EFFECT OF REDUCTIVE ALKYLATION ON THE STRUCTURE AND ACTIVITY OF GLYCOGEN PHOSPHORYLASE b

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by

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TO MY PARENTS AND MILLIE

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The author is indebted to Dr. Jerry Wang for his patience and supervision during the course of this project.

ABSTRACT

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ABSTRACT

Reductive alkylation of proteins using aliphatic aldehydes and sodium borohydride was developed by Means and Feeney (1). Reductive alkylation was shown to stabilize glycogen phosphorylase b against thermal and cold denaturation (2). The mechanism of this stabilization has been studied using aliphatic aldehydes with varying chain lengths. The modified enzymes have been characterized to learn the effect of modification on the structure and activity of the enzyme.

Glycogen phosphorylase b was modified with aldehydes ranging from the 2 carbon acetaldehyde to the 7 carbon heptanal. The enzyme was exposed to 0.5% aldehyde for 10 minutes after which sodium borohydride was added. The stable enzyme derivatives were purified by exposure to 50°C for 1 hour.

The stability of the enzyme derivatives was observed to be a function of the aldehyde chain length. The maximum stability was conferred to the enzyme by using aliphatic aldehydes with hydrocarbon chain lengths greater than 3 $-CH_2$ units. Compounds with lower chain lengths gave less protection to the enzyme.

Hydrophilic compounds such as methylglyoxal and glyceraldehyde offered no protection to phosphorylase b. These results suggest the importance of the hydrophobic moiety in conferring protection to the enzyme.

One of the most important features of reductive alkylation is its unique ability under mild conditions to specifically modify lysine ϵ-amino groups without altering the protein conformation (1). Less than 10% of the lysine residues of phosphorylase b were modified by the various aliphatic aldehydes. The modified proteins probably had the same conformation as the native form, since they could be crystallized and their ultraviolet absorption spectra, sedimentation properties and catalytic efficiency were similar to the native enzyme.

Although the gross conformational state of the modified-enzymes differed little from the native enzyme, studies of the allosteric properties of these enzymes indicated that in some cases subtle disturbances in the conformation might have taken place. Both hexanal-modified phosphorylase b and heptanal-modified phosphorylase b possessed no AMP homotropic cooperativity. In addition the affinity of AMP for the enzymes was significantly greater in these enzyme derivatives than with respect to the native and other modified-enzyme preparations. Thus the hydrophobic groups appear to play a role in subunit interactions as well as in stabilization of the enzyme.

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LIST OF ABBREVIATIONS

HSA	Human serum albumin
BSA	Bovine serum albumin
U.V.	Ultraviolet
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
EDTA	Ethylenediaminetetraacetate
PCMB	p-chloromercuribenzoate
SDS	Sodium dodecyl sulfate
PLP	Pyridoxal phosphate
TNBS	2,4,6-trinitrobenzenesulfonic acid
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
A.E.	Acetaldehyde-modified enzyme
P.E.	Propionaldehyde-modified enzyme
B.E.	Butyraldehyde-modified enzyme
V.E.	Valeraldehyde-modified enzyme
HX.E.	Hexanal-modified enzyme
HP.E.	Heptanal-modified enzyme
MeG.E.	Methylglyoxal-modified enzyme
GA.E.	Glyceraldehyde-modified enzyme
βME	2-mercaptoethanol
P_i	Inorganic phosphate
G-1-P	Glucose-1-phosphate
G-6-P	Glucose-6-phosphate
mA	Milliampere
$S_{20,w}$	Sedimentation coefficient corrected to water at 20°C
$(NH_4)_2SO_4$	Ammonium sulfate
$NaBH_4$	Sodium borohydride

SECTION I. INTRODUCTION

I. INTRODUCTION

1. Purpose of the study

Native phosphorylase b is labile to hot (2) and cold (3) extremes. The enzyme can be stabilized to these adverse environmental conditions by reductive alkylation (2). Previously, Wang and Tu (2) conducted preliminary modification experiments with monofunctional aldehydes such as propionaldehyde and butyraldehyde and the bifunctional aldehyde, glutaraldehyde. The present study examines the monoaldehyde modification of phosphorylase b. A variety of simple aliphatic aldehydes and the effect of these aldehydes on enzyme stability with respect to hot and cold extremes was studied. The optimum aldehyde concentrations necessary in protecting the enzyme were determined.

One of the prime objectives of this project was to study hydrophobicity as a factor in the preservation of protein configuration. The aldehyde-modified enzyme served as a model system for the study of hydrophobic interactions. We attempted to determine if the aldehyde carbon chain length was an important factor in stabilization and if so what the optimum chain length was. Reductive alkylation provided a simple mechanism by which the degree of hydrophobicity introduced into the enzyme could be controlled by the choice of aldehyde used.

Certainly one of the objectives of chemical modification is to determine the role of certain amino acid residues as functional parts of catalytic and allosteric sites. Since reductive alkylation is specific for lysine residues (1), the modified enzymes were studied with respect to their allosteric and catalytic properties to establish the role of the modified lysine residues in phosphorylase b. Wang et al (2) observed

that phosphorylase b when modified with the bifunctional reagent, glutaraldehyde, was devoid of AMP homotropic cooperativity yet still possessed a great deal of affinity for AMP. We attempted to determine whether the bifunctional character of the four carbon glutaraldehyde and/or the carbon chain length were critical factors in abolishing the cooperativity.

2. Organization of the thesis

Four main sections, Literature Review, Experimental Procedure, Results and the Discussion comprise the thesis. The Literature Review includes physical, chemical, catalytic and allosteric properties of glycogen phosphorylase b, chemical modifications of proteins and finally the mechanism of cold and heat denaturation of proteins. The Experimental Procedure section is subdivided into two parts which consists of the Materials and the Methods. The third section of the thesis presents the results of the experiments. The Discussion attempts to explain how the aldehydes stabilize the enzyme against heat and cold denaturation, their involvement in intra- and intermolecular interactions and the importance of hydrophobicity in maintaining protein conformation.

SECTION II. LITERATURE REVIEW

II. LITERATURE REVIEW

During the last few years numerous papers and reviews (4-8) have been published dealing with glycogen phosphorylase from rabbit skeletal muscle.

1. Properties of Glycogen Phosphorylase b.

This section of the literature review will be concerned with the physical, chemical, catalytic and allosteric properties of phosphorylase b.

a) Physical and Chemical Properties

Glycogen phosphorylase from rabbit skeletal muscle was first reported in the literature by the Coris (9-11) and consisted of two enzyme forms designated a and b. It was in the 1940's that phosphorylase a was crystallized from the rabbit muscle (12-14). Phosphorylase b was not crystallized until 1958 by Fischer and Krebs (15). The two forms of phosphorylase were found to be interconvertible by phosphorylase b kinase (16, 17) and specific phosphorylase phosphatases (18, 19). Only phosphorylase b will be discussed in this thesis.

Phosphorylase b has been reported to possess a molecular weight of 177,000 (20, 21) and 185,000 (22) with an $S_{20,w}$ value of 8.2S. Irrespective of the molecular weight, modification of the cysteinyl residues of the enzyme with PCMB dissociated the enzyme into two identical monomers (23, 24). Thus phosphorylase b is thought to be dimeric in nature. From sedimentation data carried out in 7.2M guanidine hydrochloride, the monomeric

subunit of the enzyme was found to consist of a single polypeptide chain of molecular weight 92,500 (25). With respect to the single polypeptide chain, no amino or carboxy-terminal groups have been detected in rabbit muscle phosphorylase b. Based on the occurrence of other known amino-terminal blocking groups in mammalian proteins, it appears likely that the amino blocking residue in phosphorylase is a pyrrolidone carboxylic acid derivative. The latter could be generated by the cyclisation of an amino-terminal glutamyl residue (26). While blocked amino-terminal groups are frequently observed in proteins, the apparent lack of a carboxy-terminal residue is more unusual.

Zarkadas et al in 1968 (27) showed that there was a minimum of 8 and a maximum of 9 sulfhydryl group sequences in the subunit of phosphorylase b. These sulfhydryl groups account for the total 1/2 cystine content of the enzyme which would indicate that no disulfide bonds are found. From alkylation studies using iodoacetamide, Zarkadas et al (28) proposed that 2 sulfhydryl groups were exposed on the surface of the enzyme and 2 other sulfhydryl groups were thought to be associated with enzyme activity and subunit interactions in phosphorylase b. The remaining 5 sulfhydryl groups in the monomer were thought to be buried.

Perhaps the most notable characteristic of phosphorylase b is its multiplicity of sites all involved in determining or controlling activity of the enzyme. In 1957 Madsen et al (29) found 2 AMP binding sites/mole of phosphorylase b. The same 2 sites bind ATP, an inhibitor of phosphorylase b. Two moles of PLP/mole of enzyme were noted by Cori et al (30). It is not known why PLP is essential for phosphorylase b activity, however its removal results in loss of enzyme activity (6, 30, 31). It appears that PLP which is covalently bound to the ϵ-amino group of a lysine

residue (6, 32, 33) is involved in maintaining the conformational stability of the enzyme since its removal results in dissociation of phosphorylase b to form monomers. When two moles of phosphorylase b were converted to phosphorylase a in the presence of phosphorylase kinase, four phosphate residues were introduced into the enzyme at specific serine residues (34). In addition to the nucleotide, PLP and phosphorylated sites mentioned, there is also the catalytic site to which glycogen, P_i, and G-1-P bind as well as inhibitors such as glucose. Nothing is known about the amino acid residues which form the catalytic site. Finally there must be several secondary sites on the monomer which are responsible for subunit assembly of the molecule.

b) Catalytic Properties

Phosphorylase b catalyzes the phosphorolysis of α-1-4-glucosidic bonds from glycogen releasing G-1-P (8, 35 -37). The reaction is as follows:

$$\text{Glycogen}_{(n \neq 1)} + P_i \longleftrightarrow \text{G-1-P} + \text{Glycogen}_{(n)}$$

where n represents the number of glucosyl residues in the polysaccharide.

In spite of the extensive studies on the mode of action of phosphorylase b, no detailed information exists as to the exact mechanism of catalysis. It is known that *in vivo* glycogen degradation is favoured. The relatively high P_i/G-1-P ratio which is known to exist in most animal tissues (38) does not allow the phosphorylase reaction to proceed in the direction of glycogen synthesis. *In vitro*, the reaction has been shown to be freely reversible where the P_i/G-1-P ratio at pH 6.8 is 3.6 indicating that glycogen synthesis is slightly favoured (4, 30). Unlike phosphorylase a,

phosphorylase b has an absolute requirement for AMP for activity (12, 39). Structural specificity studies (40, 41) have shown that the 5'monophosphate was essential for activation while the 2'hydroxy ribose group was important for binding as well as activation. The amino group at position 6 and the imidazole moiety of the purine ring appeared to be important for binding.

c) Allosteric Properties

Phosphorylase b has many of the properties of allosteric proteins as predicted by the model of Monod et al (42). The enzyme consists of identical subunits (27). The enzyme activity can be controlled by effectors whose molecular structure is unlike that of the substrates or products. Evidence by various authors (43-45) has shown that binding of effectors like AMP produces conformational changes. In recent years, the effect of AMP and other metabolites on the kinetics of phosphorylase b have been studied to determine the allosteric transition mechanism. Currently there is some dispute whether the allosteric mechanism of phosphorylase b can be explained by Monod's model or by Koshland's sequential model (46). No attempt will be made in this thesis to show whether the kinetics of muscle phosphorylase b can be best described by one or the other model.

Summary of the Allosteric Properties of Phosphorylase b

AMP binding

1. AMP exhibits homotropic cooperation (47).
2. AMP binding results in enhanced affinity of the enzyme towards Pi (48, 49) and G-1-P (48, 50).
3. AMP induces active conformation and aggregation to the tetrameric form (47, 51, 52).

Substrate binding

1. Homotropic cooperation occurs between G-1-P molecules in the presence

of ATP (50).

2. Homotropic cooperation occurs between Pi molecules as the AMP concentration is decreased. This also occurs in the presence of G-6-P and ATP (48).

Inhibitor binding

1. ATP exhibits increased homotropic cooperation as the concentration of Pi or AMP is decreased (48).
2. G-6-P and AMP show negative heterotropic interaction (53).

2. Chemical Modification of Proteins

There have been a number of reports which are concerned with modification of certain proteins by the introduction of a hydrophobic moiety.

a) Modification of HSA and BSA

In 1948, Duggan and Luck (54) observed that certain organic anions in appropriate concentrations prevented the rise in viscosity of BSA by 6M urea. SDS, a reagent which consists of hydrophobic as well as hydrophilic moieties, was found to be most effective in small concentrations. Markus et al (55) suggested that the native conformation of BSA was stabilized in part by detergent bridges between the hydrocarbon residues of SDS and the nonpolar groups of the protein, which are located in crevices formed by the folds of the tertiary structure. In addition, the authors noted that electrostatic interactions between $-SO_4^-$ of SDS and $-NH_3^+$ of the lysine residues in the protein are also involved in stabilization of BSA upon exposure to 6M urea. However, the importance of hydrophobic interactions should be stressed since it is one of the strongest stabilizing

factors in maintaining protein conformation (56).

