A STUDY OF PHOSPHOFRUCTOKINASE FROM *ASPERGILLUS NIGER*

BY

CHEE LING TERESA CHUNG

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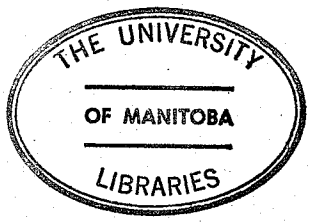

TO MY PARENTS

A B S T R A C T

ABSTRACT

Phosphofructokinase (PFK) (EC 2.7.1.11) from Aspergillus niger NRC A-1-233 (ATCC 26550) was isolated and purified.

Crude extracts from cultures grown in the presence of trace metals (Zn^{++}, Fe^{+++}, Cu^{++}) had a higher PFK activity than those from cultures grown in their absence. The 10% - sucrose cultures gave a PFK of higher activity but less stability than the 0.8% - sucrose cultures. The activity and stability of the enzyme also varied with the culture age of the cells. The possible role of PFK in the production of citrate still remained unsolved. Cultures grown in 0.8% sucrose and in the presence of trace metals at a culture age of 40 hours were used for all other experiments.

The PFK in the crude extract preparation was very unstable upon storage at $4^{o}C$. Mercaptoethanol and glycerol were protective while ATP, FDP, ADP, and NaF protected the enzyme to a lesser extent. Cyclic 3',5'-AMP had no effect and a passage through a Sepharose 6B column did not increase the stability.

Incubation of PFK at different temperatures revealed two distinct inactivation effects on the enzyme. One was thermal inactivation at 65°, which could be prevented by glycerol. The other was inactivation at 37° or 45°, which could be prevented by heat treatment at 56°, ATP, ADP, or NaF. The enzyme became much more stable during storage at 4° after heat treatment at 56° for 20 minutes.

The A. niger PFK was purified about 18 - fold after heat treatment at 58° and acetone fractionation. All other physical studies and kinetic studies were carried out with this purified enzyme preparation. Although ATP and F6P did not increase the stability of the concentrated purified enzyme, either one of them protected the enzyme from dilution effect. Activity was proportional to protein concentration. Highest activity was observed at pH 7.5 to 8.7 but with increasing initial lags with increasing pH's. K^+ stimulated PFK activity but inhibited it at higher concentrations. Na^+ inhibited PFK activity. The OH^-, Cl^-, and $SO_4^=$ anions were held responsible for the increasing lag response. Ammonium sulfate destabilized the enzyme which could be protected by the presence of ATP, FDP, or F6P.

Cyclic 3',5'-AMP did not affect the enzyme activity significantly, while 5'-AMP was found to be an allosteric activator of the enzyme. The F6P concentration affected the enzyme activity cooperatively. ATP was an allosteric inhibitor of the enzyme. A molecular model was proposed to explain the behaviours and properties of the enzyme.

True Michaelis constants were obtained for both substrates in the presence of 5'-AMP. Apparent K_m for Mg^{++} was determined with and without AMP. Kinetic results of substrate studies were consistent with Ping-Pong mechanism while those of product inhibition studies indicated a far more complex mechanism which cannot be easily comprehended with the present knowledge of the enzyme.

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ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
Cyclic 3',5'-AMP	Adenosine 3',5'-cyclic monophosphate
F6P	Fructose-6-phosphate
FDP	Fructose-1,6-diphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MES	2-(N-morpholino) ethanesulfonic acid
NADH	Nicotinamide-adenine dinucleotide, reduced
PFK	Phosphofructokinase
Tris	Tris (hydroxymethyl) aminomethane

I N T R O D U C T I O N

INTRODUCTION

Aspergillus niger has long been known as a citrate accumulator (Shu and Johnson, 1947). Composition of the liquid medium was reported to have great influence on citrate accumulation (Shu and Johnson, 1948; Wold, 1974). Growth media high in sucrose content and deficient in trace metals (Zn^{++}, Fe^{+++}, Cu^{++}) were described as favourable. Since sugar catabolism is greatly affected by its metabolism through the glycolytic pathway, this would mean that the metabolic rate through glycolysis is closely related to citrate accumulation (Wold, 1974).

Phosphofructokinase (PFK), as a key enzyme in glycolysis responsible for its regulation, was suspected to be involved, directly or indirectly, in the regulation of citrate production (Wold, 1974). It was this possibility that initiated the study of the A. niger PFK.

PFK is the enzyme which catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-diphosphate during the conversion of glucose to pyruvate. The enzyme from various sources is notorious for its re-

markable instability (Taylor, 1951; Mansour, 1965; Wakid and Mansour, 1965; Kono and Uyeda, 1973 a). An active-inactive enzyme interconversion system was described in the liver fluke enzyme (Mansour and Mansour, 1962), the mammalian enzyme (Mansour, 1965; Mansour and Ahlfors, 1968), and the yeast enzyme (Viñuela et al., 1964; Afting et al., 1971). Kinetic studies of the enzyme from these and other sources revealed an intricate and interesting nature of the enzyme. The enzyme was shown to be an allosteric enzyme and its properties were affected by the presence of some effectors or by the pH. Although the Ping-Pong mechanism was suggested in most cases, no definite conclusion could be drawn yet due to the different patterns of product inhibition.

It is the aim of this present study to isolate and purify a stable A. niger PFK with high activity and to examine some of its physical and kinetic behaviours. A molecular scheme for the enzyme was also put forth to explain certain peculiar phenomena. Kinetic results were discussed in terms of the model by Monod et al. (1965).

H I S T O R I C A L

HISTORICAL

Phosphofructokinase (PFK) was first discovered in yeast and mammalian muscle by Dische et al in 1935 (Mansour and Setlow, 1972). It was recognized as the enzyme catalyzing the phosphorylation of fructose-6-phosphate (F6P) to fructose-1,6-diphosphate (FDP):

$$F6P + ATP \xrightarrow{Mg^{++}} FDP + ADP$$

during the conversion of glucose to lactic acid, and was classified systematically as ATP : D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11.

The role of PFK as a rate-limiting enzyme in the regulation of glycolysis was first indicated by some early studies on muscle metabolism. In 1934, Hegnauer and Cori showed that epinephrine caused a marked increase in hexose monophosphate and a much smaller increase in lactic acid in frog muscle. In addition to the above evidence, C. F. Cori (1956) demonstrated that contraction of the frog gastrocnemius muscle resulted in an accumulation of hexose monophosphate and an increase in lactic acid production. He found that the sum of the hexose monophosphate and lactic acid formed during contraction was very close to the amount of glycogen

lost. This not only indicates that other intermediates of the glycolytic pathway do not accumulate to an appreciable extent, but also that "the reaction glycogen ———→ hexose monophosphate occurs more rapidly than the reaction hexose monophosphate ———→ lactic acid and points to phospho-fructokinase as the rate-limiting step for lactic acid formation during contraction" (Cori, 1956).

Besides being a rate-limiting enzyme, PFK was also suggested to be primarily responsible for the Pasteur effect in yeast, an effect in which the shift from anaerobic to aerobic conditions decreases the rate of evolution of CO_2 and of glucose utilization in glycolysis. In 1934, Hegnauer and Cori found that less lactic acid and hexose phosphate were formed under the influence of epinephrine in aerobic than in anaerobic frog muscle. In 1943, Engel'hardt and Sakov reported that PFK was sensitive to oxidative agents (Mansour and Setlow, 1972). Subsequent studies based on measurement of intracellular steady-state levels of glucose, glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and fructose-1,6-diphosphate (FDP) in yeast cells by Lynen et al. showed that anoxia, which causes an increase in the rate of glucose phosphorylation, also caused a decrease in the intracellular concentration of glucose and F6P and an increase in the intracellular level of FDP (Mansour, 1972b). This suggested an increase in the activity of PFK.

Because of its important role in the glycolytic pathway, the properties of PFK from a variety of sources have been widely investigated. The PFK from mammalian skeletal muscle, heart muscle, and yeast have been more extensively studied in various aspects than enzymes from other sources. PFK from other sources such as liver fluke (Stone and Mansour, 1967a), E. coli (Atkinson and Walton, 1965), chicken liver (Kono and Uyeda, 1973a), Ehrlich ascites tumor cells (Sumi and Ui, 1972a), human erythrocyte (Staal et al., 1972), and rat thymocyte (Yamada and Ohyama, 1972) have also been isolated, purified, and studied. The enzyme was also crystallized from skeletal muscle (Ling et al., 1966), heart muscle (Mansour et al., 1966), and chicken liver (Kono and Uyeda, 1973a).

The enzyme PFK was notorious for its remarkable instability under various conditions. The enzyme was found to be inactivated by dialysis (Taylor, 1951; Muntz, 1953), low pH (Taylor, 1951; Mansour, 1965), dilution (Kono and Uyeda, 1973a), separation from certain tissue components (Wakid and Mansour, 1965), and low temperature (Mansour, 1965; Stone and Mansour, 1967a, Kono and Uyeda, 1973b).

It was the work of Colowick on rat muscle PFK (Taylor, 1951) which first indicated that a stabilizing factor was removed by dialysis or destroyed by incubation

at pH 6 and that the enzyme could be protected by substances such as phosphate, pyrophosphate, and some other divalent anions. Then further evidence came from the work on the guinea pig heart PFK by Wakid and Mansour (1965). They found that certain tissue extracts were effective in enhancing its stability. They later identified the presence of hexose phosphates, inorganic phosphate, and nucleotides in these extracts. Further investigation on the stabilizing properties of these agents revealed that FDP was the most potent stabilizer of the enzyme while F6P, ATP, ADP, phosphate anion, sulfate anion, and mercaptoethanol, each displayed a significant stabilizing effect. Subsequent work on PFK from other sources by different workers also showed the essential requirement of either one or more than one of the above compounds for stabilizing the enzyme.

As more work was done on the purified PFK, more light was shed on the molecular forms of the enzyme, thus giving a clearer picture of how the hexose phosphates and nucleotides impose effect on the stability of the enzyme.

Early experiments on yeast PFK by Viñuela et al. (1964) demonstrated that there were two interconvertible forms of the enzyme, an active and an inactive form. They also reported the isolation of an "activating enzyme" which enhanced the PFK activity greatly. Viñuela et al. hence suggested the existence of an enzyme-catalyzed active-inactive

system similar to that known for glycogen phosphorylase.

Evidence from other sources also supported the active-inactive enzyme system. In 1962, the effect of cyclic 3′,5′-AMP on the liver fluke PFK was studied by Mansour and Mansour. The nucleotide was either added directly to the PFK assay mixture or incubated with the concentrated enzyme preparation. The enzyme was found to be activated in both cases. So they suggested that the PFK of the liver fluke might exist in active and inactive forms. Such a suggestion was later confirmed by sucrose gradient ultracentrifugal analysis of the enzyme before and after activation, indicating the involvement of an association-dissociation system (Stone and Mansour, 1967a). Nucleotides and hexose phosphates were found to be required in the activating system.

Meanwhile, studies on guinea pig heart and sheep heart PFK led Mansour (1965) and Mansour and Ahlfors (1968) to conclude that PFK from these mammalian heart muscles also underwent a reversible conversion from active to inactive form. Both groups reported that incubation of the enzyme at a mildly acidic pH resulted in a marked reduction of enzyme activity. The inactivated enzyme could be reactivated by incubation at pH 8, provided that the enzyme concentration was high enough. This reactivation was time, temperature, and enzyme concentration dependent. ATP, ADP, cyclic 3′,5′-AMP, F6P and FDP were found to enhance greatly

the reactivation of the enzyme at an alkaline pH. Of these agents, FDP was the most potent in reactivating the enzyme and a combination of a nucleotide and a hexose phosphate was much more effective than either one alone. Mansour and Ahlfors (1968) further provided the proof of the existence of a pH- and ligand-directed association-dissociation system by showing that the sedimentation coefficient of the inactive enzyme varied from 7S to 8S while that of the reactivated enzyme was 14.5S which was identical to that of the native enzyme. They also found that the presence of ATP favoured the dissociated form of the enzyme and that of FDP favoured the fully active polymerized form.

Based on information obtained from the skeletal and heart muscle PFK, Mansour and Setlow (1972) presented a molecular model for PFK to explain the inactivation and reactivation of the enzyme.

$$(EE)n \rightleftharpoons EE \rightleftharpoons 2E \longrightarrow 16e$$

They postulated that when enzyme concentration was high and FDP or F6P was present, the enzyme would mostly be in the aggregated form (EE)n. When ATP level was high, the equilibrium would be shifted to the EE form. At acidic pH, the enzyme was split into the inactive or subactive form (E). The enzyme could be further dissociated into smaller subunits (e) in the presence of 5 M guanidine HCl which were irreversibly inactive.

Besides the factors described above, temperature was found to have effect also on the inactivation and reactivation of the enzyme. In 1965, Mansour mentioned from his work on guinea pig heart PFK that the inactivated enzyme was reactivated at 25°, but not at 0°. Similar behaviour was found in the liver fluke enzyme (Stone and Mansour, 1967a). When the inactive form of the enzyme was incubated with cyclic 3',5'-AMP at 30°, the reactivation was much faster than those incubated at 0°.

The chicken liver PFK was also reported to be cold labile. Kono and Uyeda (1973b) discovered that the rates of the activation increased with temperature up to 43°, after which the enzyme activity decreased. The rate of this cold inactivation could be influenced by pH, protein concentration, glycerol and certain anions. Metabolites such as F6P, FDP, G6P and cyclic 3',5'-AMP (positive effectors) were protective against cold inactivation of the enzyme while ATP and citrate (negative effectors) increased the rate of inactivation. By further investigation into the molecular weights and the S values of the cold-inactivated and reactivated enzymes, Kono and Uyeda concluded that there also existed a dissociation-association system in the chicken liver PFK. They further proposed a structural scheme of the enzyme, of which the fully active form contains 4 subunits while the inactive form consists of completely dissociated subunits.

A thorough study of the molecular weights and sedimentation coefficients of different molecular forms of the skeletal muscle PFK was performed by Paetkau and Lardy (1967). It was shown that the enzyme ranged from 7S to 30S depending on the degree of polymerization and its molecular weights were predicted to be 1.92×10^5, 3.6×10^5, 7.7×10^5, and 1.6×10^6.

A similar association-dissociation system in yeast PFK was described by Liebe et al. (1970). They also demonstrated the presence of several interconvertible active polymeric forms of yeast PFK, of which their molecular weights were estimated to be 1×10^6, 7.5×10^5, 5.7×10^5, 3.7×10^5, and 1.8×10^5.

That PFK might possess a regulatory mechanism operated through one of its substrates, ATP, was first hinted by the work of Lardy and Parks (1956) who pointed out that ATP at high concentration could inhibit the rabbit muscle enzyme. As more research was done on this enzyme, its inhibition by ATP was confirmed in a number of systems from various sources such as yeast (Viñuela et al, 1963), liver fluke (Mansour and Mansour, 1962), sheep heart (Mansour and Ahlfors, 1968), guinea pig heart (Mansour, 1963), E. coli (Atkinson and Walton, 1965), rat thymocyte (Yamada and Ohyama, 1972), Ehrlich ascites tumor cells (Sumi and Uji, 1972b) and chicken liver (Kono and Uyeda, 1974).

In addition to the inhibitory effect of ATP, Mansour (1962) also found that in the liver fluke system the F6P substrate saturation curve was sigmoid. This sigmoidicity disappeared in the presence of cyclic 3',5'-AMP which lowered the apparent Km for F6P but did not change the V_{max} of the reaction.

Similar results of such allosteric control were subsequently discovered in other mammalian systems. Detailed studies on muscle PFK revealed that at pH 6.9, the enzyme displayed allosteric kinetics (Mansour and Setlow, 1972). The saturation curve for F6P displayed sigmoidicity which was increased at higher ATP concentrations. However, at a more alkaline pH (8.2), or at pH 6.9 in the presence of an activator, or after photo-oxidation or ethoxyformylation, the enzyme displayed the Michaelis-Menten kinetics and the ATP inhibition was relieved.

PFK from various mammalian sources has been reported to be influenced by a number of modifiers among which some have already been recognized to be required for the conversion of the enzyme from the inactive to the active form or for stabilizing the enzyme's activity. According to reports from various workers (Mansour and Setlow, 1972), AMP, ADP, cyclic 3',5'-AMP, Pi, NH_4^+, FDP, GDP, and F6P were generally described as the activators of the enzyme, while ATP, citrate, 3-phosphoglycerate, 2-phosphoglycerate, 2,3-diphosphoglycerate, phosphoenolpyruvate, and phosphocreatine were described as the inhibitors. NADH and NADPH

were also found to be potent inhibitors of the partially purified sheep liver enzyme (Brock, 1969).

PFK from different sources might not respond to each allosteric effector to the same extent. For example, recent studies by Kemp (1971) on the rabbit liver enzyme showed that, although the pH optimum, cation activation, and substrate affinity at pH 8.2 were similar to those of the rabbit muscle enzyme, the liver enzyme was more sensitive to ATP inhibition and less sensitive to deinhibition by AMP, ADP, and cyclic AMP. The rat erythrocyte PFK was reported to possess kinetic properties similar to that of muscle PFK as described above except that it was much less sensitive to citrate than the muscle enzyme (Mansour, 1972b). It was also shown that rat thymocyte PFK (Yamada and Ohyama, 1972) exhibited a greater sensitivity to allosteric effectors than the muscle enzyme.

With the exception of the PFK from the slime mold Dictyostelium discoideum (Baumann and Wright, 1968) which exhibited the Michaelis-Menten kinetics only, phosphofructokinases from other non-mammalian sources were reported to display similar allosteric kinetics as the mammalian enzymes with the effector ligands varying from one species to another. While cyclic 3',5'-AMP is an activator of the mammalian and liver fluke PFK, it does not affect the yeast and E. coli PFK at all. The enzymes from the latter organisms were

found to have very similar effector specificity (Ramaiah et al., 1964, Atkinson and Walton, 1965). Both AMP and ADP increased the apparent affinity of the enzymes for F6P while ATP served as a negative effector.

It was found that the yeast PFK, like the muscle enzyme, could be converted from the ATP-sensitive to the ATP-desensitized form in the presence of NaF, AMP, ATP, Mg^{++}, and F6P (Viñuela et al., 1964; Atzpodien et al., 1970). The so-called "activating protein" (Viñuela et al., 1964) or "desensitizing protein" (Atzpodien et al., 1970), which was earlier believed to be responsible for the event to take place, was now confirmed to have no direct enzymatic action on PFK at all (Afting et al., 1971). Rather the desensitization of PFK was due to the presence of ADP acquired from ATP hydrolysis by ATPase. Hence, ADP, F6P, NH_4^+, Mg^{++} and F^- were attributed as the essential components for desensitization of the yeast PFK to take place.

The fact that ATP is both a substrate and an inhibitor of the enzyme made it imperative to find out whether there is a separate allosteric site for ATP other than the substrate site. In the past several years, much evidence has appeared confirming the existence of a regulatory site for ATP. As early as 1964, Ramaiah et al. showed that with GTP, UTP, or ITP as phosphate donor, the yeast enzyme exhibited a normal Michaelis-Menten

kinetics for the F6P saturation curve. However, when GTP (or UTP or ITP) and ATP were both present, the allosteric kinetics appeared again. Atkinson and Walton (1965) also described that the presence of ITP alone inhibited the E. coli enzyme only weakly while the presence of both ATP and ITP together inhibited the enzyme as much as ATP alone. Thus this at least hinted that there might be another ATP site different from the catalytic site.

Another piece of supporting evidence also comes from the yeast enzyme. Salas et al. (1968) found that incubation of purified yeast PFK with trypsin would lead to desensitization to ATP inhibition. Later Liebe et al. (1970) confirmed this observation and further showed that treatment of the enzyme with trypsin at pH 6.5 did not affect the catalytic activity of the enzyme.

Further investigation of sheep heart PFK by photo-oxidation (Ahlfors and Mansour, 1969), a procedure resulting in the destruction of histidine residues, revealed that the enzyme became insensitive to ATP inhibition. The maximum catalytic activity was not much affected while the cooperativity of the F6P kinetics was abolished. Ethoxyformylation of the histidine residues of the enzyme also led to results similar to those of photo-oxidation (Setlow and Mansour, 1970). Hence, these findings not only indicate the presence of the allosteric site(s) distinct from the catalytic site,

but also give evidence that one or more histidine residues are responsible for the desensitization.

Although binding studies of various ligands to mammalian PFK have been examined by different workers (Kemp and Krebs, 1967; Lorenson and Mansour, 1969), still the entire picture of the kinetic properties of the enzyme has not yet been clarified.

The mechanism of the reaction of PFK has been studied under the conditions in which the allosteric kinetics is not observed. In 1966, kinetic evidence from the brain PFK reported by Lowry and Passonneau suggested random order of substrate binding to the catalytic site. However, detailed studies with skeletal muscle PFK by Uyeda (1970) revealed kinetic patterns consistent with the Ping-Pong mechanism in which release of one product occurs before addition of the second substrate (Cleland, 1963a). He examined the kinetic behaviour of the enzyme by using the following techniques: (a) initial velocity studies with ITP as substrate; (b) product inhibition studies; (c) studies of the ATP-ADP and FDP-F6P isotope exchange reactions; and (d) pulse labeling experiments with ^{32}P-ATP. The results after such investigations are consistent with the proposed mechanism as follows:

$$E + ATP \rightleftharpoons E..ATP \rightleftharpoons E\text{-}P..ADP \rightleftharpoons E\text{-}P + ADP$$

$$E\text{-}P + F6P \rightleftharpoons E\text{-}P..F6P \rightleftharpoons E..FDP \rightleftharpoons E + FDP$$

where E and E-P are enzyme and phosphorylated enzyme respectively.

Studies on the reverse reaction of sheep heart PFK (Lorenson and Mansour, 1968) and on the yeast enzyme (Viñuela et al., 1963; Sols and Salas, 1966) gave supporting evidence to the Ping-Pong mechanism. The enzyme from the slime mold, Dictyostelium discoideum, which showed no evidence of allosteric regulation, also presented the initial velocity pattern of Ping-Pong mechanism. The product inhibition pattern, however, was not consistent with the mechanism. ADP was a competitive inhibitor with respect to ATP and an uncompetitive inhibitor with F6P while FDP inhibited the reaction by competing with F6P but not with ATP (Baumann and Wright, 1968).

In 1971, from studies on the isotope-exchange reactions of ox heart PFK, Hulme and Tipton reported that a magnesium complex of ADP was required for phosphate exchange between F6P and FDP. Their results suggested a compulsory-order mechanism as below.

$$E + ATP \rightleftharpoons E..ATP \rightleftharpoons E^{p}_{ADP}$$

$$E^{p}_{ADP} + F6P \rightleftharpoons E^{p}_{F6P}..ADP \rightleftharpoons E^{p}_{F6P} + ADP \rightleftharpoons E..FDP \rightleftharpoons E + FDP$$

So far the proposed phosphorylated enzyme intermediates of the skeletal muscle enzyme and the ox heart enzyme have not been successfully isolated yet. The reaction mechanism of PFK still awaits to be confirmed.

How the enzyme PFK is regulated within the cell has been an issue widely questioned and speculated by different workers. Evidence based on measurements of substrate and product levels of PFK in the cell are pointing to the fact that the physiological control of PFK activity is exercised through the allosteric properties of the enzyme. It is assumed that the enzyme in the cell usually exists in the ATP-inhibited form (Mansour, 1963). When an energy demand in the cell arises, the enzyme would be activated through decrease in ATP level or increase in other modifiers' level or both together.

Several laboratories have demonstrated that during the activation of glycolysis in muscle there was a 15-30% decrease in ATP level and a more significant increase in AMP, ADP, and inorganic phosphate levels (Regen et al., 1964; Helmreich et al., 1965; Williamson, 1966). The significant role of AMP in activation of PFK has been further hinted by Krebs (1964) that there are much greater percentage changes in AMP level in the tissue than ATP or ADP level under different physiological conditions.

Another view of the role of the adenylates in the regulation of PFK has been proposed by Atkinson (1968) and Shen et al. (1968) who suggested that the ratios of AMP : ADP : ATP within the cell were controlled through an intricate mechanism of enzyme regulation which kept the ATP-forming enzymes in synchrony with the ATP-utilizing enzymes. There-

fore Atkinson formulated an "adenylate energy charge" concept by which the enzyme activity could be affected by very small changes in AMP and ATP level at different physiological conditions.

Inhibition of glycolysis is believed to be caused by citrate which is increased through fatty acid oxidation and acts as an inhibitor of PFK to block the consumption of carbohydrate reserve in the cell (Pogson and Randle, 1966; Garland et al., 1963; Williamson et al., 1964, Parmeggiani and Bowman, 1963).

It was suggested by some workers that the activity of mammalian PFK could also be regulated through hormonal control. Morgan et al. (1961) reported that treatment of the diabetic rat with insulin in vivo fully restored the phosphorylation effect, suggesting an indirect effect of insulin on PFK. Experiments on skeletal muscle extracts by Mansour (1972a) supported the idea that the activation of PFK could be mediated through the action of epinephrine which is known to increase the tissue levels of F6P, FDP, cyclic AMP and 5'AMP. Serotonin, an indolealkylamine which is recognized to stimulate both movement of the liver fluke and its metabolism of carbohydrate (Mansour, 1959), was found to be able to activate PFK in cell-free extracts (Stone and Mansour, 1967a).

Since PFK is the key enzyme of the glycolytic pathway and is responsible for its regulation, it is interesting to note how PFK acts in conjunction with other enzymes for the benefit of cellular metabolic control. In his recent review on PFK, Mansour (1972b) described that as the rate of glucose degradation increases, there is a simultaneous activation of phosphofructokinase, hexokinase, and pyruvic kinase. An increase in AMP level not only activates PFK, but also inhibits fructose-1,6-diphosphatase, thus favoring an increase in glycogen breakdown. Meanwhile, inhibition of PFK and activation of acetyl-CoA carboxylase by citrate cooperate together for energy production from fatty acids.

MATERIALS AND METHODS

MATERIALS AND METHODS

MAINTENANCE OF CULTURES

Aspergillus niger NRC A-1-233 (ATCC 26550), a citrate accumulating strain of the fungus Aspergillus, was used throughout this study. The organism was obtained from the National Research Council of Canada, Division of Biosciences, Ottawa, Ontario. Stock cultures were maintained on slants of Brain Heart Infusion Agar (Baltimore Biological Laboratory) at 4°C, and were subcultured approximately every one to five months. The agar slants, after being inoculated by loop with conidia which were spread evenly over the agar surface, were then incubated at 28°C for 3 to 4 days, with the caps of the tubes kept loose to allow conidiation.

Size of the screw-cap tubes was about 6 inches long and 3/4 inch in diameter.

GROWTH MEDIUM

The composition of the standard liquid medium was about the same (except the sucrose content) as the medium

M2 described by Choudhary and Pirt (1965).

In one litre solution, it contained sucrose, 8 g; NH_4NO_3, 2.5 g; KH_2PO_4, 2.5 g; $MgSO_4.7H_20$, 0.25 g; $FeCl_3$, 1.3 mg; $CuSO_4.5H_20$, 0.097 mg; $ZnSO_4.7H_20$, 0.444 mg; and glass distilled water. After adjusting the pH to 3.5 with HCl, the solution was autoclaved at 121°C (15 lb/ sq. in.) for 15 minutes.

A stock solution of the inorganic salts : NH_4NO_3, KH_2PO_4, and $MgSO_4.7H_20$, was usually prepared 10 times more concentrated than those in the normal medium. This was stored in the dark and diluted out appropriately when used. A fresh 100 times concentrated solution of the trace metals $FeCl_3$, $CuSO_4.5H_20$, and $ZnSO_4.7H_20$ was prepared each time when preparing the growth medium. An aliquot of this solution was then added to the growth medium to make up the correct quantity.

In case of a similar growth medium but with a high sucrose content, 100 g sucrose (per litre) was used instead (Feir and Suzuki, 1969).

INOCULATION AND INCUBATION

Five millilitres of a sterile 1% Tween 80 (Polyoxyethylene (20) Sorbitan Monooleate)-H_20 solution were added to each slant which was swirled round gently

to obtain a heavy conidia suspension. Into each 2-litre Erlenmeyer flask which contained 1 litre liquid medium, 10 ml of the conidia suspension (i.e. from 2 slants) were transferred aseptically for inoculation.

The inoculated flasks were than incubated at 28°C for 40 hours on a New Brunswick rotary shaker, model VS-100 (New Brunswick Scientific Company, N. J.), with speed-control dial set at 1.5. For some experiments, the incubation lasted for 48 hours or 72 hours.

PREPARATION OF CELL-FREE EXTRACTS

Upon harvesting, the cultures were collected on four layers of cheesecloth. After being pressed dry, the mycelia were resuspended in an equivalent volume of ice-cold Tris-HCl buffer (0.05 M, pH 7.2). After stirring, the mycelia were collected on four layers of cheesecloth, pressed dry, and stored frozen at -76°C.

To prepare cell-free extracts, the mycelia were thawed and extracted in the ratio of 1 g to 4 ml of cold 0.05 M potassium phosphate buffer (pH 7.5) which contained 20% glycerol and 3 mM mercaptoethanol. A Tris-HCl buffer (0.05 M, pH 7.5) was used in some experiments where only crude extracts were required, and substances contained in this buffer will be specified in the results

where necessary. The cells were disrupted with a Bellco tissue homogenizer (Bellco Biological Glassware, N. J.), the pestle of which had been attached to a TRI-R STIR-R instrument, model K43 (TRI-R Instruments, Inc., N. Y.), with the speed-control dial set at about 1.5. In some earlier experiments on crude extracts, a Sorvall Omni-mixer (Ivan Sorvall Inc., Connecticut) was used to break down the cells at maximum speed setting. In both cases, the homogenizing vessels were immersed in ice-water, and the time of disruption was approximately 10 minutes. The thick suspension was then centrifuged in a Sorvall RC2-B centrifuge (Ivan Sorvall Inc., Connecticut) at 48,200 x g for 30 minutes at 0-4^{o}C. The opaque supernatant was kept at about 4^{o}C for further study and purification.

ENZYME ASSAYS

Phosphofructokinase (PFK) activity was determined spectrophotometrically by coupling with aldolase, triose-phosphate isomerase, and α-glycerophosphate dehydrogenase to convert the fructose -1, 6-diphosphate (FDP) formed to α-glycerol-phosphate.

$$F6P + ATP \longrightarrow FDP + ADP$$

$$FDP \longrightarrow \text{glyceraldehyde -3-P} + \text{dihydroxyacetone -P}$$

glyceraldehyde -3-P ⟶ dihydroxyacetone -P

2 dihydroxyacetone -P + 2 NADH ⟶ 2 L-glycerol-3-P + 2 NAD

F6P + ATP + 2 NADH ⟶ ADP + 2 L-glycerol-3-P + 2 NAD.

Rate of the reaction was measured by the rate of decrease in absorbance at 340 nm due to the oxidation of NADH, with the Beckman's ACTA III spectrophotometer or the Gilford Model 2400 spectrophotometer.

In a 3 ml-cuvette with a 1-cm light path, the standard reaction mixture contained: 0.05 M Tris-HCl (pH 7.5); fructose-6-phosphate, 4 mM; ATP, 0.4 mM; $MgCl_2$, 4 mM; NADH, 0.12 mM; 0.2 ml of the coupling enzyme solution containing excess aldolase, triosephosphate isomerase (TPI), and α-glycerophosphate dehydrogenase (αGDH); and a necessary amount of the enzyme PFK, leading to a final volume of 3 ml. In most assays, unless otherwise specified, 50 µl of the crude extract (200-300 µg protein, 0.05 unit activity) or 10-20 µl of the purified PFK preparation (10-20 µg protein, 0.05 unit activity) were used. In most of the kinetic studies, 5'AMP was also added to the reaction mixture to a concentration of 1 mM.

The coupling enzyme solution was made up as follows. Into a cold Tris-HCl buffer (0.05 M, pH 7.5) of 4.5 ml or 4.55 ml, 0.3 ml of aldolase (either from Sigma or Boehringer), 0.2 ml of α-GDH-TPI (from Sigma) or 0.075 ml each of TPI and α-GDH (both from Boehringer) were added

to make a 5 ml solution.

In most assays, the reaction was initiated by the addition of the enzyme PFK preparation. However, in some kinetic studies in the absence of 5'AMP, buffer, ATP, $MgCl_2$, NADH, coupling enzymes, and PFK preparation were first allowed to be mixed and incubated in the cuvette at room temperature for 1 minute, and F6P was then added to start the reaction.

All reactions were carried out at room temperatures. Activity was expressed as the absorbance change (ΔA_{340}) per minute determined either from the initial linear rate or from the fastest linear rate when a lag existed at the initial state of the reaction.

To determine the presence of NADH oxidase and ATPase, fructose-6-phosphate was omitted from the standard reaction mixture during assay.

