GLYCOGEN DEGRADING ENZYMES AND THEIR REGULATION IN BENTHIC ANIMALS AND ESTUARINE FISH

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BY

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Cochin,
May 9, 1984.

This is to certify that the thesis entitled 'Glycogen degrading enzymes and their regulation in benthic animals and estuarine fish' herewith submitted by Smt. Sosamma Cherian in partial fulfilment of the requirement for the Ph.D. degree in Biochemistry of the University of Cochin, is an authentic record of the work carried out by her under my supervision in this department and that no part thereof has been presented before for any other degree in any University.

(Dr. George Philip)
Supervising Teacher.

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

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P R E F A C E

α-glucan phosphorylase plays a very significant role in glycolysis. The inhibition and activation of this enzyme have significant effect on the rate of glycolysis. The rate of glycolysis is also determined by the interconversion between the active a and inactive b forms of phosphorylase by two specific enzymes called phosphorylase phosphatase and phosphorylase kinase. The allosteric properties and interconversion mechanism reported for well-studied animal muscle phosphorylases do not fall under a general pattern. Studies using purified phosphorylase from marine sources are scanty. Detailed studies using specialised tissues from more marine animals are necessary to find the factors that control the properties and activities of the enzyme. This thesis is an attempt in this direction. The thesis deals with a detailed study of the control of the phosphorylase by both allosterism and interconversion between the a and b forms from four different aquatic animals of different habitat.

Phosphorylase from the four different animal muscles were purified either partially or completely and the kinetic and control properties were studied.

The animals selected for the study are seen in Cochin backwaters. They were Etroplus suratensis an estuarine fish, Metapenaeus dobsoni a marine cum estuarine prawn, Sunetta scripta a marine bivalve and Villorita cyprenoides an estuarine bivalve. Of the four animals three are benthic invertebrates and the other a vertebrate fish.

The thesis deals with the purification and properties of phosphorylase from the four animal muscles and comparison of the four phosphorylases with each other and with other animals.

A B B R E V I A T I O N S

AMP	= Adenosine-5'-monophosphate.
ATP	= Adenosine-5'-triphosphate.
DEAE-cellulose	= Diethyl aminoethyl cellulose.
FDNB	= 1-Fluoro-2,4-dinitrobenzene.
Glucose-1-P	= Glucose-1-Phosphate.
Glucose-6-P	= Glucose-6-Phosphate.
PLP	= Pyridoxal-5'-phosphate.
TCA	= Trichloro acetic acid.
TEMED	= N,N,N',N'-Tetramethyl ethylenediamine.
Tris	= Tris (hydroxy methyl) amino methane.

C O N T E N T S

CHAPTER 1

INTRODUCTION

α-Glucan phosphorylase (1,4-α-D-glucan: orthophosphate α-D-glucosyl-transferase, EC 2.4.1.1) catalyses the degradation of glycogen in animals and starch in plants. This widely distributed enzyme acts on polysaccharides and splits off glucose residues as α-D-glucose-1-P. The reaction may be written as:-

$$G_{(n)} + \text{orthophosphate} \rightleftharpoons G_{(n-1)} + \text{glucose-1-P}$$

where $G_{(n)}$ and $G_{(n-1)}$ are polysaccharides with 'n' and 'n-1' glucose residues respectively. This reaction is readily reversible. Since glucose-1-P is a stronger acid than orthophosphate, the equilibrium constant is pH dependent. In the case of rabbit muscle phosphorylase at pH 6.8, the equilibrium ratio of orthophosphate/glucose-1-P is 3.6, thus favouring synthesis rather than degradation. However, circumstantial and experimental evidences clearly show that the function of phosphorylase in vivo is degradation (1,2,3).

In animals, glucose is stored in the form of glycogen. Hence α-glucan phosphorylase from animals is generally called glycogen phosphorylases. Glycogen phosphorylase can be assayed either in the direction

of glycogen synthesis or glycogen degradation. In degradation, the liberated glucose-1-P is estimated using a coupled enzyme assay system (4). In the direction of glycogen synthesis, glucose-1-P and glycogen are used as substrates (5) and the product orthophosphate is estimated colorimetrically.

i.e. $\text{glycogen}_{(n-1)} + \text{glucose-1-P} \longrightarrow \text{glycogen}_{(n)} + \text{Pi}$.

Since the second method is easier and less expensive, it is generally preferred. This method is used for the work presented in this thesis.

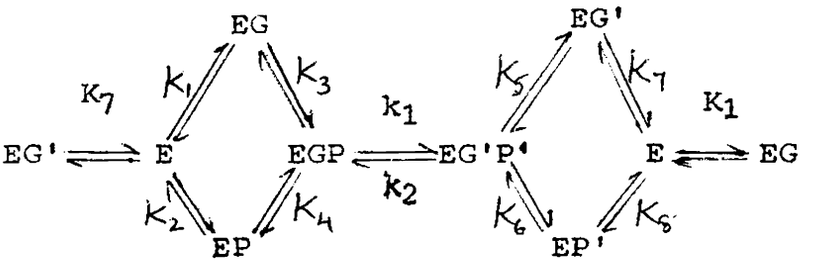
Animal phosphorylases exist in two interconvertible forms, phosphorylase a and b (6). AMP is necessary for the b form to be active, whereas the a form is active even in the absence of it. However, some activation of the a form by AMP (about 20-30%) is generally observed. Phosphorylase a is the phosphorylated form and is converted to the non-phosphorylated b form by a specific phosphatase called phosphorylase phosphatase (phosphorylase phosphohydrolase EC 3.1.3.17). Phosphorylase b is converted to the a form by phosphorylase kinase (ATP: phosphorylase phosphotransferase EC 2.7.1.38) which transfers a phosphate group from ATP to each subunit of phosphorylase b. In the regulation of glycogenolysis, the inter-conversion of the a and b forms of the enzyme plays a significant role.

The phosphorylase b kinase is activated by a hormone induced cascade system and by Ca^{+2} ions and the phosphorylase a phosphatase is regulated by conformational changes in phosphorylase induced by various ligands of phosphorylase (7,8).

Phosphorylase is highly specific for α-D-glucose-1-P for the transfer of glucosyl residue (9). Various analogues of the substrate have been found to have no action on rabbit and potato phosphorylases (9,10). In the degradative direction phosphate can be replaced by arsenate, but with a much lower rate (11). At normal assay conditions, a primer containing a minimum of 3 to 4 glucose units is essential for catalytic process (12).

The kinetic mechanism of phosphorylase from rabbit muscle (13,14) and from other sources (15-19) has been shown to be sequential. By detailed kinetic analysis including product inhibition studies and isotopic exchange, the kinetic mechanism for rabbit muscle and *E. coli* phosphorylase has been established as rapid equilibrium random bi bi (13-15). While such detailed studies have not been performed with other enzymes, no case is reported where the kinetic properties differed from what is expected for the above

mechanism. The established kinetic scheme is as given below for degradation of glycogen:



where E is the enzyme; P = orthophosphate; P' = glucose-1-P, and G and G' = glycogen with n and n-1 glucose residues. K_1, K_2 etc. are equilibrium constants and k_1 and k_2 are the rate constants for the forward and backward reaction respectively.

The velocity equation derived for this scheme differs slightly from the general equation for random mechanism, because in this case glycogen is a product as well as the substrate. This kinetic mechanism has been confirmed by isotope exchange studies and it is unaltered during allosteric transitions, when sigmoidal substrate - saturation curves are obtained (18).

$$\frac{E_0}{v} = \frac{1}{k_1} + \frac{K_4}{k_1(G)} + K_3 \frac{(1+(K_1/K_7))}{k_1(P)} + \frac{K_1K_3}{k_1(G)(P)}$$

where E_0 = total enzyme concentration; v = initial velocity.

Rabbit muscle phosphorylase b exists as a dimer. But in the mussel, Mytilus edulis it is shown that monomeric and dimeric forms of phosphorylase b exist in equilibrium (20). The subunit relationship of phosphorylase a depends on conditions like enzyme concentration, pH, temperature, ionic concentration etc. (7). At low concentrations, rabbit phosphorylase a exists as a dimer (21). Phosphorylase a from lobster (22,23), crab, (24) and Sepia (25) exists exclusively as dimers. In man, shark, rat and frog (7) also dimeric and tetrameric forms of phosphorylase a are present. The molecular weight in all these cases has been found to be in the range of 90,000 to 100,000 for the monomer.

Structural studies of the rabbit enzyme have shown similar but not identical subunits (26). From electron microscopic and X-ray crystallographic studies phosphorylase b has been found to consist of 2 ellipsoidal units bound together, with measurements 110:65:55 Å (27) and 115:75:60 Å (28,29) respectively. Because of symmetrical association of dimers, the tetramer is square in shape (30). Tubular shaped crystals have been obtained for phosphorylase a in

the presence of protamine (31).

The complete amino acid sequence of 841 amino acids in rabbit muscle phosphorylase has been derived (32). At neutral pH, the positively and negatively charged amino acids are well balanced, though it is not evenly distributed. However, starch phosphorylase from banana leaves is rich in basic and heterocyclic amino acids, but poor in acidic and aromatic amino acids (33).

Comparison of sequence of 15-19 N-terminal amino acid residues of phosphorylase from potato, E. coli, yeast and five vertebrate sources (34) showed that the first amino acid is a hydroxy amino acid. Plant phosphorylases which are non-regulated have a free α-amino group in contrast to blocked α-amino group in all other phosphorylases analysed for amino acid sequence. The sequence of potato and rabbit enzymes whose control mechanisms are different, are very similar except for the dissimilarity seen at the N-terminal residues (35,36). The sequence near the PLP site in rabbit phosphorylase is homologous to that from yeast (37), potato (36,38) and E. coli (34,39). The peptides containing SH groups obtained from the rabbit and potato phosphorylases also have highly homologous sequences (35). These authors (35)

pointed out from their studies that phosphorylase existed originally as a large catalytically active molecule and by gradual mutation, a regulatory mechanism evolved within the molecule.

All α-glucan phosphorylases contain one molecule of pyridoxal-5'-phosphate (PLP) bound to each subunit of the enzyme (7). However phosphorylase isolated from tapioca leaves (19) and sucrose phosphorylase do not contain PLP. The role of the coenzyme in phosphorylase is not clearly understood. In rabbit muscle phosphorylase, PLP is linked covalently to Lys-679 and buried inside a hydrophobic region (40,41). The PLP can be resolved by deformation of the enzyme and trapping it with a reagent like L-cysteine (42). Removal of PLP from the enzyme is accompanied by a loss of activity and a tendency to dissociate to monomers at room temperature (43). Reconstitution of the apoenzyme with PLP is attended by return of catalytic activity and dimerisation, which is temperature dependent. The quaternary structure of the apoenzyme is different from that of the holoenzyme (44).

$NaBH_4$-reduction of the enzyme from plants and animals does not abolish the catalytic activity of phosphorylase (45,46). Studies with PLP-analogues

show that except for the pyridine nitrogen and phosphate, no other group participates in the catalytic process (7). The finding by Graves et al that phosphite can activate pyridoxal reconstituted enzyme, while pyrophosphate is a competitive inhibitor of both phosphite and glucose-1-P shows the involvement of phosphate moeity in catalysis (47). The effect of pH on enzyme activity (48) and dependence of pH on the fluorescence quantum yield of PLP monomethyl ester (49) show that the phosphate group may participate as in general acid-base catalysis. Recent X-ray crystallographic analysis shows that PLP is only 6 Å away from the catalytic site. A mechanism for phosphorylase action has been proposed in which the phosphate group of PLP acts as a nucleophile and the imidazole of histidine-376 acts as a general acid (50). Reconstituted enzyme with pyridoxal-5'-phospho 1 - β-D-glucose shows no activity though the substrate binds in a similar mode as on PLP-reconstituted enzyme (51). Studies with pyridoxal-5'-diphospho 1 - α-D-glucose reconstituted enzyme have shown evidence for direct phosphate-phosphate interaction between PLP and substrate during activity. It is suggested that the positive charges in the active site may constrain

the coenzyme-phosphate into a configuration which renders the phosphorus atom electrophilic facilitating nucleophilic attack by the substrate phosphate and thereby, in the resultant quasi-pyrophosphate link transition intermediate, labilising the α-anomeric bond of glucose (52). X-ray crystallographic studies in the presence of glucose 1,2-(cyclic) phosphate show that the phosphorus atom is 6.8 Å away from the phosphorus of PLP. Detailed kinetic studies indicate that the phosphate of PLP and the substrate may be closer together in the fully activated form than observed in X-ray structure of the substrate analogue complex (53).

Spectral studies suggest that the neutral form of the enzyme is a zwitterionic addition product of some nucleophilic group on the protein with the Schiff base (54). 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 (25).

PLP also has an important effect on enzymic interconversions (55,56) between the a and b forms. Recent studies show that control of dephosphorylation

of phosphorylase a by glucose requires PLP (55-57). Since the coenzyme is present in phosphorylases of distinct species in which the control mechanisms are different, the function of PLP can not be exclusively attributed to such a control mechanism. Moreover, amino acid sequences of the PLP site of potato, yeast, E. coli and rabbit muscle phosphorylases are highly homologous (36-38) and the activity of potato phosphorylase is highly dependent on PLP; yet the former enzymes are not interconverted.

Phosphorylase b is activated by AMP and inhibited by ATP, glucose and glucose-6-P. 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. Aromatic compounds also inhibit phosphorylase, depending on their hydrophobicity (58,59).

Glycogen phosphorylase has been subjected to chemical modification studies inorder to find out the functional groups and also to study the allosteric transitions. Use of reagents modifying SH groups has shown that two surface-exposed SH groups can be modified without loss of activity, some SH groups

react slowly with loss of activity and some others are inaccessible (60,61). Lysyl residues have been shown to be essential for enzyme activity (62). An FDNB derivative which is desensitised of the allosteric properties, in which one lysyl residue and one cysteinyl residue were modified has been prepared (63). Dinitrophenylation of one amino group has also been shownto result in loss of activity (64). Two tyrosyl residues are essential for maintaining enzyme activity (65,66). Modification of phosphorylase b with potassium ferrate identified Tyr-75 as essential for catalysis (67). Presence of histidine residues at the substrate binding region is suggested (68). Carboxyl group modification also leads to loss of activity (69). Two types of essential arginine residues, one in the allosteric site and other in the active site, have been identified (70).

Direct participation of any group or groups in catalytic mechanisms of phosphorylase has not yet been confirmed from chemical modification studies. X-ray crystallographic analyses have partially revealed the environment of the active site (71). PLP is surrounded by the hydrophobic Tyr-90, Trp-490, Val-649 and Arg-138. The pyridinium nitrogen is in

close contact with the carboxyl of Glu-645 and the nitrogen of Lys-654 is associated with the phenolic oxygen. The basic residues Lys-573 and Lys-567 are near the phosphate of PLP and His-376 is found near the O-5 of bound glucose.

The X-ray crystallographic location of various ligand binding sites of phosphorylase is incomplete. According to Madsen and his collegues (71) there are four binding sites for phosphorylase a. The 'active site' (two per dimer) where the substrates, glucose-1-P, Pi, glycogen and arsenate and inhibitor glucose bind is shared between the two subunits at their interface and comprises a pocket like region with a V-shaped frame work of 2 α-helices. The second site 'the activator site' binds glucose-1-P, Pi, AMP, ATP and glucose-6-P. The third is the 'glycogen storage site' to which glycogen binds 20 times stronger than on the active site. The fourth is the 'inhibitor site' on which purines and nucleosides bind. It is suggested that in the E. coli phosphorylase the structure of the glycogen storage site determines the minimum length of the oligosaccharide primer (72). The nuecleoside inhibitor site of phosphorylase a, where a family of related compounds (purines, nucleosides, nucleotides

etc.) bind, is located at the surface of the enzyme 10 Å from the catalytic cleft (73). Philip et al have shown the presence of the glycogen storage site in rabbit phosphorylase by preparing a derivative with oligosaccharide covalently bound to the glycogen storage site (74).

X-ray studies of Johnson et al using phosphorylase b crystals grown in IMP, identified one nucleotide binding site ('Site N') and a catalytic site ('Site C') (75). The 'Site N' is very close to the active site (76). The inhibitor binding site of glucose-6-P is also very close to active site. Glycogen binds on the surface of the molecule 50-60 Å from the active site. In addition to the 'Site N', the nucleoside can bind to a second site, 'Site I' (nucleoside inhibitor site). X-ray crystal analyses have shown that the subunits may bind in different modes on the active site under different conditions (76) - a conclusion arrived at in our laboratory much earlier using kinetic analysis of chemically modified phosphorylase (77).

Two types of direct control of phosphorylase activity have been reported; (1) interconversion between a and b forms and (ii) control by allosteric inhibitor or activator (7).

All animal phosphorylases exist in two interconvertible forms *viz.* phosphorylase *a* and *b*. The *a* form is active as such while *b* form requires AMP for activity. Therefore the interconversion of the two forms plays a major role in the control of glycogen degradation (7). In rabbit muscle and other terrestrial vertebrate muscles, phosphorylase kinase (which converts the *b* form to the *a* form by transferring a phosphate group to each monomer of phosphorylase *b*) is present in an active and inactive form. The inactive form of the kinase is converted to the active form also by phosphorylation catalysed by a non-specific protein kinase which in turn is controlled by a cascade system including cyclic AMP and hormones (7). Although this system of control of phosphorylase activation is not demonstrated for all animal phosphorylases there are no reported data against it. Phosphorylase phosphatase (which converts phosphorylase *a* to *b* by removing a phosphate residue per monomer) exists in a latent form in fresh tissue extracts of rat muscle. The phosphatase is generated in the active form presumably by proteolysis (78).

Phosphorylase a phosphatase and phosphorylase b kinase from rabbit muscle have been found to interconvert most animal phosphorylases tested so far (79-82,22,23). The yeast phosphorylase which also exists in the phosphorylated and non-phosphorylated form is not acted upon by the converting enzymes from rabbit muscle (83). The kinase and phosphatase from insects act on rabbit muscle phosphorylase (84). The converting enzymes of rabbit muscle are non-specific such that purified phosphorylase phosphatase will dephosphorylate even acetyl-CoA carboxylase thereby activating it (85). Graves et al have synthesised peptides containing 14 and 8 residues which can act as substrates for the kinase and phosphatase (86,87).

The existence of two interconvertible forms have been established in a wide spectrum of animal tissues like rabbit liver, rat liver, human leukocytes, in the oocytes and embryos of the loach, in insects (88-95), in Neurospora crassa, in mollusca Biophalaria glabrata (96,97), in lamprey (82) and in mussel Mytilus edulis (20). Two or more forms of phosphorylases have been reported in blood platelets, rat chloroma, swine kidney (98-100),

in the mollusc, crab (24) and Pectan maximus (101), Brewers yeast, banana leaves, spinach leaves and pea leaves (102-104) and in a number of other plant tissues (105). In many cases, these different forms are structurally and functionally different from the a or b forms of the enzyme from rabbit muscle. For liver phosphorylase, a high concentration of salt is also necessary for activity (88,89). The human leukocyte b form is 25% active in the absence of AMP (90). In Pectan maximus an additional form of enzyme (phosphorylase c) has been reported (101). In Dictyostelium discoideum a purified dimer form of the enzyme was not activated by AMP (106). From Neurospora crassa, a phosphorylase with high specific activity has been isolated which could be classified neither as the a nor as the b form (107). In Mytilus edulis (20) monomer of the b form is present which is activated by AMP. Though both phosphorylase kinase and phosphatase are shown to be present in Sepia mantle (25), theydo not have any significant role in the control of phosphorylase activity.

The second type of control of phosphorylase activity (and hence degradation of glycogen) is by

allosteric interaction of inhibiting and activating ligands. The effect of these ligands is considerable on phosphorylase b and less significant on the a form (7). All animal phosphorylases (b form) from terrestrial and aquatic vertebrates are inhibited by glucose-6-P, ATP and glucose and activated by neucleotides like AMP (7,58,59). However, the nature of inhibition and activation differs from species to species. Inhibition observed in the presence of these ligands can be classified as allosteric or competitive (partially or purely competitive). Activation is by either positive homotropic or negative homotropic cooperativity. Inhibition by the above inhibitors is allosteric in the case of phosphorylase b from the muscle of rabbit, rat, man and other vertebrate terrestrial animals (7) whereas no such definite pattern exists for the enzyme from marine vertebrates and invertebrates (17,25,58,59). For the enzyme from the latter sources (58,59) these ligands are competitive inhibitors. Such deviations have been more evident in the case of AMP kinetics in the presence of inhibitors. While AMP exhibits positive cooperativity for rabbit muscle phosphorylase (7), phosphorylase a from the mantle

tissue of Sepia (17,25) shows negative cooperativity. Interestingly the control of phosphorylase activity in Sepia mantle is distinctly different (17,25). These aspects also point to the fact that the properties of phosphorylase are linked with the energy requirements/evolutionary status of the animals.

Many models were proposed to explain the allosteric behaviour (108-112). None completely explains all the observed facts. The partially densensitised enzyme by chemical modification of one or two groups per monomer of rabbit muscle phosphorylase has simulated many of the allosteric properties of the enzyme studied from lower forms (58,59,25). So the question naturally arises whether the differences in allosteric properties are related to the evolutionary status of the animal. Is the fine control of the enzyme from terrestrial vertebrates by allosteric effectors an acquired property? The results described in the thesis show some interesting lines of thought in this direction also.

CHAPTER 2

PHOSPHORYLASE a AND b FROM ETROPLUS SURATENSIS

Etroplus suratensis, commonly known as pearl spot is a commercial fish available at all seasons. It is an estuarine fish, but can also survive in fresh water and marine water. Being an estuarine fish, it has to adjust itself to wide variations in salinity.

The procedure used for purifying phosphorylase b from the skeletal tissue of E. suratensis is given in Chapter 7. The skeletal muscle was separated after removing the skin and kept frozen at -15 to -20°C. The enzyme in the stored muscle was stable for 3 - 4 months. After grinding the frozen or fresh muscle, extraction was attempted with distilled water and 0.01 M sodium β-glycerophosphate buffer pH 7.0. Extraction with the buffer gave a higher yield of enzyme activity than with water. The first and second extracts obtained with equal volumes of buffer were used for further purification. The third extraction yielded comparatively very little activity (less than 10% of the first two). The activity extracted

per gram of tissue was on the average 9 units* and the specific activity 0.5 to 0.6.

Ammonium sulphate fractionation gave a 4-fold purification with 95% yield.

An 18-fold purification was achieved by DEAE-cellulose chromatography. The results of a typical experiment is shown in fig.2-1. Many other chromatographic methods (such as Cellulose phosphate, CM-cellulose) did not give any purification at this stage.

After concentration and dialysis of the active fractions, the protein solution was subjected to affinity chromatography on sepharose-glycogen. Typical results obtained on affinity chromatography are shown in fig.2-2. By this procedure, a 72-fold purification was obtained with an yield of 9%. Though the yield was poor it had to be done for the sake of a homogeneous preparation.

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*Unit of activity is defined as the number of μmoles of orthophosphate liberated per minute at 30°C in the presence of 1% glycogen, 16 mM glucose-1-P and 1 mM AMP in 15 mM cysteine/20 mM sodium β-glycerophosphate buffer pH 6.8. Specific activity is expressed as units/mg of protein.

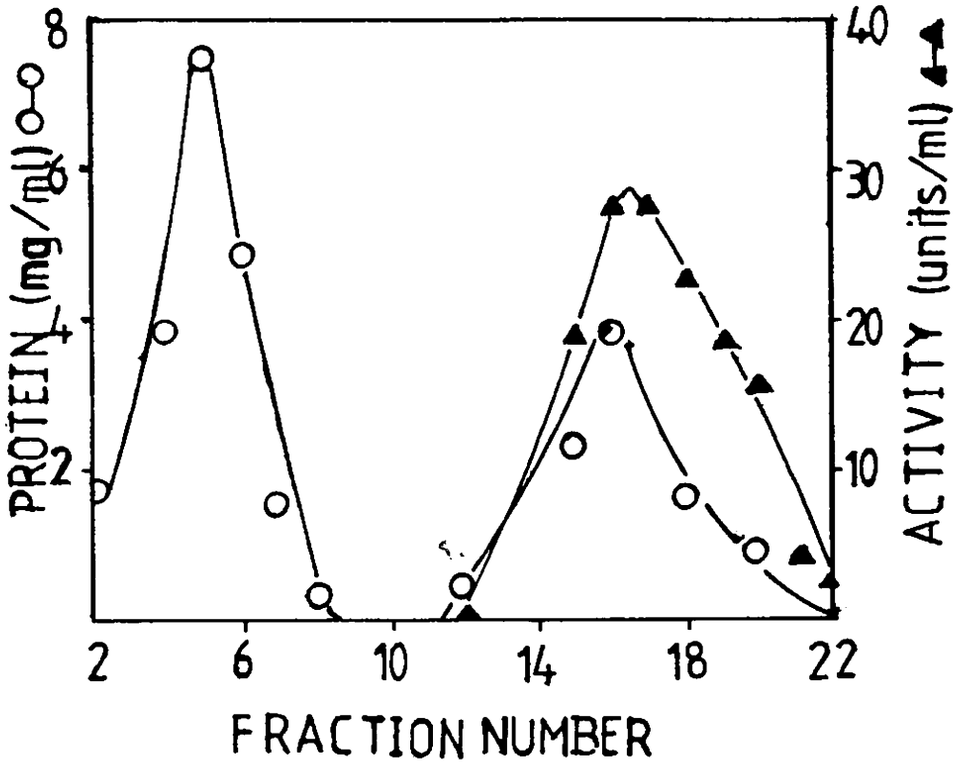

PROTEIN (mg/ml)
ACTIVITY (units/ml)
FRACTION NUMBER
2
6
10
14
18
22
8
4
2
40
30
20
10

Fig. 2-1

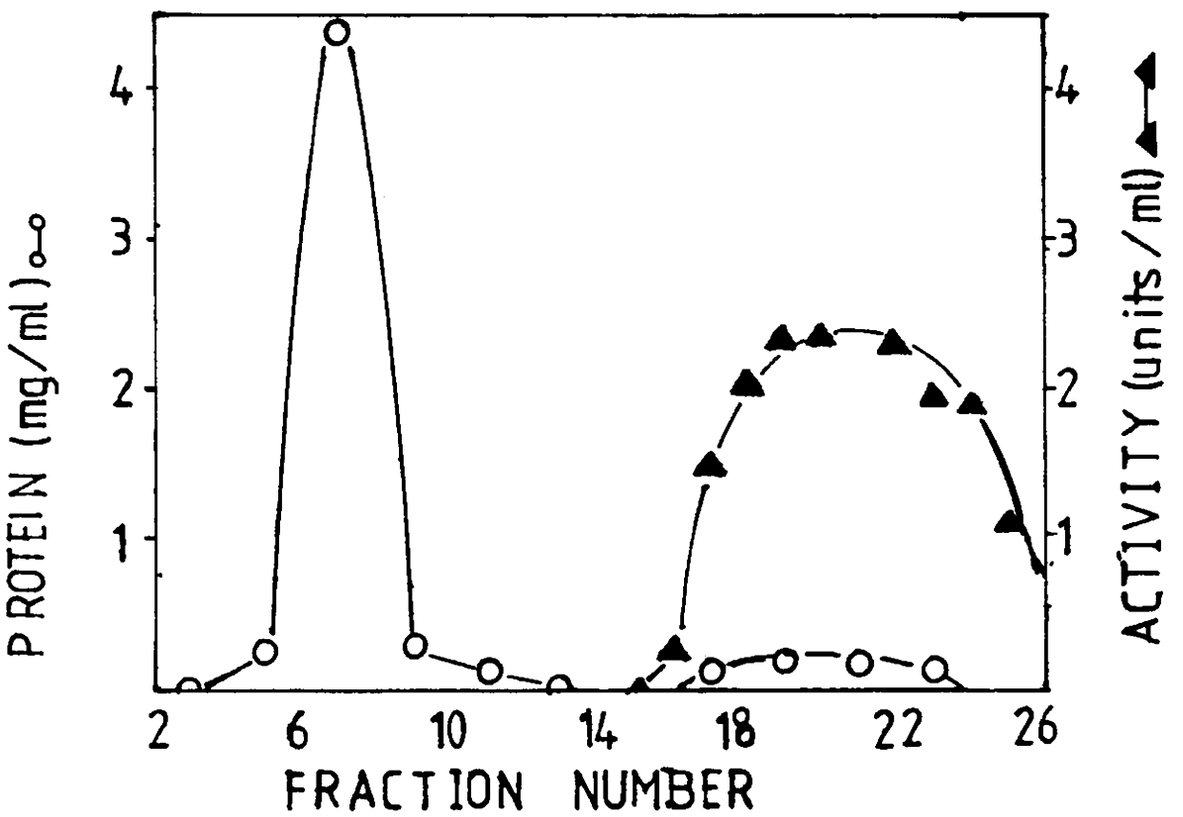

PROTEIN (mg/ml)
ACTIVITY (units/ml)
FRACTION NUMBER
2
6
10
14
18
22
26
4
3
2
1

Fig. 2-2

Fig.2-1.

Typical results of DEAE-cellulose chromatography of the protein obtained by ammonium sulphate fractionation. Experimental details are given in Chapter 7. Total protein applied in this experiment was 368 mg to a column of 16 x 1.5 cm size. The total activity yield was 75%. Fractions 15 - 22 were used for further purification. o, protein concentration (mg/ml); ▲, activity (μmoles of phosphate liberated per minute per ml of fraction).

Fig.2-2.

Affinity chromatography of the DEAE-cellulose purified E. suratensis phosphorylase b on sepharose-glycogen. Details are given in Chapter 7. Column size was 22 x 1.5 cm and 1.5 ml of protein solution (44 mg/ml) was applied. o, protein concentration (mg/ml of fraction); ▲, activity as μmoles of orthophosphate liberated/min/ml of fraction.

The results of a typical purification procedure are summarised in table 2-1. About 2 mg enzyme was obtained from 100 gm of frozen muscle. The specific activity of the purified enzyme was 40 units/mg protein which compared very well with the value

TABLE 2-1.

PURIFICATION OF PHOSPHORYLASE FROM ETROPLUS SURATENSIS

Purification step	Total volume ml.	Activity/ ml. units	Protein mg/ml	Specific activity units*	Yield	Purification
Extract	180	5	9	0.56	100	1
50% Residue (Ammonium sulphate fraction)	10	80	36.8	2.2	88.88	4
DEAE-cellulose	35	19	1.9	10	74.44	18
Affinity column	8	10	0.25	40	8.89	72

reported for the rabbit and other terrestrial vertebrate muscle phosphorylases (13) and also for the marine fish C. quttattum enzyme (119). However, the specific activity of the enzyme from the marine invertebrate mantle (25) was only half of the above value. The enzyme was concentrated by adsorption

* Specific activity is expressed as μmoles of inorganic phosphate liberated per minute per mg protein at 30°C.

using a small DEAE-cellulose column and eluted with 0.5 M sodium β-glycerophosphate pH 7.0 containing 5 mM mercaptoethanol.

Purity of the enzyme.

Polyacrylamide gel electrophoresis (113) of the preparation after DEAE-cellulose chromatography showed 4 bands of proteins. The purified enzyme after affinity chromatography, on electrophoresis showed only one protein band the corresponding activity band (see experimental). Therefore, the purified phosphorylase was apparently homogeneous.

The activity ratio (ie. the ratio of activity in the absence of AMP to that in its presence) of the enzyme at different stages of purification was as follows: The extract showed a ratio of 0.5 immediately after extraction, which decreased to 0.2 on keeping for 30 minutes at room temperature (indicating the presence of phosphorylase phosphatase in the extract). The ammonium sulphate fraction had an activity ratio of 0.14. After DEAE-cellulose chromatography the ratio decreased to 0.05. The purified preparation showed no detectable activity in the absence of AMP. Therefore the observed activity in the absence of AMP in the unpurified preparations could be due to the

presence of AMP carried from the extract or due to the presence of minor quantity of phosphorylase a.

The enzyme was stable after purification for at least 2 weeks when stored at 4°C. Etroplus suratensis phosphorylase had an optimum pH of 6.8 in 15 mM cysteine/20 mM sodium β-glycerophosphate buffer (see below) like the rabbit enzyme. In 20 mM sodium β-glycerophosphate buffer pH 6.8, the activity was found to be only 83% of that in the 15 mM cysteine/ 20 mM sodium β-glycerophosphate buffer, having the same pH indicating activation by cysteine. Similar activation by cysteine has been reported for rabbit muscle phosphorylase also (62). In the case of the rabbit enzyme the activation by cysteine has been attributed to reduction of 2-3 surface exposed SH-groups which have been shown to be easily oxidised in the absence of cysteine (62). However this effect of cysteine was not the reason for a 3-fold activation of Sepia mantle phosphorylase (25) because in this case accompanied spectral changes and time - dependent activation by cysteine were demonstrated. In the case of the Sepia enzyme, the effect of cysteine has been shown to be related to comformational changes around the PLP region which is not the case with rabbit enzyme.

Therefore, in the present case the effect of cysteine is more like its effect on the vertebrate enzyme than on the invertebrate Sepia enzyme. In the present study, cysteine was included in the assays.

The effect of various ions on the activity of the enzyme was studied. Interestingly it was found that $(NH_4)_2SO_4$ and Na_2SO_4 had concentration dependent activation and inactivation properties (table 2-2). Lamprey muscle and human leukocyte phosphorylase were shown to be activated by Na_2SO_4 (82,90). Rat liver phosphorylase b is also activated by high concentrations of sulphate (89). While Na^+, K^+ and Cl^- had no effect on the activity of the enzyme, high concentrations of $MgCl_2$, $CaCl_2$ and KI and low concentrations of $HgCl_2$ (5 mM) inhibited the enzyme activity.

The effect of various salts in the presence and absence of cysteine on enzyme activity is given in table 2-2. It is seen that in the presence of cysteine the activation by sulphate is higher than in its absence. Also in the presence of cysteine, the inhibition by $MgCl_2$, KI and $HgCl_2$ was less than in its absence. It is found that 2.5 mM $HgCl_2$ completely inhibited the activity in the absence of cysteine, whereas in the presence of cysteine only 20% inhibition

TABLE 2-2

EFFECT OF SALTS ON E. SURATENSIS PHOSPHORYLASE b

IN THE ABSENCE AND PRESENCE OF CYSTEINE

Salt	Concentration	Effect on enzyme activity in the presence of cysteine	Effect on enzyme activity in the absence of cysteine
$(NH_4)_2SO_4$	50 mM	+ 15.2%	+ 7%
	100 mM	+ 32.6%	+ 22%
Na_2SO_4	50 mM	+ 17.4%	+ 24%
	100 mM	+ 30.5%	+ 26%
$MgCl_2$	50 mM	No effect	No effect
	100 mM	- 13%	- 30.43%
$CaCl_2$	50 mM	No effect	No effect
	100 mM	- 11%	- 11%
KI	50 mM	-54.4%	- 54.4%
	100 mM	- 67.4%	-82.6%
$HgCl_2$	2.5 mM	- 19.6%	Nil activity
	5 mM	Nil activity	Nil activity
NaCl	50 mM	No effect	No effect
	100 mM	No effect	No effect
KCl	50 mM	No effect	No effect
	100 mM	No effect	No effect

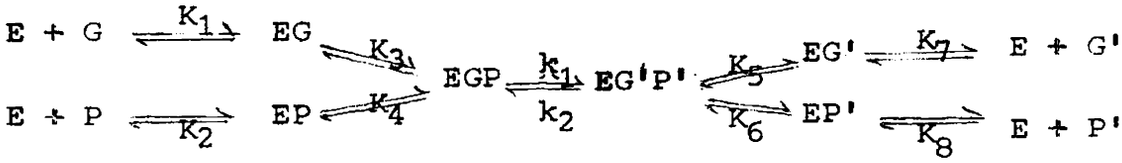
'+' indicates activation and '-' indicates inhibition.

was seen. This effect can be due to the reactivity of SH groups to Hg^{+2} and in the presence of cysteine, its SH groups react with Hg^{+2} protecting the SH groups of the enzyme. The less inhibition in the presence of cysteine for other salts than in its absence may be due to the indirect effect of cysteine on the enzyme. Cysteine may be needed for the active conformation of the enzyme and in the absence of cysteine the salts easily change the active conformation. Hence the same concentration of salt in the presence and absence of cysteine has different effects.

Kinetics.

The kinetic mechanism of rabbit muscle phosphorylase a and b (13,14,114), rabbit liver phosphorylase (115) and E. coli maltodextrin phosphorylase (15) has been shown to be rapid equilibrium random bi bi. Phosphorylase from a marine fish, Cibium guttattum (16), a marine mollusc, Sepia pharaonis (25) etc. have been reported to give results consistent with random equilibrium kinetic mechanism. The kinetic properties of Etroplus suratensis phosphorylase were studied in the direction of glycogen synthesis to compare the mechanism.

