THE EFFECT OF SPONTANEOUS VANADATE ESTER FORMATION ON REACTIONS CATALYSED BY GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND GLYCEROL-3-PHOSPHATE DEHYDROGENASE.

by

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"The Effect of Spontaneous Vanadate Ester Formation on Reactions Catalysed by Glucose-6-Phosphate Dehydrogenase and Glycerol-3-Phosphate Dehydrogenase"

Author: _____
(signature)

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July 31/1986
(date)

ABSTRACT

Evidence has been obtained that the vanadate ion reacts spontaneously with glucose and glycerol to form compounds which are detected by ^{51}V NMR. These compounds are accepted by glucose-6-phosphate dehydrogenase and glycerol-3-phosphate dehydrogenase respectively as substrates for the reduction of $NADP^+$ and NAD^+, and are thought to be vanadate ester analogues of glucose-6-phosphate and glycerol-3-phosphate, the normal substrates of these enzymes.

The rate constants for the formation of glucose-6-vanadate and glycerol-3-vanadate have been estimated to be 35 $M^{-1}s^{-1}$ and 0.6 $M^{-1}s^{-1}$ respectively. The rate constant for ester formation between vanadate and dihydroxyacetone has also been calculated; it is equal to 50 $M^{-1}s^{-1}$. Dihydroxyacetone vanadate also appears to be a good substrate for glycerol-3-phosphate dehydrogenase for the oxidation of NADH.

The equilibrium constants for the formation of the vanadate esters, as determined by ^{51}V NMR are about 10 times those of the corresponding phosphate esters.

Vanadate activates the oxidation of glucose by $NADP^+$, catalysed by glucose-6-phosphate dehydrogenase, through spontaneous ester formation with glucose. This mechanism has been compared with that of the sulphate activation of this system, which is thought to proceed through the direct binding of sulphate to the enzyme.

To Merle, Edna, and Frances.

ACKNOWLEDGEMENTS

The series of experiments which are reported in this thesis were conducted under the skillful guidance of my supervisor, Michael J. Gresser, to whom I am most grateful. The NMR results were obtained in collaboration with Alan S. Tracey. Dr. Tracey's proficiency in this field provided access to valuable experimental information about the species present in vanadate solutions. In addition, I would like to thank Dr. W. Richards for suggesting the use of 6-deoxyglucose, a compound which supplied evidence for the mechanism of the vanadate activation of the dehydrogenase enzymes.

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INTRODUCTION

Vanadium is a transition metal found in trace amounts in all biological systems. In concentrations of approximately 2 to 4 μM (0.1 to 0.2 ppm.) it is tolerated by mammals (1), and studies have indicated that it may be required to optimize growth (2). Higher concentrations are extremely toxic (3), and have been associated with pathological states such as high blood pressure (4) and renal failure (5). Atmospheric pollution by vanadium compounds is elevated by the burning of fossil fuels. This metal accumulates in organic matter through its tendency to be bound by porphyrin-type rings (6). The increased use of vanadium in recent years as a catalyst in the formation of plastics and as an agent to temper steel has increased the incidence of vanadium related disease among workers in these industries (7). In one study a correlation was found between the concentration of vanadium in the air and the mortality rate from heart disease (8).

In its highest oxidation state (+5), the vanadate anion (VO_4^{3-}) resembles the phosphate anion (PO_4^{3-}) in size and electronic structure (9). It is a potent inhibitor of many enzymes, particularly those which involve phosphorylated intermediates such as the phosphatases (6). The discovery in 1977 that a vanadium compound routinely copurified with the ATP derived from equine muscle, coupled with the evidence that vanadate inhibits the (Na^+, K^+) ATPase (10), led to the suggestion that this ion might be required for the regulation of this enzyme.

The goal of this research has been to study the interaction of vanadate with several common biomolecules and enzymes in order to understand how

this anion may be exerting its effects. The procedure was suggested by the following considerations.

The behavior of another phosphate analogue, arsenate (AsO_4^{3-}), has been studied extensively with respect to its effect on enzymic reactions. The formation of an ADP-arsenate anhydride and its subsequent rapid hydrolysis is thought to be the mechanism by which this ion uncouples oxidative phosphorylation in mitochondria (11). In addition, it has been reported that arsenate esters can form spontaneously and be accepted by the enzymes glucose-6-phosphate dehydrogenase (12) and glycerol-3-phosphate dehydrogenase (13) in lieu of their normal substrates, glucose-6-phosphate and dihydroxyacetone phosphate. Finally, it has been shown that arsenate and vanadate activate the oxidation of glyceraldehyde-3-phosphate catalysed by glyceraldehyde-3-phosphate dehydrogenase, apparently by the same mechanism (14). Arsenate and vanadate uncouple the oxidative phosphorylation of glyceraldehyde-3-phosphate by substituting for phosphate in the phosphorylation step. The products containing arsenate or vanadate are rapidly hydrolysed, their decomposition driving the reaction forward. The phosphorylated product is stable, its accumulation leads to equilibration.

Given the evidence that arsenate can react with hydroxyl groups on molecules such as glucose and dihydroxyacetone to form esters that are analogous to phosphate esters, it seems reasonable to ask if vanadate could behave similarly. It was also of interest to determine whether, as reported in the case of arsenate, these vanadate esters could be accepted as substrates by the dehydrogenase enzymes mentioned above. A description of the experiments designed to answer these questions, their results and implications forms the basis of this thesis.

EXPERIMENTAL PROCEDURE

Solution Preparation

The method used to prepare the solutions for the experiments is recorded in Table I. The solvent, except where otherwise indicated, was 50 mM Tris-acetate, pH 7.4. This was prepared by dissolving 6.05 g of Trizma base (Sigma, Reagent Grade) in distilled water to a total volume of 1.0 L; the pH was adjusted to 7.4 with glacial acetic acid (Fisher) before the final addition of water.

The pH of each solution used in the experiments was measured and adjusted to 7.4 when necessary with acetic acid or NaOH (Fisher).

The commercial preparations of the enzymes were subjected to the purification procedures described in the following section.

Enzyme Preparation

Each enzyme preparation was purified by one of two methods, a) dialysis (for large quantities of enzyme ≈10 mg) or b) desalting on a Sephadex column (for small quantities of enzyme ≈2 mg), using the procedure of Penefsky (15).

a) Dialysis

The commercial enzyme preparation which is stored in ammonium sulphate solution was placed in prepared dialysis tubing. The method for preparation of the tubing is described in reference 16. The tubing was covered with 2.0 L Tris-acetate buffer and left to dialyse overnight at 0°C with continuous stirring. After 8 hours the tubing was placed in fresh buffer and left to continue dialysis for 4 hours at 0°C.

b) Desalting on a Sephadex Column.

Sephadex granules (Sigma, G50-80, particle size 20-80 μ) were placed in distilled water, 5 g/100 mL, and left to stand for 8 hours. The swollen granules were then placed in a 1 mL plastic syringe fitted with a porous polyethylene plug, drained, refilled and drained again until the volume of drained granules was equal to 1 mL. The tube was then centrifuged in an I.E.C. clinical centrifuge for 1 minute at 3/4 of the maximum speed.

The commercial enzyme preparation was centrifuged at 10,000 rpm for 12 minutes, after which the ammonium sulphate supernatant was removed. The centrifugate was dissolved in Tris-acetate buffer (50 mM, pH 7.4) to its original volume and placed on the top of the prepared Sephadex column (approximately 1 mg of enzyme per 1 mL column). The column was then spun in the clinical centrifuge for 1 minute at 3/4 of the maximum speed.

The eluent from the Sephadex column, or the purified enzyme inside the dialysis tubing, was subjected to a protein determination by the Lowry method (17).

TABLE I

Concentrations and method of preparation for reagents used in the experiments represented by Figures 1 - 23.

REAGENT	METHOD OF PREP. STOCK SOLN. solute/solution (g/mL)	STOCK CONC. (M)	VOL. OF STOCK SOLN. (mL)	TOTAL VOL. (mL)	EXPERIMENTAL CONCENTRATION (M)	REFERENCE FIGURE
D-Glucose	0.180/100 [b]	0.010	0.030	0.30	1.0×10^{-3}	1,2,4,6,7
(Sigma, R.G. [a])	0.270/50	0.030	0.005	0.30	5.0×10^{-4}	14,15
6-Deoxy-D-glucose (Sigma, R.G.)	0.164/10	0.10	0.100	1.0	1.0×10^{-2}	8
D-Glucose-6-phosphate (Sigma, R.G.)	0.0280/100	0.001	0 - 0.070	1.0	0 - 7.0×10^{-5}	16,17
Methyl-β-D-glucopyranoside (Sigma, anhydrous)	3.0/10 (Tris-Cl, 20 mM), pH 6.9 [c]	-	-	10	1.55	10
Glycerol	23.0/50	5.0	0.005	0.40	6.25×10^{-2}	20,22
(BDH Chemicals, analytical reagent)	0.92/10 (Tris-Cl, 20 mM), pH 7.2 [c]			10	1.0	11
Dihydroxyacetone (Sigma, R.G.)	0.090/10	0.10	0.005	0.40	1.25×10^{-3}	21,23
	0.90/10 (Tris Cl, 20 mM), pH 7.5 [c]				1.0	12

[a] R.G. refers to reagent grade.

[b] The solvent is 50 mM Tris-acetate, pH 7.4, unless otherwise specified.

[c] pH adjusted with 1.0 M HCl.

TABLE I - continued

REAGENT	METHOD OF PREP. STOCK SOLN. solute/solution (g/mL)	STOCK CONC. (M)	VOL. OF STOCK SOLN. (mL)	TOTAL VOL. (mL)	EXPERIMENTAL CONCENTRATION (M)	REFERENCE FIGURE
β-NADP$^+$ • $3H_2O$ (Sigma, R.G.)	0.0830/10 [d]	0.010	0.030	0.30	1.0×10^{-3}	1,2,4,6,7
	0.0830/10 [d]	0.010	0.100	0.30	1.0×10^{-3}	14,15
	0.0497/3 [d]	0.020	0.015	1.0	1.0×10^{-3}	8,16,17
β-NAD$^+$ (Sigma, Grade III)	0.0330/10 [d]	0.005	0.040	0.40	5.0×10^{-4}	20,22
β-NADH (reduced) (Sigma, Grade III, from yeast)	0.0350/10 [d]	0.0028 [e]	0.015	0.40	1.05×10^{-4}	21,23
$MgCl_2 \cdot 6H_2O$ (Anachem, R.G.)	0.510/25	0.10	0.030	0.30	1.0×10^{-2}	1,2,4,6,7
	0.510/25	0.10	0.100	1.0	1.0×10^{-2}	8,
	1.020/25	0.20	0.015	0.30	1.0×10^{-2}	14,15,16,17
Na_2SO_4 (BDH Chemicals)	1.065/25	0.30	0 - .025	0.30	0 - 2.5×10^{-2}	2,4,6,7
	1.780/25	0.50	0 - .025	1.0	0 - 1.25×10^{-2}	8

[d] pH adjusted to 7.4 with 1 M NaOH

[e] The concentration was determined by the absorbance reading (against a blank solution) at 340 nm (ε = $6.22\ 10^3\ M^{-1}cm^{-1}$)

TABLE I - continued

REAGENT	METHOD OF PREP. STOCK SOLN. solute/solution (g/mL)	STOCK CONC. (M)	VOL. OF STOCK SOLN. (mL)	TOTAL VOL. (mL)	EXPERIMENTAL CONCENTRATION (M)	REFERENCE FIGURE
Sodium Orthovanadate						
$Na_3VO_4 \cdot 10H_2O$ [f]	0.0350/100	0.001	0 - 0.100	0.30	0 - 3.3×10^{-4}	1,4,7
(Fisher Scientific	0.0350/100	0.001	0 - 0.100	1.0	0 - 1.0×10^{-4}	8
Co., R.G.)	0.0175/50	0.001	0.030	0.30	1.0×10^{-4}	14,15
	0.0350/100	0.001	0.005	0.40	1.2×10^{-5}	20,21,22,23
	3.5/100 (Tris-Cl,[c] 20 mM), pH 7.5	0.10	0.05	10	5.0×10^{-4}	9,10,11,12
		(mg/ml)			(mg/ml)	
D-Glucose-6-phosphate	dialysis	5.1	0.010	0.30	0.17	1,2,4,6,7
dehydrogenase	desalting	10	0.005	1.0	0.05	8
(G6PDH)	dialysis	11.4	0 - 0.210	0.30	0 - 8.0	14,15
(Boehringer-	desalting	0.210	0.005	1.0	6.0×10^{-5}	16,17
Mannheim, from bakers' yeast)						
L-Glycerol-3-phosphate	dialysis	5.3	0 - 0.350	0.40	0 - 4.6	20,22
dehydrogenase	dialysis	5.6	0 - 0.250	0.40	0 - 3.5	21,23
(G3PDH)						
(Boehringer-Mannheim, from rabbit muscle)						

f The formula weight was determined by measuring the absorbance at 260 nm of a dilute solution (2.0×10^{-4} M) of vanadate in water at pH 10.5. At this pH and concentration the principal species in solution is the HVO_4^{2-} ion ($\varepsilon = 3.55 \times 10^3$ $M^{-1}cm^{-1}$). (18).

Rate Measurement

The rate of reduction of $NADP^+$ (or NAD^+) was measured by following the increase in absorbance at 340 nm caused by the formation of the product NADPH (or NADH). The molar extinction coefficient for this product at 340 nm is $6.22 \times 10^3\ M^{-1}cm^{-1}$. The reverse reaction involving the oxidation of NADH was monitored by following the decrease in absorbance at 340 nm.

The procedure for mixing the reactants was similar in all of the experiments. All of the reagents except one, which was required to initiate the reaction, were incubated for 4 minutes at 30°C in the reaction cuvette. The absorbance was measured during the incubation period to verify that no oxidation (or reduction) was taking place. The final reagent was added and mixed by blowing it into the cuvette through capillary tubing, or by syringe injection followed by several inversions of the cuvette to mix the solution. The absorbance reading was taken approximately 5 seconds after the reaction was initiated. This was the minimum time required for the pen to stabilize. The "blow-in" method was used rather than hand mixing by inversion when it was important to record the initial velocity of the reaction, as the latter procedure delayed the initial rate measurement by an additional 10 seconds. Rate determinations were performed in duplicate and were reproducible to 5%.

The Beer-Lambert law relates the change in absorbance to the change in concentration of the chromophore:

$$A = c\varepsilon l$$

A = absorbance

c = concentration of the chromophore, NADPH (or NADH)

ε = the molar extinction constant for NADPH (or NADH) at 340 nm, equal to $6.22 \times 10^3\ M^{-1}cm^{-1}$.

l = the path length of the cell, equal to 1 cm.

The change in absorbance at 340 nm per unit of time was considered to be a measure of the reaction velocity.

Instrumentation

Rate measurements were made on a Cary-17 spectrophotometer which was equipped with a temperature controlled light chamber. The high speed centrifugations required to precipitate the enzyme from its storage solution were performed on a Sorval RC-5B model centrifuge manufactured by Dupont; an I.E.C. CL clinical chemistry centrifuge was used for lower speed spins, as in column preparation and enzyme purification. A Fisher pH meter (model 620) equipped with a Trizma glass/calomel electrode (Sigma E-5003) was used to measure the pH of the experimental solutions. NMR spectra were produced by a Bruker 400 MHz (105 MHz for vanadium) nuclear magnetic resonance spectrometer.

RESULTS AND DISCUSSION

I. Effects of Vanadate and Sulphate on the Enzymic Oxidation of Glucose and 6-Deoxyglucose.

(i) Glucose

Figures 1 and 2 show the activating effect of the addition of vanadate or sulphate respectively to a reaction vessel which contains glucose, $NADP^+$, $MgCl_2$ and glucose-6-phosphate dehydrogenase* (G6PDH) at pH 7.4. G6PDH will accept glucose as a substrate as shown by the low rate of oxidation when vanadate and sulphate are absent. This rate is approximately doubled by the addition of 0.08 mM of vanadate ion or of 3 mM of sulphate ion.

Studies with glucose-6-phosphate, the normal substrate for this enzyme, have shown that sulphate (as well as bicarbonate and phosphate) inhibits the oxidation of this ester by $NADP^+$ (19). It has been suggested that these ions bind to the enzyme at the position which the phosphate group of the bound ester substrate would normally occupy. Thus glucose oxidation would be accelerated if the binding of glucose is more favourable on the enzyme/anion species, and glucose-6-phosphate oxidation would be retarded, as the phosphate moiety on the ester would be obstructed by the anion group bound to the enzyme.

The maximum vanadate concentration used in these experiments is five hundred micromolar. It was necessary to use low concentrations of vanadate in order to maintain this anion in its monomeric form (20). The marked acceleration of glucose oxidation by vanadate, and the fact that at these

* D-Glucose-6-phosphate: NADP 1-oxidoreductase, EC 1.1.1.49

Figure 1

The effect of different vanadate concentrations on the rate of $NADP^+$ reduction in the presence of glucose and G6PDH. The concentrations in the reaction mixture were: 1.0 mM $NADP^+$, 10 mM $MgCl_2$, 1.0 mM glucose, 0.17 mg mL^{-1} G6PDH and vanadate as shown. All reagents were at pH 7.4. The reaction mixture, except for the glucose was incubated at 30°C for 4 minutes, then the reaction was initiated by adding 30 µL of the glucose stock solution and inverting the cuvette eight times to mix the contents. The total volume of the reaction mixture was 0.3 mL. The glucose was added last to prevent a lag in initial velocity which was apparent when the enzyme was not preincubated with $NADP^+$ and $MgCl_2$.

