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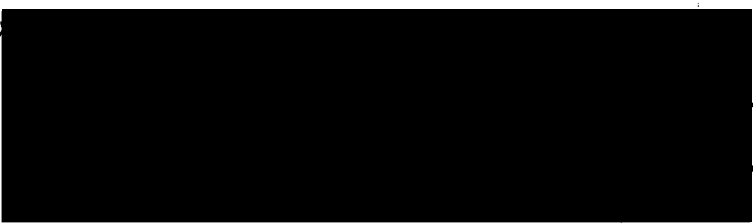
INTERACTION OF VANADIUM WITH

GLUCOSE 6-PHOSPHATE DEHYDROGENASE

by

Amani F.H. Noureldeen

B.Sc. (Biochem.), Ain Shams University, 1980

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

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ABSTRACT

Inorganic vanadate (V_i) activates catalysis by glucose 6-phosphate dehydrogenase (G6-PDH) of the oxidation of glucose by $NADP^+$. As the concentration of the enzyme is increased, the rate of the vanadate-activated glucose oxidation becomes less sensitive to increase in enzyme concentration. The rate of glucose oxidation in the absence of vanadate increases linearly with enzyme concentration. This result is interpreted in terms of nonenzymic formation of glucose 6-vanadate, an analog of the enzyme's physiological substrate, glucose 6-phosphate. At high enzyme concentration, the vanadate ester formation reaction becomes partially rate-limiting, and extrapolation to infinite enzyme concentration allows determination of the second order rate constant for formation of the ester from glucose and vanadate.

Sulphate (SO_4^{2-}) also activates catalysis by G6-PDH of the oxidation of glucose by $NADP^+$. The sulphate activation is interpreted in terms of activation of the enzyme as a glucose oxidase by binding of SO_4^{2-} to the phosphate-binding domain in the catalytic site, i.e., binding of sulphate at the region of the catalytic site occupied by the phosphate moiety of G6-P when the physiological substrate is bound at the catalytic

site. Supporting this interpretation are the observations that: a) SO_4^{2-} inhibits catalysis by G6-PDH of oxidation of both G6-P and the putative glucose 6-vanadate, while V_i does not, at the concentrations used in this study, inhibit G6-P oxidation, and b) the sulphate activation is not saturable by enzyme, i.e., the rate of the sulphate-activated glucose oxidation increases linearly with enzyme concentration.

By extrapolating to infinite G6-PDH concentration, the second order rate constant for formation of glucose 6-vanadate from glucose and vanadate at pH 7.0 was determined to be $1.93 \, M^{-1}s^{-1}$. This can be compared with the corresponding values for formation of G6-P ($9.0 \times 10^{-11} M^{-1}s^{-1}$) and G6-As $(6.3 \times 10^{-6} M^{-1}s^{-1})$. In the pH range from 7.0 to 8.4 the second order rate constant for G6-V formation increased from $1.93 \, M^{-1}s^{-1}$ to $11.2 \, M^{-1}s^{-1}$.

It is concluded that the nonenzymic formation of glucose 6-vanadate is formed in a facile nonenzymic reaction from glucose and vanadate, and that it is a substrate for glucose 6-phosphate dehydrogenase. If this is an instance of a general phenomenon, i.e., rapid formation of vanadate esters which are substrates for enzymes which catalyze reactions of the corresponding phosphate esters, then it may be a factor in some of the physiological effects of vanadium.

To Ramadan and Mohamed Ehab

ACKNOWLEDGEMENTS

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I.1 Introduction

Vanadium belongs to the Group VB transition metals that also includes niobium and tantalum. The electronic configuration for vanadium in its zero oxidation state is [Ar] $3d^3 4s^2$. The Group VB transition metals are characterized by having their d-orbitals partially filled (3 electrons in the d-orbitals in the ground state). Potential oxidation states for vanadium range from 0 to +5; however, vanadium in the +4 and +5 formal oxidation states are the most stable forms (V^{4+} and V^{5+} are not known) (1).

I.2 Distribution of Vanadium in the Environment

Vanadium has an abundance in nature of about 0.02%. It occurs commonly but not uniformly in the earth's crust where its average concentration is between 100 - 150 mg/Kg ore (1,2). In rocks and soil, vanadium does not occur as the free metal but rather as vanadate of copper, zinc, lead, uranium, iron, calcium and potassium (2). Generally, vanadium is present in the rocks as a relatively insoluble salt in the trivalent state (2).

Fresh water contains variable amounts of vanadium ranging from 0.3 to 200.0 µg/L (2). Although the concentration of vanadium in sea water is usually low (2.0 - 29.0 µg/L), the

total amount in oceans is about 7.5×10^{12} Kg (2). The con-
centrations of vanadium in a variety of plants and animals are
given in Table 1.1 (2).

Generally, marine plants and invertebrate animals contain
more vanadium than land plants, insects and vertebrates. The
vanadium concentrations in the tissues of wild animals are
listed in Table 1.2 (2).

I.3 Vanadium and Human Exposure

Exposure to vanadium pentoxide dust produces a clinical
syndrome characterized by irritation of the eyes, nose and
throat, followed by rales throughout the lungs and acute bron-
chospasm similar to bronchial asthma (3).

I.4 Biological Effects of Vanadium

Vanadium has long been known to be essential for normal
growth and development of animals. The results of a study
which was carried out on some laboratory animals (rats and
chicks) showed that retardation of growth occurred when those
animals were kept on a diet deficient in vanadium (4). It was
also found that the amount of dietary vanadate required for
the normal growth of a rat was 100 parts per billion, or 2 nM,
and the normal tissue concentration was about 0.2 n mole per g
wet weight (4).

TABLE 1.1

Vanadium in Plants and Animals[2]

Source	[V] µg/g (dry.wt)
Plants:	
Plankton	5.00
Brown algae	2.00
Bryophytes	2.30
Ferns	0.13
Gymnosperms	0.69
Angiosperms	1.60
Fungi	0.67
Animals:	
Plankton	2.30
Annelids	1.20
Molluscs	0.70
Echinoderms	1.90
Crustaceans	0.40
Insects	0.15
Fish	0.14
Mammals	< 0.40

TABLE 1.2

The Vanadium Concentration in the Tissues of Wild Animals[2]

Tissue*	[V] μg/g (dry.wt)	
	Mean	Range
Kidney	0.94	0.00 - 2.07
Liver	0.25	0.00 - 0.94
Heart	1.16	0.00 - 3.40
Spleen	1.16	___

* Of those tested animals, the only tissue in which no vanadium was detected in any instance was the lung.

Vanadium is widely distributed in many plants and animals, many of which are ingested by human beings. The discovery of a naturally occuring potent inhibitor of sodium potassium stimulated adenosine triphosphatase [(Na^+, K^+) ATPase] isolated from striated muscle (5) has raised the idea that the element may have a physiological role and many experiments have been carried out to study its effect.

The interaction of vanadate in the +5 oxidation state with purified dog kidney (Na^+, K^+) ATPase was studied (6). It was found that vanadate bound to one high affinity site and one low affinity site per enzyme molecule. Binding to the high affinity site was sufficient to cause more than 95% inhibition of the enzyme, Scheme 1.1. It was also found that in the presence of vanadium, the enzyme had a higher affinity towards potassium and this reduced its affinity towards sodium. From the above study, it seems that vanadate acted in a co-operative manner with potassium in promoting inhibition of the sodium pump which implies that vanadate may have a physiological role in controlling the rate of the sodium pump.

After the discovery of the powerful inhibitory effect of vanadate on (Na^+, K^+) ATPase, it was thought that it may be specific for this enzyme only, as it was claimed to be ineffective on sacroplasmic reticulum Ca^{2+}-ATPase (5). The
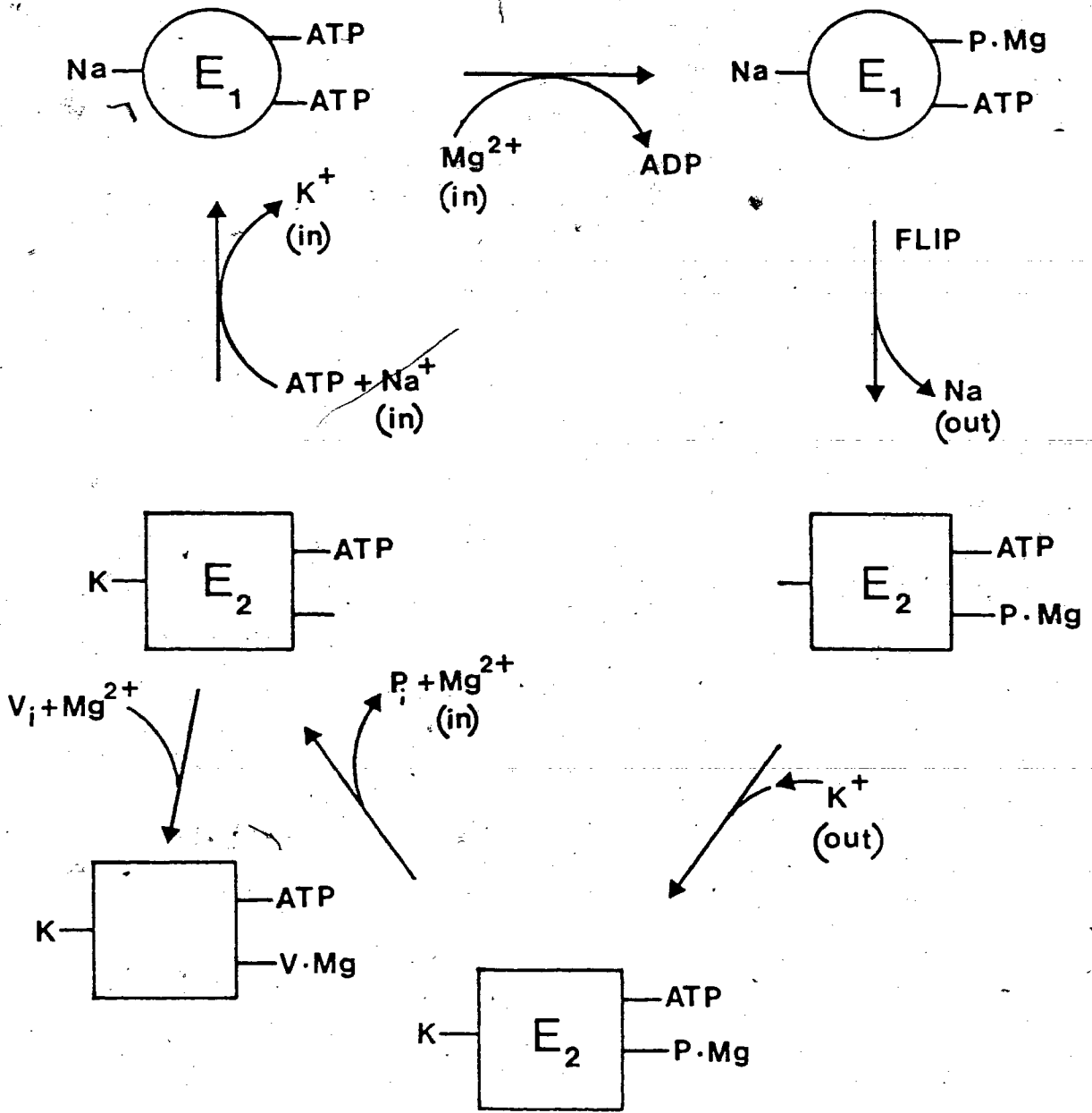
Scheme 1.1

inhibition of Ca^{2+}-ATPase of human red cell membranes by vanadate was studied. The steady-state level of the Ca^{2+}-dependent phospho-enzyme decreased and, as in the case of (Na^+, K^+)-ATPase, this may be a result of a similar $E_2 \rightarrow E_1$ conformational change. Inhibition by vanadate, thus, seems to be due to its binding to the high affinity site for ATP. Vanadate inhibition was also found to be dependent on the presence of Mg^{2+} and K^+ (7). High concentrations of Ca^{2+} were shown to decrease the inhibition by vanadate and this had made the observation of such inhibition of the enzyme difficult in the earlier studies. Vanadate inhibition was also shown by using rabbit muscle sacroplasmic reticulum Ca^{2+}-ATPase, Ca^{2+}- Mg^{2+}-ATPases of ascites plasma membrane and K^+-ATPase of E. Coli (8).

A study on the dietary ingestion of small amounts of vanadate (a potent inhibitor for (Na^+, K^+)ATPase) in rats showed that slow increases in their blood pressures occurred (9) (a similar effect was also observed in dogs (10). Increased pressure correlated positively with plasma vanadium levels.

In an experiment using red blood cells from humans, it was found that vanadate, which was taken up by these cells, was converted from the +5 to the +4 oxidation state (11).

The conversion of vanadate to the +4 oxidation state is not surprising in view of the reducing environment in the red cell cytoplasm and this can occur in the other cells which may take up vanadate.

Vanadate has an effect on many enzymes which catalyze phosphoryl transfer or release reactions. In recent studies, it was shown that vanadate acted as a potent inhibitor of many enzymes (12). The effect of vanadium IV and V on the enzyme ribonuclease, which catalyzes the hydrolysis of cyclic uridine 2',3'-phosphate, showed that the enzyme was competitively inhibited by complexes of vanadium IV and V with uridine, with dissociation constants of 10-12 μ M (13). The association constant for the binding of the vanadyl-uridine complex to ribonuclease was 1000 and 40-fold higher than that for the substrate and the product, respectively. This was rationalized by two possibilities. First, on the basis that vanadium may co-ordinate strongly with His[12] and/or His[119] at the active site of the enzyme (13). The other possibility was that the vanadium complex may adopt a structure similar to that of the substrate portion of the transition state for the ribonuclease - catalyzed hydrolysis of phosphate esters, which was thought to resemble a trigonal bipyramid of penta-oxophosphorus in which the entering and leaving groups were in axial positions (13).

Vanadium (IV nd V) was also found to be a potent inhibitor of alkaline phosphatase from E. Coli (14). Vanadate clearly showed inhibition by competition with phosphate ions over the pH range 7.0 - 10.0 with a K_i (inhibition constant) value of 2.2 μ M for vanadate compared to a corresponding value of 1.5 μ M for phosphate. Oxovanadium ion was found to bind about five to six times stronger than phosphate, with a K_i value of 0.4 μ M which indicated it to be a more potent inhibitor (14). The enzyme-bound vanadium (IV and V) ions may have some resemblance to the metastable intermediate formed during the hydrolysis of phosphate esters (14). Thus it is reasonable to hypothesize that some of the physiological effects of vanadate are due to how it acts as a phosphate analog in the biochemical reactions.

Some experimental evidence indicated that vanadate acted catalytically to stimulate the oxidation of glyceraldehyde 3-phosphate in the presence of the enzyme glyceraldehyde 3-phosphate dehydrogenase (G3-PDH) and NAD^+ (15). These results were rationalized by proposing the formation of 1-vanado-3-phosphoglycerate which then rapidly hydrolyzed to vanadate and 3-phosphoglycerate. When vanadate was replaced by phosphate, 1,3 diphosphoglycerate was formed and the reaction reached equilibrium with the accumulation of the latter compound. The

1,3 Diphosphoglycerate

$$X = VO_3^{2-} \quad \text{or} \quad AsO_3^{2-}$$
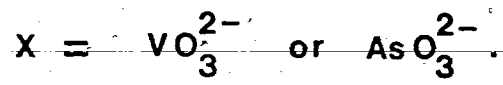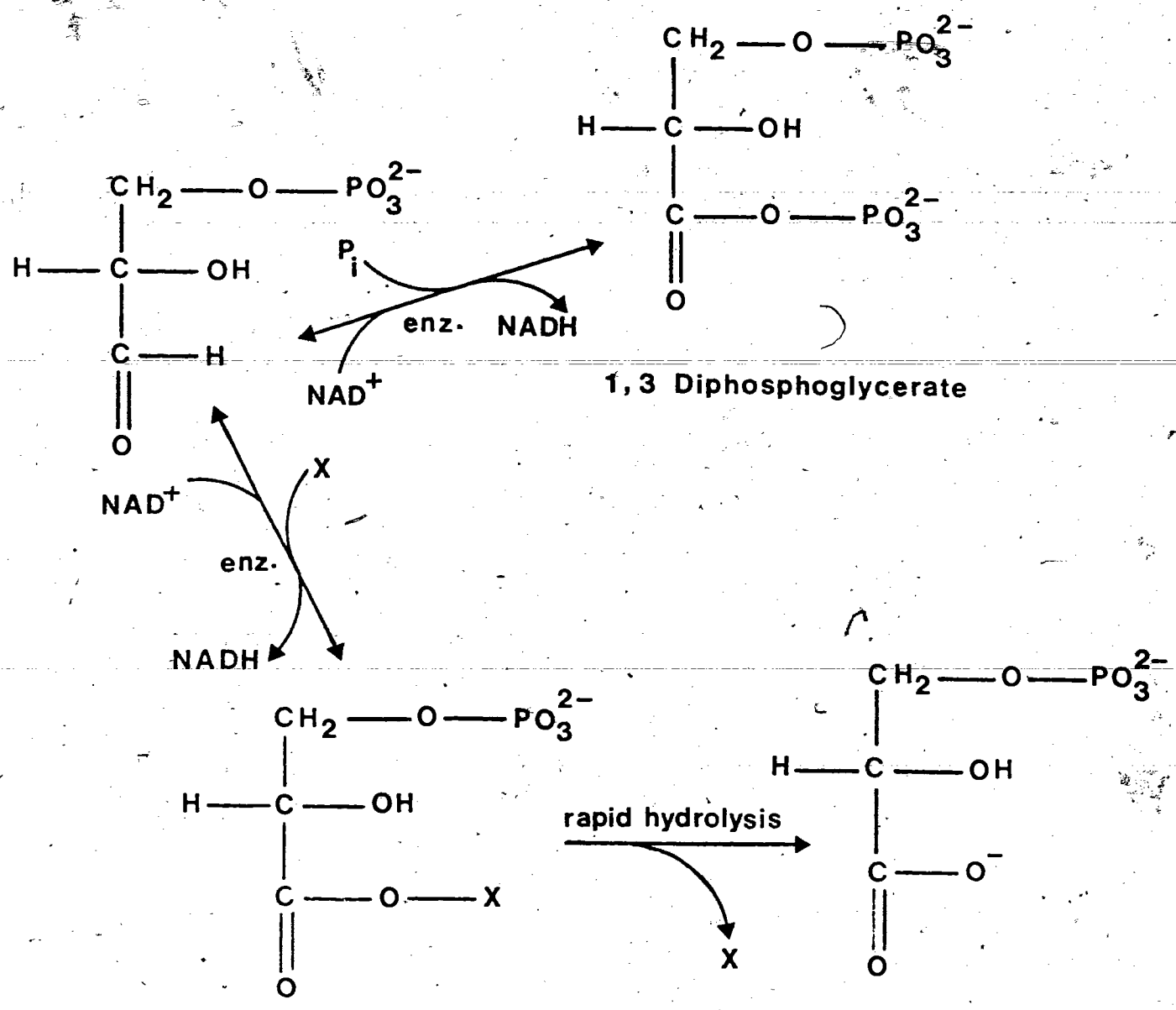
Scheme 1.2

rate of the oxidation reaction was limited by the slow hydrolysis rate of 1,3 diphosphoglycerate (Scheme 1.2). A similar mechanism was proposed to account for the stimulation by arsenate of the glyceraldehyde 3-phosphate dehydrogenase catalyzed oxidation of glyceraldehyde 3-phosphate by NAD^+ (15).

