A COMPARATIVE STUDY OF GLYCOGEN PHOSPHORYLASE FROM SELECTED CEPHALOPODS

THESIS

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Doctor of Philosophy in Biochemistry

By

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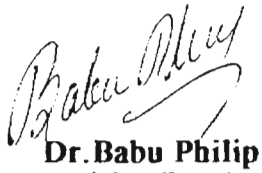
CERTIFICATE

This is to certify that the thesis entitled "**A comparative study of Glycogen phosphorylase from selected cephalopods**" herewith submitted by **Sri.Augustin Antony** in partial fulfilment of the requirement for the **Ph.D. Degree in Biochemistry** of the Cochin University of Science & Technology, is an authentic record of the research carried out by him under my supervision and guidance and that no part thereof has been presented before for any other degree in any University.



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DECLARATION

I hereby declare that the thesis entitled "**A comparative study of Glycogen phosphorylase from selected cephalopods**" submitted by me is an authentic record of research carried out under the supervision and guidance of Dr.Babu Philip, Reader in Marine Biochemistry, Division of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology in partial fulfilment of the requirements for the Ph.D. degree of Cochin University of Science and Technology and that no part of it has previously formed the basis for the award of any degree, diploma or associateship in any University.

8/12/96

AUGUSTIN ANTONY

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AUGUSTIN ANTONY

PREFACE

A comparative study of Glycogen phosphorylase from selected Cephalopods is reported in this thesis. A detailed investigation of an important glycolytic enzyme, phosphorylase, from a selected species, was undertaken. Loligo vulgaris, *commonly known as squid, is selected as the source for this study. Phosphorylase is the key enzyme in the mobilization of chemical energy from glycogen and its role in the regulation of carbohydrate metabolism, is well established. Although a good deal of information is available about phosphorylase from terrestrial animals, not much is known about the enzyme from aquatic fauna. In order to bridge this gap and also to compare the results with the findings from other sources, phosphorylase* a *was isolated from this marine mollusc and its properties studied in detail.*

Glycolysis is essentially the oldest energy yielding process in the biosphere. A comparison of the properties of glycolytic enzymes should give a fair appraisal of the features that have been preserved through the entire evolutionary process. Kinetic studies of Loligo phosphorylase a *can be regarded as an essential foundation for this comparative study.*

The thesis is divided into 14 chapters, starting with a review as introduction. Subsequent chapters deal with the mechanism and properties in a sequential order. A detailed comparative evaluation of glycolytic enzymes from marine sources is given in chapter 13. Many of the exceptional properties of glycolytic enzymes found in highly specialised marine organisms are given in this chapter. Chapter 14 deals with the experimental details. A brief summary is presented at the end of the thesis.

ABBREVIATIONS

AMP	- Adenosine-5'-monophosphate
ADP	- Adenosine-5'-diphosphate
ATP	- Adenosine 5'-triphosphate
DEAE-Cellulose	- Diethyl aminoethyl cellulose
DNP	- Dinitrophenol
DNPP	- Dinitrophenyl phosphate
DTNB	- 5, 5'-Dithio bis-(2-nitrobenzoic acid)
FDNB	- 1-Fluro-2,4-Dinitrobenzene
Glucose 1-P	- Glucose 1-Phosphate
Glucose 6-P	- Glucose 6-Phosphate
IMP	- Inosine-5'-monophosphate
NADP	- Nicotinamide adenine dinuclotide phosphate
PLP	- Pyridoxal-5'-phosphate
TCA	- Trichloroacetic acid
Tris	- Tris (hydroxy methyl) amino methane
UDP-glucose	- Uridine-5'-diphosphate glucose

CONTENTS

CHAPTER-1

INTRODUCTION

Phosphorylases are glycosyl transferring enzymes, specific for the glucose part of the molecule and the break comes next to the glucose carbon (1).

The typical phosphorylase reaction is

$$A - G + P \rightleftharpoons A + G - P$$

where G represents a glycosyl group, P represents phosphate and A the other glycosyl acceptor. The specificity towards both A and G must be considered. Each phosphorylase is highly specific for one particular glycosyl group in the role of G. Thus α-Glucan phosphorylase (EC 2.4.1.1) is completely specific for α-D glycosyl transfer. In the case of maltose phosphorylase (EC 2.4.1.8) the glycosyl group undergoes an inversion during transfer, so that in one direction the enzyme is specific for α-D glycosyl and in other direction for ß-D glycosyl residues.

A (other glycosyl acceptor) is also having a fairly high degree of specificity. Thus, for the starch phosphorylase of potato, A must be a polysaccharide chain of at least four glucose units, although very slight activity can be detected with three (2, 3, 4). The glycosyl group is transferred only to the 4-position of the non-reducing terminal unit. For good activity all the links in the chain must be 1,4-ß-glucoside links; lichenin (containing 1,4-ß-glucoside links) and dextran containing 1,6-ß-glucoside links) are not acted upon (4) and glycogen is acted on much more slowly and less completely than by the animal enzyme (5). Branched dextrins derived from amylopectin and containing one or two 1,6-ß-glucoside links instead of 1,4-ß-glucoside links, may be actually inhibitory (3). Dextrins of four or more units

containing a phosphate group on the 6-position of the second or third glucose residue can act, but the presence of this phosphate group reduces the activity to one fifth (6).

The muscle glycogen phosphorylase differs significantly from the potato enzyme in that it does not act with the small straight chain dextrins and works best with larger branched molecules. In the phosphorolysis it acts much more rapidly on amylopectin or glycogen than on amylose, and in the reverse direction it can act with the limit dextrin produced from amylopectin or glycogen by the phosphorolysis, though not with the limit dextrin produced by ß-amylase, which contains four fewer glucose residues in the main chain (7, 8). The specificity is some what influenced by the size of the substrate molecule, the liver enzyme working best with smaller glycogen molecules than the muscle enzyme (9).

Since glucose-1-P is a stronger acid than inorganic phosphate, the equilibrium constant is highly pH dependent. At pH 6.8, the Pi/glucose-1-P ratio is 3.6 (10). However, the enzyme functions in the direction of glycogen degradation *in vivo* because the ratio of Pi/glucose-1-P greatly exceeds the equilibrium constant determined *in vitro* (11, 12).

Glycogen phosphorylase assay can be done by following either the forward or the backward reaction. In the direction of glycogen degradation, the enzyme is assayed using a coupled enzyme assay system by which the liberated glucose-1-P is estimated (13). The activity is measured in the direction of glycogen synthesis using the substrate glucose-1-P and glycogen (14) and estimating the liberated inorganic phosphate colorimetrically. The convenient colorimetric method of Fiske and SubbaRow (15) for inorganic phosphate has been widely used.

Phosphorylase is the key enzyme in the mobilization of chemical energy from glycogen. It is a complex allosteric protein that is subject to activation and

inhibition by chemical stimuli, including those of two other enzymes : phosphorylase kinase (ATP : phosphorylase phosphotransferase, EC 2.7.1.38) which activates it by phosphorylation of one specific pair of Ser-14 residues and phsophorylase phosphatase (phosphorylase phospho-hydrolase, EC 3.1.3.17) which inhibits it by hydrolysis of the serine phosphate bonds. In muscle, phosphorylase kinase is activated normally by the same release of calcium ions from the sarcoplasmic reticulum that also stimulates contraction. When activated, phosphorylase catalyses the stepwise phosphorolysis of glycogen with release of glucose-1-phosphate. Under *in vivo* condition it is a dimer of two identical subunits, each containing a single polypeptide chain of 842 amino acid residues to which a pyridoxal phosphate is attached by a schiff base at lysine-680 (16).

Phosphorylase *b* is the unphosphorylated form. The molecular weight of the rabbit muscle phosphorylase monomer calculated from the amino acid sequence is 97,412 (17). This includes the N-terminal acetyl group and phosphoryl group at Ser-14. The subunit relationship of the phosphorylated *a* form of the enzyme depends on conditions like enzyme concentration, pH, temperature, ionic concentration etc. (18). At low enzyme concentrations, the rabbit phosphorylase *a* exist as a dimer (13). The phosphorylase *a* from lobster, (19, 20), crab (21) and sepia (22) exist exclusively as a dimer. The dimeric and tetrameric forms of phosphorylase *a* have been found in tissues of a number of other species like man, shark, rat and frog (18). In the mussel, *Mytilus edulis* it is shown that monomeric and dimeric forms of phosphorylase *b* exist in equilibrium (23). The molecular weight in all these cases has been found to be in the range of 90,000 to 100,000 for the monomer.

Phosphorylase *b* is inactive under *in vivo* condition, but can be activated *in vitro*, weakly by inosine monophosphate (IMP) and strongly by adenosine monophosphate (AMP), when it reaches 80 per cent of the activity of the phosphorylated

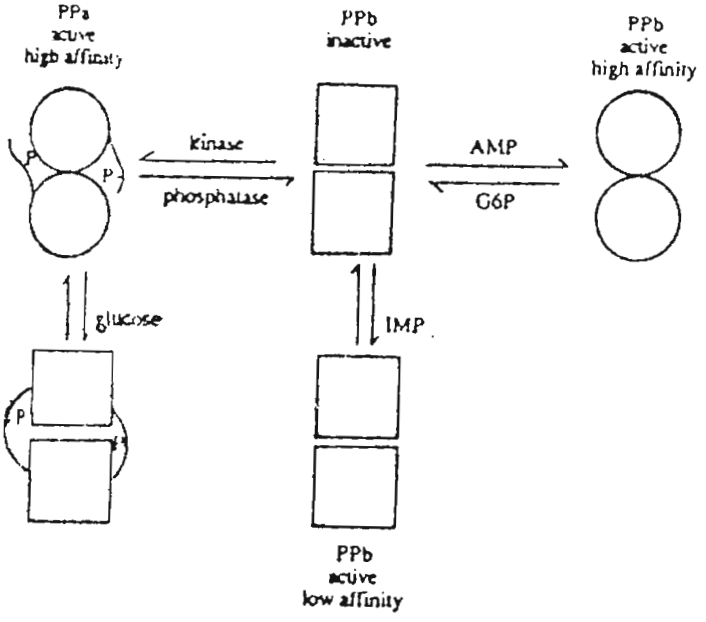


Fig.1-1

Regulation of phosphorylase a and b by effectors

form. The phosphorylated form, known as phosphorylase *a* exhibits nearly maximal activity without AMP. Each of the two forms is subject to regulation by effectors as given in Fig.1-1.

Phosphorylase *b* shows an absolute requirement of AMP for activity (24). In the absence of AMP the activity of rabbit muscle phosphorylase *b* is less than 1% of that in the presence of AMP. Phosphorylase *a*, on the other hand, is active without AMP although it is about 20-40% activated by its presence depending on the concentration of the substrate (25).

$$\text{The ratio of activity} \quad \frac{-\text{AMP}}{+\text{AMP}}$$

for phosphorlyase *a* can be as high as 80%.

The properties and interaction of phosphorylase, phosphatase and kinase have been studied in a glycogen complex isolated from rabbit muscle in which all the enzymes and glycogen are held together. Interconversion of phosphorylase *a* and *b* has been demonstrated in this complex (26). Addition of ATP, calcium and magnesium to a solution containing this complex has been shown to be accompanied by conversion of phosphorylase *b* to *a* which when all ATP is used up is reconverted to the *b* form (26, 27).

A complex of muscle phosphorylase *a* and alanine amino transferase has been purified, and it has been shown that metabolites which affect the activity of phosphorylase, such as AMP, glucose, glucose-1-phosphate and glycogen, also affect the activity of the transaminase in this complex (28).

Glycogen metabolism by mammalian tissues involves at least four enzymatically catalysed steps as given in Fig.1-2 (29). The initiating influence in the

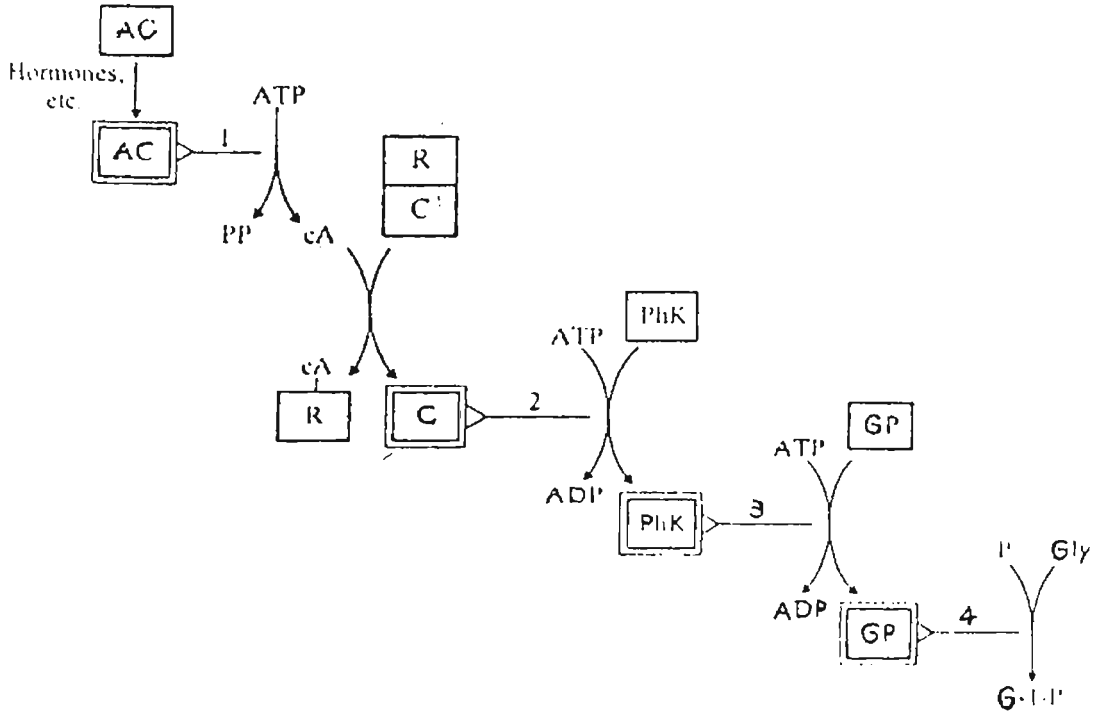


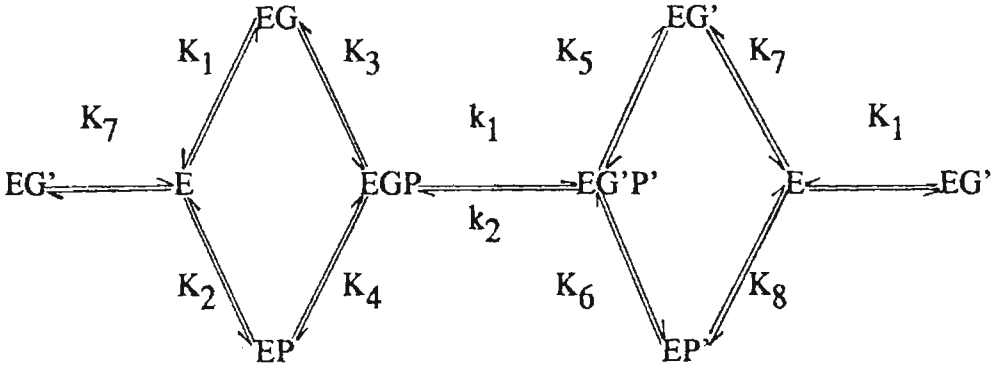
Fig. 1-2

Steps in the regulation of mammalian glycogen phosphorylase

AC is adenylate cyclase (EC 4.6.1.1): R and C are the regulatory and catalytic subunits of protein kinase (EC 2.7.1.37): PhK is phosphorylase kinase (EC 2.7.1.38). GP is glycogen phosphorylase (EC 2.4.1.1). Other abbreviations are: cA = cyclic AMP; Gly = glycogen; G-1-P = glucose-1-phosphate. The enzyme-catalysed steps are numbered 1-4.

case is the activation of the membrane-bound adenylate cyclase (AC), caused, depending on the tissue concerned, by the binding of a hormone, to a specific receptor located in the same membrane. The cyclic AMP generated interacts with an inactive protein kinase, stimulates the dissociation of an inhibitory regulatory subunit (R) from it, leaving an active catalytic unit (C) which is able to phosphorylate a number of different substrate proteins. One of these is phosphorylase kinase (PhK) which, being activated by the phosphorylation, is able to promote a further phosphorylation, that of the enzyme glycogen phosphorylase (GP). By this sequence of events, glucagon and adrenaline are able to stimulate the rate of glucose phosphate production. The cascade containing four enzyme-catalysed steps, permits a substantial amplification of the signal, so that a very small number of hormone molecules can cause a large mobilization of sugar. The molar ratio of three of the enzymes - protein kinase, phosphorylase kinase and phosphorylase, in muscle, being about 1 : 20 : 20 respectively, is in accord with the amplification concept (1).

The kinetic mechanism of phosphorylase from rabbit muscle (30, 31) and that from some other species (32, 33) has been shown to be rapid equilibrium random BiBi:



where E is the enzyme; P - orthophosphate, P' - glucose-1-P, G and G' - glycogen with n and n-1 glucose residues. K_1, K_2 etc. are the equilibrium constants and k_1 and

k_2 are the rate constants for the forward and backward reactions respectively.

The kinetic equation for this mechanism is

$$\frac{Eo}{v} = \frac{1}{k_1} + \frac{K_4}{k_1(G)} + \frac{K_3\,(1 + K_1/K_7)}{k_1(P)} + \frac{K_1\,K_3}{k_1(G)(P)}$$

where Eo = total enzyme concentration and v = initial velocity

The above kinetic mechanism has been confirmed by isotope exchange studies (34). The mechanism is unaltered during allosteric transitions, when sigmoidal substrate saturation curves are obtained. The catalytic function of phosphorylase and many of its responses to its regulators are cooperative (7, 18, 35-38).

The model predicts that homotropic and heterotropic cooperativities are interlinked functions. With phosphorylase *b* separation of these functions has been demonstrated. Several modifications to the original model have been suggested by various authors. Rubin and Changeux (39) have developed the concept of nonexclusive binding as a possibility. Some workers have tried to incorporate additional conformational states other than R and T but complementing them (40-42). Bresler and Firsow (43) preferred additional assumptions taking into account the possibility of non-exclusive binding of ligands. Wang and Tu (42) have noted that their results fit in more satisfactorily with the model of Koshland *et al.* (44). Mention may be made of the work of Will *et al.* (45) with hog muscle phosphorylase *b* for which they developed another equation to explain their results. Soman and Philip have explained their results with a model of 'right and wrong' binding of ligands on the enzyme (46).

Detailed structural studies have been carried out for the enzyme from rabbit muscle. No sulphide bridges have been found in phosphorylase. The subunits of the rabbit muscle enzyme are similar but not identifical (47). Electron microscopic measurement has shown that rabbit muscle phosphorylase *b* consists of 2 ellipsoidal units bound together with measurements of 110 : 65 : 55 A° (48). X-ray crystallographic analysis showed the dimensions as 115 : 75 : 60 A° (49, 50). The symmetrical association of the dimers gives the tetramer a square shape (51). Tubular shaped crystals have been obtained for phosphorylase *a* in the presence of protamine (52).

The complete amino acid sequence of the 841 amino acids of the rabbit muscle phosphorylase has been reported by Titani *et al.* (53). The composition derived from the sequence is as follows: Leu 79, Glu 64, Arg 63, Ala 63, Val 62, Asp 51, Ileu 49, Lys 48, Asn 45, Phe 38, Tyr 36, Thr 35, Gln 31, Ser 29, His 22, Met 21, Try 12 and CySH 9. The data shows that at neutral pH, the positively and negatively charged amino acids are well balanced. However, such a neutralization is not equally distributed. For eg. the N-terminal end has been shown to be composed of essentially basic amino acids (50, 54).

Comparison of the 15-19 amino acids of the N-terminal sequences of *E. coli* and potato phosphorylases with those from five vertebrate sources and yeast (55) showed that (i) the first amino acid is a hydroxy amino acid (threonine in potato phosphorylase and serine in all other cases), (ii) the non-regulated plant phosphorylases have a free α-amino group in contrast to all other phosphorylases studied so far which have a blocked α-amino group. The *E. coli* phosphorylase showed identity with animal phosphorylases only in position 1, 3 and 16. Nakano *et al.* (56, 57) have shown that the sequence of the potato and rabbit enzyme are very similar except for the remarkable dissimilarity seen at the N-terminal residues. These

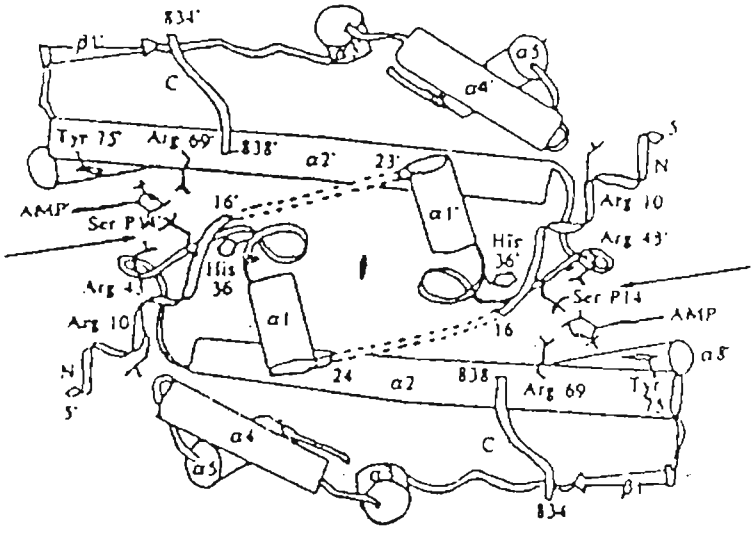
phosphorylases are similar in some of the structural and kinetic properties but their control mechanisms are different. The sequence near the PLP site in rabbit phosphorylase has been shown to be homologous to that from yeast (58), potato (57, 59) and *E. coli* (55, 60). The peptides containing SH groups obtained from the rabbit and potato phosphorylases also have highly homologous series (56). From these studies Nakano *et al.* (1980) pointed out that phosphorylase existed originally as a large catalytically active molecule and by gradual mutation a regulatory mechanism was formed within the molecule during the course of evolution.

Phosphorylase has a very complex structure. Each of its two subunits consists of two domains made up of a core of pleated-ß-sheets flanked by α-helices. The N-terminal domain includes the subunit boundary, the serine phosphate, the activating AMP and inhibiting glucose-6-phosphate (G6P) binding site, the glycogen storage site and a small part of the catalytic site. The C-terminal domain complements the catalytic site and also contains the neighbouring site where the inhibitory nucleosides and purines bind. The catalytic site lies at the head of a 12-15 A° long tunnel.

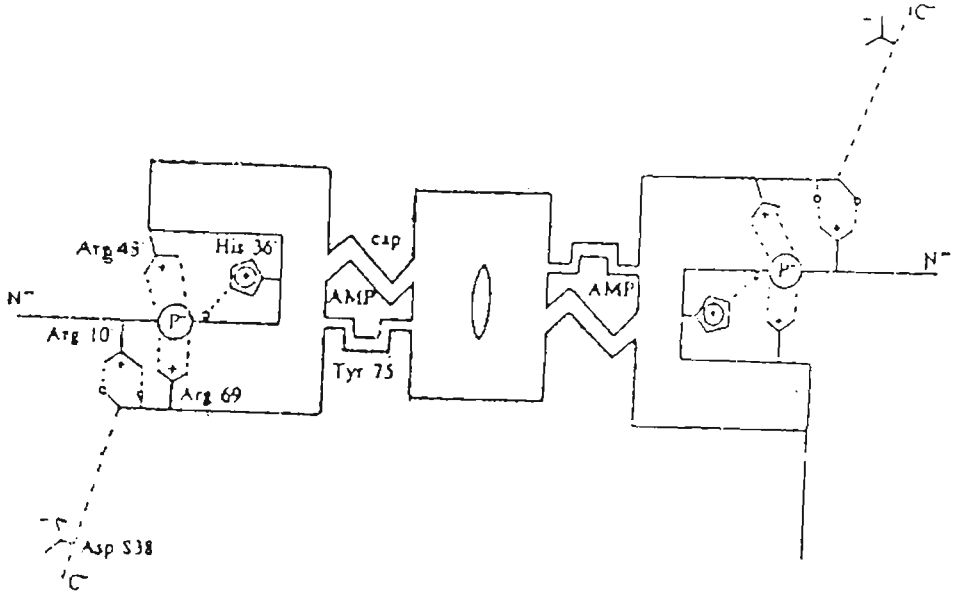
In the dimer the two subunits are joined end-to-end at a contact that is tencious for so large a protein, making up no more than 7% of its surface area in phosphorylase *b* and 10% in *a*. As in haemoglobin, the two fold symmetry axis passes through a waterfilled channel, but in phosphorylase this channel is flanked by two large grottoes, capable of holding about 150 water molecules. Unlike, haemoglobin, the channel contains no binding site for effectors. A view of the dimer perpendicular to the symmetry axis shows that its two sides are very differently constructed. One side is convex with a radius of curvature matching that of the glycogen particle (175 A°). It contains the entrance to the catalytic tunnel and the glycogen storage site, identified by its binding of maltoheptose, a 7-residue oligomer

of α-D-glucose. The side that faces away from the glycogen particle contains the regulatory, phosphorylation sites and the overlapping AMP and G6P binding sites. Most of the binding sites for substrates and effectors are widely separated. A distance of 30 A° separates the catalytic site from the one that binds maltoheptose (the glycogen storage site), which suggests that the enzyme chews away at a piece of polysaccharide chain that is far removed from the piece that attaches the glycogen particle to the enzyme. The closest distance, 15 A°, links the serine phosphate to the AMP binding site, both are over 30 A° from the nearest catalytic site, and the two catalytic sites are over 60 A° from each other. yet binding of ligands to any of these sites can be shown to affect all the others.

As a first step, X-ray analysis has revealed the changes that phosphorylation of serine 14 induces at the subunit boundary in going from the weekly activated *b* to the inhibited *a* structure. The most important of these changes counts in the burial and ordering of the amino-terminal 16 residues and the exposure and disorder of the carboxy-terminal 5 residues in phosphorylase *a*, and the reversal of these features in phosphorylase *b* as shown in Fig.1-3. These movements are accompanied by changes in hydrogen bonding. In *b* Asp 838 is tied down by a salt bridge to His 36 of the opposite subunit. On transition to *a* that salt bridge is broken and the histidine rotates about the α-ß bond to form a hydrogen bond with the carbonyl oxygen of phosphoserine 14 of the same subunit. The phosphate also forms salt bridge with two arginines, one from the same and the other from the opposite subunit. In the absence of neutralising serine phosphate, the arginines contribute to a cluster of positive charges that expel the positively charged N-terminal peptide from its binding site that spans to two protein subunits. Thus the dominant interactions responsible for the allosteric transition are electrostatic. Other changes in salt bridges and non-polar contacts between subunits follow in train, and those are transmitted to the



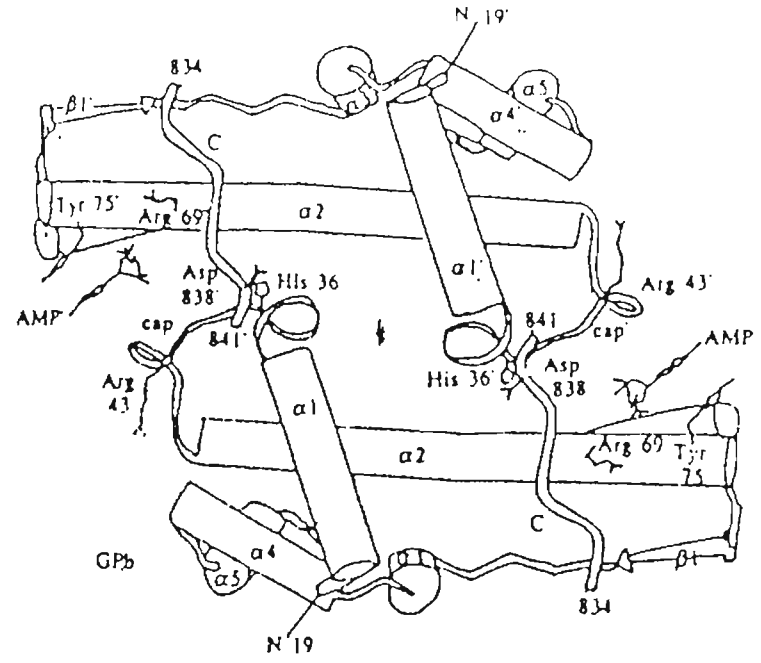
A



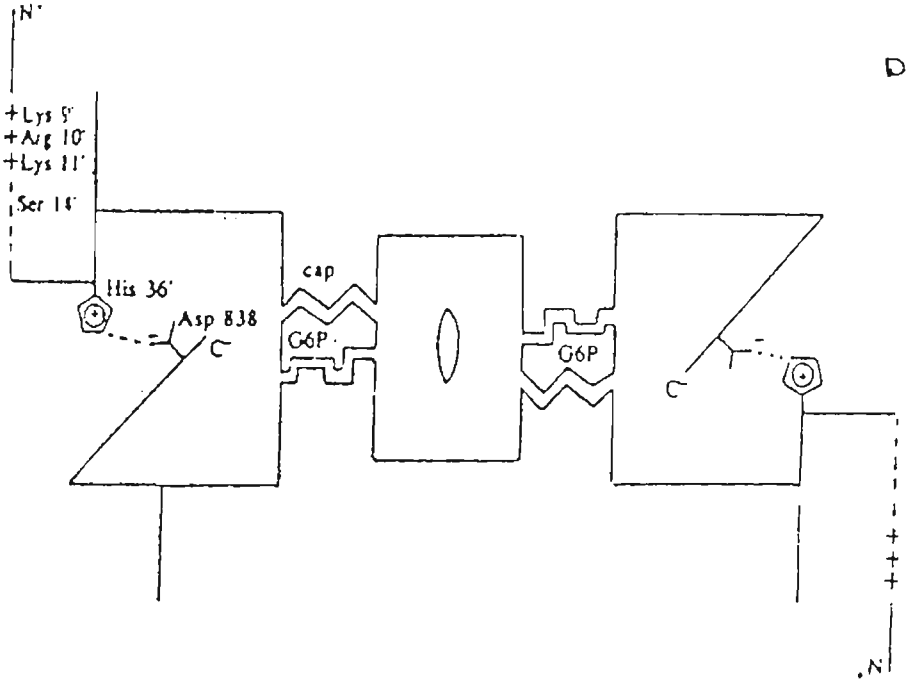
B

Fig.1-3 A
Phosphorylase a showing the N-terminal helices, marked N, with the serine phosphates 14, marked by arrows, coordinated to two arginines, one from each subunit and AMP bound firmly between helix α8 and the cap. The C-terminal peptide, marked C, is disordered.

Fig.1-3 B
Same view in diagramatic form



C



D

Fig.1-3 C
Phosphorylase b. The N-terminal residues are disordered and the C-terminal ones are ordered, with Asp 838 hydrogen bonded to His 36 of the opposite subunit. AMP is bound more losely.

Fig.1-3 D
Diagramatic view, with G6P bound at the effecter site. The plus sign stands for Lys 9, Arg 10 and Lys 11

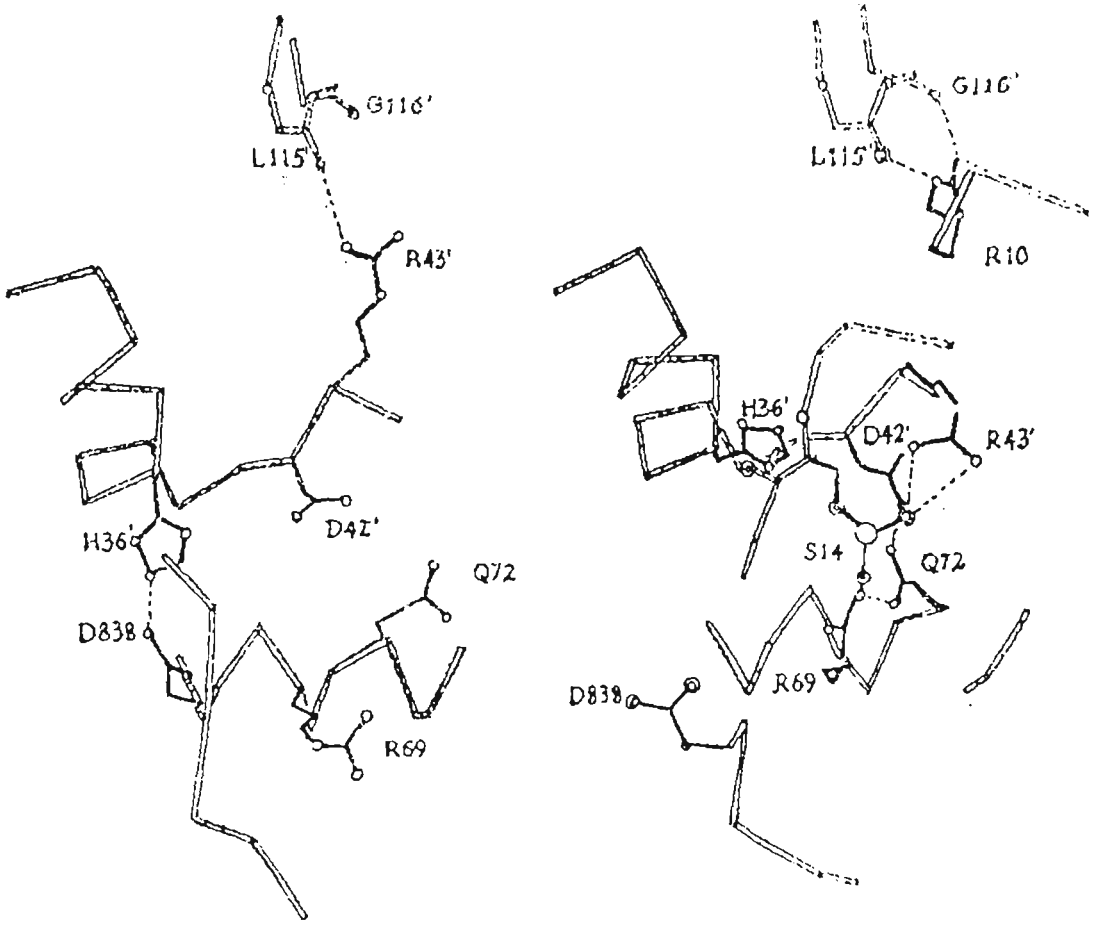


Fig.1-3 E

Enlarged views of subunit contacts in phosphorylase b (left) and a (right), showing details of some of these interactions.

AMP and G6P binding sites that lie at the subunit boundary only 15 A° from phosphoserine 14. AMP is wedged between an α-helix from one subunit and a non-helical loop from the opposite subunit, referred to as the cap; that loop is separated from phosphoserine 14 by a short helix. Comparison of the two structures shows how dephosphorylation of serine 14 and transition to phosphorylase *b* weakens both electrostatic and Van der Waals interactions with activator AMP, thus increasing its dissociation constant from the enzyme 100-fold (61, 62). The same changes strengthen binding of the inhibitor G6P. All these changes take place in the T-structure.

D.Barford and L.N.Johnson have solved the longsought structure of the active R-form of phosphorylase. It emerged from an X-ray analysis of crystals of phosphorylase *b* grown from ammonium sulfate solution; the sulfate appears to have worked as an activator in place of phosphate at the active site and at the phosphorylation site at serine 14. In these crystals the enzyme is tetrameric, a form of phosphorylase *b* also found in solution, and the glycogen storage site is buried in a subunit contact. *In vivo* attachment of the enzyme to glycogen particles causes it to dissociate into dimers.

The allosteric transition consists of rotations of each of the two monomers by 5° about axes pointing in opposite directions normal to the molecular dyad, as shown in Fig. 1-4. The transition affects the helix α 7. They interdigitate and form a bridge between the catalytic sites of neighbouring subunits; Barford and Johnson call them tower helices, because each helix protrudes from its own subunits and penetrates deeply into the neighbouring one (Fig.1-5). The angle between the two helices changes from +20° in the T structure to -80° in the R structure. Each catalytic site is flanked by six loops of chain; some from the N-terminal and some from the C-terminal domain. One of these loops links helix α 7 to α 8; it carries aspartate 283,

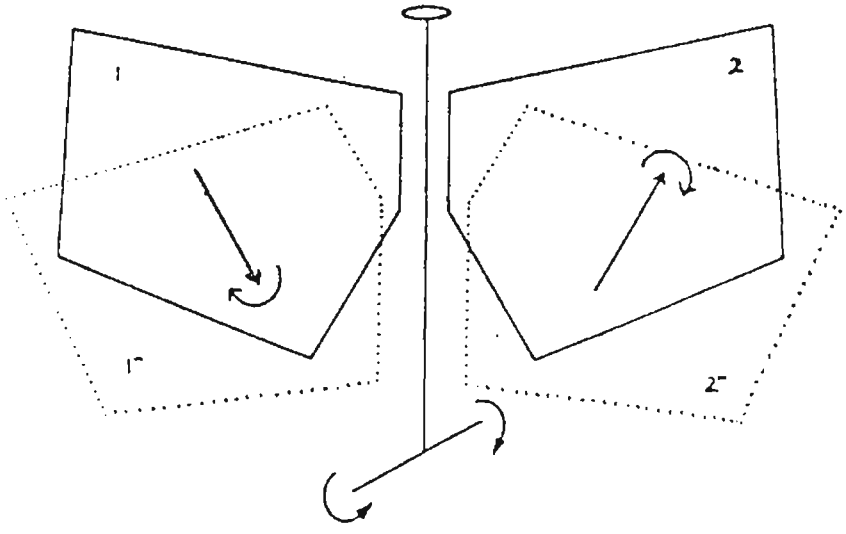


Fig.1-4

Possible changes of quaternary structure in a dimer of two identical subunits related by an axis of two fold symmetry, also called a dyad (symbol on top) (point group C2 or 2). In the simplest transition the left subunit turns anticlockwise about an axis normal to the dyad and pointing towards the observer; the right subunit turns anticlockwise about a colinear axis pointing away from the observer. More generally, the subunits can turn about any pair of axes related by the molecular dyad, for example the ones shown as bold arrows. They point into the picture, away from the observer.

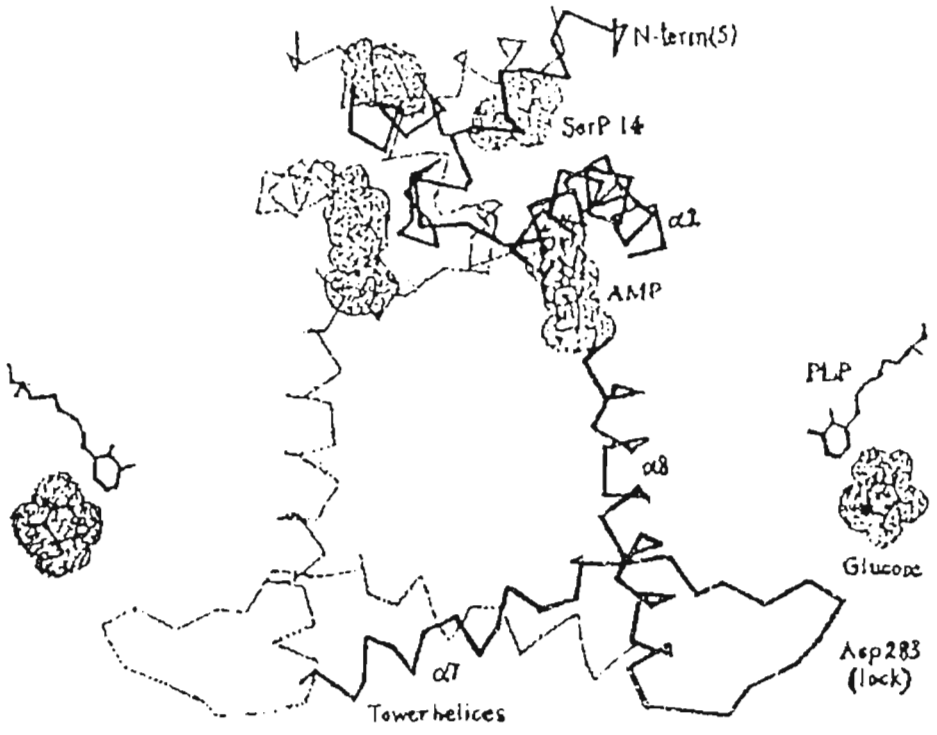


Fig.1-5

Helices linking active and regulator sites of phosphorylase in the T-structure seen perpendicular to the molecular dyad

asparagine 284 and phenyl alanine 285 as its tip, which lock access to the catalytic site when the enzyme is inhibited. Helix α 8 reaches from the catalytic site to the AMP binding site of the same subunit. At its C-terminus Arg 309 and 310 form hydrogen bonds with the phosphate of AMP. Finally helices α 2 link each of the AMP binding sites to the phosphoserine sites on the opposite subunit. Heterotropic ones may be transmitted to the regulatory sites by the tower helices and by changes at the subunit contacts of the kind shown in Fig. 1-3.

Goldsmith *et al.* (1989) have solved the structure of a crystal of phosphorylase *a* soaked in a solution of orthophosphate and maltopentaose. Phosphate ions were bound at the glycogen storage rather than the catalytic site. The binding of these molecules induced marked changes in the enzyme structure. At the active site the phosphate ion unlocked the 'gate', displaced Asp 283 and formed hydrogen bonds with imino groups at the end of the helix containing residues 133-149. The binding of the phosphates and of the oligosaccharide caused the C-terminal catalytic domain to turn by 1° and shift by 0.5 A° away from the N-terminal regulatory domain, the tower helices moved closer together. Their movement was much smaller than, and different from, the one seen by Barford and Johnson in the R-structure of phosphorylase *b*. Goldsmith *et al.* suggests that their structure may be an intermediate between the inactive T and the fully active R-structure.

X-ray studies showed how the substrate phosphate, the reaction product G1P and the inhibitory cyclic glucose 1,2-biphosphate bind to phosphorylase *b* and *a*. The most telling clues were obtained from synchrotron radiation studies of crystals of phosphorylase *b* activated by AMP and soaked in solutions of the sugar heptenitol and inorganic phosphate. The very intense X-ray beam from the synchrotron storage ring allowed the investigators to take 'Snap shots' of the diffraction pattern at successive stages of the reaction, and to analyse the changes that accompa-

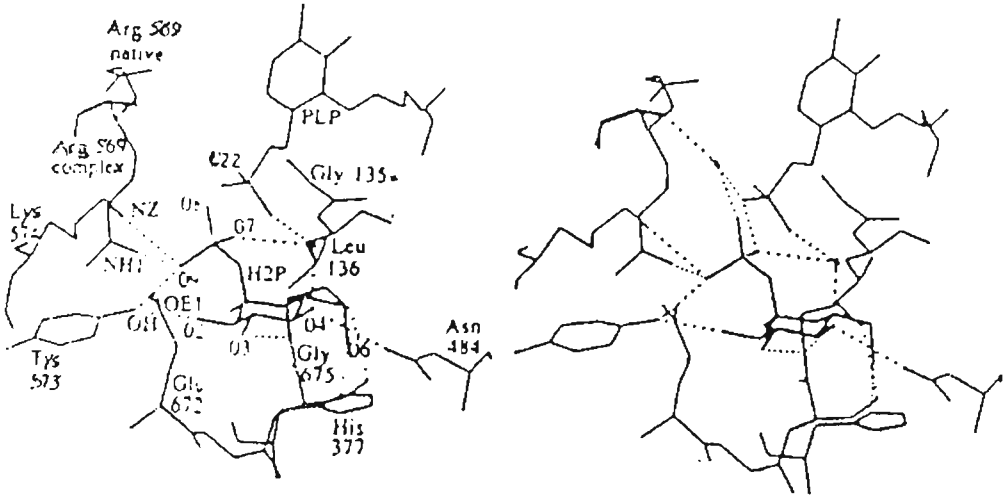


Fig.1-6

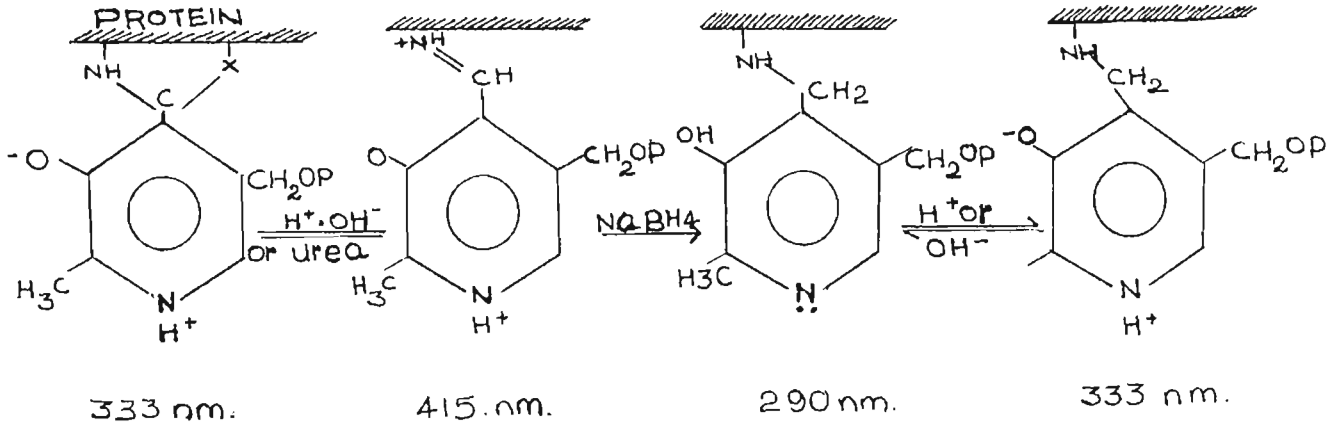
Active site showing pyridoxal phosphate (PLP)
and heptulose-2-phosphate (H_2P)
hydrogen bonded to the enzyme (63)

nied the gradual accumulation of the product heptulose-2-phosphate that remains bound in the active site as shown in Fig.1-6 (63). The conformations of the protein and coenzyme seen in this figure are similar to those found more recently in the R-structure, where the substrate phosphate is replaced by a sulphate ion and the sugar is absent.

Phosphorylases have been found to contain one molecule of pyridoxal-5'-phosphate (PLP) bound to each subunit of the enzyme. Sucrose phosphorylase however doesnot contain PLP. This prosthetic group has been shown to be covalently linked to Lys 679 and buried inside a hydrophobic region in rabbit muscle phosphorylase (64, 65). The PLP can be resolved by deformation of the enzyme and trapping it with a reagent like L-cysteine (66). The loss of PLP is accompanied by loss of activity and a tendency to dissociate to monomers at room temperature (67). The quaternary structure of the apoenzyme is different from that of the holoenzyme (68). The apophosphorylase can be reconstituted by the addition of PLP and this process has been shown to be highly temperature dependent. Unlike in other PLP containing enzymes like transaminases, $NaBH_4$ reduction of the coenzyme doesnot abolish the catalytic activity of phosphorylase (69). This finding initiated a search of the functional group in PLP that may be participating in catalysis. For this, the apophosphorylase was reconstituted with a number of PLP analogues which differ from PLP at any one of the six positions in the aromatic ring. From these studies it was concluded that except for the pyridine nitrogen and phosphate, all other groups are apparently not participating in the catalytic process (18). Out of a number of 5'-phosphate analogues tested only very closely similar compounds like pyridoxal-5'-methylene phosphate (69) restored catalytic activity. This compound has a pK 7.2 compared to 6.2 for PLP and the reconstituted enzyme showed an alkaline shift in the pH optimum. This supports the earlier assumptions that the phosphate has some

role in catalysis. The finding by Graves and his colleagues (70) that phosphite can activate pyridoxal reconstituted enzyme while pyrophosphate was a competitive inhibitor to both phosphite and glucose-1-P, shows the involvement of the phosphate moiety and its participation in catalysis. The effect of pH on enzymic activity (71) and the earlier study on the dependance of pH on the fluorescence quantum yield of PLP-monomethyl ester (72) shows that the phosphate group (with pK 6.2) may participate in general acid base catalysis.

Since the pyridoxal phosphate has an absorbance maximum at 335 nm, the study of the spectral properties under different conditions with simultaneous activity measurements can yield the structure-function relationship of the coenzyme. Eventhough the PLP site has been located in the sequence and in X-ray picture, the spectral characteristics predict a more complex environment in which the coenzyme is bound. On reduction of the coenzyme with $NaBH_4$ the 335 nm bond has been found to shift towards 290 nm (74). When the pH is shifted to the acid or alkaline side, the band again reappears. Since the absorption due to a schiff base at 415 nm, of PLP is only very less in phosphorylase and since the $NaBH_4$ reduction is highly retarded at neutral pH, it was suggested that the natural form of the enzyme is zwitterionic addition product of some nucleophilic group on the protein with the Schiff base (75). These structure can be represented as follows:



Shimomura and Fuki (76) have shown that the different spectrum of apo and holo enzyme shows, in addition to the 335 nm band, a band at 251 nm, which they have assigned to the bound PLP. By studying the factors contributing to the absorption of PLP. Veinberg *et al.* (77) have shown that the N-atom of the aromatic ring does not have to be invoked in explaining the spectral properties.

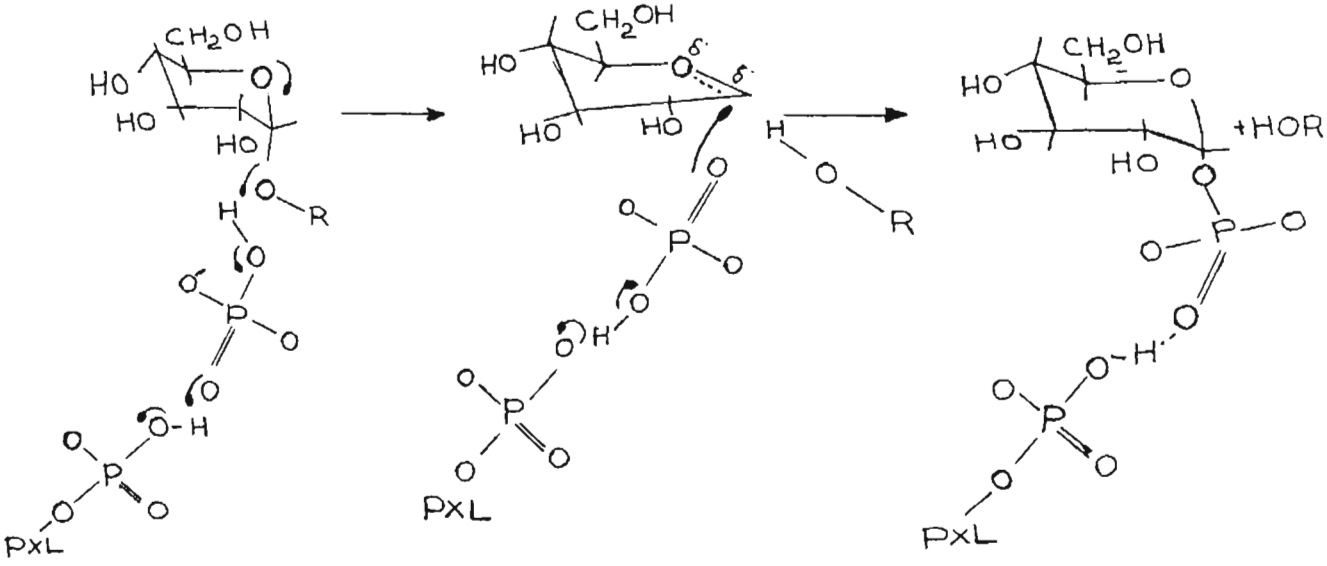
Spectral studies on *Sepia pharaonis* phosphorylase in our laboratory show that the hydrophobic character of PLP site is increased in the presence of cysteine and PLP can exist as the fully protonated form (22).

Pyridoxal phosphate has been shown to be present in phosphorylases of distinct species where the control mechanisms are different. The analysis of amino acid sequence of the PLP site in potato, yeast, *E. coli* and rabbit muscle phosphorylases shows that they are highly homologous (57, 58, 59). The activity of the potato phosphorylase has also been shown to be highly dependent on PLP (78). As Nakano *et al.* (59) have pointed out, the very strong conservation of the coenzyme binding site over approximately 1.5 million years is a good support for a catalytic role of PLP. Conservation of protein structure by the expensive way of maintaining a coenzyme, seems to be an impossibility.

In phosphorylase it is not the pyridoxal moiety but the phosphate moiety of pyridoxal-phosphate that activates the substrate (16). It assumes the monoanionic form in the inactive T-state and the dianionic form in the active R-state (79). The binding site for the substrate inorganic phosphate lies next to the pyridoxal phosphate, and both are hydrogen bonded to basic groups of the protein. The binding of heptulose-7-phosphate causes arginine 569 to move from a buried position to

another that is close to the coenzyme and product phosphates, displacing aspartate 283, the 'lock', in the process, thus substituting a buried negative, repulsive charge by a positive one that attracts the phosphates. The movements of Lysine 574 and arginine 569 are also seen in the R-structure and may stabilize the extra ionization of the pyridoxal phosphate that is essential for catalysis. Glutamate 672 has been shown to be essential as a proton acceptor.