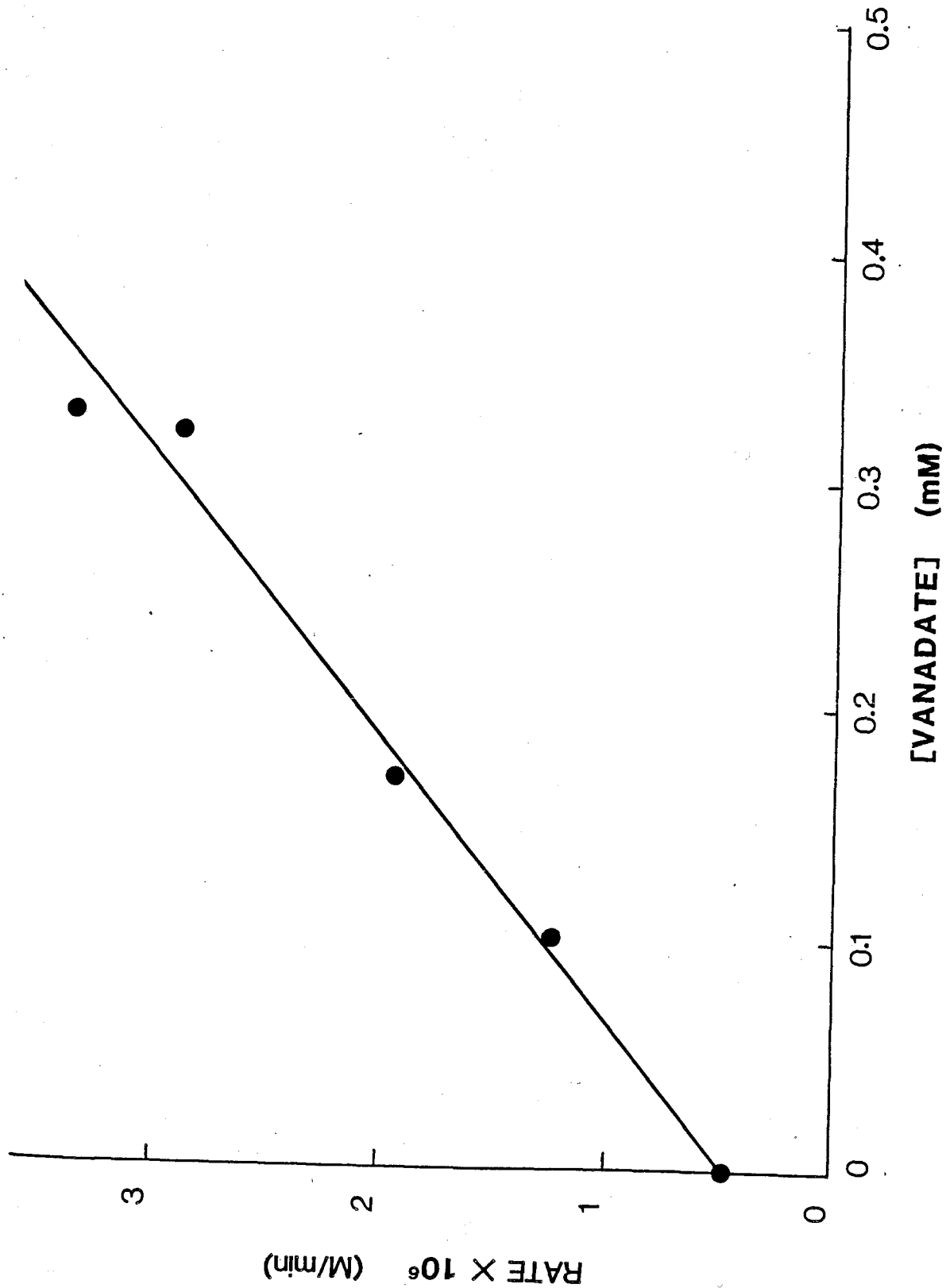
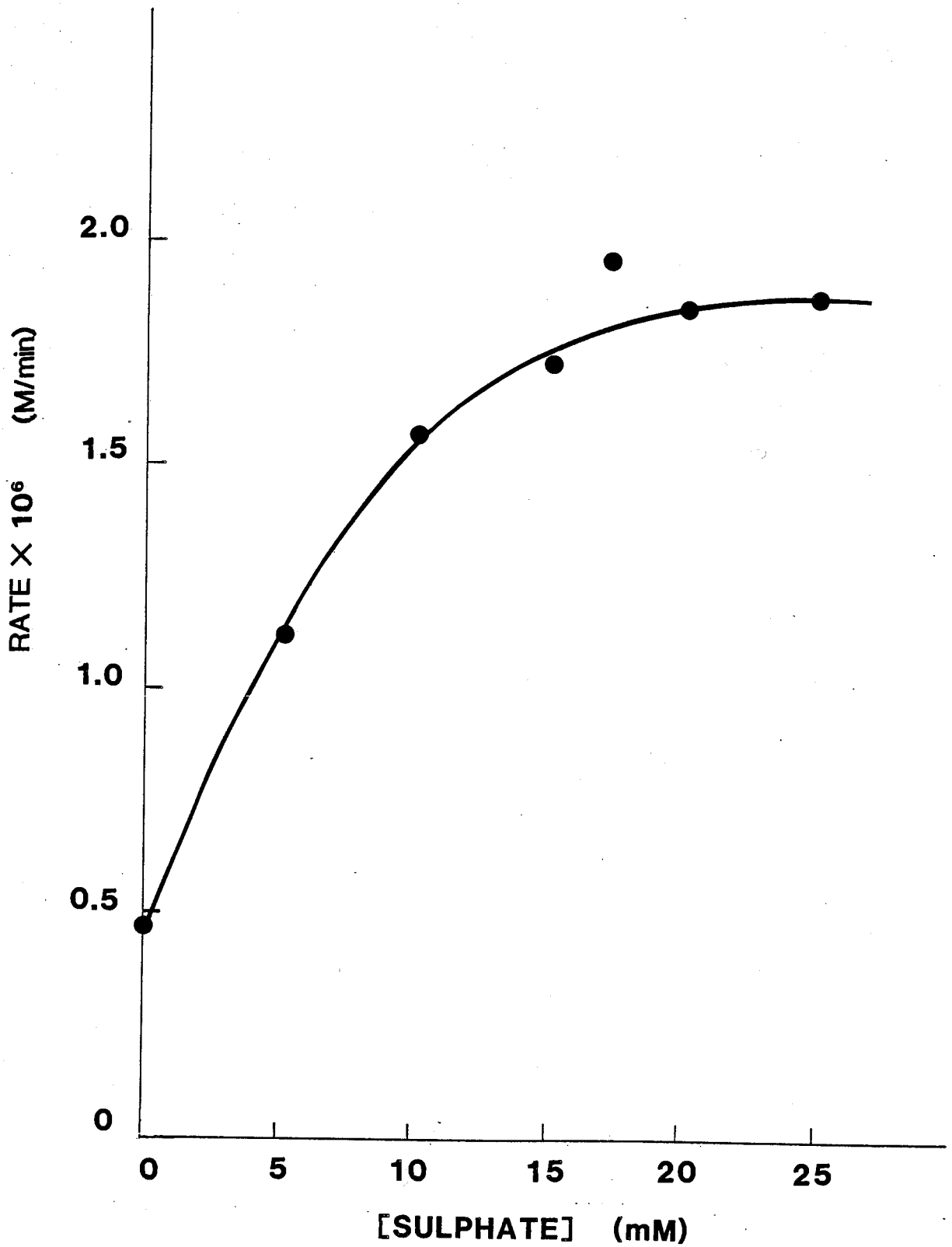

RATE X 10⁶ (M/min)
0
1
2
3
0
0.1
0.2
0.3
0.4
0.5
[VANADATE] (mM)

Figure 2

The effect of different sulphate concentrations on the rate of $NADP^+$ reduction in the presence of glucose and G6PDH at pH 7.4. Procedures and conditions were the same as those described for Figure 1, except that the concentration of sulphate was varied, as shown, and vanadate was absent.


RATE X 10⁶ (M/min)
2.0
1.5
1.0
0.5
0
0
5
10
15
20
25
[SULPHATE] (mM)

concentrations this ion has no effect on the oxidation of glucose-6-phosphate (this was shown by other investigators (21)), indicates that vandate exerts its influence by a mechanism different from that of sulphate. The observed behavior can be explained if an ester, glucose-6-vanadate, could be formed spontaneously by glucose and vanadate (22). This ester would be similar in structure to glucose-6-phosphate, the natural substrate for G6PDH, and might therefore act as a good substrate for this enzyme. Glucose-6-vanadate would then undergo oxidation more readily than glucose. Vanadate at these low concentrations would not be expected to compete significantly with glucose-6-phosphate, consequently it would not inhibit the reaction with this substrate.

The possibility that vanadate and glucose can spontaneously form an ester which is accepted by G6PDH as a substrate is supported by studies with arsenate and glucose (12). The arsenate anion is a structural analogue of phosphate and has been shown to react with glucose to form a product which undergoes oxidation by $NADP^+$ in the presence of G6PDH. The electronic similarity of vanadate to both arsenate and phosphate is reflected by the chemical properties of their oxyacids. Table II compares the size and electron configuration of these three atoms in their highest stable oxidation state (+5). Table III lists the ionization constants for the oxyacids of these elements.

The proposed enzyme/substrate/anion interactions are illustrated in Figure 3. The accelerated oxidation of the upper pathway of Figure 3 is due to the binding of a glucose ester (GX) to the enzyme, where X represents phosphate, arsenate or vanadate. This pathway is not likely to be

TABLE II

Electronic structure and physical properties of phosphate, arsenate and vanadate atoms in their +5 oxidation state(9).

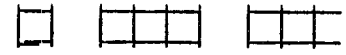
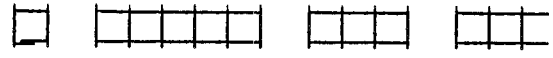
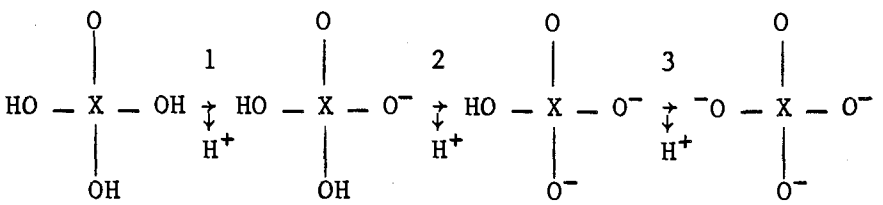
SPECIES	ELECTRON CONFIGURATION		ELECTRO-NEGATIVITY	RADIUS Å
$^{31}_{15}P^{+5}$	[Ne]	 3s 3p 3d	2.1	0.34
$^{75}_{33}As^{+5}$	[Ar]	 3d 4s 4P 4d	2.0	0.47
$^{51}_{23}V^{+5}$	[Ar]	 4s 3d 4P 4d	1.6	0.59

TABLE III

Ionization constants for the oxyacids of phosphorus, arsenic and vanadium.



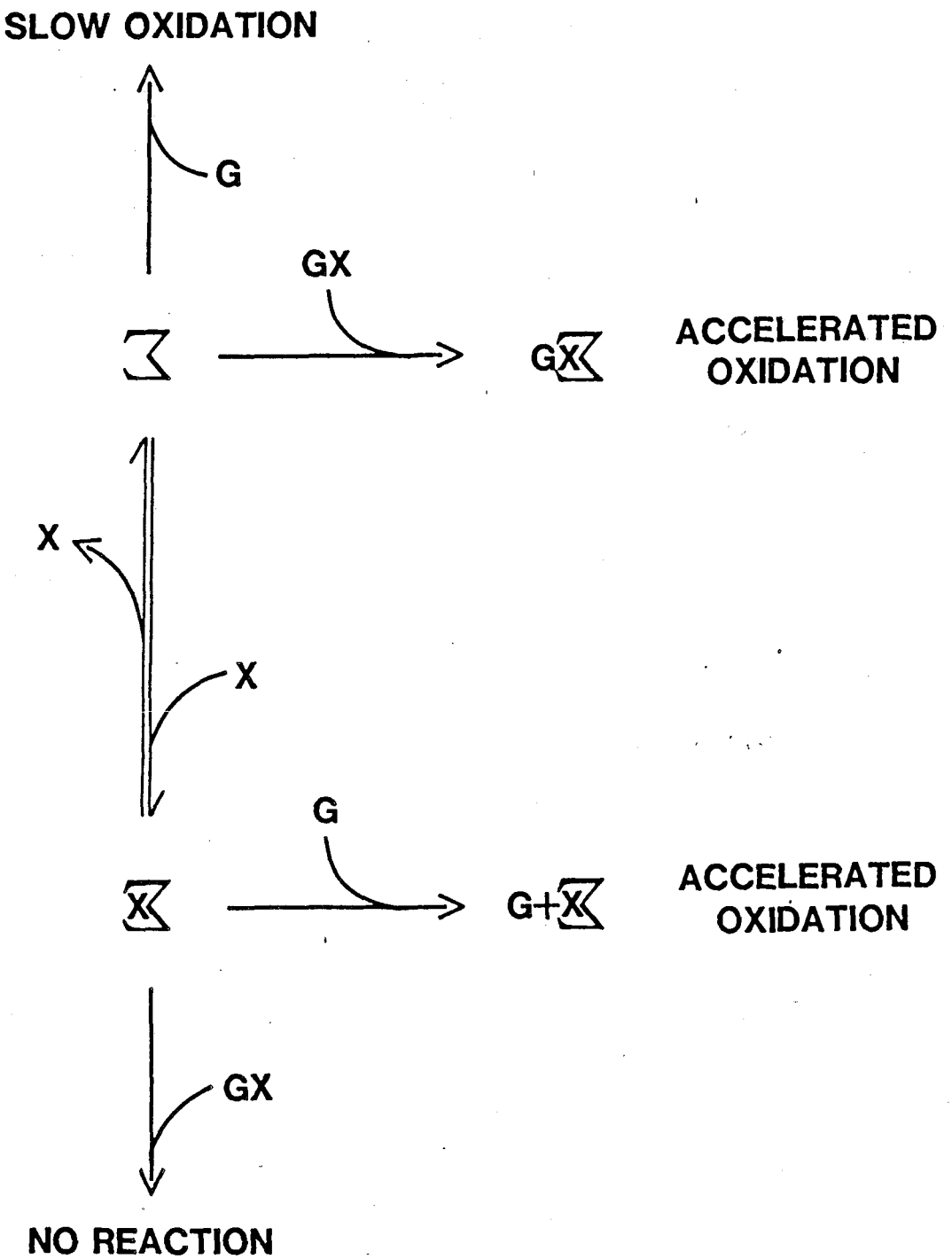
	pK_a		
X	1	2	3
P [a]	1.7	6.5	12.1
As [b]	2.2	6.8	11.5
V [a]	3.5	7.8	12.5

[a] From Reference 6

[b] From Reference 24

Figure 3

Proposed pathways for glucose (G) oxidation in the presence of G6PDH (Σ) and the anions (X) phosphate, arsenate, vanadate and sulphate. GX denotes the glucose-anion ester.


SLOW OXIDATION
G
GX
GX
ACCELERATED
OXIDATION
X
X
G
G+X
ACCELERATED
OXIDATION
GX
NO REACTION

important in the case of sulphate activation. Experiments with bacterial G6PDH (23) have shown that under these conditions glucose-6-sulphate is oxidized more slowly than glucose. Although it is possible that each of the anions, phosphate, arsenate, vanadate and sulphate can facilitate glucose oxidation through the lower pathway of Figure 3, it is proposed that rapid spontaneous ester formation is the principal mechanism by which vanadate at low concentrations enhances glucose oxidation. The following experiments were designed to test this proposal.

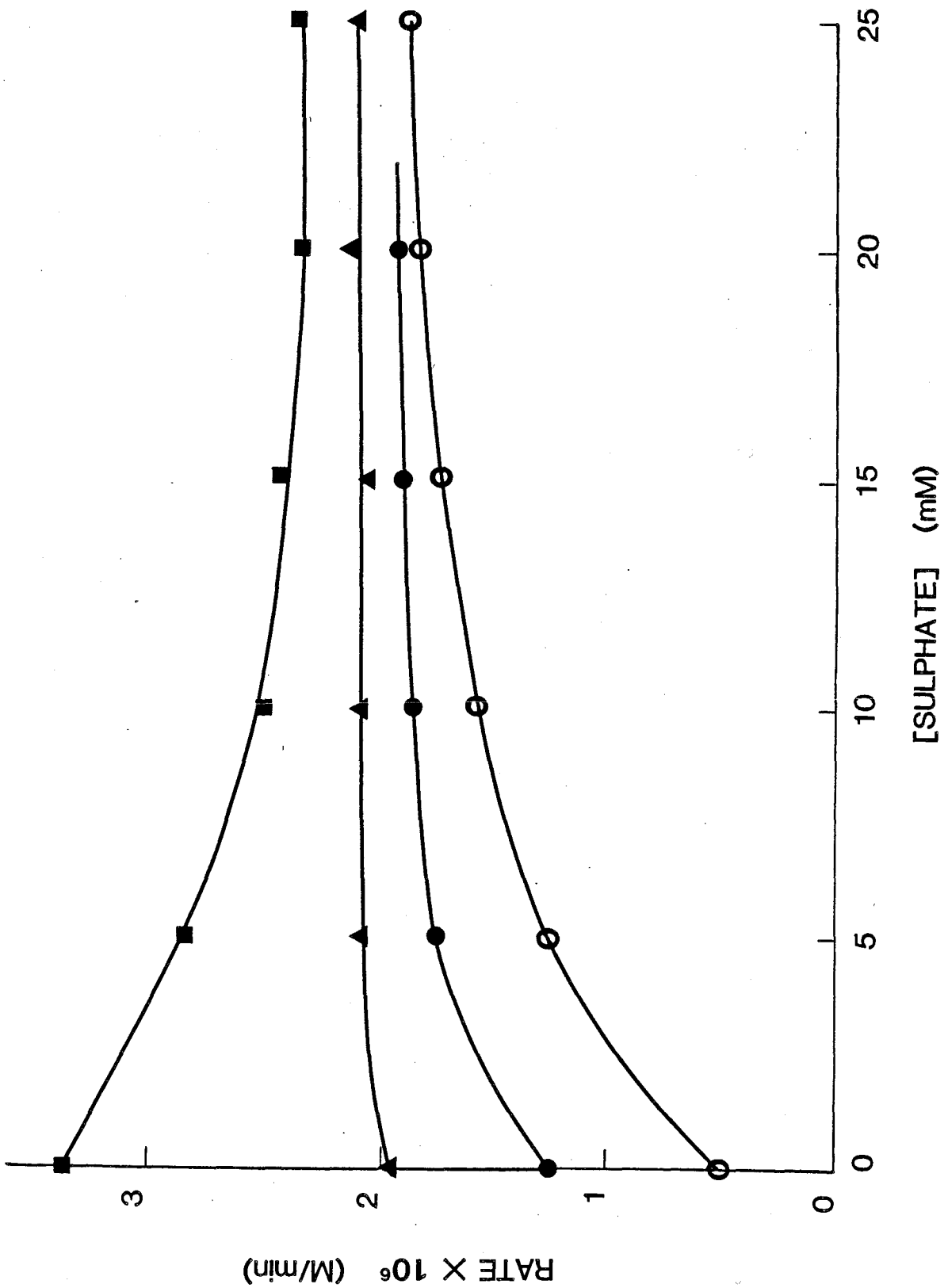
The activating effect of sulphate on glucose oxidation can be modified by the addition of vanadate as shown in Figure 4. In these experiments the reaction was initiated by the addition of glucose to a vessel which contained all of the other components.

In the absence of vanadate, the plot of rate vs. sulphate concentration shows a saturation pattern. At low vanadate concentrations, addition of sulphate increases the rate of glucose oxidation, but the effects of sulphate and vanadate are not additive. As the concentration of vanadate is increased, the sulphate activation becomes negligible compared with that of vanadate. At the highest concentration of vanadate used in these experiments (0.33 mM), the reaction is inhibited by sulphate.

If vanadate exerts its activating influence by forming an ester with glucose which is accepted by the enzyme at the same site at which it binds sulphate, and if sulphate activates by binding to the enzyme to form an adduct which can accept glucose but not a glucose ester, the curves in Figure 4 would be expected to reflect the interplay between the two different mechanisms of activation. The activating effect of the sulphate/enzyme complex is apparent at low vanadate concentrations because the rate enhancement due to the low concentration of vanadate ester is small. As vanadate is increased, empty sites can be utilized by the ester and the net rate becomes dominated by the effect of vanadate; the activation due to sulphate, while still present, is comparatively small. At higher vanadate concentrations, the binding of sulphate to the active site on the enzyme slows the net rate, as these sites must bind glucose in order for oxidation to take place. At high sulphate concentrations, the presence of the

Figure 4

The effect of different sulphate concentrations on the rate of vanadate activated $NADP^+$ reduction in the presence of glucose and G6PDH at pH 7.4. Procedures and conditions are the same as those described for Figure 2, except that vanadate was present in the following concentrations: 0 mM(o), 0.10 mM(◦), 0.17 mM(▲), and 0.33 mM(■).


RATE X 10⁶ (M/min)
0
1
2
3
0
5
10
15
20
25
[SULPHATE] (mM)

vanadate ester increases the rate of sulphate activated glucose oxidation. Vanadate ester molecules that compete successfully with sulphate ions for active sites on the enzyme, will undergo rapid oxidation. Because the process of ester oxidation is faster than that of sulphate activated glucose oxidation, the presence of ester molecules will increase the net rate.

A model for the proposed enzyme/substrate/anion interactions between G6PDH, glucose, sulphate and vanadate is presented in Figure 5. Equation 1 expresses the rate of the reaction in terms of the concentrations of the reactants, glucose [G], sulphate [S] and vanadate [V], the total enzyme concentration, e, the binding constant for the enzyme/sulphate complex, K_c, and the rate constants, k, which correspond to the pathways shown in Figure 5. The derivation of the rate equation is described in Appendix I.

