UNIVERSITY OF SOUTHAMPTON

A STUDY OF PHOSPHOFRUCTOKINASE FROM MUSCLE TISSUE OF *CARCINUS MAENAS*

by

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To my parents

for all they have done

and to Jenny

for putting up with me

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ABSTRACT

FACULTY OF SCIENCE

SCHOOL OF BIOCHEMICAL AND PHYSIOLOGICAL SCIENCES

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A STUDY OF PHOSPHOFRUCTOKINASE FROM MUSCLE TISSUE OF *CARCINUS MAENAS*

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Phosphofructokinase was purified from the leg and pincer muscle of *Carcinus maenas*, the common shore crab, to a specific activity of 19 Units/mg of protein at 25°C. The purity of the enzyme preparation was about 70%.

The molecular weight of the sub-units of *C. maenas* phosphofructokinase was 81,500 and the purified enzyme existed as two or three different molecular weight forms of undefined oligomeric composition.

At neutral pH phosphofructokinase from *C. maenas* was inhibited by concentrations of its substrate ATP above that required for maximum catalytic activity. At pH 8.0, in the presence of ammonium sulphate, the enzyme exhibited hyperbolic kinetics with respect to the concentrations of both substrates, F6P and ATP. In these respects the enzyme conforms to the steady-state kinetic properties of mammalian phosphofructokinases.

Steady-state kinetic studies of *C. maenas* phosphofructokinase were carried out in which the initial-rate of the reaction was measured as a function of the concentrations of F6P and ATP at a constant concentration of free Mg^{2+}. These measurements were repeated at eight different concentrations of free Mg^{2+} in the absence of the products, FDP and ADP, and at a single concentration of free Mg^{2+} in their presence. Although both ATP and ADP form dissociable complexes with Mg^{2+} and exist predominantly as $MgATP^{2-}$ and $MgADP^{-}$ respectively under physiological conditions, the enzyme was found to bind free Mg^{2+} and free ATP^{4-} while the nucleotide product of the reaction was the chelate $MgADP^{-}$. The kinetic data were consistent with an ordered ter-bi mechanism of reaction in which the order of binding of substrates was $F6P^{2-}$, free Mg^{2+}, ATP^{4-} and the order of release of products was $MgADP^{-}$, FDP^{4-}.

ABBREVIATIONS.

Chemicals.

ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
CTP	Cytidine-5'-triphosphate
cyclic AMP	Cyclic adenosine-3',5'-monophosphate
deoxy ATP	2'-deoxy-adenosine-5'-triphosphate
DEAE	Diethylaminoethyl
EDTA	Ethylene-diamine-tetra-acetic acid
F6P	Fructose-6-phosphate
FDP	Fructose-1-6-diphosphate
G.E.	Guanidinoethyl
GTP	Guanosine-5'-triphosphate
ITP	Inosine-5'-triphosphate
NAD^+	β-nicotinamide-adenine dinucleotide, oxidised form
NADH	β-nicotinamide-adenine dinucleotide, reduced form
$NADP^+$	β-nicotinamide-adenine dinucleotide phosphate, oxidised form
NADPH	β-nicotinamide-adenine dinucleotide phosphate, reduced form
Pyr-P	Phosphoenolpyruvate
SDS	Sodium dodecyl sulphate
Temed	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)-aminomethane
TTP	Thymidine-5'-triphosphate
UTP	Uridine-5'-triphosphate
XTP	Xanthosine-5'-triphosphate

Kinetic.

Coef	Coefficient
k	A rate constant
K_i	An inhibition constant
K_m	A Michaelis constant
K_s	A dissociation constant
v	The initial-rate or velocity of the reaction

V	The maximum rate or velocity of the reaction
V_k	The initial-rate or velocity of the reaction measured by the standard kinetic assay

General.

Å	Angström
A_{280}	Absorbance (at 280 nanometres)
i.d.	Internal diameter
MW	Molecular weight
NMR	Nuclear magnetic resonance
r.p.m.	Revolutions per minute
S	Svedberg unit
v/v	Volume for volume of solution
w/v	Weight for volume of solution

Chapter 1.

LITERATURE REVIEW.

Phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) catalyses the transfer of the terminal phosphoryl group of ATP to fructose-6-phosphate to form ADP and fructose-1-6-diphosphate. The reaction was discovered by Dische (1935) and by Ostern et al. (1936).

$$F6P + ATP \rightleftharpoons FDP + ADP + H^{+}$$

Catalysis of this reaction by phosphofructokinase is dependent on the presence of a divalent metal ion, physiologically this is Mg^{2+}. Phosphofructokinase is a soluble cytoplasmic enzyme which catalyses the only unique step in the glycolytic pathway. The enzyme is widely distributed within the tissues of multicellular organisms and is present throughout the animal and plant phyla, (Lardy, 1962).

Purification of phosphofructokinase.

Homogeneous, and in certain cases crystalline, preparations of phosphofructokinase have been obtained from various animal tissues, yeast and bacteria. These sources are listed in Table 1 together with the specific activities of the isolated enzymes.

Ling et al. (1965) first observed that extraction of phosphofructokinase from rabbit skeletal muscle in the presence of 30 mM potassium fluoride gave an increased yield of the enzyme from the tissue and enhanced its stability in phosphate buffer. This compound acted by preventing aggregation of phosphofructokinase. In the absence of potassium fluoride the enzymic activity could be restored by addition of ATP and magnesium sulphate to the enzyme extract. Phosphofructokinase from sheep, cow and pig hearts which was inactive and insoluble upon initial extraction was also reactivated by ATP and magnesium sulphate, (Mansour, 1970). This behaviour resembles that of enzymes that are regulated by phosphorylation - dephosphorylation processes, (Holzer & Duntze, 1971). Phosphorylation of

phosphofructokinase *in vivo* has recently been demonstrated, (Sorensen & Hofer, 1979; Kagimoto & Uyeda, 1979).

Phosphofructokinase from many sources is stable to heat treatment and precipitation by ammonium sulphate and these techniques are widely used in its purification. Gel filtration is also frequently employed since the molecular weight of the enzyme is high compared to that of most other cytoplasmic enzymes. However the occurrence of different molecular weight forms of the enzyme can be a difficulty with this technique, (Kemp, 1971). Massey & Deal (1973) introduced a unique method for the purification of phosphofructokinase that does not utilise conventional salt fractionation or column chromatography. The key procedure was repeated selective precipitation and dissolution of the enzyme by changing the ammonium sulphate concentration in the presence of 5 mM magnesium chloride. Phosphofructokinase precipitated at low (0-30 mM) and dissolved at slightly higher (>100 mM) concentrations of ammonium sulphate. The application of this technique as a general method for purification of mammalian phosphofructokinases has been discussed by Massey & Deal (1975). However the method has not been widely adopted.

Crystallisation of phosphofructokinase from four sources has been achieved as indicated in Table 1. The methods used are based on that first described by Parmeggiani & Krebs (1965) which consists of raising the ammonium sulphate concentration in the presence of ATP. In the absence of ATP crystals form much more slowly (Uyeda & Kurooka, 1970). Chicken liver phosphofructokinase has been crystallised in the absence of ammonium sulphate and with FDP present in place of ATP, (Kono & Uyeda, 1973).

Kopperschlager et al. (1968) first observed binding between phosphofructokinase and Blue-dextran 2000 while electrophoresing the enzyme on agarose gels. Binding was found to be due to the dye component of the dextran, Cibacron-blue F3GA, (Kopperschlager et al. 1971). The possibility of affinity chromatography of yeast phosphofructokinase by use of the dye covalently attached to Sephadex G200 was demonstrated by Bohme et al. (1972). More recent purifications of phosphofructokinase frequently use

this technique, often with specific elution of the enzyme by its substrates or products.

The use of N^6-(6-aminohexyl)carbamoyl-methyl-ATP Sepharose for affinity chromatography has been applied to the purification of phosphofructokinase from rabbit brain, rat brain and rat muscle by Ramadoss et al. (1976). Specific elution of phosphofructokinase was by a "dead-end complex" that consisted of F6P and ADP.

Recently an AMP-sepharose affinity column has been used for the purification of phosphofructokinase from E. coli, (Babul, 1978).

Structural properties of animal phosphofructokinase.

The molecular weights and sub-unit structures of various phosphofructokinases are listed in Table 2.

A. Molecular weight and self-association behaviour.

Phosphofructokinase from animal tissues is a tetrameric enzyme with a molecular weight in the range 320,000-380,000 from most sources. This molecule represents the smallest fully active form of the enzyme. Animal phosphofructokinases have a strong tendency of self-association to form aggregates with molecular weights of several millions which are also enzymically active. Self-association is dependent on pH, enzyme concentration and the presence of ligands of the enzyme.

At pH 8.0 and protein concentration in the range 5-15 mg/ml ultracentrifugation of rabbit muscle phosphofructokinase gave three peaks that corresponded to $S_{20,w}$ values of approximately 13, 19 and 30, (Ling et al. 1965; Parmeggiani et al. 1966). The 13S component was the smallest fully active form of the enzyme with a molecular weight of 320,000 -380,000. The 19S and 30S components were associated forms of the enzyme. At pH 5.8 the enzyme dissociated and sedimented as a single 7S peak (molecular weight 192,000) which was inactive. Reactivation was achieved by raising the pH to 7-8 when the enzyme re-associated. Dissociation was dependent on enzyme concentration as well as pH which explained the fact that phosphofructokinase can be inactivated by dilution especially at acid pH, (Paetkau & Lardy,

1967). Structural studies by Aaronson & Frieden (1972), Leonard & Walker (1972) and Pavelich & Hammes (1973) confirmed this basic picture of the self-association behaviour of the enzyme. However physiologically the enzyme is probably in a less aggregated state, (Johnson & Deal, 1978). These authors measured the sedimentation velocity under near physiological conditions (pH 7.4, 35^{o}C and protein concentration 1.25 mg/ml) and observed a single broad peak spanning the range 11.4-17.5S. 28S material was observed only when the temperature was lowered (3.5^{o}C) and the protein concentration increased (4.2 mg/ml). Lad & Hammes (1974) and Telford et al. (1975) showed the repeating unit in rabbit muscle phosphofructokinase aggregates was the dimer, itself inactive in free form. This was suggested by cross-linking the enzyme with the bifunctional reagent dimethyl suberimidate after which SDS gel electrophoresis and sucrose density gradient centrifugation showed the presence of tetramer and octamer and smaller amounts of dimer, hexamer and decamer. Isolated monomers, trimers or hexamers could not be observed by electron microscopy. The shapes of the enzymically active cross-linked tetramer and octamer were determined as a rectangular prism and a cube respectively, (Paradies, 1979). Recently Hesterberg & Lee (1979) studied the sedimentation behaviour of the enzymically active species of the enzyme and confirmed the tetramer (12S) is active and smaller species inactive. However no information was obtained concerning larger aggregates as these were not present under the conditions used.

The mode of aggregation is not yet clear. Leonard & Walker (1972) measured the sedimentation equilibrium of the enzyme at pH 8.0 as a function of protein concentration and suggested that the phosphofructokinase tetramer (13S) associated to give a hexamer of the tetramer (27S, molecular weight 2,000,000) probably via an intermediate dimer or trimer (18S). In contrast, measurements of the sedimentation velocity of the enzyme as a function of protein concentration at pH 8.0 by Aaronson & Frieden (1972) showed that not all the polymers are in rapid equilibrium with each other. Different polymers were separable by molecular-sieve filtration and were stable for a few days. The

authors suggested the existence of three types of tetramer (13S) at pH 8.0, one type polymerising reversibly and rapidly to 30S material, another possibly associating to a dimer of the tetramer and the third not being able to polymerise.

The influence of effectors of phosphofructokinase on the aggregation state of the enzyme was investigated by Lad et al. (1973). The Stokes' radius of the rabbit muscle enzyme was measured at pH 7.0 and low protein concentration (0.15 mg/ml) by use of frontal gel chromatography. Positive effectors such as F6P and FDP stabilised a tetramer (Stokes' radius 67 Å, 13S) while the negative effector citrate stabilised smaller forms of the enzyme, probably dimers and monomers (Stokes' radius 37 Å). Only the tetramer and larger aggregates were enzymically active. Dissociation of the tetramer was reversible for limited time periods provided dithiothreitol was present. Dissociation and aggregation of the tetramer was rapid (equilibration times of a few minutes) in the presence of citrate and activators respectively. Otherwise both processes could be considerably longer. Rate and equilibrium constants for tetramer dissociation have been determined by Hofer & Krystek (1975). Parr & Hammes (1975) found a low concentration of guanidine hydrochloride caused dissociation of the tetramer without unfolding of the polypeptide chains themselves and subsequently showed the process occurs in three phases: dissociation of tetramer to dimer with a relaxation time of a few milliseconds, dissociation of dimer to monomer in a few seconds and conformational changes of the monomer in a few minutes, (Parr & Hammes, 1976). The mechanism of the pH dependent inactivation and reactivation of rabbit muscle phosphofructokinase and the role of ligands in the process was investigated by Bock & Frieden (1976a,b). They suggested inactivation and reactivation occur by kinetically different pathways. Inactivation involves protonation of some ionisable group (presumably histidine) on the tetramer followed by isomerisation to an inactive form and then dissociation to a species of one half the molecular weight. Reactivation involves deprotonation of the dimer to a form that may either isomerise to another inactive form or

dimerise to the active tetramer. The observation of cold lability of the enzyme at less than pH 7.0 (Bock & Frieden, 1974; Bock et al. 1975) was explained by temperature dependence of the apparent pK of the ionisable group. The effect of ligands on the inactivation and reactivation was explained by preferential binding to either protonated or unprotonated forms of the enzyme. ATP and citrate bound to the protonated form and F6P, FDP and AMP bound to the unprotonated form.

Association-dissociation behaviour similar to that exhibited by the rabbit muscle enzyme has been observed for phosphofructokinase from sheep heart muscle, rabbit and human erythrocytes and chicken and pig liver. Sheep heart phosphofructokinase shows an asymmetric peak in the ultracentrifuge at pH 8.0 with $S_{20,w}$ of 15-50 depending on protein concentration. Two peaks with $S_{20,w}$ values of 8.2 and 41 are observed under some conditions, (Mansour et al. 1966). At pH 6.5 the enzyme is reversibly dissociated to a single 7.5S form which is less active, (Mansour & Ahlfors, 1968). Purified rabbit erythrocyte phosphofructokinase shows a single asymmetric 80S peak (molecular weight 5,000,000) in the ultracentrifuge at pH 8.0 and protein concentration 4 mg/ml. The state of aggregation is clearly dependent on enzyme concentration since the enzyme in a fresh haemolysate has a molecular weight of 500,000 as shown by sucrose density gradient centrifugation, (Tarui et al. 1972). Purified human erythrocyte phosphofructokinase shows a single asymmetric 57S peak in the ultracentrifuge at pH 8.0 and protein concentration 3 mg/ml. Sedimentation coefficients in the range 12S (molecular weight 330,000) to 18S (molecular weight 480,000) are observed at lower protein concentrations (0.3-10 μg/ml) with MgATP and F6P present together, (Karadsheh et al. 1977). The reversible association-dissociation behaviour of this enzyme is apparent upon Sepharose 4B chromatography when five separate peaks of activity are obtained with molecular weights that are integral multiples of 210,000. Rechromatography of the 840,000 molecular weight peak gives the five peak pattern again, (Wenzel et al. 1972b). The repeating unit in human erythrocyte phosphofructokinase aggregates is the inactive

dimer (molecular weight 190,000) at pH 7.0 (Wenzel et al. 1976) and the tetramer at pH 8.0 (Hofmann, 1976). Chicken liver phosphofructokinase is cold-labile due to dissociation of the 400,000 molecular weight tetramer to inactive 100,000 molecular weight monomers, (Kono & Uyeda, 1973). The enzyme elutes from a Bio-Gel A1.5M column as three peaks, (molecular weights 100,000, 400,000 and 800,000), when the protein concentration is less than 1 mg/ml. At higher protein concentrations a single peak is observed with a molecular weight of 1,100,000. Ultracentrifugation shows 5, 21 and 28S peaks depending on the protein concentration, (Kono et al. 1973). Pig liver phosphofructokinase does not penetrate 4.2% polyacrylamide gels unless F6P, ATP or ADP is present, (Massey & Deal, 1973). Ultracentrifugation shows a single peak of 44-104S depending on protein concentration and temperature, (Trujillo & Deal, 1977). The 104S species is largest aggregate of phosphofructokinase that has been observed with a molecular weight of at least 10,000,000.

B. Protomer composition and molecular weight.

Sub-unit molecular weights in the range 75,000-95,000 have been reported for rabbit muscle phosphofructokinase representing the protomer of this enzyme, (the identical units from which the oligomeric protein is formed). At pH 11-12 in the presence of 4 mM SDS rabbit muscle phosphofructokinase decreases to a molecular weight of 93,000 (Paetkau & Lardy, 1967). Extensive modification of amino groups by maleic anhydride caused the enzyme to dissociate to an 80,000 molecular weight species, (Uyeda, 1969). Scopes & Penny (1971) reported a molecular weight of 74,000 by SDS polyacrylamide gel electrophoresis. Leonard & Walker (1972) obtained a molecular weight in 6 M guanidine of 85,000 by a sedimentation velocity method and 76,000 by sedimentation equilibrium. Coffee et al. (1973) and Pavelich & Hammes (1973) obtained a value of 75,000 by SDS polyacrylamide gel electrophoresis and 75,000-85,000 by sedimentation equilibrium and gel chromatography in high concentrations of guanidine. Simpson et al. (1977) obtained a value of 80,000 by SDS gel electrophoresis. Walker et al. (1976) reported a single band is observed under denaturing

conditions upon gel electrophoresis at pH 8.9, 4.3 and 2.7. Since the enzyme binds one molecule of either F6P, ADP, AMP or cyclic AMP per 90,000 molecular weight (Kemp & Krebs, 1967) it seems that the protomer of rabbit muscle phosphofructokinase has a molecular weight of about 80,000 and the enzymically active 13S protein is a tetramer.

The sub-unit structure of the 80,000 molecular weight protomer of rabbit muscle phosphofructokinase has been the subject of considerable disagreement. 50 peptides were observed upon tryptic digestion of phosphofructokinase which is half the expected number for the 80,000 molecular weight protomer based on the known lysine and arginine content of the enzyme, (Paetkau et al. 1968). Similarly only 8-9 peptides containing ^{14}C-carboxymethyl cysteine were found upon tryptic digestion of phosphofructokinase carboxymethylated with ^{14}C-iodoacetic acid which is also half of the expected number based on the known cysteine content, (Coffee et al. 1973). These observations suggested the 80,000 molecular weight protomer of phosphofructokinase was comprised of two similar, if not identical, polypeptides linked in such a way as to be resistent to denaturation. The method of linkage was suggested to be a unique peptide bond since limited tryptic digestion of the enzyme gave two protein fragments with molecular weights of about 40,000 (Emerk & Frieden, 1974). However when Walker et al. (1976) repeated the ^{14}C-carboxymethylation experiments and also sequenced the ^{14}C-carboxymethyl cysteine peptides 16 cysteine containing peptides were found not more than one of which was of the same sequence. The number of arginine containing peptides was also close to the expected value. The authors obtained this result by modified conditions of peptide mapping since they found the usual two-dimensional separation method of paper electrophoresis at pH 6.5 followed by electrophoresis at pH 3.5 or chromatography at right angles was unsatisfactory for phosphofructokinase since at least 30% of the peptides remained at the origin. They suggested earlier estimates of peptide numbers were low for this reason. Simpson et al. (1977) also found more tryptic peptides than would be expected if there were a duplicated sequence within the protomer. They obtained

further evidence against any internal homology in the protomer from experiments involving specific ^{14}C-carboxymethylation of the one highly reactive thiol group per 80,000 molecular weight protomer followed by ^{3}H-carboxymethylation of the remaining thiols. After tryptic digestion the ^{14}C-peptide did not map with a ^{3}H-peptide so that the sequence around the highly reactive thiol is unique. Thus it may be concluded that rabbit muscle phosphofructokinase consists of four similar, in all probability identical, polypeptide chains of molecular weight about 80,000.

The results of total amino acid analyses of rabbit muscle phosphofructokinase by three authors are in good agreement, (Parmeggiani et al. 1966; Paetkau et al. 1968; Walker et al. 1976).

The sub-unit structure of sheep heart phosphofructokinase was investigated by Brennan et al.(1974) who concluded the protomer has a molecular weight of 80,000-85,000 and consists of two identical sub-units. However since this conclusion was based on similar experimental findings to those of the earlier studies on the rabbit muscle enzyme it is likely that it may be similarly modified. Further studies of this enzyme have not yet been reported. The N-terminal amino acid sequence has been elucidated by Fordyce et al. (1979). Rabbit erythrocyte phosphofructokinase had a molecular weight of 90,000 upon SDS gel electrophoresis but 53,000 by sedimentation equilibrium in 7 M guanidine and tryptic peptide analysis was consistent with a 100,000 molecular weight protomer being composed of two identical sub-units, (Tarui et al. 1972). The reservations already discussed must apply to these findings especially as such high concentrations of guanidine may degrade the enzyme, (Bloxham & Lardy, 1972). Similar results to those described for the rabbit erythrocyte enzyme led Kono et al. (1973) to conclude the 100,000 molecular weight protomer of chicken liver phosphofructokinase contains two identical sub-units despite the fact that SDS gel electrophoresis showed a single band at a molecular weight of 80,000. Human erythrocyte phosphofructokinase is a hybrid containing two types of protomer, one being the same as that of the human muscle

enzyme, (Karadsheh et al. 1977; Kaur & Layzer, 1977). The other protomer is the same as that of the liver enzyme, (Meienhofer et al. 1979). The enzyme shows two bands on SDS gel electrophoresis with molecular weights of 80,000 and 85,000, the higher value representing the muscle-type protomer.

Structural properties of yeast phosphofructokinase.

Phosphofructokinase from bakers' yeast, Saccharomyces cerevisiae, differs structurally from the enzyme of animal tissues in that it is octameric and does not exhibit self-association behaviour.

The enzyme was purified by Diezel et al. (1973) by a method which minimised proteolytic degredation. Ultracentrifugation gave a single symmetrical peak with an $S_{20,w}$ value of 20.5S (molecular weight 750,000) and a sub-unit molecular weight of about 130,000 by SDS gel electrophoresis was consistent with a hexameric structure of the enzyme. All previous studies to this reported molecular weights in the range 500,000-600,000 (17S), (Lindell & Stellwagen, 1968; Jauch et al. 1970; Atzpodien & Bode, 1970; Kopperschlager et al. 1972a) and a sub-unit molecular weight of about 95,000, (Wilgus et al. 1971; Kopperschlager et al. 1972b). This smaller form of the enzyme results due to proteolytic degredation which reduces the molecular weight without altering the hexameric structure of the enzyme, (Diezel et al. 1973). The 570,000 molecular weight enzyme has been shown to contain traces of a protease (Diezel et al. 1972) and the 21S enzyme can be converted to the 17S form by incubation with yeast extract and subtilisin, (Taucher et al. 1975). Thus the 750,000 molecular weight enzyme, available only since 1973, represents the native enzyme.

Degredation of the native enzyme to the lower molecular weight species by intracellular proteinases may be of regulatory significance as discussed by Holzer (1975) but this remains to be established. In this respect it is of interest that the degredation is accompanied by an increase in the activity of the enzyme (Taucher et al. 1975) and that proteinases A and B from yeast show qualitatively different degredation patterns of the enzyme, (Huse et al. 1976).

The 750,000 molecular weight enzyme was suggested to consist of two types of sub-units, α and β, of molecular weights about 130,000 and present in 1:1 ratio. They were distinguished by immunodiffusion after separation on SDS polyacrylamide gels, (Herrmann et al. 1973). Further evidence to substantiate this proposal has not yet been reported.

Evidence that bakers' yeast phosphofructokinase is octameric and not hexameric as previously supposed was first presented by Kopperschlager et al. (1976). Cross-linking of the native enzyme with dimethyl suberimidate followed by SDS gel electrophoresis to separate and characterise the products of the reaction showed that a tetramer was the largest oligomer formed. This was inconsistent with a hexameric structure of the enzyme. The molecular weight and sub-unit molecular weight of the enzyme were reassesed as 835,000 and 104,000 respectively by sedimentation equilibrium methods which is in agreement with an octameric structure, (Kopperschlager et al. 1977). The octamer is stable over a wide range of protein concentration with dissociation only becoming apparent at less than 10 μg/ml, (Kopperschlager et al. 1977). A model for the quarternary structure of the enzyme in which either the four α sub-units lie in the centre of the molecule and the four β sub-units outside, or vice-versa, was proposed, (Plietz et al. 1978). Further evidence for an octameric structure of the enzyme was presented by Tijane et al. (1979) who showed the presence of all oligomers from monomer to octamer after cross-linking the enzyme with disuccinimidyl β-hydromuconate.

Yeast phosphofructokinase was suggested to contain distinct catalytic and regulatory sub-units (Laurent & Seydoux, 1977; Laurent et al. 1978) but this has not yet been established.

The structural properties of brewers' yeast phosphofructokinase, Saccharomyces carlsbergensis, have been studied by Tamaki & Hess (1975a,b). The enzyme has a molecular weight of 720,000 (19.4S) by gel filtration and sedimentation velocity methods. Ultracentrifugation of the enzyme in the presence of 6 M guanidine gives a sub-unit molecular weight of 90,000 indicating an octameric structure. Two closely

migrating bands are obtained upon SDS gel electrophoresis indicating the occurrence of α and β sub-units as for the bakers' yeast enzyme.

Structural properties of bacterial phosphofructokinase.

Phosphofructokinase from bacteria is a tetrameric enzyme of significantly lower molecular weight than the enzyme from animal tissues and does not exhibit self-association behaviour.

The enzyme from Clostridium pasteurianum was characterised by Uyeda & Kurooka (1970). The molecular weight was 144,000 and there was no evidence of aggregation over the range of protein concentration 10 μg - 5 mg/ml. The enzyme was dissociated into 35,000 molecular weight sub-units in guanidine or urea or by maleylation. The number of peptides formed by tryptic digestion was nearly equal to the number of lysine and arginine residues per 35,000 molecular weight of the enzyme indicating the sub-units are identical.

The molecular weight of Escherichia coli phosphofructokinase is 142,000 and the sub-unit molecular weight 35,000 by SDS gel electrophoresis and 36,500 by ultracentrifugation in guanidine, (Blangy, 1968). The sub-units are identical and ADP binds to four independent sites on the enzyme, (Thornburgh et al. 1978). The tetrameric molecule is composed of a tetrahedral arrangement of the protomers, (Paradies et al. 1977).

E. coli possesses both an allosteric (type 1) and a non-allosteric (type 2) phosphofructokinase. Although originally identified in mutant strains, (Fraenkel et al. 1973), the non-allosteric enzyme is normally present at low levels (5-10% of total activity independent of growth conditions) in wild type strains, (Kotlarz et al. 1975). Babul (1978) showed the two enzymes are immunologically distinct proteins and have separate genetic loci. This author concluded type 2 phosphofructokinase is a tetramer of slightly greater sub-unit molecular weight than the type 1 enzyme. In contrast Kotlarz & Buc (1977) found an identical sub-unit molecular weight but reported the type 2 enzyme is a dimer. The normal function of the non-allosteric enzyme is not known.

E. coli exhibits a greater phosphofructokinase activity

when grown under anaerobic conditions. The enzymes in aerobic and anaerobic cells were reported to be structurally and kinetically quite different, (Doelle, 1974, 1975; Ewings & Doelle, 1976). However Babul et al. (1977) reported the enzyme was identical (type 1) in both cases.

Phosphofructokinases from Lactobacillus plantarum and Lactobacillus acidophilus are both tetrameric enzymes with a molecular weight of 154,000 and show immunological cross-reaction despite the fact that the former enzyme is non-allosteric and the latter allosteric, (Simon & Hofer, 1977).

Phosphofructokinase from the extreme thermophile Thermus X-1 is a tetramer with identical sub-units. The amino acid composition is very similar to that of E. coli and C. pasteurianum phosphofructokinase suggesting that the greater thermostability of the Thermus enzyme arises from only a small number of amino acid differences, (Cass & Stellwagen, 1975).

Crystals of phosphofructokinase from Bacillus stearothermophilus have been obtained and are suitable for detailed X-ray crystallography, (Hengartner & Harris, 1975). The tetrameric enzyme is composed of identical sub-units of 312 amino acids, (Hudson et al. 1977). There is one substrate binding site and one effector binding site per sub-unit. The effector site binds both the activator (ADP) and the inhibitor (Pyr-P) of this enzyme. Both sites are closely situated at the interface between two sub-units and it appears that cooperativity of F6P binding and allosteric control are mediated by ligand binding which bridges the interface, (Evans & Hudson, 1979).

Structural properties of invertebrate phosphofructokinase.

Only one study of the structure of phosphofructokinase from an invertebrate source has been made to date. The enzyme from the adductor muscle of the oyster, Crassostrea virginica, migrates as a single band on polyacrylamide gel electrophoresis and has a molecular weight of 340,000 by gel chromatography, (Storey, 1976).

Catalytic properties of phosphofructokinase.

A. Substrate specificity.

Rabbit muscle phosphofructokinase can phosphorylate several other sugars apart from D-F6P. These are listed in Table 3 together with their apparent K_m values and the rate of reaction relative to that for D-F6P under the same conditions.

The anomeric distribution of F6P in aqueous medium was determined by use of ^{13}C NMR spectroscopy, (Swenson & Barker, 1971; Benkovic et al. 1972). The ratio cyclic β-furanose : cyclic α-furanose : acyclic keto form is 76:19:5 respectively. Absolute stereospecificity of phosphofructokinase for the β anomer was shown by Wurster & Hess (1974) and Fishbein et al. (1974) who used stopped-flow kinetics with high enzyme concentrations to demonstrate an initial fast reaction phase consuming about 76% of the substrate and dependent on enzyme concentration followed by a slower phase dependent on the rate of spontaneous mutarotation rather than enzyme concentration. Studies using the cyclically locked analogue methyl D-fructofuranoside-6-P suggested that phosphorylation occurs without intervention of an enzyme bound acyclic keto form, (Fishbein et al. 1974). These conclusions were further supported by the observations that 2,5-anhydro-D-mannitol-6-P (structurally locked β analogue) was a substrate but 2,5-anhydro-D-glucitol-6-P (structurally locked α analogue) was not and several analogues of the acyclic keto form were neither inhibitors nor substrates, (Koerner et al. 1974). It appeared that the active site would accept α-F6P in binding but not phosphorylation since the α analogue was a competitive inhibitor. The active substrate for the reverse reaction is β-FDP (Bar-Tana & Cleland, 1974a). A model for the control of futile-cycling between phosphofructokinase and fructose-1-6-diphosphatase based on their opposite anomeric specificities was proposed by Koerner et al.(1977a).

Epimers of β-F6P were used to show that the configuration at carbon atoms C-3 (L) and C-5 (D) are important for binding but that at C-4 is not, (Koerner et al. 1976). The C-2 hydroxyl group of β-F6P is also involved in binding

since the apparent K_m and dissociation constant of 2,5-anhydro-D-mannitol-6-P are higher than those of F6P, (Bar-Tana & Cleland, 1974a). Martensen & Mansour (1976) suggested the divalent C-6 phosphoryl moiety was involved in the active site conformation from studies on the sheep heart enzyme with the alternative substrate fructose-6-sulphate. Pyrophosphate dependent phosphofructokinase from Entamoeba histolytica is specific for β-F6P, (Koerner et al. 1977b). Activation of phosphofructokinase by D-FDP was investigated with 2,5-anhydro-D-glucitol-1-6-P_2 and 2,5-anhydro-D-mannitol-1-6-P_2 and the α analogue found to be more effective than the β analogue although the observed activation was only 10% of that for D-FDP, (Benkovic & Schray, 1976).

Phosphofructokinase from rabbit muscle, yeast, E. coli and C. pasteurianum can use all the major physiologically occurring nucleoside triphosphates as phosphoryl donors. The apparent K_m values of the other nucleotides are mostly higher than that for ATP but the apparent maximum velocity is seldom greatly reduced, (see Table 3). The enzymes from Brussels sprouts (Dennis & Coultate, 1967) and Neurospora crassa (Tsao & Madley, 1972) have a narrower substrate specificity in that purine nucleotides are better substrates than pyrimidine nucleotides. Muscle phosphofructokinase can use the synthetic phosphoryl donors 1,N^6-etheno-ATP, a fluorescent analogue (Secrist et al. 1972), and 6-mercapto-9-β-D-ribofuranosylpurine-5'-triphosphate, (Bloxham et al. 1973). α,β-methylene-ATP is a substrate whereas β,γ-methylene-ATP is a competitive inhibitor with respect to ATP, (Cottam & Uyeda, 1973).

Phosphofructokinases are rather more specific for ATP as an allosteric inhibitor than as a substrate. Rabbit muscle phosphofructokinase is inhibited by ATP, CTP and UTP but not ITP even though this nucleotide is a substrate, (Uyeda & Racker, 1965). Studies with ATP analogues modified in the base moiety or the polyphosphate chain suggested both these areas of the nucleotide had to be intact for full inhibitory activity, (Barzu et al. 1977; Ngoc et al. 1979). Yeast phosphofructokinase is inhibited by ATP but not ITP or GTP, (Lindell & Stellwagen, 1968). Similarly ITP is a substrate but not an inhibitor of E. coli phosphofructo-

kinase, (Atkinson & Walton, 1965).

The divalent metal ion requirement of cow brain phosphofructokinase can be fulfilled by Mg^{2+}, Mn^{2+} or Co^{2+}, (Muntz, 1953). For muscle phosphofructokinase Lardy (1962) found that Mn^{2+} and Co^{2+} were respectively 77% and 71% as effective as Mg^{2+} at 4 mM and that Zn^{2+} did not activate. Ca^{2+} is a competitive inhibitor with respect to Mn^{2+} for rabbit muscle phosphofructokinase, (Jones et al. 1972).

B. Effect of monovalent cations on phosphofructokinase activity

Phosphofructokinase is strongly activated by K^+ and NH_4^+. This is observed for the enzyme from a wide variety of sources, (Bloxham & Lardy, 1973; Uyeda, 1979), including crustaceans, (Sugden & Newsholme, 1975). Na^+ has no effect on rabbit muscle (Paetkau & Lardy, 1967), yeast (Atzpodien & Bode, 1970) or Thermus X-1 phosphofructokinase (Stellwagen & Thompson, 1979) but it inhibits the pig liver enzyme at concentrations above 50 mM, (Foe & Trujillo, 1979). As pointed out by Seulter (1974) the general mechanism of activation of enzymes by monovalent cations involves the formation of enzyme-cation rather than substrate-cation complexes. This situation is assumed to apply with phosphofructokinase although no direct binding studies have been reported for the enzyme to date.

Yeast phosphofructokinase was completely inactive in the absence of K^+ and NH_4^+, (Hofmann, 1976). Maximum activation by NH_4^+ is achieved at lower concentrations than that for K^+. The activity of the enzyme from rabbit muscle and liver was reported to be higher when maximally activated by K^+ than when NH_4^+ was saturating, (Paetkau & Lardy, 1967; Kemp, 1971). A saturating concentration of F6P was used in these studies. However the opposite situation was observed by Abrahams & Younathan (1971) and Kuhn et al. (1974) for rabbit muscle and erythrocyte phosphofructokinase. A non-saturating concentration of F6P was present in these studies. These anomalies can be accounted for by the fact that NH_4^+ lowers the $(S)_{0.5}$ for F6P more than does K^+, (Foe & Trujillo, 1979). Thus at saturating levels of F6P the K^+-activated enzyme has a greater activity than the NH_4^+-activated enzyme

and the situation is reversed at non-saturating F6P concentrations.

Yeast phosphofructokinase has distinct high and low affinity binding sites for NH_4^+. When binding at the high affinity site NH_4^+ acts as a simple activator. At the low affinity site however NH_4^+ is a positive allosteric effector and decreases cooperativity of F6P binding. K^+ on the other hand is a simple activator only, (Mavis & Stellwagen, 1970). Similar observations were reported for rabbit and rat erythrocyte phosphofructokinase by Kuhn et al.(1974) and Otto et al. (1974) who further showed that K^+ and NH_4^+ compete for the high affinity site. NH_4^+ can still activate these enzymes at saturating concentrations of K^+ if F6P is not saturating because of its dual effect on the enzyme.

K^+ was reported as a simple activator only for rabbit muscle (Abrahams & Younathan, 1971) and pig liver phosphofructokinase (Foe & Trujillo, 1979). However for the latter two enzymes NH_4^+ does not decrease cooperativity of F6P binding although it decreases the $(S)_{0.5}$ for this substrate. In contrast Otto et al. (1976) reported that both K^+ and NH_4^+ act as both activators and allosteric effectors of rabbit muscle phosphofructokinase. NH_4^+ can activate the rabbit muscle enzyme in the presence of saturating concentrations of K^+ at pH 6.9 but not pH 8.0 since at the latter pH the enzyme is already fully allosterically activated, (Pettigrew & Frieden, 1979b).

C. Effect of anions on phosphofructokinase activity.

The SO_4^{2-} ion is an activator of phosphofructokinase from human platelets (Akkerman et al. 1974a), rat erythrocytes (Kuhn et al. 1974; Otto et al. 1977), rat jejunum (Tejwani & Ramiah, 1971), pea seeds (Kelly & Turner, 1969) and blood flukes (Bueding & Fisher, 1966). A recent study by Foe & Trujillo (1980) examined the characteristics of this activation for the pig liver enzyme. SO_4^{2-} activated by reducing the cooperativity of phosphofructokinase kinetics with respect to F6P and by counteracting ATP inhibition. The same effect was achieved with $S_2O_3^{2-}$, SO_3^{2-} and MoO_4^{2-}. All these ions are phosphate analogues and presumably sustitute for HPO_4^{2-} in allosteric activation of the enzyme.

Monovalent anions were not activators and in fact reduced the maximum velocity of the enzyme at concentrations above 50 mM.

D. Role of divalent cations in phosphofructokinase catalysis.

Phosphofructokinase requires a divalent metal ion for enzymic activity, physiologically this is Mg^{2+}. The precise role of magnesium in catalysis is not yet clear. This cation forms dissociable complexes with ATP and ADP as described in the next chapter of this thesis. Thus although the overall stoichiometric equation of the phosphofructokinase catalysed reaction can be represented as in the introduction to this chapter the precise mechanistic equation may be either, both or a combination of the following two equations depending on which substrate and product species interact with the enzyme.

$$F6P^{2-} + Mg^{2+} + ATP^{4-} \rightleftharpoons FDP^{4-} + Mg^{2+} + ADP^{3-} + H^{+}$$

$$F6P^{2-} + MgATP^{2-} \rightleftharpoons FDP^{4-} + MgADP^{-} + H^{+}$$

Since $MgATP^{2-}$ is the predominant species physiologically it has long been assumed that it is the only active substrate species and few kinetic studies on this enzyme have attempted to discriminate between the different possibilities.

A hyperbolic increase in reaction rate as a function of total Mg concentration at fixed total ATP concentration was interpreted as showing $MgATP^{2-}$ is the active substrate of E. coli phosphofructokinase, (Blangy et al. 1968). A decrease in rate at pH 8 when the total Mg : total ATP ratio exceeded 2:1 was interpreted as showing free Mg^{2+} was an inhibitor of the sheep brain enzyme, (Lowry & Passonneau, 1966). However no inhibitory effect of free Mg^{2+} up to 25 mM could be detected for yeast phosphofructokinase at 0.3 mM ATP, (Hofmann, 1976).

Several observations have indicated that low concentrations of free Mg^{2+} can limit the rate of reaction. Lardy & Parks (1956) found that the rabbit muscle enzyme was maximally active when ATP and Mg concentrations were equal and that whereas a molar excess of Mg over ATP did not

appreciably inhibit the enzyme (15% inhibition for an eighteen-fold excess), the opposite condition progressively inhibited the enzyme as the ATP concentration was increased, (up to 80% inhibition). Subsequently the enzyme was shown to be inactive in a pH-stat assay free of buffer and linking enzymes unless the total Mg concentration exceeded the total ATP concentration even though $MgATP^{2-}$ was saturating. This suggested either free Mg^{2+} is required for catalytic activity (in an unspecified role), or ATP^{4-} is a very powerful inhibitor, (Paetkau & Lardy, 1967). From experiments with yeast phosphofructokinase Mavis & Stellwagen (1970) proposed that the magnesium-nucleotide complex is the active substrate and that catalysis requires free Mg^{2+} (in an unspecified role). These authors calculated the concentrations of free Mg^{2+}, $MgITP^{2-}$ and ITP^{4-} present when total ITP concentration was varied at different fixed total Mg concentrations, total Mg concentration was varied at different fixed total ITP concentrations, or both ligands were varied in equimolar ratio. The enzymic rate was a function of the concentration of $MgITP^{2-}$ rather than ITP^{4-} and depended on free Mg^{2+} when this was low (< 60 μM) compared to $MgITP^{2-}$ (> 200 μM). Akkerman et al. (1974) assumed the substrate of human platelet phosphofructokinase was the metal-nucleotide complex and investigated kinetic effects of free Mg^{2+} and ITP^{4-} in a series of experiments holding one of the species free Mg^{2+}, ITP^{4-} or $MgITP^{2-}$ constant while varying the other two. A decrease in rate at constant $MgITP^{2-}$ was more clearly related to a decrease in free Mg^{2+} than to an increase in ITP^{4-} suggesting the former had a direct effect on enzymic activity. The authors suggested the basis of this effect was that free Mg^{2+} formed a complex with the enzyme to activate it. Jones et al. (1972) observed only a small enhancement of water proton relaxation rate in Mn^{2+} solutions of the enzyme. This rose sharply on adding ATP suggesting the metal ion must form a complex with the nucleotide before binding to the enzyme. Once bound however there was some evidence that the metal ion might interact with the enzyme. In contrast Cottam & Uyeda (1973) obtained direct evidence of binding of Mn^{2+} to rabbit muscle phosphofructokinase using NMR techniques.

There appeared to be one or two metal ion binding sites with K_s = 20 μM per molecule of enzyme and as many as fourteen binding sites with K_s about 800 μM per molecule of enzyme. It was suggested that the high affinity sites were at the active site while the low affinity sites represented non-specific binding. Formation of an enzyme-MnATP ternary complex and an enzyme-Mn-β-γ-methylene-ATP-F6P quarternary complex was also demonstrated. The type of coordination scheme of the terniary complex was not clear. Jones et al. (1974) studied binding of $MnATP^{2-}$ to rabbit muscle phospho-fructokinase using NMR techniques and suggested the ternary complex was of the type $E\begin{smallmatrix}S\\M\end{smallmatrix}$. Peters et al. (1979) studied binding of $^{54}Mn^{2+}$ to yeast phosphofructokinase by equil-ibrium dialysis and found three independent binding sites per enzyme protomer with identical affinities, (K_s = 2.26 mM). Changes in intrinsic protein fluorescence of rabbit muscle phosphofructokinase were consistent with binding of ATP^{4-} or $MgATP^{2-}$ at the active site but the latter had a higher affinity, (Pettigrew & Frieden, 1979a).

Several investigators have examined which species of ATP is the active allosteric inhibitor. Increasing the magnesium concentration at inhibitory levels of ATP decreased the inhibition, (Dennis & Coultate, 1966; Griffin et al. 1967). However since converting all the ATP to $MgATP^{2-}$ by saturat-ing with Mg does not completely overcome ATP inhibition both $MgATP^{2-}$ and ATP^{4-} must be inhibitory, (Paetkau & Lardy, 1967). For muscle and erythrocyte phosphofructokinase ATP^{4-} was reported to be a more powerful inhibitor than $MgATP^{2-}$, (Lowry & Passonneau, 1966; Otto et al. 1974). There is evidence that $MgATP^{2-}$ rather than ATP^{4-} is the inhibitory species for yeast phosphofructokinase, (Mavis & Stellwagen, 1970). Pettigrew & Frieden (1979) were able to distinguish between ATP binding at the catalytic and regulatory sites of rabbit muscle phosphofructokinase from measurements of changes in intrinsic protein fluorescence. They found ATP^{4-} bound about ten times more tightly than $MgATP^{2-}$ at the regulatory site.

E. Studies on the kinetic mechanism of phosphofructokinase.

Since changes in the free Mg^{2+} concentration can affect phosphofructokinase activity studies on the kinetic mechanism of the enzyme have employed a single constant concentration of this ion to eliminate any kinetic effects of free Mg^{2+}. At the concentrations of free Mg^{2+} usually used, (about 4 mM), ATP is about 98% $MgATP^{2-}$ which has been taken to be the only active substrate of the nucleotide.

