THE REGULATION OF HEXOKINASE

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Edward Elliston Farmer.

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TO MY PARENTS

Abstract

Hexokinase (E.C.2.7.1.1.) has been purified from pig heart by a novel procedure involving affinity-elution from an immobilised triazine dye. A similar novel procedure, also involving dye ligand chromatography, has been designed for the purification of yeast glucose 6-phosphate dehydrogenase (E.C.1.1.1.49) for use as acoupling enzyme in the hexokiase assay. The mechanism of dye-ligand chromatography of both enzymes is discussed. Also, hexokinase from the starfish Asterias rubens has been purified, for the first time, by conventional techniques.

The regulatory behaviour of both heart and Asterias hexokinases have been studied in the hope of gaining an understanding of the mechanism of regulation. Asterias hexokinase was of particular interest in this respect as, although its regulatory properties were very similar to those of the heart enzyme , it was reported to have a molecular weight of about 50,000 , roughly half that of the heart enzyme.

Data for the steady-state kinetics of heart hexokinase were consistent with those already obtained by other workers. Asterias hexokinase proved similar to heart hexokinase in its kinetics, being inhibited by glucose 6-phosphate ADP and AMP. Phosphate was a very weak inhibitor of heart hexokinase but inhibited Asterias hexokinase more strongly, the latter resembling hexokinase from skeletal muscle in this respect. Also,'double inhibitor' experiments have been undertaken allowing a study of the interrelationship of ligand binding sites. The use and limitations of double inhibitor kinetics in the study of hexokinase are discussed. The equations used in double inhibitor kinetics are also useful in predicting the activity of enzymes in vivo when metabolite levels are known; the activity of hexokinase in heart is calculated by this means and the result discussed in terms of the

physiological role of heart hexokinase.

Comparison of the amino acid compositions of heart and Asterias hexokinases shows them to be very similar. This finding is discussed in terms of the evolution of the hexokinases.

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Finally, my greatest debt is to Dr J.S.Easterby for the time and effort he has spent advising, disciplining and inspiring me during the 3 years of my research training.

Abbreviations

$A_{280\ nm}$	absorbance at 280 nm
AMP	adenosine 5' monophosphate
ADP	adenosine 5' diphosphate
ATP	adenosine 5' triphosphate
DEAE	diethylaminoethyl-
E.C.	Enzyme Commission
EDTA	ethylenediaminetetra-acetic acid
F6P	fructose 6-phosphate
$F16P_2$	fructose 1,6-bisphosphate
G6P	glucose 6-phosphate
$G16P_2$	glucose 1,6-bisphosphate
G6PDH	glucose 6-phosphate dehydrogenase (E.C.1.1.1.49)
H-8BN	Procion Red H-8BN, a monochlorotriazine dye
H-8BN-OH	Procion Red H-8BN from which the reactive chlorine atom has been removed by hydrolysis
M6P	mannose 6-phosphate
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
PAGE	polyacrylamide gel electrophoresis
PEG 6000	polyethylene glycol of average molecular weight 6000
Pi	inorganic phosphate
pI	isoelectric point
SDS	sodium dodecylsulphate
TEMED	N N N'N'-tetramethylethylene diamine
Tris	tris (hydroxymethyl)aminomethane

Contents

CHAPTER 1

GENERAL INTRODUCTION

1.1 PRELUDE

Now that most of the major metabolic pathways have been classified and the principles of intermediary metabolism established, attention has turned to the understanding of flux through these pathways. Some factors affecting the flow of metabolites through a pathway are the availability and removal of substrates, cofactors and products respectively, the kinetic behaviour of individual enzymes , interaction between various enzymes and/or their environments in the cell, the temporal response of the pathway to changing flux and the effect of other pathways on the one in question.