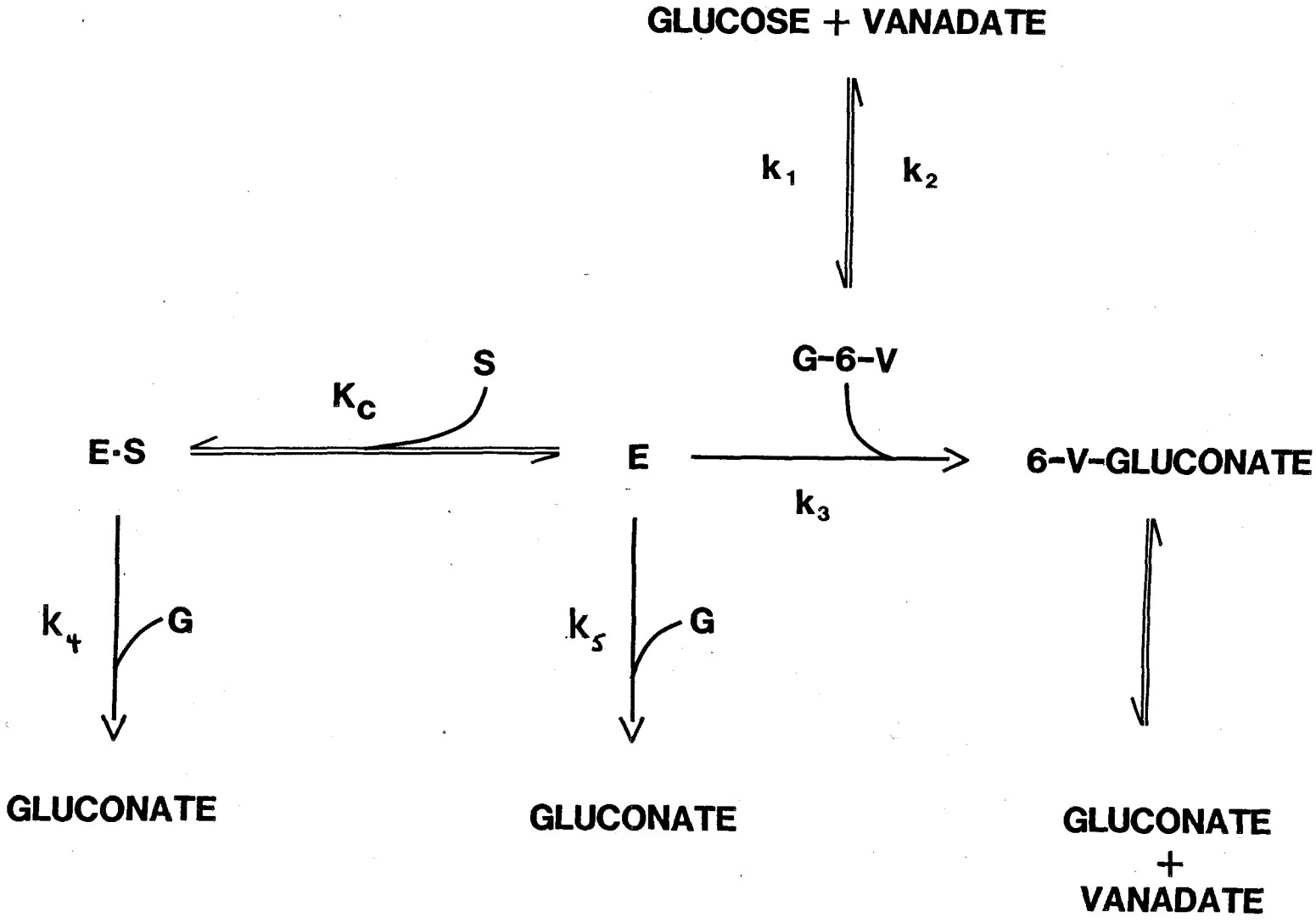
$$\text{Rate} = \frac{k_5\, e[G] + \frac{k_1 k_3}{k_2}\, e[G][V] + k_4 K_c\, e[G][S]}{1 + K_c[S]} \qquad (1)$$

The rate of the reaction was measured specrophotometrically by monitoring the increase in absorbance at 340 nm due to the production of NADPH (see Experimental Procedures). $NADP^+$, the reactant which undergoes reduction, does not appear in the rate equation as it is present at a constant saturating concentration in the reaction mixtures.

$k_5 e$ [G] is the expression for the rate of glucose oxidation in the absence of the activating anions. By setting this rate equal to zero, the

Figure 5

A model for the proposed interactions between G6PDH (E) and the anions sulphate (S) and vanadate (V) in the presence of glucose (G). Kc is the binding constant for the enzyme/sulphate complex, E•S, and lower case k's refer to the rate constants of the reactions as shown. G-6-V is the symbol for the putative ester, glucose-6-vanadate.


GLUCOSE + VANADATE
k1
k2
G-6-V
S
Kc
E·S
E
6-V-GLUCONATE
k3
kt
G
ks
G
GLUCONATE
GLUCONATE
GLUCONATE
+
VANADATE

rate of reaction in the absence of vanadate is described by Equation 2. The reciprocal of Equation 2 is 3.

$$\text{Rate} = \frac{k_4 \, K_c \, e \, [G] \, [S]}{1 + K_c[S]} \qquad (2)$$

$$\frac{1}{\text{Rate}} = \frac{1}{k_4 \, K_c \, e \, [G] \, [S]} + \frac{1}{k_4 \, e \, [G]} \qquad (3)$$

A plot of 1/Rate vs. 1/[S] is shown in Figure 6. As expected it is linear with a vertical intercept from which k_4 can be calculated. K_c can then be determined from the slope of this graph or from the horizontal intercept. In order to obtain a more accurate value of the rate at infinite sulphate concentration, the rate of unactivated glucose oxidation which was originally subtracted to obtain Figure 6, $k_5 e[G]$, is added to the rate at infinite sulphate concentration to obtain the broken line of Figure 6. The broken line represents a more accurate reciprocal rate, as the unactivated glucose oxidation will decrease as the sulphate binding increases. k_5 is estimated from the rate of oxidation when vanadate and sulphate are absent, then k_1k_3/k_2 is determined from the rate of vanadate activated oxidation in the absence of sulphate.

Figure 7 depicts rate curves which have been calculated from Equation 1, using the constants derived from Figure 6 and the values for the experimental concentrations of vanadate, sulphate, glucose and enzyme which were used to produce the curves in Figure 4. The experimental values from Figure 4 have been placed on Figure 7 for comparison with the calculated

Figure 6

The effect of different concentrations of sulphate on the rate of $NADP^+$ reduction in the presence of glucose and G6PDH at pH 7.4. Figure 6 is a double reciprocal plot of the data from Figure 2. The solid line represents the reciprocal rate vs. the reciprocal sulphate concentration after the unactivated rate of $NADP^+$ reduction has been subtracted; ie. the velocity at [S]=0. the broken line was obtained by readding the subtracted value to the rate at infinite [S]. The broken line is a more realistic representation of the reciprocal sulphate activation as the rate of unactivated glucose oxidation would be expected to decrease, becoming negligible at infinite sulphate concentration.

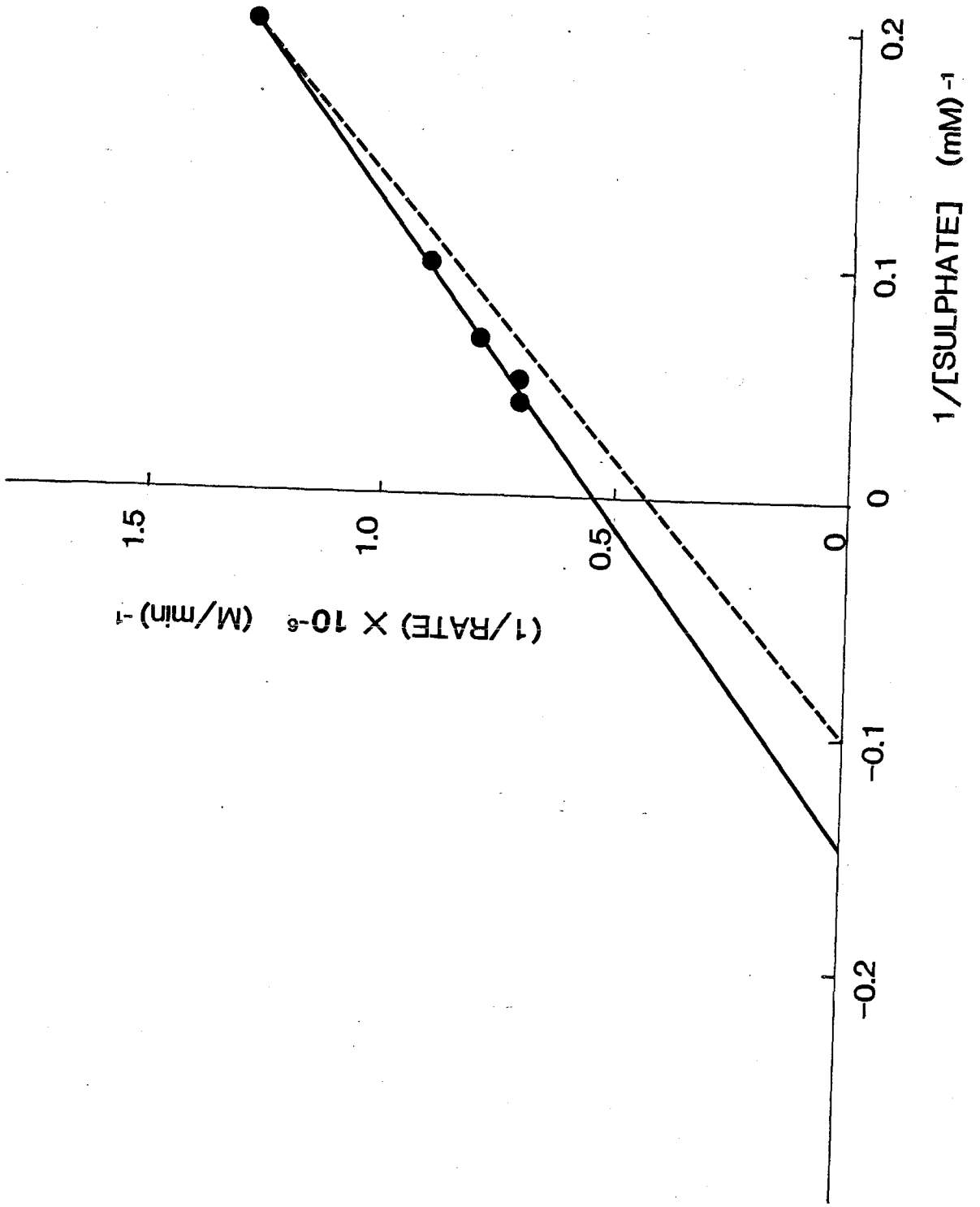
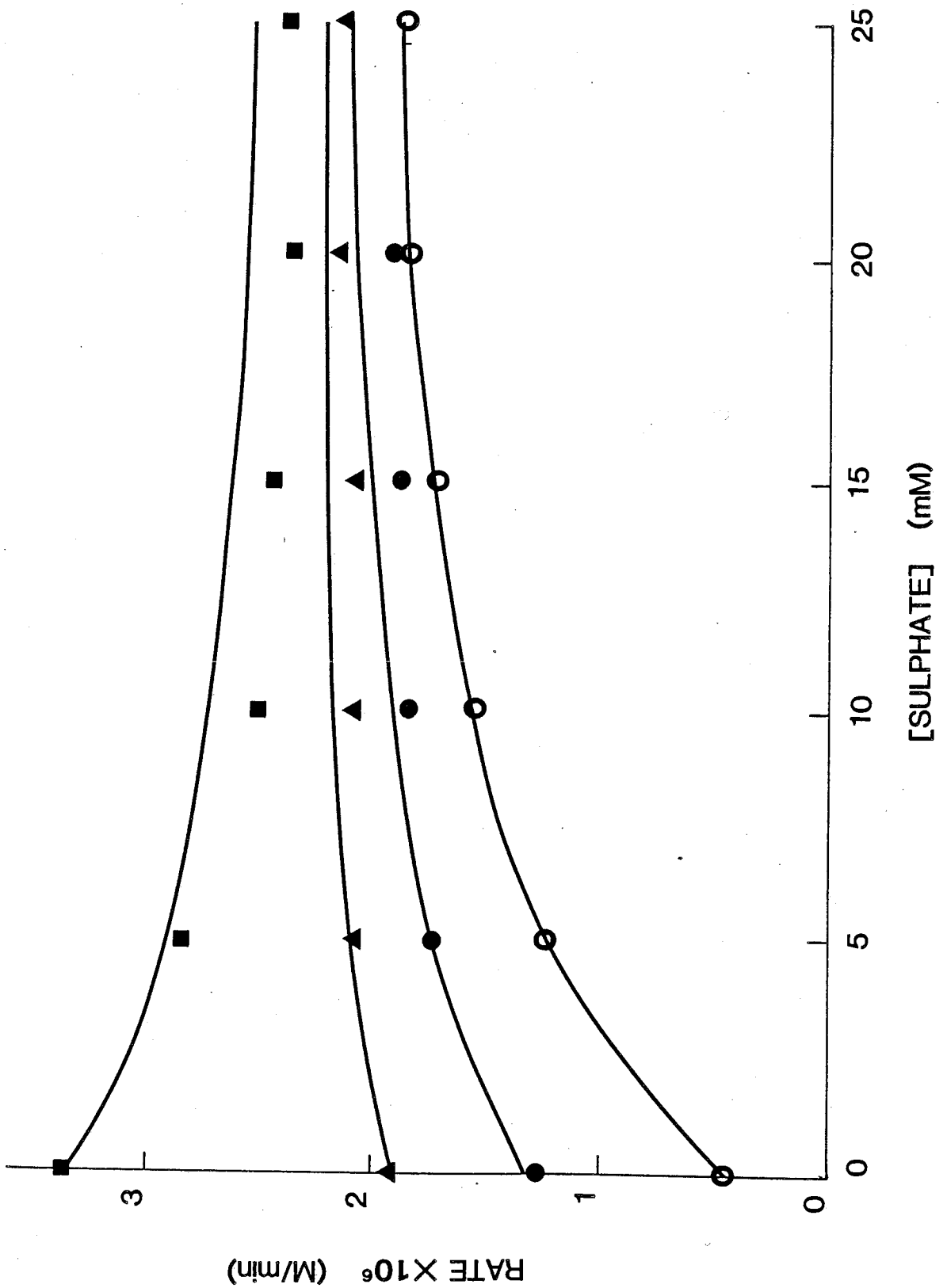

1.5
1.0
0.5
0
(1/RATE) × 10-6 (M/min)-1
0.2
0.1
0
-0.1
-0.2
1/[SULPHATE] (mM)-1

Figure 7

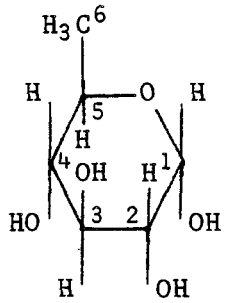
The effect of different sulphate concentrations on the rate of vanadate activated $NADP^+$ reduction in the presence of glucose and G6PDH at pH 7.4. The lines were calculated using equation 1, the constants were derived from Figure 6 as described in the text. Values for the concentration of glucose and enzyme were taken from Figure 2. The experimental points from Figure 4 correspond to vanadate concentrations of 0 (o), 0.1 mM (•), 0.17 mM (▲) and 0.33 mM (■).


RATE X10⁶ (M/min)
0
1
2
3
0
5
10
15
20
25
[SULPHATE] (mM)

curves. It can be seen that the experimental points fall close to the calculated curves at low sulphate concentration. At higher sulphate concentration the inhibition by sulphate is stronger than that calculated from Equation 1. Since the activation of glucose oxidation by vanadate is linear in this region (Figure 1), it is possible that an interaction between vanadate and sulphate, not predicted by the model in Figure 5, could account for this decrease in reaction velocity.

(ii) 6-Deoxyglucose (6-Deoxy-D-Glucose)

In order to test the hypothesis that sulphate and vanadate activate glucose oxidation by different mechanisms, it was decided to replace the glucose in the reaction vessel with 6-deoxyglucose. This compound is similar in every respect to glucose except that it lacks the hydroxyl group on the 6-carbon, as shown below.

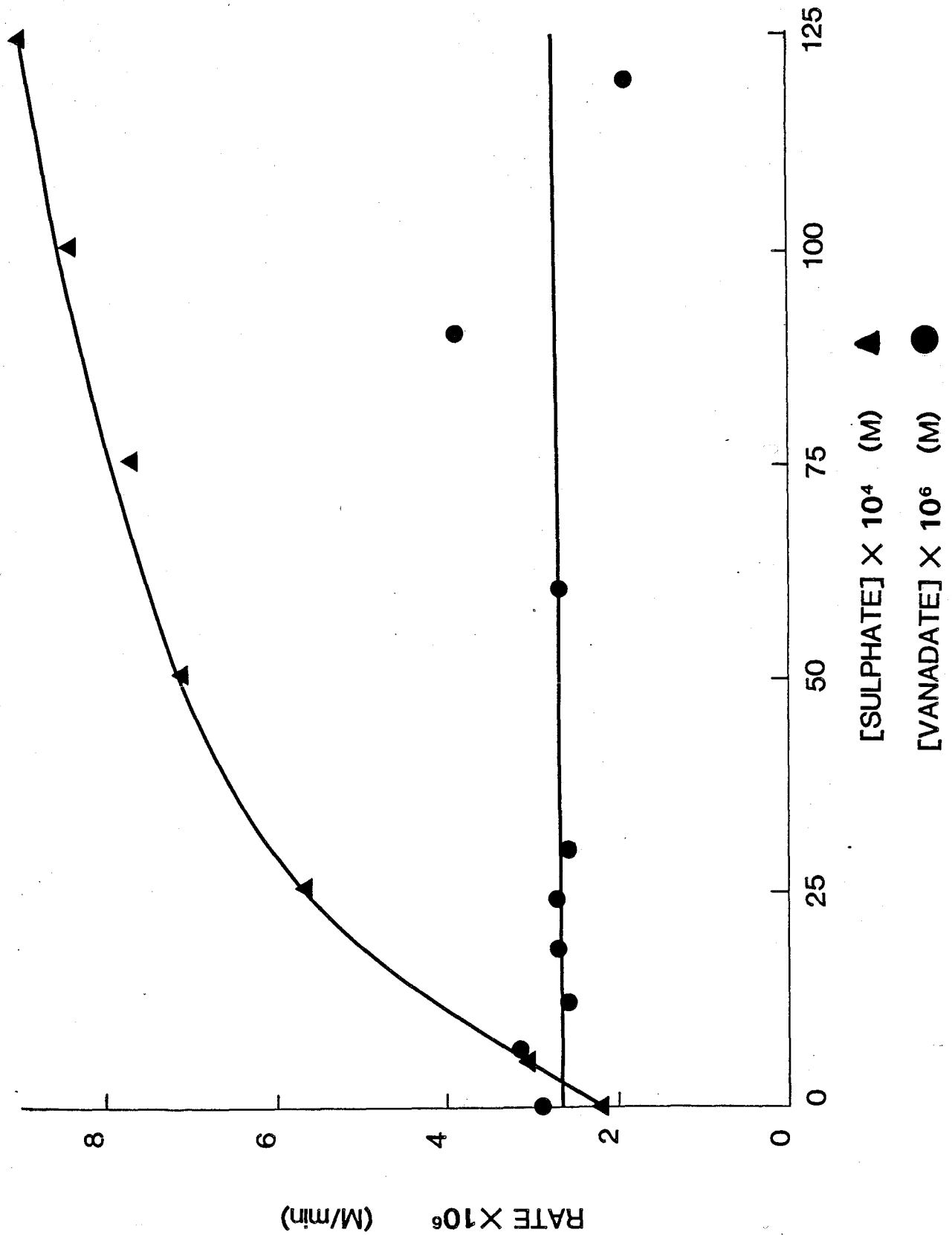


6-Deoxy-D-Glucose

Figure 8 shows that 6-deoxyglucose is oxidized slowly by $NADP^+$ in the presence of G6PDH. The addition of vanadate has no significant effect on this reaction but sulphate produces a marked acceleration. This behavior supports the proposal that vanadate activates by ester formation while sulphate activates by binding with the enzyme. Since there is no hydroxyl group on the 6-carbon of 6-deoxyglucose, the ester glucose-6-vanadate can-

Figure 8

The effect of different sulphate concentrations and of different vanadate concentrations on the rate of $NADP^+$ reduction in the presence of 6-deoxyglucose and G6PDH at pH 7.4. The reaction mixture contained 1.0 mM $NADP^+$, 10 mM $MgCl_2$, 10 mM 6-deoxyglucose, 0.05 mg mL^{-1} G6PDH and sulphate (▲) or vanadate (•) as shown. All reagents except 6-deoxyglucose were incubated at 30°C for 4 min. before the reaction was initiated by adding 0.1 mL 6-deoxyglucose to a final volume of 1.0 mL. The cuvette was inverted 8 times in order to mix the reactants before placing it in the spectrophotometer.