Initial-rate studies on phosphofructokinase yielded a series of apparently parallel lines in double-reciprocal plots for the enzymes from rabbit muscle (Uyeda, 1970; Kee & Griffin, 1972), ox heart (Hulme & Tipton, 1971), human skeletal muscle and erythrocytes (Layzer et al. 1969), ascites tumour cells (Sumi & Ui, 1972b), yeast (Vinuela et al. 1963), Dictyostelium discoideum (Baumann & Wright, 1968) and Flavobacterium thermophilum (Yoshida, 1972). Sheep heart phosphofructokinase studied in the reverse direction also yielded an apparently parallel pattern (Lorensen & Mansour, 1968). Parallel lines are often indicative of a "ping-pong" mechanism in which one substrate first reacts with the enzyme to produce the first product and a free modified enzyme which then reacts with the second substrate to yield the second product, (Cleland, 1970). On this basis and from product-inhibition and isotope exchange studies Uyeda (1970) proposed a ping-pong mechanism for rabbit muscle phosphofructokinase in which ADP was released from the enzyme before F6P bound. Isotope exchange between ^{14}C-ADP and ATP in the absence of F6P was observed to occur at about 1% of the maximum rate of the reaction when both substrates were present. A very slow exchange between ^{14}C-F6P and FDP in the absence of ATP was also demonstrated. These exchanges were catalysed by the enzyme since they were dependent on the presence of phosphofructokinase and magnesium. No direct evidence for the existence of a phosphoryl-enzyme could be obtained but a "pulse-labelling" experiment, consisting of a brief incubation of the enzyme with ^{32}P-ATP and subsequent dilution with an excess of non-radioactive ATP and F6P, yielded highly radioactive FDP suggesting an initial interaction between the enzyme and ATP (Uyeda, 1970). Ox heart phosphofructokinase catalysed

a ^{14}C-ADP-ATP exchange reaction at about 1% of the maximum forward reaction rate (Hulme & Tipton, 1971). However the ^{14}C-F6P-FDP exchange reaction for this enzyme was absolutely dependent on the presence of ADP and magnesium which is inconsistent with a ping-pong mechanism. Similarly Hanson et al. (1973) could detect no exchange between hexose phosphates in the absence of nucleotides for the rabbit muscle enzyme and Blangy (1971) did not detect any significant isotope exchange for either substrate-product pair for E. coli phosphofructokinase.

Exchange reactions between similar substrate and product pairs in the absence of the other pair are consistent with a ping-pong mechanism. However the rates of such exchanges reported for phosphofructokinase are not fast enough to indicate a kinetically significant pathway. Such low rates of isotope exchange may result from some side reaction of the enzyme. Phosphofructokinase has both a low ATP-ase and FDP-ase activity (Uyeda, 1970) and the observed exchanges may represent the reverse of these reactions, (Hanson et al. 1973).

The apparently parallel pattern of lines in double-reciprocal plots observed for many phosphofructokinases is consistent with but not proof of a ping-pong mechanism. The reciprocal form of the rate equation for a sequential mechanism involving two substrates, A and B, is:-

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m^A}{VA} + \frac{K_m^B}{VB} + \frac{K_s^A K_m^B}{VAB}$$

If the value of K_s^A is small compared to that of K_m^A and K_m^B and if the fixed substrate is used at concentrations much greater than its K_m then the slope effect due to the changing fixed substrate is small and convergence of the lines may not be detected. When initial-rate data was obtained for the rabbit muscle enzyme using low substrate concentrations (2.5 - 10 μM) relative to the Michaelis constants (K_m^{ATP} = 20 μM, K_m^{F6P} = 21 μM, K_s^{ATP} = 5 μM) a clearly intersecting pattern of lines was obtained, (Hanson et al. 1973). Bar-Tana & Cleland (1974a) studied the kinetics of rabbit muscle phosphofructokinase with a range

of nucleotide substrates and a substrate analogue of F6P as well as with the physiological substrates. Some of the double-reciprocal plots were apparently parallel but others were intersecting since the $K_s:K_m$ ratio was larger in these cases. Intersecting lines were also obtained for phosphofructokinase from sheep brain (Lowry & Passonneau, 1966), Lactobacillus casei and Lactobacillus plantarum (Doelle, 1972; Simon & Hofer, 1978), human erythrocytes (Etiemble et al. 1977) and rat liver (Brand & Soling, 1974). Uyeda (1972) reevaluated his experiments on rabbit muscle phosphofructokinase with F1P as a substrate and also found an intersecting pattern of lines. Thus initial-rate and isotope exchange studies strongly suggest the mechanism is sequential.

Product-inhibition experiments designed to distinguish whether the sequential mechanism of phosphofructokinase involves an ordered or random addition of substrates have given different results for the enzyme from different sources. The expected inhibition patterns for three different sequential mechanisms when the non-varied substrate is employed at non-saturating concentrations are as follows:-

		Variable substrate	
Mechanism	Product-inhibitor	F6P	ATP
Random	FDP	C	NC
	ADP	NC	C
Ordered	FDP	NC	NC
(ATP first substrate,	ADP	NC	C
ADP last product)			
Ordered	FDP	C	NC
(F6P first substrate,	ADP	NC	NC
FDP last product)			

For the random mechanism non-competitive inhibition between dissimilar substrate and product pairs is assumed since it is likely that the dead-end complexes Enzyme-F6P-ADP and Enzyme-ATP-FDP will form in such a mechanism.

Uyeda (1972) and Hanson et al. (1973) found that for

rabbit muscle phosphofructokinase the product inhibition patterns corresponded to that for an ordered sequential mechanism with ATP as the first substrate and ADP as the last product. In contrast Kee & Griffin (1972) and Bar-Tana & Cleland (1974b), also using the rabbit muscle enzyme, obtained product inhibition patterns corresponding to a random mechanism. The difference in these results lies in the nature of inhibition of FDP with respect to F6P. Hanson et al. (1973) considered that the mechanism was random and that formation of an Enzyme-F6P-FDP dead-end complex, possibly due to binding of the 1-phosphate of FDP at the γ-phosphate site of the ATP binding site, resulted in non-competitive FDP versus F6P inhibition. This complex would be expected to form at low but not high concentrations of ATP and in agreement with this Kee & Griffin (1972) obtained competitive FDP versus F6P inhibition at 50 μM ATP while Hanson et al. (1973) observed the pattern was non-competitive at 20 μM ATP. However Bar-Tana & Cleland (1974b) obtained competitive inhibition for this pattern at 20 μM ATP. Since product-inhibition patterns are often inconclusive in the case of a random mechanism substrate analogues which are dead-end inhibitors have been used to further study the reaction. Arabinose-5-phosphate (Hanson et al. 1973) and 1-deoxy-F6P and chromium-ATP (Bar-Tana & Cleland, 1974b)were competitive inhibitors with respect to the substrate which they resemble and non-competitive with respect to the other substrate for both the forward and reverse reactions. This is consistent with a random mechanism and rules out an ordered mechanism since in this case inhibition by the analogue resembling the second substrate to add would be un-competitive versus the first substrate to add. From a quantitative analysis of the product-inhibition and initial-rate data Bar-Tana & Cleland (1974b) suggested that rapid-equilibrium conditions occured in the reverse but not the forward reaction.

For phosphofructokinase from rat liver (Brand & Soling, 1974), human erythrocytes (Etiemble et al. 1977) and Lactobacillus plantarum (Simon & Hofer, 1978) the product-inhibition patterns obtained at non-saturating concentrations of the non-varied substrate corresponded to an ordered

mechanism with F6P as the first substrate and FDP as the last product. Since such an inhibition pattern is also consistent with a random mechanism in which a dead-end Enzyme-ATP-ADP complex is able to form (as well as the complexes Enzyme-F6P-ADP and Enzyme-ATP-FDP) the inhibition patterns were repeated at saturating concentrations of the non-varied substrate. This procedure may change the nature of an inhibition pattern depending on the kinetic mechanism, (Cleland, 1970). For the enzymes from rat liver (Brand & Soling, 1974) and erythrocytes (Etiemble et al. 1977) saturation with the non-varied substrate did not alter the FDP versus F6P or ADP versus ATP inhibition patterns. This is evidence against a random mechanism involving a dead-end Enzyme-ATP-ADP complex since in this case the saturation procedure should change the ADP versus ATP inhibition from non-competitive to competitive. Further, inhibition by ADP with respect to F6P became un-competitive at saturating ATP concentration and at saturating F6P concentration inhibition by FDP with respect to ATP was completely abolished. For Lactobacillus plantarum phosphofructokinase ADP versus F6P inhibition also became uncompetitive on saturation with the non-varied substrate, (Simon & Hofer, 1978). These results are entirely consistent with the ordered sequential mechanism proposed for these enzymes, (F6P first substrate, FDP last product).

Simon & Hofer (1978) measured the velocity of isotope exchange at equilibrium between ^{14}C-F6P and FDP and between ^{14}C-ATP and ADP when either F6P and FDP or ATP and ADP were varied in constant ratio. Linear double-reciprocal plots were obtained for both exchanges when F6P and FDP were varied. Varying ATP and ADP gave a linear double-reciprocal plot for the ^{14}C-ATP-ADP exchange and exhibited substrate inhibition of the ^{14}C-F6P-FDP exchange. This supports the ordered mechanism (F6P first substrate, FDP last product) but it was not clear if there was an alternative minor pathway since it could not be concluded whether the substrate inhibition of isotope exchange was total or partial.

The mechanism of sheep heart phosphofructokinase was reported to be rapid equilibrium ordered (ATP first substrate to bind) when the alternative substrate fructose-6-sulphate

was used, (Martensen & Mansour, 1976). The double-reciprocal plot with fructose-6-sulphate as the variable substrate and ATP as the changing fixed substrate showed a pattern of lines intersecting on the ordinate axis.

F. Thermodynamic aspects and turnover number of the phosphofructokinase reaction.

The equilibrium of the reaction catalysed by phosphofructokinase in vitro is very much in favour of FDP and ADP. In general the value of the equilibrium constant varies with temperature and pH and in the case of the phosphofructokinase reaction depends also on which substrate and product species paricipate in the enzyme catalysed reaction. Unless this latter information is known and the exact concentrations of the reactive species at equilibrium are calculated then only an apparent equilibrium constant can be determined based on the total measureable concentrations of F6P, ATP, FDP and ADP and dependent on the concentrations of the activating cations.

Hanson et al. (1973) estimated an apparent equilibrium constant at 30°C, pH 8.0 and 4 mM free Mg^{2+} of 2.3×10^3. For reaction of ATP^{4-} with $F6P^{2-}$ to produce FDP^{4-}, ADP^{3-} and H^+ the equilibrium constant was 8.5×10^3, while for reaction of $MgATP^{2-}$ with $F6P^{2-}$ to produce FDP^{4-}, $MgADP^-$ and H^+ the equilibrium constant was 1.2×10^3. The turnover numbers for the forward and reverse reactions respectively were 8.6×10^4 and 3.7×10^3 moles/minute per 380,000 g of enzyme, (Hanson et al. 1973). This is in agreement with the finding that the rate of the reverse reaction is about 5% of the forward rate for sheep heart phosphofructokinase, (Lorensen & Mansour, 1968). The apparent equilibrium constant at 20°C, pH 7.0 and 20 mM total Mg^{2+} was estimated as 4×10^2 (Simon & Hofer, 1978). Values of enthalpy, entropy and free energy at various temperatures were reported by Hofmann (1976). The Arrhenius energy of activation was determined as -10.2 kcal/mole, (Freyer et al. 1970).

Regulatory properties of phosphofructokinase.

Phosphofructokinase from animals, plants, yeast and many microorganisms is an allosteric enzyme and its activity is influenced by a variety of effectors. Only a few micro-organisms with specialised modes of nutrition possess non-allosteric forms of the enzyme. The main regulatory features of allosteric phosphofructokinases are as follows:-

(1) Inhibition by high concentrations of ATP.
(2) Inhibition by citrate in the presence of inhibitory concentrations of ATP.
(3) Counteraction of the inhibition caused by ATP by F6P, FDP, ADP, AMP, cyclic AMP and inorganic phosphate.
(4) In the presence of inhibitory concentrations of ATP increasing the concentration of F6P has a cooperative effect on the activity of the enzyme so that the rate curve is sigmoidal. Negative effectors decrease the affinity for F6P while positive effectors increase the affinity.

The enzyme shows phylogenetic variation with respect to its regulatory properties. Plant phosphofructokinases are inhibited by ADP and AMP rather than activated. AMP was also reported to be an inhibitor of phosphofructokinase from the crayfish Orconectes virilis (Freed & Kirk, 1976) but for two other crustaceans, the lobster Homarus vulgaris and the Alaskan king crab Paralithodes camtschatica, it was an activator and relieved ATP inhibition of these enzymes, (Sugden & Newsholme, 1975; Freed, 1971). Squid, Symplectoteuthis oualaniensis, muscle phosphofructokinase is activated by AMP and inorganic phosphate but ATP, ADP and FDP have no effect on the enzyme, (Storey & Hochachka, 1975). ATP inhibits phosphofructokinase from the adductor muscle of the oyster, Crassostrea virginica, (Storey, 1976). Bacterial phosphofructokinases have fewer effectors than yeast or animal phosphofructokinases, (Bloxham & Lardy, 1972). Effectors of the enzyme alter the affinity for F6P by changing the kinetics with respect to F6P from sigmoidal to hyperbolic (activators) or vice versa (inhibitors). This effect is not observed for yeast phosphofructokinase

although the affinity for F6P is still altered since the rate curve is moved either to the left (activators) or right (inhibitors).

ATP is both a substrate and an allosteric inhibitor of phosphofructokinase. Thus as the ATP concentration is increased the enzymic rate first increases and then decreases. This property of phosphofructokinase is significant for the regulation of the glycolytic pathway. The regulatory effect of ATP on the enzyme is enhanced by the fact that the metabolic degredation products of ATP, i.e. ADP, AMP, inorganic phosphate, relieve ATP inhibition. Thus the activity of the enzyme is controlled by the energy status of the cell, (Atkinson, 1968). The importance of phosphofructokinase as a control site in glycolysis in crustacean muscle was demonstrated by Hochachaka et al. (1971). The extent of inhibition by ATP is dependent on the pH of the medium. For rabbit muscle phosphofructokinase ATP is a powerful inhibitor (K_i = 2.5 mM) at pH 7.1, a weaker inhibitor at pH 7.6-8.5 and does not inhibit at pH 9.0 (Uyeda & Racker, 1965). Similar behaviour is observed for phosphofructokinase from brain (Lowry & Passonneau, 1966), diaphragm (Ui, 1966), heart (Mansour & Ahlfors, 1968) and liver (Kemp, 1971). The enzymes from yeast and E. coli show the opposite effect in that they are more sensitive to ATP inhibition at pH 8 than at pH 6, (Lindell & Stellwagen, 1968; Kopperschlager et al. 1968; Blangy et al. 1968).

Citrate is an allosteric inhibitor of phosphofructokinase. Various other TCA cycle intermediates also inhibit rat brain phosphofructokinase (Passonneau & Lowry, 1963) but not rat heart phosphofructokinase (Garland et al. 1963) and the enzymes from other tissues show intermediate specificities. The inhibitory action of citrate was found to be independent of the ATP concentration for rat heart phosphofructokinase (Pogson & Randle, 1966) but for the rat brain enzyme inhibition only occurred at inhibitory concentrations of ATP (Passonneau & Lowry, 1963). Citrate did not inhibit rabbit muscle phosphofructokinase when the non-inhibitory phosphoryl donor ITP was used, (Colombo et al. 1975).

Pyr-P, 2-P-glycerate, 3-P-glycerate, 2,3-diphosphoglycerate and creatine phosphate are inhibitors of various

phosphofructokinases, (Uyeda & Racker, 1965; Kelly & Turner, 1968 and 1970; Krzanowski & Matshinsky, 1969; Kemp, 1971; Thomas et al. 1972; Tarui et al. 1972). Their inhibition is greater at neutral than at alkaline pH and at inhibitory rather than non-inhibitory concentrations of ATP, (Kemp, 1971). Although a physiological role in enzyme control for the inhibitory action of creatine phosphate can readily be envisaged it has recently been shown that the observed inhibition *in vitro* is an artifact due to impurities in commercially available creatine phosphate, (Fitch et al. 1979). Invertebrate animals possess arginine phosphate in place of creatine phosphate and although this has been reported to inhibit invertebrate phosphofructokinase (Storey & Hochachka, 1974) it has also been found to contain impurities which may lead to inhibition of kinase enzymes, (Giles et al. 1980).

F6P, FDP, ADP, AMP, cyclic AMP and inorganic phosphate relieve the inhibition of phosphofructokinase by ATP and citrate. FDP is the most potent activator (Passonneau & Lowry, 1963) and has significant effects on the enzyme under conditions simulating those *in vivo*, (Hood & Hollaway, 1976). Its action is enhanced by AMP (Tornheim & Lowenstein, 1976). Similarly inorganic phosphate has a synergistic effect on ADP activation, (Passonneau & Lowry, 1963). Glucose-1-6-diphosphate has been shown to activate erythrocyte phosphofructokinase *in vivo*, (Rose & Warms, 1974). Cyclic GMP was reported to be an inhibitor of rat heart and skeletal muscle phosphofructokinase, (Beitner et al. 1977).

The rate curve with respect to F6P concentration is sigmoidal in the presence of inhibitory concentrations of ATP. Sigmoidal kinetics appear to be the result of ATP binding at the inhibitory site since several bacterial phosphofructokinases that are not inhibited by ATP show hyperbolic rate curves for F6P (Yoshida, 1972; Doelle, 1972; Ferdinandus & Clark, 1969). However other factors may be involved since *Clostridium pasteurianum* phosphofructokinase which is not inhibited by ATP shows a sigmoidal rate dependence on F6P concentration (Uyeda & Kurooka, 1970) and yeast phosphofructokinase still exhibits sigmoidal kinetics when the non-inhibitory substrate UTP is used, (Kopperschlager

et al. 1968). For phosphofructokinase from most sources the kinetics with respect to F6P become hyperbolic at alkaline pH. However the cooperativity of F6P binding for yeast phosphofructokinase is not affected by pH (Kopperschlager et al. 1968) and in fact increasing the pH moves the rate curve to the right so that H^+ may be regarded as an activator for this enzyme, (Hofmann, 1976).

Injection of insulin or glucagon into rats causes changes in the activity of phosphofructokinase in the liver of the animal within five minutes, (Taunton et al. 1974). Direct evidence that phosphorylation and dephosphorylation of liver phosphofructokinase may be involved in the regulation of its activity was obtained by Brand & Soling (1975). These authors showed that crude rat liver phosphofructokinase could be inactivated in the presence of high $MgCl_2$ (20 mM) and reactivated by $MgATP^{2-}$. They separated active and inactive forms of phosphofructokinase as well as enzymes catalysing the inactivation and reactivation reactions. Incubation of inactive phosphofructokinase with a partially purified activating enzyme preparation and ^{32}P-ATP resulted in incorporation of ^{32}P into phosphofructokinase. Sorensen & Hofer (1979) showed incorporation of ^{32}P into mouse skeletal muscle phosphofructokinase was produced *in vivo* by injection of ^{32}P-phosphate and *in vitro* by incubation of the enzyme with cyclic AMP dependent protein kinase and ^{32}P-ATP. Glucagon stimulated phosphorylation of rat liver phosphofructokinase *in vivo* was demonstrated by Kagimoto & Uyeda (1979).

Table 1.

Specific activities of phosphofructokinases.

Source of enzyme	Specific activity	Reference
Skeletal muscle* (rabbit)	180 (28°C)	Ling et al. (1966)
Skeletal muscle (human)	99.5 (25°C)	Layzer et al. (1969)
Skeletal muscle (human)	198 (30°C)	Cottreau et al. (1979)
Heart muscle* (sheep)	157 (25°C)	Mansour (1966)
Heart muscle (ox)	93 (25°C)	Frenkel (1968)
Adductor muscle (oyster)	112 (25°C)	Storey (1976)
Liver (sheep)	18.5 (25°C)	Brock (1969)
Liver (rabbit)	48 (26°C)	Kemp (1971)
Liver* (chicken)	134 (25°C)	Kono et al. (1973)
Liver (rat)	84 (30°C)	Brand & Soling (1974)
Liver (mouse)	63 (25°C)	Herzberg (1979)

Table 1 (contd).

Source of enzyme	Specific activity	Reference
Liver / Kidney (pig)	100 / 128 ($25^{o}C$)	Massey & Deal (1973)
Brain (sheep)	18 ($27^{o}C$)	Lowry & Passonneau (1966)
Erythrocyte (human)	136 ($25^{o}C$)	Wenzel et al. (1972)
Erythrocyte (rabbit)	139 ($25^{o}C$)	Tarui et al. (1972)
Platelets (human)	140 ($30^{o}C$)	Kahn et al. (1980)
Ascites tumour (mouse)	150 ($28^{o}C$)	Sumi & Ui (1972)
Yeast (bakers')	116 ($25^{o}C$)	Lindell & Stellwagen (1968)
Yeast (bakers')	52 ($25^{o}C$)	Atzpodien & Bode (1970)
Yeast (bakers')	103 ($25^{o}C$)	Diezel et al. (1973)
Yeast (brewers')	116 ($25^{o}C$)	Tamaki & Hess (1975a)
E. coli	170 ($28^{o}C$)	Blangy (1971)
E. coli (non-allosteric enzyme)	199 ($25^{o}C$)	Babul (1978)

Table 1 (contd).

Source of enzyme	Specific activity	Reference
Clostridium pasteurianum*	160 (28°C)	Uyeda & Kurooka (1970)
Lactobacillus plantarum	92 (25°C)	Simon & Hofer (1977)
Thermus X-1	110 (30°C)	Cass & Stellwagen (1975)

* These enzymes were crystallised.

Table 2.

Molecular weight and sub-unit structure of phosphofructokinases.

Source of enzyme	Minimum MW for full activity	$S_{20,w}$	Number of sub-units	MW of sub-units	References
Skeletal muscle (rabbit)	320,000	7, 13, 19, 30	4	80,000	Ling et al. (1965), Parmeggiani et al. (1966), Paetkau & Lardy (1967), Uyeda (1969), Scopes & Penny (1971), Aaronson & Frieden (1972), Leonard & Walker (1972), Pavelich & Hammes (1973), Coffee et al. (1973), Walker et al. (1976), Simpson et al. (1977), Johnson & Deal (1978), Hesterberg & Lee (1979)
Skeletal muscle (human)	380,000	13.7 - 14.8	4	85,000	Layzer et al. (1969), Cottreau et al. (1979)
Heart muscle (sheep)	-	7.5, 15 - 50	8	40,000	Mansour et al. (1966), Mansour & Ahlfors (1968), Brennan et al. (1974)

Table 2 (contd).

Source of enzyme	Minimum MW for full activity	$S_{20,w}$	Number of sub-units	MW of sub-units	References
Adductor muscle (oyster)	340,000	-	-	-	Storey (1976)
Liver (chicken)	400,000	5, 21, 28	8	60,000	Kono et al. (1973)
Liver (rat)	325,000	-	4	82,000	Brand & Soling (1974)
Liver (pig)	-	44 - 104	4	80,000	Massey & Deal (1973) Trujillo & Deal (1977)
Erythrocyte (rabbit)	500,000	80	-	53,000	Tarui et al. (1972)
Erythrocyte (human)	330,000	12 - 18, 57	4	85,000 (M) 80,000 (L)	Karadsheh et al. (1977)
Platelets (human)	-	-	4	85,000	Kahn et al. (1980)

Table 2 (contd).

Source of enzyme	Minimum MW for full activity	$S_{20,w}$	Number of sub-units	MW of sub-units	References
Ascites tumour (mouse)	300,000	-	-	-	Sumi & Ui (1972)
Yeast (bakers')	835,000	20.8	8	104,000	Kopperschlager et al. (1977)
Yeast (brewers')	720,000	19.4	8	90,000	Tamaki & Hess (1975a,b)
C. pasteurianum	144,000	7.8	4	35,000	Uyeda & Kurooka (1970)
E. coli	142,000	7.8	4	35,000	Blangy (1968)
L. plantarum and *L. acidophilus*	154,000	-	4	38,000	Simon & Hofer (1977)
Thermus X-1	132,000	6.1	4	33,000	Cass & Stellwagen (1975)
B. stearothermophilus	130,000	-	4	33,900	Hengartner & Harris (1975) Hudson et al. (1977)

Table 3.

Alternative substrates of phosphofructokinase.

Source of enzyme	Phosphoryl acceptors	Relative rate %	Apparent K_m and (apparent K_m for F6P under same conditions) (mM)		Reference
Rabbit muscle	D-Fructose-1-P	5	3.8	(-)	Uyeda (1972)
"	D-Glucose-1-P	0.7	-	(-)	Eyer et al. (1971)
"	D-Sedoheptulose-7-P	100	0.6	(0.055)	Karadsheh et al. (1973)
"	D-Tagatose-6-P	104	0.05	(0.043)	Koerner et al. (1976)
"	D-Psicose-6-P	45	3.0	(0.043)	Koerner et al. (1976)
"	L-Sorbose-6-P	15	11.0	(0.043)	Koerner et al. (1976)
"	2,5-anhydro-D-Mannitol-6-P	87	0.41	(0.043)	Koerner et al. (1974)
"	2,5-anhydro-D-Mannitol-6-P	132	0.15	(0.045)	Bar-Tana & Cleland (1974a)
Sheep heart	D-Fructose-6-SO_4	3	3.3	(0.033)	Martensen & Mansour (1976)
Yeast (bakers')	D-Fructose-1-P	0.5	1.0	(0.15)	Sols & Salas (1966)
Yeast (brewers')	D-Fructose-1-P	3.0	2.5	(0.26)	Kreuzberg (1978)

Table 3 (contd)

Source of enzyme	Phosphoryl donors	Relative rate %	Apparent K_m and (apparent K_m for ATP under same conditions) (mM)		Reference
Rabbit muscle	ITP	99	0.05	(0.027)	Lardy (1962), Bar-Tana & Cleland (1974a)
"	CTP	76	0.031	(0.027)	
"	UTP	111	0.051	(0.027)	"
"	GTP	64	0.016	(0.027)	"
"	XTP	73	0.119	(0.027)	"
Yeast (bakers')	ITP	100	0.2	(0.02)	Sols & Salas (1966)
"	CTP	100	0.4	(0.02)	"
"	UTP	100	0.8	(0.02)	"
"	GTP	100	0.1	(0.02)	"
E. coli	CTP	-	2.0	(0.06)	Blangy et al. (1968)
"	UTP	-	2.0	(0.06)	"
"	GTP	-	1.2	(0.06)	"
"	deoxy-ATP	-	0.08	(0.06)	"
"	TTP	-	3.5	(0.06)	"
C. pasteurianum	ITP	200	0.83	(0.065)	Uyeda & Kurooka (1970)
"	CTP	100	0.33	(0.065)	"
"	UTP	100	0.25	(0.065)	"
"	GTP	250	0.83	(0.065)	"

Chapter 2.

MATERIALS AND METHODS.

Animals.

The common shore crab, Carcinus maenas, was collected locally from Southapton Water and kept at 10-12°C in an aquarium containing circulating sea-water obtained from the same source. Only animals in the intermoult stage were used.

Chemicals.

Pyr-P (tricyclohexylammonium salt), ATP, F6P, NADH, $NADP^+$ (disodium salts), ADP (free acid), FDP (trisodium salt), rabbit muscle aldolase (EC 4.1.2.13), rabbit muscle glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), rabbit muscle lactate dehydrogenase (EC 1.1.1.27), yeast hexokinase (EC 2.7.1.1), and yeast phosphoglucose isomerase (EC 5.3.1.9) were from Boehringer Corp. Ltd. (London).

Tris base, dithiothreitol, dihydroxyacetone phosphate dimethylketal, rabbit muscle pyruvate kinase (EC 2.7.1.40), rabbit muscle glycerol-3-phosphate dehydrogenase / triose phosphate isomerase mixture (EC 1.1.1.8 / EC 5.3.1.1), and bakers' yeast glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were supplied by Sigma Chemical Co. Ltd. (London). All enzymes were purchased as crystalline suspensions in ammonium sulphate solution.

Cellulose CF11 and DEAE-cellulose were from Whatman (Maidstone, Kent), DEAE-sephadex A50 from Pharmacia (Hounslow, Middlesex) and Cellex G.E. from Bio-Rad Laboratories Ltd. (Bromley, Kent). Millipore filters (Metricell GA-6, 13 mm) were obtained through Gelman-Hawksley (Northampton).

All other chemicals were from B.D.H. Ltd. (Poole, Dorset).

Buffer Solutions.

Buffer solutions were set to the desired pH by the use of an E.I.L. (23A) pH meter calibrated with appropriate standard buffers. Buffers were stored at 0-5°C. When preparing solutions of buffer compounds which exhibited a significant change of pK' with temperature the pH was set

at the temperature at which they were to be used. Tris-phosphate buffer solutions were prepared at the desired molarity with respect to Tris.

Phosphofructokinase assays.

Phosphofructokinase activity was measured by the use of enzyme linked continuous assays in which the reaction was linked to the oxidation of NADH. This was done in one of two ways. In one method the production of ADP by the phosphofructokinase reaction was linked to NADH oxidation by the addition of excess pyruvate kinase and lactate dehydrogenase. Pyr-P was also added as the second substrate of pyruvate kinase. In the second method FDP production by the phosphofructokinase reaction was linked to NADH oxidation by the addition of excess aldolase, glycerol-3-phosphate dehydrogenase and triose phosphate isomerase. Two molecules of NADH were oxidised for each FDP formed in this method due to the presence of triose phosphate isomerase. This was taken into account in calculations of phospho-fructokinase activity.

The rate of the reaction was measured spectrophotometrically by recording the decrease in absorbance at 340 nm due to NADH oxidation. All assays were carried out at 25^{o}C in 1 cm path length cells masked with matt finish black enamel paint to eliminate light transmitted through the sides of the cells. The assays were performed in duplicate or triplicate as necessary. All components of the assays except the buffer were maintained at 0-5^{o}C.

A. Routine assay - This assay was used to follow the progress of purification and for estimation of phospho-fructokinase eluted from polyacrylamide gels. A Pye-Unicam SP1800 ultraviolet spectrophotometer fitted with an AR25 chart recorder was used.

The 1 ml reaction mixture consisted of 50 mM Tris-HCl buffer pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 4 mM F6P, 0.2 mM NADH, 1.4 Units of aldolase, 7.7 Units of glycerol-3-phosphate dehydrogenase and 8.8 Units of triose phosphate isomerase. The linking enzymes were used as suspensions in ammonium sulphate solution which was thus carried into the assay. The final concentration of ammonium sulphate was

56 mM. When measuring the elution of phosphofructokinase from calcium phosphate columns during the enzyme purification 1 mM EDTA was included in the assay.

The reaction was initiated with phosphofructokinase and was linear after an initial lag phase. The rate was measured from the linear portion of the trace. Up to 0.06 Units of enzyme were assayed in 1 ml of this reaction mixture.

B. Experimental kinetic assays - These assays were used to measure phosphofructokinase activity in kinetic experiments in which the concentration of a substrate or product of the reaction was varied. These experiments were either initial-rate or product-inhibition studies. In initial-rate studies the initial-rate of the phosphofructokinase catalysed reaction was measured at a variable concentration of F6P and different fixed concentrations of ATP at a constant concentration of free Mg^{2+}. These measurements were repeated at eight different concentrations of free Mg^{2+}. Product-inhibition studies consisted of measuring the initial-rate of the phosphofructokinase catalysed reaction at a variable concentration of F6P or ATP and different fixed concentrations of ADP or FDP at a constant 1 mM concentration of free Mg^{2+}.

For initial-rate and FDP product-inhibition experiments the 1 ml reaction mixture consisted of 25 mM Tris-HCl buffer pH 8.0, 50 mM KCl, 5 mM $(NH_4)_2SO_4$, 0.4 mM Pyr-P, 0.15 mM NADH, 10 Units of pyruvate kinase, 5 Units of lactate dehydrogenase and concentrations of ATP, F6P and free Mg^{2+} as defined in the text. The activity of pyruvate kinase in the assay may have been less than that added since its activity is influenced by the free Mg^{2+} concentration. However halving the amount of pyruvate kinase added in the assay at both the lowest (0.15 mM) and highest (16 mM) concentrations of free Mg^{2+} utilised did not change the measured rate.

For ADP product-inhibition experiments the 1 ml reaction mixture consisted of 25 mM Tris-HCl buffer pH 8.0, 50 mM KCl, 5 mM $(NH_4)_2SO_4$, 0.15 mM NADH, 1.5 Units of aldolase, 5 Units of glycerol-3-phosphate dehydrogenase and concentrations of ATP, F6P, free Mg^{2+} and ADP as defined in

the text. Triose phosphate isomerase was not included in this assay as it has been reported to be contaminated with adenylate kinase (Bar-Tana & Cleland, 1974a).

Both substrates and products of the phosphofructokinase reaction as well as Pyr-P reversibly bind Mg^{2+}. The desired free Mg^{2+} concentration in the experimental kinetic assays was obtained by adding an appropriate total amount of $MgCl_2$ to each assay to allow for complexing by the particular concentrations of ATP, F6P, ADP, FDP and Pyr-P present. This amount was calculated by use of the dissociation constants of the various metal chelates which exist at pH 8.0 as described at the end of this chapter.

The experimental kinetic assays were carried out on a Perkin-Elmer 356 dual wavelength spectrophotometer, operating in the split beam mode and linked to a Perkin-Elmer 165 chart recorder. Spectrophotometer sensitivities of 0.01-0.03 absorbance full scale deflection were used such that the rate of reaction was determined before 5% of the lowest concentration substrate was consumed. Prior to use all assay solutions were passed through a 0.45 micron Millipore filter under positive pressure to remove particulate material which otherwise caused extraneous changes in the absorbance of the reaction mixture at the high spectrophotometer sensitivities used. For the same reason the assay buffer was degassed to prevent air-bubbles forming in the cell.

C. Standard kinetic assay - This assay was used as a measure of the amount of enzymically active phosphofructokinase used in initial-rate and product-inhibition experiments. Kinetic experiments involving measurements of enzymic rates as a function of substrate or product concentrations are directly comparable only if allowance is made for the different amounts of enzyme used in different experiments. This is because the rate of an enzyme reaction depends on the concentration of enzyme as well as that of substrates or products. Solutions of phosphofructokinase used on different days were assayed under the same set of conditions in the standard kinetic assay so that the rates obtained varied only with the concentration of active enzyme and were directly proportional to this. All rates were then

normalised simply by dividing them by the rate obtained in the standard kinetic assay.

The 1 ml reaction mixture consisted of 25 mM Tris-HCl buffer pH 8.0, 50 mM KCl, 5 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 0.4 mM Pyr-P, 0.3 mM ATP, 0.5 mM F6P, 0.15 mM NADH, 10 Units of pyruvate kinase and 10 Units of lactate dehydrogenase. The concentration of free Mg^{2+} in the assay was 9.25 mM.

10 $\mu\ell$ of stock phosphofructokinase solution, 0.0017-0.0025 Units of activity, was assayed in the 1 ml assay throughout the kinetic experiments. The standard kinetic assay was carried out at the beginning and end of each day's experimentation so as to obtain an average value of the activity. Decay of the activity over the course of one day's experimentation was small and within experimental error. All assays of this type were carried out on the Perkin-Elmer spectrophotometer in conjunction with the experimental kinetic assays.

Stock phosphofructokinase solution.

For initial-rate and product-inhibition experiments, purified phosphofructokinase, activity 13.5 Units/ml as measured by the routine assay, was diluted 101-136 fold with 25 mM potassium phosphate buffer pH 8.0, containing 1 mg/ml BSA, 5 mM dithiothreitol and 1 mM F6P and stored at 0-5°C. (All initial-rate experiments were performed with a 126 fold dilution of the purified enzyme). Immediately after dilution the activity of the enzyme increased over a period of about 4 hours until it reached a stable value. No kinetic measurements were made during this period. The diluted enzyme reached a final maximum activity of 0.17-0.25 Units/ml as measured by the standard kinetic assay. 10 $\mu\ell$ of stock phosphofructokinase solution was assayed in a 1 ml reaction mixture in all initial-rate and product-inhibition experiments. The same stock enzyme solution was used for three days' experimentation during which time the activity decayed by about 10%. This was allowed for by the standard kinetic assay. The concentration of F6P in stock phosphofructokinase solutions was assayed and allowance made for F6P carried into the experimental kinetic assays with the enzyme.

Substrate assays.

The concentrations of stock solutions of the substrates and products of the phosphofructokinase reaction and also stock Pyr-P solutions were determined using coupled enzymic assays. Conditions were arranged such that the substrate being assayed limited the extent of the reaction and the assay component used to initiate the reaction did not give rise to a significant change in absorbance by itself. The concentration of the substrate was calculated from the measured change in absorbance at 340 nm due to NADH oxidation or $NADP^+$ reduction.

All substrate assays were carried out in 25 mM Tris-HCl buffer pH 8.0. ATP was assayed by the method of Lamprecht & Trautschold (1974). The 1 ml reaction mixture contained 2 mM glucose, 0.2 mM $NADP^+$, 2.8 Units of hexokinase and 3 Units of glucose-6-phosphate dehydrogenase. F6P was assayed by the method of Lang and Michal (1974). The 1 ml reaction mixture contained 0.2 mM $NADP^+$, 10 Units of phosphoglucose isomerase and 3 Units of glucose-6-phosphate dehydrogenase. ADP was assayed by the method of Jaworek et al. (1974). The 1 ml reaction mixture contained 2 mM Pyr-P, 0.2 mM NADH, 20 Units of pyruvate kinase and 20 Units of lactate dehydrogenase. FDP was assayed by the method of Michal & Beutler (1974). The 1 ml reaction mixture contained 0.2 mM NADH, 1.4 Units of aldolase and 7.7 Units of glycerol-3-phosphate dehydrogenase. Pyr-P was assayed by the method of Czok & Lamprecht (1974). The 1 ml reaction mixture contained 5 mM ADP, 0.2 mM NADH, 20 Units of pyruvate kinase and 20 Units of lactate dehydrogenase. 50 mM KCl and 10 mM $MgCl_2$ were included in assays which utilised kinase enzymes. Linking enzymes were used as suspensions in ammonium sulphate solution.

Substrate assays were performed in duplicate, the two values always corresponded within 5%. The ADP content of ATP was monitored and never exceeded 0.8% on a molar basis.

Linking enzyme assays.

The activities of dialysed linking enzymes used in the experimental and standard kinetic assays were monitored to ensure that the amounts added in these assays never fell

below the values stated.

The linking enzyme assays consisted of 25 mM Tris-HCl buffer pH 8.0, 50 mM KCl, 5 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$ and 0.2 mM NADH. Additional assay components were as follows - 5 mM ADP, 1 mM Pyr-P and 10 Units of lactate dehydrogenase for the pyruvate kinase assay, (free Mg^{2+} was 1.3 mM); 2 mM pyruvate for the lactate dehydrogenase assay; 2 mM FDP and 5 Units of glycerol-3-phosphate dehydrogenase for the aldolase assay; and 1 mM dihydroxyacetone phosphate for the glycerol-3-phosphate dehydrogenase assay.

The linking enzymes were suitably diluted with the assay buffer immediately prior to assay and then 10 $\mu\ell$ was assayed in a 1 ml reaction mixture.

Dialysis of linking enzymes.

Linking enzymes were dialysed to remove ammonium sulphate before use in the experimental and standard kinetic assays. The dialysis buffer was 25 mM Tris-HCl pH 7.4, (pH 8.0 for glycerol-3-phosphate dehydrogenase), containing 10 mM β-mercaptoethanol and 50 μM EDTA for pyruvate kinase and lactate dehydrogenase dialysis; 5 mM β-mercaptoethanol for aldolase dialysis; and 10 mM β-mercaptoethanol and 100 μM EDTA for dialysis of glycerol-3-phosphate dehydrogenase. The final concentration of EDTA carried into the experimental kinetic assays with the linking enzymes was not more than 1 μM. Linking enzyme activities were stable during dialysis and subsequent storage at 0-5°C for several days. Freezing was avoided as this greatly reduced their activity.

Standardisation of $MgCl_2$ solutions.

The delequescent hexahydrate of $MgCl_2$ was used necessitating standardisation of solutions used in phosphofructokinase assays. Two methods were used - atomic absorption spectrophotometry and direct titration against EDTA using Eriochrome Black T as the Mg^{2+} indicator, (Vogel, 1961). The standardised values of a $MgCl_2$ solution used for all initial-rate and product-inhibition experiments were within 3% agreement by the two methods.

Standardisation by atomic absorption spectrophotometry

was performed by the use of a Pye-Unicam SP90A atomic absorption spectrophotometer calibrated with standard $MgCl_2$ solutions purchased from B.D.H.

Titration of $MgCl_2$ against EDTA was performed at alkaline pH to ensure the EDTA existed predominately as its tetra-valent anion which is the species having the greatest affinity for Mg^{2+}. The buffer used consisted of 142 ml of concentrated ammonia solution (specific gravity 0.88-0.90) and 17.5 g of analar grade ammonium chloride dissolved in 250 ml of aqueous solution. The pH was 10.0

Disodium dihydrogen ethylenediaminetetra-acetate dihydrate was purified for use as the primary standard as follows. 200ml of a saturated aqueous solution of disodium dihydrogen ethylenediaminetetra-acetate (about 20 g) was prepared at room temperature. Ethanol was added slowly until a permanent precipitate just appeared and the solution was filtered. The filtrate was diluted with an equal volume of ethanol and the resulting precipitate removed by filtration through a sintered glass funnel, washed with acetone and then with ether. The precipitate was dried in air at room temperature overnight and then at 80°C for 24 hours. A 10 mM standard solution of the recrystallised salt, containing 3.7225 g/ℓ of solution, was made up in redistilled water and stored in a plastic reagent bottle. The Eriochrome Black T indicator solution consisted of 60 mg of the dyestuff dissolved in 15 ml of triethanolamine and 5 ml of absolute alcohol.

The procedure for titration was as follows. 0.9 ml of stock $MgCl_2$ solution, diluted to about 50 mM, was placed in a conical flask with 23.8 ml of water, 0.2 ml of ammonia buffer and 0.1 ml of Eriochrome Black T indicator solution. 10 mM standard EDTA solution was added from a 5 ml burette until the colour changed from red to pure blue. Titration was conducted slowly near the end-point as complex formation is not instantaneous.

Protein estimation.

Protein in concentrations greater than 1 mg/ml was measured by the Biuret method (Gornall et al. 1949). To prepare the Biuret reagent 1.5 g of $CuSO_4.5H_2O$ and 6.0 g of

sodium potassium tartrate tetrahydrate were dissolved in 500 ml of distilled water and 300ml of 10% (w/v) sodium hydroxide solution was added. The volume was taken to 1 ℓ with distilled water. An equal volume of 10% (w/v) aqueous trichloroacetic acid solution was added to a sample of the protein solution to precipitate the protein and after standing for 5 minutes the precipitate was centrifuged down in a bench centrifuge. The protein pellet was redissolved in 0.9 ml of water and 4 ml of Biuret reagent was added. After incubation at room temperature for 30 minutes the absorbance of the solution was read at 540 nm against a water-Biuret blank. Precipitation of the protein was necessary to remove Tris and NH_4^+ which were present in the solution and interfered in the Biuret assay. The method was used to estimate up to 6 mg of protein. The primary standard was previously dried BSA. This was found to give an absorbance of 0.063 for 1 mg of protein under the conditions of the assay.

The ninhydrin method (Hirs, 1967) was used to estimate protein in solutions containing less than 1 mg/ml. Samples containing 5-100 µg of protein in 0.2 ml were hydrolysed by addition of 1 ml of 2.5 M sodium hydroxide solution and autoclaving at 15 pounds/square inch for 30 minutes. Capped test-tubes were used. The samples were cooled and taken to pH 5.5 by addition of 1 ml of 30% (v/v) acetic acid. Following this 1 ml of the ninhydrin solution was added and the tubes capped and placed in boiling water for 20 minutes. The samples were cooled and 2 ml was removed and added to 6 ml of 50% (v/v) aqueous ethanol. Thorough mixing was carried out at every appropriate stage. The absorbance of the samples was read immediately at 570 nm against the blank which was 0.2 ml of water processed identically to the sample. Previously dried BSA was used as the primary standard. This was found to give an absorbance of 0.75 for 100 µg of protein under the conditions of assay. Protein was dissolved in Tris buffer which interfered in the assay. Allowance was made for this by assaying an appropriate volume and concentration of the buffer alone and subtracting the measured absorbance from that given by the protein in buffer.

The ninhydrin solution was prepared by dissolving 2 g of ninhydrin in 75 ml of methoxyethanol with gentle stirring. 0.3 g of hydrindantin was added followed by 25 ml of 4 M sodium acetate buffer pH 5.5. The final solution was bubbled with nitrogen for 20 minutes and stored at 0-5^{o}C in the dark. The solution was discarded after 12 hours.

The sodium acetate buffer was prepared by dissolving 54.4 g of sodium acetate trihydrate in 80 ml of distilled water. About 10 ml of glacial acetic acid was added to bring the pH to 5.5 and the solution was taken to 100 ml with distilled water.

Elution of protein from chromatography columns during enzyme purification was monitored by measuring the absorbance at 280 nm of samples of the eluate against a suitable buffer blank. This method was only approximate and was not used in the calculation of final specific enzyme activities. An absorbance of 1 at 280 nm was taken as representing a protein concentration of 1 mg/ml.

Preparation of dihydroxyacetone phosphate solution.

Dihydroxyacetone phosphate was supplied commercially as a dimethyl ketal which had to be hydrolysed to release the free ketone. 25 mg of the cyclohexylamine salt of dihydroxyacetone phosphate dimethyl ketal dihydrate was dissolved in 2 ml of distilled water. 0.5 g (wet weight) of Dowex 50(H^{+}) resin was added and the mixture was stirred for 30 seconds. The resin was allowed to settle and the supernatant decanted off. The resin was washed with 3 ml of water and the supernatant removed. The two supernatants were combined and incubated at 38-40^{o}C for 4 hours to hydrolyse the ketal. The acid solution was adjusted to pH 4.5 with strong potassium bicarbonate solution and stored frozen. The final concentration of dihydroxyacetone phosphate was 9.6 mM determined enzymically.

Column materials.

DEAE-cellulose, DEAE-sephadex and Cellex G.E. were precycled before use as described by their manufacturers. After use they were recycled by the same methods.