Thus the regulation of metabolism is a potentially very complex subject and in order to simplify its study it is often necessary to work on purified enzymes. The regulation of individual enzymes can often then be related to the regulation of the pathway as a whole in the context of its function in a given tissue. Study of the regulation of purified enzymes is a necessary and rewarding way of gaining information on the regulation of metabolism, but suffers those philosophical disadvantages inherent in the study of any physical entity in isolation.

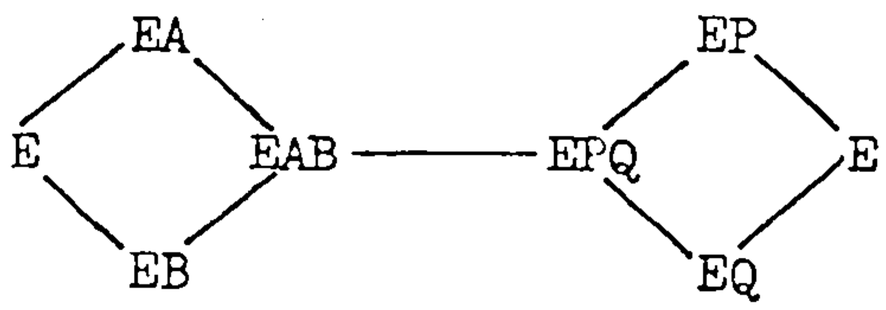
SECTION 1.2 THE REACTION CATALYSED BY HEXOKINASE

Hexokinase (ATP: D-hexose 6-phosphate transferase, E.C.2.7.1.1) catalyses the phosphorylation of hexose by adenosine triphosphate ;

$$\text{hexose} + ATP^{4-} \xrightarrow{Mg^{2+}} \text{hexose 6-phosphate} + ADP^{3-} + H^{+}$$

Magnesium is necessary for the reaction and it is thought to form a complex with the β and γ phosphates of ATP (1) thus reducing electron density at the terminal phosphate making it more susceptible to nucleophilic attack. The hexokinase reaction strongly favours the phosphorylation of hexose ; equilibrium constants for the reaction catalysed by yeast hexokinase at 30°C were 1,550 and 15,550 at pH 7 and pH 8 respectively (2) .

Use of a number of different approaches contributed to the elucidation of the mechanism of hexokinase and amongst the most important experiments were the product inhibition studies of Fromm and Zewe (3), Rose et al (4) used isotope trapping to show the importance of the ternary complex hexokinase-glucose-ATP and soon afterwards Danenberg and Cleland (5) using chromium-adenosine triphosphate as a dead end inhibitor provided evidence for a sequential, rapid equilibrium random mechanism which was consistent with the results of many other workers and other studies on the reverse reaction of hexokinase;

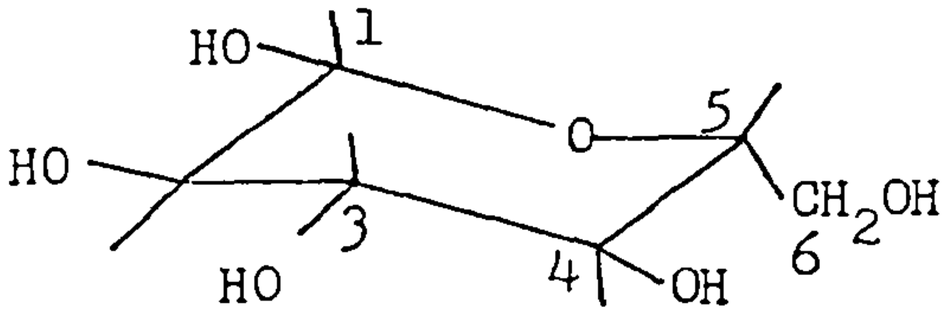


E represents hexokinase and A,B,P and Q represent glucose, ATP, glucose 6-phosphate andADP respectively. The route prefered is probably glucose binding first although this will depend on the level of both substrates. In glucokinase (E.C.2.7.1.2) the route prefered is ;

