# SELECTIVE INHIBITION BY VANADATE OF ENZYMES WHICH CATALYZE PHOSPHORYL TRANSFER REACTIONS

by

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B.Sc., Simon Fraser University, 1974

M.Sc., Simon Fraser University, 1986

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Selective Inhibition by Vanadate of Enzymes Which Catalyze Phosphoryl Transfer Reactions

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# ABSTRACT

Substrate analogues of adenosine monophosphate, adenosine diphosphate and adenosine triphosphate have been tested by kinetic methods for their ability to activate three kinases: myokinase (adenylate kinase), pyruvate kinase and hexokinase. These substrate analogues are vanadate esters and anhydrides that are formed spontaneously in solutions that contain vanadate and nucleotides. Adenosine vanadate, an analogue of AMP, was found to be a good substrate for myokinase. No activity was detected with other substrate analogues containing vanadate, for myokinase, pyruvate kinase or hexokinase. Vanadate had no observable effect on the activity of pyruvate kinase. It was found to be a weak inhibitor of myokinase and hexokinase, but the presence of nucleotides did not enhance the inhibition. The natural substrates for the kinases are $Mg^{2+}$/nucleotide complexes, an exception being myokinase which binds an uncoordinated AMP or ADP molecule at one of its catalytic sites. It has been proposed (Craig and Gresser (1988) J. Cell Biol, **107,** 189a) that the reason that vanadate in the presence of nucleotides is not a potent inhibitor of the kinases, is the failure of the phosphovanadate moiety of the spontaneously formed vanadate nucleotide to chelate a divalent cation. Thus nucleotides which are mixed phosphate/vanadate anhydrides are unable to bind to enzymes for which the normal substrates are $Mg^{2+}$/nucleotide complexes.

The ability of divanadate and of phosphovanadate to chelate $Mg^{2+}$ was tested by $^{51}V$ NMR. Divanadate and phosphovanadate were not stabilized in aqueous solutions containing $Mg^{2+}$.

The ability of vanadate to spontaneously form esters with small organic molecules in aqueous solution was tested in experiments with glyoxylate. Electrophilic catalysis of the dehydration of glyoxylate hydrate was demonstrated in a reaction coupled to lactic dehydrogenase.

## Acknowledgements

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## Abbreviations

Ad - adenosine

ADP - adenosine 5'-diphosphate

AMP - adenosine 5'-monophosphate

ATP - adenosine 5'-triphosphate

AdV - adenosine 5'-vanadate

AdVP - adenosine 5'-vanadophosphate

AdVPP - adenosine 5'-vanadopyrophosphate

$Ad_2V_2$ - $V^1,V^2$-di(adenosine-2',3')divanadate

AMPV - adenosine 5'-monophosphovanadate

AMPVP - adenosine 5'-phosphovanadophosphate

$AP_5A$ - $P^1,P^5$-di(adenosine-5')pentaphosphate

ESR - electron spin resonance

G6P - glucose-6-phosphate

G6PDH - glucose-6-phosphate dehydrogenase

HK - hexokinase

IR - infrared

LDH - lactic dehydrogenase

MK - myokinase (adenylate kinase)

NAD(H) - nicotinamide adenine dinucleotide (reduced form)

NADP(H) - nicotinamide adenine dinucleotide phosphate (reduced)

NMR - nuclear magnetic resonance

$P_i$ - phosphate ($PO_4^{3-}$)

$PP_i$ - pyrophosphate ($P_2O_7^{4-}$)

PEP - phosphoenolpyruvate

PV - phosphovanadate

PK - pyruvate kinase

Tris-OAc - tris(hydroxymethyl)methyammonium acetate

$U_2V_2$ - $V^1,V^2$-di(uridine-2',3')divanadate

$V_i$ - vanadate ($VO_4^{3-}$)

$V_2$ - divanadate ( $V_2O_7^{4-}$)

$V_4$ - tetravanadate ($V_4O_{10}$, tetracoordinate vanadium) or ($H_4V_4O_{14}^{4-}$, pentacoordinate vanadium)

## General Introduction

The transition metal vanadium is widely distributed. In trace quantities it is present in both organic and inorganic matter. The vanadium atom is so electropositive that it is usually found coordinated to electronegative atoms such as oxygen and nitrogen. In dilute aqueous solutions of vanadium oxide at neutral pH, the predominant species are the vanadate anions $HVO_4^{2-}$ and $H_2VO_4^-$. Millimolar concentrations of these ions are toxic to living organisms ($LD_{75}$ i.p. in rats 4-5 mg/Kg, Merck Index), but at lower concentrations they may be essential for growth (Swarz and Milne, 1971). Although the mechanisms for the biological behavior of vanadium are not understood, they became of more clinical interest when it was discovered that vanadate could act as an insulin mimetic in diabetic rats (Heyliger et al., 1985). In addition, evidence that incubation with vanadate increases the oxygen affinity of whole human blood indicates that it may have the potential to benefit victims of sickle-cell anaemia (Ninfali et al., 1983; Stankiewicz et al., 1987). The biological effects of vanadium may be related to some of the many different effects that vanadate ions have on enzymes. For phosphatases and nucleases it is a potent inhibitor, while it appears to enhance the activity of other enzymes such as phosphoglucomutase and adenylate cyclase. Many of the kinases and dehydrogenases are not affected, or are only slightly inhibited by vanadate. (Chasteen, 1983 discusses some relevant aspects of vanadium chemistry and its effect on a variety of enzymes). Studies of the interactions between vanadate ions

and organic molecules were originally undertaken to investigate its influence on enzymes. It was thought that the effects that were observed must reflect, in part, the similarity of vanadate ions to the phosphate anions, $HPO_4^{2-}$ and $H_2PO_4^-$. Although vanadate and phosphate are analogous with respect to physical structure and electronic properties, their behavior in aqueous solution is quite different (Hanzlik, 1976; Pope and Dale, 1968). Vanadate ions are only slightly more basic than their phosphate counterparts, but, unlike phosphate, they readily form dimers and oligomers, particularly in acidic solution. As the studies progressed it became apparent that there are other important differences between vanadate and phosphate when they are added to solutions of organic molecules. The ability of vanadate to react spontaneously at hydroxyl groups to form esters has been detected using kinetic methods (Nour-Eldeen et al, 1985; Craig, 1986) and $^{51}V$ NMR (see references listed under Gresser, M.J. and under Tracey, A.S.). In addition, vanadate forms spontaneous anhydride bonds with phosphate groups, as well as with other vanadate ions. These anhydrides have also been detected using $^{51}V$ NMR (Gresser et al., 1986; Tracey et al., 1988a). Phosphate esters and anhydrides which have thermodynamic properties similar to those of vanadate, also form spontaneously in solutions of phosphate and alcohol. However the spontaneous formation of phosphate esters and anhydrides takes place on a time scale of years, rather than milliseconds, as in the case of vanadate compounds. Under physiological conditions the formation of phosphate compounds, and their hydrolysis, is catalyzed by enzymes. The similarities and differences between vanadate and phosphate and

the implications of these differences for biological systems are discussed in more detail where they become relevant to the experiments presented in this thesis.

The work reported here represents an investigation of several aspects of vanadate organic chemistry. In Part I the rapid, spontaneous formation of a vanadate ester is a step in the catalysis of a dehydration reaction. The results of these experiments demonstrate that vanadate can act both as an electrophile and as a nucleophile in the presence of hydroxyl and carbonyl groups respectively. It has been shown previously (Nour-Eldeen et al., 1985; Craig, 1986) that vanadate esters can be recognized as substrates by enzymes which normally bind the corresponding phosphate esters. The work reported in Part II was undertaken in an attempt to understand why vanadate has little effect on the kinases, ATP and ADP utilizing enzymes. There are theoretical and empirical reasons to expect that ADP-vanadate (ADPV) and AMP-vanadate (AMPV) will bind more tightly to the active site of a kinase than will its normal substrates ATP and ADP. However, no kinetic evidence for such binding was obtained. The basis for these predictions are discussed in the introduction to Part II, and a rationale has been proposed to account for the experimental results. $^{51}V$ NMR studies of vanadate and phosphate in the presence of other inorganic ions were undertaken to investigate the interactions of these ions in aqueous solution. The results of these studies are reported in Part III. The results of the experiments in Part III provide independent evidence in support of the conclusions reached in Part II.

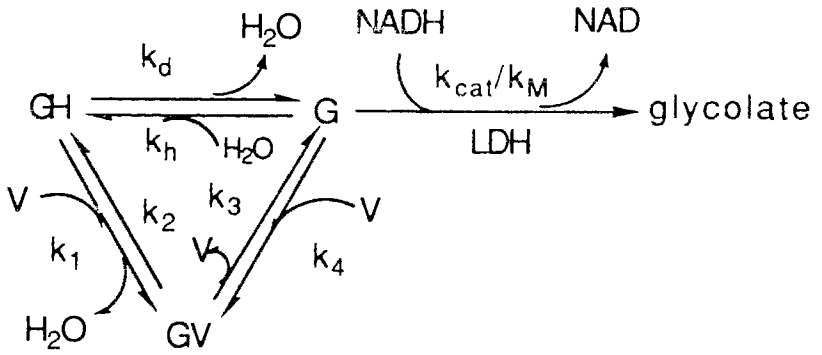
# Part I

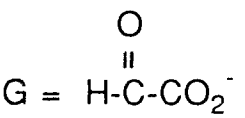
## Electrophilic Catalysis by Vanadate of the Dehydration of Hydrated Glyoxylate

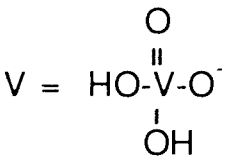
### Introduction

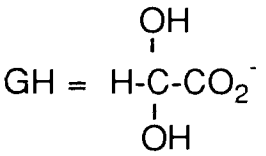
The rapid, spontaneous formation of vanadate esters has been observed in aqueous solutions of vanadate and organic molecules (Nour-Eldeen et al, 1985; also see references listed under Gresser, M.J. and under Tracey, A.S.). Therefore it is expected that the hydroxyl groups of glyoxylate hydrate will be esterified by vanadate to some extent in aqueous solutions containing glyoxylate and vanadate. In addition, the elimination of phosphate from the phosphorylated hydrate of D-glyceraldehyde has been shown to be several times faster than the dehydration of the hydrate under similar conditions (Rendina and Cleland, 1984). With these considerations in mind, vanadate ion was tested for its ability to enhance the reduction of glyoxylate by the mechanism shown in Scheme I.

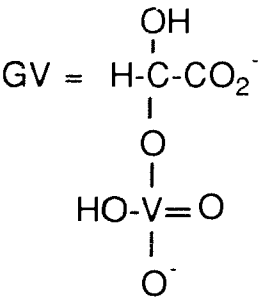
As depicted in Scheme IIb, lactate dehydrogenase (L-lactate: NAD oxidoreductase; 1.1.1.27) (LDH) has the ability to catalyze the oxidation and the reduction of glyoxylate. Nicotinamide adenine dinucleotide (NAD) is the oxidizing agent and the reduced form of NAD,



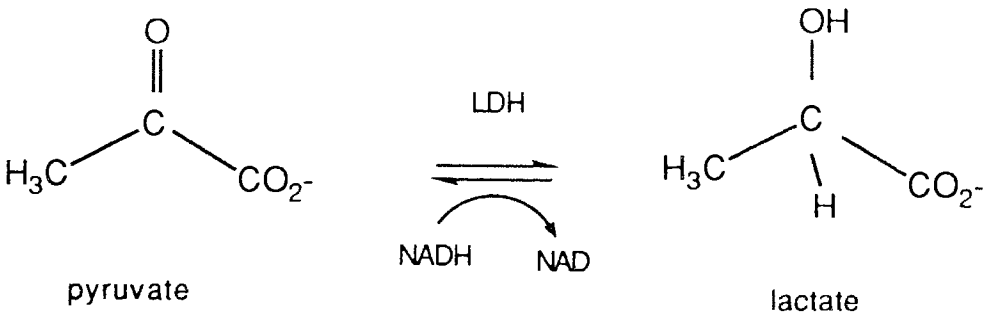

Scheme I

The uncatalyzed reversible hydration of glyoxylate and an alternative pathway which proceeds via the formation of a vanadate ester.

Scheme II

(a) The LDH-catalyzed interconversion of pyruvate and lactate

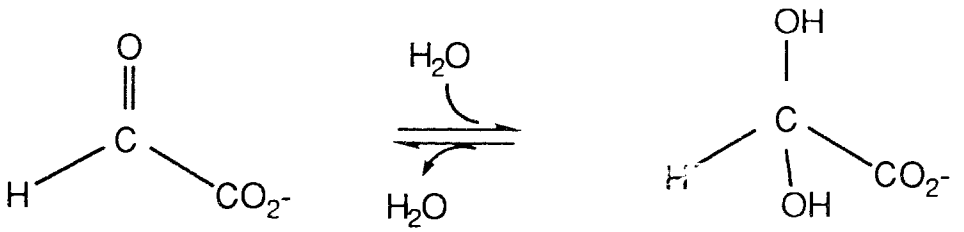
(b) The LDH-catalyzed reduction and oxidation of glyoxylate




pyruvate

lactate

(a)




glyoxylate

glyoxylate hydrate



glycolate

oxalate

(b)

NADH, is the reducing agent in the reaction catalyzed by LDH (Duncan and Tipton, 1969).

The natural substrates of LDH are pyruvate and lactate. As shown in Scheme II, the structural resemblance of glyoxylate to pyruvate and of the hydrated form of glyoxylate to lactate suggests that the anhydrous and hydrous forms of glyoxylate act as substrate analogues of pyruvate and lactate respectively. This idea has been supported by inhibition studies in which oxalate was shown to be a noncompetitive inhibitor of pyruvate reduction (Novoa et al., 1958) and of glyoxylate reduction (Duncan and Tipton, 1969). In addition oxalate was shown to be a competitive inhibitor of lactate oxidation (Novoa et al., 1958) and of glyoxylate oxidation (Duncan and Tipton, 1969). Thus it is likely that lactate and glyoxylate hydrate bind to the same site on LDH in the presence of NAD. Scheme II shows the LDH catalyzed redox reactions of lactate and pyruvate, and the proposed model of the LDH catalyzed reactions in the presence of the analogues glyoxylate hydrate and glyoxylate.

Glyoxylate is predominantly hydrated in aqueous solution (Gunshore et al., 1985). It was determined by Everse and Kaplan, 1973, that it is the anhydrous form of glyoxylate that acts as a substrate in the LDH catalyzed oxidation of NADH. The specificity of LDH for the anhydrous form of glyoxylate as a substrate in the oxidation of NADH has been put to use in the following experiments.

The rate of reduction of glyoxylate in the presence of vanadate is significantly accelerated. The lower pathway of Scheme I, the route through the vanadate ester intermediate (GV), can account for the

observed enhancement by vanadate of the reduction of glyoxylate. It is possible that the kinetically significant complex is not the vanadate ester. Aqueous solutions of vanadate and other organic acids have been shown to contain a variety of complex molecules (Tracey et al., 1987). Some of these types of complexes have been implicated in the catalysis by other transition metals of the dehydration of glyoxylate (Meany and Pocker, personal communication) and of 2,2-dihydroxypropionate (Pocker and Meany, 1970). This will be discussed in more detail after the results of the experiments with glyoxylate and vanadate have been presented.

In order to determine if the reduction of glyoxylate is enhanced in the presence of other anions, phosphate and arsenate were tested in the absence of vanadate for their ability to accelerate glyoxylate reduction.

# Procedure

## Materials

4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and nicotinamide adenine dinucleotide, reduced form (Grade II) (NADH) were obtained from Boehringer Mannheim. Crystalline L-lactate:NAD oxidoreductase (EC 1.1.27) (LDH) and glyoxylic acid from Sigma were used without further purification. Sodium metavanadate was purchased from Matheson, Coleman and Bell.

## Method

Stock solutions of NADH, glyoxylate, LDH, vanadate, phosphate and arsenate were prepared in 0.1 M HEPES buffer (pH 7.2) and adjusted to pH 7.2 with potassium hydroxide or hydrochloric acid. In a test of the effect of vanadate on the the rate of reduction of glyoxylate by NADH, reaction mixtures contained 0.1 M HEPES, 0.15 mM NADH, 0.1 mM glyoxylic acid and the appropriate concentrations of LDH and vanadate. LDH concentrations varied over a range of 0 - 1.1 mg/mL. Vanadate concentrations were 0, 0.20, 0.40 and 0.60 mM in total vanadate. The concentrations of monomeric vanadate were calculated from the total vanadium atom concentrations and the equilibrium constants for the formation of vanadate oligomers. These calculations are described in Appendix I. In other experiments 0.60 mM phosphate or

0.60 mM arsenate was substituted for vanadate in the presence of 1.0 mg/mL LDH; other conditions were the same. The reaction was initiated by adding 10 μL of the glyoxylic acid stock solution to a reaction mixture which contained all of the other reagents at 30°C, pH 7.2. The total sample volume was 1.0 mL. The rate of reaction was measured after five seconds, the minimum time required for the reactants to mix and for the recorder pen to stabilize. The dehydration rate was measured by following a decrease in absorbance at 340 nm, due to the oxidation of NADH, using a molar extinction coefficient of $6.22 \times 10^3\ M^{-1}cm^{-1}$. A Cary-17 spectrophotometer with a temperature-controlled cell holder was used for the measurements. No change in absorbance was observed before the addition of glyoxylate.

## Analysis of Data

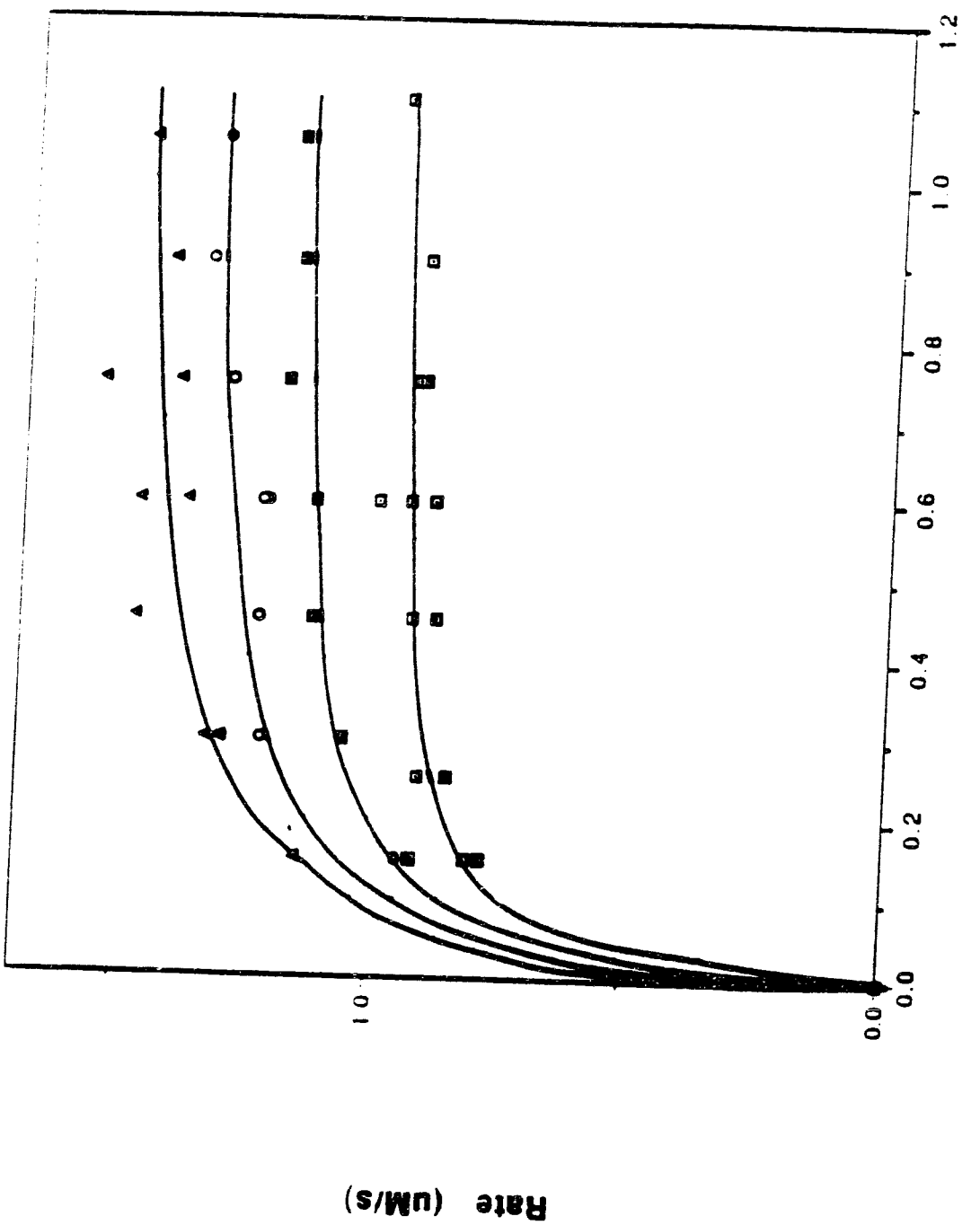
The enhancement by vanadate of the reduction of glyoxylate by NADH, catalyzed by LDH is shown in Figure 1. The points in Figure 1 were determined by experiment; the curves were calculated using equation 4 which was derived on the basis of the mechanism shown in Scheme I. The saturation behavior of the curves in Figure 1 can be explained if the dehydration step becomes rate limiting at high enzyme concentrations. The substrate, glyoxylate (G), does not appear in the denominator of equation 1 as its concentration is much lower than its $K_M$ ($4.18 \times 10^{-5}$ M, Meany and Pocker, personal communication) as a substrate for LDH The equilibrium constant for the hydration of glyoxylate has been estimated to be 163 at 25°C at pH 7.4 (Gunshore et al., 1985), Thus more than 99% exists as the hydrate (GH). The results of studies by Everse and Kaplan, 1973, indicate that it is the anhydrous glyoxylate (G) that is accepted for reduction by the enzyme. The cofactor NADH is at a constant, saturating concentration, and therefore its concentration is included in the constant term of equation 1.

$$\text{Rate} = \frac{k_{cat}}{K_m} [\text{LDH}] [\text{G}] \qquad (1)$$

The rate equation derived for the mechanism in Scheme II is shown below (2). It is assumed that the formation of the vanadate glyoxylate ester, GV, is in equilibrium with GH and $V_i$. The rapid, reversible equilibrium that is established between vanadate and its

Figure 1

The effect of different LDH concentrations on the rate of reduction of glyoxylate in the absence and presence of vanadate. Conditions were the following: 0.10 M Hepes, pH 7.2, 25°C, 0.15 mM NADH, 0.10 mM glyoxylic acid, and the indicated concentrations of LDH. Reactions were initiated by the addition of glyoxylic acid. The sample volume was 1.0 mL. Total vanadium atom concentrations (added as inorganic vanadate) were as follows: (□) 0, (■) 0.20, (○) 0.40 and (▲) 0.60 mM. The solid lines were calculated from equation 4 by using the constants $k_d$ = 0.0098 $s^{-1}$, $k_3K_{eq}$ = 12.66 $M^{-1}s^{-1}$, $k_h/(k_{cat}/K_M)$ = 0.0336 mg $mL^{-1}$, $k_4/(k_{cat}/K_M)$ = 36 mg $ml^{-1}$ $M^{-1}$. The values used in the calculations for vanadate concentrations were: (■) 0.179, (○) 0.322 and (▲) 0.437 mM. The reaction was monitored by measuring the rate of oxidation of NADH in accordance with the mechanism shown in Scheme I.


[V] – 0
[V] – 0.20 mM
[V] – 0.40 mM
[V] – 0.60 mM
Rate (uM/s)
[LDH] (mg/mL)
0.0
0.2
0.4
0.6
0.8
1.0
1.2

esters has been described in previous work (Nour-Eldeen et al., 1985; Gresser and Tracey, 1985; Tracey et al., 1987). Equation 2 can be rearranged to equation 3 which expresses the steady-state glyoxylate concentration in terms of the independent variables [GH], [LDH] and $[V_i]$, where $[GH] = [GV] / (K_{eq}[V_i])$.

$$\frac{d[G]}{dt} = 0 = k_d[GH] + k_3[GV] - k_h[G] - k_4[G][V_i] - \frac{k_{cat}}{K_m}[G][LDH] \qquad (2)$$

$$[G] = \frac{k_d[GH] + k_3K_{eq}[GH][V_i]}{k_h + k_4[V_i] + \frac{k_{cat}}{K_m}[LDH]} \qquad (3)$$

Substituting the expression for [G] from equation 3 into the rate equation and taking the reciprocal, yields equation 4.

$$\frac{1}{Rate} = \frac{k_h + k_4[V_i]}{\frac{k_{cat}}{K_m}[GH](k_d + k_3K_{eq}[V_i])} \frac{1}{[LDH]} + \frac{1}{[GH](k_d + k_3K_{eq}[V_i])} \qquad (4)$$

In equation 4 [GH] is assumed to be equal to the total glyoxylate concentration, for reasons discussed above. The value for the equilibrium constant for the formation of the vanadate ester which is present in aqueous solutions of vanadate and lactate is equal to 0.5 $M^{-1}$. This value was obtained at 1.0 M ionic strength, pH 7.35 ( Tracey et al., 1985), but it provides an approximate value for the equilibrium constant for the formation of glyoxylate vanadate. It has been

multiplied by 2 to account for the presence of 2 hydroxyl groups on glyoxylate hydrate, to obtain $K_{eq} = 1.0\ M^{-1}$.

Plots of 1/Rate vs 1/[LDH] at different concentrations of $V_i$ were linear as shown in Figure 2. The relation between the reciprocal of the vertical intercept of equation 4 and the vanadate concentration is shown in equation 5.

$$\frac{1}{V.I.} = [GH](k_d + k_3K_{eq}[V_i]) \qquad (5)$$

When the values for 1/V.I. of Figure 2 were plotted vs $[V_i]$, as shown in Figure 3, a value for $k_d$ was determined from the vertical intercept where $[V_i] = 0$. $k_3K_{eq}$ was obtained from the slope of Figure 3.

The relation between the slope divided by the vertical intercept of Figure 2 and the vanadate concentration is shown in equation 6. The plot of slope/V.I. vs $[V_i]$ is shown in Figure 4.

$$\frac{\text{slope}}{V.I.} = \frac{k_h + k_4[Vi]}{\frac{k_{cat}}{K_m}} \qquad (6)$$

When $[V_i] = 0$ equation 6 yields a value for $k_h/(k_{cat}/K_m)$. By using the published value of 163 for $k_h/k_d$ at 25 °C, pH 7.4 (Gunshore et al., 1985), and the $k_d$ determined above, a value for $k_h$ was estimated. The value of $k_h$ was used to determine $k_{cat}/K_m$ from equation 6 when $[V_i] = 0$, and to determine $k_4/(k_{cat}K_m)$ at known vanadate concentrations.

Figure 2

Double reciprocal plots of the data from Figure 1. The effect of different LDH concentrations on the rate of the reduction of glyoxylate in the absence and presence of vanadate. Total vanadium concentrations (added as inorganic vanadate) were (❑) 0, (◆) 0.20, (■) 0.40 and (◊) 0.60 mM. Conditions were as described in the legend to Figure 1.

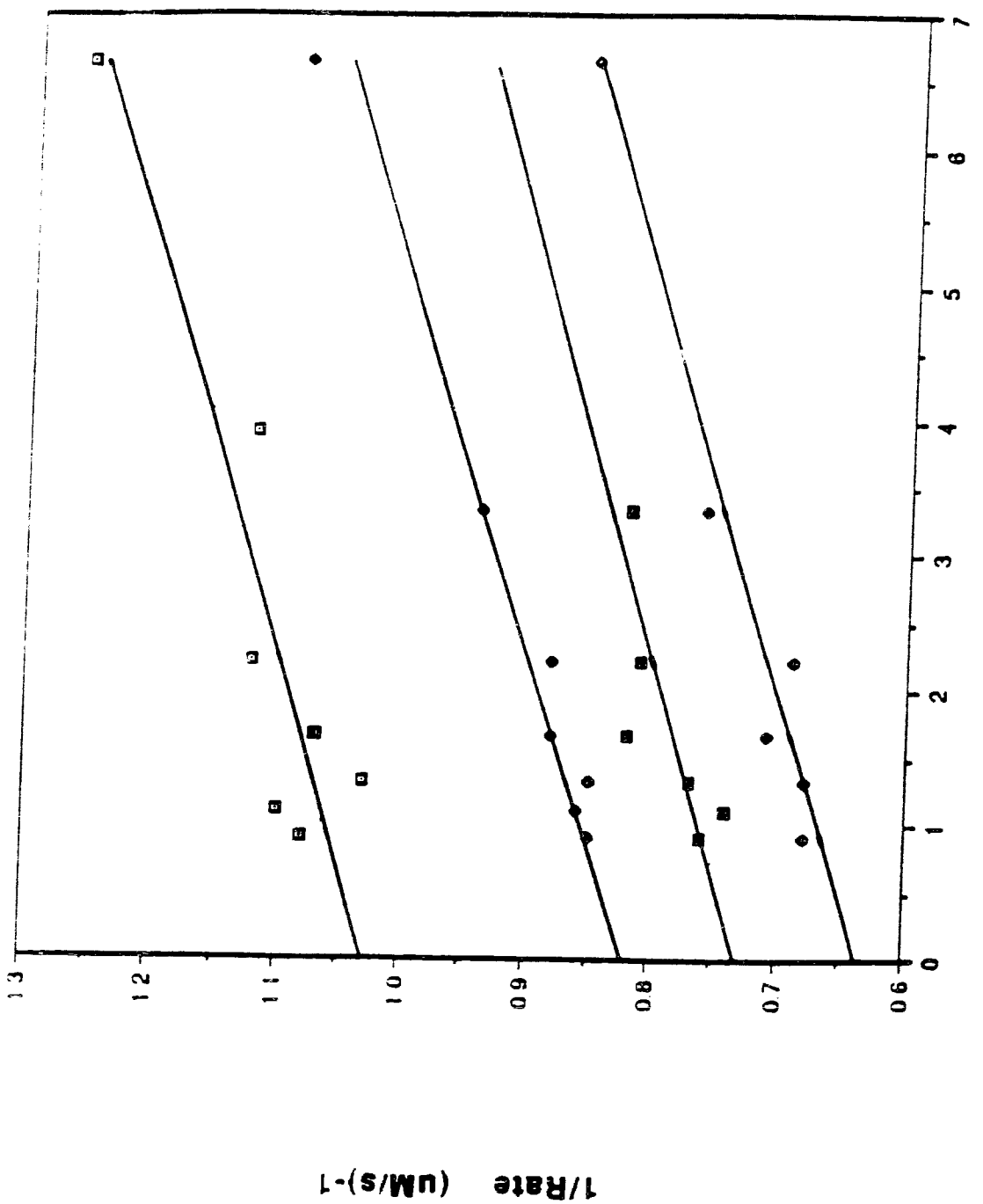

[V] = 0
[V] = 0.20 mM
[V] = 0.40 mM
[V] = 0.60 mM
1/Rate (uM/s)-1
1/[LDH] (mg/mL)-1
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
0
1
2
3
4
5
6
7

Figure 3

The reciprocals of the vertical intercepts (V.I.) of the lines in Figure 2 vs the vanadate concentration ($V_i$). Conditions were as described in the legend to Figure 1.

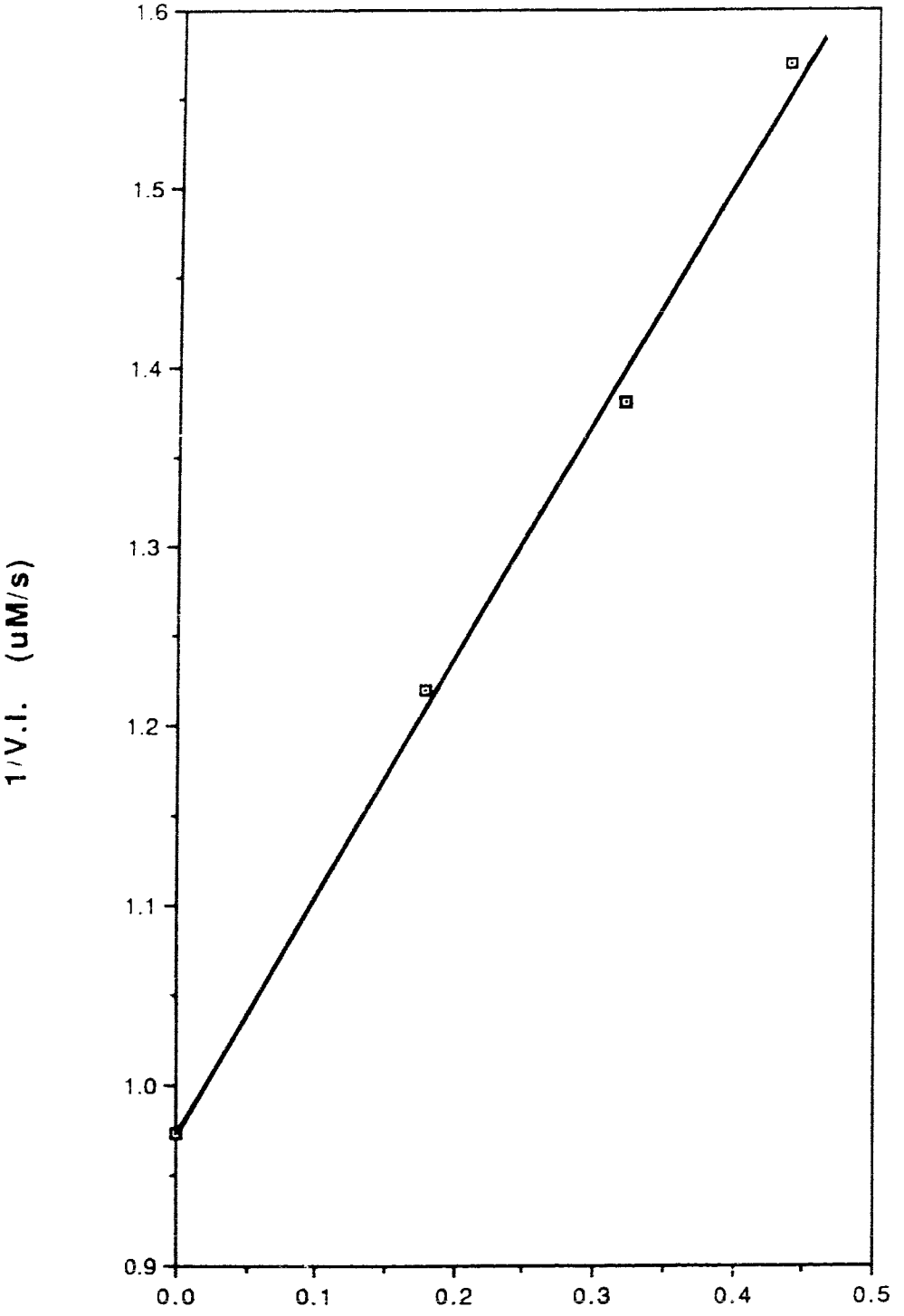
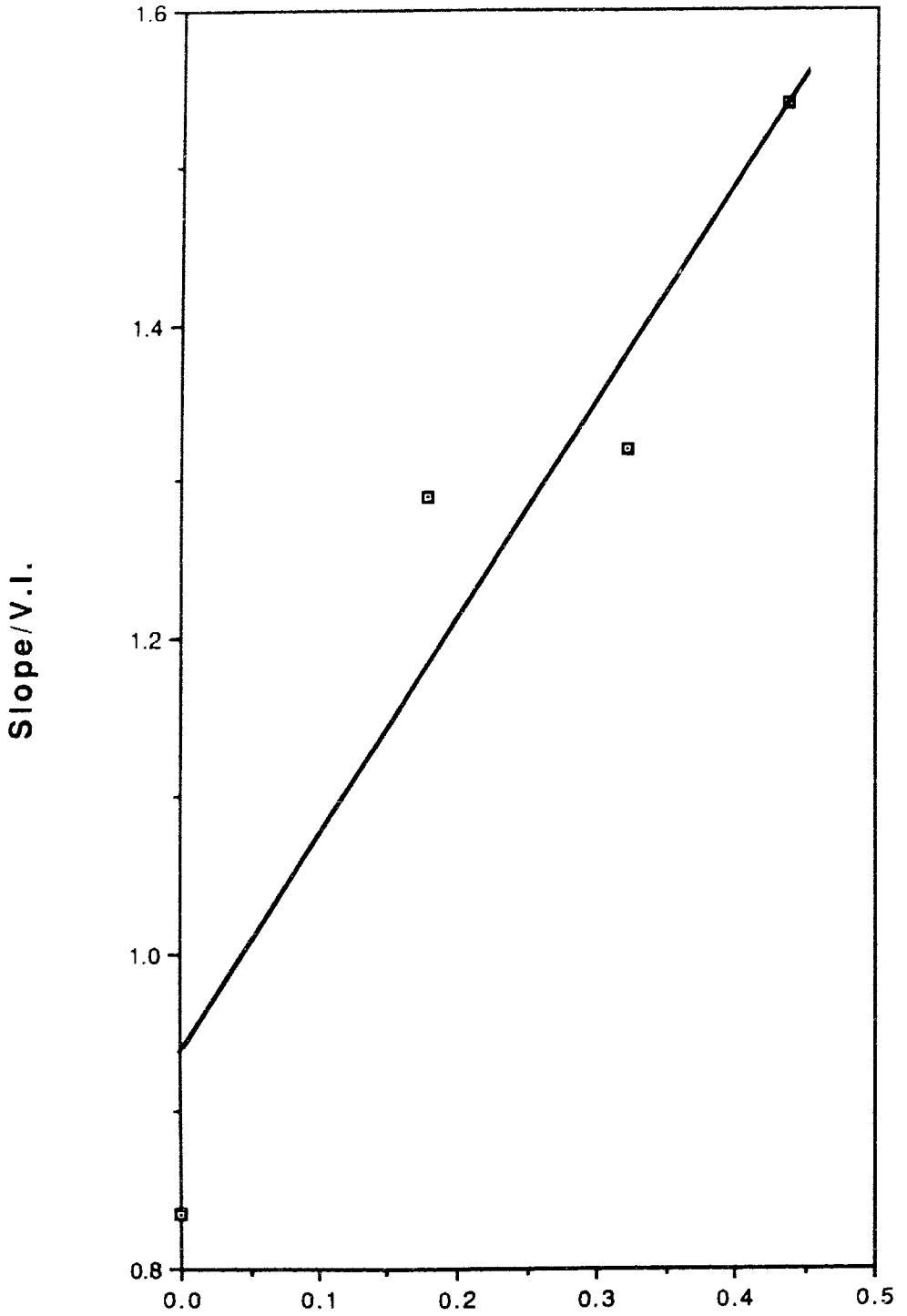

1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.0
0.1
0.2
0.3
0.4
0.5
1/V.I. (uM/s)
[V} (mM)

Figure 4

The slopes multiplied by the reciprocals of the vertical intercepts (V.I.) of the lines in Figure 2 vs vanadate concentration ($V_i$). Conditions were as described in the legend to Figure 1.


1.6
1.4
1.2
1.0
0.8
0.0
0.1
0.2
0.3
0.4
0.5
Slope/V.I.
[V] (mM)

## Results and Discussion

The values obtained from the secondary plots of the data from Figure 1 (Figures 2, 3 and 4) were used as initial estimates for the parameters in equation 4. A nonlinear, least squares program (BMDP) was used to determine the constants that provided the best fit of equation 4 to all of the data from Figure 1. The values obtained from this procedure were as follows:

$k_d = 0.0098 \pm 0.0002\ s^{-1}$

$k_3K_{eq} = 12.7 \pm 0.8\ M^{-1}s^{-1}$

$k_h/(k_{cat}/K_m) = 0.034 \pm 0.009\ mg\ mL^{-1}$

$k_4/(k_{cat}/K_m) = 36 \pm 28\ mg\ mL^{-1}M^{-1}$

Similar values were obtained when the experiment was repeated using lower concentrations of LDH, and the values were not affected by changing the concentration of glyoxylate. From the value of $k_d$ determined by the curve-fitting technique described above, and the published value of 163 for $k_h/k_d$ (Gunshore et al, 1985),

$k_h = 1.60\ s^{-1}$

$k_3 = 12.7\ s^{-1}$

$k_4 = 2.1 \times 10^3\ M^{-1}s^{-1}$

The value obtained for the rate constant for the dehydration of glyoxylate, $k_d = 0.0098\ s^{-1}$ at pH 7.2, 30°C, compares well with the $k_d$

of 0.0095 $s^{-1}$ at pH 7.0, 25°C, obtained by Rendina et al., 1984, using the same experimental method. The value of 12.7 $s^{-1}$ for $k_3$, the rate constant for the elimination of vanadate from the vanadylated glyoxylate hydrate, is more than 100 times greater than the value of 0.035 - 0.043 $s^{-1}$ reported for the elimination of phosphate from the phosphorylated glyceraldehyde hydrate at pH 7.0, 15°C (Rendina and Cleland, 1984). The presence of the carboxylate anion on glyoxylate may facilitate the elimination of the vanadate anion. Phosphate and arsenate failed to accelerate glyoxylate reduction at pH 6.0 and pH 8.5, whereas vanadate, at similar concentrations accelerated the rate of glyoxylate reduction throughout this range. These results, shown in Figures 5 and 6, indicate that the enhancement of glyoxylate reduction is specific for the presence of the vanadate ion.

Alternatively, the species which catalyses the hydrolysis of hydrated glyoxylate may be a cyclic diester in which $V_i$ is chelated by hydroxyl groups from each of the carbons on glyoxylate. Aqueous solutions of $V_i$ with lactate were found to contain cyclic diesters of 5-coordinate ($K_{eq}$ = 9.3 $M^{-1}$) and 6-coordinate ($K_{eq}$ = 2.9 x $10^{11}$ $M^{-4}$) vanadium. The 6-coordinate lactate/$V_i$ cyclic diester was binuclear in $V_i$. The 6-coordinate species was favoured at low pH as a proton was required for its formation.

In aqueous solutions of 1.0 mM $V_i$ and 0.8 mM lactate at pH 7.1, the vanadium atom concentrations for the monomeric ester,

Figure 5

The effect of different anion concentrations on the rate of reduction of glyoxylate. Conditions were 0.10 M Hepes, pH 6.0, 25°C, 0.15 mM NADH, 0.10 mM glyoxylic acid, 1.0 mg/mL LDH and the indicated concentrations of anion: vanadate ($V_i$) (□), phosphate ($P_i$) (○) arsenate ($As_i$) (X). $V_i$ concentrations are those of total $V_i$ added as inorganic vanadate. Reactions were initiated by the addition of glyoxylic acid. The sample volume was 1.0 mL. The reaction was monitored by measuring the rate of NADH oxidation in accordance with the mechanism shown in Scheme I.