A study of arsenate (As_i) showed that it reacted with glucose or gluconate to form the arsenate esters glucose 6-arsenate (G 6-As) and 6-arsenogluconate (6-AsG) (16). These esters were found to be substrates for the enzyme glucose 6-phosphate dehydrogenase (G6-PDH) and 6-phosphogluconate dehydrogenase (6-PGDH) (16). The object of the study reported in this thesis was to investigate whether vanadate and arsenate act similarly in the glucose 6-phosphate dehydrogenase catalyzed oxidation of glucose.

G6-PDH catalyzes the reaction shown below:

D-glucose 6-phosphate $\rightleftharpoons$ D-glucono-δ-lactono 6-phosphate

+ +

$NADP^+$ NADPH
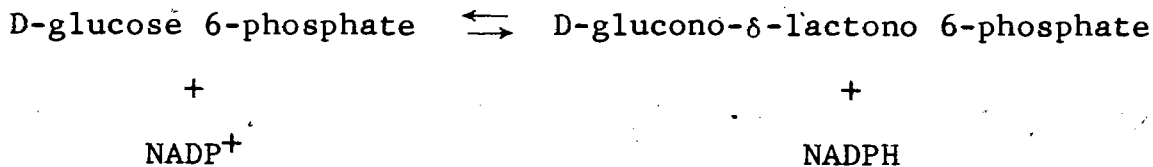
The equilibrium constant for the overall reaction is near 1.0 at pH 7 (26). The enzyme from yeast has a molecular weight of

128,000 (27).   This enzyme is used to determine directly G6-P
and NADP$^+$, and to determine many other species indirectly in
coupled enzyme assay systems.   The activity is most conven-
iently measured spectrophotometrically by the rate of forma-
tion of HADPH, which is measured by the increase in absorbance
at 340 nm.

CHAPTER II

Experimental Section and Methods of Analysis

II.1   Experimental Section

II.2   Instrumentation

II.3   Methods of Analysis

II.1 Experimental Section

II.1.1   Reagents and Solutions

Unless otherwise stated, all the reagents used in this study were commercially available (reagent grade) and were used without further purification.

a)   Trizma Base: (Sigma Reagent Grade)

50 mM T-acetate (6.055 g/1) was prepared and adjusted to the indicated pH for each experiment. The pH of the solution was adjusted by using 1M acetic acid. All the following solutions were prepared in the T-acetate buffer.

b)   Glucose: (Amachem Reagent Grade)

Stock solutions of 20 mM glucose were prepared by dissolving 0.363 gm glucose/100 ml T-acetate buffer solution.

c)   Magnesium Chloride: (Amachem Reagent Grade)

Solutions of magnesium chloride ($MgCl_2$) of concentrations of 100 mM (2.303 g $MgCl_2$/100 ml T-acetate buffer) were prepared.

c)   Nicotinamide Adenine Dinucleotide Phosphate (oxidized form): (Sigma Reagent Grade)

Stock solutions of 10 mM of nicotinamide adenine dinucleotide phosphate (B-NADP$^+$, Sigma, prepared from yeast) were prepared by dissolving 0.30 g per 3 ml T-acetate buffer solution. The pH of the solutions were then adjusted by using 1M sodium hydroxide solution.

e)   Sodium Orthovanadate: (Fisher Scientific Company Reagent Grade)

Sodium orthovanadate was used without further purification. Since the degree of hydration of the sodium vanadate was not specified, the concentrations of the stock solutions were determined by diluting to approximately 2×10^{-4} M at pH 10.5 and measuring the absorbance at $\lambda = 260$ nm. At this pH and concentration vanadium exists essentially entirely as HVO_4^{2-}, whose molar extinction coefficient (ε) at 260 nm is 3.55×10^3 (17). The formula weight of sodium orthovanadate was calculated and stock solutions of 4 mM vanadate solutions were prepared in 50 mM T-acetate buffer solutions without adjusting the pH.

f)   Glucose 6-Phosphate: (Sigma Reagent Grade)

A stock solution of 1 mM glucose 6-phosphate (0.028 g/100 ml T-acetate buffer solution) was prepared and its pH was adjusted to 7.4.

g)   Sodium Sulphate: (BDH Chemicals)

2.845 g of sodium sulphate were dissolved in 100 ml of T-acetate buffer solution, at pH 7.4, to give 0.2 M solution.

h)   Glucose 6-Phosphate Dehydrogenase:

For all the experiments, the glucose 6-phosphate dehydrogenase enzyme (Sigma type VII, prepared from Bakers yeast)

was dialyzed overnight against two liters of 50 mM T-acetate buffer solution (at the indicated pH for each experiment). The process of dialysis was carried out, in a cold room (4°C), in a beaker inserted in ice with constant stirring. For the experiments where the effect of different sulphate concentrations on the enzyme activities were checked, further treatment for the enzyme was carried out by running the enzyme twice on a Sephadex centrifuge column (using the method of Penefsky (18)).

II.1.2 Purification of the Enzyme

In the following section, purification of the enzyme by dialysis and by Sephadex centrifuge column chromatography will be given in detail as well as the preparation of the Sephadex column.

a) Method of Dialysis

This process is one of the methods which can be used for desalting the enzyme. The membrane of the dialysis tubing (Fisher Scientific Company, seamless cellulose, having an average width of 1 cm, flat) contains ultramicroscopic pores. This membrane allows small molecular weight solutes and water to pass through freely, but does not permit the passage of large molecular weight molecules (such as proteins).

The tube was prepared by soaking it, for one hour, in 1% acetic acid, then allowing it to stand, with gentle stirring,

in deionized-distilled water for a few minutes and then the water was replaced by a mixture of 1% sodium carbonate and 1×10^{-3} M EDTA (ethylene diamine tetraacetic acide disodium salt). After another few minutes of gentle stirring, the alkaline EDTA solution was replaced by a fresh solution at the same concentration and heated with stirring to about 75°C for 30 minutes. The hot solution was poured off and replaced by deionized-distilled water and stirred gently. The prepared tubing was stored in a refrigerator in deionized-distilled water containing a few drops of chloroform. The tubing was washed gently with distilled water before applying the solution that contained the enzyme.

b)   Method of Enzyme Desalting

The term desalting refers not only to the removal of salts, but also the removal of any lower molecular weight compound from a solution of macromolecules. Enzyme desalting was done by running the enzyme solution on a Sephadex centrifuge column. The preparation of the latter as well as the desalting process was done as follows:

A disposable, 1 ml plastic tuberculin syringe was fitted with a porous polyethylene disk. The syringe was filled to 1 ml mark with Sephadex G-50-80 (Sigma reagent grade, with a particle diameter 20-80 μcm and water regain of 5.0 ± 0.3 ml per g dry sephadex) previously allowed to swell in a solution

of 50 mM T-acetate buffer overnight in a refrigerator. The
column was replaced in a test tube, allowed to stand until no
further liquid drained from it, and then was transferred with
the test tube to a six-place, swinging bucket rotor in a
clinical centrifuge. The centrifuge was preset to a speed of
1000 × g (about 3/4 of the maximum speed). Centrifugation was
done for two minutes. 100 μl of the solution containing the
enzyme was applied to the top of the column and centrifugation
was immediately repeated under exactly the same conditions but
for only one minute. In this case, the solution containing
the enzyme passed down the column (the low molecular weight
molecules can penetrate into the pores and were retained in
the column, but large protein molecules cannot penetrate into
the beads and thus passed through the column).

II.2 Instrumentation

The instruments used in this study were:

1 - IEC model and CH (Clinical Chemical) centrifuge.

2 - Cary-14 spectrophotometer (λ = 340 nm) with one inch per
minute chart speed and 0.0 - 1.0 absorbance range. One
cm quartz cells were used for the measurements.

II.3 Methods of Analysis

All the kinetic measurements were carried out at a con-
stant temperature of 30°C, using the concentrations which are

given in the figure legends. The concentrations of nicotina-
mide adenine dinucleotide phosphate ($NADP^+$) and $MgCl_2$ were the
same for all the experiments (1 mM and 10 mM for $NADP^+$ and
$MgCl_2$, respectively). The total volume of each reaction
mixture was 300 μl for all the experiments.

II.3.1 Glucose Oxidation Rates

Aliquots of the incubated mixtures of glucose and vana-
date were added to reaction mixtures (containing tris acetate
buffer, $NADP^+$, $MgCl_2$ and different enzyme concentrations) to
initiate the reaction. The change in the absorbance at 340 nm
(λ_{max} for NADPH (19)) was measured spectrophotometrically.
Separate blanks with no vanadate were done for each enzyme and
glucose concentration (to measure the rate of the enzymic
oxidation of glucose alone). The rates obtained from the
blank experiments were subtracted from those obtained in the
presence of vanadate to give rates from which plots of 1/V
vs. 1/[E] were obtained (where V is the oxidation rate).

It was found that on the addition of glucose or glucose
and vanadate to the reaction mixture, a lag in the absorbance
vs. time curve occurred. Many attempts were carried out to
reduce this lag, and it was found that the optimum condition
was to incubate the enzyme with the buffer for five minutes
before the addition of the substrate.

Calculation of the rate was carried out by using Beer-Lamberts' law:

$$A = c \, \varepsilon \, \ell$$

where A = absorbance.

    c = concentration.

    ε = the molar extinction constant which equals to

       $6.22 \times 10^3 \ M^{-1}cm^{-1}$ (for NADPH) (19).

    ℓ = the light path length = 1 cm.

II.3.2 The Burst Method

This test was carried out by incubating the buffer, $NADP^+$, $MgCl_2$ glucose and vanadate for a fixed time during which the absorbance of this mixture was measured. The enzyme was then added and the absorbance of the resultant mixture was measured again. In this case, the burst was the difference between the absorbance before and after the addition of the enzyme and was proportional to the substrate concentration present at the moment when the enzyme was added. Blanks were done by leaving out vanadate.

CHAPTER III

Results and Discussion

III.1 Introduction

Addition of inorganic vanadate (V_i) to a reactionmixture that contains glucose (G), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and glucose 6-phosphate dehydrogenase (G6-PDH) resulted in an increase in the rate of glucose oxidation. Increasing the concentration of either glucose or the enzyme also caused an increase in the rate of the reaction. Omission of glucose or the enzyme from the reaction mixture resulted in no observable rate. The increase in the rate of glucose oxidation could be due to an interaction between vanadate and the alcoholic groups of glucose to form an esterlike compound (as observed in the case of V(IV) and (V) with uridine (13)) which may act as a substrate for glucose 6-phosphate dehydrogenase and therefore increase the $NADP^+$ reduction rate, or due to an activation of the enzyme (as observed in the case of sulphate, see below).

III.2 Evidence for Vanadate Ester Formation

The fact that addition of inorganic vanadate increases the rate of glucose oxidation raises the question of whether vanadate reacts with glucose to form a vanadate ester or binds to the enzyme and activates it as a glucose dehydrogenase? Suppose vanadate activates the enzyme to accelerate glucose oxidation. Thus, it binds to the inactive enzyme (E_i) to give the active enzyme (E_a), which would increase the rate of glucose oxidation as shown in equation (3.1):

$$E_i + V_i \; \underset{K_s}{\overset{}{\rightleftharpoons}} \; E_a \; \overset{G}{\rightleftharpoons} \; E_a \cdot G \longrightarrow \text{product} \quad , \quad (3.1)$$
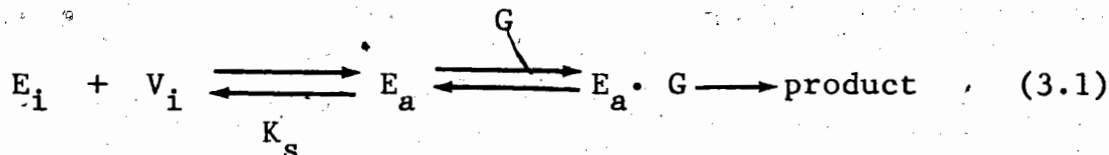
where K_s is the equilibrium constant for the dissociation of vanadate from the active enzyme. A study of the effect of vanadate on the reaction rate could be carried out by following the change in the rate of the reaction with changing enzyme concentration in the presence of a fixed vanadate concentration. So, according to equation (3.1), the rate of glucose oxidation is given by equation (3.2), the Michaelis-Menten equation:

$$V = \frac{k_{cat}[E_a][G]}{K_m + [G]} \qquad\qquad (3.2)$$

where k_{cat} is the turnover number of the enzyme (number of substrate molecules transformed to a product per minute by a single enzyme molecule at saturating substrate concentrations). K_m is the Michaelis constant or the concentration of substrate which gives half maximum velocity. By using the conservation equation (3.3), the fraction of the inactive enzyme in terms of the total enzyme and the active enzyme concentrations can be calculated as in equation (3.4):

$$[E] = [E_a] + [E_i] \tag{3.3}$$

$$[E_i] = [E] - [E_a] \tag{3.4}$$

The equilibrium constant for the dissociation of vanadate from the active site is given by equation (3.5), and by substituting for the fraction of the inactive enzyme, equation (3.6) is obtained.

$$K_s = \frac{[E_i][V_i]}{[E_a]} \tag{3.5}$$

$$K_s = \frac{[E][V_i] - [E_a][V_i]}{[E_a]} \tag{3.6}$$

Rearranging equation (3.6) gives equation (3.7) which gives the fraction of the active enzyme:

$$[E_a] = \frac{[E][V_i]}{K_s + [V_i]} \tag{3.7}$$

Substituting this expression for $[E_a]$ into the rate equation (3.2) gives equation (3.8), and taking the reciprocal of both sides gives equation (3.9):

$$V = \frac{k_{cat}[E][G][V_i]}{K_m K_s + K_m[V_i] + K_s[G] + [G][V_i]} \qquad (3.8)$$

$$\frac{1}{V} = \frac{K_m K_s + K_m[V_i] + K_s[G] + [G][V_i]}{k_{cat}[E][G][V_i]} \qquad (3.9)$$

Equation (3.9) predicts that, by plotting $1/V$ vs. $1/[E]$ a straight line through the origin will be obtained. It was found that, plotting $1/V$ vs. $1/[E]$ (using different vanadate and glucose concentrations, Figure 3.1, 3.2) gave straight lines but they did not intersect the origin. The intersection of the lines at the horizontal axis to the left of the origin means that equation (3.9) can not be applied to the present system. This also rules out the activation of the enzyme by vanadate and, therefore, the increase in the rate of glucose oxidation by vanadate is more probably due to ester formation.