RATE X10⁶ (M/min)
0
2
4
6
8
0
25
50
75
100
125
[SULPHATE] X 10⁴ (M)
[VANADATE] X 10⁶ (M)

not be formed, and vanadate cannot activate the oxidation by this mechanism. However, sulphate can activate by binding to the enzyme to form a complex which readily accepts the 6-deoxyglucose molecule for oxidation.

II. Measurement of Equilibrium Constants for Vanadate Ester Formation.

(i) Nuclear Magnetic Resonance

Evidence for vanadate ester formation has been provided by nuclear magnetic resonance (NMR) measurements of the ^{51}V nucleus. Studies of the NMR spectra of vanadate with organic molecules which contain hydroxyl groups indicate that complex formation between vanadate and these molecules is related to the concentration of reactants and the pH of the medium (25). The reference spectrum of an aqueous solution of vanadate is shown in Figure 9. Figures 10, 11 and 12 represent solutions of vanadate with methyl-β-D-glucopyranoside (1-O-methylglucose), glycerol and dihydroxyacetone respectively.

The method by which the chemical shift of the monoester has been assigned is discussed in reference 25. For the purpose of this thesis it will be assumed that the monoester peak appears as a shoulder, downfield with respect to the vanadate peak, the pair resonating at approximately 560 ppm. for 1-O-methylglucose and glycerol. The dihydroxyacetone monoester peak appears at 545 ppm.

The concentration ratio of vanadate ester to unesterified vanadate was obtained by measuring the areas under the peaks. This ratio was then used in Equation 4 with the known concentration of the organic reactant [X]. An

Figure 9

^{51}V NMR spectra of vanadate in water. The solution contained 5.0×10^{-4} M vanadate and 20 mM Tris-Cl buffer. Temperature was 21°C, pH was 7.5.

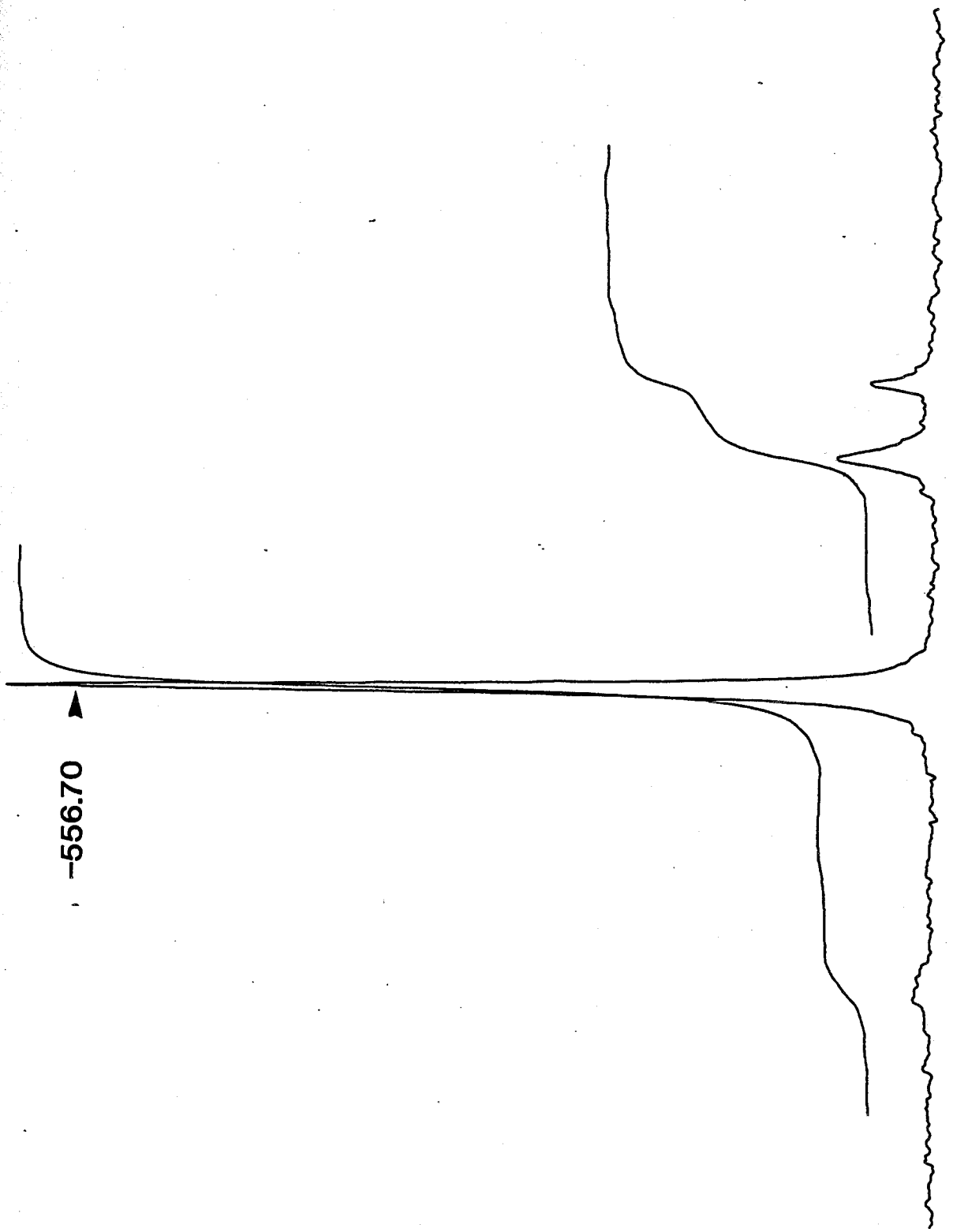

-556.70

Figure 10

^{51}V NMR spectra of a 1-O-methyl-β-D-Glucose/vanadate/water solution. Concentrations were: 1.5 M 1-O-methyl-β-D-glucose, 5.0×10^{-4} M vanadate and 20 mM Tris-Cl buffer. Temperature was 21°C, pH was 6.9.

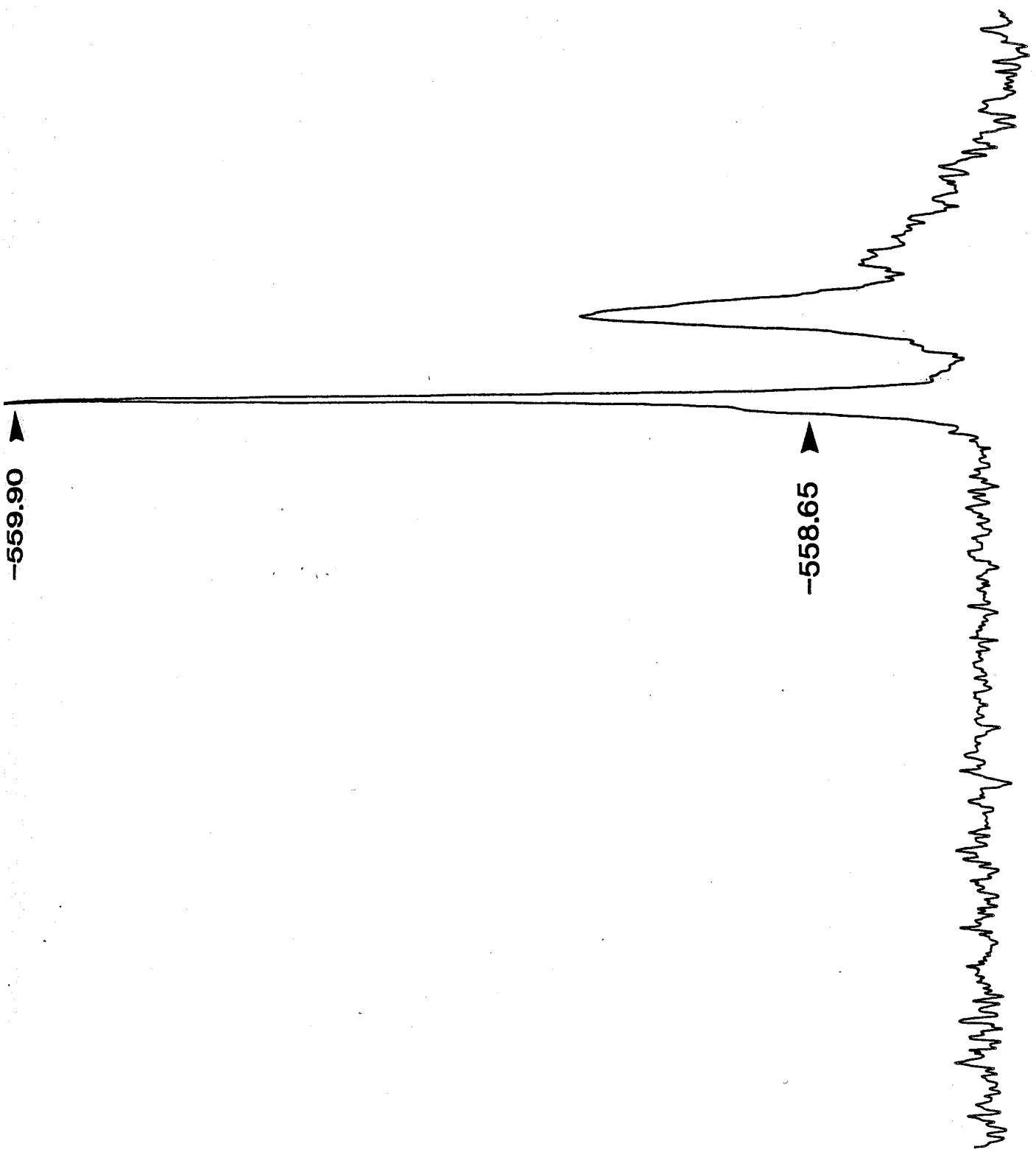

-559.90
-558.65

Figure 11

^{51}V NMR spectra of a glycerol/vanadate/water solution. Concentrations were 1.0 M glycerol, 5.0×10^{-4} M vanadate and 20 mM Tris-Cl buffer. Temperature was 21°C, pH was 7.2

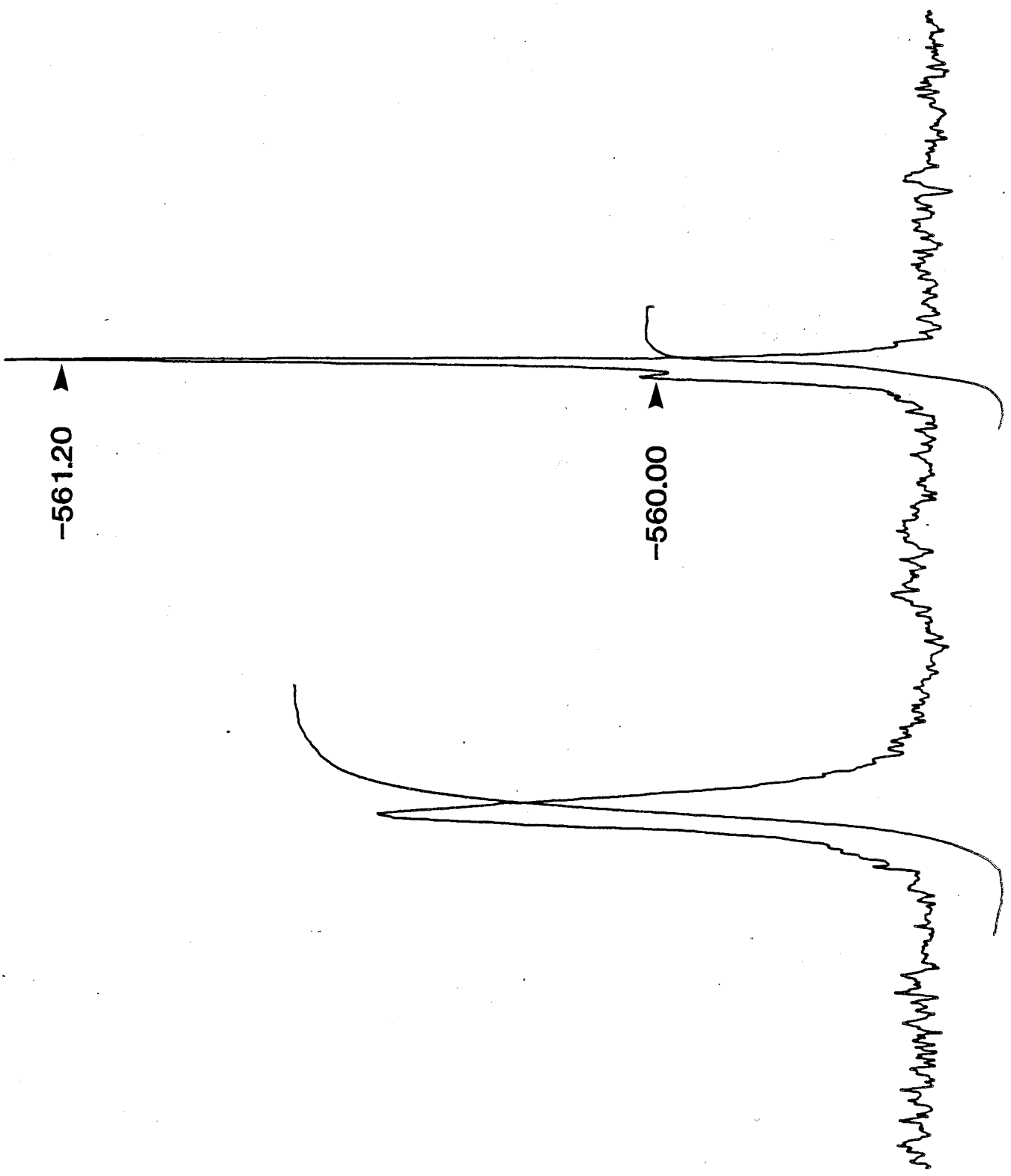
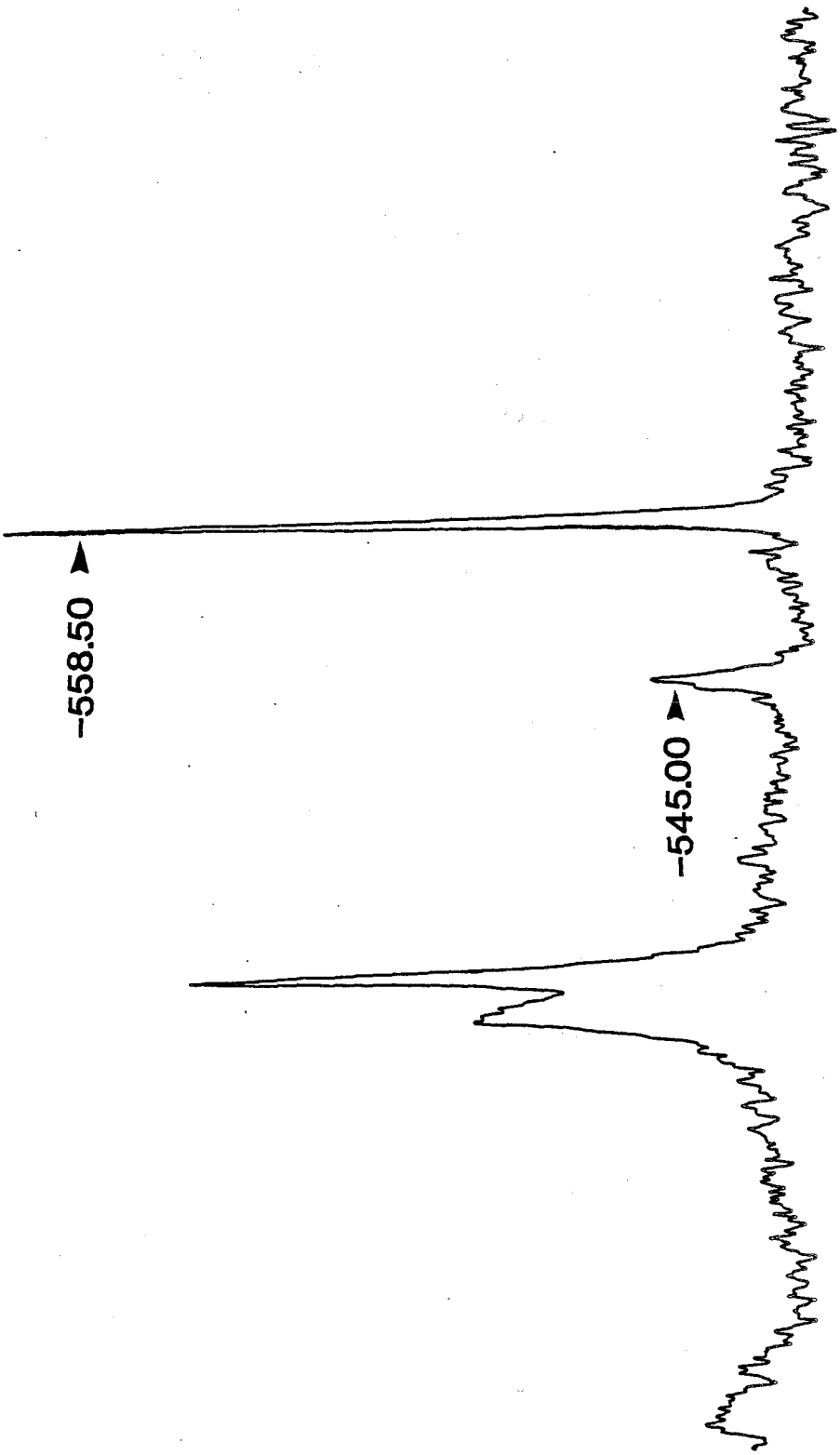

-561.20
-560.00

Figure 12

^{51}V NMR spectra of a dihydroxyacetone/vanadate/water solution. Concentrations were 1.0 M dihydroxyacetone, 5.0×10^{-4} M vanadate and 20 mM Tris-Cl buffer. Temperature was 21°C, pH was 7.5.


-558.50
-545.00

example of this calculation can be found in Appendix II.

$$K_{eq} = \frac{[\text{X-vanadate}]}{[\text{X}][\text{vanadate}]} \qquad (4)$$

The accuracy of the value for K_{eq} is restricted when the overlap of the peaks (as in 1-O-methylglucose and glycerol) makes it difficult to assign the correct area value for each species. In particular, the peaks of 1-O-methylglucose vanadate and vanadate are poorly resolved. In this case the K_{eq} value obtained from measuring the peak area ratio must be considered an approximation of the true value.

