STUDIES ON THE ENZYME α - GLUCAN PHOSPHORYLASE

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BY

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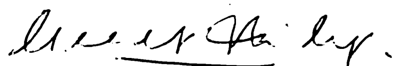
This is to certify that the thesis entitled 'Studies on the enzyme α-glucan phosphorylase' herewith submitted by Shri Thomas P. Thomas in partial fulfilment of the requirement for the Ph.D. degree in Biochemistry of the University of Cochin, is an authentic record of the work carried out by him under 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

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PREFACE

α-glucan phosphorylase is an important enzyme in glycolysis. It is the first enzyme known to exhibit allosteric properties and hence its inhibition and activation have significant effect on the rate of glycolysis. The thesis deals with a detailed study of the structure, inhibition and control of this enzyme from rabbit muscle and from a marine animal.

The thesis is divided into two parts. Part I deals with studies on rabbit muscle glycogen phosphorylase. After a review of the relevant literature (Chapter 1) the inhibition and chemical modification studies on rabbit enzyme are discussed in Chapters 2 to 5. Chapter 6 gives the methods used for the study.

The control mechanism of glycogen phosphorylase has been shown to be different in terrestrial and aquatic animals. Since detailed investigation has not been carried out on specialised tissues from marine invertebrates, phosphorylase was purified and its kinetic and control properties studied from a marine invertebrate. The mantle muscle of the mollusc *Sepia pharaonis* was selected for these studies. The mantle muscle of this species is a fast metabolising tissue like the insect flight muscle. Part II of the thesis presents the purification and

properties of phosphorylase from this species.

A summary of the results presented in part I and Part II are given at the end.

Based on the results reported in this thesis, the following papers have been published/are under publication.

1. LOCATION OF THE AROMATIC BINDING SITE AND PREPARATION OF AN AROMATIC DERIVATIVE OF GLYCOGEN PHOSPHORYLASE. Thomas,T.P.and Philip,G. Biochemica et Biophysica Acta (1980) Vol 613, pp 370-380.

2. INHIBITION OF RABBIT MUSCLE PHOSPHORYLASE BY AROMATIC COMPOUNDS. Thomas,T.P. and Philip,G. Indian Journal of Biochemistry & Biophysics (1980) Vol.17, pp 438-442.

3. PURIFICATION AND PROPERTIES OF GLYCOGEN PHOSPHORYLASE a FROM THE MANTLE TISSUE OF THE CUTTLE FISH SEPIA PHARAONIS. Thomas,T.P and Philip,G. (under publication).

4. CONTROL OF MANTLE MUSCLE GLYCOGEN PHOSPHORYLASE FROM THE CUTTLE FISH SEPIA PHARAONIS.

Thomas,T.P. and Philip,G. (under publication)

ABBREVIATIONS.

AMP	=	Adenosine-5'-monophosphate
ADP	=	Adenosine-5'-diphosphate.
ATP	=	Adenosine-5'-triphosphate.
DEAE-cellulose	=	Diethyl aminoethyl cellulose
DPE-	=	2,4-Dinitrophenylene-
DTNB	=	5,5'-Dithio bis-(2-nitro benzoic acid)
FDNB	=	1-Fluoro-2,4-dinitrobenzene.
F_2DNB	=	1,5-Difluoro-2,4-dinitrobenzene.
Glucose-1-P	=	Glucose-1-Phosphate.
Glucose-6-P	=	Glucose-6-Phosphate.
NEM	=	N-Ethyl maleimide.
PLP	=	Pyridoxal-5'-phosphate.
PCMB	=	p-Chloromercuribenzoate.
TCA	=	Trichloro acetic acid.
TEMED	=	N,N,N',N'-Tetramethylethylenediamine.
Tris	=	Tris (hydroxy methyl) amino methane.

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P A R T I

STUDIES ON RABBIT MUSCLE PHOSPHORYLASE

C H A P T E R 1

INTRODUCTION

α-GLUCAN PHOSPHORYLASE - A REVIEW

α-Glucan phosphorylase (1,4-α-D-glucan: orthophosphate α-D-glucosyl-transferase, EC 2.4.1.1) is a widely distributed enzyme that catalyses the intracellular degradation of α-glycosidic 1,4-linkages of polysaccharides such as glycogen and starch. Cori *et al.* (1) first demonstrated the reaction catalysed by α-glucan phosphorylase as

$$G_n + \text{orthophosphate} \rightleftharpoons G_{n-1} + \text{glucose-1-phosphate}$$

where G_n and G_{n-1} represent polysaccharide chains with n and n-1 glucose residues respectively. Since glucose-1-P is a stronger acid than inorganic phosphate, the equilibrium constant is highly pH dependent. At pH 6.8 the P_i/glucose-1-P ratio is 3.6(2). However the enzyme functions in the direction of glycogen degradation *in vivo* because the ratio of P_i/glucose-1-P greatly exceeds the equilibrium constant determined *in vitro* (3,4).

Glycogen phosphorylase can be assayed by following either the forward or the backward reaction. In the direction of glycogen degradation, the enzyme is assayed using a coupled enzyme assay system by which the liberated glucose-1-P is estimated (5). The activity is measured in the direction of glycogen synthesis using the substrates glucose-1-P and glycogen (6)

and estimating the liberated inorganic phosphate colorimetrically. The second method is usually employed because it is easier and less expensive. This method is used for the work presented in the thesis.

The most extensively studied α-glucan phosphorylase is that derived from rabbit skeletal muscle and is commonly referred to as glycogen phosphorylase. Crystallisation of the enzyme from this source was first reported in 1942. The enzyme was found to exist in two forms, phosphorylase a and b(7). Now it is well known that the a form is the phosphorylated form and is active without the presence of any nucleotide like AMP. The b form is the dephosphorylated form and is completely dependent on AMP *in vivo* for activity (7). Phosphorylase a is converted to the b form by phosphorylase phosphatase (phosphorylase phosphohydrolase EC 3.1.3.17) and the b form is converted back to the a form by phosphorylase b kinase (ATP: phosphorylase phosphotransferase, EC 2.7.1.38) which transfers a phosphate group from ATP to the protein. *In vivo*, the interconversion between the a and b forms of the enzyme plays a significant role in regulating glycogenolysis. The phosphorylase b kinase is activated by a hormone induced cascade system and by Ca^{++} ions and the phosphorylase a phosphatase is regulated by conformational changes in its substrate phosphorylase b induced by various ligands of phosphorylase (8,9).

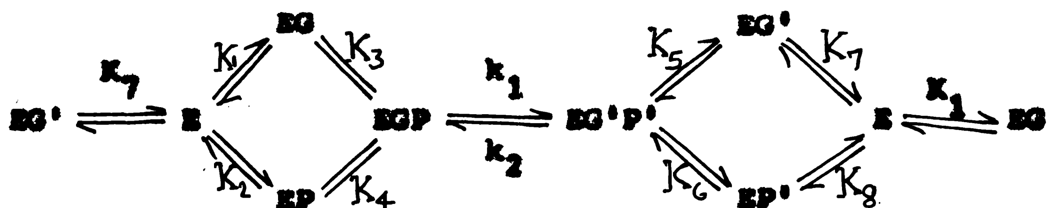
The phosphorylase *b* kinase (10,11) and phosphorylase *a* phosphatase (12-14) have been purified from muscle and liver and their properties studied in detail. The enzymes from the rabbit muscle have been found to interconvert all animal phosphorylases tested so far (15-20). In contrast to the animal phosphorylase, the yeast enzyme which also exists in the phosphorylated and non-phosphorylated form, does not serve as a substrate for the converting enzymes (21). The kinase and phosphatase from insects were found to act on rabbit muscle phosphorylase (22). Graves *et al.* have succeeded in the chemical synthesis of peptides containing 14 and 8 residues which can act as substrates for the kinase and phosphatase and also for non-specific protein kinase. (23,24). The non-specific catalysis of these enzymes is highly evident in the recent observation that acetyl CoA-carboxylase could be dephosphorylated by the phosphorylase phosphatase thereby activating it (25). The kinase reaction has been shown to be reversible and the reaction requires in addition to the phosphorylated substrates, Mg^{++}, ADP, Ca^{++} and glucose (26). The physiological importance of phosphorylase phosphatase has been questioned recently by the observation that the enzyme existed in a latent form in fresh tissue extracts of rat and that the enzyme was generated, presumably by proteolysis, during the prolonged centrifugation which is an early step in several published purification procedures (27). The non-activated phosphorylase kinase has

been shown to be dissociated to catalytically active low molecular weight species by incubation with either ATP or LiBr, at 0°C(28).

Phosphorylase is a highly specific enzyme. Only α-D-glucose-1-P reacts in the transfer of glucosyl residue (29); α-1-phosphates of mannose, galactose, L-glucose, glucosamine, xylose, allose, altrose, 6-fluoroglucose, 2-O-methyl, 3-O-methyl and 6-O-methyl glucose, 2-deoxy glucose etc. have been found to have no action either by rabbit or potato phosphorylases (29,30).

In the degradative direction, arsenate can replace phosphate, but with a much lower rate (31). Phosphorylase activity has been demonstrated in the absence of polysaccharide primer by employing a high concentration of enzyme (32). But at normal assay conditions, a primer containing a minimum of 3 to 4 glucose units is essential for catalytic process (33). The effectiveness of the primer depends on the source of the enzyme. Potato phosphorylase has been shown to utilise disaccharides having α-1,4-linkages as primers for starch formation (34).

The kinetic mechanism of phosphorylase from rabbit muscle (35,36) and that from some other species (37,38) has been shown to be rapid equilibrium random Bi Bi:



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

The kinetic equation for this mechanism is

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

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

The above kinetic mechanism has been confirmed by isotope exchange studies (39). The mechanism is unaltered during allosteric transistions, when sigmoidal substrate -saturation curves are obtained (39).

Phosphorylase **b** is a dimer. The molecular weight of the rabbit muscle phosphorylase monomer calculated from the aminoacid sequence is 97,412 (40). This includes the N-terminal acetyl group and the phosphoryl group at Ser-14. The subunit relationship of the phosphorylated **a** form of the

enzyme depends on conditions like enzyme concentration, pH, temperature, ionic concentrations etc.(8). At low enzyme concentrations, the rabbit phosphorylase a exists as a dimer (41). The phosphorylase a from lobster (15,18) and crab (42) exists exclusively as a dimer. Dimer and tetrameric forms of phosphorylase a have been shown in tissues of a number of other species like human, shark, rat and frog (8). The molecular weight in all these cases has been found to be in the range of 90,000 to 100,000 for the monomer.

Detailed structural studies have been carried out for the enzyme from rabbit muscle. No disulphide bridges have been found in phosphorylase. The subunits of the rabbit muscle enzyme are similar but not identical (43). Electron-microscopic measurement has shown that rabbit muscle phosphorylase b consists of 2 ellipsoidal units bound together with measurements of 110:65:55 A° (44). X-ray crystallographic analysis showed the dimensions as 115:75:60 A° (45,46). The symmetrical association of the dimers gives the tetramer a square shape (47). Tubular shaped crystals have been obtained for phosphorylase a in the presence of protamine (48).

The complete amino acid sequence of the 841 amino acids of the rabbit muscle phosphorylase has been recently reported by Titani et al (49). The composition derived from the sequence is as follows: Leu 79, Glu 64, Arg 63, Ala 63, Val 62, Asp 51,

Ileu 49, Lys 48, Asn 45, Phe 38, Tyr 36, Thr 35, Gln 31, Ser 29, His 22, Met 21, Try 12 and CysSH 9. The data shows that at neutral pH, the positively and negatively charged amino acids are well balanced. However, such a neutralisation is not equally distributed. For eg., the N-terminal end has been shown to be composed of essentially basic amino acids (46,50).

Comparison of the 15-19 amino acids of the N-terminal sequences of _E.coli_ and potato phosphorylases with those from five vertebrate sources and yeast (51) showed that i) the first amino acid is a hydroxy aminoacid (threonine in potato phosphorylase and serine in all other cases); ii) the non - regulated plant phosphorylases have a free α-amino group in contrast to all other phosphorylases studied so far which have a blocked α-amino group. The _E.coli_ phosphorylase showed identity with animal phosphorylases only in positions 1,3 and 16. Nakano _et al_. (52,53) have shown that the sequences of the potato and rabbit enzymes are very similar except for the remarkable dissimilarity seen at the N-terminal residues. These phosphorylases are similar in some of the structural and kinetic properties but their control mechanisms are different. The sequence near the PLP site in rabbit phosphorylase has been shown to be homologous to that from yeast (54), potato (53,55) and _E.coli_ (51,56). The peptides containing SH groups obtained from the rabbit and potato phosphorylase also have

highly homologous series (52). From these studies the authors have pointed out that phosphorylase existed originally as a large catalytically active molecule and by gradual mutation a regulatory mechanism was formed within the molecule during the course of evolution.

All α-glucan phosphorylases studied so far have been found to contain one molecule of pyridoxal-5'- phosphate (PLP) bound to each subunit of the enzyme. Sucrose phosphorylase, however does not contain PLP. The exact functions of this coenzyme in phosphorylases have not yet been established unambiguously. This prosthetic group has been shown to be covalently linked to Lys-679 and buried inside a hydrophobic region in rabbit muscle phosphorylase (57,58). The PLP can be resolved by deformation of the enzyme and trapping it with a reagent like L-cysteine (59). The loss of PLP is accompanied by loss of activity and a tendency to dissociate to monomers at room temperature (60). The quaternary structure of the apoenzyme is different from that of the holoenzyme (61). The apophosphorylase can be reconstituted by the addition of PLP and this process has been shown to be highly temperature dependent (60).

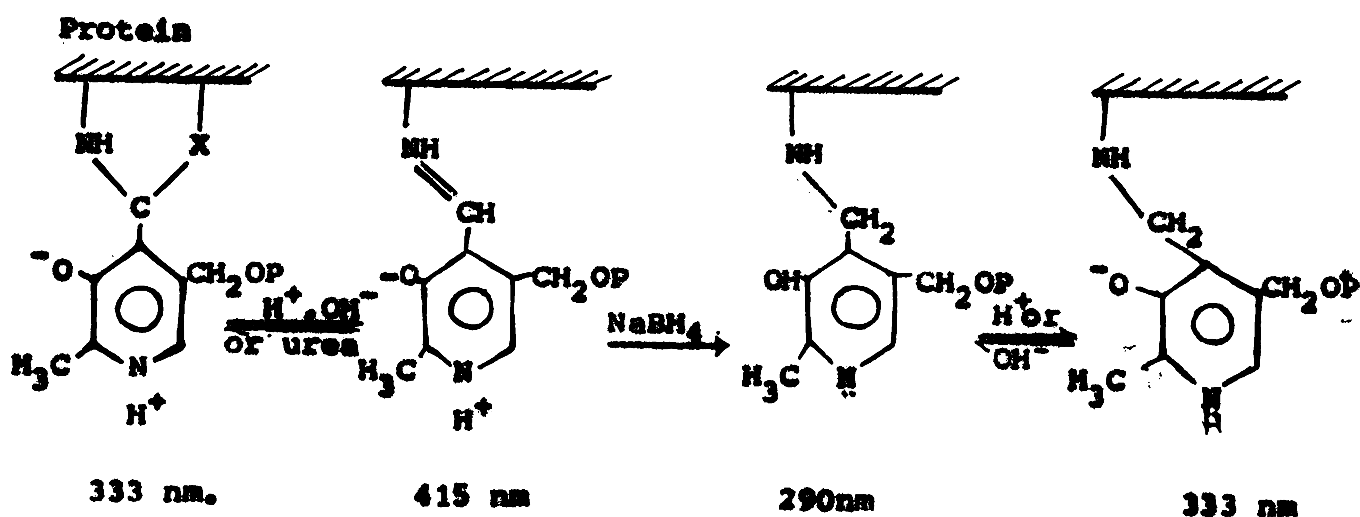
Unlike in other PLP-containing enzymes like transaminases, $NaBH_4$ reduction of the coenzyme does not abolish the catalytic activity of phosphorylase (62). This finding initiated a search for the functional group in PLP that may be participating

in catalysis. For this, the apophosphorylase was reconstituted with a number of PLP-analogues which differ from PLP at any one of the six positions in the aromatic ring. From these studies it was concluded that except for the pyridine nitrogen and phosphate, all other groups are apparently not participating in the catalytic process(8). Out of a number of 5'-phosphate analogues tested only very closely similar compounds like pyrioxal-5'- methylene phosphonate (62) restored catalytic activity. This compound has a pK 7.2 compared to 6.2 for PLP and the reconstituted enzyme showed an alkaline shift in the pH optimum. This supports the earlier assumptions that the phosphate has some role in catalysis. The finding by Graves and his colleagues (64) that phosphite can activate pyridoxal-reconstituted enzyme while pyrophosphate was a competitive inhibitor to both phosphite and glucose-1-P shows the involvement of the phosphate moeity and its participation in catalysis. The effect of pH on enzymic activity (65) and the earlier study on the dependence of pH on the fluorescence quantum yield of PLP-monomethyl ester (66) shows that the phosphate group (with pK 6.2) may participate in general acid base catalysis.

Recent X-ray crystallographic analysis by Johnson and coworkers have shown that the PLP is only 6A° away from the catalytic site (67). 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 (67).

Since the pyridoxal phosphate has an absorbance maximum at 335 nm, the study of the spectral properties under different conditions with simultaneous activity measurements can yield the structure-function relationship of the coenzyme. Eventhough the PLP site has been located in the sequence and in X-ray picture, the spectral characteristics predict a more complex environment in which the coenzyme is bound. On reduction of the coenzyme with $NaBH_4$ the 335 nm band has been found to shift towards 290 nm (68). When the pH is shifted to the acid or alkaline side, the band again reappears. Since the absorption due to a Schiff base (at 415 nm) of PLP is only very less in phosphorylase and since the $NaBH_4$ reduction is highly retarded at neutral pH, it was suggested that the natural form of the enzyme is a zwitterionic addition product of some nucleophilic group on the protein with the Schiff base (69). These structures can be represented as follows:-



Shimomura and Fukui (70) have shown that the difference spectrum of apo and holo enzyme shows, in addition to the 335 nm band, a band at 251 nm, which they have assigned to the bound PLP. By studying the factors contributing to the absorption of PLP Veinberg et al. (71) have shown that the N-atom of the aromatic ring does not have to be invoked in explaining the spectral properties. The spectral studies on *Sepia pharaonis* phosphorylase reported in the thesis throw light on the nature of PLP environment in phosphorylase.

Graves et al. (73) have studied the PLP dependent conformational states of the enzyme in relation to the interconversion between the a and b form and showed that PLP has an important effect on enzymic interconversion (72). Recent studies also show that the control of dephosphorylation of phosphorylase a by glucose requires the coenzyme PLP (73). However, the function of PLP can not exclusively be attributed to such a control mechanism because this coenzyme has been shown to be present in phosphorylases of distinct species where the control mechanisms are different. The recent analysis of the amino acid sequence of the PLP site in potato, yeast, *E.coli* and rabbit muscle phosphorylases shows that they are highly homologous (53-55). The activity of the potato phosphorylase has also been shown to be highly dependent on PLP (74). As Nakano et al. (55) have pointed out, the very strong conservation of the coenzyme binding site over approximately 1.5 billion years is a good support for a catalytic role for PLP. Conservation of

protein structure by the expensive way of maintaining a coenzyme, seems to be an impossibility.

In addition to the metabolic inhibitors, glucose-6-P, ATP and glucose, phosphorylase has been shown to be inhibited by aromatic compounds (75). The inhibition is dependent on the hydrophobicity of the aromatic compounds. The aromatic compounds have been shown to bind on a large hydrophobic region on the enzyme (76). p-Nitrophenyl phosphate has been shown to be a competitive inhibitor of AMP for phosphorylase b and is assumed to bind on the same locus where AMP binds (77). The exact location of the binding site of aromatic compounds on phosphorylase required a detailed kinetic analysis using different aromatic compounds besides the metabolic inhibitors. The first part of the thesis analyses this problem in detail.

Chemical modification studies of enzyme systems not only reveal the protein functional groups but can also be used as a tool to study allosteric transitions. Glycogen phosphorylase has been subjected to chemical modification studies using a number of reagents. p-Mercury benzoate completely dissociated the enzyme into monomers by modification of the 'SH' groups (78). Using iodoacetamide two out of the 9SH groups per enzyme monomer were modified without loss of enzymic activity and these groups were shown to be surface exposed (79,80). Some of the other 'SH' groups reacted very slowly with loss of activity and others were inaccessible to the reagent. Similar results have been obtained when reagents like DTNB (81), NEM (82) and

FDNB (83) were employed. Lysyl groups have been shown to be essential for the maintenance of enzymic activity and structure. 4-5 amino groups could be modified resulting in a totally inactive enzyme (84). Soman and Philip (85) have prepared a desensitized FDNB derivative of phosphorylase in which 1 cysteinyl and 1 lysyl residues were modified. Dinitrophenylation of 1 amino group has also been shown to result in the loss of enzymic activity (86). A number of other chemical reagents have also been shown to modify the enzyme with loss of activity. A few among them are glyoxal (87), acetylimidazole (88) glutaraldehyde and aliphatic aldehydes (89). Tyrosyl residues have also been shown to be essential for maintaining enzymic activity. Modification studies using N-acetyl imidazole has shown that 2 tyrosyl residues are essential for maintaining enzyme activity (90). Nitration also has been shown to modify 2 tyrosyl residues out of 36 per monomer, with loss of activity (91). Modification of phosphorylase b with potassium ferrate resulted in identifying tyrosine 75 as essential for catalysis (92). Studies on the protection of inactivation by AMP have suggested the nucleotide binding site near this residue. The presence of an essential tyrosine was also shown by reaction of phosphorylase b with pottassium-nitrodisulfonate salt (Fremy's radical) (93). Phosphorylase b was rapidly inactivated by 5-diazo 1 H-tetrazole or by 1-cyclohexyl-3-(2-morpholinoethyl) carbodimide metho-p-toluene sulfonate with

complete inactivation (94). The loss of activity has been attributed to carboxyl group modification. Histidine residues modified by diethyl pyrocarbonate (95) was also resulted in enzyme inactivation. The presence of histidine residues at the substrate binding region is suggested by this study. 2,3-Butane-dione has been employed to study the significance of arginine in catalysis (95a). Two types of essential arginine residues, one in the allosteric site and the other in the active site, have been identified.

Although chemical modification studies have been performed using a number of reagents, direct participation of any group or groups in the catalytic mechanism of phosphorylase has not yet been derived from these studies. Recent X-ray crystallographic analysis has partially revealed the environment of the active site (40). The carboxyl group of Glu-645 makes a close contact with the pyridinium nitrogen of PLP and the nitrogen of Lys-654 is associated with the phenolic oxygen. Surrounding the PLP include Tyr-90,Trp-490 and Val-649. Arg-138 also form part of the hydrophobic environment of PLP. The basic residues Lys-573 and Lys-567 are close to the phosphate of PLP. Near the O-5 of bound glucose is found the His-376.

The X-ray crystallographic location of various ligand binding sites of phosphorylase is still incomplete. Madsen and his colleagues (40) have classified four binding sites of

phosphorylase a. The 'active site' of which there are two per dimer, is shared between the two subunits at their interface and comprises a pocket-like region within a V-shaped frame work of 2 α-helices. This site binds the substrates glucose-1-P, phosphate, glycogen and arsenate in addition to the inhibitor glucose. A second site, 'the activator site' binds glucose-1-P, P_i, AMP,ATP and glucose-6-P. The third is the 'glycogen storage site', to which glycogen binds 20 times stronger than at the active site. The fourth site is the 'inhibitor site' to which purines and nucleosides bind. But contradictory observations have been reported by these workers in many instances. For example, glucose was first reported to bind 17 A° away from the active site in an 'interior crevice' (96), but was later reported to bind at the active site (40). AMP was first shown to bind at the active site, which is distinct from the nucleoside binding site (and the latter site was initially shown to be incapable of binding nucleotides (96)). Later it was argued that AMP and other nucleotides could bind at 2 sites, one 30 A° and the other 10 A° away from the active site (97). The former site is the 'AMP-binding site' (which seems to be the 'activator site' later reported (Ref.40) and the latter is the nucleoside binding site. The binding of AMP to this site can cause negative allosteric interaction (97). The glycogen storage site was first reported to be distinct from the active site (98) but was explained later as having 'an access' to the active site, thus making a dubious

distinction between these two sites. The crystals used for these studies were grown in high concentration of glucose (which is an allosteric inhibitor). This raises many doubts about the validity of some findings.

The X-ray crystallographic analysis by Johnson *et al*. from Oxford is also in progress. They analysed phosphorylase *b* crystal grown in IMP (unlike the preparation of Madsen *et al*. (40) which were the crystals grown in glucose and hence were of the inactive T-conformation). They also have identified one nucleotide binding site ('Site N') and the catalytic site ('Site C') (99). Unlike in phosphorylase *a* the positions of the first 19 residues of the N-terminal end was not well defined. The 'Site N' is very close to the active site (100). The inhibitor binding site of glucose-6-P is also very close to the active site. Glycogen binds on the surface of the molecule 50-60 A° from the active site. In addition to the 'Site N' described above, the nucleotide can bind to a second site, the 'Site I' (nucleoside inhibitor site). Recent X-ray crystal analysis by these workers have shown that the substrates may bind in different modes on the active site under different conditions (67). For eg., in the absence of glycogen and AMP, glucose-1-P binds on the enzyme in a 'non-productive mode'. Conversion of the enzyme to the active conformation through association with AMP may result in

conformational changes that direct the binding to the 'productive mode'. (67). Similar conclusions were arrived at earlier using kinetic studies from our laboratories (101).

The above description of the X-ray crystallographic analysis shows that further studies are essential to locate the binding sites of all the ligands of phosphorylase. The kinetic studies presented in the Ist part of the thesis provides ample support to the observed positions of ligands by crystallographic analysis, though clear discrepancies are seen with some observations, which are discussed in the text.

Phosphorylase is the first enzyme shown to exhibit allostery. A number of homotropic and heterotropic interactions have been observed with AMP, glucose-1-P, phosphate, glycogen, glucose-6-P and ATP (8). The allosteric mechanism of phosphorylase is not yet understood. Even though many workers have included phosphorylase in the 'K' system of activation and supported the two state concerted model of Monod _et al_. (102) to describe their observations, others have found discrepancies with the predictions of the model. Their observations could be satisfactorily explained by the sequential model of Koshland, Nemathy and Filmer (103). Several modifications of the original model of Monod _et al_. have been made by various authors inorder to fit in their

observations. Rubin and Changeux (104) proposed the non-exclusive binding of ligand on both the R and the T states and suggested preferential binding as a possibility. Soman and Philip have explained their results with a model of 'right and wrong' binding of ligands on the enzyme (105).

Chemical modification studies provide a useful tool to study the mechanism of allosteric interaction because the groups modified in the allosterically altered form can be analysed after modification. In fact, phosphorylase a, a naturally occurring desensitized form has entirely different allosteric properties from that of the b form. Homotropic cooperativities of AMP sites are only observed in presence of inhibitors like glucose (106,107). A glutaraldehyde modified enzyme which is devoid of all homotropic cooperativity but retaining all heterotropic interactions has been prepared (89). Phosphorylase b modified with FDNB in presence of AMP and orthophosphate has been shown to produce a desensitized enzyme derivative, the analysis of which has shown that lysyl and cysteinyl residues were modified (85). Phosphorylase b, the subunit of which was cross linked by tetroyl bis (glycylazide) has been found to be densensitized with respect to the AMP binding sites (108) showing that the effect of cross link is to restrict subunit interaction and allosteric transitions of the enzyme.

The mechanism of regulation of glycogen phosphorylase by metabolic interconversion between two forms has been demonstrated in a number of animal species besides in rabbit muscle phosphorylase. The existence of the two interconvertible forms has been established in a wide spectrum of animal tissues such as rabbit liver (109), rat liver (110), human leukocytes (111) in the lower vertebrate lamprey (20) in the oocytes and embryos of the loach (112), in insects (113-116) and in _Neurospora crassa_ (117). Two or more forms of phosphorylase have been reported in blood platelets of animals (118), in rat chloroma (119), swine kidney (120), in the molluscs crab (42) and _Pecten maximus_ (121), brewer's yeast (122), banana leaves (123), in spinach and pea leaves (124) and in a number of other plant tissues (125). However, 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 (109,110). The human leukocyte _b_ form is 25% active in the absence of AMP (111). In _Pecten maximus_ (a mollusc) an additional form of the enzyme (phosphorylase _c_) has been reported (121). In _Dictyostelium discoideum_ a purified dimer form of the enzyme was not even slightly activated by AMP (126). From _Neurospora crassa_, a phosphorylase with high specific activity has been isolated which could be classified neither in the _a_ nor in the _b_ form (126 a).

It may be noted here that Graves _et al_. (127) have prepared an active monomeric derivative of rabbit phosphorylase after reduction with $NaBH_4$.

Thus the properties of phosphorylase and its interconversion established in rabbit muscle and other terrestrial animals do not fall under a general pattern. The control of glycogen degradation could be expected to vary with the requirement of the organism. Although multiple forms of phosphorylases have been demonstrated in different tissues of animals from a wide spectrum of evolutionary status, the degree of interconversion and the extent to which it contributes to intracellular control of phosphorylase in lower forms of life is not well understood. The study of the control of phosphorylase from the cephalopoda, _Sepia pharaonis_ reported in the second part of the thesis attempts to fill this gap and opens a new chapter in the control of glycogen phosphorylase.

Several ambiguities remain to be cleared regarding the structure, role of PLP and allosteric mechanism of glycogen phosphorylase. The control properties understood for the rabbit muscle enzyme are not strictly followed by the enzyme from other sources. In order to understand this as well as to delineate the role of PLP, the enzyme from other sources should be examined. Marine animals, especially which are low

in evolutionary status compared to vertebrate animals have not been investigated in detail. From the literature already available, the allosteric properties of phosphorylase has been shown to be different in one vertebtate marine fish (105). It is thus not known whether the role of PLP would be the same in all animal phosphorylases. Also the possibility whether the control mechanism of glycogen degradation shown for rabbit ensyme is essentially common for all animal tissues. This thesis probes these aspects also üsing a specialized tissue of a marine invertebrate.

RESULTS AND DISCUSSION

CHAPTER 2

INHIBITION OF PHOSPHORYLASE BY AROMATIC COMPOUNDS.

Glycogen phosphorylase has been shown to be inhibited by aromatic compounds (75). The relative effectiveness of the various inhibitors has been shown to be dependent on the hydrophobicity of the aromatic compounds (76). However the relative position of this hydrophobic region with respect to the binding sites of other ligands like the substrates glucose-1-P and phosphate or the inhibitors like glucose and glucose-6-P has not been located.

AMP has been shown to bind on a hydrophobic region on phosphorylase b near the monomer/monomer interface where the binding sites for glucose-1-P, glucose-6-P and ATP are also located (100,123). Even though p-and-m nitrophenols are structurally dissimilar to the substrate, activator and inhibitors of phosphorylase, they have been shown to be more effective as inhibitors and bring about stronger cooperativity between substrate sites and activator sites than the naturally occurring allosteric inhibitors such as glucose-6-P and ATP.

This chapter deals with the studies on phosphorylase a and b in an attempt to locate the aromatic binding site and to delineate the mechanism of inhibition by aromatic compounds. For the investigation, the p-nitrophenyl compounds,

Fig. 2-1.

Double reciprocal plots for glucose-1-P in the presence of various p-nitrophenyl compounds for phosphorylase b. Phosphorylase b in 30 mM cysteine/40 mM β-glycerophosphate buffer pH 6.8 was mixed with an equal volume of inhibitor, also in the same buffer and incubated for 30 minutes at 30° prior to assay. The assay mixture contained 12 μg/ml enzyme, 7.5 mM of the inhibitors, 1 mM AMP, 1% glycogen and varying concentrations of glucose-1-P. ○, Control (without inhibitor); ×, p-nitrophenyl β-glucoside, ▲, p-nitrophenyl phosphate; △, p-nitrophenyl β-arabinoside; ●, p-nitrophenol.

Fig. 2-2

Hill plots for the data of Fig. 2-1

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p-nitrophenyl phosphate, p-nitrophenyl β-glucoside and p-nitrophenyl β-arabinoside were selected with a view to understand any possible difference in the kinetic response by these inhibitors. The effects of these compounds could be then compared with p and m-nitrophenols besides the metabolic inhibitors ATP and glucose-6-P.

The effect of the different inhibitors on phosphorylase b is shown in Fig. 2-1 as double reciprocal plots and in

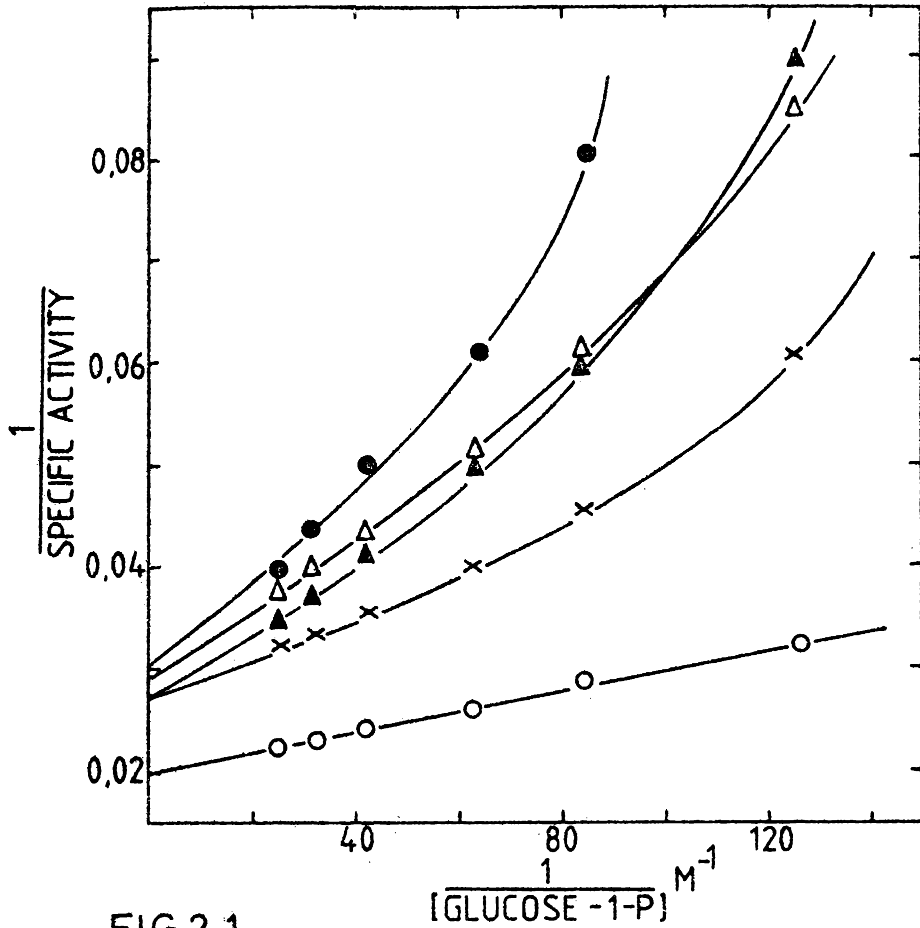

FIG. 2-1

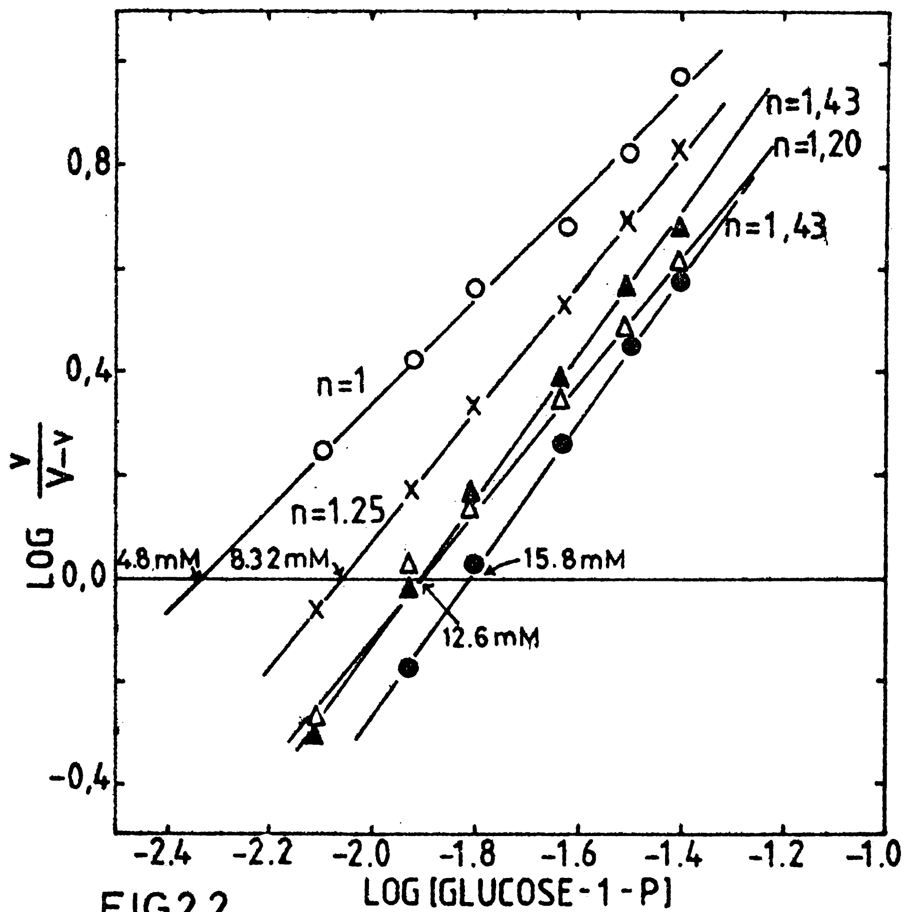

FIG.2-2

Fig. 2-2 as Hill plots. The results show that all these compounds behaved as typical allosteric inhibitors. The order of effectiveness was m-nitrophenol > p-nitrophenol > p-nitrophenyl β-arabinoside > p-nitrophenyl phosphate > p-nitrophenyl β-glucoside. The n values of p-nitrophenol and p-nitrophenyl phosphate were the same and hence an additio phosphate on the aromatic ring had no influence on the degree of cooperativity induced by the compound. It may be noted that adenosine, AMP and ATP are compounds with different allosteric properties and they vary with respect to the number of phosphates.

The inhibition by the compounds was found to be dependent on temperature. The double reciprocal plots for glucose-1-P at different temperatures using p-nitrophenyl phosphate as inhibitor is shown in Fig.2-3. At all temperatures the reciprocal plots were linear in the absence of the inhibitor. In the presence of the inhibitor, however, as the temperature was increased, the inhibition as well as homotropic cooperativ between glucose-1-P sites caused by p-nitrophenyl phosphate increased. Thus there was no inhibition at 10° and slight inhibition at 15° where as the effect became more and more pronounced at higher temperatures. The conditions employed for these experiments were such that there was only minimum dimerisation of phosphorylase b as the temperature was lowered However, the dependence of inhibition on temperature suggeste

Fig. 2-3

Double reciprocal plots for glucose-1-P in the absence and presence of p-nitrophenyl phosphate for phosphorylase b at different temperatures. Phosphorylase b in 30 mM cysteine/ 40 mM β-glycerophosphate buffer pH 6.8 was mixed with equal volume of inhibitor solution also in the same buffer and incubated at 30° for 15 minutes. The enzyme and substrate solutions were brought to the required temperatures prior to assay. The assay mixtures contained 10 μg/ml enzyme, 6.25×10^{-3} M PNPP, 1% glycogen, 10^{-3} M AMP and varying concentrations of glucose-1-P. Open symbols are data for control experiment (without inhibitor) and filled symbols are data with PNPP. ○,● 40°; □,■ 35°; ×,⊠ 30° and △,▲ 25°.

Inset: ○,● 20° and △,▲ 10°.
(PNPP = p-nitrophenyl phosphate)

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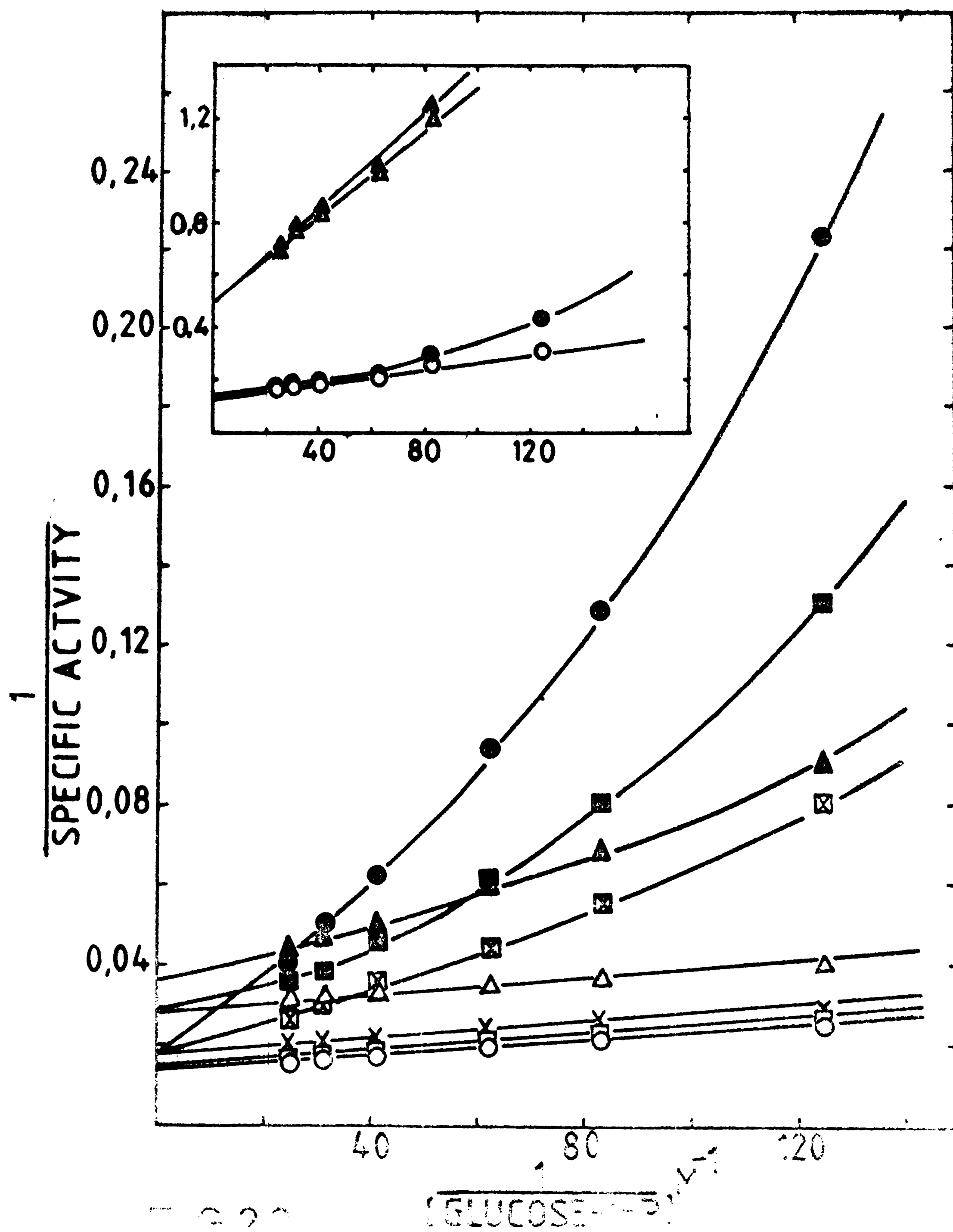
that even under assay concentrations there was dimer formation. Earlier work using centrifugal data has shown that phosphorylase b in the presence of AMP tended to form aggregates as the temperature was lowered below 20° (129). The present results showed the possibility that even at assay concentration, similar change could take place. However such aggregation arrested the ability for cooperative binding of glucose-1-P.



0,24
0,20
0,16
0,12
0,08
0,04
1
SPECIFIC ACTVITY
40
80
120
1,2
0,8
0,4
40
80
120

A detailed investigation on the location of aromatic binding site was further made using phosphorylase **a** because it is a naturally occurring desensitised form. Moreover, since the **a** form is active in the absence of AMP, the influence of the inhibitors in the presence and absence of AMP could also be studied.

In agreement with earlier reports (130) the ratio of activity of phosphorylase **a** in the absence of AMP to that in its presence at saturating concentration of AMP (1 mM) was about 0.8 at 16 mM glucose-1-P, under the assay conditions. The order of effectiveness of the inhibitors on phosphorylase **a** (in the presence and absence of AMP) was the same as that obtained for phosphorylase **b**.

The double reciprocal plots for glucose-1-P with **p**-nitrophenol and **p**-nitrophenyl phosphate as inhibitors were linear with phosphorylase **a** (Fig. 2-4). Linear plots were also obtained for AMP-kinetics both for the 1/v **vs** $\frac{1}{AMP}$ and $\frac{1}{\Delta v}$ **vs** $\frac{1}{AMP}$ plots (Fig. 2-5 and Fig. 2-6), Δv being the difference in the rate in the presence and absence of AMP. In all these cases, the inhibition was of the mixed type. From the graph it can be observed that $\frac{1}{\Delta v}$ is lower in the presence of an inhibitor and is related to the effectivenes of the inhibitor. This increase in the difference between velocity in the presence and absence of AMP can be attribut to the higher effectiveness of the inhibitors in the absen

Fig. 2-4

Lineweaver-Burk plots for glucose-1-P in the absence and presence of AMP with p-nitrophenyl phosphate and p-nitrophenol as inhibitors for phosphorylase a at 30°. The assay mixtures contained 1% glycogen, varying concentrations of glucose-1-P, 12 μg/ml enzyme, 20 mM glycerophosphate and 15 mM cysteine at pH 6.8. The concentration of AMP when present was 1 mM in the assay mixtures. Open symbols are data without AMP and filled symbols with AMP in assay. ○,●, No inhibitor (control); △,▲, with 10 mM p nitrophenyl phosphate; □,■, with 10 mM p-nitrophenol.

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of AMP. Thus AMP protected the inhibition.

Phosphorylase a was less inhibited by all the compounds tested in the presence of AMP than in its absence. This protection by AMP was seen with all inhibitors although the degree of protection varied. Protection of inactivation by AMP during reaction by the aromatic reagent fluorodinitrobenzene was reported (84). Similar protection was observed with difluorodinitrobenzene also (Chapter 3). Thus these reagents and p-nitrophenyl phosphate appear to bind on the same site.

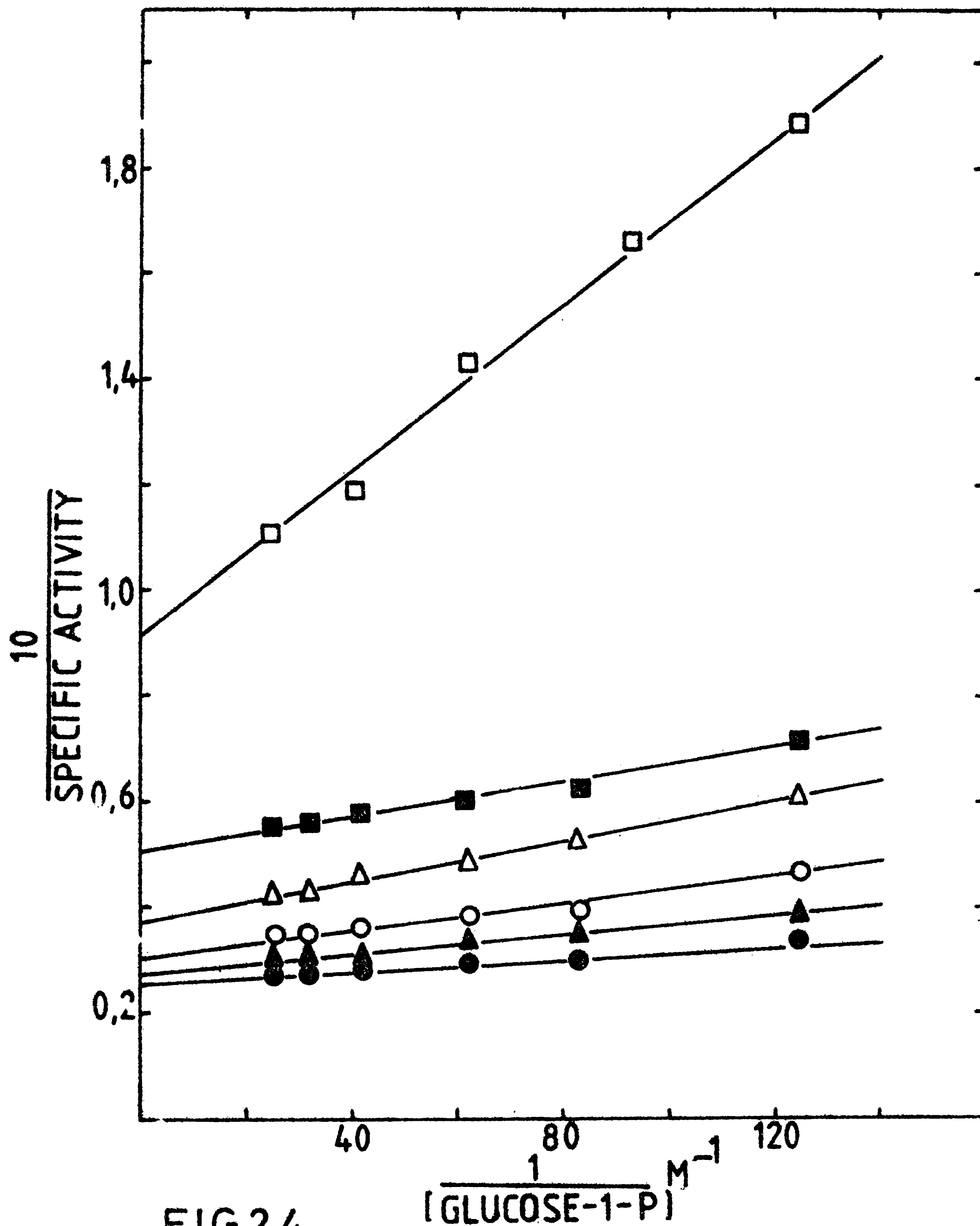

FIG.2.4

Fig. 2-5

Double reciprocal plots for AMP with p-nitrophenol and p-nitrophenyl phosphate as inhibitors for phosphorylas at 30°. The assay mixture contained 1% glycogen, 16 mM glucose-1-P and varying concentrations of AMP in 20 mM sodium β-glycerophosphate/ 15 mM cysteine buffer, pH 6.8. ○, without inhibitor; △, with 7.5 mM p-nitrophenyl phosphat with 7.5 mM p-nitrophenol.

Fig. 2-6

Plots of $\frac{1}{\Delta v}$ vs $\frac{1}{AMP}$ where Δv is the difference in activities in the presence and absence of AMP. Details are as in Fig. 2-5.

Arabinose had no inhibitory effect upto a concentration of 20 mM where as glucose was inhibitory. It appears, therefore that arabinose has no binding site on the enzyme.