Figure 3.1

The Effect of Different G6-PDH Concentrations on the
Rate of NADP$^+$ Reduction in the Presence of Glucose and Three
Different Vanadate Concentrations at pH 8.0. Rates were
determined as described in the experimental section, and the
rates observed in the absence of vanadate were subtracted
from those measured in the presence of vanadate to obtain the
values plotted in the Figure. Concentrations of the reagents
were as follows: 5.0×10^{-2} M Tris-acetate (pH 8.0), $1.0 \times$
10^{-3} M NADP$^+$, 1.0×10^{-2} M MgCl$_2$, 5.0×10^{-4} M glucose, and
vanadate at 1.0×10^{-4} M (solid circles), 2.0×10^{-4} M (solid
squares), and 3.0×10^{-4} M (solid triangles). The indicated
volumes of G6-PDH stock solution (8.6 mg/mL) were prsent in
the reaction mixtures whose final volume was 300 μL. The
error bars in this and the following double reciprocal plots
represent error limits of ±10%. It was found that when
duplicate kinetic runs were done, the rates were always
within these limits.
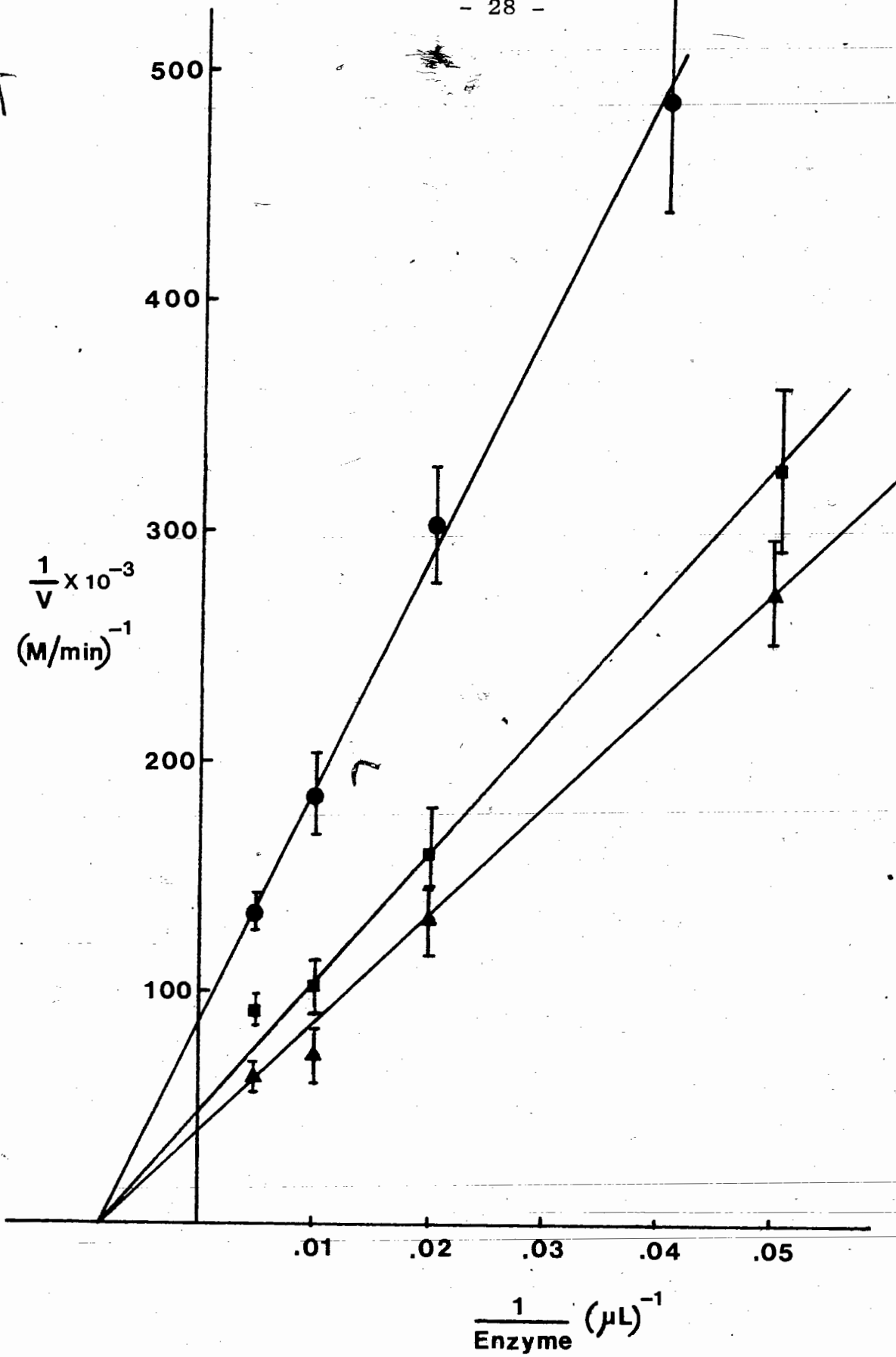
Figure 3.2

The Effect of Different G6-PDH Concentrations on the Rate of NADP$^+$ Reduction in the Presence of Vanadate and Two Different Glucose Concentrations. Procedures and Conditions were as described for Figure 3.1, except that vanadate concentration was 2.0×10^{-4} M, and the glucose concentrations were 5.0×10^{-4} M (solid triangles), and 1.0×10^{-3} M (solid circles).

Suppose that vanadate reacts with glucose to form an ester. If the present system follows Michaelis-Menten kinetics, on the basis of equation (3.10), one can derive the rate equation (3.11):

$$V_i + G \underset{k_2}{\overset{k_1}{\rightleftharpoons}} G6\text{-}V \xrightarrow[]{enzyme} 6\text{-}VG \qquad (3.10)$$

with NADP$^+$ and NADPH shown at the lower arrow.

k_1 and k_2 are the rate constants for formation and hydrolysis of G6-V (glucose 6-vanadate), respectively. 6-VG is 6-vanado-gluconate.

$$V = \frac{k_{cat}}{K_m} \; [E][G6\text{-}V] \qquad (3.11)$$

Equation (3.11) gives the rate of oxidation of G6-V (it is assumed that the reaction follows Michaelis-Menten kinetics, and that $[G6\text{-}V] \ll K_m$). By applying the steady-state assumption to G-6V (assume that the rate of formation of G6-V equals its rate of consumption) equation (3.12) is obtained which can be rearranged to equation (3.13).

$$\frac{d[G6-V]}{dt} = 0 = k_1[G][V_i] - \left(k_2 + \frac{k_{cat}}{K_m} [E] \right) [G6-V] \qquad (3.12)$$

$$k_1[G][V_i] = k_2[G6-V] + \frac{k_{cat}}{K_m} [E][G6-V] \qquad (3.13)$$

Solving equation (3.13) for the steady-state concentration of G6-V gives equation (3.14):

$$[G6-V] = \frac{k_1[G][V_i]}{k_2 + \frac{k_{cat}}{K_m} [E]} \qquad (3.14)$$

Substituting equation (3.14) into equation (3.11) results in equation (3.15), and by taking the reciprocal of both sides, one obtains equation (3.16)

$$V = \frac{\dfrac{k_{cat}}{K_m} k_1 [E][G][V_i]}{k_2 + \dfrac{k_{cat}}{K_m} [E]} \tag{3.15}$$

$$\frac{1}{V} = \frac{k_2}{\dfrac{k_{cat}}{K_m} k_1 [E][G][V_i]} + \frac{1}{k_1 [G][V_i]} \tag{3.16}$$

Equation (3.16) predicts that the plot of $1/V$ vs. $1/[E]$ is a straight line with a vertical intercept $= 1/k_1 [G][V_i]$, a horizontal intercept $= -k_{cat}/K_m k_2$ and a slope $= K_m k_2/k_{cat} k_1$ $[G][V_i]$.

By plotting the rate of G6-V oxidation (obtained from subtracting the rate of glucose oxidation from the total rate of the reaction) vs. the enzyme concentration, a hyperbolic curve was obtained (Figures 3.3 - 3.6). The hyperbolic curve is an indication of saturation by enzyme, where at saturation the rate of the reaction is independent of the enzyme concentration. This curvature was not due to decrease in the specific activity of the enzyme with increasing enzyme concentration, because in the absence of vanadate the rate of glucose oxidation increases linearly with enzyme up to the highest

Figure 3.3

The Effect of Different G6-PDH Concentrations on the Rate of NADP$^+$ Reduction in the Presence of 1.0×10^{-3} M Glucose with and without 2.0×10^{-4} M Vanadate at pH 7.0. Procedures and Conditions were as described with Figure 3.1, except that the vanadate and glucose concentrations were those indicated above, and the concentration of the enzyme stock solution was 7.8 mg/mL. The points represent, (a) measurements of the rates of NADP$^+$ reduction in the presence of glucose without vanadate (solid circles), (b) similar measurements in the presence of both glucose and vanadate (solid triangles), and (c) the difference between (b) and (a) (solid squares).

Figure 3.4

The Effect of Different G6-PDH Concentrations on the Rate
of NADP$^+$ Reduction in the Presence of 1.0×10^{-3} M Glucose
with and without 2.0×10^{-4} M Vanadate at pH 7.4. Procedures
and conditions were as described with Figure 3.3, except that
the pH was 7.4. The points represent: (a) measurements of
the rates of NADP$^+$ reduction in the presence of glucose
without vanadate (solid circles), (b) similar measurements in
the presence of both glucose and vanadate (solid squares), and
(c) the difference between (b) and (a) (solid triangles).

Enzyme, μL

$V \times 10^3$ (M/min)

Figure 3.5

The Effect of Different G6-PDH Concentrations on the Rate of $NADP^+$ Reduction in the Presence of 1.0×10^{-3} M Glucose with and without 2.0×10^{-4} M Vanadate at pH 8.0. Procedures and conditions were as described with Figure 3.3, except that the pH was 8.0. The points represent: (a) measurements of the rates of $NADP^+$ reduction in the presence of glucose without vanadate (solid circles), (b) similar measurements in the presence of both glucose and vanadate (solid triangles), and (c) the difference between (b) and (a) (solid squares).
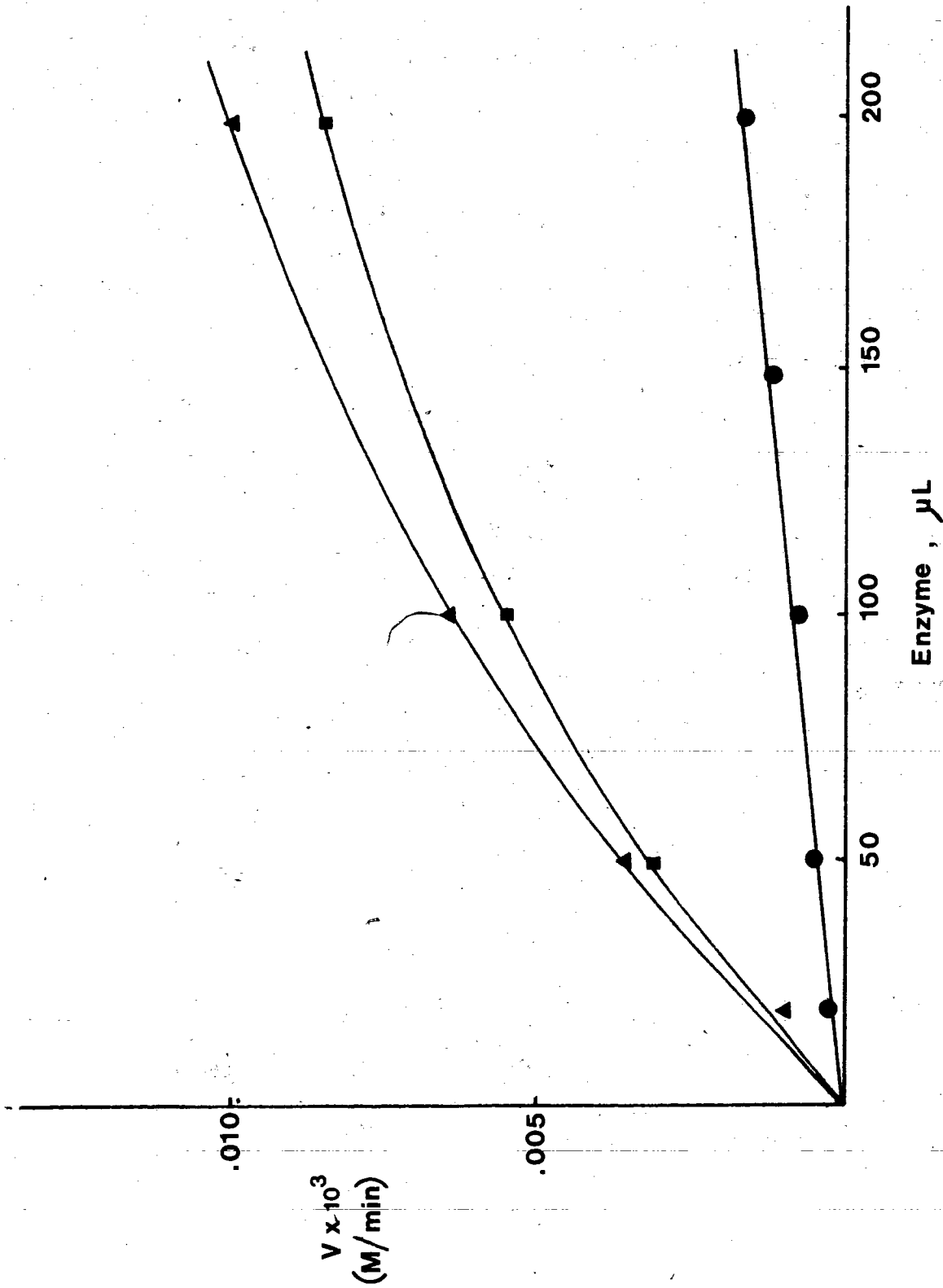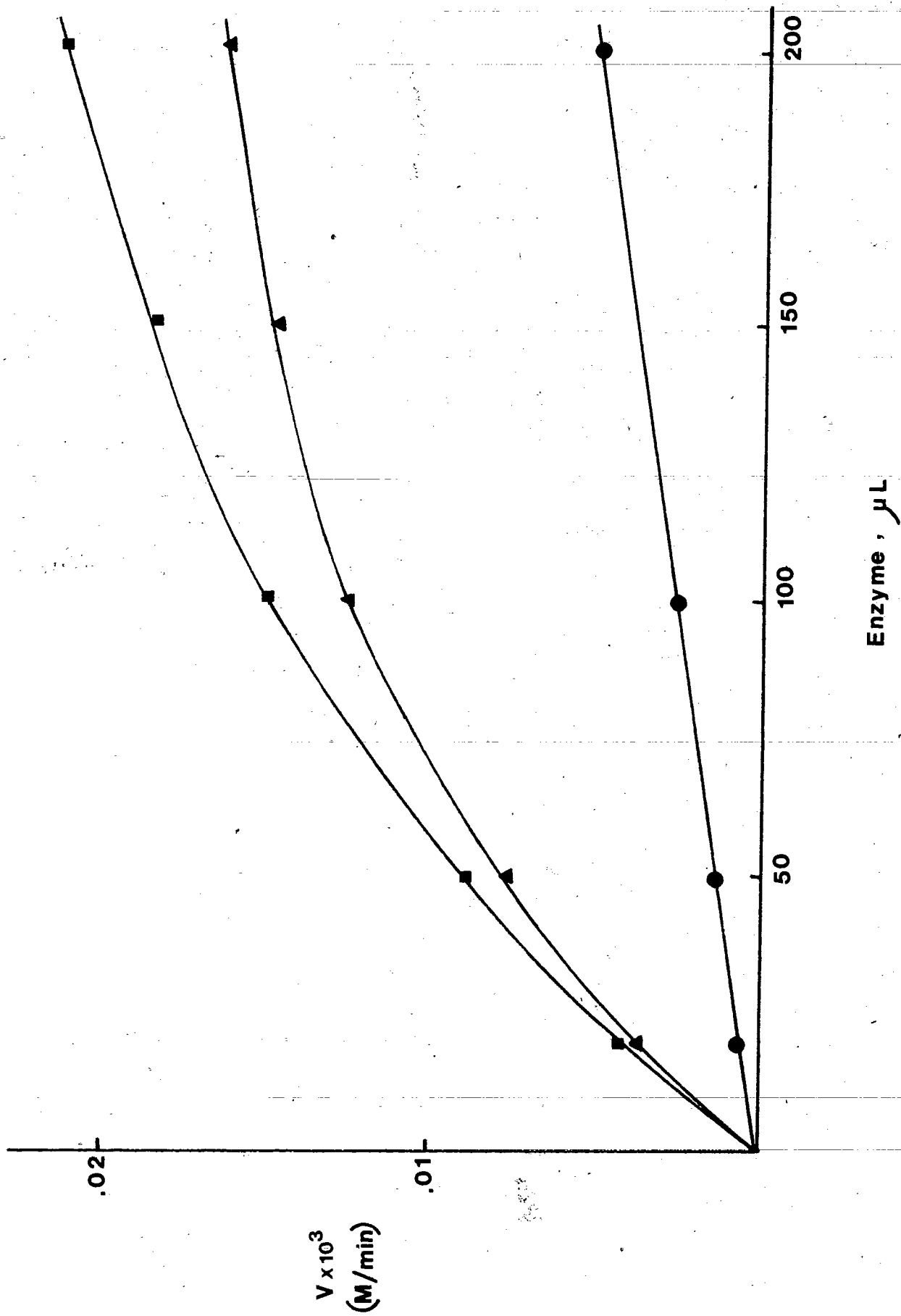
Enzyme , μL

V x 10³
(M/min)
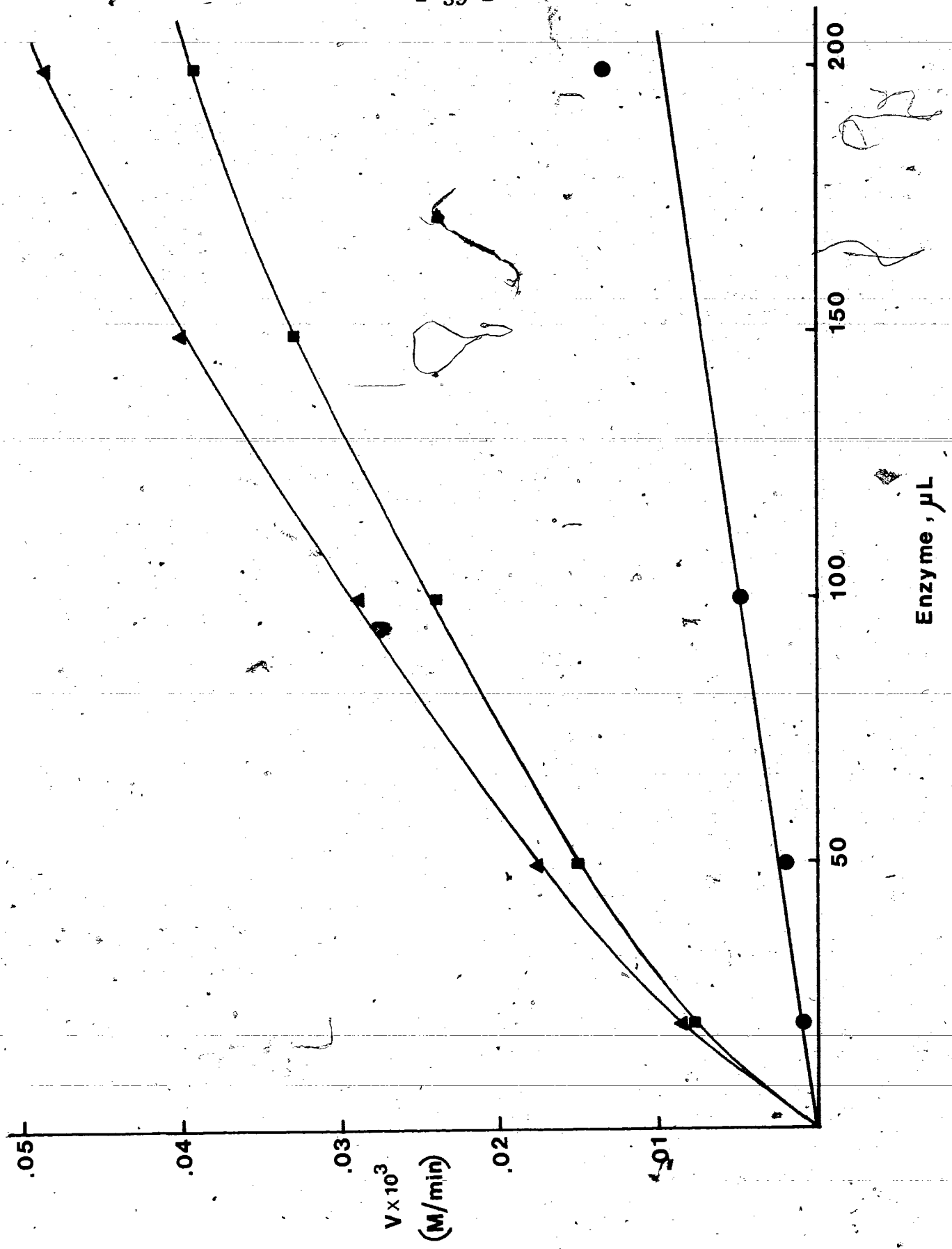
Figure 3.6

The Effect of Different G6-PDH Concentrations on the Rate of $NADP^+$ Reduction in the Presence of 1.0×10^{-3} M Glucose with and without 2.0×10^{-4} M Vanadate at pH 8.2. Procedures and conditions were as described with Figure 3.3, except that the pH was 8.2. The points represent: (a) measurements of the rates of $NADP^+$ reduction in the presence of glucose without vanadate (solid circles), (b) similar measurements in the presence of both glucose and vanadate (solid squares), and (c) the difference between (b) and (a) (solid triangles).