Chemical and structural data suggest that catalysis *in vivo* may involve the steps shown below:



First a proton may be transferred from the coenzyme phosphate to the substrate phosphate. The latter may then act as a general acid, protonating the α-(1-4) glycosidic bond that links the terminal glucose to the glycogen chain. Cleavage of that bond would lead to the formation of an oxocarbonium ion on the free sugar which would be stabilized by the newly formed orthophosphate dianion. Finally, the orthophosphate may transfer its proton to the coenzyme phosphate and simultaneously mount a nucleophilic attach on the oxocarbonium ion, forming glucose-1-phosphate. In this mechanism the coenzyme phosphate plays the dual part of a

general base catalyst (80).

Phosphorylase is a more complex allosteric system than either haemoglobin or phosphofructokinase, because both *a* and *b* can take up at least two alternative quanternary structures. Sprang and Fletterick suggest that some of the crystal structures analysed by X-rays may be chimeric in the sense that part of each subunit approaches the tertiary R-structure and another part the tertiary T-structure. This may apply to the heptulose-2-phosphate complex of *b*, where the catalytic site has the active and the subunit boundary the inactive form, or to the glucose complex of *a*, where the catalytic site has the inactive structure, while the subunit boundary has a structure close to fully active R.

Phosphorylase *b* from rabbit muscle was the first enzyme found to have allosteric property. At lower concentration the saturation curve for AMP is sigmoidal. In the presence of metabolites like G6P and ATP sigmoidicity increases. Though the substrate saturation curve for G1P is hyperbolic it is very much sensitive to the presence of the above metabolites. Thus the intracellular concentrations of glucose, glucose-6-P, AMP and ATP influence the catalytic activity of the enzyme.

Phosphorylase *b* is activated by AMP and inhibited by ATP and glucose-6-phosphate. A number of other nucleotides and sugar derivatives have been found to influence the activity of the enzyme, but since they are not present in tissues they are useful only for structural elucidation and mechanistic studies of the enzyme.

The observations from rabbit phosphorylase *b* (81) and other sources (20) show sigmoidal activation curve for AMP. Decreasing concentration of AMP increased the Km for glucose-1-phosphate or orthophosphate. Based on these observations Wyman and Changeux (82) advanced the model for allosteric transitions; the kinetic study with phosphorylase *b* received special attention mainly to analyse

the properties of this enzyme in the light of the proposed model. However some observations which did not fit in with the prediction of the model of Monod *et al.* (82) have been mentioned as satisfactorily explained by the model of Koshland, Nemathy and Fitmer (44).

Phosphorylase *b* from different sources have been analysed in the light of the model. Here also, general agreement has been recorded. The allosteric constant for rabbit muscle phosphorylase *b* was found by Buc and Bu to be 600 (83). For lobster enzyme this constant is 1200 (20). The value of the constant suggests that phosphorylase *b* exists mainly in the T state.

Hill's equation, orginally proposed for oxygen binding on haemoglobin can be used for finding the strength of cooperativity of sites (84). The Hill coefficient may be taken as a measure of the strength of cooperativity of sites. Thus in the presence of an inhibitor the *n* value will increase and approach the number of binding sites. In phosphorylase *b* the number of binding sites for the substrate and for the activator is two.

Extensive work has been done in rabbit muscle phosphorylase *b* to evaluate the strength of cooperativity of ligand sites under a variety of conditions. The following observations have been found to satisfactorily fit in with the prediction of the model: the cooperative binding of AMP (81, 85, 86) and the increase in *n* value which approaches 2 (81). Under all these conditions the saturation curves for the substrate and AMP are sigmoidal showing clearly that the Michaelis-Menten law is not obeyed. Increasing concentration of AMP or glycogen have been found to decrease the Km for glucose-1-phosphate and *vice versa*, suggesting heterotropic cooperativity of sites (81). So also, the presence of inhibitors decrease the affinity of the enzyme for substrate and activators. According to the model (82), the inhibitors

bind at sites other than the substrate or activator site. Since the reciprocal plots for substrate and activator are non-linear it can not be stated from such plots whether the different ligands bind on the same or different sites.

In addition to the metabolic inhibitors, G6P, ATP and glucose, phosphorylase has been shown to be inhibited by aromatic compounds (87). The inhibition is dependent on the hydrophobicity of the aromatic compounds. The aromatic compounds have been shown to bind on a large hydrophoic region on the enzyme (88). P-nitrophenyl phosphate has been shown to be a competitive inhibitor of AMP for phosphorylase *b* and is assumed to bind on the same locus where AMP binds (89).

Chemical modification studies of enzyme system reveals the protein functional group. It can also be used as a tool to study allosteric transition. Glycogen phosphorylase has been subjected to chemical modification studies using a number of reagents p-Mercury benzoate, completely dissociated the enzyme into monomers by modifications of the 'SH' groups (90). Using iodoacetamide two out of the 9 SH groups per enzyme monomer were modified without loss of enzyme activity and these groups were shown to be surface exposed (91, 92). Some of the other 'SH' groups reacted very slowly with loss of activity and others were inaccessible to the reagent. Similar results have been obtained when reagents like DTNB (41), NEM (93) and FDNB (94) were employed. Lysl groups have been shown to be essential for the maintenance of enzymic activity and structure. 4-5 amino groups could be modified resulting in totally inactive enzyme (95). Soman and Philip (96) have prepared a desensitised FDNB derivative of phosphorylase in which 1 cysteinyl and 1 lysl residues were modified. Dinitrophenylation of 1 amino group has also been shown to result in the loss of enzyme activity (97). Glyoxal (98), acetyl imidazole (99), glutaraldehyde and aliphatic aldehyde (100) have also been shown to modify

the enzyme with loss of activity. Modification studies using N acetyl imidazole has shown that 2 tyrosyl residues are essential for maintaining enzyme activity (101). Nitration also has been shown to modify 2 tyrosyl residues out of 36 per monomer, with loss of activity (102). Modification of phosphorylase *b* with potassium ferrate resulted in identifying tyrosine 75 as essential for catalysis (103).

Studies on the protection of inactivation by AMP have suggested the nucleotide binding site near this residue. The presence of an essential tyrosine was also shown by reaction of phosphorylase *b* with potassium-nitrodisulfonate salt (Fremy's radical) (104). Phosphorylase *b* was rapidly inactivated by 5-diazo 1 H-tetrazole or by 1-cyclohexyl-3-(2-morpholinoethyl) carbodimide metho-P-toluene sulfonate with complete inactivation (105). The loss of activity has been attributed to carboxyl group modification. Histidine residues modified by diethyl pyrocarbonate (106) also resulted in enzyme inactivation. The presence of histidine residues at the substrate binding region is suggested by this study. 2,3-Butane-dione has been employed to study the significance of arginine in catalysis (107). Two types of essential arginine residues, one in the allosteric site and the other in the active site, have been modified.

In fact, phosphorylase *a*, a naturally occurring desensitized form has entirely different allosteric properties from that of the *b* form. Homotropic cooperativities of AMP sites are only observed in presence of inhibitors like glucose (108, 109). A glutaraldehyde modified enzyme which is devoid of all homotropic cooperativity but retaining all heterotropic interactions has been prepared (100). Phosphorylase *b* modified with FDNB in the presence of AMP and orthophosphate has been shown to produce a desensitized enzyme derivative, the analysis of which has shown that lysyl and cysteinyl residues were modified (96). Phorphorylase *b*, the subunit of which was cross linked by tetroyl bis (glycylazide) has been found to be desensitized

with respect to the AMP binding sites (110) showing that the effect of cross link is to restrict subunit interaction and allosteric transitions of the enzyme.

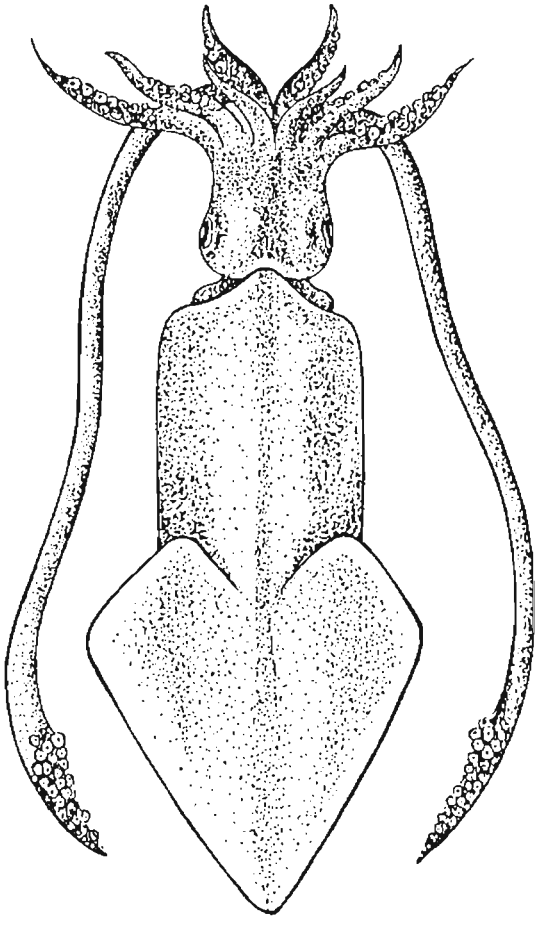
Phosphorylase *b* does not show any activity in the absence of AMP, even at increased concentration of glucose-1-phosphate or orthophosphate. This is one of the major differences from many other allosteric enzymes. If the substrate and activator can bind to the R state, at higher concentration of substrate the enzyme should show considerable activity in the absence of the activator. Yet the presence of glucose-1-phosphate in moderate concentrations has been shown to protect glucose-1-P sites against chemical modification, suggesting that it does bind on its site (95).

The Hill coefficient for AMP sites increases from about 1.4 in the absence of any inhibitor to 1.6 in the presence of ATP (81, 111). But at still higher concentrations of ATP there was no further increase in the n value (81, 111). According to the model the limit is $n = 2$.

Studies on muscle tissues of all vertebrate terrestrial and aquatic animals revealed a similar pattern of control (20, 20, 112-122). The major control device in all these cases is through the interconversion between active and inactive forms of the phosphorylase via phosphorylation or dephosphorylation. Two or more forms of phosphorylase have been reported in blood platelets, rat chloroma, swine kidney (123-125) in the mollusc, crab (21), *Pectan maximus* (126), Brewers yeast, banana leaves, spinach leaves and pea leaves (127, 128, 129) and in a number of other plant tissues (130). Structural and functional difference from the well established rabbit muscle phosphorylase was observed in many of these species. A high concentration of salt is required for activity of liver phosphorylase (131, 132). The human leukocyte *b* form is 25% active in the absence of AMP (133). An additional form of enzyme (phosphorylase c) has been reported in *Pectan maximus* (126). A purified

dimer form of the phosphorylase was not activated by AMP in *Dictyostelium discoideum* (134). A phosphorylase with high specific activity has been isolated from *Neurospora crassa* which can not be considered as *a* or *b* form (135). Monomer of phosphorylase *b* is present in *Mytilus edulis*, which is activated by AMP (23). Though both phosphorylase kinase and phosphatase are present in sepia mantle (22), they donot have any significant role in the control of phosphorylase activity.

The effect of ligands is more significant in phosphorylase *b* than phosphorylase *a* (18). Phosphorylase from all terrestrial and aquatic vertebrates are inhibited by G6P, ATP and glucose and activated by AMP (88, 136, 137). Inhibition observed in the presence of these ligands can be considered as allosteric or competitive (partially or completely competitive). Positive or negative cooperativity is also observed in the activation process. The above inhibitors show allosteric inhibition in phosphorylase *b* from rabbit, rat, man and other vertebrate terrestial animals (18). No such definite pattern of allosteric inhibition exists for the phosphorylase from marine vertebrates and invertebrates (22, 88, 136, 137). Moreover these ligands are competitive for the enzyme from marine vertebrates and invertebrates. This is very evident in the AMP kinetics in the presence of inhibitors. AMP exhibits positive cooperativity in rabbit phosphorylase (18). Phosphorylase *a* from the mantle tissue of sepia (22, 137) shows negative cooperativity. Control mechanism of phosphorylase in sepia mantle is totally different (20, 137). *Loligo vulgaris* was selected for a comparative study of glycogen phosphorylases. This invertebrate is an important cephalopod in nerve physiological research.



4 c·m

Loligo vulgaris

Systematic position of this species is as follows (138).

Superphylum	Invertebrata
Phylum	Mollusca
Class	Cephalopoda
Subclass	Coleoidea
Order	Decapoda
Suborder	Tenthoidea
Family	Loliginidae
Type	Loligo

The genus Loligo has a world wide distribution in the warmer seas. The *Loligo vulgaris* otherwise called squids, are the most active of the cephalopods occurring in coastal water, in deeper water and in the abysses. The squid has a tapering body, hence the nick name 'sea arrow'.

The mantle forms the thick, muscular and protective envelope, enclosing the visceral hump and the mantle cavity. The conical projections of the mantle, one on each side of the animal forms the fins. Ventrally, the free mantle edge forms a loose collar around the neck region, thus leaving a circular opening, through which water enters the mantle. A conical muscular tube projecting beyond the collar, beneath the head, is the funnel, through which the water of mantle cavity is expelled out. The mantle and funnel form the chief locomotory organs. The customary mode of the locomotion is slow swimming by the undulating movements of the fins, during which the arms are closely extended in front to serve for steering. But, when the animal is excited, the mantle collar closes tightly around the neck and the water is forcibly ejected through the siphon, so that the animal is propelled in the opposite direction like a rocket by the principle of jet propulsion. The rocket-like tapering body enables it to dart through water with lightning like speed.

The squids are invertebrates and low in evolutionary status from vertebrates.

Through evolution, the vertebrates have acquired the mechanisms of regulation of glycogen degradation within the phosphorylase by gradual mutation, as evidenced by the amino acid sequence analysis in a number of distinct animal species and plants (55-57). It appears that the higher demand on glycogen for energy in loligo mantle is met by maintaining a high concentration of AMP rather than evolving a structurally more active form of phosphorylase.

The above facts reveal that the nature of phosphorylase is linked with the energy requirement and/or evolutionary status of the animals. A systematic comparison of properties of phosphorylases from marine sources is presented in this thesis.

CHAPTER-2

PURIFICATION OF PHOSPHORYLASE FROM THE MANTLE MUSCLE OF *LOLIGO VULGARIS*

The procedure for purification of glycogen phosphorylase from rabbit muscle (14) was found to be not applicable in purifying the enzyme from the mantle muscle of *Loligo vulgaris*.

Usually 100 g of the frozen mantle was used in each batch for purification of the enzyme. Extraction was done at 0-5°C. Distilled water extraction was adopted for *Sepia pharaonis* in our laboratory (22). For Loligo extraction with 10 mM sodium-ß-glycerophosphate at pH 7 was preferred because with distilled water, the specific activity was comparatively low.

A second extraction of muscle yielded about 20 per cent of the activity of the first extract. Here also the specific activity was very low, only the first extract was taken for further purification. Acid treatment of the extract was reducing the activity to 60 per cent without any difference in the specific activity. So this step was abandoned.

Initially ammonium sulfate fraction of the extract was tried at different concentrations of the salt from 20 per cent to 65 per cent. The specific activity was maximum for the residue obtained between 35 per cent and 55 per cent saturation of ammonium sulfate. So the precipitate obtained between 35 per cent and 55 per cent salt solution was used for further purification. The purity obtained by this step was on the average 2 to 3 fold with 80-85 per cent yield of the enzyme.

DEAE-cellulose chromatography was used after dialysis of the residue. The results of typical DEAE- cellulose chromatography of the ammonium sulfate fraction is shown in Fig.2-1. The elution was done at 5-8°C. A gradient using 1 M NaCl in the top reservoir with 150 ml buffer in the mixing flask gave good separation of proteins. The active fractions were pooled, the proteins precipitated with enzyme grade ammonium sulfate (65% saturation) and dissolved in minimum volume of buffer.

The total purification obtained in this step was 13 to 14 fold with a total recovery of 41-42 per cent activity of the original extract.

Further purification of the DEAE-cellulose purified enzyme was tried with hydroxy apetite gel and carboxy methyl cellulose columns. Isoelectric focussing was also tried. However, the eluted enzyme showed no increase in specific activity. Therefore Agaroseglycogen hydrophobic chromatography was tried and it was found to be successful.

The dialysed enzyme was applied to agarose glycogen column (24 x 1.5 cm) equilibrated with 1 mM Sodium-ß-glycerophosphate buffer pH 7. 3.5 ml enzyme solution was used each time. The flow rate was adjusted to 3 ml/min and 1 ml fractions were collected. A gradient of 10 mM Sodium-ß-glycerophosphate was applied as second stage operation. During the second stage operation phosphorylase enzyme was eluted. Thereafter NaCl gradient was applied to test the presence of phosphorylase enzyme. Enzyme activity was not showed in the fractions of NaCl gradient.

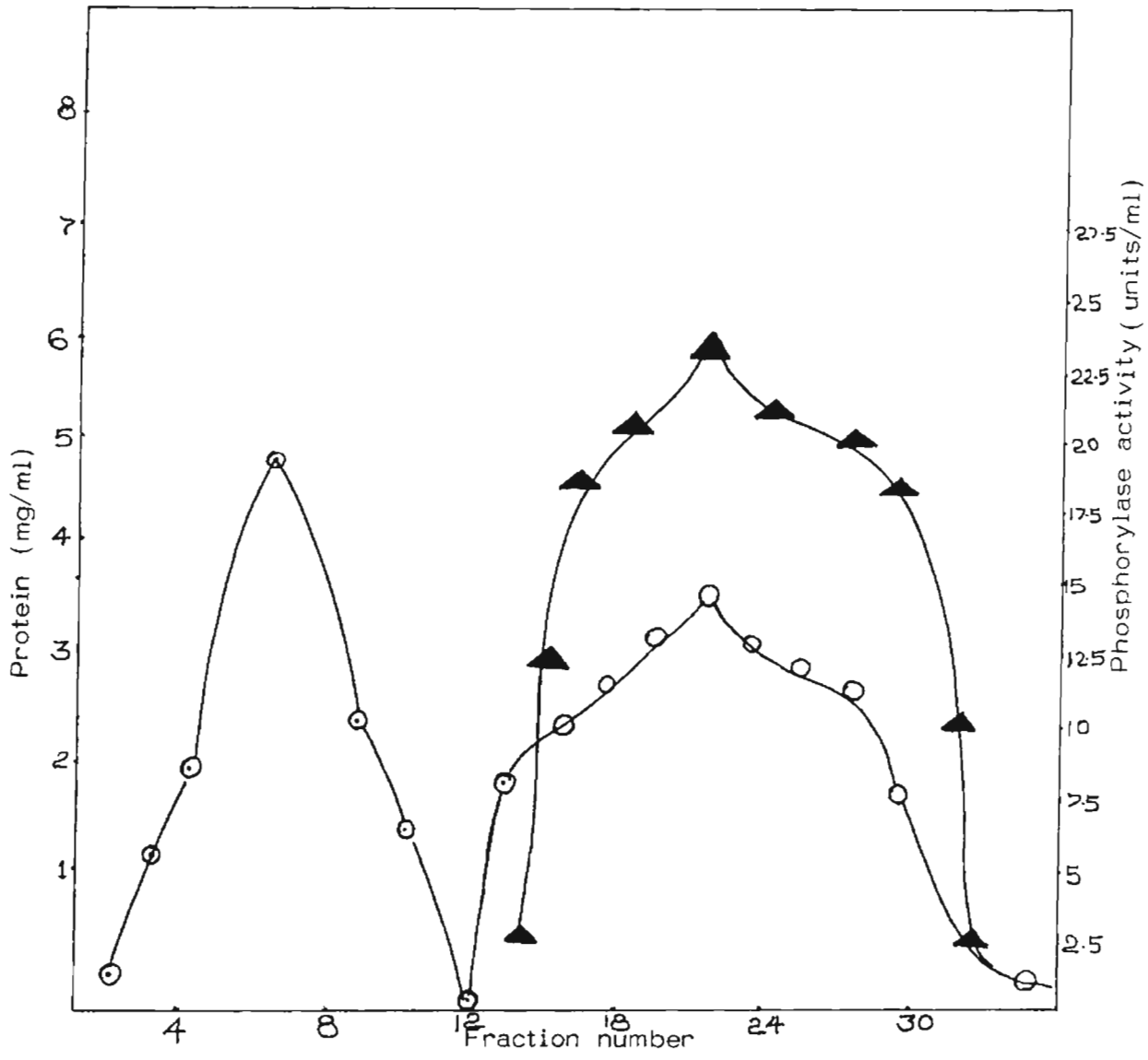

Protein (mg/ml)
Phosphorylase activity (units/ml)
Fraction number
1
2
3
4
5
6
7
8
2·5
5
7·5
10
12·5
15
17·5
20
22·5
25
27·5
4
8
12
18
24
30

Fig.2-1

Fig.2-1

DEAE - Cellulose crhomatography of protein obtained by ammonium sulfate fractionation. The experimental details are given in Chapter 14. 0 protein concentration (mg/l); ▲, activity (μ moles of orthophosphate liberated/minute/ml of fraction). The total protein applied in this typical experiment was 200 mg. The total protein and activity yield was 80-85 per cent. In this experiment fraction from 14 to 30 were taken for further purification.

The active fractions (fractions from 45 to 60) were pooled, the proteins precipitated with enzyme grade ammonium sulfate (65% saturation) and dissolved in minimum quantity of 25 mM Sodium-ß-glycerophosphate buffer, pH 7.

Removal of ammonium sulfate and other small particles was carried out by gel filtration in Sephadex G-25 column. Typical results obtained on gel filtration through Sephadex G-25 are shown in Fig.2-3. The purity obtained in this step was about 30-34 fold of the original extract with 27-28 per cent yield of the total activity.

The results of a typical purification are given in Table 2-1. About 1.5 to 2 mg enzyme was obtained from 100 g of frozen muscle. The specific activity of the purified enzyme was 36-37 units/mg protein. The presence of a phosphorylase form having a higher requirement of AMP for maximum activity has been reported in extracts of certain marine organisms (126 and 140). Such a form of phosphorylase was not observed in Loligo at any stage of purification, just as in sepia (22). A form of phosphorylase (phosphorylase *c*) was separated from Lobster tail muscle on DEAE-column (20). Phosphorylase of Loligo from DEAE-cellulose and agarose-glycogen column observed an activity ratio of 0.28 ± 0.02.

The active fractions without ammonium sulfate were pooled and stored in presence of 5 mM mercaptoethanol. The activity ratio of enzyme fractions from DEAE-cellulose column and Agarose-glycogen column were varied to a limited extent from 0.28 to 0.6 for different batches of samples. There was only one form of enzyme, phosphorylase *a* in the purified enzyme, as evidenced by the activity band after polyacrylamide gel electrophoresis.

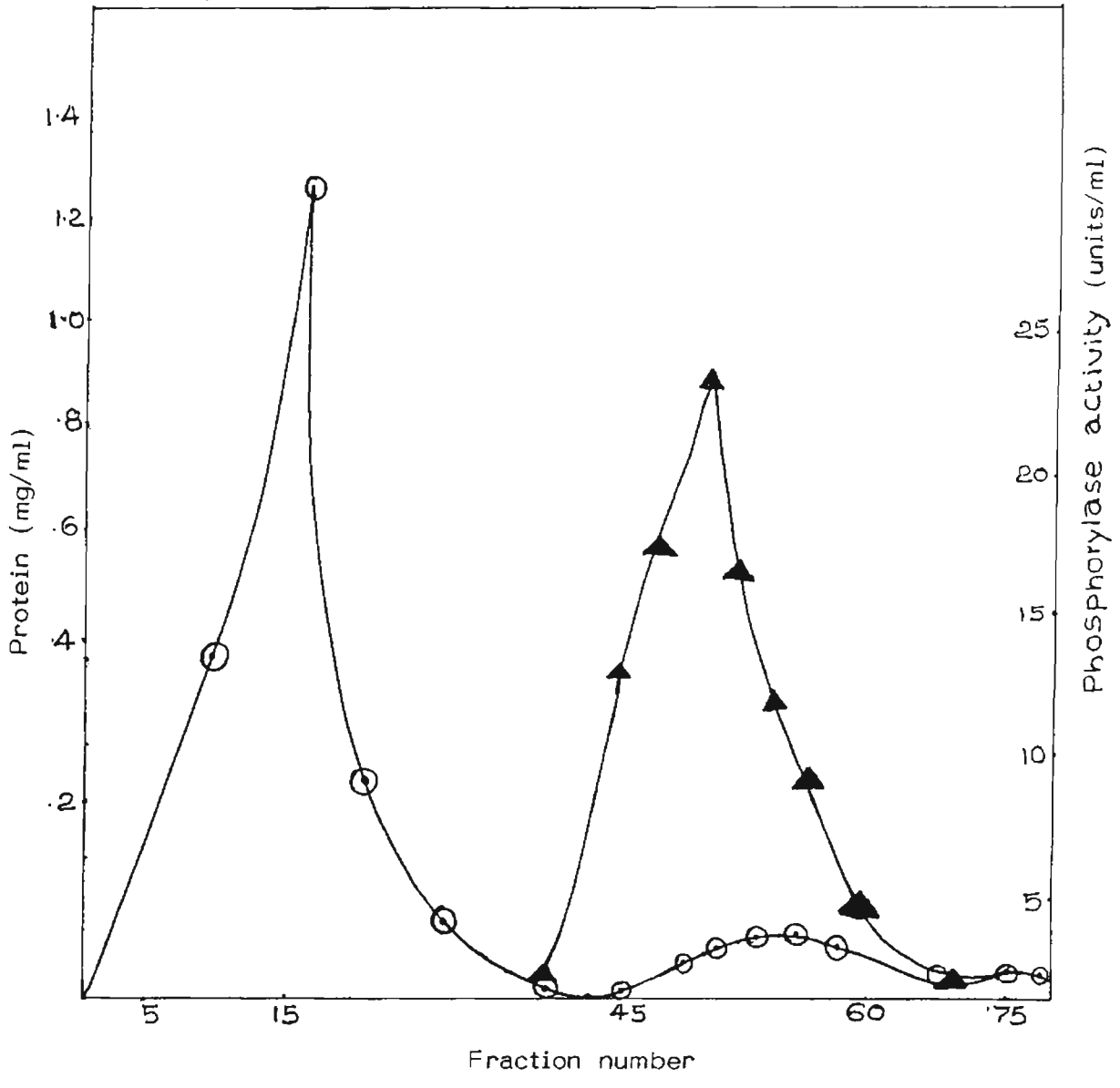

Protein (mg/ml)
1·4
1·2
1·0
·8
·6
·4
·2
Phosphorylase activity (units/ml)
25
20
15
10
5
5
15
45
60
75
Fraction number

Fig.2-2

Fig.2-2

Affinity chromatography of the DEAE-cellulose purified *Loligo vulgaris* protein on Agarose-glycogen. Details are as described in 'experimental'. In this experiment, 3.5 ml (9 mg/ml) of protein solution was applied. One ml fraction were collected at a flow rate of 3 ml/min. LKB gradient applicator, peristaltic pump, U.V.monitor, recorder and fraction collector were employed.

0. Protein concentration (mg/ml of fraction)

▲. activity as μ moles of orthophosphate liberated/min/ml of fractions

Table 2-1. Purification of glycogen phosphorylase *a* from the mantle muscle of *Loligo vulgaris*

	Total volume ml	Activity *units/ml	Protein mg/ml	Specific activity units/mg	Yield %	Purification
Extract	120	7	8	0.875	100	1
35 to 55% Ammonium sulfate fraction	12	60	25	2.4	85.71	2.74
DEAE-cellulose chromatography followed by ammonium sulfate precipitation and dialysis	3.5	100	9	12.22	41.67	13.97
Affinity chromatography followed by Ammonium sulfate precipitation and Sephadex G-25 filtration	2.5	47	1.3	36.15	14.00	41.37

* One unit of phosphorylase activity is defined as that amount of protein which will liberate one micromole of inorganic phosphate/min at 30° C, when measured in the direction of glycogen synthesis under assay conditions.

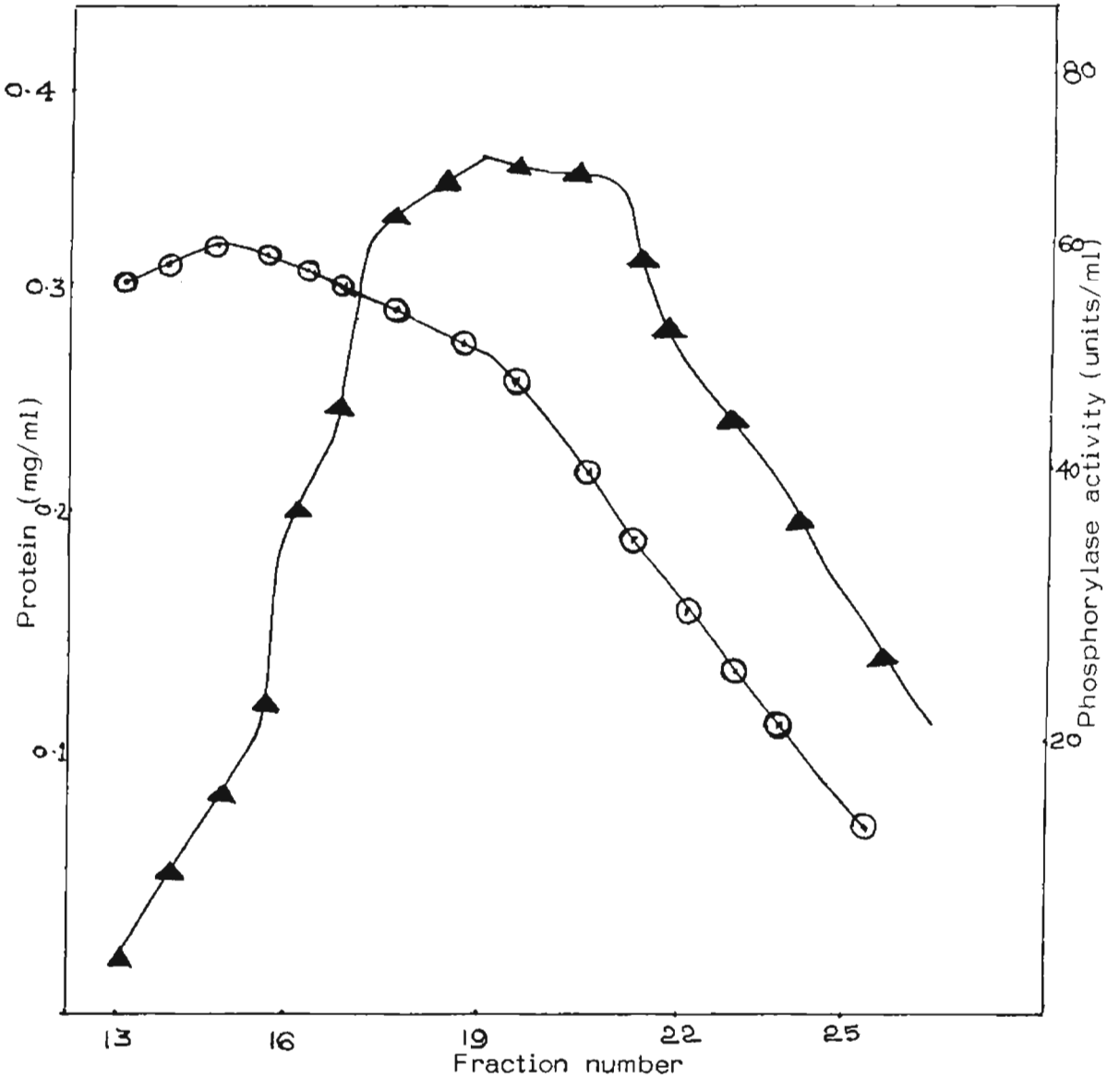

0·4
0·3
0·2
0·1
Protein (mg/ml)
80
60
40
20
Phosphorylase activity (units/ml)
13
16
19
22
25
Fraction number

Fig.2-3

Fig.2-3

Gel filtration of Loligo phosphorylase *a* on Sephadex G-25 column

Details are given in 'experimental' (Chapter 14). One ml of ammonium sulfate (65%) precipitate of Agarose-glycogen column was applied to 10 x 1 cm column. 0.5 ml fraction was collected at a flow rate of 3 ml/min LKB, peristaltic pump, U.V. monitor, recorder and fraction collector are used.

Crystallization of enzyme did not succeed even after 15 hrs at 0°C with 5 x 10^{-3}M AMP and 5 x 10^{-2}M Mg^{++}. Addition of ammonium sulfate to a 5 mg/ml preparation till the development of slight turbidity followed by dialysis at 0 to 5°C against 50 per cent saturated ammonium sulfate was also not successful in the crystallization of enzyme.

Purity of the enzyme

Polyacrylamide gel electrophoresis (141) of the enzyme preparation after purification over DEAE-cellulose column showed two major bands and one minor band of proteins. The fraction after Agarose-glycogen chromatography had only a single band as evidenced by one protein band and the corresponding activity band.

Stability of the enzyme

The purified enzyme was not stable. The activity was gradually decreasing and lost completely with in 10 days. Therefore the properties of the enzyme were studied within 3 days of preparation of each batch.

CHAPTER-3

KINETICS OF PHOSPHORYLASE *a* FROM *LOLIGO VULGARIS*

The kinetic mechanism of α-glucan phosphorylase *a* and *b* (30, 31, 142), rabbit liver phosphorylase (143) phosphorylase from a vertebrate fish *Cibium guttat-tam* (33), *E. coli* maltodextrin phospphorylase (32), phosphorylase *a* from Sepia (22), phosphorylase *a* and *b* from *Etroplus suratensis*, phosphorylase from *Metape-naeus dobsoni*, phosphorylase from foot muscle of *Sunetta scripta* and phosphorylase from the foot muscle of *Villorita cyprenoides* (140) have been shown to be rapid equilibrium random bi bi. Chao, Johnson and Graves (32) compared the data ob-tained for the *E. coli* enzyme with steady state equations for various mechanisms. Random mechanism was the best fit which was confirmed by product inhibition and isotope exchange studies (30). Inorder to compare the properties of Loligo phosphorylase with other fish enzymes and rabbit phosphorylase, its kinetics in the direction of glycogen synthesis was examined.

Glucose-1-P was used as substrate for measuring initial velocities and the liberated inorganic phosphate was estimated as discussed in 'Experimental' (Chapter 14). Reaction velocities are expressed as micromoles of inorganic phosphate/min at 30°C.

The initial rate data for the transfer of glucosyl moiety to glycogen at varying concentrations of glycogen and glucose-1-P for phosphorylase *a* from *Loligo vulgaris* are presented in Fig.3-1 and 3-2 in the form of double reciprocal plots in the presence of 1 mM AMP. The activity ratio remained at 0.2 to 0.38 at different concentrations of glucose-1-P and glycogen.

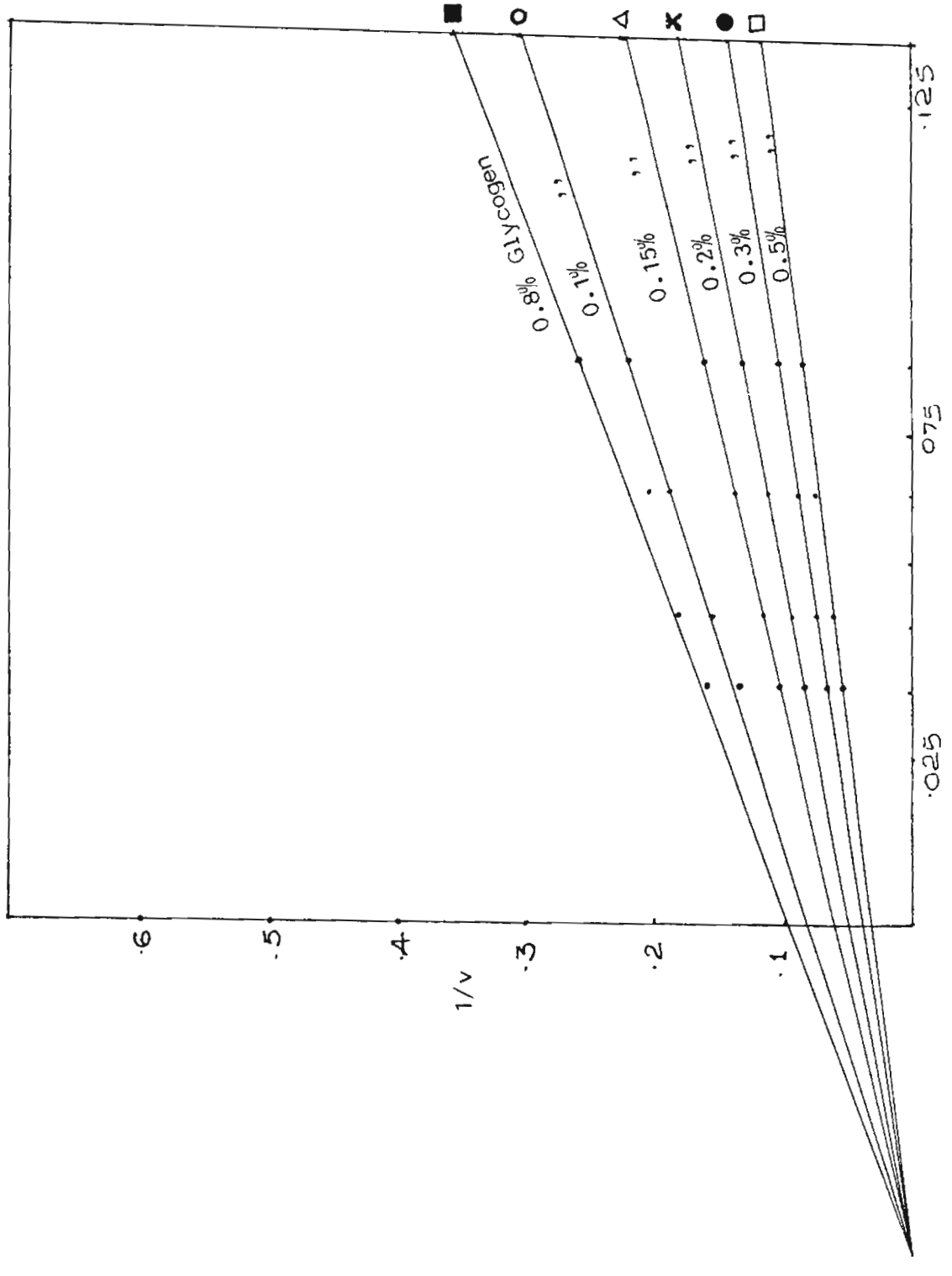


Fig.3-1

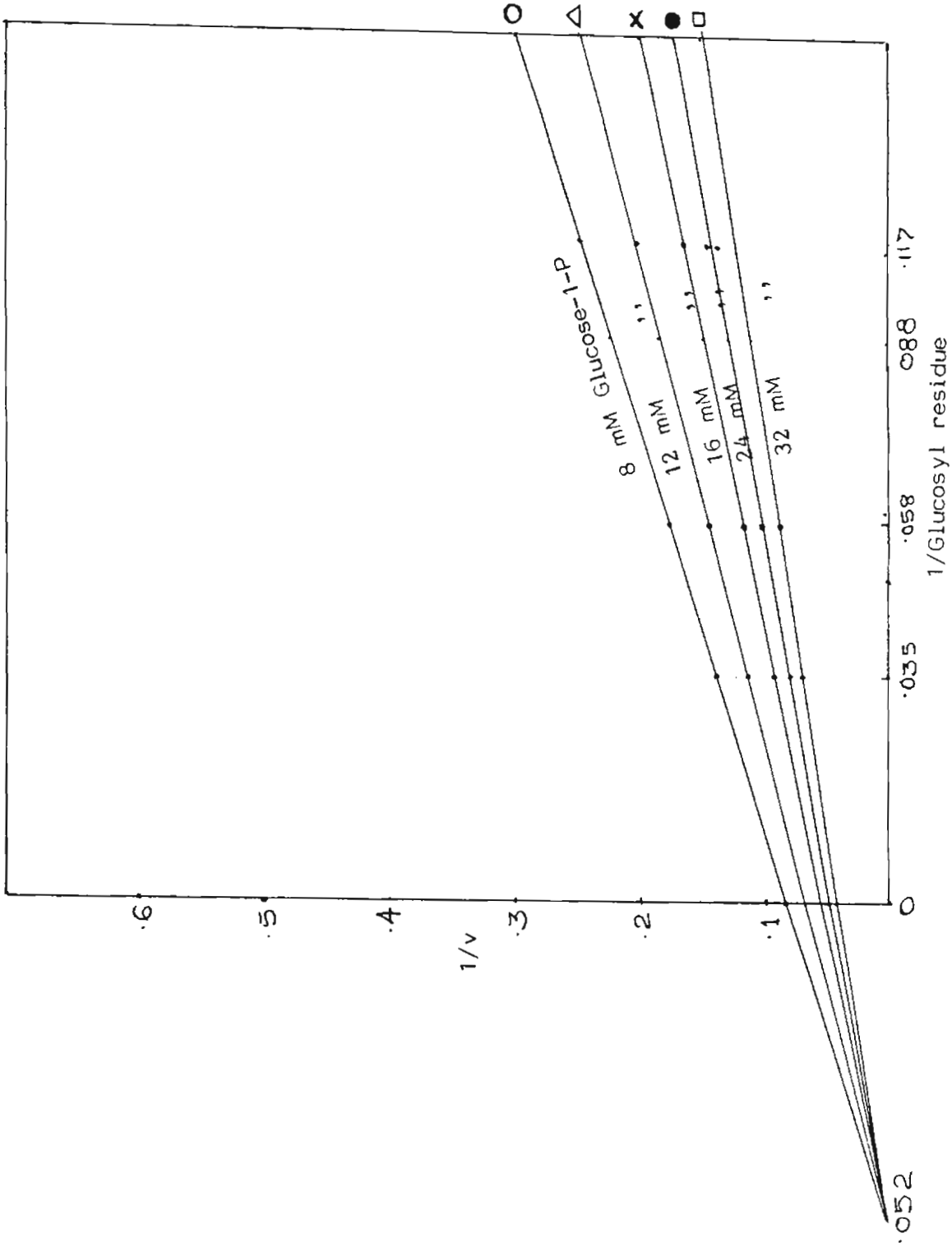

O
Δ
X
●
□
8 mM Glucose-1-P
12 mM
16 mM
24 mM
32 mM
1/v
.6
.5
.4
.3
.2
.1
0
.035
.058
.088
.117
.052
1/Glucosyl residue

Fig.3-2

The apparent Km values of glucose-1-P and glycogen calculated from the primary plots 3-1 and 3-2 are given in Table 3-1. The results illustrate the effect of glycogen and glucose-1-P on each other's affinity. Km values for glycogen remained unaltered at different concentrations of glucose-1-P but Km values for glucose-1-P decreased when glycogen concentration was increased. This suggested that binding of glycogen enhanced the affinity for glucose-1-P. The allosteric conformational changes brought about by the ligands may be different so that heterotropic interactions due to the binding of glycogen favoured binding of glucose-1-P but not vice versa. The results show inconsistency with the predictions of the model of Monod (82) where allosteric transitions are represented by T and R states and the transitions are effected through changes in the Km values only which means that the 'K' system of activation is taking place.

The reciprocal plots shows in Fig.3-1 and 3-2 are converged on the left hand side of the vertical axis showing that the kinetic mechanism of *Loligo vulgaris* phosphorylase is similar to that from other sources. Based on these results and those obtained from other sources the kinetic mechanism of *Loligo vulgaris* enzyme may be represented as follows:

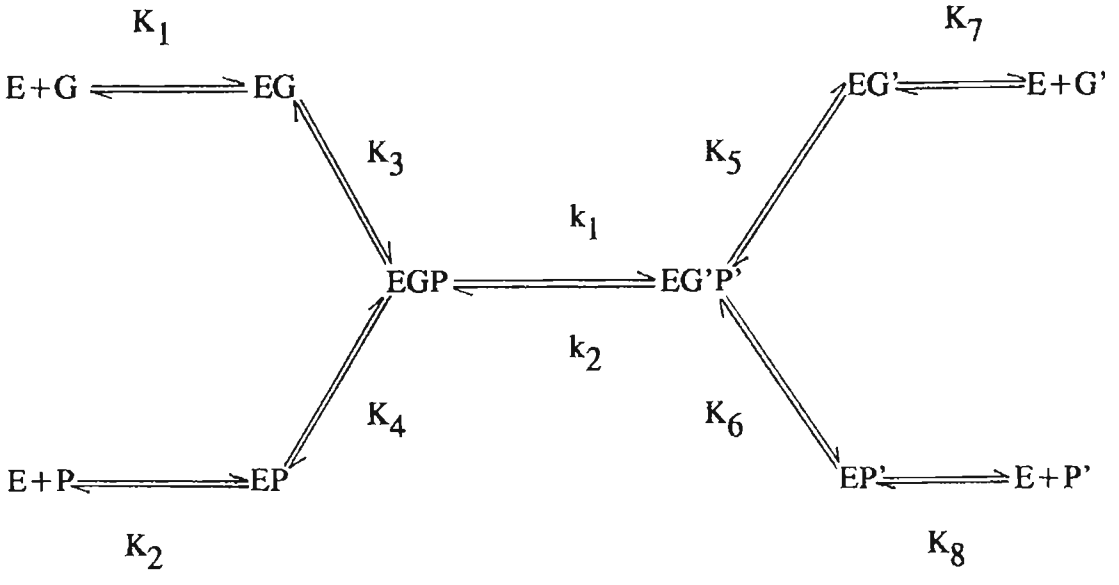


Table 3-1. Apparent Km values of Glucose-1-P and Glycogen at different concentrations of each other for Loligo phosphorylase *a* (the concentration of glycogen is expressed as mM concentration of glucosyl residue)

Glycogen (mM)	Km for Glucose-1-P	Glucose-1-P (mM)	Km for Glycogen (mM)
4.56	20.4	8	1.78
5.70	21.3	12	1.63
8.50	21.9	16	1.43
11.40	21.9	24	1.71
17.10	19.2	32	1.58
28.50	17.8		

E is the enzyme; P, orthophosphate and P', glycose-1-P. G and G' represent glycogen bound for degradation and synthesis respectively.

The velocity equation derived for the scheme differs slightly from the general equation for random *bi-bi* mechanism because one of the substrates, glycogen is both the reactant and product. Based on the above scheme, the equation for the Kinetic mechanism in the synthetic direction can be written as:

$$\frac{(Eo)}{v} = \frac{1}{k_2} + \frac{K_6}{k_2(G')} + \frac{K_5\,(1+K_7/K_1)}{k_2(P')} + \frac{K_5\,K_7}{k_2(G')(P')}$$

Eo = Total enzyme concentration, v = initial velocity

If it is assumed that the affinity of the enzyme for glycogen binding in the synthetic and degradation direction is same (ie. $K_1 = K_7$) the equation can be modified as follows:

$$\frac{(Eo)}{v} = \frac{1}{k_2} + \frac{K_6}{k_2(G')} + \frac{2K_5}{k_2(P')} + \frac{K_5K_7}{k_2(G'P')}$$

Multiplying by k_2 the equation becomes,

$$\frac{V}{v} = 1 + \frac{K_6}{(G')} + \frac{2K_5}{(P')} + \frac{K_5K_7}{(G')(P')}$$

The equation can be written in the general form of Dalziel (145) as:

$$\frac{Eo}{v} = \phi'_0 + \frac{\phi'_1}{(G')} + \frac{\phi'_2}{(P')} + \frac{\phi'_{12}}{(G')(P')}$$

ϕ'_0, ϕ'_1 etc. are representing the kinetic coefficients.

The dissociation constants K_5,K_6, K_7, K_8 and the rate constant k_2 can be calculated from Fig. 3-1 and 3-2 and from secondary plots obtained by replotting the values of the intercepts and slopes of the primary plots against reciprocal of the second substrate. The secondary plots are given in Fig.3-3 and 3-4. These four plots provided 4 x 2 equations so that the values of the dissociation constants can be checked using these different equations. This can be used for ascertaining the internal consistency of the experimental measurements. Table 3-2 shows a comparison of dissociation constants obtained by this method. The internal consistencies given in Table 3-2 also prove that the kinetic mechanism of *Loligo vulgaris* phosphorylase was consistent with that of the established phosphorylases from different sources. The average values of the kinetic constants and coefficients calculated from the primary and secondary plots are compared in Table 3-3 with that from rabbit phosphorylase *a* and *b* (30, 142), marine vertebrate fish Cibium guttattam (33), and a marine invertebrate *Sepia pharaonis* (22). The table shows that the K_5 (ie., Michaelis constant for glucose-1-P at saturating glycogen concentration) of *Loligo vulgaris* phosphorylase *a* is higher than that of Sepia and rabbit phosphorylase *a*. The dissociation constant of glucose-1-P (K_8) is much less than that in *Sepia pharaonis* and slightly higher than that in *Cibium guttattam*.