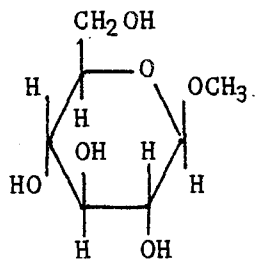
Table IV lists the values of K_{eq} that have been calculated from ^{51}V NMR spectra for the organic molecules which have been used in these experiments.

TABLE IV

Equilibrium constants (K_{eq}) for monoesters formed spontaneously in solutions of vanadate and organic molecules. K_{eq} was calculated from the peak areas obtained from ^{51}V NMR spectra as described in the text.

Vanadate Ester	K_{eq} (M^{-1})
Glucose-6-vanadate [a]	0.084 [c]
Glycerol-3-vanadate [b]	0.13 [c]
Dihydroxyacetone-vanadate	0.39

[a] Methyl-β-D-glucopyranoside (1-O-methylglucose) was used instead of glucose in the NMR determination of K_{eq}. The glucose-vanadate spectrum was complicated by numerous peaks due to the variety of esters possible. The methylated glucose cannot form an ester with vanadate at the 1-carbon, and open-chain forms, mutarotated glucose and furanoside forms are not present in solutions of 1-O-methylglucose, thus the spectrum is simplified. Also at the high glucose concentrations used in the NMR experiments, reduction of vanadium V was a problem with glucose. The 6-carbon ester is thought to be the principal species represented by the downfield shoulder on the vanadate peak at 560 ppm. The structure of 1-O-methylglucose is illustrated below.



methyl-β-D-glucopyranoside
(1-O-methylglucose)

[b] K_{eq} has been corrected for the presence of the secondary ester which is not separate from the monoester peak. The calculation is described in Appendix II.

[c] K_{eq} is an approximate value due to poor resolution of the peaks.

(ii) Burst Experiment

The equilibrium constant and the rate constant for the formation of glucose-6-arsenate have been determined by other investigators (12). The rate of glucose-6-arsenate formation was measured by adding aliquots of a solution of glucose and arsenate to an assay mixture which contained G6PDH, $NADP^+$ and $MgCl_2$. Samples of the reactant mixture were assayed at timed intervals from the moment that the glucose and arsenate were combined until the maximum concentration of glucose-6-arsenate was reached. The concentrations of glucose and arsenate in the reactant mixture were sufficently high (0.4 M and 0.2 M respectively) that the rapid oxidation by $NADP^+$ of the glucose-6-arsenate which was formed appeared as a "burst phase", followed by the steady-state oxidation of glucose after the ester had been depleted.

An attempt to measure the glucose-6-vanadate formed in a solution of glucose and vanadate was not successful. The quantity of ester formed is restricted in this case by the necessity to keep the vanadate concentration below 10^{-3} M in order to prevent oligomerization of the vanadate ions.

Since the equilibrium constants for the formation of the vanadate esters could not be obtained by the burst method, it was necessary to depend on the results from the NMR measurements described in the preceding section for the calculation of some of the kinetic parameters.

III. Kinetics of Vanadate Esterification

(i) Glucose-6-vanadate

A. Estimation of Rate Constants by Enzyme Kinetics.

It has been proposed that vanadate accelerates glucose oxidation through the formation of the ester glucose-6-vanadate, which is then accepted by the enzyme G6PDH as a substrate analogue of glucose-6-phosphate. The product of this reaction would be 6-vanadogluconate. Rapid hydrolysis of this product would release the vanadate ion for subsequent activation of other glucose molecules. Figure 13 shows the proposed reaction mechanism for glucose oxidation in the presence of vanadate.

If the reaction follows Michaelis Menten kinetics, its rate will be described by Equation 5, where [G-6-V] is the concentration of glucose-6-vanadate, e is the enzyme concentration and k_{cat}/K_m is equal to k_3 in Figure 13.

$$\text{Rate} = \frac{k_{cat}\ e\ [G\text{-}6\text{-}V]}{K_m} \qquad (5)$$

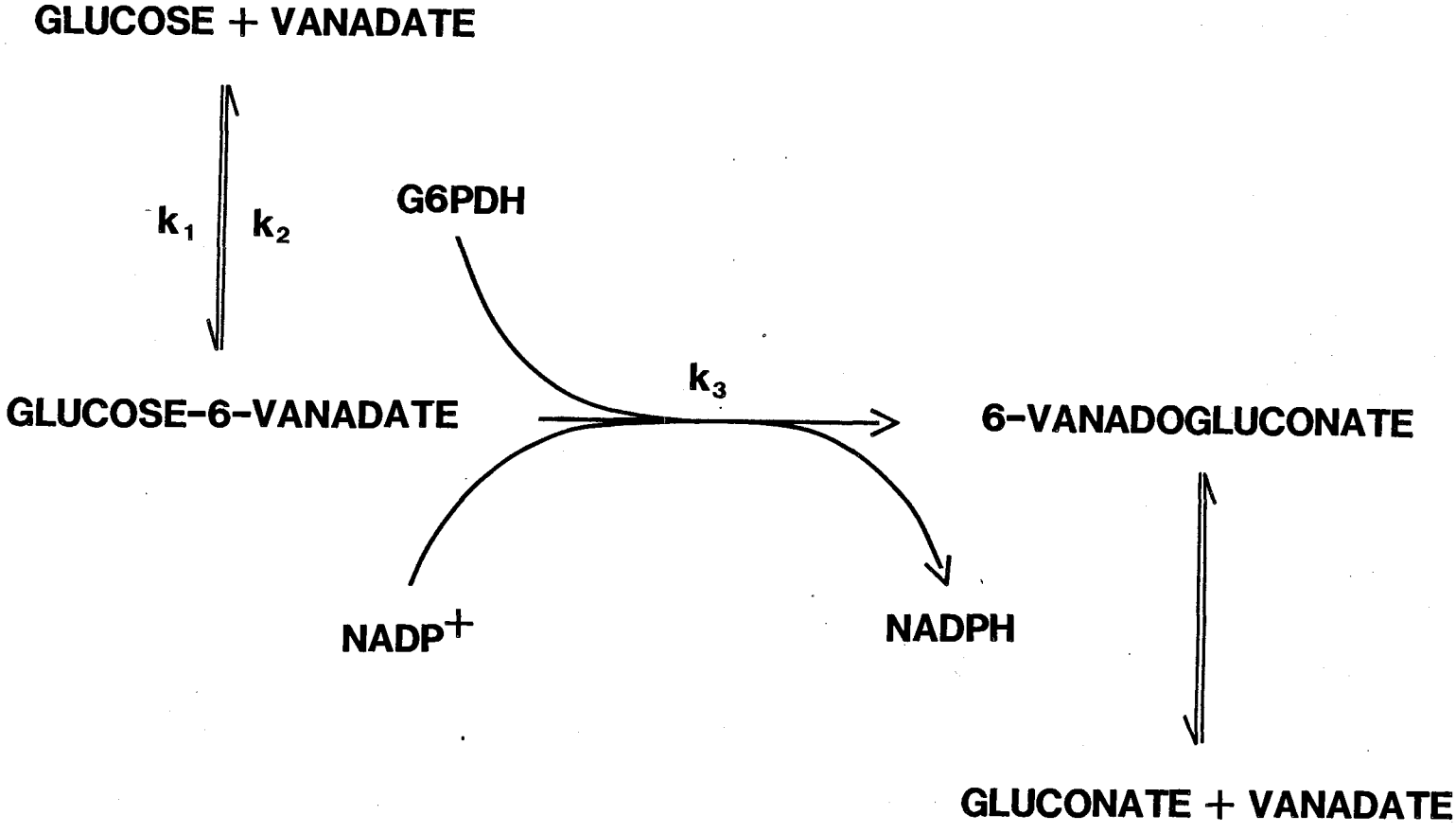
From Figure 13 the production of glucose-6-vanadate can be expressed by Equation 6, where [G] and [V] are the concentrations of glucose and vanadate respectively.

$$\frac{d[G\text{-}6\text{-}V]}{dt} = k_1[G][V] - k_2[G\text{-}6\text{-}V] - \frac{k_{cat}\ e\ [G\text{-}6\text{-}V]}{K_m} \qquad (6)$$

In Equation 5, K_m is the Michaelis constant of the ester and k_{cat} is the turnover number for the enzyme (the number of reactant molecules

Figure 13

A model of the proposed reaction mechanism for the oxidation of glucose by $NADP^+$ in the presence of vanadate, catalysed by G6PDH. k_1 and k_2 are the rate constants for the formation and hydrolysis of glucose-6-vanadate respectively. k_3 is the k_{cat}/K_m ratio for glucose-6-vanadate as a substrate for G6PDH. The reaction can be followed by observing the increase in absorbance at 340 nm due to the production of NADPH (details in Experimental Procedures).


GLUCOSE + VANADATE
k_1
k_2
G6PDH
k_3
GLUCOSE-6-VANADATE
6-VANADOGLUCONATE
$NADP^+$
NADPH
GLUCONATE + VANADATE

converted to product per unit time per active site, under conditions of saturating substrate concentration). The term for the substrate concentration has been omitted from the denominator as the assumption is made that the concentration of glucose-6-vanadate will be very small compared to its K_m. Under the conditions of the reaction ([G] = 5.0×10^{-4} M, [V] = 1.0×10^{-4} M, Figure 14), the concentration of glucose-6-vanadate can be estimated using the equilibrium constant for ester formation determined by NMR (8.4×10^{-2} M^{-1}). This gives an equilibrium concentration for glucose-6-vanadate of 4.2×10^{-9} M, or 0.0042 μM. The K_m for glucose-6-phosphate as a substrate for G6PDH has been determined by other investigators (12) to be equal to 22 μM. It must be used for comparison as the K_m for glucose-6-vanadate is not known. Since the K_m for glucose-6-arsenate (45 μM) (12) is similar in magnitude to that of glucose-6-phosphate, this substitution appears to be reasonable. The relative difference between the concentration of glucose-6-vanadate and the K_m for glucose-6-phosphate justifies the omission of the term for the substrate concentration from the denominator of the rate equation.

The rate equation does not include a term for $NADP^+$ as it is present at a constant saturating concentration.

Rearrangement of equation 6 gives equation 7, an expression of the steady state concentration of glucose-6-vanadate. When 7 is substituted into 5, the result 8 can be inverted to obtain 9.

$$[G\text{-}6\text{-}V] = \frac{k_1 [G][V]}{k_2 + \frac{k_{cat}\ e}{K_m}} \qquad (7)$$

$$\text{Rate} = \frac{k_1\ k_{cat}\ e[G][V]}{k_2 K_m + k_{cat}\ e} \qquad (8)$$

$$\frac{1}{\text{Rate}} = \frac{k_2 K_m}{k_1 k_{cat}\ e[G][V]} + \frac{1}{k_1[G][V]} \qquad (9)$$

Equation 9 predicts that plots of 1/Rate vs 1/e will be linear with a vertical intercept of $1/k_1[G][V]$. The rate constant for ester formation (k_1) can be calculated from the value of this intercept and known concentrations of glucose and vanadate. If the concentration of glucose-6-vanadate can be measured the ratio k_{cat}/K_m can be obtained from Equation 5. Using this ratio, and the value for the horizontal intercept of the reciprocal rate plot ($-k_{cat}/k_2 K_m$), the rate constant for ester hydrolysis (k_2) can be estimated.

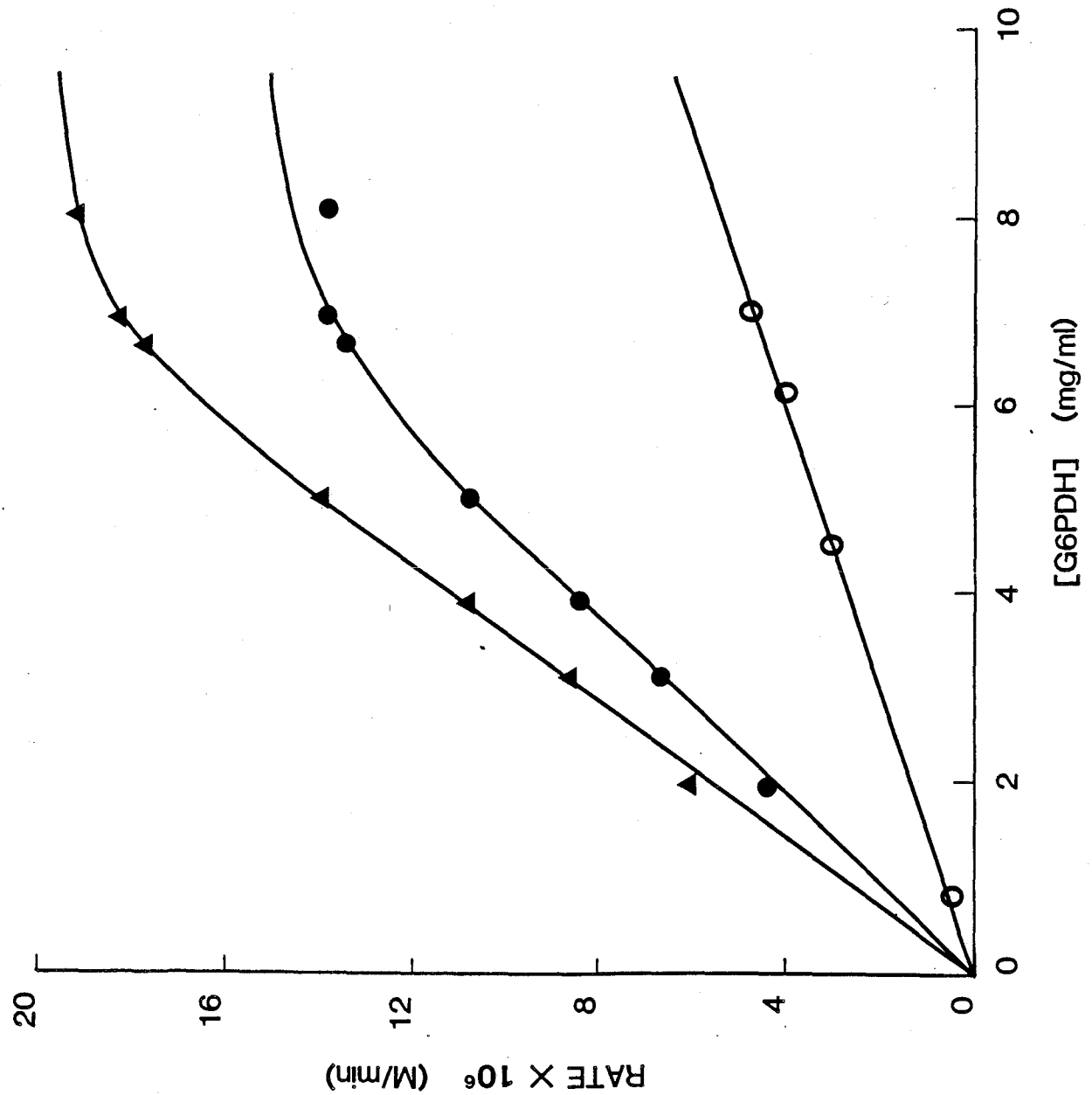
Alternatively, if K_{eq} for the ester is known, k_2 can be calculated from k_1 and the equilibrium relation $K_{eq} = k_1/k_2$. k_{cat}/K_m can then be determined from the horizontal intercept of the reciprocal rate plot.

Figure 14 shows the change in rate of the oxidation of glucose in the presence of 0.1 mM vanadate as the concentration of G6PDH is increased. The observed acceleration of the reaction is consistent with the formation of an ester, glucose-6-vanadate, which is accepted by the enzyme as a substrate. Because this compound is structurally similar to the true sub-

Figure 14

The effect of different enzyme (G6PDH) concentrations on the rate of vanadate activated $NADP^+$ reduction in the presence of glucose at pH 7.4. Reactant concentrations were: 1.0 mM $NADP^+$, 10 mM $MgCl_2$, 0.5 mM glucose and 0.1 mM vanadate. G6PDH concentrations were as shown. The reactants, except glucose, were incubated at 30°C for 4 min. before the reaction was initiated by blowing 5.0 µL of the glucose stock solution through a capillary tube into a final volume of 0.3 mL.

The concentrations of vanadate are: 0 (o) and 0.1 mM (Δ). The middle curve (•) shows the vanadate activated glucose oxidation after the unactivated glucose oxidation has been subtracted.


RATE X 10⁶ (M/min)
20
16
12
8
4
0
0
2
4
6
8
10
[G6PDH] (mg/ml)

strate for this enzyme, glucose-6-phosphate, it would be expected to bind more readily than glucose to the enzyme.

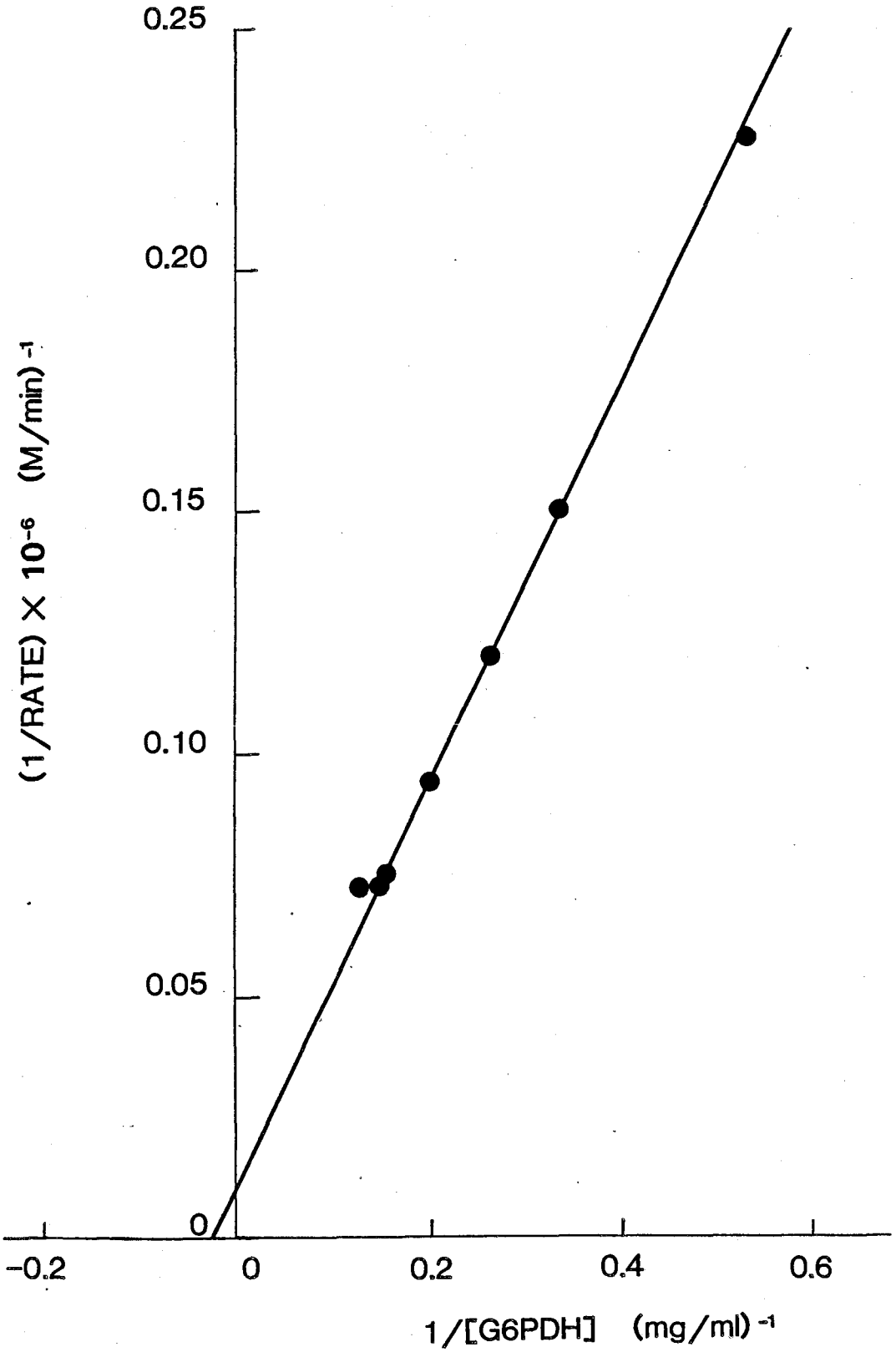
The fact that the rate increase with increasing enzyme concentration in the presence of vanadate levels off at high enzyme concentrations can be explained if the production of glucose-6-vanadate becomes rate limiting as more catalytic sites become available. Since the rate of glucose oxidation in the absence of vanadate shows a linear increase with enzyme concentration, it is likely that the enzyme maintains its activity at the highest concentrations used.