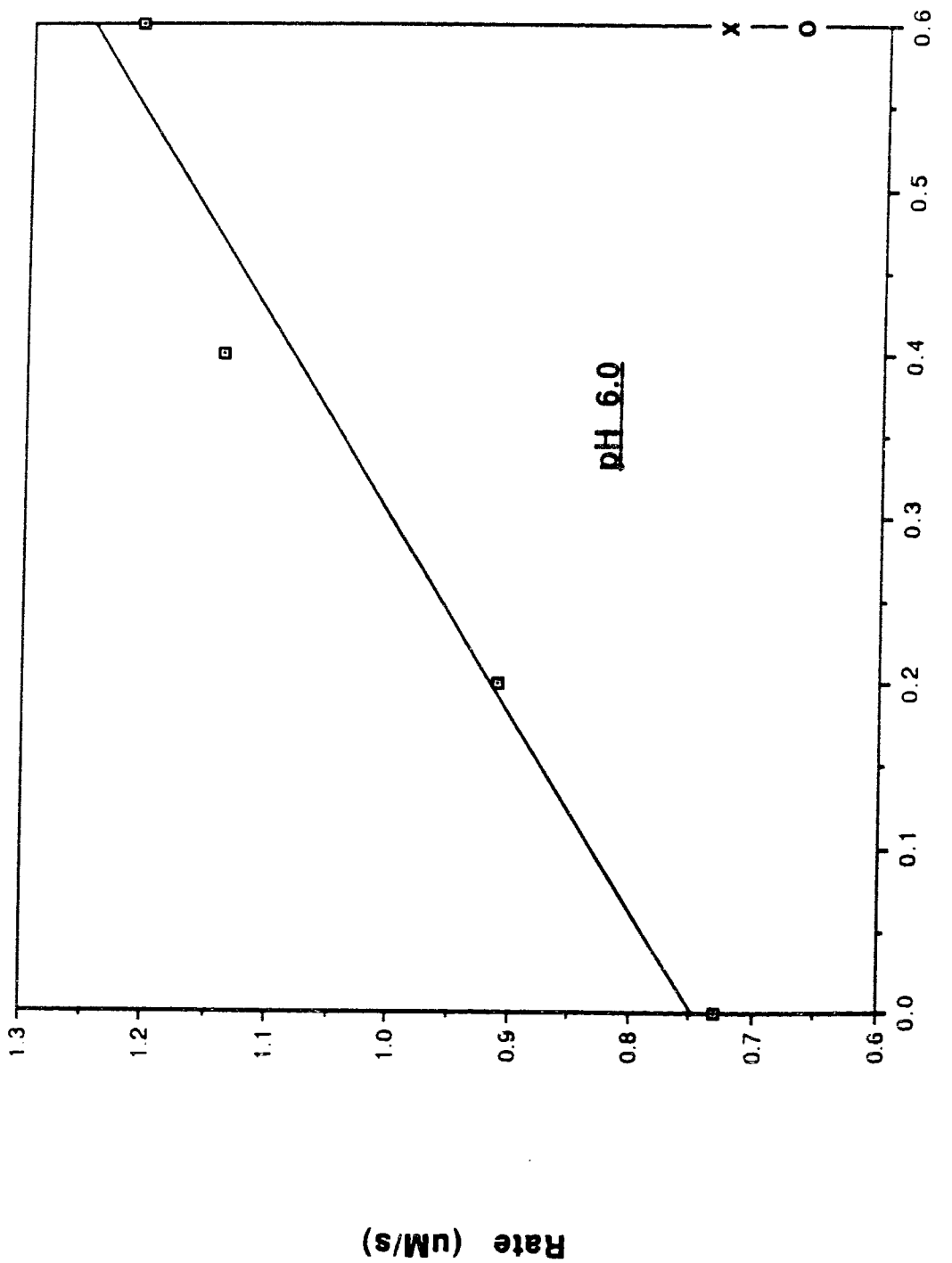
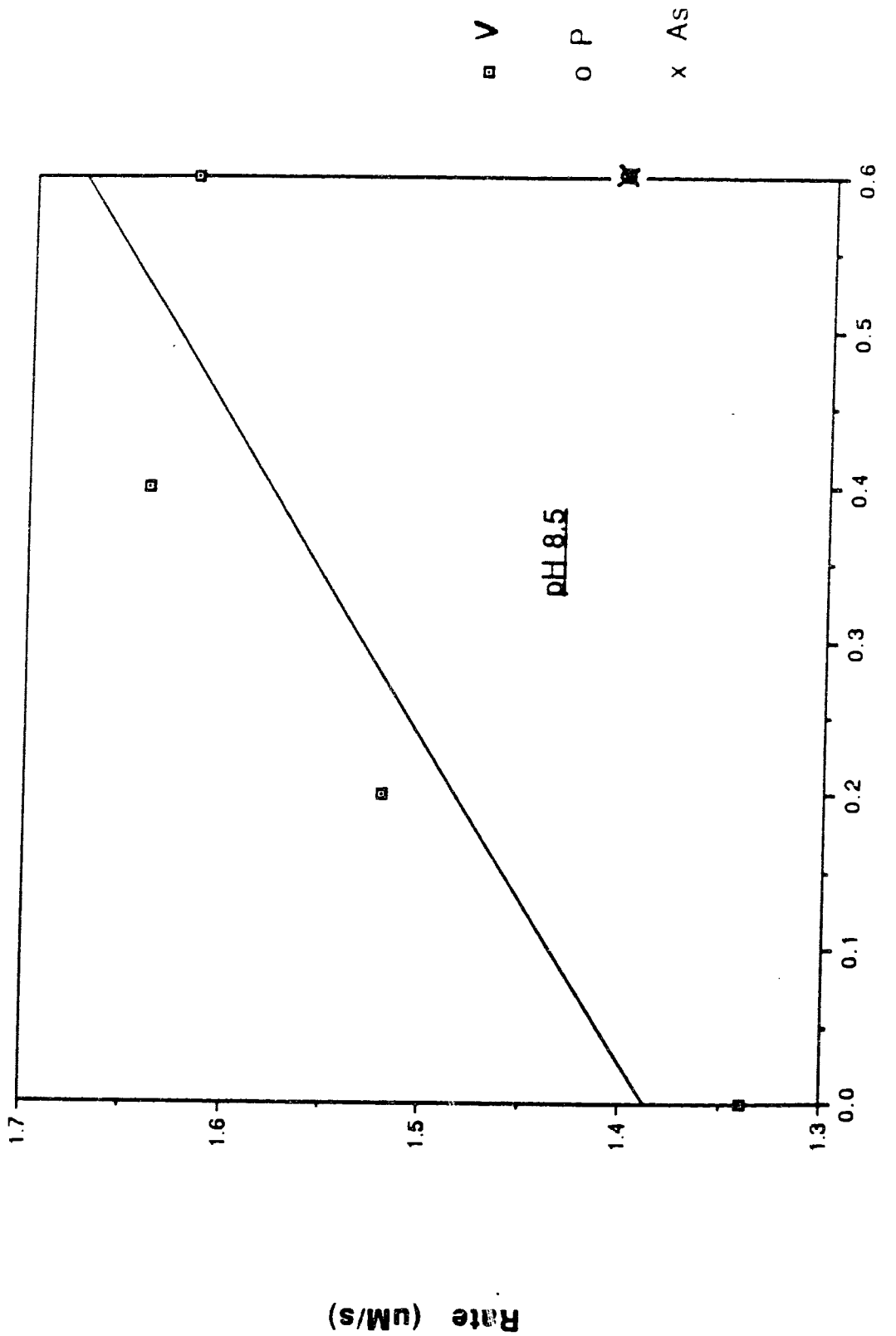

V
Pi
Asi
pH 6.0
Rate (uM/s)
[V, P, As]
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3

Figure 6

The effect of different anion concentrations on the reduction of glyoxylate. Conditions were the same as described in Figure 5, except that the pH was 8.5.


V
P
As
pH 8.5
Rate (uM/s)
[V, P, As]
1.7
1.6
1.5
1.4
1.3
0.0
0.1
0.2
0.3
0.4
0.5
0.6

the 5-coordinate bipyramidal cyclic diester and the 6-coordinate cyclic diester were 0.011, 0.230, 0.322 mM respectively. In addition there was approximately 0.060 mM $V_i$ atoms involved in various other combinations of monoester/diester bonds with lactate molecules. Presumably solutions of glyoxylate and $V_i$ contain similar complexes. Meany and Pocker (personal communication) determined that the dehydration of glyoxylate is catalyzed by other transition metal ions, the order of catalytic effectiveness being: $Zn^{2+} > Cu^{2+} > Co^{2+} > Ni^{2+} > Cd^{2+} > Mn^{2+}$. The possible mechanisms postulated by these investigators involve electrophilic attraction of the positive metal centers for the oxygen electrons on one or both of the hydroxyl groups on glyoxylate. The experiments reported here cannot differentiate between a monomeric or dimeric intermediate. However it is noted in Figure 2 that the reciprocal of the rate of reaction was linear with respect to vanadium atom concentration. This suggests that a binuclear cyclic diester of glyoxylate and $V_i$ was not significantly involved in the catalysis.

The experiments described above are significant for two reasons. First, they illustrate the versatile nature of vanadate chemistry. The vanadate ion can accept electrons at the electropositive vanadium center or can donate electrons from its oxygen ligands to other electropositive atoms. The second reason for the significance of the vanadate chemistry observed in these experiments is that it may help to explain some of the physiological effects of vanadate. Some recent developments in glyoxylate chemistry that may be relevant to some of the observed clinical effects of vanadate are discussed below.

Glyoxylate at concentrations of 10 - 100 μM is a normal component of animal cells. Glyoxylate is an inhibitor of the pyruvate dehydrogenase complex, competitive with pyruvate ($K_i$ = 80 μM, pH 7.0, Beatty and Hamilton, 1985). It has been suggested that glyoxylate partially controls the activity of the pyruvate dehydrogenase complex, an important link between glycolysis and the citric acid cycle in aerobic cells. The inhibition by glyoxylate is largely reversed in the presence of millimolar concentrations of thiols, particularly cysteine, with which glyoxylate forms spontaneous adducts, or thiohemiacetals. Gunshore et al., 1985, measured equilibrium constants of 0.2 - 1.7 $mM^{-1}$ for the formation of thiohemiacetals formed by glyoxylate and several different thiols common to the cytosol. Given that cellular thiols are normally present in millimolar concentrations, they determined that about half of the glyoxylate present in the cell will be in the form of a thiohemiacetal complex.

The thiohemiacetals formed spontaneously in the presence of glyoxylate and thiols have been shown to act as substrates for peroxisomal oxidases such as D-amino acid oxidase, D-aspartate oxidase and L-hydroxy acid oxidase which convert them to oxalyl thioesters (OTE) in the presence of oxygen (Gunshore et al., 1985). The pathway from free glyoxylate and thiol to OTE is shown below.

$$RSH + HCO\text{-}COO^- \rightleftarrows RS\text{-}CHOH\text{-}COO^-$$

$$RS\text{-}CHOH\text{-}COO^- + O_2 \rightarrow RS\text{-}CO\text{-}COO^- + H_2O_2$$

It has recently been reported (Harris and Hamilton, 1987) that OTE's are present in animal cells in concentrations of up to 50 μM. Evidence has been accumulating to support the role of OTE's as metabolic control factors in animal cells. For instance, S-oxalylglutathione, the oxalyl thioester of glyoxylate and glutathione, is a potent inhibitor of malic enzyme ($K_i$ = 5 μM at pH 7.4, 25°C, Harris and Hamilton, 1987). Malic enzyme catalyzes the oxidative decarboxlyation of malate in the presence of NADP. The products of the reaction are pyruvate and NADPH, a vital reducing agent for fatty acid synthesis. The production and specific activity of malic enzyme are both stimulated by insulin. Thus it appears that S-oxalylglutathione is a negative effector of insulin.

The relationship between OTE's and cell growth was investigated further by comparing OTE levels in resting and in rapidly growing cells. As expected, OTE levels of resting cells were significantly higher than those of rapidly proliferating transformed fibroblasts and of lectin-stimulated lymphocytes (Skorczynski et al., 1988). This effect could be a consequence of the high level of γ-glutamytransferase (GGT) that is typical of many tumour cells, as GGT catalyzes the catabolism of glutathione and its conjugates. At pH 7.4, 25°C, the $K_M$ for S-oxalylglutathione as a substrate for bovine kidney GGT was 0.36 mM for the transfer of the γ– glutamyl group to glycylglycine, and 7.0 μM for the transfer of the γ-glutamyl group to water (Hamilton et al.,1988).

Vanadium is normally present in mammals as an ultratrace element. The total-body pool of vanadium is approximately 100 μg, most of it present inside the cells. It is differentially distributed

among the tissues with the highest concentrations being found in liver (≈ 0.2 μM) (see Nechay et al., 1986, and references therein for a brief summary of the role of vanadium in biology). In vivo vanadium is distributed between the +4 (vanadyl) or the +5 (vanadate) oxidation state. Most of the intracellular vanadium is bound to proteins or to phosphates (Nechay et al., 1986). In the bound state the oxidation of $VO^{2+}$ to $VO_4^{3-}$, that would normally take place in the presence of cellular oxygen, is prevented. At normal physiological levels the effects of vanadate on glyoxylate metabolism are probably negligible. However, higher concentrations of vanadium have indisputable biological effects, some of which could involve the interference of vanadate ions in glyoxlate metabolism. For instance, the hydrolysis of an OTE could proceed through a nucleophilic attack by vanadate on the carbonyl of the thioester, with elimination of the thiol and the subsequent hydrolysis of the vanadium-oxygen bond. This is a reasonable mechanism considering the nucleophilic behavior of vanadate that has been observed in the experiments reported here and by DeMaster and Mitchell, 1973. DeMaster and Mitchell found that vanadate uncoupled the oxidative phosphorylation of glyceraldehyde-3-phosphate catalyzed by glyceraldehyde-3-phosphate dehydrogenase. The behavior of vanadate was similar to that of arsenate in that these anions compete with phosphate in a nucleophilic attack on the carbonyl carbon of the enzyme-bound 3-phosphoglycerate. Vanadium could also affect OTE levels by another mechanism. Thiols such as glutathione are complexed by oxovanadium ions. Thus an increase in vanadium concentration would decrease the concentration of thiol available for

the oxalylthiohemiacetal formation with glyoxylate. A reduction in OTE levels appears to have a similar effect on the cell as that of an increase in insulin concentration, that is, among other things, the reversal of OTE inhibition of malic enzyme and the stimulation of cell growth. Vanadate has been shown to stimulate cell growth (see below), but its effects on the OTE levels of cells and on the GGT enzyme have not yet been tested.

Vanadate behaves as an insulin mimetic, although the mechanism for this effect in not known (Chasteen, 1983, Heyliger et al., 1985). A possible mechanism for the insulin mimetic effect of vanadate is discussed in a report by Tracey and Gresser, 1986. Vanadate has also been shown to mimic and potentiate the effects of epidermal growth factor (Carpenter, 1981). It is a potent inhibitor of tyrosine phosphatase. The effects of insulin and epidermal growth factor are mediated by tyrosine phosphorylation. Thus it is possible that the mimetic effects of vanadate are produced by its ability to inhibit the tyrosine phosphatases that control the phosphorylation of the receptors for insulin and epidermal growth factor. Vanadate has the potential ability to interfere with normal metabolic processes through its spontaneous reactivity with hydroxyl groups and carbonyls. Exactly how this chemical behavior is related to the clinical effects of vanadium is interesting as a present and future subject of investigation.

# PART II

## The Effects of Vanadate on Reactions Catalyzed by the ATP-utilizing Enzymes Pyruvate Kinase, Myokinase and Hexokinase.

### Introduction

In the experiments described in this section the effect of vanadate on 3 ATP-utilizing enzymes has been investigated. In the presence of adenosine (Ad), adenosine monophosphate (AMP) and adenosine diphosphate (ADP), it is expected that vanadate will spontaneously form a variety of ester and anhydride bonds. Vanadate ester formation at the hydroxyl groups of small organic molecules has been well documented by $^{51}V$ NMR (see references listed under Gresser, M.J. and under Tracey, A.S.), and it has been shown that vanadate esters can be recognized by enzymes which normally act upon the corresponding phosphate esters (Nour-Eldeen et al., 1985, Craig, 1986). Mixed vanadate/phosphate anhydrides have been detected by $^{51}V$ NMR in aqueous solutions containing vanadate and phosphate or pyrophosphate (Gresser et al., 1986), and in solutions of vanadate and AMP (Tracey et al., 1988a). It is reasonable to expect that the mixed phosphate/vanadate anhydrides formed in these solutions can be utilized by enzymes in the same way as vanadate esters. Under physiological conditions, pyruvate kinase (PK) and hexokinase (HK)

catalyze reactions which involve the substrates ADP and ATP, while myokinase (MK) recognizes AMP, ADP and ATP. In the present work solutions which were expected to contain the substrate analogues adenosine vanadate (AdV), adenosine monophosphovanadate (AMPV), adenosine vanadophosphate (AdVP) and adenosine vanadopyrophosphate (AdVPP), substrate analogues of AMP, ADP and ATP, were tested for activity with PK, MK and HK under appropriate conditions.

In additional experiments vanadate alone and in the presence of adenine nucleotides was tested for its ability to inhibit the normal (physiological) reactions of PK, MK and HK. It has been reported that the adenosine diphosphovanadate anhydride, ADPV, is a potent inhibitor of the enzymes myosin ATPase (Goodno, 1982) and dynein ATPase (Evans et al., 1986). In the ATPase reaction a phosphoryl group is transferred from ATP to a water molecule. The inhibition has been rationalized by considering that the intermediate complex formed between the bound ADPV and the incoming water molecule should be more stable than the corresponding ATP complex. The difference in stability is thought to be due to the fact that, in order to form the nucleotide/anion/$H_2O$ complex (the anion being phosphate or vanadate), a pair of electrons from the water molecule must be accepted by the vanadate or phosphate moiety, increasing the coordination number of the central atom to 5. Vanadate has 3d orbitals of relatively low energy available, and is thus stable in a 5-coordinate configuration. Phosphate must accept electrons into relatively high energy 3d orbitals. The instability of 5-coordinate phosphate facilitates the breakdown of the complex into products, a necessary condition for the

normal reaction to take place. It is thought that the stability of the 5-coordinate vanadate in the intermediate complex is responsible for the observed inhibition of the myosin and dynein ATPases. Similar arguments have been put forward to explain the potent inhibition by vanadate of enzymes which normally pass through phosphorylated intermediates, such as the acid and alkaline phosphatases (Chasteen, 1983). Phosphatases pass through a phosphoenzyme intermediate in which phosphate is covalently bound to the enzyme. As in the ATPase reaction, the bound phosphate must accept a pair of electrons from a water molecule, forming a 5-coordinate transition state, before it is released from the enzyme. In the presence of vanadate the normal activity of the phosphatase enzyme is inhibited by the relatively stable intermediate formed by enzyme, vanadate and the incoming water molecule. In view of the evidence available about the behavior of vanadate with the phosphatases and with the myosin and dynein ATPases, it would not be surprising if vanadate were a good inhibitor of other systems which involve phosphate transfer. However, reports in the literature indicate that vanadate is not an effective inhibitor of several of the kinases. Vanadate, in millimolar concentrations, does not inhibit pyruvate kinase and is only a weak inhibitor of hexokinase (Climent et al., 1981). Moreover, even in the presence of ADP, under conditions where it would be expected to form a strong transition state analogue, it is only a weak inhibitor of glycerol kinase (Gresser and Stankiewicz, unpublished results). In the kinase reaction a phosphoryl group is transferred from ATP to an organic ligand. The transition state involves nucleotide, enzyme and incoming ligand, rather than a

phosphoenzyme/ligand intermediate (Blattler and Knowles, 1979.; Lowe et al., 1981). The presence of a divalent cation is required for the normal activity of all ATP utilizing enzymes. Both ATP and ADP have been shown by NMR to be associated with a divalent cation when bound to phosphoryl transferring enzymes (Mildvan and Fry, 1987). The presence of the cation is thought to facilitate the kinase (or ATPase) reaction by assisting the binding of the nucleotide substrate to the enzyme. In crystallographic studies of pyruvate kinase/substrate complexes, the cation has been observed to be coordinated to the nonbridging oxygens of the $\beta$ and $\gamma$ phosphate groups of ATP (Muirhead et al., 1987). In this position it can displace some of the negative charge on the oxygens away from the phosphorus atom, making it more susceptible to nucleophilic attack from basic groups on the incoming ligand. The phosphate groups of ATP and ADP have the ability to chelate divalent cations in aqueous solutions and it is the nucleotide/metal complexes MgATP and MgADP that are thought to be the true substrates for phosphoryl transferring enzymes (Connolly and Eckstein, 1981). However, $^{51}V$ NMR studies of aqueous solutions of vanadate and of vanadate plus phosphate failed to detect any stabilization of the divanadate or phosphovanadate species when magnesium chloride was added (Craig and Gresser, 1988). The experimental results for the $^{51}V$ NMR studies are presented in Part III of this thesis. The results imply that, unlike the pyrophosphate moiety of ATP and ADP, divanadate and phosphovanadate species are not good chelators of magnesium ion. An inability of ADPV to chelate magnesium ion, and thus to bind to the enzyme, would explain the lack of inhibition that was observed in the

glycerol kinase system. (Gresser and Stankiewicz, unpublished results). These considerations lead to the hypothesis that the lack of inhibition by vanadate of the kinase enzymes is due to the inability of the nucleotide vanadate anhydrides to form a complex with a divalent cation such as $Mg^{2+}$. If this hypothesis is correct, it would also be expected that these anhydrides would be poor substrates for the kinase enzymes, as well as for other ATP utilizing enzymes. The fact that the mitochondrial ATPase, an enzyme which does not include a phosphoenzyme intermediate in its reaction cycle, is inhibited poorly by vanadate ($K_i$ = 1 mM, Kao, 1990), supports this hypothesis. Vanadate is apparently unable to inhibit the enzyme in a situation where the nucleotide must be bound for the phosphoryl transfer to occur. That is, vanadate in this case would be expected to inhibit as the nucleotide vanadate anhydride, an ATP analogue, but since this species is unable to coordinate a $Mg^{2+}$ ion, it is unable to bind to the enzyme effectively.

The potent inhibition by vanadate in the presence of ADP of the myosin and dynein ATPases is difficult to explain. It has been assumed that the ADPV groups bound to these ATPases are associated with magnesium ion in a way similar to that of the normal substrate, ATP (Goodno, 1982; Evans et al., 1986), but there are no published structures of the postulated nucleotide/metal/enzyme complexes. Connolly and Eckstein, 1981 (myosin) and Shimizu and Furusawa, 1986 (dynein) have investigated the stereoselectivity of the myosin and dynein ATPases for phosphorothioate analogues of ATP in the presence of various divalent cations. These studies have provided evidence that

the divalent metal coordinates to the β– but not to the α-phosphate group. It is usual to assume β,γ-bidentate chelation if binding to the β- but not to the α-phosphate is demonstrated. This is based on the much greater thermodynamic stability of bidentate as compared to monodentate metal$^{2+}$/nucleotide triphosphate complexes. The myosin ATPase is active in the absence of divalent cation if a monovalent cation is present (Connolly and Eckstein, 1981; Lymn and Taylor, 1970). The results of stereoselectivity studies of myosin ATPase in the presence of monovalent cations were similar to the results in the presence of divalent cations. That is, metal ion coordination to the β– but not to the α-phosphate group of ATP was indicated. An example of these experiments follows.

The metal/nucleotide specificity of myosin ATPase was studied by Connolly and Eckstein, 1981. They prepared $S_p$ and $R_p$ diastereomers of ATP that had sulfur atoms substituted for oxygens in non-bridging positions on either α– or β-phosphate groups. In the case of the β analogues they observed a difference in activity between $S_p$ and $R_p$ diastereomers that was dependent on the nature of the activating divalent cation. In the presence of $Mg^{2+}$, ATPβS($S_p$) was a >3000-fold better substrate than was ATPβS($R_p$). As the metal ion was varied in the order $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Zn^{2+}$ and $Cd^{2+}$, the activities of the $S_p$ and $R_p$ diastereomers were progressively decreased and increased respectively, until with $Cd^{2+}$ both diastereomers were equally good substrates. It was only in the presence of $Cd^{2+}$ that ATPβS($R_p$) showed an appreciable activity. In contrast, ATPαS($S_p$) and ATPαS($R_p$) were both good substrates for myosin in the presence of $Mg^{2+}$ and the $S_p/R_p$

activity ratios obtained were largely independent of the activating metal ion. The high $S_p/R_p$ activity ratio that was observed in the presence of $Mg^{2+}$ with the β analogues was thought to reflect the greater affinity of the hard anion $O^-$, relative to the softer anion $S^-$, for the hard cation $Mg^{2+}$. The affinity of the soft anion $S^-$ for the metal cation would be expected to increase as the softness of the metal cation increased. As predicted, the activity of ATPβS($R_p$) was highest in the presence of $Cd^{2+}$, the softest of the metal cations tested. The difference in activity between the $S_p$ and $R_p$ β analogues in the presence of $Mg^{2+}$ and the dependence of the $S_p/R_p$ ratio on the softness of the metal cation was considered to be evidence that the β-phosphate group associates with the metal ion in the course of substrate binding. As both $S_p$ and $R_p$ diasteriomers of the phosphorothioate α analogues were good substrates for myosin ATPase in the presence of $Mg^{2+}$, it was reasonably proposed that the α–phosphate group is not significantly involved in chelation of the divalent metal cation.

In order to obtain more information about the effects of vanadate containing nucleotide analogues on the activity of phosphoryl transferring enzymes, PK, HK and MK were tested for inhibition by vanadate alone and in the presence of adenosine, AMP and ADP. The studies with PK and HK included tests of divalent cations other than $Mg^{2+}$, with the idea that the stability of complex formation between vanadate nucleotide and metal ion might be influenced by the size of the metal ion.

As the inhibition of HK by vanadate was found to be significant in preliminary tests, more detailed studies were undertaken to try to

determine which of the various species present in aqueous vanadium oxide solutions were affecting the enzyme activity. In an attempt to detect direct binding of vanadium ions to HK, $^{51}V$ NMR spectra were taken of solutions containing different concentrations of vanadium ion under conditions of high HK concentration. $^{51}V$ NMR has been a useful tool in the study of the components present in aqueous solutions of vanadium oxide, and in the characterization of complexes formed by vanadium ions and organic molecules (see references listed under Gresser, M.J. and under Tracey, A.S.). The $^{51}V$ nucleus has an abundance of nearly 100% and a sensitivity of approximately 1/3 that of the proton. The quadrupole moment of the $^{51}V$ nucleus causes line broadening, but under high field spectrometry peaks representing the various vanadate species can be resolved. Consequently, equilibrium constants for the vanadate monomer and oligomers can be estimated by integrating the peak areas of spectra obtained at different vanadate concentrations. Binding of vanadate species to a large molecule such as HK produces a shorter relaxation time for the vanadium nuclei, broadening their response to the magnetic field. The signals due to bound nuclei cannot be differentiated from the baseline of the spectrum; therefore bound species can be detected by the reduction in total peak area that takes place when the protein is added to the vanadate solution.

To summarize: vanadate containing substrate analogues of AMP, ADP and ATP have been tested as substrates and as inhibitors of PK, MK and HK by kinetic methods. These studies have been done in order to test the hypothesis that, due to their inability to chelate divalent

cations, nucleotide vanadate anhydrides are poor inhibitors of kinase enzymes. In addition, $^{51}V$ NMR was used to investigate the effect of vanadate on HK, in the absence and in the presence of the normal substrates ATP and glucose.

# Experimental Section

## Materials

All of the materials used in the experiments described in this section are listed below with their source of purchase. The chemicals were of reagent grade and all were used without further purification, with the exception of myokinase (rabbit muscle) and the hexokinase (yeast) used for $^{51}V$ NMR. These enzymes were obtained as ammonium sulfate suspensions and were treated as described in the Methods section.

### Proteins

- albumin (bovine serum)
- glucose-6-phosphate dehydrogenase (G6PDH, yeast), EC 1.1.1.49 (D-glucose-6-phosphate: NADP reductase)
- hexokinase (HK, yeast), EC 2.7.1.1. (ATP: D-hexose 6-phosphotransferase)
- lactic dehydrogenase (LDH, rabbit muscle), EC 1.1.1.27 (L-lactate: NAD oxidoreductase)
- myokinase, also known as adenylate kinase (MK, chicken muscle, rabbit muscle), EC 2.7.4.3. (ATP: AMP phosphotransferase)

- pyruvate kinase (PK, rabbit muscle), EC 2.7.1.40 (ATP: pyruvate phosphotransferase)

All proteins were purchased from Sigma Chemical Co. except for the myokinase extracted from rabbit muscle which was from Boehringer Mannheim.

Organic Reagents

- acetic acid (glacial), Fisher Scientific Co.
- adenosine (Ad)
- adenosine diphosphate, sodium salt (ADP)
- adenosine monophosphate, sodium salt (AMP)
- adenosine triphosphate, sodium salt (ATP)
- $P^1,P^5$-di(adenosine -5')pentaphosphate, sodium salt ($AP_5A$)
- glucose
- isopentyl alcohol (3-methyl-1-butanol)
- monothioglycerol
- β-nicotinamide adenine dinucleotide, reduced form, sodium salt (NADH)
- β-nicotinamide adenine dinucleotide phosphate, sodium salt (NADP)
- phosphoenolpyruvate, potassium salt (PEP)
- pyruvate, sodium salt
- Pyrophosphate Assay Reagent

All organic reagents were purchased from Sigma Chemical Co. except NADH, PEP, ATP and pyruvate which were from Boehringer Mannheim and isopentyl alcohol which was from McArthur Chemical Co.

Inorganic Ions, Acids and Bases

- ammonium molybdate ($(NH_4)_6Mo_7O_4$), American Chemicals Ltd.
- chlorides of divalent manganese, cobalt, calcium, strontium, iron, nickel, copper and zinc, Anachemia Chemicals Ltd.
- hydrochloric acid (HCl), Fisher Scientific Co.
- magnesium chloride ($MgCl_2$), Sigma Chemical Co.
- potassium phosphate ($KH_2PO_4$, $K_2HPO_4$), American Chemicals Ltd.
- sodium bisulfite ($NaHSO_3$), Anachemia Chemicals Ltd.
- sodium hydroxide (NaOH), Fisher Scientific Co.
- sodium pyrophosphate ($Na_4P_2O_7$), Anachemia Chemicals Ltd.
- sulfuric acid ($H_2SO_4$), American Chemicals Ltd.
- Trizma base (Tris), Sigma Chemical Co.
- Trizma hydrochloride (Tris-HCl), Sigma Chemical Co.
- vanadium (V) oxide ($V_2O_5$, 99.99%), Aldrich Chemical Co. Inc.

# Methods

## Reagent Preparation

Reagents were prepared in Trizma base (50 mM) which had been adjusted to pH 7.4 with glacial acetic acid. This buffer is hereafter referred to as Tris-OAc. The pH of each reagent was adjusted to pH 7.4 before it was diluted to its final concentration. The only exceptions to this procedure were the reagents for the $^{51}V$ NMR experiment which were prepared in 50 mM Tris-OAc, pH 8.0, and were adjusted to pH 8.0 before the final dilution.

### Myokinase (MK) (rabbit muscle)

Myokinase in ammonium sulfate suspension was centrifuged to remove most of the ammonium sulfate, and the precipitate was dissolved in dialysis buffer. The buffered solution was then dialyzed against 2 L Tris-OAc (50 mM, pH 7.4) for 4 hours, and against 2 L of fresh buffer for 8 hours. All procedures were performed at 4° C. Dialysis was carried out for 1 mL aliquots of the suspension mixture which contained approximately 5 mg myokinase. The concentration of protein after dialysis was determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin as a standard, or spectrophotometrically using an extinction coefficient ($\varepsilon$) equal to 0.53

$mL \cdot mg^{-1} \cdot cm^{-1}$ at 280 nm (Noda, 1973). In experiments where the concentration of MK was < 10 μg/mL, bovine serum albumin (2.0 mg/mL) was added to the enzyme preparation. Preliminary tests showed that bovine serum albumin did not alter the activity of the enzyme, but improved the stability of the enzyme preparation.

Hexokinase (HK) (yeast)

An ammonium sulfate suspension of yeast hexokinase for the $^{51}V$ NMR experiment was dialyzed against 2 L Tris-OAc (50 mM, pH 8.0) for 8 hours, and against fresh buffer for an additional 8 hours. All procedures were performed at 4° C. Dialysis was carried out with an aliquot of 7.2 ml of the suspension which contained 21 mg hexokinase. After dialysis the protein solution was concentrated by centrifuging in a Centriprep column. The final protein concentration was determined spectrophotometrically using $\varepsilon$ = 0.92 $mL \cdot mg^{-1} cm^{-1}$ at 280 nm (Lazarus et al, 1966). In experiments where HK concentrations were < 50 μg/mL, bovine serum albumin (2.0 mg/mL) was included in the enzyme preparation in order to improve the stability of the enzyme activity.

Preparation of Vanadate Solutions

Stock vanadate ($V_i$) solutions were prepared from $V_2O_5$ dissolved in Tris-OAc (50 mM, pH 7.4). A concentration of NaOH equal to that of vanadium atom was added to the buffer solution before the vanadium pentoxide was dissolved. The solutions were made basic to avoid the production of the yellow decavanadate which is formed in acidic solutions of vanadate ion. The pH of these solutions was adjusted with Tris-HCl when necessary, before they were diluted to their final concentration.

UV/Visible Spectroscopy

Absorbance measurements were made on a Hewlett-Packard 8452A spectrophotometer equipped with a temperature controlled cell holder. The multicell transport system of the HP 8452A can accommodate up to 6 cells plus 1 blank; a water-powered magnetic stirring device positioned under the light beam allows the solutions to be mixed during measurement. Conventional spectrophotometers focus a preselected wavelength of light onto the sample and measure the intensity of the transmitted beam. The diode array detector of the Hewlett-Packard 8452A passes a collimated beam of light from a deuterium lamp through the sample before the light is separated by a grating into its component wavelengths. The grating reflects the light onto an array of photodiodes, each assigned to a different wavelength

of light in 2 nm intervals. The simultaneous access to information at all wavelengths increases the rate at which data can be acquired.

Measurement of Reaction Velocity

Reaction velocities were determined by monitoring a change in the absorbance of the reaction mixtures at 340 nm. The assays used to detect activity and inhibition of the enzymes studied are described in detail in the sections appropriate to the enzyme concerned, but in every case the rate of reaction was considered to be equal to the rate of change in the concentration of pyridine nucleotide coenzymes. NADH and NADPH have an absorbance maximum at 340 nm, while the oxidized forms of these molecules, NAD and NADP respectively, do not absorb at this wavelength. NADPH and NADH both have a molar extinction coefficient ($\varepsilon$) of $6.22 \times 10^3$ $M^{-1} \cdot cm^{-1}$ at 340 nm. The change in concentration of NADPH or NADH was calculated using the Beer-Lambert law: $\Delta A = \varepsilon l \Delta c$, where $\Delta A$ is the change in absorbance measured at 340 nm, $\varepsilon$ is the molar extinction coefficient of the pyridine nucleotide, $l$ is the path length of the cell, 1 cm, and $\Delta c$ is the change in concentration of the chromophore.

Samples were prepared in a total volume of 3.0 mL by combining all of the components for the reaction except for the reagent used to initiate the reaction, incubating the mixture for 4 minutes at 25 °C, then adding the initiating reagent. The samples were mixed continually by a magnetic stir bar placed inside the cell. The absorbance at 340 nm was monitored during the period of incubation

and after initiation, for a time suitable to determine the initial velocity of the reaction. Plots were constructed by drawing the best visual line through the points.

Pyrophosphate Assay

Analysis of reaction mixtures for the presence of pyrophosphate was attempted using two different methods.

1. Molybdate forms a complex with pyrophosphate (MoPP) and with phosphate (MoP). If these complexes are reduced they absorb light at different wavelengths. In a procedure published by Grindley and Nichol, 1970, 0 - 40 nmoles pyrophosphate ($PP_i$) can be determined in the presence of a high concentration of phosphate ($P_i$). This analysis was performed on reaction mixtures which contained $P_i$ and that were thought to contain $PP_i$, as described below.

To 0.80 mL of the solution to be tested, 0.050mL $(NH_4)_6Mo_7O_4$ (0.020 M) was added to form the MoPP and MoP complexes. 0.10 mL $NaHSO_3$ (0.530M) was added to stabilize the MoPP chromophore (stable for 3 hours), and 0.050 mL thioglycerol (0.5 mL/5 mL in $H_2O$, made fresh daily) was added to reduce the molybdate complexes. After 10 minutes 1.0 mL isopentyl alcohol was added to separate the two chromophores. The solution was mixed and centrifuged at 2000 rpm for 1 minute. Two layers formed; the reduced MoP complex in the upper layer and the reduced MoPP in the lower layer. To 0.80 mL of the upper layer, 0.050 mL $H_2O$ was added to stabilize the MoP chromophore, and

0.20 mL ethanol was added to prevent the further separation of phases. After 20 minutes the absorbance of this solution was measured at 775 nm. The lower layer (containing MoPP) was treated as follows. To 0.80 mL of the lower layer 0.10 mL of 0.038 M $NaHSO_3$, 0.050 mL of the thioglycerol/water solution and 0.10 mL $H_2O$ were added. This solution was mixed and the absorbance was read at 575 nm. The procedure was performed at ambient temperature. Standard plots of absorbance vs moles of $P_i$ (0 - 40 nmoles) and $PP_i$ (0 - 80 nmoles) were prepared using stock solutions with known quantities of $P_i$ and $PP_i$. The quantity of $PP_i$ in reaction mixtures was determined by comparing the absorbance at 575 nm with that of the standard plot.

2. An enzymic assay based on the activity of $PP_i$-dependent phosphofructokinase in the presence of $PP_i$ was performed on samples thought to contain $PP_i$. The Pyrophosphate Assay Reagent used to measure the enzyme activity in the samples was purchased from Sigma Chemical Co.; reagent concentrations present in the commercial preparation and the mechanism for the reaction are described in Appendix II. If $PP_i$ is present in a solution which is to be tested with the assay mixture, the production of NAD from the assay reaction will be equal to 2 times the quantity of $PP_i$ in the test solution. As described in the section on reaction velocity, the production of NAD can be followed by a decrease in absorbance at 340 nm. The procedure for testing reaction mixtures for the presence of $PP_i$ is described below. The following solutions were prepared in spectrophotometric cells: 1) a blank containing 1.0 ml of the assay mixture and 2.0 ml $H_2O$,

2) a standard containing 1.0 ml of the assay mixture, 1.95 mL $H_2O$ and 0.050 mL of a $PP_i$ standard solution (1.0 mM, prepared in 50 mM Tris-OAc, pH 7.4), 3) test solutions containing 1.0 mL of the assay mixture, 1.95 mL $H_2O$ and 0.050 mL of the solution to be tested, 4) a control containing 1.0 mL of the assay mixture, 1.90 mL $H_2O$, 0.050 mL of the solution to be tested and 0.050 mL of the $PP_i$ standard solution. The absorbance of cells containing assay mixture and $H_2O$, at 25°C, was recorded before the introduction of $PP_i$ standard or unknown solution. After the addition of $PP_i$ and /or unknown solution the change in absorbance was recorded until no further change was observed, approximately 10 minutes.

## $^{51}V$ NMR Spectroscopy

NMR spectra were recorded at 300 K at 78.9 MHz using a 10 mm multinuclear (broadbanded) probehead on a Bruker AM 300 spectrometer. The spectral width was 41,666 Hz, the recycle time was 49 ms and 45° tip pulses were used. Starting with 16 - bit 4DC, 4 K data points were collected for 14,400 acquisitions (10 minutes). The spectrophotometer was not locked. FIDs were weighted with a 25 Hz exponential function prior to Fourier transformation, baseline correction and plotting. Chemical shifts were indirectly referenced to the internal tetrahedral vanadate signal at -536 ppm.

## Experiments with Pyruvate Kinase

### Tests for Substrate Analogues of ADP (AMPV and AdVP)*

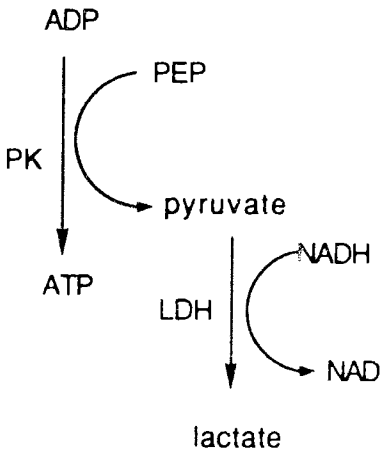
#### Tests for PK Activity with (AMP + $V_i$) in the Absence of ADP

Solutions containing a mixture of $V_i$ (1.1 mM) and AMP (up to 30 mM) were tested for their ability to activate PK (50 μg/mL) in the presence of Tris-OAc (50 mM), PEP (0.5 mM), $MgCl_2$ (1.0 mM), NADH (40 μM) and LDH (30 μg/mL). The reaction was initiated by introducing 30 μL of the PEP solution into a reaction mixture which contained all of the other reagents at 25°C, pH 7.4. Tracey et al., 1988a, detected the spontaneous formation of AMPV in aqueous solutions of AMP and $V_i$. They reported a value of 9.2 ± 0.7 $M^{-1}$ for the equilibrium constant of formation of AMPV at pH 7.4. This value was obtained at an ionic strength of 1.0 M, but it should be a good approximation for the formation constant of AMPV under the conditions of the experiments reported here. It has been used to estimate the equilibrium concentration of AMPV in reaction mixtures containing AMP and $V_i$. The highest concentration of AMPV that was estimated to be present in these solutions was 0.3 mM. Scheme IIIa shows the physiological reaction in which PK catalyzes the transfer of a phosphate group from PEP to ADP. The product pyruvate can be trapped by LDH, which

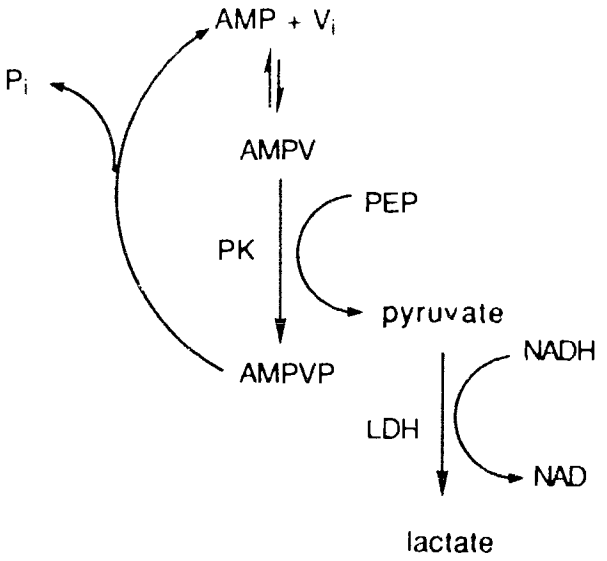
*Structures of vanadate esters and anhydrides are shown in Appendix III

Scheme III

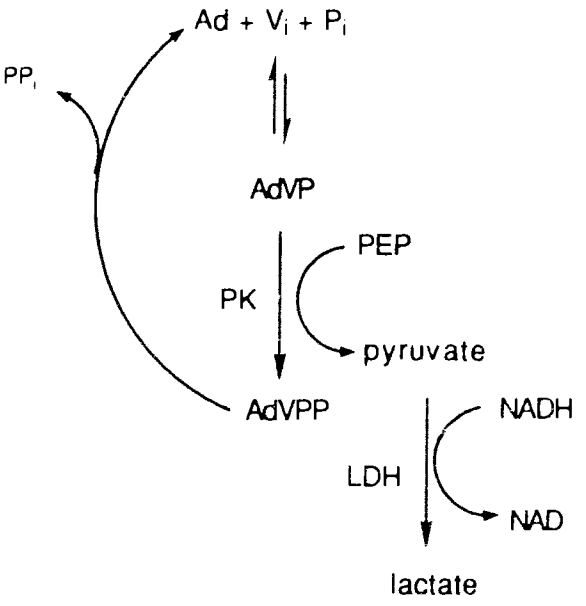
Mechanisms for the pyruvate kinase (PK) catalyzed phosphoryl transfer from (a) PEP to ADP, (b) PEP to AMPV, (c) PEP to AdVP. The product pyruvate is converted to lactate in the presence of LDH and NADH. (a) represents the physiological activity of PK, (b) and (c) represent the activity of PK that is expected in the presence of the spontaneously formed ADP analogues AMPV and AdVP respectively.


