To Peter

How does one account for the working of the vintage in the vat : of dough left to rise :.....?

Pasteur

ACTIVE CENTRE ~~RESIDUES~~ STUDIES ON YEAST HEXOKINASE ~~X~~

by

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A thesis submitted for the degree of Doctor of Philosophy of the University of London and for the Diploma of Membership of Imperial College

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ABSTRACT

This thesis describes work on the mechanism and active centre of the enzyme, Yeast Hexokinase, isoenzyme B (EC 2.7.1.1). Active centre residues have been studied in an attempt to tie these with the tentative X-ray sequence previously published.

A small scale method of purification was developed into a method for processing 20kg of yeast from which 200 - 300 mg of pure enzyme were routinely obtained.

The strategy employed has been to show substrate - protectable inactivation by reagents directed against particular amino acids. Once this had been demonstrated a radioactive form of the reagent was used and the incorporation of a single molecule of the residue per molecule of enzyme shown. The enzyme was then digested and the labelled peptide isolated using a variety of techniques including gel filtration, high voltage paper electrophoresis and high performance liquid chromatography. The peptides obtained were then sequenced using a combination of the manual Dansyl-Edman technique and a spinning cup automatic sequencer.

The main study was on the four thiols of the enzyme one of which has been implicated in the mechanism. This thiol has been identified and the peptide sequenced but its part in the mechanism is not certain. A second thiol has also been identified and sequenced.

The possibility of an essential carboxyl group has been considered but when carbodiimides were used as inactivating agents no single essential carboxyl group could be identified.

A suitable affinity label, 3', 5'-dialdehyde ATP, for the nucleotide binding site has been identified and its possibility for investigation of this site considered.

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LIST OF ABBREVIATIONS

AMP/ADP/ATP	Adenosine Mono/Di/Triphosphate
AMPPNP	Adenyl-5'-yl B imido-diphosphate
ANS	1-Anilino-8-naphthalene sulphonate
BAGA	N-Bromo-acetyl-D-galactopyranosylamine
BAWP	Butan-1-ol, Acetic Acid, Water, Pyridine
CHpA	Cycloheptaamylose
CNBr	Cyanogen Bromide
cpm	counts per minute
Dansyl chloride / DNSCl	1-dimethylaminonaphthalene-5-sulphonyl chloride
DEAE	Diethyl amino ethyl
DFP	Diisopropylphosphorofluoridate
dpm	disintegrations per minute
DTT	Dithiothreitol
ECDI	Ethyl dimethylamino propyl carbodiimide
EDTA	Ethylene diamine tetra acetic acid
fsd	full scale deflection
glc 6 P	Glucose-6-phosphate
GTP	Guanosine triphosphate
HPLC	high performance liquid chromatography
HVPE	high voltage paper electrophoresis
ITP	Inosine triphosphate
NaAc	Sodium Acetate
NSA	1,2-Naphthaquinone-4-sulphonic acid
oATP	2'3'-Dialdehyde ATP
PITC	Phenyl Isothiocyanate
PMSF	Phenyl methyl sulphonyl fluoride
PPO	2,5-Diphenyl oxazole

PTH	Phenyl thiohyd antoin
SC	Semicarbazide
SDS	Sodium dodecyl sulphate
TCA	Trichloracetic Acid
TFA	Trifluoracetic Acid
TRIS	Tris (hydroxymethyl) amino methane
UTP	Uracil triphosphate

SINGLE AND THREE LETTER AMINO ACID CODES

Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic Acid	D	Asp
Carboxymethylcysteine	CMC	CM Cys
Cysteine	C	Cys
Glutamic Acid	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

X-Ray Work Only:-

Glutamic Acid / Glutamine	Ex	Glx
Aspartic Acid / Asparagine	Dx	Asx
unknown out with 3, 4 or 5 carbon atoms in the side chain		Gam/ Del /.Eps

CHAPTER I

INTRODUCTION

Hexokinases catalyse the following reaction:-

$$MgATP^{2-} + \text{hexose} = MgADP^{1-} + \text{hexose 6 } P^{2-} + H^{+}$$

The enzyme is of interest because sugar phosphorylation is the first stage of glycolysis, and regulation of Hexokinase may be expected to affect the cell's physiological state. The enzyme occurs in many tissues - the yeast and muscle enzymes have been studied the most extensively. The interest of workers in the yeast enzyme has mainly been in the physical and kinetic properties while the muscle enzyme has been examined mainly with regard to its regulatory properties.

1.1 ISOENZYMES

Purification of the yeast enzyme (see chapter three) led to much speculation as to the existence of discrete isoenzymes. Proteolytic attack clouded the situation but with improved preparative techniques (1, 2) it has been concluded that three native isoenzymes exist. (3). These have been designated A, B and C, in order of elution from a DEAE cellulose column, with A and B corresponding to P1 and P11 described by Colowick's group. (4). A and B are structural isoenzymes having different primary structures; C has the same primary structure as B and can be converted to B by high ionic strength and low pH. (3). Studies using SDS starch gel electrophoresis (5) showed that isoenzyme A gave rise to a single molecular species of sub-unit α and isoenzymes B and C gave rise to β sub-units.

Some differences in the isoenzymes are listed in table 1.1. The physiological importance of having distinct isoenzymes is unclear. Extracts of commercial baker's yeast were found to exhibit different electrophoretic patterns depending whether the yeast was in the exponential or stationary phase of growth (6), however it is not certain how much proteolysis had occurred. The distribution of

Table 1.1

CHARACTERISTICS OF PURE ISOENZYMES (taken from reference 2)

PROPERTY	A	B	C
RATIO OF ACTIVITY ON FRUCTOSE TO THAT ON GLUCOSE, pH 8, 25°C.	2.6	1.1	1.1
pH OF ELUTION	5.6	5.4	5.15
Km GLUCOSE	0.2 - 0.3 mM	0.25 - 0.6 mM	0.6 mM
Km (ATP Mg)	0.3 mM	0.29 mM	-
V_{max} (FRUCTOSE) / V_{max} (GLUCOSE)	3.0	1.1 - 1.3	1.1 - 1.3
SPECIFIC ACTIVITY units/mg 25°C pH = 8.5	275	900	750
SPECIFIC ABSORPTIVITY $ml\,mg^{-1}\,cm^{-1}$ 280nm	1.24	0.98	0.98

isoenzymes was also found to depend on the carbon source. (7). Cells grown on ethanol appeared to have an excess of the A isoenzyme and cells grown on glucose an excess of the B isoenzyme suggesting that the isoenzymes are inducible. The work described in this thesis was performed on isoenzyme B, chosen because it has been more thoroughly investigated and it has a higher specific activity. Comparisons with Hexokinase A are possible but must be viewed with some caution because the primary structures are different.

All three isoenzymes appear to be dimers although for some time it was speculated that the native enzymes were tetramers (8, 9, 10). This was because the enzyme copurified with Protease C (11) which was activated under certain circumstances and cleaved Hexokinase very specifically to give apparent sub-units of molecular weight about 26 000. The native enzymes are also easily cleaved by very low concentrations of Trypsin (4, 9) to form the active, so called S forms, S1 and S11. The S forms are monomers and it was found that they are formed by a Tryptic cleavage 11 residues from the N terminus of the P forms. (12, 13). As well as by Tryptic cleavage, dimer dissociation is encouraged by high salt, high pH and certain substrates. Glucose alone slightly enhances dissociation of isoenzyme B while glucose and MgATP give a dramatic increase in this dissociation. (14). Since non-substrate hexoses could not replace glucose in enhancing dissociation it was concluded that dissociation of the dimer by substrates has an important part to play in the control of catalysis. High protein concentration was shown to favour association (15) and since the protein concentration in yeast is relatively high the dimeric form is thought to predominate in vivo.

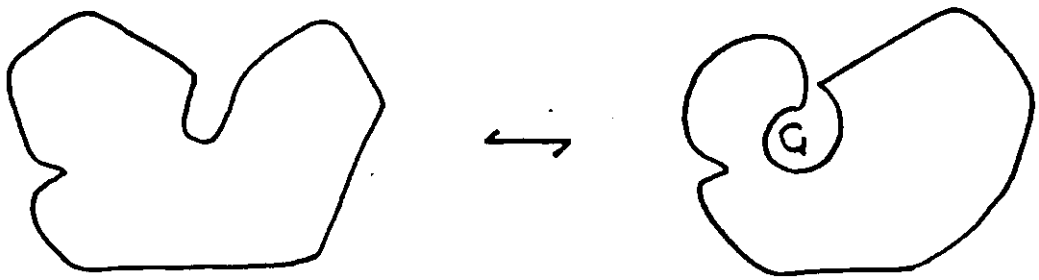
1.2 X-RAY CRYSTALLOGRAPHY

This thesis describes attempts to identify certain active centre residues and correlate them with X-Ray data generated by

Steitz's group at Yale. The X-Ray work has given some useful information about the nature of the binding sites but it is important to remember that X-Ray crystallography can only give a static picture of the active site and should be considered along with dynamic information.

Both Hexokinase A and B have been studied crystallographically with B in the greatest detail. Hexokinase B crystallizes in three forms termed B1, B11 and B111. The B111 monomer form has been studied in detail, its structure determined to 2.1 Å resolution and a tentative primary sequence generated. (16, 17). B111 will crystallize with the competitive inhibitor o-toluoyl glucosamine and a fairly detailed picture of the sugar binding site has been built up. (18). The dimer B11 has been determined to 3.5 Å resolution (19). Unfortunately the B isoenzyme will not crystallize in the presence of high glucose concentration; on the other hand Hexokinase A will not crystallize in the absence of glucose. Comparison of Hexokinase A with glucose and Hexokinase B in the absence of glucose has yielded information about a glucose induced conformational change. (20, 21, 22).

The Hexokinase B111 monomer (and indeed many other kinases) consists of two lobes separated by a deep cleft (23). The two lobes differ in their secondary structure, one being mainly α helix and the other containing substantial β structure. The binding of sugar moieties in the cleft is accompanied by extensive alterations in protein structure, the position of the lobes relative to each other changing profoundly. The polypeptide backbone of the small lobe moves as much as 8 Å towards the larger lobe, closing the cleft and bringing atoms of the small lobe in contact with both the large lobe and the substrate.

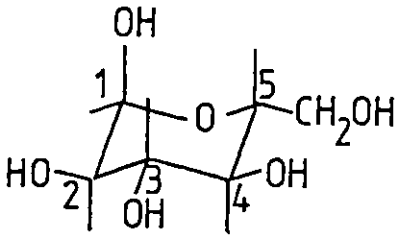


Solution work has also given evidence for this conformational change - binding of glucose or glucose-6-phosphate causes a decrease in the radius of giration of monomeric B equivalent to that calculated from native B and A complexed with glucose. (24).

X-Ray work has indicated that in the dimer the two potential glucose binding sites are not equivalent and only one is readily occupied when glucose is diffused into the crystal (25). However evidence from solution work is conflicting. Glucose quenching of intrinsic protein fluorescence fits a model in which one site is occupied in the monomer and two (non cooperatively) in the dimer. (26). Equilibrium binding of a competitive glucose derivative indicates binding of two glucose molecules per dimer without cooperativity (27). This evidence seems to indicate that the non equivalence of sites observed in the dimer is due to "crystal lattice constraints." (28)

1.3 THE SUGAR BINDING SITE

The sugar molecule binds to Hexokinase in the C1 conformer - chair, equatorial (25).



The sugar interacts with the enzyme via the 1, 3, 4 and 6 hydroxyls with the orientations of the hydroxyls as positions 3 and 4 being especially important since neither D-allose, nor D-galactose are very good substrates (29). In fact it is thought that the hydroxyls at C3 and C4 act as proton donors because the fluoro substituents are not active (30). Compounds modified at C2 are still good substrates however, e.g. mannose (29). Fructose and fructose analogues are phosphorylated showing that the furanose ring is acceptable. Modification at the anomeric hydroxyl locking the compound in the pyran ose form decreases the activity e.g. 1.5 anhydro-D-glucitol (29). However both α-D-glucose and β-D-glucose are good substrates so that the position of the anomeric hydroxyl is unimportant. A summary of these results is shown in fig 1.1.

In a model based on the 2.1 Å resolution map of B111 several amino acid side chains appear to be involved in binding the sugar (18). Especially important appear to be the electron densities relating to amino acid residues shown in table 1.2

From X-Ray data alone it is not possible to differentiate between Asp and Asn, and Glu and Gln but the residues have been assigned as such to account for the need for hydrogen bond donors. Cys 243 is too far away to play a direct role in binding or catalysis

fig. 1.1

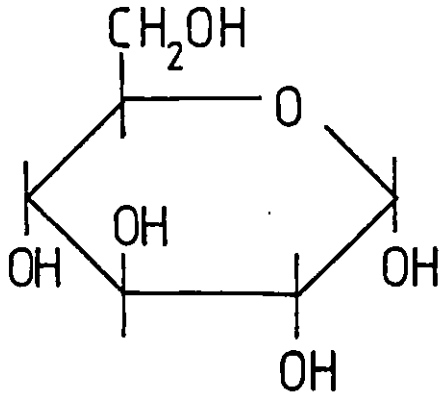
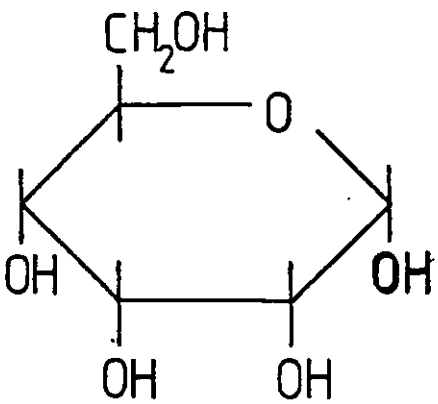
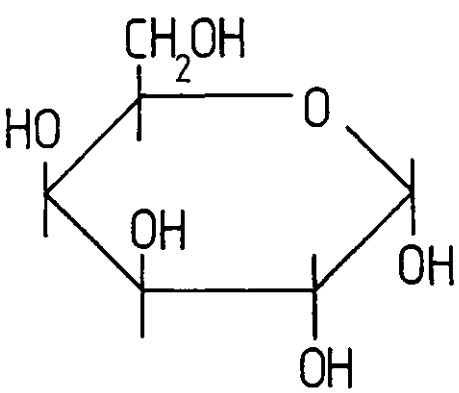
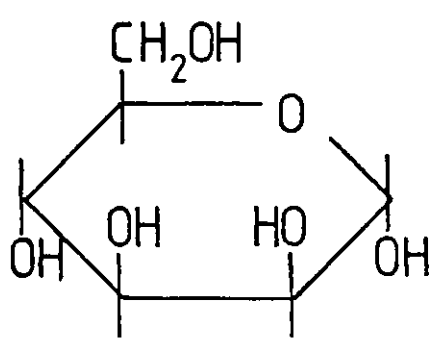
	Relative V_m	Relative K_m
 D-Glucose	100	1
 D-Allose	> 10	> 1000
 D-Galactose	> 0·2	> 500
 D-Mannose	20	0·5

fig. 1.1

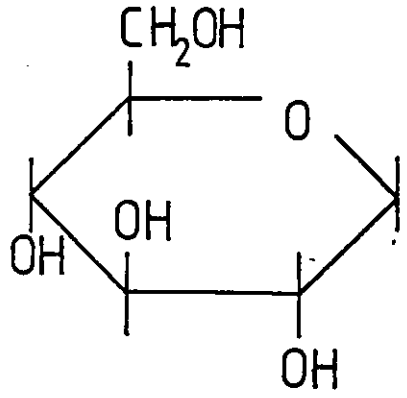
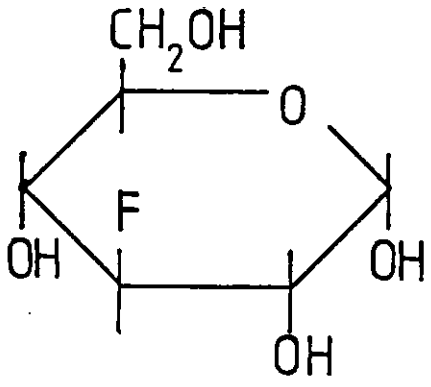
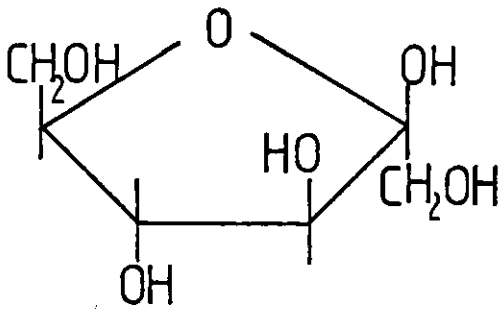
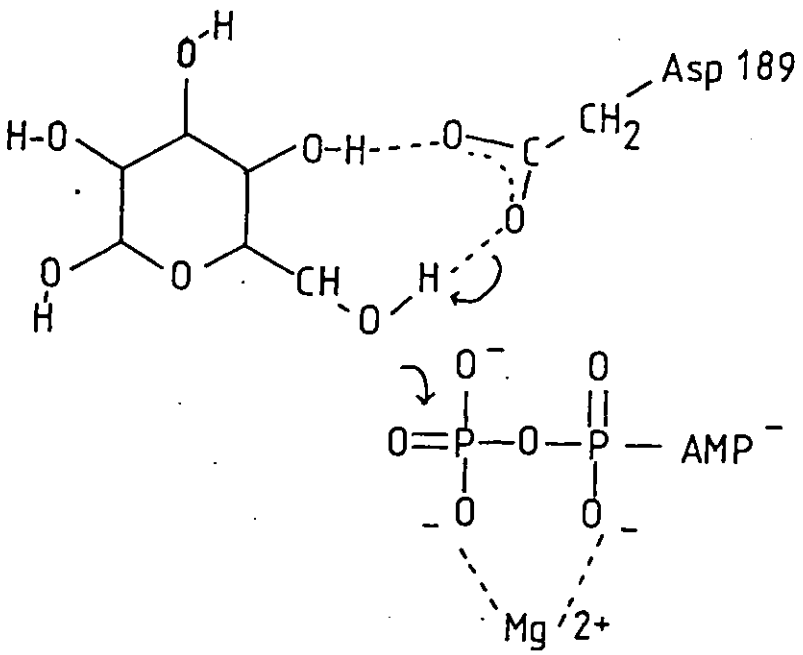
	Relative V_m	Relative K_m
 1,5-Anhydro-D-Glucitol	1	30
 3-Deoxy-3-Fluoro-D-Glucose	10	700
 D-Fructose	180	70

Table 1.2

AMINO ACID RESIDUES CLOSE TO THE SUGAR HYDROXYLS

Glucose Hydroxyl	Hexokinase Atom	Distance Å
1	Gln 277 XE	2.00
2	Cys 243 S	7.7
3	Asn 188 XD	2.39
3	Cys 243 S	5.5
3	Asn 245 XD	3.07
4	Asn 188 XD	3.12
4	Asp 189 OD2	2.57
4	Asn 215 XD	3.18
6	Asp 189 OD1	2.42
6	Cys 243 S	9.1

but it does appear to stabilize the amide groups of asparagines 188, 215 and 245 and thus chemical modification of this thiol would possibly destroy the active site. Asp 189 is supposed to be the free acid rather than the amide because of the need for a general base to be bound to the 6 hydroxyl group. This would increase the nucleophilicity of the 6 hydroxyl and facilitate its attack on the γ phosphate of ATP. A possible mechanism is outlined below:-



1.4 THE NUCLEOTIDE BINDING SITE

The nucleotide binding site is less well defined and subject to some controversy and there has been dispute as to the number of nucleotide binding sites per dimer. From the crystal structure three binding sites per dimer have been proposed. (23, 31). At 7 Å resolution the ATP analogue AMPPNP, in the presence of glucose shows a high degree of substitution at one site per dimer - the intersubunit or I site. This site is formed by both subunits, although because of apparent non equivalence of subunits, a different stretch of peptide on each subunit is involved. When ATP binds to the I site the γ- phosphate is too far from either of the two glucose-6-hydroxyls to be involved in direct

phosphoryl transfer. (25). ATP analogues binding to this site act as allosteric activators enhancing the affinity of both subunits for glucose. (31). No binding to the partial I sites in the monomer takes place. It is concluded that the I site is not involved in direct catalysis.

Two further binding sites per dimer also exist - the so called A sites which permit the binding of two AMP molecules per dimer. The A site is proposed as being the catalytic site - several lines of evidence support this. Firstly ANS and AMP competitively inhibit the monomer with respect to ATP and the A site on the monomer is the only one which binds these inhibitors. Secondly model building shows that the δ- phosphate of ATP bound to the A site is only 6 Å from the 6-hydroxyl of glucose and it is thought that a conformational change would bring the two close enough for direct phosphoryl transfer. One difficulty that arises is that is is impossible to bind an ATP analogue to the A site in either the monomer or dimer possibly because this causes the crystal to break up.

The question then arises as to the function of the I site - it has been proposed that it is some sort of regulatory site since regulatory properties have been observed at intracellular pH values (below 7). (32, 33). Negative cooperativity with respect to MgATP has been reported - double reciprocal plots being non linear below pH 7 (33). The rate of reaction is inhibited by lowering the pH especially at sub-optimal ATP concentrations although this effect is overcome by the addition of activators such as citrate, malate and 3 phosphoglycerate. Native hexokinases assayed inside semipermiable yeast cells exhibited the same properties. The possibility that this apparant activation by citrate etc., was due to chelation of contaminating heavy metal ions was ruled out because reagents pre-treated with 8-hydroxyquinoline failed to affect the results. Substrate induced slow

burst type kinetics were also observed. These regulatory affects were dependent upon the adenine moiety because ATP could not be replaced by ITP or UTP. However replacement of Mg^{2+} by Ca^{2+} and other divalent metal ions did not affect the regulatory properties.

Recently though Womack and Colowick (34) have shown that the degree of inhibition of yeast Hexokinase by protons is dependent upon the aluminium content of the ATP. It was first observed in certain experiments that different batches of ATP behaved differently. If the enzyme reaction was started by adding ATP to a system containing EDTA some ATP preparations gave a very slow initial rate which increased up to tenfold within the six minute assay period. The ATP could be 'activated' by allowing it to stand in 1 mM EDTA for six minutes prior to addition. Full activity was restored immediately by the addition of citrate. These effects were not observed above pH7.

To discover the cause of this, the ATP preparations were compared and the only significant difference was that the 'inhibited' preparation contained much higher levels of aluminium ions. Sensitivity to Al^{3+} was observed when the pH was lowered and it was concluded that the so-called 'activators' worked by chelating the Al^{3+} in the ATP preparations. In fact ATP is a much better chelator of Al^{3+} than 8-hydroxyquinoline explaining why other workers ruled out the possibility of heavy metal ion contamination. The specificity for the Adenine nucleotide was probably due to lack of Al^{3+} contamination in the ITP and UTP preparations. The mode of action of Al^{3+} is thought to be as a complex with ATP - the formation of this complex being encouraged by the titration of Al^{3+} with protons - hence the pH dependence.

In solution, using Cr . [^{3}H]-ATP as the ligand in the presence of glucose, dimeric Hexokinase B has been found to bind two Cr . [^{3}H]-ATP molecules per dimer, at the active site. (27).

Monomeric B likewise bound one per molecule. In the equilibrium binding of an ATPMg analogue at any pH, no cooperativity is found. Hence, no evidence for an additional ATP binding site in solution exists so far, nor regulatory properties attributable to such a site.

The effect of structural modifications of ATP on Hexokinase activity has been studied in some detail (fig. 1.2) (35, 36, 37). Although the 6-amino group is a major substituent of ATP, replacement of the hydrogen atoms with methyl groups or the even bulkier benzene ring appears to have relatively little effect on either the K_m or the V_{max}. (I - III). Removal of the 6-amino group (IV) or replacement with a hydroxyl group (V) has a minor effect on V_{max} but increases K_m quite considerably. It is possible that the nitrogen of the amino group is in some way important for binding - nitrogen and oxygen are of similar size and both can form hydrogen bonds; oxygen, however is more electronegative.

Replacement of nitrogen by carbon at position 7 (VI) affects K_m and V_{max} very little in spite of considerable alteration in the purine ring electron density. It is known that N7 is involved in the formation of a ring bound metal ATP complex with certain metal ions but possibly not Mg^{2+}.

The presence of an amino group at the 2 position (VII - IX) increases the K_m somewhat and decreases V_{max} substantially. The greatest effect on K_m is in the diamino compound - when no amino group is present at position 6 the effect on V_{max} is more marked.

The position of glycosidic linkage does not appear to be very important (X); however, alterations to the sugar structure bring about profound changes upon K_m and V_{max} (XI - XIV). Cis hydroxyls at the 2' and 3' positions appear to be necessary since either deoxy compound or the arabinose derivitive (where the hydroxyls are trans)

fig. 1.2

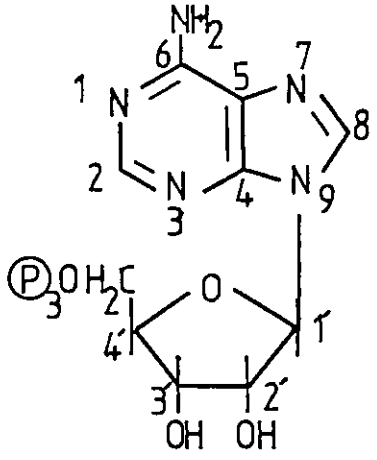
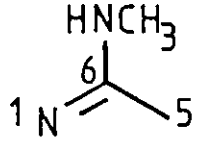
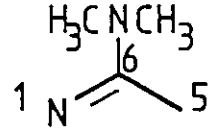
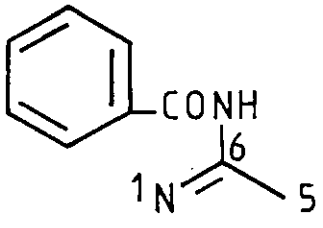
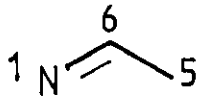
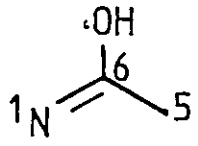
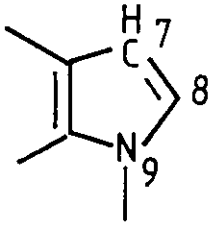
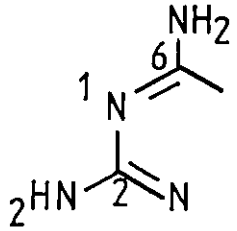
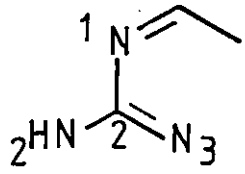
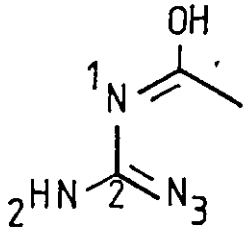
	Relative V_m	Relative K_m
 Adenosine 5′-Triphosphate	100	1
 I N^6-monomethyl ATP	83	0·96
 II N^6,N^6-dimethyl ATP	65	0·45
 III N^6-benzoyl ATP	56	0·45
 IV deamino-6- ATP	71	6

fig. 1.2

	Relative V_m	Relative K_m
 V Inosine 5′-Triphosphate	47	29·3
 VI 7-deazo ATP	94	0·78
 VII 2,6-diaminopurine riboside triphosphate	11	7·46
 VIII 2-aminopurine riboside-5′-triphosphate	1·6	1·47
 IX Guanosine 5′-Triphosphate	1·7	1·47

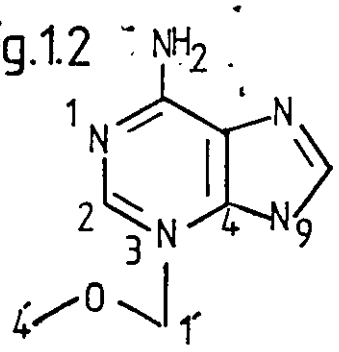
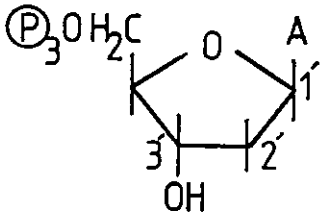
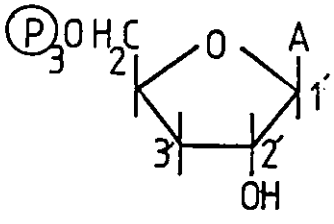
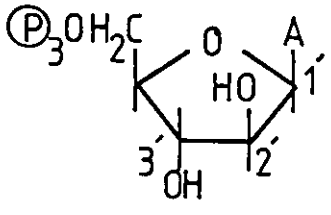
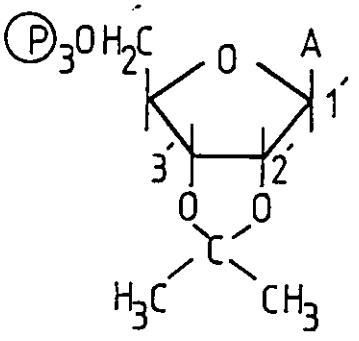
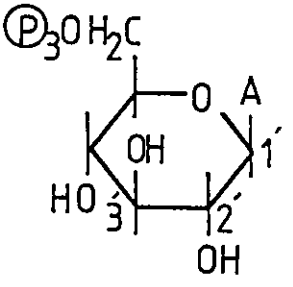
		Relative V_m	Relative K_m
fig.1.2			
X	3-(βDribofuranosyl) ATP	4.8	2·7
			
XI	2′-deoxy ATP	5·9	2·71
			
XII	3′-deoxy ATP	6·2	2·81
			
XIII	9-(β arabino – furanosyl) ATP	13	4·58

fig 1.2



XIV 9-(βD isopropylidine ribofuranosyl) ATP

Relative V_m	Relative K_m
not a substrate	



XV 9-(βDgluco-pyranosyl) ATP

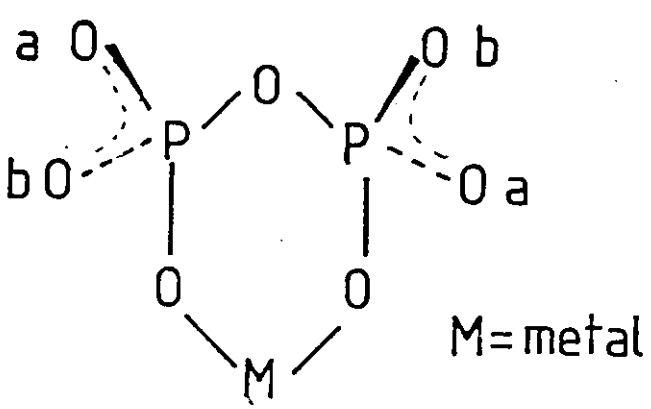
0·5	0·18

show an increase in K_m and a large decrease in V_{max}. A compound with a bulky isopropyl group substituted on the 2', 3'-hydroxyls exhibits no catalytic activity and although the glucose ATP (XV) binds well very little catalysis is observed.

It would appear, then, that the ribose moiety is important in determining whether or not a compound will be a substrate for Hexokinase. The enzyme is more tolerant to changes in the purine part of the molecule.

1.5 STEREOCHEMISTRY OF THE NUCLEOTIDE - METAL COMPLEX

The stereochemistry of the metal - ATP complex has been determined (38, 39). ATP - Mg in solution can exist in seven coordination diastereomers - four tridentate forms where α-, β-and γ-phosphates of ATP are each liganded to the metal through a single oxygen, two bidentate forms in which the β- and γ-phosphates are liganded and one monodentate form with the γ-phosphate liganded. Hexokinase shows a preference for one of the bidentate forms the absolute configuration of which has been determined using X-ray crystallography. The four sites for bidentate geometry are illustrated below:-

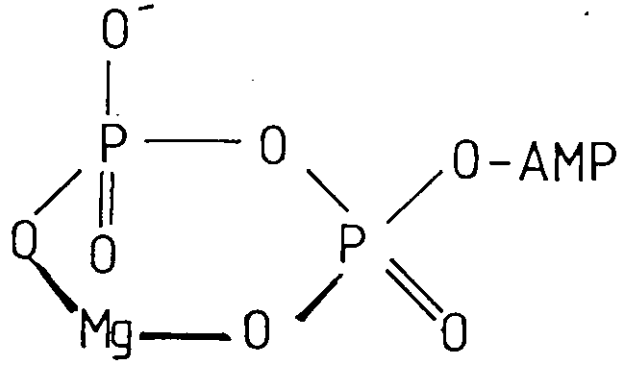


The two a sites and the two b sites are stereochemically equivalent.

When the metal involved is Mg^{2+} the interconversion of diastereomers may have a half life of 10^{-5}s but the equivalent complexes with Co (111) or Cr (111) are stable enough to allow separation and characterisation. $Co(NH_3)_4$ ATP is catalysed by Hexokinase in the following reaction:-

$$Co\ (NH_3)_4ATP + glucose \xrightarrow{Hexokinase} Co(NH_3)_4\ (glc\text{-}6\text{-}P)\ ADP$$

Only one diastereomer will react and it is this which has been used to separate the two forms. The reaction mixture is applied to cHpA column which separates the unreacted (and therefore rich in the non reactive diastereomer) $Co(NH_3)_4$ ATP from the $Co(NH_3)_4$ (glc-6-P) ATP. The latter can be enzymatically converted back to $Co\ (NH_3)_4$ ATP rich in the active diastereomer. The two separated diastereomers can now be degraded to the two forms of $Co(NH_3)_4\ H_2P_3O_{10}$ without loss of chirality and the crystals characterised by X-ray crystallography. The active form was found to have left hand screw sense, Λ coordination and thus it is thought that the active MgATP complex is of the form:-



1.6 STEREOCHEMISTRY OF PHOSPHORYL TRANSFER

Studies to determine the stereochemical course of the phosphoryl transfer have revealed interesting details about the

mechanism. Basically phosphoryl transfer can take place with either retention or inversion of configuration, and by introducing chirality into the γ- phosphate group of ATP, it is possible to say which. Using Adenosine 5' - O - ([γ ^{18}O] γ thio) triphosphate (40) stereochemical analysis showed that transfer was with inversion of configuration. This particular ATP analogue has a much reduced reaction rate and so the possibility that a different stereochemical course had been taken had to be ruled out. This was done by the synthesis of Adenosine [γ (S) ^{16}O ^{17}O ^{18}O] triphosphate (41) and stereochemical analysis which showed that this reaction also proceeded by inversion of configuration. (42). Further work has confirmed these findings and is being extended to determine in more detail the path of phosphoryl transfer. (43).

1.7 ACTIVE SITE RESIDUES

The aim of this project has been to identify amino acid residues essential for activity and where possible to sequence peptides containing them. Previous work on the active centre was used as a starting point - obvious groups to consider are those which can be relatively easily modified by known techniques or which have been implicated by the X-Ray work.

The possibility of an essential arginine residue has been chemically investigated by two groups (X-Ray work has also pointed to a catalytic role for arginine residues). One group found substrate protectable uptake of one mole of [^{14}C] - phenylglyoxal per monomer. (44). This however, was in conflict with previous results (45) which reported loss of activity concomitant with modification of 4.2 arginine residues per subunit. Much greater protection was afforded by glucose and MgATP together than either subunit separately. It was felt that modification of the arginine residues affected the distribution of the enzyme between active and less active forms

rather than the catalytic site.

Serine residues are essential for the catalytic activity of several enzymes and Hexokinase has been studied from this point of view. Non-phosphorylatable analogues of glucose, lyxose and xylose induce a slow apparently irreversible inactivation of the enzyme due to phosphorylation of the protein (46, 47). Further studies showed that a serine residue is involved. (48). The phosphorylated peptide has been isolated and sequenced (49) and corresponds to residues 128 - 146 of the X-Ray sequence. This work shows that serine-142 is phosphorylated, although the X-Ray structure clearly suggests that serine-138 is likely to be the phosphorylated residue since it is close to the 6-hydroxyl of glucose while serine-142 is buried in the interior of the small lobe. (22). This autophosphorylation is aberrant in the catalysis and absent with true substrates and so it is felt that this particular serine residue is not essential for activity.

Histidine residues have been investigated in some detail (50) since they are involved in the catalysis of several phosphoryl transfers. Diethylpyrocarbonate specifically acylates histidine residues; in Hexokinase the nine histidine residues could be acylated with only a 60% loss in activity and substrates offered no protection so it may be concluded that no histidines are involved at the catalytic site. Succinylation of 2 - 3 of the 37 amino groups per subunit decreased activity by about 50%. (51). With inactivation was a simultaneous increase in the amount of enzyme in the monomeric form and it was felt that dissociation rather than any specific lysine modification was responsible for inactivation. Tyrosine residues have also been investigated using several different reagents (52, 53) but it has proved impossible to remove activity completely and thus it seems unlikely that a single tyrosine residue is involved at the

active site.