All chromatographic materials were equilibrated batchwise

with the running buffer before they were packed into columns. The end-point was when the pH of the slurry was unchanged from the pH of the buffer alone. The columns were poured and a void volume of the running buffer passed to settle the beds and ensure complete equilibration.

Calcium phosphate gel was prepared according to the method of Swingle & Tiselius (1951). 450 g of sucrose was dissolved in 2 ℓ of water and 75 g of calcium oxide was added. The suspension was agitated periodically for several hours until most of the solid material had dissolved. Any undissolved material was allowed to settle. 800 ml of the supernatant calcium sucrate solution, chilled to 5°C, was brought to pH 9.5 over the course of an hour by dropwise addition of concentrated orthophosphoric acid (about 18 ml) with continuous stirring. Stirring was continued for a further 4 hours. A precipitate of tricalcium phosphate was formed. It was collected by filtration and washed several times with distilled water. The product, a finely divided gel, was stored at 0-5°C as a suspension in distilled water at a concentration of 21 mg/ml as determined gravimetrically.

Prior to use as a column material the calcium phosphate gel was mixed with a freshly prepared defined suspension of 10% (w/v) cellulose powder (CF11) in water to improve its flow properties. The ratio of the mixture was 4 volumes of 10% cellulose / 2.5 volumes of calcium phosphate suspension.

Polyacrylamide gel electrophoresis.

Electrophoresis was performed using a polyacrylamide gel electrophoresis tank and Vokam stabilised D.C. power supply, both products of Shandon Southern Products Ltd. (Runcorn, Cheshire). Acrylamide and bisacrylamide (NN'-methylenebis-acrylamide) were recrystallised before use to ensure polymerisation of the gel. Acrylamide was recrystallised from chloroform (solubility about 70 g/ℓ at 50°C) and bisacrylamide from acetone (solubility about 12 g/ℓ at 50°C). Recrystallised acrylamides, both solids and solutions, were stored at 0-5°C in the dark.

Normal polyacrylamide gel electrophoresis was used to estimate the purity of phosphofructokinase preparations. The proteins present in a phosphofructokinase preparation

were separated electrophoretically as bands on a gel and their relative amounts estimated from the absorbance of the bands after the gel was stained to visualise protein. The staining procedure destroyed all enzymic activity so the phosphofructokinase protein band was identified by comparing the stained gel with a duplicate unstained one in which the site of phosphofructokinase activity was located. The stained gel was used to estimate the purity of the enzyme preparation as the percentage of the total protein that was phosphofructokinase.

A neutral pH continuous buffer system in which the enzyme retained a measurable amount of its activity was used. The buffer concentration was 20 mM. This value was a compromise between a higher concentration which gave better resolution of the protein bands due to reduced diffusion and a lower concentration giving greater retention of enzymic activity due to more rapid electrophoresis. Neutral and alkaline discontinuous buffer systems were found to completely inactivate the enzyme.

The following solutions were prepared:-
Solution A was 1.938 g of Tris base and 0.23 ml of Temed dissolved in about 80 ml of water and brought to pH 7.0 with concentrated orthophosphoric acid. The final volume was taken to 100 ml with water.
Solution B was 30 g of acrylamide and 0.8 g of bisacrylamide dissolved to a final volume of 100 ml in water.
Solution C was ammonium persulphate solution prepared freshly before use. The concentration was 140 mg/100 ml in water.
The electrophoresis buffer was 2.422 g of Tris base dissolved in about 900 ml of water and brought to pH 7.0 with concentrated orthophosphoric acid. The final volume was taken to 1 ℓ with water.

Gels containing 5% (w/v) or 6.8% (w/v) acrylamide were prepared by mixing the following solutions:-

	Solution A	Solution B	Solution C	Water
	ml	ml	ml	ml
5% gel	3	4	12	5
6.8% gel	3	5.45	12	3.55

The solutions were mixed and poured immediately into 6.5 x 0.6 cm i.d. precision bore perspex running tubes sealed at one end with Parafilm and mounted vertically. The tubes were filled to a height of 6.0 cm. A small amount of water was layered carefully onto the top of each gel solution before it set by use of a 1 ml syringe fitted with a 26 guage needle. This ensured a flat gel surface. Polymerisation was complete in about 30 minutes as indicated by the development of a marked gel/water interface. The parafilm was removed from the tubes and they were mounted vertically in the electrophoresis tank with the gels in contact with both the upper (cathodal) and lower (anodal) buffers.

The gels were pre-run at 5 mA/gel for 1 hour to remove impurities that inactivate enzymes. The apparatus and gels were cooled to 0-5^{o}C before application of the enzyme and maintained at this temperature during electrophoresis.

Enough aqueous Bromophenol Blue, 0.05% (w/v), was added to the enzyme solution to colour it and act as tracking dye. This did not affect phosphofructokinase activity. Glycerol was added so that the enzyme solution could be layered on top of the gels underneath the electrophoresis buffer. A final glycerol concentration of 10-40% (v/v) was used depending on protein concentration. Up to 100 $\mu\ell$ of enzyme solution containing 25-100 μg of protein, about 1 Unit of enzyme activity, was layered carefully on top of each gel by use of a Hamilton syringe.

Electrophoresis was continued for 4 hours, (6.8% gels) or 3 hours and 15 minutes (5% gels) at a constant direct current of 2 mA/gel. The tracking dye emerged from the anodal end of the gels within this period so one gel was removed from the apparatus just before this occured to check that no protein was lost from the other gels.

As soon as electrophoresis was terminated the gels were removed from their running tubes by applying pressure with a rubber teat. Gels for which it was wished to visualise the protein bands were immersed in 5% (w/v) aqueous trichloroacetic acid for 2 hours to fix the protein. They were then stained for protein as described below. Gels for which it was wished to locate phosphofructokinase were cut

into 2.5 mm slices by use of razor blades mounted at appropriate spacings on a fixed frame. Each slice was placed in 150 μℓ of 10 mM Tris-phosphate buffer pH 7.0, containing 1 mM EDTA, 10 mM β-mercaptoethanol and 400 μM F6P. They were incubated at 0-5°C for 2 hours with intermittent agitation. Slices containing enzymic activity were identified by assaying 50 μℓ of the incubation buffer in the routine assay. Total recovery of enzymic activity after electrophoresis was about 2% of that applied to the gel.

The procedure for staining gels for protein was as follows. The gels were placed in 0.3% (w/v) Coomassie Brilliant Blue in 7% (v/v) acetic acid and left to stain overnight. After staining the background dye was removed from the gels over the course of 2-3 days by treating them with several changes of a 10% (v/v) methanol, 7% (v/v) acetic acid solution at 30-40°C. Destained gels were stored in 7% (v/v) acetic acid in the dark. The protein showed up as blue bands on the gel.

Gels stained for protein were scanned by the use of a Joyce-Loebl Chromoscan Mk.2 recording and integrating densitometer fitted with a green filter (500-560 nm). This yielded a densitometer trace of the distribution of protein along the gel. The relative amounts of protein in the various bands were quantified approximately by estimating the areas under the absorbance peaks. A visual record of the gels was obtained by photography.

SDS polyacrylamide gel disc electrophoresis.

The molecular weight of the sub-units of phosphofructokinase and the purity of the enzyme preparation were estimated by use of polyacrylamide gel electrophoresis under denaturing conditions with SDS. The discontinuous buffer system of Neville (1971) was used.

The following solutions were prepared:-
Solution A was 27.5 g of acrylamide and 0.25 g of bis-acrylamide dissolved in water to a final volume of 100 ml. Solution B, the running gel buffer, consisted of 12.85 g of Tris base and 0.375 ml of Temed dissolved in about 80 ml of water and brought to pH 9.2 with concentrated hydrochloric acid. The final volume was taken to 100 ml with water.

Solution C was 7.5 g of acrylamide and 0.5 g of bisacrylamide dissolved in water to a final volume of 100 ml. Solution D, the stacking gel buffer, consisted of 1.64 g of Tris base and 0.375 ml of Temed dissolved in about 80 ml of water and brought to pH 6.1 with concentrated sulphuric acid. The final volume was taken to 100 ml with water. Solution E was ammonium persulphate solution freshly prepared before use. The concentration was 250 mg/100 ml in water.

The buffer used in the upper (cathodal) compartment of the electrophoresis tank consisted of 4.965 g of Tris base and 1 g of SDS dissolved in about 900 ml of water and brought to pH 8.6 with solid boric acid. The final volume was taken to 1 ℓ with water. This solution was discarded after 3 days. The lower (anodal) compartment buffer was 51.39 g of Tris base dissolved in about 900 ml of water and brought to pH 9.2 with concentrated hydrochloric acid. The final volume was taken to 1 ℓ with water.

Separating gels containing 11.0% (w/v) or 7.9% (w/v) acrylamide were used, both with 2.3% (w/v) acrylamide stacking gels. The required mixtures of the stock solutions were as follows:-

	Solution A	Solution B	Solution E	Water
	ml	ml	ml	ml
7.9% separating gel	4.32	6	3	1.68
11.0% separating gel	6	6	3	0

	Solution C	Solution D	Solution E	Water
	ml	ml	ml	ml
2.3% stacking gel	1.56	2	1	0.44

9 x 0.6 cm i.d. perspex running tubes were used.

The separating gels were polymerised first. The appropriate solutions were mixed and poured immediately into the running tubes to a height of 7.5 cm. Water was layered on top of the solutions to ensure a flat gel surface and they were left to set for 30 minutes. When the separating gels had polymerised the solutions for the stacking gels were mixed and partially degassed. Surface water was removed

from the running gels with a piece of tissue and the stacking gel solution was poured on top of them to a height of 1 cm. A flat stacking gel surface was ensured by layering water on top of it before it set.

As soon as the stacking gels had polymerised (about 30 minutes) the electrophoresis was set up and the run commenced before the discontinuities of the buffer system were affected by diffusion. The electrophoresis was performed at room temperature.

Phosphofructokinase was dissociated as far as possible into sub-units by subjecting the purified enzyme to denaturing conditions. A sample of the enzyme solution was made 1% (w/v) with respect to SDS, 1% (v/v) with respect to β-mercaptoethanol and 50 mM with respect to sodium carbonate. It was placed in boiling water for 2 minutes and incubated at room temperature overnight. Glycerol was added to a final concentration of 10-40% (v/v) and aqueous Bromophenol Blue was added as tracking dye. Up to 100 μℓ of the sample, containing 25-100 μg of protein, was layered on top of each gel. The samples were run at 1 mA/gel until the protein had concentrated at the interface of the stacking and running gels, (about 1 hour and 25 minutes), after which the current was increased to 2 mA/gel. Electrophoresis was terminated when the tracking dye was close to the anodal end of the gel, (a further 1 hour and 30 minutes). The dye front was marked by inserting a piece of wire in the gel. The gels were stained for protein and destained as described previously.

The sub-unit molecular weight of phosphofructokinase was determined by use of proteins of well characterised sub-unit molecular weight as reference proteins. The standard proteins were dissolved in water and then dissociated into their sub-units and electrophoresed as described for phosphofructokinase. 5-15 μℓ of a standard containing 5-30 μg of protein was applied to each gel.

The distances moved by the protein bands and by the tracking dye were measured from the top of the separating gel. The relative electrophoretic mobility (R_m) of the protein was calculated as:-

$$R_m = \frac{\text{Distance moved along the gel by the protein}}{\text{Distance moved along the gel by the tracking dye}}$$

Expression and calculation of results.

In all relevent calculations the difference in the molar absorbance coefficients of NADH and NAD^+ and of NADPH and $NADP^+$ was taken as 6.22×10^3 $M^{-1}cm^{-1}$ at 340 nm.

One Unit of enzymic activity was defined as that amount of enzyme which converted 1 μmole of substrate into product in one minute under the conditions of assay.

The results of kinetic experiments were normalised, (allowance was made for the different concentrations of phosphofructokinase used in different experiments), by dividing the experimental rates by the rate obtained in the standard kinetic assay of the relevent enzyme sample:-

$$\text{Normalised rate} = \frac{\text{Initial rate obtained in the experimental assay}}{\text{Initial rate obtained in the standard kinetic assay}} = \frac{v}{V_k}$$

The normalised rate is a dimensionless ratio of velocities and the reciprocal initial velocity on Lineweaver-Burk plots, V_k/v, is therefore shown without units.

The initial-rate data at each fixed concentration of substrate (or product) in the Lineweaver-Burk plots was fitted to the Michaelis Menten equation:-

$$v = \frac{V\,(S)}{K_m + (S)}$$

(S) is the concentration of the variable substrate, K_m is the apparent Michaelis constant of the variable substrate and V is the apparent maximum velocity of the reaction. The hyperbolic least squares method of Wilkinson (1961) was used to obtain estimates of K_m and V with iteration proceeding until successive values of both constants were within 0.1%. The values of the constants were used to draw the lines in Lineweaver-Burk plots for visual interpretation. Calculation and graph drawing were carried out by use of a Hewlett-Packard Model 10 electronic calculator and attached x-y plotter. This method of analysing the data makes no

assumptions as to the kinetic mechanism of the reaction.

Linear regression analysis of straight line relationships in the secondary and tertiary plots was carried out by use of a Texas TI59 programmable calculator.

An ICL2970 computer was used to fit the initial-rate data to the rate equation for an ordered ter-bi mechanism. The computer programme used an extension of the method described by Cleland (1967) and utilised the Newton-Raphson iterative process to obtain best fit values of the unknowns in the rate equation.

The following equation gives the total concentration of $MgCl_2$ required in the experimental kinetic assay to yield the desired concentration of free Mg^{2+}.

$$
\begin{aligned}
Mg_{total} = {} & \frac{ATP_{total}\, Mg^{2+}_{free}\left(1 + \frac{H^{+}_{free}}{K_4}\right)}{K_2\left(1 + \frac{H^{+}_{free}}{K_1} + \frac{K^{+}_{total}}{K_5}\right) + Mg^{2+}_{free}\left(1 + \frac{H^{+}_{free}}{K_4}\right)} \\
& + \frac{ADP_{total}\, Mg^{2+}_{free}\left(1 + \frac{H^{+}_{free}}{K_9}\right)}{K_7\left(1 + \frac{H^{+}_{free}}{K_6} + \frac{K^{+}_{total}}{K_{10}}\right) + Mg^{2+}_{free}\left(1 + \frac{H^{+}_{free}}{K_9}\right)} \\
& + \frac{FDP_{total}\, Mg^{2+}_{free}\left(1 + \frac{H^{+}_{free}}{K_{16}}\right)}{K_{14}\left(1 + \frac{H^{+}_{free}}{K_{12}} + \frac{(H^{+}_{free})^2}{K_{12}\,K_{13}}\right) + Mg^{2+}_{free}\left(1 + \frac{H^{+}_{free}}{K_{16}}\right)} \\
& + \frac{Pyr\text{-}P_{total}\, Mg^{2+}_{free}}{K_{18}\left(1 + \frac{H^{+}_{free}}{K_{17}} + \frac{K^{+}_{total}}{K_{19}}\right) + Mg^{2+}_{free}} \\
& + \frac{F6P_{total}\, Mg^{2+}_{free}}{K_{11} + Mg^{2+}_{free}} + Mg^{2+}_{free}
\end{aligned}
$$

The derivation of the equation assumes the activity coefficient of $MgCl_2$ is 1.0 and that the concentration of free K^+ is equal to the total concentration of K^+. The second assumption was true to within 5%. Calculation was performed by use of the Hewlett-Packard calculator. Programmes were written to define the concentrations of the various substrate species when either a known free Mg^{2+} concentration was required and the total Mg^{2+} concentration to achieve this had to be calculated, or when the free Mg^{2+} concentration corresponding to a known total Mg^{2+} concentration was to be calculated. This latter calculation used the first calculation as a subroutine in a modified Fibonacci search procedure.

The values of the dissociation constants involved in the above equation and the equilibria which they define are listed in Table 15.

The following formula was used to calculate the amount of solid ammonium sulphate required to bring the enzyme solution to the desired level of saturation with ammonium sulphate:-

$$\text{Weight of ammonium sulphate (g)} = \frac{0.515\ v\ (S_2 - S_1)}{1 - 0.272\ S_2} \quad \text{at } 4^{\circ}C$$

v is the volume of the enzyme solution in ml.
S_1 and S_2 are the initial and final fractional saturation values.

Chapter 3.

PURIFICATION AND PROPERTIES OF PHOSPHOFRUCTOKINASE.

The enzyme was maintained at 0-5°C in all experiments unless specifically stated otherwise.

Stability of phosphofructokinase.

In order to investigate the effect of pH on the stability of the enzyme, samples of partially purified phosphofructokinase in 5 mM Tris-phosphate buffer pH 7.0 were each diluted three-fold with 50 mM Tris-phosphate buffer at pH in the range 6.7-8.5. The molarity of the dilution buffer was with respect to the phosphate so that the concentration of this was constant. The activity of the enzyme maintained in the various buffers was monitored over a period of 6 days. The routine assay was used. Enzyme stability was found to decrease with increasing pH over the range investigated. The results are summarised in Table 4. Stability of the enzyme at pH less than 6.7 was not investigated in this experiment. However other experiments indicated the enzyme was markedly unstable at pH 5.0. This observation was made while investigating the precipitation of nucleic acid at pH 5.0 as a possible step in the enzyme purification.

Phosphofructokinase was subjected to dialysis at several stages of the enzyme purification to reduce the ionic strength of the buffer. In the absence of FDP the enzyme was unstable during dialysis, about 50% of the activity was lost. The inclusion of 100 μM FDP in the dialysis buffer increased enzyme stability so that about 90% of the activity was retained.

Purified phosphofructokinase was generally not stable when the enzyme solution was stored frozen although this depended on the composition of the buffer. Purified phosphofructokinase in 5 mM Tris-phosphate buffer pH 7.0 or 25 mM Tris-HCl buffer pH 7.3 was completely inactivated on freezing and this was associated with the appearance of a protein precipitate. Inactivation occured despite the inclusion of 100 μM FDP in the buffers. In contrast phosphofructokinase was completely stable for 5 months when

frozen in 0.35 M Tris-phosphate buffer pH 7.0 as long as repeated thawing and refreezing of the enzyme solution was avoided. The most convenient condition for stable long term storage of the enzyme was at -15°C in 10 mM Tris-phosphate buffer pH 7.0 containing 100 μM FDP and 40% (v/v) glycerol. The glycerol prevented freezing. Enzyme stored in this fashion lost only 12% of its activity in 9 months.

The activity of phosphofructokinase in stock solutions of the enzyme used for initial-rate and product-inhibition experiments was not stable unless dithiothreitol and F6P were added to the solution. Table 5 shows the effect of 10 mM dithiothreitol and 1 mM F6P on the stability of the enzyme in 25 mM Tris-HCl buffer pH 8.0 containing 2 mg/ml BSA.

Phosphofructokinase purification.

Phosphofructokinase was isolated from the leg and claw skeletal muscle of Carcinus maenas. The enzyme content of the tissue was 2.5-3.0 Units/g wet weight of legs including the shell. Fresh and frozen tissues were identical in their content of active enzyme and its properties as regards purification so frozen tissue was used as it was more convenient to store. Decay of enzymic activity in frozen tissue did occur over 2 years but full activity was retained for 6 months.

The activity and protein concentration of the enzyme solution were measured at each stage of the purification. Centrifugation was at 12,000 r.p.m. for 20 minutes using an M.S.E. 18 refridgerated centrifuge driving a 6 X 250 ml rotor head.

620 g of frozen legs was briefly rinsed with distilled water and coarsely minced by use of a domestic meat-mincer directly into 1 ℓ of a solution of 30 mM potassium fluoride, 4 mM EDTA, 10 mM β-mercaptoethanol pH 7.5. The enzyme was extracted from the mince for 30-40 minutes with constant stirring by a top-drive stirrer. The extract was centrifuged and the pellet discarded. The pH of the supernatant was about 8.4, it was adjusted to pH 7.5 by dropwise addition of 1 M acetic acid with stirring.

Solid ammonium sulphate was gradually added over a period

of 20 minutes with constant stirring to a final concentration of 28% saturation, (15.61 g/100 ml). The pH was maintained at 7.5 by addition of 1 M acetic acid or 1 M Tris base as necessary. The solution was stirred for a further 10 minutes, centrifuged and the pellet discarded.

The enzyme solution was placed in a 2 ℓ stainless steel beaker and heated to 47°C in about 2½ minutes by the use of a water bath at 65°C. The enzyme solution was maintained at 47°C for 6 minutes by the use of a second water bath at this temperature. The enzyme solution was stirred manually throughout the heat treatment and finally cooled to 4°C in ice. It was centrifuged and the pellet discarded.

Solid ammonium sulphate was gradually added over a period of 25 minutes with constant stirring to increase the concentration to 53% saturation, (an additional 15.04 g/100 ml). The pH was maintained constant at 7.5 as before. The solution was stirred for a further 10 minutes, centrifuged and the supernatant discarded. The centrifugal pellet was quickly redissolved by use of a Potter-Elvehjem homogeniser in 90 ml of 10 mM Tris-phosphate buffer pH 7.0 containing 10 mM β-mercaptoethanol and 100 μM FDP. The solution was clarified by a further centrifugation.

The enzyme solution was dialysed overnight against 5 ℓ of 10 mM Tris-phosphate buffer pH 7.0 containing 10 mM β-mercaptoethanol and 100 μM FDP. It was then diluted with buffer of the same composition, dropwise over a period of 15 minutes with stirring, to a protein concentration of 8 mg/ml.

The enzyme solution was applied to a column of calcium phosphate (3.6 x 24.5 cm) equilibrated with buffer of the same composition as that used for dialysis. A Gilson peristaltic pump, attached to the column outlet, was used to give a flow rate of about 1 ml/minute. After application of the enzyme solution 150 ml of the equilibrating buffer was passed. 360 ml of 0.10 M Tris-phosphate buffer pH 7.0, containing 10 mM β-mercaptoethanol and 100 μM FDP, was passed until the A_{280} of the column eluate had fallen to 0.5. Phosphofructokinase was eluted from the column with 0.40 M Tris-phosphate buffer pH 7.0 containing 10 mM β-mercaptoethanol and 100 μM FDP. An LKB fraction collector

was used to collect 15 ml fractions from the column and the most active fractions were pooled.

The pooled fractions were dialysed by use of a Bio-Rad hollow fibre dialyser unit of 120 ml capacity and a molecular weight cut-off value of 30,000. The enzyme solution was dialysed in 70 ml portions. Each was placed in the outer compartment of the dialyser and dialysed against 2.5 ℓ of 10 mM Tris-phosphate buffer pH 7.0, containing 1 mM EDTA, 10 mM β-mercaptoethanol and 100 μM FDP, passed through the fibres in 40 minutes using a 9 ft head of buffer.

The enzyme solution was applied to a column of DEAE-cellulose (2.5 x 13.0 cm) equilibrated with buffer of the same composition as that used for hollow fibre dialysis. 115 ml of 0.15 M Tris-phosphate buffer pH 7.0, containing 1 mM EDTA, 10 mM β-mercaptoethanol and 100 μM FDP, was passed through the column at a flow rate of 1 ml/minute achieved with a buffer head of 23 cm. This brought the A_{280} of the eluate to less than 0.1. Phosphofructokinase was eluted with 0.35 M Tris-phosphate buffer pH 7.0 containing 1 mM EDTA, 10 mM β-mercaptoethanol and 100 μM FDP. 5 ml fractions were collected and the most active ones pooled and dialysed overnight against 5 ℓ of 10 mM Tris-phosphate buffer pH 7.0 containing 10 mM β-mercaptoethanol and 100 μM FDP.

The enzyme solution was applied to a column of calcium phosphate (1.2 x 10 cm) equilibrated with 10 mM Tris-phosphate buffer pH 7.0 containing 10 mM β-mercaptoethanol and 100 μM FDP. About 15 ml of the equilibrating buffer was passed until the A_{280} of the eluate fell to zero. 3 ml fractions were collected from the column and 25 ml of 0.13 M Tris-phosphate buffer pH 7.0, containing 10 mM β-mercapto-ethanol and 100 μM FDP, was passed eluting 47% of the enzyme applied to the column. Another 38% of the enzyme was then eluted with 0.35 M Tris-phosphate buffer pH 7.0 containing 10 mM β-mercaptoethanol and 100 μM FDP. The highest specific activity fractions were eluted by the 0.13 M buffer. They were pooled and dialysed for 37 hours against 10 mM Tris-phosphate buffer pH 7.0 containing 1 mM EDTA, 10 mM β-mercaptoethanol, 100 μM FDP and 40% (v/v) glycerol. The

final enzyme solution contained 85 Units at a specific activity of 14 Units/mg protein. It was stored at -15°C. All initial-rate and product-inhibition experiments were performed with this enzyme sample. The progress of the enzyme purification is shown in Table 6.

The elution of enzymic activity in two parts from the second calcium phosphate column may have been due to the concentration of the washing buffer being set too high at 0.13 M since this did not occur with the first calcium phosphate column when the washing buffer concentration was 0.10 M. This behaviour would also be expected if the enzyme existed in more than one form, each form with a different affinity for the calcium phosphate gel. A tendency of the enzyme to elute partly in the washing buffer was observed during development of the purification so that results were often difficult to reproduce. This was the case with a purification which utilised a Cellex G.E. column in place of the second calcium phosphate column. A proportion of the enzyme applied to the Cellex G.E. column was eluted by the washing buffer at a specific activity of 32 Units/mg protein, the highest value obtained. However the purification could not be reproduced. Thus further work to improve the purification was discontinued when the procedure summarised in Table 6 yielded enough enzyme to carry out all initial-rate and product-inhibition experiments.

Purity of the phosphofructokinase preparation.

The purity of the enzyme solution prepared by the method summarised in Table 6 was assessed by use of polyacrylamide gel electrophoresis. The enzyme used in these studies had a specific activity of 14 Units/mg protein.

1.3 Units of phosphofructokinase activity, 95 µg of protein, was electrophoresed on a 6.8% acrylamide gel. The gel was stained to visualise the protein bands and the phosphofructokinase bands were identified by use of a duplicate unstained gel in which the sites of enzymic activity were located. Two discrete sites of activity were found. Figure 1 compares a densitometer trace and photograph of the stained gel with the activity profile obtained

by use of the duplicate gel. The photograph shows three dark bands on the gel and several lighter ones. The three tallest peaks shown by the densitometer trace correspond with the three major protein bands. These three bands are situated within the areas of the gel where activity was found. Two of the major protein bands are situated too close together to determine whether the activity peak associated with them corresponds to both or only one of the bands. The resolution of this method of visualising phosphofructokinase on gels is limited by the size of the gel slices that can be cut for the activity profile and also by the fact that the length of a gel is changed by the staining procedure.

The result obtained when the enzyme solution was electrophoresed on a 5% acrylamide gel is shown in Figure 2. Resolution of the bands on the gel is not good but the densitometer trace shows the same number of protein peaks as in Figure 1 which indicates the 6.8% gel did not exclude any high molecular weight protein.

By estimation of the areas under the absorbance peaks in Figure 1, the three major bands represent 70% of the protein on the gel. Taking the three major bands to each contain enzymic activity, the enzyme solution can be concluded to be about 70% phosphofructokinase.

Electrophoresis on polyacrylamide gels serarates proteins according to molecular size and nett charge. The results obtained show that there were two or three different molecular weight forms of phosphofructokinase present in the purified enzyme solution. This may be due to aggregation of a phosphofructokinase sub-unit to form two or three different oligomeric forms of the enzyme. Another possibility that two or three different phosphofructokinase proteins are present is unlikely as they would have different K_m values for substrates which would give rise to non-linear double-reciprocal plots in initial-rate studies which was not observed. It cannot be concluded that there are two or three different molecular weight forms of native phosphofructokinase as these may be produced as a result of purification of the enzyme in that aggregation of a sub-unit may depend on the enzyme concentration.

Sub-unit molecular weight of phosphofructokinase.

The molecular weight of the sub-units of phosphofructokinase was estimated by use of polyacrylamide disc gel electrophoresis under denaturing conditions with SDS. This technique separates polypeptides according to their molecular weights so that the unknown molecular weight of a polypeptide can be determined by comparing its relative mobility to those of reference proteins of well characterised sub-unit molecular weights, (Weber et al. 1972). The enzyme solution used in these studies was that prepared by the method summarised in Table 6. The specific activity was 14 Units/mg protein.

The following proteins were used as reference proteins: Xenopus laevis vitellogenin, (monomer MW 220,000, Penning 1976); Xenopus laevis lipovitellin, (monomer MW 110,000, Penning 1976); bovine serum albumin (dimer MW 136,000; monomer MW 68,000); rabbit muscle pyruvate kinase (monomer MW 57,000); ovalbumin (monomer MW 43,000) and pig heart lactate dehydrogenase (monomer MW 36,000).

All reference proteins and phosphofructokinase were electrophoresed at room temperature using 7.9% acrylamide separating gels. The reference proteins were dissociated into their sub-units and electrophoresed as described in Materials and Methods. Their relative mobilities were measured and plotted versus $\log_{10}$ molecular weight. The graph is shown in Figure 3, it is a shallow curve. When purified phosphofructokinase was dissociated into sub-units and electrophoresed the result shown in Figure 4 was obtained. Several bands are visible on the gel but 70% of the protein is located in one major band as estimated from the densitometer trace. This major band is taken to be the sub-unit of Carcinus maenas phosphofructokinase because of its abundance and because it migrates almost the same distance as pure rabbit muscle phosphofructokinase obtained commercially (see Figure 5). The relative mobility of the major band in Figure 4 is 0.52 which corresponds to a molecular weight of 81,500 by use of the calibration curve.

Phosphofructokinase from Carcinus maenas was concluded to contain sub-units of molecular weight 81,500. It was not possible to determine if there were other sub-units with

different mobilities present as the enzyme preparation was not pure so that the other bands visualised on SDS gels may be either dissimilar sub-units or protein impurities. The localisation of 70% of the protein in one band on SDS gel electrophoresis suggests the three protein bands observed on normal gel electrophoresis which also accounted for 70% of the protein applied are all phosphofructokinase. The enzyme appears to exist as aggregates of a single molecular weight monomer. The different molecular weight species of the enzyme are broken down to the common monomer in denaturing conditions.

Factors affecting phosphofructokinase activity.

Phosphofructokinase was activated by ammonium sulphate, potassium phosphate, β-mercaptoethanol and dithiothreitol and inhibited by Pyr-P. The effect on phosphofructokinase activity of varying the concentrations of these effectors was investigated so as to choose conditions for assay of the enzyme under which it was maximally activated and exhibited hyperbolic kinetics with respect to the concentrations of its substrates.

A. Ammonium sulphate - Figure 6 shows the effect on the enzymic rate of varying the concentration of ammonium sulphate over the range 0-10 mM in the experimental and standard kinetic assays. In both cases the rate increases to a maximum as the concentration of ammonium sulphate is increased. Further it was found that double-reciprocal plots of reaction rate versus the concentration of F6P were linear in the presence of ammonium sulphate, (5 mM), whereas in its absence they were curved. In order to obtain values of apparent Michaelis constants and apparent maximal velocities from double-reciprocal plots it is necessary that these plots be linear. This was ensured by the inclusion of ammonium sulphate in both assays. A concentration of 5 mM was used so that the rate was unaffected by any small variations in the ammonium sulphate concentration in the assays introduced by experimental error.

B. Dithiothreitol and β-mercaptoethanol - Dithiothreitol was added to stock solutions of phosphofructokinase used for initial-rate and product-inhibition experiments to increase

the stability of the enzyme. When the concentration of dithiothreitol in the stock enzyme solution was varied in the range 0.5-20 mM the enzymic activity increased to a maximum value as the concentration of dithiothreitol was increased. A similar result was obtained when β-mercaptoethanol, (6-46 mM), was used in place of dithiothreitol except that the maximum activity observed was then less and a higher concentration of β-mercaptoethanol was required to achieve it. The activity of phosphofructokinase maximally activated by β-mercaptoethanol in the stock enzyme solution was further increased when dithiothreitol was added to the enzyme assay whereas the maximum activity of the dithiothreitol activated enzyme was not increased by this procedure. These results are shown in Figure 7.

Dithiothreitol was added to stock phosphofructokinase solutions to activate and stabilise the enzyme. A concentration of 5 mM was used so that any small variations in concentration introduced by experimental error did not affect the activity of the enzyme. Consequently dithiothreitol was carried into the experimental and standard kinetic assays with the enzyme to a final concentration of 50 μM. Dithiothreitol is known to complex divalent metal ions but a value for the dissociation constant of the Mg-dithiothreitol complex was not available. However increasing the concentration of dithiothreitol in the experimental kinetic assay three-fold at 0.15 mM free Mg^{2+} did not change the measured rate.

C. Potassium phosphate - Purified phosphofructokinase was diluted with buffer to a suitable enzyme concentration before it was used in initial-rate and product-inhibition experiments. The activity of phosphofructokinase measured by the standard kinetic assay was greater when the purified enzyme was diluted with 25 mM potassium phosphate buffer pH 8.0 than when 25 mM Tris-HCl buffer pH 8.0 was used. This difference in activities was time dependent. Immediately after dilution of the enzyme the same activity was observed in both buffers for equivalent dilutions but then the activities increased over a period of about four hours until they reached a stable maximum value which was greater in phosphate buffer than in Tris-HCl. When, after

reaching its maximum activity, different volumes of the diluted enzyme solution were assayed in the standard kinetic assay and the rate obtained plotted against the volume of stock enzyme solution assayed then the result shown in Figure 8 was obtained. The graph for phosphofructokinase maintained in potassium phosphate buffer is a straight line passing through zero but that for the enzyme in Tris-HCl is non-linear. The method of normalising enzymic rates by use of the standard kinetic assay did not work for phosphofructokinase maintained in Tris-HCl buffer in that the normalised rate varied with the amount of enzyme used. This difficulty was not experienced with the enzyme maintained in phosphate buffer.

The activity of phosphofructokinase was greater when the purified enzyme was diluted with 100 mM potassium phosphate buffer pH 8.0 than when 10 mM potassium phosphate buffer pH 8.0 was used. When different volumes of the 100 mM buffer enzyme solution were assayed in the standard kinetic assay a non-linear variation of the enzymic rate was observed. See Figure 9. This was caused by the phosphate carried into the assay with the enzyme. A phosphate concentration greater than 1 mM in the assay caused a small but significant increase in the enzymic rate. For this reason a concentration of 25 mM was chosen for the phosphate buffer used to dilute the purified enzyme for use in initial-rate and product-inhibition experiments. It was not possible to add a high concentration of potassium phosphate to the phosphofructokinase assays in order to completely activate the enzyme as this caused magnesium ammonium phosphate to precipitate. The solubility of $MgNH_4PO_4.6H_2O$ is 0.231 g/ℓ in cold water, (Weast, 1978).

As well as affecting phosphofructokinase activity, the buffer used to dilute the purified enzyme also influenced the shape of the rate progress curve obtained in the standard kinetic assay. This was linear for the enzyme maintained in phosphate buffer but curved (rate increasing with time) for that in Tric-HCl buffer.

D. Pyr-P - Pyr-P was required in the experimental and standard kinetic assays as the second substrate of pyruvate kinase. 2 mM Pyr-P inhibited the enzymic rate by 4% in the

standard kinetic assay and by 11% in the experimental kinetic assay at about 8 mM free Mg^{2+} and 50 μM of ATP and F6P. Over the range 0.1-0.5 mM Pyr-P had no effect in either assay so that a concentration of 0.4 mM Pyr-P was used in the assays for initial-rate and product-inhibition experiments.

E. ATP - to investigate whether Carcinus maenas phosphofructokinase was inhibited by concentrations of ATP above that required for maximal catalytic activity the enzyme was assayed under the conditions of the routine assay with the buffer pH reduced to 7.0 and the ATP concentration was varied in the range 0.05-4.0 mM. The result is shown in Figure 10. ATP inhibited the enzyme at concentrations above 0.3 mM.

Table 4.

Effect of buffer pH on stability of phosphofructokinase.

	% of initial activity remaining					
Time in hours	0	4	21	52	96	144
pH of buffer						
6.7	100	99	95	87	80	74
7.0	100	94	90	86	73	61
7.4	100	94	86	80	69	55
7.8	100	94	90	78	63	47
8.2	100	92	79	55	29	15
8.5	100	89	64	33	13	7

Table 5.

Effect of dithiothreitol and F6P on stability of phosphofructokinase.

	% of initial activity remaining		
Time in hours	0	3.5	6.0
Buffer composition			
25 mM Tris-HCl pH 8.0 2 mg/ml BSA	100	74	59
25 mM Tris-HCl pH 8.0 2 mg/ml BSA, 10 mM dithiothreitol	100	89	77
25 mM Tris-HCl pH 8.0 2 mg/ml BSA, 1 mM F6P 10 mM dithiothreitol	100	102	101

Table 6.

Summary of the enzyme purification.

Stage	Volume	Total activity	Total protein	Specific activity	Recovery	Purification
	ml	Units	mg	Units/mg protein	%	fold
Initial extract	895	1583	7600	0.21	100	-
28% amm. sulph. supernatant	945	1519	6525	0.23	96	1.1
Heat treatment supernatant	920	1553	4819	0.32	98	1.5
53% amm. sulph. precipitate	90	1201	1606	0.75	76	3.6
Post-dialysis and dilution	198	1178	1556	0.76	74	3.6
Calcium phosphate column	146	575	301	1.9	36	9
Post-dialysis	238	631	283	2.2	40	10
DEAE-cellulose column	16	298	35	8.5	19	40
Post-dialysis	22	270	32	8.4	17	40
Calcium phosphate column (enzyme in 0.13 M buffer)	16	113	6.1	19	7	90
Post-dialysis	6.3	85	6.1	14	5	67

Figure 1.

Electrophoresis of C. maenas phosphofructokinase on a 6.8% polyacrylamide gel. Comparison of the position of stained protein bands with the absorbance and enzymic activity profiles.

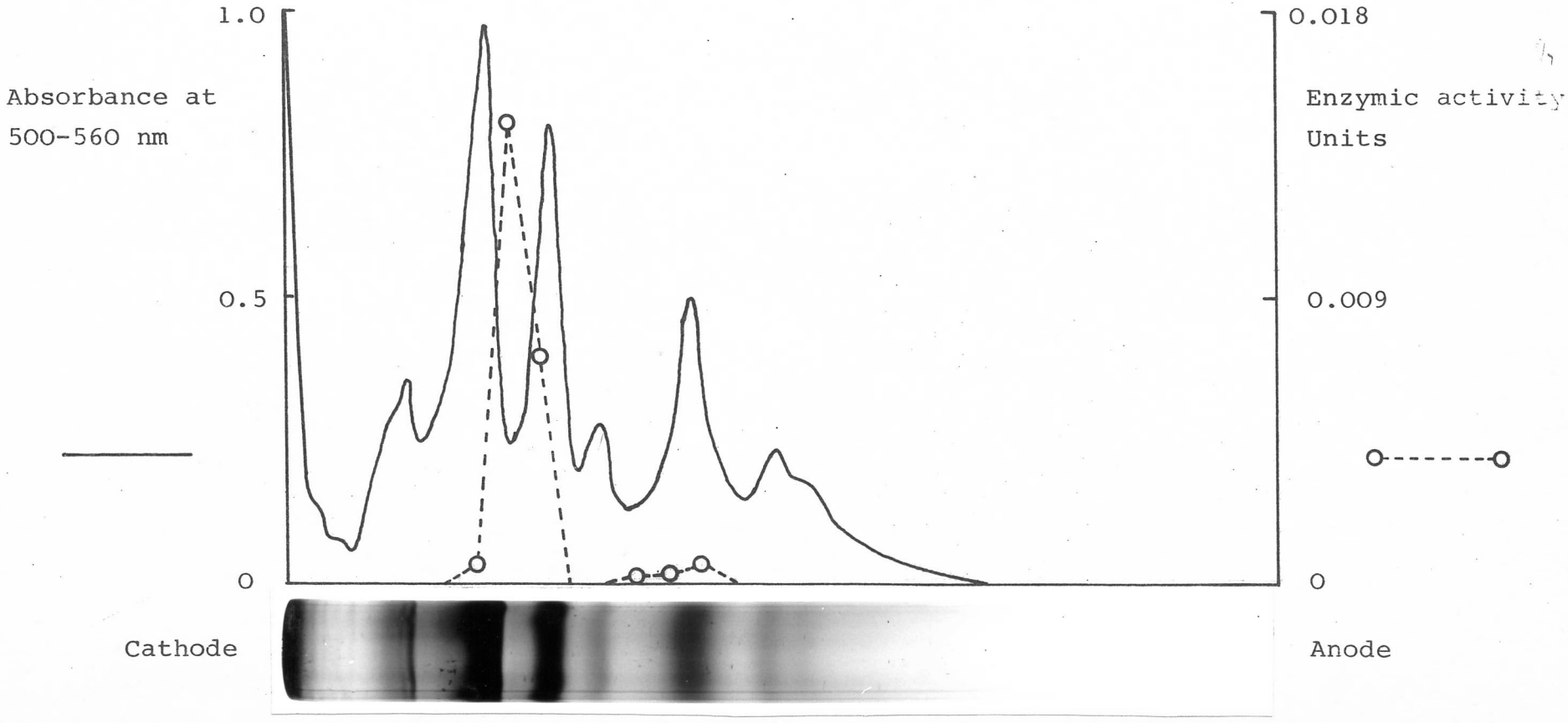


Figure 2.

Electrophoresis of C. maenas phosphofructokinase on a 5% polyacrylamide gel. Comparison of the position of stained protein bands with the absorbance and enzymic activity profiles.

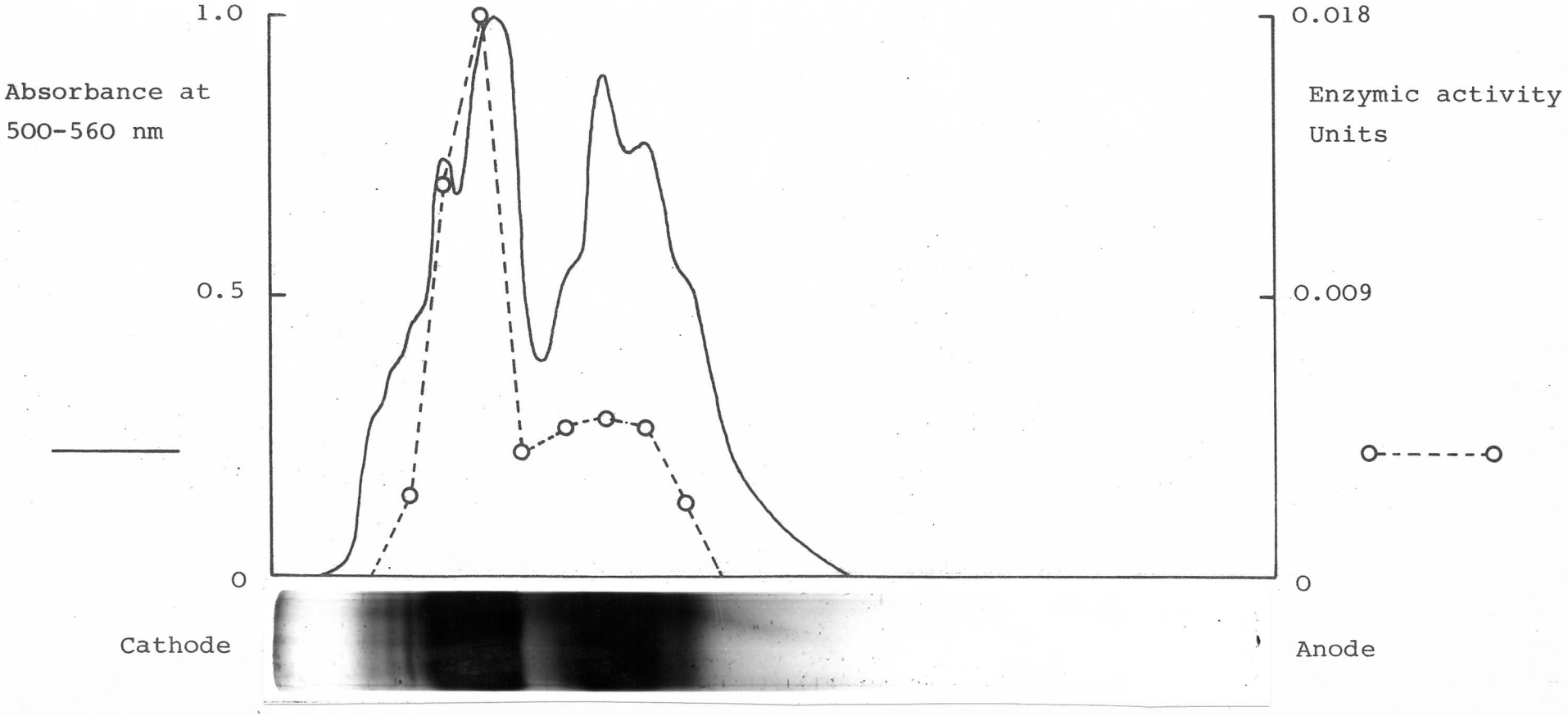


Figure 3.

Calibration curve for phosphofructokinase sub-unit molecular weight determination on 7.9% polyacrylamide gels.