Equation 9 predicts that a plot of reciprocal rate vs 1/e will yield values for the kinetic parameters of interest. From the vertical intercept of this plot, Figure 15, the second order rate constant for ester formation, k_1, was found to be equal to 35 $M^{-1}s^{-1}$. Using the value K_{eq} = 0.084 M^{-1} obtained from the NMR spectra of glucose/vanadate solutions, and the equilibrium relation $K_{eq} = k_1/k_2$, the first order rate constant for ester hydrolysis, k_2, was calculated to be equal to 420s^{-1}. The value for k_2 was introduced into the expression for the horizontal intercept of the reciprocal rate plot, k_{cat}/k_2K_m, to obtain the value for the ratio k_{cat}/K_m = 12 $mLmg^{-1}s^{-1}$.

The significance of the ratio k_{cat}/K_m, which is really an apparent second order rate constant at low substrate concentration (Equation 5), lies in the fact that its value determines the specificity of the enzyme for competing substrates (27). In order to compare this quantity with the corresponding ratio, that is the k_{cat}/K_m ratio for the physiological substrate, glucose-6-phosphate, the enzyme saturation behavior for glucose-6-phosphate was tested. The results are plotted in Figure 16; the double

Figure 15

The effect of different enzyme (G6PDH) concentrations on the rate of vanadate activated $NADP^+$ reduction in the presence of glucose, Figure 15 is a double reciprocal plot of the data from the middle curve (•) of Figure 14.


0.25
0.20
0.15
0.10
0.05
0
(1/RATE) X 10⁻⁶ (M/min)⁻¹
-0.2
0
0.2
0.4
0.6
1/[G6PDH] (mg/ml)⁻¹

reciprocal plot of Figure 16 appears in Figure 17. Equation 10 is the reciprocal of the Michaelis-Menten equation which relates the velocity of the reaction to the substrate concentration. If the oxidation of glucose-6-phosphate obeys Michaelis-Menten kinetics, the plot in Figure 17 should be described by Equation 10, where [G-6-P] is glucose-6-phosphate.

$$\frac{1}{\text{Rate}} = \frac{K_m}{k_{cat}\ e\ [G\text{-}6\text{-}P]} + \frac{1}{k_{cat}\ e} \qquad (10)$$

From the slope of the line plotted in Figure 17 the k_{cat}/K_m ratio for glucose-6-phosphate as a substrate for G6PDH was found to be equal to 120 $mLmg^{-1}s^{-1}$.

From a comparison of the values for the k_{cat}/K_m ratios of glucose-6-vanadate and glucose-6-phosphate, it can be seen that the k_{cat}/K_m ratio for glucose-6-phosphate as a substrate for G6PDH is ten times that for glucose-6-vanadate. This is a reasonable result since it would be expected that the enzyme would have a higher specificity for its natural substrate. In a published study using this enzyme (12), the k_{cat}/K_m ratio for glucose-6-phosphate was found to be about three times that for glucose-6-arsenate.

The kinetic parameters of the three glucose esters - phosphate, arsenate and vanadate are displayed in Table V. It is of interest to compare the esters in terms of their rates of formation and hydrolysis, their equilibrium constants and their k_{cat}/K_m ratios as substrates for G6PDH.

Figure 16

The effect of different concentrations of glucose-6-phosphate on the rate of $NADP^+$ reduction in the presence of G6PDH at pH 7.4. The reaction mixture contained 1.0 mM $NADP^+$, 10 mM $MgCl_2$, 6.0×10^{-5} mg mL^{-1} G6PDH and glucose-6-phosphate concentrations as shown. The reaction was initiated by the addition of 5.0 µL of the enzyme stock solution to a final volume of 1.0 mL of the reaction mixture. The temperature was 30°C.

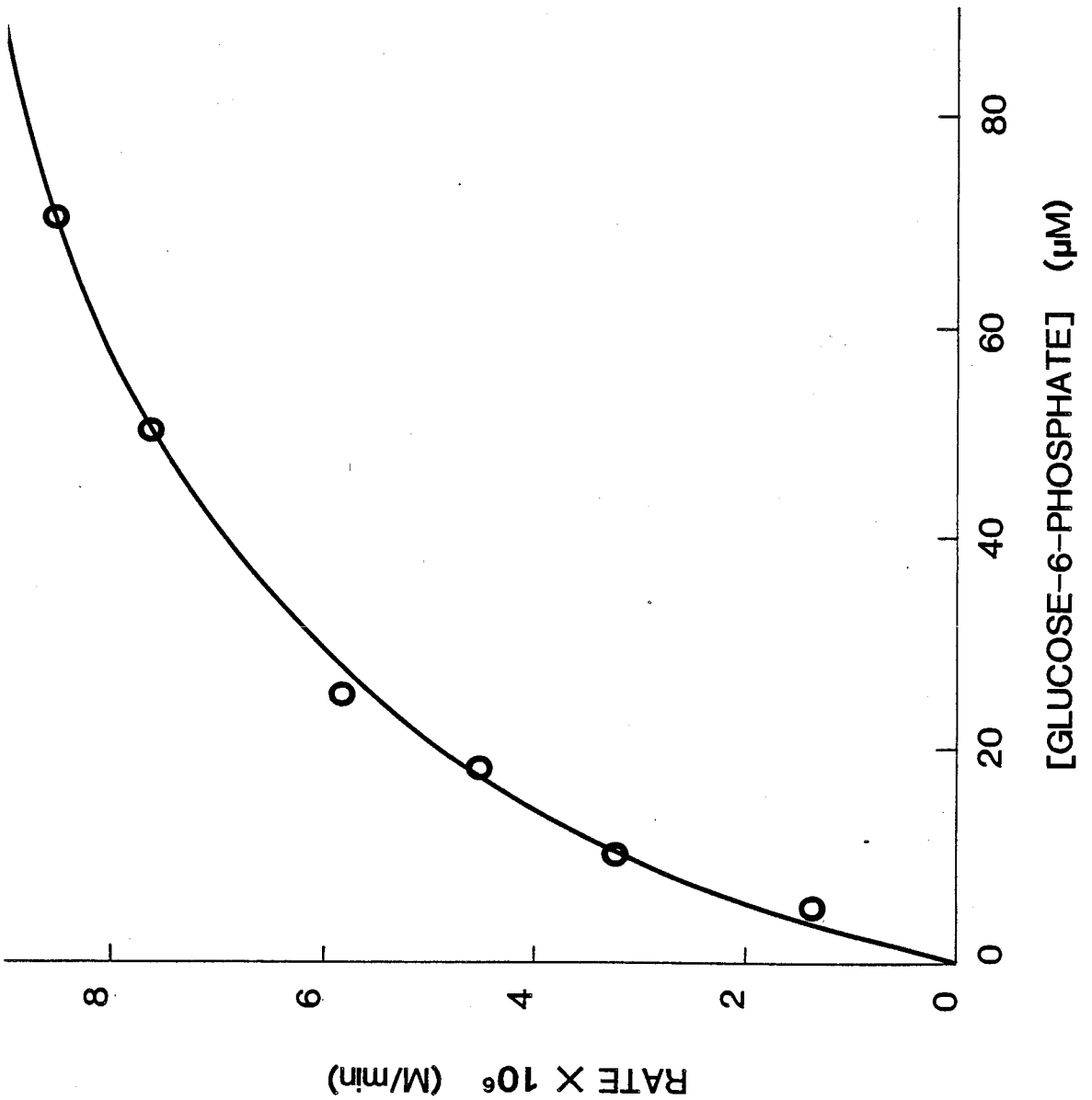

RATE X 10⁶ (M/min)
0
2
4
6
8
0
20
40
60
80
[GLUCOSE-6-PHOSPHATE] (μM)

Figure 17

The effect of different glucose-6-phosphate concentrations on the rate of $NADP^+$ reduction in the presence of G6PDH at pH 7.4. Figure 17 is a double reciprocal plot of the data from Figure 16.

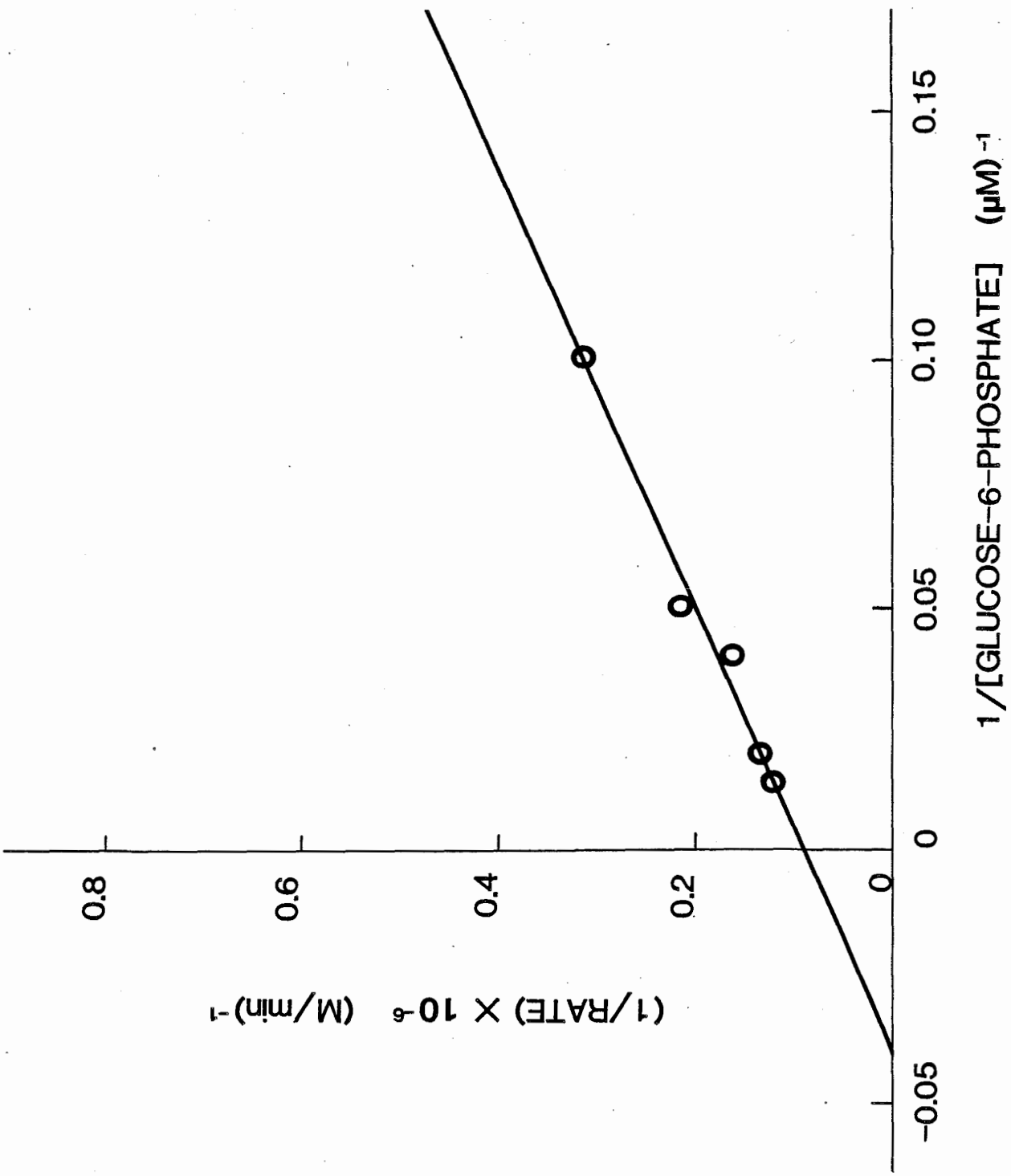

(1/RATE) X 10⁻⁶ (M/min)⁻¹
0.8
0.6
0.4
0.2
0
−0.05
0
0.05
0.10
0.15
1/[GLUCOSE-6-PHOSPHATE] (μM)⁻¹

TABLE V

Kinetic parameters for glucose esters. k_1 and k_2 are rate constants for ester formation and hydrolysis respectively. They correspond to k_1 and k_2 in Figure 13. K_{eq} is the equilibrium constant for ester formation and k_{cat}/K_m is a measure of the specificity of the enzyme for the substrate shown.

Ester	k_1 ($M^{-1}s^{-1}$)	k_2 (s^{-1})	K_{eq} (M^{-1})	k_{cat}/K_m ($mLmg^{-1}s^{-1}$)
Glucose-6-phosphate	9×10^{-11} [a]	1.5×10^{-8} [a]	4×10^{-3} [a]	120[b] 41[a]
Glucose-6-arsenate	7×10^{-6} [a]	2×10^{-3} [a]	3×10^{-3} [a]	12[a]
Glucose-6-vanadate	3.5×10^{1} [c]	4.2×10^{2} [c]	8.4×10^{-2} [c]	12 [c]

[a] From Reference 12; conditions were 25°C, pH 7.0.

[b] From Figure 17, conditions were 30°C, pH 7.4.

[c] From Figure 15, conditions were 30°C, pH 7.4.

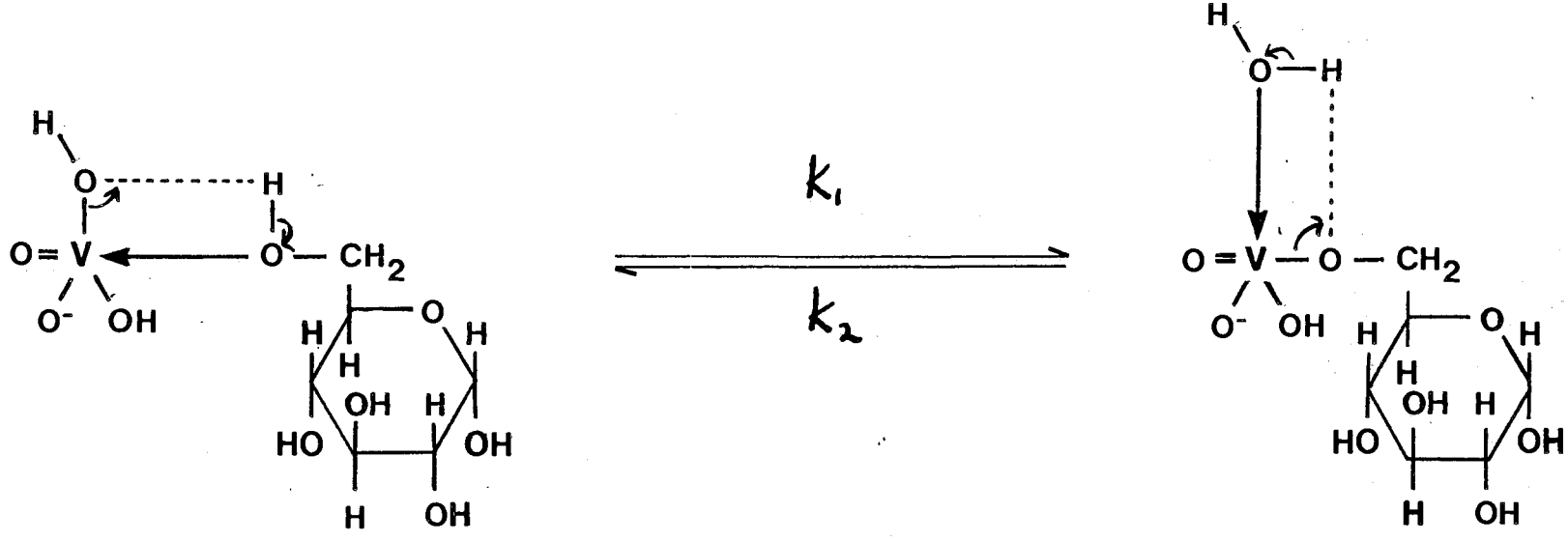
The most remarkable feature of this group of esters is the fact that although their equilibrium constants differ within a factor of thirty, their rates of formation are up to 11 orders of magnitude apart. The rate constant (k_1) for the formation of glucose-6-phosphate is extremely small. This was illustrated by a published experiment where glucose and phosphate, incubated together at room temperature for several years, produced less than one tenth of the concentration of glucose-6-phosphate expected at equilibrium (12). The k_1 for glucose-6-arsenate is 5 orders of magnitude larger than that of glucose-6-phosphate (12).

The k_1 for glucose-6-vanadate that has been determined from the reciprocal rate vs 1/(G6PDH) plot shown in Figure 15 is approximately eleven orders of magnitude larger than that for glucose-6-phosphate. The probable mechanism for ester formation involves the vanadate ion acting as an electrophile toward the hydroxyl groups of glucose. The glucose-6-phosphate analogue is the one that is the most likely to be accepted by the enzyme. One possible mechanism for ester formation and hydrolysis is shown in Figure 18.

The difference in magnitude that has been found between the formation constants of the glucose esters of phosphate, arsenate and vanadate is undoubtedly a function of the properties of the central atom of the anion. From Table II it can be seen that in the +5 oxidation state, the cation radius increases from phosphate to arsenate to vanadate (0.34, 0.47 and 0.59 respectively), while the elecronegativity decreases (2.1, 2.0 and 1.6) along this series. A larger central atom can accommodate more ligands; also a decrease in electronegativity implies a lower negative charge density close to the nucleus. From these considerations the trend for k_1 would be

Figure 18

A proposed mechanism for the spontaneous formation and hydrolysis of glucose-6-vanadate. k_1 and k_2 are the rate constants for the formation and hydrolysis respectively.


k1
k2

expected to increase from phosphate to arsenate to vanadate, as has been found (Table V).

In addition, vanadium V contains empty 3d orbitals which are at a lower energy than the empty 3d orbitals of phosphate and the empty 4d orbitals of arsenate. In aqueous solution, some five and six coordinate vanadium V complexes are known to exist, while only four coordinate phosphate complexes are stable under these conditions. The ability of vanadium to form a stable five coordinate species could significantly lower the energy of the transition state for the reactions of ester formation and hydrolysis, thus enhancing their rates.

B. Estimation of k_1 by ^{51}V Nuclear Magnetic Resonance.

The NMR spectrum shown in Figure 10 was used previously to calculate the equilibrium constant for the formation of glucose-6-vanadate from the peak areas of the vanadate and the 1-O-methyl-glucose-6-vanadate species. From the difference between the chemical shifts, $\Delta\nu$, of these peaks, an upper limit value for k_1, the rate constant for ester formation has been calculated. The presence of individual resonances for the peaks at ambient temperature placed an upper limit on the rate constant calculated by this method. Details of the procedure can be found in Appendix III.

The k_1 calculated using the NMR spectrum was equal to 43 $M^{-1}s^{-1}$, a value which is in reasonable agreement with the k_1 of 35 $M^{-1}s^{-1}$ determined kinetically. As noted earlier, the fact that there is a separation between the peaks indicates that the rate of exchange at ambient temperature will

be slower than that calculated using $\Delta\nu$.