Though the reciprocal plots were linear with p-nitrophenol and p-nitrophenyl phosphate, Dixon plots (Fig. 2-7) were curved (upwards) in the presence of the former but linear in presence of the latter. The kinetic mechanism of phosphorylase is random equilibrium random (35). For such a system the linear reciprocal plots but upwardly curved Dixon plots in the presence of competitive (or mixed)

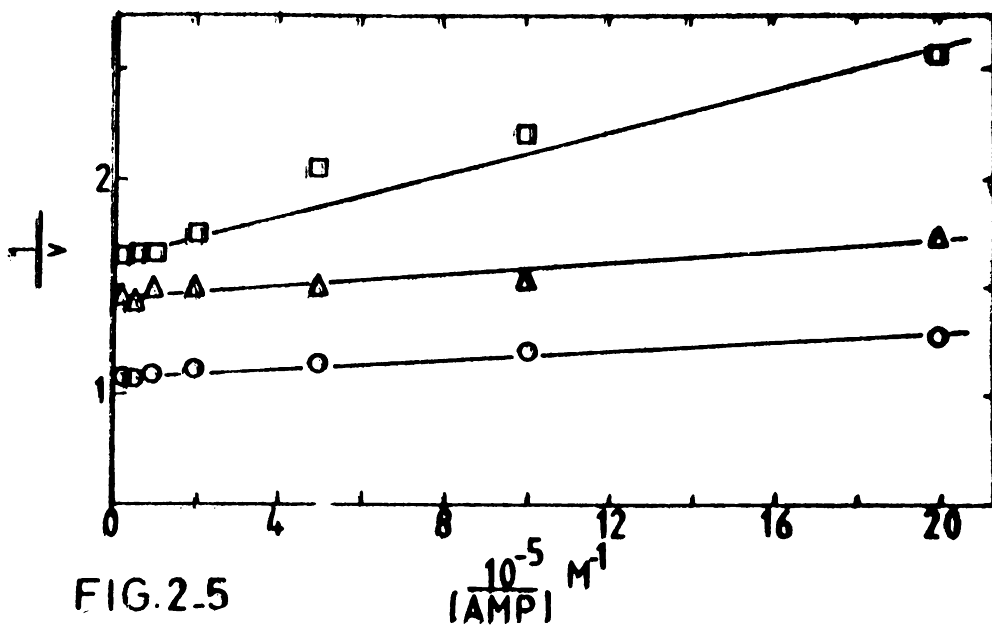

FIG.2.5

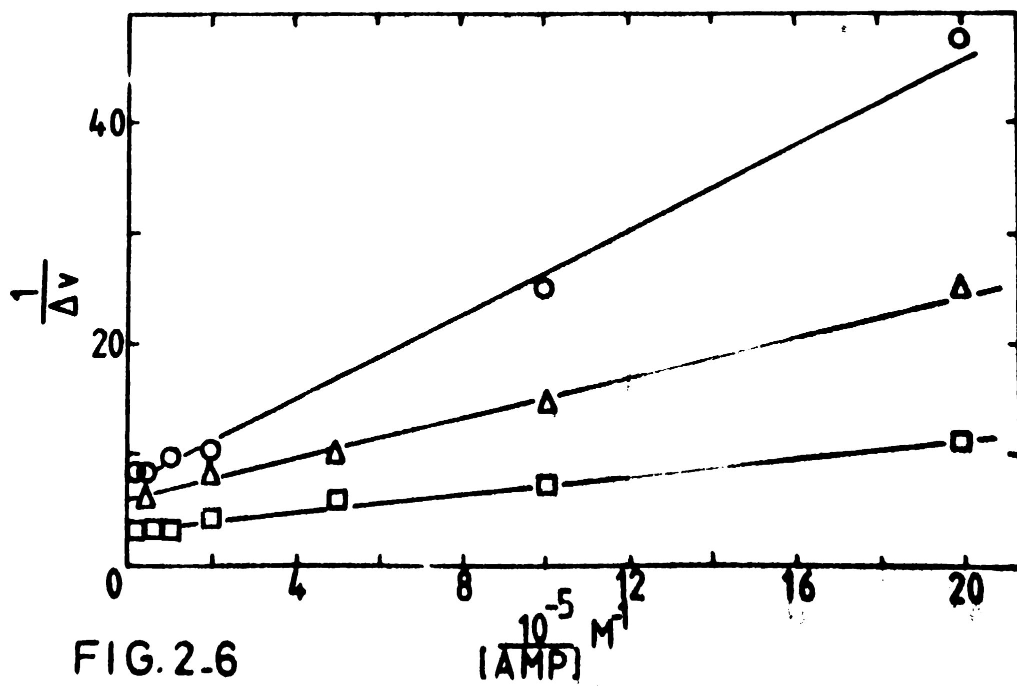

FIG.2.6

Fig. 2-7

Dixon plots for phosphorylase a with p-nitrophenyl phosphate (open symbols) and p-nitrophenol (filled symbols) as inhibitors. Phosphorylase a (60 μg/ml) in 30 mM cysteine/40 mM glycerophosphate at pH 6.8 was mixed with an equal volume of inhibitor solution in the same buffer to have final concentrations as indicated and incubated at 30° for 30 minutes prior to assay. The reaction mixtures contained 1 mM AMP, 1% glycogen and glucose 1-P; ○,●, 12 mM and △,▲ 24 mM.

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inhibitors indicated the presence of more than one inhibitor binding site. Accordingly, simultaneous binding of more than one molecule of the nitrophenol but not the nitrophenyl phosphate was possible.

In a multisubstrate system like phosphorylase, the mixed inhibition could be complicated. Since the rate of breakdown of the E-S complex to products (k_{+2}) is negligible as compared to k_{-1} in the case of phosphorylase (35), the mixed inhibition could not be due to the effect of the inhibitor on k_{+2}. Thus the variation in the rate of reaction was directly related to the binding of the inhibitors, substrate and activator.

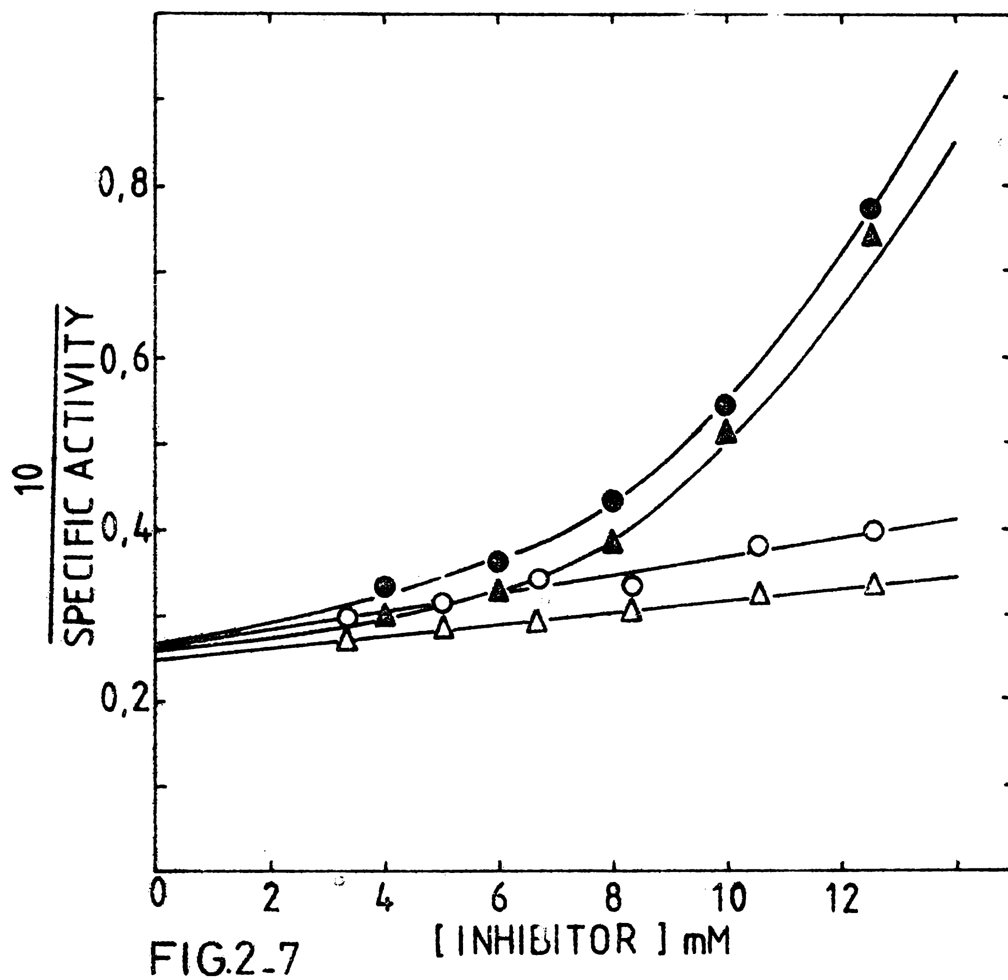

FIG.2-7

The mixed inhibition raised the question whether the aromatic compounds bound on sites entirely different from the substrate and activator sites. An answer was sought from inhibition studies using two inhibitors simultaneously using isobolograms. Isobols are lines obtained by plotting various combinations of concentrations of two inhibitors which show equal effectiveness (inhibition in the present case). This method, originally used by Loewe (131) to study the nature of interaction of drug-pairs on tissues is valid also for enzymes (132, 133). It is possible that the method is not applicable in the case of inhibitors that influence allosteric transitions. However, since mixed inhibition was understood from the use of phosphorylase a, the desensitised form of the enzyme, isobolograms have been obtained using phosphorylase a. The results are presented in Fig. 2-8 to Fig. 2-11. Fig. 2-8 and Fig. 2-9 show isobolograms obtained with two inhibitor-pairs in the absence of AMP. Here both the curves are slightl curved. Fig. 2-10 and Fig. 2-11 show the isobolograms obtained with two inhibitor-pairs in the presence of AMP. Here the isobols are linear. The isobolograms obtained with the various combinations of inhibitor-pairs could be grouped into either of the two categories presented in these figures. The results of these studies are summarised in Table 2-1 which are also shows the behaviour of glucose-6-P and ATP.

Fig. 2-8.

Isobologram with p-nitrophenol and p-nitrophenyl phosphate as the inhibitor pair for phosphorylase a assayed in the absence of AMP, at 30°. Assay mixtures contained 1% glycogen, 16 mM glucose-1-P and the inhibitors in 20 mM sodium β-glycerophosphate/15 mM cysteine buffer pH 6.8. The concentration of the enzyme was 9 µg/ml. Initial rates were measured at a constant concentration of one inhibitor (I_1) and varying concentrations of the second inhibitor (I_2) and also varying I_1 at constant I_2. The results were first plotted in the form of Yonetani-Theorell (133) plots ($\frac{1}{v}$ vs I_1 at constant I_2). (See 'experimental') From the data, various combinations of concentrations of I_1 and I_2 which gave equal inhibition were taken and plotted in the isobologram shown. □, △, ○ and × correspond to 48%, 54%, 59% and 66% inhibition respectively.

Fig. 2-9

Isobologram with p-nitrophenyl phosphate and p-nitrophenyl β-glucoside as the inhibitor pair. ○, □, ■ and × correspond to 36%, 39%, 42%, and 46% inhibition. Other details were as in Fig. 2-8.

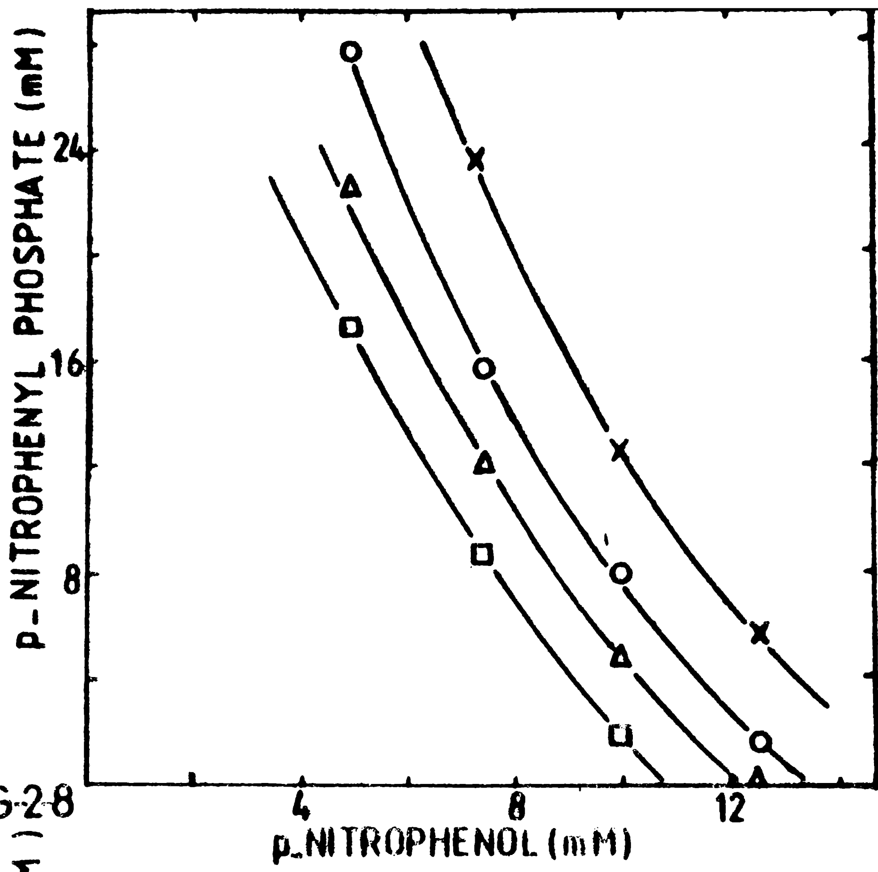

FIG-2-8

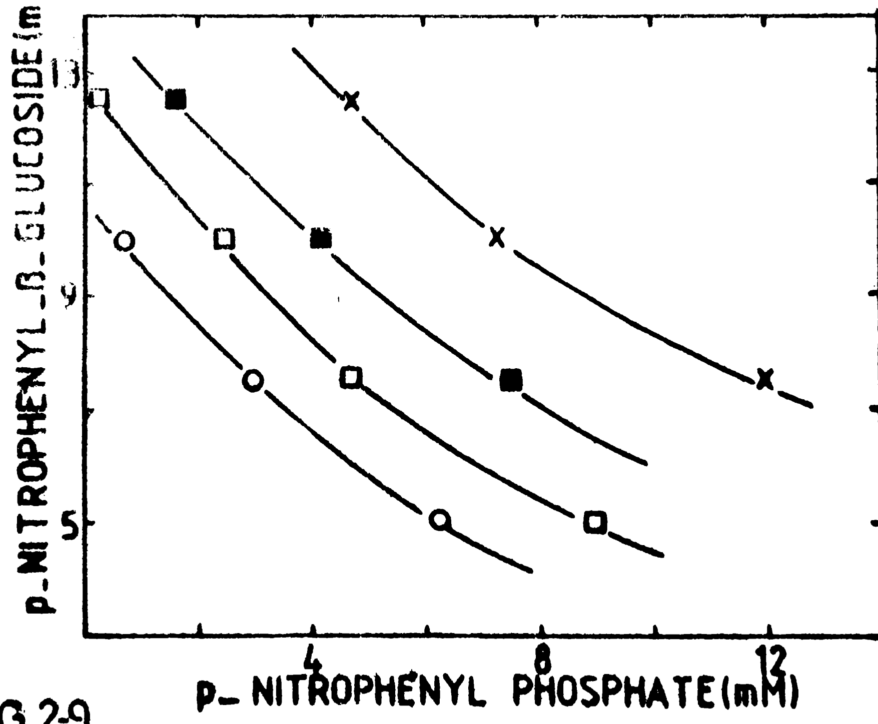

FIG 2-9

Fig. 2-10

Isobologram with p-nitrophenol and p-nitrophenyl phosphate as inhibitor pair for phosphorylase a assayed in the presence of 1 mM AMP. Other details were as in Fig. 2-8. □, △, ● and ○ correspond to 17%, 22%, 28% and 30% inhibition.

Fig. 2-11

Isobologram with p-nitrophenol and glucose-6-P as inhibitor pair. The concentration of enzyme was 11.5 μg/ml Other details were as in Fig. 2-8. □, △, ● and ○ correspond to 19%, 22%, 26% and 32% inhibition.

When isobols are linear the interaction coefficient (α) is infinity; i.e., the two inhibitors mutually and exclusively prevent each others binding (133). Minor deviations from linearity iddicate partial overlap of the binding sites. The results suggested that the binding sites of all the inhibitors studied were either the same or were located very near to each other. The presence or absence of AMP in these studies did not exhibit any uniform pattern in shape of isobols and no generalization regarding the shape of the isobols could be observed.

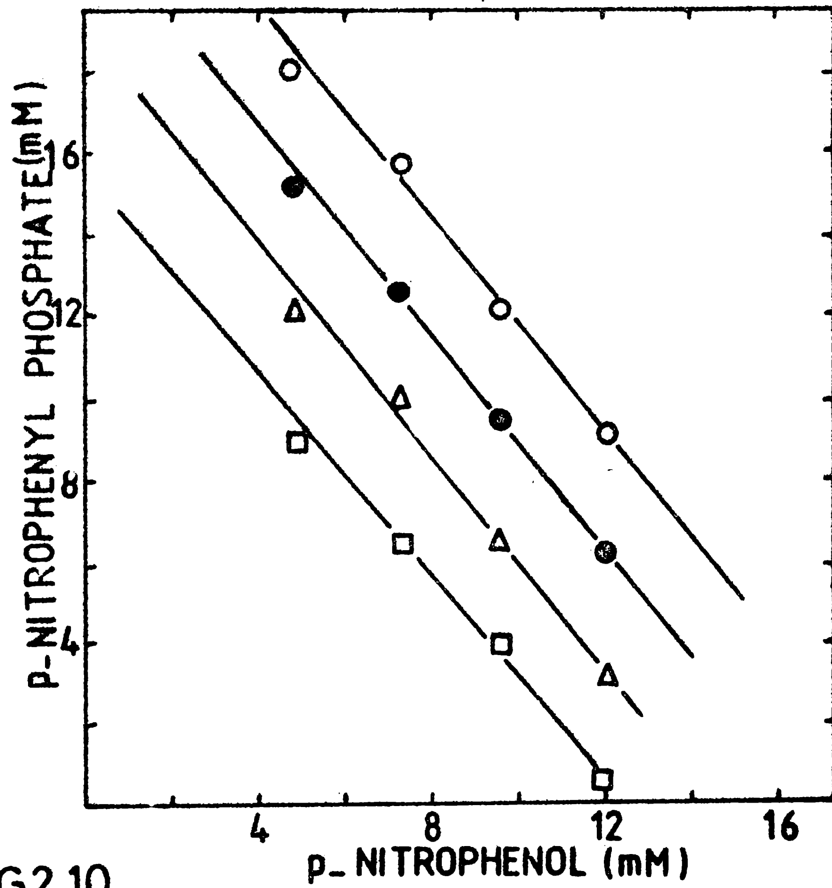

FIG.2_10

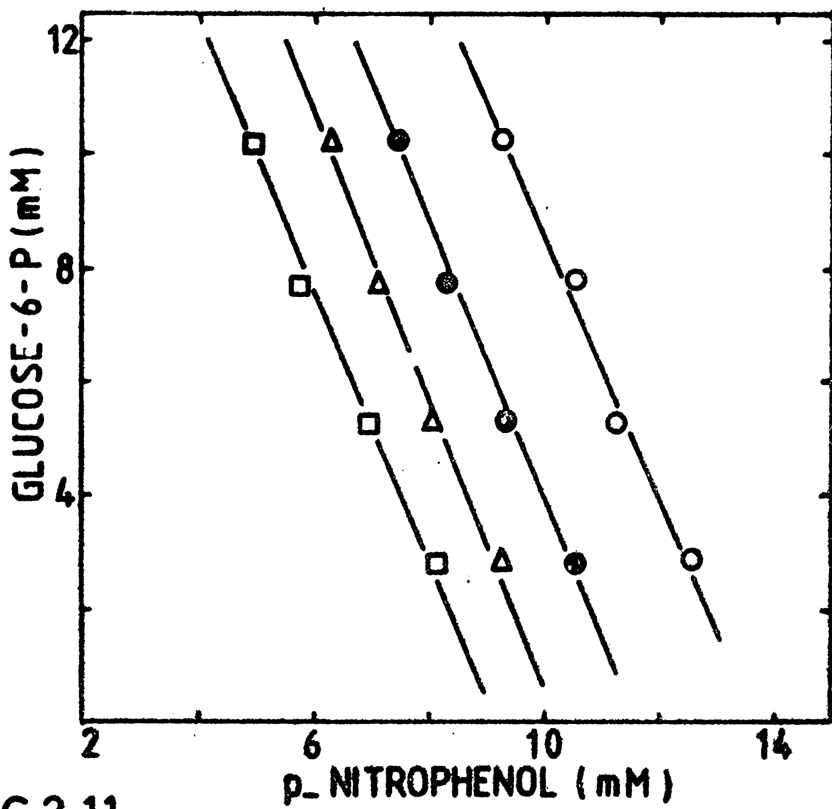

FIG.2_11

TABLE 2-1

SUMMARY OF THE ISOBOLOGRAMS OBTAINED WITH THE VARIOUS INHIBITOR-PAIRS FOR PHOSPHORYLASE a AT 30°.

(The experimental details were as in Figs. 2-8 to 2-11. The isobols were slightly curved as shown in Fig. 2-8 and 2-9. Each experiment was repeated 2 to 3 times).

Inhibitor pairs	Nature of isobols obtained when assayed in	
	presence of AMP	absence of AMP
p-Nitrophenyl phosphate and p-Nitrophenyl β-glucoside	—	curved
p-Nitrophenol and p-Nitrophenyl β-glucoside	linear	linear
p-Nitrophenol and p-Nitrophenyl β-arabinoside	linear	linear
p-Nitrophenol and ATP	linear	—
p-Nitrophenol and glucose-6-P	linear	—
m- and p-Nitrophenols	curved	curved
p-Nitrophenol and p-Nitrophenyl phosphate	linear	curved

The isobologram studies could reveal the nature and position of binding sites of aromatic compounds on phosphorylase with respect to other ligands. The proposed region consists of relatively large hydrophobic environment (76) that bind aromatic compounds and aromatic moieties including adenine part of AMP and ATP (See Chapter 4) and of hydrophilic pocket(s) that bind the phosphate and glucose moieties. Recent X-ray diffraction studies prove that the substrates glucose-1-P and orthophosphate bind on a site very near to the AMP binding site also and that this region is near the monomer/monomer interface (100). Therefore the following conclusions may be made from the results presented in this chapter; (i) the binding sites of the aromatic compounds examined are located in the same region as that of the substrate and AMP; (ii) ATP and glucose-6-P also bind in the same region (these ligands were not unambiguously located in the X-ray studies); (iii) since the binding sites a overlapping, the aromatic compounds, glucose-1-P, AMP,ATP and glucose-6-P bind on this region located near the monomer/monomer interface. This is possible only if the ligands adopt different modes of binding, the modes being determined by their structural features.

The concept of different modes of binding by the ligands on the same region in phosphorylase seemed to explain the order of effectiveness of all the inhibitors

tested as well. The curved Dixon plots exhibited by the nitrophenols and the curved isobols obtained with p-and m-nitrophenols suggested that more than one molecule of these inhibitors could simultaneously interact with the hydrophobic region. The nitrophenols were thus very powerful inhibitors.

Of the other inhibitors tested, nitrophenyl -β- glucoside was the weakest. The nitrophenyl phosphate, nitrophenyl glucoside and ATP could be envisaged as binding through the aromatic part of the hydrophobic region and through the phosphate or glucose moiety on the phosphate or glucose binding site respectively, on the enzyme in certain specific mode. Then their binding would be restricted in the sense that they could adopt only one mode of binding. Arabinose had no binding site and hence the nitrophenyl arabinoside may be expected to be more effective as an inhibitor because it is free to adopt different modes. In fact, the nitrophenyl arabinoside was an effective inhibitor next only to the nitrophenols for both phosphorylase a and b. Recent X-ray crystallographic analysis by Johnson et al. (67) supports the concept of binding of ligands in 'productive' and 'non-productive' modes.

TABLE 2-2

INFLUENCE OF VARYING RELATIVE CONCENTRATIONS OF INHIBITORS ON THE ACTIVITY DIFFERENCE OF PHOSPHORYLASE a IN PRESENCE & ABSENCE OF AMP.

(The experimental conditions were same as in Figs. 2-8 to 2-11)

Inhibitor pairs (mM)		Δv as % of activity in the absence of AMP	Δv as % of activity in the presence of AMP	Ratio
PNP	PNPP			
5.0	5.0	36	31	0.67
5.0	12.5	33	28	0.65
12.5	5.0	55	49	0.38
PNP	PNPG			
5.0	5.0	42	34	0.65
5.0	12.5	47	38	0.65
12.5	5.0	56	47	0.55
PNP	MNP			
2.5	2.5	27	22	0.75
2.5	10.0	60	48	0.32
10.0	2.5	57	46	0.47
PNPP	PNPG			
5.0	5.0	35	28	0.70
5.0	12.5	44	35	0.60
12.5	5.0	40	32	0.65

PNP, p-nitrophenol; PNPP, p-nitrophenyl phosphate; PNPG, p-nitrophenyl β-glucoside; MNP, m-nitrophenol. Δv, the difference in activity due to presence and absence of AMP; ratio, the ratio of activity in the absence of AMP to that in its presence at the concentrations of the inhibitors shown.

The difference between the activities in the presence and absence of AMP (Δv), which is a measure of protection, was influenced more by nitrophenols than by other inhibitors. The variations in Δv and the ratio of activities as a result of changing the relative concentrations of the inhibitors for a few inhibitor pairs (Table 2-2) illustrate this point. The ratio of activities remained essentially unchanged when at a fixed concentration of the nitrophenol the concentration of other inhibitors was increased whereas changing the nitrophenol concentration had considerable effect on the ratio and Δv. This effect was true for *p*-and *m*-nitrophenols. Thus when *p*- or *m*-nitrophenol was already present, the other aromatic compounds, ATP and glucose-6-P had less inhibitory effect; i.e., the effect was not additive. This also indicates the same or overlapping binding sites for the inhibitors.

CHAPTER 3

REACTION OF 1,5-DIFLUORO-2,4-DINITROBENZENE WITH PHOSPHORYLASE-b

Rabbit muscle glycogen phosphorylase b has been subjected to chemical modification studies using a few aromatic reagents which reacted with the SH groups and NH_2 groups of the enzyme (78,81-83). Phosphorylase activity was lost when the enzyme was modified with various thiol reagents. The kinetic analysis of the reaction with p-mercuribenzoate showed that modification preceded inactivation which in turn was followed by enzyme dissociation (78). Modification of SH groups using 5-5'-dithiobis-(2-nitro benzoicacid) (DTNB) has been shown to abolish the allosteric property of phosphorylase b with respect to the cooperative binding of AMP (81). Using 1-fluoro-2,4-dinitrobenzene (FDNB) 4 to 5 amino groups were modified resulting in the inactivation of the enzyme. Fasold et al. have reported that phosphorylase b could be inactivated by dinitrophenylation of one amino group per enzyme monomer (84). However, details have not been published. A partially active dinitrophenyl derivative of phosphorylase b has been shown to be devoid of its allosteric properties (85).

Chapter 2 dealt with the nature and relative position of the aromatic binding site with respect to that of other ligands of the enzyme. However, to delineate the significance and the location of the aromatic binding site, a suitable chemical derivative of the enzyme was needed. Since several residues were modified in reported derivatives and nonhomogeneous preparations only were obtained, they were not useful for the purpose of locating the binding site for aromatic compounds. We have selected 1,5-difluoro-2,4-dinitrobenzene which is a bifunctional aromatic reagent and hence can interact with the enzyme non-covalently at its aromatic binding site and cross link two regions of the enzyme. This chapter deals with studies on the reaction of rabbit muscle phosphorylase *b* by F_2DNB and with the preparation of a homogeneous dinitrophenylene derivative of phosphorylase *b* with only one aromatic group covalently incorporated per enzyme dimer.

Preliminary investigations showed that rabbit muscle phosphorylase *b* was inactivated by F_2DNB at alkaline pH. The influence of pH on the inactivation was studied in 0.05 M tris-HCl buffer (Fig.3-1). The inactivation rates at pH 7.6 and pH 8 were essentially identical and much faster than at pH 7. With a 10-fold molar excess of the reagent, almost complete inactivation occurred in 30 minutes at pH 7.6 and 8 whereas only 30% inactivation occurred at pH 7. Because of the similarity in rates and extent of inactivation at pH 7.6

Fig. 3-1

Time course of inactivation of phosphorylase $\underline{b}$ by F_2DNB at different pH. Phosphorylase $\underline{b}$ was freed from AMP by charcoal treatment and brought to pH 7, 7.6 and 8 by passage through Sephadex G-15 columns equilibrated with buffers of the respective pH. 2 mg/ml enzyme solutions were treated with 1-fold (filled symbols) and 10-fold (open symbols) molar excess at each pH. Aliquots were withdrawn at various time intervals, diluted (40 times) in 30 mM cysteine/40 mM glycerophosphate buffer pH 6.8 and incubated for 20 minutes prior to assay. The assay mixtures contained 1% glycogen, 1 mM AMP and 16 mM glucose-1-P. ○, ●, pH 7; △, ▲, pH 7.6 and □, ■ pH 8. Temperature was 30° for reaction and assay.

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and pH 8 and since pH 7.6 was used in some of the reported chemical modification studies with FDNB (84), pH 7.6 was selected for further investigations. Moreover, the enzyme was completely stable at this pH for 2 hrs. whereas at pH 8, some loss of activity was observed.

The inactivation of phosphorylase $\underline{b}$ by different concentrations of F_2DNB is shown in Fig. 3-2. The inactivation was nearly 50% when a 1:1 molar concentration of the enzyme and the reagent was employed; i.e., at an enzyme monomer to

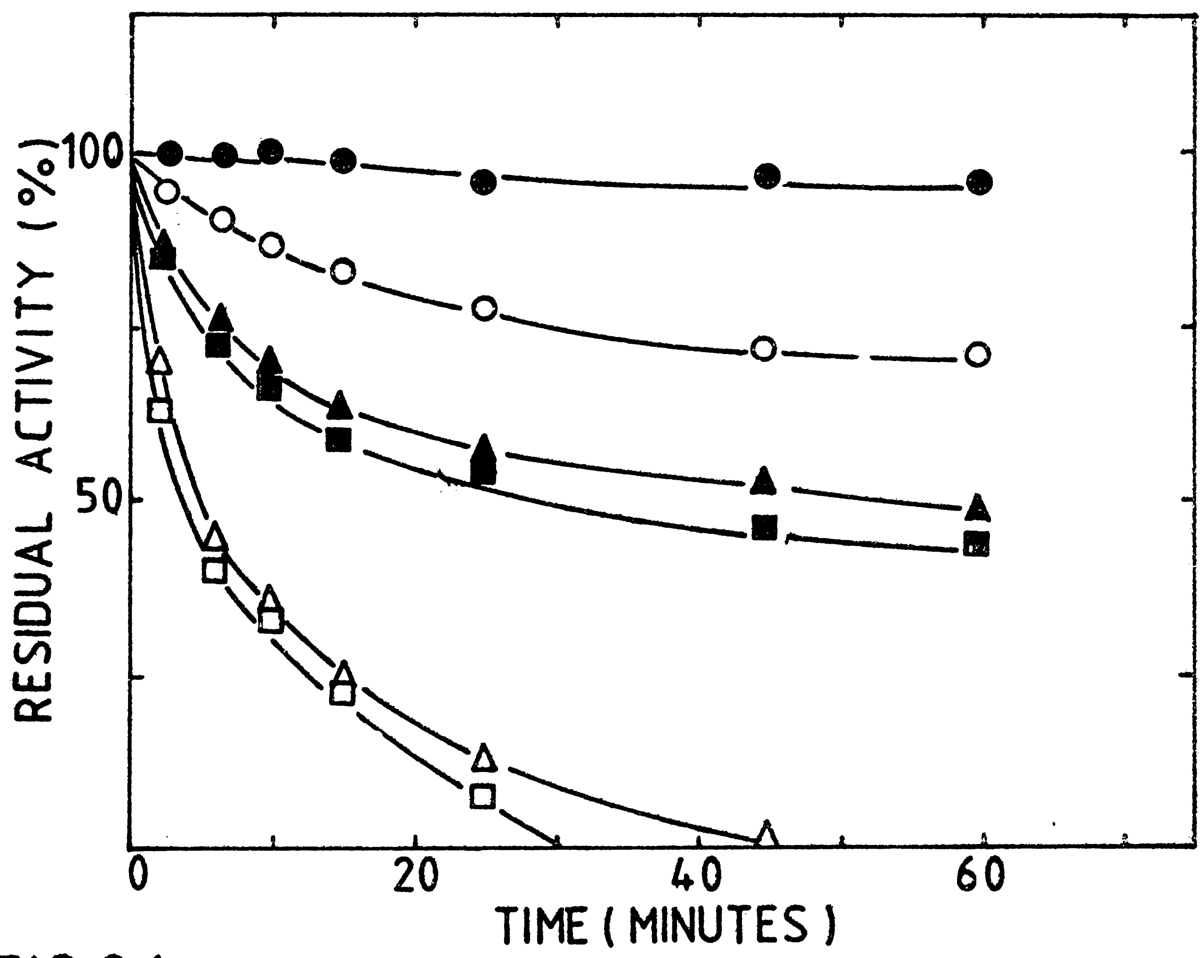

FIG. 3-1

Fig. 3-2

Comparison of the time course of inactivation of phosphorylase b using four different concentrations of F_2DNB. Phosphorylase b (2mg/ml) in 50 mM tris-HCl buffer pH 7.6 was treated with 1-fold (●), 2-fold (▲), 4-fold (○) and 10-fold (△) molar excess of F_2DNB at 30°. Aliquots were withdrawn at various time intervals, diluted in 30 mM cysteine/40 mM glycerophosphate buffer pH 6.8 and assayed as in Fig. 3-1.

Fig. 3-3

Plots of log inactivation vs. log of molar excess of F_2DNB at different time intervals of the reaction (from the data of Fig. 3-2). Curve 1, 2, 3, 4 and 5 correspond to 2.5 min., 5 min., 10 min., 20 min., and 40 min. after inclusion of the reagent, respectively.

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reagent ratio 1:0.5. Doubling the concentration of the reagent inactivated the enzyme only 65%. Even with a 4-fold molar excess of the reagent, complete inactivation of the enzyme did not occur. Estimation of the unreacted F_2DNB showed that in all these cases, the added reagent had completely reacted.

The data of Fig. 3-2 did not fit either first-order or second order kinetics satisfactorily although first-order plots showed straight lines upto 10 minutes of the reaction.

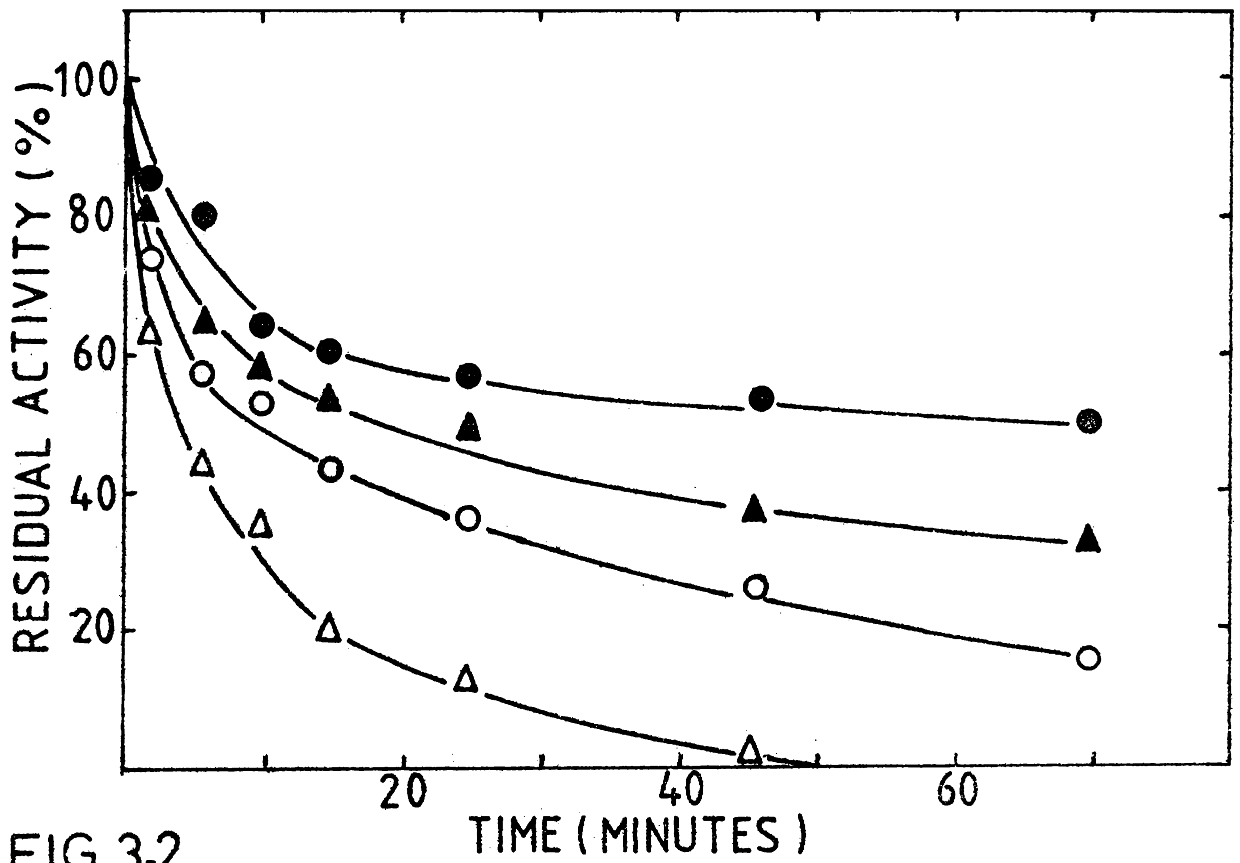

FIG. 3-2

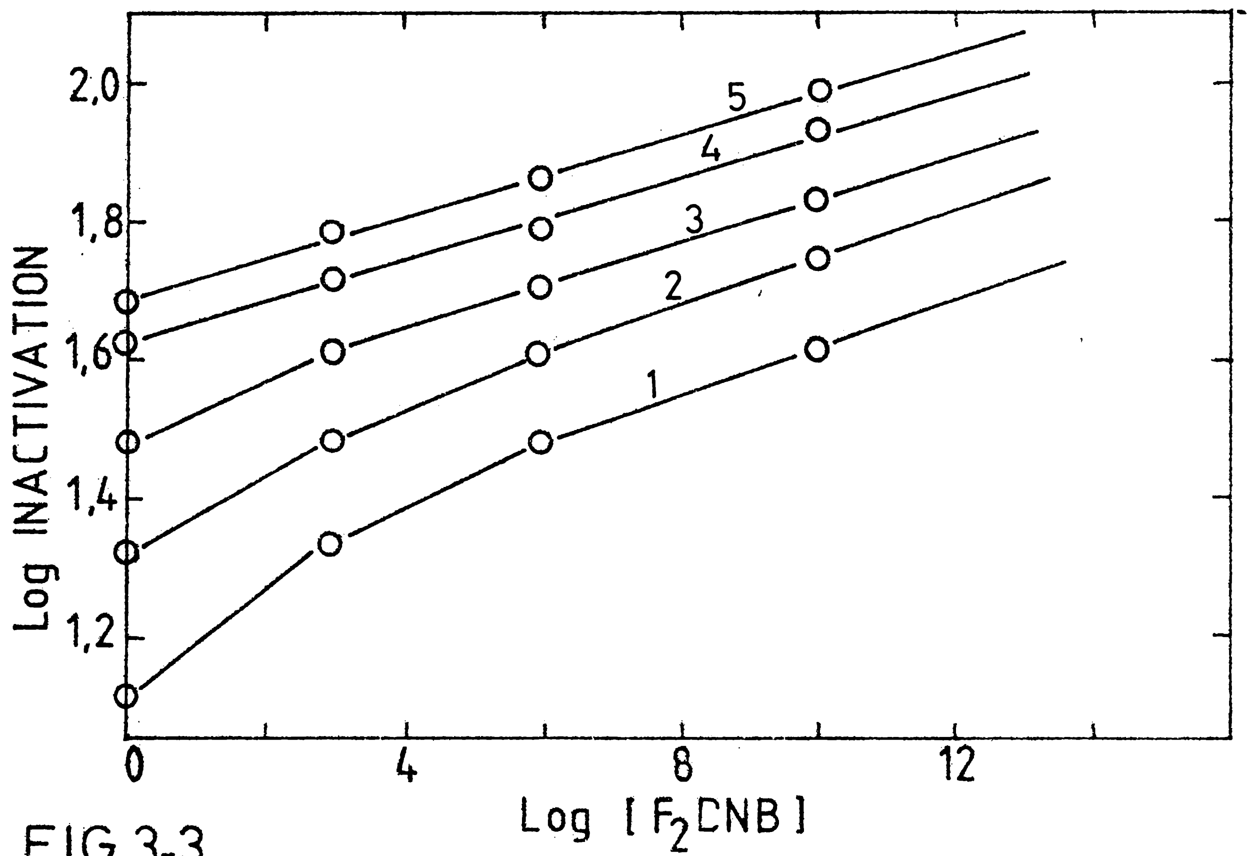

FIG. 3-3

Inorder to understand the degree of incorporation of the reagent with the enzyme, the order of the reaction was determined by plotting log concentration against log inactivation (%) at different fixed time (Fig. 3-3). Curved lines were obtained which could be approximated to two linear portions with slopes (i.e., order of reaction) of 0.8 to 1.0 and 0.33. Upto about 10 minutes of the reaction and at lower concentration of the reagent the order was 0.8 to 1.0, but changed to 0.33 at higher concentration or on continuing the reaction. These results indicated that the reaction was more specific at the initial stages and at lower concentration of the reagent. The fractional order at higher concentration of the reagent and after the initial stages suggested reaction of different types of groups on the enzyme.

The influence of substrates and effectors on the inactivation of phosphorylase b by F_2DNB was examined independently and in combinations of the various ligands. The percent inactivations in the absence and presence of the ligands at two different time intervals are given in Table 3-1. The results showed that substrates and effectors of phosphorylase b afforded some protection against inactivation. The protection by AMP against inactivation by the reagent is shown in Fig. 3-4 at three different concentrations of the reagent. The effects of all the ligands (and combinations) tested were of the same patttern as shown in Fig. 3-4, except in the case of glucose and orthophosphate (Fig.3-5). In these cases the observed protection

TABLE 3-1

INFLUENCE OF VARIOUS LIGANDS ON THE INACTIVATION OF PHOSPHORYLASE b BY F_2DNB

The enzyme (2 mg/ml) in 50 mM tris-HCl buffer pH 7.6 was treated separately with 1-fold and 4-fold molar excess of F_2DNB in the presence of the ligands for 1 hour at 30°. The reaction mixtures were passed through Sephadex G-15 columns equilibrated with 40 mM glycerophosphate pH 6.8 and the specific activities of the eluted samples were determined. The assay mixtures contained 16 mM glucose1-P, 1 mM AMP and 1% glycogen in 15 mM cysteine/20 mM glycerophosphate pH 6.8. The values are expressed as percent inactivation.

Reaction in the presence of.	Percent inactivation after 1 hour	
	with 1-fold molar excess F_2DNB	with 4-fold molar excess F_2DNB.
Nil	48.5	80.0
AMP (1 mM)	40.0	71.2
Glucose-1-P (16 mM)	42.5	70.6
Glucose-1-P (16 mM)+ AMP (1 mM)	36.6	66.5
Glucose-6-P (15 mM)	42.0	71.5
Glucose-6-P (15 mM)+ AMP (1 mM)	40.5	67.5
Phosphate (20 mM)	48.8	80.6
Phosphate (15 mM)+ AMP (1 mM)	42.0	72.0
Glucose (15 mM)	50.6	85.0
Glucose (15 mM)+ AMP (1 mM)	40.5	70.5

Fig. 3-4.

Influence of AMP on the inactivation of phosphorylase b by F_2DNB. Phosphorylase b (2mg/ml) was treated with 1-fold (○, ●), 2-fold (△,▲) and 4-fold (□,■) molar excess F_2DNB in the presence (●,▲,■) and absence of (○,△,□) of 1 mM AMP at pH 7.6 in 50 mM tris-HCl buffer. The inactivation was follwed by aliquots withdrawn at various time intervals. Experimental details for assay were as in Fig. 3-1.

Fig. 3-5.

Influence of glucose and inorganic phosphate on the inactivation of phosphorylase b. Enzyme (2mg/ml) was treated with 1-fold (open symbols) and 4-fold (filled symbols) molar excess of F_2DNB in the presence of 15 mM glucose (□,■); 15 mM phosphate (△,▲) and in the absence of any ligands (○,●) at pH 7.6 in 50 mM tris-HCl buffer. Aliquots were withdrawn at various time intervals and diluted(40 times) in cysteine/glycerophosphate buffer and assayed. Other details were as in Fig.3-1.

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was negligible. The pattern of influence of the ligands remained unchanged at 1:1, 1:2, and 1:4 molar concentrations of the enzyme dimer to reagent.

The inactivation of phosphorylase b by FDNB and F_2DNB was compared under identifical conditions. F_2DNB was much more effective than FDNB in inactivating the enzyme. The

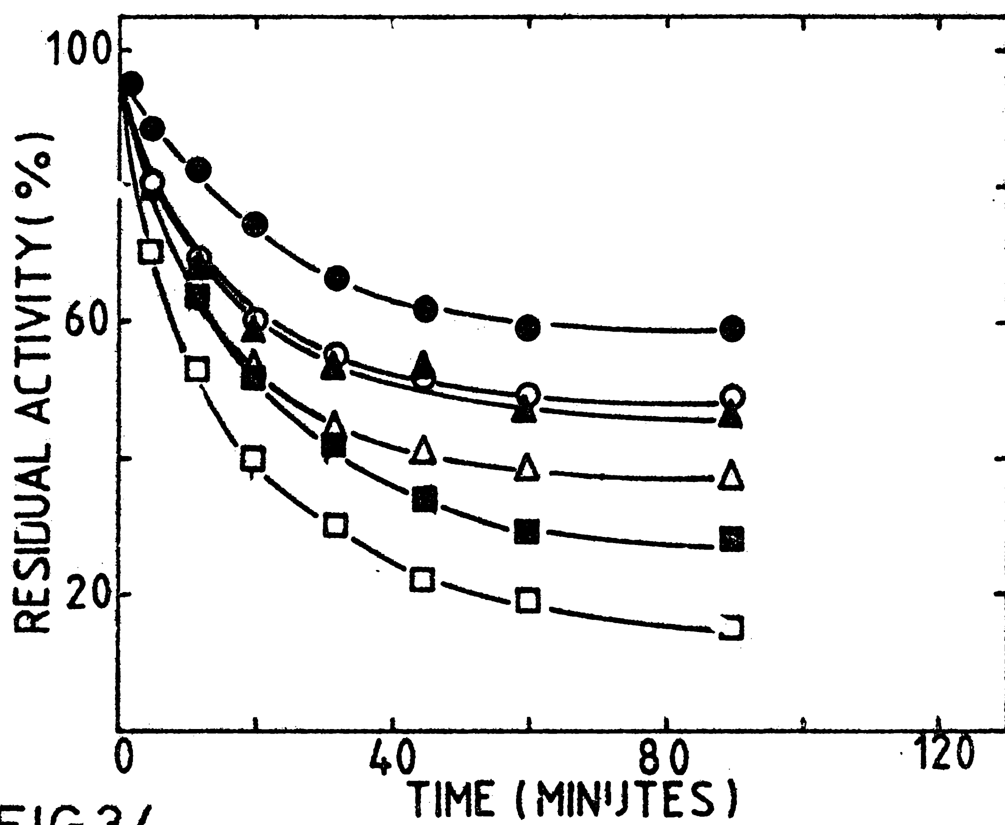

FIG.3.4

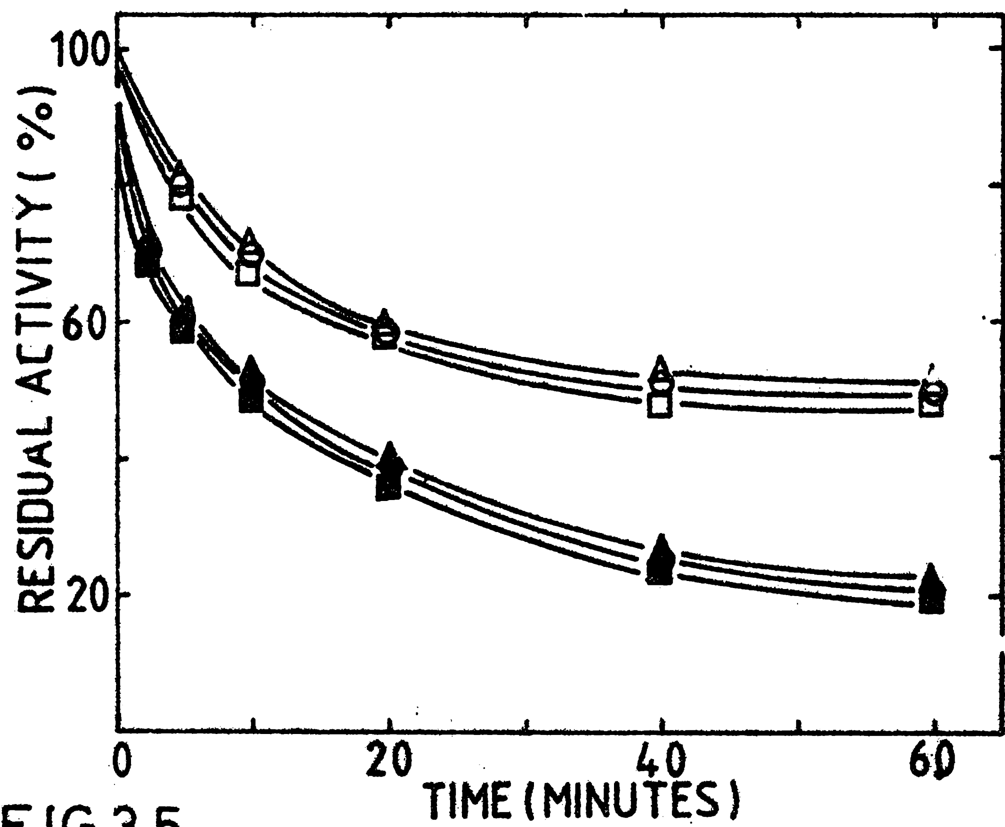

FIG.3.5

Fig. 3-6.

Comparison of the inactivation profile of phosphorylase b by FDNB and F_2DNB at pH 7.6. Enzyme concentration in the reaction mixtures was 2 mg/ml. Concentrations of FDNB were 1.3 fold (○) and 2.6 fold (●) and of F_2DNB, 1.3 fold (▲) and 2.6 fold (▲) molar excess over phosphorylase b. Other experimental details were as in Fig. 3-1.

Fig. 3-7.

Effect of adding F_2DNB in one stretch and in portions on the inactivation of phosphorylase b. 2 mg/ml enzyme in 50 mM tris-HCl buffer pH 7.6 was treated with 1.3-fold molar excess of F_2DNB added in one lot at the beginning of the reaction (△) and the same amount of reagent under identical conditions added in four portions (○). The arrows indicate the time of addition of the reagent.

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inactivation profiles using the two reagents are presented in Fig. 3-6. It can be seen that F_2DNB was much more effective than twice the concentration of FDNB., i.e., same concentration of potential reactive centres in inactivating the enzyme.