For the product inhibition studies with fructose-1, 6-diphosphate, a different coupled-enzyme system was used. Excess pyruvate kinase (6 units) and lactic dehydrogenase (36 units), which were made up in cold Tris-HCl buffer (pH 7.5, 0.05 M), were added to the cuvette instead of the aldolase system. Phosphoenol pyruvate, 0.5 mM was included in the reaction mixture.

One unit of PFK catalyzes the conversion of one μmole of fructose-6-phosphate to fructose-1,6-diphosphate

per minute under standard conditions.

Specific activity was expressed as units of PFK per mg protein. Protein was determined by the method of Lowry et al. using crystalline bovine serum albumin as the standard.

SEPHAROSE 6B COLUMN

A column of Sepharose 6B, 1.8 cm in diameter and 28 cm in length, was prepared after washing several times in glass distilled water. The column top was then covered with a serum bottle cap through which a syringe needle, connected with a rubber tubing to a bottle of 0.05 M Tris-HCl (pH 7.5, containing glycerol, 20%; mercaptoethanol, 3 mM; FDP, 10^{-4}M; ADP, 10^{-4}M; and ATP, 5×10^{-4}M), pierced to touch the wall of the column tube. After the column was equilibrated with the above buffer, a sample of 3 ml crude extract was layered gently onto the top of the column with a syringe. The flow rate was about 10 drops per 15 seconds. Fractions (about 1 ml per tube) were collected when the protein front was almost at the bottom of the column. The whole procedure was performed at room temperature.

Protein levels were detected by measuring the absorbance at 280 nm with the Beckman - ACTA III spectrophotometer.

PURIFICATION OF PHOSPHOFRUCTOKINASE

The crude extract was heated in a 58°C water bath for 20 minutes. The precipitate formed was removed by centrifugation with a Sorvall RC2-B centrifuge at 27,000 x g for 15 minutes at 0-4°C. Acetone fractionation was then carried out with the clear yellow supernatant.

To the supernatant, precooled acetone (-20°C) was added, drop by drop with stirring, up to a concentration of 30% (v/v), while the temperature was slowly lowered to -6°C. The precipitate was removed by centrifugation at the same temperature at 27,000 x g for 15 minutes. More precooled acetone was again added to the resulting supernatant in a similar manner as before, up to a final concentration of 50% (v/v) with the temperature lowered to -8°C. Centrifugation was carried out at -8°C at 27,000 x g for 15 minutes. The supernatant fraction was discarded.

The precipitate was either stored at -20°C until used, or suspended immediately in cold potassium phosphate buffer (0.05 M, pH 7.5) containing 20% glycerol and 3 mM mercaptoethanol. The suspension was stirred gently to obtain homogeneity and centrifuged at 27,000 x g for 15 minutes at 0-4°C. Phosphofructokinase remained soluble in the supernatant. This enzyme preparation was kept at

0-4°C and used for all kinetic studies and some physical studies.

The purification procedure of the enzyme is outlined in Table VII.

SOURCES OF CHEMICALS

The following chemicals were obtained from:-

The Sigma Chemical Company-

D-Fructose-6-Phosphate, Disodium salt, Grade I.

D-Fructose-1,6-Diphosphate, Tetrasodium salt, Sigma Grade.

Adenosine 5'-Diphosphate (ADP), from Equine Muscle, Sodium salt, Grade I.

Adenosine 3':5'-Cyclic-Monophosphoric Acid, Crystalline.

Adenosine 5'-Monophosphoric Acid (AMP), from Yeast, Type II.

Aldolase, from Rabbit Muscle, Grade I, Specific activity 14 units/mg, 10 mg protein/ml.

α-Glycerophosphate Dehydrogenase-Triosephosphate Isomerase (α-GDH-TPI), from Rabbit Muscle, Type III, α-GDH Activity 130 units/mg, TPI activity 1080 units/mg, 10 mg protein/ml.

Lactic Dehydrogenase, from Rabbit Muscle, Type II, Specific activity 600 units/mg, 10 mg protein/ml.

The P-L Biochemicals, Inc. -

Adenosine 5'-Triphosphate, Disodium, Crystalline (ATP).

Nicotinamide-Adenine Dinucleotide, Reduced, Disodium (NADH), Coenzyme-1, Reduced (DPNH).

The Boehringer Mannheim-

Aldolase, from Rabbit Muscle, Specific Activity approximately 9 units/mg, 10 mg protein/ml.

Glycerol-1-Phosphate Dehydrogenase, from Rabbit Muscle, Specific activity approximately 40 units/mg, 10 mg protein/ml.

Triosephosphate Isomerase, from Rabbit Muscle, Specific activity approximately 2400 units/mg, 10 mg protein/ml.

Pyruvate Kinase, from Rabbit Muscle, Specific activity approximately 150 units/mg, 10 mg protein/ml.

The Calbiochem-

2-Phosphoenolpyruvic Acid Na_3. $5\frac{1}{2}$ H_2O, A grade.

Other chemicals used were:-

Magnesium chloride, $MgCl_2.6H_2O$, 'Baker Analyzed' Reagent, J. T. Baker Chemical Co.

2-Mercaptoethanol, $HS(CH_2)_2OH$, Matheson Coleman & Bell.

Ammonium Sulfate, $(NH_4)_2SO_4$, Primary Standard, Fisher Scientific Company.

Sodium Chloride, NaCl, 'Baker Analyzed' Reagent,
J. T. Baker Chemical Co.

Potassium Chloride, KCl, 'Baker Analyzed' Reagent,
J. T. Baker Chemical Co.

Trizma Base, Reagent Grade, Sigma Chemical Company.

Zinc Sulfate, $ZnSO_4.7H_20$, 'Baker Analyzed' Reagent,
J. T. Baker Chemical Co.

Cupric Sulfate, $CuSO_4.5H_20$, Reagent Grade,
The Nichols Chemical Company.

Ferric Chloride, $FeCl_3$, Reagent Grade,
Matheson Coleman & Bell.

Magnesium Sulfate, $MgSO_4.7H_20$, Reagent Grade,
The McArthur Chemical Co.

Ammonium Nitrate, NH_4NO_3, Reagent Grade,
The McArthur Chemical Co.

Potassium Phosphate Monobasic, KH_2PO_4, Reagent Grade,
Fisher Scientific Company.

Potassium Phosphate Dibasic, K_2HPO_4, Reagent Grade,
Fisher Scientific Company.

Hydrochloric Acid, 'Baker Analyzed' Reagent,
J. T. Baker Chemical Co.

Sucrose (Saccharose), Fisher Scientific Company.

Sodium Floride, NaF, 'Baker Analyzed' Reagent,
J. T. Baker Chemical Co.

R E S U L T S

RESULTS

EFFECTS OF CULTURE AGE AND SUCROSE CONCENTRATION ON ENZYME LEVEL AND STABILITY

A. niger was grown in the liquid medium with 0.8% sucrose (8 g/ℓ) or 10% sucrose (100 g/ℓ), and harvested after 40, 48, and 72 hours of culture age. Crude extracts of mycelia were prepared in the Tris-HCl buffer and the PFK activities assayed immediately and after storage at $4^{o}C$. The results are summarized in Tables I and II.

Mycelia from the 10% - sucrose cultures yielded higher protein contents and enzyme specific activities than those from the 0.8% - sucrose cultures. The enzyme levels of the 0.8% - sucrose cultures decreased as the culture age increased, while those of the 10% - sucrose cultures remained constant. This was probably because of a limited sucrose supply in the former cultures.

Although the 10% - sucrose cultures yielded higher PFK activities in the fresh extracts (zero hour of storage), their enzyme activities were more unstable

TABLE I

Effects of culture age of A. niger on the level and stability of enzyme in 0.8% sucrose medium

Culture age (hours)	Protein concentration (mg/ml)	PFK specific activity (units/mg)			
		Fresh extract	After 24 hours	After 48 hours	After 72 hours
40	3.2	0.128	0.120	0.101	0.026
48	3.5	0.111	0.097	0.052	0.010
72	3.5	0.086	0.080	0.010	0

TABLE II

Effects of culture age of A. niger on the level and stability of enzyme in 10% sucrose medium

Culture age (hours)	Protein concentration (mg/ml)	PFK specific activity (units/mg)		
		Fresh extract	After 24 hours	After 48 hours
40	4.8	0.218	0.111	0.020
48	5.4	0.224	0.085	0
72	4.7	0.223	0.016	0

losing nearly all the activities within 48 hours of storage. In both 0.8% and 10% sucrose media, older cultures yielded more unstable enzyme preparations. The 40-hour culture grown in 0.8% sucrose medium gave a PFK preparation of highest stability retaining 80% of the activity after 48 hours at $4^{o}C$.

NADH oxidase activity was found to be much too low to interfere in the assay of PFK activity.

EFFECTS OF MEDIUM TRACE METALS ON ENZYME LEVEL

In order to test the effect of trace metals on the enzyme level, various trace metals were removed from the standard normal liquid medium. The cells were grown at either 0.8% sucrose or 10% sucrose level for 40 and 72 hours. Crude extracts were prepared as in the previous experiment.

As shown in Table III, extracts from normal cells (grown in the standard medium) had the highest PFK activity. Enzyme activities of the Zn^{++} - deficient and Fe^{+++} - deficient cells were low while those of the Zn^{++} - Fe^{+++} - Cu^{++} - deficient cells approached zero. Protein concentration also decreased with the omission of trace metals in the growth medium.

TABLE III

Effects of trace metals on the level of enzyme in 0.8% and 10% sucrose media

Trace metals removed	Culture age (hours)	0.8% - sucrose culture		10% - sucrose culture	
		Protein concentration (mg/ml)	Specific activity (units/mg)	Protein concentration (mg/ml)	Specific activity (units/mg)
None	40	2.7	0.223	5.3	0.375
None	72	2.0	0.136	4.1	0.384
Zn^{++}	40	1.6	0.057	2.4	0.105
Zn^{++}	72	1.5	0.056	2.3	0.063
Fe^{+++}	40	1.1	0.025	2.5	0.031
Fe^{+++}	72	0.96	0.068	2.5	0.007
Zn^{++}, Fe^{+++}, Cu^{++}	40	0.96	0.015	2.1	0.017
Zn^{++}, Fe^{+++}, Cu^{++}	72	0.96	0	2.2	0.017

Thus the PFK preparations of all the following experiments in this thesis were obtained from the 40-hour culture grown in 0.8% sucrose standard medium.

STABILITY OF PFK ACTIVITY IN CRUDE EXTRACT

The PFK from A. niger was found to be highly unstable. As indicated in Table I, the enzyme activity started to decline after about 24 hours of storage at 4°. Various attempts were tried to stabilize the enzyme.

Mansour (1972 b) reported that fructose diphosphate and adenine nucleotides were active stabilizers of the heart and skeletal muscle enzymes. In yeast, the enzyme was activated by incubation with NaF (Viñuela et al., 1964).

Different substances were tested on the crude extract of A. niger. The PFK stability was increased, though not significantly, by ATP, ADP, and FDP. Mercaptoethanol was found essential for the stability. Glycerol increased the enzyme stability significantly. The effect of NaF was dubious (Table IV). Cyclic 3', 5'-AMP at 1 mM did not have any effect.

In order to test if the instability was due to some factors in the crude extract (either small

TABLE IV

Effect of glycerol and NaF on stability of PFK

Addition	PFK activity (ΔA_{340}/min)		
	Fresh extract	After 24 hours	After 48 hours
None	0.205	0.175	0.010
glycerol, 20%	0.195	0.220	0.145
NaF, 10 mM	0.205	0.235	0.040

The control extract was prepared in 0.05 M Tris-HCl containing ADP, 10^{-4}M; FDP, 10^{-4}M; and mercaptoethanol, 3 mM. The storage was at 4°C.

molecular weight compounds or proteins) the extract was passed through a Sepharose 6B column.

FRACTIONATION ON SEPHAROSE 6B COLUMN

A sample of the crude extract was prepared with 0.05 M Tris-HCl buffer (pH 7.5) containing glycerol, 20%; mercaptoethanol, 3 mM; FDP, 10^{-4}M; ADP, 10^{-4}M; and ATP, 5 x 10^{-4}M. After passing through the Sepharose 6B column, the PFK activity was found in the fractions just behind the protein front (Fig. 1). This indicated that the enzyme had a fairly large molecular weight near or just below 10^{6}.

The PFK activity of the column extract was found to be even more unstable than that of the crude extract (Table V). Since most of the smaller proteins were separated from the PFK enzyme by fractionation, the possibility of inactivation by a protein factor was reduced greatly by these experiments although not eliminated entirely. Dilution of the enzyme by the column fractionation might have been responsible for the decreased stability (Table V).

Figure 1. Fractionation of crude extract on Sepharose 6B column. The extract was prepared with 0.05 M Tris-HCl containing compounds as described in the text and was applied to a column of Sepharose 6B equilibrated with the same buffer. The fractions collected were assayed for PFK activity (●) and for absorbance at 280 nm (O) as described in Materials and Methods. A volume of 200 µl of fraction was used for each assay of activity.

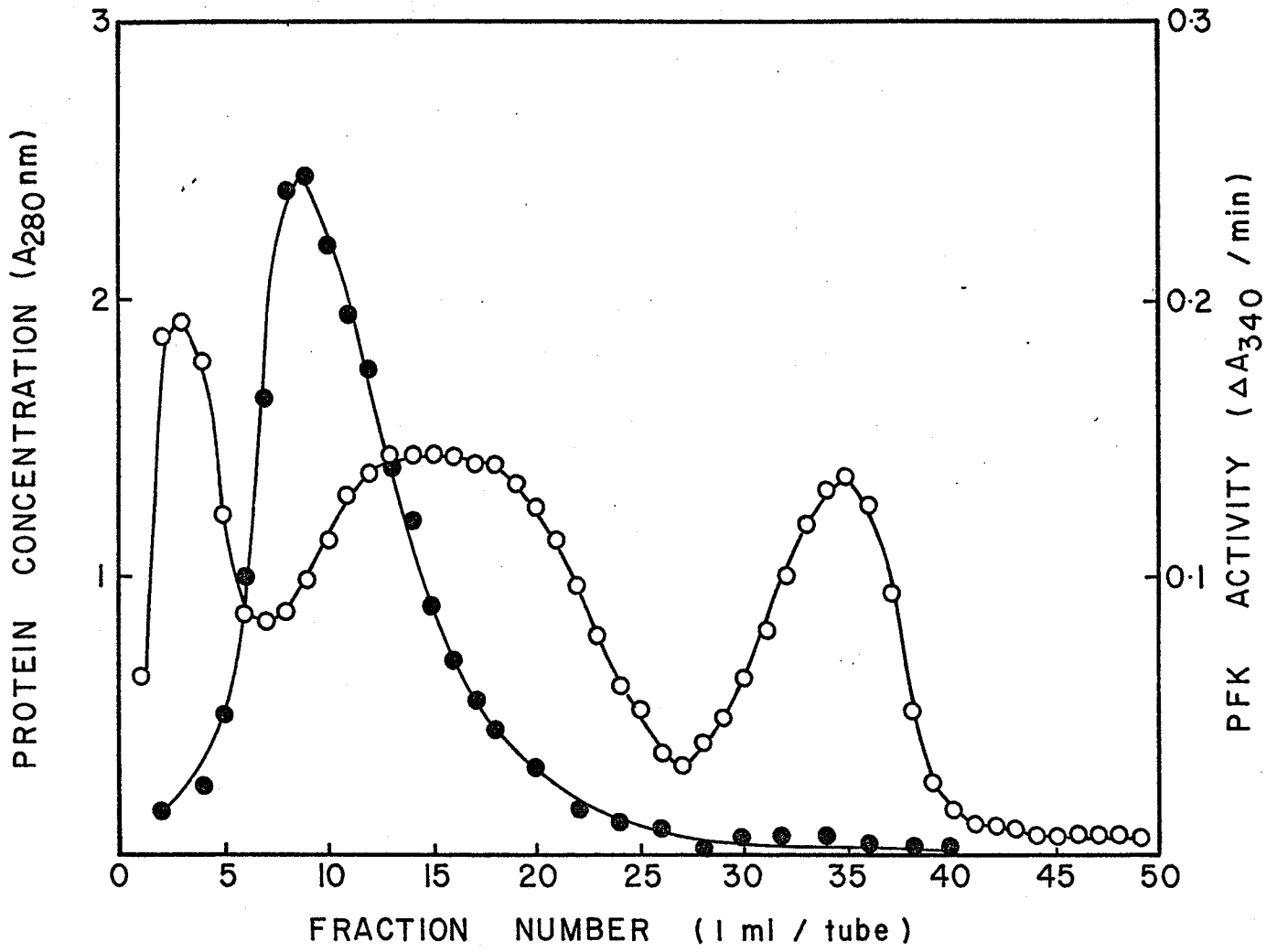

PROTEIN CONCENTRATION (A_{280} nm)
PFK ACTIVITY (ΔA_{340} / min)
FRACTION NUMBER (1 ml / tube)
0
5
10
15
20
25
30
35
40
45
50
1
2
3
0·1
0·2
0·3

TABLE V

Stability of PFK before and after passing through Sepharose 6B

Extract	Protein concentration (mg/ml)	Volume of extract used for assay	Activity (ΔA_{340}/min)	
			Fresh extract or fraction	After 24 hours at 4°C
Crude extract	5.568	50 µl	0.24	0.235
Fraction No. 8	0.573	200 µl	0.24	0.07

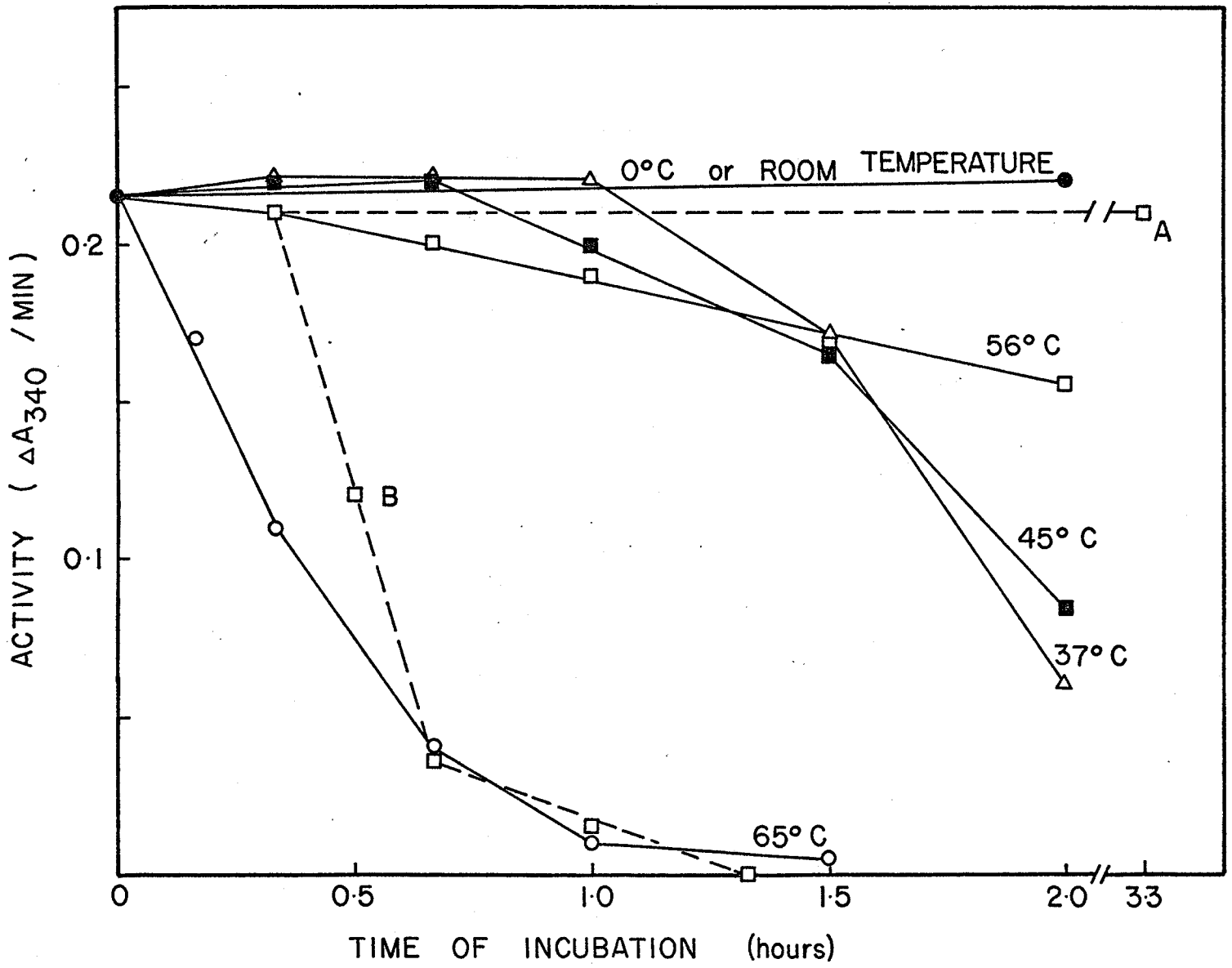
EFFECT OF TEMPERATURE ON STABILITY OF PFK

The guinea pig heart PFK (Mansour, 1965), liver fluke PFK (Stone and Mansour, 1967 a), and chicken liver PFK (Kono and Uyeda, 1973 b) were reported to be cold labile enzymes. Experiments on the A. niger PFK did not reveal such a characteristic.

To see if the stability of the A. niger PFK could be affected by temperature, a crude extract, prepared with 0.05 M potassium phosphate buffer (pH 7.5) containing 3 mM of mercaptoethanol, was divided into ½-ml portions into 6-inch test tubes. The tubes were incubated separately at 0^{o}, room temperature, 37^{o}, 45^{o}, 56^{o}, or 65^{o}C. The enzyme activity was assayed at various time intervals.

The enzyme was rather thermostable. Fig. 2 shows that the enzyme activity was rapidly destroyed at 65^{o} (50% activity lost in 20 minutes) while the inactivation rate at 56^{o} was slow. There was some protein precipitation at both these temperatures. At 37^{o} and 45^{o}, a rapid inactivation started after about one hour of incubation. At the end of 2 hours, the activities of the enzyme at these two temperatures were much lower than that at 56^{o}. Further incubation at 56^{o} after inactivation at 37^{o} did not reactivate the enzyme. There was

Figure 2. Effect of temperature on the stability of PFK in potassium phosphate buffer containing mercaptoethanol (3 mM). The crude extract prepared in the above buffer was divided into 0.5 ml portions and was incubated in test tubes at 0^{o} or room temperature (●), 37^{o} (Δ), 45^{o} (■), 56^{o} (□), and 65^{o} (O). At given time intervals, tubes were removed and 50 µl samples were assayed for enzyme activity as described in Materials and Methods. Line A and line B represent the activities of the enzyme after a heat treatment at 56^{o} for 20 minutes and a further incubation at 37^{o} (A) or 65^{o} (B).


0°C or ROOM TEMPERATURE
A
56° C
B
45° C
37° C
65° C
0·2
0·1
ACTIVITY (ΔA340 /MIN)
0
0·5
1·0
1·5
2·0
3·3
TIME OF INCUBATION (hours)

no loss in enzyme activity after 2 hours at 0^{o} or room temperature.

In order to find out whether the heat treatment at 56^{o} would further affect the stability of the enzyme at other temperatures, the crude extract was first heated at 56^{o} for 20 minutes, then was further incubated at 4^{o}, 37^{o}, or 65^{o}. As indicated in Fig. 2, the enzyme became perfectly stable at 37^{o} retaining its full activity even after a further incubation for 3 hours, but it became less stable at 65^{o} losing more than 80% of the activity in 20 minutes. The enzyme after the 56^{o} treatment was found to be more stable than the crude extract when stored at 4^{o} (Table VI, part A).

The experiment was repeated with the addition of glycerol (20% v/v) to the crude extract. (In all the experiments followed, 3 mM of mercaptoethanol were included in the crude extracts). It was found that the enzyme was highly protected by glycerol from inactivation at 65^{o} and to a lesser degree at 56^{o}, but was relatively unaffected by glycerol at other temperatures (Fig. 3).

When ATP (5×10^{-4}M) was added instead of glycerol, PFK at 37^{o} and 45^{o} was protected from inactivation while PFK at 65^{o} was inactivated at a faster rate (Fig. 4). ADP, at the same concentration, had the same effect as ATP (not shown).

Figure 3. Effect of temperature on the stability of PFK in potassium phosphate buffer containing mercaptoethanol (3 mM) and glycerol (20% v/v). The experimental procedures were the same as in Fig. 2. Room temperature (●), 37° (Δ), 45° (■), 56° (□), and 65° (O).

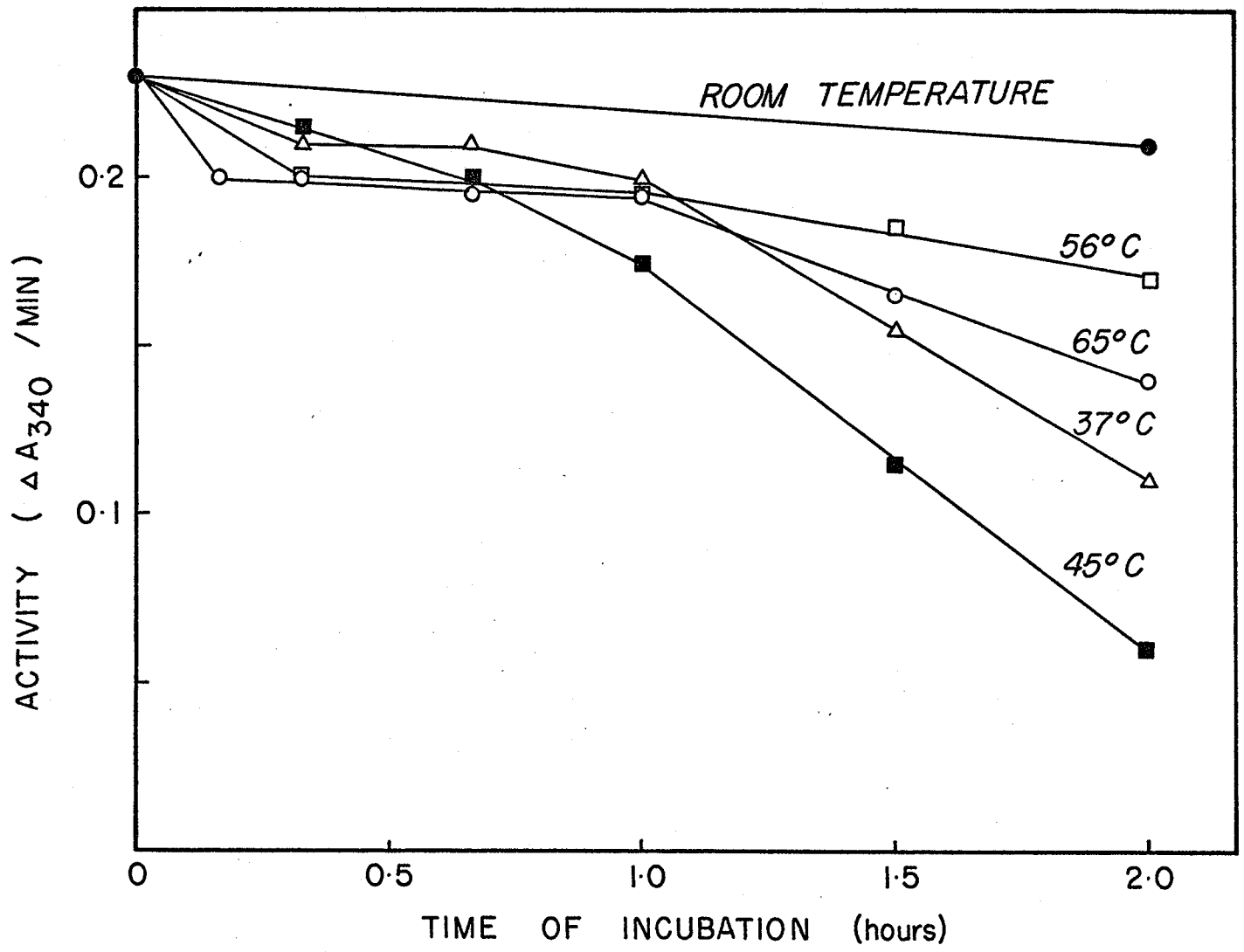

ROOM TEMPERATURE
56° C
65° C
37° C
45° C
0·2
0·1
ACTIVITY (ΔA340 /MIN)
0
0·5
1·0
1·5
2·0
TIME OF INCUBATION (hours)

Figure 4. Effect of temperature on the stability of PFK in potassium phosphate buffer containing mercaptoethanol (3 mM) and ATP (5×10^{-4}M). The experimental procedures were the same as in Fig. 2. Room temperature (●), 37° (△), 45° (■), 56° (□), and 65° (○).

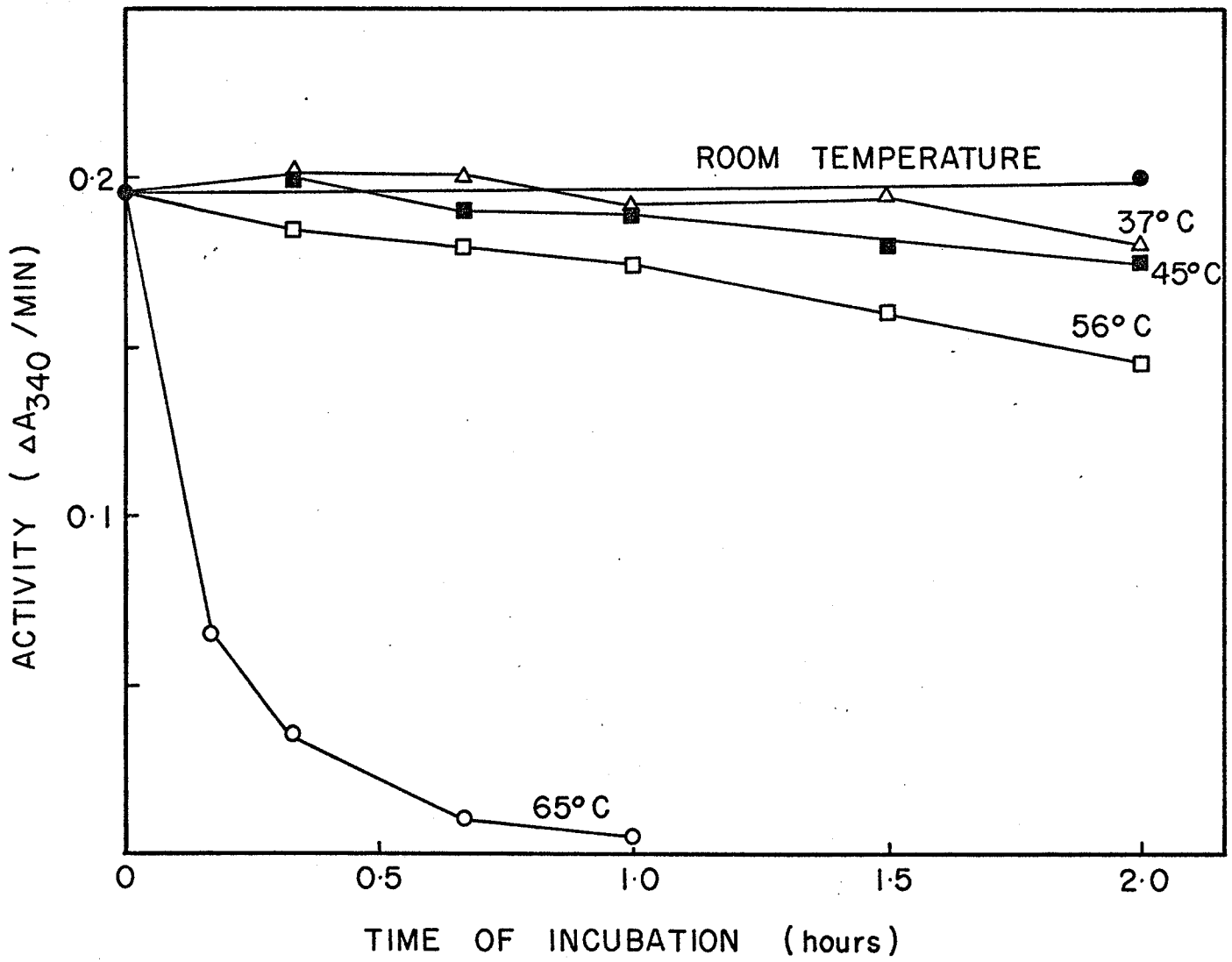

ROOM TEMPERATURE
37° C
45° C
56° C
65° C
0·2
0·1
0
0·5
1·0
1·5
2·0
ACTIVITY (ΔA340 /MIN)
TIME OF INCUBATION (hours)

TABLE VI

Stability of PFK before and after heat treatment at 56°C

Addition in extract	Treatment	Activity (ΔA_{340}/min) after storage at 4°C for				
		0 day	3 days	4 days	6 days	7 days
(A) None	None	0.206	0.01	-	-	0
	20 minutes at 56°C	0.19	0.21	-	-	0.145
(B) ATP, ADP, FDP (each 5×10^{-4}M); glycerol (20%)	None	0.205	-	0.06	0.02	0.015
	20 minutes at 56°C	0.175	-	0.19	0.20	0.205

Mercaptoethanol, 3 mM, was present in both extracts.