ADP
PEP
PK
pyruvate
ATP
NADH
LDH
NAD
lactate

(a)


AMP + Vi
Pi
AMPV
PEP
PK
pyruvate
AMPVP
NADH
LDH
NAD
lactate

(b)


Ad + Vi + Pi
PPi
AdVP
PEP
PK
pyruvate
AdVPP
NADH
LDH
NAD
lactate

(c)

catalyzes the reduction of pyruvate in the presence of NADH. With the assay enzymes and their substrates present at saturating concentrations, the rate of oxidation of NADH to NAD will reflect the rate of phosphoryl transfer catalyzed by PK. The rate of NADH oxidation was monitored as described in the section titled Measurement of Reaction Velocity. Scheme IIIb shows how AMPV would be expected to activate PK. If PK can accept AMPV as a substrate, a phosphate group from PEP will be transferred to AMPV to form a labile AMPVP species that will hydrolyze spontaneously to AMP, $V_i$ and $P_i$. Pyruvate, the other product of the reaction is a substrate for the LDH-catalyzed oxidation of NADH.

Tests for PK Activity with (Ad + $V_i$ + $P_i$) in the Absence of ADP

Solutions containing a mixture of $V_i$ (1.1 mM), adenosine (Ad) (6.7 mM) and $P_i$ (up to 30 mM) were tested for their ability to activate PK-catalyzed phosphoryl transfer in assay mixtures that contained Tris-OAc (50 mM), PEP (0.50 mM), $MgCl_2$ (1.0 mM), PK (0.12 mg/mL), NADH (40 μM) and LDH (10 μg/mL). The reaction was initiated by introducing 30 μL of the PEP stock solution into a reaction mixture that contained all of the other reagents at 25°C, pH 7.4. Because of the evidence for the spontaneous formation of vanadate esters (Gresser and Tracey, 1985; Nour-Eldeen et al., 1985; Craig, 1986) and vanadate anhydrides (Gresser et al.,1986; Tracey et al., 1988a), it is reasonable to assume that aqueous solutions containing Ad, $V_i$ and $P_i$ will contain small quantities of the vanadate ester anhydride AdV*P*. Tracey et al.,

1988 obtained evidence for the formation of a methanol/vanadate/phosphate complex in $^{51}V$ NMR studies of aqueous solutions containing methanol, vanadate and phosphate. The highest concentration of AdVP that could be present in these solutions was calculated to be equal to 0.6 μM. The concentration of AdVP was calculated by assuming that the equilibrium constant for the formation of AdVP can be approximated by multiplying the formation constant of a vanadate ester, ethyl vanadate, by the formation constant of a vanadate anhydride, vanadophosphate. The formation constant of ethyl vanadate, 0.19 ± 0.04 $M^{-1}$, was obtained at pH 7.5 in solutions of 1.0 M ionic strength by Gresser and Tracey, 1985. The formation constants for vanadophosphate, obtained at an ionic strength of 1.0 M, were reported to be 25 ± 1.0 $M^{-1}$ at pH 6.69 and 5.8 ± 0.2 $M^{-1}$ at pH 7.98, by Gresser et al., 1986. From these values the formation constant of vanadophosphate was estimated to be 15 $M^{-1}$ under the conditions of the experiments reported here, pH 7.4. Thus the formation constant for AdVP was calculated to be equal to 0.19 $M^{-1}$ x 15 $M^{-1}$ = 2.8 $M^{-2}$. Scheme IIIc shows how AdVP would be expected to activate PK to produce pyruvate. If PK is able to catalyze the transfer of a phosphate group from PEP to AdVP, the products will be pyruvate and the labile species AdVPP. AdVPP will undergo a spontaneous, rapid hydrolysis to Ad, $V_i$ and $PP_i$. This hypothesis was tested in separate experiments where similar reaction mixtures were tested for the presence of $PP_i$.

## Tests for the Presence of $PP_i$ due to the Formation of AdVPP Catalyzed by PK

Tests for the presence of $PP_i$ were performed on samples containing $V_i$ (0.66 mM), Ad (6.7 mM), $P_i$ (0.1 mM), Tris-OAc (50 mM), PEP (0.5 mM), $MgCl_2$ (1.0 mM) and PK (0.50 mg/mL) which had been incubated for times of 20 minutes to 72 hours. Samples were stored at 4°C for incubations that were longer than 20 minutes in order to preserve enzyme activity. The samples were tested for the presence of $PP_i$ using the molybdate-complexing procedure described under Pyrophosphate Assay (1). In addition, reaction mixtures containing $V_i$ (1.0 mM), adenosine (16 mM), $P_i$ (30 mM), PEP (0.5 mM), $MgCl_2$ (1.0 mM) and PK (0.5 mg/mL), which had been incubated at 4°C for times of 20 minutes to 120 hours, were tested with the $PP_i$-dependent phosphofructokinase assay reagent purchased from Sigma. The procedure that was followed is described in the section titled Pyrophosphate Assay (2).

## Tests of Divalent Cation Effects on Substrates and Substrate Analogues

It has been shown that for kinases the coordination of a divalent cation to the nucleotide is an important factor in the binding of the nucleotide to the enzyme (Mildvan and Fry, 1987; Muirhead et al., 1987). In addition to the experiments described above, where AMPV and AdVP were tested for their ability to activate PK in the presence of $Mg^{2+}$, these ADP analogues were also tested without $Mg^{2+}$ ion in the

presence of the divalent cations of: Ca, Sr, Mn, Fe, Co, Ni, Cu and Zn. The natural substrate ADP (33 μM) was also tested under the same conditions. The procedure was the same as that described previously in the tests for PK activity in the presence of substrate analogues, except that in some of the trials an appropriate aliquot of either buffer solution or another chloride salt (1.0 mM) was substituted for $MgCl_2$.

## Tests for the Inhibition of PK by Anions and Nucleotides

### Tests for the Inhibition of PK Activity in the Presence of ADP and PEP by $V_i$, $P_i$ and $PP_i$

The anions $V_i$, $P_i$ and $PP_i$ were tested for their ability to inhibit the normal PK reaction. Because they were involved in the tests for the substrate analogues of ADP, it was necessary to know that these anions, at similar concentrations, did not inhibit the activity of PK in the presence of ADP and PEP. Reaction mixtures containing Tris-OAc (50 mM), ADP (0.10 mM), PEP (0.5 mM), $MgCl_2$ (1.0 mM), PK (50 μg/mL), NADH (46 μM) and LDH (30 μg/mL) were tested with and without 2.0 mM $V_i$. The experiment was repeated under the same conditions except that the concentration of ADP was 33 μM and 30 mM $P_i$ was substituted for $V_i$. A third experiment was performed in order to test 10 mM $PP_i$. The conditions were the same as those used to test $V_i$ and $P_i$ except that concentrations of up to 1.0 mM ADP were included in reaction mixtures. The reaction was initiated by the addition of 30 μL of the PEP stock solution into a reaction mixture which contained all

of the other reagents at 25°C, pH 7.4. The reaction velocity was measured by following a decrease in absorbance due to the oxidation of NADH.

The addition of 30 mM $P_i$ or 10 mM $PP_i$ increases the ionic strength of the reaction mixture to 84 mM and 67 mM respectively, a factor which could itself account for observed effects on the rate of reaction. The effect on the reaction velocity of increasing the ionic strength of the reaction mixture with KCl, a salt which is likely to be inert with respect to an enzyme that catalyzes phosphoryl transfer, was tested by including 84 mM KCl (ionic strength = 84 mM) in assay mixtures which did not contain $V_i$, $P_i$ or $PP_i$.

Tests for the Inhibition of PK Activity in the Presence of ADP and PEP by AMP and ($V_i$ + AMP)

AMP was tested for the ability to inhibit PK activity in the presence of ADP and PEP. Reaction mixtures contained Tris-OAc (50 mM), ADP (0.10 mM), PEP (0.50 mM), $MgCl_2$ (1.0 mM), PK (50 µg/mL), NADH (46 µM) and LDH (30 µg/mL) were tested with and without 10 mM AMP. The procedure was the same as that described for the tests for $V_i$, $P_i$ and $PP_i$ inhibition. In a separate experiment a mixture of AMP (10 mM) and $V_i$ (1.8 mM) was tested by the same procedure. AMPV could be expected to act as a competitive inhibitor of ADP binding. Synergistic effects between AMP and $V_i$ would suggest that the AMPV complex had a higher affinity for the ADP site than AMP or $V_i$ alone.

## Results and Discussion

### The Activity of PK with Substrate Analogues

#### The Effect of (AMP + $V_i$) and (Ad + $V_i$ + $P_i$) on PK Activity in the Absence of ADP: Analysis of Pyruvate Kinase Activity

There was no PK activity detected in the absence of ADP in the kinetic tests for ADP analogues. Using the observed rate of 4.3 μM/minute in the presence of 0.84 μM enzyme (PK = 50 μg/mL, $M_{PK}$ = 59,250) and 33 μM ADP, a $k_{cat}/K_M$ of 0.15 $\mu M^{-1}minute^{-1}$ was calculated for ADP. The calculation was based on the Michaelis-Menten rate equation $R = (k_{cat}/K_M)$ [E][S], where E and S represent PK and substrate respectively. The value of 0.15 $\mu M^{-1}minute^{-1}$ for ADP can be compared with the upper limit for $k_{cat}/K_M$ for ADP analogues that can be estimated if the minimum observable change in absorbance ($\Delta A = 1 \times 10^{-3}$/minute, approximately) was measured. A $\Delta A$ of $1 \times 10^{-3}$/minute corresponds to a rate of 0.16 μM/minute as calculated from the Beer-Lambert law using $6.22 \times 10^3$ $M^{-1}cm^{-1}$ for the molar extinction coefficient of NADH. Given a rate of 0.16 μM/minute, 0.84 μM PK and 0.3 mM AMPV, $k_{cat}/K_M = 6.3 \times 10^{-4}$ $\mu M^{-1}minute^{-1}$ for AMPV. As calculated previously (Tests for PK Activity with AMP + $V_i$), the estimated equilibrium concentration of the ADP analogue AMPV, 0.3

mM, was comparable to the Michaelis constant of the natural substrate ADP, 0.3 mM, measured under similar conditions (McQuate and Utter, 1959). Since the lowest observable $\Delta A$ was not measured in the presence of the ADP analogue AMPV, it can be concluded that the $k_{cat}/K_M$ of ADP is at least 200 times greater than that of AMPV.

The equilibrium concentration of the ADP analogue AdVP was estimated to be very low, 0.6 $\mu$M, and the rate of NADH oxidation may be too slow to be detected under these conditions. However, the activity of enzymes in the presence of submicromolar concentrations of vanadate esters has been demonstrated (Nour-Eldeen et al., 1985; Craig, 1986). The lability of vanadate esters and anhydrides should ensure that the starting materials, $V_i$ and AMP or Ad, would be released from the AMPVP or AdVPP complexes that might form in the presence of PEP and PK; that is they would be continuously recycled through the phosphorylation reaction. The absence of activity that was observed cannot be attributed to the inhibition of PK in the presence of AMP, Ad, $V_i$ or $P_i$ under the conditions of these experiments. The effects of anions and nucleotides are discussed under the appropriate headings. Further discussion of the activity tests follows the presentation of the results of the inhibition studies.

## The Effect of Divalent Cations Other than $Mg^{2+}$ on PK Activity with Substrate Analogues

When other dications were substituted for $Mg^{2+}$, it was found that only $Mn^{2+}$ and $Co^{2+}$ were able to support the PK catalyzed

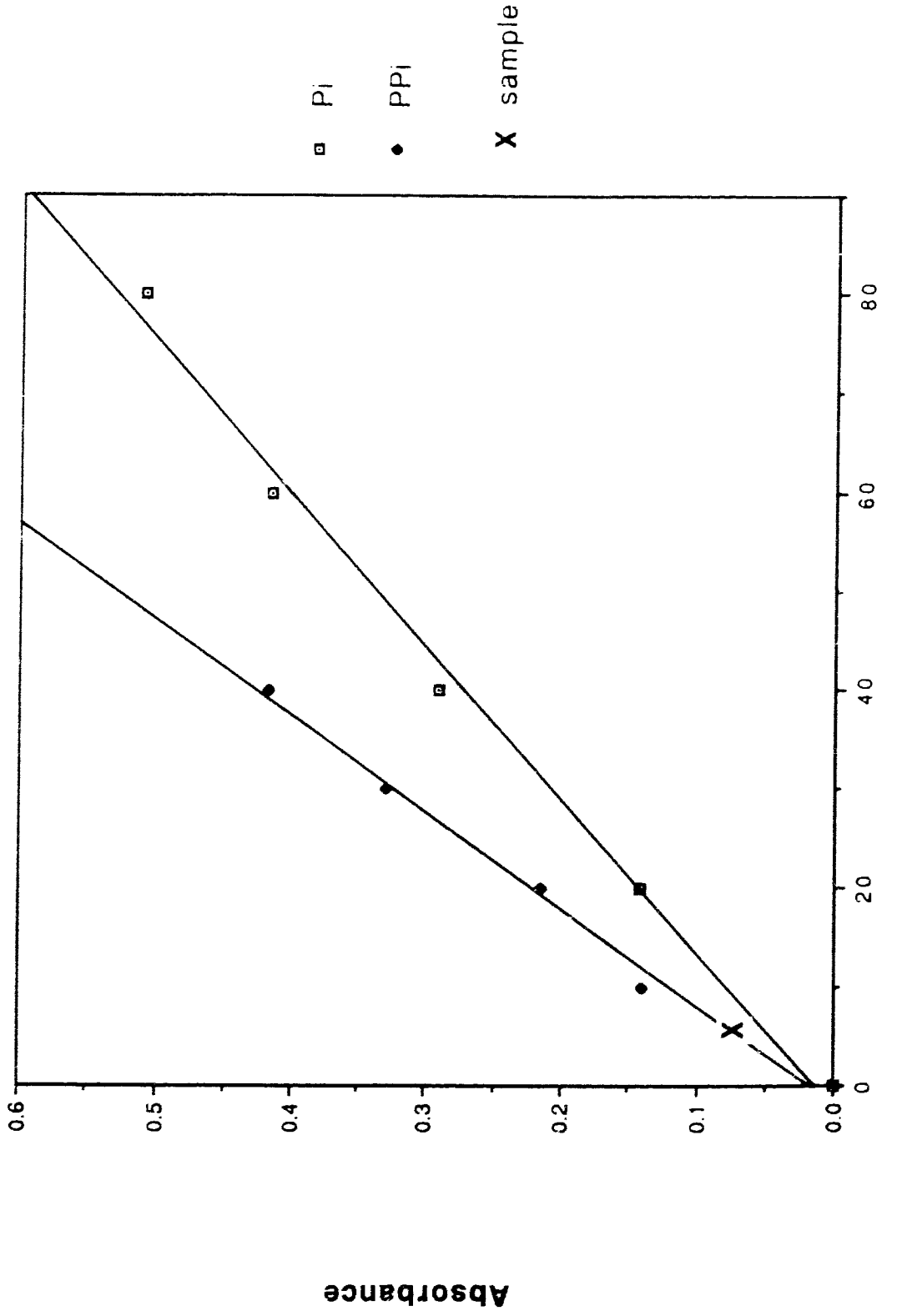
phosphorylation of ADP in the absence of $Mg^{2+}$. $Ca^{2+}$ and $Sr^{2+}$ inhibited the normal reaction in the presence of $Mg^{2+}$. In the absence of ADP, in solutions that were expected to contain ADP analogues, PK activity was not detected in the presence of any of the divalent cations studied. The lowest observable rate of reaction was approximately 0.16 μM/minute. Thus the highest activity that could have occurred, but not been detected, was less than 0.16 μM/minute.

## The Effect of (Ad + $V_i$ + $P_i$) on PK Activity in the Absence of ADP: $PP_i$ Analysis

Attempts to detect $PP_i$ in reaction mixtures containing Ad, $V_i$ and $P_i$ and the phosphorylating assay system were unsuccessful. The quantity of phosphate detected in the test solutions was larger than the amount that had been added to produce the AdVP. The cause of the greater-than-expected absorbance values of the test solutions was determined to be an interaction between Tris-OAc and the reagents used for the molybdate assay. When the $P_i$ stock solution was prepared in Tris-OAc (50 mM), the absorbance of the standard solutions was much higher than that of standards that were prepared from a $P_i$ stock solution made up with water. Figure 7 shows plots of absorbance vs quantity of $P_i$ and $PP_i$ for the preparation of the standard solutions in $H_2O$. In four separate experiments a sample was removed from the lower layer of the sample mixture after isopentyl alcohol extraction, the layer that contained the MoPP complex. The average value for the absorbance of these samples was 0.080 ± 0.021. This value, indicated

Figure 7

Standard plots of absorbance vs phosphate ($P_i$) (❑) and pyrophosphate ($PP_i$) (◆) concentrations. $P_i$ and $PP_i$ were detected through their ability to form a complex with molybdate. The procedure is described in the Methods section, under the heading Pyrophosphate Assay (1). (**X**) represents the average absorbance of four unknown samples that were tested for the presence of $PP_i$. The procedure for preparing the unknowns was the same as for the standard samples.


Pi
PPi
X sample
Absorbance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
20
40
60
80
[Pi] or [PPi] (nmoles)

on the standard $PP_i$ plot in figure 7, could be interpreted to represent 8 ± 3 nmoles $PP_i$. However it is more likely that the small amount of absorbance observed in the MoPP assay is the result of the intense color produced by the $P_i$/Tris-OAc/Mo interaction coupled with an incomplete extraction by the isopentyl alcohol of the $P_i$ chromophore. It is possible that the presence of $PP_i$ in the test samples would produce a $PP_i$/Tris-OAc/Mo interaction that would result in an intense absorbance similar to that due to the presence of $P_i$. If $PP_i$ behaved in this manner the absorbance of $PP_i$ in the test solutions would be enhanced relative to that of the standard solutions. The low value that was observed indicates that the upper limit to the quantity of $PP_i$ that was present in the reaction mixtures was 11 nmoles. A reading of 5 - 11 nmoles was consistent for all trials and it did not change with the time of incubation of the test solution. It is therefore unlikely to be due to the presence of $PP_i$, which would be expected to increase with the time of incubation. The conclusion from this experiment was that, in spite of the interference by Tris-OAc, the absence of a significant quantity of $PP_i$ in the test solutions was clearly demonstrated.

The enzymatic test for $PP_i$ was based on the rate of decrease and on the total decrease in the absorbance at 340 nm due to the oxidation of NADH to NAD in the presence of $PP_i$ and the assay reagents (see Appendix II for the reaction mechanism). The number of moles of NADH oxidized is equal to twice the number of moles of $PP_i$ present when the reaction is initiated. The enzymatic test for $PP_i$ was negative for all of the reaction mixtures that were being monitored for the activity of the substrate analogue AdVP. There was no evidence from the rate of

decrease in absorbance or from the total change in absorbance that test solutions contained $PP_i$. The enzyme activity in samples that had been incubated for 120 hours at 4°C was found to be approximately 2/3 the initial activity. In 2 experiments the rate of change in absorbance was similar for blank and test samples; blanks contained only assay reagent while test samples contained assay reagent plus test solution. This value could not be differentiated from the normal baseline drift under these conditions. In addition, the rate of decrease in absorbance was similar for standard and control samples; standards contained 50 nmoles $PP_i$ plus assay reagent, while control samples contained 50 nmoles $PP_i$, assay reagent and test solution. The standard samples decreased in absorbance at a rate of (0.27 ± 0.07)/minute; the control samples decreased at a rate of (0.19 ± 0.02)/minute, independent of the time of incubation of the test solution before measurement. The total decrease in absorbance of the test samples (2 experiments) was not significantly higher than that of blank samples. This value was similar for all test samples and did not change with the time of incubation of the test solution before measurement. The total decrease in absorbance for standard samples was 0.26 ± 0.01 (the expected value in the presence of 50 nmoles $PP_i$ is 0.21). The value for control samples was 0.27 ± 0.01 and was independent of the time of incubation of the test solution. These results indicate that $PP_i$ was not produced in significant quantities in solutions that were being tested for PK catalyzed phosphoryl transfer from PEP to AdVP.

## The Effects of Anions and Nucleotides on PK Activity

### The Effects of $V_i$, $P_i$ and $PP_i$ on PK Activity in the Presence of ADP and PEP

The activity of PK in the presence of 0.10 mM ADP was not significantly inhibited by 2.0 mM $V_i$. In one experiment rates of reaction of 17 μM/minute in the absence of $V_i$ and 18 μM/minute in the presence of vanadate were recorded. Another experiment gave rates of 14 μM/minute without and 13 μM/minute with $V_i$. The difference in rates in the absence and presence of $V_i$ in the latter experiment was within the experimental error of the measurements. The activity of PK was totally inhibited by 10 mM $PP_i$ in the presence of 1.0 mM ADP. The inhibition by $PP_i$ could not be attributed to an inhibition of LDH, as in the presence of pyruvate (3.3 μM) and NADH (46 μM), LDH (30 μg/mL) was not inhibited by 10 mM $PP_i$. The rate of reaction in the absence of $PP_i$ was 71 μM/minute and in the presence of $PP_i$ was 74 μM/minute. Although PK activity is inhibited by 10 mM $PP_i$, this cannot account for the failure to detect PK activity in solutions containing Ad, $V_i$ and $P_i$. There was no evidence of a significant quantity of $PP_i$ in any of the test samples, whether they were measured by the Mo-complexing assay or by the $PP_i$-dependent enzyme assay.

The activity of PK in the presence of 33 μM ADP and a saturating concentration of PEP was enhanced by a factor of 26 ± 10 (average of 4 trials) when 30 mM $P_i$ was included in the reaction mixture. However, when 84 mM KCl was substituted for $P_i$ under the same conditions, the

activity was enhanced by a factor of 35 ± 12 (average of 3 trials). Therefore it may be that it is the increase in ionic strength which accelerates the reaction, rather than a specific effect of $P_i$ on the enzyme. However, Collier and Webb, 1958, found that AMP and $P_i$ were activators of reaction systems in which PK was coupled to LDH, and both of these effects have been observed in the experiments reported here (see the following section for the effects of AMP on PK catalyzed phosphoryl transfer). It was not determined whether the enhancing effect of $P_i$ was acting upon PK or LDH.

Effect of AMP and ($V_i$ + AMP) on PK Activity in the Presence of ADP and PEP

The activity of PK in the presence of 0.10 mM ADP was enhanced by a factor of 2.2 ± 0 2 (average of 3 trials) when 10 mM AMP was included in the reaction mixtures. This result is consistent with a report by Collier and Webb, 1958, who found that AMP was an activator of PK/LDH coupled reactions. In the presence of 1.8 mM $V_i$ the enhancement was 1.8 ± 0.1 (average of 2 trials). Thus the enhancement was not changed significantly in the presence of 1.8 mM $V_i$. This result provides additional evidence that the AMPV species does not bind to the enzyme at the ADP site; it is consistent with the hypothesis that the inability of the PV moiety to chelate $Mg^{2+}$, or some other factor prevents the binding of AMPV to the kinase.

## Summary and Hypothesis

The fact that PK was not active in the absence of ADP in solutions which would be expected to contain the ADP analogues AMPV and AdVP indicates that, either these analogues were not formed in concentrations sufficient to demonstrate an observable activity, or that they were formed but were not accepted by the enzyme as substrates for the phosphoryl transfer reaction. The absence of PK activity in reaction mixtures which contained mixtures of AMP and $V_i$, and in which ADP was absent, was not a result of inhibition of the enzyme by $V_i$, by AMP,. or by a mixture of $V_i$ and AMP. PK activity was not inhibited by $V_i$ and was enhanced by AMP under the conditions of these experiments. In experiments that tested solutions containing mixtures of Ad, $V_i$ and $P_i$ in the presence of a phosphorylating assay, $PP_i$ produced in the initial stages of the reaction would be present in a concentration too low to significantly inhibit the enzyme. In addition it has been shown that the presence of 30 mM $P_i$ significantly enhances PK activity. Since there is evidence that AMPV is present in significant concentrations in aqueous solutions of AMP and $V_i$ (Tracey et al., 1988a), it is likely that the AMPV species is not accepted as a substrate by PK. This could be explained if the PV moiety on AMPV was unable to chelate $Mg^{2+}$, and thus to bind to the protein. Similarly, there is evidence that AdV is formed in solutions of Ad and $V_i$ (see experiments in this thesis), and there is strong evidence for the presence of PV in solutions containing $P_i$ and $V_i$ (Gresser et al., 1986).

Therefore it is likely that AdVP is present in aqueous solutions containing Ad, $V_i$ and $P_i$.

Although the equilibrium concentration of AdVP has been estimated to be very low (0.6 μM), if it could act as a substrate for PK, it is unlikely that there would be no evidence of $PP_i$ formation after 120 hours, as was observed. The equilibrium constant for the transfer of a phosphate group from PEP to phosphate is favourable. Relevant equilibrium constants for the experiments reported here are listed in Appendix V. In other experiments NADH and LDH were included in the reaction mixtures. The equilibrium constant for the transfer of a phosphate from PEP to phosphate, coupled to the reduction of the product pyruvate catalyzed by LDH, is several orders of magnitude greater than that of the uncoupled reaction. However, no $PP_i$ formation was detected in the coupled assay. The concentration limit for the reliable detection of $PP_i$, as suggested by Sigma, is 40 μM. However, no $PP_i$ was detected in the reaction mixture after 120 hours. The absence of PK activity in the presence of AdVP may be a consequence of the inability of the VP moiety of AdVP to chelate $Mg^{2+}$, and thus to bind to the enzyme.

The idea that the PV moiety is unable to chelate $Mg^{2+}$ is supported by $^{51}V$ NMR evidence. Phosphovanadate anhydride formation is accompanied by a change in chemical shift and an increase in intensity of the signal for monovanadate relative to the signal in the absence of phosphate (see Part III of this thesis). The addition of $MgCl_2$ to solutions containing vanadate and phosphate failed to produce the

spectral changes that would indicate the stabilization of the PV anhydride.

Further evidence that nucleotide vanadate anhydrides are not accepted as substrates or as inhibitors by kinases is presented in the following sections: Experiments with Myokinase and Experiments with Hexokinase.

## Experiments with Myokinase

### Tests for Substrate Analogues of AMP (AdV),* ATP (AdVPP) and ADP (AdVP)

### Tests for MK Activity with (Ad + $V_i$) in the Absence of AMP

Reaction mixtures containing fixed concentrations of Ad (0.80, 4.0 and 12 mM) were tested at different concentrations of $V_i$ (0 - 1.0 mM) for their ability to activate the phosphoryl transfer potential of MK. An assay mixture containing Tris-OAc (50 mM), ATP (1.5 mM), $MgCl_2$ (2.0 mM), MK (chicken muscle, 14 µg/mL), PEP (0.50 mM), PK (50 µg/mL), NADH (0.13 mM) and LDH (30 µg/mL) was included in each sample. The reaction was initiated by adding 30 µL of the stock PEP solution to a reaction mixture which contained all of the other reagents at 25°C, pH 7.4. The reaction was also studied using fixed concentrations of $V_i$ (0.10 and 1.0 mM) and different concentrations of Ad (0 - 12.5 mM). MK normally catalyzes the transfer of a phosphate group from ATP to AMP, producing two molecules of ADP. In the presence of PK and PEP the ADP product of this reaction is converted back to ATP by a PK catalyzed phosphoryl transfer from PEP. The other product, pyruvate is converted to lactate in the presence of LDH and NADH. The rate of the reaction can be followed by monitoring a decrease in absorbance due to the oxidation of NADH. Schemes IVa and

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* Structures of vanadate esters and anhydrides are shown in Appendix.III

IVb show the mechanism for the normal MK-catalyzed reaction and the mechanism expected in the presence of the AMP analogue AdV. AdV would be expected to form spontaneously in aqueous solutions containing Ad and $V_i$ (Gresser and Tracey, 1985). If AdV is accepted as a substrate by MK, phosphoryl transfer from ATP will produce a labile AdVP species which will rapidly hydrolyze to Ad, $V_i$ and $P_i$. The other product of the phosphoryl transfer reaction is ADP. In the presence of PK and PEP, ATP will be regenerated as ADP accepts a phosphate group from PEP. The other product of the PK-catalyzed reaction is pyruvate, and the rate of the reaction can be followed by monitoring the reduction of pyruvate by NADH, as previously described.

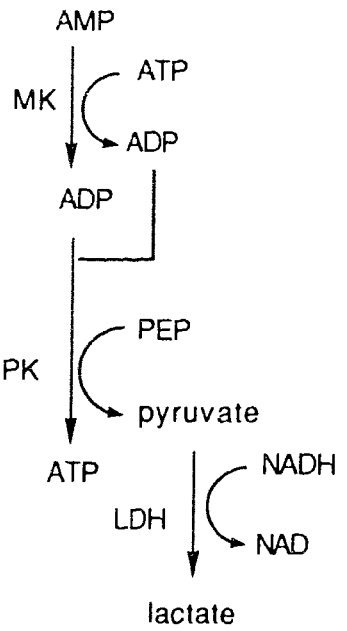
In an experiment similar to that described for the detection of AdV, the rate of the MK-catalyzed reaction was tested as a function of MK concentration. Reaction mixtures contained Ad (8.5 mM), $V_i$ (1.0 mM), Tris-OAc (50 mM), ATP (1.0 mM), $MgCl_2$ (1.0 mM), PEP (0.50 mM), PK (50 μg/mL), NADH (50 μM), LDH (30 μg/mL) and different concentrations of MK (rabbit muscle, 0 - 15 μg/mL).

Tests for MK Activity with (Ad + $V_i$ + $PP_i$) in the Absence of ATP

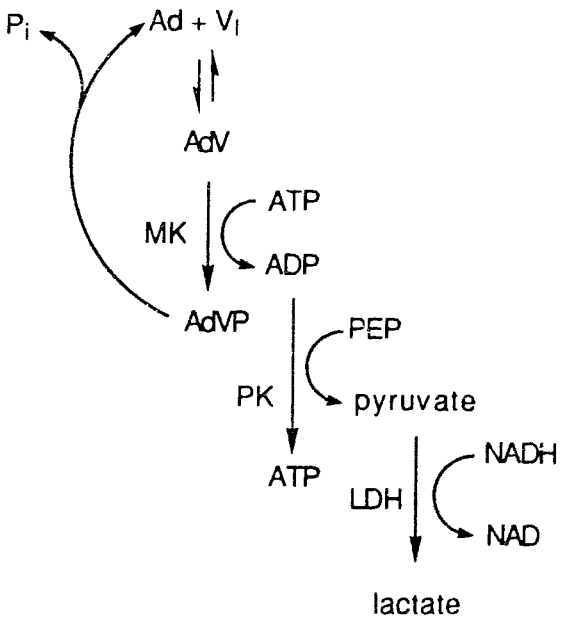
The spontaneous formation of AdVPP in aqueous solutions containing Ad, $V_i$ and $PP_i$, and its acceptance as a substrate by MK, was tested by measuring the PPase activity of MK. The reaction mixtures contained Ad (8.0 mM), $V_i$ (1.0 mM), $PP_i$ (10 mM), Tris-OAc (50 mM), AMP (1.0 mM), $MgCl_2$ (1.0 mM), MK (rabbit muscle, 50 μg/mL,

Scheme IV

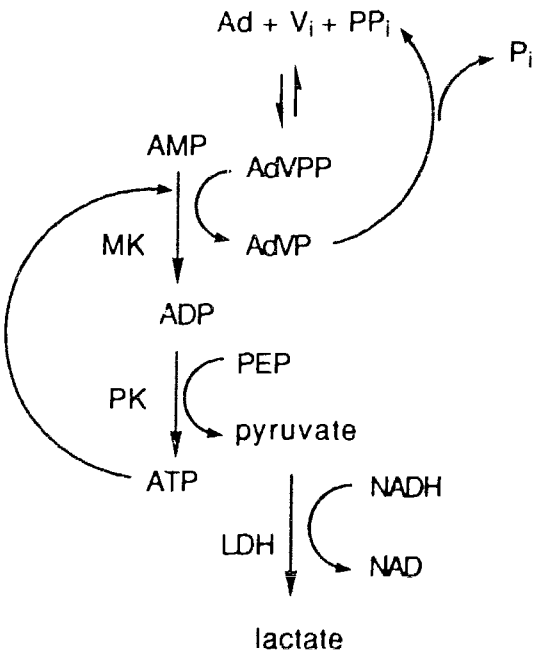
Mechanisms for the myokinase (MK) catalyzed phosphoryl transfer from (a) ATP to AMP, (b) ATP to AdV, and (c) AdVPP to AMP. (a) represents the physiological activity of MK, (b) represents the MK activity that is expected in the presence of ATP and the spontaneously formed AMP analogue AdV and (c) represents the MK activity that is expected in the presence of AMP and the spontaneously formed ATP analogue AdVPP. In each case the product ADP is converted to ATP in the presence of PEP and PK. Pyruvate, produced by the PK-catalyzed phosphorylation of ADP by PEP is reduced to lactate by NADH in a reaction catalyzed by LDH.


AMP
MK
ATP
ADP
ADP
PK
PEP
pyruvate
ATP
LDH
NADH
NAD
lactate

(a)


Pi
Ad + Vi
AdV
MK
ATP
ADP
AdVP
PEP
PK
pyruvate
ATP
LDH
NADH
NAD
lactate

(b)


Ad + Vi + PPi
Pi
AMP
AdVPP
MK
AdVP
ADP
PEP
PK
pyruvate
ATP
LDH
NADH
NAD
lactate

(c)

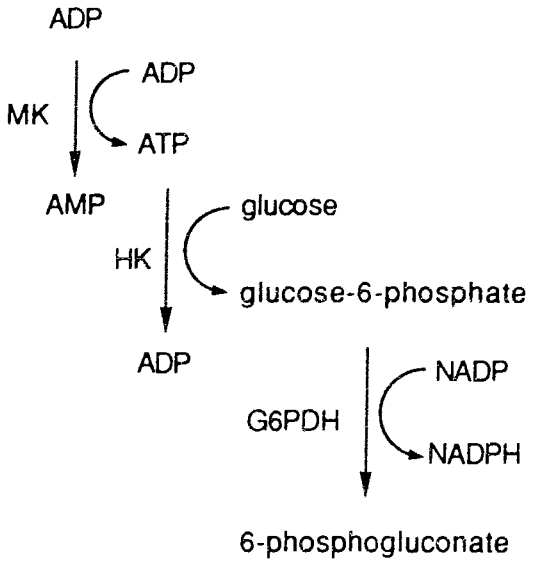
PEP (0.50 mM), PK (50 μg/mL), NADH (50 μM) and LDH (30 μg/mL). The experiment was repeated without the mixture of Ad, $V_i$ and $PP_i$, in the presence of ATP (1.0 μM). The reaction was initiated by introducing 30 μL of the PEP stock solution into a reaction mixture containing all of the other reagents at 25°C, pH 7.4. Scheme IVc shows how MK might be activated by AdVPP in the presence of AMP. The transfer of a phosphate group from AdVPP to AMP, catalyzed by MK, would produce a labile species AdVP which is rapidly hydrolyzed to Ad, $V_i$ and $P_i$. Under these assay conditions the other product of the phosphoryl transfer, ADP, will accept a phosphate group from PEP to produce ATP. ATP is the natural substrate for MK and would be expected to have a greater affinity for the enzyme than the analogue AdVPP. ATP produced by PK activity would compete with AdVPP for the active site of MK. However, initially the ATP produced would be dependent on the activity of the enzyme with the substrate analogue AdVPP.

Tests for MK Activity with (Ad + $V_i$ + $P_i$) in the Presence of ADP

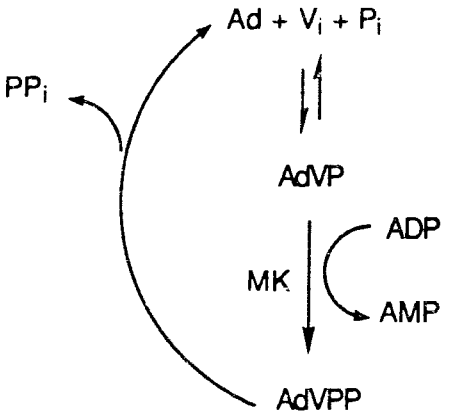
The phosphoryl transfer reaction catalyzed by MK is reversible. In the absence of ATP and AMP, and in the presence of ADP, MK catalyzes the transfer of a phosphate group between 2 ADP molecules to produce a molecule of AMP and a molecule of ATP. This reaction was followed by using an assay mixture which contained glucose, HK, NADP and glucose-6-phosphate dehydrogenase (G6PDH). The mechanism for this reaction is shown in Scheme Va. HK catalyzes a phosphoryl

Scheme V

Mechanisms for the MK-catalyzed phosphoryl transfer from (a) ADP to another ADP molecule, (b) AdVP to ADP. (a) represents the physiological activity of MK; (b) represents the activity that would be expected in the presence of the ADP analogue AdVP. The product of the MK-catalyzed reaction shown in (b) is AdVPP, a labile molecule that breaks down into Ad, $V_i$ and $PP_i$.


ADP
MK
ADP
ATP
AMP
glucose
HK
glucose-6-phosphate
ADP
NADP
G6PDH
NADPH
6-phosphogluconate

(a)


Ad + Vi + Pi
PPi
AdVP
ADP
MK
AMP
AdVPP

(b)

transfer from ATP to glucose. The product, glucose-6-phosphate (G6P), is oxidized to 6-phosphogluconate (6PG) by NADP in a reaction catalyzed by G6PDH. The reduction of NADP to produce NADPH can be followed by monitoring an increase in absorbance at 340 nm. A kinetic test for the activity of MK in the presence of a mixture of Ad, $V_i$ and $P_i$, a mixture which is thought to contain the ADP analogue AdVP, was not possible because of the necessity of including ADP in the reaction mixture. A phosphate group is transferred from one ADP molecule to another in the presence of MK, therefore it could not be determined if any of the activity observed was produced by the AdVP species. The formation and utilization of AdVP was tested by analyzing reaction mixtures for the presence of $PP_i$ as described in the next section.

Tests for the Presence of $PP_i$ due to the Formation of AdVPP Catalyzed by MK

The formation of the ADP analogue AdVP, and its acceptance as a substrate by MK, was tested by measuring $PP_i$ formation in reaction mixtures which contained Ad (16 mM), $V_i$ (0.40 mM), $P_i$ (30 mM), Tris-OAc (50 mM), ADP (0.25 mM), $MgCl_2$ (1.0 mM) and MK (rabbit muscle, 29 μg/mL). As shown in Scheme Vb, the transfer of a phosphate group from ADP to AdVP, catalyzed by MK, would be expected to produce the labile species AdVPP, which would rapidly hydrolyze to Ad, $V_i$ and $PP_i$. The equilibrium constant for the transfer of a phosphate group from ADP to phosphate is favourable, as shown in Appendix V. The reaction mixtures were tested for the presence of $PP_i$ with the $PP_i$-dependent

phosphofructokinase assay reagent from Sigma. Samples of the reaction mixture were tested at 25°C, pH 7.4, after incubation times of 20 minutes to 72 hours. Test samples were stored at 4°C for incubations of longer than 20 minutes in order to preserve the activity of the enzymes. The procedure that was followed is described in the section titled Pyrophosphate Assay 2.

Test to Compare MK Activity in the Presence of AMP and (Ad + $V_i$)

The rates of MK-catalyzed phosphoryl transfer were compared for samples which contained AMP or a mixture of Ad and $V_i$. Reaction mixtures contained Tris-OAc (50 mM), ATP (1.0 mM), $MgCl_2$ (1.0 mM), MK (rabbit muscle, 4.9 μg/mL), PEP (0.50 mM), PK (50 μg/mL), NADH (50 μM), LDH (30 μg/mL) and either AMP (0.83 μM) or a mixture of Ad (8.3 mM) and $V_i$ (1.0 mM). The reaction was initiated by adding 30 μL of the stock PEP solution to a reaction mixture which contained all of the other reagents at 25°C, pH 7.4. The same procedure was used to test reaction mixtures which contained a mixture of AMP (0.83 μM), Ad (8.3 mM) and $V_i$ (1.0 mM).

Tests for the Inhibition of MK

The activity of MK was tested in the presence of the normal substrates, AMP and ATP, for inhibition by $V_i$. In addition, mixtures which were thought to contain species with the potential to form tightly bound transition state analogues were tested for their effect on

the normal activity of the enzyme. Finally, the potent MK inhibitor $P^1,P^5$-di(adenosine-5')pentaphosphate ($AP_5A$) (Lienhard and Secemski, 1973) was tested for its effect upon MK activity in the absence of AMP in reaction mixtures which contained Ad and $V_i$.

Tests for the Inhibition of MK Activity (AMP + ATP = 2ADP) by $V_i$, Ad and ($V_i$ + Ad)

The rate of MK catalyzed phosphoryl transfer from ATP to AMP was measured for different concentrations of ATP. Reaction mixtures contained Tris-OAc (50 mM), AMP (2.5 mM), $MgCl_2$ (2.0 mM), MK (chicken muscle, 75 ng/mL), PEP (0.50 mM), PK (50 µg/mL), NADH (0.15 mM), LDH (30 µg/mL) and different concentrations of ATP (0 - 1.5 mM). The reaction was initiated by introducing 30 µL of the PEP stock solution into a reaction mixture which contained all of the other reagents at 25°C, pH 7.4. The experiment was repeated in the presence of Ad (15 mM), in the presence of $V_i$ (2.0 mM) and in the presence of a mixture of Ad (15 mM) and $V_i$ (2.0 mM).

