HEXOKINASE

STUDIES OF HEXOKINASE FROM HEART

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CHAPTER I

INTRODUCTION

INTRODUCTION

The main pathway of metabolism of glucose in many organisms involves the formation of phosphorylated sugars. Meyerhof first used the term hexokinase for his preparation of yeast autolysate which catalyzed the phosphorylation of glucose by ATP to give hexose-6-phosphate. Hexokinase has been reported to be present in a wide variety of sources: animal tissues, plants and micro-organisms. Several workers have obtained hexokinase in crystalline form from yeast (Berger et al. 1946; Bailey and Webb, 1948; Kunitz and McDonald, 1946; Darrow and Colowick, 1962; and Schulze, Gazith and Gooding, 1966) and the properties of pure yeast hexokinase have been studied in great detail. Little progress has, however, been made in obtaining highly purified hexokinase from animal tissues. This is mainly due to the low activity of the starting material, its attachment to particulate material or its instability. However in recent years considerable progress has been made in the study of animal tissue hexokinases especially from brain (Joshi and Jagannathan, 1966; Schwartz and Basford, 1967). The present work deals with the preparation of hexokinase in soluble form from the particulate fraction of ox heart, its purification and the study of its properties and kinetics.

Historical

In 1927 Meyerhof observed an increased rate of glucose utilization when the alcohol precipitated fraction of yeast autolysate was added to the extracts of frog or rabbit muscle. Lutwak-Mann and Mann (1935) reported the presence of an enzyme that phosphorylated glucose in the presence of ATP in yeast. According to Bauer (1936) the enzyme phosphorylated glucose, fructose and mannose, but not ribose or galactose, at a Mg^{2+} concentration of 1 to 10 mM. The enzyme was active over a wide range of glucose concentrations and had a broad pH optimum between pH 5.3 and 7.9. In 1937 von Euler and his colleagues reported the presence of hexokinase in brain extracts.

Hexokinase was shown to be present in a wide variety of animal tissues, plants and microorganisms. In animal tissues hexokinase was found to occur in two forms - soluble and particulate.

Berger et al and Kunitz and McDonald isolated crystalline yeast hexokinase in 1946. Detailed studies were carried out on the mechanism of action, properties, specificity and kinetics of the pure enzyme.

Progress in the study of animal tissue hexokinases was relatively slow. The observation that these enzymes are generally inhibited by glucose-6-phosphate (Crane and Sols, 1953) and that the inhibition is reversed by inorganic phosphate (Rose, Warms and O'Connell, 1964) is of considerable interest. The isolation of purified brain hexokinase (Joshi and Jagannathan, 1966) (Schwartz and Basford,1967) and the discovery of several isoenzymes, including a

glucokinase in animal tissues are among other recent advances in this field.

A brief survey of the relevant literature is given in the following sections.

Literature Survey

The name for the group of enzymes included under the term hexokinase (recommended by the International Union of Biochemistry) is "Adenosine triphosphate-D-hexose-6-phosphotransferase (EC 2.7.1.1)."

$$ATP + D\text{-hexose} \longrightarrow ADP + D\text{-hexose-6-phosphate} + H^+$$

The name 'hexokinase' will be used to describe the group of enzymes which catalyze the phosphorylation of glucose and other D-hexoses in the presence of ATP to form hexose-6-phosphate and ADP. Kinases which phosphorylate hexoses, namely fructose and galactose, in the presence of ATP to form the corresponding hexose-1-phosphates will not be discussed. It will not be possible to include in this survey the interesting enzymes which are active with phosphate donors other than nucleotides e.g. acetyl phosphate and phosphoenol pyruvate. Only brief references will be made to enzymes of narrow specificity such as glucokinase. This survey is mainly restricted to hexose (glucose, fructose and mannose) - ATP transphosphorylases, and especially of heart and other animal tissues and of yeast.

SECTION I

ANIMAL TISSUES

The presence of hexokinase has been reported in the following tissues: brain, heart, adipose tissue, ascites cells, erythrocytes, intestine, kidney, liver, muscle (skeletal), pancreas, retina, testis, skin, uterus and mammary gland.

As stated earlier, animal tissue hexokinases have been difficult to obtain in homogeneous form since they are highly unstable or attached to insoluble particles. However, in recent years considerable progress has been made in the study of these hexokinases. A characteristic feature of these animal tissue hexokinases is that glucose-6-phosphate at low concentrations inhibits them. This inhibition is partially reversed by inorganic phosphate.

Particulate and soluble hexokinase

Crane and Sols (1953) have estimated the relative amounts of particulate and soluble hexokinase in different tissues. The relative amounts of enzyme sedimented and remaining in the supernatant liquid (on centrifugation at 18,000 x g for 60 minutes) varied considerably for extracts from different tissues. Rat brain had almost 90% of the enzyme in the particulate fraction, whereas all the hexokinase of red blood corpuscles was in the soluble fraction. Heart muscle contained equal amounts of enzyme in the two forms whereas liver and stomach had about 35%, kidney 47% and intestinal mucosa 73% of the hexokinase activity in the particulate fraction.

Brain

Hexokinase from brain has been studied extensively by various workers (von Euler et al. 1937), Geiger (1940), Ochoa (1941), Meyerhof (1947), Crane and Sols (1953), Schwartz and Basford (1967), Fromm and Zewe (1962), Joshi and Jagannathan (1966, 1968), Moore (1968).

Bennet et al (1962) have studied the hexokinase from different parts of the brain. They reported the relative hexokinase activity of different parts to be:visual cortex, somesthetic cortex 106%, remainder of dorsal cortex 93%, hypothalamus 79%, cerebellum 100%, ventral cortex 86%, caudate 73%, olfactory bulbs 70%, remainder of subcortex 67%, medulla and pons 57%.

Crane and Sols (1953) reported that 90% of the hexokinase activity of homogenates of rat brain and calf brain is associated with the particulate fraction. They separated the particulate fraction by centrifugation and by treatment with lipase and deoxycholate and obtained a 45-50-fold increase in specific activity. Bachelard (1967) stated that more than 80% of the mitochondrial activity was extracted by freezing and thawing the suspension in 50 mM glycylglycine (which contained 5 mM KCl, 5 mM $MgCl_2$, 2 mM cysteine and 5 mM ATP, pH 7.0).

Biesold (1965) determined hexokinase activity in subcellular fractions of rat brain tissue. Fractions were obtained by fractional centrifugation of 10% tissue homogenate but 67% of the total hexokinase was found in the mitochondria.

Teichgraber and Biesold (1968) reported that the total mitochondrial hexokinase activity can be solubilized completely by incubation

with salt and Triton X-100. This activity could not be entirely released by washing with sucrose or by freezing and thawing.

Thompson and Bachelard (1969) solubilized the enzyme from mitochondria of ox brain in the following way. Purified ox brain mitochondria were suspended in 0.32 M sucrose at 1-2 mg protein/ml and were then treated with 0.5% Triton X-100 and 1 M KCl at pH 6.5 or 7.5 at 0° and then centrifuged at 80,000 x g for 20 minutes. They stated that almost all the hexokinase activity was obtained in the supernatant fraction.

Wilson (1968) reported that the particulate hexokinase activity of rat brain is interconvertible among soluble, particulate and latent (particulate) forms. Glucose-6-phosphate, ATP, AMP, ADP, various chelating agents and high salt concentrations caused solubilization of the particulate enzyme.

Though there are several reports of "solubilization" of animal tissue hexokinases, there are very few reports on the purification of the soluble material. From the point of view of isolation of pure enzyme, the ability to purify an enzyme is more important than more inability to sediment an enzyme at 20,000 x g or 100,000 x g. The presence of detergents frequently makes an enzyme difficult to sediment, but does not always permit purification. There are only two reports on the isolation of animal tissue hexokinases of high purity (Joshi and Jagannathan, 1966 and Schwartz and Basford, 1967). Both the groups used ox brain. Joshi and Jagannathan solubilized the enzyme by treatment with crystalline pancreatic elastase and then purified it by freezing and thawing, treatment with protamine sulfate and fractionation with

ammonium sulfate, calcium phosphate gel and DEAE-cellulose. The final preparation had a specific activity of 63 units/mg and was 90% pure by ultracentrifugal analysis.

Schwartz and Basford solubilized the enzyme by chymotrypsin digestion and Triton-X-100 treatment and purified it by chromatography twice on DEAE cellulose and fractionation with ammonium sulfate. The final preparation had a specific activity of 80 units/mg and was ultracentrifugally homogeneous. It is of interest, however, that the preparation of Joshi and Jagannathan had a $S_{20,w}$ of 5.86 whereas the latter authors reported an uncorrected sedimentation constant of 4.44 S at 21°.

The addition of sucrose at 0.5-1.0 M in the presence of 0.1-0.2 M phosphate buffer (pH 7.5) and 0.005-0.010 M thioethanol was found to have an extraordinary stabilizing effect on hexokinase by Joshi and Jagannathan. In the presence of very high sucrose concentrations the enzyme could be heated at 50° without loss of activity, whereas even in 0.1 M sucrose or glucose it could not be stored even for 1-2 days at 0° or -20°.

The K_m values for different carbohydrates were reported to be glucose 8×10^{-6} M, fructose 1.6×10^{-3} M, 2-deoxyglucose 2.7×10^{-5} M, glucosamine 8×10^{-5} M, mannose 5×10^{-6} M, 1,5 sorbitan 3×10^{-2} M (Crane and Sols (1954,1955). The relative activities with glucose, 2-deoxyglucose and 1-5 sorbitan were nearly the same, whereas mannose and glucosamine gave about half the activity and fructose about 1.5 times the activity with glucose. The enzyme was competitively inhibited by N-acetyl glucosamine, 6-deoxy-D-glucose and D-xylose. The K_m value for Mg^{2+} was 8×10^{-4} M at 10 mM ATP and the K_m for ATP 1.3×10^{-4} M in the presence of 5 mM Mg^{2+}. The brain enzyme had a broad pH optimum

between pH 6 and 8. The activity increased2.2 fold over the range 30° to 40°.

Fromm and Zewe (1962a) determined the K_m values for glucose at different ATP concentrations and the K_m values for ATP at different glucose concentrations.

Schwartz and Basford (1967) reported that solubilized particulate brain hexokinase was homogeneous with respect to ultra-centrifugation and electrophoresis on cellulose acetate paper. Their partial amino acid analysis of brain hexokinase indicated a low content of tyrosine, proline and histidine and a high content of glycine, leucine, aspartic acid and glutamic acid. The K_m values for glucose were 5×10^{-5}M (at 2×10^{-3}M ATP), 3×10^{-5}M (at 7.5×10^{-4}M ATP) and 1.3×10^{-5}M (at 2.5×10^{-4}M ATP). Similarly the K_m values for ATP were 4.89×10^{-3}(at 1.2×10^{-3}M glucose) and 2.83×10^{-3}M (at 5×10^{-5}M glucose).

Joshi and Jagannathan (1968) reported a broad pH optimum between 5.5 and 8.0. K_m values for ATP, Mg^{2+}, glucose, mannose and fructose were 0.5, 2.8, 0.038, 0.03 and 1.5 mM respectively. They studied glucose-6-phosphate inhibition and its reversal by Pi in detail. The Ki values for glucose-6-phosphate were 0.03, 0.11 and 0.16 mM at 0.05, 10 and 30 mM Pi respectively. Insulin, growth hormone and other hormones had no effect on the hexokinase activity with or without glucose-6-phosphate and Pi. Moore (1968) studied the kinetics of soluble and particulate beef brain hexokinase. The kinetic constants are listed in Table 1.

Crane and Sols (1954) reported that brain hexokinase was inhibited by ADP and that this inhibition was competitive with respect to ATP. Fromm and Zewe (1962a) reported that the addition of ADP caused a decrease in maximal velocity as well as an increase in K_m for ATP. The inhibition by ADP was uncompetitive with respect to glucose. Bachelard and Goldfarb (1969) stated that the cerebral cortex hexokinase was inhibited by ADP. This inhibition was dependent on Mg^{2+}/ATP ratio. ADP at Mg^{2+}/ATP ratios 2:1 exhibited mixed type inhibition while at Mg^{2+}/ATP ratios 1:1 the inhibition appeared to be competitive with respect to Mg ATP^{2-}.

Weber (1969) reported that phenyl pyruvic acid is a competitive inhibitor of brain hexokinase. Newsholme, Rolleston and Taylor (1968) reported that 50% inhibition was produced by 0.023, 0.046 and 0.068 mM glucose-6-phosphate for soluble, particulate and crude homogenate respectively. The optimum Mg^{2+} concentration was 10 mM irrespective of ATP concentration. 1 mM Ca^{2+} inhibited the enzyme with or without glucose-6-phosphate. They have reported that the glucose-6-phosphate inhibition could be relieved by Pi and α-glycerol phosphate.

Thompson and Bachelard (1970) reported the following kinetic properties of the purified mitochondrial hexokinase. They obtained two peaks. Peak I K_m for ATP 0.60 mM and K_m for glucose 0.042 mM. Peak II K_m for ATP 0.66 mM, and K_m for glucose 0.043 mM. They also obtained two peaks of purified soluble hexokinase. For peak I K_m for ATP was 0.56 mM and K_m for glucose was 0.048 mM; for peak II the K_m for ATP was 0.68 mM and K_m for glucose 0.062 mM.

Tuttle and Wilson (1970) studied the characteristic of soluble and particulate forms of rat brain hexokinase. The K_m for glucose (at either 4 mM or 8 mM ATP) was 0.029 mM for the soluble and 0.053 mM for the particulate form. The K_m values for ATP (at either 2 or 25 mM glucose) were 0.24 and 0.20 mM for the soluble and particulate fractions respectively. N-acetylglucosamine inhibited competitively with respect to glucose. Both fractions were equally sensitive to this inhibition.

Skeletal muscle

In skeletal muscle, hexokinase is mostly present in the soluble fraction. Crane and Sols (1955), Hanson and Fromm (1965), Toews (1966) and Grossbard and Schimke (1966) described methods for the partial purification of the enzyme. Toews (1966) partially purified the rat skeletal muscle enzyme by ammonium sulphate fractionation and gel filtration. Grossbard and Schimke (1966) reported a final specific activity of about 13 units per mg at 37° of rat skeletal muscle hexokinase, type II.

The hexokinase of Lousta migratoria muscle was studied by Kerly and Leaback (1957). They reported that the enzyme was inhibited by 5 mM glucose-6-phosphate or fructose-6-phosphate. It is not known whether the inhibition by fructose-6-phosphate was due to its conversion to glucose-6-phosphate by phosphofructoisomerase. Walass and Walass (1962) observed maximum activity of rabbit muscle hexokinase when the Mg:ATP ratio was 1. Ca^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+} activated the enzyme.

Karpatkin (1966, 1967) reported that the particulate and soluble enzyme of frog skeletal muscle and nearly the same activities

and that several properties (like K_m for glucose and ATP, optimum Mg^{2+}, Q_{10}, inhibition by p-CMB and NaCl) of the two forms were identical. 1-Deoxyglucose-6-phosphate inhibited both particulate (P) and soluble (S) forms, but only in the case of (P) form was this inhibition reversed by inorganic phosphate. In the pH range 6.5-7.0 the (P) enzyme had 50% more activity. The (S) enzyme was 4.5 times more resistant to heat at 40° for 10 min.

Toews (1966) observed that glucose-6-phosphate was a non-competitive inhibitor of glucose and ATP. ADP was a non-competitive inhibitor of glucose and competitive inhibitor of ATP. Hanson and Fromm (1967) studied the kinetics and mechanism of action of partially purified hexokinase from rat skeletal muscle. They claimed that this enzyme (Type II) differed from the other types of hexokinase in muscle.

Ilin et al (1970) purified and studied the properties of hexokinase in normal or tendotomized rabbit skeletal muscle. The ratio of hexokinase I to hexokinase II in normal or tendotomized rabbit skeletal muscle was 1:2. Denervated muscle had a ratio of 1:15 which was similar to the ratio for rabbit embryonic muscle. They reported that mature, embryonic or denervated muscle hexokinase II had a K_m 2.1×10^{-4}M - 2.3×10^{-4}M for glucose and a K_m for 7.1×10^{-4} - 7.3×10^{-4}M for ATP. They compared Hexokinase I and II and found that hexokinase II was more heat-labile and less resistant to tryptic proteolysis and phosphate inhibition. Kozlov and Protchenko (1968) reported that when rats were heated at 40-45° until heat shock developed which was accompanied by an abrupt decrease in glucokinase of liver and noticeable decrease of hexokinase activity in skeletal muscle. Hexokinase activity in cardiac muscle and brain tissue was

not noticeably changed.

Liver

Kildema and Teras (1969) determined hexokinase, glucokinase and fructokinase activities in the soluble and mitochondrial fraction in liver homogenates from rats and mice during daily administration of diethylnitrosamine. The hexokinase activity increased in the mitochondrial fraction during the course of malignization.

Lamothe et al (1970) reported that injections of L-tyrosine into rats were found to increase hepatic hexokinase activity, though the amino acid had no effect in the enzyme in vitro. The glucokinase of liver has been extensively studied, but the literature on this enzyme is beyond the scope of this review. The kinetic properties of some isoenzymes of liver are presented in Table 1 .

Adipose tissue

Adipose tissue contains both glucokinase and hexokinase. Dipietro (1963), Hernandez and Sols (1963), Grossbard and Schimke (1966) studied the hexokinase of adipose tissue. Dipietro's (1963) studies showed that the adipose tissue hexokinase requires ATP and is activated by Mg^{2+} and Mn^{2+}. He reported K_m values for fructose of 3 mM and for glucose of 0.07 mM.

Hernandez and Sols (1963) reported that hexokinase of rat epididymal adipose tissue phosphorylated glucose, mannose and fructose. They claim that its substrate specificity is broadly similar to that of brain hexokinase, though it can phosphorylate mannose and 2-deoxyglucose faster than glucose. The enzyme is inhibited by glucose-6-phosphate

and ADP. 2-Hydroxymethylglucose strongly inhibits this hexokinase but N-acetylglucosamine does not inhibit it.

Spydevold&Bøgrebaek (1968) carried out differential centrifugations with sucrose homogenates of rat epididymal adipose tissue and observed that hexokinase activity was associated with both mitochondria and microsomes. The bound hexokinase was slowly released when the suspension was incubated for 50 min at 4° with $MgCl_2$, KCl or $(NH_4)_2SO_4$. Glucose-6-phosphate also released hexokinase activity. Bøgrebaek (1970) claimed that the total hexokinase activity which was associated with mitochondria varied with pH. This variation was caused by a variation in the stability of binding. He observed higher adipose tissue hexokinase in carbohydrate fed than in fasted rats.

Erythrocytes

Henessey et al (1962) reported a preliminary separation of the enzyme from hemoglobin by DEAE-cellulose chromatography. The hexokinase of erythrocytes occurs almost entirely in soluble form. Glucose and glutathione partially stabilize the enzyme. Brewer and Powell (1963) stated that the older cells have a lower activity than the younger ones.

Rakitzis and Mills (1967) presented data on human erythrocyte hexokinase. They stated that the effect of pH on hexokinase activity was indirect and the major factors that govern the hexokinase activity are the levels of glucose-6-phosphate and Pi.

Gerber and Elsner (1968) investigated the effects of glucose-6-phosphate, inorganic phosphate, ADP and AMP on purified hexokinase of red cells. They reported that glucose-6-phosphate

inhibited hexokinase competitively with respect to ATP. The Ki for glucose-6-phosphate was 30 μM. The K_m for ATP in the absence of glucose-6-phosphate was 3 mM. Glucose-6-phosphate inhibition was pH-independent between pH 6.6 and 8.2 and it was practically unchanged between 25° and 37°. 2 mM ADP or AMP inhibited hexokinase about 50% and 0.1 mM ADP or AMP about 10%.

Brewer (1969) stated that hexokinase is a rate limiting step in erythrocytes. 2,3-Diphosphoglycerate inhibits hexokinase and this inhibition is relieved by increasing concentrations of ATP and Mg^{2+}.

Intestine

Sols (1956) reported that a homogenate of rat intestinal mucosa phosphorylated glucose, fructose, mannose, glucosamine and 2-deoxyglucose. Lange and Kohn (1962) prepared cell-free extracts of rat intestine, kidney and liver. The K_m values for glucose, deoxyglucose and glucosamine were 6.5×10^{-5}M, 9×10^{-5}M, 3.3×10^{-4}M respectively for intestinal hexokinase. Srivastava et al (1968) studied the intracellular distribution of hexokinase activity in the mucosa of rat and guinea pig small intestine. In rat 60% and in guinea pig 45% of the hexokinase activity of the homogenates was recovered in the particulate fraction. Manunta and Marongiu (1967) reported that hexokinase activity in the intestinal mucosa of dog and sheep had marked variations within both species, but the average activities were not statistically different between the species.

Pancreas

Hexokinase of dog pancreas was present in the soluble fraction. It is not known whether this was due to the action of proteolytic enzymes on the particulate fraction. Villar-Palasi (1957) prepared the enzyme by centrifuging the extract at 23,000 x g for 60 minutes. This enzyme phosphorylated glucose, fructose, mannose, 2-deoxyglucose and 1,5-sorbitan. The hexokinase activity of normal and alloxan diabetic dogs showed no significant difference.

Retina

Hoare and Kerly (1954) demonstrated the presence of hexokinase in retinal extracts. It phosphorylates glucose, fructose, mannose and glucosamine in the presence of ATP. Mg^{2+}, Mn^{2+} and to a less extent Co^{2+} activate the enzyme. Lowry et al (1961) reported that hexokinase within the first neuron of retina is almost entirely confined to the inner segments of the rods and cones. Pottinger (1967) showed the presence of hexokinase in extracts of eye lens from cow, calf, rabbit, rat and guinea pig and in human cataractous lenses. Hockwin and Gassner (1968) showed the presence of hexokinase in calf and ox lens.

Etingof and Biesold (1969) reported that about 40% of the total hexokinase activity is concentrated in the structural components of the bovine retina. Retinal mitochondria show high hexokinase activity. They activated and solubilized the mitochondrial enzyme with 0.9% NaCl solution and 0.5% solution of Triton X-100. They also observed that a mixture of salt solution and detergent proved most effective for this purpose.

Uterus

Smith and Gorsk (1967) showed that the immature rat uterus contains a higher concentration of hexokinase than mature rat liver. Unlike the liver the uterine tissue did not contain glucokinase. Uterine hexokinase activity significantly and steadily increased from 8 to 24 hours after 17-β- estradiol treatment.

Kidney

Fomina (1968) studied hexokinase in three fractions isolated from renal cortex, soluble, mitochondrial and eluted from mitochondria. No essential differences were found in the affinity of hexokinase to 2-deoxy-D-glucose and in the inhibition of the enzyme by glucose-6-phosphate and N-ethylmaleimide.

Grossbard and Schimke (1966) have purified Type I hexokinase of rat kidney (specific activity 6.8 units/mg at 37°). The properties of the enzyme are different from those of Type II and Type III with respect to K_m values.

Balyabina (1968) reported that the hexokinase activity of extracts from the cortex and medulla of the kidney in rats with alloxan induced diabetes was lower than the activity of extracts from normal rat kidney.

Testis

Akaeda (1956) claimed that rabbit testis contain glucokinase which gives glucose-1-phosphate as a reaction product. This result has not been confirmed or extended.

Skin

Smirnova (1959) has shown the presence of hexokinase in aqueous extracts of the skin of rabbit, mice and guinea pigs. The enzyme had a pH optimum of 8.0 and was inactive below pH 6.0.

Mier and Cotton (1966) reported hexokinase activity in homogenate of mouse skin. They stated that this activity was about equally divided between soluble and particulate fractions. The pH curve was virtually flat from pH 6 to 8.5.

Ascites cells

McComb and Yushik (1959) partially purified hexokinase from Krebs 2-ascites carcinoma cells. They found the properties of this enzyme were similar to those of brain hexokinase. It was observed by Rose and Warms (1965) that the hexokinase of ascites cells was solubilized by glucose-6-phosphate or anhydro-glucitol-6-phosphate. Mannose-6-phosphate did not solubilize the enzyme. Uyeda and Racker (1965) purified ascites hexokinase about 100-fold. The purified enzyme was unstable in the absence of glucose, whereas 10 mM glucose stabilized the enzyme for two months at 0°. It had higher stability at neutral pHs than at pH 5.5 or 9.0. It was inhibited by ADP and this inhibition was reversed by ATP. Glucose-6-phosphate inhibited this enzyme and the inhibition was partially reversed by inorganic phosphate.

Rose and Warms (1967) reported that the hexokinase of ascites tumor, sarcoma 37, was a mitochondrial one. The activity was readily solubilized by glucose-6-phosphate, ribonucleoside triphosphates, or high salt concentrations at different pHs. They

suggested that the effect of glucose-6-phosphate was related to the regulator site on the enzyme. The enzyme can be bound again to mitochondria in a Mg^{2+} dependent equilibrium. The interaction follows a simple overall equation: (binding sites) + (enzyme) + (Mg^{2+}) $\rightleftharpoons$ complex, represented by a single equilibrium constant.

Kosow and Rose (1968) purified hexokinase II of ELD ascites tumor cells from soluble and particulate fractions. They studied the initial rate and product inhibition of both the fractions. The inhibitions by AMP and ADP were similar for the two forms. Anhydroglucitol-6-phosphate (a non-competitive inhibitor of glucose) was competitive with respect to ATP only with the soluble form.

Some of the kinetic constants of animal tissue hexokinases are given in the accompanying Table 1.

TABLE 1

K_m VALUES OF SUBSTRATES AND Ki VALUES OF INHIBITION OF HEXOKINASE FROM DIFFERENT ANIMAL TISSUES

Source	K_m glucose	K_m ATP	K_m Mg^{2+}	Ki ADP	Ki Glucose-6-P	References
	M	M	M	M	M	
Ox brain particulate	8.0×10^{-6}	1.3×10^{-4}	8×10^{-4}	1.3×10^{-4}	4.0×10^{-4}	Crane & Sols (1954, 1955)
Ox brain particulate	6.4×10^{-5}	3.4×10^{-4}	-	-	-	Fromm & Zewe (1962a)
Ox brain soluble	2.67×10^{-4} (at 2.5×10^{-3}M ATP)	1.5×10^{-4} (at 4×10^{-3}M glucose)	-	-	-	Moore (1968)
Ox brain particulate	4.4×10^{-5} (at 1.0×10^{-5}M ATP)	6.0×10^{-5} (at 4×10^{-7}M glucose)	-	-	-	Moore (1968)
Ox brain particulate	3.8×10^{-5}	5.0×10^{-4}	2.8×10^{-3}	-	3.0×10^{-5} to 1.6×10^{-4} (at 0.05-30 mM Pi)	Joshi and Jagannathan (1968)
Ox brain particulate	5.0×10^{-5} (at 2×10^{-3}M ATP)	4.89×10^{-3} (at 2×10^{-3}M glucose)	-	-	-	Schwartz and Basford (1967)
Ox brain soluble peak I	4.8×10^{-6}	5.0×10^{-4}	-	-	-	Thompson and Bachelard (1970)
Ox brain soluble peak II	6.0×10^{-6}	6.8×10^{-4}	-	-	-	Thompson and Bachelard (1970)
Ox brain particulate peak I	4.2×10^{-6}	6.0×10^{-4}	-	-	-	Thompson and Bachelard (1970)
Ox brain particulate peak II	4.3×10^{-6}	6.6×10^{-4}	-	-	-	Thompson and Bachelard (1970)
Rat brain soluble	4.5×10^{-5}	4.0×10^{-4}	-	6.2×10^{-4}	2.6×10^{-5}	Grossbard and Schimke (1966)

TABLE I (CONTD.)

Source	K_m glucose M	K_m ATP M	K_m Mg^{2+} M	Ki ADP M	Ki glucose-6-P M	References
Rat brain soluble	2.9×10^{-6}	2.4×10^{-4}	-	-	-	Tuttle and Wilson (1970)
Rat brain particulate	5.3×10^{-6}	2.0×10^{-4}	-	-	-	Tuttle and Wilson (1970)
Rat liver	4.0×10^{-5}	-	-	-	-	Lange & Kohn (1972)
Rat liver soluble	7.0×10^{-6}	9.8×10^{-4}	-	8.8×10^{-4}	7.4×10^{-5}	Grossbard and Schimke (1966)
Rat liver isozyme A	4.4×10^{-5}	4.2×10^{-4}	-	-	-	Carmen et al (1964)
Rat liver isozyme B	1.3×10^{-4}	7.0×10^{-4}	-	-	-	Carmen et al (1964)
Rat liver isozyme C	6.0×10^{-6}	-	-	-	-	Carmen et al (1964)
Rat liver isozyme D	1.8×10^{-2}	4.9×10^{-4}	-	-	-	Carmen et al (1964)
Rat skeletal muscle	1.8×10^{-4}	4.6×10^{-4}	-	-	-	Hanson and Fromm (1965)
Rat skeletal muscle	1.1×10^{-4}	8.0×10^{-4}	-	8.0×10^{-4}	-	Toews (1966)
Rat muscle soluble	2.3×10^{-4}	7.8×10^{-4}	-	2.2×10^{-5}	2.1×10^{-3}	Grossbard and Schimke (1966)
Rabbit skeletal muscle	$2.1\text{-}2.3 \times 10^{-4}$	7.1 / 7.3 $\times 10^{-4}$	-	-	-	Ilin et al.(1970)
Rat heart muscle (soluble)	4.5×10^{-5}	5.0×10^{-4}	-	6.8×10^{-4}	1.6×10^{-4}	England and Randle (1967)
Rat heart muscle (particulate)	4.5×10^{-5}	5.0×10^{-4}	-	6.0×10^{-4}	3.3×10^{-4}	England and Randle (1967)

TABLE I (CONTD.)

Source	K_m glucose	K_m ATP	K_m Mg^{2+}	Ki ADP	Ki glucose-6-P	References
	M	M	M	M	M	
Adipose tissue	3.0×10^{-5}	5.0×10^{-5}	-	-	-	Hernandez & Sols (1963)
Adipose tissue soluble	2.8×10^{-4}	7.5×10^{-4}	-	-	-	Grossbard and Schimke (1966)
Adipose tissue	7.0×10^{-5}	-	-	-	-	Diepietro (1963)
Ascites cells	-	4.3×10^{-4}	-	5.1×10^{-5}	-	Uyeda and Racker (1965)
Ascites cells	2.8×10^{-5}	1.7×10^{-4}	1.0×10^{-3}	-	4.0×10^{-4}	McComb & Yushok (1959)
Rat erythrocyte	-	3.0×10^{-3}	-	-	3.0×10^{-5}	Gerber & Elsner (1968)
Rat intestine	6.5×10^{-5}	-	-	-	-	Lange & Kohn (1962)
Rat intestinal mucosa	2.0×10^{-4}	-	-	-	6.0×10^{-3}	Sols (1956)
Rat kidney	4.8×10^{-5}	-	-	-	-	Lange & Kohn (1962)
Rat kidney soluble	4.9×10^{-5}	4.4×10^{-4}	-	-	-	Grossbard and Schimke (1966)
Islets of langerhans of fish	1.1×10^{-5}	-	-	-	-	Renold (1961)

SECTION II

HEART

Hexokinase from heart muscle was reported to be present in both soluble and particulate forms in approximately equal amounts (Crane and Sols,1953). Pigeon ventricle muscle contained half of the hexokinase in particulate form, which was sedimented at 18,000 x g (Bargoni, 1958). Mayer, Mayfield and Haas (1966) determined the intracellular distribution of hexokinase from hearts of several species. When homogenates were prepared in sucrose medium most of the hexokinase was present in the non-particulate fraction, but on differential centrifugation in isotonic KCl a large fraction of hexokinase activity was associated with mitochondria.

It was observed by Rose and Warms (1965) that mitochondrial hexokinase could be released in soluble form by glucose-6-phosphate. Hernandez and Crane (1966) studied in great detail the effect of pH, salts of mono- and divalent metals and glucose-6-phosphate in solubilizing particulate heart hexokinase. They determined the amount of enzyme obtained in soluble form at different pHs and ionic strengths. They observed that at pH 7.0 the solubilization was 35% with 0.4 M KCl or 0.2 M ammonium sulphate and 90% with 0.2 M $MgCl_2$. At pH 5.5 the solubilization was 70% with KCl and 80% with $MgCl_2$. Low concentrations of glucose-6-phosphate also solubilized the particulate enzyme. At pH 7.5 the solubilization was 19% at 0.1 mM; 31% at 1 mM but only 19% at 5 mM glucose-6-phosphate. If the monovalent cation

salts were removed from the solubilized enzyme, then the enzyme was again bound to the particles. They state that this rebinding was not due to aggregation. With divalent cations the solubilization was irreversible.

Crane and Sols (1955) described a method of preparing soluble and particulate hexokinase from calf or rat heart muscle. Homogenates in phosphate buffer pH 7.4 containing KCl and EDTA were centrifuged at 1500 x g to remove the inactive debris. The particulate enzyme was obtained from the 1500 x g supernatant by centrifuging it at 18,000 x g. The 18,000 x g supernatant contained the soluble enzyme and the precipitate contained the particulate enzyme. The particulate enzyme was solubilized by treatment with 0.1% Triton X-100 and then centrifuged at 50,000 x g. This supernatant contained the solubilized particulate hexokinase.

Crane and Sols (1955) partially purified their solubilized preparation as follows. They removed insoluble proteins at pH 5.5 and then fractionated the enzyme with ammonium sulphate. They reported that this enzyme was free from phosphofructokinase activity. Its specific activity was 1.5 units/mg protein (All units refer to the conversion of 1 micromole of substrate per min.).

England and Randle (1967) partially purified the soluble hexokinase of rat heart by DEAE-cellulose chromatography and ammonium sulphate fractionation.

Very little work has been done on obtaining highly purified soluble or insoluble hexokinases of heart muscle and the preparations obtained so far have been of very low specific activity.

England and Randle (1967) reported the total activity of rat heart homogenate was 21.5±1.0 units/g of dry heart ventricle at 25°. The Q_{10} over the range 22° to 38.5° was 1.93. The hexokinase values were unaffected by extraction with buffer containing 2-mercaptoethanol or in diabetes. They studied the kinetics of the soluble and particulate forms. The K_m values for both the forms were 0.045 mM for glucose and 0.5 mM for ATP. No enzyme with a high K_m for glucose could be found. With 0.20 mM AMP the K_m for glucose for the particulate enzyme was 25 µM. AMP and ADP were competitive inhibitors with respect to ATP for the soluble enzyme (Ki values 0.36 mM and 0.68 mM respectively). Glucose-6-phosphate was a non-competitive inhibitor with respect to glucose (Ki 0.16 mM) and a mixed inhibitor with respect to ATP (Ki 80 µM). When the glucose-6-phosphate concentration was below 0.3 mM, 15 mM Pi and 0.8 mM AMP partially relieved the glucose-6-phosphate inhibition.

ADP and AMP were also competitive inhibitors with respect to ATP for the particulate enzyme of rat heart (Ki values 0.6 mM and 0.16 mM respectively). Glucose-6-phosphate inhibition was non-competitive with respect to glucose (Ki 0.33 mM) and mixed with respect to ATP (Ki 40 µM).

From the concentrations of substrates and effectors of rat heart hexokinase in perfused heart and the kinetics of the enzyme (Newsholme and Randle (1964) concluded that the main control of hexokinase activity in rat heart was through changes of glucose-6-phosphate concentration and that ADP and AMP inhibitions had only a slight effect.

Mayer, Mayfield and Haas (1966) reported that glucose-6-phosphate was a non-competitive inhibitor with respect to ATP or

glucose. The Ki values for glucose-6-phosphate ranged from 3 x 10^{-5}M to 1.3 x 10^{-4}M in various preparations. They observed a change in hexokinase activity with pH. Inorganic orthophosphate antagonized the glucose-6-phosphate inhibition in concentrations comparable to physiological ones. This effect was lost on ageing of dog heart mitochondria.

Postupaev (1963) studied the effect of ACTH and ATP injection on hexokinase activity of skeletal muscle and heart in hypoxia. He found that hexokinase activity was decreased when animals were exposed to lower atmospheric pressure (190 mm Hg) for 1.5 hours. Daily injections of ACTH and ATP for six days did not change the hexokinase activity in either tissue. The injections of ACTH and ATP prevented the decrease of hexokinase in hypoxia.

Belchenko (1964) studied the hexokinase reaction in febrile condition and hyperthyroidism caused by bacterial infection. Rats were infected with a pathogenic strain of E.coli. During the development of hyperthyroidism and an experimental febrile condition the change of hexokinase activity of cardiac and skeletal muscle was investigated. Hexokinase activity of cardiac muscle decreased from the first day of intoxication. This fall was not observed until the second day in the case of skeletal muscle. The hexokinase of cardiac muscle showed a greater lability than that of skeletal muscle.

Belchenko, Shaplakov and Sokolova (1964) observed that inhibitors, such as β-lipoprotein, depress the hexokinase activity in myocardium and skeletal muscle. Dubinina (1966) studied the hexokinase activity in skeletal and cardiac muscles of rabbits two hours after the application of pyrogenal. At the moment of maximum temperature increase

the hexokinase activity of skeletal muscle was significantly decreased. Insulin increased the hexokinase activity in heart and not in skeletal muscle and partially reversed the hexokinase inhibition caused by pyrogenal. ACTH decreased hexokinase in skeletal muscle and increased it in heart. Pyrogenal did not influence it in heart. Pyrogenal did not influence the enzyme activity in heart but prevented the stimulating effect of insulin.

Dimitrieva (1967) reported that strophanthin (0.1 mg/100 g) increased the myocardial hexokinase activity in normal rats.