Table 3-2. The values of Kinetic constants obtained using different equations from the secondary plots

	Fig.3-3A	Fig.3-3B	Fig.3-4A	Fig.3-4B
Vmax (μ moles/min/mg)	33.33	-	33.33	-
K_5 (mM)	-	8.33	7.98	-
K_6 (mM)	1.15	-	-	1.467
K_7 (mM)	-	3.43	-	3.6

Table 3-3. Comparison of Kinetic constants and coefficients of phosphorylase from marine sources with Rabbit muscle phosphorylase

	Loligo vulgaris phosphory-lase	*Sepia pharanois* phosphory-lase	*Cibium guttattom* phosphorylase	Rabbit muscle phosphorylase *a*	*b*
ϕ'_3 (mg x min/μ mole)	0.03	0.0185	0.0163	0.0145	0.0155
ϕ'_1 (min x mg)	39.3	14.6	36	7.25	14
ϕ'_2 (min x mg)	49.2	266.7	120	2.9×10^{-3}	51
ϕ'_{12} (mole x min x mg)	0.87	0.6	0.53	$3 \times 12 \times 10^{-3}$	0.104
Kinetic constants					
K_3 (μ moles/mg/min)	33.33	54	61.4	69	64
K_5 (mM Glucose-1-P)	8.2	7.2	3.6	0.1	1.5
K_6 (mM glycogen)	1.31	0.79	2.8	0.5	0.9
K_7 (mM glycogen)	3.51	4.5	9.0	2.2	4.4
K_8 (mM Glucose-1-P)	20	50	15.3	0.8	7.4

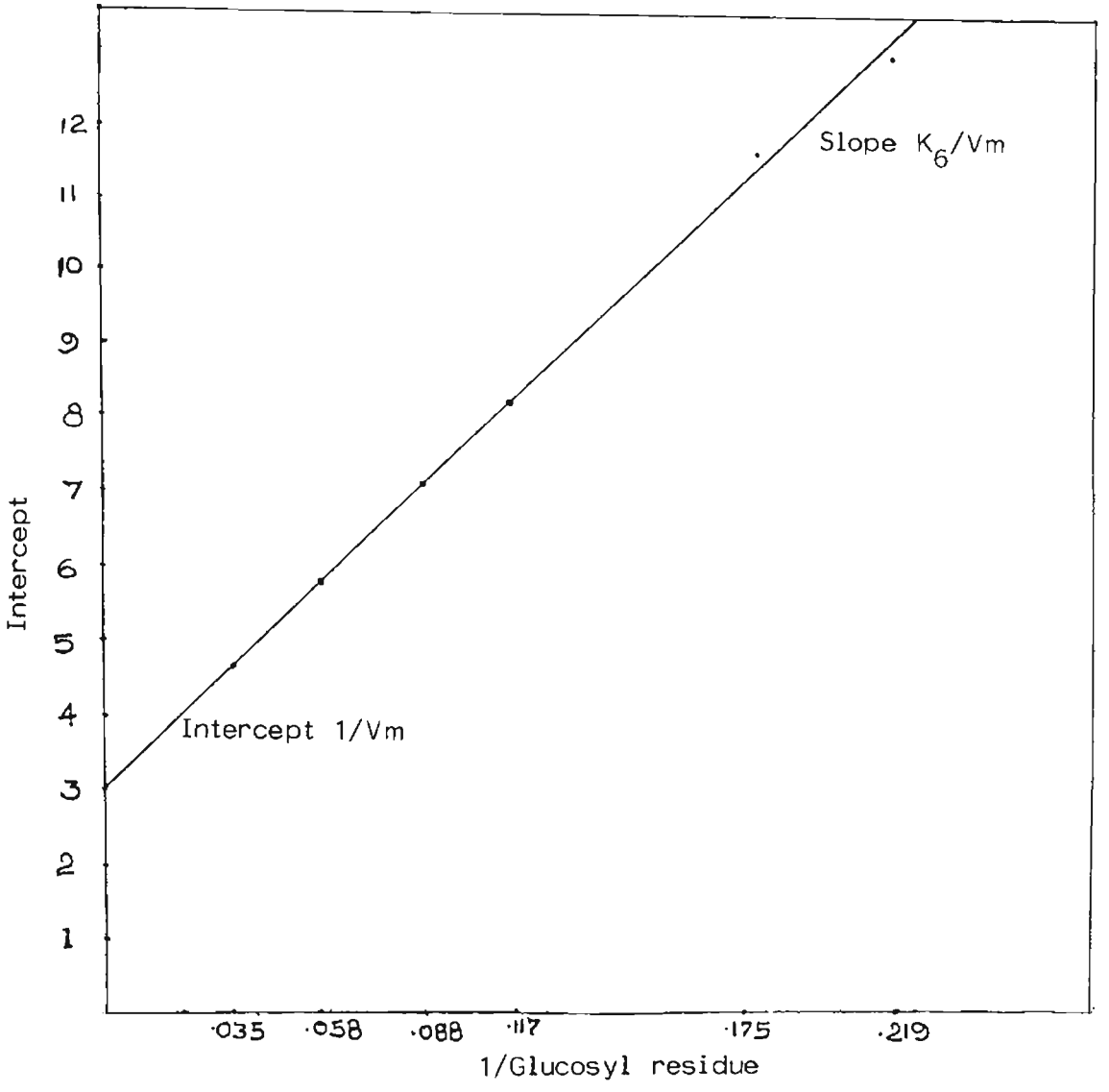

Slope K_6/Vm
Intercept 1/Vm
Intercept
12
11
10
9
8
7
6
5
4
3
2
1
·035
·058
·088
·117
·175
·219
1/Glucosyl residue

Fig.3-3 A

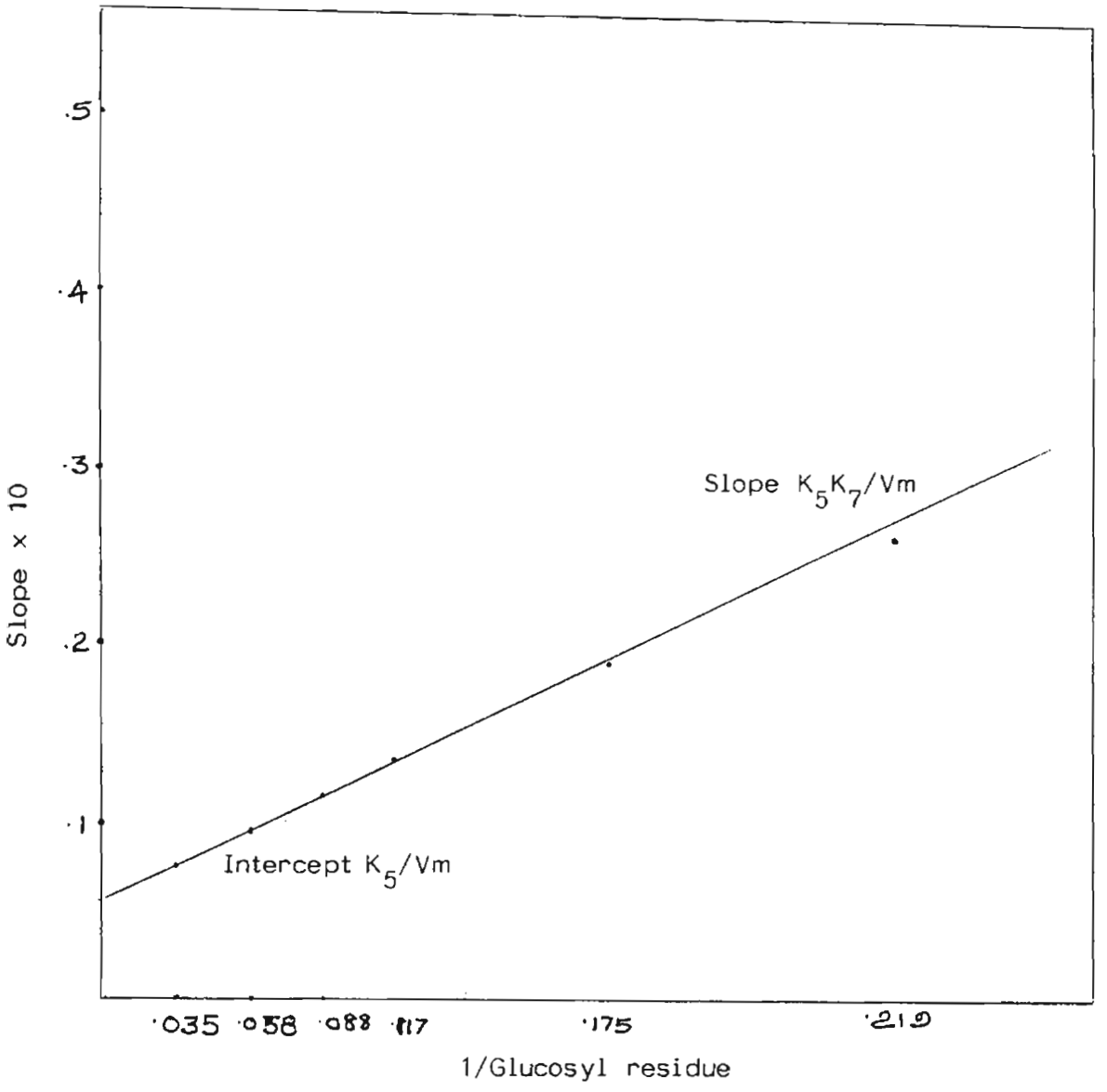

Slope x 10
·5
·4
·3
·2
·1
Slope K_5K_7/Vm
Intercept K_5/Vm
·035
·058
·088
·117
·175
·219
1/Glucosyl residue

Fig.3-3 B

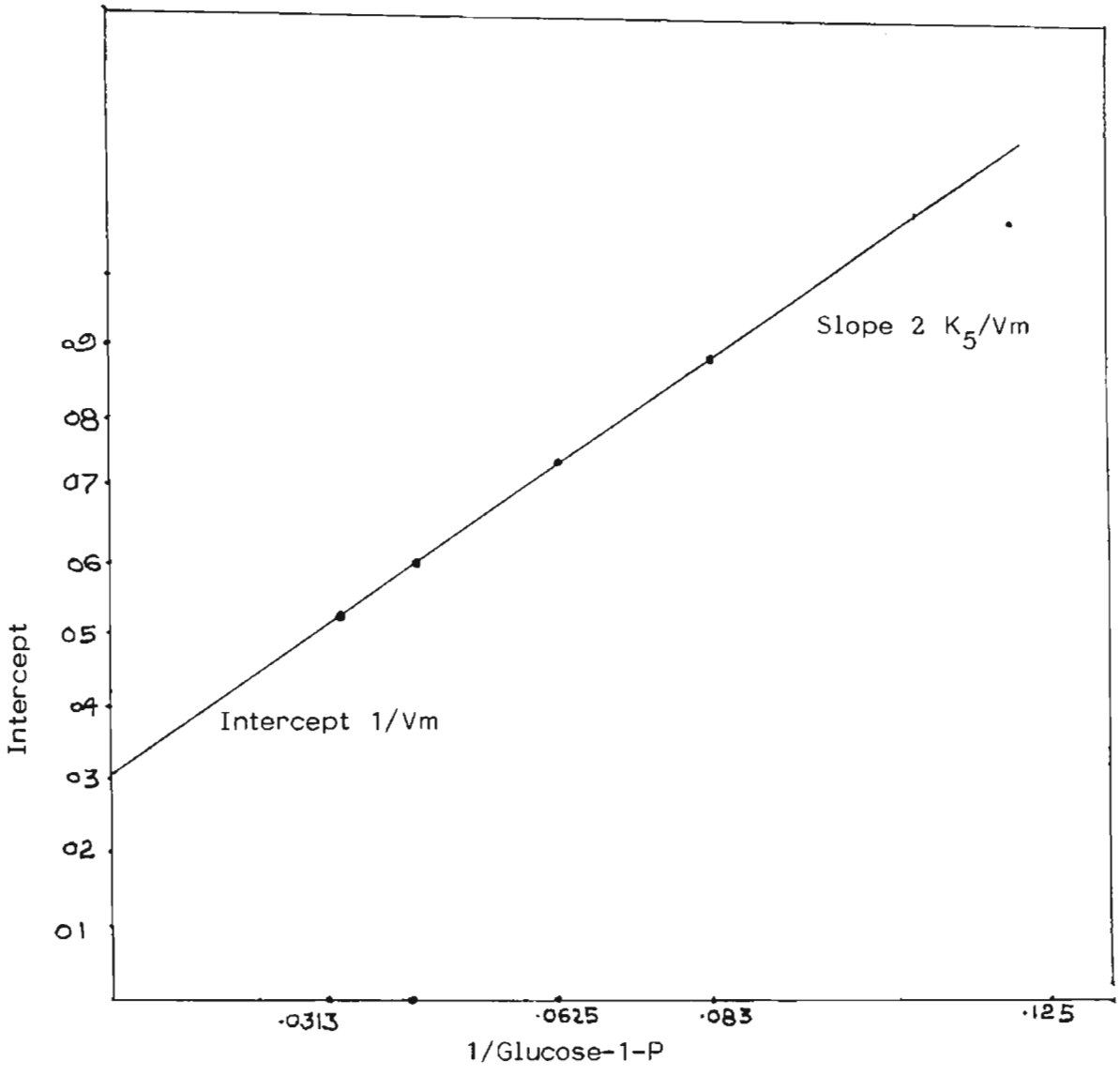

Slope 2 K_5/Vm
Intercept 1/Vm
Intercept
09
08
07
06
05
04
03
02
01
·0313
·0625
·083
·125
1/Glucose-1-P

Fig.3-4 A

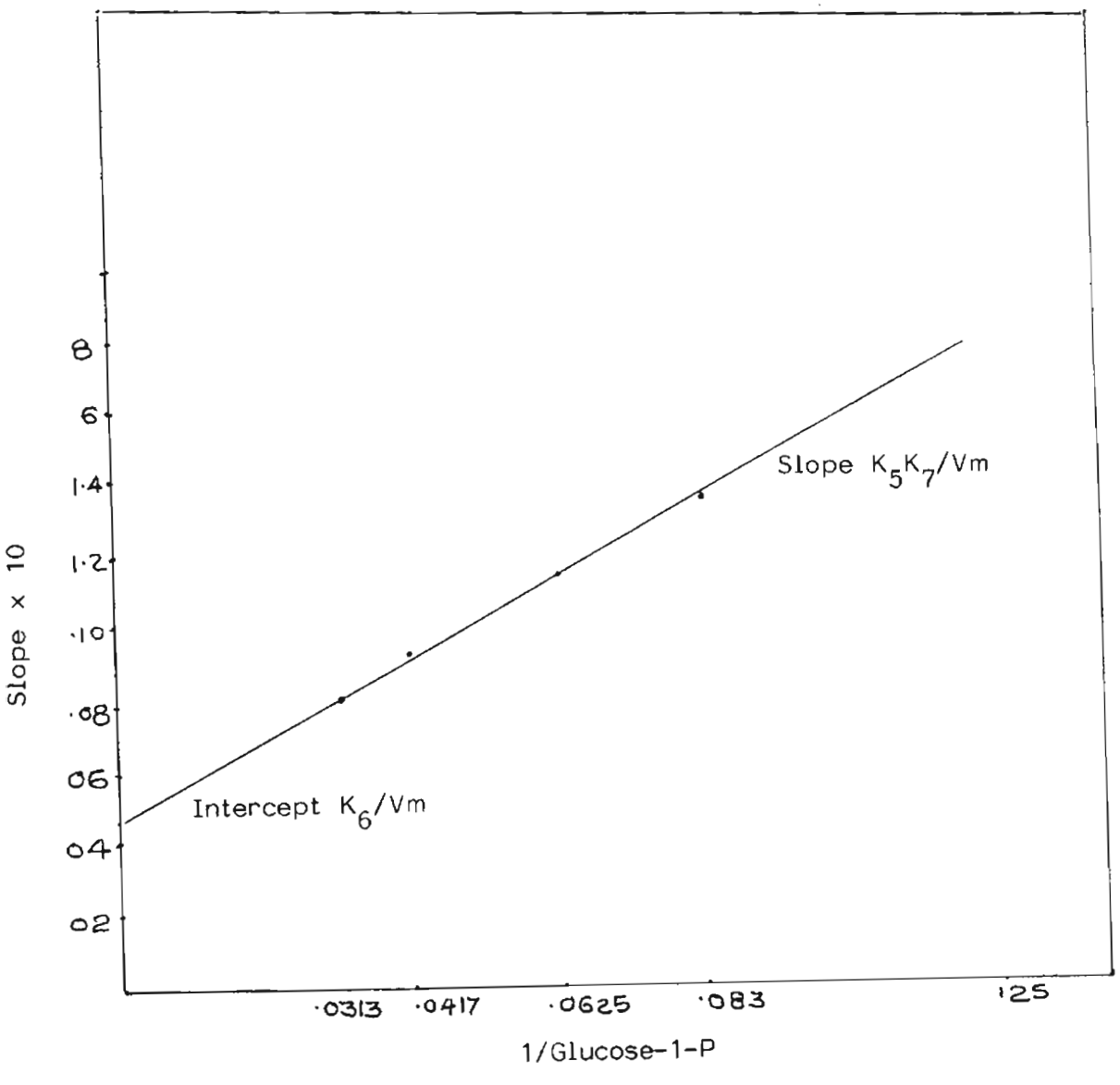

Slope x 10
8
6
1·4
1·2
·10
·08
06
04
02
Slope K_5K_7/Vm
Intercept K_6/Vm
·0313
·0417
·0625
·083
·125
1/Glucose-1-P

Fig.3-4 B

Fig.3-1

Lineweaver-Burk plots for glucose-1-P at different levels of glycogen for *Loligo vulgaris* phosphorylase *a* at pH 7. The reaction mixture contained 1 mM AMP, 10 mM Sodium-ß-glycerophosphate and glucose-1-P as indicated. The enzyme concentration was 200 μg/ml. The concentration of glycogen were ▪, 4.56 mM (expressed as glycosyl residue); 0, 5.7 mM, ▲ 8.5 mM, x 11.4 mM, •, 17.1 mM and ▫28.5 mM.

Fig.3-2

Lineweaver-Burk plots for glycogen at different levels of glucose-1-P for *Loligo vulgaris* at pH 7.

0, ▲, x, • and ▫ correspond to 8, 12, 16, 24 and 32 mM concentrations of glucose-1-P. Other details were as in Fig.3-1.

Fig.3-3A

Secondary plots of intercepts (mg enzyme vs min per micromole from Fig. 3-1.

Fig.3-3B

Secondary plots of the slopes from Fig.3-1

Fig.3-4A

Secondary plots of intercepts (mg enzyme vs min per micromole) from Fig.3-2

Fig.3-4B

Secondary plots of the slopes from Fig.3-2

CHAPTER-4

AMP KINETICS OF LOLIGO PHOSPHORYLASE *a*

The reciprocal plots for glucose-1-P and AMP at different levels of each other are shown in Fig.4-1 and 4-2. The apparent Km values for glucose-1-P and AMP were calculated and tabulated in Table 4-1. Increasing concentration of substrate slightly decreased the Km for AMP and *vice versa* which shows the limited similarity of Loligo phosphorylase to that from other sources.

The reciprocal plots for glucose-1-P were linear at different concentrations of AMP, whereas those for AMP at different levels of glucose-1-P were not linear. Moreover, the reciprocal plots for glucose-1-P converge to a point above the horizontal axis. This suggests that AMP has a mixed effect on the enzyme affinity to glucose-1-P above 0.25 mM AMP. This result is different from the results obtained for other animal phosphorylases, where AMP increased the affinity of the enzyme for glucose-1-P. Hence in this case, it is shown that from 0.25 mM concentration onwards, AMP does not have any heterotropic effect on glucose-1-P binding sties.

Linear reciprocal plots were also obtained for AMP-Kinetics both for

$$1/v \text{ } vs \text{ } \frac{1}{\text{G-1-P}} \quad \text{and} \quad \frac{1}{\blacktriangle v} \text{ } vs \text{ } \frac{1}{\text{G-1-P}} \quad \text{plots}$$

(Fig.4-1 and 4-3), ▲v being the difference in the rate in the presence and absence of AMP. In all these cases, the activation was of the mixed type. From the graph it can be observed that 1/▲v is lower in the presence of activator and substrate at higher concentration and is related to the effectiveness of the activator. This increase in the difference between the velocity in the presence and absence of AMP can be attributed to the higher effectiveness of the AMP protection of enzyme.

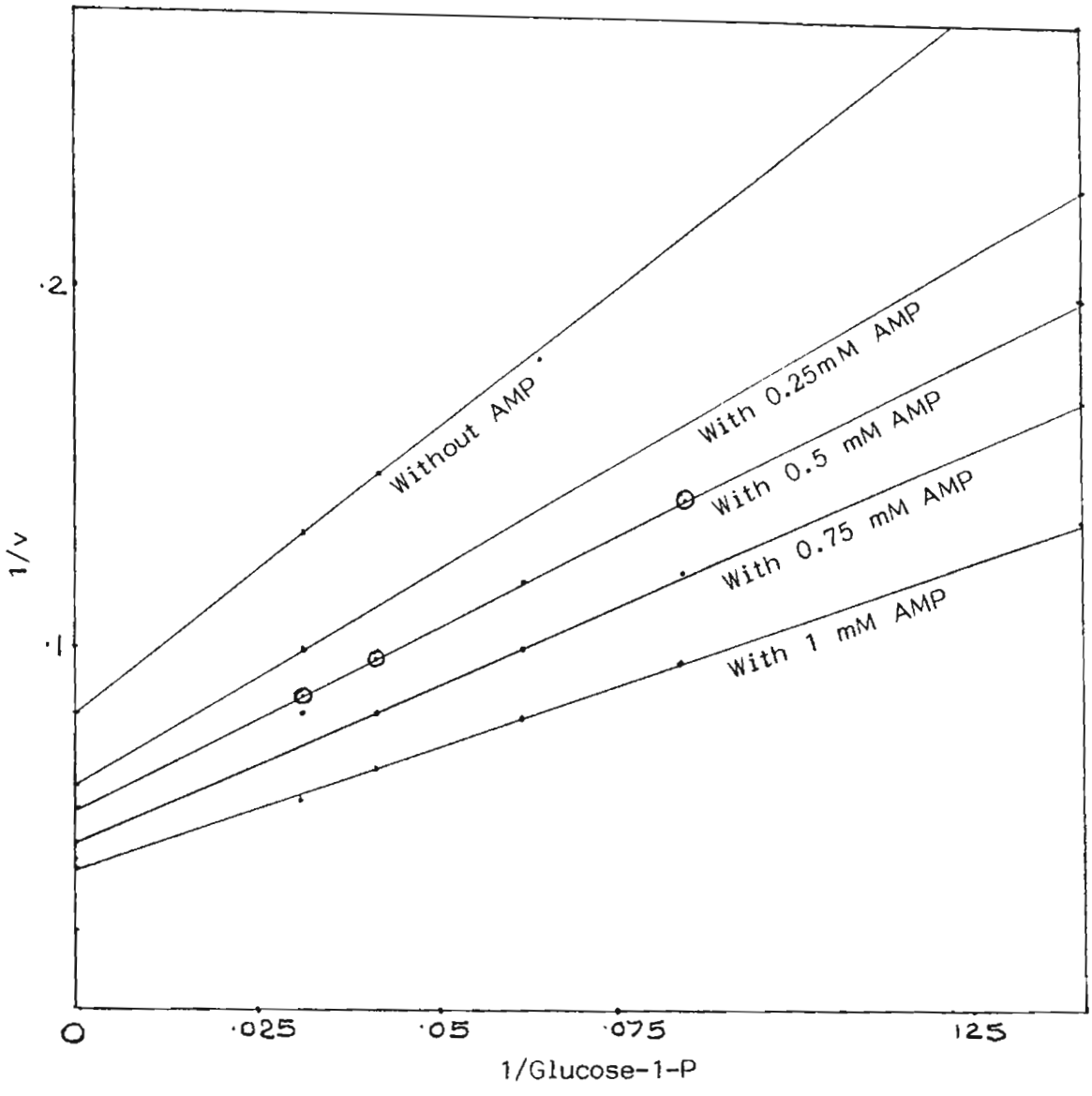

1/v
·2
·1
0
·025
·05
·075
125
1/Glucose-1-P
Without AMP
With 0.25mM AMP
With 0.5 mM AMP
With 0.75 mM AMP
With 1 mM AMP

Fig.4-1

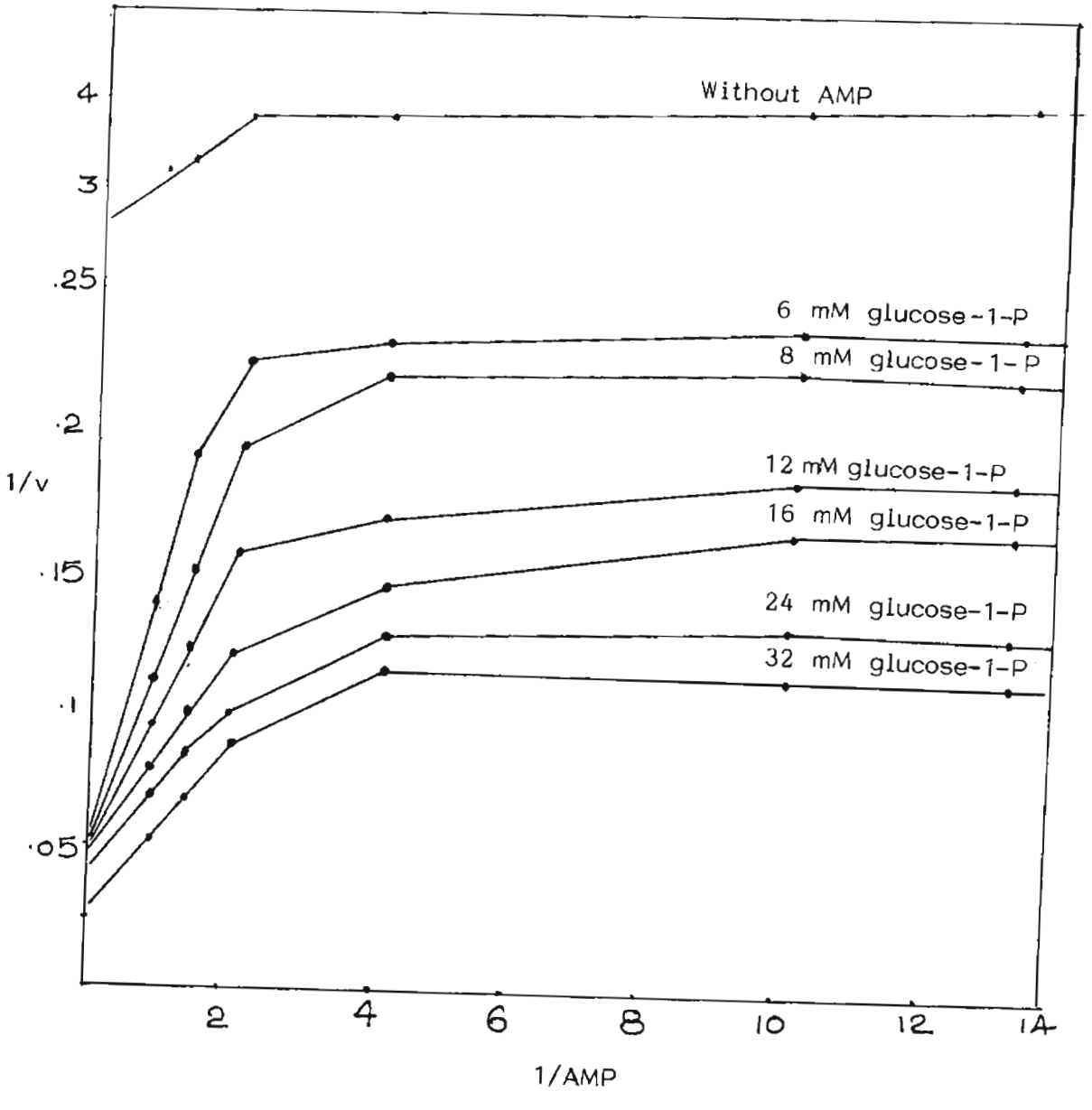

Without AMP
6 mM glucose-1-P
8 mM glucose-1-P
12 mM glucose-1-P
16 mM glucose-1-P
24 mM glucose-1-P
32 mM glucose-1-P
1/v
1/AMP
4
3
.25
.2
.15
.1
.05
2
4
6
8
10
12
14

Fig.4-2

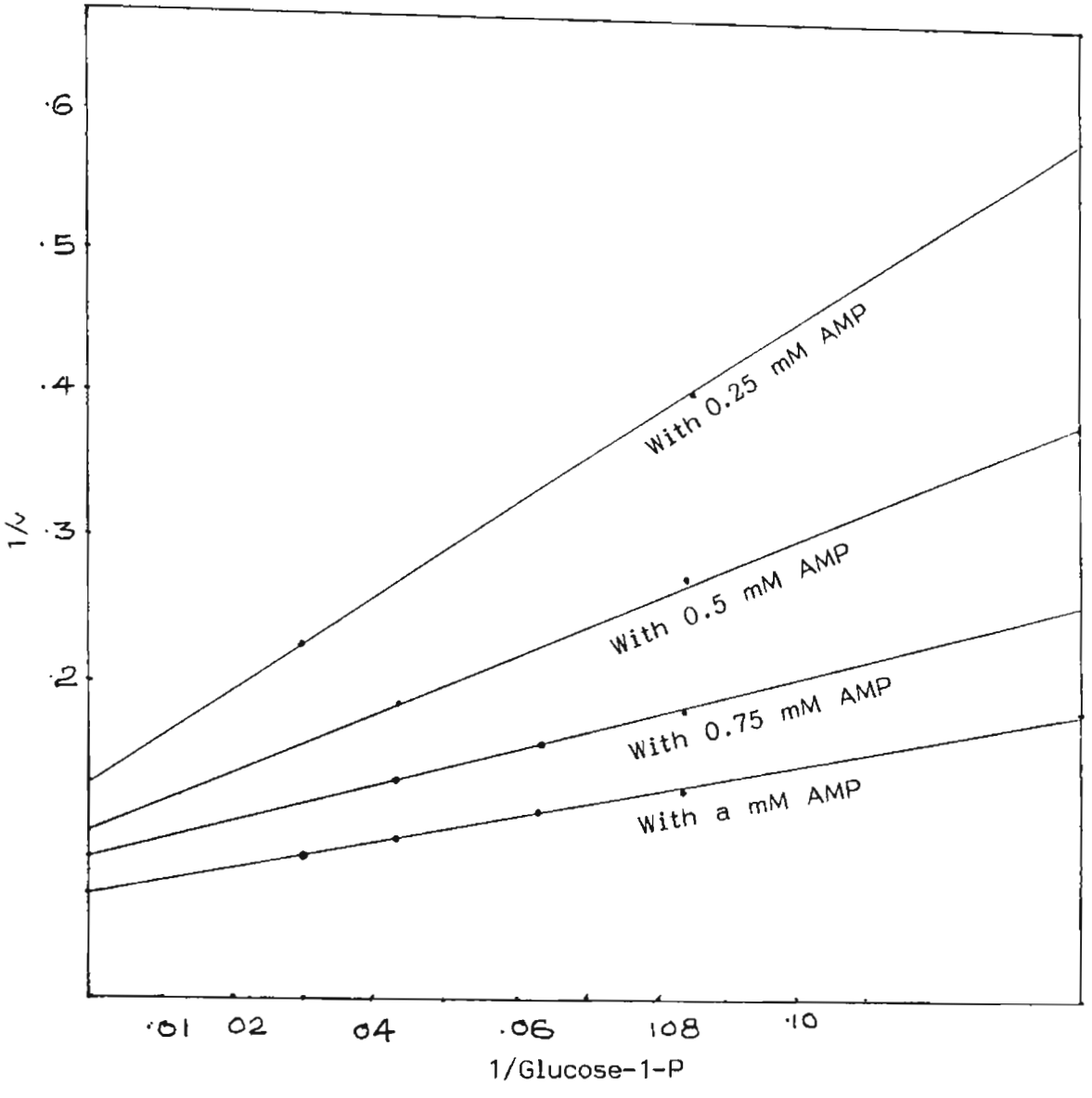

1/v
·6
·5
·4
·3
·2
With 0.25 mM AMP
With 0.5 mM AMP
With 0.75 mM AMP
With a mM AMP
·01
02
04
·06
108
·10
1/Glucose-1-P

Fig.4-3

Table 4-1. Apparent Km values for AMP and Glucose-1-P at different concentrations of each other for *Loligo vulgaris* phosphorylase *a*

Glucose-1-P (mM)	Km for AMP (mM)	AMP (mM)	Km for Glucose-1-P (mM)
32	0.63	1.00	21
24	0.63	0.75	21
16	0.98	0.50	22
12	1.14	0.25	22
8	1.18	Without AMP	31

If the dissociation constants from the ternary complex are different from the binary complex both the slopes and intercepts of double reciprocal plots will be altered by a change in the concentration of activator and the activation may be termed 'mixed' (1). The secondary plots of the reciprocal plots of glucose-1-P and AMP are given in Fig.4-4 and 4-5. The apparent Km values were calculated (Table 4-2). From these plots and table, it is observed that the dissociation constant of ternary complex and reciprocal plots are different for Loligo phosphorylase. Again it is confirming the mixed type of AMP activation.

The reciprocal plots for AMP were curved showing negative cooperativity between AMP binding sites. Similar negative cooperativity has been found for the phosphorylase form Sepia (22), *Metapenaeus dobsoni* and Villorita (142). This property may be an adaptation to energy need.

From the above observations it is evident that the control mechanism of Loligo phosphorylase is different from that of the enzymes from other sources. Although AMP was the only natural activator that we have found so far, the physiological significance of the slight shift in kinetic constant is not clear.

Table 4-2. The values of kinetic constants obtained using different equations from secondary plots of Fig.2-4 and 2-5

	Values obtained from			
	Fig.2-4A	Fig.2-4B	Fig.2-5A	Fig.2-5B
Vmax (μ moles/min/mg)	30.3		47.62	
K_5 (mM)		3.5	7.86	
K_6 (mM)	0.24			0.68
K_7 (mM)		5.74		2.71

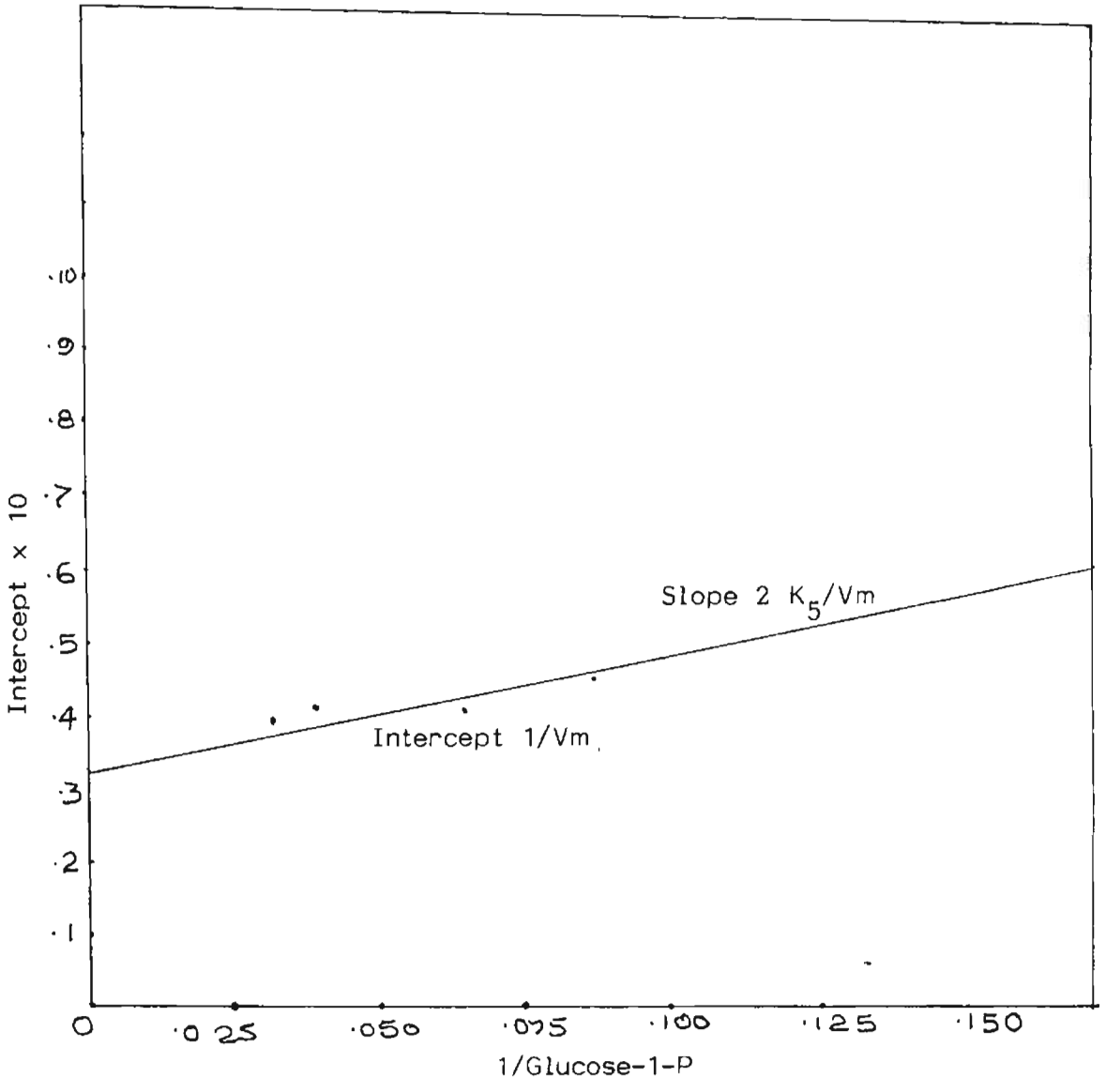

Intercept x 10
·1
·2
·3
·4
·5
·6
·7
·8
·9
·10
Slope 2 K_5/Vm
Intercept 1/Vm
0
·025
·050
·075
·100
·125
·150
1/Glucose-1-P

Fig.4-4 A

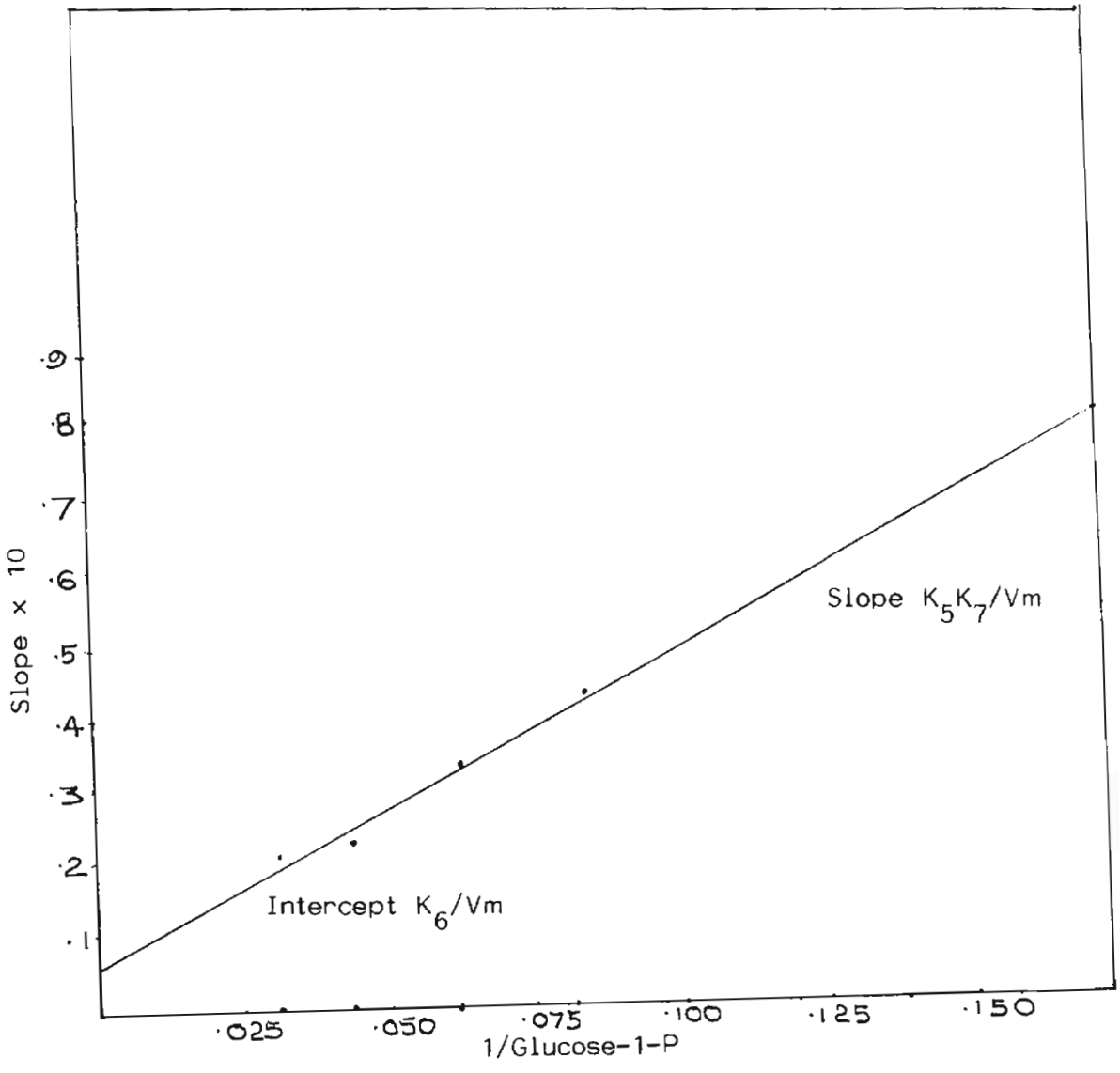

Slope x 10
·9
·8
·7
·6
·5
·4
·3
·2
·1
Slope K_5K_7/Vm
Intercept K_6/Vm
·025
·050
·075
·100
·125
·150
1/Glucose-1-P

Fig.4-4 B

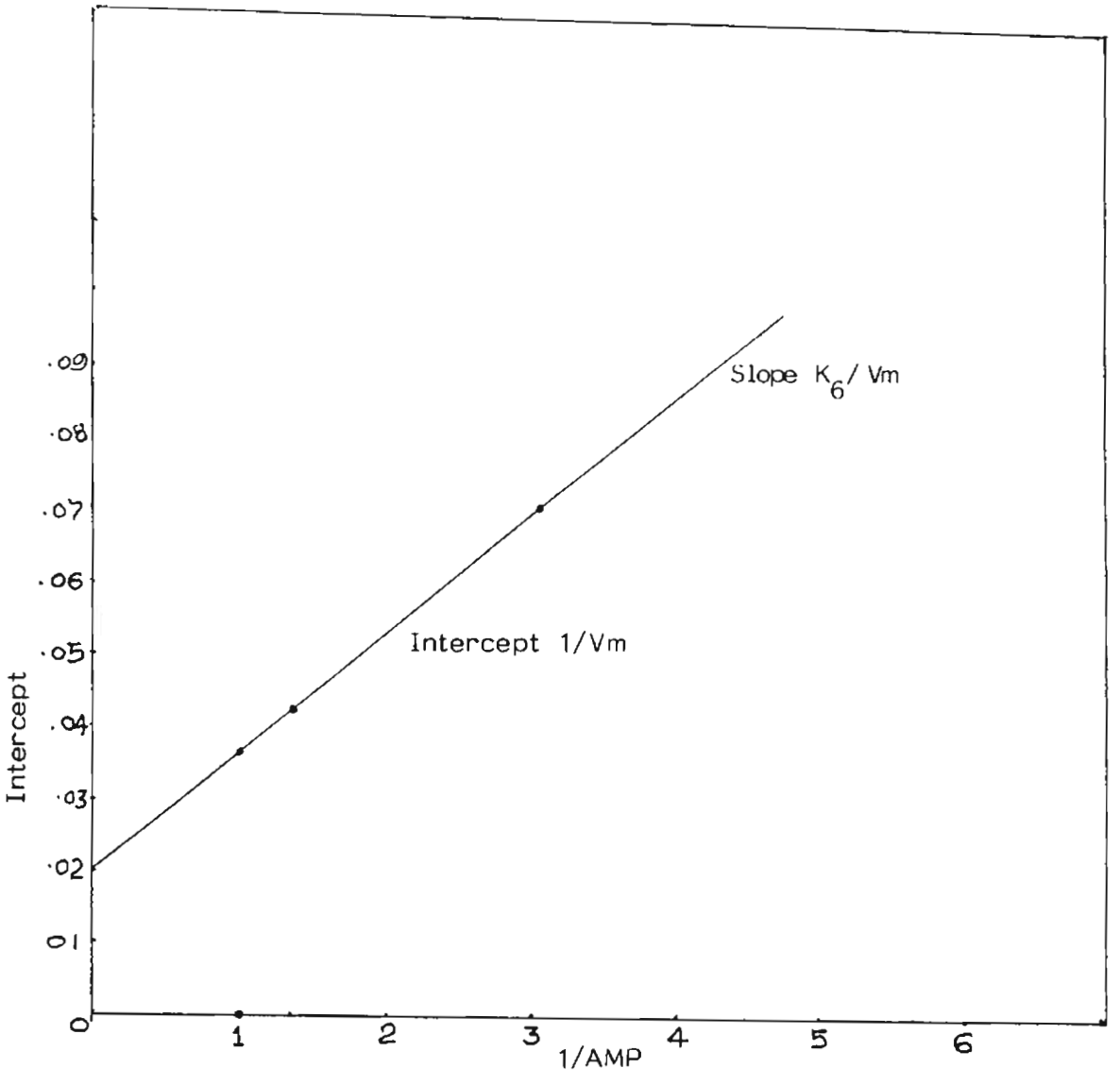

Slope K_6/ Vm
Intercept 1/Vm
Intercept
.09
.08
.07
.06
.05
.04
.03
.02
.01
0
1
2
3
4
5
6
1/AMP

Fig.4-5 A

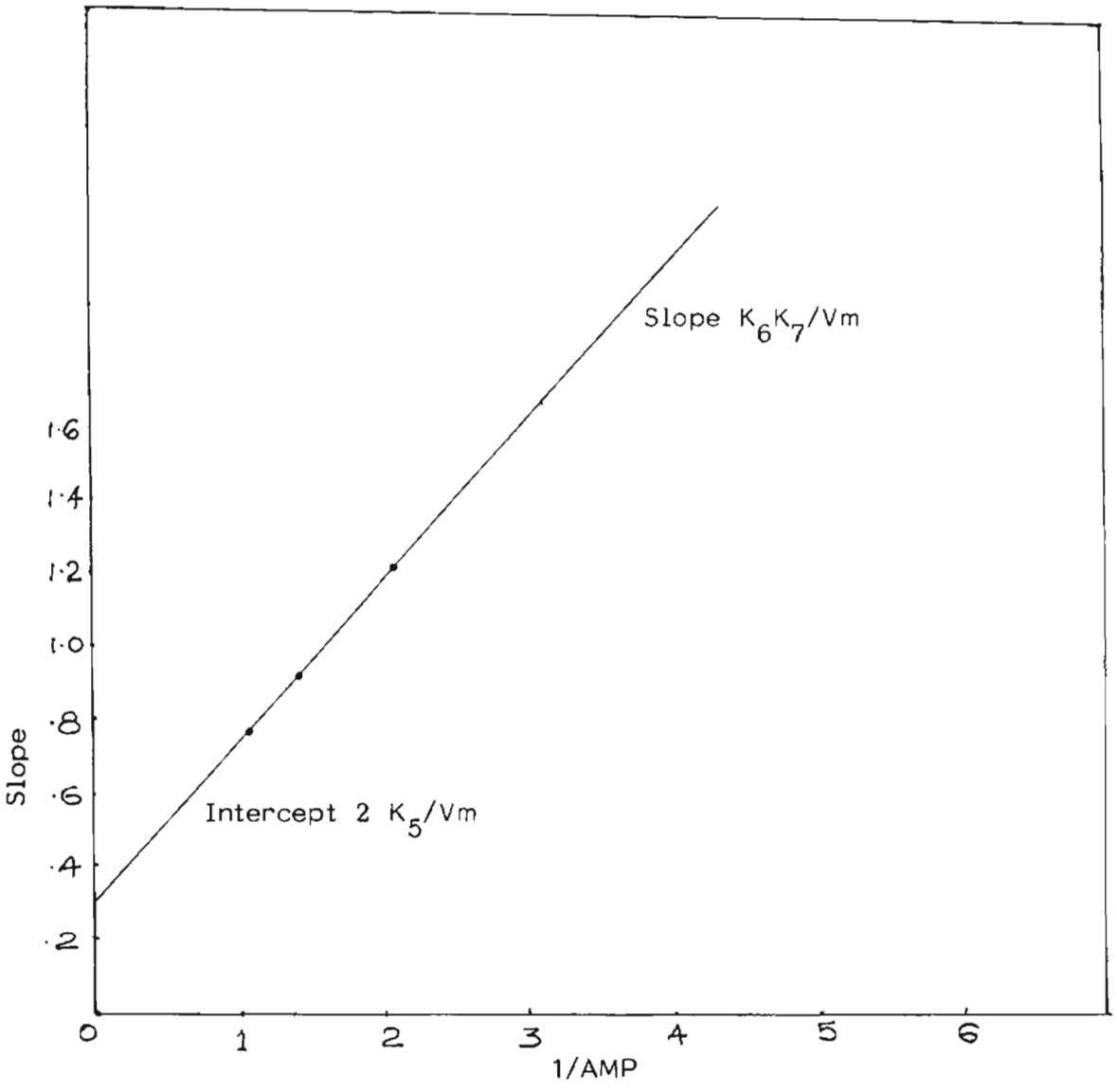

Slope K_6K_7/Vm
Intercept 2 K_5/Vm
Slope
1·6
1·4
1·2
1·0
·8
·6
·4
·2
0
1
2
3
4
5
6
1/AMP

Fig.4-5 8

Fig.4-1

Reciprocal plots for glucose-1-P at different levels of AMP for Loligo phosphorylase *a*. The assay mixture contained 10 mM Sodium-ß-glycerophosphate buffer (pH 7) and 0.5% glycogen. The concentration of enzyme in the assay mixture was 31.2 μg/ml. The concentrations of AMP were 0.25 mM, 0.5 mM, 0.75 mM and 1.00 mM respectively.

Fig.4-2

Reciprocal plots for AMP at different levels of Glucose-1-P for Loligo phosphorylase *a*. The assay mixture contained 10 mM Sodium-ß-glycerophosphate buffer (pH 7) and 0.5% glycogen. The concentration of enzyme in the assay mixture was 31.2 μg/ml. The concentration of glucose-1-P were 8 mM, 12 mM, 16 mM and 32 mM respectively.

Fig.4-3

Plots $\frac{1}{\blacktriangle v}$ *vs* $\frac{1}{AMP}$ where $\blacktriangle v$ is the difference in activities in the presence of AMP. Details as in Fig.4-2.

Fig.4-4A

Secondary plots of the intercepts from Fig.4-1

Fig.4-4B

Secondary plots of the slopes from Fig.4-1

Fig.4-5A

Secondary plots of the intercepts from Fig.4-2

Fig.4-5B

Secondary plots of the slopes from Fig.4-2

CHAPTER 5

INFLUENCE OF GLUCOSE-6-P ON THE KINETICS OF LOLIGO AND SEPIA PHOSPHORYLASE *a*

The saturation curve for glucose-1-P for rabbit phosphorylase *b* is hyperbolic in the absence of an allosteric inhibitor (144). In the presence of an inhibitor like glucose-6-P, the curve becomes sigmoidal and the double reciprocal plot is curved upwards (145).

The influence of varying concentrations of glucose-1-P on Loligo phosphorylase a, in the presence and absence of glucose-6-P is shown in Fig.5-1A. The figure shows that with the Loligo enzyme in the presence and absence of glucose-6-P the dependence of initial velocity on glucose-1-P concentration is hyperbolic. This hyperbolicity is not at all comparable with that of rabbit phosphorylase. The same data is shown in Fig. 5-1B in the form of Lineweaver-Burk plot (146). The presence of glucose-6-P may be seen here as having the effect of inducing linear plots only.

Even if the linearity of reciprocal plot is high in loligo enzyme, it is clear from other evidences that the enzyme doesnot obey the Michaelis-Menten Kinetics in the presence of glucose-6-P as from other sources.

The sigmoidal substrate saturation curve and the nonlinear reciprocal plots are characteristic of an allosteric enzyme showing positive cooperativities(83).

Fig. 5-1 shows hyperbolicity of substrate saturation curve and linearity in reciprocal plot of glucose-1-P sites in the presence of glucose-6-P. The strength of cooperativity can be checked by Hill plot.