Tests for the Inhibition of MK Activity (2ADP = AMP + ATP) by $V_i$ and ($V_i$ + AMP)

The rate of MK activity, under conditions of the reverse reaction where ADP is converted to AMP and ATP, was measured at different concentrations of $V_i$. It was necessary to use a low concentration of ADP as inhibition of the reaction was observed at concentrations in

excess of 0.25 mM ADP. (A Michaelis constant for ADP of 1.6 mM has been determined under similar conditions, pH 7.5, Tris, 30°C, by Callaghan and Weber, 1959.) Reaction mixtures contained Tris-OAc (50 mM), ADP (0.25 mM), $MgCl_2$ (1.0 mM), MK (chicken muscle, 0.17 µg/mL), glucose (1.0 mM), HK (50 µg/mL), NADP (50 µM), G6PDH (3.0 µg/mL) and different concentrations of $V_i$ (0 - 0.60 mM). The reaction was initiated by introducing 10 µL of the MK stock solution into a reaction mixture that contained all of the other reagents at 25°C, pH 7.4.

AMP is an inhibitor of MK ($K_i$ = 0.50 mM, Callaghan and Weber, 1959), competitive with ADP. Because of the potiential for the species AMPV to inhibit MK as a transition state analogue of ADP, the effect of AMP on the rate of reaction was tested at 0 - 0.60 mM $V_i$ in the presence of 0.05, 0.10, and 0.40 mM AMP.

Tests for the Inhibition of MK Activity (2ADP = AMP + ATP) by $V_i$, $P_i$, Ad, ($V_i$ + $P_i$), ($V_i$ + Ad), ($P_i$ + Ad) and ($V_i$ + $P_i$ + Ad)

The effect of Ad and $V_i$ on the rate of phosphoryl transfer between ADP molecules was tested in the presence of $P_i$. AdVP, although not a transition state analogue of ADP, is a potential inhibitor of MK activity because of its structural resemblance to ADP. Reaction mixtures contained Tris-OAc (50 mM), ADP (0.25 mM), $MgCl_2$ (1.0 mM), MK (chicken muscle, 0.17 µg/mL), glucose (1.0 mM), HK (50 µg/mL), NADP (50 µM), G6PDH (3.0 µg/mL) and 0 - 0.60 mM $V_i$. In separate experiments $V_i$ was varied in the presence of the assay mixture and 30

mM $P_i$, or 15 mM Adenosine, or a mixture of $P_i$ and Adenosine. In addition, $P_i$ and Adenosine were tested with the assay mixture in the absence of vanadate. In this case adenosine was varied 0 - 14 mM at fixed concentrations of $P_i$, 0, 15 and 30 mM. The reaction was initiated by introducing 10 µL of the MK stock solution into a reaction mixture that contained all of the other reagents at 25°C, pH 7.4. The reaction rate was tested in the presence of 84 mM KCl with and without 30 mM $P_i$, in order to assess the effect on the rate of an increase in the ionic strength of the reaction mixture. The reaction rate was not affected by the addition of 84 mM KCl to the reaction mixture.

Tests for the Inhibition of MK Activity (2ADP = AMP + ATP) by $PP_i$ and (Ad + $PP_i$)

The concentration of PV in aqueous solutions containing 30 mM $P_i$ and 0.60 mM $V_i$ was estimated to be equal to 0.27 mM. The concentration of PV was calculated using a value of 15 $M^{-1}$ for the equilibrium constant for the formation of the PV anhydride obtained under similar conditions by Gresser et al., 1986. It was of interest to compare the quantity of inhibition produced by 0.27 mM PV and the same concentration of $PP_i$. A mixture of 0.27 mM $PP_i$ and 15 mM adenosine was also tested. The conditions were the same as those described in the inhibition tests of $V_i$, $P_i$ and adenosine. In this experiment $PP_i$ was varied 0 - 0.35 mM in the absence of and in the presence of 15 mM adenosine.

Tests for the Inhibition of MK Activity in the Presence of (Ad + $V_i$) by $AP_5A$

$P^1P^5$-di(adenosine-5')pentaphosphate ($AP_5A$) is a potent inhibitor of MK, competitive with AMP and ATP. It is thought that $AP_5A$ binds to MK as a multisubstrate analogue of ATP and AMP. Lienhard and Secemski, 1973 reported inhibition constants of 2.0 nM and 3.6 nM with respect to AMP and ATP respectively. These values were obtained at pH 8.0, 24°C, in 50 mM Tris-HCl buffer. The activity of MK in the absence of AMP, in solutions containing Ad and $V_i$, was measured in the presence and absence of $AP_5A$. Reaction mixtures contained Tris-OAc (50 mM), Ad (4.0 mM), $V_i$ (1.0 mM), ATP (1.5 mM), $MgCl_2$ (2.0 mM), MK (rabbit muscle, 18 µg/mL), PEP (0.50 mM), PK (50 µg/mL), NADH (0.22 mM), LDH (30 µg/mL) and different concentrations of $AP_5A$ (0 - 0.17 µM). The reaction was initiated by introducing 30 µL of the PEP stock solution into a reaction mixture which contained all of the other reagents at 25°C, pH 7.4.

# Results and Discussion

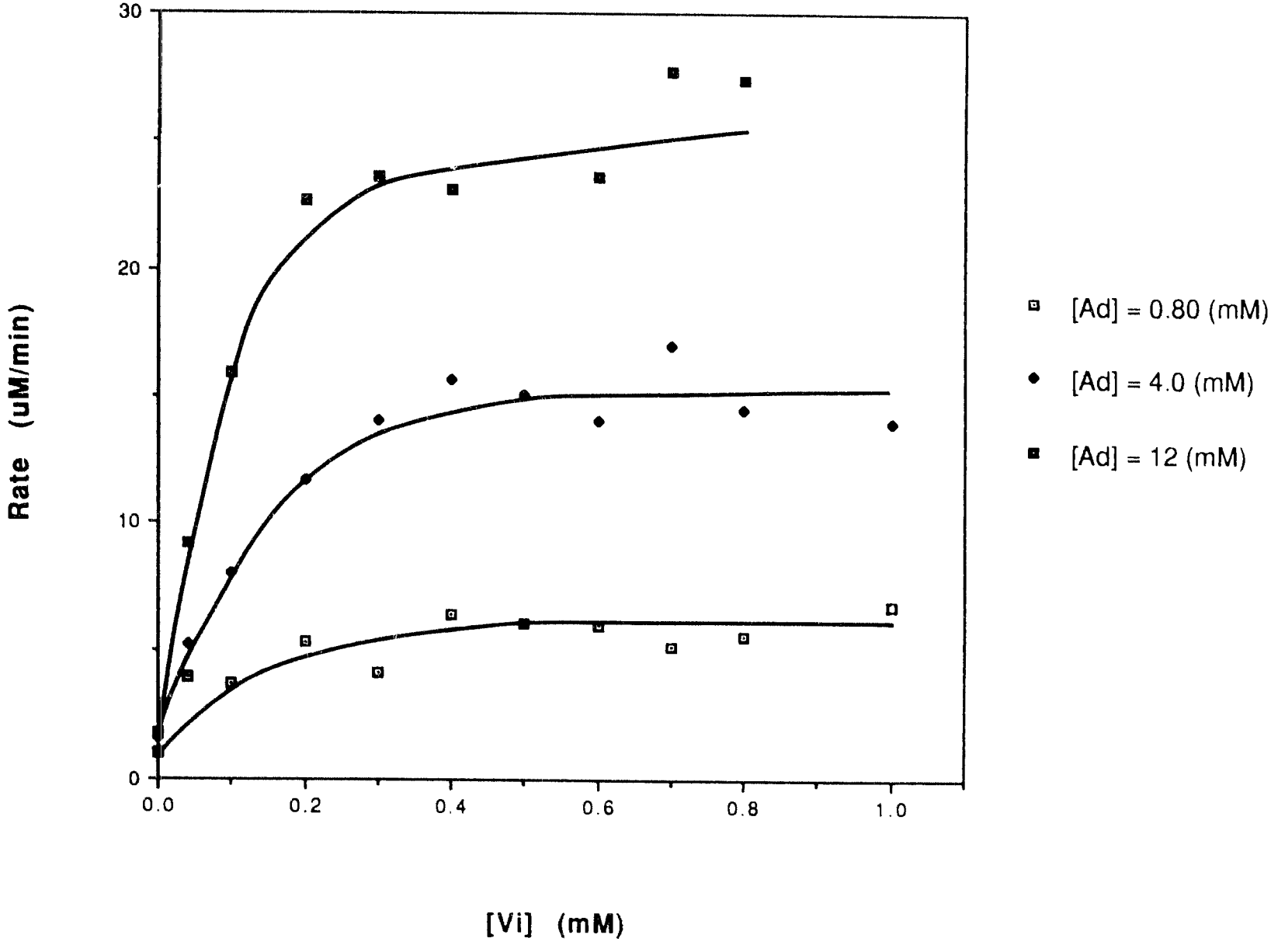
## The Activity of MK with Substrate Analogues

### The Effect of (Ad + $V_i$) on MK Activity in the Absence of AMP

Figure 8 shows the effect of different $V_i$ concentrations on the rate of NADH oxidation in a reaction which can be accounted for by the mechanism shown in Scheme IVb. The presence in the reaction mixtures of 0.8, 4.0 or 12 mM Ad is represented by the lower, middle and upper curves respectively. From the mechanism shown in Scheme IVb, it might be expected that the species ADP could accumulate if Ad, $V_i$, ATP and MK were incubated together. The high concentration of assay reagents would be expected to consume any accumulated ADP in a burst of activity when PEP was added to the reaction mixture. The size of burst would be limited by the quantity of NADH present in the reaction mixture, 0.13 mM, the equilibrium concentration of ADP, or by the rate of ADP production. At the rates measured, it would be expected that, during the 4 minute incubation period required to stabilize the temperature of the reaction mixture, a significant quantity of ADP would accumulate. However, the activity observed did not have the appearance of a burst. That is the rates of reaction observed were slower than would be expected for a reaction burst. The absence of a burst of activity was observed also when AMP was the

Figure 8

The effect of different $V_i$ concentrations on the rate of NADH oxidation in a reaction that is thought to proceed by an MK catalyzed phosphoryl transfer from ATP to the spontaneously formed AMP analogue AdV. The proposed mechanism is shown in Scheme IVb. Reaction mixtures contained 50 mM Tris-OAc, 1.5 mM ATP, 2.0 mM $MgCl_2$, 14 μg/mL MK (chicken muscle), 0.50 mM PEP, 50 μg/mL PK, 0.13 mM NADH, 30 μg/mL LDH, the indicated concentrations of $V_i$ and Ad in the following concentrations: 0.80 mM (□), 4.0 mM (◆), 12 mM (■). The reaction was initiated by adding 30 μL of the stock PEP solution to a reaction mixture that contained all of the other reagents at 25°C, pH 7.4. The sample volume was 3.0 mL. The reaction was monitored by measuring the rate of NADH oxidation in accordance with the mechanism shown in Scheme IVb.

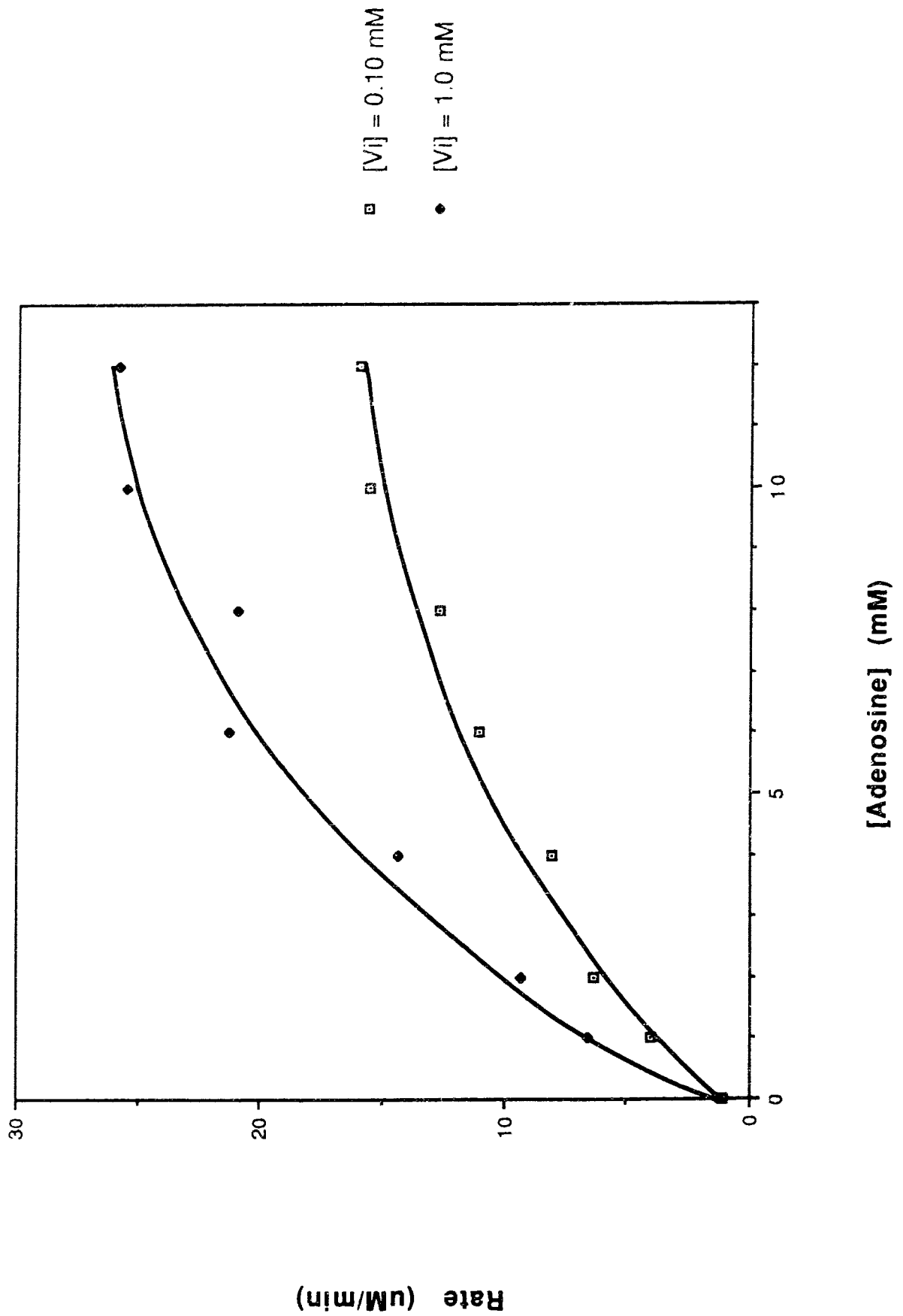

30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
Rate (uM/min)
[Vi] (mM)
[Ad] = 0.80 (mM)
[Ad] = 4.0 (mM)
[Ad] = 12 (mM)

substrate for the reaction. A discussion of this experiment appears in the section titled The Effect of $V_i$, Ad and ($V_i$ + Ad) on MK Activity.

Figure 9 shows data collected from a similar experiment in which the concentration of Ad was varied and the concentration of $V_i$ was fixed at 0.10 mM (lower curve) and 1.0 mM (upper curve). It can be seen from the figures that, in the absence of AMP, MK is activated in the presence of a mixture of Ad and $V_i$ and that the rate of NADH oxidation increases with an increase in either Ad or $V_i$. The low rate observed (1 - 2 μM/minute) in the absence of either $V_i$ or Ad was due to the presence in the reaction mixtures of small quantities of AMP and ADP which co-purify with ATP (the supplier specifies < 0.5%). The concentration of AdV in the reaction mixtures was estimated to range from 0 - 2 μM. MK activity was observed at concentrations as low as 15 nM AdV, in solutions which contained 0.80 mM Ad and 0.10 mM $V_i$. The values for the concentration of AdV were calculated using the equilibrium constant for the formation of ethyl vanadate, 0.19 $M^{-1}$, published by Gresser and Tracey, 1985. This value was obtained at pH 7.5 at an ionic strength of 1.0 M, and it is thought to be a reasonable approximation for the formation constant of a vanadate ester under the conditions of the experiments reported here. The curvature of the lines in Figures 8 and 9 is discussed after the results of the inhibition studies have been presented (see The Formation of $Ad_2V_2$).

Figure 9

The effect of different Ad concentrations on the rate of NADH oxidation in a reaction that is thought to proceed by an MK-catalyzed phosphoryl transfer from ATP to the spontaneously formed AMP analogue AdV. The mechanism is shown in Scheme IVb. Conditions were the same as those described in Figure 8, except that the concentration of $V_i$ was fixed at 0.10 mM (❑) and 1.0 mM (◆).


[Vi] = 0.10 mM
[Vi] = 1.0 mM
Rate (uM/min)
[Adenosine] (mM)
0
10
20
30
0
5
10

Comparison of AMP-Induced MK Activity with (Ad + $V_i$)-Induced MK Activity

Since AdV can act as a substitute for AMP in a MK catalyzed phosphoryl transfer reaction, it was of interest to compare the rates of the reaction for similar concentrations of the two substrates. Solutions containing a mixture of 8.3 mM Ad and 1.0 mM $V_i$ (AMP = 0), which were estimated to contain 0.62 μM AdV, were tested for their ability to stimulate MK activity. The concentration of AdV has been corrected for the presence of $Ad_2V_2$, using a formation constant of 2.8 x $10^7$ $M^{-3}$ (see Figure 16 and section titled The Formation of $Ad_2V_2$).

These results were compared with the activity of MK that was observed in the presence of 0.83 μM AMP (Ad = $V_i$ = 0). An average value is reported for 3 trials which were performed under each set of conditions. The concentration of enzyme was the same in each case, 0.23 μM (4.9 μg/mL; $M_{MK}$ = 21,700). The rate of reaction measured in the presence of 0.62 μM AdV was approximately equal to 4.1 ± 0.8 μM/minute, while the rate of reaction in the presence of 0.83 μM AMP was approximately 1.1 ± 0.1 μM/minute. The rates of reaction when both substrates were present in the reaction mixture, AdV (0.62 μM) and AMP (0.83 μM), were additive within experimental error; an average value of 5.8 ± 0.7 μM/minute was obtained for two trials. These rates correspond to $k_{cat}/K_M$ values of 29 $\mu M^{-1}minute^{-1}$ for AdV and 5.8 $\mu M^{-1}minute^{-1}$ for AMP. It is rare that the $k_{cat}/K_M$ value for a substrate analogue is greater than that of the true substrate. However, when the experiment was repeated at a higher concentration of $Mg^{2+}$

(10 mM) and a different enzyme preparation (the enzyme from chicken rather than rabbit muscle was used), $k_{cat}/K_M$ values of 54 $\mu M^{-1}$ $minute^{-1}$ for AdV and 12 $\mu M^{-1} minute^{-1}$ for AMP were obtained. It is difficult to obtain an accurate rate for the experiment with a low concentration of AMP as the total $\Delta A$ is very small. When AdV is the substrate the absorbance continues to change until the limiting reagent, usually NADH, is used up as Ad and $V_i$ can be recycled in the phosphorylation reaction. The $k_{cat}/K_M$ for AMP was obtained at 0.5 mM AMP for this reason and is thought to be a more reliable value than the 5.8 $\mu M^{-1} minute^{-1}$ obtained previously. Therefore it appears that the $k_{cat}/K_M$ of the substrate analogue AdV is of a comparable magnitude to, if not greater than, the kinetic constant of the normal substrate. These results indicate that AdV is a very good substrate for MK. It will be shown later that the presence of 8.0 mM Ad does not significantly affect the rate of MK catalyzed phosphoryl transfer from ATP to AMP. However, under conditions which approached saturation by the substrates AMP and ATP, the inhibition by 2.0 mM $V_i$ was 54%. Thus it is likely that the presence of 1.0 mM $V_i$ inhibited the activity of the enzyme about 30%. It is possible that the product AdVP does not inhibit the reaction to the same extent as ADP. The labile $V_i$ bonds would prevent a build-up of AdVP.

When MK catalyzed phosphoryl transfer was tested in the absence of AMP in solutions containing Ad and $V_i$ the rate of NADH oxidation was found to be dependent on the concentration of MK included in the reaction mixture. The linear dependence of the rate on MK concentration that was found in this experiment supports the

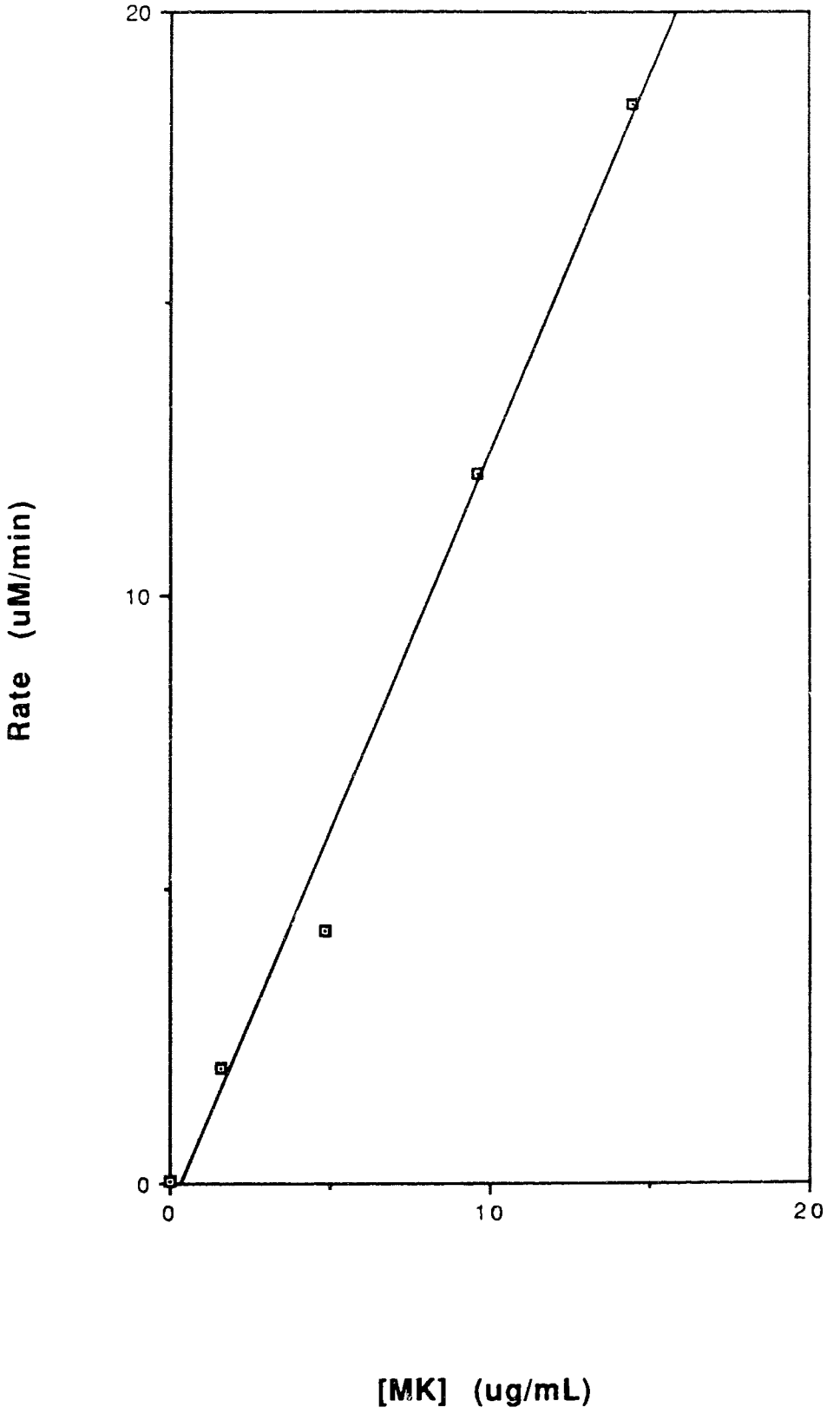
conclusion that AdV acts as a substrate for MK. The results are shown in Figure 10.

The Effect of (Ad + $V_i$ + $PP_i$) on MK Activity in the Absence of ATP

There was no evidence that AdVPP was accepted as a substrate by MK in the absence of ATP. The concentration of AdVPP present in solutions containing 8.0 mM Ad, 1.0 mM $V_i$ and 10 mM $PP_i$ has been estimated to be approximately equal to 0.6 μM. This value was calculated using an equilibrium constant of 7.4 $M^{-2}$ for the formation of AdVPP. The $K_{eq}$ for AdVPP was estimated by multiplying the equilibrium constant for the formation of ethyl vanadate, 0.19 $M^{-1}$ (Gresser and Tracey, 1985) by the formation constant for vanadopyrophosphate (VPP), 39 ± 3 $M^{-1}$ obtained at pH 7.98 in solutions of 1.0 M ionic strength (Gresser et al., 1986). It can be seen from Scheme IVc that if AdVPP is accepted as a substrate by MK, a phosphate group could be transferred to AMP to form ADP. The phosphorylation of ADP catalyzed by PK would produce ATP, the natural substrate for PK. Thus the nucleotide would be recycled through the phosphorylation system made up of the two enzymes, MK and PK. The estimated concentration of AdVPP is very low (0.6 μM), but a high activity was observed In the presence of 1.0 μM ATP ( Ad = $V_i$ = $PP_i$ = 0) due to the regeneration of the ATP substrate by the PK-catalyzed reaction. However, in the presence of 10 mM $PP_i$ the reaction with ATP was totally inhibited under the same conditions. The total inhibition of

Figure 10

The effect of different MK concentrations on the rate of NADH oxidation in a reaction that is thought to proceed by an MK catalyzed phosphoryl transfer from ATP to the spontaneously formed AMP analogue AdV. The mechanism is shown in Scheme IVb. The conditions are the same as those described in Figure 8, except that the concentrations of Ad (8.5 mM) and $V_i$ (1.0 mM) were constant.


20
10
0
0
10
20
Rate (uM/min)
[MK] (ug/mL)

PK activity by $PP_i$ under the same conditions (except for the presence of Ad and $V_i$) as those used in these experiments was reported in the section titled Experiments with PK. Because of the inhibition by $PP_i$ there can be no conclusion as to the viability of AdVPP as a substrate for MK. The fact that no activity was observed in solutions of Ad, $V_i$ and $PP_i$ can be explained by the inhibition of the auxiliary enzyme PK by $PP_i$, or by AdVPP not being accepted as a substrate.

The Effect of (Ad + $V_i$ + $P_i$) on MK Activity in the Presence of ADP: $PP_i$ Analysis

There was no evidence of $PP_i$ formation in any of the tests which involved the spontaneous formation of AdVP and its subsequent phosphorylation by ADP, catalyzed by 29 µg/mL MK. These tests were run under the same conditions as the tests which involved the phosphorylation of AdVP by PEP catalyzed by PK (see Experiments with Pyruvate Kinase, Results and Discussion). A background rate of $\Delta A$ of $\pm$ $9 \times 10^{-4}$ $minute^{-1}$ was recorded during the experiment. If this $\Delta A$ was due to a concentration change in NADH, it would correspond to a value of $\pm$ 0.14 µM. Therefore the minimum concentration change of NADH, due to the presence of $PP_i$, that could be measured would have to be greater than 0.14 µM. This would correspond to the presence of 0.07 µM $PP_i$, as the reaction produces 2 molecules of NAD from 1 molecule of $PP_i$. The values for the rate of decrease in absorbance due to the oxidation of NADH and for the total decrease in absorbance for the

reaction mixtures containing 29 μg/mL MK were, within experimental error, equal to the values for the reaction mixtures containing PK. That is the measurements for the test solutions were similar to the blanks, and the control solutions were similar to the standards. The rate of change in absorbance and the total change in absorbance did not depend on the time of incubation of the reaction mixture before measurement with the $PP_i$ assay.

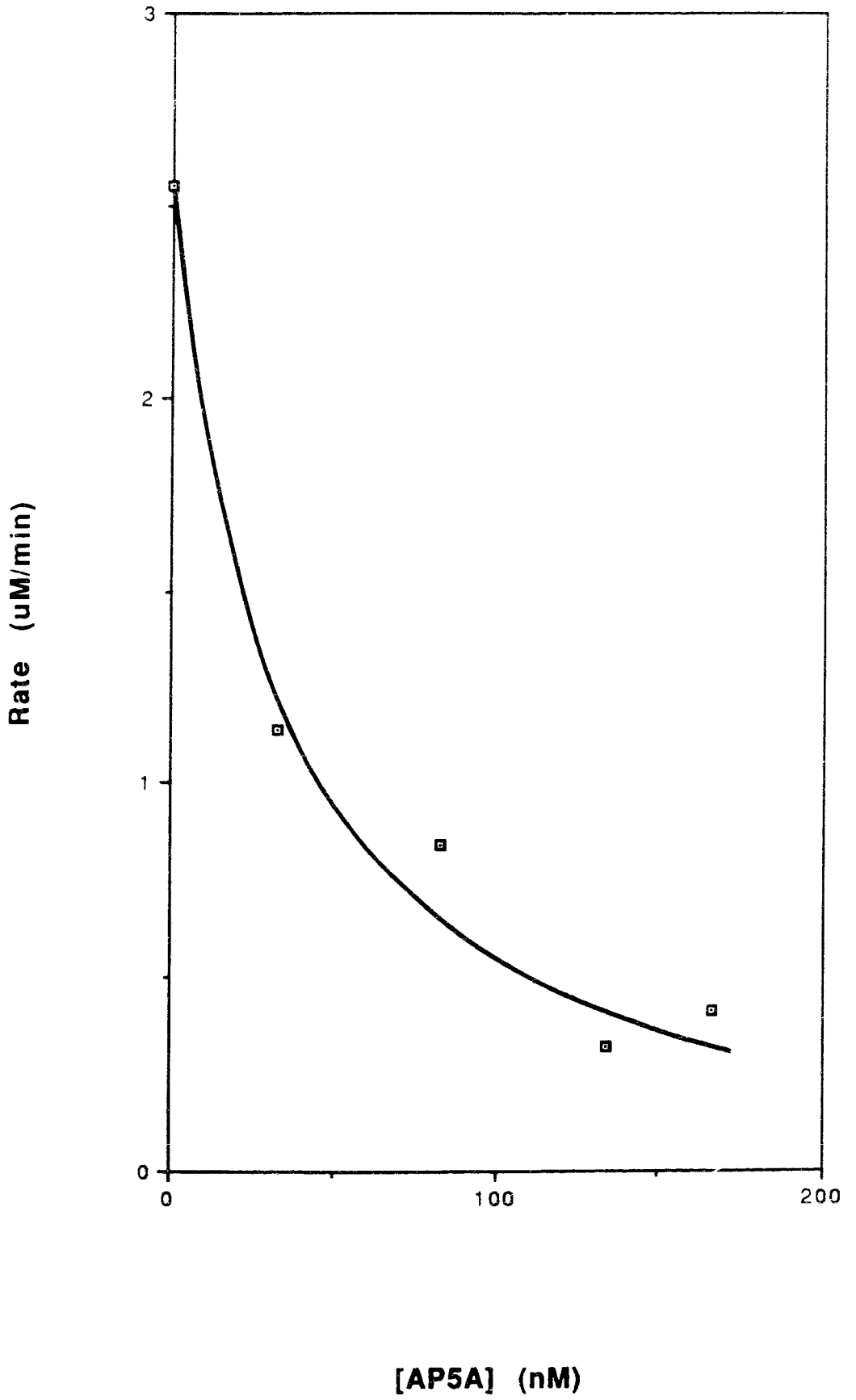
The lack of MK activity cannot be explained by inhibition of the enzyme in the presence of Ad, $V_i$ or $P_i$. As described in the inhibition studies, it was shown that the inhibition of MK catalyzed phosphoryl transfer between ADP molecules is inhibited by approximately 50% in the presence of a mixture of 15 mM Ad, 1.0 mM $V_i$ and 30 mM $P_i$. Thus the enzyme should retain 50% of its activity under these conditions.

The Inhibition of MK Activity in the Presence of (Ad + $V_i$) by $AP_5A$

$AP_5A$ was a good inhibitor of MK activity in the presence of Ad (4.0 mM) and $V_i$(1.0 mM). Figure 11 shows a plot of the rate of NADH oxidation vs the concentration of $AP_5A$, for an experiment in which AMP was absent and ATP was present at 1.5 mM. The results clearly demonstrate the dependence of the rate of the MK catalyzed reaction on $AP_5A$ concentration. Lienhard and Secemski, 1973, tested the inhibition of MK activity in the presence of the natural substrates (0.20 mM AMP, 0.15 mM ATP). They reported an inhibition of 55% of the MK activity at 0.03 μM $AP_5A$. The experiment described here was conducted in reaction mixtures that contained 1.5 mM ATP, 4.0 mM Ad

Figure 11

The effect of different $AP_5A$ concentrations on the rate of NADH oxidation in a reaction that is thought to proceed by an MK catalyzed phosphoryl transfer reaction from ATP to AdV. The mechanism is shown in Scheme IVb. Reaction mixtures contained 50 mM Tris-OAc, 4.0 mM Ad, 1.0 mM $V_i$, 1.5 mM ATP, 2.0 mM $MgCl_2$, 18 μg/mL MK (rabbit muscle), 0.50 mM PEP, 50 μg/mL PK, 0.22 mM NADH, 30 μg/mL LDH and the indicated concentrations of $AP_5A$. The reaction was initiated by adding 30 μL of the stock PEP solution to a reaction mixture that contained all of the other reagents at 25°C, pH 7.4. The sample volume was 3.0 mL. The reaction was monitored by measuring the rate of oxidation of NADH in accordance with the mechanism shown in Scheme IVb.


3
2
1
0
0
100
200
Rate (uM/min)
[AP5A] (nM)

and 1.0 mM $V_i$. Under these conditions an inhibition of 55% was recorded for 0.04 μM $AP_5A$. The concentration of $AP_5A$ required to achieve 55% inhibition of MK activity was higher in the experiment reported here than that reported by Lienhard and Secemski, 1973. The higher concentration of ATP used in this experiment (1.5 mM vs 0.15 mm for the experiment of Lienhard and Secemski) would compete more successfully for the active sites on the enzyme. In the presence of a saturating concentration of ATP a higher concentration of $AP_5A$ would be required to produce the same amount of inhibition. Lienhard and Secemski performed the experiment at pH 8.0 while the work reported here was done at pH 7.4. Since there is a broad optimum for the activity of MK between pH 7 and pH 9 (Noda, 1958), the activity of the enzyme is expected to be comparable at pH 7.4 and pH 8.0.

The vertical intercept of a Dixon plot (1/rate vs inhibitor concentration) of the data from Figure 11 gave a value of 27 nM for the inhibition constant ($K_i$) of $AP_5A$. This value can be compared with the value of 10 nM for the dissociation constant of $AP_5A$ to myokinase reported by Mannherz et al. 1974, as the unpublished results of R.S. Goody, T. Frohlich, B. Walter and H. Schirmer.

The maximum rate of NADH oxidation in reaction mixtures containing 4.0 mM Ad and 1.0 mM $V_i$ was approximately equal to 16 μM/minute in the experiment which was designed to test the formation and activity of AdV (Figure 8). In the experiment which tested $AP_5A$ inhibition of this activity, the maximum rate in the absence of inhibitor in reaction mixtures that contained 4.0 mM Ad and 1.0 mM $V_i$ was approximately equal to 2.5 μM/minute (Figure 11). The apparent

difference in the maximum rates of reaction under similar conditions of MK concentration (14 μg/mL and 18 μg/mL respectively) is a consequence of the fact that different preparations of the enzyme were used for each experiment. Activity tests in the presence of saturating concentrations of AMP and ATP gave values for the activity of the enzyme preparations of 745 units/mg protein for the AdV activity study and 29 units/mg protein for the $AP_5A$ inhibition study. The ratio of (activity x enzyme concentration) should be equal to the ratio of the rates of reaction in the absence of inhibitor for reaction mixtures containing 4.0 mM Ad and 1.0 mM $V_i$. These values, 26 and 6.4 respectively, do not agree well. Qualitatively however, the fact that the activity of the enzyme preparation used in the test for AdV was much higher than that used for the $AP_5A$ inhibition study can account for the difference in the maximum rates that were observed in these experiments.

## The Inhibition of MK Activity by $V_i$ and Nucleotide Analogues

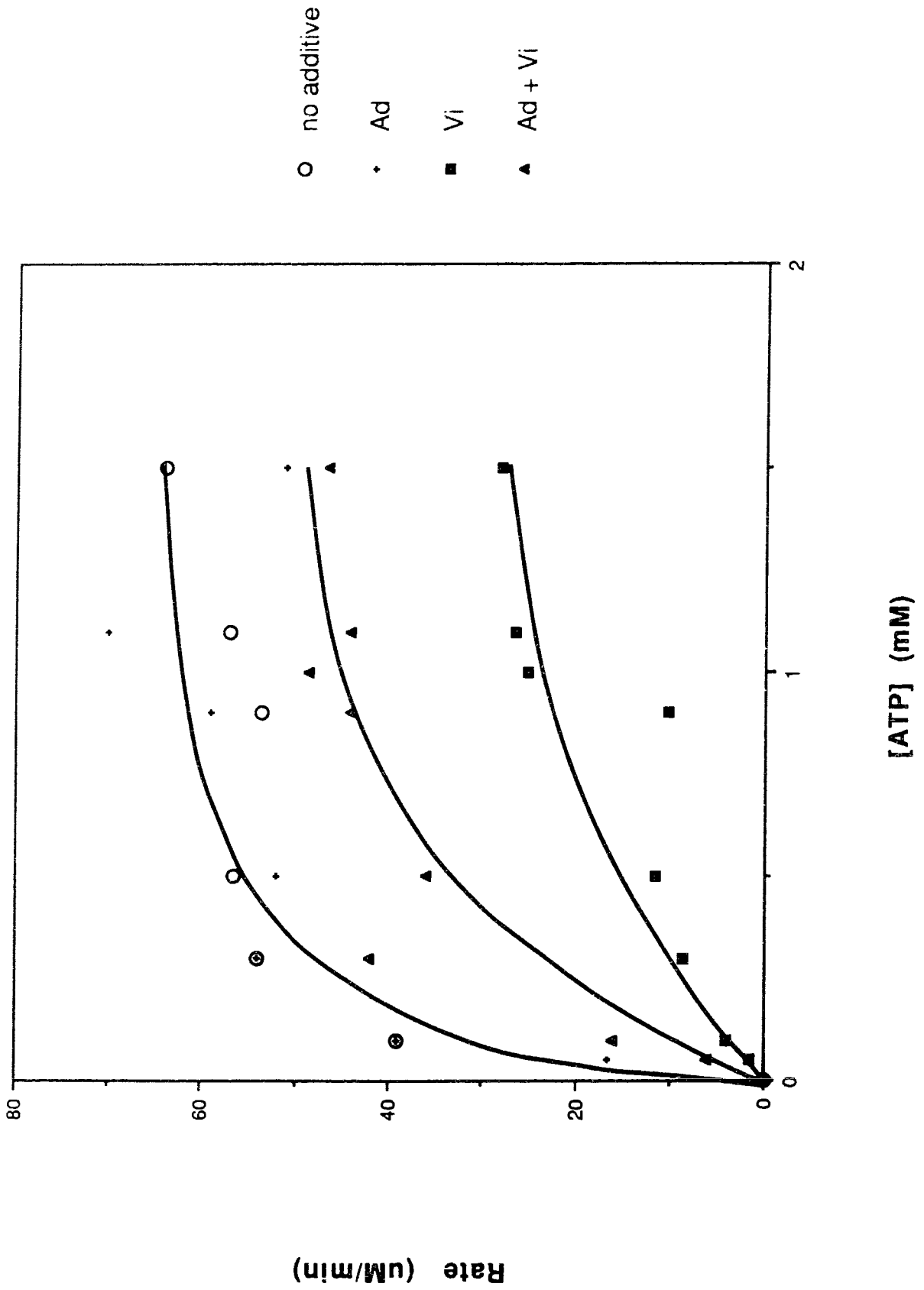
The most important results of the experiments performed to detect inhibition of MK activity by $V_i$ and by $V_i$ in the presence of nucleotides can be summarized briefly. Although $V_i$ alone behaved as an inhibitor, the inhibition was not particularly potent, and it was not enhanced in the presence of adenosine or of adenine nucleotides.

The Effect of $V_i$, Ad and ($V_i$ + Ad) on MK Activity (AMP + ATP = 2ADP)

The uppermost curve of Figure 12 is a plot of the rate of MK catalyzed phosphoryl transfer at different concentrations of ATP in the presence of 2.5 mM AMP. The maximum rate of reaction in the absence of any inhibitor is 60 μM/minute in the presence of 75 ng/mL MK, thus the activity of MK was equal to 800 units/mg protein. ADP would be expected to accumulate when a reaction mixture containing ATP, AMP and MK was incubated for 4 minutes (reaction mixtures were incubated for 4 minutes in order to stabilize the temperature). Accumulated ADP would be consumed in a burst of activity on the initiation of the PK catalyzed reaction by the addition of PEP, due to the high concentrations of assay reagents present in the reaction mixture. At an equilibrium concentration of 0.15 mM ADP (a rate of 38 μM/minute could produce 0.15 mM ADP in 4 minutes), it would be expected that all of the NADH present, 0.15 mM, would be consumed in a burst of activity. From the $K_{eq}$ of 2.26 for the formation of ADP from ATP and AMP published by Noda, 1973, it is estimated that a reaction mixture of 2.5 mM AMP and 0.079 mM ATP will produce 0.15 mM ADP at equilibrium. Thus all of the samples which had rates greater than 38 μM/minute and greater than 79 μM ATP would be expected to proceed entirely as a burst of activity on the addition of PEP to the reaction mixture. However, as with the studies in which AdV was the substrate, the progress curves did not have the appearance of a burst. The $K_{eq}$ of 2.26 reported by Noda, 1973, was obtained at 10 mM $MgCl_2$. A high $Mg^{2+}$ concentration would tend to favour the formation of 2 ADP

Figure 12

The effect of different ATP concentrations on the rate of MK-catalyzed phosphoryl transfer from ATP to AMP in the absence and in the presence of Ad and $V_i$. Reaction mixtures contained 50 mM Tris-OAc, 2.5 mM AMP, 2.0 mM $MgCl_2$, 75 ng/mL MK (chicken muscle), 0.50 mM PEP, 50 μg/mL PK, 0.15 mM NADH, 30 μg/mL LDH, the indicated concentrations of ATP and the following additives: 0 (○), 15 mM Ad (◆), 2.0 mM $V_i$ (■), 15 mM Ad and 2.0 mM $V_i$ (▲). The reaction was initiated by adding 30 μL of the PEP stock solution to a reaction mixture that contained all of the other reagents at 25°C, pH 7.4. The sample volume was 3.0 mL. The reaction was monitored by measuring the rate of NADH oxidation in accordance with the mechanism shown in Scheme IVa.


no additive
Ad
Vi
Ad + Vi
Rate (uM/min)
[ATP] (mM)
0
20
40
60
80
0
1
2

molecules over the formation of 1 AMP and 1 ATP. However, an experiment performed at 10 mM $Mg^{2+}$ produced the same results with respect to an absence of burst activity.