The residues considered above were not chosen for further study because they had been shown not to be essential for activity. Thiol groups, of which there are four, had been studied in depth (see chapter four) and so it was decided to continue this work further. Carboxyl groups were also studied - aspartic acid 189 had been implicated at the active site (18) and chemical evidence had been claimed for an active site glutamic acid (54). It therefore seemed logical to attempt to identify the active site carboxyl group. Work was also directed towards developing a suitable ATP analogue as an affinity label.

Table 2.1

Material	Supplier
Acetic Acid	Hopkin and Williams Ltd.
Acetone	Fisons Scientific Apparatus.
Adenosine Diphosphate	Sigma Chemical Co., Ltd.
Adenosine Triphosphate	Sigma Chemical Co., Ltd.,
Ammonium Bicarbonate	BDH Chemicals Ltd.
Ammonia	BDH Chemicals Ltd.
Biogels (P30 etc)	BioRad Laboratories Ltd.
Butan-1-ol	Fisons Scientific Apparatus.
Chromatography Paper	Whatman Ltd.
Coomassie Brilliant Blue G250	Sigma Chemical Co., Ltd.
Cresol Red	Fisons Scientific Apparatus.
Dansyl Chloride.	Sigma Chemical Co., Ltd.
Dialysis Tubing.	Medicell International Ltd.
DE52, DE23	Whatman Ltd.
DFP	Sigma Chemical Co., Ltd.
DTT	Sigma Chemical Co., Ltd.
EDTA	Fisons Scientific Apparatus.
Ethanol	James Burrough Ltd.
Formic Acid	Fisons Scientific Apparatus.
Fructose	Fisons Scientific Apparatus.
Glucose	Fisons Scientific Apparatus.
Glycine	Fisons Scientific Apparatus.
Glycyl-Glycine	Sigma Chemical Co., Ltd.
Guanosine Triphosphate	Sigma Chemical Co., Ltd.
Guanidine	BDH Chemicals Ltd.
Hydrochloric Acid AR	BDH Chemicals Ltd.
Hydrochloric Acid Aristar	BDH Chemicals Ltd.

Table 2.1 contd.

Material	Supplier
Imidazole	Sigma Chemicals Co., Ltd.
Iodoacetic Acid	Nutritional Biochem. Corp.
Magnesium Chloride	Fisons Scientific Apparatus.
Ninhydrin	Sigma Chemical Co., Ltd.
NSA	BDH Chemicals Ltd.
PITC	Fluorochem Ltd.
Polyamide Layer Sheets	BDH Chemicals Ltd.
Potassium Chloride	BDH Chemicals Ltd.
Potassium Cyanate	BDH Chemicals Ltd.
Potassium Hydroxide	BDH Chemicals Ltd.
PPO	Fisons Scientific Apparatus
Propan-2-ol	Fisons Scientific Apparatus
Pyridine	Rathburn Chemicals Ltd.
Semicarbazide	Sigma Chemical Co., Ltd.
Sephacel	Pharmacia Fine Chemicals Ltd.
Sephadex	Pharmacia Fine Chemicals Ltd.
Sodium Bicarbonate	Fisons Scientific Apparatus.
Sodium Borohydride	Sigma Chemical Co., Ltd.
Sodium Carbonate	BDH Chemicals Ltd.
Sodium Chloride	BDH Chemicals Ltd.
Sodium Cyanoborohydride	Sigma Chemical Co., Ltd.
Sodium Hydroxide	BDH Chemicals Ltd.
Sodium Periodate	Sigma Chemical Co., Ltd.
Sodium Succinate	Fisons Scientific Apparatus.
TFA	BDH Chemicals Ltd.
Triton X100	Fisons Scientific Apparatus.
Tris	Sigma Chemical Co., Ltd.

Table 2.1 contd.

Material	Supplier
Xylene	May and Baker Ltd.
Yeast	United Yeast Co., Ltd.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

The chemicals used were of Analar grade except for the hydrochloric acid used for peptide hydrolysis and N-terminal determination which was Aristar grade. Chemicals were used without further purification except for the reagents used for sequencing which were re-distilled, and the iodoacetic acid for carboxymethylation which was re-crystallized from light petroleum ether. A list of suppliers is shown in table 2.1.

Fractions were collected using an LKB Ultrarac fraction collector and all spectrophotometric readings were performed on a Unicam 500 spectrophotometer using a Gilford 2000 chart recorder. Amino acid analysis was with a Beckman 121 MB analyser while automatic peptide sequencing was on a Beckman Spinning Cup Sequencer (890B). High performance liquid chromatography was on a Walters instrument and freeze drying was carried out on a Virtis freezemobile II.

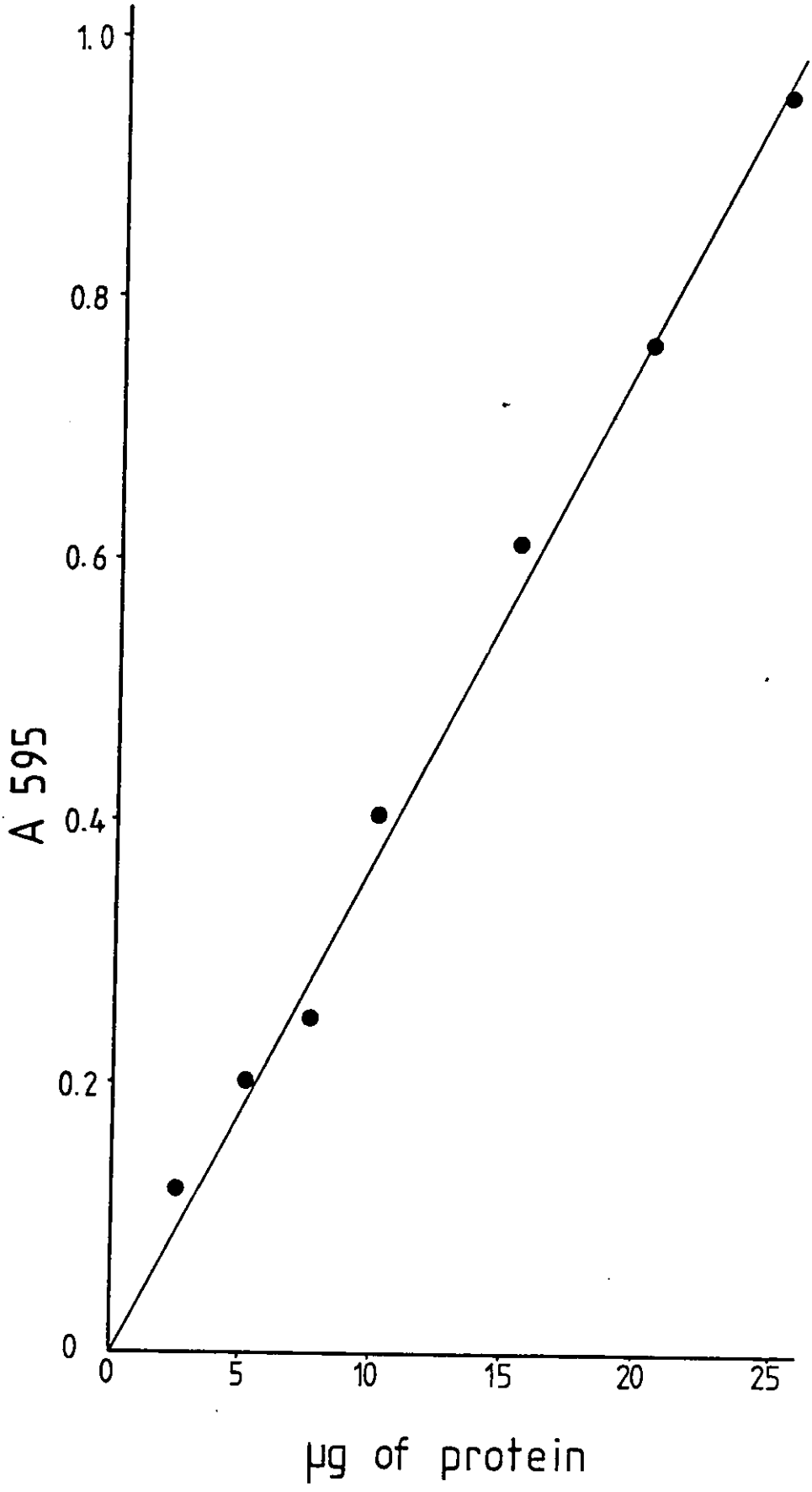
2.2 PROTEIN ESTIMATION

This was spectrophotometrically at low levels of purification assuming a specific absorptivity at 280 nm of 1 $mlmg^{-1}cm^{-1}$. At higher levels of purification and when accurate estimation of very low protein concentrations was required a method employing Coomassie Brilliant Blue G250 was used (55):-

Reagent — 100mg Coomassie Brilliant Blue G250
50ml 95% ethanol
100ml 85% (W/V) phosphoric acid

The above solution was diluted to 1000 ml with water. Protein solution containing 10 - 100 µg protein was mixed with 5 ml reagent and the absorbance at 595 nm was read between 2 and 60 minutes later

fig. 2.1 Standard Curve For Protein Determination



against a blank of reagent and buffer. It was possible to measure even lower amounts of protein by using 1 ml of reagent. In either case standard curves were constructed using Bovine Serum Albumin. (fig. 2.1).

2.3 ENZYME ASSAY

The method was based on that of Darrow and Colowick (56) with some modification. The Hexokinase reaction liberates protons which neutralize an equivalent amount of basic buffer (glycyl-glycine) - at the same time a proportional amount of an acid-base indicator (Cresol Red) is neutralised, the colour change (red to yellow) being monitored at 574 nm.

Reagents

1. 0.006% (W/V) Cresol Red, 1.8% (W/V) $MgCl_2.6H_2O$ stirred overnight to dissolve and filtered through glass wool. This reagent keeps indefinitely in the dark at 4°C.
2. ATP - disodium salt.
3. 0.1M glycyl-glycine buffer, pH=9
4. 0.1M NaOH.
5. 0.2M glucose.

Assay Solution

30 ml Cresol Red reagent.
3 ml glycyl-glycine buffer.
110 mg ATP.

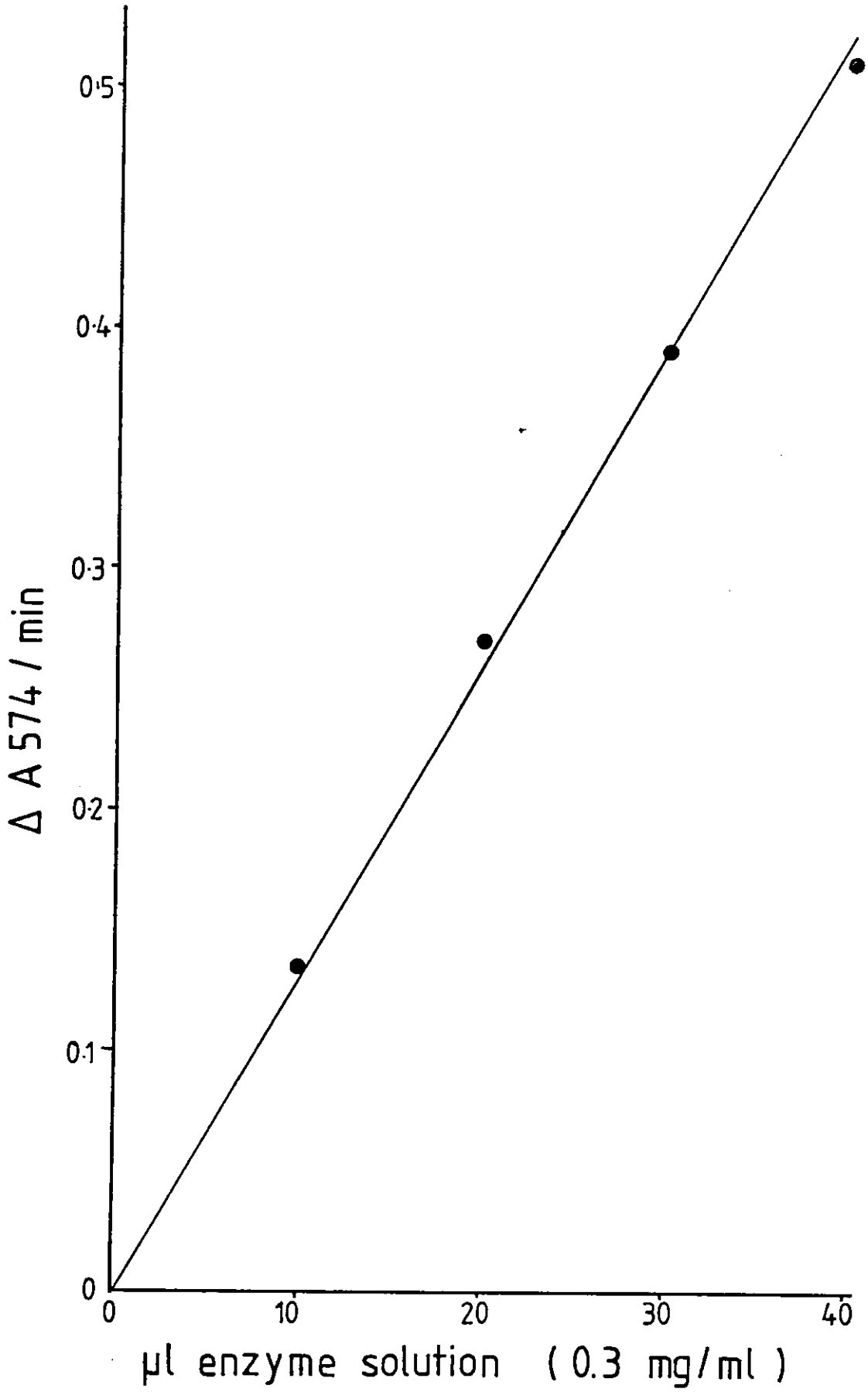
The assay solution was adjusted to pH=8.6 and diluted to 60 ml with distilled water.

Assay

2.5 ml assay solution.
0.4 ml 0.2M glucose.
10-100 µl enzyme.

The assay is very temperature sensitive and all reagents were maintained at 25°C. Standardisation was by adding 0.1 ml 0.1M HCl and measuring the change in absorbance at 574 nm. One unit of Hexokinase was equivalent to the liberation of 1 µmole protons per minute. The proportionality of the assay is shown in fig. 2.2.

fig. 2.2 Hexokinase Assay



2.4 DIALYSIS

Dialysis tubing was prepared by boiling in 5% (w/v) sodium bicarbonate solution, washing in distilled water, boiling in 5mM EDTA and washing thoroughly in distilled water. Tubing was stored in 1mM EDTA. Dialysis of protein (to exchange buffers or remove contaminating small molecules) was by stirring the tubing containing protein against large volumes of the appropriate liquid. Removal of radioactive contaminants was usually by dialysis against either 5% (v/v) formic acid or 1mM HCl.

2.5 RADIOACTIVITY - HANDLING AND PREPARATION

All isotopes used in this project were of carbon 14 and were purchased from Amersham International Ltd. In all cases the small quantity of high specific activity isotope was diluted with a larger known quantity of non-radioactive material. Great care was taken to remove all radioactive material from the vials supplied. In some instances further reactions had to be carried out on the radioactive material before it could be used to modify the protein.

The scintillation cocktail used to count the material was of the following composition:-

P.P.O.	3g
Xylene	750 ml
Triton X100	250 ml

In order to obtain a homogeneous system it was necessary that aqueous samples be $\leq 50\mu l$ or $\geq 450\ \mu l$ in 5 ml of cocktail. Samples were counted on an Intertechnique Scintillation Counter. Efficiency of counting was always greater than 90% as shown by counting 10 µl of a C14-nhexodecane standard which was known to contain 8735 dpm.

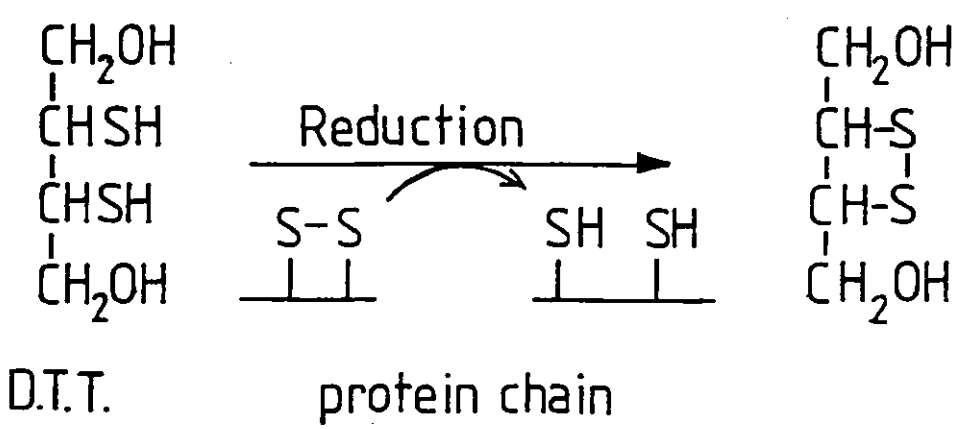
Radioactive material present on paper was identified by autoradiography. The paper was marked with radioactive ink and placed against Kodak Blue Brand film, in the dark, for two or three

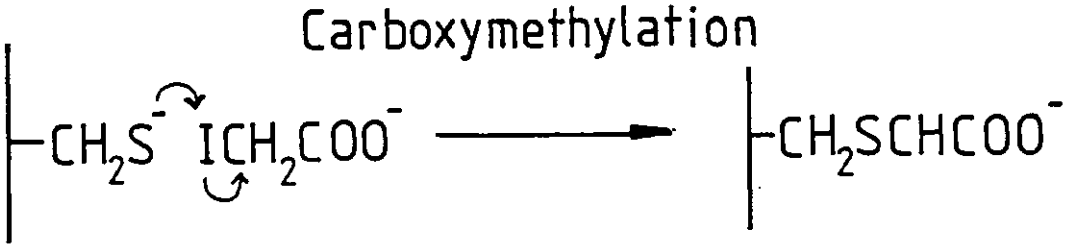
days. The exposed film was developed in a Kodak Xomat and lined up on the paper, using the radioactive ink spots. Radioactive material showed up black against the blue background and its position could be marked on the paper.

2.6 REDUCTION AND CARBOXYMETHYLATION

Carboxymethylation was used as a method of alkylating cysteine groups either to provide a specific label for cysteine or to block cysteines to prevent them reacting with e.g. cyanogen bromide. Normally carboxymethylation was carried out under denaturing conditions but obviously this was not the case when specific cysteines were being investigated. After denaturation, reduction by DTT was performed (57); although not strictly necessary because Hexokinase does not contain disulphide bridges, reduction was carried out as a precaution in case some oxidation had occured.

Reactions





Method

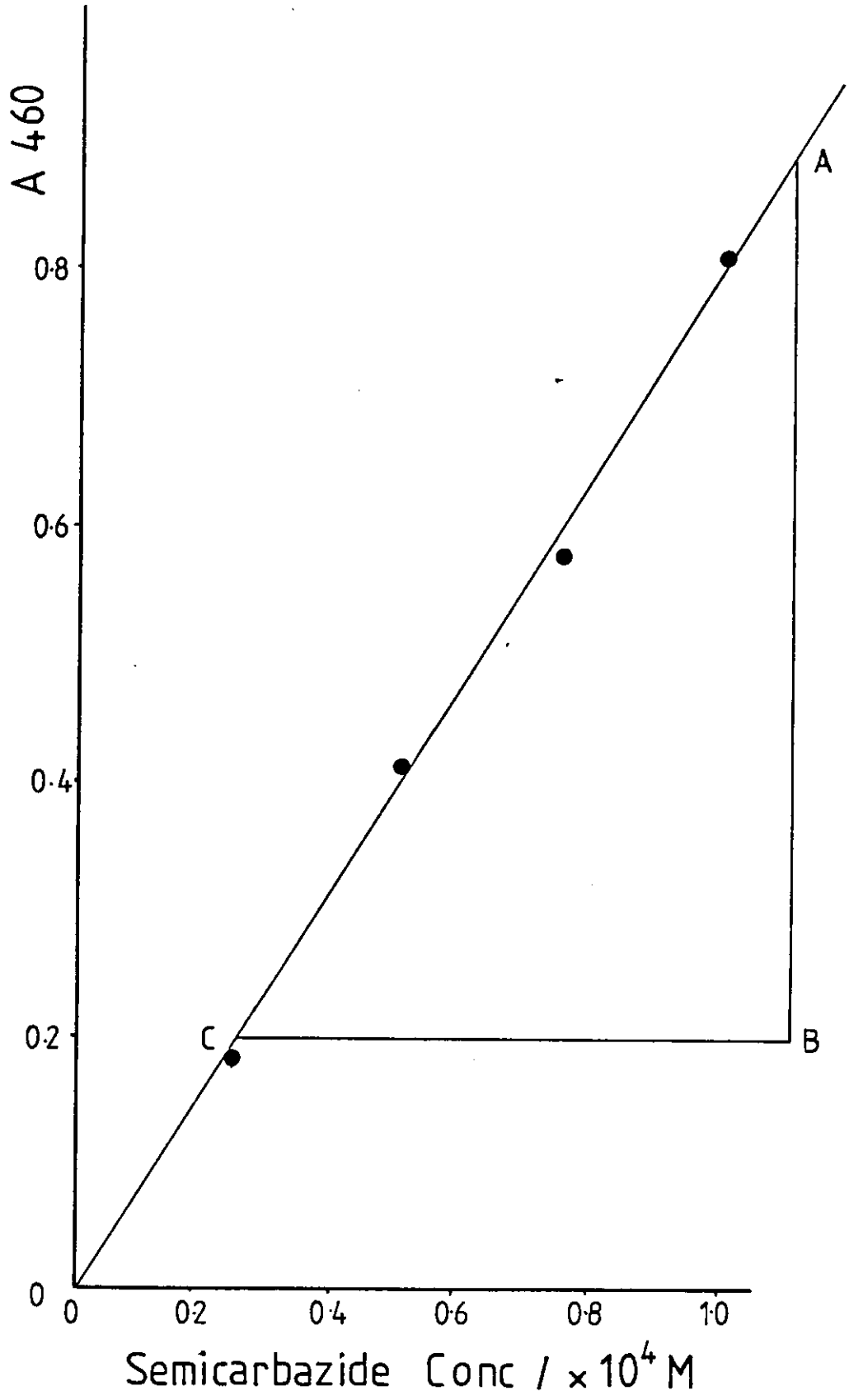
The protein was dissolved in Tris-guanidine hydrochloride buffer pH=8.5 (6M guanidine, 0.3M Tris) to a concentration of about 5 $mgml^{-1}$. The resulting solution was flushed with nitrogen gas and DTT added in two-fold molar excess over thiol groups, the solution flushed with nitrogen and allowed to stand for one hour for reduction to occur. Recrystallised iodoacetic acid was added in ten-fold molar excess and the pH maintained above 8.5 by the addition of solid TRIS. After 30 minutes in the dark at room temperature the excess reagent was removed from the carboxymethylated protein by dialysis against 5% formic acid.

2.7 PREPARATION AND ASSAY OF RADIOACTIVE SEMICARBAZIDE

Unlabelled semicarbazide was easily obtained but it was necessary to prepare radioactive semicarbazide from ^{14}C-potassium cyanate. (58). The semicarbazide was prepared by adding 161 mg potassium cyanate containing 250 μCi of ^{14}C-material to a solution comprising of 0.1 ml hydrazine hydrate, 0.4 ml 5M HCl and 1.4 ml water at pH=7. The pH was maintained at 7 by the addition of acid over two hours and the solution left standing overnight. The white precipitate was removed by centrifugation and the supernatant assayed for semicarbazide and counted for radioactivity. The assay (59)

fig. 2.3

Standard Curve to Calculate Extinction Coefficient of Semicarbazide



was based on the fact that semicarbazide forms an adduct with 1:2 napthaquinone-4-sulphonic acid (NSA) giving a visible maxima at 460 nm. The assay mixture was of the following composition:-

	ml
$1x10^{-3}$M NSA	5
0.1M NaOH	1
sample	x
water	4-x

The absorbance was measured after 2.0 minutes at 25°C and it was found that Beer's Law was obeyed up to $2x10^{-4}$M semicarbazide. The absorbance of the blank was about 0.06 and the extinction coefficient of semicarbazide was calculated as being 8.0×10^{-3}M (see fig. 2.3). Typically yields of semicarbazide were in the order of 75% (lit. 93%).

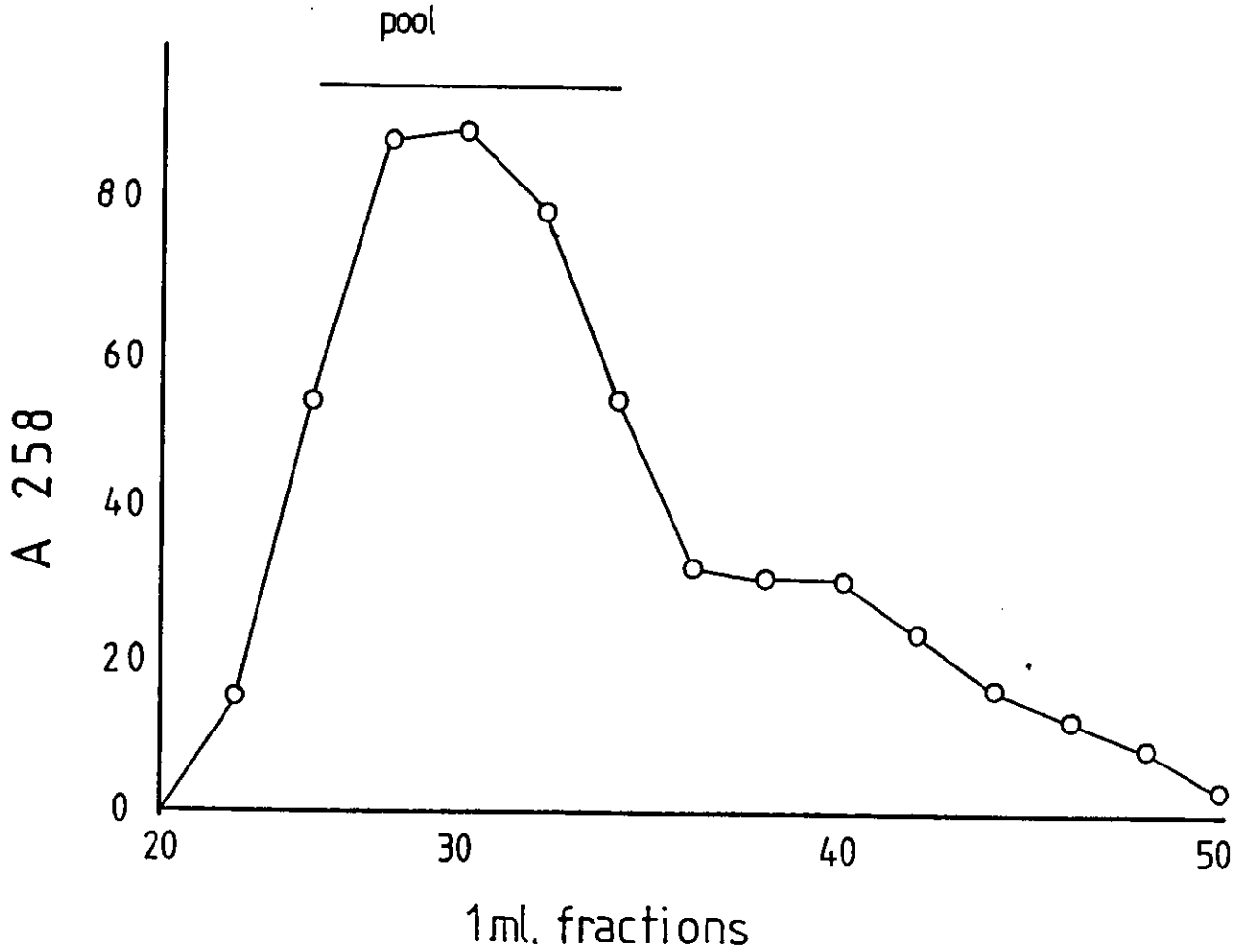
2.8 PREPARATION OF 2',3'-DIALDEHYDE ATP

Neither labelled nor unlabelled oATP were available commercially and thus they were prepared by the method of Easterbrook-Smith et al (60).

ATP (0.1 mmoles) was dissolved in water and the pH adjusted to 7 at 0°C with dilute sodium carbonate. Sodium periodate (0.11 mmoles) was added and the solution stirred for one hour in the dark at 0°C. The reaction was stopped by the addition of 0.05 mmoles of ethane diol and the oATP separated on a sephadex G10 column (25 x 2.5 cm) equilibrated with 25mM KCl. (61 fig. 2.4). The fractions were monitored at 258 nm and the peak tubes pooled. The absorption coefficient was 14 900 cm^{-1} M^{-1}. Typical yields were 50% and the purity was checked by two methods (60):-

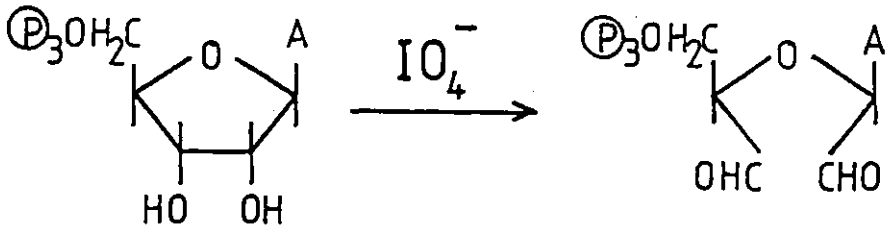
1. Thin layer chromatography on polyethyleneamine sheets, using 0.8M ammonium bicarbonate and ultra violet light to visualise any spots. One spot was observed with a mobility of 0.02.

fig. 2.4 oATP Isolated on Sephadex G10



2. Ascending paper chromatography on Whatmann 3mm paper using a solvent system of Butanol : Acetic Acid : Water, (4:1:5). One spot with a mobility of 0.1 was observed.

Reaction



The radioactive compound was prepared using the $U^{14}C$ - ammonium salt of ATP in a small quantity and high specific activity and diluting it by the unlabelled disodium salt of ATP before addition of periodate..

2.9 DIGEST STRATEGY

The aim of digestion was to obtain suitable peptides in a pure form in order to sequence them and identify labelled residues. Manual sequencing by the Dansyl-Edman technique and an automated Beckman Spinning Cup Sequenator were used. The two types of digest used were peptic and cyanogen bromide.

Peptic.

Pepsin gave a large number of small peptides which could be easily purified using gel filtration and HVPE. The large number of peptic 'cuts' meant that many overlaps were obtained and so yields were small. In spite of this a fairly large amount of manual sequence was obtained and overlaps proved useful.

Cyanogen Bromide

Cleavage at the 11 methionine residues gave a small number of large peptides with relatively few overlaps. After gel filtration the peptides were not suitable for separation on paper and a mixture of ion exchange chromatography and H.P.L.C. was used. One disadvantage of the CNBr digest is that an equimolar mixture of peptides with C-terminal homoserine and homoserine-lactone is obtained. (fig.2.5), (62,63). These peptides had similar molecular weights but the 'homoserine peptide' had an extra negative charge and would behave differently when separation on the basis of charge was used. Ring opening was performed by incubating in 0.2M ammonium bicarbonate at 21°C for four hours. (64). Homoserine is more suitable for amino acid analysis than the lactone and is also stable in alkali which is useful for ion exchange chromatography.

Details of the digest methods are shown in table 2.2.

2.10. SEPARATION AND IDENTIFICATION OF PEPTIDE MATERIAL ON PAPER

All separations of peptides, both analytical and preparative were carried out on Whatman 1mm paper which contained few contaminating amino acids and could be loaded up to 0.1 umole per cm. Separations were carried out in volatile buffers because it was not possible to desalt on a microscale.

High voltage paper electrophoresis (HVPE) was carried out at pH=6.5 and pH=2.1 in apparatus shown in fig. 2.6 (65). At pH=6.5 peptides (and amino acids) fell into three classes : acidic, basic and neutral. Acidic and basic peptides separated well at pH=6.5 and could be further purified on the basis of hydrophobicity by descending chromatography. Neutral peptides migrated by about one cm due to electroendosmosis but did not separate at pH=6.5. The neutral band was cut out and stitched onto a fresh sheet of Whatman No 1, the backing removed (so no double layer of paper existed) and re-run at

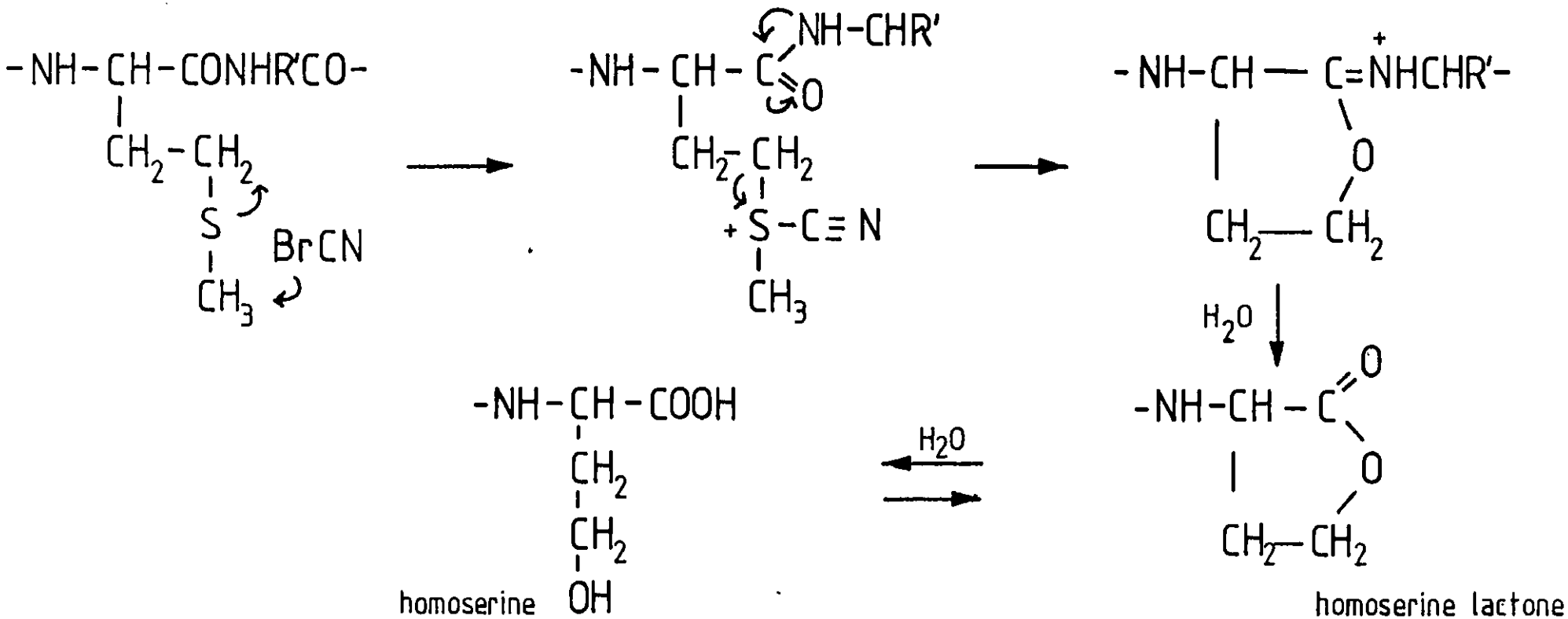


fig. 2.5

The Cyanogen-Bromide Reaction

$NH_2CHR'CO-$ peptide chain

Table 2.1

DIGEST	CLEAVAGE POINTS	Pretreatment	RATIO	CONDITIONS	PEPTIDE PURIFICATION
PEPSIN	X - F - X X - L - X X - Y - X Plus other groups with bulky non-polar side chains.		1:100 w/w pepsin: Hexokinase	5% Formic 2 hours 37°C Halt reaction by freezedrying Hexokinase at 5mg/ml	Biogel P4 (- 400 mesh) sizing column followed by separation by HVPE and descending chromatography.
CNBr	at M to give an equimolar mixture of C terminal homoserine and homoserine lactone Partial cleavage due to M sulphoxide not being cleaved, M-S, M-T, M-C-C, M-E only partially cleaved. Additional cleavage at D-P & N-P oxidative cleavage at W	carboxy-methylation	CNBr in 100 x molar excess over M residues.	70% Formic 24 hours RT in dark remove CNBr by rotary evapo ration and freezedrying	Biogel P30 (- 400 mesh) sizing column followed by either ion exchange chromatography or HPLC

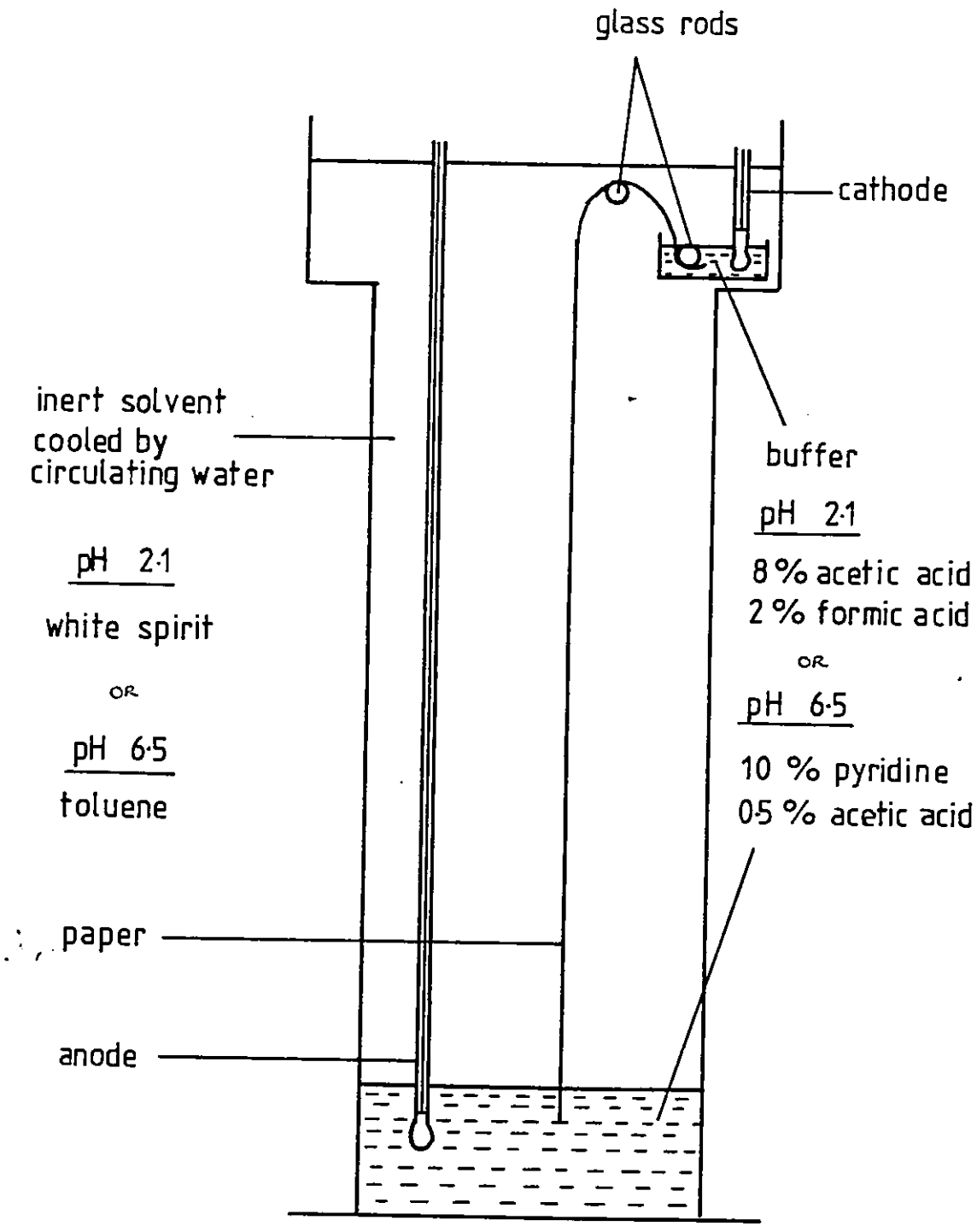
pH=2.1 whence separation was achieved. Further purification was by descending chromatography.