The effect of FDP (5×10^{-4}M) under these various temperatures was less dramatic. Fig. 5 shows that the enzyme was protected only at 45^{o}.

When the experiment was carried out with glycerol, ATP, ADP, and FDP all present in the crude extract, the pattern of inactivation (Fig. 6) was similar to that of Fig. 3 (glycerol alone present). The PFK at 37^{o} and 45^{o} was rapidly inactivated after about 1½ hours of incubation. The protection afforded at these temperatures by ATP, ADP, and FDP (Figs. 4 and 5) was not evident in Fig. 6. The 56^{o} treatment, however, was again effective in protecting the enzyme from inactivation at 37^{o} (Fig. 6).

The stability of PFK at 4^{o}C, with and without the 56^{o} treatment, was studied in the above experiment (Table VI, part B). As before, the enzyme after heat treatment was more stable than the untreated crude extract.

The experiment was also repeated in the presence of NaF. Fig. 7 indicates that NaF seemed to act in a similar manner as ATP and ADP.

All these findings indicate that there are at least two different effects involved in these stability experiments. One is the thermal inactivation effect which was most striking at 65^{o} in the absence of glycerol. Glycerol seemed to increase the thermal stability of the

Figure 5. Effect of temperature on the stability of PFK in potassium phosphate buffer containing mercaptoethanol (3 mM) and FDP (5×10^{-4}M). The experimental procedures were the same as in Fig. 2. Room temperature (●), 37° (△), 45° (■), 56° (□), and 65° (○).

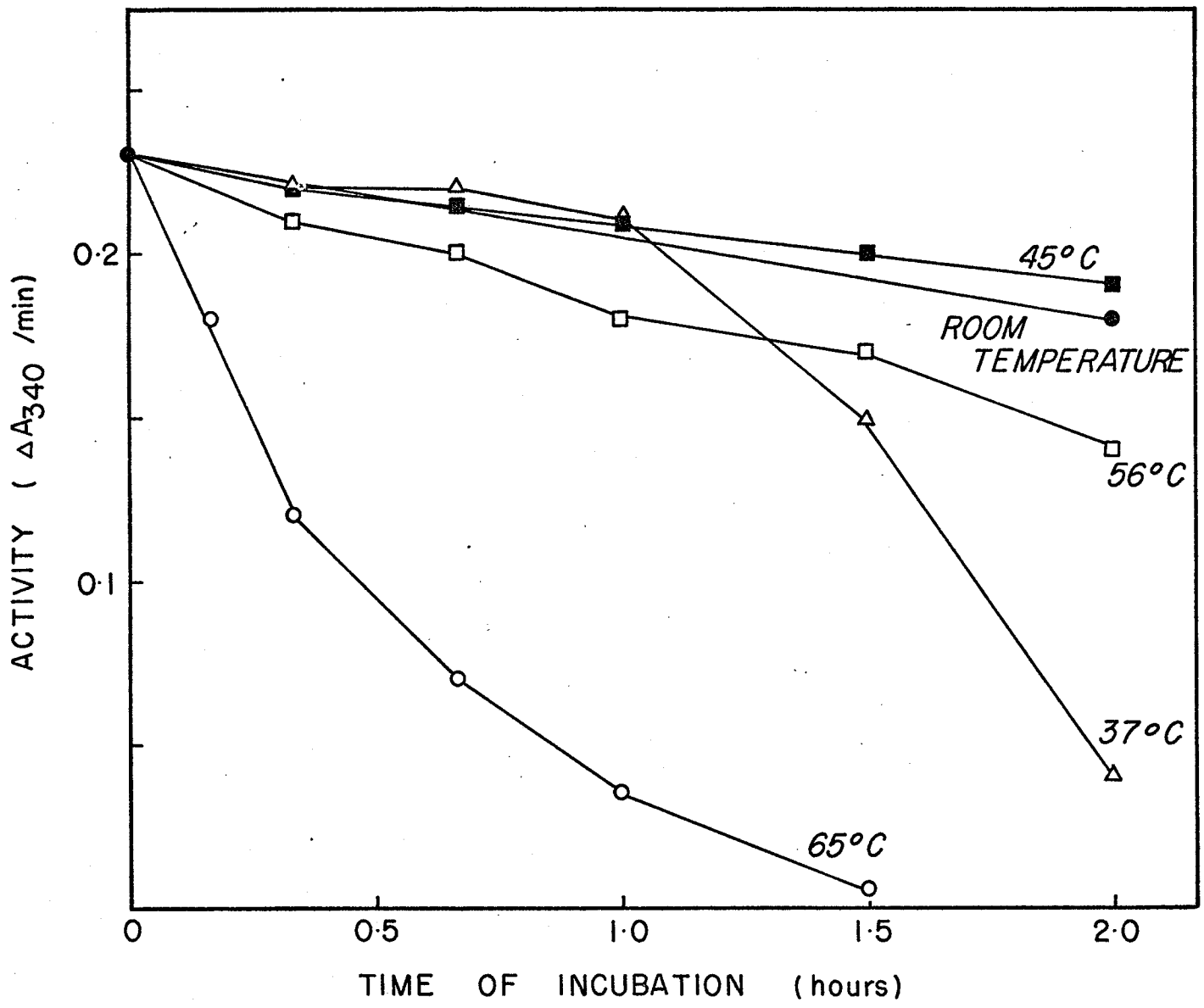

ACTIVITY (ΔA340 /min)
0·2
0·1
0
0·5
1·0
1·5
2·0
TIME OF INCUBATION (hours)
45°C
ROOM TEMPERATURE
56°C
37°C
65°C

Figure 6. Effect of temperature on the stability of PFK in potassium phosphate buffer containing mercaptoethanol (3 mM), glycerol (20% v/v), ATP (5×10^{-4}M), ADP (5×10^{-4}M), and FDP (5×10^{-4}M). The experimental procedures were the same as in Fig. 2. Room temperature (●), 37° (△), 45° (■), 56° (□), and 65° (○).
Line A represents the activity of the enzyme after heat treatment at 56° and a further incubation at 37° for 3 hours.

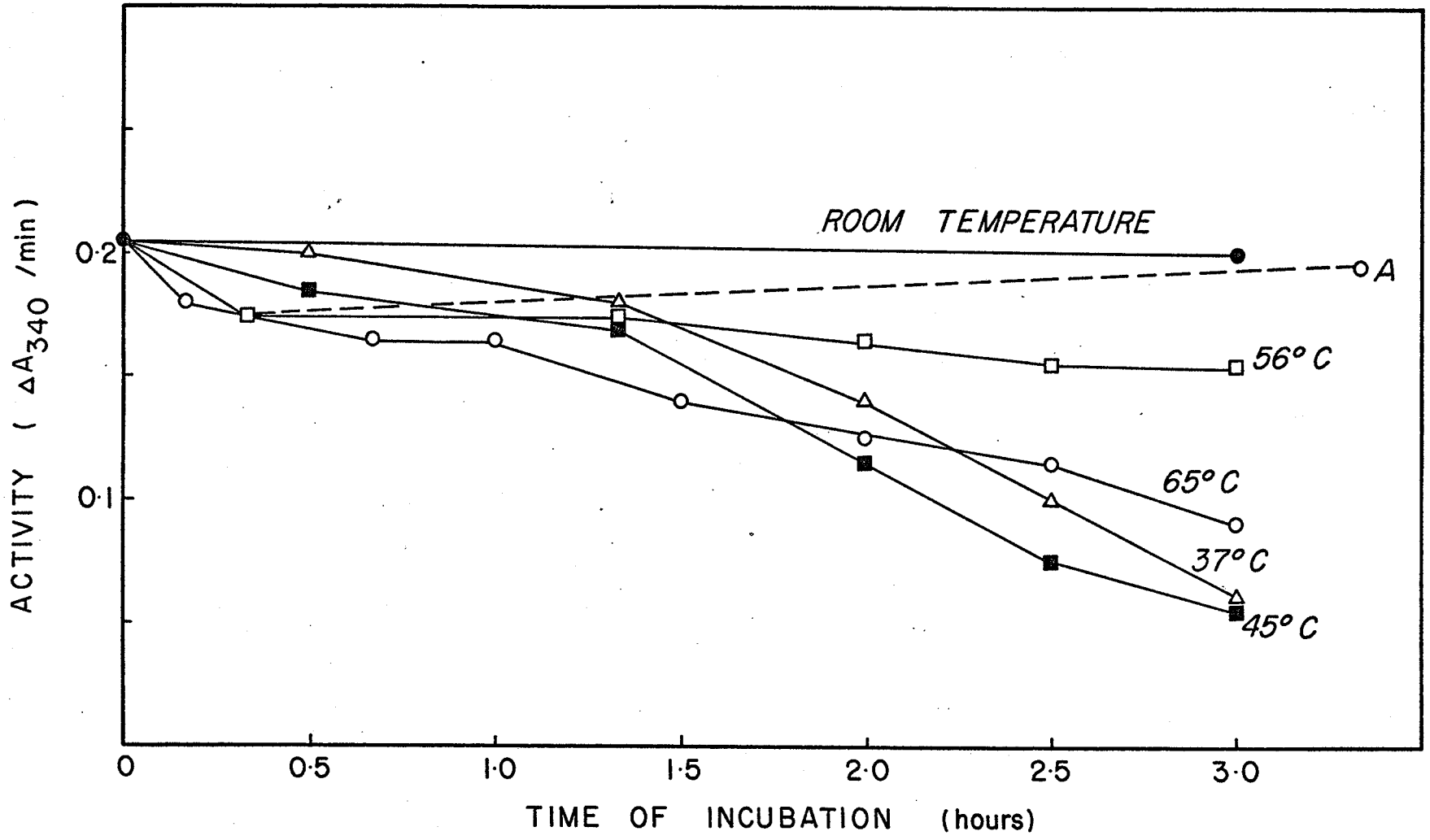
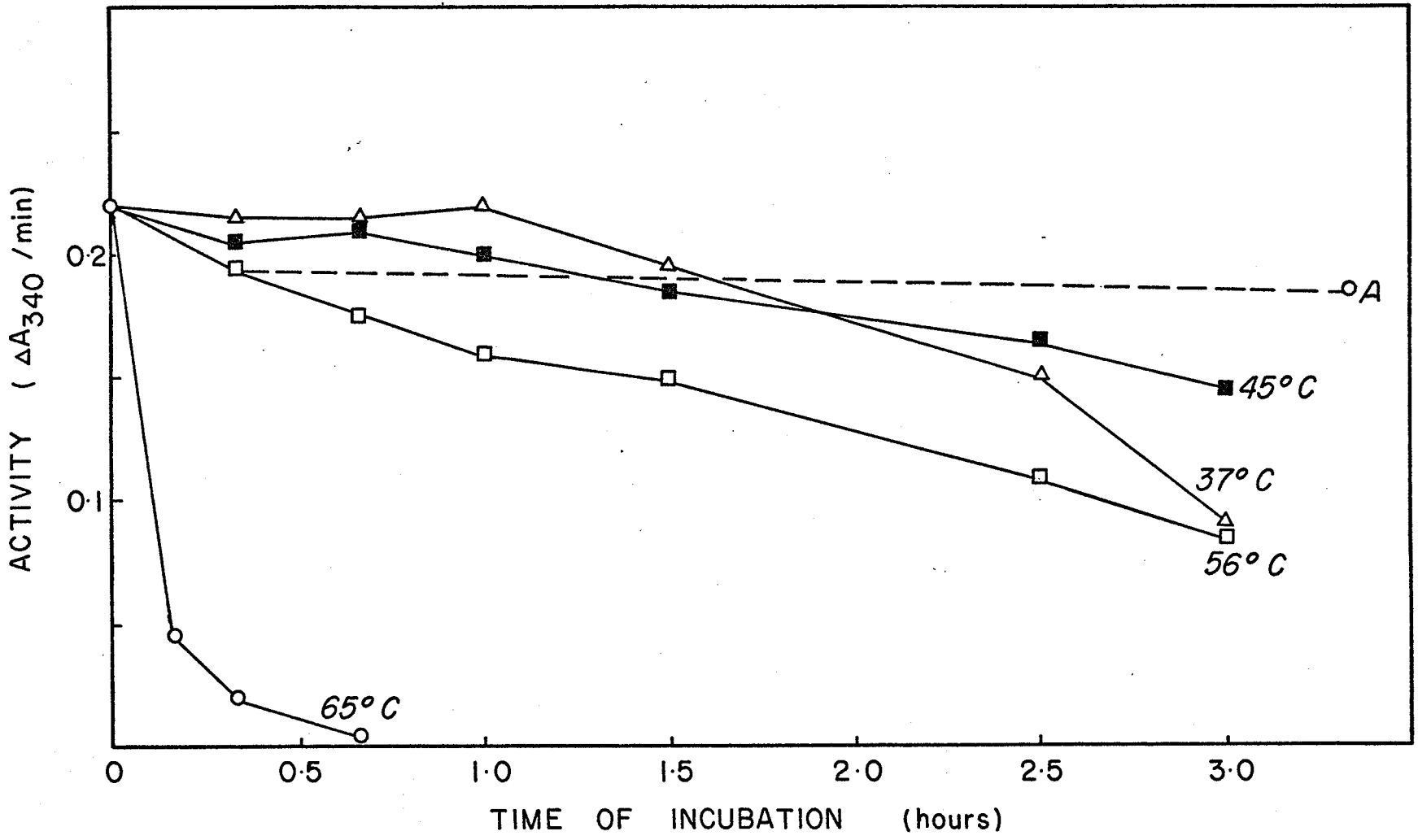

ROOM TEMPERATURE
A
56° C
65° C
37° C
45° C
ACTIVITY (ΔA340 /min)
0·2
0·1
0
0·5
1·0
1·5
2·0
2·5
3·0
TIME OF INCUBATION (hours)

Figure 7. Effect of temperature on the stability of PFK in potassium phosphate buffer containing mercaptoethanol (3 mM) and NaF (10 mM). The experimental procedures were the same as in Fig. 2. 37^{o} (△), 45^{o} (■), 56^{o} (□), and 65^{o} (○).
Line A represents the activity of the enzyme after heat treatment at 56^{o} and a further incubation at 37^{o} for 3 hours.


ACTIVITY (ΔA_{340} /min)
0·2
0·1
0
0·5
1·0
1·5
2·0
2·5
3·0
TIME OF INCUBATION (hours)
A
45° C
37° C
56° C
65° C

enzyme, but ATP and NaF seemed to decrease it. The other is related to the inactivation at 37° or 45° and is more complex than simple thermal inactivation. The enzyme became resistant to this effect after the heat treatment at 56° or in the presence of ATP, ADP or NaF.

PURIFICATION OF PHOSPHOFRUCTOKINASE

Phosphofructokinase was purified from the crude extracts of A. niger by heat treatment at 58° C and acetone fractionation as described in the Materials and Methods. The results of purification are presented in Table VII where each figure shown represents an average of the results from 4 batches of cells. The supernatant of the suspension of the 30-50% acetone precipitate was used as the purified PFK preparation in the following experiments.

ASSAY OF PHOSPHOFRUCTOKINASE

No activities of NADH oxidase and ATPase were detected in the purified preparation.

The enzyme PFK, either in the crude extract or in the purified preparation, exhibited at least two

TABLE VII

Purification of phosphofructokinase from A. niger

Procedure	Volume (ml)	Total protein (mg)	Total activity (units)	Recovery (%)	Specific activity (units/mg)
crude extract	54	275	50.3	100	0.183
supernatant after 58° treatment	54	106	44.7	88.9	0.421
supernatant of the 30-50% acetone precipitate suspension	9	12.1	39.2	77.9	3.266

unusual time-course behaviour under the standard assay conditions for the activity measurement. The enzyme activity either showed an initial lag in velocity before achieving a linear rate of reaction (Fig. 8a) or showed a slight levelling off of activity after an initial linear rate of reaction (Fig. 8b). Sometimes the activity was entirely linear throughout the reaction. The enzyme seemed to oscillate between the initially slow and fast states during storage at 4^{o} since the same enzyme preparation showed a lag on one day and a slight levelling off the next day or vice versa.

In both cases, however, the activity of the purified PFK as measured at a linear portion of reaction was found to be proportional to the amount of protein (Figs. 9a, b).

STABILITY OF PURIFIED PHOSPHOFRUCTOKINASE

In the presence of mercaptoethanol (3 mM) and glycerol (20% v/v), the purified enzyme did not lose any activity during storage at 4^{o} for about 5 days, after which there was a gradual loss of activity. Compounds such as ATP, FDP, and F6P at 5×10^{-4}M had no effect on the stability of the enzyme. Table VIII is a comparison of stabilities among the crude extract,

Figure 8a. Time course relationship of PFK assay showing a lag. Assay mixtures were prepared as described in Materials and Methods.

○ —— 5 µl purified PFK

● —— 10 µl purified PFK

▲ —— 20 µl purified PFK

△ —— 30 µl purified PFK

Figure 8b. Time course relationship of PFK assay showing a levelling off. Assay mixtures were prepared as described in Materials and Methods.

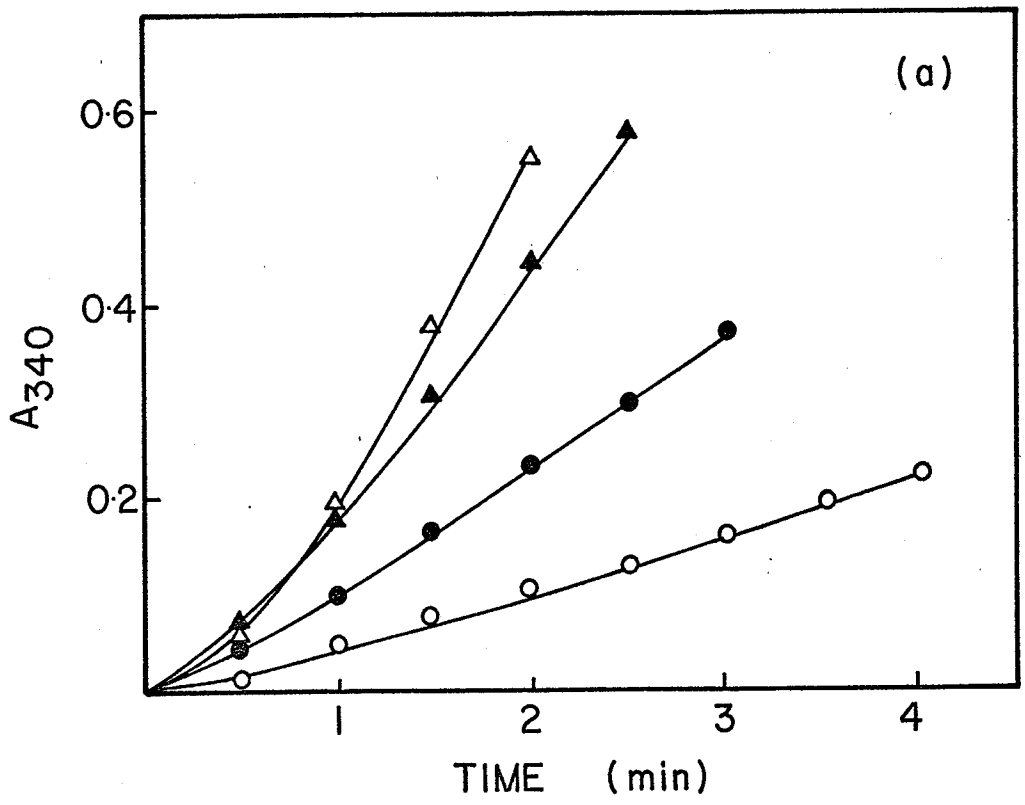
○ —— 5 µl purified PFK

● —— 10 µl purified PFK

△ —— 15 µl purified PFK

▲ —— 20 µl purified PFK

(Figure 8a and 8b were from different batches of cells.)


(a)
0·6
0·4
0·2
A340
1
2
3
4
TIME (min)

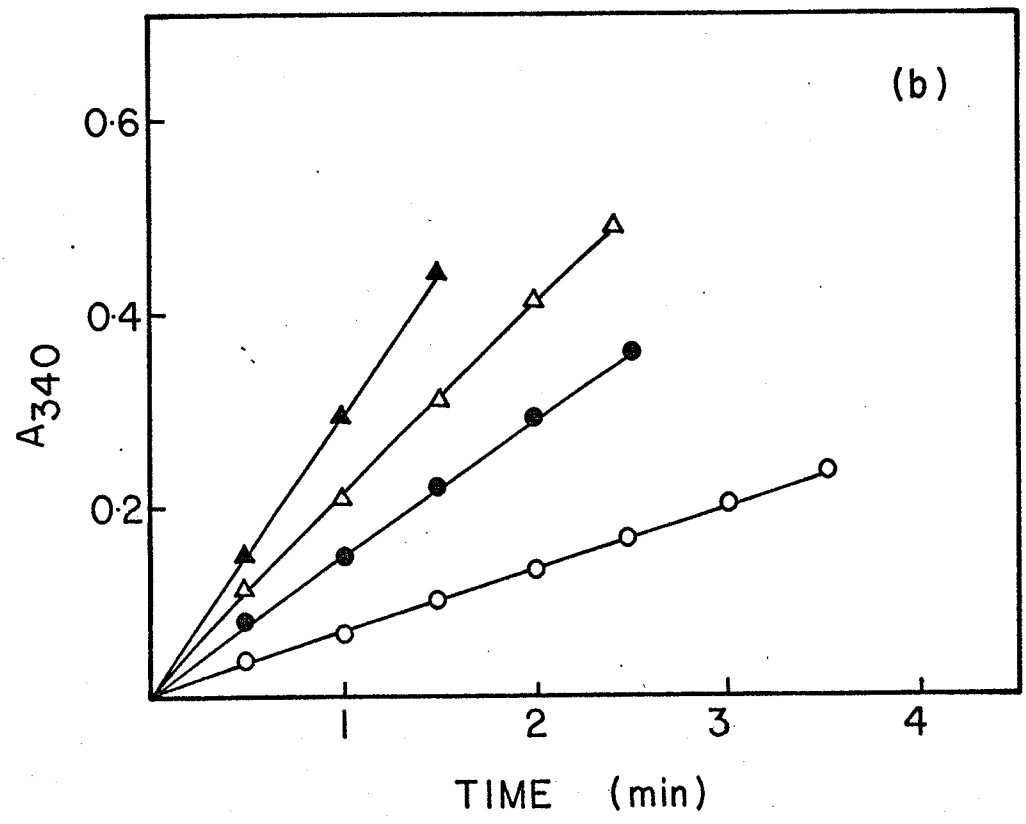

(b)
0·6
0·4
0·2
A340
1
2
3
4
TIME (min)

Figure 9 (a, b). Proportionality of PFK activity with amount of enzyme. The data were obtained from Figs. 8a and 8b for Figs. 9a and 9b, respectively.

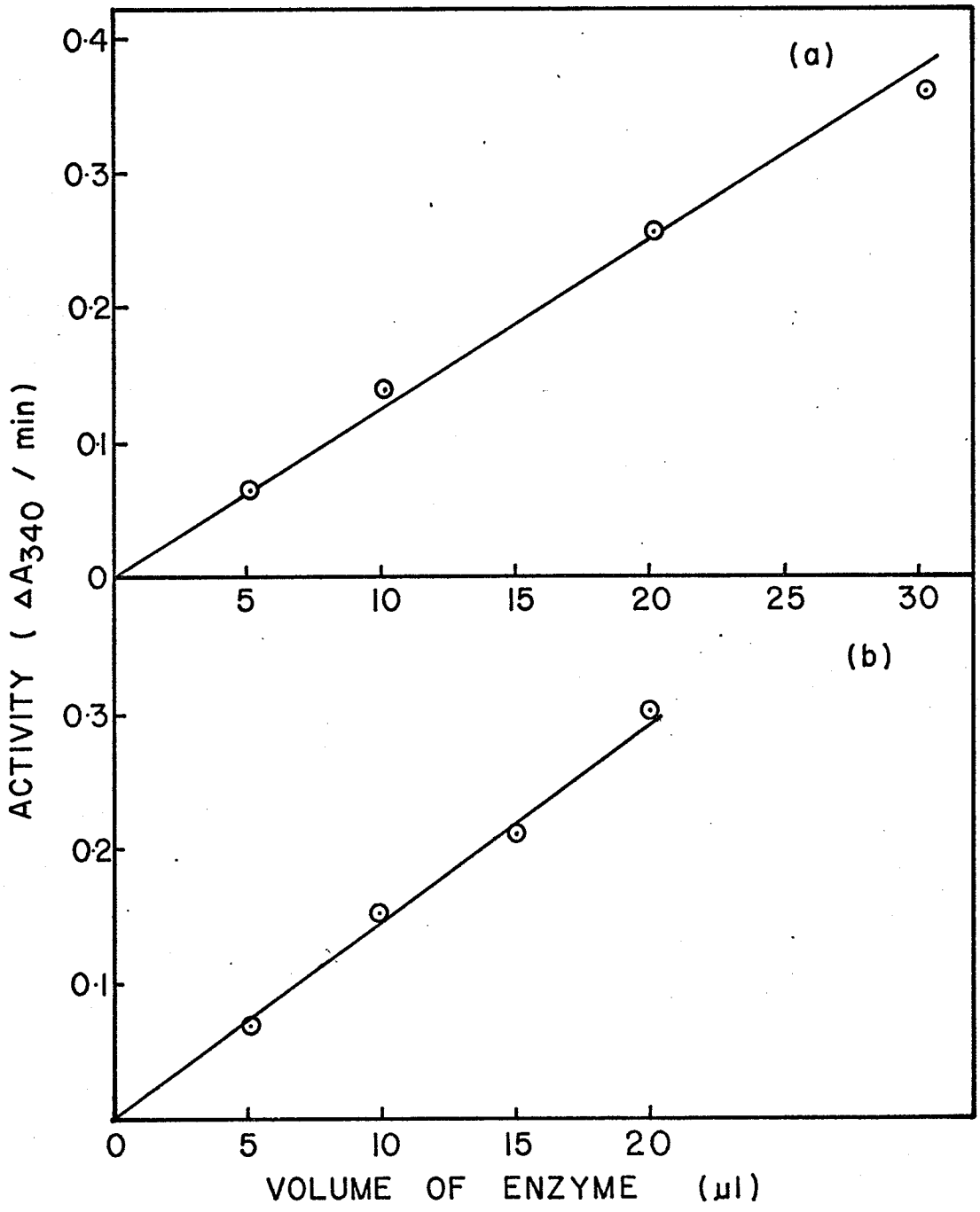

(a)
(b)
0·4
0·3
0·2
0·1
0
5
10
15
20
25
30
ACTIVITY (ΔA340 / min)
VOLUME OF ENZYME (µl)

TABLE VIII

Comparison of stabilities of PFK in different preparations

Preparation	Activity (ΔA_{340}/min) after storage at 4° for					
	0 day	2 days	5 days	7 days	9 days	16 days
crude extract	0.165	0.11	0.01	-	-	-
supernatant after 58° treatment	0.15	0.165	0.14	0.125	0.11	0.10
purified enzyme	0.175	0.185	0.18	0.135	0.12	0.08
purified enzyme + ATP	-	-	-	0.14	0.11	0.075
purified enzyme + FDP	-	-	-	0.135	0.125	0.09
purified enzyme + ATP + FDP	-	-	-	0.155	0.13	0.07
purified enzyme + F6P	-	-	0.17	0.13	0.125	0.08

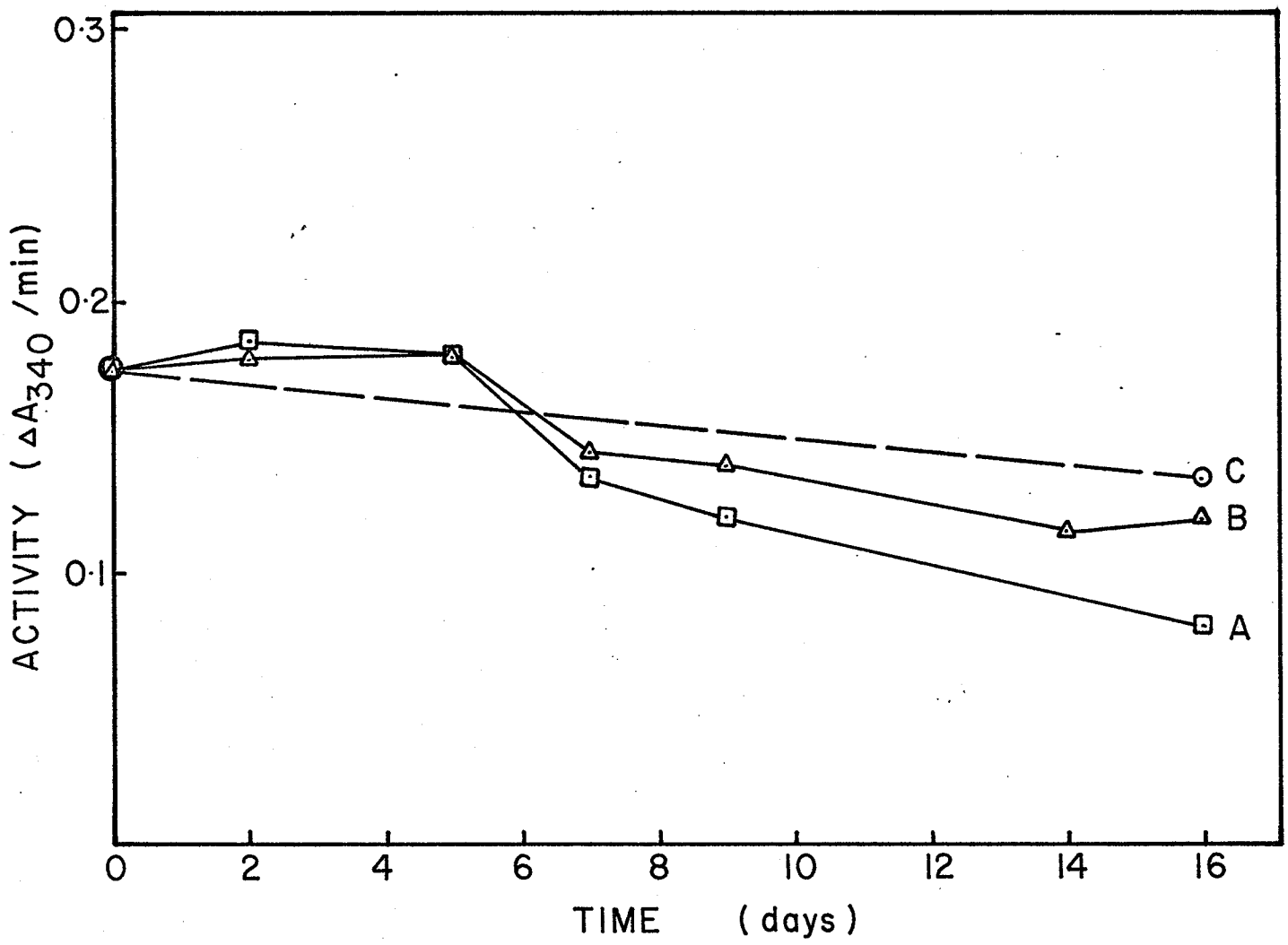
Concentrations of ATP, FDP, and F6P were 5×10^{-4}M.
Mercaptoethanol (3 mM) and glycerol (20%) were present in all preparations.

the fraction after heat treatment, and the final purified enzyme with different additions. The purified enzyme was just as stable as the 58° supernatant during storage at 4° and both were much more stable than the crude extract.

The purified enzyme preparation when stored at -76° was slightly more stable than at 4°, losing only 20% of activity after 16 days as compared to 50-60% loss at 4° (Fig. 10, C and A). Daily freezing and thawing reduced the stability resulting in 30-40% loss of activity after 16 days (Fig. 10, B). There was no loss of activity during the first 5 days at -76°, even with daily thawing. These results showed that, though the enzyme at the frozen state was a little more stable than that at 4°, there was still a gradual loss of activity at -76° within 16 days.

The purified enzyme in the pellet form after acetone precipitation could be stored at -20°C for 2 weeks without loss of activity. However, this stability varied with the length of storage period of the intact cells (at -76°C). When cells stored longer than 3 months were used, the 30-50% acetone precipitate was unstable either in the pellet form at -20° or in solution form at 4°, losing 40% of activity after 24 hours at 4° in contrast to its normal stability shown in Table VIII.

Figure 10. Effect of freezing during storage on the stability of PFK. The purified PFK enzyme was stored at 4^{o} (A), stored at -76^{o} and thawed approximately daily (B), stored at -76^{o} and thawed after 16 days of storage (C). Assay mixtures were as described in Materials and Methods.