Since the reaction with F_2DNB was specific at lower concentration of the reagent, the effect of adding the same amount of the reagent at one stretch or in portions was studied, (Fig. 3-7). In either case, the extent of inactivation was

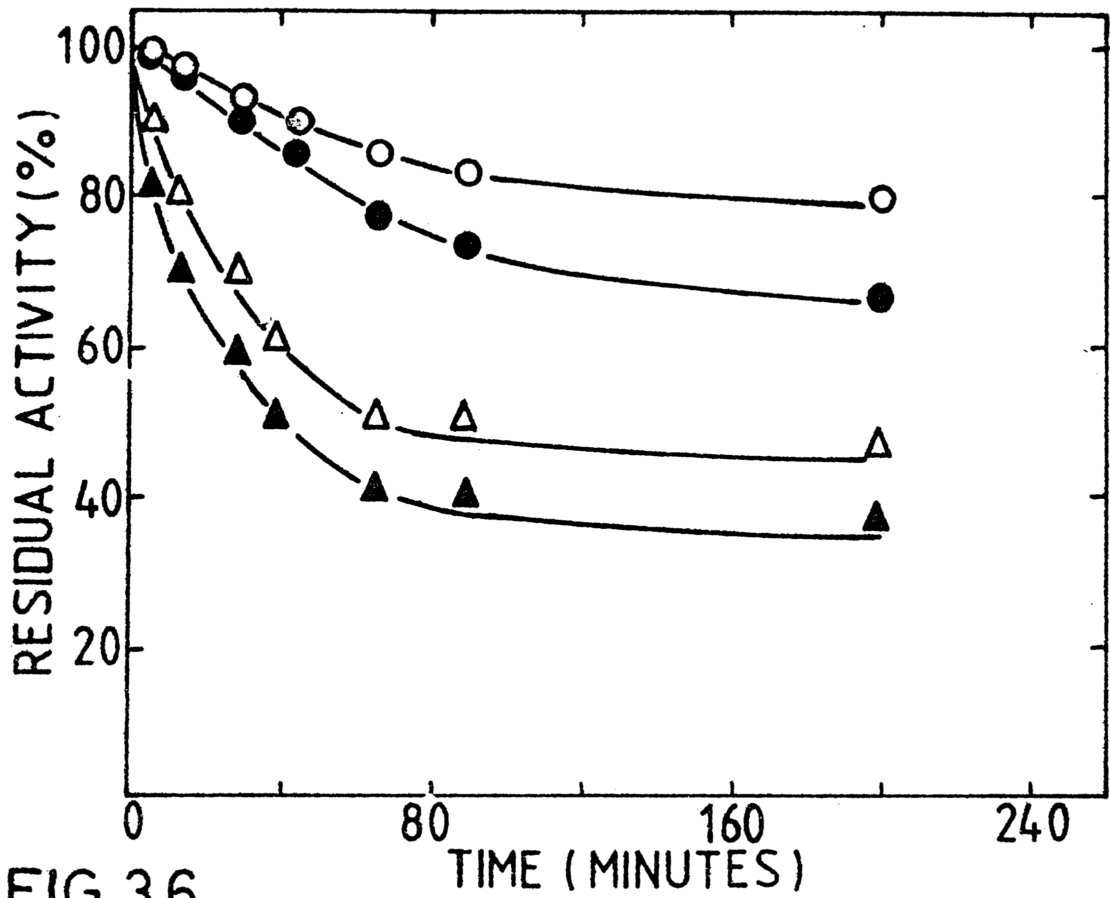

FIG. 3.6

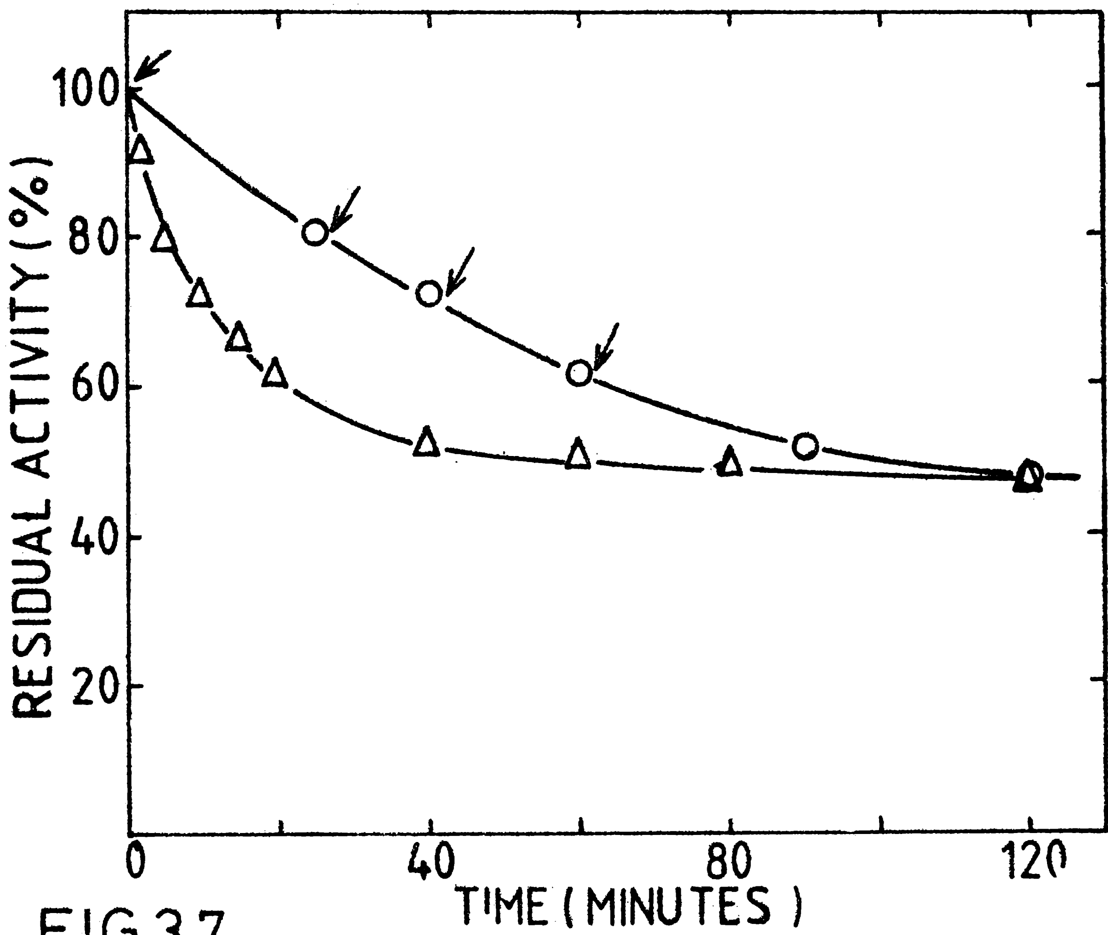

FIG. 3.7

found to be the same. It was also found that when a further molar quantity of the reagent was added to the 50% inactivated sample (prepared by reaction of the enzyme with equivalent molar quantity of the reagent for 1 hour) there was no further decrease in the activity, although the added reagent had completely reacted. These observations confirmed the specificity of the reaction at 1:1 molar concentrations of the reagent and enzyme dimer and suggested that at higher concentrations, the reagent reacted with groups unimportant for catalytic activity.

The binding site of F_2DNB.

Since phosphorylase has a binding site for aromatic compounds (Chapter 2), F_2DNB at lower concentrations will interact non-covalently before reacting with amino acid residues. Protection against inactivation observed with the ligands AMP, glucose-1-P, glucose-6-P, or their combinations had been more or less similar at different molar concentrations of the reagent suggesting that the protection was likely to be of the specific inactivation process. The concentration of the ligands was much more than the concentration of the reagent. Therefore the aromatic site was not completely hindered by the ligands. It is possible that the conformation induced by the ligands had resisted inactivation to some extent. However, it seems unlikely that the activato

the substrate and allosteric inhibitor and their combinations would bring about the same conformational change. Therefore, partial hindrance to the binding of the reagent is more likely to be the reason for the observed protection. Kinetic studies presented in the previous chapter had shown that, the sites of the above ligands were situated very near to each other. Recent X-ray crystallographic studies (100) show that all these ligands bind on the same location in phosphorylase _b_. Therefore it can be concluded that F_2DNB binds on the aromatic binding site through the aromatic moiety of the reagent and by covalent binding through the functional groups of the reagent. Since the latter seems to be retarded by the binding of substrates and effectors (except glucose and orthophosphate) these ligands should bind very near the aromatic binding site. Glucose can be envisaged to be binding on a separate binding site on the enzyme (Chapter 4).

F_2DNB-inactivation and 'SH' groups.

Several authors have reported faster reaction of a few SH groups of phosphorylase _b_ with chemical reagents like iodoacetamide and DTNB (78-83). On the basis of reactivity of the SH groups toward iodoacetamide, Madsen and associates (78) have concluded that two groups reacted without loss of activity and structural alteration. Even more SH groups reacted with chlorodinitrobenzene without any loss of activity (83). These fast reacting groups may be surface

exposed (84). Prolonged dialysis of the enzyme in tris-HCl buffer was shown to destroy these surface exposed groups (84). Therefore in the present study, the enzyme was exhaustively dialysed for 2 days with 3 changes of 25 mM sodium β-glycerophosphate buffer pH 7. The rate and extent of inactivation of the dialysed enzyme was found to be identical with those of the undialysed enzyme showing that the surface exposed SH groups either did not react or if reacted, did not influence the activity. The result also supported the specific nature of the reaction. The specific activity of the enzyme remained unchanged in the presence of 1 mM 2-mercaptoethanol indicating that there was no interaction of thiol groups leading to activity change.

Reaction of F_2DNB with monomerised phosphorylase b.

Inorder to study any influence of SH groups on F_2DNB reaction with phosphorylase b, the reagent was allowed to react with phosphorylase b, monomerised with p-chloromercuribenzoate (78). For this, the enzyme in 0.05 M tris-HCl buffer pH 7.6 was treated with a 15-fold molar excess of PCMB. When the activity was completely lost, (in 90 minutes) it was divided into 3 portions. Two portions were treated with 1- and 2-fold molar excess of F_2DNB and the third portion was used as control. After 1 hour at 30°, 20-fold molar excess of neutral cysteine was added and the reversal of activity was followed. The

Fig. 3-8.

PCMB-monomerized rabbit phosphorylase b (see text) (2 mg/ml) in 50 mM tris-HCl buffer was treated with 1-fold and 2-fold molar excess of F_2DNB. After 1 hour at 30° neutral cysteine was added (20-fold molar excess). Aliquots were withdrawn, diluted (50-fold) in 0.04 M glycerophosphate buffer pH 6.8 and assayed at 30°. The assay mixture contained 16 mM glucose-1-P, 1 mM AMP and 1% glycogen and 18 ug/ml enzyme. ○ ,Control (monomerized enzyme not reacted with F_2DNB); ▲ and ● correspond to enzyme reacted with 1-fold and 2-fold molar excess F_2DNB respectively.

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activity of the control was regained to nearly 100% within 20 minutes. The specific activity of the enzyme treated with 1-fold molar excess F_2DNB reached 50% of the native enzyme within 15 minutes and thereafter remained constant. Activity of the sample treated with 2-fold molar excess F_2DNB reached 30% within 10 minutes and thereafter remained constant. The results are shown in Fig. 3-8. These results showed that inactivation was not due to reaction with SH-groups and that the inactivation was to the same extent with the dimeric or monomeric enzyme.

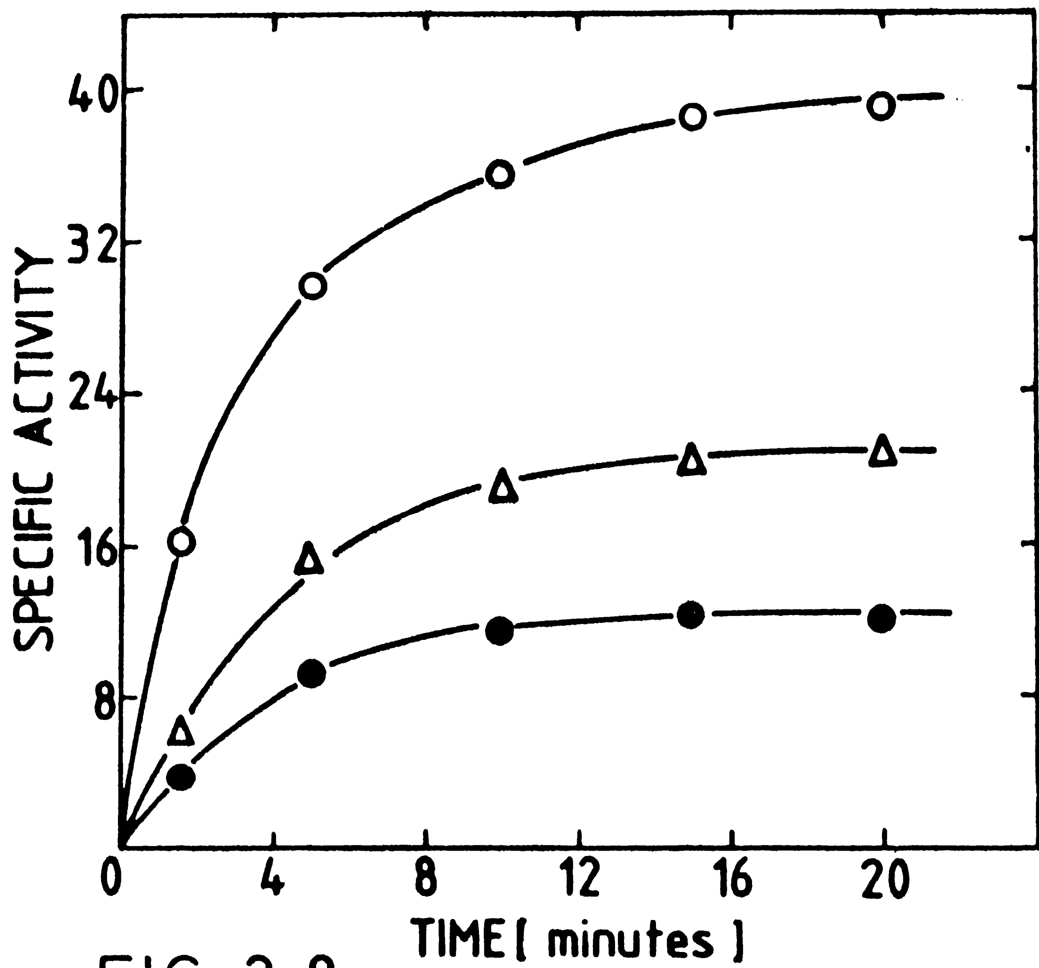

FIG. 3-8

Estimation of number of amino acid residues modified.

The number of DNP- groups incorporated into the enzyme was estimated using benzylamine reagent, originally employed for the estimation of FDNB (134). Benzylamine reacts with unreacted F_2DNB giving a colored DNP derivative, the concentration of which can be colorimetrically estimated. The method obeyed Beer Lambert law in the case of F_2DNB also (Fig. 3-9). By estimating the concentration of F_2DNB before and after its reaction with the protein, the number of F_2DNB incorporated can be calculated. Analysis by this method showed that 0.95 and 1.8 mol of groups were incorporated when 1:1 and 1:2 molar concentrations of phosphorylase b and F_2DNB respectively were employed. The rate of incorporation of the reagent estimated by this method is shown in Fig. 3-10.

Preparation of a homogeneous dinitrophenylene-phosphorylase b

For further studies on the modification of phosphorylase b with F_2DNB, a derivative of the enzyme was prepared using a 1.3-fold molar excess of the reagent due to the following reasons: (i) it was found that the incorporation of the reagent was exactly 1 mol in 60 minutes when a 1.3-fold molar excess of the reagent was employed (ii) at this concentration the inactivation was repeatedly

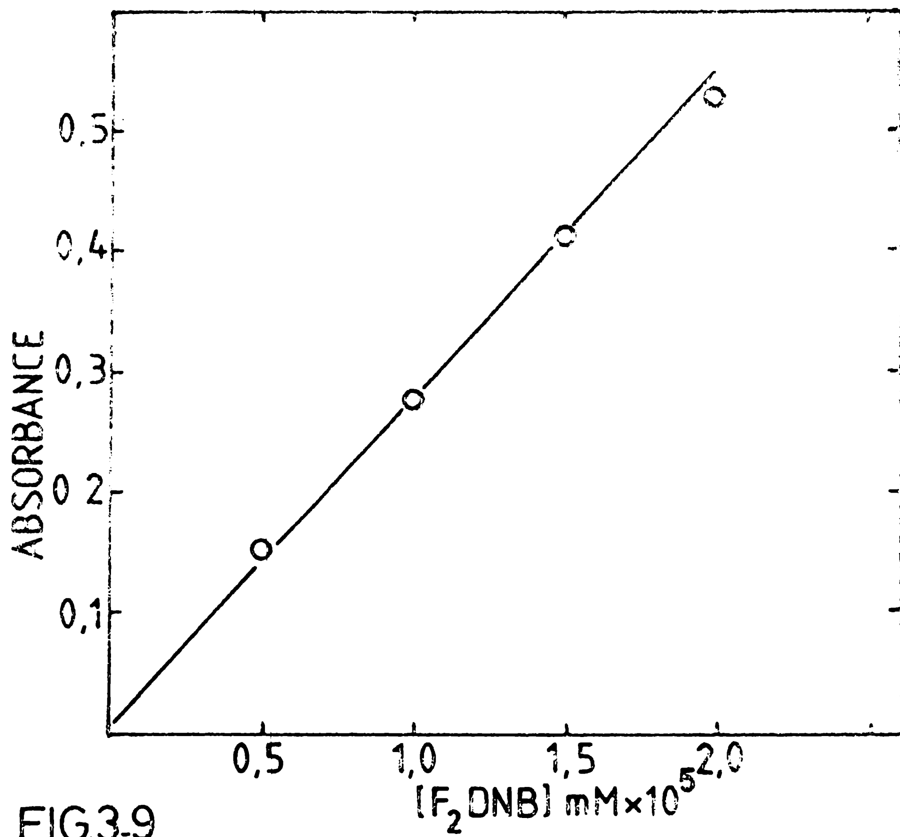

FIG.3-9

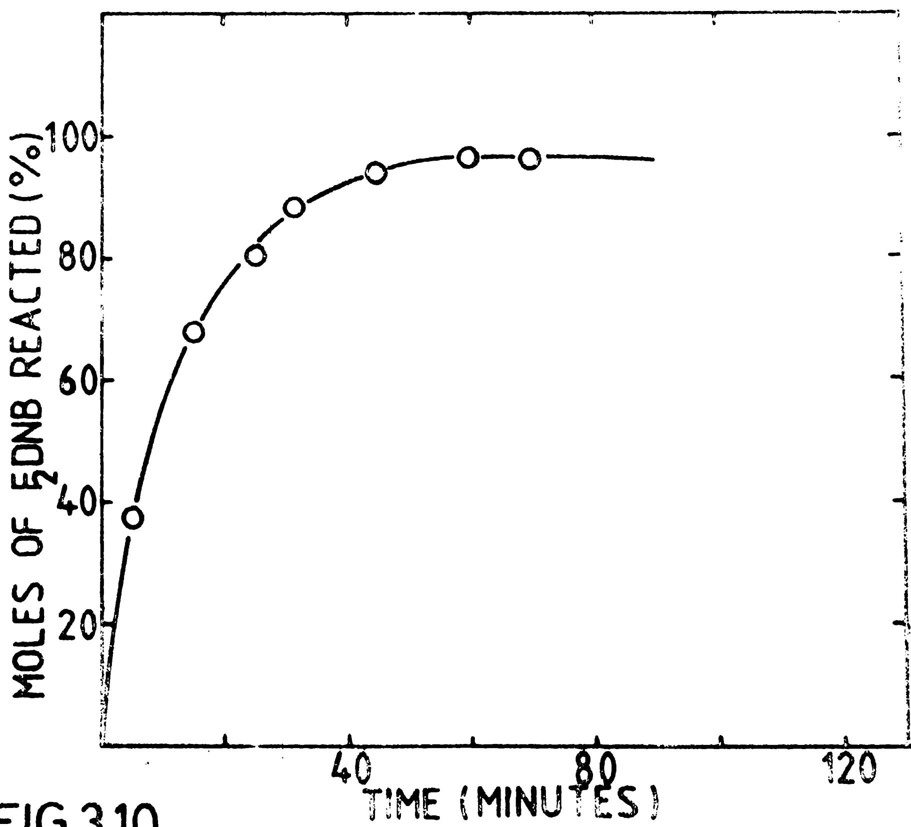

FIG. 3-10

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Fig. 3-9

Calibration curve for F_2DNB using benzylamine reagent. Different concentrations of F_2DNB were reacted with 2% benzylamine in toluene and the absorbance measured at 328 nm.

Fig. 3-10

The number of moles of F_2DNB incorporated per mol of enzyme dimer at various time intervals. 2 mg/ml phosphorylase b in 50 mM tris-HCl buffer pH 7.6 was incubated with 1.0-fold molar excess of F_2DNB. 2 ml portions were withdrawn from the reaction mixture at various time intervals and added to 2.5 ml of a 2.5% benzylamine in toluene, shaken for 30 minutes, toluene layer removed and the absorbance measured at 328 nm. The number of moles of F_2DNB reacted were calculated using the calibration curve of Fig. 3-9.

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found to be exactly 50% (iii) since some hydrolysis of the reagent occurred the slight excess accounted for the hydrolysis and ensured incorporation of 1 mol/mol of the enzyme dimer.

The homogeneity of the derivative was checked by chromatographic methods. Phosphorylase concentration was

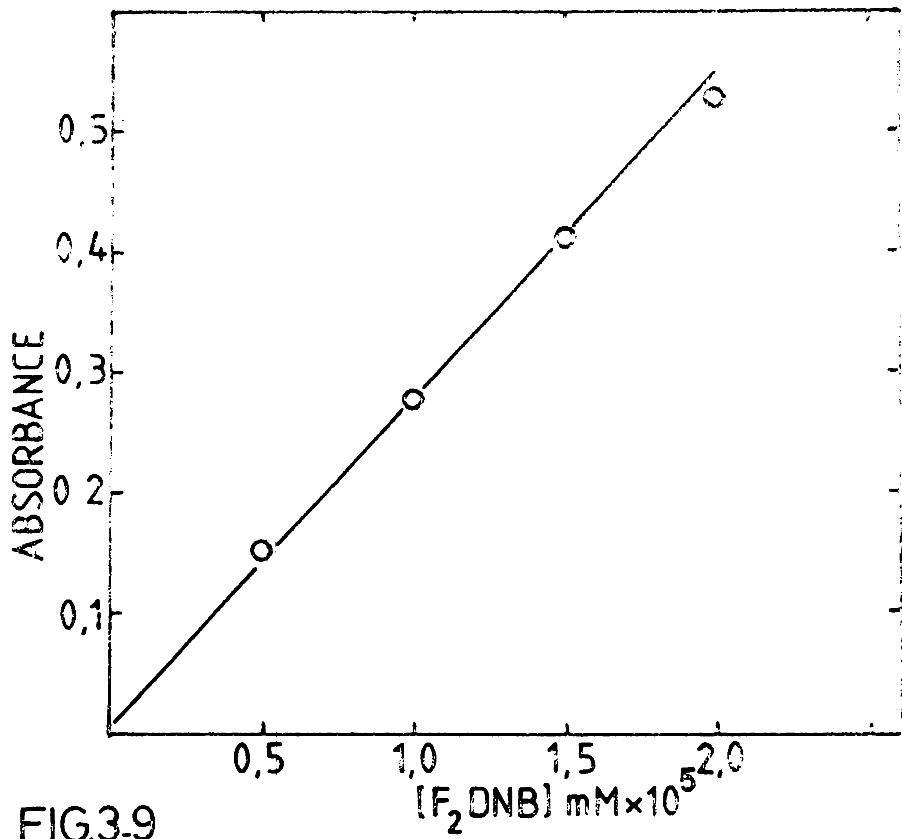

FIG.3-9

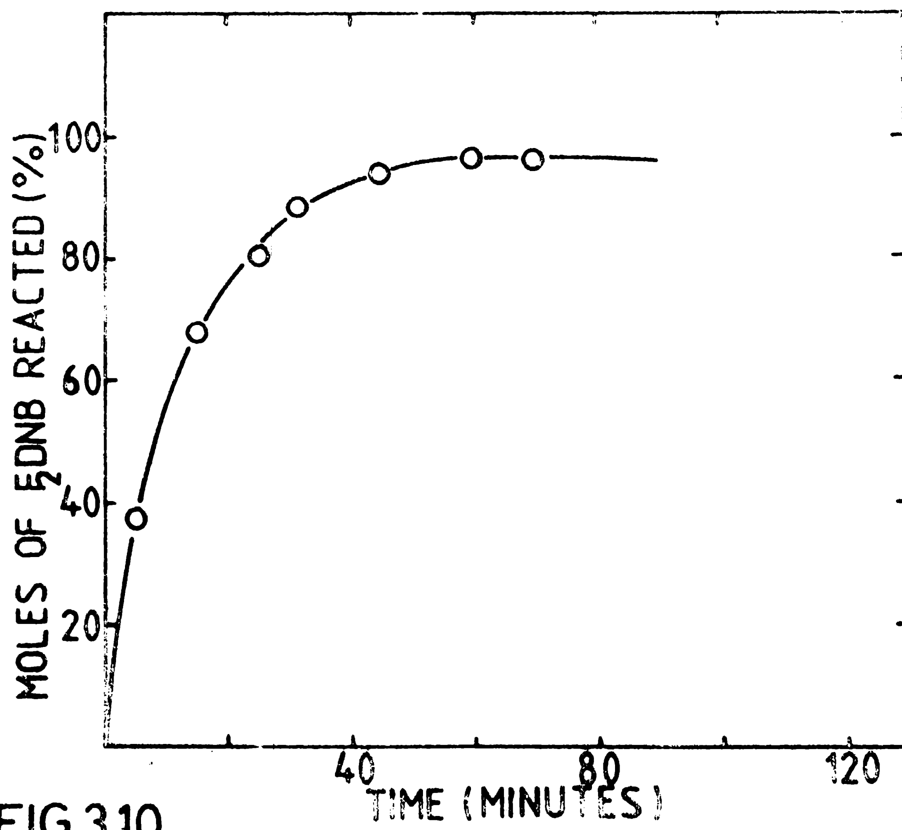

FIG.3-10

maintained at 2 mg/ml of the reaction mixture. The reaction was stopped by precipitating the protein with ammonium sulphate. After removing the salt by passing the enzyme through Sephadex G-15 column, the enzyme was exhaustively dialysed against distilled water and hydrolysed in 6M hydrochloric acid in a sealed tube for 24 hours at 110° ± 5°. The hydrolysate was thoroughly extracted with ether. The ether layer which contained no DNP- amino acids was rejected. The aqueous layer was evaporated under 60°C and the residue was dissolved in water and again dried. After repeating this process, the residue was dissolved in a small amount of distilled water and spotted on a Whatman No.3 filter paper and chromatographed using isobutanol: formic acid: water (75: 14.5:10.5). Three spots with Rf values 0.235, 0.35 and 0.72 were obtained on ascending chromatography at room temperature. The spot with R_f = 0.72 showed on uv-vis. spectroscopic analysis a maximum of 343 nm. The other spots gave broad peaks. The results suggested that the derivative contained more than one species.

Polyacrylamide gel electrophoresis of the 50% inactivated derivative prepared using 1.3-fold molar excess reagent showed a diffused band. Kinetic studies also suggested that the derivative consisted of more than one population of molecules.

Chromatography on DEAE-cellulose, CM-cellulose and Amberlite IRC-50 were not successful in purifying the

the reaction mixture. With the cellulose derivatives the total amount of the protein fed was eluted in a single peak on elution using a concentration or pH gradient. With the amberlite, the protein could be eluted out only under conditions that led to partial inactivation.

If the F_2DNB-reacted phosphorylase b consisted of tetrameric forms formed as a result of intermolecular cross-linking they could be detected by gel filtration on Sephadex G-200. Fig. 3-11 shows gel filtration profiles of the modified and native enzyme. The figure shows that some cross-linking had occurred and that the tetramer (or oligomers, if any) was eluted out in the void volume of the column. The major portion of the derivative was eluted with the same elution volume as that of the native enzyme dimer.

Since reaction with p-chloromercuribenzoate would convert phosphorylase completely into monomers, this reaction was used to find the extent of intersubunit cross linking in the derivative. These results are also given in Fig. 3-11. The major portion of the derivative was eluted with the same elution volume of the native enzyme monomers. The minor band with lower elution volume appeared to represent cross-linked protein which probably came from the protein eluted out in the void volume.

Fig. 3-11.

Gel filtration on Sephadex G-200 of native and F_2DNB-reacted phosphorylase *b* before and after reaction with *p*-chloromercuribenzoate. ●, F_2DNB-reacted phosphorylase *b* (35 mg) prepared by reacting with a 1.3-fold molar excess of F_2DNB., ○, native phosphorylase *b* (40 mg); ▲, F_2DNB-reacted phosphorylase *b* after treatment with p-chloromercuribenzoate (35 mg); △, native phosphorylase *b* after treatment with *p*-chloromercuribenzoate (18 mg); X--X, enzyme activity profile of the F_2DNB-reacted phosphorylase *b*. Fraction volume in all cases were 5 ml. Other details are given in 'experimental'. The figures in brackets refer to the amount of protein fed on the column.

The fractions 19-23 from the F_2DNB-reacted enzyme (Fig. 3-11) were pooled, adsorbed in a small DEAE-cellulose column at pH 6.8 in 0.01 M sodium glycerophosphate. Concentration of the enzyme was achieved by elution with the same buffer of higher concentration (0.5 M). The concentrated enzyme when treated with *p*-mercuribenzoate and rechromatographed on the same G-200 column, the total amount of protein was eluted with an elution volume of the monomer.

The purified enzyme thus obtained was used for further studies. Polyacrylamide gel electrophorsis of

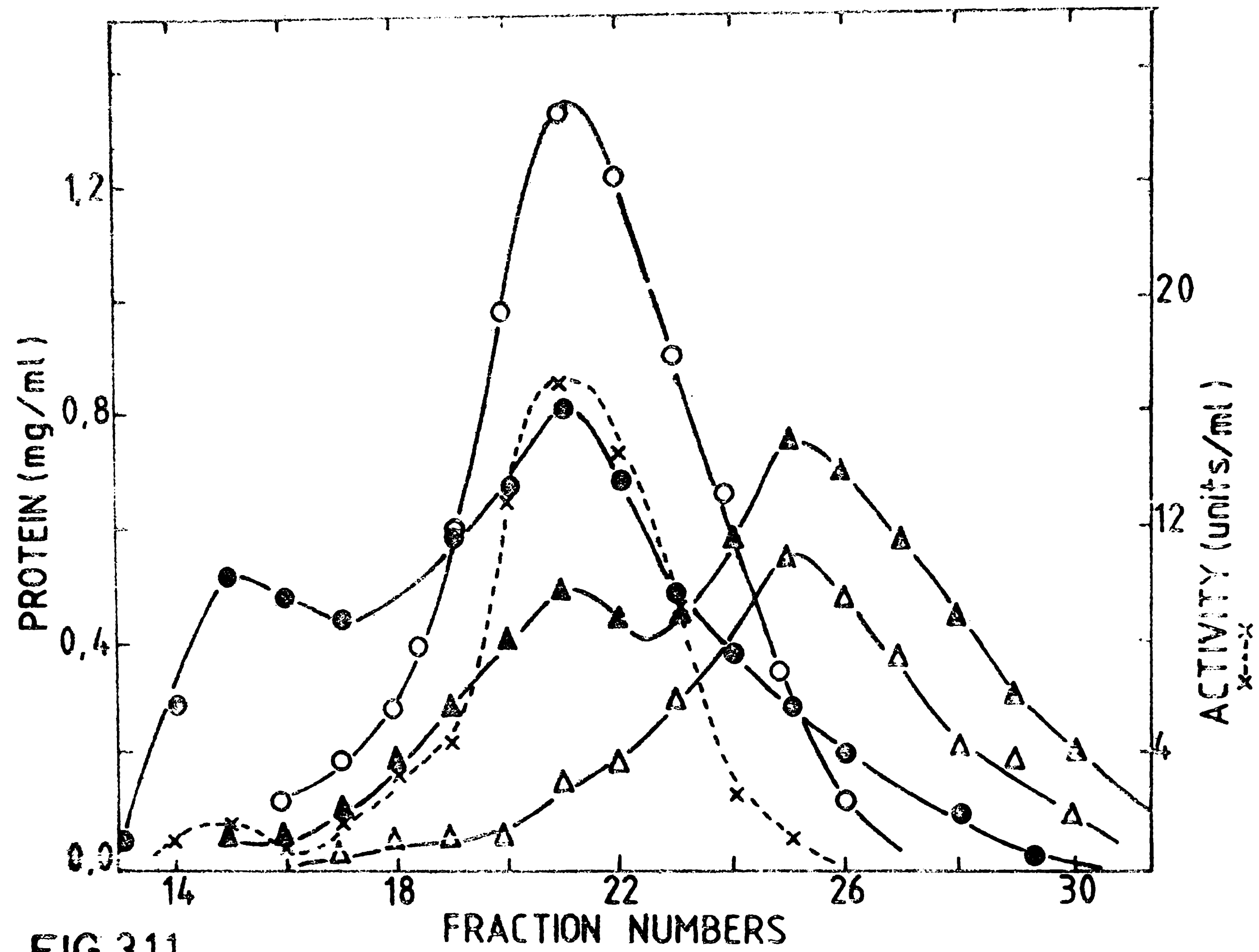

FIG.3-11

the purified derivative showed a single band. It was presumed that the fractions contained only one species. Identical results were obtained on repeating the reaction and the gel filtration several times. The dinitrophenylene phosphorylase b thus obtained showed a specific activity of 20 (μmoles of inorganic phosphate liberated/minute /mg of protein) when assayed in the presence of 1% glycogen, 16 mM glucose-1-P and 1 mM AMP at 30°C. Under these conditions, the specific activity of the native enzyme was 40.

Did the dinitrophenylene phosphorylase (fractions 19-23 of Fig. 3-11) consist of an equimolar mixture of completely inactive and fully active dimeric enzyme molecules or of a homogeneous population of 50% inactive dimeric molecules? Kinetics with modified enzyme did not show major changes in K_m for AMP or glucose-1-P (See Chapter 4), ruling out the possibility of affinity changes as the reason for the decreased activity of the derivative. Electrophoresis of the derivative gave only one band. If it were a mixture, further addition of the reagent would have inactivated the enzyme completely. Infact, the added reagent had reacted without influencing the activity. Therefore, the dinitrophenylene phosphorylase b obtained by Sephadex gel filtration seems to represent a homogeneous population of dimeric molecules with only 50% of the activity of the native enzyme

and with 1 mol of the aromatic group/mol of the dimeric phosphorylase *b*.

The dinitrophenylene phosphorylase could be monomerised completely and reconstituted like native enzyme showing that the subunits were not cross-linked in the derivative. The specificity of the reaction, the results of Fig. 3-7, and the fact that 1 mol of group was incorporated per mol of dimeric enzyme suggested that phosphorylase *b* dimer has only one exposed site where the reagent could bind and react, resulting in inactivation. The subunits of phosphorylase are similar and are arranged with a center of symmetry (97-100). Hence the incorporation of the group was likely to be at the subunit interface.

It is possible that one subunit in the dinitrophenylene phosphorylase *b* dimer was completely inactive and the other subunit was potentially active. It has been reported (135) from hybridisation experiments that an inactive monomer can induce activity in a potentially active monomer of phosphorylase so that an active monomer-inactive monomer hybrid has 50% activity of the native enzyme.

There is considerable similarity in the inhibition pattern of phosphorylase *b* by aromatic compounds (76,77) and AMP analogues (136, 137). Phosphorylase from *Escherichia coli* which was not affected by AMP was not inhibited by phenol or nitrophenol at concentrations where

the rabbit enzyme was 50% inhibited (unpublished observation). Therefore it seemed likely that the binding site for aromatic compounds and AMP is the same in phosphorylase b. Combining the earlier conclusion from our results, the binding site of glucose-1-P, AMP, ATP, glucose-6-P and aromatic compounds are all located in the same region. The recent crystallographic studies (67,100) are in agreement with the conclusions arrived from our studies.

There is a second site in phosphorylase a which binds nucleosides and purines preferentially and is located towards the centre of the monomer near the pyridoxal-5'-phosphate (40). Even if such a site exists in phosphorylase b the results of these studies are not in agreement with assigning the aromatic binding site to this nucleoside site.

CHAPTER 4

PROPERTIES OF THE

DINITROPHENYLENE PHOSPHORYLASE.

The homogeneous preparation of the dinitrophenylene phosphorylase b (DPE-phosphorylase b) was found to be as stable as the native phosphorylase b, at 0-5° in 25 mM sodium β-glycerophosphate/10 mM mercaptoethanol buffer, pH 6.9. A 5 mg/ml solutions of DPE-phosphorylase b and native phosphorylase b retained their respective specific activity for at least 10 days.

The derivative was, however, less stable in the presence of urea as compared to the native enzyme (Fig. 4-1). The stability of the enzyme was considerably less at elevated temperatures (Fig. 4-2). The rate of inactivation of the native enzyme and the derivative by trypsin was nearly the same (Fig. 4-3). These results suggested that the tertiary structure of the protein was affected only slightly as a result of the modification. Hydrophobic regions of proteins are generally stable till about 60° (138). The increased sensitivity towards temperature of the derivative thus indicated a sli ht loosening of the hydrophobic region near the active site. However, such effect did not expose considerably the regions of tryptic attack.

Fig. 4-1.

Urea stability of the DPE-phosphorylase b. Native enzyme (open symbols) and DPE-enzyme (filled symbols) in 0.05 M glycerophosphate buffer pH 6.8 were incubated with 0.5 M (△,▲) and 1M (○,●) urea at 30°. Aliquots were diluted in cysteine/glycerophosphate buffer and assayed immediately. The assay mixture contained 16 mM glucose-1-P, 1 mM AMP, 1% glycogen, 15 mM cysteine and 20 mM glycerophosphate at pH 6.8.

Fig. 4-2.

Thermal stability of DPE-phosphorylase b. Native enzyme (open symbols) and DPE-enzyme (filled symbols) (2 mg/ml) in 40 mM glycerophosphate buffer pH 6.8 were incubated at 40° (○,●); 50° (△,▲).and 60° (□,■). Aliquots were diluted in 40 mM glycerophosphate/30 mM cysteine at pH 6.8 and incubated for 20 minutes and 30° prior to assay. The assay mixtures contained 16 mM glucose-1-P, 1 mM AMP and 1% glycogen.

The total number of SH groups in the DPE-enzyme was estimated using Ellman's reagent (DTNB)(139). For this, the enzyme was freed from mercaptoethanol by passage through a Sephadex G-15 column equilibrated with 0.05 M tris-HCl buffer pH 8. This was made 6 molar in urea and treated with 25-fold molar excess DTNB and the SH groups were

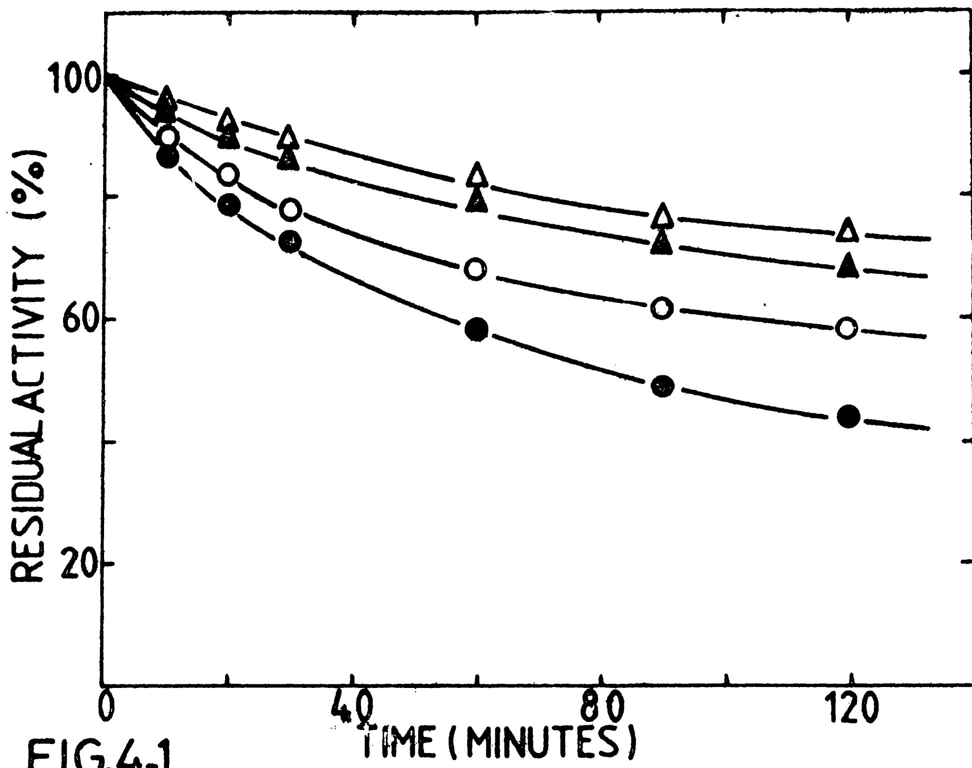

FIG.4-1

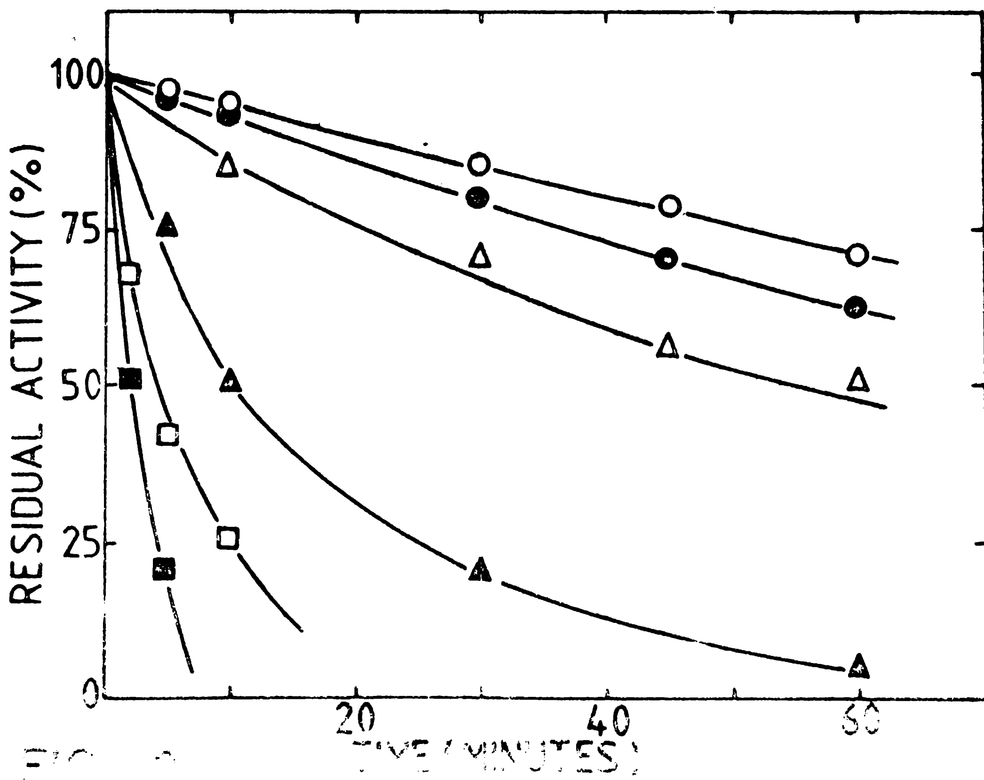

Fig. 4-3.

Rate of trypsin inactivation of native and dinitrophenylene phosphorylase b. Native and DPE-enzymes were taken in 40 mM mercaptoethanol pH 6.8 by passing the enzyme through Sephadex G-15 column equilibrated with the buffer. To 1 ml each (2.5 mg/ml), added 20 μlitre of trypsin solution (2 mg/ml) in 10^{-3} M HCl. Aliquots were transferred to 40 mM glycerophosphate pH 6.8 containing trypsin inhibitor. An aliquot from this was again diluted (10-fold) in 40 mM glycerophosphate/30 mM cysteine buffer pH 6.8 and assayed. Two controls were also assayed (without addition of trypsin).
● ,control (native enzyme), ○ ,trypsin added native enzyme; ▲ , control (DPE-enzyme) and △ , trypsin added DPE-enzyme.

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estimated using cysteine as standard. The total number of SH groups in both the native and DPE-enzymes were repeatedly found to be 16.2 ± 0.2 moles per mol of the enzyme dimer (M=200,000). Thus above experiments showed that SH groups were not modified in the DPE-enzyme and confirmed the earlier observations given in Chapter 3.

The coenzyme pyridoxal-5'-phosphate in the DPE-phosphorylase b was resolved in imidazole citrate in presence of L-cysteine according to the procedure of

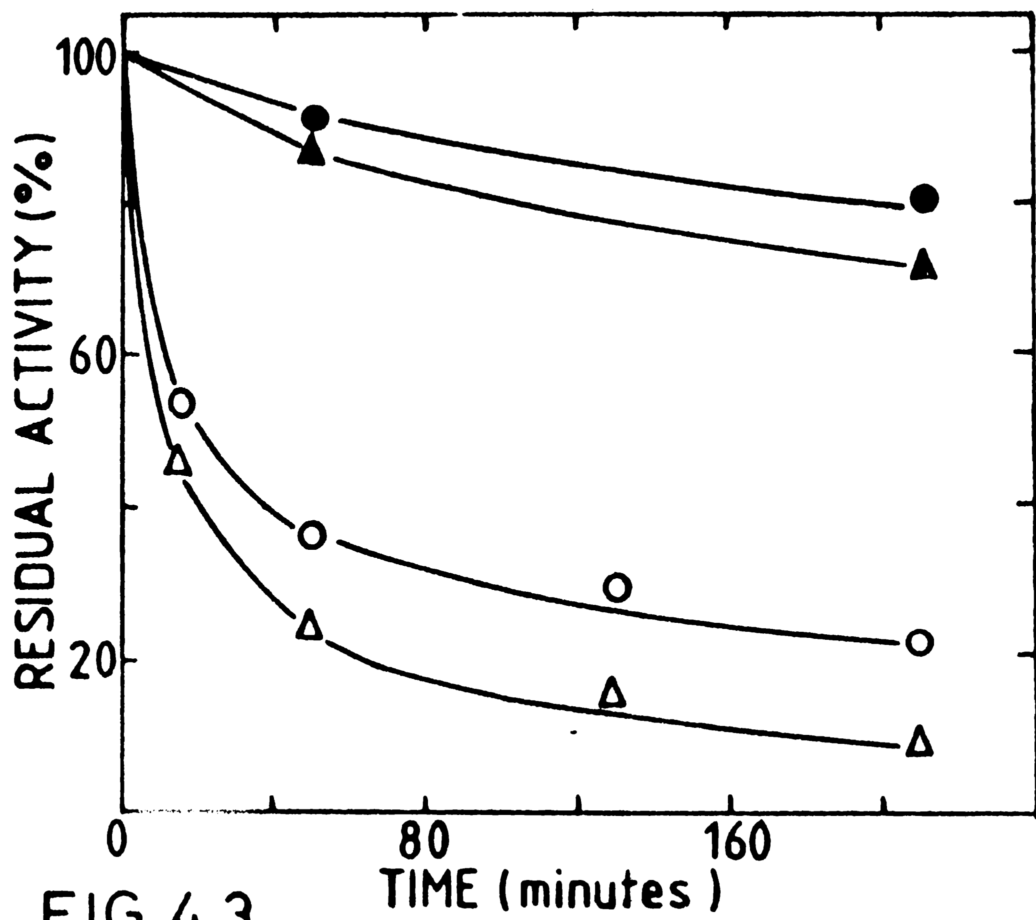

FIG. 4.3

Fig. 4-4.

Rate of reconstitution of pyridoxal phosphate in DPE-apophosphorylase in comparison to the native apophosphorylase. The apoenzymes were prepared as described in 'experimental'. To the native and DPE-apoenzymes (1.5 mg/ml) in 50 mM glycerophosphate-mercaptoethanol buffer pH 6.8 were added pyridoxal-5'-phosphate solution (also at the same pH) to a final concentration of 5×10^{-5} M. Aliquots were withdrawn at various time intervals, diluted (40-fold) in 30 mM cysteine /40 mM glycerophosphate pH 6.8 and assayed immediately. ○ ,native enzyme and ● ,DPE-enzyme.

Shaltiel *et al.* (59). After resolution, PLP and other molecules were removed by passage through Sephadex G-15 column equilibrated with 25 mM β-glycerophosphate buffer pH 6.9. The dinitrophenylene apoenzyme was completely devoid of its activity like the native apoenzyme. However, the rate and extent of reconstitution of PLP in DPE-enzyme using pure pyridoxal phosphate was found to be considerably less than in the native enzyme under the same experimental conditions (Fig.4-4). Within 40 minutes, 90% of the activity was regained in native enzyme whereas only 25% of the activity was regained in the DPE-phosphorylase.

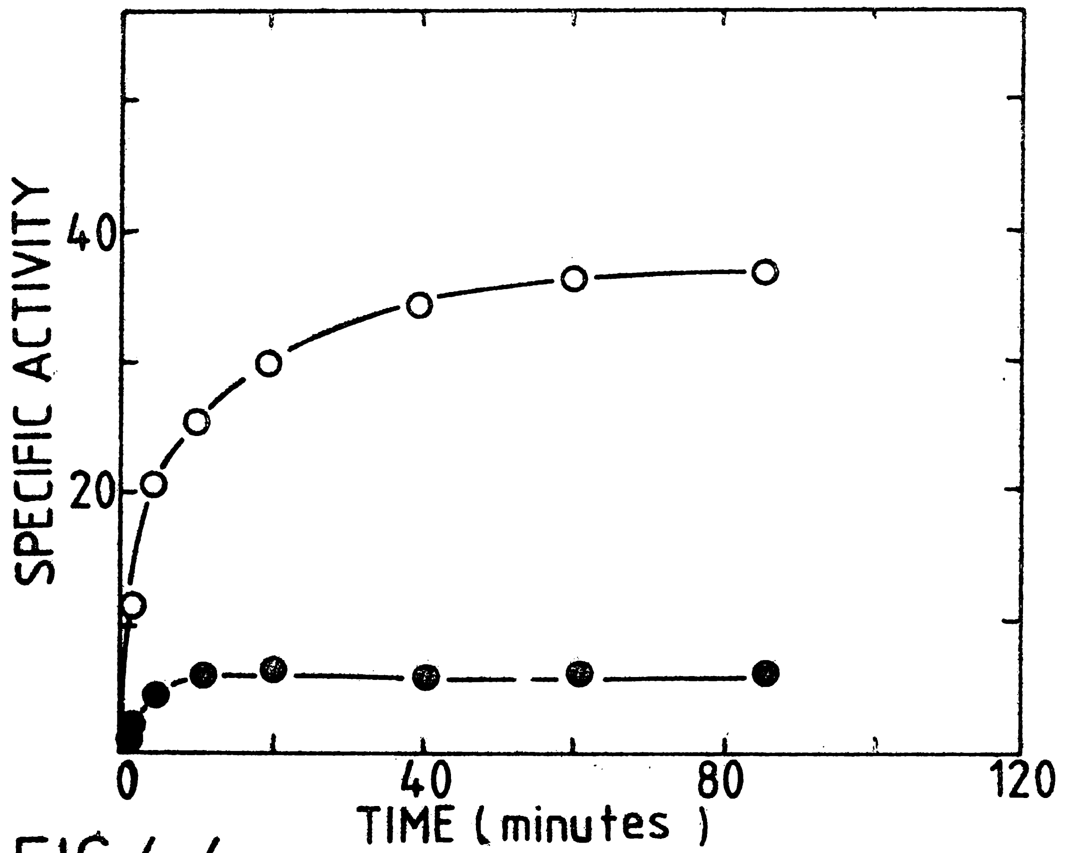

FIG.4-4

The specific activity of the reconstituted DPE-enzyme was 5 units/mg and that of the native enzyme was 35 units/mg (The initial specific activities were 20 and 40 for the DPE-and native enzymes respectively). Thus the reconstitution of DPE- apophosphorylase did not lead to the original conformation completely.