The peptide material was applied to the paper in a narrow band at a suitable distance from the edge (10 cm for pH=2.1 and 25 cm for pH=6.5). Between applications the peptides were dried using cold air from a hair dryer. Dansyl markers (DNSOH, DNSARG, DNSARGARG) were applied in a similar way so that after HVPE the mobilities of the peptides could be calculated (66, 67). The appropriate buffer (see fig. 2.6) was used to wet the paper either side of the sample and allowed to flow to concentrate the sample by capillary action. Excess buffer was blotted off and the paper arranged as shown in fig. 2.6. A potential difference of 3KV was applied for between 45 and 60 minutes, the paper removed, dried and stained by the appropriate method. During an analytical separation all the material was stained but during a preparative separation only a narrow guide strip was stained because stains tended to quench radiation and may have prevented sequencing in certain cases when the stain blocked the N terminus. Before staining, peptides exhibiting natural fluorescence in ultra violet light, were marked - these peptides contained aromatic amino acids particularly Tryptophan and Tyrosine. Cadmium-ninhydrin was the primary staining method.

Separation by descending chromatography was carried out before staining. The solvent mixture (usually butan-1-ol, acetic acid, water, pyridine, 15 : 3 : 12 : 10) was placed in the upper trough of a tank similar to that shown in fig. 2.6 and allowed to run down the paper by capillary action overnight. The paper was dried and treated as for HVPE.

In preparative work it was necessary to elute unstained peptide material from the paper. A strip containing a single peptide detected by staining a small spot of the material run in parallel was cut out

fig. 2.6 Apparatus For HVPE



and arranged so that the eluting buffer ran down the paper by capillary action. Basic peptides were eluted using 20mM Acetic acid and acidic peptides using 20mM Ammonia. Only a small quantity of eluant was collected because most of the material eluted is in the first drop. Peptide separation on paper gave poor yields - in the order of 25 - 30%.

2.11 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Separation of a mixture by liquid chromatography depends upon each component (solute) having a different distribution coefficient between a stationary, and mobile liquid phase. In H.P.L.C. a dilute solution of the sample is pumped through a column packed with small diameter particles - high pressures (1000 p.s.i. and greater) are used in order to speed up the separation. The aim of H.P.L.C. is to optimise resolution of solutes, speed of elution and economic use of pressure (68).

To separate peptide mixtures it is usual to use a column with a chemically bonded stationary phase, prepared by bonding an organic moiety to the surface of an adsorbant. Gradient elution techniques are usually employed.

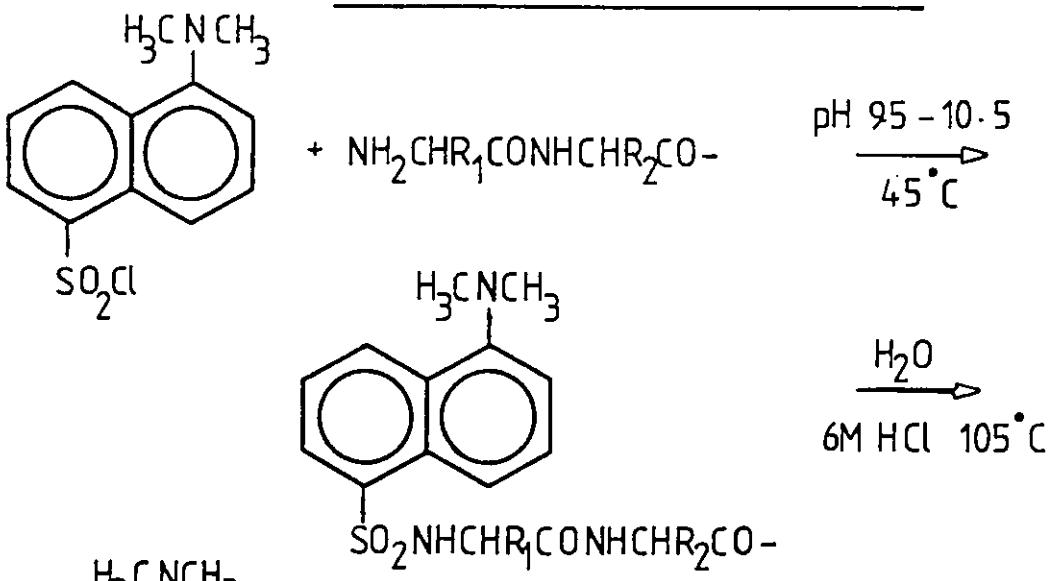
The separation work carried out for this thesis used the 'reverse phase technique'. The polarity of the packed medium is low and a gradient starting with a fairly high polarity (e.g. acetic acid) moving to lower polarity (e.g. propanol) is used. Obviously the most polar solute will elute first.

2.12. N-TERMINAL DETERMINATION

Dansyl chloride is a typical aromatic sulphonyl chloride which reacts with a wide variety of bases forming fluorescent, easily identifiable derivitives. It reacts with the amino group of amino acids and peptides as shown(69) fig. 2.7. Optimum conditions for the reaction are several-fold excess of 5mM Dansyl chloride for

fig.2.7

N-Terminal Determination



Dansyl Amino Acid

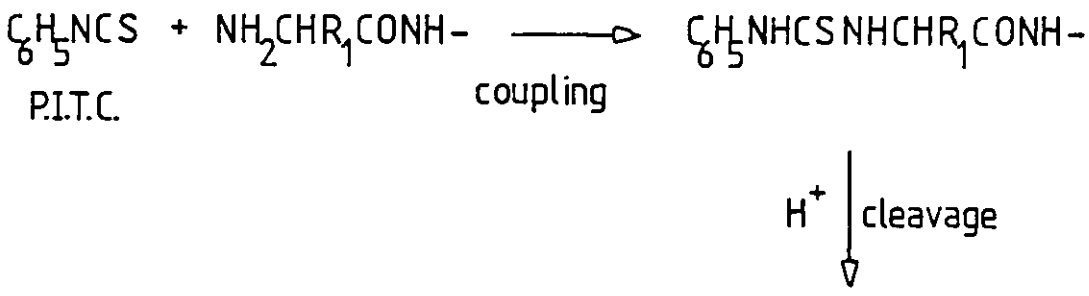
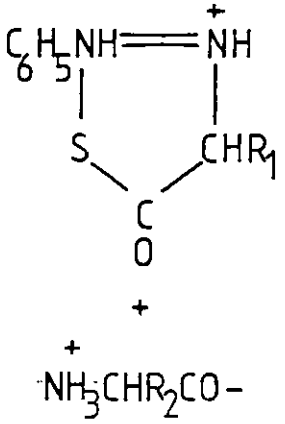


fig. 2.8

Subtractive Edman Degradation



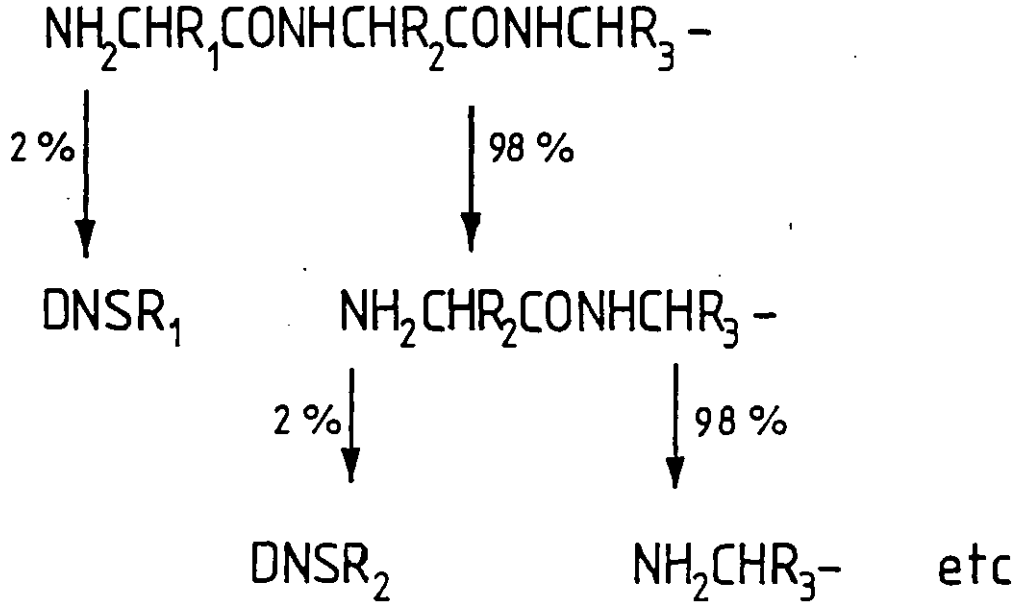
45 minutes, 45°C, pH 9.5 - 10.5. The pH is important because above pH=10.5 the reagent is hydrolysed too rapidly and below pH=9.5 the unreactive protonated forms of the amino acid are present in too high a concentration.

Procedure (70)

1 - 2 nmoles of peptide were dried down in a small thick walled glass tube and 5 µl of sodium bicarbonate solution 0.2M added. To this 5 ul of 2.5 mg/ml solution (in acetone) of Dansyl chloride were added - the acetone increased the pH to above 9.5, surpresses amino group ionisation and solubilises the Dansyl chloride. The tubes were sealed with parafilm and incubated for 45 minutes at 45°C - the yellow colour had disappeared and the clear solution was dried down. To the residues 15 µl 6M HCl were added and the tubes drawn and sealed in an oxygen flame. After incubation at 105°C for either 6 hours (non N terminus) or overnight (N-terminus) the tubes were opened and dried down. The residues were dissolved in about 1 µl of 95% ethanol and spotted out on both sides of a polyamide sheet. On one side only a mixture of marker Dansylamino acids was spotted so that identification of the sample could take place. The plates were run in a series of solvents of increasing alkalinity and the Dansyl amino acid identified. (71).

2.13 SEQUENTIAL EDMAN DEGRADATION PLUS DANSYLATION AS A SEQUENCING TECHNIQUE

The phenyl isothiocyanate reaction (72), fig. 2.8 can be used to degrade a peptide from the N-terminus, one residue at a time, leaving the remainder of the peptide intact. This method can be used directly to sequence peptides by identifying the PITC amino acids formed but this is not as easy as identifying Dansyl amino acids. A procedure combining the Dansyl-Edman methods has proved most effective:- (73).



The Edman reaction mechanism is shown in fig. 2.8 and can be divided into a coupling reaction and a cleavage reaction. During the coupling reaction a high pH is required because the PITC reacts with unprotonated amino groups only; it is also necessary to choose a solvent system in which the peptide and PITC are both soluble. A reaction pH of 9 at 45°C for one hour was used - the solvent system chosen was 1 : 1, water : pyridine. The reaction was carried out under nitrogen to prevent oxidative desulphuration of the phenylthiocarbamyl group. After the coupling reaction had occured excess PITC was removed by evacuating in a heated dessicator over P_2O_5 and NaOH - this completely removed PITC, pyridine, water and volatile side products such as phenol and aniline.

The cleavage reaction is nonhydrolytic and relatively rapid - it was necessary for relatively mild conditions to be used to prevent cyclisation of nitrogen which would block the reaction. Other side reactions (74) are also prevented by using relatively mild conditions.

100 µl of TFA are added and the solution incubated for 10 minutes at 45°C and then dried over NaOH. The resulting peptide is now one amino acid residue shorter, but before dansylation of the new N-terminus is carried out, the diphenylthiourea must be removed by extracting with a fairly polar solvent such as n-butylacetate. The TFA residues were taken up in 200 ul water and extracted three times with 1 ml redistilled n-butyl-acetate. At this stage an aliquot is removed for dansylation before drying down for a further Edman round.

2.14. AUTOMATED SEQUENCING TECHNIQUE

The advantages of sequencing a peptide automatically are chiefly those of ease of handling and high repetitive yields. The method used in the department is that of the spinning cup (75) using a Beckman Spinning Cup Sequencer (890B) with improved vacuum system which carries out what is basically the Edman reaction automatically (section 2.13).

Coupling of the sample takes place in 0.25M QUADROL TFA buffer pH9 for reasons of solubility with the addition of 5% PITC in heptane. After reacting for 20 - 30 minutes at 54°C the coupled peptide is dried to a film and washed with a benzene/ethyl acetate mixture, the latter containing 0.1% acetic acid. If less than 50 nmoles of peptide is available a carrier polymer, Polybrene is required to minimize losses during the washing stages.

The phenylthiocarbamyl derivitive is dried and subjected to acid cleavage with anhydrous heptafluorobutyric acid - if preliminary work has shown the sequence to be acid stable a second addition of acid may be made for a double cleavage reaction. The peptide material is taken to near dryness (while allowing solvent penetration) and extraction with 1-chlorobutane containing 1 mg DTT per 100 ml carried out. The extract is converted to the corresponding phenylthiohydrantoin using 1M HCl containing 1.5 mg/l DTT under N_2

at 80°C. The PTH was then identified by HPLC using the following system:-

25mM sodium acetate, pH 5.15 and 96% ethanol

18% ethanol	6 / 8'	43% ethanol
82% NaAc		57% NaAc

This separates all PTH's except those of valine and proline which can be distinguished by their A 280/ A 254 ratios.

2.15 AMINO ACID ANALYSIS

Before sequencing a peptide it is useful to know the amino acid composition - this gives some idea of size, purity and quantity of material. The initial step is hydrolysis of the peptide into its constitutive amino acids.

The sample (1 - 10 nmole) was dried down in a hydrolysis tube and 50 ul Aristar 6M HCl added. The acid contained norleucine (an internal standard) and phenol (to prevent tyrosine destruction). The tube was drawn, evacuated (to prevent formation of cysteic acid, methionine sulphoxide and chlorotyrosine) and then sealed. Hydrolysis was at 105°C for 18 - 24 hours after which the tube was opened, the contents dried and the sample analysed.

The analyser separates the amino acids on an ion exchange column of sulphonated polystyrene - a strongly acid resin. A three buffer salt gradient was used:-

buffer	pH	
0.2M citrate	3.28	all containing
0.35M citrate	3.9	isopropanol
1.4M citrate	4.95	
0.2M NaOH wash		

The most acidic amino acids were eluted first - about 90%

of the separation was due to the salt gradient but norleucine, leucine and isoleucine were separated on the basis of temperature. The isopropanol aided separation of glycine and alanine but hampered that of serine and threonine so the correct balance of isopropanol had to be found. (76). The amino acids were detected using ninhydrin bearing in mind that hydrolysis destroys some amino acids. (77).

Tryptophan	completely destroyed
Serine	15% decomposition
Threonine	6% decomposition
Cysteine	4% decomposition
Tyrosine	10% decomposition
Valine	apparent increase with time
Isoleucine	because hydrolysis of side chain is slow.

CHAPTER 3.

ENZYME PURIFICATION

The main problem found in yeast enzyme purification is that of proteolysis. In order to achieve a homogenous preparation and to avoid the formation of artifactual "isoenzymes", (56, 78), it is necessary to take steps to prevent this proteolysis.

3.1 DEVELOPMENT

Classical methods of yeast Hexokinase preparation (56, 79) involved drying the yeast cells at room temperature for 10 - 14 days, followed by autolysis at 37°C for about three hours. Although not much activity is lost by this process, proteolytic enzymes may be released during the slow drying, when the cells maintain many of their enzyme systems, and during autolysis, when vigorous acid production occurs. The Hexokinase will then be partially degraded either at this stage, or after isolation in an apparently pure form, due to adhering traces of protease.

Methods of preventing proteolysis have included chromatography on DEAE cellulose (4), freeze thawing (80) and use of the serine protease inhibitors PMSF (81) and DFP (80). These methods were partially successful but did not prevent some initial attack on native Hexokinases by non-serine proteases which have been isolated and characterized. (82). No known inhibitor exists for the acid protease of yeast and its natural substrate is not known.

In order to overcome this problem the slow freeze thawing technique which could permit some proteolysis was replaced by rapid mechanical cell disruption in a French press or Manton Gaulin Homogenizer (1). The preparation was treated with DFP initially, and at critical early stages while pH and temperature were controlled to keep exposure to DFP insensitive proteases to a minimum. Instead of slow dialysis rapid gel filtration was used to remove the ammonium

sulphate; ion exchange chromatography removed the proteases during the final stages of purification.

The large scale method used in Imperial College Pilot Plant was based on that of Barnard (2). It was not possible to use DFP during the early stages so PMSF was substituted; also ammonium sulphate fractionation was not step-wise because of the problems posed in carrying out several large scale spins. Two-stage ion chromatography was carried out but instead of the second stage being on DE52 the newly available DEAE sephacel was found to give better results.

3.2 METHOD

Initial cell breakage and large volume spins were carried out by Imperial College Pilot Plant Staff. Other operations were on a laboratory scale.

Cell Disruption 20kg yeast (commercial packed wet yeast) were suspended in 60 l. of chilled 20mM Tris HCl buffer pH 7.5 containing 10^{-4}M PMSF and 10^{-3}M EDTA using a Silverson Homogenizer. The final volume of cell suspension was approximately 70 l. PMSF was used at this stage to avoid pilot plant staff having to handle large amounts of DFP.

The cells were disrupted by passing the chilled suspension through a Manton Gaulin Homogenizer operating at 8000 p.s.i. It was necessary to homogenize twice to achieve 80 - 90% enzyme release. The cell suspension and homogenate were kept chilled at all times; in particular the homogenate was rapidly re-chilled after each cell-breakage pass and the machine was pre-chilled by passing iced water through it.

Acid Precipitation The pH of the homogenate was adjusted to 5.0 with 12% acetic acid. Since the enzyme is rapidly inactivated below pH 4.6 the acid was added slowly with efficient stirring (but

avoiding froth formation).

The precipitate formed contained nucleic acids, cell debris and impurities and was removed using a Sharples continuous flow centrifuge. Two cycles were given, the supernatent retained and the discharged solids checked to contain zero activity. The pH was adjusted to 7.5 by addition of solid Tris. and the conductivity adjusted to that of 50mM Tris HCl (i.e. 3mS) using chilled distilled water.

DE23 Fractionation 14kg DE23 were added and the cooled suspension stirred for 45 minutes. The resin was removed by spinning in a gauze bag in a domestic spin dryer. The resin was immediately washed in cooled 100mM Tris HCl pH 7.5, 30 l and collected in the spin dryer. The resin was now stirred in 35 l of cold 500mM Tris HCl, pH 7, 10^{-3}M EDTA, 10^{-4}M PMSF and the supernatant collected after 35 minutes.

At this stage about 50% of the initial units is recovered. Normally there is negligible activity in the 20mM and 100mM supernatants, thus most of the activity must be retained on the resin. If higher ionic strengths were used more protein would be eluted and thus the purification would drop.

Ammonium Sulphate Precipitation The supernatant was adjusted to pH 5.0 with 12% acetic acid and a good grade of solid ammonium sulphate added to 0.8 saturation (490 g/l). After cooling for two hours two spins on the Sharples centrifuge were sufficient to give a small precipitate ($1\frac{1}{2}$kg) containing all of the Hexokinase. The precipitate was dissolved in the minimum volume of 10mM succinate pH 5.9 and the pH adjusted to 7.0 with solid Tris. DFP was added to 10^{-4}M and the slurry left at 4°C for 20 minutes to react. DFP does not inactivate acid proteases and is ineffective below pH.7. The addition of DFP was done with great care in a fume cupboard because it is highly toxic.

After reacting with DFP, the pH of the slurry was lowered to 5.0, which served to precipitate remaining debris. Low pH and high ionic strength convert isoenzyme C to isoenzyme B (1). The slurry was spun in a Sorvall centrifuge at 8000 r.p.m. (11 000g) for one hour.

The supernatent was desalted on a G25 column equilibrated with 10mM succinate pH 5.8. The brown protein band separated clearly from the green salt band and was collected. The protein pool was acidified to pH 5.0 and spun again at 8000 r.p.m. for one hour to give a clear solution which would not clog the ion exchange columns.

Ion Exchange Chromatography The pH was adjusted to 5.8 with Tris and the conductivity to that of 10mM succinate (1.5mS). The pool was pumped onto two DE52 ion exchange columns previously equilibrated with 10mM succinate, mM EDTA pH 5.8. Elution was with a pH gradient consisting of 3 l of equilibrating buffer and 3 l of the same buffer pH 4.6. The fractions were assayed for enzyme activity, OD280 and ionic strength. A typical profile is shown in fig. 3.1.

The second peaks of activity eluted from the column were pooled, the pH and conductivity adjusted and the pool applied to a DEAE sephacel column. A similar gradient was applied consisting of two lots of 600ml. The main peak of activity was pooled and consisted of pure Isoenzyme B which was concentrated and stored. Fig. 3.2.

Concentration A small DE52 column was prepared (1ml/25mg protein) and equilibrated with 10mM Tris HCl pH 8.3. The enzyme (pH adjusted to 8) was passed onto the column and a little of the Tris buffer run through. Elution was with 0.1M succinate 1mM EDTA pH 5.5 1M NaCl and 1ml fractions collected. These were tested for protein content by mixing 10ul aliquots from each tube with 500 ul cold 10% TCA; protein presence was indicated by turbidity.

Protein containing fractions were pooled and assayed. The

fig. 3.1

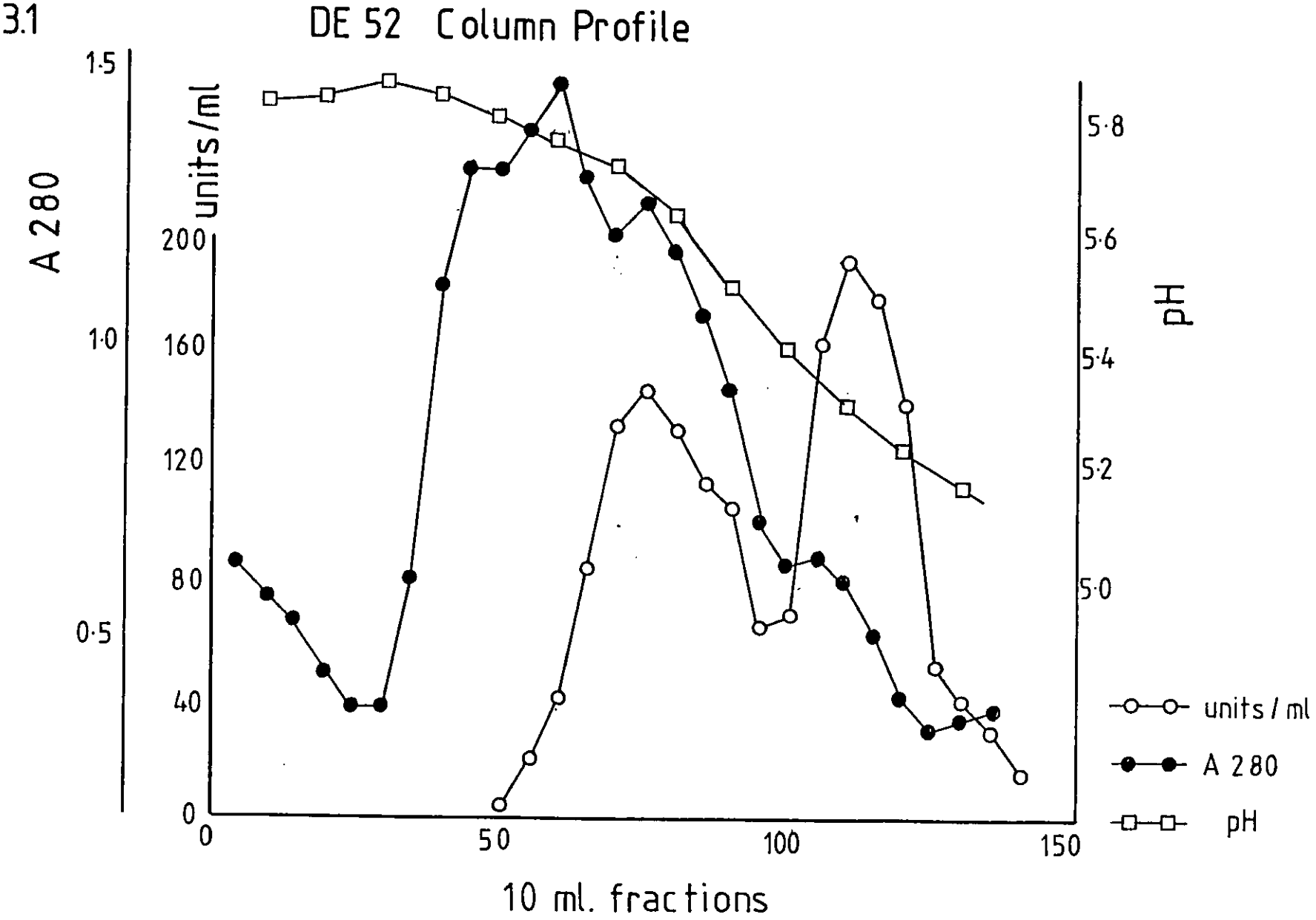
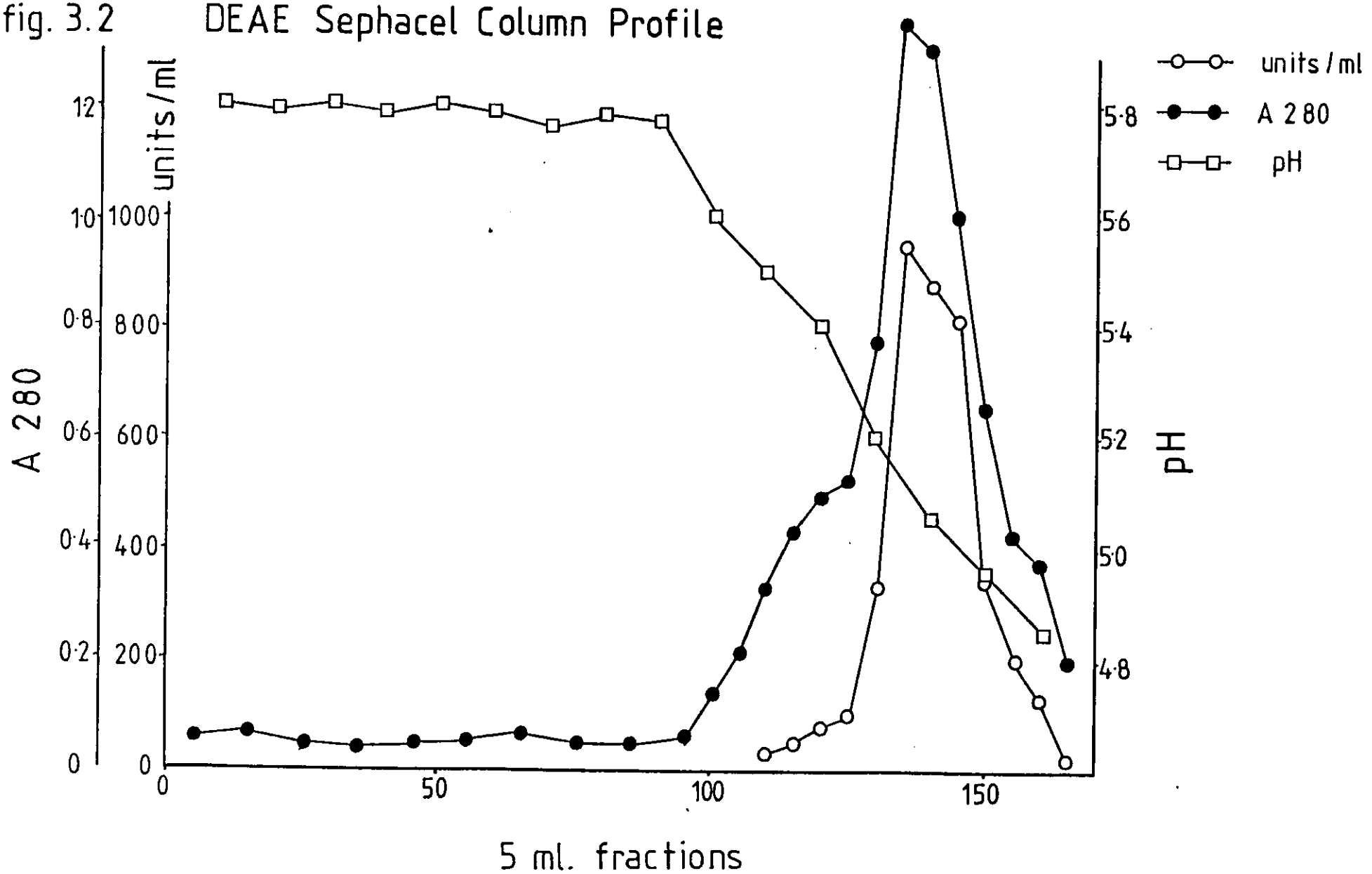

DE 52 Column Profile
A 280
1·5
1·0
0·5
units/ml
200
160
120
80
40
0
pH
5·8
5·6
5·4
5·2
5·0
0
50
100
150
10 ml. fractions
units / ml
A 280
pH

fig. 3.2 DEAE Sephacel Column Profile



preparation was routinely of specific activity 800 units/mg. Experience showed that the best concentration at which to store the enzyme was about 15mg/ml. Storage at higher concentrations for long periods led to the enzyme coming out of solution and losing activity. The enzyme was, therefore, diluted where necessary and stored at $4^{\circ}C$ - it must not be frozen because activity is lost on thawing.

If pure Isoenzyme A was required the first activity peak from the DE52 column was passed onto a DEAE sephacel column (as for Isoenzyme B). The concentrated enzyme had a specific activity of about 250 units/mg.

A summary of a typical preparation is shown in table 3.1.

Table 3.1

RESULTS OF A TYPICAL PREPARATION OF HEXOKINASE B

STAGE	VOLUME l	ACTIVITY unitsml^{-1}	TOTAL UNITS	(PROTEIN) $mgml^{-1}$	TOTAL PROTEIN mg	SPECIFIC ACTIVITY units mg^{-1}	PURIFICATION	YIELD %
EXTRACT	47	54	2.3×10^6	405	1.9×10^7	0.12	1	100
DE23	40	25	1×10^6	54	2.8×10^6	0.36	3	43.5
AMMONIUM SULPHATE	2	500	1×10^6	137	2.7×10^5	3.6	30	43.5
DE52 ISOENZYME A	0.6	450	2.7×10^5	3.2	1.9×10^3	140	1166	11.7
DE52 ISOENZYME B	0.75	293	2.2×10^5	1.3	9.4×10^2	240	1983	10
TOTAL A + B			4.9×10^5		2.9×10^3			21
SEPHACEL ISOENZYME B	0.05	4200	2.1×10^5	4.5	2.2×10^2	937	7812	9

CHAPTER 4

THIOL RESIDUES IN HEXOKINASE

4.1 INTRODUCTION

Cysteine residues are commonly found at the active centres of enzymes - the - SH group is a good nucleophile and often acts in a similar way to the - OH group of serine. Thus the proteases, Papain and Ficin, contain essential thiols which act in a similar way to the serine in Chymotrypsin and form thioesters. Several methods for investigating thiol groups are available, including alkylation and the use of organomercurials.

4.2 THIOLS IN HEXOKINASE

The cysteine content of Hexokinase has been subject to some speculation. Early work on heterogeneous preparations gave variously six thiols/dimer (83,84) 7.8 thiols/dimer (85) and six/dimer in Hexokinase B and eight/dimer in Hexokinase A. (9). However later work, (86,87) on purer preparations indicated that four sulphydryls were present per monomer and eight per dimer in both Hexokinase A and B. The methods used included p.mercurobenzoate titration, alkylation followed by carboxymethyl cysteine determination, reaction with methyl mercuric iodide and performic acid oxidation followed by cysteic acid determination. All workers agreed that no disulphide bridges were present.

4.3 ESSENTIAL AND NON-ESSENTIAL THIOLS

The cysteine determinations were carried out under denaturing conditions so all thiols would be equally accessible to the reagents. This of course gave no indication as to the relative reactivity of the thiols, nor the effect upon enzyme activity of thiol modification. Preliminary experiments with various thiol reagents, (88) showed that at temperatures above 30°C activity of native Hexokinase was abolished as cysteine

groups were modified. This suggested that one - four - SH groups per monomer are required for activity - it was then necessary to investigate this more fully.

Reaction with iodacetate and iodoacetamide, (both non-active centre directing reagents), at 35°C showed that the thiols in Hexokinase fall into two classes. (89) Using ^{14}C-labelled reagent two thiols reacted very quickly and two only very slowly. (see fig 4.1). Activity was 96% abolished when only two thiols had reacted. The inactivation was strongly pH and ionic strength dependant. At pH values up to 8.9 increase in rate of inactivation was due to dissociation of - SH groups while above pH 8.9 the enzyme spontaneously inactivated so the rate of alkylation could not be accurately determined. At very low ionic strengths the rate of inactivation was slow, increasing to a maximum at I = 0.4 and falling off at higher ionic strengths. The elevation of rate is much greater than expected from stabilisation of the transition state or modification of the pk of the reacting group and possibly involves the monomer-dimer equilibrium of the enzyme. The decline in rate at high I suggests a conformational change which makes the apparently essential thiols less available to the alkylator.