Enzyme, μL

$V \times 10^3$
(M/min)

Figure 3.7

The Effect of Different G6-PDH Concentrations on the Rate of NADP$^+$ Reduction in the Presence of 1.0×10^{-3} M Glucose with and without 2.0×10^{-4} M Vanadate at pH 8.4. Procedures and conditions were as described with Figure 3.3, except that the pH was 8.4. The points represent: (a) measurements of the rates of NADP$^+$ reduction in the presence of glucose without vanadate (solid circles), (b) similar measurements in the presence of both glucose and vanadate (solid squares), and (c) the difference between (b) and (a) (solid triangles).
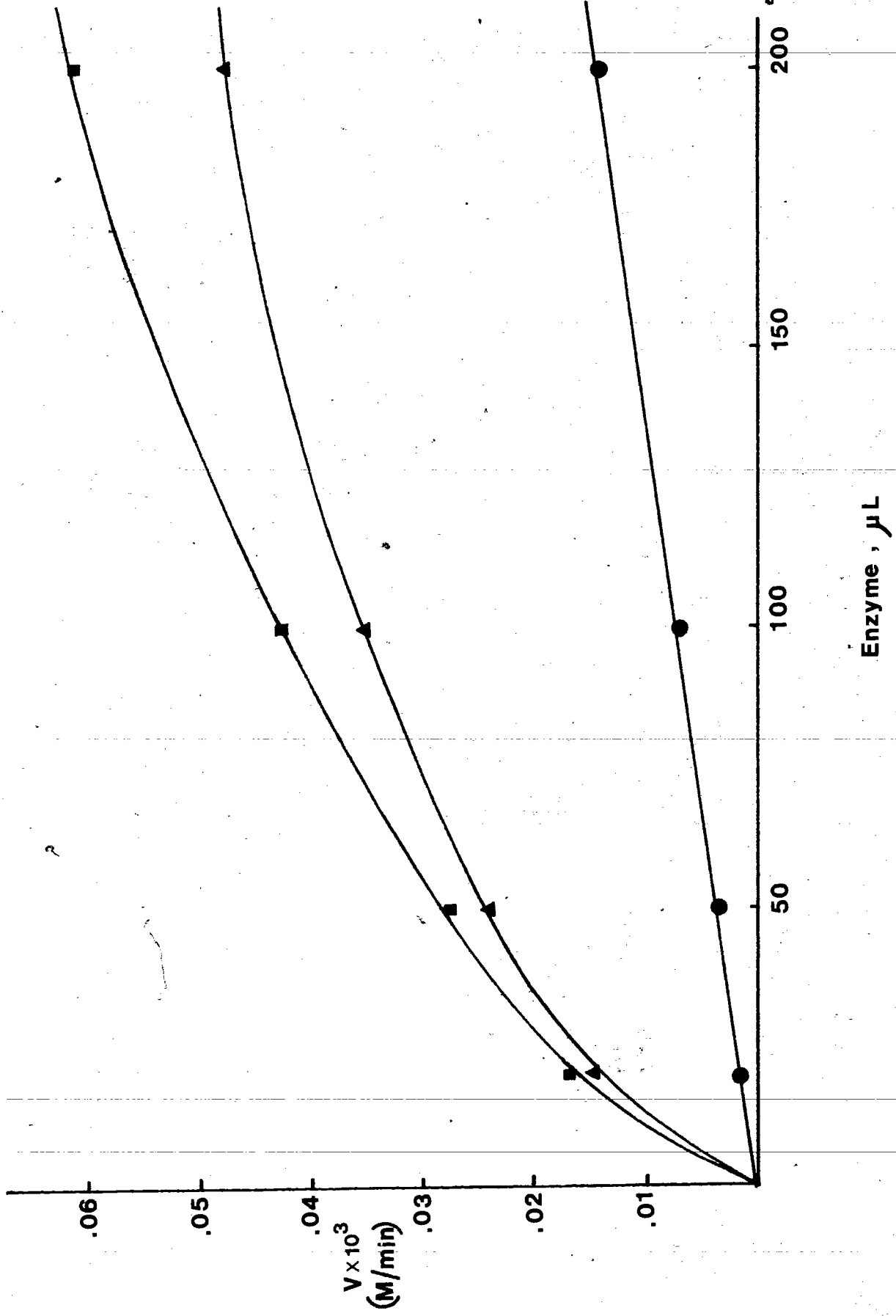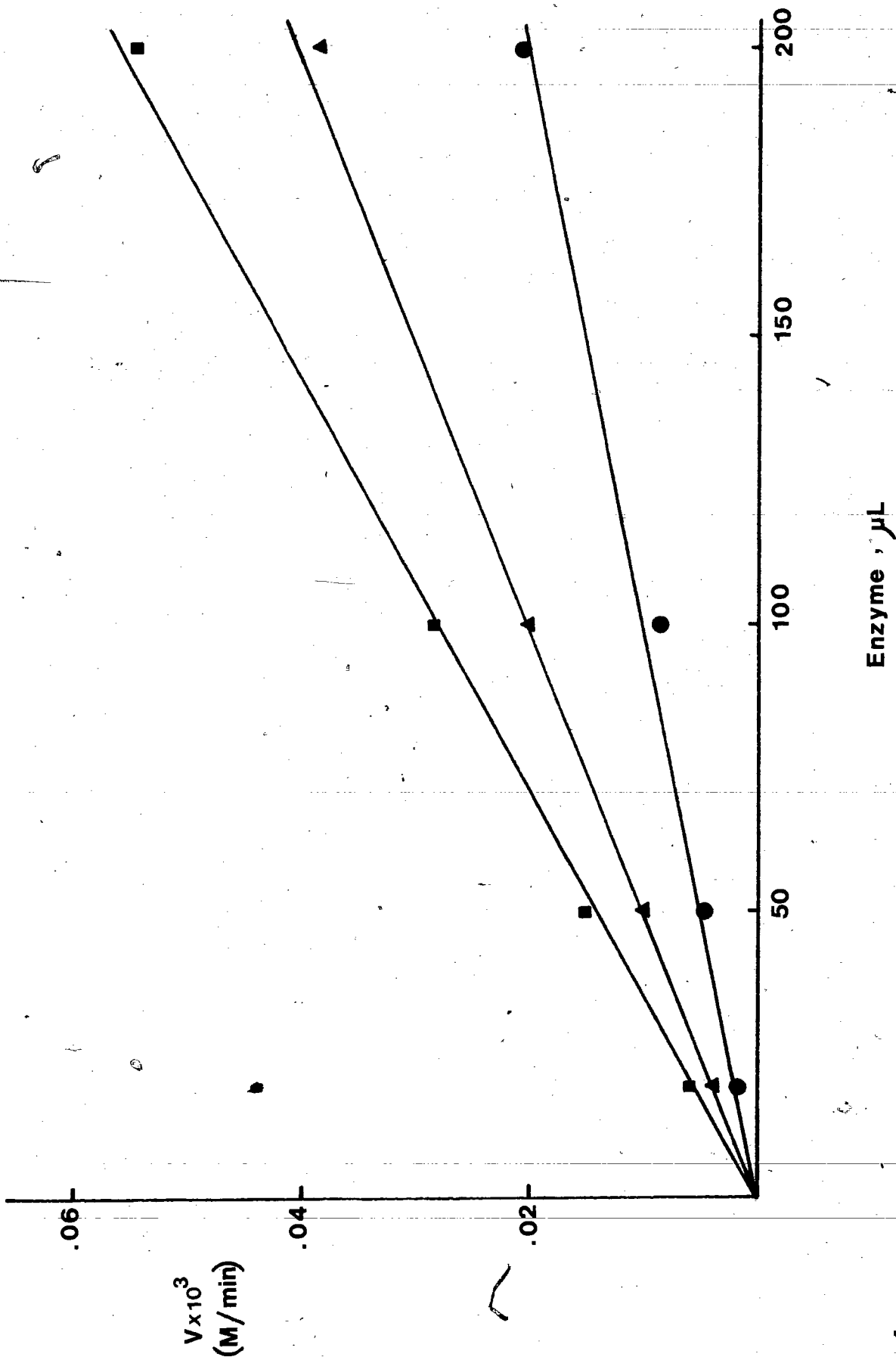
enzyme concentrations used. This saturation can be rational-
ized in terms of a step in the reaction mechanism before the
enzymic oxidation, which becomes rate-limiting at saturating
enzyme concentrations. This step which becomes rate-limiting
at saturating enzyme concentrations is believed to be forma-
tion of G6-V, which is similar to G6-P (glucose 6-phosphate)
and G6-As (glucose 6-arsenate) whose oxidation reactions are
catalyzed by G6-PDH (16).

Furthermore, a ^{51}V NMR study of a mixture of vanadate and
ethanol showed that the vanadate formed a compound with ethan-
ol with a chemical shift for vanadium different from that for
the inorganic vanadate. Thus, when ethanol was added to di-
lute aqueous vanadate two peaks were observed in the ^{51}V NMR
spectrum, while only one major peak was observed in the ab-
sence of ethanol (20). At ambient temperature, two separate
resonances due to the free V_i and the putative ethyl ester
were observed. On raising the temperature, the two resonances
broadened and then coalesced to a single peak, indicative of a
rapid exchange process (20). This observation could be repre-
sented by equation (3.17) which supports the hypothesis that
vanadate esters form rapidly from V_i and compounds, such as
glucose, which contain hydroxyl groups.

$$HVO_4^{2-} + CH_3 \cdot CH_2OH \rightleftharpoons CH_3CH_2O\text{-}VO_3^{2-} + H_2O \qquad (3.17)$$
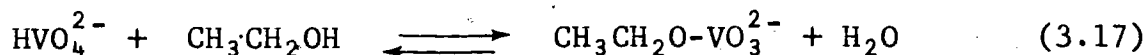
III.3   Effect of pH on G6-V Formation

It is known that the concentration of the monovanadate species in solution is very pH dependent at constant vanadium atom concentration (21). As the pH of the solution increases, the fraction of total vanadate which exists as monovanadate species increases and it is the predominant species above pH 7.0 with total vanadium concentration less than about $1.0 \times 10^{-3} M$ (21). A study of the effect of pH on the rate of G6-V formation from pH 7.0 to pH 8.4 at a total vanadate concentration of $2.0 \times 10^{-4} M$ was carried out. As can be seen from the figures (3.3 - 3.7), the rate of the G6-V oxidation increased as the pH of the solution increased.

Saturation curves (V vs. [E] plot) were observed at pH values less than 8.4 (Figures 3.3 - 3.6) while no such curve was obtained at that pH (Figure 3.7). This could be due to the monoprotonated species, HVO_4^{2-} (the predominant species at pH ranges 8.3 - 13.0 (21)), being the most reactive species (21) towards ester formation. (The pK_a for loss of a proton from $H_2VO_4^-$ is 8.3 (22).) Thus, at pH 8.4 G6-V ester was formed faster than at the lower pH's and in turn more enzyme was required to reach the saturation point.

These experimental results were supported by an estimated V vs. [E] plot (Figure 3.8) based on the following: the rate

Figure 3.8

Estimated Rates of NADP$^+$ Reduction at Different G6-PDH Concentrations. The rates were calculated from equation 3.15 using the following values for the constants and concentrations: [Glucose] = 1.0×10^{-3} M, [Vanadate] = 2.0×10^{-4} M, k_1 = 21.6 M^{-1}s^{-1}, k_2 = 5.0×10^3s^{-1}, k_{cat}/K_m = 3.6 μL^{-1}s^{-1}, pH 8.4.

Enzyme , μ L

$V \times 10^6$
$M \cdot s^{-1}$
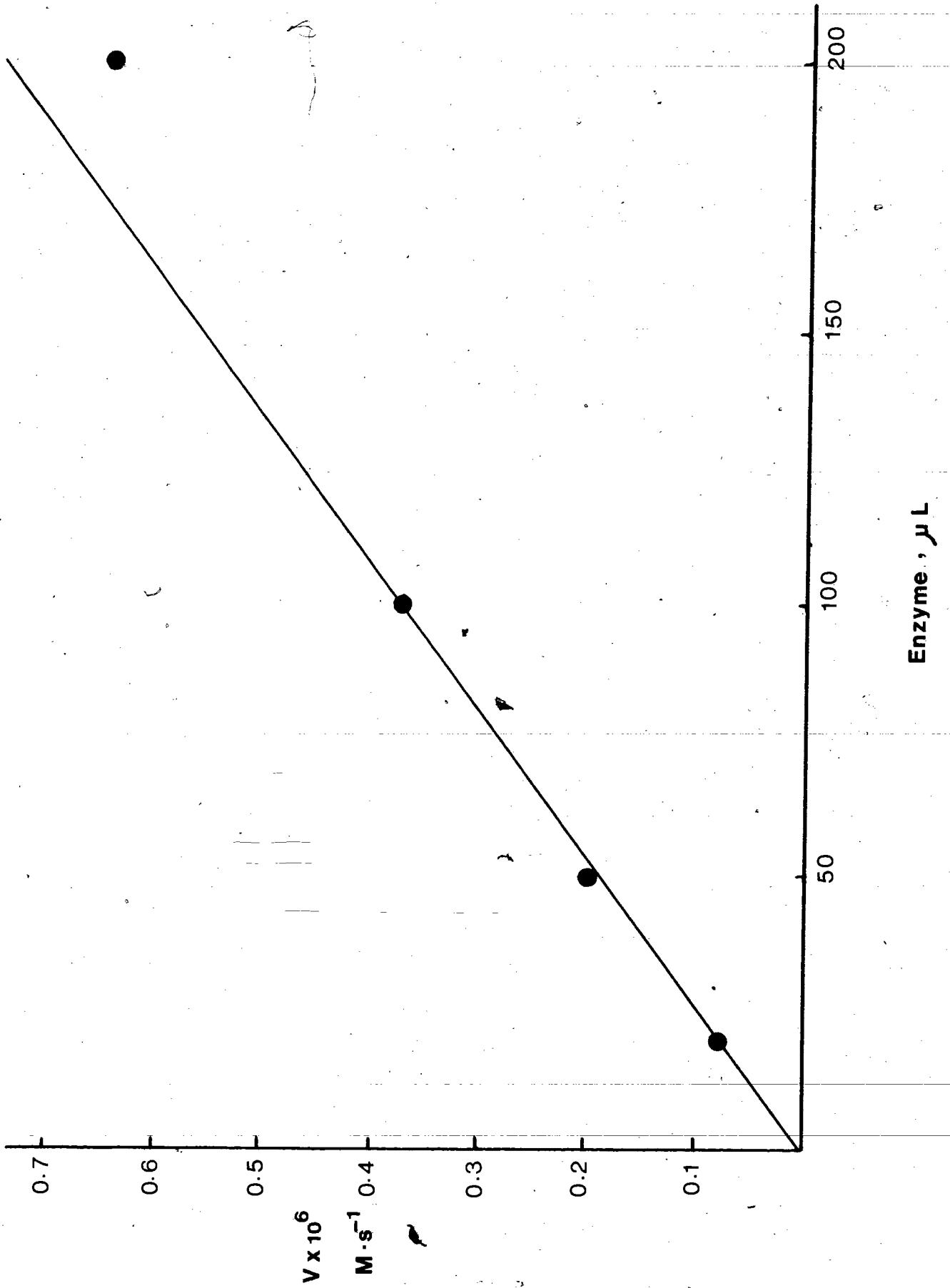
constant of G6-V formation, k_1, at pH 8.4 (11.2 $M^{-1}s^{-1}$) was obtained from the vertical intercept of the plot 1/V vs. 1/[E] (Figure 3.13). The rate constant of G6-V hydrolysis (k_2 = 1.8 $\times$ 10^3s^{-1}) was then calculated by assuming a K_{eq} value equal to that of G6-P (6.2 $\times$ $10^{-3}M^{-1}$)(16). The k_{cat}/K_m ratio (3.6 μL^{-1}) was estimated from the horizontal intercept (-0.002 $\mu L^{-1} \cdot s^{-1}$) of Figure (3.13). The estimated rate, V, was then obtained by applying the k_1, k_2 as well as k_{cat}/K_m values to equation (3.15) and using the same experimental values of [E] at pH 8.4. The results (which are shown in Figure 3.8) indicate that it is not unreasonable that the plot of V vs. [E] is only very slightly curved at pH 8.4.