Further evidence to support the importance of hydrophobic groups in stabilizing native conformation was supplied by G. Ballou et al (57). The authors studied the effect of salts of fatty acids on HSA with respect to their thermal stability. They observed that the fatty acid salts conferred stability to the enzyme and that the chain length of the fatty acid salt was a factor in determining the degree of stability. The binding energy associated with the binding of fatty acid salts to BSA was found to increase markedly with the length of the hydrocarbon chain used (58).

b) Modification of trypsin and chymotrypsin

Epstein et al (59) introduced varying amounts of alanine into the structure of trypsin and chymotrypsin producing poly-DL-alanyl trypsin and poly-DL-alanyl chymotrypsin. The derivative formed by the introduction of the hydrophobic moieties showed a marked resistance to inactivation upon heating at 38°C compared to the native enzyme forms. The proteolytic and esterolytic activities of the two derivatives were preserved.

c) Aldehyde modifications of proteins

Means and Feeney treated protein solutions with low concentrations of simple aliphatic aldehydes as well as ketones (1). Formaldehyde and acetaldehyde were the aldehydes used in the alkylation of RNase, insulin, α-chymotrypsin and other proteins. The authors found that upon amino acid analysis only one type of amino acid was modified in these proteins by reductive alkylation. When formaldehyde and acetaldehyde were used as modifying reagents and the modified proteins

subjected to amino acid analysis, ε-N, N-dimethyllysine and ε-N-monomethyllysine respectively, were the only amino acid derivatives formed. Both derivatives were stable in 6M HCl at 110°C for 22 hours. It was observed that sodium borohydride had to be added so that appreciable amounts of the lysine derivatives were found. Reductive alkylation of the proteins occurred with minimal changes in the gross physical properties. The mechanism for reductive alkylation presumably involves the formation of an intermediate Schiff base, the reduction of which yields the corresponding alkyllysine. The aliphatic carbonyl compounds were found to bind covalently as their alkyl groups to the ε-amino groups of lysine in proteins. Although exposure of RNase to formaldehyde and acetaldehyde resulted in significant activity loss, in general exposure to low concentrations of carbonyl compounds is not harmful to proteins.

d) Chemical modification of phosphorylase b

Little work has been undertaken in the chemical modification of phosphorylase b. The effect of chemical modification of lysine groups on the structure and activity of phosphorylase b was carried out by Huang and Madsen (60). They showed that phosphorylase b was inactivated by KCNO which resulted in the modification of 20 amino groups. Sedimentation information showed that a partial dissociation occurred. Philips and Graves (61) observed that complete inactivation of phosphorylase b occurred after dinitrophenylation of 4-5 lysine residues. Chemical modification has been used to study the role of the sulfhydryl groups in phosphorylase b (24, 27, 62).

The project which is described in this thesis derived its roots from the work of Wang and Tu (2, 63). The authors incubated phosphorylase

b in the presence of .05% glutaraldehyde, a bifunctional reagent and produced an enzyme form which still possessed activity. The form was stable to heat, cold and urea inactivation and possessed different allosteric properties than native phosphorylase b. As in Feeney's work (1) sodium borohydride was essential for the modification. The enzyme after modification was by gel electrophoretic criteria heterogenous. Heating at 50°C for 5 hours resulted in denaturation and precipitation of 90% of the enzyme. Gel electrophoretic analysis of the remaining protein, which consisted of thermally the most stable enzyme species, indicated a degree of purification had been achieved. Gel electrophoretic analysis showed only 1 major and 1 minor protein component.

Various methods were employed to quantitate the modification. Titration with TNBS, and amino acid analysis indicated that the modification was specific for approximately 10% of the lysine residues. Titration of the enzyme with DTNB suggested that sulfhydryl groups had not reacted.

Phosphorylase b was also modified with monofunctional aldehydes such as butyraldehyde and propionaldehyde by the same authors (2). The enzyme was modified with 1% and 3% aldehyde concentrations using the same procedure as in the glutaraldehyde modification. The purification procedure was also similar. Preliminary results indicated that the modified enzymes possessed heat stability.

3. Theory of Cold and Heat Inactivation of Phosphorylase b

Since the thesis discusses the stability of aldehyde treated phosphorylase b under conditions of cold and heat exposure, the mechanisms of denaturation by cold and heat will be considered.

a) Cold Inactivation

It has been shown that exposure of protein molecules to cold temperatures can disrupt their native conformations (64, 65). The instability of phosphorylase a in high ionic strength solutions below pH 7.0 during dialysis at 3-4°C (66) prompted an investigation of the effect of cold temperature on glycogen phosphorylase b. Graves et al (3) found phosphorylase b to be more sensitive to storage at 0°C than 20°C in .04M glycerophosphate-.03M-cysteine buffer at pH 6.0. After storage at cold temperatures, enzyme activity was lost and inhomogenous material was detected in the ultracentrifuge. In addition a blue shift occurred with respect to pyridoxal phosphate absorbance. Inactivation could be reversed by warming. At pH 6.8 in the glycerophosphate-cysteine buffer little enzyme activity is lost by exposure to 0°C. Inactivation of phosphorylase b was slowed by glycogen, pyridoxal phosphate, AMP, ATP and organic solvents. A number of authors (67, 68) have shown that hydrophobic interactions are sensitive to cold temperatures. It is known that these interactions are one of the most stable forces in maintaining protein conformation (56). It is quite likely that the cold temperatures cause the protein to unfold because of the disturbance of hydrophobic bonds. The disruption of hydrogen bonding between peptide groups and hydrogen bonds other than those between peptide groups, ion bond pairs and other electrostatic forces are also thought to be involved in cold denaturation of proteins. It is thought that enzyme activity is lost because of a conformational change and as a result the prosthetic group is exposed to a more polar environment. The reversibility of the inactivation would indicate that little PLP is lost. However some PLP may be lost because PLP when added to cold inactivated phosphorylase b results in some degree

of reactivation. The lack of sensitivity to cold at pH 6.8 in buffer suggests that other forces are involved in stabilizing the protein conformation. The mechanism of cold inactivation is still uncertain.

b) Heat Inactivation

The theory regarding thermal denaturation of proteins is thought to be similar to the mechanism proposed for cold mediated denaturation of proteins. As mentioned previously the importance of hydrophobic bonds have been established. Generally the non polar groups and slightly polar groups are largely buried in the molecular interior and are out of contact with the solvent (69). The hydrophobic bonding reaction as it pertains to heat or cold denaturation studies, involves the transition of a non polar or slightly polar side chain from its folded state in the protein interior to its unfolded state in water. A disruption of hydrogen bonds and electrostatic interactions involved in maintaining the secondary and tertiary structure of the protein will also occur and results in unfolding of the protein.

SECTION III. EXPERIMENTAL PROCEDURE

III. EXPERIMENTAL PROCEDURE

1. Materials

a) Chemicals

Cysteine hydrochloride, sodium glycerophosphate, glucose-1-P, shellfish glycogen, AMP, DL-glyceraldehyde, methylglyoxal and TNBS were obtained from Sigma Chemical Company. Glycogen was purified with Norit A according to the procedure of Sutherland and Wosilait (70). Sodium borohydride and ammonium sulfate were purchased from Fischer Scientific Company. Chemicals for gel electrophoresis were from Canalco Ind. Co. with the exception of Amido Black which was obtained from Matheson Coleman and Bell. Acetaldehyde, butyraldehyde, valeraldehyde, hexanal and heptanal were purchased from J.T. Baker Chemical Co. Propionaldehyde was obtained from Matheson Coleman and Bell. The ninhydrin was purchased from Biorad.

b) Preparation of Phosphorylase b

Phosphorylase b was purified from commercial frozen rabbit muscle (Pel-Freeze Biologicals, Inc., Rogers, Arkansas) by the method of Fisher and Krebs (71, 72). Phosphorylase b preparation was crystallized three times before use and treated with Norit A to remove nucleotides and other impurities (73).

2. Methods

a) Protein concentration

Phosphorylase concentrations were determined spectrophotometrically

at 280mμ . An absorbancy index of 11.9 (74) for a 1% enzyme solution was used.

b) Phosphorylase b assay

Routine enzyme activity measurements were carried out in the direction of glycogen synthesis as described by Illingworth and Cori (74). The assay time was 5 minutes and the activity was measured at 30°C.

c) Modification procedure

The procedure used is a slight modification of that of Wang and Tu (2). Phosphorylase b was dialyzed overnight in .04M glycerophosphate buffer, pH 7.5, prior to modification. The protein concentration was adjusted to approximately 15mg/ml. The enzyme preparation was incubated on ice with various concentrations of aldehydes on a v/v or w/v basis. At times it was necessary to dilute the aldehyde in ethanol prior to modification to achieve the correct aldehyde concentration. Control experiments were conducted to insure that the ethanol was not responsible for the altered properties of the modified enzymes. After a 10 minute incubation of enzyme and aldehyde, an equal volume of 2mg./ml. of $NaBH_4$ in .3M glycerophosphate buffer, pH 7.5, was added. The addition of $NaBH_4$ was repeated 20 minutes later. The modified enzyme preparations were dialyzed overnight in .04M glycerophosphate-.03M-cysteine buffer at pH 7.0. Modifications performed without the addition of $NaBH_4$ were not successful.

d) Purification of the modified enzyme preparations

The modified enzyme preparations were initially concentrated by adding neutral and saturated $(NH_4)_2SO_4$. The pellet was dissolved

in .04M glycerophosphate-.03M-cysteine buffer, pH 7.0 and dialyzed overnight in the same buffer to remove the $(NH_4)_2SO_4$. The concentrated modified enzyme preparations were then heated for 1 hour at 50°C. The soluble enzyme remaining is known as the purified preparation. The impure enzyme preparations refer to the modified enzyme which has not been heated for 1 hour yet is devoid of $NaBH_4$ and excess aldehyde.

e) Heat inactivation of the modified phosphorylase b preparations

The procedure is similar to that used by Wang and Tu (2) except that the enzyme (2-5mg./ml.) was diluted 25-150 fold in .04M glycerophosphate-.03M-cysteine. The routine substrate employed in the assay contained 2% glycogen, 32mM G-1-P and 5mM AMP.

f) Cold inactivation of modified phosphorylase b

The time course of cold inactivation was slightly different from that employed by Wang and Tu (2) as outlined by Graves et al (3). The enzyme (2-5mg./ml.) was diluted 25-150 fold in .04M glycerophosphate-.03M-cysteine, pH 6.0 and the test tubes were placed on ice. At various times aliquots (.2ml.) were removed and added to test tubes placed in a 30°C water bath. The aliquots were assayed immediately with the same substrate used for assays of heat treated enzyme.

g) Titration of amino groups by TNBS

Enzyme preparations were dialyzed overnight in .04M glycerophosphate-.001M-EDTA buffer, pH 8.5. Titration of amino groups was according to the method of Habeeb (75). Protein concentration for the native and modified enzyme preparations were almost identical.

h) Ninhydrin titration

Enzyme preparations were dialyzed against two changes of .04M glycerophosphate-.03M- βME-.1M-KCL buffer, pH 7.0, followed by two changes of .04M glycerophosphate-.03M- βME buffer, pH 7.0. Total time for dialysis was 48 hours. Enzyme was titrated with ninhydrin solutions as outlined by H. Fraenkel-Conrat (76).

i) Amino acid analysis

Amino acid analysis was performed on a Spinco automatic amino acid analyzer. Enzyme samples were first dialyzed against two changes of .1M KCL, followed by two changes of deionized water. Total time for dialysis was five days. Hydrolysis of the protein was carried out at 110°C in 6N HCL under vacuum for 24 hours.

j) Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out on 7% gels at pH 9.5 and room temperature with the procedure of Ornstein (77). Amido Black was used as the protein stain. The sample load was approximately 100μg; the runs were 2 1/4 hours at 2.5mA/tube.

k) Ultracentrifuge experiments

Ultracentrifuge runs were performed on a Spinco Model E analytical ultracentrifuge at a temperature of 20°C and a speed of 60,000 r.p.m. Sedimentation coefficients were determined with the aid of a Nikon Model 6C microcomparator.

l) Ultraviolet absorption spectra

Enzyme (7-11mg./ml.) contained in .04M glycerophosphate-.03M-

cysteine was diluted 15 fold and the U.V. absorption spectra was determined manually using a Beckman DB U.V. and visible light spectrophotometer.

m) Determination of kinetic parameters

K_m and K_a, the affinity of G-1-P and AMP respectively for the different enzyme preparations were determined by plotting the reciprocal of the G-1-P or AMP concentrations against the reciprocal of the velocity.

SECTION IV. RESULTS

IV. RESULTS

1. Optimum Aldehyde Concentration for Modification

To determine the optimum aldehyde concentration to use in the reductive alkylation of phosphorylase b, the enzyme was treated with varying amounts of butyraldehyde. Heat inactivation profiles for the modified enzymes are illustrated in Fig. 1. 100% activity is the activity of the enzyme at zero time.[1] As can be seen the modified enzymes possess significant thermal stability upon heating at 50°C for up to 60 minutes. With respect to their stabilities, .3%, .5%, .75%, and 1% butyraldehyde-modified enzymes can be grouped together with .5% B.E. possessing the highest degree of protection. .1% and the native enzyme appear distinct from the other group with respect to their thermal stability. The modified-enzyme preparations were purified by heating for 1 hour at 50°C, except for the .1% B.E. which was only heated for 30 minutes. The same experiment was performed with the enzyme that remained soluble. The results can be seen in Fig. 2. Again the various enzyme preparations can be grouped as a function of their stability towards heat denaturation. .3%, .5%, .75% and 1% modified enzyme comprise one group while .1% B.E. and the native enzyme make up the two remaining groups. No particular modified enzyme appears to be the most stable to heat denaturation on the basis of the experiments conducted although .3%, .5%, .75% and 1% B.E.'s were grouped together and were the most stable at all times. Similar experiments using the same aldehyde

[1] Absolute values in terms of $O.D._{660}$ units representing the original activity for the enzyme preparations are given in the legends of the first two Fig.'s.