The fact that the inactivation by non-substrate-like alkylating reagents was prevented by the addition of both sugar and nucleotide substrates led to the view that an active centre thiol(s) may be involved. These ideas had to be confirmed by using substrate like alkylating reagents and for this purpose N-bromoacetyl-D-galactopyranosylamine (BAGA) and N-bromoacetyl-D-glucopyranosylamine were synthesised. (90) BAGA was shown to inactivate Hexokinase B readily and at a faster rate than the non-substrate-like bromoacetate while inactivation with the glucose-based alkylator was much slower. (fig 4.2). The incorporation of ^{14}C into the

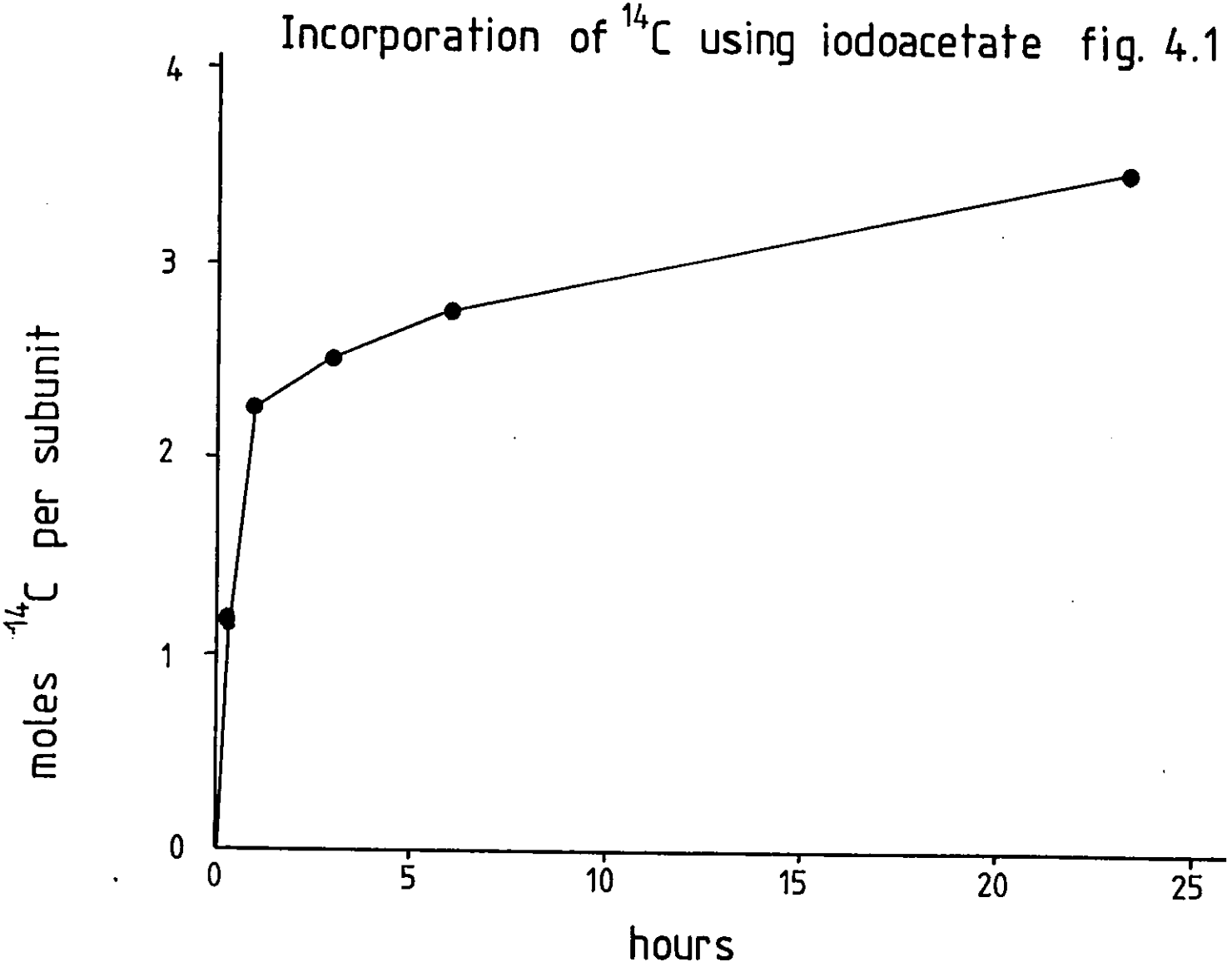
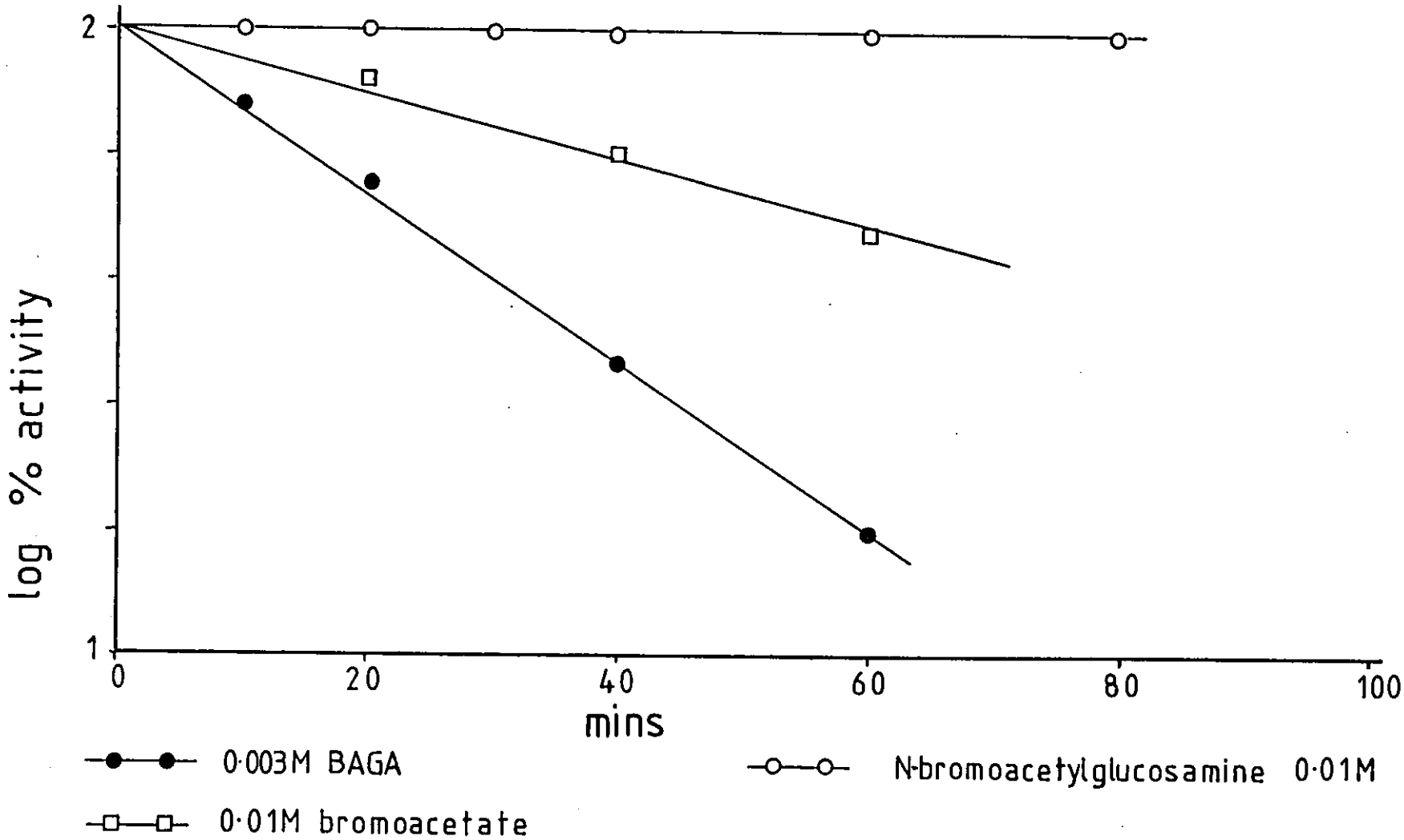

Incorporation of ^{14}C using iodoacetate fig. 4.1
moles ^{14}C per subunit
0
1
2
3
4
0
5
10
15
20
25
hours

fig. 4.2 Inactivation by affinity labels



protein was similar to that described previously (89) with two thiols reacting very quickly; however with BAGA prolonged reaction did not increase the incorporation above two. The substrate protection pattern was also very similar to that of iodoacetate and it was concluded that both alkylating reagents acted in the same way. It was felt that the glucose-based alkylator acted more slowly because it was protecting against inactivation, as well as alkylating (galactose does not protect against inactivation). Although two thiols are reactive towards active centre-directing reagents it does not necessarily mean that both are directly involved at the active site. Indeed one, or both could be involved in holding the enzyme in the active conformation rather than taking part directly in the enzyme reaction.

More detailed work showed that only one of these apparently essential thiols was actually needed for activity (91). Reaction with unlabelled BAGA in the presense of saturating mannose gave no activity loss; after the reagents were removed by gel filtration or dialysis the fully active enzyme could be reacted with ^{14}C-BAGA to give complete inactivation and an incorporation of one alkyl group per subunit. Iodoacetate gave the same effect. The active trialkylated enzyme exhibited the same kinetic properties as the active non-alkylated enzyme and thus it was concluded that when the three non-essential thiols were alkylated the active centre was unchanged.

The alkylation introduces a fairly bulky carboxymethyl group into the enzyme and it has been found that alkylation can indicate an 'essential' cysteine which turns out to be an artifact of the alkylation procedure. (92). In these cases the only thiol reagent which did not inactivate was 2-nitro, 3-cyanobenzoic acid which cyanylates thiol groups. If the

introduction of the small uncharged - CN group abolished activity it would be a good indication that a truly essential thiol was involved. Cyanylation of the trialkylated enzyme completely abolished activity and it was also shown that inactivation due to BAGA and that due to cyanylation involve the same thiol.

Cyanylated peptides can undergo an intramoleculor cleavage upon incubation at 35°C in slightly alkaline medium. Hexokinase B was trialkylated with ^{14}C-iodoacetamide and then cyanylated to inactivation and cleaved. The cleaved products were separated by gel filtration on a calibrated column and three peaks isolated. The first corresponded to uncleaved protein, the second contained radioactive material and the last peak contained protein but no radioactivity. Because the final peak had a blocked N-terminus it was at the C-terminal of Hexokinase indicating that the essential thiol was the last in the chain; from the calibration of the column this thiol was thought to be 80% along the protein chain from the N-Terminus.

4.4 THE FOUR THIOLS OF HEXOKINASE B

The cysteine residues are numbered arbitrarily I, II, III and IV and can be classified according to their chemical behaviour.

Thiol I is essential for activity since even the introduction of the small uncharged - CN group inactivates. Bound substrates (both sugar and nucleotide) protect thiol I from alkylation; thiol I also undergoes affinity labelling with BAGA. This leads to the belief that thiol I is close to the active centre but need not be directly in it. It is thought to be about 80% along the protein chain from the N-terminus and preceeded by thiols II, III and IV. This thiol is only accessible above 31°C indicating that some sort of conformational change involving loosening of the protein structure

occurs above the transition temperature.

Thiol II is highly reactive when thiol I has been alkylated but is not essential. It is not in the vicinity of either of the substrate binding sites since it is not protected by substrates and can be blocked without affecting the enzyme activity. The region of the molecule containing thiol II is subject to the same transition as that which exposes thiol I.

Thiols III and IV are non-essential and must be well outside the binding site - they are relatively inaccessible since even at 35°C iodoacetate reacts with them slowly and BAGA not at all. Thus the transition exposing thiols I and II is not a general unfolding of the protein. These thiols are thought to be buried in a hydrophobic region of the molecule because they are reactive to methylmercuric iodide. (87)

4.5 LABELLING OF THE THIOLS

In order to identify the individual thiol groups unambiguously it would be necessary to singly label the protein at each of the thiols in turn, then digest the protein and sequence the labelled peptide. Thiol I can be so labelled by alkylating with unlabelled iodoacetate in the presence of saturating mannose and then inactivating with ^{14}C-iodoacetate. Thiol II can also be uniquely labelled by protecting and reacting with ^{14}C-BAGA and then either denaturing and reacting with unlabelled alkylator or inactivating with unlabelled iodoacetate. It is not possible to distinguish between thiols III and IV but they could both be labelled by inactivating with unlabelled BAGA and then denaturing and reacting with ^{14}C-alkylator.

4.6 STRATEGY

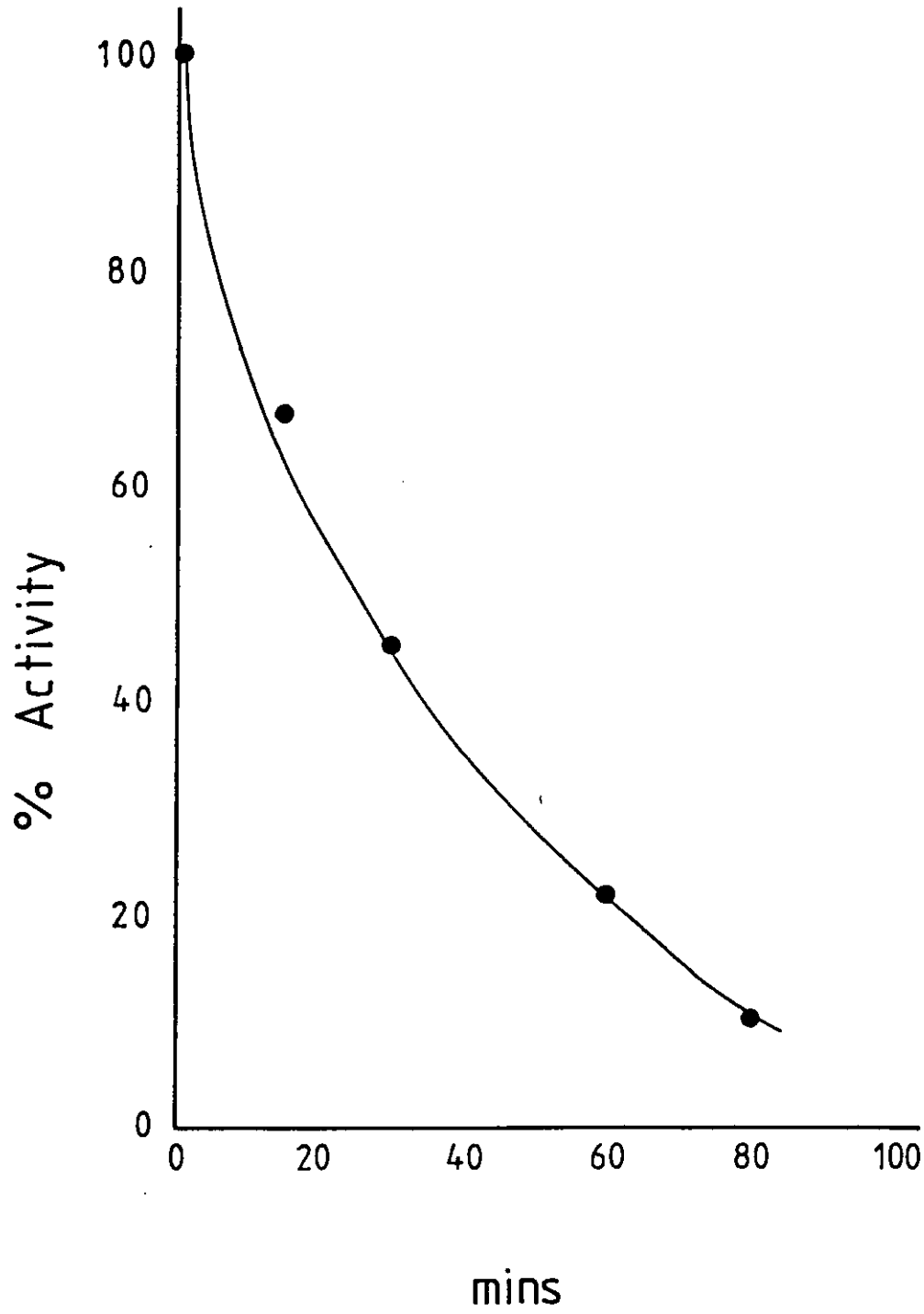
Because, as yet, there is no definitive primary structure of Hexokinase B the identification of thiol I was

considered to be the most important, leaving identification of thiols II - IV until the sequence is known. The X-Ray work of Steitz (17, 18) shows five cysteine residues per subunit in conflict with the chemical evidence which points to four. Of the five cysteines only one, cys 243, lies close to the glucose-binding site although even at its closest it is 5.5 Å from the 3-OH of glucose, not close enough to play a direct role in binding or catalysis. It does however appear to stabilize the proper orientation of the amide groups of three asn groups (188, 215, 245) that do bind directly to glucose. Any modification of this thiol would probably disrupt the glucose-binding site. This thiol also lies in a cleft which would be consistent with the transition temperature of Hexokinase B. Unfortunately this thiol lies in the middle of the peptide chain, not 80% from the N-terminus as predicted. There are two cysteines 80% along (372 and 378) but both are about 20 Å from the glucose binding site and it would seem unlikely that they would be involved in binding or catalysis.

4.7 METHOD

Two different digests of Hexokinase B, singly-labelled at thiol I, were carried out, the peptides obtained separated and the labelled material sequenced so as to obtain information about thiol I. To singly label the enzyme at thiol I, 1 - 2 umoles (50 - 100 mg) of Hexokinase B were dialysed against four litres of 0.05M glycine buffer, 0.1M NaCl (to obtain a high enough ionic strength), pH = 8.6. The protection experiment was carried out in the glycine buffer using an enzyme concentration of about 2 mg/ml. The enzyme was preincubated with 6 mM mannose at 35°C for 30 minutes, before the addition of unlabelled iodoacetate to a concentration of 7 mM and incubation for three hours. Unreacted iodoacetate and mannose were removed by dialysis against 15 litres

fig. 4.3 Inactivation by ^{14}C-iodoacetate



of glycine buffer before inactivation with 7 mM, ^{14}C iodoacetate, 2 μCi/μmole. In all experiments using iodoacetate the pH was maintained above 8.0 using solid Tris. The course of a typical inactivation is shown in fig 4.3. Incorporation of radioactive iodoacetate was 0.95 umole/umole subunit.

In theory the enzyme is now fully alkylated with no free thiols but as a precaution (especially before the CNBr digest) the inactive enzyme was denatured, reduced and carboxymethylated (see 2.6). The now fully alkylated enzyme could be digested.

4.8 PEPTIC DIGEST

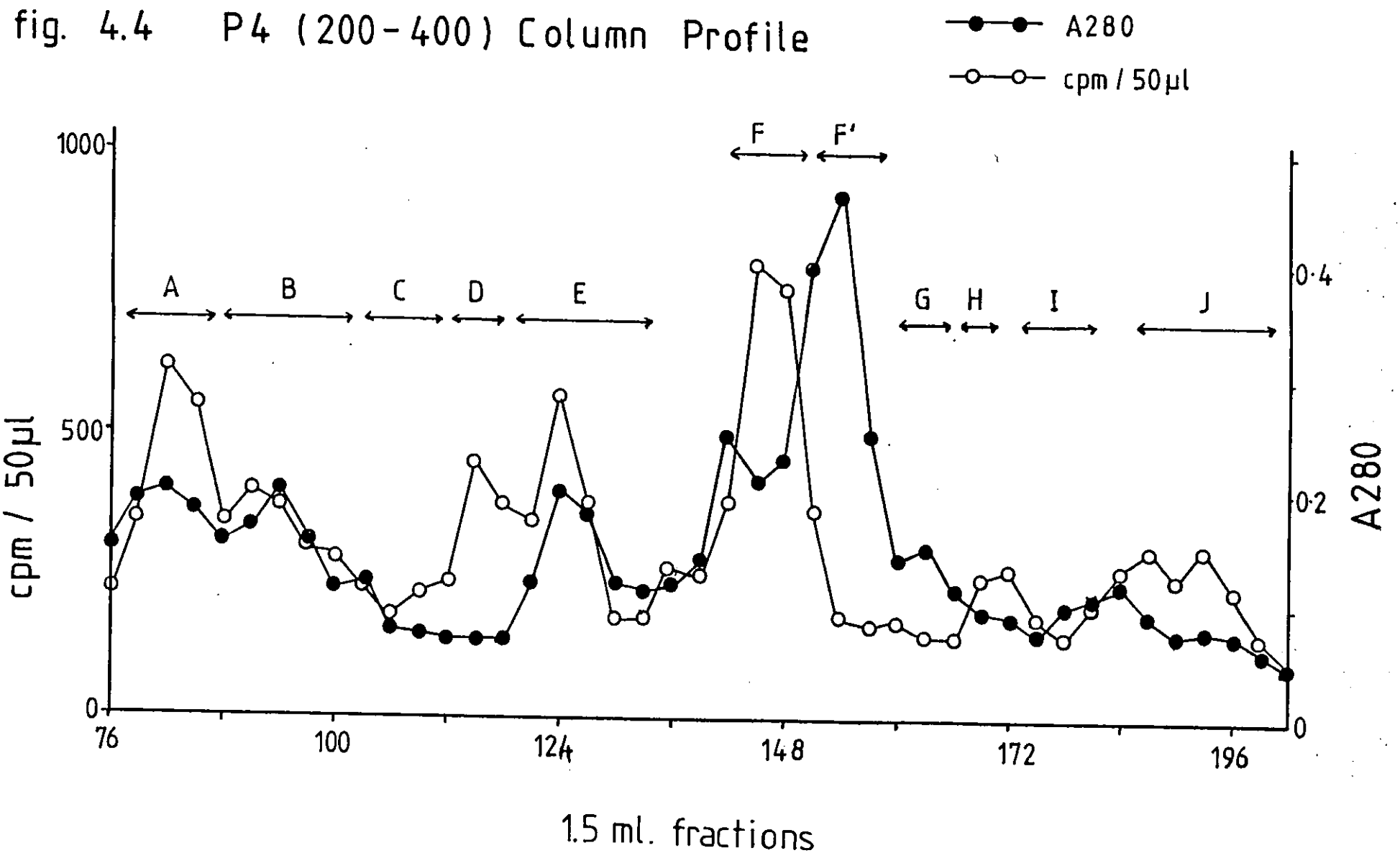
Initially it was decided to carry out a fairly non-specific digest to obtain short peptides that would be easy to separate and sequence. The peptic digest was carried out under mild conditions in an attempt to minimize the overlaps. The freeze-dried enzyme dissolved easily in 5% formic acid and solid pepsin was added to give a ratio of 1 : 100 w/w. Digestion was carried out for two hours at 37°C and the material then freeze dried. Initial separation was by gel filtration on a Biogel P4 (200 - 400) column (80 x 2.5 cm) equilibrated with 5% formic acid and 1.5 ml fractions collected. The column profile obtained is illustrated in fig 4.4 and suitable pools made.

After freeze-drying the pools they were dissolved in 5% formic acid and spotted out in anthin strip on Whatmann No 1 paper for HVPE as described in section 2.10. HVPE was carried out at pH = 6.5 with the neutral band rerun at pH = 2.1. Peptide material was now subjected BAWP (section 2.10) and the peptides cut out and eluted (section 2.10). These peptides were pure according to N-terminal determination and amino acid analysis and could be sequenced manually.

4.9 CNBr DIGEST

The digestion of singly-labelled enzyme was carried out in

fig. 4.4 P4 (200-400) Column Profile



70% formic acid as described in section 2.9 . The CNBr was removed by rotary evaporation and freeze drying after which the material was applied to a Biogel P30 (- 400) column (80 x 2.5 cm) equilibrated with 20% formic acid. The 1.5 ml fractions were analysed and the profile illustrated in fig 4.5

The small pools, V and VI were considered suitable for HPLC while the main large pool I was subjected to ion exchange chromatography. All the radioactive material was retained on a DE52 column which had been equilibrated with 20 mM ammonium bicarbonate, pH = 8.6 indicating that the radioactive peptides were acidic. Three gradients were then applied to the column:-

20 mM - 200 mM } ammonium bicarbonate
200 mM - 600 mM } pH = 8.6
600 mM - 1000 mM } 2 x 80 ml.

The column profile (fig 4.6) showed several non-radioactive peaks and two radioactive peaks, the first of which (IA) consisted of one very large protein fragment, presumably predominantly undigested material which was not sequenced. Peak IC was separated by HVPE into several radioactive spots which were eluted. Unfortunately the material obtained was in very poor yield ($<$ 10 nmoles) and each spot was only very weakly radioactive and thus not suitable for sequencing. It was therefore decided to concentrate on the HPLC of peaks V and VI from the P30 column, using the same conditions described in section 2.11.

HPLC of Peak V from the P30 column gave three peptide peaks the least polar of which was strongly radioactive. Peak VI also gave one radioactive peptide - this was much less strongly radioactive than that obtained from peak VI. Both peptides, named CV and CVI respectively were in sufficient quantity to be sequenced on the Beckman Spinning Cup Sequencer.

fig. 4.5 P30 (-400) Column Profile

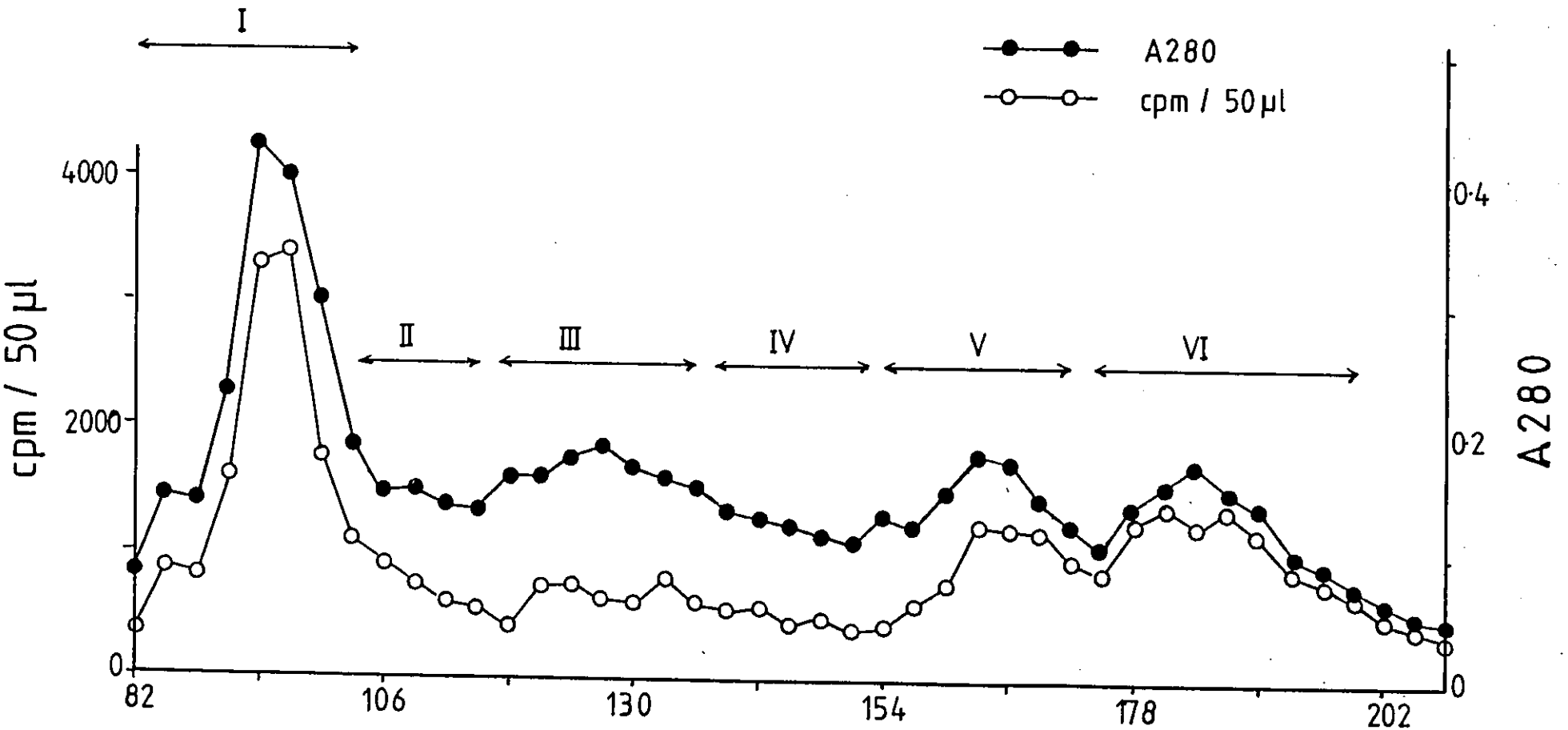
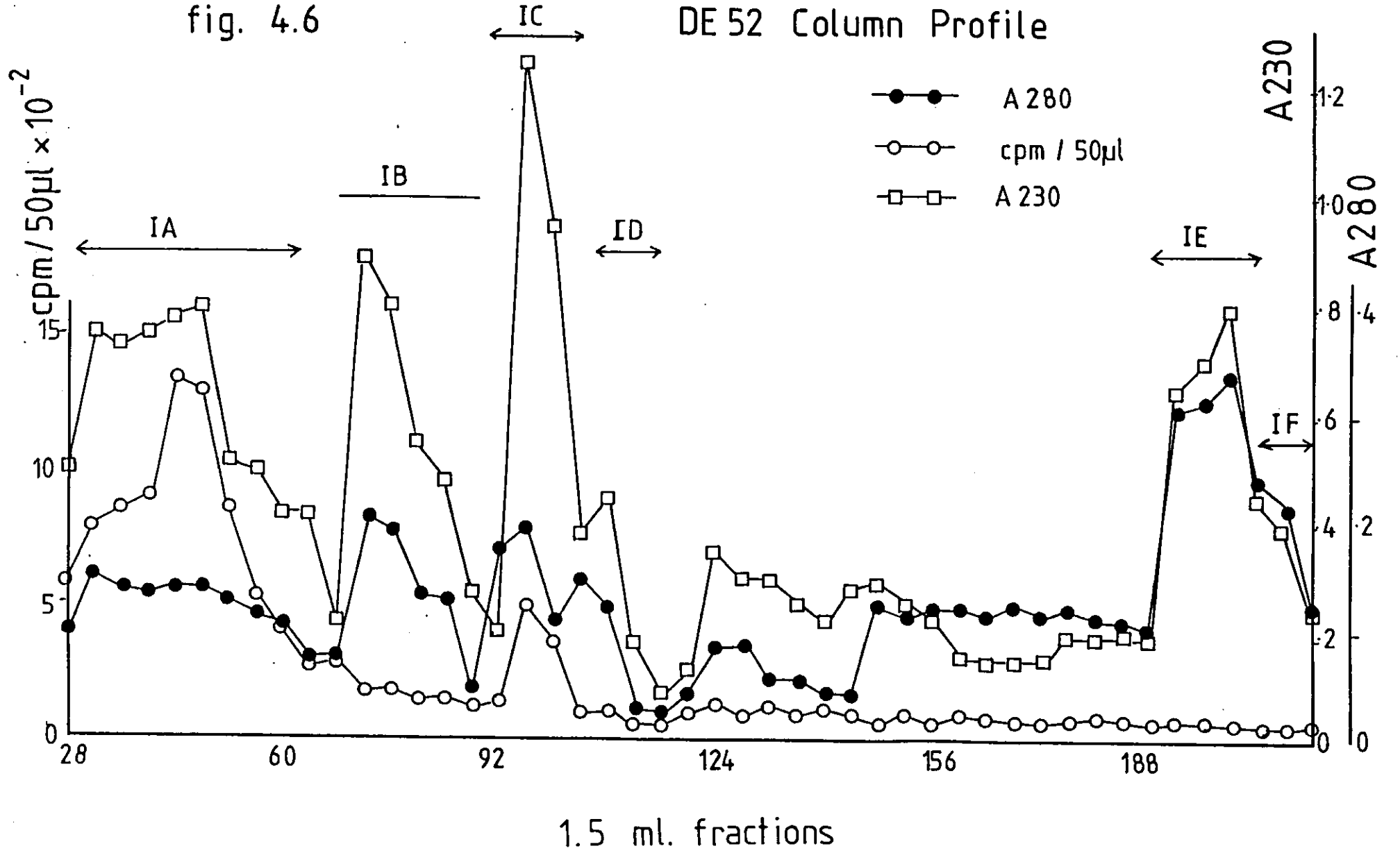


fig. 4.6


DE 52 Column Profile
A 280
cpm / 50µl
A 230
cpm/50µl ×10⁻²
A230
A280
IA
IB
IC
ID
IE
IF
0
5
10
15
28
60
92
124
156
188
0
·2
·4
·6
·8
1·0
1·2
·2
·4
1.5 ml. fractions

The pattern of digestion from the CNBr digest indicated that digestion was not complete. In other words some methionine residues were more susceptible to digestion than others.

4.10 SEQUENCE DATA

Manual sequencing was less successful than automatic sequencing probably owing to the relative impurity of peptides separated on paper. During the elution of peptides from paper (section 2.10) contaminating amino acids from the paper itself are often eluted. These are primarily serine, alanine and glycine which can cause problems in later identification of the dansylated amino acids. Because of the uncertainty of the manual sequence greater importance was put on the sequence obtained from the cyanogen bromide digest and where doubt existed the cyanogen bromide digest was assumed to be correct. Comparison with the X-Ray sequence of Steitz (17) was useful in identifying cysteine residues although it was important to bear in mind that the X-Ray sequence was unlikely to be more than 60 - 70% correct. According to Steitz many of the incorrectly identified residues are disordered because they are on the surface of the protein. The main differences between the X-Ray composition and chemical composition are due to charged and long chain residues such as lysine. The difference in chemical and X-Ray composition is shown in Table 4.1.

The major radioactive peptide obtained from the peptic digest was from peak B of the Biogel P4 column and was further purified by HVPE at pH 6.5 and descending chromatography. The peptide was basic, contained tyrosine or tryptophan but was not present in sufficient quantity to be sequenced beyond the seventh residue. This sequence was as follows:-

Table 4.1

The Amino Acid Composition of Hexokinase B (17)

Amino Acid	X-Ray (2.1 Å)	Chemical
Gly	34	39
Ala	58	32
Ser	48	25
Cys	5	4
Gam*	28	-
Val	23	23
Thr	14	29
Leu	26	34
Asx	32	53
Ile	32	36
Del*	33	-
Met	8	11
Eps*	17	-
Glx	21	53
Lys	17	32
Arg	10	17
His	5	4
Phe	19	23
Tyr	8	15
Trp	4	4
Pro	15	27
Residues	457	461
Protein Atoms	3293	3596

*Gam, Del and Eps are unidentified amino acids with 3,4 and 5 carbon atoms respectively in their side chains.

Cycle	Main Spot	Secondary Spots
1	K	A/OY/G
2	I	OY/G
3	no clear	OY/CMC/N } all
4	main spot	OY/CMC/E } faint
5	D/E	OY
6	Y/E	-
7	-	G/S

The peptide was no longer radioactive after cycle 4 but it was difficult to decide which of cycle 3 or cycle 4 CMCys represented the radioactive cysteine. The difficulty of assigning a definite sequence from this data is obvious and it was clear that more information was required.

The main radioactive peptide from the cyanogen bromide digest, CV had the following unambiguous sequence:-

M - A - I - N - CMC - E - Y - G - S - F - D - N - E - - - -

This tied up reasonably well with the information from the peptic digest although some spots assigned secondary in the manual sequence would appear to have been primary.

The slightly radioactive peptide, CV1 could also be sequenced:-

G - V - I - F - G - T - G - V - N - G - A - Y - Y - D - V - CMC - S - D - I - - - -

With this sequence information available it was now possible to attempt to match the chemical sequence with that obtained by X-Ray crystallography. The main sequence from each digest was presumably that around the so called "essential thiol", Thiol I. Again it must be emphasised that this thiol may not be involved in the actual

catalysis but could be involved in holding the enzyme structure in the active conformation. The X-Ray data pointed to Cys 243 being Thiol I and the X-Ray sequence around this thiol is as follows:-

240 250
M - Eps - I - C - C - Dx - Ex - S - S - F - R - K

The chemical sequence around the active thiol is similar but certainly not identical to that from the X-Ray data. Because previous work (section 4.3) indicated that the essential thiol was last in the sequence and 80% from the N-terminus of the protein this region of the X-Ray structure was studied. Confirmatory chemical data also existed in this area (17) which proved useful.

X-Ray Sequence:-

370 380
L - V - V - C - Gam - I - Del - A - I - C - Ex - K - K - G - Y - S

Chemical Data:-

370
L - S - V - C - G - I - A - A - V

By comparing the chemical data from the active centre work with that of the X-Ray and chemical data around the last thiols the evidence implies that the apparently essential thiol is not in the final 20% of the molecule. From this work (fig 4.7) it would seem that the essential thiol is either 243 or 244 in the X-Ray sequence although this is by no means certain.