The symbols in Figure 12 represent the rates that were observed when 15 mM Ad was included in the reaction mixtures containing the natural substrates. The addition of 15 mM Ad did not have a significant effect on the reaction rates. The lowermost curve of Figure 12 is a plot of the rate vs ATP concentration in the presence of 2.5 mM AMP and 2.0 mM $V_i$ (Ad = 0). When the concentration of ATP was equal to 1.5 mM, the presence of 2.0 mM $V_i$ inhibited the maximum rate by approximately 54%. At lower concentrations of ATP the inhibition was considerably higher. At 0.30 mM ATP the inhibition was equal to 82%. (A value of 0.30 mM for the $K_m$ of ATP was determined by Callaghan and Weber, 1959, under conditions similar to those of the experiments reported here: pH 7.5. Tris buffer, 30°C.). The fact that the % inhibition decreases as the concentration of ATP increases suggests that $V_i$ competes with ATP for a site on the enzyme. The middle curve of Figure 12 shows the rate of the phosphoryl transfer reaction vs ATP concentration in the presence of 2.5 mM AMP, 2.0 mM $V_i$ and 15 mM Ad. The inhibition of the maximum MK activity that was observed in the presence of 2.0 mM $V_i$ is reduced to about 18% when 15 mM Ad is included in the reaction mixture. This reduction in $V_i$-induced inhibition in the presence of Ad is not likely to be due to an increase in reaction rate caused by the formation of the substrate analogue AdV. The concentration of the substrate analogue AdV is estimated to be equal to 6 μM in solutions containing 15 mM Ad and 2.0 mM $V_i$. This

value was calculated as previously described, using $K_{eq}$ = 0.19 $M^{-1}$ (Gresser and Tracey, 1985). The activity of 6 μM AdV would be expected to have a negligible effect on the rate of reaction in the presence of 2.5 mM AMP. Therefore it has been assumed in this experiment that the NADH oxidation observed is a function of the MK catalyzed phosphoryl transfer from ATP to AMP. A rationale for the reduction in $V_i$ inhibition that was observed when Ad was added to the reaction mixtures is presented at the end of the inhibition studies (see The Formation of $Ad_2V_2$).

The Effect of Different Concentrations of $V_i$ and of ($V_i$ + AMP) on MK Activity (2ADP = AMP + ATP)

The reaction velocity vs $V_i$ concentration for MK catalyzed phosphoryl transfer between ADP molecules is represented in the uppermost curve of Figure 13. The reaction rate in the absence of inhibitor is estimated from the figure to be 23.8 μM/minute, while in the presence of 0.60 mM $V_i$ the rate is 22.2 μM/minute. Thus 0.60 mM $V_i$ inhibits the enzyme activity by 7%. The maximum concentration of $V_i$ did not exceed 0.60 mM, as higher concentrations produce curvature of the plots due to vanadate oligomer formation.

Figure 14 is a reciprocal plot of the data from Figure 13. The data was analyzed using the procedure of Yonetani and Theorell, 1964. They showed that when 2 competitive inhibitors bind at the same site on an enzyme, their interaction can be quantitated by using equation 1. The derivation of equation 1 can be found in Appendix IV.

Figure 13

The effect of different $V_i$ concentrations on the rate of MK catalyzed phosphoryl transfer reaction between ADP molecules, in the absence and in the presence of AMP. Reaction mixtures contained 50 mM Tris-OAc, 0.25 mM ADP, 1.0 mM $MgCl_2$, 0.17 μg/mL MK (chicken muscle), 1.0 mM glucose, 50 μg/mL HK, 50 μM NADP, 3.0 μg/mL G6PDH and the indicated concentrations of $V_i$. The reaction was initiated by adding 10 μL of the MK stock solution into a reaction mixture that contained all of the other reagents at 25°C, pH 7.4. The sample volume was 3.0 mL. The rate of reaction was measured in the absence of AMP (❑) and in the presence of 0.050 mM (◆), 0.10 mM (■) and 0.40 mM (◊) AMP. The reaction was monitored by measuring the rate of NADP reduction in accordance with the mechanism shown in Scheme Va.

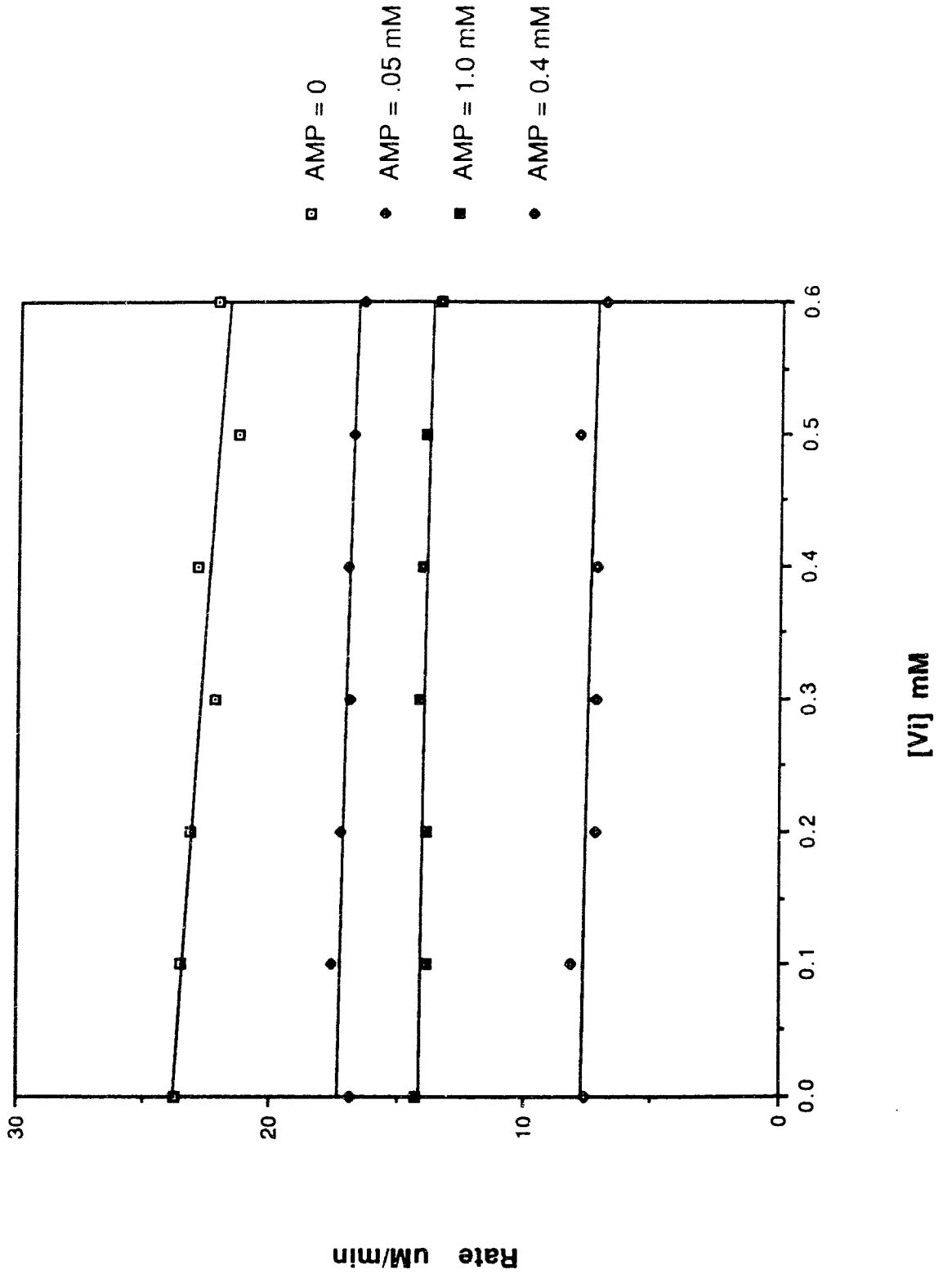
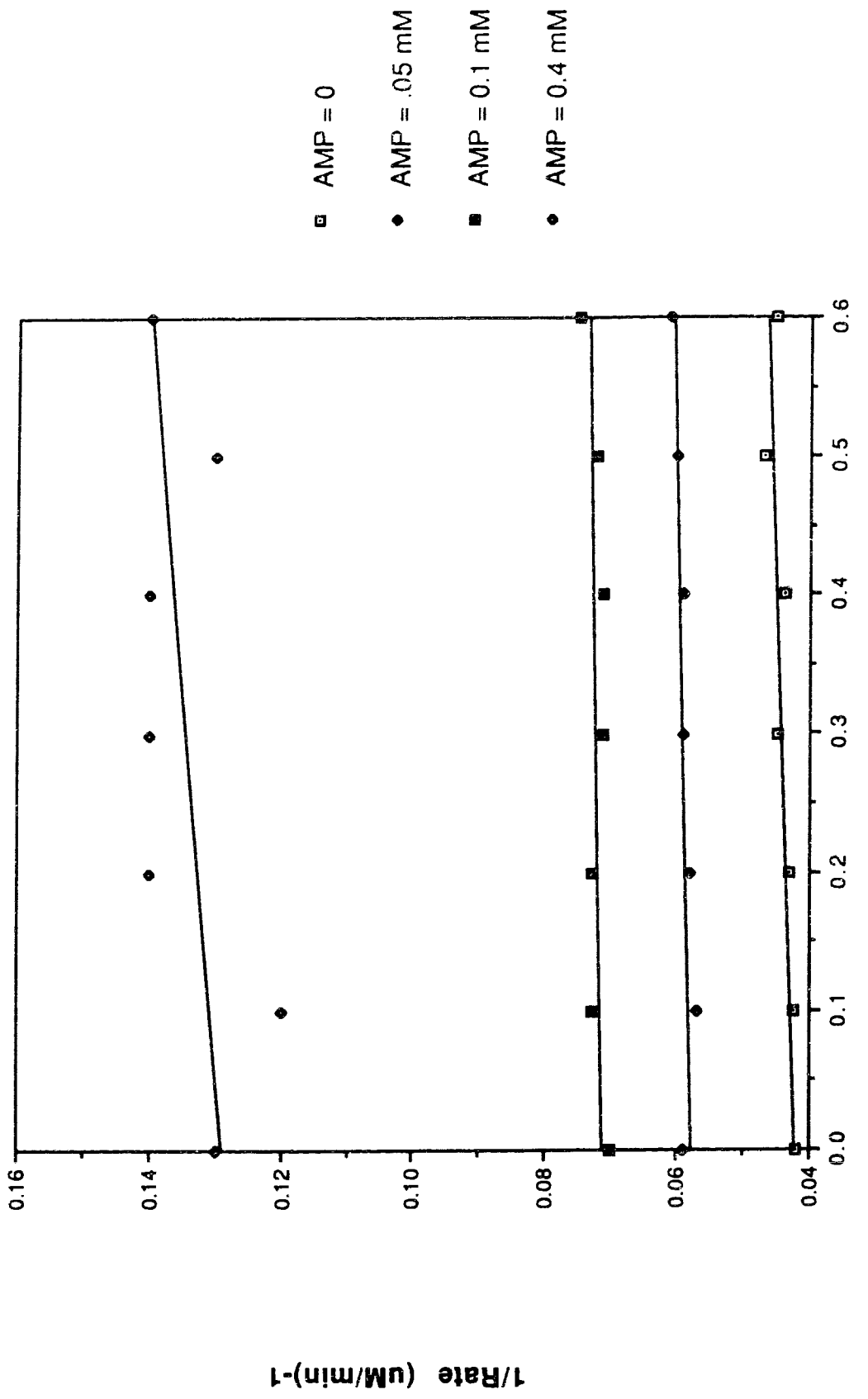

AMP = 0
AMP = .05 mM
AMP = 1.0 mM
AMP = 0.4 mM
Rate uM/min
30
20
10
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
[Vi] mM

Figure 14

The effect of different $V_i$ concentrations on the rate of MK catalyzed phosphoryl transfer reaction between ADP molecules, in the absence and in the presence of AMP. A reciprocal plot of the data from Figure 13.


AMP = 0
AMP = .05 mM
AMP = 0.1 mM
AMP = 0.4 mM
1/Rate (uM/min)-1
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.0
0.1
0.2
0.3
0.4
0.5
0.6
[Vi] (mM)

$$\frac{1}{v} = \frac{K_M}{V_m S}\left(1 + \frac{I_2}{K_2}\right) + \frac{K_M I_1}{V_m S K_1}\left(1 + \frac{I_2}{\alpha K_2}\right) + \frac{1}{V_m} \qquad (1)$$

$v$ = initial velocity

$K_M$ = Michaelis constant of substrate

$V_m$ = maximum velocity

$S$ = substrate concentration

$I_1$, $I_2$ = concentrations of inhibitors 1 and 2

$\alpha$ = interaction constant

$K_1$, $K_2$ = dissociation constants of inhibitors 1 and 2

Equation 1 is symmetrical for inhibitors 1 and 2. The symbol $\alpha$ expresses the influence of one inhibitor on the dissociation constant of the other inhibitor. An $\alpha$ value between 0 and 1 indicates that two inhibitors behave synergistically; that is the $K_i$ of one inhibitor is reduced in the presence of the other inhibitor. An $\alpha$ value between 1 and $\infty$ represents repulsion between two inhibitors. An $\alpha$ value of 1 indicates that both inhibitors can bind to the enzyme simultaneously, but binding of neither inhibitor affects the binding of the other. An $\alpha$ value of $\infty$ indicates that the binding is mutually exclusive. A fast visual analysis of inhibitor interaction can be obtained graphically. Plots of 1/v vs $I_1$ at several fixed concentrations of $I_2$, at constant substrate concentration, appear parallel when the inhibitor binding is mutally exclusive, that is when the presence of one inhibitor at the

binding site completely prevents binding of the other inhibitor. This can be seen from the relation between the slope and $I_2$, shown in equation 2. At $\alpha = \infty$ the slope is a constant value, independent of the concentration of $I_2$.

$$\text{Slope} = \frac{K_M}{V_m S K_1}\left(1 + \frac{I_2}{\alpha K_2}\right) \qquad (2)$$

When synergism is present between two inhibitors, the lines converge and meet at a point where the value of $-I_1$ is equal to $\alpha K_1$. At this point the value of 1/v is independent of the concentration of $I_2$. This can be seen when equation 1 is rearranged by collecting the terms which contain $I_2$, as shown in equation 3.

$$\frac{1}{v} = \frac{K_M}{V_m S} + \frac{K_M}{S V_m K_2}\left(I_2 + \frac{I_1 I_2}{\alpha K_1}\right) + \frac{1}{V_m} \qquad (3)$$

The plots in Figure 14 are parallel. This indicates that there is no potentiation of $V_i$ inhibition by AMP. This result was somewhat surprising in view of the fact that myokinase has a binding site which does not require a metal cation cofactor for nucleotide binding. At concentrations of 0.4 mM AMP and 0.6 mM $V_i$, the concentration of the AMPV anhydride is 2.2 μM. The concentration of AMPV was estimated using a $K_{eq}$ of 9.2 $M^{-1}$, obtained by Tracey et al., 1988a. AMPV is not expected to bind to a site which requires a metal cation, due to the apparent inability of the PV moiety to chelate the divalent cation. However, it might be expected that the enzyme would have a greater affinity for AMPV than for $V_i$ alone, at the site which normally binds

uncoordinated AMP and ADP. These results will be considered again after the effects of other inhibitors have been presented.

The Effect of $V_i$, $P_i$, Ad, ($V_i$ + $P_i$), ($V_i$ + Ad), ($P_i$ + Ad) and ($V_i$ + $P_i$ + Ad) on MK Activity (2ADP = AMP + ATP)

A weak inhibition of MK catalyzed phosphoryl transfer between ADP molecules was observed in the presence of 0.6 mM $V_i$ (5% - 11%) and a moderate inhibition was observed in the presence of 30 mM $P_i$ (20% - 36%). The presence of 84 mM KCl did not affect the reaction rate. Inhibition was not observed in the presence of 15 mM Ad. The quantity of inhibition observed in mixtures of $P_i$ + $V_i$, adenosine + $V_i$, adenosine + $P_i$ and adenosine + $V_i$ + $P_i$, could be accounted for by the presence of 30 mM $P_i$, or by the additive inhibition of $P_i$ and 0.60 mM $V_i$. The inhibition observed in the presence of 0.27 mM $PP_i$ was approximately that observed in mixtures of $P_i$ and $V_i$, where the concentration of the PV anhydride was estimated to be equal to 0.27 mM. The presence of adenosine did not change the amount of inhibition by $PP_i$. The averaged results of several experiments are shown in Table I. In two cases weak synergistic effects were observed when the data were analysed with the method of Yonetani and Theorell, 1964. This method is described in the previous section. The pairs of inhibitors: $P_i$ + $V_i$ and Ad + $P_i$ produced slightly convergent lines when 1/v vs $I_1$ was plotted at different fixed concentrations of $I_2$. However, the synergism did not significantly effect the total % inhibition of MK activity, which could be accounted for by the presence of 30 mM $P_i$, or

by a mixture of $P_i$ and $V_i$. The presence of 15 mM adenosine actually reduced the amount of inhibition demonstrated by $V_i$. This was a very slight but consistent observation in these experiments. The reduction

Table I

The Effects of Adenosine, Vanadate and Phosphate on MK Activity in the Presence of ADP

| Conditions | % Inhibition | No. of Experiments |
|---|---|---|
| $P_i$ (30 mM) | 29 ± 8 | 3 |
| $V_i$ (0.60 mM) | 7 ± 4 | 4 |
| Ad (15 mM) | 0 | 1 |
| Ad + $V_i$ | 5 | 1 |
| $P_i$ + $V_i$ | 41 ± 1 | 2 |
| Ad + $P_i$ | 37 | 1 |
| Ad + $P_i$ + $V_i$ | 44 | 1 |
| $PP_i$ (0.27 mM) | 5 | 1 |
| Ad + $PP_i$ | 5 | 1 |

in inhibition by $V_i$ in the presence of adenosine is likely a result of the formation of the cyclic diester $Ad_2V_2$, as discussed in the following section.

It is not possible to obtain an accurate $K_i$ for $V_i$, due to the curvature of plots of velocity vs $[V_i]$. However, it appears that, as an inhibitor of MK activity, $V_i$ has a potency similar to that of $P_i$. The $K_i$ of $PP_i$ was also similar to that of $P_i$. Evidently the ease with which $V_i$ can form a 5-coordinate species does not significantly enhance its ability to inhibit this enzyme.

It is clear that myokinase retains at least 50% of its activity in the presence of a mixture of 30 mM $P_i$, 0.6 mM $V_i$ and 15 mM adenosine. Therefore the attempt to detect effects which could be attributed to the presence of the ADP analogue AdVP did not fail due to inhibition by the mixture of $P_i$, $V_i$ and adenosine at these concentrations.

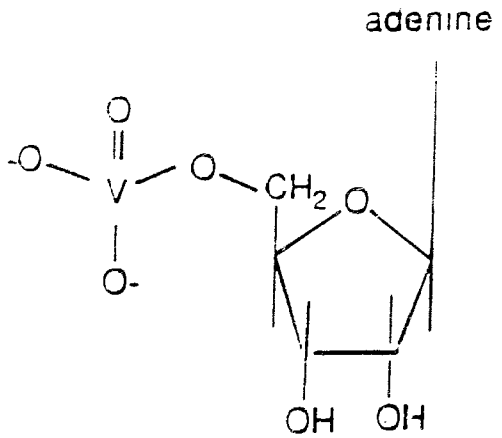
## The Formation of $Ad_2V_2$ : Effects on $V_i$ Inhibition of MK and Effects on MK Activity in the Presence of (Ad + $V_i$)

The partial removal of the $V_i$ inhibition of MK activity in the presence of high concentrations of adenosine (Ad) is likely due to a reduction in the concentration of free $V_i$ through the formation of a cyclic diester containing $V_i$ and adenosine. Similar complex formation has been detected by $^{51}V$ NMR studies of aqueous solutions containing $V_i$ and AMP and solutions containing $V_i$ and uridine (Tracey et al., 1988a). The change in signal intensities that were observed as the ligand (AMP or uridine) concentration was changed lead to the

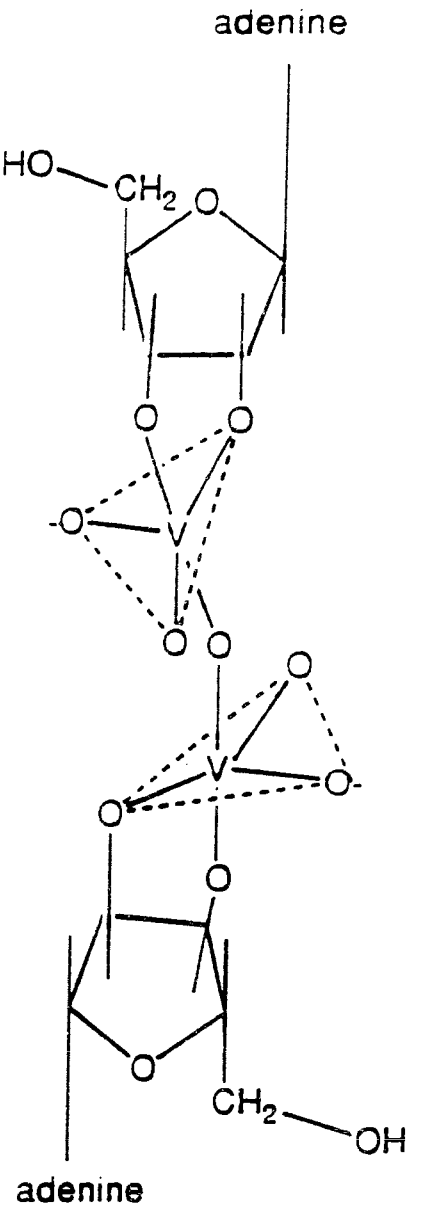
conclusion that these complexes were binuclear in vanadium and that each binuclear complex contained two ligands. Binuclear cyclic diesters were differentiated from vanadate-containing noncyclic esters and anhydrides by their chemical shift. Noncyclic $V_i$ esters and anhydrides have a tetrahedral configuration around the vanadium atom. Peaks representing noncyclic esters in $^{51}V$ NMR spectra are superimposed on the peak which represents monomeric vanadate, a species consisting of four oxygens tetrahedrally arranged around a vanadium nucleus. Cyclic diesters of vanadate appear at relatively lower field than their noncyclic counterparts. The configuration around the vanadium nucleus in a cyclic diester is trigonal bipyramidal due to the acceptance by the vanadium atom of a pair of electrons in excess of the four pairs that make up its tetrahedral structures. Figure 15 shows the structures of (a) the vanadate ester AdV that is thought to act as a substrate analogue of AMP and (b) the cyclic vanadate diester $Ad_2V_2$ that is thought to be responsible for the reduction of $V_i$ inhibition of MK activity at high adenosine concentrations. The equilibrium constant for the formation of the cyclic diester containing $V_i$ and uridine (U), $U_2V_2$, has been reported by Tracey et al., 1988a to be equal to $(2.8 \pm 0.3) \times 10^7$ $M^{-3}$. This value, obtained at pH 7.47 in a solution of 1.0 M ionic strength, is thought to be a good approximation for the formation constant of a similar complex $Ad_2V_2$ that is likely to be present in aqueous solutions containing $V_i$ and adenosine. Figure 16 shows plots

Figure 15

The structures of two of the complexes that are formed spontaneously in aqueous solutions containing adenosine and vanadate: (a) AdV, an ester that is a substrate analogue of AMP, (b) $Ad_2V_2$, a binuclear cyclic diester, the formation of which is favoured at high concentrations of Ad and $V_i$.


adenine
O
-O
V
O
CH2
O
O-
OH
OH

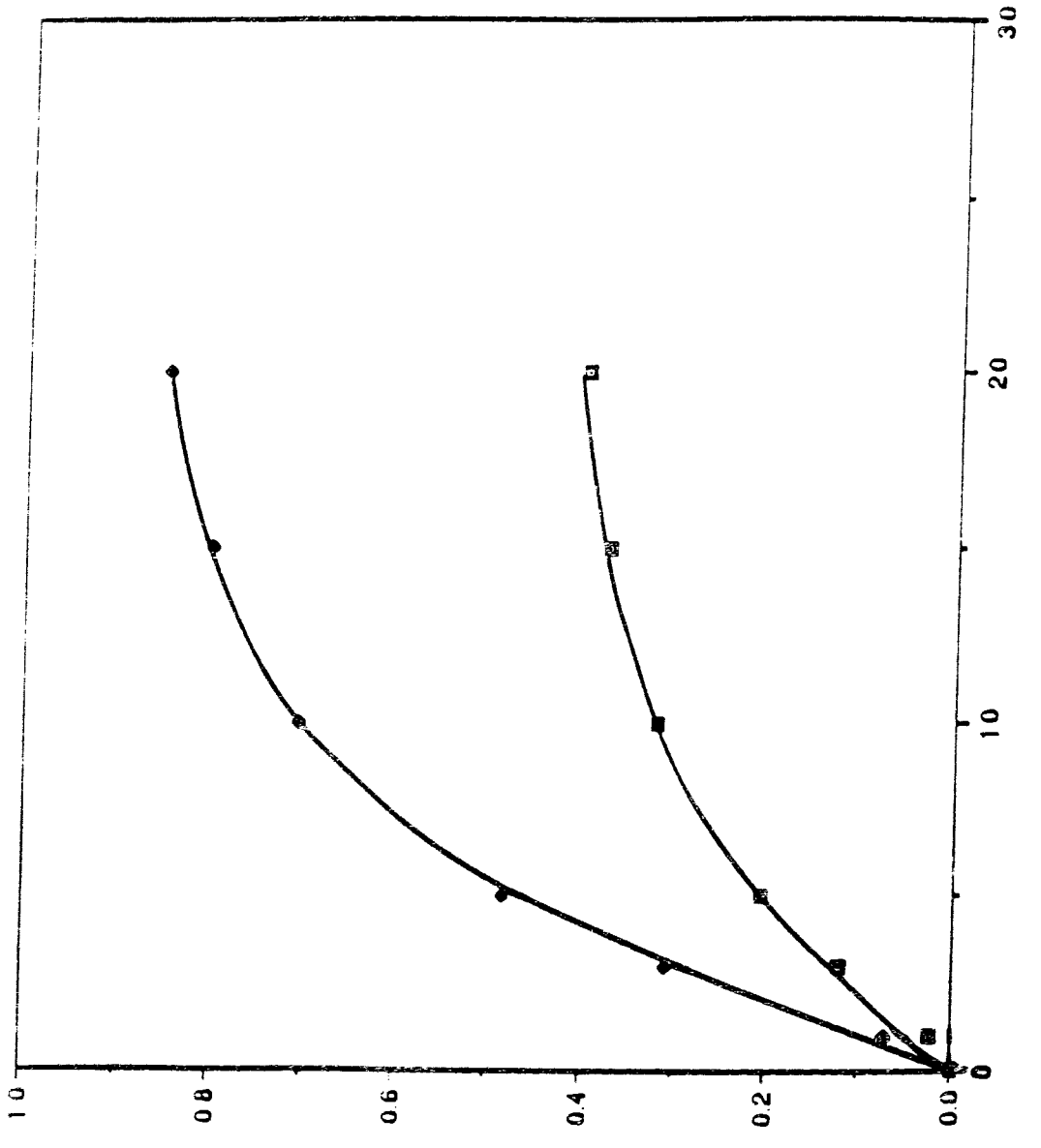
(a) AdV


adenine
HO
CH2
O
O
O
-O
V
O
O
V
O-
O
O
O
CH2
OH
adenine

(b) $Ad_2V_2$

Figure 16

The effect of different Ad concentrations on the formation of the binuclear cyclic diester $Ad_2V_2$ at the following concentrations of $V_i$: 1.0 mM (□), 2.0 mM (◆). The lines were calculated using a formation constant for $Ad_2V_2$ equal to $(2.8 \pm 0.3) \times 10^7$ $M^{-3}$ (Tracey et al., 1988a).


[Vi] – 1.0 mM
[Vi] – 2.0 mM
[Ad2V2] (mM)
[Adenosine] (mM)
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30

of $Ad_2V_2$ concentration vs adenosine concentration at two different concentrations of $V_i$, calculated by using the $K_{eq}$ of $U_2V_2$ published by Tracey et al., 1988a. If it is assumed that $Ad_2V_2$ complex formation is comparable to that of $U_2V_2$, the concentration of $Ad_2V_2$ in solutions containing 15 mM adenosine and 2.0 mM $V_i$ is approximately equal to 0.80 mM. This value for $Ad_2V_2$ represents a value of 1.6 mM for $V_i$ bound by the complex. It is likely to be uncomplexed $V_i$ that is responsible for the inhibition of MK activity. Free $V_i$ could bind to the enzyme at sites which normally accept the phosphate moiety of the nucleotides AMP and ATP. A reduction in the concentration of free $V_i$ from 2.0 mM to 0.4 mM can account for the reduction in the inhibition of MK activity from 54% to 18% as observed in Figure 12. That is the inhibition was reduced to 1/3 when the concentration of free $V_i$ was reduced to 1/5 of its original value, by the addition of 15 mM Ad.

Figures 8 and 9 have the appearance of typical Michaelis-Menten plots of rate vs substrate concentration. As substrate concentration increases, active sites on the enzyme are filled until saturation is achieved. At high substrate concentrations the rate cannot be increased by increasing the substrate concentration, as all of the active sites on the enzyme are occupied. This is unlikely to be the explanation for the curvature of the plots of Figures 8 and 9, in which the substrate is thought to be AdV. A maximum concentration of 2 µM AdV was calculated for reaction mixtures that contained 12 mM Ad and 1.0 mM $V_i$. The Michaelis constant ($K_M$) for the true substrate AMP is equal to 0.5 mM at pH 7.5 in Tris-OAc buffer at 30°C (Callaghan and Weber, 1959), a value which represents the concentration of AMP that

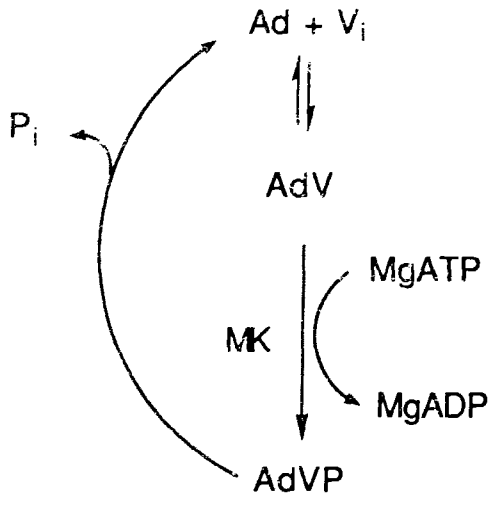
is necessary to obtain half of the maximum rate possible under saturating conditions of ATP. The results of the study that compared the rate of reaction for AMP and AdV suggest that the $K_M$ for AdV is of the same order of magnitude as that of AMP. Therefore a concentration of 2 μM AdV would not be sufficient to saturate the active sites on MK. The fact that the rates do not increase with Ad or $V_i$ concentration in a linear manner may be explained by the depletion of substrate through the formation of the cyclic $Ad_2V_2$ diester. As the concentration of $V_i$ or of Ad increases, there is a corresponding decrease in the proportion of these reactants available to form the AdV ester substrate.

## Summary

The fact that the AMP analogue AdV is accepted as a substrate by MK is not surprising when it is considered that other $V_i$ esters (glucose-6-vanadate, Nour-Eldeen et al., 1985; glycerol-3-vanadate, Craig, 1986) are accepted by enzymes in lieu of their natural phosphorylated substrates. The absence of MK activity in the presence of the $V_i$ anhydride AdVPP, an analogue of ATP, may be due to the inhibition by $PP_i$ of PK, an enzyme used to assay MK activity. The equilibrium constant for the MK/PK/LDH coupled assay shown in Scheme IVc, calculated under standard conditions, was equal to $5.28 \times 10^9$ (Appendix V). Thus product formation would be expected to be favourable in the absence of inhibition. However, the absence of MK activity in the presence of the ADP analogue AdVP cannot be accounted

for by the inhibition of MK by Ad, $V_i$, or $P_i$, or by a combination of these reagents. Some inhibition of phosphate transfer between ADP molecules was evident under the same conditions that were employed to test the activity of the substrate analogue. This inhibition was mainly due to the additive effects of $V_i$ and $P_i$ and was not sufficient to completely inactivate the enzyme. No $PP_i$ was detected after 120 hours, although the equilibrium constant for the transfer of a phosphate group from ADP to $P_i$ is favourable, as shown in Appendix V. Since, as discussed previously, it is reasonable to assume the presence of AdVP in solutions that contain a mixture of Ad, $V_i$ and $P_i$, the failure of AdVP to activate MK requires another explanation. An explanation can be found if it is accepted that nucleotides which are vanadate anhydrides do not bind to sites which require a metal/nucleotide complex, as the VP moiety is a poor chelator of divalent cations. However it may be that the simultaneous binding of Ad, $V_i$ and $P_i$ to form the substrate analogue AdVP is simply too improbable at the reagent concentrations employed.

MK has two binding sites for nucleotides, only one of which has a requirement for a divalent cation. One of the binding sites accepts MgATP and one accepts uncomplexed AMP. In the reverse reaction, an uncomplexed ADP molecule is bound to the AMP site, while MgADP binds to the MgATP site. This mechanism is shown in Scheme VI, where the AMP analogue AdV has been substituted for the normal substrate.




Scheme VI

A mechanism for the MK-catalyzed phosphoryl transfer from MgATP to AdV. The metal cation $Mg^{2+}$ is required for ATP binding, but is not required for AdV binding.

According to the hypothesis presented here, AdVP would not be expected to bind to the MgADP site, as the hypothesis is based on the suggestion that VP is a poor chelator of $Mg^{2+}$. However there is no such restriction for AdVP at the ADP site, as this site does not require that the nucleotide be coordinated with a metal ion in order to bind. The MK-catalyzed phosphoryl transfer between 2 ADP molecules requires that MgADP accept a phophate group from the uncomplexed ADP. Since it is unlikely that AdVP would bind at the MgADP site, AdVP bound to the ADP site will be the phosphate donor in an MK-catalyzed reaction, the reverse direction to the reaction shown in Scheme VI. The products of this reaction will be MgATP and AdV, rather than AMP and AdVPP.

Thus it is understandable that no $PP_i$ was detected in the assay of the reaction mixture. In order for $PP_i$ to be produced, it would be necessary that AdVP coordinate $Mg^{2+}$ and bind to the MgADP site on the enzyme. These results support the hypothesis that the reason that AdVP is not accepted as a substrate is that the VP moiety of AdVP is unable to chelate $Mg^{2+}$, and thus is not bound by the enzyme at sites that require metal ion coordination to the nucleotide. It is unfortunate that it was not possible to measure the results of the kinetic test for the ability of AdVP to act as a substrate in the reverse reaction, due to the activity of ADP. In future work the ability of AdVP to donate a phosphate group to ADP could be tested by adding labelled $P_i$ to the reaction mixture and measuring the quantity of label that is present on the ATP produced.

In order to be covalently transferred from one molecule to another, $P_i$ must go through a high-energy 5-coordinate transition state. It has been rationalized that the potent inhibition of some enzymes by $V_i$ is due to the stability of 5-coordinate $V_i$ anion. Thus an enzyme is effectively immobilized in the transition state when $V_i$ is substituted for $P_i$ in a donor position. Therefore it follows that molecules which contain $V_i$ as a potential donor group will be more potent inhibitors of phosphate transfer than those which contain $V_i$ as a nondonor $P_i$ substitute. An example of a substrate analogue containing $V_i$ in a nondonor position is AdVP, which, if it is able to bind to MK at the non-Mg site, would transfer a $P_i$ group to ADP, bound at the Mg site. Another example is AdV, which would be an acceptor in the forward reaction when bound to the uncoordinated site. Although AdVP

could possibly act as a substrate for MK, as AdV does, the potency of AdVP inhibition would not necessarily be enhanced by the presence of $V_i$ in the molecule. Likewise the presence of $V_i$ in AdV would not be expected to enhance the inhibition by AdV above that effected by AMP. These considerations may at least partially account for the failure of $V_i$ to enhance the inhibition by Ad + $P_i$ and by adenosine. An additional factor which can account for the weak inhibition by AdVP is that the formation of the cyclic diester $Ad_2V_2$ would be expected to significantly reduce the quantity of free $V_i$, and thus of AdVP in the reaction mixture. The inhibition of the forward reaction was reduced from 54% in the absence of adenosine to 18% in the presence of 15 mM adenosine. The inhibition by $V_i$ observed in the reverse reaction (7% at 0.6 mM $V_i$) was proportionally weaker than that observed in the forward reaction (54% at 2.0 mM $V_i$). The proportional increase in inhibition at higher $V_i$ concentrations suggests that $V_i$ oligomers may inhibit the enzyme more effectively than the $V_i$ monomer. The concentration of various $V_i$ species present in aqueous media is shown in Figure 22. The effects of $V_i$ oligomers on the activity of enzymes is discussed in more detail after the experiments with hexokinase have been presented.

Another factor that may partially account for the difference in $V_i$ inhibition of the forward and reverse reactions is discussed below. The concentration of ADP (0.25 mM) in the reverse reaction was much greater than the $K_M$ of this substrate. The $K_M$ for ADP measured in the experiments reported here was 25 μM for the enzyme from chicken muscle, considerably lower than the $K_M$ of 1.6 mM reported by Callaghan and Weber, 1959 for rabbit muscle MK under similar

conditions. The difference in the values of $K_M$ may be due to the difference in source of MK; a $K_M$ of 4 μM has been reported for the enzyme isolated from Escherichia coli (Reinstein et al., 1990), obtained under similar conditions. The inhibition by $V_i$ of the reverse reaction would have been stronger and could be more accurately measured if lower ADP concentrations were employed. However, it is not likely that the conclusions of the study would have been seriously affected.

The studies of the inhibition of MK activity by $V_i$ + AMP showed that the enzyme does not have a high affinity for the AMPV molecule. As in the case of AdVP, the inhibition observed would have been reduced by the high substrate concentration due to the competitive nature of ADP. However, as the AMPV molecule would most likely bind to the non-Mg site, the donor site of the reverse reaction, the $V_i$ moiety would be positioned to act as a transition state analogue of $P_i$. The failure of $V_i$ to enhance the effect of AMP on the inhibition of the reverse reaction suggests that $V_i$ cannot attain the stable 5-coordinate geometry that is so effective in reducing the enzyme activity.

The site on MK which accepts the uncoordinated nucleotide has been shown to be quite specific for adenosine nucleotides (Noda, 1973). Also it has been shown by other investigators that $PP_i$ is accepted by the Mg site, but not by the uncoordinated site (Zabern, 1972). MK catalyses the production of AMP and $PPP_i$, but not ATP and $P_i$, in the presence of $PP_i$ and ADP. The reaction rate of the phosphorylation of $PP_i$ by ADP is several orders of magnitude smaller than the phosphorylation of ADP by another molecule of ADP. However, the fact

that ADP cannot accept a phosphate group from $PP_i$ indicates that $PP_i$ does not act as a substrate at the noncoordinated site of the enzyme, as it is the Mg site that accepts the phosphate group in the reverse reaction. The specificity of the AMP/ADP site undoubtedly has a negative effect on the binding of substrate analogues and can be added to the list of factors that may prevent analogue binding. It may be this specificity which prevents the formation of the stable 5-coordinate $V_i$ structure in AMPV.

## Experiments with Hexokinase

### Tests for Substrate Analogues of ATP (AdVPP and AMPVP)*

### Tests for HK Activity with (Ad + $V_i$ + $PP_i$) in the Absence of ATP

The spontaneous formation of the ATP analogue AdVPP, in aqueous solutions containing Ad, $V_i$ and $PP_i$, was discussed in the section titled Experiments with MK. In physiological systems HK catalyzes the transfer of a $P_i$ group from ATP to glucose. If AdVPP is accepted as a substrate by HK, a phosphate group will be transferred from AdVPP to glucose. The acceptance of AdVPP as a substrate by HK was tested by measuring the PPase activity of HK. Reaction mixtures contained Ad (4.0 mM), $V_i$ (1.0 mM), Tris-OAc (50 mM), glucose (1.0 mM), $MgCl_2$ (1.0 mM), HK (0.83 mg/mL), NADP (50 μM), G6PDH (2.9 μg/mL) and either 0.50 mM or 5.0 mM $PP_i$. The reaction was initiated by introducing 30 μL of the glucose stock solution into a reaction mixture which contained all of the other reagents at 25°C, pH 7.4. The experiment was repeated under the same conditions except that the concentration of Ad was 8.0 mM and the concentration of $PP_i$ was 10 mM.

The value for the concentration of AdVPP in solutions containing 8.0 mM Ad, 1.0 mM $V_i$ and 10 mM $PP_i$ has been estimated to be equal to

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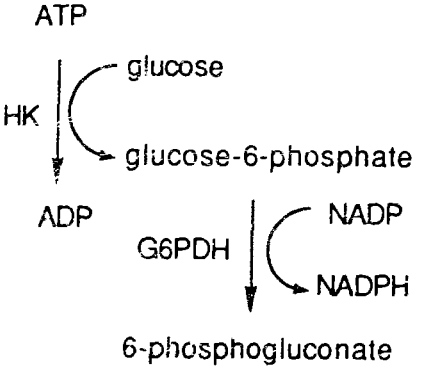
* Structures of vanadate esters and anhydrides are shown in Appendix III

0.6 μM. This value was calculated by using a formation constant for AdVPP equal to 7.4 $M^{-2}$, as described in Experiments with Myokinase. In order to demonstrate that it is possible to observe activity at such low substrate concentrations, a third experiment was performed under the same conditions except that the mixture of Ad, $V_i$ and $PP_i$ was substituted with 1.0 μM ATP. The experiment with 1.0 μM ATP was repeated at a lower HK concentration (50 μg/mL) in order to observe the effect over a longer time.