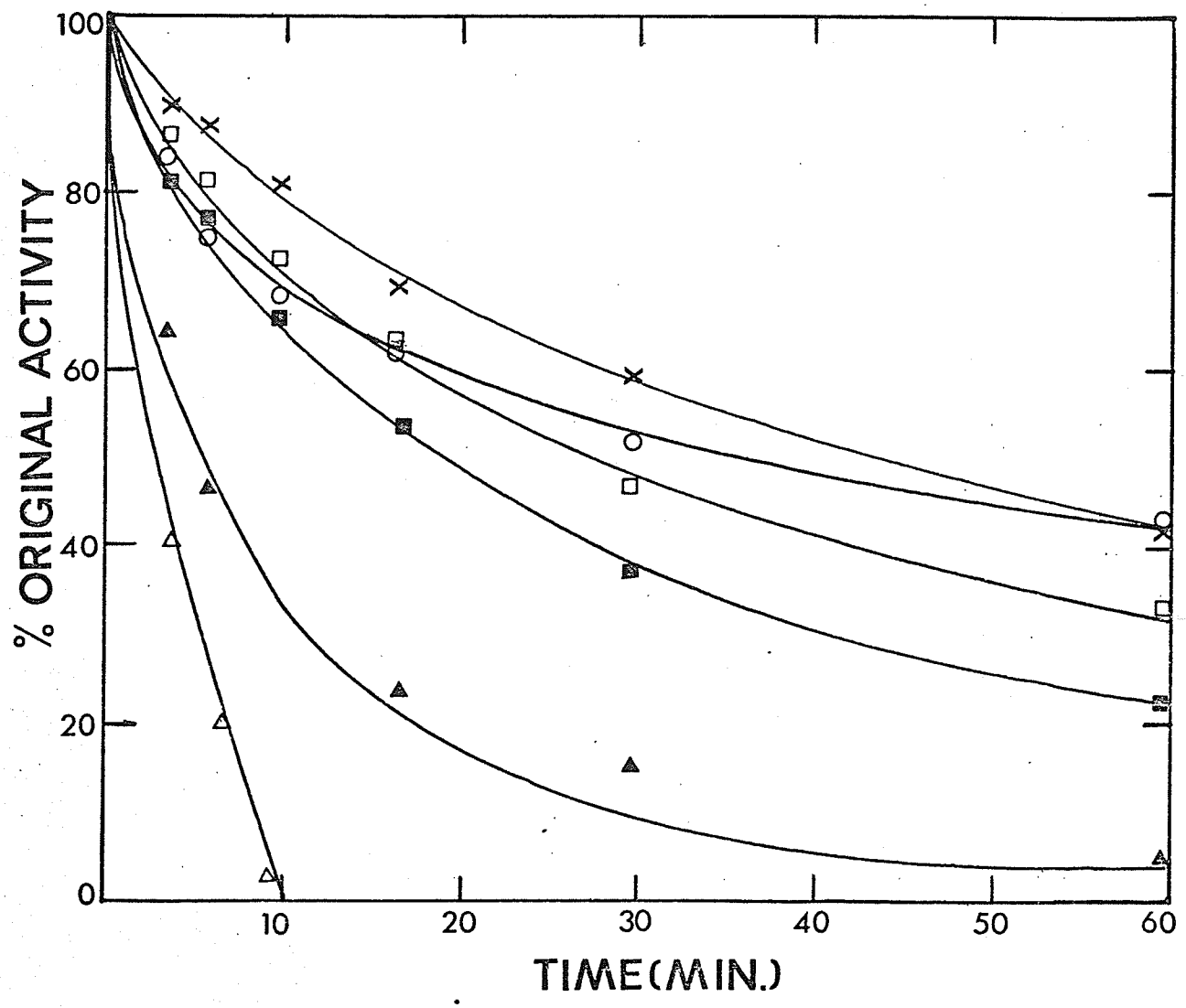


Fig. 1

The kinetics of heat inactivation for native phosphorylase b (△), .1% (▲), .3% (▣), .5% (X), .75% (○) and 1% (□) impure butyraldehyde-modified phosphorylase b preparations. Activity of phosphorylase b was plotted as per cent original activity as a function of incubation time at 50°C.

Original activity of the native enzyme, .1%, .3%, .5%, .75% and 1% B.E.'s in $O.D._{660}$ units/5 minute assay were .54, .59, .57, .45, .41, and .33.

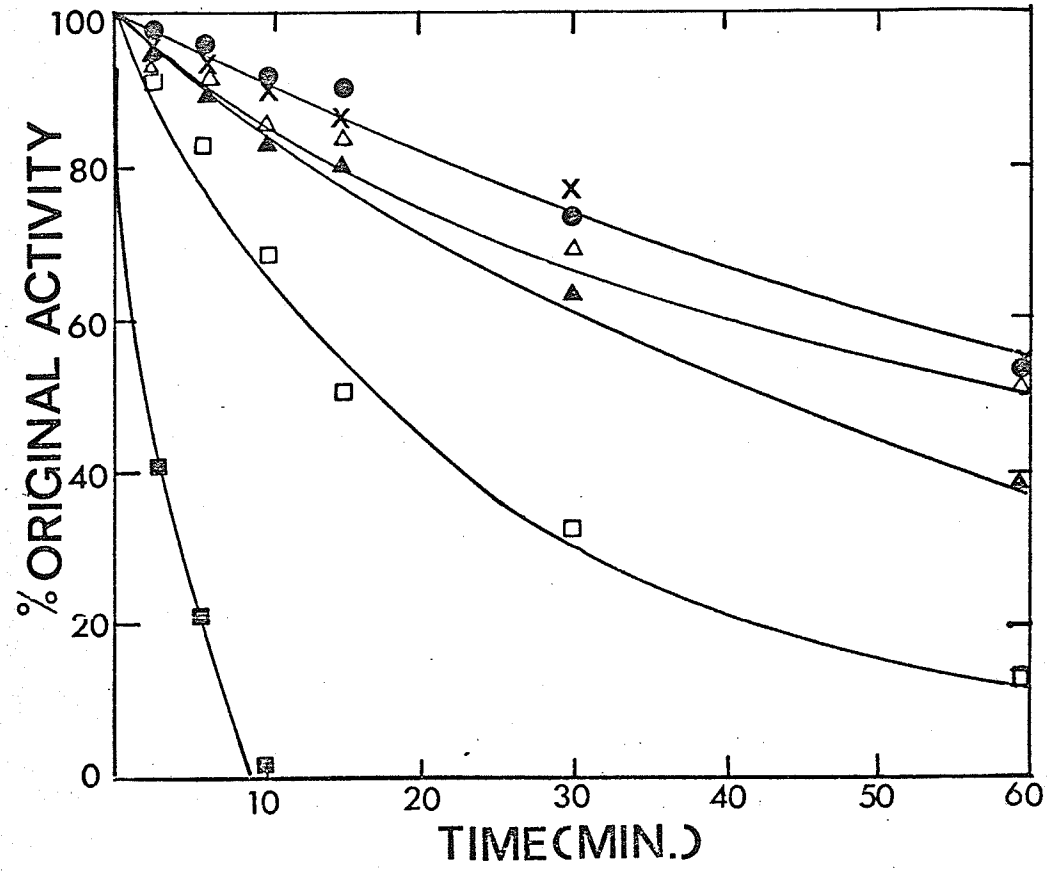


Fig. 2

Heat inactivation for native enzyme (■) and purified preparations of .1% (□), .3% (●), .5% (X), .75% (△) and 1% (▲) butyraldehyde-modified phosphorylase b. Activity of phosphorylase b was plotted as per cent original activity as a function of incubation time at 50°C. Original activity of the native enzyme, .1%, .3%, .5%, .75%, and 1% B.E.'s in $O.D._{660}$ units/5 minute assay were .52, .44, .39, .74, .86 and .58.

concentrations were conducted with acetaldehyde and heptanal. The results were similar to those obtained by the previously described experiments. After purification, a significant increase in stability occurred with all heptanal modifications but with only the .5% A.E. The .5% aldehyde concentration was used arbitrarily in subsequent modifications since this concentration resulted in one of the most thermally stable enzyme derivatives.

2. Kinetics of the Heat Inactivation of Modified Phosphorylase b

Phosphorylase b was modified with .5% acetaldehyde, propionaldehyde, butyraldehyde, valeraldehyde, hexanal and heptanal. The resulting impure enzyme derivatives were studied and compared with respect to their thermal stabilities. Initially, diluted portions of A.E., B.E., HP.E. and P.E. were heated up to 1 hour. Samples were removed at various times and assayed. The kinetics of heat inactivation can be seen in Fig. 3. The A.E., B.E., HP.E. and P.E. appear to be more stable to heat inactivation than the native enzyme with B.E. and HP.E. possessing the highest stability among the enzyme derivatives studied.

To determine the degree of homogeneity, log. of activity versus time of exposure to 50°C was plotted (Fig. 4). The B.E. and HP.E. in addition to the native enzyme display a linear relationship between log. activity and time. The P.E. and A.E. produced non linearity.

Heat inactivation experiments were also undertaken with V.E. and HX.E. (Fig. 5). The B.E. and P.E. were also included in this experiment as "marker enzymes" so that thermal stability profiles of all enzyme derivatives could be compared. The valeraldehyde derivative possessed the

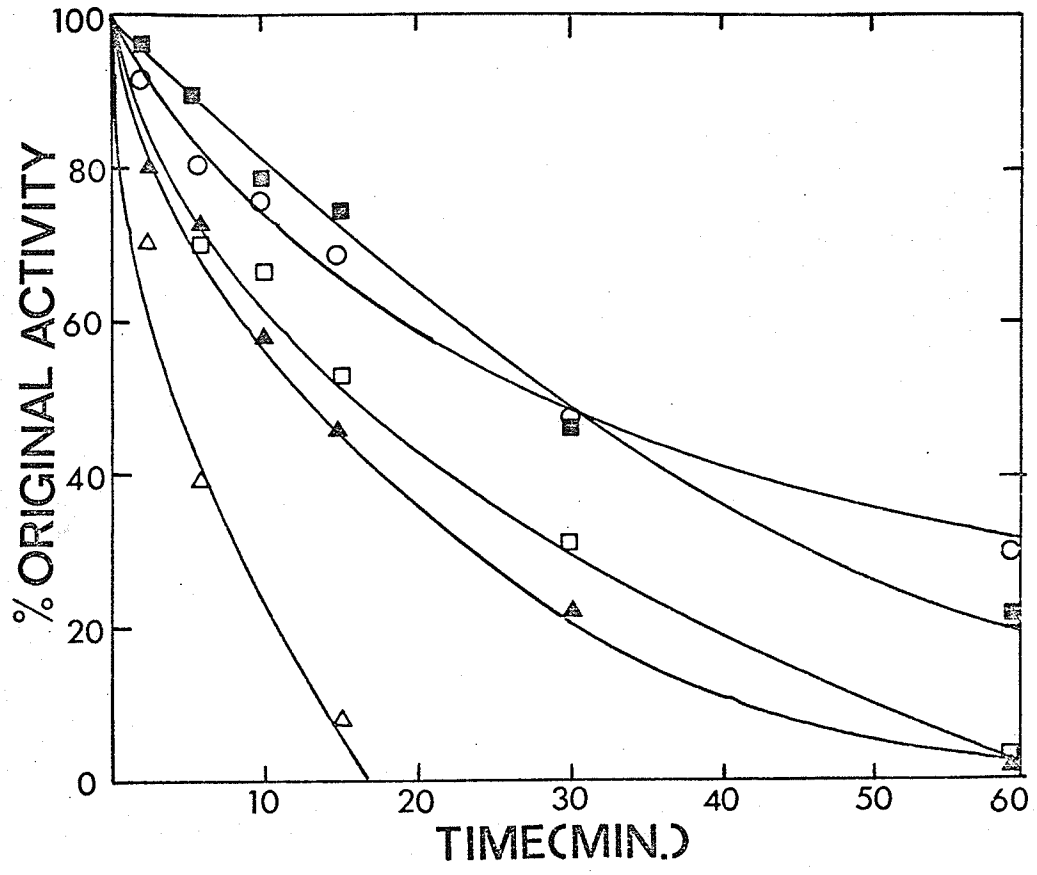


Fig. 3

Heat inactivation profiles for native enzyme (△) and impure preparations of A.E. (▲), P.E. (□), B.E. (○), and HP.E. (■). Activity of phosphorylase b was plotted as per cent original activity as a function of incubation time at 50°C.

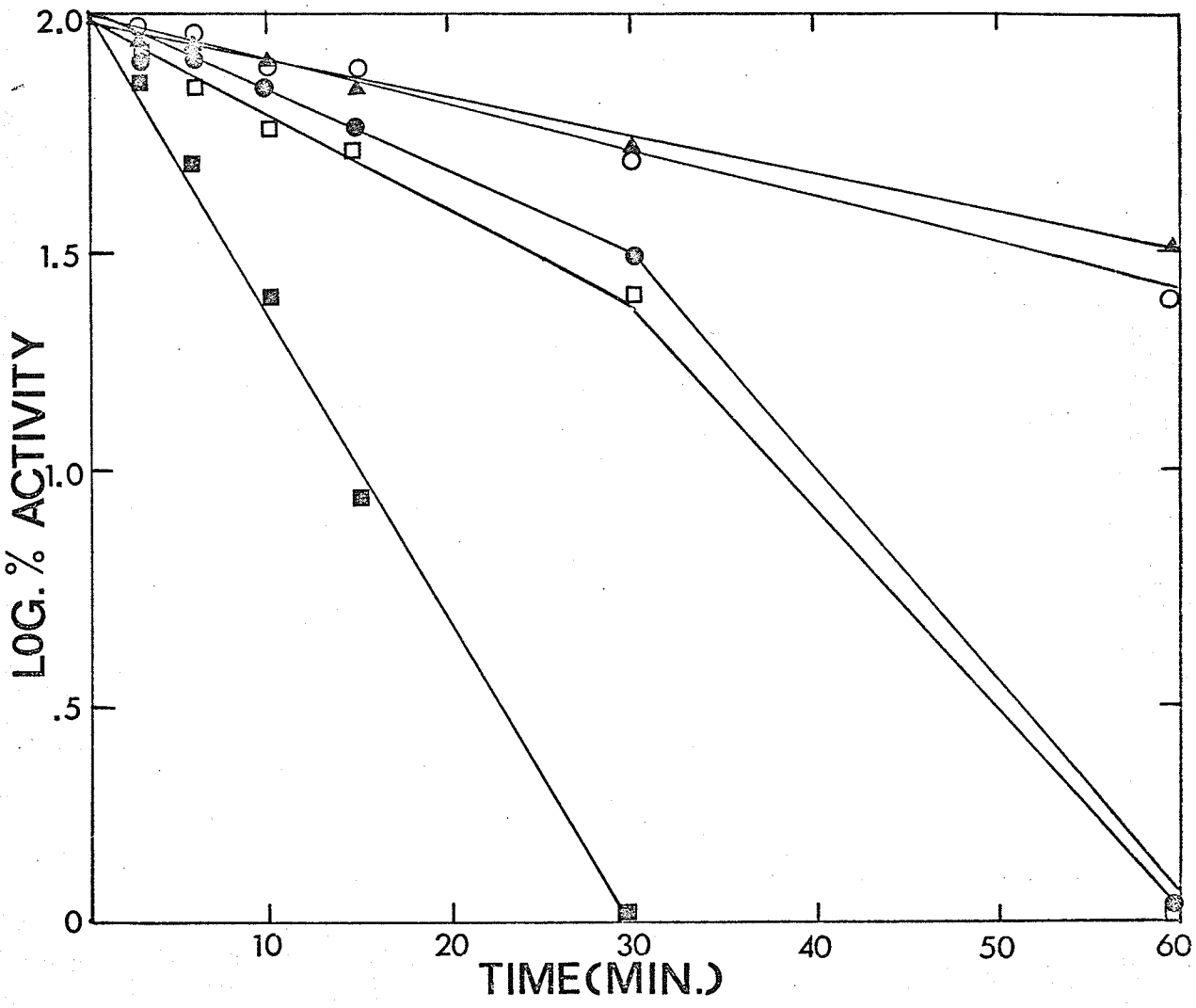


Fig. 4

Data for native enzyme (■), A.E. (□), P.E. (●), B.E. (▲), and HP.E. (○) in Fig. 3 was expressed as log. per cent original activity as a function of incubation time at 50°C.