The data from peptide CV1 is far less ambiguous. Because it was only lightly labelled it is probably Thiol II. A comparison of X-Ray and chemical data shows that it is likely to be the first thiol in the peptide chain. The similarity between X-Ray and chemical data is clearly shown in figure 4.8.

Figure 4.7

Thiol I - Chemical and X-Ray Data

	240									250	
X-Ray Sequence	Met	- Eps -	Ile	- Cys -	Cys	- Asx -	Glx	- Ser -	Ser	- Phe -	Arg	- Lys
CV - peptide	Met	- Ala -	Lle	- Asn -	Cmc	- Glu -	Tyr	- Gly -	Ser	- Phe -	Asp	- Asn - Glu
Peptic Digest		K/A	I	C/N	C/E	D/E	Y/E	G/S				

Figure 4.8

Thiol II (?) Chemical and X-Ray Data

X-Ray Gly - Ile - Ile - Phe - Gly - Ser - Gly - Val - Asn - Ala - Ala - Tyr - Trp - Cys - Asx - Ser - Thr - Eps - Ile

CV1
Peptide Gly - Val - Ile - Phe - Gly - Thr - Gly - Val - Asn - Gly - Ala - Tyr - Tyr - Asp - Val - Cys - Ser - Asp - Ile

369 LSVCGIAAV

When a complete chemical sequence is available it will be possible to compare the data and come to some conclusions about the exact position and nature of all four thiols. Until then the likely situation would appear to be:-

Thiol I	cys 243/244
Thiol II	at the beginning of the chain, probably in position 222 not 220 as indicated by the X-Ray data
Thiols III and IV	one of cys 372 (chemically identified) or cys 376 (X-Ray data only)

CHAPTER 5

CARBOXYLIC ACID RESIDUES IN HEXOKINASE

5.1 INTRODUCTION

Acids commonly act as catalysts in chemical reactions and enzymes are no exception. Many reactions are susceptible to catalysis by proton transfer from general acids and proton transfer to general bases; this general acid-base catalysis is often seen in enzymology eg. Lysozyme (93).

The mechanism of Lysozyme enzyme activity (fig. 5.1) shows how the microenvironment of the enzyme can promote general acid-base catalysis. The working pH of the enzyme is about 6, at which point carboxyl groups would normally be in the ionised form. However, Glutamic Acid 35 is located in a hydrophobic region which serves to increase its pK to about 6.3 and therefore at pH 6 it is protonated and can act as a general acid. Aspartic Acid 52 in its polar environment has the normal pK of about 4-4.5 and in its ionised form acts as a proton accepting, general base. Lysozyme is a good example of how carboxylic acids can act as general acids or general bases depending on their state of ionisation.

Carboxylic acids can coordinate with other groups on the protein or substrate and thus be essential for activity. Metal ions are often involved eg. in Carboxypeptidase A Glutamic Acid 72 is one of the residues coordinating with the zinc ion which is essential for activity. (94). Ionised carboxyl groups can also act as nucleophiles attacking carbon centres, as in one of the mechanisms proposed for Carboxypeptidase A. (94).

In Hexokinase, Mg^{2+}, or some other divalent metal is required for activity by forming a coordination complex with ATP (see section 1.5). It is conceivable however that the metal ion could coordinate to an acidic residue on the enzyme. It is also possible that acidic

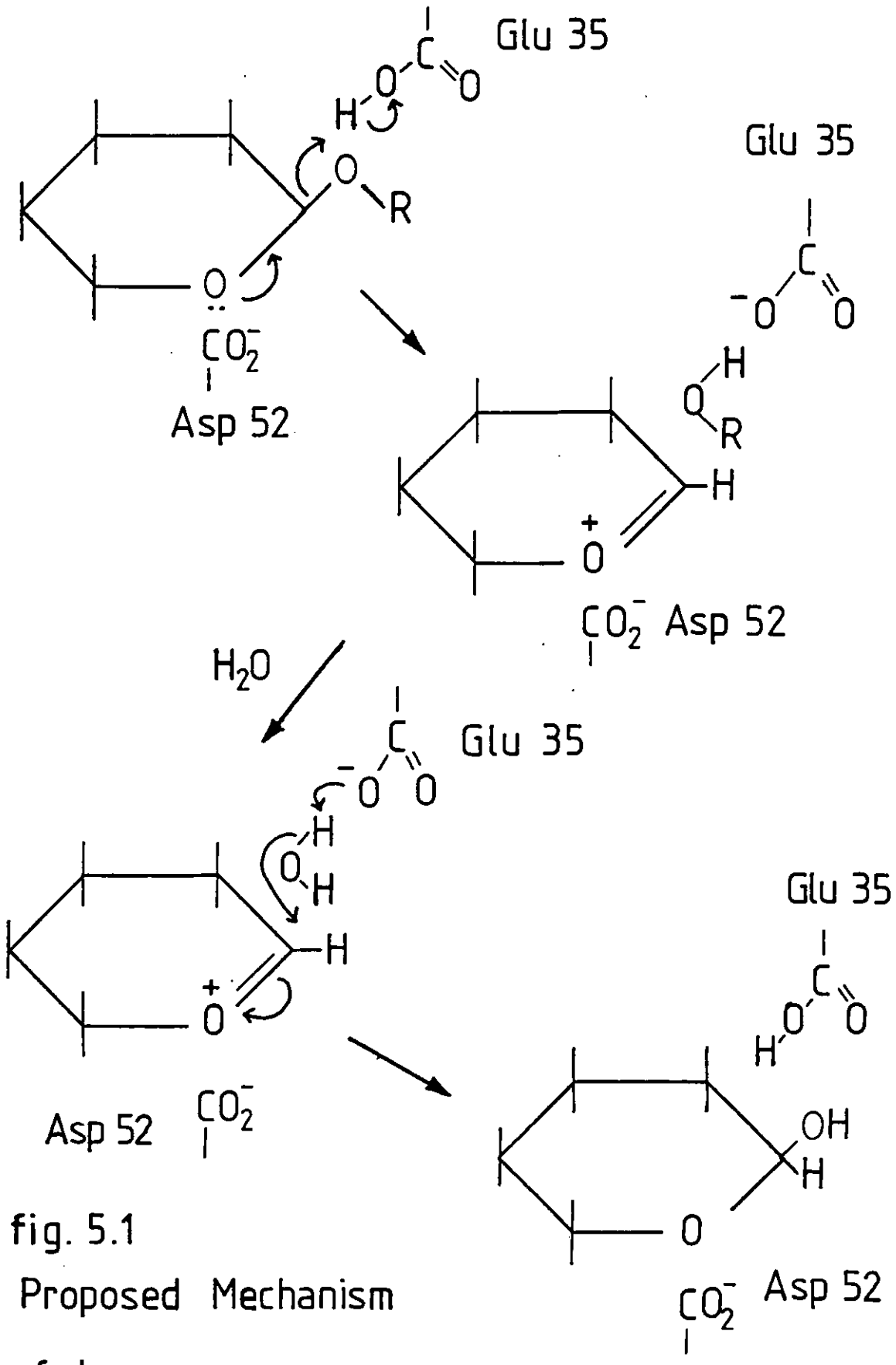


fig. 5.1
Proposed Mechanism
of Lysozyme
Activity

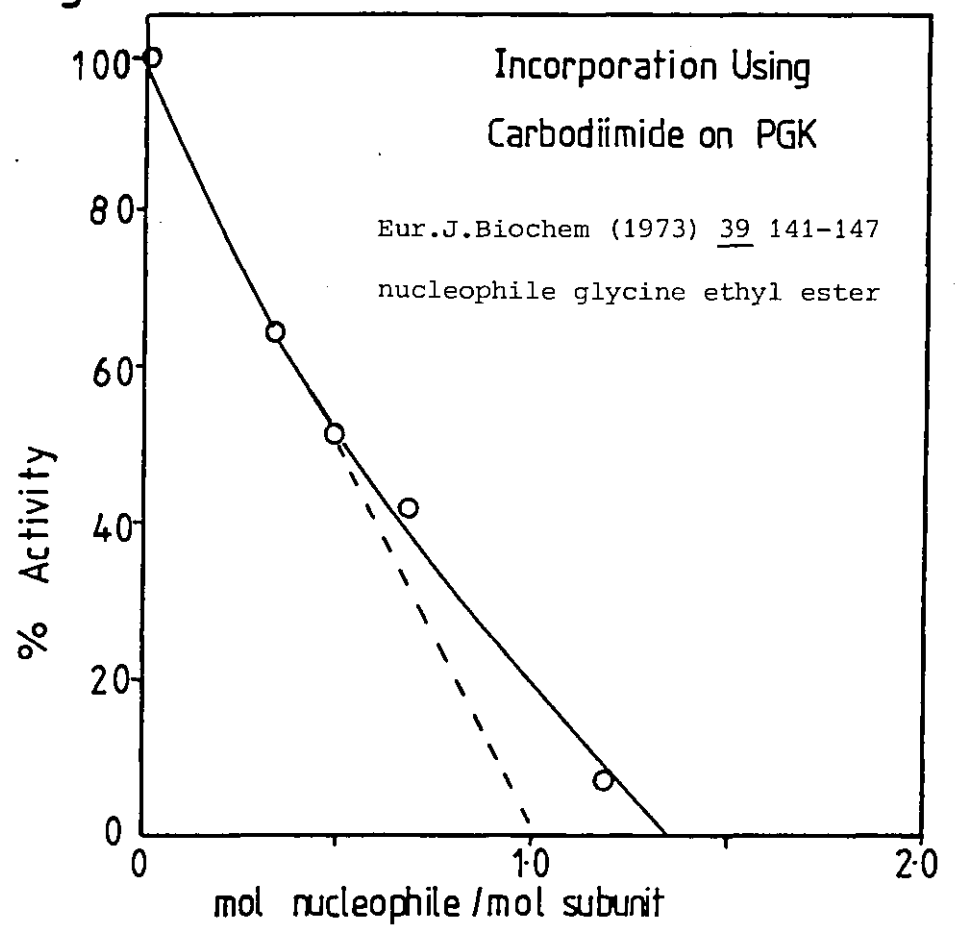
groups are required to maintain the enzyme in the active conformation. The most likely role though is in acid base catalysis facilitating the attack of the 6-OH of glucose onto the -δ phosphate of ATP (see section 1.3) (18).

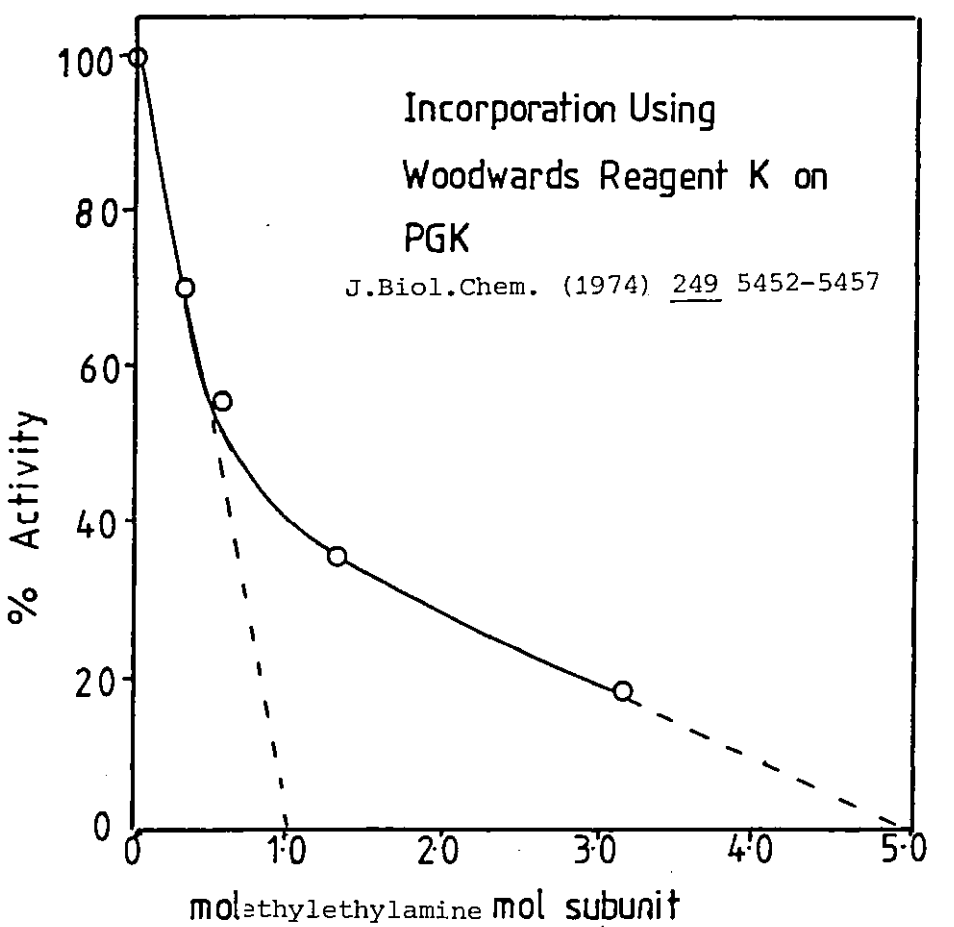
As well as the implication from X-Ray crystallography that acidic residues are involved in catalysis there is a fair amount of evidence from solution work that carboxylic acids, in particular, glutamic acid are involved in the mechanism of Hexokinase A and Yeast Phosphoglycerate Kinase.

With Yeast Phosphoglycerate Kinase reaction with carboxylic acid specific reagents, namely water soluble carbodiimides followed by nucleophilic displacement (see section 5.2) were found to cause complete inactivation (95). This inactivation was protectable by substrates although only to a small extent, 100% inactivation was concomitant with the incorporation of 1 mole nucleophile/mole enzyme and so it was concluded that an active centre carboxyl group exists. Later, further work on the enzyme (96) led to somewhat different conclusions. A different reagent, N-ethyl-5 phenylisoxalonium - 3' - sulphonate (woodward's Reagent K) was used, which because it slightly mimics ATP (having an aromatic ring and negative charge) was thought to be active centre directing, 100%, substrate protectable inactivation was obtained but the pattern of incorporation of radioactive material was considerably different. (fig. 5.2). Because of its more specific nature and because substrate protection was greater with Woodwards Reagent K one might expect the results to be more representative. The conclusions reached were that five carboxyl groups could be modified but only one was essential for activity.

Work on Hexokinase pointed to a similar situation. Preliminary results (54) using the carbodiimide method indicated protectable inactivation with 100% inactivation concomitant with the incorporation

fig 5.2

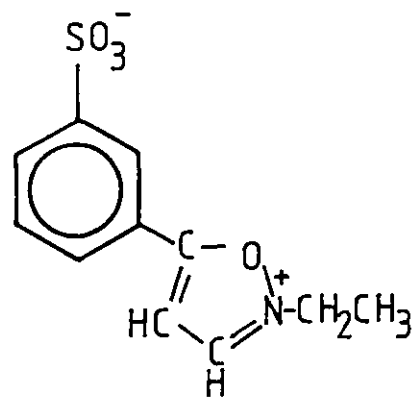

Incorporation Using
Carbodiimide on PGK
Eur.J.Biochem (1973) 39 141-147
nucleophile glycine ethyl ester
% Activity
100
80
60
40
20
0
0
1·0
2·0
mol nucleophile / mol subunit


Incorporation Using
Woodwards Reagent K on
PGK
J.Biol.Chem. (1974) 249 5452-5457
% Activity
100
80
60
40
20
0
0
1·0
2·0
3·0
4·0
5·0
mol ethylethylamine mol subunit

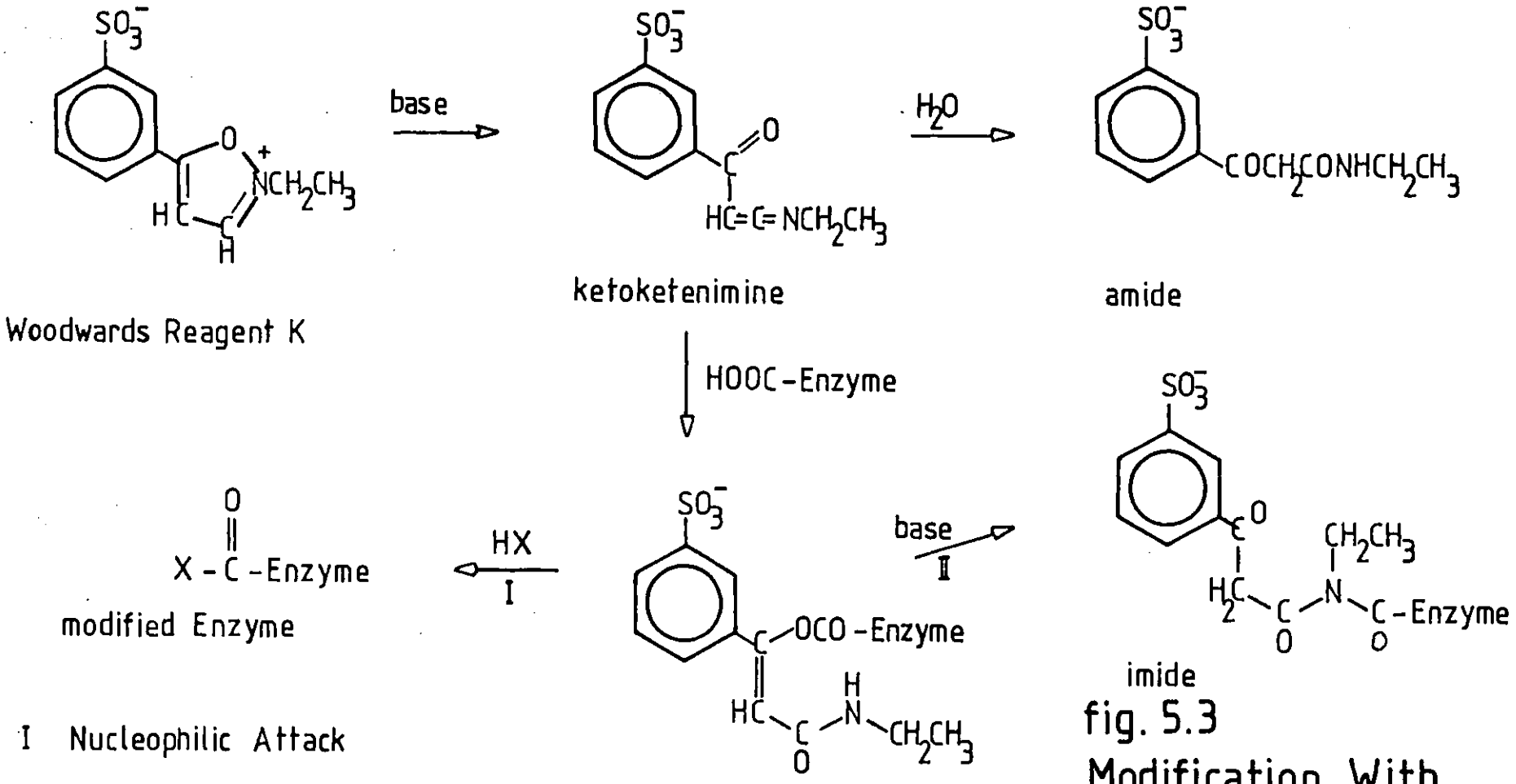
of 2 moles of nucleophile per mole sub-unit. More detailed work (97) using 0.1M NaOH dialysis indicated that only 1 mole of nucleophile was incorporated per mole sub-unit and so the conclusion that a single carboxyl residue was required for activity was reached. Extensive proteolytic digestion indicated that a glutamic acid residue was involved; this did not correspond with the X-Ray work which implicated Aspartic Acid 189. However the X-Ray sequence is obviously liable to error and moreover the X-Ray work was on the B isoenzyme while the solution work was on the A isoenzyme.

5.2 METHODS

Before studying the situation further it was necessary to decide on a method of carboxyl group modification. Two methods were possible, using a water soluble carbodiimide or Woodwards Reagent K. The latter has the advantage of being a slight substrate mimic:-



However in everyday use it has several disadvantages. To incorporate a radioactive label successfully a very high (1-2M) concentration of radioactive nucleophile is required. (98) presumably because the rearrangement reaction (fig. 5.3) is predominant. This would mean that a large amount of a suitable radioactive nucleophile would be required in order to have a reagent of high enough specific activity to incorporate measurable counts into the protein. This method has also been reported to cause precipitation of the protein if more



I Nucleophilic Attack

II Rearrangement

fig. 5.3
Modification With
Woodwards Reagent K

than five groups are incorporated (99) and for these reasons was rejected.

The water soluble carbodiimide method is well known (100), the reaction being shown in fig. 5.4. This method can be used to modify all the carboxyl groups in a protein without the problems of precipitation found using Woodwards Reagent K. A suitable nucleophile in fairly low concentrations can be used to direct the overall reaction away from hydrolysis and towards the modified protein. Carbodiimides react with groups other than acidic residues (53, 101) including the phenolic hydroxyl of tyrosine and uncharged lysine residues although below pH 7 this can probably be discounted. Therefore it was decided that modification of carboxyls using carbodiimides was preferable; the next step was the choice of nucleophile.

Ideally the nucleophile should react specifically with the O-Acylurea to give a high yield of modified protein. The nucleophile should also be available in the radioactive form with a high specific activity. Ethyl esters of the amino acids tyrosine and glycine have been used with some success (95, 54) and nitrotyrosine ethyl ester has also been used to estimate the incorporation of nucleophile into the protein (97). Using nitro-tyrosine ethyl ester it was necessary to measure nucleophile incorporation spectroscopically, however it was felt that a radioactive measurement would be more suitable. Amino acid ethyl esters increased the rate of inactivation about threefold over the rate using carbodiimide alone. The nucleophile, semicarbazide has been reported as being superior to the ethyl esters in this respect. (58). The low pK of the semicarbazide means that it is present in the unionized, reactive form at lower pH values and it also acts as a buffer for the system. Because semicarbazide could easily be made in the radioactive form (see section 2.7) it was used in place of the ethyl esters.

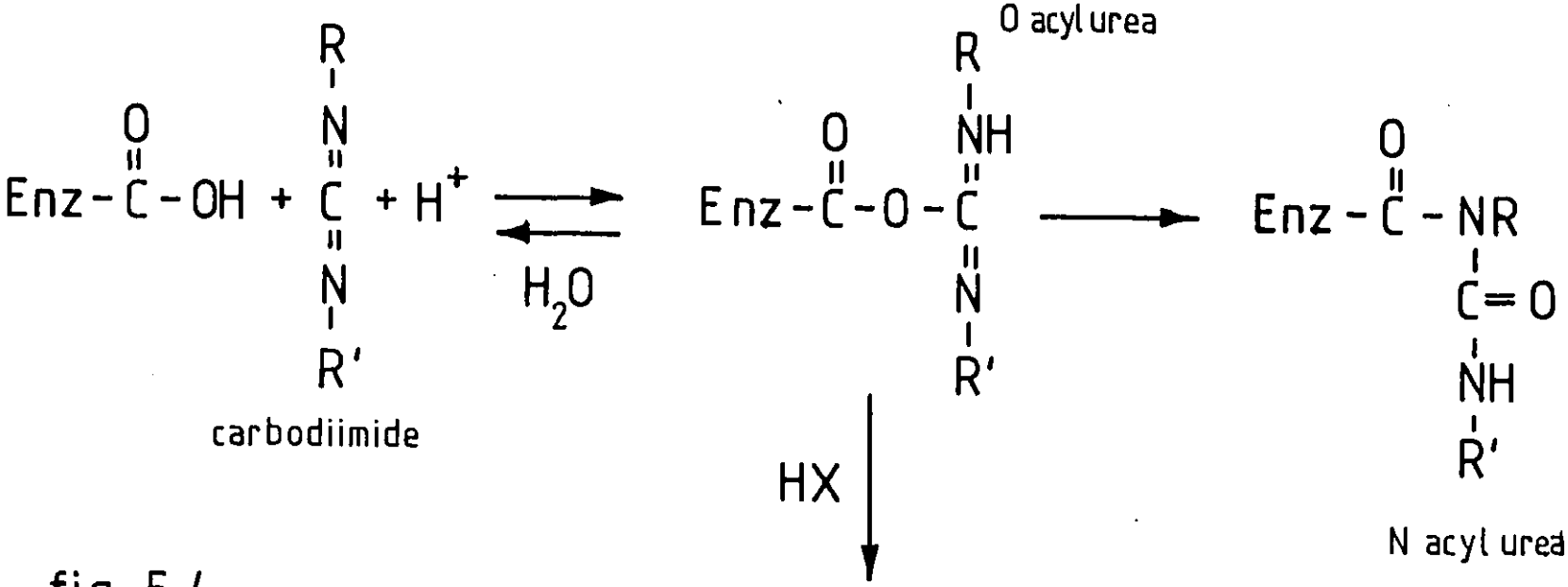


fig. 5.4

Reaction With Carbodiimide & Nucleophile

In all cases the inactivation was started by the addition of ethyl dimethylamino propyl carbodiimide and was monitored by assaying aliquots of reaction mixture at suitable time intervals. Unless otherwise stated inactivations were carried out at pH=6 and at 20°C.

5.3 KINETICS

A discussion of the kinetics of inactivation is presented here by applying the steady state approximation to the following scheme:-

$$E + CDI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ECDI$$

$$ECDI + SC \xrightarrow{k_2} E^{+} \qquad 5.1$$

This simplified scheme assumes that nucleophilic attack competes effectively with the rearrangement to the N acylurea which is ignored.

$$\text{Reaction Rate, } v = k_2 [ECDI] [SC] \qquad 5.2$$

At the beginning of the inactivation we can approximate that

$$[E_T] = [ECDI] + [E] \qquad 5.3$$

and $$\frac{d[ECDI]}{dt} = 0$$

$$\frac{d[ECDI]}{dt} = 0 = k_1[E][CDI] - k_{-1}[ECDI] - k_2[ECDI][SC] \qquad 5.4$$

Substituting for E

$$0 = k_1 [E] [CDI] - k_1 [ECDI][CDI] - k_{-1} [ECDI] - k_2 [ECDI][SC]$$

$$[ECDI] = \frac{k_1 [E_T] [CDI]}{k_1 [CDI] + k_{-1} + k_2 [SC]} \qquad 5.5$$

Substituting into equation 5.2

$$v = \frac{k_1 k_2 [E_T] [CDI] [SC]}{k_1 [CDI] + k_{-1} + k_2 [SC]} \qquad 5.6$$

It is known that in many cases k_1 approximates to k_{-1} (100) and so the relative values of k_2 and k_1, k_{-1} become important. Consider the two extreme cases:-

1. $k_1, k_{-1} \gg k_2 [SC]$

Equation 5.6 becomes $v \longrightarrow \frac{k_1 k_2 [E_T][CDI] [SC]}{k_1 [CDI] + k_{-1}}$

2. $k_2 [SC] \gg k_1, k_{-1}$

Equation 5.6 becomes $v \longrightarrow k_1 [E_T] [CDI]$

So unless the second case applies the reaction rate is going to be dependent upon the concentration of enzyme, carbodiimide and semicarbazide. For most enzyme reactions the substrate concentration is very large compared with enzyme concentration and therefore it is valid to regard the substrate concentrations as constant. The rate can be considered as:-

$$v = k_1' [E_T]$$

where k_1' is the observed rate constant for the reaction.

$$-\frac{d [E_T]}{dt} = k_1' [E_T]$$

separating the variables and integrating gives:-

$$\ln \frac{[E_T]o}{[E_T]} = k_1' t \qquad 5.7$$

where $[E_T]_o$ o is the initial enzyme concentration and $[E_T]$ is the concentration of E + ECDI after t minutes. A plot of $\ln [E_T] o / [E_T]$ against t should therefore give a straight line of slope k_1'. Since $[E_T]$ is proportional to the % activity of the enzyme.

$[E_T]_o$ = 100%

$[E_T]$ = % activity remaining after time, t

From equation 5.6 we know that k_1' is some function of semicarbazide concentration and at constant carbodiimide concentration we can re-write equation 5.7 to give:-

$$\ln[E_T]_o / [E_T] = k_2[SC]\,t \qquad 5.8$$

and a plot of k_1' against [SC] should give a straight line slope k_2.

This fairly simple kinetic scheme was shown to be obeyed. Figs. 5.5 and 5.6 depict inactivation by carbodiimide and semicarbazide at four semicarbazide concentrations, and plots of $\ln[E_T]_o / [E_T]$ against t giving four values of k_1'. These values are shown in table 5.1.

A plot of k_1' against [SC] is shown in fig. 5.7. From fig. 5.7.

k_2 = slope

= $1.0 M^{-1}\ min^{-1}$

5.4 EFFECT OF pH

The effect of pH on the reaction rate was investigated (fig. 5.8) using imidazole buffer. Up to about pH=6 the rate of inactivation is proportional to pH but above pH 6 the rate decreases dramatically. This is predicted by the reaction scheme (fig. 5.4) because the reaction is dependent upon the carboxyl groups being protonated and upon the presence of protons. Below the pH when the carboxyl groups are protonated the rate will be proportional to hydrogen ion concentration but as soon as most of the carboxyl groups ionise the rate will fall off rapidly. Since the semicarbazide is only reactive in its unionised form (58) at pH values below its pK (3.9) the rate should again fall. However, Hexokinase is not active below pH = 4.6 and so it was not possible to show this.

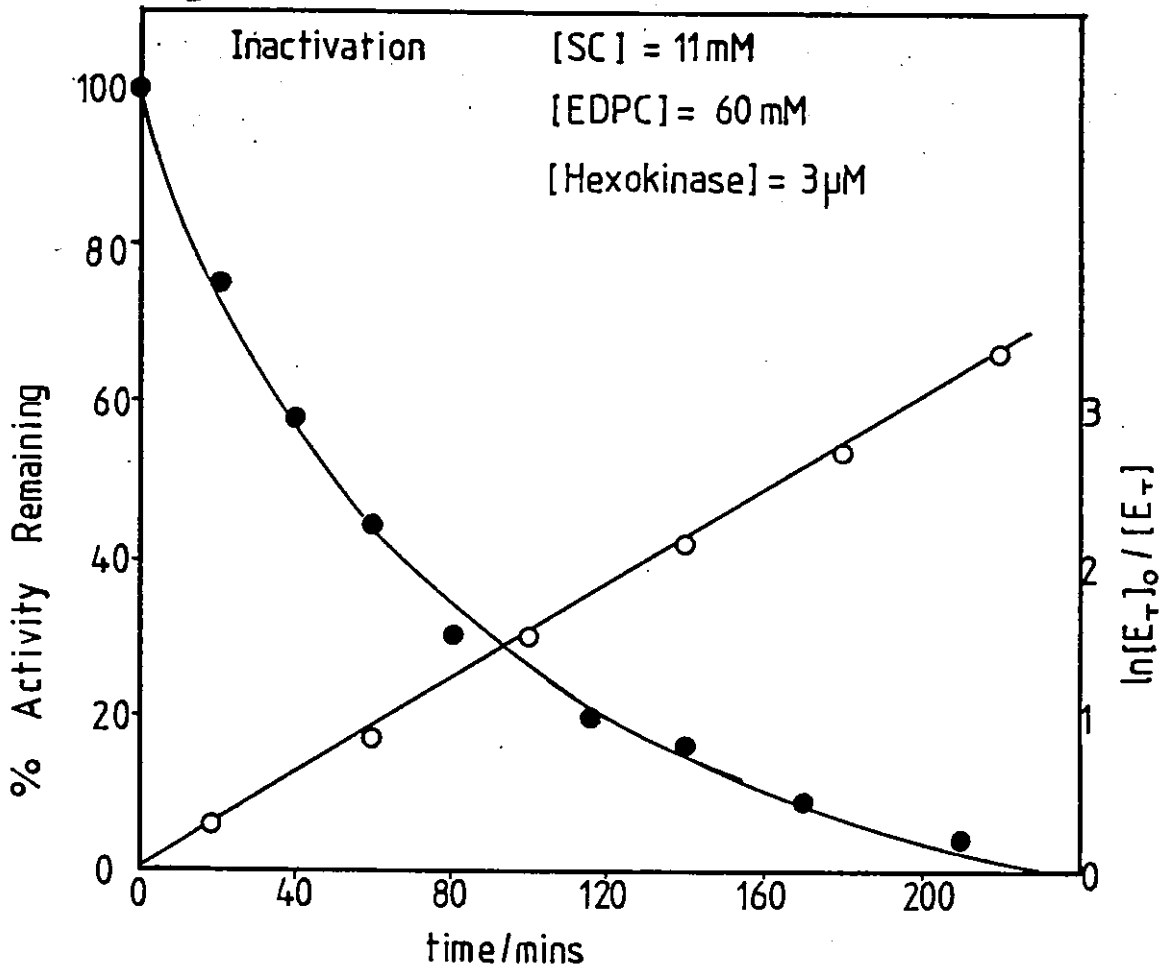
Since the normal pK of carboxylic acids is about 4 - 4.5 at the pH values at which Hexokinase is active one would expect all the

Table 5.1

VALUES OF k_1 AT VARIOUS SEMICARBAZIDE CONCENTRATIONS

SC / M	k_1' min^{-1}
0.011	0.015
0.025	0.022
0.07	0.069
0.40	0.40

fig. 5.5


Inactivation
[SC] = 11mM
[EDPC] = 60 mM
[Hexokinase] = 3μM
% Activity Remaining
ln[E_T]o / [E_T]
time/mins
0
20
40
60
80
100
0
40
80
120
160
200
1
2
3