A finger-print analysis of the DPE-phosphorylase b was attempted after completely hydrolysing with trypsin and comparing it with native phosphorylase b. The hydrolysed samples were concentrated by evaporation and spotted on one end of a 25x25 cm Whatman no.1 paper (8 cm away from the anode). Electrophoresis was conducted using pyridine-acetic acid buffer pH 6.5. After drying the paper, ascending chromatography was performed in perpendicular direction using isobutanol: formic acid: water (75:14.5:10.5) as solvent. No yellow spots could be identified after the analysis. When a larger amount of the peptide mixture was applied, an initial trailing of the yellow color was observed which disappeared on continuing the electrophoresis. When the spots were identified using Ninhydrin, the position corresponding to one spot in the native enzyme appeared in the DPE-enzyme paper as two nearer spots. Also there was one more additional spot in the finger-print of the DPE-enzyme towards the middle of the paper. The positions of all other spots were identical (Fig. 4-5). The results

Fig. 4-5

Finger-print analysis of native and DPE-phosphorylases. The native and DPE-phosphorylases were separately hydrolysed with trypsin and a concentrated sample of the hydrolysate was spotted on one end of a Whatman No.1 paper (25x25 cm). Electrophoresis was conducted using p yridine: acetic acid buffer at pH 6.5 using a constant voltage (8 volts/cm) for 10 hours. Ascending chromatography was conducted in the perpendicular direction using isobutanol: formic acid: water as solvent. The separated peptides were developed by Nihydrin. (Details are given in experimental') Fig. A: Native phosphorylase b Fig.B: DPE-phosphorylase b. The points indicated by arrows show the points of application

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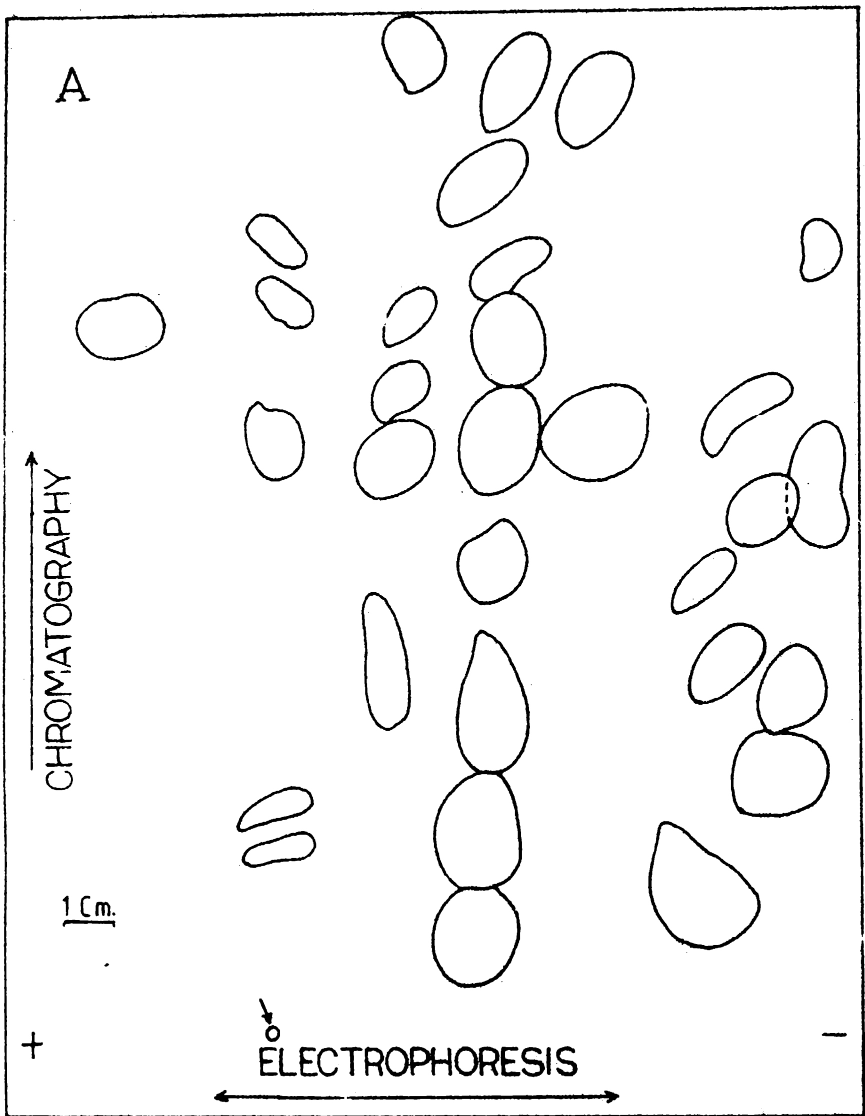
showed that the conformational change brought about by the incorporation of the reagent was not considerable.

The absorption spectrum of the DPE-phosphorylase showed a peak around 330 nm. To correct the effect due to PLP, the difference spectrum of the DPE-enzyme was taken using native enzyme as the reference (Fig.4-6). The spectrum showed a broad peak with 2 shoulders at 330 nm and 360 nm. The difference spectrum taken after treatment with 5 molar urea also gave a spectrum similar to untreated



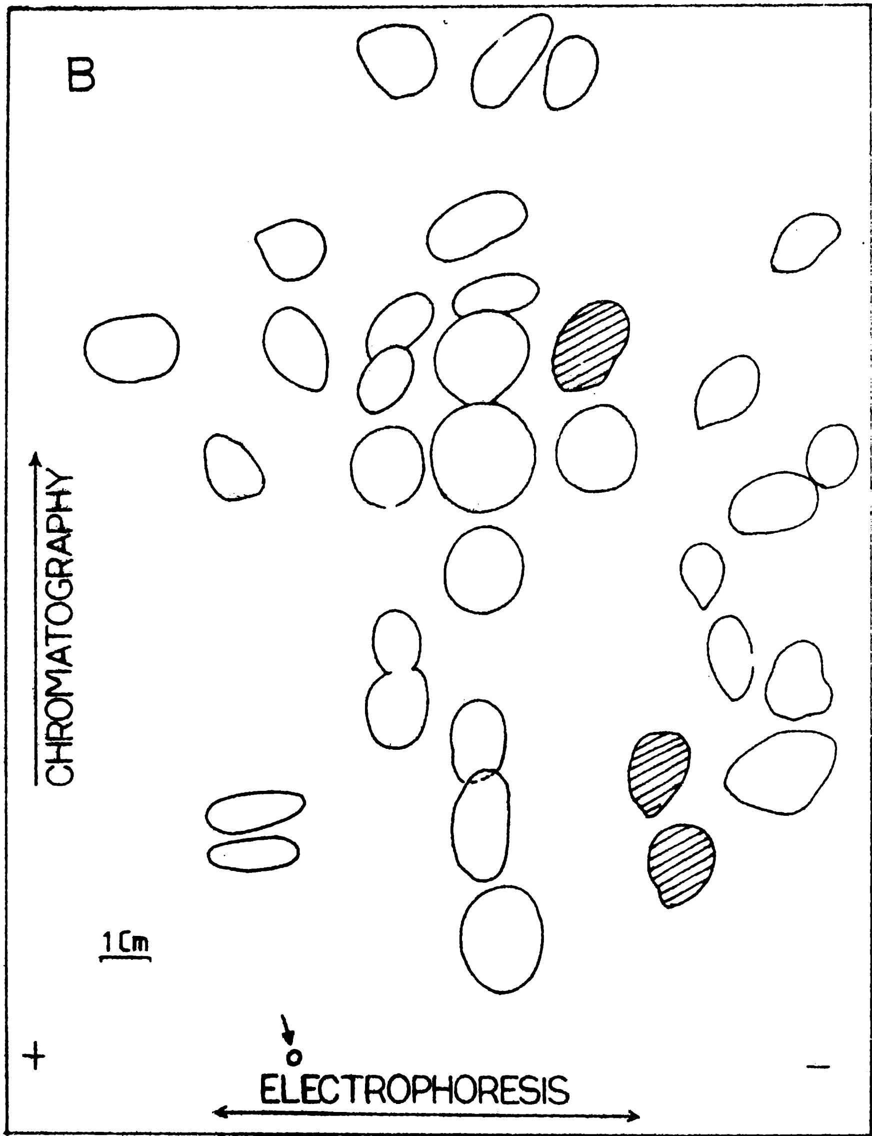


FIG 4-5

Fig. 4-6

Difference spectrum of DPE-phosphorylase against native phosphorylase as reference, in 40 mM glycerophosphate buffer pH 6.8. The enzyme concentration was 1.45 mg/ml. The spectrum was taken using a Hitachi model 200 recording uv-visible spectrophotometer.

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derivative. PCMB- monomerised enzyme also gave a similar spectrum. The difference spectrum (Fig. 4-6) compares well with the spectrum reported for ϵ-N-lysyl,O-tyrosyl, dinitrodiphenylene (140,141). Thus one lysyl and one tyrosyl residues were modified in DPE-enzyme. (The spectrum of Fig. 4.6 was identical to the spectrum for the spot with R_f= 0.235 obtained by hydrolysis of the unpurified F_2DNP-derivative mentioned in Chapter 3).

Kinetics of dinitrophenylene phosphorylase b

The kinetics for glucose-1-P and AMP of the DPE-phosphorylase b in the presence and absence of glucose-6-P ATP and glucose are presented in Figs. 4-7 and 4-8. The K_m values and Hill coefficients obtained from Hill plots (not shown) for the derivative in the presence and absence of these inhibitors are compared with those of the native enzyme in Table 4-1. From figs. 4-7 and 4-8 and from the table the following observations could be made;

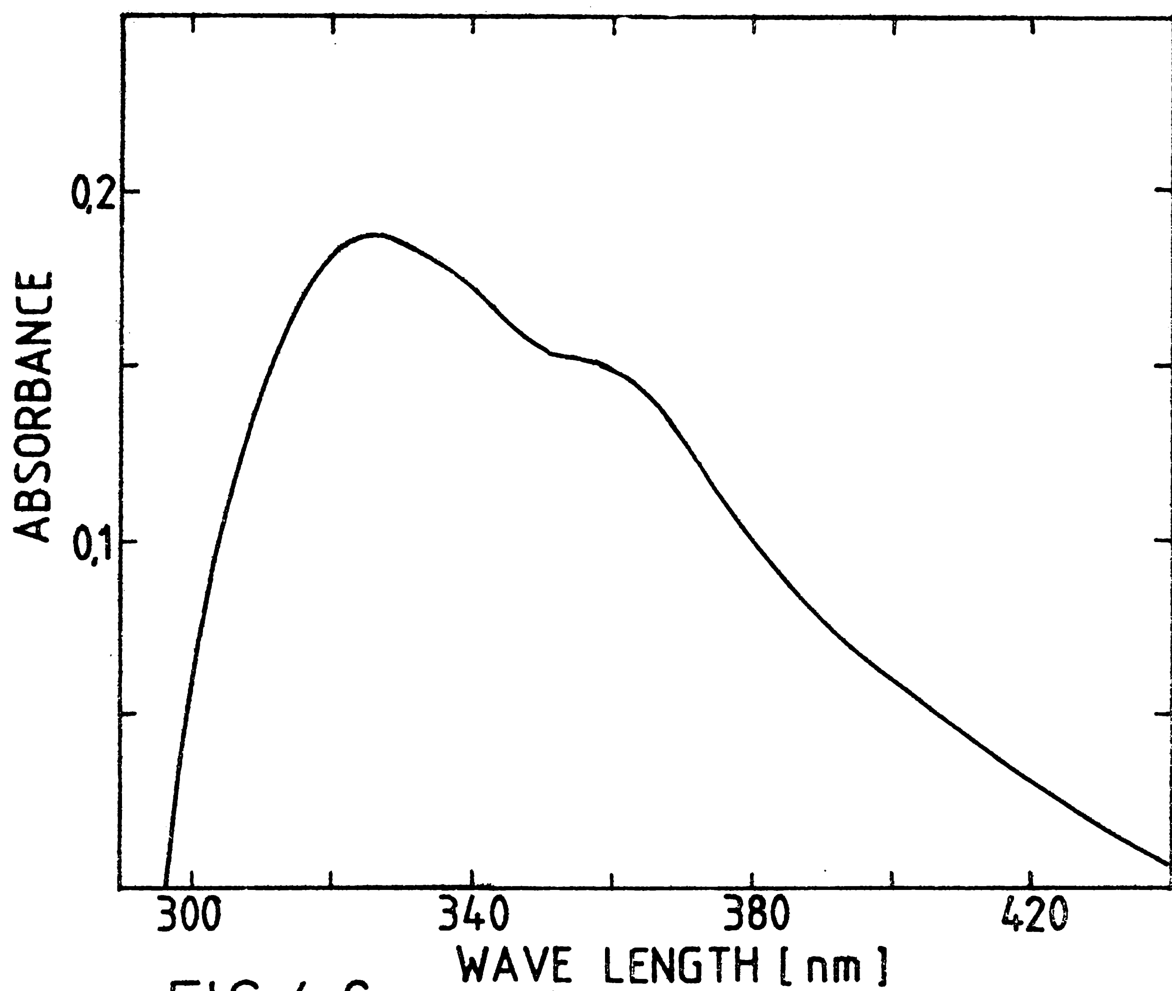

FIG.4-6

Fig. 4-7.

Lineweaver-Burk plots for glucose-1-P in the absence and presence of glucose, glucose-6-P and ATP for DPE-phosphorylase b. The assay mixtures contained 18 μg/ml enzyme, 1% glycogen, 1 mM AMP and varying concentrations of glucose-1-P in 15 mM cysteine/20 mM glycerophosphate buffer pH 6.8. ○, no inhibitor; ●, 10 mM glucose-6-P; △, 10 mM ATP and, ▲, 10 mM glucose. Assay temperature was 30°.

Fig. 4-8.

Lineweaver-Burk plots for AMP in the absence and presence of glucose, glucose-6-P and ATP for DPE-phosphorylase b. The assay mixtures contained 1% glycogen, 16 mM glucose-1-P and varying concentrations of AMP. ○, no inhibitor; ●, 10 mM glucose-6-P; ▲, 10 mM ATP and △, 10 mM glucose. Others details were as in Fig. 4-7.

(i) the dinitrophenylene phosphorylase b did not show any homotropic cooperativity between substrate sites and between activator sites; (ii) the inhibition of the derivative by ATP and glucose-6-P was competitive in nature with respect to the kinetics of AMP and glucose-1-P. (iii) Glucose, unlike other ligands, induced cooperativity of sites like in the native enzyme. From these results and the results in

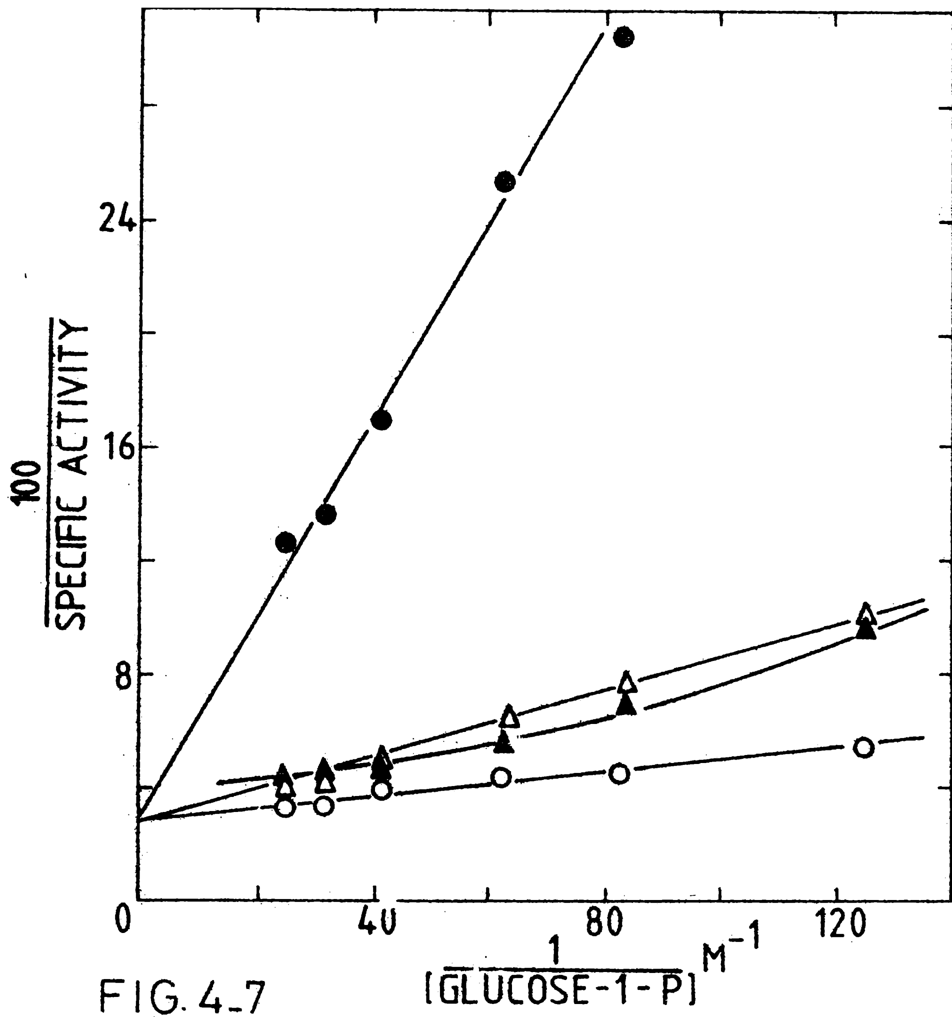

FIG. 4-7

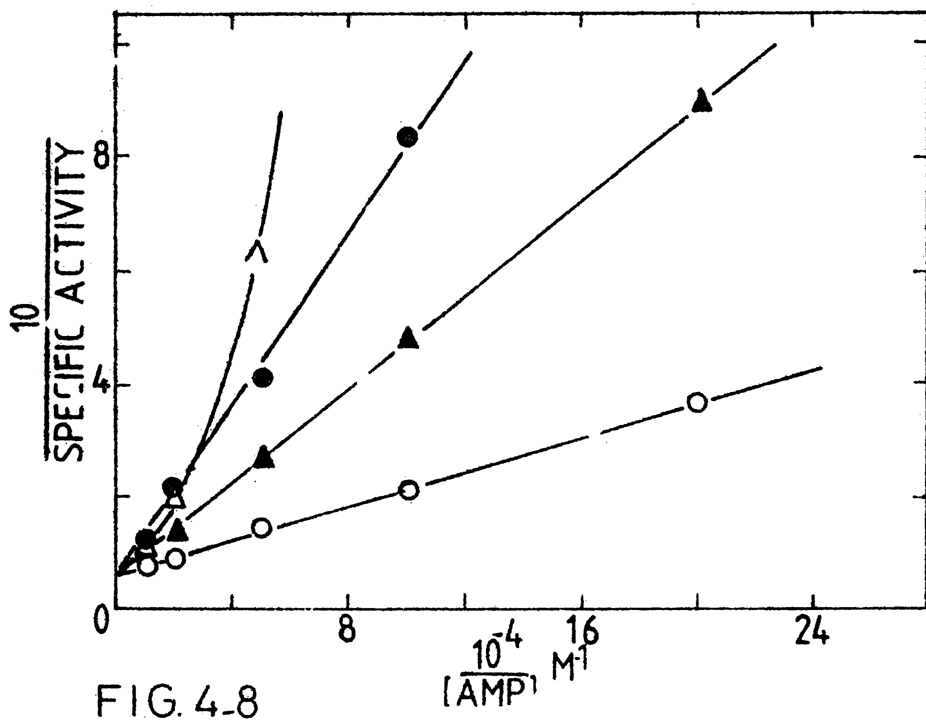

FIG. 4-8

TABLE 4-1

INFLUENCE OF GLUCOSE, GLUCOSE-6-P AND ATP ON THE K_m VALUES AND HILL COEFFICIENTS FOR GLUCOSE-1-P AND AMP FOR THE NATIVE AND DINITROPHENYLENE PHOSPHORYLASE b

Conditions of experiments were the same is in Fig.4-7 and Fig.4-8. The K_m and n values were taken from Hill plots.

	Dinitrophenylene phosphorylase b		Native phosphorylase b	
	K_m (M)	n	K_m (M)	n
Glucose-1-P kinetics				
No inhibitor	7.1×10^{-3}	1.1	7.5×10^{-3}	1.0
10 mM glucose-6-P	1.0×10^{-1}	1.0	1.5×10^{-2}	1.6
10 mM ATP	1.7×10^{-2}	1.1	1.3×10^{-2}	1.5
10 mM glucose	1.1×10^{-2}	1.5	1.8×10^{-2}	1.5
AMP kinetics				
No inhibitor	2.5×10^{-5}	1.0	5.0×10^{-4}	1.[illegible]
10 mM glucose-6-P	1.0×10^{-4}	1.0	2.5×10^{-4}	1.6
10 mM ATP	6.3×10^{-5}	1.1	1.0×10^{-4}	1.5
10 mM glucose	8.3×10^{-5}	1.6	7.7×10^{-5}	1.8

the previous chapter it becomes clear that, except glucose, all other ligands bound on the enzyme on the same region and the binding of glucose caused allosteric transition of the enzyme.

It may be noted that native phosphorylase b behaved differently under these conditions. ATP and glucose-6-P have been shown to increase the homotropic cooperativity of phosphorylase b (8). The Hill coefficients for native enzyme in the presence of ATP and glucose-6-P for the kinetics with respect to both AMP and glucose-1-P would be in the range 1.5 to 2. But for DPE-enzyme the coefficients were nearly 1 indicating desensitization. However since glucose induced cooperativity, the derivative could be considered as partially desensitized. Glucose-6-P which has been shown to be an allosteric inhibitor for native phosphorylase b became a competitive inhibitor of the DPE-enzyme. Since the structural deformations in the modified enzyme were not considerable, the changes observed for the inhibition pattern could be assigned to the presence of the hydrophobic dinitrophenylene group in a specific region on the enzyme. That this region is in the neighbourhood of AMP-binding site is significant. Then the changes in allosteric properties and the observed desensitization were due to the incorporated group interferring with the binding

of the inhibitors. Such a situation could be envisaged if the dinitrophenylene group would be disallowing certain modes of binding by the inhibitors.

Therefore, the inhibitors would bind on the native enzyme and the DPE-enzyme in different modes. Whether the binding involved both the monomers of the native enzyme simultaneously by one molecule of the inhibitor was not clear.

Glucose, on the other hand, inhibited the native and modified enzyme in a similar fashion suggesting that it bound on a site different from ATP and glucose-6-P. The site could be the active site. Recent X-ray crystallographic studies have suggested that the glucose binding site is same as the glucose-1-P site and different from glucose-6-P site (40). It may be recalled that two sites are assigned for glucose-1-P one in the 'active site' and the other in or near the AMP site (40,101). A small pocket where many compou having a phosphate group could interact was earlier suggested to be present in the monomer/monomer interface (142). Earlier studies from this laboratory also confirmed such a view (101).

Conversion of dinitrophenylene phosphorylase b to a

The DNP-phosphorylase b could be converted to the a form using partially purified phosphorylase b kinase according to the procedure of Krebs and Fischer (10).

Like the native enzyme, the modified phosphorylase a was active in the absence of AMP and showed a specific activity of 15.5 in the absence of the nucleotide. In the presence of 1 mM AMP the specific activity was 22 under standard assay conditions. The a form was also equally stable to the b form of the enzyme at 2-5°C in 25 mM β-glycerophosphate buffer. The a form also moved as a single band in polyacrylamide gel electrophoresis. The spectral properties were also similar to that of the b form.

The kinetics of the a form for glucose-1-P in the presence of glucose, glucose-6-P and ATP (in the absence of AMP) is presented in Fig. 4-9. The K_m values calculated from these plots are given in Table 4-2. In the presence of 1 mM AMP, the inhibition obtained in the presence of 10-15 mM concentrations of the inhibitors was negligible at the various substrate concentrations for both the native and dinitrophenylene phosphorylase a.

The a form is a naturally occurring desensitised form. Native phosphorylase a is much less inhibited by ATP, glucose and glucose-6-P than the b form. In the presence of saturating concentration of AMP, inhibition has been earlier shown to be negligible (40). In these respects the derivative and native phosphorylase a showed similar behaviour. Thus the effect of modification was on the allosteric properties of phosphorylase b. Since the

Fig.4-9.

Lineweaver-Burk plots for glucose-1-P in the absence (●) and presence of 7.5 mM glucose(×); 10 mM ATP (△); and 10 mM glucose-6-P (○) for DPE-phosphorylase a (Fig.A) and native phosphorylase a (Fig.B). The assay mixtures contained 10 μg/ml enzyme, 1% glycogen, 15 mM cysteine, 20 mM glycerophosphate and varying concentrations of glucose-1-P. Assay temperature was 30°.

TABLE 4-2

INFLUENCE OF GLUCOSE-6-P, GLUCOSE AND ATP ON THE K_m VALUES FOR GLUCOSE-1-P FOR THE NATIVE AND DPE-PHOSPHORYLASE a

	K_m values (mM)	
	Dinitrophenylene phosphorylase a	Native phosphorylase a
No inhibitor	5.2	5.2
10 mM Glucose-6-P	12.3	16.1
10 mM ATP	6.9	7.8
15 mM glucose	7.7	9.1

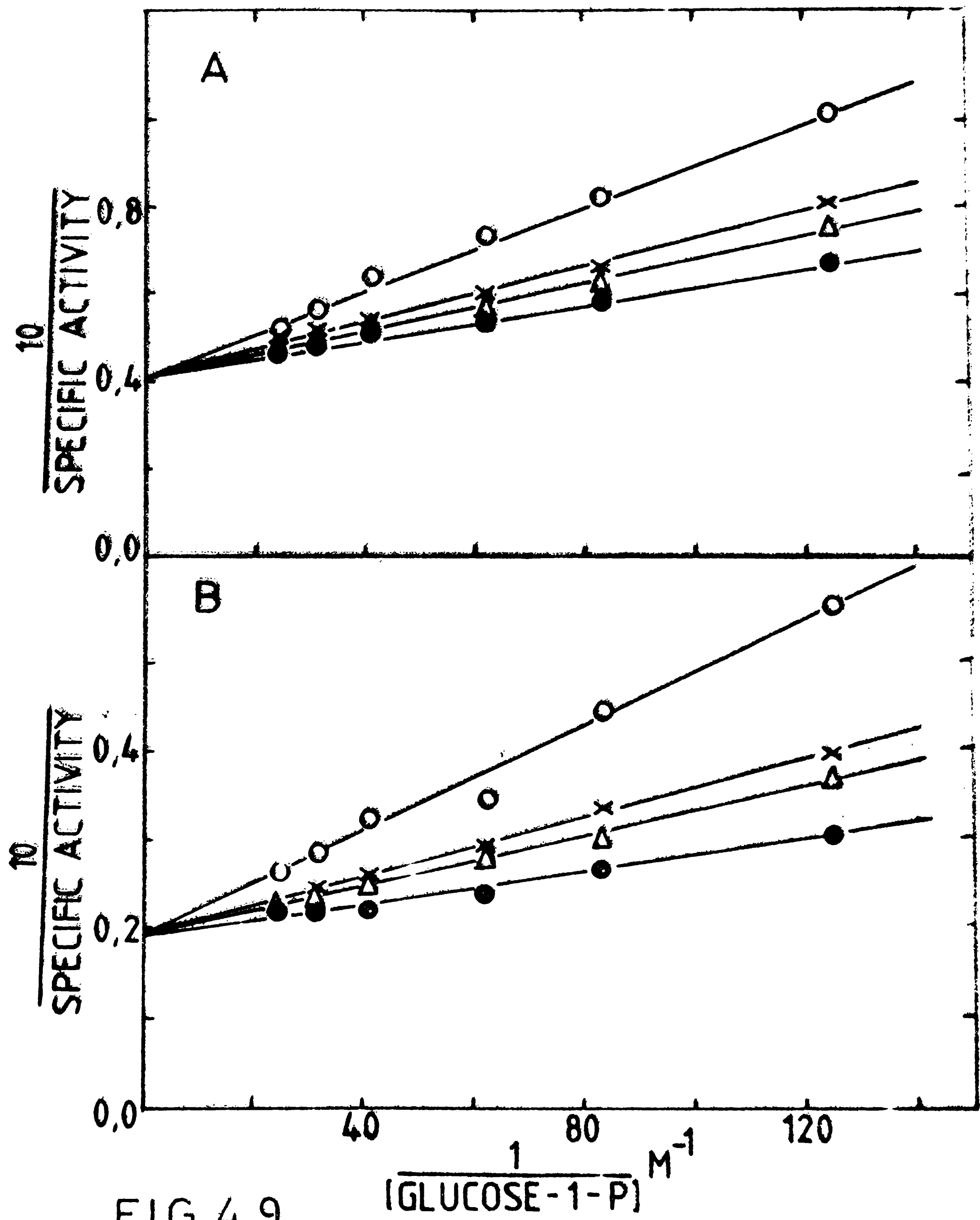

FIG. 4-9

specific activity of the modified phosphorylase *a* also was half that of the native *a* form, the incorporated group or minor conformation always brought about by the incorporation had caused a permanent change on the enzyme protein.

In conclusion, major differences were observed only in the specific activity and allosteric properties. Thus incorporation of a single aromatic group per enzyme dimer had affected the activity by binding on or in the neighbourhood of the AMP site.

CHAPTER 5

SEARCH FOR OTHER CATALYTIC FUNCTIONS OF GLYCOGEN PHOSPHORYLASE

Glycogen phosphorylase is a highly specific enzyme with respect to its substrate glucose-1-P. Since the reaction catalysed is the transfer of a glycosyl residue to polyaccharide, the requirement of the latter has been established to be a pre-requisite for catalysis (8). Illingworth *et al*. (32) have demonstrated the *de novo* synthesis of glycogen at very high enzyme concentration after incubation for a long time (this *de novo* synthesis was later disproved by Watkins *et al*. (141) who concluded that the observed synthesis was due to the presence of trace amounts of oligosaccharides in glucose-1-P). In Chapter 2, it was established that the aromatic compounds *p*-nitrophenyl phosphate (PNPP) and *p*-nitrophenyl glycosides bind on a site very near to the substrate binding site. Hence it was doubted if the enzyme possesses the function of hydrolysing compounds like PNPP. Interestingly we found that the enzyme could catalyse a very slow hydrolysis of these compounds at high enzyme concentration. This chapter deals with the preliminary studies made to understand the nature of this catalysis by the enzyme.

The progress curve for the reaction using PNPP as substrate is given in Fig. 5-1. Linear activity profile

Fig. 5-1.

Progress curve for the hydrolysis of p-nitrophenyl phosphate by phosphorylase b. The reaction mixture contained 0.25 mg/ml enzyme and $5x10^{-3}$M PNPP in 50 mM phosphate buffer pH 5.5. The reaction was maintained at 30°. 0.5 ml each were withdrawn from the reaction mixture at various time intervals and added to 2.5 ml of 0.5 M sodium carbonate and the absorbance measured at 400 nm against a blank which didn't contain the enzyme. The absorbance was converted to μ moles of p-nitrophenol using a molar extinction coefficient of $1.5x10^{4}$, found out using pure p-nitrophenol, at similar conditions.

Fig. 5-2

Effect of enzyme concentration on the hydrolysis of PNPP by phosphorylase b. The reaction mixture contained $5x10^{-3}$M PNPP in 0.05 M phosphate buffer pH 5.5 and different concentrations of enzyme. This was incubated for 4 hours and the absorbance converted to μ moles of p-nitrophenol. Other details were as in Fig. 5-2.

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was obtained upto 5-6 hours at room temperature. The activity was directly proportional to the enzyme concentration. (Fig. 5-2). The results showed that at μg quantities of enzyme, the activity was very low even when

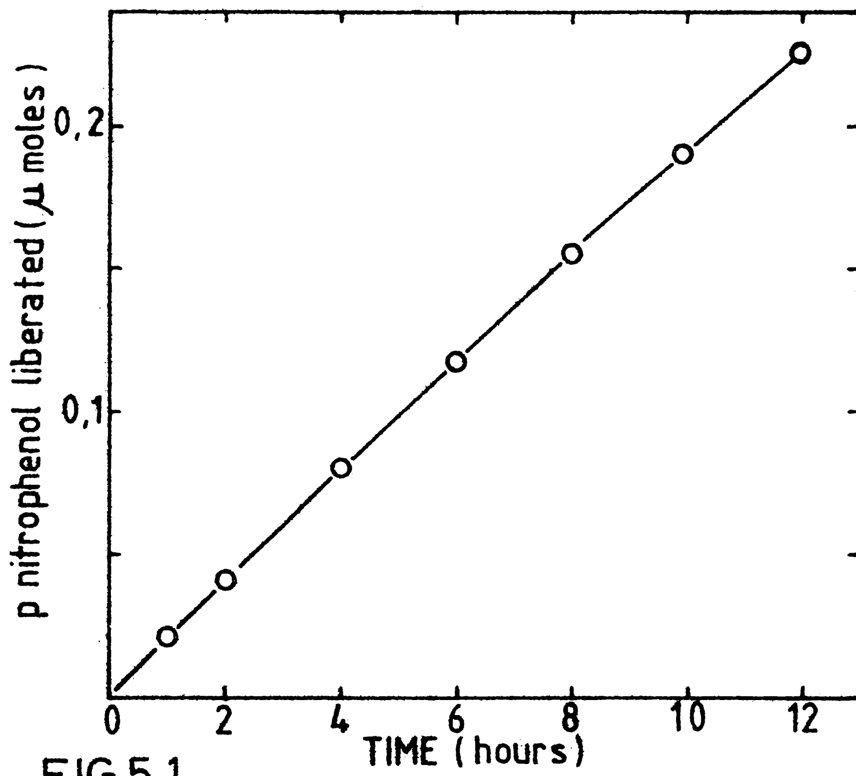

FIG.5-1

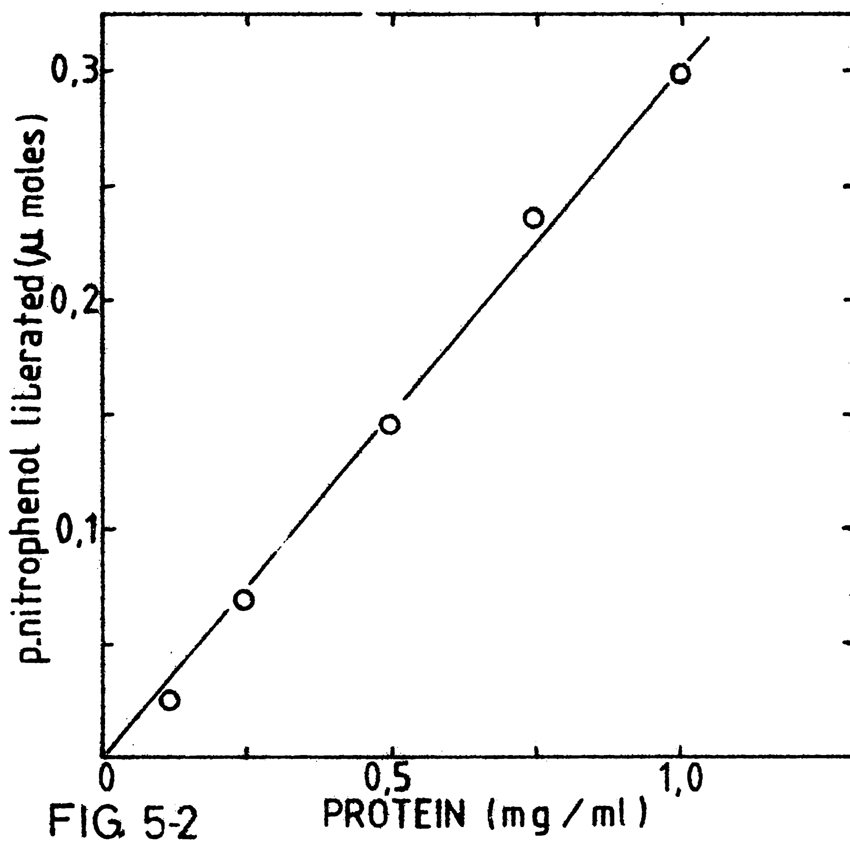

FIG. 5-2

assayed for 5-6 hours. Hence, this non-specific hydrolytic function of phosphorylase is insignificant metabolically.

Phosphorylase b showed nearly the same specific activity even after 3 times recrystallization of the enzyme. The specific activity after the third recrystallization was 0.08 units*/mg. The results showed that the effect was not due to contamination of phosphatase in the phosphorylase preparation. The enzyme showed a maximum activity near pH 5.5. This could mean that the functional groups involved in catalysis were different from those involved in phosphorylase activity.

The enzyme activity was intact up to a urea concentration of 5 molar, at pH 5.5, in 0.05 M phosphate buffer. Above 5 molar, the activity was decreased because of protein precipitation. Under the same conditions, the phosphorylase activity was zero, above 2 molar urea. The enzyme treated with p-chloromercuribenzoate (PCMB) was also active up to 20-fold molar excess of the reagent. Here also the phosphorylase activity was lost above 6-fold molar excess PCMB in 2 hours. Since the urea and PCMB inactivated enzymes were also active in hydrolysing PNPP, it was doubted that the catalytic activity could be due to nonspecific interaction

* 1 unit of the enzyme is defined as the number of μ moles of p-nitrophenol liberated from PNPP in 1 hour at standard assay condition (See 'experimental')

with certain amino acid residues present in the protein chain rather than contamination by phosphatase. The effect of different amino acids were therefore tested individually and in combinations. Out of all the naturally occurring amino acids tested, arginine and lysine showed slight activity after incubation for a long period; but the rate was not comparable with that of the native or urea denatured enzyme.

Pyridoxal phosphate and imidazole had also no effect in hydrolysing PNPP. A combination of PLP and lysine were also ineffective. Under the same conditions employed for phosphorylase b, bovine serum albumin had no effect on PNPP upto a concentration of 0.5 mg/ml even after 10 hours.

The specificity of the reaction was tested with different p-nitrophenyl compounds. All the compounds tested were hydrolysed by the enzyme with minor changes in the rate of hydrolysis.

From the results reported here and from several studies not presented, it was concluded that the observed hydrolysis of PNPP and other compounds was due to some non-specific effect of some amino acid residues of the primary structure of the protein.

E X P E R I M E N T A L

CHAPTER 6

EXPERIMENTAL

A. MATERIALS

Glucose-1-P (dipotassium salt), rabbit liver glycogen and ATP used for the work described in chapters 2 to 4, AMP (Chapter 2), p-nitrophenyl phosphate and bovine serum albumin were purchased from V.P.Chest institute, New Delhi.

L-cysteine hydrochloride (Chapters 2-4), AMP (Chapter 3 and 4), sodium β-glycerophosphate and nitrophenols (Chapter 2) and mercaptoethanol were products of E.Merck, Germany.

Trypsin, F_2DNB, FDNB, p-chloromercuribenzoate (or p-hydroxymercuribenzoate) and trypsin inhibitor were from Sigma Chemical Company, USA.

Sodium β-glycerophosphate (Chapters 3 and 4) and glucose-6-P were obtained from Koch Light or British Drug House. The Folin-Ciacalteu reagent was from the BDH.

Pyridoxal-5'-phosphate was the product of Sisco, Bombay.

The nitrophenyl glycosides used in Chapters 2 and 5 were a kind gift from Prof.O.P.Malhotra of the Banaras Hindu University.

All other chemicals used were of analytical grade. Distilled water from an all glass assembly was used throughout and the pH was measured using glass electrodes.

B. REAGENTS

i) Biuret reagent.

9 g of sodium potassium tartarate was dissolved in 500 ml of 0.2 N NaOH. To this was added 2 g of copper sulphate and dissolved by stirring. After dissolving 5 g of potassium iodide in to it, the volume was made up to 1 litre with 0.2 N NaOH.

ii) ANSA reagent.

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

iii) 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.

C. METHODS

1. Preparation of phosphorylase b from rabbit skeletal muscle.

Rabbit muscle glycogen phosphorylase b was prepared according to the procedure of Fischer and Krebs (144) with the substitution of mercaptoethanol for cysteine.

Step 1. Extraction.

Medium to large size 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 stored frozen. The procedure described below is for 400 g of frozen muscle. When smaller quantity of muscle was employed, the volume, concentration etc. of further additions were reduced accordingly. The frozen muscle was weighed and ground finely in an ordinary meat grinder at room temperature. The ground muscle from 400 g frozen muscle was stirred well with 400 ml distilled water for 10-20 minutes at room temperature and filtered through two thickness of cheese cloth in to a beaker cooled in ice. The muscle was again extracted for a second time with another 400 ml of water and finally using 200 ml water, each extraction taking 10-15 minutes. 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 (to - 20°C) centrifuge bottles and centrifuged. The supernatant was filtered through large coarse fluted filter paper into a beaker cooled in ice and the 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 (saturated at 28° ; added 700 ml to 1 litre of the protein solution). The mixture was kept in a refrigerator overnight. Most of the supernatant was decanted and the residue collected by centrifugation at room temperature (The centrifugation was made in pre-cooled centrifuge bottles). The residue was dissolved in 40-50 ml water and dialysed against pre-cooled 10^{-3} M tris-HCl buffer pH 7.6, at 3-5° . The dialysis was performed for 15 hours with 2 changes of 2 litres each of the buffer.

Step. 4 Heat treatment at high pH

The following solutions were added to the dialysed protein solutions: (1) sufficient mercaptoethanol (1:1 diluted in water) 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° for 1 hour, cooled to 0° and pH readjusted to 7.0 by careful addition of 1 M acetic acid. The solution was centrifuged and the precipitate was discarded.

Step 5. Crystallisation and recrystallisation.

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°, the mixture was centrifuged to collect crystals of phosphorylase b. The crystals were dissolved at 30°C in 10-20 ml 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. These processes were repeated at least two times. Results obtained for a typical purification under these conditions are summarised in Table 6-1.

TABLE 6-1.

SUMMARY OF PURIFICATION OF PHOSPHORYLASE b FROM RABBIT SKELETAL MUSCLE.

Step	Volume (ml)	Activity (Units/ml)	Protein (mg/ml)	Specific Activity (units/mg)	Yield (%)
Combined extract.	930	46.5	29.5	1.58	100
Ammonium sulphate fraction (after dialysis)	26	815	28	29.1	49
First crystallisation	16	805	21	38.3	29.8
Second crystallisation	12	840	21	40	23.3
Third crystallisation	10	835	20	41.8	19.3

2. Partial purification of phosphorylase b kinase from rabbit skeletal muscle.

The phosphorylase b kinase was partially purified from rabbit skeletal muscle according to the procedure of Krebs and Fischer (10).

The muscle from rabbits were taken as given above for phosphorylase purification 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 2×10^{-3}M EDTA (pH 7) for 1 minute in an electric blendor. The extract was centrifuged for 10 minutes at 7000xg and the supernatant was filtered through glass wool into a beaker cooled in ice.

The pH of the extract was lowered to 6 by the addition of 1N acetic acid. The precipitated protein was collected by centrifugation at 8000xg for 10 minutes. The residue was dissolved in 0.08 M sodium β-glycerophosphate and pH adjusted to 7 with careful addition of 0.5N NaOH. The enzyme solution was stored at -20°. 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.

3. Sephadex gel filtration.

For separation of enzyme from mercaptoethanol and other small molecules, Sephadex G-15 or Sephadex G-25 were employed. The Sephadex gels were allowed to swell in distilled water

for several days. The gel was washed by decanting the water layer and adding further volumes of water. A slurrey of the gel was poured in to column and allowed to settle while water was allowed to slowly pass through the gel. For the separation of small molecules, a column size of 1 x 20 cm was usually employed. Before passing the enzyme solution the column was repeatedly washed with the required buffer. The enzyme solution to be chromatographed was layered on top of the gel without disturbing the gel surface. When the enzyme layer completely entered the gel, the eluting buffer was carefully filled and the column connected to a reservoir containing the buffer. The flow rate was usually about 0.5 ml/minute. Fractions of approximately 2-5 ml were collected, depending on the concentration of enzyme applied. This method gave good separation of the enzyme from small molecules.

The Sephadex G-200 used for the purification of dinitrophenylene phosphorylase was also subjected to a similar treatment. The column size was 2.5 x 48 cm. Here the protein was applied in a different manner. The enzyme was made 20% in sucrose and layered on top of the column where 2-3 cm layer of the eluting buffer was retained. This allowed the enzyme to enter the gel as a sharp band without getting diluted. 5 ml fractions were collected and the flow rate was 1 ml/ 2minutes.

4. Estimation of phosphorylase activity.

The work presented in the thesis employs the procedure of Illingworth and Cori (6) for determining the phosphorylase activity. The inorganic phosphate produced in the direction of glycogen synthesis was estimated according to the method of Fiske and Subbarow (145).

For routine assay, phosphofylase was properly diluted in 30 mM cysteine / 40 mM glycerophosphate pH 6.8 for 20-30 minutes prior to assay*. To 0.2 ml of substrate solutio 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°. 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 color 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 μ moles of inorganic phosphate using a calibration curve obtained with KH_2PO_4 under the same conditions (Fig 6-1).

The time of incubation of enzyme assays in various experiments were adjusted so that the optical density of the developed solution was well in the linear part of a

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*without the prior incubation, the kinetic properties of the enzyme have been found to be not fully reproducible.

Fig. 6-1.

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

Fig. 6-2.

Progress curve for phosphorylase activity. Phosphorylase b (30 µg/ml) in 30 mM cysteine / 40 mM glycerophosphate pH 6.8 was incubated for 30 minutes. At zero time, 2 ml of the enzyme was added to 2 ml of substrate solution containing 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 color was developed and optical density measured as given in Section 4 of this chapter.

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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. The progress curve thus obtained is shown in Fig. 6-2.

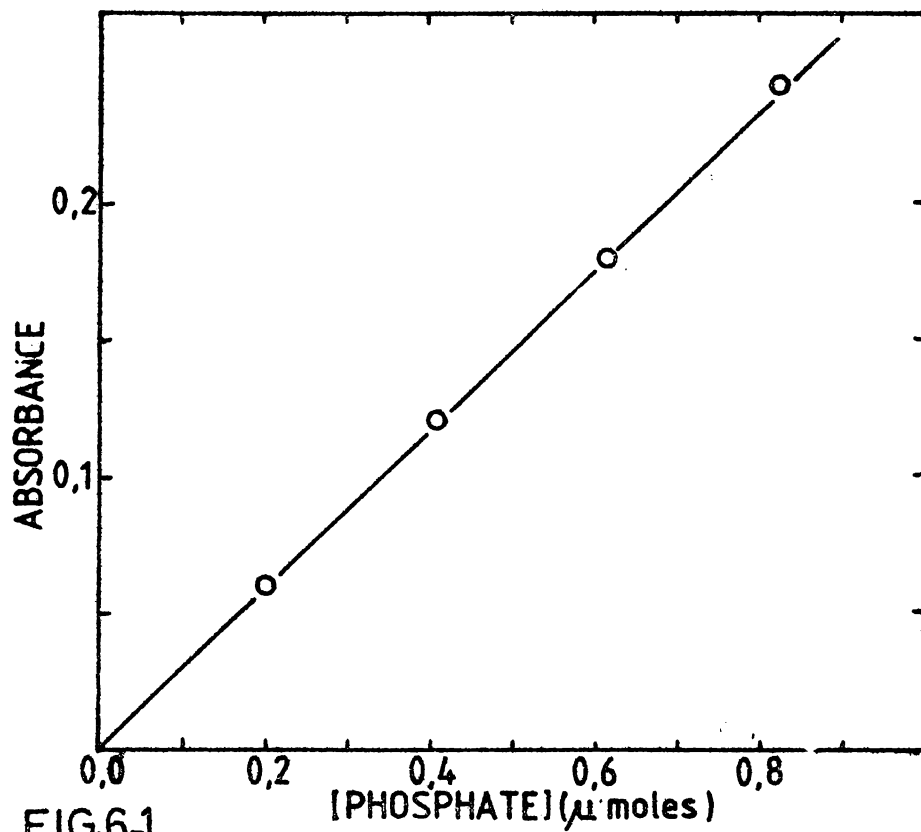

FIG.6-1

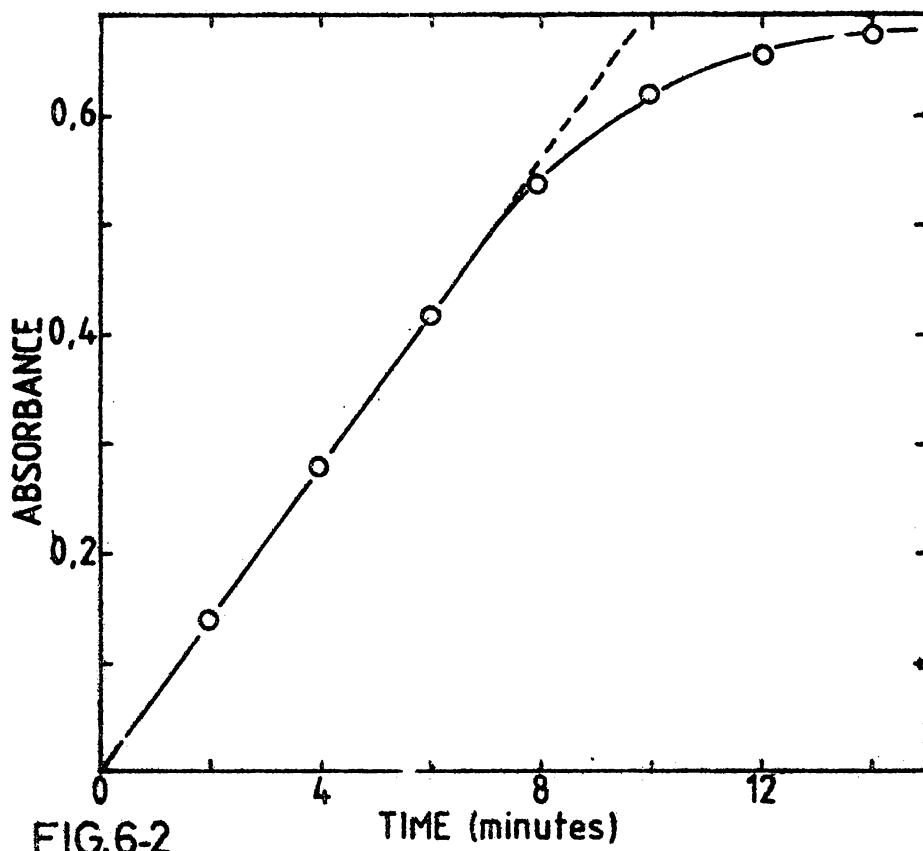

FIG.6-2

5. Estimation of protein.

i) Estimation by Lowry's method using Folin - Ciocalteu reagent (146).

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 quntity 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 (15-20 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) 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 500nm in a spectrocolorimeter. The instrument was calibrated using bovine serum albumin as standard (Fig. 6-3).

Fig. 6-1

Calibration curve for protein estimation using Folin-Ciocalteu reagent. The optical density was measured at 500 nm in a spectrocolorimeter.

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ii) Biuret method (147)

To 1ml of solution containing 1-3 mg of protein, 1.7 ml of biuret reagent was added, mixed well and the absorbance measured at 550 nm against a similarly treated blank. Bovine serum albumin or crystalline phosphorylase was used as standard, whose concentration was determined spectrophotometrically (see below). The protein concentration in dinitrophenylene phosphorylase was estimated using this method.

iii) Spectrophotometric measurement. (148).

The concentration of crystalline phosphorylase was also determined using an absorption coefficient of 1.32 for a 1 mg/ml solution at 280 nm.

6. Kinetic studies.

For kinetic studies with varying glucose-1-P concentrations, the glycogen and AMP concentration in the substrate solutions were maintained constant (2% and 2 mM respectively) and glucose-1-P concentration varied from 16 mM to 80 mM. Similarly, for kinetics with respect to AMP, glucose-1-P and glycogen concentrations of the substrates were

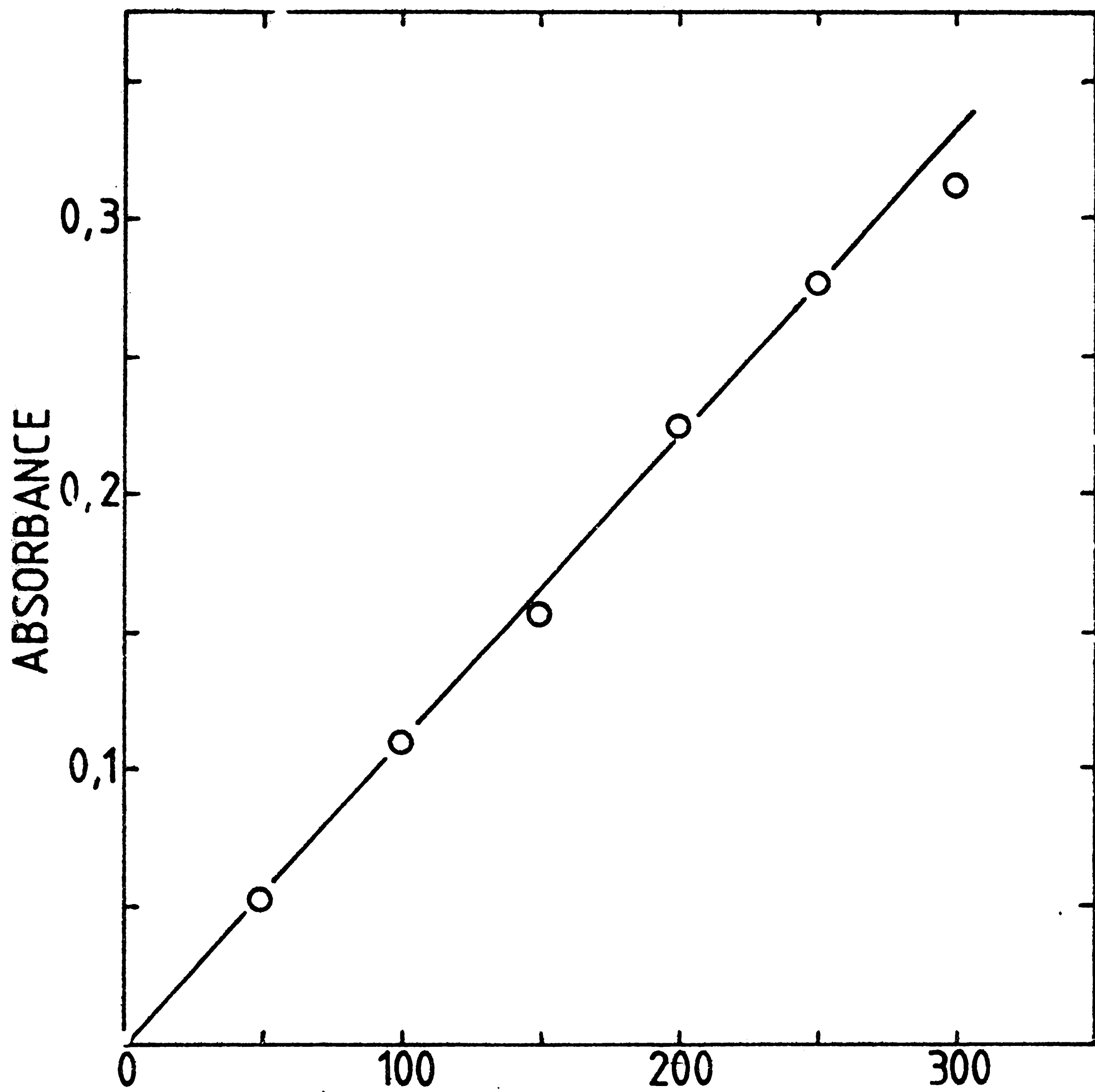


FIG.6-3

32 mM and 2% respectively and AMP concentration varied from 2 x 10^{-3} mM to 2 mM. The enzyme solution was properly diluted and added to an equal volume to the substrate solutions at 30°C and assayed as given in section 4. Separate blanks were taken for each substrate concentrations.