Lȣgovoi (1969) reported that when adrenaline at 300-350 µg/100 g body weight was used to induce heart necrosis in rats, hexokinase activity increased progressively and it was suggested that increase in hexokinase activity was probably caused by hypoxia and increased transport and utilization of blood.

Mori (1958) reported that the activity of heart muscle hexokinase was increased by δ-aminovalerate, γ-aminobutyric acid and butylamine by 105, 69 and 72% respectively and markedly suppressed by N-acetyl-γ-aminobutyric acid. α- and β-hydroxy derivatives of γ-aminobutyrate and β-alanine had no significant effect. The $(NH_2-CH_2)_3$-group was postulated to be necessary for enzyme activation.

Regen et al (1963) reported that hexokinase activity was found to be inversely related to the glucose-6-phosphate level suggesting that hexokinase from rat heart may be partly under the inhibitory control of glucose-6-phosphate. The glucose-6-phosphate levels were controlled to a large extent by the activity of phosphofructokinase.

SECTION III

PLANTS

Hexokinase has been reported to be present in grape berries (Hawker, 1969), wheat germ (Saltman, 1953), potato tuber (Meeuse et al. 1952), sugar beet, pea cotyledons (Brown and Wray, 1968) and rubber latex (Jacob and d'Auzac, 1967), lentils and in pollen of several species, but very little progress has been made in the purification and study of plant hexokinase.

Katsumata and Togasawa (1968) detected hexokinase in the pollen of Pinus densiflora, Zea mays and Cucurbita maxima. Maximum activity was found at pH 7.5 and 37°. Hexokinase activities increased on germination on sucrose-agar. Gussin and McCormack (1970) described the presence of hexokinase in Lilium longiflorum pollen. They reported that the pellet contained 73% of the total hexokinase in particulate form whereas the supernatant liquid contained 19% of the activity.

Kursanov et al (1969) have studied the localization and properties of hexokinase in the conducting bundles of sugar beet leaf stalks. The mitochondrial and microsomal fraction contained 20 to 30 per cent of the total hexokinase activity. The remaining activity was in the soluble portion. The hexokinase of mitochondria and microsomes phosphorylated both glucose and fructose but there was much greater affinity for glucose. Hence, phosphorylation of fructose by these fractions was completely inhibited by the presence of glucose, but fructose did not suppress the phosphorylation of glucose. In the soluble subcellular fraction corresponding to the cytoplasm phosphorylation of each of the hexoses proceeded irrespective of the presence

of the other sugar.

Meunier, Moustacas and Ricard (1968) reported that root cells of the lentil Lens culinaris contain hexokinase in the cytoplasmic fraction and on the exterior of the mitochondrial membrane.

SECTION IV

MICROORGANISMS
(other than yeast)

Several workers have reported the presence of hexokinase in microorganisms: A.niger (Tewari and Krishnan, 1961), Azotobacter vinelandii (Mortensen and Wilson, 1954), Aeromonas formicans (Pivinic and Sabina, 1957), E.coli (Cardini, 1951), Leishmania donovani (Chatterjee and others, 1958), Pseudomonas putrefaciens (Klein and Doudroff, 1950), Plasmodium gallinaceum (Speck and Evans, 1945), Salmonella typhosa (Perova, 1961), Spirochaeta recurrentis (Smith, 1960), Trypanosoma gambiense (Seed and Baquero, 1965), A.variabilis (Pearce and Carr, 1969), a prototrophic non-sporogenic strain of B.subtilis (Moses and Sharp, 1968) and Rhizobium japonicum (Keele, Hamilton and Elkan, 1969).

Medina and Nicholas (1957) claimed that the enzyme from Neurospora crassa requires Zn^{2+} for its activity. Davidson (1960) purified the enzyme from A.parasiticus about 200-fold. The enzyme also phosphorylated D-galactose and galactosamine to yield the respective -6-phosphates. With galactose the relative activity was 0.86 with respect to glucose and with galactosamine the relative activity was 0.46 whereas the yeast and brain enzymes were inactive with galactose and galactosamine.

Sapico and Anderson (1967) have reported a hexokinase specific for D-mannose and D-fructose from L.mesenteroides. The specific activity of the purified enzyme was 76 units per mg. Its

molecular weight was 47,000. D-mannose and D-fructose were phosphorylated at equal rates whereas D-glucose and 2-deoxyglucose and other sugars were not phosphorylated and also did not inhibit the enzyme.

Risby and Seed (1969) have purified hexokinase 350-fold from crude extracts of Trypanosomes brucei, T.gambiense and T.equiperdur. The purified enzyme had a temperature optimum of 45°-50° and pH optimum between 6.5-7.0. It required Mg^{2+} and ATP and was inhibited by ADP and p-hydroxymercuribenzoate and glucose-6-phosphate. Mannose, glucosamine, N-acetyl-D-glucosamine and xylose inhibited the enzyme competitively. It phosphorylated glucose, fructose, mannose, 2-deoxyglucose and glucosamine. Glucose was required for stabilization of the enzyme.

Glucokinase from microorganisms

Kamel, Allison and Anderson (1966) purified glucokinase over 1000-fold from extracts of A.aerogenes PRL R_3 and Coffee and Hu Alfred (1970) purified glucokinase from a Pseudomonad. This enzyme was homogeneous by ultracentrifugation and agarose gel electrophoresis. Mitra (1970) has purified glucokinase about 100-fold from crude extracts of S.cerevisiae

Szymona and Ostrowski (1964) have partially purified the glucokinase from Mycobacterium phlei which phosphorylates glucose by utilizing inorganic polyphosphate. Its molecular weight was 100,000. Mitra (1970) reported the K_m value for glucose and ATP as 28 and 50 µM respectively for glucokinase of yeast. The maximum velocity of the enzyme towards fructose was 0.4% of that towards glucose. This enzyme showed a marked heterogeneity when sedimented in a sucrose gradient. Its molecular weight ranged from 144,000 to

a value in excess of 200,000. Yeast glucokinase had a number of properties similar to those of yeast hexokinase. Its mode of reaction with glucose and ATP was random, as indicated by kinetic studies. Coffee and Hu Alfred (1970) reported the molecular weight of glucokinase from a Pseudomonad to be 112,000. This enzyme lost activity very rapidly in the presence of pCMB and more slowly in the presence of oxidized glutathione and carboxymethyl disulfide. The enzyme was not protected against heat inactivation either by the substrates or the products of the reaction.

Kamel, Allison and Anderson (1966) reported that with glucokinase from A.aerogenes only D-glucose ($K_m = 8 \times 10^{-5}$M) and D-glucosamine ($Ki = 4 \times 10^{-4}$M) were phosphorylated. It was inhibited by D-xylose ($Ki = 3 \times 10^{-3}$M) and by ADP (competitive with respect to ATP, $Ki = 4 \times 10^{-4}$M) but not by D-glucose-6-phosphate or D-mannose-6-phosphate. The pH optimum was 7.5 in glycylglycine buffer and about 8.9 in glycine buffer.

Phosphorylation with phosphate donors other than ATP

Hexokinases which act with phosphate donors other than ATP have been reported from several microorganisms, e.g. Corynebacterium diphtheriae and Mycobacterium phlei. These enzymes transfer phosphate from inorganic polyphosphate to glucose and glucosamine. The enzyme from Aerobacter aerogenes can utilize acetyl phosphate as a phosphate donor. The enzyme from E.coli which utilizes phosphoramidate has been partially purified. E.coli contains an enzyme which phosphorylates glucose in the presence of phosphoenol pyruvate.

SECTION V

YEAST

Methods for the preparation of yeast hexokinase have been reported by several workers. Agren et al (1960, 1963), Berger et al (1946), Kunitz and McDonald (1946); Bailey and Webb (1945), Darrow and Colowick (1962), Lazarus et al (1946), Sols and de la Fuente (1958), Schulze, Gazith and Gooding (1966), Schulze and Colowick (1969).

Darrow and Colowick's modified method which includes adsorption on bentonite is notable for the relatively high yields (15%) of crystalline enzyme. Schulze, Gazith and Gooding (1966) introduced several modifications in this method. Proteolytic activity was reduced by the addition of phenylmethyl sulfonyl chloride and proteolytic enzymes were removed by chromatography on DEAE-cellulose. The presence of traces of proteolytic activity was responsible for several apparent "isoenzymes" obtained by earlier methods, which were really artefacts and formed during the isolation of the enzyme. The availability of large quantities of pure crystalline yeast hexokinase was important in facilitating detailed studies both on the enzyme as well as on the hexokinase reaction. Thermodynamic data are especially useful since they are independent of the catalyst.

Mg^{2+} is essential for hexokinase activity. Trayser and Colowick (1961) found several elements such as Al^{3+}, Co^{2+} etc. in the first crystals of yeast hexokinase, but these elements were negligible after repeated recrystallization. Fluoride did not inhibit the enzyme with or without phosphate. Mg^{2+} could be

partially replaced by Mn^{2+} but not by Ca^{2+}. The activation of yeast hexokinase by Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+} and Cd^{2+}, which was reported by Brintzinger *et al* (1959), requires confirmation. Controversial reports regarding biotin and hexokinase were made by different workers. Strauss and Moat (1958) reported stimulation of yeast fermentation by biotin but Trayser and Colowick (1961) found no biotin in several times recrystallized yeast hexokinase. However biotin may have been present in the glucose used for estimation of the enzyme and the absence of biotin in the enzyme does not necessarily show the absence of an effect of biotin added with the other reagents. Joshi and Jagannathan found no effect of avidin on brain hexokinase. Trayser and Colowick (1961) found no phosphorus in their preparation while Kunitz and McDonald (1946) observed phosphorus in their purified preparation.

Yeast hexokinase phosphorylates several D-hexoses. The relative rates of phosphorylation of some of the hexoses are: fructose 1.8, glucose 1.0, 2-deoxyglucose 1.0, mannose 0.8, glucosamine 0.7 and glucosone 0.2. The relative rates (in micromoles of glucose phosphorylated per mg protein) for different nucleotides are ATP 300, deoxy-ATP 400, ITP 23, UTP 3, CTP 1.

The K_m values for some of the substrates are as follows: fructose 7×10^{-4}M, glucose 1×10^{-4}M, 2-deoxyglucose 3×10^{-4}M, mannose 5×10^{-5}M and glucosamine 1.5×10^{-3}M. The K_m value for ATP was reported to be 9.5×10^{-5}M (McDonald, 1955) and 2×10^{-4}M (Fromm and Zewe, 1962b) and a 1.6×10^{-4}M with (Mg^{2+}:ATP ratio 2.5:1) and 1.3×10^{-4}M with (Mn^{2+}:ATP ratio 2.5:1) (Zewe *et al*, 1964). More detailed studies on the K_m values of one substrate at

different concentrations of the other substrate are reported in a later section.

The temperature coefficient of activity of the enzyme between 0° and 30° was 1.9 to 2.2.

In the hexokinase reaction one proton is involved and the equilibrium varies with the pH. Robbins and Boyer (1957) determined the equilibrium constant $\frac{(ADP)(Glucose\text{-}6\text{-}phosphate)}{(ATP)(Glucose)}$ at different pH and Mg^{2+} concentrations. At pH 6, 7 and 8 and at 30° with Mg^{2+} the values of K were 155, 1550, 15,500 respectively. These correspond to free energy changes of -3.2, -4.7 and -6.2 Kcal/mole enzyme respectively.

According to Kunitz and McDonald the isoelectric point is 4.8. The diffusion constant D^{o}_{20} at 1° in acetate buffer, pH 5.5 was 2.7×10^{-7} $cm^2 \times sec^{-1}$ and the sedimentation constant at the same temperature and pH was 3.1 S. The molecular weight of the enzyme was reported to be 96,000. The turnover number of the enzyme with glucose at pH 7.5 and at 30° was 13,000 moles per minute per 100,000 g of protein.

The specific activity of the crystalline enzyme of Darrow and Colowick was found to vary with the method of estimation. In the manometric assay it was about 130 units per mg whereas with the cresol red indicator method it was 600 units per mg. It was suggested that there was inactivation in the former method, though this was not established. Lazarus *et al* claimed to have obtained a preparation with a specific activity of 800 units/mg at 25°. The presence of protease modified enzyme may explain the differences in specific activity of the enzyme obtained by different methods, but

not the differences in the activity by different methods with the same enzyme preparation. The enzyme prepared with minimum proteolysis (Schulze et al. 1966) however had a specific activity of only 600 units/mg. This corresponds to a turnover number of 57,000 with glucose at 30° (hexokinase molecular weight of 95,000).

Several carbohydrates inhibit yeast hexokinase and the Ki values for some of them are: α-methylglucoside $>$ 0.1 M; N-acetylglucosamine 1 x 10^{-3}M, mannoheptulose 1.5 x 10^{-4}M; 2-hydroxymethylglucose 1.5 x 10^{-3}M; xylose 1 x 10^{-2}M. Yeast hexokinase was markedly inhibited by 10^{-3}M palmitic, stearic, myristic and ricinoleic acids and by higher concentrations of oleic and lauric acids. Digitonin, saponin, deoxycholate and different Tweens had no effect on yeast hexokinase. Bargoni (1959) reported that palmitic acid inhibition was irreversible.

Yeast hexokinase is inhibited by several other compounds. The inhibition by ADP and sodium tripolyphosphate is reversed by ATP but not by glucose. Glucose-6-phosphate was reported to be inhibitory by Wajzer (1953) and non-inhibitory by Weil-Malherbe and Bones (1951) and Sols and Crane (1953). Glucose-6-phosphate is a potent inhibitor of animal tissue hexokinases. Pattabiraman (1968) showed that yeast hexokinase is inhibited by LiCl, Li_2SO_4, NaF, NaCl, $NaHCO_3$, KF, KCl, KBr, KI and ammonium chloride and Tris-HCl at high concentrations. Domagk et al (1967) reported that diisopropylphosphorofluoridate at 10^{-4}M - 10^{-2}M inhibited crystalline yeast hexokinase. Brolin et al (1969) studied the inhibitory effects of adenine, adenosine, thiaminepyrophosphate, mono- or disodium triphosphate and glucose-6-phosphate on yeast hexokinase. Both adenine and adenosine

were weakly inhibitory, while the other compounds showed moderate inhibition. Inhibition was dependent on Mg^{2+} concentration and was less at higher Mg^{2+} concentrations. de la Fuente and Sols (1970) reported that yeast hexokinase was inactivated specifically by xylose in the presence of Mg-ATP. The nucleotide and divalent metal specificities were similar to those of the hexokinase reaction. The dissociation constants were $\backsim$ 0.2 mM for Mg ATP and 10 mM for xylose and resemble the K_m and Ki values for these compounds as substrate and competitive inhibitor respectively in the hexokinase reaction. Hexokinase activity was markedly decreased when resting yeast was incubated aerobically with 0.1 M xylose and 10% ethanol. Kosow and Rose (1970) reported that ADP^{3-} and $ADP\ Mg^{-}$ caused mixed inhibition when ATP-Mg is the variable substrate.

According to Berger et al yeast hexokinase does not appear to contain any reactive -SH groups. However it was irreversibly inactivated by treatment with mustard gas or nitrogen mustards. Bernard and Ramel (1962), Colowick and coworkers (Kenkare and Colowick, 1965; Kaji, 1966) clarified the role of -SH groups. Bernard and Ramel (1962) found that four -SH groups per mole could be titrated. Amino acid analysis indicated the presence of four cysteine groups per mole of enzyme. Yeast hexokinase was completely inactivated by 5×10^{-5} M methylmercurinitrate and this inactivation was reversed by 0.01 M cysteine. Similarly sodium bromoacetate irreversibly inactivated the enzyme at temperatures above 30°. Glucose protected the enzyme against bromoacetate inactivation to a considerable extent. These results suggest that the sulphydryl reagents do not have direct access to the -SH groups of yeast

hexokinase, but that the changes occurring at high temperatures and pHs expose these groups to the action of sulphydryl reagents. Kaji (1966) has suggested the presence of six -SH groups per mole of hexokinase on the basis of spectrophotometric assay with pCMB.

Lazarus, Derechin and Barnard (1968) isolated two enzyme species, hexokinase A & B, from yeast in homogeneous form. They claimed that each form contains 8 -SH groups per mole (102,000 molecular weight) and no disulfides. Four of these thiols react readily with methylmercuric iodate without enzyme inactivation. Only after that reaction do the other four become reactive to this and to other thiol reagents. The reaction of the four latter -SH groups inactivates the enzyme.

Yuan, Hui-Yin (1969) studied the reaction of -SH groups in hexokinase with pCMB. One mole of yeast hexokinase contains 5.4 -SH groups (determined with pCMB). The reaction of hexokinase with pCMB was influenced by the presence of glucose, the pH of the buffer and irradiation with X-rays. Jones (1969) studied the inactivation of yeast hexokinase B with iodoacetate at high ionic strength. These studies indicated that two thiol groups/mole (molecular weight 50,000) are probably located at the active site. The recently discovered presence of hexokinase "isoenzymes" which are formed by proteolytic activity complicates the interpretation of some of the earlier studies. The reason for the discrepancies in the number of -SH groups per mole of enzyme reported by different workers is not known.

Kenkare and Colowick (1965) made detailed physico-chemical studies of yeast hexokinase. They found that its molecular weight

was about 95,000. It dissociates into four subunits of molecular weight 24,000. The subunits lost a large part of the helical structure but not their tertiary structure. Gooding and Colowick (1966) have also reported six cysteine residues per molecule of enzyme of molecular weight of 96,000. Easterby and Rosemeyer (1969) reported the molecular weight of yeast hexokinase as 111,000. They treated the enzyme with phenylmethylsulfonylfluoride and reported that yeast hexokinase existed predominantly as tetramers of molecular weight 111,000 in equilibrium with dimers of molecular weight 55,000. In high concentrations of NaCl the enzyme underwent further dissociation towards a monomer of molecular weight 28,000.

Gazith et al (1968) studied the multiple forms of yeast hexokinase. They observed two forms of hexokinase, P I and P II, which differed in chromatographic and electrophoretic behaviour. These forms also differed in amino acid composition, catalytic activity, substrate binding capacity and susceptibility to various reagents. Forms P I and P II were both converted to more acidic forms S-I and S-II respectively. The S-I and S-II forms had electrophoretic mobilities similar to those of the parental forms. This conversion by protease or trypsin was carried out in the presence of glucose or salt. Protease or trypsin promoted the dissociation of the P forms from tetramer to dimer. The K_m for glucose was about the same for all four forms. The S forms showed markedly stronger glucose binding than P forms. Form P I and P II contained 4 and 3 -SH groups per dimer respectively. The addition of thiols at slightly alkaline pH caused inactivation of hexokinase and drastic structural alterations.

Kopperschlager and Hofmann (1969) detected multiple forms of hexokinase by agar gel and polyacrylamide gel electrophoresis of crude extracts of different yeast strains. Electrophoretic mobility and number of enzyme forms depended on the conditions of cell disruption and varied with the yeast strain. Commercial baker's yeast contained two or three isoenzymes when the yeast extract was prepared by short mechanical disruption. Cell lysis by proteolytic procedure changed the electrophoretic properties. Polyacrylamide gel electrophoresis indicated that the differences were not due to their molecular sizes. Trypsin increased the electrophoretic mobility of one or both forms of baker's yeast hexokinase.

Schulze and Colowick (1969) modified the method of isolation of yeast hexokinase and removed protease on a DEAE-cellulose column. They obtained two forms of yeast hexokinase P I and P II. Both forms were more basic by chromatography and electrophoresis than the protease modified enzyme (S). P II showed the same specific activity as that reported by Darrow and Colowick while P I showed less. P I and P II mainly existed as tetramers of molecular weight $\backsim$100,000 at low ionic strength and neutral pH as measured by ultracentrifugation, light scattering or gel filtration. They dissociated to dimer as the ionic strength, temperature or pH was raised or when glucose was added in the presence of phosphate. They could be converted to S form by trypsin or yeast protease only when glucose or salts are present, suggesting that the enzyme must be in dimer state for modification to occur. The protease modified enzyme tends to exist mainly as a dimer of molecular weight $\backsim$50,000 at neutral pH. Thus modification by trypsin seems to remove some groups which normally play a role in the association of dimer molecules to form the tetramer.

S E C T I O N VI

MULTIPLE FORMS OF HEXOKINASE

It has been reported in recent years that several animal tissues contain multiple forms of hexokinase.

Glucokinase (ATP-D-glucose-6-phosphotransferase, EC 2.7.1.2)
(ATP + D-glucose ⟶ D-glucose-6-phosphate + ADP)

Glucokinase has a higher K_m value for glucose than hexokinase. This enzyme is soluble and has been purified by Salas and Salas (1965), McLean and Brown (1966), Parry and Walker (1966).

Salas and Salas have purified glucokinase from rabbit liver 200-fold by fractionation with alcohol, DEAE-Sephadex and ammonium sulphate. Parry and Walker (1966) purified rat liver glucokinase about 870-fold relative to the original extract by ammonium sulphate fractionation and the use of DEAE-Sephadex,DEAE-cellulose and polyacrylamide columns.

Parry and Walker (1966) reported the K_m value of glucokinase for glucose as 2×10^{-2}M (as compared to 10^{-4}M or 10^{-5}M for hexokinase). It was active with glucose, mannose and 2-deoxyglucose, the relative rates being 1, 0.9 and 0.5 respectively. The K_m for fructose was 2 M. This enzyme was not inhibited by glucose-6-phosphate upto 10 mM. It was competitively inhibited by ADP. The optimum pH was 7.7 to 8.0. It was activated by sulphydryl compounds and inhibited by pCMB. It occurs predominantly in liver. Adipose tissue and several other tissues also contain glucokinase. Glucokinase content is very low in alloxan diabetics, in starvation and on high fat diets. The

normal enzyme level is restored after treatment with insulin or high carbohydrate diets.

Pilkis, Hansen and Krahl (1968) studied glucokinase in livers of several amphibians, reptiles and mammals. The enzyme behaves adaptively in rats, hamsters, guinea pigs, gerbils and squirrel monkeys. An antibody to yeast hexokinase did not react with any animal tissue hexokinases. An antibody to rat liver glucokinase reacted with glucokinase from several species but it did not inhibit the hexokinases of rat, other animals or yeast. This suggested the fundamental structural differences between glucokinase and hexokinase.

Other forms of hexokinases

Multiple forms of hexokinase are present in several tissues. Gonzalez et al (1964) separated rat liver extracts on DEAE-cellulose and showed three isozymes A, B and C (which had low K_m for glucose) in addition to glucokinase. Under physiological conditions major changes occurred in glucokinase levels. Katzen and Schimke (1965) also observed four different types of hexokinases in liver by using starch gel electrophoresis and chromatography on DEAE-cellulose or hydroxyapatite. Kidney contained three types of hexokinase. Eaten et al (1966) reported that two isoenzymes were present in white blood cells and seven isoenzymes in red blood corpuscle hemolysate.

Grossbard and Schimke (1966) have reported different isoenzymes in various rat tissues based on differences in electrophoretic mobilities. Grossbard et al (1966) described different isoenzymes in brain, skeletal muscle and liver from several species

(rat, mice, hamster, guinea pig, rabbit, monkey and cow). They claimed that the electrophoretic mobilities of one or more of the hexokinases vary significantly from species to species. They carried out the work on soluble enzymes present in the 100,000 x g supernatant fraction by starch gel electrophoresis. Hence this represented studies of only the soluble and not of the particulate forms.

Katzen et al (1968) reported that rat heart contained two isoenzymes of which isoenzyme type II was predominant.

Grossbard et al (1966) observed that brain contained two forms, the major fraction having lower mobility (Type I). Skeletal muscle and adipose tissue also showed two types of hexokinase,whereas liver contained several types of hexokinase varying from three in the case of mouse and rabbit to five in hamster. Kidney contained three isoenzymes.

As stated in an earlier section, Karpatkin (1966) studied the particulate and soluble fractions of frog muscle hexokinase. He separated these fractions by centrifugation at 105,000 x g. He observed equal distribution of hexokinase in soluble and particulate fractions. Both the enzymes were inhibited by 1-deoxyglucose-6-phosphate. Only in the case of the particulate enzyme was this inhibition reversed by inorganic phosphate. The soluble enzyme was more stable to heat inactivation.

Grossbard and Schimke (1966) purified the enzyme from rat kidney, muscle, adipose tissue, liver and brain. The specific activities of the purified enzymes ranged from about 1.4 units per mg

for liver to about 13 units per mg for muscle and adipose tissue enzymes. The purified preparations were similar with respect to their specificity, pH optimum and molecular weight. Kinetic studies demonstrated that the isoenzymes, irrespective of tissue origin, differed in relation to their K_m values for glucose and ATP, their Ki values for glucose-6-phosphate and ADP and their heat stabilities. These types also differed in their mobility on starch gel electrophoresis. They concluded that the observed differences could not be due to the difference in the degree of aggregation.

McLean, Brown, Walters and Greenslade (1967) distinguished hexokinase type I and II in adipose tissue by their different stabilities to heat treatment. In alloxan diabetic rats the hexokinase type II was markedly depressed in adipose tissue. Lung contained hexokinase type I, II and III, type I predominating. They observed no significant change in these multiple forms in lungs from alloxan diabetic rats.

Bensen and Jong (1968) observed a hexokinase (embryonic and adult human tissue) not previously described, the anodal migration of which was between types II and III. It stained clearly at 0.1 M glucose but was absent or stained weakly in 5×10^{-4} M glucose indicating a K_m value higher than that of types I-III but lower than that of type IV.

Brewer and Knutsen (1968) presented evidence that the seven bands reported previously for human erythrocyte (Eaten et al.1966) are not artefacts as stated by Holmes et al (1967).

Katzen et al (1968) found a new type of hexokinase in rat testis and tentatively designated it as sperm type. They stated that hexokinase type I, II and III appeared in most rat tissues whereas type IV was found only in the liver. A relative deficiency of type I hexokinase in conjunction with a deficiency in hexokinase types II and III may be correlated with insulin sensitivity of the tissue.

In the absence of β-mercaptoethanol the loss of hexokinase type IIa was seen in heart and gastrocnemius muscles and diaphragm muscle of the drug induced diabetic rat, as well as in these tissues of the genetically diabetic Chinese hamster. Types IIa and IIb appeared equally to diminish or disappear in diabetic tissue extracts.

Schimke and Grossbard (1968) reported that rat tissues contained at least four forms of hexokinase including both a liver specific, high K_m glucokinase (type IV isoenzyme) and three low K_m hexokinases (Type I-III). Electrophoretic mobilities of purified enzymes were unaffected by mercaptoethanol or glucose in buffer. In general the three low K_m hexokinases had certain similar properties. The isoenzymes were distinguished by differences in the K_m values for glucose, deoxyglucose and fructose, apparent Ki values for glucose-6-phosphate, hexose-specificities molecular weight and heat stability and proteolytic inactivation. The presence of glucose prevented inactivation of all hexokinases. Other mammalian species have hexokinase isozymes with tissue distributions and kinetic parameters similar to those of rat, but the migratory properties on starch gel differed slightly.

Kaplan and Beutler (1968) studied the electrophoretic mobility of hexokinase from human erythrocytes. The hexokinase of cord blood erythrocyte had slightly different electrophoretic properties from that of the adult red cells. The type I enzyme was split into type IA and IF; the latter was more intense in cord blood. In hemolysates of adult blood the activity of the two bands was usually equal. No type II enzyme was found in cordd blood. The double type I band was present in red cells from rabbits.

Gumaa and Greenslade (1968) studied the patterns of multiple forms of hexokinase in hepatomas and ascites cells using starch gel electrophoresis. In tumors Type II was predominant in both the soluble as well as particulate fractions. In some hepatomas Type III was present in the soluble fraction.

Vizet (1969) separated and purified three hexokinase isozymes from beef brain homogenates. Tillmann and Schroeter (1969) demonstrated hexokinase isoenzymes type I to IV in human tissues and rat livers. Only type I and III could be found in the hemolyzates from new-born infants and adults by high voltage starch gel electrophoresis.

Katzen, Soderman and Wiley (1970) studied the multiple forms of hexokinase, which were associated with the soluble and several subcellular particulate fractions of various tissue extracts of normal and diabetic rats. Hexokinase types were separated by DEAE-cellulose column chromatography and electrophoresis.

Baquer and McLean (1969) studied the effect of estrogen on the activity of binding of hexokinase type I and II in the rat uterus. They found that 4 hours after the administration of estradiol

there was no change in the total tissue content of hexokinase. However, there was a change in the distribution between the particulate and soluble fractions. There was 80% increase in the amount of hexokinase type I bound to the particles. There was no change in the binding of hexokinase type II. At 96 hours hexokinase type I had risen 3-fold in the soluble fraction and 15-fold in the particulate fraction; hexokinase type II increased 10-fold in both fractions.

Machiya and Hosoya (1969) reported that the activity of type II isoenzyme in alloxan diabetic rat liver and muscle and the activity of type IV isoenzyme in diabetic liver were markedly decreased. Insulin injection 24 hours before sacrifice increased the levels of type II and type IV in liver and the level of type II in muscle.

Pilkis and Hansen (1968) reported that rat liver supernatant fraction contained four hexokinase isozymes I-IV respectively. Type I, II and III were typical animal hexokinases, while type IV was found only in liver extracts. They carried out starch gel electrophoresis of liver extracts under well-defined conditions and found two types of type IV band, IVf (fast form) and IVs (slow form). Brown et al (1967) reported the presence of hexokinase with a low K_m not previously found in extracts of human and dog liver but not of rat liver.

Galton and Jones (1967) carried out disc electrophoretic studies on acrylamide gel to examine the isoenzymes of several human tissues. The band pattern suggested that at least two types of hexokinases were present in several human tissues. An infant

with hemolytic anemia was found to have an unusual band that migrated faster than hemoglobin. The infant's father displayed the same abnormality, but his mother's zymogram was normal.

Walters and McLean (1968) described the distribution of hexokinase I and II in soluble and particulate fractions of mammary glands from alloxan diabetic and antiinsulin serum treated rats. In rats nearly half the total hexokinase activity was in the particulate fraction, but this was decreased in both alloxan diabetic and anti-insulin serum treated rats to 10%. During anti-insulin serum treatment the hexokinase was released from the particulate to the soluble fraction. The hexokinase activity in the soluble fraction increased from 38 to 61 units per gram of tissue whereas there was no change in the total activity of the whole homogenate. There was a greater increase in hexokinase I than of hexokinase II in the soluble fractions suggesting that more type I was released from the mitochondria. After longer periods of insulin deprivation as in alloxan diabetic rats, total hexokinase in the whole homogenate fell and the activity of the soluble fraction returned to normal suggesting a secondary decrease in the hexokinase activity in the soluble fraction.

SECTION VII

ACTIVATORS AND INHIBITORS

There are several reports on activation and inhibition of hexokinase some of which have been referred to in earlier section. Much of the earlier work was carried out with crude enzyme preparations. No attempt will be made to cover earlier literature in this field especially on the effect of hormones. Only some of the more recent references will be briefly surveyed in the following section.

$BeCl_2$ was reported to inhibit hexokinase in Y cells in continuous culture of embryonic mouse liver (Santacroce and Costabile, 1966). Inhibition by substituted benzoates related to salicylate at 5 to 20 mM (Sturman and Smith, 1966,1967) in human red cells and by fatty acids (Weber et al. 1966; Lea and Weber, 1967) have been described. Rat liver and rat hepatoma hexokinases were inhibited 50 per cent by low concentrations of octanoate and laurate in the case of rat hepatoma. Similar inhibitions were also observed with normal liver and with normal and tumorous kidney tissue. It is not clear whether the observed effects are due to inhibition or inactivation of the enzyme.

Diisopropylphosphorofluoridate at 10^{-4} to 10^{-2}M inhibited crystalline yeast hexokinase (Domagk et al. 1967). Pyridoxal phosphate (Grillo, 1968) at 0.3 to 12 mM was found to inhibit yeast hexokinase 10 to 60 per cent whereas pyridoxal and pyridoxamine phosphate were found to have no effect. The possibility that these

reagents act at a serine and lysine residues at the active centre requires further investigation. It was claimed that hydrocortisone inhibits yeast hexokinase, that this inhibition can be prevented by zinc chloride silver nitrate and p-CMB and that these effects were related to changes in the quaternary structure of the enzyme (Titova, 1968). Crystalline yeast hexokinase was inactivated on incubation with hydroxycortisone at 4°. The presence of insulin or oxytocin prevented this inactivation possibly by preventing the formation of a complex between enzyme and the steroid (Titova,1970).

The observation that oxamate also inhibits hexokinase in sarcoma 37 ascites cells deserves further investigation in view of the studies with this inhibitor on lactic dehydrogenase in glycolysis (Elwood,1968).

There have been several reports on the activation of hexokinase by detergents and lecithin. Most of this work was done with crude enzyme preparations and it is not possible to assess the effects on latent enzyme. In some cases even an effect on the assay system cannot be ruled out. Care has has not always been taken to ascertain that 6-phosphogluconate dehydrogenase was absent or present in excess when the enzyme was assayed with glucose-6-phosphate dehydrogenase and NADP. The addition of a freshly prepared solution of lecithin to yeast hexokinase after the addition of ATP, glucose and Mg^{2+} was found to enhance the activity of the enzyme whereas aged lecithin was found to be inhibitory, possibly due to oxidation products. It was claimed that 2-fold increase in activity was obtained and that the effect was dependent upon the concentration of hexokinase and decreased with increase in concentration of ATP (Serebrovskaya *et al.* 1969). Rat brain mitochondrial hexokinase activity was found to be

increased when the extraction was carried out with Triton X-100 NaCl or sodium deoxycholate. The detergent caused a greater increase in activity than NaCl (Dovedova and Bigl, 1969).

Injection with streptomycin was reported to increase hexokinase activity of skeletal muscle and brain whereas the activity of liver decreased. Streptomycin had no effect on yeast hexokinase in vitro but the addition of plasma from animals treated 10 days previously with streptomycin increased the activity of yeast hexokinase in vitro. The effect was diminished on dialysis of the plasma. It was claimed that streptomycin induces the formation of a low molecular substance in rats which stimulates yeast hexokinase (Barna et al. 1970).

It was stated that cholesterol and monoiodoacetate (Shpigel, 1969) increased rapidly liver hexokinase activity in vitro by about 33 per cent whereas they had relatively little or no effect on the muscle enzyme.

The hexokinase activity of homogenates of myocardial and skeletal muscle from normal rats was strongly inhibited by the addition of serum of febrile rats, whereas it was unaffected by serum from adrenalectomized febrile rats. It was suggested that the hexokinase is apparently inhibited by the high levels of corticosteroids in the blood of febrile rats (Bel'chenko, 1967). The level of hexokinase in connective tissue of sham operated rats (Foster,1967) which had been treated with corticosterone or hydrocortisone was found to increase. In the case of polyvinyl sponge implants the change in enzyme activity in adrenalectomized animals could be restored to normal by treatment with corticosteroids. The restoration

of hexokinase activity, which had been diminished by injection of cortisone to chick embryos and mice, was reported to have been restored by treatment with insulin (Pertseva, 1967). The hexokinase of blood lymphocytes was reported to increase 2-fold by continued administration of cortisone for six days (Emel'yantsev and Svestinikova, 1969). Insulin had no direct effect on the enzyme, but it prevented the effect of cortisone when administered along with it.

Administration of hydrocortisone and especially of ACTH of starved rabbits prevented the decrease of hexokinase activity of muscle, heart and liver due to starvation. However, other reports (Kildema, 1966) claimed no effect of starvation on hexokinase activity.

When thyroxine was administered to rabbits for five successive days the enzyme activity was found to increase by about 25 per cent. Experimental hypothyroidism (induced by chemicals or by thyrodectomy) reduced hexokinase and glucokinase activity (Fomina and Vasil'eva, 1968) in rat liver fractions. This effect was accentuated by starvation but administration of thyroxine did not reverse these effects. An interesting observation was made on the hormonal control of hexokinase in seminal vasicles on orchidectomized rats (Singhal and Ling,1969). Castration caused a marked decrease in hexokinase activity whereas administration of testosterone propionate increased activity to about 4.5 times that of the control. This increase was inhibited by the administration of 17 β estradiol though the estrogen by itself had no significant effect. Inhibitors

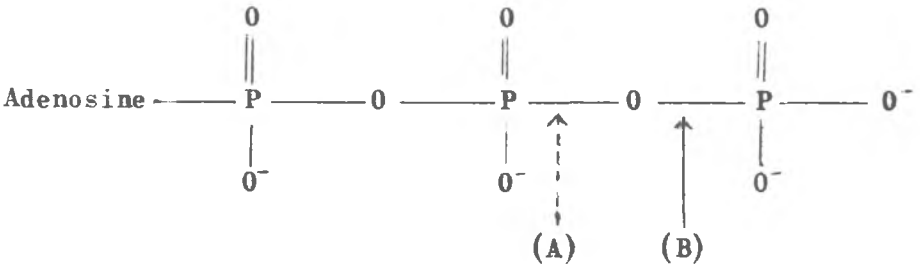
of protein synthesis actinomycin, cycloheximide, ethionine effectively prevented the androgen-induced increase of hexokinase.

Much of this work on the effect of hormones requires careful reexamination.

S E C T I O N VIII

MECHANISM OF ACTION OF HEXOKINASE

The mechanism of action of yeast hexokinase has been studied in great detail but relatively little work has been carried out with animal tissue hexokinases. Cohn (1956) studied the mechanism of action of yeast hexokinase using $H_2{}^{18}O$.



Neither ADP nor glucose-6-phosphate contained excess ^{18}O. If cleavage occurs at (A), ADP will be labelled. Cleavage occurs, therefore, at (B). The oxygen atom of glucose-6-phosphate links C_6 of glucose with P, is derived from glucose and not from ATP.