0·3
0·2
0·1
ACTIVITY (ΔA340 /min)
0
2
4
6
8
10
12
14
16
TIME (days)
C
B
A

EFFECT OF pH

The purified PFK (in phosphate buffer) was assayed in 3 different buffers: Tris-HCl, HEPES, and MES, each ranging within its own effective buffer range. All three buffers had a concentration of 0.05 M and were adjusted to the desired pH with HCl or NaOH.

It was found that pH 7.5 to 8.7 gave the highest enzyme activity and that the higher the pH of the assay buffers, the greater was the initial lag of the reaction. This lag was more pronounced in Tris-HCl buffer. In order to find out whether the lag was caused by the abrupt change in the buffer suspending the enzyme (from phosphate to other buffers), the 30-50% acetone precipitate was solubilized in Tris-HCl (0.05 M, pH 8.0). After centrifugation, the supernatant was used for the following pH study. The results were essentially identical to those obtained above with the PFK solubilized in phosphate buffer.

Among the three buffers used for the assay, Tris-HCl gave the highest activity (Fig. 11). Although the enzyme attained the same final linear reaction rate from pH 7.5 to 8.7, the initial lag period became more pronounced as the pH of the buffer increased (Fig. 12).

Figure 11. Effect of pH on PFK activity. The usual standard assay method as described in Materials and Methods was followed with different buffers.

○ — Tris-HCl buffer

△ — HEPES buffer

□ — MES buffer

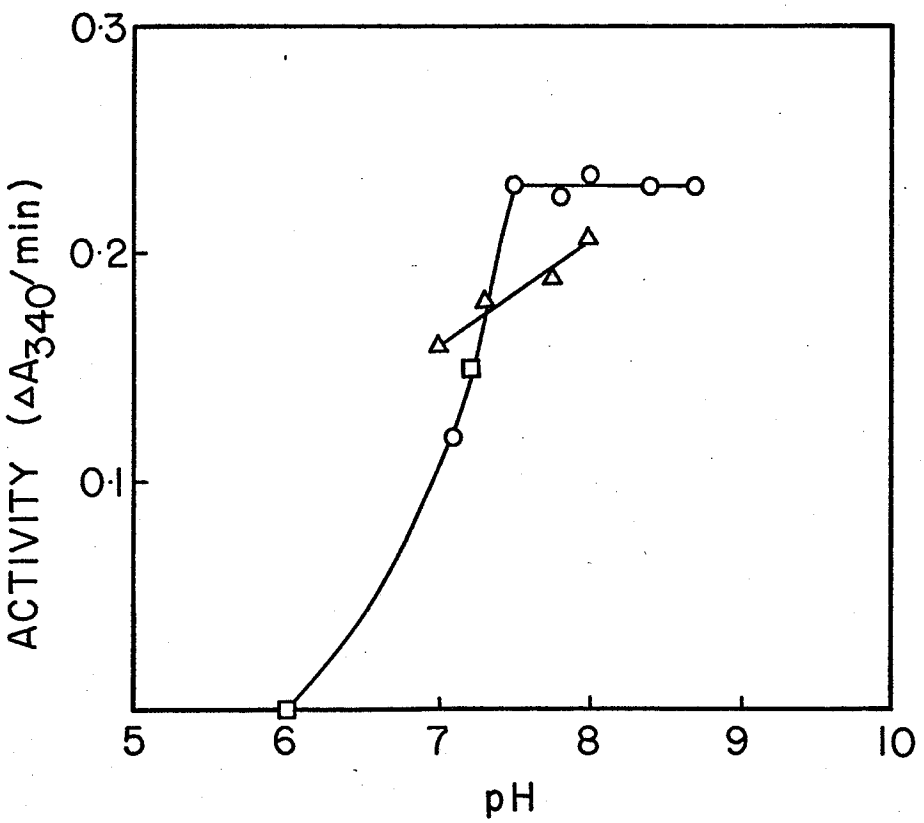
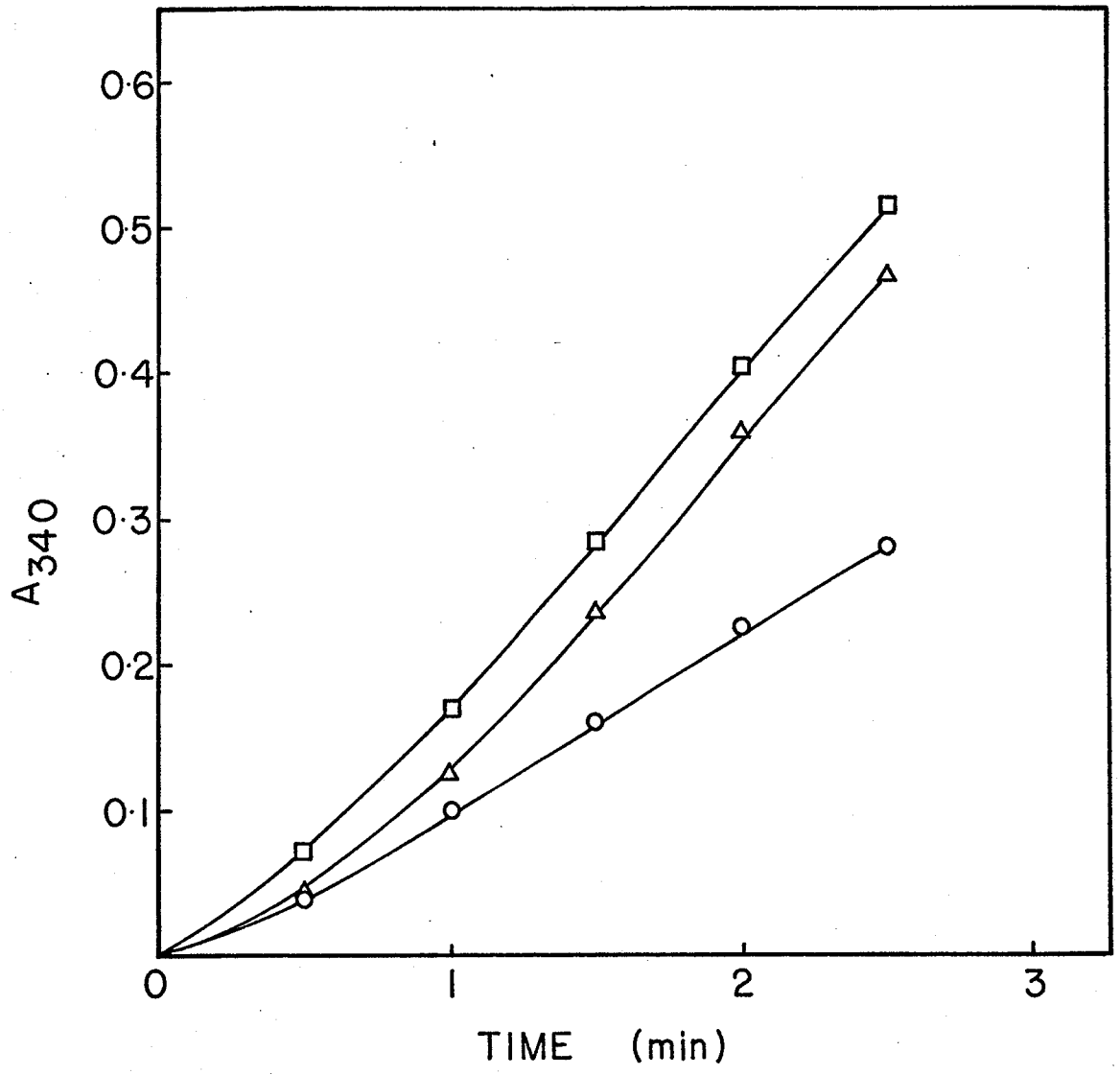

0·3
0·2
0·1
ACTIVITY (ΔA340/min)
5
6
7
8
9
10
pH

Figure 12. Effect of pH on the initial lag of PFK assay. Assay mixtures were prepared as described in Materials and Methods using a Tris-HCl buffer at different pH's as indicated.

○ — pH 7.1

□ — pH 7.5

△ — pH 8.7


0·6
0·5
0·4
0·3
0·2
0·1
A340
0
1
2
3
TIME (min)

In order to minimize possible "environmental" effects to the enzyme of a sudden change in pH when the enzyme was added to the reaction mixture to initiate the reaction, enzyme assays were also carried out as follows. The PFK was first added to a cuvette containing buffer, F6P, $MgCl_2$, NADH, and coupling enzymes and the mixture was incubated at room temperature for 1 minute. ATP was then added to start the reaction. The results remained unchanged with increasing lag periods with increasing pH's.

EFFECT OF DILUTION ON PFK WITH AND WITHOUT ATP AND F6P

When the enzyme was diluted in the assay mixture without ATP and F6P the activity was lost rapidly, but the presence of ATP or F6P protected the enzyme from this inactivation by dilution (Table IX).

The presence of 1 mM AMP either during or after the 15 minutes incubation did not protect the enzyme from inactivation or reverse the inactivation. The degree of inactivation by dilution was the same at both pH's tested. The effect of pH was only found in the extent of initial lag period as in Fig. 12. F6P was more potent than ATP in the protection.

TABLE IX

Effect of dilution on PFK

Dilution mixture *	pH	Activity (ΔA_{340}/min)
Buffer alone	7.5	0
	8.7	0
Assay mixture minus F6P and coupling enzymes	7.5	0.16
	8.7	0.16
Assay mixture minus ATP and coupling enzymes	7.5	0.195
	8.7	0.19
Assay mixture minus ATP, F6P, and coupling enzymes	7.5	0

*The enzyme was diluted 130 fold in the dilution mixture and incubated for 15 minutes at room temperature before starting the reaction by the addition of missing assay components. Buffer used : Tris-HCl. All the concentrations of reagents used in the assay mixture were as described in Materials and Methods.
The enzyme gave an activity of 0.23 ΔA_{340}/min. in both pH's under the standard assay conditions without pre-incubation.

EFFECT OF K^+ AND NA^+ IONS ON PFK ACTIVITY

The standard assay mixtures were prepared and KCl or NaCl were added as indicated in Table X. As the concentration of salt increased, the lag period increased as well, regardless of a stimulation effect or an inhibition effect. At 100 mM, KCl produced a 14% stimulation while NaCl increased the lag only. At higher concentrations both KCl and NaCl inhibited the enzyme.

The increasing lag by these salts was perhaps due to the anionic effect similar to the effect of increasing pH's (OH^- ions). The stimulation or inhibition was probably caused by the K^+ or Na^+ ions.

EFFECT OF AMMONIUM SULFATE ON PFK ACTIVITY

It had been noticed in some preliminary experiments that ammonium sulfate $((NH_4)_2SO_4)$ increased the initial lag of a PFK reaction and accelerated the rate of inactivation during storage. Further experiments, therefore, were carried out to investigate these effects.

Immediate effect on PFK activity. The purified enzyme in phosphate buffer was used to study the effect of

TABLE X

Effect of K^+ and Na^+ concentrations on PFK activity

KCl (mM)	NaCl (mM)	Activity (ΔA_{340}/min)
0	0	0.255
20	0	0.265
70	0	0.27
100	0	0.29
200	0	0.26
300	0	0.21
0	20	0.255
0	100	0.26
0	200	0.215
0	300	0.205

ammonium sulfate in the PFK assay at two different ATP concentrations. As shown in Figs. 13 and 14, increasing concentrations of ammonium sulfate increased the initial lag of the enzyme reaction, although the final rate of reaction approached that of no ammonium sulfate. The results were the same at the usual assay concentration of 0.4 mM ATP (Fig. 13) and at the inhibitory concentration of 4.4 mM ATP (Fig. 14).

It seemed that the increasing lag was probably due to the anionic effect exerted by $SO_4^=$ ions similar to Cl^- and OH^- ions described earlier. Somehow these anions seemed to prolong the lag period possibly by delaying the conversion of PFK to the fully active state as presented in Discussion.

Effect on PFK storage. Stability of the enzyme preparations during storage at 4^o was studied in the presence of ammonium sulfate with and without ATP and FDP as shown in Figs. 15a and b. The PFK activity was assayed one hour after these additions and during storage afterwards.

The enzyme was fairly stable for 6-7 days in the absence of ammonium sulfate, but it became remarkably unstable in its presence losing all the activity after 4 days of storage in 100 mM $(NH_4)_2SO_4$ (Fig. 15a). The

instability of the enzyme was less drastic in 50 mM $(NH_4)_2SO_4$ than in 100 mM $(NH_4)_2SO_4$ (Fig. 15b). FDP not only reduced the effect of ammonium sulfate (Fig. 15a), but also increased the activity slightly during storage with a lower concentration of ammonium sulfate present (Fig. 15b). Addition of ATP to the PFK preparation containing $(NH_4)_2SO_4$ produced an immediate drop in activity (about 75% drop in Fig. 15a or 55% drop in Fig. 15b) which, however, gradually increased during storage reaching the original level (Fig. 15a) or higher (Fig. 15 b). The presence of FDP in addition to ATP and $(NH_4)_2SO_4$ prevented this initial inactivation (Fig. 15a). Although both ATP and FDP protected the enzyme during storage in the presence of ammonium sulfate, their effects were clearly different.

The effect of F6P in the presence of $(NH_4)_2SO_4$ (data not shown) was found to be similar to that of FDP. Since enzyme preparations containing ATP, FDP, or F6P (at 5×10^{-4}M) without $(NH_4)_2SO_4$ yielded a stability pattern similar to the control (no addition), these three compounds seemed to show their effect on PFK only in the presence of $(NH_4)_2SO_4$.

The effect of ATP was further investigated (Fig. 16a). By following the enzyme activity immediately

Figure 13. Effect of $(NH_4)_2SO_4$ on the PFK assay with 0.4 mM ATP. Concentrations of $(NH_4)_2SO_4$ were: 0 mM (○), 5 mM (△), 20 mM (□), 50 mM (●), and 100 mM (▲). Other assay components were as described in Materials and Methods.

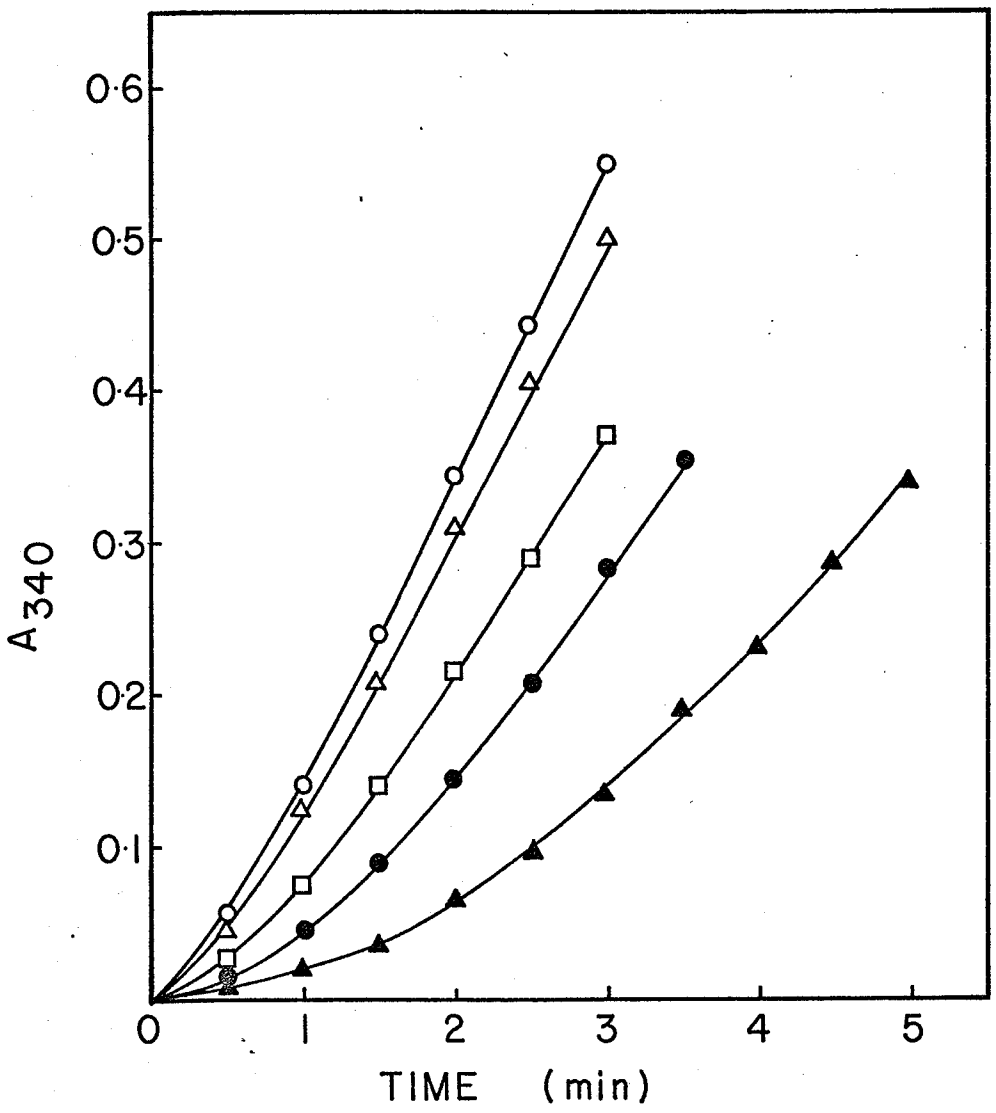

0·6
0·5
0·4
0·3
0·2
0·1
A340
0
1
2
3
4
5
TIME (min)

Figure 14. Effect of $(NH_4)_2SO_4$ on the PFK assay with 4.4 mM ATP. Concentrations of $(NH_4)_2SO_4$ were: 0 mM (◒—◒), 5 mM (◒···◒), and 100 mM (●). Other assay components were as described in Materials and Methods.

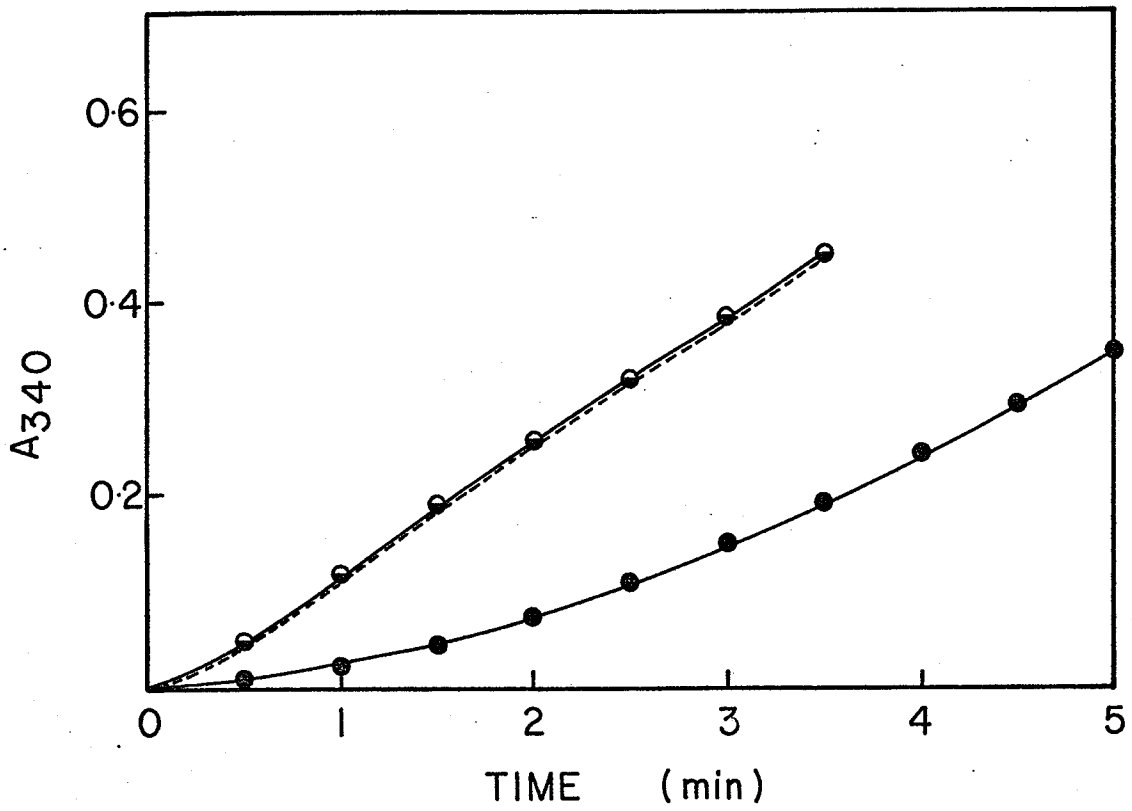

0·6
0·4
0·2
A340
0
1
2
3
4
5
TIME (min)

Figure 15a. Effect of 100 mM $(NH_2)_2SO_4$ on PFK preparations with different compounds present during storage at 4^{o}. Assay mixtures were prepared as described in Materials and Methods. Compounds contained in PFK preparations were:

⊙ ——— control (no addition)

▲ ——— 100 mM $(NH_4)_2SO_4$

⊡ ——— 100 mM $(NH_4)_2SO_4$ + 5×10^{-4}M ATP

● ——— 100 mM $(NH_4)_2SO_4$ + 5×10^{-4}M FDP

△ ——— 100 mM $(NH_4)_2SO_4$ + 5×10^{-4}M ATP + 5×10^{-4}M FDP

Figure 15b. Effect of 50 mM $(NH_4)_2SO_4$ on PFK preparations with different compounds present during storage at 4^{o}. Assay mixtures were prepared as described in Materials and Methods. Compounds contained in PFK preparations were:

⊙ —— control (no addition)

● —— 50 mM $(NH_4)_2SO_4$

⊡ —— 50 mM $(NH_4)_2SO_4$ + 5×10^{-4}M ATP

△ —— 50 mM $(NH_4)_2SO_4$ + 5×10^{-4}M FDP

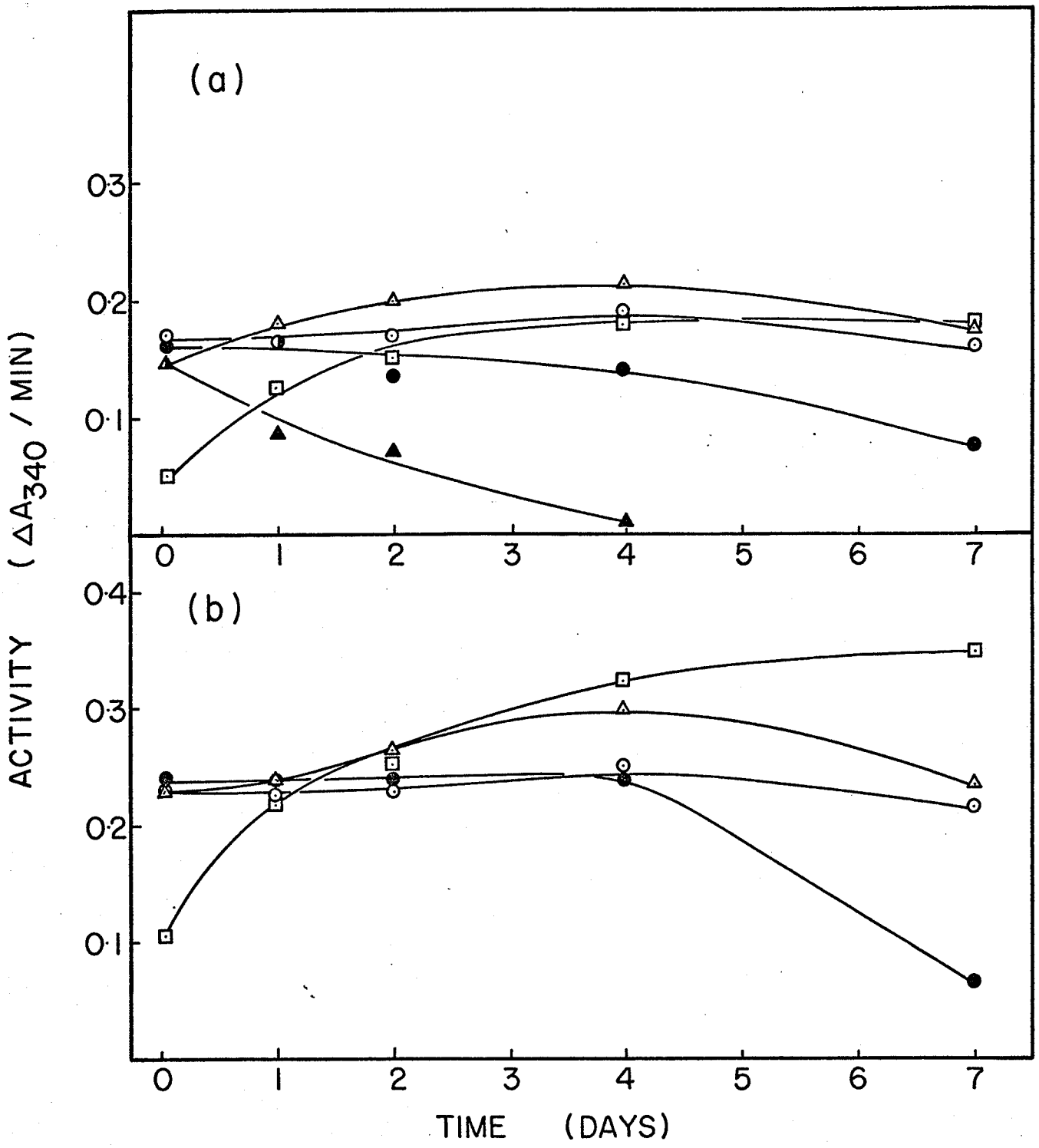
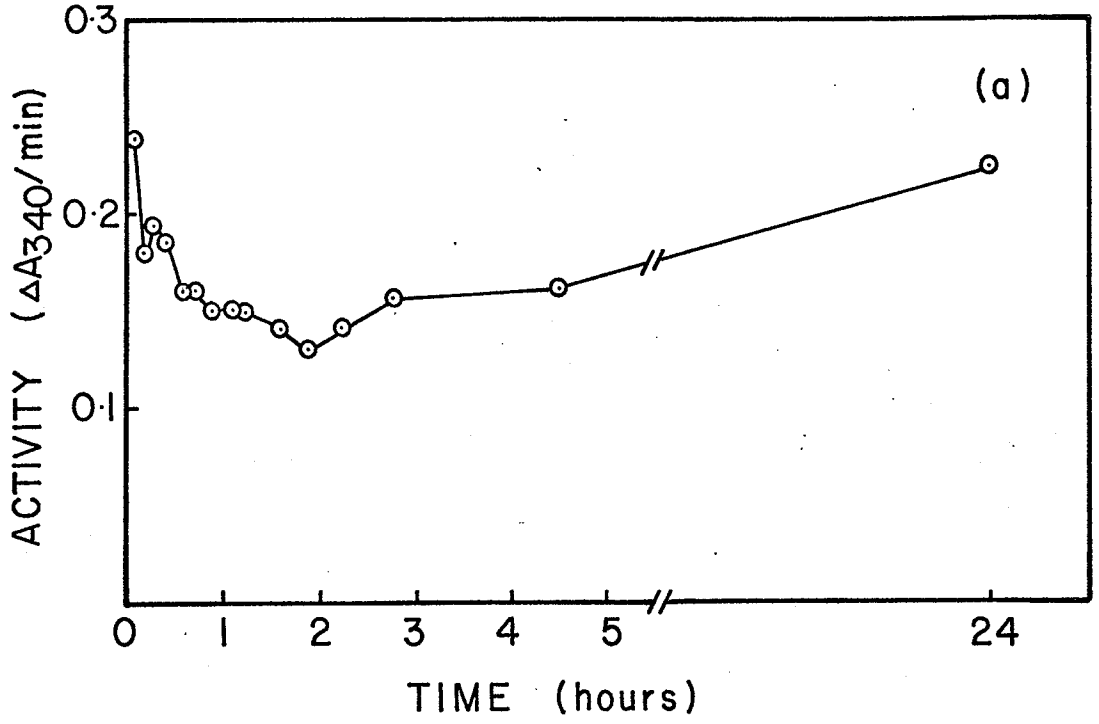
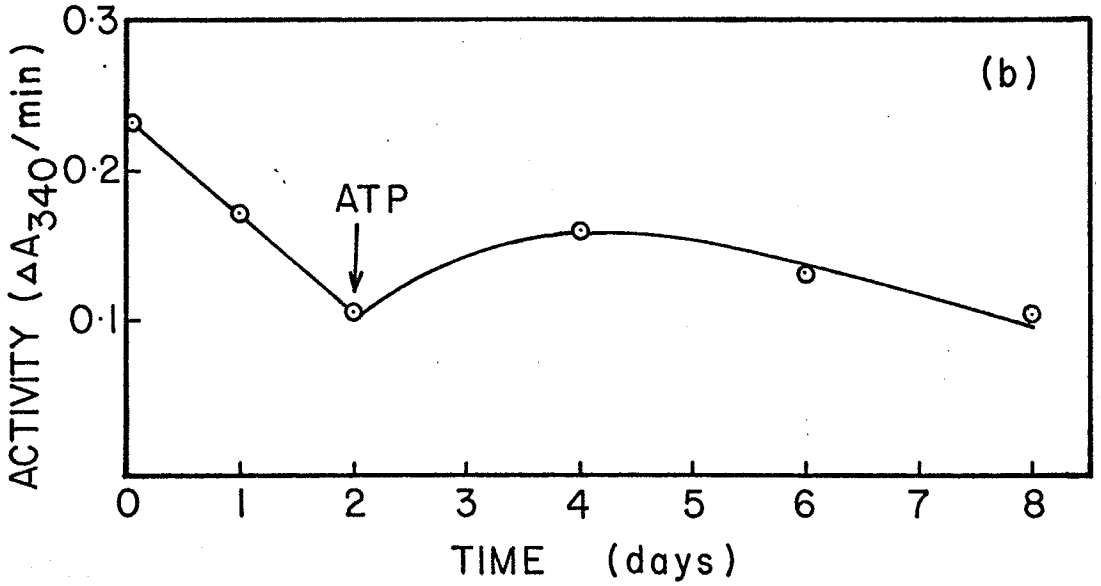

(a)
(b)
0·3
0·2
0·1
0·4
0·3
0·2
0·1
0
1
2
3
4
5
6
7
ACTIVITY (ΔA_{340} / MIN)
TIME (DAYS)

Figure 16a. Effect of ATP on a PFK preparation containing 100 mM $(NH_4)_2SO_4$ during storage at 4^{o}. ATP (5×10^{-4}M) was added at zero time. Assay mixtures were prepared as described in Materials and Methods.

Figure 16b. Reactivation by ATP of a PFK preparation after storage at 4^{o} in the presence of 100 mM $(NH_4)_2SO_4$. ATP (5×10^{-4}M) was added to the enzyme preparation 2 days after the addition of $(NH_4)_2SO_4$. Assay mixtures were prepared as described in Materials and Methods.


0·3
0·2
0·1
ACTIVITY (ΔA340/min)
(a)
0
1
2
3
4
5
24
TIME (hours)


0·3
0·2
0·1
ACTIVITY (ΔA340/min)
(b)
ATP
0
1
2
3
4
5
6
7
8
TIME (days)

after the addition of ATP to an enzyme preparation containing $(NH_4)_2SO_4$ (100 mM), the lowest activity was found to occur within 2 hours, after which a gradual reactivation took place. The initial inactivation rate was comparatively much faster than the reactivation rate. Fig. 16b shows an experiment where ATP was added to an enzyme preparation containing 100 mM $(NH_4)_2SO_4$ which had lost over a half of its activity during storage for two days. ATP stopped further inactivation of the enzyme and a 50% recovery of the lost activity occurred within 2 days of further storage.

These results show that the PFK of A. niger responds to various ligands and environments in a very complex manner.

EFFECT OF CYCLIC 3', 5'-AMP

It was reported in the liver fluke and other mammalian systems that cyclic 3',5'-AMP not only affected the kinetics of PFK, that is, activated PFK in the assay system, but also produced marked activation after incubation with a concentrated enzyme preparation which was then assayed after several fold dilution (Mansour and Mansour, 1962; Stone and Mansour, 1967 a;

Mansour, 1963; Mansour and Ahlfors, 1968). Although preincubation of the crude yeast extract with the cyclic nucleotide could activate the yeast PFK (Viñuela et al., 1964), neither the yeast PFK (Sols and Salas, 1966; Ramaiah et al., 1964) nor the E. coli PFK (Atkinson and Walton, 1965) was affected by its presence in the assay system.