Table (3.1) gives the values of k_1 at different pH values obtained from Figures (3.9 - 3.13). It was found that, as the pH increased, the value of k_1 increased, and this is shown in the plot of log k_1 vs. pH (Figure 3.14).

III.4 Effect of Vanadate and Glucose Concentrations on the Rate of G6-V Formation

The second order rate constant (k_1) for the formation of G6-V was obtained using the same procedure which was used for the pH dependence studies in the preceding section. The values of k_1 which were obtained from using different vanadate (Figure 3.1) and different glucose (Figure 3.2) concentrations at pH 8.0 are listed in Table (3.2). It was found that, k_1

Figure 3.9

The Effect of Different G6-PDH Concentrations on the Rate
of G6-V Oxidation at pH 7.0. This Figure is a double recipro-
cal plot of data from Figure 3.3. The values used are from
line (c) in Figure 3.3, i.e. the rates of NADP$^+$ reduction in
the presence of glucose and vanadate, minus the rates in the
absence of vanadate.

Figure 3.10

The Effect of Different G6-PDH Concentrations on the Rate
of G6-V Oxidation at pH 7.4. ·This Figure is a double recipro-
cal plot of data from line (c) in Figure 3.4., i.e. the rates
of $NADP^+$ reduction in the presence of glucose and vanadate,
minus the rates in the absence of vanadate.
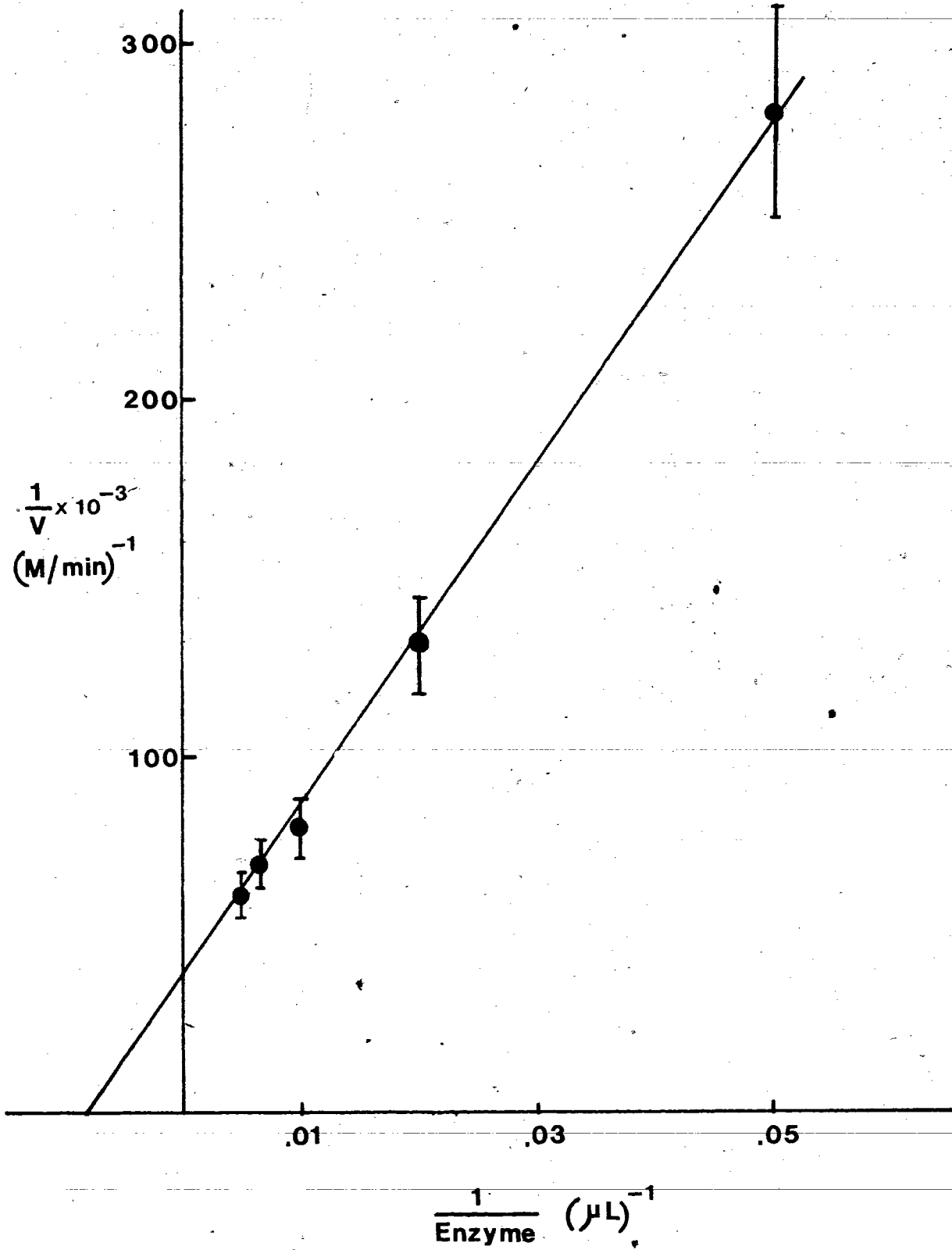
Figure 3.11

The Effect of Different G6-PDH Concentrations on the
Rate of G6-V Oxidation at pH 8.0. This Figure is a double
reciprocal plot of data from Fig. 3.5. The values used are
from line (c) in Fig. 3.5, i.e. the rates of $NADP^+$ reduction
in the presence of glucose and vanadate, minus the rates in
the absence of vanadate.
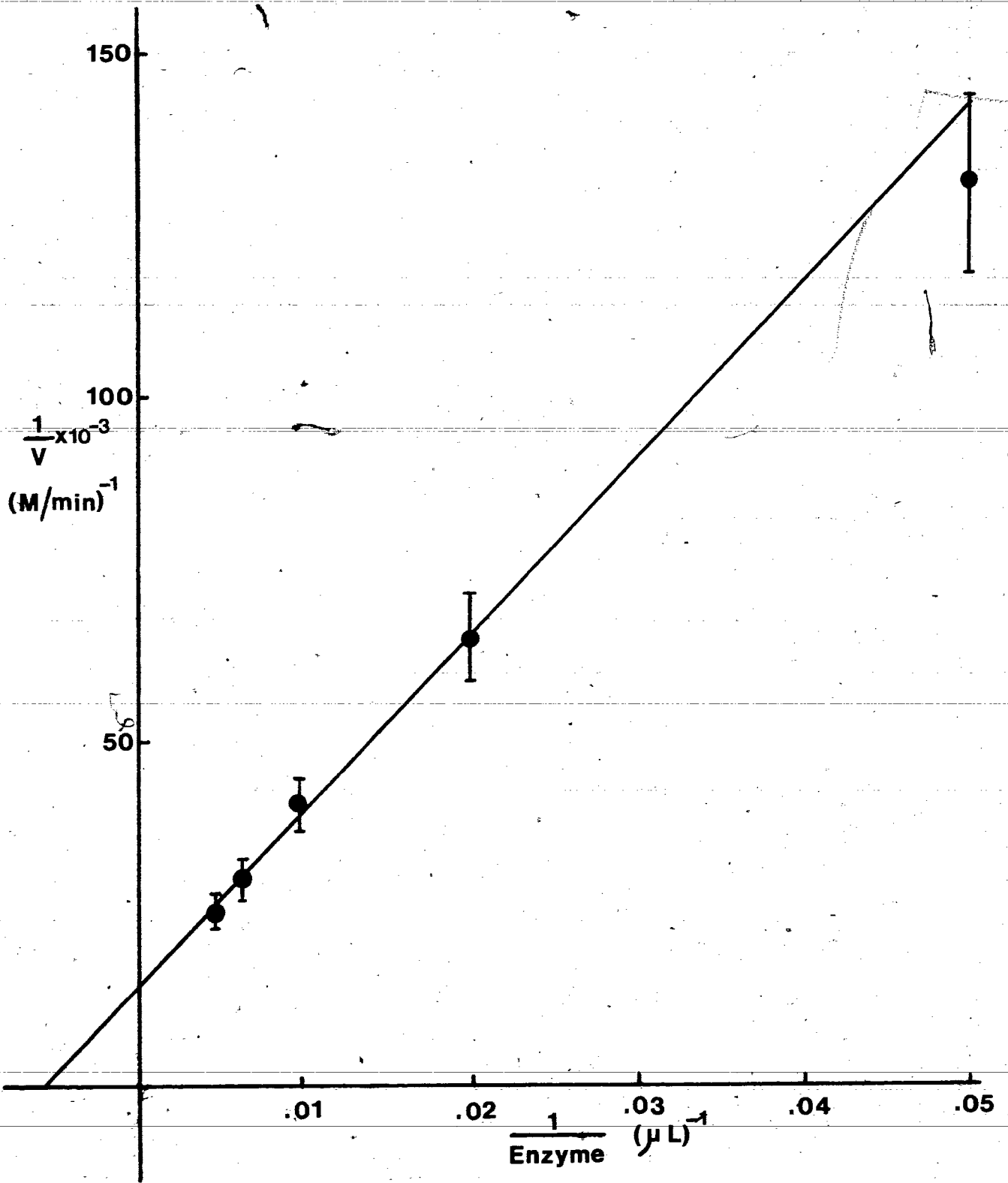
Figure 3.12

The Effect of Different G6-PDH Concentrations on the Rate of G6-V Oxidation at pH 8.2. This Figure is a double reciprocal plot of data from Figure 3.6. The values used are from line (c) in Figure 3.6, i.e. the rates of $NADP^+$ reduction in the presence of glucose and vanadate, minus the rates in the absence of vanadate.
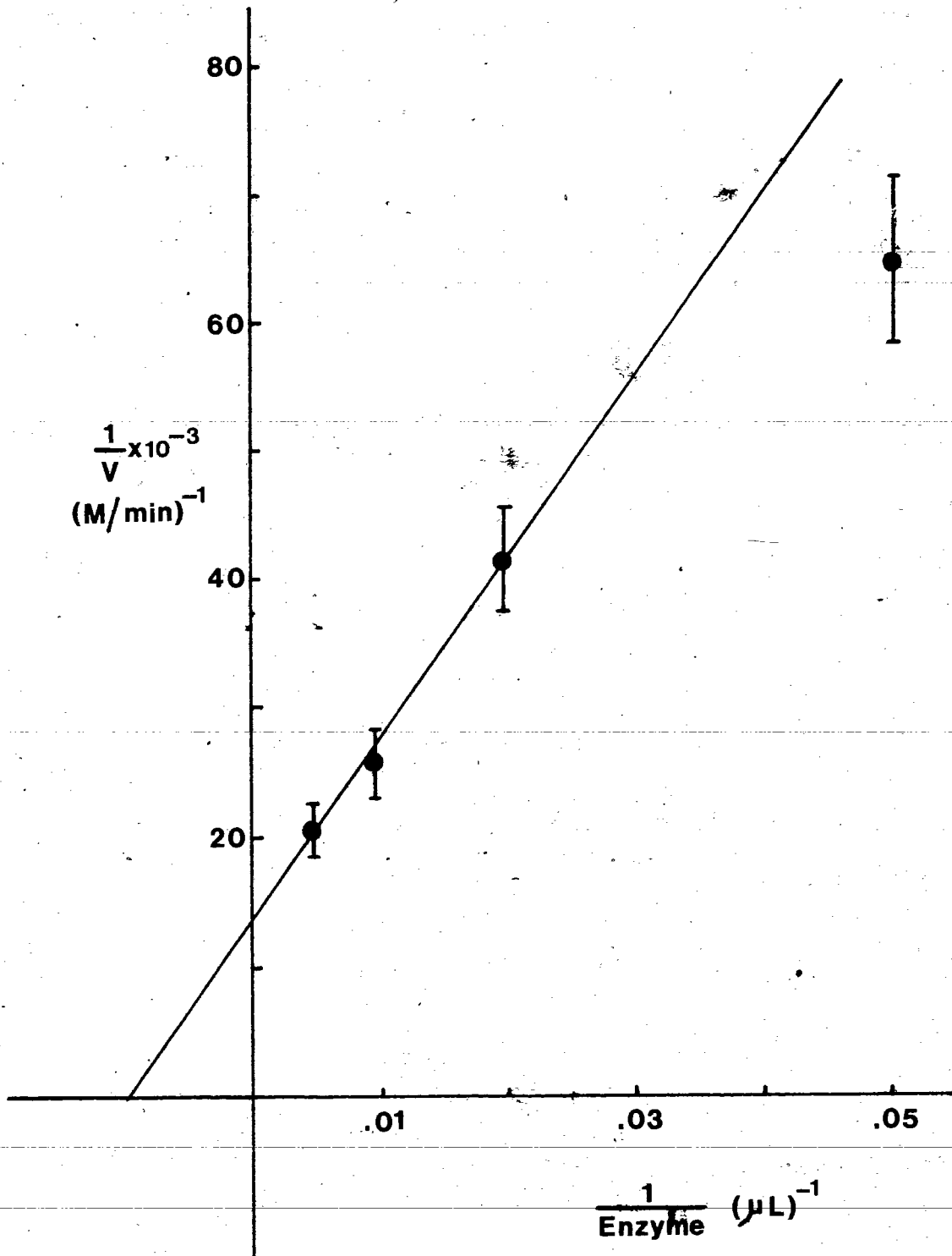
Figure 3.13

