pH optimum for the analogue XCVII was lower than that of the natural substrate

Table 20

enzyme source	compound	KmM	K_m(NADH)mM	V_{max} (relative to DHAP)
rabbit muscle	Dihydroxyacetone phosphate	0.13	2.9	0.61
	XCVII	0.18	2.2	0.024
E. coli	Dihydroxyacetone phosphate	0.4	—	1.0
	XCVII	0.4	—	0.1

which is not consistent with explanations of low analogue V_{max} values in terms of high pKa. Similar results were obtained by Stribling (1974) using E. coli as a source of glycerol 3-phosphate dehydrogenase (Table 20).

No significant differences in the ratio of V_{max} values at pH 7.2 and pH 8.2 were observed for either the natural substrate or its analogue. It is accordingly unlikely that the ionic form of the substrate is an important factor in binding to the enzyme. The decrease in V_{max} values for XCVII , like that of X for glucose 6-phosphate dehydrogenase and of LXXXI for aldolase can be attributed to very small changes in the geometry or to stereo electronic effects of the phosphate group, which has an essential role in catalysis.

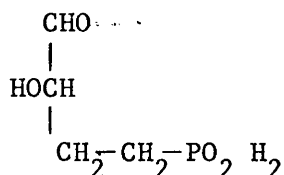
In contrast, triose phosphate isomerases from chicken muscle or from *Bacillus stearothermophilus* showed no interaction with analogue XCVII (Dixon & Sparkes, 1974). The second pKa value of XCVII was found to differ by 1.1 units from that of dihydroxyacetone phosphate (7.1 and 6.0 respectively). If differences in interaction of XCVII and the natural substrate with triose phosphate isomerase were solely the result of a preference for a particular ionic form no more than a 13 fold discrimination against XCVII could be achieved at any pH. The total lack of substrate activity of XCVII could (could as in other cases) be expained on the basis that the enzyme normally interacts with the C-O-P moiety. Alternatively Dixon & Sparkes suggested that the inactivity of XCVII may result from small geometrical differences (including the preference of the methylene group for the staggered conformation). This is a further example where substitution of a methylene group for oxygen prevents enzymic action even though the phosphate moiety is not directly involved in the catalysed reaction.

Analogue XCVII was shown to be incorporated into the cell wall of *Bacillus subtilus* when it acted as a bacteriocidal or bacterio-

static agent depending on the strain used (Klein *et al.*, 1977).

(iii) Glyceraldehyde 3-phosphate

Goldstein *et al.* (1974) carried out *in vitro* and *in vivo* biochemical investigations on the isosteric analogue XCIX

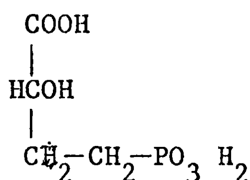


XCIX

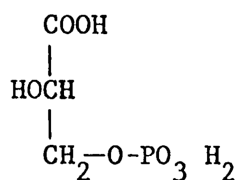
No kinetic parameters were determined, but racemic XCIX was reported to be a substrate for rabbit muscle glyceraldehyde phosphate dehydrogenase, and to totally inhibit growth of a mutant strain of *E. coli*. The mechanism of this inhibition is unknown but glyceraldehyde 3-phosphate has been shown to inhibit sn glycerol 3-phosphate transport, and if the L form of XCIX were to enter the cell through this transport system it could interfere with cell metabolism. Conversion of the L-form into the D form might inhibit the glycolytic pathway. Alternatively the L form itself could act as an inhibitor in other pathways.

(iv) Analogues of Glycerate 3-phosphate

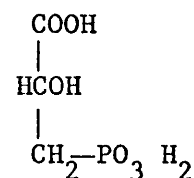
The isosteric analogue C was found to be a substrate for yeast phosphoglycerate kinase (Orr & Knowles, 1974). At pH 8.5 both the analogue C and glycerate 3-phosphate itself (CI) are essentially fully ionized and have similar K_m values. At pH 6.9, however, C was found to have a much larger K_m value compared to that of the natural substrate (Table 21). This suggests that one of the main contributions



C



CI



CII

to the specificity of substrate binding for Glycerate 3-phosphate kinase is the electrostatic interaction between the dianionic phosphoryl groups of the substrate and one or more positively-charged

Table 21

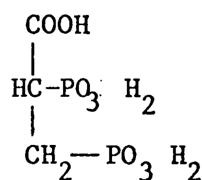
pH	pH 6.9		pH 8.5	
substrate	glycerate 3-phosphate	C	glycerate 3-phosphate	C
K_m (mM)	0.1	6.3	0.19	0.49

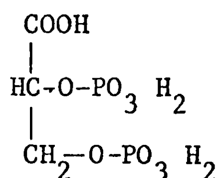
groups on the enzyme (in contrast to the situation found for other glycolytic enzymes mentioned so far).

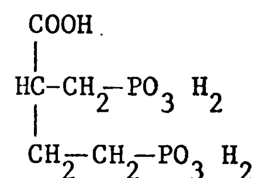
No enzymic or antibacterial activity was observed for either of the analogues C and CII against several strains of Gram positive and Gram negative bacteria (Pfeiffer et al., 1974).

(v) Analogues of Glycerate 2,3-bisphosphate

The nonisosteric analogue CIII was found to be incapable of replacing Glycerate 2,3-bisphosphate (CIV) as a regulator of the oxygen affinity of haemoglobin (Bensesch et al., 1973) and it was concluded that the flexibility of the binding site is insufficient to adjust to the smaller and less acidic phosphonate. Pfeiffer et al. (1974) further demonstrated that neither the isosteric (CV) nor the non-isosteric (CIII) analogue had any effect on the oxygen dissociation curve of human red cell suspensions.



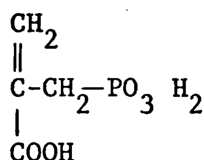


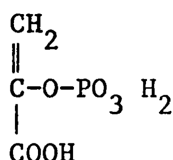


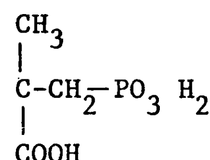
CIII CIV C V

(vi). Analogues of phosphoenolpyruvate

The isosteric phosphonate analogue CVI of phosphoenol-pyruvate CVII was found to be a substrate for rabbit muscle enolase and, as in the case of several other glycolytic enzymes, the K_m values for the phosphonate and natural phosphate were similar whereas the V_{max} value of the phosphonate was substantially lower (Table 22) (Stubbe & Kenyon, 1972). Nowak et al. (1973) obtained similar results with yeast enolase. Not surprisingly the saturated analogue CVIII showed no







CVI CVII CVIII

substrate activity with enolase.

Table 22

compound	K_m (μM)	V_{max}
Phosphoenolpyruvate	92	1.5
CVI	250	0.14

Although Stubbe & Kenyon (1972) detected no substrate activity of phosphonate CVI with rabbit muscle pyruvate kinase, Reed & Cohn (1973) showed by electron paramagnetic resonance (EPR) that compound

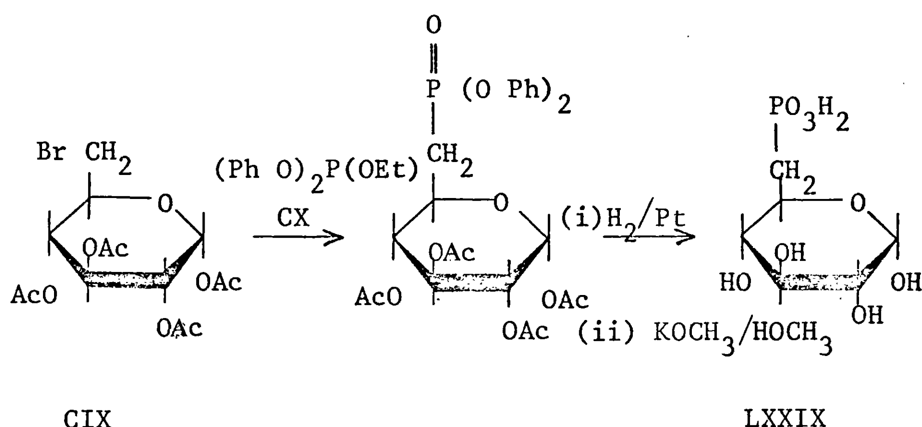
CVI forms a ternary substrate - Mn^{2+}- enzyme complex. The analogue-Mn^{2+}-enzyme ternary complex gave only an isotropic spectrum whereas the corresponding phosphoenolpyruvate -Mn^{2+}-enzyme ternary complex displayed a superposition of spectra for two distinct species, one anisotropic and one isotropic. The latter matched the spectrum obtained for the analogue CVI ternary complex. Analogue CVI was found to bind more weakly to the enzyme than did phosphoenolpyruvate. This allowed proton and paramagnetic relaxation studies which afforded quantitative structural information about the enzyme-analogue complexes. Further studies by James & Cohn (1974) demonstrated that analogue CVI competed with the natural substrate for the same binding site on the enzyme. They concluded that, although competition of an analogue and natural substrate for the same binding site, as demonstrated by kinetic or binding experiments, is a necessary condition for similarly-bonded enzyme complexes, it is by no means a sufficient condition. At least two other criteria must be met; the proton relaxation rates of water and EPR spectra should be similar for the natural substrate and the analogue before distances in the analogue-enzyme complex may be considered applicable to the natural substrate-enzyme complex.

Synthesis of 6,7 dideoxy α-D-*gluco*-heptose 7-phosphonic acid, the isosteric phosphonate analogue of glucose-6-phosphate

Synthesis of phosphonate analogues of carbohydrate phosphates

Several approaches are now available for the introduction of a C-P bond into carbohydrates.

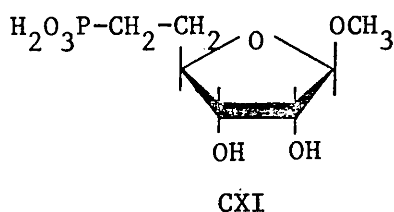
Griffin & Burger (1956) first reported the synthesis of a phosphonate analogue of a naturally-occurring carbohydrate phosphate, the nonisosteric analogue LXXIX of glucose 6-phosphate. The phosphorus-containing moiety was introduced into the protected 6-bromo-6-deoxy-D-glucose CIX by means of an Arbuzov reaction with diphenyl ethyl-phosphite CX. Hydrogenolysis of the phenyl groups and hydrolysis of the acetate ester groupings gave analogue LXXIX. The Arbuzov reaction



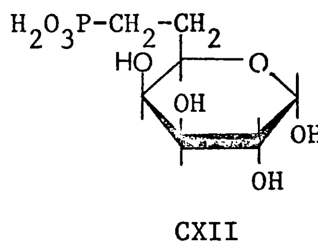
of triethyl phosphite with compound CIX yielded the diethyl phosphonate ester of analogue LXXIX but all attempts to remove the ethyl esters by hydrolysis resulted in decomposition.

Any successful synthetic introduction of a phosphonate grouping necessarily involves the protection of the P-OH moiety by blocking groups which can be subsequently removed under mild conditions. Phenyl esters can sometimes, as above, be removed by hydrogenolysis but the reaction is unpredictable and often does not proceed. Benzyl esters are in general, readily removed by hydrogenolysis but the corresponding phosphites are difficult to prepare and use. Alkyl esters are stable and

easy to prepare but are not easily converted into the free acid. The conversion of the diethyl ester of analogue LXXIX to the free acid was claimed by Moffatt & Jones (1971) in a patent which reported heating the phosphonate diester to 150°C with sodium iodide in N.N-dimethyl formamide (DMF) followed by treatment with aqueous acetic acid. The successful use of this technique was claimed for the synthesis of a series of isosteric and nonisosteric analogues of carbohydrates and other phosphates, although experimental details were presented for only a few systems (e.g. CXI and CXII the isosteric analogues of methyl riboside 5-phosphate and galactose 6-phosphate respectively).



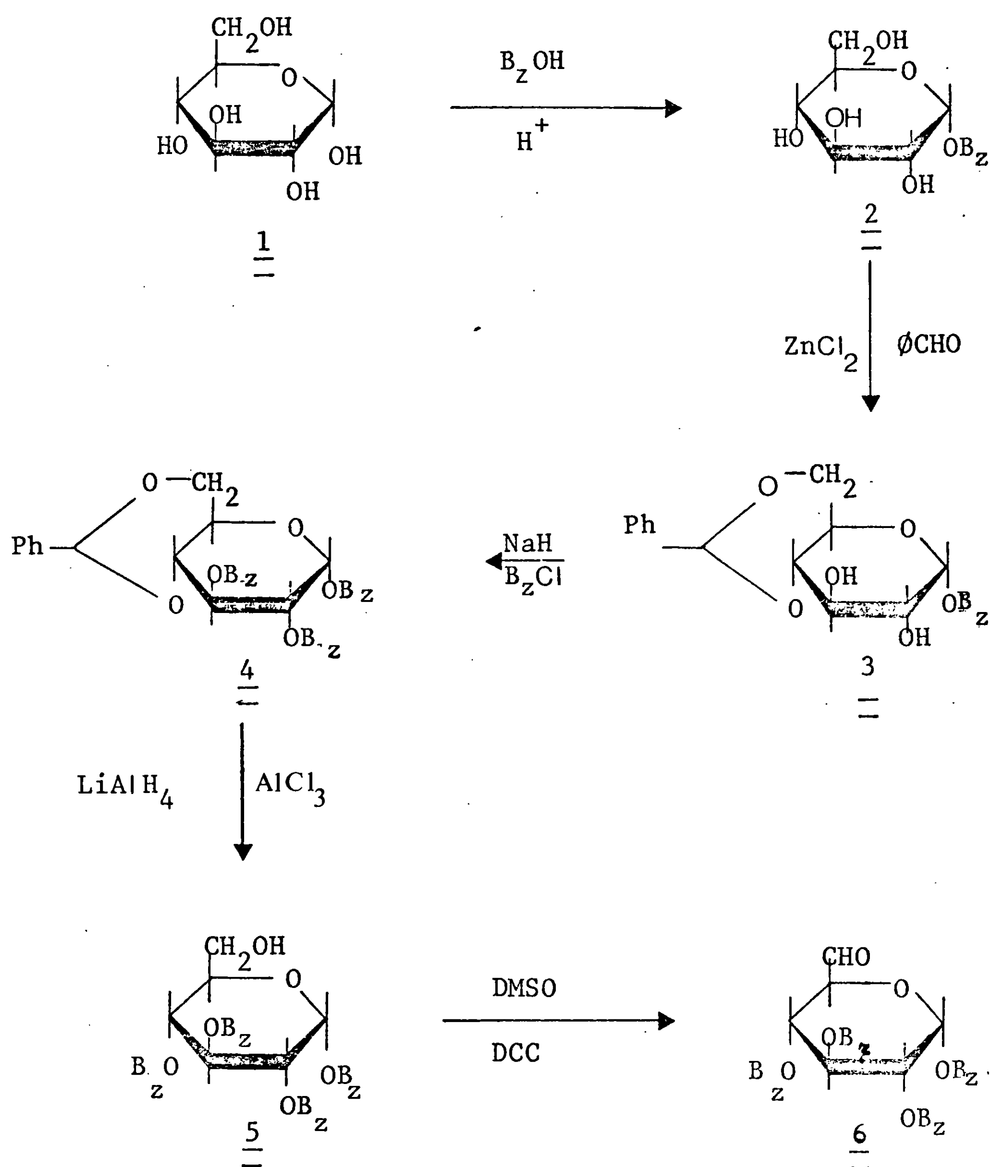
CXI



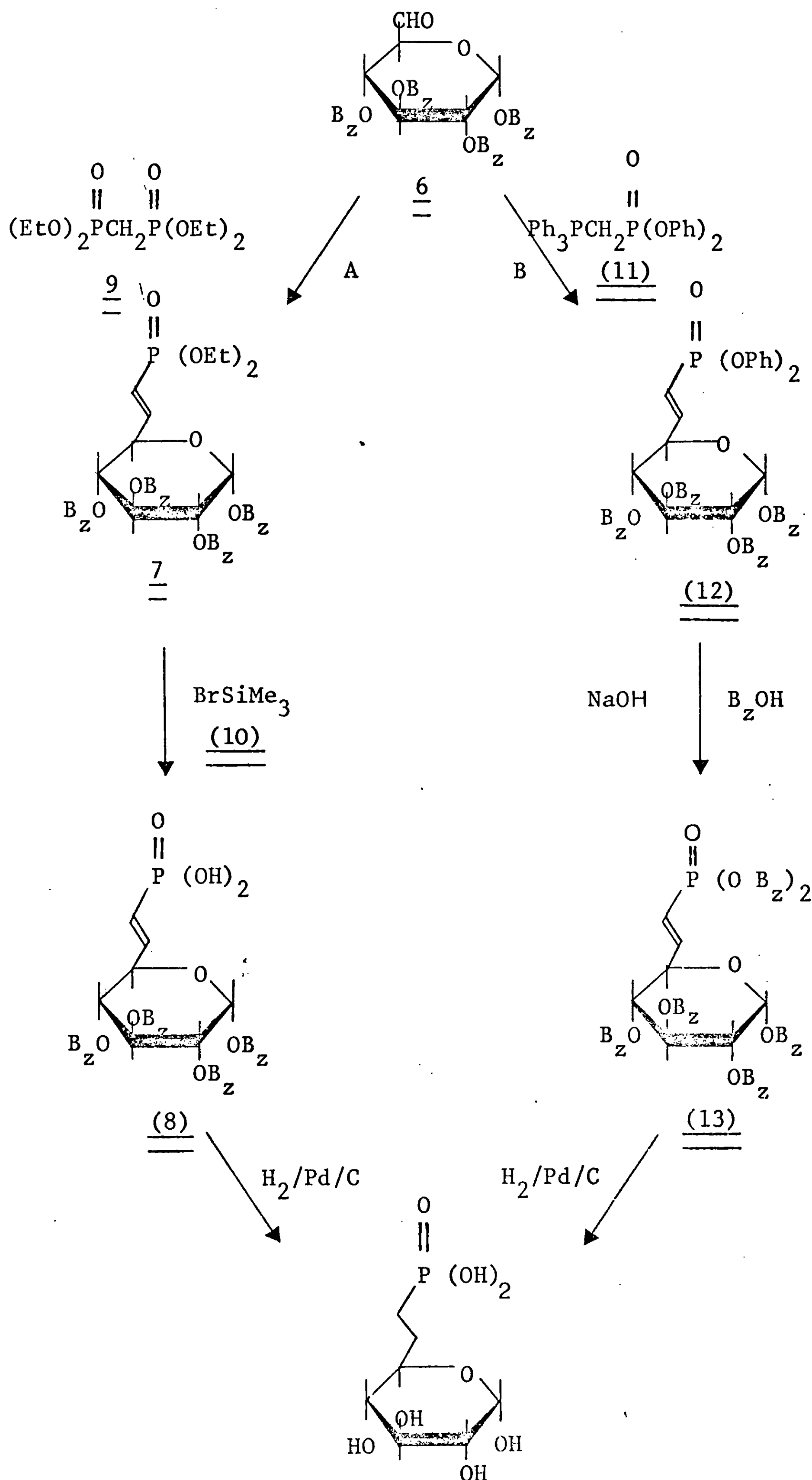
CXII

An alternative, more convenient route for phosphorus introduction involving the reaction of a stabilized Wittig reagent with an aldehydo group was claimed in another patent by Jones & Moffatt(1971). The authors reported the use of this approach in the preparation of isosteric phosphonate analogues of D-xylose 5-phosphate,D-lyxose 5-phosphate, D-arabinose 5-phosphate, 2 deoxy D-ribose 5-phosphate, D-glucose 6-phosphate and D-mannose 6-phosphate. Again however, complete experimental details for each analogue were not given in the patent. More recently Adams et al. (1976) have described the synthesis of the isosteric analogue of glucose 6-phosphate (X) using the stabilised Wittig reagent of Jones & Moffatt(1971). This is described in the Experimental and Results section (Scheme 1B, pages 79-80). Tang et al. (1978) have prepared the isosteric analogue (LXXXIV) of fructose 1-phosphate

Scheme 1 (A + B)

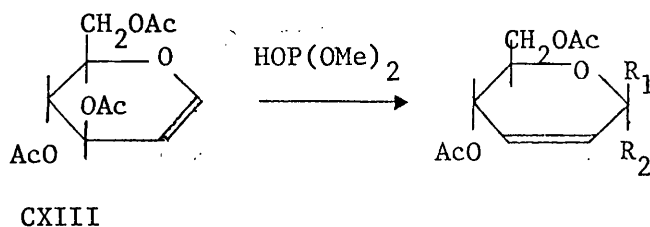


Scheme 1 A+B (cont)



using a similar procedure (Scheme 2) which involves Pfitzner-Moffatt (Pfitzner & Moffatt 1965) oxidation of an unprotected alcohol grouping prior to reaction of the resulting aldehyde with the Wittig reagent. Unger et al. (1978) in preparing the isosteric analogue (LXXXVIII)of aribinose 5-phosphate, again introduced the C-P bond by way of the Wittig reagent (diphenyl triphenylphosphoroanylidene-methylphosphonate) but generated the aldehydo precursor by use of pyridinium chlorochromate (Fetizon & Golfier, 1968) in preference to the Pfitzner Moffatt oxidation, which in their hands gave inferior yields.

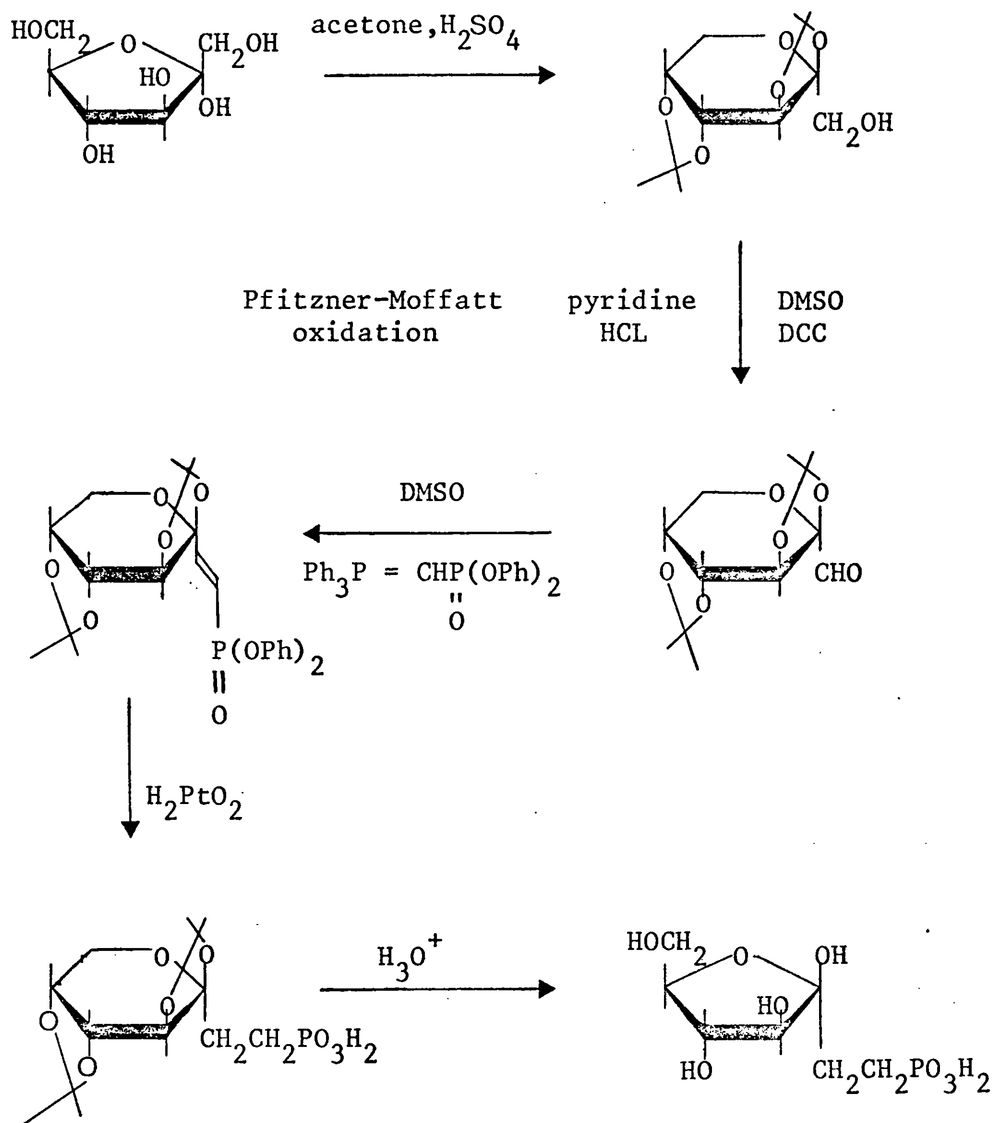
Phosphonylation has been effected by the use of phosphite addition across activated olefinic linkages under both acidic (Paulsen & Thiem, 1973; Thiem et al., 1975) and basic (Paulsen et al., 1971; Paulsen & Greve, 1973) conditions in the preparation of various deoxy-, dehydro-, nitro- and amino deoxy phosphonocarbohydrate derivatives. The reaction of 3,4,6-tri-O-acetyl-1,2-dideoxy hex-1-eno-pyranoses (glycals)(e.g. the arabino compound CXIII with dimethyl phosphite in the presence of BF_3 gave dimethyl (4,6,-di-O-acetyl-2,3-dideoxy hex-2 enopyranosyl) phosphonates (CXIV + CXV) by way of an allylic arrangement. Transesterification of the dimethyl esters to bis trimethyl silyl phosphonates led to the free phosphonic acids under very mild conditions



CXIV (R_1=H, R_2 = $PO(OMe)_2$) 50%

CXV (R_1=$PO(OMe)_2$ R_2 = H) 50%

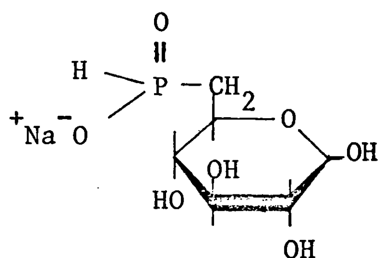
Scheme 2



LXXXIV

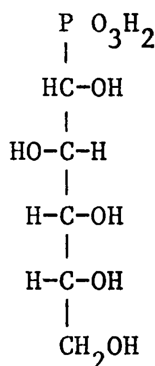
(Paulsen & Thiem, 1973). Free phosphonic acids were not reported for the nitro and amino deoxyphosphonocarbohydrate derivatives.

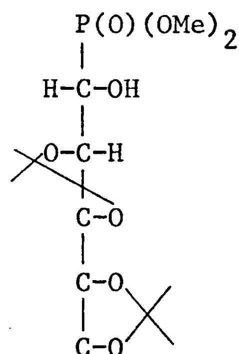
The preparation of the nonisosteric phosphinate analogue (CXVI) of glucose 6-phosphate has been reported (Kochemkov et al., 1967) to result from heating of hypophosphorous acid with levoglucosan and sodium hypophosphite.



CXVI

Paulsen & Kuhne (1974) have synthesised the phosphonate CXVII in high yield by reaction of 2,3:4,5-di-O-isopropylidene-D-arabinose with $HPO(OMe)_2$ to give compound CXVIII followed by deblocking.





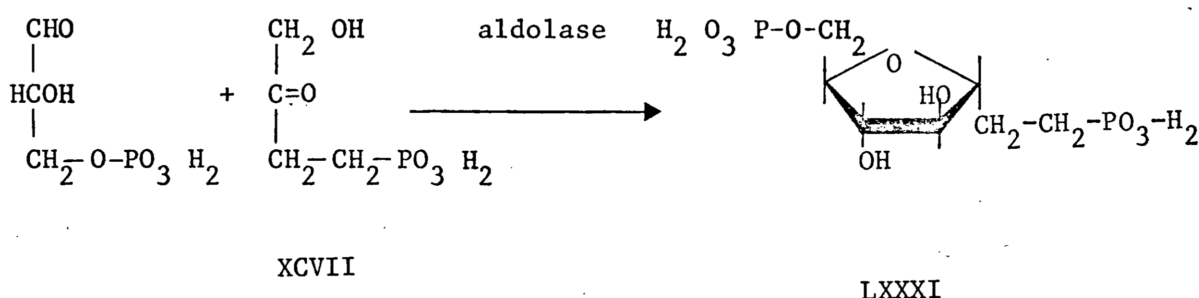
CXVII CXVIII

Enzymic synthesis

Stribling (1974) prepared the 1-phosphonomethyl isostere (LXXXI) of fructose 1,6-bisphosphate (LXXXII) by way of the aldolase-catalysed condensation of 4-hydroxy 3 oxobutyl-1-phosphonic acid

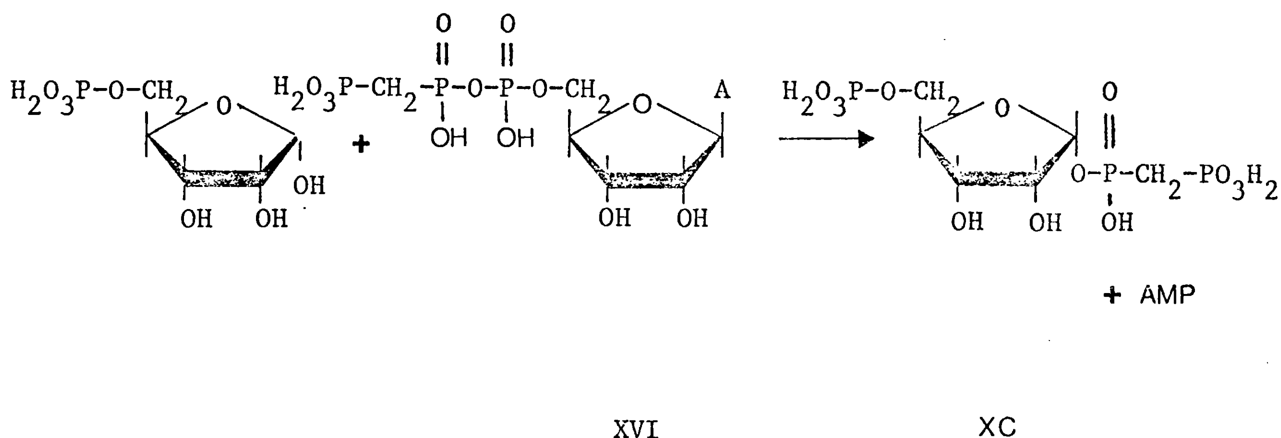
(XCVII) (the isosteric analogue of dihydroxyacetone phosphate), with glyceraldehyde 3-phosphate (Scheme 3).

Scheme 3

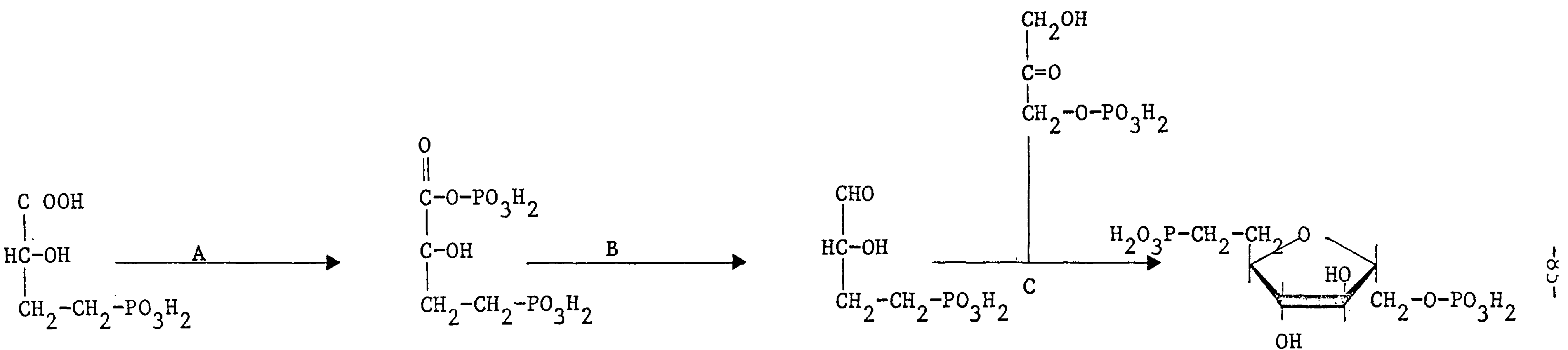


More recently Webster *et al.* (1976) prepared the 6-phosphonomethyl isostere (LXXXIII) of fructose 1,6-bisphosphate from the corresponding isosteric analogue (C) of glycerate 3-phosphate (Scheme 4).

Murray *et al.* (1969) prepared 5-phosphoribosyl 1-methylene diphosphonate (XC) the isosteric analogue of 5-phosphoribosyl pyrophosphate via the reaction of ribose 5-phosphate with the commercially-available isosteric phosphonic analogue of ATP (XVI) catalysed by 5-phosphoribosyl pyrophosphate synthetase from Ehrlich ascites-tumour cells.



Scheme 4



C XCIX LXXXIII

A glycerate 3-phosphate kinase (yeast)

B Glyceraldehyde 3-phosphate DH (rabbit muscle)

C Aldolase (rabbit muscle)

EXPERIMENTAL AND RESULTS

General - Melting points are uncorrected. Thin layer chromatography (t.l.c.) was performed on microscopic slides coated with silica gel G (Merck) and the chromatoplates were developed with 50% sulphuric acid or iodine vapour. Column chromatography was performed with silica gel (Merck) of particle size 0.063 → 0.2mM, mesh 70 → 230 ASTM. N.m.r. spectra were measured with a Jeol-JNM4H-100 n.m.r. spectrometer at 100mH with deuteriochloroform as solvent and tetramethylsilane as the internal standard. D line optical rotations was determined with a Bellinger and Stanley polarimeter, with a 1.0dm tube. IR spectra were measured with a Perkin-Elmer infrared spectrometer.
Solutions were concentrated under diminished pressure (rotary evaporator or vacuum pump) at bath temperatures below 40°C. Light petroleum refers to the fraction b.p. 60°-80°.

Benzyl 4,6-O-benzylidene-α-D-glycopyranoside [3]

This compound was prepared according to the procedure of Inch & Lewis (1972). A solution of D-glucose [1] (800g) and toluene-p-sulphonic acid (40g) in benzyl alcohol (1500ml) was heated at 80°-100°C for 5 hr, cooled to 0°C and poured into an excess of ether (2ℓ). The ether solution was stirred for 15 min and stored overnight at room temperature. The supernatant was then decanted from a syrupy residue which contained inter alia benzyl-α-D-glucopyranoside [2], detected by t.l.c. [Rf = 0.2, (benzene-methanol 12:1)]. Zinc chloride (500g) was added slowly to a solution of the syrupy residue in benzaldehyde (1500ml) which was stirred vigorously at room temperature for 3hr before being poured into a mixture of water (3ℓ) and light petroleum (4ℓ) and allowed to stand overnight at 5°C. The solid product was

collected by filtration, (further crops were obtained by resuspension of the oily filtrate in water (3ℓ) and light petroleum (4ℓ) and storage overnight at 5°C prior to refiltration) dissolved in the minimal volume of hot ethanol (containing ammonia) and poured into an excess of water. The precipitate was removed by filtration and recrystallised from ethanol to afford benzyl 4,6-O-benzylidene-α-D-glucopyranoside [3] (50g, yield 7%) the I.R. and n.m.r. spectra of which were identical to that previously obtained by Inch & Lewis (1972) [Rf 0.65, (chloroform-methanol 12:1)].