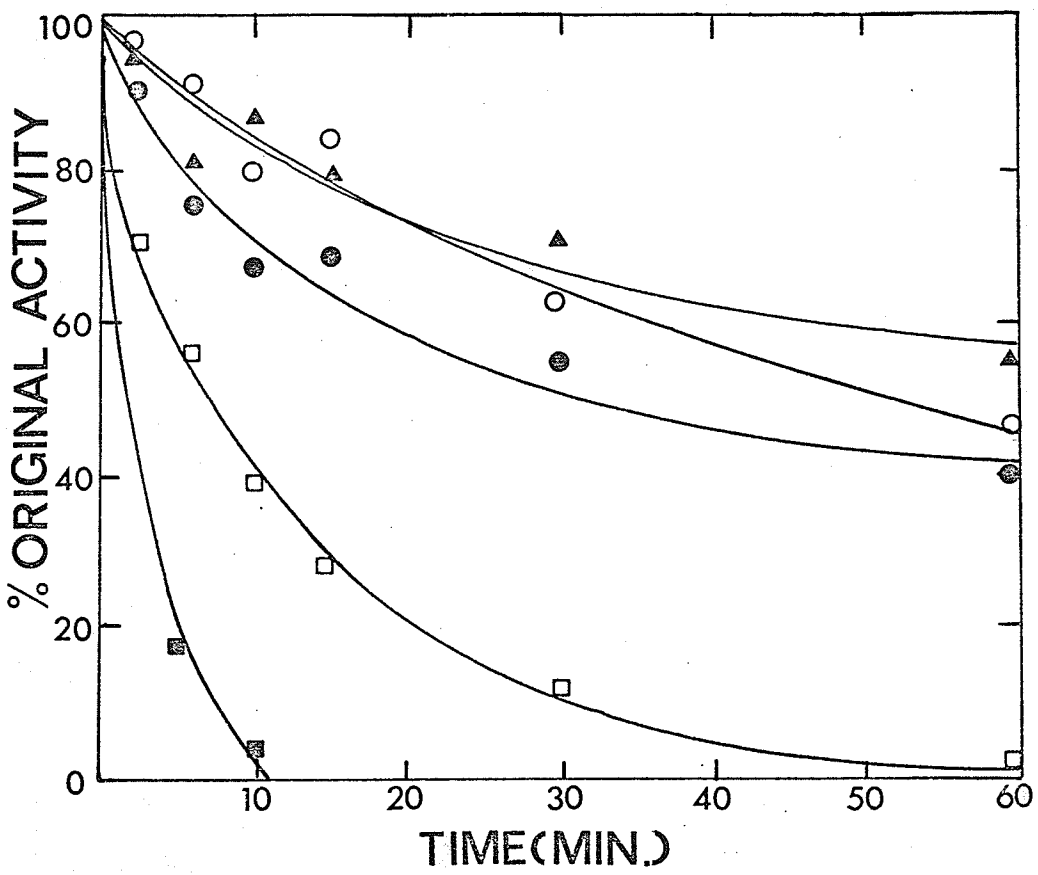


Fig. 5

Heat inactivation profiles for native enzyme (■) and impure preparations of P.E. (□), B.E. (○), V.E. (▲), and HX.E. (◍). Activity of phosphorylase b was plotted as per cent original activity as a function of incubation time at 50°C.

highest degree of stability although B.E. and HX.E. were also highly resistant to denaturation. The results of a log. activity versus time plot are shown in Fig. 6. Note the linearity associated with V.E., B.E., HX.E. and the native enzyme in addition to the non linearity observed with P.E.

The same enzyme preparations i.e. A.E., P.E., B.E., V.E., HX.E. and HP.E. were studied after purification. The purification procedure resulted in the precipitation of 45-80% of the protein. The kinetics of heat inactivation with respect to the purified enzyme species was studied (Fig. 7). The B.E. is the most stable of the enzyme derivatives studied in the experiment. HP.E., P.E., A.E. and native enzyme follow in decreasing order of thermal stability. Log. activity versus time plots (See Fig. 8) indicate a linear relationship between log. of activity and time of exposure to 50°C for all enzyme species studied. Note also the increased stability of P.E. and to a small extent A.E., B.E. and HP.E. after purification compared to Fig. 4.

Fig. 9 indicates that V.E. possesses the greatest level of thermal stability followed by B.E., HX.E. and P.E. In comparison with Fig. 5, the purification technique appeared to increase the stabilities of HX.E. and P.E. but V.E. and B.E. stability profiles remained for the most part unchanged. The log. activity versus time plot did not change appreciably after heating the enzyme preparations in the purification procedure and therefore was not included.

The kinetics of heat inactivation was studied over a 4 hour period of time using the purified enzyme preparations (Fig. 10). Even after a 4 hour exposure to 50°C an appreciable amount of activity remains. The graph allows for a direct comparison of all six modified enzyme

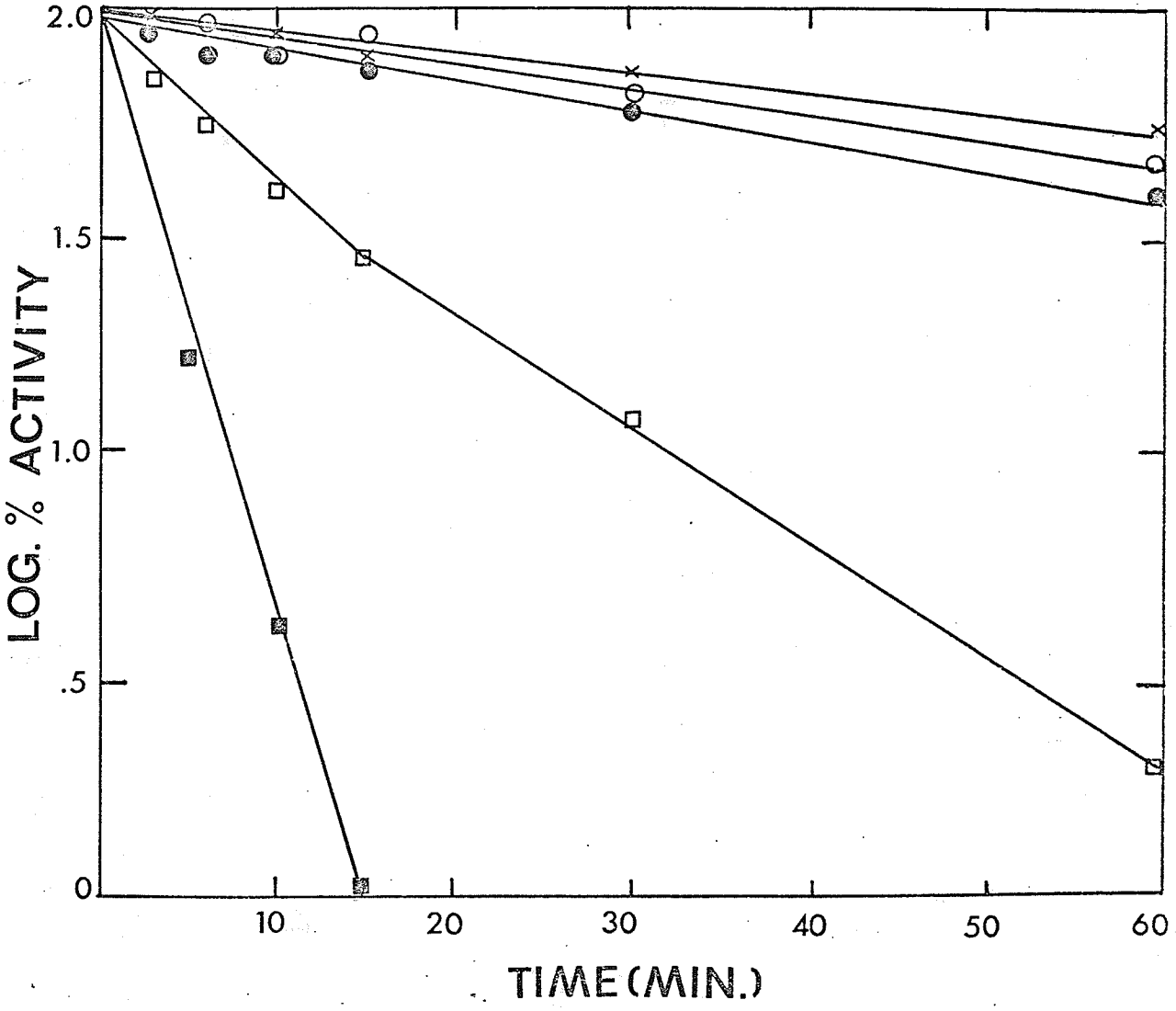


Fig. 6

Data for native phosphorylase b (■), P.E. (□), B.E. (○), V.E. (X), and HX.E. (●) in Fig. 5 was expressed as log. per cent original activity as a function of incubation time at 50°C.

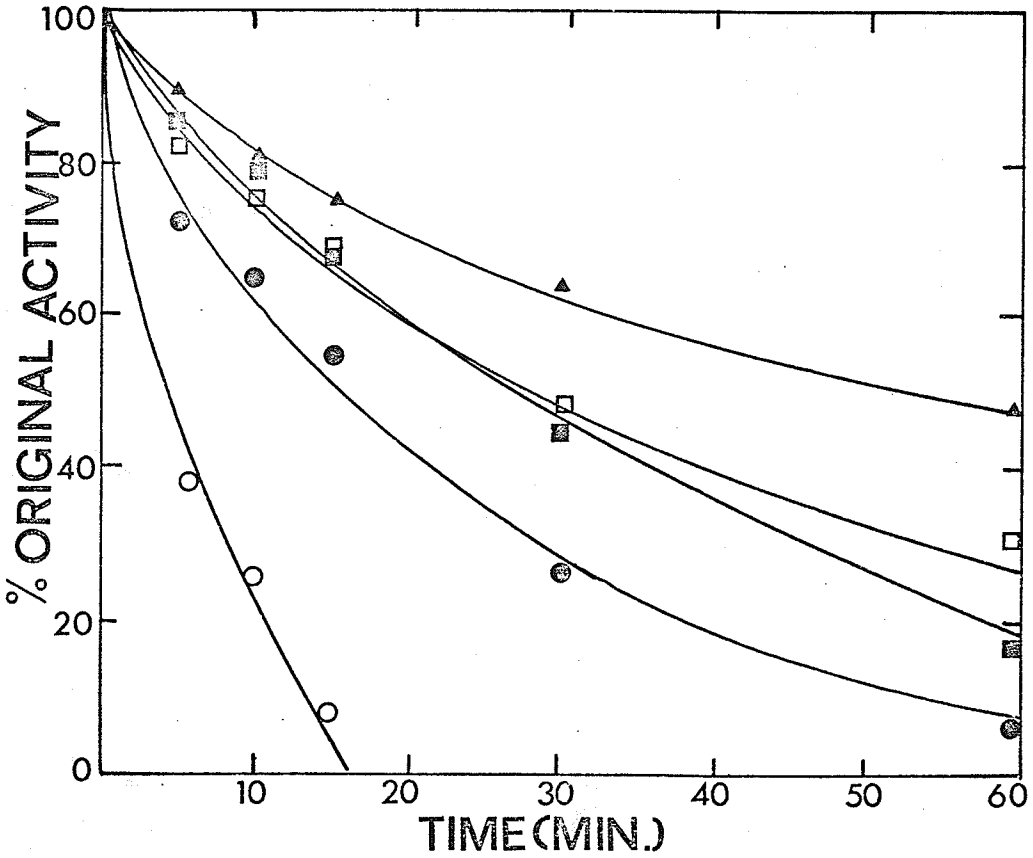


Fig. 7

Heat inactivation profiles for the native enzyme (○), and purified preparations of A.E. (●), P.E. (■), B.E. (▲), and HP.E. (□). Activity of phosphorylase b was plotted as per cent original activity as a function of incubation time at 50°C.