The Effect of Different G6-PDH Concentrations on the Rate of G6-V Oxidation at pH 8.4. This Figure is a double reciprocal plot of data from Figure 3.7. The values used are from line (c) in Figure 3.7, i.e. the rates of $NADP^+$ reduction in the presence of glucose and vanadate, minus the rates in the absence of vanadate.
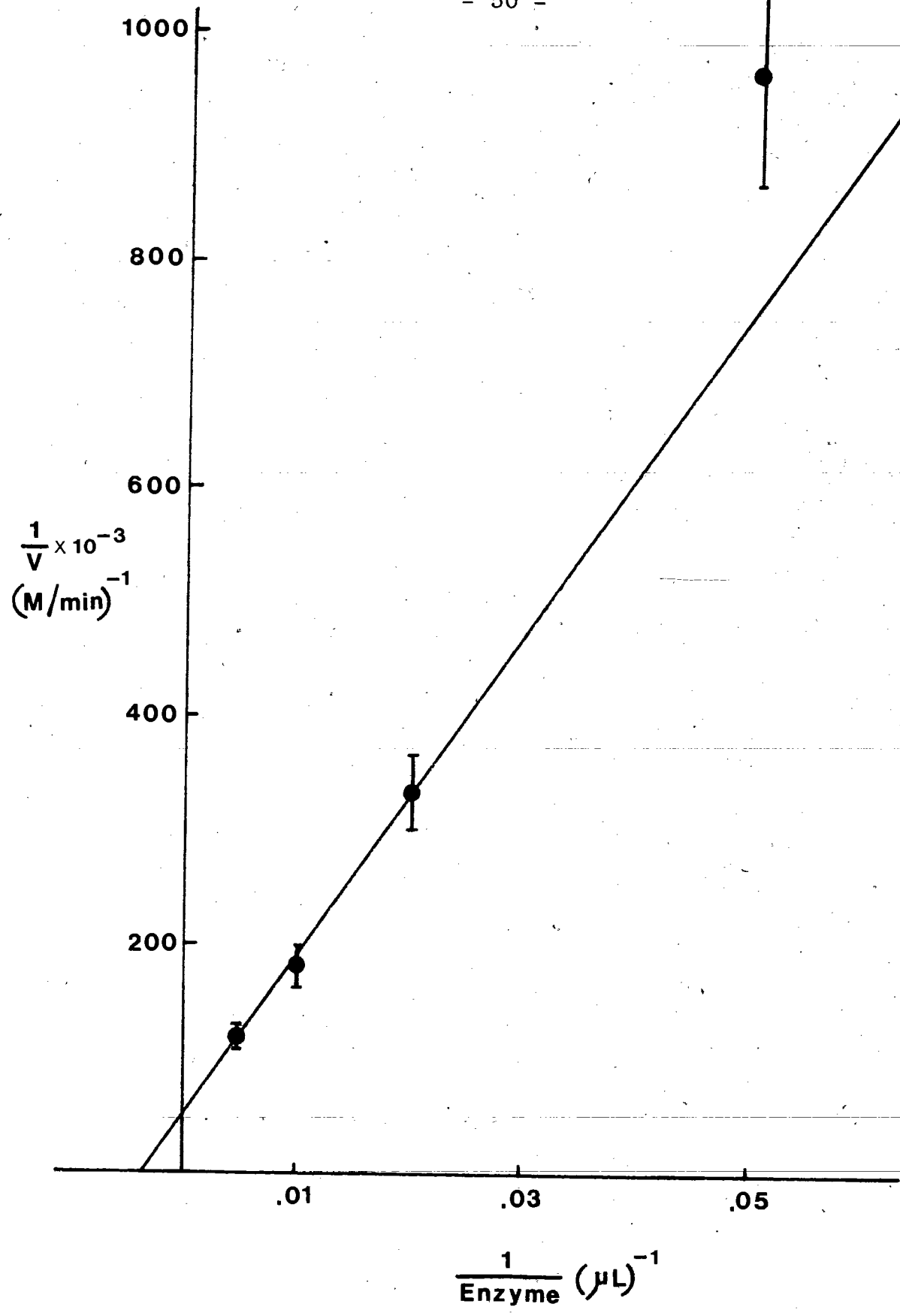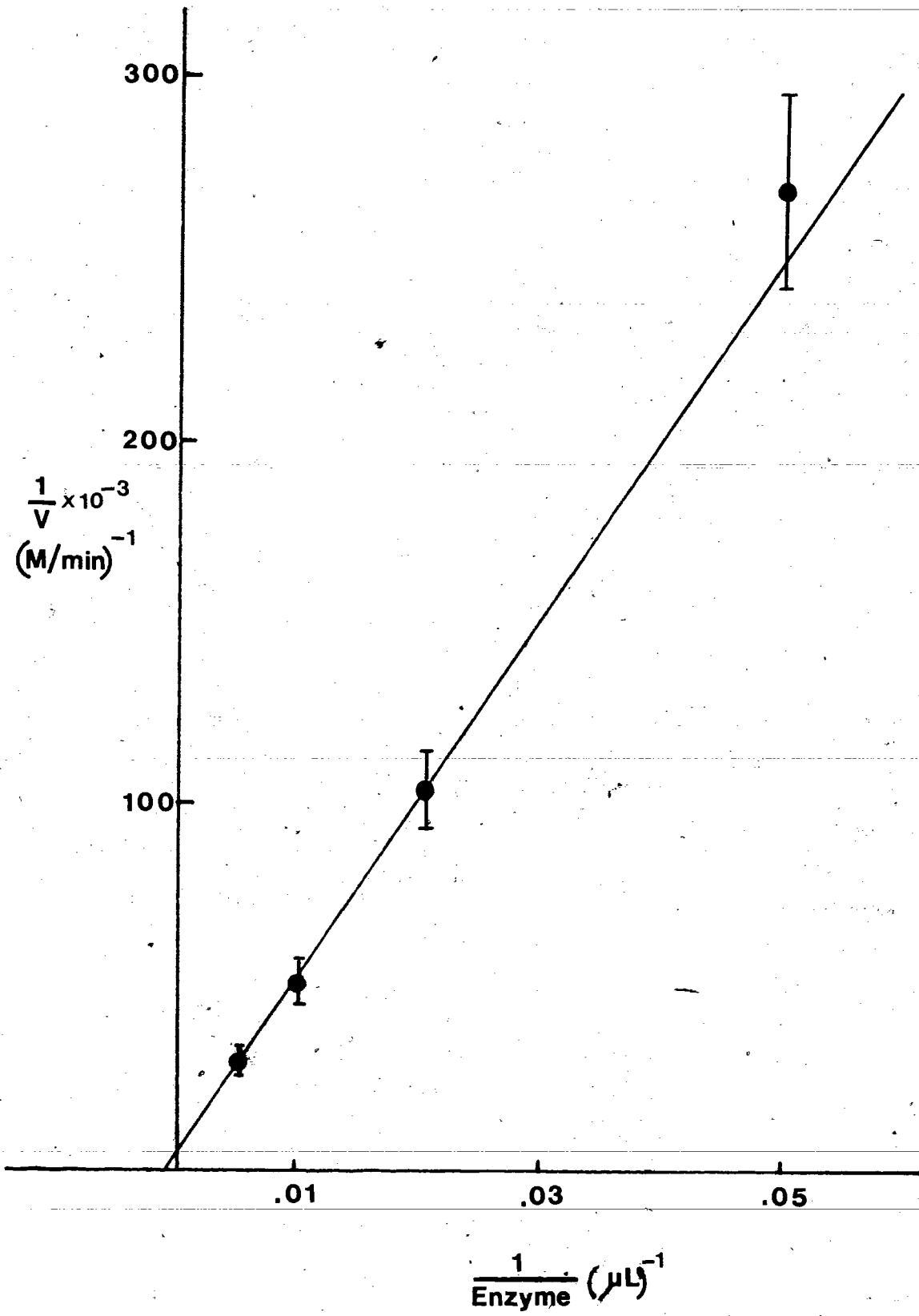
Figure 3.14

The Effect of pH on the Rate of G6-V Formation. The apparent second order rate constants for G6-V formation were calculated from the data shown in Figures 3.9 - 3.13 using equation 3.16.
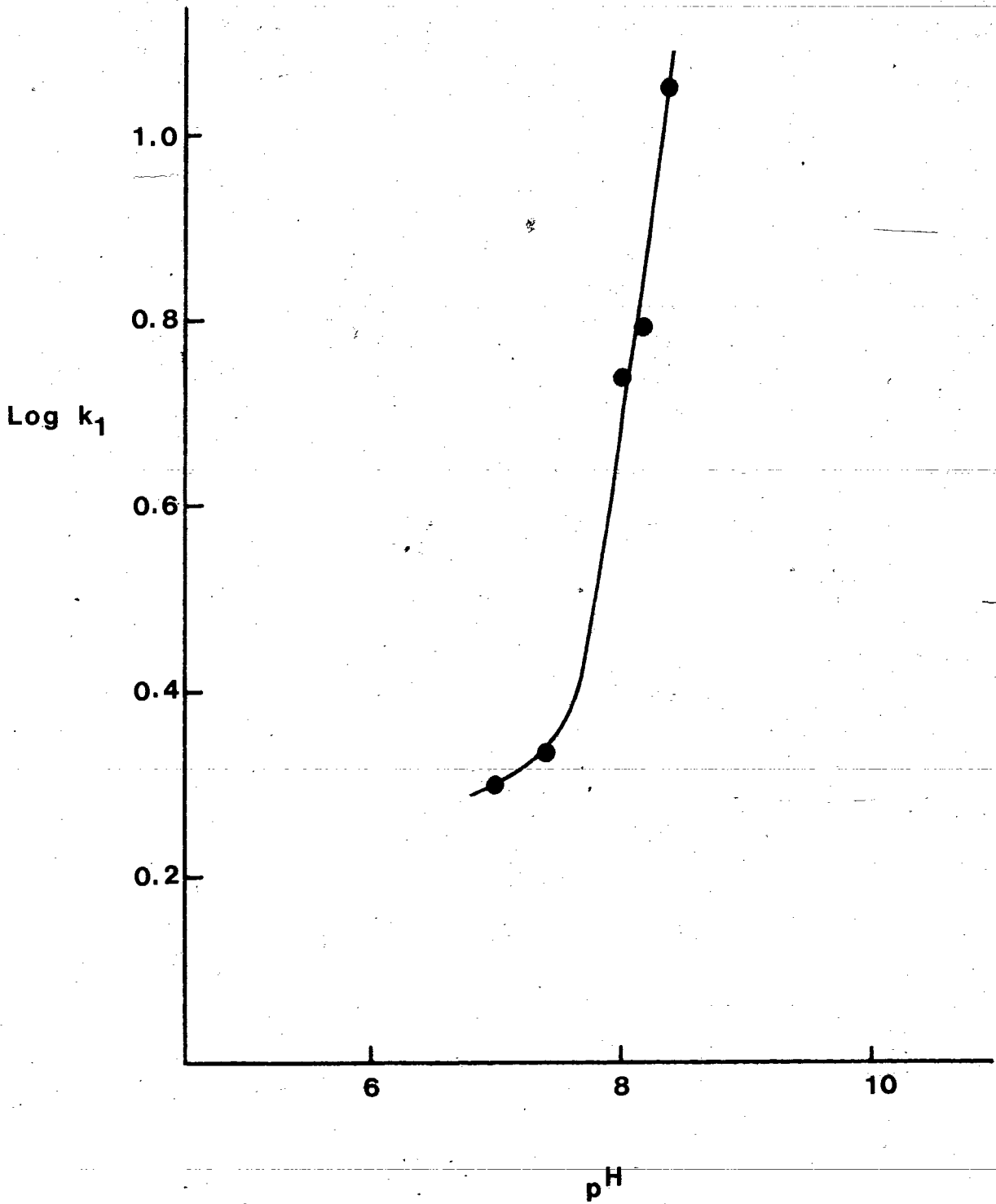
TABLE 3.1

The rate constant (k_1) values for G6-V ester formation at different pH values.

pH	k_1 $(M^{-1}s^{-1})$*
7.0	1.9
7.4	2.2
8.0	5.6
8.2	6.2
8.4	11

* Obtained from plots of $\dfrac{1}{V}$ vs. $\dfrac{1}{[E]}$ (Figures 3.9 - 3.13).

TABLE 3.2

The rate constant (k_1) values for formation of G6-V ester at different vanadate and glucose concentrations, pH 8.0.

$[V_i]$ M	$[G]$ M	$k_1 (M^{-1}s^{-1})^*$
1.0×10^{-4}	5.0×10^{-4}	3.9
2.0×10^{-4}	5.0×10^{-4}	3.4
3.0×10^{-4}	5.0×10^{-4}	2.8
2.0×10^{-4}	1.0×10^{-3}	3.3

* Obtained from plots of $\dfrac{1}{V}$ vs. $\dfrac{1}{[E]}$ (Figures 3.1 - 3.2).

decreases slightly as the concentration of vanadate increases. For example, at 1.0×10^{-4}M vanadate concentration, k_1 was 3.9 $M^{-1}s^{-1}$, while at 3.0×10^{-4}M vanadate it was 2.78 $M^{-1}s^{-1}$ (see Table 3.2). This could be rationalized by increased polymerization of vanadate as vanadate concentration increases, resulting in a decrease in the concentration of monomeric vanadate. This explanation was supported by calculating the percentage decrease of the monovanadate species in solution as the vanadium concentration increases, using the value of K_{eq} (which is equal to $10^{-3.2}$ (21)) for vanadate dimerization according to equation (3.18) and comparing it to the percentage decrease in k_1 at the same concentrations.

$$2 \; HVO_4^{2-} \; \rightleftharpoons \; HV_2O_7^{3-} \; + \; {}^-OH \qquad \qquad (3.18)$$

$$K_{eq} \; = \; \frac{[HV_2O_7^{3-}][{}^-OH]}{[HVO_4^{2-}]^2} \qquad \qquad (3.19)$$

Substituting the value of $K_{eq} = 10^{-3.2}$ and the $[{}^-OH] = 10^{-6}$ at pH 8.0, therefore,

$$\frac{[HV_2O_7^{3-}]}{[HVO_4^{2-}]^2} \; \simeq \; 10^3 M^{-1} \qquad \qquad (3.20)$$

The total vanadium concentration will be:

$$V_{i_{tot}} = \left[HVO_4^{2-} \right] + 2 \left[HV_2O_7^{3-} \right]$$

Then

$$\left[HV_2O_7^{3-} \right] = \frac{V_{i_{tot}} - \left[HVO_4^{2-} \right]}{2}$$

Substituting for the $[HV_2O_7^{3-}]$ into equation (3.19)

$$2K_{eq}' \left[HVO_4^{2-} \right]^2 + \left[HVO_4^{2-} \right] - V_{i_{tot}} = 0 \qquad (3.21)$$

From equation (3.21), the concentration of the monoprotonated monovanadate species could be calculated as follows:

$$HVO_4^{2-} = \frac{-1 + \sqrt{1 + 8K_{eq}' V_{i_{tot}}}}{4 K_{eq}'}$$

Thus, the equilibrium concentrations of HVO_4^{2-} in 1.0×10^{-4} and 3.0×10^{-4} M vanadate solution at pH 8.0 are 8.54×10^{-5} and 2.11×10^{-4} M, respectively. This means that about 85.5 and 70.3% of the total vanadate are expected to exist as the monomeric form at 1.0×10^{-4} and 3.0×10^{-4} M vanadate concentrations, respectively. It is clear that as the concentration of vanadate increases threefold, the monovanadate species decreases by about 15.2%.

The values of k_1 were found to be several orders of magnitude larger than those reported for the reaction of glucose with arsenate and phosphate (16). For example, at pH 7.4, k_1 for G6-V formation was 2.16 $M^{-1}s^{-1}$, where the corresponding second order rate constants for arsenate and phosphate ester fromation were 6.3×10^{-6} and about 9.0×10^{-11} $M^{-1}s^{-1}$, respectively. The change in the rate of glucose oxidation in the presence of arsenate with changing enzyme concentration, using 4.0×10^{-4} M arsenate is shown in Figure 3.15 It is clear from the figure that only a slight activation of glucose oxidation by As_i was observed compared to that by vanadate at similar concentrations, Figure (3.5).

Calculation of the rate of G6-As oxidation at different As_i concentrations (4.0×10^{-4} to 3.0×10^{-1} M) using the published values of the kinetic constants (k_1, k_2 and k_{cat}/K_m) gave very small values which were negligible compared to the experimental values for the glucose oxidation rate. On this basis, it was expected that addition of As_i to a reaction mixture containing glucose and the enzyme would not increase the rate of glucose oxidation by glucose 6-arsenate formation. However, the small increase in the rate of the reaction on addition of As_i (which is shown in Figure 3.15) could be due to activation of the enzyme by arsenate, since an inhibition constant, K_i, of 12 mM for As_i with respect to G6-P was previously reported (16). This is consistent with binding of As_i at the phosphate binding domain of the catalytic site, which would be expected to inhibit catalysis of G6-P oxidation by the enzyme, but might enhance glucose oxidation. That glucose alone can bind at the catalytic site is indicated by the fact that the enzyme catalyzes its oxidation, and that it is a competitive inhibitor with respect to G6-P. No such inhibition was observed by vanadate with respect to G6-P at the vanadate concentrations used in these experiments, in agreement with published work (12).

Figure 3.15

The Effect of Different G6-PDH Concentrations on the Rate of $NADP^+$ Reduction in the presence of 1.0×10^{-3} M Glucose and 4.0×10^{-4} M arsenate at pH 8.0. Procedures and conditions were as described with Figure 6.1, except that the pH was 8.0, vanadate was not present, 4.0×10^{-4} M arsenate was present, and the concentration of the G6-PDH stock solution was 2.0 mg/mL. The points represent: (a) measurements of the rates of $NADP^+$ reduction in the presence of glucose without arsenate (solid circles), and (b) similar measurements in the presence of both glucose and arsenate.
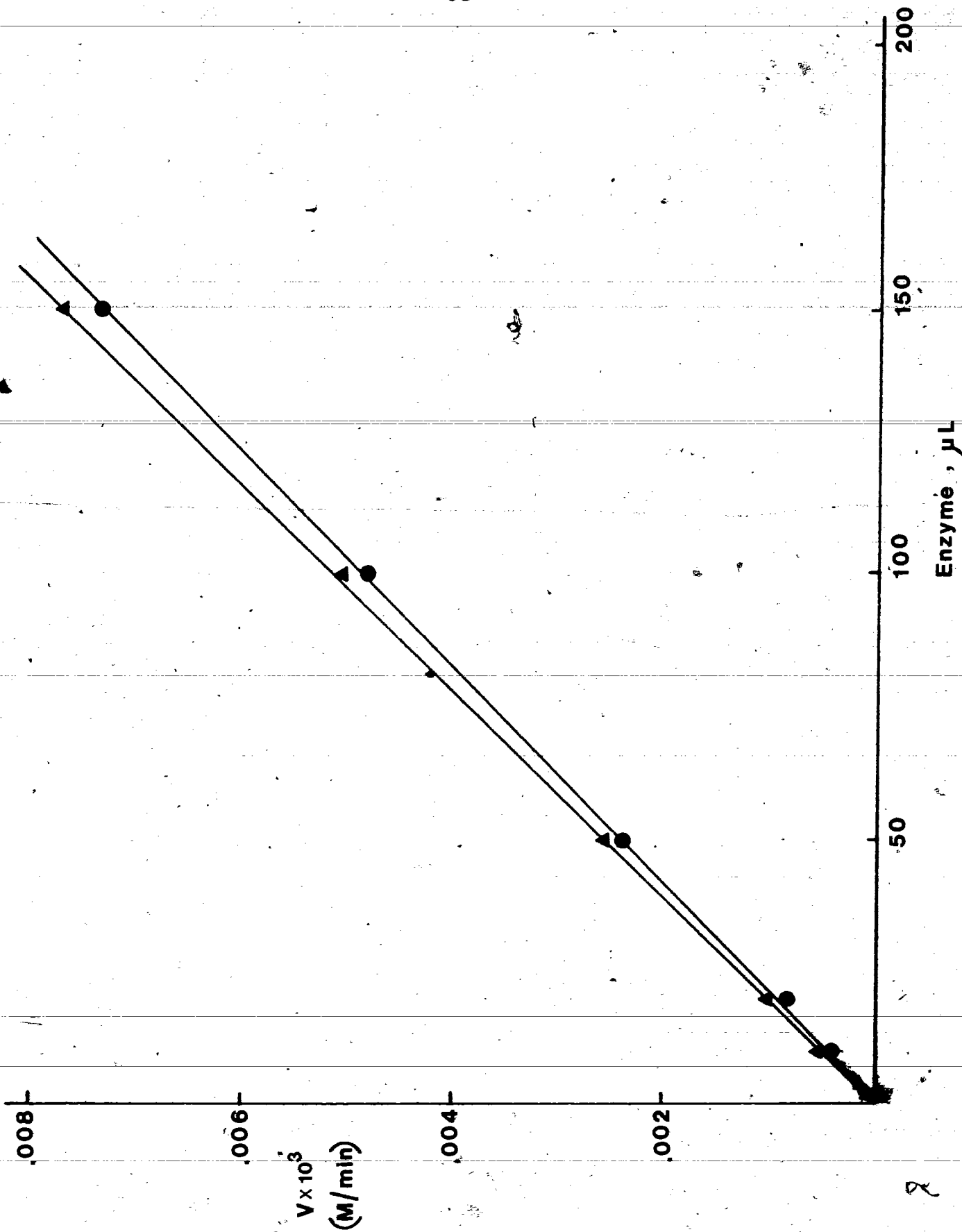
III.5   Attempts to Determine Directly the Concentration of G6-V

Several attempts were made to measure the concentration of G6-V in solutions containing glucose and V_i, using the method reported by others for determining G6-As (16). Briefly, the approach was as follows: enzyme was added to a solution containing glucose, V_i and $NADP^+$. In the case of arsenate ester, essentially, all of the G6-As was consumed in a "burst" reaction. Subsequent formation of G6-As was slow, so the burst phase was clearly separated from the subsequent slow phase of $NADP^+$ reduction.