Initial rates were measured for E. suratensis phosphorylase b in the presence of 1 mM AMP by varying the concentrations of glycogen and glucose-1-P. The data obtained are presented in figs. 2-3 and 2-4 in the form of double reciprocal plots. These primary plots were all linear in the range of glycogen and glucose-1-P employed. The lines converged on the left hand side of the vertical axis, indicating that the kinetic mechanism of E. suratensis phosphorylase also is consistent with that for other phosphorylases from other sources. On this basis, the kinetic mechanism of E. suratensis enzyme may be represented as it has been proved for rabbit enzyme and other well studied phosphorylases (13).



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

The velocity equation derived for this scheme differs from the general equation for rapid equilibrium

Fig. 2-3.

Reciprocal plots for glucose-1-P at different levels of glycogen for glycogen phosphorylase b from E. suratensis. Phosphorylase b was assayed in 15 mM cysteine/20 mM sodium β-glycerophosphate pH 6.8 at 30°C. The reaction mixtures contained 1 mM AMP and glycogen and glucose-1-P as indicated. The enzyme concentration was 5.25 μg/ml. The concentration of glycogen was ▲, 42.5 mM (expressed as glucosyl residues); △, 17 mM; ●, 4.25 mM and ○, 1.7 mM.

---

random bi bi mechanism, because glycogen is both the reactant and a product. The equation for the kinetic mechanism is

$$\frac{(E_0)}{v} = \frac{1}{k_2} + \frac{K_6}{k_2\,(G')} + K_5\frac{(1+K_7/K_1)}{k_2\,(P')} + \frac{K_5\,K_7}{k_2(G')\,(P')}$$

Where E_0 is the total enzyme concentration.

If it is assumed that the affinity of the enzyme for glycogen is the same for the synthetic and degradative direction, the equation can be written as (13)

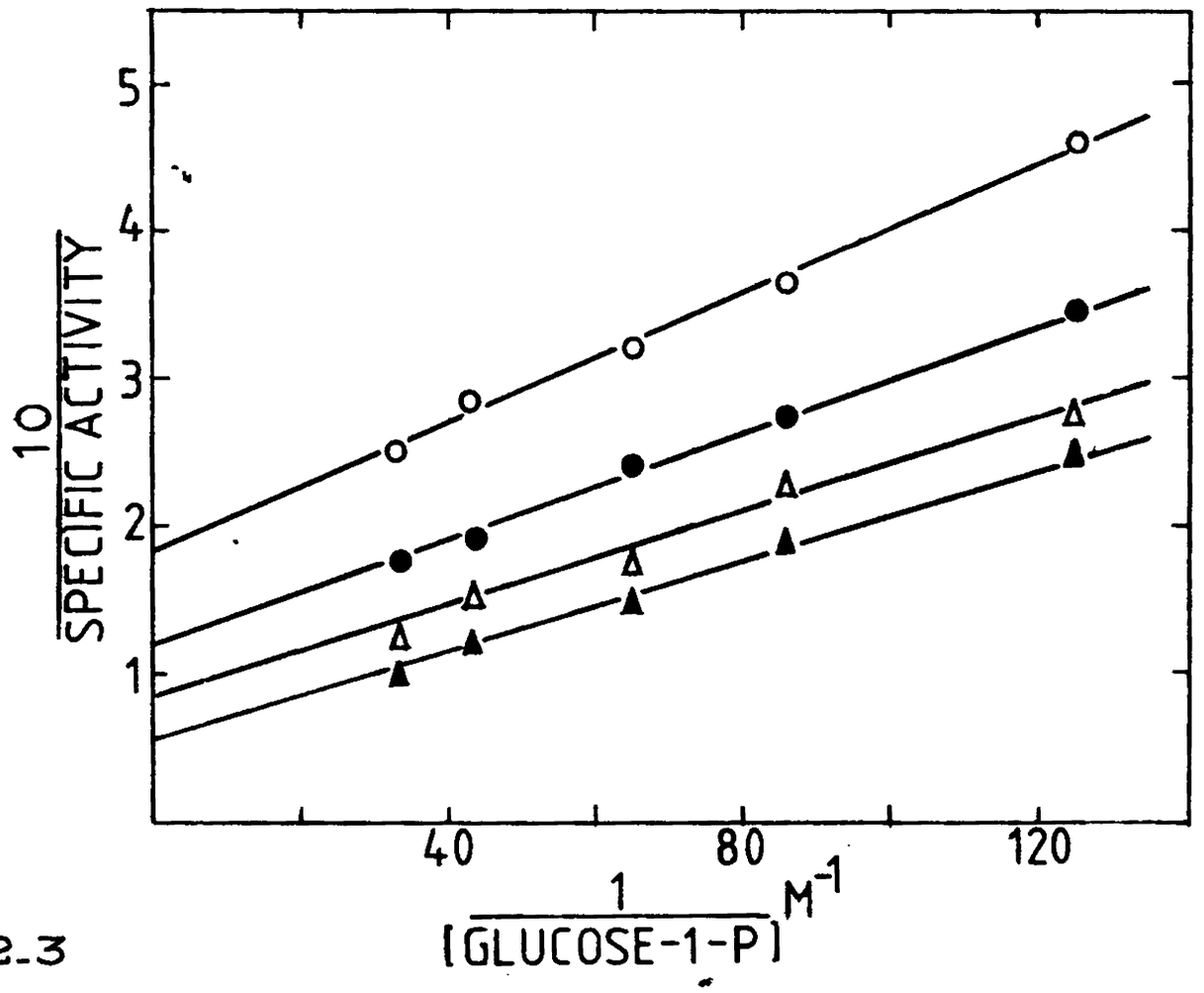


Fig. 2-3

Fig. 2-4.

Lineweaver - Burk plots for glycogen at different levels of glucose-1-P for E. suratensis phosphorylase b. ■, ●, △, □ and ○ correspond to 32,24,16,12 and 8 mM concentrations of glucose-1-P respectively. Other conditions were same as in fig. 2-3.

$$\frac{(E_0)}{v} = \frac{1}{k_2} + \frac{K_6}{k_2(G')} + \frac{2K_5}{k_2(P')} + \frac{K_5 K_7}{k_2(G')(P')}$$

The random mechanism can be proved by data obtained from reciprocal plots ie., secondary plots of intercepts and slopes derived from the primary plots (figs.2-3 and 2-4). For random mechanism, the secondary plots should be linear (116,117). In the present case of E. suratensis also, the secondary plots were found to be linear (figs.2-5 and 2-6). Therefore, the kinetic mechanism of E. suratensis phosphorylase was consistent with that proved for other cases ie., rapid equilibrium random bi bi.

The apparent K_m values of glucose-1-P and glycogen calculated from primary plots (figs.2-3 and 2-4) are given in table 2-3.

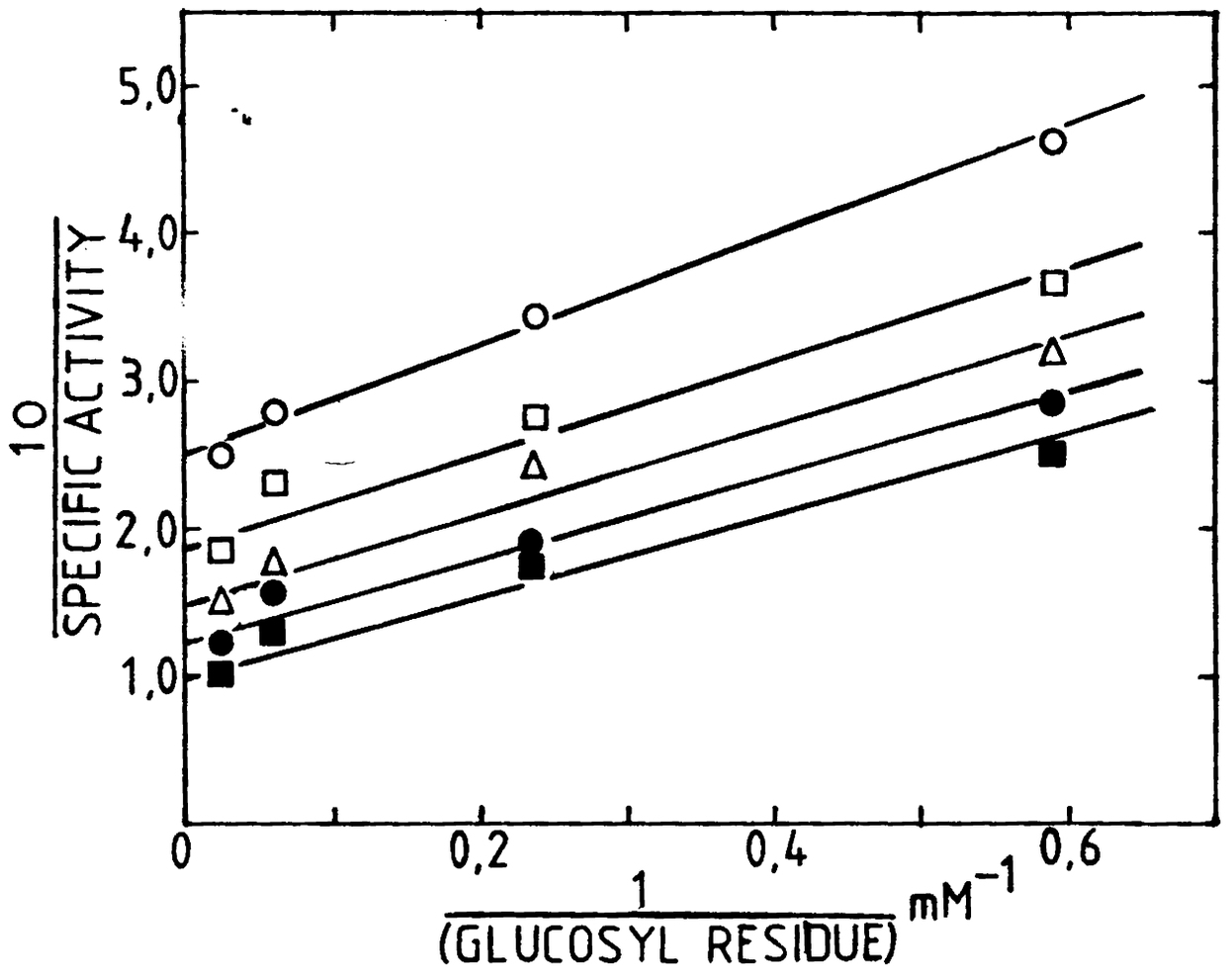

10
SPECIFIC ACTIVITY
5,0
4,0
3,0
2,0
1,0
0
0,2
0,4
0,6
1
(GLUCOSYL RESIDUE)
mM⁻¹

Fig. 2-4

Fig. 2-5.

Secondary plots from fig. 2-3.

A. Intercept of each plot versus the reciprocal of the concentration of glycogen.

B. Slope of each plot versus the reciprocal of the concentration of glycogen.

TABLE 2-3

APPARENT K_m VALUES FOR GLYCOGEN AND GLUCOSE-1-P AT DIFFERENT CONCENTRATIONS OF EACH OTHER FOR PHOSPHORYLASE b FROM E. SURATENSIS. (The concentration of glycogen is expressed as mM concentration of glucosyl residues)

Glucose-1-P (mM)	K_m for glycogen (mM)	Glycogen (mM)	K_m for glucose-1-P (mM)
8	1.45	1.7	11.5
12	1.67	4.25	14.7
16	2.00	17.0	17.24
24	2.38	42.5	25.00
32	2.7		

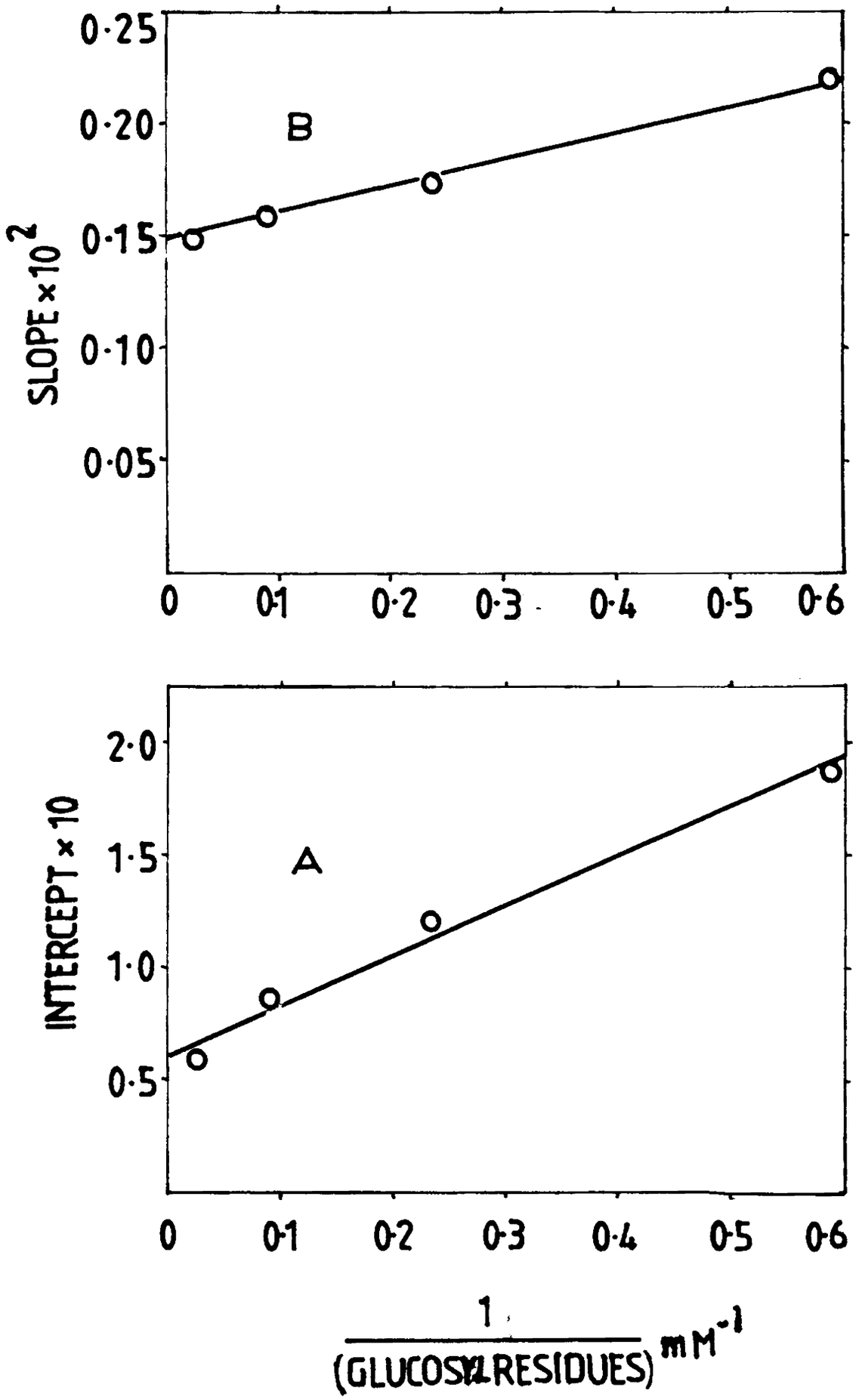

0·25
0·20
0·15
0·10
0·05
SLOPE × 10²
B
0
0·1
0·2
0·3
0·4
0·5
0·6
2·0
1·5
1·0
0·5
INTERCEPT × 10
A
0
0·1
0·2
0·3
0·4
0·5
0·6
1
(GLUCOSYL RESIDUES)
mM⁻¹

Fig.2-5

Fig. 2-6.

Secondary plots from fig.2-4.

A. Intercept of each plot versus the reciprocal of the concentration of glucose-1-P.

B. Slope of each plot versus the reciprocal of the concentration of glucose-1-P.

The heterotropic effect observed with the E. suratensis enzyme is contrary to all other cases of animal phosphorylases reported. The table 2-3 shows that increasing the concentration of glucose-1-P increases the K_m for glycogen and vice versa. Similarly increasing the concentration of glycogen increases the K_m for glucose-1-P. The kinetic mechanism of phosphorylase being random equilibrium, this means that increasing the concentration of glucose-1-P decreases the affinity for glycogen and vice versa. The role of phosphorylase being degradation of glycogen, this property is easily explained ie., when the concentration of glucose-1-P increases in muscle, it tends to decrease further degradation of glycogen by decreasing the affinity of the enzyme for glycogen. However, in all other well

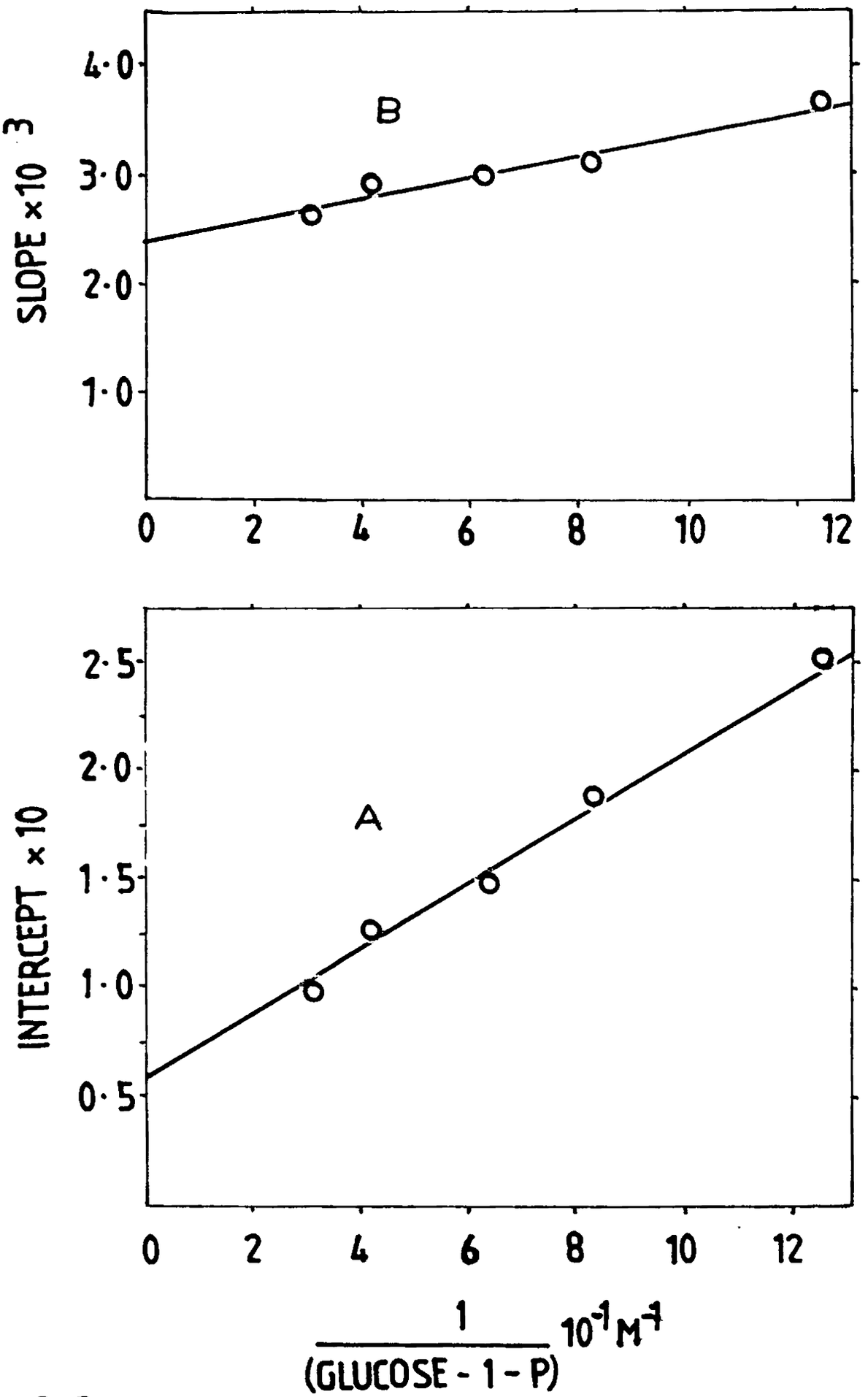

SLOPE ×10 3
4·0
3·0
2·0
1·0
B
0
2
4
6
8
10
12
INTERCEPT ×10
2·5
2·0
1·5
1·0
0·5
A
0
2
4
6
8
10
12
1
(GLUCOSE - 1 - P)
10⁻¹ M⁻¹

Fig. 2.6

studied cases, the opposite effect has been reported. Muscle phosphorylase from rabbit, rat, other terrestrial animals and Sepia, Cibium and other aquatic animals show increased affinity for glucose-1-P on increasing the concentration of glycogen and vice versa. Thus this property of E. suratensis phosphorylase is distinctly different from (and opposite to) other reported cases.

Activation by AMP.

The reciprocal plots for the substrate at different constant concentrations of AMP and those for AMP at different levels of glucose-1-P are given in figs.2-7 and 2-8 respectively. In both the cases, the lines are linear showing lack of homotropic cooperativity between activator sites and substrate sites. In the absence of inhibitors, rabbit enzyme also showed similar properties. It may be noted that the kinetics for AMP with rabbit phosphorylase differ at concentrations of AMP below 0.01 mM and above. Below 0.01 mM AMP, the reciprocal plot has been shown to be non linear (7). At higher concentrations the lines are linear. Similar results have been reported for lobster tail phosphorylase (23) and Cibium phosphorylase (16). However Sepia phosphorylase a has been shown to behave quite differently. The Sepia enzyme gives downwardly

Fig. 2-7.

Reciprocal plots for glucose-1-P at different concentrations of AMP for α-glucan phosphorylase b from E. suratensis. The concentration of glycogen in assay mixture was 1%. The concentrations of AMP in the mixtures were ○, 2×10^{-5} M; ●, 5×10^{-5} M; □, 1×10^{-4} M and ■, 1×10^{-3} M. Other conditions were same as in fig. 2-3.

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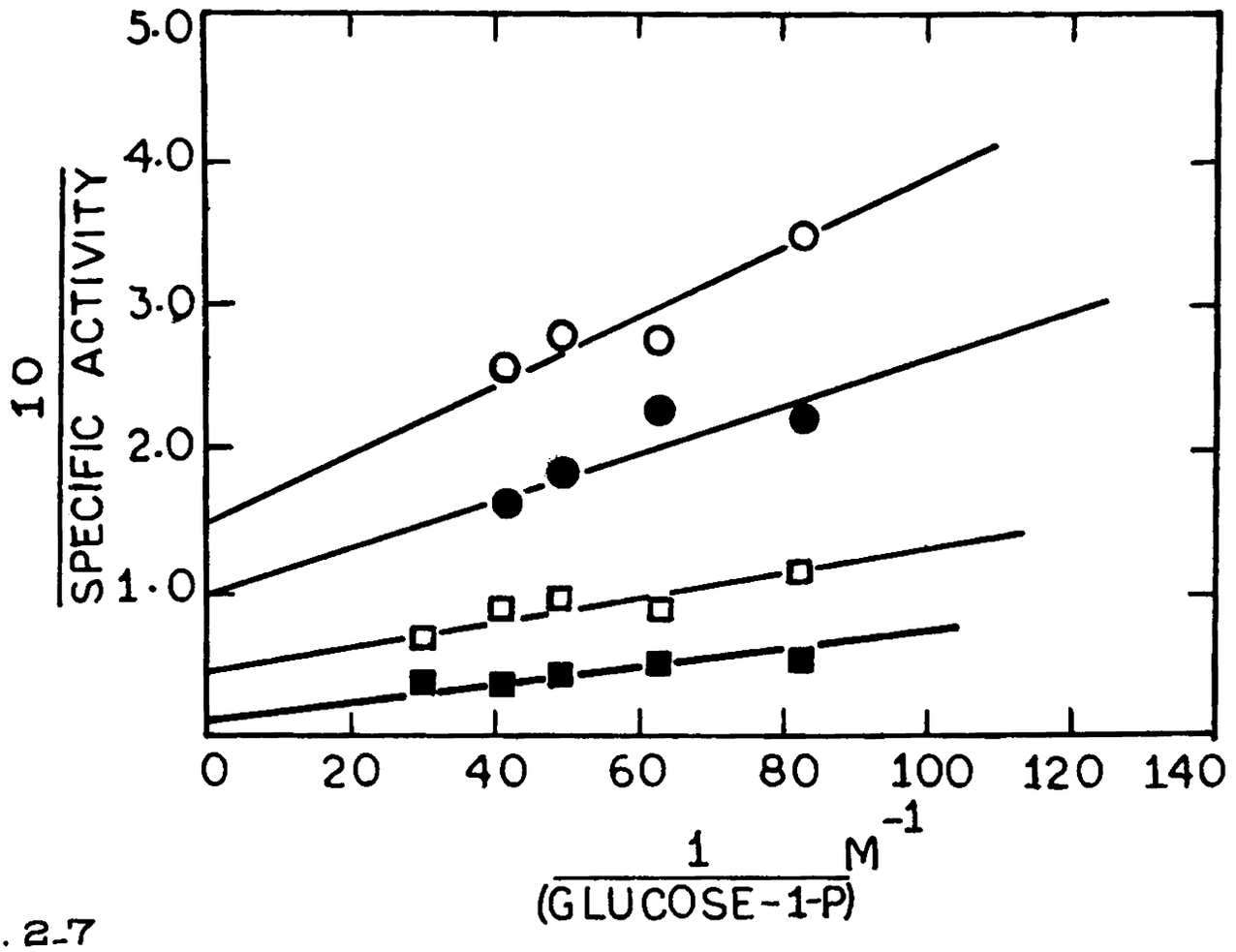
curved reciprocal plots for AMP which has been shown to be due to negative homotropic cooperativity or sites (25). As will be discussed later in the thesis differences of AMP kinetics have been observed for the enzymes from other sources. Therefore in this respect also no generalised pattern of property exists for muscle phosphorylases.

The apparent K_m values for glucose-1-P and AMP calculated from figs. 2-7 and 2-8 are tabulated in table 2-4. Increasing the concentration of the substrate increases the K_m for AMP and vice versa - a finding not reported so far for any animal muscle phosphorylases. In the case of rabbit enzyme and other terrestrial animal enzymes and in aquatic animal muscle enzyme the opposite was observed (ie., positive


5.0
4.0
3.0
2.0
1.0
10 / SPECIFIC ACTIVITY
0
20
40
60
80
100
120
140
1 / (GLUCOSE-1-P)
M^-1

g. 2-7

Fig. 2-8.

Reciprocal plots for AMP at different levels of glucose-1-P for E. suratensis phosphorylase b. Concentration of glucose-1-P in the assay mixtures were o, 12 mM; ■, 20 mM and □, 24 mM. The rest of the conditions were same as in fig. 2-3.

TABLE 2-4

APPARENT K_m VALUES FOR AMP AND GLUCOSE-1-P AT DIFFERENT CONCENTRATIONS OF EACH OTHER FOR E. SURATENSIS PHOSPHORYLASE b

Glucose-1-P (mM)	K_m for AMP (M)	AMP (M)	K_m for Glucose-1-P (mM)
12	1.88×10^{-4}	2×10^{-5}	16.6
20	2.5×10^{-4}	5×10^{-5}	17.5
24	3.3×10^{-4}	1×10^{-4}	22.8
		1×10^{-3}	40.0

heterotropic cooperativity). The negative heterotropic effect observed here for the E. suratensis enzyme along

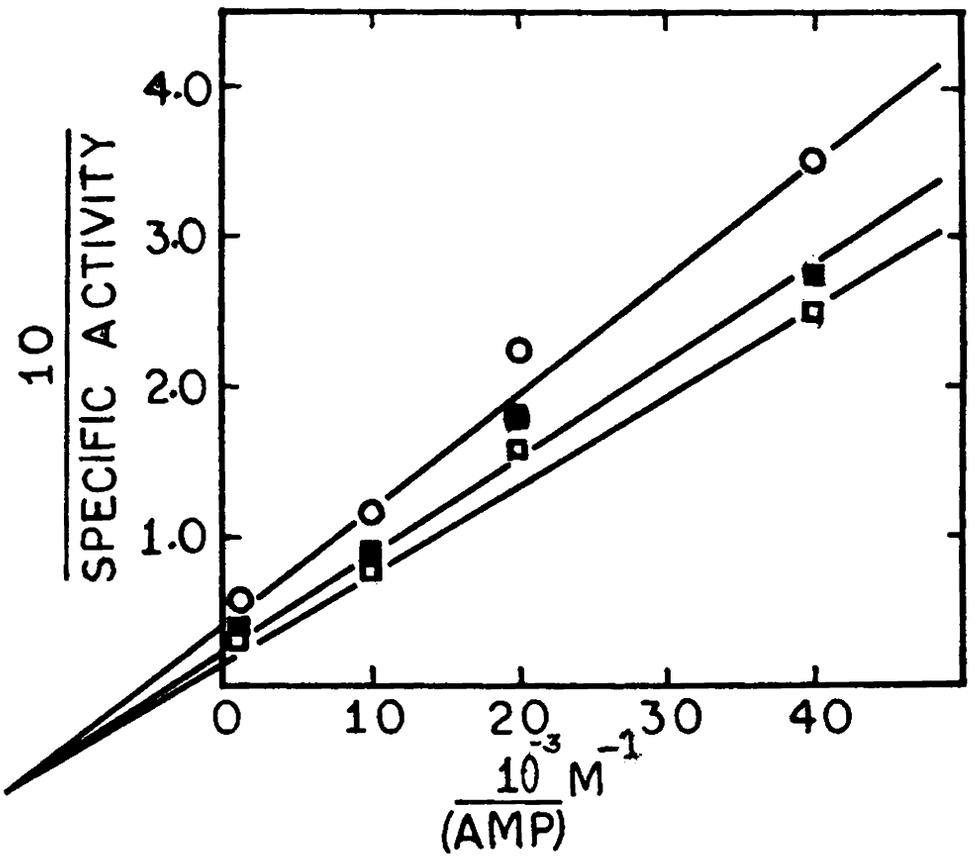


Fig. 2-8

with similar effect observed for the substrate sites, clearly show the structural changes (or changes of the binding sites). Here also, no generalisation can be made. It appears to be the result of minor adapted variation to adjust to different energy needs of the animal.

Inhibition by metabolic ligands

α-Glucan phosphorylase b from different sources has been shown to be inhibited by the metabolites glucose, glucose-6-P and ATP (25,118,119). The nature of inhibition is different in different animal species and varied with the structural difference of phosphorylase (25,118,119). The inhibition of the a form has been found to be negligible in the case of the rabbit enzyme in the presence of 1 mM AMP at 10-15 mM concentration of the inhibitors (7). Similar results were observed for the a form from other animal sources also (25,106). However in the absence of the nucleotide, these ligands inhibit to an extent of 20% or less in the case of the rabbit enzyme (71). Phosphorylase b from a marine fish and from Sepia have been shown to be inhibited competitively by these ligands (25,119). In the absence of AMP, Sepia phosphorylase is inhibited by them, whereas in the

presence of AMP, no inhibition was observed. The Dictyostelium discoideum phosphorylase a is not inhibited by glucose-6-P or glucose (106).

Etroplus suratensis phosphorylase b is inhibited competitively by glucose and glucose-6-P (Fig. 2-9) like the C. guttattum enzyme. The double reciprocal plots for AMP in the absence and presence of inhibitors are shown in fig. 2-10. Glucose exhibited mixed type inhibition. However in the presence of glucose-6-P, the reciprocal plots became curved upward. Thus glucose-6-P induces cooperativity of AMP sites. In these respects, the enzyme is similar to the C. guttattum enzyme. For rabbit muscle phosphorylase b using AMP as the varying substrate and 2 mM glucose-6-P as inhibitor, an upwardly curved line was reported (118). The Hill coefficient for this was 1.6 (77). Using chemically modified and partially desensitised rabbit phosphorylase b, the Hill coefficient has been found to be 1.1 ie., almost nearing competitive inhibition (77). The naturally occuring 'desensitised form' ie., the a form of rabbit enzyme has a Hill coefficient of 1 just like competitive inhibition. It was concluded that the slight cooperativity exhibited by the desensitised b form

Fig. 2-9.

Reciprocal plot for glucose-1-P in the presence of glucose and glucose-6-P for α-glucan phosphorylase b from E. suratensis. The inhibitors were added to the enzyme solution and the mixture was added to an equal volume of substrate and assayed. The assay mixture contained 15 mM cysteine/20 mM sodium β-glycerophosphate, 1% glycogen, 1 mM AMP and varying concentrations of glucose-1-P. The concentration of enzyme was 5.25 μg/ml ○, no inhibitor, ●, 10 mM glucose-6-P and △, 10 mM glucose.

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and the C. guttattum enzyme showed very similar allosteric properties and hence the inhibitors would bind on the above at the substrate or activator sites (ie., the same regions) in different modes rather than at distinctly different allosteric sites (77). Such conclusions were arrived at also by recent X-ray crystallographic studies and NMR studies. Considering the above findings reached by exhaustive investigation suggest similar situation of inhibitor binding of the E. suratensis enzyme also. It may also be noted that the above inhibitors do not induce any cooperativity for rabbit a and also for the a form of the

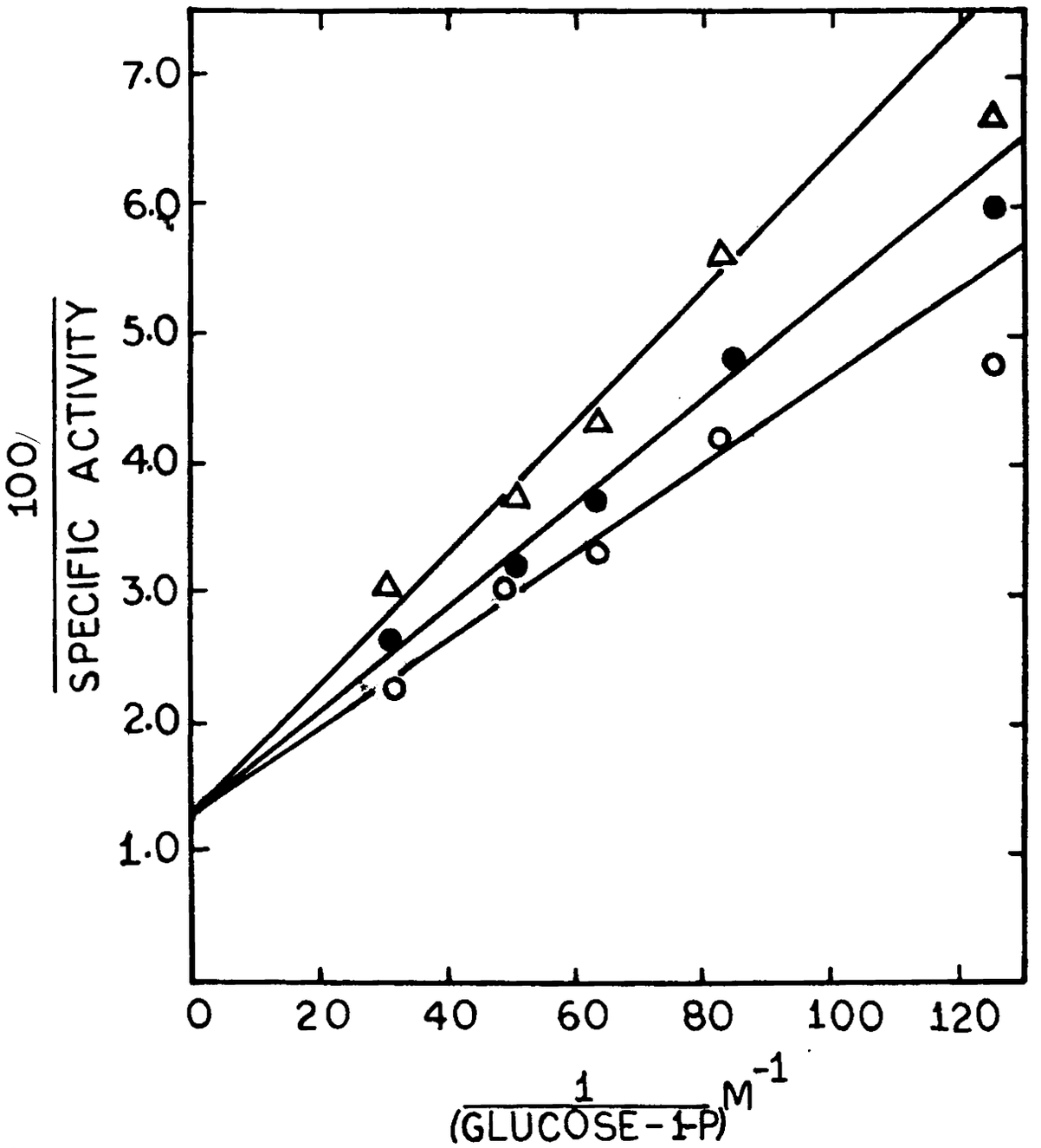
100/
SPECIFIC ACTIVITY
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
20
40
60
80
100
120
1
(GLUCOSE-1-P)
M-1

Fig. 2.9

Fig. 2-10.