Benzyl 2,3-di-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside [4]

Benzyl 4,6-O-benzylidene-α-D-glucopyranoside [3] (50g) was added to a suspension of NaH (10g) in dry NN - dimethylformamide (500ml) at 10°C for 30 min. Benzyl chloride (50ml) was added dropwise to the stirred mixture, which was left to stand overnight at room temperature. Excess NaH was destroyed by addition of methanol (75ml) and the mixture was poured into water (3ℓ). The solid precipitate was removed by filtration and recrystallized from methanol to give benzyl 2,3-di-O-benzyl-4,6-O-benzylidene α-D-glucopyranoside [4] (4.5g, yield 60%) with identical I.R. and n.m.r. spectra to that previously obtained by Inch & Lewis (1972).

Benzyl 2,3,4-tri-O-benzyl-α-D-glucopyranoside [5]

$LiAlH_4$ (10g) was added portionwise to a solution of the benzylidene acetal, [4] (40g) in dry ether (400ml) and dichloromethane (400ml). The mixture was heated under reflux in an atmosphere of N_2, and a solution of $AlCl_3$ (40g) in diethyl ether (400ml) was added dropwise. The final mixture was boiled under reflux for 3½hr. Excess of $LiAlH_4$ in the cooled reaction mixture was decomposed by the sequential

addition of ethyl acetate (10ml) and water (50ml) and the resultant precipitate ($Al(OH)_3$) was removed by filtration. The filtrate was concentrated leaving a residue which was recrystallised from petroleum ether to give benzyl 2,3,4-tri-O-benzyl-α-D-glucopyranoside [5] (29g, yield 75.0%) with identical I.R. and n.m.r. spectra to that previously obtained by Adams et al. (1976). [Rf 0.5 (light petroleum-ether 1:3)].

Benzyl 2,3,4-tri-O-benzyl-α-D-gluco-hexodialdo-1,5-pyranoside [6]

Benzyl glycoside 5 (15g) in dimethylsulphoxide (150ml) was added slowly to a solution of dicyclohexylcarbodi-imide (30g) in benzene (150ml). Phosphoric acid (1.6ml) was added dropwise to the reaction mixture which was left to stand at room temperature. A white precipitate of dicyclohexylurea quickly formed and production of the aldehyde 6 was monitored by t.l.c. [Rf 0.4 (light petroleum-ether, 1:3) cf 5 Rf. 0.5]. After 15hr the reaction mixture was filtered (to remove the bulk of the dicyclohexylurea) and the filtrate was poured into a solution of oxalic acid (30g) in methanol (150ml). Sufficient diethyl ether was added to effect complete precipitation of salts which were removed by filtration and the filtrate was concentrated, poured into an aqueous solution of Na_2CO_3 and extracted with ether. The combined ether extracts were washed once in water, dried ($MgSO_4$) and concentrated to give crude benzyl 2,3,4-tri-O-benzyl-α-D-gluco-hexodialdo-1,5-pyranoside, 6 (16g)(40% yield by n.m.r.) n.m.r. (chloroform-d):δ, 9.6 (-CHO); 7.25 (Ø).

Benzyl 2,3,4, tri-O-benzyl-6,7 dideoxy-α-D-gluco-hept-6-eno pyranoside, 7-phosphonic acid diethyl ester [7]

Crude aldehyde 6 (16g) was used immediately without further purification, n-Butyl lithium (15ml) was added dropwise to a solution of tetraethyl methylene diphosphonate 9 (9g) in tetrahydrofuran (150ml) at -10°C. After 20 min the crude aldehyde 6 (17g) was added to the reaction mixture which was gently shaken and allowed to stand overnight under an atmosphere of N_2 at room temperature. Production of the diethyl phosphonate 7 [Rf 0.3 (light petroleum-ether, 1:3)] was monitored by t.l.c. and aliquots of n-butyl lithium were added until the reaction was complete. The completed reaction mixture was poured into water and extracted with ether. The ether extracts were combined, dried ($MgSO_4$) and concentrated to give benzyl 2,3,4-tri-O-benzyl-6,7, dideoxy-α-D-gluco-hept-6-eno pyranoside, 7-phosphonic acid diethyl ester [7]. The crude product was applied to a column of silica gel and eluted with light petroleum:ether (1:3). Fractions containing the product 7 (monitored by t.l.c.) were collected and concentrated to give benzyl 2,3,4-tri-O-benzyl-6,7-dideoxy-α-D-gluco hept-6-eno pyranoside, 7 phosphonic acid diethyl ester [7] (7.0g)(yield 96%). N.m.r. (chloroform-d): δ, 7.3 (Ø); 6-7 (CH = CH); 4.1, 1.3 (OEt). I.R.: 8.0μ (P=O); 9.7 + 10.3μ (P-OEt).

Benzyl 2,3,4 tri-O-benzyl 6,7 dideoxy α-D-gluco-hept-6-eno-pyranoside 7 phosphonic acid [8]

Partially-purified diethyl ester 7 (70g) was mixed with bromotrimethylsilane 10 (2.3g, 1.5 mol.equiv.) and allowed to stand under an atmosphere of N_2 overnight at room temperature. Disappearance of starting material 7 [Rf 0.7 (ether)] was monitored by t.l.c. and after

15 hr aliquots of bromotrimethylsilane were added until starting material was completely absent. The reaction mixture was poured into a large excess of water (300ml) and acetone was added to obtain a homogeneous solution which was extracted with chloroform. The combined chloroform extracts were dried ($MgSO_4$) and concentrated to give a brown residue which was crystallized from benzene: light petroleum to give benzyl 2,3,4 tri-O benzyl 6,7 dideoxy α D-gluco-hept-6-eno-pyranoside 7-phosphonic acid [8] (3.55g yield 59%) mp 165-168° $[\alpha]^{20}_{D}$ + 83.5°. N.m.r. (chloroform-d); δ, 7.2 (Ø); 6-7 (HC=CH). IR: 3-5μ (P-OH).

6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid [X]

A solution of the unsaturated benzyl ether 8 (3.4g) in ethanol (150ml) containing a few drops of trifluoroacetic acid was hydrogenolysed over 10% palladium charcoal at room temperature and atmospheric pressure until hydrogen uptake ceased (uptake 700ml, theoretical value 653ml). The solution was filtered through Celite and concentrated to give 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid (X)(1.3g yield 90%) as a hygroscopic white solid. Both n.m.r. and I.R. spectra showed the complete absence of benzyl groups, and were identical to the spectra obtained by Adams et al. (1976)

The product X was dehydrogenated in the presence of $NADP^+$ and yeast glucose 6-phosphate dehydrogenase showing a K_m value (232mM) identical to that obtained by Adams et al. (1976).

Tetraethyl methylene diphosphonate [9]

Tetraethyl methylene diphosphonate 9 was prepared according to the procedure of Ford-More & Williams (1947) as modified by Kosolapoff (1953). Methylene iodide (20g) was added to triethyl phosphite (40g) and the temperature of the mixture was rapidly brought to 150°C by immersion in a preheated oil bath. Ethyl iodide (b.p. 60-70°C) was allowed to distil from the reaction mixture which was then fractionally distilled under reduced pressure to give the following fractions: 60-70°C [diethyl methane phosphonate (85%) and diethyl iodomethane phosphonate (15%)]; 70-120°C [diethyl iodomethane phosphonate (80%) and tetraethyl methylene diphosphonate (10) 15%]; 120-135°C [tetraethyl methylene diphosphonate (10)]. N.m.r. (chloroform-d): δ, 2.4 ($P-CH_2-P$); 4.2, 1.1 (OEt). IR: 7.95μ (P = O); 9.7, 10.3 (POEt).

Bromotrimethylsilane [10]

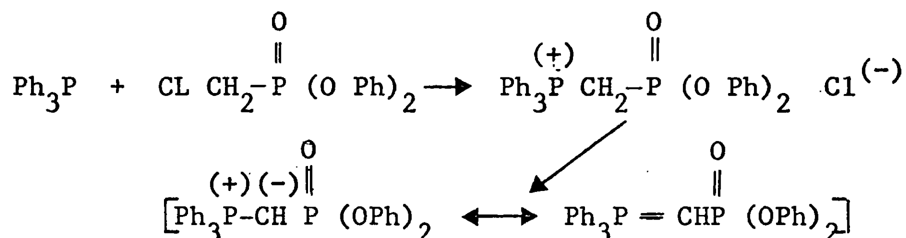
Bromotrimethylsilane was produced according to the procedure of Gilliam *et al*. (1946). Hexamethyldisiloxane (10g), phosphorus tribromide (2.8g) and ferric chloride hexahydrate (60mg) were mixed thoroughly, sealed into a stoppered flask, and allowed to stand at room temperature for 48 hr. The reaction mixture was distilled under reduced pressure and bromotrimethylsilane 10 was collected at 78°C.

n Butyl lithium

n-Butyl lithium was prepared from n-butyl bromide and lithium wire, and the concentration of the resulting solution was determined by hydrolysis and titration against HCl as described in Organic Reactions Vol. VI (ed. R. Adams *et al*., 352-353) after the procedure of Gilman *et al*. (1949).

DISCUSSION

A synthesis of 6,7-dideoxy-α-D-gluco-heptose 7-phosphonate (X) has been reported by Adams et al. (1976) who followed Scheme 1b. This Scheme has two major disadvantages, the first of which concerns the use of diphenyl triphenylphosphororanylidene-methylphosphonate (11). This reagent is prepared by reaction of triphenylphosphine with diphenyl chloromethylphosphonate to give a quaternary salt which can be converted by base to the ylid. Thus:-

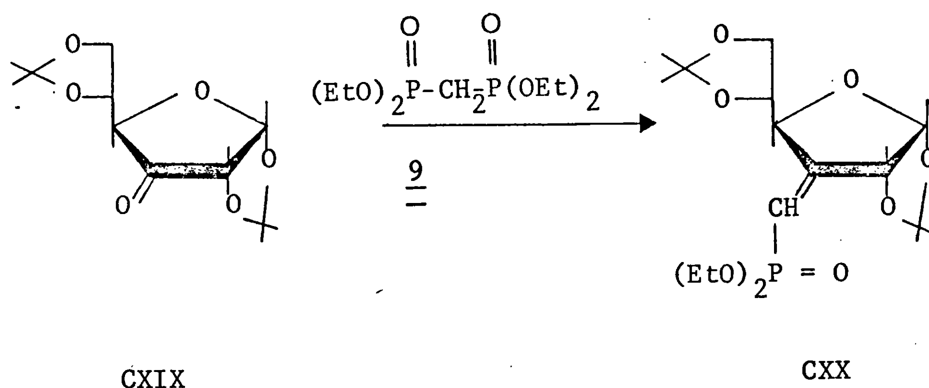


The reaction involving unreactive triphenylphosphine, presents a number of difficulties and the synthesis of diphenyl chloromethylphosphonate itself is not straightforward. The second major problem with Scheme 1b concerns the removal of the phenyl groups from the ester 12 to give the phosphonic acid X. Phenyl ester groupings can occasionally (e.g. Adams et al., 1974) be removed directly by hydrogenolysis but this reaction is unpredictable and Adams et al. (1976) were obliged to trans-esterify the phenyl ester (12) in 48% yield to the dibenzyl ester (13) which was then hydrogenolysed under mild conditions.

An alternative route to the glucose 6-phosphate analogue (X) was accordingly investigated in the presently-described work. In view of the problems involved in the use of phenyl ester blocking groups it would be preferable to use the more easily-prepared and stable alkyl esters. These are, however, traditionally difficult to remove but in 1977 McKenna et al. reported a facile dealkylation procedure using bromo-

trimethylsilane and it was decided to make use of this reaction in the synthesis of phosphonate X.

The first five steps of the synthetic sequence were followed essentially as described by Adams et al. (1976) with only minor modifications. Crystalline benzyl 4,6-O-benzylidene-α-D-glucopyranoside (3) was obtained in low yield from D-glucose by direct benzylidination of a crude benzyl-α-D-glucopyranoside preparation as described by Inch & Lewis (1972). Further benzylation of the acetal 3 under standard conditions afforded benzyl 2,3-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside (4), the acetal ring of which was cleaved by using $LiAlH_4/AlCl_3$ (Liptak et al., 1975) to give a high yield (75%) of crystalline benzyl 2,3,4-tri-O-benzyl-α-D-glucopyranoside (5). The primary alcohol 5 was now oxidised to the free aldehyde 6 by the procedure of Pfitzner & Moffat (1965). Adams et al. (1976) did not attempt to isolate the aldehyde 6 but allowed it to react immediately with the ylid 11 as described above in Scheme 1b. Attempts were made in the present work to purify the intermediate 6 both by column chromatography on silica gel and by extraction in aqueous metabisulphite solution. Neither method was successful and so the free aldehyde was allowed to react directly with the stable Wittig reagent tetraethyl methylene diphosphonate (9). This reagent had been used by Albrecht et al. (1970) in the conversion of 1,2:5,6-di-O-isopropylidene-α-D-ribohexofuranose-3-ulose CXIX to the vinyl phosphate ester (CXX), and in the present work was found to be effective in converting aldehyde 6 into the diethyl phosphonate 7 which was purified on a column of silica gel. As was hoped, compound 7 could be readily dealkylated by the method of McKenna et al. (1977) giving the free



acid 8 which was readily debenzylated over palladium/charcoal at atmospheric pressure and room temperature to give the required product X.

The overall yield of the final product X from benzyl 2,3,4-tri-O-benzyl-α-D-glucopyranoside 5 quoted by Adams et al. (1976) was 14%. The alternative synthesis of analogue X described here was done twice with overall yields from intermediate 5 of 23% and 17%.

The alternative synthesis of phosphonate analogue X (Scheme 1a) offers a number of advantages over that described by Adams et al. (1976): the Wittig reagent 9 and the diethyl phosphonate 7 are more easily prepared and stable than the corresponding intermediates in Scheme 1b while preparation of the free acid from 7 proceeds reliably under mild conditions; the overall yield is better and could undoubtedly be improved, minor modifications of reaction conditions, work-up procedures, etc.; finally the overall synthesis of compound X from 5 is relatively rapid taking, in the second run, only 7 days.

The two batches of phosphonate Analogue X prepared by the above method were homogeneous, with the same I.R. and n.m.r. spectra as those of the material prepared previously by Adams _et al_. (1976) and showed kinetic parameters identical with those of the latter product when examined as a substrate for yeast glucose 6-phosphate dehydrogenase.

6,7 Dideoxy-α-*D*-*gluco*-heptose 7-phosphonic acid, product inhibition of bovine brain hexokinase

INTRODUCTION

The synthesis of 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid (X) has been described earlier. In this compound the C-O-P moiety of glucose 6-phosphate is replaced by a $C-CH_2-P$ grouping and the analogue might be expected to mimic glucose 6-phosphate in acting as a product inhibitor of brain hexokinase. This should clarify the nature of the contribution of the C-6 oxygen atom of glucose 6-phosphate to binding of the phosphate to hexokinase. This section of the thesis describes the purification of bovine brain hexokinase I (after the procedure of Redkar & Kenkare, 1972) and a comparative study of the activities of glucose 6-phosphate and 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid as product inhibitors of the purified enzyme.

Early studies by Weil-Malherbe & Bone (1951) demonstrated that a feature of the regulation of mammalian hexokinases was the potent inhibition of the reaction by the product glucose 6-phosphate. Crane & Sols (1954) reported the sugar phosphate product to be a noncompetitive inhibitor of both substrates (glucose and $MgATP^{2-}$) and postulated that the observed inhibition was the result of action at a regulatory site discrete from the catalytic site. This view was one of the earliest expressions of the concept of allosteric control. However, the question of whether glucose 6-phosphate is a classical allosteric inhibitor or a normal product inhibitor is still in debate (Colowick, 1973; Casazza & Fromm, 1976; Wilson, 1978, 1979).

The original basis for the proposal of a separate regulatory site (Crane & Sols, 1954) was based on the observation that the configurational and hydroxyl-group requirements of the hexose ring were

different for substrate activity on the one hand or for product inhibitory activity on the other (Figure 1)(Crane & Sols 1954)

Figure 1 ● represents in each case the configuration or hydroxyl-group requirement for the specified activity.

suggesting discrete sites of different specificity for the substrate (glucose) and the inhibitor (glucose 6-phosphate). Further evidence (Rose & Warms, 1967) showed that glucose 6-phosphate has the special effect of solubilizing the enzyme and (Redkar & Kenkare, 1972) that glucose 6-phosphate causes marked conformational changes as measured by sedimentation and protection of the enzyme against Ellman's reagent, again indicating that the inhibition by glucose 6-phosphate is effected at a discrete regulatory site. The fact that analogous inhibition (by glucose 6-phosphate) is not seen with yeast hexokinase also suggests evolution of a special regulatory site.

As pointed out by Colowick (1973) and Wilson (1978) while this argument for a specific allosteric regulatory site is "compelling" it is by no means definitive. Fromm and his colleagues have, on the basis of binding and kinetic studies, consistently maintained that there is no discrete regulatory site for glucose 6-phosphate (Purich et al., 1973; Ellison et al., 1975a,b; Casazza & Fromm, 1976) and that the inhibition is the result of binding at, or overlapping the active site. From binding studies, Ellison et al. (1975a)

showed that there is only one glucose 6-phosphate binding site per molecule of enzyme and that the kinetics of inhibition are not sigmoidal. Furthermore glucose has been shown to encourage the dissociation of glucose 6-phosphate. The fact that an enzyme-glucose-glucose 6-phosphate ternary complex forms indicates that the hexose rings of glucose and of glucose 6-phosphate must bind to separate sites and this would explain the results of differing specificity for glucose and glucose 6-phosphate binding sites (Crane & Sols, 1954). These results and the fact that a number of workers have shown that glucose 6-phosphate is a linear competitive inhibitor of ATP and a linear noncompetitive inhibitor of glucose (Fromm & Zewe, 1962; Grossbard & Schimke, 1966; Kosow _et al._, 1973; Gerber _et al._, 1974; Casazza & Fromm, 1976) for mammalian hexokinase led Casazza & Fromm (1976) to propose the following model for the mechanism of action of glucose 6-phosphate inhibition of mammalian hexokinase. After phosphorylation of glucose to glucose 6-phosphate the C-2 hydroxyl group becomes bound to the enzyme, at a site distinct from the glucose binding site, and in so doing permits the binding of a molecule of glucose. The phosphate moiety of glucose 6-phosphate remains anchored to the γ-phosphoryl portion of the ATP pocket during this sequence of events and serves to explain why glucose 6-phosphate is a linear competitive product inhibitor of ATP. This proposed mechanism would seem to exclude a distinct allosteric site for glucose 6-phosphate. Wilson (1978) in proposing an overall mechanism of action for mammalian hexokinase adopted the same view.

Regardless of the question of the site of action of glucose 6-phosphate, its importance as a regulator of the action of the mammalian

hexokinases is not in doubt and the finer details of the mechanism of inhibition were not considered in the following comparative studies.

MATERIALS AND METHODS

Chemicals and enzymes

Glucose 6-phosphate dehydrogenase (yeast, grade II) (EC. 1.1.1.49), glucose oxidase (fungal)(EC 1.1.3.4), peroxidase (horse-radish)(EC 1.11.1.7), pyruvate kinase/lactate dehydrogenase (rabbit muscle)(EC 2.7.1.40/EC 1.1.1.27), 3' phosphodiesterase (calf spleen)(EC 3.1.4.18), 5' phosphodiesterase (snake venom)(EC 3.1.4.1.), glucose 6-phosphate, phosphoenolpyruvate, $NADP^+$, NADH and ATP were purchased from Boehringer Corp. (London) Ltd., London, W.5., U.K. *n*-Octonal was purchased from Koch-Light Laboratories Ltd., Colnbrook, Buck., U.K. Bovine serum albumin (used as a standard for measurement of protein concentration) *O*-dianisidine dihydrochloride and 2'-deoxythymidine-3-p-nitrophenol phosphate were obtained from Sigma Chemical Co. Ltd., London, U.K. DE52 DEAE cellulose was purchased from Whatman Ltd., Kent, U.K. All other chemicals were from BDH Chemicals, Poole, Dorset, U.K.

Beef brains from freshly-killed animals were obtained from Bristol slaughterhouse and immediately chilled on ice.

All spectrophotometric determinations were done using a Unicam SP 1800 spectrophotometer at 25°C and all centrifugations were carried out using an MSE 18 centrifuge. $MgCl_2 \cdot 6H_2O$ solution was titrated against 0.1M EDTA and the titrated value of Mg^{2+} was found to be 100% pure by weight (Vogel p434).

Buffer A: 10mM-potassium phosphate, pH 7.0, containing 5mM-EDTA, 5mM-mercaptoethanol and 10mM-glucose.

Measurement of hexokinase activity during the purification procedure

Hexokinase activity was measured by coupling the formation of glucose 6-phosphate to the reduction of $NADP^+$ in the presence of glucose 6-phosphate dehydrogenase and by measuring the increase in absorbance at 340nm. Reaction mixtures contained 0.16mM-$NADP^+$, 6mM-ATP, 8mM-$MgCl_2$, 27mM-glucose, 5mM- mercaptoethanol, glucose 6-phosphate dehydrogenase (0.5 IU) and varying concentrations of enzyme sample in 100mM-Tris-HCl buffer adjusted to pH 7.5 with 2M-NaOH (total volume 3.015-3.11ml). Reactions were carried out in 1cm path length quartz cuvettes and were routinely started by addition of enzyme (5µl-100µl). Control experiments were done to ensure that the rate-limiting step was that catalysed by hexokinase.

Inhibition of hexokinase by glucose 6-phosphate or 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid

The initial rates of enzyme reactions were followed by coupling the rate of ADP formation to the oxidation of NADH, using phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase. The assay system was essentially that developed by Ureta (1976) and measures the decrease in absorbance at 340nm. Reaction mixtures contained, 13mM-NADH, 2.5mM-phosphoenolpyruvate, lactate dehydrogenase (11 I.U.), pyruvate kinase (12 I.U.) and varying concentrations of substrates and inhibitors in 100mM-Tris HCl adjusted to pH 7.5 with 2M-NaOH (total volume 3.0ml). The concentration of Mg^{2+} was adjusted in each case according to the ATP concentration so that 1mM-free divalent ion was always present. 0.04 units purified bovine brain hexokinase I (0.04 I.U.) was added to start the reaction. Control experiments were carried out to ensure that the rate-limiting step was that catalysed by hexokinase and also that glucose 6-phosphate and its isosteric phosphonate analogue

had no effect on the coupling enzymes. One unit of hexokinase activity is defined as that amount of enzyme catalysing the transformation of 1μmole of glucose/min at 25°C. Specific activity is defined as units/ mg of protein.

Determination of glucose 6-phosphate and 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid (X)

Glucose 6-phosphate (or analogue X) was measured by the amount of excess $NADP^+$ (at least 10 times the concentration of glucose 6-phosphate) that was reduced in the presence of glucose 6-phosphate dehydrogenase, as shown by total increase in absorbance at 340nM. Reaction mixtures contained 0.8mM-$NADP^+$, glucose 6-phosphate dehydrogenase (1.4 U.I.) and approximately 0.05-mM glucose 6-phosphate (or analogue X) in 100mM-Tris-HCl buffer adjusted to pH 7.5 with 2M-NaOH (total volume 1.22ml). Reactions were started by addition of enzyme.

$NADP^+$ concentration was determined in an analogous manner, with a reaction mixture containing 10 times excess of glucose 6-phosphate in relation to the $NADP^+$ concentration.

K_m value of 6,7 dideoxy-α-D-gluco-heptose, 7-phosphonic acid as substrate for yeast glucose 6-phosphate dehydrogenase

Initial rates of enzyme reactions were followed by monitoring the reduction of $NADP^+$ as shown by increase in absorbance at 340nm in the presence of glucose 6-phosphate dehydrogenase. Reaction mixtures contained 0.5mM-NADP, glucose 6-phosphate dehydrogenase (0.7 I.U.) and varying concentrations (0.1mM-1.0mM) of 6,7 dideoxy-α-D-gluco-heptose, 7-phosphonic acid (X) in 100mM-Tris-HCl adjusted to pH 7.5 with NaOH (total volume 1.005ml).

Tests for phosphodiesterase activity

A. 3' nucleotidohydrolases

Phosphodiesterase activity was followed by measuring formation of p-nitrophenol resulting from the hydrolysis of 2'deoxythymidine-3' p-nitrophenol phosphate. Reaction mixtures contained 3mM-2' deoxythymidine-3'-p-nitrophenol phosphate and varying concentrations of enzyme in 650mM-citrate buffer (650mM trisodium citrate adjusted to pH 6.0 with 650mM-citric acid)(total volume 0.1ml). The reaction mixture was incubated for 5 min. before addition of 0.1M-NaOH (2.9ml). The absorbance at 405nm was read against a control solution lacking enzyme.

B. 5' nucleotidohydrolases

Phosphodiesterase activity was followed by measuring formation of p-nitrophenol resulting from the hydrolysis of bis p-nitrophenol phosphate essentially as described above. Reaction mixtures contained bis-p-nitrophenyl phosphate (3.3mg/ml) and enzyme sample (20µl) in 0.2MTris-base buffer adjusted to pH 8.9 with 1MHCl (total volume 3.0ml). The reaction was initiated by addition of enzyme.

Determination of glucose (glucose oxidase/peroxidase assay)

Glucose was measured by following the oxidation of o-dianisidine dihydrochloride via hydrogen peroxide in the presence of glucose oxidase and peroxidase. Reaction mixtures contained glucose solution (1ml), solution A [containing, glucose oxidase (0.333g), peroxidase (5mg), o-dianisidine dihydrochloride (20mg) in 500mM sodium phosphate buffer, pH 7.0 (100ml)]. The reaction mixture was incubated for 30 min at 25°C before addition of 60% H_2SO_4 (10ml) and reading the absorbance at 530nm.

Protein determinations

Enzyme elution from DEAE cellulose columns during enzyme purification was monitored by the spectrophotometric procedure of Warburg & Christian (1941). Otherwise protein concentrations were determined by the method of Lowry et al. (1951).

Values of K_m and V_{max} were obtained by extrapolation to infinite concentration of alternate susbstrate as described by Florini & Vestling (1957), by using, slopes from [S]/Vo versus [S] plots which were calculated by weighted regression (assuming simple error see page 113) using a computer programme based on the procedure described by Cornish-Bowden (1976).

EXPERIMENTAL, RESULTS, AND DISCUSSION

Purification of bovine brain mitochondrial hexokinase isoenzyme type I

Extraction and purification of bovine brain mitochondrial hexokinase was based on the procedure published by Redkar & Kenkare (1972). Unless otherwise indicated all steps were carried out at 0-4°C. Table 23 summarises the yields and specific activities throughout the procedure.

1. Homogenate and combined supernatants

Cortical tissue was carefully scraped from the white myelin of the brain of a freshly-killed cow and kept on ice overnight. Cortical tissue (150g) was suspended in 250mM-sucrose (750ml) containing 5mM-EDTA and several drops of _n_-octanol. The suspension was homogenised (3 x 1 min) in a Braun homogeniser at maximum speed and the homogenate (A) was centrifuged (800 _g_, 10 min). The resulting supernatant (B_1) was separated and stored and the pellet was resuspended in 250mM-sucrose (500ml), containing 5mM-EDTA and rehomogenised (1 min) before centrifugation (800 _g_, 10 min). The sediment was discarded and the supernatant (B_2) was combined with supernatant B_1.

2. Mitochondrial fraction

The combined supernatant fractions (B_1 and B_2) (1160ml) were centrifuged (13,000 _g_, 15 min) and the mitochondrial pellet was dispersed (ultra Turex homogeniser) in buffer A (450ml) containing 100mM-NaCl and centrifuged (13,000 _g_, 10 min). The supernatant was discarded. The mitochondrial pellet was resuspended in buffer A (150ml) and mixed with an equal volume of 50% (v/v) glycerol in Buffer A. The suspension was dispersed (ultra Turex homogeniser, ½ speed, 30 sec.) stirred for 15 min, and centrifuged (13,000 _g_, 30 min). The pellet

in 25% (v/v) glycerol in buffer A (150ml) was dispersed (ultra Turex) and centrifuged as before. The pellet was dispersed in 0.2M-sodium acetate buffer, pH 5.0 (268ml), containing 5mM-EDTA, 5mM-mercaptoethanol, 10mM-glucose and 900mM NaCl.

3. Acetate extract

The mitochondrial suspension (268ml) was stirred for 2 hr and dialysed overnight against 0.2M sodium acetate buffer, pH 8.0 (5ℓ) containing 5mM-EDTA, 5mM-mercaptoethanol, 10mM-glucose and 900mM-NaCl. The non-diffusible material was then further dialysed for 4 hr against the same buffer but lacking NaCl, stirred for 1½ hr. and centrifuged (20,000 g, 30 min). The pellet was discarded and the supernatant was adjusted to pH 7.0 with 8-80 liquor ammonia. The neutralised acetate extract (390ml) was turbid in appearance.

4. $(NH_4)_2SO_4$ fraction

$(NH_4)_2SO_4$ was added in small quantities with constant stirring to the neutralised acetate extract, (390ml) to 45% saturation (0.264 g/ml, total addition 103 g). During the addition the pH of the solution was maintained at approximately pH 7.0 by addition of ammonium hydroxide. After 30 min. a cloudy suspension formed which was removed by centrifugation (16,000 g, 15 min). The supernatant solution was adjusted to 70% $(NH_4)_2SO_4$ saturation (0.159 g/ml, total addition 62.01 g) by stepwise addition of $(NH_4)_2SO_4$ as before. The solution was allowed to stand for 30 min, centrifuged (16,000 g, 15 min.) and the supernatant was discarded. The pellet was resuspended in buffer A (25ml) and dialysed overnight against the same buffer (2ℓ).

5. First DEAE - cellulose column

The dialysed $(NH_4)_2SO_4$ fraction (26ml) was applied to a DEAE-cellulose column (2.1 cm x 41 cm; pre-equilibrated with buffer A). The column was eluted with buffer A containing 100mM-KCl with a flow rate 60 ml/hr maintained by using an LKB peristaltic pump. Fractions (5ml) were collected and assayed for protein and hexokinase activity.

Most of the inactive protein emerged from the column in the first column volume of the eluting buffer and the hexokinase was eluted immediately afterwards (see Figure 2).

Hexokinase-active fractions (25-32 inclusive) were combined (43.5ml) and purified by addition of $(NH_4)_2SO_4$ to 45% and then 70% saturation exactly as described above except that the precipitate was allowed to stand for 1 hr (instead of 30 min) in 70% saturated $(NH_4)_2SO_4$ before centrifugation. The precipiate obtained between 45% and 70% saturation with $(NH_4)_2SO_4$ was taken up in buffer A (16ml) and dialysed overnight against buffer A (1ℓ).

6. Second DEAE cellulose column

The dialysed solution (18ml) obtained from 45%-70% $(NH_4)_2SO_4$ precipitation of hexokinase-active fractions from the first DEAE-cellulose column was immediately applied to a second DEAE-cellulose column (1.5cm x 18.5cm pre-equilibrated with buffer A). The elution was effected with a linear gradient of 0-120mM-KCl in Buffer A (200ml) with a flow rate 22ml/hr. Fractions (5.5ml) were collected and assayed for protein and hexokinase activity. The enzyme was eluted from the column with 4-8 column volumes of eluant, the peak of enzyme activity emerging at 35mM-KCl (Figure 3). Fractions 25 to 40 (inclusive) were combined and subjected to $(NH_4)_2SO_4$ fractionation as

described in step 5. The 45%-70% $(NH_4)_2SO_4$ precipitate was taken up in buffer A (12.5ml) diluted with doubly-distilled water (12.5ml) and dialysed for 24 hr in buffer A (lacking glucose).