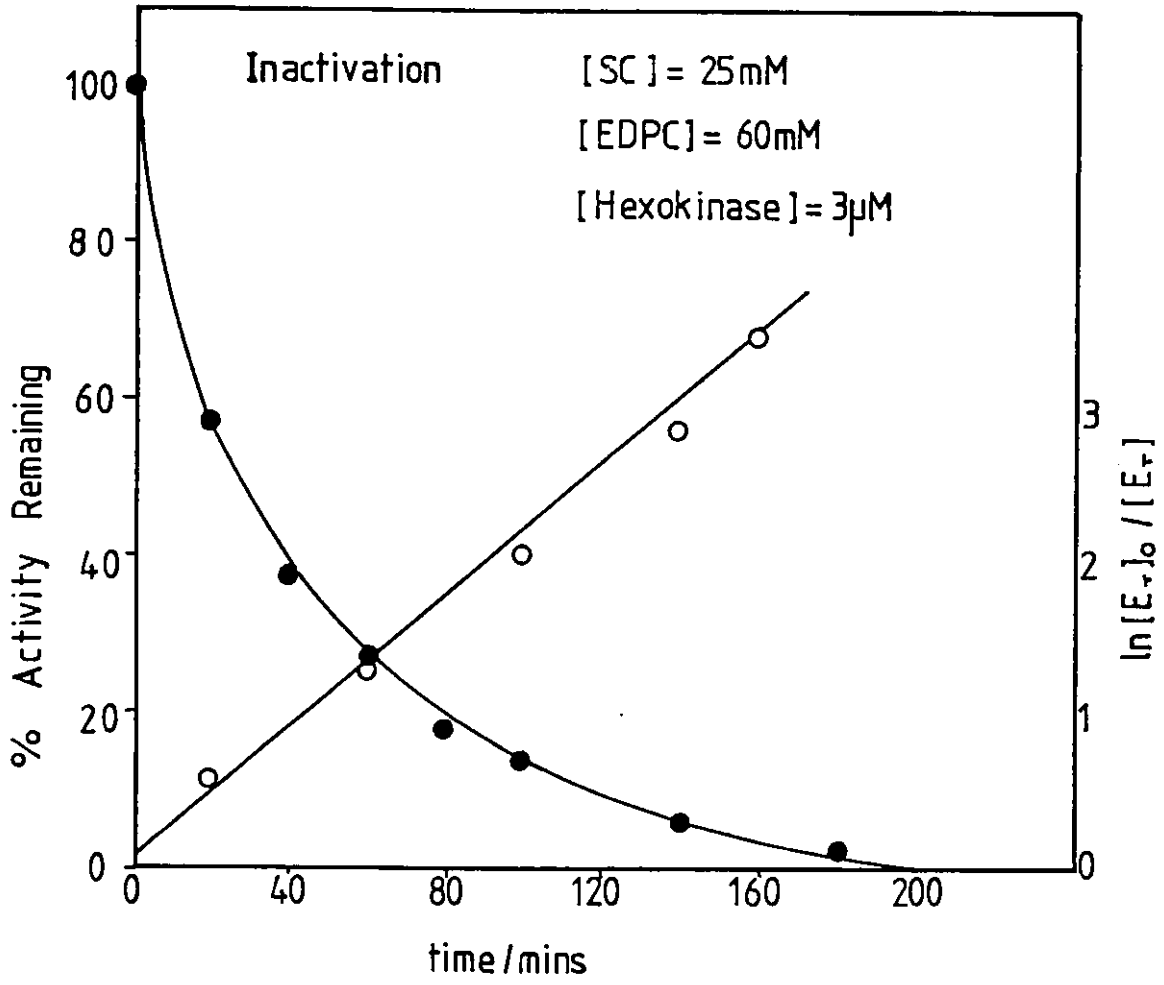
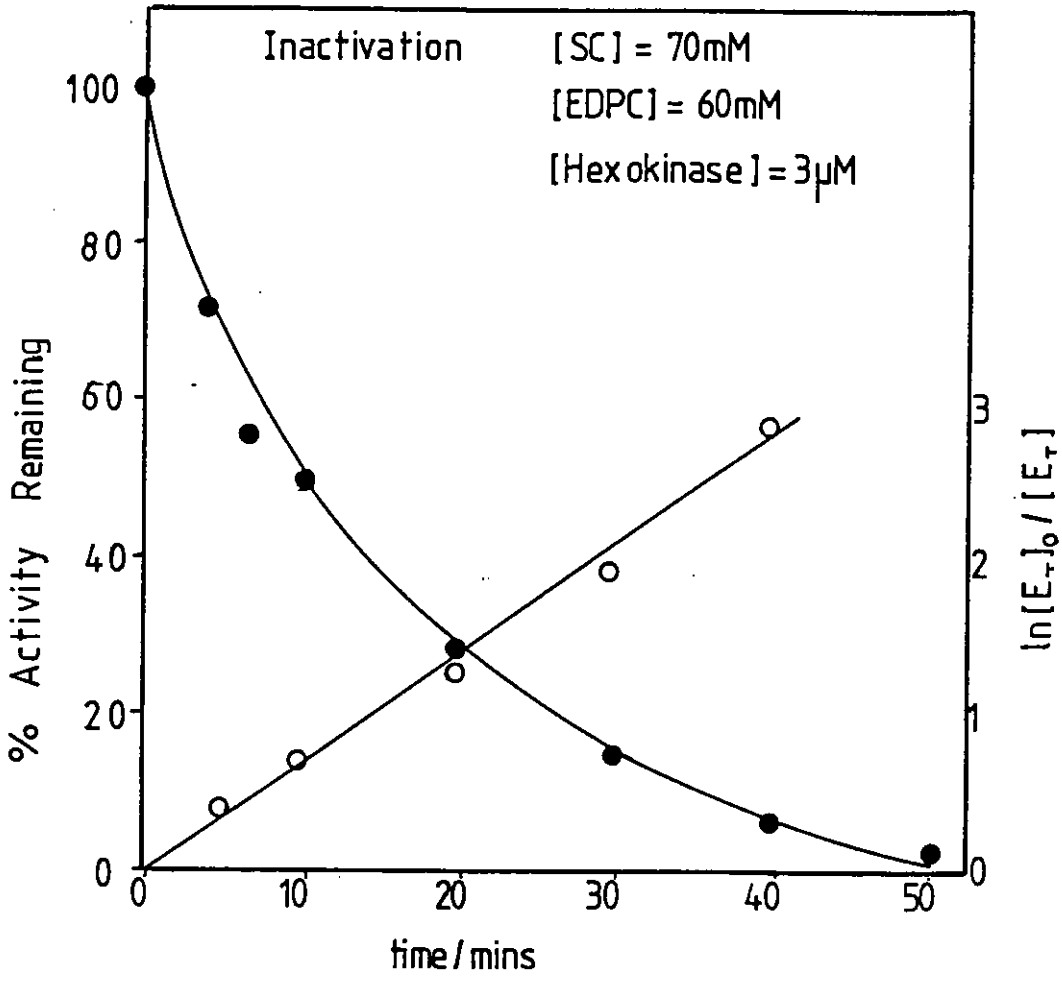

Inactivation
[SC] = 25mM
[EDPC] = 60mM
[Hexokinase] = 3μM
% Activity Remaining
ln[E_T]o / [E_T]
time/mins
0
20
40
60
80
100
0
40
80
120
160
200
1
2
3

fig. 5.6


Inactivation
[SC] = 70mM
[EDPC] = 60mM
[Hexokinase] = 3μM
% Activity Remaining
ln[E_T]o / [E_T]
time / mins
0
10
20
30
40
50
0
20
40
60
80
100
1
2
3

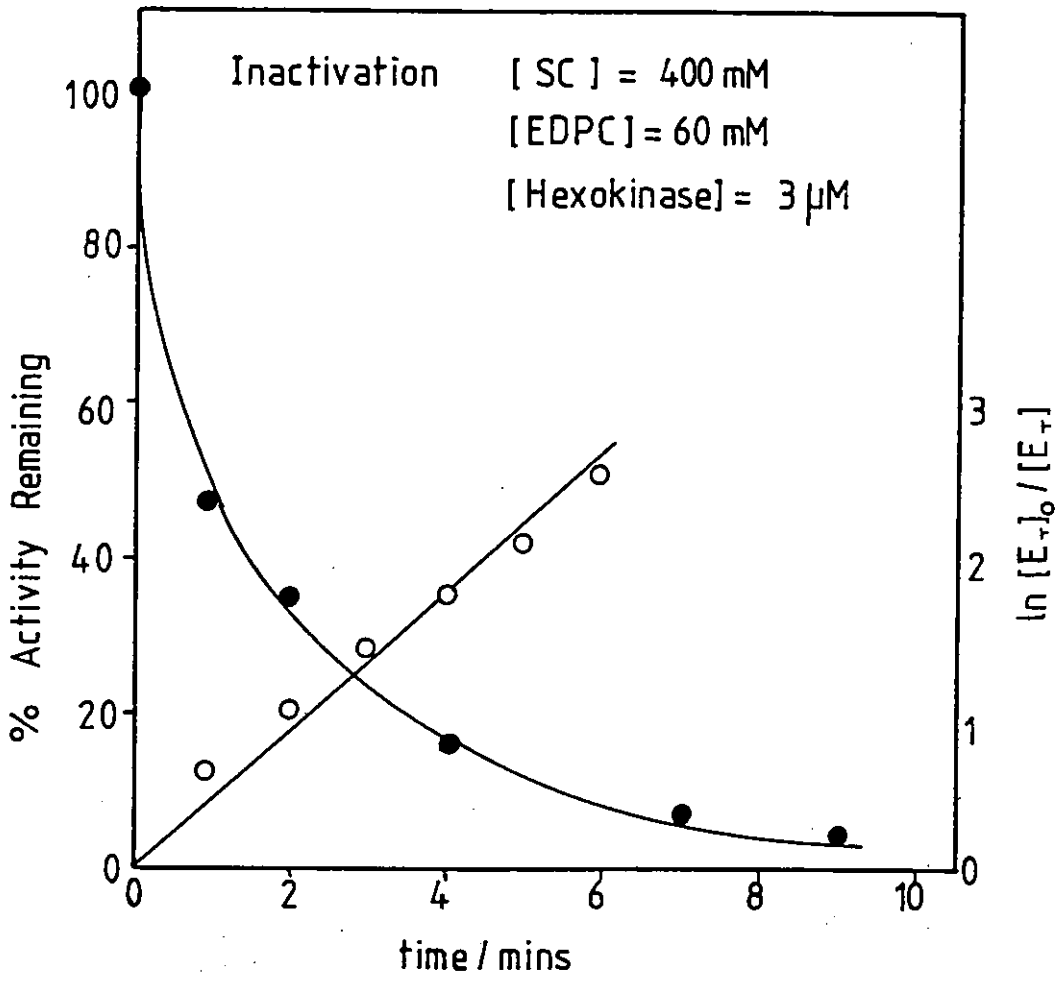

Inactivation
[SC] = 400 mM
[EDPC] = 60 mM
[Hexokinase] = 3 μM
% Activity Remaining
ln [E_T]o / [E_T]
time / mins
0
2
4
6
8
10
0
20
40
60
80
100
1
2
3

fig. 5.7 Variation of $k_1' min^{-1}$ with [SC]

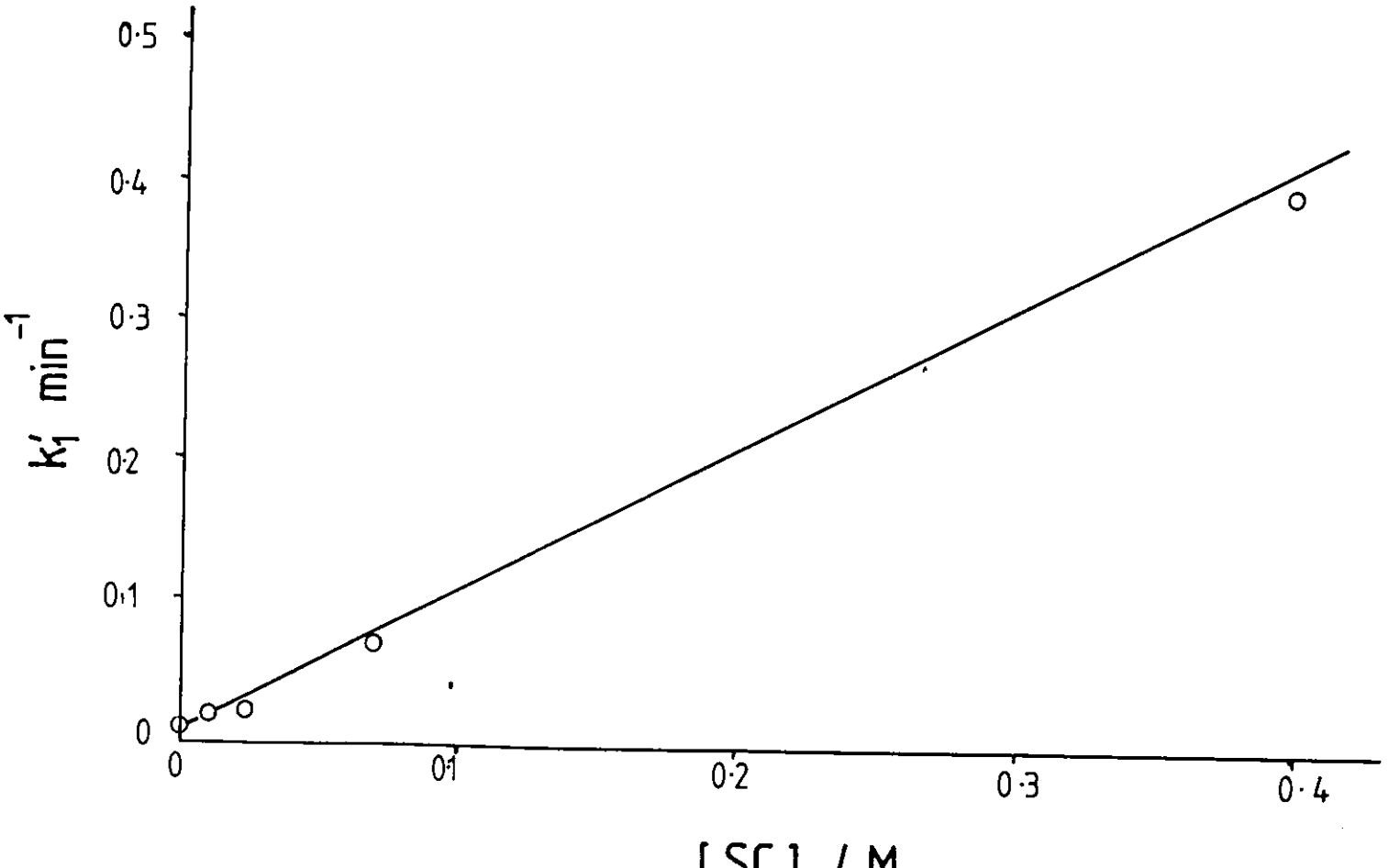
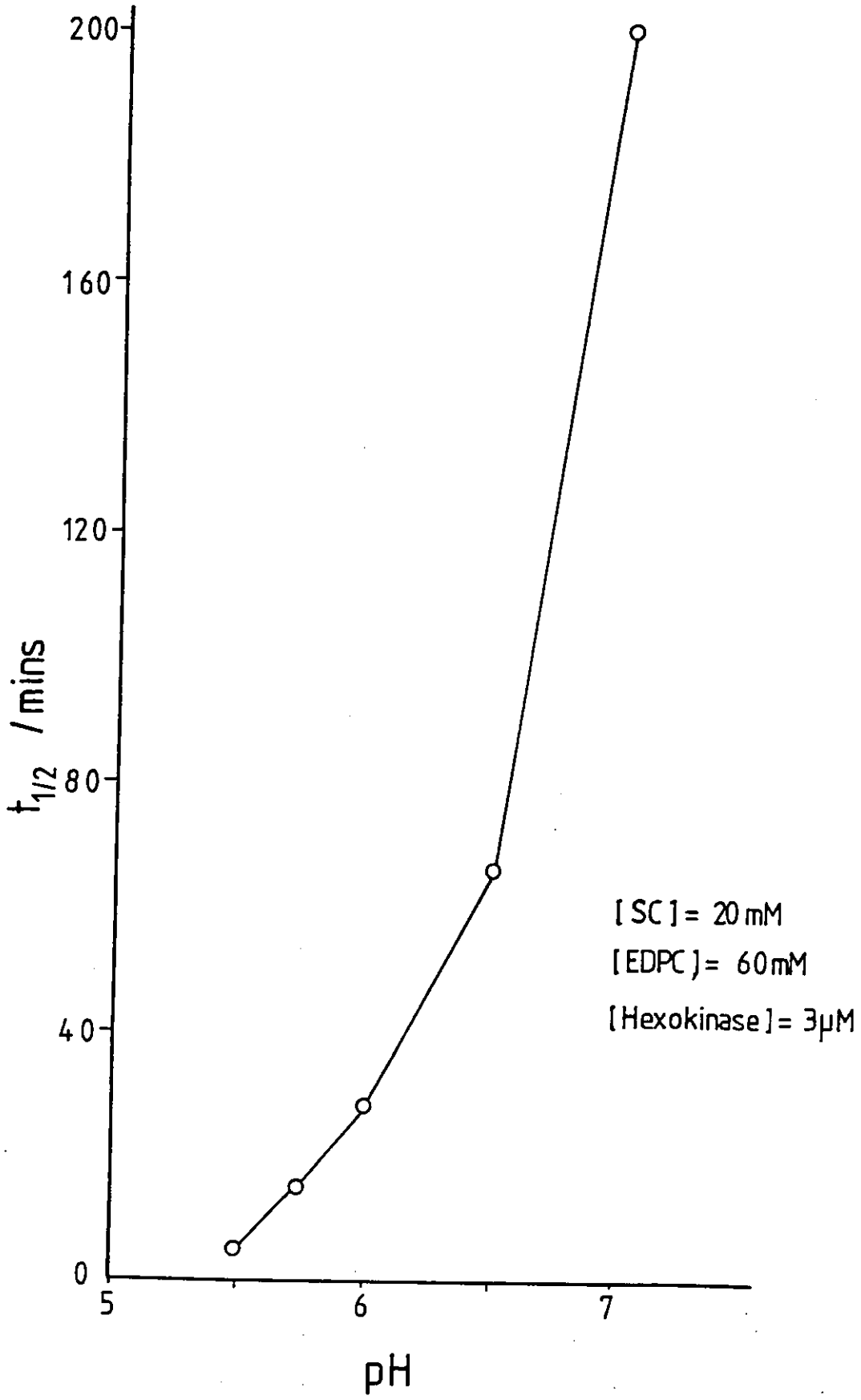


fig. 5.8
Effect of pH on Inactivation



acidic residues to be ionised. Solvent effects previously discussed could increase the pK of certain residues and permit inactivation. A critical residue in Hexokinase with a pKa of 6.8 has been reported which is suggestive of either a carboxylic acid or a histidyl residue.
(102). The presence of a critical histidyl residue has been ruled out (50) and so a carboxyl group is indicated. At pH = 6 this group would be protonated while any carboxyl group of pK 4 - 4.5 would be ionised and thus not react with carbodiimide. Any carboxylic acid in the protonated form could act as a general acid comparable to glutamic acid 35 in Lysozyme.

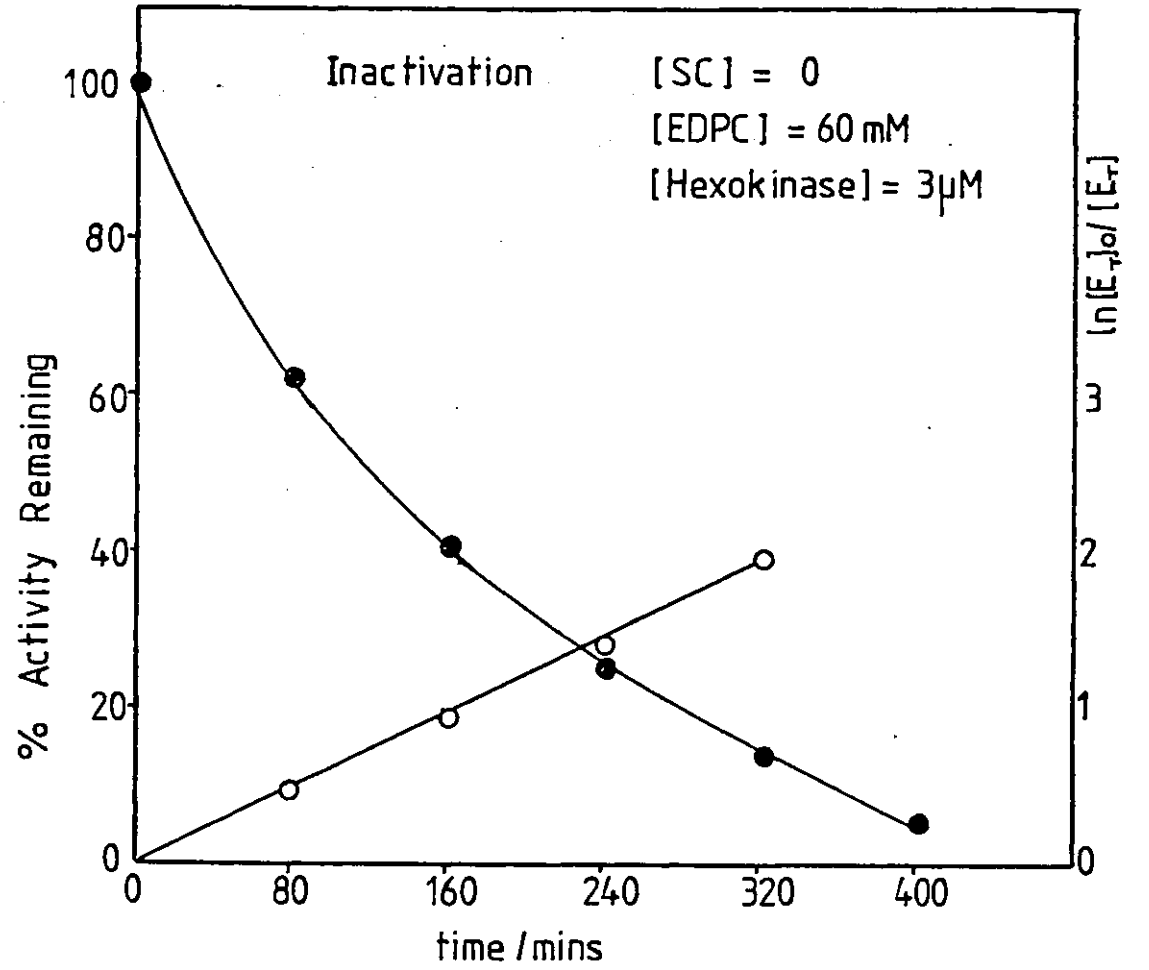
The role of the essential aspartate group suggested by Steitz is however as a general base (18) to increase the nucleophilicity of the 6' -OH in glucose. This would indicate that the pK would be normal and the residue not reactive to carbodiimide at pH values above 4.5.

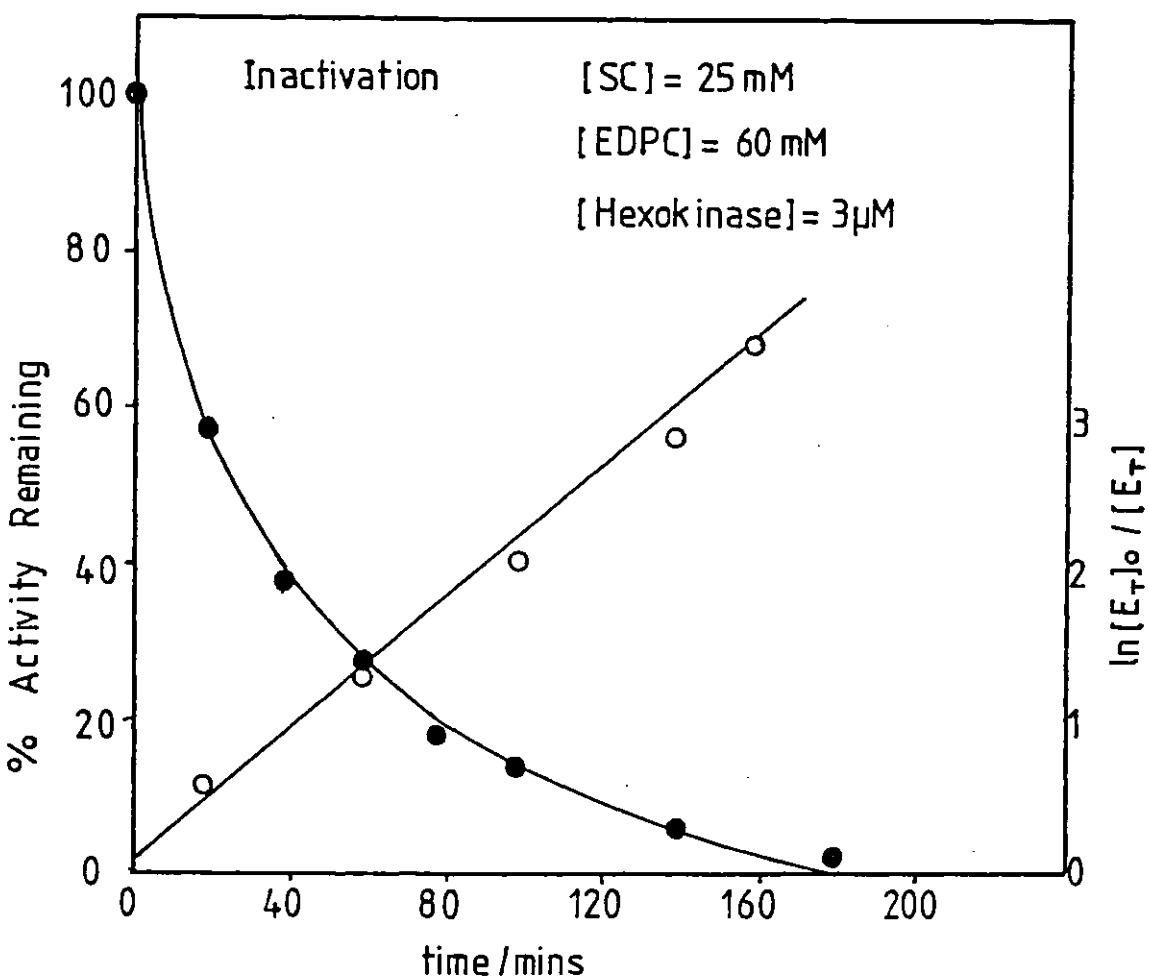
In order to investigate the inactivation pH = 6 seemed the most appropriate since the reaction is still relatively fast but it might be expected to favour the essential carboxyl if that residue has a high pK.

5.5 EFFECT OF NUCLEOPHILE AND SUBSTRATES

When no nucleophile is present the carbodiimide alone will inactivate the enzyme. This is because some stable but inactive N-acyl urea is formed even though the hydrolysis reaction (fig. 5.4) is much faster than the rearrangement. Of course the hydrolysis has a cyclic effect reforming the carboxylic acid which can react with carbodiimide again. Presumably in this way a stock of N-acyl urea is built up. The addition of 25mM semicarbazide does increase the rate of inactivation considerably. The inactivation (fig. 5.9) shows that

fig. 5.9


Inactivation
[SC] = 0
[EDPC] = 60 mM
[Hexokinase] = 3μM
% Activity Remaining
ln[E_T]o/[E_T]
time / mins
0
20
40
60
80
100
0
80
160
240
320
400
1
2
3


Inactivation
[SC] = 25 mM
[EDPC] = 60 mM
[Hexokinase] = 3μM
% Activity Remaining
ln[E_T]o / [E_T]
time / mins
0
20
40
60
80
100
0
40
80
120
160
200
1
2
3

$$Ra = 3.5$$

where $Ra = k_1'$ (60mM EDPC + 25mM SC) / k_1' (60mM EDPC).

Pho et al.(97) found that most nucleophiles they tried gave Ra = 1 although 30mM tyrosine ethyl ester increased the rate by a factor of 3. Semicarbazide would therefore appear to be a suitable nucleophile to use in the inactivation reaction.

If an active centre carboxyl group is reacting, substrates would be expected to protect against inactivation. Figs. 5.10 and 5.11 show that protection was reasonable although on no occasion did it reach 100%. Table 5.2 shows that glucose and mannose offered a similar, low degree of protection with the addition of MgADP affording much greater protection.

In all cases the degree of protection was greater than that obtained by Pho et al.(97) with nitrotyrosine ethyl ester as nucleophile. However this group used phosphate buffer and phosphate is known to be an activator of Hexokinase below pH=7 (33) and thus results obtained in this buffer would be expected to be inaccurate.

5.6 EFFECT OF TEMPERATURE ON THE RATE OF INACTIVATION

Arrhenius found that over a moderate range of temperature there was a relationship between the temperature of the reaction and the rate constant given by:-

$$k = A\, e^{-Ea / RT} \qquad 5.10$$

where k is the rate constant, A the pre-exponential factor, Ea the activation energy, R the Gas Constant and T the absolute temperature. Equation 5.10 can be re-written:-

$$\ln k = \ln A - Ea / RT \qquad 5.11$$

and a plot of $\ln k$ against $1/T$ should yield a straight line slope $-Ea / R$. Arrhenius plots are usually linear although in certain instances they may not be and a discontinuity can exist. Some of the reasons for this could be that the overall process involves two

fig 5.10 Effect of substrates on inactivation

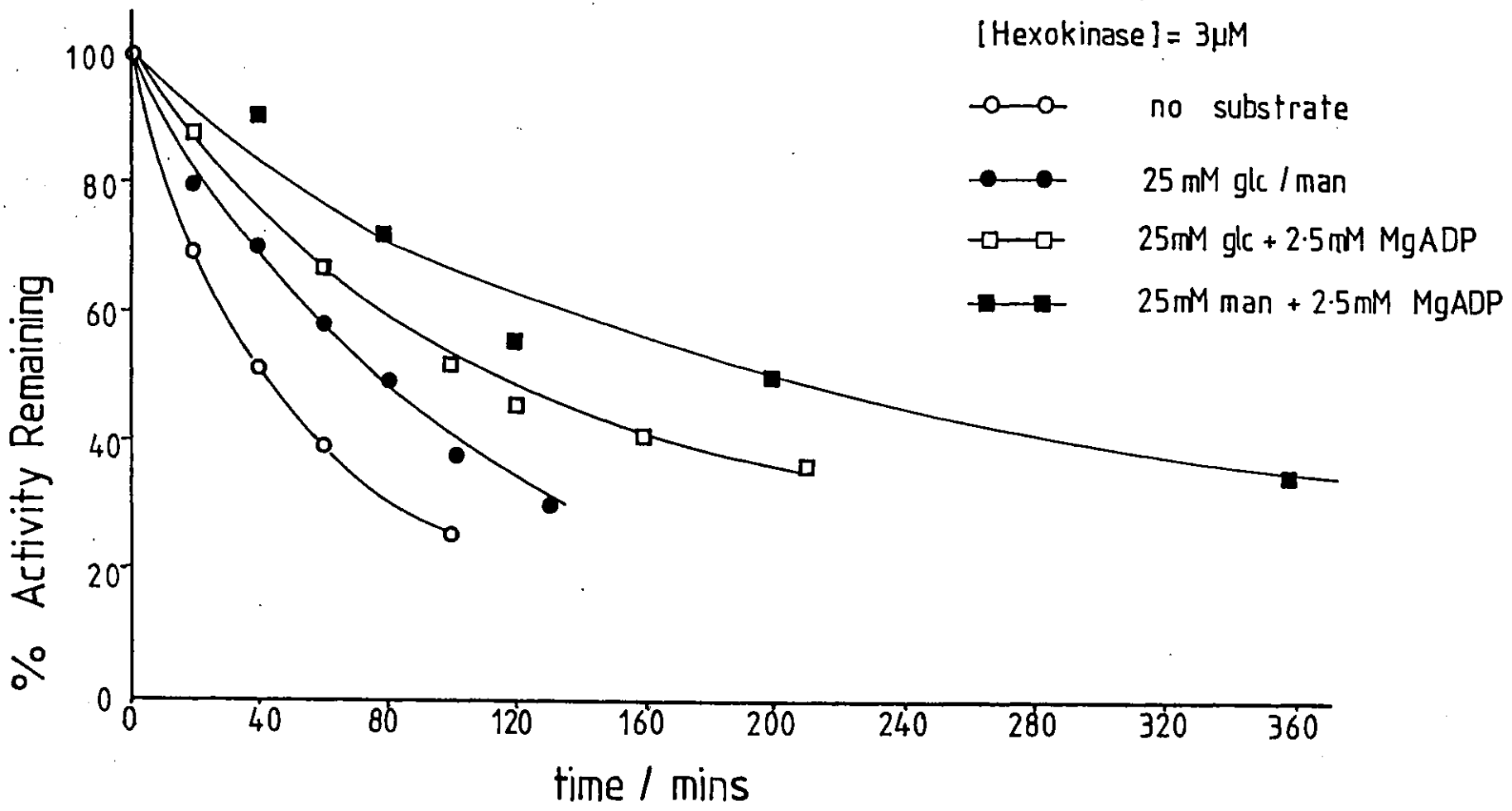


fig. 5.11 ln $[E_\tau]_o / [E_\tau]$ against time / mins from fig 5.10

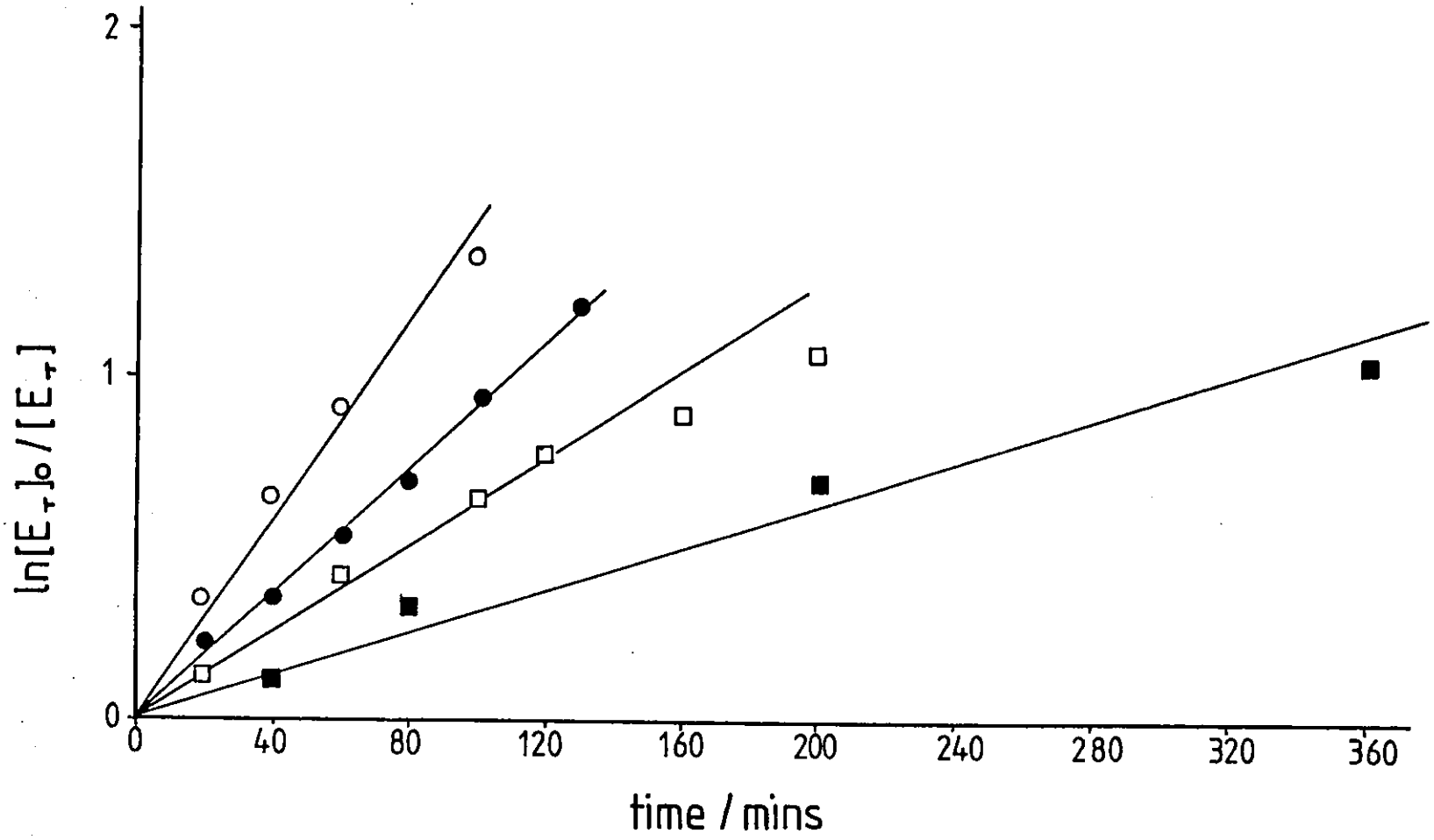


Table 5.2

EFFECT OF SUBSTRATES ON INACTIVATION OF HEXOKINASE

added substrate	nucleophile = SC		NTEE +
	k_1' min $^{-1}$	% protection	% protection
none	0.0144	0	-
25mM glucose	0.0092	36	19
25mM mannose	0.0092	36	21
25mM glucose + 2.5mM MgADP	0.0064	56	42
25mM mannose + 2.5mM MgADP	0.0032	78	40

+ taken from reference 97.

successive reactions with different temperature coefficients or a temperature dependant change in the structure of the reactants.

The inactivation of Hexokinase by iodoacetate shows a marked temperature dependence. (91). Below 30°C the inactivation is very slow but it increases rapidly above this temperature presumably because there is a major structural change exposing the reactive thiol.