(ii) Glycerol-3-vanadate and Dihydroxacetone Vanadate

Glycerol-3-phosphate dehydrogenase* (G3PDH) catalyses both the oxidation of glycerol-3-phosphate by NAD^+ and the reverse reaction, the reduction of dihydroxyacetone phosphate by NADH. Unlike the glucose/G6PDH system, G3PDH shows only a slight activity with the nonphosphorylated substrates glycerol and dihydroxyacetone. However, the addition of vanadate to a solution of G3PDH and glycerol or dihydroxyacetone along with the appropriate coenzyme produces a considerable enhancement of the reaction velocity.

The progress of the oxidation of glycerol by NAD^+, catalysed by G3PDH in the presence of vanadate can be followed by measuring the increase in absorbance at 340 nm. due to the production of NADH. The reverse reaction, reduction of dihydroxyacetone by NADH in the presence of vanadate, is followed by observing the decrease in absorbance as the NADH becomes oxidized.

The observed rate acceleration by vanadate is interpreted in terms of spontaneous vanadate ester formation; the vanadate ester, glycerol-3-vanadate or dihydroxyacetone vanadate, being accepted by G3PDH as a substrate analogue of glycerol-3-phosphate or dihydroxyacetone phosphate respectively. As the position of equilibrium was found to be independent of the quantity of vanadate used in the experiment, it is expected that the product of the reaction undergoes rapid hydrolysis, releasing the vanadate ion into solution for the activation of other reactant molecules.

Similar behavior has been reported for the reduction of dihydroxyacetone by NADH, catalysed by G3PDH in the presence of arsenate (13). In this case the ester dihydroxyacetone arsenate has been suggested to account for

* L-Glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8

the enhancement of the reaction rate by arsenate.

A model which describes the proposed mechanism for the activation by vanadate is presented in Figure 19.

The relationship between the kinetic parameters in Figure 19 is exactly analogous to that derived for the glucose/G6PDH system. Equation 9, restated below, was derived by considering a similar model for the acceleration of glucose oxidation in the presence of vanadate. It is used here to predict the behavior of the 1/Rate vs 1/e plots which describe the activation of glycerol oxidation by vanadate (Figure 20), and the activation of dihydroxyacetone reduction by vanadate (Figure 21). In Equation 9, X symbolizes either glycerol or dihydroxyacetone, according to which direction of reaction is being considered. Other symbols have the meanings previously defined; they appear unprimed in reference to glycerol oxidation and primed for dihydroxyacetone reduction in Figure 19. k_{cat}/K_m is referred to as k_3 in the figure.

$$\frac{1}{\text{Rate}} = \frac{k_2 K_m}{k_1 k_{cat}\, e[X][V]} + \frac{1}{k_1[X][V]} \qquad (9)$$

Figures 22 and 23 show the rate vs enzyme concentration curves for the forward and reverse reactions illustrated in Figure 19. The leveling-off of the acceleration at high enzyme concentrations is a recurrent characteristic of these experiments. In the case of the glucose/G6PDH system, it was suggested that ester formation becomes rate limiting at high enzyme concentration, as the enzyme activity was shown to be proportional to its concentration in studies with glucose. The rates of glycerol oxidation and dihydroxy acetone reduction, catalysed by G3PDH are very low, and the

Figure 19

The mechanism proposed to account for the acceleration of the enzymic oxidation of glycerol by NAD^+, and the reverse reaction, the reduction of dihydroxyacetone by NADH, in the presence of vanadate ion. G3PDH symbolizes the enzyme, glycerol-3-phosphate dehydrogenase. The k's indicate rate constants; unprimed for glycerol oxidation and primed for dihydroxyacetone reduction.

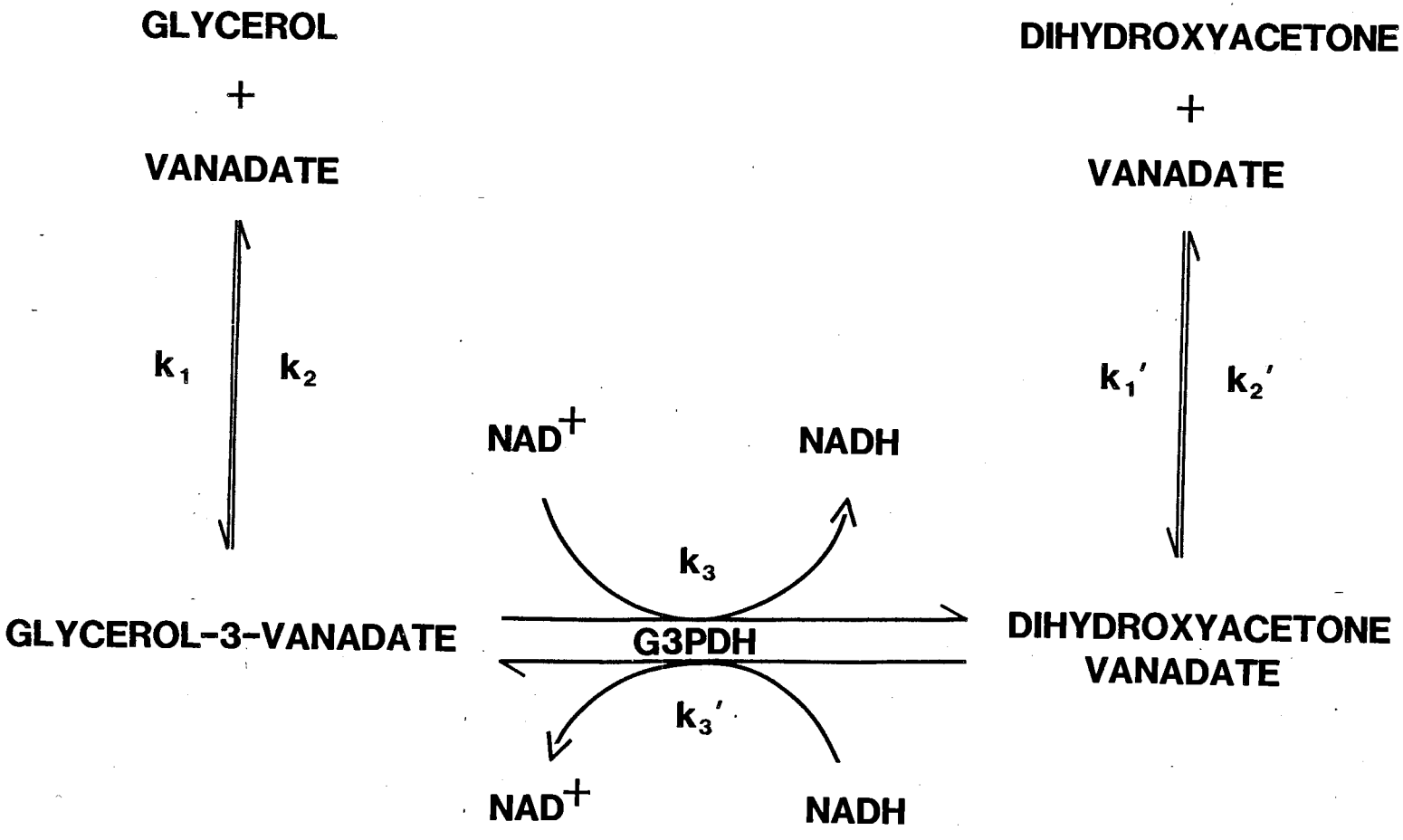

GLYCEROL
+
VANADATE
DIHYDROXYACETONE
+
VANADATE
k1
k2
k1'
k2'
NAD+
NADH
k3
GLYCEROL-3-VANADATE
G3PDH
DIHYDROXYACETONE
VANADATE
k3'
NAD+
NADH

Figure 20

The effect of different enzyme (G3PDH) concentrations on the rate of vanadate activated NAD^+ reduction in the presence of glycerol at pH 7.4. The reaction mixture contained 0.5 mM NAD^+, 62.5 mM glycerol, 12 μM vanadate and G3PDH concentrations as shown. The reaction was initiated by the addition of 5.0 μL of the stock vanadate solutions, blown in through capillary tubing, to a final volume of 0.4 mL of the reaction mixture. Figure 20 is a double reciprocal plot of the data from Figure 22.

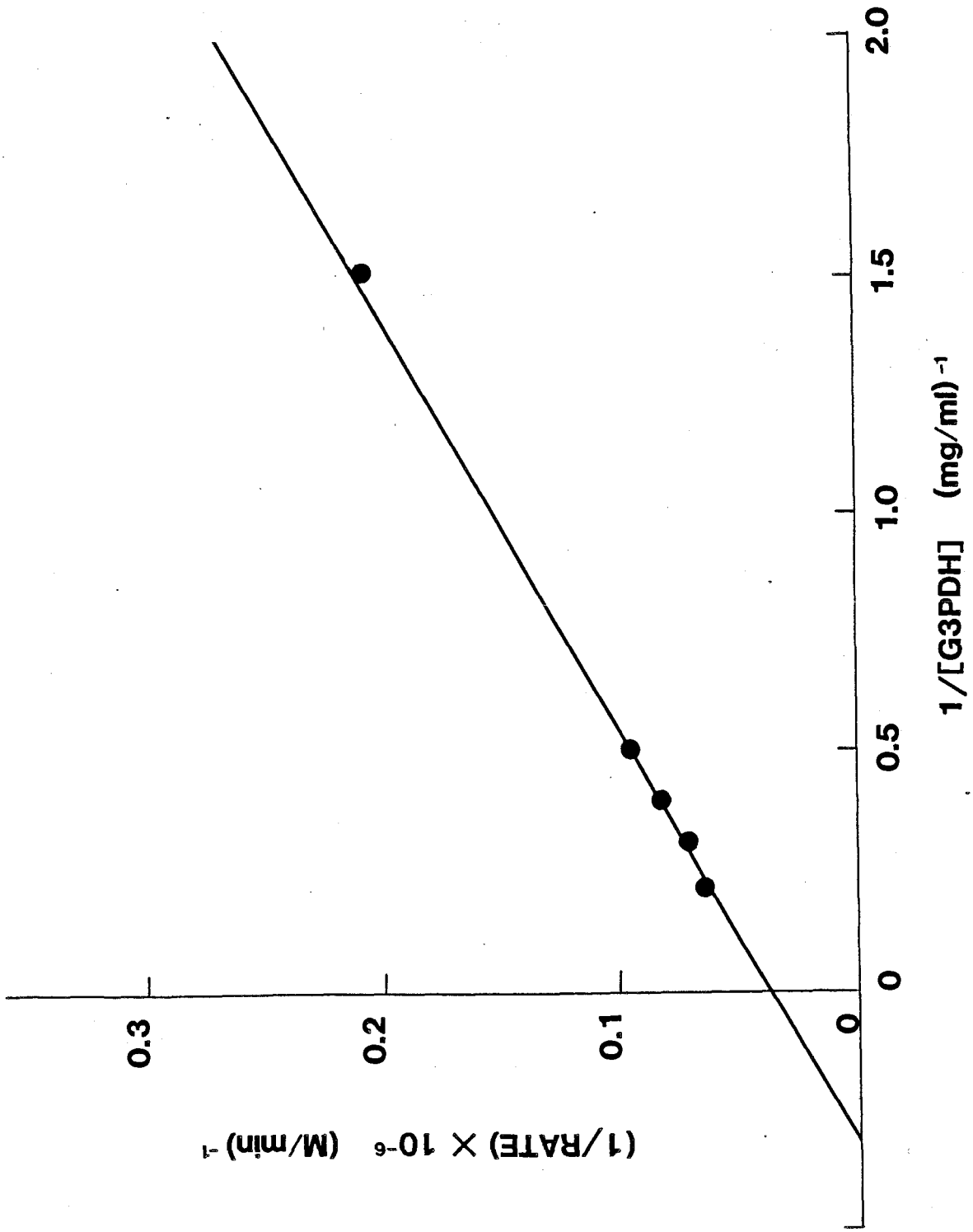

0.3
0.2
0.1
0
0
0.5
1.0
1.5
2.0
(1/RATE) × 10-6 (M/min)-1
1/[G3PDH] (mg/ml)-1

Figure 21

The effect of different enzyme (G3PDH) concentrations on the rate of vanadate activated NADH oxidation in the presence of dihydroxyacetone at pH 7.4. The reaction mixture contained 0.105 mM NADH, 1.25 mM dihydroxyacetone, 12 µM vanadate and G3PDH concentrations as shown. The reaction was initiated by the addition of 5.0 µL of the stock vanadate solution, blown in through capillary tubing, into a total volume of 0.4 mL of the reaction mixture at 30°C. Figure 21 is a double reciprocal plot of the data from Figure 23.

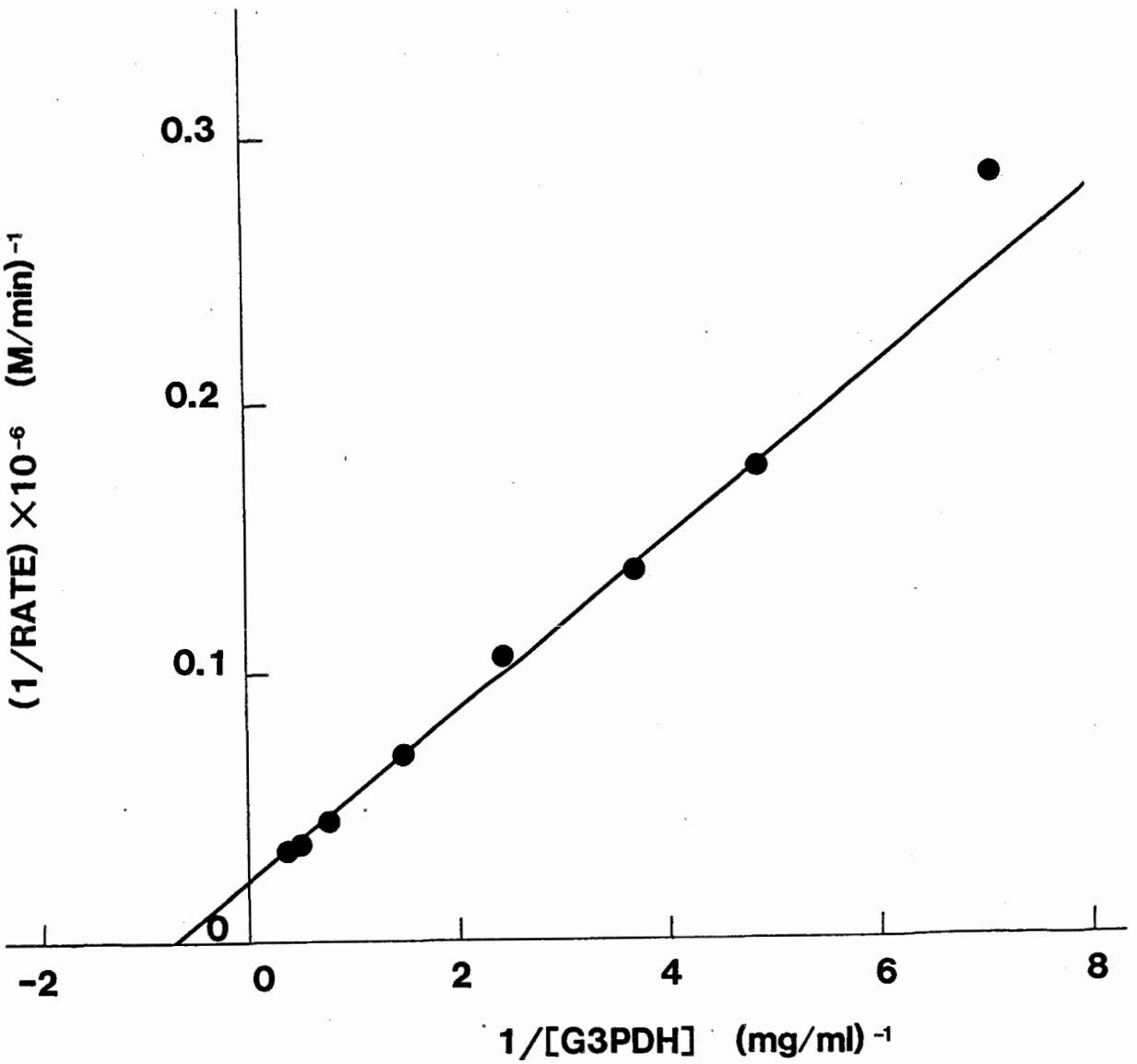

0.3
0.2
0.1
0
(1/RATE) ×10⁻⁶ (M/min)⁻¹
-2
0
2
4
6
8
1/[G3PDH] (mg/ml)⁻¹

Figure 22

The effect of different enzyme (G3PDH) concentration on the rate of vanadate activated NAD^+ reduction in the presence of glycerol. Procedures and concentrations are the same as those described in Figure 20.