Ågren and Engstrom (1956) reported the formation of an enzyme phosphate intermediate in yeast hexokinase reaction. They isolated phosphoserine from the acid hydrolysate of the enzyme which was incubated with ^{32}P-labeled ATP in the presence of glucose or glucose-6-phosphate. However the results, which suggested the formation of an enzyme-phosphate intermediate, could not be confirmed by other workers.

Najjar and McCoy (1958) showed that there was no exchange between ^{14}C labeled glucose and glucose-6-phosphate in the absence of adenine nucleotide. Such exchange should occur if phosphoenzyme

were to react with glucose to form dephosphoenzyme and glucose-6-phosphate.

Hass, Boyer and Reynard (1961) observed that when yeast hexokinase was incubated with ^{32}P labeled ATP, the specific activity of the isolated enzyme was less than 1/500 th of that of ATP. They demonstrated that within experimental error no stable phosphoryl enzyme was formed. Trayser and Colowick (1961) also ruled out the possibility of formation of a phosphoenzyme. They incubated excess hexokinase with ATP, PEP, pyruvate kinase, lactic dehydrogenase and NADH and observed no burst of ADP formation. However a slow formation of ADP was noted but this was attributed to the ATPase activity and traces of glucose in the enzyme. It is of interest that the crystalline yeast enzyme has a feeble ATPase activity, which is probably due to hexokinase and not due to an impurity. Ågren *et al* (1956) have also later reported that their highly purified preparation did not form a phosphoenzyme intermediate on incubation with ATP. It may be concluded that the formation of a phosphoenzyme intermediate is ruled out at least for yeast hexokinase. Similar studies have not been carried out with animal tissue hexokinases.

Najjar and McCoy (1958) suggested a second mechanism in which the formation of a glucose-enzyme complex was postulated. This complex then reacts with ATP to form glucose-6-phosphate enzyme and ADP. The glucose-6-phosphate enzyme complex then reacts with glucose to form glucose-enzyme and glucose-6-phosphate. However, the work of Trayser and Colowick (1961) did not support this hypothesis. If the isolated enzyme existed as an enzyme-glucose complex according to Najjar and McCoy, then the complex should react in the absence of glucose with AT^{32}P and Mg^{2+} to give enzyme-glucose-6-phosphate and ADP. No such

complex formation was detected and no exchange between ADP and ATP was observed in the absence of added glucose or glucose-6-phosphate.

Hammes and Kochavi (1962) studied the transfer of phosphate group from ATP to D-glucose over a wide range of ATP, Mg^{2+}, MgATP and glucose concentrations. They claim that these studies indicated that the most probable mechanism is the combination of MgATP and enzyme-glucose complex to form a quaternary intermediate which in turn decomposes to MgADP and a dissociable enzyme-glucose complex. They found the binding constant of D-glucose to hexokinase to be 2.5×10^{-3}M.

Fromm and Zewe (1962b) studied the mechanism of yeast hexokinase kinetically. The effect of mannose and product inhibition of the forward reaction were studied. They showed that the substrates, glucose and ATP, add randomly to the enzyme and suggested that the participation of enzyme-phosphate or enzyme-glucose intermediates in yeast hexokinase reaction is unlikely. Hence it may be concluded that there is no definite proof for the formation of phosphoenzyme or enzyme-glucose as an intermediate in the yeast hexokinase reaction. The formation of a quaternary complex of enzyme, Mg^{2+}, ATP and glucose, as suggested by paramagnetic resonance studies, appears probable (Cohn, 1963).

Noat et al (1967) made a detailed kinetic study of the mechanism of yeast hexokinase. This study showed a reaction scheme with an orderly sequence. The first step involves the combination of glucose with hexokinase. Noat, Ricard and Buc (1968) reported that simulation of the catalytic mechanism of hexokinase using an analogue computer permitted theoretical analysis of the evolution of different enzyme-substrate complexes and enzyme-product complexes during the

first stages of the reaction. Fromm (1969) later suggested that yeast hexokinase may react in random fashion with its substrates before forming a ternary complex of enzyme-ATP-glucose.

de la Fuente, Lagunas and Sols (1970) have examined some properties of the ATPase activity of yeast hexokinase with ATP, ITP and GTP as substrates. In the presence of xylose or lyxose as non-phosphorylable analogues of glucose and mannose respectively, the phosphatase activity was modified with respect to K_m and Vmax. The effects indicate the occurrence of an "induced fit" by which certain sugars specifically modify the binding of the triphosphate substrates and enhance the labilizing action of the enzyme on their terminal phosphoryl bond. They observed a single pattern of the double reciprocal plots of the forward and backward reactions of yeast hexokinase, even when the concentration of acceptor and donor varied over a wide range. With ITP or ATP as donor substrate it appeared that in the forward or backward reactions the interconversions of the ternary complex of the enzyme with both substrates is the rate limiting step. The binding of sugar to an enzyme-nucleotide complex apparently results in an abortive ternary complex. Thus formation of an active ternary complex in hexokinase reaction requires the sequential addition of a nucleotide substrate to a binary complex of the enzyme with sugar substrate.

Fromm and Rudolph (1970) used alternative substrates to study the mechanism of action of yeast hexokinase. D-fructose was the alternative substrate. They concluded from experiments with alternative and isotopic substrates that the mechanism appears to be Random Bi Bi.

The mechanism of action of animal tissue hexokinases has been investigated by Fromm and his colleagues (Fromm and Zewe,1962a; Hanson and Fromm,1965; Zewe and Fromm, 1964).

Fromm and Zewe (1962a) studied the mechanism of action of brain hexokinase kinetically. They concluded that animal tissue hexokinases differ from yeast hexokinase. With these enzymes interaction with one substrate and dissociation of a product occurs before the second substrate combines with the enzyme. This differs from the quaternary complex postulated for yeast hexokinase. Since a glucose-glucose-6-phosphate exchange did not occur with skeletal muscle hexokinase in the absence of adenine nucleotide, an enzyme-phosphate intermediate was ruled out. The initial formation of an enzyme-glucose intermediate was suggested.

Fromm et al (1966) showed that skeletal muscle hexokinase catalyzes an ADP-ATP exchange in the absence of hexose. Hanson and Fromm (1967) studied the kinetics and mechanism of action of partially purified hexokinase from rat skeletal muscle. This enzyme (II) differs from the other types of hexokinase in muscle. Random addition of the substrates to the enzyme was postulated where all enzyme and substrate interactions equilibrate rapidly relative to the interconversion of the central ternary complexes, Enzyme-ATP-glucose and enzyme-ADP-glucose-6-phosphate. The other enzyme present in the muscle tissue exhibits a reaction mechanism in which substrate reacts with the enzyme to produce a product prior to the addition of the second substrate. Kinetic experiments with AMP, ADP, mannose and mannose-6-phosphate suggested that muscle hexokinase type II is similar, but not identical in its mechanism of action to yeast hexokinase, pyruvic kinase and creatine kinase.

Ning et al (1969) carried out initial rate studies using solubilized brain hexokinase. Both ATP^{4-} and β,γ-5'-adenylmethylene-diphosphonate are competitive inhibitors of $MgATP^{2-}$ and mixed inhibitors of the sugar substrate while N-acetylglucosamine, a competitive inhibitor of fructose, is a mixed inhibitor of $MgATP^{2-}$. They claim that their results are consistent with Random Bi-Bi mechanism for the brain enzyme. Purine nucleotides, with the exception of ATP, bind at the catalytic site as well as allosteric site on the enzyme. ATP and pyrimidine nucleotides bind only at the catalytic site of brain hexokinase. All allosteric effectors were inhibitory. These include ADP, AMP, GTP, GDP, ITP, IDP, IMP, 3',5' cyclic AMP and 3-PGA. The most potent inhibitor appears to be ADP according to them.

Machiya and Hosoya (1969) studied the reaction mechanism of hexokinase of rat liver. The reaction of the type I isoenzyme appeared to be of the ping-pong type, while that of type II was of the sequential type where both ATP and glucose bind to the enzyme before any products are released.

Kosow and Rose (1970) reported that ADP^{3-} and $ADPMg^{-}$ were found to cause mixed inhibition of purified yeast hexokinase isoenzymes and ascites hexokinases I and II when ATPMg was the varied substrate. It was claimed that the effect of ADP-Mg on Vmax results from the reaction at the ADP product site of the enzyme-glucose-6-phosphate complex. This type of inhibition, which is rare among the kinases, is attributed to relatively slow release of the second product glucose-6-phosphate. In the case of yeast hexokinase the replot of the intercepts of $\frac{1}{V}$ against $\frac{1}{ATPMg}$ as a function of ADPMg concentration suggested an alternative sequence of product release with

glucose-6-phosphate being released first. However the sequence is probably of minor significance except in the presence of high concentrations of ADPMg. No exchange was observed between glucose-6-^{32}phosphate and ATPMg under conditions of glucose-6-phosphate inhibition with free ADP kept low by a trapping reaction. It was suggested that glucose-6-phosphate acts at a specific modifier site especially in tumor hexokinase II.

S E C T I O N IX

ESTIMATION OF HEXOKINASE ACTIVITY

$$\text{Hexose} + \text{ATP} \longrightarrow \text{Hexose-6-phosphate} + \text{ADP} + H^+$$

Hexokinase activity can be estimated by the disappearance of hexose or ATP or the formation of hexose-6-phosphate or ADP or the liberation of protons. All these methods have been used for the assay of the enzyme.

1) The reaction can be followed by measuring the utilization of ATP. The amount of ATP utilized is determined by hydrolysis with 1 N HCl at 100° for 7 minutes. The phosphorylation of 1 µmole of ATP corresponds to the decrease of one equivalent of 7 minute acid hydrolysable phosphate. The disappearance of hexoses can be followed by the procedure of Nelson after the precipitation of hexose-6-phosphate and nucleotide by zinc hydroxide.

2) The methods based on the estimation of liberated protons are more sensitive. These methods are useful in the absence of enzymes which hydrolyze glucose-6-phosphate or adenine nucleotides. The hydrogen ions formed can be estimated manometrically by measuring CO_2 evolution in the presence of bicarbonate (Berger et al, 1946). The acid produced can also be directly titrated with alkali using phenol red as indicator. (Kunitz and McDonald, 1946). A third method for measuring the liberated protons is based on the use of an indicator with the same pKa as that of the buffer, the change in colour being measured spectrophotometrically as suggested by Wajzer (1953). Darrow and Colowick have used cresol red in glycylglycine buffer at 25° and measured the change in the optical

density at 560 mμ. The change in the optical density was proportional within limits to the acid produced. Temperature variations must be avoided. The method is sensitive, rapid and does not require the use of other enzymes.

3) The ADP formed can be measured in the presence of pyruvic kinase, lactic dehydrogenase and PEP, Mg^{2+}, K^{+} and NADH.

$$PEP + ADP \xrightarrow[\text{Pyruvate kinase}]{K^{+},\ Mg^{2+}} \text{Pyruvate} + ATP$$

$$\text{Pyruvate} + NADH \xrightarrow[\text{genase}]{\text{Lactate dehydro-}} \text{Lactate} + NAD$$

It is evident from the above two reactions, that in the presence of the two enzymes one mole of ADP formed is equivalent to the oxidation of one mole of NADH, which can be measured spectrophotometrically at 340 mμ. This method is sensitive and accurate provided ATPase and other interfering enzymes are absent from the test system.

4) Glucose-6-phosphate formation can be measured spectrophotometrically in the presence of NADP and excess of glucose-6-phosphate dehydrogenase and the rate of NADP reduction measured at 340 mμ. The presence of 6-phosphogluconic dehydrogenase in hexokinase or glucose-6-phosphate dehydrogenase gives erroneously higher readings. The details of the above methods especially of (3) and (4) are given in the "Materials and Methods" chapter. The choice of the method is dependent on the enzyme and properties to be studied. Yeast hexokinase can be estimated by any of these methods. Animal tissue hexokinases, which are markedly inhibited by glucose-6-phosphate, are not assayed by methods (2) and (3) if glucose is used as the substrate and are

best assayed by method (4).

5) Uyeda and Racker (1965) have measured the exchange of isotopically labelled glucose with glucose-6-phosphate for studying hexokinase activity, especially in the presence of glucose-6-phosphate which inhibits the enzyme.

SECTION X

PRESENT WORK

The work in this thesis deals with the preparation of soluble hexokinase from the particulate fraction of ox heart, its purification and the study of its properties and kinetics.

This work is based on earlier studies (Jagannathan, 1963) which indicated that the particulate enzyme from brain and heart can be solubilized by treatment of the enzyme with crystalline pancreatic elastase. After treatment with elastase the hexokinase of ox heart was not sedimented at 100,000 x g in one hour. The particulate ox heart hexokinase was prepared by a method based on that of Crane and Sols (1955). The particulate enzyme was solubilized by digestion with crystalline pancreatic elastase followed by repeated freezing and thawing. It was then treated with salmine sulphate and fractionated with ammonium sulfate and calcium phosphate gel and then chromatographed on DEAE cellulose and Sephadex G-200. The maximum specific activity obtained by this procedure was 58 micromoles of glucose phosphorylated per mg protein at 30°.

The properties and kinetics of this enzyme, which include the stability of the enzyme, the effect of pH, temperature and the concentrations of Mg^{2+}, ATP, glucose, fructose and mannose on the activity and its specificity towards different sugars, nucleotides and metals, were studied. The inhibition of heart hexokinase by glucose-6-phosphate and the reversal of this inhibition by inorganic

phosphate were investigated. The effect of several compounds on hexokinase activity either directly or in the presence of glucose-6-phosphate and Pi was determined.

Chapter II of the thesis deals with the materials and experimental methods used in these studies.

Chapter III describes the preparation of soluble hexokinase from the particulate fraction of ox heart and the purification of the solubilized hexokinase.

Chapter IV deals with the properties and kinetics of the purified enzyme.

Chapter V deals with the discussion of the results of these studies.

Chapter VI contains a summary of the results and conclusions of this work.

A bibliography of the literature references cited in this thesis is given in the final section.

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

The following chemicals and enzymes were obtained from Sigma Chemical Company, U.S.A. : D-glucose-6-phosphate (dipotassium salt, hydrate), crystalline disodium salts of ATP and ADP, NADP and NADH, phenazine methosulphate, DEAE-cellulose, CM-cellulose,elastase (EC 3.4.4.7) from pancreas (twice crystallized), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (from yeast, Type XI, crystalline, specific activity 325 units/mg), pyruvate kinase (EC 2.7.1.40) (from rabbit muscle, Type II), crystalline, (specific activity 30 units/mg), lactate dehydrogenase (EC 1.1.1.27) (from rabbit muscle, Type II, crystalline, specific activity 630 units/mg), crystalline glyceraldehydephosphate dehydrogenase (EC 1.2.1.12), trypsin inhibitor (from soybean 2 x crystallized), cytochrome c (from horse heart).

Salmine sulphate, nitrotetrazolium blue and bromophenol blue were obtained from British Drug Houses, U.K.

The chemicals used for acrylamide gel electrophoresis viz., acrylamide N,N'-methylene-bis-acrylamide and tetraethylmethylethylene-diamine and amido black 10B were obtained from Eastman Kodak Company, U.S.A. Glycine was obtained from Koch Light Laboratories, U.K.

The following chemicals which were used in this study were of analytical grade: Tris (hydroxymethyl)amino methane, calcium carbonate, phosphoric acid, sodium and potassium hydroxide, potassium chloride, ethylene diaminetetraacetate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium pyrophosphate, ammonium sulphate, magnesium chloride, manganese chloride, sodium acetate, acetic acid,

2-mercapto ethanol, glucose and sucrose.

The following sugars used as substrates were of high purity: D-arabinose, cellobiose, dulcitol, fructose, D-galactose, mannose, mannitol, mellibiose, α-methylglucoside, rhamnose, L-sorbose, trehalose and D-xylose.

5-Thio-D-glucose was obtained as a gift from Dr. U. G. Nayak (the author wishes to gratefully acknowledge this gift). Phosphoenol pyruvic acid was prepared by the method of Clark and Kirby (1963). The method of Swingle and Tiselius (1951) was used for preparing calcium phosphate gel. The final preparation was suspended in glass distilled water to give a concentration of 60 mg dry weight/ml.

Sephadex G-200, was obtained from Pharmacia Fine Chemicals, Sweden. It was suspended in water and kept on a boiling water bath for 5 hr, cooled and deaerated before use. DEAE-cellulose (100-200 mesh; 0.5 meq/g) and CM-cellulose (0.7 meq/g) were washed according to Peterson and Sober (1962). Amberlite IRC-50 (XE-64) (mesh 200-400) was obtained from Rohm and Haas, U.S.A. It was washed as described by Hirs et al (1953). The chromatographic columns were used with flow of liquid under gravity without the application of external pressure.

Methods

All the reagents and buffers were prepared in glass-distilled water.

Glass apparatus was routinely soaked overnight in sodium carbonate solution and then brushed, washed with tap water and then rinsed with nitric acid. After washing successively with tap water, distilled water and finally with glass distilled water, the glassware was dried.

Hexokinase activity and protein were determined by using a Beckman DU spectrophotometer fitted with a constant temperature attachment. Silica cuvettes of 10 mm light path were used. pH estimations were carried out by using the glass electrode.

For ammonium sulfate fractionations powdered ammonium sulfate was added slowly to the enzyme solution over a period of 15 to 25 min avoiding rapid stirring and frothing. Ammonium sulfate saturations were calculated according to Jagannathan et al (1956). The precipitates obtained after ammonium sulfate precipitation were dissolved in known volumes of the buffer. The final volume was noted for the calculation of ammonium sulfate saturation of the enzyme preparation.

Centrifugations were carried out at 0°. The following centrifuges were used: International Refrigerated Centrifuges (Models PR-1 and PR-2), Sorvall (Model SS-1), Spinco ultracentrifuge (Model L). Fractions from column chromatographic runs were collected on a Technicon automatic fraction collector.

Protein was routinely estimated by the two methods described below.

(1) Protein was estimated by a modification of Warburg and Christian's method (1941) according to Jagannathan et al (1956). By this method the absorption due to nucleic acids and other impurities were corrected. It was assumed that 0.1% protein solution has an optical density of 1 at 280 mμ.

$$\frac{4}{7}\left[2.3\ (O.D._{280\ m\mu} - O.D._{340\ m\mu}) - (O.D._{260\ m\mu} - O.D._{340\ m\mu})\right]$$

= mg protein per ml

Optical densities of the enzyme solution were determined at 340, 280 and 260 mμ. A blank was run by using a solution of the same composition (e.g. with respect to TE, phosphate and sucrose) and determining its optical density at the above wavelengths. The values of the blank were subtracted from the corresponding readings of the enzyme solution.

2) Protein was routinely estimated by the method of Lowry et al (1951). Serum albumin was used as the standard. The concentration of serum albumin was calculated from its extinction coefficient at 280 mμ according to Long (1961). Glucose, ammonium sulphate, phosphate and TE interfere with this method. Hence it was necessary to dialyse the enzyme preparation till it was free from interfering substances before estimating the protein. A blank with the dialysate was also run. This blank was used for correcting for any residual interfering material in the enzyme solution. While calculating the protein concentration the change in volume after dialysis was taken into consideration.

Hexokinase assay

Crude heart preparations were diluted in 0.05 M phosphate buffer pH 7.5 and the purified preparations were diluted with 0.05 M phosphate buffer pH 7.5 containing 5 mg serum albumin/ml and 0.005 M TE.

The extinction coefficient of NADPH and NADH was taken as 6.22×10^{6} cm^{2} moke^{-1} (Horecker and Kornberg, 1948) for calculating the hexokinase activity.

All enzyme units are expressed as the amount of enzyme required to convert one micromole of substrate per min under the experimental condition.

A unit of hexokinase is defined as the amount of enzyme required to phosphorylate one micromole of glucose per min at 30° under the experimental conditions as stated in the experimental section.

The specific activity of hexokinase is defined as the activity per mg protein. Hexokinase activity was estimated by using the following three methods.

Assay I: Glucose-6-phosphate dehydrogenase assay
(Based on that of Slein, Cori and Cori,1950)

This assay was used throughout the purification procedure and in some kinetic studies. The reaction mixture contained the following ingredients, glucose (15 mM), $MgCl_2$ (20 mM), ATP (10 mM), Tris-HCl buffer, pH 7.5 (50 mM), EDTA (0.1 mM), NADP (0.13 mM) and 0.10 unit of glucose-6-P dehydrogenase in a total volume of 1 ml. The reaction was initiated by adding ATP last and the change in O.D. at 340 mμ was recorded. Here the amount of enzyme used was such as to give an O.D. of 0.005-0.025 per min. The change in O.D. was measured from the second to the tenth min. The reaction was linear and the average change in O.D. was used for calculating the hexokinase activity. Blanks without ox heart hexokinase and ATP were run. These blanks were necessary to ensure that the glucose-6-P dehydrogenase does not contain hexokinase and glucose-NADP reductase. Care was taken to use sufficient amount of glucose-6-P dehydrogenase in the case of high salt concentrations or varying amount of Mg^{2+} which affect the dehydrogenase activity.

Assay II - Direct hexokinase assay

To study the effect of pH and temperature on hexokinase activity this method was used. The reaction mixture consisted of glucose, $MgCl_2$, ATP, EDTA, Tris-HCl buffer and enzyme at a definite temperature as stated in the experimental section. Acetic acid was added to stop the reaction and the reaction mixture was then neutralized by adding an equivalent amount of KOH solution. The glucose-6-P formed was then estimated by the addition of NADP and glucose-6-P dehydrogenase to the reaction mixture.

The composition of the reaction mixture was as follows: glucose (5 µM), $MgCl_2$ (6.6 µM), ATP (3.3 µM), EDTA (0.03 µM), Tris-HCl buffer pH 7.5 (17 µM) and enzyme in a total volume of 0.34 ml. After equilibration at the desired temperature the reaction was started by adding ATP last. 240 micromoles of acetic acid were added after the incubation period to stop the reaction. This reaction mixture was then neutralized to pH 7.5 with the addition of an equivalent amount of KOH solution. The total amount of glucose-6-P formed was then estimated. For this 10 µM $MgCl_2$ and 50 uM Tris HCl buffer pH 7.5 and 0.3 units of glucose-6-P dehydrogenase and 0.52 mM NADP were added to the reaction mixture. The final volume was 1 ml. The O.D. was recorded before and after the addition of NADP. The change in O.D. was proportional to the amount of glucose-6-P formed. A blank was run in which enzyme was added after the addition of acetic acid. Glucose-6-P being a strong inhibitor of hexokinase the reaction is not linear and the amount of hexokinase taken was such as to keep the reaction approximately in the initial linear period.

Assay III - Pyruvic kinase and lactic dehydrogenase assay (Ochoa, 1961)

In this assay the rate of formation of ADP was measured enzymically by adding pyruvic kinase and lactic dehydrogenase and NADH. The ADP formed was rephosphorylated by phosphoenol pyruvate. The oxidation of NADH by the pyruvate formed was recorded at 340 mμ. This method was used for carrying out some of the kinetic experiments, especially on the effect of glucose-6-P on hexokinase activity. Here mannose and not glucose was used as the substrate. Mannose-6-P had no effect on hexokinase activity under the experimental conditions.

The reaction mixture consisted of KCl (10 mM), $MgCl_2$ (10 mM); mannose (5 mM), Tris-HCl buffer (50 mM) pH 7.5, ATP (5 mM), EDTA (0.1 mM), PEP (5 mM); pyruvic kinase (1.2 units); lactic dehydrogenase (1.5 units); NADH (0.25 mM) in a final volume of 1 ml. The reaction mixture was equilibrated for 3 to 5 min so that traces of ADP present in the ATP were converted to ATP. The reaction was then initiated by adding heart hexokinase. The decrease in O.D. was proportional to the amount of hexokinase. Blanks were run without heart hexokinase and without ATP. The amount of enzyme used was such as to give O.D. of 0.020-0.060 per min.

Acrylamide gel electrophoresis

Acrylamide gels were prepared by following the method of Davis (1964) with slight modifications. Riboflavin was used instead of ammonium persulphate. 7% acrylamide gels were used unless otherwise stated. The pH of the gel was generally 8.5 and pH of the running buffer was 8.5. Tris-glycine buffer was used, the concentration of the running buffer being 0.02 M with respect to

glycine. The current applied was 4 milliamps/tube and the period of the run 90 min. Protein was stained by using 0.5% Amido Black 10B in 7% acetic acid.

The hexokinase bands were located by the method of Dewey and Conklin (1960) as follows: After the run the polyacrylamide gel was incubated with the reaction mixture at 30° for 30 min. (The composition of the reaction was as stated in Assay I). The polyacrylamide gel was then washed with glass distilled water and immersed in a dyeing mixture which consisted of phenazine methosulphate (0.5 mg/ml) 0.15 ml, nitrotetrazolium blue (5 mg/ml) 0.30 ml and Tris-HCl buffer 0.1 M pH 8.4, 2.55 ml. Violet bands indicated the presence of hexokinase.

C H A P T E R III

PURIFICATION OF PARTICULATE OX HEART HEXOKINASE

PURIFICATION OF PARTICULATE OX HEART HEXOKINASE

Preliminary experiments on the solubilization of particulate heart hexokinase were carried out with beef heart mitochondria and with electron transport particles (ETP prepared according to Green and Zeigler, 1963). There was very little or no solubilization of hexokinase from mitochondria when the mitochondrial suspension in 0.25 M sucrose was incubated with elastase as described later in the section on "solubilization". But when the same experiment was carried out with ETP, about 50 per cent of the enzyme was solubilized and was not sedimented on centrifugation at 100,000 x g for one hour. Owing to the difficulty of preparing large quantities of ETP, these experiments were repeated with particulate heart hexokinase prepared according to Crane and Sols (1955). The enzyme was readily solubilized from this preparation of particulate heart hexokinase. All further experiments were carried out with the particulate enzyme prepared by this procedure, which was slightly modified so that larger quantities of heart muscle could be conveniently handled.

Preparation of the particulate heart hexokinase

All operations in this and in subsequent steps were carried out at 0° unless otherwise stated.

The following procedure for the preparation of the particulate enzyme from beef heart is based on that of Crane and Sols (1955).Fresh beef hearts (4 or 5) were washed with cold distilled water, the connective tissue was removed and the fat trimmed off. The ventricles were excised and passed through a meat mincer. The mince was

homogenized (in two lots) with 500 ml of 0.115 M KCl-0.02 M phosphate and 0.01 M EDTA buffer pH 7.5, for 3 min in a Waring blendor. During this operation the temperature rose to 10° to 12°. It was then diluted with the same buffer ($KCl-PO_4$-EDTA) to 2.5 l. The homogenate was centrifuged at 1,500 x g for 10 min. The supernatant liquid was collected and the residue discarded. The former was recentrifuged at 13,000 x g for 30 min. The supernatant liquid was kept for studies on soluble enzyme. The sediment was suspended in 500 ml of the same buffer to obtain a smooth suspension free from lumps. It was centrifuged at 13,000 x g for one hour. This washing procedure was repeated and the sediment was finally suspended in $KCl-PO_4$-EDTA buffer, pH 7.5, containing 0.25 M sucrose.

Solubilization of hexokinase (Fraction I)

On the basis of several preliminary trials the following procedure was adopted for solubilizing the particulate enzyme. To 100 mlof the suspension of particulate heart hexokinase 12 ml of 1 M phosphate buffer, pH 7.5, and 24 ml of 0.09 M Na pyrophosphate-HCl buffer, pH 8.4, were added and twice crystallized pancreatic elastase was then added (1 mg of elastase for enzyme corresponding to 100 g of heart muscle) and the suspension kept for 24 hr with occasional stirring. (Elastase is almost completely insoluble at this pH and tends to settle down and occasional stirring is essential.) The enzyme was kept at -20° for about a day and then thawed slowly at room temperature with occasional mixing without allowing the temperature to rise above 5°. The liquid was again frozen and thawed in the same manner five to six times. The enzyme at this stage became highly viscous and jelly-like. It was centrifuged at 13,000 x g for one hour. Approximately 50% of the activity was obtained in

soluble form. The soluble enzyme was not sedimented at 100,000 x g in one hour.

In forty experiments the yield of particulate enzyme was on the average 0.30 unit/g muscle. The yield on solubilization was about 50 per cent and the amount of soluble enzyme obtained from 100 g of muscle was about 15 units.

The results of a typical experiment on solubilization are shown in Table 2.

The enzyme at this stage slowly lost activity and the second step (salmine sulphate treatment) was carried out immediately after the solubilization of the enzyme (Fraction I).

Salmine sulphate treatment (Fraction II)

The supernatant liquid obtained after elastase treatment was mixed with 0.1 volume of 1 M phosphate buffer, pH 7.5, followed by 0.1 volume of 5 per cent salmine sulphate solution (5 g dissolved in 100 ml of 0.05 M phosphate buffer, pH 7.5) with continuous stirring. The final pH was about 7.3 to 7.4. It was kept for 15 to 20 min and then centrifuged at 13,000 x g for 30 min. The precipitate was discarded. The highly viscous supernatant liquid obtained after elastase treatment became water-clear and non-viscous after treatment with salmine sulphate. The recovery of the enzyme at this stage was nearly quantitative but there was no significant increase in the purity of the enzyme. Table 3 shows the results of a typical experiment on salmine sulphate treatment. It will be noted that there was no significant change in the ratio of optical density at 280 mμ to that at 260 mμ. The decrease in viscosity was not due to the removal of nucleic acids to any significant extent. However, without this

step further purification of the enzyme was difficult. The soluble enzyme, which was not treated with salmine sulfate, gave very little increase in purity on calcium phosphate gel adsorption or ammonium sulphate fractionation, whereas the salmine sulphate treated enzyme could be readily purified. The nature of the various impurities removed at this stage is not known. Fraction II enzyme could be stored for several weeks at -20° without significant loss of activity.

TABLE 2

SOLUBILIZATION OF HEART HEXOKINASE

Particulate preparation				Elastase treated enzyme (Fraction I)			
Volume	Activity	Total activity	Activity	Volume	Activity	Total activity	Solubilization
ml	units/ml	units	units/g tissue	ml	units/ml	units	%
300	0.65	195	0.32	290	0.33	96	49

TABLE 3

EFFECT OF SALMINE SULPHATE TREATMENT (FRACTION II)

(Fraction I was treated with salmine sulphate as described in the text)

Description	Volume	Activity	Total activity	Protein	Total protein	Specific activity	O.D. 280 mu / 260 mu	Recovery
	ml	units/ml	units	mg/ml	mg	units/mg protein		%
Fraction I	290	0.33	96	6.0	1740	0.055	0.88	
Fraction II	320	0.28	90	4.4	1408	0.063	0.80	93

PURIFICATION OF HEART HEXOKINASE

Preliminary experiments were carried out to determine which methods were suitable for the purification of the enzyme. Fractionation with ammonium sulphate and the use of different adsorbents were studied.

Calcium phosphate gel treatment

The amount of calcium phosphate gel required for the adsorption of the enzyme was determined in preliminary experiments. About 45 to 50 mg of gel were required for the adsorption of one unit of hexokinase. This proportion of gel to the enzyme was used in all subsequent experiments. The enzyme was in 0.05 M phosphate buffer and the final composition of the buffer during gel treatment was 0.05 M phosphate, 0.25 M sucrose and 0.005 M TE. The pH was 7.5 unless otherwise stated and sucrose and TE were added at the above concentrations in all the buffers. After the adsorption of the enzyme the gel was successively eluted with phosphate buffer of different concentrations and the specific activities of the eluates were determined.

Gel treatment was initially carried out on Fraction II directly. The results are shown in the next section (Procedure I). In later experiments an ammonium sulphate fractionation step was introduced as described in Procedure II.

Procedure I

9.5 ml of enzyme (Fraction II) (0.57 units) were treated with 0.4 ml of calcium phosphate gel (20 mg gel) mixed well and kept in the cold for 15 min and then centrifuged. The gel was then successively eluted with 5 ml each of 0.1 M, 0.15 M and 0.20 M phosphate buffer and

finally with 10 ml of 0.20 M phosphate buffer. The results are summarized in Table 4. In this procedure there was only two to three fold purification relative to the fraction II enzyme.

Procedure II

With some batches the specific activity obtained after calcium phosphate gel treatment was low. The salmine sulphate also precipitated due to a slight increase in pH or lowering of temperature. Hence an ammonium sulfate fractionation step was introduced prior to gel treatment.

The enzyme (Fraction II) was fractionated with ammonium sulphate as follows: To every 100 ml of enzyme solution 22.5 g of powdered ammonium sulphate were added with stirring and the liquid was kept for 20 to 30 min at 0°. The solution was then centrifuged at 13,000 x g for 30 min. The sediment was discarded. To every 100 ml of the supernatant liquid 29 g of powdered ammonium sulphate were added. After about 30 min it was centrifuged at 13,000 x g for 60 min. The precipitate was dissolved in 0.05 M phosphate buffer, pH 7.5, containing 0.25 M sucrose and 0.005 M TE and dialysed against the same buffer for 3 to 4 hr with three changes of buffer. The dialysed enzyme was centrifuged to remove any turbidity (Fraction III).

The results are shown in Table 5. The recovery of enzyme was about 80-90% and the specific activity increased from about 0.08 units/mg to 0.3 - 0.8 units/mg.

TABLE 4

CALCIUM PHOSPHATE GEL TREATMENT

Fraction	Volume	Activity	Protein	Total activity	Total protein	Specific activity
	ml	units/ml	mg/ml	units	mg	units/mg protein
Fraction II enzyme	9.5	0.060	0.94	0.57	8.9	0.06
0.15 M eluate	5.0	0.022	0.14	0.11	0.7	0.15
0.20 M eluate I	5.0	0.033	0.34	0.17	1.7	0.10
0.20 M eluate II	10.0	0.022	0.10	0.22	1.0	0.22

TABLE 5

AMMONIUM SULPHATE PRECIPITATION (FRACTION III)

Description	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg	units/mg protein	%
Fraction II	620	0.25	155	3.0	1860	0.08	-
Fraction III	26.5	5.20	138	6.3	166	0.82	89

Calcium phosphate gel treatment (Fraction IV)

26 ml enzyme (Fraction III) (135 units) were mixed with 125 ml of calcium phosphate gel (7.5 g gel). (The final concentration of the buffer was 0.05 M phosphate, pH 7.5.) After 30–40 min with occasional stirring the liquid was centrifuged and the supernatant fraction discarded. The gel was then washed with 150 ml of 0.10 M phosphate buffer, pH 7.5, containing 0.25 M sucrose and 0.005 M TE and the supernatant liquid was discarded after centrifugation. The enzyme was then eluted successively with 230 ml of 0.15 M phosphate buffer (eluate I) and 220 ml of 0.15 M phosphate buffer (eluate II) containing the same amount of sucrose and TE.

The calcium phosphate gel eluates were combined and to every 100 ml of eluate 60.2 g of powdered ammonium sulphate were added. After 30 min the precipitate was collected by centrifugation at 13,000 x g for one hr. The precipitate was dissolved in 0.05 M phosphate buffer containing 0.25 M sucrose and 0.005 M TE. The results are summarized in Table 6.

Fractionation of the enzyme with different ion exchangers was then studied.

Adsorption on IRC-50 (Fraction Va)

a) 2 g resin (IRC-50) previously equilibrated with 0.01 M phosphate buffer, pH 6.8, (containing no sucrose or TE) were added to 4 ml enzyme (Fraction II) (1 unit) mixed well, kept in the cold for 20 min and then centrifuged. The enzyme was then eluted successively with 5 ml of 0.1 M phosphate buffer, pH 8.0, and then twice with 10 ml of 0.2 M phosphate buffer, pH 8.0. The results are summarized in Table 7. It will be seen that there

TABLE 6

CALCIUM PHOSPHATE GEL TREATMENT OF HEXOKINASE

Fraction	Volume	Total activity	Total protein	Specific activity	Recovery
	ml	units	mg	units/mg protein	%
Fraction III	26.0	135	164	0.82	-
0.15 M eluate I	220	77	44	1.85	76
0.15 M eluate II	215	26	14	1.84	
Fraction IV	6.5	98	53	1.83	72

TABLE 7

ADSORPTION OF HEXOKINASE ON IRC-50

(Fraction II enzyme was used for this experiment)

Fraction	Volume	Total units	Total protein	Specific activity
	ml		mg	units/mg protein
Fraction II	4	1.00	14.8	0.06
0.1 M eluate	5	0.12	0.9	0.13
0.2 M eluate I	10	0.34	2.6	0.13
0.2 M eluate II	10	0.20	0.3	0.66

was two to ten-fold increase in the purity in different fraction of the eluates with 66 per cent recovery of the enzyme.

b) This experiment was repeated with Fraction III enzyme obtained after ammonium sulfate fractionation.

2 g resin (IRC-50) equilibrated with 0.01 M phosphate buffer, pH 6.8, (without sucrose or TE) were added to 0.5 ml enzyme (Fraction III)(1.1 unit) and kept with occasional stirring for 20 min. After centrifugation the enzyme was eluted successively with 10 ml of 0.05 M phosphate buffer (twice), pH 7.5, containing sucrose (0.25 M) and TE (0.005 M) and twice with 10 ml of 0.1 M phosphate buffer, pH 7.5, containing same amount of sucrose and TE. The results are summarized in Table 8.

The enzyme obtained after IRC-50 adsorption was very dilute and extremely unstable. Hence the procedure was not used for subsequent work, though there was significant purification.