This approach could not be used successfully with the vanadate system for two reasons:

a)   The facile oligmerization of V_i at concentrations above about 10^{-3} M made it impossible to achieve formation of an equilibrium concentration of G6-V which could be readily detected in a burst.

b)   The nonenzymic formation of G6-V is so fast that the separation between burst and subsequent slow phases of $NADP^+$ reduction would not be expected to be very clear. No burst of $NADP^+$ reduction was observed in these experiments at the low enzyme concentrations used.

III.6 Comparison of G6-P, G6-As and G6-V as Substrate for G6-PDH

If it were possible to determine the concentration of G6-V during catalysis its oxidation by G6-PDH of, then from the oxidation rate and the G6-V concentration it would be possible to calculate a k_{cat}/K_m ratio for G6-V as a substrate for G6-PDH, using the rate equation (equation 3.11). However, as indicated in Section III.5, it has not thus far been possible to measure the concentration of G6-V under any conditions. Alternatively, if a value for the rate constant, k_2, for hydrolysis of G6-V were known, it would be possible to calculate k_{cat}/K_m from the horizontal intercept of $1/V$ vs. $1/[G6-PDH]$ plot (horizontal intercept = $-k_{cat}/K_m k_2$). However, k_2 has not been determined.

It is perhaps worthwhile to make an estimate of the k_{cat}/K_m ratio for G6-V as a substrate for G6-PDH for comparison with the k_{cat}/K_m ratio for G6-As and G6-P, because it is the k_{cat}/K_m ratio which determines (or is the best indication of) the specificity of an enzyme for a given substrate (19).

Such an estimate can be made if it is assumed that the value of K_{eq} for formation of G6-V from glucose and V_i is similar to that for G6-As formation. This assumption is reasonable as a first approximation in view of the fact that the K_{eq} value for formation of G6-As is the same as that for

G6-P (16). Thus, assuming that K_{eq} for formation of G6-V at pH 7.4 is equal to 4.3×10^{-3} M^{-1}, and using the k_1 value 2.16 $M^{-1}s^{-1}$ (from Table 3.1), one can calculate a value of $k_2 = 5.0 \times 10^2$ s^{-1}. From the horizontal intercept of Figure 3.10 (8.25 $\times 10^{-3}$ μL^{-1}), the fact that the volume of the reaction mixture was 300 μL, and the G6-PDH stock solution was at a concentration of 7.8 mg/mL, one calculates a value of 0.318 $(mg/mL)^{-1}$ for its value of $k_{cat}/K_m k_2$. If $k_2 = 5.0 \times 10^2$ s^{-1}, then $k_{cat}/K_m = 159$ $(mg/mL)^{-1}s^{-1} = 9540$ $(mg/mL)^{-1} \cdot min^{-1}$.

A value of k_{cat}/K_m for G6-P can be calculated from the data in Figure 3.20. The rate of G6-P oxidation is 3.2×10^{-6} $M \cdot min^{-1}$ at 2.6×10^{-5} mg/mL G6-PDH and 5.0×10^{-4} M G6-P. Using a value of 2.4×10^{-4} M for the K_m for G6-P (16), and the Michaelis-Menten equation, a value of 760 $(mg/mL) \cdot min^{-1}$ is calculated. Since there appears to be no case known in which the k_{cat}/K_m ratio for a nonphysiological substrate is larger than that for the physiological substrate (19, 23), the large value estimated above for the k_{cat}/K_m for G6-V may indicate that the value used for the rate constant for G6-V hydrolysis (k_2) is larger than the real value. This, in turn, would indicate that the equilibrium constant for formation of G6-V is larger than the corresponding values for G6-P and glucose 6-arsenate. Values of k_{cat}/K_m for G6-P and G6-As calculated from published work (16) indicate that k_{cat}/K_m for G6-P is 3.4 times that for glucose 6-arsenate.

The units for the k_{cat}/K_m ratio are the same as those of a second order rate constant. The use of the units $(mg/mL)^{-1}.min^{-1}$ in the above calculations is due to the choice of concentration units for the enzyme of 1.0 mg/mL rather than 1.0 M. Such a choice is common, and is essential in cases where the molecular weight of the enzyme is not known.

III.7 Inhibition by Sulphate of Enzymic Oxidation of G6-V

It is well established that sulphate (SO_4^{2-}) inhibits the oxidation of G6-P by the enzyme (G6-PDH) in the presence of $NADP^+$ (24). The study of the effect of sulphate on the present system provided some interesting results as well as additional evidence for G6-V formation. The effect of differ-ent sulphate concentrations ($0.0 - 2.0 \times 10^{-2}$ M) on the rate of glucose oxidation in the presence and absence of vanadate was studied (Figure 3.16). It was found that the rate of glucose oxidation increased with increasing sulphate concen-tration. In the absence of vanadate, the rate started to be independent of sulphate at higher sulphate concentrations, showing saturation behaviour (Figure 3.16). Two possibilities can account for this observation: either sulphate reacted with glucose to form an ester-like compound, which could act as a substrate for the enzyme, or it bound to the enzyme and activated it as a glucose oxidase. A plot of the rate of glucose oxidation vs. sulphate concentration in the presence

Figure 3.16

The Effect of Different Sulphate Concentrations on the Rate of Glucose Oxidation in the Presence and Absence of 2.0×10^{-4} M Vanadate at pH 7.4. Procedures and conditions were as described with Figure 3.1, except that the pH was 7.4, glucose was at 1.0×10^{-3} M, G6-PDH was at 0.12 mg/mL, and sulphate concentrations were as indicated. The points represent: (a) measurements of the rates of NADP^{+} reduction in the presence of glucose without vanadate (solid circles), (b) similar measurements in the presence of both glucose and vanadate (solid triangles), and (c) the difference between (b) and (a) (solid squares).

$\left[SO_4^{2-}\right]$, M

$V \times 10^3$
(M/min)
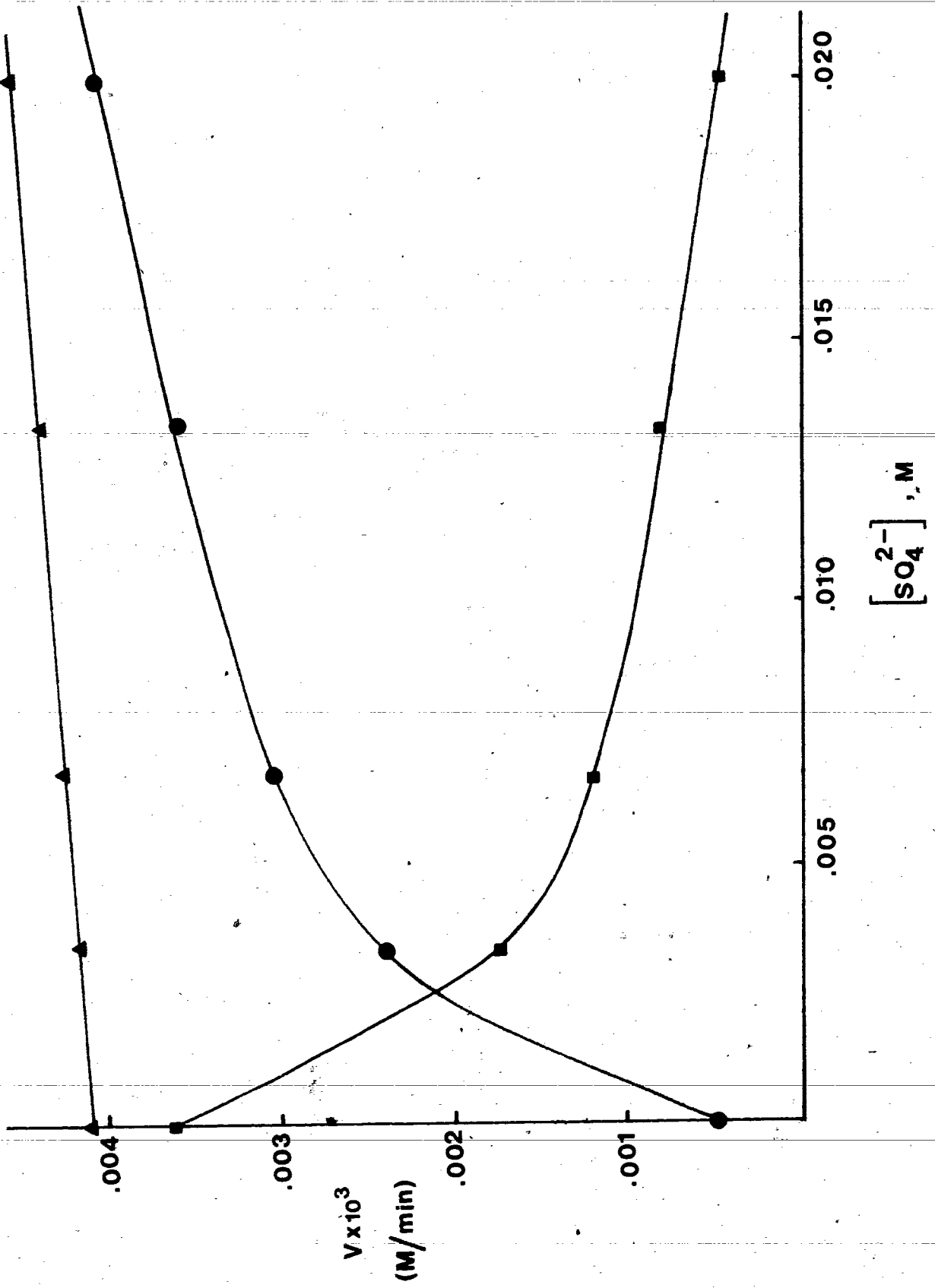
of a fixed vanadate concentration was linear (Figure 3.16).
The plot of the rate of glucose 6-vanadate oxidation (differ-
ence between oxidation rates in the presence and absence of
vanadate) vs. sulphate concentration indicates that the rate
of glucose 6-vanadate oxidation by the enzyme decreased as the
sulphate concentration was increased.

The effect of sulphate on glucose oxidation is most like-
ly due to enzyme activation by sulphate since no curvature was
observed on plotting the difference in the reaction rate in
the presence and absence of sulphate vs. enzyme concentration
(Figure 3.17). Also there was a straight line through the
origin, which is consistent with equation 3.9 obtained by
assuming enzymic activation by the anion. This indicated that
SO_4 binds to the enzyme and activates it as glucose dehydro-
genase. This result is similar (as mentioned above) to the
resulted obtained with arsenate, and this suggeted that arsen-
ate and sulphate are both acting with the same mechanism,
while vanadate a different mechanism. However, very fast
ester formation could not be completely ruled out. The effect
of sulphate on the rate of glucose oxidation (Figure 3.17) was
similar to the effect of arsenate on the same system (Figure
3.15) except that higher activation by sulphate occurred.
This was due to the higher sulphate concentration used (0.01₂
(0.01 M SO_4 vs 4.0×10^{-4} M As_i). This suggested that SO_4

and As_i both had the same effect on the enzyme. Thus, the high activation of glucose oxidation in the presence of SO_4^{2-} was more likely due to enzyme activation by binding of sulphate to the enzyme at the P_i binding domain of the catalytic site.

The above reactions can be summarized by the following scheme:

Figure 3.17

The Effect of Different Enzyme Concentrations on the Rate of Glucose Oxidation in the Presence of 1.0×10^{-3} M glucose with and without 1.0×10^{-2} M Sulphate at pH 7.4. Procedures and conditions were as described with Figure 3.1, except that the pH was 7.4, glucose was at 1.0×10^{-3} M, vanadate was not present, sulphate (where indicated) was present at 1.0×10^{-2} M, and the G6-PDH stock solution was at 2.0 mg/mL. The points represent: (a) measurements of the rates of $NADP^{+}$ reduction in the presence of glucose without sulphate (solid circles), (b) similar measurements in the presence of both glucose and sulphate (solid triangles), and (c) the difference between (b) and (a) (solid squares).
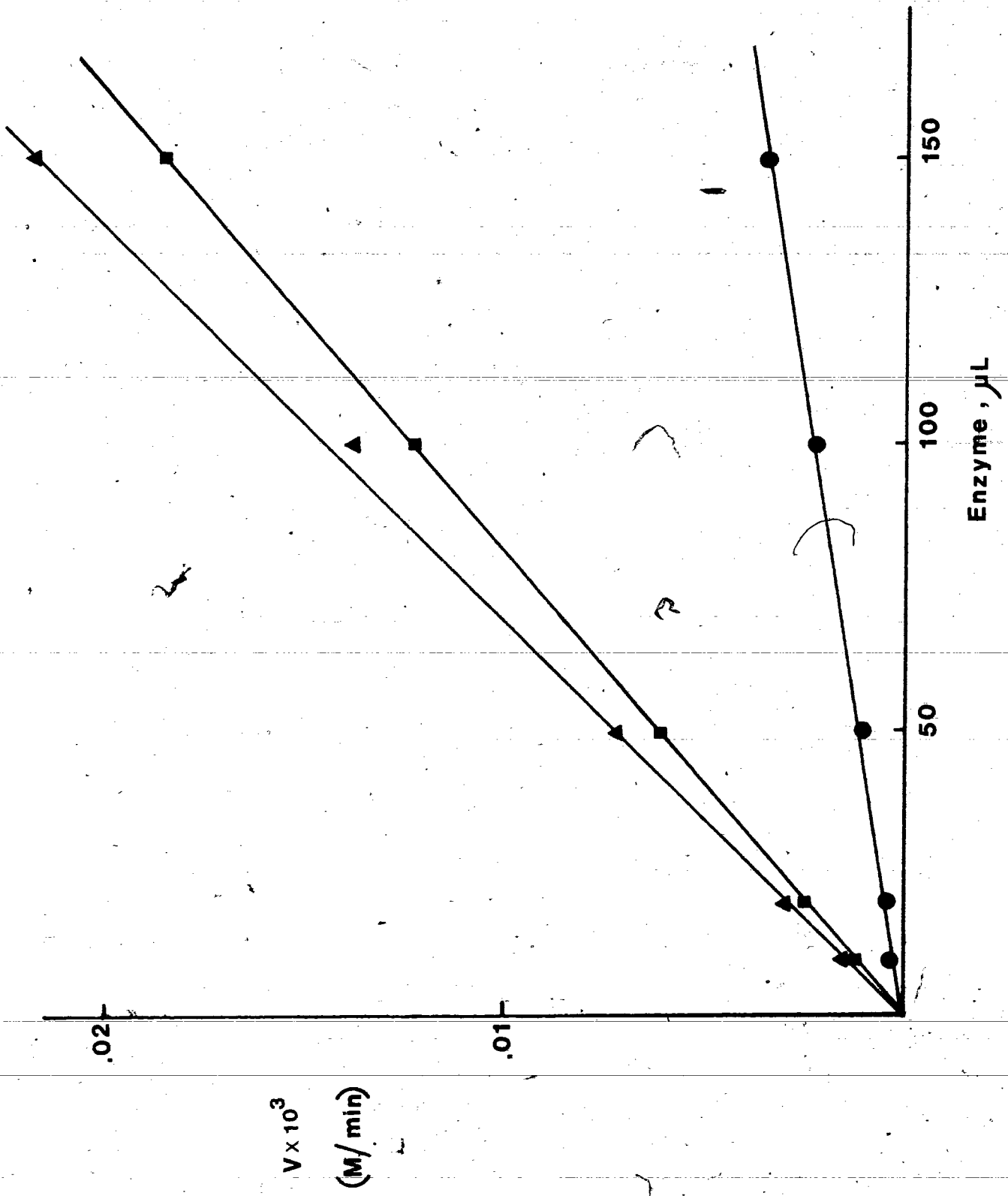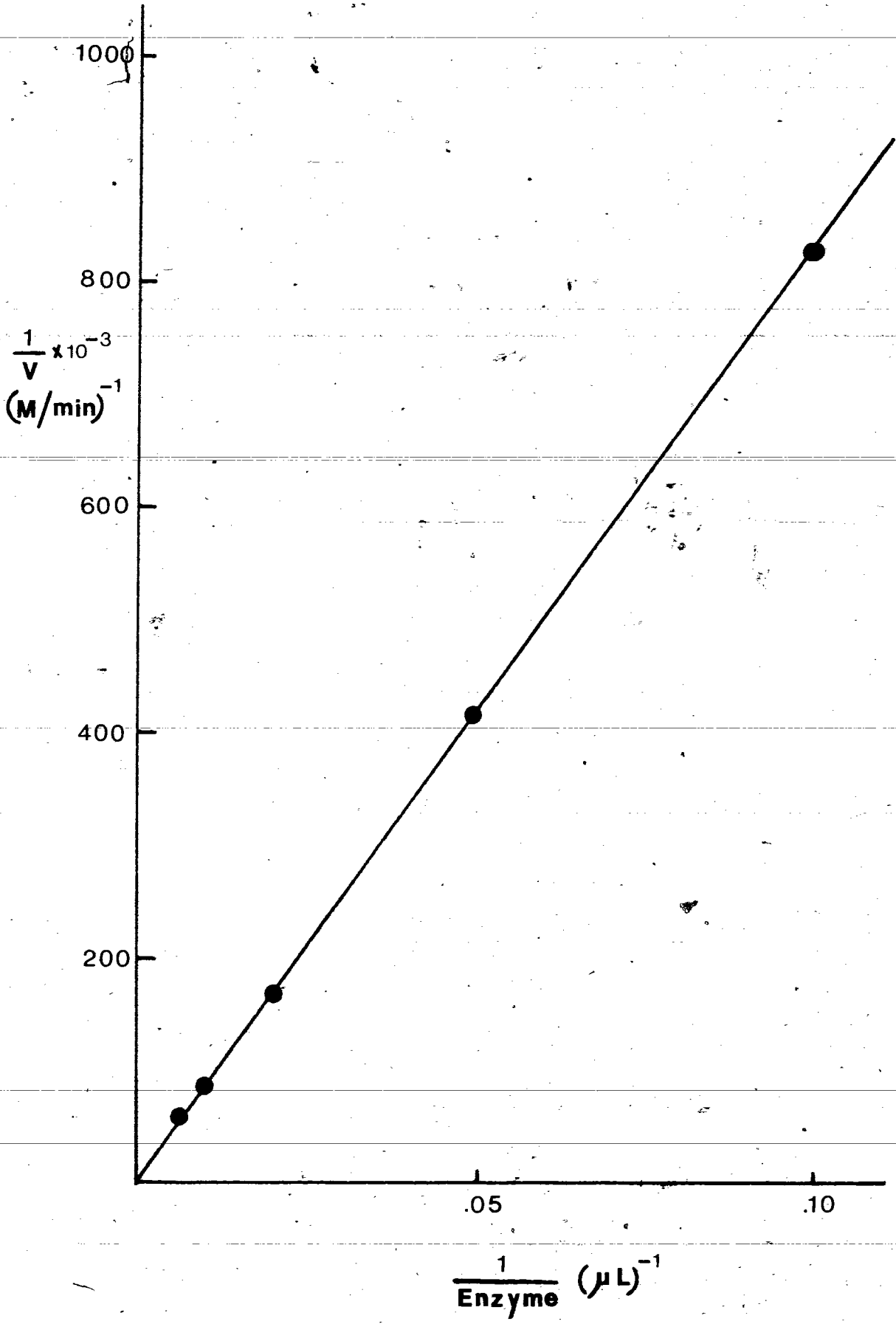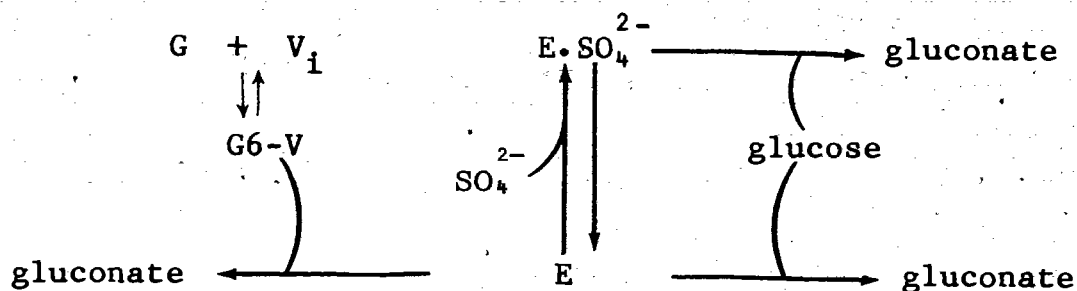
Figure 3.18