Reciprocal plots for AMP in the presence of glucose and glucose-6-P for E. suratensis phosphorylase b. The concentrations of glycogen and glucose-1-P were 1% and 16 mM respectively in the assay mixture. Assay mixture contained 5.75 µg/ml protein. ○, no inhibitor, △, 10 mM glucose and ●, 10 mM glucose-6-P. Other conditions were same as in fig. 2-9.

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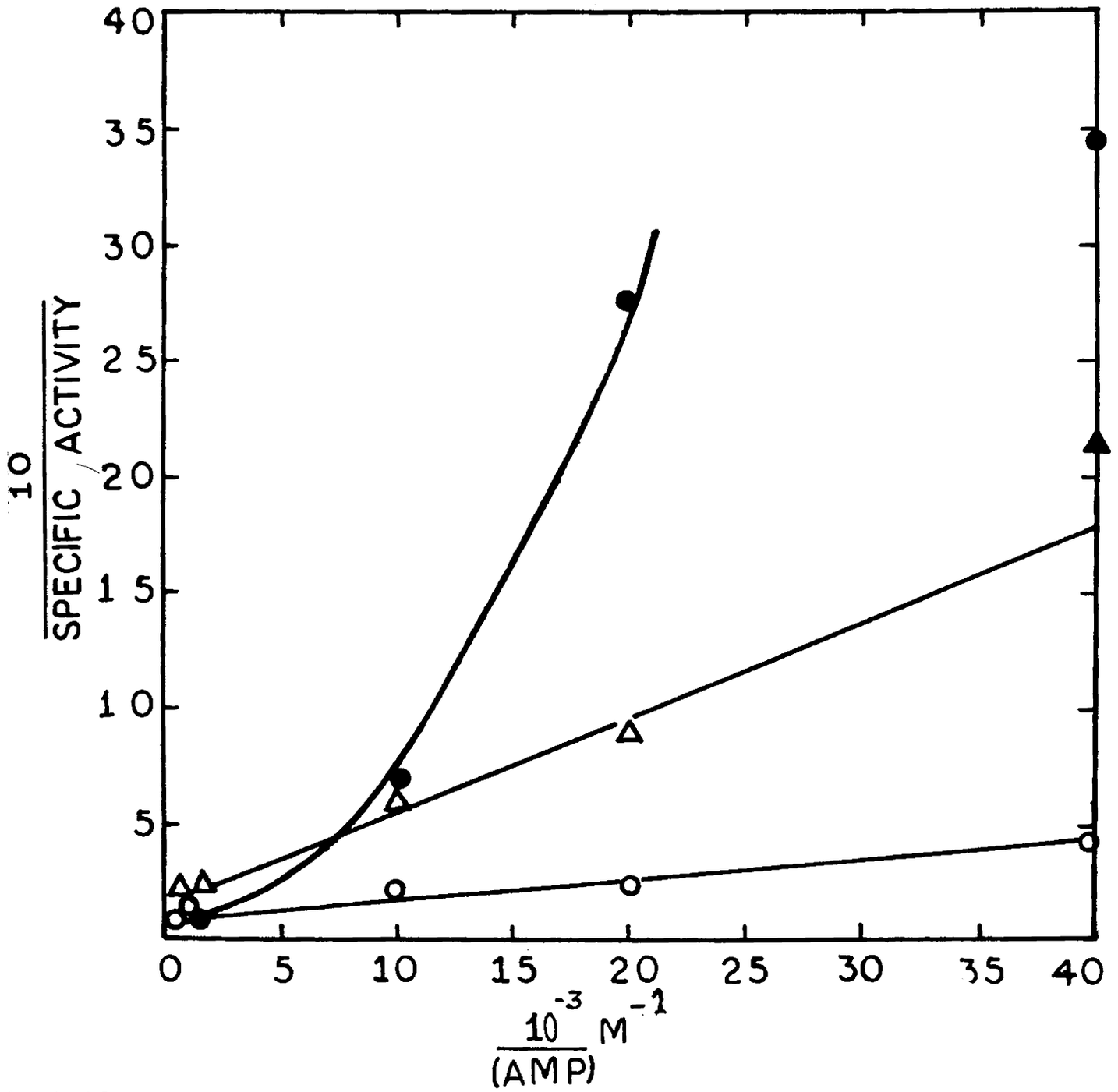
invertebrate Sepia, supporting further the location of the substrate and inhibitor sites to be in the same loci.

E. suratensis phosphorylase a

E. suratensis phosphorylase b after purification by DEAE-cellulose column chromatography was converted to phosphorylase a by partially purified rabbit phosphorylase kinase by inoculation with ATP and $MgCl_2$ at pH 8.0 (120). The enzyme after passing through sephadex G-15 had activity ratio 0.6. The inhibition kinetics were done with this sample. Initial velocity data in the presence of glucose and glucose-6-P are shown in fig. 2-11. It is seen that in the absence of AMP, E. suratensis phosphorylase a is inhibited to a slightly higher degree than the b form by both glucose


40
35
30
25
20
15
10
5
0
5
10
15
20
25
30
35
40
10 / SPECIFIC ACTIVITY
10^-3 / (AMP) M^-1

ig. 2.10

Fig. 2-11.

Reciprocal plots for glucose-1-P for α-glucan phosphorylase a from E. suratensis in the presence of glucose and glucose-6-P and absence of AMP. The activity ratio was 0.6. Phosphorylase a (35 μg/ml) was assayed in the presence of 1% glycogen and glucose-1-P as indicated. ○, no inhibitor; □, 10 mM glucose-6-P and △, 10 mM glucose. Other conditions were same as in figs. 2-9 and 2-10.

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and glucose-6-P. For eg. at 0.0125 M glucose-1-P, the inhibition of the b form by glucose-6-P was 11% whereas for the a form it was 15%. Under similar conditions, glucose inhibition was 26% and 36% respectively. In the presence of 1 mM AMP, however, Sepia phosphorylase a is not inhibited by these metabolites. AMP had overcome the slight inhibition of the a form. Sepia phosphorylase a was not inhibited by these inhibitors in the presence of AMP. Therefore, here in the case of E. suratensis phosphorylase a also AMP afforded protection of inhibition by glucose-6-P and glucose.

Effect of pH on E. suratensis phosphorylase b

The pH activity curve for E. suratensis

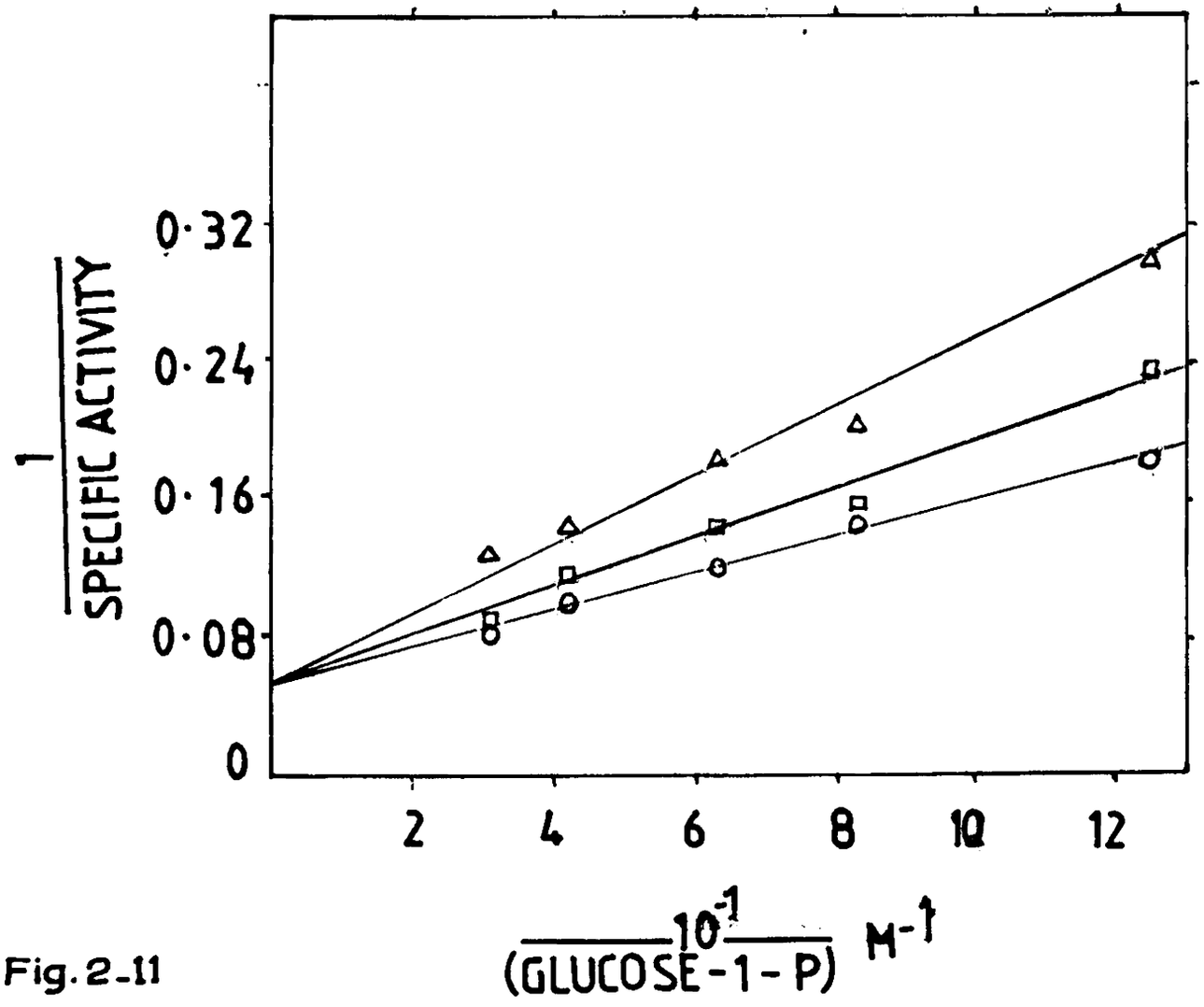
1/SPECIFIC ACTIVITY
0·32
0·24
0·16
0·08
0
2
4
6
8
10
12
10⁻¹/(GLUCOSE-1-P) M⁻¹

Fig. 2-11

phosphorylase b in 15 mM cysteine/20 mM sodium β-glycerophosphate buffer is shown in fig. 2-12. The optimum pH for the enzyme was 6.8. The pH profile is very similar to that of rabbit phosphorylase and Sepia phosphorylase (ie., pK values 6.2 & 7.4). Therefore the same groups might be expected to be involved in catalysis. From pH kinetics a protein functional group with pK_a of 6.2 was suggested to be involved in binding and catalysis in the cases of rabbit enzyme and potato phosphorylase (12,122-124). This group could be the phosphate group of PLP among others. Later the direct involvement of this phosphate group in catalysis has been demonstrated using apophosphorylase and phosphite (47). The identification of the group with pK_a of 7.4 is not yet clear. From the available data it may be stated that one of the active groups in E. suratensis phosphorylase also is the phosphate of PLP.

From the results presented in this chapter the following conclusions may be made with respect to other well studied animal phosphorylases.

1) (i) Sulphate ions activate the phosphorylase activity as in lamprey muscle phosphorylase.

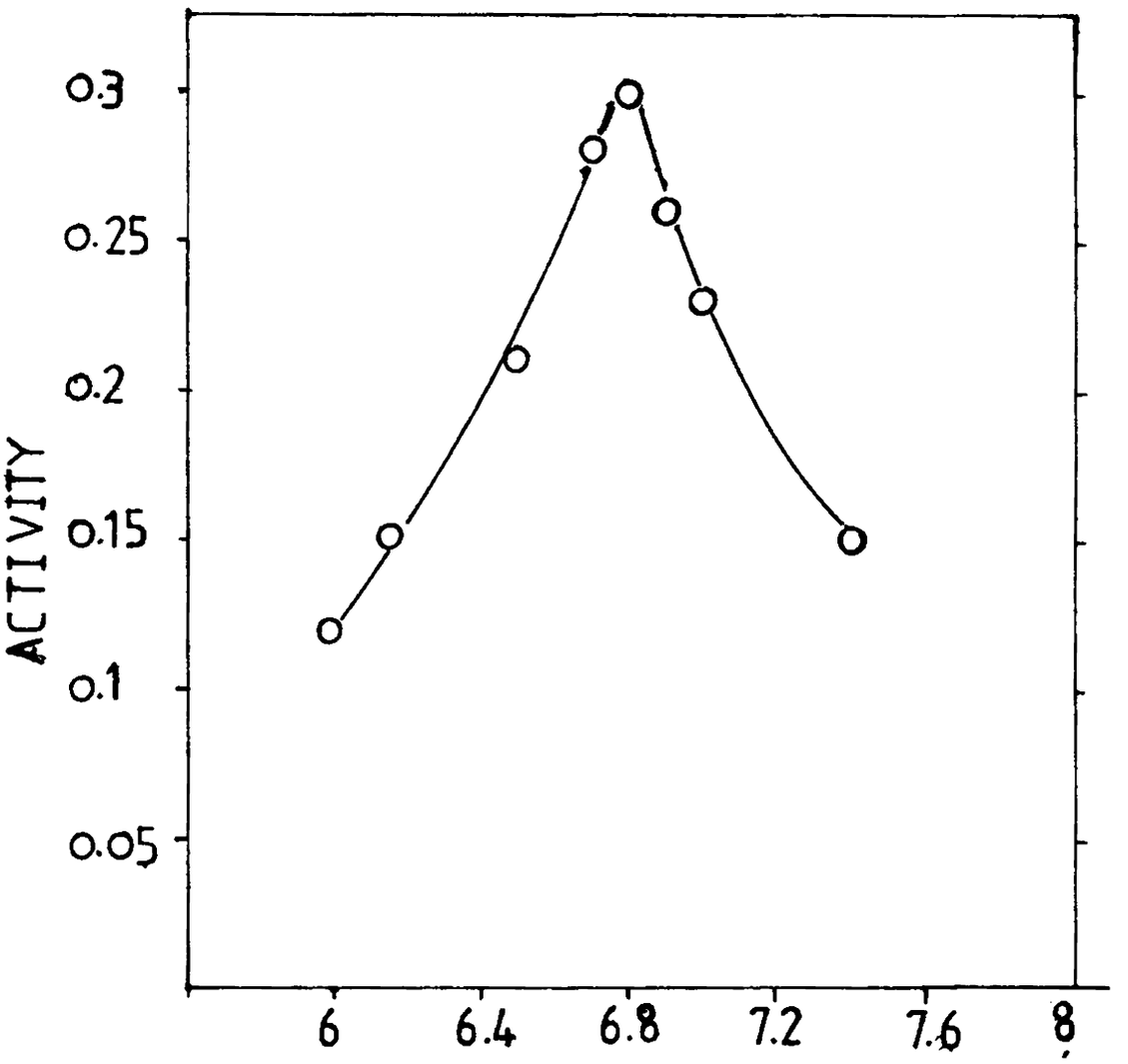

Fig. 2-12

Fig. 2-12.

E. suratensis phosphorylase b activity as a function of pH. The enzyme was diluted in 30 mM cysteine/40 mM sodium β-glycerophosphate of required pH and added to an equal volume of substrate solution containing 32 mM glucose-1-P, 2% glycogen and 2 mM AMP.

(ii) High concentrations of Mg^{+2} and Ca^{+2} are inhibitory.

(iii) 5 mM Hg^{+2} inhibited the enzyme activity completely.

(iv) Cysteine was found to have some protective action against the inhibition by Mg^{+2}, Hg^{+2} and KI.

(v) Na^{+}, K^{+}, Cl^{-} etc. have no effect on the enzyme activity.

2) The kinetic mechanism seems to be same as for other phosphorylases.

3) The heterotropic cooperativity between glucose-1-P and glycogen sites is negative in E. suratensis enzyme, whereas in other cases it is positive.

4) The heterotropic cooperativity between AMP and glucose-1-P is also negative unlike in other animal phosphorylases.

5) Homotropic cooperativity between AMP sites in the presence of inhibitors does not conform to the rabbit and other terrestrial animal muscle phosphorylases, but is similar in some cases to vertebrate fish muscle enzyme and dissimilar in some others.

6) But for the kinetic mechanism, some properties reported here in this chapter are unique. Some properties are qualitatively similar to other vertebrate fish enzyme. Therefore, no generalised pattern can be evolved for all the properties of phosphorylases.

CHAPTER 3

PHOSPHORYLASE FROM METAPENAEUS DOBSONI.

Metapenaeus dobsoni is a small prawn which comes under the class crustacea. Crustaceans come under the phylum Arthropoda and are distinguished from other athropodes by the possession of two pairs of pre-oral antennae and most possess at least 3 pairs of post-oral appendages functioning as jaws. Most of them are aquatic (125). M. dobsoni is found in the estuary during its juvenile or post larval stage and when it becomes mature, it goes back to the sea for breeding. It is highly tolerant to salinity variations. It is a commercially important edible prawn abundant in Cochin backwaters.

Fresh, though not live prawns were collected and peeled to remove the exoskeleton within a couple of hours after capture. After removing the thread-like intestine along with other parts, the muscle tissue was either used immediately or kept forzen at -15 to -20°C in a deep freezer. Glycogen phosphorylase in the stored muscle was stable for one month after which gradual decrease of specific activity was found in the extracts prepared under same conditions. By extracting with 0.01 M sodium β-glycerophosphate, pH 7.0,

the activity per gram tissue was found to be 10 units (μmoles of phoshate liberated per minute at 30°C). The first extract with buffer, 5 times the weight of muscle had a specific activity of 0.5, whereas the second extract with buffer, 2 times the weight of muscle orginally used had only a specific activity of 0.2 and 2% yield of that of the first extract.

The $\frac{-AMP}{+AMP}$ activity of the extract was 0.9, which indicated the presence of the a form of phosphorylase in the M. dobsoni extract. On keeping the extract under conditions which are known to convert the a form completely to the b form (as observed in the case of rabbit muscle extract and fish muscle extracts) no detectable change in the ratio of $\frac{-AMP}{+AMP}$ activity was observed. However a gradual change from 0.9 to 0.74 was noticed during 7 days at pH 7.0 and 0°C in the extract. This shows that phosphorylase a phosphatase exists in a latent form as observed in some cases (78). The gradual change in activity ratio can be due to the activation of phosphorylase a phosphatase presumably by proteolysis during the 7 days' period.

Since the phosphorylase phosphat$^{a}_{\wedge}$se and kinase of most animals have been known to effect interconversion

of rabbit muscle phosphorylase (7,8) the muscle extract of M. dobsoni was used to study its effect on purified rabbit muscle phosphorylase. Incubation of the M. dobsoni extract (1 ml) with crystalline rabbit muscle phosphorylase b (2 ml, 2 mg/ml) and 10 mM ATP for 30' resulted in complete conversion of the b form to the a form as found by the activity ratio becoming 0.8 (under the assay conditions, purified rabbit muscle kinase gives the same ratio). This result showed that active phosphorylase kinase was present in the extract of M. dobsoni. However, the extract didn't convert crystalline rabbit muscle phosphorylase a back to the b form, confirming the earlier conclusion that the phosphatase is present in an inactive form.

Purified rabbit muscle enzymes could be preserved for months. The same was true for other well-studied phosphorylases from terrestrial animals. But the enzyme from the marine fish was less stable and that from the marine invertebrate was very unstable. Partially purified phosphorylase from M. dobsoni was extremely unstable (see below). Therefore the stability of the enzyme was checked at different stages of purification.

The activity of the enzyme in the extract itself

could not be maintained even for a day in the cold. Therefore attempts were made to stabilise the enzyme activity by adding different substances to the extract. This had to be tried because the stability problem became more and more serious as purification of the enzyme progressed. When the extract was kept for 7 days, in glycerophosphate buffer pH 7 in the refrigerator, the activity loss was found to be 40%. Glycerol was tested to preserve the activity. The optimum preservation of activity was found if the extract was kept in buffer containing 5% glycerol. Here also there was loss of activity, but the loss was reduced to 22% on the average. Mercaptoethanol which has been found to be a good preserving agent for rabbit enzyme and phosphorylase from aquatic source was not only ineffective, but increased the rate of loss of activity. In the presence of 10 mM mercaptoethanol the loss of activity was 54%. However, EDTA was found to be effective in preserving the activity. In the presence of 10 mM EDTA, only 5% loss of the activity was observed in the extract in 7 days in the cold. It is not known whether the metal ion in the extract was responsible for the activity loss. It appears unlikely in view of the time-dependence of activity loss. However, (see below) EDTA was included in the

buffer during further purification procedures.

For purifying the enzyme, ammonium sulphate fractionation was tried. The activity obtained at 0-40% ammonium sulphate precipitation had a higher percentage of activity than the 41-60% fraction. The residue precipitated at 0-55% saturation with ammonium sulphate was found to be most ideal and gave 2-fold purification with 75% yield.

For further purification, removal of the salt was necessary. Dialysis of the residue dissolved in minimum amount of 0.005 M sodium β-glycerophosphate buffer pH 7.0 containing 10 mM EDTA, for 5 hours in the cold resulted in complete loss of activity whereas the undialysed part of the same sample had not shown any loss of activity. To check whether the activity was lost due to removal of some dialysable substance, the dialysed protein was assayed in the presence of the buffer, in which it was dialysed. But no activity could be detected in the presence of the dialysate. It was found that phosphorylase b from skate muscle was inactivated in the course of the reaction due to the mechanical destruction of the enzyme molecule due to stresses induced by comformational changes caused by substrate binding (126).

Therefore here also the mechanical stress may be the reason for the inactivation of the enzyme.

Gel filtration on sephadex G-25 (15x0.9 cm) at room temperature was sought as the next step to remove the salt. This method was successful in removing the ammonium sulphate with only about 20% loss of activity and took only less than 20 minutes. After gel-filtration the active fractions were adsorbed on to a DEAE-cellulose column (30x1.2 cm) and the unabsorbed proteins were washed off with 5 mM sodium β-glycerophosphate pH 7.0, containing 10 mM EDTA. An exponential gradient of 1 M NaCl in the same buffer with 100 ml buffer in the lower flask was applied. Activity was found from the 5th fraction onwards when 3 ml fractions were collected. An overall purification of 6-fold with 15% yield was achieved.

Affinity chromatography using ~~sepharose~~-glycogen was the next logical step for further purification probably to homogenity as it was successful with E. coli phosphorylase (127), Sepia phosphorylase (25) and E. suratensis phosphorylase, (Chapter 2). However, apparently because of the unstability of the M. dobsoni phosphorylase, direct application of the DEAE-cellulose fractions from the previous step was

unsuccessful, because no activity could be eluted from the affinity column. Concentration of the protein by ammonium sulphate followed by dialysis failed because no activity could be detected after dialysis. Gel-filtration, through sephadex G-15, of the ammonium sulphate precipitate was also equally ineffective. Not knowing the cause of total inactivation of the enzyme and its probable dependence on salt concentration and time of dialysis, as a last resort, concentration of the enzyme from the DEAE-cellulose column fractions by the use of dry sephadex G-200 was tried. This was done by taking the fractions in a dialysis bag and putting the bag always covered with dry sephadex. Although the process of reducing the volume to 1/10th took only 1-2 hours, almost complete loss of activity was repeatedly observed.

Further studies were carried out with the partially purified enzyme obtained after DEAE-cellulose column chromatography. The partially purified enzyme was highly unstable and hence the properties were studied on the same day of purification. It may be emphasised here that the whole procedure from extraction to DEAE-cellulose chromatography and kinetic studies were done on the same day. This means that for each or

a couple of kinetic analysis, a different preparation was used.

The $\frac{-AMP}{+AMP}$ activity of the purified enzyme was 0.7 invariably. Hence the purified sample is also the a form. In the absence of cysteine in the assay mixture (in 20 mM sodium β-glycerophosphate pH 6.8) 25-100 mM Na_2SO_4 and $(NH_4)_2SO_4$ had no effect. Also NaCl and KCl had no effect. 50 mM and 100 mM KI inhibited the enzyme activity 35%&50% respectively, 50 mM $MgCl_2$ and 100 mM $MgCl_2$ showed 50% and 65% inhibition respectively. 5 mM concentrations of $Zn(Ac)_2$ and $HgCl_2$ completely inhibited the phosphorylase activity. The inhibition by KI is not high for the M. dobsoni enzyme as for the Etroplus phosphorylase.

Cysteine had no activation of phosphorylase activity. In the case of Sepia phosphorylase 2-3 fold increase in activity was found in the presence of cysteine, and about 15% activation was seen for Etroplus phosphorylase under the same conditions.

However, in the presence of cysteine 14% activity loss was found after 1½ hours, whereas under the same conditions and absence of cysteine 62% loss was found for the phosphorylase activity. This means

that the cysteine is having some protective action against the loss of activity. Thus, the inactivation of the enzyme may be due to the oxidation of surface exposed SH groups and cysteine protects against such inactivation.

The optimum pH of the M. dobsoni phosphorylase was found to be 6.8 like the rabbit and Etroplus phosphorylases.

Kinetics

Like that of Etroplus phosphorylase b the kinetics of Metapenaeus dobsoni phosphorylase a was also studied in the direction of glycogen synthesis. Since the enzyme was the a form, glycogen and glucose-1-P kinetics were done in the absence and presence of AMP. Initial velocity data obtained by varying the concentration of glucose-1-P and glycogen in the absence of AMP are given in figs. 3-1 and 3-2. Like all other animal phosphorylases, the primary plots were linear, converging at a point on the left hand side of the vertical axis. Hence the kinetic mechanism of M. dobsoni phosphorylase is the same as for other animal phosphorylases ie., rapid equilibrium discussed in Chapter 2. In this respect phosphorylase

Fig. 3-1

Double reciprocal plots for glucose-1-P at different levels of glycogen for α-glucan phosphorylase a from M. dobsoni. Phosphorylase a was assayed in the direction of glycogen synthesis in 15 mM cysteine/20 mM sodium β-glycerophosphate buffer, pH 6.8. The assay mixture contained 90 μg protein in 0.4 ml of the assay mixture. The $\frac{-AMP}{+AMP}$ activity of the sample was 0.7. Glycogen concentrations were (expressed in terms of glucosyl residues) ○, 61.5 mM; ●, 24.6 mM; □, 6.15 mM; ■, 4.92 mM and △, 2.46 mM.

Fig. 3-2

Lineweaver - Burk plots for glycogen at various levels of glucose-1-P for phosphorylase a from M. dobsoni. The conditions were same as in fig. 3-1. The concentrations of glucose-1-P in the assay mixtures were ●, 32 mM; ■, 24 mM; ○, 16 mM; △, 12 mM and □, 8 mM.

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from M. dobsoni also falls into the only one general pattern observed for all the phosphorylase studied so far.

The values of K_5, K_6 and K_7 (Chapter 2) were calculated from the secondary plots figs. 3-3 A & B.

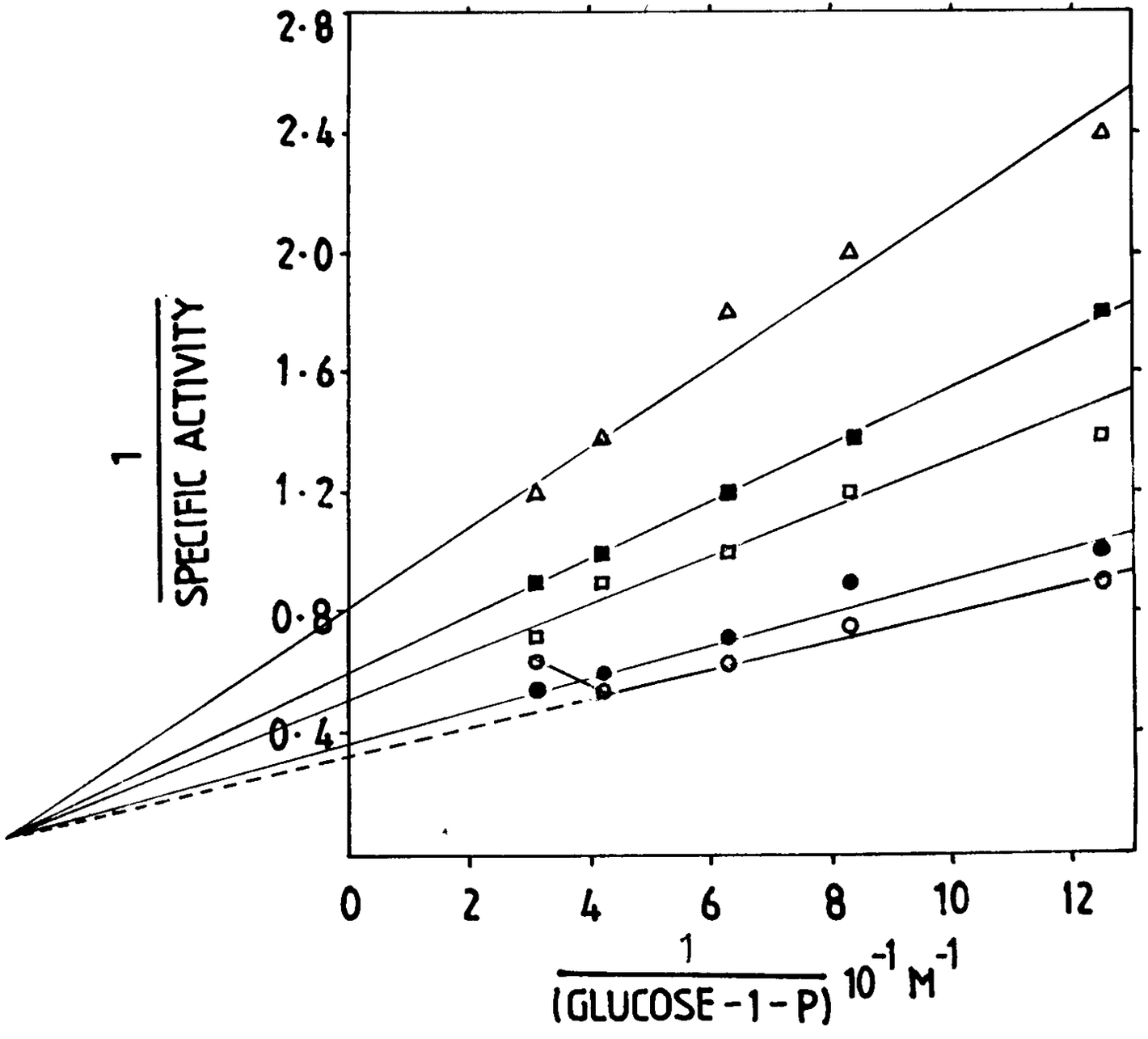
2·8
2·4
2·0
1·6
1·2
0·8
0·4
1 / SPECIFIC ACTIVITY
0
2
4
6
8
10
12
1 / (GLUCOSE-1-P) 10⁻¹ M⁻¹

FIG. 3.1

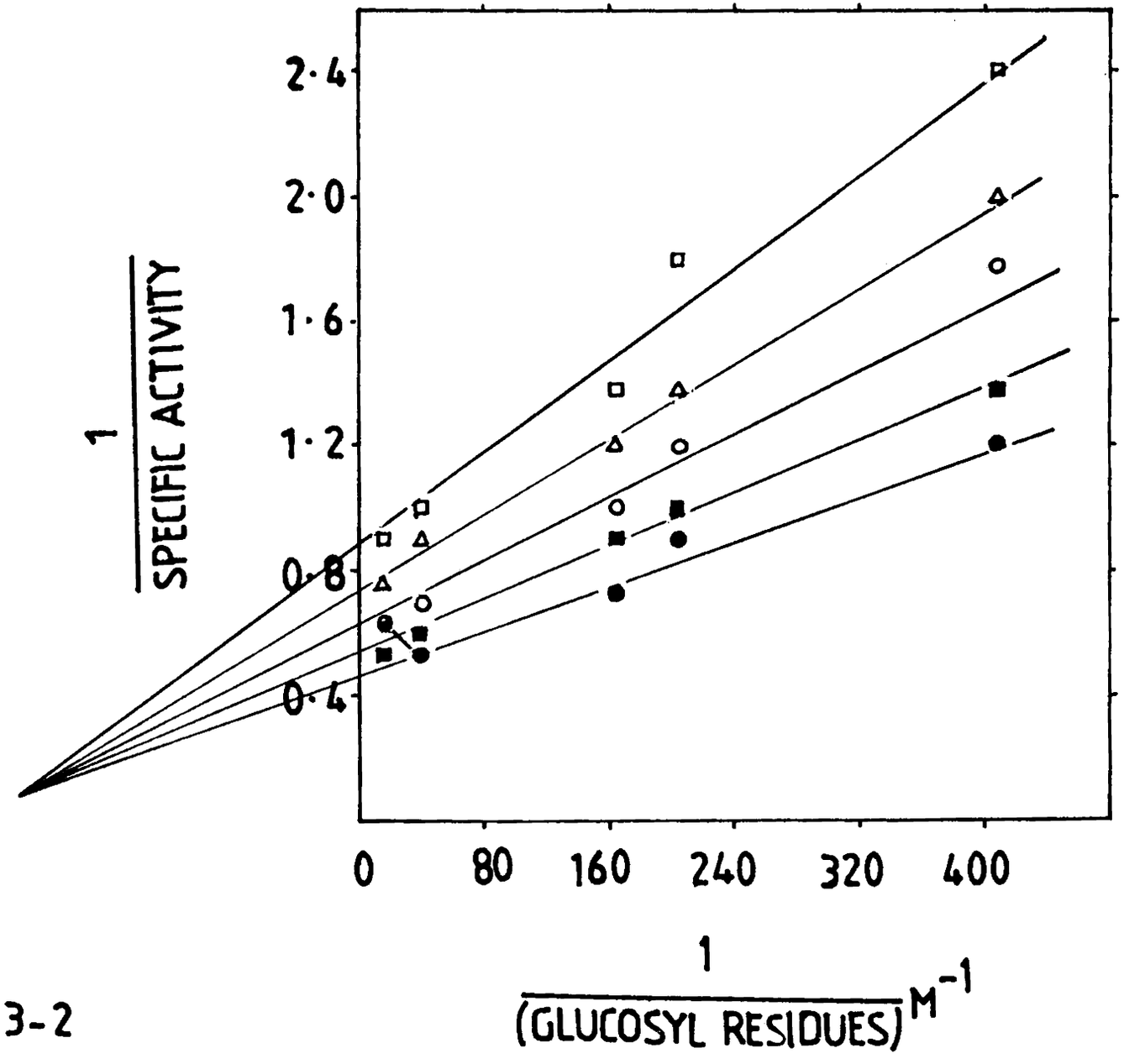
2·4
2·0
1·6
1·2
0·8
0·4
1 / SPECIFIC ACTIVITY
0
80
160
240
320
400
1 / (GLUCOSYL RESIDUES) M⁻¹

FIG. 3-2

Fig. 3-3

A. Secondary plots of intercept and slope versus glycogen concentration from fig. 3-1.

B. Secondary plots of intercept and slope versus glucose-1-P concentration from fig. 3-2.

The values of these constants and Vmax are almost the same when calculated using different equations from the four different secondary plots as given in table 3-1.

TABLE 3-1

VALUES OF KINETIC CONSTANTS OBTAINED USING DIFFERENT EQUATIONS FROM THE SECONDARY PLOTS OF PHOSPHORYLASE a FROM M. DOBSONI.

	Values obtained from fig.3-3.			
	A(1)	A(2)	B(1)	B(2)
Vmax (μmoles/min/mg)	3.131		2.94	
K_5 (mM glucose-1-P)		6.72	6.47	
K_6 (mM glycogen)	4.03			3.38
K_7 (mM glycogen)		10.7		9.55

The consistency of these values further support that the mechanism is rapid equilibrium random bi bi.