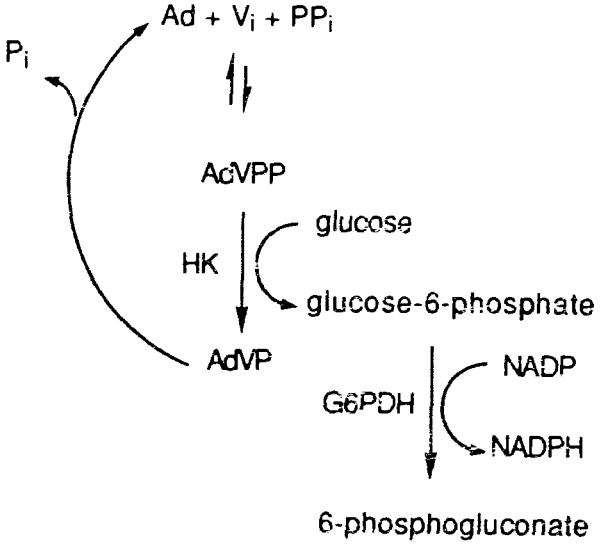
Scheme VIIa shows the physiological reaction in which a phosphate group is transferred from ATP to glucose in a reaction catalyzed by HK. The products of the reaction are ADP and glucose-6-phosphate (G6P). In the presence of NADP and G6PDH, G6P is oxidized to 6-phosphogluconate while NADP is reduced to NADPH in a reaction catalyzed by G6PDH. The rate of the HK-catalyzed reaction can be monitored by following the rate of production of NADPH from NADP, as described in the section titled Measurement of Reaction Velocity. Scheme VIIb shows how AdVPP might substitute for ATP. In the presence of glucose, if AdVPP is accepted as a substrate by HK, a phosphate group will be transferred from AdVPP to glucose to produce G6P and the labile intermediate AdVP. The rate of production of G6P can be monitored by an assay system which contains NADP and G6PDH as described above. The rapid hydrolysis of AdVP will free Ad and $V_i$ for further reaction with $PP_i$; that is Ad and $V_i$, as well as HK will behave as catalysts in the transfer of phosphate from $PP_i$ to glucose.

Scheme VII

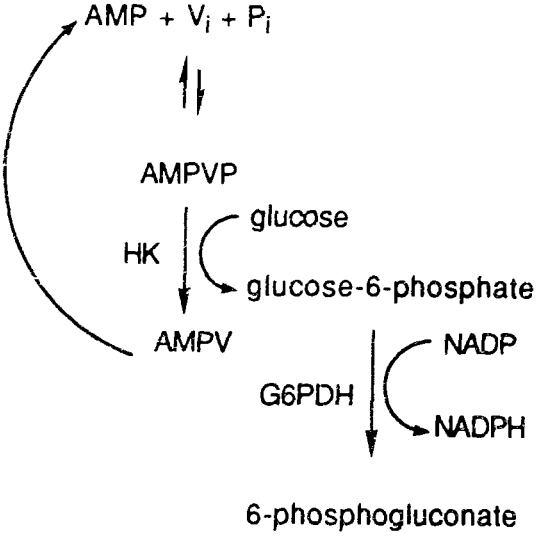
Mechanisms for the hexokinase (HK) catalyzed phosphoryl transfer from (a) ATP to glucose, b) AdVPP to glucose and (c) AMPVP to glucose. (a) represents the physiological activity of HK, (b) represents the HK activity that is expected in the presence of glucose and the spontaneously formed ATP analogue AdVPP and (c) represents the HK activity that is expected in the presence of glucose and the spontaneously formed ATP analogue AMPVP. In each case the product glucose-6-phosphate is oxidized to 6-phosphogluconate in the presence of NADP and G6PDH.


ATP
glucose
HK
glucose-6-phosphate
ADP
G6PDH
NADP
NADPH
6-phosphogluconate

(a)


Ad + Vi + PPi
Pi
AdVPP
glucose
HK
glucose-6-phosphate
AdVP
G6PDH
NADP
NADPH
6-phosphogluconate

(b)


AMP + Vi + Pi
AMPVP
glucose
HK
glucose-6-phosphate
AMPV
G6PDH
NADP
NADPH
6-phosphogluconate

(c)

Tests for HK Activity with (AMP + $V_i$ + $P_i$) in the Absence of ATP

The spontaneous formation of AMPV in aqueous solutions containing $V_i$ and AMP (Tracey et al., 1988a) and the formation of the mixed phosphovanadate anhydride PV in solutions containing $V_i$ and $P_i$ (Gresser et al., 1986), suggests that a small amount of the mixed anhydride AMPVP is present in solutions which contain AMP, $V_i$ and $P_i$. If HK can accept AMPVP as a substrate in the absence of ATP, in the presence of HK, AMPVP and glucose a phosphate group will be transferred from AMPVP to glucose to produce G6P. The mechanism for this reaction is shown in Scheme VIIc. The rate of formation of G6P can be followed as described previously. The formation of AMPVP and its acceptance as a substrate by HK was tested in reaction mixtures which contained AMP (10 mM), $V_i$ (1.0 mM), $P_i$ (10 mM), Tris-OAc (50 mM), glucose (1.0 mM), $MgCl_2$ (1.0 mM), HK (50 μg/mL), NADP (50 μM) and G6PDH (2.9 μg/mL). The reaction was initiated by introducing 30 μL of the glucose stock solution into a reaction mixture which contained all of the other reagents at 25°C, pH 7.4.

The equilibrium constant for the formation of AMPVP is estimated to be equal to 138 $M^{-2}$. This value was calculated by multiplying the formation constant of AMPV, 9.2 $M^{-1}$ (Tracey et al, 1988a), by the formation constant of VP, 15 $M^{-1}$ (Gresser et al., 1986). The procedure for obtaining these constants was discussed in Experiments with Pyruvate Kinase (AMPV) and Experiments with Myokinase (VP). Using $K_{eq}$ = 138 $M^{-2}$, the concentration of AMPVP that

is estimated to be present in solutions containing a mixture of 10 mM AMP, 1.0 mM $V_i$ and 10 mM $P_i$ is 14 μM.

## Tests of Divalent Cation Effects on HK Activity with Substrates and Substrate Analogues

The potential for divalent cations such as $Mg^{2+}$ to support HK catalyzed phosphoryl transfer from ATP to glucose is likely to be related to the ionic radius of the cation as well as to the bond angles of the coordinating phosphate groups. The bond angles of the PV moiety of the nucleotide are likely to be different from those of the PP group of ADP and ATP. The effects of the structure of the chelating groups on cation coordination will be considered later in this thesis. The possibility that substrate analogues might be activated in the presence of cations other than $Mg^{2+}$, in cases where no activity was observed in the presence of $Mg^{2+}$, was tested in the following experiment. The effects of different divalent cations on the reaction rate with and without $Mg^{2+}$ was first tested in the presence of ATP with the chloride salts of Mn, Co, Fe, Ni, Cu, Zn, Ca and Sr. Reaction mixtures contained Tris-OAc (50 mM), ATP (33 μM), glucose (1.0 mM), HK (1.7 μg/mL), NADP (50 μM), G6PDH (3.0 μg/mL) and either $MgCl_2$ (1.0 mM) or the chloride salt of another divalent cation (1.0 mM). In other experiments the rate of reaction was measured under the same conditions except that instead of ATP, a mixture of Ad (15 mM), $V_i$ (2.0 mM) and $PP_i$ (10 mM) or a mixture of AMP (20 mM), $V_i$ (2.0 mM) and $P_i$ (30 mM) was included in the reaction mixture. In the experiments

where substrate analogues were used instead of ATP, the concentration of HK was increased to 50 μg/mL.

## Tests for the Inhibition of HK

The anions $V_i$, $P_i$ and $PP_i$ are similar in structure and electronic properties to the phosphate moieties of ATP. Thus they have the potential to compete with ATP for binding sites on HK. The presence of these anions in the experiments designed to detect the presence of ATP analogues might reduce or eliminate the ability of HK to bind the analogues. Therefore the effects of these anions on the rate of HK-catalyzed phosphoryl transfer from ATP to glucose was examined. In cases where inhibition of HK was detected, similar tests were performed on the auxiliary enzyme G6PDH. In addition, a mixture of ADP and $V_i$ was tested for its ability to inhibit the enzyme. The species ADPV has been shown to be a potent inhibitor of other phosphoryl transferring enzymes, as discussed in the Introduction.

## Tests for the Inhibition of HK Activity by $V_i$, $P_i$ and ($V_i$ + $P_i$)

The effects of $V_i$, $P_i$ and a mixture of $V_i$ and $P_i$ on HK catalyzed phosphoryl transfer from ATP to glucose was studied at different concentrations of ATP. The presence of 30 mM $P_i$ in some of the reaction mixtures increased the ionic strength of these solutions significantly; therefore KCl was added to all of the reaction mixtures in appropriate concentrations to maintain an ionic strength of 0.10 M.

Preliminary tests showed that the presence of KCl up to 0.10 M did not affect the reaction velocity. The rate of reaction was tested in the absence of inhibitor in reaction mixtures which contained Tris-OAc (50 mM), KCl (0.10 M), glucose (1.0 mM), $MgCl_2$ (2.0 mM), HK (0.20 μg/mL), NADP (50 μM), G6PDH (2.9 μg/mL) and different concentrations of ATP (0 - 0.70 mM). The reaction was initiated by introducing 15 μL of the HK stock solution into a reaction mixture which contained all of the other reagents at 25°C, pH 7.4. In separate experiments 1.0 mM $V_i$ or 30 mM $P_i$ or a mixture of $V_i$ (1.0 mM) and $P_i$ (30 mM) was included in the reaction mixture. In the tests for inhibition by 30 mM $P_i$ (ionic strength = 70 mM), instead of 0.10 M KCl, 30 mM KCl was included in the reaction mixtures to bring the ionic strength to 0.10 M.

The effect of a mixture of $V_i$ and $P_i$ on the rate of reaction in the presence of a concentration of ATP comparable to that estimated for the substrate analogue AMPVP (14 μM) was tested in a reaction mixture that contained Tris-OAc (50 mM), ATP (1.0 μM), $V_i$ (1.0 mM), $P_i$ (10 mM), glucose (1.0 mM), $MgCl_2$ (2.0 mM), HK (40 μg/mL), NADP (50 μM) and G6PDH (2.9 μg/mL).

Tests for the Inhibition of HK Activity by $V_i$, $PP_i$ and ($V_i$ + $PP_i$)

The effects of $V_i$, $PP_i$ and a mixture of $V_i$ and $PP_i$ on HK catalyzed phosphoryl transfer from ATP to glucose was studied at different concentrations of ATP. The presence of 10 mM $PP_i$ in some of the reaction mixtures caused an increase of 46 mM in the ionic strength of these solutions. In order that all of the reaction mixtures would be

equal in ionic strength, KCl, (46 mM, ionic strength = 46 mM) was added to reaction mixtures which did not contain $PP_i$. KCl is a nonreactive species under these conditions (see preceeding section). The rate of reaction in the absence of inhibitor was tested in reaction mixtures which contained Tris-OAc (50 mM), glucose (1.0 mM), $MgCl_2$ (2.0 mM), KCl (46 mM), HK (0.20 μg/mL), NADP (50 μM), G6PDH (2.9 μg/mL) and different concentrations of ATP (0 - 0.70 mM). The reaction was initiated by introducing 15 μL of the HK stock solution into a reaction mixture which contained all of the other reagents at 25°C, pH 7.4. In separate experiments 1.0 mM $V_i$ or 10 mM $PP_i$ or a mixture of $V_i$ (1.0 mM) and $PP_i$ (10 mM) was included in the reaction mixture. KCl was not included in the reaction mixtures which contained $PP_i$.

The effect of a mixture of $V_i$ and $PP_i$ on the rate of reaction in the presence of a concentration of ATP comparable to that of the substrate analogue AdVPP (0.6 μM) was tested in reaction mixtures which contained Tris-OAc (50 mM), ATP (1.0 μM), $V_i$ (1.0 mM), $PP_i$ (10 mM), glucose (1.0 mM), $MgCl_2$ (1.0 mM), HK (50 μg/mL), NADP (50 μM) and G6PDH (2.9 μg/mL).

Tests for the Inhibition of G6PDH Activity by $V_i$, $P_i$ and $PP_i$

It was observed that, in the presence of the substrates ATP and glucose, the anions $V_i$, $P_i$ and $PP_i$ had inhibitory effects on HK activity. Since the assay for HK activity was coupled to the G6PDH-catalyzed reduction of NADP, these anions were tested for their ability to inhibit G6PDH. The rate of NADP reduction by G6P, catalyzed by G6PDH, was

measured without inhibitor in reaction mixtures that contained Tris-OAc (50 mM), NADP (50 μM), G6P (33 μM) and G6PDH (1.0 μg/mL). The reaction was initiated by introducing 10 μL of the G6PDH stock solution into a reaction mixture that contained all of the other reagents at 25°C, pH 7.4. In separate experiments, the rate of reaction in the presence of each of the anions was measured under the same conditions, except that in the test for $V_i$ inhibition the concentration of G6PDH was 2.9 μg/mL. $V_i$ was tested at one concentration, 2.0 mM; $P_i$ was tested over a range of 0 - 125 mM; $PP_i$ was tested over a range of 0 - 100 mM.

Tests for the Inhibition of HK Activity by $V_i$, ADP and ($V_i$ + ADP)

The rate of HK-catalyzed phosphoryl transfer from ATP to glucose was measured at different concentrations of $V_i$ (0 - 0.60 mM) in the presence of 0, 0.40 and 0.80 mM ADP. Reaction mixtures contained Tris-OAc (50 mM), ATP (0.1 mM), glucose (1.0 mM), $MgCl_2$ (2.0 mM), HK (0.17 μg/mL), NADP (50 μM), G6PDH (3.0 μg/mL) and the appropriate concentrations of $V_i$ and ADP. The reaction was initiated by introducing 10 μL of the stock HK solution into a reaction mixture which contained all of the other reagents at 25°C, pH 7.4.

$^{51}V$ NMR Spectroscopy: Test for HK/$V_i$ Binding

The inhibition of HK in the presence of $V_i$ was investigated by attempting to determine the extent to which the enzyme was able to bind $V_i$. The binding of the $V_i$ ion to a large molecule such as HK

increases the rate of relaxation of the $V_i$ nuclei. The increase in relaxation time has the effect of broadening the peaks to the point where they cannot be differentiated from the baseline of the $^{51}V$ NMR spectra. $^{51}V$ NMR spectra of aqueous solutions of V(V) are composed of several peaks, each representing a different species of oxovanadium ion. The quantity of free $V_i$ present in a given solution is estimated by adding the integrated peak areas for all of the species represented in the spectrum of that solution. In the presence of a large molecule such as HK, some of the $V_i$ may be bound and thus removed from the free $V_i$ pool. The extent of this binding is estimated by calculating the difference in total peak area between $V_i$ spectra recorded with and without enzyme present. If $V_i$ binds to the enzyme at active sites, the introduction of substrates which have affinity for these sites would be expected to have the effect of displacing some of the bound anion. The position of the bound $V_i$ was examined by measuring the total peak area of the HK/$V_i$ solution in the presence of the natural substrates, and comparing these values with the values for the total peak area of HK/$V_i$ solutions in the absence of substrates.

Details of how the spectra were acquired can be found in the section on Methods. The experiments were conducted at ambient temperature.

HK Activity Tests

The preparation of HK for the $^{51}V$ NMR spectroscopic study has been described under Reagent Preparation. An estimate of the activity

of the preparation was performed before and after the NMR spectra were taken. Reaction mixtures for the activity tests contained Tris-OAc (50 mM), ATP (2.0 mM), glucose (1.0 mM), $MgCl_2$ (2.0 mM), HK (87 nM), NADP (50 μM) and G6PDH (3.0 μg/mL). The reaction was initiated by introducing 30 μL of the glucose stock solution into a reaction mixture which contained all of the other reagents at 25°C, pH 7.4.

Preparation and Measurement of $V_i$ Standard Solutions

$V_i$ standard solutions were prepared as follows. 1.5 mL Tris-OAc (50 mM) was added to each of 4 weighed NMR tubes. The volume of buffer solution in the NMR tube was determined by its weight, assuming that the density of the solution was equivalent to that of water, 1.0 g/mL. This procedure was necessary because of the difficulty of introducing a precise volume of fluid into the long narrow tubing. An aliquot of the stock $V_i$ solution (100 mM) was then added to each NMR tube to achieve the desired concentration of $V_i$ and the solution was mixed on a vortex mixer. In order to maintain an approximately constant volume of solution, the volume of the aliquots of $V_i$ stock solution were small (1 or 2 microliters) relative to the volume of buffer in the NMR tube. Four standard solutions were prepared: 0.14, 0.27, 0.48 and 0.68 mM $V_i$. The range of $V_i$ concentrations used to test for binding of the anion to HK was chosen by considering the results of kinetic experiments, where the inhibition of HK by 1.0 mM $V_i$ was approximately 40%. The highest $V_i$ concentration was lower than the concentration necessary to saturate active sites on the enzyme, but high enough to be observed if

displaced from the enzyme in the presence of substrate. If saturating concentrations of $V_i$ were present when the substrate was added, the substrate might not compete effectively for active sites on the enzyme. Furthermore, high $V_i$ concentrations might result in some nonspecific binding of $V_i$ to the protein. $^{51}V$ NMR spectra were recorded for each of the standard solutions and the area under all of the peaks was estimated for each of the spectra. A plot of total peak area vs $V_i$ concentration was prepared in which each point on the standard plot represented the total peak area for a given concentration of $V_i$. The computing facility of the Bruker AM 300 spectrometer provides automatic integration of peak area. The estimation of relative peak area by cutting out and weighing the peaks gave results similar to those recorded by the spectrometer.

Preparation and Measurement of HK/$V_i$ Solutions

The enzyme solution was prepared and its activity was measured as described in the sections titled Reagent Preparation and HK Activity Tests. The concentration of HK used in the $^{51}V$ NMR study was 27 mg/mL, which is converted to 0.27 mM if a value of 100 kD is used as the molecular weight of the protein (Colowick, 1972). HK is a dimer with a binding site for ATP and glucose on each of its subunits. Therefore the HK used in this experiment contained a total concentration of 0.54 mM catalytic sites. A volume of 1.5 mL of the prepared enzyme solution (27 mg/mL) was placed in an NMR tube. As in the case of the buffer solution the volume was determined by weight,

assuming that the density of the solution was 1.0 g/mL. A small aliquot of 100 mM $V_i$ was added to the enzyme solution in the NMR tube, the tube was mixed gently by vortex and the $^{51}V$ NMR spectra was recorded. This procedure was repeated as successive aliquots of the $V_i$ solution were introduced into the same enzyme solution. Spectra were recorded for concentrations of 0.14, 0.20, 0.27, 0.34, 0.48 and 0.55 mM $V_i$. The total peak area for each of the spectra was determined as described previously. A plot of peak area vs $V_i$ concentration was superimposed on the standard plot in order to compare the $V_i$ spectra obtained with and without HK.

After 0.55 mM $V_i$ had been added to the HK solution in the NMR tube and the spectra had been recorded, $MgCl_2$ (2.0 mM), ATP (1.5 mM) and glucose (1.4 mM) were added, serially, to the $V_i$/HK solution and a spectrum was recorded after each addition. The concentrations of the substrates added was 5 - 10 times as high as their $K_m$ values to ensure their effective competition with $V_i$ ions for the active sites on the enzyme.

# Results and Discussion

## The Activity of HK with Substrate Analogues

### The Effect of ($Ad+V_i+PP_i$) and ($AMP+V_i+P_i$) on HK Activity in the Absence of ATP

There was no evidence that either AdVPP or AMPVP was accepted as a substrate by HK. In solutions which contained mixtures of 8.0 mM Ad, 1.0 mM $V_i$ and 10 mM $PP_i$, the concentration of AdVPP is estimated to be 0.6 μM. Solutions containing a mixture of 10 mM AMP, 1.0 mM $V_i$ and 10 mM $P_i$ are expected to contain 14 μM AMPVP. These values were calculated previously in the experimental section. Product formation in the HK/G6PDH coupled assay shown in Scheme VII was estimated to be thermodynamically favourable (Appendix V). The failure of ADVPP and AMPVP to be accepted by HK may be due to the low concentration of these substrate analogues. However, it was possible to observe the activity of 1.0 μM ATP, the natural substrate for HK. The change in absorbance for the conversion of 1.0 μM ATP to 1.0 μM ADP is expected to be 0.006. A change of this magnitude (approximately 0.005) could be observed in solutions which contained ATP, glucose, HK and the assay reagents. The rate of this change was dependent on HK concentration; that is although it was very rapid at 0.83 mg/mL HK, it could be observed to take place over several minutes at 50 μg/mL HK (approximately 0.001/minute). Moreover, the change in absorbance was

reproducible when additional aliquots of ATP were added to the reaction mixture after equilibrium was established. The rate of change of absorbance in the presence of the substrate analogues is expected to be slow but continuous. The labile nature of the $V_i$ ester and $V_i$ anhydride bonds allow the starting materials, $V_i$, $PP_i$, Ad and AMP, to be released after the substrate analogue has been dephosphorylated (see Scheme VII, b and c).

It will be shown later that the anions $P_i$ and $PP_i$ have an inhibitory effect on the HK/G6PDH coupled assay. Activity in the presence of 1.0 μM ATP was not observed when a mixture of $V_i$ and $P_i$ or a mixture of $V_i$ and $PP_i$ was present under the same conditions as those used to test for activity of the substrate analogues. Therefore it is possible that the failure to observe activity of HK in the presence of the substrate analogues AdVPP and AMPVP was due to the presence of inhibitory anions. Although the inhibition is reduced at low concentrations of $V_i$, $P_i$ and $PP_i$, experiments performed at low anion concentrations have the disadvantage that the concentration of the substrate analogue is also reduced. For instance, no activity was observed in solutions containing 4.0 mM Ad, 1.0 mM $V_i$ and 0.50 mM $PP_i$, in which the concentration of AdVPP is expected to be 15 nM. Under these conditions the inhibition of HK by $V_i$ and $PP_i$ would be expected to be much lower. Although the $V_i$ concentration was 1.0 mM, the $PP_i$ concentration was reduced to 5% of the $PP_i$ concentration that was tested in the presence of ATP. However, the concentration of the substrate analogue is so low (15 nM, using $K_{eq}$ = 7.4) that activity, if it was present, probably could not be measured.

## The Effects of Divalent Cations Other than $Mg^{2+}$ on HK Activity with Substrate Analogues

None of the divalent cations studied supported HK activity in the absence of ATP in solutions which were expected to contain substrate analogues of ATP. HK-catalyzed phosphoryl transfer from ATP to glucose was supported slightly by the chloride salts of Mn and Co in the absence of $MgCl_2$. In the presence of 1.0 mM $MgCl_2$ the reaction was inhibited about 50% by the chloride salts (1.0 mM) of Ni and Zn. The chloride salts (1.0 mM) of Ca, Sr, Fe and Cu had no effect on the rate of the normal reaction in the presence of $MgCl_2$.

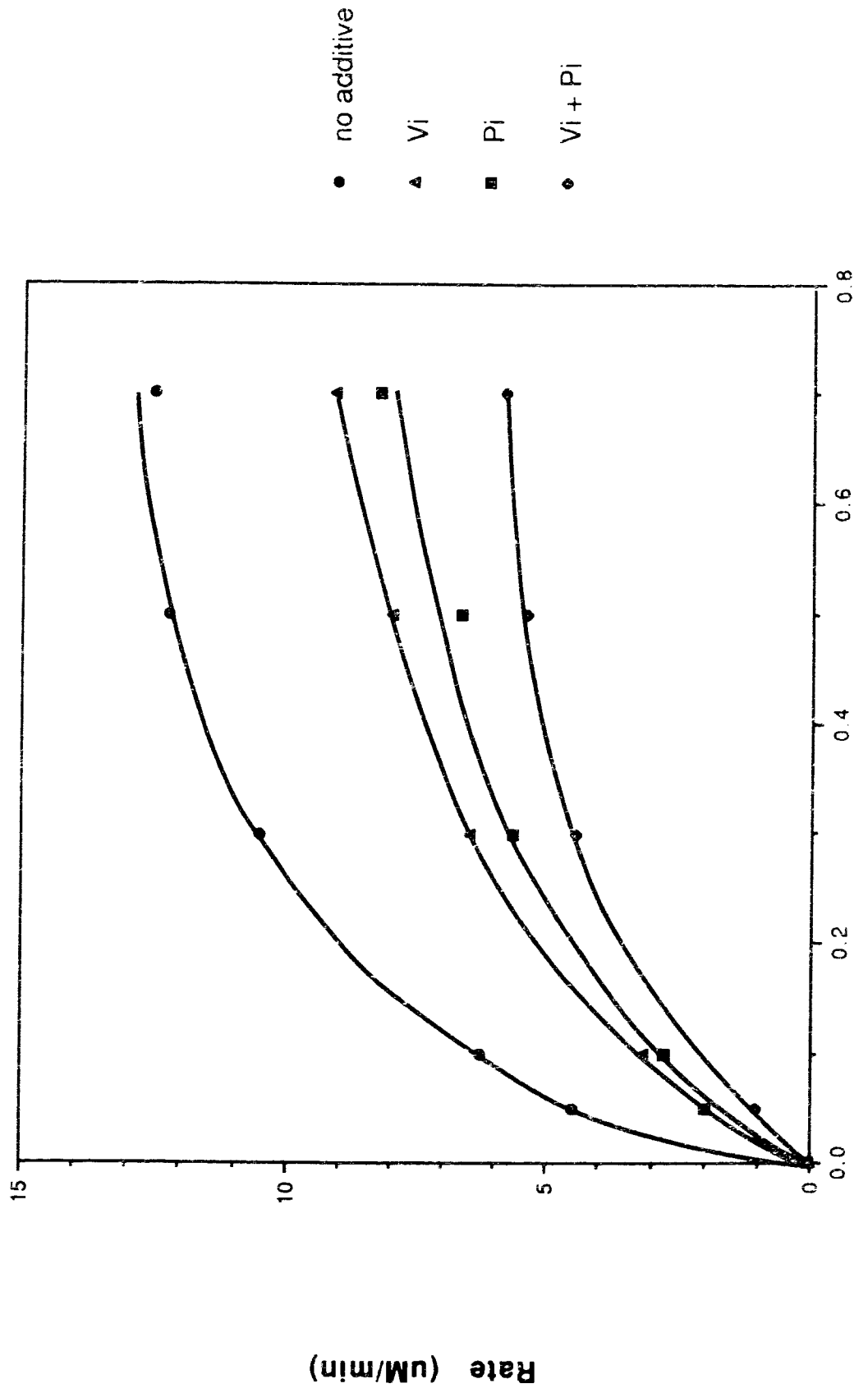
## The Effects of Anions and Nucleotides on HK Activity

### The Effects of $V_i$, $P_i$ and $PP_i$ on HK Activity in the Presence of ATP and Glucose

Figure 17 shows that both $V_i$ and $P_i$ inhibit HK-catalyzed phosphoryl transfer from ATP to glucose. Fromm and Zewe, 1962, have reported Michaelis constants of 0.17 mM and 0.20 mM for glucose and ATP respectively, acting as substrates for HK. These values were obtained at pH 7.6, 28°C, in Tris buffer, under conditions similar to those of the experiments reported here. At saturating concentrations of glucose (1.0 mM), in the presence of 0.20 mM ATP, the inhibition by 1.0 mM $V_i$, 30 mM $P_i$ and a mixture of $V_i$ (1.0 mM) and $P_i$ (30 mM) was 42%, 50% and 60% respectively.

Figure 17

The effect of different ATP concentrations on the rate of HK catalyzed phosphoryl transfer from ATP to glucose in the absence and presence of $V_i$ and $P_i$. Reaction mixtures contained 50 mM Tris-OAc, 1.0 mM glucose, 2.0 mM $MgCl_2$, 0.20 μg/mL HK, 50 μM NADP, 2.9 μg/mL G6PDH, the indicated concentrations of ATP and the following additives: 0.1 M KCl (●), 1.0 mM $V_i$ and 0.1 M KCl (▲), 30 mM $P_i$ and 30 mM KCl (■), 1.0 mM $V_i$, 30 mM $P_i$ and 30 mM KCl (◊). (KCl was added to control the ionic strength of the solutions.) The reaction was initiated by adding 15 μL of the HK stock solution into a reaction mixture that contained all of the other reagents at 25°C, pH 7.4. The sample volume was 3.0 mL. The reaction was monitored by measuring the rate of NADP reduction in accordance with the mechanism in Scheme VIIa.


no additive
Vi
Pi
Vi + Pi
Rate (uM/min)
[ATP] (mM)
0
5
10
15
0.0
0.2
0.4
0.6
0.8

The results of tests for the inhibition of HK-catalyzed phosphoryl transfer from ATP to glucose by $V_i$, $PP_i$ and a mixture of $V_i$ and $PP_i$ are shown in Figure 18. These tests were performed under the same conditions as the tests for $V_i$ and $P_i$. In the presence of 1.0 mM $V_i$, 10 mM $PP_i$ and a mixture of $V_i$ (1.0 mM) and $PP_i$ (10 mM), the inhibition was 36%, 89% and 93% respectively.

The ability of $V_i$, $P_i$ and $PP_i$ to significantly inhibit the HK/G6PDH coupled assay system suggests that it would be unlikely that HK activity in the presence of low concentrations of ATP or of ATP analogues could be observed. In fact the activity of 1.0 μM ATP, measurable in the absence of these anions, was no longer observable when the anions were present at the concentrations indicated in the figures.

The Effects of $V_i$, $P_i$ and $PP_i$ on G6PDH Activity

$V_i$ (2.0 mM) did not inhibit the G6PDH-catalyzed reduction of NADPH in the presence of a saturating concentration of NADP and 33 μM G6P. The other conditions of the experiment were the same as those present in the tests for HK activity with AdVPP and AMPVP. $P_i$ and $PP_i$ were weak inhibitors of G6PDH activity; a plot of % inhibition vs anion concentration is shown in Figure 19. In the presence of 50 μM NADP and 33 μM G6P the rate of NADP reduction was inhibited 16% by 30 mM $P_i$ and 38% by 10 mM $PP_i$.

Figure 18

The effect of different ATP concentrations on the rate of HK-catalyzed phosphoryl transfer from ATP to glucose, in the absence and presence of $V_i$ and $PP_i$. Reaction mixtures contained 50 mM Tris-OAc, 1.0 mM glucose, 2.0 mM $MgCl_2$, 0.20 μg/mL HK, 50 μM NADP, 2.9 μg/mL G6PDH, the indicated concentrations of ATP and the following additives: 46 mM KCl (□), 1.0 mM $V_i$ and 46 mM KCl (◆), 10 mM $PP_i$ (■), 1.0 mM $V_i$ and 10 mM $PP_i$ (◇). (KCl was added to control the ionic strength of the solutions.) The reaction was initiated by adding 15 μL of the HK stock solution to a reaction mixture that contained all of the other reagents at 25°C, pH 7.4. The sample volume was 3.0 mL. The reaction was monitored by measuring the rate of NADP reduction in accordance with the mechanism shown in Scheme VIIa.

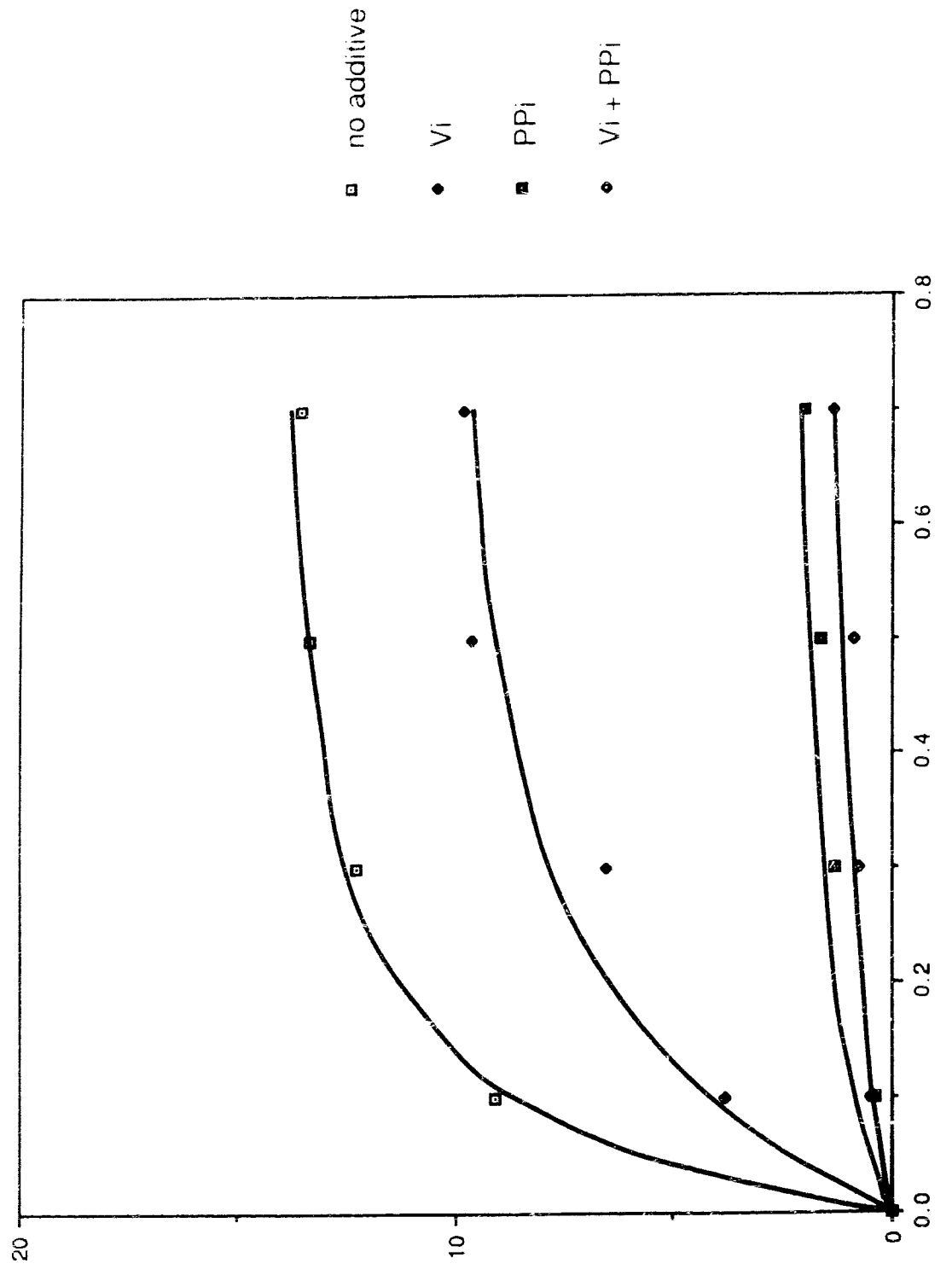
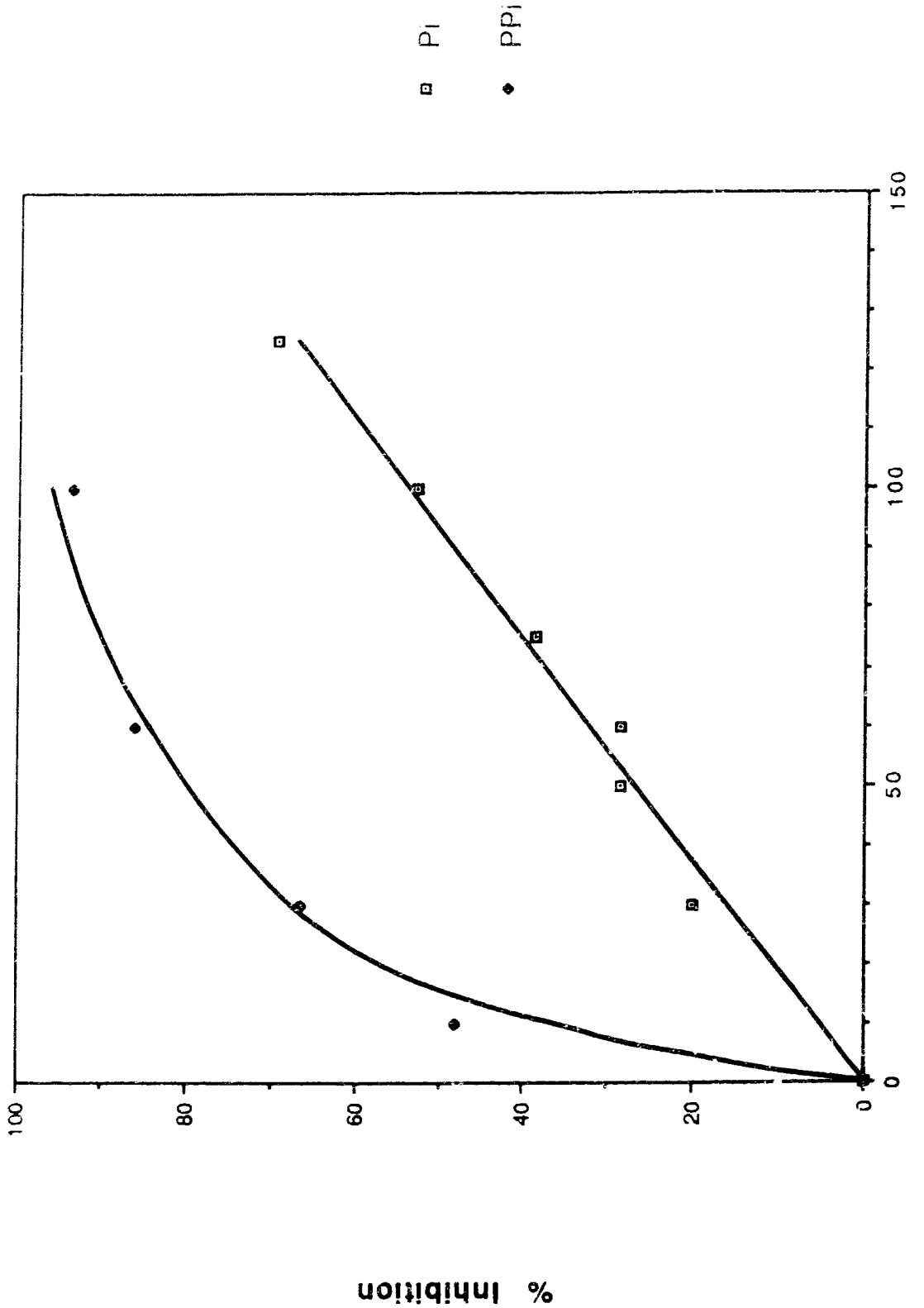

no additive
Vi
PPi
Vi + PPi
Rate (uM/min)
[ATP] (mM)
0
10
20
0.0
0.2
0.4
0.6
0.8

Figure 19

The effect of different concentrations of $P_i$ (□) or $PP_i$ (◆) on the rate of G6PDH catalyzed oxidation of glucose-6-phosphate (G6P). Reaction mixtures contained 50 mM Tris-OAc, 50 μM NADP, 33 μM G6P, 1.0 μg/mL G6PDH and the indicated concentrations of $P_i$ or $PP_i$. The reaction was initiated by adding 10 μL of the G6PDH stock solution to a reaction mixture that contained all of the other reagents at 25°C, pH 7.4. The sample volume was 3.0 mL. The reaction was monitored by measuring the rate of NADP reduction in accordance with the mechanism shown in Scheme VIIa.

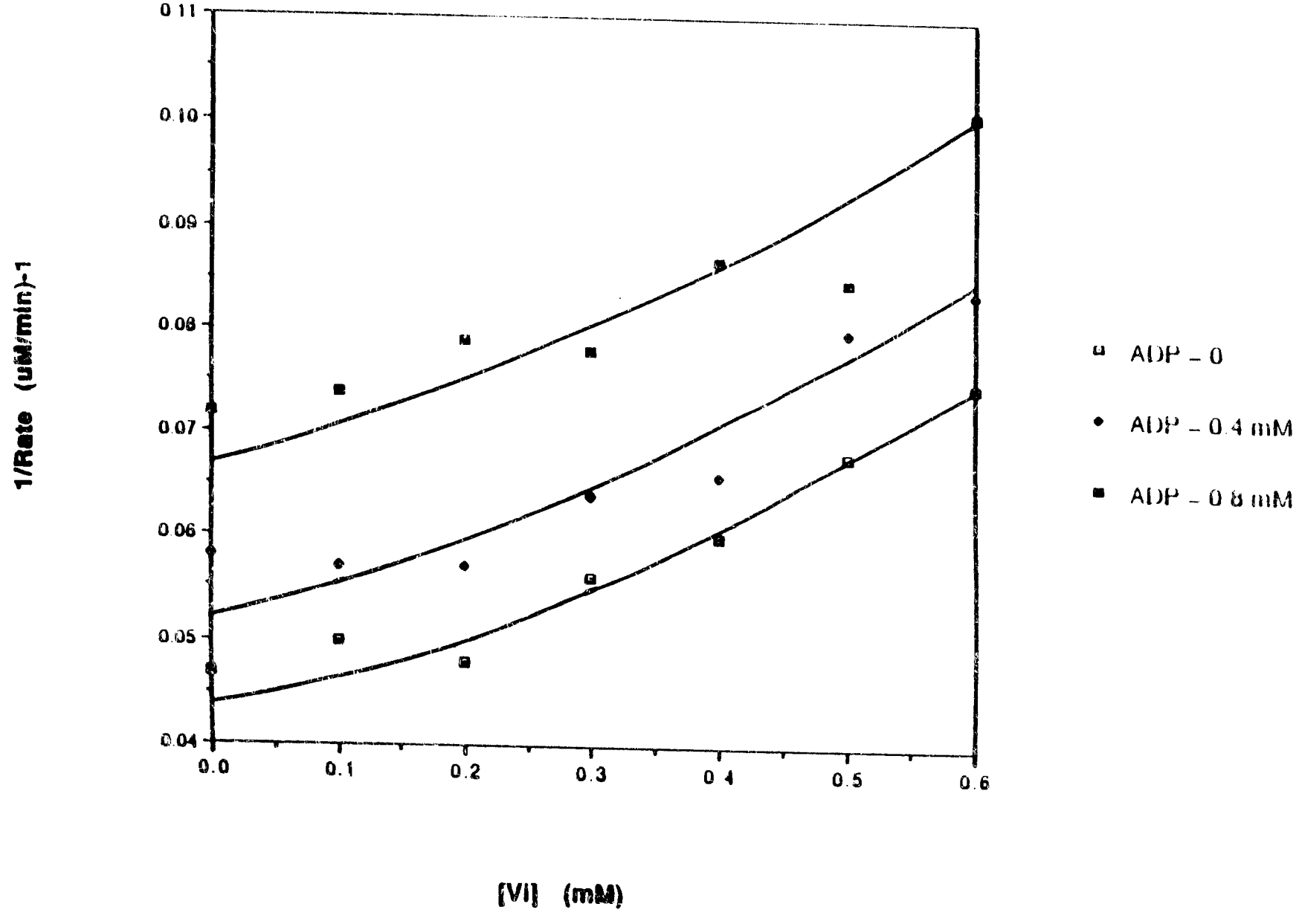

Pi
PPi
% Inhibition
[Pi] or [PPi] mM
0
20
40
60
80
100
0
50
100
150

The Effects of $V_i$, ADP and ($V_i$ + ADP) on HK Activity in the Presence of ATP

$V_i$ and ADP are both weak inhibitors of HK catalyzed phosphoryl transfer from ATP to glucose, but there was no evidence that their effects were synergistic. Data from an experiment in which the concentration of $V_i$ was varied in the absence of ADP and in the presence of two different concentrations of ADP are presented in Figure 20. The fact that the plots are parallel indicates that the binding of the two inhibitors is mutually exclusive, according to the method of analysis of Yonetani and Theorell, 1964. The method is described in the section titled "The Effect of Different Concentrations of $V_i$ and of ($V_i$ + AMP) on MK Activity". The substrate analogue of ATP, ADPV, is expected to be present at a concentration of approximately 4.4 μM in aqueous solutions containing a mixture of 0.80 mM ADP and 0.60 mM $V_i$. The concentration of ADPV is an estimate based on the formation constant of AMPV of 9.2 $M^{-1}$ (Tracey et al., 1988a). ADPV is the species which has been postulated to inhibit the myosin and dynein ATPases by forming a tightly bound transition state analogue of ATP. The inhibition of the myosin and dynein ATPases was significant at concentrations of 400 μM $V_i$ and less than 10 μM ADP (Goodno, 1982; Evans et al., 1986). It is clear from the results of the experiments reported here that ADPV does not affect HK in a manner similar to the ATPases of myosin and dynein. HK utilizes MgATP as phosphate donor. Therefore this result supports the hypothesis that the PV moiety is not

Figure 20

The effect of different $V_i$ concentrations on the rate of HK catalyzed phosphoryl transfer from ATP to glucose in the absence of ADP (□) and in the presence of 0.40 mM (◆) and 0.80 mM (■) ADP. Reaction mixtures contained 50 mM Tris-OAc, 0.10 mM ATP, 1.0 mM glucose, 2.0 mM $MgCl_2$, 0.17 μg/mL HK, 50 μM NADP, 3.0 μg/mL G6PDH and the indicated concentrations of $V_i$ and ADP. The reaction was initiated by adding 10 μL of the HK stock solution to a reaction mixture that contained all of the other reagents at 25°C, pH 7.4. The sample volume was 3.0 mL. The reaction was monitored by measuring the rate of NADP reduction according to the mechanism shown in Scheme VIIa.