Purification with ECTEOLA cellulose (Vb)

100 mg ECTEOLA cellulose equilibrated with 0.005 M phosphate buffer, pH 8.0, were added to 5.0 ml enzyme in (0.15 M phosphate buffer, pH 7.5) (Fraction II) (1 unit) and kept with stirring for 30 min. On centrifugation it was found that almost all the activity (85%) was in the supernatant liquid. The increase in purity was 1.3 fold in this batchwise treatment. Since better results were obtained with DEAE-cellulose, further work with ECTEOLA-cellulose column was not carried out.

TABLE 8

ADSORPTION OF HEXOKINASE ON IRC-50

Fraction	Volume	Total units	Total protein	Specific activity
	ml		mg	units/mg protein
Fraction III	0.5	1.1	3.4	0.32
0.05 M eluate I	10	0.13	0.25	0.52
0.05 M eluate II	10	0.14	0.16	0.88
0.1 M eluate I	10	0.29	0.40	0.72
0.1 M eluate II	10	0.25	0.22	1.10

Adsorption on CM-cellulose (Fraction Vc)

CM-cellulose was equilibrated with 0.01 M phosphate buffer, pH 6.8. 200 mg of CM-cellulose (in 0.01 M phosphate buffer, pH 6.8) were mixed with 1 ml enzyme (1.6 unit, of Fraction III) and centrifuged after 30 min. It was observed that 75% of the enzyme activity was adsorbed on CM-cellulose. The CM-cellulose was then eluted with 10 ml of 0.05 M phosphate buffer, pH 7.5 (twice), 10 ml of 0.1 M phosphate buffer and finally with 10 ml of 0.2 M phosphate buffer. Only 25% of the adsorbed activity was eluted and the enzyme obtained was in very dilute form. Further work with this adsorbent was not carried out due to the instability of the enzyme in very dilute solution.

Alcohol precipitation

5 ml enzyme (Fraction II) (1 unit) 0.15 M phosphate buffer, pH 7.5 were mixed with 90 mg glucose and 7.5 ml of ethanol were slowly added to the solution at 0°. The precipitate was dissolved in 0.2 ml of 0.1 M phosphate buffer, pH 7.5. It was found that the enzyme lost its activity completely.

The enzyme was also precipitated with 3 volumes of cold ethanol in the presence of 0.5 M glucose and 0.01 M TE. In this experiment also the enzyme was completely inactivated.

DEAE-cellulose column chromatography

The enzyme used for DEAE-cellulose adsorption experiments was Fraction IV obtained after ammonium sulphate precipitation of the gel eluates. It was dialysed against 0.005 M phosphate buffer, pH 7.5, containing 0.25 M sucrose and 0.005 M TE. All buffers used

in this experiment were K-phosphate buffers at pH 7.5 and contained the same amount of sucrose and TE unless otherwise stated. From preliminary experiments it was found that 35 mg to 40 mg of DEAE-cellulose were required for the adsorption of one unit of hexokinase.

Washed DEAE-cellulose was equilibrated with phosphate buffer by suspending it in 0.005 M phosphate buffer for 30 min. It was then filtered and washed four or five times with glass distilled water. The suspension was de-aerated under vacuum. It was finally suspended in 0.005 M phosphate buffer and poured into a column (2.5 cm x 20 cm) with a layer of acid washed glass-wool at the bottom. After it had settled down the DEAE-cellulose was finally equilibrated by passing 500 ml of 0.005 M phosphate buffer through it.

The dialysed enzyme was loaded on the column and the sides of the tube were rinsed thrice with about 6 ml of 0.005 M buffer. The cellulose was then washed with 300 to 400 ml of 0.01 M buffer. The enzyme was then eluted with 0.05 M buffer. The fractions were collected using an automatic fraction collector at 30 min intervals and tested for enzyme activity and protein. Protein was estimated by Lowry's method. Fractions having maximum specific activity (about 10 units/mg or higher) were pooled and precipitated with ammonium sulphate by adding 60.2 g of solid ammonium sulphate for every 100 ml of the eluate (Fig. 1). It was kept in the cold for 40-50 min and the precipitate was collected by centrifugation at 13,000 x g for one hr. The precipitate was dissolved in 0.05 M phosphate buffer, pH 7.5, containing sucrose and TE, centrifuged to remove any insoluble material and stored at -20° (Fraction V).

In five different experiments the final specific activities at this step ranged from 9 to 12 units/mg and the total recovery of

TABLE 9

PURIFICATION OF HEXOKINASE BY DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

Fraction No.	Volume	Activity	Total activity	Protein	Total protein	Specific activity
	ml	units/ml	units	mg/ml	mg	units/mg protein
Enzyme loaded	9.0	8.8	79.2	4.8	43.2	1.8
1 - 4	16.0	0.08	1.0	-	-	-
5 - 8	16.0	0.16	2.6	0.13	2.08	1.5
9 - 11	22.0	0.33	7.3	0.07	1.54	4.8
12 - 16	32.5	0.45	17.6	0.035	1.14	13.0
17 - 21	32.5	0.34	11.1	0.030	0.98	11.5
22 - 23	13.0	0.28	3.6	0.025	0.33	11.2
24 - 27	26.0	0.14	5.5	0.030	0.78	5.8
28 - 33	39.0	0.12	4.3	-	-	-
Enzyme recovered after ammonium sulphate precipitation	3.0	11.5	34.5	1.26	3.78	9.1

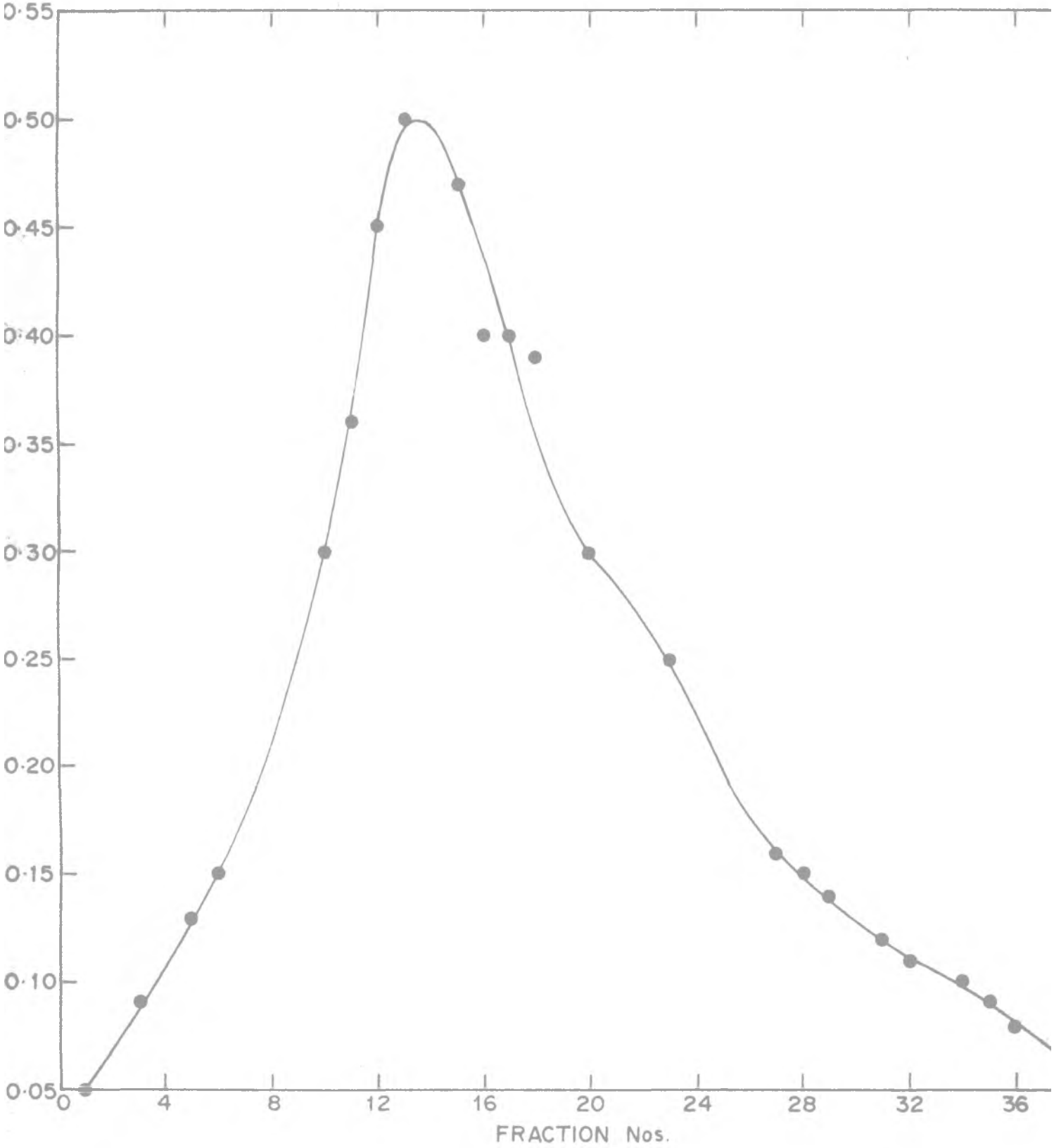


FIG. 1 DEAE-CELLULOSE COLUMN CHROMATOGRAPHY.

TABLE 10

DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF HEXOKINASE

Experiment No.	Enzyme loaded	Initial specific activity	Enzyme recovered	Final specific activity	Total recovery
	units	units/mg protein	units	units/mg protein	%
1	10	1.0	3	10.3	30
2	25	1.0	7.5	9.4	30
3	80	1.8	33	9.1	41
4	70	1.8	22	9.1	30
5	40	2.0	16	12.0	40

enzyme in all fractions was 60 to 75% and the recovery of enzyme of specific activity higher than 10 units per mg was about 50 per cent of the total. The results are shown in Tables(9)and(10).

Sephadex G-200 column

Sephadex G-200 was deaerated and then poured into a column (2 cm x 100 cm). It was equilibrated with 0.05 M phosphate buffer, pH 7.5, containing 0.25 M sucrose and 0.005 M TE.

The enzyme/(Fraction V) (10 units) was dialysed against 0.05 M phosphate buffer, pH 7.5, containing 0.25 M sucrose and 0.005 M TE. The enzyme was slowly loaded on the column and the sides of the column were rinsed with 3 ml of the same buffer thrice. The enzyme was then eluted with buffer of the same composition. The initial 50 to 60 ml were collected in a measuring cylinder and then 4 ml fractions were collected by using an automatic fraction collector (flow rate 8 ml/hr). The activity and protein of the fractions were also determined, the protein being estimated by Lowry's method after dialysis of aliquots. The results are shown in Fig. 2 and Tables 11. The maximum specific activity in any fraction was 58 units/mg protein and the total yield of enzyme of specific activity 45 units per mg was 4 units.

The results of five different experiments are shown in Table 12. The final specific activity ranged from 43 to 54 units/mg and the recovery of the enzyme in this step was 35 to 45%.

The enzyme obtained after this step was very unstable. It rapidly lost its activity, about 30% being lost within a day even at -20°. The concentration of sucrose, phosphate and TE were increased

TABLE 11

PURIFICATION OF HEXOKINASE ON SEPHADEX G-200

Fraction No.	Volume	Activity	Total activity	Protein	Total protein	Specific activity
	ml	units/ml	units	mg/ml	mg	units/mg protein
Enzyme loaded	0.9	11.5	10.35	1.26	1.13	9.1
7	4.0	0.03	0.12	-	-	-
8	4.0	0.14	0.56	0.015	0.060	9.3
9	4.0	0.18	0.72	0.011	0.044	16.3
10	4.0	0.23	0.92	0.004	0.016	58.0
11	4.0	0.27	1.08	0.005	0.020	54.0
12 & 13	8.0	0.25	2.00	0.009	0.072	28.0
14	4.0	0.20	0.80	0.015	0.060	13.3
15	4.0	0.13	0.52	-	-	-
16 & 17	8.0	0.055	0.44	-	-	-

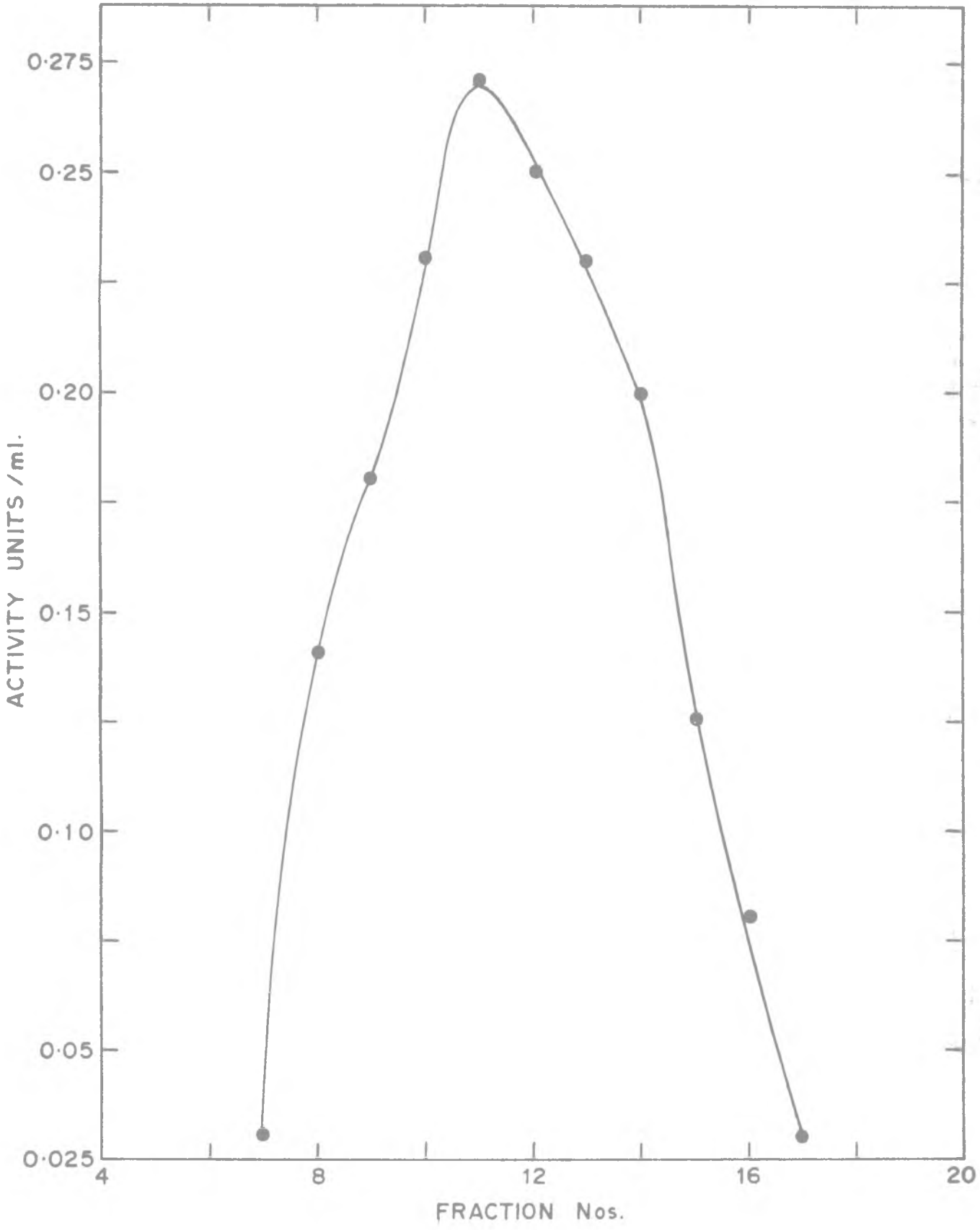


FIG. 2. SEPHADEX G-200 COLUMN CHROMATOGRAPHY

TABLE 12

SEPHADEX G-200 COLUMN CHROMATOGRAPHY OF HEXOKINASE

Expt. No.	Enzyme loaded	Initial specific activity	Enzyme recovered	Specific activity	Recovery of enzyme
	units	units/mg protein	units	units/mg protein	%
1	10	10.3	3.4	46	34
2	10	9.4	4.0	45	40
3	10	9.1	3.5	54	35
4	10	9.1	4.5	43	45
5	10	12.0	4.0	45	40

to 1 M, 0.2 M and 0.01 M respectively but even then the loss of activity at -20° was 30-35% in 24 hr. But when 5 mg serum albumin was added per ml of enzyme containing 0.5 M sucrose, 0.2 M phosphate and 0.01 M TE, pH 7.5, there was no loss of activity even after several months of storage at -20°.

In one or two experiments the Sephadex eluates were pooled and precipitated with ammonium sulphate. In these experiments it was observed that only 25-30% of the enzyme activity could be precipitated by ammonium sulphate. The ammonium sulphate precipitated enzyme was also found to lose its activity rapidly on storage.

In one experiment the enzyme obtained after Sephadex treatment was kept with varying concentrations of sucrose at different temperatures (viz. 0° and -20°). The enzyme lost 30-40% of activity within 48 hr in all cases.

In another experiment the enzyme was concentrated with dry Sephadex G-200. The enzyme was kept in a dialysis tube and the dialysis tube was then immersed in solid Sephadex. In this case the loss in volume was rapid but there was almost complete loss of activity. It was unfortunately not possible to concentrate the enzyme by filtration (e.g. through an Amicon filter) and test whether the rapid loss of activity was due to its high dilution.

Since it was difficult to concentrate the enzyme obtained after Sephadex treatment and since the enzyme was highly unstable in the absence of serum albumin, further purification of the enzyme was not attempted.

On the basis of the previous studies, the following was the final procedure for the purification of particulate ox heart hexokinase.

Purification of heart hexokinase

Elastase treatment (Fraction I)

All operations were carried out at 0° unless otherwise specified.

To 100 ml of the suspension of particulate heart hexokinase 12 ml of 1 M phosphate buffer, pH 7.5, and 24 ml of 0.09 M Na-pyrophosphate-HCl buffer, pH 8.4, were added. Twice crystallized pancreatic elastase was then added (1 mg of elastase for enzyme corresponding to 100 g of heart muscle) and the suspension was kept for 24 hr with occasional stirring. The enzyme was then frozen by keeping at -20° for 24 hr and then thawed slowly at 5° and the freezing and thawing were repeated five or six times. The enzyme, which became jelly-like at this stage, was centrifuged at 13,000 x g for 1 hr.

Salmine sulphate treatment (Fraction II)

The supernatant liquid (Fraction I) was mixed with 0.1 volume of 1 M phosphate buffer, pH 7.5, and 0.1 volume of 5% salmine sulphate solution (5 g dissolved in 100 ml of 0.05 M phosphate buffer, pH 7.5) with continuous stirring. After 20 min it was centrifuged at 13,000 x g for 30 min and the sediment was discarded. The highly viscous supernatant liquid obtained after fraction I became water-clear and non viscous after salmine sulphate treatment.

Ammonium sulphate fractionation (Fraction III)

Ammonium sulphate fractionation was carried out by adding 22.5 g for every 100 ml of enzyme (Fraction II), centrifuging off the precipitate and adding 29 g to 100 ml of the supernatant liquid. The

precipitate was collected by centrifugation and dissolved in a small volume of 0.05 M phosphate buffer (containing 0.25 M sucrose and 0.005 M TE), pH 7.5, and was dialysed against three changes of the same buffer. The dialysed enzyme was centrifuged to remove any turbidity.

Calcium phosphate gel adsorption (Fraction IV)

In all subsequent operations K-phosphate buffer, pH 7.5 containing 0.25 M sucrose and 0.005 M TE was used unless otherwise specified.

The enzyme (Fraction III) was mixed with calcium phosphate gel (final concentration of phosphate was 0.05 M and gel concentration was 45 to 50 mg/unit of enzyme). The liquid was gently stirred for 30 to 40 min and then centrifuged. The gel was washed with 100 to 150 ml of 0.1 M and the supernatant liquid discarded. The enzyme was then eluted twice with 200 ml of 0.15 M phosphate. The enzyme was precipitated from the combined eluates by the addition of 60.2 g of ammonium sulphate/100 ml of enzyme. The liquid was centrifuged and the precipitate was dissolved in 0.05 M phosphate buffer and then dialysed against three changes of 0.005 M phosphate buffer (Fraction IV).

DEAE-cellulose column chromatography (Fraction V)

The enzyme was loaded on a DEAE-cellulose column (2.5 cm x 20 cm), which was previously equilibrated with 0.005 M phosphate buffer by passing 500 ml of the buffer through the column. The column was washed with 300 to 400 ml of 0.01 M phosphate buffer and the enzyme was eluted with 0.05 M phosphate buffer. 5 ml fractions were collected and analysed for activity and protein.

Fractions having a specific activity of 10 units/mg or more were pooled and precipitated with ammonium sulphate as before. The precipitate was dissolved in 0.05 M phosphate buffer, pH 7.5 and dialysed against several changes of the same buffer.(Fraction V).

Sephadex G-200 chromatography

The Sephadex G-200 column (2 cm x 100 cm) was equilibrated with 0.05 M phosphate buffer. The enzyme (10 units, Fraction V) was then loaded and elution carried out with the same buffer. After discarding the first 50 ml, 4 ml fractions were collected (flow rate 8 ml/hr). The enzyme activity and protein were estimated in aliquots of the eluates and fractions having maximum specific activity were pooled. Then 8 mg of serum albumin/ml was added to the enzyme. The final concentrations of sucrose, phosphate and TE were 1, 0.2 and 0.01 M respectively. This enzyme was then stored at -20°.

The results of a typical fractionation are shown in the Table 13. The purification of ox heart hexokinase was about 500-fold.

TABLE 13

PURIFICATION OF PARTICULATE OX HEART HEXOKINASE

Step	Description	Protein	Activity	Specific activity
		mg	units	units/mg protein
1	Fraction II	383.0	39.0	0.10
2	Fraction III	42.0	34.5	0.80
3	Fraction IV	13.5	24.5	1.80
4	Fraction V	1.05	10.05	10.0
5	Fraction VI	0.082	4.00	48.0

PARTIAL PURIFICATION OF SOLUBLE OX HEART HEXOKINASE

The soluble hexokinase of beef heart was present in the supernatant liquid obtained after centrifuging the homogenate at 13,000 x g. This fraction was used for partial purification of the soluble enzyme. 10 ml of the enzyme was mixed with 16 ml of calcium phosphate gel (960 mg) (final phosphate concentration 0.01 M, pH 7.5). The suspension was centrifuged for 30 min and the gel was washed once with 50 ml of 0.01 M phosphate, pH 7.5, containing 0.5 M glucose and 0.005 TE and twice with 50 ml each of 0.03 M phosphate containing glucose and TE as above. The enzyme was finally eluted with 0.05 M phosphate buffer of the same composition and pH. The enzyme was fractionated on DEAE-cellulose batchwise. 0.5 M sucrose and 0.005 M TE was present in all solutions used subsequently and the pH was 7.5. DEAE cellulose was added (1.7 g) to the gel eluate and diluted so that the final concentration was 0.02 M phosphate. The cellulose was centrifuged, washed with 50 ml buffer of the same composition and eluted with 120 ml of 0.075 M phosphate. The eluate was precipitated with ammonium sulfate (90% saturation) and the precipitate dissolved in 0.1 M phosphate buffer pH 7.5 containing glucose and TE. The results are shown in Table 13a. A 25-fold purification was achieved.

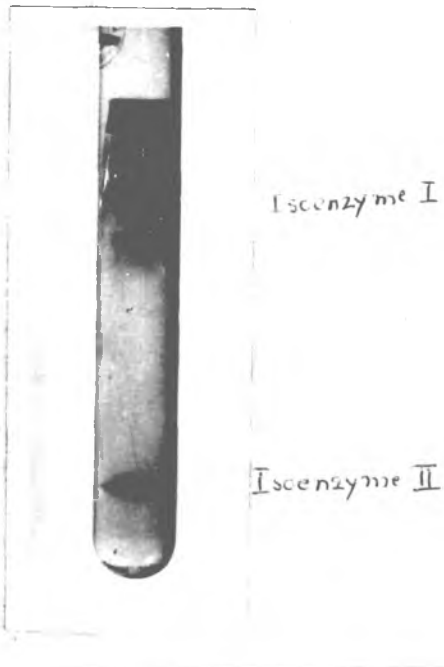
Acrylamide gel electrophoresis of soluble and particulate hexokinase

About 0.1 unit of soluble enzyme in the original 13,000 x g supernatant was used for this experiment. The acrylamide gel concentration was 7% and the time of the run was 1.5 hr. The hexokinase bands were detected by the method of Dewey and Conklin (1960). Two hexokinase bands were detected which moved towards the anode showing

showing the presence of two isoenzymes in the soluble fraction.

Particulate enzyme

The solubilized particulate hexokinase obtained after DEAE-cellulose chromatography was used for this work. About 0.1 unit was loaded on acrylamide. It was observed that the hexokinase band was present only at the origin. Since no movement of the enzyme band was observed the following variations in conditions were tested: 7% acrylamide at pH 7 in phosphate, 7% acrylamide at pH 8.5 and 5% acrylamide at pH 8.5. In all the experiments mentioned above the hexokinase band was located at the origin.



Acrylamide gel electrophoresis of soluble hexokinase

TABLE 13a

PARTIAL PURIFICATION OF SOLUBLE OX HEART HEXOKINASE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity
	ml	units/ml	units	mg/ml	mg	units/mg protein
13,000 x g sup. (soluble)	10	0.22	2.2	18	180	0.012
0.05 M eluate	100	0.014	1.40	0.18	18	0.079
DEAE-cellulose eluate precipitated with ammonium sulphate	4.3	0.15	0.65	0.45	1.93	0.33

CHAPTER IV

PROPERTIES AND KINETICS

PROPERTIES AND KINETICS

Properties

Stability of heart hexokinase

The stability of hexokinase obtained after Sephadex G-200 chromatography was tested under different conditions. The experimental results are summarized in Table 14. It will be seen that the enzyme obtained after Sephadex treatment was extremely unstable. Even using high concentration of sucrose, phosphate and TE the stability of the enzyme was not improved. On the contrary if bovine serum albumin was added (1 mg-5 mg/ml) no loss in activity was observed.

The effect of temperature on hexokinase activity was determined with Fraction IV enzyme. The experimental results are summarized in Table 15. It will be seen that high concentration of sucrose significantly stabilizes enzyme activity at temperature upto 55°. Above this temperature sucrose had very little stabilizing effect.

Ultraviolet absorption spectrum

Purified hexokinase (Fraction V) (without serum albumin) was dialysed against 0.001 M phosphate buffer, pH 7.5, and its optical densities were recorded at various wavelengths in the ultraviolet. The blank cuvette contained 0.001 M phosphate buffer, pH 7.5. (Fig.3).

The spectrum of the purified enzyme was that of a typical protein. However the ratio of the O.D. at 280 mu to that at 260 mμ (1:12) is lower than that of most proteins (1.80). It is not known whether it is due to traces of TE which were not completely removed by dialysis. It is of interest that the absorption of a 0.1% solution

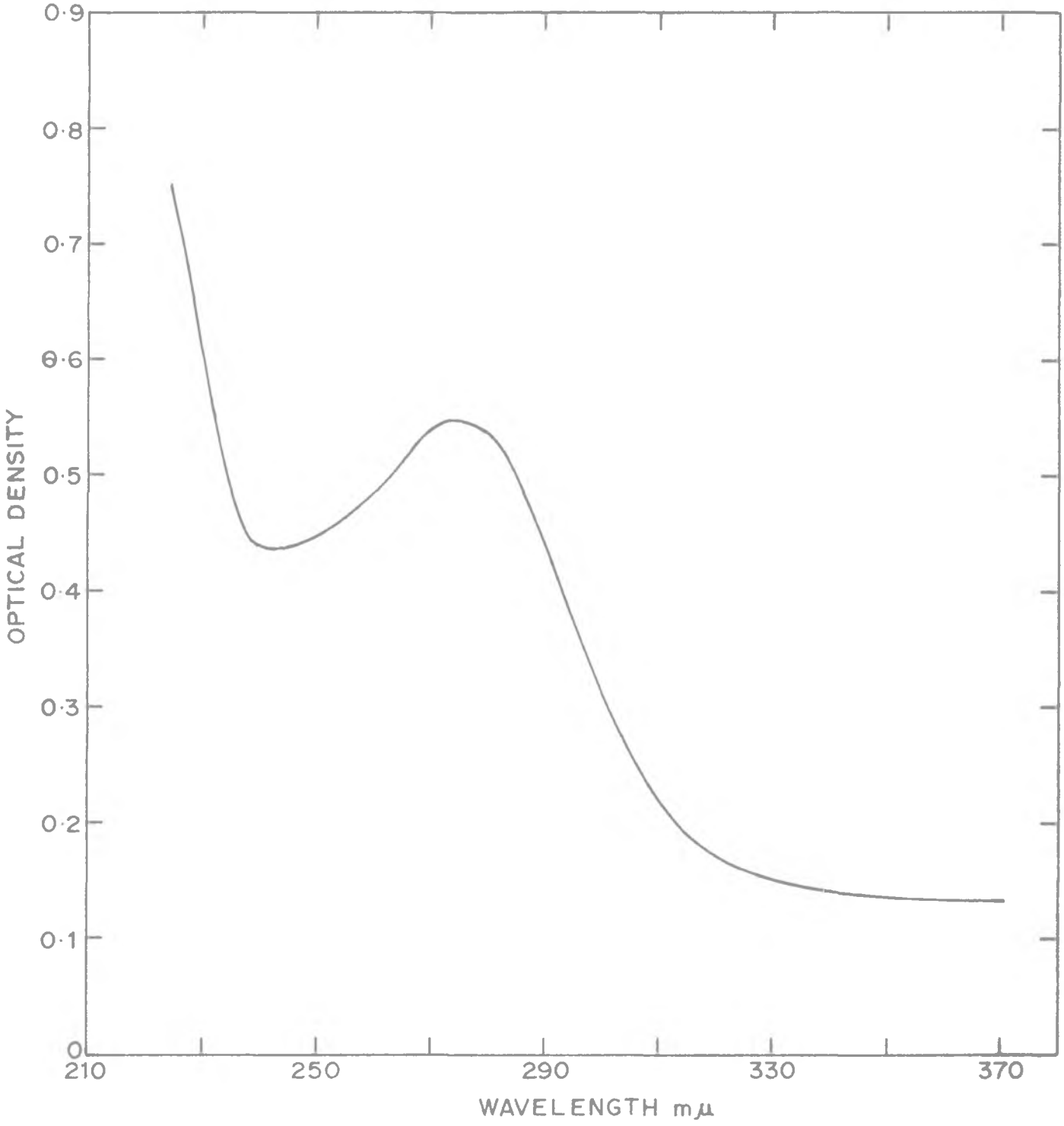


FIG. 3. ULTRAVIOLET ABSORPTION SPECTRUM OF HEART HEXOKINASE.
(Protein concentration 0.5 mg/ml).

TABLE 14

STABILITY OF HEXOKINASE

Sucrose concentration	Phosphate pH 7.5	TE	Serum albumin	Ammonium sulphate	Temperature	Time	Loss in activity
M	M	M	%	saturation	°C	hr	%
1.0	0.2	0.01	-	-	-20	24	28
1.0	0.2	0.01	0.5	-	-20	24	0
1.0	0.2	0.01	-	0.6	-20	24	50
0.5	0.2	0.01	-	0.5	-20	24	50
0.5	0.2	0.01	-	-	0	24	40
0.5	0.2	0.01	-	-	-20	24	40
1.0	0.2	0.01	-	-	0	24	20
1.0	0.2	0.01	-	-	-20	24	20
1.0	0.2	0.01	-	-	0	24	30
0.25	0.2	0.01	0.1	-	0	48	0

Enzyme obtained after Sephadex G-200 chromatography without the addition of serum albumin was used for these studies. Aliquots of enzyme (final concentration 0.20 units/ml) were mixed with the substances to give the final concentrations shown in the Table, stored for 24-48 hr at the specified temperature and then retested for activity (Assay I).

TABLE 15

EFFECT OF TEMPERATURE ON THE STABILITY OF HEXOKINASE

No.	Temperature	Time	Sucrose concentration	Loss in activity
	°C	min	M	%
1	55	10	0.25	60
2	55	20	0.25	84
3	55	30	0.25	90
4	60	10	0.25	90
5	65	5	0.25	90
6	55	10	0.50	44
7	55	20	0.50	70
8	55	30	0.50	83
9	60	10	0.50	90
10	65	5	0.50	95
11	55	10	1.0	34
12	55	20	1.0	47
13	55	30	1.0	60
14	60	10	1.0	90
15	65	5	1.0	95

Fraction IV enzyme was used. [0.15 M phosphate, pH 7.5, containing 0.25 M sucrose and 0.005 M TE (0.05 units/ml)]. After heating for the specified time at the temperature shown in the Table the enzyme was rapidly cooled and assayed for activity.(Assay I).

of the enzyme is about 1.1 which is similar to that reported by Joshi and Jagannathan (1968) for purified brain hexokinase, whereas Schwartz and Basford (1967) stated that the purified brain hexokinase obtained by their procedure had an O.D. of only about 0.72 at the absorption maximum for a solution containing 1.32 mg/ml (i.e., about 0.53 O.D. for a 0.1% solution). The reason for this striking discrepancy is not known. The sedimentation constant of the latter enzyme was 4.44 S whereas that of Joshi and Jagannathan was 5.86 S. It is possible that a peptide fragment high in aromatic acids is present in the latter enzyme which is not essential for activity and which is removed during the chymotrypsin treatment used by Schwartz and Basford.

Molecular weight by Sephadex G-200 filtration

The molecular weight of purified ox heart hexokinase was determined by Sephadex G-200 filtration (Andrews,1965). A suspension of Sephadex G-200 was kept in a boiling water bath for 30 min, cooled and packed in a column (2 cm x 100 cm). The column was then equilibrated by passing about 500 ml of 0.05 M phosphate buffer, pH 7.5, containing 0.25 M sucrose and 0.005 M TE. The flow rate of the column was 6 ml/hr. The following reference proteins were used, the molecular weights of which are shown in parenthesis: catalase (2.5×10^5), rabbit muscle glyceraldehyde phosphate dehydrogenase (1.4×10^5), soybean trypsin inhibitor (2.15×10^4), horse heart cytochrome c (1.3×10^4). Dextran blue was used to determine the void volume of the column.

A mixture of the above proteins and purified heart hexokinase and dextran blue in 1 ml of 0.05 M phosphate buffer, pH 7.5, was loaded on the Sephadex G-200 column. The effluent fractions were

collected and assayed for the different proteins.

1) Cytochrome c was estimated by determining the absorption at 412 mμ.

2) Glyceraldehyde 3-phosphate dehydrogenase was estimated by using the aldolase assay (optical method of Warburg and Christian, 1953) wherein rabbit muscle aldolase was used.

3) Catalase was estimated by determining the absorption at 412 mμ.

4) Trypsin inhibitor was estimated by the casein digestion method (Kunitz,1947) and absorption at 280 mμ.

5) Hexokinase was estimated by using assay I.

The molecular weight of hexokinase was then determined by plotting the eluant volume vs logarithm of molecular weight (Fig.4).

From the graph it was found that the molecular weight of ox heart hexokinase was approximately 90,000.

K i n e t i c s

Enzyme of maximum specific activity was used for all kinetic experiments unless otherwise stated.

Proportionality of enzyme activity with time

It will be seen from Fig. 5 that the activity of the enzyme is proportional to time when the total change in O.D. per minute is less than 0.25 in Assay I and 0.55 in Assay III. Enzyme activity was also proportional to enzyme concentration (Fig. 6).

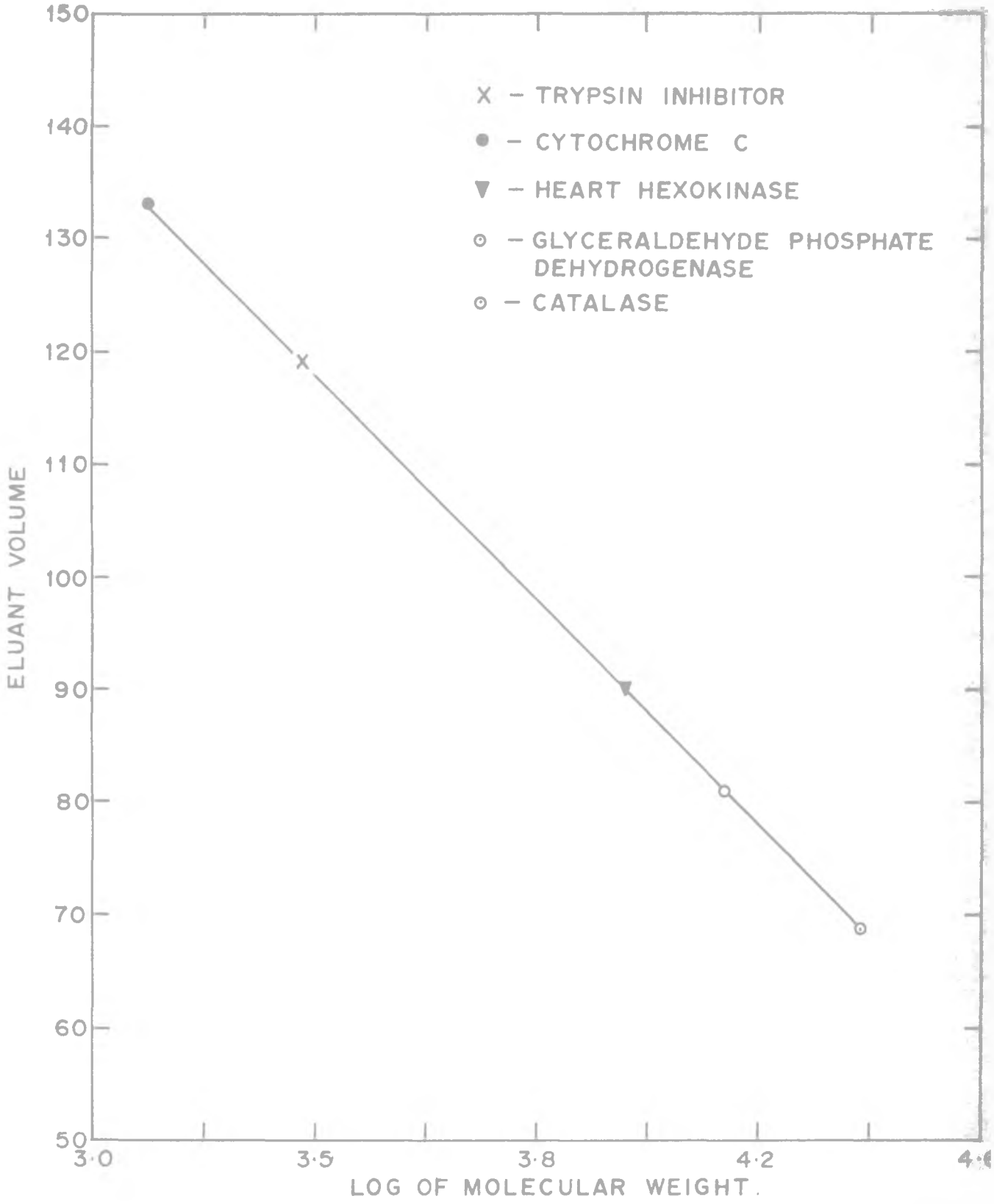


FIG. 4 PLOT OF ELUANT VOLUME VS LOG OF MOLECULAR WEIGHT.