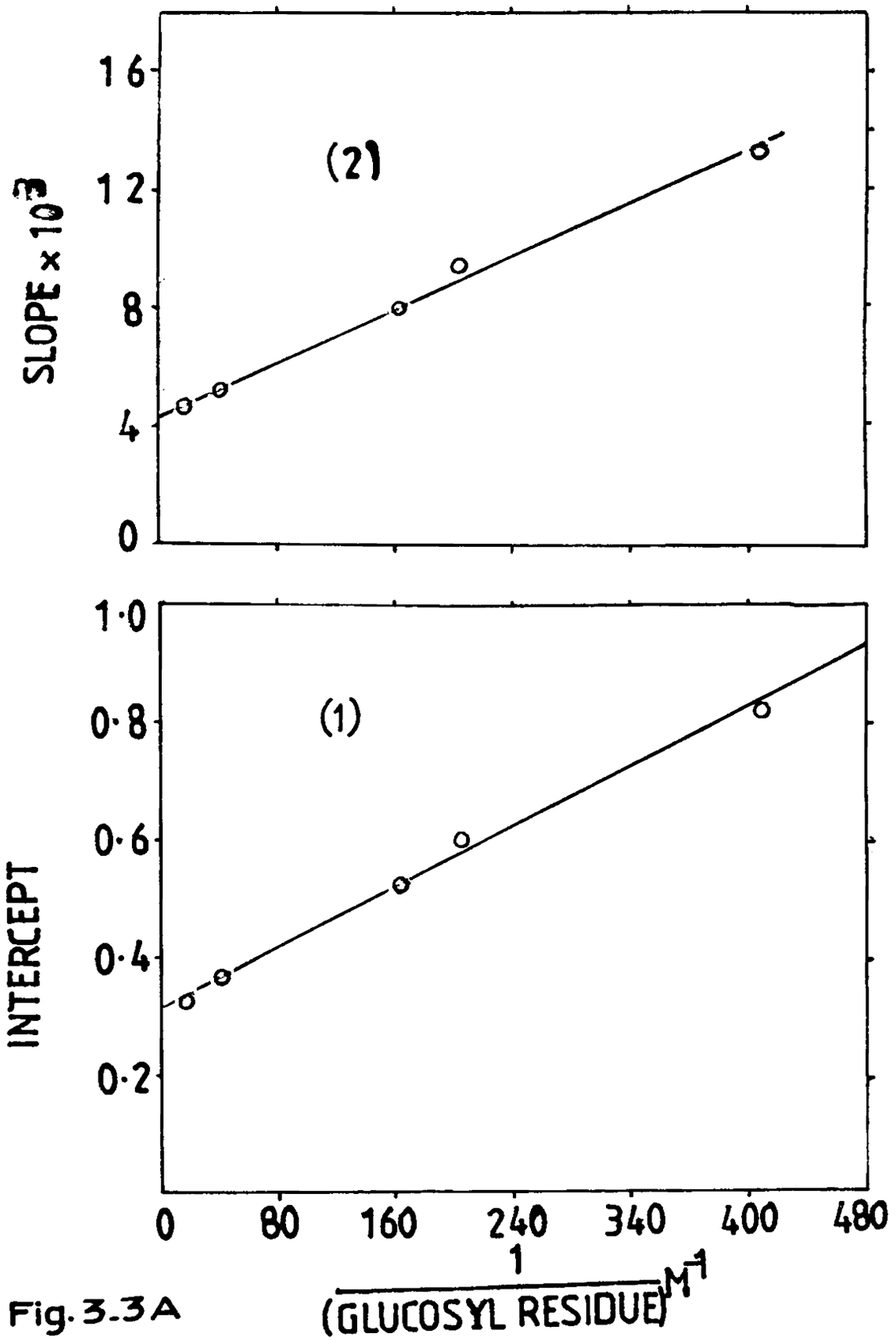
16
12
8
4
0
SLOPE x 10³
(2)
1·0
0·8
0·6
0·4
0·2
INTERCEPT
(1)
0
80
160
240
340
400
480
1
(GLUCOSYL RESIDUE)M⁻¹

Fig. 3.3A

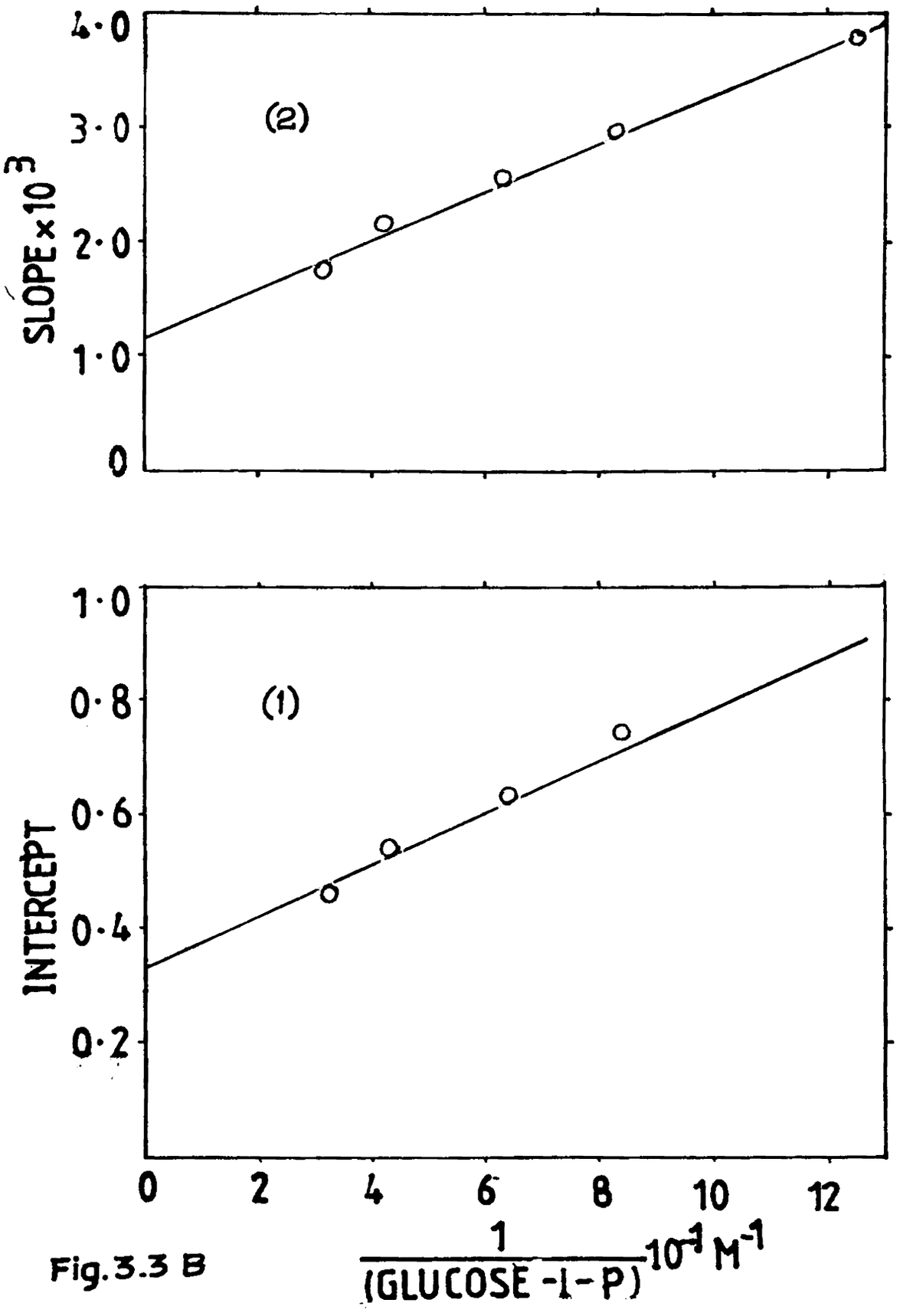
4·0
3·0
2·0
1·0
0
SLOPE×10³
(2)
1·0
0·8
0·6
0·4
0·2
INTERCEPT
(1)
0
2
4
6
8
10
12
1
(GLUCOSE -1- P)
10⁻¹ M⁻¹

Fig.3.3 B

The apparent K_m values for glucose-1-P at various levels of glycogen and for glycogen at various levels of glucose-1-P obtained from figs. 3-1 and 3-2 are given in table 3-2.

TABLE 3-2

APPARENT K_m VALUES FOR GLUCOSE-1-P AND GLYCOGEN AT DIFFERENT CONCENTRATIONS OF EACH OTHER FOR M. DOBSONI PHOSPHORYLASE a. (Concentration of glycogen is expressed as mM glucosyl residues)

Glycogen (mM)	K_m for glucose-1-P (mM)	Glucose-1-P (mM)	K_m for glycogen (mM)
61.5	13.74	32	3.78
24.6	14.22	24	3.97
6.15	15.15	16	4.11
4.92	15.74	12	4.19
2.46	16.25	8	4.24

The K_m values for glucose-1-P decreases as the concentration of glycogen increases showing positive heterotropic cooperativity like in the case of rabbit enzyme (7) and other muscle phosphorylases from animals

like lobster (23) Cibium and Sepia (16,25) but unlike E. suratensis phosphorylase (Chapter 2). In the latter case the cooperativity was negative. Similarly the K_m values for glycogen decreases as the concentration of glucose-1-P increases (table 3-2). Here also, the E. suratensis phosphorylase behaved differently.

Thus the M. dobsoni phosphorylase a exhibits positive heterotropic interaction between the substrate sites. The effects have been shown to be marginal for other phosphorylase a also (25). The kinetic mechanism of M. dobsoni being random equilibrium as in other cases, the decrease of K_m can be interpreted as increase in affinity and vice versa.

Kinetics in the presence of AMP

To check the effect of AMP on the kinetic mechanism of phosphorylase a from M. dobsoni, the initial velocities were measured with varying concentrations of glycogen and glucose-1-P in the presence of 1 mM AMP. Figs. 3-4 and 3-5 show the data obtained. The plots were also linear and converged to a point on the left side of the vertical axis similar to that obtained in the absence of AMP. In the absence of AMP, the plots at 1% glycogen (fig. 3-1) and 32 mM glucose-1-P (fig. 3-2)

Fig. 3-4

Reciprocal plots for glucose-1-P at different levels of glycogen for phosphorylase a from M. dobsoni in the presence of 1 mM AMP. Assay was conducted in the presence of 15 mM cysteine/20 mM sodium β-glycerophosphate pH 6.8, 1 mM AMP and glucose-1-P as indicated. The concentrations of glycogen were ●, 61.5 mM; ○, 24.6 mM; ■, 6.15 mM; □, 4.92 mM and △, 2.46 mM glucosyl residues. Assay mixture contained 90 μg protein per 0.4 ml.

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showed substrate inhibition ie., at higher concentration the enzyme was inhibited by the substrate. In the presence of AMP, this inhibition was not observed. Substrate inhibition (at higher concentration) can be due to misoriented modes (unproductive binding) of binding also. The fact that observed substrate inhibition in the absence of AMP had been overcome by the presence of AMP indicated some kind of reorientation of binding sites (probably at the interface of monomer-monomer interaction). Substrate inhibition has been shown for Pi in the direction of glycogen degradation for phosphorylase b from cod muscle (128).

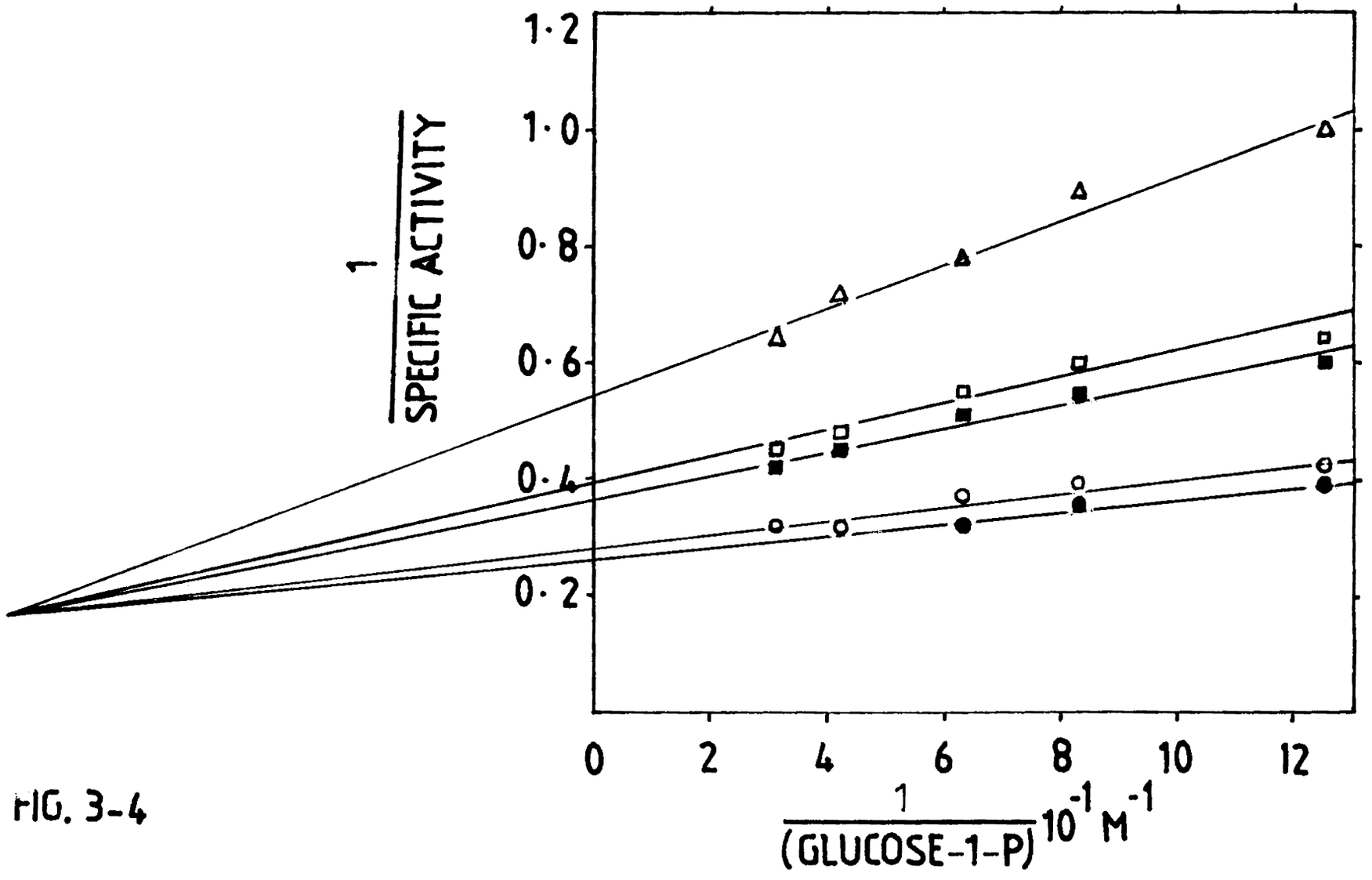
1·2
1·0
0·8
0·6
0·4
0·2
1
SPECIFIC ACTIVITY
0
2
4
6
8
10
12
1
(GLUCOSE-1-P)
10^-1 M^-1

FIG. 3-4

Fig. 3-5

Reciprocal plots for glycogen at different levels of glucose-1-P for M. dobsoni phosphorylase a in the presence of AMP. The concentrations of glucose-1-P were ●, 32 mM; ○, 24 mM; ■, 16 mM, □, 12 mM and △, 8 mM. Other conditions were same as in fig. 3-4.

The kinetic constants were calculated from figs. 3-4 and 3-5 and from the secondary plots and are shown in table 3-3.

TABLE 3-3

VALUES OF KINETIC CONSTANTS OBTAINED USING DIFFERENT EQUATIONS FROM THE SECONDARY PLOTS OF PHOSPHORYLASE a FROM M. DOBSONI IN THE PRESENCE OF AMP.

	Values obtained from the secondary plots (not shown in the thesis)			
	1	2	3	4
Vmax (μmoles/min/mg)	4.0		4.35	
K_5 (mM glucose-1-P)		2.0	2.48	
K_6 (mM glycogen)	2.83			3.74
K_7 (mM glycogen)		13.7		8.56

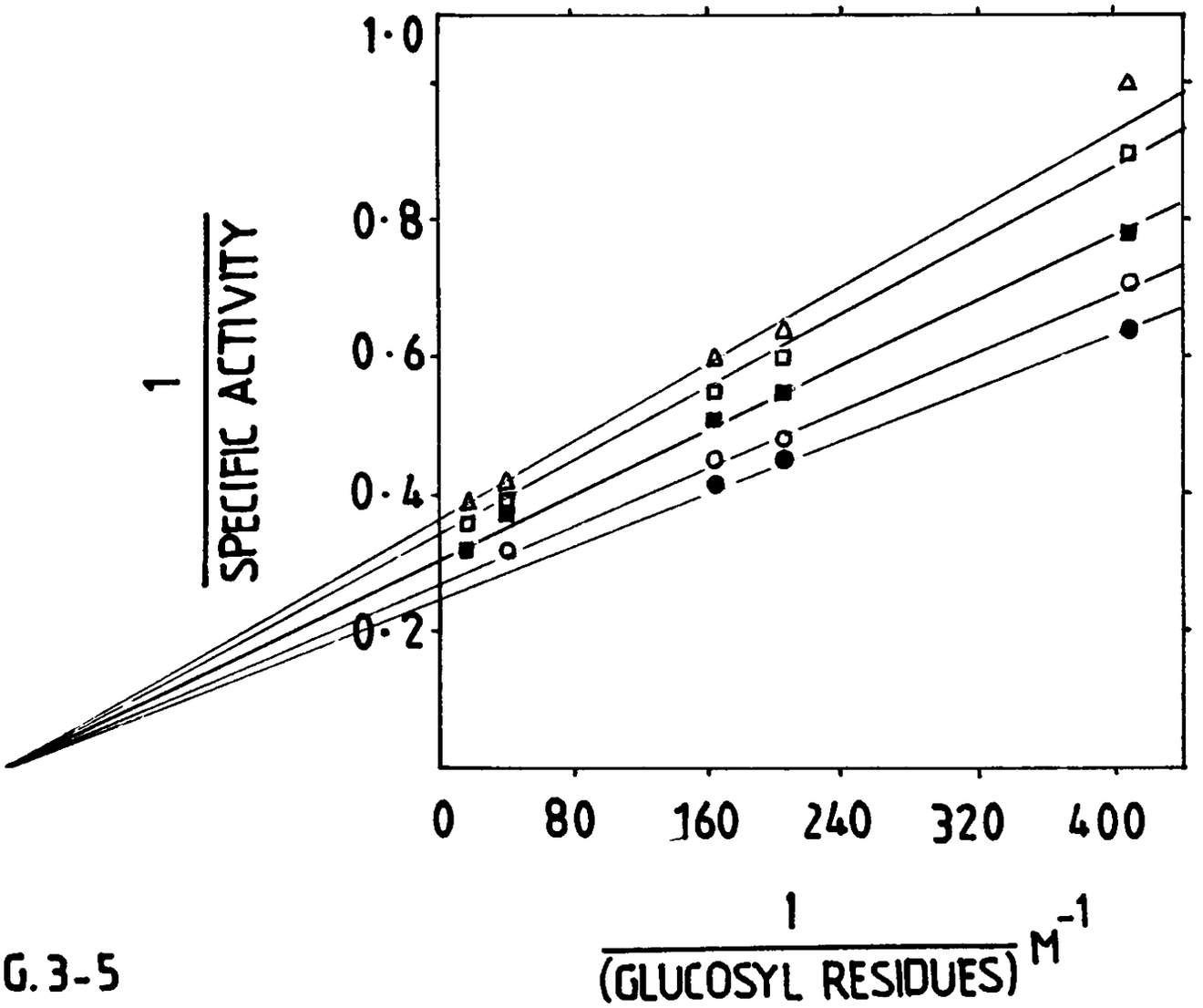
1·0
0·8
0·6
0·4
0·2
1
SPECIFIC ACTIVITY
0
80
160
240
320
400
1
(GLUCOSYL RESIDUES)
M-1

FIG. 3-5

The consistency suggested the kinetic mechanism to be same as rapid equilibrium in the presence or absence of the activator.

The apparent K_m values in the presence of 1 mM AMP for glucose-1-P and glycogen as a function of each other obtained from figs. 3-4 and 3-5 are given in table 3-4.

TABLE 3-4

APPARENT K_m VALUES FOR GLUCOSE-1-P AND GLYCOGEN AT DIFFERENT CONCENTRATIONS OF EACH OTHER FOR M. DOBSONI PHOSPHORYLASE a IN THE PRESENCE OF AMP.

(Concentration of glycogen is expressed as mM glucosyl residues)

Glycogen (mM)	K_m for glucose-1-P (mM)	Glucose-1-P (mM)	K_m for glycogen (mM)
61.5	3.77	32	3.75
24.6	4.16	24	3.85
6.15	5.59	16	3.95
4.92	5.97	12	4.01
2.46	7.06	8	4.06

The results are apparently same as that in the absence of AMP (table 3-2). In the presence or absence of AMP, the heterotropic cooperativity between glucose-1-P and glycogen sites remained positive. However, in both the cases the influence of one substrate on the affinity of the other was less in magnitude compared to other animal muscle phosphorylases (table 3-2 and 3-4, 16,25).

The kinctic constants for M. dobsoni phosphorylase a were calculated from the secondary plots and the primary plots (figs. 3-1, 3-2, 3-4 and 3-5). The values are given in table 3-5.

TABLE 3-5

COMPARISON OF THE KINETIC CONSTANTS FOR M. DOBSONI PHOSPHORYLASE a IN THE ABSENCE AND PRESENCE OF AMP

Kinetic constants	In the presence of 1 mM AMP	In the absence of AMP
k_2 (μmoles/min/mg)	4.15	3.03
K_5 (mM glucose-1-P)	2.24	6.56
K_6 (mM glycogen)	3.29	3.7
K_7 (mM glycogen)	8.56	10.1
K_8 (mM glucose-1-P)	10.1	17.54

The kinetic constants are having a comparatively lesser value.. in the presence of AMP, than in its absence except k_2, which is having a higher value in the presence of AMP. The value of K_5 is only one third in the presence of the activator than in the absence of it. The low values of K_5, K_6, K_7 and K_8 indicate that the affinity of the enzyme for its substrate is increased in the presence of AMP. The affinity of the enzyme for glucose-1-P is 3 times in the presence of AMP, than in its absence. Thus AMP increases the efficiency of the enzyme by increasing its affinity for the substrate and also the velocity of the reaction (k_2 is greater in the presence of AMP).

Kinetics with respect to AMP.

Initial velocity data with varying concentrations of glucose-1-P and AMP are given in figs. 3-6 and 3-7. Though the reciprocal plots for glucose-1-P are linear, the plots for AMP are curved. The Hill plots of the data of fig. 3-7 gave Hill coefficients (in values) 0.625 to 0.66 showing negative homotropic cooperativity between AMP sites. Of all the muscle phosphorylases studied, only the Sepia enzyme (25) and phosphorylase b' (129) from rabbit have been shown to exhibit negative homotropic cooperativity of AMP

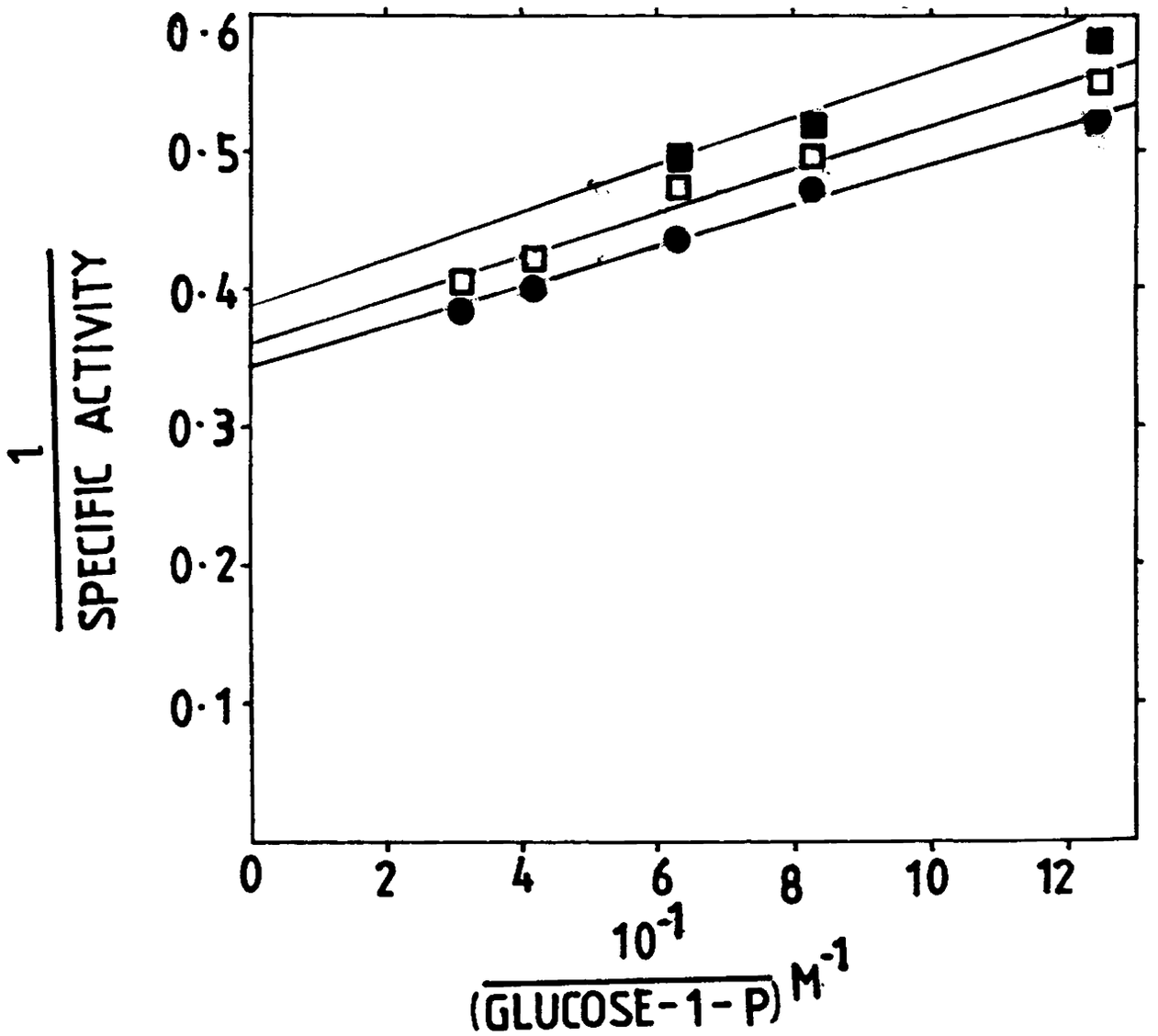
0·6
0·5
0·4
0·3
0·2
0·1
1/SPECIFIC ACTIVITY
0
2
4
6
8
10
12
10⁻¹/(GLUCOSE-1-P) M⁻¹

Fig.3-6

Fig. 3-6

Reciprocal plots for glucose-1-P at different levels of AMP for α-glucan phosphorylase a from M. dobsoni. The assay mixtures contained 15 mM cysteine/20 mM sodium β-glycerophosphate pH 6.8, 1% glycogen and 140 μg protein per 0.4 ml. The concentrations of AMP were ●, 1×10^{-4} M; □, 5×10^{-5} M; and ▩, 2.5×10^{-5} M AMP.

Fig. 3-7

Reciprocal plots for AMP at different levels of glucose-1-P for M. dobsoni phosphorylase a. The concentrations of glucose-1-P were ○, 32 mM; ●, 24 mM; △, 16 mM; □, 12 mM and ▩, 8 mM. Other conditions were same as in fig. 3-6.

Inset: Hill plots of the above fig. Concentrations of glucose-1-P were ●, 8 mM, ○, 12 mM and □, 16 mM.

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sites. Rabbit muscle b' is an artificially made enzyme form by limited tryptic digestion of phosphorylase a (129). But the purified Sepia phosphorylase a ($\frac{-AMP}{+AMP}$ ratio = 0.4) has been found to

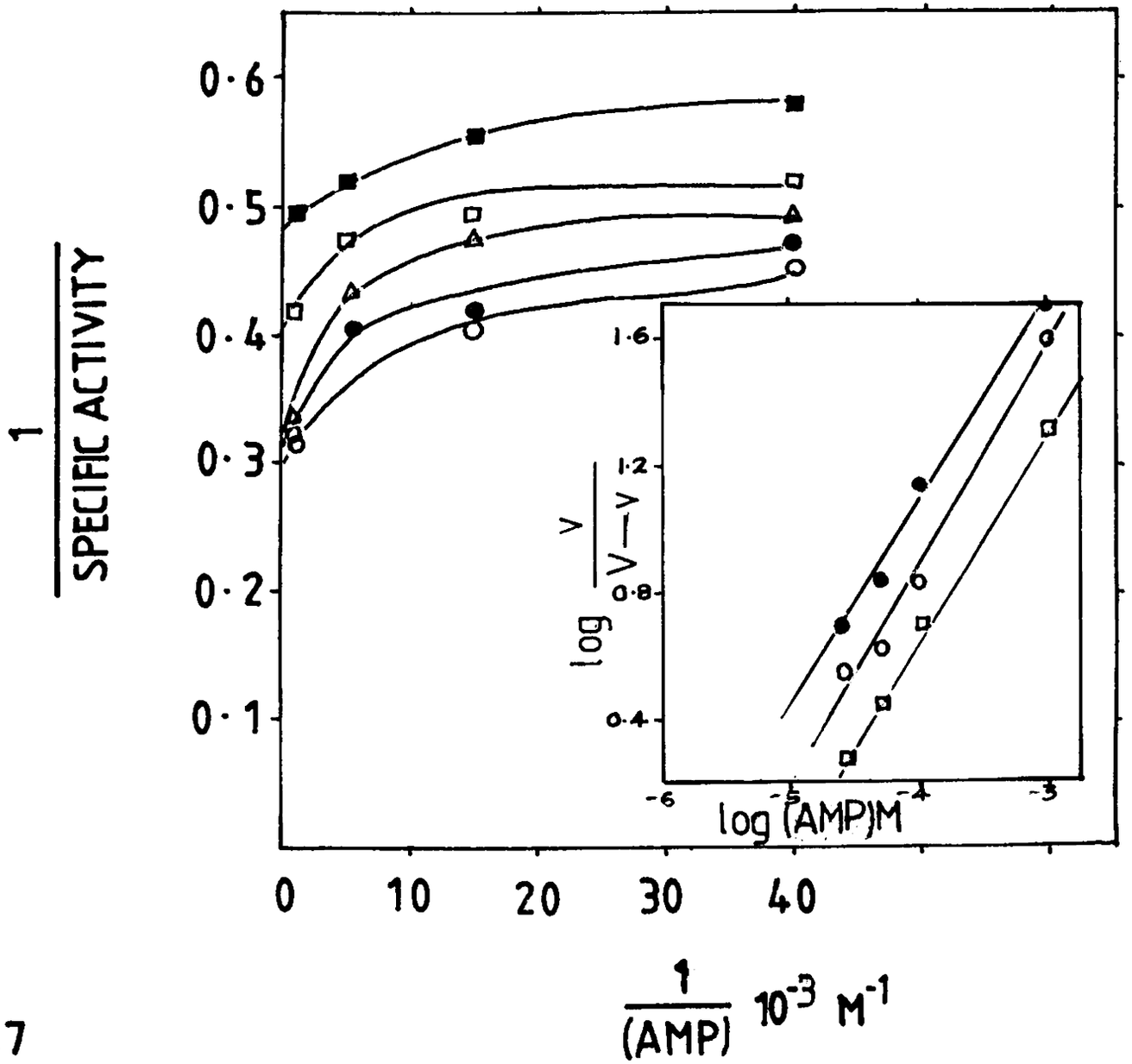
1 / SPECIFIC ACTIVITY
0·6
0·5
0·4
0·3
0·2
0·1
0
10
20
30
40
1 / (AMP) 10⁻³ M⁻¹
log V / V—V
1·6
1·2
0·8
0·4
-6
-5
-4
-3
log (AMP)M

FIG. 3-7

show negative cooperativity (25). In the case of the Sepia enzyme, the control mechanism of phosphorylase activity has been unambiguously shown to be distinctly different from other reported cases (25). Sepia is an invertebrate marine mollusc whose mantle has an extremely high energy requirement. The demand for energy had to be met by such a special control mechanism (25). It is interesting to note that M. dobsoni, also an invertebrate which thrives in marine water, exhibited similar activation (negative cooperativity) by AMP. However, being a bottom feeder, with moderate energy requirement for the animal muscle the M. dobsoni has similar AMP kinetics as the Sepia, but without any specialised way of adaptation. For example the Sepia mantle has been shown to have more than saturating concentration of AMP, but the dobsoni muscle has negligible AMP concentration.

Inhibition kinetics

Substrate inhibition was found for M. dobsoni phosphorylase a by both glycogen and glucose-1-P at higher concentrations in the absence of AMP. Glycogen at 1% showed inhibition at 32 mM glucose-1-P concentration. Hence inhibition studies were

conducted at 0.4% glycogen concentration, where no substrate inhibition was found.

Glucose-6-P and glucose exhibit a mixed type of inhibition for M. dobsoni phosphorylase, (fig. 3-8) in the absence of AMP. In the presence of 1 mM AMP the effect was different (fig. 3-9). Presence of 10 mM glucose-6-P gave rise to downwardly curved line showing negative cooperativity between glucose-1-P sites in the presence of this inhibitor. Reciprocal plots for glucose-1-P in the presence of ATP and glucose were linear and converged to a point on the left side of the vertical axis, showing mixed type of inhibition. The effect of glucose-6-P calls for special comments in two respects.

(i) it gives downwardly curved plots for glucose-1-P kinetics in the presence of AMP (fig. 3-9) and linear plots in the absence of AMP (fig. 3-8) and

(ii) for AMP kinetics (fig. 3-10) (the range of AMP concentration used is much less than 1 mM) also glucose-6-P behaves similarly as it induces negative cooperativity between AMP sites. These effects by glucose-6-P have not been reported for phosphorylase from any source.

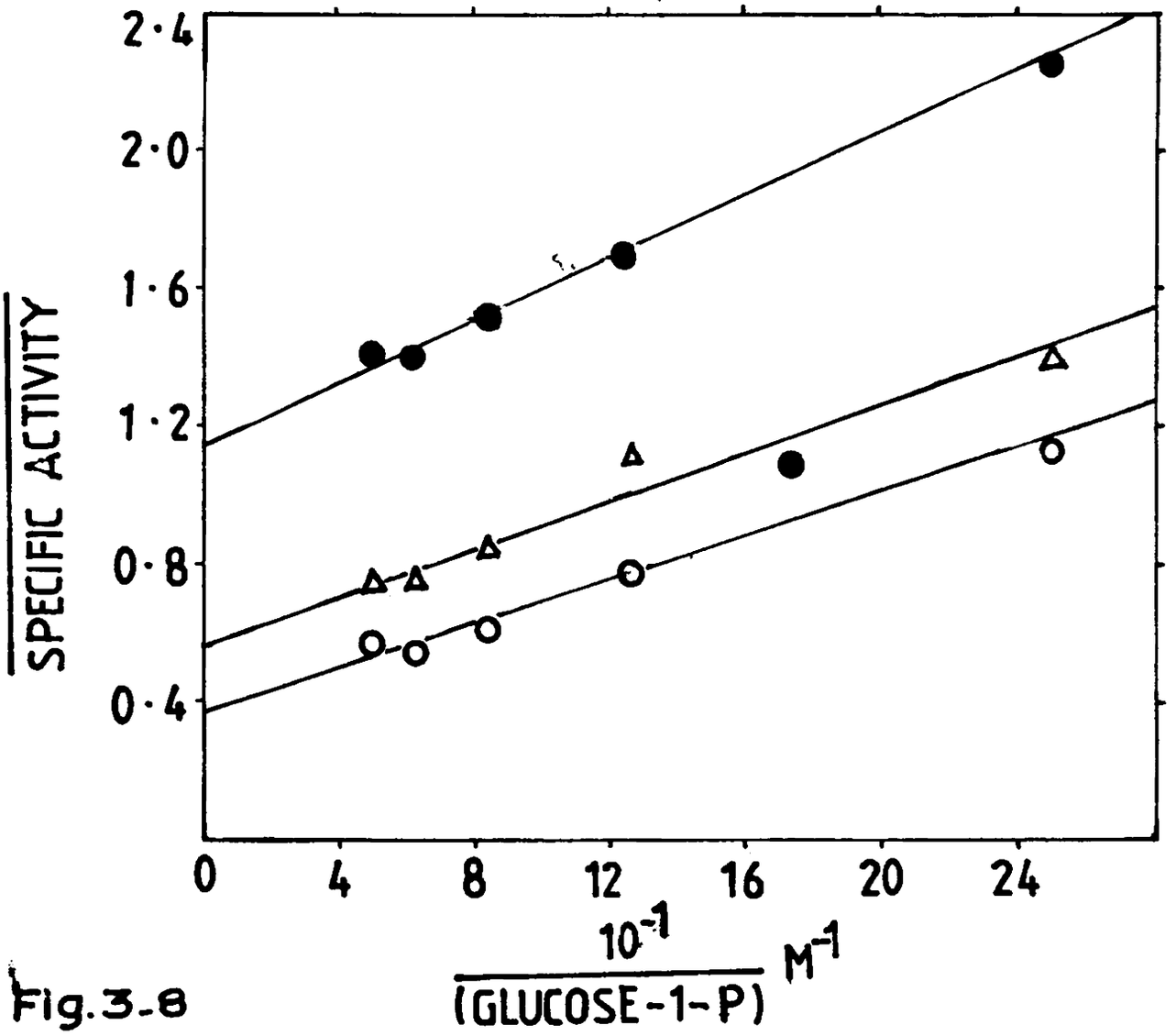
2·4
2·0
1·6
1·2
0·8
0·4
1/SPECIFIC ACTIVITY
0
4
8
12
16
20
24
10⁻¹/(GLUCOSE-1-P) M⁻¹

Fig.3-8

Fig. 3-8

Reciprocal plot for glucose-1-P for phosphorylase a from M. dobsoni in the absence and presence of glucose, and glucose-6-P. The assay mixture (0.4 ml) contained 0.4% glycogen, 140 µg protein, 15 mM cysteine/20 mM sodium β-glycerophosphate and glucose-1-P as indicated ○, no inhibitor; △, 10 mM glucose and ●, 10 mM glucose-6-P.