7. Inhibition studies.

a) Inhibition by aromatic compounds.

Stock solutions of the aromatic compounds were prepared in water (0.1 M) 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 diluted in 30 mM cysteine / 40 mM glycerophosphate buffer pH 6.8 to the required concentrations. Equal volume of this solution was mixed with enzyme and assayed as given in section 4.

b) Dixon plots.

The enzyme-inhibitor mixture was prepared as in Section 7(a) with varying concentrations of the inhibitor and assayed using the standard assay method given in section 4.

c) Temperature studies.

The substrates and enzyme solutions (with or without inhibitors) were separately brought to the required temperature, mixed and incubated in the same bath for a fixed time and assayed as described above. The color was

developed after bringing the solutions to room temperature by placing in a water bath.

d) Isobologram studies.

For the isobologram studies, to 0.1 ml of enzyme solution was added 50 μ litre each of the inhibitors I_1 and I_2 using λ-pipettes (the enzyme and inhibitor solutions were prepared as in 7(a). The concentrations of I_1 and I_2 were varied so that a set of mutually varying concentrations of the inhibitors were obtained. These solutions were incubated at 30°C for 20-30 minutes and to this was added 0.2 ml each of substrate solutions containing 32 mM glucose-1-P and 2% glycogen with or without the presence of 2 mM AMP. The liberated inorganic phosphate was estimated as in section 4. The results thus obtained were plotted as described by Yonetani and Theorell (133). For this, the reciprocal of velocity was plotted against concentration of I_1 for different fixed concentration of I_2. From this, the concentrations of the two inhibitors required to effect the same extent of inhibition were chosen and plotted as isobols in the isobologram.

8) Preparation of dinitrophenylene phosphorylase b

A 4 mg/ml stock solution of F_2DNB was prepared in methanol and stored in a brown bottle at -20°C, in a desiccator.

The phosphorylase b was treated with acid washed charcoal at 0° to remove bound AMP. This was then passed through Sephadex G-15 equilibrated with 0.05 M tris-HCl buffer pH 7.6. The protein solution was diluted in the same buffer to make it a 2 mg / ml solution.

The F_2DNB was properly diluted in methanol and 25-50 μ litre was added to 5-10 ml of the enzyme solution so that final concentration was 1.30-fold molar excess of the protein, assuming a molecular weight of 200,000 for the enzyme. (For eg. added 50 μ litre of 2.6×10^{-3}M F_2DNB to 10 ml of 2 mg/ml phosphorylase b). The solution was stirred well and kept at 30° in the dark with occasional stirring. After 1 hour, when the reaction was completed the protein was cooled to 0° and precipitated using solid ammonium sulphate. This was centrifuged and the protein was dialysed in the required buffer.

9. Treatment of phosphorylase b with p-chloromercuribenzoate

A stock solution of p-chloromercuribenzoate was prepared in water by adjusting to pH 7 with careful addition of 0.1 N NaOH. This solution was diluted in a standard flask using 25 mM tris-HCl buffer. Further dilutions were made in the same buffer such that by mixing 1 volume of PCMB with 9 volume of the enzyme solution yielded the desired molar excess of PCMB as mentioned in the text.

10. Estimation of amino acid residues modified by F_2DNB.

The number of DNP-groups incorporated in to the protein was determined by the method of Hill and Davis (134) using benzyl amine, originally employed for the estimation of FDNB. The reaction obeyed Beer- Lambert law in the case of F_2DNB also. Free F_2DNB in solution was estimated before and after reaction with the enzyme, from a calibration curve obtained using F_2DNB and benzyl amine. The absorption maximum was found to be 328 nm and the molar absorptivity at this wave length was 2.73×10^4.

To 4 ml of 2 mg/ml phosphorylase *b* in 50 mM tris-HCl buffer pH 7.6 was added 25 μ litre of F_2DNB to get the required molar excess of the reagent. A control was simultaneously run by adding 25 μ litre of the reagent to 4 ml of tris-HCl buffer. The samples were kept at 30° for 1 hour and added 5 ml of 2% benzylamine in toluene. The mixture was shaken for 30 minutes, the tolene layer removed and the absorbance measured at 328 nm against a reagent blank obtained by shaking 5 ml of the benzylamine solution against 4 ml of tris-HCl buffer. The difference in the F_2DNB concentration in the test and blank correspond to that reacted with enzyme, from which the number of F_2DNB incorporated were calculated.

11. Polyacrylamide gel electrophoresis.

The homogeneity of the dinitrophenylene phosphorylase was checked by polyacrylamide gel electrophoresis according to

the procedure of Ornstein and Davis (149) (Experimental details are given in Chapter 12).

12. Resolution and reconstitution of pyridoxal-5'-phosphate.

The resolution was carried out by the method of Shaltiel *et al.* (59,150) with some modifications to avoid partial denaturation of protein. The native and dinitrophenylene enzymes were passed through Sephadex G-15 columns equilibrated with 20 mM β-glycerophosphate, 5 mM mercaptoethanol pH 6.2. To 1 ml of this enzyme solution (containing about 4 mg phosphorylase) was added an equal volume of 0.8 M imidazole citrate containing 0.4 M cysteine (pH 6.2) at 0°. After 40 minutes at 0°, the mixture was passed through Sephadex G-15 column equilibrated with 0.4 M imidazole citrate-0.2 M cysteine buffer (pH 6.2). The PLP-free protein thus obtained was again passed through a second Sephadex G-15 column equilibrated with 25 mM β-glycerophosphate/25 mM mercaptoethanol, pH 7.

For the reconstitution of apoenzymes, the native and dinitrophenylene enzymes were diluted in glycerophosphate/mercaptoethanol buffer so that both the samples were exactly of the same concentration. To this was added pyridoxal-5'- phosphate to a final concentration of 5×10^{-5}M and the rate of reactivation was followed by assaying the

phosphorylase activity after diluting in the mercaptoethanol glycerophosphate buffer.

13. Finger-print analysis (151)

Reagents:

1. Volatile solvent for paper electrophoresis: (152) 125 ml pyridine + 5 ml glacial acetic acid diluted to 2.5 litre with water.

2. Isobutanol : formic acid : water (75:14.5:10.5) (140).

3. 0.2% Ninhydrin in accetone.

Method:

The native and dinitrophenylene enzymes were hydrolysed by trypsin under exactly the same conditions and the resulting mixture of peptides were characterised by a combination of paper electrophoresis and paper chromatography. 5 mg each of the enzyme samples in 0.05 molar tris-HCl buffer pH 7.8 were denatured by keeping in a water bath at 60° for 10 minutes. To 5 ml each of the denatured enzyme was added 0.1 ml of 0.5% trypsin in 0.001 N HCl. The pH was occasionally adjusted to 8 by using 0.5 N NaOH. After about 2 hours, when there was no pH change, another 10 μ litre of trypsin was added, kept for 30 minutes and pH readjusted to 6.5 with 1 N HCl. The digested solutions were concentrated to about 0.5 ml by evaporation at 50-60°.

Two rectangular Whatman No.1 papers (25 x 25 cm) were used. About 0.05-0.1 ml of the trypsin digest was applied to one end of the paper, 8 cm away from the anodal side. The papers were completely soaked with pyridine:acetic acid buffer and placed in an electrophoresis tank, in between two fibre glass supports. The two ends of the wicks were dipped in the tanks and was tightly closed with the cover lid. A constant voltage (8 volts/cm) was applied for 10 hours. After the electrophoresis, the papers were dried at 50-60°, keeping it in a stretched horizondal position. Ascending chromatography of the dried papers were conducted in the other direction using isobutanol: formic acid: water as solvent.

The yellow band due to the DNP-amino acid was not visible after electrophoresis. The finger-prints were developed by spraying the dried paper with ninhydrin solution. When the paper was heated in an oven at 80°, the spots became clear.

15. Reaction of p-nitrophenyl phosphate and p-nitrophenyl glycosides with phosphorylase.

The hydrolytic action of phosphorylase on the above compounds were tried using the following method.

p-nitrophenyl phosphate, p-nitrophenyl α-glucoside, p-nitrophenyl β-glucoside and p-nitrophenyl β-arabinoside were prepared in 50 mM phosphate buffer, pH 5.5. 0.5 ml

of enzyme solution (diluted in the same buffer) was added to 0.5 ml of the solutions of the above compounds to get the final concentrations given in the text. After incubation for 4-5 hours at 30°, 5 ml of 0.5 molar sodium carbonate was added and the color was measured at 400 nm. The absorbance was corrected to μ moles of p-nitrophenol using an extinction coefficient of 1.5×10^4, found out using pure p-nitrophenol, under same conditions.

P A R T II

PURIFICATION AND PROPERTIES OF GLYCOGEN PHOSPHORYLASE a FROM *SEPIA PHARAONIS*

INTRODUCTION

The structure, function and control of α-glucan phosphorylase from a wide spectrum of vertebrate animal tissues of terrestrial and aquatic origin have been studied in various laboratories (15-20, 109-111, 118-120, 153-157). Eventhough some studies on phosphorylase from marine invertebrate sources have been reported (42,121), detailed studies using purified enzyme have not been made so far. Information on properties of phosphorylase in highly metabolising active tissues of invertebrates is lacking. To fill this gap, the cuttle fish *Sepia pharaonis* was selected for a detailed study of the enzyme, after its isolation.

Sepia pharaonis is available at all seasons. The taxonomy of this species is as follows (158):

Super phylum	:	Invertebrata
Phylum	:	Mollusca
Class	:	Cephalepoda
Sub-class	:	Coleoidea
Order	:	Sepioidea
Family	:	Sepiidae
Genus	:	*Sepia*, Linnaeus, 1758.

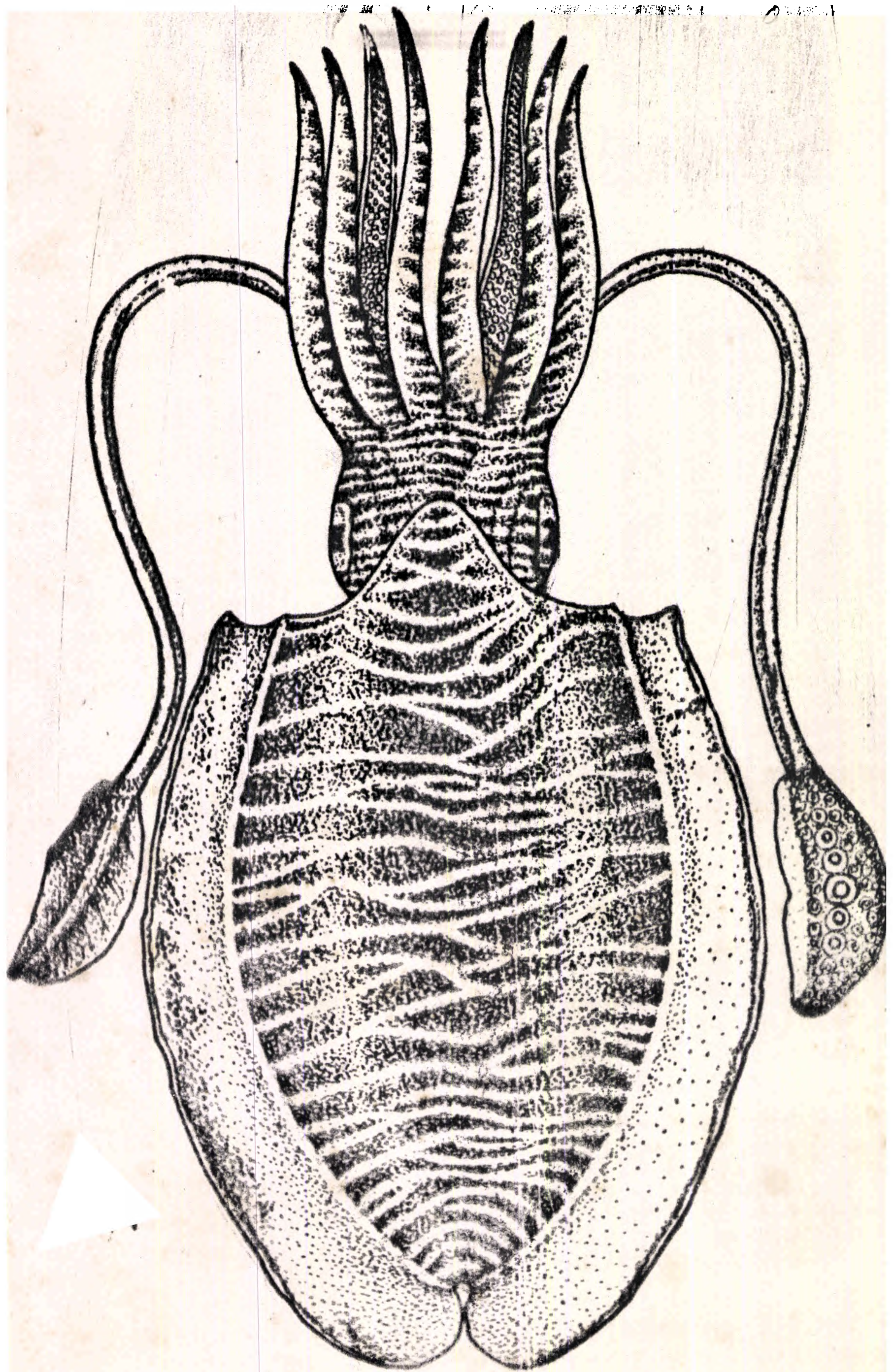
Sepia pharaonis, Ehrenberg, 1831.

The Cephalopods are far removed in evolutionary status when compared to aquatic and terrestrial vertebrates. However, among invertebrates, they are the most active, elaborately evolved and specialized groups of molluscs, living in the open waters of the ocean on more or less equal terms with fish and aquatic mammals. They are almost all fast-moving carnivores. (158).

Cuttle fishes belong to the order Sepioidea. Three species of cuttle fish dominate the catches obtained from the south west coast of India, viz. _Sepiella inermis_, _Sepia aculeata_ and _Sepia pharaonis_.

S. pharaonis is widely distributed in the Indo-Pacific area and it occurs all along the coasts of India. This species can be readily identified from all other cuttle fish available from our coast by presence of (i) conspicuous transverse stripes on the dorsal surface of the mantle, head and anus. (ii) enlarged suckers on the tentacular club (iii) ∧-shaped striae and the distinct medium furrow on the ventral side of the cuttle bone.

The mantle tissue of the _Sepia_ is a fast metabolising tissue. The animal moves using its mantle muscle. Water is taken in laterally by contraction of radial fibers in the pallial wall. By pushing out the water through a funnel by contracting the circular muscles of the mantle, the



Sepia pharaonis Ehrenberg

animal moves with lightning speed (159). Thus the mantle muscle of the cuttle fish has an extremely high energy demand like the insect flight muscles. This prompted the study of glycogen phosphorylase which regulates the glycolytic pathway in all animal species studied so far. This part of the thesis deals with studies on the structure and control of this enzyme from *S. pharaonis*.

R E S U L T S   A N D   D I S C U S S I O N

CHAPTER 7

PURIFICATION OF PHOSPHORYLASE FROM THE MANTLE MUSCLE OF THE CUTTLE FISH, SEPIA PHARAONIS

The procedure for purification of glycogen phosphorylase from rabbit muscle (144) was found to be not applicable in purifying the enzyme from the mantle muscle of *S. pharaonis*.

Usually 100-200 g of the frozen mantle was employed in each batch for purification of the enzyme. After grinding, extraction was attempted at 0°, 10° and at 30°. Although there were no differences in the total activity in extracts prepared at these temperatures, the specific activity* was found to be higher when extracted at 0-5°. Extraction with distilled water was preferred because with 20 mM β-glycerophosphate pH 7, the specific activity was comparatively low without any increase in the yield.

A second extraction of the muscle yielded about 30% of the activity of the first extract. Here also, since

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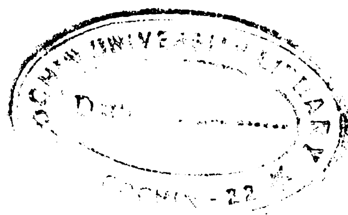
* One unit of phosphorylase is defined as the amount of enzyme that produces 1 μ mole of inorganic phosphate in one minute at 30° when assayed in the direction of glycogen synthesis under the conditions given in 'experimental'. Specific activity is defined as units/mg protein.



the specific activity was considerably lower, only the first extract was taken for further purfication. Unlike the preparation from rabbit muscle, acid treatment of the extract at pH 5.5 only decreased the activity to 75% without any difference in the specific activity. So this step was abandoned.

Initially ammonium sulphate fractionation of the extract was tried at different concentrations of the salt from 30% to 60% saturation. The specific activity was maximum for the residue obtained between 45% and 50% saturation of ammonium sulphate. Since a good fraction of the total activity was carried over also by the residues obtained at 40 to 45% saturation, the precipitate obtained between 40 and 55% salt saturation was used for further purification. The purity obtained by this step was on the average 2.5-to 3-fold with 65 to 70% yield of the enzyme.

Affinity chromatography was tried after dialysis of the residue. Since only 4-to 5-fold purification could be achieved by this procedure, other methods were sought to procede this. Of the procedures tried, DEAE-cellulose chromatography was successful.

The results of typical DEAE-cellulose chromatography of the ammonium sulphate fraction is shown in Fig.7-1.

Fig. 7-1.

DEAE-cellulose chromatography of the protein obtained by ammonium sulphate fractionation. The experimental details are given in Chapter 12. ○, protein concentr tion (mg / ml); ▲, activity (as μ moles of orthophosphate liberated/minute/ml of fractions). The total protein applied in this typical experiment was 190 mg. The total protein and activity yield was 90-95%. In this experiment, fractions from 30 to 40 were taken for further purification.

The elution was at 5-8°C. A gradient using 1.5 molar NaCl in the top reservoir with 150 ml buffer in the mixing flask gave good separation of proteins. However, repeated experiments showed that a continuous gradient using 0.8 M NaCl in the top reservoir with 150 ml of buffer in the mixing flask yielded a better resolution of the protein and increased the specific activity of the phosphorylase fractions. The active fractions were pooled, the proteins precipitated with solid ammonium sulphate (at 65% saturation) and dissolved in minimum volume of buffer. The total purification obtained in this step was 12-15-fold with a total recovery of 30-35% activity of the original extract.

Further purification of the DEAE-cellulose purified

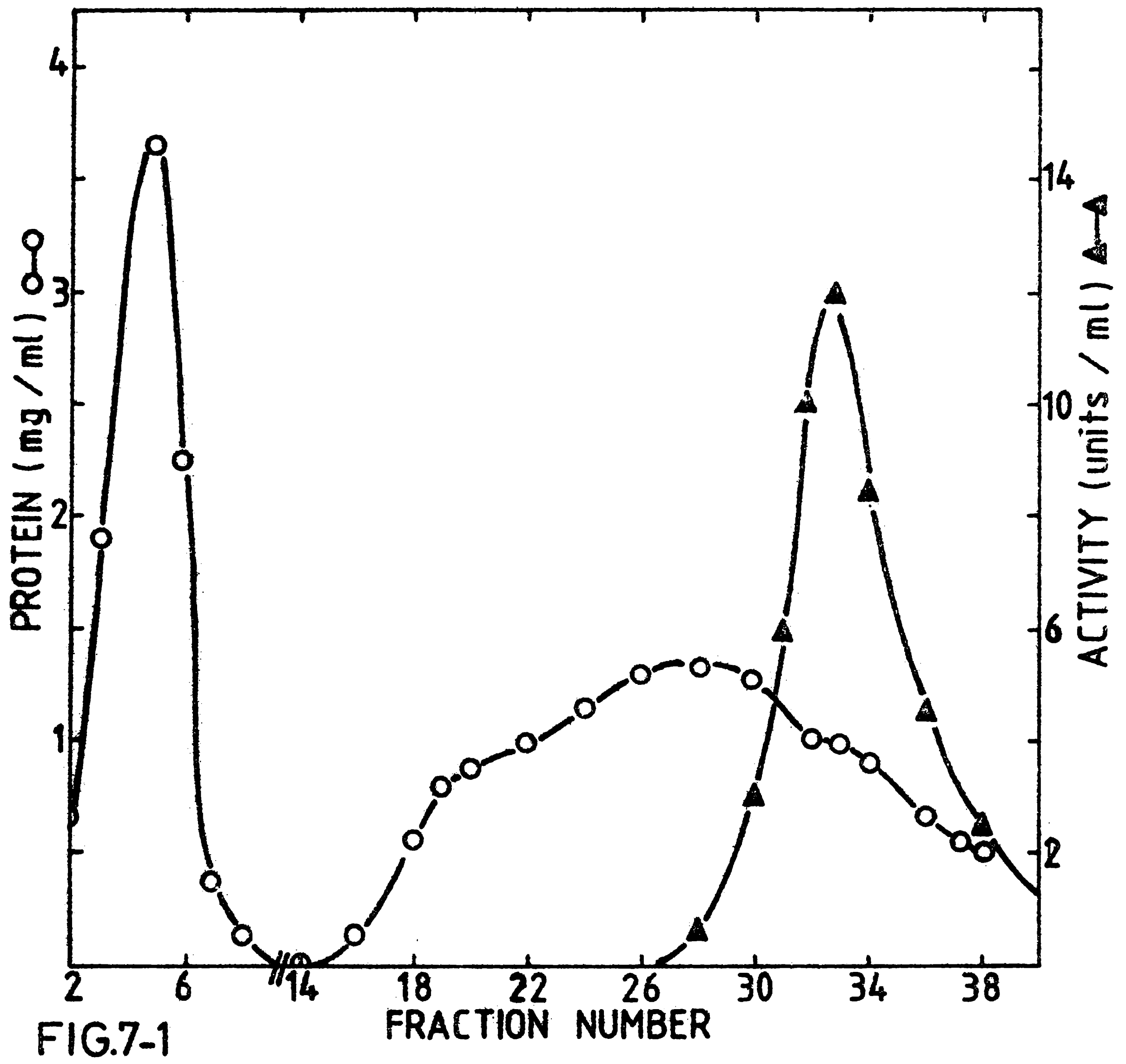


FIG.7-1

enzyme was tried with hydroxyl apetite gel and carboxymethy cellulose column. However, the eluted enzymes showed no increase in specific activity. Therefore affinity chromatography using Sepharose-glycogen was tried and it was found to be successful. A homogenous preparation of phosphorylase b from bovine uterus, using Sepharose-glycogen, has been reported (156).

Typical results obtained on affinity chromatography through Sepharose-glycogen are shown in Fig. 7-2. The purity obtained in this step was about 35-fold of the original extract with 25% yield of the total activity.

The results of a typical purification are given in Table 7-1. About 10-15 mg enzyme was obtained from 100 g of frozen muscle. The specific activity of the purified enzyme was 25 to 28 units/mg protein. The enzyme was concentrated by adsorption and elution using a small DEAE-cellulose column. The active fractions were pooled and stored in 25 mM glycerophosphate pH 7 containing 10 mM mercaptoethanol.

The presence of a phosphorylase form having a higher requirement of AMP for maximum activity has been reported in extracts of certain marine organisms (121). We did not observe such a form in the muscle extract of Sepia or at any stage of the purification of phosphorylase. Such a form (phosphorylase c) was also separated from the lobster tail muscle on DEAE-cellulose column (18).

Fig. 7-2.

Affinity chromatography of the DEAE-cellulose purified *Sepia* phosphorylase on Sepharose-glycogen. Details were as described in 'experimental'. In this experiment, 2 ml (12 mg/ml) of protein solution was applied. Volume of each fraction was 5 ml. ○, protein concentration (mg/ml of fractions) ▲, activity as μ moles of orthophosphate liberated/min/ml of fractions.

TABLE 7 - 1

PURIFICATION OF GLYCOGEN PHOSPHORYLASE *a* FROM THE MANTLE MUSCLE OF *SEPIA PHARAONIS*.

	Total Volume ml.	Activity (units/ ml).	Protein (mg/ml)	Specific activity (units/ mg)	Yield (%)	Purification.
Extract.	150	11	13	0.83	100	1
40 to 55% ammonium sulphate fraction	15	70	31	2.25	64	2.7
DEAE cellulose chromatography followed by concentration by ammonium sulphate precipitation and dialysis	4	120	10	12.0	29.5	14.5
Affinity chromatography followed by concentration using DEAE- cellulose column (See 'experimental').	8	50	1.8	28.0	24.0	34.0

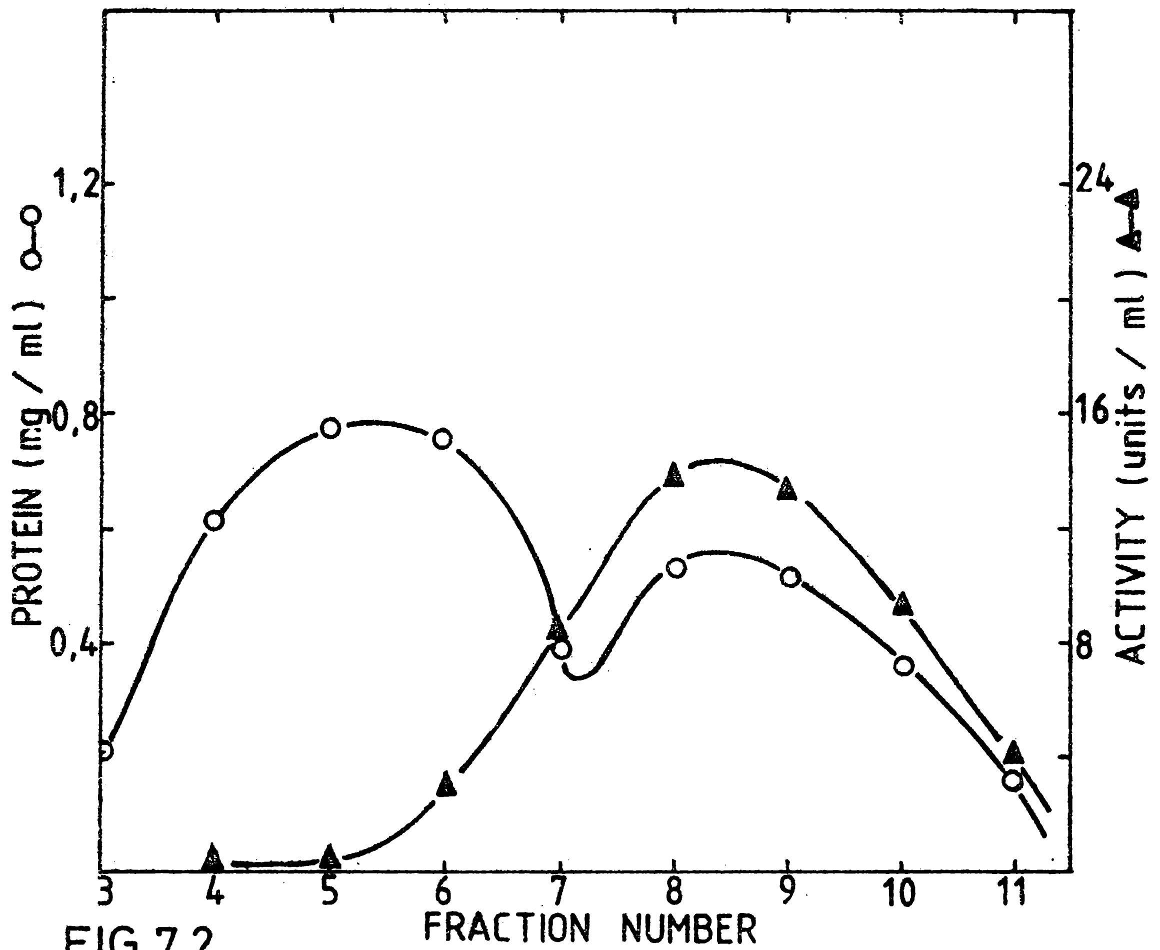


FIG. 7-2

In the present case only one form could be detected on DEAE-cellulose as well as on Sepharose-glycogen columns.

Crystallization of the phosphorylase was tried using AMP and Mg^{++}. Even after 10 hours at 0° with 5×10^{-3}M AMP and 5×10^{-2}M Mg^{++}, no crystallization of the enzyme was observed. Addition of ammonium sulphate to a 5 mg/ml preparation till the development of slight turbidity followed by dialysis at 0 to 5° against 50% saturated ammonium sulphate was not successful. Inclusion of mercaptoethanol or cysteine was also unsuccessful in obtaining the enzyme in crystalline form.

Purity of the enzyme.

Polyacrylamide gel electrophoresis (149) of the enzyme preparation after purification over DEAE-cellulose column showed two major bands and two minor bands of proteins. The preparation after affinity chromatography moved as a single band as evidenced by one protein band and the corresponding activity band (Fig. 7-3).

The purified enzyme showed an activity ratio* of 0.4± 0.02.

Stability of the enzyme.

The purified enzyme was stable for 3-4 days. Thereafter the activity gradually decreased and lost completely in two weeks. Therefore the properties of the enzyme were studied within 3-4 days of each batch of preparation.

* The activity ratio is defined as the ratio of phosphorylase activity in the absence of AMP to that in presence of saturating AMP.

Fig. 7-3.A.

Polyacrylamide gel electrophoretic pattern of the DEAE-cellulose purified S.pharaonis phosphorylase scanned in a Hitachi model 200 gel-scanner at 500 nm.

Curve-1: Protein bands developed by amido black.

Curve-2: The corresponding activity bands. (Experimental detai were as given in Chapter 12).

Fig. 7-3.B.

Polyacrylamide gel electrophoretic pattern of phosphorylase purified by affinity chromatography using Sepharose-glycogen.

Curve-1: Protein bands; Curve-2, activity bands. Other detail were as in Fig. 7-3.A

Interestingly, inclusion of cysteine was found to result in faster inactivation on storage whereas mercaptoethanol had no effect. (Fig.7-4).

The molecular weights of the S.pharaonis phosphorylase and rabbit phosphorylase_b were compared using gel filtration through Sephadex G-200 (Fig. 7-5). It is seen that rabbit muscle phosphorylase b (M = 194,000 (40) and the S.pharaonis phosphorylase a were eluted with the same elution volume. Assuming that the S.pharanois enzyme has comparable shape as

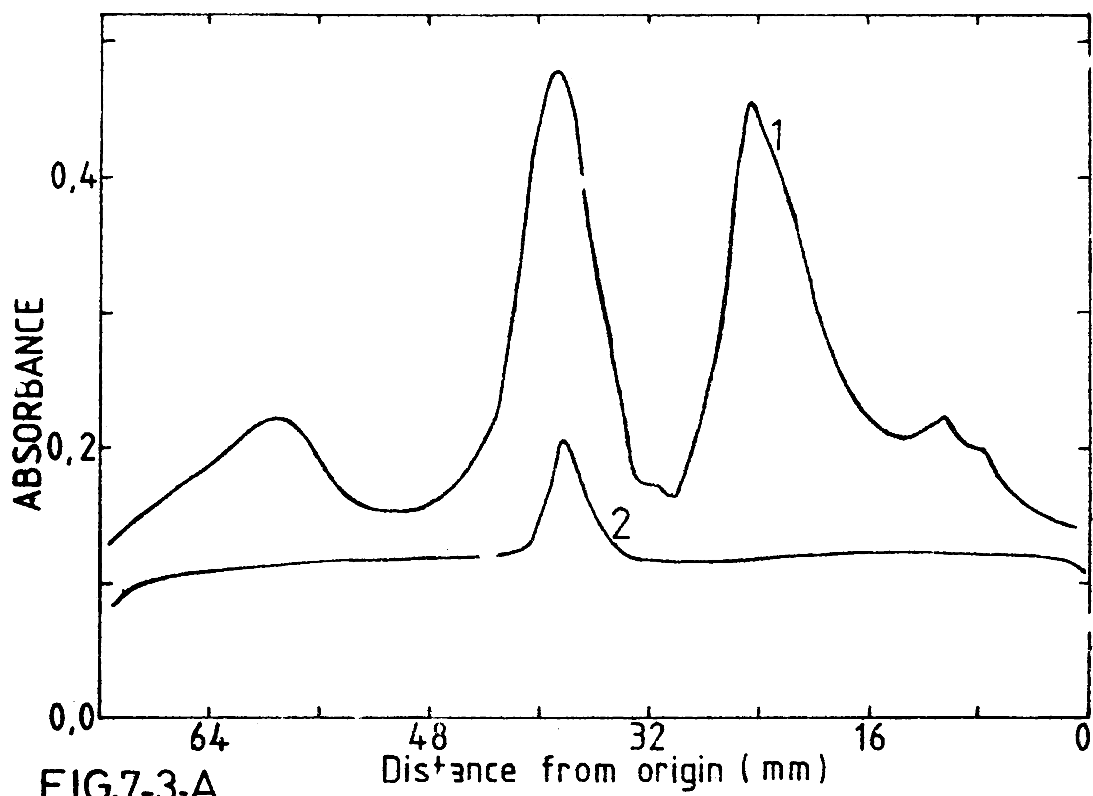


FIG.7-3-A

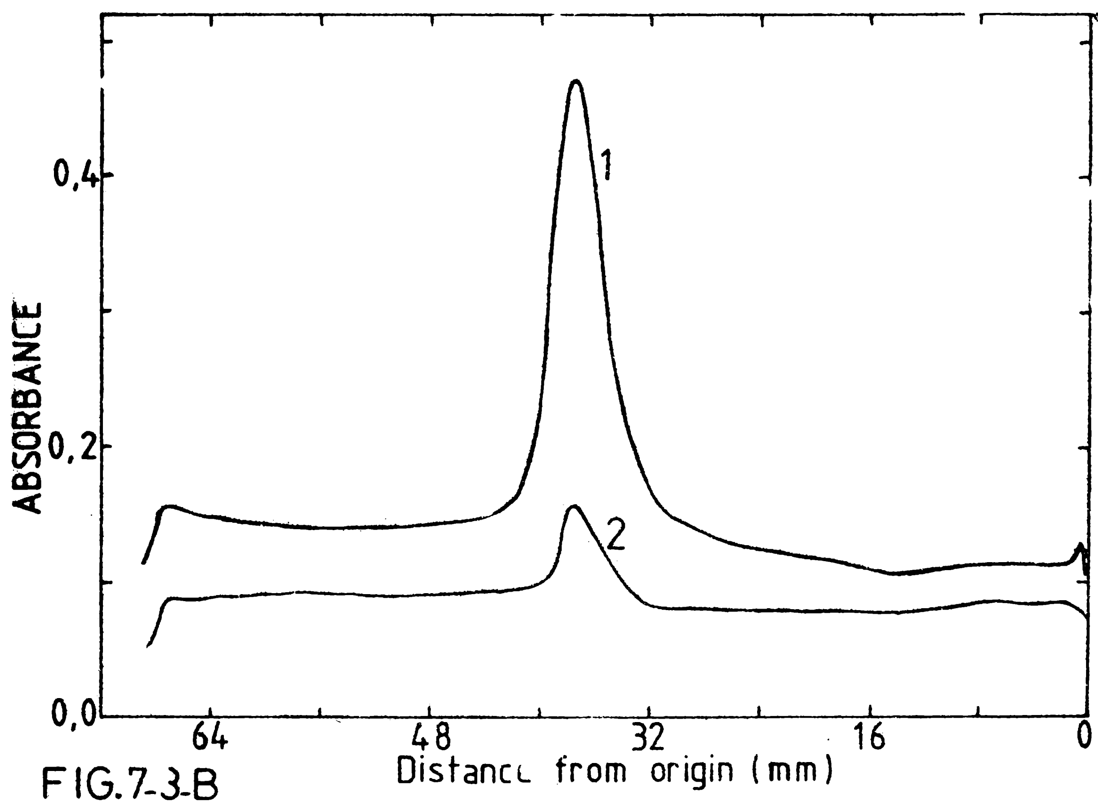


FIG.7-3-B

Fig. 7-4.

Stability of the S.pharaonis phosphorylase a on storage at 3-5°. The enzyme solutions (4mg/ml) were stored in 25 mM sodium β- glycerophosphate pH 7 in presence of 10 mM mercaptoethanol, (△); 15 mM cysteine (□) and in the absence of any other additions, (○). The enzyme was assayed after diluting in 30 mM cysteine/ 40 mM glycerophosphate, pH 6.9.

Fig. 7-5.

Sephadex G-200 chromatography of rabbit phosphorylase b and Sepia phosphorylase. Column size was 2.5 x 50 cm. 5 ml fractions were collected. Flow rate was 0.5 ml per minute. The gel was equilibrated with 25 mM β-Glycerophosphate buffer pH 6.8 containing 15 mM mercaptoethanol and eluted with the same buffer. ○, rabbit muscle enzyme; △, Sepia enzyme.

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the rabbit enzyme, their molecular weights would be similar i.e about 200,000. Thus S.pharaonis phosphorylase a is a dimer unlike rabbit phosphorylase a. Phosphorylase a prepared from Crab muscle (42) (M= 180,000) and Dictyostelium discoideum (126) (M = 210,000) are similar to the S.pharaonis enzyme in this respect.

Estimation of total sulphydryl groups.

The total 'SH' groups in the Sepia enzyme were estimated

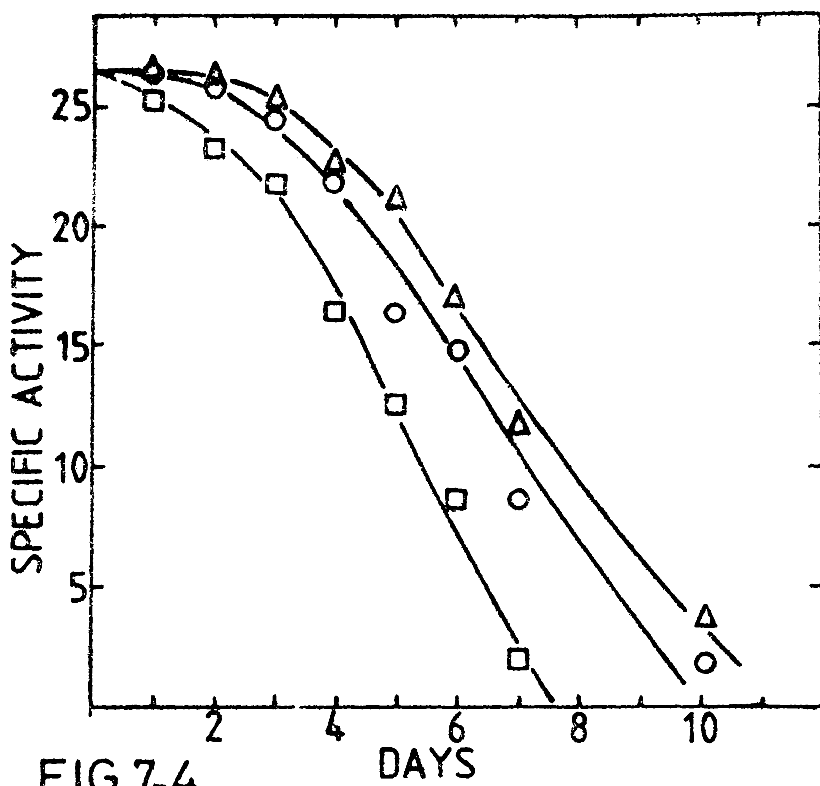

FIG.7-4

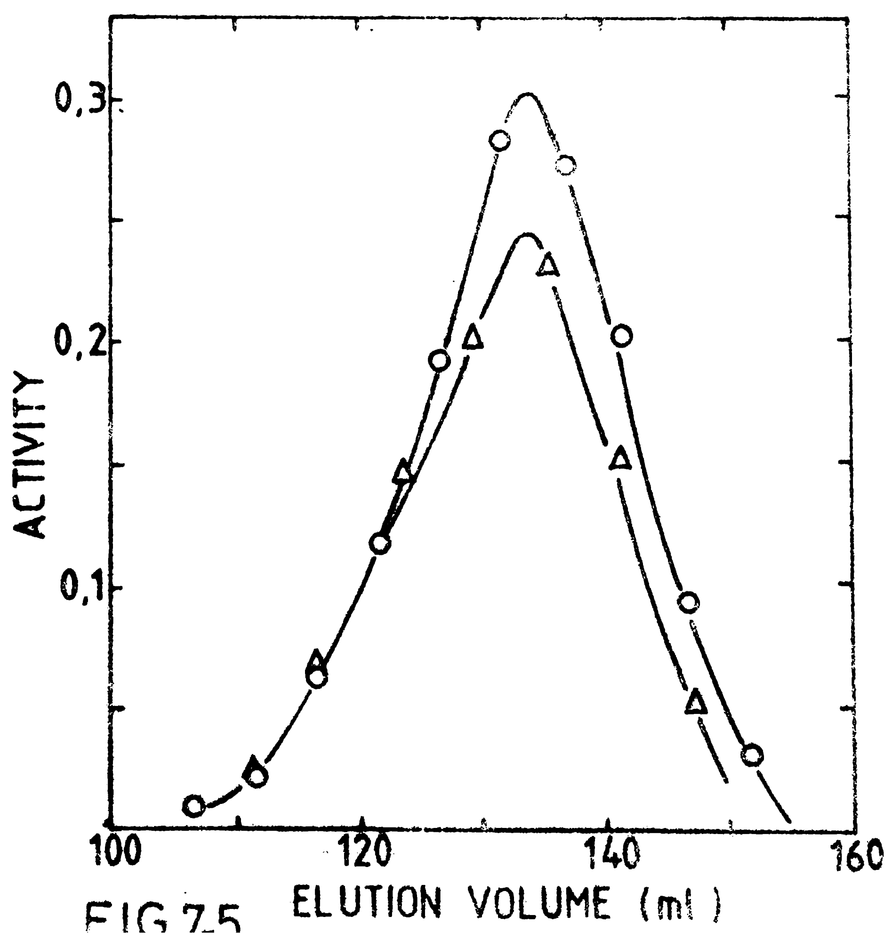

FIG.7-5

Fig. 7-6.

Effect of Ca^{++} and Mg^{++} on the activity of *Sepia* muscle phosphorylase. The enzyme was diluted in 40 mM glycerophosphate/ 30 mM cysteine and incubated with different concentrations of $CaCl_2$ or $MgCl_2$ (prepared in the same buffer) for 15 minutes. The assay mixtures contained, 40 µg/ml enzyme, 16 mM glucose-1-P, 1% glycogen and 1 mM AMP and varying concentrations of $CaCl_2$ (○) or $MgCl_2$ (△).

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using Ellman's reagent (DTNB) (139). The enzyme was first treated with urea at pH 8 to expose all the SH groups. This was allowed to react with DTNB and the absorbance measured at 412 nm. Using cysteine as standard, the SH groups in the enzyme were calculated. Assuming a molecular weight for the enzyme to be 2×10^5, the total SH groups present were repeatedly found to be 8.6 mole per mole of the enzyme.

The effect of the following ions on the activity of *S.pharaonis* phosphorylase was tested upto a concentration of 0.2 molar of the salts: Na^+, K^+, Ca^{++}, Mg^{++}, NH_4^+, Cl^-, $SO_3^=$, $SO_4^=$, acetate and citrate. Except for Ca^{++} and Mg^{++}, all other ions tested had no effect. For Ca^{++} and Mg^{++} (as $CaCl_2$ and $MgCl_2$) there were slight activation upto a concentration of 20 mM. Thereafter these ions inhibited the enzyme. The inhibition was about 90% at 0.2 M concentration of the salt. (Fig. 7-6). Na_2SO_4 has been shown to be an

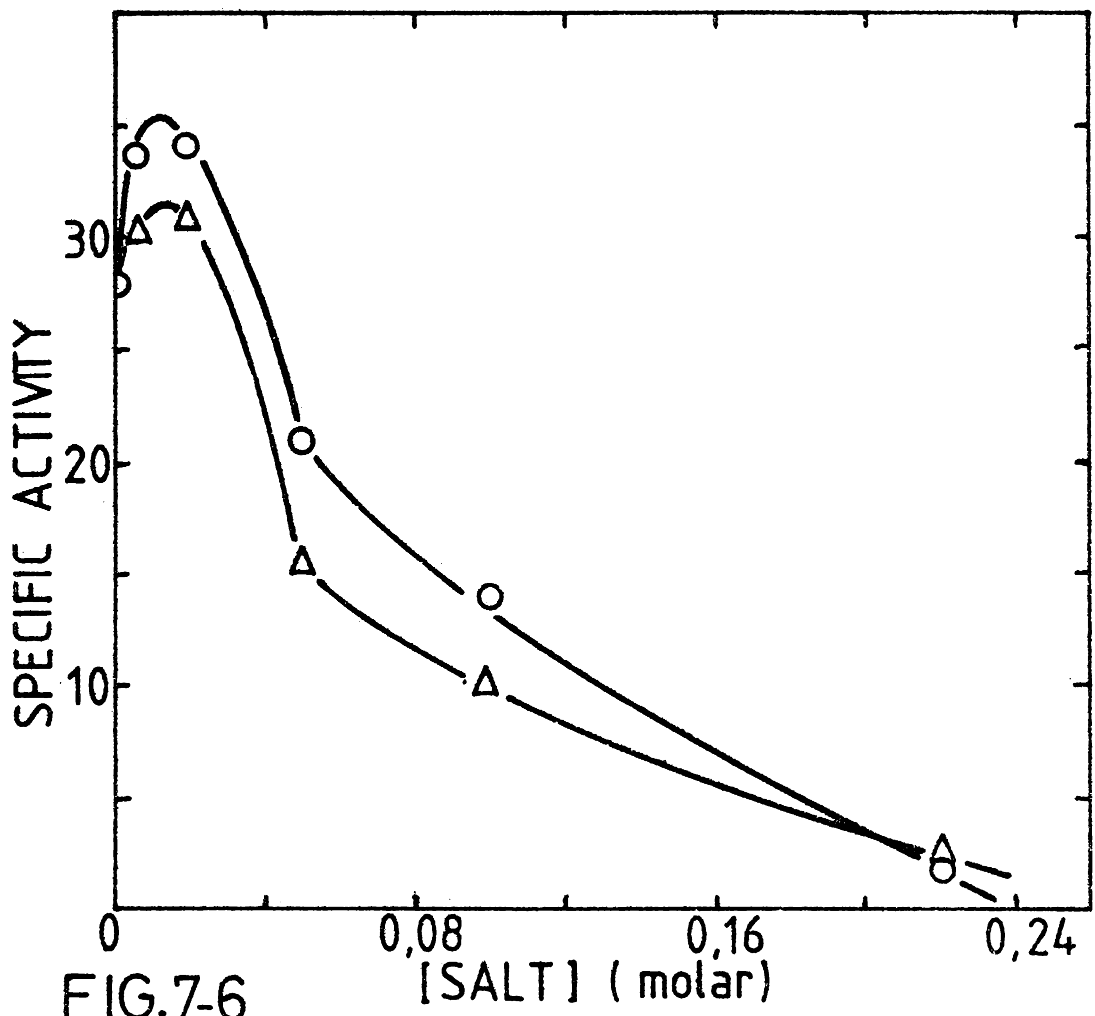


FIG.7-6

activator of lamprey (a lower vertebrate) phosphorylase (20). No such activation was observed for the S.Phoraonis enzyme.

Estimation of pyridoxal phosphate.

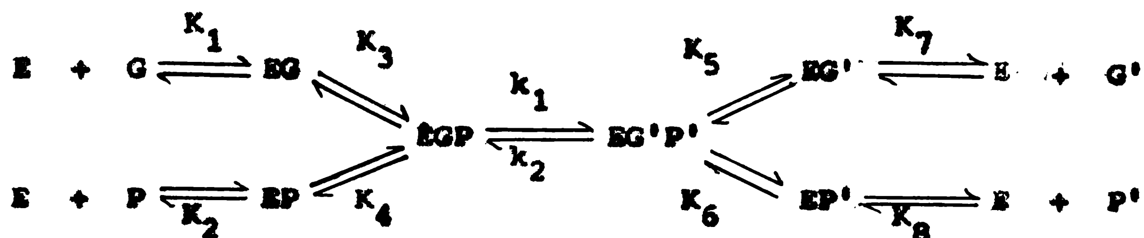
Pyridoxal-5'- phosphate has been shown to be present in all α-glucan phosphorylases studied so far. Like in the phosphorylases from other sources, the S.pharaonis enzyme also showed an absorption maximum around 335 nm. (The absorption maximum observed was presumed to be due to the presence of PLP. Further characterization and identification of the coenzyme were not tried). The number of pyridoxal phosphate in the S.pharaonis enzyme was estimated colorimetrically using phenyl hydrazine (160). One mole of PLP was found to be present in 102,000 g of the enzyme or roughly 100,000 g of the enzyme. Since the molecular weight of the S.phraonis enzyme was found to be similar to that from the rabbit phosphorylase b (dimer) and since a number of species have phosphorylase a in dimeric form with a similar molecular weight, it was presumed that the S.pharaonis phosphorylase a was a dimer and 1 mole of PLP was present in each monomer.

CHAPTER 8

KINETICS OF PHOSPHORYLASE a FROM SEPIA PHARAONIS.

The kinetic mechanism of rabbit muscle phosphorylase a and b (35, 36, 161), rabbit liver phosphorylase (162), phosphorylase b from a vertebrate fish Cibium guttattam (38) and E.coli maltodextrin phosphorylase (37) has been shown to be rapid equilibrium random bi bi . The kinetic properties of the S.pharaonis phosphorylase a were studied in the direction of glycogen synthesis inorder to compare the mechanism.

The kinetics of the Sepia phosphorylase a was analysed in the presence of 1 mM AMP by varying the glycogen and glucose-1-P concentrations (Figs. 8-1 and 8-2). The lines in both cases converged on the left hand side of the vertical axis showing that the kinetic mechanism of S.pharaonis phosphorylase is similar to that from other sources. Based on these results and those obtained for other animal phosphorylases the kinetic mechanism for S.pharaonis enzyme may be represented as follows:-



E is the enzyme; P, orthophosphate and P', glucose-1-P.

<u>Fig. 8-1.</u>

Lineweaver-Burk plots for glucose-1-P at different levels of glycogen for *S. pharaonis* phosphorylase *a* at pH 6.9. The reaction mixtures contained 1 mM AMP, 15 mM cysteine, 20 mM sodium β-glycerophosphate and glucose-1-P as indicated. The enzyme concentration was 65 μg/ml. The concentration of glycogen was ○, 0.2 mM (expressed as glucosyl residues); △, 0.5 mM; ●, 2.0 mM and □, 5.0 mM.

<u>Fig. 8-2.</u>

Lineweaver-Burk plots for glycogen at different levels of glucose-1-P for *S. pharaonis* phosphorylase *a* at pH 6.9 ○, △, ×, ● and □ correspond to 8, 12, 16, 24 and 32 mM concentrations of glucose-1-P. Other details were as in Fig. 8-1.