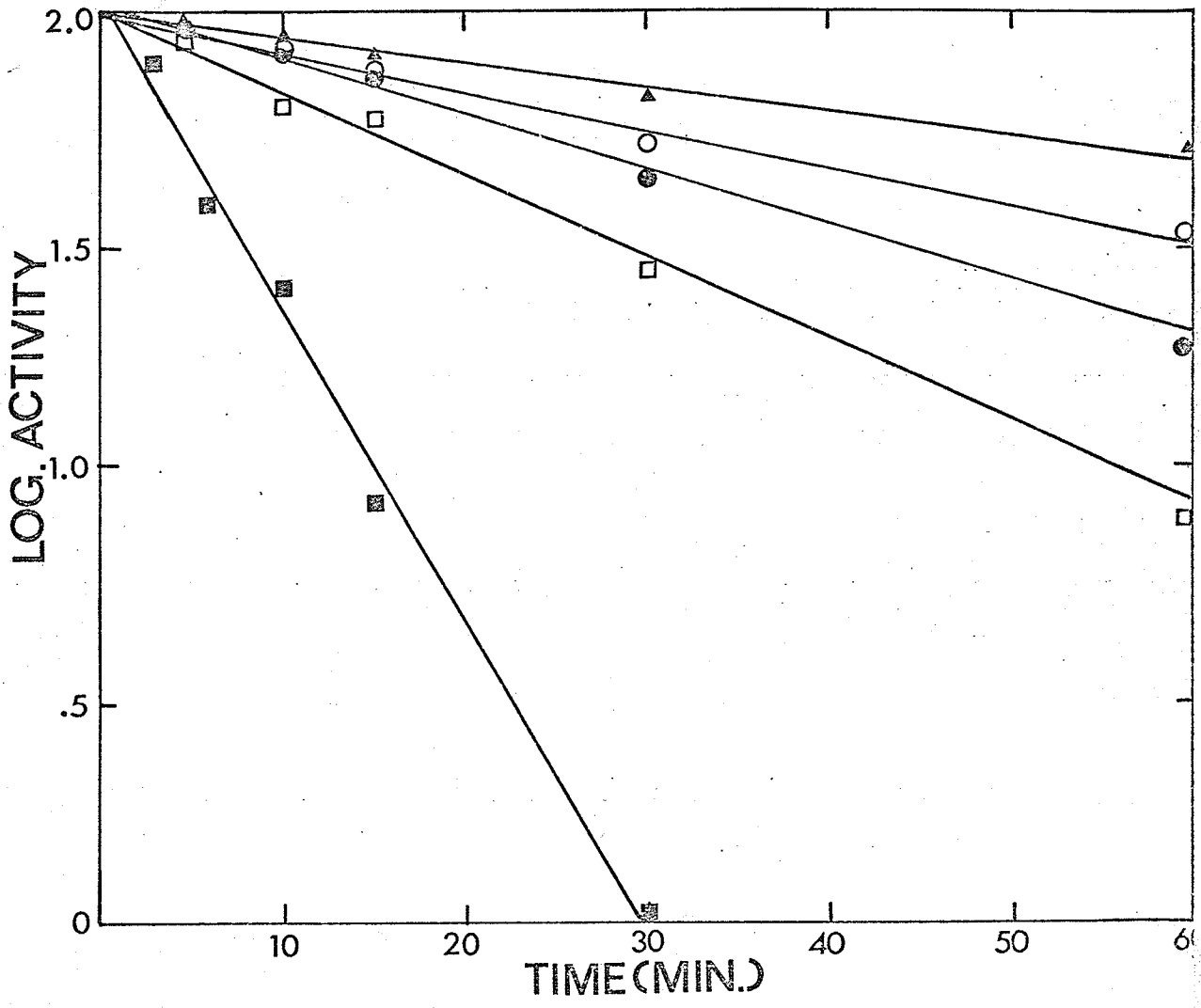


Fig. 8

Data for the native enzyme (■), A.E. (□), P.E. (◉), B.E. (▲), and HP.E. (○) in Fig. 7 was expressed as log. per cent original activity as a function of incubation time at 50°C.

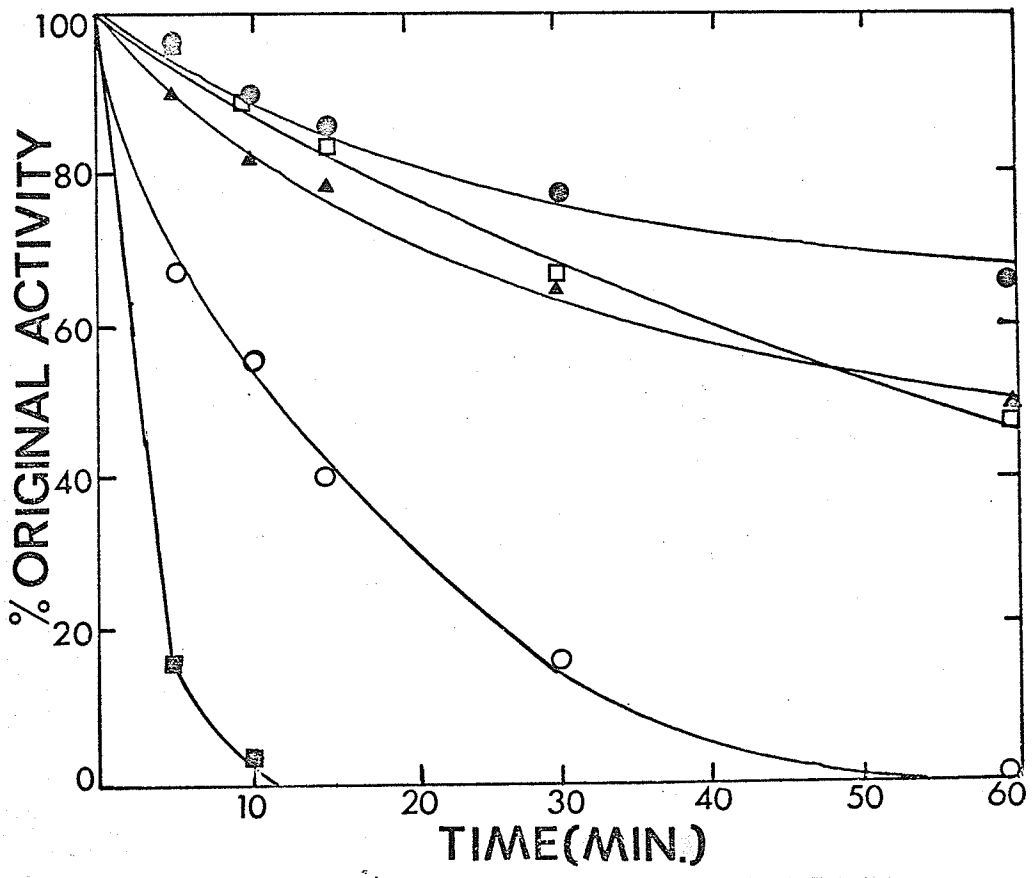


Fig. 9

Kinetics of heat inactivation for native phosphorylase b (▨) and purified preparations of P.E. (○), B.E. (▲), V.E. (●), and HX.E. (□). Activity of phosphorylase b was plotted as per cent original activity as a function of incubation time at 50°C.

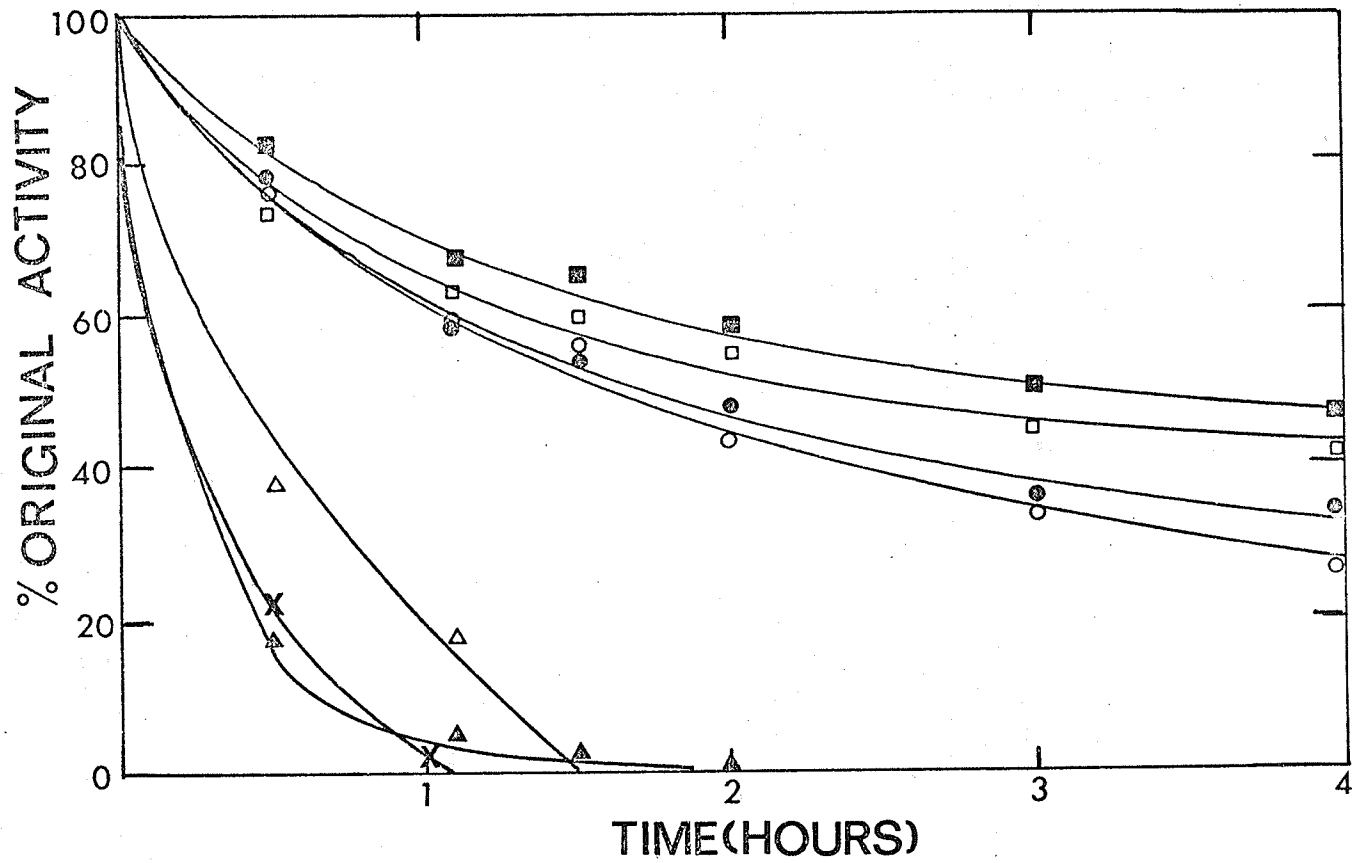


Fig. 10

Heat inactivation of native phosphorylase b (X), A.E. (▲), P.E. (△), B.E. (□), V.E. (▣), HX.E. (◉) and HP.E. (○) over a 4 hour period of time. Activity of phosphorylase b was plotted as per cent original activity as a function of incubation time at 50°C.

preparations. V.E., B.E., HX.E. and HP.E. differ only slightly in their thermal stabilities while A.E. and P.E. possess significantly more lability to heat denaturation than the other modified preparations.

The aldehyde-modified phosphorylase b preparations were also studied with respect to their susceptibility to cold inactivation. Both impure and purified preparations were subjected to cold temperatures. Only the profiles of inactivation for the purified preparations will be illustrated because of the similarity with respect to the impure enzyme profiles. Fig. 11 indicates that hexanal, valeraldehyde and butyraldehyde offer similar degrees of protection. Log. activity versus time plots were linear for all enzyme preparations studied. Problems occurred when A.E. and HP.E. were studied. More information will be contained in the Discussion.

3. Kinetic Parameters

The K_m's of the purified enzyme preparations for G-1-P were studied (Table I). The K_m's were obtained in 5 different experiments. The constant for the native enzyme in each experiment was calculated.

An AMP kinetic study was also undertaken. (See Fig. 12). The double reciprocal plots for AMP activation were linear for HX.E. and parabolic for B.E., V.E. and the native enzyme. The non linearity made it difficult for the calculation of K_a's. AMP activation for A.E., P.E. and the native enzyme (Figs. 13 and 14) resulted in parabolic curves while HP.E. displayed linearity. The linearity is associated with an absence of homotropic cooperation while the parabolic curves are characteristic of homotropic cooperation. The K_a's for the different

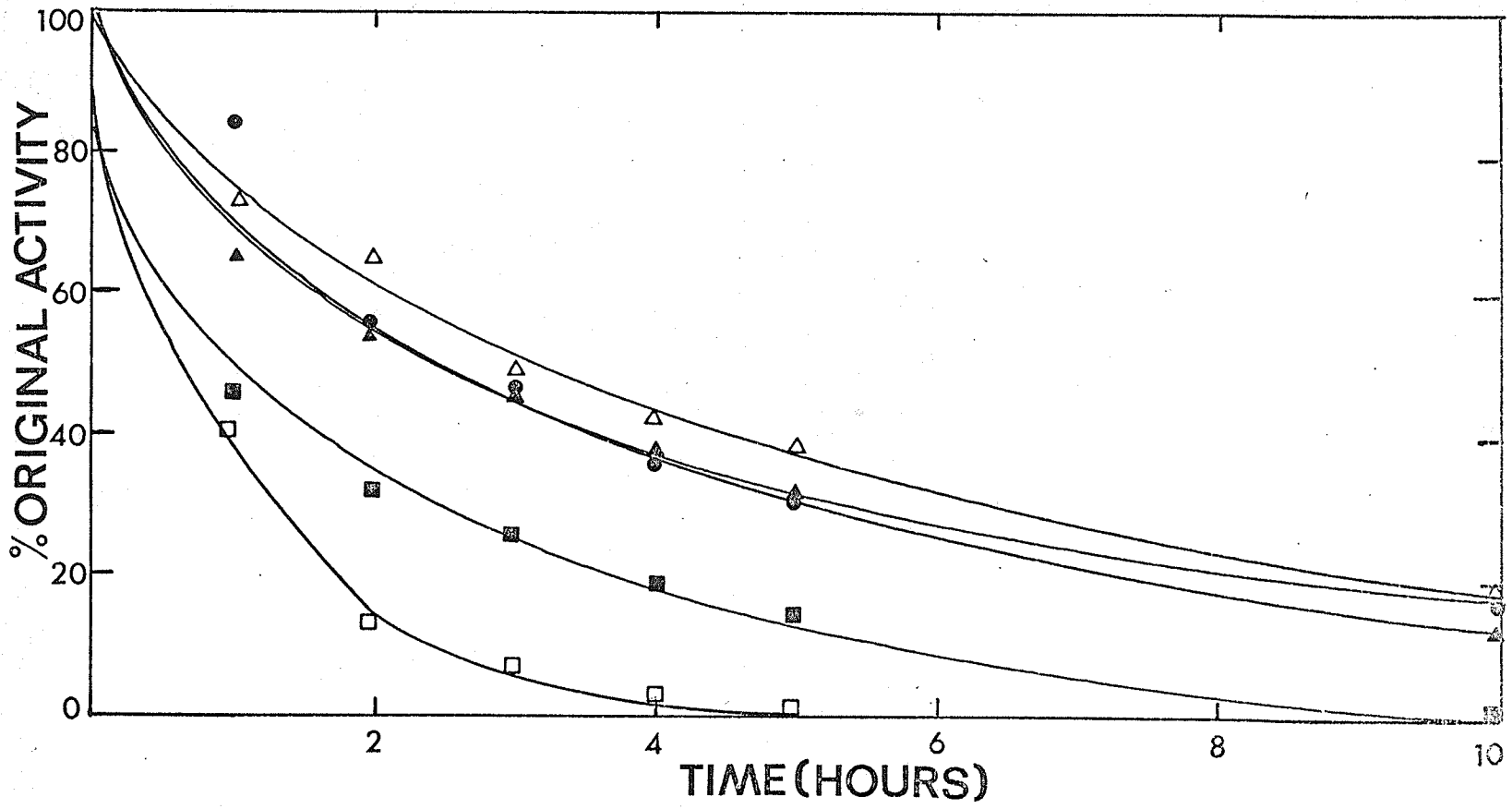


Fig. 11

Cold inactivation of native phosphorylase b (□), P.E. (■), B.E. (▲), V.E. (△), and HX.E. (●). Activity of phosphorylase b was plotted as per cent original activity as a function of incubation time at 50°C.