0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
1/Rate (uM/min)-1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
[VI] (mM)
ADP - 0
ADP - 0.4 mM
ADP - 0.8 mM

a good chelator of $Mg^{2+}$, and thus does not bind to the enzyme. It does however raise questions about differences between HK and the myosin and dynein ATPases. These ATPases, which also utilize MgATP, have a high affinity for ADPV.

A notable difference between myosin ATPase and hexokinase is the value of the dissociation constant for ATP. At pH 8, 23°C the dissociation constant of ATP from myosin has been reported to be in the region of 0.10 pM (Mannherz et al., 1974). Danenburg and Cleland, 1975 report a dissociation constant for ATP from hexokinase of 4 - 6 μM at pH 7, 17°C. The difference between the affinities of the two enzymes for ATP is of the order of $10^7$, in favor of the binding of ATP to myosin. It is possible that the factors which are responsible for this difference in affinity can account for the fact that ADPV is a potent inhibitor of myosin ATPase while it has very little effect, if any, on hexokinase. The presence of a $Mg^{2+}$ ion undoubtedly contributes to the remarkable affinity of myosin for ATP. However, it is likely that other factors of structure and charge density are involved in maintaining the stability of the nucleotide/enzyme complex. For instance, myosin may have a high affinity for groups on the adenosine moiety and thus permit tight binding of the vanadate/nucleotide, irrespective of its ability to chelate the divalent cation. Alternatively there might be enough binding energy available to distort the ADPV structure so that it can bind $Mg^{2+}$ correctly. In general, dissociation constants for nucleotides that are the substrates of the enzymes studied here are in the micromolar to millimolar range. Therefore the forces involved in nucleotide binding to pyruvate kinase and myokinase

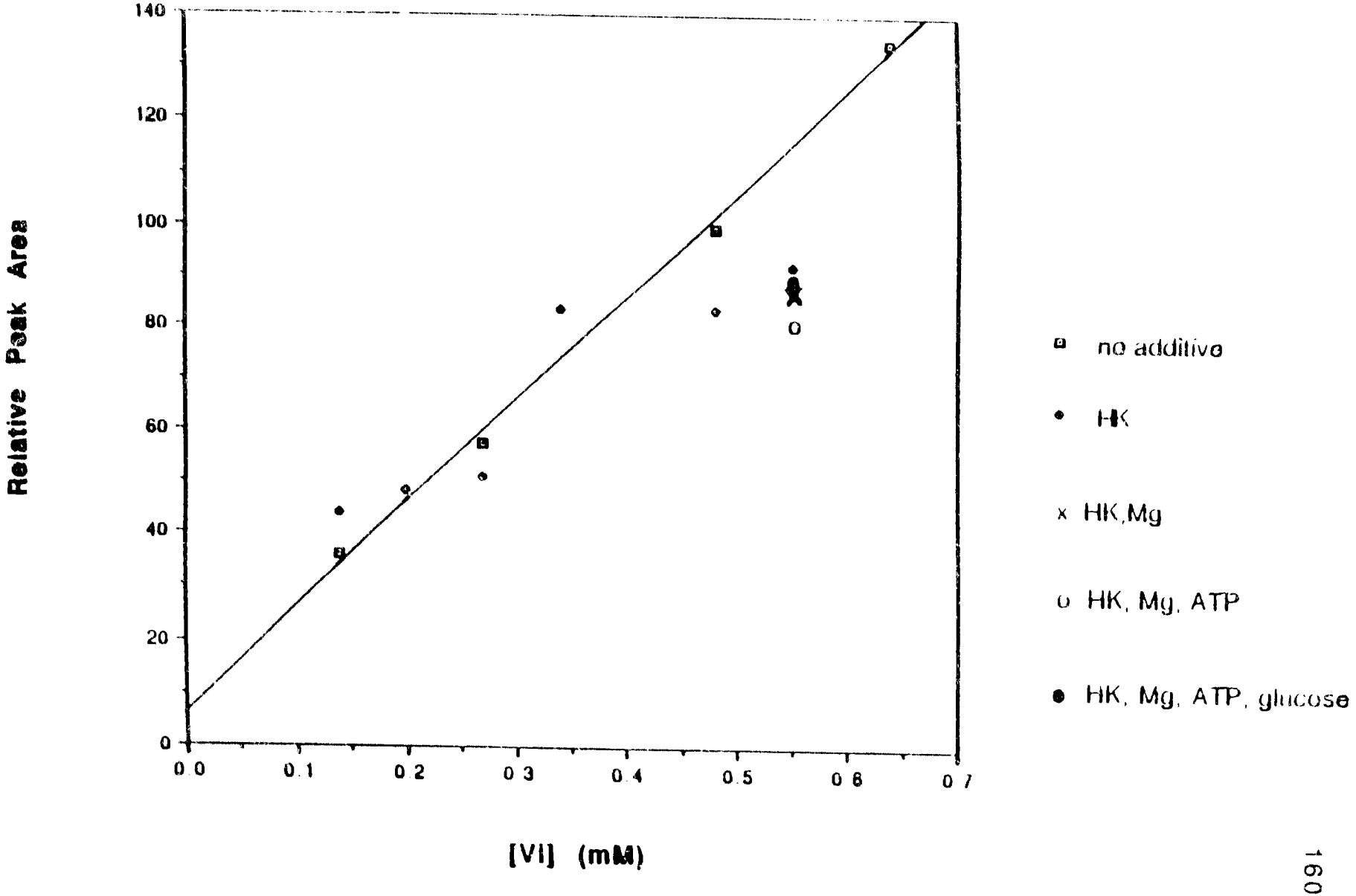
are of an order similar to that of hexokinase, rather than to that of myosin. Therefore, with regard to their interactions with substrate analogues, it is not unreasonable that they behaved more like hexokinase than like myosin ATPase.

## $^{51}V_i$ NMR Spectroscopy: $V_i$/HK Binding Study

The total peak areas for each of four $^{51}V$ NMR spectra representing $V_i$ concentrations of 0.14, 0.27, 0.48 and 0.68 mM are plotted in Figure 21. The symbols represent the values for total peak areas that were measured at the given concentrations of $V_i$ ion in the absence and the presence of 0.27 mM (27 mg/mL) HK. The line is drawn through the values for $V_i$ alone. The values for the activity of the HK preparation, measured before and after the $^{51}V$ NMR experiment, were 263 and 209 units/mg protein respectively. Thus 20% of the HK activity was lost during the experiment. This loss was probably due to a partial denaturation of the protein during the vortex mixing. In addition the enzyme was subjected to ambient temperature for the period of testing, about 8 hours. The enzyme solution grew slightly cloudy as the experiment progressed, an indication that some of the protein was precipitating. Since the results of this experiment are of a qualitative nature, the loss of 20% of the enzyme activity is not likely to significantly affect the analysis of the data. At low concentrations of $V_i$ (< 0.4 mM) there was no significant difference between the peak areas with and without HK. The low values of the points which appear at higher concentrations of $V_i$, 0.48 mM and 0.55 mM, may represent

Figure 21

The effect of different vanadate ($V_i$) concentrations on the total peak area of $^{51}V$ NMR spectra in the absence and presence of 27 mg/mL (0.27 mM) HK, and in the presence of HK, $MgCl_2$, ATP and glucose. Reaction mixtures contained 1.5 mL of 50 mM Tris-OAc, pH 7.4, the indicated concentrations of $V_i$ and the following additives: 0 (❑), 0.27 mM HK (◆), 0.27 mM HK and 2.0 mM $MgCl_2$, (X), 0.27 mM HK, 2.0 mM $MgCl_2$ and 1.5 mM ATP (○), 0.27 mM HK, 2.0 mM $MgCl_2$, 1.5 mM ATP and 1.4 mM glucose (●). Spectra were measured at ambient temperatures.


Relative Peak Area
140
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
[VI] (mM)
no additive
HK
HK,Mg
HK, Mg, ATP
HK, Mg, ATP, glucose

approximately 20% $V_i$ binding to the protein. In preliminary work this effect was observed when the experiment was performed at higher concentrations of $V_i$. The symbols in Figure 21, (X), (○) and (●) represent the relative peak areas that were measured after the addition of $MgCl_2$ (1.0 mM), ATP (1.5 mM) and glucose (1.4 mM), respectively, to the solution containing HK (0.27 mM) and $V_i$ (0.55 mM). All of the values which represent the addition of substrates to the $V_i$/HK mixture are significantly lower than those of the standard plot. This indicates that some of the $V_i$ was bound to the enzyme under these conditions. It is clear that the addition of substrate did not decrease the tendency of $V_i$ to bind to the enzyme. The lower total peak areas measured in the presence of substrate can be interpreted as a slight increase in the binding of $V_i$ to HK, but the effect is small, almost within the experimental error. Because the addition of substrates does not increase the quantity of free $V_i$, it was concluded that the small amount of $V_i$/HK binding that was observed at the highest $V_i$ concentrations was of a nonspecific nature. There was no evidence from the results of this experiment that $V_i$ binds preferentially to the active sites of HK.

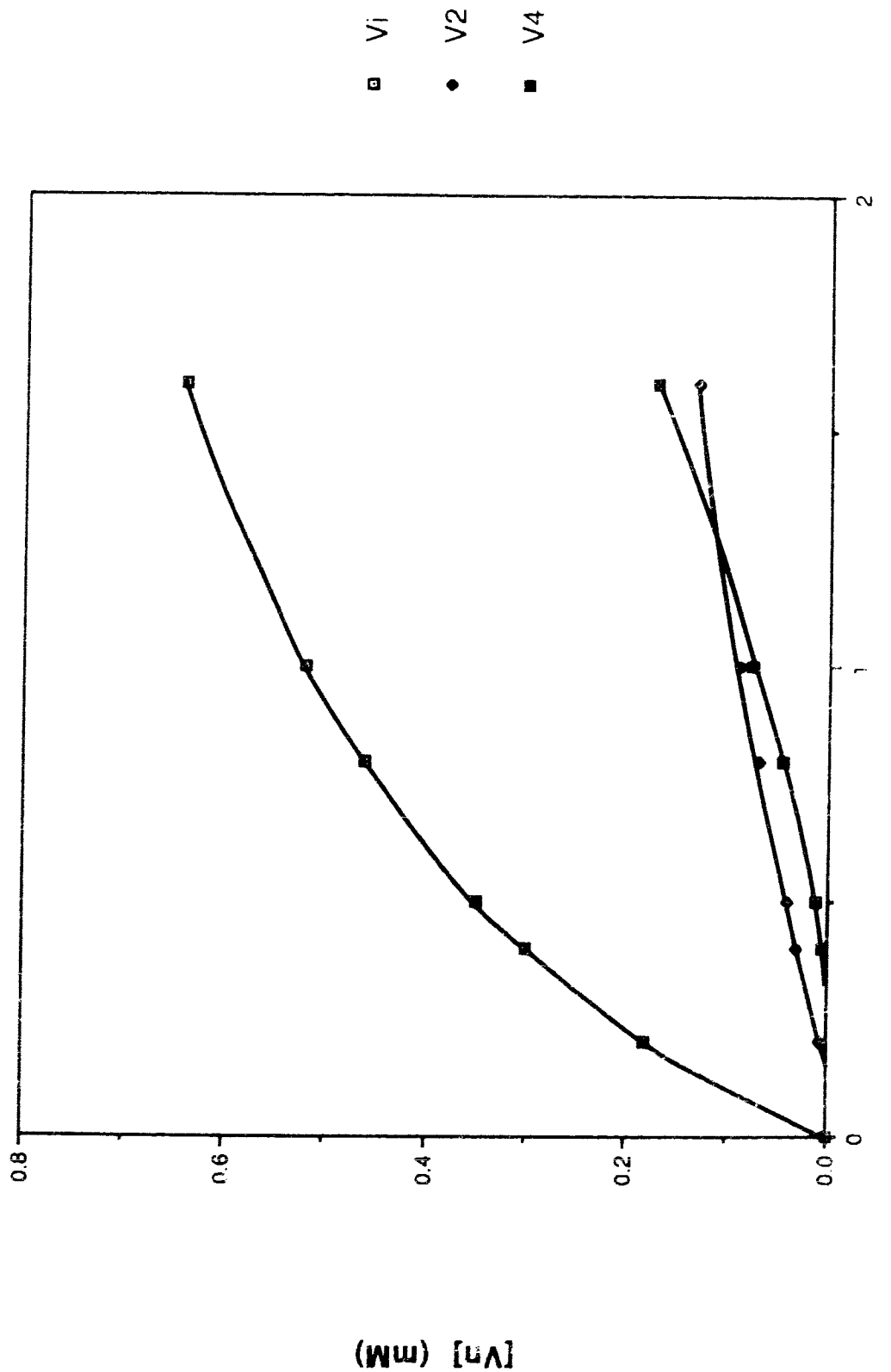
Inhibition by $V_i$ of HK catalyzed phosphoryl transfer from ATP to glucose was demonstrated in the kinetic studies, where 0.6 mM $V_i$ inhibited the rate of reaction by approximately 30%. The simplest explanation for this inhibition is that $V_i$, an analogue of $P_i$, competes with ATP for the active site of the enzyme. However, the failure of the $^{51}V$ NMR studies to show that $V_i$ is released from the enzyme in the presence of $Mg^{2+}$, ATP and glucose suggests that $V_i$ anions are not

bound to sites which normally accommodate substrate molecules. The results of the $^{51}V$ NMR experiment do not support the hypothesis that $V_i$ acts competitively with the $P_i$ moiety on ATP. However, the conditions of the NMR experiment were significantly different from those of the kinetic studies. The HK concentration in the kinetic experiments was 2.0 nM (0.20 µg/mL), while the enzyme concentration in the NMR experiment was 0.27 mM (27 mg/mL). High concentrations of protein can lead to the oligomerization of protein molecules, and a consequent reduction in the activity of the enzyme. The results of the kinetic studies, acquired at low enzyme concentrations, are considered to be more reliable as an indication of the nature of $V_i$ inhibition.

The absence of $V_i$/HK binding at low $V_i$ concentrations and the appearance of approximately 20% binding at 0.6 mM $V_i$ suggests that $V_i$ oligomers, may be involved in the inhibition of HK. The distribution of monomeric ($V_i$), dimeric ($V_2$) and tetrameric ($V_4$) vanadate species in an aqueous solution containing 50 mM Tris-OAc, 2.0 mM $MgCl_2$ and the indicated vanadium atom concentrations (V), is shown in Figure 22. The procedure for calculating the concentrations of the different vanadate species from $^{51}V$ NMR spectra is described in Appendix 1. In the presence of 50 mM Tris-HCl, 2.0 mM $MgCl_2$, 1.0 mM $V_i$, at pH 7.4, 25°C, the equilibrium constants for the formation of $V_2$ and $V_4$ were 319 $M^{-1}$ and 1.0 x $10^9$ $M^{-3}$ respectively. Figure 22 shows that in the range of concentrations employed in the $^{51}V$ NMR experiments, 0.14 - 0.55 mM $V_T$ (added as inorganic vanadate, $V_i$), the predominant species

Figure 22

The effect of different total vanadium atom (V) concentrations on the concentrations of monomeric ($V_i$) (□), dimeric ($V_2$) (◆) and tetrameric ($V_4$) (■) vanadate in aqueous solutions at pH 7.4. The lines were calculated using equilibrium constants for the formation of $V_i$, $V_2$ and $V_4$ that were estimated by measuring the peak ratios of $^{51}V$ NMR spectra, as described in Appendix I. The $^{51}V$ NMR spectrum used to estimate the formation constants was obtained in a solution containing 50 mM Tris-OAc, pH 7.4, 2.0 mM $MgCl_2$ and 1.0 mM vanadium added as inorganic vanadate.


Vi
V2
V4
0.8
0.6
0.4
0.2
0.0
0
1
2
[V] (mM)
[Vn] (mM)

is the monovanadate ion. The concentrations of $V_2$ and $V_4$, negligible at 0.1 mM $V_i$, become significant at 0.5 mM $V_i$. The absence of $V_i$ binding at low concentrations of $V_i$ and the appearance of approximately 20% binding at 0.55 mM $V_i$, may indicate that there is very little binding of the monomer, and that binding of the dimer and/or tetramer is observed when their concentrations become significant. This behavior may also be indicated by the curvature of the plots in Figure 20. Since the small amount of bound vanadate is not released on the addition of the natural substrates, the binding either does not take place at the active sites, or it is irreversible. In either case it could be responsible for the inhibition that was observed in the kinetic studies.

The inhibition of G6PDH from Leuconostoc mesenteroides by dimeric and tetrameric species of $V_i$ has been investigated by D.C. Crans and S.M. Scheible (personal communication). By analyzing their results in terms of the concentrations of $V_i$, $V_2$ and $V_4$ present in their samples, they obtained $K_i$ values of 0.16 mM (0.32 mM total vanadium atom concentration) and 0.53 mM (2.1 mM total vanadium atom concentration) for the dimer and tetramer respectively, both competitive with the NADP cofactor for the G6PDH-catalyzed reaction. No inhibition was observed at low monomer concentrations and it was concluded that inhibition by the monomer ($V_i$) in this system was within the experimental uncertainty of the measurements. It is interesting that G6PDH derived from yeast, tested in the experiments reported in this thesis, showed no sign of inhibition at 2.0 mM. $V_i$ (total vanadium atom concentration).

In kinetic studies which were an extension of those reported here, an attempt to correlate the inhibition of HK-catalyzed phosphoryl transfer with the concentration of dimer or tetramer present in $V_i$ solutions did not give consistent results. In a study of HK-catalyzed phosphoryl transfer from ATP to glucose, with glucose at a saturating concentration and ATP as the varied substrate, the inhibition of HK activity was measured at 0.40, 1.0 and 1.6 mM $V_i$. The data were fitted reasonably well to a model in which monomer, dimer and tetramer species of $V_i$ were considered to be distinct inhibitors. However, the same model did not fit the data obtained at saturating concentrations of ATP, with glucose as the varied substrate. These results have not been presented because of their inconclusive nature. However, they did suggest that the oligomers of $V_i$ are involved in the inhibition and it is likely that the inconsistencies could be resolved if a sufficient quantity of data were accumulated.

The question of which $V_i$ species is involved in the inhibition of HK can be postponed to future work, as the question more relevant to this thesis has already been answered. That is that the inhibition of HK in the presence of $V_i$ and ADP is not enhanced in excess of the added effect of the individual inhibitors.

## Part III

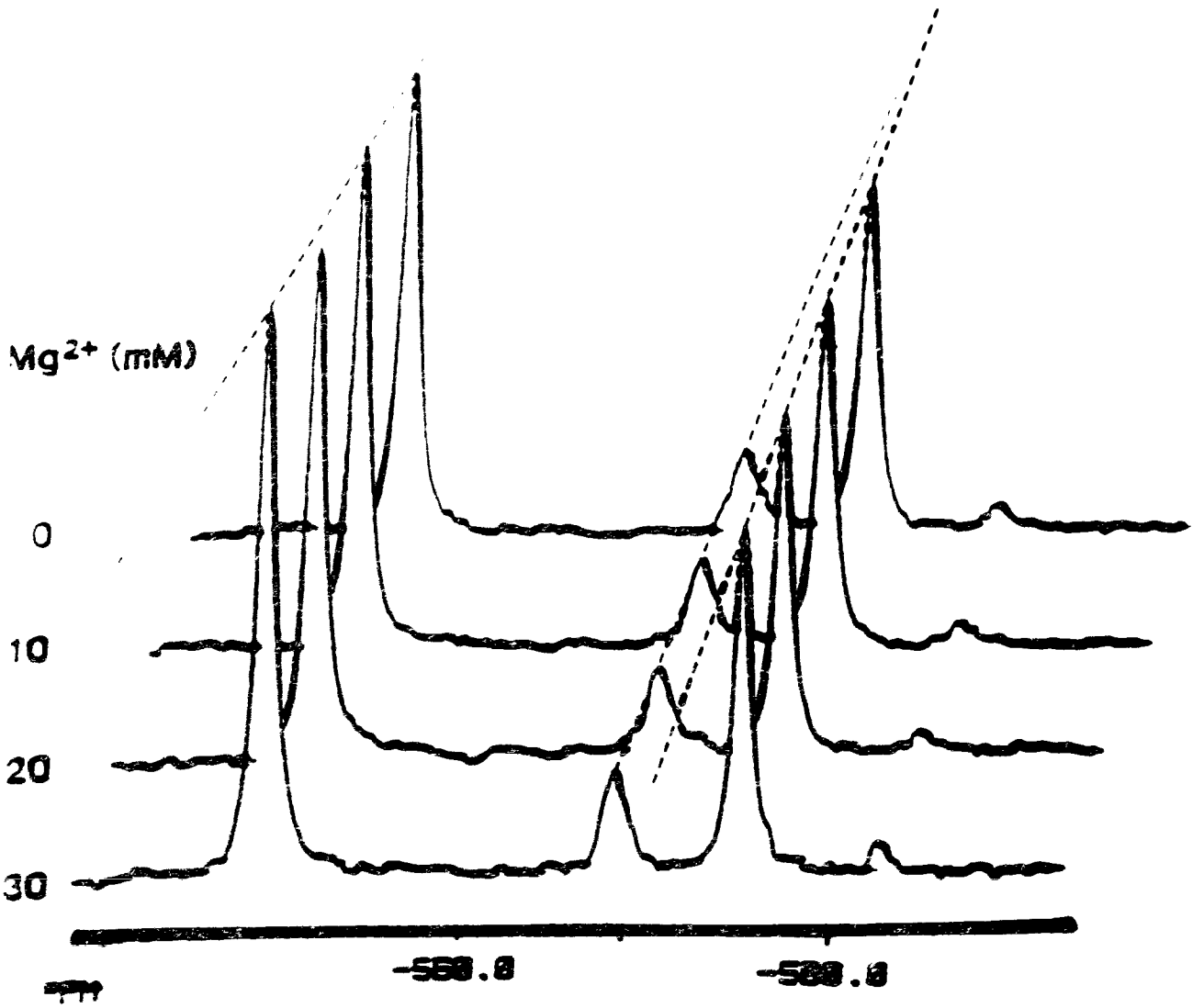
### Interactions Between Vi, Pi and $Mg^{2+}$; a $^{51}V$ NMR Study

#### $V_i$ and $Mg^{2+}$

It has been postulated that $Mg^{2+}$ is not significantly chelated by the vanadate dimer $V_2$, or by the phosphovanadate anhydride PV. In order to detect possible stabilization of the dimer in the presence of $Mg^{2+}$, NMR spectra were recorded for aqueous solutions of $V_i$ with and without $MgCl_2$. Reaction mixtures at pH 8.0 contained 20 mM Tris-Cl, 1.0 mM $V_i$ and 0, 10, 20 or 30 mM $MgCl_2$ in a total volume of 50 mL. The ionic strength was 1.0 M, adjusted with KCl as follows: 3.728 g KCl (0 $MgCl_2$), 3.616 g KCl (10 mM $MgCl_2$), 3.504 g KCl (20 mM $MgCl_2$), 3.392 g KCl (30 mM $MgCl_2$). Spectra were recorded at ambient temperature. $^{51}V$ NMR spectra of $V_i$ in the absence of $MgCl_2$ and in the presence of 10, 20 and 30 mM $MgCl_2$ are shown in Figure 23. The signals were assigned as follows: -551 ppm, -569 ppm, -579 and -582 ppm represent the monomer ($V_i$), dimer ($V_2$), tetramer ($V_4$) and pentamer ($V_5$) respectively. The relative concentration of each vanadate species was quantitated by integrating the area under each peak and it was determined that peak height was a relatively accurate reflection of the integrated areas for the $^{51}V$ spectra. The peak heights of the tetramer ($V_4$), connected by a dashed line, appear to decrease relative to that of the monomer ($V_i$), as the concentration of $Mg^{2+}$ is

Figure 23

The effect of different $Mg^{2+}$ concentrations on the $^{51}V$ NMR spectrum of vanadate ion at pH 8.0. Reaction mixtures contained 20 mM Tris-Cl, 1.0 mM $V_i$ and $MgCl_2$ as indicated. KCl was added to a total ionic strength of 1.0 M. Spectra were recorded at ambient temperature.


Mg2+ (mM)
0
10
20
30
-568.8
-568.8

increased. Thus it appears that as $Mg^{2+}$ concentration increases, the concentration of tetramer decreases relative to that of the monomer. The peak height of the dimer ($V_2$) appears to diminish also as the $Mg^{2+}$ concentration increases, but the broadness of the $V_2$ peaks makes them a less reliable measure of relative concentration. If the dimer is stabilized by $Mg^{2+}$ a decrease in concentration of all other species of vanadate would be expected, as vanadium atoms from the monomer, tetramer and pentamer would be used to form the dimer. The apparent decrease in $V_2$ and $V_4$ relative to $V_i$ indicates that as $Mg^{2+}$ concentration is increased, monomer concentration increases at the expense of dimer and tetramer concentration. The observed stabilization of the vanadate monomer in the presence of $Mg^{2+}$ is supported by the work of other investigators. An association constant of 76 $M^{-1}$ has been reported for $MgHPO_4$ in aqueous solutions of $Mg^{2+}$ and $HPO_4^{2-}$ at 25°C (de Meis, 1984).

## $V_i$, $P_i$ and $Mg^{2+}$

Interactions between vanadate and phosphate were studied by Gresser et al., 1986, using $^{51}V$ NMR. In the presence of phosphate the signal for the monovanadate ion is broadened and shifted to higher magnetic field. In the experiments reported here $^{51}V$ NMR spectra were recorded for mixtures of vanadate and phosphate in the absence of and in the presence of $MgCl_2$. Reaction mixtures at pH 7.98 contained 20 mM Tris-Cl, 1.0 mM $V_i$, 52 mM $P_i$, 3.170 g KCl (to adjust the ionic strength to 1.0 M) and either 0 or 2.0 mM $MgCl_2$ in a total volume of 50

mL. In the presence of 52 mM $P_i$, it was necessary to limit the concentration of $MgCl_2$ to 2 mM as the precipitation of $Mg_3(PO_4)_2$ was observed at higher concentrations of $Mg^{2+}$.

In other experiments performed under similar conditions of vanadate concentration and ionic strength, 0.10 M $Na_2SO_4$ or 0.10 M $Na_2CrO_4$ was substituted for $P_i$ in the absence of $Mg^{2+}$. $Na_2SO_4$ and $Na_2CrO_4$ were tested at pH 7.0 and pH 8.0 respectively. In addition the divalent cations $Ca^{2+}$ and $Sr^{2+}$ were substituted for $Mg^{2+}$ in reaction mixtures containing $V_i$ and $P_i$.

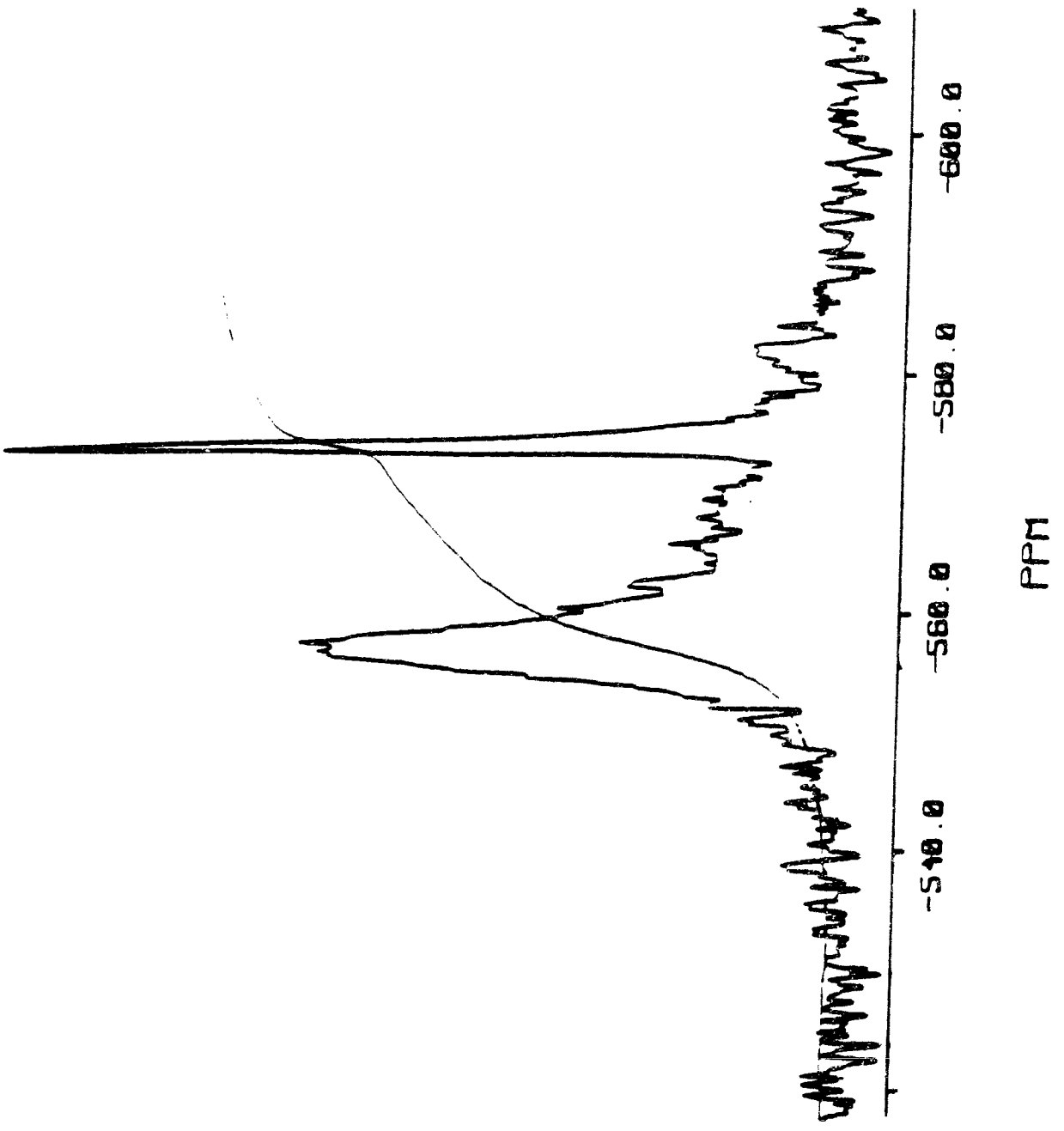
The broad signal at 557.9 ppm in Figure 24 represents the vanadate/phosphate interaction at 52 mM $P_i$. In the presence of 2.0 mM $Mg^{2+}$ the PV signal was observed at 558.6 ppm (spectra not shown). The difference in chemical shift was not considered to be significant as the broadness of the signal introduced some uncertainty in the measurement.

There was no observable broadening or change in chemical shift of vanadate signals in the presence of $CrO_4^{2-}$ or $SO_4^{2-}$ ions. Similarly, no changes in $^{51}V$ spectra were detected in the presence of the divalent cations $Ca^{2+}$ and $Sr^{2+}$.

Since it has been reported that the presence of $Mg^{2+}$ has a stabilizing effect on $PP_i$ (Alberty, 1969), it was of interest to estimate the size of the chemical shift that would be expected if the stabilization of the PV molecule by $Mg^{2+}$ was equivalent to its stabilization of $PP_i$.

Figure 24

$^{51}V$ NMR spectrum of vanadate ion in the presence of phosphate at pH 7.98, ambient temperature. The reaction mixture contained 20 mM Tris-Cl, 1.0 mM $V_i$ and 52 mM $P_i$. The ionic strength was 1.0 M, adjusted with KCl.


-540.0
-560.0
-580.0
-600.0
PPM

From the work of Alberty, 1969, the change in free energy observed ($\Delta G°_{obs}$) for the hydrolysis of $PP_i$ was -9.6 Kcal/mol in the absence of $Mg^{2+}$, and -6.9 kcal/mol in the presence of 2.0 mM $Mg^{2+}$. Since all other conditions were the same, it is likely that the decrease in free energy of hydrolysis of 2.7 Kcal/mol in the presence of $Mg^{2+}$ was due to the stabilization of the $PP_i$ species by the $Mg^{2+}$ ion. An equilibrium constant ($K_{PV}$) of 5.8 ± 0.2 for the formation of the PV anhydride at pH 7.98 has been reported by Gresser et al., 1986, This value corresponds to a change in free energy of -1.0 Kcal/mol for the formation of phosphovanadate. If the the PV anhydride is stabilized in the presence of 2 mM $Mg^{2+}$ to the same extent as $PP_i$, $\Delta G°_{obs}$ for the formation of PV in the presence of 2 mM $Mg^{2+}$ would be equal to [-1.0 + (-2.7)] = -3.7 Kcal/mol. This leads to a value of $K_{PV}$ = 551 $M^{-1}$.

The equation below shows how $K_{PV}$ is related to $P_i$ concentration and to the chemical shifts for $V_i$ in the absence of $P_i$ ($\delta_V$), $V_i$ in the presence of 52 mM $P_i$ ($\delta$) and $V_i$ in the presence of a saturating concentration of $P_i$ ($\delta_{PV}$) (Gresser et al., 1986).

$$\delta = \frac{[P_i]\, K_{PV}\delta_{PV} + \delta_V}{1 + K_{PV}[P_i]}$$

Values of -550.6 ppm and -582.0 ppm for $\delta_V$ and $\delta_{PV}$ respectively have been estimated by Gresser et al., 1986. These values were obtained at pH 7.98, 20 mM Tris-Cl, 1.0 mM $V_i$, $\mu$ = 1.0 M, in the absence of $Mg^{2+}$ ion. Under these conditions, using $K_{PV}$ = 5.8 $M^{-1}$, the chemical shift for $V_i$ in the presence of 52 mM $P_i$ was estimated from the above equation to be equal to -558 ppm, a value in accordance with

the observed chemical shift. Thus for $K_{PV}$ = 551 $M^{-1}$, at 52 mM $P_i$, the chemical shift of PV in the presence of 2 mM $Mg^{2+}$ would be expected to equal to -581 ppm. It is noted that the values of $\delta_V$ and $\delta_{PV}$ were obtained in the absence of $Mg^{2+}$. It has been shown (Figure 23) that the presence of $Mg^{2+}$ has a negligible effect on the chemical shift of vanadate in the absence of phosphate. Protonation of the PV molecule has the effect of shifting the resonance to a higher field (Gresser et al., 1986). Coordination with $Mg^{2+}$ would be expected to have a similar effect. Since a significant chemical shift change of the PV signal was not detected in the presence of $Mg^{2+}$, it is probable that $Mg^{2+}$ ions are not chelated by the PV molecule.

The work of Alberty was performed at 0.2 M ionic strength, a value considerably lower than the 1.0 M ionic strength present in the experiments reported here. It could be argued that the formation of complexes of PV with $Mg^{2+}$ would be less favourable at high ionic strength due to the partial neutralization of the oxygen anions by counter ions other than $Mg^{2+}$. In order to test the effect of a change in ionic strength, spectra of $V_i$ and of $V_i$ in the presence of $P_i$, with and without $Mg^{2+}$ were obtained at 0.2 M ionic strength. Reaction mixtures at pH 8.0 contained 20 mM Tris-Cl, 1.0 mM $V_i$, 52 mM $P_i$, 0.20 g KCl (to adjust the ionic strength to 0.20 M), and either 0 or 2.0 mM $MgCl_2$ in a total volume of 50 mL.

The observed chemical shifts for $V_i$ and for $V_i$ in the presence of 52 mM $P_i$ were 548 ppm and 551 ppm respectively. The difference of 3 ppm was smaller than the difference of 7 ppm observed at an ionic strength of 1.0 M. This is a reasonable result as the proximal anions of

the PV molecule would be stabilized at higher ionic strength. In the presence of 52 mM $P_i$ and 2.0 mM $Mg^{2+}$ the chemical shift at 0.20 M ionic strength was 551 ppm, the same value as that obtained in the absence of $Mg^{2+}$. Thus the apparent inability of the PV molecule to chelate $Mg^{2+}$ ion at 1.0 M ionic strength was supported by the results obtained at 0.20 M ionic strength.

## General Discussion

The results presented in this thesis indicate that the $V_i$ nucleotide anhydrides that were tested did not interact significantly, either as substrates or as inhibitors, with the phosphoryl transferring enzymes PK, MK and HK. All of the mixed PV nucleotides tested were inactive as substrates for these enzymes. Where inhibition was observed it could be accounted for by $V_i$ alone, or by the additive effect of $V_i$ and the nucleotide. There were no instances where the inhibition by $V_i$ was enhanced in the presence of a nucleotide. The only species for which enzymic effects of vanadate complexes could be demonstrated in solutions containing $V_i$ and adenosine or adenine nucleotides was the putative ester AdV. AdV is an analogue of AMP, a nucleotide which binds to MK at a site that does not require that the nucleotide be coordinated with a divalent cation. Thus the hypothesis that $V_i$ nucleotides are unable to chelate $Mg^{2+}$ ions, and therefore cannot bind to kinases is supported by these results.

This hypothesis provides a reasonable explanation for the failure of $V_i$ to affect the activity of kinases, but the evidence for the mechanism is indirect. $^{51}V$ NMR experiments in aqueous solution showed that millimolar quantities of the divalent cations $Mg^{2+}$, $Ca^{2+}$ and $Sr^{2+}$ did not stabilize the PV anhydride, and that the $V_i$ dimer, divanadate ($V_2$), was slightly destabilized in the presence of $Mg^{2+}$. Alberty, 1969 reported that the stabilization of $PP_i$ in the presence of $MgCl_2$ significantly reduced the change in free energy of $PP_i$ hydrolysis. Kinetic studies presented here show that the effects of $V_i$ on PK, MK

and HK are not enhanced in the presence of nucleotides, and that a change of divalent cation does not affect these results. The evidence from the NMR experiments suggests that in aqueous solution divalent cations are not chelated by PV. The results of the kinetic studies are consistent with the evidence from NMR experiments. By studying the activity of the kinases in the presence of phosphate and mixed phosphovanadate nucleotides, it was shown that when the chelation of a divalent cation is necessary for activity, nucleotides bearing PP moieties are active while those bearing PV moieties are not active.

The reasons for the differences between the PV and $PP_i$ anhydrides with respect to their coordination of divalent cations are not clear. The geometric and electronic structures of PV and $PP_i$ are very similar, while their chemical natures are very different. In the solid state, the bond lengths between the oxygens and the central atoms are shorter in $MgP_2O_7$ than in $MgV_2O_7$. The mean P-O distances are 159 pm (bridging) and 152 pm (terminal) (Calvo, 1967), while the mean V-O distances are 180 pm (bridging) and 169 pm (terminal) (Holloway and Melnick, 1986). POP and VOV bond angles at temperatures below 70°C are similar, 144° (Calvo, 1967) and 141° (Holloway and Melnick, 1986) respectively. Presumably an intermediate value would be observed for the bond angle of POV. It would appear necessary that the nonbridging oxygens be in reasonable proximity in order to chelate the metal ion. Although the slightly smaller VOV angle tends to offset the effect that the longer V-O bond lengths would have on the distance between the nonbridging oxygens, their distance apart is greater in $V_2O_7^{2-}$ than in $P_2O_7^{2-}$. Likewise there is likely to be a

greater average distance between the nonbridging oxygen atoms of PV compared with those of $PP_i$ in the solid state, and also in aqueous solutions of these ions. Possibly $Mg^{2+}$ is not effectively coordinated by PV for this reason. The larger cations $Ca^{2+}$ and $Sr^{2+}$ might be expected to fit into coordination sites that are too large for $Mg^{2+}$, but $Ca^{2+}$ and $Sr^{2+}$ did not stabilize the PV anhydride in $^{51}V$ NMR experiments, and did not lend activity to any of the $V_i$ nucleotide substrate analogues. However, the attraction of $Ca^{2+}$ and $Sr^{2+}$ for oxygen is smaller than that of $Mg^{2+}$, due to the softer nature of the larger cations. Therefore they would be expected to be chelated less effectively than $Mg^{2+}$. It may be that structural differences are not adequate to explain the differences between PV and $PP_i$ cation coordination.