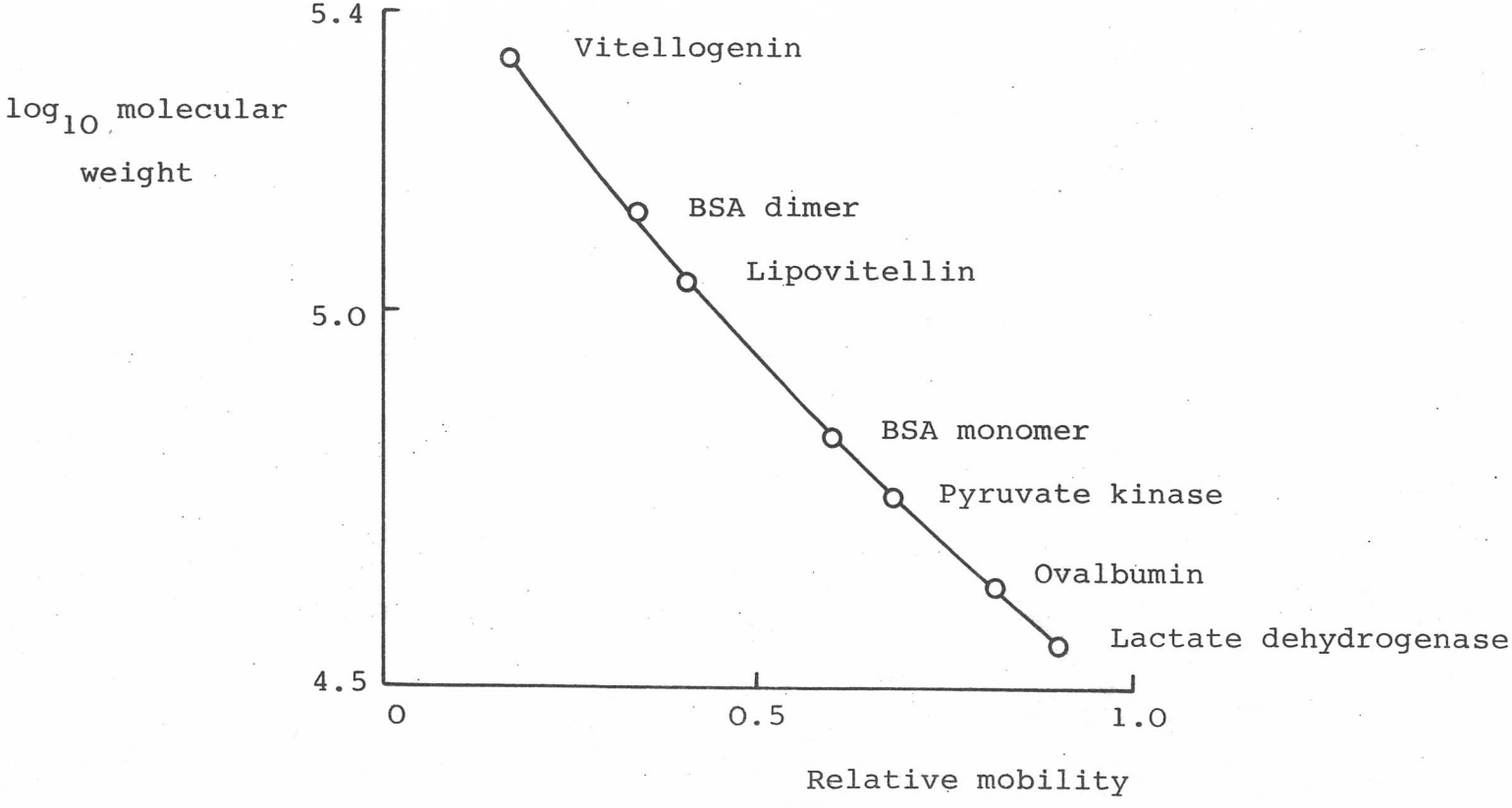


Figure 4.

Electrophoresis of C. maenas phosphofructokinase on a 7.9% polyacrylamide gel under denaturing conditions with SDS. 50 µg of protein was applied to the gel.

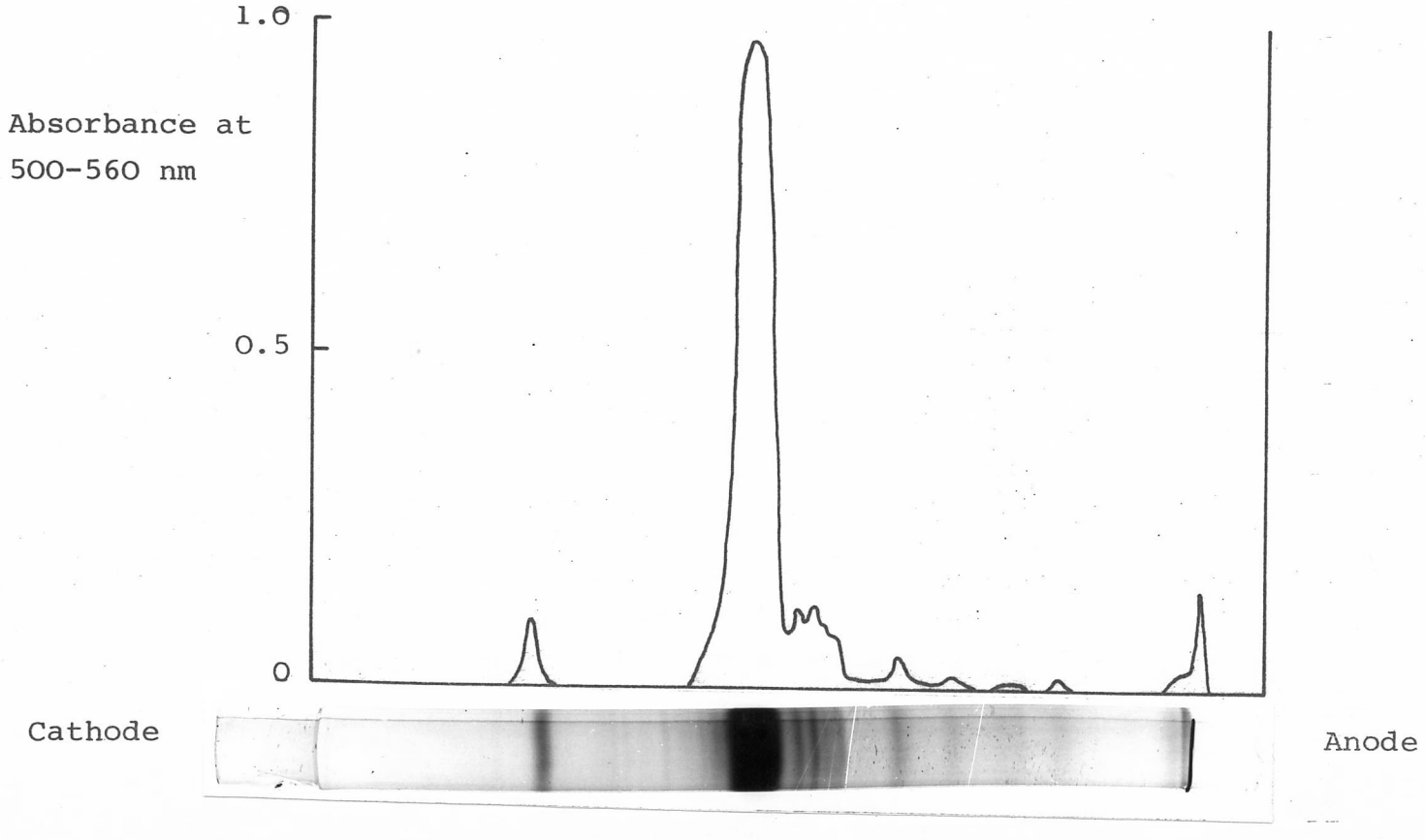


Figure 5.

Comparison of C. maenas phosphofructokinase and rabbit muscle phosphofructokinase electrophoresed on 11% polyacrylamide gels under denaturing conditions with SDS. The absorbance profile is for the C. maenas gel to which 40 μg of protein was applied.

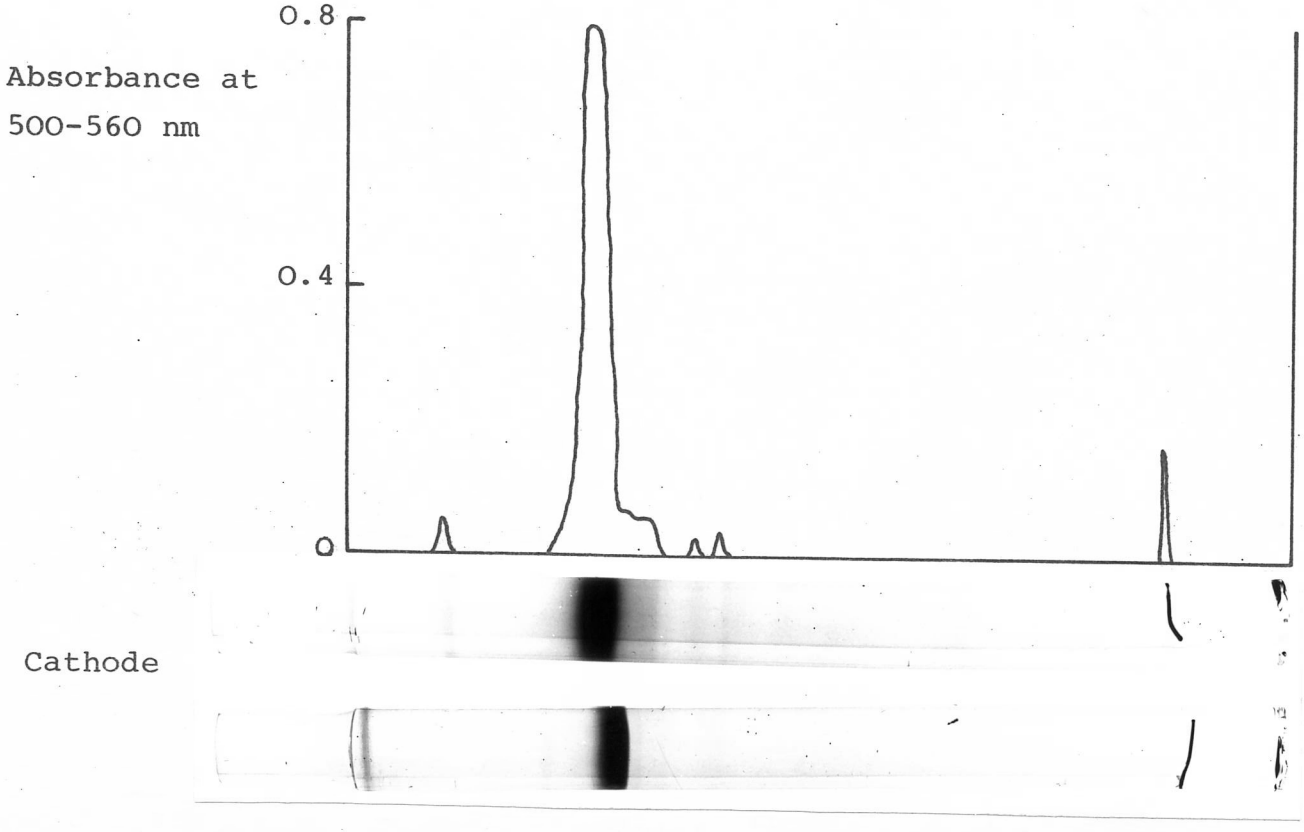


Figure 6.

Effect of ammonium sulphate on phosphofructokinase activity in (a) the standard kinetic assay and (b) the experimental kinetic assay at 4 mM free Mg^{2+} 42 μM F6P and 46 μM ATP.

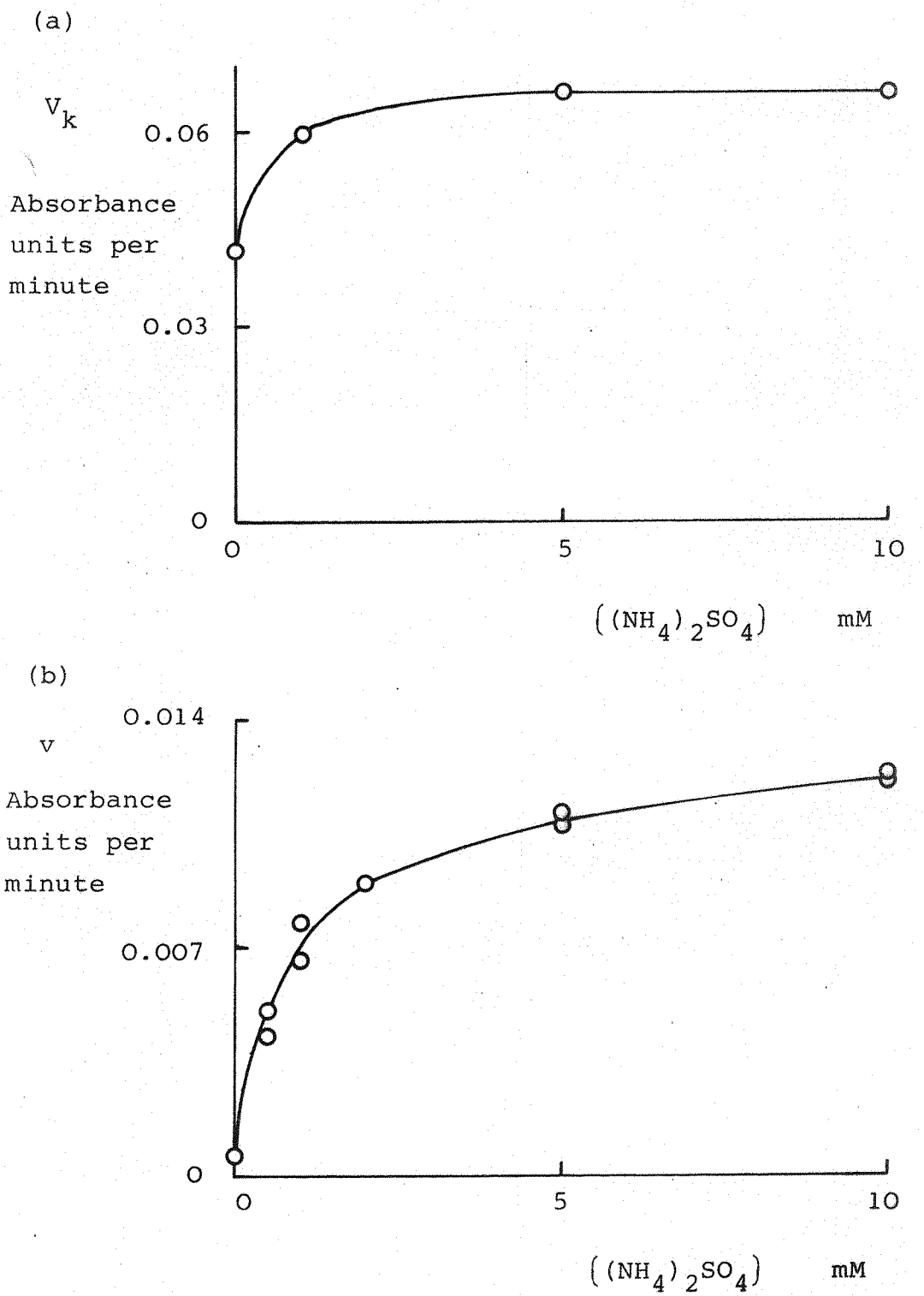


Figure 7.

Activity of phosphofructokinase maintained in 25 mM Tris-phosphate buffer pH 8.0, 1 mg/ml BSA, 1 mM F6P and a variable concentration of either dithiothreitol or β-mercaptoethanol. The enzyme was assayed in the standard kinetic assay in the presence (▽) or absence (O) of 2 mM dithiothreitol.

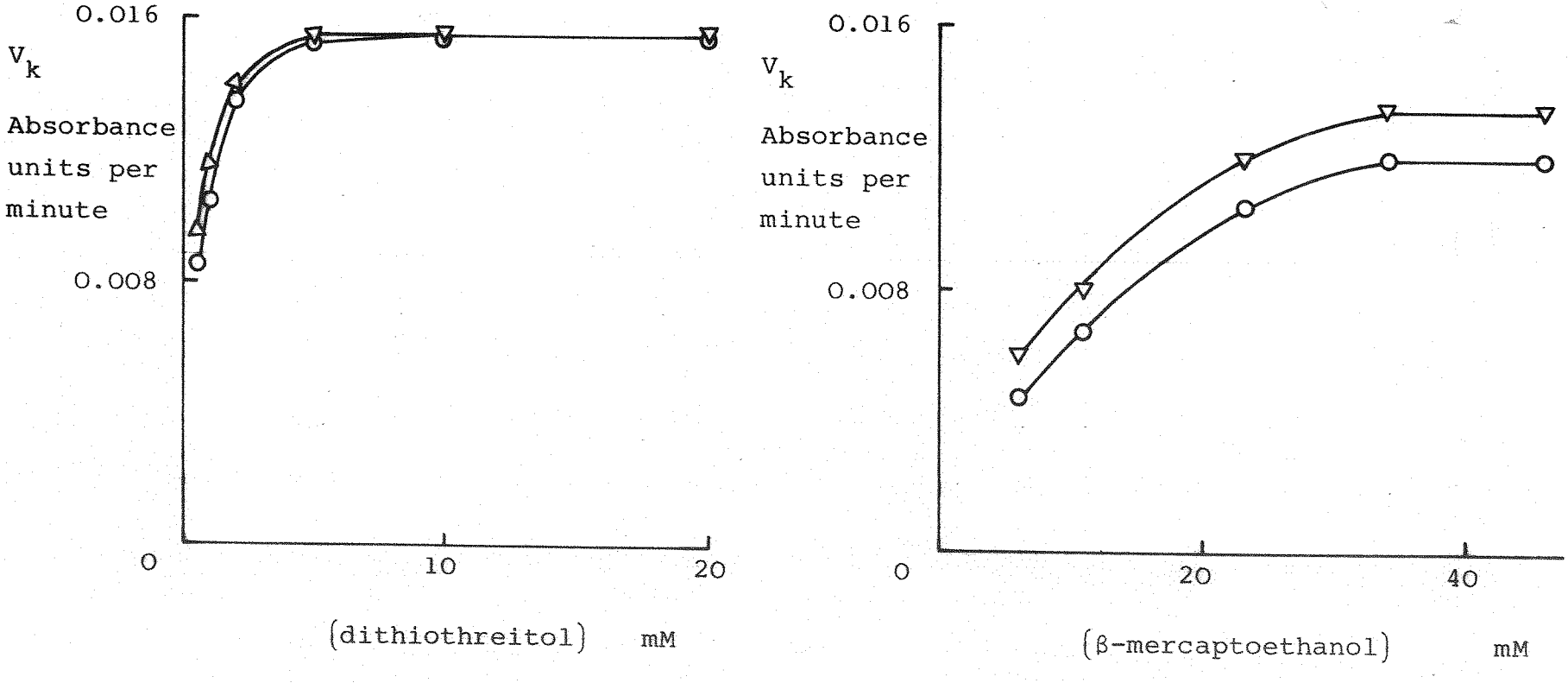


Figure 8.

Activity of phosphofructokinase maintained in 25 mM Tris-HCl buffer pH 8.0 (▽) and 25 mM potassium phosphate buffer pH 8.0 (○) each containing 1 mg/ml BSA, 5 mM dithiothreitol and 1 mM F6P.
Purified phosphofructokinase, specific activity 14 Units/mg protein, was diluted 38 fold with each buffer and 5-40 μℓ of each solution was assayed in the standard kinetic assay.

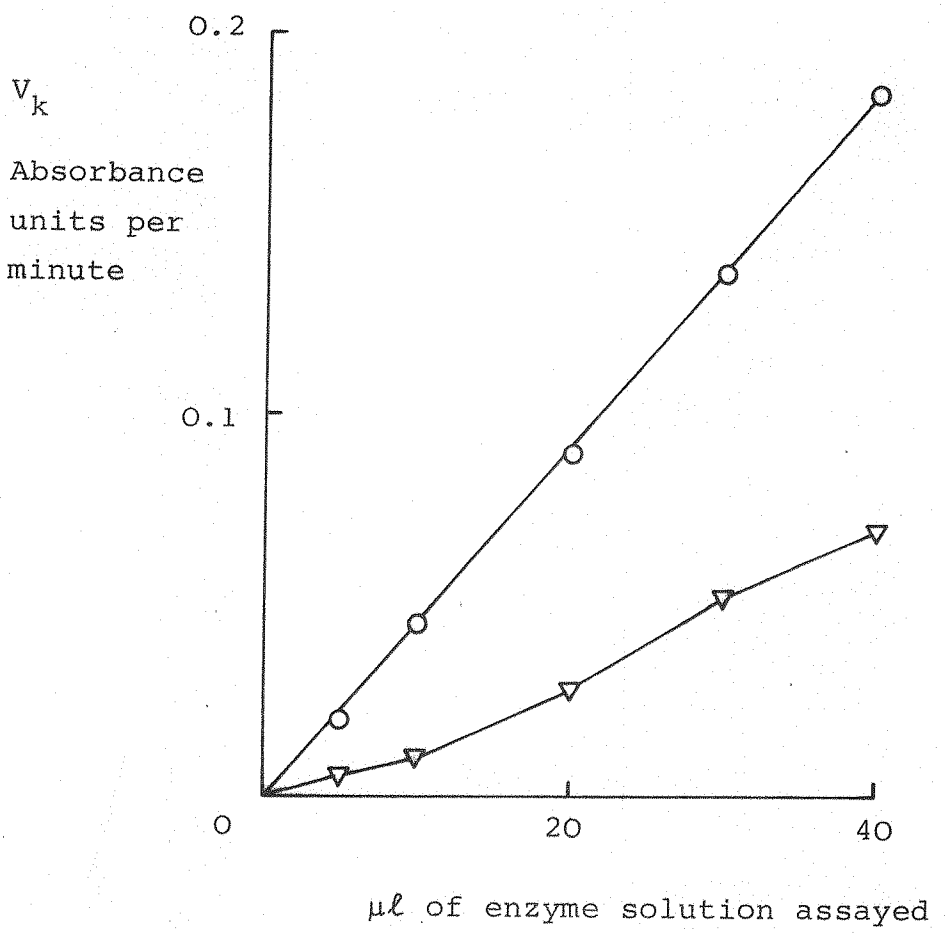


Figure 9.

Activity of phosphofructokinase maintained in 10 mM potassium phosphate buffer pH 8.0 (▽) and 100 mM potassium phosphate buffer pH 8.0 (○) each containing 1 mg/ml BSA, 5 mM dithiothreitol and 1 mM F6P. Purified phosphofructokinase, specific activity 14 Units/mg protein, was diluted 81 fold with each buffer and 10-40 μℓ of each solution was assayed in the standard kinetic assay.

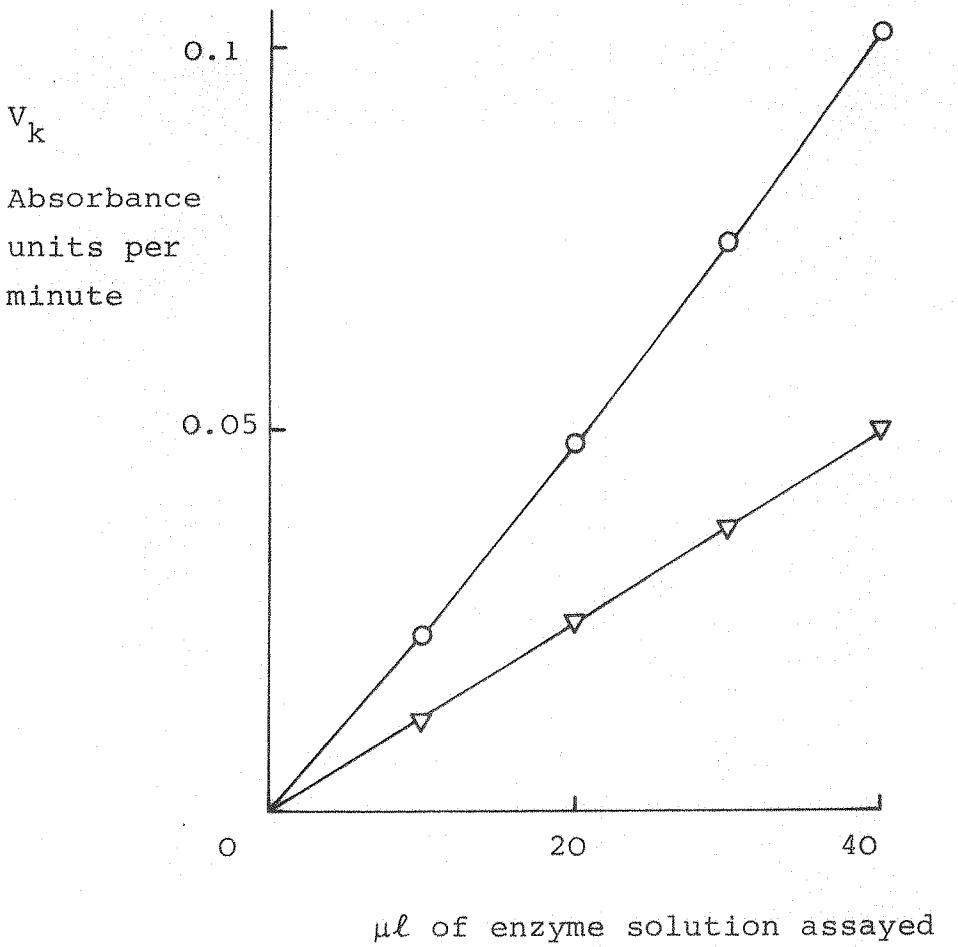
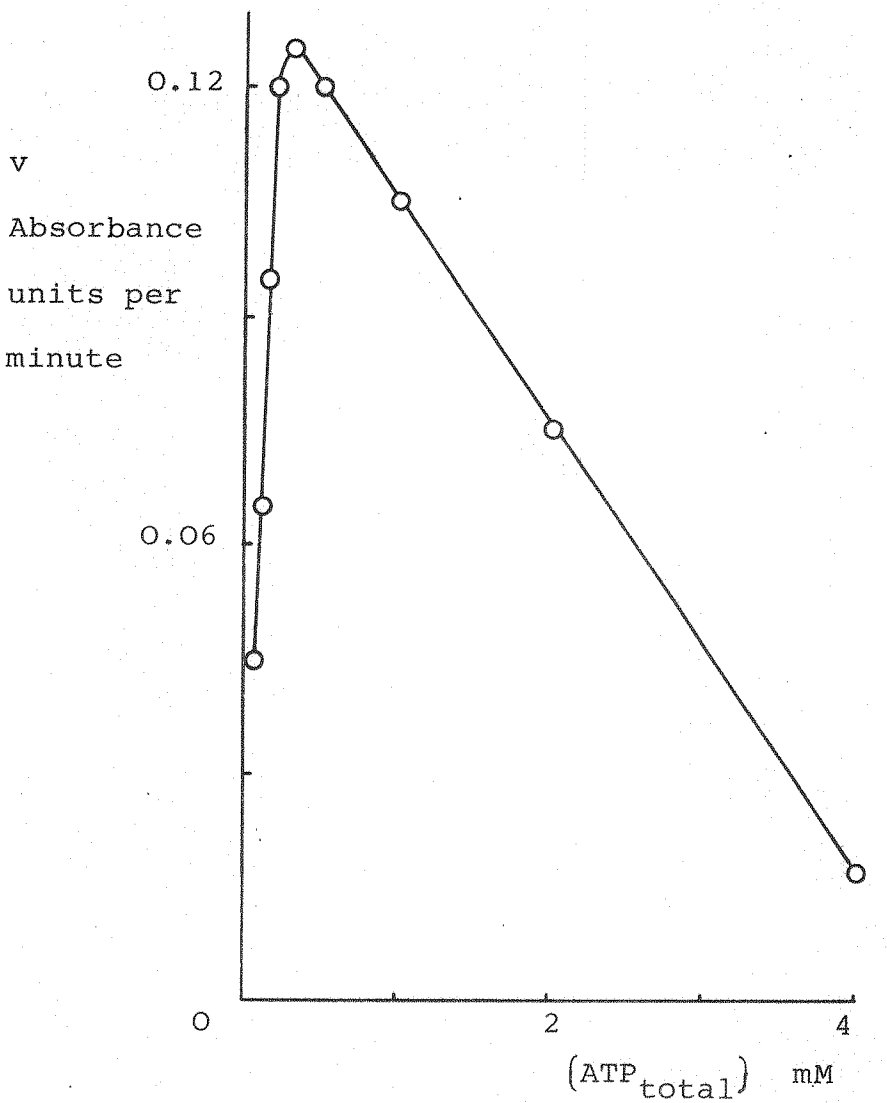


Figure 10.

Activity of phosphofructokinase as a function of ATP concentration at pH 7.0. The other assay conditions were as in the routine assay. The enzyme sample used had a specific activity of 9.1 and was dissolved in 0.35 M Tris-phosphate buffer pH 7.0. 10 $\mu\ell$ of enzyme was assayed throughout.



Chapter 4.

KINETIC PROPERTIES OF PHOSPHOFRUCTOKINASE.

Introduction.

Steady-state kinetic studies of enzyme catalysed reactions give information about the kinetic mechanism of the enzyme, (the order in which substrates and products bind to and are released from the enzyme). The maximum velocity of the reaction and the values of dissociation and Michaelis constants governing the binding of substrates and products to the enzyme are also obtained. Tentative conclusions regarding the *in vivo* concentrations of substrates and products and the physiological role of the enzyme may be drawn from this information, (Cleland, 1967). The types of kinetic experiments that can be performed and the information obtained from each have been described by Cleland (1970).

Experimentally steady-state kinetic studies involve measurement of the reaction rate as a function of the concentration of one substrate with the concentrations of any other substrates held constant. In the case of the phosphofructokinase reaction these measurements are complicated by the fact that two of the ligands required for the reaction, ATP and F6P, reversibly bind the third, Mg^{2+}. In addition ATP also binds K^{+} which is present in the assay. The nature of the complexes formed at the pH of the phosphofructokinase assay is shown in Figure 44. It is not possible to vary the concentration of a single free or complexed species of ATP or F6P while maintaining the concentrations of all the other species constant. The binding of Mg^{2+} by ATP and F6P is governed by the relevent dissociation constant, e.g.

$$K_2 = \frac{(Mg^{2+})\ (ATP^{4-})}{(MgATP^{2-})} = 25\ \mu M$$

so that it is only possible to keep one of the species in such an equilibrium constant while varying the other two. Experimentally it is easiest to maintain a constant concentration of free Mg^{2+}. This fixes the ratio of the free to complexed species of ATP and F6P so that when total ATP or

F6P concentration is varied the concentrations of free and complexed species vary together in a constant ratio. Primary double reciprocal plots of reaction rate against the total concentration of ATP or F6P are always linear and the data can be analysed in terms of the free or complexed species of ATP and F6P without making assumptions as to which interacts with the enzyme. The method of calculation of the individual species concentrations was described in Chapter 2. Repitition of the kinetic measurements at different concentrations of free Mg^{2+} gives information about the role of this ion in the enzyme catalysed reaction. The effect of varying the free Mg^{2+} concentration on the ratio of the free to complexed species of ATP and F6P is shown in Figure 45.

Derivation of the rate equation.

The rate equation for the ordered ter-bi mechanism shown in Scheme 1 was derived assuming that steady-state conditions applied, i.e. the change in concentration with time of the enzyme substrate and enzyme product complexes and free enzyme was zero compared to the change in concentration with time of the substrates and products. In the following discussion of results the letters A, B, C, P, Q, E_t and E represent the concentrations of $F6P^{2-}$, free Mg^{2+}, ATP^{4-}, $MgADP^{-}$, FDP^{4-}, total enzyme and free enzyme respectively. The letters v and V represent the velocity of the forward reaction and the maximum velocity of the forward reaction respectively.

The change in concentration with time of the enzyme substrate and enzyme product complexes is given by the following equations:-

$$\frac{d(EA)}{dt} = k_1(E)A + k_4(EAB) - k_2(EA) - k_3(EA)B = 0 \quad (a)$$

$$\frac{d(EAB)}{dt} = k_3(EA)B + k_6(EABC) - k_4(EAB) - k_5(EAB)C = 0 \quad (b)$$

$$\frac{d(EABC)}{dt} = k_5(EAB)C + k_8(EQ)P - k_6(EABC) - k_7(EABC) = 0 \quad (c)$$

$$\frac{d(EQ)}{dt} = k_7(EABC) + k_{10}(E)Q - k_8(EQ)P - k_9(EQ) = 0 \quad (d)$$

For initial-rate studies carried out in the absence of added products all terms containing P or Q approximate to zero since the concentrations of products are much less than the concentrations of substrates. The concentrations of the enzyme substrate complexes (EA), (EAB) and (EABC) can be expressed in terms of the concentration of (EQ) as follows:-

From (d):

$$(EABC) = \frac{k_9(EQ)}{k_7}$$

From (c):

$$(EAB) = \frac{(k_6+k_7)(EABC)}{k_5C} = \frac{k_9(k_6+k_7)(EQ)}{k_5k_7C}$$

From (b):

$$(EA) = \frac{k_4(EAB) + k_5(EAB)C - k_6(EABC)}{k_3B}$$

$$= \frac{k_4k_9(k_6+k_7)(EQ)}{k_3k_5k_7BC} + \frac{k_5k_9(k_6+k_7)C(EQ)}{k_3k_5k_7BC} - \frac{k_6k_9(EQ)}{k_3k_7B}$$

The concentration of free enzyme is given by:-

$$(E) = E_t - (EQ) - (EABC) - (EAB) - (EA)$$

Equation (a), the conservation equation, can now be written in terms of the concentration of (EQ) by substituting the values of (E), (EA), (EAB) and (EABC) in terms of (EQ):-

$$k_1E_tA - k_1A(EQ)$$

$$- \frac{k_1k_9A(EQ)}{k_7} - \frac{k_1k_9(k_6+k_7)A(EQ)}{k_5k_7C} - \frac{k_1k_4k_9(k_6+k_7)A(EQ)}{k_3k_5k_7BC}$$

$$- \frac{k_1k_5k_9(k_6+k_7)AC(EQ)}{k_3k_5k_7BC} + \frac{k_1k_6k_9A(EQ)}{k_3k_7B} + \frac{k_4k_9(k_6+k_7)(EQ)}{k_5k_7C}$$

$$- \frac{k_2k_4k_9(k_6+k_7)(EQ)}{k_3k_5k_7BC} - \frac{k_2k_5k_9(k_6+k_7)C(EQ)}{k_3k_5k_7BC} + \frac{k_2k_6k_9(EQ)}{k_3k_7B}$$

$$- \frac{k_3k_4k_9(k_6+k_7)B(EQ)}{k_3k_5k_7BC} - \frac{k_3k_5k_9(k_6+k_7)BC(EQ)}{k_3k_5k_7BC} + \frac{k_3k_6k_9B(EQ)}{k_3k_7B} = 0$$

Solving this equation for (EQ) we obtain:-

$$(EQ) = \frac{k_1 k_3 k_5 k_7 E_t ABC}{k_1 k_3 k_5 (k_7+k_9) ABC + k_1 k_3 k_9 (k_6+k_7) AB + k_1 k_5 k_7 k_9 AC + k_3 k_5 k_7 k_9 BC + k_1 k_4 k_9 (k_6+k_7) A + k_2 k_5 k_7 k_9 C + k_2 k_4 k_9 (k_6+k_7)}$$

The initial-rate of the forward reaction is given by:-

$$v = k_9 (EQ)$$

Thus the initial-rate can be expressed in terms of the concentrations of the substrates A, B and C by substituting the value of (EQ) in terms of A, B and C. The rate equation can be simplified by expressing it in the coefficient manner of Cleland (1963).

$$v = \frac{(\text{numerator})ABC}{(\text{coef ABC})ABC + (\text{coef AB})AB + (\text{coef AC})AC + (\text{coef BC})BC + (\text{coef A})A + (\text{coef C})C + \text{constant}} \qquad (1)$$

The following relationships are true for an ordered ter mechanism:-

$$\frac{(\text{numerator})}{(\text{coef ABC})} = V \qquad \frac{(\text{coef AB})}{(\text{coef ABC})} = K_m^C \qquad \frac{(\text{coef AC})}{(\text{coef ABC})} = K_m^B$$

$$\frac{(\text{coef BC})}{(\text{coef ABC})} = K_m^A \qquad \frac{(\text{coef A})}{(\text{coef ABC})} = K_s^B K_m^C \qquad \frac{(\text{coef C})}{(\text{coef ABC})} = K_s^A K_m^B$$

$$\frac{\text{constant}}{(\text{coef ABC})} = K_s^A K_s^B K_m^C$$

The rate equation can be written with kinetic constants by dividing equation (1) by (coef ABC).

$$v = \frac{V A B C}{ABC + K_m^A BC + K_m^B AC + K_m^C AB + K_s^A K_m^B C + K_s^B K_m^C A + K_s^A K_s^B K_m^C} \qquad (2)$$

The reciprocal form of the rate equation is:-

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m^A}{VA} + \frac{K_m^B}{VB} + \frac{K_m^C}{VC} + \frac{K_s^A K_m^B}{VAB} + \frac{K_s^B K_m^C}{VBC} + \frac{K_s^A K_s^B K_m^C}{VABC} \qquad (3)$$

Analysis of initial-rate data.

The initial-rate data was analysed in terms of the concentrations of free Mg^{2+}, ATP^{4-} and $F6P^{2-}$. Plots of reciprocal initial velocity versus the reciprocal concentration of $F6P^{2-}$, with ATP^{4-} concentration constant for each line and the concentration of free Mg^{2+} constant for the family of lines, were constructed at eight different concentrations of free Mg^{2+}, (Figures 11-18). The form of the rate equation describing this data is obtained by rearrangement of equation (3).

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m^B}{VB} + \frac{K_m^C}{VC} + \frac{K_s^B K_m^C}{VBC} + \left(\frac{K_m^A}{V} + \frac{K_s^A K_m^B}{VB} + \frac{K_s^A K_s^B K_m^C}{VBC} \right) \frac{1}{A} \qquad (4)$$

In equation (4) the terms forming the coefficient of the reciprocal concentration of A represent the slopes of the lines in the primary plots and the other terms represent the ordinate intercepts. This equation is consistent with the intersecting patterns of the lines in Figures 11-18. A similar intersecting pattern is observed if the reciprocal concentration of ATP^{4-} is taken as the variable in the primary plot, (Figure 19). These intersecting patterns are diagnostic of a sequential mechanism, (all substrates must be combined with the enzyme before any products can be released).

Secondary plots of the slopes and ordinate intercepts of the primary plots at the different concentrations of free Mg^{2+} versus the reciprocal concentration of ATP^{4-} were constructed, (Figures 20 and 21). Both the slopes and ordinate intercepts of the lines in the secondary plots decrease as the concentration of free Mg^{2+} increases as predicted by slope and intercept functions in equation (4).

Analysis of the slopes and ordinate intercepts of the two secondary plots to give four tertiary plots allowed calculation of the kinetic constants. The values of the slopes and ordinate intercepts of the lines in the two secondary plots were plotted against the reciprocal concentration of free Mg^{2+}, (Figures 22-25).

The slopes of the lines in the secondary plot of primary slopes show a linear dependence on the reciprocal concentration of free Mg^{2+}, (Figure 22). This is predicted by the corresponding function from equation (4).

$$\text{Slope of secondary plot of primary slopes} = \frac{K_s^A K_s^B K_m^C}{V} \cdot \frac{1}{B}$$

There is no ordinate intercept term in this equation and in accordance with this the ordinate intercept of Figure 22 is not statistically different from zero. The lack of an ordinate intercept in this plot shows that there is no (coef Mg^{2+}) term in any rate equation which is consistent with the data. For an ordered ter mechanism this is observed only if Mg^{2+} is the second substrate to bind to the enzyme.

The tertiary plot of ordinate intercepts of the secondary plot of primary slopes is a straight line with positive slope and ordinate intercept values, (Figure 23). This is in agreement with the corresponding function from equation (4).

$$\text{Ordinate intercept of secondary plot of primary slopes} = \frac{K_m^A}{V} + \frac{K_s^A K_m^B}{V} \cdot \frac{1}{B}$$

The tertiary plot of slopes of the secondary plot of primary ordinate intercepts is a straight line with positive slope and ordinate intercept values, (Figure 24). This is in agreement with the corresponding function from equation (4).

$$\text{Slope of secondary plot of primary ordinate intercepts} = \frac{K_m^C}{V} + \frac{K_s^B K_m^C}{V} \cdot \frac{1}{B}$$

The ordinate intercept in Figure 24 is statistically greater than zero at the 85-90% level of probability. The

value of the intercept is small compared to the range of the ordinate axis because K_m^C is small compared to K_s^B. A positive ordinate intercept in this plot indicates the presence of a (coef $F6P^{2-}$ Mg^{2+}) term in any rate equation consistent with the data. For an ordered ter mechanism a (coef $F6P^{2-}$ Mg^{2+}) term is formed if free enzyme binds $F6P^{2-}$ followed by free Mg^{2+} to form an E.F6P.Mg complex.

The function from equation (4) describing the tertiary plot of ordinate intercepts of the secondary plot of primary ordinate intercepts is:-

$$\text{Ordinate intercept of secondary plot of primary ordinate intercepts} = \frac{1}{V} + \frac{K_m^B}{V} \cdot \frac{1}{B}$$

In agreement with this Figure 25 shows a straight line with positive slope and ordinate intercept values. This shows V is dependent on the concentration of free Mg^{2+}. In the case of an ordered ter-ter mechanism in which free Mg^{2+} and ADP^{3-} leave the enzyme separately, (in either order), the appropriate rate equation predicts a non-linear plot for Figure 25 with a minimum in the line at the higher concentrations of free Mg^{2+}. As this was not observed $MgADP^-$ was taken to be the product of the reaction.

The parameters which describe the tertiary plots are listed in Table 7. The kinetic constants were calculated by taking ratios of the parameters of the tertiary plots. The ratios used and the values of the kinetic constants are shown in Table 8.

Thus analysis of the form of secondary and tertiary plots of the primary data shows that the results of the initial rate experiments are in agreement with the predictions of the rate equation and hence they are consistent with the ordered ter-bi mechanism shown in Scheme 1.

The experimental data can also be shown to be in agreement with the proposed reaction mechanism by fitting the primary data directly to the full rate equation for Scheme 1 expressed in coefficient form. For this purpouse the rate equation (1) was divided through by (numerator) to reduce the number of unknowns in the equation:-

$$v = \frac{A\ B\ C}{(\text{coef ABC})'\ ABC + (\text{coef AB})'\ AB + (\text{coef AC})'\ AC + (\text{coef BC})'\ BC + (\text{coef A})'\ A + (\text{coef C})'\ C + \text{constant}'} \quad (5)$$

Where: $(\text{coef ABC})' = \frac{(\text{coef ABC})}{(\text{numerator})}$ $(\text{coef AB})' = \frac{(\text{coef AB})}{(\text{numerator})}$ etc.

The measured rates of reaction and the corresponding concentrations of ATP^{4-}, $F6P^{2-}$ and free Mg^{2+} used in the initial-rate experiments, (618 data points), were fitted to equation (5) by a non-linear regression method. The values of the seven unknowns in the equation and their standard errors were obtained along with the variance for the fit and the value of sigma. These are listed in Table 9. The standard errors of the unknowns are not more than 10% of the values of the unknowns except for the standard error of (coef AC)' which is 20% of its value. This shows the data is a good fit to the rate equation. Furthermore this rate equation gave a better fit to the data than the rate equations for two other mechanisms which were investigated, (see Chapter 5). These were a random mechanism for which the variance was higher than that for the ordered ter-bi mechanism, (Table 14), and an ordered ter-ter mechanism for which negative or insignificant values of coefficients were obtained, (Table 13).

The kinetic constants in equation (2) were calculated by taking ratios of the values of the coefficients in Table 9. The ratios used and the values of the kinetic constants are shown in Table 10.

Comparison of the initial-rate data with the predictions of the rate equation.

The rate equation for Scheme 1 was used to predict the pattern of lines in primary plots in which the concentration of $F6P^{2-}$ was maintained constant rather than the concentration of free Mg^{2+}. The variable and changing fixed substrates in these alternative primary plots were ATP^{4-} and free Mg^{2+}. The alternative primary plots were constructed from the initial-rate data and qualitatively examined to see if they were consistent with the predictions of the rate

equation.

The appropriate points needed to construct the alternative primary plots were interpolated from the primary plots in Figures 11-18 by use of the apparent K_m and apparent V values of the lines. The rate for any desired substrate concentration, (within the experimental range), was calculated by use of the Michaelis-Menten equation.

Figure 26 shows a primary plot constructed with ATP^{4-} as the variable substrate and free Mg^{2+} as the changing fixed substrate. The concentration of $F6P^{2-}$ is constant. The form of the rate equation describing this plot is obtained by rearrangement of equation (3).

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m^A}{VA} + \frac{K_m^B}{VB} + \frac{K_s^A K_m^B}{VAB} + \left(\frac{K_m^C}{V} + \frac{K_s^B K_m^C}{VB} + \frac{K_s^A K_s^B K_m^C}{VAB} \right) \frac{1}{C}$$

This equation predicts that as the concentration of free Mg^{2+} is increased both the slopes and ordinate intercepts of the lines in Figure 26 will decrease. This behaviour is observed and can be seen more clearly by inspection of the appropriate secondary plots, (Figures 27 and 28). The equations describing the secondary plots are:-

$$\text{Primary slope} = \frac{K_m^C}{V} + \left(\frac{K_s^B K_m^C}{V} + \frac{K_s^A K_s^B K_m^C}{VA} \right) \frac{1}{B}$$

$$\text{Primary ordinate intercept} = \frac{1}{V} + \frac{K_m^A}{VA} + \left(\frac{K_m^B}{V} + \frac{K_s^A K_m^B}{VA} \right) \frac{1}{B}$$

These equations were used to calculate the positions of the lines in the secondary plots predicted by the rate equation and the weighted fit values of the kinetic constants listed in Table 10, (an average value for K_s^A was taken). The predicted lines are shown in Figures 27 and 28 in broken type. The positions of the predicted and experimental lines in Figure 28 are in good agreement despite the error which must arise when plots are constructed

using interpolated data and calculated kinetic constants. The predicted value of the slope in Figure 27 is twice the experimental value. This discrepancy arises due to the uncertainty in the value of K_s^A. A better correspondence between the predicted and experimental lines in Figure 27 is obtained if the lower value of K_s^A is used in calculation.