Fig. 3-9

Reciprocal plots for glucose-1-P for M. dobsoni phosphorylase a in the presence of 1 mM AMP in the presence of glucose, glucose-6-P and ATP. The assay mixture contained 1 mM AMP and 112 µg protein (in 0.4 ml) and glucose-1-P as indicated. ○, no inhibitor △, 10 mM glucose; ■, 10 mM ATP and ●, 10 mM glucose-6-P. Other conditions were same as in fig. 3-8.

This is a special case and invoking models of allosterism and their modifications will not be of any use because they simply can not explain the property. Phosphorylase from M. dobsoni being an extremely labile enzyme, the ligands may have specific influences in stabilising (or destabilising)

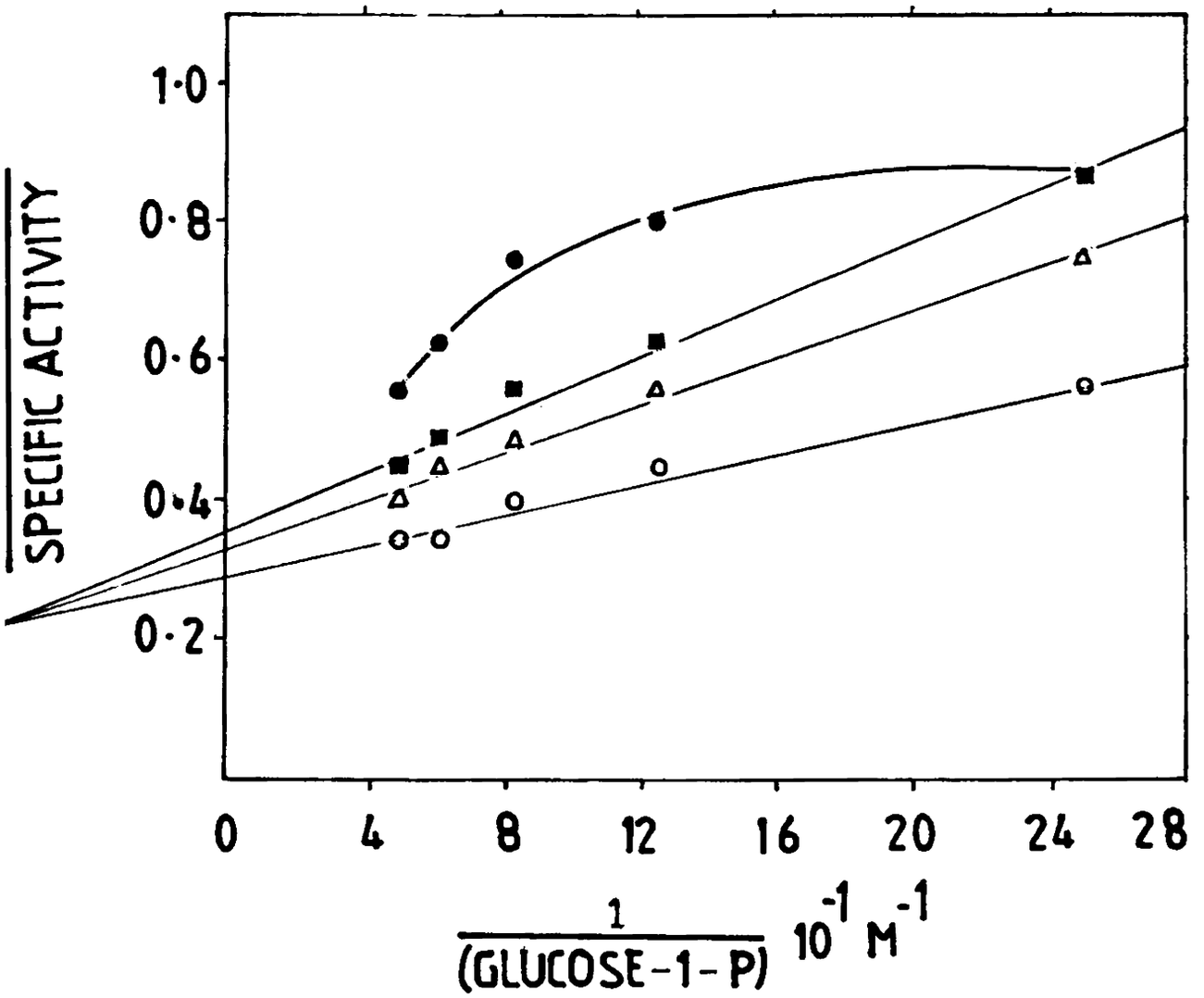
SPECIFIC ACTIVITY
1·0
0·8
0·6
0·4
0·2
0
4
8
12
16
20
24
28
1
(GLUCOSE-1-P)
10^-1 M^-1

FIG. 3-9

Fig. 3-10

Reciprocal plot for AMP for phosphorylase a from M. dobsoni in the presence of glucose, glucose-6-P and ATP. The assay mixture contained 16 mM glucose-1-P, 0.4% glycogen and 112 µg protein (in 0.4 ml) and AMP as indicated. ○, control; □, 10 mM ATP; △, 10 mM glucose and ●, 10 mM glucose-6-P. Other conditions were same as in fig. 3-8.

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a given active conformation. Since the effect could be overcome by 1 mM AMP, it could be deduced that glucose-6-P was interfering with the different possible modes of binding (or stability of conformation). The substrate inhibition observed may also be viewed in this perspective. These observations add further support to the suggestions presented from our lab (77) that right and wrong binding induced by substrate, activator and inhibitors determine the allosteric properties of phosphorylases.

In the case of the fish enzymes (C. guttattum and Etroplus) and Sepia phosphorylase, competitive inhibition was found with glucose-6-P, ATP and glucose when glucose-1-P was used as substrate. In the present case of M. dobsoni enzyme, a mixed type

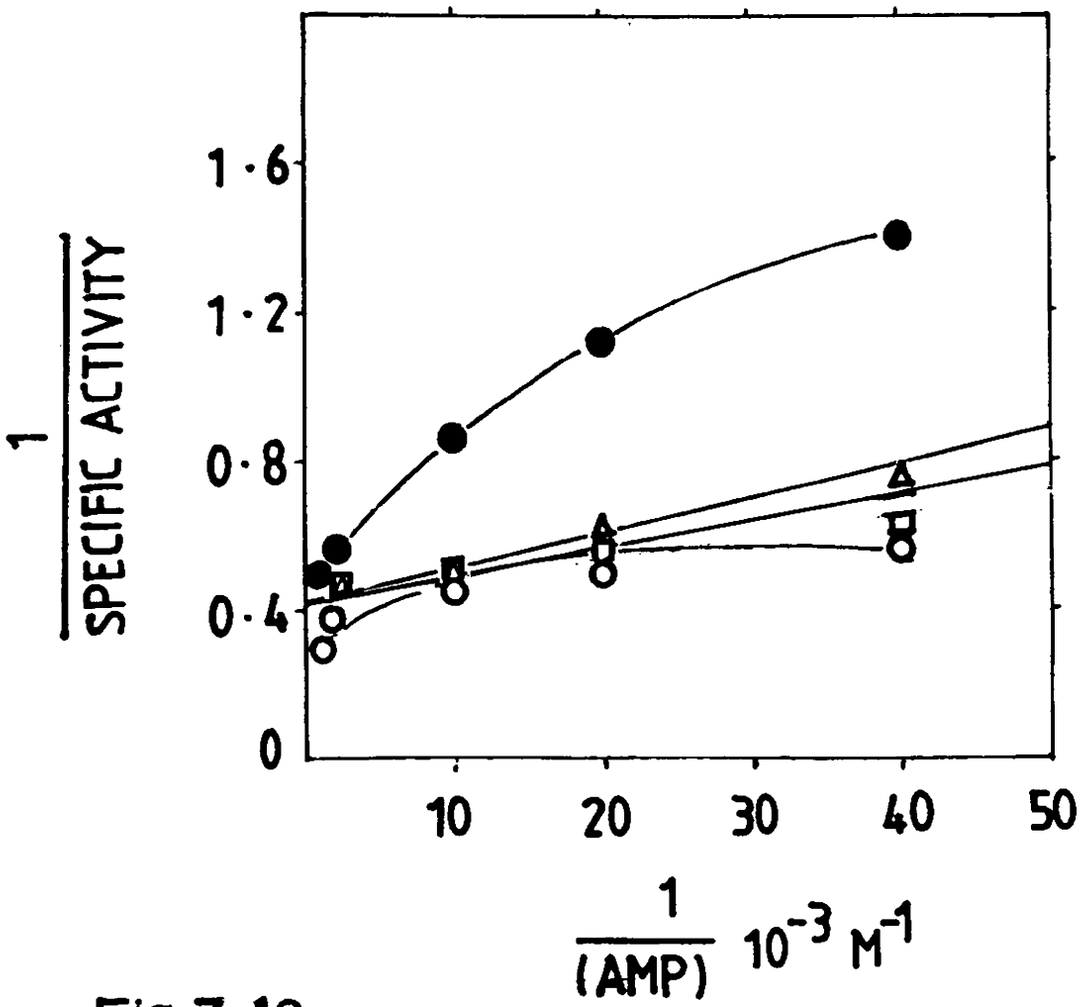

Fig.3.10

of inhibition was found for these ligands. That is, these inhibitors affected the affinity of the enzyme for the substrate and also the Vmax.

In conclusion, the following are the properties of M. dobsoni phosphorylase which differ from other well studied phosphorylases.

(i) In stability:- M. dobsoni phosphorylase was very unstable. The purified enzyme was extremely unstable. So unstable was the purified preparation, that it could not be preserved for even 24 hours without loss of activity. Such unstability has not been reported for phosphorylase from the muscle tissue of any other animal.

(ii) Effect of ions:-

1. 25-100 mM concentrations of Na_2SO_4, $(NH_4)_2SO_4$, NaCl and KCl had no activation or inhibition effect on enzyme activity.
2. KI & $MgCl_2$ (50 mM) were inhibitory.

(iii) Negative homotropic cooperativity of AMP sites:- In this respect M. dobsoni phosphorylase behaved like Sepia enzyme. All other muscle phosphorylases exhibit positive cooperativity of AMP sites. The Sepia enzyme is controlled by the presence of high concentration of AMP in the mantle, whereas in M. dobsoni tissue, AMP concentration was negligible. Both being invertebrate aquatic animals, this property could be seen as adaptation of the animals to energy needs.

(iv) Negative cooperativity of glucose-1-P sites in the presence of glucose-6-P:-
This property has not been reported in any other phosphorylase. The fact that the presence of AMP brings about the change may be significant, but not easily explained.

CHAPTER 4

PHOSPHORYLASE FROM THE FOOT MUSCLE OF SUNETTA SCRIPTA

Adults of benthic molluscs do not and cannot migrate from one habitat to another as and when seasonal variations in salinity, pH etc. in their habitats occur. Because of this, these molluscs are used for monitoring pollution in different locations.

The benthic bivalves use their foot muscle occassionally to change position (not to migrate). Two bivalves (commonly known as clams) were selected for our studies because of this special function of their foot muscle. It was thought that the property and control of phosphorylase of the foot muscle might provide interesting comparison with those studied from the extremely high energy requiring mantle of Sepia (25), from the muscle of Cibium (111) (both marine, the former an invertebrate and the latter a fish) and from the muscle of two animals reported in this thesis. (Chapters 2 & 3).

The two clams selected for study of phosphorylase and its control were Sunetta scripta and Villorita cyprenoides. Sunetta scripta is essentially a marine benthic bivalve with some tolerance to salinity decrease

but cannot survive in fresh water. On the contrary Villorita cyprenoides which survives in fresh water can tolerate certain salinity increase but cannot survive in marine water. Tolerance to changing salinity involves special energy requirements. Because of this and the special function of the foot muscle, studies of the property of phosphorylase and the control of its activity should give interesting information for comparison with other animal phosphorylases and among themselves with regard to adaptive changes and kinetic differences.

This chapter deals with phosphorylase from the foot muscle of Sunetta scripta. The salinity tolerance of S. scripta is 5-34‰. It is seen in sandy shallow waters.

Live animals (S. scripta) were collected and the foot muscle from medium to fully grown animals separated while the animals were still alive. The excised muscle was either stored at -15°C or used immediately. The foot muscle of a fully grown animal weighs approximately 0.3 g. The activity in the stored tissue was found to decrease gradually after 2-3 weeks of storage.

Extraction of ground tissue with 10 mM sodium β-glycerophosphate pH 7.0 gave a higher yield of phosphorylase activity than extraction with water. After the first extraction with 4 volumes of buffer, the second extract with 2 volumes of buffer yielded only 7% activity and one-third specific activity of the first extract. Hence only first extract was used. The specific activity of the first extract was 0.3 units*. Phosphorylase activity per gram tissue was only 4 units compared to 9 and 10 units for Etroplus suratensis and M. dobsoni respectively in the presence of AMP in the assay mixture. The enzyme content in terms of activity (in the presence of AMP) per gram tissue of Sunetta foot muscle from two extractions was about 40% only of the Etroplus and M. dobsoni muscles.

The activity ratio (defined as the activity in the absence of AMP to that in the presence of 1 mM AMP when assayed using 1% glycogen and 16 mM glucose-1-P) was 0.5. Incubation of the extract for 2 hours either at room temperature or in the cold at pH 7.8 or 8.0 changed the ratio only to 0.23. This means

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* Units of activity is defined as μmoles of Pi liberated/min. under the given assay conditions.

that the extract had no active phosphorylase phosphatase or that the extract contained considerable concentration of AMP. When ATP and Mg^{+2} were added to the extract and incubated at room temp. the activity ratio increased to 1.0 indicating the presence of phosphorylase kinase in the extract. Maintaining the extract at 45°C for 1 hour followed by incubation with ATP and Mg^{+2} did not change the ratio suggesting that the kinase was labile at increased temperature. Dialysis of the extract in the cold (for 16 hours) decreased the ratio from 0.5 to 0.23. Incubation of the dialysed extract with ATP & Mg^{+2} also increased the ratio to 1.0. Again on further dialysis in cold the ratio changed to 0.23 and further incubation with ATP & Mg^{+2} changed the ratio to 1.0. Hence phosphorylase kinase is present in an active form in the extract. Also phosphatase may be very active, but the activity ratio remained constant, ie., the phosphatase cannot act on the Sunetta phosphorylase so as to completely abolish the activity measured in the absence of AMP. This indicated that either phosphorylase b from Sunetta is active in the absence of AMP or, there is an equilibrium between the a and b forms such that they remain in a constant ratio. It was shown that the

human leukocyte phosphorylase b is active in the absence of AMP and activity ratio was 0.2 (90). Similarly Sepia phosphorylase a has an activity ratio 0.4 (25). Thus Sunetta phosphorylase is qualitatively similar to human leukocyte and Sepia phosphorylases in this respect.

Purified rabbit muscle phosphorylase has been reported to be convertible between the a and b forms by almost all animal muscle extracts (7). Similarly phosphorylase from the animal muscles have been shown to be converted between the a and b forms by rabbit muscle phosphorylase phosphatase and kinase (7). An exception to this was found in the case of Sepia mantle tissue. Purified Sepia mantle phosphorylase was not interconverted by partially purified rabbit muscle kinase and phosphatase. Crystalline rabbit muscle phosphorylase b (activity ratio 0) was converted to the a form (ratio 0.8) by extracts of Sunetta scripta confirming the presence of active kinase in extract.

Stability of the extract was checked for 7 days in the presence of 10 mM concentrations of each of EDTA and mercaptoethanol and 5% glycerol. It was found that the enzyme was stable in the extract for

seven days in the absence or presence of these substances and no loss of activity was found after 7 days when stored at 4°C. Hence in the extract the enzyme is stable. The activity ratio was changed from 0.5 to 0.23 in all these samples, showing the presence of phosphorylase phosphatase and its slow action at pH 7.0 in the extract. Here again the activity ratio came only to 0.23 indicating that the Sunetta phosphorylase b was active to some extent in the absence of AMP.

Ammonium sulphate fractionation was tried to purify the enzyme. It was found that 41-50% fraction had 20% yield with the same specific activity of the extract whereas the 51-60% fraction had 17% yield with 4.5-fold purification. Fraction obtained at 46-60% saturation had 40% yield and 4-fold purification. After dialysis of the ammonium sulphate fraction, crystallisation with AMP & Mg^{+2} was tried (as was tried for rabbit muscle phosphorylase), but was not successful. Dialysis of the fraction overnight in buffer at 5°C resulted in 66% activity loss. Hence like M. dobsoni phosphorylase, the Sunetta phosphorylase was also unstable. However at this stage, Sunetta phosphorylase was not as unstable as

the M. dobsoni phosphorylase (M. dobsoni phosphorylase was inactivated completely under the same conditions). Gel-filtration requiring only 15-30 min. was used to remove ammonium sulphate from the preparation. On gel-filtration the sample was diluted by 2-3 fold and about 25% activity was lost. This loss was only apparent as sulphate ions were found to activate the enzyme (see below).

Among other purification methods sought, column chromatography using cellulose phosphate, CM-cellulose etc., resulted only in loss of activity without any increase in specific activity. On DEAE-cellulose column chromatography using a gradient elution with NaCl and buffer, an overall purification of 10-fold with 15% yield was obtained. The results of a typical purification procedure are summarised in table 4-1. About 20 g of muscle was used for each experiment.

The partially purified enzyme after DEAE-cellulose chromatography was found to be extremely unstable. Concentration of fractions with ammonium sulphate followed by dialysis or gel-filtration on G-15 and G-25 resulted in almost complete inactivation. The fraction after the chromatography could not be preserved in the cold for

TABLE 4-1

PURIFICATION OF PHOSPHORYLASE FROM SUNETTA SCRIPTA

Purification step	Total volume ml.	Activity/ ml. units.	Protein mg/ml.	Specific activity units*	Total units	Yield %	Purification
Extract	66	1.2	4	0.3	80	100	1
46-60% Ammonium sulphate fraction	5	6.4	5.3	1.2	32	40	4
Gel-filtration	15	1.6	1.3	1.2	24	30	4
DEAE-cellulose column	9	1.33	0.44	3	12	15.	10

* Specific activity is expressed as μmoles of inorganic phosphate liberated per minute per mg. protein at 30°C.

more than a day without considerable loss of activity. Due to the high unstability of the preparation after DEAE-cellulose chromatography, further purification was found to be impossible. It may be noted that the Sunetta phosphorylase was comparatively more stable

than the M. dobsoni enzyme, because in the former case the activity in the preparation was stable for one day. It may be recalled that the M. dobsoni enzyme was completely inactivated under these conditions.

Since further attempts to purify the enzyme from S. scripta failed because of the inherent unstability of the enzyme, the preparation after the DEAE-cellulose chromatography stage was used for further studies on the same day of purification. The purified sample had activity ratio 0.23 (activity in the absence of AMP to that in the presence of 1 mM AMP when assayed with 1% glycogen and 16 mM glucose-1-P) as that of the extract. Human leukocyte phosphorylase b has been reported to have an activity ratio of 0.2 (90) and Sepia phosphorylase 0.4 (25) in contrast to vertebrate (both terrestrial and aquatic) animals.

The stabilising effects of various ions on the purified enzyme activity was checked. 0.2 M concentrations of $(NH_4)_2SO_4$, $CaCl_2$, $MgCl_2$ and NaCl all resulted in loss of activity when the enzyme was kept overnight in the presence of them, 80% activity loss was found in the presence of $(NH_4)_2SO_4$ by overnight period and complete loss was seen in the

presence of $CaCl_2$ and $MgCl_2$. However in the presence of NaCl the activity loss was 12% of the control, in which any of these ions was absent by the same period.

Purified Sunetta phosphorylase was found to be activated by $(NH_4)_2SO_4$; 50% activation was found when 50 mM $(NH_4)_2SO_4$ was included in the assay mixture. NaCl and KCl had no activation or inhibition effect on enzyme activity upto 0.1 M concentration. In the presence of 50 mM $MgCl_2$ 33% inhibition was found. The activation by sulphate is seen also in lamprey muscle phosphorylase, human leukocyte phosphorylase and Etroplus phosphorylase.

The optimum pH of the Sunetta phosphorylase was found to be 6.8 in 20 mM sodium β-glycerophosphate/15 mM cysteine buffer.

Kinetics with glucose-1-P and glycogen

Like the E. suratensis and M. dobsoni enzyme, the initial velocity measurements of the S. scripta phosphorylase were carried out in the direction of glycogen synthesis and the figs. 4-1 and 4-2 are drawn from the data obtained. The double reciprocal plots were straight lines for both glucose-1-P and glycogen as varying substrates which converged to a point on

Fig. 4-1

Lineweaver - Burk plots for glucose-1-P for Sunetta scripta phosphorylase b at different levels of glycogen assayed at 30°C. Assay mixture contained 15 mM cysteine/20 mM sodium β-glycerophosphate pH 6.8, 1 mM AMP, glycogen and glucose-1-P as indicated. The assay mixture has got 210 µg/ml protein. The concentrations of glycogen (expressed as mM glucosyl residues) were ○, 61.5 mM; ●, 34.6 mM; □, 16 mM and ■, 4.92 mM.

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the left of the vertical axis and above the horizontal axis. Secondary plots of intercepts and slopes against the reciprocal of the substrate concentration were also linear. Thus the mechanism of S. scripta phosphorylase is also consistent with that observed for other phosphorylases (7,25,111). The kinetic constants (table 4-2) calculated using different equations from these secondary plots were also consistent with the mechanism.

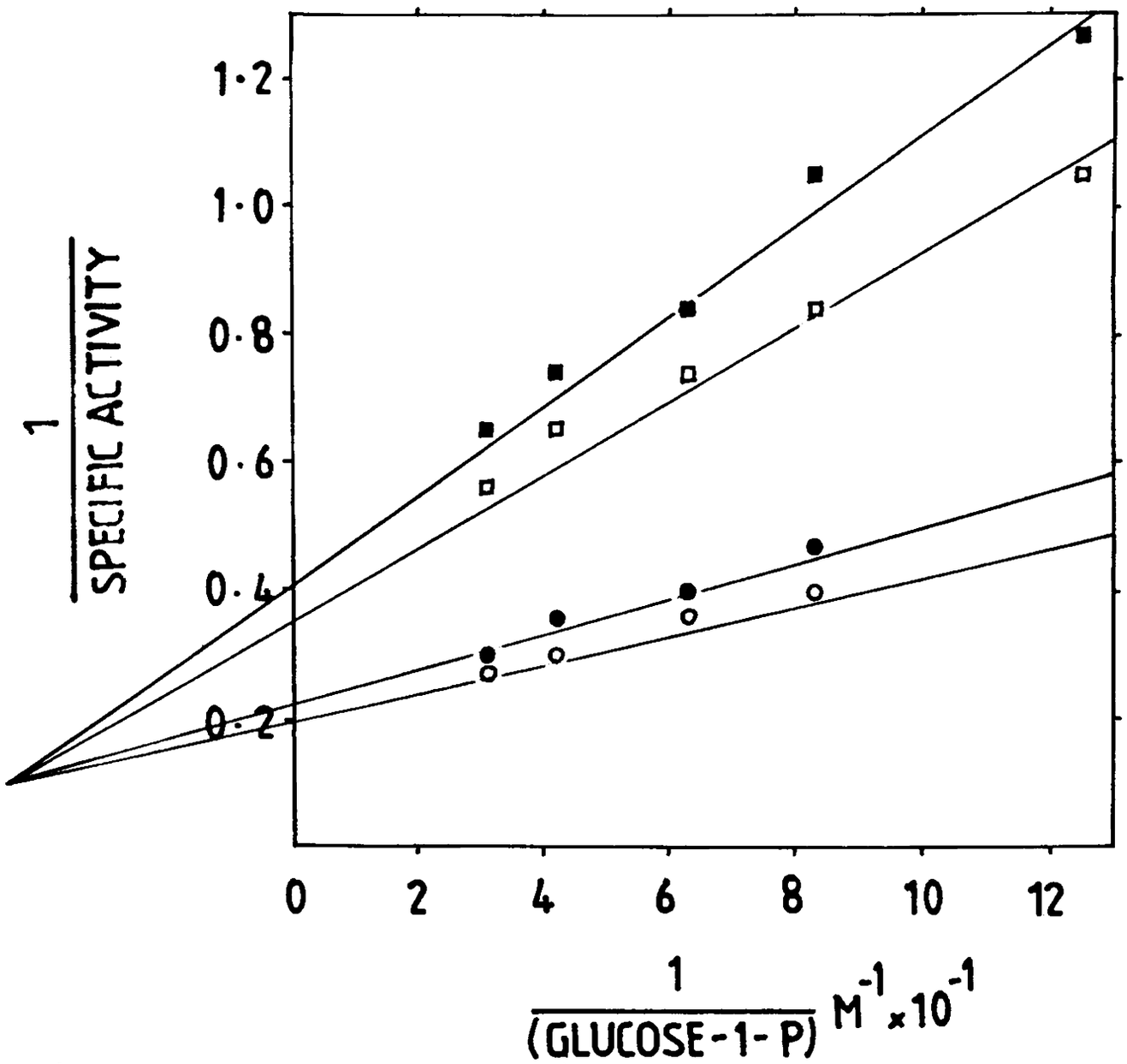
1·2
1·0
0·8
0·6
0·4
0·2
1
SPECIFIC ACTIVITY
0
2
4
6
8
10
12
1
(GLUCOSE-1-P)
M⁻¹ x10⁻¹

FIG. 4_1

Fig. 4-2

Reciprocal plots for glycogen at different levels of glucose-1-P for phosphorylase b from S. scripta. Assay mixture contained 1 mM AMP, glucose-1-P and glycogen as indicated. The concentrations of glucose-1-P were ▲, 32 mM; ●, 24 mM; ○, 16 mM; ■, 12 mM and □, 8 mM. Other conditions were same as in fig. 4-1.

TABLE 4-2

VALUES OF KINETIC CONSTANTS OBTAINED USING DIFFERENT EQUATIONS FROM THE SECONDARY PLOTS OF PHOSPHORYLASE b FROM SUNETTA SCRIPTA.

	Values obtained from the seconday plots (not shown in the thesis)			
	A	B	C	D
Vmax (μmoles/min/mg)	5.56		5.26	
K_5 (mM glucose-1-P)		5.28	4.62	
K_6 (mM glycogen)	6.59			7.1
K_7 (mM glycogen)		26		24.2

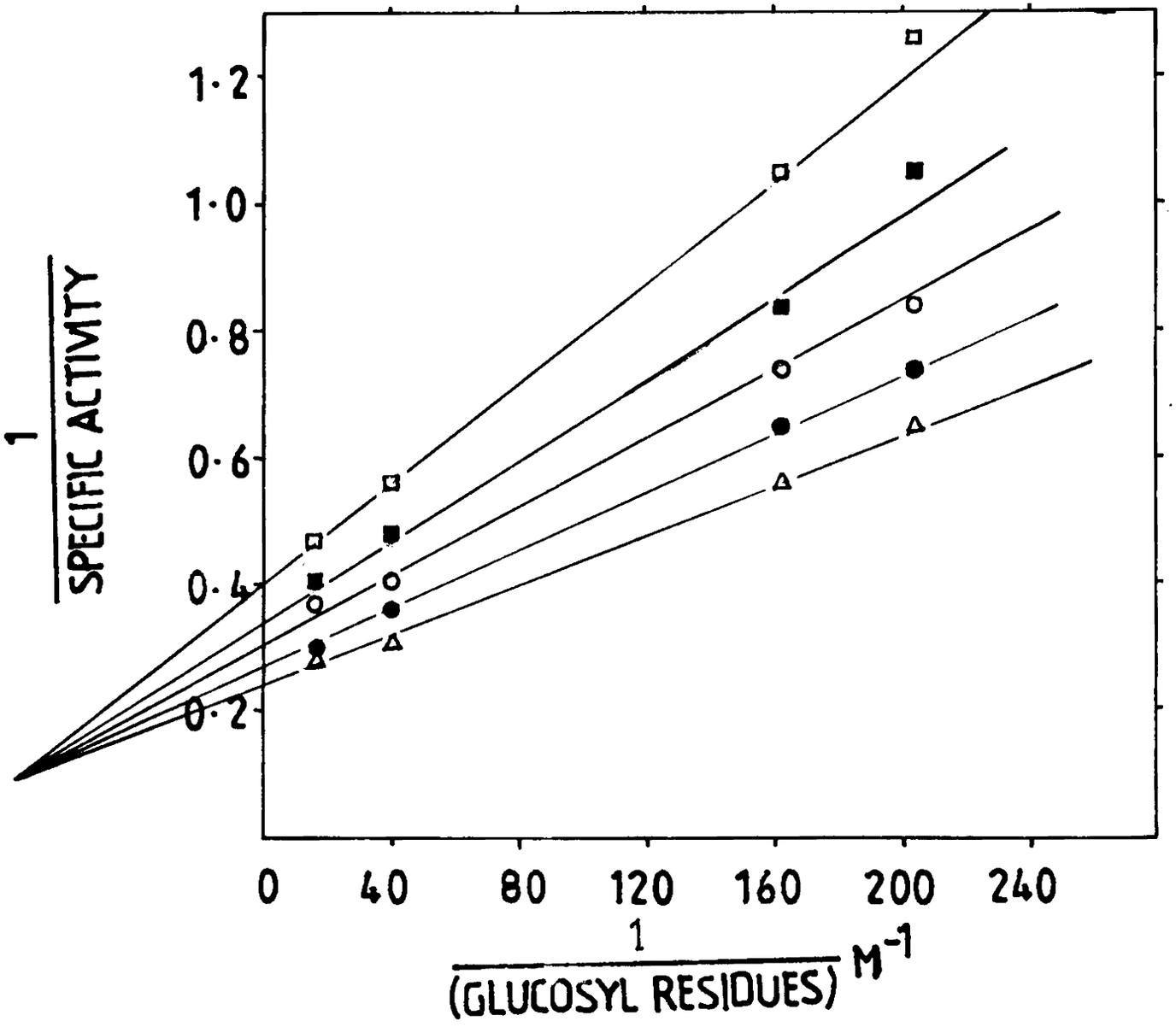
1·2
1·0
0·8
0·6
0·4
0·2
1/SPECIFIC ACTIVITY
0
40
80
120
160
200
240
1/(GLUCOSYL RESIDUES) M⁻¹

TABLE 4-3

KINETIC CONSTANTS FOR PHOSPHORYLASE b FROM S. SCRIPTA.

K_5 (mM glucose-1-P)	4.95
K_6 (mM glycogen)	6.8
K_7 (mM glycogen)	25.0
K_8 (mM glucose-1-P)	11.36

Table 4-3 shows the values of kinetic constants for phosphorylase b from Sunetta scripta. The values of K_6 and K_7 are 7.5 and 6 times (average values) higher than the values for rabbit muscle phosphorylase b and K_5 and K_8 3 and 1.5 times greater respectively.

The apparent K_m values calculated from figs. 4-1 and 4-2 are given in table 4-4. The table (table 4-4) shows that increasing concentrations of glycogen resulted in decrease of the K_m values for glucose-1-P (ie., the kinetic mechanism being sequential, decrease of K_m implies increased affinity) and vice versa. Here also like in the case of M. dobsoni enzyme (Chapter 3) the change of K_m values of glycogen on varying the concentration of glucose-1-P was not

TABLE 4-4

APPARENT K_m VALUES FOR GLUCOSE-1-P AND GLYCOGEN AT DIFFERENT LEVELS OF EACH OTHER FOR PHOSPHORYLASE b FROM S. SCRIPTA.

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

Glycogen (mM)	K_m for glucose-1-P	Glucose-1-P (mM)	K_m for glycogen (mM)
61.5	11.25	32	8.08
24.6	12.99	24	8.52
6.15	16.10	16	9.05
4.92	17.60	12	9.28
		8	9.78

considerable. However a consistent change of K_m of glycogen was observed (table 4-4). Therefore, the Sunetta phosphorylase exhibited minor positive heterotropic cooperativity between the substrate sites.

Kinetics with respect to AMP

The reciprocal plots for glucose-1-P at different levels of AMP and those for AMP at different constant

concentrations of glucose-1-P are given in figs. 4-3 and 4-4. The lines in both cases are linear showing lack of homotropic cooperativity between AMP sites and substrate sites at the AMP concentration (0.025-1 mM) and glucose-1-P used. Similar results were obtained for phosphorylase from the marine fish Cibium (16). In the case of rabbit muscle phosphorylase also the reciprocal plots at these concentrations of AMP are linear. However, the reciprocal plots for AMP for Sepia phosphorylase a (25) and M. dobsoni phosphorylase a (Chapter 3) are different. They show negative homotropic cooperativity between AMP sites.

Apparent K_m values for glucose-1-P and AMP were calculated from figs. 4-3 and 4-4. They are tabulated in table 4-5. Increasing concentration of the substrate decreased the K_m for AMP and vice versa, which shows similarity to phosphorylases from other sources.

In this respect also E. suratensis phosphorylase (Chapter 2) stands out as an exception which showed opposite properties. It showed increased K_m values for AMP/glucose-1-P for increased glucose-1-P/AMP concentrations and vice versa.

Fig. 4-3

Reciprocal plots for glucose-1-P at different levels of AMP for glycogen phosphorylase from S. scripta. Phosphorylase was assayed in 15 mM cysteine/20 mM sodium β-glycerophosphate pH 6.8 at 30°C in the presence of 1% glycogen, AMP and glucose-1-P as indicated. Assay mixture contained 210 µg/ml protein. Concentrations of AMP were ○, $1x10^{-3}$ M; ●, $1x10^{-4}$ M; △, $1x10^{-5}$ M and □, $2.5x10^{-5}$ M.

Fig. 4-4

Reciprocal plots for AMP at different levels of glucose-1-P for S. scripta phosphorylase. The assay mixture contained 1% glycogen, glucose-1-P and AMP as indicated. The concentrations of glucose-1-P were ○, 8 mM; ●, 12 mM, □, 16 mM, ■, 24 mM and △, 32 mM. Other conditions were same as in fig. 4-3.

Inhibition by Metabolites.