The results of experiments to test the temperature-dependence of Hexokinase inactivation by carbodiimide and semicarbazide are shown in fig. 5.12. There is no discontinuity in the Arrhenius plot and thus it is likely that no substantial temperature-dependant structural change takes place to expose a particular carboxyl group. Thus a single activation energy for the reaction can be calculated.

$$\text{Slope} = -0.117 \times 10^5 \text{ K}$$

$$= -\text{EaR}.$$

$$\text{Ea} = 0.117 \times 10^5 \times 8.3143 \text{ J mol}^{-1}$$

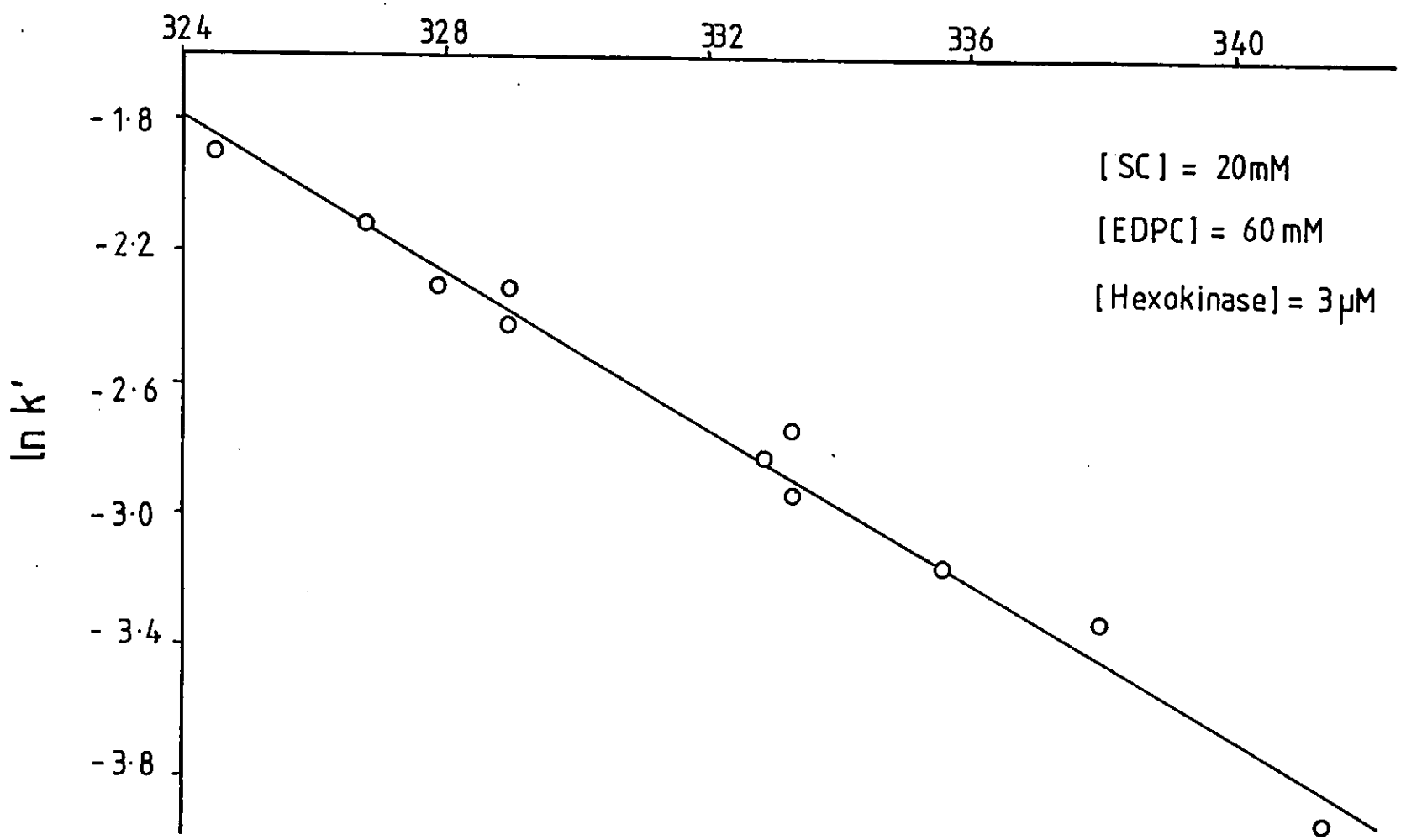
$$= 97 \text{ KJ mol}^{-1}$$

5.7 RADIOACTIVE LABELLING EXPERIMENTS

Since previous workers had found a single essential carboxyl group in Hexokinase A and preliminary experiments with carbodiimide and semicarbazide indicated that this could be the case in Hexokinase B it was decided to test this theory using radioactive semicarbazide. Inactivation experiments had shown that a concentration of semicarbazide as low as 10mM would produce inactivation in a reasonable time and so it was not necessary to use a large amount of radioactive material. Using ^{14}C-KCNO it was relatively easy to prepare ^{14}C-semicarbazide of specific activity 1.8 μCi / μmole. (see section 2.7).

Initial labelling was with unprotected enzyme, samples being taken at suitable times and dialysed extensively against 1mM HCl before incorporation was measured. It was thought necessary to continue

fig. 5.12 Arrhenius Plot for Inactivation by carbodiimide & semicarbazide



the experiment after 100% inactivation had been reached - if a single essential carboxyl group was reacting the incorporation at 100% inactivation would be unity with no increase even if the reaction was continued for longer. However as fig. 5.13 shows this was not the case. At 100% inactivation the incorporation achieved was two - this increased to three at double the inactivation time (t2) and levelled off at ten when t3 was reached. At t_∞ the incorporation was still ten. Although these results were somewhat unexpected they were not as anomolous as first appeared.

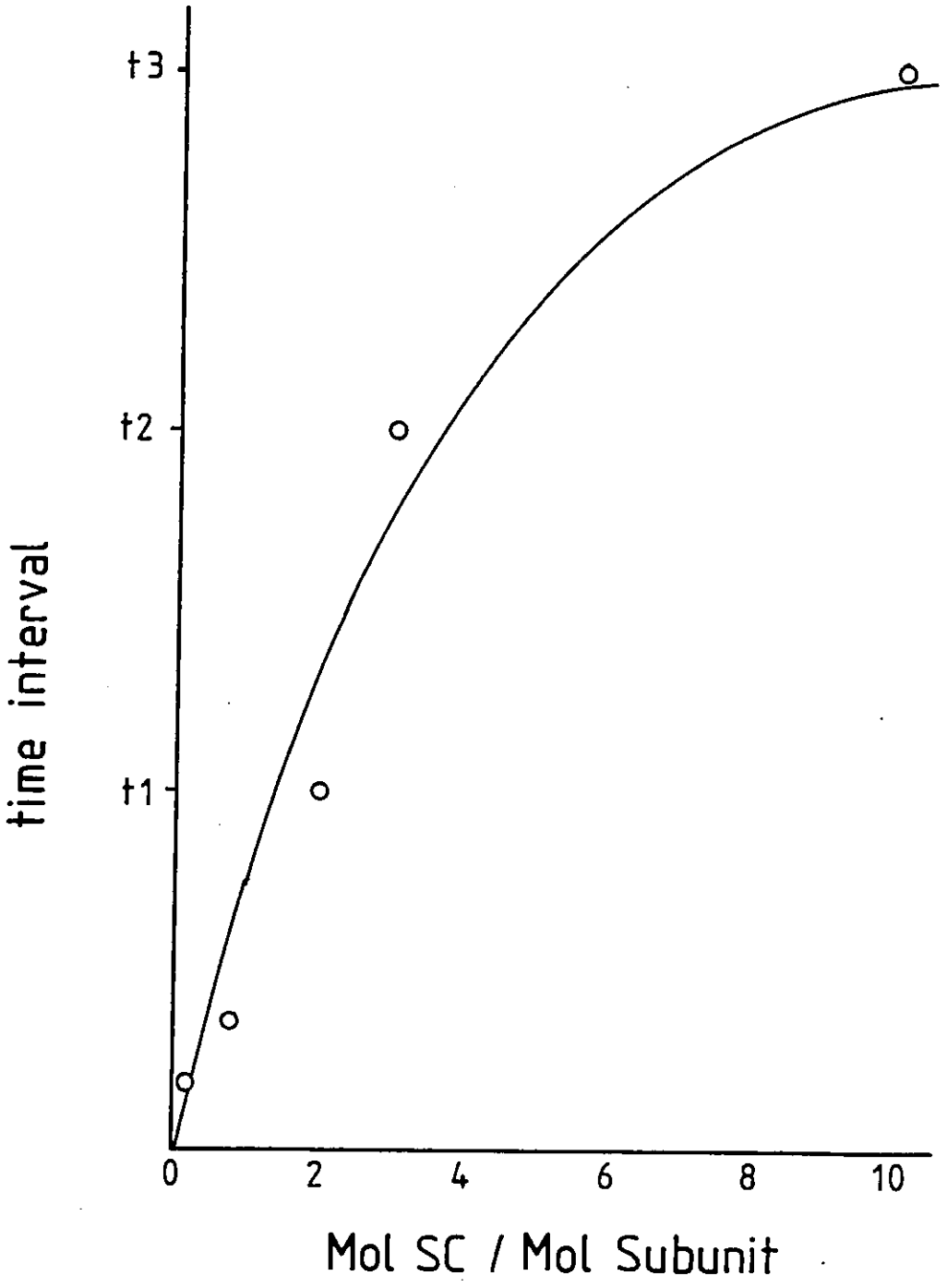
Examination of the preliminary results obtained by Pho et al. (54) with Hexokinase A were similar to those obtained with Hexokinase B. When Hexokinase A was completely inactivated the incorporation was two. An incorporation of three was also noted although not explained. No details of the incorporation at longer time intervals were reported and so it is possible that the two sets of results are compatible.

Since substrate protection had been observed it was decided to find out whether single incorporation could be achieved by using the substrate protection. The enzyme was reacted with carbodiimide and unlabelled semicarbazide for half an hour in the presence of 25mM mannose and 2.5mM MgADP. After this time the small molecules were removed by dialysis and the still active enzyme reacted with carbodiimide and radioactive semicarbazide. At 1t the incorporation was unity but this doubled at 2t. Clearly the system was not behaving as expected. Nevertheless an attempt to identify a single labelled carboxyl group was made.

The protection experiment was repeated using a larger quantity of enzyme (4 mg). The inactivation using radioactive semicarbazide was taken to 80% and an incorporation of 0.7 obtained. The protein was then freeze-dried and taken up in 6M guanidine 0.3M Tris HCl buffer

fig. 5.13 Incorporation of ^{14}C semicarbazide into Hexokinase

t1 = time for 100 % inactivation



for reduction and carboxymethylation (see section 2.6). The enzyme was then digested using CNBr (1:100 W/W, 24 hours, 70% formic acid) and after freeze-drying and re-dissolving in 20% formic acid the peptide mixture was applied to a P30 (100-200) biogel column. As is shown in fig. 5.14 the radioactive material was spread right across the column and did not seem to be concentrated into any particular peptide pool.

Four pools of peptide material were made corresponding to fractions (19-26), (27-44), (45-53) and (54-65) and labelled I-IV respectively. These pools were then subjected to HPLC using a C8 column with isocratic conditions of 5% acetic acid and a gradient followed by of 0-40% propan-1-ol. Although the separation of peptide material could have been better it was easy to identify zones of peptide material from the A280, A254 traces and pool accordingly. A typical trace is shown in fig. 5.15 along with a profile showing the distribution of radioactivity. No single peptide was well-labelled above any other in any of the pools.

The results seem to indicate that by this method it is not possible to label the single essential carboxyl group if indeed one exists. It may be that with this method the essential carboxyl group reacts very quickly and induces some sort of conformational change allowing other carboxyl groups, up to a maximum of ten, to react. If reaction at the essential carboxyl group does not bring about complete inactivation it is quite likely that more than one group will be incorporated. Another possibility is that several groups are hit concomitantly and that an average of two carboxyl groups per monomer - distributed through a variety of positions in different molecules - are modified when activity is removed.

This differs from the results with Hexokinase A (97) and could presumably be due to either the difference in primary structure

fig. 5.14 P10 Column - Separation of CNBr Digest

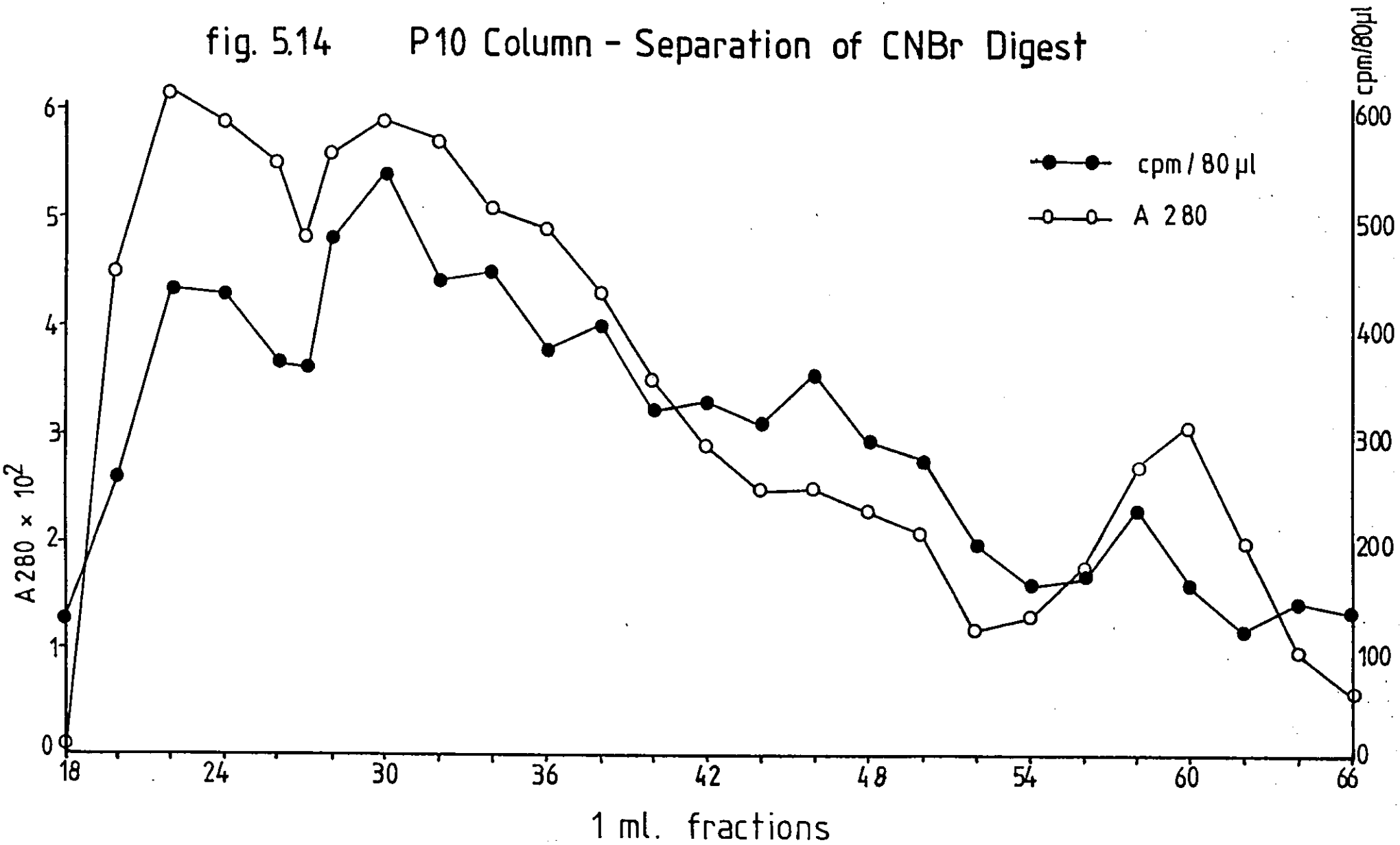
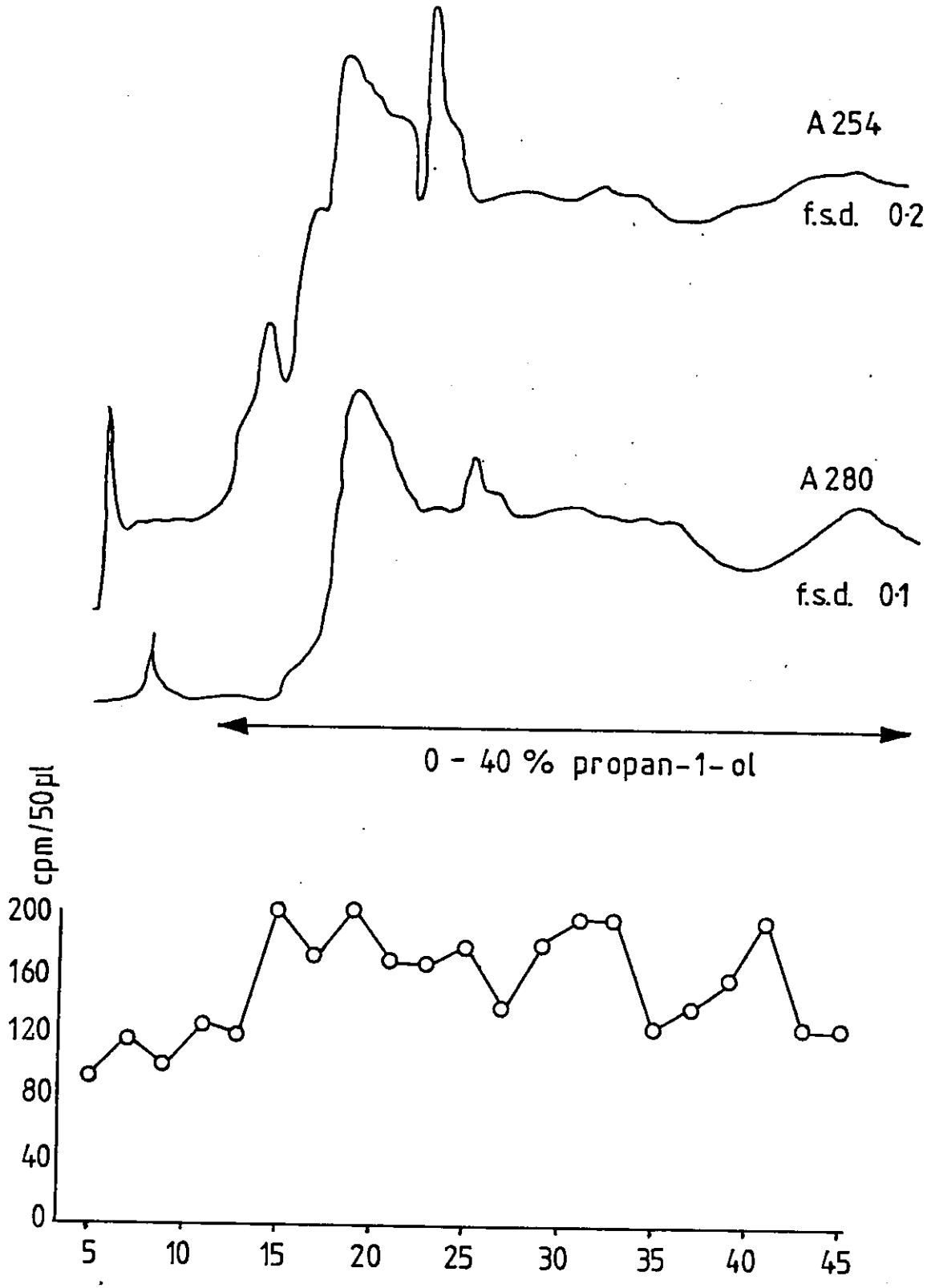


fig. 5.15 H.P.L.C. of Pool II



or the different reagents used. However it seems unlikely because the reagents are not active site-directing nor does it seem probable that the other isoenzyme should react completely differently. In fact the evidence for a single essential glutamic acid is not from the isolation of a single labelled peptide but from the isolation of the glutamic acid residues after complete proteolytic digest. In the case of a complete digest it would be more difficult to unequivocally state that only one residue had been modified. The lack of complete substrate protection would also seem to indicate that a single carboxyl residue is not involved.

In order to investigate the matter more fully some other chemical method would have to be used possibly by developing an active centre-directing reagent.

CHAPTER 6

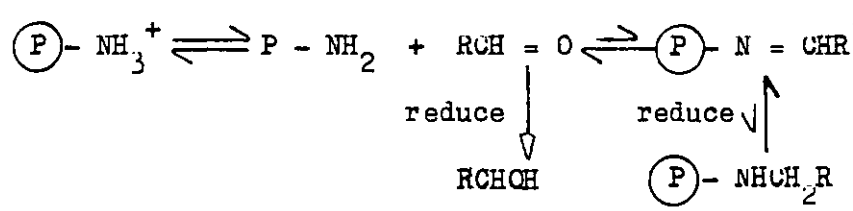
AN AFFINITY LABEL FOR THE ADENINE NUCLEOTIDE SITE

6.1 INTRODUCTION

An affinity reagent is designed to label the active centre of an enzyme by using the specificity of that enzyme. Usually, the affinity label is a competitive inhibitor with a close resemblance to the normal substrate of the enzyme, in this case ATP. Adenosine analogues which have been used are Adenosine 5'-(2 bromoethyl)-phosphate (103) which was used to label NAD dependent Isocitrate Dehydrogenase and 3' p - fluorosulphonyl benzoyl adenosine (104) which labelled a regulatory site of Glutamate Dehydrogenase. For the work on Hexokinase, it was decided to use a more easily synthesised label.

Dialdehyde nucleotide derivatives have been used successfully to label a fairly wide variety of enzymes including Pyruvate Carboxylase (60), Pyruvate Phosphate Dikinase (61) and Aminoacyl - tRNA synthetases (105). Both the preparation of (see section 2.8) and inactivation of the enzyme with oATP were straight forward so that oATP seemed a suitable compound to investigate.

The inactivation of the enzyme is thought to proceed via the following mechanism:- (106, 107)



1 = Schiff's base.

6.2 REACTION CONDITIONS

Inactivation was brought about by incubating the enzyme with oATP in the appropriate buffer for five minutes, after which time a 5 - 10 fold excess of reducing agent was added and the solution allowed

to stand for 35 minutes before assaying. Initial experiments were carried out in imidazole buffer which contains no amino groups to interfere with the reaction. The concentration of oATP used initially was 1mM .

Normally in experiments using nucleotides the appropriate divalent cation (M^{2+}) must be used (see section 1.5) but opinion is divided when using dialdehyde derivatives. A group using the Uridine derivative (108) have shown that optimum inactivation is found when $5\ uM \leq M^{2+} \leq 100\ uM$ - most other workers have shown that the active species is M-oATP. The requirement for Mg^{2+} ions was therefore explored. In all cases control experiments replaced oATP with the same concentration of ATP. Table 6.1 shows that the difference between experiments carried out with and without Mg^{2+} is minimal so it would appear that the inactivation has no particular requirement for divalent cations, nor do they have an inhibitory effect.

6.3 CHOICE OF REDUCING AGENT

Both $NaBH_4$ (60) and $NaCNBH_3$ (61) have been used with some success although there are some theoretical implications which should make $NaCNBH_3$ a better choice. $NaBH_4$ is a very strong reducing agent and reduces aldehydes with ease i.e., reduction of the aldehyde could occur before the Schiff's base formed. The reaction with $NaBH_4$ is pH dependent (109) and the reagent reduces disulphide bonds and may cleave peptide linkages. (110). On the other hand $NaCNBH_3$ is a much weaker reducing agent which will not reduce aldehydes at neutral pH but readily reduces Schiff's bases (imines). (111).

The differences between the reducing agents were explored and $NaCNBH_3$ found to be far superior (table 6.2). Not only did the $NaBH_4$ inactivate the ATP-containing control, it also failed to produce any greater inactivation with the addition of oATP. It seems clear that this reducing agent affects the activity of the enzyme and also prevents significant formation of the Schiff's

Table 6.1

DEPENDENCE ON DIVALENT CATION

ATP 1mM	√	–	√	–	√	–
oATP 1mM	–	✓	–	✓	–	✓
$MgCl_2$ 1mM	–	–	✓	✓	–	–
EDTA 1mM	–	–	–	–	✓	✓
Activity % Control	100	31	100	30	100	32

All reaction mixtures contained 5×10^{-4} µmoles Hexokinase in 20mM Imidazole pH 7.6. Reducing agent $NaCNBH_3$

Table 6.2

INVESTIGATION OF REDUCING AGENT I

ATP 1mM	✓	✓	–	✓	–
oATP 1mM	–	–	✓	–	✓
$NaCNBH_3$	–	✓	✓	–	–
$NaBH_4$	–	–	–	✓	✓
% Control.	100	100	30	51	45

Conditions as table 6.1 - reducing agents present in 5 fold molar excess.

base. $NaCNBH_3$ was therefore, the chosen reducing agent.

6.4 INACTIVATION AND PROTECTION

The concentration dependence of the inactivation (fig 6.1) does not show the pseudo first order kinetics exhibited by Pyruvate Phosphate Dikinase (4) and 100% inactivation was not obtained. However the inactivation was irreversible and probably covalent because gel filtration on G25 to remove small, non-covalently bound molecules did not give an increase in enzyme activity.

One of the criteria for an affinity label is that the normal substrate or a substrate analogue should offer substantial protection against inactivation. The substrates/substrate analogues used were MgATP, MgADP, MgGDP, mannose and glucose. Only the nucleotides offered any significant protection (figs 6.2 - 6.4) with MgATP offering the greatest degree of protection. The fact that MgADP protects presumably indicates that the binding position of the phosphate group is not important in determining specificity. MgGTP affords less protection which is to be expected because it is a much poorer substrate than MgATP (see section 1.4). Neither sugar substrate tested offered much protection (figs 6.5, 6.6) which indicates that oATP is specific for the nucleotide binding site because sugar substrates do protect against more general inactivating reagents.

Since glucose offered no significant protection against inactivation and since glucose is known to enhance the binding of ATP to Hexokinase (112) an experiment was carried out to see if glucose enhanced the inactivation of Hexokinase by oATP (fig. 6.7). No appreciable enhancement of rate of inactivation was observed which is somewhat surprising in view of the very definite effect glucose has on the binding of ATP to Hexokinase. Presumably the oATP binds slightly differently and a glucose-induced conformational change has

fig. 6.1

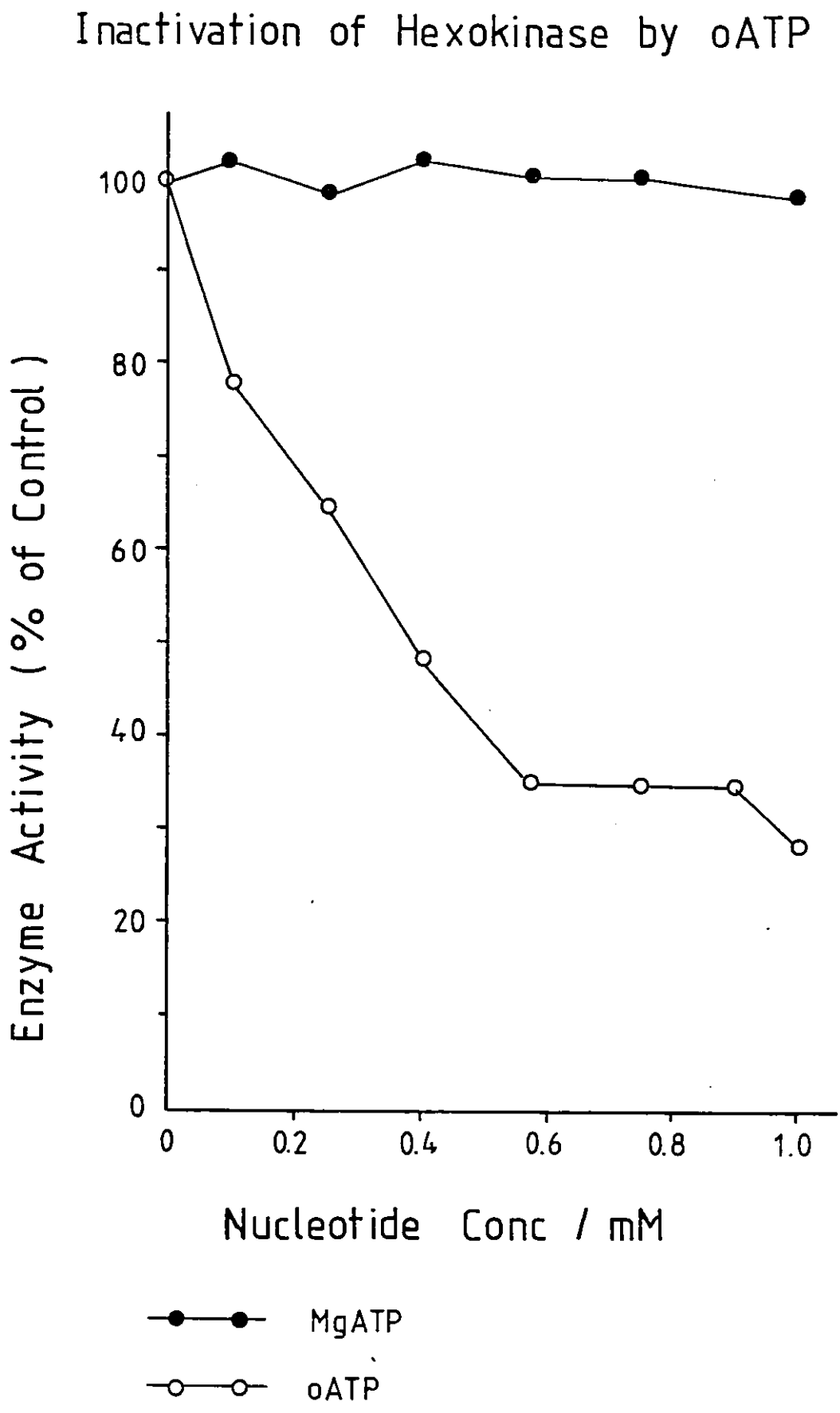

Inactivation of Hexokinase by oATP
Enzyme Activity (% of Control)
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
Nucleotide Conc / mM
MgATP
oATP

fig. 6.2

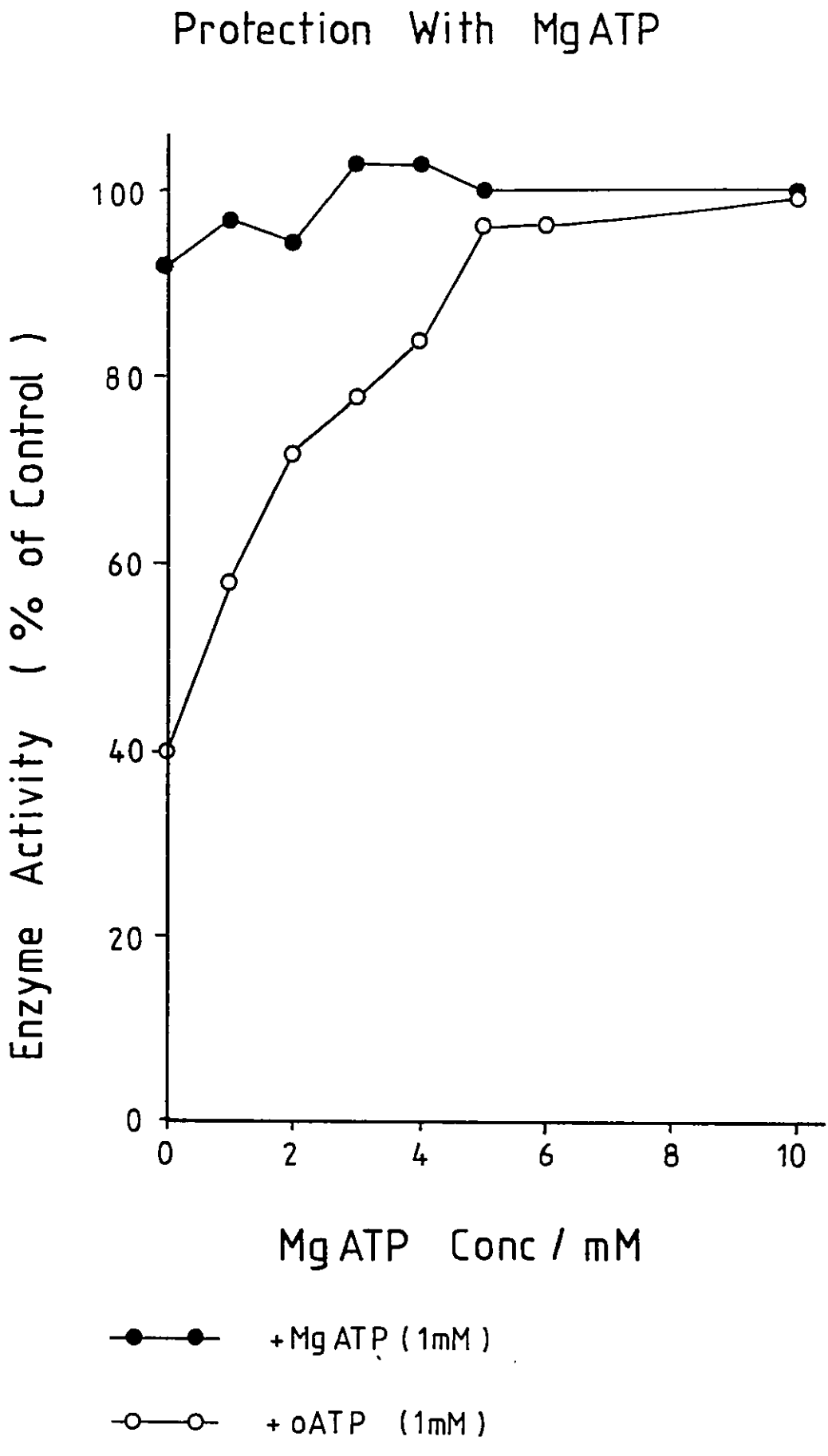

Protection With MgATP
Enzyme Activity (% of Control)
100
80
60
40
20
0
0
2
4
6
8
10
MgATP Conc / mM
+MgATP (1mM)
+oATP (1mM)

fig. 6.3

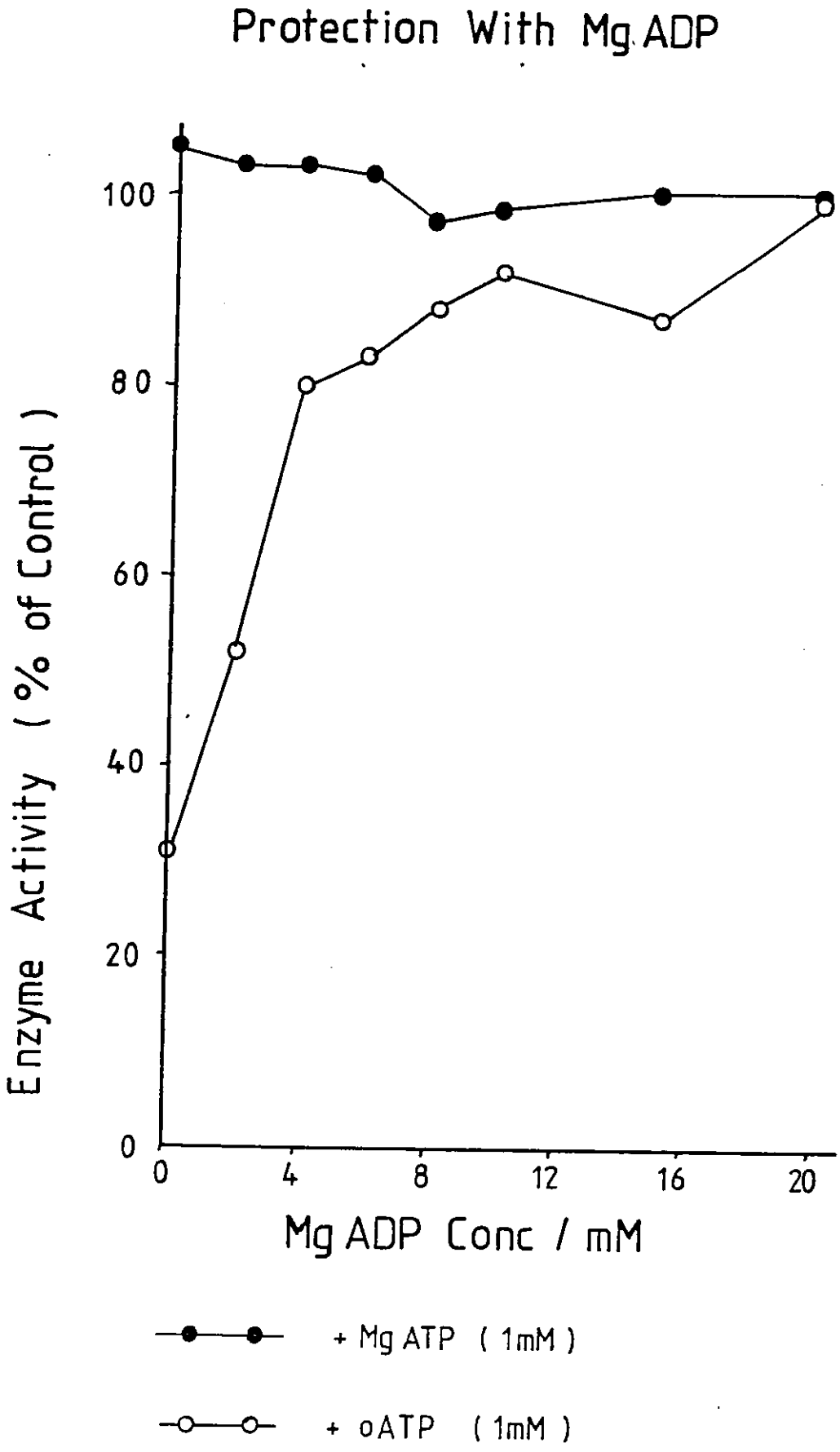

Protection With Mg.ADP
Enzyme Activity (% of Control)
100
80
60
40
20
0
0
4
8
12
16
20
Mg ADP Conc / mM
+ Mg ATP (1mM)
+ oATP (1mM)