Table 23. Summary of purification procedure

Purification step	Volume ml	Total Protein mg	Total Hexokinase Activity I.U.	Specific Activity I.U./mg protein	Yield %	Specific Activity I.U./mg protein *	Yield % *
1 Homogenate	900	15,200	1107	0.073	100	0.07	100
2 Combined supernatants	1160	6,380	797	0.125	72	0.10	72
3 Mitochondrial fraction	268	3,618	517	0.143	46.1	0.35	58
4 Acetate extract	390	234	585	2.5	52.6	1.30	53
5 $(NH_4)_2 SO_4$ fraction	26	96.2	151	1.56	13.0	4.50	39
6 First DEAE-cellulose column chromatography	18	33.3	100	3.0	9.0	53.00	27
7 Second DEAE-cellulose column chromatography	20	4.14	9 8	24.0	9.0	83.0	18

* results published by Redkar & Kenkare (1972)

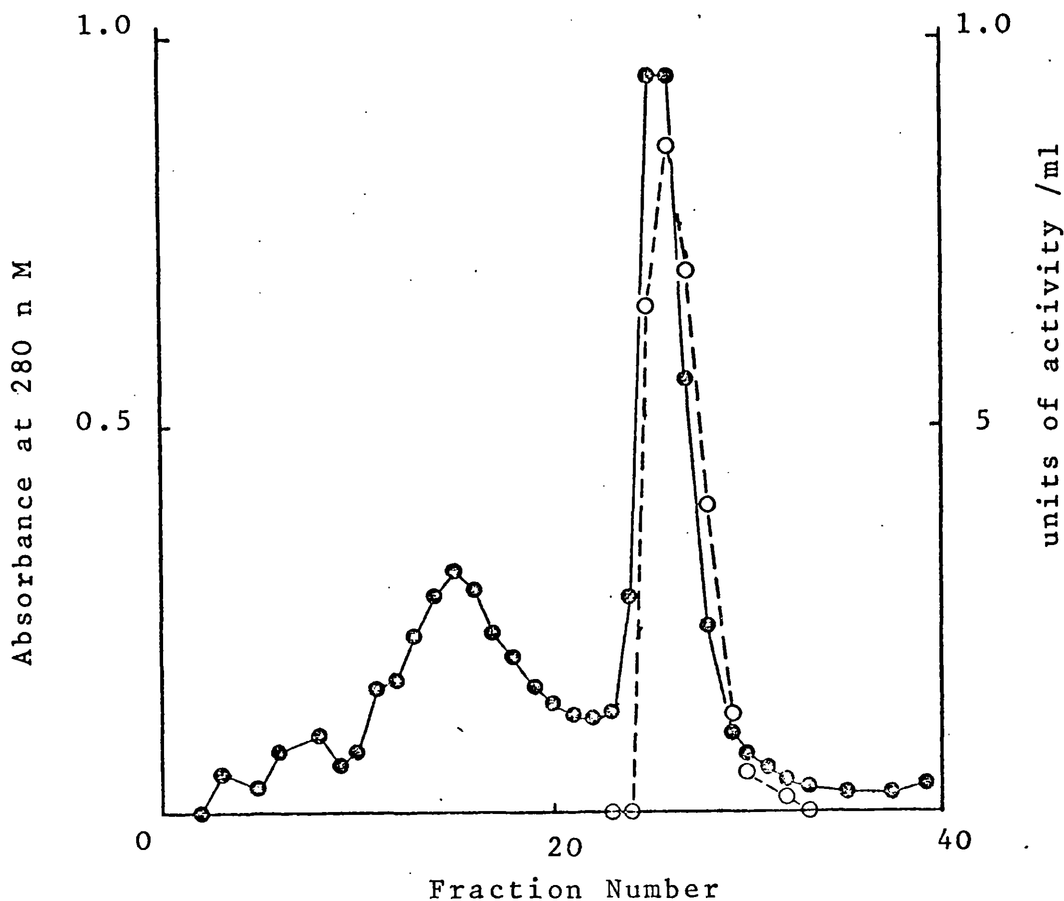


Figure 2 Step 5 First DEAE - cellulose column

Dialysed material (26 ml, 96.2 mg protein) from $(NH_4)_2SO_4$ precipitation step was loaded on the column and eluted with buffer A containing 100 mM-KCl. Fractions (5ml) were collected and assayed for proteins (●—●) and hexokinase activity (○--○). Tubes 25 - 32 inclusive were pooled (43.5ml)

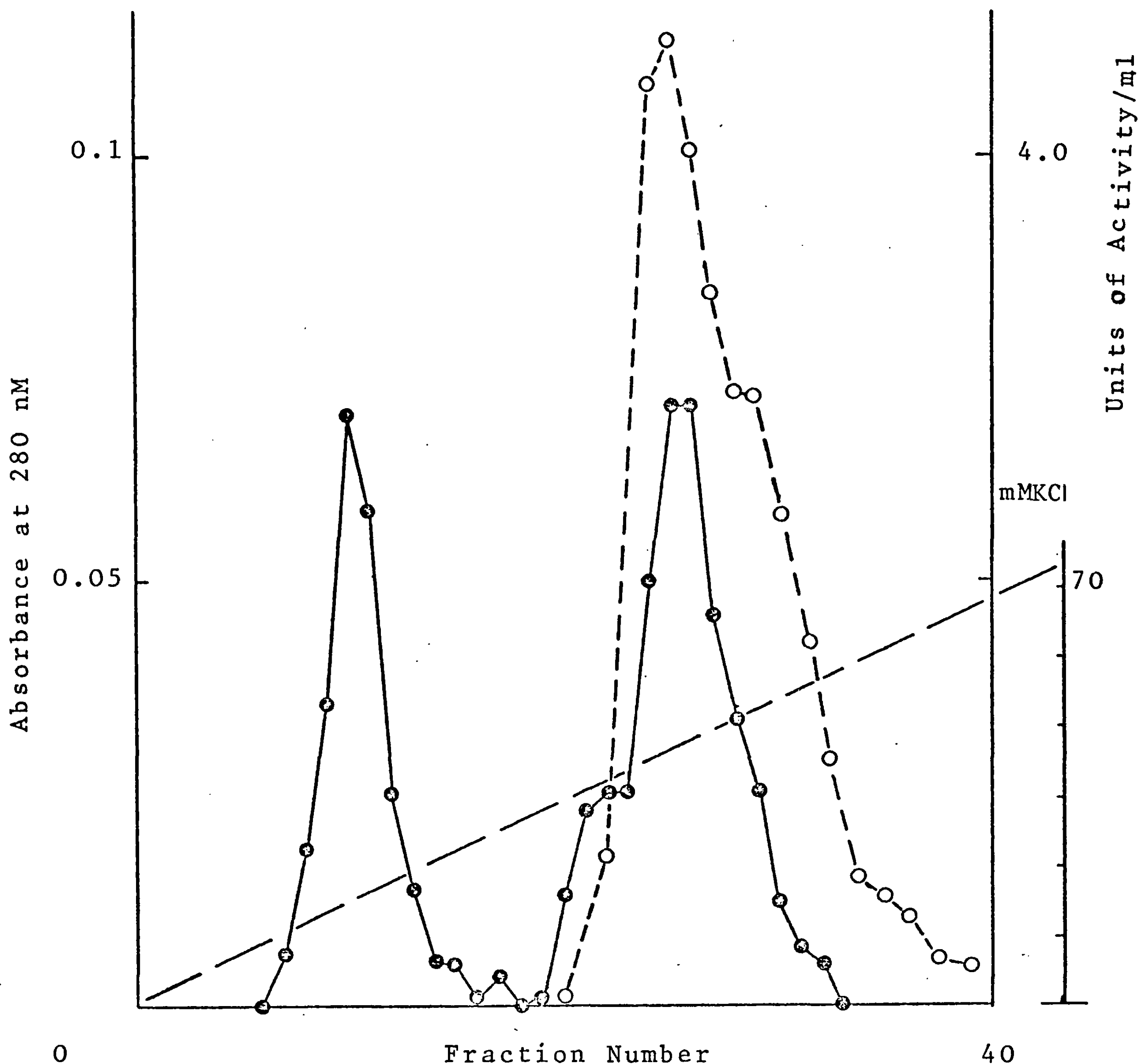


Figure 3   Step 6   Second DEAE - cellulose column

Dialysed material (18 ml, 33.3 mg protein) from step 5 was loaded on the column and eluted with a linear gradient 0 - 120 mM KCI (— —) in buffer A (200ml). Fractions (5.5 ml) were collected and assayed for protein (●—●) and hexokinase activity (○--○). Tubes 25-40 inclusive were pooled.

Inhibition of brain hexokinase by glucose 6-phosphate and its isosteric phosphonic analogue

Enzymic purity of 6,7, dideoxy-α-D-*gluco*-heptose 7-phosphonic acid (X) as measured by glucose 6-phosphate dehydrogenase was found to be 75% compared to its value determined by weight. This is in agreement with the result obtained for compound X prepared by Adams *et al.* (1976) (P. Adams personal communication). Furthermore the K_m value for compound X as a substrate for glucose 6-phosphate dehydrogenase was in close agreement to that reported by Adams *et al.* (1976) (Table 24).

Table 24. Comparison of K_m for 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid

	K_m (μM) (pH 7.5)
Adams *et al.*(1976)	227±6
observed	232

The coupled-enzyme assay system used to measure hexokinase activity in the presence of product inhibitor was found to have a small initial rate which rapidly tailed off. This effect was observed in the assay medium containing only buffer, phosphoenolpyruvate, lactate dehydrogenase, pyruvate kinase, NADH, ATP and $MgCl_2$. As a result of a series of experiments in which one constituent at a time was omitted, the observed initial rate was attributed to the ATP sample and was assumed to result from the presence of a small percentage of contaminating ADP. Before addition of hexokinase to start the reaction, each assay was accordingly incubated for 15 min to allow enzymic conversion of ADP to ATP. A very small but constant endogenous

rate was also observed with the purified hexokinase in the absence of substrates. The purified enzyme showed no contaminating phosphodiesterase activity and a glucose oxidase/peroxidase coupled assay indicated the absence of glucose. In view of this, substrate concentrations were used such that the initial rate of the reaction was at least 50 times greater than the endogenous rate. Prior to any kinetic experiments the purified hexokinase was dialysed overnight in buffer A minus glucose.

Bovine brain hexokinase (isoenzyme I) was prepared after the procedure of Redkar & Kenkare (1972) as described above and had a specific activity of 24 units/mg.

The mean and standard deviations of intial velocities through a wide range of glucose concentrations (Table 25) indicated that the standard deviations in initial velocity measurements are "simple" (i.e. the standard deviation of initial velocity values is independent of glucose concentrations) as apposed to the situation where each velocity has a standard deviation proportional to its true value (i.e. "relative") (Cornish Bowden, 1976; Henderson, 1978). All kinetic parameters determined in this section were calculated with a weighting procedure assuming "simple" errors in initial velocity measurements.

Figures 4A and 4B illustrate inhibition of glucose 6-phosphate relative to $MgATP^{2-}$ and glucose respectively and Figures 6A and 6B illustrate inhibition of 6,7 dideoxy α-D-gluco-heptose 7-phosphonic acid relative to $MgATP^{2-}$ and glucose respectively. Qualitatively glucose 6-phosphate and 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid act in an identical manner with respect to bovine brain hexokinase I,

Table 25. Variation of the initial velocities obtained over a wide range of glucose concentrations

[glucose] mM	number of determinations	Initial Velocity (mean) ΔAbs/min	Standard deviation of initial velocity	Standard deviation of initial velocity as a % of [glucose]
0.05	6	0.00515	0.00059	12.0
0.1	6	0.00753	0.00048	6.0
0.25	5	0.01070	0.00042	3.9
0.5	5	0.01396	0.00065	4.6
1.0	6	0.01695	0.00046	2.7

ATP concentration held constant, 2.0mM.

both being competitive inhibitors of $MgATP^{2-}$ and non competitive inhibitors of glucose. Apparent dissociation constants (K_i^{slopes} and $K_i^{intercepts}$) were obtained from the secondary plots of slopes and intercepts versus the relevant inhibitor (Table 26).

Quantatively 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid appears to be a less potent inhibitor of brain hexokinase I compared to the natural product inhibitor glucose 6-phosphate, with apparent K_i values 50 to 100 fold larger for the phosphonate analogue. The K_i values obtained for glucose 6-phosphate were of the same order as those obtained by Grossbard & Schimke (1966) for rat brain hexokinase I [$K_i^{intercept}$, 0.21mM and K_i^{slope} ($MgATP^{2-}$ varied substrate) 0.026mM].

True K_i values cannot be derived directly from any primary or secondary kinetic plot of product inhibition where the reaction mechanism is rapid equilibrium random order [as shown for this enzyme; Ning et al. (1969), Bachelard et al. (1971) and Gerber et al. (1974)] The derived apparent K_i^{slope} and $K_i^{intercept}$ values are complex factors including substrate kinetic constants in addition to true K_i values. Therefore additional kinetic studies were performed on the purified brain hexokinase (Figures 8A and 8B) to determine the substrate kinetic constants necessary to calculate the true K_i and K_{ii} values for glucose 6-phosphate and 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid [K_i, dissociation constant of enzyme inhibitor complex (EI); K_{ii}, dissociation constant of inhibitor from enzyme-glucose-inhibitor complex (ESI), where inhibitor is glucose 6-phosphate or analogue X] (Table 27).

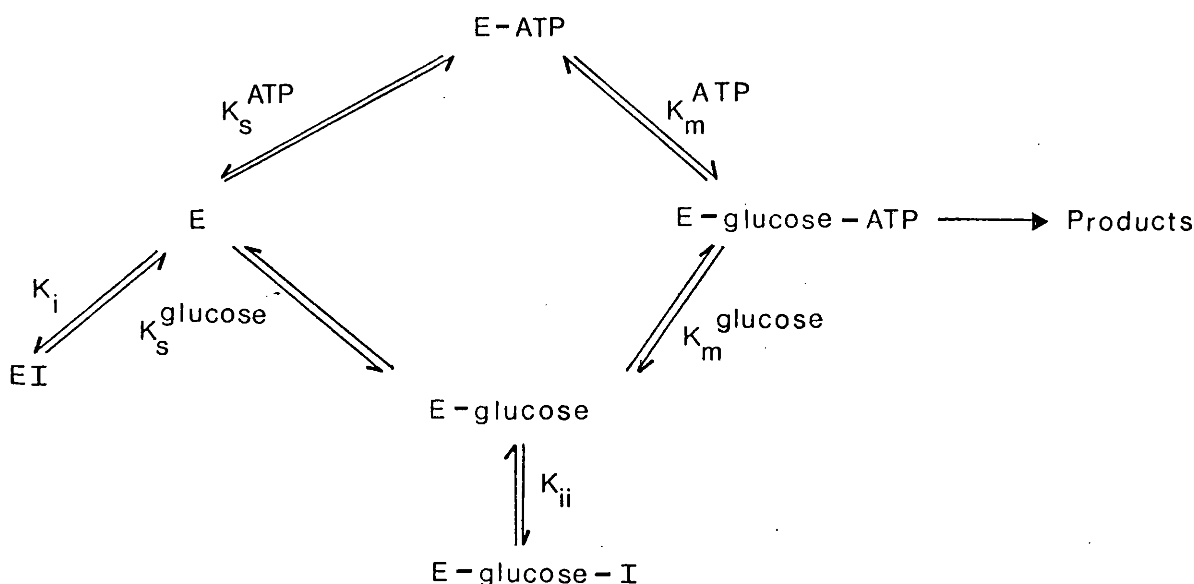
Table 26. Apparent K_i constants for glucose 6-phosphate and 6,7 dideoxy-α-D-*gluco*-heptose 7-phosphonic acid (X)

Substrate Varied	Inhibitor	K_i^{slope} mM	$K_i^{intercept}$ mM	K_i^{slope} mM (relative to that of glucose 6 phosphate)	$K_i^{intercept}$ mM (relative to that of glucose 6 phosphate)
glucose	glucose 6 phosphate	0.127 ± 0.018	0.076 ± 0.01	1	1
$MgATP^{2-}$	glucose 6 phosphate	0.032 ± 0.009	———*	1	—*
glucose	analogue X	7.0 ± 1.53	4.83 ± 0.33	55	63
$MgATP^{2-}$	analogue X	3.4 ± 0.83	———*	106	—*

K_i^{slopes} were obtained as the abscissal intercept from the secondary plot of slopes (of plots of I/V versus 1/[substrate], Figures 4A, 4B, 6A and 6B) versus the relevant inhibitor concentration (glucose 6-phosphate Figures 5A and 5B; analogue X Figures 7A and 7B). Similarly $K_i^{intercepts}$ were obtained from the secondary plot of intercepts (from Figure 4B and 6B for glucose 6-phosphate and analogue X respectively) versus inhibitor concentration (glucose 6-phosphate, Figure 5C; analogue X, Figure 7C). Values are ± S.E.

____* common intercept on ordinate.

Scheme 5



I = glucose 6-phosphate or analogue X

K_i and K_{ii} values obtained (Table 28) show clearly that qualitatively inhibition by glucose 6-phosphate and 6,7 dideoxy-α-D-*gluco*-heptose 7-phosphonic acid act in an identical manner with respect to bovine brain hexokinase I, but quantitatively the phosphonate analogue appears to be a much less potent "product" inhibitor, with K_i and K_{ii} values 88 and 63 times larger than the corresponding values for glucose 6-phosphate.

The K_{ii} value for glucose 6-phosphate obtained by me differed significantly from that obtained by Casazza & Fromm (1976)(35μM and 1μM respectively). This is possibly a consequence of larger K_m and K_s^{ATP} values (Table 27) obtained and used in the presently described work. The K_i and K_{ii} values for glucose 6-phosphate and its analogue (X) were accordingly determined using the kinetic constants obtained for bovine brain hexokinase by Casazza & Fromm (1976) (also shown in Table 27). The K_i and K_{ii} values for glucose 6-phosphate computed on this basis

Table 27. Substrate kinetic constants obtained from secondary plots of slopes and intercepts from Figures 8A and 8B (secondary plots, Figures 9 A, B, C and D)

substrate kinetic constant	determined value, mM	Casazza & Fromm (1976), mM
$K_m^{glucose}$	0.102 ± 0.015	0.030
$K_m^{MgATP^{2-}}$	1.12	0.200
$K_s^{MgATP^{2-}}$	0.667	0.087

Assuming the model of glucose 6-phosphate product inhibition as proposed by Casazza & Fromm (1976)(Scheme 5), the results obtained for glucose 6-phosphate inhibition (Figures 4A and 4B) and 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid inhibition (Figures 6A and 6B) were fit to the following rate expression.

$$V_o = \frac{V_{max}}{1 + \frac{K_m^{ATP}}{[ATP]}\left(1 + \frac{[I]}{K_{ii}}\right) + \frac{K_m^{glucose}}{[glucose]} + \frac{K_s^{ATP}\,K_m^{glucose}}{[ATP]\,[glucose]}\left(1 + \frac{[I]}{K_i}\right)}$$

[I] = glucose 6-phosphate or 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid.

Table 28. Comparison of true K_i values for glucose 6-phosphate and its phosphonate analogue X

			relative to glucose 6-phosphate		Casazza & Fromm (1976)	
	K_i mM	K_{ii} mM	K_i mM	K_{ii} mM	K_i mM	K_{ii} mM
glucose 6-phosphate	0.021	0.035	1	1	0.01	0.001
6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid	1.85	2.2	88	63	-	-

Table 29. Comparison of true K_i values for glucose 6-phosphate and its phosphonate analogue X

			relative to glucose 6-phosphate		Casazza & Fromm (1976)	
	* K_i mM	* K_{ii} mM	K_i mM	K_{ii} mM	K_i mM	K_{ii} mM
glucose 6-phosphate	0.006	0.0008	1	1	0.01	0.001
Analogue X	0.393	0.042	66	53	-	-

* calculated from results from Figures 4 and 6, and from kinetic parameters determined by Casazza & Fromm (1976)

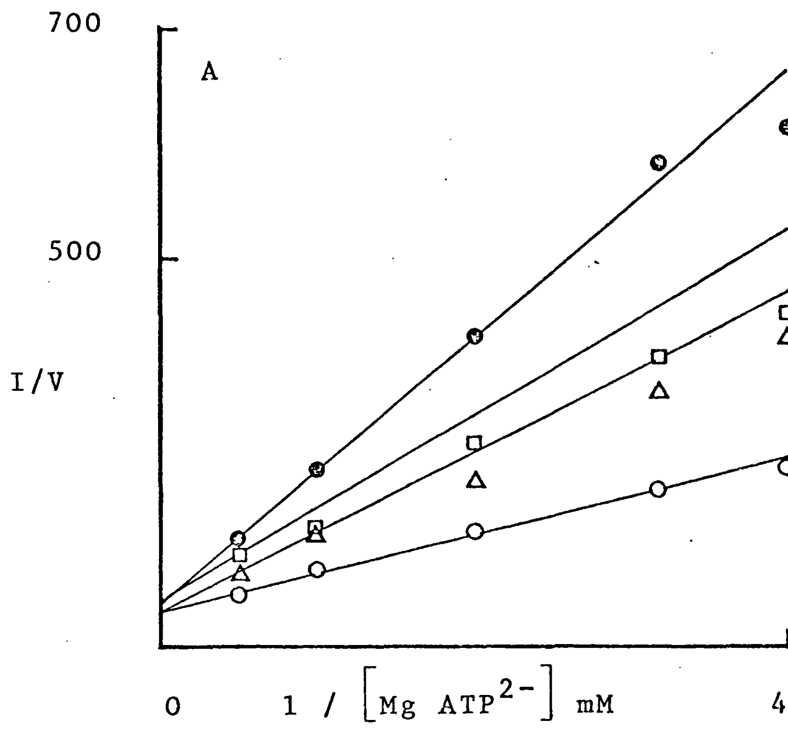
are seen to be in much closer agreement with those obtained by Casazza & Fromm (1976)(Table 29).

Most importantly, 6,7 dideoxy α-D-*gluco*-heptose 7-phosphonic acids acts in an identical manner to glucose 6-phosphate as a product inhibitor of bovine brain hexokinase I, but less potently (K_i and K_{ii} values being 50-80 times greater than for glucose 6-phosphate) irrespective of the treatment of the results obtained.

Glucose 6-phosphate has been shown not to inhibit brain hexokinase to the same extent at pH 4.6 as at higher pH values (Crane & Sols, 1954) and it was suggested that the mono anionic glucose 6-phosphate form is not an inhibitory species. As the second pK of the phosphonate analogue (X) (7.46) differs by only 1.25 units from that of the natural substrate (6.21)(Adams *et al.*, 1976) no more than a 15-fold discrimination could be achieved at any pH by the preference for a particular ionic form (Dixon & Sparkes, 1974). It is accordingly unlikely that the ionic state of the analogue is a major factor in determining its higher K_i values compared with those of glucose 6-phosphate. The apparent decrease in affinity caused by replacing the C-O-P grouping of the natural substrate by C-CH_2-P could result from specific interactions of the oxygen atoms or from minor geometric changes resulting from the substitution. The decreased inhibitory capacity of the phosphonate compared with that of the natural phosphate clearly reflects the high degree of specificity for the binding site for the phosphate moiety of glucose 6-phosphate in product inhibition of hexokinase.

Figure 4 Lineweaver-Burk plots for the inhibition of hexokinase by glucose 6-phosphate. Reaction conditions as described in the Materials and Methods.
(A) Glucose concentration held constant (0.5mM).
Concentration of glucose 6-phospate; ○ , 0 ; △ , 0.028mM; □ , 0.042mM; ● , 0.084 mM.

(B) $MgATP^{2-}$ concentration held constant (2.0 mM).
Concentration of glucose 6-phosphate; ○ , 0 ; △ , 0.028mM; □ , 0.048 mM; ● , 0.084 mM; ▲ , 0.168 mM.



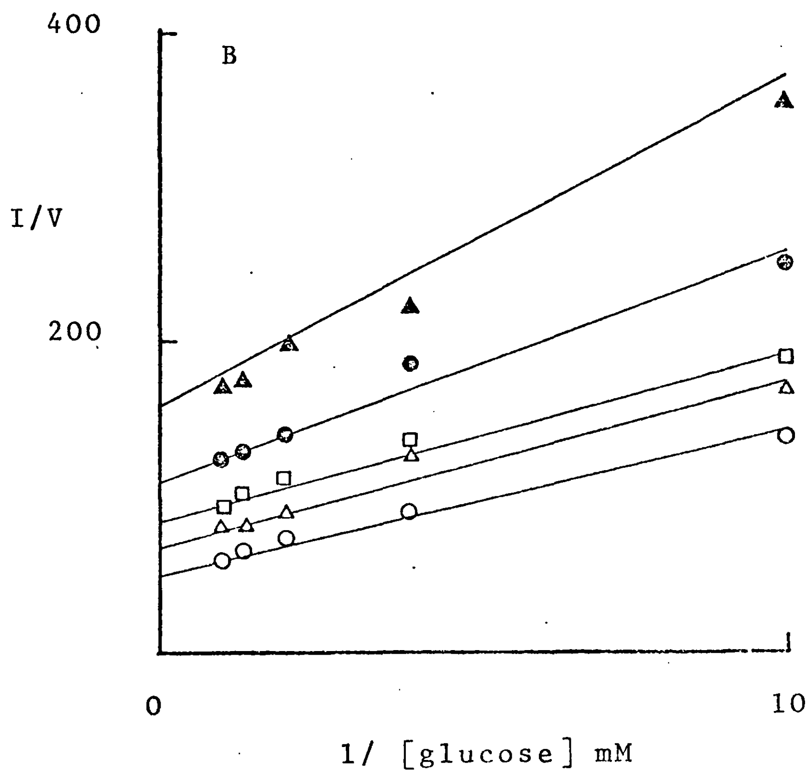


Figure 4

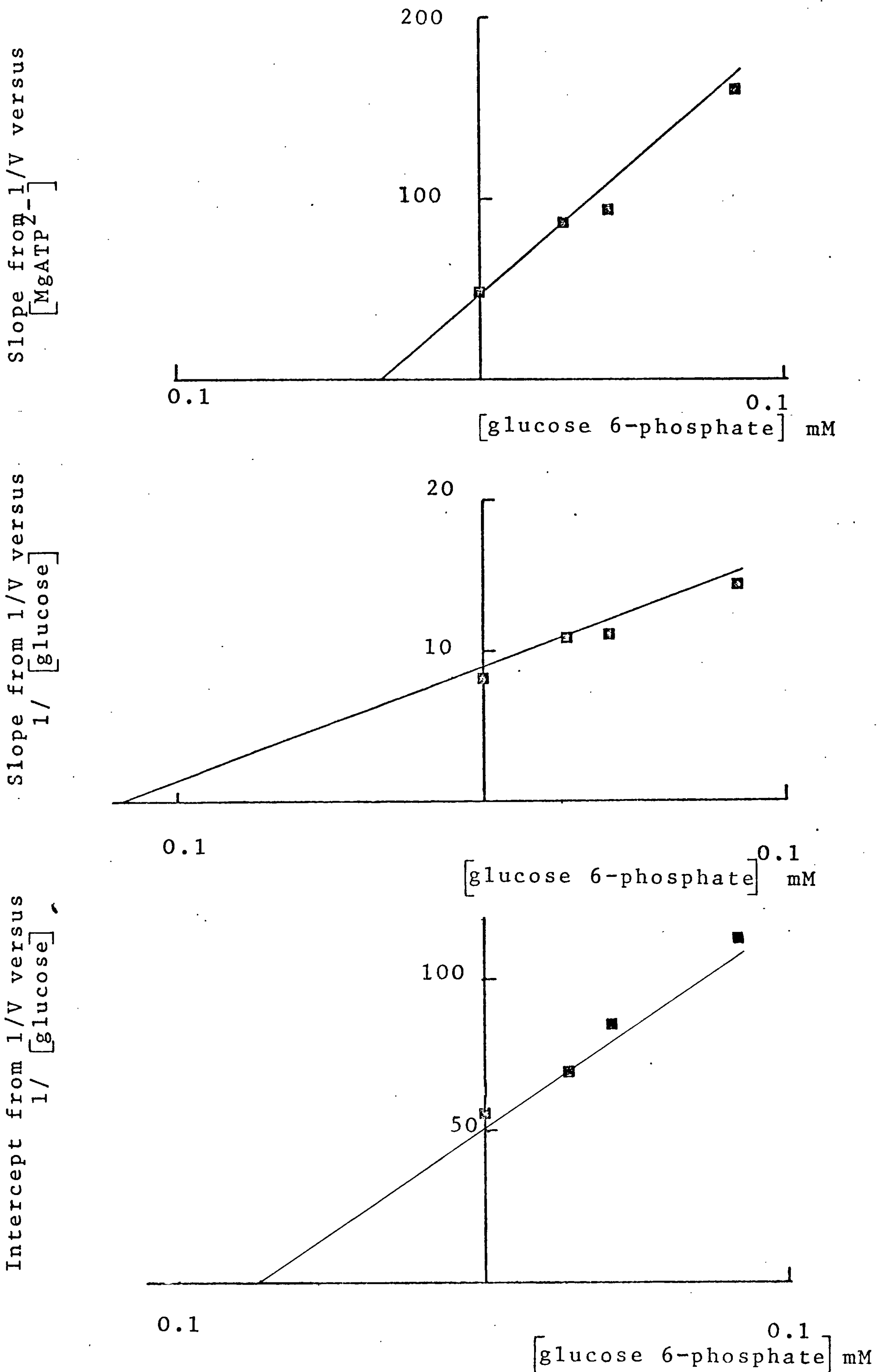
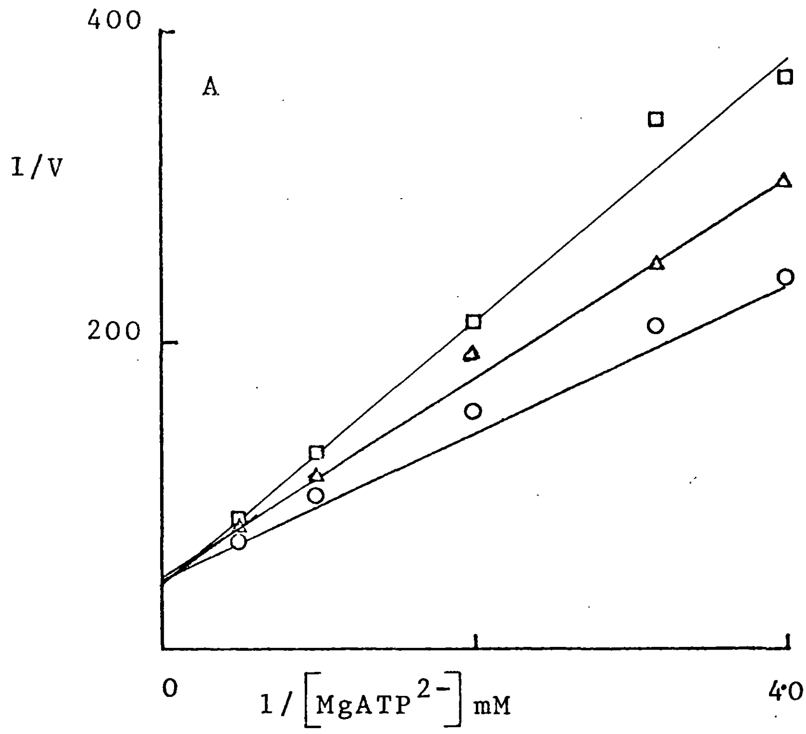


Figure 5 Secondary plots of slopes and intercepts (from Figure 4) versus glucose 6-phosphate concentration.

Figure 6 Lineweaver-Burk plots for the inhibition of hexokinase by analogue X. Reaction conditions as described in the Materials and Methods.

(A) Glucose concentration held constant (0.5mM). Concentration of analogue X; ○ , 0 ; △ , 1.432mM; □ , 2.86mM.

(B) $MgATP^{2-}$ concentration held constant (2.0mM). Concentration of analogue X; ○ , 0; △ , 0.692mM; □ , 1.39 mM; ● , 2.77 mM.



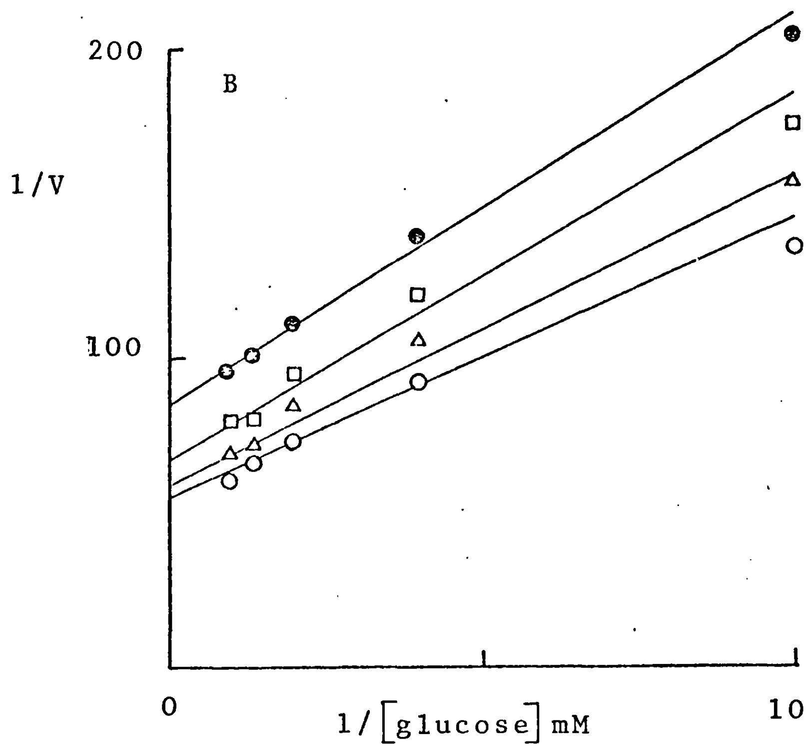


Figure 6

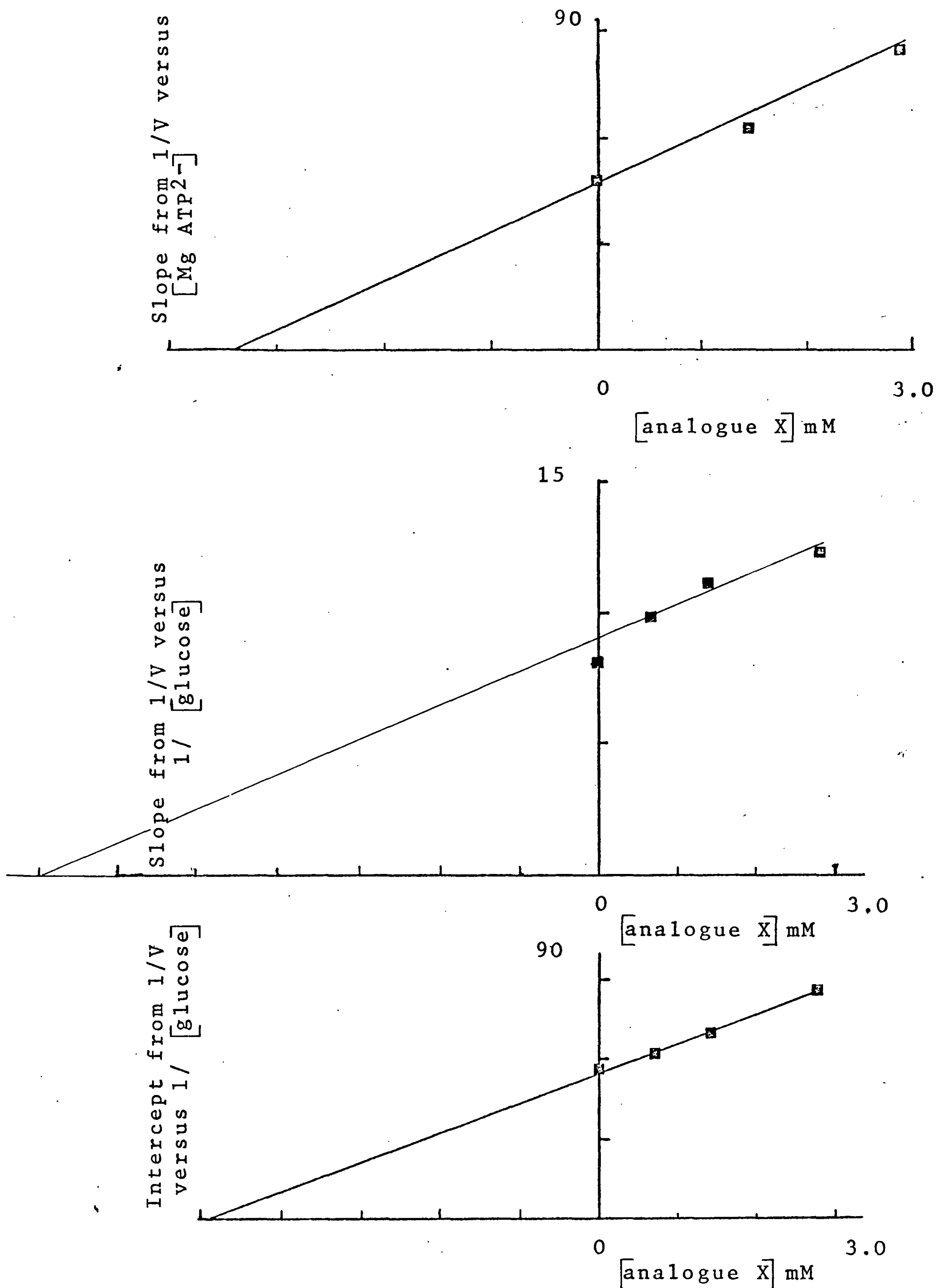


Figure 7 Secondary plots of slopes and intercepts (from figure 6) versus analogue X concentration.

<u>Figure 8</u> Lineweaver-Burk plots.