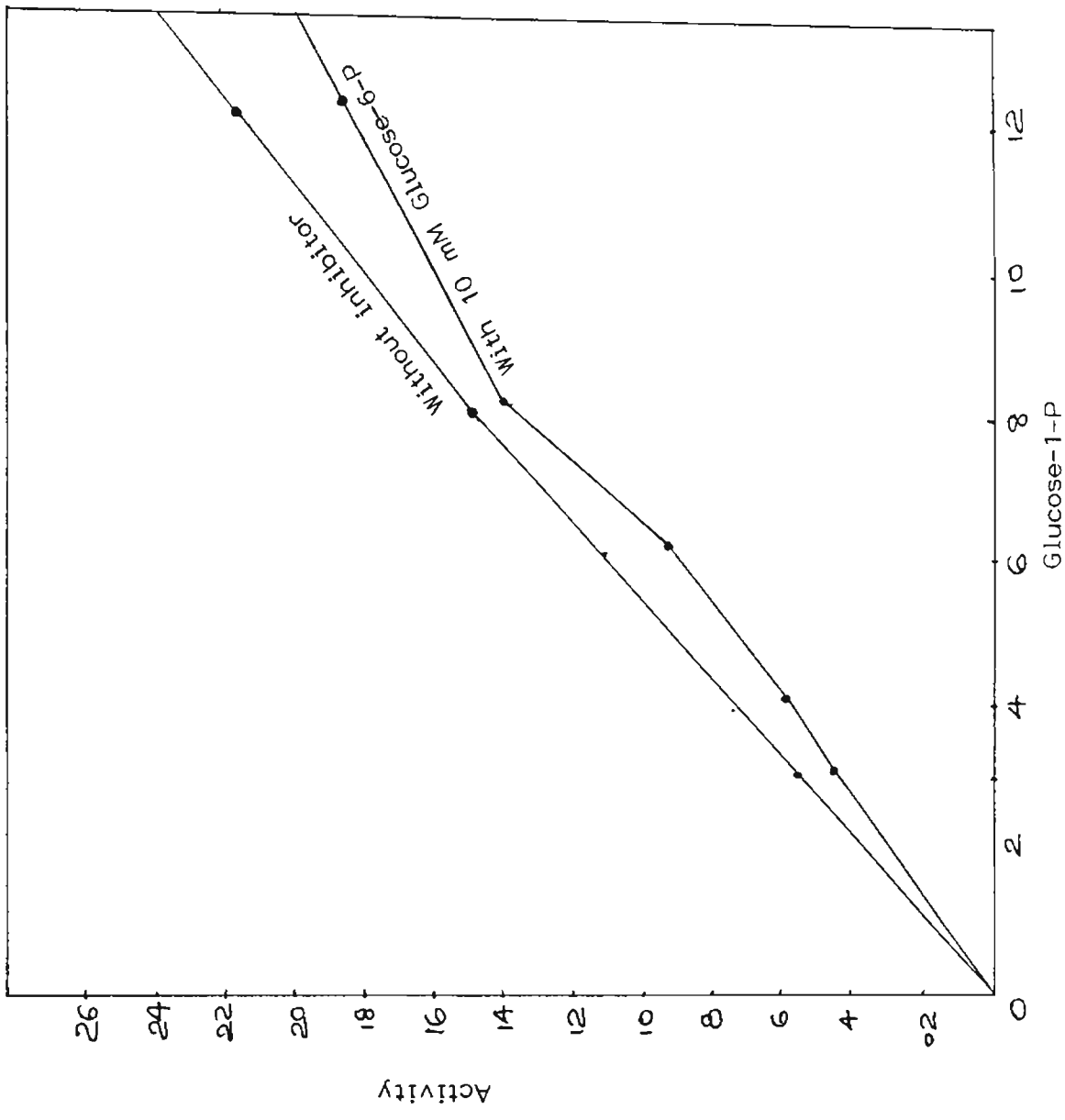
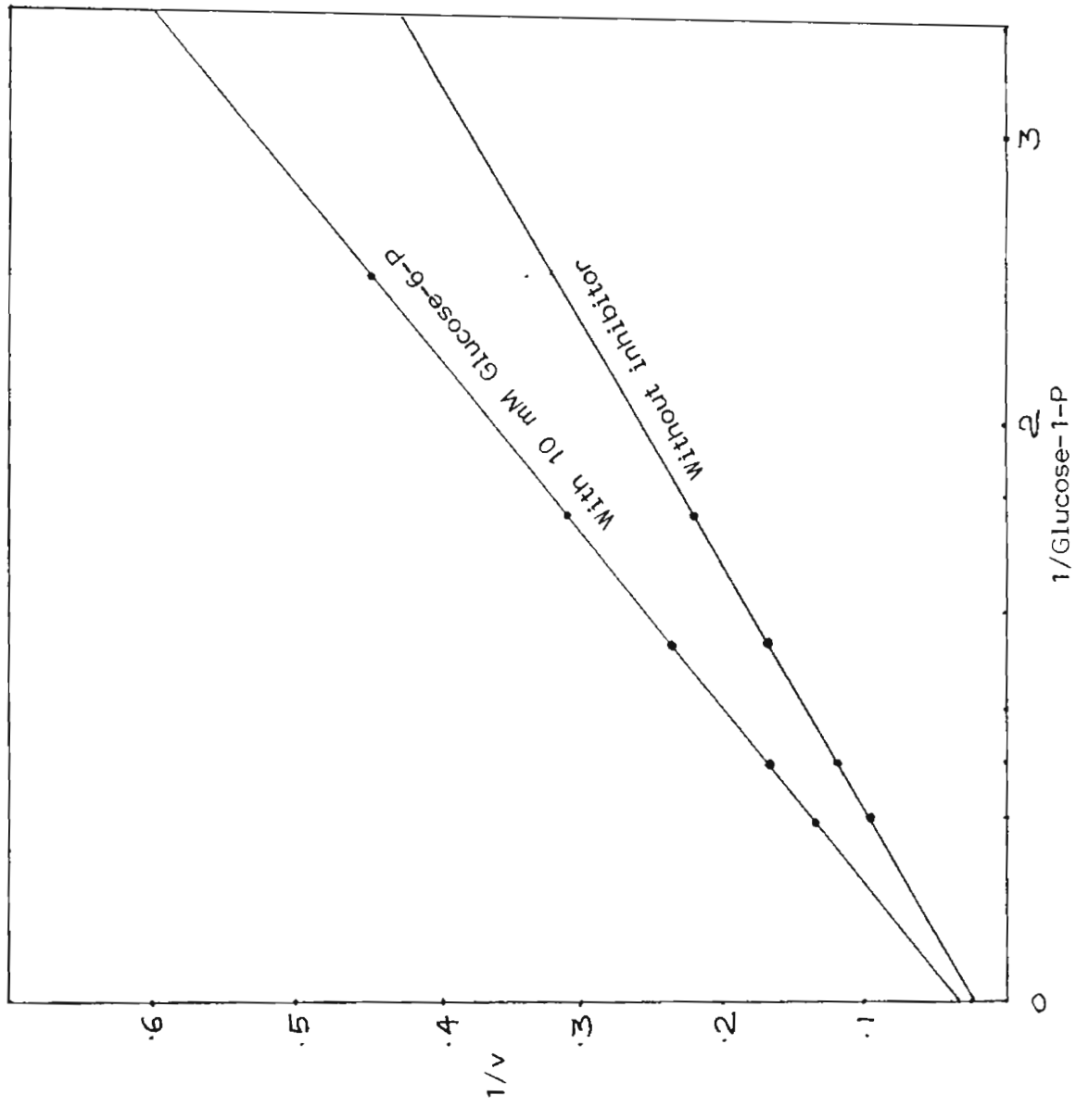

Activity
Glucose-1-P
Without inhibitor
With 10 mM Glucose-6-P

Fig.5-1 A


1/v
.6
.5
.4
.3
.2
.1
0
2
3
1/Glucose-1-P
With 10 mm Glucose-6-P
Without inhibitor

Atkinson *et al.* (147) Rubin and Changeux (39) have suggested the use of Hill plots to measure the cooperativity of binding sites. The Hill equation in its useful form may be written as

$$\text{Log}\ \frac{v}{V-v} = \log K - n \log(s)$$

where v is the initial velocity at a particular substrate concentration, V is maximal velocity, K is a constant and

n is Hill coefficient. Thus a plot of Log $\frac{v}{V-v}$ against log(s) will give a straight line with slope equal to *n*. The value of *n* is a measure of the strength of cooperativity of sites. If kinetic analysis is carried out in the presence of an allosteric inhibitor the value of *n* will rise and approach the number of binding sites as the concentration of inhibitor is increased. Phosphorylase *b* has two binding sites, each for glucose-1-P and AMP. Madsen and Sheckosky (82) have applied this method to study the strength of cooperativity of sites in phosphorylase *b*. Since the present chapter deals with the influence of the metabolic inhibitor glucose-6-P on the kinetic properties of Loligo phosphorylase *a* it is convenient to use Hill plots for the analysis of data.

The data of Fig.5-1 are given in the form of Hill plot in Fig.5-2. The Loligo enzyme gives a Hill coefficient of unity, indicating lack of cooperativity of glucose-1-P sites in the presence and absence of glucose-6-P. The *n* value is 1, even in the presence of 10 mM glucose-6-P, indicating that under these conditions, the sites become non-cooperative. This is not in agreement with the usual characteristics of allosteric enzymes from other sources.

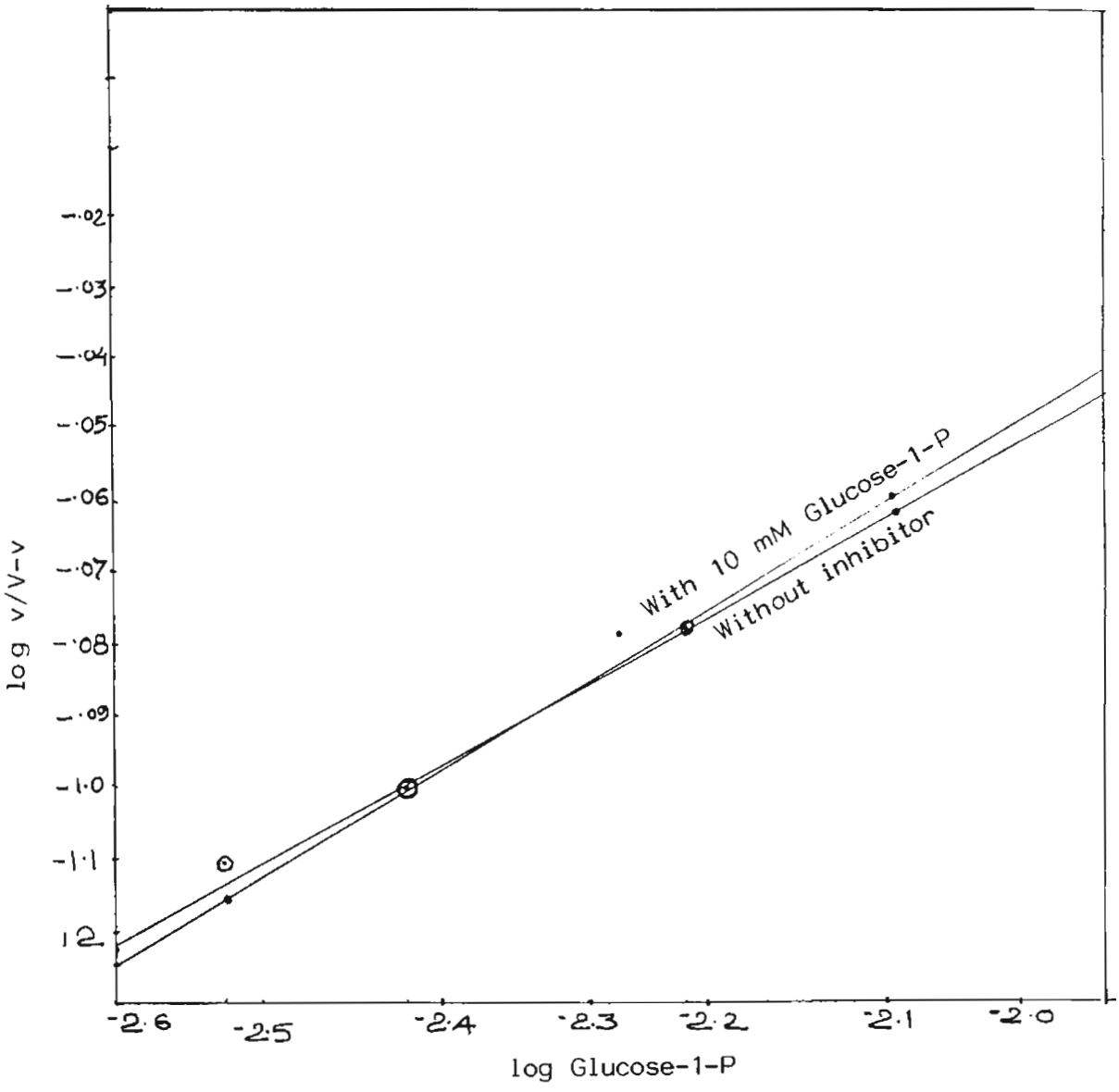

log v/V-v
-·02
-·03
-·04
-·05
-·06
-·07
-·08
-·09
-1·0
-1·1
12
With 10 mM Glucose-1-P
Without inhibitor
-2·6
-2·5
-2·4
-2·3
-2·2
-2·1
-2·0
log Glucose-1-P

Fig.5-2

The effect of glucose-6-P in Sepia phosphorylase *a* also was studied in varying concentrations of glucose-1-P. Extraction of phosphorylase was carried out just as in Loligo sample. Linear reciprocal plots with mixed inhibition were observed (Fig.5-3). Hill plots of Fig.5-4 gave an *n* value of unity which shows the lack of cooperativity. Apparent Km value of Sepia phosphorylase was lower (4) than the Loligo enzyme (37.5) which implies the low degradative efficiency of Loligo enzyme.

The influence of varying concentrations of AMP on Loligo phosphorylase *a*, in the presence and absence of glucose-6-P is shown in Fig.5-5A. The figure shows that with the Loligo enzyme in the absence and presence of glucose-6-P, the dependence of initial velocity of AMP concentration is hyperbolic. The same data is shown in Fig.5-5B in the form of lineweaver-Burk plot. Linear plots were obtained. The data of Fig.5-5 are given in the form of Hill plot in Fig.5-6. The Loligo enzyme gives a Hill coefficient of unity. The *n* value is less than one even in the presence of 10 mM glucose-6-P, indicating that under these conditions also sites are negatively cooperative.

Linear plots were also obtained $\frac{1}{\blacktriangle v}$ vs $\frac{1}{AMP}$ plots (Fig.5-7),

▲v being the difference in the rate in the presence and absence of glucose-6-P. In all these cases, the inhibition was of mixed type. From the graph, it can be observed that $\frac{1}{\blacktriangle v}$ is lower in lower substrate concentration and is related to the effectiveness of the inhibitor. This increase in the difference between the velocity in the presence and absence of glucose-6-P can be attributed to the higher effectiveness of the inhibitors.

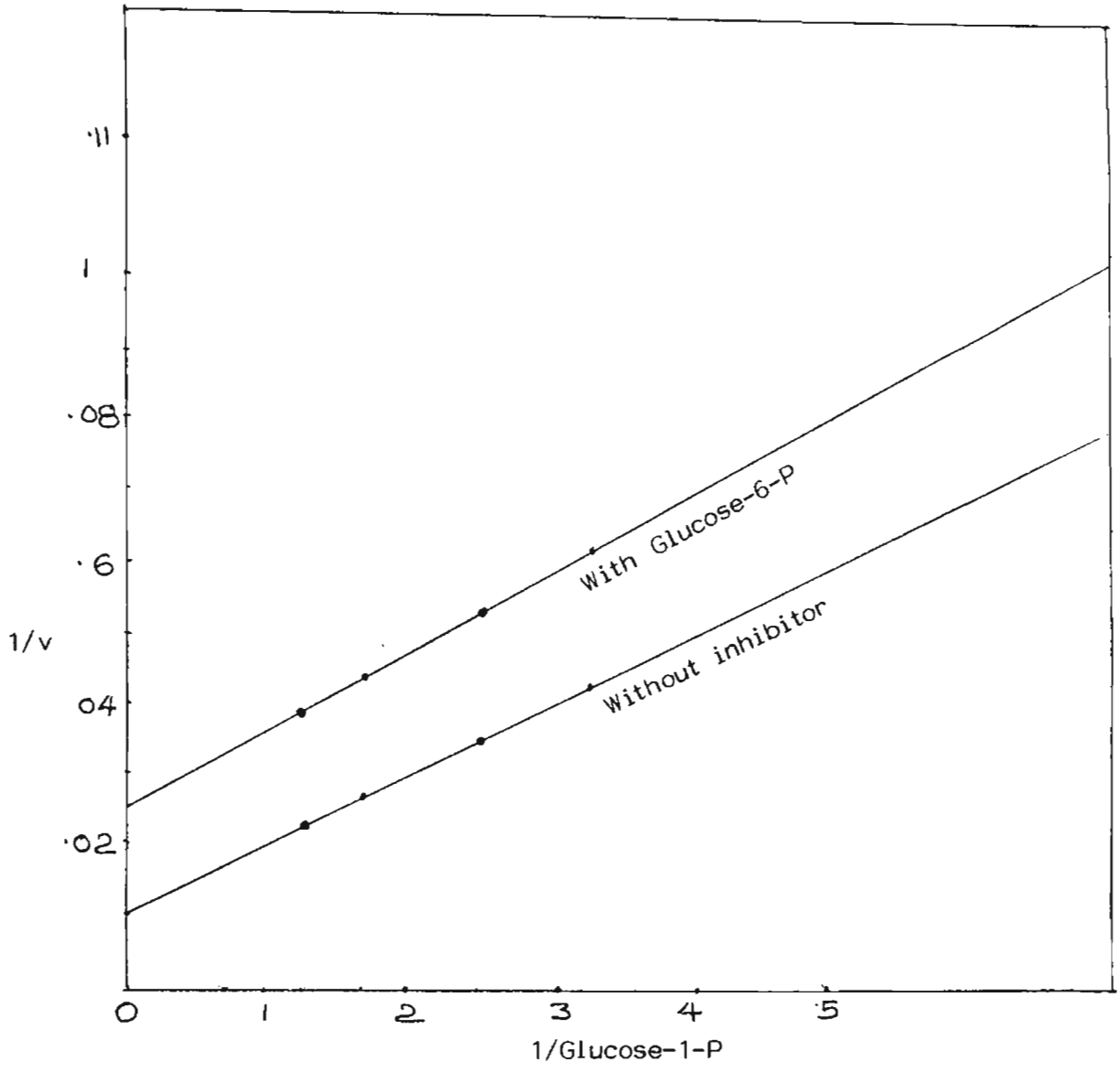

·11
1
·08
·6
1/v
04
·02
0
1
2
3
4
5
1/Glucose-1-P
With Glucose-6-P
Without inhibitor

Fig.5-3

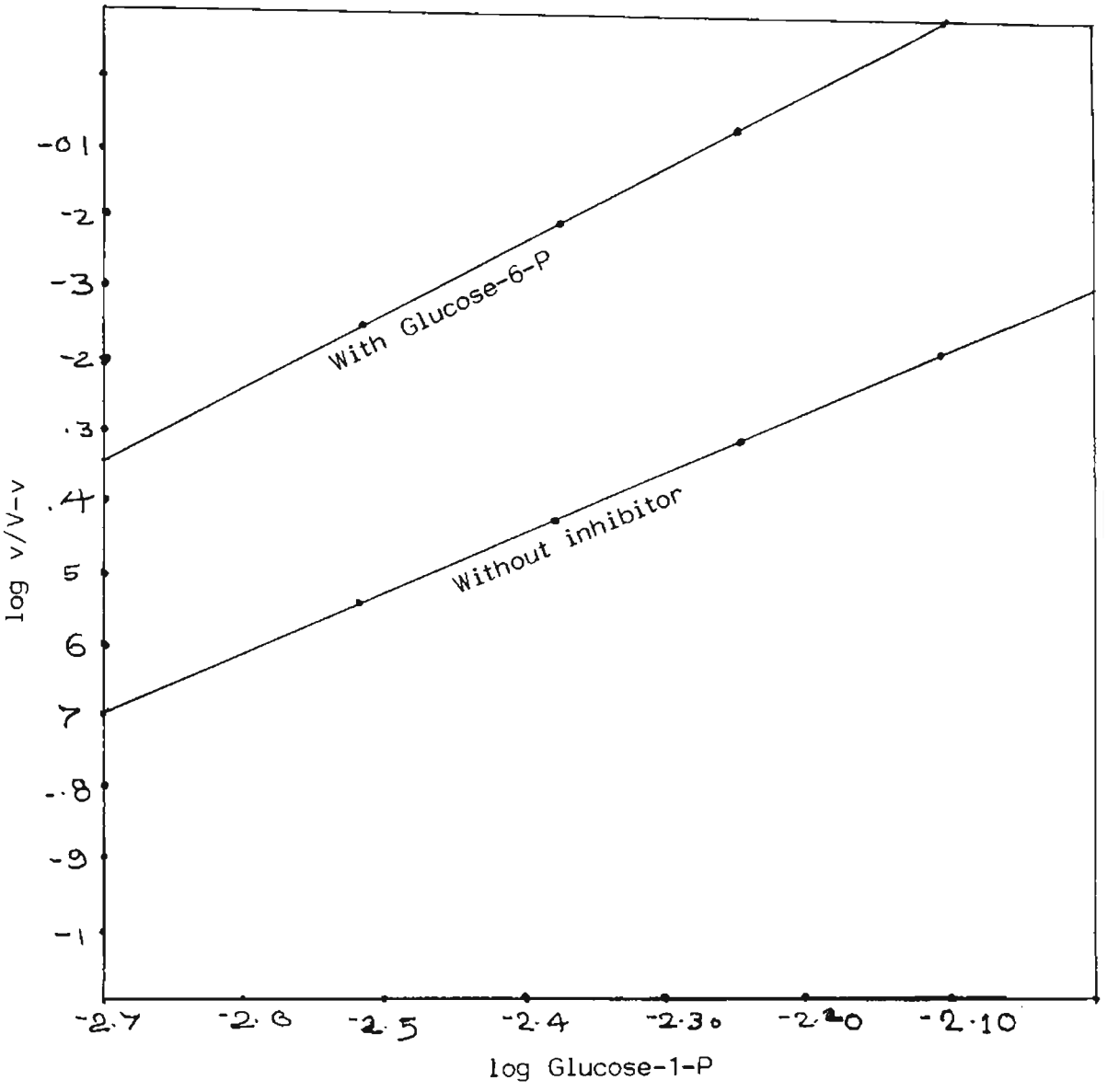

With Glucose-6-P
Without inhibitor
log v/V-v
-0 1
-2
-3
-2
.3
.4
5
6
7
-.8
-9
-1
-2.7
-2.6
-2.5
-2.4
-2.30
-2.20
-2.10
log Glucose-1-P

Fig.5-4

Fig. 5-1

(A) Substrate saturation curve for Loligo phosyphorylase *a* in the absence and presence of glucose-6-P. Phosphorylase was diluted in 10 mM sodium-ß-glycerophosphate buffer (pH 7) at 30°C prior to assay. The concentration of enzyme in the mixture was 27.5 μg/ml and that of glycogen and AMP were 0.5% and 1 mM respectively. Specific activities are expressed as micromoles of inorganic phosphate liberated/min/mg protein at 30°C.

(B) Double reciprocal plot for the same data (same symbols)

Fig.5-2

Hill plot for the data Fig.5-1 (same symbols)

Fig.5-3

Double reciprocal plots for Sepia phosphorylase *a* in the absence and presence of glucose-6-P. Phosphorylase was diluted in 10 mM Sodium-ß-glycerophosphate buffer (pH 7) at 30°C prior to assay. The concentration of enzyme in the mixture was 13.3 μg/ml and that of glycogen and AMP were 0.5 per cent and 1 mM respectively. Specific activities are expressed as micromoles of inorganic phosphate liberated/min/mg protein at 30°C.

Fig.5-4

Hill plot for the data of Fig. 5-3 (same symbols)

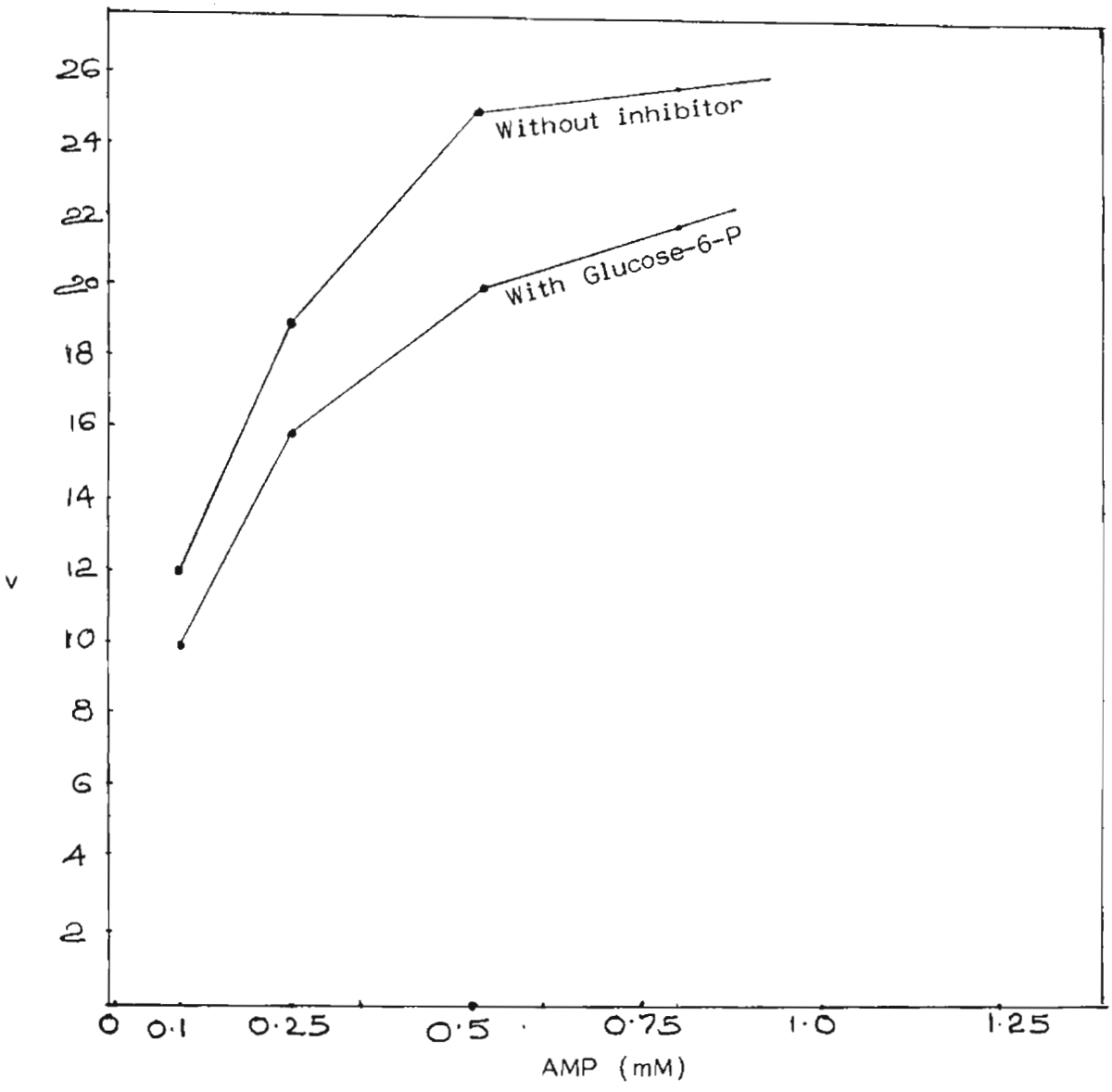

26
24
22
20
18
16
14
12
10
8
6
4
2
v
Without inhibitor
With Glucose-6-P
0
0·1
0·25
0·5
0·75
1·0
1·25
AMP (mM)

Fig.5-5 A

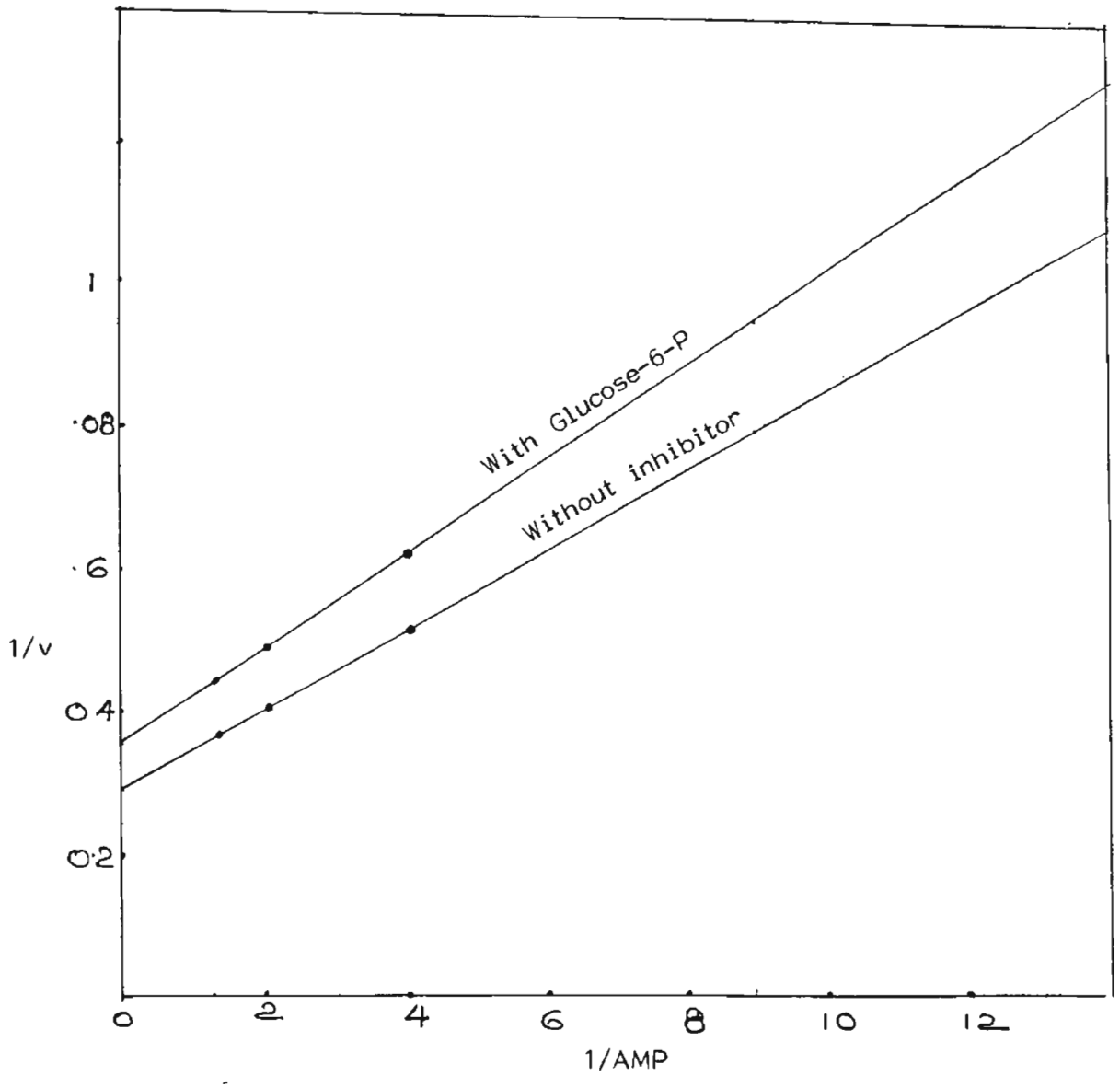

1
·08
·6
1/v
0·4
0·2
With Glucose-6-P
Without inhibitor
0
2
4
6
8
10
12
1/AMP

Fig.5-5 B

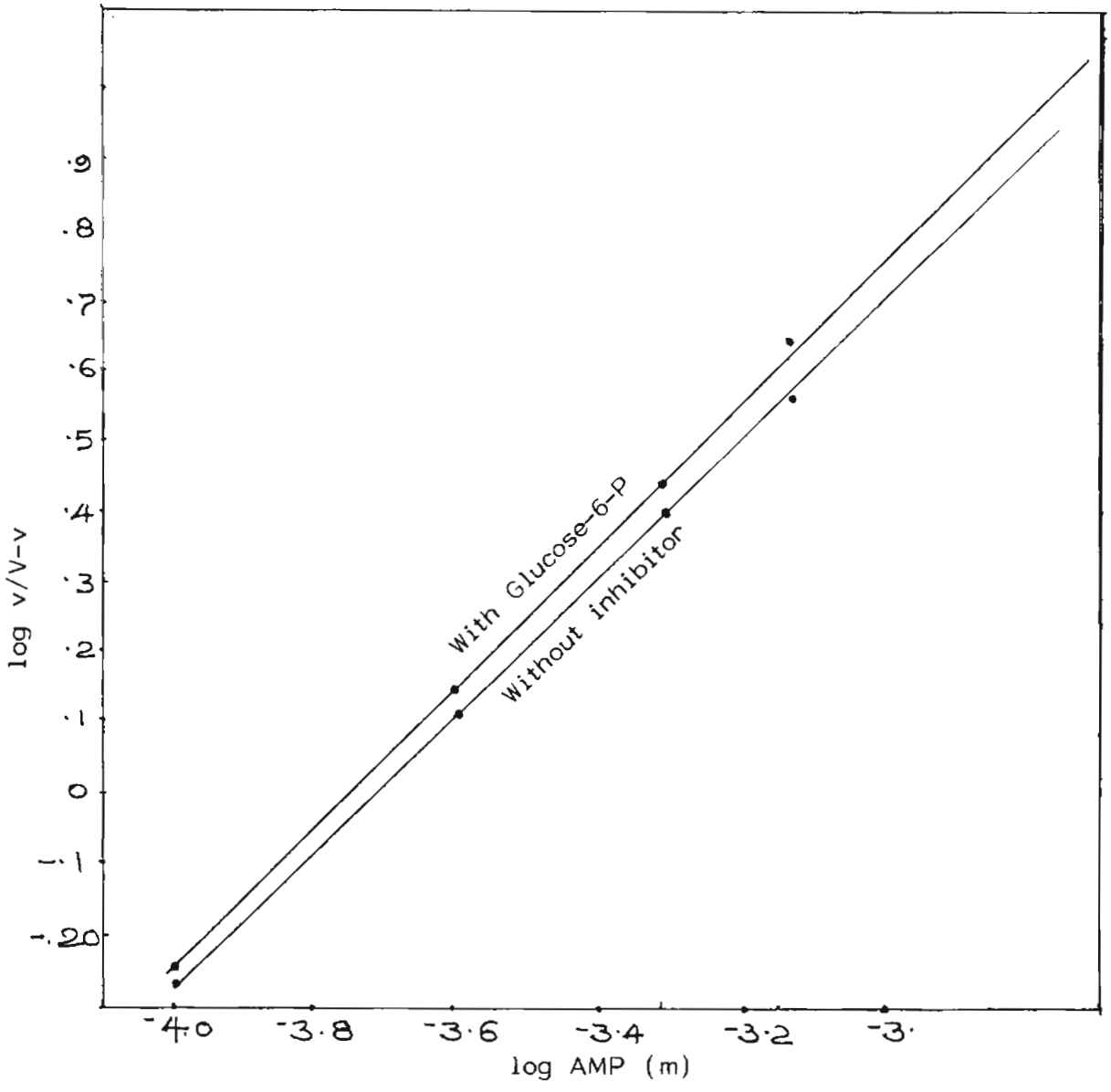

log v/V-v
·9
·8
·7
·6
·5
·4
·3
·2
·1
0
-·1
-·20
With Glucose-6-P
Without inhibitor
-4·0
-3·8
-3·6
-3·4
-3·2
-3·
log AMP (m)

Fig.5-6

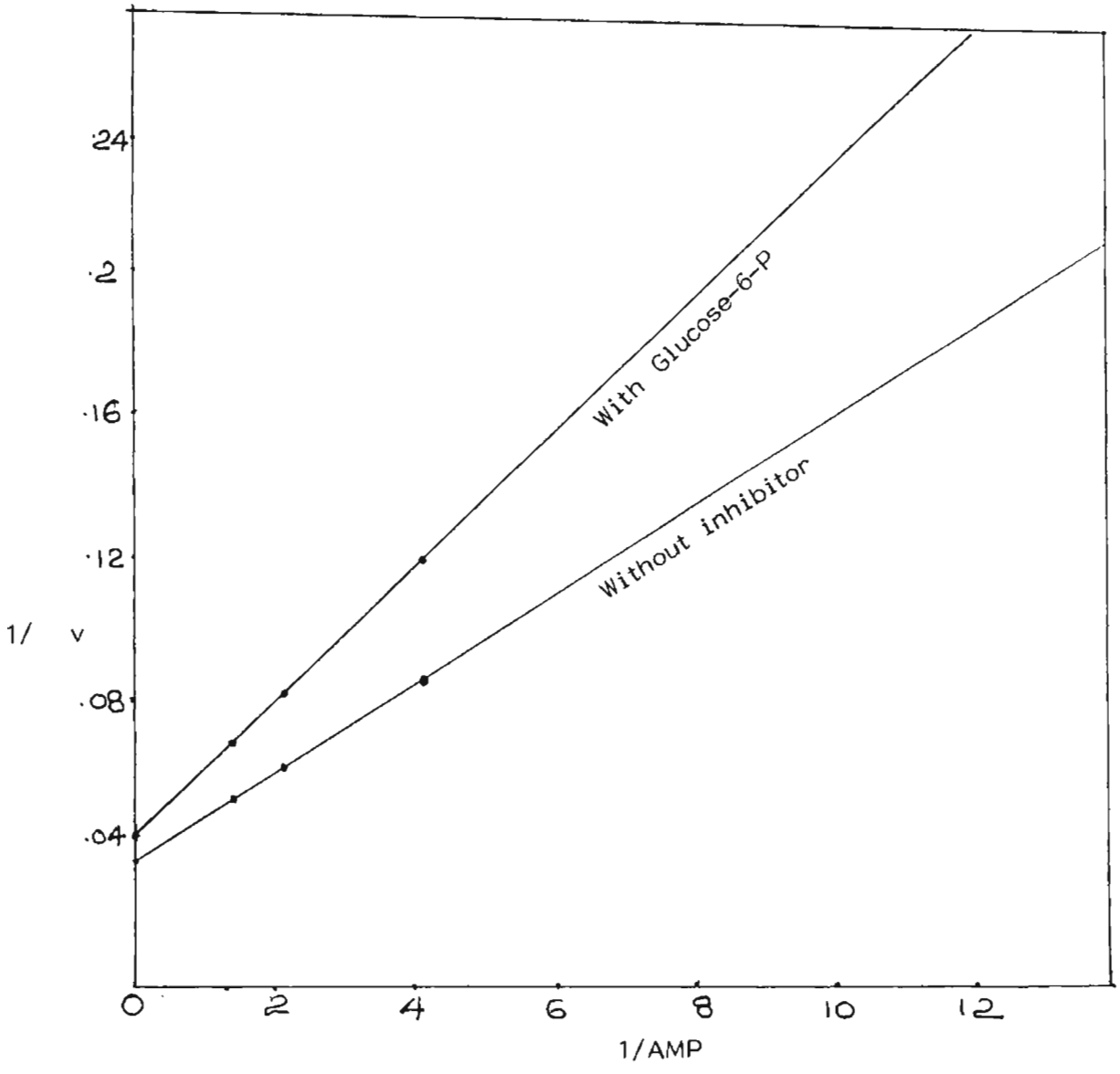

.24
.2
.16
.12
.08
.04
1/ v
With Glucose-6-P
Without inhibitor
0
2
4
6
8
10
12
1/AMP

Fig.5-7

Fig.5-5

(A) Activator saturation curve for Loligo phosphorylase *a* in the absence and presence of glucose-6-P. Phosphorylase was diluted in 10 mM Sodium-ß-glycerophosphate buffer (pH 7) at 30°C prior to assay. To concentration of enzyme in the mixture was 10 μg/ml and that of glycogen and AMP were 0.5 per cent and 1 mM respectively.

(B) Double reciprocal plot for the same data (same symbols)

Fig.5-6

Hill plot for the data of Fig.5-5 (same symbols)

Fig.5-7

Plots of $\frac{1}{\blacktriangle v}$ vs $\frac{1}{AMP}$ where $\blacktriangle v$ is the difference

in activities in the presence and absence of glucose-6-P. Details as in Fig.5-5.

CHAPTER-6

INFLUENCE OF GLUCOSE ON THE KINETICS OF LOLIGO AND SEPIA PHOSPHORYLASES

Rabbit muscle phosphorylase *a* is known to be activated by glucose due to dissociation of phosphorylase *a* to a more active dimer (109, 110, 148). Effect of glucose in the dimer-tetramer equilibrium of phosphorylase *a* was observed as a sigmoidal saturation curve in rabbit phosphorylase (46). The reciprocal plot (Fig.6-1) shows that the Vmax for loligo phosphorylase is low (14.29) in presence of glucose than in its absence (33.33 μ moles/mg/min), at lower glucose-1-P concentration glucose inhibition is clearly evident. The decrease in Vmax can be due to glucose influencing a dimer $\rightleftharpoons$ tetramer equilibrium: (i) under the assay conditions. Phosphorylase *a* is known to be completely dissociated (148) and (ii) glucose-1-P is known to protect the enzyme from glucose induced dissociation and therefore, one would expect less dissociation and less activation at higher glucose concentration.

Glucose inhibition was reported to be overcome by AMP (110). Therefore its effect on the kinetic behaviour was tested in the presence of glucose. The double reciprocal plot in the presence of saturating concentration of AMP with and without glucose is shown in Fig.6-1. These results are typical of mixed inhibition and suggest that glucose interacts with sites other than glucose-1-P site in the presence of AMP. n value of Hill plot (Fig.6-2) is 1.25 with glucose and 1 without glucose. It seems that the inhibitor glucose had a positive co-operativity.

Since Loligo phosphorylase is activated by AMP, it was of interest to compare the influence of glucose on the activity in the presence of varying amounts of AMP. From the reciprocal plots shown in Fig.6-3, it is clear that glucose inhibits

the activity more in lower concentration of AMP than in its higher concentration. Ten mM glucose decreased the activity to a level of 40 per cent, in the presence of 1 mM AMP. The reciprocal plots show that the protection afforded by AMP increases with increasing concentration of AMP. Since the increment in activity due to AMP is more at higher AMP concentration, it is possible that AMP binding brings about a conformational change in the enzyme and that this conformation is less sensitive to glucose. Kinetic analysis of Loligo phosphorylase in presence of glucose shows no increase in n value of Hill plot of data from experiments at varying AMP concentrations (Fig.6-4) and negative co-operativity was observed even in the presence of glucose.

Cori *et al.* (109) and Wang *et al.* (148) have concluded from their studies that glucose competes only for the glucose-1-P sites in rabbit phosphorylase. If glucose was competing only for the site without any other effect, the double reciprocal plot for glucose-1-P in the presence of AMP with glucose (Fig.6-1) should have shown results consistent with such an inhibition. But the results of Loligo phosphorylase show mixed inhibition.

It is likely, therefore, that glucose is either competing or interacting with other sites, glycogen site is a possible one.

$$\text{The reciprocal plots of } \frac{1}{v} \text{ vs } \frac{1}{\text{glucosyl residue}}$$

shows mixed inhibition for the glucose (6-5) and the Hill plot of same data shows no co-operativity (n = 1) (Fig.6-6). However, the effect of glucose in causing a conformational change cannot be ruled out.

Glucose is a potent inhibitor for Loligo enzyme, just as in *Cibium guttatam* (56) and *Etroplus suratensis* phosphorylases (140). Glucose exerts a higher degree of inhibition than glucose-6-P.

In order to compare the nature of glucose inhibition, sepia phosphorylase was tested with 10 mM glucose. Under the same condition of Loligo phosphorylase, sepia showed competitive inhibition (Fig.6-7). Sepia and Loligo are from the same family. Even though both are under the same family, the phosphorylases from both species showed different kinetic properties with respect to glucose. The *n* value of Hill plots (Fig.6-8) with glucose in Sepia phosphorylase was below unity (0.8) which means that there is a negative co-operativity with glucose in the experimental condition where as Loligo phosphorylase showed no co-operativity at all.

The double reciprocal plots for AMP in the presence of glucose was shown in Fig.6-9. The reciprocal plots were non-linear for the whole range of AMP tested and the Hill plots (Fig.6-10) have slopes of 0.5 compared to the value of n = 1.4 for rabbit phosphorylase at lower AMP concentration. Here also, the inhibition was of mixed type and the co-operativity was found to be negative.

The reciprocal plots of Sepia phosphorylase with and without glucose in varying concentrations of glycogen showed competitive type of inhibition (Fig.6-11), where as Loligo enzyme showed mixed type of inhibition. *n* value of Hill plots of the same data was unity which indicates the lack of co-operativity (Fig.6-12).

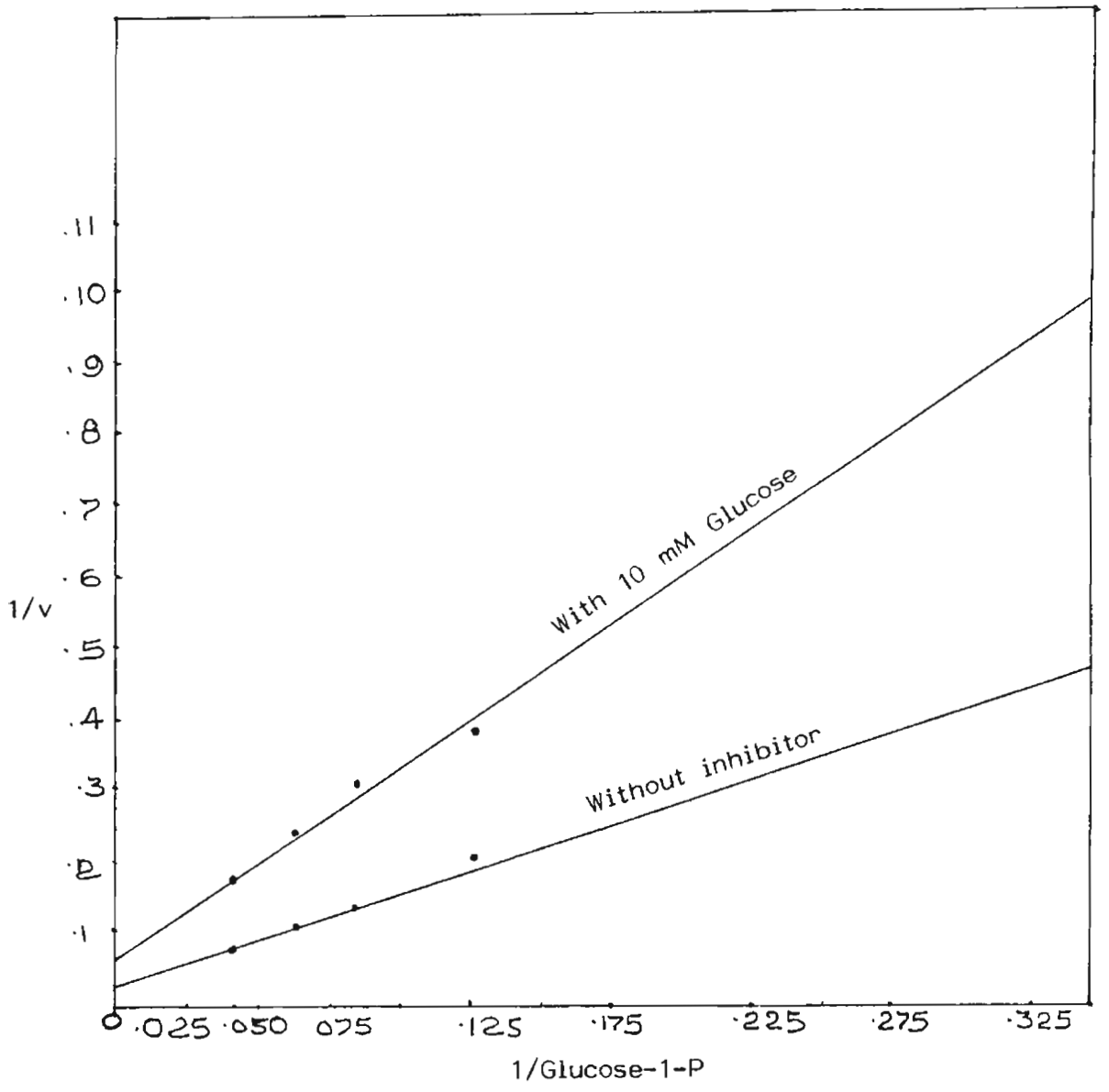

1/v
.11
.10
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
.025
.050
.075
.125
.175
.225
.275
.325
1/Glucose-1-P
With 10 mM Glucose
Without inhibitor

Fig.6-1

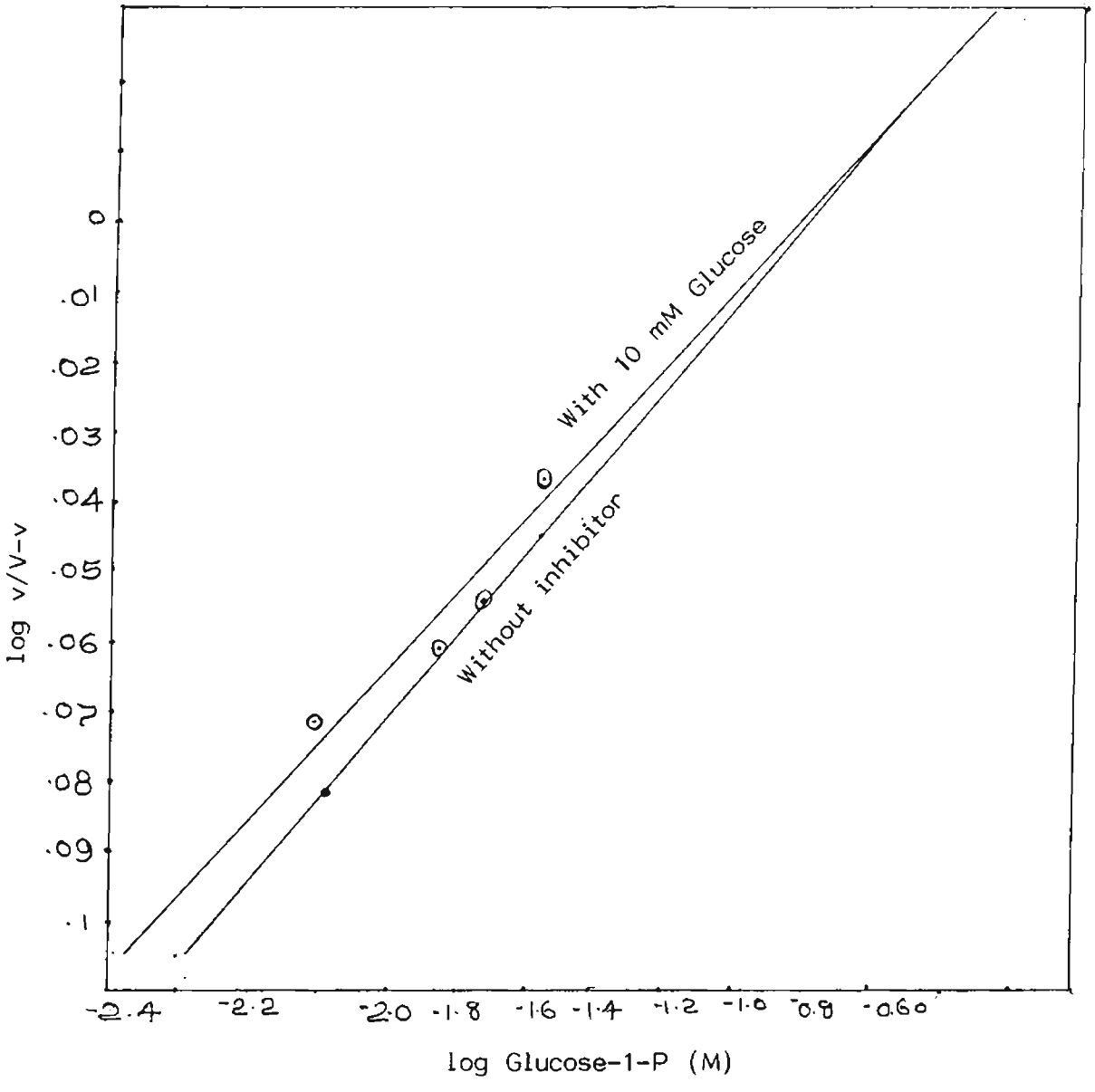

With 10 mM Glucose
Without inhibitor
log v/V-v
0
.01
.02
.03
.04
.05
.06
.07
.08
.09
.1
-2.4
-2.2
-2.0
-1.8
-1.6
-1.4
-1.2
-1.0
-0.8
-0.60
log Glucose-1-P (M)

Fig.6-2

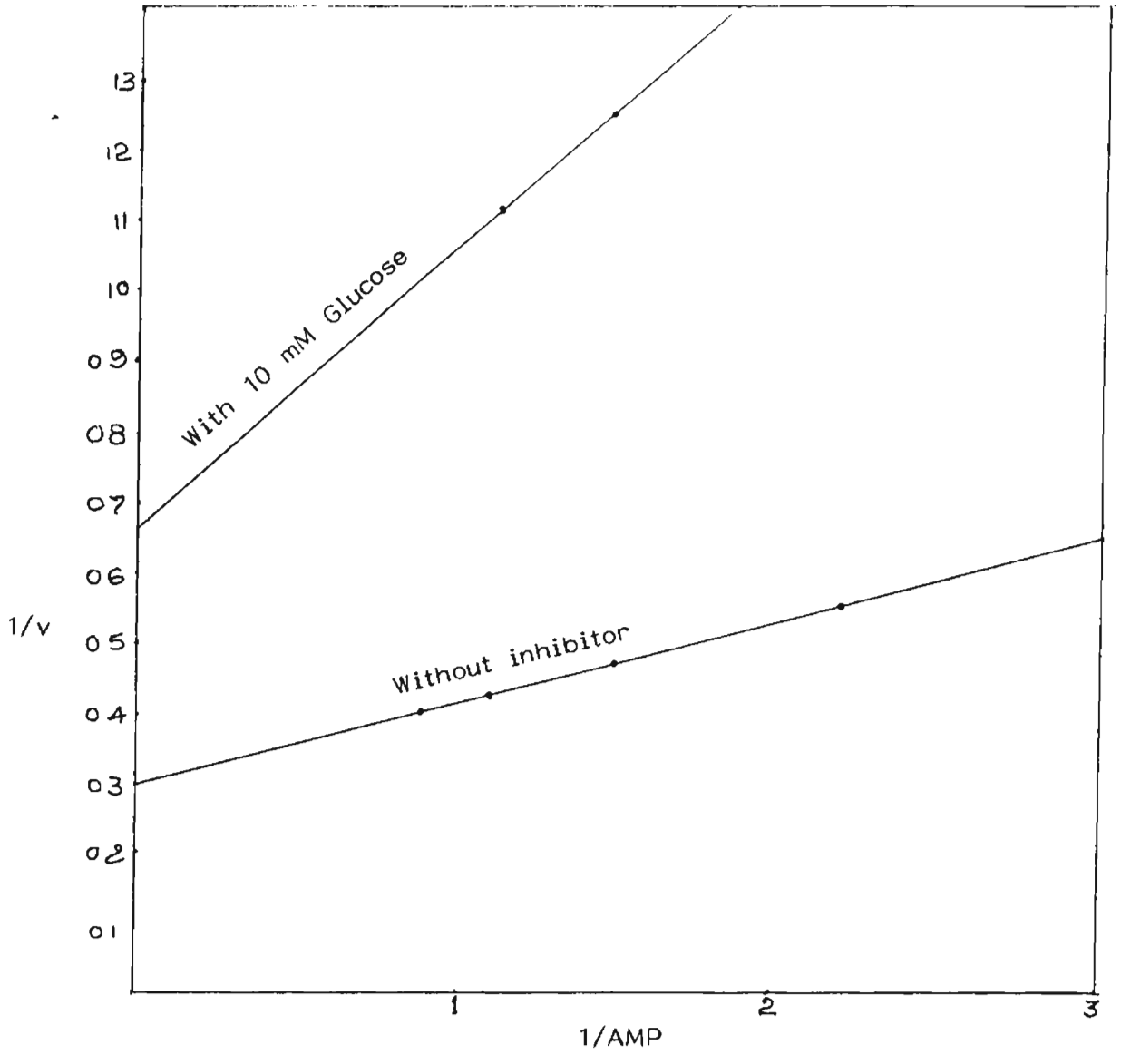

1/v
13
12
11
10
09
08
07
06
05
04
03
02
01
With 10 mM Glucose
Without inhibitor
1
2
3
1/AMP

Fig.6-3

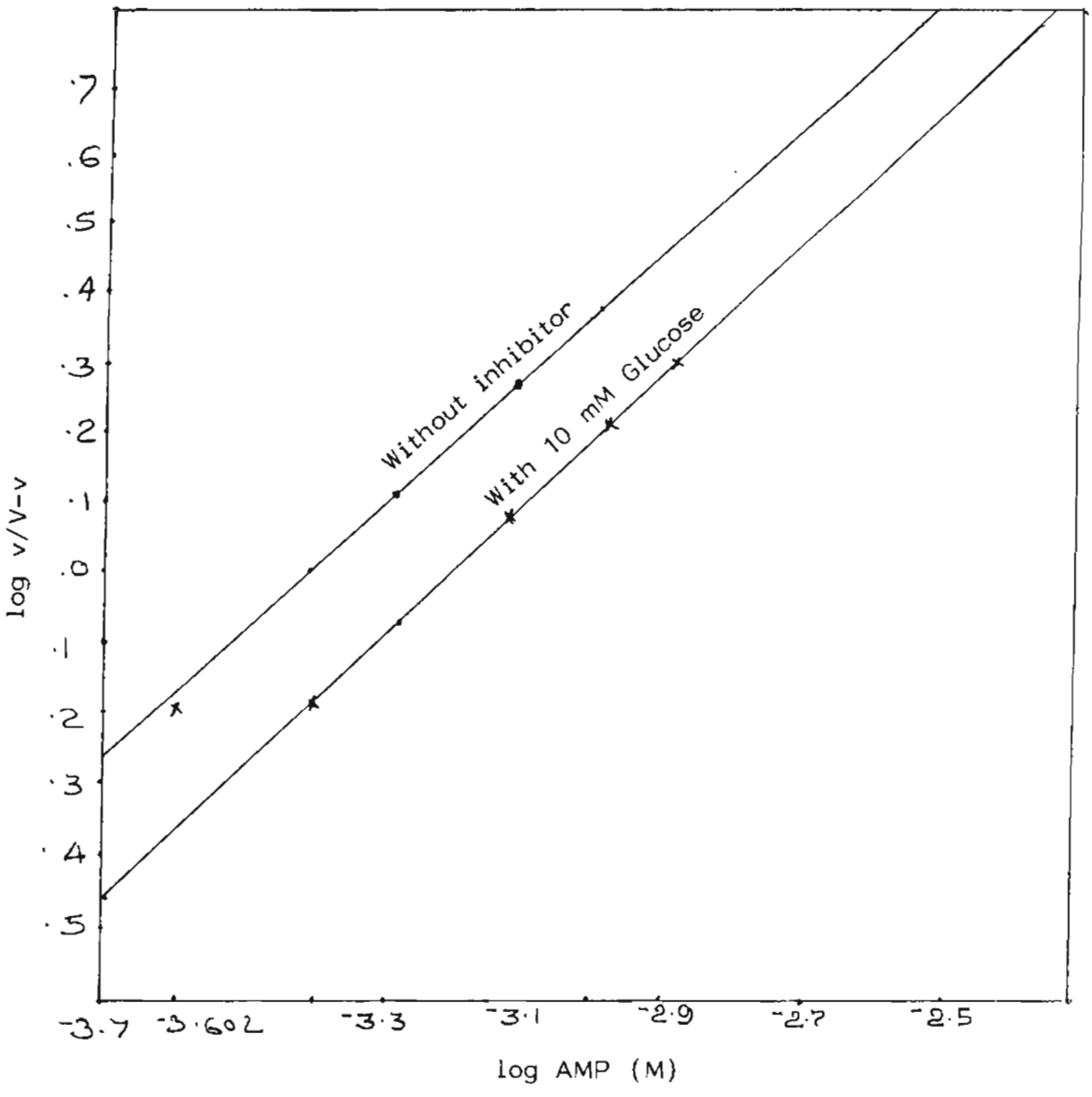

Without inhibitor
With 10 mM Glucose
log v/V-v
·7
·6
·5
·4
·3
·2
·1
·0
-·1
-·2
-·3
-·4
-·5
-3.7
-3.602
-3.3
-3.1
-2.9
-2.7
-2.5
log AMP (M)

Fig.6-4

Fig.6-1

Reciprocal plot for glucose-1-P for Loligo phosphorylase *a* in the presence of glucose and AMP. Enzyme solution was diluted with 10 mM Sodium-ß-glycerophosphate buffer and assayed with 1 mM AMP and varying concentrations of glucose-1-P in the presence of glucose and absence of glucose. Incubation of reaction mixture was at 30°C for 10 minutes.