Table I

K_m's of the enzyme preparations for G-1-P

Experiment	Enzyme	K_{m_n}[1] (mM)	K_{m_m}[2] (mM)
1.	Native	6.1[3]	
	A.E.		7.0
2.	Native	8.1	
	P.E.		9.0
	V.E.		15.1
3.	Native	7.0	
	HX.E.		9.0
4.	Native	7.9	
	HP.E.		6.7
5.	Native	8.6	
	B.E.		13.5

[1,2] K_{m_n} and K_{m_m} are the Michaelis-Menten constants for the native and modified enzymes respectively.

[3] The variation in the K_m's is not thought to be significant.

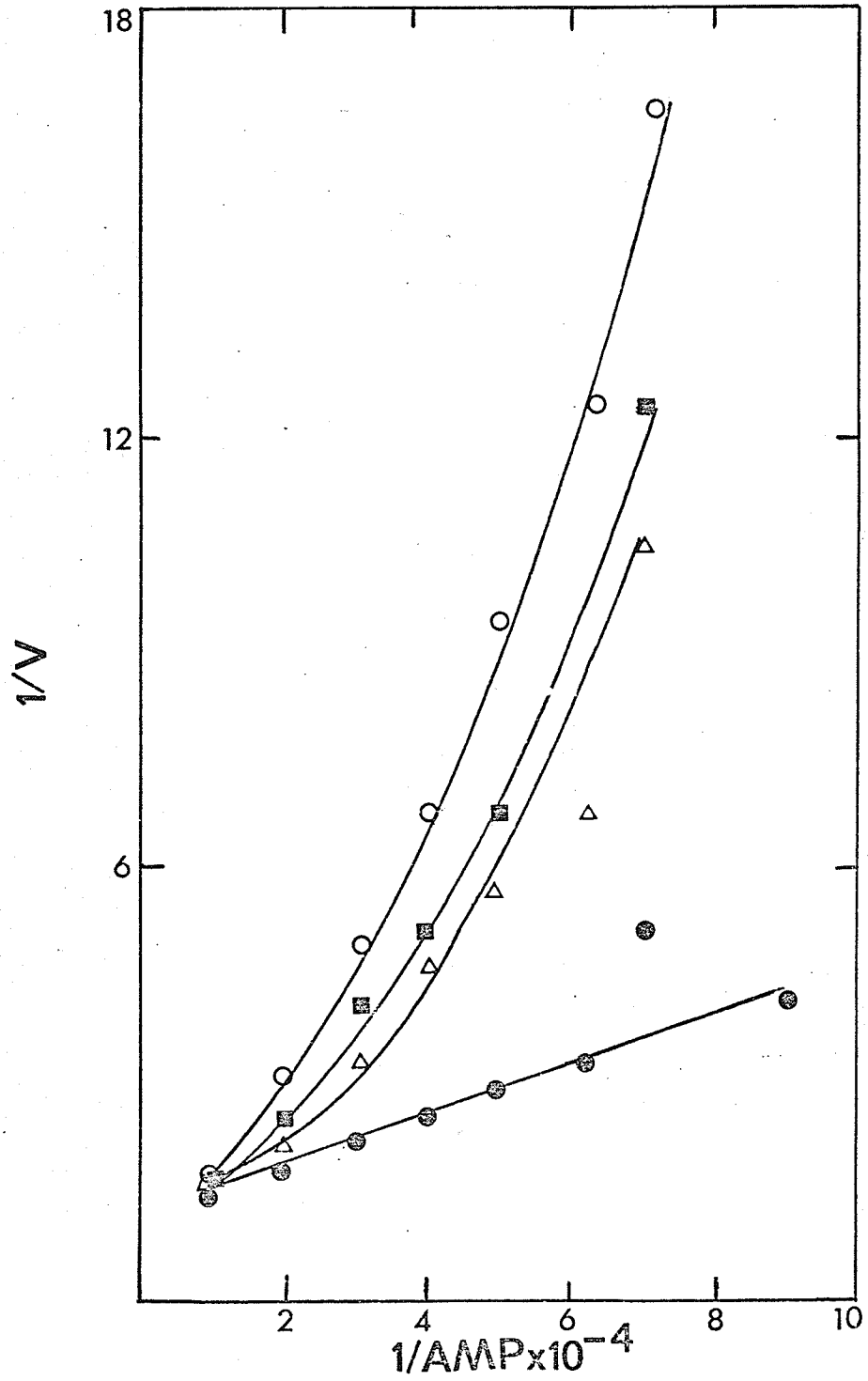


Fig. 12

Reciprocal plot for phosphorylase b (△), B.E. (○), V.E. (■), and HX.E. (●) with respect to AMP. Assay mixture contained 1% glycogen-.016M G-1-P with varying concentrations of AMP.

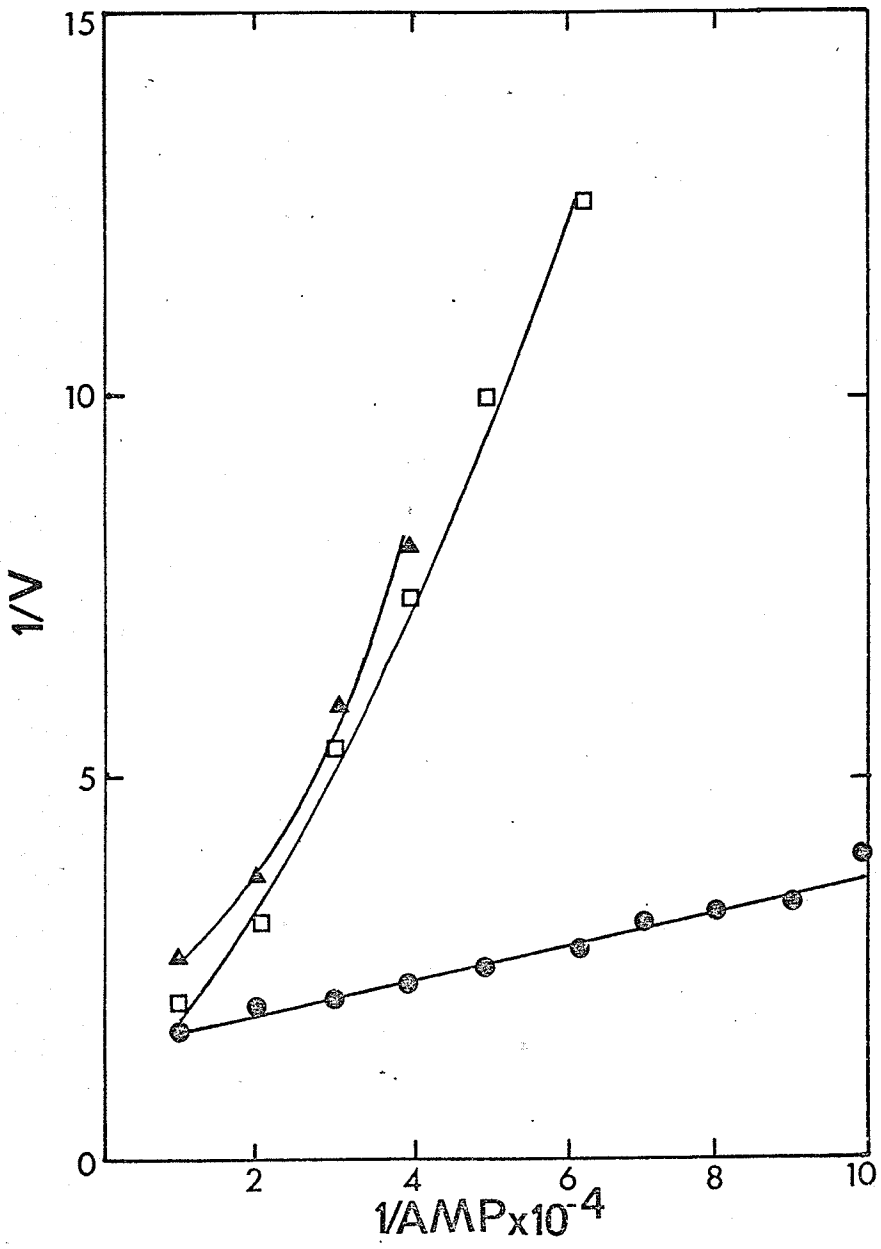


Fig. 13

Reciprocal plot for native phosphorylase b (□), A.E. (▲), and HP.E. (●) with respect to AMP. Assay mixture contained 1% glycogen-.016M G-1-P with varying concentrations of AMP.

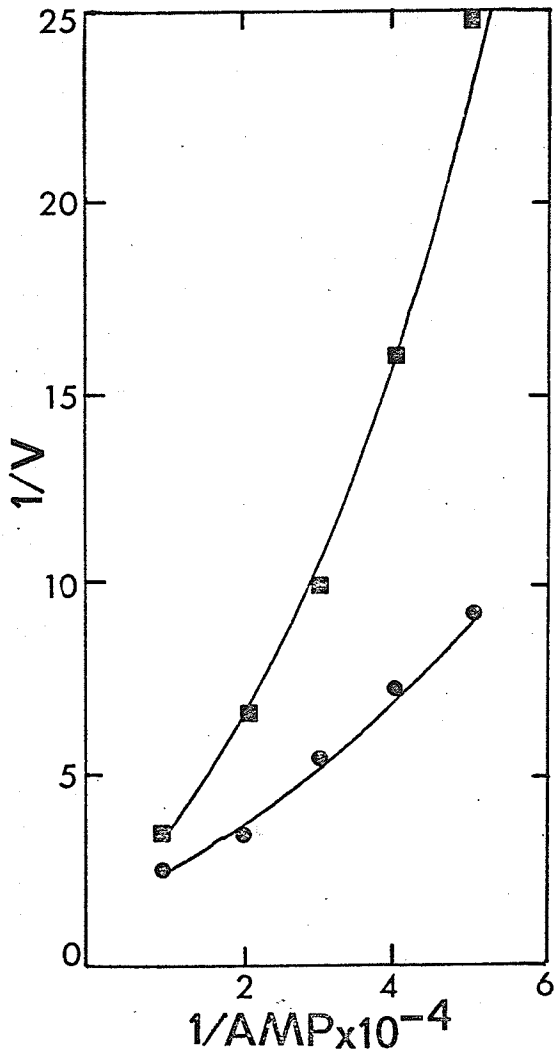


Fig. 14

Reciprocal plot for native phosphorylase b (●) and P.E. (■) with respect to AMP. Assay mixture contained 1% glycogen-.016M G-1-P with varying concentrations of AMP.

enzyme preparations were calculated (See Table II). HX.E. and HP.E. appear to have the lowest constants and therefore the greatest affinity of AMP for the enzyme. The other constants were similar or slightly higher than those of the native enzyme.

Table III indicates the effect of aldehydes with respect to inhibition of phosphorylase b activity. The aldehydes resulted in little or no inhibition of enzyme activity.

Compounds similar in chain length to propionaldehyde such as methylglyoxal and glyceraldehyde were employed in chemical modification studies. All three compounds possess the reactive aldehyde portion but methylglyoxal and glyceraldehyde differ in their hydrophobic contents with respect to propionaldehyde i.e. they possess less hydrophobicity than propionaldehyde. Phosphorylase b was modified with methylglyoxal and glyceraldehyde. The heat stability of the MeG.E. and GA.E. were compared with the native enzyme, P.E. and HX.E. (Fig. 15). Impure preparations were used. Methylglyoxal and glyceraldehyde did not protect the enzyme from heat denaturation. MeG.E. and GA.E. possessed the same heat inactivation profiles as the native enzyme. P.E. on the other hand possessed considerably more stability. Cold inactivation studies produced similar results.

The activity of MeG.E. and GA.E. expressed as % of the native enzyme was 96 and 89.5 of the activity of the native form.

The enzyme preparations studied in Fig. 15 were concentrated with $(NH_4)_2SO_4$ and eventually modified with .5% butyraldehyde according to the method outlined in the Experimental Procedure section. Kinetics of heat inactivation were studied (Fig. 16). The HX.E. possesses approximately the same activity before and after butyraldehyde treatment. The native

Table II

K_a's of the enzyme preparations for AMP

Experiment	Enzyme	K_a (mM)
1.	Native	.05[1]
	A.E.	.066
	HP.E.	.014
2.	Native	.046
	B.E.	.066
	V.E.	.052
	HX.E.	.029
3.	Native	.047
	P.E.	.076

[1] K_a's for the native enzyme, A.E., P.E., B.E., and V.E. are apparent K_a's.

Table III

The effect of reductive alkylation on phosphorylase b activity

Reagent[2]	Enzyme activity (%)[1]
None	100
Acetaldehyde	100
Propionaldehyde	100
Butyraldehyde	95
Valeraldehyde	100
Hexanal	94
Heptanal	100

[1]Enzyme activity is expressed as a % of native enzyme activity

[2]All reagent concentrations used were .5%.