(A) 1/V versus 1/ [Mg ATP^{2-}] mM with the following glucose concentrations: ○ , 1.0mM; △ , 0.5mM; □ , 0.25mM; ● , 0.1mM; ▲ , 0.05mM.

(B) 1/V versus 1/ [glucose] mM with the following $MgATP^{2-}$ concentrations; ○ , 2.0mM; △ , 1.0mM; □ , 0.5mM; ● , 0.25mM; ▲ , 0.1mM.

Reaction conditions as described in Materials and Methods.

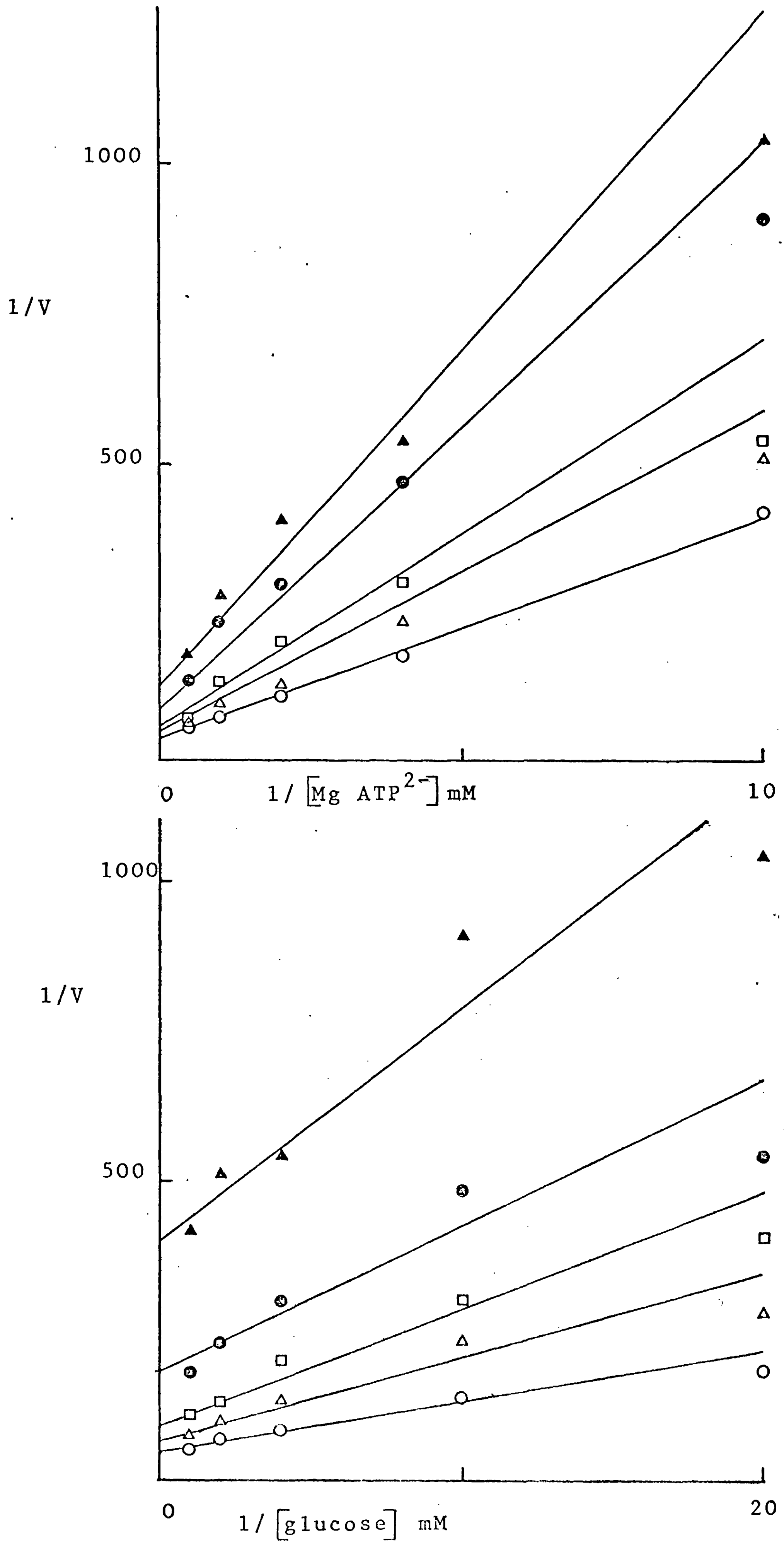


Figure 8

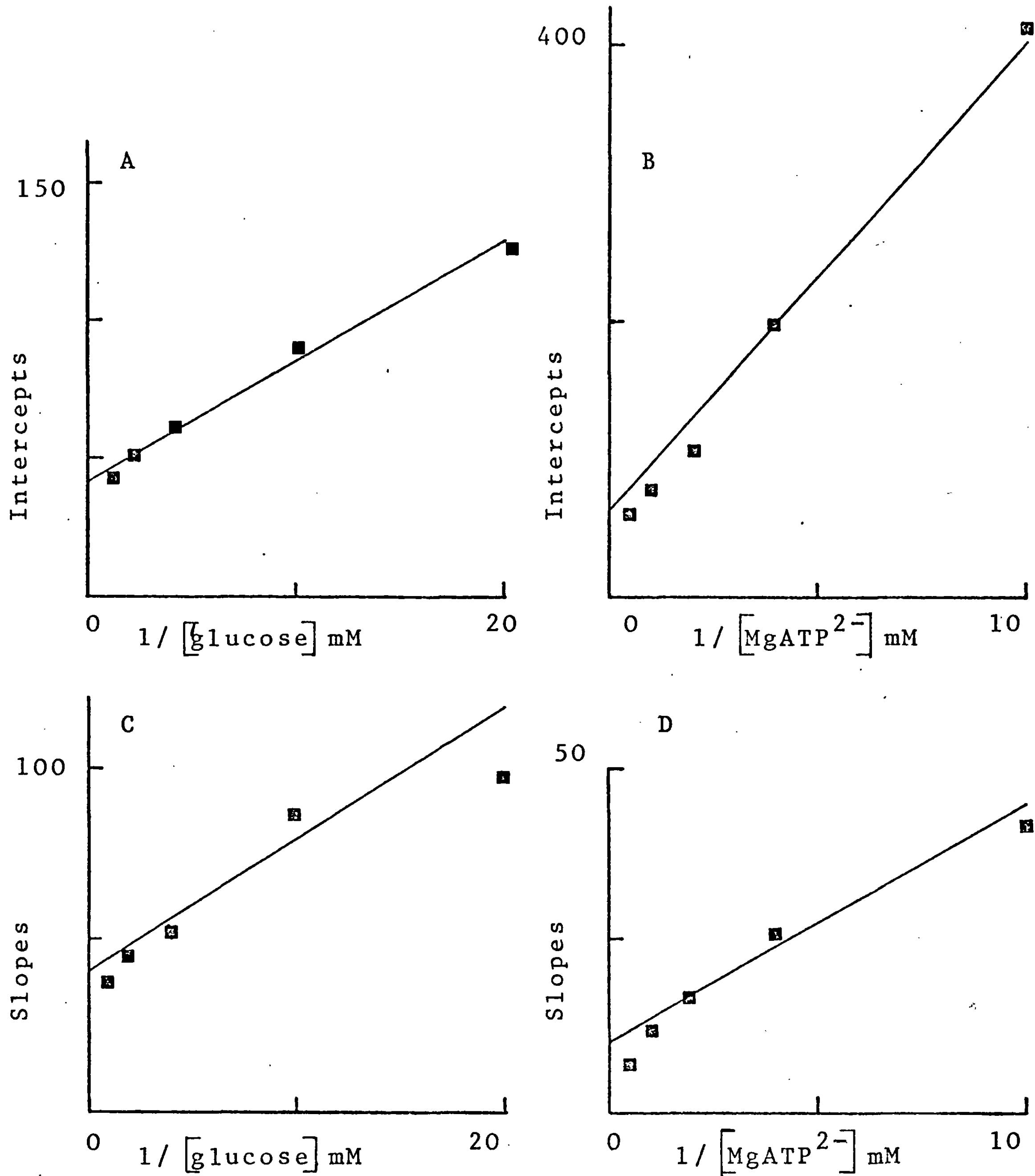


Figure 9 Secondary Plots

(A) Intercepts from 1/V versus 1/ [MgATP^{2-}] mM (Figure 8A) versus 1/ [glucose] mM.

(B) Intercepts from 1/V versus 1/ [glucose] mM (Figure 8B) versus 1/ [MgATP^{2-}] mM.

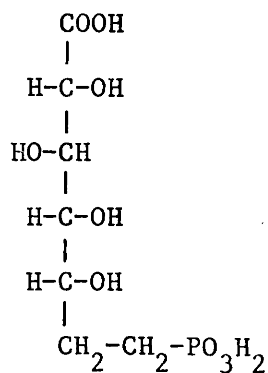
(C) Slopes from 1/V versus 1/ [MgATP^{2-}] mM (Figure 8A) versus 1/ [glucose] mM.

(D) Slopes from 1/V versus 1/ [glucose] mM (Figure 8B) versus 1/ [MgATP^{2-}] mM.

Enzymic synthesis of 6,7 dideoxy-D-*gluco*-heptonic 7-phosphonic acid, its purification and interaction with gluconate 6-phosphate dehydrogenase

INTRODUCTION

Adams et al (1976) reported that analogue X mimics glucose 6-phosphate in acting as a substrate for yeast glucose 6-phosphate dehydrogenase (see page 62). Furthermore use of a coupled assay system suggested that the oxidation product of this enzymic dehydrogenation [presumed to be the isosteric phosphonate analogue (CXXI) of gluconate 6 phosphate] is in its turn a slow substrate for



CXXI

yeast gluconate 6-phosphate dehydrogenase. A similar result was obtained by Webster et al. (1976) who showed that the isosteric analogue of fructose 6-phosphate is a substrate for the sequential action of glucose 6-phosphate isomerase, glucose 6-phosphate dehydrogenase, and gluconate 6-phosphate dehydrogenase. On the basis of their results both groups of workers pointed out the possibilities of enzymic conversion of the glucose 6-phosphate analogue (X) into the corresponding analogue of ribose 5-phosphate and, even into analogues of nucleotides. Introduction of any of these analogues into a living cell by way of permeable derivatives could lead to intracellular release of the phosphonate analogues which might be converted into nucleotide analogues capable of preferentially inhibiting nucleic

acid metabolism, e.g. for therapeutic purposes.

The work to be reported in this section concerns; (A) the enzymic synthesis of 6,7 dideoxy D-*gluco*-heptonic 7-phosphonic acid (CXXI) from the isosteric analogue of glucose 6-phosphate (X) via a cyclic assay system. (B) The purification of compound CXXI by anion exchange column chromatography. (C) A comparison of the kinetics of the dehydrogenation (at pH 7.5 and pH 8.0) by yeast gluconate 6-phosphate dehydrogenase of gluconate 6-phosphate and of 6,7 dideoxy-D-*gluco*-heptonic 7-phosphonic acid.

MATERIALS AND METHODS

Chemicals and Enzymes

Gluconate 6-phosphate dehydrogenase (yeast)(EC 1.1.1.44.), glutamate dehydrogenase (beef liver)(EC 1.4.1.3.), gluconate 6-phosphate, and α-keto glutarate were purchased from Boehringer Corp. (London) Ltd., W.5. U.K. MN 300 cellulose powder, particle size ≤ 10μM was obtained from Machery, Nagel and Co., Düren, Germany. Sephadex A-25, anion-exchange beads, particle size 40-120μm, were purchased from Pharmacia (Great Britain), Hounslow, Middlesex, U.K. Reagents used in work described earlier in this thesis were obtained from sources previously quoted and all other chemicals were purchased from BDH Chemicals, Pool, Dorset, U.K.

Glucose 6-phosphate, 6,7 dideoxy α-D-*gluco*-heptose 7-phosphonic acid and NADP were determined as described earlier (page 102). Gluconate 6-phosphate and 6,7 dideoxy α-D-*gluco*-heptonic 7-phosphonic acid were determined as described for glucose 6-phosphate except gluconate 6-phosphate dehydrogenase (0.48 I.U) replaced glucose - 6 phosphate dehydrogenase.

Kinetic determinations

Initial rates of gluconate 6-phosphate dehydrogenase reactions were followed by determining the formation of NADPH from $NADP^+$. The increase in E_{340} was measured in a Unicam 8-100 spectrophotometer at 25°C. Reaction mixtures contained varying concentrations of substrates in 100mM-Tris-base buffer adjusted to the required pH with 2M-HCl in a 4cm-pathlength cell (total volume 4.005ml). Reactions were initiated by the addition of enzyme [1μg (for glucose 6-phosphate) or 5μg (for analogue CXXI)] in buffer 5μl) containing 1% bovine serum

albumin. A standard assay for gluconate 6-phosphate dehydrogenase activity using 0.45mM-$NADP^+$ and 1.2mM-gluconate 6-phosphate, was performed at regular intervals during each experiment to check the stability of the enzyme. Initial rates were determined for five concentrations of one substrate at each of five different fixed concentrations of the alternate substrate. Values of K_m and V_{max} were obtained by extrapolation to infinite concentration of alternate substrate as described by Florini & Vestling (1957), by using slopes from [S]/Vo versus [S] plots which were calculated by weighted regression (assuming simple error) using a computer programme based on the procedure described by Cornish-Bowden (1976).

Phosphate or phosphonate determination

(i) Ashing of phosphate (or phosphonate) sample with Mg $(NO_3)_2$

This procedure follows that described by Ames & Dubin (1960). The phosphate (or phosphonate) sample (10μl-200μl) was mixed with 10% $Mg(NO_3)_2 \cdot 6H_2O$ (w/v) in 95% ethanol in a pyrex test tube. The mixture was evaporated to dryness over a strong bunsen flame with rapid shaking and further heated in the flame until the brown fumes had disappeared. 0.1M HCl (0.3ml) was added to the cooled tube which was capped with silver foil and heated in a boiling water bath for 15 min.

(ii) Phosphate assay

Inorganic phosphate was then determined using the sensitive procedure described by Chen *et al.* (1956). The phosphate sample was diluted (to 4m) with doubly-distilled water, reagent C (4ml) was added and the tube was capped with parafilm. The contents of the tube were mixed, incubated for 2 hr. at 37°C and then cooled to room temperature when the absorbance was read (Unicam SP 8-100, or SP 500 spectro-

photometer) at 820nm (1·0cm path-length cell) against a blank solution. K_2HPO_4 was used to obtain standard curves which were linear in the range I-0.35 μmoles phosphorus (see Figure 10). Solution C was made up daily and stored on ice. It contained 6M-sulphuric acid (1 vol.), 2.5mM-ammonium molybdate (1 vol.), 10% ascorbic acid (stable for one month at 0^oC) (1 vol.) and doubly-distilled water (2 vol.). All glassware used was cleaned in chromic acid and rinsed twice with distilled and once with doubly-distilled water.

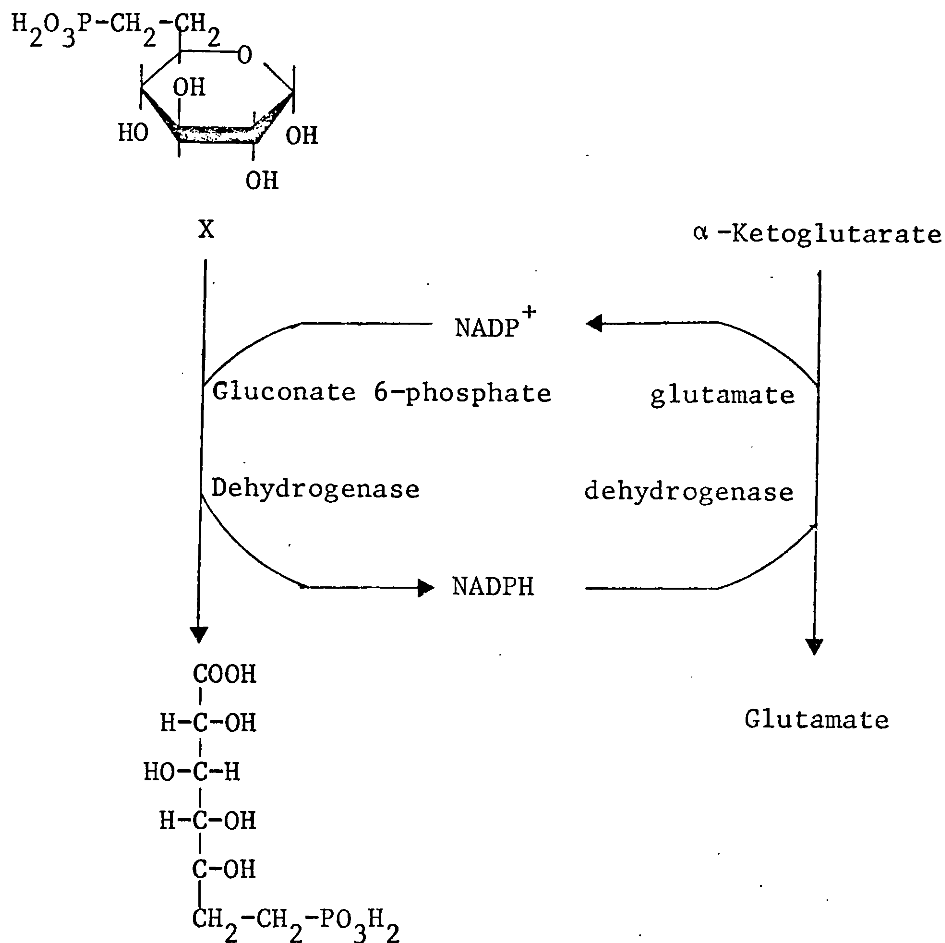
Thin layer chromatography

Following the procedure of Waring & Ziporin (1964), cellulose MN300 t.l.c. plates were spotted with aliquots of glucose 6-phosphate and gluconate 6-phosphate and developed in either solvent A [water - poor phase from a mixture of t-amyl alcohol (60ml), doubly-distilled water (30ml) and p-toluenesulphonic acid acid (2g)] or solvent B [isobutyric acid (66ml), concentrated ammonium hydroxide (1 ml) and doubly-distilled water (33ml)]. 6-8 hr were required for the solvent front of solvent A to move 18cm, and 3-4 hr for the solvent front of solvent B to move 14-16cm. Detection of the sugar phosphates was accomplished with spray reagent A [10% $SnCl_2$ H_2O in HCl (concentrated HCl freshly-distilled 200 fold with 0.5M-H_2SO_4)] or spray B [cysteine hydrochloride (0.75g), 50% ethanol (20ml) and concentrated H_2SO_4 (5ml)]. After spraying, the plates were heated for 5 min at 100^oC. Commercially-prepared silica gel G plates were also used.

EXPERIMENTAL, RESULTS, AND DISCUSSION

The chemical oxidation of 6,7 dideoxy-α-D-gluco-heptose 7-phosphonic acid (X) by bromine was known to present difficulties (P. Adams, personal communication) and so an enzymic preparation of the analogue CXXI was sought. Scheme 6 illustrates the reaction sequence that was devised in order to minimise levels of NADPH which is known to act as a product inhibitor of glucose 6-phosphate dehydrogenase (Afolayan 1972).

Scheme 6



CXXI

Preparative and separation methods were established using glucose 6-phosphate as substrate in order to conserve the phosphonate analogue during experimental stages.

Enzymic synthesis of gluconate 6-phosphate or 6,7-dideoxy-D-gluco-heptonic 7-phosphonic acid (CXXI)

Preparation

The cycling procedure used was based on that described by Lowry *et al*. (1961). The reaction mixture contained 5mM- α keto-glutarate, 3mM-glucose 6-phosphate (or 6,7 dideoxy α-D-*gluco*-heptose 7-phosphonic acid), 25mM-ammonium acetate, 0.2mM-ADP, 2mM-$NADP^+$, bovine serum albumin (2mg/ml), glutamate dehydrogenase (80μg/ml) and glucose 6-phosphate dehydrogenase (6μg/ml) in 50mM-Tris-base buffer adjusted to pH 8.0 with 2M-HCl (total volume, 50ml). The cyclic reaction was initiated by the addition of $NADP^+$ and the mixture was incubated for 16 hr at room temperature. The reaction was terminated by heating the mixture at 100°C (boiling water bath) for 2-6 min, and the resulting mixture was allowed to cool to room temperature over a period of 3 hr when a precipitate of deactivated enzymes appeared. Charcoal (100mg) was added and the mixture was allowed to stand overnight at 4°C. The charcoal, denatured proteins and nucleotides were removed by filtration and washed with doubly-distilled water (63 ml). Filtrate and washings were combined. Samples (1ml) of the reaction mixture were monitored at 340nm and showed an initial slight increase in E_{340} followed by a drop to a steady level somewhat below that of the starting value. It is possible that these changes could result from contamination of $NADP^+$ by NADPH which was initially oxidised. Such an explanation would lead to a value of approximately 13% contamination

of $NADP^+$ by NADPH.

α Ketoglutarate concentration was found to be optimal at 5mM, as lower levels led to decreased overall rates of product formation, whereas higher levels apparently inhibited the dehydrogenation of glucose 6-phosphate or its analogue. Ammonium ion concentrations up to 100mM were found not to affect the reaction and both enzymes were accordingly added together with their ammonium sulphate-containing stock solutions. The system described above was found to be suitable for glucose 6-phosphate or analogue concentrations of up to 5mM. ADP was present in order to stabilize glutamate dehydrogenase.

Separation of glucose 6-phosphate from gluconate 6-phosphate

Separation of commercial glucose 6-phosphate from commercial gluconate 6-phosphate was initially investigated using the thin layer chromatographic system described by Waring & Ziporin (1964) (Materials and Methods) which was found not to be satisfactory.

The separation of glucose 6-phosphate and gluconate 6-phosphate from the components of the cyclic reaction mixture was examined using Amberlite CG 400 anion - exchange resin in a procedure based on that of Lefebvre *et al*. (1964). Amberlite CG 400 anion exchange (Cl^- form) was converted into the borate form by washing with 0.8M potassium tetraborate (20 bed volumes) until all the chloride ions had been displaced. It was then washed with doubly-distilled water until the eluent gave no precipitate on addition of silver nitrate. The resin was equilibrated by passing 0.1M-ammonium tetraborate (20 bed volumes) through the column. The completed reaction mixture (containing approximately 1mM gluconate 6-phosphate) was adjusted to pH 8.0 with

ammonium hydroxide, applied to the column, and eluted with a linear gradient of ammonium tetraborate (0.1-0.4M, 800ml) at a flow rate of 1.4ml/min at room temperature. Fractions (10ml) were collected and aliquots of every third fraction were taken for phosphate determination. Phosphate-containing fractions were pooled and freeze-dried to give a white powder to which methanol was added and removed by evaporation in attempts to obtain sugar phosphates freed from salts.

A good separation of glucose 6-phosphate and gluconate 6-phosphate from each other and from components of the reaction mixture was obtained by this method (Figure 11A). However, all attempts to free the sugar phosphates from borate salts were unsuccessful and the procedure was abandoned.

A more useful separation of glucose 6-phosphate and its oxidised product was achieved by use of a DEAE-Sephadex A-25 column and elution with potassium bicarbonate based on a method described by Loesche _et al_. (1974).

DEAE-Sephadex A-25 (Cl^- form)(20g) was allowed to stand in 1M-$KHCO_3$ for several days and then in 50mM-$KHCO_3$ for 48 hr before packing into a column (30cm x 1.7cm) which was equilibrated by elution with 50mM-$KHCO_3$ (1500ml). A mixture of commercial glucose 6-phosphate (66μmoles) and gluconate 6-phosphate (58 μmoles) in 50mM $KHCO_3$ (10 ml) was applied to the column and eluted with a linear gradient (50mM-400mM, 400ml) at a flow rate of 1.0ml/min. Fractions (7.5ml) were collected and aliquots of every third fraction were taken for phosphate determination (Figure 12A). Phosphate-rich fractions were enzymically-

assayed by using glucose 6-phosphate dehydrogenase and gluconate 6-phosphate dehydrogenase. Fractions showing the latter activity were combined and stirred with Dowex 50 W-X1 (H^+ form, 50-100 mesh) cation-exchange beads until bubbling ceased. This procedure was performed under vacuum to facilitate the escape of CO_2 and thus to minimize any consequent acidification. The Dowex resin was removed by filtration and the filtrate was shell-frozen and freeze dried to give a pure sample of the free sugar phosphate acid.

In view of the successful separation of commercial phosphates (Figure 12A), the method was applied to purification of the reaction mixture used to convert glucose 6-phosphate into gluconate 6-phosphate. The completed reaction mixture (generated from approximately 300µmol glucose 6-phosphate) was adjusted to pH 8.4 with 2M-Tris-base, applied to a Sephadex column and eluted as described above (Figure 12B, typical separation achieved). Gluconate 6-phosphate was so obtained in a suitable state of purity and in 57% overall yield (by enzymic assay). Phosphate and enzymic determinations at various stages of the preparation are shown in Table 30.

Table 30. Enzymic synthesis and purification of gluconate 6-phosphate

fraction	Phosphate Determination µmoles	Enzymic Determination µmoles
Glucose 6-phosphate added to cyclic mixture	184.7	184.6
Gluconate 6-phosphate, inactivated filtered cycling mixture	250.0	145.0
Gluconate 6-phosphate, peak from Sephadex anion exchange column	122.0	111.4
Gluconate 6 phosphate free acid, from cation exchange	122.5	105.0

Separation of 6,7-dideoxy-D-gluco-heptonic 7-phosphonic acid (CXXI) from its precursor 6,7 dideoxy α D-gluco-heptose 7-phosphonic acid (X)

The chemically-prepared sample of analogue X used as substrate in the enzymic synthesis of CXXI was purified on Sephadex A-25 prior to the reaction. A single major peak of "phosphate" was eluted at 330mM-$KHCO_3$ (Figure 13A) and was shown by enzymic assay to represent 93% recovery of starting material (Table 31).

Table 31. Elution and recovery of analogue X from Sephadex A-25 anion exchange column chromatography

Fraction	Phosphate Determination μmoles	Enzymic Determination μmoles
analogue X added to column	179	164
analogue X, peak from Sephadex anion exchange column	134	159
analogue X free acid, after cation exchange	134	151

The purified phosphonate (X) was dehydrogenated by means of the cyclic reaction system and the completed reaction mixture was separated on Sephadex A-25 (Figure 13B) as described for gluconate 6-phosphate prepared as above. The overall yield of analogue CXXI, by enzyme assay was 56% (Table 32).

Table 32. Enzymic synthesis and purification of 6,7 dideoxy D-gluconic-heptose 7-phosphonic acid

Fraction	Phosphate Determination μmoles	Enzymic Determination μmoles
analogue X added to cycling mixture	245	289
analogue CXXI inactivated filtered cycling mixture	288	181
analogue CXXI, peak from Sephadex anion exchange column	172	161.3
analogue CXXI, free acid from cation exchange	173	161

The high values obtained by phosphate determination for the inactivated cyclic mixtures clearly reflect the presence of phosphate-containing constituents of the cyclic mixture as well as gluconate 6-phosphate or its phosphonate analogue (Table 30 and Table 32 respectively). A comparison of the yields at various stages in the purification of gluconate 6-phosphate and of its phosphonate analogue (CXXI) as determined by enzymic assay is shown in Table 33.

Table 33. Comparison of yields for the synthesis and purification of gluconate 6-phosphate and its phosphonate analogue (CXXI)

Fraction	Yield for invididual step % gluconate 6-phosphate	analogue CXXI
enzymic synthesis	79	70
separation from other inactivated cyclic mixture constituents by Sephadex anion exchange	79	89
free acid form, after cation exchange	77	99
overall yield	57	56

Comparison of activities of gluconate 6-phosphate and its analogue CXXI as substrate for gluconate 6-phosphate dehydrogenase

In order to check that the kinetic parameters of enzymically-synthesised gluconate 6-phosphate (and by implication those of its phosphonate analogue) were not affected by any impurities not detected by phosphate assay, the parameters were compared with those of the commercial compound and with literature values. The kinetic parameters, at pH 7.5, for enzymically-synthesised gluconate 6-phosphate and commercial 6-phosphate (trisodium salt) were found to be in reasonable agreement with each other (Table 34, Figures 14-17) and with those published by Pontremoli et al. (1961).

Table 34. Comparison of kinetic parameters of commercial gluconate-6-phosphate, enzymically synthesised gluconate 6-phosphate and literature values of commercial gluconate 6-phosphate as substrates for gluconate 6-phosphate dehydrogenase

pH 7.5

substrate	gluconate 6-phosphate K_m (μM)	$NADP^+$ K_m μM	V_{max} (arbituary units)
commercial gluconate 6-phosphate	119 ± 6	4.3 ± 0.5	1
synthetic gluconate 6-phosphate	156 ± 12	4.3 ± 1.4	1
commercial gluconate 6-phosphate (reported by Pontremoli *et al.*, 1961)	160	25	-

Values are ± SE.

These latter authors found that the Michealis constants for gluconate 6-phosphate and $NADP^+$ of the concentration of the fixed substrate, and concluded that their data fitted a random order mechanism (where all the equilibria are adjusted rapidly except for the rate-determining step, consisting of the breakdown of a single ternary complex). It was, therefore, assumed that the enzymically-synthesised and purified gluconate 6-phosphate, was essentially pure, containing no contaminants that would significantly alter its kinetic parameters for yeast gluconate 6-phosphate dehydrogenase. A similar case is accordingly argued for its phosphonate analogue (CXXI).

Analogue CXXI was dehydrogenated by $NADP^+$ in the presence of gluconate 6-phosphate dehydrogenase showing Michaelis-Menton kinetics (Figures 20-23). The kinetic parameters for this reaction are compared with those for the natural substrate, gluconate 6-phosphate (commercial) in Table 35. The similarity of K_m^{NADP} values for gluconate 6-phosphate and its phosphonate analogue (CXXI) suggests that the mechanisms of the enzymic reactions do not differ greatly in the two cases. If K_m is regarded as a crude measure of affinity of the substrate for the enzyme then it is apparent that at both pH values examined, the binding of the analogue CXXI is significantly weaker than that of gluconate 6-phosphate. The Introduction section of this thesis contains a number of examples in which replacement of a naturally-occurring phosphate by its phosphonate analogue led to a reduced enzyme-substrate interaction as shown by standard kinetic parameters. A number of rationalizations were offered in these cases and such explanations can be examined here also. The second pKa values of phosphonates are generally found to exceed those of their corresponding phosphates by approximately 1.0 pKa unit (Freedman & Doak, 1957; Engel, 1977) and different kinetic parameters can sometimes be attributed to different states of ionisation of a naturally substrate and its analogue under the conditions of the assay. At pH 7.5 the K_m value for analogue CXXI is over three times higher than that of gluconate 6-phosphate which might be thought to reflect, at least partially, the lower degree of ionization of the analogue at this pH (assuming pKa values of 6 and 7 respectively, gluconate 6-phosphate and its analogue would contain approximately 97% and 76% of the trianionic forms at pH 7.5). In this case raising the pH of the assay from 7.5 to 8.0 would be expected to decrease the K_m value for

Table 35. Comparison of kinetic parameters for gluconate 6-phosphate and its phosphonate analogue CXXI

	gluconate 6-phosphate or CXXI K_m (μM)		$NADP^+$ K_m (μM)		V_{max} (relative to that of gluconate 6-phosphate)	
substrate	pH 7.5	pH 8.0	pH 7.5	pH 8.0	pH 7.5	pH 8.0
analogue CXXI	435 ± 64	736 ± 74	5.4 ± 0.5	6.5 ± 2.5	0.14	0.12
gluconate 6-phosphate (commercial)	119 ± 6	276 ± 18	4.3 ± 0.5	3.6 ± 0.7	1.0	1.0

analogue CXXI (corresponding to an increase in trianionic form from 76% to 90%) while changing that of gluconate 6-phosphate very little (trianionic form increases from 97% to 99%). If in fact the ionisation of substrate does effect the kinetic parameters in this way the effect is masked by a general two fold increase of K_m of both substrates attributable to other factors (Table 35). At pH 8.0 the K_m of analogue CXXI is still some three fold greater than that of gluconate 6-phosphate and the conclusion must be that these differences are not mainly caused by the relatively small differences in content of trianionic form (90% versus 99%) at this pH. The situation is far from clear, however as the pH profile of initial reaction rates (Figure 24) would be consistent with an increasing rate of dehydrogenation of both substrates as their contents of trianionic form increased to approximately 90% followed by decreasing rates as the pH increases further.

The apparent decrease in affinity caused by replacing the C-O-P grouping of the natural substrate by C-CH_2-P could result either from specific interactions of the oxygen atoms or from minor geometric changes resulting from the substitution. These changes are reflected in the markedly lower V_{max} values of the analogue compared with those of gluconate 6-phosphate (Table 35).

Enzymic synthesis of ribulose 5-phosphate

A preliminary investigation of the conversion of glucose 6-phosphate, by two enzymic cycling steps and one anion exchange column, to demonstrate the formation of ribulose 5-phosphate was carried out (Figure 11B). The inactivated constituents of a first cycling mixture (containing no glucose 6-phosphate but only gluconate

6-phosphate by enzymic assay) were not separated by anion-exchange chromatography but were subjected to a second cyclisation procedure under the same conditions as the first (page 133) except that gluconate 6-phosphate dehydrogenase was added in place of glucose 6-phosphate dehydrogenase. The inactivated constituents of the second cyclisation mixture displayed neither glucose 6-phosphate dehydrogenase nor gluconate 6-phosphate dehydrogenase activity and were passed down on anion exchange column (Figure 11B). The major phosphate peak was eluted in a position which might be expected to correspond to that of ribulose 6-phosphate (around 200mM ammonium tetraborate). This result suggests the possibility of the enzymic conversion of the glucose 6-phosphate analogue (X) into the phosphonate analogue of ribulose 5-phosphate and its purification on a preparative scale.