Figure 29 is a primary plot of reciprocal initial velocity against the reciprocal concentration of free Mg^{2+} with ATP^{4-} concentration constant for each line and $F6P^{2-}$ concentration constant for the family of lines. The corresponding form of the rate equation is obtained by rearrangement of equation (3).

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m^A}{VA} + \frac{K_m^C}{VC} + \left(\frac{K_m^B}{V} + \frac{K_s^A K_m^B}{VA} + \frac{K_s^B K_m^C}{VC} + \frac{K_s^A K_s^B K_m^C}{VAC} \right) \frac{1}{B}$$

The equations describing the slopes and ordinate intercepts of the lines are:-

$$\text{Primary slope} = \frac{K_m^B}{V} + \frac{K_s^A K_m^B}{VA} + \left(\frac{K_s^B K_m^C}{V} + \frac{K_s^A K_s^B K_m^C}{VA} \right) \frac{1}{C}$$

$$\text{Primary ordinate intercept} = \frac{1}{V} + \frac{K_m^A}{VA} + \frac{K_m^C}{V} \cdot \frac{1}{C}$$

Thus both the slopes and ordinate intercepts of the primary plot are predicted to decrease as the concentration of ATP^{4-} increases. This is observed and is shown clearly by the secondary plots, (Figures 30 and 31). The slope of the secondary plot of primary ordinate intercepts against the reciprocal concentration of ATP^{4-} is very small. This is because the value of K_m^C is small compared to the values of the other kinetic constants.

It was not possible to interpolate primary plots in which ATP^{4-} was constant for the family of lines as there is no one concentration of ATP^{4-} which is present in all the primary plots in Figures 11-18.

Product-inhibition studies.

The results of product-inhibition studies are consistent with the ordered ter-bi mechanism shown in Scheme 1. The rate equation for this mechanism which includes those terms which are introduced when either one of the products is present in the absence of the other was taken from Plowman (1972).

$$v = \frac{V\,A\,B\,C}{ABC + K_m^A BC + K_m^B AC + K_m^C AB + K_s^A K_m^B C + K_s^B K_m^C A + K_s^A K_s^B K_m^C + \frac{K_s^A K_s^B K_m^C}{K_i^Q}.Q + \frac{K_m^Q K_s^A K_s^B K_m^C}{K_m^P K_i^Q}.P + \frac{K_m^Q K_s^B K_m^C}{K_m^P K_i^Q}.AP + \frac{K_s^A K_m^B}{K_i^Q}.CQ + \frac{K_m^Q K_m^C}{K_m^P K_i^Q}.ABP + \frac{K_m^A}{K_i^Q}.BCQ + \frac{K_m^Q K_m^C}{K_s^C K_m^P K_i^Q}.ABCP}$$

The reciprocal form of the rate equation is:-

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m^A}{VA} + \frac{K_m^B}{VB} + \frac{K_m^C}{VC} + \frac{K_s^A K_m^B}{VAB} + \frac{K_s^B K_m^C}{VBC} + \frac{K_s^A K_s^B K_m^C}{VABC} + \frac{K_s^A K_s^B K_m^C}{VABC\,K_i^Q}.Q + \frac{K_m^Q K_s^A K_s^B K_m^C}{VABC\,K_m^P K_i^Q}.P + \frac{K_m^Q K_s^B K_m^C}{VBC\,K_m^P K_i^Q}.P + \frac{K_s^A K_m^B}{VAB\,K_i^Q}.Q + \frac{K_m^Q K_m^C}{VC\,K_m^P K_i^Q}.P + \frac{K_m^A}{VA\,K_i^Q}.Q + \frac{K_m^Q K_m^C}{V\,K_s^C K_m^P K_i^Q}.P \qquad (6)$$

Inhibition of the phosphofructokinase catalysed reaction was studied with only one of the products $MgADP^-$ or FDP^{4-} present at a time. The rate equation is modified for this situation simply by the omission of terms in P or Q as appropriate.

Analysis of product-inhibition data.

The product-inhibition data was analysed in terms of the concentrations of $MgADP^-$ and FDP^{4-} for inhibition by ADP and FDP respectively.

Figure 32 shows a plot of reciprocal initial velocity against the reciprocal concentration of $F6P^{2-}$ with FDP^{4-} concentration constant for each line and the concentrations of ATP^{4-} and free Mg^{2+} constant for the family of lines. The plot shows FDP^{4-} is a competitive inhibitor with respect to $F6P^{2-}$. The form of the rate equation describing this plot is obtained by rearrangement of equation (6) with omission of terms in P.

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m^B}{VB} + \frac{K_m^C}{VC} + \frac{K_s^B K_m^C}{VBC} + \left(\frac{K_m^A}{V} + \frac{K_s^A K_m^B}{VB} + \frac{K_s^A K_s^B K_m^C}{VBC} + \frac{K_s^A K_s^B K_m^C}{VBC K_i^Q} . Q + \frac{K_s^A K_m^B}{VB K_i^Q} . Q + \frac{K_m^A}{V K_i^Q} . Q \right) \frac{1}{A}$$

This equation predicts that as the concentration of FDP^{4-} is increased the slopes of the lines in Figure 32 will increase while their ordinate intercepts remain constant. This is observed and is clearly shown by the secondary plots, (Figures 33 and 34). The value of K_i^Q can be calculated from the parameters of the slope replot.

A primary plot of reciprocal initial velocity against the reciprocal concentration of ATP^{4-} with FDP^{4-} concentration constant for each line and $F6P^{2-}$ and free Mg^{2+} concentrations constant for the family of lines is shown in Figure 35. Both the slopes and ordinate intercepts of the lines increase as FDP^{4-} is increased showing that FDP^{4-} is a non-competitive inhibitor with respect to ATP^{4-}. The corresponding form of the rate equation is:-

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m^A}{VA} + \frac{K_m^B}{VB} + \frac{K_s^A K_m^B}{VAB} + \frac{K_s^A K_m^B}{VAB K_i^Q} . Q + \frac{K_m^A}{VA K_i^Q} . Q + \left(\frac{K_m^C}{V} + \frac{K_s^B K_m^C}{VB} + \frac{K_s^A K_s^B K_m^C}{VAB} + \frac{K_s^A K_s^B K_m^C}{VAB K_i^Q} . Q \right) \frac{1}{C}$$

The predictions of this equation are in agreement with the pattern of lines in the primary plot. The secondary plots, (Figures 36 and 37), show that the slopes and ordinate intercepts of the lines in the primary plot increase linearly with the increasing concentration of FDP^{4-} as predicted by the equation.

$MgADP^{-}$ is a non-competitive inhibitor with respect to $F6P^{2-}$. This is shown by a primary plot of reciprocal initial velocity against the reciprocal concentration of $F6P^{2-}$ with $MgADP^{-}$ concentration constant for each line and the concentrations of ATP^{4-} and free Mg^{2+} constant for the family of lines, (Figure 38). Both the slopes and ordinate intercepts of the lines increase with increasing concentration of $MgADP^{-}$ as predicted by the appropriate form of the rate equation.

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m^B}{VB} + \frac{K_m^C}{VC} + \frac{K_s^B K_m^C}{VBC} + \frac{K_m^Q K_s^B K_m^C}{VBC K_m^P K_i^Q} \cdot P$$

$$+ \frac{K_m^Q K_m^C}{VC K_m^P K_i^Q} \cdot P + \frac{K_m^Q K_m^C}{V K_s^C K_m^P K_i^Q} \cdot P$$

$$+ \left(\frac{K_m^A}{V} + \frac{K_s^A K_m^B}{VB} + \frac{K_s^A K_s^B K_m^C}{VBC} + \frac{K_m^Q K_s^A K_s^B K_m^C}{VBC K_m^P K_i^Q} \cdot P \right) \frac{1}{A}$$

The kinetic constants K_m^P and K_m^Q are present together in the inhibitory terms of the equation so that they cannot be calculated from the parameters of the secondary plots, (Figures 39 and 40).

$MgADP^{-}$ is a non-competitive inhibitor with respect to ATP^{4-}. This is shown by a primary plot of reciprocal initial velocity against the reciprocal concentration of ATP^{4-} with $MgADP^{-}$ concentration constant for each line and the concentrations of $F6P^{2-}$ and free Mg^{2+} constant for the family of lines, (Figure 41). Both the slopes and ordinate intercepts of the lines increase with increasing concentration of $MgADP^{-}$ as predicted by the appropriate form of the rate equation.

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m^A}{VA} + \frac{K_m^B}{VB} + \frac{K_s^A K_m^B}{VAB} + \frac{K_m^Q K_m^C}{V K_s^C K_m^P K_i^Q} \cdot P$$

$$+ \left(\frac{K_m^C}{V} + \frac{K_s^B K_m^C}{VB} + \frac{K_s^A K_s^B K_m^C}{VAB} + \frac{K_m^Q K_s^A K_s^B K_m^C}{VAB K_m^P K_i^Q} \cdot P + \right.$$

$$\left. \frac{K_m^Q K_s^B K_m^C}{VB K_m^P K_i^Q} \cdot P + \frac{K_m^Q K_m^C}{V K_m^P K_i^Q} \cdot P \right) \frac{1}{C}$$

In agreement with this equation secondary plots of both primary slopes and ordinate intercepts against the concentration of $MgADP^-$ are linear with positive slopes and intercepts, (Figures 42 and 43). These secondary plots cannot be used to obtain values for K_m^P, K_m^Q and K_s^C since none of the inhibitory terms in the equation contain one of these constants in the absence of the other two. Initial-rate studies of the reaction in the reverse direction are required to obtain values for these constants.

The parameters which describe the secondary plots of the four product-inhibition patterns are listed in Table 11. The method of calculation of K_i^Q and its value are shown in Table 12.

Scheme 1.

Ordered ter-bi mechanism.

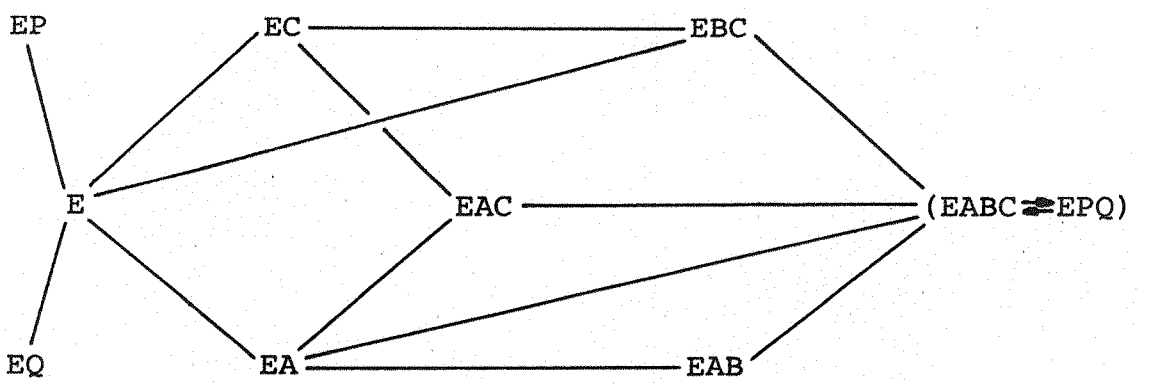
$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EAB + C \underset{k_6}{\overset{k_5}{\rightleftharpoons}} (EABC \rightleftharpoons EPQ) \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EQ + P \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E + Q$$

A = $F6P^{2-}$. B = free Mg^{2+}. C = ATP^{4-}. P = $MgADP^{-}$. Q = FDP^{4-}.

This reaction scheme is consistent with the results of the initial-rate and product inhibition studies and gives the best fit to the data of the mechanisms tested.

Scheme 2.

Rapid-equilibrium random mechanism.



A = $F6P^{2-}$. B = free Mg^{2+}. C = ATP^{4-}. P = $MgADP^{-}$. Q = FDP^{4-}.

This reaction scheme is also consistent with the results of the initial-rate experiments but not those of the product-inhibition experiments.

Table 7.

Slope and ordinate intercept values of tertiary plots and their kinetic identities according to the rate equation (2).

Tertiary plot	Parameter	Kinetic identity	Value	Standard error
Fig. 22				
(1)	Intercept	None	-1.65×10^{-6} mM^2	1.77×10^{-6}
(2)	Slope	$\frac{K_s^A K_s^B K_m^C}{V}$	3.29×10^{-5} mM^3	6.18×10^{-7}
Fig. 23				
(3)	Intercept	$\frac{K_m^A}{V}$	4.05×10^{-2} mM	2.94×10^{-3}
(4)	Slope	$\frac{K_s^A K_m^B}{V}$	5.57×10^{-3} mM^2	1.03×10^{-3}
Fig. 24				
(5)	Intercept	$\frac{K_m^C}{V}$	5.24×10^{-5} mM	3.28×10^{-5}
(6)	Slope	$\frac{K_s^B K_m^C}{V}$	5.46×10^{-4} mM^2	1.14×10^{-5}
Fig. 25				
(7)	Intercept	$\frac{1}{V}$	0.828	3.57×10^{-2}
(8)	Slope	$\frac{K_m^B}{V}$	2.60×10^{-2} mM	1.25×10^{-2}

Calculations were performed using values expressed to 6 significant figures. The final values of the parameters are expressed to 3 significant figures.

Table 8.

Calculation of the kinetic constants in equation (2) using values and relationships in Table 7.

Kinetic constant	Method of calculation	Value (mM)
K_s^A	$\frac{(2)}{(6)}$	60.2×10^{-3}
K_s^A	$\frac{(4)}{(8)}$	214×10^{-3}
K_m^A	$\frac{(3)}{(7)}$	48.9×10^{-3}
K_s^B	$\frac{(6)}{(5)}$	10.4
K_m^B	$\frac{(8)}{(7)}$	31.4×10^{-3}
K_m^C	$\frac{(5)}{(7)}$	0.0633×10^{-3}
V	$\frac{1}{(7)}$	1.21 (dimensionless ratio)

The numbers in parentheses refer to the values of the parameters listed in Table 7. Values expressed to 6 significant figures were used in calculation and the final values for the kinetic constants then expressed to 3 significant figures.

Table 9.

Values of the coefficients in equation (5) obtained by fitting the initial-rate data to this rate equation.

	Unweighted fit		Weighted fit	
Coefficient	Value	Standard error	Value	Standard error
(coef ABC)'	0.776	0.0194	0.857	0.0232
(coef AB)'	0.0639×10^{-3} mM	0.00546×10^{-3}	0.0402×10^{-3} mM	0.00432×10^{-3}
(coef AC)'	32.0×10^{-3} mM	6.44×10^{-3}	21.2×10^{-3} mM	7.04×10^{-3}
(coef BC)'	37.1×10^{-3} mM	1.34×10^{-3}	37.1×10^{-3} mM	0.891×10^{-3}
(coef A)'	0.560 μM mM	0.0347	0.556 μM mM	0.0295
(coef C)'	6.24 μM mM	0.576	6.16 μM mM	0.428
constant'	31.0 μM^2 mM	2.64	31.1 μM^2 mM	1.29
Variance	0.001137		0.000360	
Sigma	0.033716		0.018982	

For the weighted fit to the rate equation the weight for each data point was proportional to $1/v^2$ such that the sum of the weights was equal to the number of data points. This method of weighting is that applicable where there is a constant percentage error in v.

Table 10.

Calculation of the kinetic constants in equation (2) using values of coefficients in Table 9.

Kinetic constant	Method of calculation	Unweighted fit		Weighted fit	
		Value (mM)	Standard error	Value (mM)	Standard error
K_s^A	$\frac{(\text{coef C})'}{(\text{coef AC})'}$	195×10^{-3}	54.7×10^{-3}	290×10^{-3}	112×10^{-3}
K_s^A	$\frac{\text{constant}'}{(\text{coef A})'}$	55.3×10^{-3}	7.68×10^{-3}	56.0×10^{-3}	4.85×10^{-3}
K_m^A	$\frac{(\text{coef BC})'}{(\text{coef ABC})'}$	47.8×10^{-3}	2.77×10^{-3}	43.3×10^{-3}	2.04×10^{-3}
K_s^B	$\frac{(\text{coef A})'}{(\text{coef AB})'}$	8.77	0.973	13.8	1.77
K_m^B	$\frac{(\text{coef AC})'}{(\text{coef ABC})'}$	41.2×10^{-3}	8.77×10^{-3}	24.8×10^{-3}	8.52×10^{-3}
K_m^C	$\frac{(\text{coef AB})'}{(\text{coef ABC})'}$	0.0824×10^{-3}	0.00762×10^{-3}	0.0469×10^{-3}	0.00546×10^{-3}
V	$\frac{1}{(\text{coef ABC})'}$	1.29 (dimensionless ratio)	0.0323	1.17 (dimensionless ratio)	0.0316

Values expressed to 5 significant figures were used in calculation and the final values of the kinetic constants then expressed to 3 significant figures.

Table 11.

Slope and ordinate intercept values of secondary plots from the product inhibition experiments and their kinetic identities according to the rate equation (6).

Secondary plot	Parameter	Kinetic identity	Value	Standard error
Fig. 33				
(9)	Intercept	$\frac{K_m^A}{V} + \frac{K_s^A K_m^B}{VB} + \frac{K_s^A K_s^B K_m^C}{VBC}$	1.95×10^{-1} mM	2.89×10^{-2}
(10)	Slope	$\frac{K_s^A K_s^B K_m^C}{VBC K_i^Q} + \frac{K_s^A K_m^B}{VB K_i^Q} + \frac{K_m^A}{V K_i^Q}$	2.62×10^{-1}	3.92×10^{-2}
Fig. 34				
(11)	Intercept	$\frac{1}{V} + \frac{K_m^B}{VB} + \frac{K_m^C}{VC} + \frac{K_s^B K_m^C}{VBC}$	1.30	3.58×10^{-2}
(12)	Slope	None	-8.01×10^{-3} mM^{-1}	4.84×10^{-2}

Table 11 (contd).

Secondary plot	Parameter	Kinetic identity	Value	Standard error
Fig. 36				
(13)	Intercept	$\frac{K_m^C}{V} + \frac{K_s^B K_m^C}{VB} + \frac{K_s^A K_s^B K_m^C}{VAB}$	1.26×10^{-3} mM	3.65×10^{-4}
(14)	Slope	$\frac{K_s^A K_s^B K_m^C}{VAB K_i^Q}$	2.70×10^{-3}	4.60×10^{-4}
Fig. 37				
(15)	Intercept	$\frac{1}{V} + \frac{K_m^A}{VA} + \frac{K_m^B}{VB} + \frac{K_s^A K_m^B}{VAB}$	1.88	1.01×10^{-1}
(16)	Slope	$\frac{K_s^A K_m^B}{VAB K_i^Q} + \frac{K_m^A}{VA K_i^Q}$	7.16×10^{-1} mM^{-1}	1.28×10^{-1}

Table 11 (contd).

Secondary plot	Parameter	Kinetic identity	Value	Standard error
Fig. 39				
(17)	Intercept	$\frac{K_m^A}{V} + \frac{K_s^A K_m^B}{VB} + \frac{K_s^A K_s^B K_m^C}{VBC}$	6.86×10^{-2} mM	4.28×10^{-3}
(18)	Slope	$\frac{K_m^Q K_s^A K_s^B K_m^C}{VBC K_m^P K_i^Q}$	1.13×10^{-1}	7.45×10^{-3}
Fig. 40				
(19)	Intercept	$\frac{1}{V} + \frac{K_m^B}{VB} + \frac{K_m^C}{VC} + \frac{K_s^B K_m^C}{VBC}$	1.66	8.38×10^{-2}
(20)	Slope	$\frac{K_m^Q K_s^B K_m^C}{VBC K_m^P K_i^Q} + \frac{K_m^Q K_m^C}{VC K_m^P K_i^Q} + \frac{K_m^Q K_m^C}{V K_s^C K_m^P K_i^Q}$	1.55 mM^{-1}	1.46×10^{-1}

Table 11 (contd).

Secondary plot	Parameter	Kinetic identity	Value	Standard error
Fig. 42				
(21)	Intercept	$\frac{K_m^C}{V} + \frac{K_s^B K_m^C}{VB} + \frac{K_s^A K_s^B K_m^C}{VAB}$	1.54×10^{-3} mM	2.79×10^{-4}
(22)	Slope	$\frac{K_m^Q K_s^A K_s^B K_m^C}{VAB K_m^P K_i^Q} + \frac{K_m^Q K_s^B K_m^C}{VB K_m^P K_i^Q} + \frac{K_m^Q K_m^C}{V K_m^P K_i^Q}$	3.31×10^{-3}	4.33×10^{-4}
Fig. 43				
(23)	Intercept	$\frac{1}{V} + \frac{K_m^A}{VA} + \frac{K_m^B}{VB} + \frac{K_s^A K_m^B}{VAB}$	8.79×10^{-1}	3.57×10^{-2}
(24)	Slope	$\frac{K_m^Q K_m^C}{V K_s^C K_m^P K_i^Q}$	6.06×10^{-1} mM^{-1}	5.54×10^{-2}

Calculations were performed using values expressed to 6 significant figures. The final values of the parameters are expressed to 3 significant figures.

Table 12.

Calculation of the kinetic constants for products using values and relationships in Table 11.

Kinetic constant	Method of calculation	Value (mM)	Standard error
K_i^Q	$\frac{(9)}{(10)}$	0.744	0.157

The numbers in parentheses refer to the values of the parameters listed in Table 11. Values expressed to 6 significant figures were used in calculation and the final value of the kinetic constant then expressed to 3 significant figures.

Table 13.

Fit of the initial-rate data to equation (5) with a (coef AB^2C)' AB^2C term added to the denominator.

	Unweighted fit		Weighted fit	
Coefficient	Value	Standard error	Value	Standard error
(coef AB^2C)'	0.885×10^{-3} mM	3.47×10^{-3}	-7.07×10^{-3} mM	3.74×10^{-3}
(coef ABC)'	0.775	0.0197	0.875	0.0250
(coef AB)'	0.0622×10^{-3} mM	0.00833×10^{-3}	0.0485×10^{-3} mM	0.00625×10^{-3}
(coef AC)'	32.0×10^{-3} mM	6.45×10^{-3}	19.7×10^{-3} mM	7.08×10^{-3}
(coef BC)'	37.0×10^{-3} mM	1.44×10^{-3}	37.6×10^{-3} mM	0.924×10^{-3}
(coef A)'	0.561 μM mM	0.0349	0.543 μM mM	0.0302
(coef C)'	6.25 μM mM	0.581	6.09 μM mM	0.429
constant'	31.2 μM^2 mM	2.74	30.6 μM^2 mM	1.31
Variance	0.001138		0.000359	
Sigma	0.033742		0.018947	

Table 14.

Fit of the initial-rate data to equation (5) with the (coef AC)' AC term omitted from the denominator.

	Unweighted fit		Weighted fit	
Coefficient	Value	Standard error	Value	Standard error
(coef ABC)'	0.813	0.0181	0.887	0.0213
(coef AB)'	0.0604×10^{-3} mM	0.00549×10^{-3}	0.0373×10^{-3} mM	0.00424×10^{-3}
(coef BC)'	35.1×10^{-3} mM	1.28×10^{-3}	36.3×10^{-3} mM	0.849×10^{-3}
(coef A)'	0.582 μM mM	0.0349	0.567 μM mM	0.0295
(coef C)'	8.61 μM mM	0.365	7.12 μM mM	0.300
constant'	30.0 μM^2 mM	2.66	31.0 μM^2 mM	1.29
Variance	0.001182		0.000365	
Sigma	0.034380		0.019113	

Table 15.

Values for the dissociation constants used in the calculation of the free Mg^{2+} concentration.

Dissociation constant	Value (M)	Reaction	Reference
K_1	8.55×10^{-8}	$ATP^{4-} + H^+ \rightleftharpoons HATP^{3-}$	Phillips et al. (1966)
K_2	2.50×10^{-5}	$ATP^{4-} + Mg^{2+} \rightleftharpoons MgATP^{2-}$	"
K_3	1.59×10^{-3}	$HATP^{3-} + Mg^{2+} \rightleftharpoons MgHATP^{-}$	"
K_4	5.44×10^{-6}	$MgATP^{2-} + H^+ \rightleftharpoons MgHATP^{-}$	"
K_5	7.1×10^{-2}	$ATP^{4-} + K^+ \rightleftharpoons KATP^{3-}$	Smith & Alberty (1956)
K_6	1.47×10^{-7}	$ADP^{3-} + H^+ \rightleftharpoons HADP^{2-}$	Phillips et al. (1966)
K_7	3.32×10^{-4}	$ADP^{3-} + Mg^{2+} \rightleftharpoons MgADP^{-}$	"
K_8	1.09×10^{-2}	$HADP^{2-} + Mg^{2+} \rightleftharpoons MgHADP$	"
K_9	4.83×10^{-6}	$MgADP^{-} + H^+ \rightleftharpoons MgHADP$	"
K_{10}	1.81×10^{-1}	$ADP^{3-} + K^+ \rightleftharpoons KADP^{2-}$	Smith & Alberty (1956)

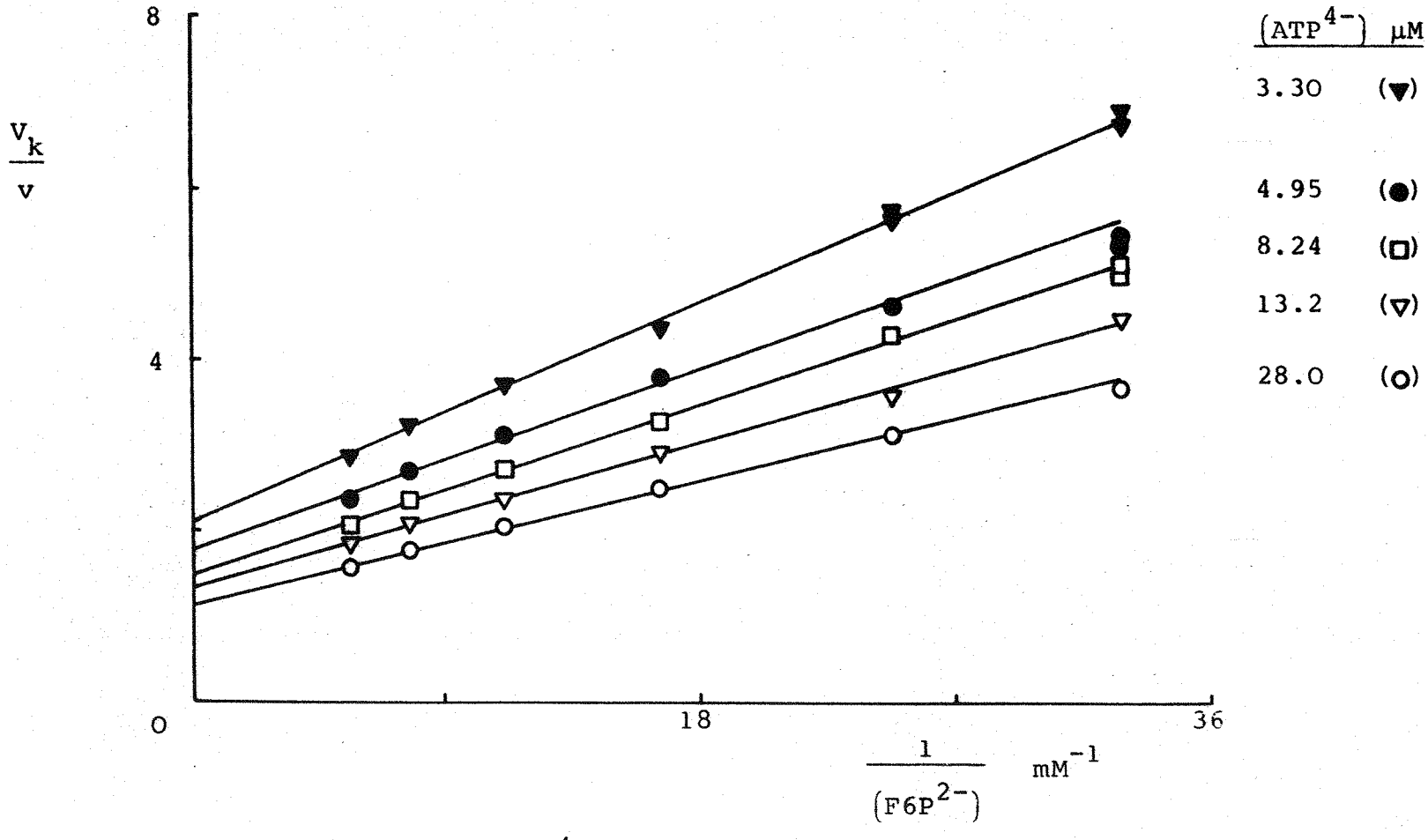
Table 15 (contd).

Dissociation constant	Value (M)	Reaction	Reference
K_{11}	9.52×10^{-3}	$F6P^{2-} + Mg^{2+} \rightleftharpoons MgF6P$	Hanson et al. (1973)
K_{12}	1.7×10^{-7}	$FDP^{4-} + H^{+} \rightleftharpoons HFDP^{3-}$	McGilvery (1965)
K_{13}	1.1×10^{-6}	$HFDP^{3-} + H^{+} \rightleftharpoons H_2FDP^{2-}$	"
K_{14}	1.8×10^{-3}	$FDP^{4-} + Mg^{2+} \rightleftharpoons MgFDP^{2-}$	"
K_{15}	7.1×10^{-3}	$HFDP^{3-} + Mg^{2+} \rightleftharpoons MgHFDP^{-}$	"
K_{16}	6.7×10^{-7}	$MgFDP^{2-} + H^{+} \rightleftharpoons MgHFDP^{-}$	"
K_{17}	4.47×10^{-7}	$Pyr\text{-}P^{3-} + H^{+} \rightleftharpoons HPyr\text{-}P^{2-}$	Wold & Ballou (1957)
K_{18}	5.50×10^{-3}	$Pyr\text{-}P^{3-} + Mg^{2+} \rightleftharpoons MgPyr\text{-}P^{-}$	"
K_{19}	8.32×10^{-2}	$Pyr\text{-}P^{3-} + K^{+} \rightleftharpoons KPyr\text{-}P^{2-}$	"

The values of the dissociation constants are those at 25°C and an ionic strength of 0.2 except those for the FDP equilibria which are at an ionic strength of 0.1. The variation in the ionic strength of the phosphofructokinase assay due to varying the substrate and free Mg^{2+} concentrations was not great enough to significantly alter the values of the dissociation constants.

Figure 11.

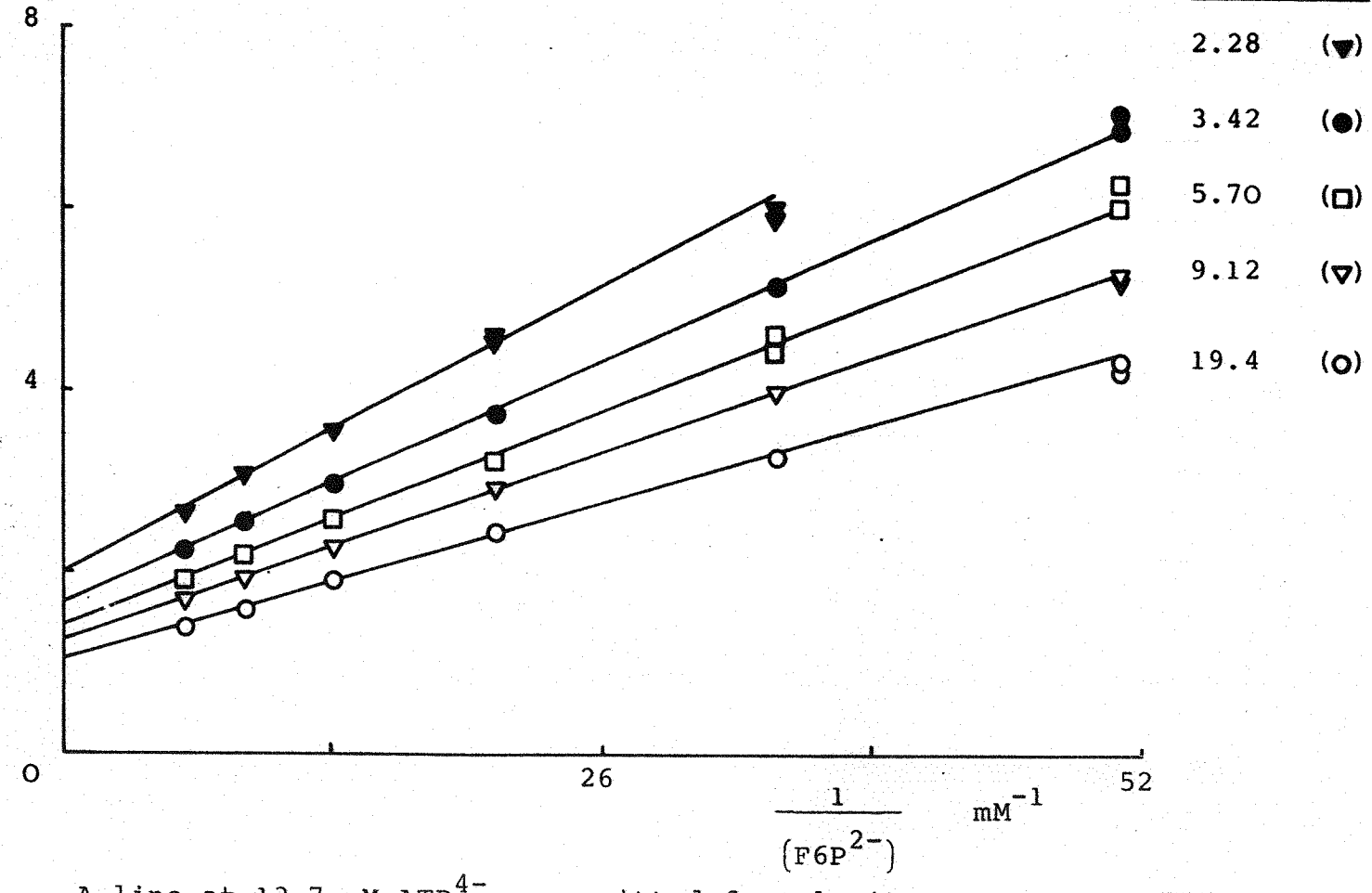
Lineweaver-Burk plot at 0.15 mM free Mg^{2+}.



A line at 19.8 µM ATP^{4-} was omitted for clarity.

Figure 12.

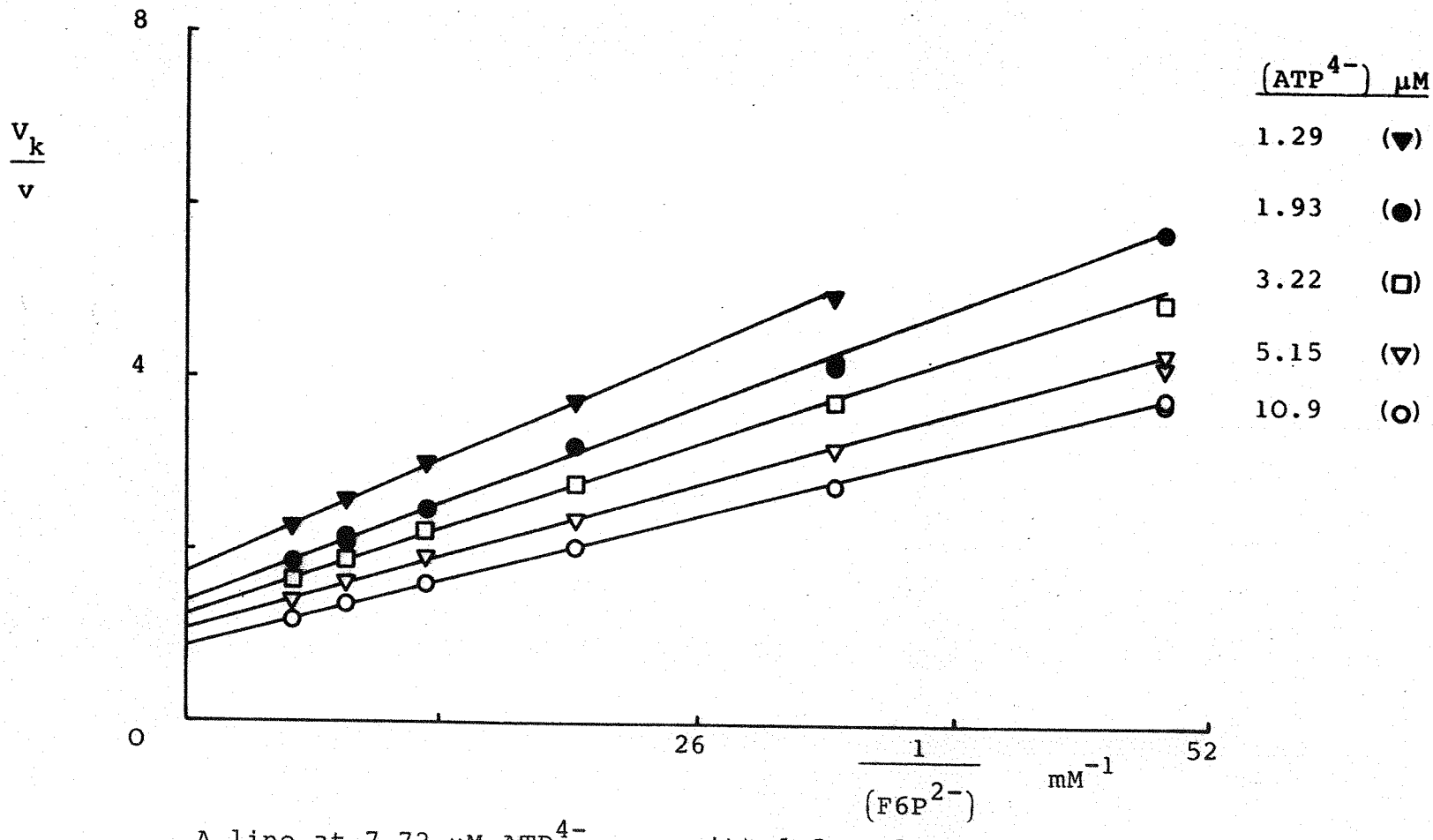
Lineweaver-Burk plot at 0.25 mM free Mg^{2+}.



A line at 13.7 μM ATP^{4-} was omitted for clarity.

Figure 13.

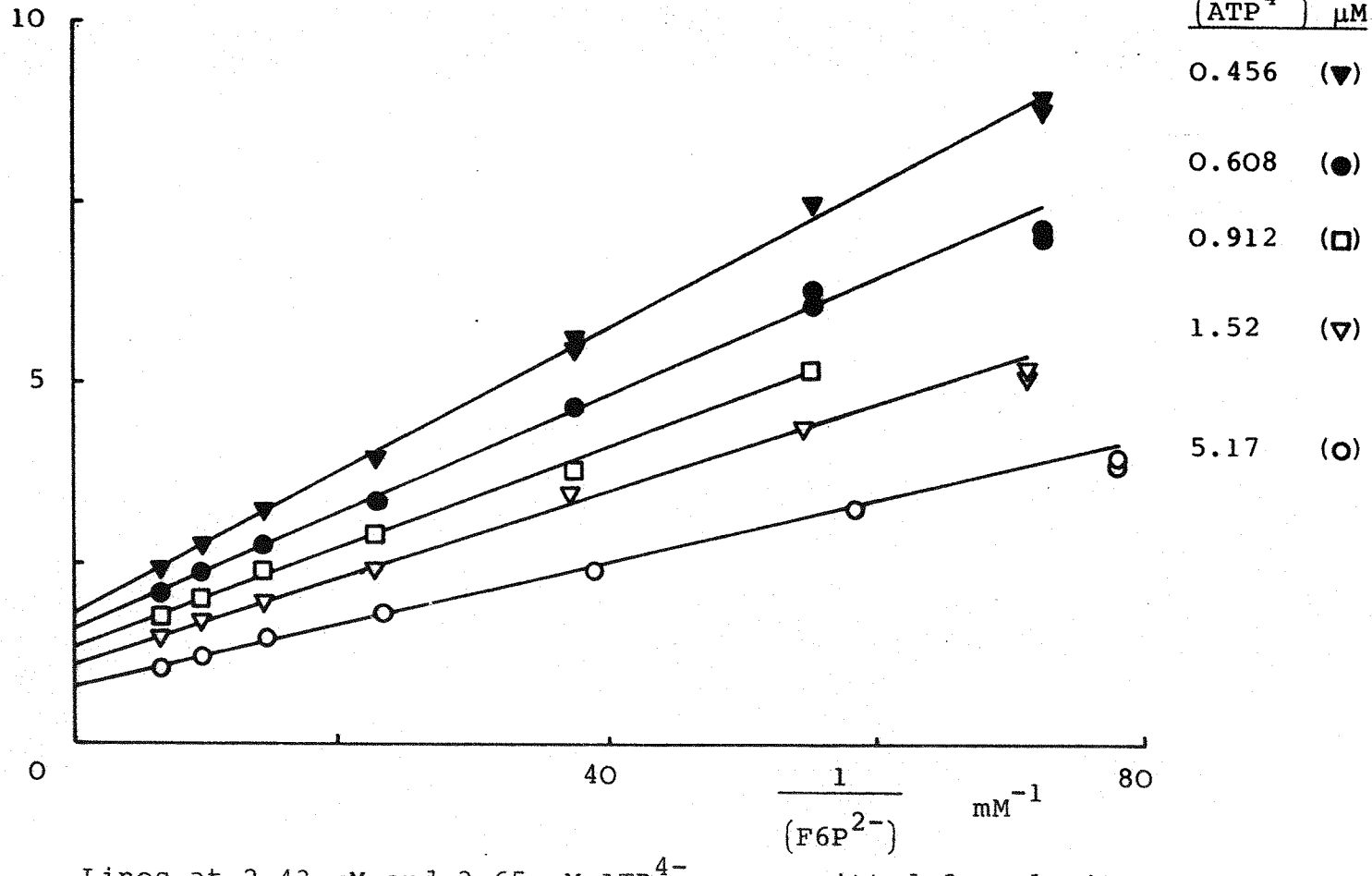
Lineweaver-Burk plot at 0.5 mM free Mg^{2+}.



A line at 7.72 µM ATP^{4-} was omitted for clarity.

Figure 14.

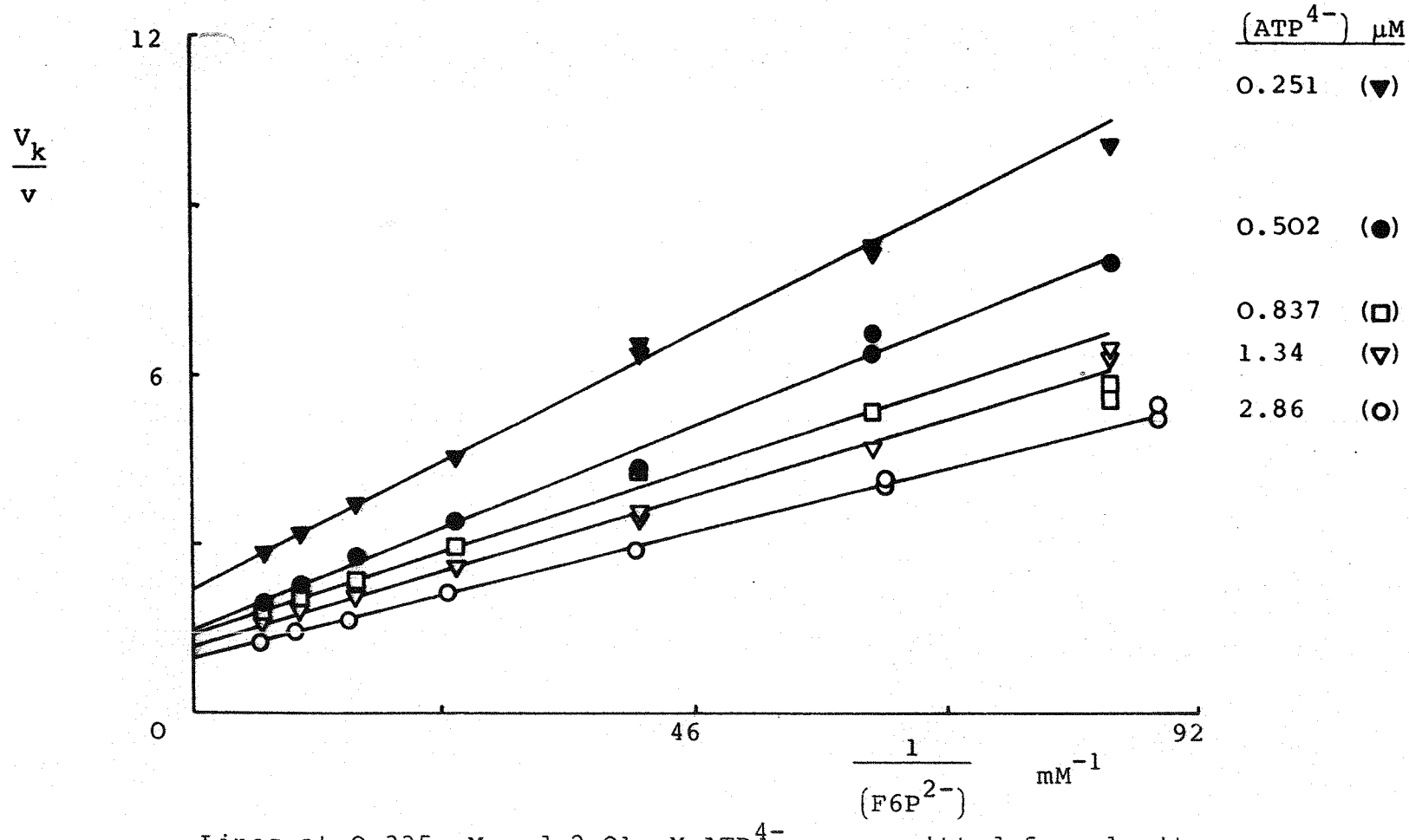
Lineweaver-Burk plot at 1 mM free Mg^{2+}.