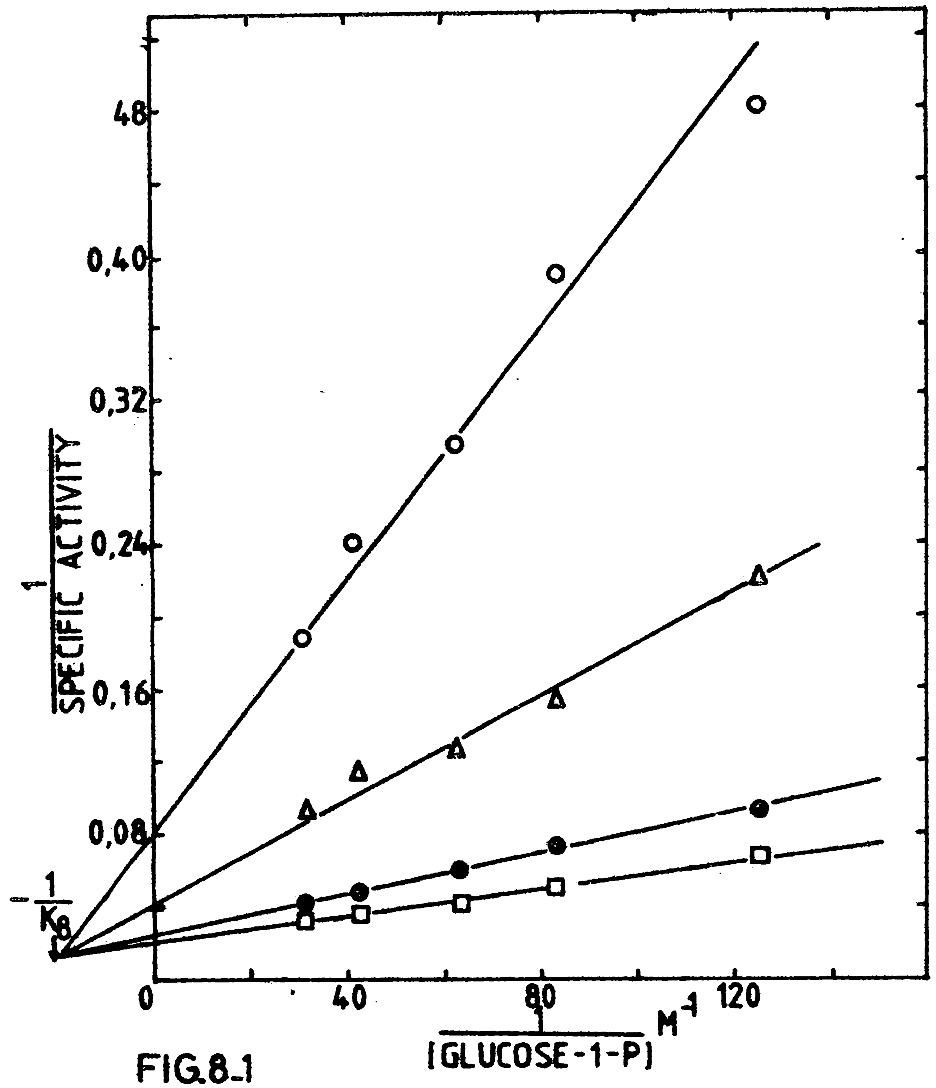


FIG.8.1

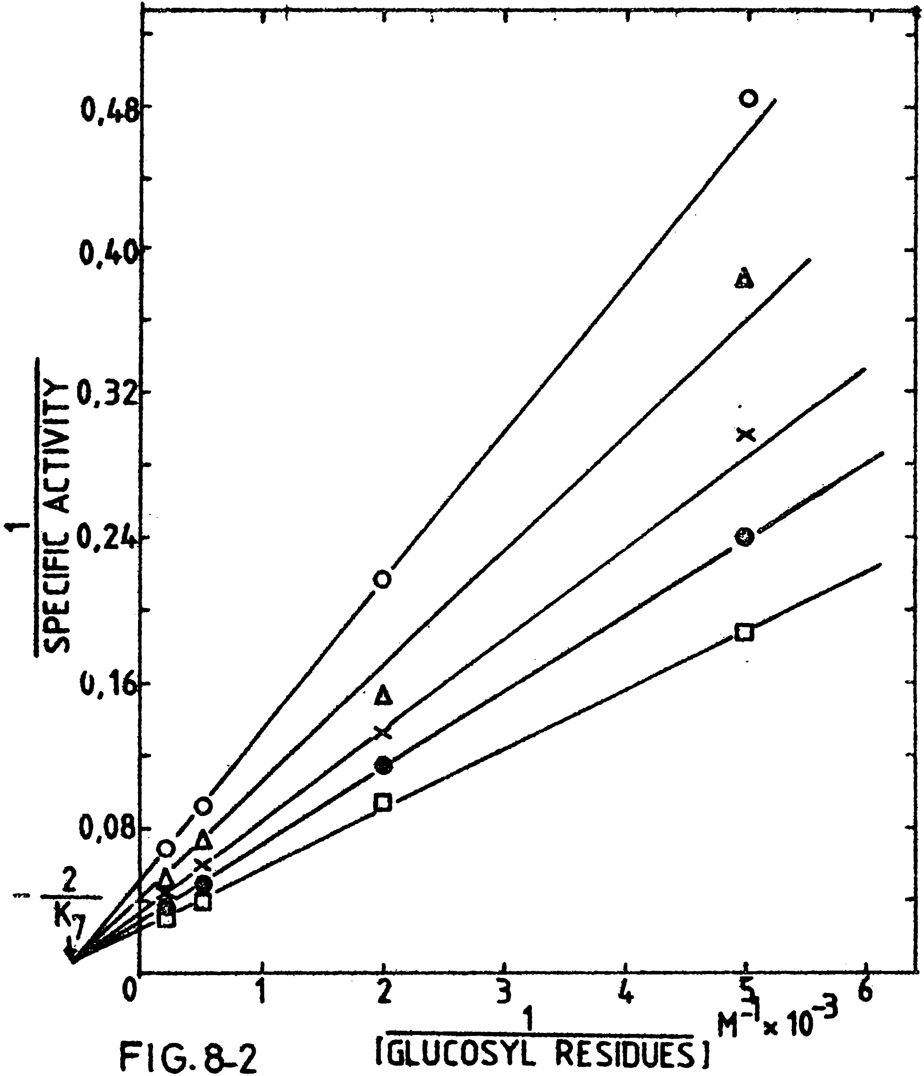


FIG. 8-2

G and G' represent glycogen bound for degradation and synthesis respectively.

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

$$\frac{(E)_0}{v} = \frac{1}{k_2} + \frac{K_6}{k_2\,(G')} + K_5\left(\frac{1 + K_7/K_1}{k_2\,(P')}\right) + \frac{K_5\,K_7}{k_2\,(G')\,(P')}$$

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

$$\frac{(E)_0}{v} = \frac{1}{k_2} + \frac{K_6}{k_2\,(G')} + \frac{2K_5}{k_2\,(P')} + \frac{K_5\,K_7}{k_2\,(G'\;P')}$$

multiplying by k_2 the equation becomes,,

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

The equation can be written in the general form of Dalziel (163) as:

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

φ'_0, φ'_1 etc. represent the kinetic coefficients.

The apparent K_m values evaluated as a function of each other from the reciprocal plots (Figs. 8-1 and 8-2) are given in Table 8-1.

TABLE 8 - 1

APPARENT K_m VALUES FOR GLUCOSE-1-P AND GLYCOGEN AT DIFFERENT CONCENTRATIONS OF EACH OTHER FOR S. PHARAONIS PHOSPHORYLASE a. (The 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)
0.2	43	8	1.6
0.5	33.3	12	1.6
2.0	23.2	16	1.55
5.0	18.2	24	1.5
		32	1.5

K_m values for glycogen remained unaltered at different concentrations of glucose-1-P but K_m values for glucose-1-P decreased when glycogen concentration was increased. This suggested that binding of glycogen enhanced the affinity for glucose-1-P. The allosteric conformational changes brought about by the two ligands may be different so that heterotropic interactions due to the binding of glycogen favoured binding of glucose-1-P but not _vice versa_. The result shows inconsistency with the predictions of the Monod model (102) where allosteric transitions are represented by 2 states (T and R) and the transitions are effected through changes in the K_m values only (i.e the 'K' system of activation).

The dissociation constants K_5, K_6, K_7 and K_8 and the rate constant k_2 can be calculated from Figs. 8-1 and 8-2 and from secondary plots obtained by replotting the values of the intercepts and slopes of the primary plots against reciprocal of the second substrate. The secondary plots are given in Figs. 8-3 and 8-4. These four plots provide 4 x 2 equations, so that the values of the dissociation constants can be checked using these different equations. The internal consistency of the experimental measurements can be ascertained by this. Table 8-2 shows a comparison of the dissociation constants obtained by this method. The internal consistencies

Fig. 8-3. A

Secondary plots of intercepts (mg enzyme vs min per micromole) from Fig.8-1.

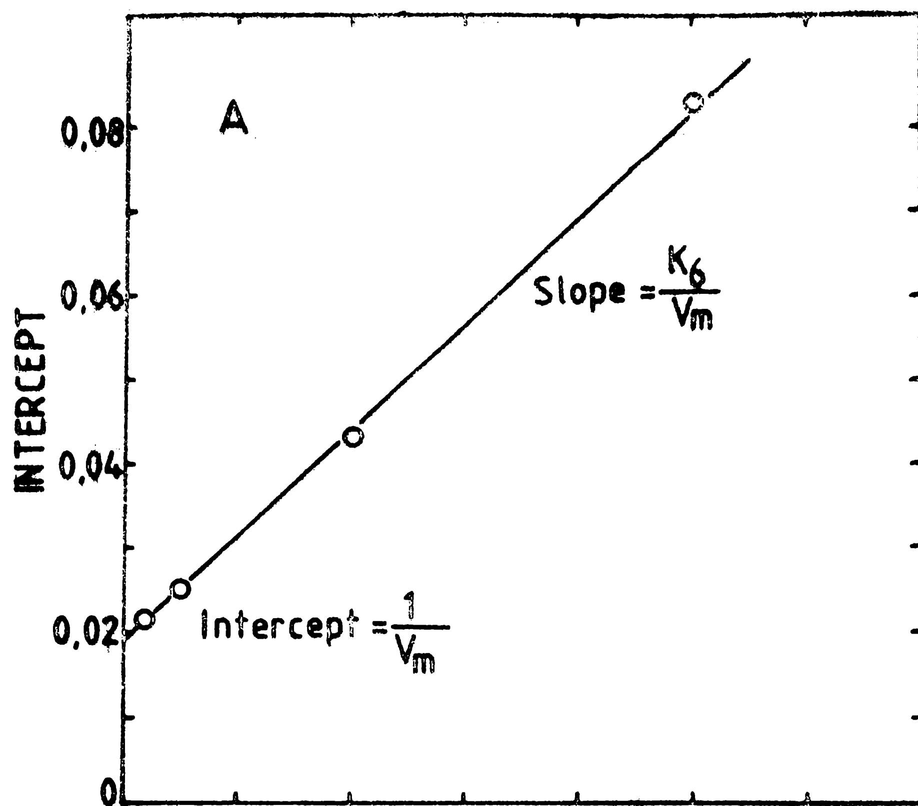
Fig. 8-3. B

Secondary plots of the slopes from Fig. 8-1.

TABLE 8 - 2.

THE VALUES OF KINETIC CONSTANTS OBTAINED USING DIFFERENT EQUATIONS FROM THE SECONDARY PLOTS.

	Values obtained from			
	Fig. 8-3.A	Fig. 8-3.B	Fig. 8.4.A	Fig. 8-4.
V_{max} (μ moles/min/mg)	52.6	-	55.6	-
K_5 (mM)	-	7.0	7.3	-
K_6 (mM)	0.67	-	-	0.9
K_7 (mM)	-	4.8	-	4.2



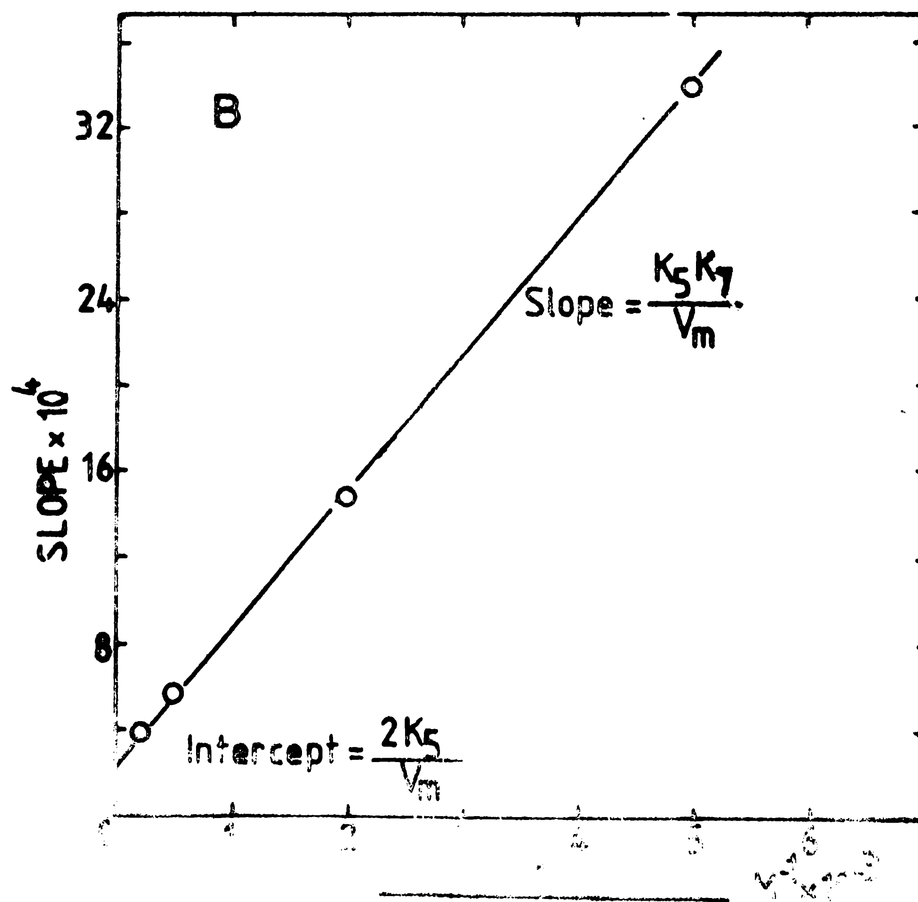


FIG.8.1

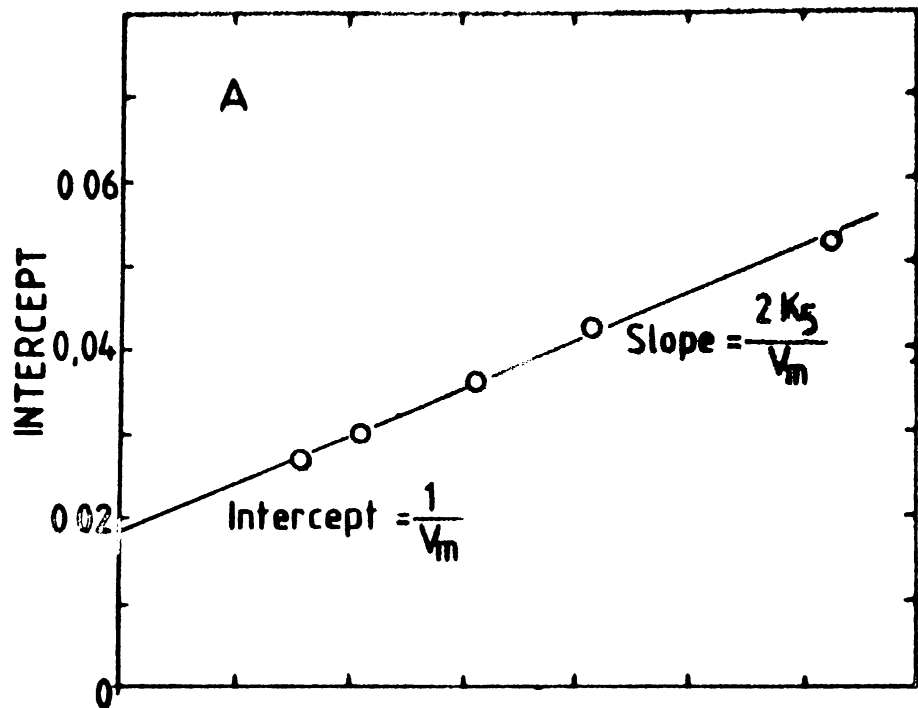
Fig. 8-4.A

Secondary plots of the intercepts from Fig.8-2 (mg enzyme *vs* min per micromole).

Fig. 8-4.B.

Secondary plots of the slopes from Fig. 8-2.

(Table 8-2) also prove that the kinetic mechanism of *S. pharaonis* phosphorylase was consistant with that established for phosphorylase from different sources. The average values of the kinetic constants and coefficients calculated from the primary and secondary plots are compared in Table 8-3 with that from rabbit muscle phosphorylase *a* and *b* (35, 161) and that from a marine vertebrate fish *Cibium guttattam* (38). The table shows that the value of K_5 (i.e., Michaelis constant for glucose-1-P at saturating glycogen concentration) of the *S. pharaonis* phosphorylase *a* is 2 times higher than the *C. guttattam* phosphorylase *b* and about 5 times and 70 times higher than rabbit muscle phosphorylase *b* and *a* respectively. The dissociation constant of glucose-1-P (K_8) is also much higher in the *S. pharaonis* enzyme. The results show that the *S. pharaonis* phosphorylase *a* and the rabbit muscle phosphorylase *a* varied greatly with respect



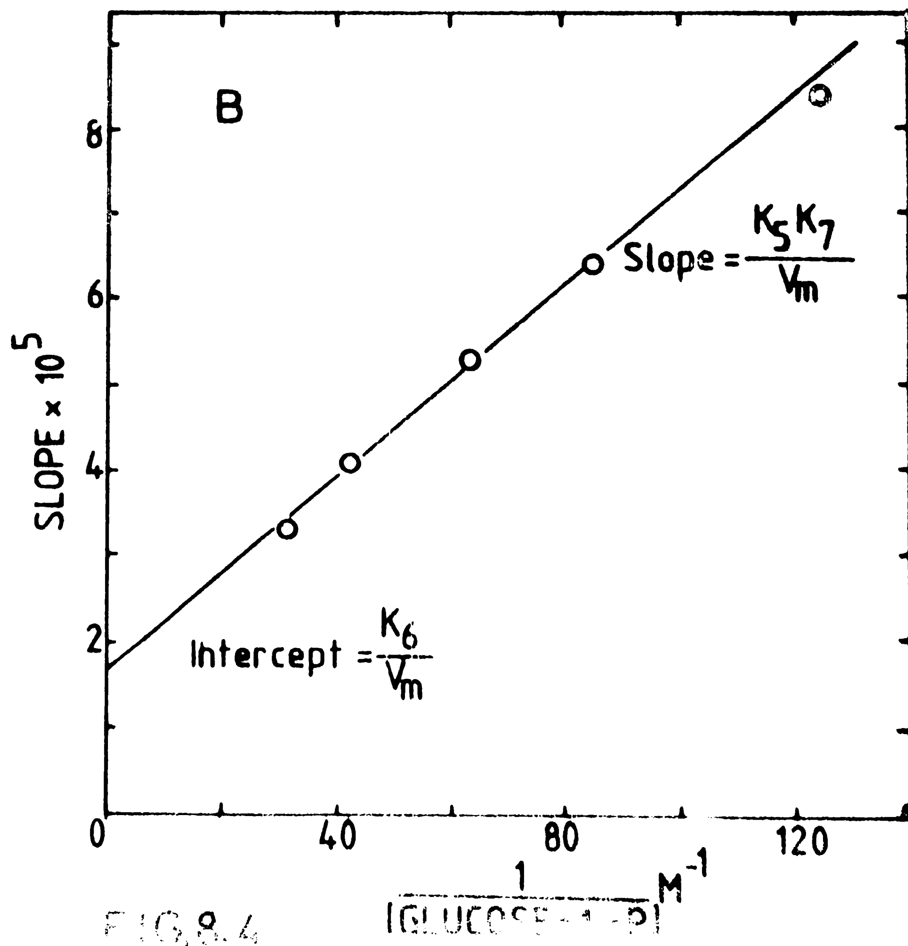


FIG. 8.4

TABLE 8 -3

COMPARISON OF THE KINETIC CONSTANTS AND COEFFICIENTS FOR *SEPIA PHARAONIS* PHOSPHORYLASE *a* WITH THE VALUES REPORTED FOR THE ENZYMES FROM RABBIT MUSCLE AND *C. GUTTATTAM*.

	S. pharaonis phosphorylase *a*	*C. guttattam* phosphorylase *b* (From Ref. 38)	Rabbit muscle phosphorylase (From Ref. 35 & 161)	
			a	*b*
Kinetic Coefficients				
φ'_0 (mg x min/μ mole)	0.0185	0.0163	0.0145	0.0155
φ'_1 (min x mg)	14.6	36.0	7.25	14.0
φ'_2 (min x mg)	266.7	120.0	2.9×10^{-3}	51.0
φ'_{12} (min x mg)	0.60	0.53	3.12×10^{-3}	0.104
Kinetic Constants.				
k_3 (μ moles/mg/min)	54.0	61.4	69	64
K_5 (mM glucose-1-P)	7.2	3.6	0.1	1.5
K_6 (mM glycogen)	0.79	2.8	0.5	0.9
K_7 (mM glycogen)	4.5	9.0	2.2	4.4
K_8 (mM glucose-1-P)	50.0	15.3	0.8	7.4

Fig. 8-5.

S. pharaonis phosphorylase a activity as a function of pH. The enzyme was diluted in 50 mM β-glycerophosphate/ 30 mM cysteine of the required pH and added to an equal volume of substrate solution containing 32 mM glucose-1-P, 2 mM AMP and 2% glycogen adjusted to the required pH.

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to the affinity for substrates suggesting structural changes at the region of the active site.

Effect of PH

The pH-activity curve for S.pharaonis phosphorylase a in cysteine-glycerophosphate buffer is shown in Fig.8-5. The optimum pH for the enzyme was 6.9. The pH profile was very much similar to that of rabbit phosphorylase. Therefore the same active groups might be expected to be involved in catalysis. Although the exact nature of the groups in rabbit enzyme has not been unambiguously identified, the 5'-phosphate group of the PLP has been shown to directly participate in catalysis (64,65). Since kinetic mechanism of the Sepia enzyme was the same as for the rabbit enzyme, the reaction mechanism could also be the same.

Influence of temperature on S.pharaonis phosphorylase.

The effect of temperature on the velocity of

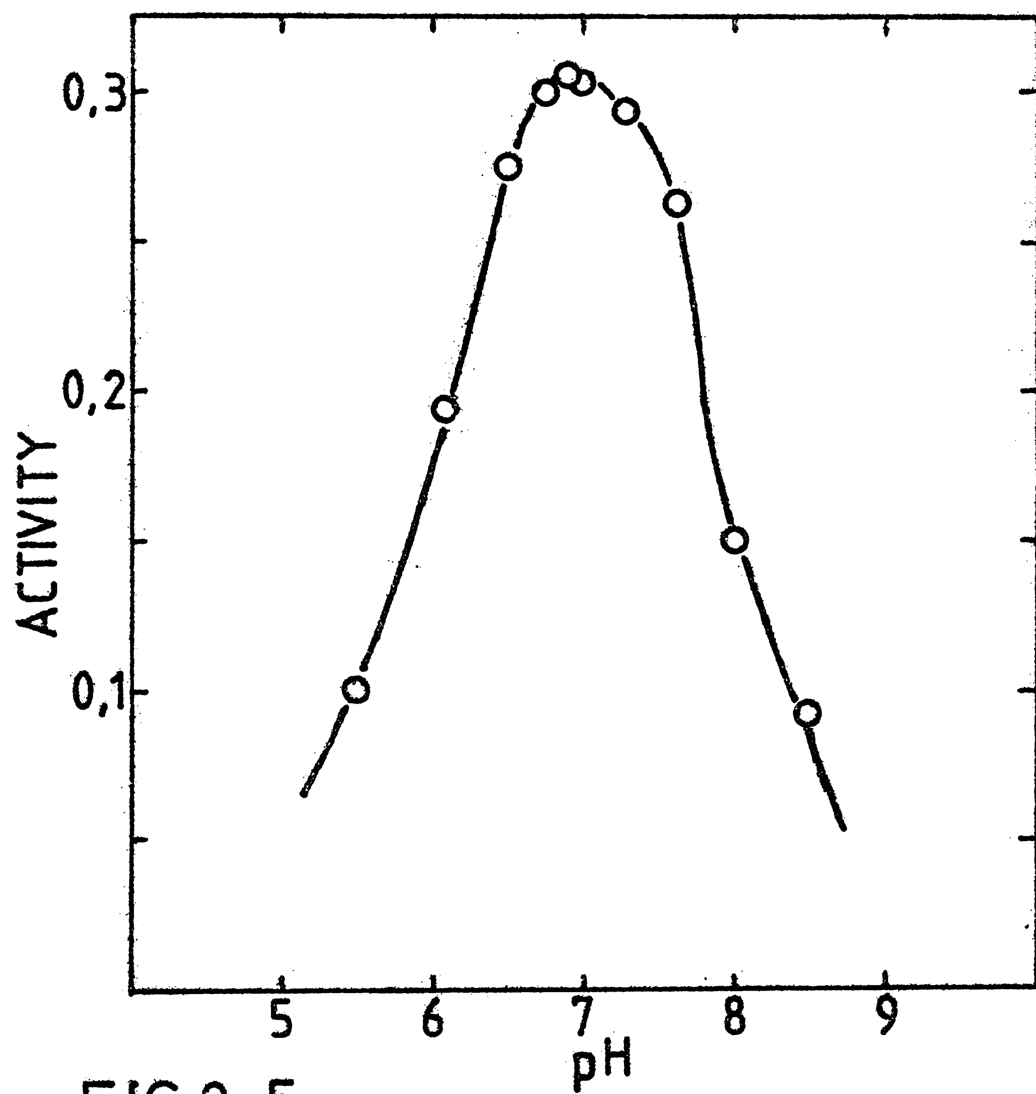

FIG.8-5

Fig. 8-6.

Double reciprocal plots for glucose-1-P at different temperatures; Fig. A: At 20°, in the absence (△) and presence (○) of 1 mM AMP. Fig. B: At 30° (open symbols) and at 35° (filled symbols) in the absence (△, ▲) and presence (○ , ●) of 1 mM AMP.

Fig. 8-7.

Arrhenius plots for the data in Fig. 8-6; in the absence (●) and presence (○) of 1 mM AMP.

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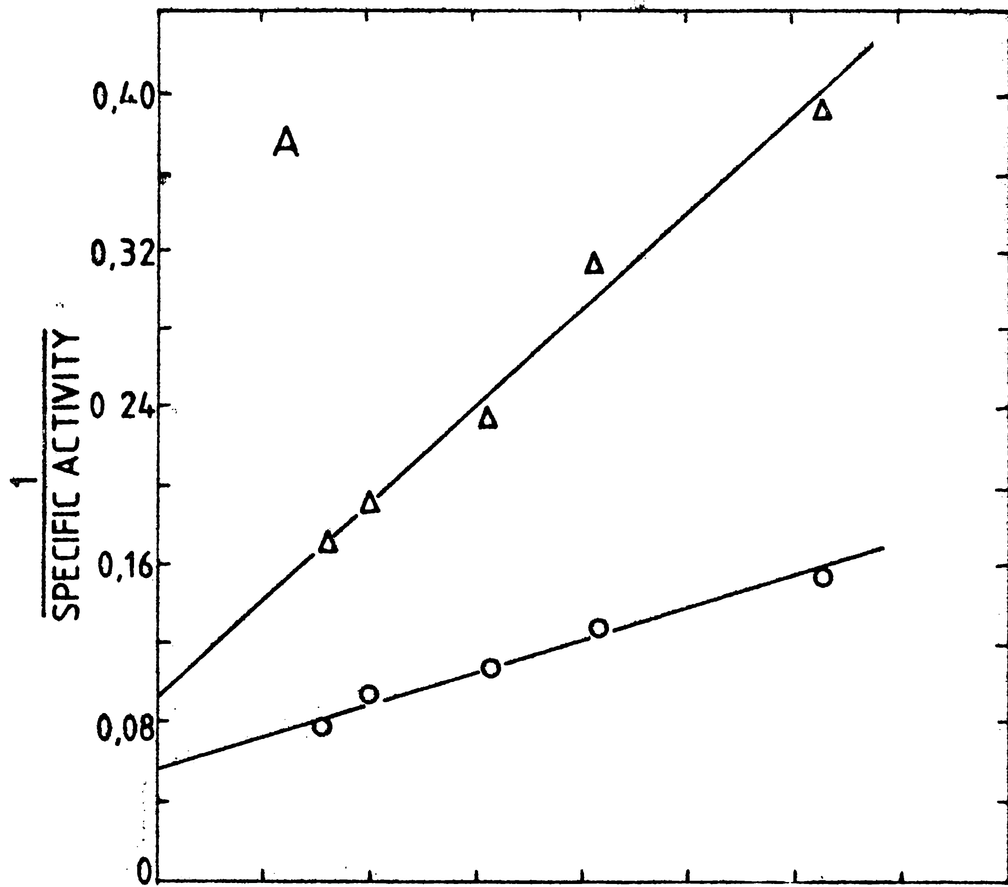
phosphorylase reaction was studied at varying substrate concentration. Since the activity ratio of S.pharaonis phosphorylase a was 0.4, the K_m and V_{max} could be evaluated in the presence and absence of AMP. Fig. 8-6 shows the Lineweaver-Burk plots obtained at three different temperatures. The V_{max} was increased with rise in temperature, but there was no ordered change for K_m. Fig. 8-7 shows the Arrhenius plot obtained from the values of Fig.8-6. The linear plots obtained indicated that the temperature coefficient of this reaction did not change in this temperature range. It also confirmed that the enzyme contained only one active form. The activation energy of the S.pharaonis enzyme calculated from the Arrhenius plots was 21 kcals in the absence and in the presence of 1 mM AMP. This value is comparable to that reported for rabbit muscle enzyme (164) and for the enzyme for C.guttattam (105).



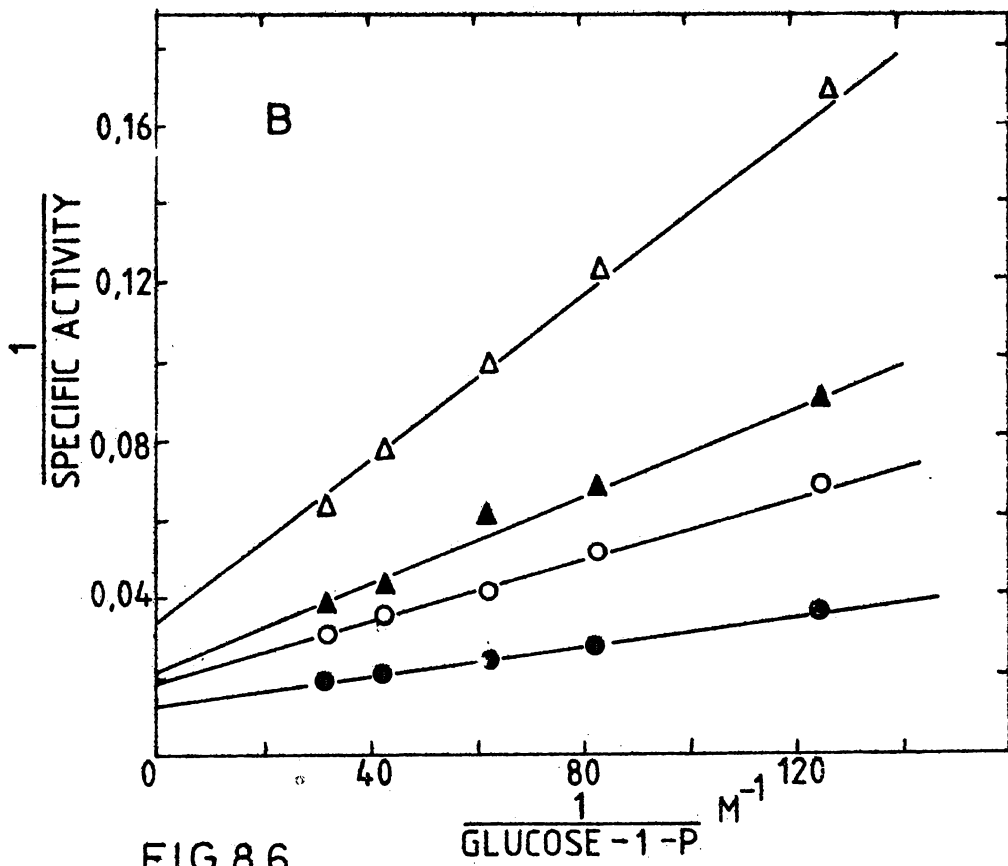


FIG.8-6

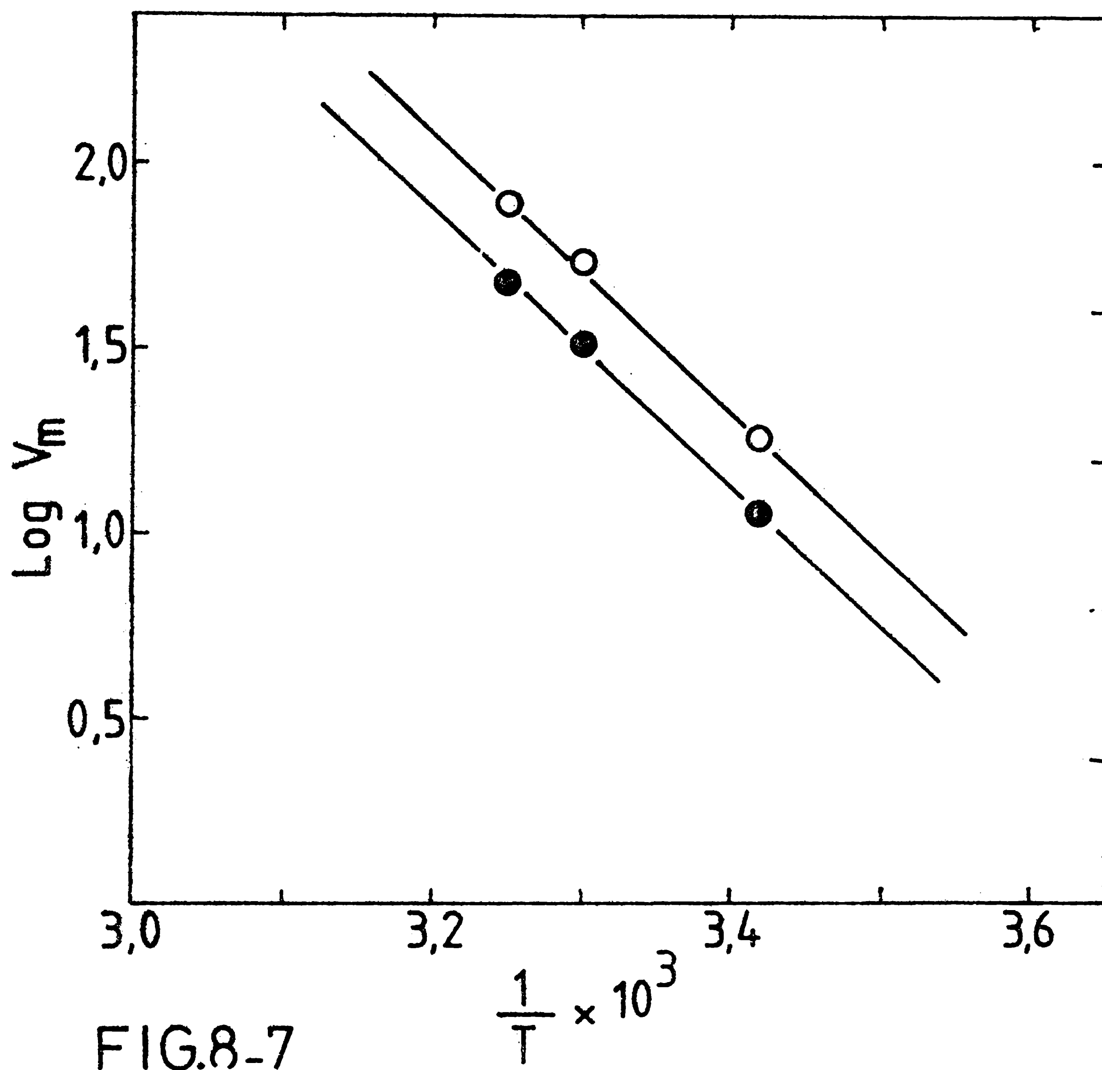


FIG.8-7

CHAPTER 9

EFFECT OF CYSTEINE ON THE STRUCTURE AND ACTIVITY OF SEPIA PHOSPHORYLASE a.

α-glucan phosphorylases studied so far contain the coenzyme pyridoxal-5'- phosphate (PLP). The enzyme has an absorption band near 335 nm due to the presence of PLP. This band has been assigned to a zwitterionic addition product of the Schiff base form of the PLP formed with Lysine-679 (8,40). The enzyme was reduced with sodium borohydride without loss of activity and this reduced enzyme shows an absorption maximum near 290 nm (68,165). However, since removal of the coenzyme is followed by loss of enzyme activity, PLP has been believed to have some definite role. Any part of the PLP might be directly involved in catalysis or it could be that it is responsible for the active conformation of the enzyme. The significance of PLP in the interconversion between the a and b forms has also been investigated (72). The 5'-phosphate group of PLP in rabbit enzyme has been shown to participate directly or indirectly in catalysis. The *S.pharaonis* phosphorylase was found to be specifically activated by L-cysteine considerably (see below) unlike phosphorylase from other sources reported. This demanded a detailed study of the effect of cysteine on *Sepia* phosphorylase as well as of the interaction of cysteine if any, with the PLP. This chapter

describes studies carried out in these directions.

The purified phosphorylase a showed an absorption maximum at 335 nm in 40 mM sodium β-glycerophosphate buffer pH 7. However, unlike the phosphorylase from rabbit muscle, this peak was completely lost in the presence of neutral cysteine (Fig. 9-1). Such a property has not been reported in any other phosphorylases studied so far. The effect of cysteine was time dependent (Fig. 9-1). In 10 minutes the absorption band at 335 nm of a 4 mg/ml enzyme solution was completely lost on inclusion of 5 mM neutral cysteine. Lowering the cysteine concentration (below 1mM) reduced the rate of spectral change, but there was no increase in the rate above 10 mM cysteine. The effect of cysteine on the spectral properties in relation to the enzyme concentration will be discussed below.

Fig. 9-2 shows the difference spectrum for the cysteine treated enzyme (native enzyme was used as reference). The difference spectrum exhibited a trough at 343 nm and a peak around 300 nm. This suggested that the effect of cysteine was to shift the apparent 335 nm band to 300 nm. Thus cysteine had the effect of converting the PLP into a form other than that present in native phosphorylases from other sources. No such change was observed in the rabbit muscle phosphorylase a or b on addition of cysteine under the same experimental conditions employed for the *Sepia* enzyme.

Fig. 9-1.

Absorption spectra of *Sepia* mantle muscle phosphorylase *a* in the absence and presence of neutral L-cysteine. Spectra were taken using a Hitachi model 200 recording uv-visible spectrophotometer.

Curve-1. Phosphorylase (4 mg/ml) in 40 mM β-glycerophosphate pH 7.

Curve-2. Spectrum 1 min. after inclusion of 5 mM neutral L-cysteine. No correction for volume change was made. The volume increase was 2.5%.

Curve-3. Same as above after 5 min.

Curve-4. Same as above after 10 min.

Rabbit muscle phosphorylase has been shown to be inactivated by cold temperature in the presence of cysteine at pH 6.2 and at high concentration of salt (0.2 to 0.5 M). This inactivation was shown to be followed by a slight shift of the 333 nm absorption band towards a lower wave length, but with a significant increase in absorbance at 333 nm (166). The *Sepia* phosphorylase was found to be more sensitive to cold temperature (Chapter 10). However, the effect of cysteine in shifting the absorption band of *Sepia* enzyme was not related to inactivation of the enzyme because the spectral shift was

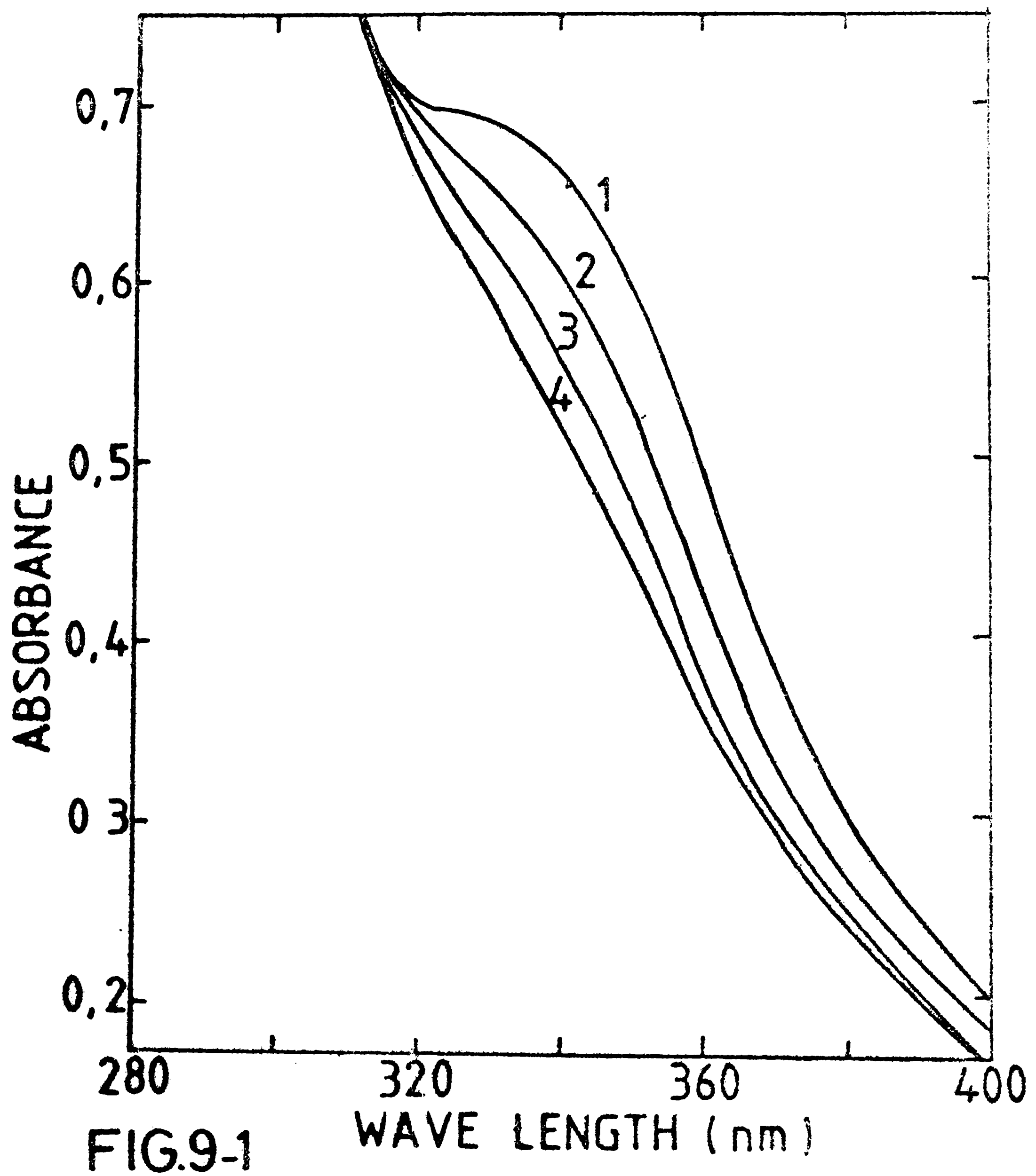


FIG.9-1

Fig. 9-2.

Difference spectrum for the cysteine treated enzyme against native enzyme as reference. 4 mg/ml phosphorylase in 40 mM glycerophosphate pH 7 was made 10 mM in neutral cysteine and spectrum taken with untreated enzyme as reference, after adding a corresponding volume of water to the reference enzyme.

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also observed at 30° , without any loss of activity. There was about 10% loss of activity for the enzyme at 25° after 2 hours in the presence of 30 mM cysteine and 0.5 M NaCl. The *Sepia* enzyme might be more sensitive to such conditions so that the structural changes brought about by cold temperature in rabbit enzyme was slightly favoured in the *Sepia* enzyme at neutral pH and at room temperature (See Chapter 10). However, the spectral shift in *Sepia* enzyme could not be directly attributed to such structural changes and inactivation because the effect was seen without the presence of salt and without the enzyme undergoing inactivation.

Enzyme treated with mercaptoethanol (under the same conditions employed for cysteine) did not show any significant effect. The substrates glucose-1-P and partially hydrolysed starch also had no effect on the spectral properties of the enzyme. Also the substrates did not affect the spectral

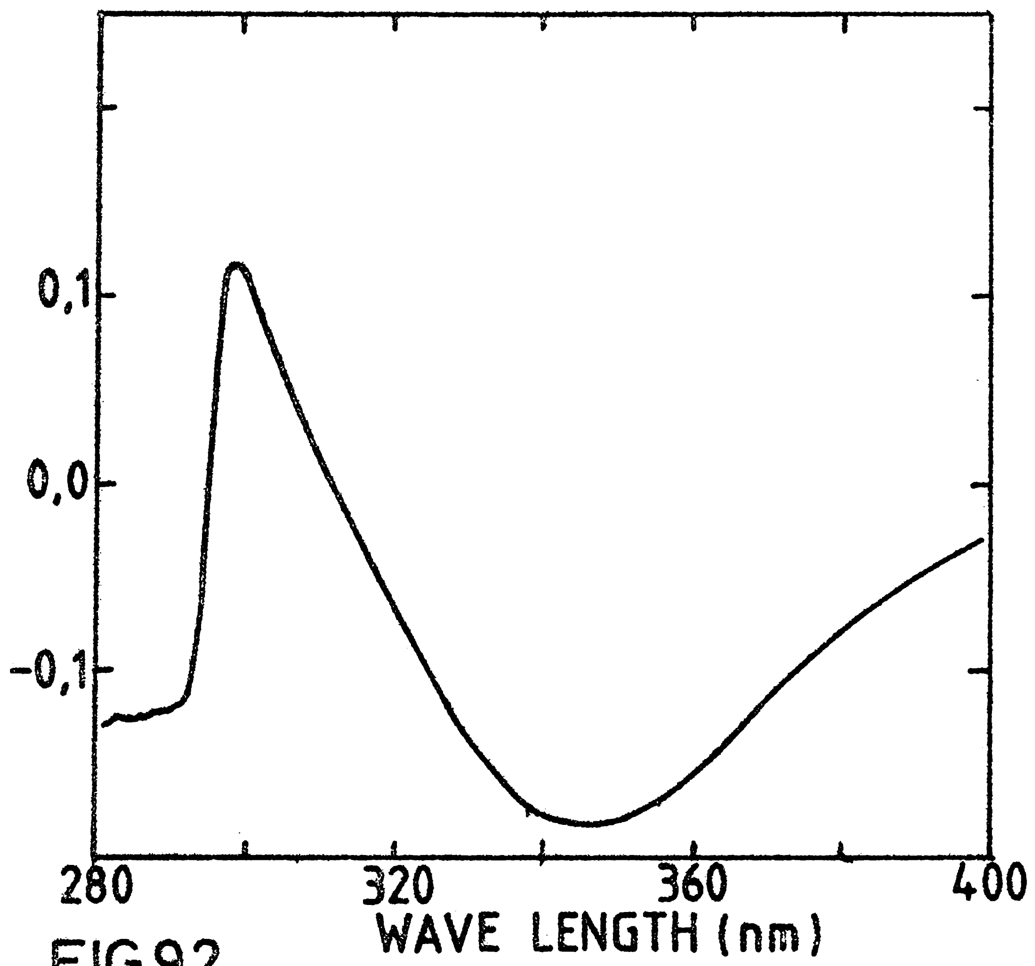

FIG.9-2

Fig. 9-3.

Activation of *Sepia* phosphorylase *a* by L-cysteine. The enzyme was freed from mercaptoethanol by passage through Sephadex G-25. It was then diluted in buffers containing different concentrations of cysteine and assayed by adding to to an equal volume of substrate solution. The assay mixture contained 16 mM glucose-1-P, 1% glycogen in the absence (O) and presence (△) of 1 mM AMP.

Fig.9-4.

Double reciprocal plots for glucose-1-P in the absence and presence of cysteine. The enzyme was diluted in 0.06 M glycerophosphate and in 30 mM cysteine/40 mM glycerophosphate, pH 6.9 and assayed by adding an equal volume to substrate solutions containing different concentrations of glucose-1-P. The assay mixture contained 1 mM AMP, 1% glycogen and 15 mM cysteine (O) and no cysteine (△).

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changes brought about by cysteine.

Effect of cysteine on the activity of Sepia Phosphorylase.

Cysteine was found to activate the *Sepia* phosphorylase. The effect was not time dependent as evidenced by the observation that there was the same extent of activation when the assay was completed within 45 seconds or for longer periods after addition of cysteine. The activation, however was dependent on

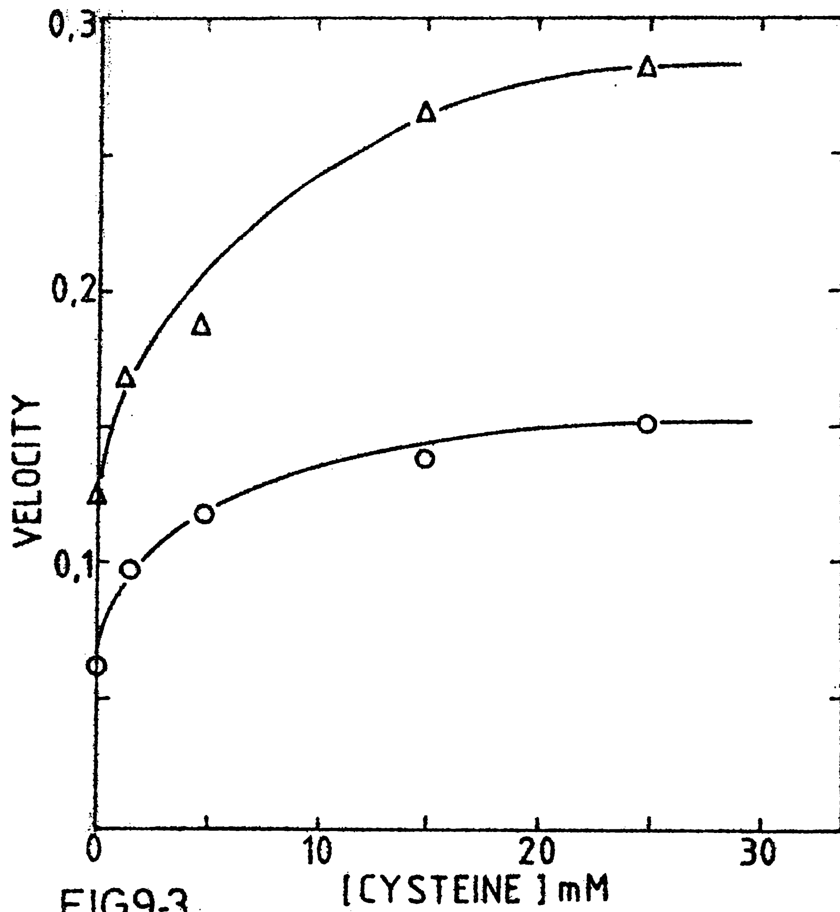

FIG.9-3

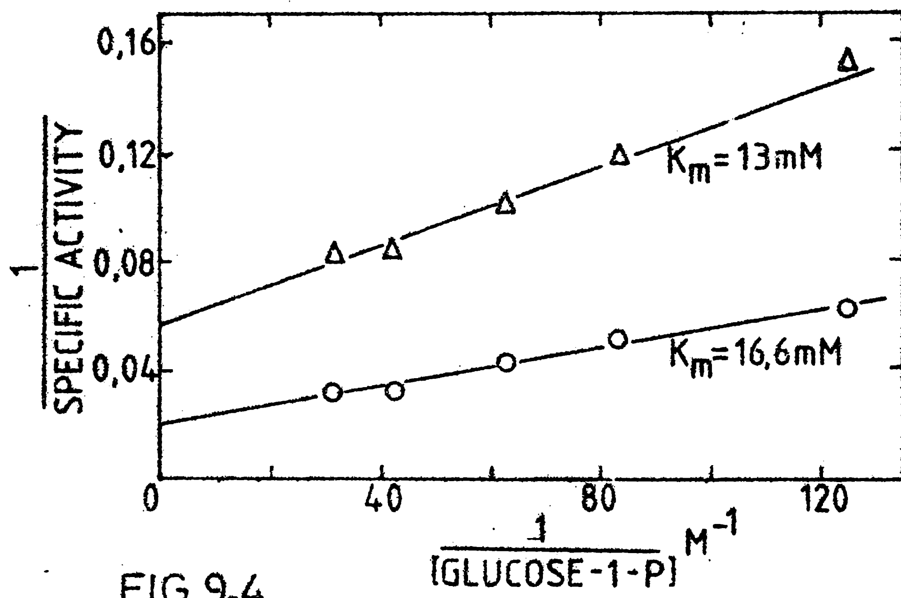

FIG.9-4

the concentration of cysteine and about 2.5-to 3-fold activation was observed at 15-20 mM cysteine. The enzyme activity as a function of cysteine concentration in the presence and absence of AMP is shown in Fig. 9-3. The activity ratio was not affected suggesting that the influences of cysteine and AMP were independent. The K_m values for glucose-1-P were nearly the same in the presence or absence of cysteine showing that the activation by cysteine was not due to change in affinity for glucose-1-P (Fig.9-4)

The activation by cysteine, like the spectral change was specific. Comparable concentration of mercaptoethanol showed only a 1.3-fold activation, whereas 3-fold activation was showed by cysteine. Thus the activation by cysteine was not dependent on the presence of sulphydryl compounds. Rabbit muscle phosphorylase contains 2-3 surface exposed SH groups which undergo oxidation in the absence of sulphydryl compounds (84). Hence cysteine (or mercaptoethanol) was used in the assay of rabbit phosphorylase to maintain the SH groups in the reduced state so that the native conformation of the protein is retained.