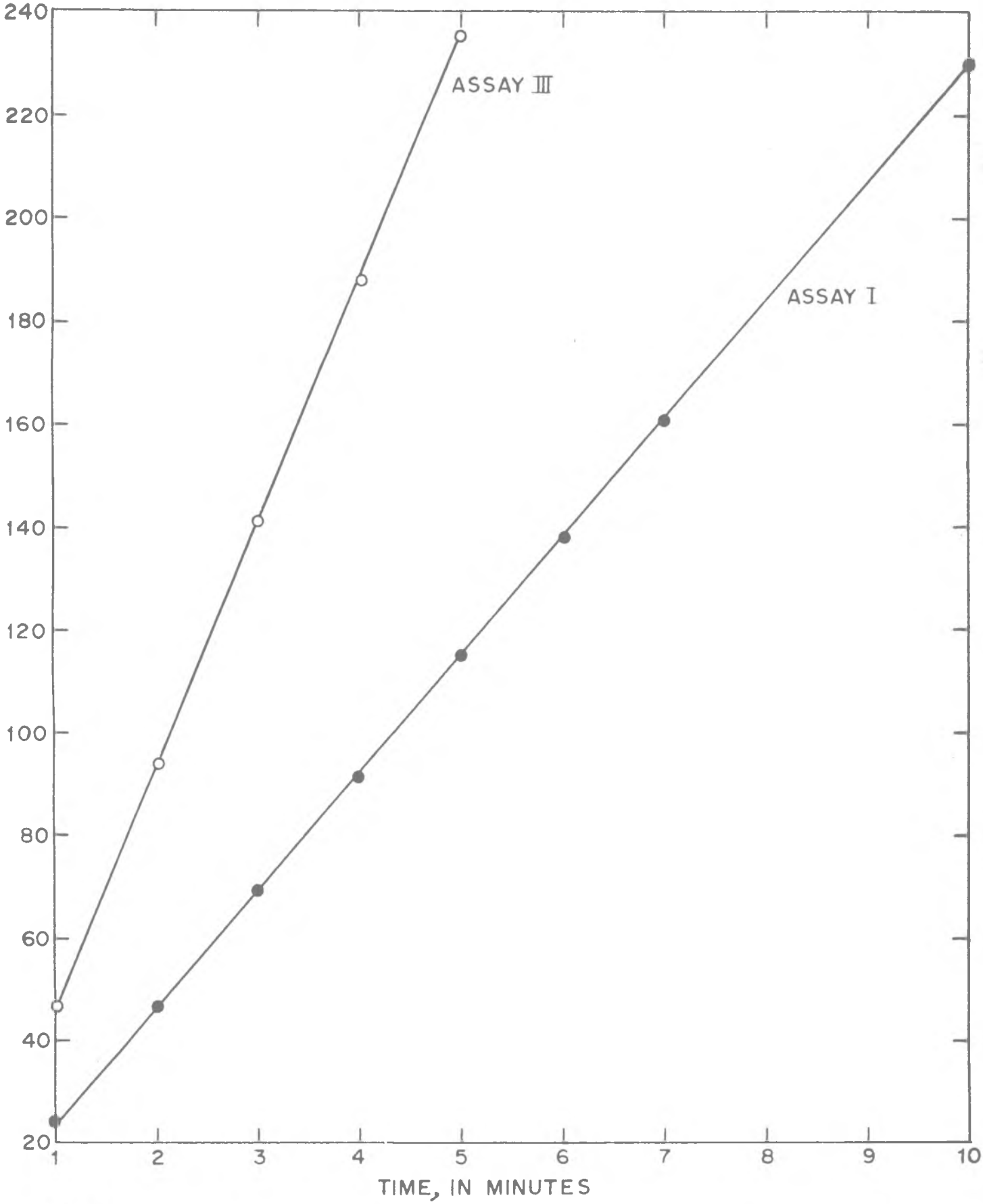


FIG. 5. EFFECT OF TIME OF REACTION ON THE ACTIVITY OF HEXOKINASE.

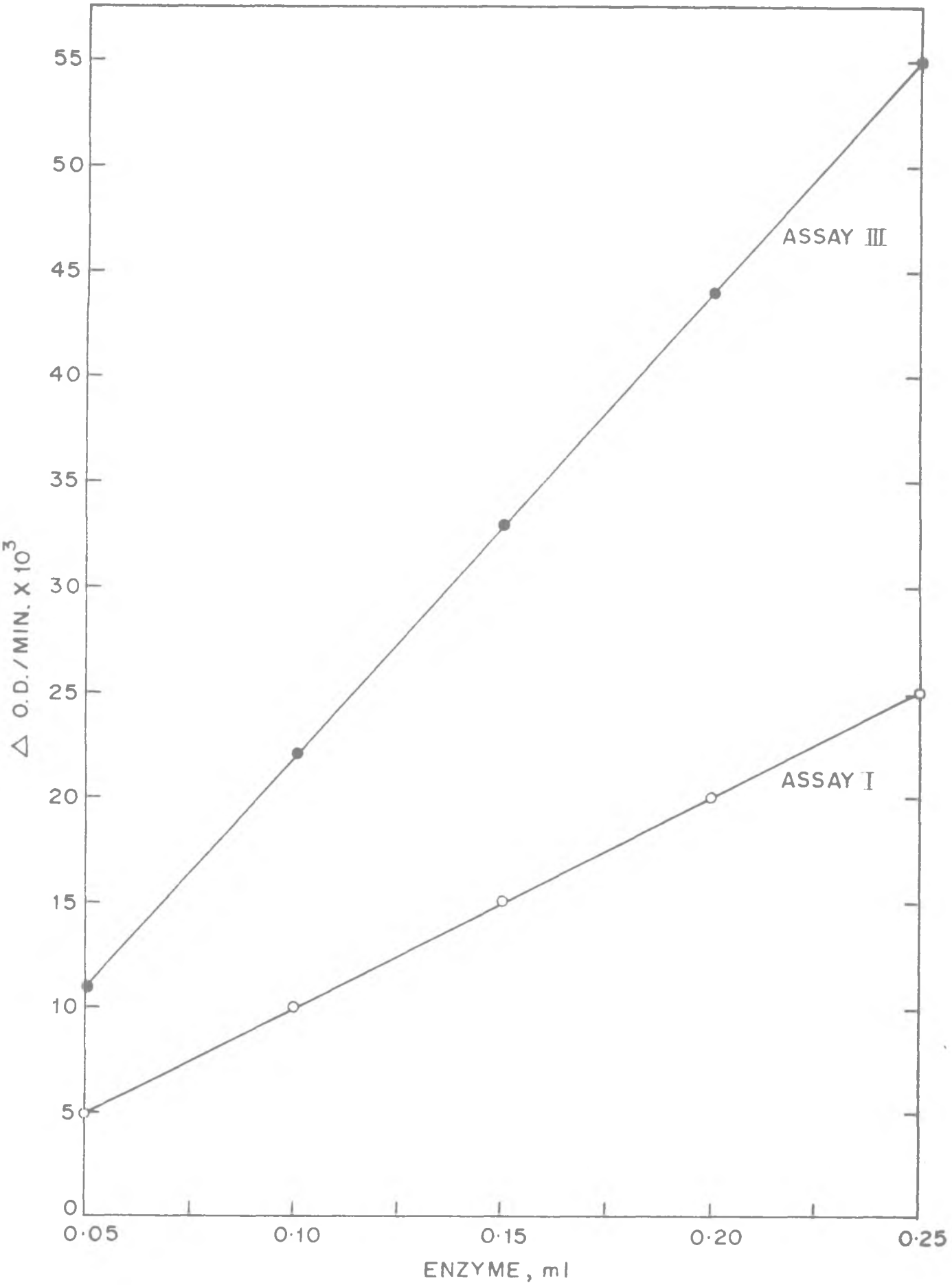


FIG. 6. EFFECT OF ENZYME CONCENTRATION ON THE ACTIVITY OF HEXOKINASE.

Effect of temperature on hexokinase activity

The effect of temperature on hexokinase activity was studied using Assay II. From Table 16 it will be seen that there was marked inactivation at temperatures above 40°. The relative activities of the enzyme at 20°, 30°, 40°, 50° and 60° were 100, 196, 330, 250 and 230 respectively.

Effect of pH

Enzyme activity was determined at different pHs using Assay-II. The composition of the buffers is shown in Table 17 and Fig. 7. It will be seen that hexokinase had a broad pH optimum between pH 5.8 and 8.0 with a falling off in activity at pH 5.5.

Effect of Mg^{2+}

The effect of Mg^{2+} concentration on hexokinase activity was determined the ATP concentration being kept constant at 10 mM. The results are shown in Table 18 and Figure 8. The K_m for Mg^{2+} was 3×10^{-3}M (at 10 mM ATP).

Effect of mannose concentration on hexokinase activity

This study was carried out using Assay-III (Table,19 and Fig. 9). The K_m value for mannose was found to be 3.8×10^{-5}M (at 5 mM ATP and Mg^{2+} ATP ratio of 2:1).

Effect of fructose on heart hexokinase activity

The effect of fructose concentration on hexokinase activity was studied using Assay III (Table 20 and Fig. 10). The Mg:ATP ratio was kept constant at 2:1. The K_m value for fructose was 2.0×10^{-3}M (at 5 mM ATP).

TABLE 16

EFFECT OF TEMPERATURE ON HEXOKINASE ACTIVITY

Temperature (°C)	20	30	40	50	60	65
△O.D./5 min	0.050	0.098	0.165	0.125	0.115	0.030

The composition of the reaction mixture was as stated for Assay-II. Enzyme was diluted in 0.05 M phosphate buffer (pH 7.5), 5 mg/ml SA and 0.005 M TE. Incubation period 5 min.

TABLE 17

EFFECT OF pH ON HEXOKINASE

pH	5.5	5.8	6.8	7.5	8.0
△ O.D./10 min	0.205	0.220	0.219	0.220	0.221

The reaction mixture contained 20 μmoles of buffer of the appropriate pH and other components as described in Assay II. Sodium acetate-acetic acid buffer, pH 5 to 6, phosphate buffer for pH 6.8, Tris-HCl buffer for pH 7.5 to 8.0. Incubation period 10 min.

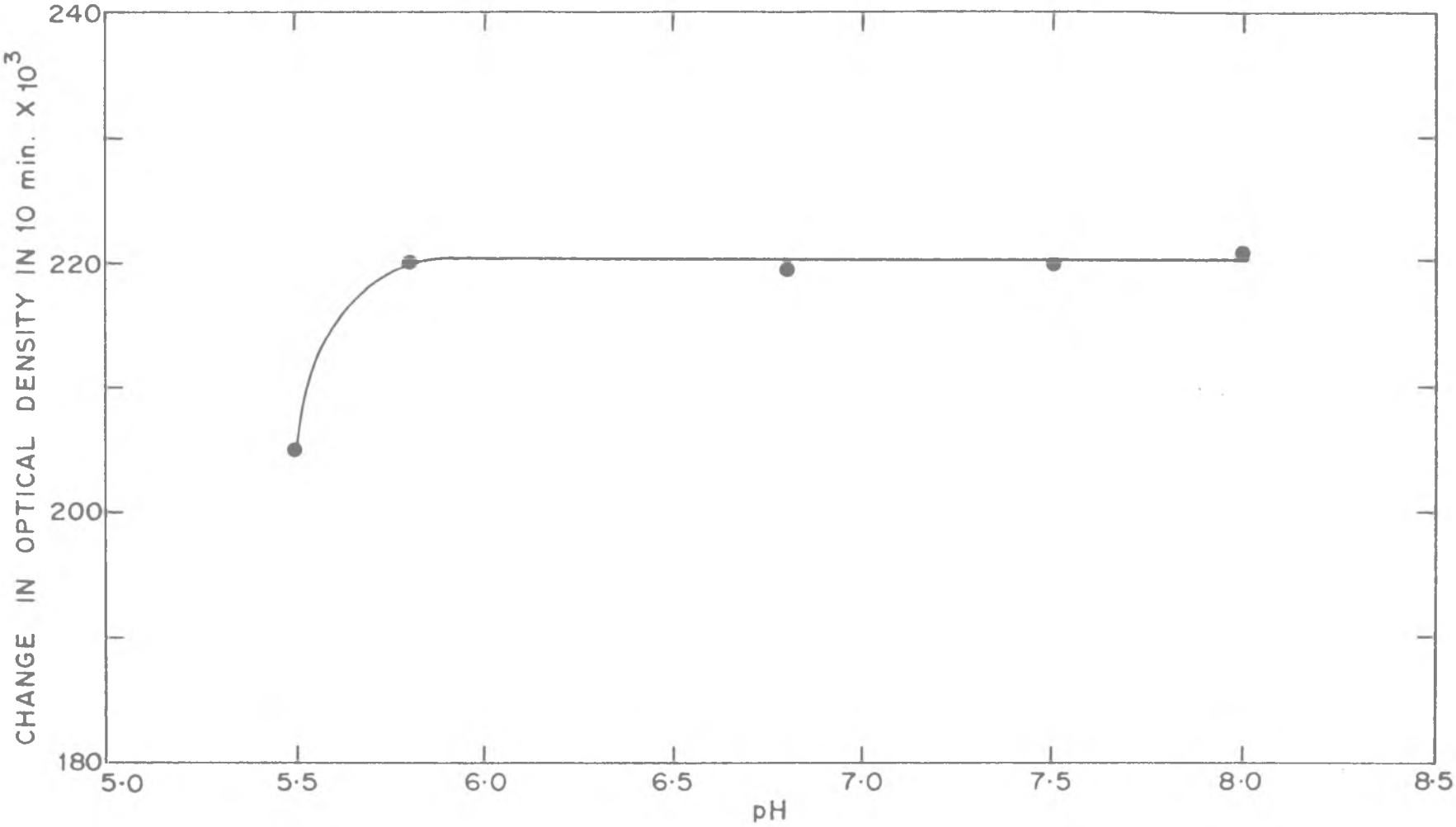


FIG. 7. EFFECT OF pH ON HEXOKINASE ACTIVITY. (Assay II)

TABLE 18

EFFECT OF Mg^{2+} CONCENTRATION ON HEXOKINASE ACTIVITY

Mg^{2+} concentration $Sx10^{3}M$	1	2	3	4	5	10
$\triangle$ O.D./5 min	0.037	0.050	0.080	0.095	0.100	0.120
$\frac{S}{V}$	0.1351	0.2000	0.1875	0.2150	0.2500	0.4166

The composition of the reaction mixture except for $MgCl_2$ and glucose-6-phosphate dehydrogenase was as stated in Assay I. (0.2 unit of glucose-6-phosphate dehydrogenase was used). Enzyme was diluted in 0.05 M phosphate buffer, pH 7.5, containing 0.5% S.A. and 0.01 M TE.

TABLE 19

EFFECT OF MANNOSE CONCENTRATION ON HEXOKINASE ACTIVITY

Mannose S x $10^{5}M$	0.5	1	2	3	4	5	10
$\triangle$ O.D./5 min	0.015	0.030	0.050	0.060	0.078	0.090	0.120
$\frac{S}{V} \times 10^{2}$	0.1666	0.1662	0.2000	0.2500	0.2564	0.2777	0.4166

The composition of the reaction mixture, except for mannose was according to Assay III. Enzyme was diluted in 0.05 M phosphate buffer, pH 7.5, containing 8 mg/ml S.A. and 0.005 M TE.

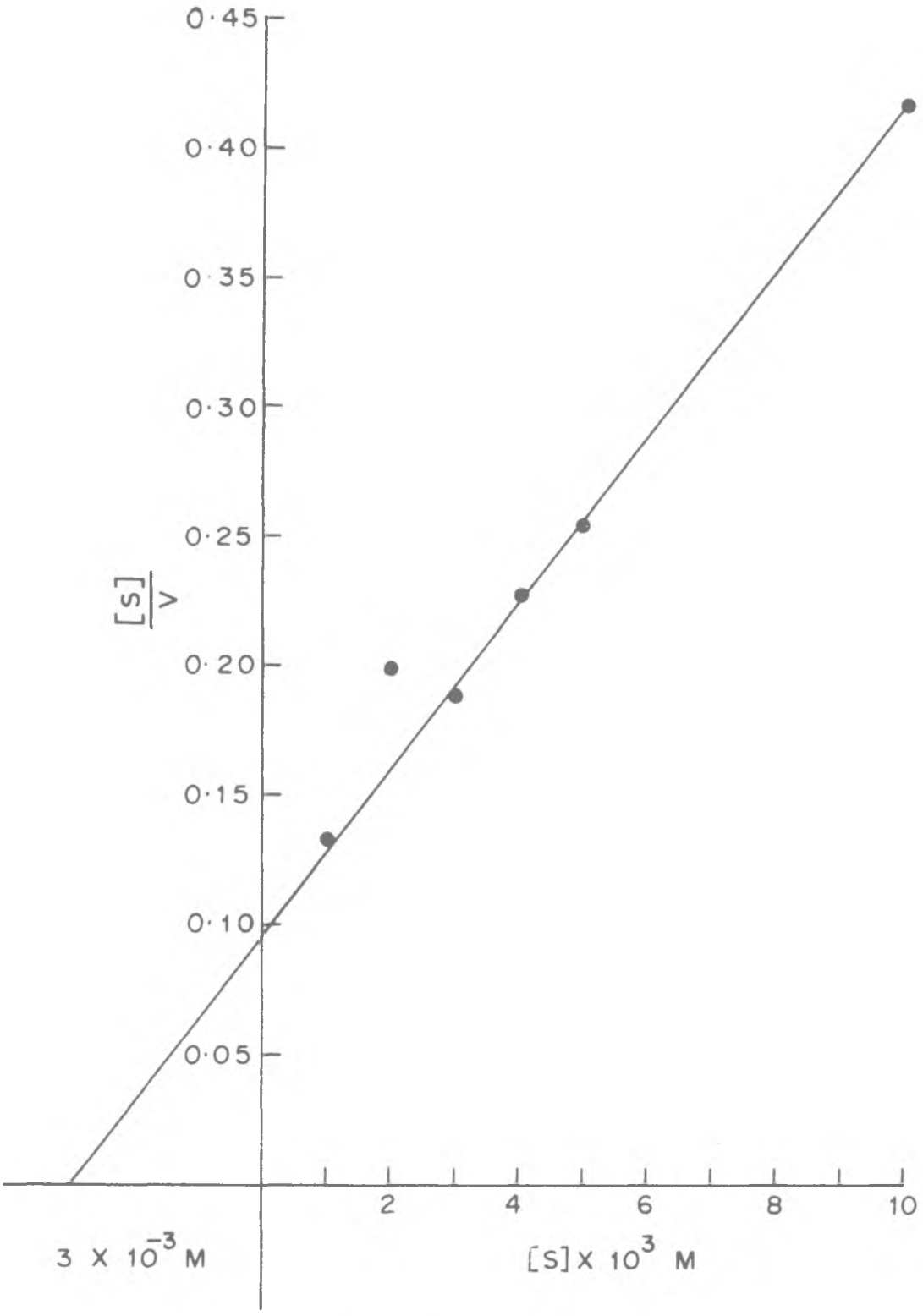


FIG. 8: PLOT OF $\frac{[S]}{V}$ VS [S], WHERE [S] IS THE MOLAR CONCENTRATION OF Mg^{2+} V THE INITIAL VELOCITY. Assay I.

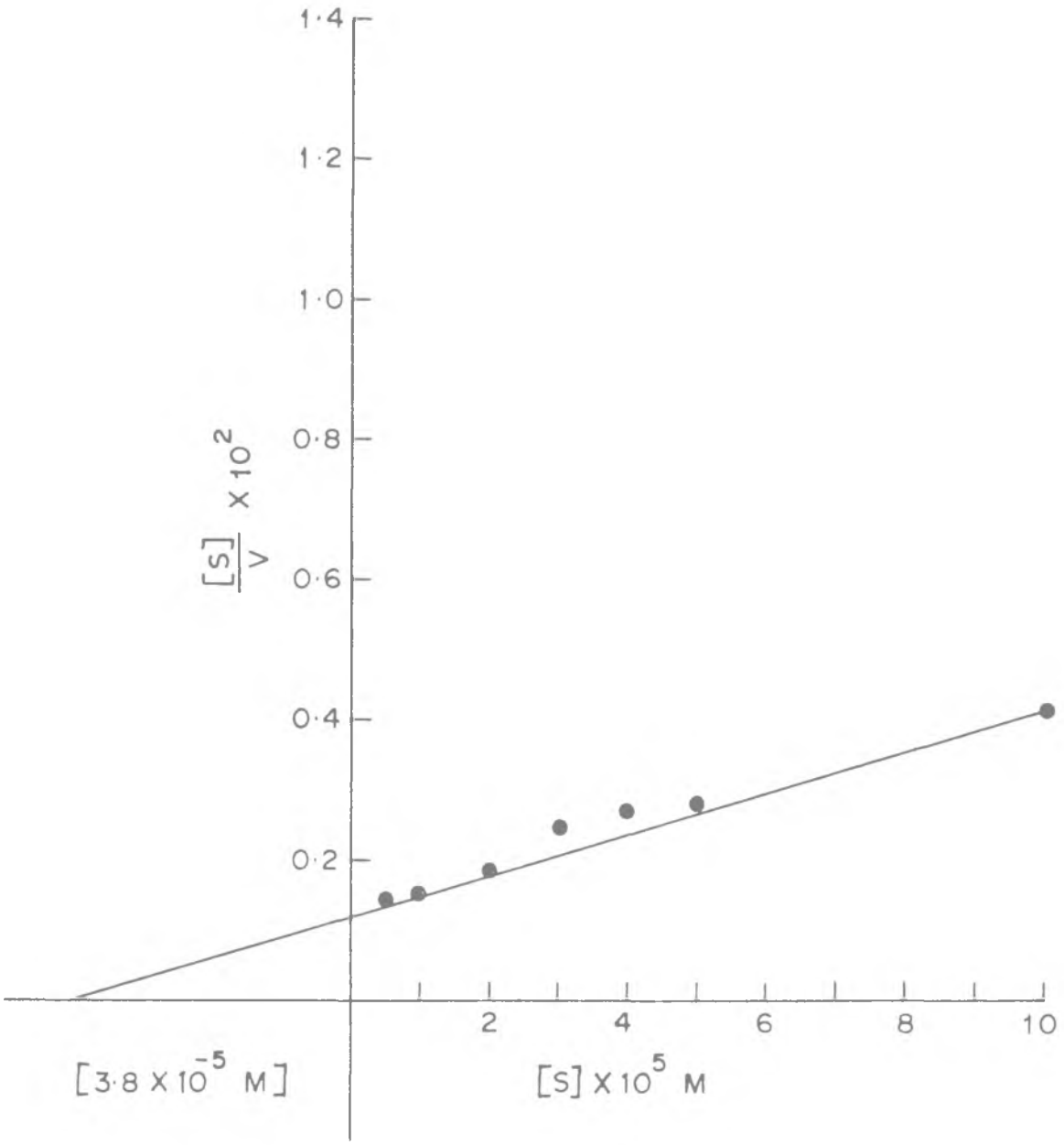


FIG. 9. PLOT OF $\frac{[S]}{V}$ VS $[S]$, WHERE $[S]$ IS THE MOLAR CONCENTRATION OF MANNOSE AND V THE INITIAL VELOCITY. (Assay III).

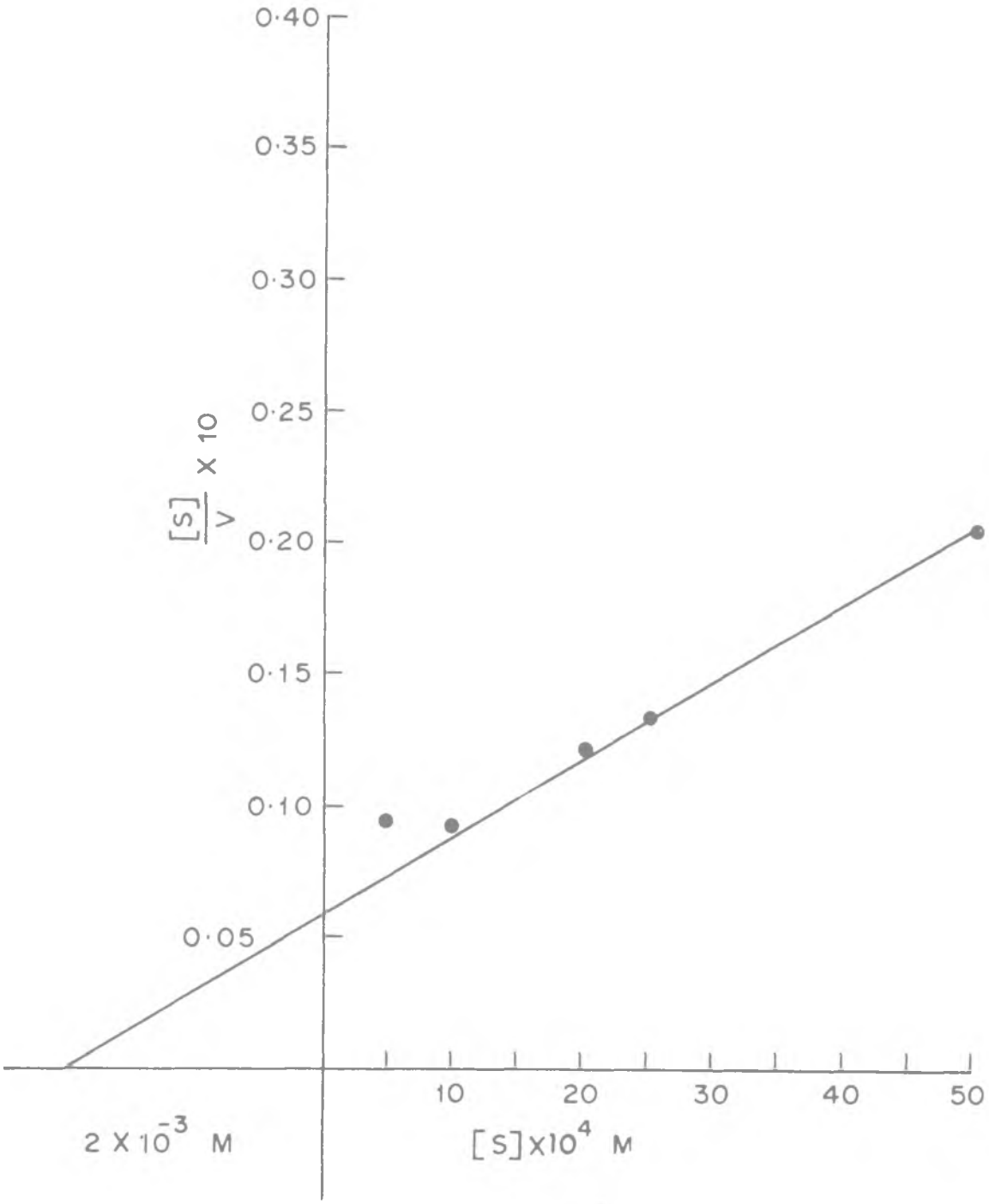


FIG. 10. PLOT OF $\frac{[S]}{V}$ VS $[S]$, WHERE $[S]$ IS THE MOLAR CONCENTRATION OF FRUCTOSE AND V THE INITIAL VELOCITY. (Assay III).

Effect of ATP at different glucose concentrations

This study was carried out using Assay I, the Mg:ATP ratio being kept constant at 2:1. The effect of ATP concentration on hexokinase activity was determined at five different glucose concentrations. The results are shown in Table 21 (Fig.11a,b,c,d and e). It will be seen that the K_m value for ATP increased as the glucose concentration was lowered.

Effect of glucose on heart hexokinase at different ATP concentrations

The results obtained in the previous section were p repletted to show the effect of glucose concentration on activity at different ATP concentrations. The K_m value for glucose was found to increase as the ATP concentration was lowered (Table 22 and Fig.12a, b,c,d and e). The double reciprocal plots of concentration vs activity for glucose at different ATP concentrations and for ATP at different glucose concentrations are shown in Fig. 13 and 14. It will be seen that the plots are nearly parallel to each other in both cases.

Effect of Mn^{2+} on hexokinase activity

The effect of different divalent ions instead of Mg^{2+} on enzyme activity was determined (Table 23). Mn^{2+} could replace Mg^{2+} but there was no activity with Ca^{2+} or Zn^{2+} (2-10 mM).

Effect of 5-thio-D-glucose on heart hexokinase

The effect of 5-thio-D-glucose concentration on hexokinase activity was studied using Assay III at different concentrations of mannose and 5-thio-D-glucose. 5-Thio-D-glucose inhibited heart hexokinase competitively with respect to mannose. The Ki value for 5-thio-D-glucose was 1.4×10^{-3} M. (Fig. 15a and b)

TABLE 20

EFFECT OF FRUCTOSE ON HEXOKINASE ACTIVITY

Fructose $Sx10^{4}M$	0.5	1	2	2.5	5
$\triangle$ O.D./5 min	0.025	0.054	0.083	0.095	0.120
$\frac{S}{V} \times 10$	0.1000	0.0925	0.1225	0.1315	0.2083

The composition of the reaction mixture was as described in Assay III except that mannose was omitted. The enzyme was diluted in 0.05 M phosphate buffer, pH 7.5 containing 8 mg/ml S.A. and 0.005 M TE.

TABLE 21. K_m FOR ATP AT DIFFERENT GLUCOSE CONCENTRATIONS

K_m values for ATP	Glucose concentration
M	mM
6.0×10^{-4}	15.0
7.8×10^{-4}	3.0
9.6×10^{-4}	1.5
1.1×10^{-3}	1.0
1.12×10^{-3}	0.5

Enzyme was diluted in 0.05 M phosphate buffer, pH 7.5, containing 0.5% S.A. and 0.01 M TE. The composition of the reaction mixture except ATP and Mg^{2+} was as stated in Assay I.

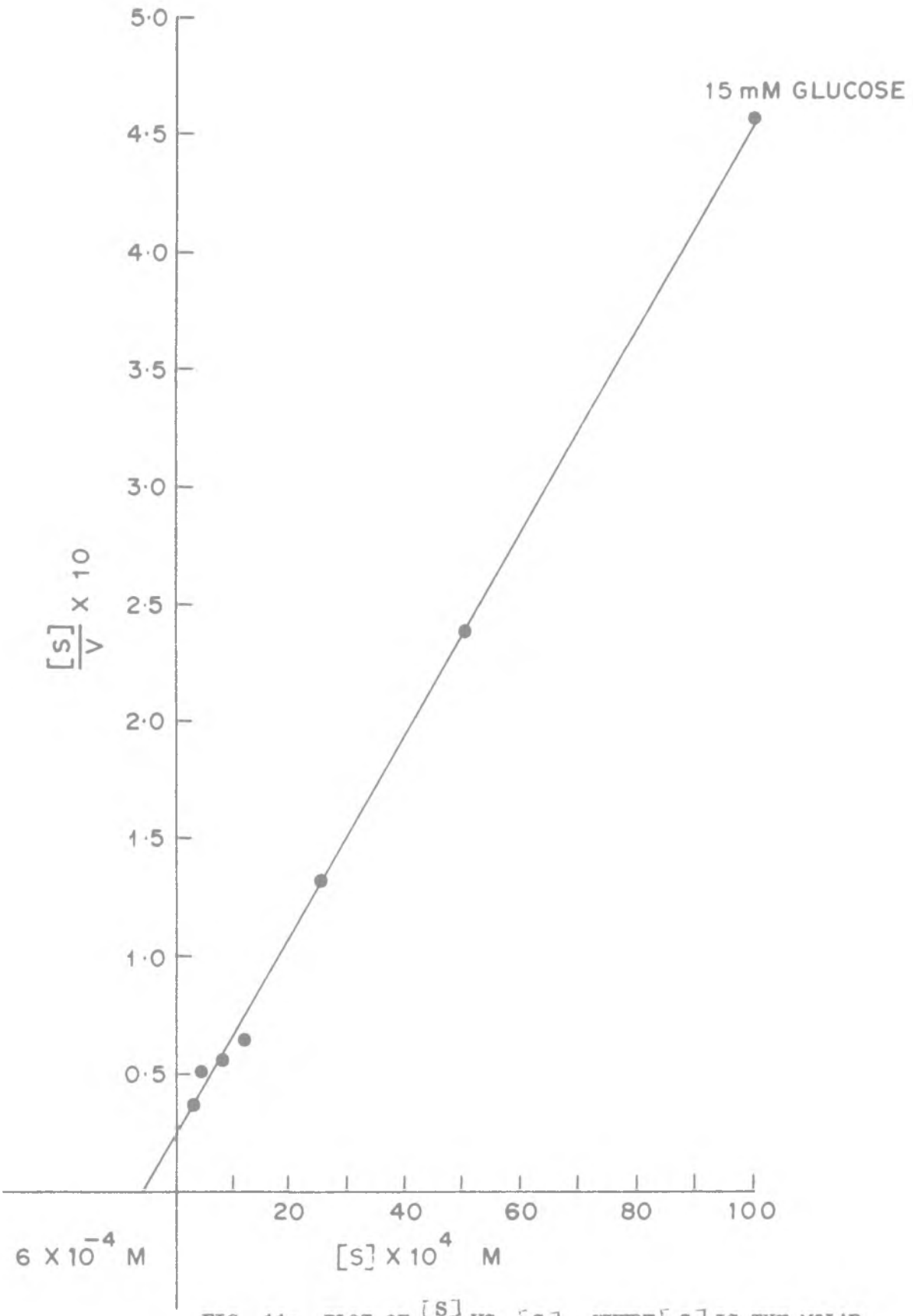


FIG. 11a. PLOT OF $\frac{[S]}{V}$ VS $[S]$, WHERE $[S]$ IS THE MOLAR CONCENTRATION OF ATP AND V THE INITIAL VELOCITY. (Assay I).

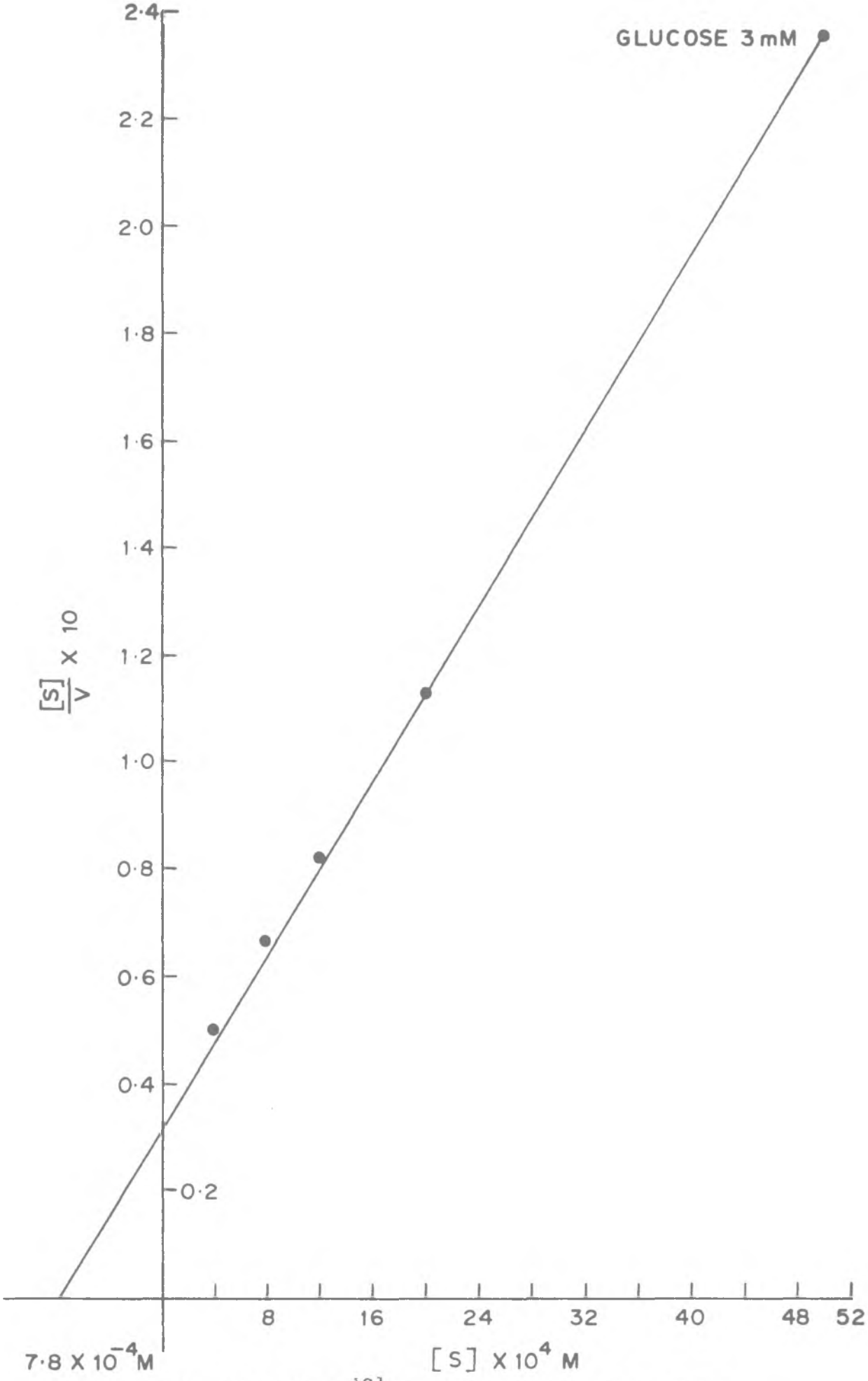


FIG. 11b. PLOT OF $\frac{[S]}{V}$ VS [S] WHERE [S] IS THE MOLAR CONCENTRATION OF ATP AND V THE INITIAL VELOCITY. (Assay I).