Fig.6-2

Hill plot of the data from Fig.6-1

Fig.6-3

Reciprocal plot for AMP for Loligo phosphorylase *a* in presence of glucose and AMP. Enzyme was diluted with 10 M Sodium-ß-glycerophosphate buffer (pH 7) and assayed with 16 mM glucose-1-P and varying concentrations of AMP in the presence of 10 mM glucose and absence of glucose. Incubation of the reaction mixture was at 30°C for 10 minutes.

Fig.6-4

Hill plot of the data from Fig.6-3

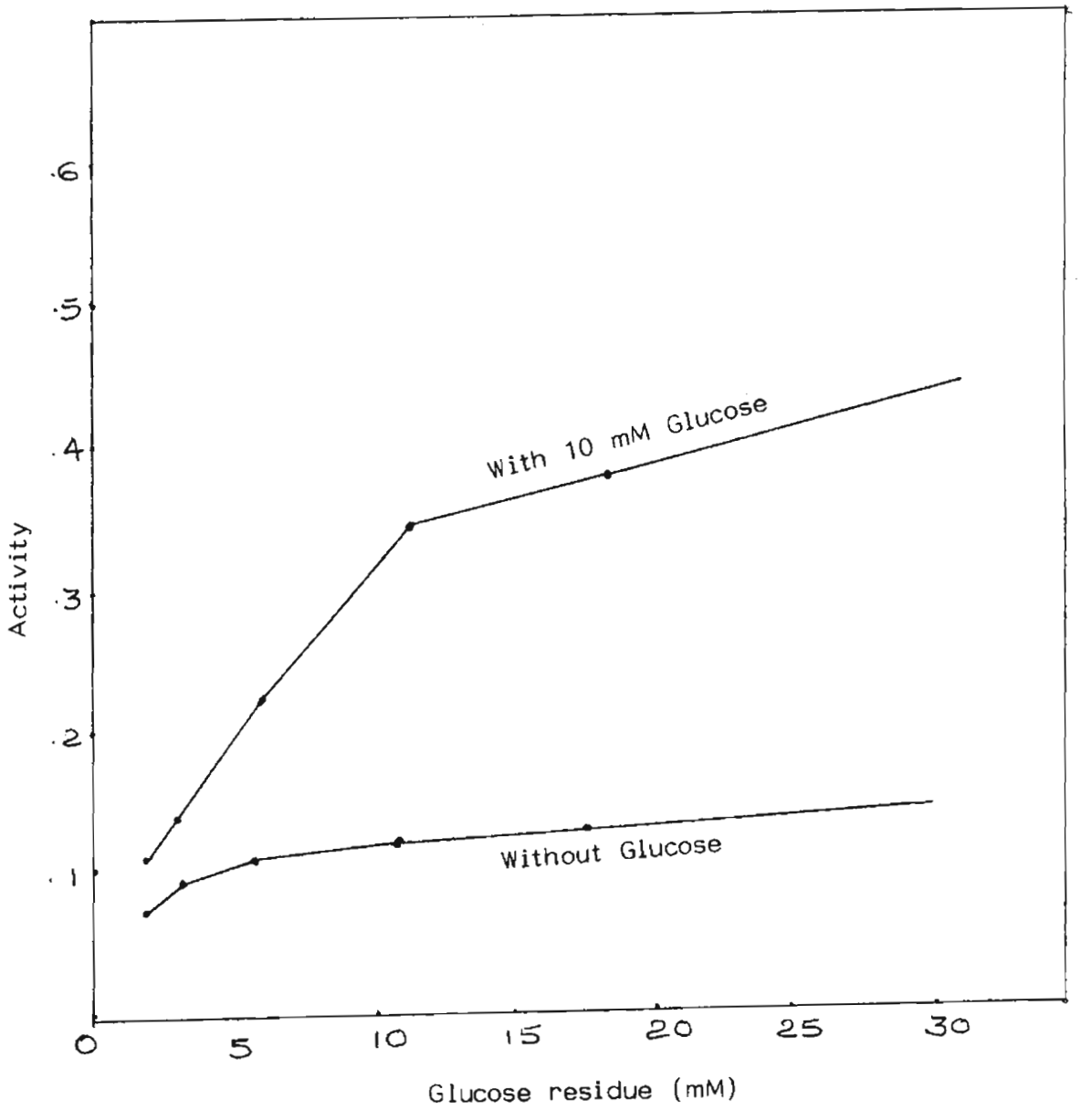

Activity
.6
.5
.4
.3
.2
.1
With 10 mM Glucose
Without Glucose
0
5
10
15
20
25
30
Glucose residue (mM)

Fig.6-5 A

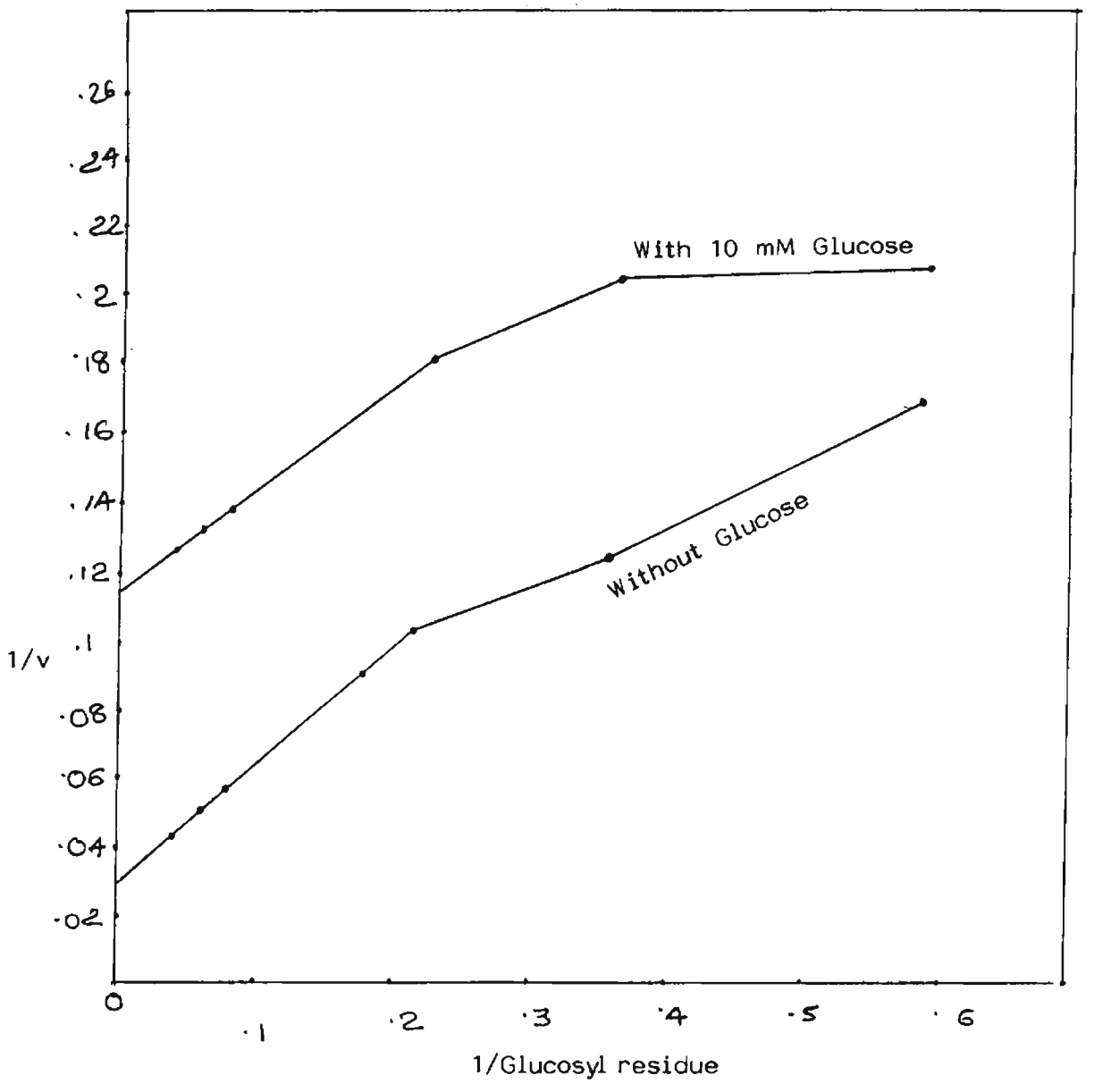

With 10 mM Glucose
Without Glucose
1/v
.26
.24
.22
.2
.18
.16
.14
.12
.1
.08
.06
.04
.02
0
.1
.2
.3
.4
.5
.6
1/Glucosyl residue

Fig.6-5 B

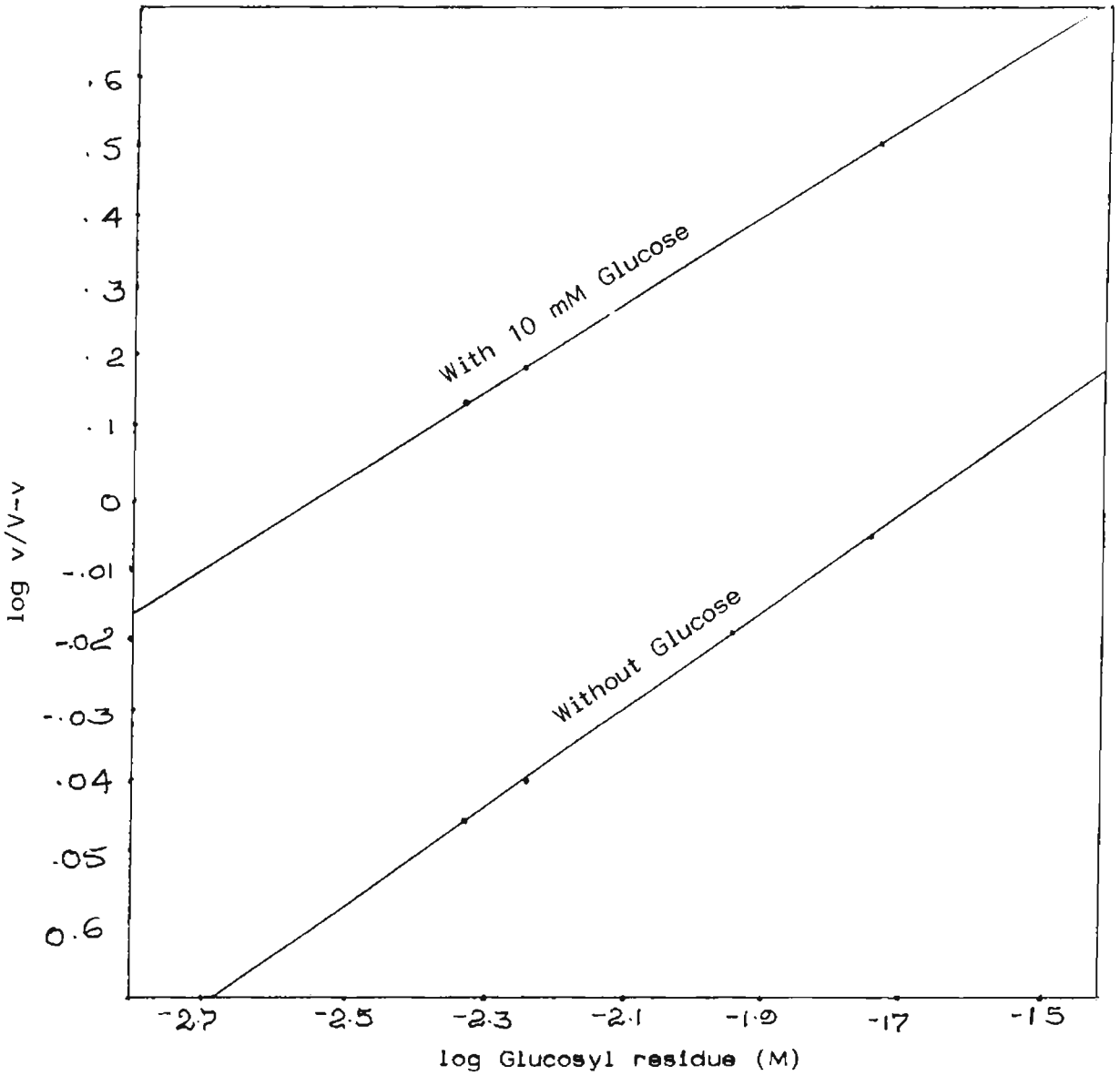

log v/V-v
·6
·5
·4
·3
·2
·1
0
-·01
-·02
-·03
·04
·05
0·6
With 10 mM Glucose
Without Glucose
-2·7
-2·5
-2·3
-2·1
-1·9
-17
-15
log Glucosyl residue (M)

Fig.6-6

Fig.6-5A

Glycogen saturation curve for Loligo phosphorylase activity. Enzyme was diluted in 10 mM Sodium-ß-glycerophosphate buffer (pH 7) and assayed with 1 mM AMP and varying concentrations of glycogen in the presence and absence of glucose. Incubation of reaction mixture was done at 30°C for 10 minutes.

Fig.6-5B

Reciprocal plot for glycogen for Loligo phosphorylase *a* in the presence of glucose and AMP. Enzyme solution was diluted with 10 mM Sodium-ß-glycerophosphate buffer (pH 7) and assayed with 1 mM AMP and varying concentrations of glycogen in the presence and absence of 10 mM glucose. Incubation of reaction mixture was done at 30°C for 10 minutes.

Fig.6-6

Hill plot of the data from Fig.6-5

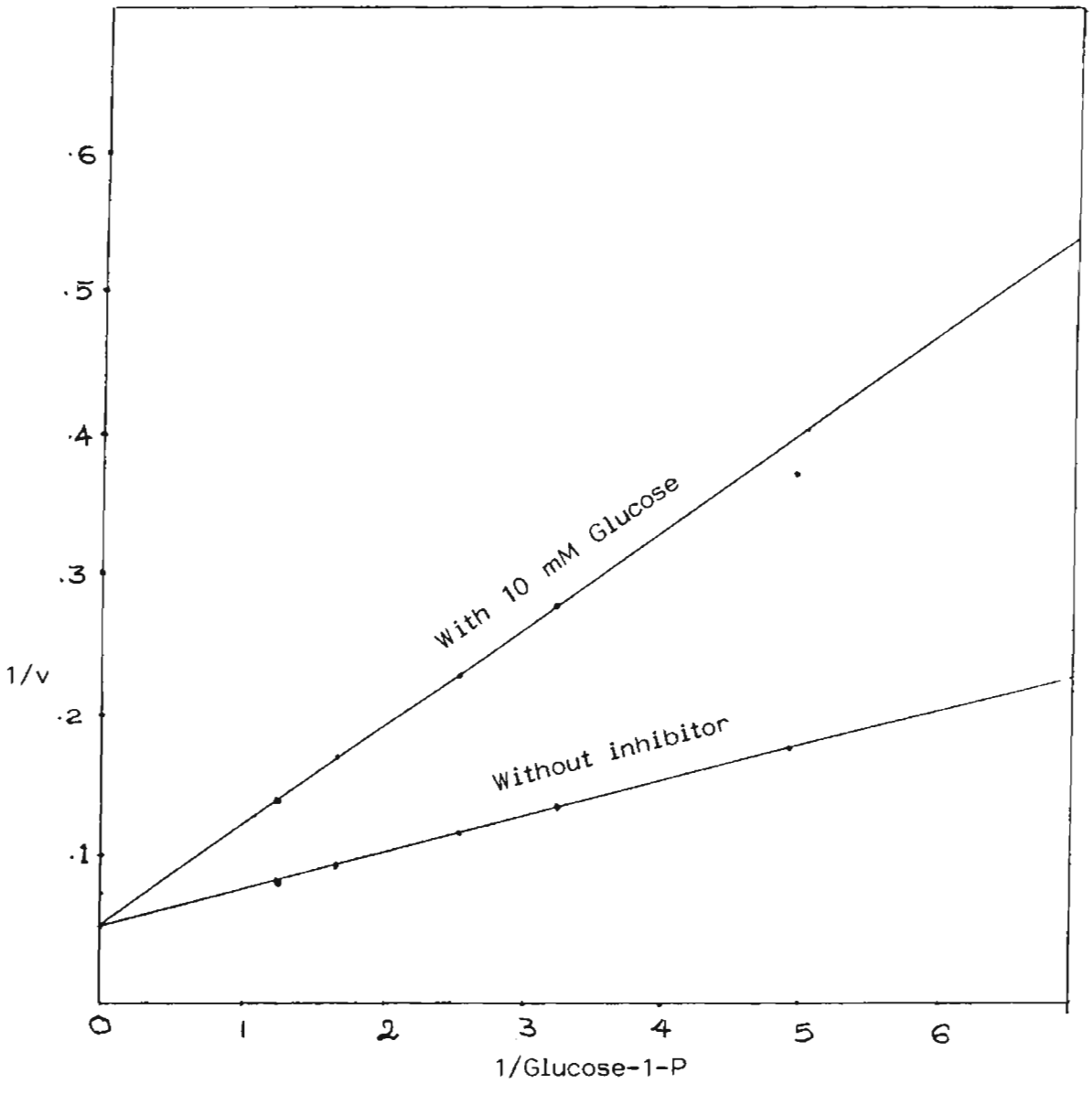

.6
.5
.4
.3
.2
.1
1/v
With 10 mM Glucose
Without inhibitor
0
1
2
3
4
5
6
1/Glucose-1-P

Fig.6-7

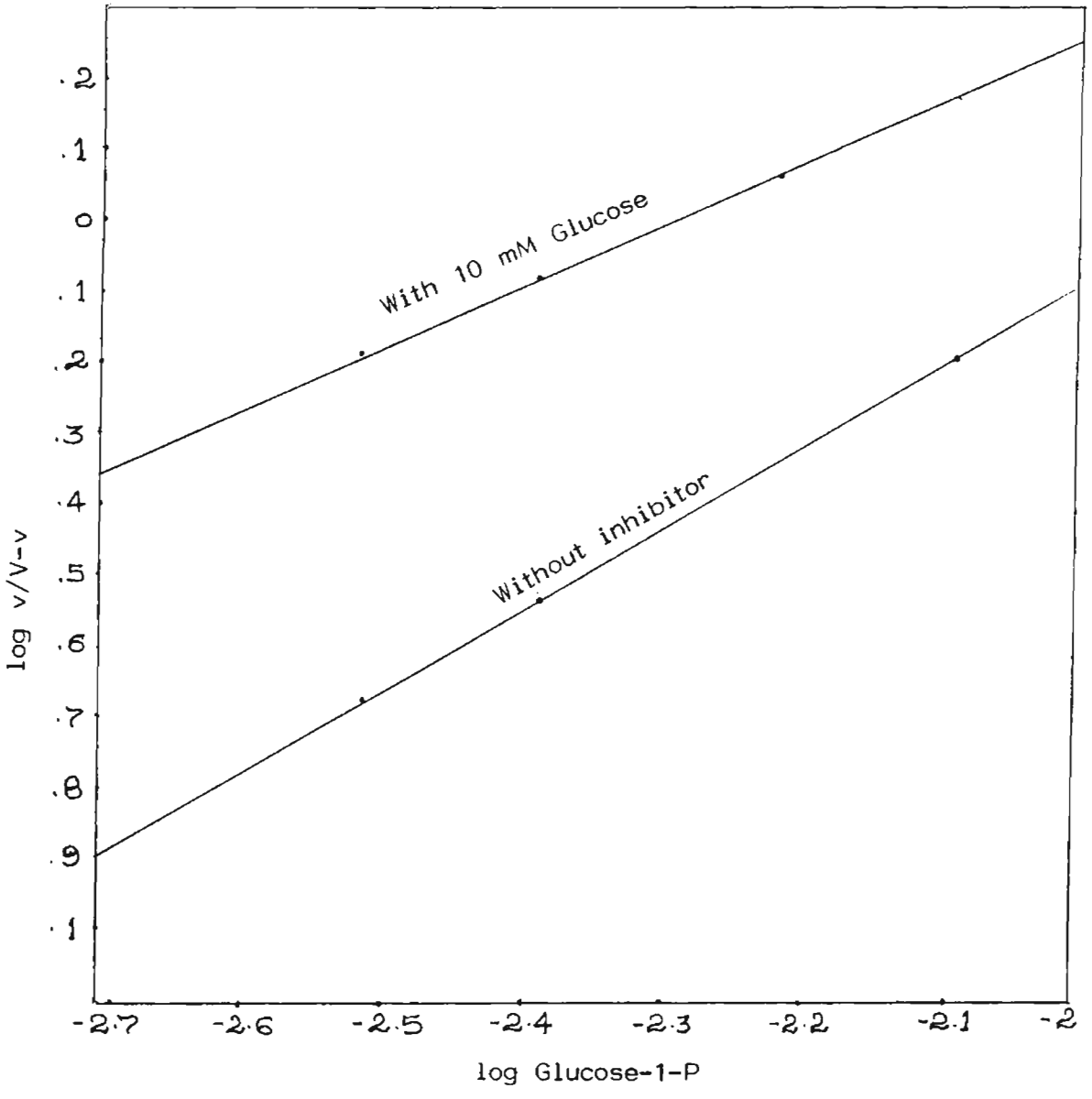

With 10 mM Glucose
Without inhibitor
log v/V-v
.2
.1
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1
-2.7
-2.6
-2.5
-2.4
-2.3
-2.2
-2.1
-2
log Glucose-1-P

Fig.6-8

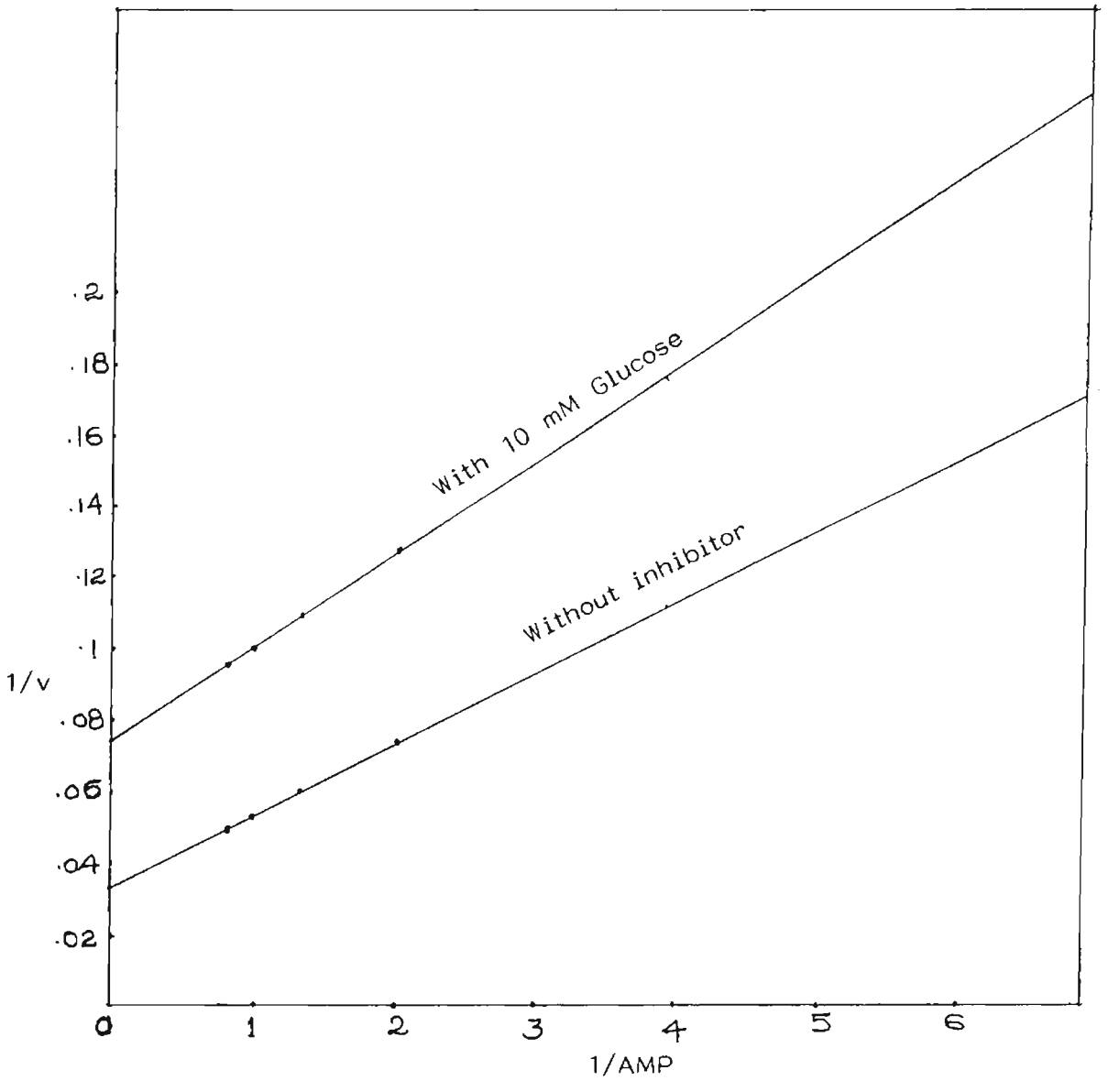

.2
.18
.16
.14
.12
.1
.08
.06
.04
.02
1/v
With 10 mM Glucose
Without inhibitor
0
1
2
3
4
5
6
1/AMP

Fig.6-9

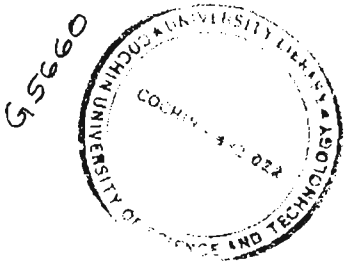


Fig.6-7

Reciprocal plot for glucose-1-P for Sepia phosphorylase *a* in the presence of glucose and AMP. Enzyme solution was diluted with 10 mM Sodium-ß-glycerophosphate buffer and assayed with 1 mM AMP and varying concentrations of glucose-1-P in the presence and absence of glucose. Incubation of reaction mixture was at 30°C for 10 minutes.

Fig.6-8

Hill plots of the data from Fig.6-7

Fig.6-9

Reciprocal plots for AMP for Sepia phosphorylase *a* in presence and absence of glucose. Enzyme was diluted with 10 mM Sodium-ß-glycerophosphate buffer (pH 7) and assayed with 16 mM glucose-1-P, 0.5 per cent glycogen and varying concentrations of AMP in the presence and absence of 10 mM glucose. Incubation of the reaction mixture was at 30°C for 10 minutes.

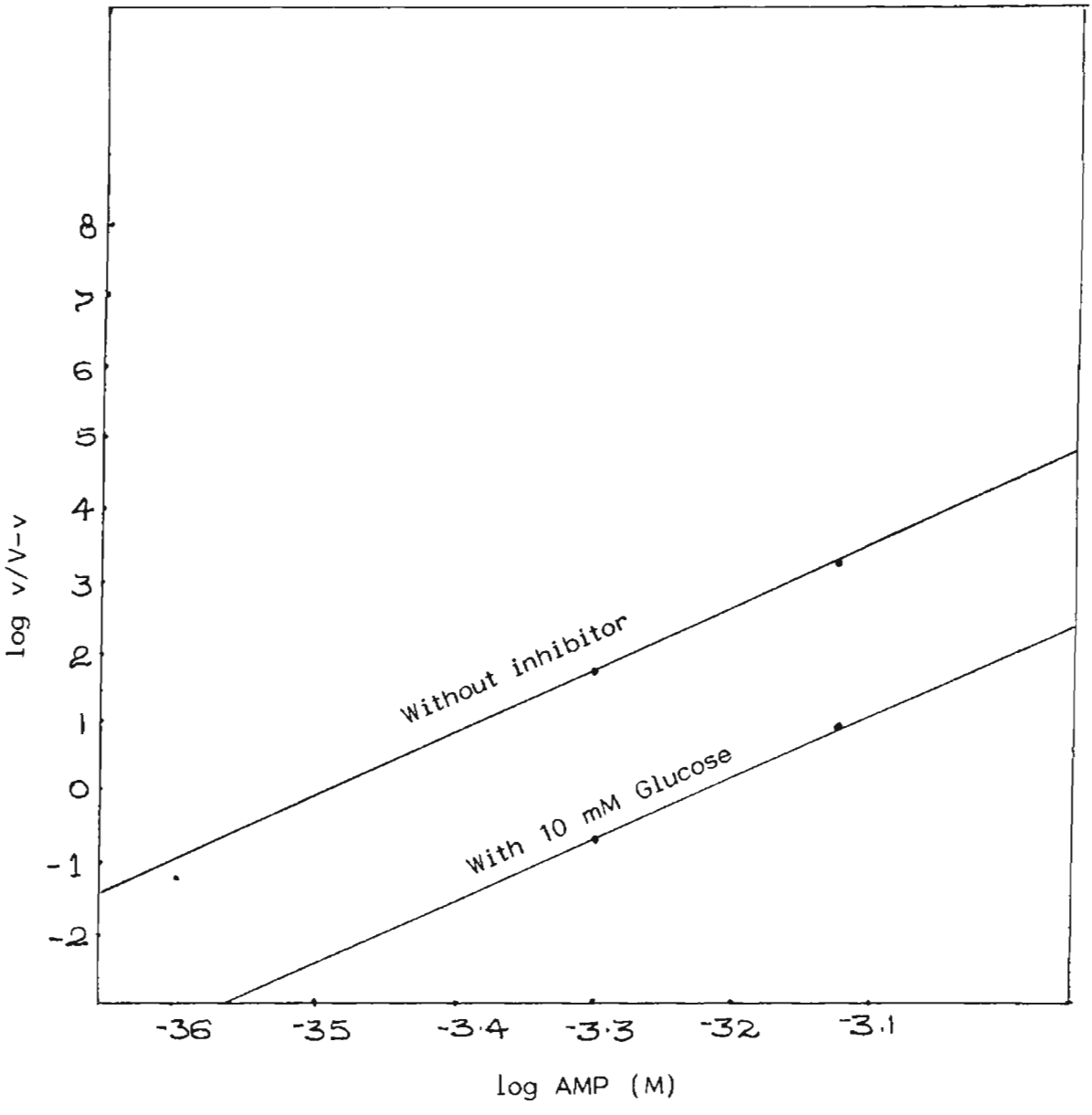

log v/V-v
8
7
6
5
4
3
2
1
0
-1
-2
Without inhibitor
With 10 mM Glucose
-3·6
-3·5
-3·4
-3·3
-3·2
-3·1
log AMP (M)

Fig.6-10

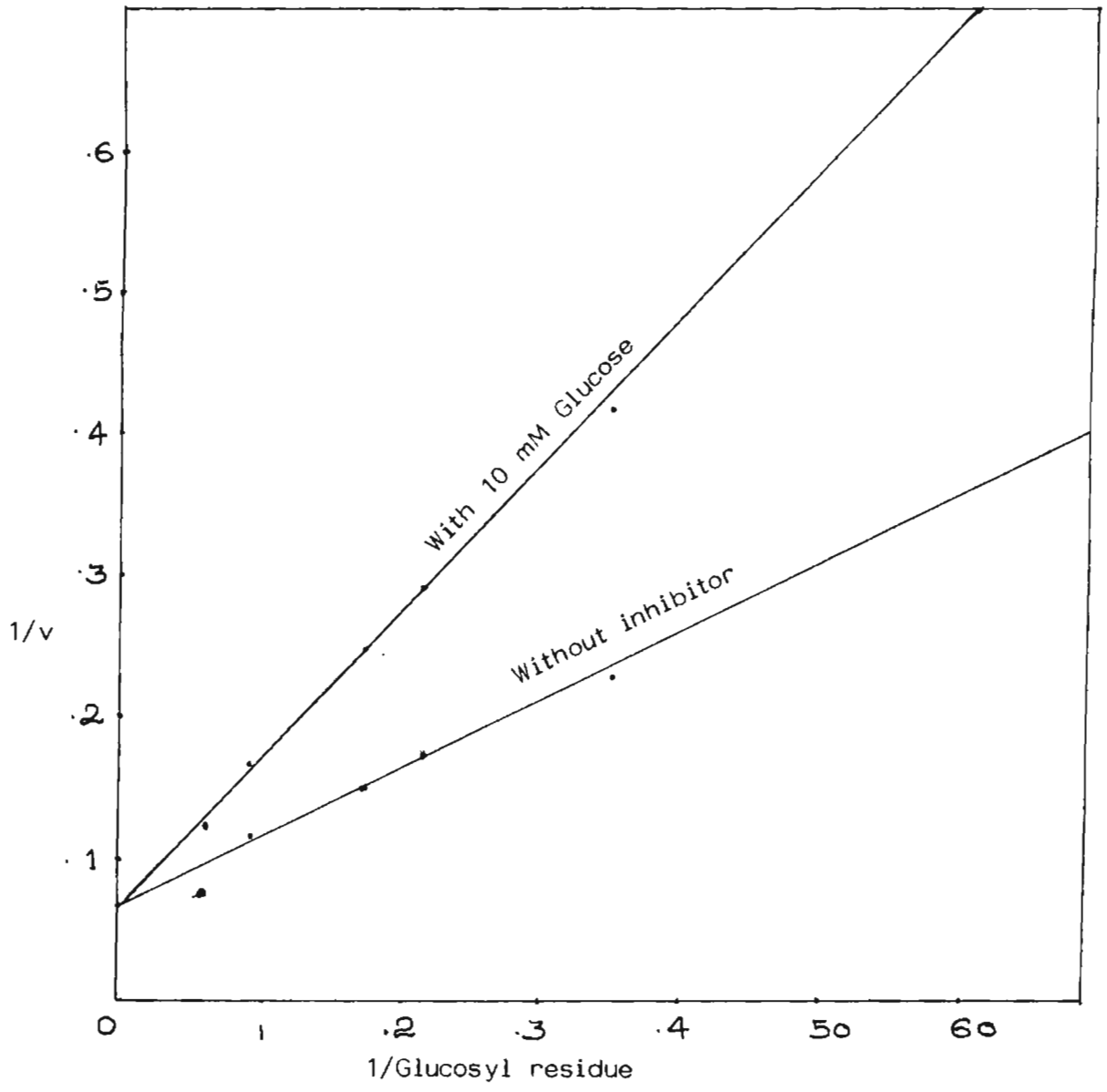

1/v
·6
·5
·4
·3
·2
·1
With 10 mM Glucose
Without inhibitor
0
1
·2
·3
·4
50
60
1/Glucosyl residue

Fig.6-11

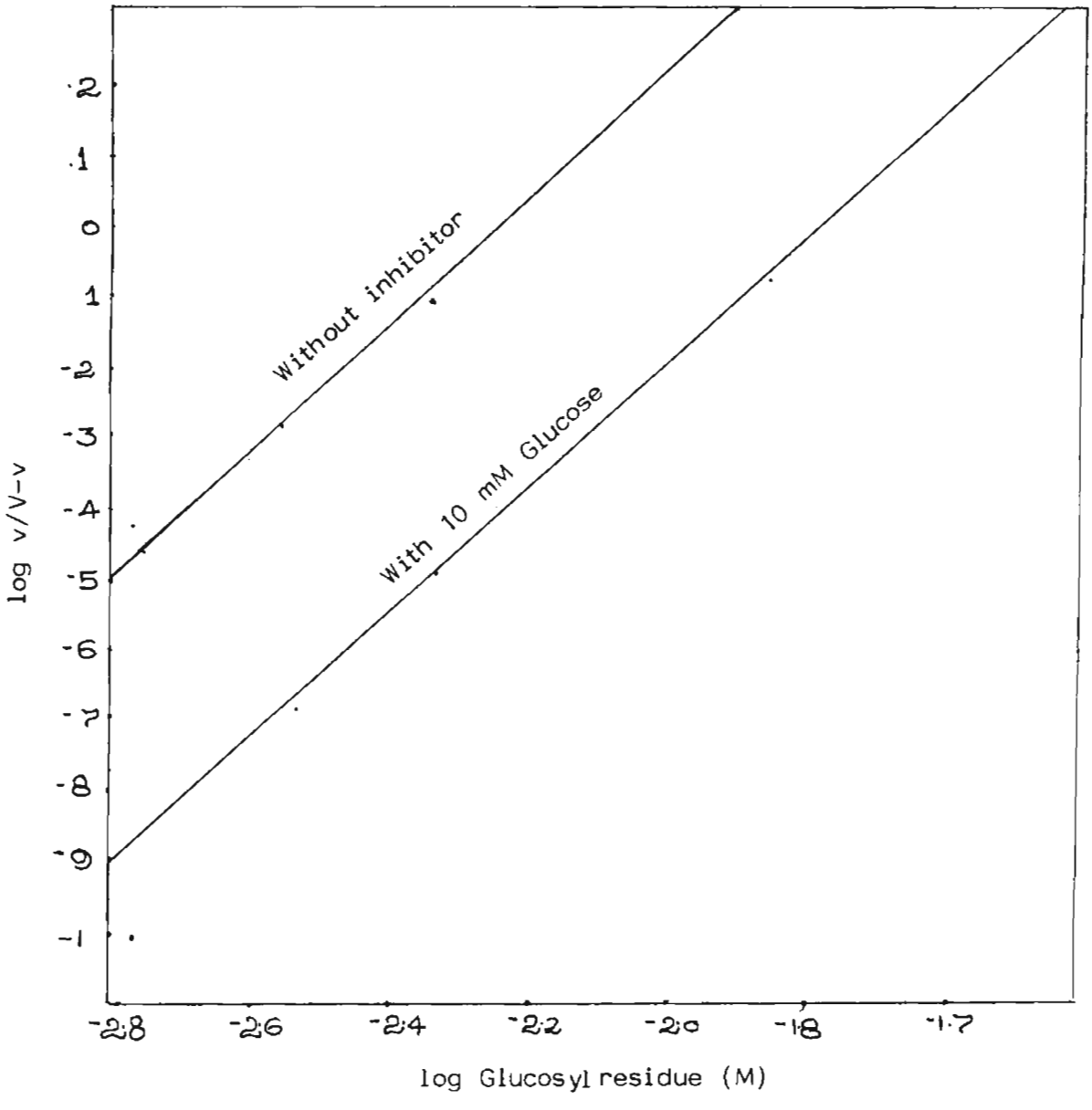

Without inhibitor
With 10 mM Glucose
log v/V-v
log Glucosyl residue (M)
-2.8
-2.6
-2.4
-2.2
-2.0
-1.8
-1.7

Fig.6-12

Fig.6-10

Hill plot of the data from Fig.6-9

Fig.6-11

Reciprocal plots of glycogen for Sepia phosphorylase *a* in presence and absence of glucose. Enzyme was diluted with 10 mM Sodium-ß-glycerophosphate buffer (pH 7) and assayed with 16 mM glucose-1-P, 1 mM AMP and varying concentrations of glycogen in the presence and absence of glucose. Incubation of the reaction mixture was at 30°C for 10 minutes.

Fig.6-12

Hill plots of the data from Fig.6-11

CHAPTER-7

INHIBITION OF LOLIGO PHOSPHORYLASE *a* BY PARANITROPHENOL (PNP) AND PARANITROPHENYL PHOSPHATE (PNPP)

Glycogen phosphorylase has been shown to be inhibited by aromatic compounds (87). The relative effectiveness of the various inhibitors has been shown to be dependent on the hydrophobicity of the aromatic compounds (88). AMP has been shown to bind on a hydrophobic region on phosphorylase *b* near the monomer/monomer interface where the binding sites for glucose-1-P, glucose-6-P and ATP are also located (42, 149). Eventhough PNP and PNPP are structurally dissimilar to the substrate, activator and inhibitors of phosphorylase, they have been shown to be more effective inhibitors than the naturally occuring allosteric inhibitor glucose-6-P (22). Chemical modification of apparently the same kind of amino acid residues of phosphorylase *b* with different reagents has been shown to yield partially active enzyme derivatives with distinctly different and some times contradictory catalytic and allosteric properties (41, 94, 95, 97).

This chapter deals with the studies on Loligo phosphorylase *a* to delineate the mechanism of inhibition by PNP and PNPP.

The influence of varying concentrations of glucose-1-P on loligo phosphorylase *a*, in the presence and absence of PNP and PNPP are shown in Fig.7-1A. The figure shows that with the loligo enzyme in the presence and absence of PNP or PNPP, the dependence of initial velocity on glucose-1-P concentration is hyperbolic. The same data is shown in Fig.7-1B in the form of Lineweaver-Burk-plot. The presence of PNP and PNPP may be seen here as having the effect of inducing linear plots.

The data of Fig.7-1 is given in the form of Hill plots in Fig.7-2. The loligo enzyme gives a Hill coefficient of unity, indicating lack of co-operativity of glucose-1-P sites in the presence and absence of PNP or PNPP.

Linear plots were also obtained for $\frac{1}{\blacktriangle v}$ vs $\frac{1}{\text{G-T-P}}$ plots (Fig.7-3), ▲v being the difference in the rate in the presence and absence of AMP. In all these cases, the inhibition was of mixed type. From the graph, it can be observed that $\frac{1}{\blacktriangle v}$ is lower in the higher concentration of G-1-P. The decrease in activity ratio during increase in substrate concentration can be attributed to the higher effectiveness of these inhibitors in the absence of Glucose-1-P (Table 7-1).

The influence of varying concentrations of AMP on Loligo phosphorylase *a* in the presence and absence of PNP and PNPP are shown in Fig.7-4A. The figure shows that with the Loligo emzyme in the absence of PNP or PNPP, the dependence of initial velocity on AMP concentration is hyperbolic. The same data is shown in Fig.7-4B in the form of Linewear-Burkplot, linearity can only be obtained with limited range of AMP concentration. Km and Vmax are varying at different range of AMP concentration. AMP at different concentration gives a Hill coefficients of 0.22 and 0.31 (Fig.7-5), indicating that under these conditions also Loligo enzyme shows negative co-operativity.

Activity ratio of Loligo phosphorylase *a* in the absence of AMP to that of in presence at saturating concentration of AMP (1 mM) was about 0.30 at 16 mM glucose-1-P under the assay condition. The order of effectiveness of the inhibition was PNP > PNPP.

Table 7-1. Activity ratio of Loligo phosphorylase *a* at different levels of glucose-1-phosphate calculated from the data of Fig.7-1B

Concentration of Glucose-1-P mM	Activity ratio, v in presence of 5 mM		
	Control	PNP	PNPP
3	0.41	0.53	0.70
4	0.35	0.47	0.62
6	0.34	0.43	0.50
8	0.33	0.43	0.52
12	0.31	0.45	0.41
16	0.34	0.50	0.51
24	0.37	0.47	0.47
32	0.37	0.48	0.53

Loligo phosphorylase *a* was less inhibited by all inhibitors tested in the presence of AMP than in its absence. This protection by AMP was seen with all inhibitors although the degree of protection varied. Protection of inactivation by AMP during reaction by the aromatic reagent fluorodinitrobenzene was reported (95). Similar protection was observed with difluorodinitrobenzene also (22). The activity ratios at different levels of glucose-1-P and AMP in the presence and absence of PNP and PNPP are shown in Table 7-1 and 7-2. The data shows that the activity ratios are higher in the presence of the inhibitor at all levels of glucose-1-P tested, indicating no protection by AMP against inhibitor. This suggests that a Loligo phosphorylase *a*, PNP and PNPP bind at a site other othan AMP site. Kinetic mechanism of Loligo phosphorylase *a* is rapid equilibrium random *bi bi*. For such a system the linear reciprocal plots in the presence of mixed inhibitors indicated the presence of more than one inhibitor binding site. In a multisubstrate system like phosphorylase, the mixed inhibition could be complicated. Since the rate of breakdown of the ES complex to products k_{+2} is negligible as compared to k_{-1} in the case of phosphorylase (30), the mixed inhibition could be due to the effect of the inhibitor on k_{+2}. Thus the variation in the rate of reaction is directly related to the binding of the inhibitors, substrates and activators.

The n value of Hill plots of PNP and PNPP were same and hence an additional phosphate on the aromatic ring had no influence on the co-operativity. Accordingly, simultaneous binding of more than one molecule of the nitrophenol but not the nitrophenyl phosphate was possible.

It may be noted that in contrast to the rabbit enzyme, the reciprocal plots are linear with Loligo enzyme. No co-operativity of the substrate site in Loligo

Table 7-2. Activity ratio of Loligo phosphorylase *a* at different levels of AMP (calculated from the data of Fig.7-4B)

Concentration of AMP mM	Activity ratio, v in presence of 5 mM		
	Control	PNP	PNPP
0.025	0.84	0.75	1.00
0.050	0.60	0.64	1.00
0.075	0.47	0.56	1.00
0.100	0.41	0.53	1.00
0.250	0.33	0.45	0.75
0.500	0.31	0.41	0.70
0.750	0.27	0.37	0.67
1.000	0.26	0.36	0.63

phosphorylase was induced by PNP or PNPP. The inhibition of Loligo phosphorylase *a* by PNP or PNPP are of mixed type. Apparent Km values in presence of PNP and PNPP were lower than the Km value without PNP and PNPP suggesting that affinity was increasing in presence of PNP and PNPP. The figures also point out that the inhibition by PNP and PNPP were due to mixed type inactivation of the enzyme and not pure competition for substrate or activator. The influence of PNP and PNPP in affecting allosteric transition in Loligo enzyme suggest conformational changes caused by the binding of inhibitor. Conformational changes are producing the 'productive' and 'nonproductive modes'.

X-ray crystallographic analysis by Johnson *et al.* (73) supports the concept of binding of ligands in 'productive' and 'nonproductive modes'. When the concentration of p-nitrophenol was increased to 0.01 M in the assay mixture the loligo enzyme was found to be completely inactived. p-nitrophenol was added to 2 mg/ml solution of loligo phosphorylase *a* until turbidity developed, which disappeared on keeping. On dilution the diluted enzyme was devoid of any enzyme activity. The inactivation did not appear to be due to protein denaturation followed or preceeded by dissociation and aggregation.

Therefore the following conclusion may be made from the results presented in this chapter (i) Increase in activity ratio at all levels of glucose-1-P, suggested that there is no protection by AMP against inhibition. So binding of PNP and PNPP in Loligo phosphorylase *a* is at a site other than AMP site. Decrease in activity ratio during increase in substrate concentration can be attributed to the higher effectiveness of these inhibitors in the absence of glucose-1-P. (ii) Since the binding sites are overlapping, PNP, PNPP, glucose-1-P, AMP and glucose-6-P may bind on the region located near the monomer/monomer interface. This is possible only if the ligands adopt different modes of binding, the modes being determined by their struc-

tural features. (iii) The inhibition of PNP or PNPP are not time dependent whereas modifications by the aromatic reagents have been shown to be time dependent (95, 97). Therefore, these reagents undoubtedly bind on the enzyme prior to reaction.