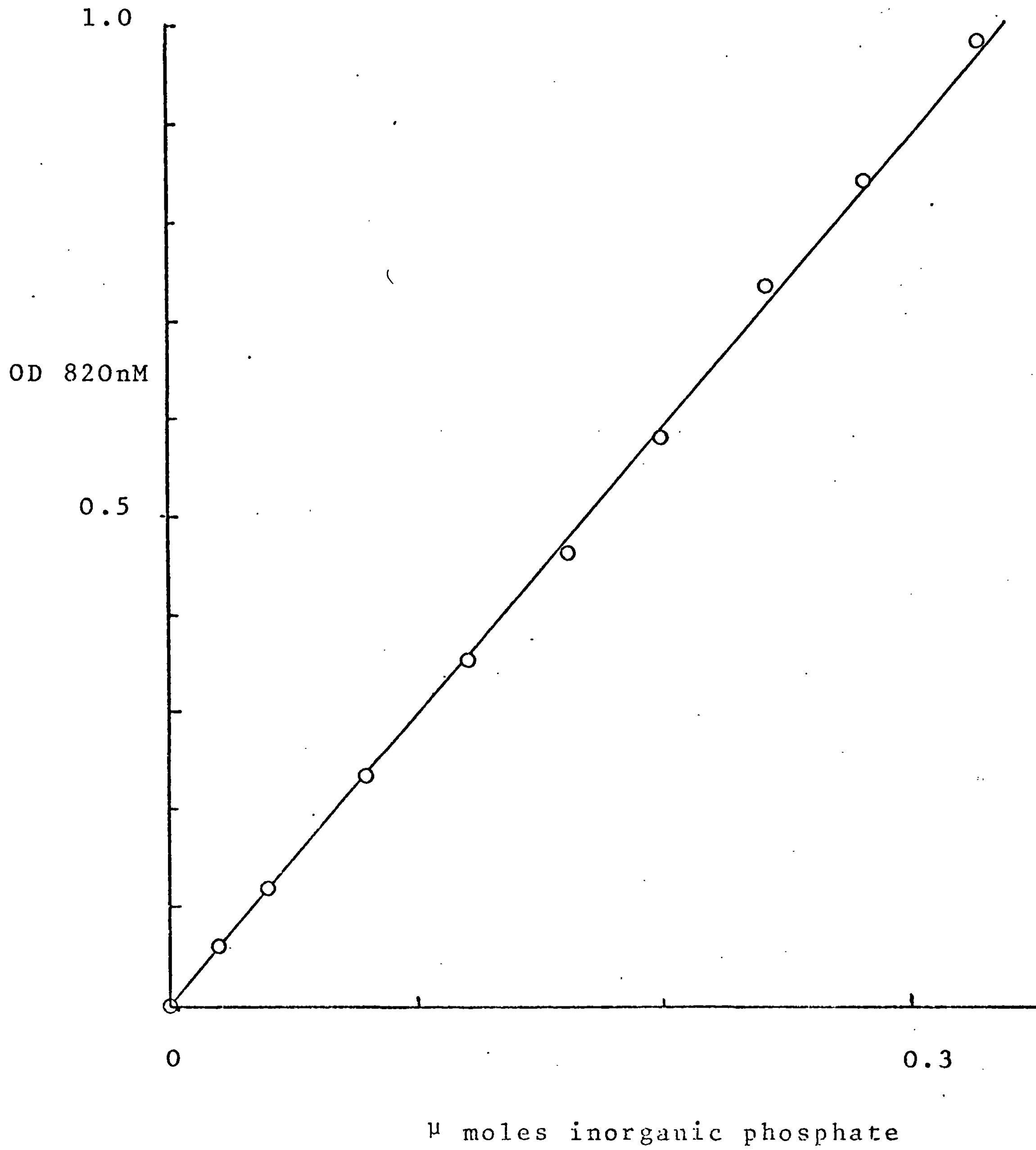


Figure 10 Phosphate determination standard curve.

Figure 11 Elution of phosphate-containing fractions from CG400 amberlite anion exchange column.
(A) separation of gluconate 6-phosphate enzymically synthesised from glucose 6-phosphate

(B) separation of ribulose 5-phosphate enzymically synthesised from glucose 6-phosphate.
Ammonium tetraborate elution gradient (---)

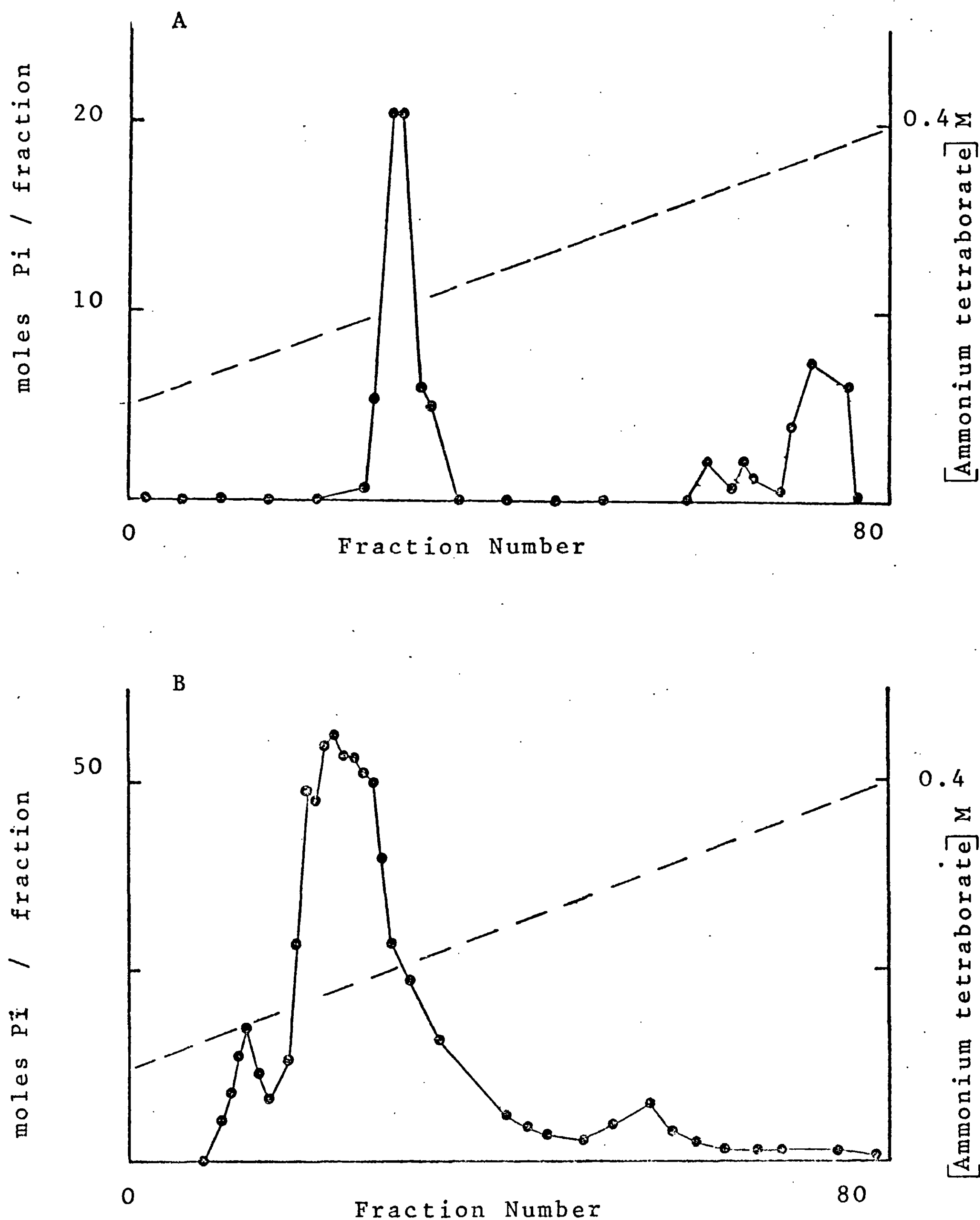
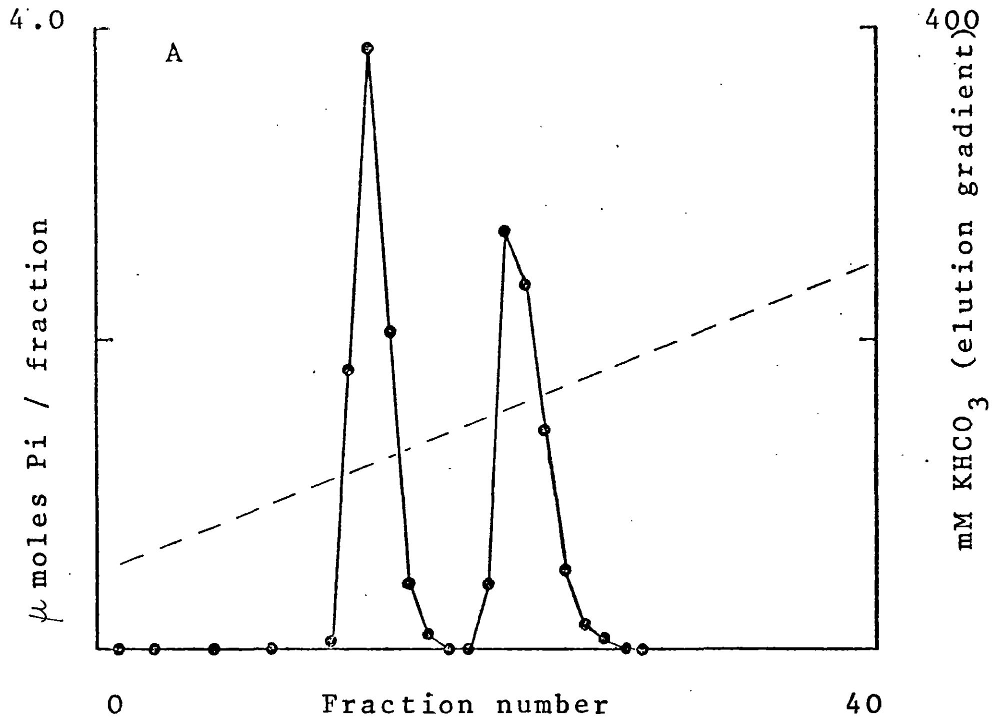


Figure 11

Figure 12 Elution of phosphate-containing fractions from Sephadex A-25 anion exchange column.
(A) Separation of commercial glucose 6-phosphate and gluconate 6-phosphate using a linear gradient of $KHCO_3$ (50mM-400mM, 400ml). Column dimensions 1·7cm x 30cm. flow rate 1ml/min.
(B) Separation of enzymically prepared gluconate 6-phosphate from other cyclic reaction constituents using a linear gradient of $KHCO_3$ (50mM-400mM, 500ml). Glucose 6-phosphate (25μmoles) was added to cyclic mixture prior to elution to act as a marker in the elution profile. Column dimensions 1.7cm x 35 cm, flow rate 1.5ml/min.



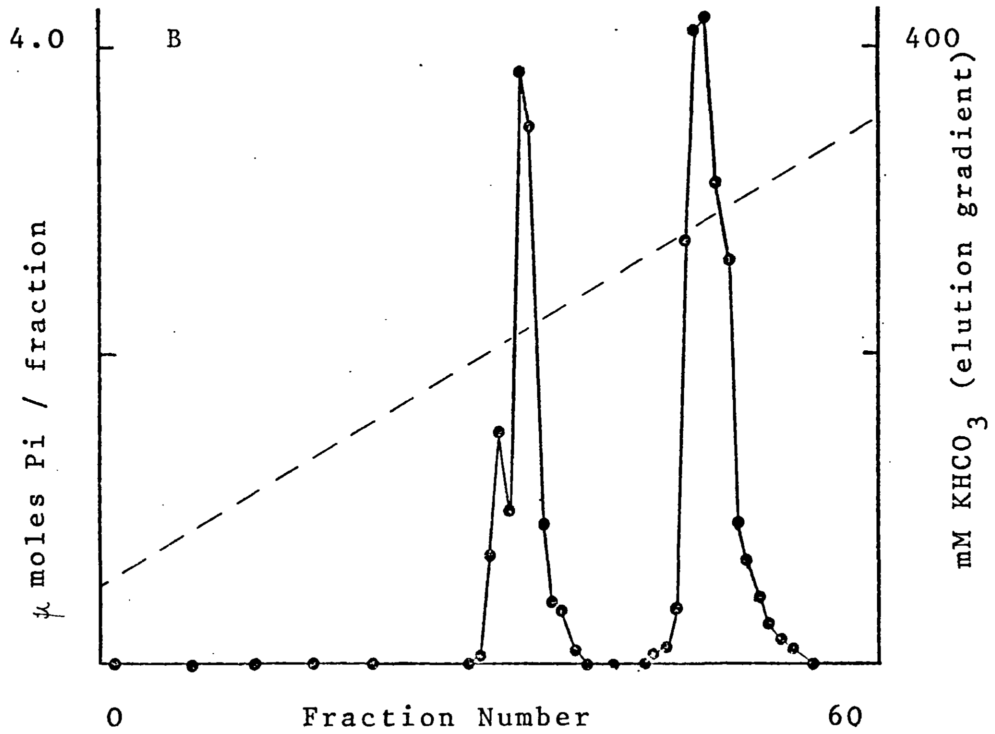
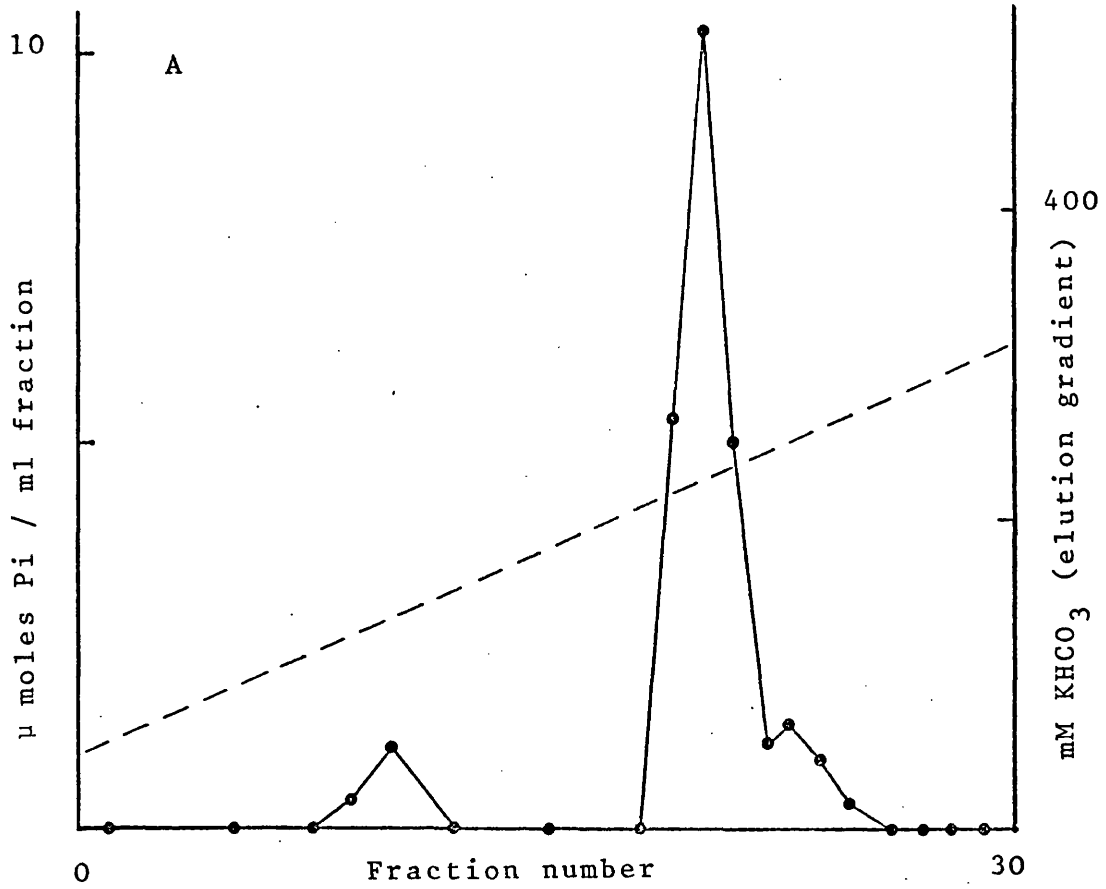


Figure 12

Figure 13 Elution of phosphate-containing fractions from Sepadex A-25 anion exchange column.
(A) Purification of analogue X, using a linear gradient of $KHCO_3$ (50m-400mM, 300ml). Column dimensions 1.4cm x 20cm, flow rate 1.4ml/min.
(B) Separation of 6,7, dideoxy D - *gluco* - heptonic 7-phosphonic acid (CXX1) produced enzymically from 6,7 dideoxy-α-D *gluco*-heptose 7-phosphonic acid (X), using a linear gradient of $KHCO_3$ (50mM-400mM, 500ml) Column dimensions 1.7 cm x 35cm, flow rate 1.4ml/min. The first peak eluted showed no glucose 6-phosphate dehydrogenase activity.



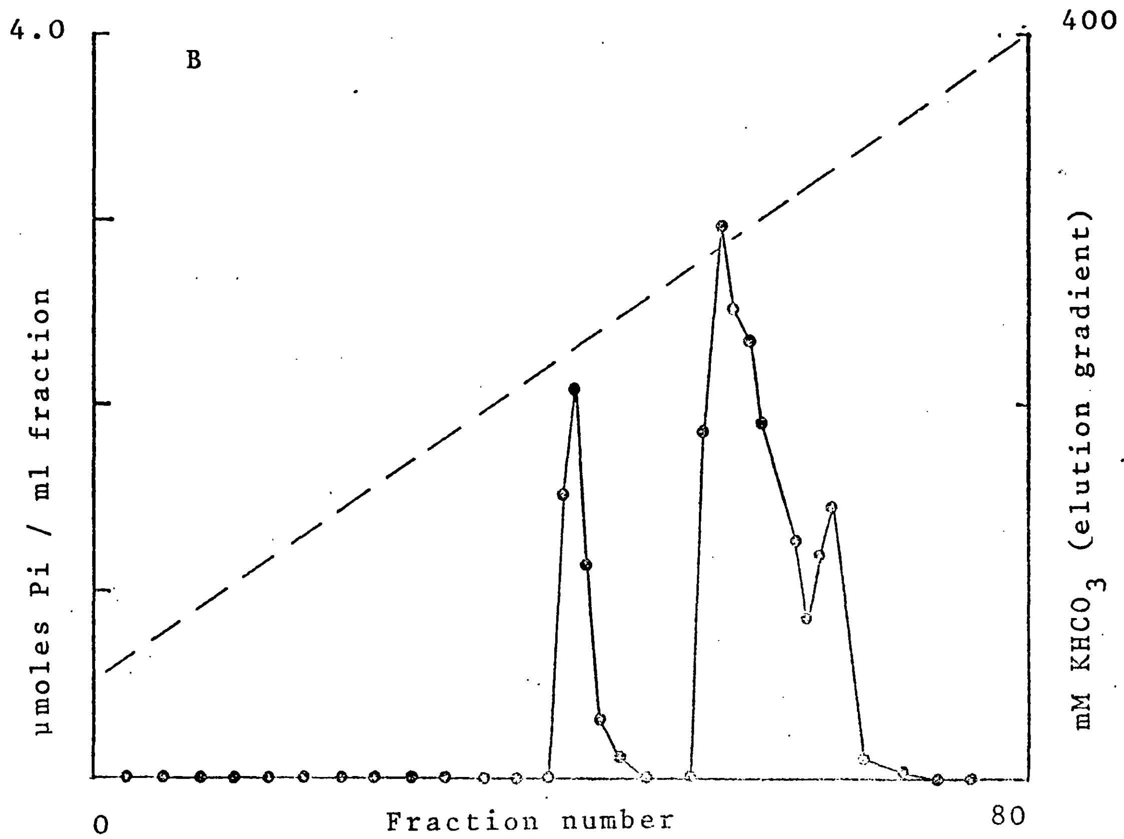


Figure 13

Figure 14 Lineweaver-Burk plots. All assays were carried out at pH 7.5.
(A) 1/V versus 1/ [gluconate 6-phosphate] mM with the following $NADP^+$ concentrations;
○, 0.448mM; △, 0.135mM; □, 0.045mM; ●, 0.0089mM.
(B) 1/V versus 1/ [$NADP^+$] mM with the following gluconate 6-phosphate concentrations; ○, 0.657mM;
△, 0.219mM; □, 0.164mM; ●, 0.0985mM; ▲, 0.049mM.
Gluconate 6-phosphate was enzymically synthesised and purified.

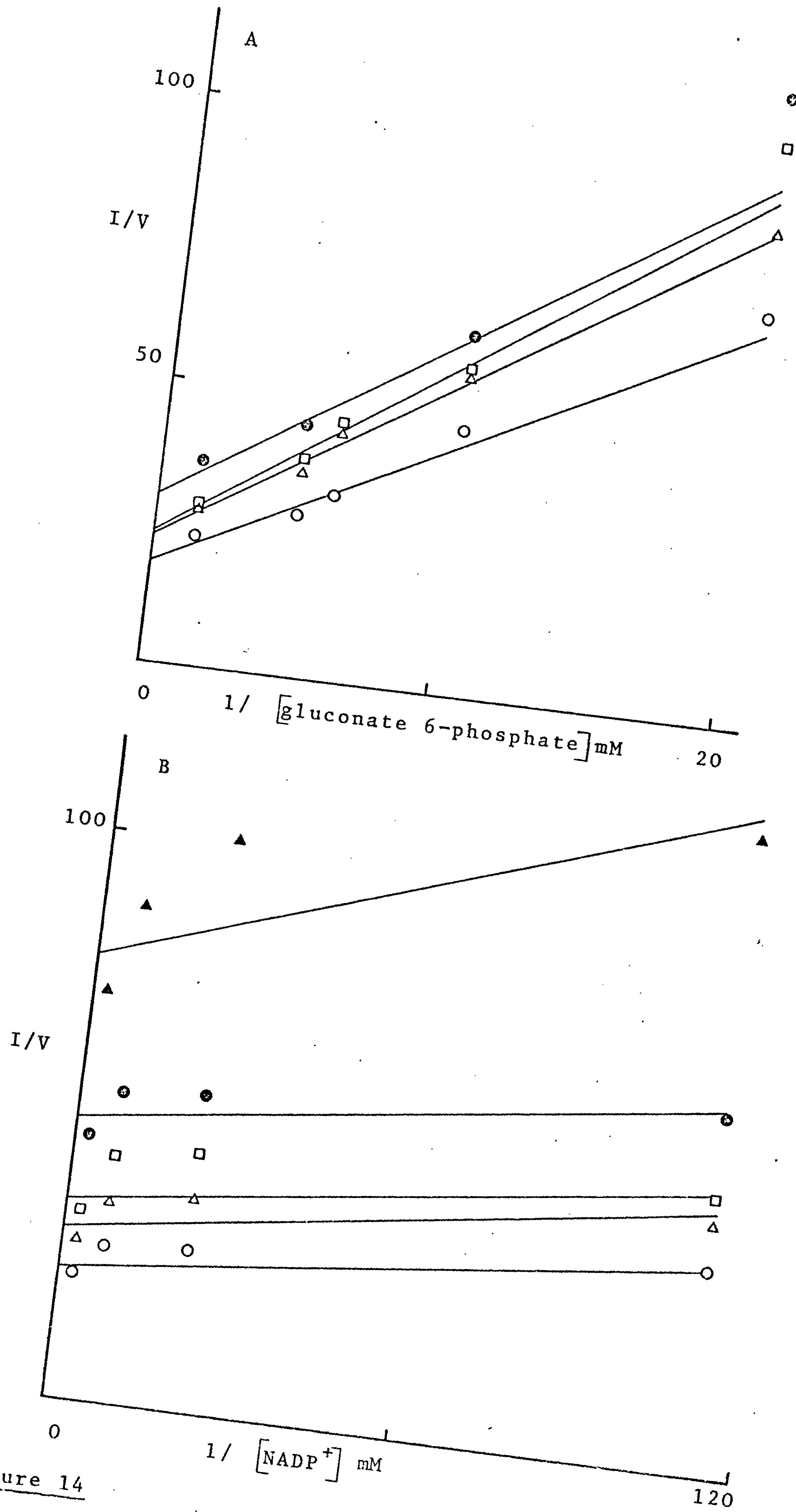


Figure 14

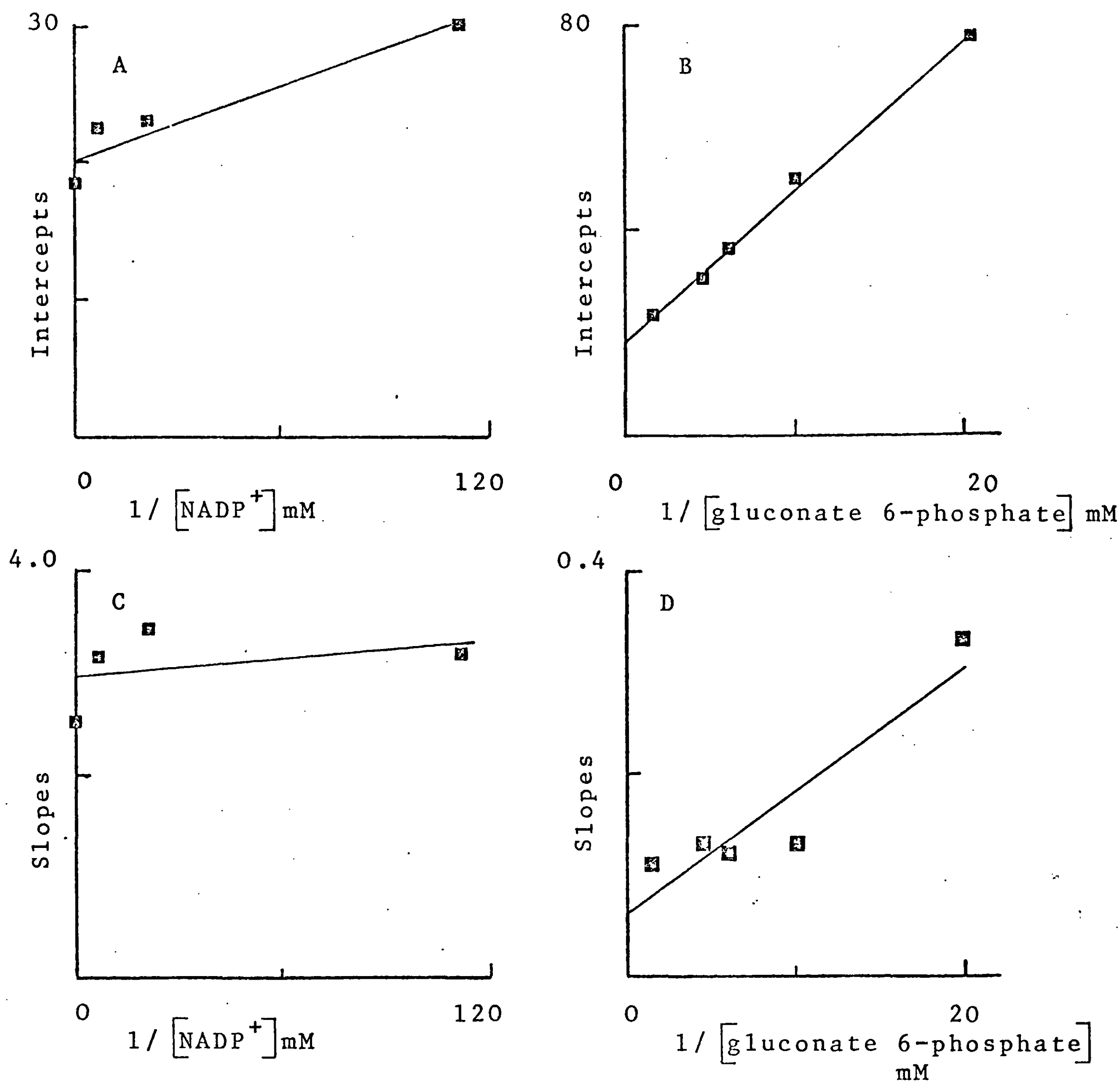


Figure 15 Secondary Plots

(A) Intercepts from 1/V versus 1/ [gluconate 6-phosphate] (Figure 14A) versus 1/ $[NADP^+]$.

(B) Intercepts from 1/V versus 1/ $[NADP^+]$ (Figure 14B) versus 1/ [gluconate 6-phosphate].

(C) Slopes from 1/V versus 1/ [gluconate 6-phosphate] (Figure 14A) versus 1/ $[NADP^+]$.

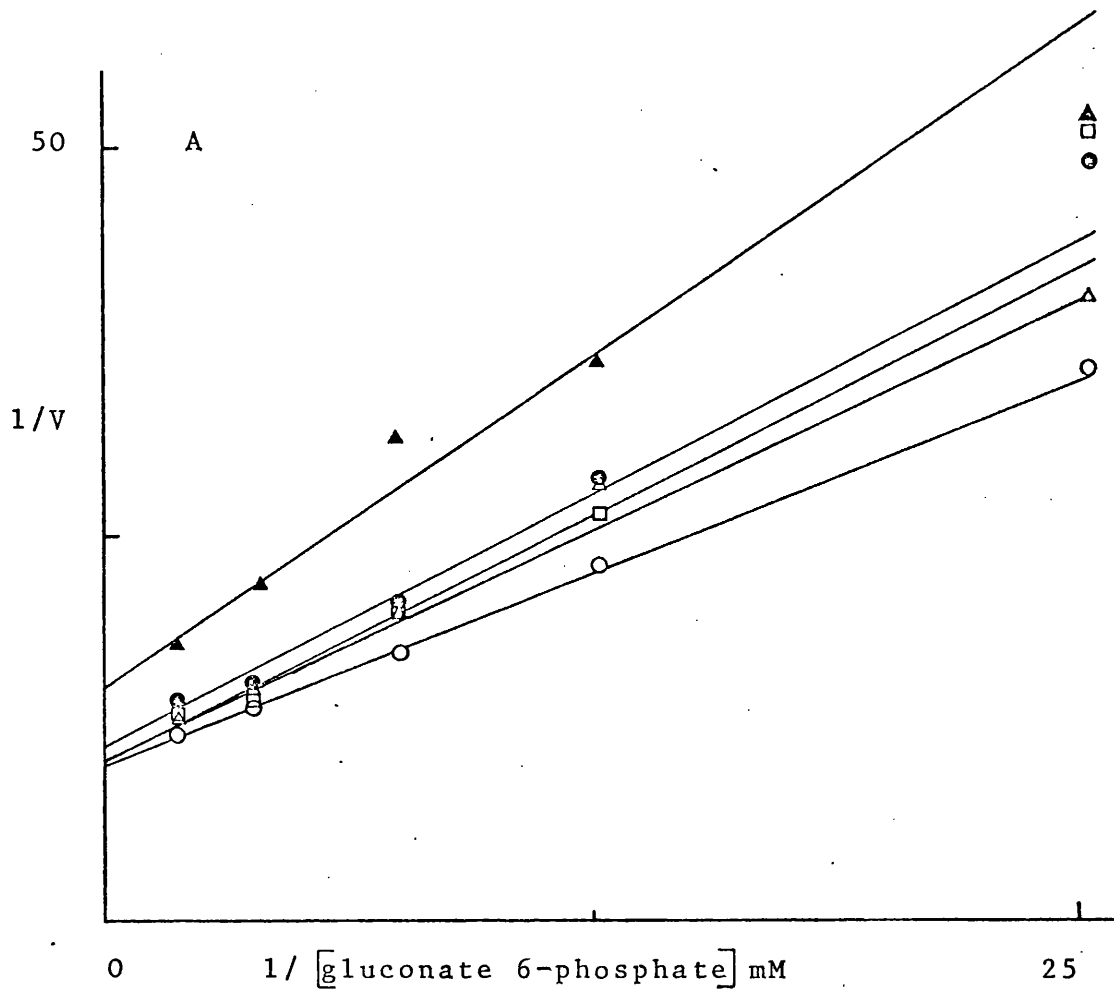
(D) Slopes from 1/V versus 1/ $[NADP^+]$ (Figure 14B) versus 1/ [gluconate 6-phosphate].

Figure 16 Lineweaver-Burk plots. All assays were carried out at pH7.5.

(A) 1/V versus 1/ (gluconate 6-phosphate) mM with the following $NADP^+$ CONCENTRATIONS: O, 0.883 mM; △, 0.353 mM; □, 0.087 mM; ●, 0.022 mM; ▲, 0.009 mM.

(B) 1/V versus 1/($NADP^+$) mM with the following gluconate 6-phosphate concentrations; o, 0.526 mM; △, 0.263 mM; □, 0.131 mM; ●, 0.079 mM; ▲, 0.04 mM.

Commercial gluconate 6-phosphate was used.