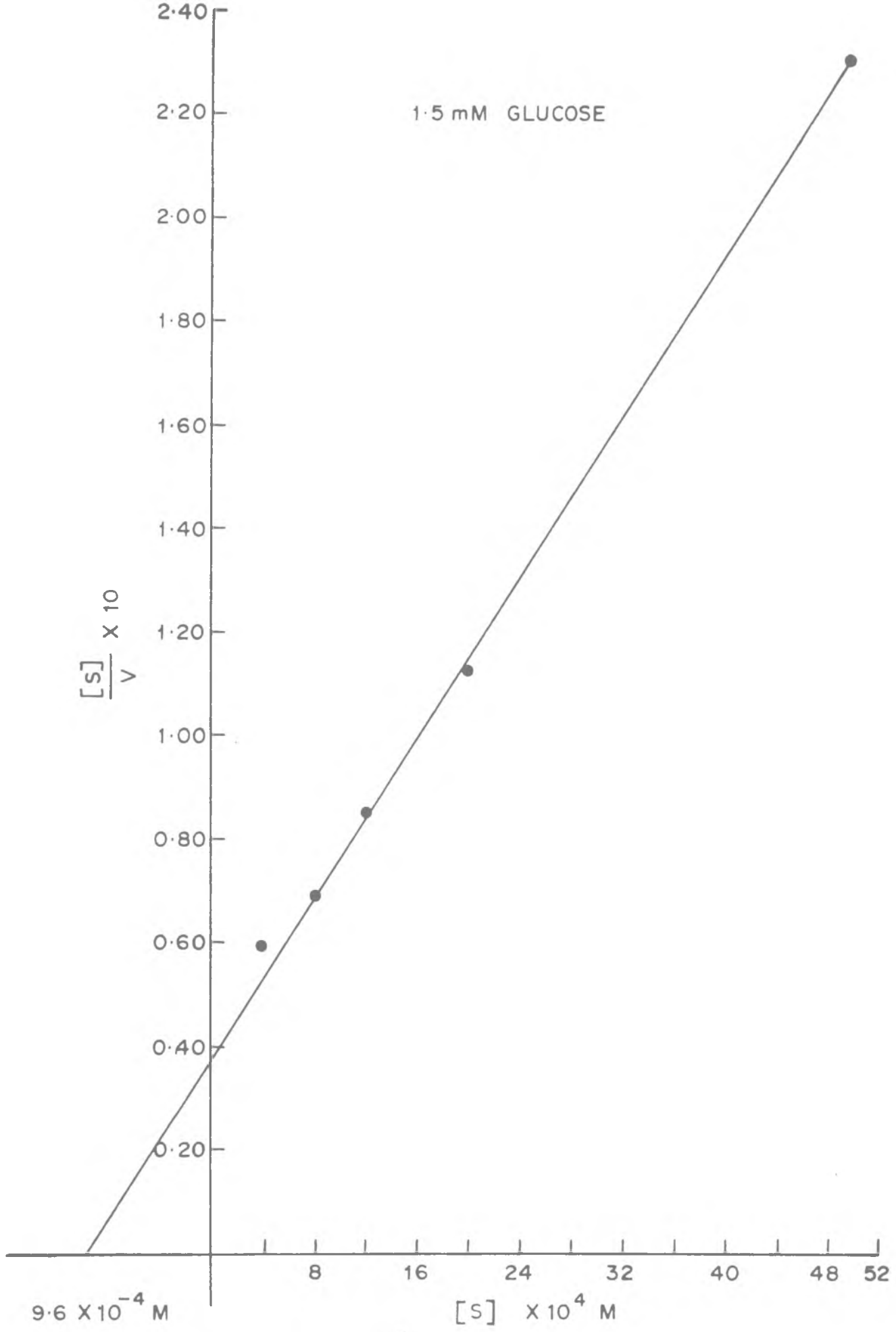


FIG. 11c. PLOT OF $\frac{[S]}{V}$ VS [S], WHERE [S] IS THE MOLAR CONCENTRATION OF ATP AND V THE INITIAL VELOCITY. (Assay I).

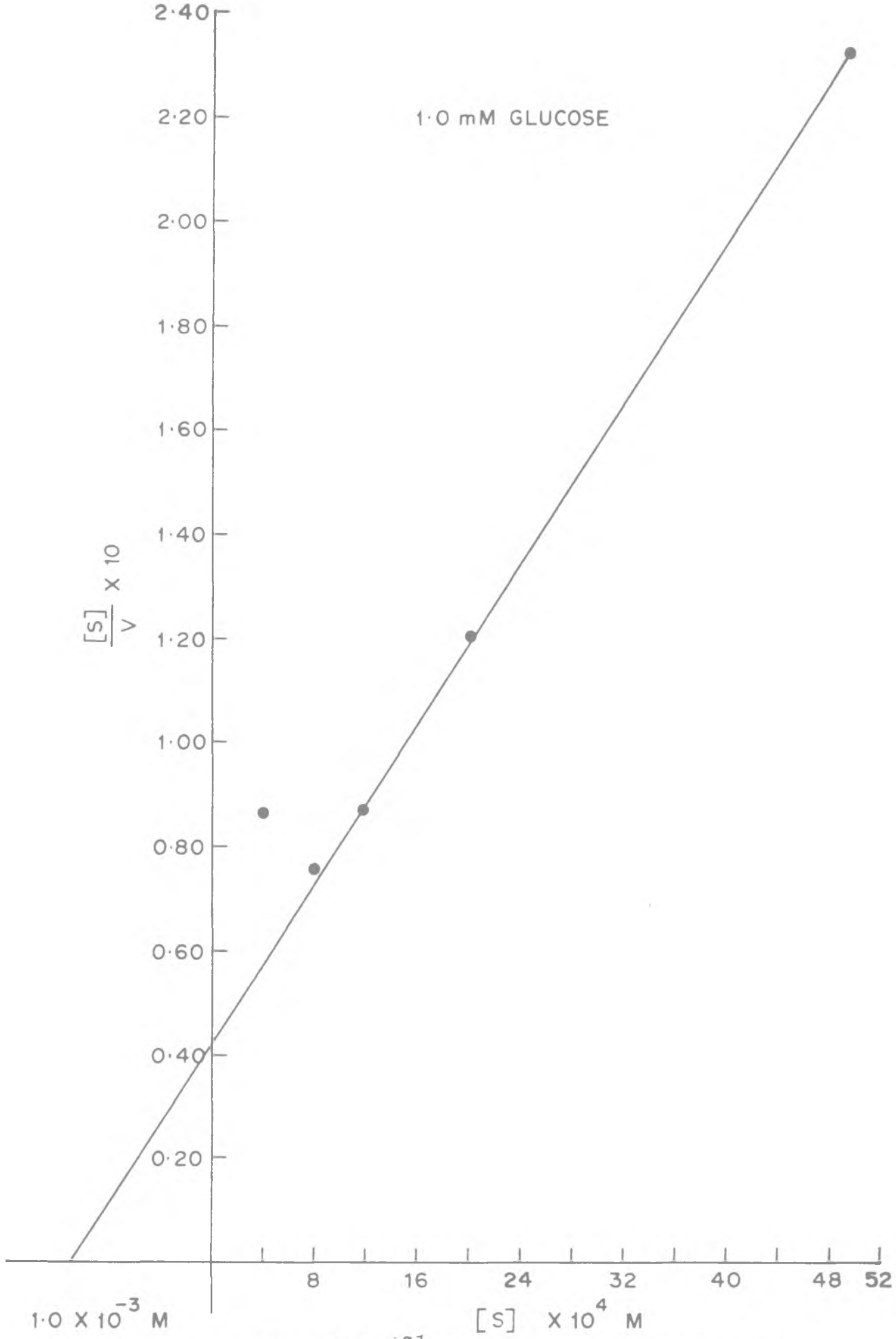


FIG.11d. PLOT OF $\frac{[S]}{V}$ VS $[S]$, WHERE $[S]$ IS THE MOLAR CONCENTRATION OF ATP AND V THE INITIAL VELOCITY. (Assay I).

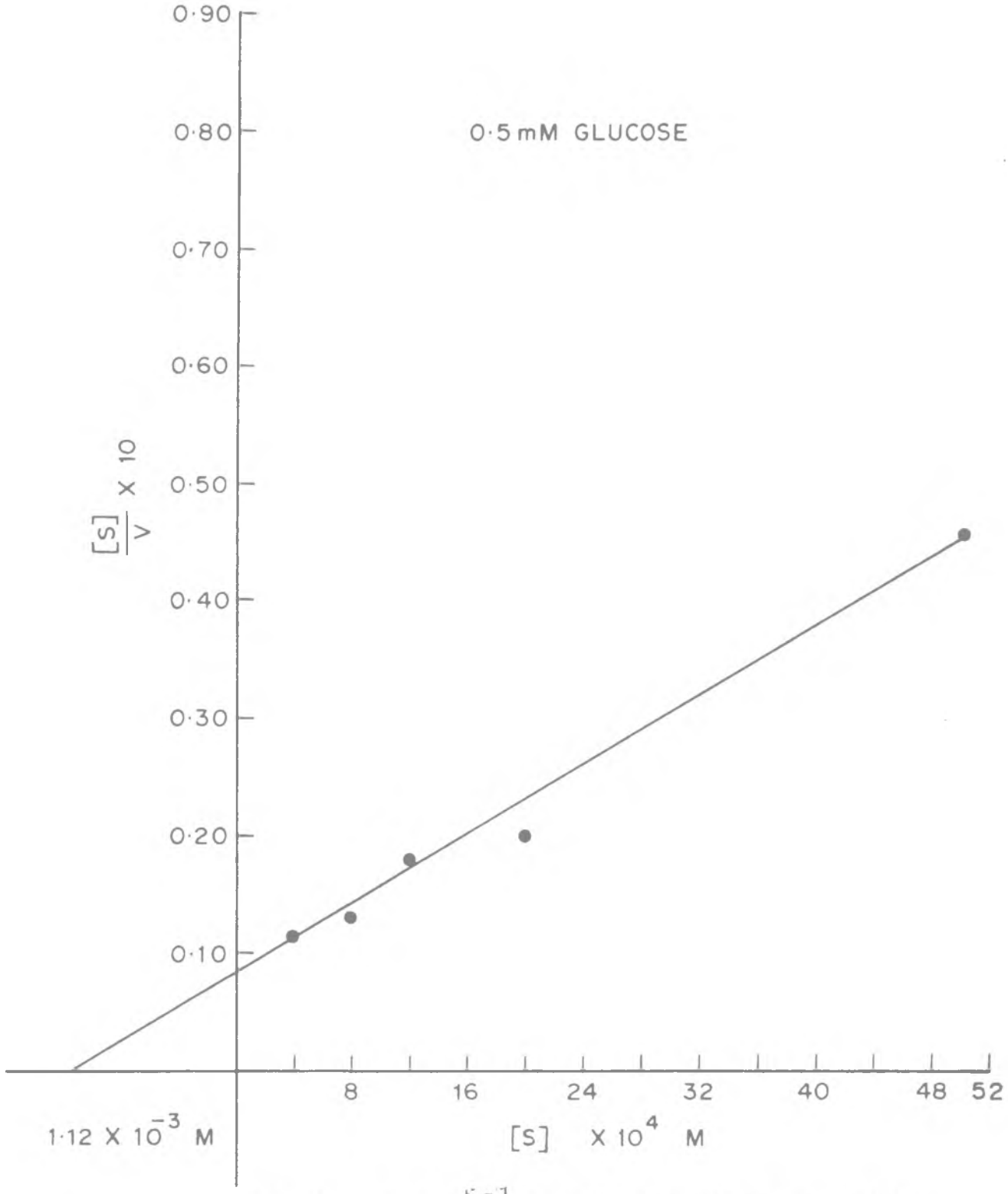


FIG. 11e. PLOT OF $\frac{[S]}{V}$ VS $[S]$, WHERE $[S]$ IS THE MOLAR CONCENTRATION OF ATP AND V THE INITIAL VELOCITY. (Assay I).

TABLE 22

K_m FOR GLUCOSE AT DIFFERENT ATP CONCENTRATIONS

K_m value for glucose	ATP concentration
M	mM
5.20×10^{-5}	10.0
6.26×10^{-5}	5.0
6.25×10^{-5}	2.0
1.00×10^{-4}	1.2
1.12×10^{-4}	0.8

The enzyme was diluted in 0.05 M phosphate buffer, pH 7.5, containing 0.5% serum albumin and 0.01 M TE. The composition of the reaction mixture except for glucose was according to Assay I.

TABLE 23

EFFECT OF Mn^{2+} ON HEXOKINASE ACTIVITY

Concentration of Mn^{2+} mM	2	4	5	6
△ O.D./5 min	0.015	0.030	0.042	0.056

The composition of the reaction mixture except Mg^{2+} was as stated in Assay I. Enzyme was diluted in 0.05 M phosphate buffer, pH 7.5, containing 8 mg/ml S.A. and 0.005 M TE. The activity with 20 mM Mg^{2+} under the same conditions was a change in O.D. of 0.090/5 min.

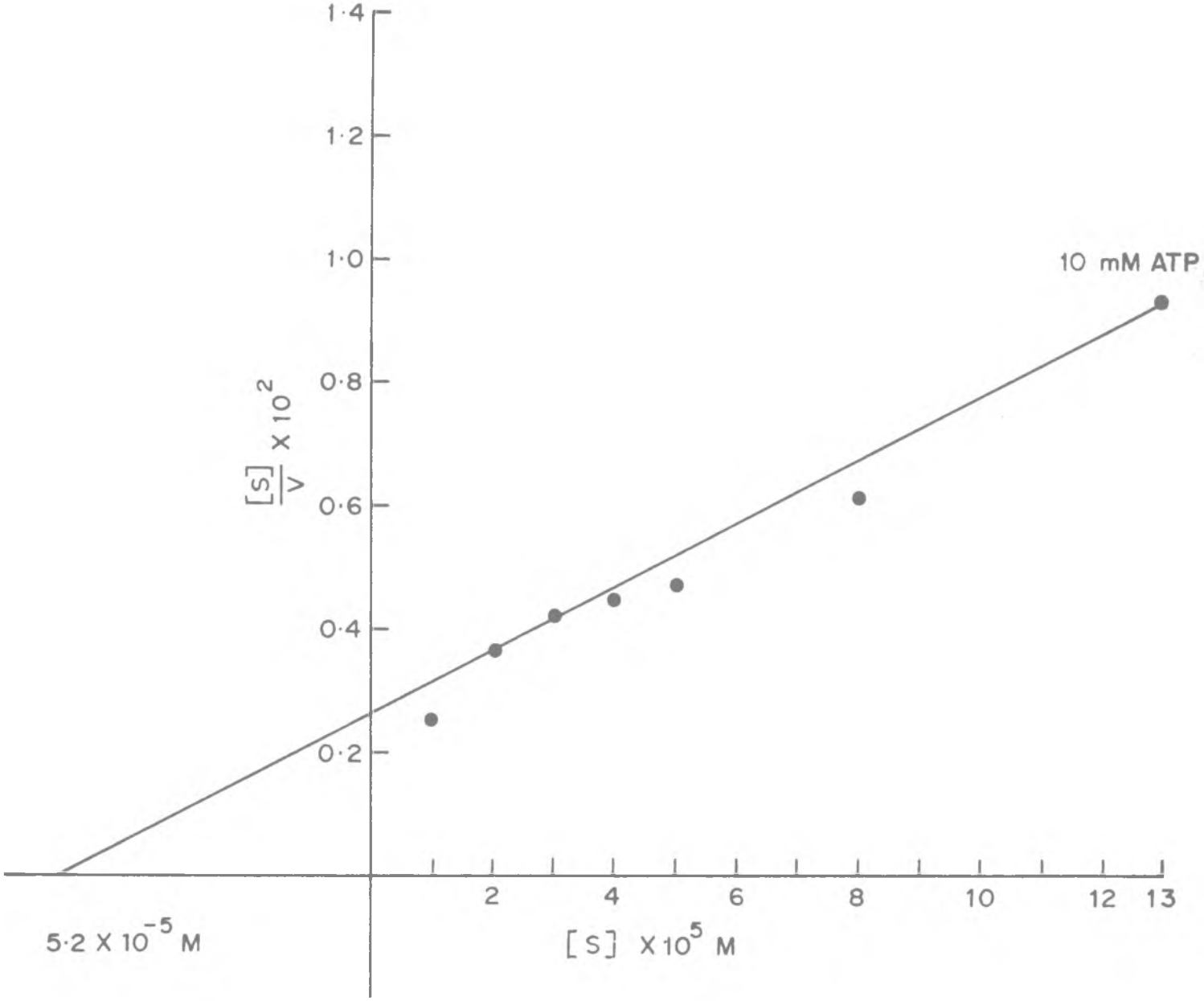


FIG. 12a. PLOT OF $\frac{[S]}{V}$ VS $[S]$, WHERE $[S]$ IS THE MOLAR CONCENTRATION OF GLUCOSE AND V THE INITIAL VELOCITY. (Assay I)

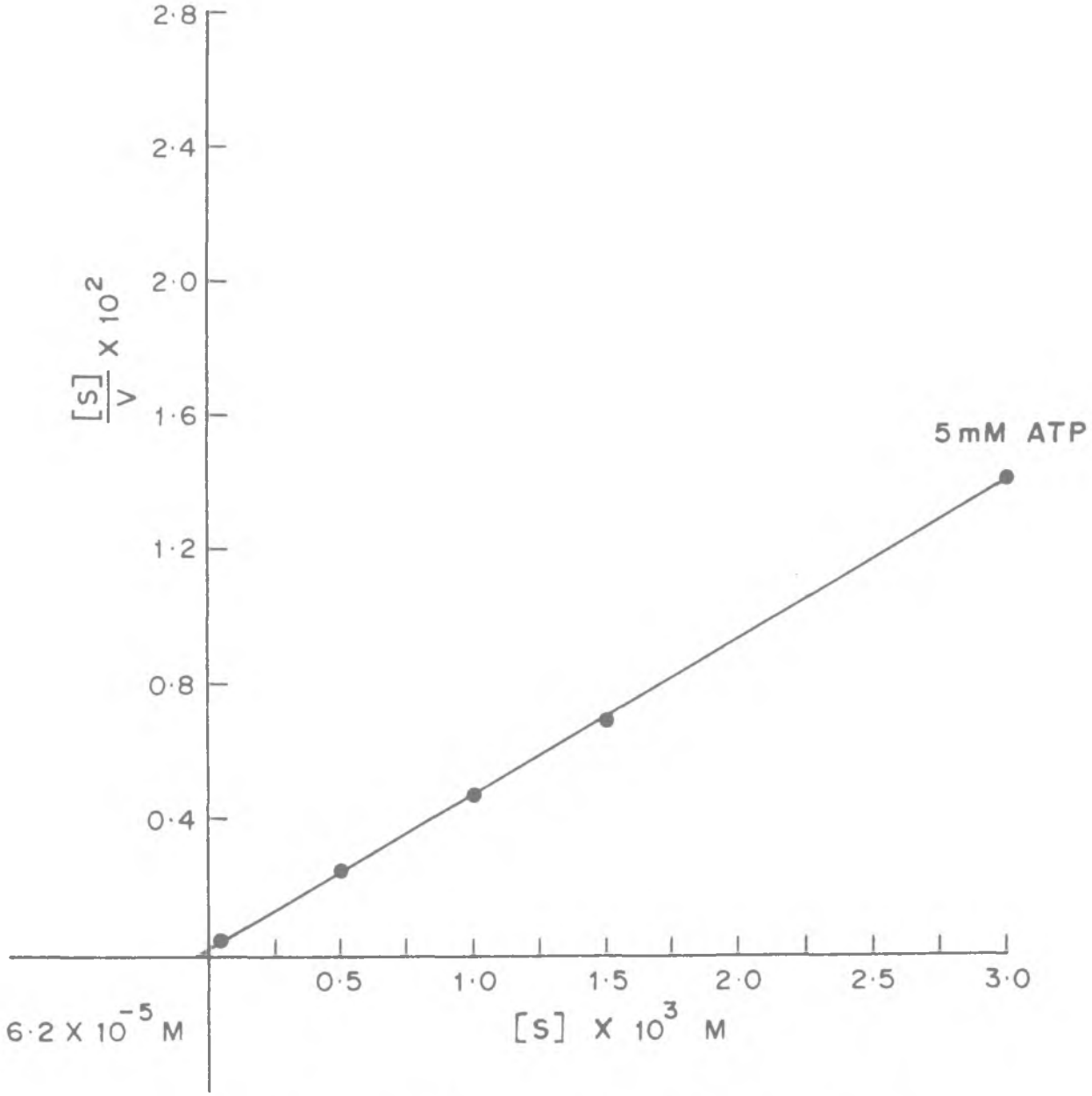


FIG.12b. PLOT OF $\frac{[S]}{V}$ VS $[S]$, WHERE $[S]$ IS THE MOLAR CONCENTRATION OF GLUCOSE AND V THE INITIAL VELOCITY. (Assay I)

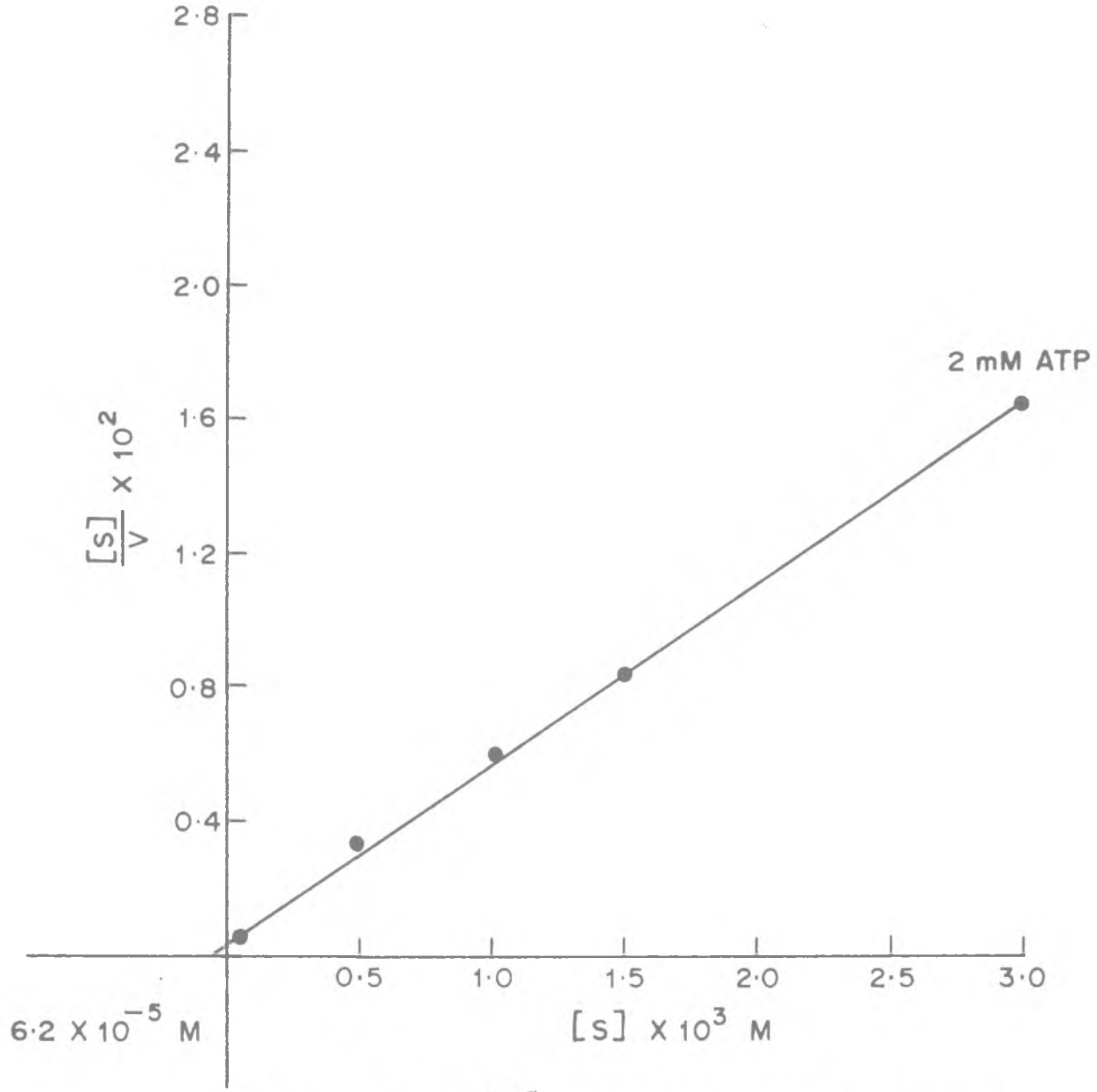


FIG.12c. PLOT OF $\frac{[S]}{V}$ VS $[S]$, WHERE $[S]$ IS THE MOLAR CONCENTRATION OF GLUCOSE AND V THE INITIAL VELOCITY. (Assay I).

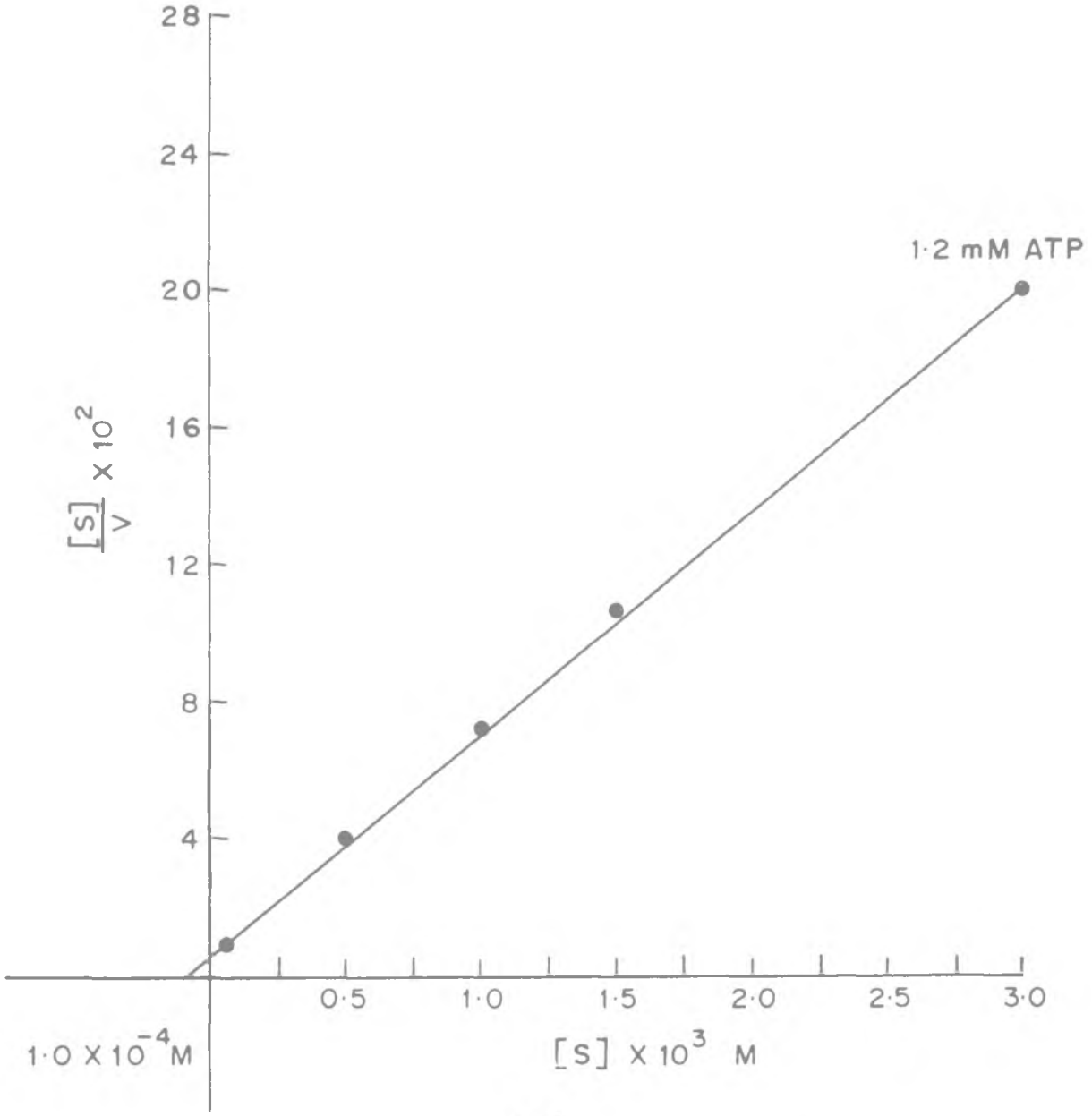


FIG. 12d. PLOT OF $\frac{[S]}{V}$ VS $[S]$, WHERE $[S]$ IS THE MOLAR CONCENTRATION OF GLUCOSE AND V THE INITIAL VELOCITY. (Assay I).

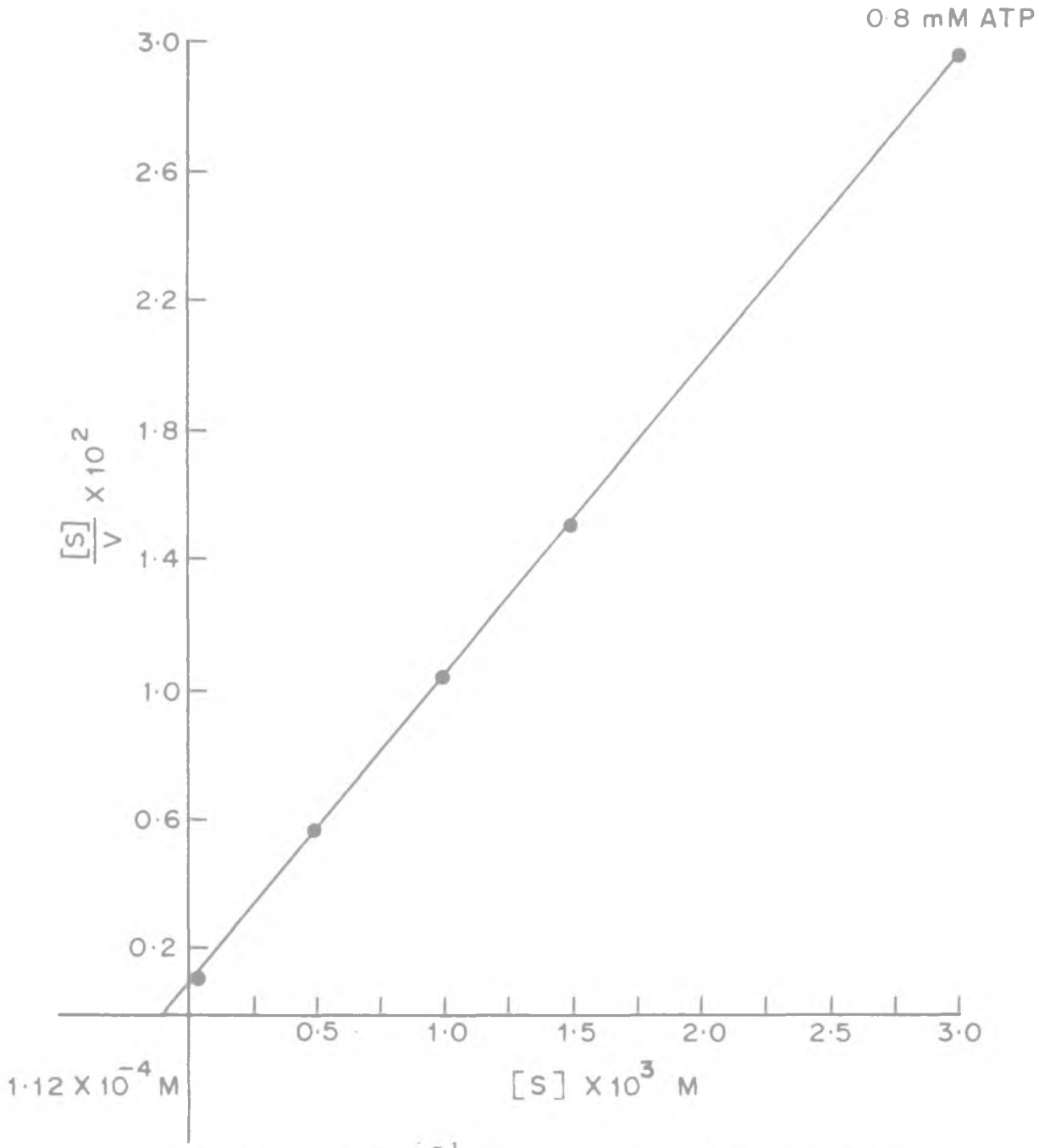


FIG.12e. PLOT OF $\frac{[S]}{V}$ VS [S], WHERE [S] IS THE MOLAR CONCENTRATION OF GLUCOSE AND V THE INITIAL VELOCITY. (Assay I).

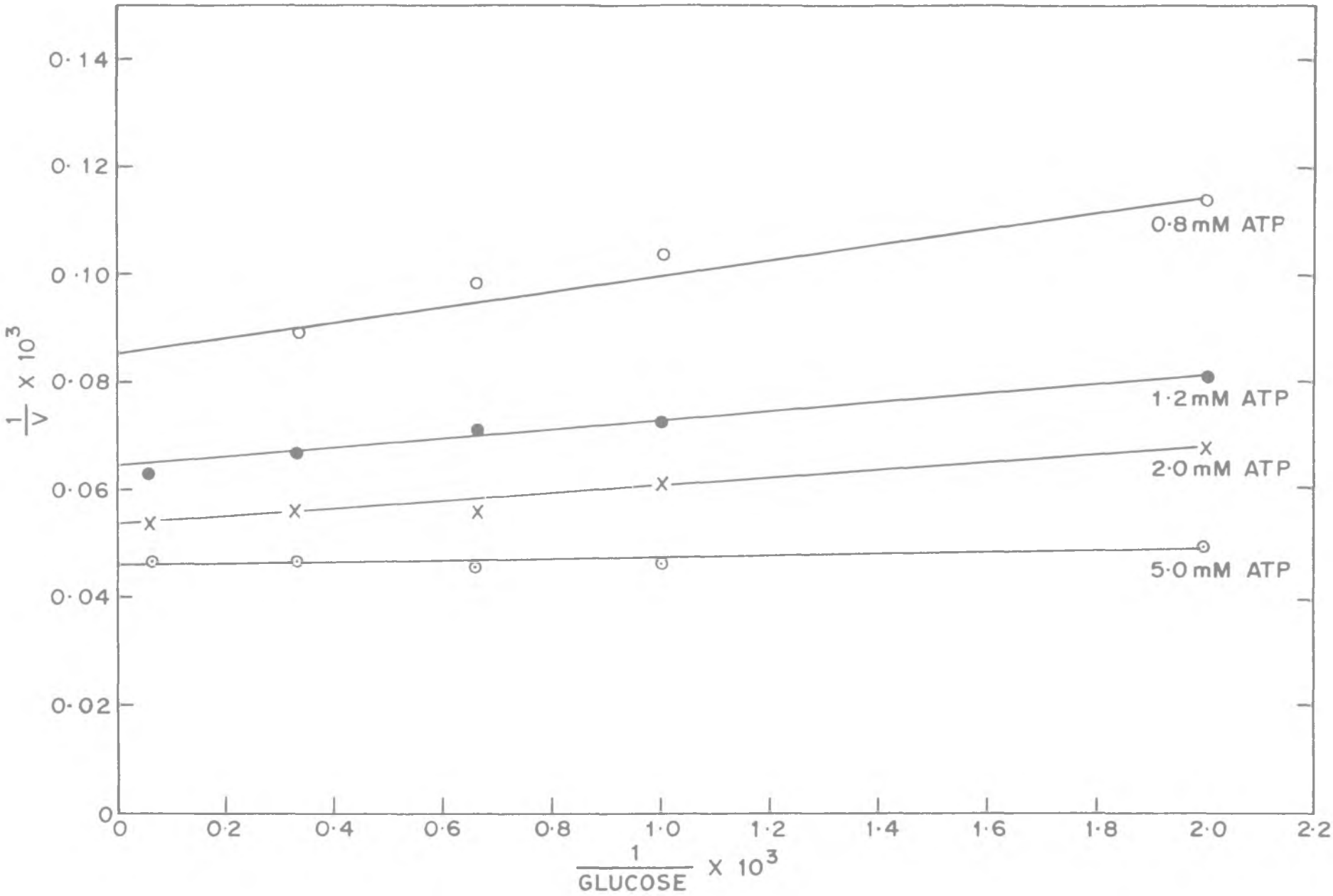


FIG. 13. PLOT OF RECIPROCAL OF INITIAL VELOCITY 'V' VS RECIPROCAL OF GLUCOSE AT DIFFERENT ATP CONCENTRATIONS. (Assay I).