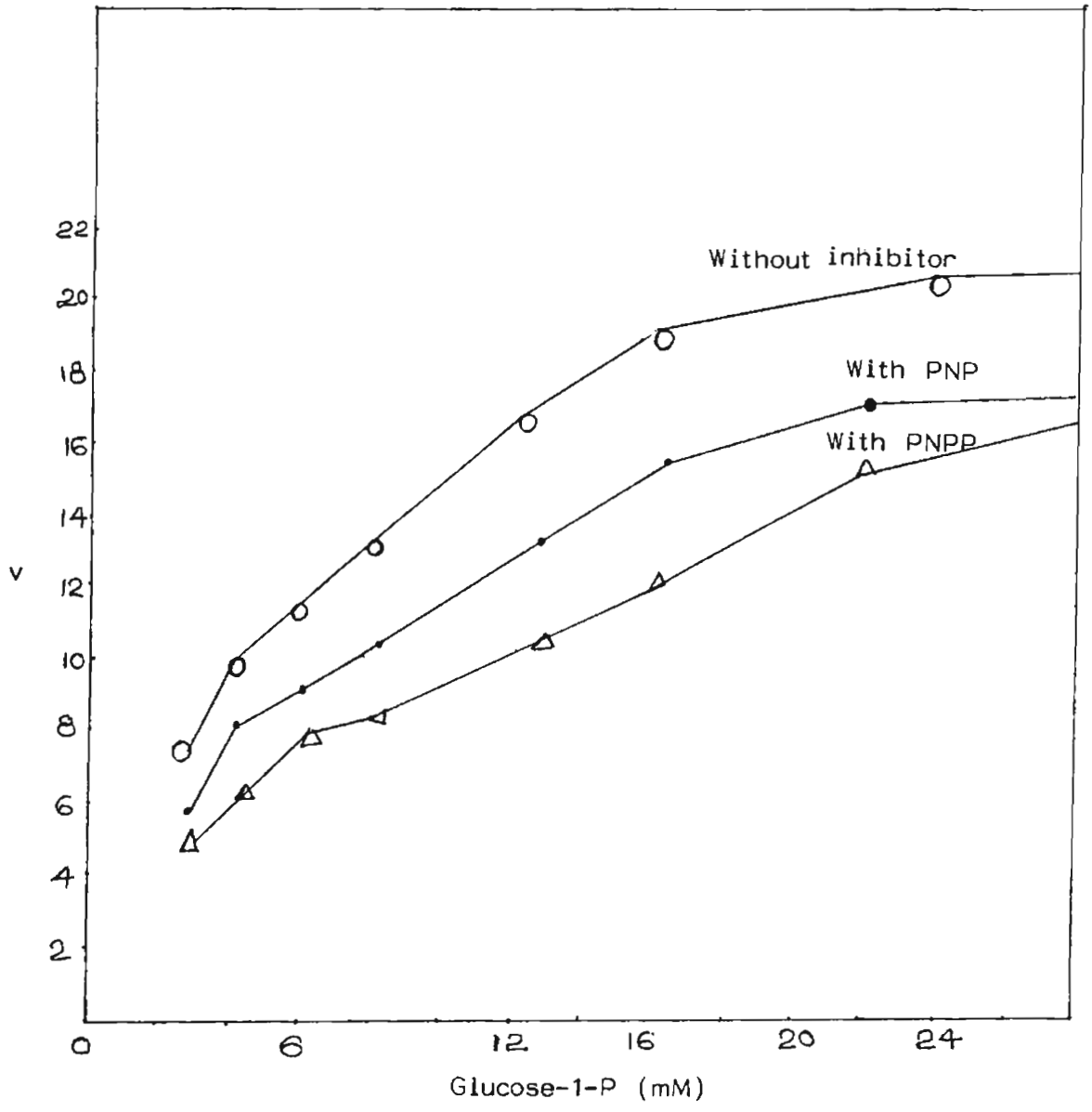

Without inhibitor
With PNP
With PNPP
V
22
20
18
16
14
12
10
8
6
4
2
0
6
12
16
20
24
Glucose-1-P (mM)

Fig.7-1A

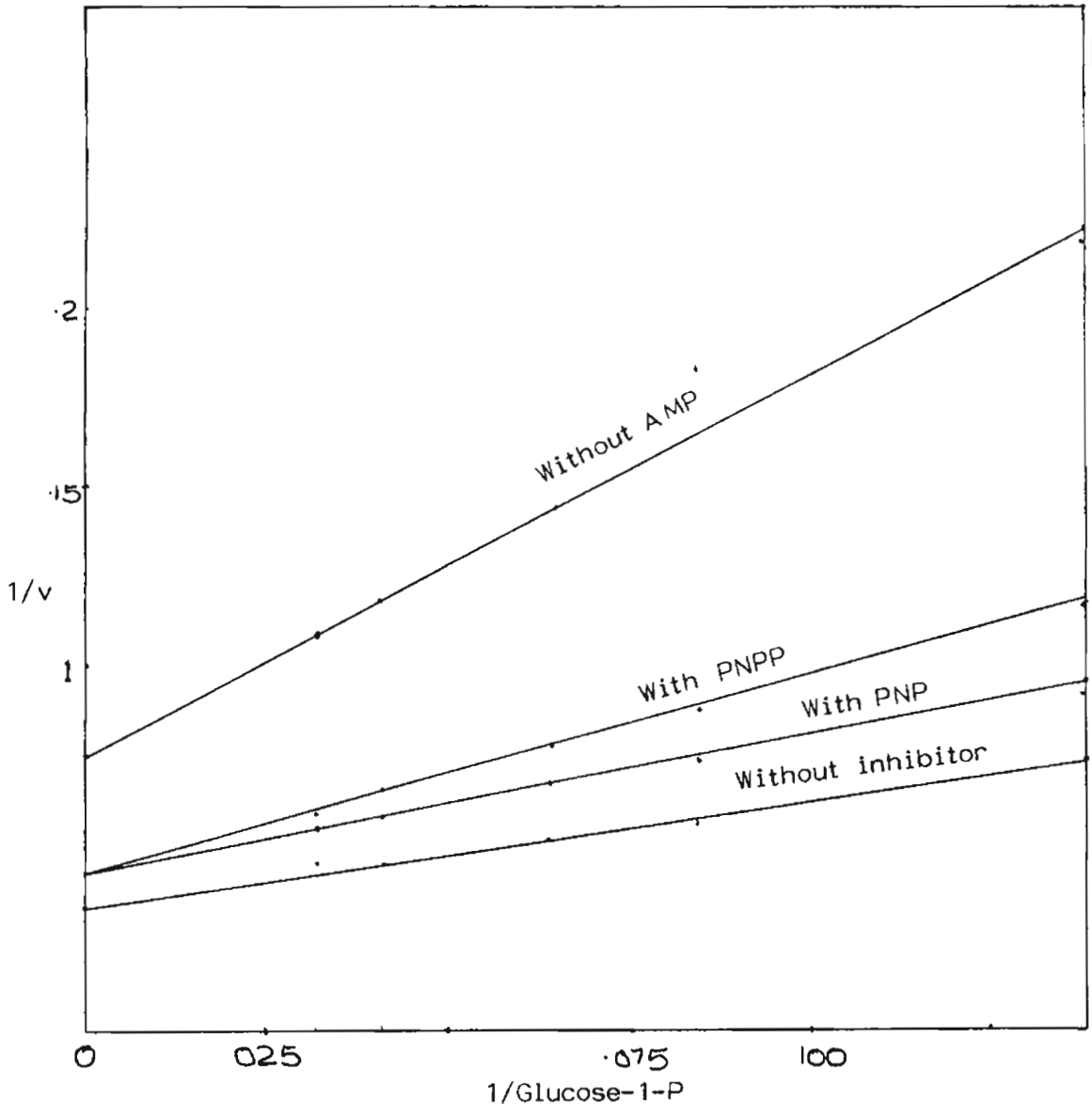

·2
·15
1/v
1
Without AMP
With PNPP
With PNP
Without inhibitor
0
025
·075
100
1/Glucose-1-P

Fig.7-1B

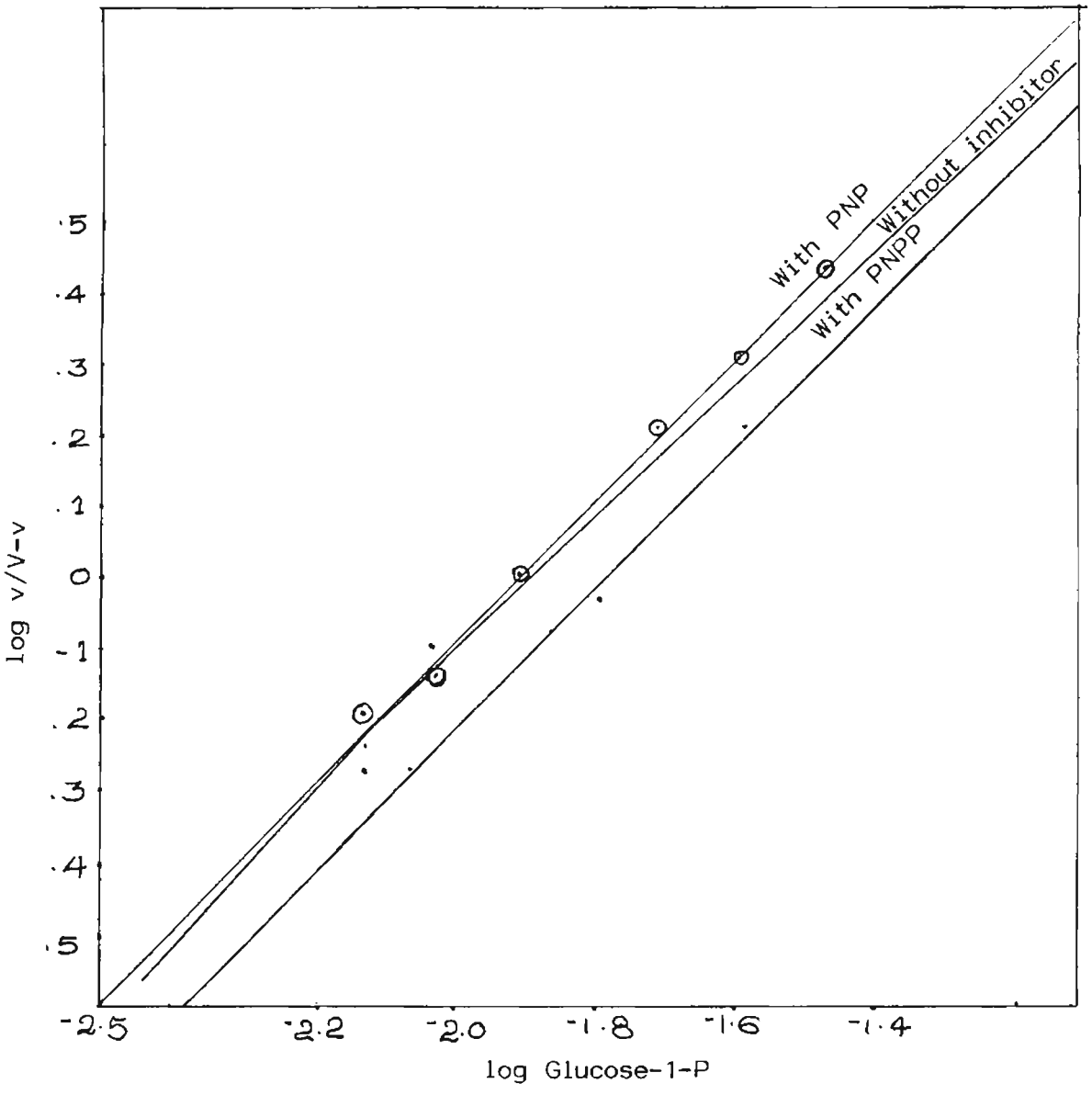

With PNP
Without inhibitor
With PNPP
log v/V-v
·5
·4
·3
·2
·1
0
·1
·2
·3
·4
·5
-2·5
-2·2
-2·0
-1·8
-1·6
-1·4
log Glucose-1-P

Fig.7-2

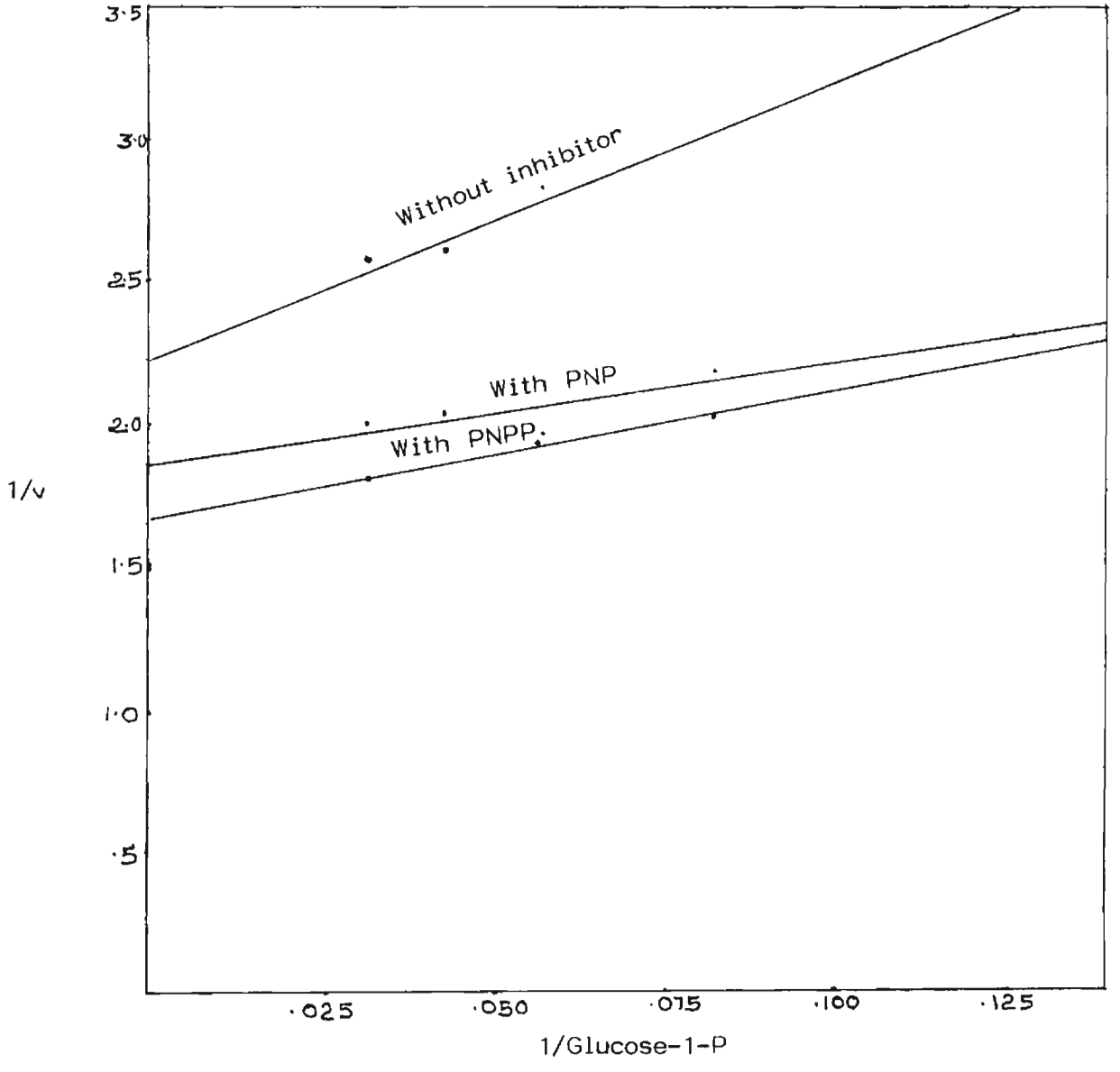

3·5
3·0
2·5
2·0
1·5
1·0
·5
1/v
Without inhibitor
With PNP
With PNPP
·025
·050
·075
·100
·125
1/Glucose-1-P

Fig.7-3

Fig.7-1A

Effect of preincubation with PNP and PNPP on the substrate saturation curve of glucose-1-P for Loligo phosphorylase *a*. Enzyme was preincubated with PNP and PNPP (5 mM each) respectively, for 30 minutes at 30°C prior to initial rate measurements. The assay mixture contained 0.5 per cent glycogen and varying concentrations of glucose-1-P in the presence and absence of AMP.

Fig.7-1B

The double reciprocal plot of the data from Fig.7-1A

Fig.7-2

Hill plot from the data of Fig.7-1B

Fig.7-3

Plots of $\frac{1}{\blacktriangle v}$ vs $\frac{1}{\text{G-1-P}}$ where $\blacktriangle v$ is the ratio in the presence and absence of inhibitors (Data from Fig.7-1)

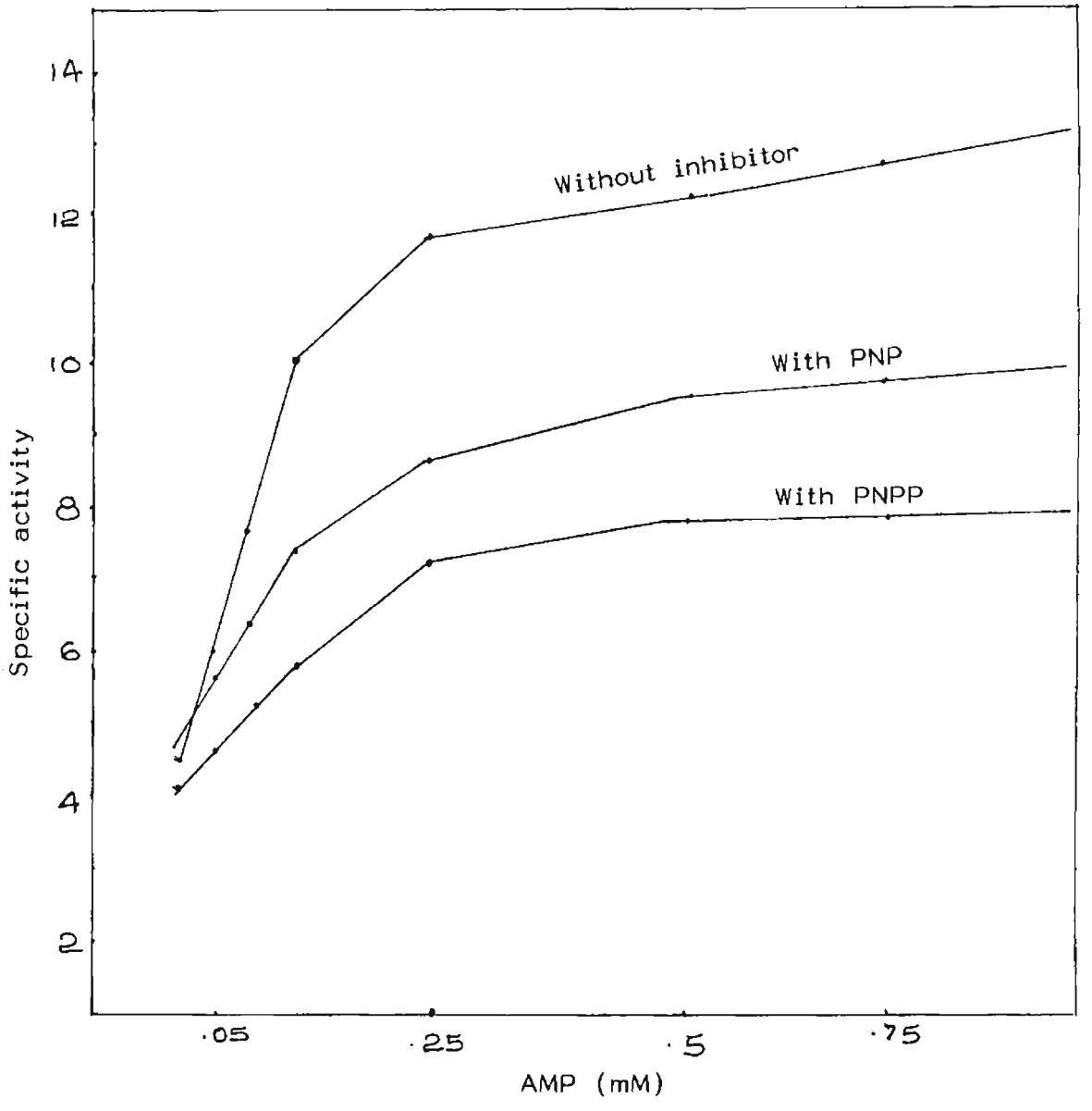

14
12
10
8
6
4
2
Specific activity
Without inhibitor
With PNP
With PNPP
.05
.25
.5
.75
AMP (mM)

Fig.7-4A

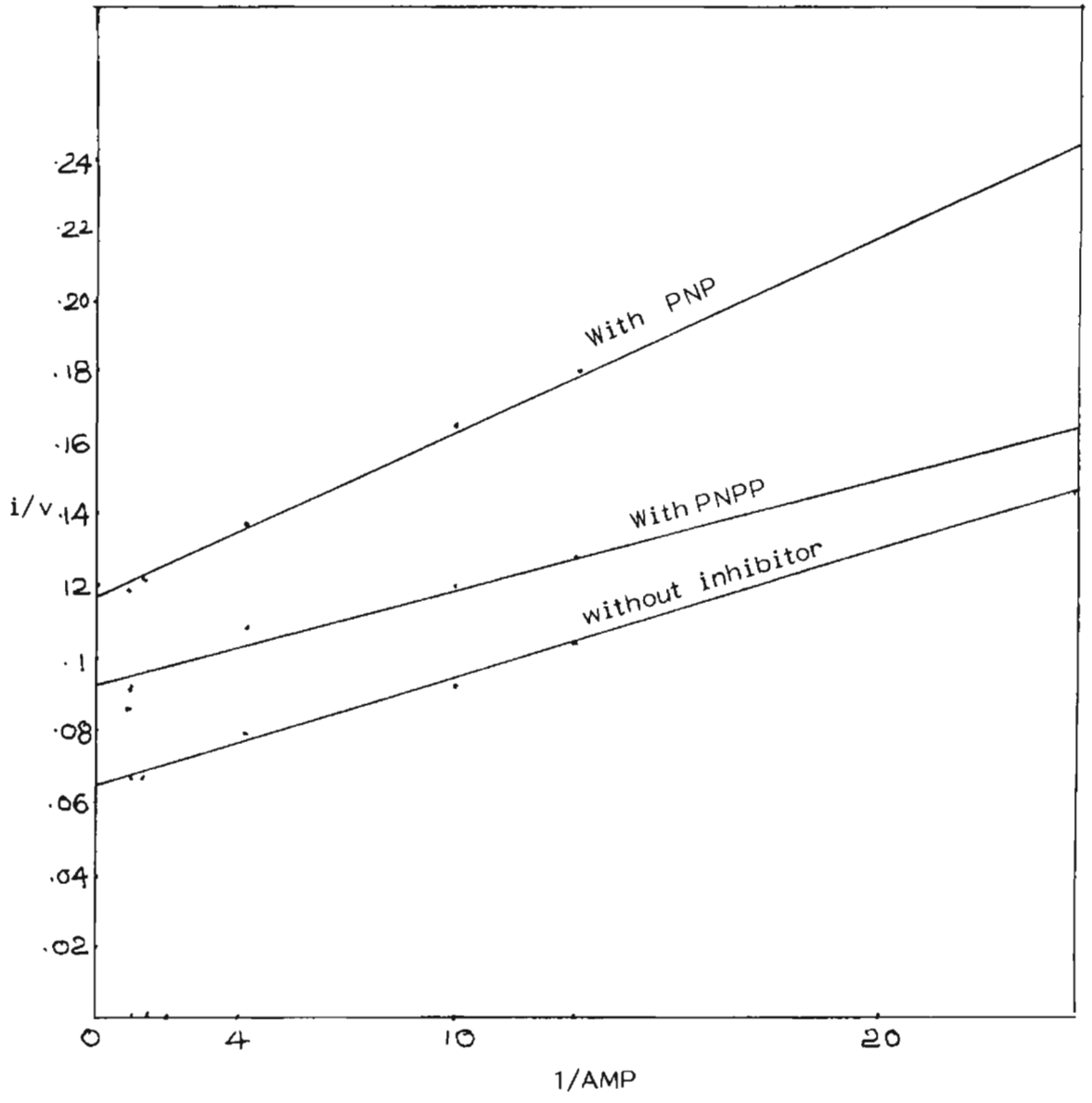

.24
.22
.20
.18
.16
i/v .14
12
.1
.08
.06
.04
.02
0
4
10
20
1/AMP
With PNP
With PNPP
without inhibitor

Fig.7-4B

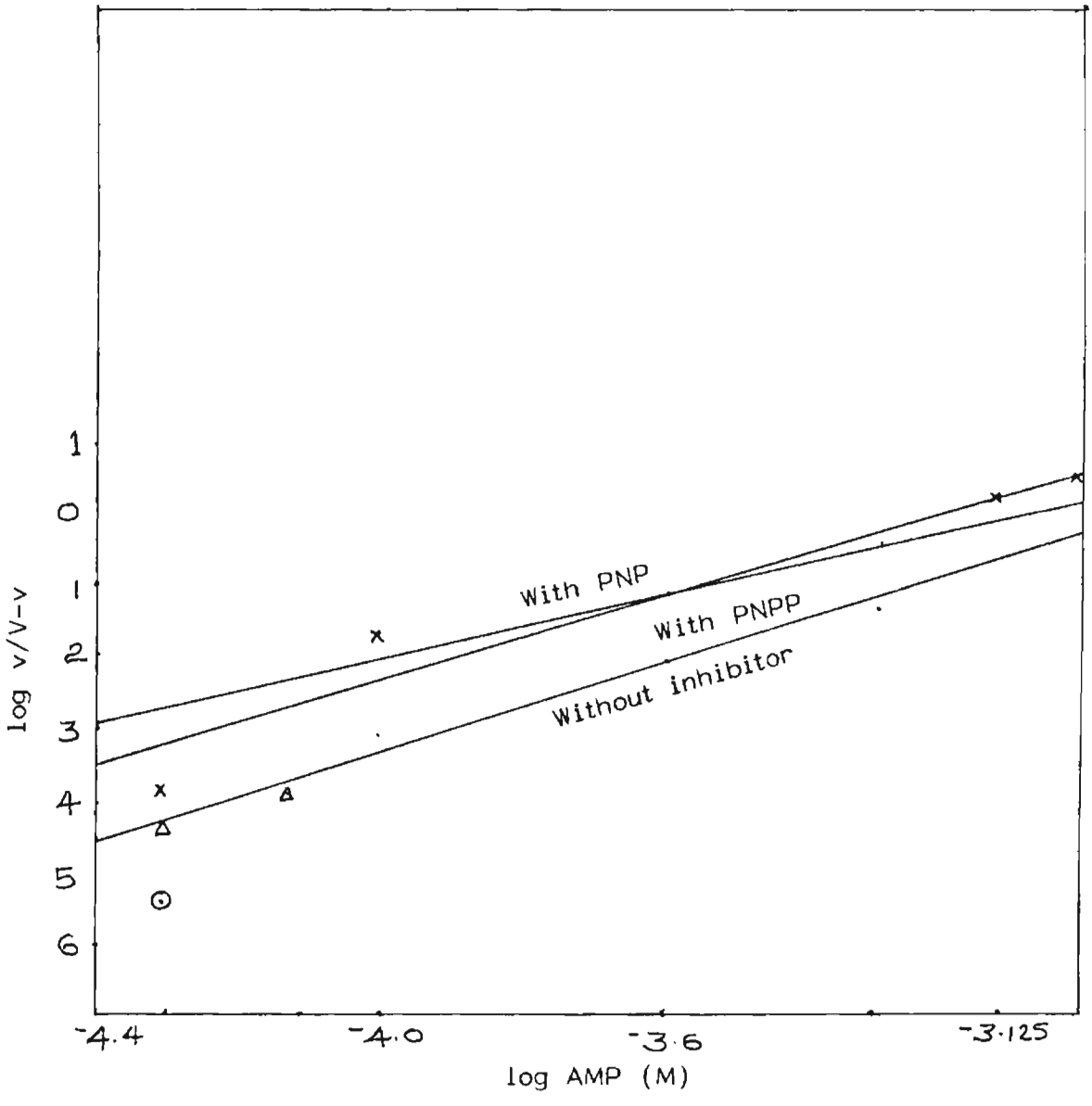

log v/V-v
1
0
1
2
3
4
5
6
With PNP
With PNPP
Without inhibitor
-4.4
-4.0
-3.6
-3.125
log AMP (M)

Fig.7-5

Fig.7-4A

Effect of preincubation with PNP and PNPP on the activator (AMP) saturation curve for Loligo phosphorylase *a*. Enzyme was preincubated with PNP and PNPP (5 mM each) separately for 30 minutes at 30°C prior to initial rate measurements. The assay mixture contained 0.5 per cent glycogen, 16 mM glucose-1-P and varying concentrations of AMP in the absence and presence of PNP and PNPP.

Fig.7-4B

The double reciprocal plot of the data from Fig.7-4A

Fig.7-5

Hill plots for the data of Fig.7-4

CHAPTER-8

EFFECT OF TEMPERATURE ON Vmax AND Km OF LOLIGO PHOSPHORYLASE *a*

The effect of temperature on velocity of enzyme reaction may be due to several different factors. It may be due to an effect on the stability of the enzyme; to an effect on the actual velocity of breakdown on the complex (i.e. on k+2) determined by the heat of activation of the reaction; to an effect on the enzyme - substrate affinity (i.e., k+1 and k-1); to an effect on the pH function of any or all the components, due to an alteration of their pKs which is determined by the heat of ionization; to an affinity of the enzyme for activators or inhibitors, if any; to a transfer of rate-limiting function from one enzyme to another, in a system involving two or more enzymes with different temperature coefficients, or even to such subsidiary causes such as a change in concentration of dissolved O_2 due to a change of solubility in manometric experiments, or a change in the pH of the buffers used (1).

Effect of the affinity of enzyme to substrate and activator was studied in Loligo mantle phosphorylase *a*. The reciprocal plots for glucose-1-P are given in Fig.8-1. Optimum temperature at saturation level of substrate was 30°C.

Chemical reaction proceeds through energy rich activated state. The activation energy is less for catalysed reaction than for uncatalysed reaction and is a function of the catalyst. Therefore, measurement of the activation energy is useful for the comparison of the property of phosphorylases from different sources. Since in the case of Loligo phosphorylase *a* Km is essentially a dissociation constant, the temperature studies will be useful for an understanding of the thermodynamic parameter also.

Arrhenius plots for glucose-1-P is given in Fig.8-3. The plot is linear at temperatures ranging from 20° to 35°C. Km is a function of both enthalpy and entropy changes during binding. If one assumes that Km for glucose-1-P at saturating level of AMP is more or less equal to the dissociation constant, free energy may be calculated from the relation

$$\blacktriangle F^{\circ} = -RT \ln \frac{1}{Km}$$

Since $\frac{d(\ln K)}{d(1/T)} = \frac{\blacktriangle H}{RT^2}$,

it follows that a plot of ln K vs 1/T would yield a straight line with slope = ▲H/R from which enthalpy change for the formation of enzyme substrate complex can be calculated. Knowing the values of ▲F and ▲H, entropy change for binding can be calculated (46). The activation energy of Loligo phosphorylase *a* at saturation level of AMP was calculated as 4.3 Kcal/mol which is less than that for the phosphorylase of Sepia (21 Kcal/mol) (22). Knowing the values of ▲F and ▲H entropy change for binding can be calculated. Since Km is a dissociation constant in this case.

$$\blacktriangle F^{\circ} = -RT \ln \left(\frac{1}{Km}\right)$$

(Here the system is not at equilibrium and proceeds spontaneously only in the direction of negative free energy change)

i.e. $\blacktriangle F^{\circ} = -2 \times 303 \times 2.303 \times \frac{1}{12.86}$

$= -111.65$ cal/mol at 30°C

Since the Km does not vary continuously with rise in temperature, ▲H may be taken as negligible. Therefore ▲F° is the function of entropy change

$$\blacktriangle S = - \frac{\blacktriangle F^{\circ}}{T}$$

$$\text{i.e.} \quad \blacktriangle S = \frac{0.112 \times 10^{3}}{303} + 0.368 \text{ Cal/mol/degree at } 30^{\circ}C$$

The Hill coefficients (Fig.8-2) for glucose-1-P sites also not having co-operativity at different temperature ranging from 20 to 35°C.

The positive entropy change for the formation of the complex suggests possible exclusion of water molecules when the substrate glucose-1-P binds the Loligo enzyme. Such a situation arises when there is charge neutralization in amino groups of the enzyme. This is quite possible in the case of Loligo phosphorylase *a* because the binding of the charged substrate on the enzyme is likely to involve neutralization of charge. The most likely amino groups in the enzyme sites are lysyl and arginyl residues which are positively charged under assay condition. In this respect, Loligo enzyme is very much identical to rabbit phosphorylase (46).

Arrhenius plot for AMP at saturation level of glucose-1-P is given in Fig.8-6, which is linear at temperatures from 20 to 35°C. The activation energy at saturation level of glucose-1-P was 3.69 Kcal/mol which is less than the activation energy at saturation level of AMP (4.38 Kcal/mol) under the same condition. Knowing the values of ▲F and ▲H entropy change for binding can be found.

Here also, Km is the dissociation constant and the system is in equilibrium and proceeded spontaneously only in the direction of negative free energy change

$$\text{So} \quad \blacktriangle F^{\circ} = -2 \times 303 \times 2.303 \times \frac{1}{0.17}$$

$$= -8.206 \text{ Kcal/mol}$$

Temperature can obviously contribute to the observed entropy change in the conformation of the enzyme. Such a change could be either a loosening or a tightening of the structure, and thus could result in increase or decrease entropy (1). As a result of the loosening or R-state of Loligo phosphorylase *a* exothermic reaction takes place.

The reciprocal plots for Loligo phosphorylase at saturating levels of glucose-1-P (16 mM) and varying concentration of AMP is given in Fig.8-4. Hills plot (Fig.8-5) from the same data shows *n* values in an increasing order of 0.6, 0.8 and 0.83 at 20, 30, 35 °C respectively.

Since the Km does not vary continuously with rise in temperature, ▲H may be taken as negligible. ▲F is the function of entropy change

$$\text{i.e.} \quad \blacktriangle S = \frac{8.26 \times 10^{3}}{303} = + 27.08 \text{ Cal/mole/degree at } 30^{\circ}\text{C}$$

In any bimolecular reaction there will always be an entropy change resulting from 'unmixing', that is from the loss of a solute species when activated complex is formed; this effect is however, quite small (1). Since the entropy change in Loligo phosphorylase *a* is quite small (27 Cal/mol/degree at 30 °C), there may be loss of water when activated complex is formed. AMP reciprocal plots are curved downward after a particular concentration which shows the negative heterotropic co-operativity. *n* value of Hill plots of the same reciprocal plot is nearing one when saturation level is attaining. This reveals the absence of co-operativity at optimum condition.

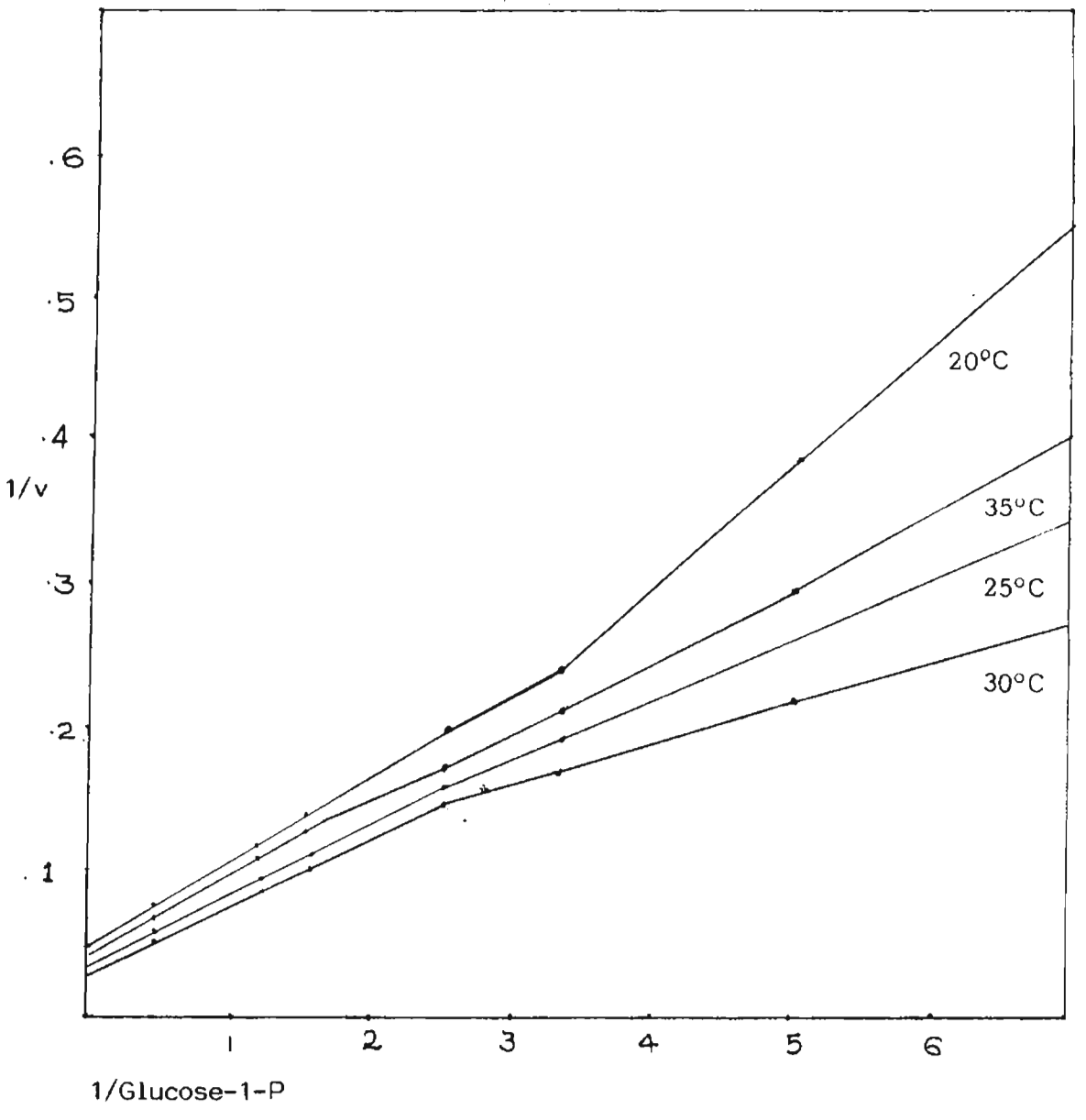


Fig.8-1

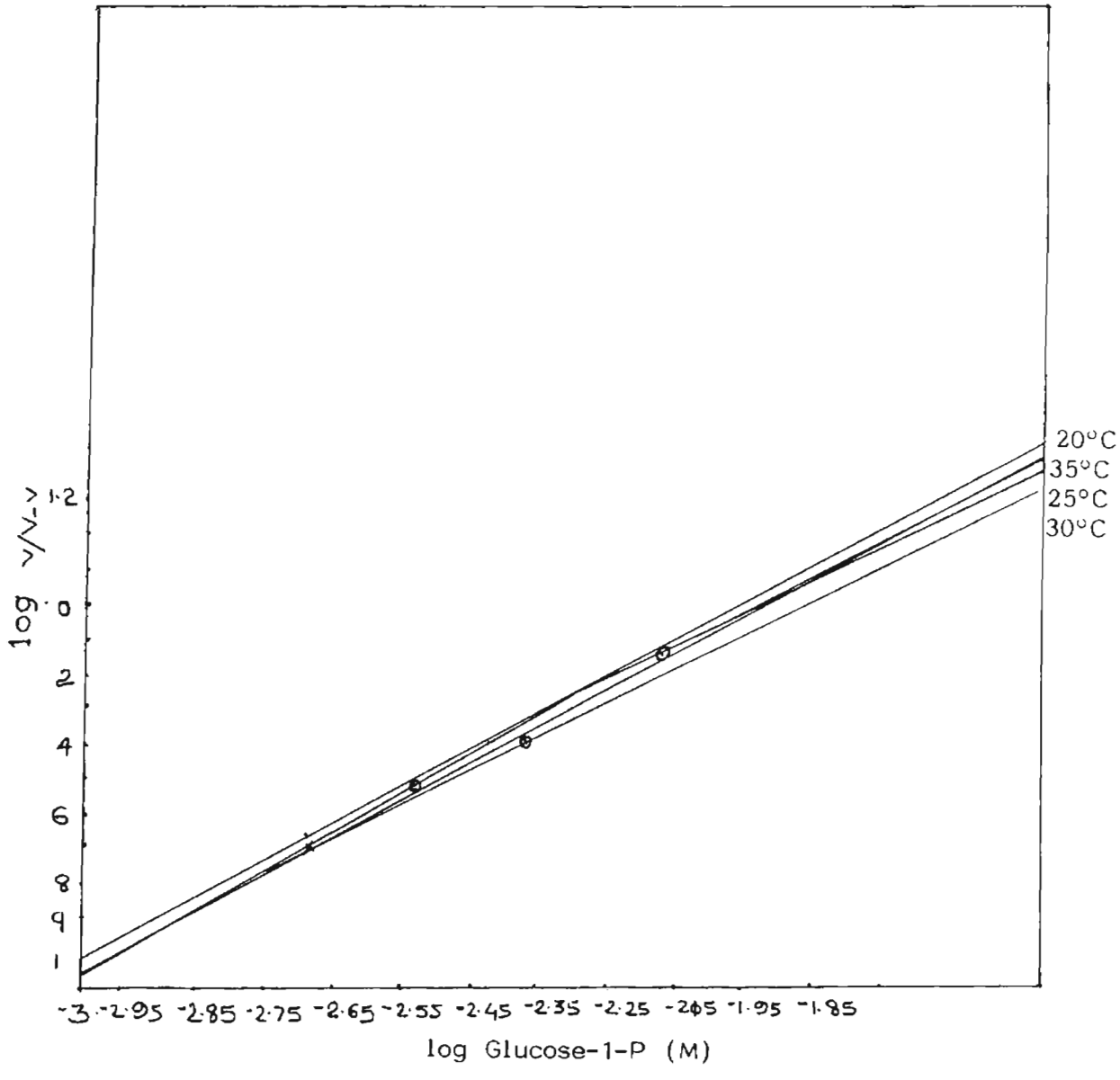

20°C
35°C
25°C
30°C
log v/v-v
1·2
1·0
·8
·6
·4
·2
·0
-3 -2·95 -2·85 -2·75 -2·65 -2·55 -2·45 -2·35 -2·25 -2·05 -1·95 -1·85
log Glucose-1-P (M)

Fig.8-2

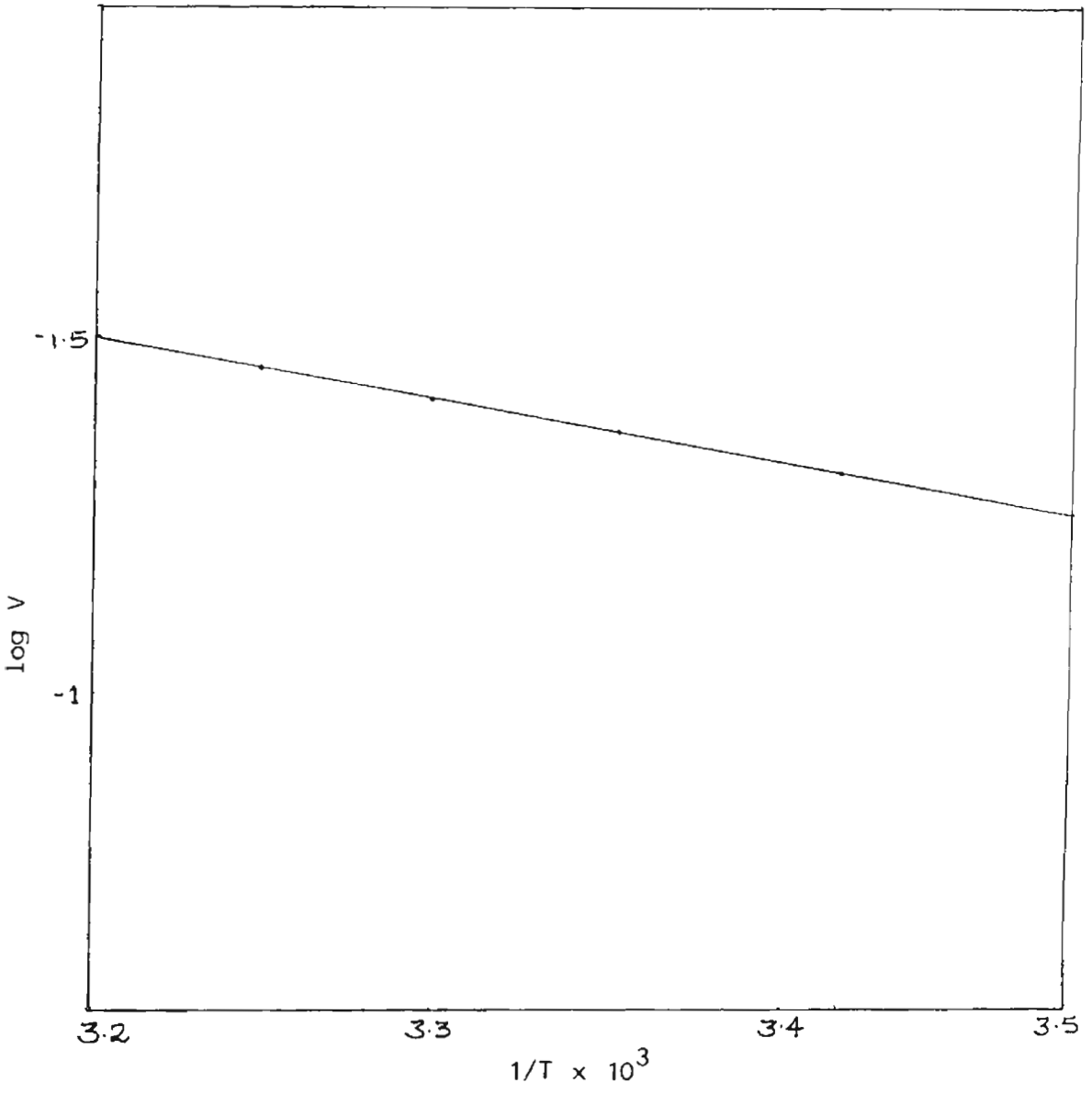


Fig.8-3

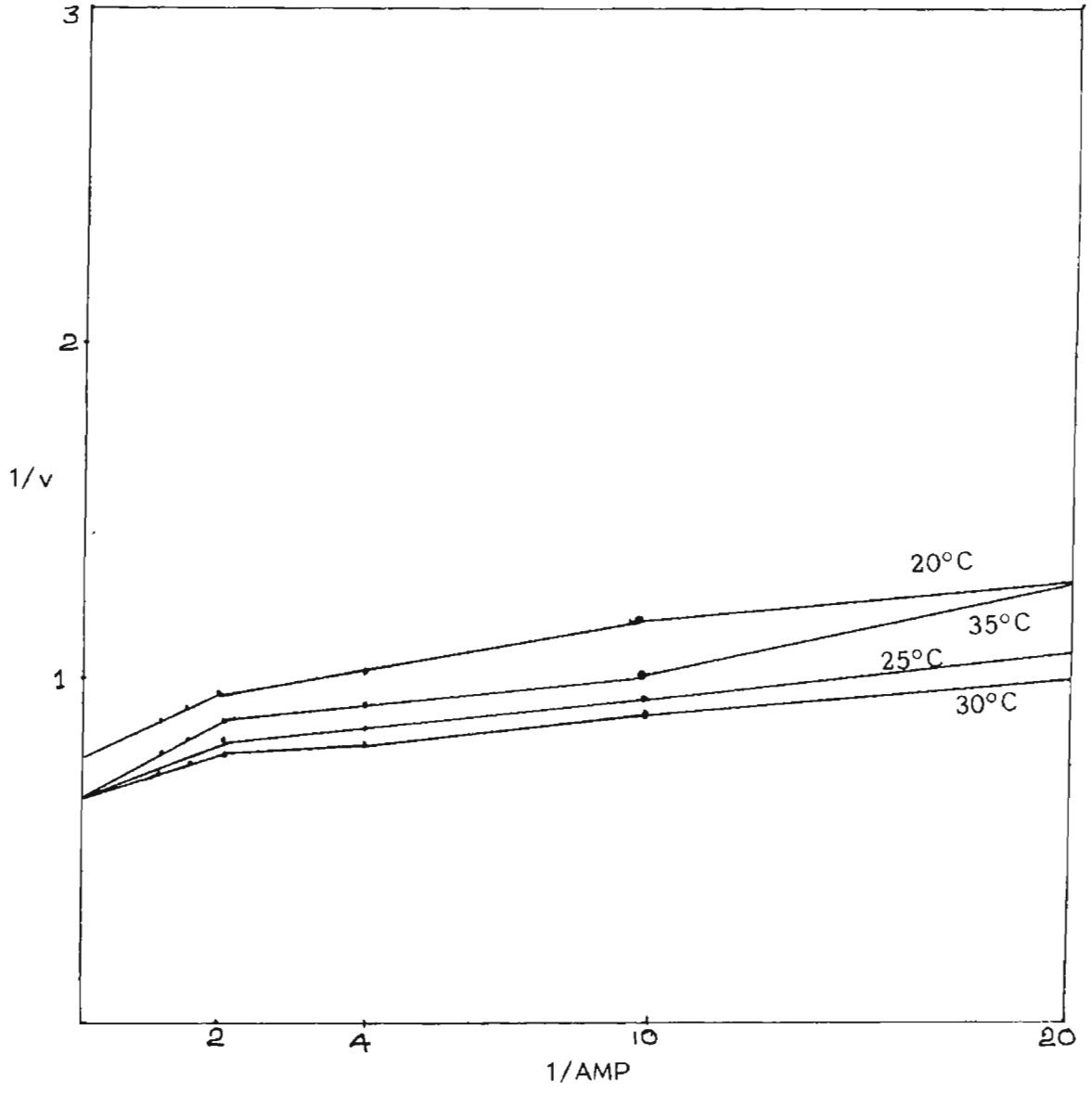


Fig.8-4

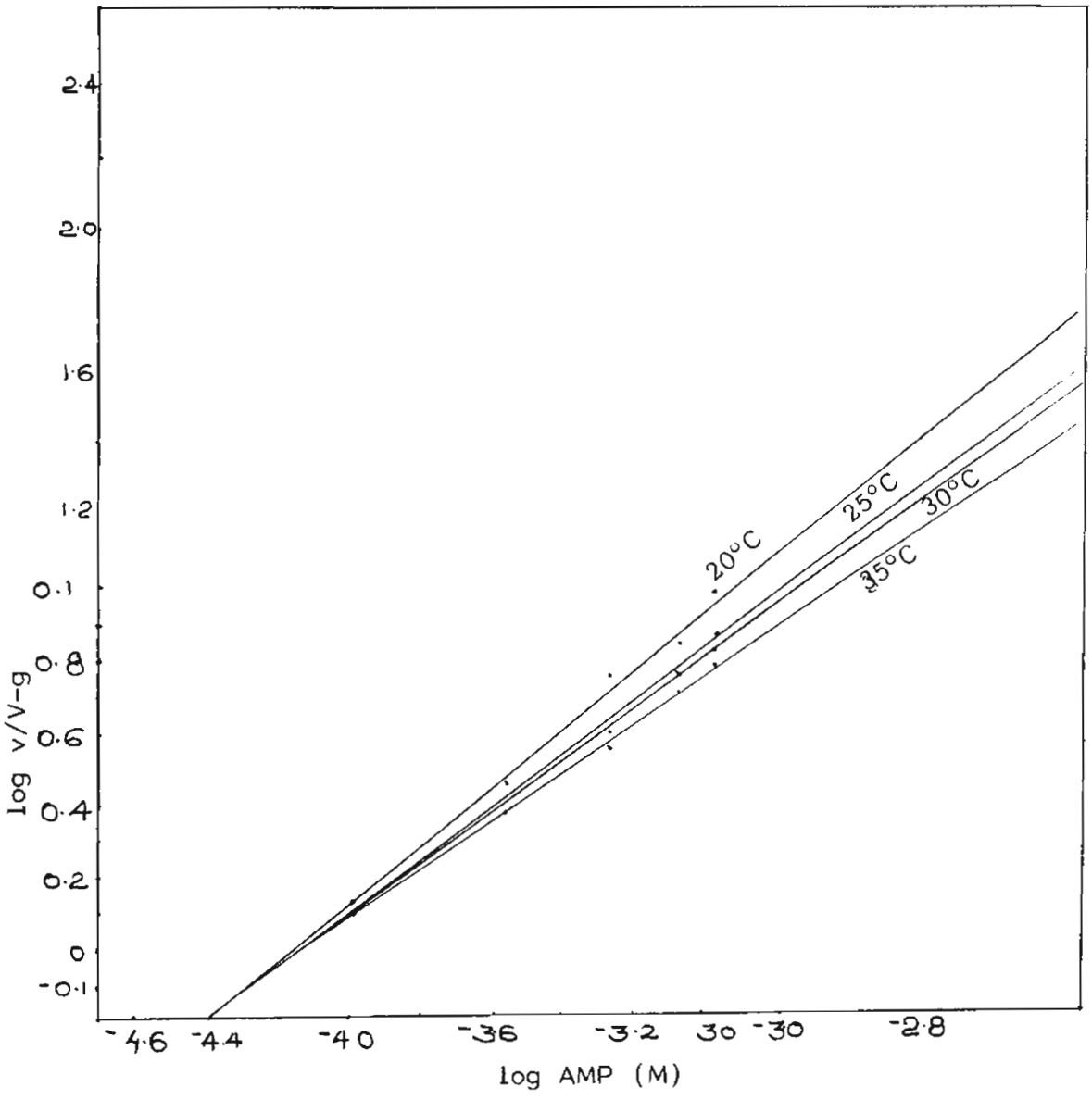

2·4
2·0
1·6
1·2
0·8
0·6
0·4
0·2
0
-0·1
log v/v-g
-4·6
-4·4
-4·0
-3·6
-3·2
-3·0
-2·8
log AMP (M)
20°C
25°C
30°C
35°C

Fig.8-5

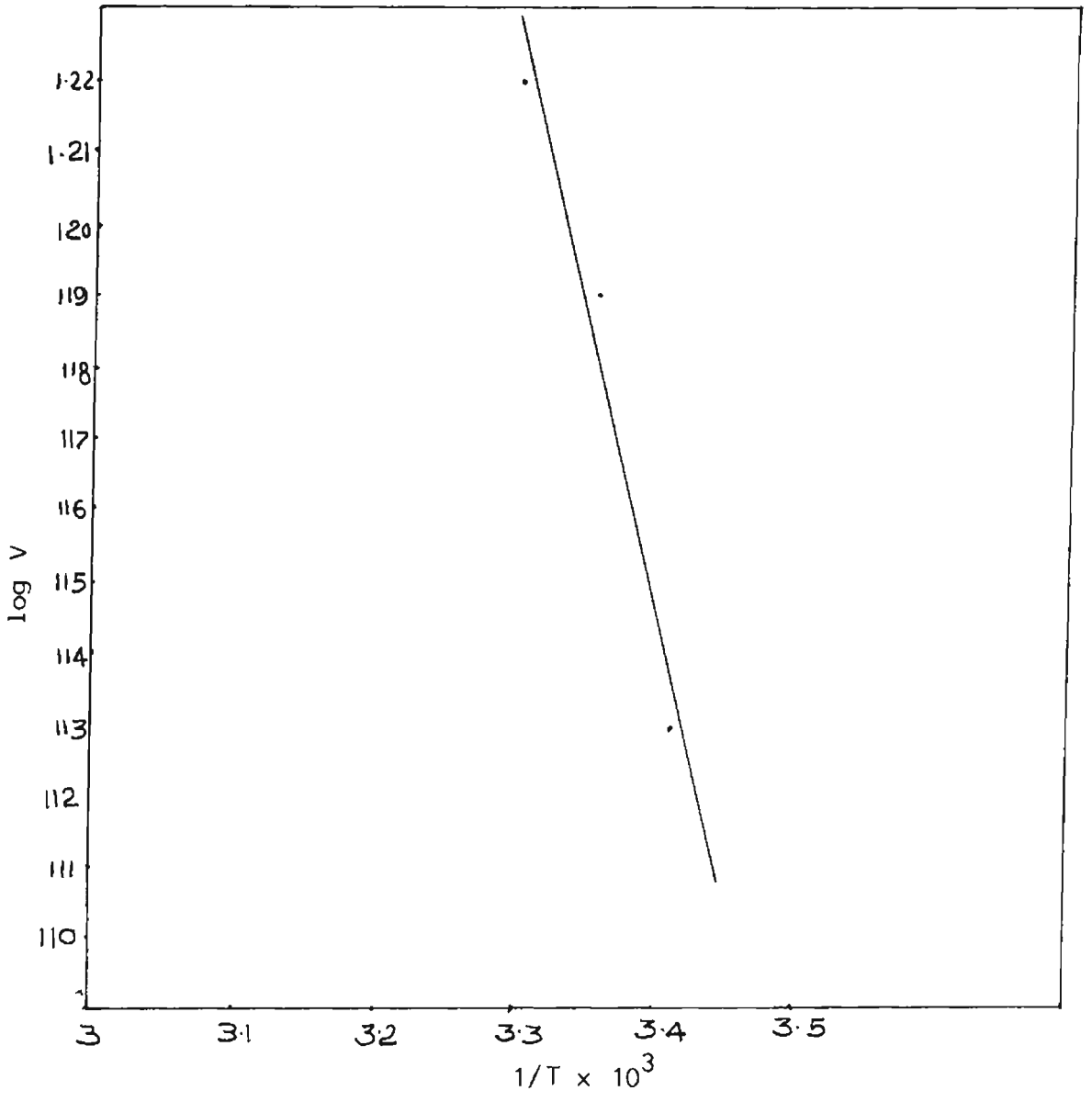

1·22
1·21
1·20
1·19
1·18
1·17
1·16
1·15
1·14
1·13
1·12
1·11
1·10
log V
3
3·1
3·2
3·3
3·4
3·5
1/T x 10^3

Fig.8-6

Fig.8-1

The double reciprocal plot for glucose-1-P at different temperatures of Loligo phosphorylase *a*. Enzyme was incubated at 20, 25, 30 and 35°C respectively for 30 minutes prior to initial rate measurements. The assay mixture contained 0.5 per cent glycogen, 1 mM AMP and varying concentration of glucose-1-P.