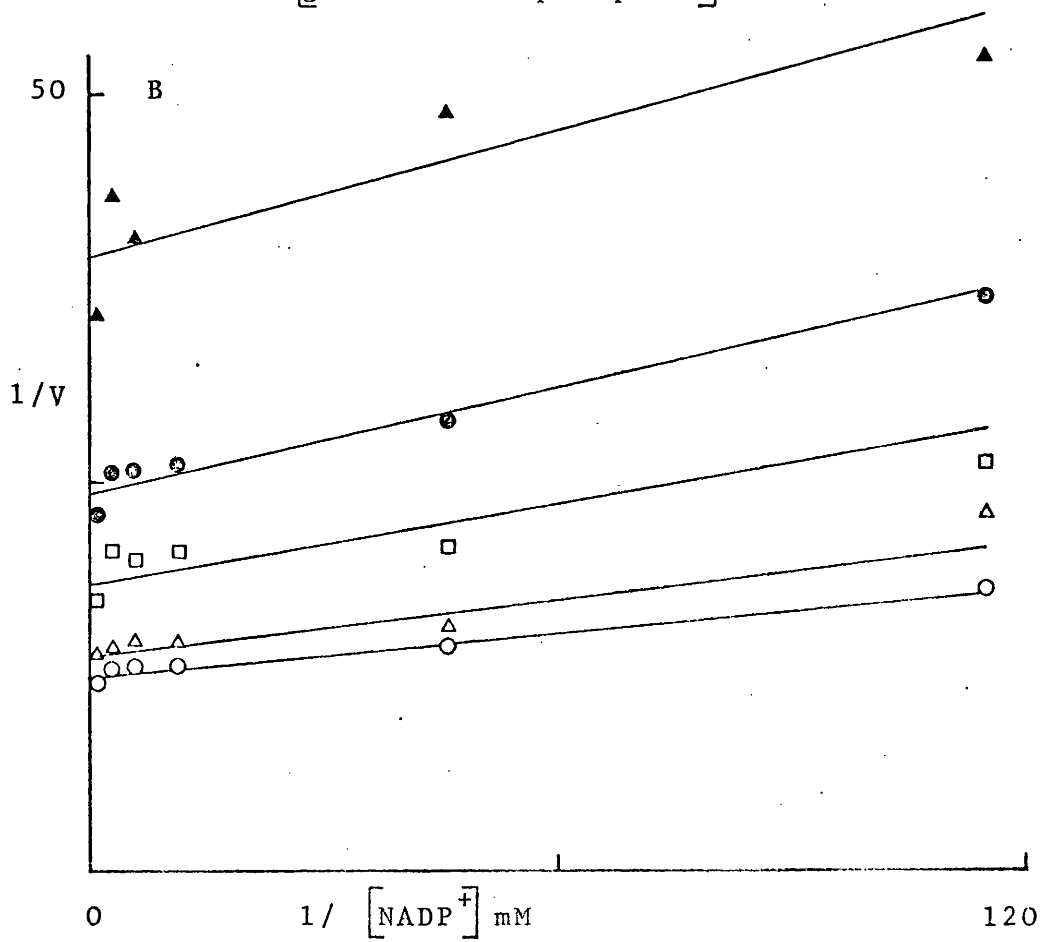


Figure 16

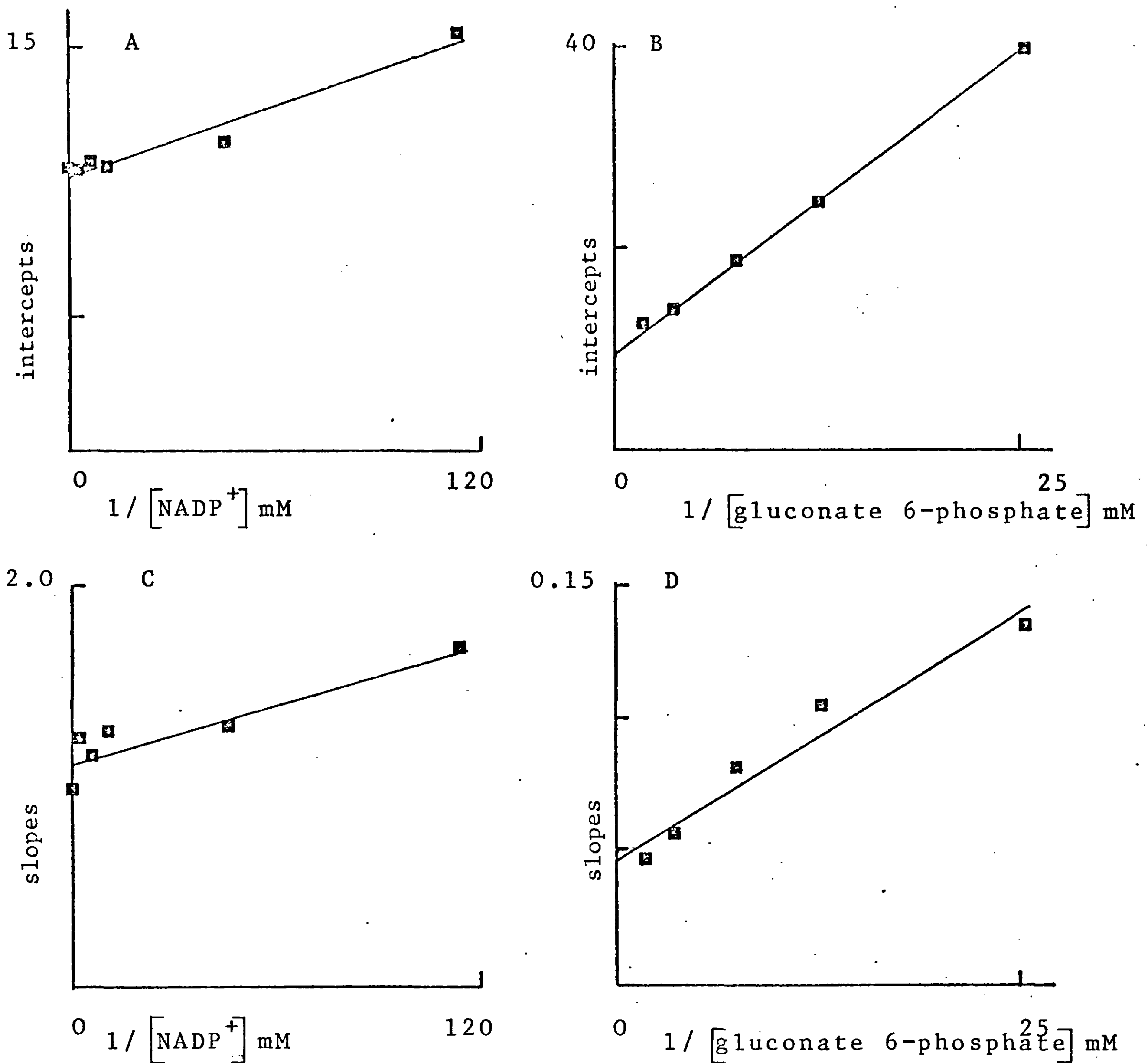


Figure 17 Secondary plots

(A) Intercepts from 1/V versus 1/ [gluconate 6-phosphate] (Figure 16A) versus 1/ [$NADP^+$]

(B) Intercepts from 1/V versus 1/ [$NADP^+$] (Figure 16B) versus 1/ [gluconate 6-phosphate]

(C) Slopes from 1/V versus 1/ [gluconate 6-phosphate] (Figure 16A) versus 1/ [$NADP^+$]

(D) Slopes from 1/V versus 1/ [$NADP^+$] (Figure 16B) versus 1/ [gluconate 6-phosphate].

Figure 18 Lineweaver-Burk plots. All assays were carried out at pH 8.0.

(A) 1/V 1/(gluconate 6-phosphate) mM with the following $NADP^+$ concentrations; o, 0.446 mM; △ , 0.149 mM; □, 0.045 mM; ● , 0.023 mM; ▲ , 0.009 mM.

(B) 1/V versus 1/($NADP^+$) mM with the following gluconate 6-phosphate concentrations; o, 0.58 mM; △ , 0.29 mM; □ , 0.144 mM; ● , 0.087 mM; ▲ , 0.058 mM.

Commercial gluconate 6-phosphate was used.

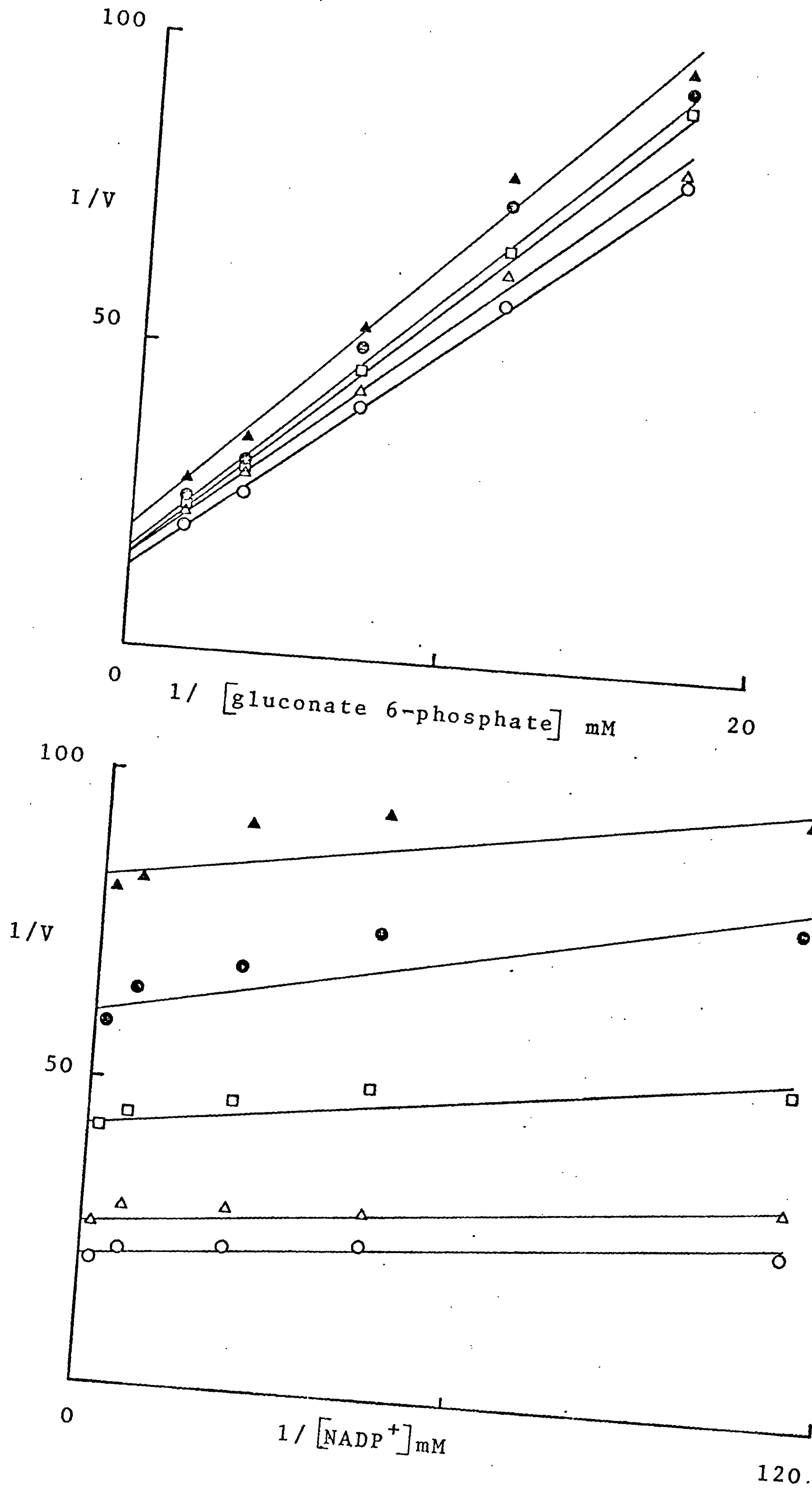


Figure 18

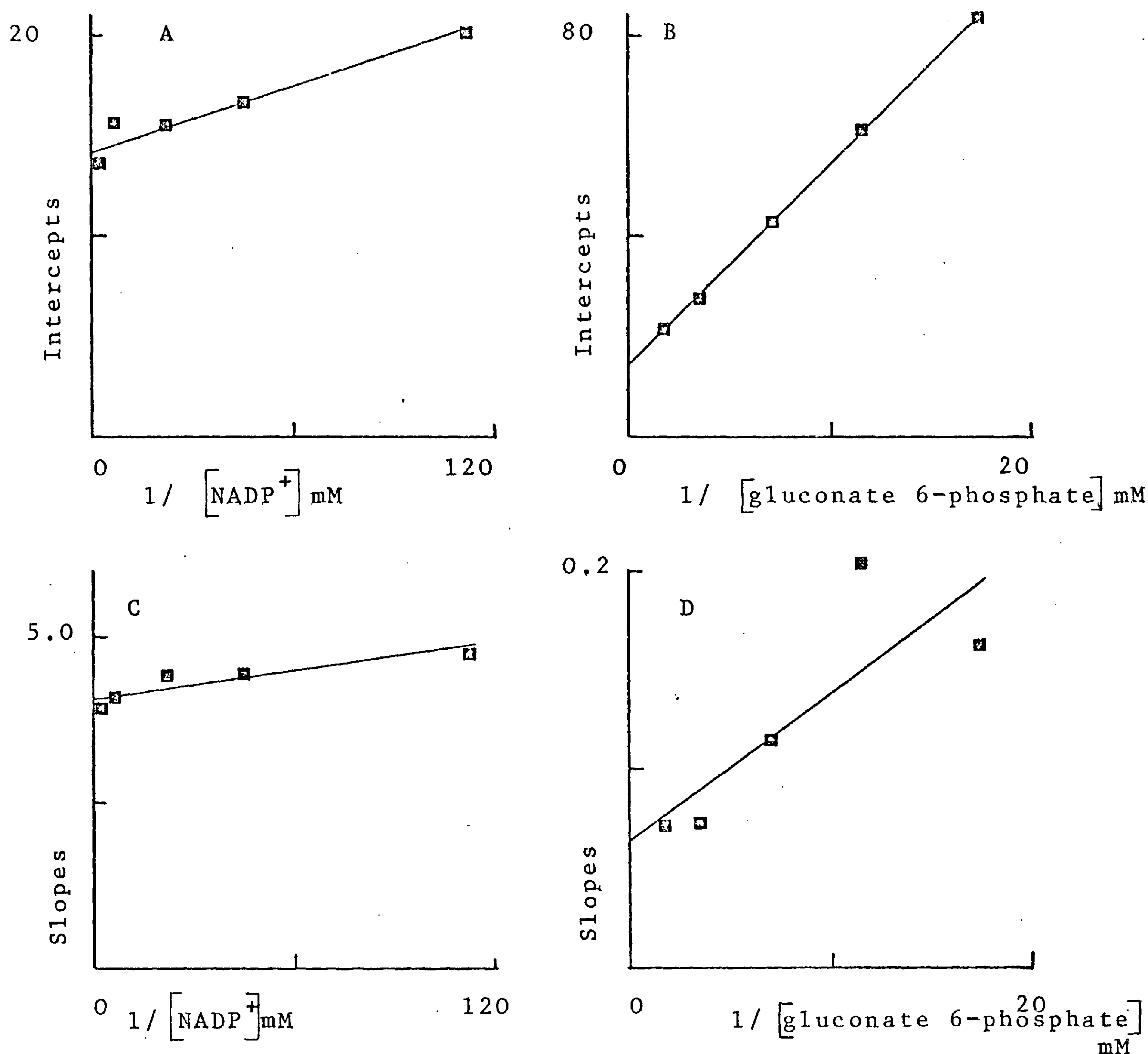


Figure 19 Secondary plots.

(A) Intercepts from 1/V versus 1/ [gluconate 6-phosphate] mM (Figure 18A) versus 1/ [$NADP^+$]

(B) Intercepts from 1/V versus 1/ [$NADP^+$] mM (Figure 18B) versus 1/ [gluconate 6-phosphate] mM.

(C) Slopes from 1/V versus 1/ [gluconate 6-phosphate] mM (Figure 18A) versus 1/ [$NADP^+$]

(D) Slopes from 1/V versus 1/ [$NADP^+$] (Figure 18B) versus 1/ [gluconate 6-phosphate] .

Figure 20 Lineweaver-Burk plots. All assays were carried out at pH 7·5

(A) 1/V versus 1/[analogue CXX1] mM with the following $NADP^+$ concentrations; ○ , 0.347 mM; △, 0.105 mM; □ , 0.035mM; ● , 0.017 mM; ▲ , 0.007 mM.

(B) 1/V versus 1/[$NADP^+$]mM with the following analogue CXX1 concentrations; ○ , 1.06 mM; △ , 0.709 mM; □ , 0.496 mM; ● , 0.248 mM; ▲ , 0.142mM.

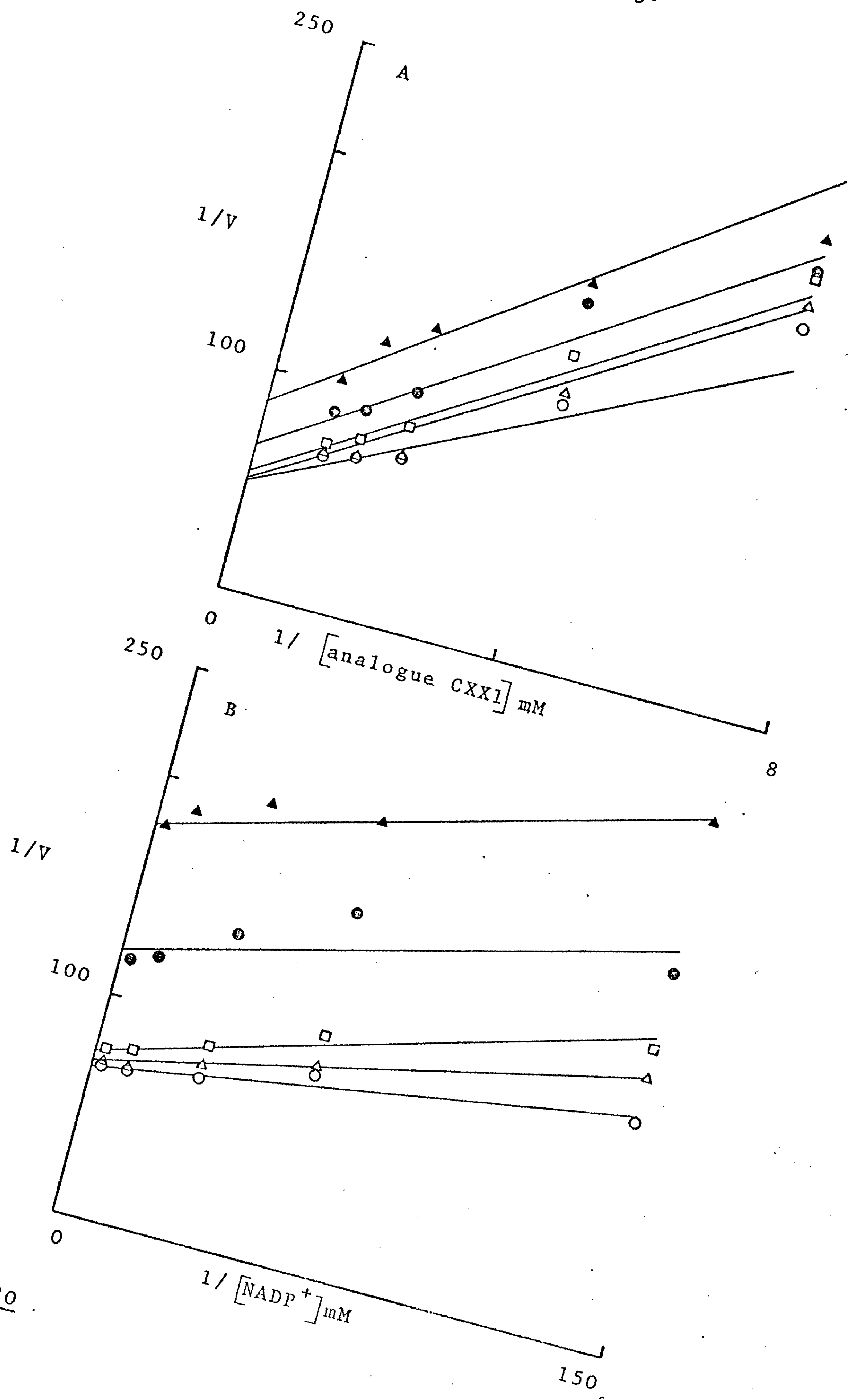


Figure 20

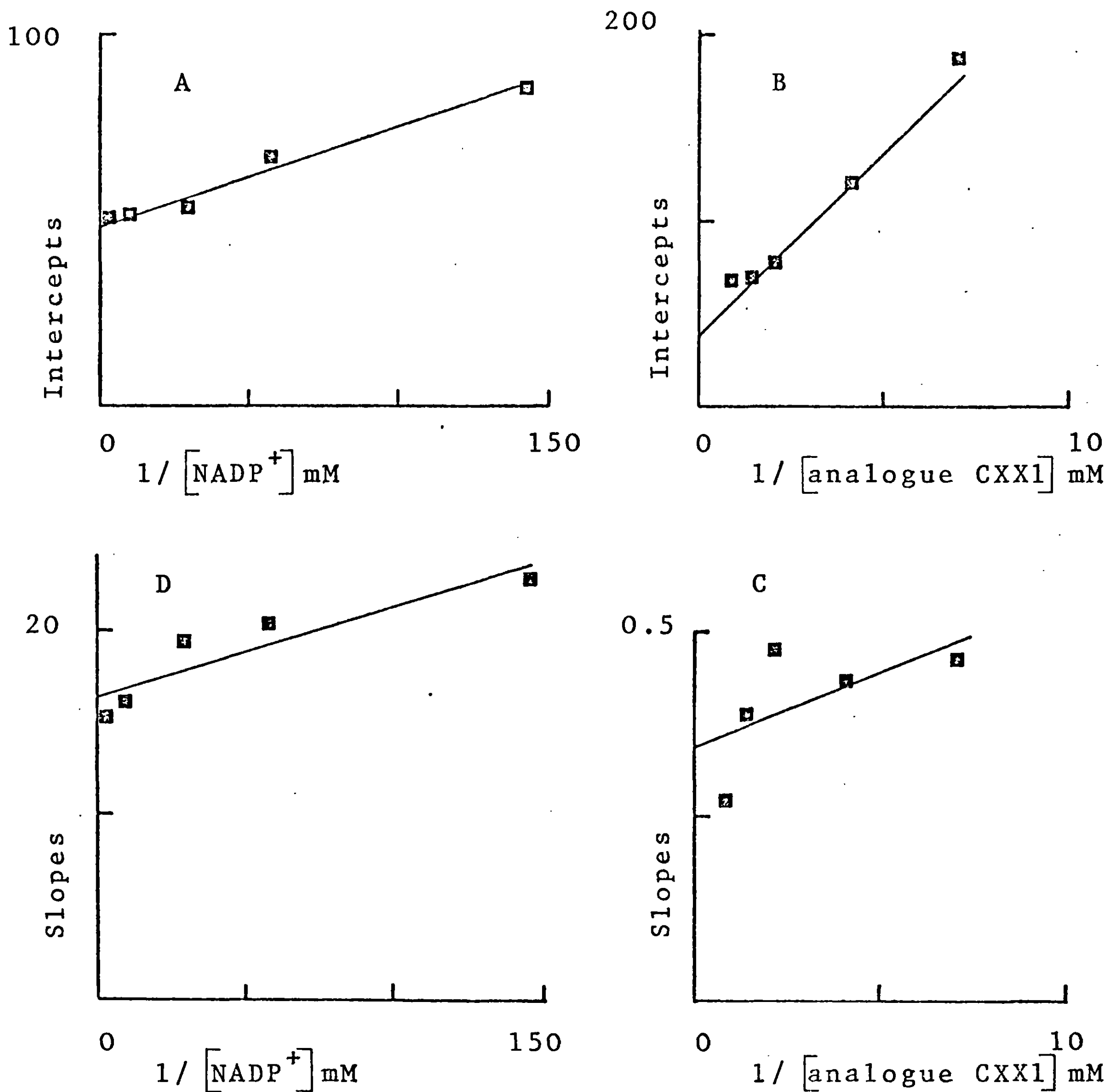
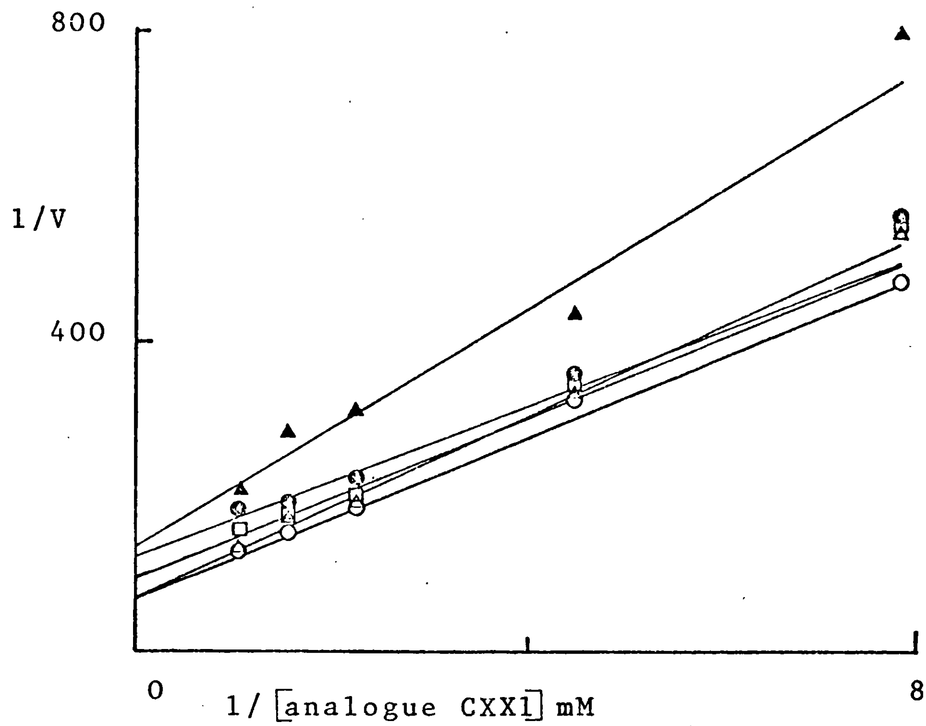


Figure 21 Secondary plots.

(A) Intercepts from 1/V versus 1/ [analogue CXX1] mM (Figure 20A) versus 1/ [$NADP^+$] mM.
(B) Intercepts from 1/V versus 1/ [$NADP^+$] mM (Figure 20B) versus 1/ [gluconate 6-phosphate] mM.
(C) Slopes from 1/V versus 1/ [analogue CXX1] mM (Figure 20A) versus 1/ [$NADP^+$] mM.
(D) Slopes from 1/V versus 1/ [$NADP^+$] mM (Figure 20B) versus 1/ [gluconate 6-phosphate] mM.



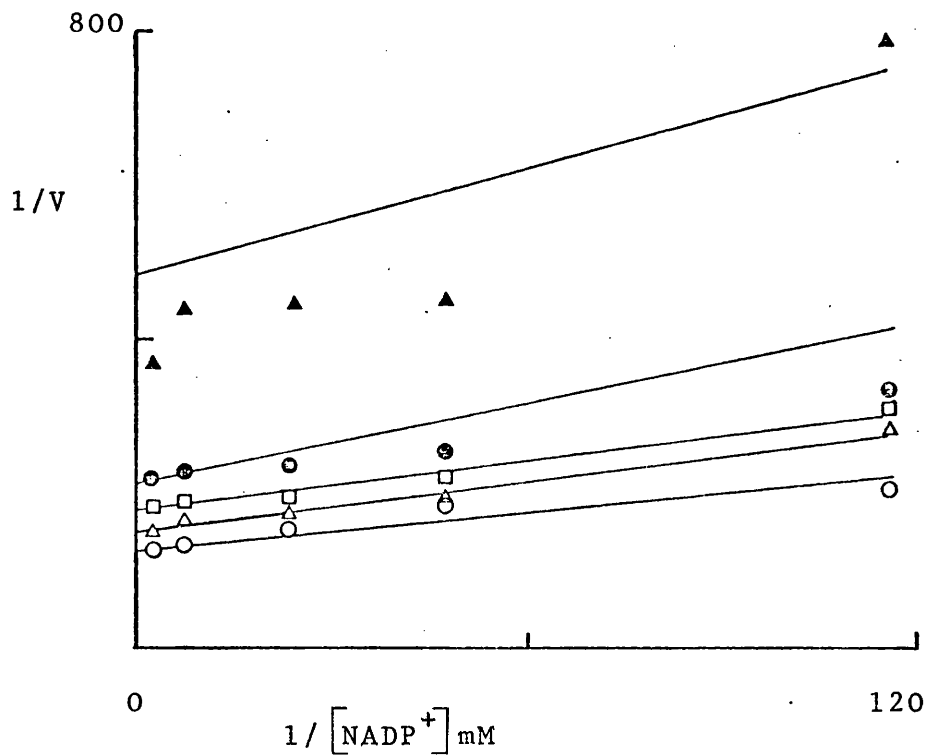


Figure 22 Lineweaver-Burk plots. All assays were carried out at pH 8.0. (A) 1/V versus 1/ [analogue CXX1] mM. with following $NADP^+$ concentrations; ○ , 0.426mM; △, 0.142mM; □ , 0.043mM; ◉ , 0.021mM; ▲ , 0.009mM. (B) 1/V versus 1/ [$NADP^+$]mM with the following analogue CXX1 concentrations; ○ , 0.956mM; △ , 0.637mM; □ , 0.446mM; ● , 0.223mM; ▲ , 0.128mM.

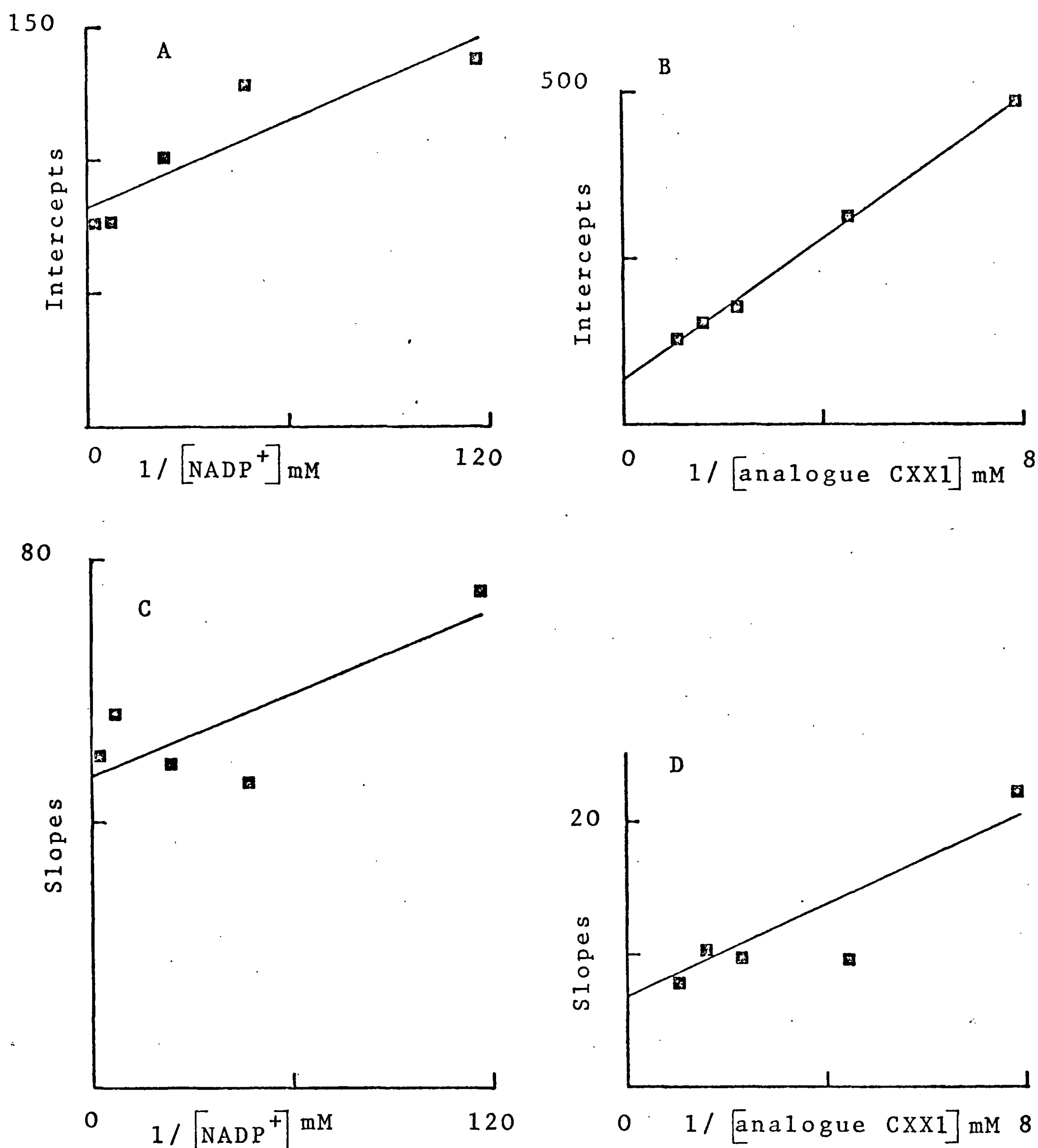


Figure 23 S condary plots

(A) Intercepts from 1/V versus 1/ [analogue CXX1] mM (Figure 22A) versus 1/ [NADP+] mM.
(B) Intercepts from 1/V versus 1/ [NADP+] mM (Figure 22B) versus 1/ [analogue CXX1] mM.
(C) Slopes from 1/V versus 1/ [analogue CXX1] mM (Figure 22A) versus 1/ [NADP+] nM.
(D) Slopes from 1/V versus 1/ [NADP+] mM (Figure 22B) versus 1/ [analogue (XXI)] mM.

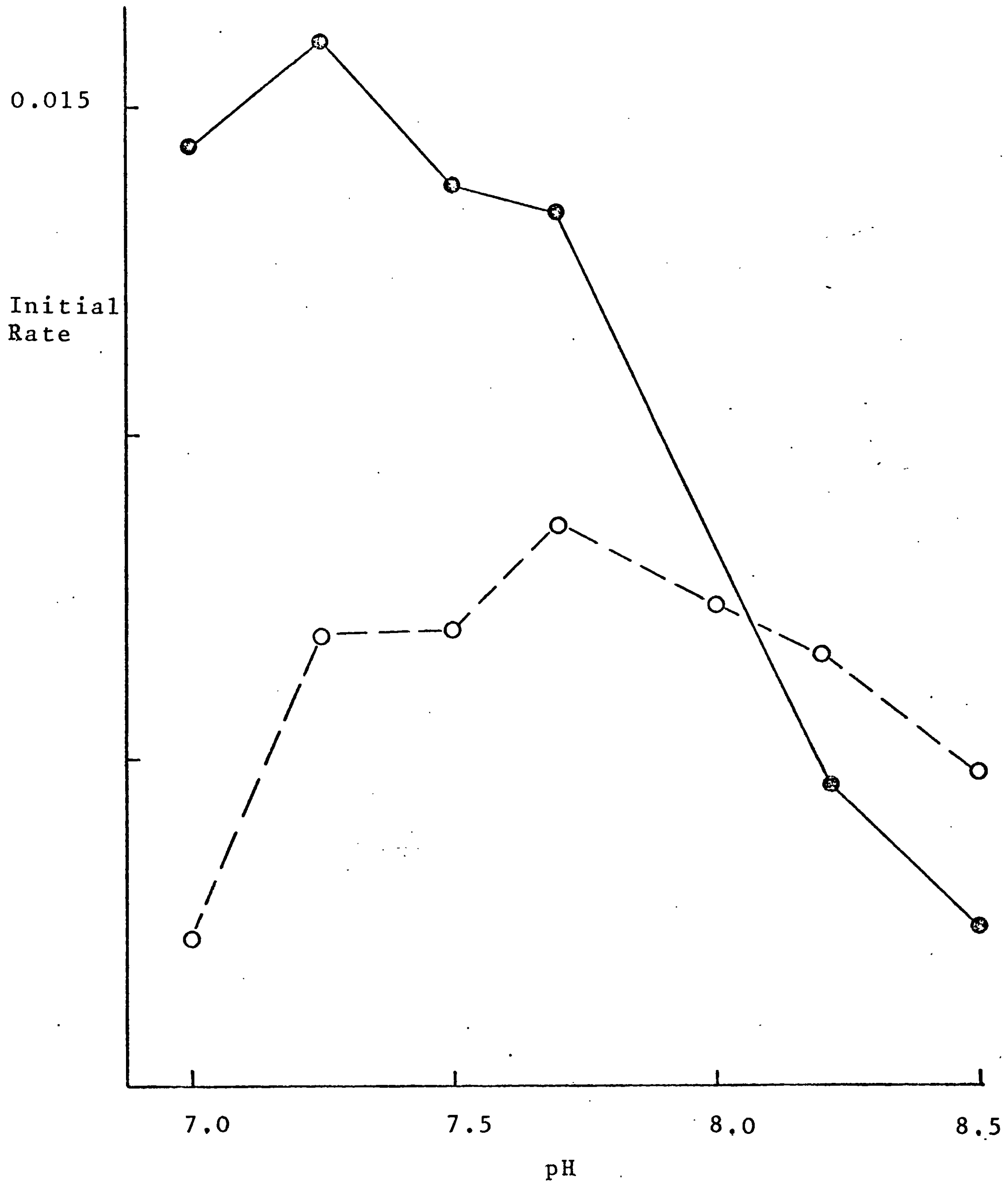


Figure 24 Effect of pH on the initial rate of dehydrogenation of 77µM gluconate 6-phosphate (●——●), and 218µM analogue CXXI (○- - - -○). All assays contained 136 µM $NADP^+$. The initial rates are expressed as the change in absorbance/min/25µg gluconate 6-phosphate dehydrogenase.