Experiments with the crude extract of A. niger showed that neither preincubation at room temperature for 10 minutes nor storage at 4°C with the cyclic 3',5'-AMP at 1 mM activated the PFK. The effect of cyclic AMP present in the PFK assay mixtures seemed to vary depending on the concentrations of substrates used. The effect was further investigated with the purified enzyme at low as well as at high F6P and ATP levels (Figs. 17 a, b). It was observed that, at a low F6P level (0.5 mM) (Fig. 17b), the cyclic nucleotide inhibited the PFK activity resulting in 50% inhibition at 8 mM cyclic AMP. At a high F6P level (4 mM), on the other hand, the PFK activity was activated at low, but inhibited at high cyclic AMP concentrations when ATP was held at 0.4 or 4 mM (Fig. 17a). At 0.05 mM ATP there was only an inhibition by cyclic AMP.

Figure 17a. Effect of cyclic 3',5'-AMP on the PFK activity at a high F6P level (4 mM). Various concentrations (0-8 mM) of cyclic AMP were included in the assay mixtures which were prepared as described in Materials and Methods. The ATP concentrations used for assay were varied:

● ——— 0.05 mM

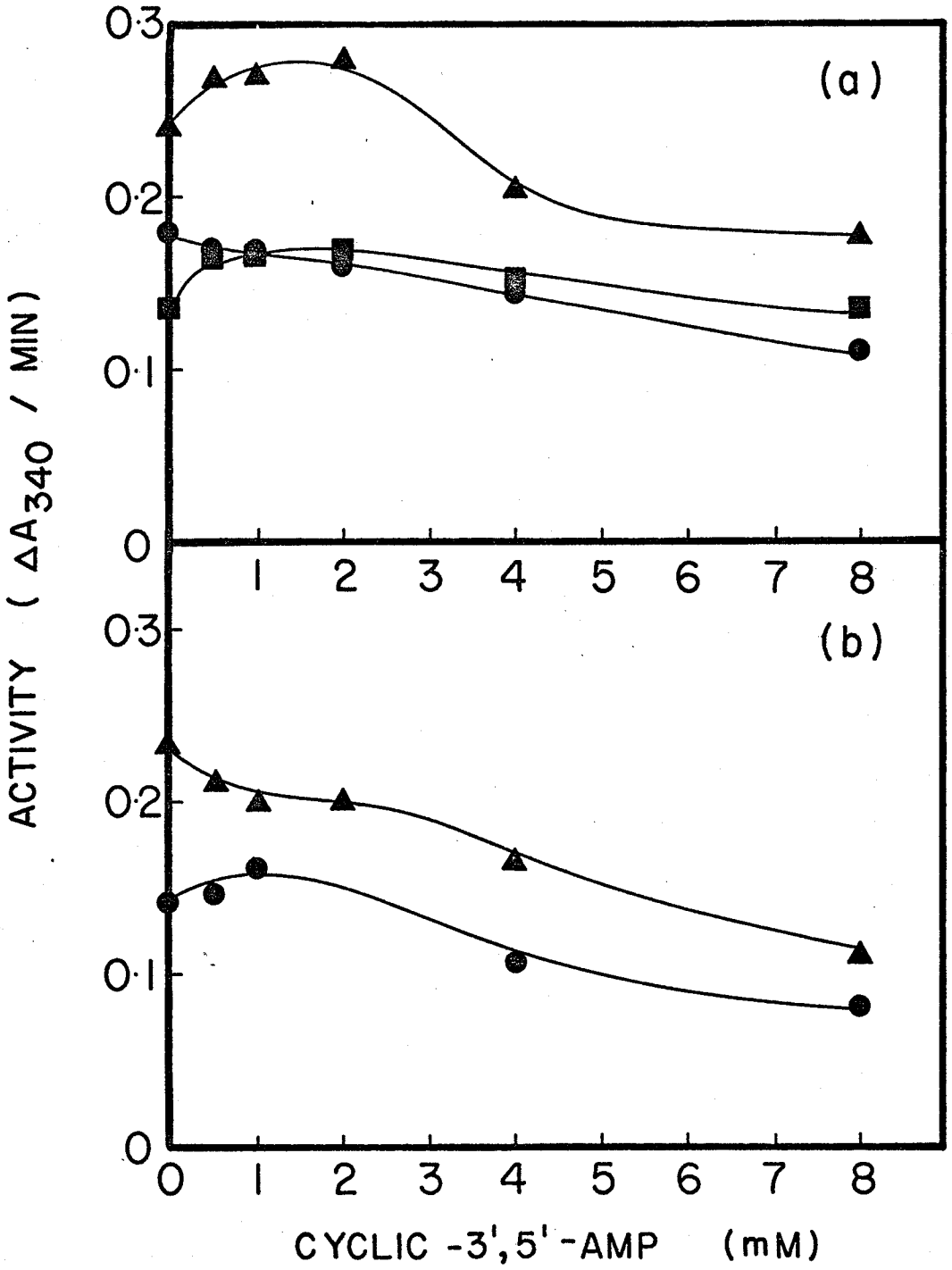
▲ ——— 0.4 mM

■ ——— 4.0 mM

Figure 17b. Effect of cyclic 3',5'-AMP on the PFK activity at a low F6P level (0.5 mM). Various concentrations (0-8 mM) of cyclic AMP were included in the assay mixtures which were prepared as described in Materials and Methods. The ATP concentrations used for assay were varied:

● ——— 0.05 mM

▲ ——— 0.4 mM


0·3
0·2
0·1
0
(a)
1 2 3 4 5 6 7 8
0·3
0·2
0·1
0
(b)
0 1 2 3 4 5 6 7 8
ACTIVITY (ΔA340 / MIN)
CYCLIC -3',5'-AMP (mM)

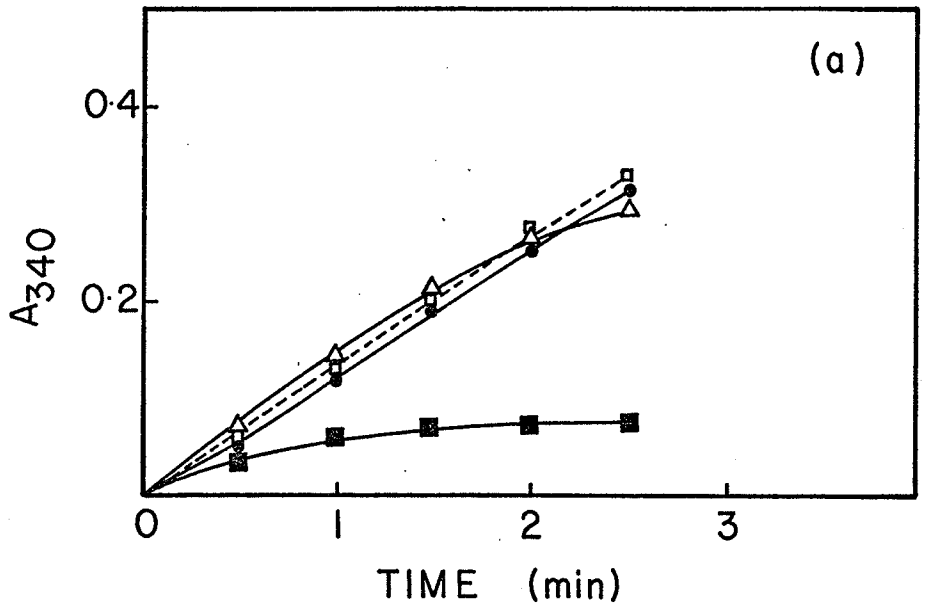
EFFECT OF 5'-AMP

When PFK was assayed at a low F6P level either with the optimal ATP concentration (0.4 mM) or the inhibitory ATP concentration (4 mM), the reaction levelled off very quickly (Fig. 18a, b). In the presence of 1 mM AMP, however, the linear reaction period was much more extended.

At relatively high levels of F6P, AMP had little effect on the PFK activity with 0.4 mM ATP, but had a stimulatory effect when the enzyme was assayed with inhibitory high concentrations of ATP (Fig. 19). Both Fig. 19 and 20 show that AMP could release the inhibition by 4 mM ATP to a considerable extent, but as the ATP level was raised to 6 mM, the relieving effect of AMP was greatly reduced. Increasing the concentration of AMP up to 4 mM did not increase the relieving effect (Fig. 20). Such relieving effect of the ATP inhibition by AMP on the A. niger enzyme was similar to that of the E. coli PFK (Atkinson and Walton, 1965) and the liver fluke PFK (Stone and Mansour, 1967 b).

Figure 18a. Effect of AMP on the PFK assay with 0.4 mM ATP and 0.05 mM F6P. Concentrations of AMP were: 0 mM (■), 0.5 mM (△), 1 mM (▫), and 2 mM (•). Other assay components were as described in Materials and Methods.

Figure 18b. Effect of AMP on the PFK assay with 4 mM ATP. F6P concentrations were 0.1 mM (● , ○), 0.5 mM (▲ , △), and 4 mM (■ , □). Assays were carried out either in the presence of 1 mM AMP (○ , △ , □) or in the absence of AMP (● , ▲ , ■). Other assay components were as described in Materials and Methods.


(a)
0·4
0·2
A340
0
1
2
3
TIME (min)

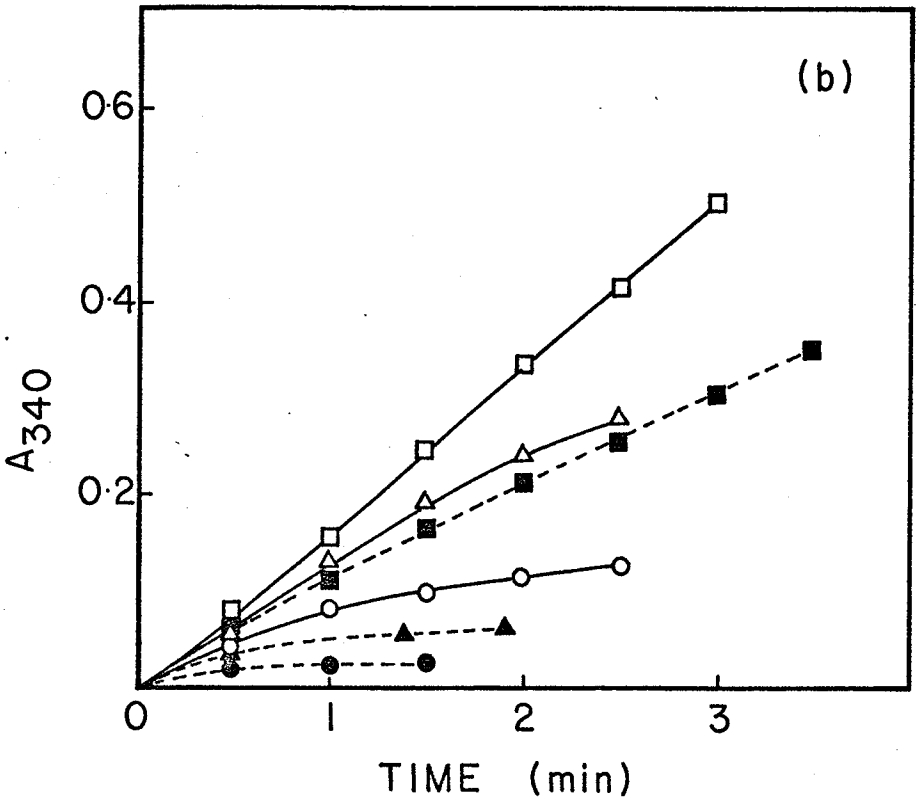

(b)
0·6
0·4
0·2
A340
0
1
2
3
TIME (min)

Figure 19. Effect of AMP (1 mM) on the PFK activity at different concentrations of ATP. Assays were carried out either in the presence of AMP (○ , □ , △) or in its absence (● , ■ , ▲). Other assay components were as described in Materials and Methods except that the concentrations of F6P and ATP were varied as shown.

Concentrations of ATP were:

○ , ● ——— 0.4 mM

□ , ■ ——— 4.0 mM

△ , ▲ ——— 6.0 mM

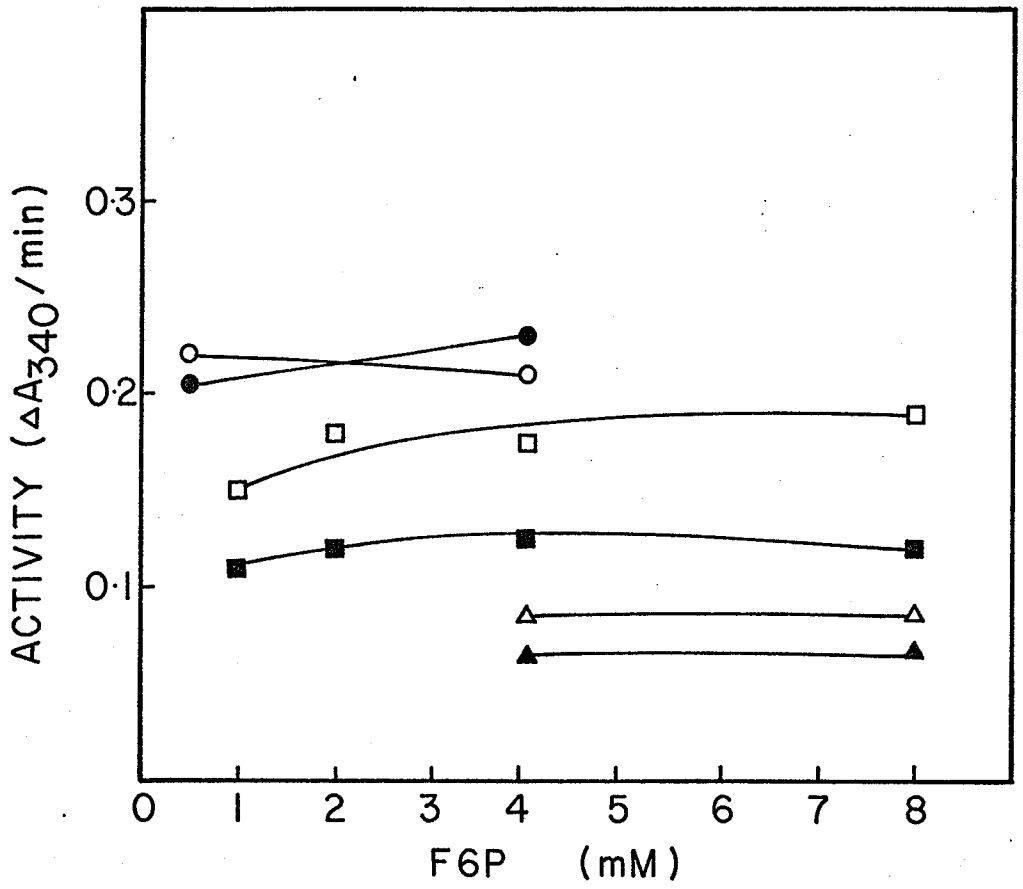
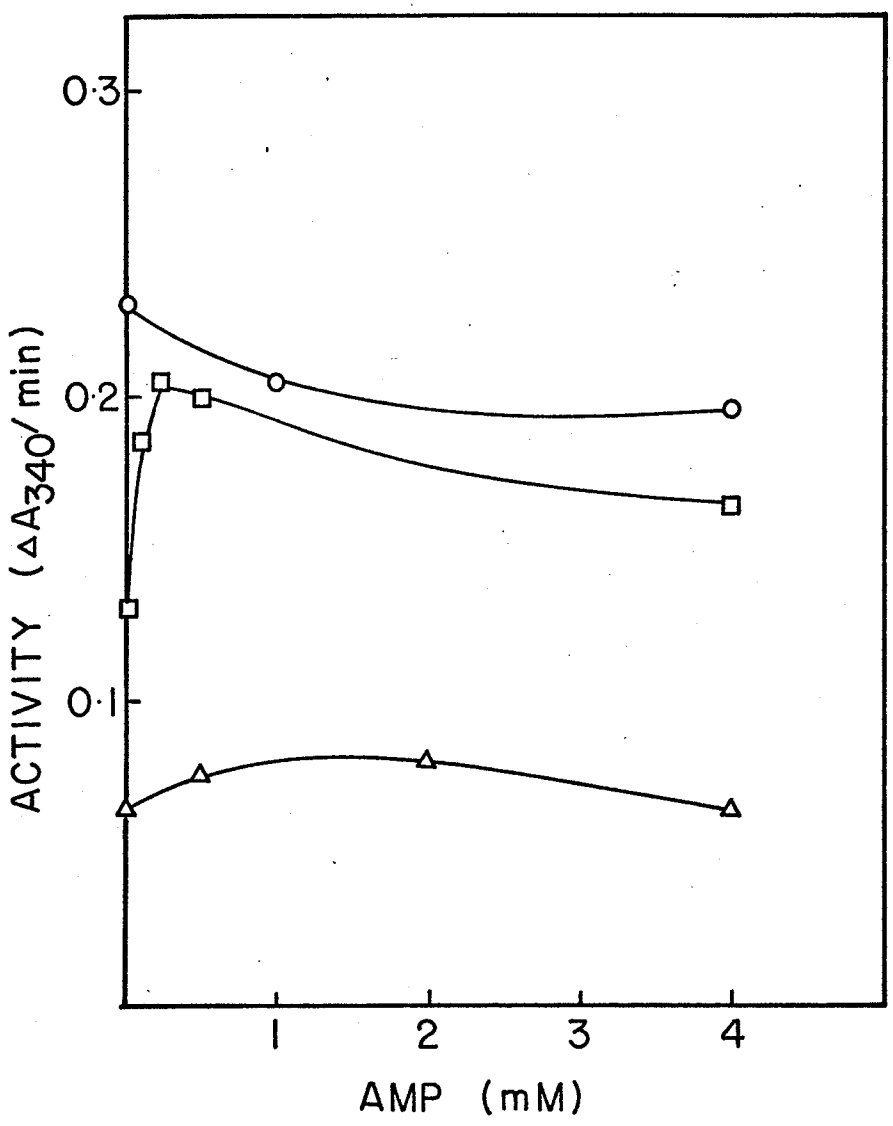

ACTIVITY (ΔA_{340}/min)
0·3
0·2
0·1
0
1
2
3
4
5
6
7
8
F6P (mM)

Figure 20. Effect of various concentrations of AMP on the PFK activity. The F6P concentration was 4 mM. Other assay components were as described in Materials and Methods except that the concentrations of ATP were varied as follows:

○ ——— 0.4 mM

□ ——— 4.0 mM

△ ——— 6.0 mM


0·3
0·2
0·1
ACTIVITY (ΔA340/min)
1
2
3
4
AMP (mM)

KINETICS OF PHOSPHOFRUCTOKINASE

All velocity data were obtained from either the initial linear rate of reaction or the fastest linear rate when a lag was present. These were plotted either in the form of reaction velocity (v) versus substrate concentration (S) or in the double reciprocal form (1/v versus 1/S) according to Lineweaver and Burk (1934). The nomenclature of reaction mechanisms and definitions of kinetic constants followed Cleland's proposal (1963 a).

When the standard assay procedure (i.e., the initiation of reaction by the addition of enzyme) was used at low concentrations of F6P, the enzyme activity levelled off at such a rate that it was difficult to measure the initial velocity of the reaction. If, however, the enzyme was preincubated with the reaction components first except F6P and the reaction was started with the addition of F6P, the originally fast initial rate was eliminated and a much lower linear initial rate was obtained. Such a phenomenon only appeared at low F6P levels. There was little difference in the reaction rate between the two assay methods at high F6P levels.

Hence two slightly different methods of assay were employed. A) The enzyme was preincubated for

1 minute at room temperature with all reaction components except F6P. The required amount of F6P was then added to start the reaction. B) One mM of 5'-AMP was included in each assay and the reaction was started with the addition of enzyme.

Effect of F6P concentrations. The reaction velocities were measured at varied concentrations of F6P and several fixed concentrations of ATP. The plots of v versus S are shown in Figs. 21 and 23.

The data of Fig. 21 were obtained from the pre-incubation assays. The sigmoid nature of F6P concentration - velocity plots was evident. The concentration of F6P required for a half maximal velocity ($S_{0.5}$) (Koshland et al., 1966) increased from 0.3 mM at 0.025 mM ATP to 2.2 mM at 4 mM ATP. The phosphofructokinase from A. niger is obviously an allosteric enzyme (Monod et al, 1965) and the F6P concentration affects the enzyme activity co-operatively. Increasing ATP concentrations seemed to make the binding of F6P more difficult, thus raising the $S_{0.5}$ values. Very high ATP concentrations (over 2 mM), in addition, lowered the maximal velocity.

The degree of cooperativity of the binding sites for F6P under different ATP concentrations may be estimated by the Hill equation (Changeux, 1963) which

is expressed as:

$$\log \left(\frac{v}{V_m - v} \right) = n \log S - \log K$$

where S is the substrate (F6P) concentration, K is a constant, and n is a function of the strength of co-operative interaction. The maximum velocity (V_m) was determined at 4 mM F6P and 0.4 mM ATP. In Fig. 22, the data at 0.025, 0.4, 2, and 4 mM ATP from Fig. 21 were plotted according to the Hill equation. The values of n were obtained from the maximum slopes. It is seen that as the ATP concentrations increased, the values of n also increased, from 1.88 at 0.025 mM ATP to 3.5 at 4 mM ATP. Hence, raising the ATP concentrations increased the co-operative interaction for F6P binding.

The data of Fig. 23 were obtained from the assays containing 5'-AMP (1 mM). Instead of sigmoid curves, normal hyperbolic curves were obtained. The $S_{0.5}$ values of F6P were reduced 30- to 40-fold in the presence of AMP (0.6 ——→ 0.02 mM at 0.4 mM ATP and 2.2 ——→ 0.05 mM at 4 mM ATP). Therefore the presence of AMP increased the affinity of the enzyme for F6P at low ATP as well as at high ATP levels. The Hill plot in 0.4 mM ATP gave a value of 1.2 for n, approximating the Michaelis-Menten kinetics (Fig. 24).

Figure 21. Effect of F6P concentrations on the PFK activity at various ATP concentrations in the absence of AMP. F6P was varied in the range of 0.1 mM to 8 mM. The ATP concentrations were 0.01 mM (◒), 0.025 mM (○), 0.05 mM (△), 0.1 mM (▲), 0.4 mM (□), 2 mM (■), and 4 mM (●). Other assay components were as described in Materials and Methods. The enzyme was incubated in the assay mixture at room temperature for 1 minute, after which F6P was added to start the reaction.

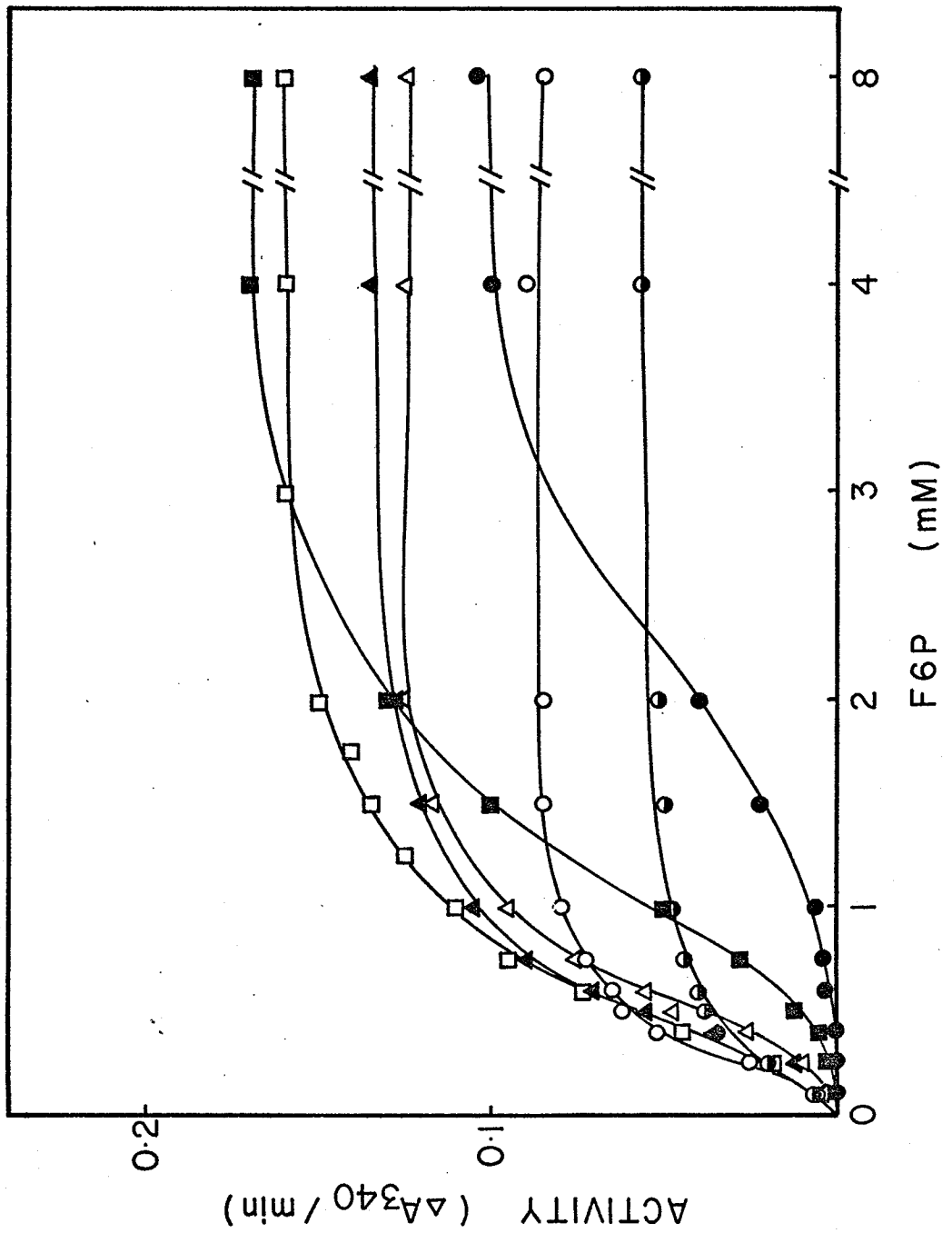

ACTIVITY (ΔA_{340} / min)
0·2
0·1
0
1
2
3
4
8
F6P (mM)

Figure 22. Hill plots of the data from Fig. 21.

Concentrations of ATP were:

○ ——— 0.025 mM

● ——— 0.4 mM

△ ——— 2.0 mM

□ ——— 4.0 mM

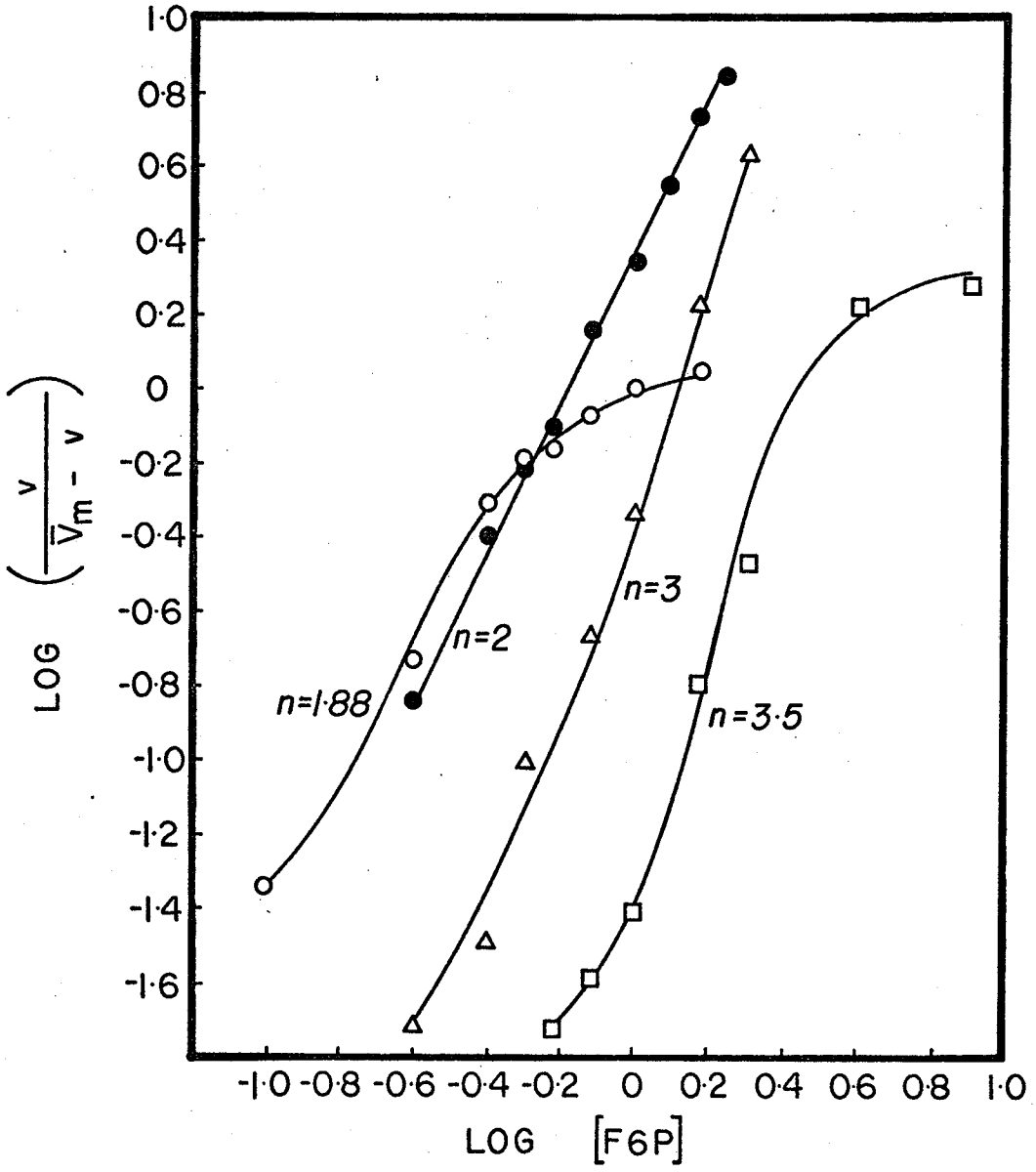

1·0
0·8
0·6
0·4
0·2
0
-0·2
-0·4
-0·6
-0·8
-1·0
-1·2
-1·4
-1·6
LOG (v / (Vm - v))
n=1·88
n=2
n=3
n=3·5
-1·0 -0·8 -0·6 -0·4 -0·2 0 0·2 0·4 0·6 0·8 1·0
LOG [F6P]

Figure 23. Effect of F6P concentrations on the PFK activity at various ATP concentrations in the presence of AMP. F6P was varied in the range of 0.01 mM to 4 mM. The ATP concentrations were 0.01 mM (▽), 0.025 mM (◇), 0.05 mM (△), 0.1 mM (□), 0.4 mM (●), 4 mM (▲), and 6 mM (○). Other assay components were as described in Materials and Methods. Reactions were started by the addition of PFK preparation.