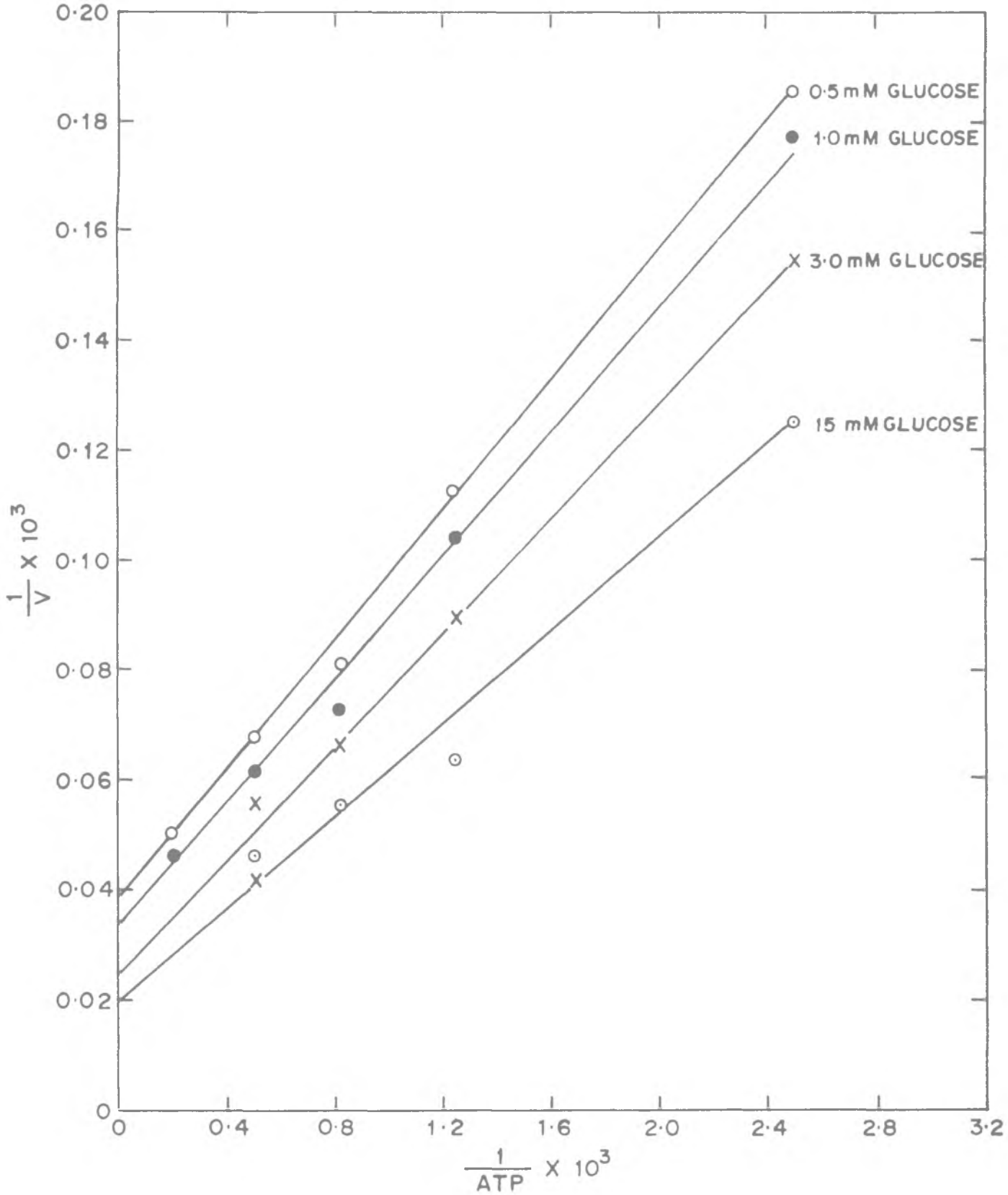


FIG.14. PLOT OF RECIPROCAL OF INITIAL VELOCITY 'V' VS RECIPROCAL OF ATP AT DIFFERENT GLUCOSE CONCENTRATIONS. (Assay I).

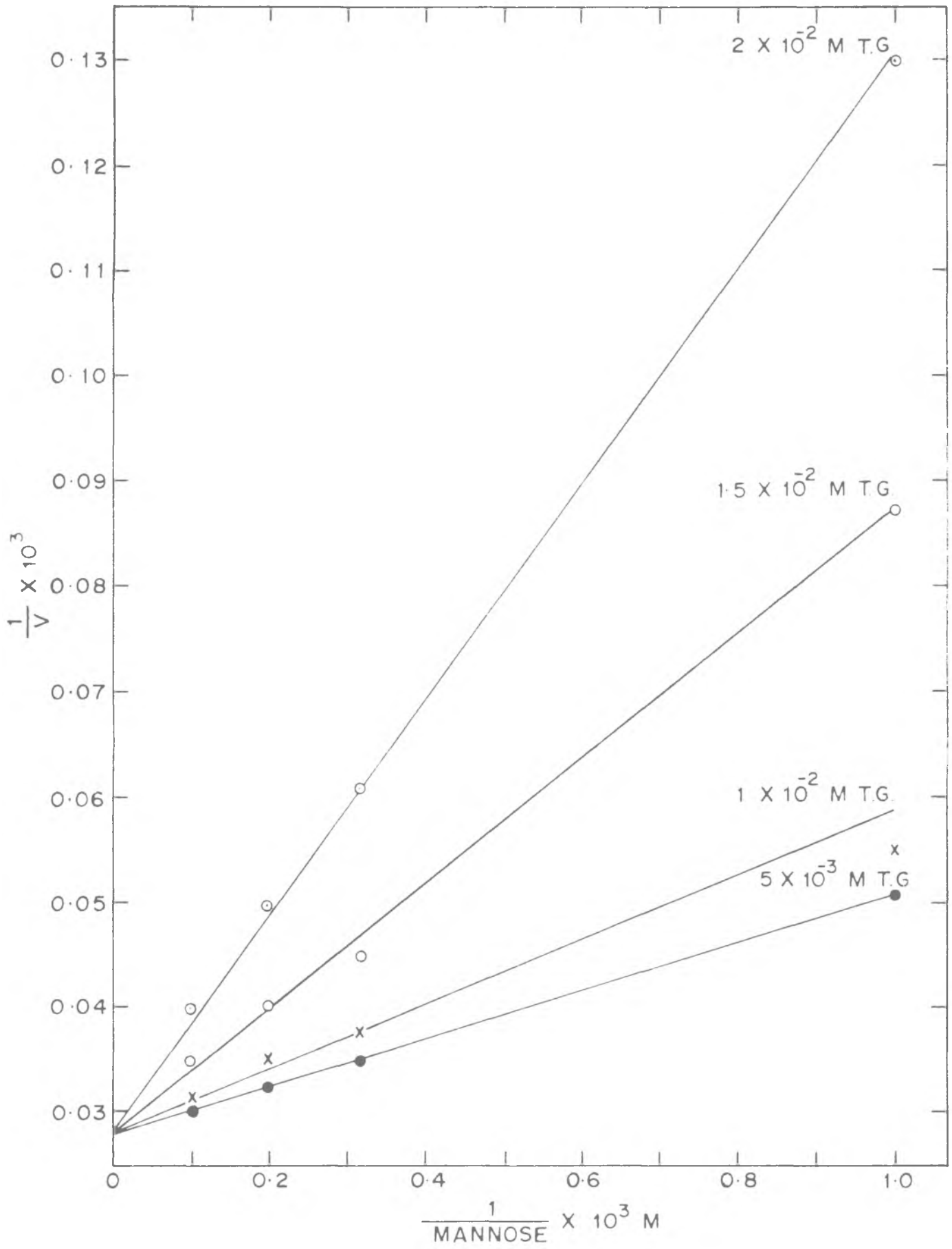


FIG. 15a. PLOT OF RECIPROCAL OF INITIAL VELOCITY 'V' VS RECIPROCAL OF MANNOSE AT DIFFERENT COCENTRATIONS OF 5-THIO-D-GLUCOSE. (Assay III).

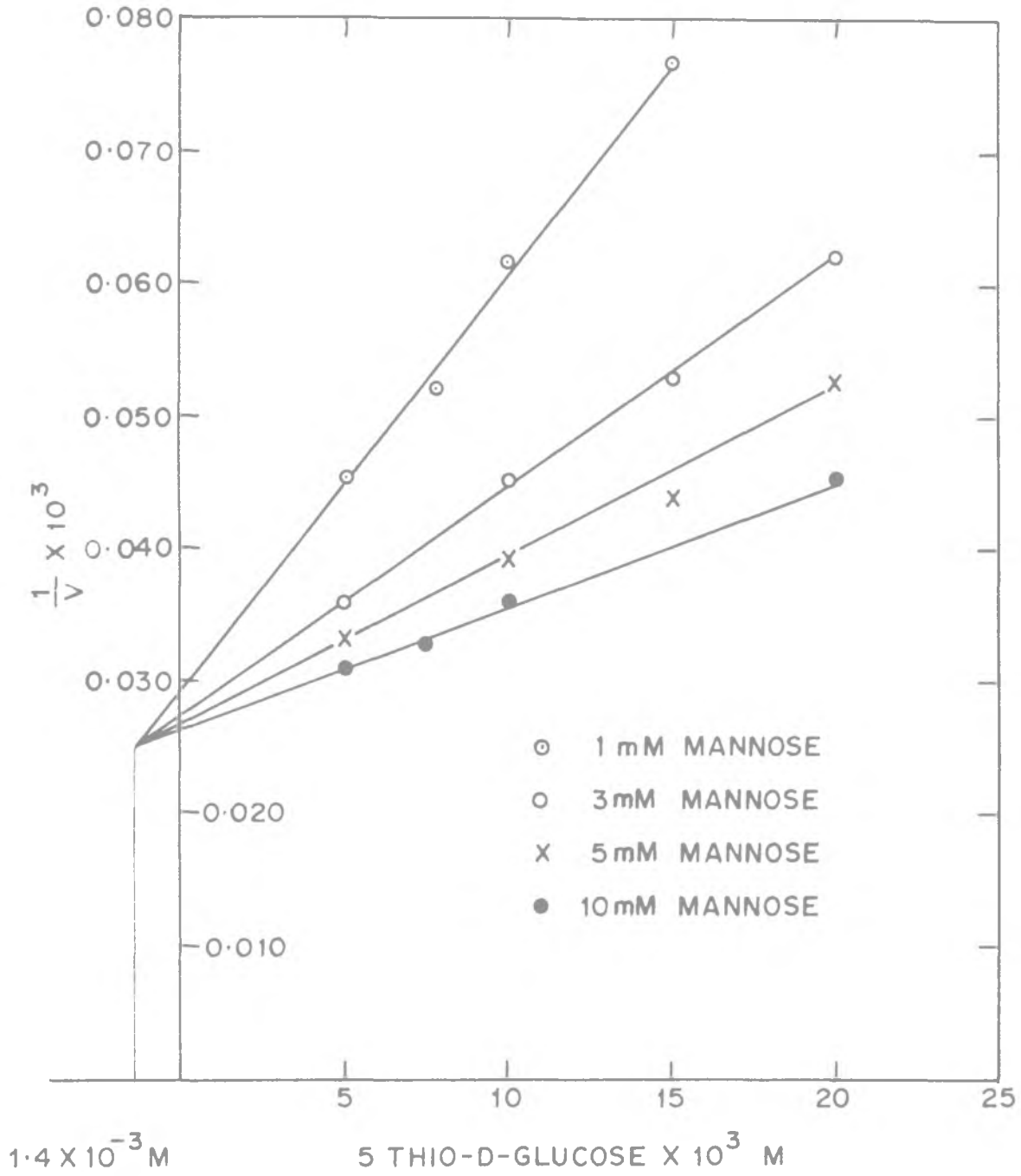


FIG. 15b. PLOT OF RECIPROCAL OF INITIAL VELOCITY (V) VS MOLAR CONCENTRATION OF 5-THIO-D-GLUCOSE AT VARIOUS MANNOSE CONCENTRATIONS.

Effect of ADP on heart hexokinase

The effect of varying ADP concentration on activity at different ATP concentrations was determined. This study was carried out using Assay I. The results are shown in Fig.17. ADP inhibition of hexokinase was non-competitive with respect to ATP. The Ki values for ADP at different ATP concentrations are shown in Table 24.

Effect of high concentration of glucose on heart hexokinase

Assay I was used to carry out this experiment. The glucose concentration was varied from 15 mM to 150 mM (Table 25). It will be seen that the activity was the same at all glucose concentrations showing the absence of any glucokinase with a high K_m for glucose.

Effect of glucose-6-P and inorganic phosphate on heart hexokinase

The effect of glucose-6-P and inorganic phosphate were studied by using Assay III. Five different glucose-6-P concentrations at four Pi concentrations were tested. The experimental results are summarized in Table 26. For this study Assay III was used with mannose as substrate since the mannose-6-phosphate formed did not inhibit the hexokinase under the experimental conditions as shown by the proportionality of activity with time or enzyme concentration (see Table 28). Fig. 16 a,b & c.

Since glucose-6-P is an inhibitor of animal tissue hexokinases and Pi reverses this inhibition, these studies were carried out to obtain quantitative kinetic data for purified heart hexokinase.

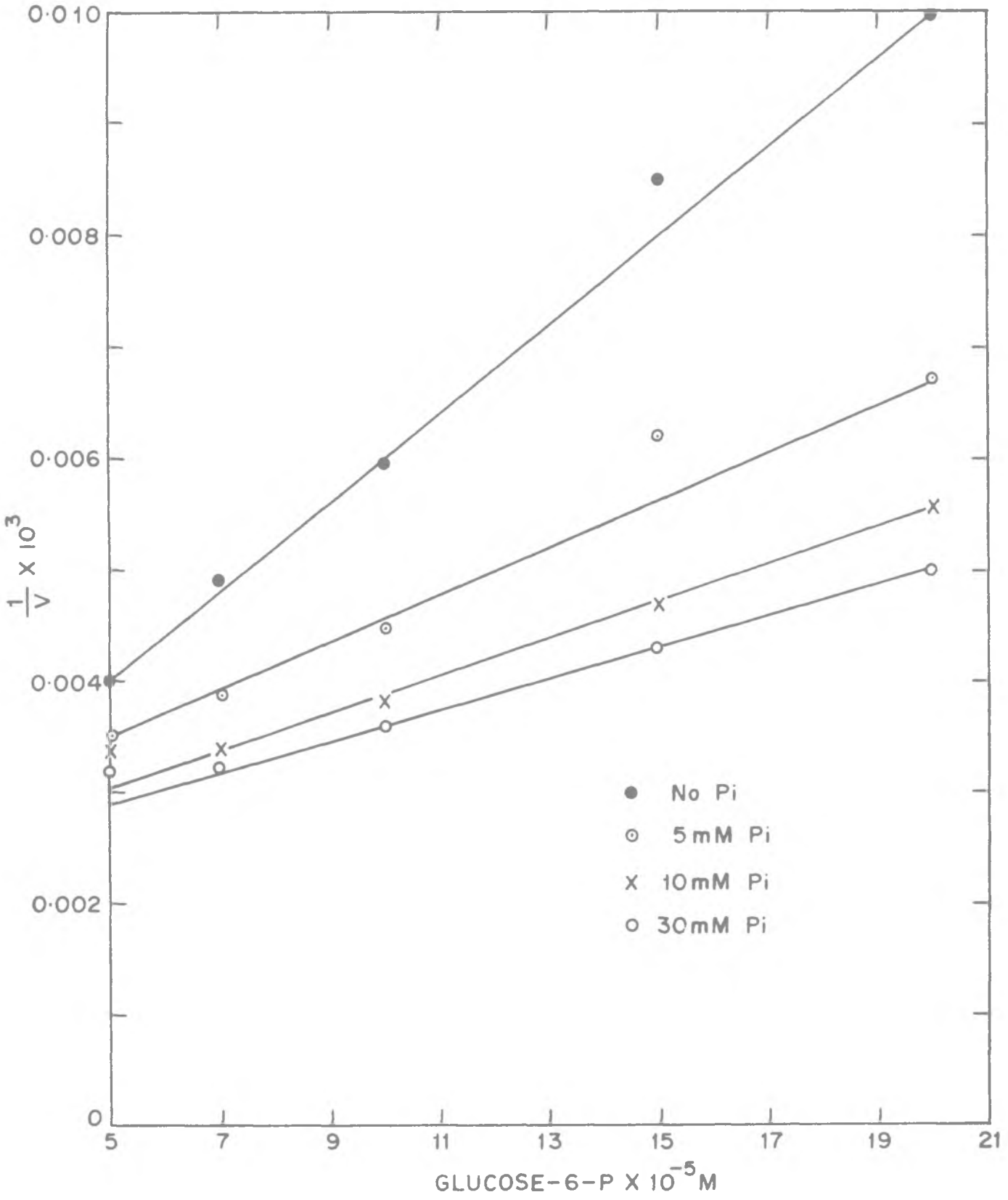


FIG. 16a. PLOT OF RECIPROCAL OF INITIAL VELOCITY (V) VS GLUCOSE-6-P CONCENTRATION AT DIFFERENT Pi CONCENTRATIONS ASSAY III

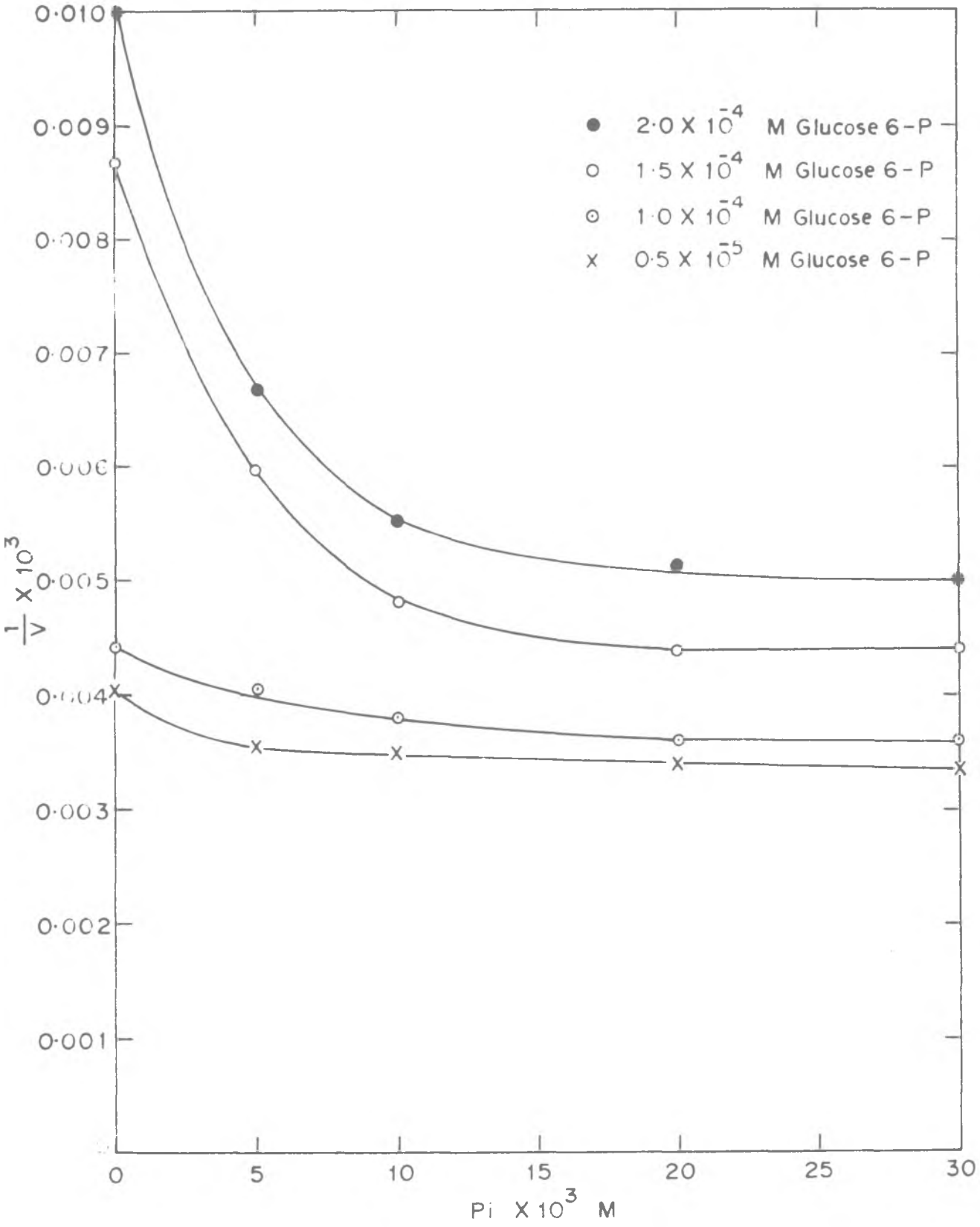


FIG. 16b. PLOT OF RECIPROCAL OF INITIAL VELOCITY (V) VS Pi CONCENTRATION AT DIFFERENT GLUCOSE 6-CONCENTRATIONS ASSAY III

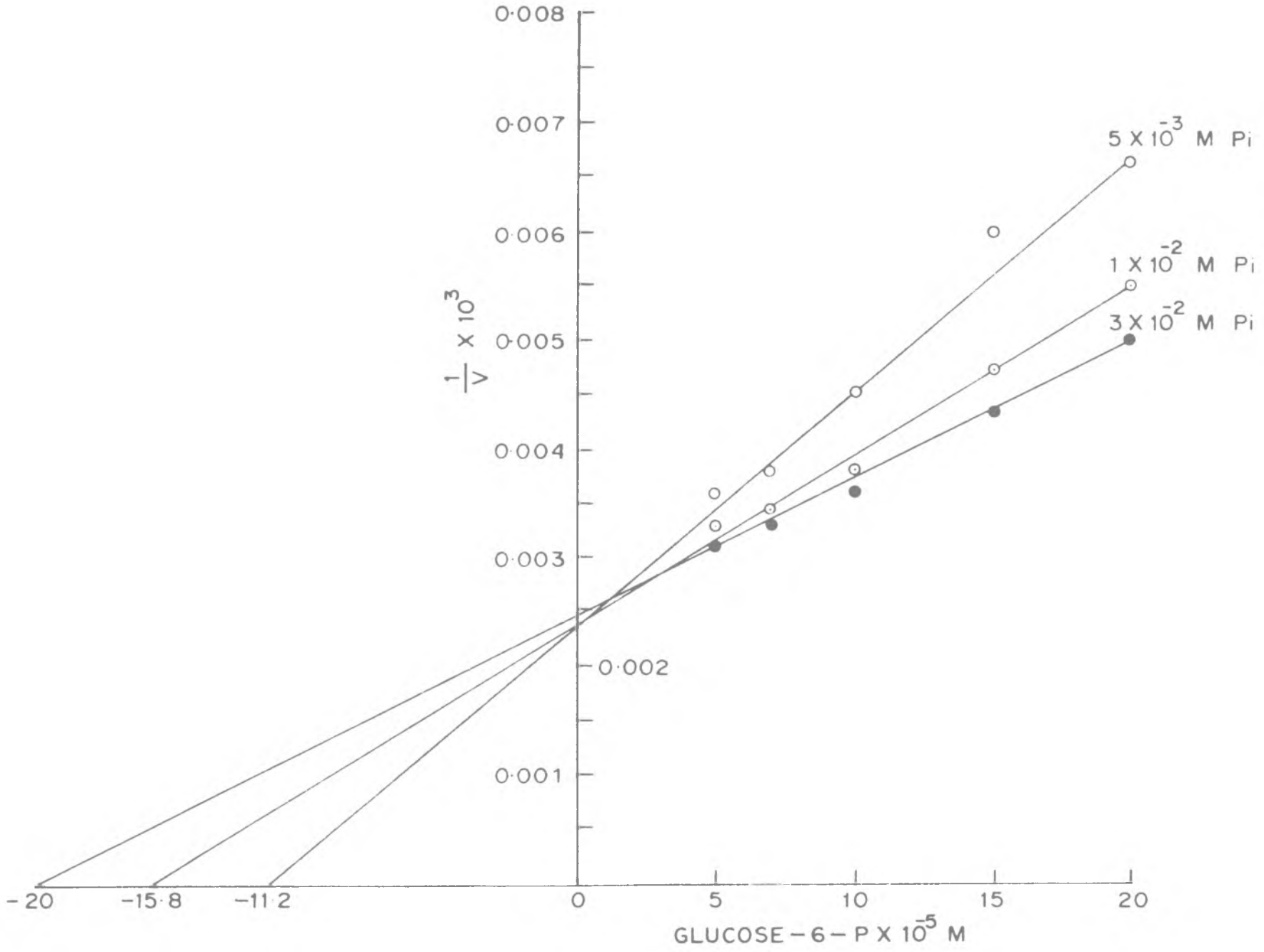


FIG.16c. PLOT OF RECIPROCAL OF INITIAL VELOCITY 'V' VS GLUCOSE-6-P CONCENTRATIONS AT THE Pi CONCENTRATIONS SHOWN IN THE FIGURE. (Assay III).

TABLE 24

Ki VALUES FOR ADP

ATP concentration	Ki for ADP
mM	M
1	1.9×10^{-3}
2	1.7×10^{-3}
5	1.0×10^{-3}
10	7.5×10^{-4}

TABLE 25

EFFECT OF HIGH CONCENTRATION OF GLUCOSE ON HEXOKINASE ACTIVITY

Concentration of glucose mM	15	50	100	150
$\triangle$ O.D./10 min	0.218	0.218	0.218	0.220

The composition of the reaction except glucose was as stated in Assay I.

TABLE 26

EFFECT OF GLUCOSE-6-PHOSPHATE AND INORGANIC PHOSPHATE ON HEXOKINASE ACTIVITY

No.	Concentration of glucose-6-P	Concentration of phosphate	△ O.D./10 min	Inhibition
	mM	mM		%
1	0.05	-	0.250	29
2	0.05	5	0.280	20
3	0.05	10	0.290	17
4	0.05	20	0.295	16
5	0.05	30	0.300	15
6	0.07	-	0.230	34
7	0.07	5	0.273	22
8	0.07	10	0.295	17
9	0.07	20	0.300	14
10	0.07	30	0.305	14
11	0.10	-	0.170	52
12	0.10	5	0.225	30
13	0.10	10	0.262	25
14	0.10	20	0.280	20
15	0.10	30	0.285	19
16	0.15	-	0.115	67
17	0.15	5	0.161	54
18	0.15	10	0.210	40
19	0.15	20	0.230	34
20	0.15	30	0.230	34
21	0.20	-	0.100	72
22	0.20	5	0.150	57
23	0.20	10	0.180	49
24	0.20	20	0.190	43
25	0.20	30	0.200	39

The composition of the reaction mixture was as stated in Assay III. The amount of inorganic phosphate present in the hexokinase was 0.05 μM.

Effect of ATP on glucose-6-P inhibition and its reversal by Pi

This study was carried out using Assay-III. Five different ATP concentrations were tested (Fig.16d, e, and). The Ki values for glucose-6-P at different ATP and Pi concentrations are summarized in Table 27. From the Figure it will be seen that inhibition by glucose-6-P was non-competitive with respect to ATP.

Effect of mannose and enzyme concentrations on glucose-6-P inhibition and its reversal by Pi

This experiment was carried out using Assay III (Table 28). Three different enzyme concentrations and two different mannose concentrations were tried. The results are summarized in Table 28. This experiment was carried out to show that high concentrations of mannose had no effect on enzyme activity. Mannose-6-phosphate neither inhibits nor has it any effect in the presence of glucose-6-P alone or of glucose-6-P and Pi. The inhibition due to glucose-6-P and its reversal by Pi remained unaffected by mannose concentration.

Effect of hormones on glucose-6-P inhibition and its reversal by Pi

The effect of hormones on enzyme activity or on glucose-6-P inhibition and its reversal by Pi was determined. No direct effect of hormones on enzyme activity in vitro has hitherto been demonstrated unequivocally (except for the effect of 3',5'-AMP and other compounds on the interconversion of phosphorylases a and b). However the inhibition of hexokinase by low concentrations of glucose-6-P and the effect of Pi on this inhibition suggested that the effect of different compounds on purified heart hexokinase activity requires investigation in the presence of or absence of glucose-6-P and Pi. These studies were carried out with Assay III using mannose as substrate. The hormones were tested at different concentrations.

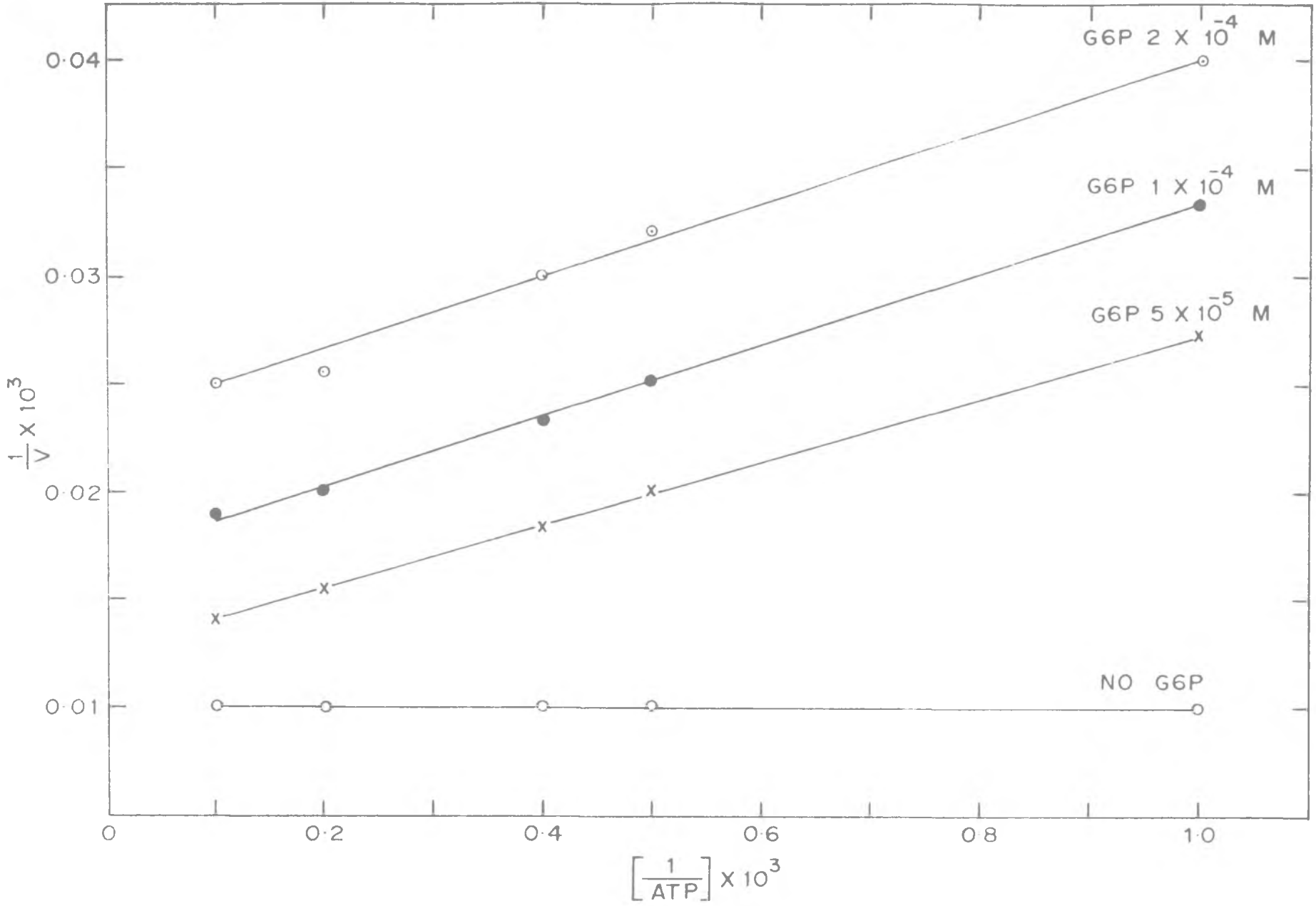


FIG.16d. PLOT OF RECIPROCAL OF INITIAL VELOCITY 'V' VS THE RECIPROCAL OF ATP AT DIFFERENT CONCENTRATIONS OF GLUCOSE-6-P. (Assay III).

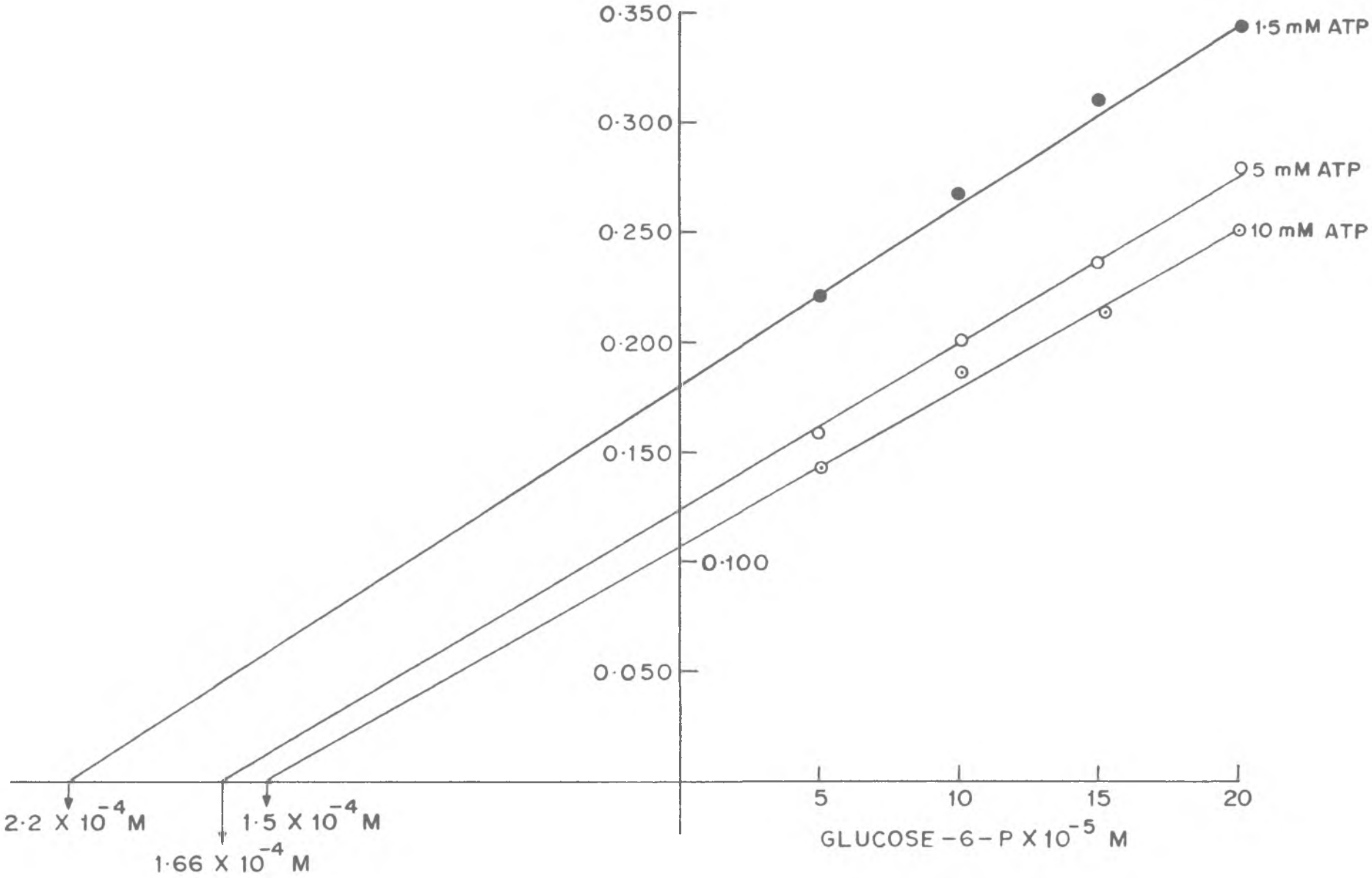


FIG. 16e. PLOT OF RECIPROCAL OF INITIAL VELOCITY 'V' VS GLUCOSE-6-P CONCENTRATIONS AT THE ATP CONCENTRATIONS SHOWN IN THE FIGURE. (Assay III).

TABLE 27

Ki VALUES FOR GLUCOSE-6-P FOR PARTICULATE HEXOKINASE

	Ki values M
Pi 5 mM	1.12×10^{-4}
Pi 10 mM	1.58×10^{-4}
Pi 30 mM	2.00×10^{-4}
ATP 1.5 mM	2.20×10^{-4}
ATP 5 mM	1.66×10^{-4}
ATP 10 mM	1.50×10^{-4}

Composition of the reaction mixture was same as stated for Assay III. The amount of Pi in the enzyme used for estimations was 0.05 mM.