Glycogen phosphorylase b from different sources has been shown to be inhibited by the metabolites glucose, glucose-6-P and ATP (25,118,119). The a form of rabbit muscle phosphorylase is inhibited by these ligands negligibly in the presence of 1 mM AMP

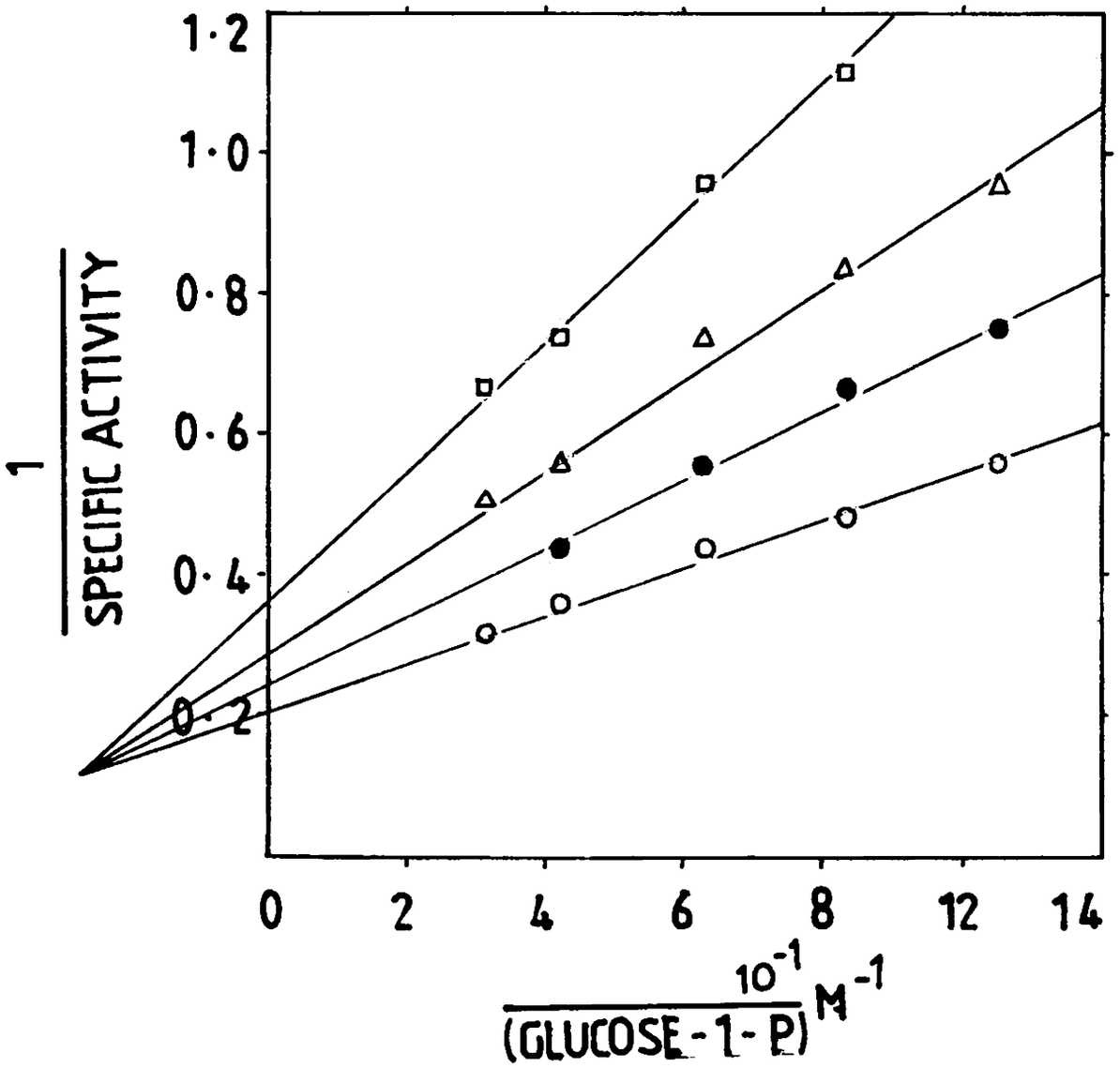
1·2
1·0
0·8
0·6
0·4
0·2
1 / SPECIFIC ACTIVITY
0
2
4
6
8
12
14
10⁻¹ / (GLUCOSE-1-P) M⁻¹

FIG. 4-3

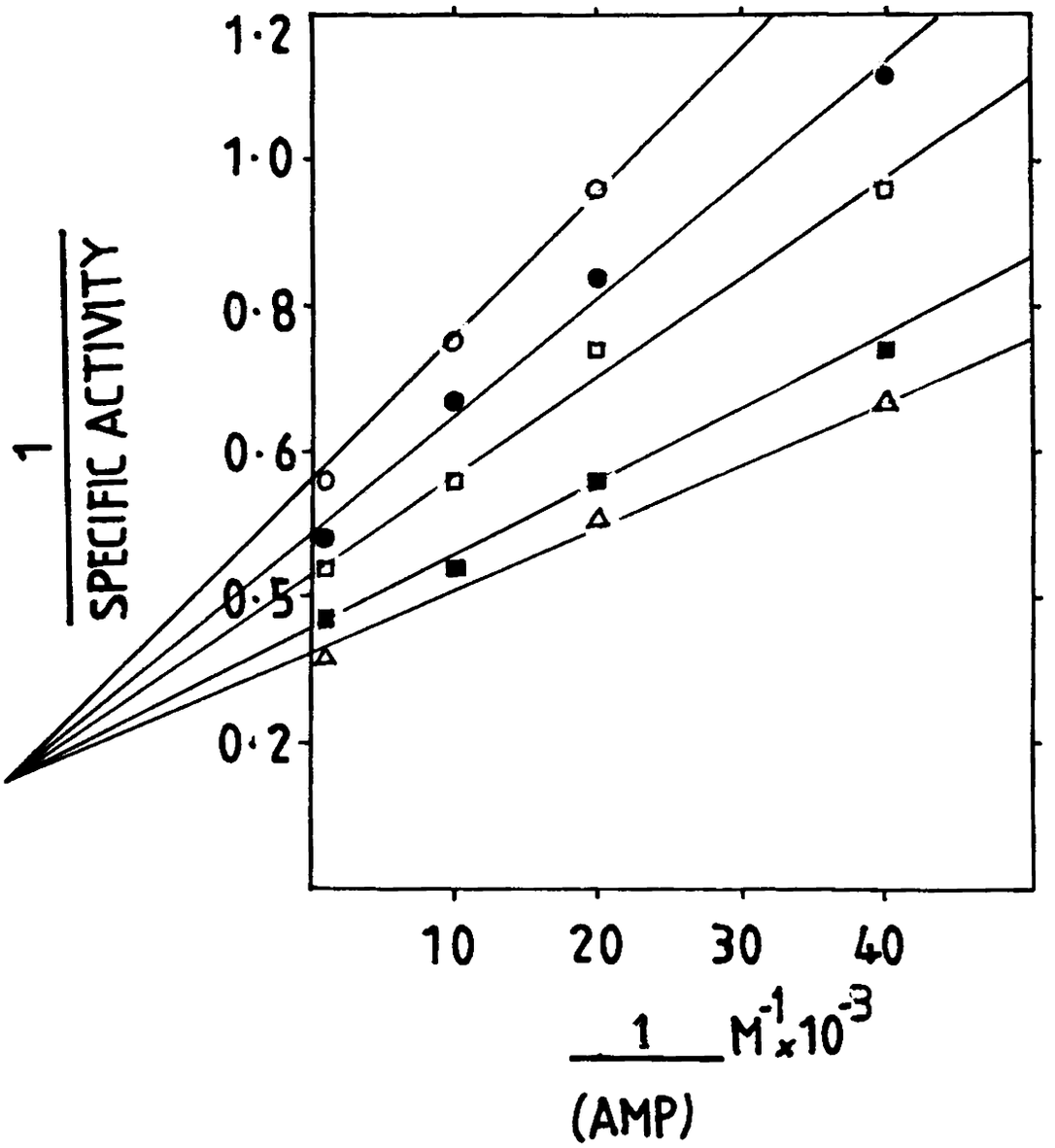

FIG. 4_4

Fig. 4-5

Reciprocal plots for glucose-1-P in the presence and absence of glucose, glucose-6-P and ATP for glycogen phosphorylase from S. scripta. The inhibitors were mixed with the enzyme solution and was added to an equal volume of substrate solution and assayed. The assay mixture contained 1% glycogen, 1 mM AMP, varying concentrations of glucose-1-P, 15 mM cysteine/20 mM sodium β-glycerophosphate and 330 µg/ml protein. ○, no inhibitor; ●, 10 mM glucose; △, 10 mM ATP and □, 10 mM glucose-6-P.

TABLE 4-5

APPARENT K_m VALUES FOR GLUCOSE-1-P AND AMP AT DIFFERENT LEVELS OF EACH OTHER FOR S. SCRIPTA PHOSPHORYLASE b.

AMP (M)	K_m for glucose-1-P (mM)	Glucose-1-P (mM)	K_m for AMP (M)
$1x10^{-3}$	15.9	32	$2.7x10^{-5}$
$1x10^{-4}$	19.3	24	$2.95x10^{-5}$
$5x10^{-5}$	23.0	16	$3.1x10^{-5}$
$2.5x10^{-5}$	25.5	12	$3.3x10^{-5}$
		8	$3.6x10^{-5}$

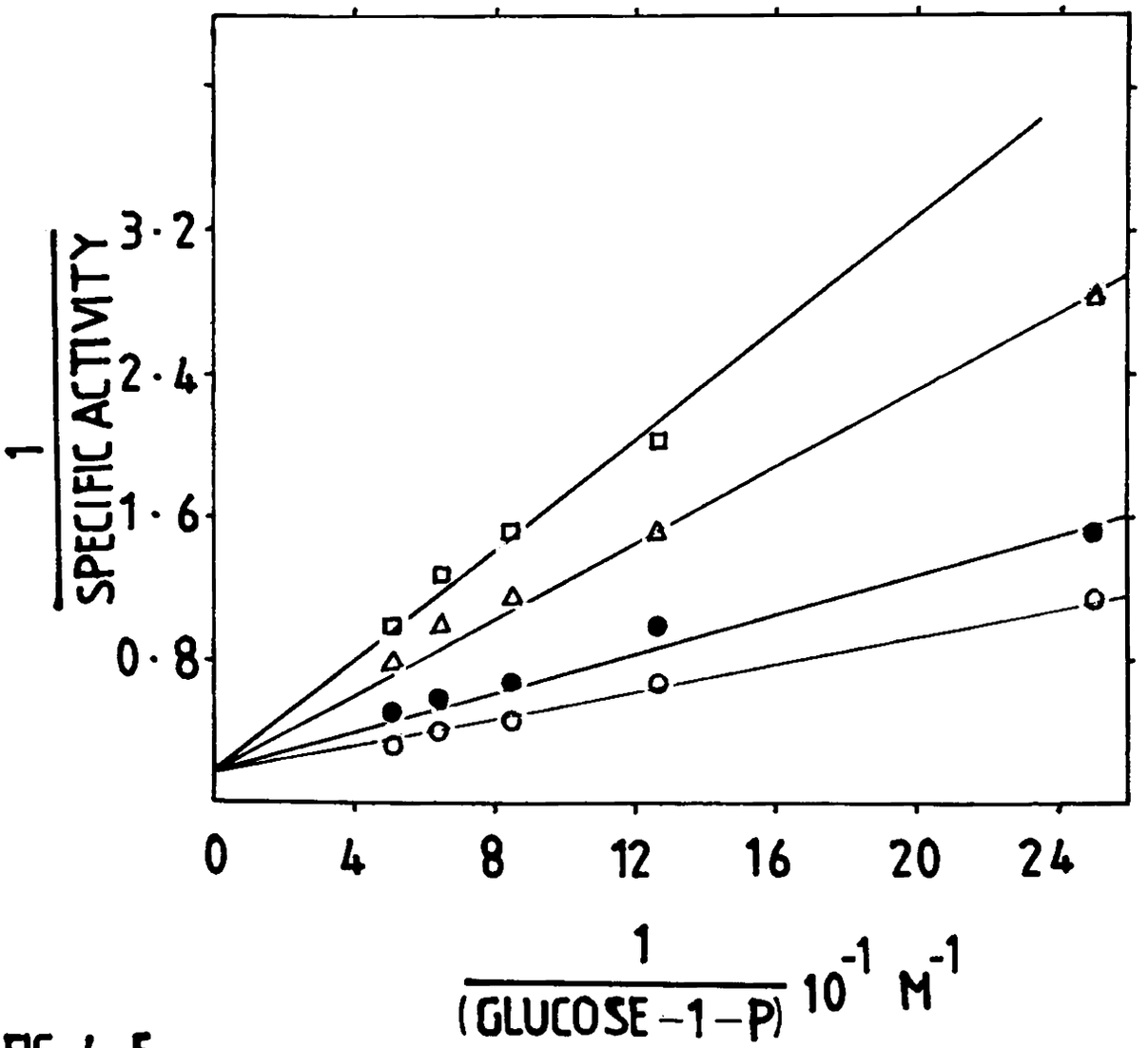
1 / SPECIFIC ACTIVITY
3·2
2·4
1·6
0·8
0
4
8
12
16
20
24
1 / (GLUCOSE-1-P) 10⁻¹ M⁻¹

FIG. 4_5

and at 10-15 mM concentrations of inhibitors (7). Sepia phosphorylase a is not inhibited by them in the presence of AMP whereas in the absence of AMP, they behave as competitive inhibitors (25). The marine fish Cibium phosphorylase (119) and estuarine fish E. suratensis phosphorylase (Chapter 2) also showed competitive inhibition with these ligands for glucose-1-P.

Reciprocal plots for glucose-1-P and AMP for S. scripta phosphorylase in the presence and absence of glucose, glucose-6-P and ATP are shown in fig. 4-5 and 4-6. Competitive inhibition was exhibited by these ligands for glucose-1-P. In the case of the fish enzymes (Cibium and Etroplus) glucose is a potent inhibitor. Glucose exerts a higher degree of inhibition than glucose-6-P and ATP for the fish phosphorylases (119, Chapter 2). For the Sunetta phosphorylase, glucose is a weaker inhibitor as compared to glucose-6-P and ATP. Sepia enzyme is also inhibited to a lesser extent by glucose than glucose-6-P and ATP. This difference in degree of inhibition by glucose and glucose-6-P shows the structural dissimilarity between the phosphorylases from fishes and the invertebrates. But it was stated

Fig. 4-6

Reciprocal plots for AMP in the presence of glucose, glucose-6-P and ATP for S. scripta phosphorylase. Concentrations of glycogen and glucose-1-P in the assay mixture were 1% and 16 mM respectively and AMP as indicated. The assay mixture contained 15 mM cysteine/20 mM sodium β-glycerophosphate buffer pH 6.8 and 330 µg/ml protein. ○, no inhibitor; ●, 10 mM glucose, △, 10 mM ATP and □, 10 mM glucose-6-P.

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earlier in this chapter that rabbit phosphorylase b kinase acted on the Sunetta phosphorylase b converting it to its a form. Sunetta phosphorylase kinase also converted rabbit phosphorylase b showing the similarity in the structure around the region where phosphorylation and dephosphorylation occur, whereas the binding sites of phosphorylase where glucose and glucose-6-P bind appear to be different in fishes and the aquatic invertebrates.

These metabolites show mixed type of inhibition with respect to AMP. In the case of the phosphorylase from the fishes, Cibium and Etroplus, the reciprocal plots for AMP in the presence of

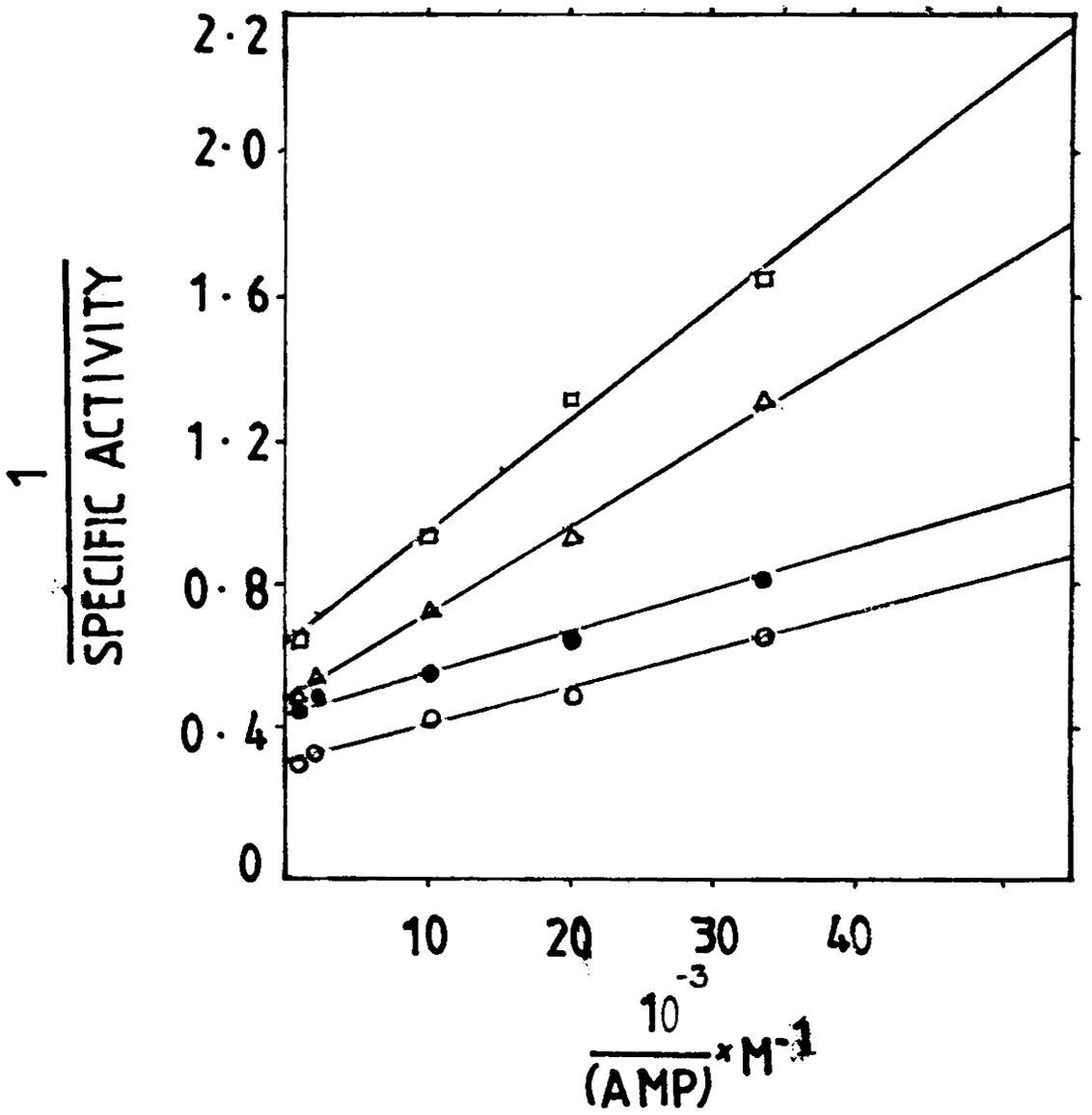

Fig. 4-6

glucose-6-P were upwardly curved, which was suggested as due to the different modes of binding at the substrate binding site itself like in the case of the chemically desensitised rabbit enzyme (119) rather than different allosteric sites. This suggestion was proved recently by X-ray crystallographic studies (76).

Thus in the inhibition pattern, the phosphorylases from fish are slightly different from the Sepia and Sunetta (invertebrate) phosphorylases.

The following conclusions may be made from the results presented in this chapter with respect to other well studied animal phosphorylases.

i) Sulphate ions activate the phosphorylase from Sunetta scripta like the lamprey, human leukocyte and Etroplus phosphorylases.

ii) 50 mM concentrations of Mg^{+2} and Ca^{+2} are inhibitory.

iii) Na^{+}, K^{+}, Cl^{-} etc. have no activation or inhibition effect on the enzyme activity.

iv) The enzyme is similar to all other animal phosphorylases, in the positive heterotropic cooperativity between glucose-1-P and glycogen sites and unlike the Etroplus

phosphorylase.

v) Positive heterotropic cooperativity exists between glucose-1-P and AMP sites also, for the Sunetta phosphorylase whereas the cases of *Sepia*, *M. dobsoni* and *Etroplus* phosphorylases are different.

vi) *Sunetta* phosphorylase is different from the fish (*Cibium* and *Etroplus*) phosphorylases in the inhibition pattern by glucose-6-P.

vii) Unlike the fish phosphorylases, glucose is comparatively a weaker inhibitor than ATP and glucose-6-P.

CHAPTER 5

PHOSPHORYLASE FROM THE FOOT MUSCLE OF VILLORITA CYPRENOIDES

The most common and abundant benthic mollusc in estuary is the bivalve Villorita. The shells are the raw material of lime and white cement industry. The flesh is a good quality food. Villorita cyprenoides (var.) cochinensis is found in muddy places and its salinity tolerance is 0-12‰. This clam thrives in slightly saline estuarine condition but tolerates fresh water also. However it cannot survive in marine water.

After studying the properties of phosphorylases from an estuarine fish, estuarine prawn and a marine bivalve, Villorita cyprenoides was taken as the next species for study. The two clams, Villorita and Sunetta are almost alike in their structure, mode of living etc. and the difference between them is, while Villorita has estuarine muddy habitat, the habitat of Sunetta is sandy and marine. Two closely similar animals in two slightly different habitats were taken for the phosphorylase study, to see whether

there is any difference between them, or whether this slightly different habitat can influence the properties of the enzyme.

Foot muscle was selected as the source of the enzyme, because of its special function, and for comparative study with the Sunetta foot muscle phosphorylase. Fresh live clams were collected and the foot muscle was taken. The separated foot muscle which was not contaminated with the intestinal parts were kept frozen at -15 to -20°C. Phosphorylase activity in the muscle was stable in the frozen stage for 2-3 weeks. When cold water (double distilled) was used for extraction of the enzyme only a very low yield of phosphorylase activity was obtained. Sodium β-glycerophosphate (0.01 M pH 7.0) also was tried. Higher yield was obtained when the ground tissue was extracted with 6 volumes of 0.01 M sodium β-glycerophosphate pH 7.0 containing 1 mM EDTA. The specific activity of the extract was 0.05* and activity per gram tissue was 2 units. Thus the lowest specific activity and activity per gram tissue were found for Villorita among the four species

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* Specificativity is no. of μmoles of Pi liberated per minute per mg protein at 30°C.

studied. This may have significance to the relatively sedentary life of the animal with low energy requirement.

EDTA was found to have some stabilising effect on the enzyme activity of the extract. The extract when kept overnight in the absence of EDTA, complete loss of activity was observed, whereas in the presence of 1 mM EDTA only 30% loss was found.

Attempts made to purify the enzyme include ammonium sulphate fractionation and adsorption on various ion exchangers. It was found that in the 41-60% ammonium sulphate fraction 40% of the activity was present with 2-fold purification. Enzyme was found to be highly unstable like the M. dobsoni and Sunetta phosphorylases. On dialysis overnight the activity was found to be lost. Hence the ammonium sulphate fraction was passed through sephadex G-25 gel column to remove the ammonium sulphate.

The active fractions obtained from the gel column, when passed through a column of DEAE-cellulose, the activity was completely adsorbed. On elution with 1 M NaCl, the yield was very low and the enzyme preparation was highly unstable. On keeping the sample the activity was completely lost. Hence the

DEAE-cellulose chromatography was not a successful method for the purification of Villorita phosphorylase. Attempt to purify the enzyme by crystallisation in the presence of AMP was also failed. Hence the properties of the enzyme were studied with ammonium sulphate fraction, after passing through the sephadex G-25 column.

The activity ratio (activity in the absence of AMP to that in the presence of AMP) of the extract was found to be 0.5 whereas that of the ammonium sulphate fraction was 0.3. The ratio did not decrease from this value. On incubating the extract and also the ammonium sulphate fraction with ATP and $MgCl_2$, the ratio changed from 0.5 to 1.0. But on ammonium sulphate fractionation of the extract, the ratio changed only to 0.6 unlike in the case of the Sunetta where under the same conditions, the ratio changed to the constant value 0.23.

Purified rabbit muscle phosphorylase b was acted upon by the phosphorylase kinase of the Villorita foot muscle extract changing the activity ratio to 0.75. The sample (containing the extract) on dialysis overnight at pH 6.8 in 3 mM cysteine or on keeping at room temperature for 2 hours the ratio

changed to 0.2. Hence both phosphorylase kinase and phosphorylase phosphatase of the Villorita foot muscle act on rabbit phosphorylase b and a respectively. Other animal phosphorylase kinases and phosphorylase phosphatases also act in the same fashion. This shows the similarity in structure between the rabbit muscle phosphorylase and Villorita foot muscle phosphorylase like the other animal phosphorylases.

Villorita phosphorylase is active in the absence of AMP and the activity ratio was found to be 0.3. Sunetta phosphorylase also exhibited an activity ratio of 0.23. Human leukocyte phosphorylase b has an activity ratio 0.2 (90) and Sepia phosphorylase a has 0.4 (25). Thus all these phosphorylases have slight activity in the absence of AMP. Generally the a forms of terrestrial animal phosphorylases are having an activity ratio 0.7 to 1.0 and the b forms are inactive in the absence of AMP. Thus the three phosphorylases from the molluscan phylum ie., from Villorita, Sunetta and Sepia, and the human leukocyte phosphorylase are having slight activity in the absence of AMP. Here the three phosphorylases from the molluscan phylum are alike in this respect.

Effect of various ions on the activity of the

enzyme was studied in detail. Like other phosphorylases mentioned in this thesis, 0.1 M concentrations of NaCl and KCl had no activation or inhibition effect. Na_2SO_4 and $(NH_4)_2SO_4$ (0.1 M) activated the enzyme by 12%. 50 mM $MgCl_2$ had no action whereas 100 mM concentration inhibited the activity by 63%. 50 mM $CaCl_2$ was inhibitory (16%) and 5 mM $HgCl_2$ inactivated the enzyme activity completely. 50 mM and 100 mM KI were also inhibitory (50% and 72% inhibition respectively). The activation by sulphate was similarly seen in Sunetta, lamprey muscle (82), human leukocyte (90) and Etroplus phosphorylases. One major difference in the inhibition by ions was seen in the case of Mg^{2+}. While Mg^{2+} (50 mM $MgCl_2$) had no effect on the Villorita phosphorylase, the same concentration of Mg^{2+} inhibited the Sunetta enzyme by 33%.

The optimum pH for Villorita phosphorylase was found to be 6.8 in 15 mM cysteine/20 mM sodium β-glycerophosphate buffer.

Kinetics

The initial velocity data of the Villorita cyprenoides phosphorylase were analysed in the direction of glycogen synthesis as in the other three cases given in the thesis. The data are given in figs. 5-1 and 5-2. Here also the lines converged to a point left to the vertical axis, similar to the other animal phosphorylases and those that we studied. Therefore the results obtained with Villorita phosphorylase are also consistent with rapid equilibrium random mechanism. The secondary plots of slopes and intercepts versus the reciprocal of glycogen and glucose-1-P were also linear (figs. 5-3 and 5-4) confirming the consistency. The scheme of the mechanism and the equation for the scheme for phosphorylase is given in Chapter 2. Kinetic constants were calculated using different equations from the figs. 5-3 and 5-4 and tabulated in table 5-1.

The values are the same when calculated using different equations. This also is consistent with the mechanism suggested, ie., rapid equilibrium random.

The kinetic constants of Villorita phosphorylase are tabulated in table 5-2.

Fig. 5-1

Reciprocal plots for glucose-1-P for Villorita cyprenoides phosphorylase b at different levels of glycogen assayed at 30°C at pH 6.8. Assay mixture had 15 mM cysteine/20 mM sodium β-glycerophosphate 1 mM AMP, glycogen and glucose-1-P as indicated and 290 µg/ml protein. The concentrations of glycogen (expressed in mM glucosyl residues) were ■, 61.5 mM; □, 24.6 mM; ●, 6.15 mM and ○, 2.46 mM.

Fig. 5-2

Reciprocal plots for glycogen at different constant concentrations of glucose-1-P for phosphorylase b from Villorita cyprenoides. The concentrations of glucose-1-P were ○, 8 mM; ●, 12 mM; □, 16 mM; ■, 24 mM and △, 32 mM. Other conditions were same as in fig. 5-1.

The kinetic constant k_2 is very less compared to other animal phosphorylases. However, this result can be compared only if the enzyme is pure. Here in this case, because of the unstability and very low activity of phosphorylase in the muscle, complete purification could not be achieved. Hence

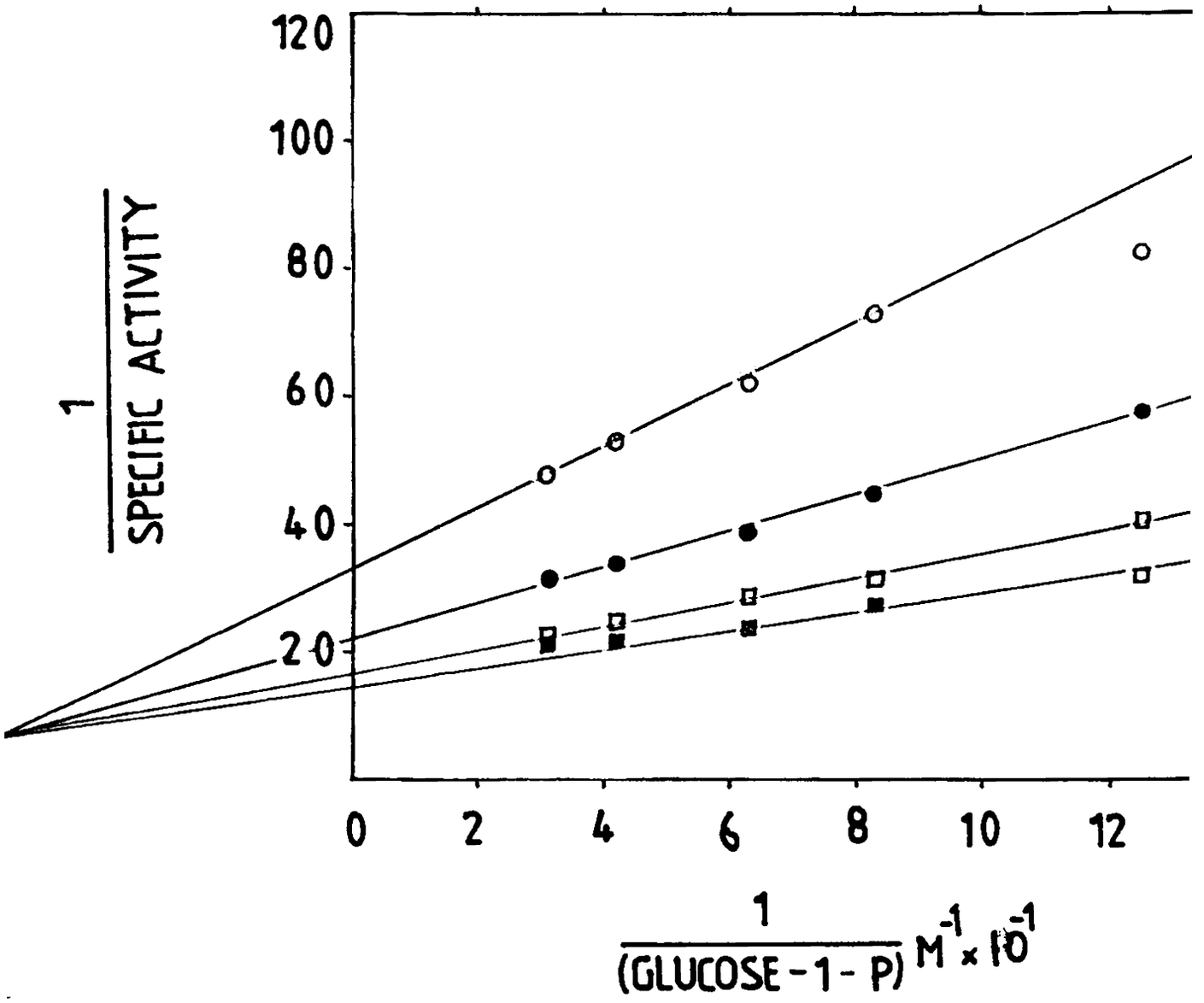
120
100
80
60
40
20
0
2
4
6
8
10
12
1 / SPECIFIC ACTIVITY
1 / (GLUCOSE-1-P) M⁻¹ × 10⁻¹

FIG. 5_1

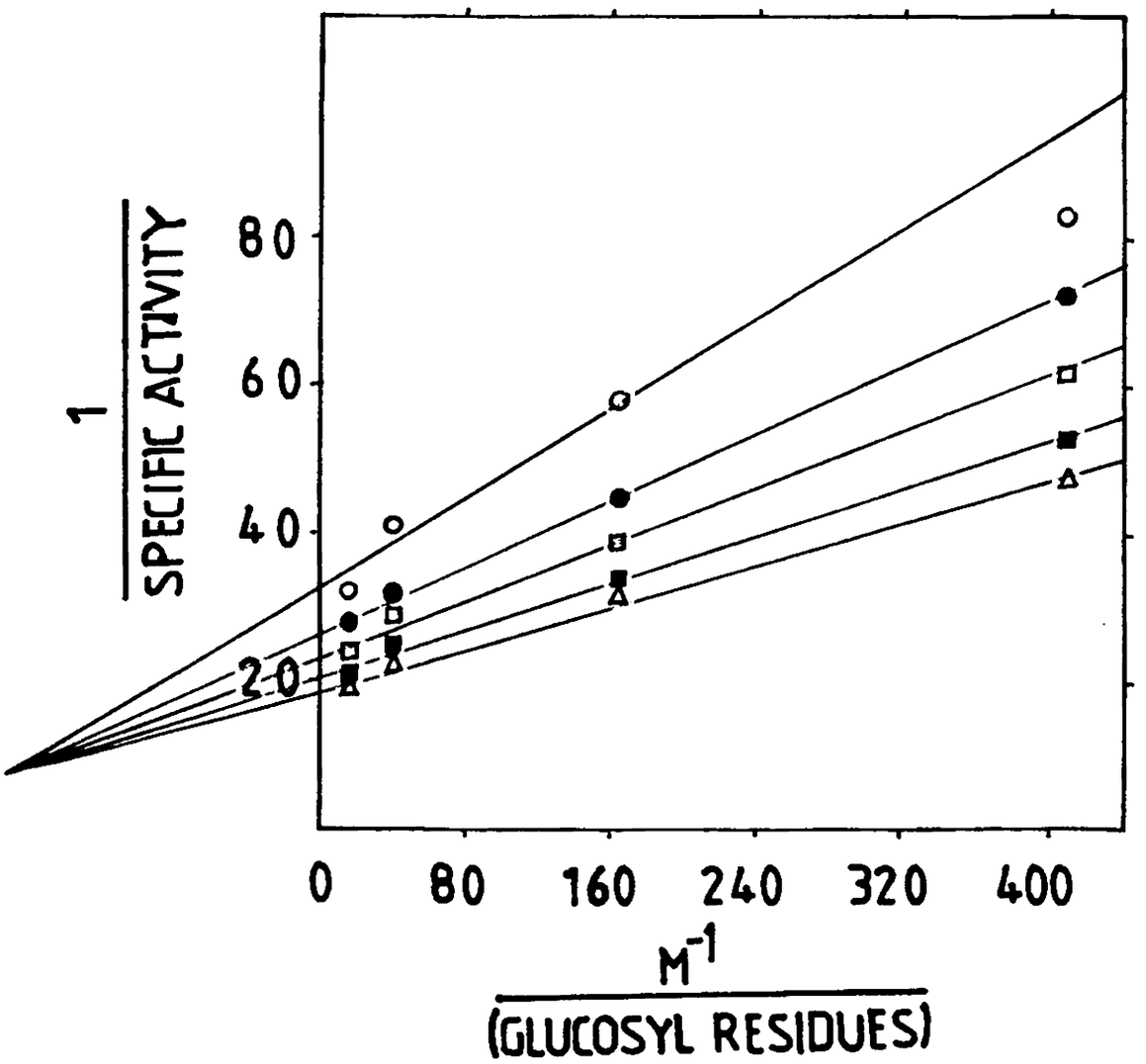
1
SPECIFIC ACTIVITY
80
60
40
20
0
80
160
240
320
400
M-1
(GLUCOSYL RESIDUES)

FIG. 5.2

Fig. 5-3

Secondary plots of intercepts (A) and slopes (B) against the concentration of glycogen from fig. 5-1.

Fig. 5-4

Seconday plots of intercepts (A) and slopes (B) against the concentration of glucose-1-P from fig. 5-2.