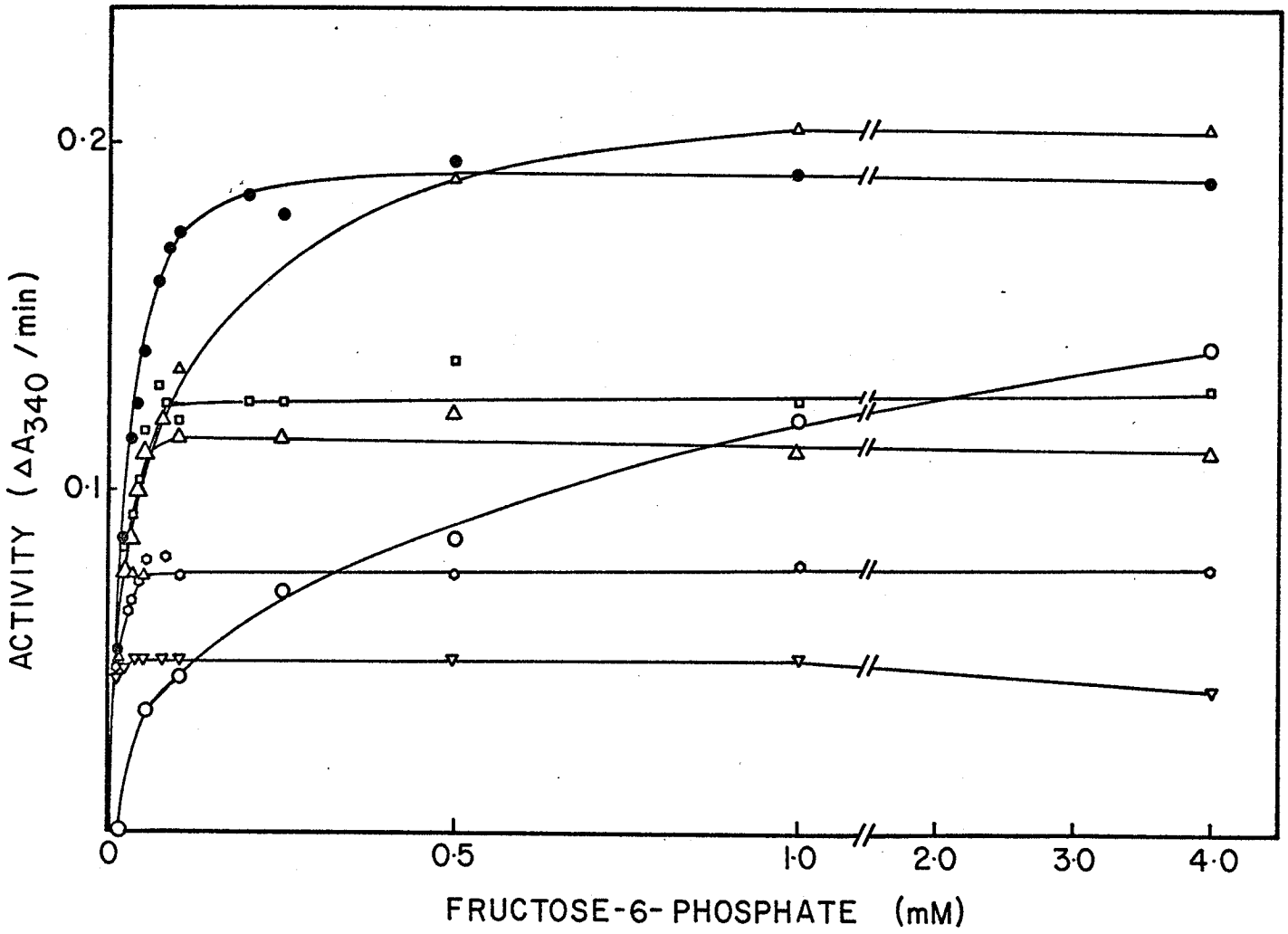
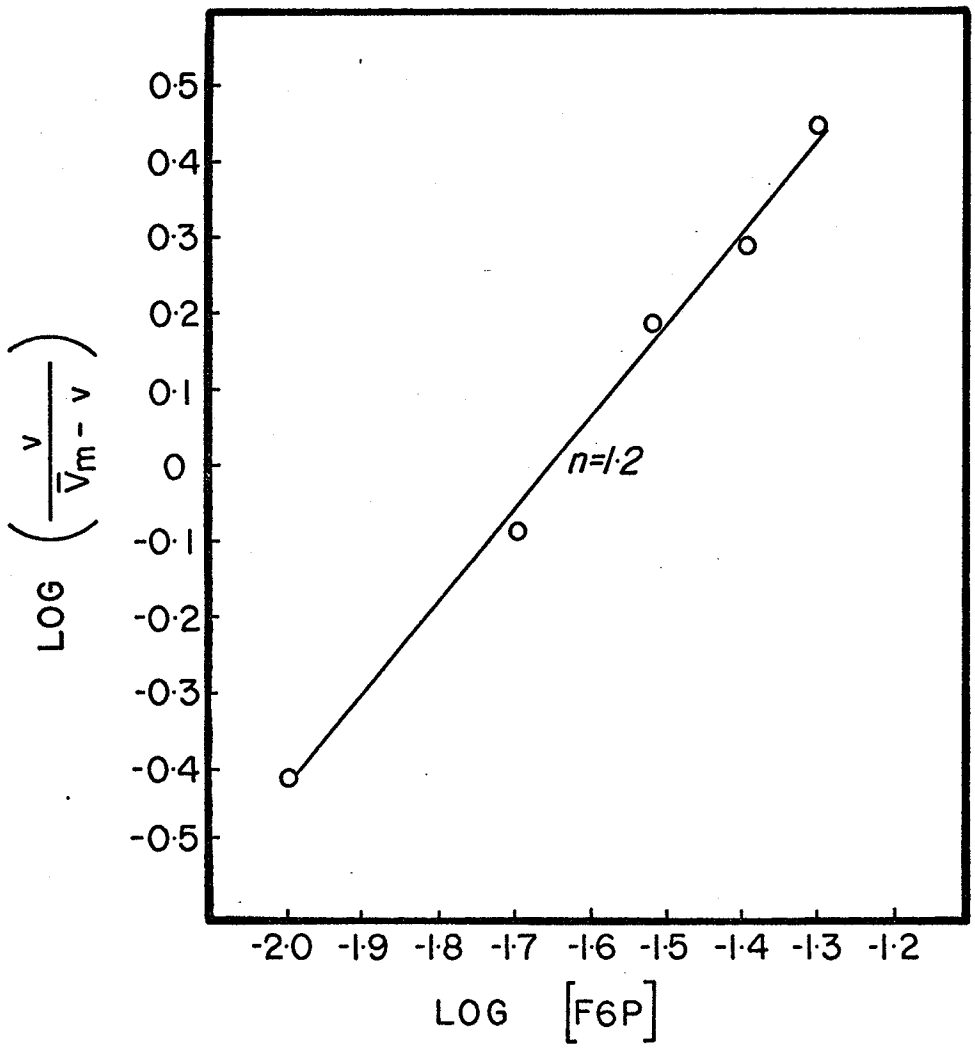

0·2
0·1
ACTIVITY (ΔA340 /min)
0
0·5
1·0
2·0
3·0
4·0
FRUCTOSE-6-PHOSPHATE (mM)

Figure 24. A Hill plot of the data from Fig. 23. The concentration of ATP was 0.4 mM.


0·5
0·4
0·3
0·2
0·1
0
-0·1
-0·2
-0·3
-0·4
-0·5
LOG (v / (Vm - v))
n=1·2
-2·0 -1·9 -1·8 -1·7 -1·6 -1·5 -1·4 -1·3 -1·2
LOG [F6P]

Effect of ATP concentrations. Both Figs. 25 and 26 are the ATP concentration - velocity plots at several fixed concentrations of F6P, obtained from the preincubation assays and the assays containing AMP respectively. It is clearly shown in both figures that ATP acts as a substrate as well as an inhibitor of the enzyme.

As indicated in Fig. 25, when assayed at a F6P concentration of 4 mM, the enzyme showed maximal activity at an ATP concentration range of 0.4 to 2 mM (optimal concentrations). At higher concentrations of ATP, the enzyme was inhibited, resulting in 40% inhibition at 4 mM. The $S_{0.5}$ of ATP at 4 mM F6P was 0.02 mM. As F6P concentrations decreased, the degree of ATP inhibition increased (more than 90% inhibition by 4 mM ATP at 1 mM F6P). The concentration of F6P had only a slight effect on the $S_{0.5}$ value of ATP.

The activity pattern of Fig. 26, obtained in the presence of AMP, was similar to that of Fig. 25. The $S_{0.5}$ of ATP at 4 mM F6P was about 0.04 mM. The optimal concentration range of ATP at 4 mM F6P was extended to 4 mM. The degree of inhibition by ATP was much reduced in the presence of AMP.

Determination of true K_m values. Double reciprocal plots (Figs. 27a and 28a) were obtained from the data

Figure 25. Effect of ATP concentrations on the PFK activity at various F6P concentrations in the absence of AMP. ATP was varied in the range of 0.01 mM to 4 mM. The F6P concentrations were 1 mM (△), 1.5 mM (○), and 4 mM (□). Other assay components were as described in Materials and Methods. The enzyme was incubated in the assay mixture at room temperature for 1 minute, after which F6P was added to start the reaction.

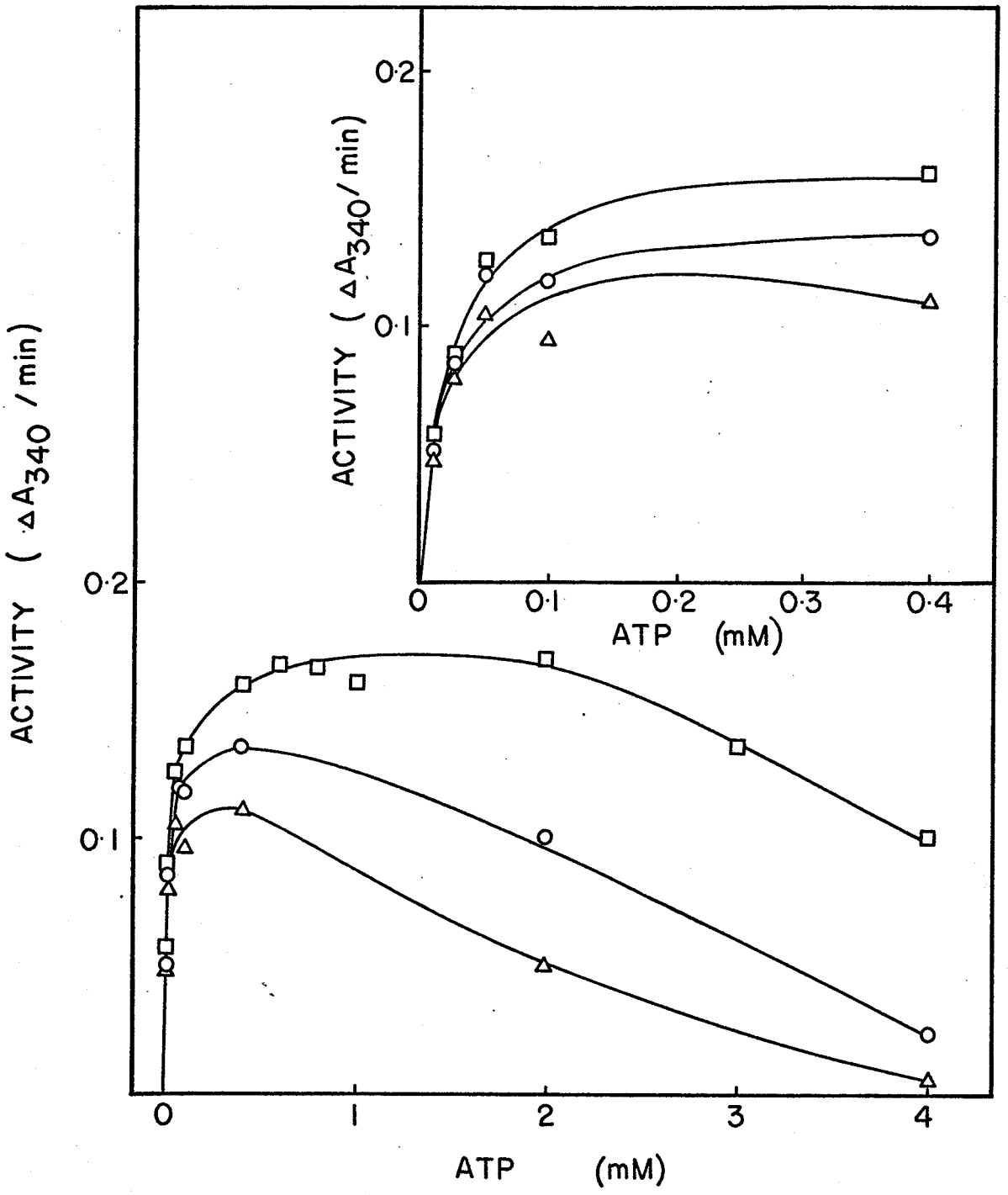

ACTIVITY (ΔA340 / min)
0·2
0·1
0
1
2
3
4
ATP (mM)
ACTIVITY (ΔA340/ min)
0·2
0·1
0
0·1
0·2
0·3
0·4
ATP (mM)

Figure 26. Effect of ATP concentrations on the PFK activity at various F6P concentrations in the presence of AMP. ATP was varied in the range of 0.01 mM to 6 mM. The F6P concentrations were 0.05 mM (□), 0.1 mM (△), 0.5 mM (○), and 4 mM (●). Other assay components were as described in Materials and Methods. Reactions were started by the addition of PFK preparation.

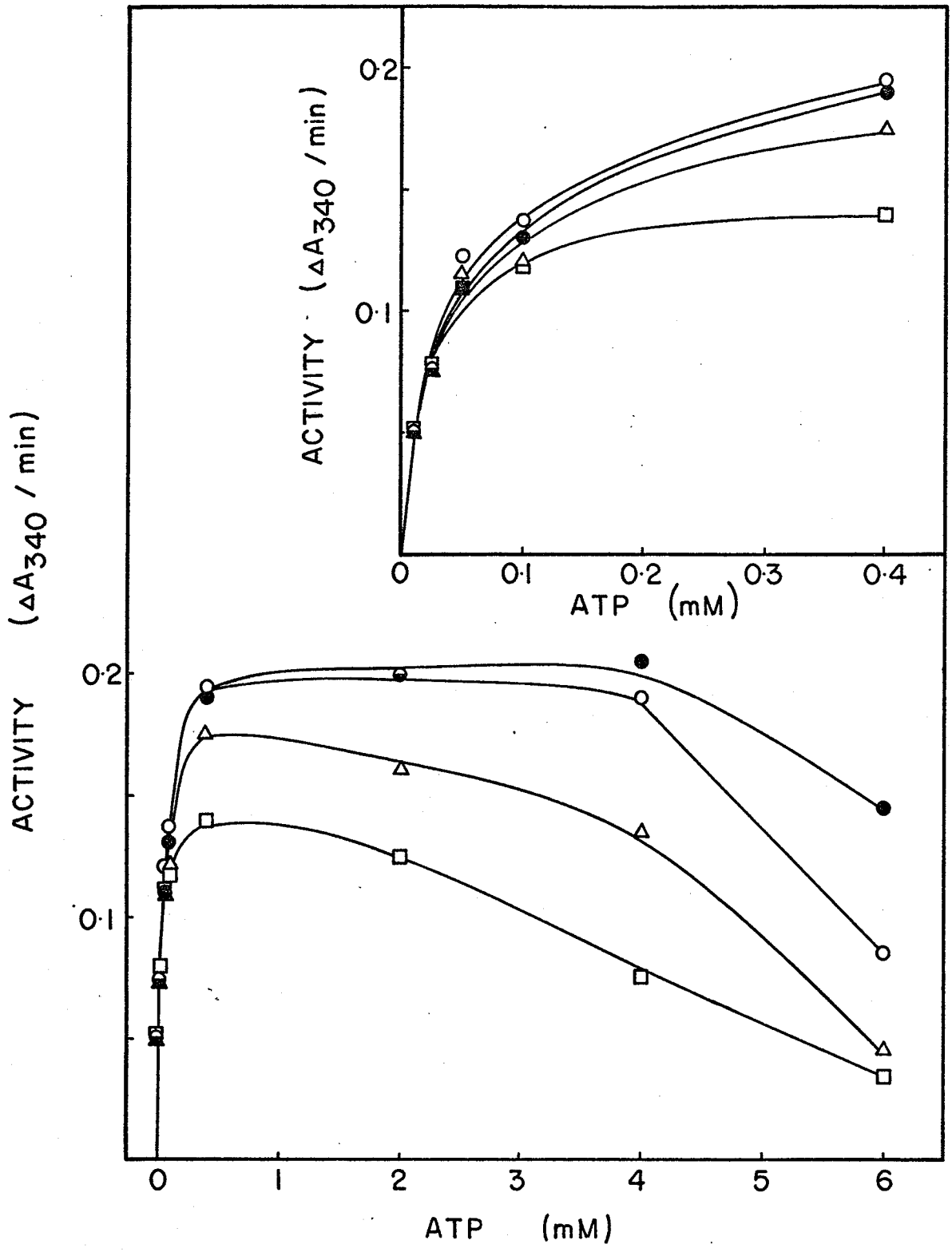

ACTIVITY (ΔA340 / min)
0·2
0·1
0
1
2
3
4
5
6
ATP (mM)
ACTIVITY (ΔA340 / min)
0·2
0·1
0
0·1
0·2
0·3
0·4
ATP (mM)

Figure 27a. Double reciprocal plots of velocity versus varying F6P concentrations with ATP as fixed variable (in the presence of AMP).

ATP concentrations:

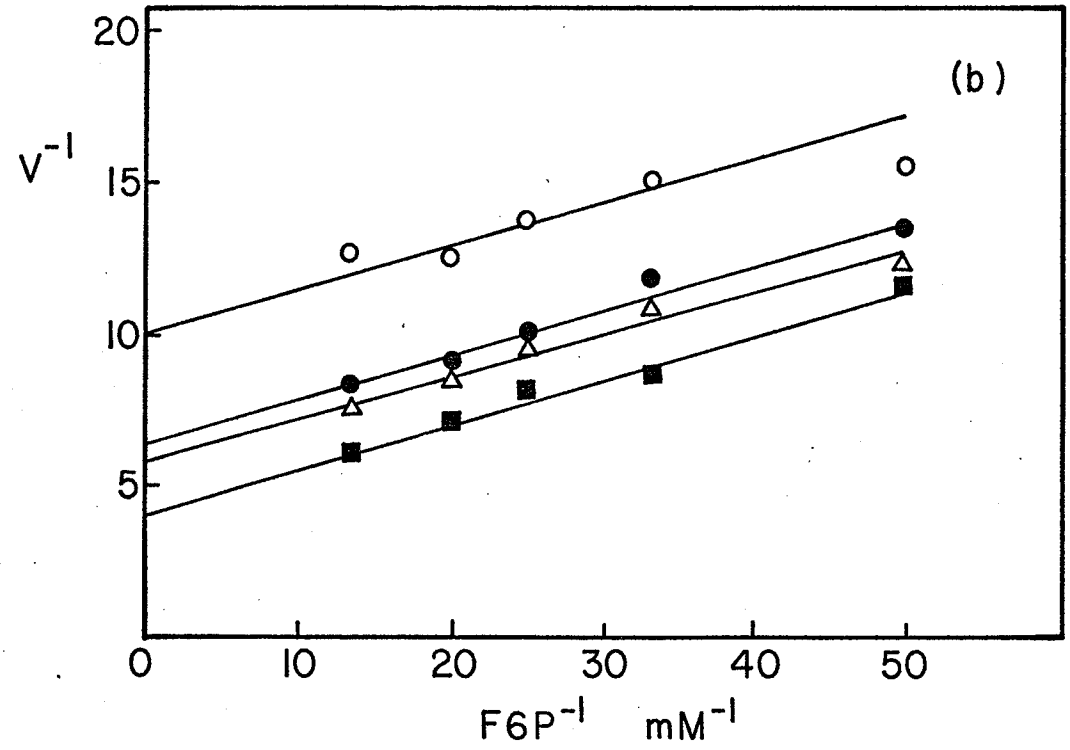
○ ———— 0.025 mM

● ———— 0.05 mM

△ ———— 0.1 mM

■ ———— 0.4 mM

Figure 27b. Replot of intercepts versus reciprocals of ATP from Fig. 27a.


20
15
10
5
V^{-1}
(b)
0
10
20
30
40
50
$F6P^{-1}$ mM^{-1}

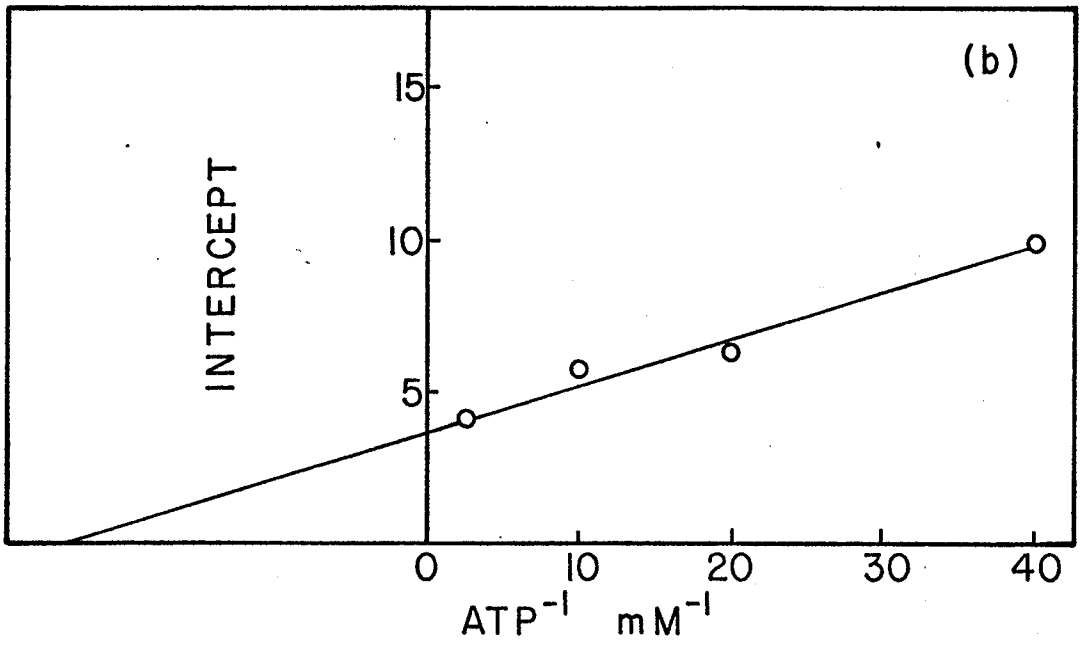

(b)
15
10
5
INTERCEPT
0
10
20
30
40
ATP^{-1} mM^{-1}

Figure 28a. Double reciprocal plots of velocity versus varying ATP concentrations with F6P as fixed variable (in the presence of AMP).

F6P concentrations:

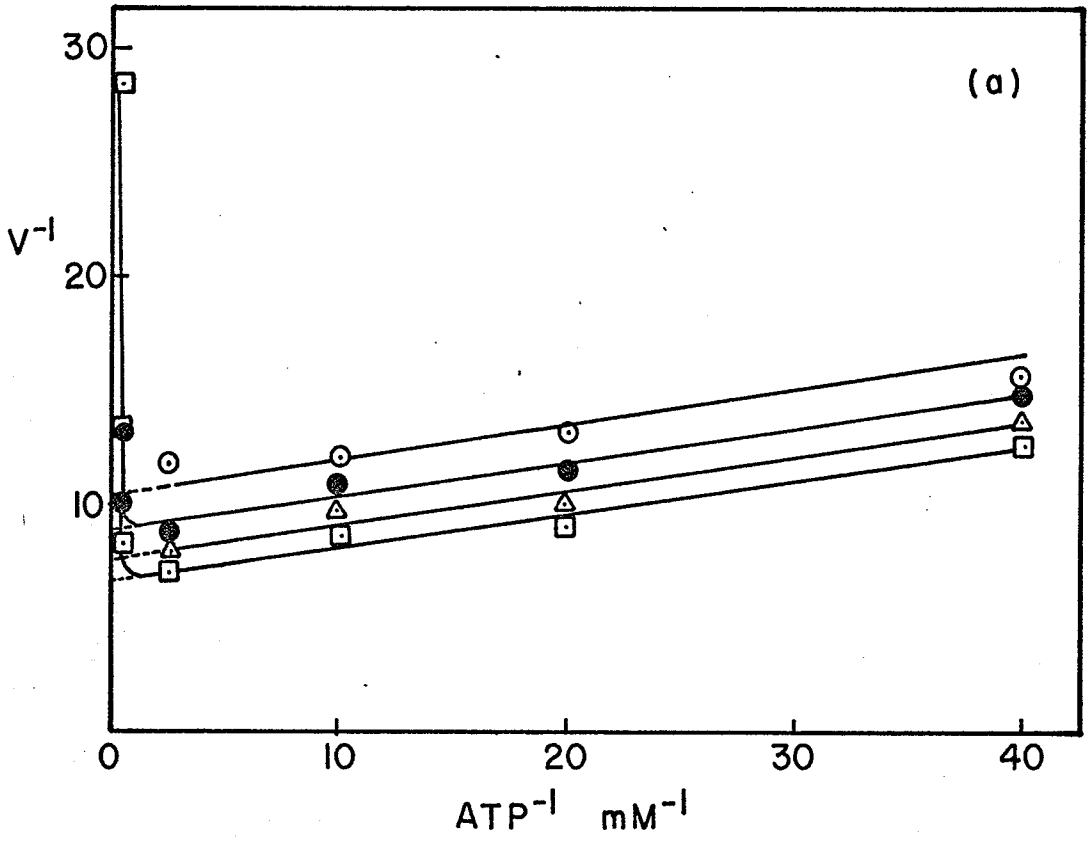
⊙ —— 0.02 mM

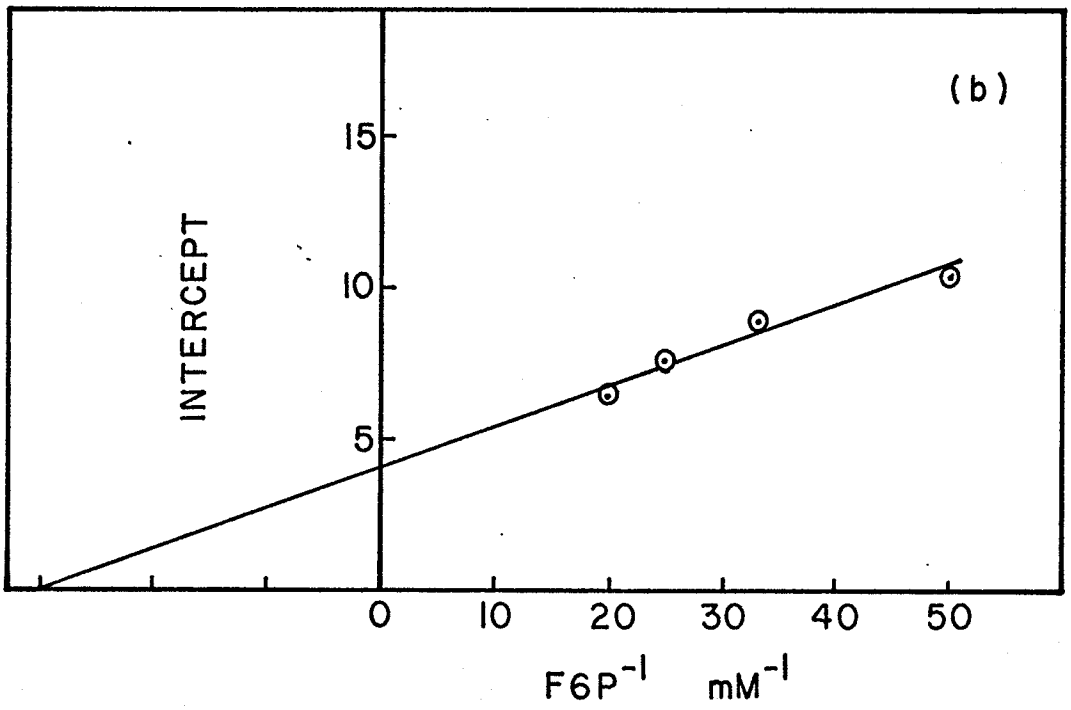
● —— 0.03 mM

△ —— 0.04 mM

⊡ —— 0.05 mM

Figure 28b. Replot of intercepts versus reciprocals of F6P from Fig. 28a.


(a)
30
20
10
V⁻¹
0
10
20
30
40
ATP⁻¹ mM⁻¹


(b)
15
10
5
INTERCEPT
0
10
20
30
40
50
F6P⁻¹ mM⁻¹

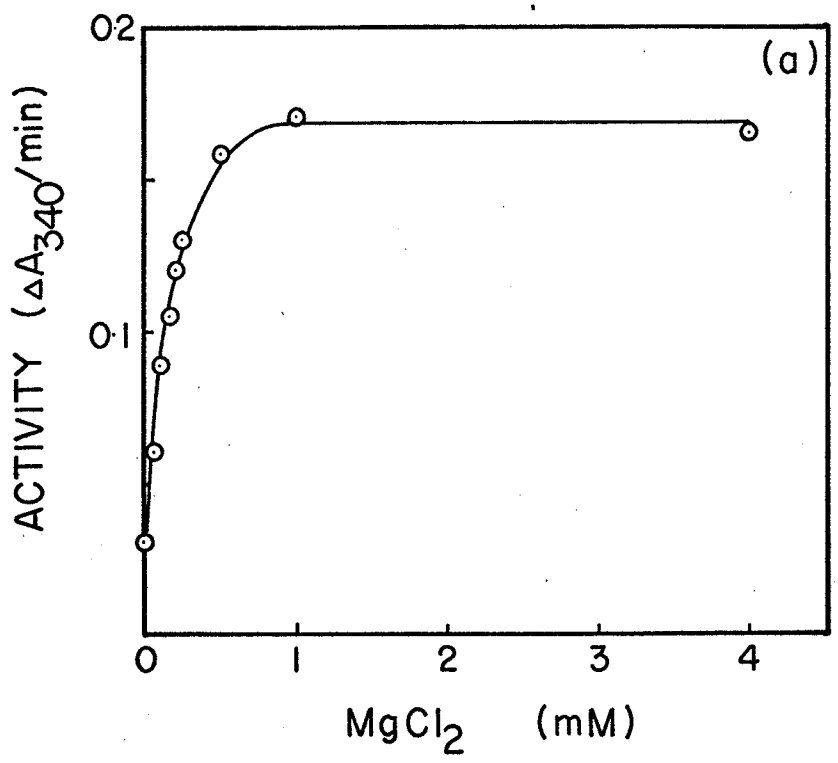
of Figs. 23 and 26 (in the presence of AMP) and the intercepts on the vertical axis were then replotted against the reciprocals of the fixed concentrations of the respective substrate (Florini and Vestling, 1957) in Figs. 27b and 28b. True Michaelis constants (Km) were then determined from the intercepts at the horizontal axis of the replots.

Parallel lines were obtained in the double reciprocal plots of velocity versus F6P concentrations (Fig. 27a) or ATP concentrations (Fig. 28a) and true Km values of 0.042 mM ATP and 0.033 mM F6P were obtained from the replots (Fig. 27b and Fig. 28b). A typical substrate inhibition pattern was observed in Fig. 28a where the lines curved upward towards the vertical axis asymptotically when ATP concentrations were increased to very high levels.

Apparent K_m for Mg^{++}. The plots for v versus S ($MgCl_2$) are shown in Figs. 29a (no AMP) and 30a (with 1 mM AMP). Concentrations of F6P and ATP were those under the standard conditions as stated in Materials and Methods (4 mM and 0.4 mM, respectively). Both figures gave hyperbolic curves. The presence of activity in the absence of Mg^{++} hinted that Mg^{++} was probably required

Figure 29a. Effect of Mg^{++} concentrations on the PFK activity in the absence of AMP. $MgCl_2$ was varied in the range of 0 mM to 4 mM. Other assay components were as described in Materials and Methods. The enzyme was incubated in the assay mixture at room temperature for 1 minute, after which F6P was added to start the reaction.

Figure 29b. A double reciprocal plot of Fig. 29a.