fig. 6.4

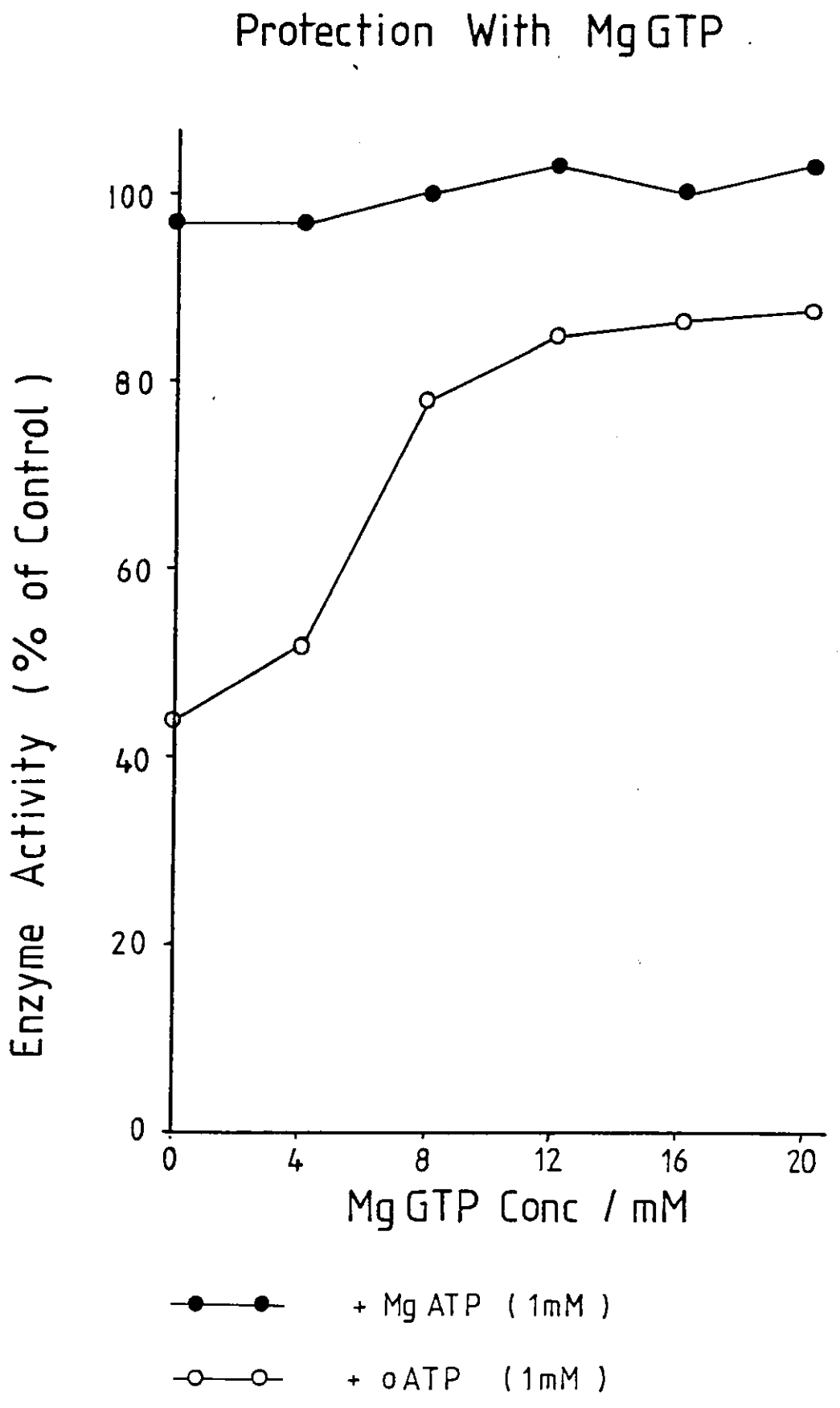

Protection With Mg GTP
Enzyme Activity (% of Control)
100
80
60
40
20
0
0
4
8
12
16
20
Mg GTP Conc / mM
+ Mg ATP (1mM)
+ oATP (1mM)

fig. 6.5

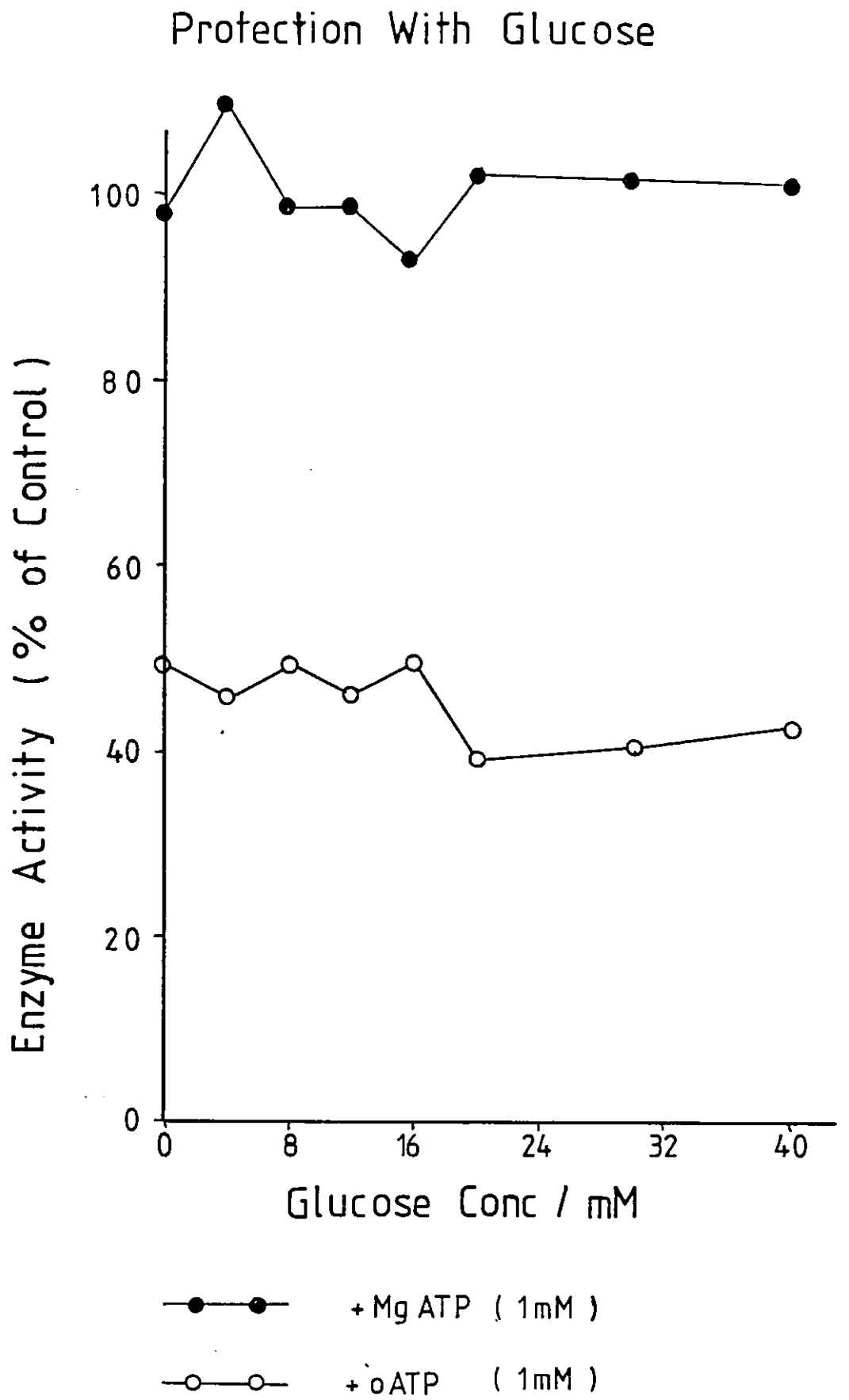

Protection With Glucose
Enzyme Activity (% of Control)
100
80
60
40
20
0
0
8
16
24
32
40
Glucose Conc / mM
+ Mg ATP (1mM)
+ oATP (1mM)

fig. 6.6

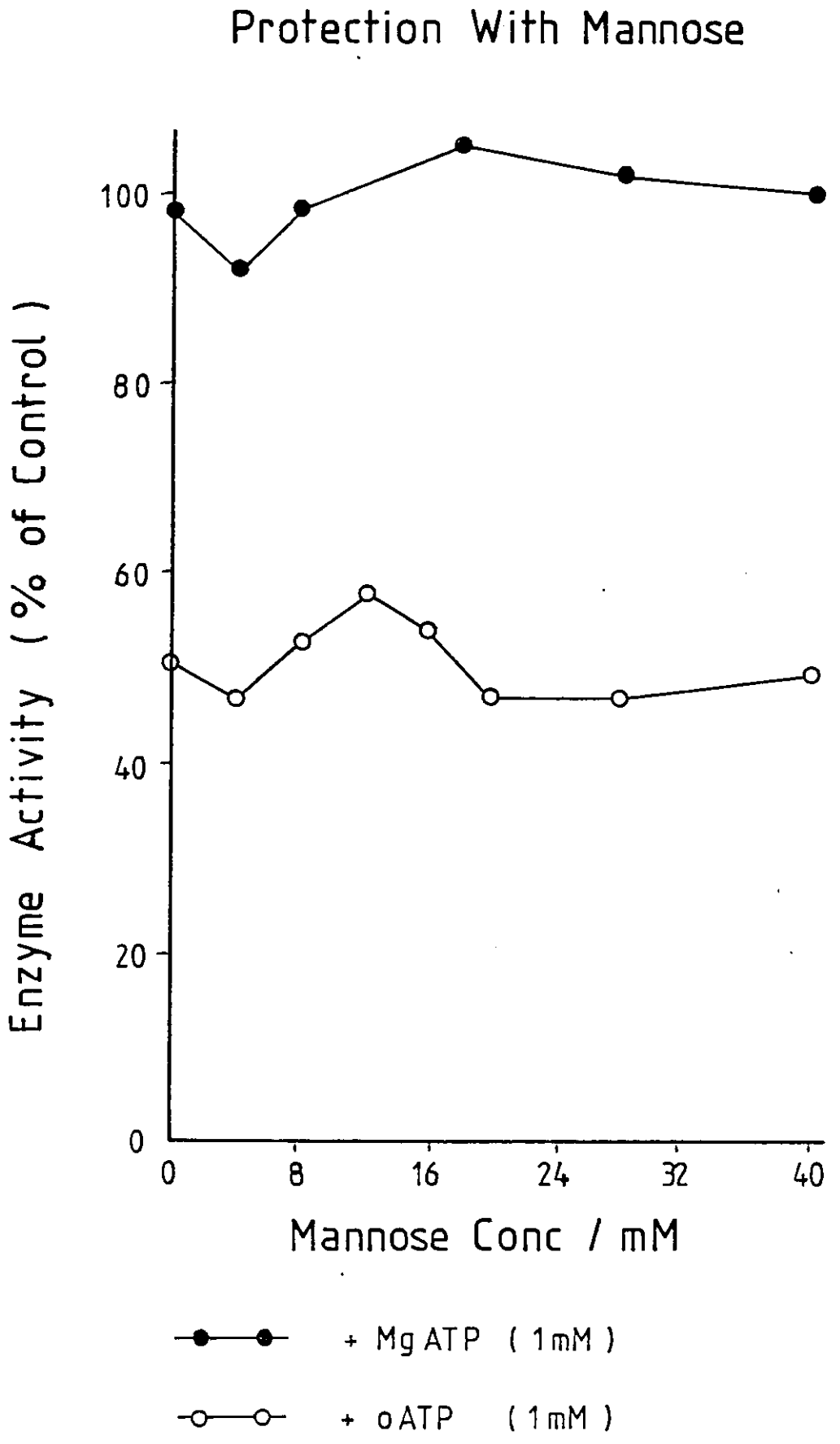
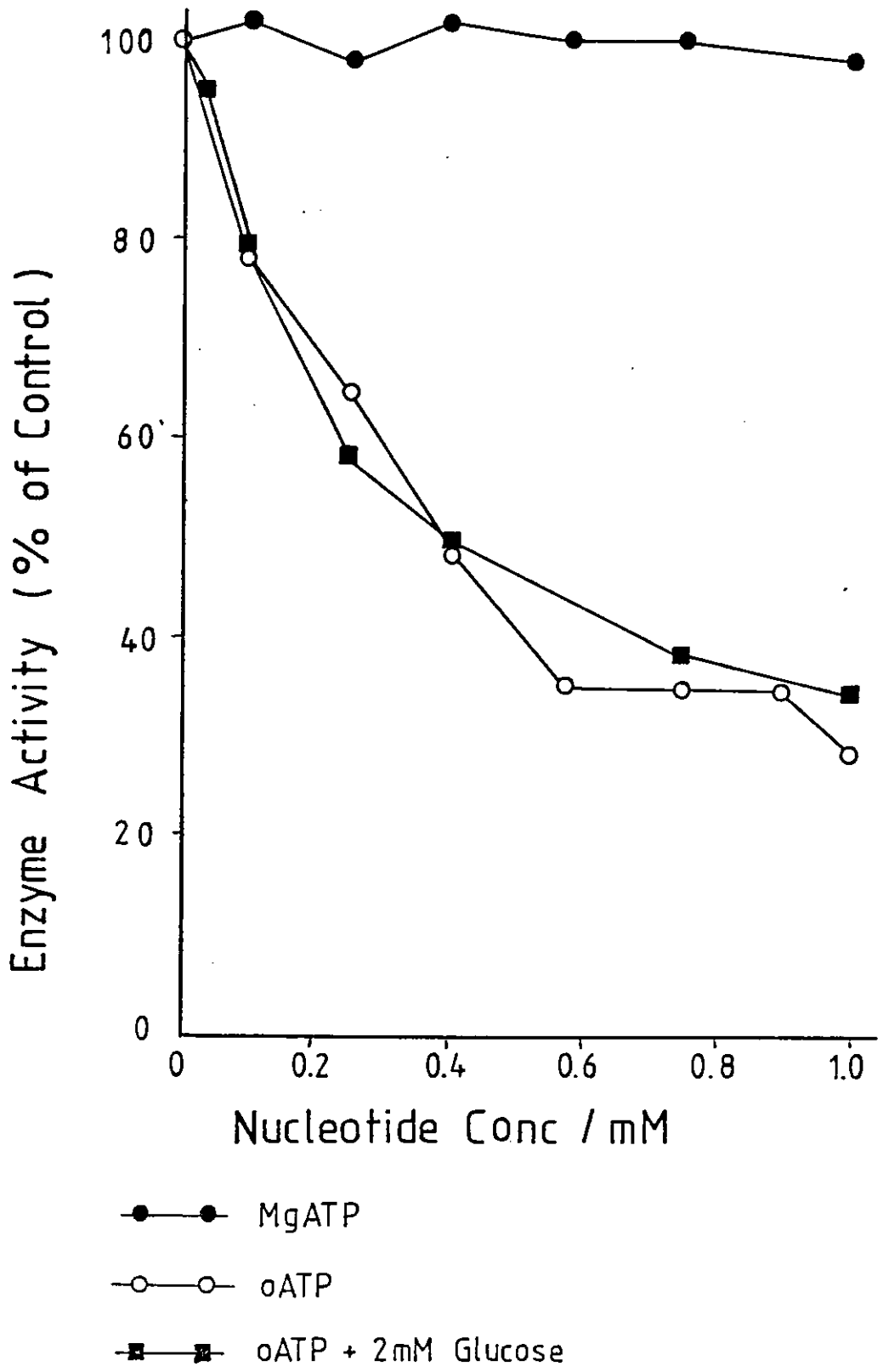

Protection With Mannose
Enzyme Activity (% of Control)
100
80
60
40
20
0
0
8
16
24
32
40
Mannose Conc / mM
+ Mg ATP (1mM)
+ oATP (1mM)

fig. 6.7

Effect of Glucose on Inactivation By oATP



no effect.

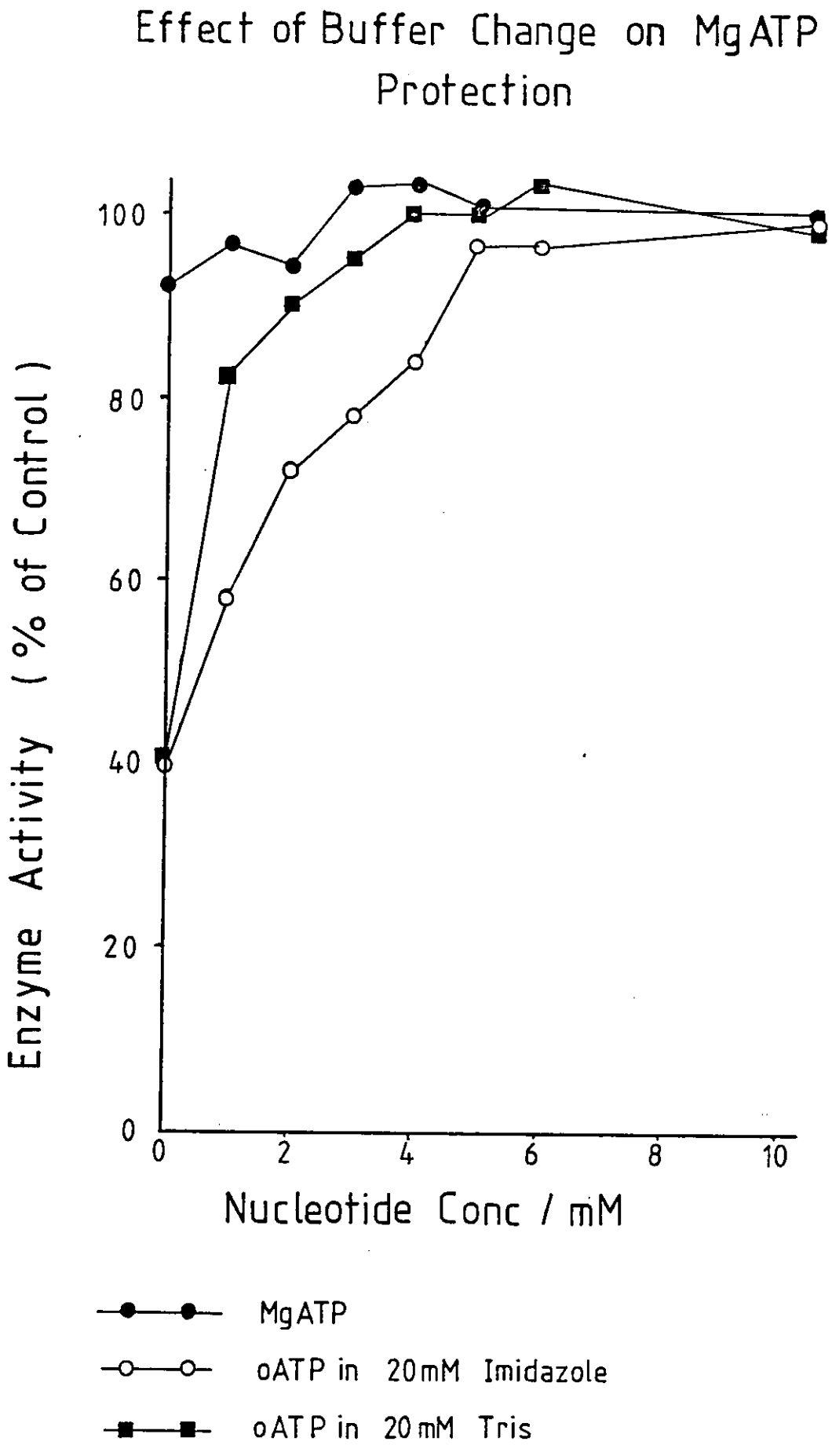
6.5 RADIOACTIVE LABELLING EXPERIMENTS

All the previous experiments indicated oATP would be a suitable affinity label for Hexokinase - this view had to be confirmed by an experiment using radioactive oATP. One problem was that the high cost of ^{14}C-ATP meant that very small quantities of protein and radioactive oATP had to be used introducing some sources of error. The initial experiments were in 20mM imidazole buffer and excess label was removed by dialysis against 1mM HCl. Fig. 6.8 shows that extremely high levels of incorporation were obtained - extrapolation to complete inactivation showed an incorporation of 10 mol oATP per mol subunit. In view of the previous work on protection it seemed extremely unlikely that this level of incorporation could be specific. The oATP molecule is relatively bulky and it seemed improbable that there were ten binding sites per monomer. Of course if the so called I site was involved it might be expected that $\geq$ 1 and $\leq$ 2 sites per monomer would be occupied. However, as discussed in 1.4 there is no evidence for partial binding to the I site in the crystal and no indication that the I site exists in solution. Therefore it was concluded that a large amount of non-specific binding was taking place.

Workers with Pyruvate Phosphate Dikinase (61) had found similar problems using imidazole buffer which they attributed to lysine residues, other than those at the active site reacting. By changing to Tris buffer, they eliminated these problems presumably because the amino group on the Tris buffer acts as an effective 'scavenger' competing with the enzyme for the oATP molecules.

Firstly it was necessary to perform a series of non-radioactive experiments to see if Tris was a suitable buffer.

fig. 6.8


Effect of Buffer Change on MgATP Protection
Enzyme Activity (% of Control)
100
80
60
40
20
0
0
2
4
6
8
10
Nucleotide Conc / mM
MgATP
oATP in 20mM Imidazole
oATP in 20mM Tris

The suitability of the two reducing agents was checked (table 6.3). Results were similar to those obtained with imidazole buffer of table 6.2 with the $NaCNBH_3$ being the most appropriate. When $NaBH_4$ is present alone it appears to have no significant effect on the activity of the enzyme unlike this reducing agent in the presence of imidazole. There does not appear to be any obvious reason for this effect. MgATP in Tris buffer offers protection against inactivation to a greater extent than in imidazole buffer (fig. 6.9). The curve is displaced to the left and is much steeper than the curve produced with imidazole buffer. This would be expected if fewer lysine residues were reacting in the Tris buffer. This system would, therefore, seem suitable for trying radioactive incorporation (fig. 6.10).

Incorporation at 100% inactivation was found to be five mol oATP/mol subunit by extrapolation. This was much lower than in imidazole buffer but still rather high. It was decided to repeat the experiment using 0.1M Tris as the buffer which should entirely eliminate non specific binding. The results (fig. 6.11) show an incorporation of 1.2 for a completely inactive enzyme. The fact that the reaction led to inactivation indicated that an essential lysine was reacting. Lysine residues which react non-specifically are likely to be on the surface of the enzyme and reaction at a single surface lysine would not be likely to affect the activity although ten such lysines might well do.

It would therefore appear that, given the correct reaction conditions oATP is a very suitable affinity label for the adenine nucleotide site of Hexokinase. It exhibits protectable inactivation of the enzyme, Complete inactivation is synchronous with an incorporation of about 1 mol oATP per mol subunit and it is also easy to prepare in a pure form.

Table 6.3

INVESTIGATION OF REDUCING AGENT II

ATP 1mM	✓	✓	-	✓	-
oATP 1mM	-	-	✓	-	✓
$NaCNBH_3$	-	✓	✓	-	-
$NaBH_4$	-	-	-	✓	✓
% Control	100	100	35	100	78

All reactions mixtures contained 5×10^{-4} umoles Hexokinase in 20mM TRIS pH 7.6

fig. 6.9

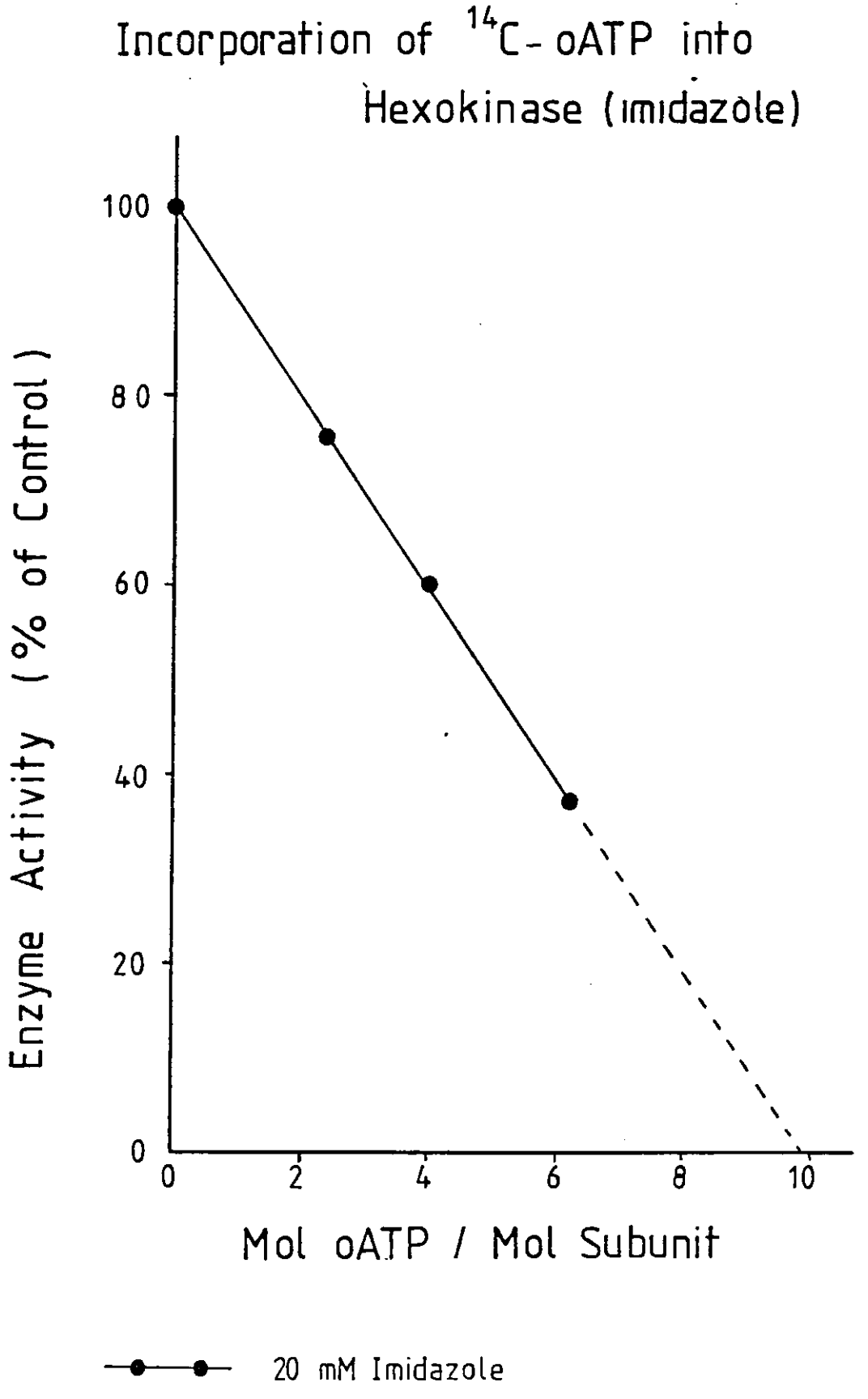

Incorporation of ^{14}C-oATP into
Hexokinase (imidazole)
Enzyme Activity (% of Control)
100
80
60
40
20
0
0
2
4
6
8
10
Mol oATP / Mol Subunit
20 mM Imidazole

fig. 6.10

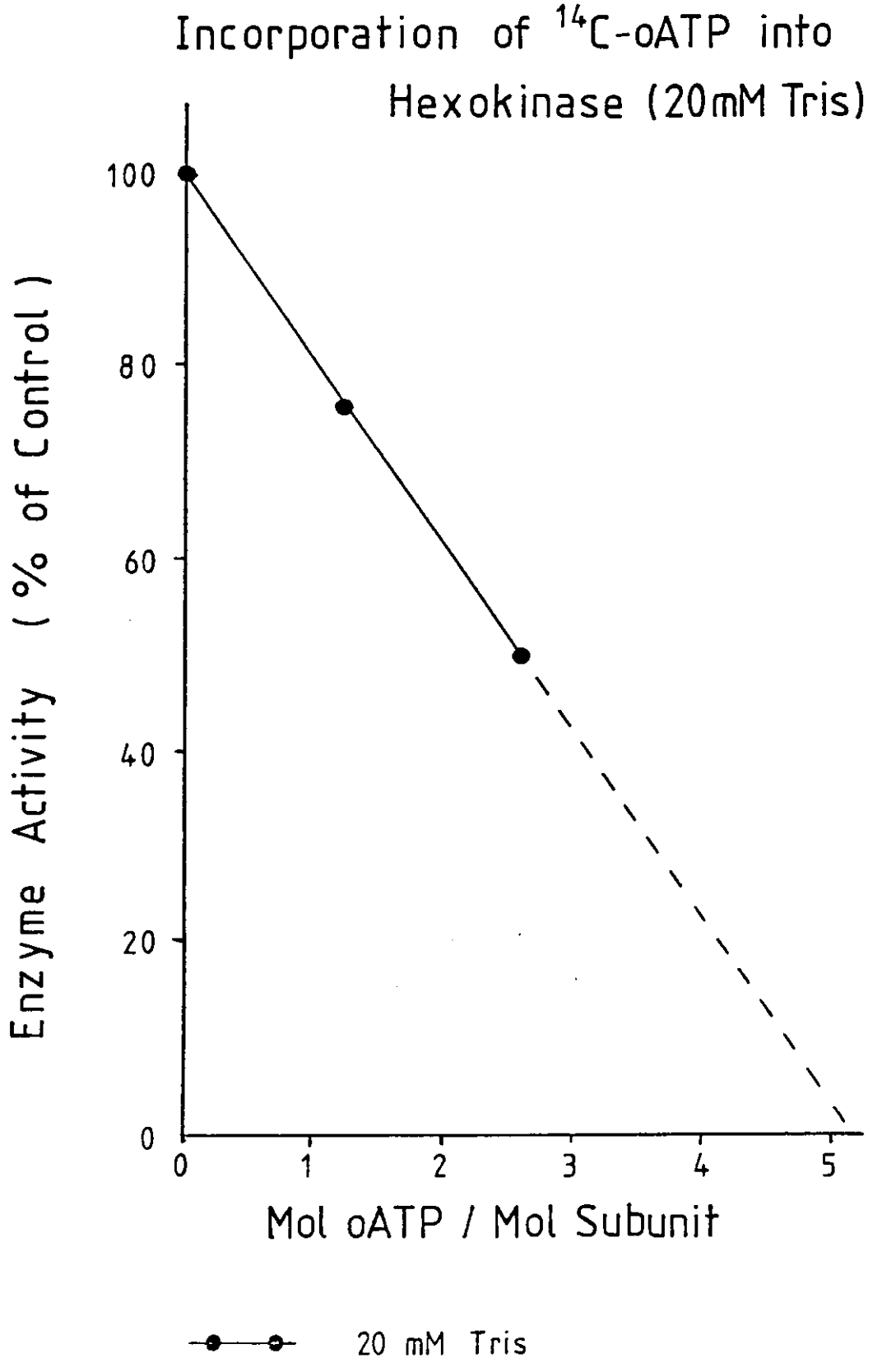
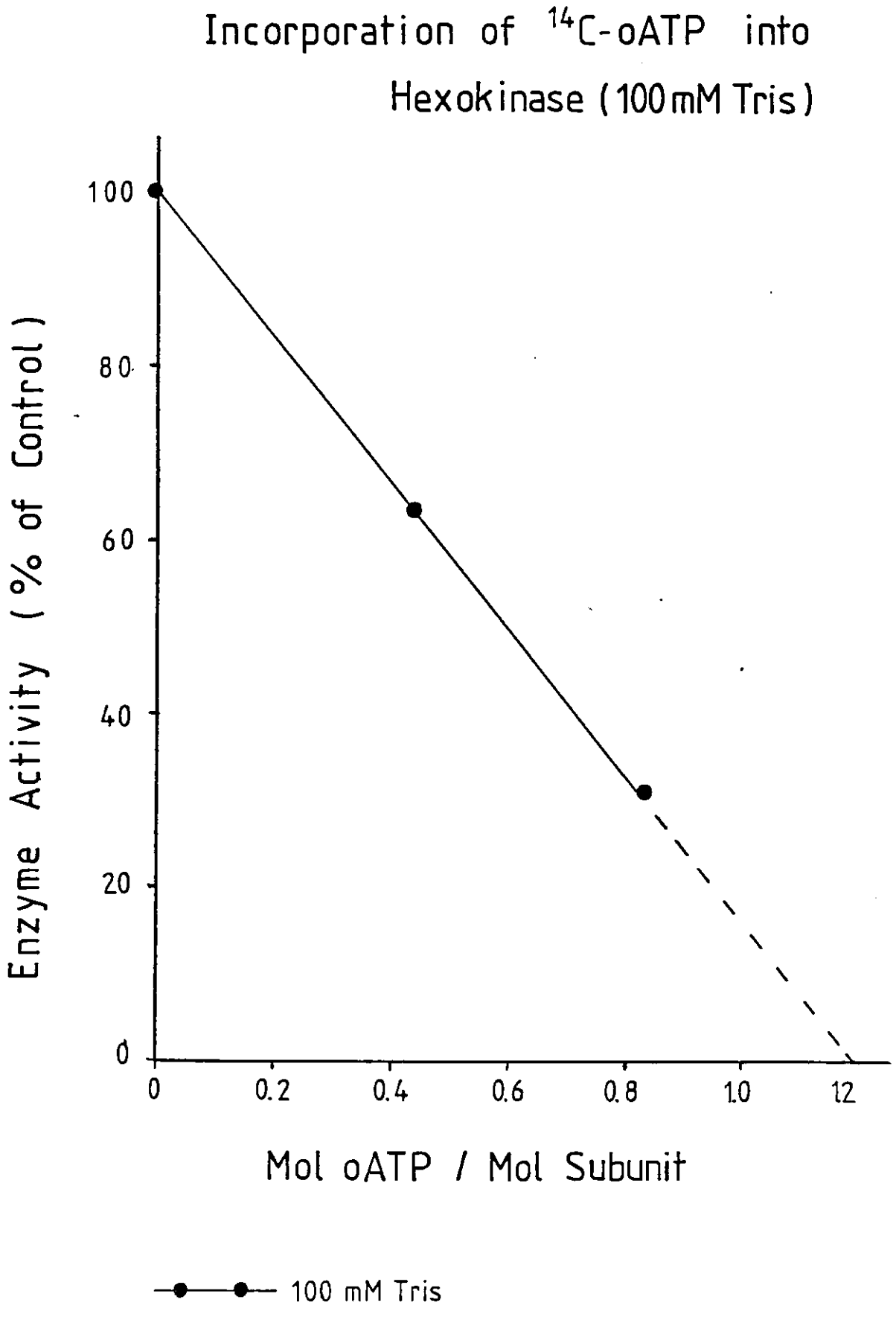

Incorporation of ^{14}C-oATP into
Hexokinase (20mM Tris)
Enzyme Activity (% of Control)
100
80
60
40
20
0
0
1
2
3
4
5
Mol oATP / Mol Subunit
20 mM Tris

fig.6.11


Incorporation of ^{14}C-oATP into
Hexokinase (100 mM Tris)
Enzyme Activity (% of Control)
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
1.2
Mol oATP / Mol Subunit
100 mM Tris

CONCLUSIONS AND PERSPECTIVES

The successful development of a large scale method of purification of yeast Hexokinase has made available large enough quantities of pure Hexokinase B for sequencing and active centre studies.

The lack of availability of a definitive primary sequence has hampered the work undertaken for this thesis.When such a sequence is available the clear identification of thiols I and II will be possible. However it seems likely that thiol I is not 80% along the peptide chain from the N-terminus but is in position 243 or 244 as indicated by the X-Ray structure. Further work on the thiol residues could confirm this and also determine the position of thiols III and IV.

It is clear that simple carboxylic acid specific reagents are not suitable for the identification of the active centre carboxylic acid group in Hexokinase B (if one exists). Since aspartic acid 189 is implicated in the X-Ray structure further study of acidic groups would be interesting. The obvious route to follow would be to use an active-centre directing reagent. Identification of a peptide containing an active-centre carboxyl may give valuable clues as to the mechanism of action of the enzyme.

2',3'-dialdehyde ATP has been identified as a suitable affinity agent for the nucleotide binding site of Hexokinase B. Identification of the lysine residue involved and comparison of the peptide with the 3-dimensional X-Ray structure would indicate any further studies which would be useful in characterising the binding site.

Cross-linking reagents may be useful in the study of the active centre groups since if two side chains in a protein can be linked with a small bifunctional reagent, the span length of the reagent defines the maximum distance apart for the two reidues in the 3-dimentional structure. Reagents are available to link cysteine residues,e.g. bifunctioal maleimide derivitives and these could be useful in determining the relationship of the thiols to each other. In particular thiols I and II may be close to each other in the region of the active site. Linkage of cysteine to histidine or lysine residues is also potentially useful , this could be accomplished by the use of bifunctional aryl or alkyl halides.

The availability of a definitive primary sequence and the use of active-centre directing reagents should enable the mechanism of action of Hexokinase B to be more clearly elucidated. It would then be possible to compare the mechanism of action of Hexokinase with that of other kinases, particularly those in the Glycolytic pathway.

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