The Effect of Different Enzyme Concentrations on the Rate
of Glucose Oxidation in the presence of 1.0×10^{-3}M Glucose
and 1.0×10^{-2}M Sulphate of pH 7.4. The data from Figure 3.17
(solid square) are plotted as a double reciprocal plot.

$$G + V_i$$

$$\updownarrow$$

$$G6\text{-}V$$

$$E\cdot SO_4^{2-} \longrightarrow gluconate$$

$$glucose$$

$$SO_4^{2-}$$

$$gluconate \longleftarrow E \longrightarrow gluconate$$

Scheme 3.1

From Scheme 3.1 one predicts that increasing sulphate concentration will decrease the concentration of the free enzyme present in the reaction mixture which in turn will result in a decrease in the rate of G6-V oxidation catalyzed by the enzyme, as was confirmed by the results of Figure 3.16.

In terms of scheme 3.1, the results shown in Figure 3.16 are rationalized as follows: in the absence of V_i, SO_4^{2-} activates G6-PDH as a glucose oxidase. The levelling off of the SO_4^{2-} activation plot (solid circles, Figure 3.16) is due to the saturation of the SO_4^{2-} binding site, at which point most of the enzyme has been converted to the $E\cdot SO_4^{2-}$ form. In the absence of SO_4^{2-}, V_i causes considerable activation of glucose oxidation, as shown by the difference between the solid circle and the triangle on the vertical axis. However, as shown by the line through the triangles, the activation by

SO_4^{2-} in the presence of V_i is considerably less than in the absence of V_i. The two activating effects are not additive, even though they involve different mechanisms.

The nonadditivity of the vanadate and sulphate activation of glucose oxidation is also shown in Figure 3.18. This figure shows that the activation of glucose oxidation by increasing concentrations of vanadate (lower line, solid circles) is considerably less in the presence of sulphate (upper line, solid triangles). In the absence of other information, the results shown in Figure 3.18 would be consistent with an inhibition by vanadate of the sulphate activation. However, in terms of Scheme 3.1, exactly the converse is true. Adding sulphate decreases the concentration of the enzyme species (E in Scheme 3.1) which catalyzes the oxidation of G6-V.

The lack of any curvature in the velocity vs. enzyme concentration plot for glucose oxidation in the presence of sulphate (Figure 3.17) is also consistent with Scheme 3.1 as mentioned earlier. It was also mentioned that this lack of curvature could be explained by a very fast sulphate ester formation, so that the enzymic oxidation step remained rate-limiting even at the highest enzyme concentrations used. However, if this were the mechanism of sulphate activation of glucose oxidation one would not expect to see the curvature in the velocity vs. sulphate concentration plot (Figure 3.16, solid circles) indicating saturation by sulphate.

Figure 3.19

The Effect of Different Vanadate Concentrations on the

Rate of $NADP^+$ Reduction in the Presence and Absence of

1.0×10^{-2}M Sulphate at pH 7.4. Procedures and conditions

were as described with Figure 3.16, except that the vanadate

concentration was varied and sulphate, when present, was at a

constant concentration of 1.0×10^{-2} M. Also, G6-PDH was at

0.2 mg/mL. The points represent: (a) measurements of the

rates of $NADP^+$ reduction in the presence of glucose, without

sulphate (solid circles), and (b) similar measurements in the

presence of both glucose and sulphate (solid triangles).

$v \times 10^3$ (M/min)

.003

.002

.001

.05

.10

.15

.20

[Vanadate] $\times 10^3$, M
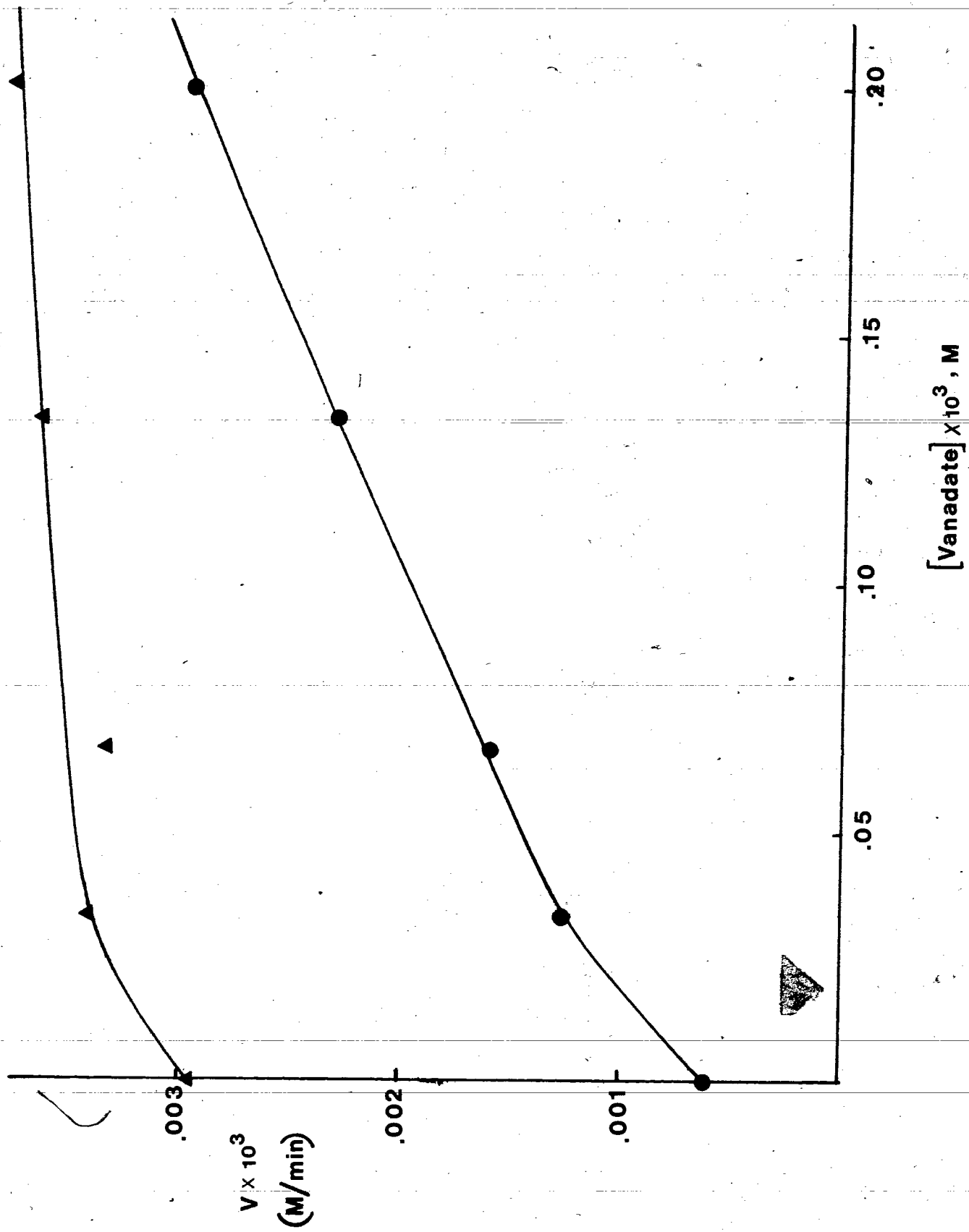
Also, consistent with Scheme 3.1 is the observation that sulphate at the concentrations used in this study, inhibits oxidation of G6-P catalyzed by G6-PDH, while vanadate does not (12). These results are discussed below.

When the effect of SO_4^{2-} concentration was checked on the rate of G6-P oxidation, the same effect as in the case of G6-V was observed (Figure 3.19). The similarity in the effect of sulphate concentration on both the rates of oxidation of G6-P and glucose with vanadate supported the formation of G6-V. Inhibition by 20 mM sulphate was about 87% in case of G6-V and about 52% in case of G6-P. The weaker inhibition of G6-P oxidation is probably because the steady-state concentration of G6-V is lower than that used for G6-P.

When the effect of increasing the concentration of vanadate ($0.0 - 2.0 \times 10^{-4}$ M) on the rate of G6-P oxidation (5.0×10^{-5} M G6-P) was studied, it was found that vanadium has no effect, consistent with published results (12). This does not exclude the possibility that vanadate might bind to the enzyme significantly at higher V_i concentrations. In the case of As_i, an inhibition constant ($K_i = 12$ mM) was reported (16). Thus, if the K_i values for arsenate and vanadate are similar, inhibition by vanadate would be insignificant at the low V_i concentrations used in the present study. Because of the facile oligmerization of V_i, it is not feasible to

Figure 3.20

Inhibition by Sulphate of Catalysis by G6-PDH of G6-P Oxidation at pH 7.4. G6-PDH was present at 2.6×10^{-5} mg/mL, G6-P was at 2.5×10^{-4} M (solid circles) and 5.0×10^{-4} M (solid squares), $MgCl_2$ was at 1.0×10^{-2} M, Tris-acetate (pH 7.4) was at 4.0×10^{-2} M, and sulphate was present at the indicated concentrations. Procedures were as described under methods.

$[SO_4^{2-}]$, M

$V \times 10^3$ (M/min)

.020

.015

.010

.005

.003

.002

.001
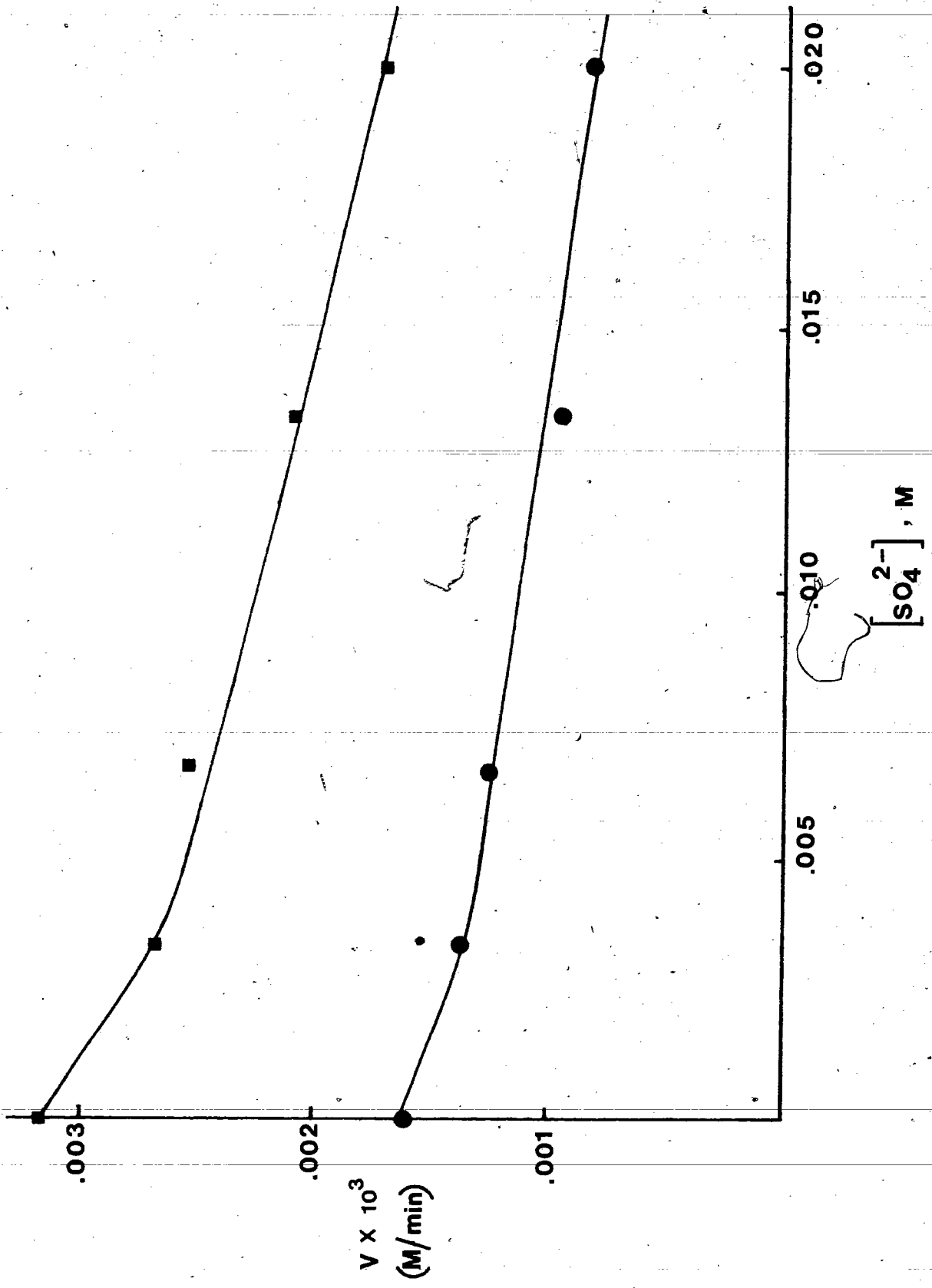
extend the study to higher V_i concentrations; monomeric

vanadate would then no longer be the predominant species.

III.8   Conclusion and Physiological Implications

In this study, it was found that glucose and vanadate

react to yield a product which can be oxidized by $NADP^+$ in a

reaction catalyzed by glucose 6-phosphate dehydrogenase. It

is likely that the product of the reaction of glucose and

vanadate is glucose 6-vanadate. Similar behaviour was observ-

ed previously in the case of phosphate and arsenate. The

values of k_1 were found to be several orders of magnitude

larger than those reported for the reaction of glucose with

arsenate and phosphate (16). A value for k_{cat}/K_m of 9540

$(mg/mL)^{-1} \cdot min^{-1}$ was estimated for glucose 6-vanadate compared

to 990 $(mg/mL)^{-1} \cdot min^{-1}$ for G6-P. This is very likely an over-

estimate, but it is consistent with the hypothesis that G6-V

is a good substrate for G6-PDH.

If the rapid reaction of vanadate with hydroxyl groups is

a general phenomenon, then vanadate esters are expected to

come to equilibrium rapidly under physiological conditions.

If vanadate esters are generally good analogs to phosphate

esters, as is apparently the case with G6-V as a substrate for

G6-PDH, then nonenzymic vanadate ester formation may be physi-
ologically important.

Previous work has been interpreted as indicating that
vanadium compounds have a regulatory role in carbohydrate
metabolism as revealed by their effect on glucose metabolism
in vitro (25). It was found that vanadium has an insulin-
mimetic action in isolated rat adipocytes. The incubation of
adipocytes with orthovanadate stimulated the rate of glucose
oxidation as well as glucose transport. The effects were
dependent on the concentration of vanadate and the time of the
incubation. The authors suggested that vanadate may enter the
cells and is chemically altered and/or sequestered, but in the
course of these intracellular reactions hexose transport is
stimulated. This could be due to the involvement of vanadate
on phosphorylation of key membrane proteins in the insulin-
mimetic effect of vanadate.

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