(a)
0·2
0·1
ACTIVITY (ΔA_{340}/min)
0
1
2
3
4
$MgCl_2$ (mM)

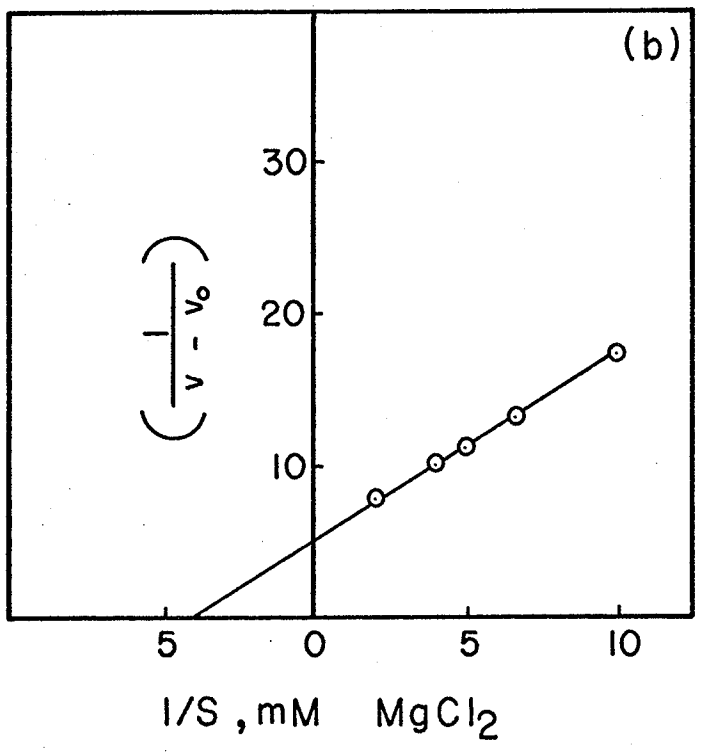
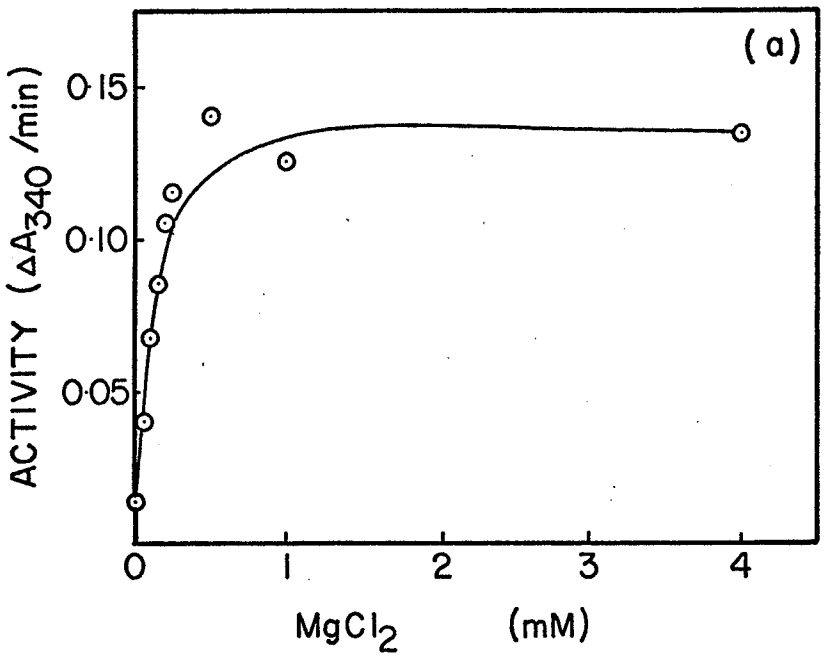
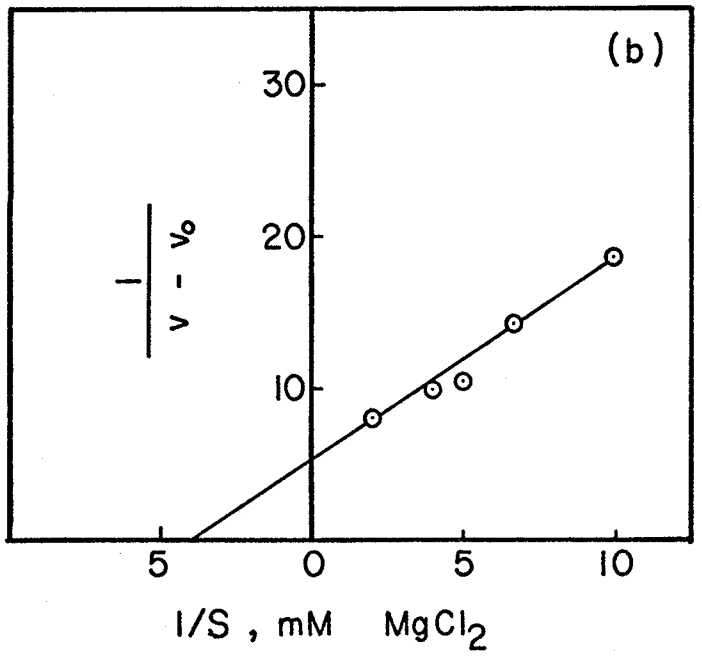

(b)
30
20
10
$\left(\frac{1}{v - v_0}\right)$
5
0
5
10
1/S, mM $MgCl_2$

Figure 30a. Effect of Mg^{++} concentrations on the PFK activity in the presence of AMP. $MgCl_2$ was varied in the range of 0 mM to 4 mM. Other assay components were as described in Materials and Methods. Reactions were started by the addition of PFK preparation .

Figure 30b. A double reciprocal plot of Fig. 30a.


(a)
ACTIVITY (ΔA_{340} /min)
0·15
0·10
0·05
0
1
2
3
4
$MgCl_2$ (mM)


(b)
$\frac{1}{v - v_0}$
30
20
10
5
0
5
10
1/S , mM $MgCl_2$

for activation of the enzyme.

The double reciprocal plots of $\frac{1}{v - v_o}$ (where v_o is the velocity at 0 mM $MgCl_2$) versus S were linear. An apparent K_m value of 0.23 mM (Fig. 29b) or 0.25 mM Mg^{++} (Fig. 30b) was obtained. Thus the apparent K_m for Mg^{++} was not affected by the presence of AMP.

PRODUCT INHIBITION STUDIES

Inhibition by the products, ADP and FDP, was studied at varied concentrations of one substrate and several fixed concentrations of one product. Other conditions were the same as those in the standard assay with 1 mM AMP. The inhibition constants (K_i) were determined by replotting the intercepts and slopes from the double reciprocal plots against the inhibitor concentration. The intercepts at the horizontal axis gave the values of K_i intercept (K_{iI}) and K_i slope (K_{is}).

The terminology used for various types of inhibition is that of Cleland (1963 b). If only the slope changes with the concentration of the inhibitor in the double reciprocal plots, the inhibition is competitive. If both the slope and the vertical intercept change, the inhibition is noncompetitive. If only the intercept changes, it is uncompetitive.

ADP as inhibitor. When F6P was varied at several fixed concentrations of ADP, normal hyperbolic curves as shown in Fig. 31 were obtained. The double reciprocal plots were of linear parallel pattern indicating uncompetitive inhibition at low concentrations of F6P (Fig. 32a). The replot of intercepts versus ADP concentrations was linear and yielded a K_{iI} value of 1.75 mM (Fig. 32b).

The inhibition by ADP showed a complex pattern when ATP was a variable substrate because of PFK inhibition at inhibitory ATP level (Fig. 33). The double reciprocal plots of the data at non-inhibitory concentrations of ATP gave a biphasic pattern (Fig. 34a). ADP was a competitive inhibitor with respect to ATP at high ATP concentrations. At low ATP concentrations, however, the double reciprocal plots levelled off increasingly as the ADP concentration increased, indicating some kind of mixed inhibition. The K_{is} for the competitive portion was 0.3 mM (Fig. 34b).

FDP as inhibitor. Assays were carried out as stated in Materials and Methods. Fig. 35a is the F6P-velocity plots at different FDP concentrations. Effect of FDP as an inhibitor depended on the concentrations of F6P used. At low F6P concentrations (0.01 to 0.1 mM), the enzyme activity was inhibited by FDP at a concentration as low as 0.25 mM. When F6P concentrations were raised,

however, the concentration of FDP required to induce any inhibition also increased. In Fig. 35a, the F6P-velocity plot at 0.5 mM FDP displayed negative co-operativity at higher F6P concentrations. At 4 mM F6P, no inhibition was shown at a concentration of FDP as high as 6 mM. The effect of FDP inhibition at several fixed concentrations of F6P were compared and shown in Fig. 35b.

Linear parallel double reciprocal plots were obtained when F6P concentrations were varied from 0.025 to 0.1 mM, suggesting uncompetitive inhibition (Fig. 36a). Replot of intercepts was linear (Fig. 36b) and gave a K_{iI} value of 1.05 mM for FDP. Although the effect of FDP at higher F6P concentrations was not thoroughly studied, it is speculated from the data of Fig. 35a and b that competitive inhibition took place at high F6P concentrations.

With the concentrations of F6P being kept constant at 1 mM, ATP was the variable at several fixed concentrations of FDP (Fig. 37). The double reciprocal plots (Fig. 38a) were of linear parallel pattern indicating uncompetitive inhibition. Replot of intercepts versus FDP concentrations was linear (Fig. 38b) and gave a value of 9.3 mM for K_{iI}.

Figure 31. Effect of ADP on the PFK activity at varied F6P concentrations. F6P was varied in the range of 0.01 mM to 8 mM. The ADP concentrations were 0 mM (●), 0.5 mM (△), 1 mM (○), and 2 mM (□). Other assay components were as described in Materials and Methods.

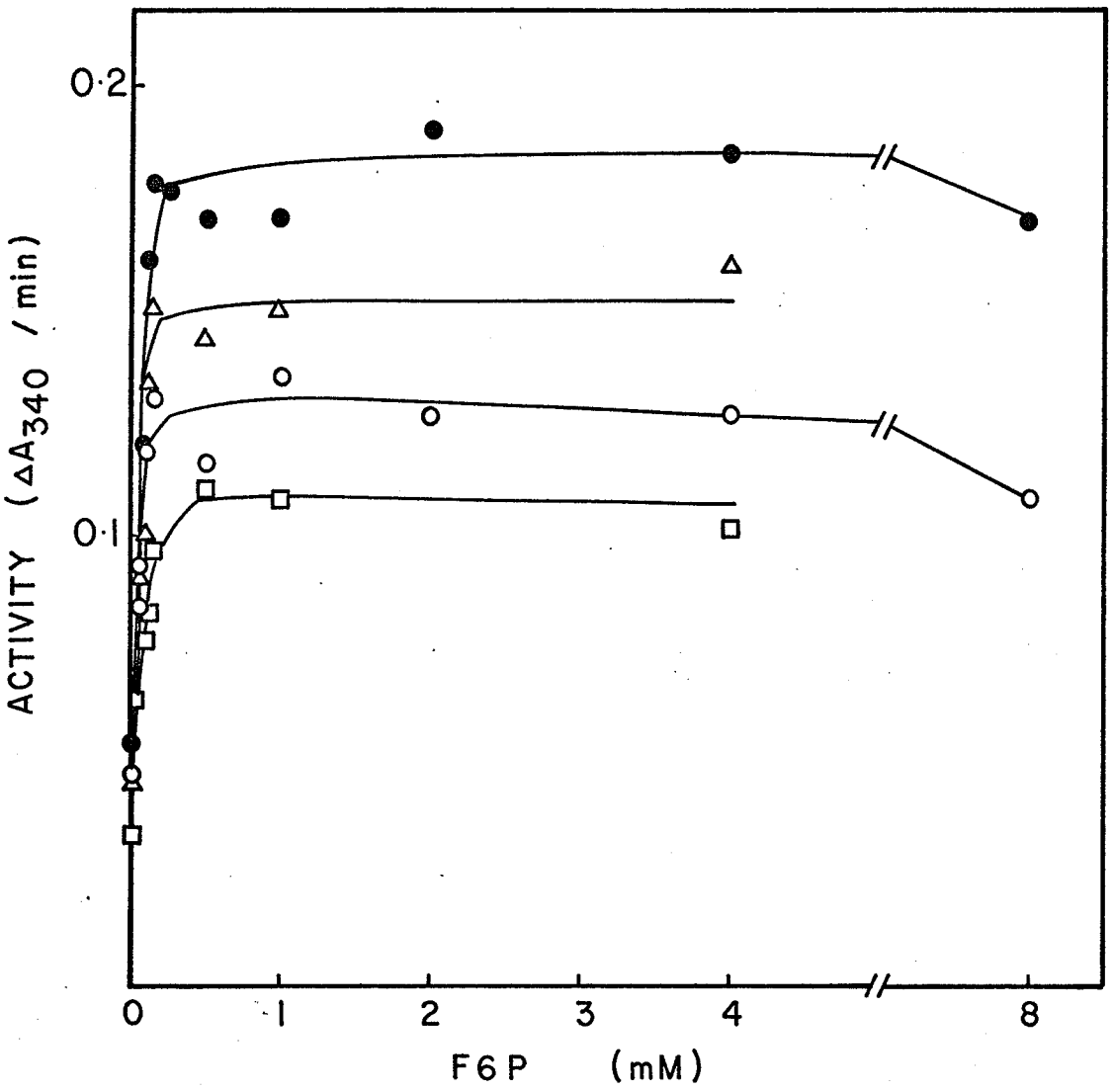

0·2
0·1
ACTIVITY (ΔA340 / min)
0
1
2
3
4
8
F6P (mM)

Figure 32a. Double reciprocal plots of velocity versus varying F6P concentrations with ADP as fixed variable product. ADP concentrations:

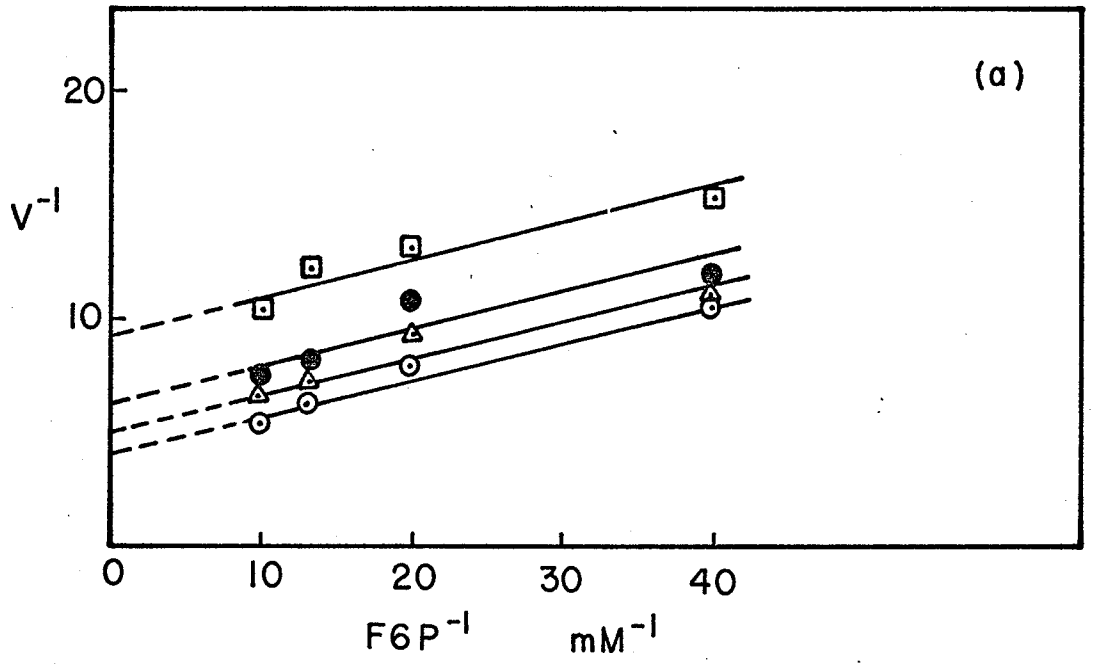
⊙ —— 0 mM

△ —— 0.5 mM

● —— 1.0 mM

⊡ —— 2.0 mM

Figure 32b. Replot of intercepts versus ADP concentrations from Fig. 32a.


(a)
20
10
V^{-1}
0
10
20
30
40
$F6P^{-1}$ mM^{-1}

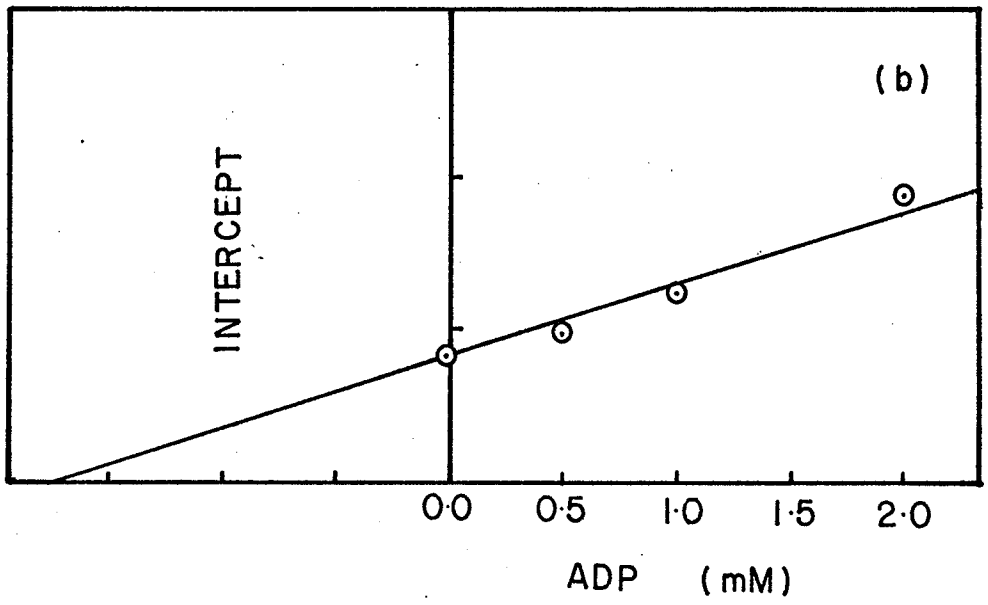

(b)
INTERCEPT
0·0
0·5
1·0
1·5
2·0
ADP (mM)

Figure 33. Effect of ADP on the PFK activity at varied ATP concentrations. ATP was varied in the range of 0.025 mM to 4 mM. The ADP concentrations were 0 mM (●), 0.5 mM (○), 1 mM (△), and 2 mM (□). Other assay components were as described in Materials and Methods.

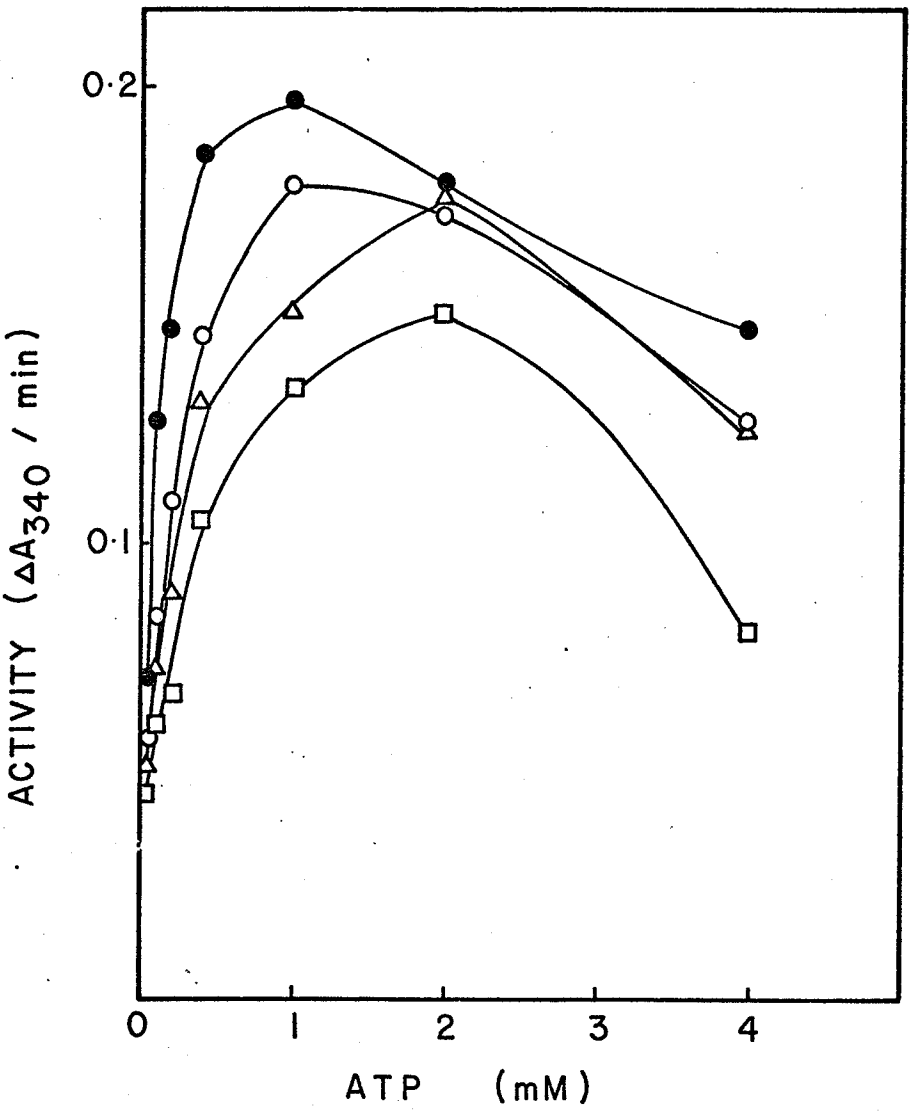

0·2
0·1
ACTIVITY (ΔA340 / min)
0
1
2
3
4
ATP (mM)

Figure 34a. Double reciprocal plots of velocity versus varying ATP concentrations with ADP as fixed variable product. ADP concentrations:

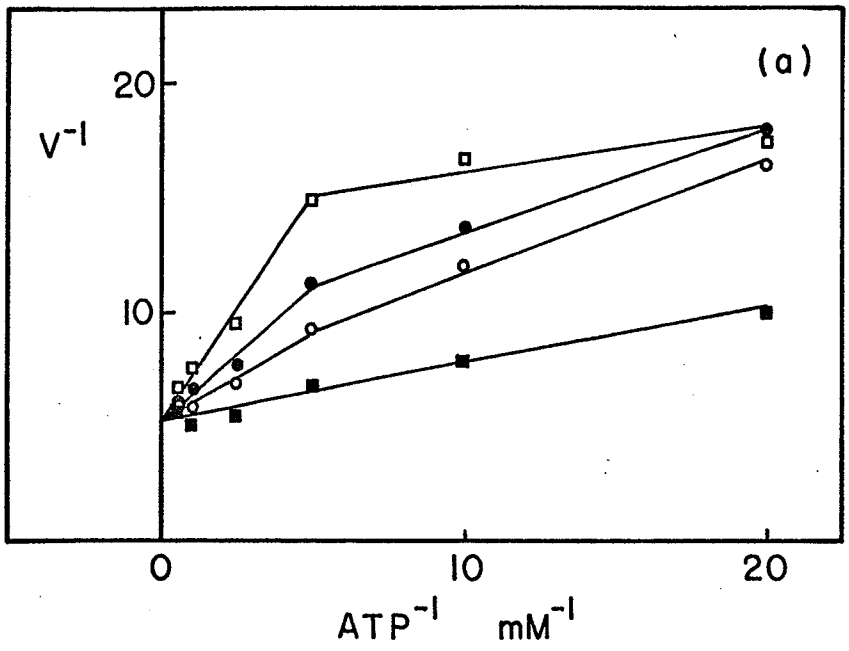
■ ——— 0 mM

○ ——— 0.5 mM

● ——— 1.0 mM

□ ——— 2.0 mM

Figure 34b. Replot of slopes (at high ATP concentrations) versus ADP concentrations from Fig. 34a).


(a)
20
10
V⁻¹
0
10
20
ATP⁻¹ mM⁻¹

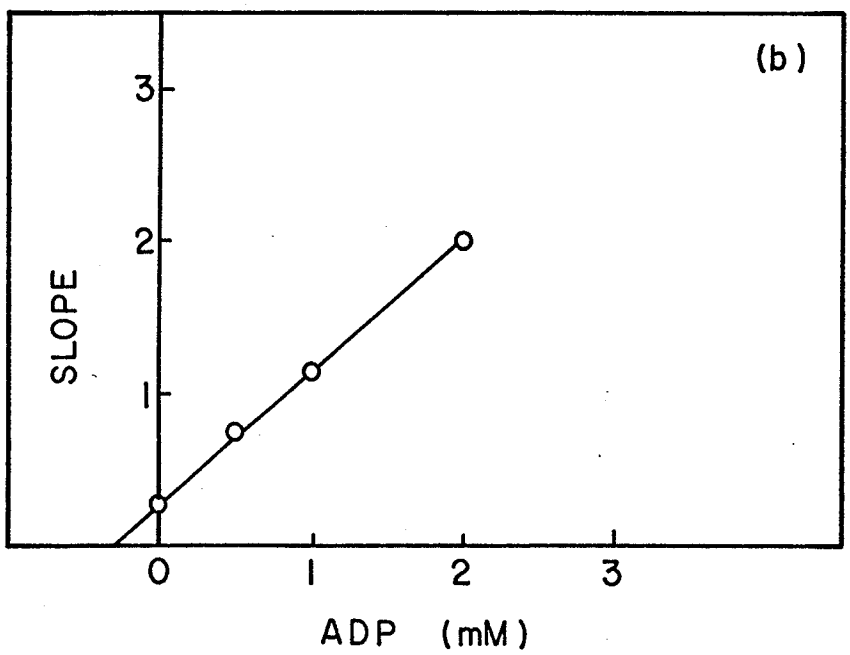
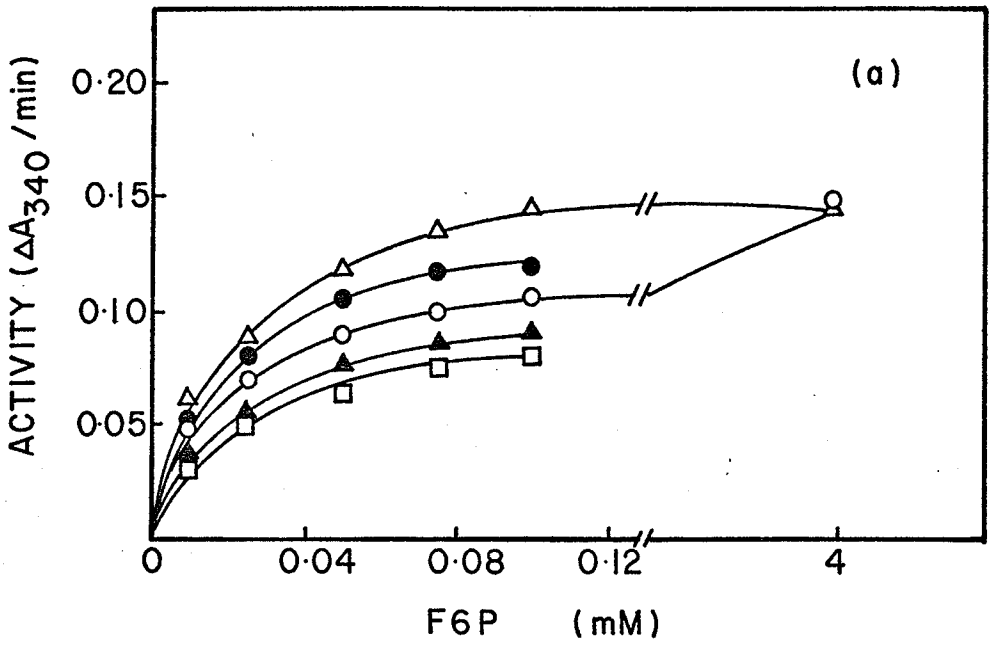

(b)
3
2
1
SLOPE
0
1
2
3
ADP (mM)

Figure 35a. Effect of FDP on the PFK activity at varied F6P concentrations. F6P was varied in the range of 0.01 mM to 4 mM. The FDP concentrations were 0 mM (△), 0.25 mM (●), 0.5 mM (○), 1 mM (▲), and 1.5 mM (□). Other assay components were as described in Materials and Methods.

Figure 35b. Different inhibition effects of FDP at different F6P concentrations. FDP was varied in the range of 0 mM to 5 mM. The F6P concentrations were 0.1 mM (○), 1 mM (●), and 4 mM (△). Other assay components were as described in Materials and Methods. Activity was expressed as % of the activity at 0 mM FDP.


(a)
ACTIVITY (ΔA_{340}/min)
0·20
0·15
0·10
0·05
0
0·04
0·08
0·12
4
F6P (mM)

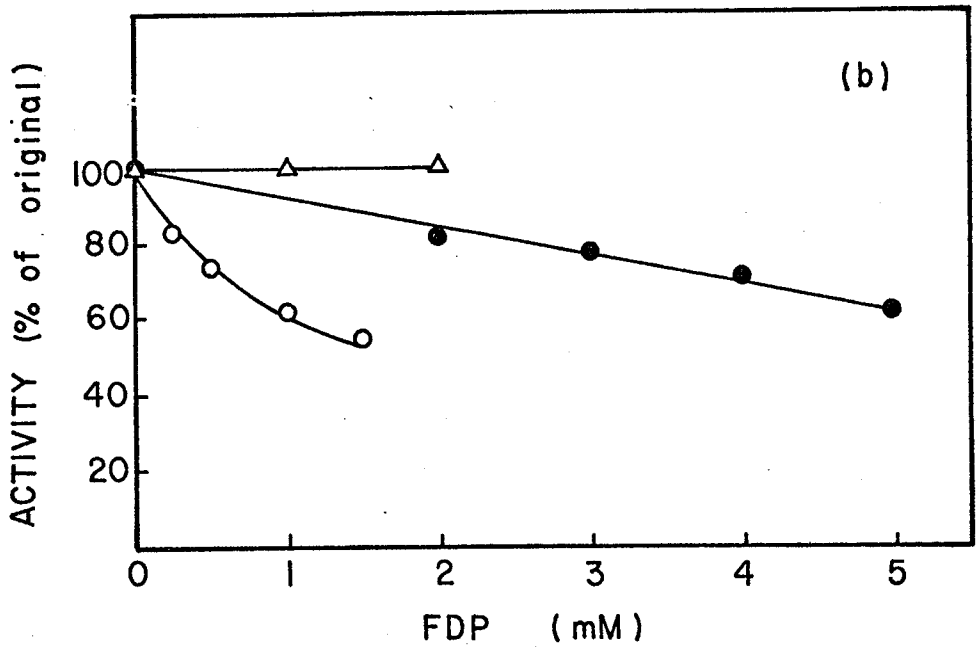

(b)
ACTIVITY (% of original)
100
80
60
40
20
0
1
2
3
4
5
FDP (mM)

Figure 36a. Double reciprocal plots of velocity versus varying F6P concentrations with FDP as fixed variable product.

FDP concentrations:

□ —— 0 mM

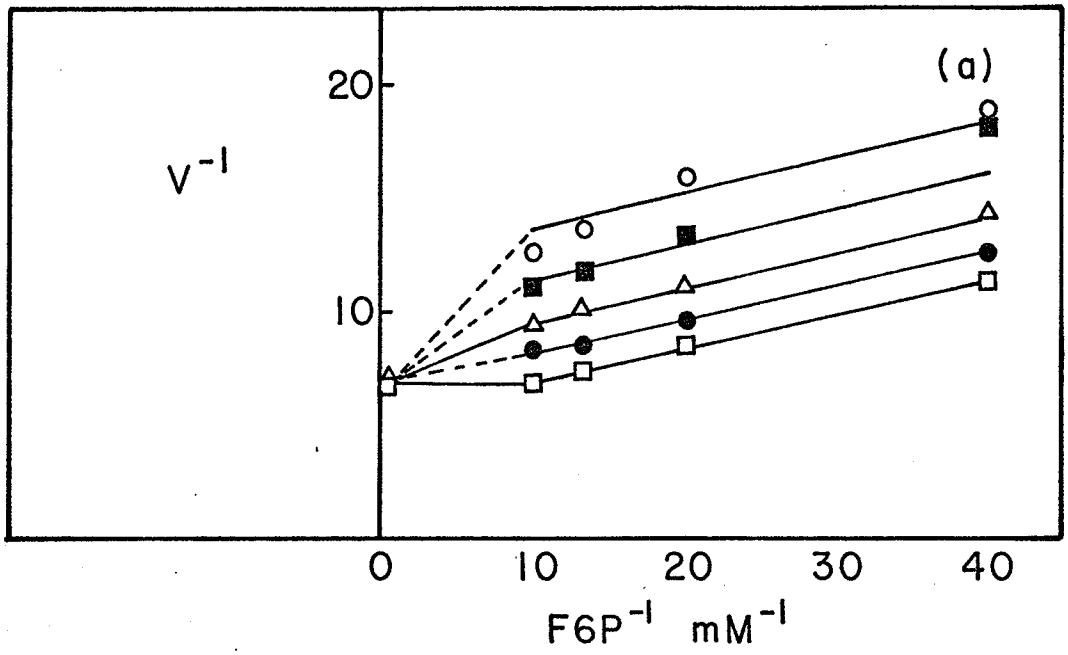
● —— 0.25 mM

△ —— 0.5 mM

■ —— 1.0 mM

○ —— 1.5 mM

Figure 36b. Replot of intercepts versus FDP concentrations from Fig. 36a.


(a)
20
10
V^{-1}
0
10
20
30
40
$F6P^{-1}$ mM^{-1}

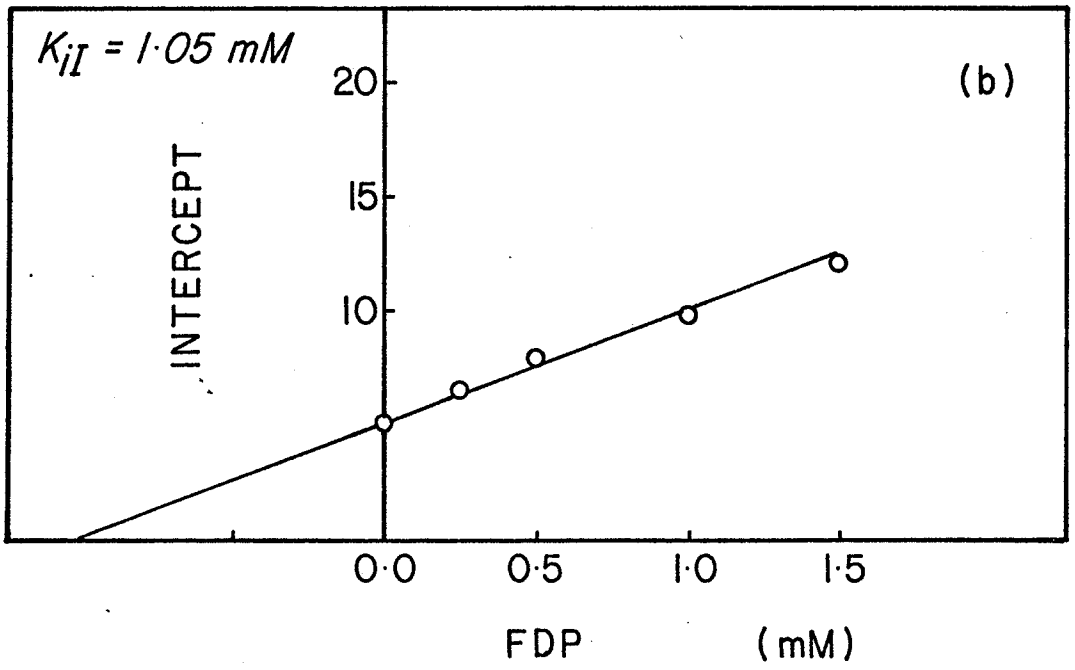

K_{iI} = 1·05 mM
(b)
20
15
10
INTERCEPT
0·0
0·5
1·0
1·5
FDP (mM)

Figure 37. Effect of FDP on the PFK activity at varied ATP concentrations. ATP was varied in the range of 0.025 mM to 0.4 mM. The F6P concentration was kept at 1 mM. The FDP concentrations were 0 mM (□), 3 mM (O), and 5 mM (Δ). Other assay components were as described in Materials and Methods.