TABLE 5-1

VALUES OF KINETIC CONSTANTS OBTAINED FROM THE DIFFERENT SECONDARY PLOTS OF V. CYPRENOIDES PHOSPHORYLASE b

	Values obtained from fig. 5-3		fig. 5-4	
	A	B	A	B
Vmax (μmoles/min/mg)	0.05		0.05	
K_5 (mM glucose-1-P)		3.55	3.87	
K_6 (mM glycogen)	2.4			2.3
K_7 (mM glycogen)		12.63		7.15

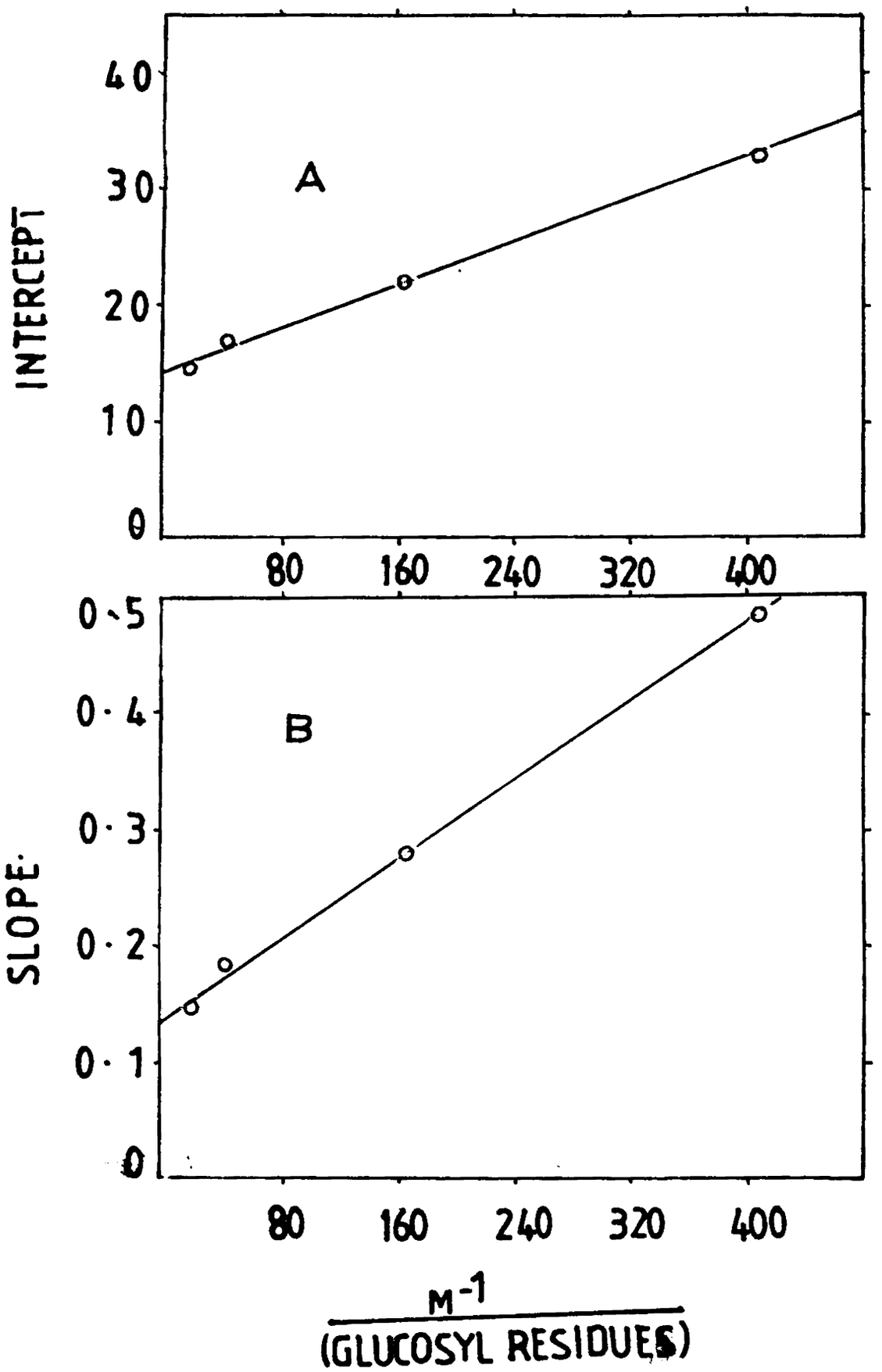
A
INTERCEPT
40
30
20
10
0
80
160
240
320
400
B
SLOPE
0·5
0·4
0·3
0·2
0·1
0
80
160
240
320
400
M⁻¹
(GLUCOSYL RESIDUES)

Fig. 5.3

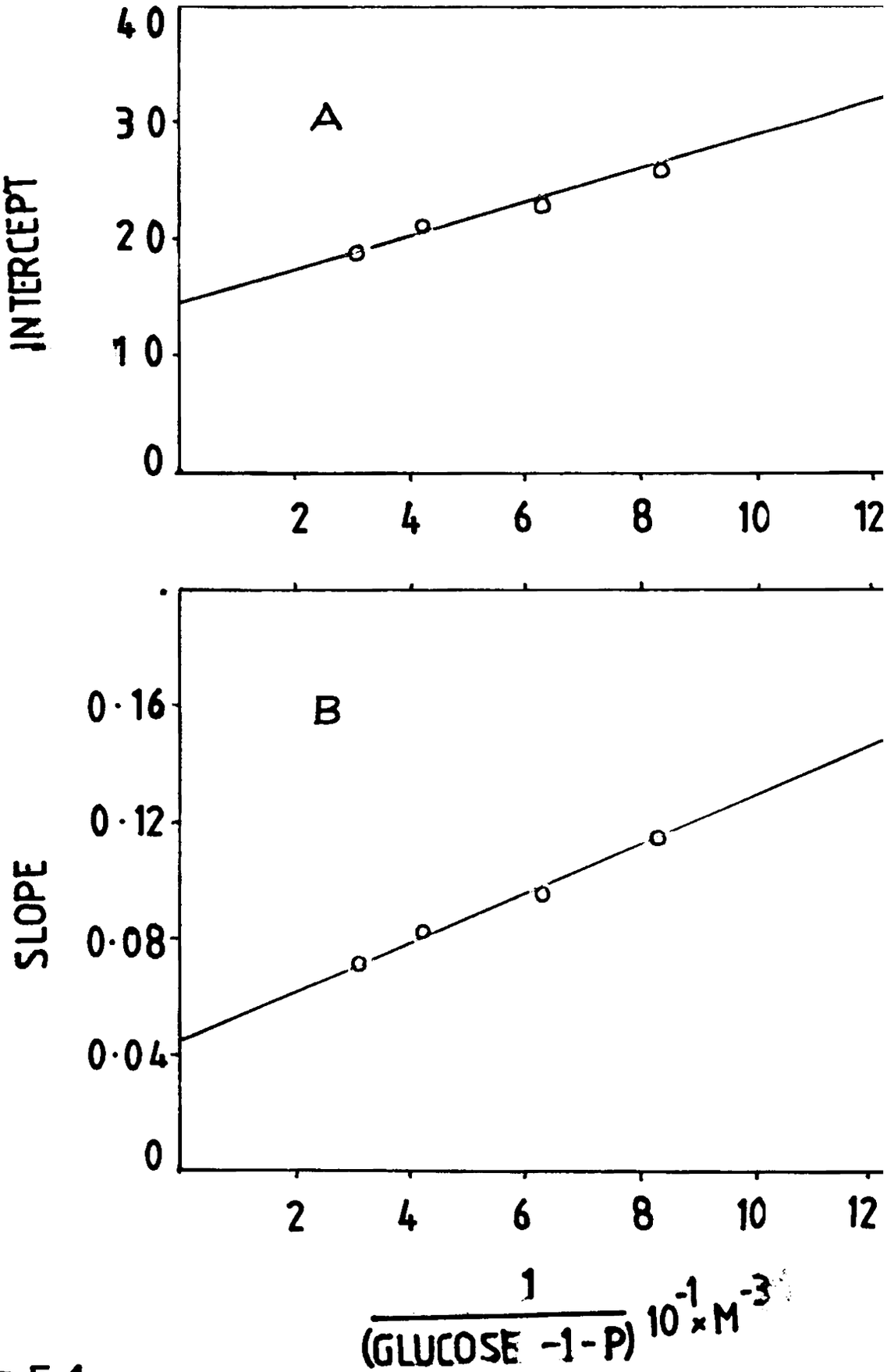
A
INTERCEPT
40
30
20
10
0
2
4
6
8
10
12
B
SLOPE
0·16
0·12
0·08
0·04
0
2
4
6
8
10
12
1
(GLUCOSE -1-P)
10^-1 x M^-3

Fig. 5.4

TABLE 5-2

KINETIC CONSTANTS FOR VILLORITTA CYPRENOIDES PHOSPHORYLASE b

K_5 (mM glucose-1-P)	3.7
K_6 (mM glycogen)	2.4
K_7 (mM glycogen)	9.7
K_8 (mM glucose-1-P)	18.35

the value of k_2 cannot be compared to that of other completely purified animal phosphorylases. However the values K_5, K_6, K_7 and K_8 are not affected by the purification as far as there is no other enzymes, which act on the substrate. These kinetic constants were found to be almost the same as that of the fish enzyme C. guttattum and less than that of Sunetta phosphorylase except the value of K_8.

The apparent K_m values for glycogen and glucose-1-P at different levels of each other obtained from figs. 5-1 and 5-2 are given in table 5-3.

In the case of Villorita the heterotropic effect observed between glucose-1-P and glycogen are similar to that observed for other animal (except Etroplus)

TABLE 5-3

APPARENT K_m VALUES FOR GLUCOSE-1-P AND GLYCOGEN AT DIFFERENT CONCENTRATIONS OF EACH OTHER FOR PHOSPHORYLASE b FROM VILLORITTA CYPRENOIDES.

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

Glycogen (mM)	K_m for glucose-1-P (mM)	Glucose-1-P (mM)	K_m for glycogen (mM)
61.5	9.8	32	3.8
24.6	11.0	24	3.9
6.15	12.7	16	4.1
2.46	14.7	12	4.3
		8	4.6

phosphorylases. For Villorita phosphorylase as the concentration of glycogen (glucose-1-P) increases, the affinity of the enzyme for glucose-1-P (glycogen) also increases and vice versa. Here the apparent K_m values for glycogen is only half of that of Sunetta enzyme and K_m values for glucose-1-P is also slightly less than the latter enzyme. Thus though the enzyme

activity is very low in Villorita, the affinity of the enzyme for the substrate is higher for the Villorita enzyme.

Activation by AMP

The reciprocal plots for glucose-1-P and AMP at different levels of each other are shown in figs. 5-5 and 5-6. 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 converged to a point almost on the horizontal axis. This suggests that the AMP does not have any effect on the enzyme affinity to glucose-1-P above 0.025 mM AMP. This result is different from the results obtained for other animal phosphorylases, where AMP increases the affinity of enzyme for glucose-1-P or AMP decreases the affinity of the enzyme for glucose-1-P as in the case of Etroplus phosphorylase under the same concentrations of AMP. Hence in this case it is shown that from 0.025 mM concentration onwards, AMP doesn't have any heterotropic effect on the glucose-1-P binding sites.

The reciprocal plots for AMP were curved showing negative cooperativity between AMP binding sites.

Fig. 5-5

Reciprocal plots for glucose-1-P at various levels of AMP for Villorita phosphorylase at 30°C and pH 6.8. The assay mixtures had, 15 mM cysteine/20 mM sodium β-glycerophosphate, 345 μg/ml protein, 1% glycogen, glucose-1-P and AMP as indicated. The concentrations of AMP were ○, $1x10^{-3}$ M; ●, $1x10^{-4}$ M; □, $5x10^{-5}$ M and △, $2.5x10^{-5}$ M.

Fig. 5-6

Reciprocal plots for AMP at different levels of glucose-1-P for phosphorylase from Villorita cyprenoides. The concentrations of glucose-1-P were ●, 32 mM; □, 24 mM; ▣, 16 mM; ○, 12 mM and △, 8 mM. Other conditions were same as in fig. 5-5.

Similar negative cooperativity has been found for Sepia (25) and M. dobsoni phosphorylases (Chapter 3). Hence the control mechanism of the Villorita phosphorylase must be different, since it doesn't alter the K_m for glucose-1-P and negative homotropic cooperativity exists between AMP sites.

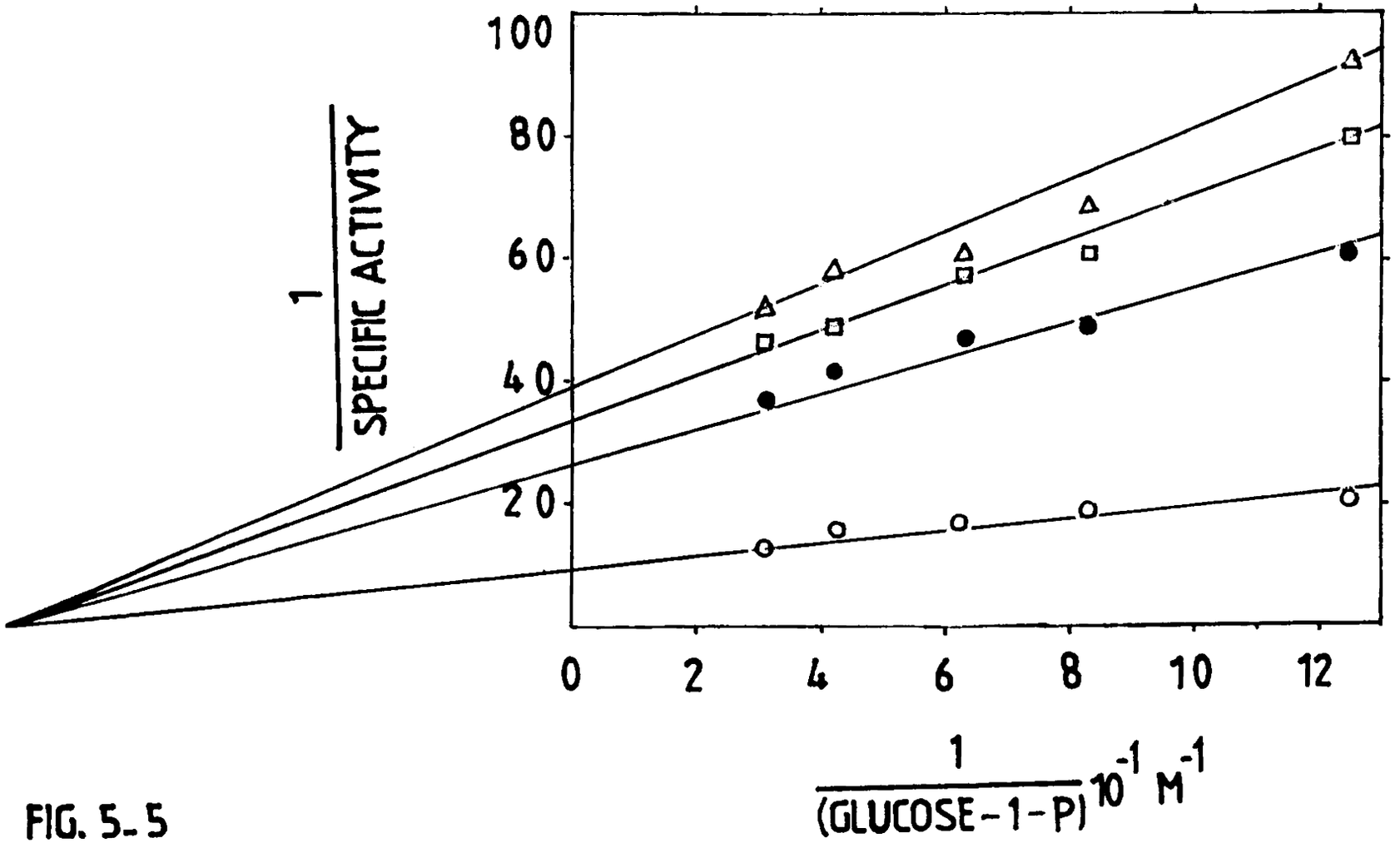
100
80
60
40
20
0
2
4
6
8
10
12
1 / SPECIFIC ACTIVITY
1 / (GLUCOSE-1-P) 10^-1 M^-1

FIG. 5_5

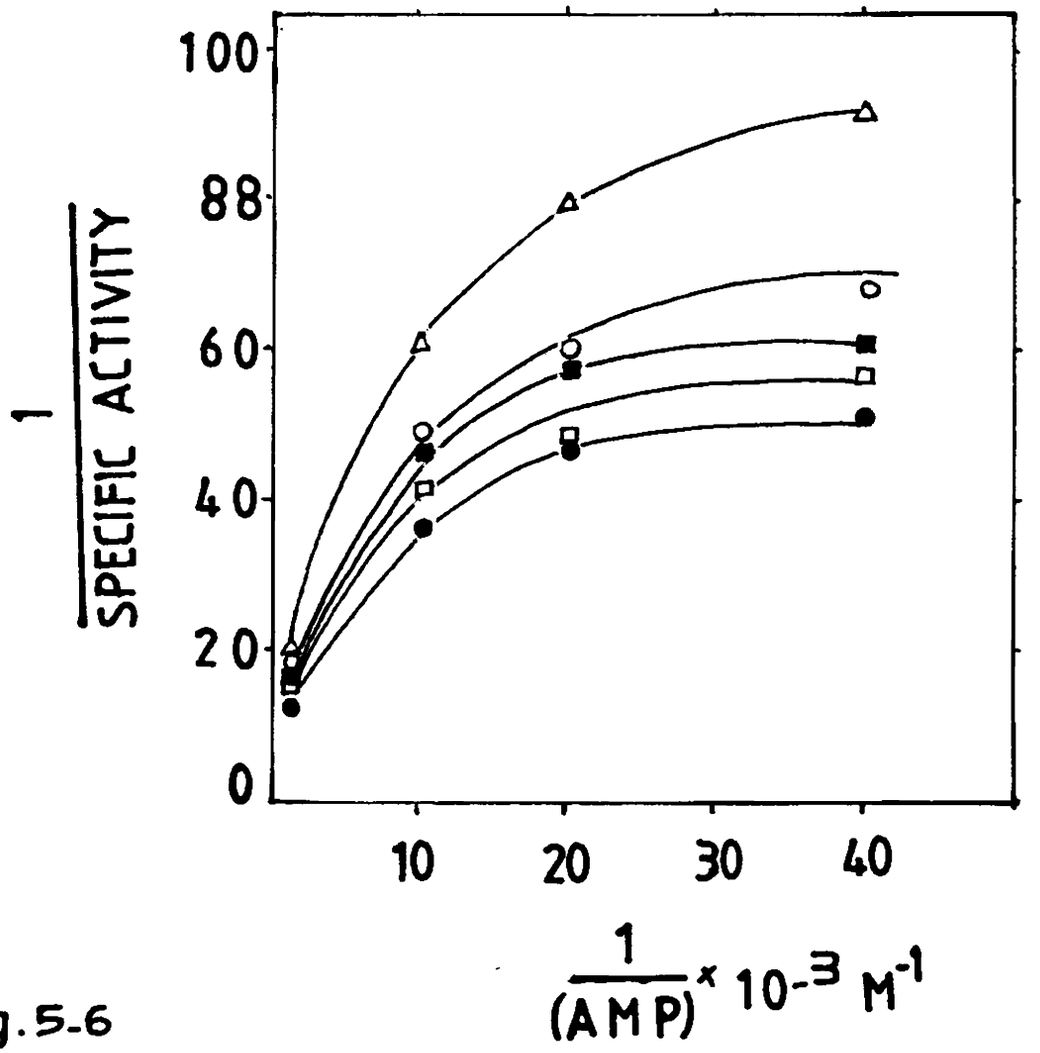

Fig. 5-6

Inhibition by metabolites

The metabolites, glucose, glucose-6-P and ATP are found to be inhibitors of glycogen phosphorylase b (25,118,119), whereas the a form is inhibited by these ligands negligibly in the presence of 1 mM AMP and 10-15 mM concentrations of them (7). The Sepia phosphorylase is not inhibited by them in the presence of AMP whereas in the absence of AMP, competitive inhibition was observed with respect to glucose-1-P (25). Competitive inhibition was observed by these ligands in the fish (Cibium and Etroplus) phosphorylases also. Sunetta scripta phosphorylase also is inhibited competitively by these ligands (Chapter 4).

The reciprocal plots for glucose-1-P in the presence and absence of these inhibitors are shown in fig. 5-7. In the Villorita cyprenoides phosphorylase also the inhibition by these ligands was competitive with respect to glucose-1-P. Like in the case of Sunetta, Sepia and M. dobsoni glucose is a less potent inhibitor than glucose-6-P and ATP ie., it competes less with the glucose-1-P for the substrate binding site than the glucose-6-P or ATP. In the case of the fish enzymes, glucose

Fig. 5-7

Reciprocal plots for glucose-1-P for Villorita phosphorylase in the presence and absence of glucose, glucose-6-P and ATP. The assay was conducted at 30°C in the presence of 15 mM cysteine/20 mM sodium β-glycerophosphate pH 6.8 in the presence of 1% glycogen, 1 mM AMP and glucose-1-P. The assay mixture contained 312 µg/ml protein. ○, control; ●, 10 mM glucose; □, 10 mM ATP and △, 10 mM glucose-6-P.

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competes with glucose-1-P more for the substrate binding site than glucose-6-P and ATP. Hence, generally saying, for the invertebrate enzyme, glucose-6-P is a more potent competitive inhibitor than glucose.

The reciprocal plots for AMP in the presence of these metabolites are also peculiar for Villorita phosphorylase as shown in fig. 5-8. In the presence and absence of these inhibitors, negative homotropic cooperativity was seen between AMP sites. Hence in this case also the Villorita phosphorylase is different from all other enzymes studied.

The following conclusions were arrived at from

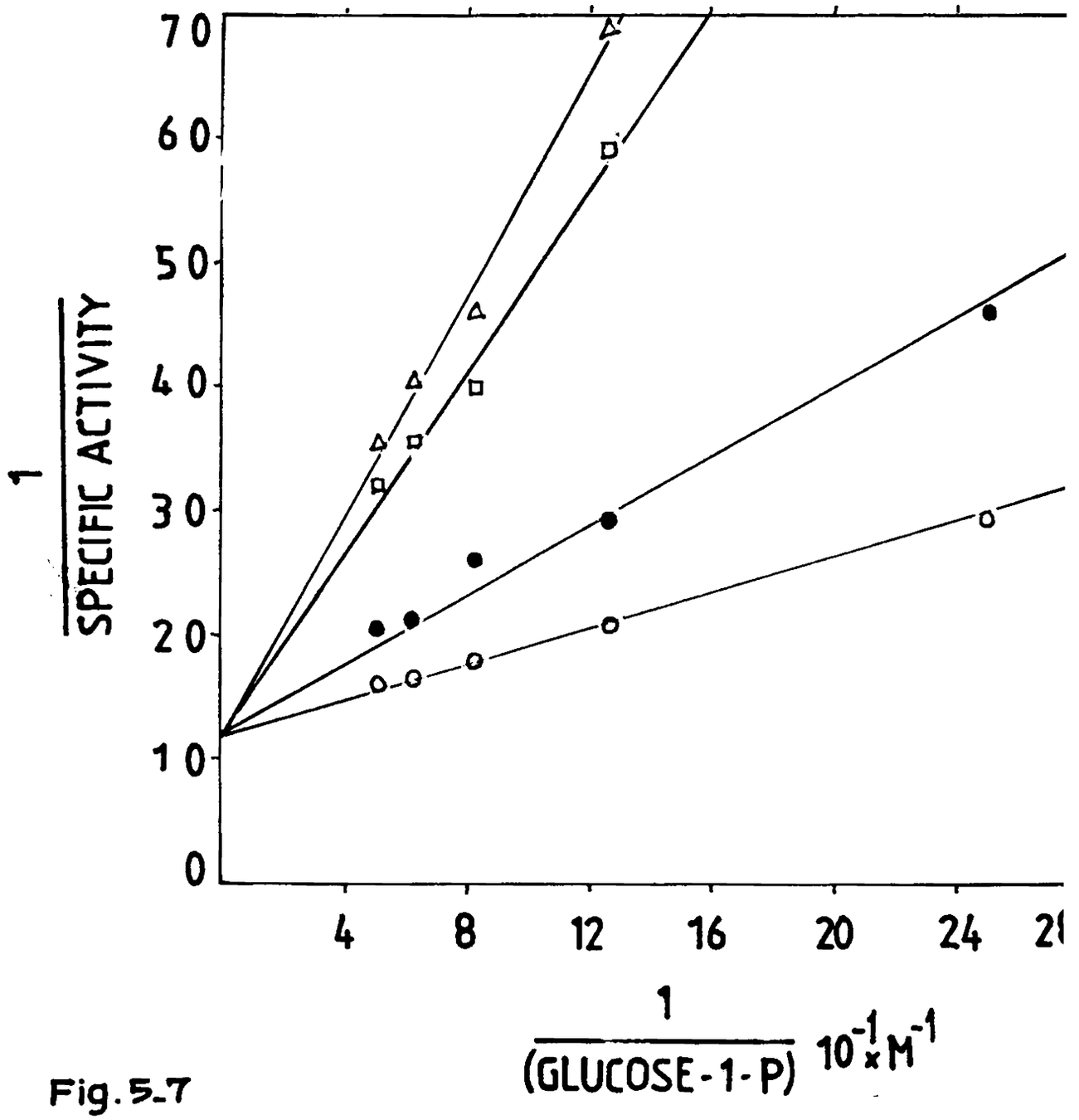
70
60
50
40
30
20
10
0
4
8
12
16
20
24
1
SPECIFIC ACTIVITY
1
(GLUCOSE-1-P)
$10^{-1} \times M^{-1}$

Fig. 5-7

Fig. 5-8

Reciprocal plots for AMP in the presence and absence of glucose, glucose-6-P and ATP for Villorita phosphorylase. The assay mixture had 1% glycogen, 16 mM glucose-1-P, AMP and 260 µg/ml protein. Other conditions were same as in fig. 5-7. ○, control; □, 10 mM glucose; ●, 10 mM ATP and △, 10 mM glucose-6-P.

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the results presented in this chapter with respect to other well studied animal phosphorylases and the E. suratensis phosphorylase.

i) Sulphate ions activate the Villorita phosphorylase like the lamprey, human leukocyte, Etroplus and Sunetta phosphorylases.

ii) It is resistant to low concentration (50 mM) $MgCl_2$.

iii) Positive heterotropic cooperativity exists between glucose-1-P and glycogen sites like all other animal phosphorylases and unlike E. suratensis phosphorylase.

iv) No heterotropic cooperativity is seen between AMP and glucose-1-P sites ie., K_m for

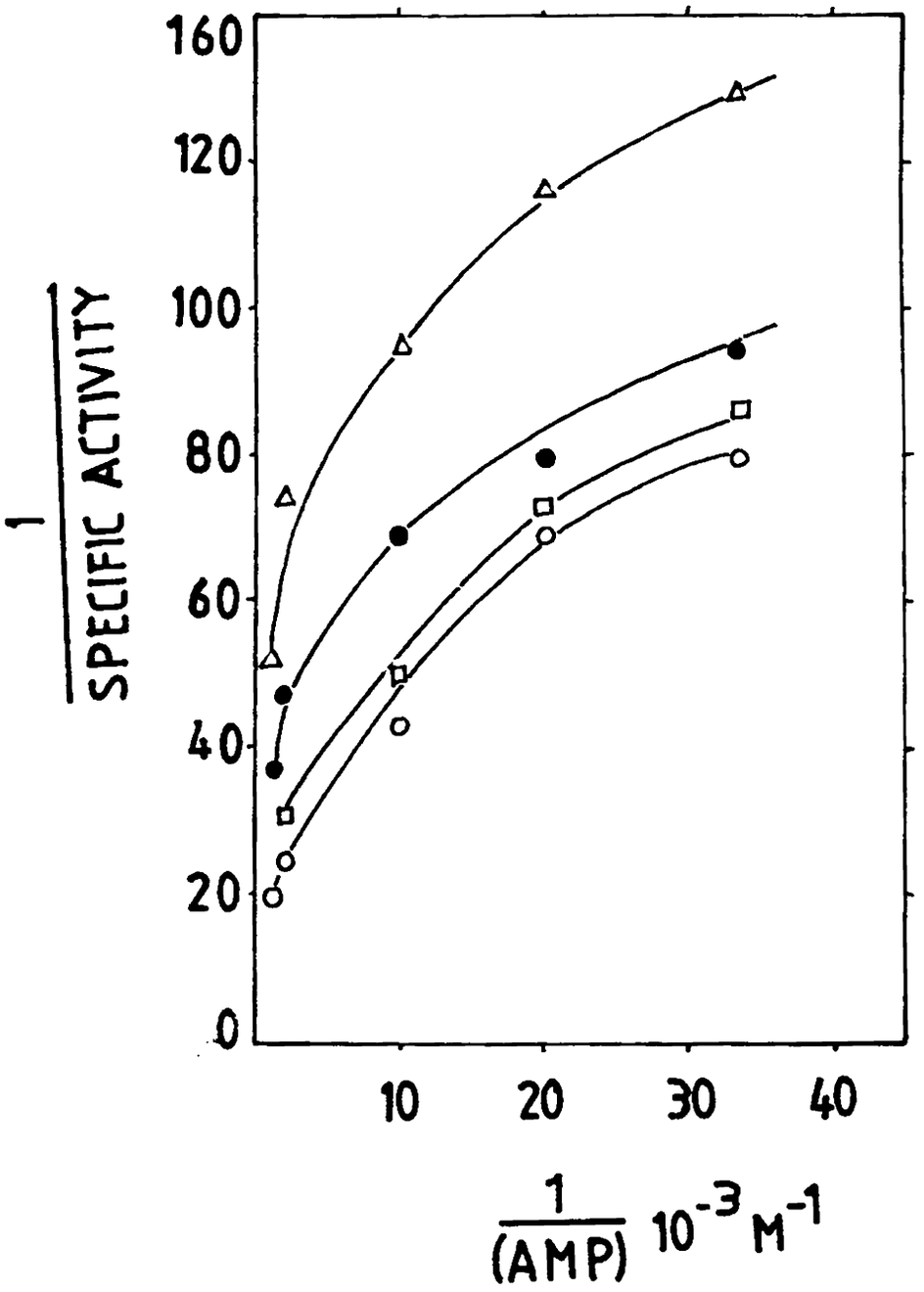
160
120
100
80
60
40
20
0
1
SPECIFIC ACTIVITY
10
20
30
40
1
(AMP)
10-3 M-1

Fig. 5.8

glucose-1-P at different concentrations of AMP is the same.

v) Negative cooperativity exists between the AMP sites like the Sepia and M. dobsoni enzymes, whereas positive cooperativity is found in all other animal phosphorylases, except Etroplus phosphorylase.

vi) In the presence of inhibitors, the homotropic cooperativity between AMP sites is negative which is different from all other animal phosphorylases and fish phosphorylases.

vii) Similar to Sunetta, Sepia and M. dobsoni phosphorylases the inhibition by glucose is comparatively less than by glucose-6-P and ATP, the opposite of which is seen in the fish enzymes.

CHAPTER 6

COMPARISON OF THE PROPERTIES OF PHOSPHORYLASES FROM ETROPLUS SURATENSIS, METAPENAEUS DOBSONI, SUNETTA SCRIPTA AND VILLORITA CYPRENOIDES

The above four species of animals are found in the estuary near Cochin. Among them *E. suratensis* is a fish (vertebrate), *M. dobsoni*, a crustacean and the other two belong to mollusca (bivalves). All the four can be called benthic animals since all are bottom feeders. The fish and the bivalve *Villorita cyprenoides* can tolerate fresh water as well as slightly saline water, but not sea water, whereas *M. dobsoni* can survive well in brackish water and sea water, but not in fresh water. *Sunetta scripta* thrives well in sea water and brackish water.

The foot muscle of the bivalves is the least energy requiring among the muscles of the four animals since the foot muscle is used only occassionally to change position of the animal. While *M. dobsoni* and *E. suratensis* are found at the bottom as well as the surface of the estuary, their muscle tissues require considerably higher constant energy supply than the foot muscles of the clams. *Etroplus* is a vertebrate

(fish) whereas the other three are invertebrates.

The comparison of general properties of these four species are summarised in table 6-1. The activity per gram tissue extracted under same conditions appears to vary according to the energy requirement of the muscle. Among them M. dobsoni and E. suratensis muscles have higher activity (10 and 9 units per g. respectively). The bivalves have very low value, Sunetta having 4 units/g and Villorita having 2 units/g. The activity content per gram tissue thus appears to reflect the relative energy requirement of the animal muscle.

The specific activity of the extract also varies from species to species. E. suratensis and M. dobsoni extracts have the same specific activity whereas the clams (Sunetta and Villorita foot muscle extracts) have much lower specific activities, the least being found for Villorita extract. Thus the lowest specific activity and activity per gram tissue for glycogen phosphorylase were found for Villorita. This suggests that Villorita is the least energy utilising animal among the four species.

The activity ratio of the extracts of Etroplus, Sunetta and Villorita was 0.5 whereas that of M. dobsoni

TABLE 6-1

Comparison of general properties of phosphorylases from *E. suratensis*, *M. dobsoni* *V. cyprenoides* and *S. scripta*.

Property	*Etroplus suratensis*	*Metapenaeus dobsoni*	*Sunetta scripta*	*Villorita*	Remarks
1. Habitat	Estuarine	Estuarine	Marine	Estuarine	
2. Class	Vertebrate (Fish)	Invertebrate (Prawn)	Invertebrate (clam)	Invertebrate (clam)	
3. Activity/g wet tissue (Units)	9	10	4	2	
4. Specific activity	0.5	0.5	0.3	0.05	
5. Activity ratio of the extract	0.5	0.9	0.5	0.5	
6.*Activity ratio of the purified enzyme	0.0 (*b* form)	0.7 (*a* form)	0.3	0.23	
7. Convertibility of the crystalline rabbit muscle *b* *a* in the presence of Mg^{+2} and ATP by the extract	Yes	Yes	Yes	Yes	Phosphorylase kinase is present in all.

* Only Etroplus phosphorylase was purified to homogenity.

(Contd..)

TABLE 6-1 (Continuation)

Property	E. suratensis	M. dobsoni	S. scripta	V. cyprenoides	Ramraks
8. Interconvertibility of a and b forms in the extract					
i) Conversion to the a form	Yes	Yes	Yes	Yes	
ii) *Conversion to the b form	Yes	No	Yes	Yes	
9. Optimum pH	6.8	6.8	6.8	6.8	
10. Activation by cysteine	Yes	No	No	No	
11. Stability of the purified enzyme	Stable	Unstable	Unstable	Unstable	Invertebrate phosphorylases are unstable.
12. Activation by Ammonium sulphate (0.1 M) and Sodium sulphate (0.1 M)	32%	0%	50%	12%	
13. Effect on 50 mM $MgCl_2$	No effect	50% Inhibition	33% Inhibition	No effect	
14. Activation or Inhibition by 0.1 M NaCl & KCl	No effect	No effect	No effect	No effect	

In the case of M. dobsoni, phosphatase is present apparently in a latent form in the extract and for Sunetta and Villorita conversion takes place upto activity ratios of 0.23 and 0.3 respectively.

was 0.9. This indicates that in the fish and the bivalves both a and b forms of phosphorylase are present whereas in the case of the crustacean, phosphorylase is present as its a form. This may be due to the presence of phosphorylase phosphatase in a latent form in M. dobsoni.

The activity ratio of the purified enzyme was different for each species. E. suratensis phosphorylase b was purified and the b form was not active in the absence of AMP. M. dobsoni phosphorylase was purified as its a form having an activity ratio of 0.7. Sunetta and Villorita phosphorylases come in between these two, having activity ratios 0.23 and 0.3 respectively. Purified human leukocyte phosphorylase b was similarly found to have an activity ratio of 0.2 (90). In the case of rabbit muscle phosphorylase, hybrid forms of phosphorylase a and b have been isolated in which the phosphorylase was partially phosphorylated (7). Hence in the case of the bivalve phosphorylases also, the enzyme may be existing as a hybrid variety of a and b forms. Work in this direction requires the use of ^{32}P labelled ATP. We could not undertake the work in this laboratory.

The presence of phosphorylase kinase was detected in the extract of all the four species of animals described in this thesis. Rabbit muscle phosphorylase b was converted to the a form by the extract of all the four species. Similarly phosphorylase phosphatase was also detected in all the species. However in M. dobsoni it exists in an inactive (latent) form like in some other animals (78). Etroplus phosphorylase a can be converted to the b form which is inactive in the absence of AMP. However, Sunetta and Villorita phosphorylases could be converted to forms having activity ratios 0.23 and 0.3 respectively by the respective phosphatases. Thus all the four species are qualitatively similar in that the interconversion between the a and b forms exists in all of them like in other animals, by phosphorylase kinase and phosphorylase phosphatase.

For all the phosphorylases, a pH optimum of 6.8 has been found in 15 mM cysteine/20 mM sodium β-glycerophosphate buffer. In the case of the Etroplus phosphorylase, some activation was found, (about 20%) in the presence of cysteine whereas for the other three phosphorylases, no such activation by cysteine was found.

The stability of the enzyme also differs in each case. It was found that the Etroplus phosphorylase was stable for at least 2 weeks after purification, whereas the other three phosphorylases were highly unstable, the most unstable being the M. dobsoni phosphorylase. Due to their high unstability, they could not even be subjected to dialysis at 0-5 °C after the ammonium sulphate fractionation. The unstability (loss of activity) was more pronounced after each step of purification. This property seems related to the evolutionary status of the animals: M. dobsoni (lowest in status among the four) enzyme is the most unstable and the fish (vertebrate) enzyme comparatively very stable. The molluscan (status in between the above two) phosphorylases are unstable but more stable than M. dobsoni enzyme.

Rabbit
↑
Fish (vertebrate)
↑
bivalves (mollusc)
↑
M. dobsoni (crustacean)

The effect of various ions on phosphorylase was different for each species. Ammonium sulphate and sodium sulphate had an activating effect on Sunetta,

Villorita and Etroplus phosphorylases, whereas the M. dobsoni phosphorylase activity was not affected by 0.1 M of both the salts.