Fig.8-2

Hill plot from the data of Fig.8-1

Fig.8-3

Arrhenius plot from the data of Fig.8-1

Fig.8-4

The double reciprocal plot of effect of temperature on the AMP saturation for Loligo phosphorylase *a*. Enzyme was incubated at 20, 25, 30 and 35°C respectively for 30 minutes prior to initial rate measurements. The assay mixture contained 0.5 per cent glycogen, 16 mM glucose-1-P and ranging concentrations of AMP.

Fig.8-5

Hill plot from data of Fig.8-4

Fig.8-6

Arrhenius plot from data of Fig.8-4

CHAPTER-9

COLD INACTIVATION OF LOLIGO PHOSPHORYLASE *a*

Rabbit phosphorylase *a* and *b* have been found to be sensitive to cold temperature at pH 6. On storage at 5° or below at pH 6, the rabbit enzyme has been shown to be inactivated (22). Phosphorylase *b* from muscle of trout fish (113), lobster (20) and *Cibium guttattam* (150) has been shown to be sensitive to cold conditions but only to a lesser extent than Sepia phosphorylase (22).

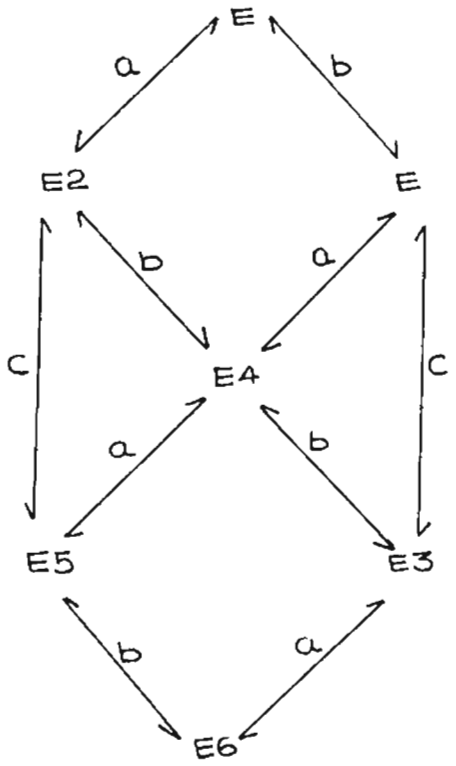
The Loligo phosphorylase *a* was also found to be sensitive to cold temperature. The stability of Loligo enzyme was not influenced at any condition by cysteine where as Sepia phosphorylase was influenced by cysteine at different conditions of pH and salt concentration (22). The effect of cold temperature on Loligo enzyme was therefore studied in detail. Fig.9-1 and 9-2 show the cold inactivation of Loligo phosphorylase at pH 6 and 7.

In contrast to rabbit muscle phosphorylase *a* and *b*, the Loligo enzyme was inactivated at cold temperature at neutral pH also. Similar result was obtained for Sepia phosphorylase (22). Within six hours there was 76 to 79 per cent activity loss at 10, 15 and 25°C, in presence of 1 M NaCl at pH 7, in the absence of NaCl, the activity loss was 63 to 68 per cent. At pH 6, the inactivation rate was high (80 to 83% in presence of 1 M NaCl and 82 to 89% in the absence of salt). This shows that the loligo enzyme is sensitive to cold in a distinct manner.

At pH 6, the Loligo phosphorylase *a* was more sensitive to cold temperature than rabbit phosphorylase. At 0°C, in the absence of 1 M NaCl, there was about 88 per cent inactivation in 4 hours where as there was 60 per cent inactivation of rabbit phosphorylase of 1.2 M NaCl and less than 10 per cent at 0.2 M NaCl

under same condition (151). At pH 6 also, the inactivation was less in the presence of sodium chloride than in its absence at 0, 15 and 25°C respectively. In this respect Loligo phosphorylase was not similar to rabbit phosphorylase *b* where the inactivation at pH 6 in presence of salt was higher than in its absence under the same condition.

Based on the above mentioned observations, it is easy to arrive at a conclusion that the cold inactivation of Loligo enzyme cannot exclusively be attributed to cold temperature because inactivation and hence conformational change, also occurs at 0, 15 and 25°C under the conditions used for the cold inactivation study. Since the cold inactivation was observed at pH 7 also, this property may not be solely due to instability of the enzyme at pH 6. The inactivation was pronounced at low pH and also at high ionic concentration, it could be due to the unstable ionization state of the protein and under these conditions and when the temperature is lowered, this partially inactive enzyme tends to undergo further conformational changes resulting in irreversible structural alteration. The activity of the rabbit enzyme also was not completely regained on rewarming the enzyme (151). Such an irreversible structural alteration was reported in Sepia phosphorylase also (22). It was proposed that the shift to an apparently protonated form of PLP could be due to the structural alteration resulting in an increased hydrophobic character conferred on the PLP site. Hydrophobic bonds and regions have been shown to be sensitive to cold temperature (152, 153, 154) and the hydrophobic portion is easily susceptible to conformation change. The Loligo enzyme undergoes inactivation at high ionic concentration, low pH/or at low temperature unlike enzymes from other sources, which is a distinguishing character of Loligo phosphorylase *a*. Based on the above observations different forms of phosphorylase during cold inactivation can be schematically represented as given in Scheme 9-1.



Scheme 9-1

A schematic representation of different forms of Loligo phosphorylase a under different conditions. Details are given in the text.

In the scheme, E is the native enzyme and E_1, E_2 etc. are inactive forms, a, b and c represent low pH, high ionic strength and low temperature respectively. The stability of these forms decreases from E to E_6. The proposed scheme shows that the process of cold inactivation of phosphorylase could be due to the conformational changes induced by different parameters like ions, pH and low temperatures on phosphorylase. As observed on the rabbit phosphorylase enzyme (151), these changes can lead to enzyme dissociation and hence these forms are unstable.

Cold-labile enzymes generally appear to be composed of subunits and to be active in the polymerised state. A polymerization process would be expected to be associated with a decrease in entropy, but if water molecules are liberated on the formation of hydrophobic bonds between the subunits the process could be accompanied by increase in entropy and thus a positive heat of polymerization (1). In this case liberation of water molecule and thus the dissociation and loss of activity would be favoured by low temperature in Loligo phosphorylase.

Since the cold inactivation was high in Loligo enzyme, it has a much more flexible conformation than other well studied systems. This can be related to the evolutionary status because the cephalopods are very low in evolutionary status, and hence several fine control mechanisms identified as well developed in rabbit and man are still in a much lower level in Loligo mantle phosphorylase.

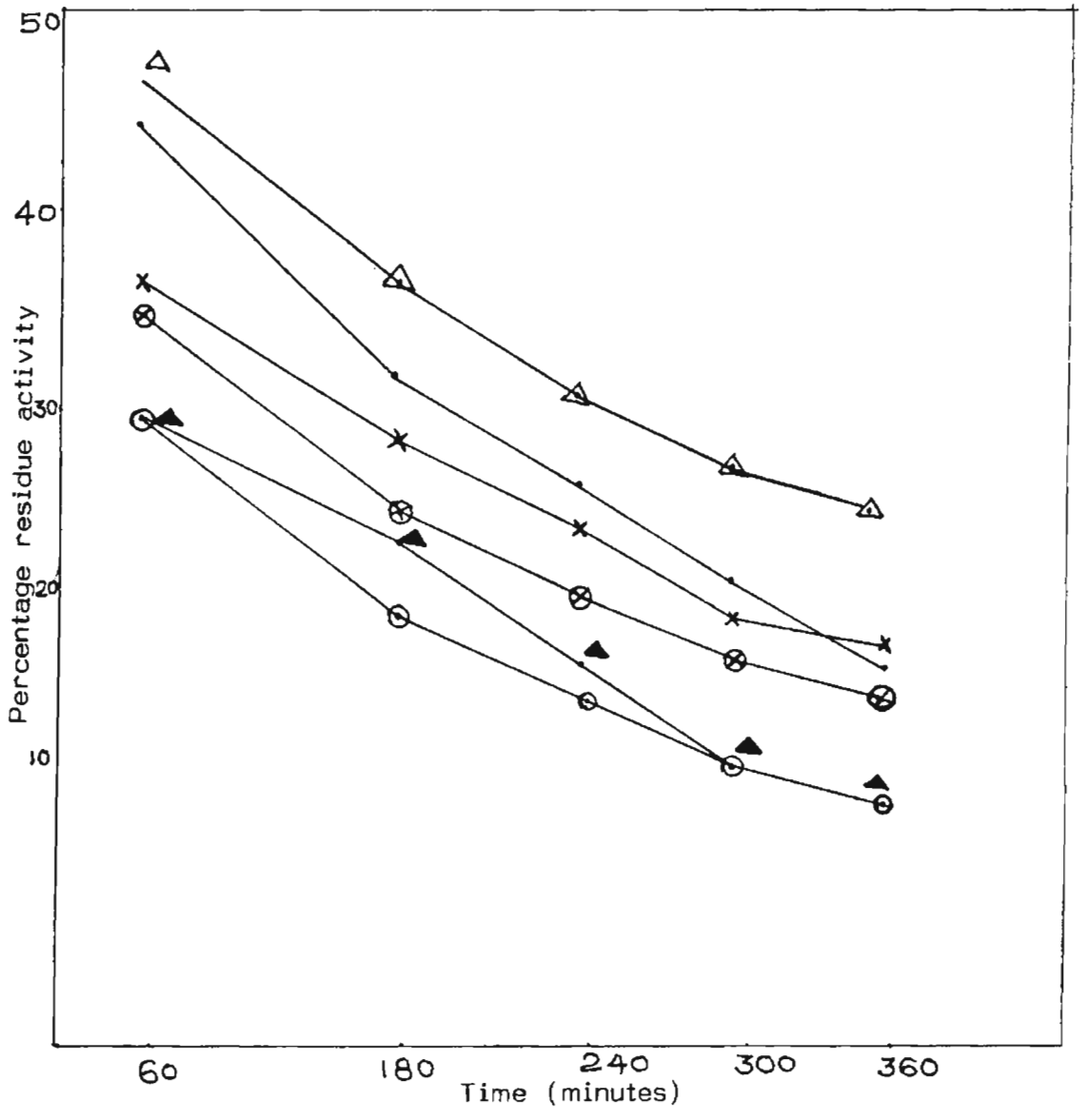

50
40
30
20
10
Percentage residue activity
60
180
240
300
360
Time (minutes)

Fig.9-1

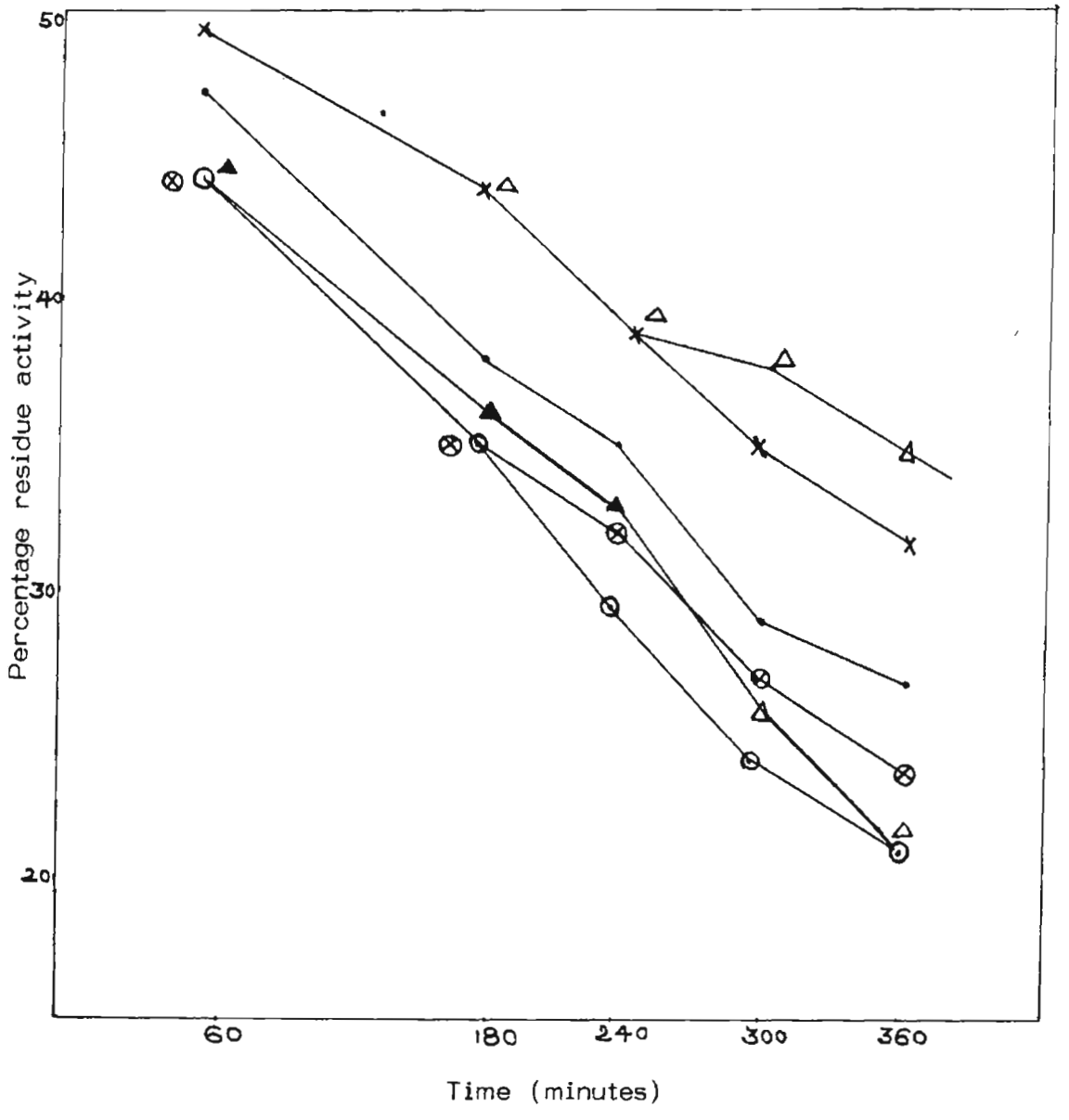

50
40
30
20
Percentage residue activity
60
180
240
300
360
Time (minutes)

Fig.9-2

Fig.9-1

Cold inactivation of Loligo muscle phosphorylase *a* at pH 7. Phosphorylase *a* (194 μg/ml) in 10 mM Sodium-ß-glycerophosphate pH 7, was incubated at 0, 15 and 25° in the presence and absence of 1 M NaCl. Aliquots were withdrawn and diluted in the same buffer and assayed immediately at 30° (10 minutes assay). • incubation at 0°C in the absence of salt, ⊙ incubation in the presence of salt, x incubation at 15°C in the absence of salt, (x) incubation at 15°C in the presence of salt, △ incubation at 25°C in the absence of salt, ▲ incubation at 25°C in the presence of salt.

Fig.9-2

Cold inactivation of Loligo phosphorylase *a* at pH 6. The enzyme (194 μg/ml) was incubated in 10 mM Sodium-ß-glycerophosphate buffer pH 6. Aliquots were withdrawn and diluted on the same buffer assayed (10 minutes). The details of the symbols are as shown in Fig.9-1.

CHAPTER-10

EFFECT OF SALTS/IONS CONCENTRATION ON THE ACTIVITY OF LOLIGO PHOSPHORYLASE *a*

The sulphydryl groups of enzymes, in general, can be roughly classified into (i) those in the active sites which participate in the catlytic reaction, (ii) those which are not in the active site but are involved in maintenance of structure. Almost all enzymes have many such 'SH' groups and their extensive modification will lead to structural changes and hence inactivation. α-glucan phosphorylases from various sources are believed to belong to the second class of enzymes (46). In rabbit muscle phosphorylase *b* 4 'SH' groups, could be modified without loss of activity. Further modification brings about dissociation and inactivation. Therefore the 'SH' groups in it are significant in the maintenance of structure. Heavy metals are known to inhibit and inactivate enzymes containing 'SH' groups (155). However, their effect may not be specific. Therefore, the influence of heavy metals on the activity of the Loligo enzyme was studied. 2.5 mM $HgCl_2$ was sufficient to inactivate phosphorylase of Loligo mantle (Table 10-1). Lamprey muscle, human leukocyte and *Etroplus suratensis* phosphorylases were shown to be activated by Na_2SO_4 (115, 133, 140). Rat liver phosphorylase *b* is also activated by high concentration of sulfate (100). *Eschericia coli* phosphorylase was strongly activated by fluoride and sulfate (156). Activation by sulfate ion was observed in Loligo phosphorylase also (Table 10-1). X-ray analysis revealed that sulfate ion appears to have worked as an activator in place of phosphate at the active site and at the phosphorylation site at serine 14 (16).

The effect of various salts/ions on the enzyme activity is given in Table 10-1. The Loligo phosphorylase was strongly inhibited by Hg^{++}. Zn^{++}, Fe^{++}, K^{+}, Na^{+} and Ag^{+} were not inhibitory, while Mg^{++} and Ca^{++} showed inhibition. All metals except Ag^{+} were added as chloride because Cl^{-} is not an activator. Ag^{+} was added as nitrate whereas Hg^{++} was used in the Cl^{-} form only. The table serves only as a rough data of inactivation by different salts. A solution of Loligo phosphorylase of 0.25 mg/ml in 10 mM Sodium-ß-glycerophosphate pH 7 was incubated with salts at different concentration and assayed after 30 minutes. Inactivation rate differs from source to source, concentration of enzyme and other factors where the rate of inactivation may be specific for each condition.

Table 10-1
Effect of salt/ions concentration in the activity of Loligo phosphorylase *a*

Loligo phosphorylase *a* (250 μg/ml) in 10 mM sodium-ß-glycerophosphate pH 7, was incubated with salts at 25, 75, 150 mM (2.5, 5, 10 mM for $HgCl_2$) for 10 minutes assay at 30°C. 0.5% glycogen, 16 mM glucose 1-P and 1 mM AMP were there in the reaction mixture

Salt	Concentration mM	Effect on enzymes %
$(NH_4)_2SO_4$	25	+15
	75	+37
	150	+37
$Na_2(SO_4)$	25	+10
	75	+12
	150	+15
$HgCl_2$	2.5	No activity
	5	No activity
	10	No activity
$CaCl_2$	25	-30
	75	-50
	150	-63
$MgCl_2$	25	No effect
	75	-20
	150	-50
$AgNO_3$	25	-22
	75	-30
	150	-45
$ZnCl_2$	25	No effect
	75	No effect
	150	No effect
$FeCl_3$	25	No effect
	75	No effect
	150	No effect
KCl	25	No effect
	75	No effect
	150	No effect
NaCl	25	No effect
	75	No effect
	150	No effect

CHAPTER-11

EFFECT OF pH ON THE ACTIVITY OF LOLIGO PHOSPHORYLASE *a*

The hydrogen ion concentration affects the affinity of an enzyme for its substrate, the maximum rate of reaction and the protein stability. The resultant of these effects is generally a narrow pH range of activity with an optimum pH. The contribution of each of these can be separated and experimentally evaluated relatively. Construction of Michaelis Menten-Henri profiles and derived plots such as those of Lineweaver and Burk Plot at each pH enables the individual pH - dependencies of Km and Vmax to be ascertained. Such plots also allow the analysis of the other phenomena which may arise at certain hydrogen ion concentrations, such as substrate inhibition or activation (157).

Effect of pH on the macromolecular stability of the enzyme can be quantitated by exposing it to different hydrogen ion concentrations for varying times, followed by assay at a fixed intermediate value. For most enzymes, inactivation and denaturation become significant only at pH extremes, where the catalytic activity would be very low. At intermediate values, the environmental hydrogen ion concentration determines the state of ionization of the amino acid side chains in the protein, the enzyme-substrate interaction and the charged state of the substrate.

For the interpretation of the observed pH profile in terms of side chain ionizations at the active site, the bell shaped graph (Fig.11-1) may be treated as the composite of two sigmoidal curves. Consider the left hand 'S' shape ascending from the lower pH values to the optimum pH. This could be taken as corresponding to the ionization of acidic groups in the active site. As the pH was raised this ionised to R-$-C\overset{O}{\underset{O^-}{<}}$ Similarly second sigmoid could be considered to be due to the conjugate acid

(H^+B) of a basic species (B) at the active site. As the pH increased, concentration of the unprotonated base increased which was inactive, resulted in a progressive decrease in activity. Combination of these two constitutes Fig.11-1.

The rate of inactivation of enzyme, like other proteins is in most cases greatly dependent on the pH of the solution. The effect of pH varies greatly from one enzyme to another. In general there is a zone of maximum stability, not necessarily around the isoelectric point, and the inactivaton increases on the acid and basic sides. Many enzymes are inactivated even at room temperature. Factors other than pH may also have a considerable effect on the inactivation rate, for example ionic strength, protein concentration and the protective action of the substrate, the inhibitors and other substances. The concentration of the water is also important and enzymes are comparatively heat stable. From the previous chapters, it is observed that the Loligo enzyme is not an exception to the above mentioned factors.

Fig.11-1 shows the pH profile of Loligo phosphorylase *a*. The optimum pH (6.9-7.0) is quite similar to that of rabbit phosphorylase *a* and *b*, a little higher than that of liver phosphorylase (pH 6.2-6.4) and a little lower than that of the *E. Coli* maltodextrin phosphorylase (pH 7.2) (158).

Since the pH profile of Loligo phosphorylase is almost similar to that of Sepia and rabbit phosphorylase (22), the same active groups might be expected to be involved in catalysis. Although the actual nature of the groups in rabbit enzyme has not been unambiguously identified, the 5'-phosphate group of the PLP has been shown to directly participate in catalysis (70-71). The kinetic mechanism of Loligo enzyme is similar to that of rabbit enzyme and the reaction mechanism including effect of pH may be the same.

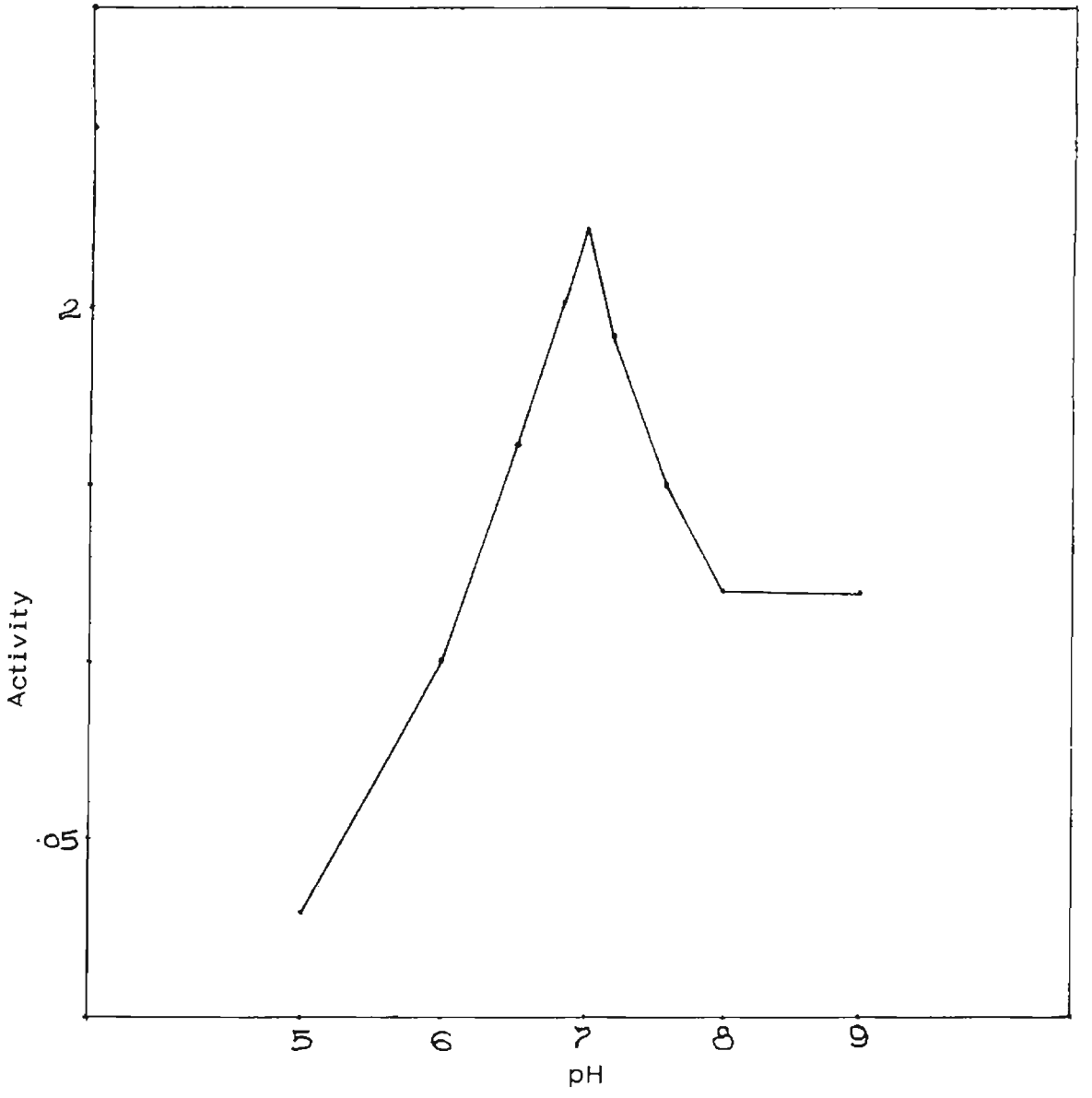


Fig. 11-1

Fig.11-1

Loligo vulgaris phosphorylase *a* activity as a function of pH. The enzyme was diluted in 10 mM Sodium-ß-glycerophosphate of the required pH and added to the substrate solution containing 16 mM glucose 1-P, 1 mM and 0.5 per cent glycogen adjusted to the required pH. The assay was done at 30°C under standard conditions.

CHAPTER-12

CONTROL OF GLYCOGEN DEGRADATION BY LOLIGO MANTLE MUSCLE PHOSPHORYLASE *a*

The degradation of glycogen involves phosphorolysis-breakdown by adding phosphate rather than water. Glycogen phosphorylase is a cooperative homodimer that catalyses the degradative phosphorolysis of glycogen. The enzyme is regulated by covalent phosphorylation and by substrates and effectors. The substrate-phosphate, glucose-1-P and glycogen bind co-operatively and, in terms of the two state model, drive the conformational equilibrium from the catalytically inactive T-state to the active R-state conformation (159).

Rabbit muscle phosphorylase *a* crystals, grown in glucose, are of the inactive T-state and crack when exposed to substrate. Crystallographic analysis of this presumed T → R transition shows conformational changes linking all the ligand binding sites and the two subunits. Conformational changes around the Ser-14 phosphate suggest a molecular basis for the glucose activation of phosphorylase phosphatase and for the glucose control of glycogen metabolism. The asymmetric distribution of changes on the control face suggests a binding domain for the inter-converting enzymes (17).

In brief glycogen degradation in muscle tissues is generally controlled by (i) the concentration of phosphorylase, (ii) the hormone regulated interconversion between the AMP dependent phosphorylase *b* and the AMP independent *a* form and (iii) allosteric activation and inhibition of phosphorylase. Investigation using muscle tissues of all vertebrates, terrestrial and aquatic animals revealed a similar pattern of control (91, 20, 112-122). The major control device in all these cases is through the

interconversion between active and inactive forms of the enzyme via phosphorylation and dephosphorylation. Even though the existence of different forms of phosphorylase has been detected in lower forms of animals and in some plants (21, 126-130, 134, 135, 160), the participation of these enzyme forms in the interconversion process is a universal control device. In many of these species these different forms of phosphorylase are structurally and functionally different from the rabbit muscle phosphorylase *a* and *b*. This chapter deals with the *in vitro* studies on the degradation of glycogen by the mantle muscle phosphorylase of *Loligo vulgaris*.

The loligo fish uses its stronge pallial musculature for its every movement. Water is taken in by contraction of radial fibres in the pallial wall and by pushing out water with force through a funnel by contracting the circular muscles of the mantle, the animal moves with lightning speed (161). Hence the mantle muscle of loligo has an extremely high energy demand. Even though the loligo is primitive in evolutionary status, it is independently a highly evolved invertebrate when compared to the other molluscs (162). The unique properties of cold inactivation, effect of aromatic compounds and other properties suggested structural difference of the Loligo phosphorylase *a* from other purified phosphorylase studied so far.

The initial velocity data obtained at different levels of AMP and glucose-1-P were analysed by using reciprocal plots as shown in the previous chapter (Fig.4-1 and 4-2). These results show absence of homotropic co-operativity between glucose-1-P sites, as in other phosphorylase studied (30-33). However, unlike in other cases, the plots for AMP were curved downwards. The Kinetic mechanism of Loligo phosphorylase being the same as reported for other phosphorylases, the curved plots would mean either that the preparation contained more than one active form of the enzyme having requirements for AMP or that the enzyme exhibits negative co-operativity between AMP sites.

The Loligo enzyme was inhibited by glucose and glucose-6-P and activated by ATP (2 to 10 mM) in the absence and presence of 1 mM AMP (Fig.12-1 and 2). The rabbit phosphorylase with fully co-operative glucose-1-P sites as seen in the presence of glucose-6-P on modification of 2-3 amino acid residues with DTNB yields a derivative which showed not only lack of co-operativity of the site but also competitive kinetics for glucose-1-P and glucose-6-P (46). Loligo enzyme is behaving as a modified phosphorylase which implies different modes of binding by these ligands on the same sites rather than distinctly different binding sites in the Loligo enzyme. This may be one of the reasons for increase of activity when 10 mM ATP was added in the reaction mixture of Loligo enzyme. Considering the functional difference of Loligo enzyme, it is tempting to speculate that the specific influence of modified groups in the binding sites promote ATP as an activator at 10 mM level.

AMP and ATP compete for same site in Loligo enzyme just as the DTNB modified enzyme (46). Since AMP is not a substrate, Km represents the dissociation constant. The Km for AMP (0.18) is considerably smaller than the apparent Km for ATP (3.75) showing that AMP has greater affinity for the enzyme than ATP in Loligo phosphorylase. Since both these ligands bind on the same site in Loligo enzyme, the decreased affinity of ATP may be due to steric resistance for access to the site or due to electrostatic repulsion or both. The metabolic role of this inhibitor seems to be significant as it was found that loligo muscle was maintaining a very low concentration of AMP.

The probability of AMP in the enzyme preparation was ruled out on the basis of the following observations

$$\text{(i) the activity ratio } \left(\frac{\text{Activity without AMP}}{\text{Activity with AMP}}\right) \text{ remains}$$

unchanged at different stages of purification of the enzyme, (ii) the activity ratio was same at different stages of heat inactivation at different temperatures, (iii) the preparation was electrophoretically homogeneous and (iv) the Arrhenius plot was linear. Thus Loligo phosphorylase exhibited negative co-operativity between AMP sites. The negative co-operativity was reported in Sepia and Sunnetta (140) phosphorylases. This is significant not only because such behaviour has not been reported for other animal muscle phosphorylases, but also because it suggests the possibility of a different control of glycogen degradation in Loligo mantle. This is supported by the studies on inhibition by metabolic inhibitors. Even though Sepia phosphorylase was slightly inhibited by glucose in the absence of AMP, the presence of 1 mM AMP showed no inhibition at all (22). Loligo enzyme was found to be inhibited by glucose in presence of 1 mM AMP at a higher rate. The inhibition rate of Loligo enzyme by glucose is comparable to that of rabbit phosphorylase. Phosphorylase purified from the frozen Loligo mantle showed an activity ratio of 0.28 ± 0.02.

The presence of phosphorylase phosphatase and kinase in the mantle muscle extract was checked by using the purified phosphorylase as substrate. For this the frozen muscle was extracted with twice its weight of distilled water at $5^{\circ}C$. The homogenate was centrifuged and passed through Sephadex G-25, equilibrated with 10 mM Sodium-ß-glycerophosphate buffer (pH 7) to remove small molecules. The phosphorylase phosphatase action of this extract was checked at pH 6.5, 7 and 7.5 in 10 mM Sodium-ß-glycerophosphate buffer at $30^{\circ}C$. The activity was unaffected at different concentrations of the extract. By performing a similar set of experiments with further inclusion of 2 mM ATP and 10 mM Mg^{++} in the reaction

mixture, it was found that the frozen mantle tissue of loligo did not contain phosphorylase kinase activity at a pH range 6.5 to 7.5.

The above experiments showed that phosphorylase phosphatase and kinase became inactivated on freezing of the mantle tissue. The activity ratio of the loligo mantle was usually near 0.6 and the ratio gradually decrease during various stages of enzyme purification and came to a constant value of 0.28 $\pm$ 0.02 (Table 12-1).

Slight variations were observed in the activity ratios at the initial steps of enzyme purification in different batches of preparation. The values given in the table (Table 12-1) shows the range of such variation.

The extent of allosteric activation of phosphorylase depends not only on the concentration of AMP, but also on the condition such as the concentration of substrate and effectors, divalent metal ions, pH, temperature etc. The concentration of AMP in the resting rabbit muscle was first determined by Lohman and Schuster (163) and found to be apparently 0.01 mM. With improvements in the technique of "quick freezing", the AMP concentration was estimated to be 0.5 M (given per ml intracellular water, taken as 50% tissue wet weight) in the rabbit resting muscle (164). Even if such a high concentration of AMP is present in the resting rabbit muscle, the high concentration of ATP, ADP and glucose-6-P (97 mM, 2 mM and 0.3 mM respectively in 1 ml of intracellular water) inhibit the allosteric activation of phosphorylase *b* in the rabbit muscle and *in vivo* it has been found out that the order of activity in the rabbit resting muscle is 1/10,000 of its total potential activity (165). Therefore in the rabbit resting muscle, the presence of AMP does not serve as a controlling factor of phosphorylase.

Table 12-1
Activity ratio of Loligo phosphorylase at different stages enzyme purification

The fractions obtained from different purification steps were diluted in 10 mM glycerophosphate buffer (pH 7) and assayed at 30°C. The assay mixture contained 16 mM G-1-P, 0.5 per cent glycogen in the presence and absence of 1 mM AMP.

Step	Activity ratio
Extract	
35 to 55% ammonium sulfate fraction	0.56 ± 0.04
The above fraction after dialysis	0.44 ± 0.04
DEAE cellulose purified enzyme	0.34 ± 0.02
Sepharose-glycogen purified enzyme	0.28 ± 0.02

Since the activity ratio of Loligo phosphorylase *a* was only 0.28 $\pm$ 0.02 the presence of AMP in the contracting muscle may not be critical. The loligo caught in the trawling nets may fastly contract its mantle tissue which may result in maximum kinase activity and the *a* form must be highly dominating in the frozen muscle of loligo caught by this method. Loligo enzyme was also shown to be highly AMP dependent and because a continuous production of lactate is not a common physiological phenomenon, the control of breakdown of glycogen in Loligo muscle must either be on the regulation of AMP production or on a separate site of the glycolytic pathway or by both the process. Because of the low specific activity and of the narrow range of variation in the activity ratio, the control of the phosphorylase activity of loligo mantle muscle phosphorylase could not be due to the mere interconversion between *a* and *b*. Through evolution, the vertebrates have acquired the mechanisms of regulation of glycogen degradation within the phosphorylase molecule by gradual mutation, as evidenced by the amino acid sequence analysis of phosphorylase in a number of distinct animal species and plants (55-57). *Loligo vulgaris* might have developed such a mechanism of regulation of glycogen degradation by gradual mutation for evolving a structurally more active form of phosphorylase to meet the extremely high energy demand of the mantle muscle for moving with lightning speed.

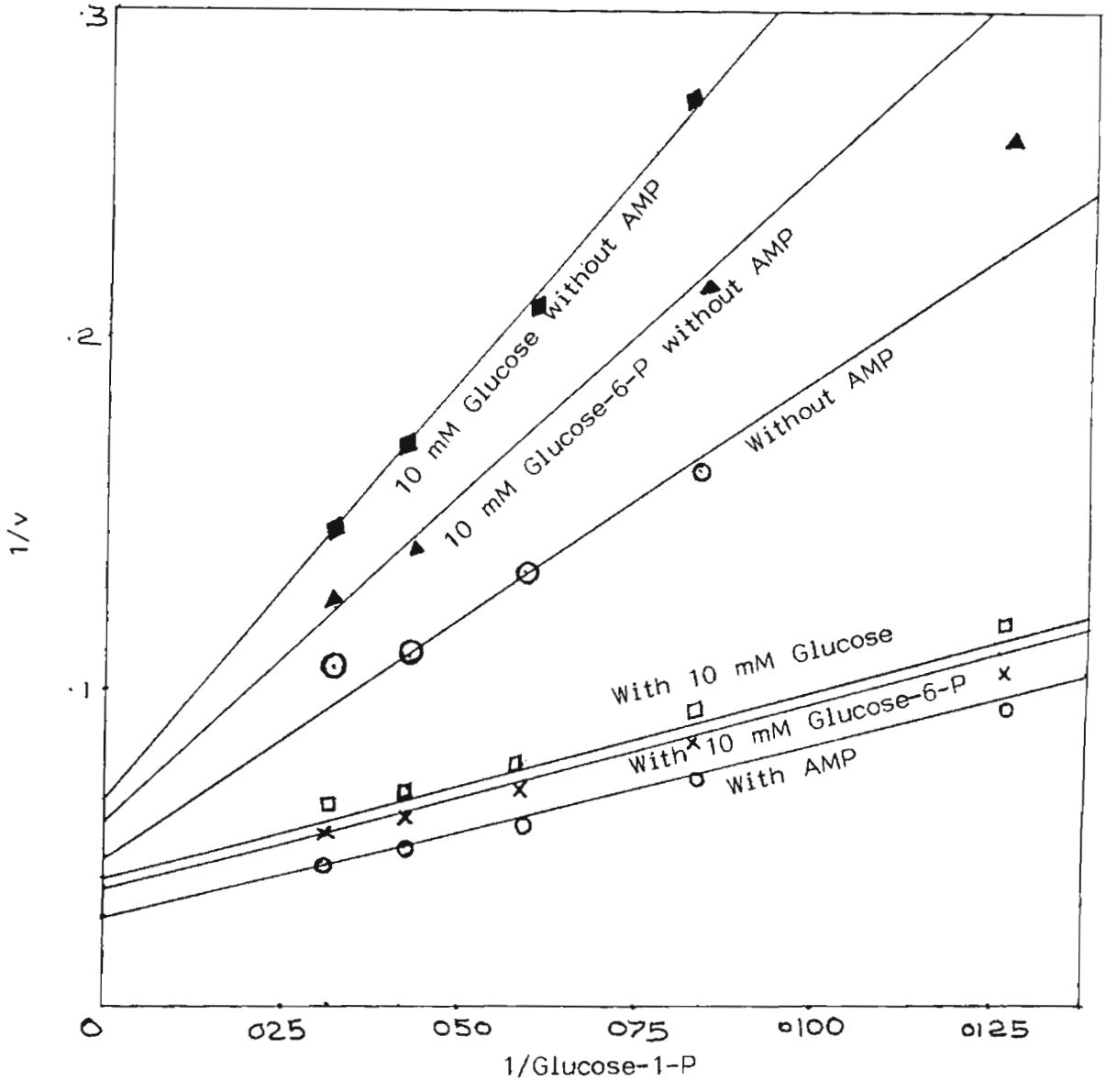

1/v
.3
.2
.1
0
025
050
075
0100
0125
1/Glucose-1-P
10 mM Glucose without AMP
10 mM Glucose-6-P without AMP
Without AMP
With 10 mM Glucose
With 10 mM Glucose-6-P
With AMP

Fig.12-1

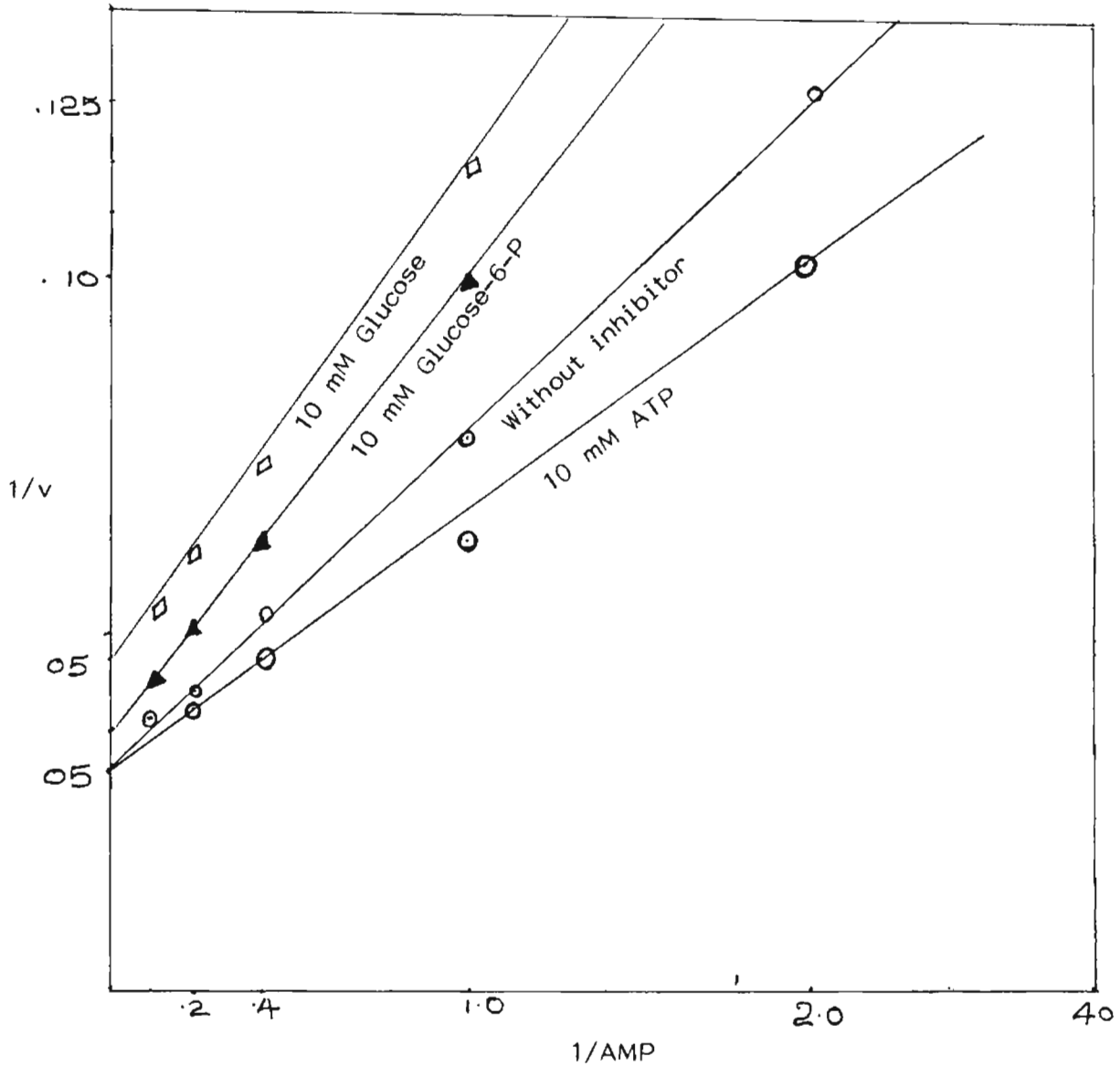

.125
.10
1/v
05
05
10 mM Glucose
10 mM Glucose-6-P
Without inhibitor
10 mM ATP
·2
·4
1·0
2·0
40
1/AMP

Fig. 12-2

Fig. 12-1

Reciprocal plot for glucose-1-P for Loligo phosphorylase *a* in the presence and absence of glucose (10 mM), glucose-6-P (10 mM) and AMP (1 mM). Enzyme solution was diluted with Sodium-ß-glycerophosphate buffer (pH 7) and assayed with varying concentrations of glucose-1-P in the presence and absence of AMP. Incubation of reaction mixture was at 30°C for 10 minutes.

O with AMP, ○ without AMP, x with AMP and glucose-6-P, ▲ without AMP and with glucose-6-P, ▱ with AMP and glucose, ■ without AMP and with glucose.

Fig. 12-2

Reciprocal plot for AMP for Loligo phosphorylase *a* in the absence and presence of glucose, glucose-6-P and ATP at 10 mM level. Enzyme solution was diluted with Sodium-ß-glycerophosphate buffer (pH 7). Reaction mixture contained 16 mM glucose-1-P, 0.5 per cent glycogen and varying concentrations of AMP. Incubation of the reaction mixture was at 30°C for 10 minutes.

O No inhibition, ○ with ATP, with glucose-6-P, with glucose.

CHAPTER-13

COMPARATIVE EVALUATION OF GLYCOLYTIC ENZYMES FROM MARINE SOURCES

Purification of glycolytic enzymes differs from animal to animal, cell to cell and tissue to tissue. The well studied rabbit muscle phosphorylase was prepared in our laboratory according to the procedure of Fischer and Krebs (13) with the substitution of mercaptoethanol for cysteine (26). The muscle from the hind leg and back of medium size rabbit was removed and stored frozen. 400 g of frozen muscle was weighed out and finely minced mechanically and ground well in a mortar at room temperature. Finely minced muscle was stirred well with 400 ml water for 15-20 minutes at room temperature and filtered through two thickness of cheese cloth into a beaker cooled in ice. The muscle was again extracted for a second time with another 400 ml water and finally using another 200 ml of water. The combined extract was filtered through glass wool and taken for acid precipitation. Acid precipitation was carried out at pH 5.1 to 5.3. The pH of the cold extract was adjusted carefully by adding 1N acetic acid. The mixture was immediately transferred to pre-cooled centrifuge tubes and centrifuged at 7000 rpm for 15 minutes at 5°C. The supernatant was filtered through a large coarse fluted filter paper and the pH of the filtrate was readjusted to 6.8 by adding solid potassium bicarbonate. The solution was made 41 per cent saturation with ammonium sulfate by adding saturated and neutral ammonium sulfate. The mixture was kept in a refrigerator overnight at 5°C. Most of the supernatant was decanted and the percipitate was collected by centrifugation at 5°C for 15 minutes at 7000 rpm and dissolved in 50 ml water and dialysed against pre-cooled 0.001 M tris-HCl buffer pH 7.6, for 24 hours at 5°C to free it from ammonium sulfate and small ions.

The following solutions were added to the dialysed protein solution; (1) sufficient mercaptoethanol (1:1 diluted in water) to a final molarity of 3 x 10^{-2} M; (2) neutral 0.1 M EDTA to a final molarity of 5 x 10^{-4} M; (3) enough non-neutralised 2 M tris to bring the pH to 8.8. The mixture was incubated at 37°C for 1 hour, cooled to 0° and the pH readjusted to 7 by careful addition of 1N acetic acid. The solution was centrifuged at 5°C for discarding the precipitate.

To the solution was added 1/100th of its volume each of 0.1 M AMP and 1 M magnesium acetate solution and the mixture was cooled in a refrigerator. The next day the mixture was centrifuged to collect crystals of phosphorylase *b*. The crystals were dissolved at 30°C in 15 ml water containing 0.03 M mercaptoethanol and again AMP and magnesium acetate were added to final molarities of 10^{-3} and 10^{-2}M respectively. On cooling, crystals separated out which were collected by centrifugation. These processes were repeated two times and the enzyme could be kept in the refrigerator for months together without any appreciable loss of activity.

The procedure for purification of glycogen phosphorylase from rabbit muscle was not applicable to the purification of enzyme from *Loligo vulgaris*. Instead of distilled water 10 mM Sodium-ß-glycerophosphate buffer (pH 7) was used for Loligo mantle muscle. Water extraction of Loligo mantle muscle showed appreciable loss of activity. 60 per cent of inactivation was observed when acid precipitation trial was carried out in Loligo extract in the second stage of purification. So this step was abandoned.

The presence of phosphorylase phosphatase and kinase in mantle muscle extract was checked by using purified phosphorylase (as substrate), ATP and Mg^{++} ions. No phosphorylase phosphatase and kinase activity was observed. So further step of increasing the pH of extract and heat treatment were avoided.

Loligo phosphorylase was further purified by dialysis, DEAE cellulose column chromatography and Agarose-glycogen hydrophobic chromatography.

Crystallization of phosphorylase from Loligo mantle extract was not successful with AMP and Mg^{++} unlike rabbit muscle phosphorylase. The purified enzyme was not stable, gradually losing the activity within 10 days after final purification.

Loligo enzyme had a specific activity of 32-36 units/mg protein where as Sepia showed only 25 to 28 units/mg protein (32).