Effect of 6,7 dideoxy-α-*D*-*gluco*-heptose 7-phosphonic acid on cell proliferation of mouse lymphoma cells (LS 1210)

INTRODUCTION

The Introduction section of this thesis contained a number of examples in which phosphonate analogues were shown to be capable of inhibiting mutant strains of *E. coli* and *B. subtilus* (pages 68-70.) Through the efforts of Engel and his co-workers (Kabak *et al.*, 1972; Shopsis *et al.*, 1972, 1973) it was shown that the isosteric phosphonate analogue (XCIII) of glycerol 3-phosphate was capable of inhibiting the growth of mutant strains of *E. coli* at low concentrations. The use of the tritiated form of analogue XCIII showed that such inhibition resulted from perturbation of normal phospholipid production. In treated *E. coli* there was a rapid inhibition of the rate of phosphatidylglycerol synthesis, a slower but almost as pronounced inhibition of the rate of phosphatidylethanolamine synthesis (Shopsis *et al.*, 1974) and the appearance of a new phosphoglyceride, the phosphonate analogue (XCVI) of phosphatidyl glycerophosphate (Tyhach *et al.*, 1976). Analogue XCVI was a substrate and inhibitor of CDP diglyceride: *sn*-glycerol-3 phosphate phosphatidyltransferase (Cheng *et al.*, 1975). More recently Leifer *et al.* (1977) have demonstrated that analogue XCIII is actively transported into mutant strains of *E. coli*.

The isosteric phosphonate analogue (XCVII) of dihydroxyacetone-phosphate has been shown (Klein *et al.*, 1977) to be incorporated into the cell wall of *B. subtilus*, acting either as a bacteriocidal or bacteriostatic agent depending on the strain investigated. Goldstein *et al.* (1974) demonstrated that the phosphonate analogue (XCIX) of glyceraldehyde 3-phosphate totally inhibited the growth of a mutant strain of *E. coli*. Most recently Tang *et al.* (1978) reported that the isosteric analogue (XCIV) of fructose 1-phosphate was an inhibitor of growth of mutant strains of *E. coli* capable of transporting phos-

phates. On the other hand the nonisosteric and isosteric phosphonate analogues (C and CII respectively) of glycerate 3 phosphate had no observable effect on several strains of Gram positive and Gram negative bacteria (Pfieffer _et al._, 1974).

In view of the above results a preliminary investigation of the effects of analogue X on the cell proliferation of mouse lymphoma cells (LS1210) was attempted.

METHODS AND MATERIALS

Chemicals

Dulbecco's modification of Eagles medium, feotal calf serum, glutamine, trypan blue and pencillin streptomycin were obtained from Flow Laboratories Ltd., Irvine, Ayrshire, U.K. Reagents used in work described earlier in this thesis were obtained from sources previously quoted and all other chemicals were purchased from BDH Chemicals, Poole, Dorset, U.K.

Mouse lymphoma cells (LS1210) were the generous gift of Dr. W.J.D. Whish. The cells were cultured in Medium A; (Dulbecco's modification of Eagles medium supplemented with 10% feotal calf serum, 2mM-glutamine and 100 units/ml pencillin, streptomycin) and grown at 37°C under 5% CO_2 in air. Asceptic technique was used throughout the procedure.

Typical procedure:- A stock solution of cells, approximately 1-2 x 10^{6} cells/ml, in stationary phase of growth were diluted 10 fold (10ml → 100ml, in medium A) and transferred to bijou bottles (9ml). Cell proliferation was monitored by counting cell number on an improved Neubauer haemocytometer (average of eight counts). When cells entered the exponential phase of growth, 10mM-glucose 6-phosphate or 10mM-analogue X or 150mM saline (1ml) (all solutions were made up in 5mM-Tribase buffer adjusted to pH 7.5 with 2M-HCl, and autoclaved for 5 min at 10 psi, prior to addition) was added to each bijou bottle (in duplicate) and cell proliferation regularly monitored.

Glucose 6-phosphate and analogue X were determined as described earlier (page 102).

RESULTS AND DISCUSSION

Experiment 1.

The stock solutions of glucose 6-phosphate and analogue X were made up in 5mM-Tris-base buffer pH 7.5, and the solutions were added to diluted suspensions of mouse lymphoma cells entering exponential growth phase. Cell suspensions containing glucose 6-phosphate (0.71mM) had no effect on cell proliferation displaying a growth curve identical to that of the control suspensions (in 150mM saline) (Figure 25). Addition of analogue X (0.95mM) on the other hand, appeared to have a cytostatic effect on cell proliferation (Figure 25). 20% of cells incubated in the presence of analogue X were dead as judged by uptake of trypan blue. Centrifugation and resuspension in fresh medium demonstrated that even at concentrations of analogue X as low as 30μM (by enzymic assay) no cell proliferation was observed.

After 150 hr incubation no glucose 6-phosphate was shown to be present (determined enzymically) in cell suspensions initially containing glucose 6-phosphate. In contrast after 150 hr incubation 0.85mM analogue X was present in cell suspensions initially containing 0.95mM analogue (representing 11% loss of phosphonate).

Experiment 2.

After dissolving analogue X in 5mM-Tris-base buffer pH 7.5, the pH was read (ph 4.0) and adjusted with Tris-base to pH 7.5. The stock solutions were added to diluted suspensions of mouse lymphoma cells entering exponential growth phase. Neither glucose 6-phosphate nor analogue X had any effect on cell proliferation with growth curves identical to those of control samples (Figure 26).

Again glucose 6-phosphate present in the medium had decreased, 0.016mM being present after 60 hr (a decrease of 85% from its initial concentration of 1.08mM). Control experiments in which 1.0mM glucose 6-phosphate was added to medium A and incubated without cells at 37°C showed that the concentration of glucose 6-phosphate dropped to 0.74mM after 45 hr. The greater loss of glucose 6-phosphate in the presence of cells clearly points to a cell-mediated effect which could result either from extracellular phosphatase action or from transport into the cells. The concentration of analogue X fell from 1.02mM to 0.88mM (a fall of 14%) over the 70 hr incubation in the presence of mouse lymphoma cells. It is possible that the 14% reduction in analogue concentration reflects its transport into lymphoma cells but this was not further investigated in view of the apparent lack of effect of the analogue on cell growth and the limited supplies of sample available.

Addition of glucose 6-phosphate and its analogue to lymphoma cells in late exponential growth gave similar results (Figure 27) in which neither compound appeared to affect cell proliferation. Incubation again resulted in major loss of glucose 6-phosphate and much less reduction of analogue in the cell medium.

The inhibition of cell growth effected by analogue X in experiment 1 clearly resulted from pH changes following addition of analogue (in the free acid form) in contrast glucose 6-phosphate was added as the disodium salt to the buffered cell suspension and the cytostatic effects of lower pH (4) were subsequently confirmed in control experiments. Experiment 1 is included here mainly as a cautionary note. The conclusion to be drawn from the exploratory

in vivo study of analogue X is that although some uptake into cells might occur there are no dramatic inhibitory metabolic effects. The greater loss of glucose 6-phosphate compared with that of its analogue in the extracellular medium could arise from resistance of the analogue of phosphatase cleavage or to its reduced transport into the cells. Resistance to phosphatase action on the part of analogue X is, of course, to be expected, is common to phosphonate analogues of this type, and most likely explains the above differences. There is some evidence for transport of analogue X into the lymphoma cells from these experiments and it may be that use of a suitably-blocked non-polar derivative might give rise to significant cytostatic action. Overall, however, the initial results were not considered to be particularly promising and the line of investigation was not pursued.

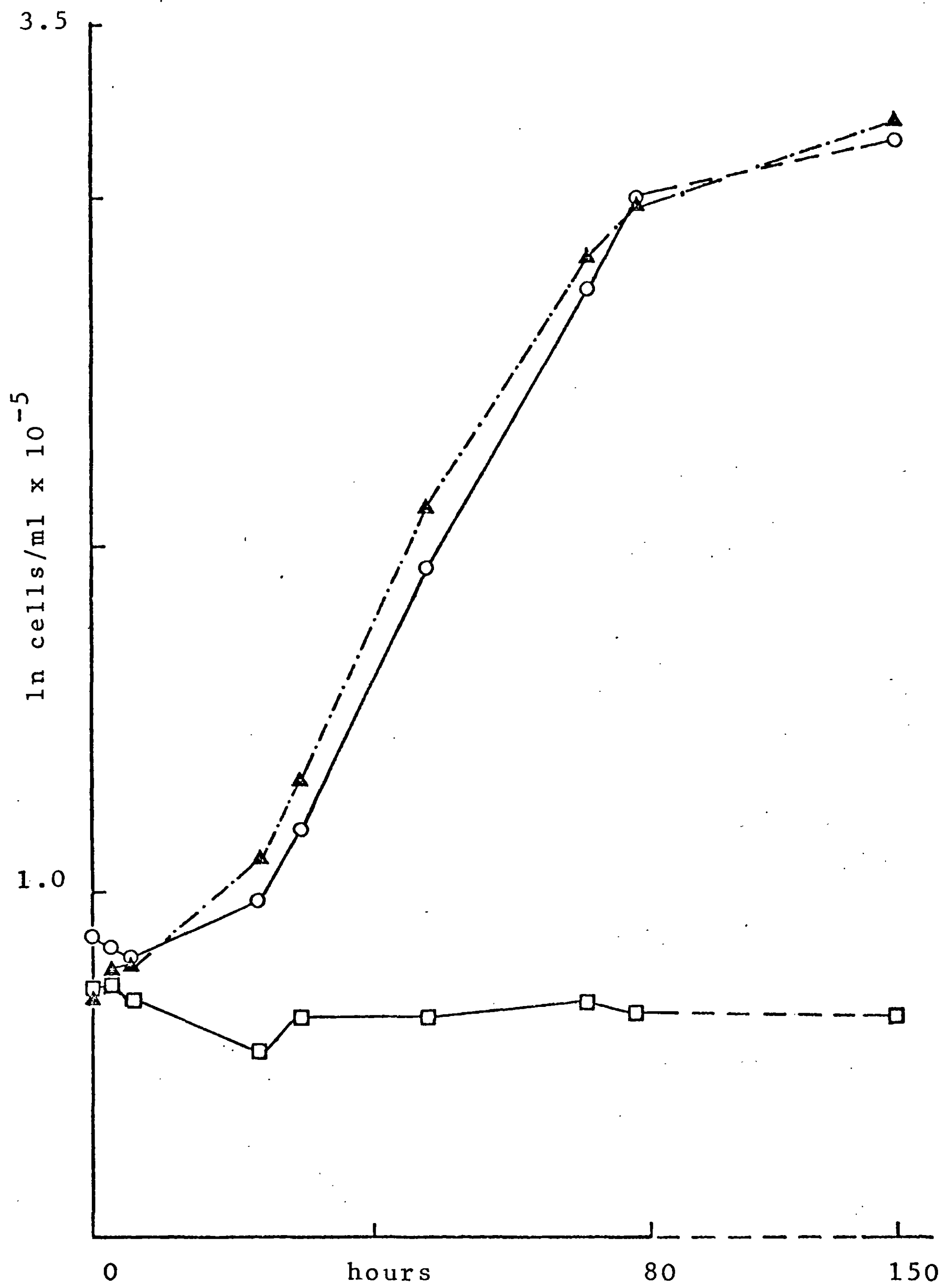


Figure 25 Experiment 1. Effect of glucose 6-phosphate and analogue X on cell proliferation of mouse lymphoma cells (LS1210). Grown in medium A plus; 0.15M NaCl (▲- - -▲); 0.71 mM glucose 6-phosphate (○——○); 0.95mM analogueX(□——□).

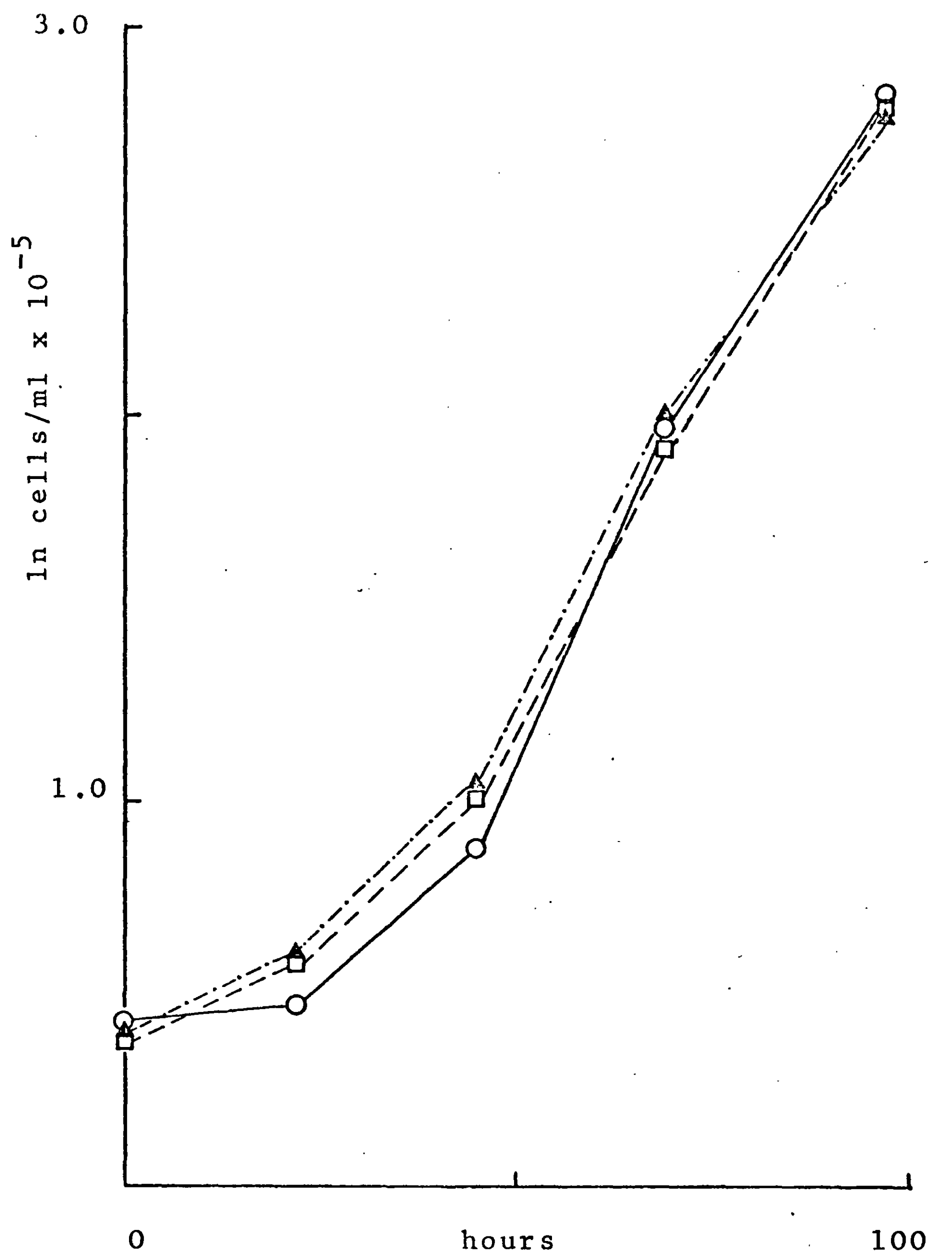


Figure 26 Experiment 2. Effect of glucose 6-phosphate and analogue x on cell proliferation of mouse lymphoma cells (LS1210). Grown in Medium A; 0.15m NaCl (▲-·-·-·-▲); 1.08mM glucose 6-phosphate (○——○); 1.02mM analogue x (□- - - -□).

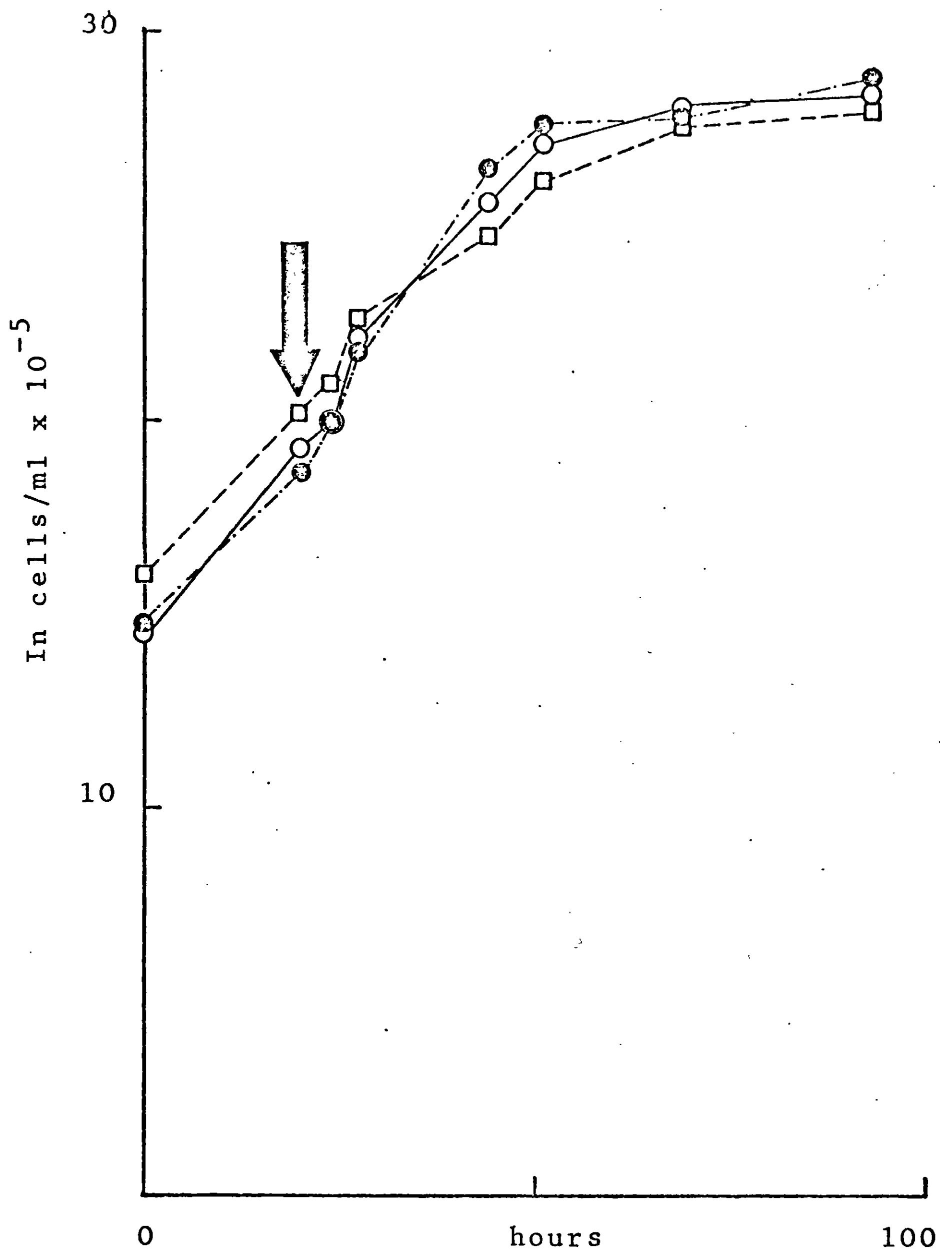


Figure 27 Experiment 3. Effect of glucose 6-phosphate and analogue X on cell proliferation of mouse lymphoma cells (LS1210). Grown in medium A; 0.15M NaCl (●-·-·-●) 1.0 mM glucose 6-phosphate (○——○); 1.02 mM analogue X(□-----□). Addition after 20hr exponential growth.

Preliminary study of the interaction of *D*-glucose 6-fluorophosphate with glucose 6-phosphate dehydrogenase

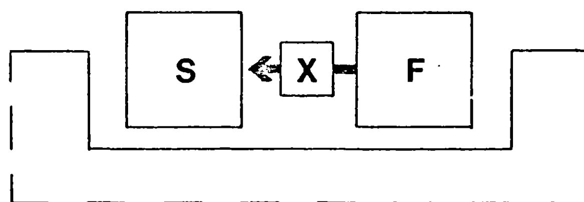
INTRODUCTION

Enzymic transferase reactions using a cofactor can be generalised in the following form

$$S + FX \rightleftharpoons SX + F$$

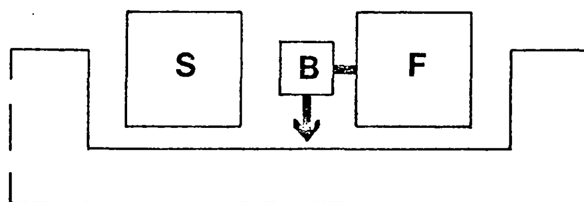
where S is the substrate and F is the cofactor bearing a group X that is to be transferred to the substrate as shown in Figure 28.

Figure 28



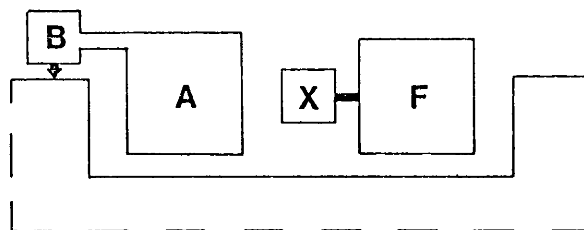
When the group X is replaced by B, a group that cannot be transferred, an inhibitor results. If B has the ability to alkylate some nucleophilic group within the active site (endoalkylation) to form a covalent bond the active site becomes selectively inhibited, as shown in Figure 29.

Figure 29



Similar inhibition of the active site can occur if an alkylating group (B) is positioned on the substrate, so as not to interfere with substrate binding at the active site, but in a place such that it can bridge to some nucleophilic group on the enzyme surface outside the active site, (exo-alkylation), then covalent-bond formation might take place as shown by the arrow in Figure 30. Once

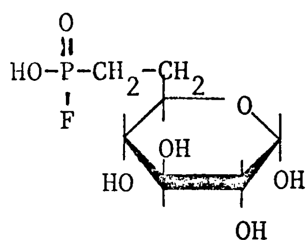
Figure 30



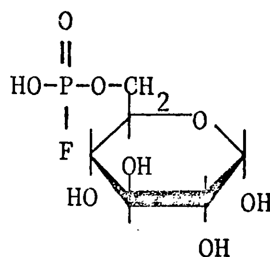
this covalent bond between inhibitor and enzyme has formed, the enzyme is blocked from any further reactions with the natural substrate. Thus inactivation tends to be very fast (accelerated-neighbouring group reaction) and as only one molecule of the irreversible inhibitor is necessary to block one enzyme molecule, inhibition constants tend to be very small. The potential of this concept as a general method for enzyme study was appreciated by Baker (1967) who coined the phrase "active-site-directed irreversible enzyme inhibitors." Baker used this concept extensively in investigations designed to produce therapeutically-useful enzyme inactivators.

Thus active-site-directed irreversible inhibitors of enzymes are designed to combine two requirements; specific binding at the active site and covalent attachment to a group on the enzyme in or near the active site.

The fluoro-phosphonate analogue CXXII is an analogue of glucose 6-phosphate and, in view of its steric similarities to the latter, might be expected to mimic the natural substrate in its interaction with an enzyme active site. Furthermore the fluoro-phosphonate moiety is a potential acylating agent which is capable of forming a covalent linkage with a suitably-placed nucleophilic group on the enzyme. Phosphonate CXXII is potentially a more useful metabolic inhibitor than the fluoro-phosphate CXXIII which is likely to be much more susceptible to inactivation by phosphatase activity in an <u>in vivo</u> situation. Nevertheless a preliminary investigation of enzyme inhibitory activity



CXXII



CXXIII

was carried out by using the fluoro-phosphate CXXIII in preference to phosphonate CXXII in view of the more ready availability of a synthetic sample of the former compound.

MATERIALS AND METHODS

Chemicals

Glucosamine 6-phosphate was purcheased from Boehringer Corp (London) Ltd, W.5. U.K. Thin layer chromatography was carried out with silica gel, particle size 0.05 - 0.2 mM obtained from Merck, Darmstadt W. Germany. 2, 3, 4 tribenzyl α D-gluco fluorophosphoramidate (CXXV) was the generous gift of Dr. C.R. Hall. Reagents used in work described earlier in this thesis were obtained from sources previously quoted and all other chemicals were purchased from BDH Chemicals, Poole, Dorset, UK. Thin layer chromatograms were visualised with spray reagent C (0.75 gm cysteine di-hydrochloride in 20 ml 50% ethanol, 25 ml 60% concentrated H_2SO_4).

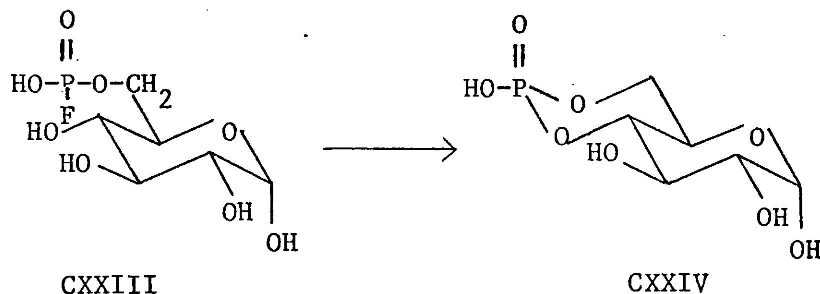
Initial rates of glucose 6-phosphate dehydrogenase assays were followed as described earlier (page102). Difference spectra were measured on a SP 1800 Unicam spectrophotometer.

All concentrations of α D-glucose 6-fluorophosphate quoted in the text assume 100% conversion of the benzylated derivative (CXXVI) and an initial product ration of 2:1 (desired product: cyclic product) (See next section.)

EXPERIMENTAL AND RESULTS

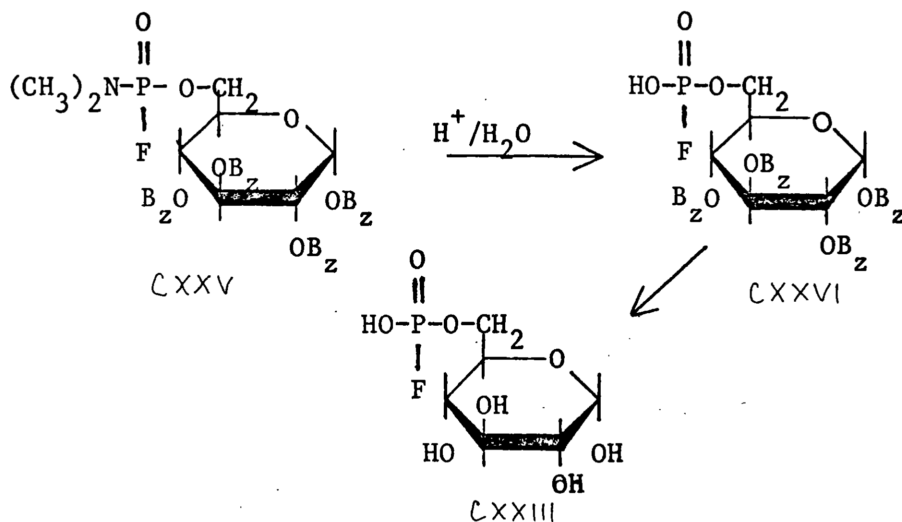
The synthesis of α-D-gluco 6-fluorophosphate (CXXIII) was (initially) carried out by Dr. C.R. Hall, at C.D.E. Porton Down, Wiltshire, UK. The ^{31}P n.m.r. suggested that the final product (CXXIII) had a half-life measured in hours; immediately after isolation of the product it contained a 2:1 ratio of expected product (CXXIII): cyclised product (CXXIV). After 2 days this ratio had changed to approximately 1:4 (CXXIII:CXXIV) and after 6 days to 1:25 (CXXIII:CXXIV) (scheme 7) (Personal communication C.R. Hall.)

Scheme 7



As the intermediate, benzyl 2, 3, 4 - tri O-benzyl α-D -gluco 6-fluorophosphoramidate (CXXV) was stable it was convenient to store this compound which represents the starting material for the work described here. When experiments were ready to be carried out with the fluoro-acid (CXXIII) the benzylated fluorophosphoramidate deritative (CXXV) was hydrogenolysed and the product, CXXIII, was worked up rapidly as possible (excess quantities of palladium catalyst were used in the hydrogenalysis in order to minimise the effect of internal cyclisation to the cyclic product (CXX) (scheme 8).

Scheme 8



Benzyl 2, 3, 4-tri-O-benzyl- α -D-gluco 6-fluorophosphate (CXXVI)

A mixture of the diastereoisomers (at the phosphorus atom) of the flurophosphoramidate (CXXV) (39 mg) was dissolved in acetone (10 ml) and 4 M - HCl (1 ml) was added dropwise. The solution was allowed to stand at room temperature for 2 hr., poured into an excess of doubly-distilled water and extracted with chloroform. The organic phase was dried ($MgSO_4$) and concentrated to give benzyl 2,3,4-tri-O-benzyl - α - D-gluco 6-fluorophosphate (CXXVI) (20 mg, yeild 60%). The production of CXXVI was monitored by t.l.c (Rf CXXVI, 0.0; CXXV 0.45 (ether : light petroleum, 2:3)).

α -D-gluco 6-fluorophosphate (CXXIII)

The benzylated derivative (CXXVI) (20 mg) was immediately dissolved in ethanol (10 ml), hydrogenolysed over 10% palladium on charcoal (0.1 g) at room temperature and atmospheric pressure, until hydrogen uptake ceased (uptake 5.2 ml, theoretical 3.05 ml. 40 mins, figure 28). The solution was filtered (over celite), washed with hot ethanol and concentrated to 7.3 ml to give a solution of α -D-gluco 6-fluorophosphate CXXIII. There was at this point no trace of startling material CXXVI by t.l.c. (Rf CXXVI, 0.3; CXXIII 0.0 (benzene:methanol:acetone, 8:2:2)).

1. Effect of α-D-glucose 6-fluorophosphate (CXXIII) on glucose 6-phosphate dehydrogenase at various pH values.

Glucose 6-phosphate dehydrogenase was diluted to 1 x 10^{-7} M in 100 mM-Tris-HCl buffer containing 1% bovine serum albumin and adjusted to the required pH (7.0, 7.5 or 8.0) with 2 M-NaOH. Each enzyme solution was incubated in the presence of 0.28 mM - α-D-gluco 6-fluorophosphate (0.48 mM in the case of pH 7.0)

The activity of the enzyme solutions containing the fluoro-acid (CXXIII) was measured with a standard assay containing 0.4 mM - glucose 6-phosphate and 0.1 mM-$NADP^+$ in 100 mM-Tris-HCl, adjusted to the required pH (7.0, 7.5 or 8.0) with 2 M NaOH in a 1 cm - pathlength cell (total volume 3.005 ml). Activities were assayed at intervals of time and compared with that of a control enzyme solution lacking the fluoro-acid. Reactions were initiated by the addition of enzyme (5 μl). (Figure 32). At none of the three pH values tested did α-D-glucose 6-fluorophosphate (CXXIII) inhibit the activity of glucose 6-phosphate dehydrogenase over a period of 24 hours (Figure 29). Even when in nearly 5000 fold excess (48 mM / 0.0001 mM, CXXIV / enzyme) over the enzyme, α-D glucose 6-fluorophosphate did not detectably inhibit the enzyme. These results were repeated in a series of 5 experiments.

2. Difference Spectra

If α-D-glucose 6-fluorophosphate reacts at the active site of the enzyme in a similar manner to that of glucose 6-phosphate one would expect a close similarity in the difference spectra for (1) native enzyme versus enzyme plus excess glucose 6-phosphate and (2)

native enzyme versus enzyme plus excess α-D-glucose fluorophosphate. However no meaningful difference spectra were observed in either case between the absorbances at 200 and 700 nM.

3. Substrate Activity of α-D-glucose 6-fluorophosphate

Within 2hr of hydrogenolysis 0.18mM α-D-glucose 6-fluorophosphate showed no substrate activity towards glucose 6 phosphate dehydrogenase in assays run at 3 different pH values (7.0, 7.5 and 8.0). A small endogenous rate (0.001 OD/min) was observed in assays containing α-D-glucose 6-fluorophosphate prior to addition of enzyme.

4. Competitive inhibition of glucose 6-phosphate by α-D-glucose 6-fluorophosphate on glucose 6-phosphate dehydrogenase

Reaction mixtures contained varying concentrations of glucose 6-phosphate (12.5 - 243 μM) and α-D-glucose 6-fluorophosphate (0, 130 or 260 μM), $NADP^+$ concentration was held constant (62 μM) in 100 mM-Tris-base buffer adjusted to pH 7.5 with 2 M-HCl in a 5 cm pathlength cell (total volume 5.005 ml). Reactions were initiated by the addition of enzyme (0.05 μg in 5 μl of buffer containing 1% bovine serum albumin). A small endogenous rate (0.003 OD/min) was observed in assays containing α-D-glucose 6-fluorophosphate prior to addition of enzyme. 0.13 and 0.26 mM concentrations of αD-glucose 6-fluorophosphate appeared not to competitively inhibit glucose 6-phosphate dehydrogenase.

5. Competitive inhibition of glucose 6-phosphate dehydrogenase by glucosamine 6-phosphate.

Reaction conditions were as described in 4 (above), except that

glucosamine 6-phosphate replaced α-D-glucose 6-fluorophosphate. Glucosamine 6-phosphate was shown to be a competitive inhibitor with respect to glucose 6-phosphate (Figure 33) with a K_i value of 1.48 mM in general agreement with the findings of Glaser and Brown (1956).

DISCUSSION

α-D-glucose 6-fluorophosphate was found not to be an active site - directed irreversible inhibitor of glucose 6-phosphate dehydrogenase. Even when the compound was used in 5000 fold excess of the enzyme no inhibition was observed over a 24 hr period. In contrast Bromohydroxyacetone phosphate, a potent active side directed irreversible inhibitor, inactivated triose phosphate isomerase from chicken muscle in 1.2 fold excess within 5 min and the inactivation reaction was too fast for convenient study of the inactivation kinetics. (De La Mare et al 1972) The failure of the analogue to act as an inhibitor cannot be explained by its internal cyclisation reaction, for even if it cyclised 1000 times faster than the rate observed (by C.R. Hall), inhibition of the enzyme should be easily detected.

Furthermore, α-D-glucose 6-fluorophosphate did not appear to be a substrate for or competitive inhibitor of glucose 6-phosphate dehydrogenase at 10^{-4}m concentrations. One possible explanation for lack of observed inhibition could be the result of adding α-D-glucose 6-fluorophosphate in a solution of ethanol. However controls containing only ethanol were used to counterbalance any effect ethanol might have on the assays. To check that the assay system worked, glucosamine 6-phosphate was shown to produce product inhibition of glucose 6-phosphate dehydrogenase. (Figure 30) The cause of the endogenous rates observed prior to addition of glucose 6-phosphate dehydrogenase in assays containing 10^{-4}m quantities of α-D-glucose 6-phosphate is not known, however it could possibly be the result of ethanol mixing with aqueous solution. (Occasionally control assays containing ethanol alone displayed similar endogeneous

rates.)

The work-up of the benzylated fluorophosphoramidate (CXXV) was repeated 5 times, on no occasion did the product behave as an active site directed irreversible inhibitor of glucose 6-phosphate dehydrogenase.