Lines at 2.43 μM and 3.65 μM ATP^{4-} were omitted for clarity.

Figure 15.

Lineweaver-Burk plot at 2 mM free Mg^{2+}.



Lines at 0.335 μM and 2.01 μM ATP^{4-} were omitted for clarity.

Figure 16.

Lineweaver-Burk plot at 4 mM free Mg^{2+}.

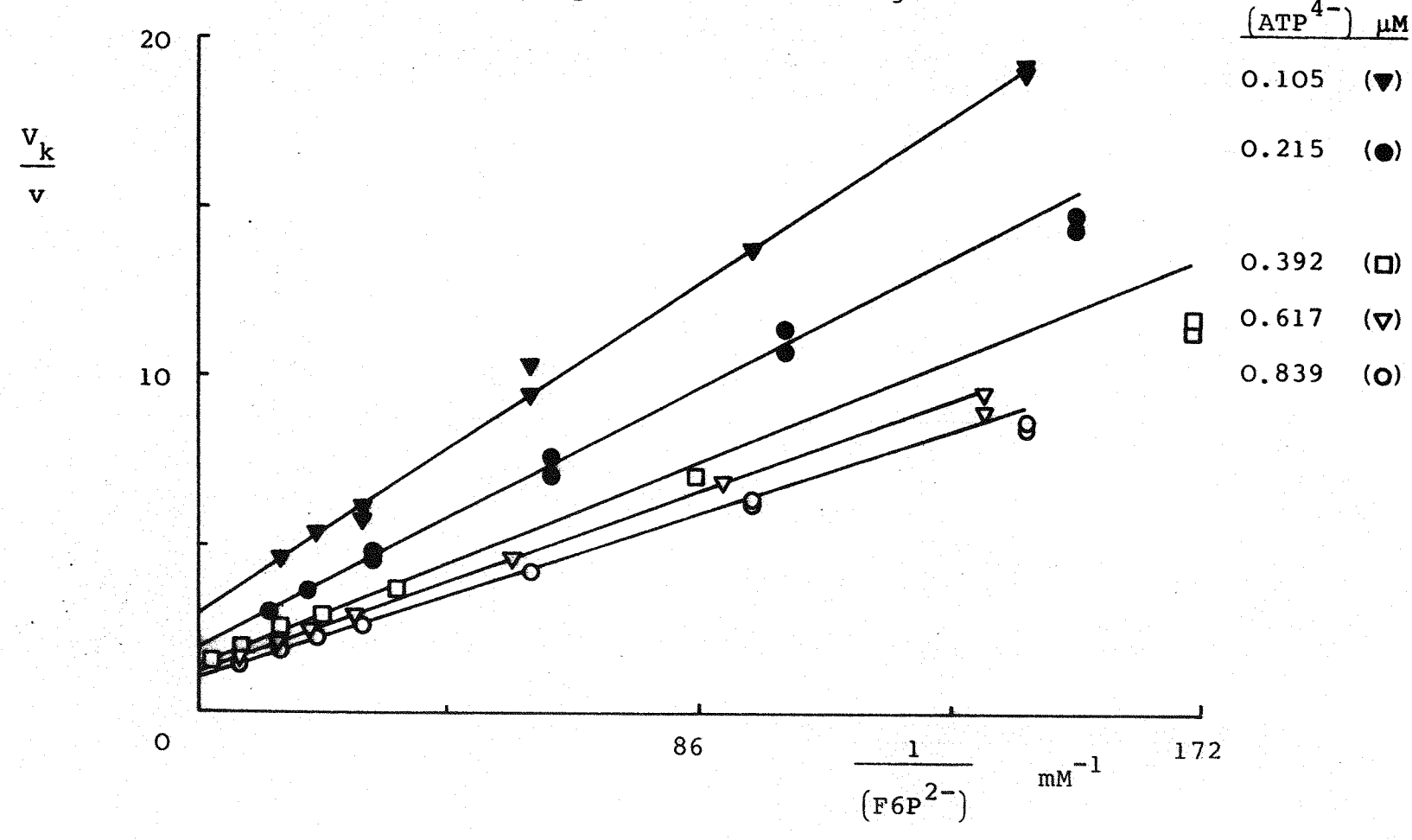
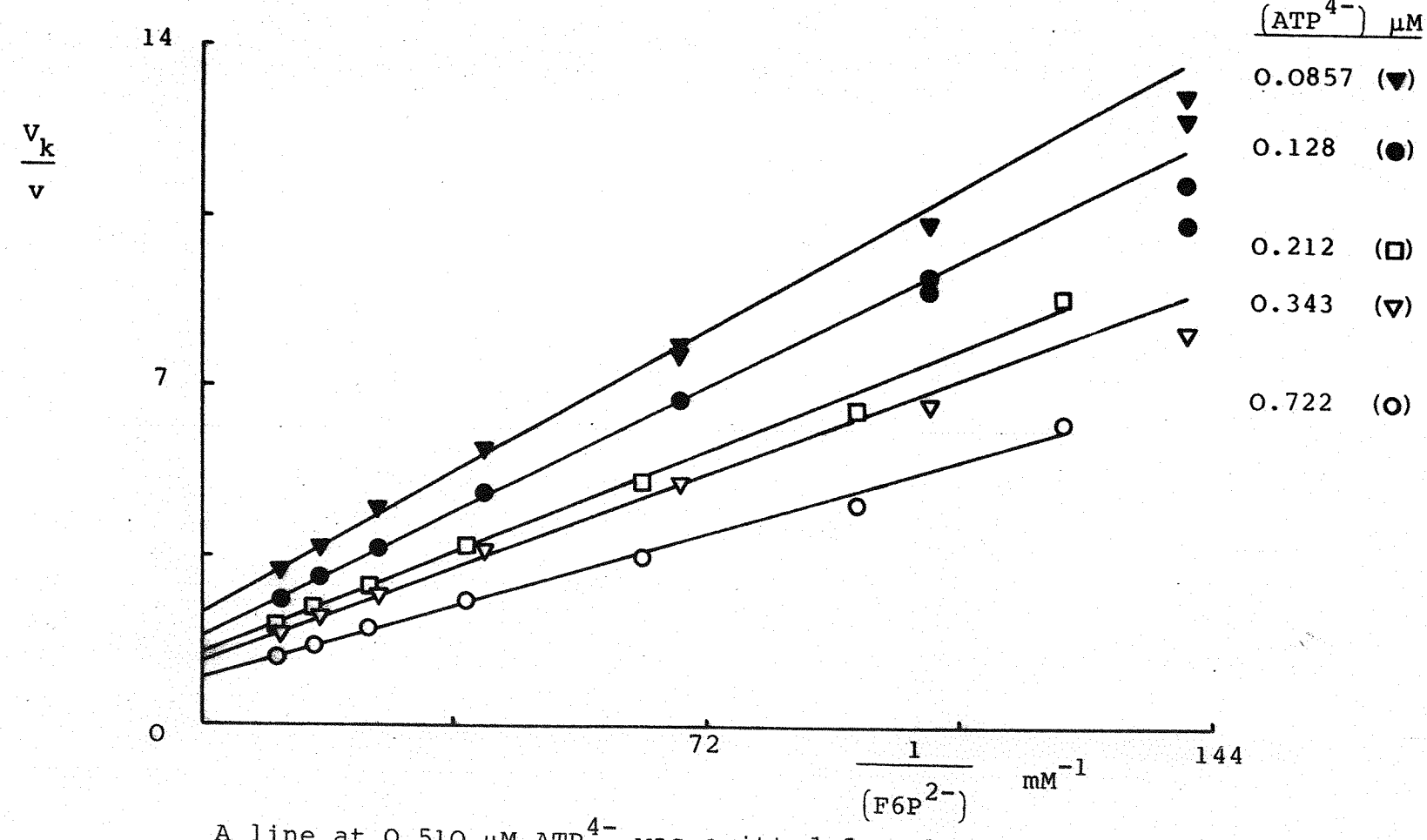


Figure 17.

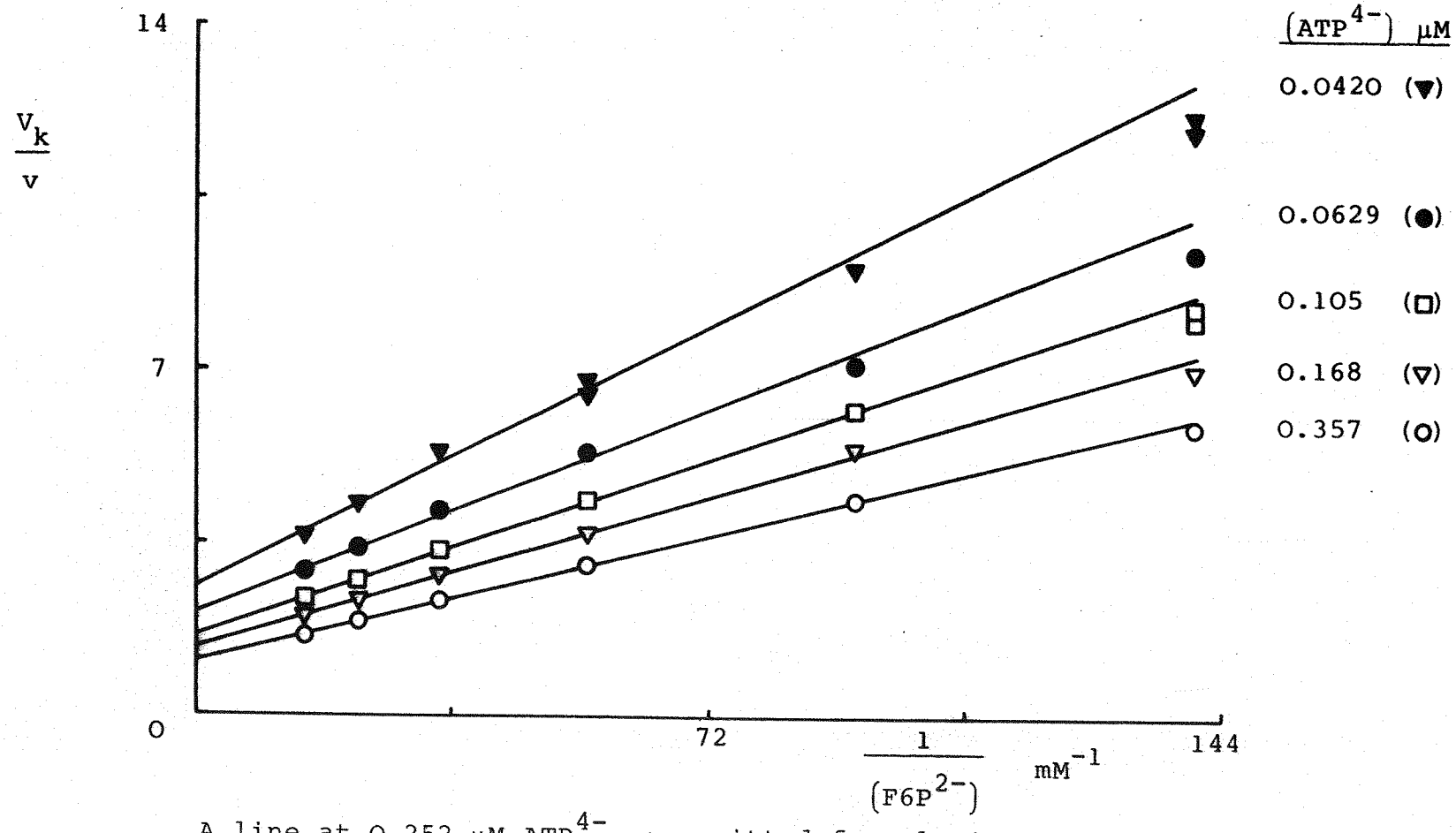
Lineweaver-Burk plot at 8 mM free Mg^{2+}.



A line at 0.510 μM ATP^{4-} was omitted for clarity.

Figure 18.

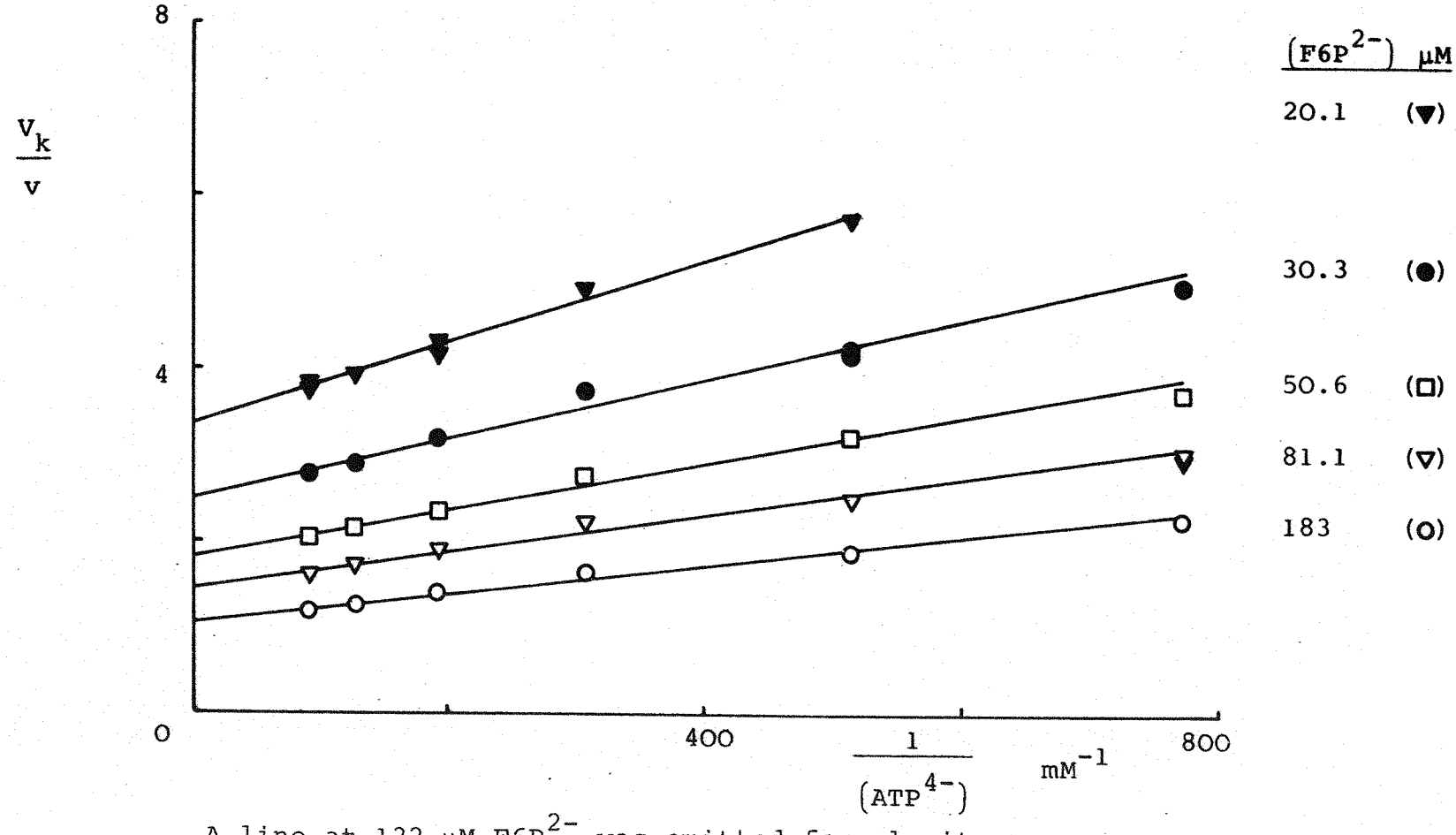
Lineweaver-Burk plot at 16 mM free Mg^{2+}.



A line at 0.252 μM ATP^{4-} was omitted for clarity.

Figure 19.

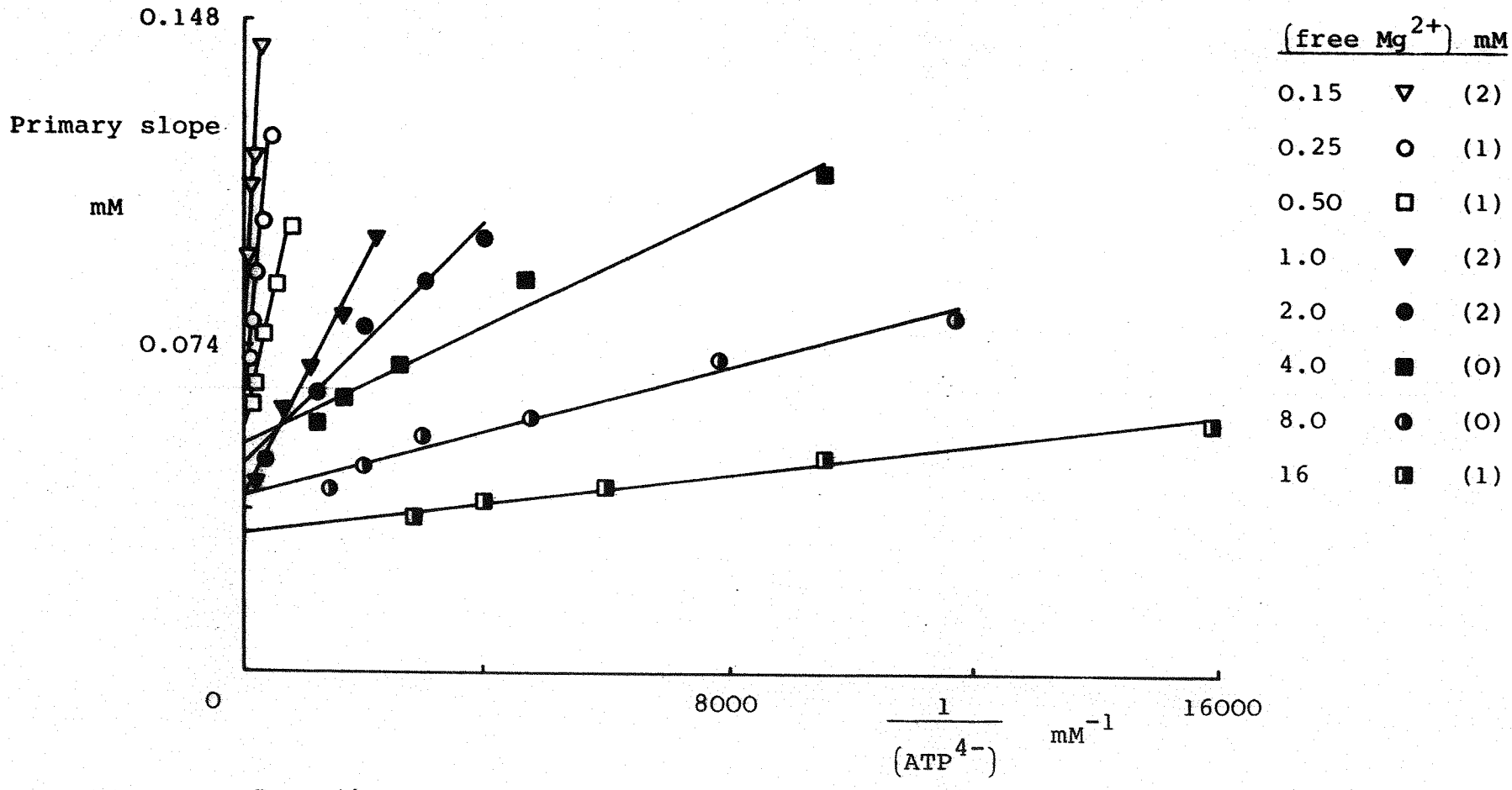
Lineweaver-Burk plot at 0.5 mM free Mg^{2+}.



A line at 122 μM $F6P^{2-}$ was omitted for clarity.

Figure 20.

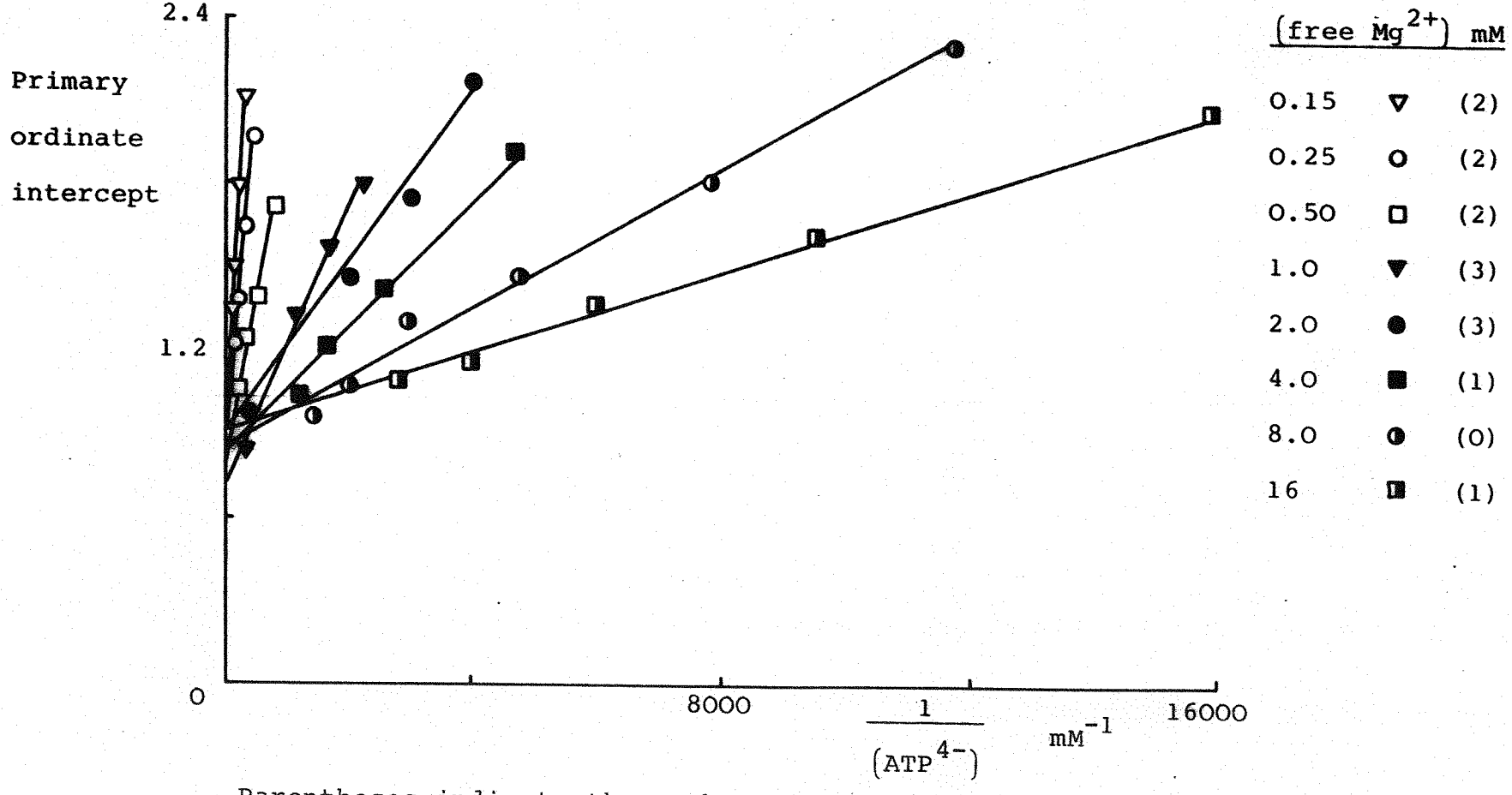
Secondary plot of primary slopes against the reciprocal concentration of ATP^{4-}.



Parentheses indicate the number of points omitted from each line for clarity.

Figure 21.

Secondary plot of primary ordinate intercepts against the reciprocal concentration of ATP^{4-}.



Parentheses indicate the number of points omitted from each line for clarity.

Figure 22.

Tertiary plot of slopes (taken from the secondary plot of primary slopes against the reciprocal concentration of ATP^{4-}) versus the reciprocal concentration of free Mg^{2+}.

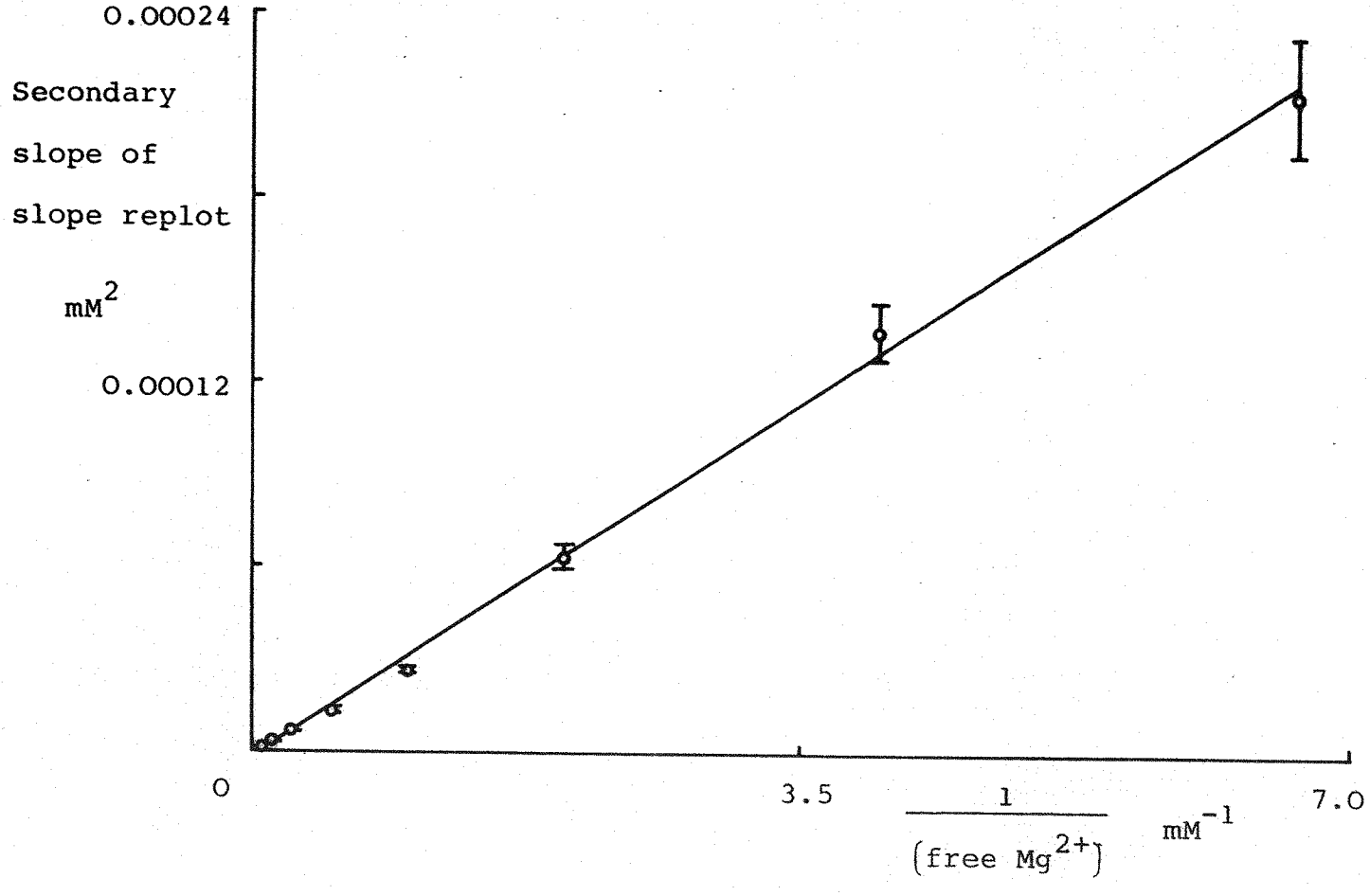


Figure 23.

Tertiary plot of ordinate intercepts (taken from the secondary plot of primary slopes against the reciprocal concentration of ATP^{4-}) versus the reciprocal concentration of free Mg^{2+}.

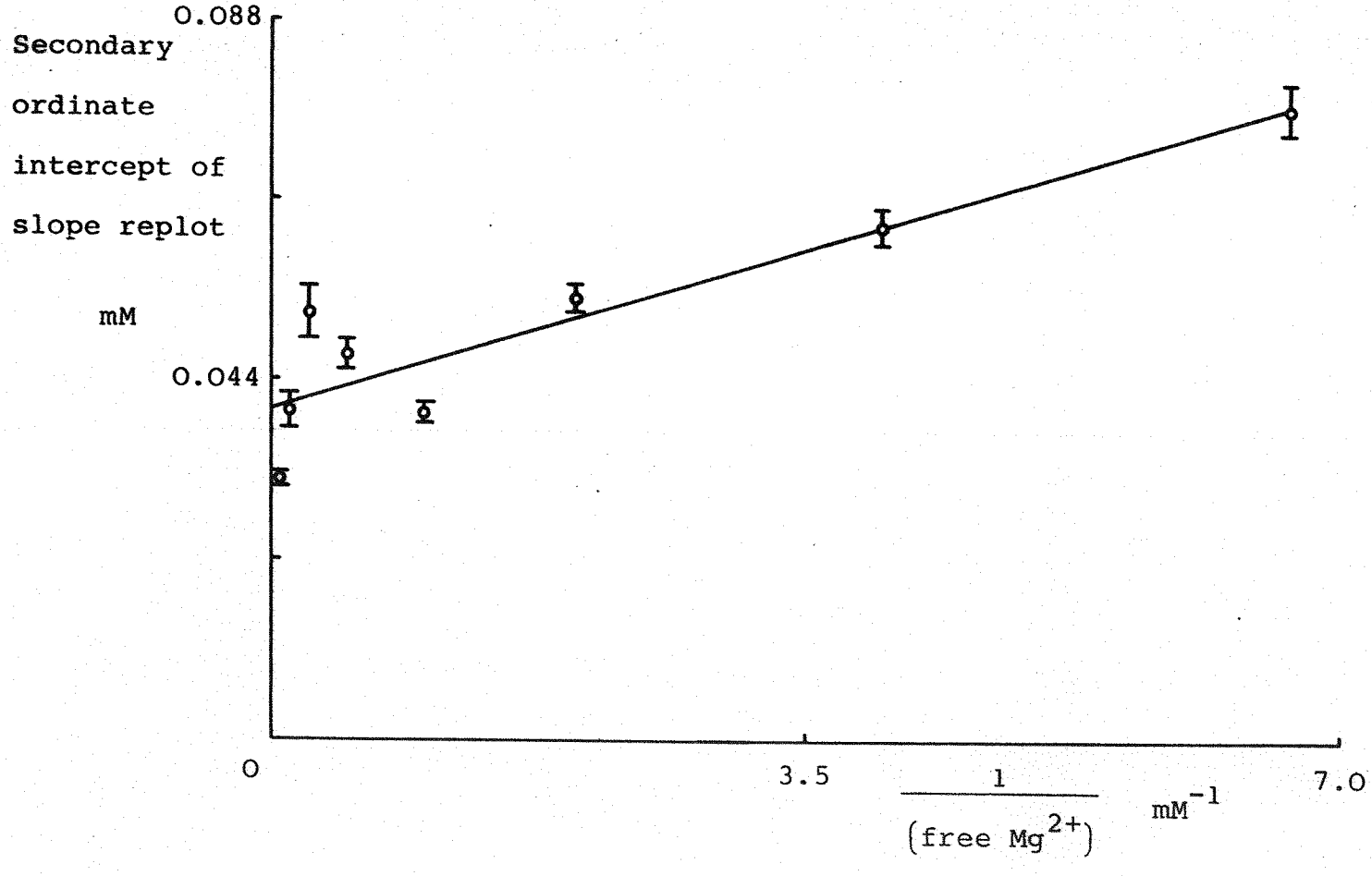


Figure 24.

Tertiary plot of slopes (taken from the secondary plot of primary ordinate intercepts against the reciprocal concentration of ATP^{4-}) versus the reciprocal concentration of free Mg^{2+}.

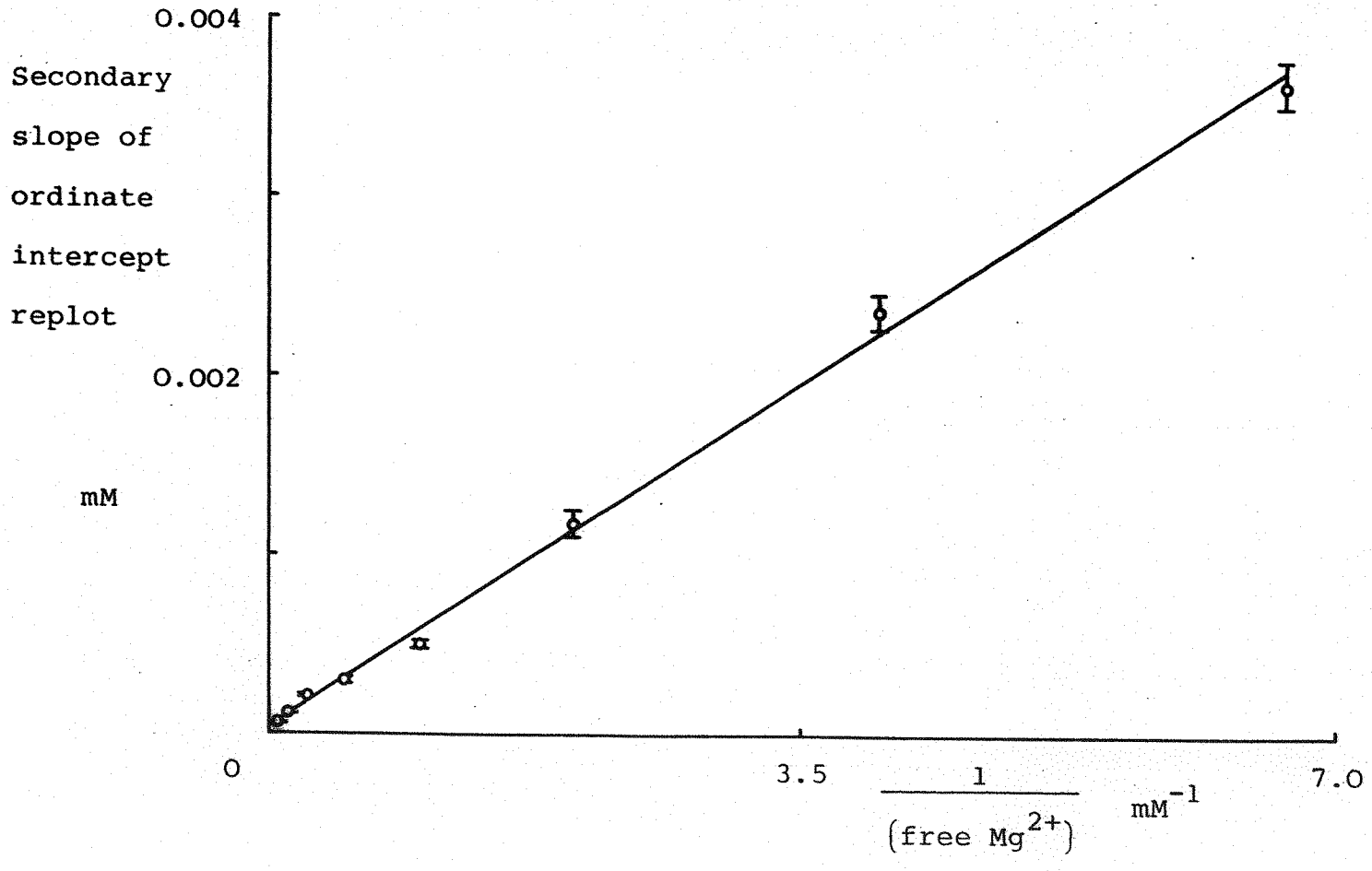


Figure 25.

Tertiary plot of ordinate intercepts (taken from the secondary plot of primary ordinate intercepts against the reciprocal concentration of ATP^{4-}) versus the reciprocal concentration of free Mg^{2+}.

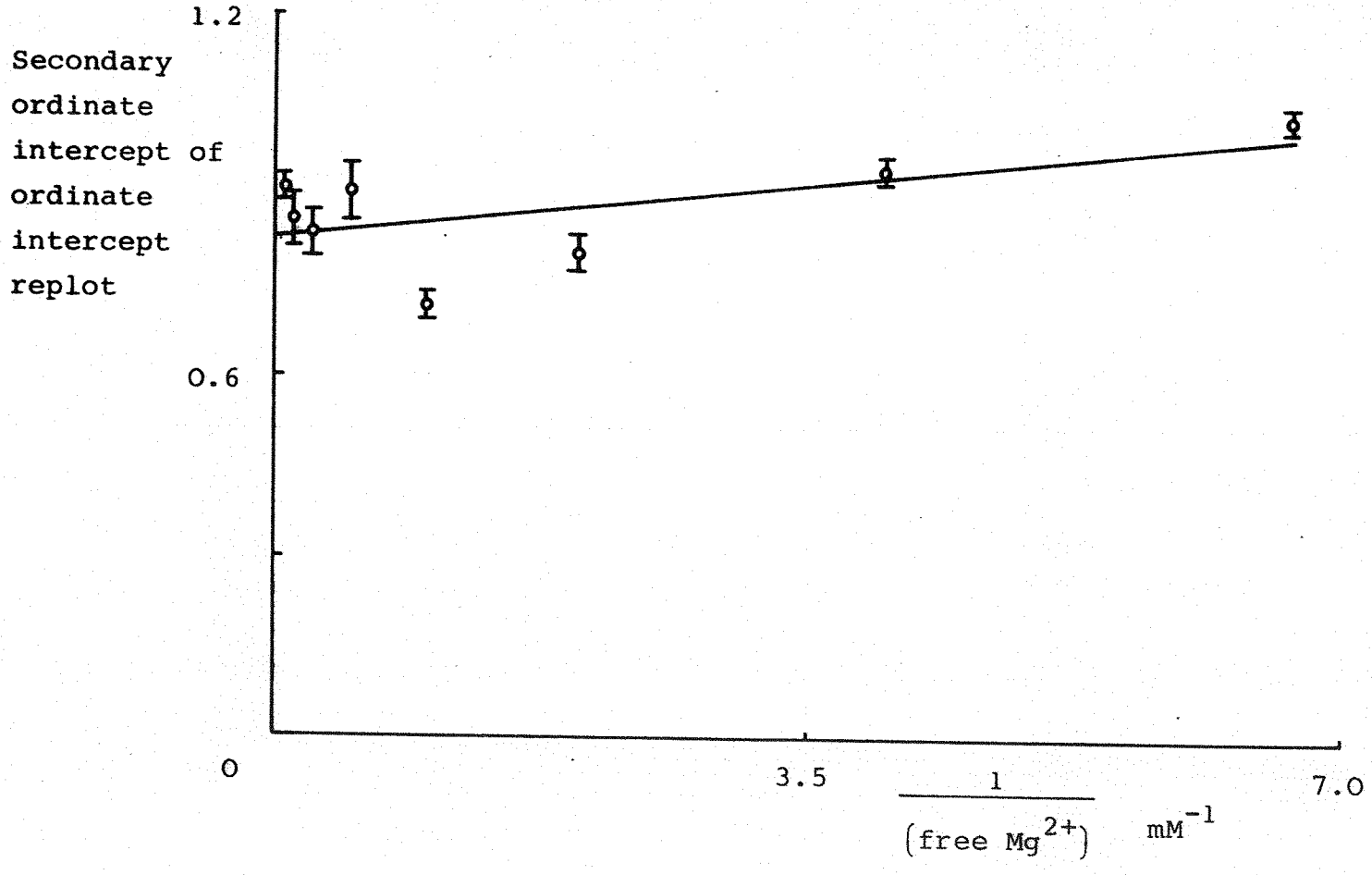
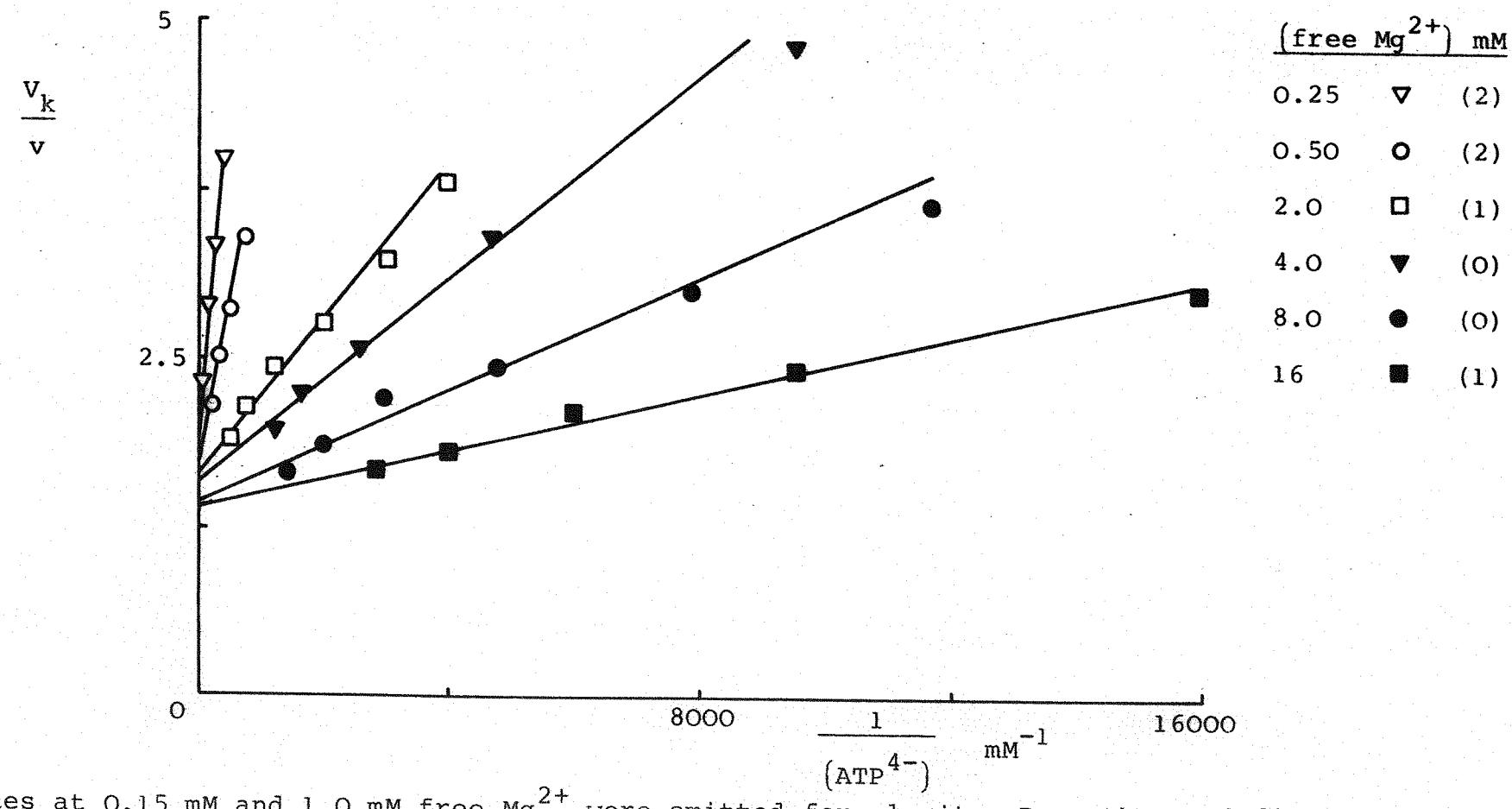


Figure 26.