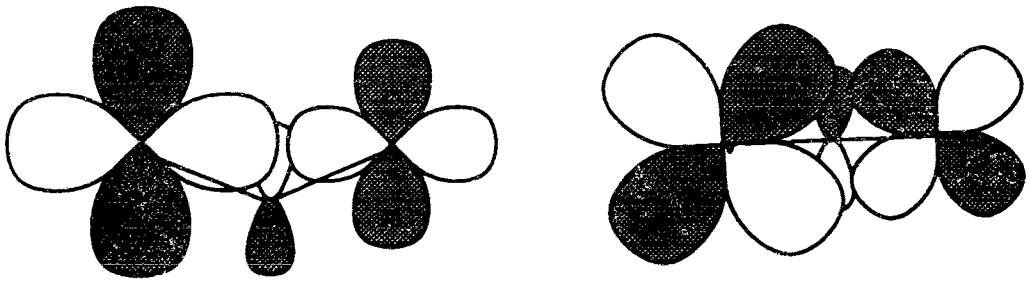
The chemical differences between PV and $PP_i$ are substantial, probably mostly due to the fact that $V_i$ has a surplus of low energy empty orbitals and $P_i$ does not. The equilibrium constants for the formation of $V_2$, PV and $PP_i$ are 370 $M^{-1}$ (pH 7.8, Tracey and Gresser, 1986), 15 $M^{-1}$ (pH 7.4, Gresser et al., 1986) and $1.3 \times 10^{-6}$ $M^{-1}$ (pH 7.0, Jencks, 1968) respectively. This means that the thermodynamic stabilities of $V_i$ anhydrides are greater than the stability of $PP_i$ by 6 - 7 orders of magnitude. The large difference in the thermodynamic stability between $PP_i$ and the $V_i$ anhydrides $V_2$ and PV can be rationalized, in part, in terms of the sizes and electronic environments of the central atoms. $V^{5+}$ (59 pm) is somewhat larger than $P^{5+}$ (34 pm), a property that is reflected by longer V-O vs P-O bond lengths. The longer V-O bond length allows for a reduction in negative charge density, and therefore a greater thermodynamic

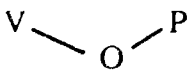
stability for anhydrides that contain $V_i$. However, bond length is not likely to be the only factor responsible for the large difference in stability between $PP_i$ and the $V_i$ anhydrides. The availability of low energy d orbitals on the vanadium atom may account for some of the stability of $V_i$ anhydrides over that of $PP_i$. The force constants and bond lengths between oxygen ligands and acceptor atoms of the third row of the periodic table (Si, P, S, Cl) indicate the presence of double bond character of various strengths in molecules such as $SiO_4^{4-}$, $PO_4^{3-}$, $SO_4^{2-}$ and $ClO_4^-$ (Cotton and Wilkinson, 1972). The higher bond strengths are thought to be a consequence of the donation of electrons from the filled 2p orbitals of the oxygen atoms to the empty 3d orbitals of the central atoms. A similar case of $d\pi$-$p\pi$ bonding has been observed for $(SiH_3)_2O$, a nonlinear molecule with a Si-O-Si bond angle of 144°C. In this molecule the overlap between oxygen p orbitals and silicon d orbitals has a shortening effect on both of the Si-O bonds. It is reasonable to suppose that $d\pi$-$p\pi$ bonding would be much more effective in an oxyanion containing vanadium as a central atom, as the 3d orbitals of vanadium, as well as the 4s and 4p orbitals, lie much closer in energy to the valence electrons than do the 3d orbitals of the third row elements. Thus the PV anhydride, and more so the $V_2$ anhydride, would be stabilized in comparison with $PP_i$.

The bond angles between the central atoms and bridging oxygens in the ions $V_2O_7^{2-}$ and $P_2O_7^{2-}$ depends largely on the geometry of the bonding orbitals of the central atoms. The necessity to accommodate steric requirements at the lowest energy possible will determine which orbitals on the central atoms are available to accept electrons

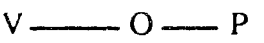
from ligand atoms. Thus the bond angle between the central atom of a $V_2$ dimer and the bridging oxygen atom partially depends on how much $d\pi$-$p\pi$ overlap can be achieved in the final structure. For instance, for phosphate in a tetrahedral field, $d_z2$ and $d_x2 - _y2$ orbitals are lower in energy than $d_{xy}$, $d_{yz}$ and $d_{xz}$ orbitals. Electrons in filled p orbitals on oxygen atoms will be preferentially donated to the empty $d_z2$ and $d_x2 - _y2$ orbitals of phosphorus under these conditions. Cruickshank, 1961, examined bond lengths between phosphorus and oxygen, obtained by x-ray crystallography. He proposed that $d_z2$ and $d_x2 - _y2$ orbitals of phosphate are best suited for $p\pi$ overlap with oxygen electrons. The relationships between d orbital geometry, bond angle and $d\pi$-$p\pi$ overlap are shown in Figure 25. Overlap of oxygen p orbitals with phosphorus $d_z2$ and $d_x2 - _y2$ orbitals (Figure 25a) is consistent with the bent POP bond of 144° that has been detected in crystals of $MgP_2O_7$ (Calvo, 1967). Since a VOV bond angle of 141° has been measured in $MgV_2O_7$, it is likely that there is a similar arrangement of bonding electrons in the $V_2$ dimer. However, a $d\pi$-$p\pi$ overlap between oxygen p electrons and the $d_{xy}$, $d_{yz}$ and $d_{xz}$ orbitals of vanadium (Figure 25b) would involve an interaction between two lobes of the bridging oxygen p orbitals with two lobes of the vanadium d orbitals. Such an increase in $d\pi$-$p\pi$ overlap would be certain to increase the stability of the dimer, but would require that the bond angle between the central and bridging atoms be linear.

Figure 25






(a)



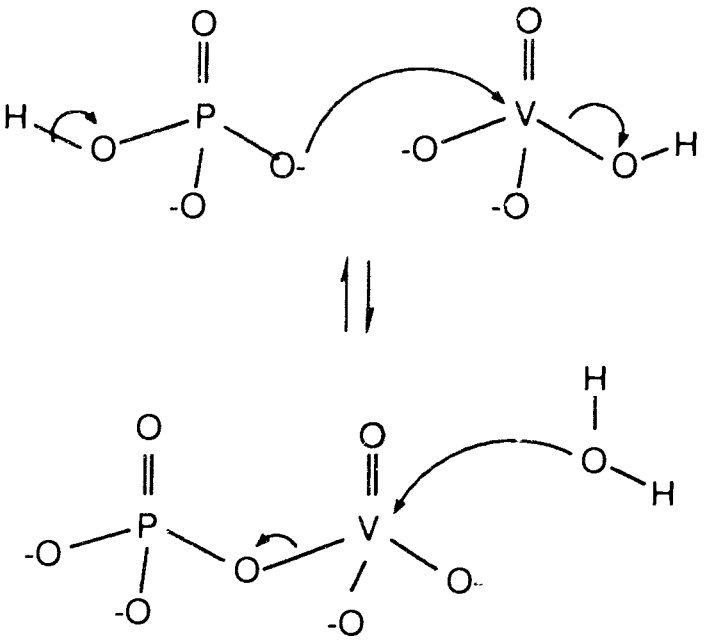

(b)

Overlap of p orbitals of the central oxygen atom with d orbitals of vanadium and phosphorus.

The empty 3d orbitals of vanadium are more accessible to the electrons on bonded oxygen atoms than those of phosphorus. The low energy of the $d_{xy}$, $d_{yz}$ and $d_{xz}$ orbitals of vanadium, coupled with the increased stability of $d\pi$-$p\pi$ overlap involving these orbitals, apparently does not significantly influence the bond geometry of the $MgV_2O_7$ crystal, however it may provide the conditions necessary for a linear bond in aqueous solution. When the divalent cation is solvated by water molecules its ability to interact with the oxygen electrons is diminished. Furthermore the necessity for the presence of a divalent

cation proximal to the anion will be reduced in solution; this will be particularly true for $V_2O_7^{2-}$, as the VO bond length is greater than the PO bond length. It is possible that under these conditions the forces in favour of stabilizing the anhydride through $p\pi$-$d\pi$ overlap become dominant in the $V_2O_7^{2-}$ ion. That is the most stable structure for $V_2O_7^{2-}$ in aqueous solution may involve a linear VOV bond, a structure that does not favour the chelation of a cation. The same forces would be present in the PV anhydride, although they would not be as intense as those present in $V_2$, where both of the central atoms have empty orbitals of low energy. Thus the presence of the low energy d orbitals may account for the thermodynamic stability of the $V_i$ anhydrides and the failure of these anhydrides to chelate $Mg^{2+}$ ion.

The kinetics of formation and hydrolysis of $V_i$ anhydrides are also strikingly different from those of $PP_i$. $V_2$ and PV are rapidly formed and hydrolyzed in aqueous solution, while the formation and hydrolysis of $PP_i$ is very slow at room temperature. The mechanisms for formation and hydrolysis of the anhydrides, $PP_i$, $V_2$ and PV is probably similar to that for phosphate esters (Butcher and Westheimer, 1955). Formation of the anhydride bond likely involves a nucleophilic attack of an oxygen of one monomeric anion on the electropositive center of another, with the subsequent release of a water molecule. In the reverse reaction the nucleophilic attack of a water molecule on a central atom of the binuclear anhydride anion would release a monomeric anion. The mechanism is shown below.



The reversible formation of the phosphovanadate anhydride

The availability of low energy orbitals facilitates the donation of electrons from the nucleophile in both directions of the reaction. When vanadium is the central atom of the monomer, or one of the central atoms of the anhydride, its low energy 3d orbitals readily accept electrons from the nucleophile. There is no low energy pathway for this mechanism when phosphorus is the electron acceptor, as there are no empty orbitals that are at a low enough energy to accept the donated electrons. In biological systems, where the kinetic stability of phosphate anhydrides is vital, formation and hydrolysis of the pyrophosphate bond is controlled enzymically. The thermodynamic

instability of $PP_i$ is used biologically to pull energetically unfavourable reactions to completion.

Examples of synergistic relations between anions and enzyme-bound nucleotides have been reported. Certain small anions, including $V_i$, have been observed to affect the activity of phosphoryl transferring enzymes in conjunction with nucleotides. In some cases the relationship between these anions and the obligatory metal cations of the binding sites have been investigated. This work will be discussed briefly as it is related to the question of how divalent cations are coordinated by enzyme-bound species.

It was mentioned earlier in this thesis that in the instances of the ATPases myosin and dynein, evidence has been obtained that is inconsistent with the hypothesis proposed in this thesis. $V_i$ binds very tightly to these enzymes in the presence of ADP (Goodno, 1982; Evans et al., 1986). The tight binding of the ADPV complex has been rationalized in terms of the stability of the $V_i$ ion in the trigonal bipyramidal configuration which mimics the transition state of the phosphoryl transfer reaction. In the case of the myosin and dynein ATPases, interaction between the metal cation and $V_i$ has not yet been investigated. It appears that in the case of myosin ATPase the presence of $Mg^{2+}$ is required for formation of the enzyme/ADP/$V_i$ complex (C.R. Cremo, personal communication). Furthermore, evidence has been obtained that $Mg^{2+}$ coordinates to the β-phosphate, but not to the α-phosphate group of ATP during ATP hydrolysis catalysed by myosin ATPase (Connolly and Eckstein, 1981) and dynein ATPase (Shimizu and Furusawa, 1986). However, as discussed previously, the

fact that the affinity of myosin ATPase for ATP is approximately 7 orders of magnitude higher than the affinity of hexokinase for ATP, makes it possible that as yet unknown factors play an important role in ATP binding to myosin.

Reports of other anions which inhibit phosphoryl transferring enzymes in conjunction with a bound nucleoside diphosphate can be found in the recent literature (Milner-White and Watts,1971; Reed et al., 1978; Combeau and Carlier, 1988; Dupuis et al., 1989; Chabre, 1990). Small anions such as $BeF_3(H_2O)^-$, $AlF_4(H_2O)_2^-$, $SCN^-$, $N_3^-$, $NO_3^-$, $HCO_3^-$, $HCO_2^-$ and some halide ions reversibly inhibit enzyme catalyzed phosphoryl transfer reactions by attaching to the nucleoside triphosphate sites of ADP- or GDP-bound enzymes. In general it is believed that tetrahedral anions that cannot acquire a pentavalent conformation, such as $BeF_3(H_2O)^-$ inhibit phosphoryl transfer competitively with the tetrahedral form of the transferring phosphate group (Combeau and Carlier, 1988; Dupuis et al., 1989). It is suggested that planar anions such as $NO_3^-$ inhibit because they fit into the equatorial plane of the pentacoordinate transition state of the migrating phosphate group, the nucleotide diphosphate and the phosphate acceptor completing the 5-coordinate structure (Reed et al., 1978). Anions such as $AlF_4(H_2O)^-$ can bind to a nucleoside triphosphate site in conjunction with a nucleoside diphosphate by exchanging an $H_2O$ ligand for the phosphate group of the nucleotide diphosphate. Thus it is postulated that the $AlF_4^-$/nucleotide complex inhibits via the pentacoordinate transition state of the phosphoryl transferring enzyme (Dupuis et al., 1989). The structures of tetrahedral and pentacoordinate

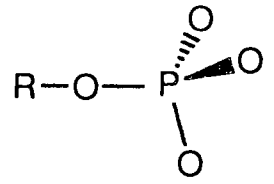
phosphate and some analogues of these phosphate conformations are shown in Figure 26.

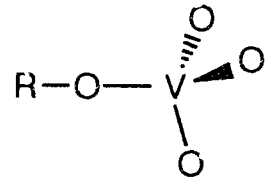
Several planar anion inhibitors of creatine kinase, $NO_3^-$, $N_3^-$ and $SCN^-$ have been studied with respect to the interaction between these anions and metal ions such as $Mg^{2+}$, $Mn^{2+}$ and $Co^{2+}$. Creatine kinase normally catalyzes the transfer of a phosphate group from ATP to creatine. The formation of an abortive quaternary complex consisting of enzyme/MeADP/anion/creatine, where Me refers to the divalent cation, has been postulated to explain the inhibition of this reaction by the planar anion inhibitors. Infrared absorption frequencies for metal anion complexes were compared in solutions of metal/anion salts and in solutions which included enzyme, creatine and ADP (Reed et al., 1978). It was found that the affinities of the anions for the divalent cations were much stronger in the presence of the enzyme complex. In addition, the similarity between the infrared absorption frequencies of enzyme-bound anions in the presence of metal cations and those of the free metal/anion complexes suggests that the mode of metal/anion attachment is the same for both cases; that is that the anions are directly coordinated to the divalent metal cation at the active site of the enzyme complex.

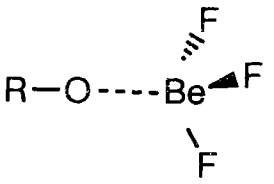
The phosphate analogues discussed above differ in their potency as inhibitors of phosphoryl transferring enzymes. The dissociation constants for beryllium and aluminum fluorides to F-ADP-actin were reported to be 2 μM and 25 μM respectively (Combeau and Carlier, 1988). The planar anions $SCN^-$, $N_3^-$ and $NO_3^-$ have dissociation constants to the Mn/ADP creatine kinase complex of 7.0, 0.6 and 0.1 mM

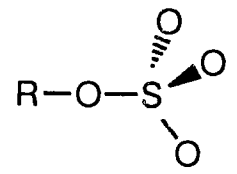
Figure 26

Anions which inhibit phosphoryl transfer reactions because of structural similarities to different configurations of phosphate: (a) tetrahedral phosphate and anions which inhibit by assuming tetrahedral configurations, (b) pentacoordinate phosphate (an unstable transition state) and anions which inhibit by forming pentacoordinate structures.

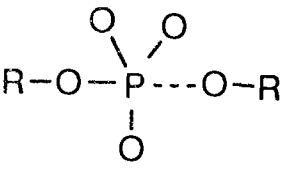


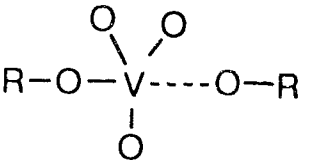


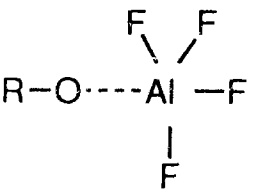


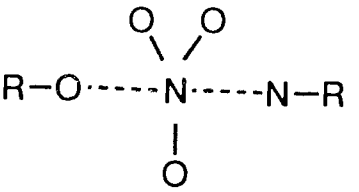


(a)









(b)

respectively (Reed et al., 1978). These values can be compared with a dissociation constant of 0.1 μM that has been calculated for $V_i$ from the enzyme/ADP/$V_i$ complex in the myosin ATPase system (Goodno,1982).

The evidence that the phosphoryl group analogues $SCN^-$, $N_3^-$ and $NO_3^-$ are directly liganded to the metal cation in the enzyme/MeADP/anion/creatine complex of creatine kinase suggests that the γ phosphate group of ATP is coordinated to the cation in the normal phosphoryl transfer reaction. Such mechanistic information is useful, as the role of the divalent cation in activating these reactions is not completely understood. Although the planar anion inhibitors are directly chelated to the metal ion, and their binding mode is thought to mimic the transition state of the phosphoryl transfer reaction, the dissociation constants of $SCN^-$, $N_3^-$ and $NO_3^-$ are much higher than that expected for a true transition state analogue, which is generally in the micromolar range or lower. $NO_3^-$ has the lowest dissociation constant, and it is the only anion of the three in which the coordination to the metal cation is through oxygen, $SCN^-$ and $N_3^-$ coordination being through N atoms (Reed et al., 1978). Because of its lower electronegativity, nitrogen would be less strongly attracted to $Mg^{2+}$ than oxygen, and this may account for the higher dissociation constants of the N-coordinated anions. In addition, Gutterman and Gray, 1971, have shown that the coordination of the transition metal cation $Co^{2+}$ to $NCS^-$ is linear, while the coordination of metal to $N_3^-$ is angular as shown below.

Linear and angular configurations adopted by metal cation complexes with nitrogen ligands

The lower dissociation constant of $N_3^-$ may be accounted for if the configuration of the $Mg/N_3$ species provides a better fit in the active site of the enzyme complex.

Coordination of the metal ion to the aluminum fluoride inhibitors has not been demonstrated. It would be expected that fluorine would be a better ligand of $Mg^{2+}$ than oxygen, and this might partially account for the fact that these inhibitors are active at micromolar concentrations. However, the beryllium fluoride inhibitors are also active at micromolar concentrations, but in this case the tetraheral structure of these ions does not allow them to mimic the five coordinate transition state. Thus the tight binding of the aluminum and beryllium fluorides may be mainly due to their effective, fluorine-mediated, coordination to the metal cation. Experiments without inhibitor are commonly performed in the presence of millimolar concentrations of $Cl^-$ ion, in glass vessels which would normally contain trace quantities of aluminum. Apparently the complexes of aluminum ion and chloride ion do not inhibit with the same intensity as

those of aluminum and fluoride. Although size may be an important factor in anion binding, $Cl^-$ being a larger ion than $F^-$, coordination of the metal cation would be less effective for $Cl^-$ than for $F^-$ due to the softer nature of the $Cl^-$ ion.

The interplay of many factors determines the binding constants of anions to the active site of an enzyme. Most of them have not been considered here because they involve the active groups on the enzyme, groups that in many cases have not yet been identified. The anion inhibitors have been useful in helping to clarify the interactions that take place, but as yet only a small fraction of the data that can be obtained in this way have been collected. The ultimate goal of this research is to understand how protein molecules can achieve their remarkable catalytic effects.

## Future Work

Information about the interactions between proteins and their substrates and cofactors has been obtained through many different approaches, only a few of which have been discussed in this thesis. In addition to the uv/visible, NMR and infrared techniques described here, neutron diffraction (Lindquist et al., 1975) and ESR (Reed and Cohn, 1972; Lodato and Reed, 1987), have been used successfully to study the aqueous solutions of protein/substrate complexes. Examination of the solid state of enzyme/substrate complexes by x-ray crystallography provides a direct method for visualizing atomic interactions between reactive species (Bennett and Steitz, 1978).

Most of these techniques are more difficult to apply than the uv/visible spectroscopy that was employed in the experiments reported here. The interpretations of NMR, ESR, IR and x-ray crystallographic data, particularly where proteins are involved, usually requires that the investigator have a specialized background. Clearly it would be useful to be able to visualize the enzyme/MgADP/$V_i$ complex of the myosin and dynein ATPases, as they appear to behave differently from other phosphoryl transferring enzymes. X-ray diffraction is the most direct method of observation, but the difficulties associated with the crystallization of proteins often limit the information that can be obtained by this technique. It would be interesting also to study the metal/anion interactions in the many inhibitory complexes that appear to bind both metals and anions. The high quantity of protein required for NMR and IR studies can be a problem when the purified protein is difficult to obtain. This is likely to be true for the myosin and dynein ATPases, as they are not available commercially. This is probably the reason that the nucleotide-binding proteins adenylate cyclase, transducin, tubulin, actin and $F_1$ATPase, all of which are inhibited by aluminum and beryllium fluorides (Chabre, 1990; Combeau and Carlier, 1988; Dupuis et al., 1989), have been studied by spectroscopic methods. It is noteworthy that the effects of $V_i$ have been tested on several of these enzymes: $V_i$ mimics phosphate in the stabilization of F-actin filaments, and has about the same affinity as phosphate for F-ADP-actin (Combeau and Carlier, 1988); $V_i$ inhibits the binding of tubulin to dynein (Shimizu and Furusawa, 1986); $V_i$ is a weak inhibitor of $F_1$ATPase ($K_i$ = 1.0 mM) (Kao, 1990). Some of these effects of $V_i$

have been rationalized in terms of competitive inhibition with respect to tetrahedral phosphate groups, some have been rationalized in terms of a tight enzyme/ADP/$V_i$ transition state complex, and for some there is no rationale, as yet.

Although the supply of data that can be obtained from spectroscopic studies is far from exhausted, application of the more direct methods of structural determination would contribute information that is indispensible to our comprehension of the mechanisms by which these enzymes exert their effects.

# Appendix i

## Calculation of Vanadate Monomer Concentrations

$^{51}V$ NMR spectra of dilute vanadate solutions (≤1 mM) display peaks for several different vanadate species: monomer ($V_i$), dimer ($V_2$) and tetramer ($V_4$). The chemical shift of the various species can be assigned relative to a vanadate pentamer ($V_5$) which appears at higher concentrations of vanadate (> 3 mM). The method by which these assignments were made is described in Gresser et al., 1986. The signals that represent the different vanadate species are well resolved and the integrated peak area ratios can be used to calculate the concentrations of $V_i$, $V_2$ and $V_4$ with respect to the known concentration of total vanadium ion as shown below, where $V_n$ represents the peak area of $V_i$ or $V_2$ or $V_4$ and $[V_T]$ is the total vanadium atom concentration. Square brackets denote molar concentration.

$$\frac{(V_n)}{(V_i + V_2 + V_4)} \times [V_T] = [V_n] \qquad (1)$$

The relationships between the formation constants of the dimer and tetramer to $[V_i]$ are shown in equations 2 and 3.

$$K_d = \frac{[V_2]}{[V_i]^2} \qquad (2)$$

$$K_t = \frac{[V_4]}{[V_i]^4} \qquad (3)$$

$K_d$ and $K_t$ were estimated by the following procedure. $[V_i]$, $[V_2]$ and $[V_4]$ were obtained by applying equation 1 to the $^{51}V$ NMR spectra obtained under the following conditions: 50 mM Tris-HCl, 2.0 mM $MgCl_2$, 1.0 mM $V_i$, pH 7.4, 25°C. Equation 4, shown below, relates the concentrations of the various vanadate species to the total vanadium ion concentration.

$$[V_T] = [V_i] + 2[V_2] + 4[V_4] \qquad (4)$$

Rearrangement of equations 2 and 3 gives expressions for $[V_2]$ and $[V_4]$ which can be substituted into equation 4 to give equation 5.

$$[V_T] = [V_i] + 2(K_d)[V_i]^2 + 4(K_t)[V_i]^4 \qquad (5)$$

$[V_T]$ is a known quantity, therefore $[V_i]$ can be estimated from equation 5 and the calculated values of $K_d$ and $K_t$. The concentrations of $V_2$ and $V_4$ for other values of $[V_T]$ and $[V_i]$, obtained under similar conditions can be calculated from equations 2 and 3.

## Appendix II

### Enzymic Pyrophosphate Assay

The mechanism for the enzymic pyrophosphate ($PP_i$) assay is shown below. In the presence of $PP_i$, $PP_i$-dependent phosphofructokinase ($PP_i$-PFK) catalyzes the phosphorylation of fructose-6-phosphate (F-6-P). The product, fructose-1,6-diphosphate is cleaved to produce glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), in a reaction catalyzed by aldolase. The GAP produced in the aldolase-catalyzed reaction is isomerized to DHAP in the presence of triosephosphate isomerase (TPI). The 2 DHAP molecules that are produced from one $PP_i$ ion are oxidized in the presence of NADH in a reaction catalyzed by glycerol-3-phosphate dehydrogenase (GDH). 2 molecules of NAD are produced for 1 molecule of $PP_i$ present in the starting material.

$$PP_i + \text{F-6-P} \xrightarrow{PP_i\text{-PFK}} \text{F-1,6-DP} + P_i$$

$$\text{F-1,6-DP} \xrightarrow{\text{aldolase}} \text{GAP} + \text{DHAP}$$

$$\text{GAP} \xrightarrow{\text{TPI}} \text{DHAP}$$

$$2\ \text{DHAP} + 2\ \beta\text{-NADH} + 2\ H^+ \xrightarrow{\text{GDH}}$$

$$2\ \text{glycerol-3-phosphate} + 2\ \beta\text{-NAD}$$

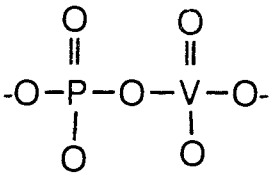
Abbreviations:

$PP_i$ = pyrophosphate
F-6-P = D-fructose-6-phosphate
$PP_i$-PFK = fructose-6-phosphate kinase, pyrophosphate-dependent
F-1,6-DP = D-fructose1,6-diphosphate
$P_i$ = inorganic phosphate
GAP = D-glyceraldehyde-3-phosphate
TPI = triosephosphate isomerase
DHAP = dihydroxyacetone phosphate
GDH = glycerophosphate dehydrogenase
β-NAD(H) = β-nicotinamide adenine dinucleotide (reduced form)

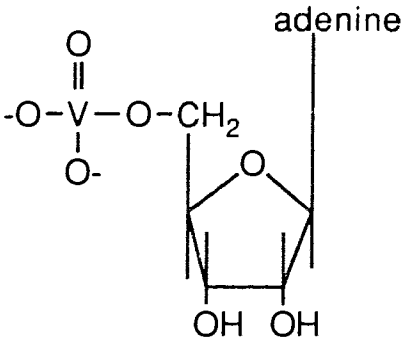
Final Concentrations of Reagents in Reaction Mixture

| Reagent | Concentration |
|---|---|
| imidazole/HCl, pH 7.4 | 45 mM |
| citrate | 0.5 mM |
| EDTA | 0.10 mM |
| $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$ | 2 mM, 0.2 mM, 0.02 mM |
| β-NADH | 0.8 mM |
| F-6-P | 12 mM |
| bovine serum albumin | 5 mg/mL |
| sugar stabilizer | 5 mg/mL |
| $PP_i$-PFK | 0.5 units/mL |
| aldolase | 7.5 units/mL |
| GDH | 5 units/mL |
| TPI | 50 units/mL |

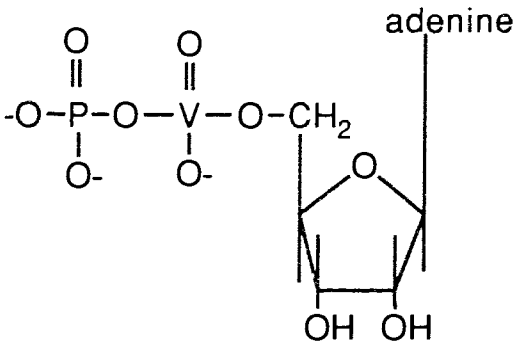
Appendix III



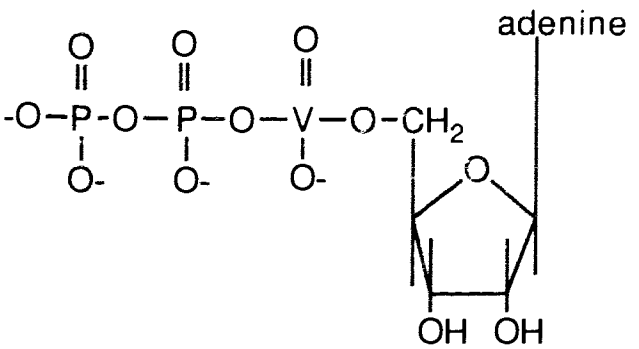
PV



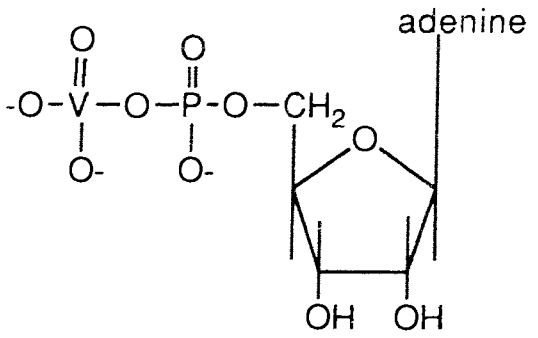

AdV



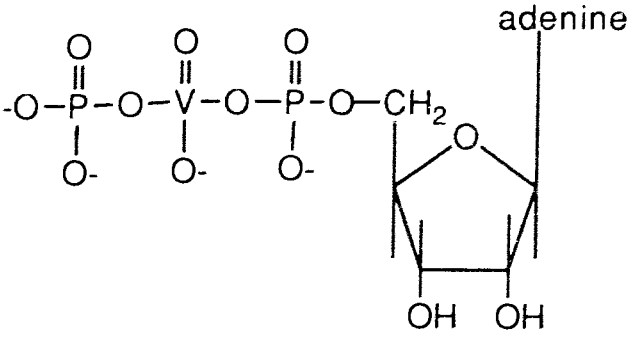

AdVP



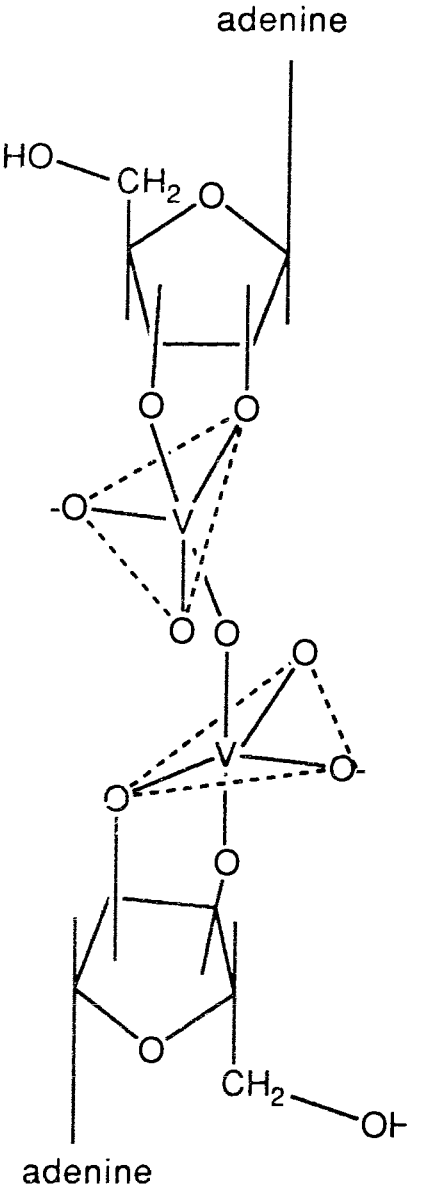

AdVPP




AMPV



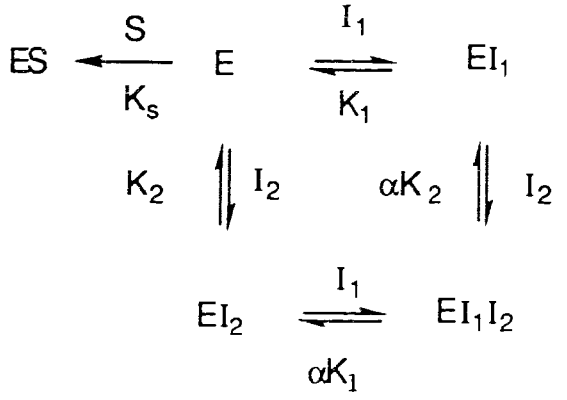

AMPVP




$Ad_2V_2$

## Appendix IV

### Kinetics of Inhibition with 2 Inhibitors (Yonetani & Theorell, 1964)




E - enzyme
S - suostrate
P - product
I - inhibitor
K - dissociation constant
$\alpha$ - interaction constant

$$K_1 = \frac{[E][I_1]}{[EI_1]}; \quad K_2 = \frac{[E][I_2]}{[EI_2]}; \quad \alpha K_1 = \frac{[EI_2][I_1]}{[EI_1I_2]}; \quad \alpha K_2 = \frac{[EI_1][I_2]}{[EI_1I_2]}; \quad K_S = \frac{[E][S]}{[ES]}$$

The enzyme concentration [E] can be expressed in terms of the total enzyme concentration $[E_T]$.

$$[E_T] = [E] + [ES] + [EI_1] + [EI_2] + [EI_1I_2]$$

$$= [E] + \frac{[E][S]}{K_S} + \frac{[E][I_1]}{K_1} + \frac{[E][I_2]}{K_2} + \frac{[E][I_1][I_2]}{\alpha K_1 K_2}$$

$$= [E]\left\{1 + \frac{[S]}{K_S} + \frac{[I_1]}{K_1} + \frac{[I_2]}{K_2} + \frac{[I_1][I_2]}{\alpha K_1 K_2}\right\}$$

$$[E] = \frac{[E_T]}{1 + \frac{[S]}{K_S} + \frac{[I_1]}{K_1} + \frac{[I_2]}{K_2} + \frac{[I_1][I_2]}{\alpha K_1 K_2}} \qquad (1)$$

The velocity of the reaction is proportional to the concentration of the enzyme/substrate complex [ES], which is expressed in terms of the dissociation constant $K_S$ in equation (2).

$$v = k_{cat}[ES]$$

$$v = \frac{k_{cat}[E][S]}{K_S} \qquad (2)$$

Substitution of the expression for [E] from equation (1) into equation (2) gives equation (3). The reciprocal of equation (3) is equation (4), where $K_M = K_S$ and $k_{cat}[E_T]$ = the maximum velocity of reaction $V_m$.

$$v = \frac{k_{cat}[E_T][S]}{K_S\left\{1 + \frac{[I_1]}{K_1} + \frac{[I_2]}{K_2} + \frac{[I_1][I_2]}{\alpha K_1 K_2} + \frac{[S]}{K_S}\right\}} \qquad (3)$$

$$\frac{1}{v} = \frac{K_M}{V_m[S]}\left\{1 + \frac{[I_1]}{K_1} + \frac{[I_2]}{K_2} + \frac{[I_1][I_2]}{\alpha K_1 K_2}\right\} + \frac{1}{Vm} \qquad (4)$$

Rearrangement of equation (4) gives equation (5).

$$\frac{1}{v} = \frac{K_M}{V_m[S]}\left(1 + \frac{[I_2]}{K_2}\right) + \frac{K_M[I_1]}{V_m K_1[S]}\left(1 + \frac{[I_2]}{\alpha K_2}\right) + \frac{1}{V_m} \qquad (5)$$

# Appendix V

## Equilibrium Constants for Reagents in Coupled Assays

$$\frac{[PP_i]}{[P_i]^2} = 1.7 \times 10^{-6}\ M^{-1}$$ (pH 7, Rawn, 1989)

$$\frac{[\text{pyruvate}][P_i]}{[PEP]} = 2.9 \times 10^{6}\ M$$ (pH 7, Rawn, 1989)

$$\frac{[AMP][P_i]}{[ADP]} = 2.0 \times 10^{6}\ M$$ (pH 7, Rawn, 1989)

$$\frac{[PV]}{[V_i][P_i]} = 5.8\ M^{-1}$$ (pH 8, Gresser et al.,1986)

$$\frac{[ADP][P_i]}{[ATP]} = 9.8 \times 10^{5}\ M$$ (pH 7, Rawn, 1989)

$$\frac{[\text{glucose-6-phosphate}]}{[\text{glucose}][P_i]} = 2.6 \times 10^{2}\ M^{-1}$$ (pH 7, Rawn, 1989)

## Equilibrium Constants for Enzyme-Coupled Assays

### Pyruvate Kinase (PK) (EC 2.7.1.40)

$$\frac{[ATP][pyruvate]}{[ADP][PEP]} = 6.45 \times 10^3$$ (pH 7.4, 30°C, Tris, McQuate and Utter, 1959)

$$\frac{[PP_i][pyruvate]}{[P_i][PEP]} = (1.7 \times 10^{-6}\ M^{-1})(2.9 \times 10^6\ M) = 4.9$$

$$\frac{[pyruvate][PV]}{[PEP][V_i]} = (2.9 \times 10^6\ M)(5.8\ M^{-1}) = 1.7 \times 10^7$$

### Myokinase (MK) (EC 2.7.4.3)

$$\frac{[ADP]^2}{[ATP][AMP]} = 2.26$$ (pH 7.4, 25°C, 10mM $MgCl_2$, Noda, 1973)

$$\frac{[AMP][PP_i]}{[ADP][P_i]} = (1.7 \times 10^{-6}\ M^{-1})(2.0 \times 10^6\ M) = 3.4$$

$$\frac{[ADP][VP]}{[ATP][V_i]} = (9.8 \times 10^5\ M)(5.8\ M) = 5.7 \times 10^6$$

Hexokinase (HK) (EC 2.7.1.1)

$$\frac{[ADP][D\text{-glucose-6-phosphate}]}{[ATP][D\text{-glucose}]} = 3.86 \times 10^2, \text{ (no } Mg^{2+}\text{)}; \ 1.55 \times 10^2$$

($Mg^{2+}$ = 17 - 79 mM); (pH 6.0, 30°C, Robbins and Boyer, 1957)

$$\frac{[\text{glucose-6-phosphate}][P_i]}{[\text{glucose}][PP_i]} = (2.6 \times 10^2 \ M^{-1})(2.3 \times 10^5 \ M) = 6.0 \times 10^7$$

Lactic Dehydrogenase (LDH) (EC 1.1.1.27)

$$\frac{(\text{L-lactate})(NAD^+)}{(\text{pyruvate})(NADH)} = 3.62 \times 10^5$$ (pH 7.0, 25°C, $P_i$, Hakala et al., 1956)

Glucose-6-Phosphate Dehydrogenase (G6PDH) (EC 1.1.1.49)

$$\frac{(\text{D-glucono-}\delta\text{-lactone})(NADPH)}{(\text{glucose-6-phosphate})(NADP)} = 2.03 \times 10^6$$ (pH 6.4, 28°C, Tris, Noltmann and Kuby, 1963)

## Appendix VI

### Substrate Analogues of Pyruvate Kinase, Myokinase and Hexokinase that were Tested for their Ability to Activate these Enzymes

| Enzyme | Substrates | Substrate Analogues* |
|---|---|---|
| | | |
| hexokinase | ATP | AdVPP (Ad + $V_i$ + $PP_i$)<br>AMPVP (AMP + $V_i$ + $P_i$) |
| | glucose | |
| | | |
| pyruvate kinase | ADP | AdVP (Ad + $V_i$ + $P_i$)<br>AMPV (AMP + $V_i$) |
| | phosphoenolpyruvate | |
| | | |
| myokinase | AMP | AdV (Ad + $V_i$) |
| | ATP | AdVPP (Ad + $V_i$ + $PP_i$) |
| | ADP | AdVP (Ad + $V_i$ + $P_i$) |

* All substrate analogues were inactive except for AdV, which activated myokinase.

# Appendix VII

## Inhibitors of Pyruvate Kinase, Myokinase and Hexokinase

Inhibition of Pyruvate Kinase  ADP + PEP → ATP + pyruvate

([ADP] = 0.10 mM; [PEP] = 0.50 mM)

| Reagent | Effect |
|---|---|
| | |
| | |
| $V_i$ (2.0 mM) | no inhibition |
| | |
| $P_i$ (30 mM) | enhancement of activity* |
| | |
| $PP_i$ (10 mM) | 100% inhibition |
| | |
| AMP (10 mM) | enhancement of activity |
| | |
| AMP + $V_i$ | same effect as AMP alone |

* KCl at the same ionic strength had a similar effect.

Inhibition of Myokinase $\quad AMP + ATP \rightarrow 2ADP$

([ATP] = 2.5 mM; [AMP] = 2.5 mM)

| Reagent | Effect on MK Activity |
|---|---|
| | |
| | |
| $V_i$ (2.0 mM) | inhibition = 54% |
| | |
| Ad (15 mM) | inhibition = 0 |
| | |
| PPi (10 mM) | inhibition = 100% |
| | |
| $V_i$ (2.0 mM) + Ad (15 mM) | inhibition = 18%* |

* Ad removed some of the inhibition by $V_i$ through the formation of the binuclear diester $Ad_2V_2$.

Inhibition of Myokinase $2ADP \rightarrow AMP + ATP$

([ADP] = 0.25 mM)

| Reagent | Effect on MK Activity |
|---|---|
| | |
| | |
| AMP (0.40 mM) | inhibition = 68% |
| | |
| $V_i$ (0.60 mM) | inhibition = 7.0% |
| | |
| AMP (0.40 mM) + $V_i$ (0.60 mM) | inhibition = 70% |

| Conditions | % Inhibition | No. of Experiments |
|---|---|---|
| $P_i$ (30 mM) | 29 ± 8 | 3 |
| $V_i$ (0.60 mM) | 7 ± 4 | 4 |
| Ad (15 mM) | 0 | 1 |
| Ad + $V_i$ | 5 | 1 |
| $P_i$ + $V_i$ | 41 ± 1 | 2 |
| Ad + $P_i$ | 37 | 1 |
| Ad + $P_i$ + $V_i$ | 44 | 1 |
| $PP_i$ (0.27 mM) | 5 | 1 |
| Ad + $PP_i$ | 5 | 1 |

Inhibition of Hexokinase ATP + glucose → ADP + glucose-6-P

([ATP] = 0.20 mM; [glucose] = 1.0 mM)

| Reagent | Effect on HK Activity |
|---|---|
| | |
| | |
| ADP (0.80 mM) | inhibition = 34% |
| | |
| $V_i$ (0.60 mM) | inhibition = 40% |
| | |
| ADP (0.80 mM) + $V_i$ (0.60 mM) | inhibition = 56% |
| | |
| $V_i$ (1.0 mM) | inhibition = 36% - 42% |
| | |
| $P_i$ (30 mM) | inhibition = 50% |
| | |
| $V_i$ (1.0 mM) + $P_i$ (30 mM) | inhibition = 60% |
| | |
| $PP_i$ (10 mM) | inhibition = 89%* |
| | |
| $V_i$ (1.0 mM) + $PP_i$ (10 mM) | inhibition = 93%* |
| | |

* $P_i$ and $PP_i$ inhibit glucose-6-phosphate dehydrogenase 16% and 38% respectively under these conditions.

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