In favourable cases, where the amino acid composition around the active site is known, it may be possible to design the reagent to react with a particular postulated catalytic function. In this instance such information is not know at the present time and it is possible that no suitably-reactive nucleophilic groups are available for neighbouring group-type reactions.

A less likely hypothesis for the lack of observed inhibition by α-D-glucose 6-fluorophosphate stems from the fact this compound spontaneously cyclises (indicating the reactivity of the fluorine leaving grouping). Thus there is the possibility that α-D-glucose 6-fluorophosphate may react indiscriminately with any suitably-reactive nucleophilic groups present on the enzyme, which would have no direct effect on the activity of the enzyme.

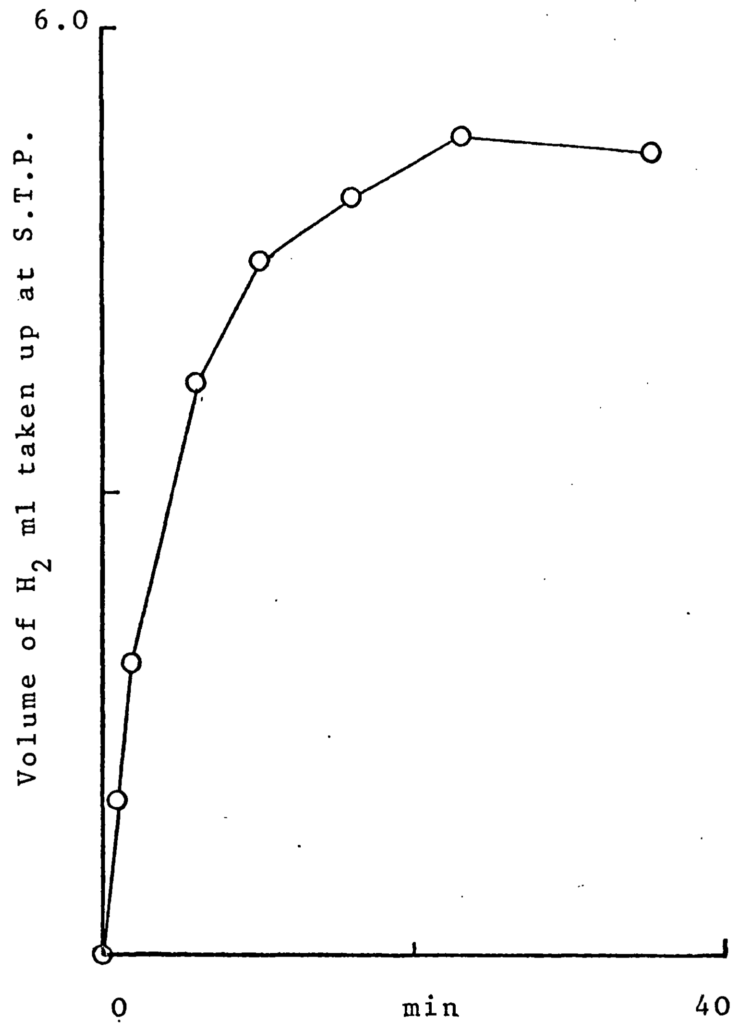


Figure 31 Typical hydrogen uptake of the final hydrogenolysation step.

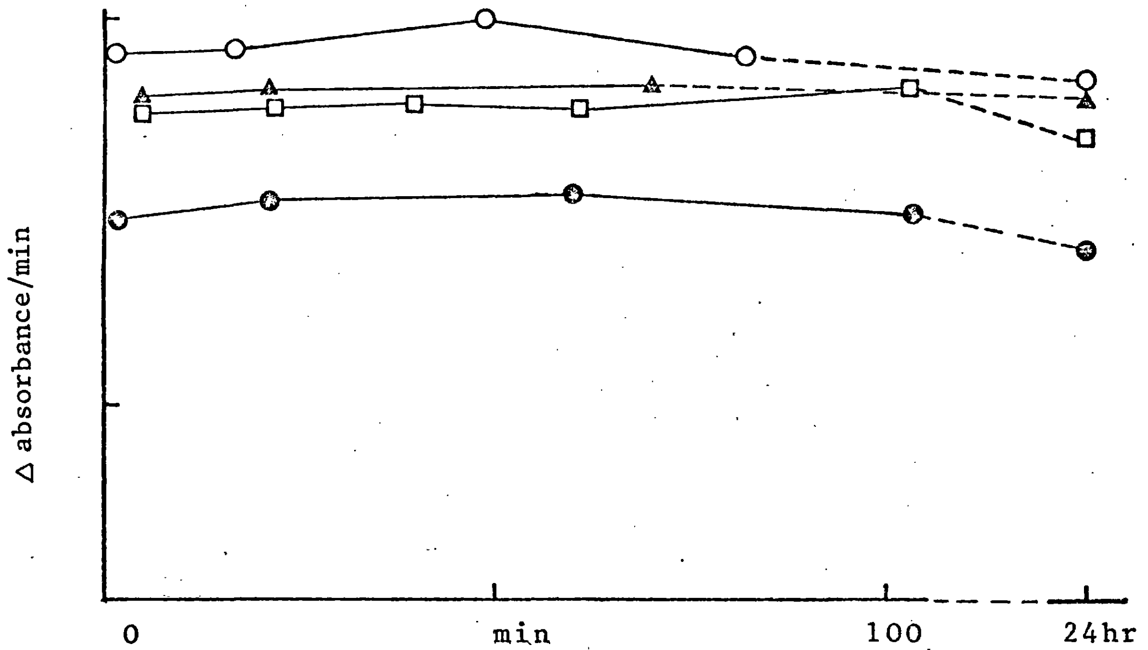


Figure 32 Effect of α- D - glucose 6-fluorophosphate (CXXIII) on glucose 6-phosphate dehydrogenase.

pH	addition		
7.0	0.48mM	CXXIII	(●——●)
7.5	0.24mM	CXXIII	(□——□)
8.0	0.24mM	CXXIII	(○——○)
7.5	100 μl	ethanol	(▲——▲)

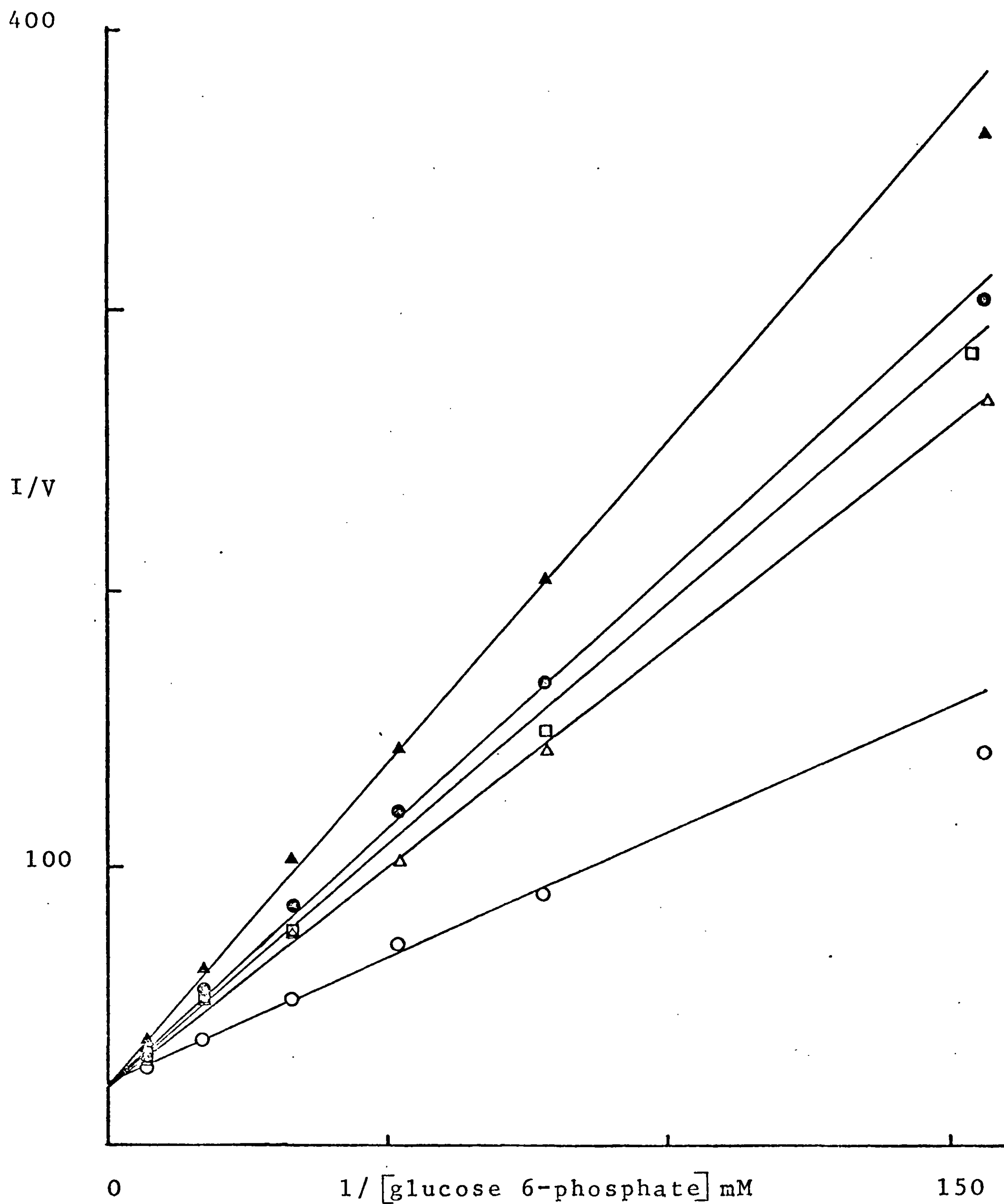


Figure 33 Lineweaver-Burk plots for inhibition of glucose 6-phosphate dehydrogenase by glucosamme 6-phosphate. Cencentration of glucosamine 6-phosphate, ○, 0; △ ,1.54 mM; □ , 1.89mM; ⊚ , 2.06mM; ▲, 2.57mM.

GENERAL CONCLUSIONS

The phosphonate analogues synthesised and tested in the presently-described work have been shown to behave qualitatively like their respective natural-occurring phosphates in enzymic interactions but less effectively. Similar instances of such behaviour have been quoted in the Introduction section to this thesis and the most likely explanations in all cases involve assumptions of slightly-changed geometry around the CH_2-CH_2-P linkage region compared with that of CH_2-O-P, together with possible loss of oxygen-enzyme interactions in the phosphonate case. Such changes might well be expected to lead to decreased affinity of the analogue for the enzyme active site. As has been detailed in the Introduction section, however, this is not always the case and some phosphonate analogues have been shown to behave as effectively as the natural-phosphates in their interactions with certain enzymes. No consistent pattern has emerged and it seems that although decreased interaction with enzymes is the most likely result of replacing CH_2-O-P of a natural phosphate with CH_2-CH_2-P each system should be individually checked experimentally. The most likely potential of phosphonate analogues of naturally-occurring phosphates probably lies in their use in clear-cut situations such as their application as probes,to decide whether a biochemical effect observed using a natural phosphate results from phosphate-cleavage or simply from its binding to a suitable receptor site. The stability of the C-P bond in biochemical systems certainly allows the possibility of prolonging the <u>in vivo</u> life of any phosphonate metabolic inhibitor and despite the negative results obtained

using the glucose 6-phosphate dehydrogenase system described here the potential of phosphonates as the basis of active-site directed irreversible inhibitors remains real at the present time.

REFERENCES

Adams, P.A., Harrison, R. and Inch, T.D. (1974) Biochem. J. 141, 729-732.

Adams, P.A., Harrison, R., Inch, T.D. and Rich, P. (1976) Biochem. J. 155, 1-4.

Adams, R. in Organic Reactions VI, 352-353.

Albrecht, H.P., Jones, G.H. and Moffatt, J.G. (1970) J. Amer. Chem. Soc. 92, 5511-5513.

Afolayan, A. (1972) Biochemistry 11, 4172-4178.

Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769-775.

Aria, T., Ihara, Y. and Kaziro, Y. (1975) J. Biochem. 77, 647-658.

Atkinson, M.R. and Murray, A.W. (1967) Biochem. J. 104, 10C-12C.

Atkinson, M.R. and Polya, G.M. (1967) Aust. J. Biol. Sci. 20, 1069-1080.

Bachelard, H.S., Clark, A.G. and Thompson, M.F. (1971) Biochem. J. 123, 707-715.

Baer, E., Nazir, D.J. and Basu, H. (1969) Can. J. Biochem. 47, 992-994.

Baer, E. and Stanacev, N.Z.(1966) Can. J. Biochem. 44, 893-897.

Baker, B.R. (1967) Design of Active-Site-Directed Irreversible Enzyme Inhibitors. John Wiley & Sons, N.Y.

Bax, P.C., Morris, F. and Rammler, D.H. (1970) Biochim. Biophys. Acta 201, 416-424.

Benesch, R.E., Benesch, R., Yung, S., Baer, E. and Robinson, R. (1973) Can. J. Biochem. 51, 1120-1122.

Bennett, R., Burger, A. and Umbrelt, W.W. (1959) J. Med. Pharm. Chem. 1, 213-221.

Berman, K.M. and Cohn, M. (1970) J. Biol. Chem. 245, 5319-5324.

Binet, A. and Volfin, P. (1974) Arch. Biochem. Biophys. 164, 756-764.

Bittman, R. and Blau, L. (1972) Biochemistry 11, 4831-4839.

Bjerve, K.S. (1972) Biochim. Biophys. Acta 270, 348-363.

Burger, R.M. and Lowenstein, J.M. (1970) J. Biol. Chem. 245, 6274-6280.

Burger, R.M. and Lowenstein, J.M. (1975) Biochemistry 14, 2362-2370.

Buttlaire, D.H. and Reed, G.H. (1975) J. Biol. Chem. 250, 261-270.

Casazza, J.P. and Fromm, H.J. (1976) Arch. Biochem. Biophys. 177, 480-487.

Chen, P.S.(Jr.), Toribara, T.Y. and Warner, H. (1956) Analytical Chemistry 28, 1756-1758.

Cheng, P-J., Hickey, R., Engel, R. and Tropp, B.E. (1974) Biochim. Biophys. Acta 341, 85-92.

Cheng, P-J., Nunn, W.D., Tyhach, R.J., Goldstein, S.L., and Tropp, B.E. (1975) J. Biol. Chem. 250, 1633-1639.

Chou, J.Y. and Singer, M.F. (1970) J. Biol. Chem. 245, 995-1004.

Chou, J.Y. and Singer, M.F. (1971) J. Biol. Chem. 246, 7497-7504.

Colowick, S.P. (1973) in The Enzymes (Boyer, P.D. ed.) Vol.9, pp.1-48, Academic Press, N.Y.

Cook, P. and Murdoch, L. (1973) Biochemistry 12, 3927-3932.

Corey, E.J. and Volante, R.R. (1976) J. Amer. Chem. Soc. 98, 1291-1293.

Cornish-Bowden, A. (1976) Principles of enzyme kinetics, Chap.10, pp.168-193.

Crane, R.K. and Sols, A. (1954) J. Biol. Chem. 210, 597-606.

Cuatrecasas, P., Jacols, S. and Bennett, V. (1975) Proc. Nat. Acad. Sci. U.S. 72, 1739-1744.

Curthoys, N.P. and Rabinowitz, J.C. (1971) J. Biol. Chem. 246, 6942-6952.

Dahms, A.S. and Boyer, P.D. (1973) J. Biol. Chem. 248, 3155-3162.

DeKruyff, B., Demel, A., Slotboom, A.J., VanDeenen, L.L.M. and Rosenthal, A.F. (1973) Biochim. Biophys. Acta 307, 1-9.

Dixon, H.B.F. and Sparkes, M.J. (1973) Biochem. J. 141, 715-719.

De La Mare, S., Coulson, A.F.W., Knowles, J.R., Priddle, J.D. and Offord, R.E. (1972) Biochem. J. 129, 321-331.

Duree, E.D., Vignais, P.V. and Moreau, M. (1968) Biochem. Biophys. Res. Commun. 30, 420-427.

Ellison, W.R., Lueck, J.D. and Fromm, H.J. (1975a) Biochem. Biophys. Res. Commun. 57, 1214-1220.

Ellison, W.R., Lueck, J.D. and Fromm, H.J. (1975b) J. Biol. Chem. 250, 1864-1871.

Engel, R. (1977) Chem. Reviews 77, 349-367.

England, P.T., Huberman, J.A., Jovin, T.M. and Kornberg, A. (1969) J. Biol. Chem. 244, 3038-3044.

Evans, W.H. and Gurd, J.W. (1973) Biochem. J. 133, 189-199.

Fetizon, M. and Golfier, M. (1968) C.R. Acad. Sci. 267, 900-903.

Fleisch, H., Fast, D., Rizzoli, R., Trechsel, U. and Bonjour, J-P. (1977) Adv. Exp. Med. Biol. 81, 279-289.

Flesher, J.W., Oester, Y.T. and Myers, T.C. (1960) Nature 185, 772-773.

Florini, J.R. and Vestling, C.S. (1957) Biochim. Biophys. Acta 25, 575-578.

Ford-Moore, A.H. and Williams, J.H. (1947) J. Chem. Soc. Part II, 1465-1467.

Freedman, L.D. and Doak, G.O. (1957) Chem. Reviews 57, 479-523.

Fromm, H.J. and Zewe, V. (1962) J. Biol. Chem. 237, 1661-1667.

Geider, K. (1972) Eur. J. Biochem. 27, 554-563.

Gerber, G., Preissler, H., Heinrich, R. and Rapoport (1974) Eur. J. Biochem. 45, 39-52.

Gilliam, W.F., Meals, R.N. and Sauer, R.O. (1946) J. Amer. Chem. Soc. 68, 1161-1163.

Glaser, L. and Brown, D.H. (1955) J. Biol. Chem. 216, 67-79.

Goody, R.S., Holmes, K.C., Mannherz, H.G., Barrington-Leigh, J. and Rosenbaum, G. (1975) Biophys. J. 15, 687-705.

Gough, G.R., Maguire, M.H. and Penglis, F. (1972) Mol. Pharm. 8, 170-177.

Griffin, B.S. and Burger, A. (1956) J. Amer. Chem. Soc. 78, 2336-2338.

Griffin, J.H., Schechter, A.N. and Cohen, J.S. (1973) Ann. N.Y. Acad. Sci. 222, 693-708.

Grossbard, L. and Schimke, R.T. (1966) J. Biol. Chem. 241, 3546-3560.

Gulyaev, N.N. and Holy, A. (1972) FEBS Lett. 22, 294-296.

Hampton, A. and Chu, S.Y. (1970) Biochim. Biophys. Acta 198, 594-600.

Hampton, A., Perini, F. and Harper, P.J. (1973a) Biochemistry 12, 1730-1736.

Hampton, A., Sasaki, T. and Paul, B. (1973b) J. Amer. Chem. Soc. 95, 4404-4414.

Haselkorn, R. and Rothman-Denes, L.B. (1973) Ann. Rev. Biochem. 42, 397-438.

Henderson, P.J.R. (1978) Techniques in Lifes. Biochem. Vol. B1, part 11, Stat. analysis of enzyme kinetics, pp. 1-43.

Hendlin, D., Stapley, E.O., Jackson, M., Wallick, H., Miller, A.K., Wolf, F.J., Miller, T.W., Chaiet, L., Kahan, F.M., Foltz, E.L., Woodruff, H.B., Mata, J.M., Hernandez, S. and Mochales, S. (1969) Science 166, 122-123.

Hendrickson, S., Rustad, D.G., Scattergood, E.M. and Engle, D.E. (1974) Chem. Phys. Lipids 13, 63-70.

Hershey, J.W.B. and Monro, R.E. (1966) J. Mol. Biol. 18, 68-76.

Hershey, J.W.B. and Thach, R.E. (1967) Proc. Nat. Acad. Sci. U.S. 57, 759-766.

Holland, P.C., Labelle, W.C. and Lardy, H.A. (1974) Biochemistry 13, 4549-4553.

Holy, A. (1967) Tetrahedron lett., 881-884.

Holy, A. and Hong, N.D. (1971) Collect. Czech. Chem. Commun. 36, 316-317: Chem. Abstr. 74 76623d. (1971).

Holy, A. and Hong, N.D. (1972) Collect. Czech. Chem. Commun. 37. 2066-2076: Chem. Abstr. 102108y. (1972).

Holy, A. and Vortruba, I. (1974) Collect. Czech. Chem. Commun. 39. 1646-1661: Chem. Abstr. 81 116499W. (1974)

Horak, H. and Barton, P.G. (1974) Biochim. Biophys. Acta 373, 471-480.

Horiguchi, M. and Kandatsu, M. (1959) Nature 184, 901-902.

Hullar, T.L. (1967) Tetrahedron Lett., 4921-4923.

Inch, T.D. and Lewis, G.T. (1972) Carbohyd. Res. 22, 91-101.

Issaly, A.S., Issaly, I.M. and Reissig, J.L. (1973) Biochim. Biophys. Acta 331, 410-420.

James, T.L. and Cohn, M. (1974) J. Biol. Chem. 249, 3519-3526.

Jebeleann, G., Ty, N.G., Mentsch, H.H., Barzu, O., Niac, G. and Abrudan, I. (1974), Proc. Nat. Acad. Sci. U.S. 71, 4630-4634.

Johnson, N.P. and Schleich, T. (1974) Biochemistry 13, 981-987.

Jones, D.H. and Boyer, P.D. (1969) J. Biol. Chem. 244, 5767-5772.

Jones, G.H. and Moffatt, J.G. (1969) U.S. patent 3 446 793 (27/5/1969): Chem. Abstr. 71 70903m (1969).

Jones, G.H. and Moffatt, J.G., U.S. patent 2 583 974 (8/6/1971): Chem. Abstr. 75 130091q (1971).

Jones, G.H., Murthy, D.V.K., Tegg, D., Golling, R. and Moffatt, J.G. (1973) Biochem. Biophys. Res. Commun. 53, 1338-1343.

Kabuk, J., Defilippe, L., Engel, R. and Tropp, B.E. (1972) J. Med. Chem. 15, 1074-1075.

Kinsky, S.C., Bonsen, P.P.M., Kinsky, C.B., VanDeenen, L.L.M. and Rosenthal, A.F. (1971) Biochim. Biophys. Acta 233, 815-819.

Kittredge, J.S. and Roberts, E. (1969) Science 164, 37-42.

Klein, D.A., Engel, R. and Tropp, B.E. (1977) J. Bacteriol. 129, 550-553.

Kochemkov, N.K., Nifant'ev, E.E. and Gudkova, L.P. (1967) Zh. Obshch. Khim. 37, 260.

Koretz, J.F. and Taylor, E.W. (1975) J. Biol. Chem. 250, 6344-6350.

Kosolapoff, G.M. (1953) J. Amer. Chem. Soc. 75, 1500-1501.

Kosow, D.P., Oski, F.A., Warms, J.V.B. and Rose, I.A. (1973) Arch. Biochem. Biophys. 157, 114-124.

Kosow, D.P. and Rose, I.A. (1971) J. Biol. Chem. 246, 2618-2625.

Kuo, J.F. and Greengard, P. (1970) Biochem. Biophys. Res. Commun. 40, 1032-1038.

Kuwano, M., Kwan, C.N., Apirion, D. and Schlessinger, D. (1969) Proc. Nat. Acad. Sci. U.S. 64, 693-700.

La Nauze, J.M., Rosenberg, H. and Shaw, D.C. (1970) Biochim. Biophys. Acta 212, 332-350.

Landt, M., Boltz, S.C. and Butler, L.G. (1978) Biochemistry 17, 915-919.

Larsen, M., Willet, R. and Yount, R.G. (1969) Science 166, 1510-1511.

Laugin, G. and Vignais, P.V. (1973) Biochim. Biophys. Acta 305, 534-556.

Le Blanc, P. and Clauser, H. (1974) Arch. Biochem. Biophys. 347, 87-101.

Lefevre, M., Gonzalez, N.S. and Pontis, H.G. (1964) J. Chromatog.15, 499-500.

Leifer, Z., Engel, R. and Tropp, B.E. (1977) J. Bacteriol.130, 968-971.

Letendre, C.H. and Singer, M.F. (1974) J. Biol. Chem. 249, 7383-7389.

Li, H-C. and Buchanan, J.M. (1971) J. Biol. Chem. 246, 4720-4726.

Lin, M.C., Salomon, Y., Rendell, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 4246-4252.

Lippmann, F. (1969) Science 164, 1024-1031.

Liptak, A., Jodál, I. and Nánási, P.(1975) Carbohyd. Res. 44, 1-11.

Loesche, W., Bublitz, R., Horn, A., Koehler, W., Peterman, H. and Till, U. (1974) J. Chromatog. 92, 166-170.

Lowry, O.H., Passonneau, J.V., Schulz, D.W. and Rock, M.K. (1961) J. Biol. Chem. 236, 2746-2755.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

Lucas-Lenard, J. and Lippmann, F. (1971) Ann. Rev. Biochem. 40, 409-448.

Maccioni, R. and Seeds, N.W. (1977) Proc. Nat. Acad. Sci. U.S., 462-466.

Malchow, D., Fuchila, J. and Jastorff, B. (1973) FEBS Lett. 34, 5-9.

Marutzky, R., Flossdorf, J. and Kula, M-R. (1976) Nucl. Acid Res. 3, 2067-2077.

Mato, J.M. and Konijn, T.M. (1977) FEBS Lett. 75, 173-176.

McKenna, C.E., Higa, M.T., Cheung, N.H. and McKenna, M.C. (1977) Tetrahedron Lett. 155-158,

Moffatt, J.G. and Jones, G.H., U.S. Patent 3 524 846 (18 Aug. 1970): Chem. Abstr. 74, 31940p (1971).

Montgomery, J.A. and Thomas, H.J. (1979) J. Med. Chem. 22, 109-111.

Moos, C., Alpert, N.R. and Myers, T.C. (1960) Arch. Biochem. Biophys. 38, 183-192.

Murray, A.W. (1968) Biochem. J. 106, 549-555.

Murray, A.W., Wong, P.C.L. and Friedrichs, B. (1969) Biochem. J. 112, 741-746.

Nichol, A.W., Nomura, A. and Hampton, A. (1967) Biochemistry 6, 1008-1015.

Ning, J., Purich, D.L. and Fromm, H.J. (1969) J. Biol. Chem. 244, 3840-3846.

Nowak, T., Mildran, A.S. and Kenyon, G.L. (1973) Biochemistry 12, 1690-1701.

Ohta, T., Sarkar, S. and Thach, R.E. (1967) Proc. Nat. Acad. Sci. U.S. 58, 1638-1644.

Orr, G.A. and Knowles, J.R. (1974) Biochem. J. 141, 721-732.

O'Sullivan, W.J., Reed, G.H., Marsden, K.H., Gough, G.R. and Lee, C.S. (1972) J. Biol. Chem. 247, 7839-7843.

Panitz, N., Ricke, E., Morr, M., Wagner, K.G., Roesler, G. and Jastorff, B. (1975) Eur. J. Biochem. 55, 415-422.

Papas, T.S. and Case, R.V. (1970) Fed. Proc. Fed. Amer. Soc. Exp. Biol. 29, 915.

Paulsen, H. and Greve, W. (1973) Chem. Ber. 106, 2114-2123: Chem Abstr. 79 126721K (1973).

Paulsen, H., Greve, W. and Kuhne, H. (1971) Tetrahedron Lett., 2109-2112.

Paulsen, H. and Kuhne, H. (1974) Chem. Ber. 107, 2635-2643: Chem. Abstr. 81 136404q (1974).

Paulsen, H. and Thiem, J. (1973) Chem. Ber. 106, 3850-3876: Chem. Abstr. 80 96246K (1973).

Pfeiffer, F.R., Mier, J.D. and Weisbach, J.A. (1974) J. Med. Chem. 17, 112-115.

Pfeuffer, T. and Helriech, E.J.M. (1975) J. Biol. Chem. 250, 867-876.

Pfitzner, K.E. and Moffatt, J.G. (1965) J. Amer. Chem. Soc. 87, 5661-5670.

Pontremoli, S., DeFlora, E.G., Mangiarotti, G., Bonsignore, A. and Horecker, B.L. (1961) J. Biol. Chem. 236, 2975-2980.

Purich, D.L., Fromm, H.J. and Rudolph, F.B. (1973) in Advances in Enzymology (ed. A. Meister) vol.39, p.287 (Wiley N.Y.).

Rammler, D.H., Bagdasarian, A. and Morris, F. (1972) Biochemistry 11, 9-12.

Raue, H.A. and Cashel, M. (1974) Biochim. Biophys. Acta 340, 40-51.

Ravel, J.M., Shorey, R.L., Froehner, S. and Shive, W. (1968) Arch. Biochem. Biophys. 125, 514-526.

Redkar, V.D. and Kenkare, U.W. (1972) J. Biol. Chem. 247, 7576-7584.

Reed, G.H. and Cohn, M. (1973) J. Biol. Chem. 248, 6436-6442.

Robinson, J.D. (1975) Biochim. Biophys. Acta 384, 250-264.

Rodbell, M., Birnbaumer, L., Pohl, S.L. and Krans, H.M.J. (1971b) J. Biol. Chem. 246, 1877-1882.

Rodbell, M., Krans, H.M.J., Pohl, S.L. and Birnbaumer, L. (1971a) J. Biol. Chem. 246, 1872-1876.

Rose, I.A. and Warms, J.V.B. (1967) J. Biol. Chem. 242, 1635-1645.

Rosenthal, A.F. and Chodsky, S.V. (1974) Lipids 9, 77-80.

Rosenthal, A.F. and Han, S.C-H (1968) Biochim. Biophys. Acta 152, 96-103.

Rosenthal, A.F. and Han, S.C-H (1970) Biochim. Biophys. Acta 218, 213-220.

Rosenthal, A.F. and Pousada, M. (1966) Biochim. Biophys. Acta 125, 265-276.

Rosenthal, A.F. and Pousada, M. (1968) Biochim. Biophys. Acta 164, 226-237.

Rudolph, F.B. and Fromm, H.J. (1969) J. Biol. Chem. 244, 3832-3839.

Salomon, Y., Lin, M.C., Londos, C., Rendell, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 4239-4245.

Santi, D.V., Danenberg, P.V. and Montgomery, K.A. (1971) Biochemistry 10, 4821-4824.

Santi, D.V. and Pena, V.A. (1973) J. Med. Chem. 16, 273-280.

Sarin, V., Tropp, B.E. and Engel, R. (1977) Tetrahedron lett. 351-354.

Seidel, J.C. (1975) J. Biol. Chem. 250, 5681-5687.

Shopsis,C., Engel, R. and Tropp, B.E. (1972) J. Bacteriol. 112, 408-412.

Shopsis, C.S., Engel, R. and Tropp, B.E. (1974) J. Biol. Chem. 249, 2473-2477.

Shopsis, C.S., Nunn, W.D., Engel, R. and Tropp, B.E. (1973) Antimicrob. Agents Chemother. 4, 467-473.

Simon , L.N. and Myers, T.C. (1961) Biochim. Biophys. Acta 51, 178-180.

Simon , L.N., Myers, T.C. and Mednieks, P. (1965) Biochim. Biophys. Acta 103, 189-195.

Southgate, C.B. and Dixon, H.B.F. (1978) Biochem. J. 175, 461-465.

Spiegel, A.M., Downs, R.W.(Jr.) and Aurbach, G.D. (1977) Biochem. Biophys. Res. Commun. 76, 758-764.

Stansifeld, D.A. and Franks, D.J. (1971) Biochim. Biophys. Acta 242, 606-616.

Stoffel, W. and Grol, M. (1974) Chem. Phys. Lipids 13, 372-388.

Stribling, D. (1974) Biochem. J. 141, 725-728.

Stubbe, J.A. and Kenyon, G.L. (1972) Biochemistry 11, 338-345.

Sutherland, J.W.H. (1976) Biochem. Biophys. Res. Commun. 72, 933-938.

Taketa, K., Sarngadharan, M.G., Watanabe, A., Aoe, H. and Pogell, B.M. (1971) J. Biol. Chem. 236, 5676-5683.

Tang, J-C., Tropp, B.E. and Engel, R. (1977) Chem. Phys. Lipids 19, 99-106.

Tang, J-C., Tropp, B.E. and Engel, R. (1978) Tetrahedron lett., 723-726.

Thach, R.E., Dewey, K.F. and Mydolajewycz, N. (1967) Proc. Nat. Acad. Sci. U.S. 57, 1103-1109.

Thiem, J., Gunther, M. and Paulsen, H. (1975) Chem. Ber. 108, 2279-2289: Chem. Abstr; 83 114567j (1975).

Tobin, T., Akera, T., Hogg, R.E. and Brody, T.M., (1973) Mol. Pharmacol. 9, 278-281.

Tonomura, Y., Imamura, K., Ikehara, M., Uno, H. and Harada, F. (1967) J. Biochem. 61, 460-472.

Tyhach, R.J., Engel, R. and Tropp, B.E. (1976) J. Biol. Chem. 251, 6717-6723.

Tyhach, R.J., Rosenthal, A.F. and Tropp, B.E. (1975) Biochim. Biophys. Acta 388, 29-37.

Unger, F.M., Stix, D., Moderndorfer, E. and Hammerschmid, F. (1978) Carbohyd. Res. 67, 349-356.

Ureta, T. (1976) J. Biol. Chem. 251, 5035-5042.

Vidgoff, J.M., Pocker, A., Hullar, T.L. and Fischer, E.H. (1974) Biochem. Biophys. Res. Commun. 57, 1166-1174.

Vogel, A.I., A Textbook of Inorganic Quantative Analysis, p.434. (Longmans 1957).

Warburg, O. and Christian, W. (1941) Biochem Z.310 384.

Waring, P. and Ziporin, Z.Z. (1964) J. Chromatog. 15, 168-172.

Webster, D., Jondorf, W.B. and Dixon, H.B.F. (1976) Biochem. J. 155, 433-441.

Weil-Malherbe, H. and Bone, A.D. (1951) Biochem. J. 49, 339-347.

Werber, M.M., Szent-Gyorgi, A.G. and Fasman, G.D. (1972) Biochemistry 11, 2872-2883.

Wigler, P.W. and Lozzio, C.B. (1972) J. Med. Chem. 15, 1020-1024.

Wilson, J.E. (1978) Arch. Biochem. Biophys. 185, 88-99.

Wilson, J.E. (1979) Arch. Biochem. Biophys. 196, 79-87.

Wong, P.C.L. and Murray, A.W. (1969) Biochemistry 8, 1608-1614.

Yengoyan, L. and Rammler, D.H. (1966) Biochemistry 5, 3629-3638.

Yount, R.G., Babcock, D., Ballantyne, W. and Ojala, D. (1971a) Biochemistry 10, 2484-2489.

Yount, R.G., Ojala, D. and Babcock, D. (1971b) Biochemistry 10, 2490-2496.

Zeleznick, L.D., Myers, T.C. and Titchener, E.B. (1963) Biochim. Biophys. Acta 78, 546-547.