The specific influence of the cysteine in abolishing the 335 nm band and activating the enzyme suggested the possibility that these effects were directly related. However, it was not the case as the spectrum at assay concentrations (25-250 µg/ml) in the absence of cysteine showed no detectable

Fig. 9-5.

Absorption spectra of *Sepia* phosphorylase *a* at assay concentration. The spectra were taken using the expanded scale (10 times) in a Hitachi model 200 spectrophotometer.

Curve-1. *Sepia* phosphorylase *a* (240 µg/ml) in 20 mM glycerophosphate pH 7.

Curve-2. After inclusion of 15 mM neutral cysteine.

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band near 335 nm (Fig 9-5). This shows that on dilution of the enzyme to assay concentration in the absence or presence of cysteine converts the coenzyme to a form other than the 335 nm form. This form in dilute solutions (diluted with glycerophosphate buffer) was also activated 2.5-to 3-fold by 30 mM cysteine without showing any change in the spectrum or the absorbance at 335 nm. These results clearly suggested that the influence of cysteine in enhancing the activity was not directly related to its effect on reducing or abolishing the 335 nm band. The activation however was dependent on the concentration of cysteine in the final assay mixture, whether it was added before or after dilution of the enzyme in the assay buffer. Table 9-1 shows the specific activities of the cysteine treated enzyme compared to untreated enzyme when diluted in glycerophosphate or cysteine/glycerophosphate buffers.

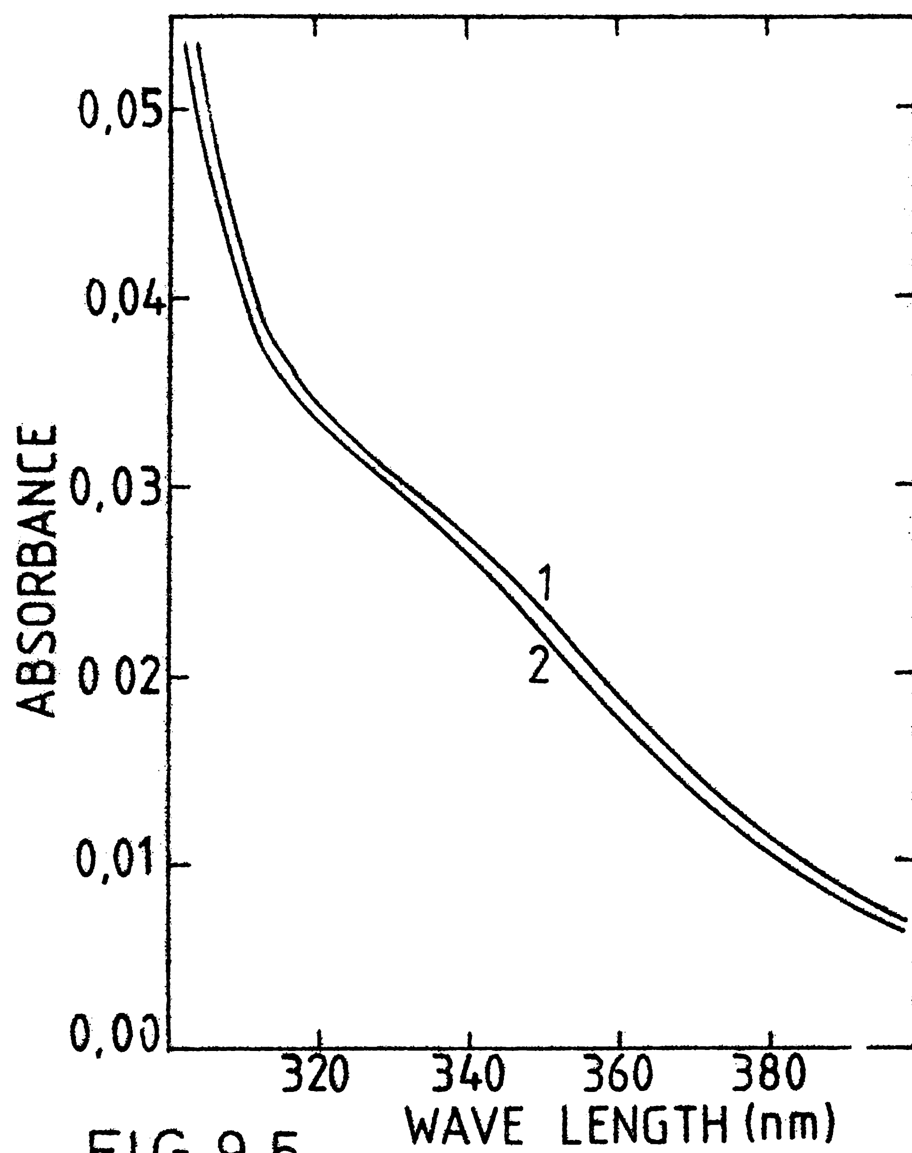

FIG. 9-5

TABLE 9 - 1.

SPECIFIC ACTIVITY OF SEPIA PHOSPHORYLASE IN THE ABSENCE AND PRESENCE OF CYSTEINE.

4 mg/ml phosphorylase in 40 mM glycerophosphate pH 6.9 (in the presence and absence of added cysteine) was diluted 50-fold in 60 mM glycerophosphate pH 6.9 and in 30 mM cysteine/40 mM glycerophosphate pH 6.9 and assayed for phosphorylase activity.

Enzyme 4 mg/ml. present in.	Specific activity after 50-fold dilution in			
	60 mM glycerophosphate pH 6.9		30 mM cysteine / 40 mM glycerophosphate, pH 6.9	
	Assayed in the absence of AMP.	Assayed in the presence of 1 mM AMP	Assayed in the absence of AMP	Assayed in the presence of 1 mM AMP
In 40 mM glycerophosphate-13.5 mM cysteine pH 6.9 (absorption maximum at 300 nm)	5.55	13.6	10.5	27.8
In 40 mM glycerophosphate pH 6.9 (absorption maximum at 335 nm and lost on dilution).	4.85	10.0	10.4	26.5

Since the 335 nm band was absent in dilute enzyme solutions (even in the absence of cysteine) it was checked

if added substrates could bring back the 335 nm band. The band was not reappeared and this suggested that the 335 nm form of PLP in *Sepia* phosphorylase was not necessarily the active form of the coenzyme. Although this form has been reported to be the one present in all α-glucan phosphorylases studied so far, the above results are not surprising in view of the fact that $NaBH_4$-reduced rabbit muscle phosphorylase in which the 333 nm band is absent is catalytically active. However it can not be deduced that the PLP should be present in the 300 nm form to be active.

If the PLP in *Sepia* enzyme and in phosphorylase from other sources participate in catalysis by identical mechanism, similar spectral changes could be expected in all α-glucan phosphorylases. This was tested using crystalline rabbit muscle phosphorylase *a* and *b*. Rabbit muscle enzymes showed no differences in the 333 nm/ 280 nm absorbance ratio upto a dilution of 150 μg/ml. Further dilution decreased the ratio from 0.07 at 150 μg/ml to 0.02 at 40 μg/ml. Addition of substrates to 150 μg/ml of the rabbit enzyme did not change the ratio whereas the enzyme at this concentration was fully active. No change was observed when substrates were added to more dilute solutions (50 μg/ml). Therefore, in rabbit enzyme also, the PLP is not necessarily in the 333 nm form at normal

assay concentrations and hence this form of the coenzyme is not needed for the enzyme to be active. Even though similar type of spectral changes are apparent in rabbit and *Sepia* at assay concentration, the spectral properties of the former was unaffected by the addition of cysteine at lower or higher enzyme concentrations.

$NaBH_4$-reduced rabbit muscle phosphorylase which is catalytically active has no 333 nm band but instead has a band at 290 nm which shifts to 330 nm on acidification (165). It was observed that the 290 nm band of the reduced *Sepia* enzyme was unaffected on dilution to assay concentration, even in the presence of substrates. Apparently an equilibrium between the 290 nm and 330 nm form was not attained in the case of the reduced enzyme, whereas such an equilibrium may be assumed to be existing in native *Sepia* enzyme.

Since substrates did not shift the 333 nm band of a 150 µg/ml solution of rabbit phosphorylase to 290 nm either partially or completely, PLP need not remain in the 290 nm form to be active. No decrease of the 333 nm absorption in the rabbit enzyme was observed as a consequence of the presence of substrates. Combining the observations obtained using *Sepia* and rabbit enzymes, it becomes clear that neither of the two forms of PLP represented by 290 nm(300 nm in *Sepia* enzyme) and 333 nm (335 nm) band is directly responsible

Fig. 9-6.

Absorption spectrum of pyridoxal-5'- phosphate at different pH.

Curve-1. 200 μ molar PLP in 0.01 M β-glycerophosphate pH 6.8.

Curve-2. 175 μ molar PLP at pH 2.

Curve-3. 175 μ molar PLP at pH 12.

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for activity. The 290 nm band in reduced phosphorylase has been assigned to the neutral tautomer of the pyridoxamine derivative (165). Since the effect of cysteine on the Sepia enzyme was reversible, the 300 nm band could not be assigned to any pyridoxamine derivative of phosphorylase. This band could be due to a form of PLP either less conjugated than the 335 nm form or in an environment different from the 335 nm form or both. Pure PLP at acid pH (pH 3 or less) showed only one major band at 295 nm (Fig 9-6). This protonated form of PLP obviously would be less conjugated. If one assumes a similar structure of PLP in Sepia phosphorylase in the presence of cysteine, it should be present as a fully protonated form (ie, the nitrogen and the OH group at position 3 are both protonated). Cysteine treated PLP (ie. PLP in Schiff base with the amino acid) has an absorption maximum near 330 nm and shifted to 295 nm on acidification (Fig. 9-7). The effect of cysteine could be explained by a

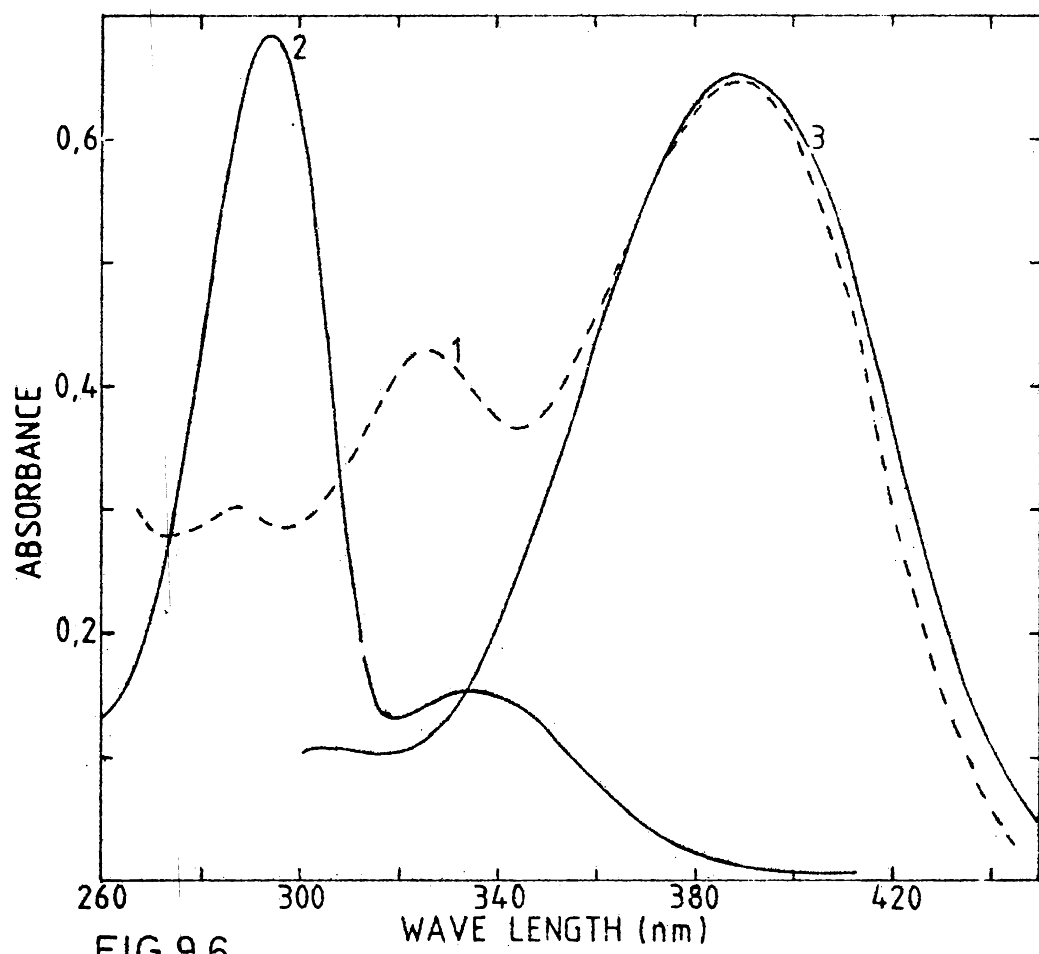

FIG. 9-6

Fig. 9-7.

Absorption spectrum of PLP (100 μ molar) in the presence of 5 mM cysteine at different pH (in 10 mM glycerophosphate). Curve-1, pH 7; Curve-2, pH 4; Curve-3, pH 3; Curve-4, pH 2.5; Curve-5, pH 1.7.

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a specific interaction with the enzyme leading to a structural change affecting the region around PLP. Such a structural change might confer an increased hydrophobic character on the region, which, thus, would maintain a fully protonated form of PLP.

It is not known whether the 333/335 band was shifted to 290/300 nm on dilution of the enzyme also. Cysteine has been earlier shown to stimulate both the resolution and reconstitution of pyridoxal phosphate under different conditions (59,60). It has been also shown that there exists a site on rabbit phosphorylase for one mole of L-cysteine per mole of monomer (167). It was suggested that both resolution and reconstitution occur through a common thazolidne derivative (167). The formation of such a derivative in *Sepia* enzyme under the conditions of the present study is unlikely. Also no inactivation of the enzyme was observed. However, the cysteine activation might be explained by the

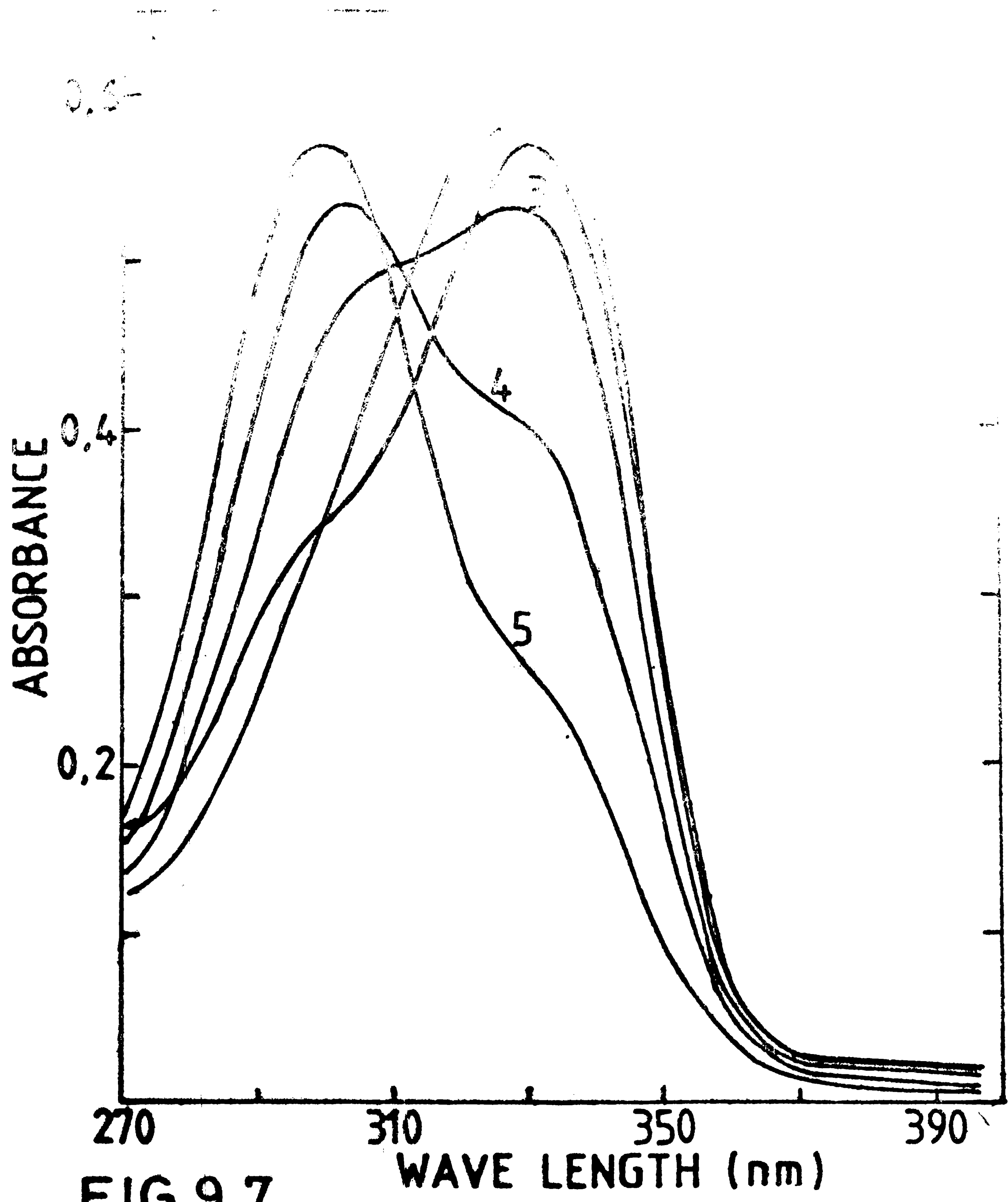

FIG.9-7

binding of the amino acid on a specific site on the enzyme and the spectral shift seemed to be a secondary effect unrelated to catalytic activity of the enzyme but related to conformational alteration of the PLP site by cysteibe interaction.

C H A P T E R 10

INHIBITION AND INACTIVATION OF SEPIA PHARAONIS PHOSPHORYLASE

A. Inhibition by metabolic inhibitors.

Glycogen phosphorylase b from a number of animal species has been shown to be inhibited by the metabolites glucose-6-P, ATP and glucose. The nature of inhibition, however, is different in different animal species and varied with the structural differènce of phosphorylase. The inhibition of rabbit muscle phosphorylase a by these ligands has been shown to be comparatively lesser than the b form. The inhibition of the a form is negligible in presence of 1 mM AMP at 10-15 mM concentrations of the inhibitors (8). In the absence of the nucleotide, however, these ligands exhibit competitive (or mixed) inhibition (40). The inhibition of the a form by glucose is dependent on its time of incubation with the enzyme prior to assay (168). Preincubation of phosphorylase a with glucose for 30-45 minutes converts it to a form devoid of homotropic cooperativity between glucose-1-P sites whereas without the prior incubation glucose induces cooperativity between the substrates sites. Phosphorylase b from a vertebrate fish has been shown to be competitively inhibited by these ligands (105). The a form of the enzyme from *Dictyostelium discoideum* has been found to be not inhibited by glucose-6-P or glucose (126).

The *Sepia* phosphorylase *a* was not inhibited by glucose, glucose-6-P or ATP in the presence of 1 mM AMP at 15 mM concentrations of the inhibitors and at different levels of glucose-1-P. In the absence of AMP however, glucose-6-P and glucose inhibited the enzyme competitively (Fig.10-1). In the presence of ATP, there was increased activity at all concentrations of glucose-1-P. Since the activity ratio (for defenition, see page 103) of *Sepia* phosphorylase was only 0.4, contamination of ATP by AMP (produced by autolysis) could produce an apparent activation. That this increased activity was only apparent could be seen from the figure where the activity is constantly higher at all glucose-1-P concentrations. The competitive inhibition (Fig. 10-1) suggested that, like in rabbit phosphorylase *b* (101), glucos and glucose-1-P would bind at a site very close to the glucose-6-P site. The metabolic role of these inhibitors, however, seemed to be insignificant as it was found that *Sepia* muscle maintained a high concentration of AMP (see Chapter 11).

B. Inhibition by aromatic compounds

Rabbit muscle phosphorylase *a* has been shown to be inhibited by aromatic compounds and the inhibition was of the 'mixed' type (Chapter 2). The inhibition of *Sepia* phosphorylase *a* by *p*-nitrophenol was checked in the presence and absence of AMP and the results were similar

Fig. 10-1.

Lineweaver-Burk plots for glucose-1-P in the absence and presence of the inhibitors glucose, glucose-6-P and ATP. *Sepia* phosphorylase a was incubated in 30 mM cysteine/ 40 mM glycerophosphate pH 6.9 for 20 minutes in he absence and presence of the inhibitors and assayed by adding to an equal volume of substrate solution. The assay mixture contained 1% glycogen, and varying concentrations of glucose-1-P. The concentration of enzyme was 42 μg / ml. O, no inhibitor; ▲, 10 mM glucose, Δ, 10 mM glucose-6-P and X, 10 mM ATP.

Fig. 10-2.

Inhibition of *Sepia* phosphorylase a by p-nitrophenol. The enzyme (84 μg/ml) in 30 mM cysteine / 40 mM glycerophosphate pH 6.9 was incubated with 10 mM p-nitrophenol for 20 minutes and assayed by adding to an equal volume of substrate solution containing 2% glycogen, varying concentrations of glucose-1-P in the absence (filled symbols) and presence of (open symbols) of 1mM AMP. O, ●, control; Δ, ▲, in presence of 5 mM p-nitrophenol.

---

to those obtained for rabbit phosphorylase a (Fig. 10-2). The activity ratios at different levels of glucose-1-P in the absence and presence of p-nitrophenol is shown in Table 10-1. The data show that the activity ratio was slightly lesser in the presence of the inhibitor at all

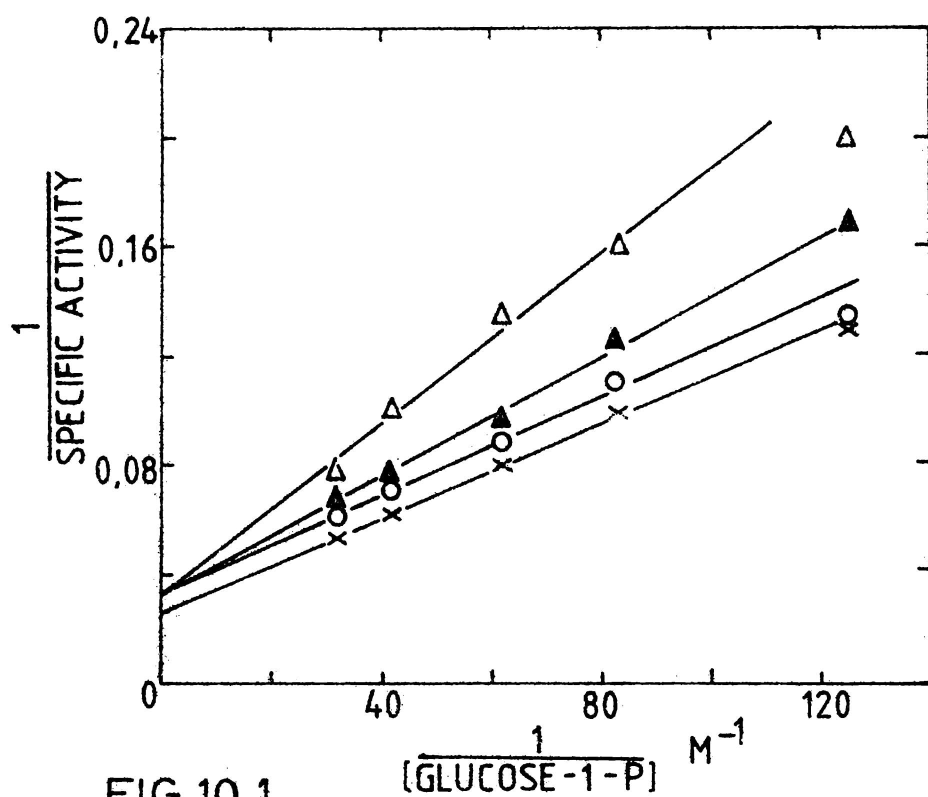

FIG.10-1

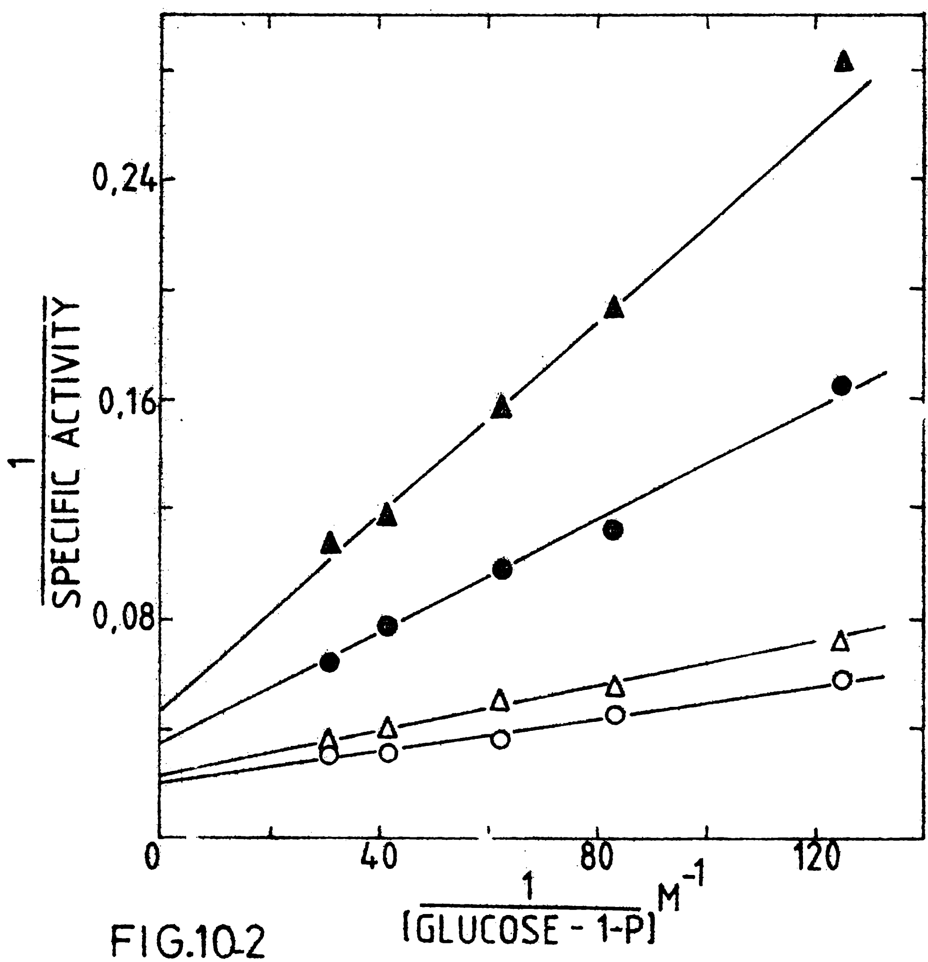

FIG.10.2

TABLE 10-1

THE ACTIVITY RATIO OF *SEPIA* PHOSPHORYLASE *a* AT DIFFERENT LEVELS OF GLUCOSE-1-P.

Glucose-1-P (mM)	Control (No inhibitor).	In presence of 5 mM *p*-nitrophenol
8	0.35	0.27
12	0.36	0.30
16	0.39	0.31
24	0.41	0.33
32	0.42	0.34

levels of glucose-1-P tested, indicating protection by AMP against inhibition. This suggested that in *Sepia* phosphorylase also, *p*-nitrophenol and other aromatic compounds bind at the AMP-binding site.

C3 Inactivation by temperature and urea.

The inactivation of *Sepia* phosphorylase *a* at three different temperatures is shown in Fig.10-3. At pH 6.9, 50% of the activity was lost in 85 minutes, 27 minutes and 6 minutes at temperatures 45°, 50° and 55° respectively. The inactivation by urea is shown in Fig. 10-4. The enzyme was completely inactivated by 5 M urea.

Fig. 10-3.

Effect of temperature on *Sepia* phosphorylase a

Enzyme (0.5 mg/ml) in 0.04 Mβ-glycerophosphate/ 0.03 M mercaptoethanol pH 7 was brought to the required temperatures by immersing in a water bath 10° higher than the needed temperatures for 10 seconds. After immersion in water bath of the required temperatures at zero time, aliquots were withdrawn at various time intervals, diluted in 0.04 M glycerophosphate/0.03 M cysteine pre-cooled in ice and incubated at 30° for 10-15 minutes prior to assay. The assay mixtures contained 16 mM glucose-1-P, 1 mM AMP and 1% glycogen. ○, △ and ● correspond to inactivations at 45°, 50° and 55° respectively.

Fig. 10-4.

Urea inactivation of *Sepia* phosphorylase. Enzyme (0.75 mg/ml) in 0.03 M cysteine/0.04 M glycerophosphate pH 6.9 and varying concentrations of urea (0 to 8 M) was incubated at 30° for 20 minutes. Aliquots were withdrawn and diluted in 0.03 M cysteine/0.04 M glycerophosphate pH 6.9 so that the final concentration of urea was 0.1 M in the dilution buffer and incubated in the buffer for 15 minutes and assayed. The assay mixture contained 16 mM glucose-1-P, 1 mM AMP and 1% glycogen.

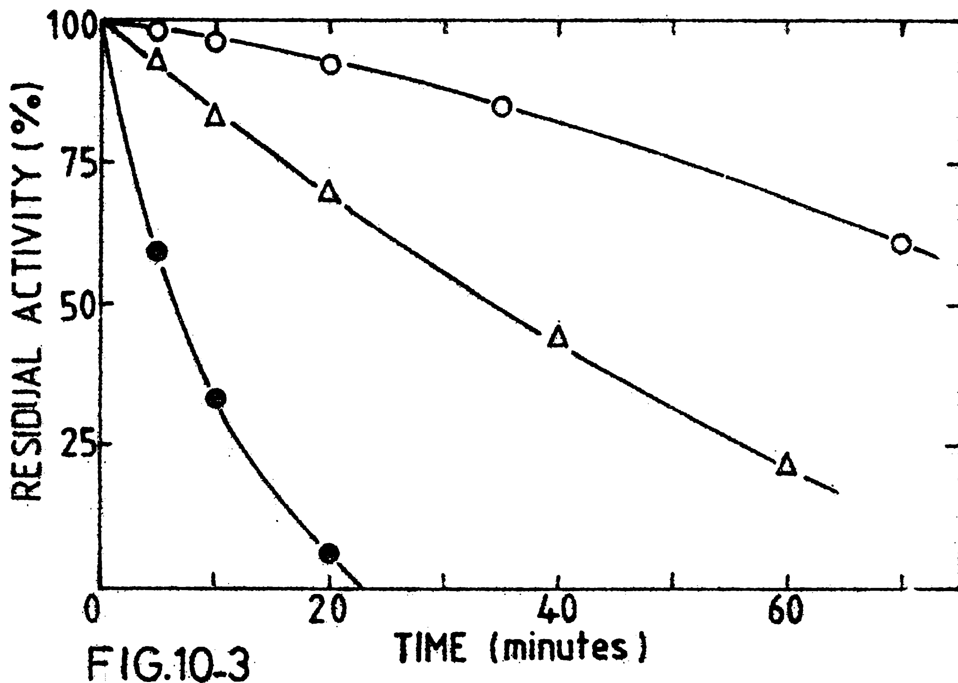

FIG.10-3

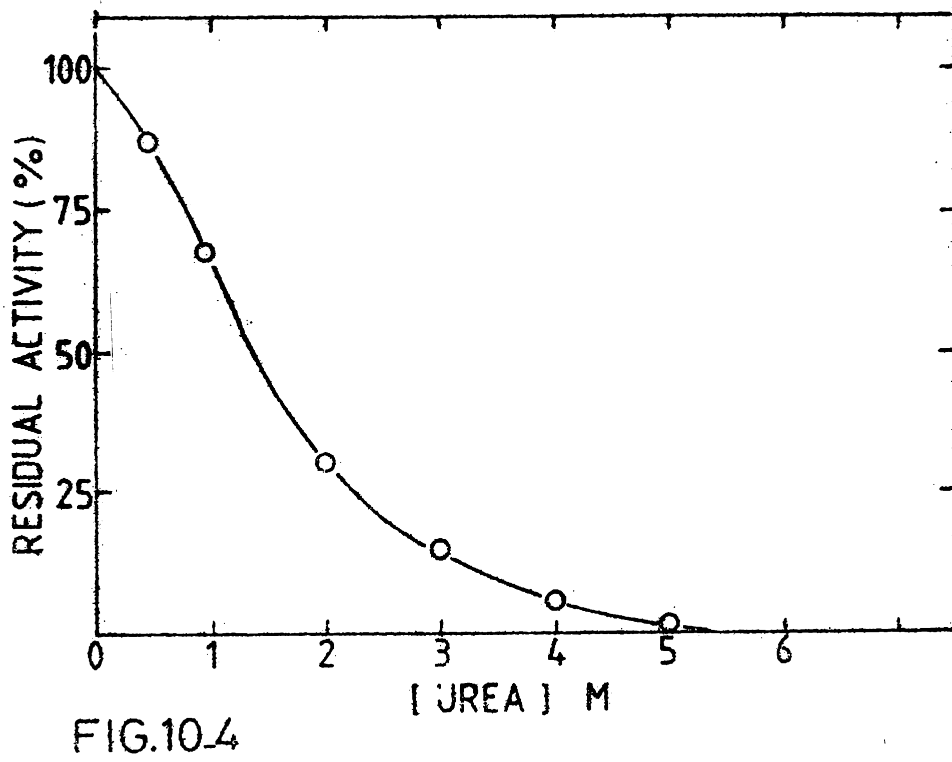

FIG.10-4

A similar pattern of temperature inactivation (Fig.10-3) was obtained when the enzyme was assayed in the absence of AMP also. The activity ratio was the same at different stages of inactivation at the three temperatures tested.

D. Inactivation by heavy metals and sulphydryl reagents.

Rabbit muscle phosphorylase and other enzymes having sulphydryl groups essential either for catalytic process or for maintenance of protein structure have been shown to be inactivated by heavy metals. The effect of the following heavy metal ions were tested: Zn^{++}, Cd^{++}, Pb^{++}, Hg^{++} and Ag^{+}. All these heavy metals completely inactivated the enzyme at 5×10^{-5}M. Table 10- 2 shows the percent inactivations at 5×10^{-6}M concentration of the heavy metals.

The *Sepia* phosphorylase *a* contains 8.6 mol of cysteinyl residues per 2×10^{5}g of the enzyme (Chapter 7). The relation between SH group modification and enzyme inactivation was studied using DTNB. Since reaction of SH group with DTNB releases stoichiometric amount of nitrothiophenolate ion, the inactivation and extent of modification could be studied by assaying at different time intervals and by noting the optical density at 412 nm (139). For this, the *Sepia* phosphorylase was freed from mercaptoethanol by passage through a Sephadex G-25 column.

TABLE 10-2

INACTIVATION OF SEPIA PHOSPHORYLASE a BY HEAVY METAL IONS

Phosphorylase a (150 µg/ml) in 20 mM glycerophosphate pH 6.9 was incubated with the heavy metal ions (at a final concentration of 5 x 10^{-6} molar) for 30 minutes and assayed by adding to an equal volume of substrate solution containing 32 mM glucose-1-P, 2% glycogen and 2 mM AMP.

Heavy metal ions.	Residual activity (%)
None	100
Hg^{++}	52
Pb^{++}	61
Cd^{++}	64
Zn^{++}	64
Ag^{+}	67

The mercaptoethanol - free enzyme in 50 mM glycerophosphate/ 2 mM EDTA pH 7.2 was treated with 25 -fold molar excess of DTNB and the absorbance at 412 nm was measured at different time intervals. The number of moles of SH groups modified per mol of the enzyme (using a molecular weight 2 x 10^{5}) was calculated using a calibration curve obtained under the same conditions using cysteine as standard. The SH groups determined by this method is compared to loss of enzyme

Fig. 10-5.

Reaction of *Sepia* phosphorylase *a* with DTNB. Phosphorylase (1mg/ml) in 40 mM glycerophosphate/ 2 mM EDTA pH 7.2 was treated with 25-fold molar excess of DTNB prepared in the same buffer. Aliquots were removed from the reaction mixture and assayed after diluting in 40 mM glycerophosphate/2 mM EDTA pH 6.9. Also shown the number of SH groups modified during the course of inactivation by the method given in the text.

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activity in Fig.10-5. Modification of about 5.5 SH groups completely inactivated the enzyme and at 50% inactivation, about 3.8 residues were modified (the presence of any fast-reacting SH groups which did not affect activity could not be identified). In this respect the *Sepia* enzyme is clearly different from rabbit enzyme. In the latter case, it has been shown that 4 SH groups undergo reaction without any loss of activity.

E. Cold inactivation of *Sepia* muscle phosphorylase *a*

Glycogen phosphorylase *b* and *a* have been found to be sensitive to cold temperature at pH 6 (166). On storage at cold temperature (5° or below) at pH 6, the rabbit enzyme has been shown to be inactivated. Phosphorylase *b* from muscle of trout fish (17) lobster (18) and

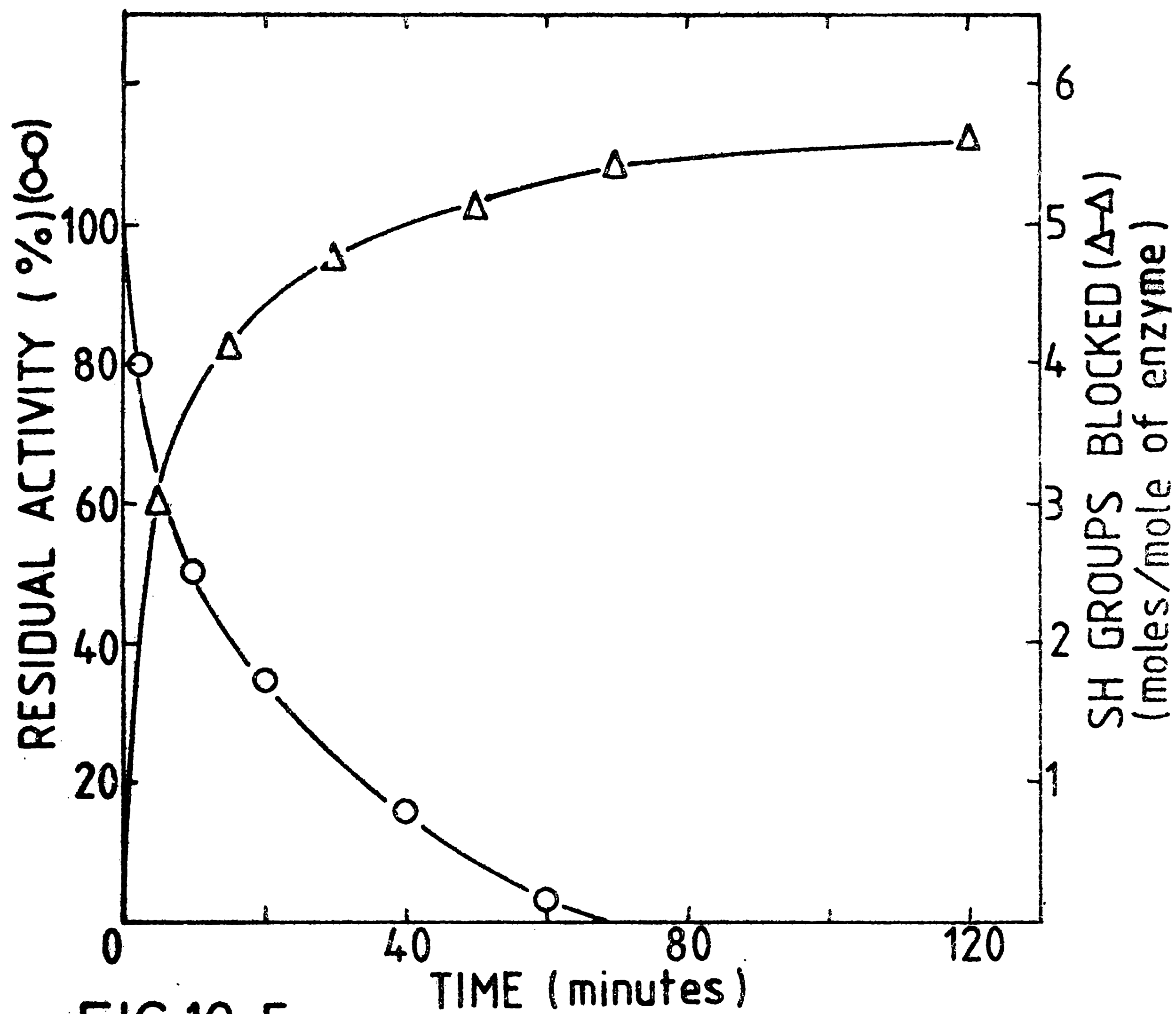

FIG.10-5

Cibium guttattam (169) has been shown to be sensitive to cold conditions but only to a lesser extent than the rabbit enzyme.

The *Sepia* phosphorylase *a* was found to be highly sensitive to cold temperature. The enzyme was stable in the presence of cysteine only for 1-2 days, whereas it was more stable in the presence of mercaptoethanol or in the absence of any sulphydryl compounds (Chapter - 7). Rabbit phosphorylase *a* or *b* kept under similar conditions was stable for more than 2 weeks. The effect of cold temperature on the *Sepia* enzyme was therefore studied in detail. Figs. 10-6 and 10-7 show the cold inactivation of the *Sepia* enzyme at two different pH .

In contrast to rabbit muscle phosphorylase *a* and *b* the *Sepia* enzyme was inactivated at cold temperatures at neutral pH also. In 4 hours there was 30% activity loss at 0° compared to 15% at 25° in the presence of 0.5 M NaCl. In the absence of any salt, there was 5% of activity loss at both temperatures. In 30 mM mercaptoethanol/ 40 mM glycerophosphate buffer pH 7, containing 0.5 M NaCl there was only 15% inactivation at 0° after 4 hours. This shows the specificity of cysteine for the enzyme to make the latter sensitive to cold temperatures.

At pH 6, the *Sepia* phosphorylase *a* was more sensitive to cold temperatures than its rabbit counterpart. At 0°

Fig. 10-6.

Cold inact vation of <u>Sepia</u> muscle phosphorylase <u>a</u> at pH 7 Phosphorylase <u>a</u> (4 mg/ml) in 30 mM cysteine/ 40 mM glycerophosphate pH 7 was incubated at 0° and 25° in the presence and absence of 0.5 M NaCl. Aliquots were withdrawn and diluted in the same buffer 60-fold and assayed immediately at 30° (2 min. assay)., ●, Incubation at 25° in the absence of any salt. △, Incubation at 0° in the absence of any salt. ○, Incubation at 25° with 0.5 M NaCl. ▲, Incubation at 0° with 0.5 M NaCl.

Fig. 10-7.

Cold inactivation of <u>Sepia</u> phosphorylase <u>a</u> at pH 6. The enzyme (4mg/ml) was incubated in 30 mM cysteine/ 40 mM glycerophosphate pH 6. Aliquots were withdrawn and diluted in the same buffer (30-fold) and assayed (2 min. assay). The details of the symbols are as shown in Fig.10-6.

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in presence of 0.5 M sodium chloride, there was about 90% inactivation in 2 hours (in the case of rabbit phosphorylase <u>a</u> there was only 60% inactivation at 1.2 M NaCl and less than 10% at 0.2 M NaCl under the same conditions (166). At pH 6 also, the inactivation was greater in the presence of sodium chloride than in its absence, at 0° and at 25°.

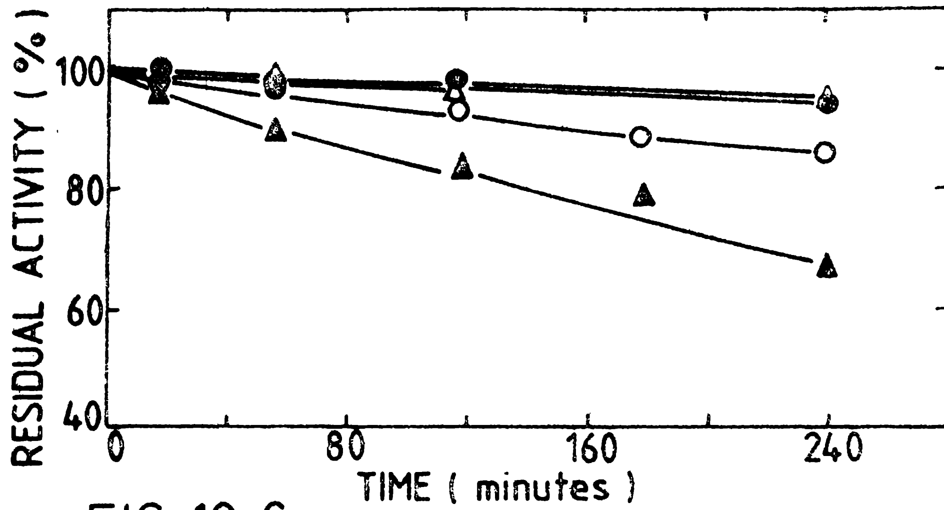

FIG. 10-6

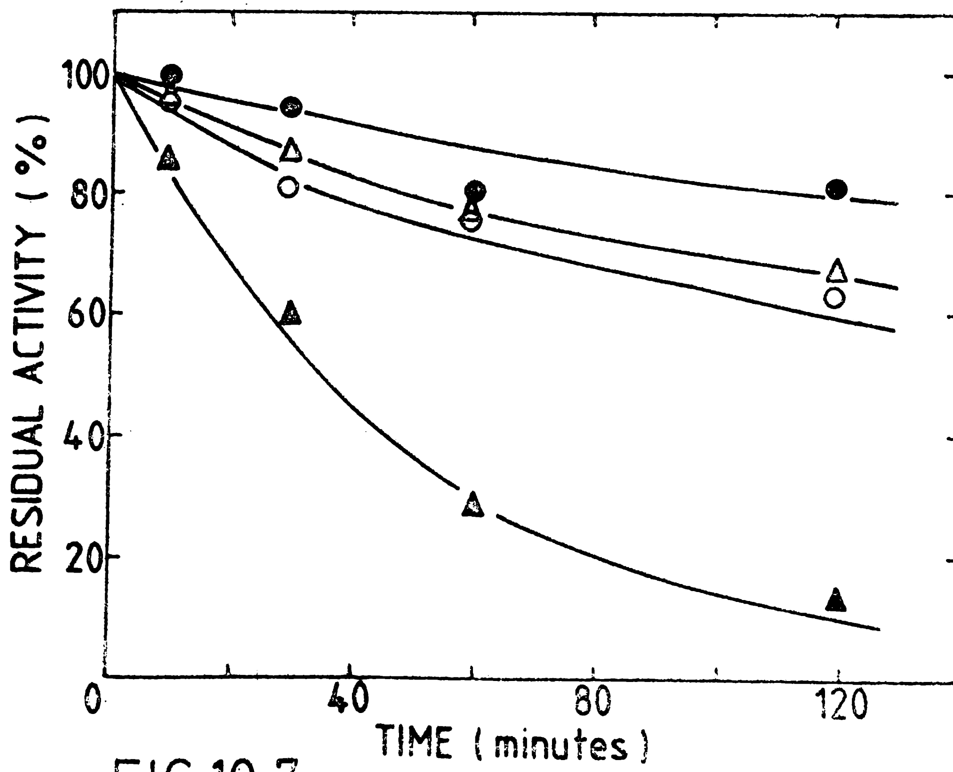

FIG. 10-7

In this respect, *Sepia* phosphorylase a was more similar to rabbit phosphorylase b where the inactivation at pH 6 and at 0° in the absence of any salt was 70% (in 4 hours). The rabbit phosphorylase a was only slightly inactivated under the same conditions.

The following conclusions were made from these studies:-

i) The 'cold inactivation' of the enzyme can not exclusively be attributed to cold temperatures because inactivation (and hence conformational changes) also occurs at 25° under the conditions used for the 'cold inactivation' study.

ii) Since the cold inactivation was observed at pH 7 also, this property may not be solely due to instability of the enzyme at pH 6. However, since the inactivation was pronounced at low pH and at high ionic concentration, it could be due to the unstable ionization state of the protein and under these conditions and when temperature is lowered, this partially inactive enzyme tends to undergo further conformational changes resulting in irreversible structural alteration. (In fact, the activity of cold inactivated rabbit enzyme also was not completely regained on rewarming the enzyme (166).

iii) Cysteine has some specific effect on inactivating the enzyme. Cysteine was earlier shown to interact with the

Sepia enzyme thereby shifting the 335 nm band of the enzyme to 290 nm (Chapter 9). It was proposed that the shift to an apparently protonated form of PLP could be due to structural alteration resulting in an increased hydrophobic character conferred on the PLP site due to cysteine interaction. Hydrophobic bonds and regions have been shown to be sensitive to cold temperature (138, 170,171). The hydrophobic character induced by cysteine on the phosphorylase could convert it to an easily susceptible conformation. (which in *Sepia* enzyme led to a spectral shift also). This cysteine-treated enzyme undergoes inactivation at high ionic concentration, low pH and/or at low temperature. The specific interaction of phosphorylase with cysteine distinguishes it from other enzymes where such 'cold inactivation' has not been observed. The *Sepia* phosphorylase is more susceptible to cysteine interaction and hence more sensitive to cold temperature. Based on these observations the different forms of the cysteine treated enzyme can be schematically represented as in Fig. 10-8.

In the scheme, E is the native enzyme E', the cysteine interacted enzyme and E'_1, E'_2 etc. are inactive enzyme forms. a, b and c represent low pH, high ionic concentration and low temperature respectively. The stability of these forms decreases from top to bottom (the positions, however, could not be absolutely represented). The proposed scheme shows

Fig. 10-8.

A schematic representation of the different forms of cysteine-treated *Sepia* phosphorylase under different conditions. Details are given in the text.

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that the process of cold inactivation of phosphorylase could be due to conformational changes induced by different parameters like ions, pH and low temperatures on phosphorylase in presence of cysteine. As observed in the rabbit enzyme (166) these changes can lead to enzyme dissociation and hence these form are unstable.