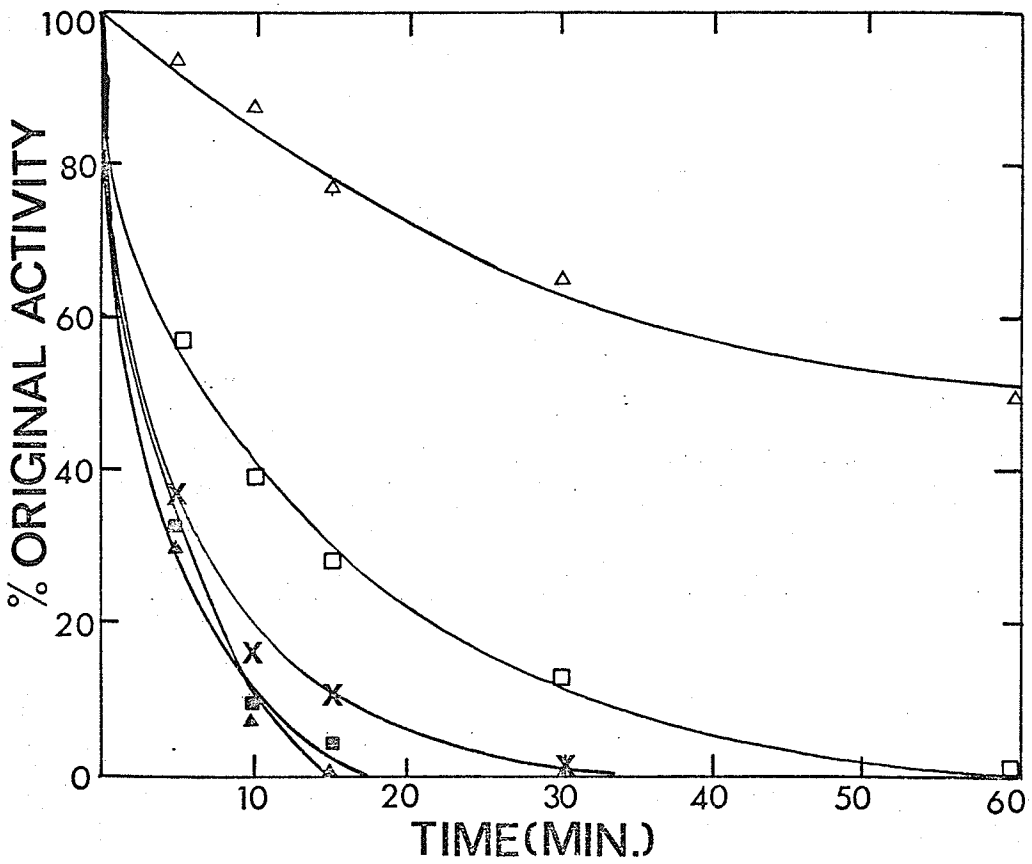


Fig. 15

Comparison of the thermal inactivation of phosphorylase b modified with hydrophilic and hydrophobic compounds. Activity of the native enzyme (▲), MeG.E. (▣), GA.E. (X), P.E. (□), and HX.E. (△) were plotted as per cent original activity as a function of incubation time at 50°C.

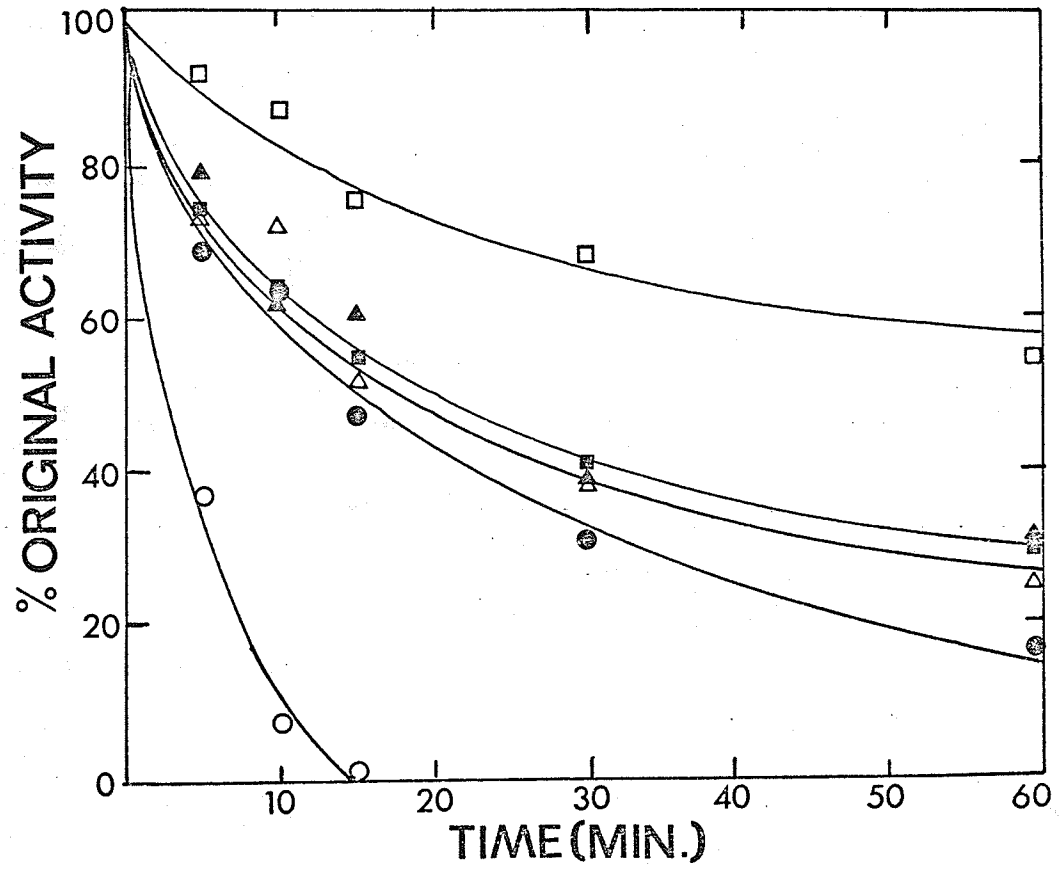


Fig. 16

Thermal inactivation profiles of enzyme preparations in Fig. 15 modified with .5% butyraldehyde. Activity of native phosphorylase b (○), and the butyraldehyde modified native enzyme (△), MeG.E. (◒), GA.E. (◙), P.E. (▲), and HX.E. (□) was plotted as per cent original activity as a function of incubation time at 50°C.

enzyme, P.E., MeG.E. and GA.E. are characterized by increased activity. P.E. and GA.E. possess similar profiles. Cold inactivation experiments produced identical results.

4. Quantitation of the Modification

Attempts were made to quantitate the reaction using three different methods. TNBS, ninhydrin and amino acid analysis of modified and native phosphorylase b failed to yield any evidence for modification. In the TNBS and amino acid analysis methods, the modified to native enzyme ratios were almost identical. The ninhydrin method failed to produce any conclusive results.

5. Ultracentrifuge Experiments

.1% and .5% B.E. sedimented in the ultracentrifuge as molecular species with a $S_{20,w}$ value of 8.58. This value for the purified species agreed with the value of 8.2S calculated for the impure .1% and .5% B.E. 8.2S is also the Svedberg value for native phosphorylase b.

6. Crystallization Experiments

All modified enzyme preparations were crystallized. Examination of the crystals under a light microscope indicated that the native and modified enzymes contained long, thin crystalline forms.

7. Phosphorylase b Kinase Experiments

Experiments in this laboratory conducted by J. Wang indicated

that B.E. could be converted to the phosphorylase a form by the action of rabbit muscle phosphorylase b kinase.

8. Ultraviolet Absorption Spectra

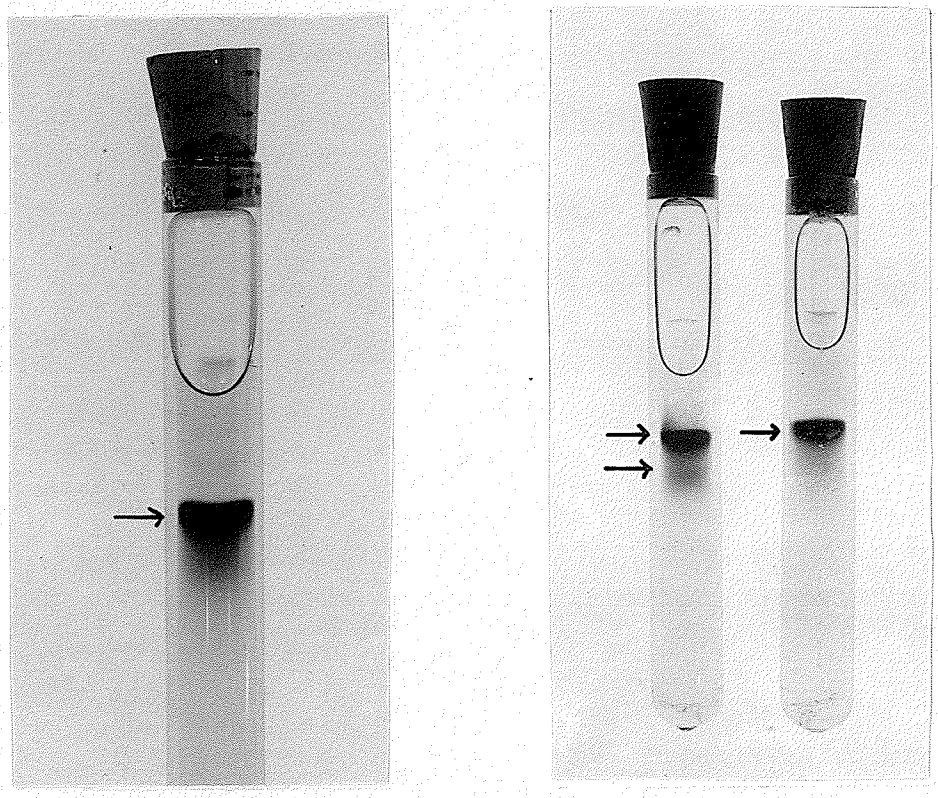
Native and aldehyde-modified phosphorylase b were observed with respect to their U.V. absorption properties between 200-400mμ. All enzyme species were characterized with maximum absorption peaks at 230 and 280mμ and a minimum absorption peak at 250 mμ.

9. Gel Electrophoresis Runs

Disc gel electrophoresis on polyacrylamide gel showed that certain modified phosphorylase b preparations were heterogeneous before purification and homogeneous after purification. This can be seen in Fig. 17. Both A and C show that native and purified A.E. appear homogeneous displaying 1 band. The A.E. which had not been heated appeared heterogeneous consisting of 1 major and 1 minor band. P.E. and V.E. although not shown displayed similar results. In Fig. 18 B.E. possesses 1 major and 1 minor band before and after heating. HP.E. produced the same results. HX.E. before heating could be separated into 1 major and 1 minor band. Only one band, indicating homogeneity, is seen after purification.

Fig. 17

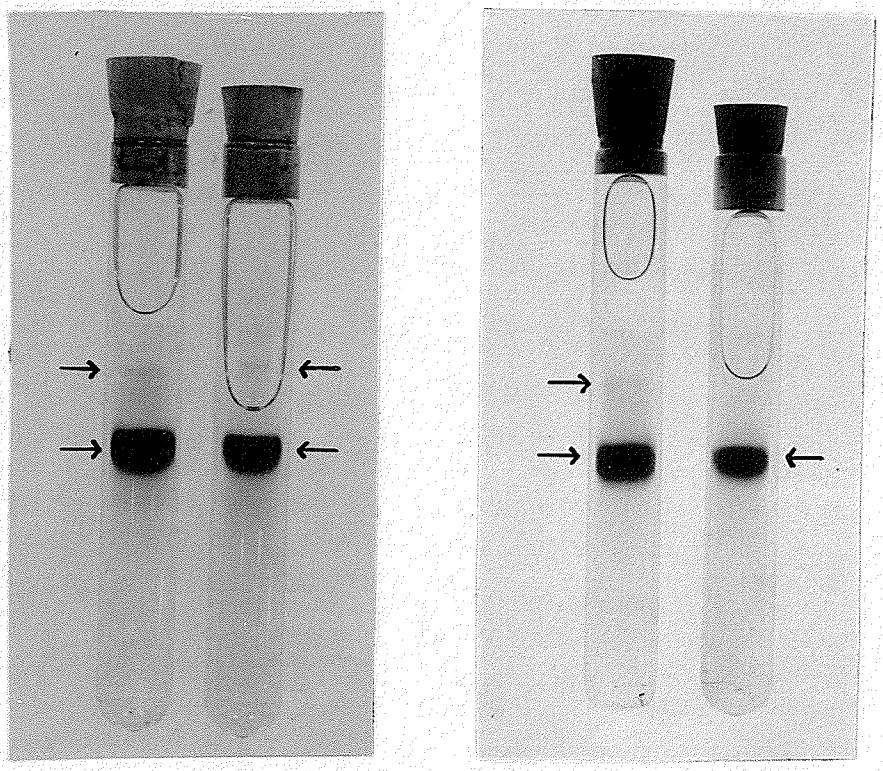
Polyacrylamide gel electrophoresis of native phosphorylase b and A.E. (A) native phosphorylase b, (B) A.E. prior to heating for 1 hour at 50°C, (C) A.E. after heating.



A B C

Fig. 18

Polyacrylamide gel electrophoresis of B.E. and HX.E. (A) B.E. prior to heating at 50°C. for 1 hour (B) B.E. after heating (C) HX.E. prior to heating at 50°C. for 1 hour (D) HX.E. after heating.



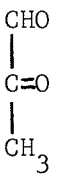
A B C D

SECTION V. DISCUSSION

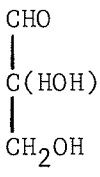
V. DISCUSSION

Our results indicate that 0.5% monofunctional aldehydes are capable of stabilizing phosphorylase b to hot and cold extremes. Evidence by Means and Feeney (1) indicates that aldehyde modifications are characterized by the covalent binding of the carbonyl portion of the aldehyde to the ε-amino group of lysine residues. The protection of the enzyme against hot and cold extremes is due in part to the covalent binding of the aldehyde with a Schiff base intermediate. This is apparent because of the importance of $NaBH_4$ in the expression of enzyme stability.