The kinetic and allosteric properties of the four animal muscle phosphorylases are summarised in table 6-2. The kinetic data obtained for all the four animal phosphorylases were consistent with rapid equilibrium random bi bi kinetic mechansim. However differences in many other kinetic properties could be noticed. M. dobsoni and Villorita phosphorylases showed negative homotropic cooperativity between AMP sites whereas for Etroplus and Sunetta no such homotropic cooperativity was found.

Except for Etroplus suratensis phosphorylase, the other three phosphorylases showed positive heterotropic cooperativity between glycogen (glucose-1-P) and glucose-1-P (glycogen) sites. Only Etroplus phosphorylase showed negative cooperativity between AMP and glucose-1-P sites. While M. dobsoni and S. scripta phosphorylases showed positive cooperativity between AMP and glucose-1-P sites Villorita did not show any. The apparent K_m values are also different for each species. The apparent K_m values for glucose-1-P at different levels of glycogen

TABLE-6-2

Comparison of Kinetic and allosteric properties of phosphorylases from *E. suratensis*, *M. dobsoni* *V. cyprenoides* and *S. scripta*

Property	*E. suratensis*	*M. dobsoni*	*S. scripta*	*V. cyprenoides*
1. Kinetic mechanism	Rapid equilibrium random	Rapid equilirbium random	Rapid equilibrium random	Rapid equilibrium random
2. Homotropic cooperativity between				
i) glucose-1-P sites	Nil	Nil (Substrate inhibition)	Nil	Nil
ii) glycogen sites	Nil	Nil (Substrate inhibition)	Nil	Nil
iii) AMP sites	Nil	Negative	Nil	Negative
3. Heterotropic cooperativity between				
i) glycogen and glucose-1-P sites and *vice versa*	Negative	Positive	Positive	Positive
ii) AMP and glucose-1-P sites	Negative	Positive	Positive	Nil

(Contd..)

TABLE 6-2 (Continuation)

Property	E. suratensis	M. dobsoni	S. scripta	V. cyprenoides
4. Inhibition by metabolites				
i) Nature of inhibition with respect to glucose-1-P by				
a) glucose	Competitive	Mixed	Competitive	Competitive
b) glucose-6-P	Competitive	Mixed (in the absence of AMP) & negative homotropic cooperativity in the presence of 1 mM AMP	Competitive	Competitive
c) ATP	...	Mixed	Competitive	Competitive
5. Nature of Inhibition with respect to AMP by				
a) glucose	Mixed	Mixed	Mixed	Negative homotropic cooperativity
b) glucose-6-P	Positive heterotropic cooperativity	Negative homotropic cooperativity	Mixed	Negative homotropic cooperativity
c) ATP	....	Mixed	Mixed	Negative homotropic cooperativity.

are 2-3 times higher for the Villorita and Sunetta phosphorylases than that of M. dobsoni phosphorylase in the presence of AMP. Also the apparent K_m values for glucose-1-P is slightly higher for Sunetta phosphorylase than that for Villorita phosphorylase. The apparent K_m values for glycogen for M. dobsoni and Villorita phosphorylases are almost the same whereas that for Sunetta phosphorylase is 2 times higher than them. However, for Etroplus phosphorylase the K_m values for glycogen is very less when compared to these values, whereas K_m values for glucose-1-P come almost in the same range. Thus quantitatively all the four phosphorylases are different. The kinetic constants are also different for each of them.

Inhibition kinetics also showed differences between each species. The inhibition with respect to glucose-1-P by the metabolites glucose and glucose-6-P were competitive in the case of Villorita, Sunetta and Etroplus phosphorylases whereas M. dobsoni phosphorylase showed mixed type of inhibition with respect to glucose-1-P. In the presence of 1 mM AMP the inhibition by glucose-6-P was different for M. dobsoni phosphorylase a giving rise to a hyperbolic curve. Inhibition by ATP with respect to glucose-1-P

was competitive for Villorita and Sunetta phosphorylases, whereas mixed type of inhibition was seen for M. dobsoni phosphorylase.

The Etroplus phosphorylase is different from the other three phosphorylases in the extent of inhibition by glucose and the other two metabolites. While glucose is the most potent inhibitor among the three for Etroplus phosphorylase, for the other three invertebrate phosphorylases glucose-6-P was found to have more inhibitory effect than glucose. Thus there is definite differences in structure around the binding site/active site between the fish phosphorylase and the other three invertebrate phosphorylases.

In the case of inhibition with respect to AMP for Villorita and M. dobsoni enzymes, negative homotropic cooperativity was seen for the AMP sites in the presence and absence of glucose-6-P. For Etroplus phosphorylase glucose-6-P gave an upwardly curved plot whereas for Sunetta phosphorylase, glucose-6-P showed mixed type of inhibition. Inhibition by glucose and ATP were mixed for Sunetta, Villorita and Etroplus (by glucose alone), and for M. dobsoni negative homotropic cooperativity was seen also in

the presence of them.

The following conclusions may be arrived at by comparing the four benthic muscle phosphorylases.

i) The specific activity and activity/g tissue vary according to the energy requirement of the animal. Here, the least energy-utilising animals, (the bivalves), have the lowest activity/g tissue and specific activity whereas the highest value is seen for the M. dobsoni muscle and moreover, according to meet its higher energy requirement, the phosphorylase is present as the more efficient a form.

ii) The interconversion of phosphorylase between a and b forms takes place in all the four animals, though the phosphatase is latent in the M. dobsoni muscle, (may be due to its high energy utilising nature) and thus forms a major regulatory mechanism of glycogen degradation.

iii) The invertebrate phosphorylases are highly unstable compared to the fish phosphorylase apparently due to the low evolutionary status of the animals.

iv) Sulphate ion activates the phosphorylases, except M. dobsoni phosphorylase.

v) The kinetic mechanism is same as in the case of other animals, ie. rapid equilibrium random

vi) Except Etroplus phosphorylase the other three phosphorylases show positive heterotropic cooperativity between glycogen and glucose-1-P sites and vice versa. Only Etroplus phosphorylase shows negative heteroptropic cooperativity.

vii) The metabolites, glucose, glucose-6-P and ATP are inhibitors of the phosphorylases. Except for M. dobsoni phosphorylase a they are competitive inhibitors with respect to glucose-1-P. For M. dobsoni phosphorylase mixed type of inhibition is observed. But for this similarity, the nature of inhibition (ie., homotropic, heterotropic etc.) can not be brought into a general pattern.

Comparison of the four Benthic phosphorylases given in the thesis with other Animal muscle phosphorylases.

The phosphorylases described here are from four

benthic (aquatic) animals. The specific activity and activity per gram of the comparatively less energy requiring clams were found to be less than that of other animal muscle phosphorylases. The bivalve phosphorylases also showed constant activity ratio (0.23 and 0.3) in between the a and b forms like Sepia phosphorylase (25) and human leukocyte phosphorylase (90) (activity ratios 0.4 and 0.2 respectively). All other phosphorylases exist either as a or b form like the rabbit or fish enzymes (7,119).

The interconversion between the a and b forms takes place in all the muscle phosphorylases like in other animal phosphorylases. The kinases of all these animals convert rabbit phosphorylase b. The phosphorylase phosphatase is present as a latent (inactive) form in the M. dobsoni muscle like in some other animals (78). This may be due to its high energy requirement compared to other animals and hence the enzyme is present as its more efficient form. It is likely that a different control of activation of the phosphatase exists in this form.

Rabbit muscle phosphorylase and other well studied terrestrial animal muscle phosphorylases

are very stable (ie., can be preserved even in purified state without loss of activity for months). The fish phosphorylase from E. suratensis and Cibium (111) are fairly stable. The invertebrate enzyme (the three phosphorylases described here and Sepia (25)) are unstable. In the partially purified form the invertebrate phosphorylase could not be stored even for a day. This gradation in the stability (apparently related to the overall structure) parallels the evolutionary status.

Fish phosphorylases and Sunetta and Villorita phosphorylases show apparent competitive inhibition with glucose-1-P by glucose-6-P, glucose and ATP. This effect is different from that of the rabbit muscle phosphorylase. For M. dobsoni phosphorylase mixed type inhibition was found for glucose-1-P by these metabolites. For fish phosphorylases and other vertebrate enzymes glucose is a more potent inhibitor than the other two metabolites whereas for the invertebrate (Sepia, M. dobsoni, Sunetta and Villorita) phosphorylases glucose is comparatively less potent than the other two metabolites. Thus this property seems to be a characteristic difference between the vertebrate and

invertebrate muscle phosphorylases.

A combination of two factors appears to explain the differences viz. the evolutionary status of the animal and the energy requirement of the muscle.

C H A P T E R 7

EXPERIMENTAL

A. MATERIALS.

Glucose-1-P (dipotassium salt), glucose-6-P and AMP used for the work described in this thesis were the products of SISCO research laboratory, Bombay. Sodium β-glycerophosphate was obtained from Koch-Light (London). Oyster glycogen and bovine serum albumin were the products of Sigma chemical company, U.S.A and DEAE-cellulose and ATP were from V.P. Chest Institute, New Delhi. L-cysteine hydrochloride and mercaptoethanol were products of E. Merck, Germany. The sepharose-glycogen was a generous gift from Prof. Dieter Palm of the University of Wuerzburg, Germany. Folin - Ciocalteu reagent was from BDH. All other chemicals used were of analytical grade. Distilled water from an all glass assembly was used for the work.

B. REAGENTS.

(i) 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 distilled water. The reagent was stored in a brown bottle at 0-5 °C.

(ii) Stopping reagent for phosphorylase assay

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.

(iii) Preparation of DEAE-cellulose column

Coarse DEAE-cellulose was suspended in 1 N NaOH and washed with it repeatedly as recommended in Methods in Enzymology (130). This was washed with water and suspended in 1 N HCl and filtered immediately and washed with distilled water till free from the acid. It was again suspended in 1 N 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 1 N 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 equilibrated with the required buffer was filled in columns of required sizes and flow was adjusted by compressing the column with a glass rod and also by manipulating the pinch cork.

(iv) Preparation of sepharose-glycogen column

The sepharose-glycogen was filled in a column of the required size and washed with 0.2 M sodium chloride and equilibrated with the eluting buffer by passing about 200 ml of the buffer to flow through the column.

(v) Preparation of polyacrylamide gel

a) Buffer solution

6 g of Tris and 28.8 g of glycine dissolved in 100 ml of water (pH 8.6)

b) Stock solutions

i) Stock A (pH 9) : 38.3 g of Tris and 0.46 ml of N,N,N',N'-Tetramethylethylenediamine (TEMED) dissolved in 48 ml of 1 N HCl and made up to 200 ml.

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

iii) Stock C : 0.14 g of crystalline ammonium persulphate dissolved in 100 ml of water. This solution was preserved only for one week.

c) Preparation of the 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 glacial 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.

(vi) Preparation of anthrone-sulphuric acid reagent

500 mg anthrone and 10 g of thiourea were dissolved in 1 litre of 72% sulphuric acid and warmed to 80-90°C.

C. METHODS

1. Purification of glycogen phosphorylase b from Etroplus suratensis

Medium to large sized fish were sorted out, their skin removed, the sketetal muscle was separated

and used immediately or kept frozen at -20°C in a deep freezer.

The typical purification procedure given below was for 100 g of frozen muscle. Unless otherwise mentioned, all the purification steps were carried out at 2-5°C.

Step 1. Extraction

The frozen muscle was cut into small pieces and ground in a mincer. This was then immediately homogenised in an electric blendor for 30 seconds with 100 ml of 0.01 M sodium β-glycerophosphate buffer pH 7.0. After keeping in ice (external cooling) for 10 minutes with occassional stirring, the homogenate was centrifuged at 7000 rpm for 10 minutes and the supernatent collected. The residue was again mixed thoroughly with another 100 ml of buffer, kept for 10 minutes at 0°C and centrifuged. The supernatent obtained was pooled with the previous one.

Step 2. Ammonium sulphate fractionation

The pooled supernatent from step 1 was brought to 0.5 saturation by adding 180 ml of neutralised saturated ammonium sulphate solution

(saturated at room temperature) to 180 ml of the supernatent. After 30 minutes at 0°C, (in an 'ice-bath) the precipitate was collected by centrifugation at 8000 rpm for 20 minutes. It was dissolved in 3-5 ml of cold 0.01 M sodium β-glycerophosphate pH 7.0 containing 5 mM mercaptoethanol (buffer-A) and dialysed against two changes of 1 litre each of the same buffer.

Step 3. DEAE-cellulose chromatography

The dialysed enzyme solution was fed on a DEAE-cellulose column of size 16x1.5 cm equilibrated with buffer-A and washed with it till the eluate was devoid of protein. A concentration gradient was applied using 1 M sodium chloride dissolved in buffer-A in the top reservoir and 100 ml of the same buffer alone in the mixing flask. The solution in the mixing flask was occassionally stirred using a magnetic stirrer placed beside the flask. 5 ml fractions were collected at a flow rate of 0.8 ml/minute.

The fractions having specific activity above 5 units/mg were pooled and the protein precipitated with solid ammonium sulphate (60% saturation). After keeping for 30 minutes in ice-bath, the precipitate was collected by centrifugation,

dissolved in 1-2 ml of buffer-A and dialysed against two changes (1 litre each) of the same buffer.

Step 4. Affinity chromatography using sepharose-glycogen

The dialysed enzyme (1.6 ml) was applied on a column of sepharose-glycogen (22x1.5 cm) equilibrated with buffer-A and eluted using the same buffer. 5 ml fractions were collected at a flow rate of 0.4 ml/minute. Phosphorylase which was retarded in the column came out as a single peak (see results)

Step 5. Concentration and storage of the enzyme

The enzyme eluted out from the affinity column was applied on a 2x1 cm DEAE-cellulose column equilibrated with buffer-A. When the enzyme was completely adsorbed in the column, 0.5 M sodium β- glycerophosphate pH 6.8 was applied. The complete phosphorylase activity was obtained in 3-4 ml of the buffer. The enzyme was stored at 4°C in a refrigerator in the presence of 2.5 mM mercaptoethanol.

2. Purification of glycogen phosphorylase a from Metapenaeus dobsoni

Freshly caught prawns were collected and peeled. The intestine and head parts were also

removed along with the exoskeleton. The muscle was either used immediately or kept frozen at -20°C. The purification procedure described below was for 30 g muscle tissue.

Step 1. Extraction

The muscle was ground well using a pre-cooled mortar and pestle. 150 ml of 0.01 M sodium β-glycerophosphate pH 7.0 at 0°C was immediately added and mixed throughly. The mixture was then kept in an ice-bath for 10 minutes with occassional mixing. The homogenate was centrifuged at 7000 rpm for 20 minutes and the supernatent was collected.

Step 2. Ammonium sulphate fractionation

The supernatent was cooled to 0°C and saturated solution of ammonium sulphate neutralised with ammonia (saturated at room temperature) added to it in the ratio 45:55 with constant stirring. After keeping 30 minutes in an ice-bath, the solution was centrifuged at 10,000 rpm for 30 minutes. The residue was dissolved in 1-2 ml of 0.01 M sodium β-glycerophosphate buffer pH 7.0.

Step 3. Gel filtration

1 ml samples of the ammonium sulphate fraction were applied to a sephadex G-25 column of size (15x0.9 cm) at room temperature, equilibrated with 5 mM sodium β-glycerophosphate pH 7.0 containing 10 mM EDTA. The protein was eluted using the same buffer at a rate of 1 ml/minute and the first 3 ml just after the appearance of protein in the fraction was taken for further purification.

Step 4. DEAE-cellulose chromatography

The active salt-free samples collected were pooled together and fed on a DEAE-cellulose column of size (30x1.2 cm) equilibrated with 5 mM sodium β-glycerophosphate pH 7 containing 10 mM EDTA and washed with the same buffer till the eluate was free from protein. A concentration gradient was then applied using 1 M NaCl in buffer in the top reservoir and 100 ml of the above buffer in the mixing flask. The solution in the mixing flask was stirred occassionally using a magnetic stirrer, 3 ml fractions were collected at the rate of 0.5 ml per minute and checked for activity and protein. The fraction having maximum specific activity was directly used for further studies.

3. Purification of glycogen phosphorylase from Sunetta scripta

Live animals were chosen for taking the foot muscle. The shells were opened with the help of a knife and the foot muscle separated using scissors taking care not to cut the intestinal parts. The cut muscle was stored immediately in a deep freezer at -15 to -20°C or used immediately. For each batch of purification 20 g foot muscle tissue was used.

Step 1. Extraction

After cutting the muscle into small pieces, it was ground well in a pre-cooled mortar and pestle. 80 ml of cold 0.01 M sodium β-glycerophosphate pH 7 was added to it and mixed thoroughly. The homogenate was then kept at 0°C for 10 minutes with occassional stirring and centrifuged at 10,000 rpm for 20 minutes. The supernatent collected was kept cooled in ice-bath.

Step 2. Ammonium sulphate fractionation

To the cold supernatent obtained from step 1, neutralised saturated ammonium sulphate solution (saturated at room temperature) was added so that the saturation was 0.45 (to 55 ml of the extract 45 ml ammonium sulphate solution was added). The

solution was then kept in an ice-bath for 30 minutes and centrifuged at 10,000 rpm for 30 minutes. The supernatent was collected and again saturated solution of ammonium sulphate was added to it so that its saturation became 0.6 (to 100 ml of above solution 37.5 ml of saturated ammonium sulphate solution was added). The solution was again kept in an ice-bath for 30 minutes and centrifuged for 30 minutes at 10,000 rpm. The residue was dissolved in 1-2 ml of 0.01 M sodium β-glycerophosphate pH 7.0.

Step 3. Gel filtration

The enzyme fraction obtained by ammonium sulphate fraction was applied to sephadex G-25 column of size 15x0.9 cm in batches of 1 ml each and collected the fraction as in the case of M. dobsoni phosphorylase. The buffer used here was 0.01 M sodium β-glycerophosphate pH 7.

Step 4. DEAE-cellulose chromatography

The procedure was same as in the case of M. dobsoni phosphorylase. The buffer used was 0.01 M sodium β-glycerophosphate pH 7.

4. Purification of glycogen phosphorylase from Villorita cyprenoides

The foot muscle of Villorita was separated from live animals as in the case of Sunetta foot muscle and kept frozen at -15 to -20°C in a freezer or used immediately. For each batch of experiment 10 g muscle was used.

Step 1. Extraction

The muscle was cut into small pieces and ground well using a mortar and pestle. To the ground tissue 60 ml of cold 0.01 M sodium β-glycerophosphate pH 7, containing 1 mM EDTA was added and mixed well. The homogenate was then kept in the cold for 10 minutes with occassional mixing and centrifuged at 10,000 rpm for 20 minutes. The supernatent was taken and kept in an ice-bath.

Step 2. Ammonium sulphate fractionation

To the cold supernatent, neutralised saturated solution of ammonium sulphate (saturated at room temperature) was added slowly with continuous stirring so that the saturation became 0.4 (to 30 ml extract 20 ml ammonium sulphate solution was added). It was kept in an ice-bath

for 30 minutes and centrifuged at 10,000 rpm for another 30 minutes. The supernatent was collected and the saturation of ammonium sulphate was increased to 0.6 (to 50 ml of the supernatent, 25 ml ammonium sulphate solution was added). The solution was again kept in an ice-bath for 30 minutes and centrifuged at 10,000 rpm for 30 minutes. The residue was dissolved in 1-2 ml of 0.01 M sodium β-glycerophosphate pH 7.0.

Step 3. Gel filtration

The procedure was same as in the case of M. dobsoni and Sunetta phosphorylases. The buffer used was 0.01 M sodium β-glycerophosphate pH 7. The 3 ml fraction obtained just after the appearance of protein (in the eluate) was directly used for further studies.

5. Polyacrylamide gel electrophoresis

The polyacrylamide gel electrophoresis was performed according to the procedure of Ornstein and Davis (113). Electrophoresis was performed at 5-10°C for 2 hours in the presence of bromophenol blue as the tracking dye using a current of 5 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 20 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 2 N 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 40 mM and heated in a 60°C water bath for 5-10 minutes. The activity bands were developed as clear discs of calcium phosphate (62).

6. Estimation of protein

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 sulphate 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 (15 minutes at 3000 rpm) and washed 2-3 times with 5% TCA. The precipitate was dissolved in 0.1 N NaOH.

To 1 ml of the protein solution (containing 50-300 µg of protein) was added 5 ml of reagent (c), mixed well and allowed to stand at room temperature for 10 minutes. To this was added 0.5 ml of reagent (d) and mixed thoroughly. After 30 minutes, the optical density was measured at 500 nm in a spectrophotometer/ colorimeter. The instruments were calibrated using bovine serum albimin as standard.

7. Estimation of phosphorylase activity

The work presented in this thesis employed the procedure of Illingworth and Cori (5) for determining phosphorylase activity. The inorganic phosphate produced when assayed in the direction of glycogen synthesis was estimated according to the method of Fiske and Subbarow (133)

For routine assay, phosphorylase was properly

diluted in 30 mM cysteine/40 mM glycerophosphate pH 6.8 prior to assay. To 0.2 ml of substrate solution containing 2% glycogen, 32 mM glucose-1-P and 2 mM AMP at 30°C was added 0.2 ml of the diluted enzyme solution. It was incubated for a fixed time interval at 30°C. The reaction was arrested by the addition of 8.2 ml of stopping reagent (see section on 'Reagents'). To this was added 0.9 ml of 5 N H_2SO_4 followed by 0.5 ml of ANSA reagent. The blue colour developed was measured in a spectrocolorimeter at 660 nm. The optical density obtained was corrected for blank (the blank was prepared by a similar procedure, but the stopping reagent was added before the addition of enzyme) and converted to µmoles of inorganic phosphate using a calibration curve obtained with KH_2PO_4 under the same conditions (fig. 7-1).

The time of incubation of enzyme assay in various experiments were adjusted such that the optical density of the developed solution was well in the linear part of a reference curve obtained for the reaction. The reference curve was obtained by mixing an equal volume of enzyme and substrate and plotting a product vs. time curve (For this 2 ml of the enzyme was added to 2 ml of the substrate solution containing

Fig. 7-1

Calibration curve for determination of phosphate in phosphorylase assay. To 0.4 ml of KH_2PO_4 (having concentrations as indicated) was added to 8.2 ml of the stopping reagent, followed by the addition of 0.9 ml of 5 N H_2SO_4 and 0.5 ml of ANSA reagent. The optical density at 660 nm was measured after 20 minutes in a spectrocolorimeter.

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32 mM glucose-1-P, 2% glycogen and 2 mM AMP and incubated at 30°C. 0.4 ml aliquots were withdrawn at different time intervals and added to 8.2 ml of stopping reagent. The colour was developed and optical density measured as above)

8. Kinetic studies

For kinetic studies with glucose-1-P as the varying substrate, glycogen and AMP concentrations in the substrate solution were maintained constant (2% and 2 mM respectively) and glucose-1-P concentration varied from 16 mM to 64 mM. Similarly for kinetics with respect to AMP, glucose-1-P and glycogen concentrations of the substrates were 32 mM and 2% respectively and AMP concentration varied from

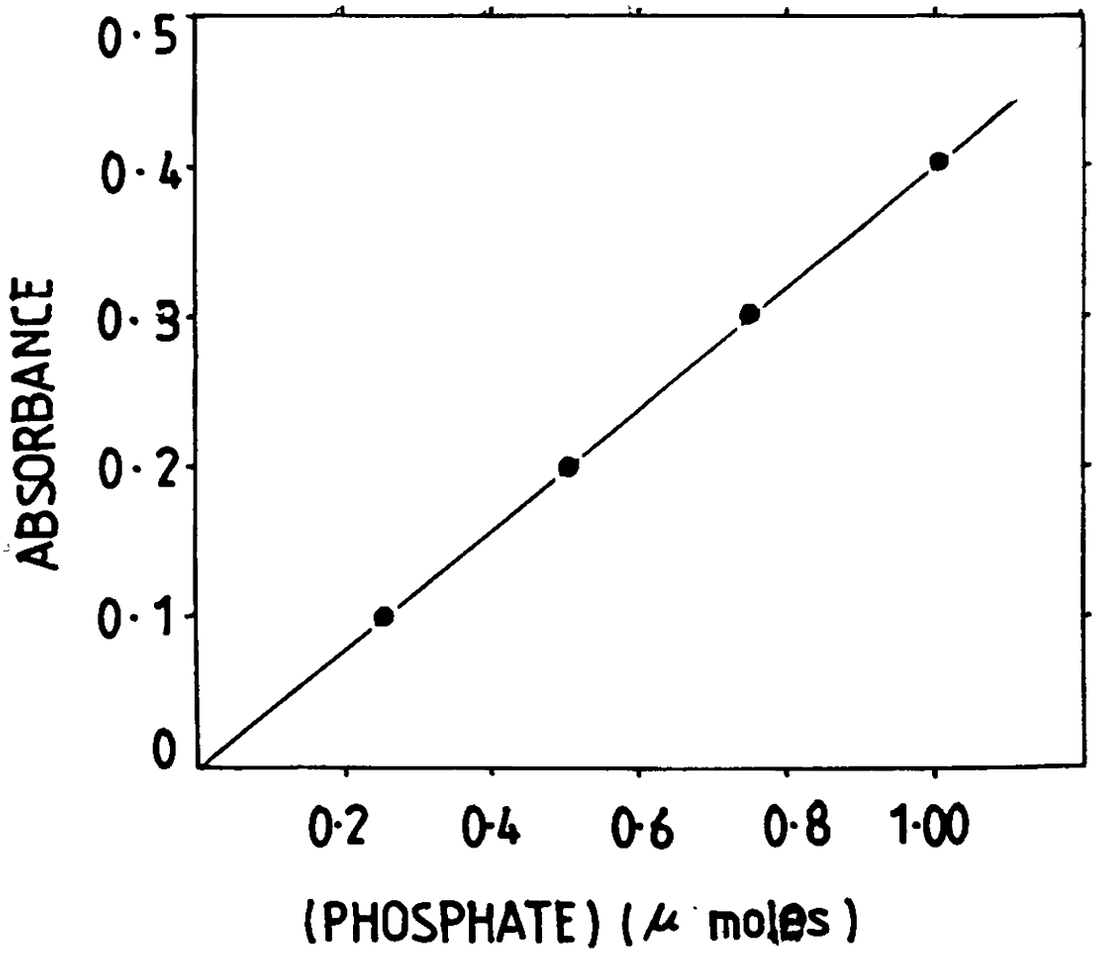

Fig. 7.1

$2x10^{-3}$ M to $5x10^{-5}$ M. The enzyme solution was properly diluted and added to an equal volume of the substrate solutions at 30°C and assayed as given in section 7. Separate blank was taken for each substrate concentration.

9. Inhibition studies

Stock solutions of the metabolites (0.1 M) were prepared in water the pH of which was adjusted to 6.8 with 0.1 N NaOH or with 0.1 N HCl as the case may be. This was then mixed with the diluted enzyme solution in 30 mM cysteine/40 mM glycerophosphate buffer pH 6.8 to the required concentrations. For the control, instead of the metabolite solution equal volume of distilled water was added. Activity was measured as given in section 7.

10. Estimation of glucosyl residues in glycogen

The glucosyl residues in glycogen were estimated using anthrone-sulphuric acid reagent.

To 5 ml each of the anthrone-sulphuric acid reagent kept in ice-bath was added 1 ml each of a standard solution containing 50-220 µg of glucose and 1 ml (0.02-0.05%) of glycogen to be estimated. These solutions were heated in a boiling water bath

for 16 minutes, cooled and the absorbance measured at 670 nm against a blank treated with 1 ml of water. The number of glucosyl residues were calculated using the calibration curve obtained with glucose as standard.

11. Preparation of rabbit muscle phosphorylase b

Phosphorylase b from rabbit muscle was prepared according to the procedure of Fischer and Krebs (132) with the substitution of mercaptoethanol for cysteine.

Step 1. Extraction

Medium to large sized rabbits were incapicitated by a single stroke on the back head and the blood was drained from the jugular veins. The muscles from the hind legs and back are excised and stored frozen. The procedure described below is for 100 g of frozen muscle. The frozen muscle was weighed and ground finely in an ordinary meat grinder at room temperature. The ground muscle was stirred well with 100 ml distilled water for 10-20 minutes at room temperature and filtered through two thickness of cheese cloth into a beaker cooled in ice. The muscle was again extracted with another 100 ml of water for a second time and finally using 50 ml water.

The combined extract was filtered through glass wool to remove fat particles.

Step 2. Acid precipitation of other enzymes

The pH of the cold extract was adjusted to 5.1-5.3 by careful addition of 1 N acetic acid. The mixture was immediately transferred to pre-cooled centrifuge bottles and centrifuged. The supernatent was filtered through large coarse fluted filter paper into a beaker cooled in ice and pH of the filtrate was immediately adjusted back to 6.8 by adding solid potassium bicarbonate.

Step 3. Ammonium sulphate precipitation

The solution was brought to 41% saturation with ammonium sulphate using saturated ammonium sulphate solution (saturated at room temperature; added 700 ml to 1 litre of the protein solution). The mixture was kept in a refrigerator overnight. Most of the supernatent was decanted and the residue collected by centrifugation. The residue was dissolved in 10 to 12 ml of water and dialysed against pre-cooled 10^{-3} M tris-HCl buffer pH 7.6, at 3-5 °C. The dialysis was performed for 15 hours with 2 changes of 500 ml each of the buffer.

Step 4. Heat treatment at higher pH

The following solutions were added to the dialysed protein solution: (1) 1:1 diluted (in water) mercaptoethanol to a final molarity of 3×10^{-2} M (2) neutral 0.1 M EDTA to a final molarity of 5×10^{-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 °C and pH readjusted to 7.0 by careful addition of 1 N acetic acid. The solution was centrifuged and the precipitate was discarded.

Step 5. Crystallization and recrystallization

To the solution were added 1/100 its volume each of 0.1 M AMP and 1 M magnesium acetate solutions and the mixture was kept in crushed ice. After 8-10 hours at 0 °C, the mixture was centrifuged to collect crystals of phosphorylase b. The crystals were dissolved at 30 °C in 2-5 ml of water containing 0.03 M mercaptoethanol and again AMP and magnesium acetate were added to final molarities of 10^{-3} M and 10^{-2} M respectively. On cooling, crystals separated which were collected by centrifugation.

Step 6. Gel filtration to remove mercaptoethanol and other small molecules

A small portion of the crystals of rabbit phosphorylase b thus prepared was dissolved in minimum amount of 0.01 M sodium β-glycerophosephate pH 7, layered on top of a sephadex G-25 column (15x0.9 cm) equilibrated with the same buffer just before use, and 2 ml fractions were collected after connecting the column to a reservoir containing the same buffer.

12. Partial purification of phosphorylase kinase from rabbit skeletal muscle

Phosphorylase b kinase was partially purified from rabbit skeletal muscle according to the procedure of Krebs and Fischer (120).

For taking the muscle, medium to large sized rabbits were incapicitated by a single stroke on the back head and the blood was drained from the jugular veins. The muscles from the hind legs and back were excised and packed in crushed ice. 100 g of the muscle was passed through a meat grinder previously cooled to -10°C and extracted with 200 ml

cold $2x10^{-3}$ M EDTA (pH 7) for 1 minute in an electric blendor. The extract was centrifuged for 10 minutes at 7000 rpm and the supernatent was filtered through glass wool into a beaker cooled in ice.

The pH of the extract was lowered to 6 by the addition of 1 N acetic acid. The precipitated protein was collected by centrifugation at 8000 rpm for 10 minutes. The residue was dissolved in 0.08 M sodium β-glycerophosphate and pH adjusted to 7 with careful addition of 0.5 N NaOH. The enzyme solution was stored at -20°C. This preparation was stable for 2-3 months. The enzyme solution was diluted in 15 mM cysteine buffer, pH 7 (500 to 600 times) before reaction with phosphorylase b.

13. Phosphorylase phosphatase and kinase reaction

The phosphorylase phosphatase reaction was carried out according to the procedure of Keller and Cori (133). For this, the muscle extract (at pH 6.5 and 7) was incubated at 30°C. Aliquots were removed from this, diluted in 30 mM cysteine/40 mM glycerophosphate buffer pH 6.8 and assayed for phosphorylase activity at 30°C in the absence and presence of 1 mM AMP.

The phosphorylase kinase reaction was carried out by the method of Krebs and Fischer (120). For this, to 1 ml of the extract (or 1 ml of rabbit phosphorylase b) at pH 7 containing 15 mM cysteine was added 1 ml of 0.125 M tris-glycerophosphate buffer (at pH 7, 7.5 or 8), (ii) 0.5 ml of $6x10^{-2}$ M Mg $(Ac)_2$ /$1.8x10^{-2}$ M ATP, pH 7 and (iii) 0.5 ml of phosphorylase kinase (40 fold diluted rabbit phosphorylase kinase in 15 mM cysteine buffer pH 7 or the muscle extract as the case may be). This was then incubated at 30°C, aliquots removed, diluted in 30 mM cysteine/40 mM glycerophosphate buffer pH 6.8 (so that the concentration of ATP in assay mixtures was below 0.5 mM). The activity ratio was determined.

SUMMARY

The major control of glycogen degradation at the enzyme level is by glycogen phosphorylase in animals. To study the regulation of glycogen degradation in different aquatic animals, four different species were selected. They are Etroplus suratensis, a brackish water fish, Metapenaeus dobsoni an estuarine cum marine prawn and two bivalves Sunetta scripta (marine) and Villorita cyprenoides (estuarine). Among them M. dobsoni occupies the lowest evolutionary status and the bivalves are in between the fish and M. dobsoni.

Phosphorylase activity per gram tissue and specific activity in the original extract were found to vary in the different animal tissue extracts in a definite manner consistent with the energy requirement of the tissues.

The stability of the enzymes (measured by activity loss) in the extracts and at different stages of purification were found to correlate well with the evolutionary status of the animal; ie, higher the animal's status, the more stable the enzyme.

Phosphorylase was purified either completely or partially from the muscle of each of these species and the kinetic properties were studied. The

presence of phosphorylase phosphatase and phosphorylase kinase were also tested in all these animals and also their action on crystalline rabbit muscle phosphorylase. From these it was found that both phosphorylase kinase and phosphatase were present in these animals like the other animals and hence the interconversion reaction plays an important role in the regulation of glycogen degradation in these animals also. Only the M. dobsoni muscle was found to have a latent form of phosphorylase phosphatase.

The specific activity and activity per gram of phosphorylase in the muscles of the different animals were different, the lowest being found for the bivalves. Activity ratios (activity in the presence of 1 mM AMP to that in its absence) were 0.23 and 0.3 for Sunetta and Villorita respectively.

Ammonium sulphate and sodium sulphate activated the Sunetta, Villorita and Etroplus phosphorylases. Heavy metals inhibit all the enzymes. Sodium chloride and potassium chloride had no activating or inhibiting effect. It was also found that except for Etroplus phosphorylase the other three phosphorylases were highly unstable, M. dobsoni

being the least stable.

The kinetic analyses gave data consistent with rapid equilibrium mechanism for all phosphorylases studied. In the case of glycogen/glucose-1-P kinetics, except Etroplus phosphorylase the other three phosphorylases showed positive heterotropic cooperativity between the two substrate sites whereas Etroplus phosphorylase showed negative heterotropic cooperativity. Quantitatively the three phosphorylases were again different. Kinetics with the activator AMP was different for different species. Villorita and M. dobsoni phosphorylases showed negative homotropic cooperativity between AMP sites, Etroplus phosphorylase showed negative heterotropic cooperativity between glucose-1-P and AMP sites and Sunetta phosphorylase showed positive heterotropic cooperativity between them.

The metabolites ATP, glucose-6-P and glucose inhibited all the four enzymes though the pattern of inhibition was different. For Sunetta, Villorita and Etroplus these metabolites were competitive to to glucose-1-P whereas for M. dobsoni phosphorylase a, in the absence of AMP, mixed type of inhibition was observed. In the presence of AMP, for M. dobsoni

phosphorylase a glucose-6-P induced negative homotropic cooperativity between glucose-1-P sites. Another difference observed was that while glucose was the most potent inhibitor (among the three metabolites mentioned) for the fish phosphorylase, glucose-6-P was found to be the most potent inhibitor for the invertebrate phosphorylases.

The properties of the four animal phosphorylases were compared with respect to each other and with other well-studied animal muscle phosphorylases. Thus the interconversion between the a and b forms is a general mechanism of regulation of glycogen degradation in these animals also. Moreover the allosteric regulation by the metabolites glucose, glucose-6-P and ATP and activator AMP is also involved in the control of degradation of glycogen. Comparison of the properties of the phosphorylases reveals that the properties of the enzyme and its control can be explained by two factors viz. the evolutionary status of the animals and the energy requirement of the muscle.

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