The specific effect of cysteine should be viewed in different perspectives; (i) its effect on the spectrum (ii) its role in cold inactivation and (iii) its influence in activating the enzyme. While the second aspect was observed in rabbit enzyme also, the other two effects have not been reported in any other phosphorylases. Since the spectral change and activation were unrelated, we maintain that the *Sepia* phosphorylase has a much more flexible conformation that other well studied system. It is not surprising in view of the evolutionary status of *Sepia*. This Cephalopod

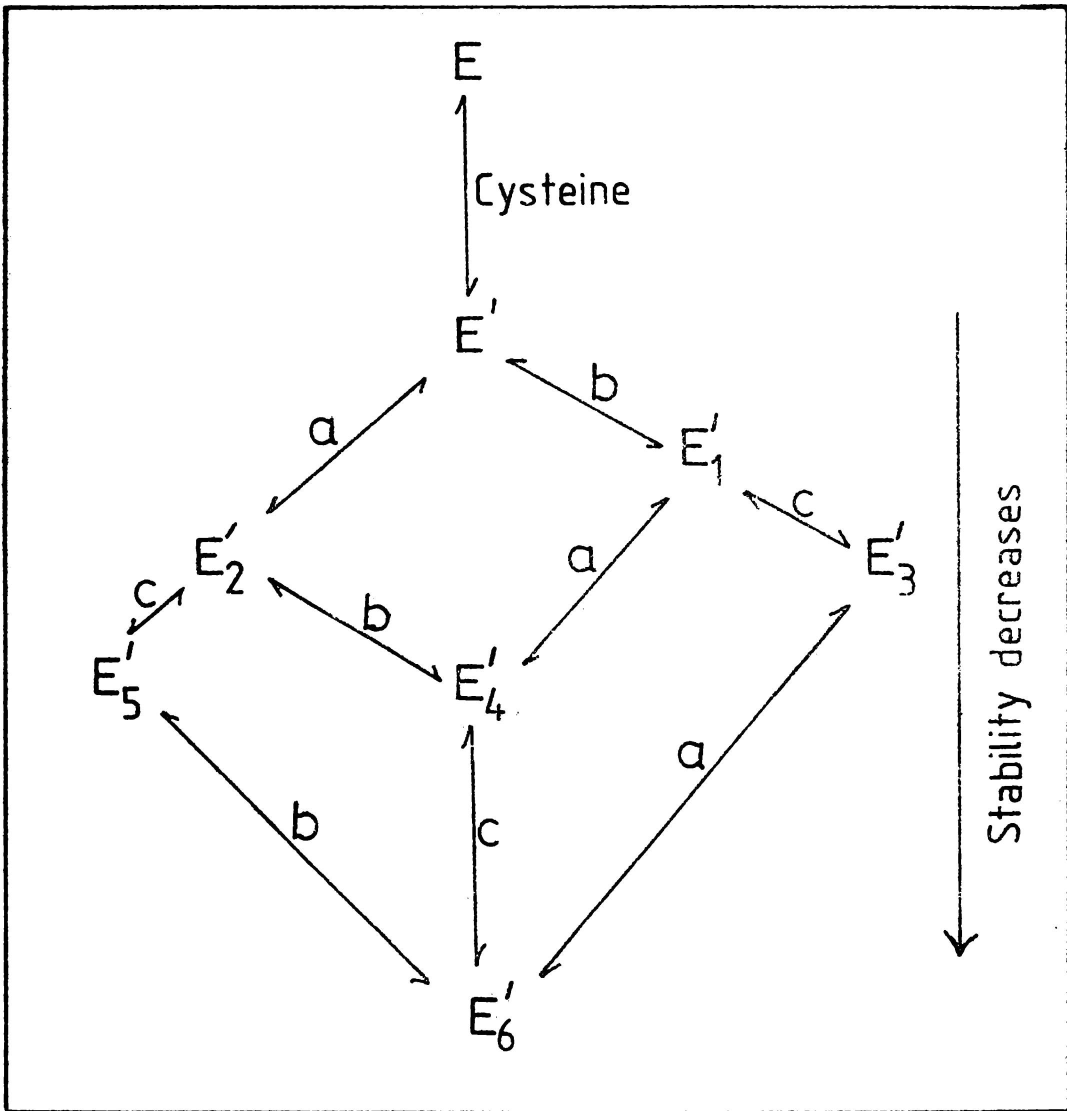

FIG.10-8

is far below in evolutionary status, and hence several fine control mechanisms identified as well developed in rabbit and man are still in a much lower level in *Sepia*. This point becomes more clear in the next chapter.

CHAPTER 11

CONTROL OF GLYCOGEN DEGRADATION BY PHOSPHORYLASE IN THE MANTLE MUSCLE OF *SEPIA PHARAONIS*.

Glycogen degradation in muscle tissues, in general, is controlled by (i) the concentration of phosphorylase, (ii) the hormone regulated interconversion between the AMP-dependent phosphorylase b and the AMP-independent a form and (iii) allosteric activation and inhibition of phosphorylase b. Investigation using muscle tissues of all vertebrate terrestrial and aquatic animals revealed a similar pattern of control (15-20, 113 -116, 153, 154, 157). The major control device in all these cases is through the interconversion between active and inactive forms of the enzyme *via* phosphorylation or dephosphorylation. Even though the existence of different forms of phosphorylase has been detected in lower forms of animals and in some plants (42, 117, 121-126a), the participation of these enzyme forms in the interconversion process as a universal control device lacks experimental support. In many of these species, these different forms of phosphorylase are structurally and functionally different from the rabbit muscle phosphorylase a and b. Detailed studies on the regulatory mechanism of phosphorylase of marine invertebrates have not so far been reported. This chapter deals with the *in vitro* studies on

the control aspect of the mantle muscle phosphorylase of *Sepia pharaonis*.

The *Sepia* belongs to the Class Cephalopoda and this class stands apart from other molluscs in their larger size, higher metabolic rate and faster tempo of life (159). The *Sepia* uses its strong pallial musculature for its every movement. Water is taken in by contraction of radial fibres in the pallial wall and by pushing out water with force through a funnel, (by contracting the circular muscles of the mantle) the animal moves with lightning speed (159). Hence the mantle muscle of *Sepia* has an extremely high energy demand.

Even though the *Sepia* is primitive in evolutionary status, it is independently a highly evolved invertebrate when compared to other molluscs (158). The unique properties of cysteine activation, cysteine induced spectral change and cold inactivation suggested structural difference of the *Sepia* phosphorylase from other purified phosphorylases studied so far. These prompted the study of the control properties of the enzyme.

The initial velocity data obtained at different levels of AMP and glucose-1-P were analysed using reciprocal plots and are shown in Figs. 11-1 and 11-2. These results show absence of homotropic cooperativity between glucose-1-P

Fig. 11-1.

Double reciprocal plots for glucose-1-P at different constant levels of AMP for phosphorylase a from S. pharaonis. Phosphorylase a (65 µg/ml) in 30 mM cysteine/ 40 mM glycerophosphate pH 6.9 was incubated for 10-15 minutes at 30° prior to assay and mixed with an equal volume of substrate. The reaction mixture contained 1% glycogen and varying concentrations of glucose-1-P. The concentration of AMP was 2.5×10^{-5} M (△), 5×10^{-5} M (▲); 10^{-4} M (○); 10^{-3} M (●) and no AMP (×).

Fig. 11-2.

Double reciprocal plots for AMP at different constant levels of glucose-1-P. Details are as in Fig.1. The concentrations of glucose-1-P were 12 mM (○); 16 mM (▲); 20mM (×); 24 mM (●) and 32 mM (△).

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sites, like in other phosphorylases studied (35-38). However, unlike in other cases, the plots for AMP were curved downwards. The kinetic mechanism of Sepia phosphorylase being the same as reported for other phosphorylases (Chapter 8) the curved plots would mean either that the preparation contained more than one active form of the enzyme having different requirements for AMP or that the enzyme exhibited

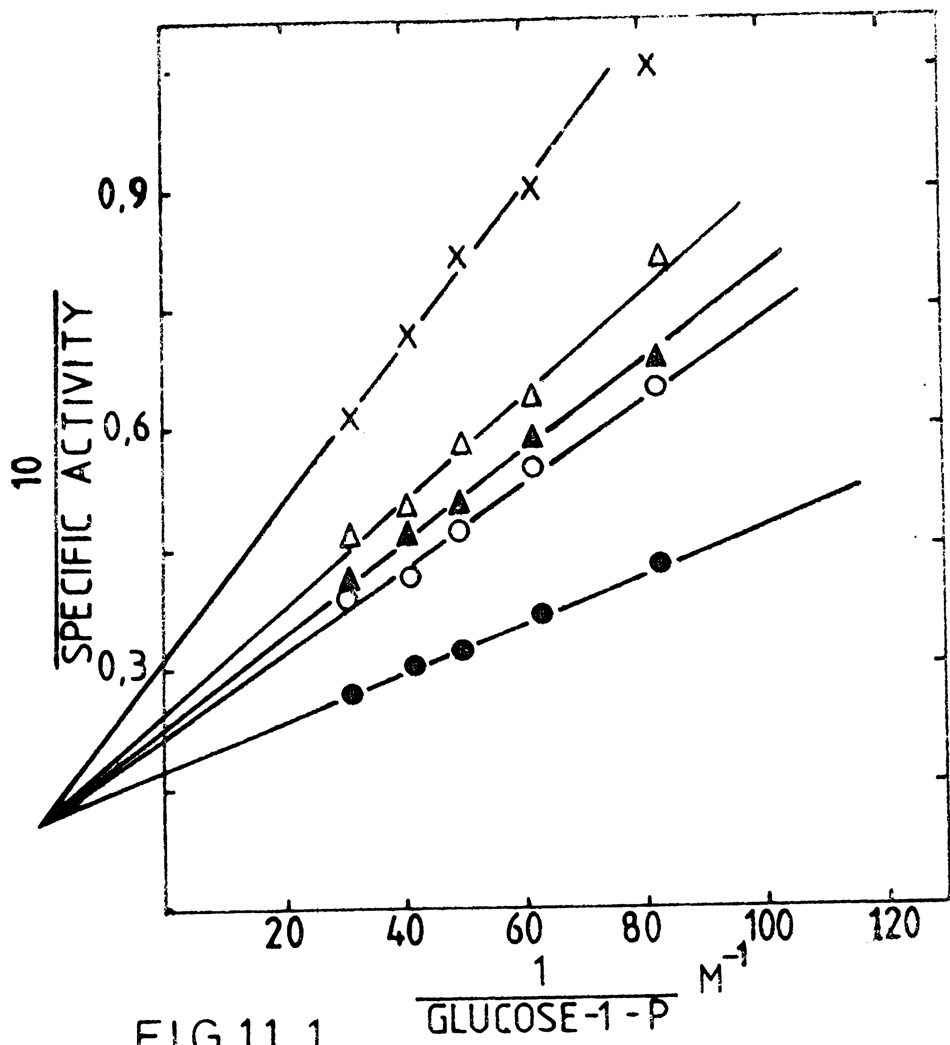
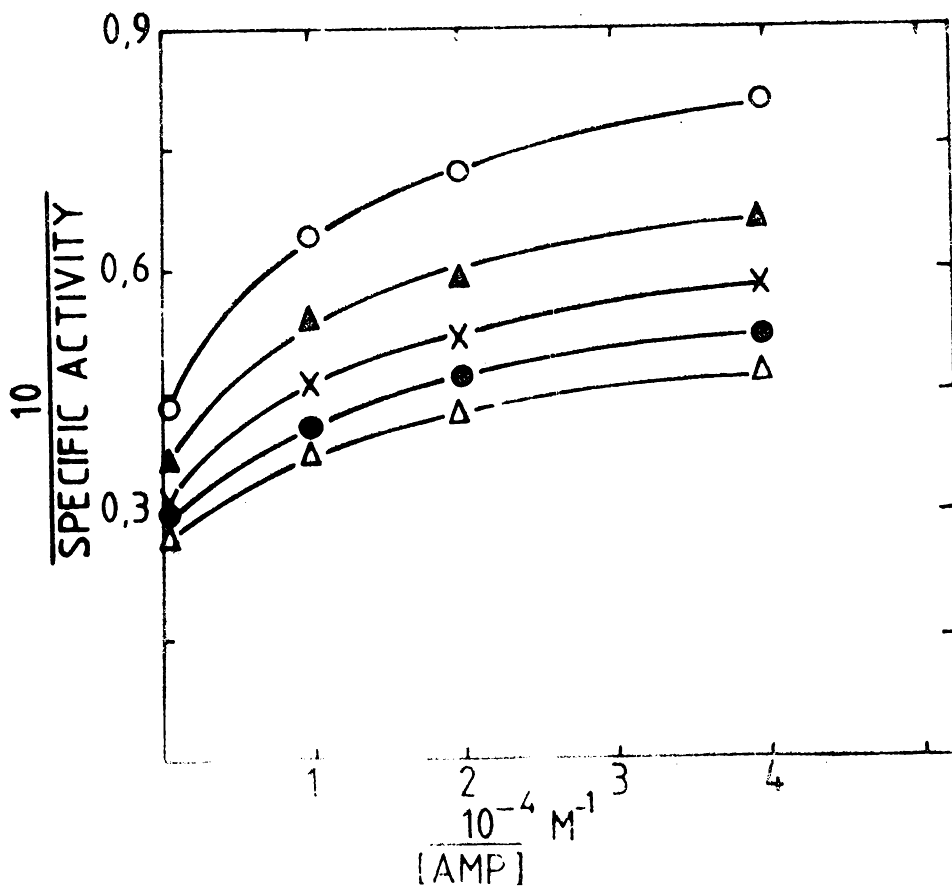


FIG.11_1



negative cooperativity between AMP sites. The former possibility was ruled out by the following observations: (i) the activity ratio* remained unchanged at different stages of purification of the enzyme, (ii) the activity ratio was the same at different stages during heat inactivation at different temperatures, (iii) the ratio did not change on activation by cysteine, (iv) the preparation was electrophoretically homogeneous and (v) the Arrhenius plot was linear. Thus *Sepia* phosphorylase exhibited negative cooperativity between AMP sites. This is significant not only because such behaviour has not been reported for other animal muscle phosphorylases, but also it suggested the possibility of a different control of glycogen degradation in *Sepia* mantle. This is supported by the studies on the inhibition by metabolic inhibitors. Unlike in rabbit enzyme, the *Sepia* phosphorylase *a* was only very slightly inhibited by glucose (and apprently no inhibition by ATP). Under the same conditions, the rabbit enzyme was found to be inhibited 25 to 30 % by these inhibitors. This also shows that the *Sepia* phosphorylase may be controlled in a distinct manner from that of the rabbit enzyme. In the case of rabbit phosphorylase, Fletterick and Madsen (40) have shown that glucose binds on the active site. The negligible inhibition of the *Sepia* enzyme by glucose thus indicated a structurally different active site.

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* For defenition, see page.103.

Phosphorylase purified from the frozen _Sepia_ mantle showed an activity ratio of 0.4 ± 0.02.

The presence of phosphorylase phosphatase and kinase in the mantle muscle extract was checked using the purified phosphorylase as substrate. For this the frozen muscle was extracted with twice its weight of distilled water at 2-5°. The homogenate was centrifuged and passed through Sephadex G-25 equilibrated with 10 mM glycerophosphate pH 6.9 to remove AMP and other small molecules (the extract contained a high concentration of AMP; see below). The phosphorylase phosphatase action of this extract was checked at pH 6.5, 7 and 7.5 in 25 mM sodium β-glycerophosphate / 15 mM cysteine buffer at 30°. The activity ratio was found to be unaffected even at a high concentration of the extract (10-fold dilution). The participation of any small molecules in catalysis was tested by allowing the reaction with the original extract without purification through Sephadex and removing the AMP and other molecules by Sephadex G-25 after incubation of the enzyme -extract mixture for 15-20 minutes. Even then, the ratio was found to be unaffected.

By performing a similar set of experiments with further inclusion of 3 mM ATP and 10 mM Mg^{++} in the reaction mixture, it was found that the frozen mantle tissue of the _Sepia_ didn't contain phosphorylase kinase activity at a pH range

of 6.5 to 8.5.

However, when the purified phosphorylase was incubated at pH 6.5 (under the conditions mentioned above) with extracts from fresh muscle cut out of live *Sepia* (after removing small molecules from the extract as above) the activity ratio decreased to a minimum of 0.15. When ATP and Mg^{++} were introduced into the reaction mixture at pH 8, the ratio rose to maximum of 0.43.

The above experiments showed that, (i) phosphorylase phosphatase (and possibly kinase) became inactivated on freezing of the muscle tissue; (ii) although both kinase and phosphatase were present is *Sepia* muscle, the activity ratio of phosphorylase changed only between 0.15 and 0.4 unlike in other known cases where such change was from essentially zero to approximately 1 under the experimental conditions used for the standard assay (see chapter 12). The possible requirement of any additional factors for exhibiting maximum phosphatase and kinase activity was checked using the original extract of live *Sepia* muscle (without prior purification through Sephadex G-25). Here also, when the reaction products were passed through Sephadex G-25, the activity ratio was 0.15. The maximum activity could not be ascertained by this method because removal of ATP and Mg^{++} through Sephadex reduced the

activity ratio of the eluted sample to about 0.15 again. This showed that the whole extract did not contain any activator or inhibitor that might alter the rate of both kinase and phosphatase reaction. The observed activity ratio was unaffected by the presence of upto 1 M NaCl.

The experiments conducted for the purified enzyme were repeated using the crude muscle extract to know if any additional protein factors which might be required at a high concentration could influence the activity ratio. The activity ratio of the AMP-free extract prepared from frozen muscle remained unchanged at about 0.4 when incubated at pH 6.5 to 7 or with ATP and Mg^{++} at pH 7 to 8. The AMP-free extract obtained from fresh muscle (prepared at 0-2°) showed a ratio of 0.35 which decreased to 0.15 when incubated at 30° and pH 6.5 and increased to about 0.42 in the presence of ATP and Mg^{++} at pH 8 (assayed after dilution in sodium β-glycerophosphate buffer pH 6.7). This experiment was also performed with extracts containing AMP and removing the latter prior to phosphorylase assay. Here also, the activity ratio was in the range of 0.15-0.4. This showed that the presence of AMP in the extract (see below) did not affect the kinase and phosphatase activity.

The activity ratio of purified Sepia phosphorylase was not affected by partially purified rabbit muscle phosphorylase kinase (10) and phosphatase (172) at

different pH values. Under the same conditions, these enzymes interconverted the rabbit phosphorylase *a* and *b*. All animal muscle phosphorylases studied so far have been shown to be interconvertible between the *a* and the *b* form by the rabbit muscle kinase and phosphatase (15-20). Thus the *Sepia* enzyme is clearly different from other animal muscle phosphorylases studied so far.

The specific activity of the purified *Sepia* muscle phosphorylase was 10 units/min/mg protein in the absence of AMP and about 25 units/min/mg protein in the presence of saturating concentration of AMP (at 16 mM glucose-1-P) as against 50 and above for other reported cases (40). It was surprising that the *Sepia* mantle having a very high energy requirement functions using phosphorylase having such a low activity. Therefore other control mechanisms were searched.

The activity ratio of the muscle extract was usually 1 and this ratio gradually decreased during the various stages of enzyme purification and came to a constant value of 0.40 ± 0.02 (Table 11-1).

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

TABLE 11-1.

ACTIVITY RATIO OF SEPIA PHOSPHORYLASE AT DIFFERENT STAGES OF ENZYME PURIFICATION.

The fractions obtained from different purification steps were diluted in cysteine / glycerophosphate buffer pH 6.9 and assayed at 30°. The assay mixture contained 16 mM glucose-1-P, 1% glycogen in the presence and absence of 1 mM AMP.

Step	Activity ratio.
Extract	0.95± 0.05
39 to 55% ammonium sulphate fraction.	0.64± 0.04
The above fraction after dialysis.	0.48± 0.02
DEAE-cellulose purified enzyme.	0.43± 0.02
Sepharose-glycogen purified enzyme.	0.40± 0.02

The observed decrease in the ratio was not due to phosphorylase phosphatase action as evidenced from the following experimental observations; (i) gel filtration of the extract (at 2-4°) through Sephadex G-25 followed by

treatment with charcoal decreased the ratio to 0.4; (ii) dialysis of the extract (at 2-4°) resulted in the gradual decrease to almost the same ratio; (iii) the activity ratio of the purified *Sepia* phosphorylase increased from 0.4 to 1.0 in the presence of twice diluted dialyzate (the dialyzate was prepared as given in Chapter 12) of the muscle extract in assay. The extract was deproteinized by heating in a water bath at 70° for 10 minutes and this deproteinized extract also increased the activity ratio to 1.

The identity of the dialyzable, non proteinous activating substance was established as AMP by the following experiments;

1) The AMP concentration of the dialyzate was determined using the enzyme, AMP-deaminase. For this, the AMP-deaminase was partially purified from rabbit muscle according to the procedure of Nikiforuk and Collowick (173). About 10-12-fold purification of this enzyme was achieved by fractionation with ammoniacal ammonium-sulphate. The AMP-deaminase is a highly specific enzyme and acts only on 5'-AMP among the various nucleotides (173). The enzyme deaminates AMP resulting in the shift of the ultraviolet spectrum of the nucleotide. At 265 nm, the molar extinction of the product, 5'-IMP is only 40% of that of 5'-AMP. The concentration of AMP in the dialyzate was estimated by

employing this method, using pure AMP as standard. The AMP-content of the homogenate prepared by extraction of the mantle with twice its weight of water was found to be between 1 and 1.5 mM.

2) The extent of activation of *Sepia* phosphorylase with different concentrations of pure AMP tallied with the activation observed by different dilution of dialysate whose AMP concentration was estimated using AMP-deaminase (Fig.11-3).

3) The dialysate activated crystalline rabbit phosphorylase b to the same level expected from the estimated AMP concentration of the dialyzate (Table 11-2).

TABLE 11-2

SPECIFIC ACTIVITY OF RABBIT MUSCLE GLYCOGEN PHOSPHORYLASE b USING THE DIALYZATE OF SEPIA MUSCLE EXTRACT AS THE NUCLEOTIDE ACTIVATOR.

The assay mixture contained 16 mM glucose-1-P, 1% glycogen, 15 mM cysteine, 20 mM glycerophosphate (pH 6.9), 7.5 μg/ml rabbit phosphorylase b and AMP as given

Concentration of AMP in assay (mM).	Specific activity using dialysate AMP*.	Specific activity using pure AMP.
0.01	28.5	20.0
0.10	35.0	36.5
1.00	38.0	41.0

* The AMP concentration was determined by AMP-deaminase.

Fig. 11-3.

Activation of phosphorylase a by dialysate of *Sepia* mantle muscle extract. The AMP concentration in the dialysate was estimated using AMP-deaminase (see Chapter 12) and was diluted in 40 mM sodium β-glycerophosphate pH 6.9 and incubated in the reaction mixtures containing 16 mM glucose-1-P, 1% glycogen, 20 mM β-glycerophosphate and enzyme (35 μg/ml). △, Activation obtained using dialysate whose AMP concentration was determined using AMP deaminase; ○, Activation obtained using pure AMP.

The above experiments clearly showed that *Sepia* mantle muscle maintained a high concentration of AMP. The AMP content in the extracts of frozen mantle tissues of several specimens was independently determined using the AMP-deaminase from rabbit muscle. The AMP-concentration was found to be consistently high which on the average was 1.2 mM in the muscle extract prepared by homogenising the tissue with twice its weight of water. The concentration of AMP in the wet mantle tissue would therefore be about 3.5 mM. The saturating concentration of AMP for the purified enzyme was about 1 mM so that the enzyme would be in fully saturated state in the tissues.

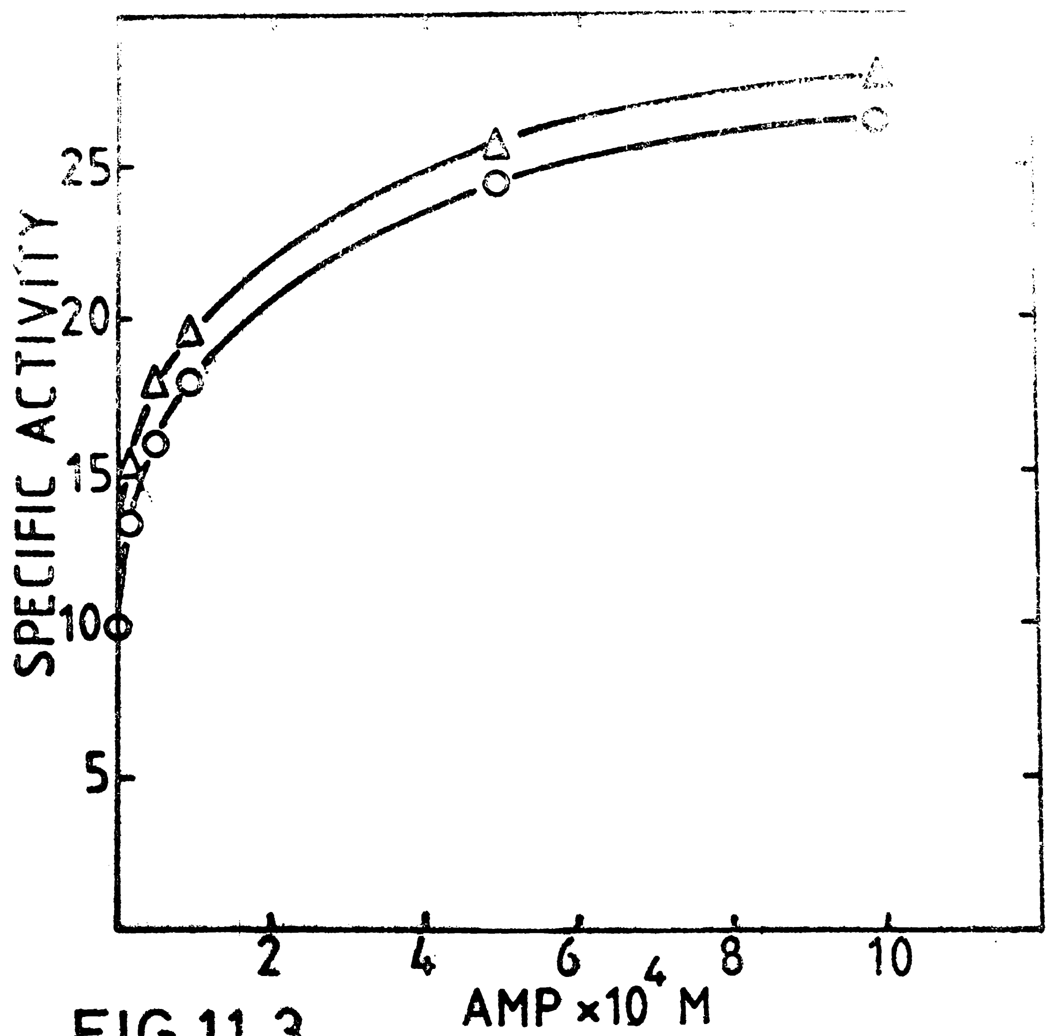

FIG.11-3

The extent of allosteric activation of phosphorylase b depends not only on the concentration of AMP, but also on the conditions such as the concentration of substrates and effectors, divalent metal ions, pH, temperature etc. The concentration of AMP in the resting rabbit muscle was first determined by Lohman and Schuster (174) and found to be approximately 0.01 mM. With improvements in the technique of 'quick freezing' the AMP concentration was estimated to be 0.5 mM (given per ml. intracellular water, taken as 50% tissue wet weight) in the rabbit resting muscle (175). Even if such a high concentration is present in the resting rabbit muscle, the high concentrations of ATP, ADP and glucose-6-P (97 mM, 2mM and 0.3 mM respectively / ml of intracellular water) inhibits the allosteric activation of the phosphorylase b in the rabbit muscle and *in vivo*, it has been found out that the order of activity in the rabbit resting muscle is 1/10,000 its total potential activity (176). Therefore in the rabbit resting muscle, the presence of the AMP does not serve as a controlling factor of phosphorylase.

Since the *Sepia* phosphorylase a was not even slightly inhibited by glucose-6-P, ATP, or glucose in the presence of 1 mM AMP (see chapter 10), even if these compounds are present in the mantle tissue, the AMP activation remains unaffected. Since the activity ratio of the *Sepia* phosphorylase a was only 0.4, the presence of AMP in the contracting muscle is highly significant. The *Sepia* caught

in trawling nets may fastly contract its mantle tissue which may result in maximum kinase activity and the a form must be highly dominating in the frozen muscle of *Sepia* caught by this method. In fact, the mantle extract always showed an activity ratio of about 0.4. The high concentration of AMP present in the contracting muscle must convert the phosphorylase a to a form which shows maximum potential activity under the physiological conditions. The concentration of AMP in the resting *Sepia* muscle is not known at present. Since the b form of the *Sepia* enzyme was also shown to be highly AMP-dependent and because a continuous production of lactate (even while resting) is not a common physiological phenomena, the control of breakdown of glycogen in *Sepia* muscle must either be on the regulation of AMP-production or on a separate site of the glycolytic pathway or by both the processes Anyhow, the control on phosphorylase activity could not be due to interconversion between a and b form (unlike in other vertebrate species studied in detail so far) because of the low specific activity observed for both a and b form and of the narrow range of variation in the activity ratio.

Using the partially purified AMP-deaminase, the concentration of AMP in the extracts of the muscle of a vertebrate fish (*Etroplus suratensis*) and the frozen foot muscle of a clam (*Sunetta scripta*) and rabbit muscle were determined and are compared with that of *Sepia* muscle in

TABLE 11-3.

CONCENTRATION OF AMP IN THE EXTRACTS OF MUSCLE TISSUES OF DIFFERENT ANIMALS OF AQUATIC ORIGIN COMPARED TO RABBIT MUSCLE.

The muscle was ground (at 0-2°) with twice its weight of water and centrifuged. The supernatant was dialysed (at 2-5°) and the dialysate was used for the estimation of AMP as described in chapter 12. The concentration of AMP expressed are those found in the dialysate. The values given are averages of 3 to 6 estimations.

Source of muscle tissue.	Concentration of AMP in the extract (mM)
Mantle tissue of *Sepia pharaonis*	1.25 ± 0.25
Foot muscle of the clam, *Sunetta scripta*	0.01 ± 0.01
Skeletal muscle of the vertebrate fish, *Etroplus suratensis*	0.12 ± 0.03
Rabbit skeletal muscle.	0.02 ± 0.01

Table 11-3. In all the cases, except for the *Sepia* muscle, the AMP concentration was negligible. This shows the distinction of *Sepia* species from oth r vertebrate and the

slow moving invertebrates in its metabolic regulation of phosphorylase activity. The observed cysteine activation, the fact that the purified *Sepia* phosphorylase was not interconvertible between the *a* and *b* forms by active rabbit phosphorylase phosphatase and kinase and the negative cooperativity observed for AMP sites also suggested that this enzyme was structurally different from other vertebrate phosphorylases studied in detail so far.

Sepia which is an invertebrate, is far removed and low in evolutionary status from vertebrates. However, the Class Cephalopods have independently evolved elaborately and they comprise a specialised group of molluscs which have evolved to be fast-moving (159). They live in the open waters of the ocean on "more or less equa terms with such creatures as fish and aquatic mammals" (158). Through evolution, the vertebrates have acquired the mechanism of regulation of glycogen degradation within the phosphorylase molecule by gradual mutation, as evidenced by the amino acid sequence analysis of phosphorylase in a number of distinct animal species and plants (51-53). It appears, however, that the higher demand on glycogen for energy in *Sepia* mantle is met by maintaining a high concentration of AMP rather than evolving a structurally more active form of phosphorylase. The source and cause of AMP flux may be sought elsewhere.

E X P E R I M E N T A L

C H A P T E R 12

EXPERIMENTAL

A. MATERIALS AND REAGENTS

1. Sources of materials

Sodium β-glycerophosphate used in this part of the thesis was from Koch Light (London). AMP, glucose-1-P and sodium borohydride were from E.Merck, Germany. Oyster glycogen was obtained from Sigma Chemical Company, U.S.A. DEAE-cellulose was from V.P.Chest Institute, New Delhi. The Sepharose-glycogen was a generous gift from Prof. Dieter Palm of the University of Würzburg, West Germany. The sources of all other chemicals were as given in Chapter 6. The reagents used for estimation of protein and assay of phosphorylase were also as in Chapter 6.

2. Preparation of DEAE-cellulose column.

Coarse DEAE-cellulose was washed with 1 N NaOH till no more yellow color was removed. This was washed with water and suspended in 1 N HCl and filtered immediately and washed with water till free from the acid. It was then 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 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 was filled in the column of the required

size and equilibrated with the eluting buffer connected from a reservoir.

3. 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 allowing about 200 ml of the buffer to flow through the column.

4. Preparation of polyacrylamide gel.

a) Buffer solution.

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

b) Stock solutions

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

5. 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°.

6. Preparation of phenylhydrazine reagent.

Phenylhydrazine hydrochloride (2 g) was dissolved in 100 ml of 10 N sulphuric acid. The reagent was preserved in a refrigerator.

7. Preparation of alumina C_γ gel

The alumina C_γ gel used for the purification of AMP-deaminase was prepared as given in ref. 177.

A hot solution of 85 g of aluminium ammonium sulphate in 500 ml of water was poured at once into 800 ml of ammonium sulphate-ammonia water at 60°. The latter reagent contained 25 g of ammonium sulphate and 54 ml of 20% ammonia. During the precipitation and for an additional 15 minutes the mixture was stirred vigorously, care being taken to see that the temperature did not fall below 60°. The first voluminous precipitate gradually became flocculent. The

mixture was then diluted to 5 litres with de-ionised water and the supernatant fluid was decanted as soon as the precipitate had settled down. The washing with water by decantation was repeated two more times. To destroy any remaining basic aluminium sulphate, 10 ml of 20% ammonia was added during the fourth washing and washing with pure water resumed for several days.

The reagents used for reduction of the coenzyme, assay of kinase and AMP-deaminase are given along with the 'methods'

B. METHODS

1. Purification of glycogen phosphorylase a from *Sepia pharaonis*.

The *Sepia* caught along with other fishes were stored in ice-tanks of fishing vessels for 2 days before brought to shore. Medium to large sized *Sepia pharaonis* were sorted out and their mantle muscle separated. The muscle was stored at -20°.

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

Step.1. Extraction

The frozen mantle muscle was cut in to small pieces and ground in a mincer. This was then immediately homogeni

in an electric blendor for 30 seconds with 250 ml of cold distilled water. After keeping at 0° for 15 min. with occasional stirring, the homogenate was centrifuged at 7000 x g for 10 min. and the supernatant collected.

Step.2. Ammonium sulphate fractionation

The supernatant from Step 1 was brought to 0.39 saturation by the addition of 96 ml of saturated and neutralised ammonium sulphate solution to 150 ml of the supernatant. After 20 min. at 0°, the precipitate was removed by centrifugation and the supernatant collected. To 240 ml of the supernatant was added 86 ml of saturated ammonium sulphate solution (0.55 saturation). After 30 min. at 0°, the precipitated protein was collected by centrifuging at 8000 x g for 15 min. It was dissolved in 10-15 ml cold 10 mM β-glycerophosphate/ 2.5 mM mercaptoethanol pH 6.9 (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 (20 x 1.6 cm) equilibrated with buffer-A. The column was washed with buffer-A till the eluate was devoid of any protein. A concentration gradient was applied using 0.8 molar sodium chloride dissolved in buffer-A in the top reservoir and 150 ml of buffer-A in the mixing flask.

The solution in mixing flask was continuously stirred using a magnetic stirrer placed beside the flask. The flow rate was 0.6 ml/min and 5 ml fractions were collected.

The fractions having specific activity of 5 units/mg or above were pooled and the protein precipitated with solid ammonium sulphate (65% saturation). This was dissolved on 1-2 ml. of 7.5 mM sodium β-glycerophosphate pH 6.9 containing 2.5 mM mercaptoethanol (buffer-B) and dialysed against two changes of 1 litre each of buffer-B.

Step.4. Affinity chromatography using Sepharose-glycogen

The dialysed enzyme was applied to Sepharose- glycogen column (24 x 1.6 cm) equilibrated with buffer-B. About 3 ml of the enzyme was used each time. The flow rate was adjusted to 0.3 ml/min. and 5 ml fractions were collected. Phosphorylase, which was retarded in the column came out as a single symmetrical peak (see results).

2. Concentration and storage of the enzyme.

The enzyme eluted out from the affinity column was applied on 5 x 0.8 cm DEAE-cellulose column equilibrated with 10 mM glycerophosphate buffer pH 6.9. When the enzyme was completely adsorbed in the column, 0.5 M NaCl in 0.05 molar glycerophosphate buffer pH 6.9 (or 0.5 molar glycerophosphate pH 6.9) was applied. The phosphorylase moved as a clear

yellow band and was collected in a total 3-4 ml of the eluting buffer. The solution was dialysed in 25 mM glycerophosphate/ 10 mM mercaptoethanol buffer for 4-5 hours and stored at 2-5° in a refrigerator.

3. Polyacrylamide gel electrophoresis.

The polyacrylamide gel electrophoresis was performed according to the procedure of Ornstein and Davis (149). Electrophoresis was performed at 5-10° for 4 hours using a current of 4 mA per tube. A 7.5% gel was used. The buffer employed was Tris-glycine pH 8.5. The protein bands were stained with amido black and the gels were washed with 7% acetic acid at 50-60°.

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

4. Estimation of protein and activity.

These are described in chapter 6.

5. Estimation of total 'SH' groups.

The total 'SH' groups in the *Sepia* phosphorylase

were determined using Ellman's reagent (139). The details were as given in chapter 6.

6. Estimation of pyridoxal phosphate

Pyridoxal-5'-phosphate in the *Sepia* phosphorylase was estimated according to a modified procedure of Wada and Snell (160). To 1 ml of phosphorylase containing 1.5 mg of protein was added 0.5 ml of 1.5 M perchloric acid. After 30 minutes at 30°, 2 ml of 0.15% phenylhydrazine in 0.75 N sulphuric acid was added. The solution was centrifuged and the absorbance measured at 410 nm. Pure PLP (2-10 µg) was used as standard (The molar absorptivity of the pyridoxal-5'-phosphate phenylhydrazone under these condition was 2.85×10^4).

7. Estimation of glucosyl residues in glycogen.

The glucosyl residues in glycogen were estimated using the 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.

8. Reduction of coenzyme in *Sepia* phosphorylase by sodium borohydride.

Reduction of the coenzyme in phosphorylase was performed by the variant II method of ref. 178, a procedure based essentially on the method of Graves *et al*. (166).

(i) Reagents.

a) 0.02 M sodium borohydride prepared in a plastic tube with cold distilled water just before use.

b) Ammonium sulphate solution, saturated at 0° in 0.1 M glycerophosphate buffer (pH 7.0).

c) 3.0 M sodium chloride in 0.2 M imidazole-citrate buffer, pH 6.5.

(ii) method: To 2ml of the reagent (c) cooled to -5° was added 2ml of charcoal-treated *Sepia* phosphorylase (3 mg/ml). Then over a 10 minute period, 0.5 ml portions of the $NaBH_4$ solution was added 3 times with stirring. An equal volume of the ammonium sulphate solution was added and the solution was centrifuged at 0°. The residue was dissolved in 5 ml of water and dialysed in 0.05 M sodium β-glycerophosphate buffer pH 7. The specific activity of this reduced enzyme was about 20 units / mg under the standard assay conditions used for the present work.

9. Inactivation by heavy metals.

The *Sepia* phosphorylase was freed from mercaptoethanol by passage through a Sephadex G-15 column equilibrated with 25 mM sodium β-glycerophosphate pH 6.9 and was diluted in the same buffer to make it a 150 μg/ml solution. To this was added the required volume of a 5×10^{-4}M heavy metal salt solution to get the required final molarity, incubated for 30 minutes and assayed by adding to an equal volume of substrate solution containing 32 mM glucose-1-P, 2% glycogen and 2mM AMP.

10. 'SH' groups modification and inactivation by DTNB.

The relation between modification of SH groups and activity loss was studied using DTNB. For this, the enzyme was taken in 30 mM glycerophosphate/ 2 mM EDTA buffer, pH 6.9. To 2 ml of enzyme solutions (1 mg/ml) in a cuvette, was added 10 μlitre of 25 mM DTNB prepared in the same buffer and the increase in the absorbance at 412 nm was recorded using a Hitachi model 200 spectrophotometer. The activity of the enzyme solution was also noted with aliquots withdrawn from the reaction mixture at different time intervals and assaying for phosphorylase activity after diluting the samples in the same buffer. The number of SH groups reacted was calculated from a calibration curve obtained at pH 6.9 under the same conditions, using cysteine as standard.

11. Cold inactivation study.

Phosphorylase a (4 mg/ml) in 30 mM cysteine / 40 mM glycerophosphate in the absence and presence of 0.5 M NaCl (at pH 7 and pH 6) were incubated at 0° and 25°. Aliquots were withdrawn from these samples and diluted in the same buffer used for incubation and again incubated at 30° for 1 minute and assayed by adding to an equal volume of substrate solution containing 2% glycogen, 32 mM glucose-1-P and 2 mM AMP, also at the same pH of the assay.

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 (10). Details were as given in Chapter 6.

13. Partial purification of phosphorylase phosphatase from rabbit skeletal muscle.

The phosphorylase phosphatase was partially purified by the method of Keller and Cori (172). Fresh rabbit muscle (100 g) was passed through a chilled meat grinder and the ground muscle was homogenised for 3 minutes in a mincer with 200 ml of cold distilled water. The homogenate was centrifuged at 8000 x g at 0° for 10 minutes and the supernatant fluid filtered through a coarse filter paper.

The pH of the extract was lowered to 5.65 by the addition of 1.0 M acetate buffer, pH 4.6. After 10 minutes at 5° it was centrifuged at 8000 x g for 10 minutes at 0°. The precipitate was collected in to two tubes, washed once with 80 ml of cold distilled water and dissolved in 20 ml of a cold solution of 0.08 M sodium β-glycerophosphate. The pH of the solution (at 0-5°) was raised to 9.4 by the addition of 1.0 M sodium carbonate solution and was incubated at 37° for 60 minutes. The enzyme was cooled to 5° and pH readjusted to 7 by the addition of 1 M acctate buffer, pH 4.6. The flocculent precipitate was discarded after centrifugation. This solution was kept frozen. The phosphatase activity of this preparation was stable for 1 month.

14. Preparation of crude extract for phosphatase and kinase reaction.

The extraction was carried out at 2-4°. 40 g of the *Sepia* muscle was ground in a mortar and homogenised in an electric blendor with 80 ml of cold distilled water for 40 seconds. This was centrifuged at 7000 x g and the supernatant collected. The phosphatase and kinase activity of this extract was checked by incubating 0.1 ml of the extract with 0.9 ml of purified

Sepia phosphorylase under the conditions given below.

15. The phosphorylase phosphatase and kinase reaction.

The phosphorylase phosphatase reaction was carried out according to the procedure of Keller and Cori (172). For this, the *Sepia* enzyme (2-3 mg/ml) in 30 mM cysteine (at pH 6.5 and 7) was incubated with phosphatase solution (using rabbit phosphorylase phosphatase diluted in the same buffer or using the *Sepia* muscle extract as the case may be) and incubated at 30°. Aliquots were removed from this, diluted in 30 mM cysteine/ 40 mM glycerophosphate buffer pH 6.9 and assayed for phosphorylase activity at 30° in the absence and presence of 1 mM AMP. The rabbit phosphorylase phosphatase at a 40-fold final dilution or extract from frozen mantle muscle at a 10-fold final dilution could not alter the activity ratio of *Sepia* phosphorylase. The extract from fresh *Sepia* at a 40-fold final dilution was effective in the conversion of the a to b form.

The phosphorylase kinase reaction was carried out by the method of Krebs and Fischer (10). For this, to 1 ml (2-3 mg) of the enzyme in 15 mM cysteine pH 7 was added (i) 1 ml of 0.125 M tris-glycerophosphate buffer (at pH 7, 7.5 or 8 as the case may be), (ii) 0.5 ml of 6×10^{-2} M $Mg(Ac)_2$/ 1.8×10^{-2} M ATP, pH 7 and (iii) 0.5 ml of phosphorylase kinase (using rabbit muscle phosphorylase kinase

which was diluted 40-fold in 15 mM cysteine buffer pH 7 or using the *Sepia* muscle extract, as the case may be). This was incubated at 30°, aliquots removed, diluted in 30 mM cysteine/ 40 mM glycerophosphate buffer pH 6.9 (so that the concentration of ATP in assay was below 0.5 mM) and the activity ratio found out. The activity ratio of the *Sepia* phosphorylase *a* (= 0.4) was unaffected by the rabbit phosphorylase kinase or by the extract from frozen mantle (at a final 15-fold dilution). The phosphorylase *b* formed by reaction with extract from fresh *Sepia* muscle (at pH 6.5) could be converted back to the *a* form by incubation of the enzyme at pH 8 under these conditions (see chapter 11).

16. Determination of AMP by AMP-deaminase.

i) Purification of AMP-deaminase from rabbit skeletal muscle.

A 10-fold purification AMP-deaminase from rabbit skeletal muscle was achieved by the method of Nikiforuk and Colowick (173). Fresh rabbit muscle (150 g) was passed through a chilled meat grinder and washed four times by occasional stirring with 4 vol. of cold 0.85% NaCl for 20 minutes and squeezed through a cheese cloth. The deaminase was extracted from this colorless residue by occasional stirring with 150 ml or cold 2% $NaHCO_3$ for 1 hour and filtered

through a coarse filter paper.

To 100 ml of $NaHCO_3$ extract was slowly added about 20 ml of partially decanted alumina C_γ gel with continuous stirring. The suspension was kept at 8° for 15 minutes with occasional stirring. It was centrufged at 10° and supernatent fluid was discarded. The residue was suspended in about 10-15 ml 1.0 M Na_2HPO_4, stirred for 10 minutes at room temperature, centrifuged and the supernatant collected. This solution was stored in a refrigerator when Na_2HPO_4 crystallised, which was discarded.

A solution of ammonium sulphate (saturated at 0° and adjusted to pH 7.6 with 0.01 vol. of 18% ammonia) was added slowly to the eluate at 0° to give 0.27 saturation. After 10 minutes, the precipitate was removed by centrifugation in the cold at 15,000 r.p.m. The supernatant fluid was brought to 0.45 saturation and centrifuged. The residue was dissolved in a minimum volume of 0.1 M Na_2HPO_4. This solution was kept in a refrigerator at 2-6°.

ii) Assay of AMP by AMP-deaminase

To 4 ml of dialyzate in 0.01 M citrate buffer pH 6.5 was added 1 ml of diluted enzyme solution (diluted the enzyme stock solution 150 times in 0.01 M citrate buffer pH 6.5). The change in absorbance at 265 nm was noted in

a Hitachi model 200 uv-spectrophotometer, until there was no decrease in the color. 4 ml of pure AMP (2-10 mM) was similarly treated and the difference in absorbance was noted. The AMP concentrations were calculated from the difference in absorbance for complete conversion of AMP to IMP in the known volume of the dialyzate, using the calibration curve obtained for the standard AMP.

17. Preparation of dialyzate.

The dialyzates of the muscle tissues of different species were prepared by the following method: The muscle was ground with twice its weight of water and the homogenate was centrifuged. The supernatant was then dialysed against equal volume of 5 mM glycerophosphate pH 7 for 12 hours and the dialyzate collected. The whole experiment was performed at 0-4°.

S U M M A R Y

S U M M A R Y

The thesis has two parts. Part I deals with studies on rabbit muscle glycogen phosphorylase and Part II deals with glycogen phosphorylase from the mantle muscle of the marine invertebrate *Sepia pharaonis*.

Part I

Inhibition of the rabbit muscle phosphorylase *a* and *b* was examined using *m* and *p*-nitrophenols, *p*-nitrophenyl phosphate, *p*-nitrophenyl β-glucoside, *p*-nitrophenyl β-arabinoside, ATP and glucose-6-P. Sigmoidal saturation curves were obtained for phosphorylase *b* in the presence of each of these inhibitors. Inhibition of phosphorylase *a* by the above compounds was of the mixed type. Location of the binding domain in the enzyme was analysed using isobologram. The results indicated that all these ligands bind either mutually exclusively or nearly so on the same location in the enzyme near the monomer/monomer interface where the binding sites of glucose-1-P and AMP are also located. AMP afforded some protection of activity. Nitrophenols were the most effective inhibitors. Based on the degree of inhibition by these inhibitors, a mechanism of different modes of binding by ligands on the same region in the enzyme was presented to explain the special properties of phosphorylase.

The bifunctional reagent 1,5-difluoro-2,4-dinitrobenzene was used for further studies to locate the aromatic

binding site and to prepare an aromatic derivative of the enzyme. The reaction with phosphorylase b was found to be very specific at low concentration of the reagent and resulted in inactivation of the enzyme at a rate much faster than by 1-fluoro-2,4-dinitrobenzene.

Glucose-1-phosphate, glucose-6-P, AMP and ATP afforded some protection against inactivation by 1,5-difluoro-2,4-dinitrobenzene. The above ligands and aromatic compounds were shown to bind on the enzyme in the same region which is located near the monomer/monomer interface.

An apparently homogeneous dinitrophenylene derivative of phosphorylase b with only one group incorporated per dimeric enzyme and having 50% of the catalytic activity was prepared. In the derivative, subunits were not cross-linked. It was devoid of the homotropic cooperativity for the substrate or activator site even in the presence of allosteric inhibitors. Spectroscopic analysis of the derivative showed that one lysyl and one tyrosyl residues were modified, which resulted in the desensitisation of the enzyme. The derivative was structurally different from the native enzyme as evidenced from its comparatively higher instability to urea, temperature and the lower reconstitution rate of apodinitrophenylene enzyme. The derivative could be converted to the a form like the native enzyme.

Glucose behaved quite differently from other ligands in its effect on inactivation by the reagent and on the kinetics of the modified enzyme derivative. The results showed that glucose, unlike the other ligands did not bind on the aromatic binding site. Also, the binding of glucose was found to cause allosteric transitions.

Rabbit muscle glycogen phosphorylase at higher concentrations was found to effect a slow hydrolysis of p-nitrophenyl phosphate and similar nitrophenyl compounds. This effect was established as due to non-specific effect of some amino acid residues on the enzyme.

Part II.

α-glucan phosphorylase from specialised tissues of marine and estuarine invertebrates have not been subjected to detailed studies. Glycogen phosphorylase a was purified to homogeneity from the mantle muscle of the Cephalopod *Sepia pharaonis*. The mantle tissue is very much similar to the flight muscle of insects. The molecular weight of the phosphorylase purified was about 200,000. Estimation of the pyridoxal-5'-phosphate and the molecular weight suggested that the enzyme was a dimer. The kinetic mechanism was consistent with rapid equilibrium random bi bi, similar to the enzyme from other sources. The kinetic constants in the direction of glycogen synthesis were evaluated.

The *S. pharaonis* phosphorylase was found to be specifically activated by L-cysteine considerably (about 3-fold) unlike phosphorylases from other sources reported so far. Cysteine shifted the 335 nm band of the *Sepia* enzyme to 300 nm. These two effects of cysteine were found to be independent. The effect of cysteine was explained by a specific structural change which conferred an increased hydrophobic character near the PLP site. Such a change should aid in maintaining a fully protonated form of PLP.

The *Sepia* phosphorylase *a* was not inhibited by glucose-6-P, glucose or ATP in the presence of 1 mM AMP, like its rabbit counterpart. However, unlike in rabbit phosphorylase *a*, in the absence of the nucleotide, the *Sepia* enzyme was only slightly inhibited by glucose, and not at all by ATP. As with rabbit phosphorylase *a*, aromatic compounds showed mixed inhibition with the *Sepia* enzyme. The enzyme was found to be more sensitive to cold inactivation . Based on the results, a mechanism for cold inactivation was suggested. It may be noted that cold inactivation first observed for the rabbit enzyme has not yet been satisfactorily explained.

The control mechanism of the glycogen phosphorylase from *Sepia* was found to be different from other animal muscle phosphorylases. The *Sepia* enzyme exhibited negative homotroic cooperativity between AMP site. Fresh *Sepia* muscle contained

active phosphorylase phosphatase and kinase which became inactivated on freezing of the muscle. Rabbit muscle phosphorylase phosphatase and kinase did not interconvert the *Sepia* phosphorylase suggesting structural differences. The animal was found to maintain a very high concentration of AMP (about 3.5 mM) in its mantle tissue. The high energy demand of the mantle was met by this high concentration of AMP such that the enzyme would be fully active whether it was present in the *a* or *b* form. Therefore interconversion between AMP-dependent and AMP-independent forms of phosphorylase did not appear to be a major controlling factor of glycogen degradation, unlike in other cases studied so far.

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