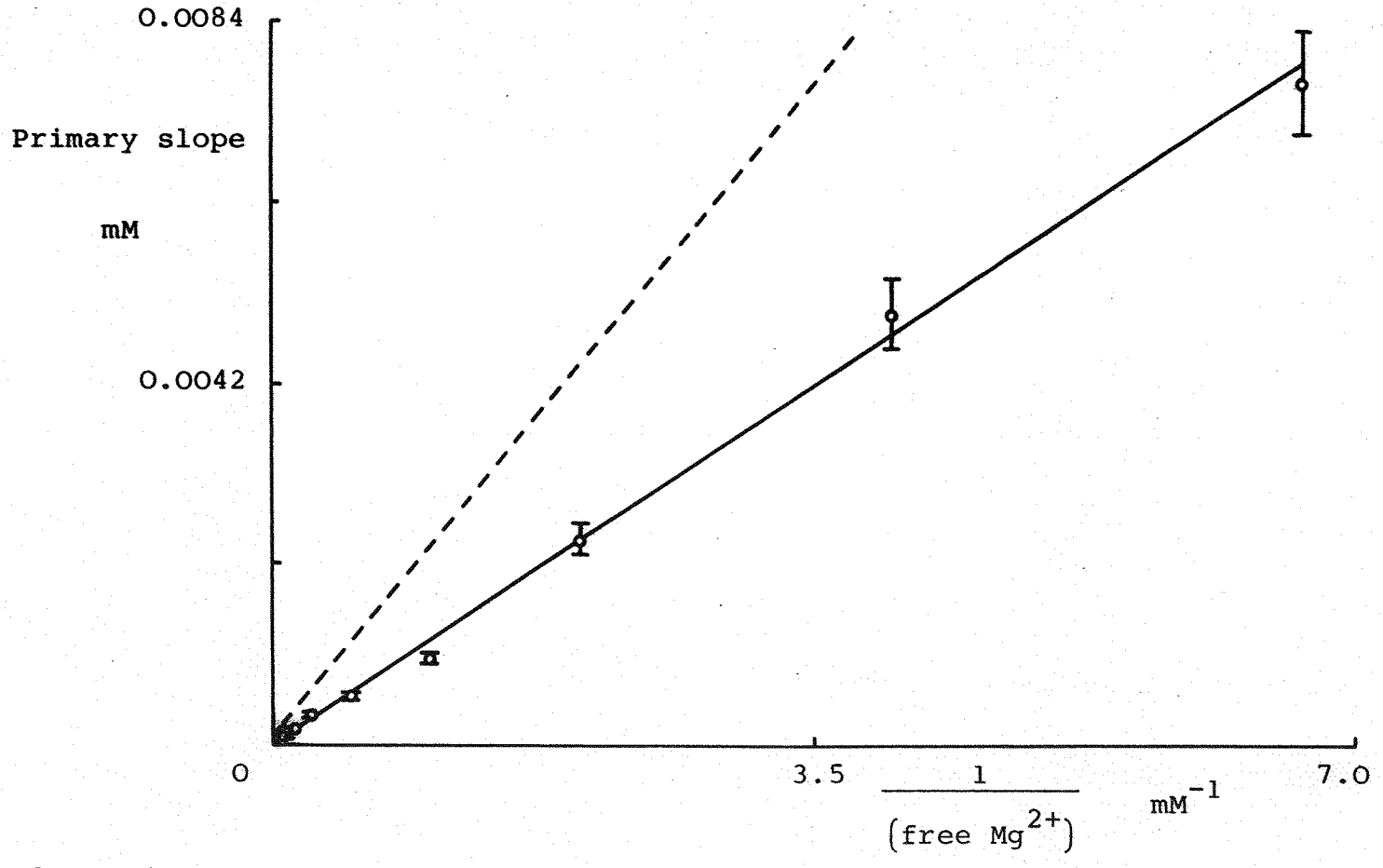
Lineweaver-Burk plot at 60 μM $F6P^{2-}$.



Lines at 0.15 mM and 1.0 mM free Mg^{2+} were omitted for clarity. Parentheses indicate the number of points omitted from each line. The data for each line was interpolated from the appropriate primary plot at constant free Mg^{2+} with $F6P^{2-}$ as variable substrate and ATP^{4-} as changing fixed substrate.

Figure 27.

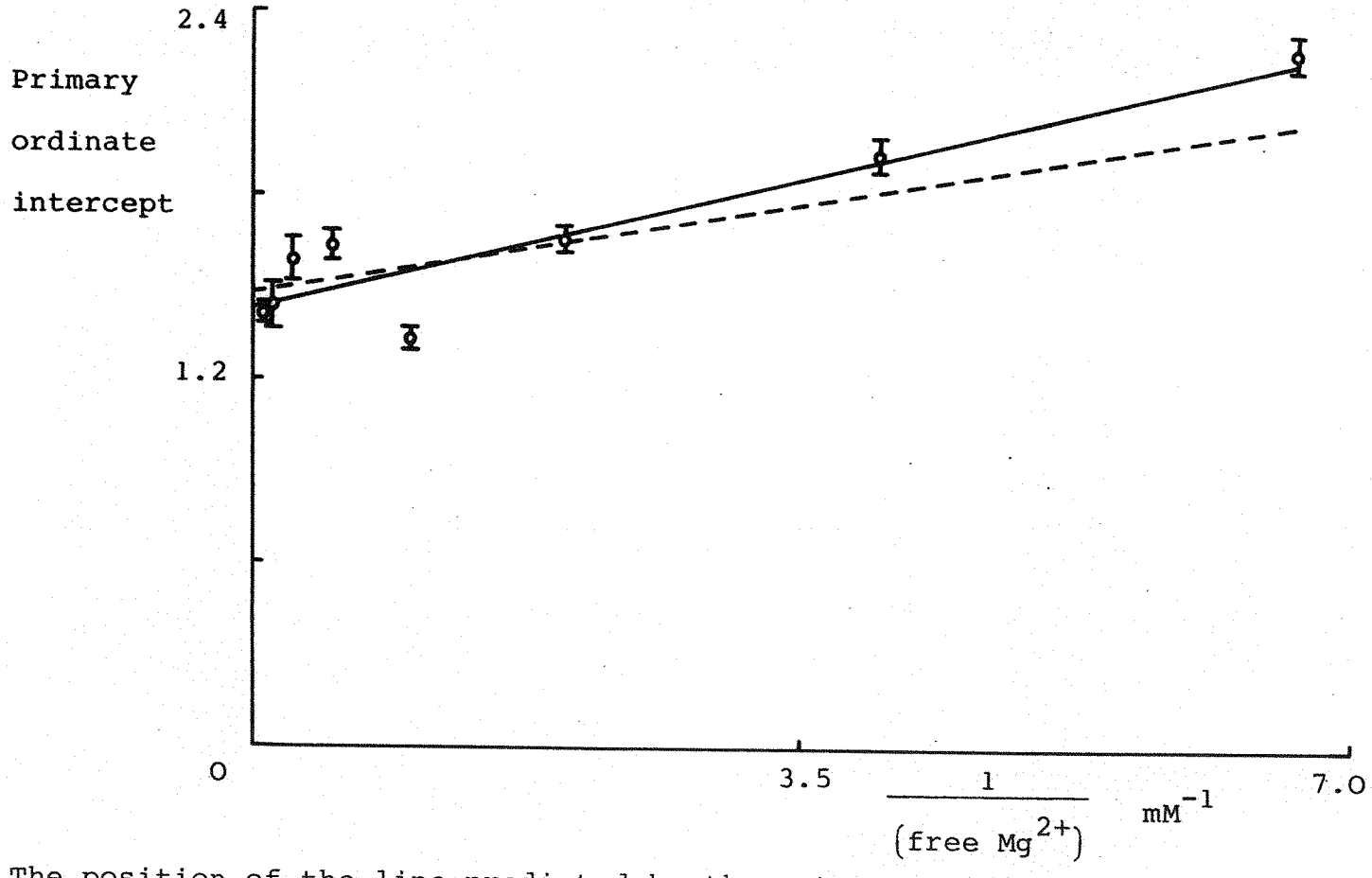
Secondary plot of primary slopes against the reciprocal concentration of free Mg^{2+}. $F6P^{2-}$ was 60 μM.



The position of the line predicted by the rate equation and the values of kinetic constants from Table 10 (weighted fit) is shown in broken type.

Figure 28.

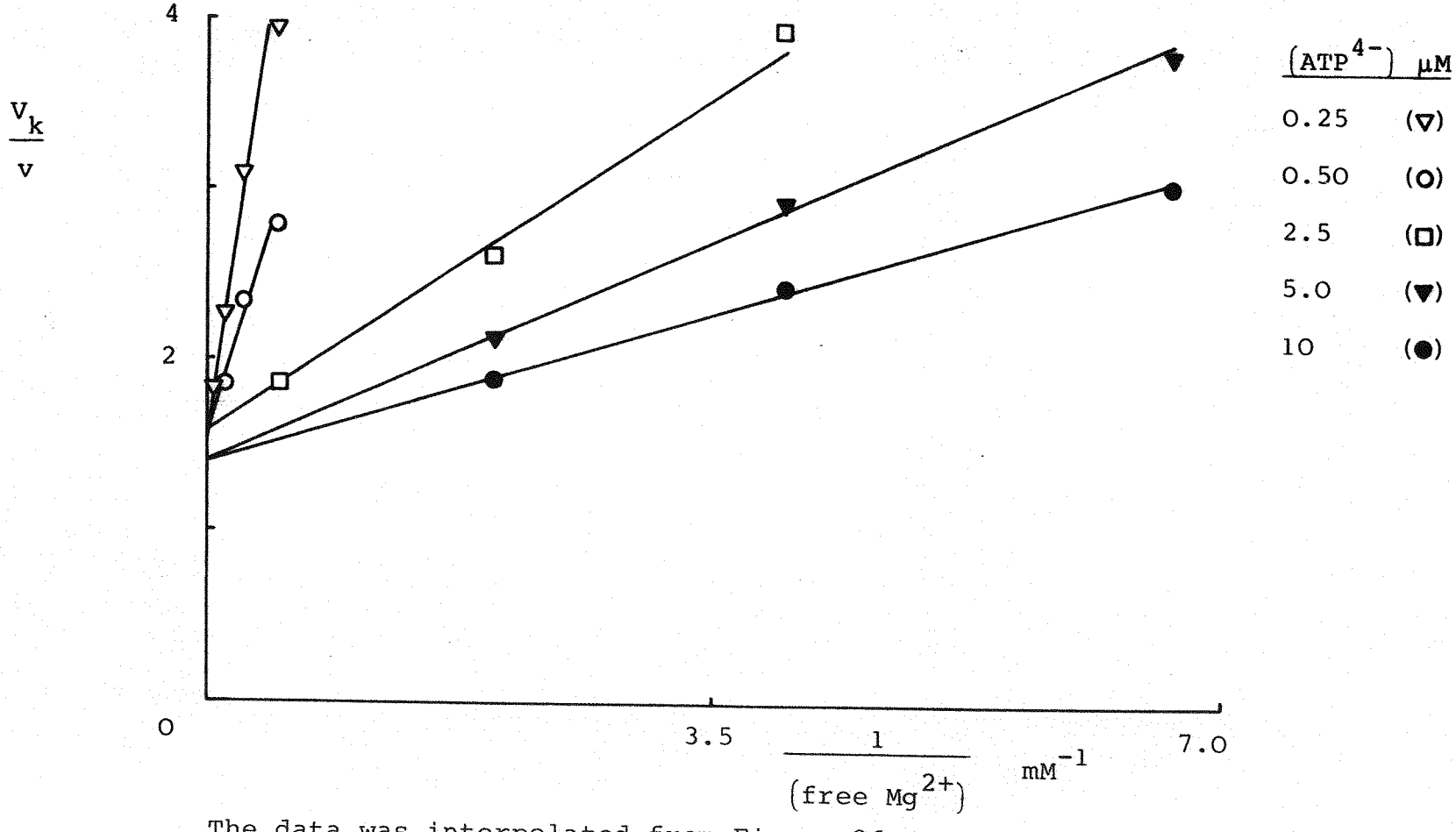
Secondary plot of primary ordinate intercepts against the reciprocal concentration of free Mg^{2+}. $F6P^{2-}$ was 60 μM.



The position of the line predicted by the rate equation and the values of kinetic constants from Table 10 (weighted fit) is shown in broken type.

Figure 29.

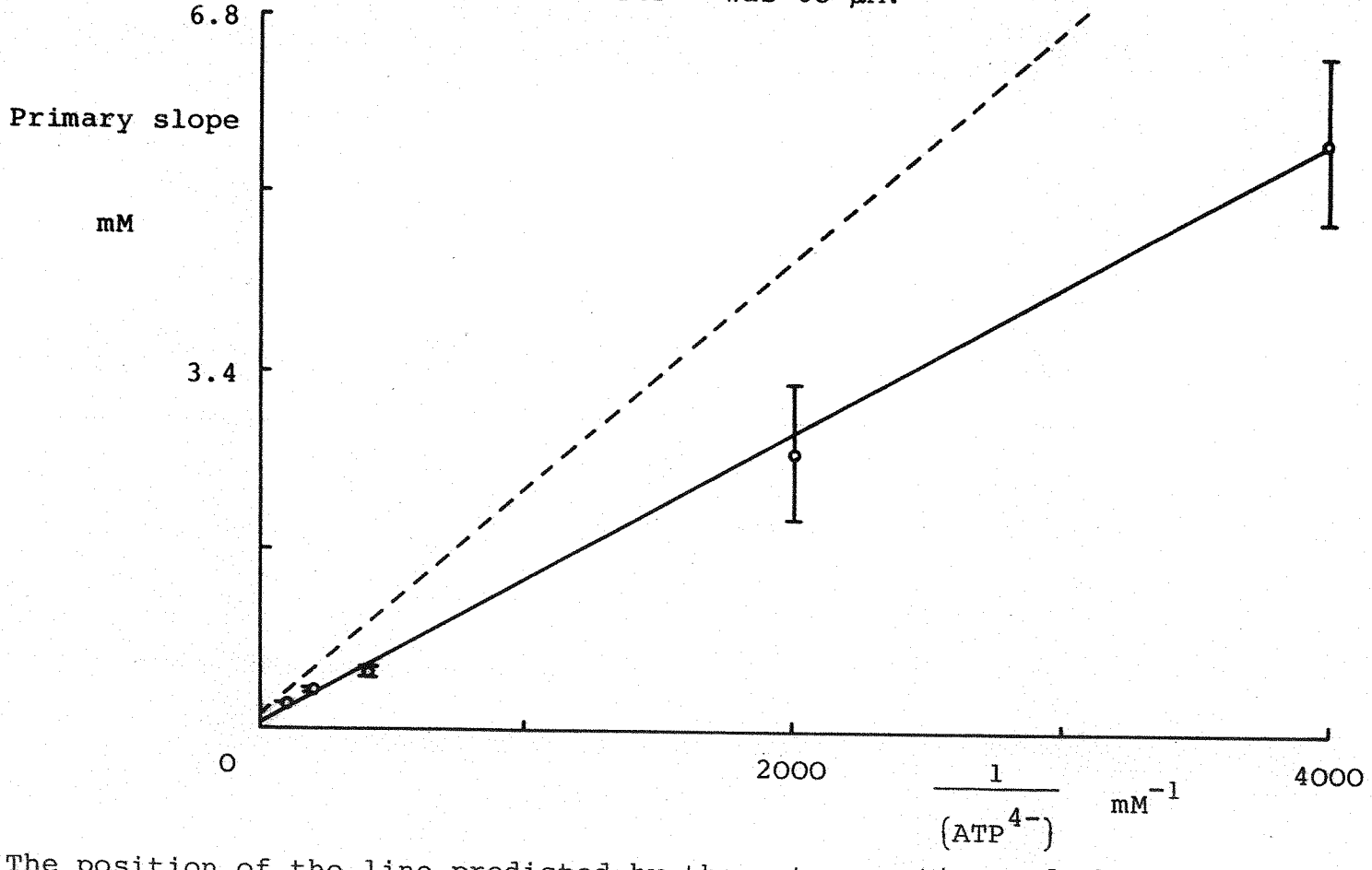
Lineweaver-Burk plot at 60 μM $F6P^{2-}$.



The data was interpolated from Figure 26.

Figure 30.

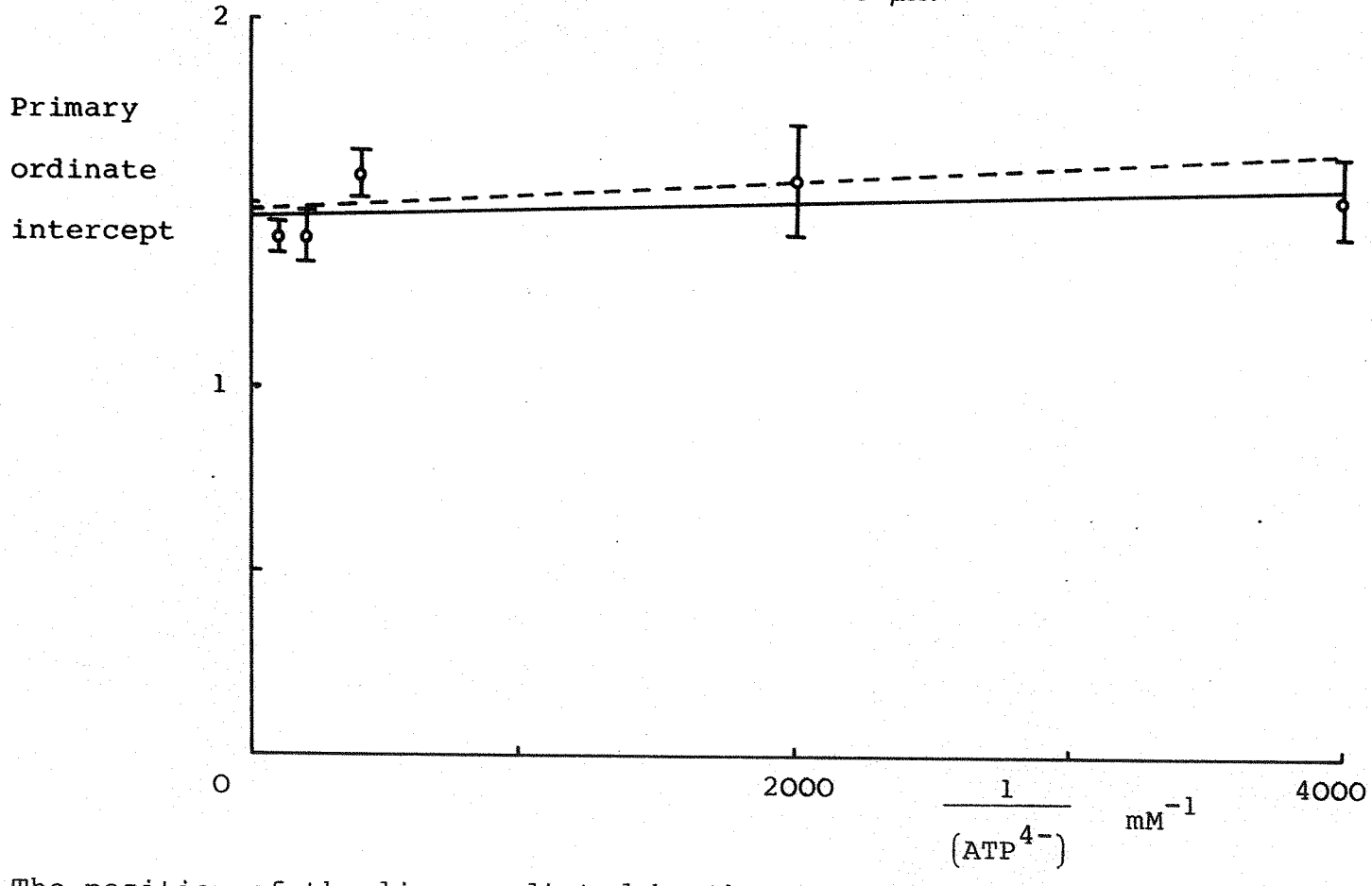
Secondary plot of primary slopes against the reciprocal concentration of ATP^{4-}. $F6P^{2-}$ was 60 μM.



The position of the line predicted by the rate equation and the values of kinetic constants from Table 10 (weighted fit) is shown in broken type.

Figure 31.

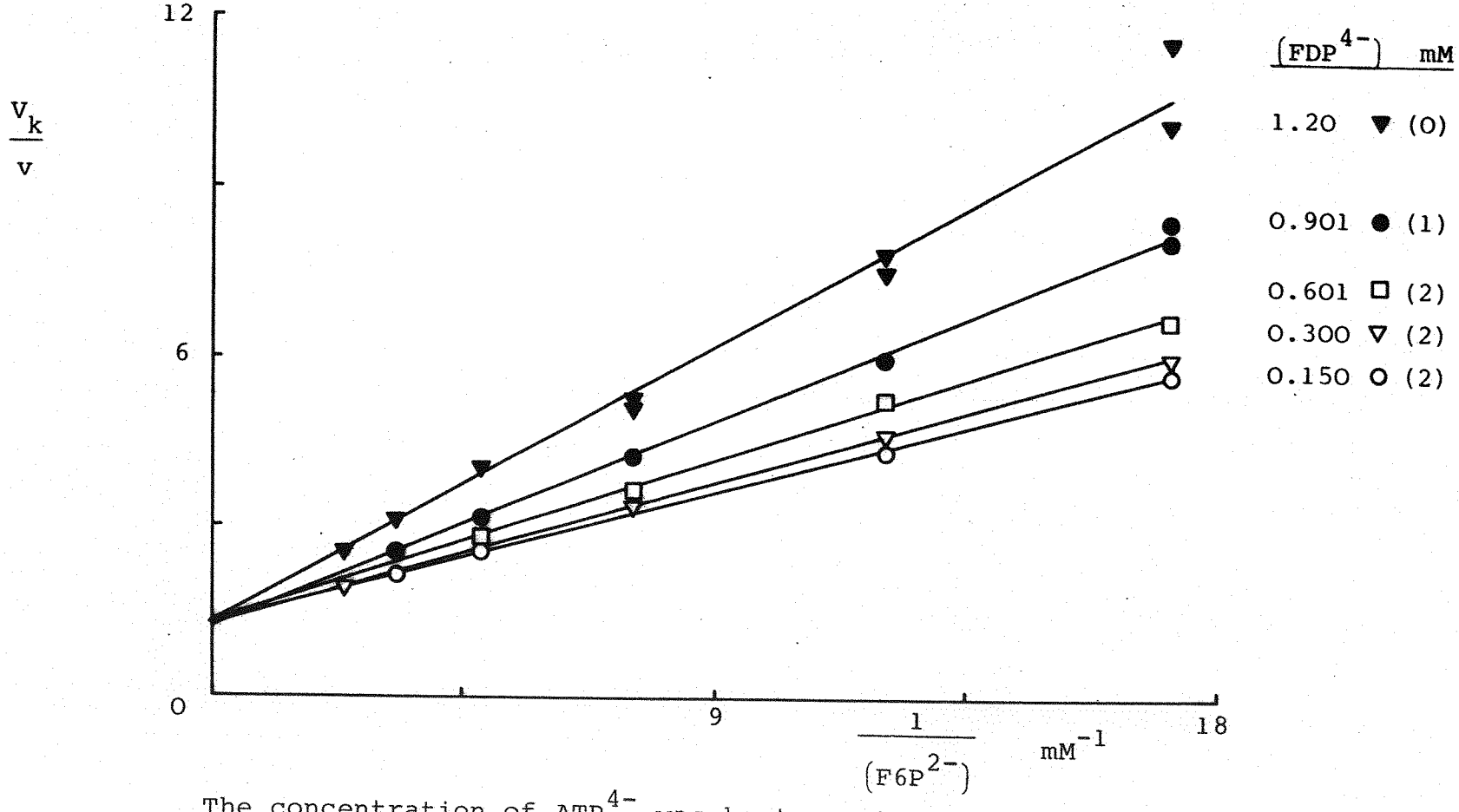
Secondary plot of primary ordinate intercepts against the reciprocal concentration of ATP^{4-}. $F6P^{2-}$ was 60 μM.



The position of the line predicted by the rate equation and the values of kinetic constants from Table 10 (weighted fit) is shown in broken type.

Figure 32.

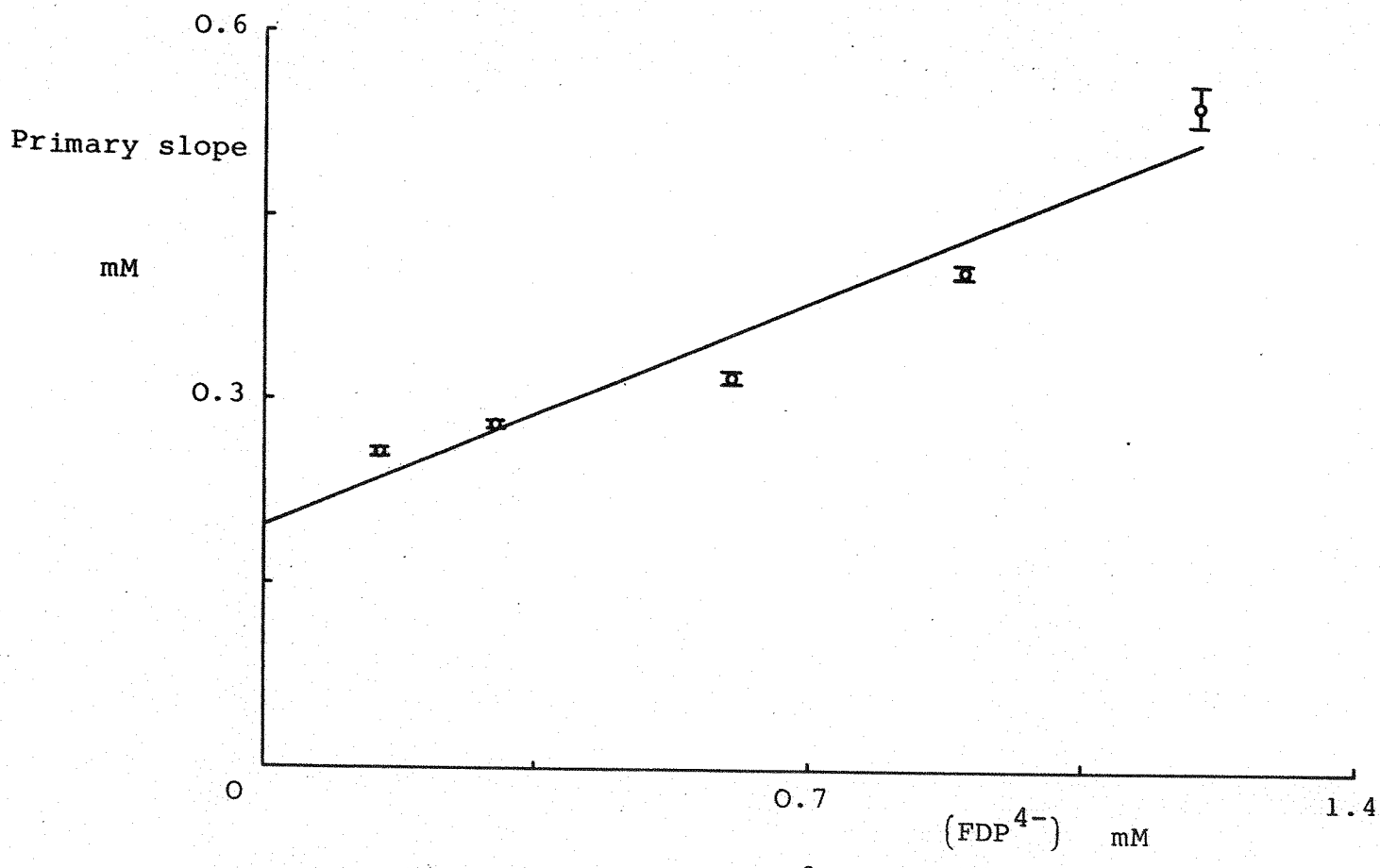
Lineweaver-Burk plot at 1 mM free Mg^{2+} showing competitive inhibition by FDP^{4-}.



The concentration of ATP^{4-} was kept constant at 0.642 μM.
Parentheses indicate the number of points omitted from each line for clarity.

Figure 33.

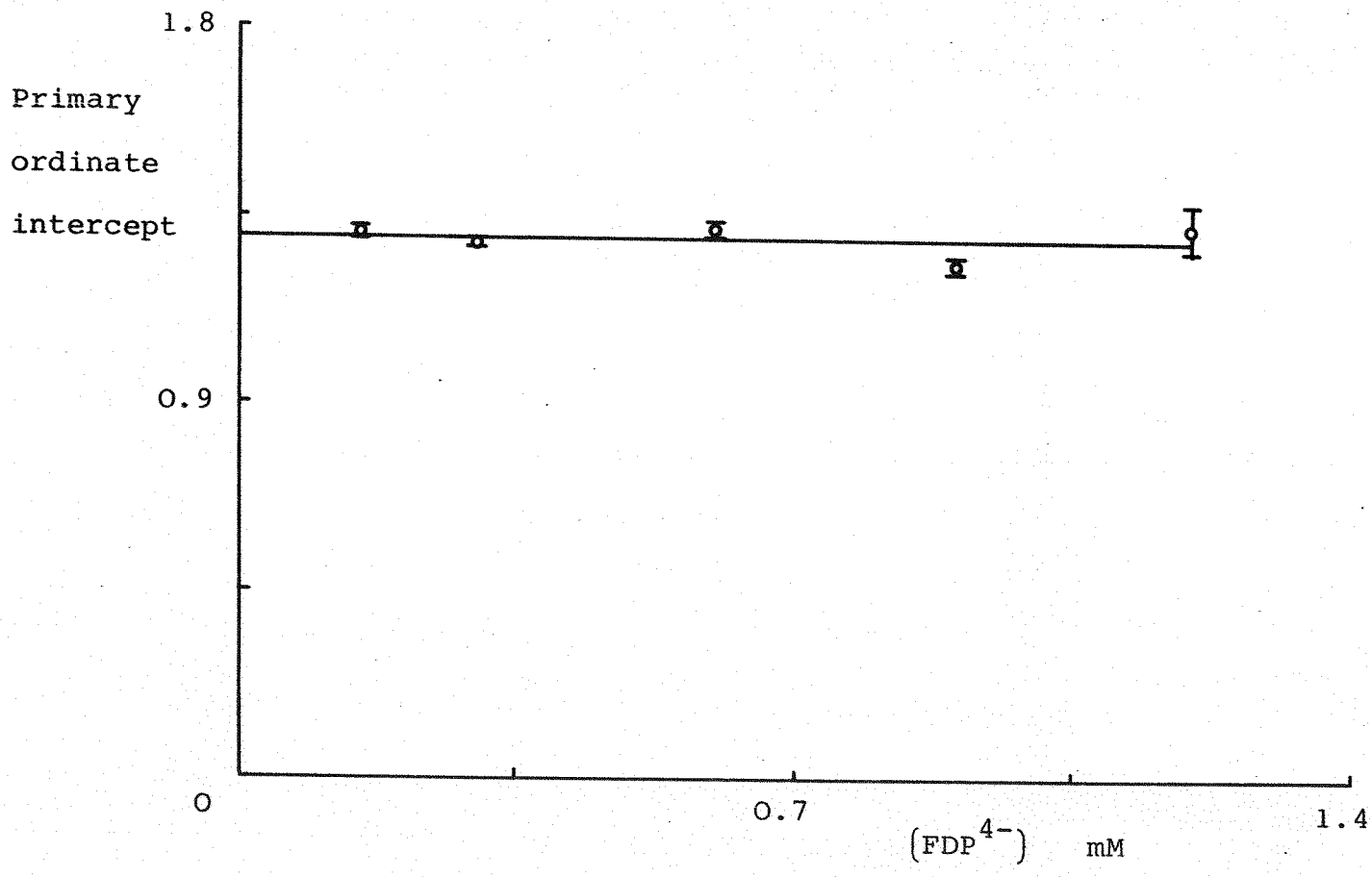
Secondary plot of primary slopes against the concentration of FDP^{4-}.



The concentrations of free Mg^{2+} and ATP^{4-} were 1 mM and 0.642 μM respectively.

Figure 34.

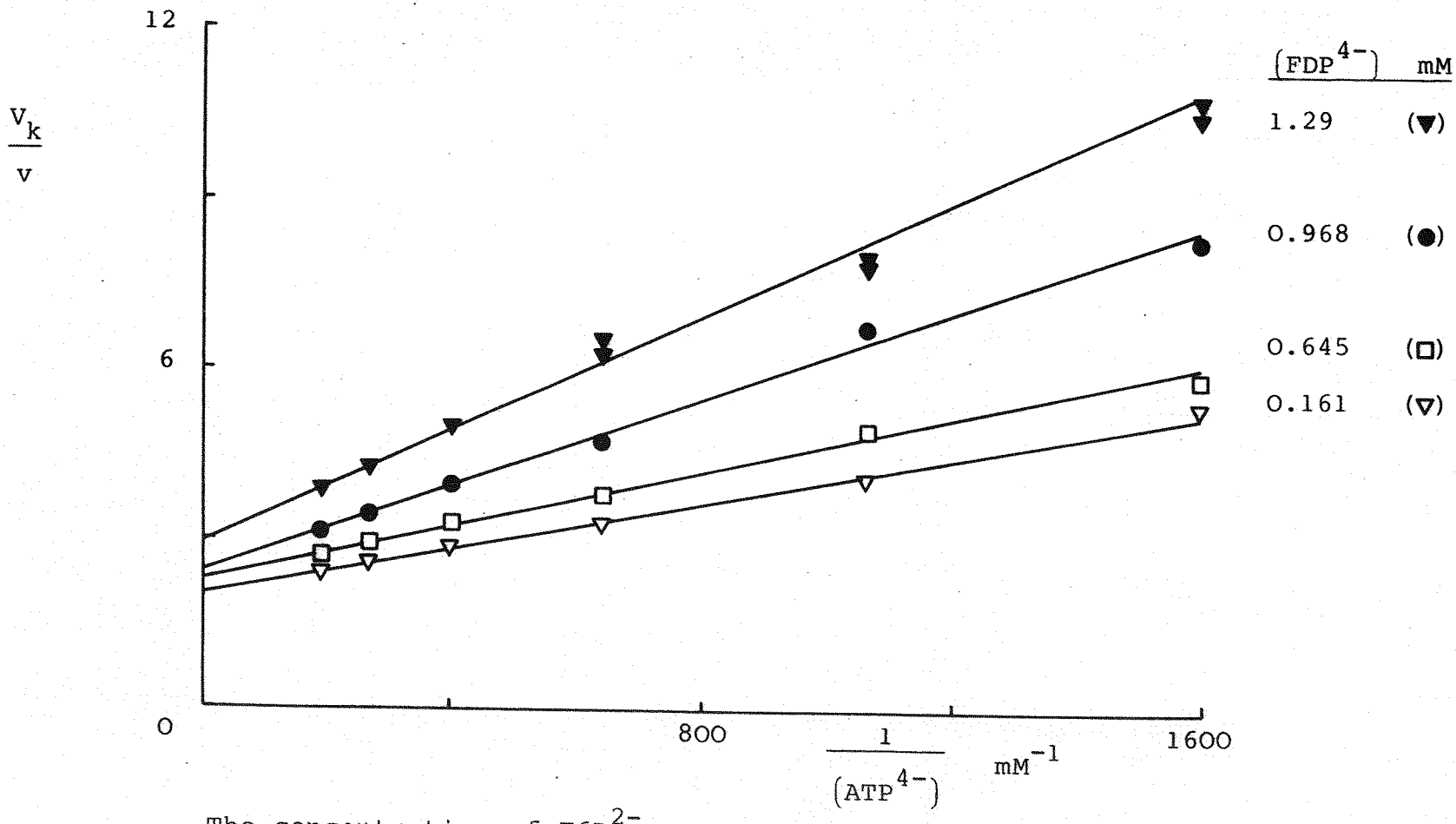
Secondary plot of primary ordinate intercepts against the concentration of FDP^{4-}.



The concentrations of free Mg^{2+} and ATP^{4-} were 1 mM and 0.642 μM respectively.

Figure 35.

Lineweaver-Burk plot at 1 mM free Mg^{2+} showing non-competitive inhibition by FDP^{4-}.

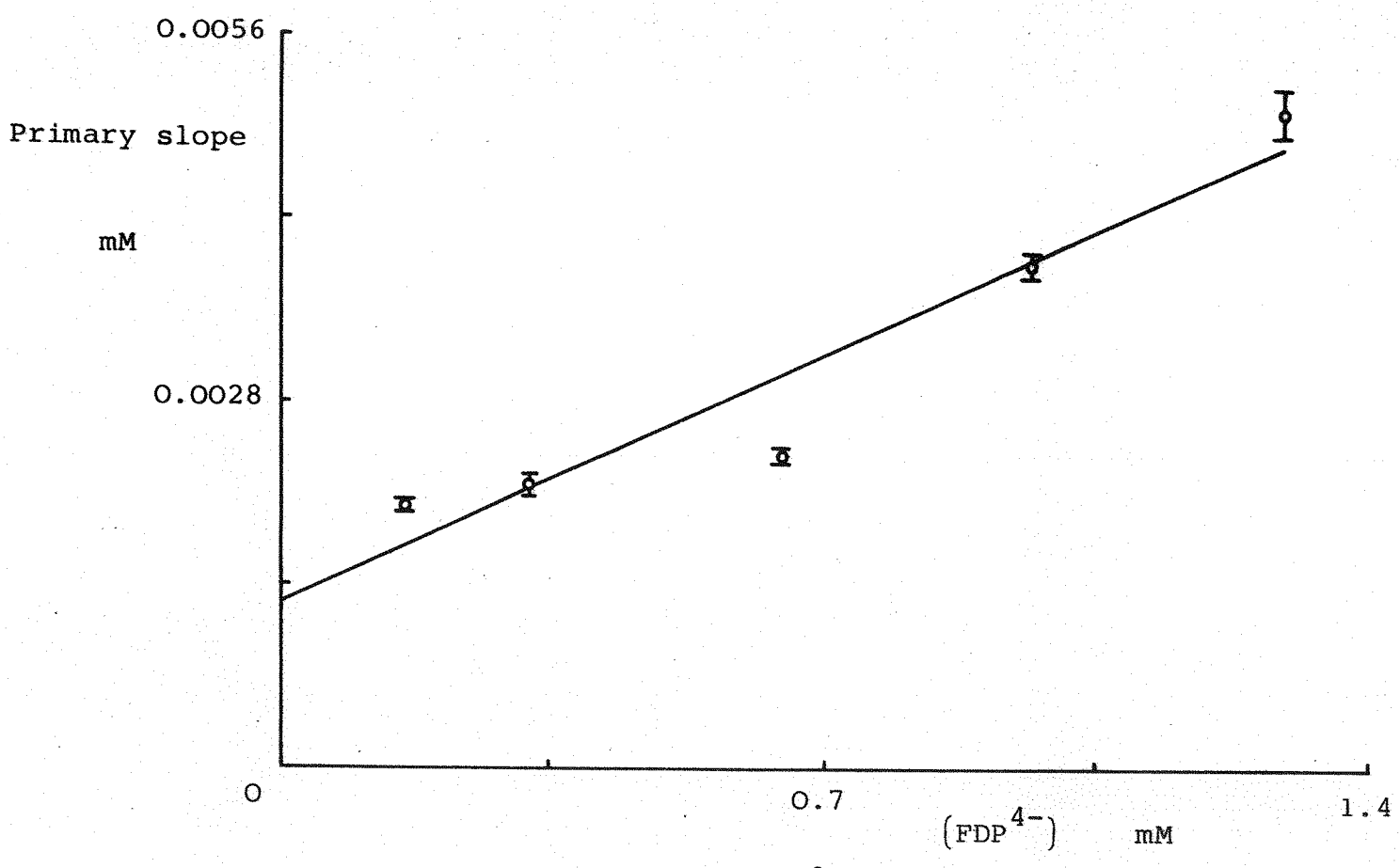


The concentration of $F6P^{2-}$ was kept constant at 63.2 μM.

A line at 0.323 mM FDP^{4-} was omitted for clarity.

Figure 36.

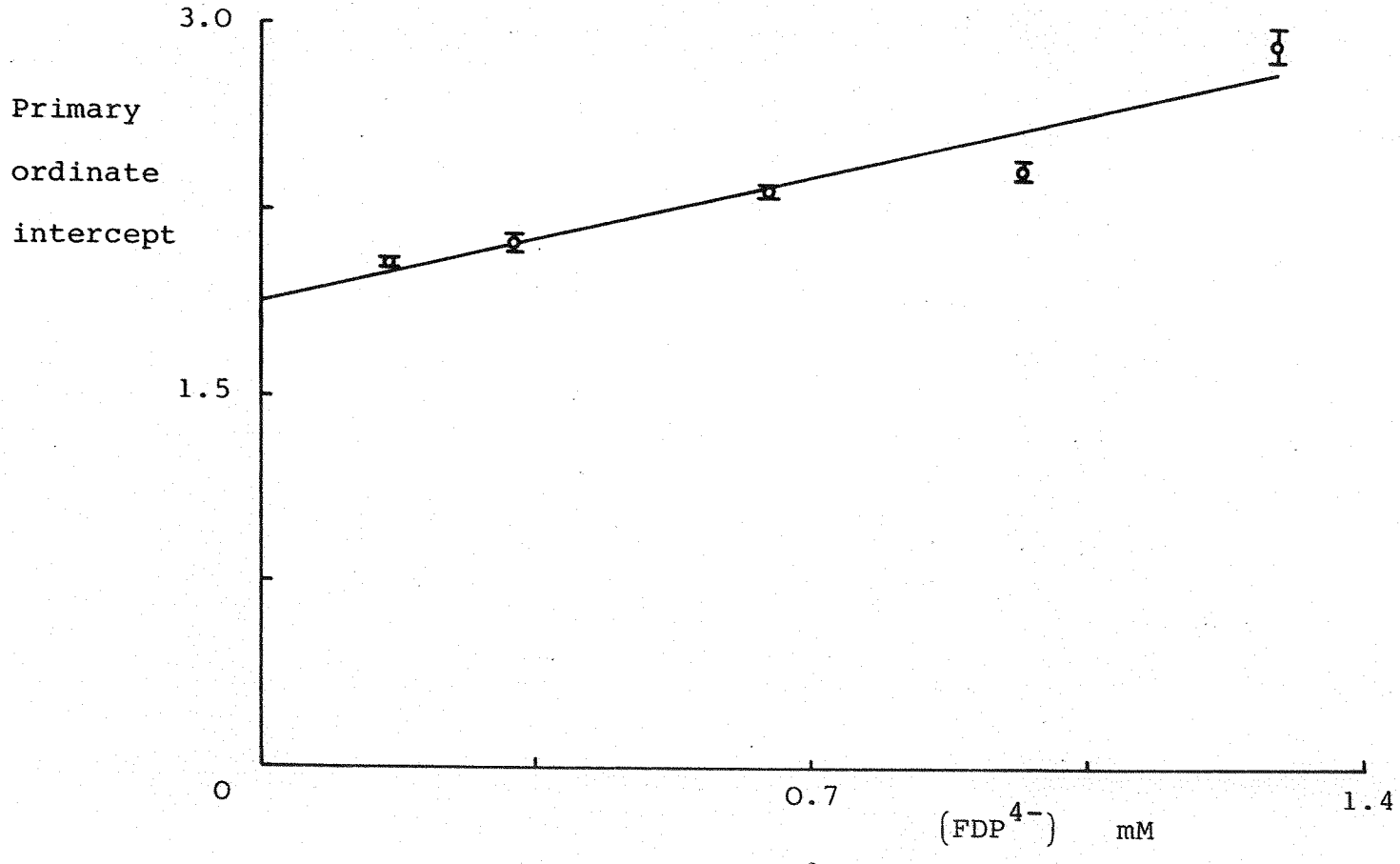
Secondary plot of primary slopes against the concentration of FDP^{4-}.



The concentrations of free Mg^{2+} and $F6P^{2-}$ were 1 mM and 63.2 μM respectively.

Figure 37.

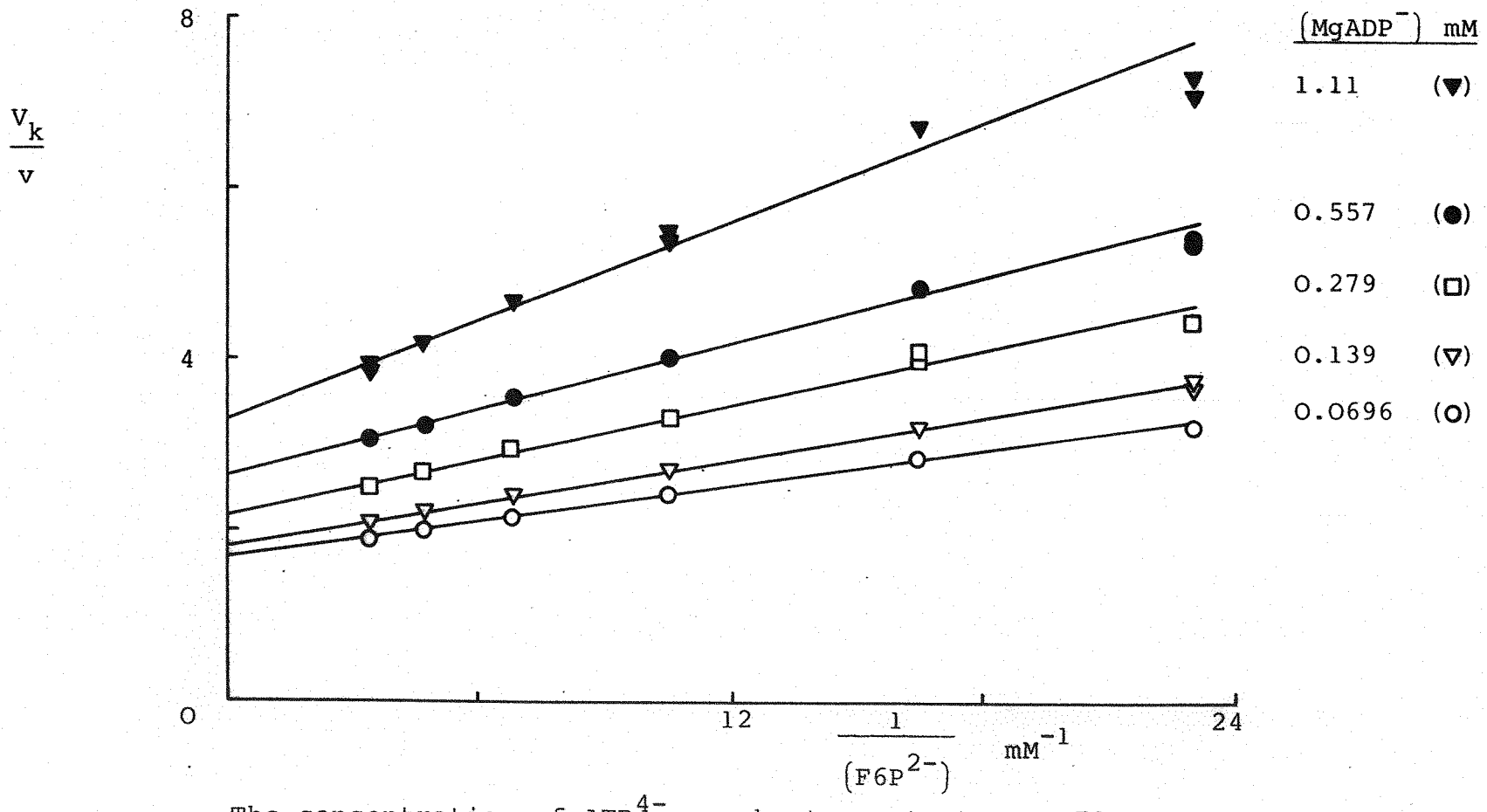
Secondary plot of primary ordinate intercepts against the concentration of FDP^{4-}.