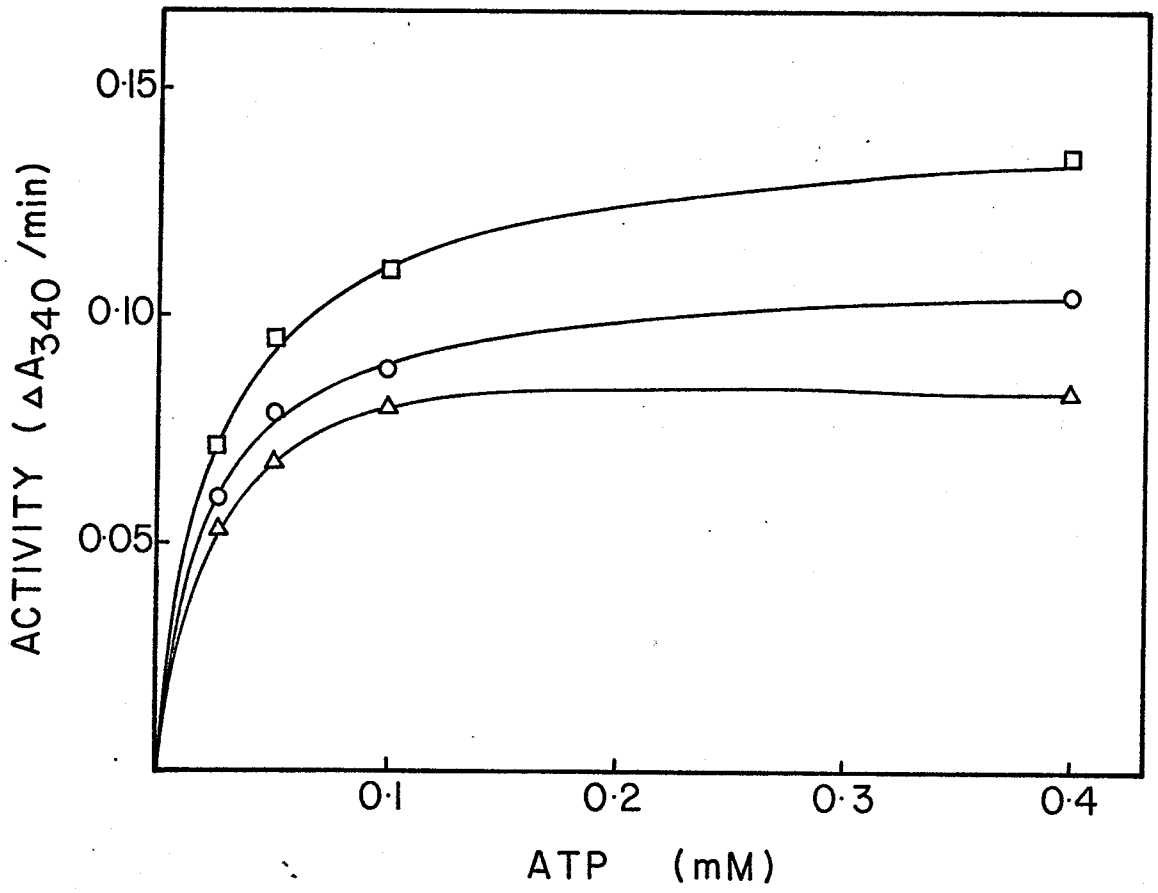

0·15
0·10
0·05
ACTIVITY (ΔA_{340} /min)
0·1
0·2
0·3
0·4
ATP (mM)

Figure 38a. Double reciprocal plots of velocity versus varying ATP concentrations with FDP as fixed variable product. (The F6P concentration was kept at 1 mM).

FDP concentrations:

▲ —— 0 mM

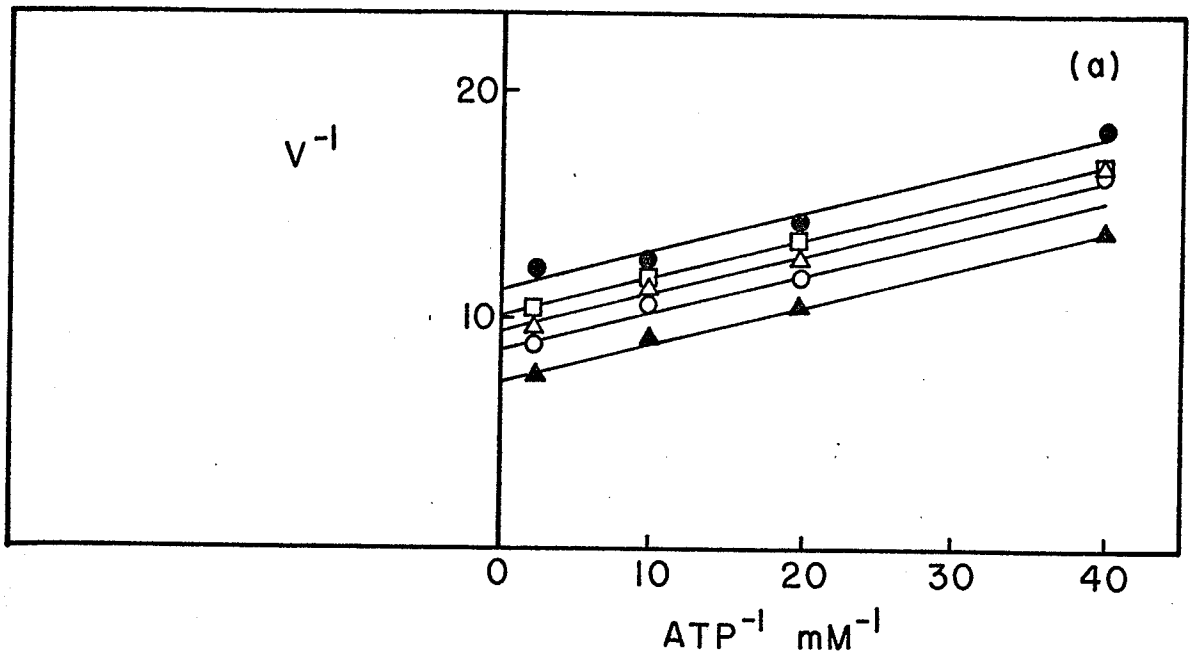
○ —— 2 mM

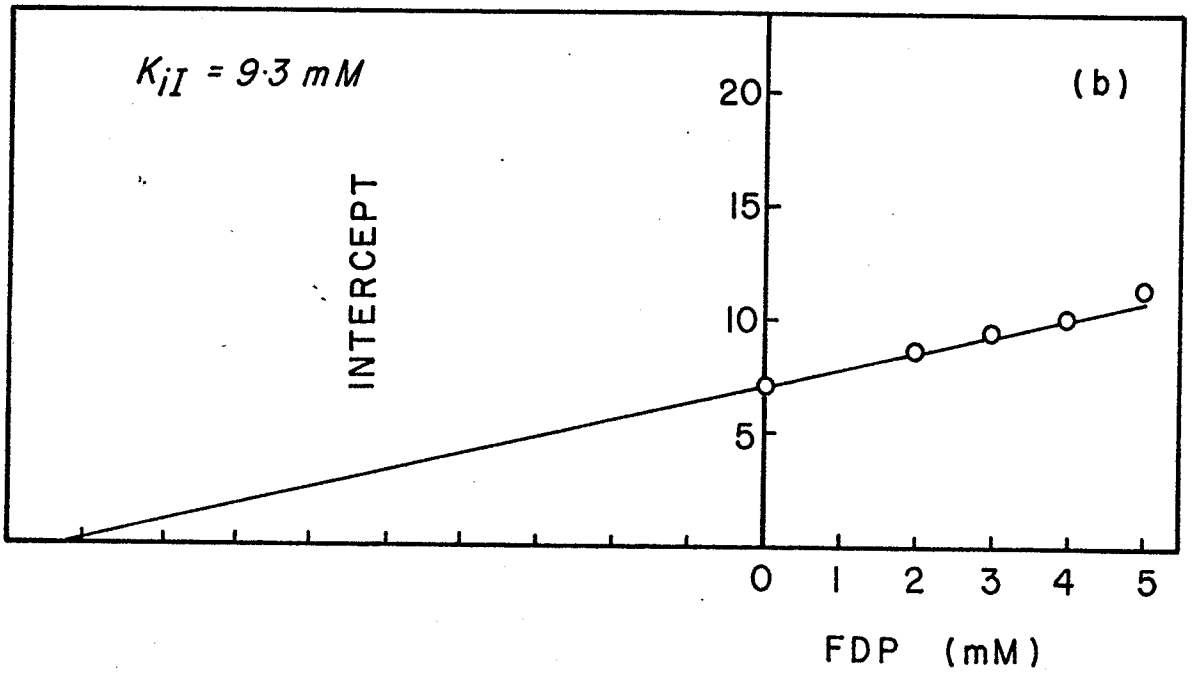
△ —— 3 mM

□ —— 4 mM

● —— 5 mM

Figure 38b. Replot of intercepts versus FDP concentrations from Fig. 38a.


(a)
V^{-1}
20
10
0
10
20
30
40
ATP^{-1} mM^{-1}


K_{iI} = 9·3 mM
(b)
INTERCEPT
20
15
10
5
0
1
2
3
4
5
FDP (mM)

D I S C U S S I O N

DISCUSSION

GROWTH CONDITIONS IN RELATION WITH THE PHYSIOLOGICAL ROLE OF PFK

A. niger is a citrate accumulating organism and has been extensively used for industrial citrate production. The concentrations of trace metals (Zn^{++}, Fe^{+++}, Cu^{++}) in the growth medium were found to affect citrate production of the organism (Wold, 1974). It was also reported that A. niger, grown in high-sucrose medium, gave a much higher yield of citrate than that grown in low-sucrose medium. Since sugar catabolism is influenced greatly by the metabolism of the glycolytic pathway, it was postulated that A. niger PFK, which is a key enzyme in glycolysis and is responsible for its regulation, might play a certain role in regulating citrate production (Wold, 1974). In the work of this thesis, there is certain evidence suggesting that the concentrations of sucrose and trace metals in the liquid medium would possibly affect citrate production either directly or indirectly through the activity of the enzyme PFK.

Tables I, II, and III show how the different growth conditions affected the activities of PFK. The specific activity of PFK in the crude extract from the low-sucrose (0.8%) culture was only about half of that from the high-sucrose (10%) culture, while the former enzyme was more stable than the latter. A culture age of 40 hours gave the highest activity and stability of the enzyme, while the enzyme from aging cells (72 hours of culture age) was the least active and stable.

Cells grown in the presence of Zn^{++}, Fe^{+++}, and Cu^{++} gave fluffy pellets (not reported in Results) and higher PFK activity, while those grown in their absence, gave less fluffy, smaller pellets (not reported in Results) and much lower PFK activity (Table III). The enzyme was also less stable in the latter (not reported in Results). According to Wold's report (1974), citrate was produced in large quantities only in the absence of the above trace metals.

Since in this study it was essential to obtain a stable PFK preparation with a high activity in order to study the properties of the enzyme, A. niger had to be grown under conditions in which citrate accumulation was not favoured (low sucrose, presence of

trace metals). Although the PFK activity in extracts was found to be low in the absence of trace metals in the growth medium, it is possible that the in vivo PFK activity might have been higher. This possibility is supported by the unusual instability of PFK from A. niger cells grown in the absence of trace metals or in the presence of high sucrose concentration, the conditions favorable to the accumulation of citrate.

It is concluded, therefore, that a further physiological and comparative study of PFK of A. niger grown under different conditions is necessary to correlate the properties of PFK and citrate accumulation.

EFFECT OF TEMPERATURE ON THE CRUDE EXTRACT

Phosphofructokinase from A. niger is a labile enzyme upon storage and dilution. This lability is not only characteristic of the A. niger enzyme alone, but also of PFK from other sources. It was due to this instability that isolation, purification and studies of the A. niger enzyme became difficult. Many treatments had been tried earlier to stabilize the enzyme. Few were successful except mercaptoethanol and glycerol which were found to be essential and ATP, ADP, FDP, and NaF which increased stability to a minor extent. It was not

until the effects of temperatures were examined that a procedure for obtaining a stable enzyme was found.

Unlike guinea pig heart PFK (Mansour, 1965), liver fluke PFK (Stone and Mansour, 1967a), and chicken liver PFK (Kono and Uyeda, 1973b), the A. niger enzyme was not found to be cold labile. Instead, Fig. 2 indicates clearly that apart from the simple thermal inactivation at 65°C, there is another inactivation effect on the enzyme at 37°C or 45°C. The enzyme, however, was immune to this effect after the heat treatment at 56° or in the presence of ATP, ADP, or NaF (Figs. 2, 4, 6, and 7). Further incubation at 56° after inactivation at 37° did not reactivate the enzyme. The instability of PFK at 37° or 45°, but not at 56° (Fig. 2) suggests a possibility of a heat labile factor, perhaps protein, being present in the crude extract. This factor is most active at 37° or 45° and inactivates the PFK at these two temperatures, but is itself destroyed at 56°. ATP, ADP, or NaF may prevent the factor from inactivating the enzyme.

The possibility of this factor being a phosphatase was at first considered because of the work by Viñuela et al. (1964) who suggested the presence of a phosphatase in the yeast crude extract which converted the yeast PFK from the ATP-insensitive form to the ATP-

sensitive form and the activity of which on the yeast PFK was inhibited in the presence of NaF. If such were the case for A. niger PFK, at 37° or 45° the ATP or ADP might serve to furnish phosphate group to protect the enzyme while NaF might inhibit the action of the phosphatase. So far, however, no such protein has yet been isolated from the A. niger system. Also the protection effect of NaF was not as pronounced as expected at both 4° and 37°. Moreover, the results of the Sepharose 6B fractionation (Table V) imply that either the above theory is incorrect or the factor is not separated from PFK by the Sepharose treatment.

An alternate possibility is that PFK can exist at least in two different states, one stable at 37° or 45° and the other unstable at these temperatures. ATP, ADP, or NaF may shift the equilibrium from the unstable to the stable state and the 56° treatment may 'fix' the enzyme in the stable state. Stability due to conformational change of the enzyme may well be explained by the model proposed in the following section.

POSSIBLE MOLECULAR STRUCTURES OF PFK

It has been found that PFK's from many sources have a tendency to aggregate and the degree of aggrega-

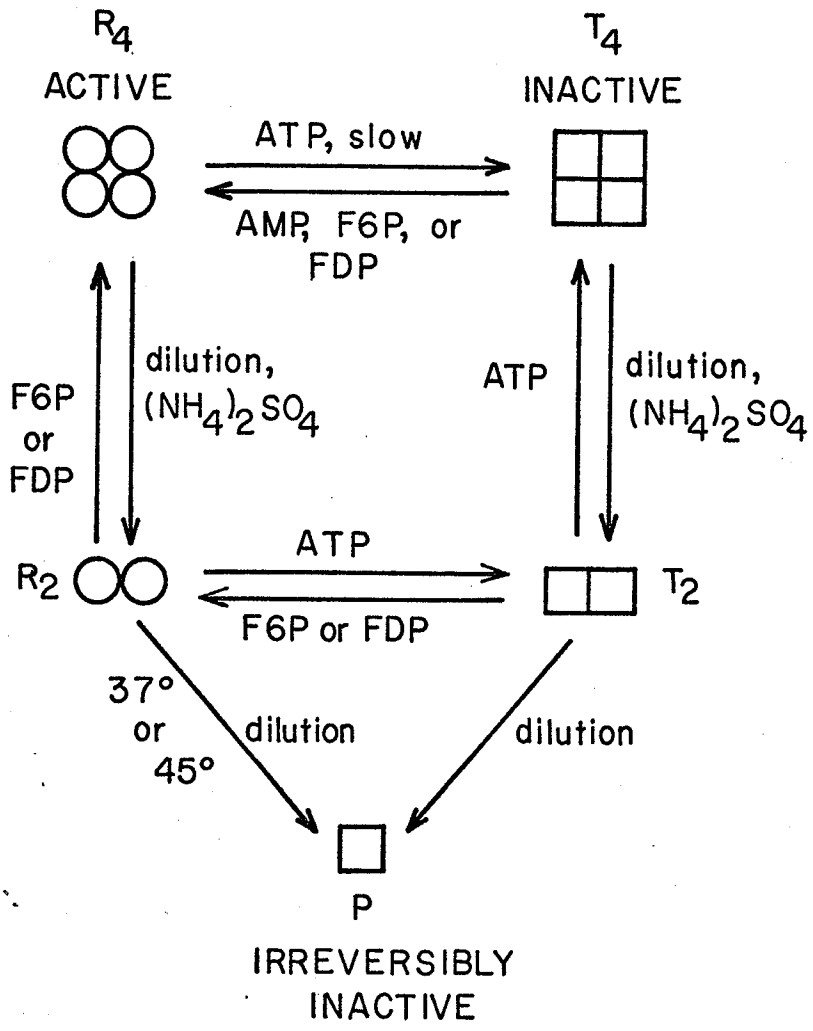
tion depends on the presence of the enzyme in high concentration. For example, skeletal muscle PFK (Paetkau and Lardy, 1967) and yeast PFK (Liebe et al., 1970) were reported to exist in several interconvertible polymeric forms. Mansour and Ahlfors (1968) have even postulated a molecular model to account for the peculiar behaviour of the enzyme. Although no sedimentation studies were done, according to the results obtained in the present study it is speculated that aggregation and deaggregation of the A. niger enzyme also took place.

A molecular model of the A. niger PFK was therefore considered in order to explain certain peculiar phenomena observed in this study such as the instability properties, the presence of lag at the initial state of the reaction, the pH and anion effects, etc. Based on models proposed for the PFK in other sources (Mansour and Ahlfors, 1968; Kono and Uyeda, 1973 b), the molecular scheme of A. niger PFK is postulated as follows (Fig. 39).

The enzyme is assumed to exist in several forms : 4-subunit molecules (R4, T4), dimers (R2, T2), and protomer (P). The state of association and dissociation of the enzyme molecules is influenced by enzyme concentration, pH, ionic strength, temperature and some effector

FIGURE 39

Molecular scheme of A. niger PFK



molecules. R4, T4, R2, and T2 are usually present in equilibrium in enzyme preparations. R4 is active while T4, R2, and T2 are inactive. These four states are interconvertible and the interconversion is regulated by the factors as shown in the scheme. It is assumed that the interconversion is not instantaneous leading to the hysteretic behaviour of the enzyme (Frieden, 1970).

In the concentrated purified preparation, the PFK would be present mostly in R4 and T4 states, while in the less concentrated crude extract preparation, there would be more R2 and T2 present. In the former preparation, the enzyme was stable in the presence of mercaptoethanol and glycerol, and metabolites such as ATP, ADP, F6P, and FDP had no influence on its stability (Table VIII). In the latter preparation, however, the above metabolites were effective in enhancing the stability of the enzyme (Table VI). This effectiveness increased when the enzyme was highly diluted (Table IX). Dilution in the absence of ATP and F6P would cause the dissociation of the enzyme to the protomer state, P, which is the irreversibly inactive state.

Whether the enzyme appears predominantly in R4 or T4 state in the enzyme preparation would determine the pattern of the time-course behaviour during the

activity measurement : an initial lag in velocity before achieving a linear rate of reaction (Fig. 8a), a slight levelling off of activity after an initial linear rate of reaction (Fig. 8b), or a linear activity throughout the reaction. This means that there is a possibility that the enzyme molecules oscillate between these states in a cyclic manner since R4, T4, R2, and T2 are interconvertible as presented in the scheme. The lag or levelling off is then explained by the slowness of interconversions reaching equilibrium states under given conditions.

The effect of anions may be explained by assuming that the conversion of T4 $\longrightarrow$ R4 can be slowed down by anions. As the concentrations of the OH^-, Cl^-, or $SO_4^=$ ions were increased, the initial lags of the reactions were also increased because the formation of active enzyme state (R4) from T4 would be slowed down.

R4 and T4 are assumed to be the two kinetic forms mainly involved during kinetic studies. ATP would bind to both R4 (at the catalytic site) and T4 (at the regulatory site), while F6P, FDP, and AMP would bind only to R4. The conversion of R4 $\longrightarrow$ T4 is assumed to be slower than the conversion of T4 $\longrightarrow$ R4. Hence, when the concentrated enzyme was added to a assay mixture

containing low F6P and high ATP in the absence of AMP, the equilibrium was shifted gradually towards the T4 state, resulting in a fast levelling off of the reaction (Fig. 18a). Preincubation of the enzyme with ATP in the assay system (neither F6P nor AMP present) would render the enzyme to result completely in the T4 conformation. As F6P concentration is increased, more and more T4 would be driven back to the R4 state.

Besides dilution, the addition of $(NH_4)_2SO_4$ to the concentrated enzyme preparation is assumed to result in an immediate dissociation of the enzyme to the dimers which can either be associated back to the tetramers by ATP or F6P or dissociate slowly to P losing activity. Hence, during storage of the above system at 4^{o}, addition of ATP reassociated the dimers to T4, resulting in a partial recovery of activity (Fig. 16b). It is probable that the action of $(NH_4)_2SO_4$ is on the ionic bonds of the enzyme molecule, thus rendering its subsequent dissociation.

Addition of ATP to the enzyme preparation containing $(NH_4)_2SO_4$ produced an immediate drop in activity (Figs. 15 a,b). This can be explained as below. Ammonium sulfate would dissociate the enzyme from R4 and T4 to R2 and T2 respectively, and subsequent addition of ATP would convert all the R2 molecules to T2 molecules.

In the presence of $(NH_4)_2SO_4$, the conversion of T2 to T4 is assumed to be very slow compared to other conversions. Hence the reactivation rate of the enzyme was comparatively much slower than its inactivation rate (Fig. 16a). No immediate drop in activity was observed when FDP or F6P was present and the enzyme was stable during storage (Figs. 15a, b). This is to be expected if the conversions T2 ⟶ R2 and R2 ⟶ R4 are reasonably fast counteracting the effect of ATP and $(NH_4)_2SO_4$. However, the gradual increase in activity during storage to a level higher than the original (Fig. 15b) in the presence of $(NH_4)_2SO_4$ and ATP and/or FDP cannot be explained by this simple model.

The model is also capable of explaining the temperature effect on the crude extract. Incubation at 37^{o} or 45^{o} would accelerate the dissociation of R2 to P, resulting in an irreversible inactivation of the enzyme activity (Fig. 2). Presence of ATP, ADP, or NaF would protect the enzyme at these temperatures by keeping it at the T2 and T4 states (Figs. 4, 7). FDP and possibly glycerol, on the other hand, would keep the enzyme to the R4 and R2 states which would easily dissociate to P at these temperatures leading to the loss of activity (Figs. 5, 6). Heat treatment at 56^{o} might change the

enzyme in such a way that the passage from R2 to P would become unavailable, thus not only protecting the enzyme at 37° but also increasing its stability at 4° during storage. T2 and T4 are possibly less thermostable (rapid destruction at 65°) than R2 and R4 since glycerol or FDP protected the enzyme at 65° (Figs. 3, 5, 6), while ATP accelerated the inactivation (Figs. 4, 7).

Nevertheless, the molecular scheme proposed here is by no means a perfect model. It is only an attempt to explain certain peculiar phenomena of the A. niger enzyme. There are still behaviours not understood and questions unanswered. For example, what governs the oscillation of the molecular states of the enzyme? What was the cause of the gradual activation of the enzyme in the presence of $(NH_4)_2SO_4$ and ATP and/or FDP? How was the passage from R2 to P blocked? Further detailed experiments on dissociation-association of the enzyme through sedimentation studies, ligand binding studies, etc. are required before a clearer picture can be drawn.

KINETICS AND MECHANISMS OF THE ENZYME REACTION

A. niger PFK is an allosteric enzyme. Its kinetic effects described in the Results can be ex-

plained by the model by Monod et al. (1965).

Each of the three ligands : F6P, ATP, and 5'-AMP, was able to exert an effect on the saturation curve (ligand concentration - velocity curve) of the other. In the absence of AMP, the saturation curve for F6P was sigmoidal (Fig. 21) suggesting that there was a cooperative interaction between the F6P binding sites of a PFK molecule (T4 ⟶ R4 in Fig. 39). The affinity of the enzyme for F6P (or the sigmoidicity of the curve) could be altered at different levels of ATP (Fig. 21) and in the presence of AMP (Fig. 23). Increasing ATP concentrations would increase the sigmoidicity, thus increasing the $S_{0.5}$ values for F6P, while AMP had the opposite effect. The degree of cooperative interaction of different F6P binding sites on the enzyme was further illustrated by the Hill plots (Figs. 22, 24). The higher the ATP concentration used, the higher was the value of n, indicating an increase in homotropic interactions. The presence of AMP decreased the n value. Thus the results agree with the model where ATP converts R4 to T4 and AMP T4 to R4 and F6P binds only to the R4 state as shown in Fig. 39.

Since AMP did not affect the V_{max} of the reaction but affected its K_m for F6P, the A. niger PFK, according

to Monod et al. (1965), appears to be a "K system" of the allosteric enzymes.

That ATP is an allosteric inhibitor of PFK is revealed by its influence on the $S_{0.5}$ value for F6P (Fig. 21) and its inhibition at high levels (Figs. 25, 26). Varying the concentration of F6P or the presence of AMP did not have much significant effect on the $S_{0.5}$ value for ATP at the catalytic site (Figs. 25, 26). The presence of AMP increased the K_i for ATP. Hence the inhibition of ATP must be due to the binding of ATP at a second, regulatory site. The effect of AMP might perhaps be achieved either by direct competition with ATP at the ATP regulatory site or as an indirect consequence of binding at another site. The fact that increasing AMP did not completely relieve ATP inhibition (Fig. 20) indicates that the AMP site is different from the ATP regulatory site. The presence of an ATP regulatory site other than the catalytic site in PFK has been confirmed in yeast and E. coli by the use of UTP, GTP, and ITP as phosphate donors (Ramaiah et al., 1964; Atkinson and Walton, 1965); and in sheep heart muscle by photooxidation and ethoxyformylation (Ahlfors and Mansour, 1969; Setlow and Mansour, 1970).

Besides A. niger PFK, PFK from other sources such as yeast (Ramaiah et al., 1964), E. coli (Atkinson and Walton, 1965), liver fluke (Stone and Mansour, 1967 b), heart muscle (Mansour and Setlow, 1972), Ehrlich ascites tumor cells (Sumi and Ui, 1972), and a number of other systems, all display similar allosteric kinetics with various effector ligands although their specific effects vary from one species to another, from one source to another source.

Besides AMP, cyclic 3', 5'-AMP was also tested on the A. niger PFK. Although the cyclic nucleotide is an activator of the mammalian and liver fluke PFK (Mansour and Setlow, 1972; Stone and Mansour, 1967 b), it has no effect on the yeast and E. coli PFK at all, and its effect on the A. niger PFK was dubious (Figs. 17a, 17b).

Lardy and Parks (1956) first proposed that the inhibition of PFK by ATP was probably due to the decrease in the concentration of free Mg^{++} as a result of chelation with ATP. Such a suggestion has been rejected by Ramaiah et al. (1964) from the work on the yeast PFK. As for A. niger PFK, there is also certain similar evidence as in the yeast enzyme to disagree with the proposal by Lardy and Parks. Both Figs. 25 and 26 show that the inhibition by ATP depended on the

F6P concentrations and the presence of AMP. Also, the inhibition was present even when the concentration of Mg^{++} greatly exceeded that of ATP.

The initial lag of the reaction velocity is not limited to the A. niger PFK. The rabbit muscle PFK, glyceraldehyde 3-phosphate dehydrogenase from yeast, glutamate dehydrogenase from bovine liver, phosphorylase a from rabbit muscle, and acetyl-CoA carboxylase from rat adipose tissue (Frieden, 1970) were also reported to exhibit such behaviour which was defined by Frieden (1970) as the hysteretic response of the hysteretic enzymes. The hysteretic response is believed to occur when the enzymes respond slowly to rapid changes in ligand concentration, pH, or dilution, thus resulting in a time lag of the kinetic behaviour.

Mechanisms such as isomerization of the enzyme, displacement of ligands, and polymerization and depolymerization system were proposed by Frieden to explain the hysteresis. The molecular model (Fig. 39) described earlier could involve all these three possible mechanisms to explain the initial lag of the reaction observed in the A. niger enzyme.

Kinetic interactions of substrates and products on A. niger PFK are rather complex and not easy to

TABLE XI

Comparison of kinetic data, K_m and K_i for PFK from several sources

Source	K_m for F6P (mM)	K_m for ATP (mM)	$1/v_b$ vs $1/F6P$	$1/v$ vs $1/ATP$	ADP-ATP	ADP-F6P	FDP-ATP	FDP-F6P
Rabbit skeletal muscle (1)	0.2	1.0	parallel	parallel	NC K_i=2.1mM	Comp K_i=1.4mM	Comp	NC
Chicken liver (2)	0.051	0.041	parallel	parallel	-	-	-	-
Yeast (3)	-	-	parallel	parallel	-	-	-	-
Calf-lens (4)	0.19	0.007	-	parallel	-	-	-	-
Dictyostelium discoideum (5)	0.25	0.031	parallel	parallel	Comp	UC	UC	Comp
Human erythrocyte (6)	0.045	0.12	parallel	parallel	-	-	-	-
Human skeletal muscle (6)	0.083	0.15	parallel	parallel	-	-	-	-
Brain (7)	0.04	0.1	intersecting	intersecting	-	-	-	-

Table XI Continued

Ehrlich ascites tumor(8)	1.0	0.2	parallel	parallel	Comp	NC	- -	-
Aspergillus niger	0.033	0.042	parallel	parallel	*	UC K_i=1.75mM	UC UC MI K_i=9.3 mM	*

The abbreviations used are: Comp, competitive inhibition; UC, uncompetitive inhibition; NC, noncompetitive inhibition; MI, mixed inhibition.

* Two types of inhibition depending on the substrate concentration (Figs. 34a, 36a).

(1) Uyeda, 1970.
(2) Kono and Uyeda, 1974.
(3) Viñuela et al., 1963.
(4) Lou and Kinoshita, 1967.
(5) Baumann and Wright, 1968.
(6) Layzer et al., 1969.
(7) Lowry and Passonneau, 1966.
(8) Sumi and Ui, 1972.

comprehend with the present knowledge of the enzyme. The results of substrate studies and product inhibition studies, and the K_m and K_i values for substrates and products of the PFK from several different sources (including A. niger) are compared in Table XI.

The reaction mechanism of the A. niger PFK was studied in the presence of AMP, that is, when the allosteric kinetics were not observed. Linear parallel double reciprocal plots were obtained for both substrates (Figs. 27a, 28a). Such a kinetic pattern is consistent with the Ping-Pong mechanism in which release of one product occurs before addition of the second substrate (Cleland, 1963a). With the exception of the brain PFK, substrate studies from PFK's of other sources gave evidence consistent with the Ping-Pong mechanism (Table XI).

However, product inhibition studies of the A. niger enzyme indicated a more complex mechanism rather than the simple Ping-Pong. ADP was an uncompetitive inhibitor with respect to F6P (Fig. 32a) but a competitive inhibitor with ATP at high concentrations (Fig. 34a). As the ATP concentrations were lowered, it seemed that a mixed type of inhibition was present (Fig. 34a). FDP was an uncompetitive inhibitor with respect to ATP (Fig. 38a) and an uncompetitive inhibitor

with F6P at lower concentrations (Fig 36a), but a competitive inhibitor with F6P at high concentrations (speculation from Figs. 35a, 36a). Product inhibition studies of PFK's from other sources (Table XI) also revealed kinetic patterns inconsistent with the Ping-Pong mechanism.

Judging from the product inhibition studies, it seemed that there is a possibility that the enzymatic reaction operates differently at high and at low concentrations of ATP and F6P. Since the PFK is an important regulatory enzyme in the glycolytic pathway, it would not be too surprising that the enzyme might possess more than one reaction mechanism at different levels of ligands for the benefit of cellular metabolism.

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