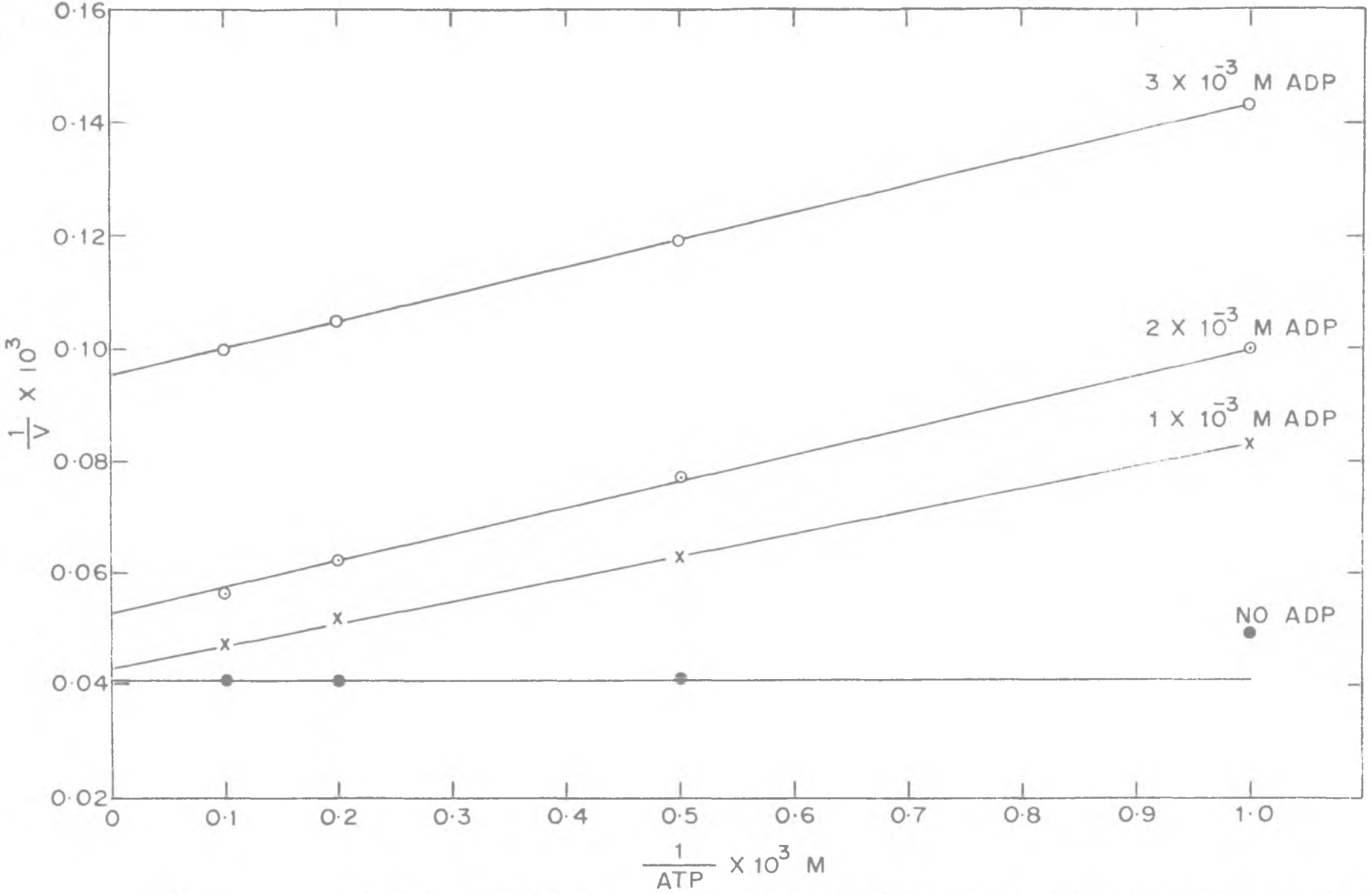


FIG. 17. PLOT OF RECIPROCAL OF INITIAL VELOCITY 'V' VS RECIPROCAL OF ATP AT DIFFERENT CONCENTRATIONS OF ADP. (Assay I).

TABLE 28

EFFECT OF MANNOSE AND ENZYME CONCENTRATION ON GLUCOSE-6-P INHIBITION AND ITS REVERSAL BY INORGANIC PHOSPHATE

No.	Enzyme	Mannose	Glucose-6-P	Inorganic P	△O.D./5 min	Inhibition
	ml	µM	mM	mM		%
1	0.05	5	-	-	0.150	-
2	0.05	5	0.10	-	0.070	53
3	0.05	5	0.10	10	0.100	34
4	0.10	5	-	-	0.300	-
5	0.10	5	0.10	-	0.150	50
6	0.10	5	0.10	10	0.200	34
7	0.15	5	-	-	0.450	-
8	0.15	5	0.10	-	0.225	50
9	0.15	5	0.10	10	0.270	40
10	0.10	30	-	-	0.300	-
11	0.10	30	0.10	-	0.155	48
12	0.10	30	0.10	10	0.210	30

The composition of the reaction mixture was as stated in Assay III except mannose. The enzyme was diluted with 0.05 Tris-HCl buffer, pH 7.5, containing 8 mg/ml S.A. and 0.01 M TE.

The results (Table 29) show that none of the compounds had any effect on hexokinase activity in the presence or absence of glucose-6-P with or without Pi.

Effect of nucleotides on hexokinase

The activity of hexokinase with different nucleotides was determined (Table 30). There was no activity with GTP, CTP, UTP or ADP. The absence of activity with ADP showed the absence of significant amounts of myokinase in the purified enzyme.

Relative activities of heart hexokinase with different carbohydrates

Assay III was used for determining the relative activities of heart hexokinase with different substrates. The experimental results are summarized in Table 31. It will be seen that dulcitol, cellobiose, lactose and mannitol were ineffective as substrates for heart hexokinase. The observed activities with galactose, xylose, sorbitol and α-methylglucoside are suspect since they contain appreciable amounts of glucose as shown in the next section.

This preliminary study was carried out using Assay I. Mg:ATP (2:1) ratio was kept constant. The results are summarized in Table 32. It is not clear from earlier studies on the specificity of hexokinase whether the substrates were tested for the presence of traces of other carbohydates. This experiment was intended to test for glucose in some of the compounds tested in the preceding section. There should be no activity in this test except with glucose since the products of the reaction (mannose-6-phosphate etc.) are not oxidized by glucose-6-P dehydrogenase. However, galactose, xylose, sorbitol and α-methyl glucoside contained appreciable amounts of glucose. Further work is needed to show whether

TABLE 29

EFFECT OF HORMONES ON GLUCOSE-6-P INHIBITION AND ITS REVERSAL BY INORGANIC PHOSPHATE

Hormone	Quantity	Control	Glucose-6-P	Inhibition	Glucose-6-P + Pi	Inhibition
	µg	△O.D./10 min	△O.D./10 min	%	△O.D./10 min	%
Bovine growth hormone	1	0.300	-	-	-	-
" "	5	0.300	-	-	-	-
" "	55	0.305	-	-	-	-
" "	1	-	0.100	67		
" "	1	-	-	-	0.210	30
" "	5	-	0.100	67	-	-
" "	5	-	-	-	0.212	30
" "	55	-	0.102	67	-	-
" "	55	-	-	-	0.210	30
Adrenalin	5	0.300	-	-	-	-
"	50	0.303	-	-	-	-
"	5	-	0.100	67	0.200	34
"	50	-	0.100	67	0.205	32
Serotonin	5	0.305	-	-	-	-
"	55	0.300	-	-	-	-
"	5	-	0.100	67	0.200	32
"	55	-	0.100	67	0.205	34
Insulin	5	0.306	-	-	-	-
"	55	0.310	-	-	-	-
"	5	-	0.100	67	0.210	30
"	55	-	0.100	67	0.213	29
Prednisolone	5	0.300	-	-	-	-
"	50	0.305	-	-	-	-
"	5	-	0.100	67	0.200	34
"	50	-	0.103	66	0.205	32

The composition of the reaction mixture was as stated in Assay III. Enzyme was diluted in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.01 M TE and 8 mg S.A./ml. (Glucose-6-P was 0.15 mM and Pi 10 mM).

TABLE 30

EFFECT OF NUCLEOTIDES ON HEXOKINASE

No.	Nucleotide	ΔO.D./10 min
1	ATP	0.200
2	GTP	0
3	CTP	0
4	UTP	0
5	ADP	0

The composition of the reaction mixture was as stated in Assay I except that ATP was replaced by the respective nucleotides and the amount of nucleotide used for determining the activity was 10 μM.

TABLE 31

RELATIVE ACTIVITIES OF HEXOKINASE WITH DIFFERENT SUBSTRATES

No.	Substrate	△O.D./10 min	Relative activity
1	Mannose	0·210	100
2	Fructose	0.380	179
3	Mellibiose	0.020	10
4	l-Sorbose	0.015	8
5	Galactose	0.084	40
6	l-Xylose	0.050	24
7	α-Methylglucoside	0.075	34
8	Sorbitol	0.020	10
9	Dulcitol	-	-
10	Rhamnose	0.013	6
11	Trehalose	0.050	24
12	Cellobiose	-	-
13	Raffinose	0.020	10
14	Lactose	-	-
15	Mannitol	-	-

The composition of the reaction mixture except for substrate was as described in Assay III. Enzyme dilution was made as in Table 19. The amount of substrate used for determining the activity was 10 μM.

TABLE 32

ACTIVITY OF HEXOKINASE WITH DIFFERENT SUBSTRATES

No.	Substrate	△ O.D./10 min
1	Glucose	0.230
2	Galactose	0.120
3	D(+)-Xylose	0.065
4	Sorbitol	0.045
5	α-Methylglucoside	0.103
6	Mellibiose	-
7	Fructose	-
8	Mannose	-
9	Sorbose	-
10	Mannitol	-

The composition of the reaction mixture for glucose was as described for Assay I. The enzyme was diluted as in Table 19. The amount of substrate used for determining the activity was 10 μM.

some of the compounds showing activity (Table 34) contain fructose or mannose as impurities.

The effect of fructose 1,6-diphosphate on hexokinase activity was determined using Assay III at varying concentrations of Mg^{2+}. It was found to be inhibitory to the enzyme (Table 36) (Fig.18). The Plot of $\frac{1}{Mg}$ against $\frac{1}{\text{activity}}$ at different FDP concentrations appears to show that the inhibition is noncompetitive with respect to Mg^{2+}. Further work with different Mg concentrations is essential to substantiate this result.

Effect of various inhibitors on hexokinase

The effect of diisopropylfluorophosphate on hexokinase activity was studied using Assay I. The enzyme (in 0.05 M phosphate, pH 7.5 containing 0.25 M sucrose and 0.005 M TE) was incubated with DFP (10 mM) at 30° for 30 min and then activity was determined. It will be seen that there was no change on hexokinase activity after incubation with DFP (Table 33).

The effect of different compounds on hexokinase activity was studied by using Assay I (Table 34). The composition of the reaction mixture was as stated in Assay I. The different compounds shown in the Table had no effect on hexokinase activity at the specified concentrations.

The effect of SH reagents was determined using Assay I. The results are summarized in Table 35. The adequacy of glucose-6-P dehydrogenase in the presence of the inhibitors was established in separate experiments. It will be seen that hexokinase was inhibited by pCMB and iodoacetate.

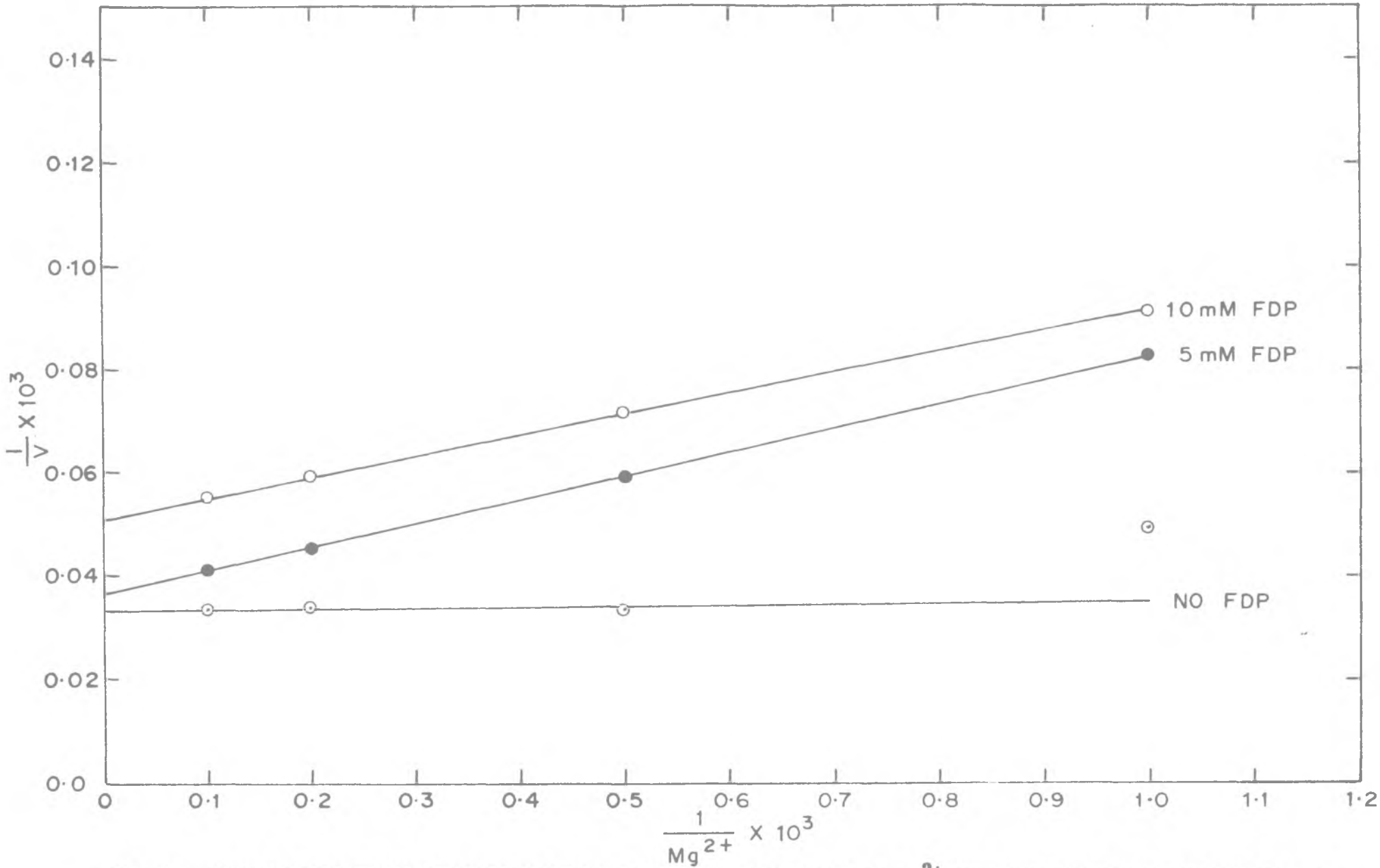


FIG. 18. PLOT OF RECIPROCAL OF INITIAL VELOCITY 'V' VS RECIPROCAL OF Mg^{2+} AT DIFFERENT CONCENTRATIONS OF FDP. (Assay III).

TABLE 33

EFFECT OF DIISOPROPYLFLUOROPHOSPHATE ON HEXOKINASE ACTIVITY

Control	Control + DFP
△ O.D./10 min	△ O.D./10 min
0.286	0.290

The composition of the reaction mixture was as stated for Assay-I. Enzyme was in 0.05 M phosphate buffer, pH 7.5, containing 0.25 M sucrose and 0.005 M TE. 1 ml enzyme was incubated with 1 ml DFP (10 μM) at 30° for 30 min and then tested for activity.

TABLE 34

EFFECT OF DIFFERENT COMPOUNDS ON HEXOKINASE ACTIVITY

Compound	Concentration
	mM
Sodium fluoride	10
Potassium chloride	5
Ammonium sulphate	10
3-Phosphoglyceric acid	5
Phosphoenolpyruvic acid	5
Thioethanol	5
Oxamate	1-3

The composition of the reaction mixture was as stated for Assay I.

TABLE 35

EFFECT OF VARIOUS INHIBITORS ON HEXOKINASE ACTIVITY

Compound	Concentration	Enzyme activity	Inhibition
	M	ΔO.D./10 min	%
p-Chloromercuribenzoate	1×10^{-6}	0.090	50
Iodoacetate	1×10^{-3}	0.160	10
Iodoacetate	4×10^{-3}	0.140	22
Control	-	0.180	-

The composition of the reaction mixture was as stated for Assay I.

TABLE 36

EFFECT OF FRUCTOSE 1,6-DIPHOSPHATE ON HEXOKINASE

No.	Magnesium	Fructose 1,6-diphosphate	△O.D./5 min	Inhibition
	mM	mM		%
1	1	-	0.100	-
2	1	2.5	0.070	30
3	1	5.0	0.060	40
4	1	10.0	0.045	55
5	2	-	0.155	-
6	2	2.5	0.125	25
7	2	5.0	0.085	44
8	2	10.0	0.070	52
9	5	-	0.150	-
10	5	5.0	0.110	27
11	5	10.0	0.085	44
12	10	-	0.150	-
13	10	5.0	0.120	20
14	10	10.0	0.090	40

Composition of the reaction mixture was as stated in Assay III.

CHAPTER V

DISCUSSION

D I S C U S S I O N

Purification and properties

Ox brain hexokinase is the only animal tissue hexokinase which has been purified to a significant extent. The specific activity of the purified enzyme obtained by Joshi and Jagannathan (1968) was about 63 units/mg and that of Schwartz and Basford (1967) was 80 units/mg. The former was about 90 per cent pure and the latter was ultracentrifugally homogeneous. The specific activities reported for other animal tissue hexokinases are comparatively very low (Grosbard and Schimke, (1966) 13 units/mg for skeletal muscle hexokinase Type II, 6.8 units/mg for rat kidney type I at 37°). The maximum specific activity of ox heart particulate hexokinase obtained in the present work was 58 units/mg at 30°. This activity is 70 per cent of that of the maximum specific activity reported for ox brain hexokinase. However a comparison between the enzyme from different tissues may not be valid, especially if solubilization of a particulate enzyme involves formation of fragments of different sizes having the same catalytic activity per molecule but with different maximum specific activities in terms of activity per mg. The quantity of hexokinase present in heart is far less than that in brain. The instability of the enzyme at the final stages prevented any further attempts at purification. The procedure described in this report gives the purest preparation of particulate heart hexokinase obtained hitherto.

The procedure used for the solubilization of the enzyme is essentially similar to that used with ox brain. However, several

points of similarity are noteworthy. Unless the enzyme was frozen and thawed and the viscous material removed by salmine sulfate further purification of the enzyme by ammonium sulfate fractionation etc. was not possible. However at this stage the hexokinase is not sedimented at 100,000 x g in 1 to 2 hours showing that it is solubilized. Similar results were reported by Joshi and Jagannathan (1968) for brain hexokinase. The nature of the impurity which prevents further purification in the initial elastase treatment stage is not known. It is also of interest that elastase has no action at all on ox heart hexokinase activity and even prolonged incubation with elastase for several days causes no significant loss in hexokinase activity compared to controls without elastase. The retention of properties such as inhibition by glucose-6-P and reversal of the inhibition by Pi etc. also suggests that these properties of the particulate enzyme are not significantly altered by solubilization with elastase and subsequent purification. Treatment with trypsin, chymotrypsin etc. however caused significant loss of the enzyme when the digestion was carried beyond a particular period.

The yield of enzyme from the particulate preparation on solubilization was approximately 50 per cent. More enzyme could be recovered by repeating the elastase treatment, but owing to the danger of bacterial contamination this procedure was not followed.

A further point of interest with regard to the particulate hexokinase of heart muscle is its firm attachment to mitochondria. Even sonication of the mitochondria and preparation of the electron transport particle does not remove the hexokinase from the particulate material. Some of the enzymes of the citric acid cycle and of fatty

acid oxidation are removed in the supernatant liquid by this procedure. Treatment of whole mitochondria with elastase does not yield any significant amount of soluble hexokinase. On the other hand treatment of ETP readily gives about 50 per cent of the enzyme in soluble form on treatment with elastase. The hexokinase is probably inaccessible to elastase in the form in which it is present in the heavy beef heart mitochondria. The attachment of hexokinase to the electron transport system is of interest but the significance if any of this is not known.

The ready solubilization of heart hexokinase by glucose-6-P, ATP etc. has been described earlier in the Introduction. This is in striking contrast to the difficulty of removing hexokinase from mitochondria by sonication and centrifugation. The soluble enzyme obtained by glucose-6-P treatment of mitochondria requires further characterization.

A comparison with the soluble enzyme obtained by this procedure with that obtained by elastase treatment may throw light on the mode of attachment of hexokinase to mitochondria.

The total purification of the enzyme after solubilization was approximately 500-fold. The methods used are conventional ones and require no comment. The instability of the enzyme in dilute solution prevents its purification by other methods such as the use of IRC resin etc.

About half of the hexokinase in heart muscle is present in soluble form. Preliminary work on gel electrophoresis of the soluble enzyme reported here shows the existence of two different isozymes. The purification of the enzyme was unfortunately not

considerable and the two isozymes were not separated. However these results show that the mobilities of the soluble isozymes are different from that of the particulate enzyme after solubilization. The latter does not show any significant mobility under a variety of conditions in acrylamide gel (e.g. change of pH, gel concentration etc.). The reason for this is not clear. The data on the molecular weight on Sephadex columns show that the solubilized hexokinase has a molecular weight of about 90,000. Its lack of mobility is not due to high molecular weight. It is possible that hexokinase is adsorbed on acrylamide, but this requires confirmation. These results show that the soluble enzymes of heart are not merely insoluble particulate enzymes which have been released in the presence of Mg etc. Though the evidence available so far is insufficient to prove it conclusively the present work suggests the existence of three distinct hexokinases in ox heart (one in the particulate enzyme and two soluble isoenzymes).

Properties

The heart enzyme also resembles ox brain hexokinase in being stabilized by high concentrations of glucose. Concentrations of about 1 M sucrose are required to stabilize the enzyme against heating. The stability on storage is also markedly increased in the presence of high concentrations of the sugars except with the final fraction. Heart hexokinase however differs from brain hexokinase in its marked instability to organic solvents such as alcohol even in the presence of high glucose concentrations. The ox brain enzyme was unstable to alcohol and acetone in the absence of glucose but was completely stable in the presence of 1 M glucose.

The stability of the enzyme was also increased in the presence of TE as shown by rapid loss of activity on dialysis against buffer free from TE. The marked inhibition of the enzyme by compounds such as pCMB explains the stabilizing effect of TE.

The instability of the final purified enzyme appears to be due mainly to its high dilution. The addition of serum albumin completely stabilized the enzyme. In the absence of serum albumin it could not be stored even for a few days without very rapid loss of activity, whereas in the presence of serum albumin the purified enzyme was stable for several months at -20° and at least for a day or two at 0°. It is improbable that the instability of the enzyme is due to the presence of traces of elastase in the final purified enzyme. This was shown by the fact that treatment with DFP has no effect on enzyme activity or on its stability. The fluoro-phosphate should inhibit traces of elastase if present in the enzyme, but there was no significant change in the stability of the purified enzyme by this treatment.

Ultraviolet absorption

The ultraviolet absorption spectrum of the enzyme is that of a typical protein. However there is considerable divergence in the data on the ultraviolet absorption of the purified hexokinase. The ratio of absorption at 280 mμ to 260 mμ varies from about 1.8 for brain hexokinase (Joshi and Jagannathan, 1968) to only about 1.1 in the present work. The optical density of a solution of 1 mg of purified brain hexokinase in 1 ml of buffer was approximately 1.08 for the preparation of Joshi and Jagannathan whereas in the case of the enzyme obtained from ox brain by Schwartz and Basford it

was nearly 0.5. These divergences are possibly due to impurities in the final preparations or to the presence of a fragment rich in aromatic amino acids in the preparation of Joshi and Jagannathan which is removed during the isolation of the enzyme than according to Schwartz and Basford. The latter enzyme is also a larger molecule as shown by its larger sedimentation constant compared to that of the enzyme prepared by Schwartz and Basford. Further elucidation of the reason for these discrepancies is required.

Molecular weight

It was unfortunately not possible to get a sufficient quantity of purified enzyme free from serum albumin for ultracentrifugal studies. The molecular weight of the enzyme as determined by Sephadex G-200 filtration gave an approximate molecular weight of 90,000. This is similar to the molecular weight of yeast hexokinase.

K i n e t i c s

Substrate specificity

The metal, carbohydrate and nucleotide specificity of purified heart hexokinase is similar to that of brain hexokinase and several other animal tissue hexokinases. The enzyme is active with Mg^{2+} or Mn^{2+} and inactive with other divalent metal ions which were tested.

Nucleotide

The enzyme was also specific for ATP and there was no activity with UTP, CTP, GTP and ADP. The enzyme phosphorylates glucose, fructose, mannose at relative rates of 1.0, 1.8 and 0.7 respectively. Higher activity with fructose than with glucose

than with mannose is characteristic of type I animal tissue hexokinases. The results presented in this section show the impurities present in several apparently pure carbohydrates and very rigorous purification will be required to establish the true carbohydrate specificity of purified hexokinases. This is especially important in the case of low activities which have been reported in the literature with some carbohydrates.

pH

The broad pH optimum between 5.8 to 8.0 of ox heart hexokinase is similar to that reported for brain hexokinase (Joshi and Jagannathan,1968; Crane and Sols, 1955). A lowering in activity at pH 8 or higher has been reported for some animal tissue isoenzymes, but confirmation of these results with purified preparations is desirable.

Temperature

The increase in activity of heart hexokinase between 20 and 30° is about 1.96. There is undoubtedly inactivation at higher temperature. The Q_{10} of about 2 is similar to that described by several workers for other hexokinases (Joshi and Jagannathan, 1968; Berger et al. 1946; England and Randle,1967; Bennet et al. 1962; Hernandez et al. 1963).

K_m Values

The K_m values for glucose and fructose were similar to those of other animal hexokinases (except glucokinase). The K_m value for fructose was markedly high. Hence the rate of fructose phosphorylation, though higher than that of glucose under conditions

of substrate saturation, is likely to be very low at physiological fructose concentrations. It has been suggested that the high K_m value for fructose is due to the fact that only fructofuranose which constitutes about 20 per cent of fructose, is acted upon by the enzyme. But the concentration of fructose required for half saturation is not 5 times higher than that of glucose but nearly 30 times.

The K_m value for Mg^{2+} is similar to that of other animal tissue hexokinases.

The data on the effect of varying ATP at different fixed concentrations and that of varying glucose concentration at different fixed ATP concentrations is of some interest with regard to the mechanism of action of purified heart hexokinase. The double reciprocal plots for one substrate at different fixed concentrations of the other substrate give nearly parallel lines in both cases. This appears to suggest a "ping-pong" mechanism. A similar mechanism has been suggested for brain hexokinase by Fromm and Zewe (1962a). It has, however, been pointed out by Alberty (cited in Fromm and Zewe,1962a) that the data also fit a symmetrical mechanism:

$$\text{ATP} + \text{E} \rightleftharpoons \text{EX} \rightleftharpoons \text{E}' + \text{ADP}$$

$$\text{Glucose} + \text{E}' \rightleftharpoons \text{EY} \rightleftharpoons \text{E} + \text{glucose-6-P}$$

This mechanism would give different rate constants but would not alter the form of the kinetic equations.

An ordered pathway for the formation of a ternary complex (Hames and Kochavi,1962; Noat et al. 1968) has been suggested for yeast hexokinase, glucose being added first and Mg-ATP later.

Fromm and coworkers postulate random addition of substrates. Cohn (1963) showed by NMR studies that Mn ATP binds with yeast hexokinase only when glucose is present. de la Fuente and Sols (1970) suggest that the interconversion of a ternary complex of the enzyme with substrates is the limiting step and that an abortive ternary complex is also formed. Some of the earlier results have been critically examined in a review by Cleland (1967). Much of the earlier work on the mechanism of action of hexokinases has been carried out without proper statistical analysis. A visual fit of the different compounds has generally been made in these studies and statistical analysis either by the least squares method or preferably by that of Wilkinson (1961) is essential for evaluating the data. In addition to the inherent limitations of the kinetic method in distinguishing between different mechanisms the possible errors in the estimations also need to be emphasized. Some of these have been pointed out by Cleland (1967). In addition the yeast zwischenferment used for these assays generally contains traces of hexokinase for which corrections should be made. It also contains a glucose-NADP reductase, which is markedly active at higher concentrations of glucose. The possible effect of high concentrations of yeast glucose-6-P dehydrogenase as well as serum albumin in binding glucose, ATP and Mg especially at low concentrations of these substrates requires study. The results are least accurate at very low concentrations of glucose and ATP. In view of these errors no attempt at statistical analysis of the kinetic data obtained in this work on the effect of ATP and glucose on enzyme activity has been made. The calculation of individual constants

such as K_a and K_b would also be of doubtful value, unless a much larger number of experiments at different concentrations of the two substrates are carried out with the precautions indicated above.

Inhibition

Inhibition of hexokinase by pCMB and iodoacetate shows that the enzyme requires -SH groups for its activity as in the case of other animal tissue hexokinases and in contrast to yeast hexokinases the -SH groups of which are relatively inaccessible.

The enzyme is not inhibited by NaF in the presence or absence of phosphate or by ammonium sulfate and KCl. High concentrations of some of the salts inhibit glucose-6-P dehydrogenase and unless sufficient amounts of the dehydrogenase are present erroneous results regarding the inhibition of hexokinase by these salts are likely to be obtained.

3-Phosphoglyceric acid and phosphoenol pyruvate do not have any effect on hexokinase activity. It would be desirable to study the effect of 2,3, diphosphoglyceric acid on hexokinase activity at different Mg concentrations in view of the reports on the effect of these compound on erythrocyte hexokinase.

Fructose 1,6-diphosphate is, however, inhibitory to heart hexokinase, especially at low concentrations of Mg^{2+}. Inhibition by FDP does not appear to be competitive with respect to Mg though it would be desirable to confirm these results over a wider range of Mg^{2+} and FDP concentrations and Mg:ATP ratios. Further work is needed to confirm whether the FDP inhibition is of physiological significance.

Inhibition by ADP was found to be non-competitive with respect to ATP. This may be compared to reports that ADP inhibition is competitive with respect to ATP for yeast hexokinase (Fromm and Zewe, 1962) for brain hexokinase (Crane and Sols, 1954) for ascites hexokinase (Uyeda and Racker,1965) and for rat heart hexokinase (England Randle,1967). In the case of brain hexokinase Fromm and Zewe reported that ADP inhibition is more complex in nature and depends partly on the Mg ADP ratio. Further work on different Mg ADP ratios is required to elucidate the effect of ATP and ADP on enzyme activity. Purified heart hexokinase was free from myokinase as shown by the fact that there was no activity when ATP was replaced by ADP. Toews (1966) reported that with rat skeletal muscle hexokinase glucose-6-P was non-competitive with respect to glucose as well as ATP. England and Randle (1967) found that glucose-6-P was a non-competitive inhibitor of particulate as well as soluble rat heart hexokinase with respect to glucose and a mixed inhibitor with respect to ATP. On the other hand Mayer, Mayfield and Haas (1966) reported that glucose-6-P was a non-competitive inhibitor with respect to either ATP or glucose in the case of the hexokinase of heart of other species. The present results show that glucose-6-P inhibition with purified ox heart hexokinase is non-competitive with respect to ATP.

The inhibition of heart hexokinase by glucose-6-P is also reversible by Pi. The results on the effect of Pi and glucose-6-P with heart hexokinase are similar to those with brain hexokinase

(Joshi and Jagannathan, 1968). Inhibition by glucose-6-P and reversal of this inhibition by Pi are undoubtedly of considerable physiological significance and the concentration of these two compounds would influence the rate of glucose metabolism. Wu (1965) suggested that the control of aerobic glycolysis with Ehrlich ascites cells was strongly influenced by Pi, hexokinase and phospho-fructokinase.

Earlier results with brain hexokinase showed no effect of insulin, bovine growth hormone, adrenalin etc. on brain hexokinase (Joshi and Jagannathan, 1968) either directly or in the presence of glucose-6-P with or without Pi. It was however suggested that these studies should be repeated with an insulin-responsive tissue since the absence of any effect with the bfain enzyme could conceivably be due to the fact that brain is probably relatively insensitive to insulin. The results with purified heart hexokinase essentially confirmed those obtained with brain hexokinase. The literature on the effect of pituitary hormones and insulin on hexokinase is extensive and controversial, but confined to a direct effect on the enzyme. Though a direct effect on the enzyme could not be demonstrated it was considered possible that an indirect effect of relieving glucose-6-P inhibition or in altering the effect of Pi and glucose-6-P may be dependent on the hormones. The present results make it probable that these hormones have no such effect in the case of heart hexokinase. It would, however, be desirable to repeat these studies especially in the case of cortico steroids at different Mg and ATP concentrations preferably at physiological levels in view of the extensive literature (already referred to in the Introduction)

on the effect of these compounds on hexokinase activity. It is, however, also probable that these hormones have an effect not on hexokinase activity but on hexokinase content of tissues.

5-Thio-D-glucose is an interesting new inhibitor of hexokinase. It acts on hexokinase at relatively low concentrations with a Ki value of 1.4 mM. This inhibition is competitive with respect to the carbohydrate (mannose).

CHAPTER VI

SUMMARY AND CONCLUSIONS

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The particulate hexokinase of ox heart prepared according to the procedure of Crane and Sols was solubilized with a yield of about 50 per cent by treatment with pancreatic elastase followed by repeated freezing and thawing. The solubilized enzyme remained in the supernatant fraction when centrifuged at 100,000 x g for 1 to 2 hours.

The soluble enzyme was purified 500-fold by treatment with salmine sulfate, fractionation with calcium phosphate gel and ammonium sulfate and chromatography on DEAE-cellulose and Sephadex G-200. The maximum specific activity obtained was about 58 μmoles of glucose phosphorylated per milligram of enzyme at 30°. The yield of enzyme was, however, only 10 per cent.

The addition of sucrose (0.5 to 1 M) in the presence of phosphate (0.1 to 0.2 M) and TE (0.005 to 0.01 M) was required to stabilize the enzyme when its specific activity was more than 10 units/mg. Lower concentrations of sucrose were relatively ineffective. The final purified enzyme was, however, very unstable at 0° or -20° and lost most of its activity within a few days. In the presence of 8 mg of serum albumin per ml the purified enzyme could be stored for several months at -20° without loss of activity.

The ultraviolet absorption of the purified enzyme was that of a typical protein.

Purified particulate hexokinase showed no mobility towards the anode at pH 8.5 or 7 in 5-7% acrylamide gel.

Soluble ox heart hexokinase showed the presence of two isoenzymes on acrylamide gel electrophoresis.

The molecular weight of the solubilized enzyme as determined by Sephadex G-200 chromatography was approximately 90,000.

The pH optimum of the enzyme was unaltered between 5.8 and 8.

The increase in activity of the enzyme between 20° and 30° was approximately 2. At higher temperatures there was inactivation of the enzyme.

Hexokinase was active with Mg^{2+} or Mn^{2+} but not with Ca^{2+} and Zn^{2+}.

The enzyme was active only with ATP and not with GTP, UTP, CTP or ADP.

The relative activities with glucose, fructose and mannose were 1, 1.8 and 0.7 respectively. There was no activity with L-sorbose, D-sorbitol, rhamnose, cellobiose, lactose or mannitol.

K_m for Mg^{2+} was 3 mM at 10 mM ATP.

K_m for mannose was 0.038 mM and for fructose 2 mM at 5 mM ATP.

K_m values for glucose were 0.052, 0.062, 0.062, 0.100 and 0.112 mM at 10, 5, 2, 1.2 and 0.8 mM ATP respectively.

K_m values for ATP were 0.6, 0.78, 0.96, 1.10, 1.12 mM at 15, 3, 1.5, 1 and 0.5 mM glucose respectively.

Double reciprocal plots of activity vs substrates at fixed concentrations suggested a possible "ping-pong" mechanism but further work will be needed to elucidate the mechanism.

The enzyme was inhibited by 5-thio-D-glucose competitively with respect to mannose. Ki for 5-thio-D-glucose was 1.4 mM.

The enzyme was inhibited by ADP non-competitively with respect to ATP at a Mg:ATP ratio of 2:1.

Glucose-6-P inhibited heart hexokinase and this inhibition was reversed partially by Pi. Ki values for glucose-6-P were 0.11, 0.16, 0.2 mM at 5, 10, 30 mM Pi respectively. Ki values for glucose-6-P at 1.5, 5 and 10 mM ATP were 0.22, 0.17 and 0.15 mM respectively.

Insulin, bovine growth hormone, adrenalin and prednisolone and serotonin had no effect on hexokinase activity either directly or in the presence of 0.15 mM glucose-6-P with or without 10 mM Pi.

NaF, ammonium sulfate, KCl, 3 phosphoglyceric acid, phosphoenol pyruvate and TE had no effect on enzyme activity. The activity and stability of the enzyme were not influenced by treatment with 1 mM DFP.

The enzyme was inhibited10 and 20% at 1 and 4 mM iodoacetate respectively. pCMB inhibited the enzyme 50% at 0.001 mM.