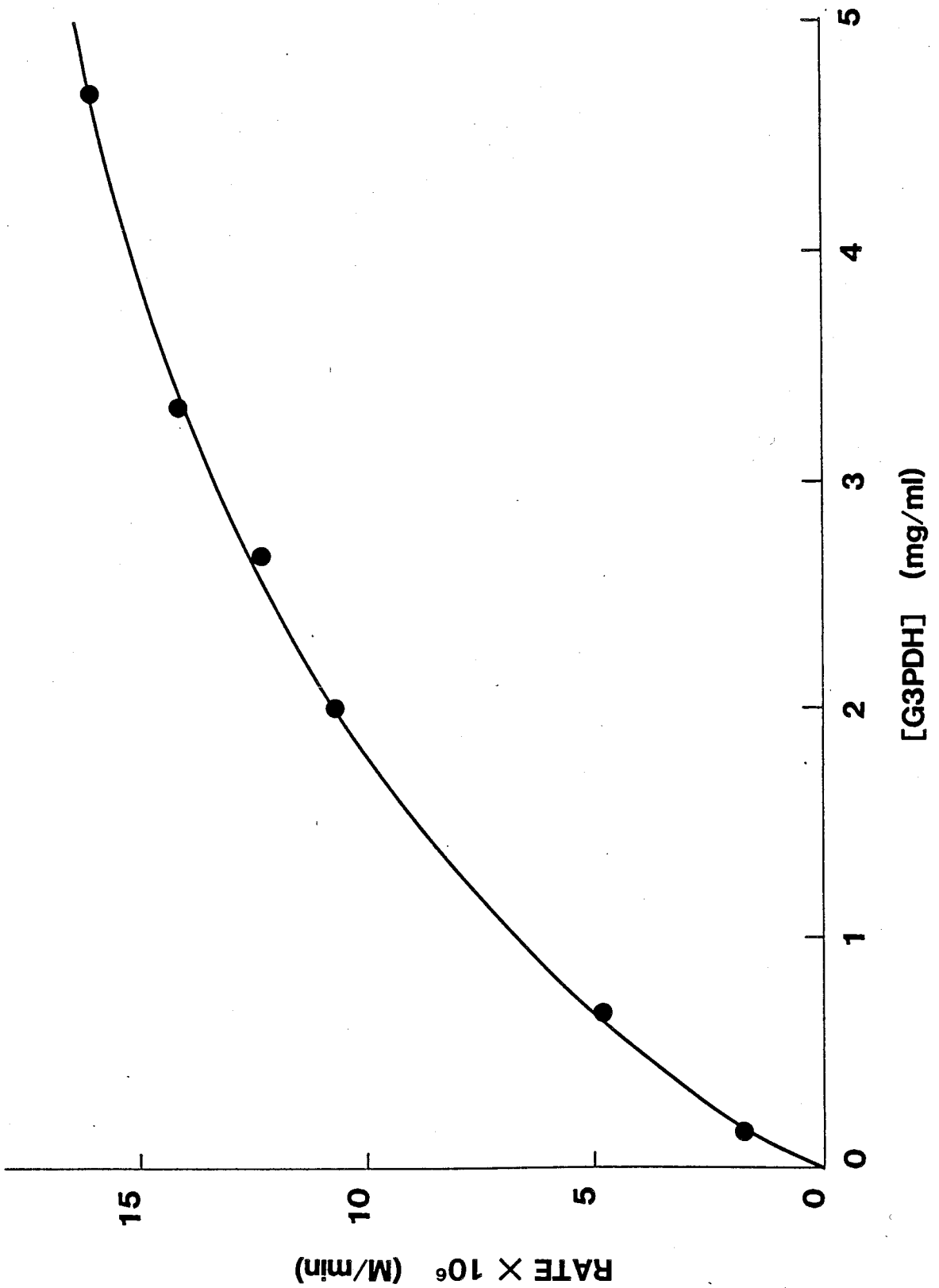
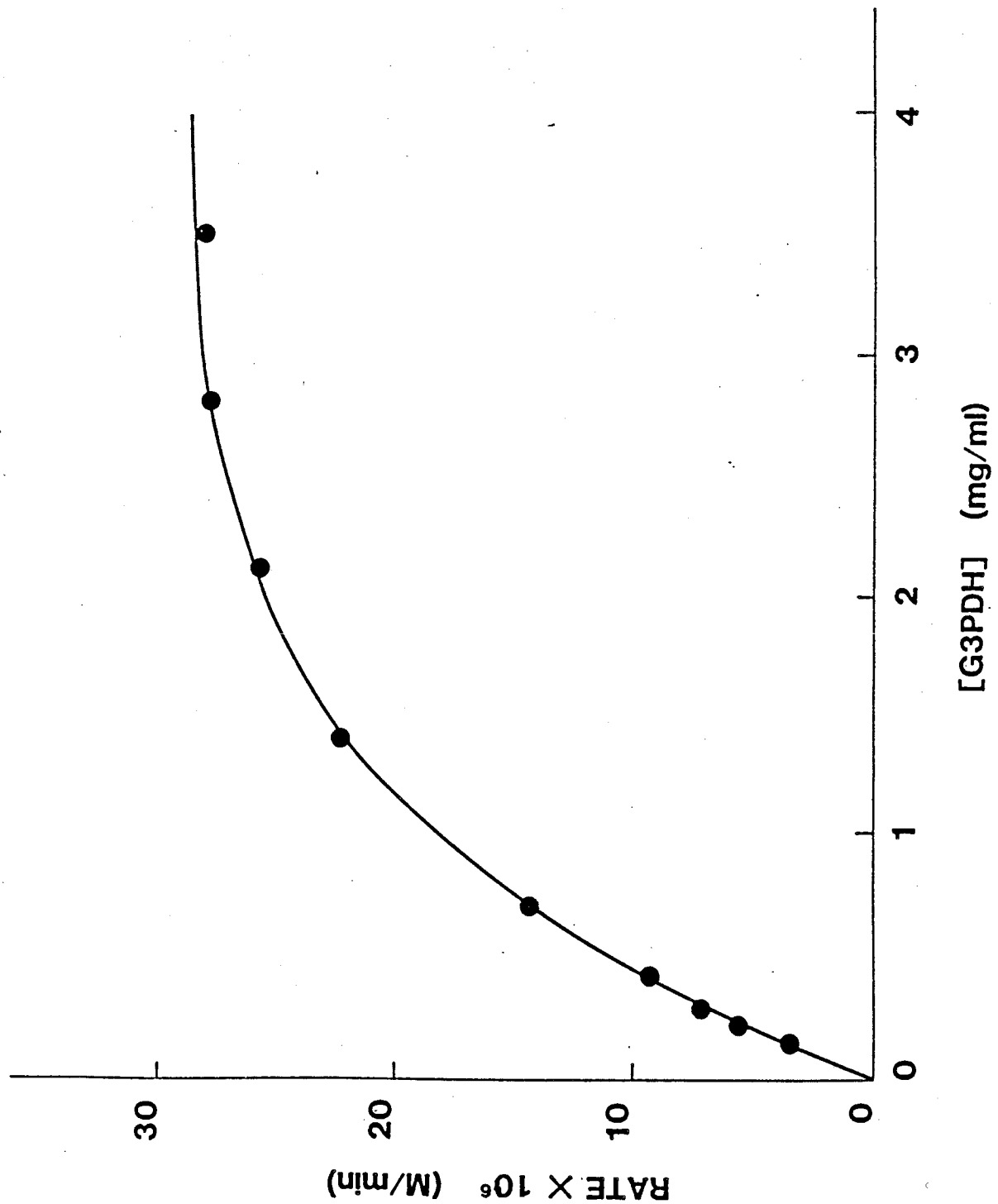

RATE × 10⁶ (M/min)
0
5
10
15
[G3PDH] (mg/ml)
0
1
2
3
4
5

Figure 23

The effect of different enzyme (G3PDH) concentrations on the rate of vanadate activated NADH oxidation in the presence of dihydroxyacetone. Procedures and conditions are those which were described for Figure 21.


RATE X 10⁶ (M/min)
0
10
20
30
0
1
2
3
4
[G3PDH] (mg/ml)

activity vs. enzyme concentration was not examined. It is possible that the curvature of the rate vs. enzyme concentration plot for G3PDH is a reflection of a decrease in enzyme activity. Alternatively, the curvature could be explained if ester formation becomes rate limiting at high enzyme concentrations.

The rate constants for the formation (k_1 and k_1') of the putative esters glycerol-3-vanadate and dihydroxyacetone vanadate have been calculated from the vertical intercepts of Figures 20 and 21 respectively. Rate constants for ester hydrolysis (k_2 and k_2') were calculated using the equilibrium relation $K_{eq} = k_1/k_2$. Values for the equilibrium constants were obtained from the peak area ratios of vanadate ester to vanadate recorded on the ^{51}V NMR spectra (Figures 11 and 12). An example of this calculation can be found in Appendix II. The values for k_2 and k_2' were used to determine the k_{cat}/K_m ratios for glycerol-3-vanadate and dihydroxyacetone vanadate as substrates for G3PDH, from the horizontal intercepts of Figures 20 and 21 respectively. The calculated constants are displayed in Table VI, along with the corresponding values for glycerol-3-phosphate and dihydroxyacetone phosphate that have been reported in the literature.

TABLE VI

Kinetic and equilibrium constants for the phosphate and vanadate esters of glycerol and dihydroxy acetone. k_1 and k_2 are rate constants for ester formation and hydrolysis respectively. Conditions were 30°C, pH 7.4 unless otherwise indicated.

Ester	k_1 ($M^{-1}s^{-1}$)	k_2 (s^{-1})	K_{eq} (M^{-1})	k_{cat}/K_m ($mLmg^{-1}s^{-1}$)
Glycerol-3-vanadate	0.60	4.6	0.13	1.5
Glycerol-3-phosphate	-	-	0.024 [a]	0.50 [b]
Dihydroxyacetone vanadate	50	128	0.39	84
Dihydroxyacetone Phosphate	-	-	-	7.6 [b]

[a] From Reference 24: conditions were 25°C, pH 7.0.

[b] From Reference 30: conditions were 25°C, pH 7.0.

It is apparent from Table VI that the rate constants for the formation of glycerol-3-vanadate and dihydroxyacetone vanadate are within two orders of magnitude of each other and of that of glucose-6-vanadate (Table V). It is expected that the k_1 values for the corresponding phosphate esters will be many orders of magnitude smaller, as is the case with glucose-6-phosphate, but in this case literature values are not available for comparison.

The k_{cat}/K_m values that have been calculated for glycerol-3-vanadate and dihydroxyacetone vanadate are unexpectedly higher than those published by Bentley and Dickinson (29) for glycerol-3-phosphate and dihydroxyacetone phosphate. Since the k_{cat}/K_m ratio expresses the specificity of an enzyme for its substrate, it is usual for the natural substrate to produce the highest value for this ratio. A truly quantitative comparison can be made only when both experiments have been performed under the same conditions. Since Bentley and Dickinson used a phosphate buffer and a different enzyme preparation, their results may not reflect the k_{cat}/K_m values for glycerol-3-phosphate and dihydroxyacetone phosphate under the conditions for which this ratio has been determined for glycerol-3-vanadate and dihydroxyacetone vanadate. Because of these considerations an attempt was made to obtain the k_{cat}/K_m values for glycerol-3-phosphate and dihydroxyacetone phosphate under the conditions of the vanadate experiments. This attempt was not successful for the reasons discussed below.

The enzyme G3PDH is strongly inhibited by its own substrates and coenzymes (29). Therefore, a large extrapolation is necessary to obtain a value for the rate of reaction at infinite substrate concentration. The low

concentrations essential to prevent enzyme inhibition lead to low rates of reaction. Although these low rates were detected by absorbance spectroscopy, they could not be differentiated. In order to measure the kinetic parameters correctly, it will be necessary to use more sensitive instrumentation, such as the fluorescence spectrophotometer and rapid-mixing apparatus used in the experiments done by Bentley and Dickinson (29).

Another reason for the high values obtained for the k_{cat}/K_m ratios of the vanadate esters could be that glycerol and dihydroxyacetone do not inhibit the enzyme to the same extent as the phosphate esters. In a study with G3PDH by Jaffé and Apitz-Castro (13), in which the activation by arsenate of dihydroxyacetone reduction was measured, dihydroxyacetone did not exhibit the enzyme inhibition characteristic of dihydroxyacetone phosphate.

IV. Margin of Error

The k_{cat}/K_m ratio calculated for the natural substrate is dependent upon measurement of the true initial velocity of the reaction, as the value for the total enzyme concentration ($e = [E]_o$) has been used in the calculations. Since a period of approximately five seconds elapsed before the velocity was measured in these experiments, it is possible that this factor could lead to some error in the k_{cat}/K_m value. The result of a delay in measurement would be that the initial velocities detected would be lower than their true values. Since the slope of the 1/Rate *vs* 1/(Substrate) plot is equal to $K_m/k_{cat}e$, the steeper slope resulting from the lower velocities would lead to a lower value for k_{cat}/K_m. Therefore, it is possible that the k_{cat}/K_m calculated for glucose-6-phosphate

($120\ mL mg^{-1}\ s^{-1}$) has been underestimated.

As noted in the previous section, the use of a rapid-mixing device would improve the measuring technique considerably.

Both the k_{cat}/K_m ratio and the k_1 calculated for the vanadate esters are dependent upon the measurement of the steady-state velocity, as it is the steady-state concentration of the ester which leads to Equation 9. A progress curve which clearly delineates the linear, steady-state phase of the reaction is desirable, but in many cases product inhibition causes the linear portion to become curved. This effect was prevalent in the case of glycerol oxidation, less so with glucose oxidation, and only slight with dihydroxyacetone reduction.

The fact that the steady-state velocity is difficult to locate accurately leads to uncertainties in the calculation of kinetic parameters. In the experiments performed on glucose, glycerol and dihydroxyacetone, the assumption was made that the steady-state velocity was equivalent to the velocity measured approximately five seconds after the reaction was initiated; this was the earliest measurement possible given the equipment at hand. This is a reasonable assumption, since the equilibrium concentration would be reached in the order of milliseconds due to the high rate constants for ester formation. However, there is a possibility that even only five seconds after initiation, product inhibition could result in low velocity readings. If this is the case, the true values of k_{cat}/K_m and k_1 will be higher than those reported here.

CONCLUSION

Rapid, spontaneous vanadate ester formation with the hydroxyl groups on glucose, glycerol and dihydroxyacetone is supported by evidence from NMR and from enzymic rate studies. The fact that vanadate accelerates the oxidation of glucose catalyzed by glucose-6-phosphate dehydrogenase, but not that of 6-deoxyglucose indicates that vanadate requires a free hydroxyl group on the 6-carbon of glucose to express its activity. The failure of vanadate to inhibit the oxidation of glucose-6-phosphate under similar conditions (21) shows that, at these concentrations, this ion does not significantly interact with the enzyme.

Sulphate also activates the enzymic oxidation of glucose. However, its behavior with other substrates implies that it activates by a different pathway than that of vanadate. Sulphate accelerates the oxidation of 6-deoxyglucose and inhibits the oxidation of glucose-6-phosphate (19), results consistent with the formation of sulphate/enzyme complex which facilitates binding with 6-deoxyglucose, but blocks binding with glucose-6-phosphate.

The rate constants of formation for vanadate esters have been found to be many orders of magnitude higher than those of phosphate esters. Physiologically, the concentration of phosphate esters is controlled by enzymes which catalyze phosphorylation and dephosphorylation reactions according to the requirements of the cell. The ability of vanadate to spontaneously form compounds which can substitute for phosphate substrates in enzymic reactions may be responsible for some of its toxic effects.

Although the physiological concentration of vanadate is normally much lower than that of phosphate, the equilibrium constants for the formation of vanadate esters are about 10 times greater than those of the corresponding phosphate esters, for the compounds that were measured in this study. The more favorable equilibrium constants for the formation of vanadate esters make it more likely that their concentrations will become significant if the level of vanadate is increased, as it might be under toxic conditions.

The ability of the vanadate ion to react with hydroxyl groups, its facile reduction at cell potentials, its tendency to oligomerize and its ionization behavior combine to make it a useful investigative tool for biological systems.

APPENDIX I

From the model illustrated in Figure 5, the rate of reaction (R) is:

$$R = \frac{d[\text{Gluconate}]}{dt} = k_5[G][E] + k_3[E][G\text{-}6\text{-}V] + k_4[E\cdot S][G] \qquad (1a)$$

In order to express [E], [ES] and [G-6-V] in terms of measurable variables, three additional equations are necessary,

- The association constant for the enzyme binding with sulphate:

$$K_c = \frac{[E\cdot S]}{[E][S]} \qquad (1b)$$

- The total enzyme concentration, e:

$$e = [E] + [E\cdot S] \qquad (1c)$$

- The rate of formation of G-6-V:

$$\frac{d[G\text{-}6\text{-}V]}{dt} = k_1[G][V] - k_2[G\text{-}6\text{-}V] - k_3[E][G\text{-}6\text{-}V] \qquad (1d)$$

At the steady state, the rate of formation of G-6-V is equal to its rate of hydrolysis, therefore $d(G\text{-}6\text{-}V)/dt = 0$. Rearrangement of 1d gives the value for the steady state concentration of G-6-V.

$$[G\text{-}6\text{-}V] = \frac{k_1[G][V]}{k_2 + k_3[E]} \qquad (1e)$$

Rearranging 1b and substitution with 1c leads to expressions for [E] and [ES] in terms of total enzyme concentrations, sulphate concentration and K_c.

$$[E\cdot S] = K_c[E][S]$$

$$= K_c[S](e - [E\cdot S])$$

$$[E\cdot S] = \frac{K_c[S]e}{1 + K_c} \qquad (1f)$$

$$[E] = \frac{[E\cdot S]}{K_c[S]}$$

$$= \frac{K_c[S]e}{1 + K_c[S]} \times \frac{1}{K_c[S]}$$

$$[E] = \frac{e}{1 + K_c[S]} \qquad (1g)$$

Substitution of 1g into 1e gives:

$$[G\text{-}6\text{-}V] = \frac{k_1[G][V]}{k_2 + k_3\left(\frac{e}{1 + K_c[S]}\right)} \qquad (1h)$$

When 1f, 1g and 1h are substituted into 1a and the simplifying assumption is made that $k_2 >> k_3e\left(1 + K_c[S]\right)$, which would be true at low enzyme concentration, Equation 1 is obtained.

$$R = \frac{k_5[G]\,e + \frac{k_1k_3}{k_2}\,e\,[G][V] + k_4K_c\,e\,[G][S]}{1 + K_c[S]} \qquad (1)$$

APPENDIX II

The peak at 560 ppm. on the NMR spectrum of the glycerol/vanadate solution (Figure 11) which is assigned to the monoester glycerol-3-vanadate, is actually composed of a mixture of the primary and secondary monoesters. Since the secondary ester is unlikely to contribute significantly to the vanadate activation, it is desirable to separate its quantity from the ester peak in order to obtain a value for the primary monoester/vanadate ratio.

Fordham and Wang (26) have calculated the equilibrium ratio of primary to secondary phosphate esters which would be undergoing exchange in a solution of glycerol phosphate in water. Assuming that the ratio of vanadate esters is the same as that of the phosphate esters, the equilibrium constant for the formation of the primary ester can be calculated from the value for the total ester obtained from Figure 11.

The equilibrium constant for the total amount of monoester formed (K_{eq}) is calculated from the peak area ratio of monoester to vanadate (P_{GV}/P_V), where G stands for glycerol and V for vanadate. [G] is the concentration of glycerol.

$$K_{eq} = \frac{P_{GV}}{P_V[G]}$$

$$= \frac{(387)}{(1176)(1.0\ M)}$$

$$= 0.33\ M^{-1}$$

The concentration of primary vanadate monoester (p) can be calculated using the value for the primary/secondary ratio (p/s) obtained by Fordham and Wang (26) for phosphate esters.

$$\frac{p}{s} = 0.67$$

$$K_{eq} = \frac{(p+s)}{[G]([V]-(p+s))}$$

$$0.33 = \frac{(p + p/0.67)}{(1.0\ M)(5.0 \times 10^{-4}\ M - (p+p/0.67))}$$

$$p = 0.5 \times 10^{-4}\ M$$

The equilibrium constant for the formation of the primary vanadate monoester (K'_{eq}) can now be calculated.

$$K'_{eq} = \frac{p}{[G]([V] - (p+p/0.67))}$$

$$= \frac{(0.5 \times 10^{-4}\ M)}{(1.0\ M)(5.0 \times 10^{-4} - 1.2 \times 10^{-4})M}$$

$$= 0.13\ M^{-1}$$

APPENDIX III

The Gutowsky-Holm equation (28) provides the rate constant (k_{ex}) for an exchange between two equally populated sites at the coalescence temperature, where $\Delta\nu$ is the difference in chemical shift between the two species.

$$k_{ex} = \frac{\pi}{\sqrt{2}} \Delta\nu$$

Using this relation and their new approximation formula, Fraser et al. (28) obtained rate constants for the case of unequal singlet peaks within 4% of the more accurate but cumbersome method of complete line shape analysis. For the process

$$G + V \rightleftarrows GV + H_2O$$

where G = 1-O-methylglucose

V = vanadate

GV = Glucose vanadate monoester

At equilibrium:

$$k_1[G][V] = k_2[H_2O][GV]$$

where k_1 and k_2 are the rate constants for vanadate ester formation and hydrolysis respectively.

The Fraser et al. formulations are:

$$k_V = 2k_{ex}P_{GV} \quad \text{for vanadate going to glucose vanadate ester}$$
$$= k_1(G)$$

$$k_{GV} = 2k_{ex}P_V \quad \text{for ester hydrolysis}$$
$$= k_2(H_2O)$$

where P is the relative population ratio of the species denoted by the subscript. The population ratio of each vanadate species is determined by the ratio of its peak area to the total peak area of vanadate species.

From Figure 10,

$$\Delta\nu = 131 \text{ Hz}$$

$$k_{ex} = \frac{\pi}{\sqrt{2}}(131 \text{ s}^{-1}) = 291 \text{ s}^{-1}$$

$$P_V = 0.885$$

$$P_{GV} = 0.115$$

$$k_V = 2(291 \text{ s}^{-1})(0.115) = 67.1 \text{ s}^{-1}$$

$$k_{GV} = 2(291 \text{ s}^{-1})(0.885) = 515 \text{ s}^{-1}$$

$$k_1 = \frac{k_V}{[G]} = \frac{67.1 \text{ s}^{-1}}{1.55 \text{ M}} = 43 \text{ M}^{-1} \text{ s}^{-1}$$

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