The presence of a phosphorylase form having a higher requirement of AMP for maximum activity has been reported in extract of certain marine organisms (126). Such a form (Phosphorylase *c*) was also separated from the lobster tall muscle on DEAE-cellulose column (20). Sepia was not requiring high quantity of AMP (22) while phosphorylase from *Cibium guttattam* had only 1 per cent activity in the absence of AMP (46). AMP requirement for Loligo enzyme was inevitable for activity eventhough requirement of AMP in certain organisms is selective and minimum. Phosphorylase activity per gram tissue and specific activity in the original extract of *Etroplus suratansis, Metapenaeus dobsoni, Sunetta scripta* and *Villorita cyprenoides* were varying in a definite manner, consistent with the energy requirement of the tissues (140). The stability of the enzyme, measured by activity loss in the extracts at different stages of purification were found to correlate with the evolutionary status (146).

Presence of phosphorylase phosphatase and kinase was not detected in Sepia (22) and Loligo mantle. Latent form of phosphorylase phosphatase was reported in *Metapenaeius dobsoni* (140). The presence of phosphorylase phosphatase and

kinase enzymes was established in all other reported animals and hence the interconversion reaction plays an important role in the regulation of glycogen degradation in all animals, even though certain exceptions are there, like Sepia (22), Sunetta (140) and Loligo.

The comparison of general properties of Loligo, Sepia and Sunetta is summarised in Table 13-1. The table shows that the activity per gram tissue may vary according to the energy requirement of the muscle. Among the three, Sepia mantle muscle extract has higher activity (13 units/g) (22). The bivalve Sunnetta has very low activity (4 units/g) (140). The specific activity of the extract also varies from animal to animal. Sunetta has a much lower specific activity (3), suggesting that it is the least energy utilizing animal among the three.

Sepia showed high activity ratio (0.95) and high content of AMP (3.5 mM) in the muscle extract (22) where as Loligo had an activity ratio of 0.56. The presence of AMP content in Loligo mantle extract was negligible, practically nil. Even though the activity ratios of Sepia and Loligo were high, the phosphorylases were present in the *a* form just as in the sunetta. The activity ratio of Sunetta (0.23) can be compared with the purified human leukocyte phosphorylase *b* which has an activity ratio of 0.2 (124). In the case of rabbit muscle phosphorylase, hybrid forms of phosphorylase *a* and *b* have been isolated in which the phosphorylase was partially phosphorylated (18).

Influence of pH on phosphorylases varies from source to source. Optimum pH for activity of phosphorylase of Sunetta, Sepia and Loligo was 6.8, 6.9 and 7 respectively. Cysteine activation was observed in Sepia (22) where as Sunetta (140) and Loligo showed no such activation in presence of cysteine.

Table 13-1. Comparison of general properties of phosphorylases from marine source

Property	Loligo vulgaris	Sepia pharaonis (22)	Cibium guttattam (46)	Sunetta scriota (140)
1) Activity/g wet tissue	7.00	13.00	22.50	4.00
2) Specific activity	0.89	0.83	1.67	0.30
3) Activity ratio of the extract	0.56	0.95	-	0.50
4) Activity ratio of purified enzyme	0.28	0.40	0.001	0.23
5) Convertability of the crystalline rabbit muscles b to a in the presence of Mg^{+2} and ATP* by extract	No	No	-	Yes
6) Interconvertability of a and b forms in the extract				
1. Conversion to the a form	No	No	-	Yes
2. Conversion to the b form	No	No	-	Yes
7) Optimum pH	7.00	6.90	6.80	6.80
8) Activation by cysteine	No	Yes	-	No
9) Protection by mercaoto etnanol	Yes	No	No	-
10) Stability of the purified enzyme	Not stable, decreasing the activity and lossing within 10 days	Stable for 3-4 days and decline	30 to 40% activity was lossing on storage	Highly unstable
11) Activation by Ammonium sulfate and sodium sulfate	Yes	Yes	-	Yes
12) Effect of $MgCl_2$, NaCl and KCl	No effect	-	-	Yes
13) Effect of heavy metal like Hg^{2+} and Ag^{+}	Inactivate	Inactivate	Inactivate	Inactivate
14) Inactivation by cold when compared with rabbit phosphorylase	Less	Less	Less	-

Phosphorylases show variation in stability (loss of activity). Rabbit muscle phosphorylase can be stored for months together. Stability of the Etroplus phosphorylase was for 2 weeks after purification (140), where as Loligo phosphorylase was gradually decreasing activity after purification and completely lossing the same within 10 days. *Metapenaeus dobsoni* phosphorylase could not even be subjected to dialysis at 0-5 °C after ammonium sulfate fractionation (140). The unstability was more pronounced after each step of purification. This property seems to be related to the evolutionary status of the animals.

The kinetic studies of most of the animal phosphorylases are consistent with rapid equilibrium random *bi bi* mechanism. However differences have been observed in many properties which are related to the need of each animal. The kinetics and allosteric properties of phosphorylase from four marine animals are summarised in Table 13-2. Sepia (22) and Loligo enzymes showed negative heterotropic co-operativity where as no co-operativity was shown by Sunetta phosphorylase (140). Similar reports were there in *M. dobsoni* and Villorita (negative heterotropic) and Etroplus (no co-operativity) (140). At the same time the above mentioned phosphorylases showed positive heterotropic co-operativity between glycogen (glucose-1-P) and glucose-1-P (glycogen) sites. The kinetic constants of Sepia, Sunetta and Loligo enzymes are also different (Table 13-3). The Sunetta K_6 value was two times lower and K_7 value 5 times higher than Loligo phosphorylase. K_5 and K_8 values of Sunetta phosphorylase are 7 and 2 times greater than Loligo phosphorylases. Difference is there in the apparent Km value of the above mentioned phosphorylases. Km value of Sunetta phosphorylase is five to six times higher than that of Loligo and Sepia phosphorylases.

Table 13-2. Comparison of allosteric properties of phosphorylases from marine sources

Property	Loligo vulgaris	Sepia pharaonis	Cibium guttattam	Seunnetta scripta
1) Kinetic mechanism	Rapid equilibrium random bi bi	Rapid equilibrium random bi bi	Rapid equilibrium random bi bi	Rapid equilibrium random bi bi
2) Homotropic cooperativity between				
1) Glucose-1-P sites	Nil	Nil	Nil	Nil
2) Glycogen sites	Nil	Nil	Nil	Nil
3) AMP sites	Negative	Negative	Nil	Nil
3) Heterotropic cooperativity between				
1) Glycogen and glucose-1-P sites and vice versa	Positive	Positive	Positive	Positive
2) AMP and glucose-1-P sites	Negative	Negative	Positive	Positive
4) Inhibition by metabolites				
1) Nature of inhibition with respect to glucose-1-P by				
a) Glucose	Mixed	Competitive	Competitive	Competitive
a) Glucose-6-P	Mixed	In the absence of AMP competitive	Competitive	Competitive
b) Glucose-6-P	Mixed			
c) ATP	Activation at 2 mM level	No inhibition	Competitive	Competitive
5) Nature of inhibition with respect to AMP by				
a) Glucose	Mixed	Competitive	Mixed	Mixed
b) Glucose-6-P	Mixed	Competitive	Competitive	Mixed
c) ATP	Activation	Activation	-	Mixed
6) Nature of inhibition by aromatic compounds	Mixed	Mixed	Mixed	-

Table 13-3. Kinetic constants of phosphorylases from marine source

Kinetics constants	*Loligo vulgaris*	*Sepia pharanois*	*Cibium guttattom*	*Sunnetta scripta*
K_5 (mM glucose-1-P)	8.20	7.20	3.6	4.95
K_6 (mM glycogen)	1.31	0.79	2.8	6.80
K_7 (mM glycogen)	3.52	4.50	9.0	25.00
K_8 (mM glucose-1-P)	20.00	50.00	15.3	11.60

Table 13-4 reveals that the increase in concentration of glucose-1-P resulted in decrease of Km value for glucose-1-P (i.e. the kinetic mechanism being sequential, decrease of Km implies increased affinity) and *vice versa*. Again, Km values acertain the distinction among the phosphorylases from marine sources.

Inhibition kinetics also showed differences in phosphorylases from source to source. Loligo exhibited mixed inhibition with respect to glucose-1-P by the metabolites glucose and glucose-6-P where as Sunetta (140) showed competitive inhibition in presence of 1 mM AMP. In the absence of AMP, sepia showed minor competitive inhibition to the above metabolites (22). Sunetta phosphorylase exhibited competitive inhibition by ATP with respect to glucose-1-P (140) where as phosphorylase from Sepia (22) and Loligo showed activation and no inhibition at all. Loligo phosphorylase was different from other two phosphorylase in the extent of inhibition by glucose and the other two metabolites. Thus there is a definite difference in structure at the binding site/active sites of these four phosphorylase from marine sources.

It may be recalled the Loligo phosphorylase *a* in the presence of AMP shows a Hill coefficient of one for glucose-1-P, glucose-6-P (Chapter 5). The influence of glucose, glucose-6-P, PNP and PNPP on the kinetics with respect to glucose-1-P for Loligo phosphorylase is given in Chapter 5, 6 and 7 in the form of Hill plots. The *n* values for glucose-1-P site in the presence of glucose-6-P is 1 except for glucose where the *n* value is 1.25. Therefore, as far as the effect of glucose is concerned, the Loligo phosphorylase differs in the co-operativity of glucose-1-P towards positive co-operativity. However, the results bring out one important point i.e. glucose-6-P and the other inhibitors behave differently making a clear deviation from the property of rabbit phosphorylase. The difference between these inhibitors can be seen in double reciprocal plots presented in figures of Chapter 5, 6

Table 13-4 Apparent Km values for glucose-1-P and glycogen at different levels of each other for phosphorylases from marine sources

(Concentration of glycogen is expressed as mM concentration of glycosyl residues)

I - Loligo vulgaris, II - Sepia pharaonis, III - Cibium guttattam, IV - Sunnetta scripta

Glycogen (mm)				Km values for glucose-1-P				Glucose-1-P for I, II, III & IV	Km for glycogen			
I	II	III	IV	I	II	III	IV		I	II	III	IV
28.50	-	-	-	17.8	-	-	-	32	1.58	1.50	-	8.08
17.10	-	57.00	61.50	19.2	-	6.0	11.25	24	1.71	1.50	3.0	8.52
11.40	50	28.50	24.60	21.9	18.2	6.4	12.99	16	1.43	1.55	3.2	9.05
8.50	20	14.28	6.15	21.9	23.2	7.4	16.10	12	1.63	1.60	3.3	9.28
5.70	5	5.70	4.92	21.3	33.3	8.5	17.60	8	1.78	1.60	3.8	9.78
4.56	2	2.28	-	20.4	43.0	10.5	-					

and 7. All inhibitors show mixed type of inhibition. The results presented so far suggest that these inhibitors interact with Loligo phosphorylase differently. Such variations in the effect of different ligands is usually explained as due to their binding on different sites.

The influence of inhibitors on the kinetics with respect to the activator for Loligo phosphorylase is shown in the figures of the same chapters mentioned above. The Loligo enzyme has been found to exhibit negative heterotropic co-operativity with AMP sites. The results show that the presence of inhibitors also show difference in the degree of negative co-operativity as evidenced from the *n* values (Table 13-5). The reciprocal plots in the presence of these inhibitors show different Km, typical of mixed inhibition, indicating that these ligands do not bind on AMP sites.

The results presented in Chapter 5, 6 and 7 therefore reveal that the Loligo phosphorylase enzyme is a typical desensitised enzyme by its origin as evidenced by kinetic studies of various ligands.

Mixed type of inhibition, for glucose and glucose-6-P and activation for ATP was seen with respect to AMP for Loligo phosphorylase *a*. Sunnetta exhibited mixed type of inhibition for all the three metabolities. These inhibition properties support the difference in structure of phosphorylases from marine sources.

Glycolysis is essentially the oldest energy yielding process in the biosphere. A comparison of the properties of glycolytic enzymes should give a fair appraisal of the features that have been preserved through the entire evolutionary process. The results of the survey of subunit structure of glycolytic enzymes by R.C. Ruth and Finword are presented in the Table 13-6 (166).

Table 13-5. Hill coefficients, apparent Km and Ki values of Loligo phosphorylase *a* in the absence and presence of inhibitors

	n values for glucose-1-P	Km values for glucose-1-P mM	Ki values for glucose-1-P mM
Without inhibitor	1.00	31.00	-
With glucose-6-P (10 mM)	1.00	37.50	30.0
With glucose (10 mM)	1.25	3.72	7.5
With PNP (5 mM)	1.00	10.00	15.0
With PNPP (5 mM)	1.00	16.00	15.0
	n values for AMP	Km values for AMP	Ki values for AMP
Without inhibitor	0.31	0.17	-
With glucose-6-P (10 mM)	0.81	0.62	10.1
With glucose (10 mM)	0.80	0.53	7.9
With PNP (5 mM)	0.22	0.51	11.9
With PNPP (5 mM)	0.31	0.32	21.3

Table 13-6. Comparison of subunit structure of glycolytic enzymes in different organisms and tissues (166) a

	Phosphorylase	Phosphoglucomutase	Hexokinase	Phosphoglucose isomerase	Phosphofructokinase	Aldolase	Triose phosphate isomerase	Triose phosphate dehydrogenase	Phosphoglycerate kinase	Phosphoglycerate mutase	Enolase	Pyruvate kinase	Lactate dehydrogenase	Alcohol dehydrogenase
Mammalian tissues														
Muscle	185 93	62 31b		132 64	360 85	160 40	55 27	145 36	45 --	57 27	82 41	237 57	145 35	
Erythrocyte				125 62			56 --	-- 33	50 --			225 --		
Liver, Kidney	185 80-90		96 50c		360 --	153 39	56 27				90 52	265 62	145 35	80 40
Fish muscle	200 100	63 --				160 --		145 35			93 45		140 35	
Lobster muscle	180 (100)					150 --		145 36			87 --			
Higher plants	207 100	63 --		110 --		120 30		600 145		54 --				
Yeasts	200 --		104 52	120 60	590 100	80 40	53 27	148 38	47 --	112 28	88 44	151 42		150 37
Bacteria: E. coli	250 --	62 --						144 35	44 --	56 --	92 46	100 (50)		
Other species[d]	250 --	(60) --			134 68	75 --					330 44		150 --	
Number of subunits observed	2	1	2	2	2-6	2-4	2	4	1	2-4	2	2-4	4	2-4

a The upper number represents native enzyme mass and the lower number subunit mass, both in 1000 Dalton. Molecular weight established by indirect methods are given in brakets.

b Nonidentical subunits. Because of the failure to demonstrate subunits in any other forms, this enzyme should perhaps be considered as predominantly monomeric.

c The low molecular weight form was identified as glucokinase.

d Individual organism can be identified from reference (166)

Of the 14 enzymes surveyed, only one, phosphoglycerate kinase, appears to be a monomer. All the others are dimers or higher multimers, and moreover, with only a few exceptions, a given subunit pattern appears to be quite constant for each enzyme. The main deviations from the constant pattern are high molecular weight triosephosphate dehydrogenase from spinach chloroplasts (167) the monomeric aldolase (168) and the thermophile enolase (169 and 170). Phosphoglucomutase is reported to be a dimer of two different subunits (171). The multimeric proteins have multiple substrate binding sites-often one site per unit (172-175), which is important in the general pattern of glycolytic enzymes. Most of the glycolytic enzymes have preserved a subunit structure through evolution and the multimeric enzymes seem to be made up of identical or very similar subunits, each containing one active site. Many of the exceptions are found in highly specialised organisms, tissues or cells. If the multimeric structures of glycolytic enzymes were undesirable or irrelevant, they would have been randomly distributed. It is fair to assume that a fairly narrow spectrum of subunit aggregation is advantageous and thus has been preserved. Subunit interaction is an absolute prerequisite for regulation of enzyme action. For some of the glycolytic enzymes this possibility is valued. The phosphorylase *a* and *b* interconversion through phosphorylation - dephosphorylation is an excellent example of it. When we compare the glycolytic enzymes from marine sources, the above assumption is again complex. Phosphorylase from Sepia (22) and Loligo exhibit, in most cases the nature and properties of desensitised rabbit phosphorylase. It may be due to the adaptation of phosphorylases of these animals to meet definite needs.

CHAPTER-14

EXPERIMENTAL

A. MATERIAL

Glucose-1-P, Glucose-6-P, Glycogen, Sephadex G-25, CM-cellulose and Dialysing tube were obtained from Sigma Chemical Co., USA.

AMP, ATP, PNP, PNPP, DEAE-Cellulose, Cyanogen bromide, ANSA, L-cysteine hydrochloride, Bovine serum albumin were the products of SISCO Laboratories, Bombay

Sodium-ß-glycerophosphate was obtained from Koch Light Lab., London.

All other chemicals were of analytical grade.

B. METHODS

i) Estimation of protein by Lowry's method using Folin-ciocalteu reagent (25)

Reagents

a) 2% Sodium carbonate in 0.1 N NaOH

b) 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartarate

c) Alkaline copper solution: mixed 50 ml of reagent (a) with 1 ml of reagent (b)

d) Diluted Folin's reagent: Folin-ciocalteu reagent was diluted 1:2 in water to make it 1 N in acid

Method

A small quantity of the protein (containing approximately 1 to 2 mg) was precipitated by adding an equal volume of 10% Trichloroacetic acid. The precipitate was collected by centrifugation at 5000 rpm for 15 minutes and washed 2-3 times with 5% TCA. The pricipitate was dissolved in 0.1 N NaOH.

To 1 ml of protein solution (containing 50-300 μg of protein), added 5 ml of reagent (c), mixed well and allowed to stand at room temperature fro 10 minutes. To this was added 0.5 ml of reagent (d) and mixed well. After 30 minutes, the optical density was measured at 500 nm in a spectrophotometer. The instrument was calibrated using bovine serum albumin as standard (Fig.14-1). Calibration curve for protein estimation using Folin-ciocalteu reagent. The optical density was measured at 500 nm in a spectrophotometer.

Dialysing

Dialysis was used both for removing excess low-molecular-weight solute, including ammonium sulphate and simultaneously introducing 10^{-3} M tris buffer containing 5 mM mercaptoethanol to the enzyme solution. Complete removal of salt and small molecules could not be achieved by single dialysis. A large volume (1 to 2 litres) of buffer was preferred to avoid fewer buffer changes. This was a convenient step to be carried out overnight as it needed no attention and equilibrium was reached by morning. Dialysis was carried out at 0-5°C. The tube was directly used from the roll and avoided the boiling of EDTA-$NAHCO_3$ solution as recommended elsewhere. After wetting, two knots were tied in one end and a filter funnel placed in the other for pouring in the sample. With the end of the tubing resting on a convenient surface, the sample was poured in. It was best to have the tubing flat to start with so that air did not get out through the funnel. The tubing was then closed with a further knot and placed in the dialysis buffer. Some space was left for expansion during dialysis. After dialysis enzyme solution was applied to Agarose-glycogen column or DEAE cellulose column.

Preparation of DEAE-cellulose column

Coarse DEAE-cellulose was washed with 1N NaOH till no more yellow colour was removed. This was washed with water and suspended in 1N HCl and filtered immediately and washed with water till free from the acid. It was then suspended in 1N NaOH, filtered and washed free of alkali with water and finally suspended in 10 mM glycerophosphate buffer (pH 7) and adjusted to pH 7 using a few drops of 1N HCl with stirring. The DEAE-cellulose after one experiment was

reused after washing with 0.5 N NaOH and adjusting to pH 7 as above. A suspension of the DEAE-cellulose was filled in the column of the required size and equilibrated with the eluting buffer connected from a reservoir.

Preparation of Agarose-glycogen column

Five gm cyanogen bromide was mixed with 125 ml of 4% agarose beads previously washed with distilled water. After equilibration for a few minutes under gentle stirring with a smooth magnetic stirrer bar, 4 M NaOH was added at a rate that maintains pH of 11.0-11.5 for 6 to 10 minutes. The reaction versel was cooled in an ice bath, as formation of reactive imidocarbonate was favoured at low temperature at the expense of side reaction giving nonreactive derivatives. Crushed ice was added in the reaction mixture to aid dissipation of heat. Local over heating was avoided by controlling the NaoH concentration at 4 N. The gel was washed on a Buchner funnel with 5-6 volumes of chilled distilled water.

Agarose was then added to a solution containing 2-10 mmoles of ligand (1.25 g glycogen in 125 ml water) and the mixture was adjusted to pH 10 and stirred at 4 $^{\circ}$C for 12 to 18 hrs. The glycogen-agarose derivative was washed exhaustively with water by filtration and equilibrated with 1 mM sodium-ß-glycerophosphate buffer (pH 7) for hydrophobic chromatography.

Sephadex gel filtration

For separation of enzyme from mercaptoethanol ammonium sulfate and other small molecules Sephadex G-25 was employed. The Sephadex gel was allowed to swell in distilled water for several days. The gel was washed by decanting the water layer and adding further volume of water. Slurry of the gel was poured into column and allowed to settle while water was allowed to slowly pass through the

gel. For separation of small molecules, a column size of 1 x 20 cm was used. Before passing the enzyme solution, the column was repeatedly washed with 2 mM Sodium-ß-glycerophosphate buffer (pH 7). The enzyme solution was layered on the top of the gel without disturbing the gel surface. When the enzyme layer completely entered the gel, the eluting buffer (10 mM Sodium-ß-glycerophosphate) was carefully filled and the column connected to a reservoir containing buffer and LKB UV-monitor, recorder and fraction collector. The flow rate was adjusted to 0.5 ml/minute. Ammonium sulfate free (tested with Nesslers reagent) fractions having phosphorylase activity were collected.

Estimation of glycosyl residue in glycogen

Phenol sulfuric acid reagent

Phenol 5%: Redistilled (reagent grade) phenol (50 g) dissolved in water and diluted to one litre.

Sulfuric acid 96% reagent grade.

A weighed quantity (10 mg) of glycogen was hydrolysed in 0.6 N HCl by boiling in a water bath for 2 to 3 hrs (172). The contents were cooled, neutralized using 0.5 N NaOH with phenol red as indicator and quantitatively made upto 10 ml. The glucose concentration in the hydrolysate was estimated according to the procedure (173), using glucose as standard. Calibration curve of glucose standard is given in Fig.14-4.

Polyacrylamide gel electrophoresis

The polyacrylamide gel electrophoresis was performed according to the procedure of Ornstein and Davis (141).

Preparation of gel

a) Buffer solution

Six gram of Tris and 28.8 g of glycine dissolved in 1000 ml of water (pH 8.6)

b) Stock solution

i) Stock A (pH 9): 38.3 g of Tris and 0.46 ml of TEMED dissolved in 48 ml of 1N HCl and made upto 200 ml.

ii) Stock B: 30 g of acrylamide and 0.9 g of bisacrylamide dissolved in water and made upto 100 ml.

iii) Stock C: 0.14 g of crystalline ammonium persulfate dissolved in 100 ml of water. Fresh preparation is advisable.

c) Preparation of gel column

Stock A, B and C were pipetted out (in that order) in the ratio 1:1:2, mixed well and the solution was allowed to polymerise in the electrophoretic tubes.

d) Preparation of Amido black

The stain solvent was a mixture of 5 parts of water, 5 parts of methanol and 1 part of acetic acid. 1 g of amido black was dissolved in 100 ml of the solvent.

e) Washing solution

The gel stained with amido black was washed with 7% acetic acid.

Electrophoresis was performed at 5-10 $^{\circ}$C for 4 hours using a current of 4 mA per tube. A 7.5% gel was used. The buffer employed was Tris-glycine pH

8.5. The protein bands were stained with amido black and the gels were washed with 7% acetic acid at 50-60°C.

For the activity band, the gel was immersed in a substrate solution containing 40 mM glucose-1-P, 1 mM AMP and 2% partially hydrolysed starch. (The starch was prepared by boiling 10 ml of 4% starch solution with 2-3 drops of 1N HCl for a few seconds and neutralising to pH 7 with solid Na_2CO_3). After incubation at 35°C for 30 min, calcium chloride solution was added to a final concentration of 0.04 M and heated in a 60°C water bath for 5-10 min. The activity band was developed as clear disc of calcium phosphate.

Kinetic studies

For Kinetic studies with varying glucose-1-P concentration, the glycogen and AMP concentration in the substrate solution was maintained constant (0.5% and 1 mM respectively) and glucose-1-P concentration varied from 1 mM to 32 mM. Similarly for kinetics with respect to AMP, glucose-1-P and glycogen concentrations of the substrate were 16 mM and 0.5% respectively and AMP concentration varied from 2×10^{-3} mM to 1.25 mM. The enzyme solution was properly diluted and added to substrate solution at 30°C and assayed as given in previous chapters. Separate blanks were taken for each substrate concentration.

Inhibition by glucose-6-P and glucose

For inhibition studies, a stock solution of the inhibitor was first prepared in water by adjusting the pH to 7. This inhibitor solution was suitably diluted in the buffer to be used in the assay. The enzyme solution was diluted in 10 mM Sodium-ß-glycerophosphate buffer (pH 7) and the solutions of the inhibitors glucose-6-P; glucose and ATP (pH 7) were added so as to have the specified concentration

of the inhibitors indicated in text when mixed with substrate solution. The enzyme concentration in the incubation mixture was 18 to 30 μg/ml. Preincubated (at 30°C) enzyme-inhibitor mixture was mixed with substrate and incubated for 10 minutes. The reaction was arrested by the addition of stopping reagent.

Inhibition by PNP and PNPP

For studies with PNP and PNPP as inhibitors, stock solution of these compounds (0.1 M) were prepared in water after adjusting the pH to 7. Further dilutions were made in 10 mM Sodium-ß-glycerophosphate pH 7. A suitably diluted inhibitor solution mixed with enzyme solution and assayed by adding substrate as mentioned above. In all the cases specific activities were calculated from comparison with a calibration curve obtained with KH_2PO_4. All the optical densities were corrected for blank readings obtained under similar assay conditions.

The phosphorylase phosphatase and kinase reaction

The phosphorylase phosphatase reaction was carried out according to the procedure of Keller and Cori (174). For this, the Loligo enzyme (1-2 mg/ml) in 10 mM Sodium-ß-glycerophosphate buffer at pH 6.5 and 7) was incubated with phosphatase solution using the Loligo muscle extract and incubated at 30°C. Ali-quots were removed from this, diluted in 10 mM Sodium-ß-glycerophosphate buffer pH 7 and assayed for phosphorylase activity at 30°C in the absence and presence of 1 mM AMP. The extract from frozen muscle at a 10-fold final dilution could not alter the activity ratio of Loligo phosphorylase.

The phosphorylase kinase reaction was carried out by the method of Krebs and Fisher (1975). For this, to 1 ml (1-2 mg) of the enzyme in 10 mM

Sodium-ß-glycerophosphate (pH 7) was added to (i) 1 ml of 0.125 M tris/glycerophosphate (at pH 8.6), (ii) 0.5 ml of 6 x 10^{-2} M Mg (Ac) and 1.8 x 10^{-2} M ATP, pH 7 and (iii) 0.5 ml of phosphorylase kinase, using the loligo muscle extract. This was incubated at 30°C, aliquots removed, diluted in 10 mM Sodium-ß-glycerophosphate buffer (pH 7) so that the concentration of ATP in assay medium was below 0.5 mM and the activity ratio found out. The activity ratio of loligo phosphorylase *a* (0.6) was unaffected by the extract from frozen mantle at a final dilution of 5 fold.

Temperature studies

The substrate and enzyme solutions (with or without inhibitors) were separately brought to the required temperature in a thermostat. The solutions were mixed and incubated in the same temperature. After the required period of incubation, stopping reagent was added and the solution cooled (in the case of incubation at temperature above 30°C) to room temperature by placing the test tubes in water at room temperature. Blanks at each substrate concentration were also similarly treated except that the enzyme solutions were added after the addition of the stopping reagent.

Effect of pH

The enzyme was diluted in 10 mM Sodium-ß-glycerophosphate buffer of the required pH and added to the substrate solution containing 16 mM glucose-1-P, 1 mM AMP and 0.5% glycogen adjusted to the required pH. The optical density was measured after 10 minutes at 30°C as described earlier.

Cold inactivation studies

Phosphorylase from *Loligo vulgaris* (19.4 µg/ml) in 10 mM Sodium-ß-

glycerophosphate in the presence and absence of 1 M NaCl (at pH 7 and pH 6) were incubated at 0°, 15°C and 25°C. Aliquots were withdrawn from these samples at intervals and again incubated at 30°C for 1 minute and assayed the enzyme activity in usual procedure by adding 16 mM glucose-1-P, 0.5% glycogen and 1 mM AMP, maintaining the same pH of the assay.

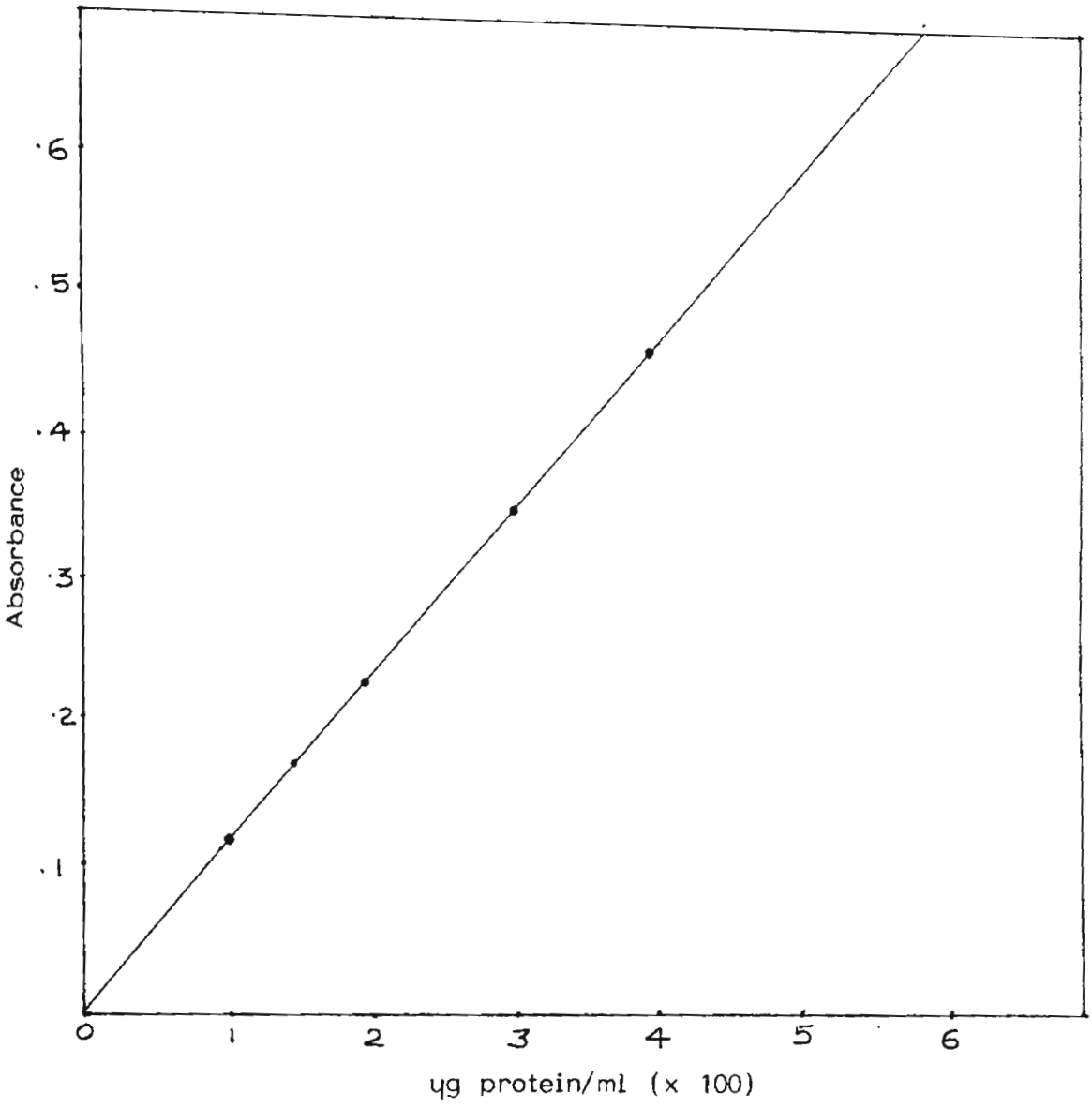


Fig. 14-1

Fig. 14-1

Estimation of phosphorylase activity

Procedure of Illingworth and Cori (14) was adopted for determining the phosphorylase activity. The inorganic phosphate was estimated according to the method of Fiske and Subbarow (15). For routine assay phosphorylase was diluted in 10 mM Sodium-ß-glycerophosphate (pH 7) for 20 to 30 minutes prior to assay for getting reproducible properties of enzyme.

Reagents

ANSA reagent

A mixture of 12 g of sodium metabisulphite, 1.2 g of sodium sulphite and 200 mg of 1 amino-2 naphthol-4-sulphonic acid (ANSA) was well powdered and dissolved in 100 ml of glass distilled water. The reagent was stored in a brown bottle at 0-5°C.

Stopping reagent

To 2.5 g of ammonium molybdate dissolved in 100 ml of glass distilled water, was added 10 ml of 5 N H_2SO_4 and 710 ml of water. At zero time 1 ml of enzyme solution was added to 1 ml substrate containing 16 mM glucose-1-P, 0.5%, glycogen and 1 mM AMP and incubated at 30°C, 2 ml of aliquots were withrawn at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 minutes interval added 0.2 ml water and 8.2 ml stopping reagent. To this was added 0.9 ml of 5 N H_2SO_4, followed by 0.5 ml of ANSA reagent. A blank was similarly treated (in this case enzyme solution was added after addition of the stopping reagent). The blue colour developed was measured after 20 minutes at 660 nm in a spectrophotometer. The optical density was

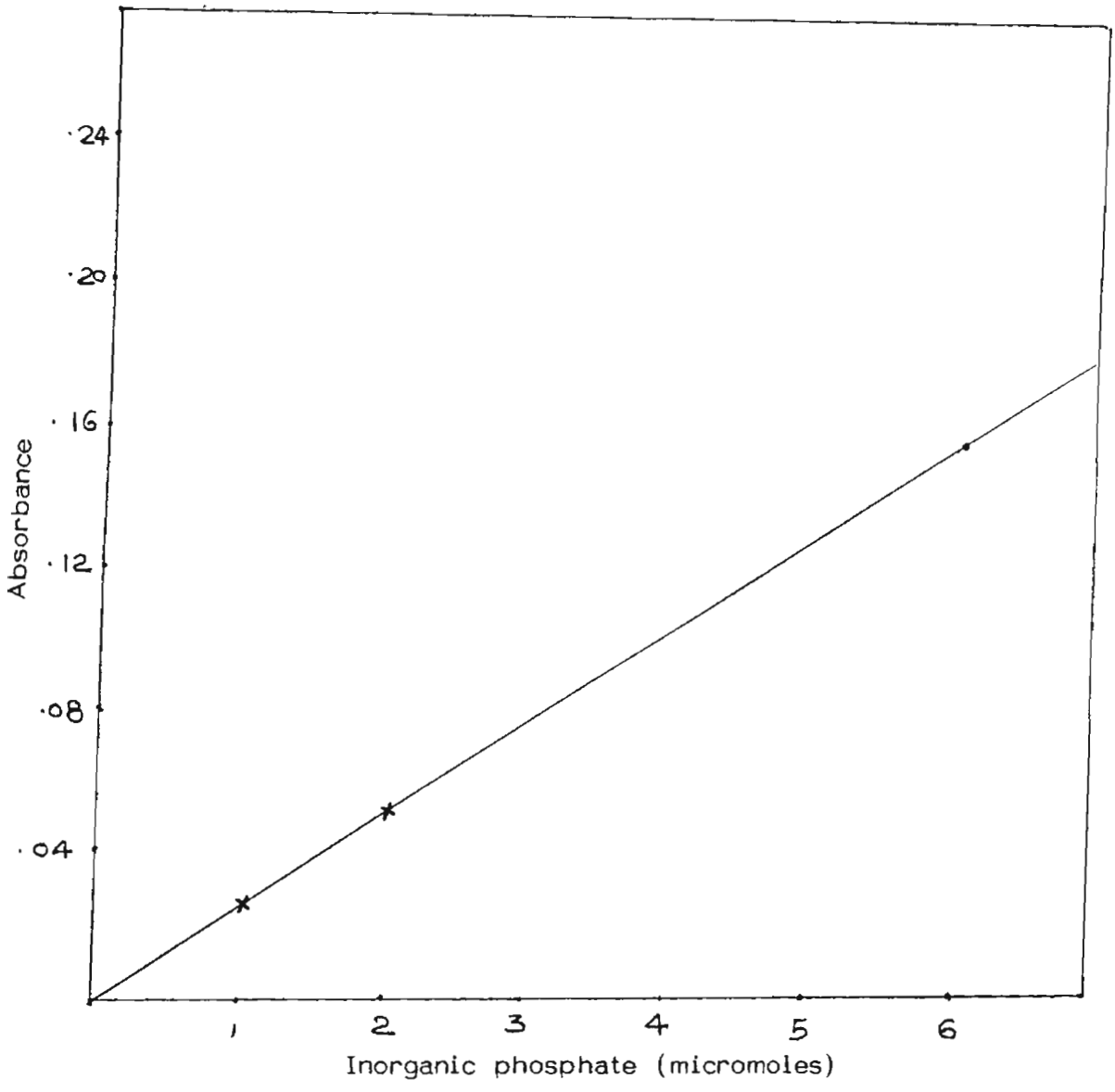

Absorbance
·24
·20
·16
·12
·08
·04
1
2
3
4
5
6
Inorganic phosphate (micromoles)

Fig.14-2

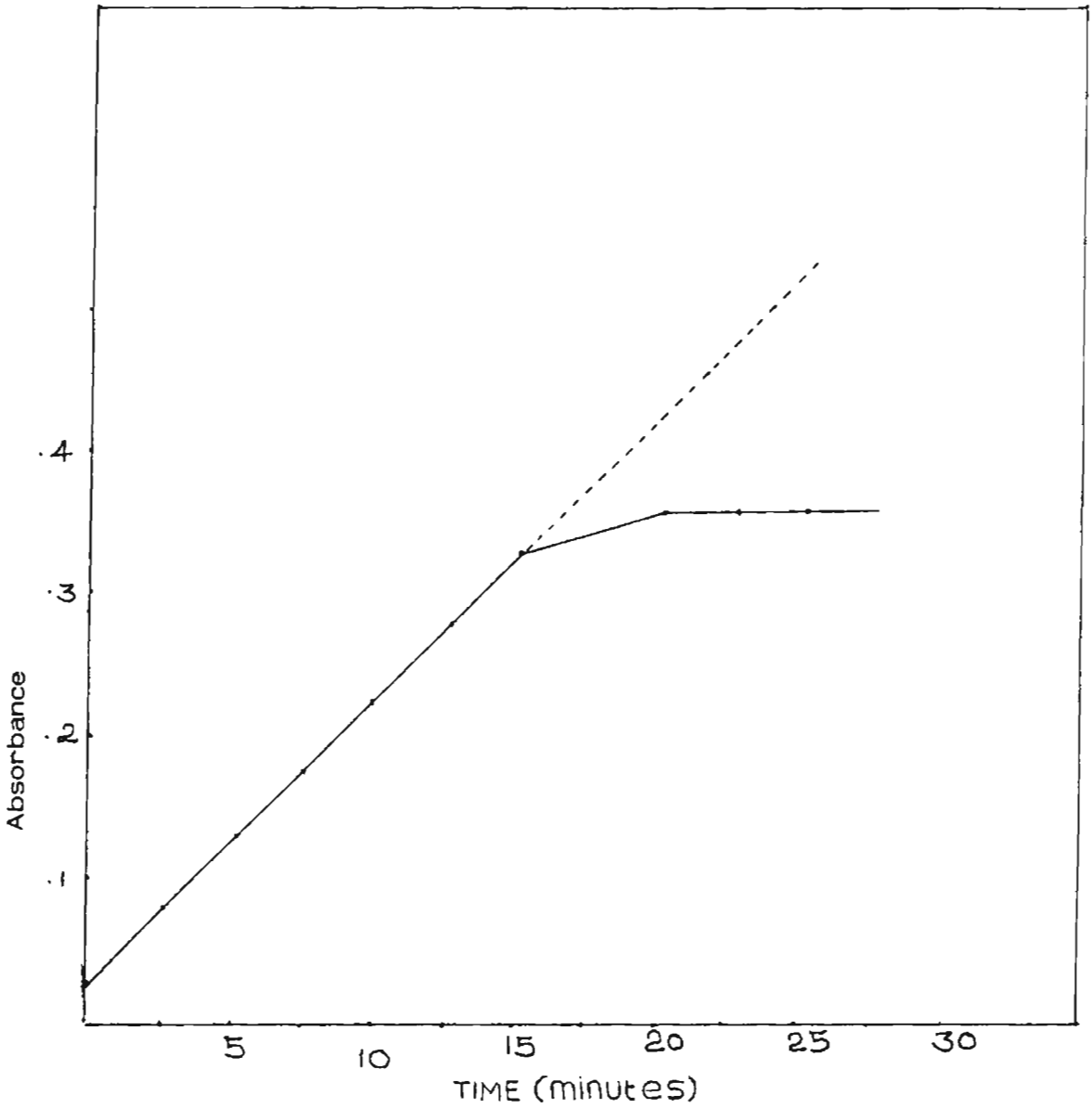


Fig.14-3
Progress curve of phosphorylase activity of Loligo vulgaris

converted to micromoles of inorganic phosphate liberated using a calibration curve obtained with KH_2PO_4 under the same condition (Fig.14-2).

The time of incubation of enzyme assays in various experiments were adjusted so that the optical density of the developed solution was well in the linear part of a reference curve obtained for the reaction. The progress curve was obtained by plotting a product *vs* time curve as shown in Fig.14-3.

Fig.14-2

Calibration curve for determination of phosphate in phosphorylase assay. To 0.4 ml of KH_2PO_4 (containing different concentrations as indicated) was added 8.2 ml stopping reagent, 0.9 ml of 5 H_2SO_4 and 0.5 ml of ANSA reagent. The optical density at 660 nm was measured after 20 minutes, in a spectrophotometer.

Fig.14-3

Progress curve for phosphorylase activity. Phosphorylase *a* (10 μg/ml) in 10 mM Sodium-ß-glycerophosphate buffer (pH 7) was incubated along with substrate at 30°C (At zero time 1 ml of the enzyme was added to 1 ml of 32 mM glucose-1-P, 1% glycogen and 2 mM AMP). 0.2 ml aliquots were withdrawn at different time intervals, added 0.2 ml distilled water and 8.2 ml of stopping reagent. The colour development and reading were taken as mentioned in the calibration curve of phosphate determination.

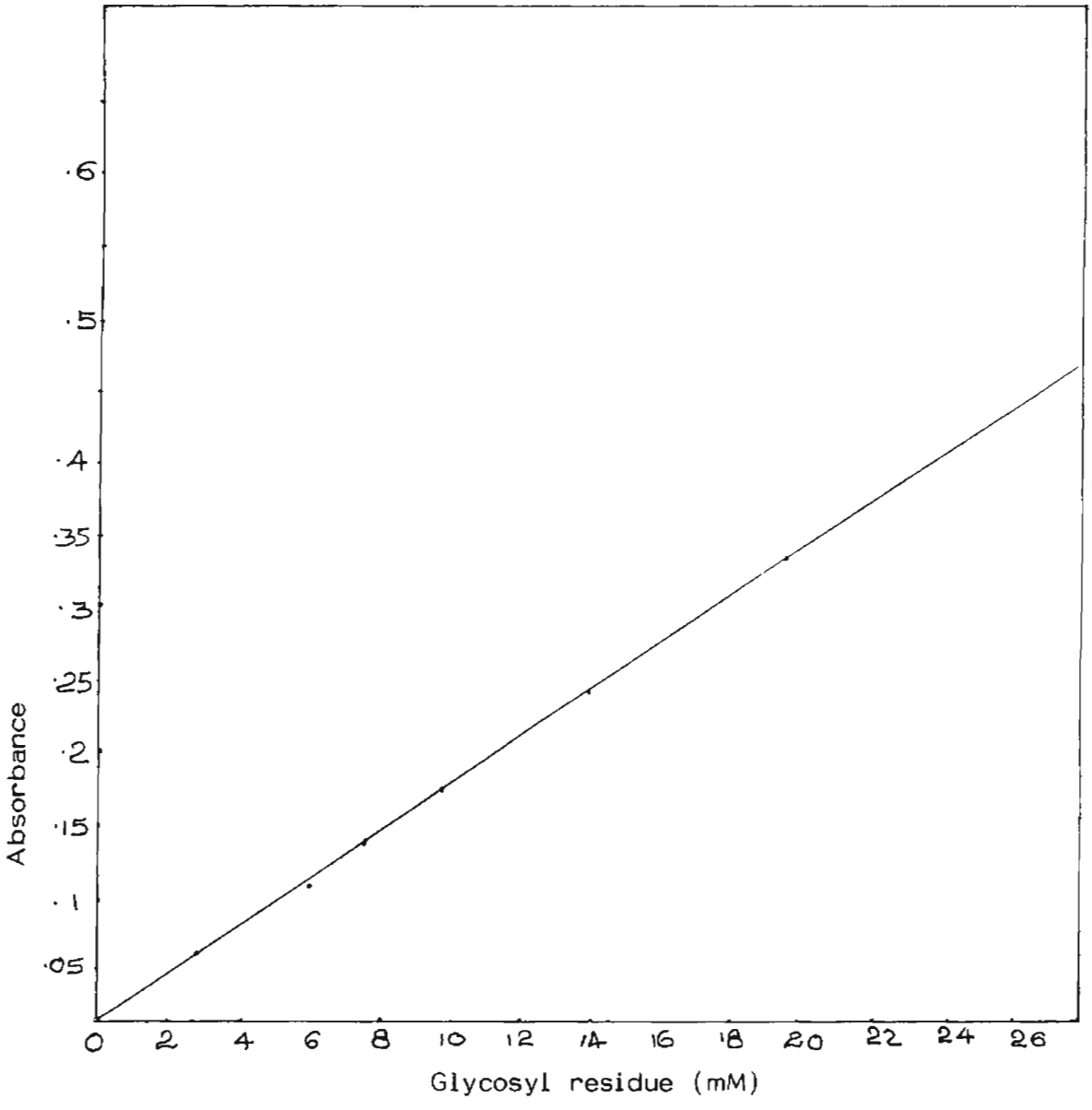


Fig. 14-4
Calibration curve of glucose

Fig.14-4

Calibration curve for glycosyl residues using phenol sulfuric acid reagent. 1 ml solution containing various concentration of glucose were used for estimation. Optical density read at 490 nm using a spectrophotomer.

SUMMARY

The thesis deals with the comparative study of glycogen phosphorylase from selected cephalopods, leading from the detailed study of *Lolio vulgaris* Mantle muscle phosphorylase *a*.

The apparent Km values for glucose-1-P and glycogen of Loligo phosphorylase illustrate the effect of glycogen and glucose-1-P on the affinity. Km values for glycogen remain unaltered at different concentrations of glucose-1-P but Km value for glucose-1-P decreases when glycogen concentration is increased, suggesting that the binding of glycogen enhanced the affinity for glucose. Heterotropic interaction due to the binding of glycogen favoured binding of glucose-1-P but not *vice versa*. The results show inconsistency with the predictions of the model of Monod where allosteric transitions are represented by T and R states and the transitions are affected through changes in the Km values only.

The Kinetic content K_5 (i.e., Michael constant of glucose-1-P at saturating glycogen concentration) of Loligo phosphorylase *a* is higher than Sepia and rabbit phosphorylase *a*. The dissociation constant for glucose-1-P (K_8) is much less than that in *Sepia pharaonis* and slightly higher than that in *Cibium guttattam*.

The reciprocal plots of AMP are curved downward showing negative cooperativity between AMP binding sites. Similar negative cooperativity has been reported for Sepia, *Metapenaeas dobsoni* and Villorita. This negative cooperativity may be an adaptation to the energy need of the animal. Allthough AMP is the only

natural activator that we have found so far, the physiological significance of the slight shift in Kinetic constant is not clear.

The influence of varying concentrations of Glucose-1-P on Loligo phosphorylase *a*, in the presence and absence of glucose-1-P is not at all comparable with that of rabbit phosphorylase. Apparent Km value of glucose-1-P in the presence of glucose-6-P in Sepia phosphorylase is lower (4) than in the Loligo enzyme (37.5) which implies the low degradative efficiency of Loligo enzyme.

Since the increment in activity due to AMP is more at higher AMP concentration, it is possible that AMP binding brings about a conformational change in the enzyme and that this conformation is less sensitive to glucose. The glucose inhibition in Loligo enzyme is of mixed type which again is different from that in rabbit phosphorylase. It is likely, therefore, that glucose is either competing or interacting with other sites, glycogen binding site is a possible one. However, the effect of glucose, in causing a conformational change, cannot be ruled out.

Paranitrophenol and Paranitrophenyl phosphate increase the activity ratio at all levels of glucose-1-P. This suggests that there is no protection by AMP. So binding of PNP and PNPP in Loligo phosphorylase *a* is at a site other than the AMP site. Since the binding sites are overlapping, PNP, PNPP, glucose-1-P, AMP and glucose-6-P may bind on the region located near the monomer/monomer interface. This is possible only if the ligands adopt different modes of binding, the modes being determined by their structural features. The inhibition of PNP and PNPP are not time dependent where as modification by aromatic compounds like FDNB has been shown to be time dependent. These reagents undoubtedly bind on the enzyme prior to reaction.

Studies on temperature effect reveal that charge neutralization and exclusion of water is possible in Loligo phosphorylase. The most likely amino groups in the enzyme sites are lysyl and arginyl residues which are positively charged under assay condition. The activation energy at saturation level of glucose-1-P is 3.69 Kcal/mol which is less when compared to that of saturation levels of AMP (4.35 Kcal/mol). *n* values of Hill plots in loligo phosphorylase *a* (saturating levels of glucose-1-P (16 mM) and varying concentration of AMP) are in an increasing order of 0.6, 0.8 and 0.83 at 20, 30 and 35°C respectively. Since the entropy change in loligo phosphorylase is quite small, there may be a loss of water when activated complex is formed.

Cold inactivation of loligo phosphorylase cannot exclusively be attributed to cold temperature because inactivation and hence conformational changes occur under different temperatures and other conditions used for the studies. Based on the observations, different forms of phosphorylase are proposed in a scheme for explaining cold inactivation. Since cold inactivation is high in loligo enzyme, it has a more flexible conformation than other well studied systems. This can be related to the evolutionary status because the cephalopods are far below rabbit and man in evolutionary status, and hence several fine control mechanisms identifed as well developed in rabbit and man operate at a much lower level in loligo phosphorylase.

Effect of ionic concentration in the activity of loligo phosphorylase is found to be the same as that in the well-studied rabbit enzyme. 2.5 mM $HgCl_2$ is sufficient to inactivate the enzyme. Activation by Ammonium sulfate and sodium sulfate is predominant in loligo phosphorylase. This is in agreement with X-ray analysis of Barford and Johnson which shows that sulfate ions appear to have worked as an activator in place of phosphate at the active site and at the phosphorylation site at serine 14.

The optimum pH for phosphorylase activity is 6.9-7.0 which is quite similar to that of rabbit phosphorylase *a* and *b*, a little higher than that of liver phosphorylase (pH 6.2-6.4) and a little lower than that of *E. coli* maltodextrin phosphorylase (pH 7.2). Since the pH profile of Loligo phosphorylase *a* is almost similar to that of Sepia and rabbit phosphorylase, the 5'-phosphate group of PLP has been shown to be directly participating in catalysis.

Even though the *Loligo vulgaris* is primitive in evoluationary status, the extremely high energy demand of the mantle muscle for moving with lightning speed is operated by the modified enzyme system of glycolysis. The unique property of cold inactivation, effect of aromatic compounds and other properties suggest the structural difference of Loligo phosphorylase *a* from other purified phosphorylases studied so far. Considering the functional difference of Loligo enzyme, it is possible to speculate that the specific influence of modified groups in the binding sites promote ATP as an activator at 10 mM level. The metabolic role of ATP seems to be significant as it is found that Loligo muscle is maintaining a very low concentration of AMP. In brief loligo phosphorylase *a* exhibiting the nature and properties of desensitised rabbit phosphorylase.

A comparative study of glycolytic enzymes from marine sources is given in chapter 13. Glycolysis seems to be the oldest energy yielding process in the biosphere. A fairly narrow spectrum of subunit aggregation is advantageous and thus has been preserved. The phosphorylase *a* and *b* interconversion through phosphorylation - dephosphorylation is an excellent example of subunit interaction which is an absolute prerequisite for regulation of enzyme action. A survey of 14 glycolytic enzymes is given in Table 13-5. Of the 14 enzymes surveyed, only phosphoglycerate kinase appears to be a monomer. All the others are dimers or higher multimers, and

moreover with only a few exceptions, a given subunit pattern appears to be quite constant for each enzyme. Most of the glycolytic enzymes have preserved a subunit structure through evolution and the multimeric enzymes seem to be made up of identical or very similar subunits, each containing one active site. Many of the exceptions are found in highly specialised organisms like *Loligo vulgaris*, commonly known as squid.

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