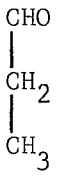
However our experiments indicated the possible importance of hydrophobicity in stabilization as well as covalent attachment. Methylglyoxal and glyceraldehyde possess 3 carbon units including a carbonyl group as does propionaldehyde, but the two compounds differ from propionaldehyde in that the hydrophobic content of propionaldehyde is greater. The GA.E. and MeG.E. possessed no enhanced stability with respect to hot or cold extremes unlike P.E.



Methylglyoxal



Glyceraldehyde



Propionaldehyde

Assuming that a covalent attachment of methylglyoxal and glyceraldehyde occurred, it would seem that the hydrophobic portions of aldehydes are important in stabilization by fixing the conformation of phosphorylase b. Many workers (56) have shown that hydrophobic interactions contribute to the rigidity of protein structure. Brandt (78) noted that the

hydrophobic contribution was found to stabilize native proteins at all temperatures but considerably more so at high than low temperatures.

The significance of hydrophobicity has already been established therefore it is not surprising that the carbon chain length is also a factor in stabilization. We observed this in heat and cold inactivation experiments noting that the optimum chain length of aldehydes yielding the greatest stability ranged from 4-7. A.E. and P.E. possessed less stability. The modified-enzyme preparations served as model systems illustrating the role of hydrophobic groups in maintaining protein conformation. Aldehydes with carbon contents greater than 7 were not studied because of solubility problems incurred.

One of the most important features of reductive alkylation is its unique ability under mild conditions to modify amino groups while retaining the same three dimensional structure (1). Ultraviolet absorption studies indicated that no significant conformational changes had occurred to phosphorylase b subsequent to modification. The absorbance due to chromophoric amino acids like tryptophan and tyrosine did not change after modification. Our ultracentrifuge experiments also supported the absence of structural changes because of the similarity in $S_{20,w}$ values before and after modification. In addition all modified enzyme preparations possessed the same crystalline structure as the native form.

The results of Fig. 16 indicated that the propionaldehyde, methylglyoxal and glyceraldehyde binding sites on phosphorylase b were different from the butyraldehyde binding sites on the enzyme. This is evident when the stability of MeG.E., GA.E. and P.E. increased after butyraldehyde modification. Evidently the butyraldehyde binds to different lysine residues which are not masked by the aldehyde from the initial

modification. This is not the situation with respect to the aldehyde binding sites on HX.E. Butyraldehyde modification of HX.E. did not change the heat and cold stability profiles. Thus the butyraldehyde and hexanal modifications are probably similar with respect to the lysine groups modified.

AMP kinetic studies with HX.E. and HP.E. indicated that these two enzyme derivatives had been "desensitized" in the sense that homotropic cooperation between AMP molecules was abolished even though the affinity of AMP for these derivatives was greater than with the native enzyme. Wang and Tu (2) observed similar results with glutaraldehyde-modified phosphorylase b except that the K_a's for the enzyme were slightly higher than the activation constant for the native form. Desensitization of the enzymes can be explained by a disruption or alteration of intermolecular forces. A.E., P.E., B.E., and V.E. were characterized by homotropic cooperation between AMP molecules while the affinity of the enzyme for AMP was similar and in some cases slightly lower than for the native enzyme. Thus no alteration in inter-subunit interactions appears to have occurred. However the stability of all the enzyme derivatives may be due to inter- and/or intra-subunit interactions.

The B.E. with its AMP cooperativity showed that the length of the bifunctional reagent was not the only factor in abolishing homotropic cooperativity in glutaraldehyde-modified enzyme preparations. Our results with HX.E. and HP.E. indicated that the bifunctional nature of the reagent was not an absolute requirement for the disruption of intermolecular forces in the enzyme. However the bifunctional nature and not the length of the reagent was critical in the specific case of the glutaraldehyde-modified phosphorylase b.

Wang and Tu (2) found the degree of modification associated with

1% B.E. by the TNBS method to be 10% i.e. 10% of the lysine residues were modified. It is quite likely that .5% B.E., as well as the other enzyme derivatives studied, possessed less than a 10% modification. This would explain why the three methods of quantitation employed failed to yield any positive results because of their lack of sensitivity to modifications that were less than 10%. On the basis of 86 moles of lysine/dimer of phosphorylase b (79) less than 8.6 lysine residues can be expected to be modified. These modified residues were not essential for catalysis.

The ability of rabbit muscle phosphorylase kinase to convert the B.E. to phosphorylase a indicated that the modification did not mask by steric hindrance the specific serine residues, which are phosphorylated in the kinase reaction.

During the course of the project some modified enzyme preparations were encountered which possessed little more stability than the native enzyme. A variability would appear to exist between modified enzyme preparations because when other enzyme preparations were similarly modified the stability of the enzyme derivatives were greater than that of the native enzyme forms. However A.E. and HP.E. subjected to cold inactivation possessed little more stability than the native enzyme on all occasions studied. Heat stability profiles of these enzyme derivatives conducted earlier in the year showed marked differences in stability compared to the unmodified enzyme. One would expect a similar relationship with cold inactivation studies. It is possible that a variation in phosphorylase b preparations was a factor due to the age of the muscle tissue.

With respect to the log. activity versus time plots, linearity indicat homogeneity. However it may be argued that a homogeneous modified enzyme could give nonlinear log. plots of thermal inactivation if protein collapse occurs as a multistage process with different rate constants. However in Fig.'s 4 and 8, A.E. and P.E. display nonlinearity before purification and linearity after.

VI. BIBLIOGRAPHY

VI BIBLIOGRAPHY

1. G. Means and R. Feeney, Biochemistry, 7, 2192 (1968).

2. J.H. Wang and Jan-I Tu, Biochemistry, 8, 4403 (1969).

3. D. Graves, R. Sealock, and J.H. Wang, Biochemistry, 4, 290 (1965).

4. D. Brown and C.F. Cori, in The Enzymes, P. Boyer, H. Lardy, and K. Myrback (eds.), Vol. 5, Academic Press, New York (1960), p. 207.

5. E.G. Krebs and E.H. Fischer, Adv. Enzymol., 24, 263 (1962).

6. E.H. Fischer and E.G. Krebs, Federation Proc., 25, 1511 (1966).

7. E.H. Fischer, A. Pocker, and J. Saari, Essays in Biochem., P.N. Campbell and G.D. Grenville (eds), Vol. 6, Academic Press (1970) (in press).

8. G.T. Cori, S.P. Colowick, and C.F. Cori, J. Biol. Chem., 123, 381 (1938).

9. G.T. Cori and C.F. Cori, Proc. Soc. Exp. Biol. Med., 36, 119 (1937).

10. G.T. Cori and S.P. Colowick, J. Biol. Chem., 121, 465 (1937).

11. C.F. Cori and G. Schmidt, Science, 89, 464 (1939).

12. G.T. Cori and A.A. Green, J. Biol. Chem., 151, 31 (1943).

13. A.A. Green and G.T. Cori, J. Biol. Chem., 151, 21 (1943).

14. C.F. Cori and G.T. Cori, J. Biol. Chem., 158, 341 (1945).

15. E.H. Fischer and E.G. Krebs, J. Biol. Chem., 231, 65 (1958).

16. E.H. Fischer and E.G. Krebs, J. Biol. Chem., 216, 121 (1955).

17. E.G. Krebs, D.J. Graves, and E.H. Fischer, J. Biol. Chem., 234, 2867 (1959).

18. S.S. Hurd, Ph.D. Thesis, University of Washington (1967).

19. D.J. Graves, E.H. Fischer, and E.G. Krebs, J. Biol. Chem., 235, 805 (1960).

20. M.H. Buc and H. Buc, in Regulation of Enzyme Activity and Allosteric Interactions, Fed. Eur. Biochem. Soc. Proc., Fourth Meeting, Oslo, Academic Press, New York (1968), p. 109.

21. V. Seery, E.H. Fischer, and D. Teller, Biochemistry, 6, 3315 (1967).

22. A. Ullmann, M. Goldberg, D. Perrin, and J. Monod, Biochemistry, 7, 261 (1968).

23. N.B. Madsen, J. Biol. Chem., 223, 1067 (1956).

24. N.B. Madsen and C.F. Cori, J. Biol. Chem., 223, 1055 (1956).

25. V. Seery, E.H. Fischer and D. Teller, Biochemistry, 9, 3591 (1970).

26. A. Parmeggiani and H. Morgan, Biochem. Biophys. Res. Commun., 9, 252 (1962).

27. C. Zarkadas, C. Smillie and N.B. Madsen, J. Mol. Biol., 38, 245 (1968).

28. M. Battell, L. Smillie, and N.B. Madsen, Can. J. Biochem., 46, 609 (1968).

29. N.B. Madsen and C.F. Cori, J. Biol. Chem., 224, 899 (1957).

30. C.F. Cori and B. Illingworth, Proc. Natl. Acad. Sci. U.S.A., 43, 547 (1957).

31. T. Baranowski, B. Illingworth, D.H. Brown, and C.F. Cori, Biochim. Biophys. Acta, 25, 16 (1957).

32. E.H. Fischer, A.B. Kent, E. Snyder and E.G. Krebs, J. Amer. Chem. Soc., 80, 2906 (1958).

33. C. Nolan, W.B. Novoa, E.G. Krebs and E.H. Fischer, Biochemistry, 3, 542 (1964).

34. E.G. Krebs, A.B. Kent, and E.H. Fischer, J. Biol. Chem., 231, 73 (1958).

35. C.F. Cori, G.T. Cori, and A.A. Green, J. Biol. Chem., 151, 39 (1943).

36. G.T. Cori and C.F. Cori, J. Biol. Chem., 135, 733 (1940).

37. G.T. Cori and C.F. Cori, J. Biol. Chem., 151, 57 (1943).

38. R.R. Traut, Ph.D. Thesis, The Rockefeller Institute (1962).

39. G.T. Cori, J. Biol. Chem., 158, 333 (1945).

40. T. Okazaki, A. Nakazawa, and O. Hayasihi, J. Biol. Chem., 243, 5266 (1968).

41. W.J. Black, Ph.D. Thesis, University of Manitoba (1969).

42. J. Monod, J. Wyman, and J.-P. Changeux, J. Mol. Biol., 12, 88 (1965).

43. E. Helmreich, M. Michaelides, and C.F. Cori, Biochemistry, 6, 3695 (1967).

44. H. Engers and N.B. Madsen, Biochem. Biophys. Res. Commun., 33, 49 (1968).

45. W.J. Black and J.H. Wang, J. Biol. Chem., 243, 5892 (1968).

46. D. Koshland, G. Nemethy, and D. Filmore, Biochemistry, 5, 365 (1966).

47. R.W. Sealock and D.J. Graves, Biochemistry, 6, 201 (1967).

48. N.B. Madsen and S. Schechosky, J. Biol. Chem., 242, 3301 (1967).

49. E. Helmreich and C.F. Cori, Proc. Natl. Acad. Sci. U.S.A., 51, 131 (1964).

50. N.B. Madsen, Biochem. Biophys. Res. Commun., 15, 390 (1964).

51. M.M. Appelman, Ph.D. Thesis, University of Washington (1962).

52. A.B. Kent, E.G. Krebs, and E.H. Fischer, J. Biol. Chem., 232, 549 (1958).

53. J.H. Wang, Jan-I Tu and F.M. Lo, J. Biol. Chem., 245, 3115 (1970).

54. E. Duggan and F. Luck, J. Biol. Chem., 172, 205 (1948).

55. G. Markus, R. Lane, and F. Wissler, J. Biol. Chem., 239, 3687 (1964).

56. J. Brandt, in Thermobiology, A. Rose (ed.), Academic Press, London and New York (1967).

57. G. Ballou, P. Boyer, F. Luck, and F. Lum, J. Biol. Chem., 153, 589 (1944).

58. A. Ray, J. Reynolds, H. Polet, and J. Steinhardt, Biochemistry, 5, 2606 (1966).

59. C. Epstein, C. Anfinsen, and M. Sela, J. Biol. Chem., 237, 3458 (1962).

60. L. Huang and N.B. Madsen, Biochemistry, 5, 116 (1966).

61. G. Philips and D.J. Graves, Biochemistry, 7, 2093 (1968).

62. S. Damjanovich and K. Kleppe, Biochim. Biophys. Acta, 122, 145 (1966).

63. J.H. Wang and Jan-I Tu, J. Biol. Chem., 245, 176 (1970).

64. A. Rosenberg and R. Lumry, Biochemistry, 3, 1055 (1964).

65. M. Scrutton and M. Utter, Federation Proc., 23, 162 (1964).

66. J.H. Wang and D.J. Graves, J. Biol. Chem., 238, 2386 (1963).

67. W. Kauzmann, Adv. in Pr. Chem., 14, 1 (1959).

68. G. Nemethy and H. Scheraga, J. Phys. Chem., 66, 1773 (1962).

69. J. Kendrew, Brookhaven Symp. Biol. 15, 216 (1962).

70. E. Sutherland and W. Wasilait, J. Biol. Chem., 218, 459 (1956).

71. E.H. Fischer and E.G. Krebs, in Methods in Enzymology, S.P. Colowick and N.O. Kaplan (eds.), Vol. 5, Academic Press, New York (1962), p. 369.

72. E.H. Fischer and E.G. Krebs, J. Biol. Chem., 231, 65 (1958).

73. E.G. Krebs and E.H. Fischer, in Methods in Enzymology, S.P. Colowick and N.O. Kaplan (eds.), Vol. 5, Academic Press, New York (1962), p. 373.

74. B. Illingworth and G.T. Cori, Biochem. Prep., 3, 1 (1953).

75. A. Habeeb, Anal. Biochem., 14, 328 (1966).

76. H. Fraenkel-Conrat, in Methods in Enzymology, S.P. Colowick and N.O. Kaplan (eds.), Vol. 4, Academic Press, New York (1957), p. 252.

77. L. Ornstein, Ann. N.Y. Acad. Sci., 121, 321 (1964).

78. J. Brandt, J. Amer. Chem. Soc., 86, 4302 (1964).

79. C. Sevilla and E.H. Fischer, Biochemistry, 8, 2161 (1969).