The concentrations of free Mg^{2+} and $F6P^{2-}$ were 1 mM and 63.2 μM respectively.

Figure 38.

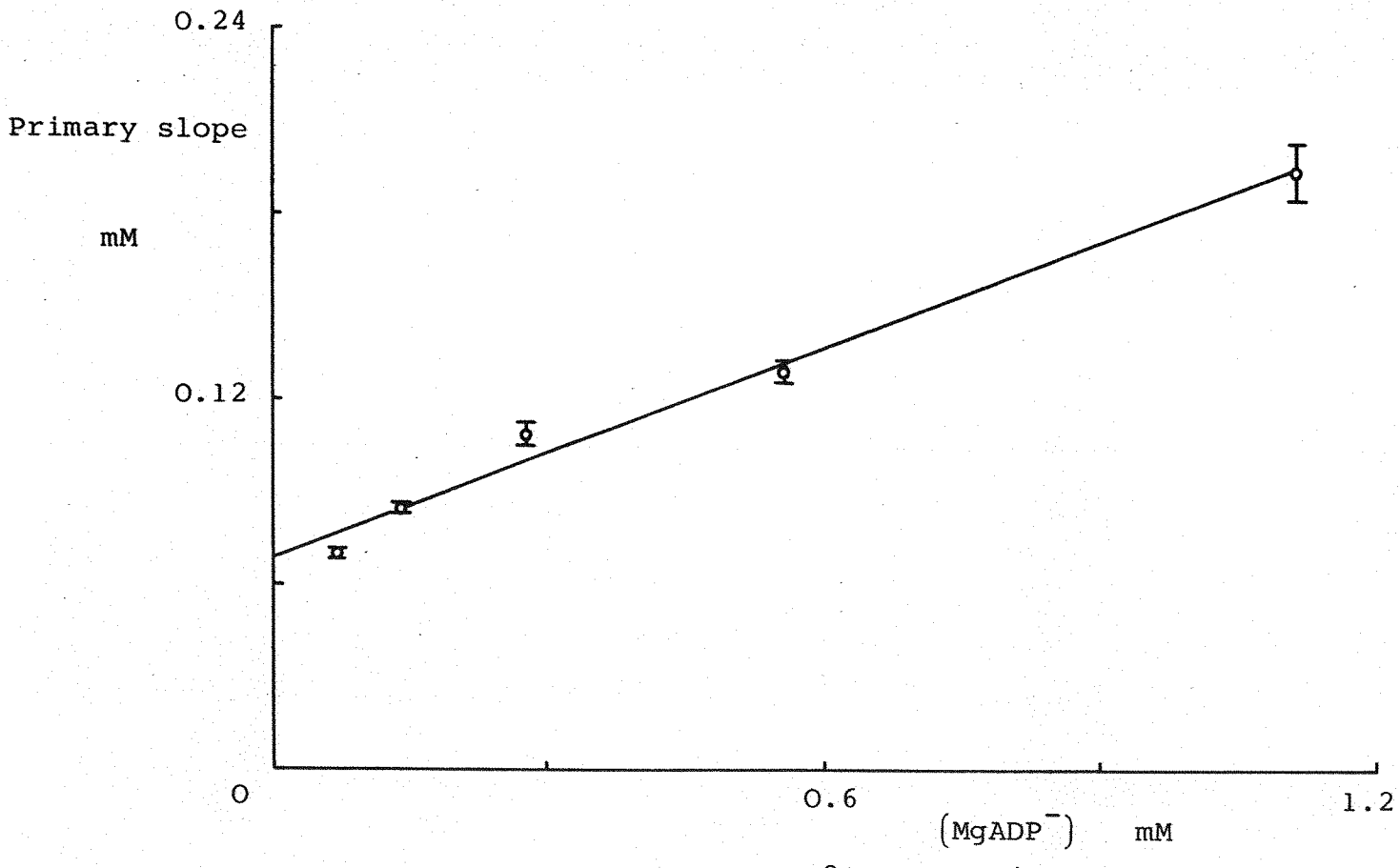
Lineweaver-Burk plot at 1 mM free Mg^{2+} showing non-competitive inhibition by $MgADP^-$.



The concentration of ATP^{4-} was kept constant at 0.721 μM.

Figure 39.

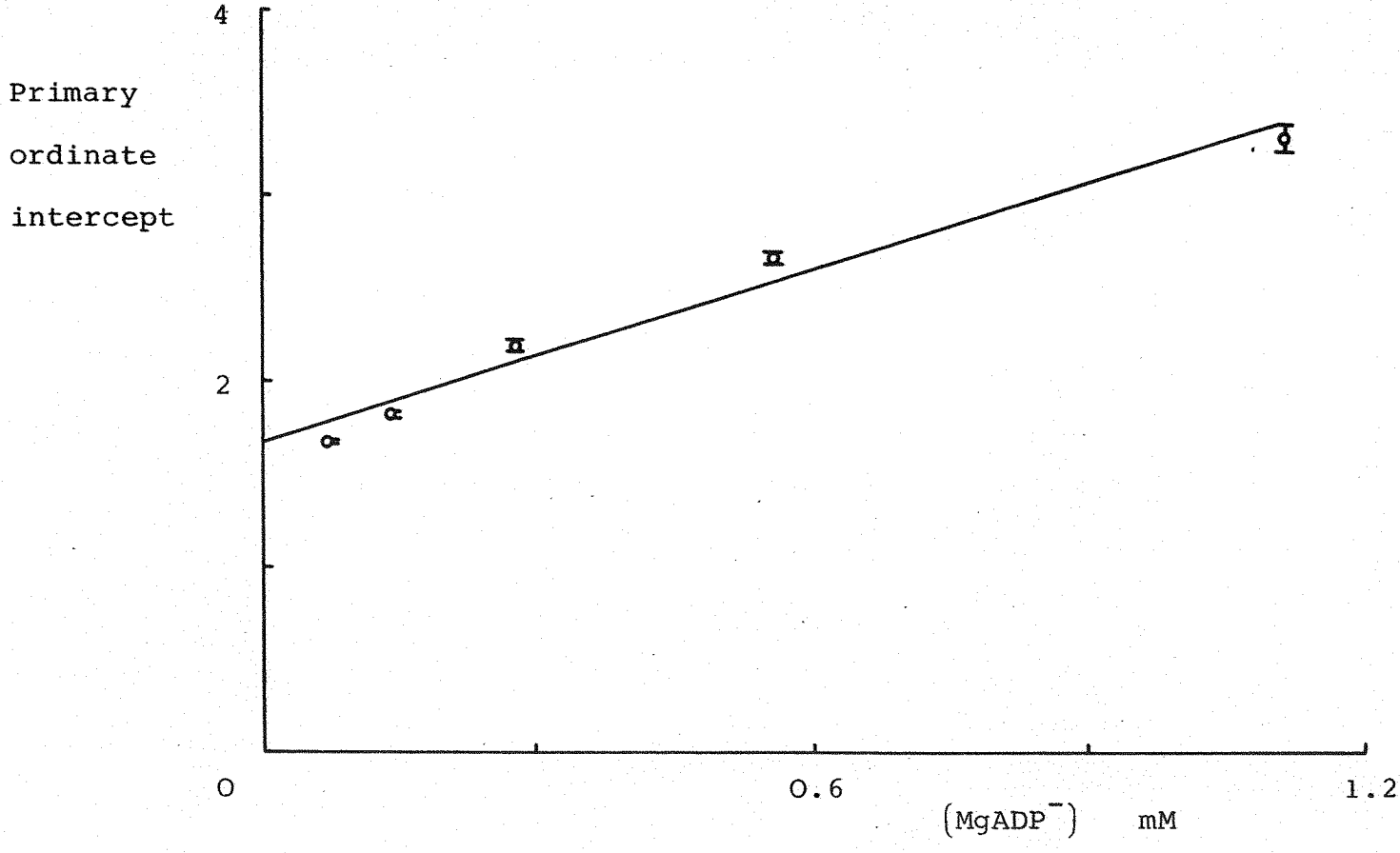
Secondary plot of primary slopes against the concentration of $MgADP^-$.



The concentrations of free Mg^{2+} and ATP^{4-} were 1 mM and 0.721 μM respectively.

Figure 40.

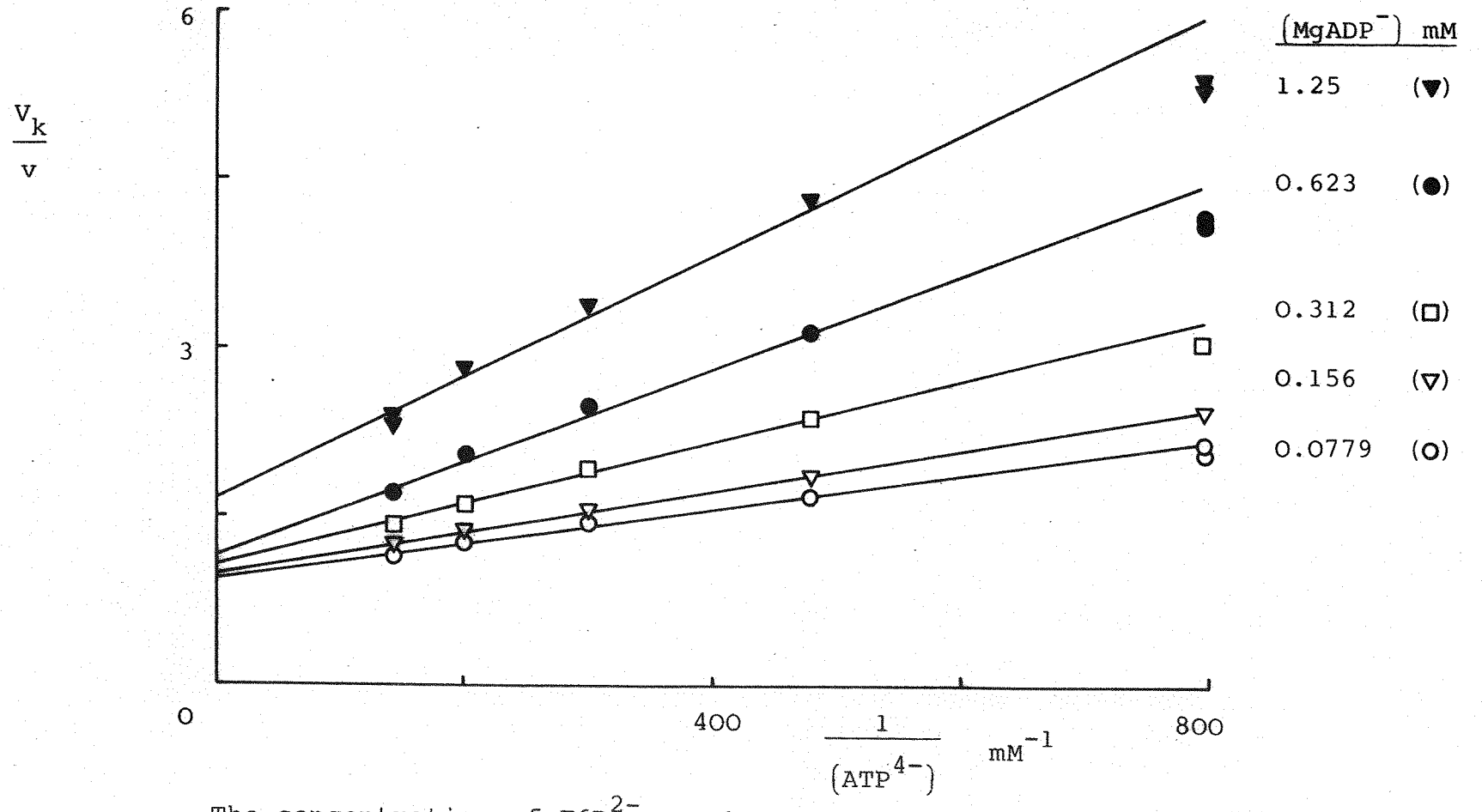
Secondary plot of primary ordinate intercepts against the concentration of $MgADP^-$.



The concentrations of free Mg^{2+} and ATP^{4-} were 1 mM and 0.721 μM respectively.

Figure 41.

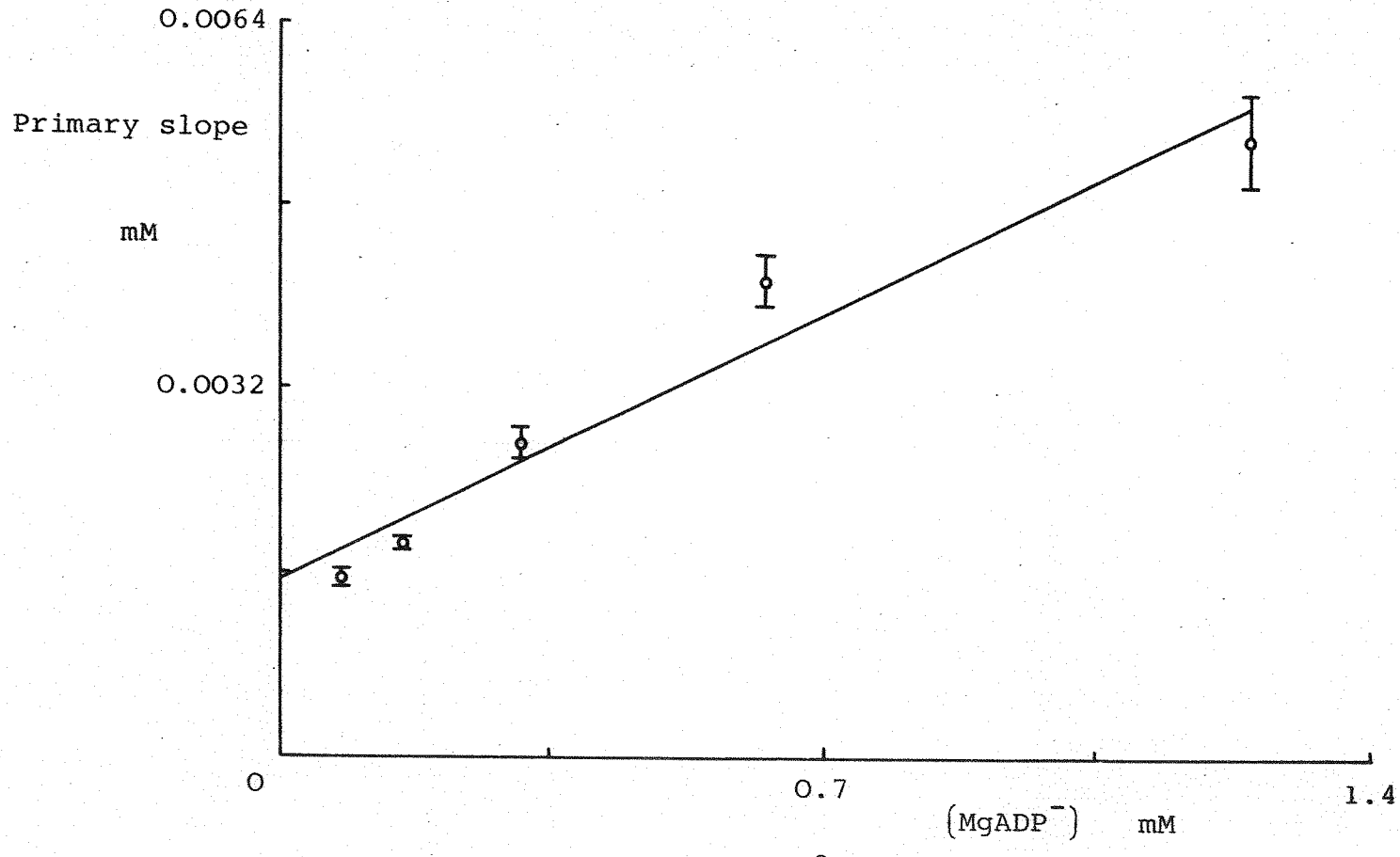
Lineweaver-Burk plot at 1 mM free Mg^{2+} showing non-competitive ihibition by $MgADP^-$.



The concentration of $F6P^{2-}$ was kept constant at 68.7 μM.

Figure 42.

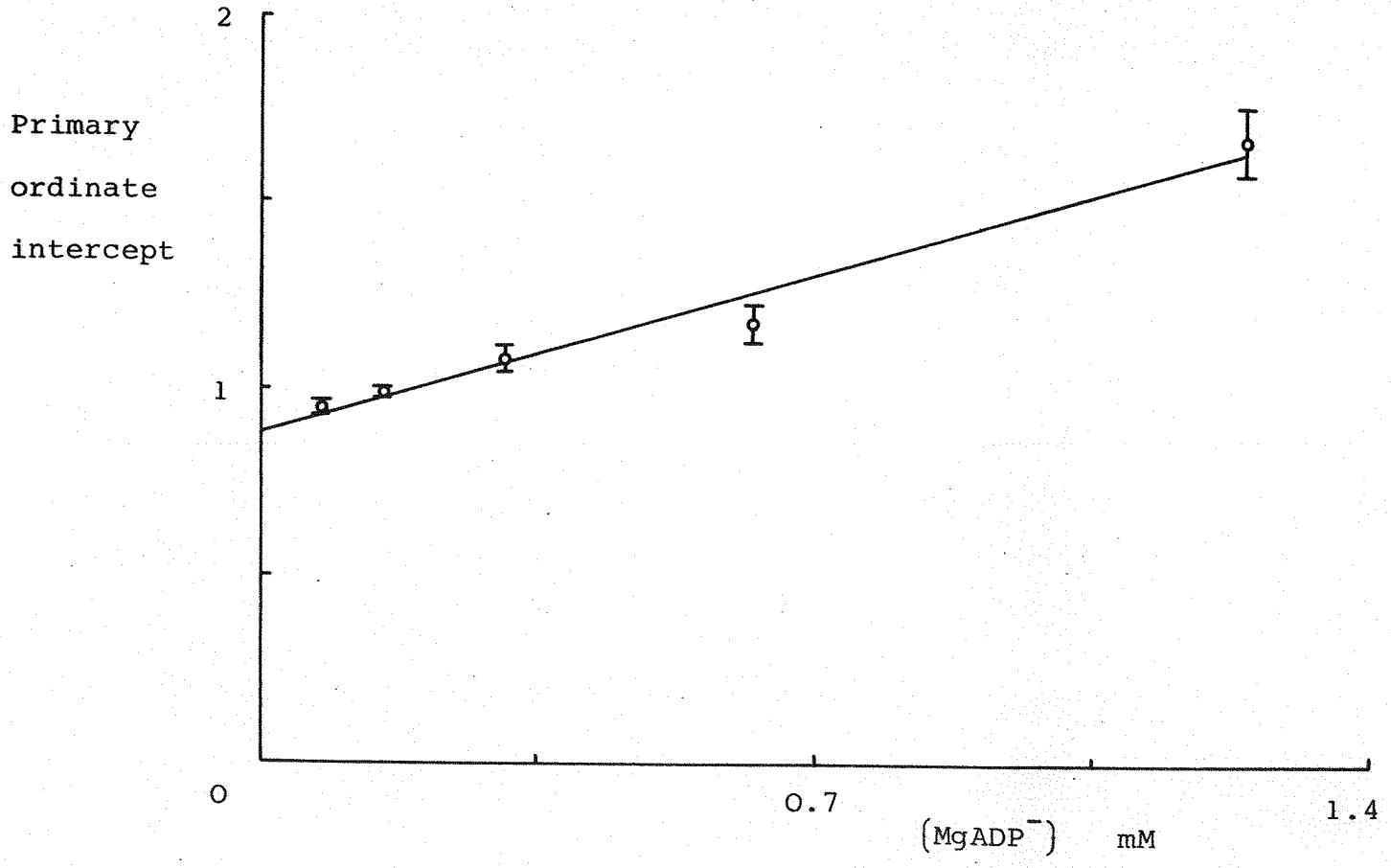
Secondary plot of primary slopes against the concentration of $MgADP^-$.



The concentrations of free Mg^{2+} and $F6P^{2-}$ were 1 mM and 68.7 μM respectively.

Figure 43.

Secondary plot of primary ordinate intercepts against the concentration of $MgADP^-$.



The concentrations of free Mg^{2+} and $F6P^{2-}$ were 1 mM and 68.7 μM respectively.

Figure 44.

K^+ and Mg^{2+}-substrate complexes present in the phosphofructokinase assay at pH 8.0.

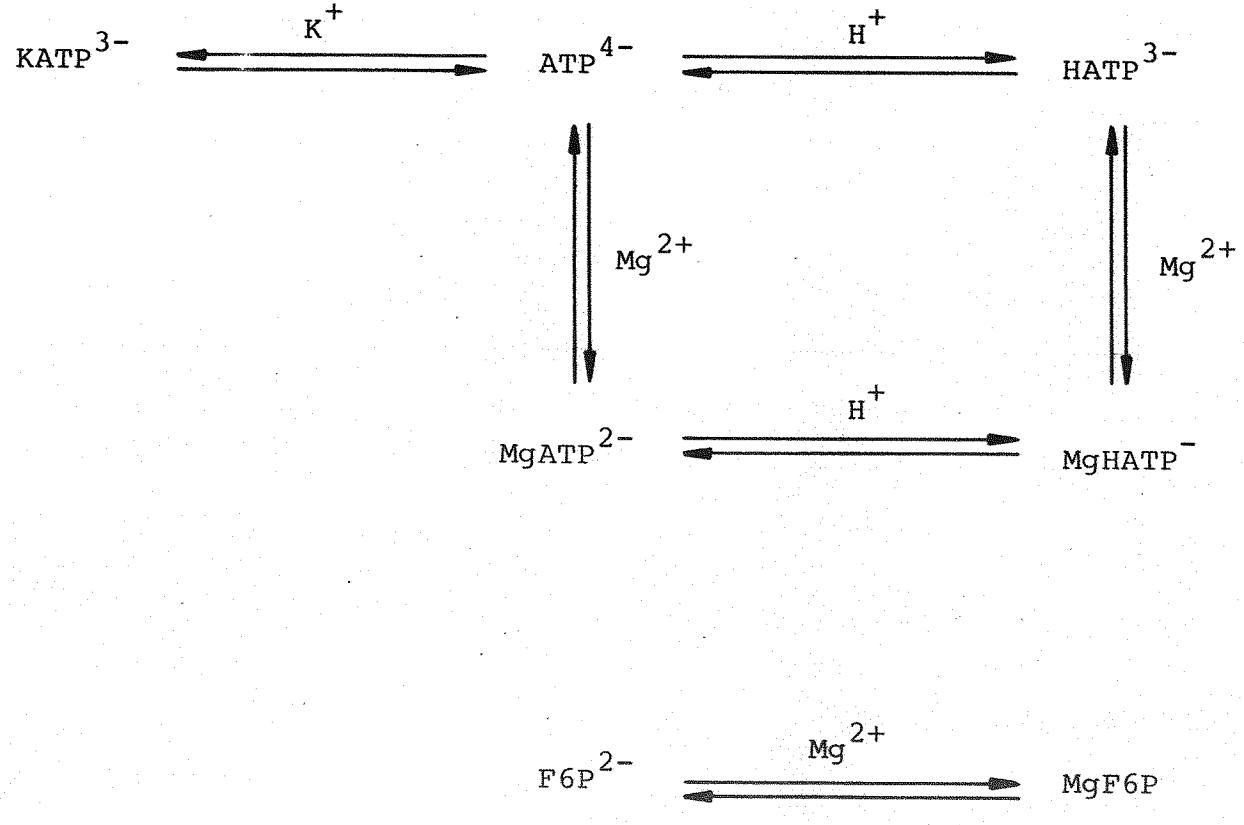
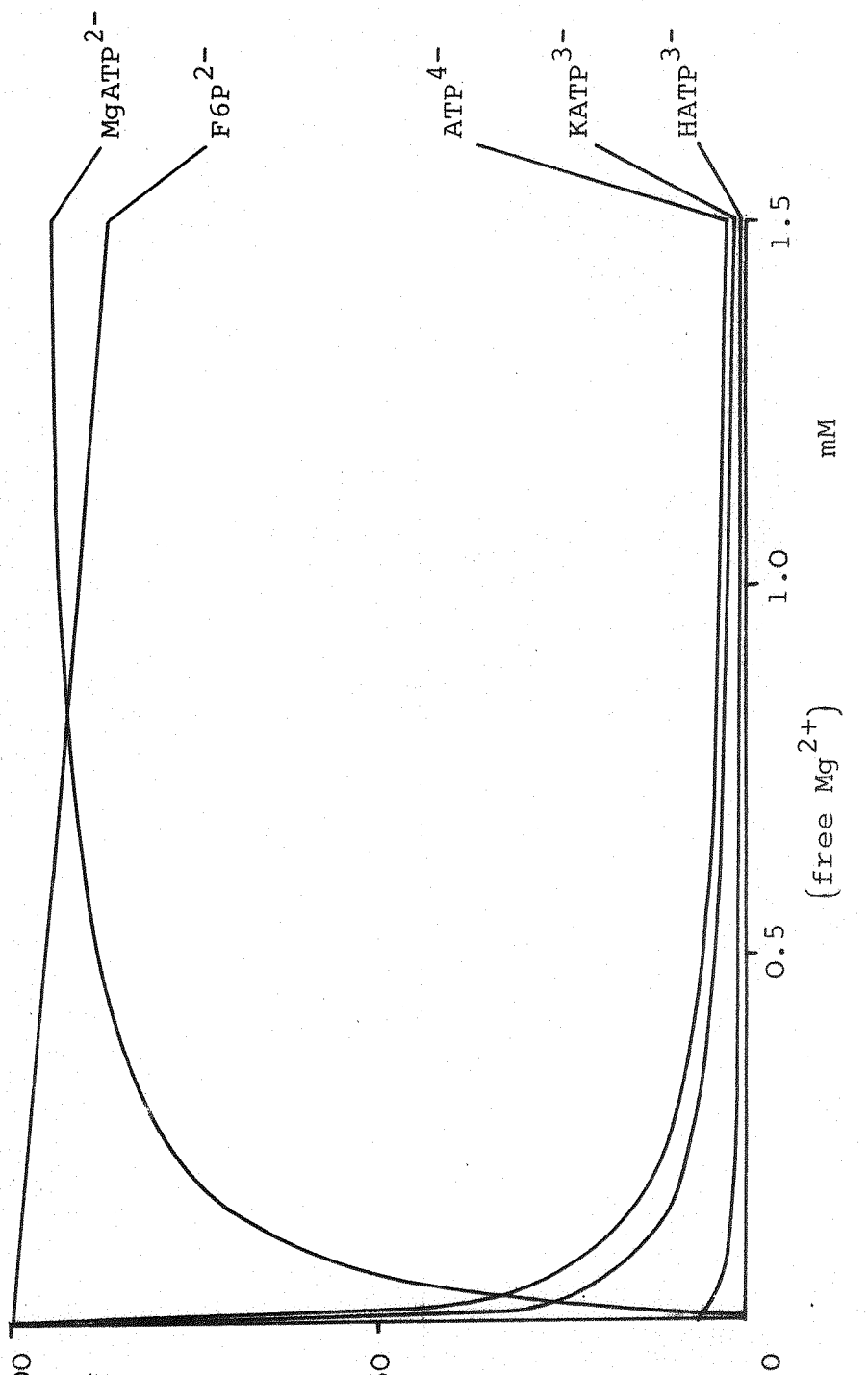


Figure 45.

Effect of varying the free Mg^{2+} concentration on the relative amounts of the K^+ and Mg^{2+}-substrate complexes present at pH 8.0 and 50 mM total K^+.



Chapter 5.

DISCUSSION.

Factors affecting phosphofructokinase activity.

The inhibition of C. maenas phosphofructokinase by high concentrations of ATP is evidence for the allosteric nature of this enzyme. At pH 8.0 and under the conditions of the experimental kinetic assay phosphofructokinase from C. maenas exhibited hyperbolic kinetics with respect to the concentration of F6P. This is similar to the enzyme from mammalian sources for which cooperative effects of F6P are weakened at alkaline pH and in contrast to the yeast enzyme which exhibits sigmoidal kinetics with respect to F6P at neutral and alkaline pH, (Hofmann, 1976).

The activation of C. maenas phosphofructokinase by ammonium sulphate is as expected since the enzyme from a wide variety of sources is activated both by NH_4^+ (Bloxham & Lardy, 1973) and SO_4^{2-} (Foe & Trujillo, 1980). The effect of this compound on F6P cooperativity indicates one or both of these ions acts as a positive allosteric effector of the enzyme.

The increase in C. maenas phosphofructokinase activity in the presence of thiol reagents is similar to that first observed for the rabbit muscle enzyme, (Paetkau & Lardy, 1967). Although chemical modification of thiol groups on phosphofructokinase inactivates the enzyme (Younathan et al. 1968; Froede et al. 1968; Mathias & Kemp, 1972; Schwartz et al. 1976) it is unlikely that there is a thiol group involved in catalysis since the inactivation is not complete, (Uyeda, 1979). Probably the redox status of thiol groups on the enzyme is important for its conformation.

Activation of C. maenas phosphofructokinase by inorganic phosphate added to the enzymic assay is similar to the effect of this compound on most phosphofructokinases for which it is an allosteric activator. However the gradual increase in activity of the purified enzyme diluted with phosphate buffer is more likely to reflect a change in the association state of the enzyme than a shift in the allosteric equilibrium considering the rate of the process and that

activation also occurs to a lesser extent in Tris-HCl buffer. The association state of mammalian phosphofructokinases depends on the enzyme concentration (Johnson & Deal, 1978) and effectors of the enzyme have been observed to influence both the association state of rabbit muscle phosphofructokinase and the rate of equilibration of different aggregates of this enzyme, (Lad et al. 1973).

Structural properties of phosphofructokinase.

The separation of C. maenas phosphofructokinase into three bands by electrophoresis on polyacrylamide gels suggests this enzyme exhibits association-dissociation behaviour similar to that of mammalian phosphofructokinases. It is not possible to define the oligomeric nature of the three bands since the mobility of proteins on polyacrylamide gels is not directly proportional to their molecular weights. One may speculate that if the middle of the three enzyme bands is an active tetramer then the band with the greatest mobility may be a dimer and that with the least mobility may be a hexamer or octamer. The low activity of the dimer (presumably inactive) could reflect reassociation under the conditions of assay.

The molecular weight of the sub-units of C. maenas phosphofructokinase (81,500) is similar to those values reported for mammalian enzymes and different from yeast (bakers' 104,000; brewers' 90,000) and bacterial phosphofructokinases (about 35,000). The molecular weight of C. maenas phosphofructokinase was not determined as all the purified enzyme was required for the kinetic work so that the number of sub-units it contains cannot be calculated. Since C. maenas phosphofructokinase resembles mammalian phosphofructokinases rather than the yeast enzyme as regards its sub-unit molecular weight and self-association behaviour it may also be a tetrameric enzyme.

Substrates and products of phosphofructokinase.

Initial-rate data obtained for C. maenas phosphofructokinase is consistent with the binding of $F6P^{2-}$, free Mg^{2+} and ATP^{4-} by the enzyme and the release of $MgADP^{-}$ and FDP^{4-} as products of the reaction.

As discussed in Chapter 1 phosphofructokinase activity has been observed to decrease at low concentrations of free Mg^{2+}, in some cases to zero. The concentration of free Mg^{2+} below which kinetic effects of this ion on the enzyme became apparent was 60 μM for yeast phosphofructokinase (Mavis & Stellwagen, 1970), 500 μM for human platelet phosphofructokinase (Akkerman et al. 1974) and about 400 μM for rabbit muscle phosphofructokinase (calculated from data for Figure 2 of Paetkau & Lardy, 1967). A more recent report for the latter enzyme stated free Mg^{2+} had no effect on enzymic activity in the range 0.1-4.0 mM (Bar-Tana & Cleland, 1974a). The proposed role of free Mg^{2+} as a substrate for C. maenas phosphofructokinase is entirely consistent with such observations since the enzyme must be less active at non-saturating concentrations of any one of its substrates. The K_m for free Mg^{2+} from the present study is 24.8 μM which is in the concentration range at which kinetic effects of this ion have been reported.

The observations of an apparent inhibitory action of Mg^{2+} on phosphofructokinase when total Mg^{2+} is in molar excess over total ATP (Lowry & Passonneau, 1966; Lardy & Parks, 1956) can be explained by the proposed role of ATP^{4-} as a substrate of the enzyme since under these conditions ATP^{4-} concentration may be low enough to limit the rate of reaction. In fact for C. maenas phosphofructokinase simply varying the total Mg^{2+} concentration in the experimental kinetic assay at 50 μM of both total ATP and total F6P showed inhibition of the enzymic rate above 2 mM total Mg^{2+} (data not shown). At 2 mM total Mg^{2+} the concentration of ATP^{4-} in the assay was 0.8 μM which is only twenty times greater than the K_m for ATP^{4-} (0.047 μM). Since the initial-rate data gave no evidence that Mg^{2+} is a dead-end inhibitor this observation probably results from "substrate depletion" as described above.

The kinetic effects of low free Mg^{2+} concentrations may also be interpreted as showing free Mg^{2+} forms a complex with the enzyme to activate it (e.g. Akkerman et al. 1974). The activated $E\text{-}Mg^{2+}$ complex then presumably combines with the substrates $F6P^{2-}$ and $MgATP^{2-}$ (or free Mg^{2+} and ATP^{4-}) so that two Mg^{2+} ions are involved in the reaction. This

situation is known to occur for pyruvate kinase (Newton, 1977, and references therein). The studies of Cottam & Uyeda (1973) and Peters et al. (1979) which show direct binding of free Mn^{2+} to phosphofructokinase are consistent with activation of the enzyme by free Mg^{2+}. Unfortunately no information about the kinetic mechanism is yielded by these studies so the role for free Mg^{2+} is not clarified. No evidence that two Mg^{2+} ions are involved in the reaction was obtained for C. maenas phosphofructokinase. If the sequential mechanism of phosphofructokinase involved binding of $MgATP^{2-}$ (or free Mg^{2+} and ATP^{4-}) to an activated E-Mg^{2+} complex then the appropriate rate equation would have VAB^2C as numerator and the tertiary plot of slopes of the secondary plot of primary slopes would show a parabola, (Figure 22). However this plot is clearly linear. This does not exclude the possibility of such a mechanism since if the dissociation constant of Mg^{2+} from the E-Mg^{2+} complex was much lower than 150 μM (the lowest free Mg^{2+} concentration studied) then non-linearity in Figure 22 might not be apparent. The dissociation constant of the E-Mn^{2+} complex observed for rabbit muscle and yeast phosphofructokinase was 20 μM (Cottam & Uyeda, 1973) and 2.26 mM respectively, (Peters et al. 1979).

The K_m for $F6P^{2-}$ for C. maenas phosphofructokinase (43.3 μM) is similar to values obtained for rabbit muscle phosphofructokinase - 21 μM and 70 μM, both at 4 mM free Mg^{2+} (Hanson et al. 1973; Bar-Tana & Cleland, 1974a) and rat liver phosphofructokinase - 77 μM at 3 mM free Mg^{2+} (Brand & Soling, 1974). These other values were obtained in terms of total F6P concentration so that the K_m for $F6P^{2-}$ is 70% of the values at 4 mM free Mg^{2+} and 76% of the value at 3 mM free Mg^{2+}, (see Figure 45). A similar correction is required in all cases to obtain the K_m for β-F6P which is the anomeric form of the substrate that binds to the enzyme and is 76% of total F6P, (Fishbein et al. 1974). Since studies of the kinetic mechanism of phosphofructokinase from other sources have taken $MgATP^{2-}$ to be the nucleotide substrate of the enzyme no K_m values for free Mg^{2+} or ATP^{4-} are available for comparison with those of the present study.

Physiologically $MgATP^{2-}$ is the predominant species of ATP. Measurements of the intracellular concentration of free Mg^{2+} have indicated values of 0.57 mM for human erythrocytes (Bunn et al. 1971), 3.0-3.5 mM for squid axon (Brinley & Scarpa, 1975) and 0.6-1.3 μmoles per gram wet weight for rat liver, brain and kidney (Veloso et al. 1973). This last concentration range is 1.2-2.6 mM if tissue water content is taken as 0.5 ml/g. The total ATP concentration of the leg muscle of C. maenas has been measured in this laboratory as 4.4 mM for animals maintained in 100% sea-water, (P.C. Poat, personal communication). The total K^+ concentration of C. maenas muscle is 112 mM (Shaw, 1955). At pH 7.4, 112 mM K^+ and 1 mM free Mg^{2+}, ATP is 90% $MgATP^{2-}$ and 3% ATP^{4-}. Thus if free Mg^{2+} concentration in C. maenas muscle is about 1 mM then physiological ATP^{4-} is about 137 μM and phosphofructokinase can utilise ATP^{4-} as a substrate in vivo since the K_m for ATP^{4-} is very low (0.047 μM).

Although free Mg^{2+} and ATP^{4-} bind separately to C. maenas phosphofructokinase the initial-rate data in the absence of added products also shows that the nucleotide product after phosphoryl transfer is the chelate $MgADP^-$, (see Scheme 1). If free Mg^{2+} and ADP^{3-} leave the enzyme separately so that the reaction is ter-reactant in both directions then free Mg^{2+} would be both a substrate and a product of the reaction and the initial-rate experiments could only be performed in the presence of both. In such a situation apparent substrate inhibition by free Mg^{2+} would be observed. The rate equation for the appropriate ter-ter mechanism (i.e. Scheme 1 with free Mg^{2+} and ADP^{3-} leaving the enzyme separately rather than as $MgADP^-$) is equation (5) with additional terms in the denominator which arise due to the fact that the initial-rate experiments are performed in the presence of a product of the reaction (free Mg^{2+}).

If the order of release of products is free Mg^{2+}, ADP^{3-} and FDP^{4-} the additional terms in the denominator of equation (5) are:-

$$(\text{coef } B)' B + (\text{coef } AB)' AB + (\text{coef } AB^2)' AB^2 + (\text{coef } AB^2C)' AB^2C$$

The modified rate equation would predict a positive ordinate

intercept in the tertiary plot in Figure 22 and apparent substrate inhibition by free Mg^{2+} in the tertiary plots in Figures 24 and 25. Inspection of these plots shows that this is not the case.

If the order of release of products is ADP^{3-}, free Mg^{2+} and FDP^{4-} then only one additional term arises in the denominator of equation (5):-

$$(\text{coef } AB^2C)' AB^2C$$

The predictions of this modified rate equation are the same as those of equation (5) except that apparent substrate inhibition by free Mg^{2+} should be observed in the tertiary plot in Figure 25. That is this graph should show a minimum at the higher concentrations of free Mg^{2+} as observed in substrate inhibition. Visual inspection of the data in Figure 25 suggests that there was a possibility of substrate inhibition in this plot. However when the initial-rate data was fitted directly to the appropriate rate equation the value of $(\text{coef } AB^2C)'$ was not significantly greater than zero so that this mechanism was discounted, (Table 13).

Thus the species of ADP which is the product of the reaction can be deduced from the kinetic data despite the fact that product-inhibition was studied at only one concentration of free Mg^{2+}. Repitition of the product-inhibition data at different concentrations of free Mg^{2+} would allow confirmation that $MgADP^-$ is the reaction product since in this case inhibition by free Mg^{2+} should not be observed.

The kinetic mechanism of phosphofructokinase.

Although all phosphofructokinases so far examined appear to have sequential mechanisms kinetic studies have indicated either a random or ordered addition of substrates depending on the source of the enzyme. As discussed in Chapter 1 a random mechanism seems probable for rabbit muscle phosphofructokinase (Kee & Griffin, 1972; Hanson et al. 1973; Bar-Tana & Cleland, 1974a,b) whereas an ordered bi-bi mechanism with F6P as the first substrate and FDP as the last product has been proposed for the enzymes from rat liver (Brand & Soling, 1974), human erythrocytes (Etiemble

et al. 1977) and Lactobacillus plantarum (Simon & Hofer, 1978).

Initial-rate and product-inhibition data for C. maenas phosphofructokinase are consistent with an ordered ter-bi mechanism for this enzyme. The competitive inhibition of FDP^{4-} with respect to $F6P^{2-}$ indicates that $F6P^{2-}$ is the first substrate to bind and FDP^{4-} the last product to leave the enzyme. The other three inhibition patterns are all non-competitive which is consistent with the nucleotide substrate binding after $F6P^{2-}$ and the nucleotide product leaving before FDP^{4-}. The true substrate and product species of ATP and ADP were identified as described above. In view of the different sequential mechanisms proposed for phosphofructokinases from different sources an alternative mechanism which is consistent with the initial-rate data for C. maenas phosphofructokinase is shown in Scheme 2. It is a rapid-equilibrium partially random ter-reactant mechanism. The rate equation for this mechanism when no products are present is the same as that for Scheme 1 when expressed in coefficient form, i.e. equation (5), so that the two mechanisms are not distinguished by the initial-rate data. However competitive inhibition between all substrate-product pairs is predicted for the random mechanism. Since such product-inhibition data was not obtained this is evidence against the random alternative. Unfortunately random binding of substrates and products makes the formation of mixed substrate-product dead-end complexes likely (Cleland, 1970) and if the dead-end complexes Enzyme-ATP-FDP, Enzyme-F6P-ADP and Enzyme-ATP-ADP were able to form in the random mechanism (i.e. dead-end ECQ, EAP and ECP complexes occur in Scheme 2) then the product-inhibition data for C. maenas phosphofructokinase would be consistent with such a random mechanism as well as with the ordered mechanism. In that case repitition of the product-inhibition experiments at a saturating concentration of the non-varied substrate would be necessary to distinguish the ordered and random alternatives. If the non-competitive inhibition patterns obtained for C. maenas phosphofructokinase arise due to dead-end complex formation in a random mechanism then saturation with the non-varied

substrate should yield four competitive inhibition patterns. This would not be the case for the ordered mechanism. The ordered mechanism shown in Scheme 1 is favoured at present since it is consistent with the product-inhibition data without assuming dead-end complex formation and as such is the simpler mechanism. This is a application of Occam's razor, (one version of which states: it is vain to do with more what can be done with fewer - William of Occam, early fourteenth centuary). Finally, although the dead-end complexes Enzyme-ATP-FDP and Enzyme-F6P-ADP have been suggested to form with rabbit muscle phosphofructokinase (Kee & Griffin, 1972; Hanson et al. 1973; Bar-Tana & Cleland, 1974b), the dead-end complex Enzyme-ATP-ADP has not been suggested to occur for any phosphofructokinase.

If the EAC complex in Scheme 2 does not exist then the rate equation for the resulting partially random mechanism would be equation (5) minus the (coef AC)' AC term in the denominator. The predicted plots of this rate equation are as those of equation (5) with the exception that a straight line with a positive ordinate intercept and zero slope should be observed in the tertiary plot represented by Figure 25. Although visually there appears to be some credence for this suggestion since the slope of the line in Figure 25 is small compared to the range of the ordinate axis fitting the initial-rate data directly to the rate equation gave an error variance greater than that for the ordered mechanism, (Table 14). Again the ordered mechanism is favoured.

An explanation for the different kinetic mechanisms indicated by steady-state kinetic studies of phosphofructo-kinase from different sources is not readily apparent. It may be that the enzymes studied do indeed have different kinetic mechanisms. An evolutionary change of the kinetic mechanism might however be expected and none is apparent when the proposed mechanisms for the enzymes studied are examined with respect to phylogenetic variation. Furthermore if a phosphofructokinase were to have a random mechanism within which there is a kinetically preferred pathway then steady-state kinetic studies (which measure the overall reaction rate) might only indicate this major

pathway. That is to say that an alternative minor pathway that does not contribute significantly to the overall reaction flux might exist but an ordered mechanism would be observed experimentally. Another enzyme for which the flux through the minor pathway is more significant might then exhibit a random mechanism. Measurements of the rate of isotope exchange at equilibrium between one substrate-product pair as a function of the concentration of the other substrate-product pair (varied together in a constant ratio) would distinguish a random mechanism with a preferred pathway from a strictly ordered mechanism. Consider an ordered mechanism in which the order of substrate binding is F6P, ATP and the order of product release is ADP, FDP. Linear double-reciprocal plots are predicted for the ATP-ADP exchange when either ATP and ADP or F6P and FDP are varied and also for the F6P-FDP exchange when F6P and FDP are varied. The F6P-FDP exchange however will show substrate inhibition as the concentration of ATP and ADP is increased. The substrate inhibition will be total (i.e. the exchange rate becomes zero when the ATP and ADP concentration is infinite) if the mechanism is strictly ordered but if an alternative minor pathway exists the inhibition will be partial (i.e. a finite exchange still occurs when the ATP and ADP concentration is infinite). Total and partial substrate inhibition can be distinguished in a double-reciprocal plot as an ordinate intercept is observed only in the latter case. Such isotope exchange studies should yield further information about the kinetic mechanism of phosphofructokinase and may help to resolve the apparently contradictory results reported in the literature.

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