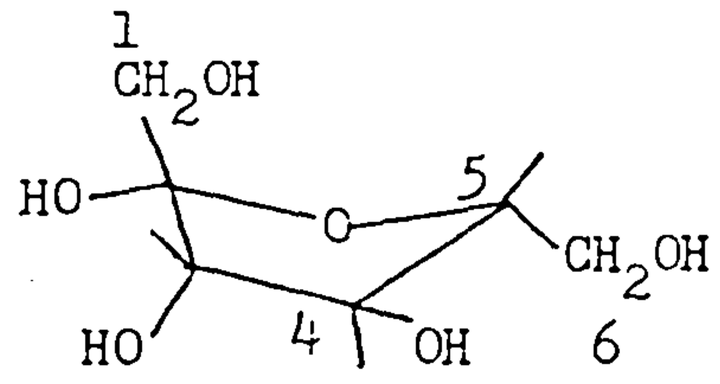
E ——EA——EAB——EPQ——EP——E

although there can be random addition of substrates and random loss of products (6).

Generally hexopyranoses and fructofuranose are phosphorylated in the 6 position. For hexokinase, carbon atoms 1,3,4 and 6 of glucose and 2,3, 4 and 6 of fructose impart specificity into the interaction of hexokinase with its hexose substrate (7)

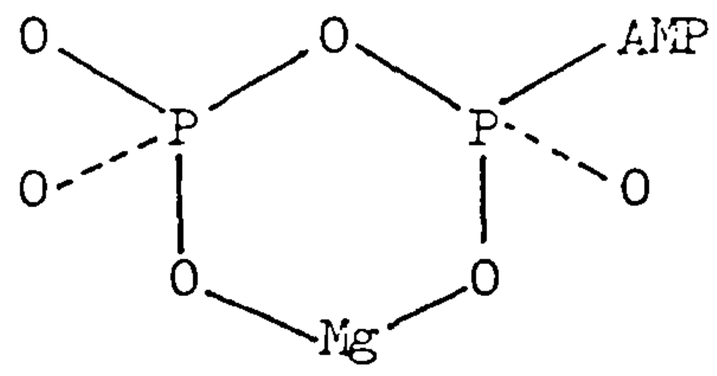


β-D-glucopyranose



β-D-fructofuranose

The reaction involves phosphoryl transfer rather than phosphate transfer (8) and recently Darby and Trayer (9) using phosphorothionate analogues of ATP showed that the bidentate complex $MgATP^{2-}$ in the Λ-screw configuration ;



was the true substrate for glucokinase. The stereochemical course of the yeast hexokinase reaction has recently been elucidated using adenosine 5'[γ(S)-^{16}O,^{17}O,^{18}O] triphosphate as a substrate (10). An inversion of configuration of the chiral phosphoryl group occurred during phosphorylation.

SECTION 1.3 THE OCCURRENCE AND PROPERTIES OF HEXOKINASE

Hexokinase has been identified in a large range of life forms although it has been purified from relatively few. Some microorganisms have been found to contain hexokinase (11) but in many bacteria hexose is phosphorylated as it is transported into the cell by the phosphoenol-pyruvate-dependent sugar transport system (12), the biochemistry of which is totally different to any hexokinase. Hexokinase has been identified in Protozoa (13) but not purified from this source. Hexokinase from the roundworm Ascaris was shown to have a molecular weight of 100,000 and was inhibited by ADP and glucose 6-phosphate (14). An enzyme of roughly properties has been purified from the tapeworm Hymenolepis (15).

Two forms of hexokinase have been identified in lobster (16), both having molecular weights of about 50,000. Lobster hexokinase form II was strongly inhibited by both ADP and glucose 6-phosphate and glucose was the preferred hexose substrate. In contrast lobster hexokinase form I had a preference for mannose and fructose over glucose and was not inhibited by physiological levels of ADP or glucose 6-phosphate.

Trout liver was found to contain two forms of hexokinase (17) one of which showed substrate inhibition by glucose and could use a wide range of nucleotides including ADP as a phosphoryl donor; this form was strongly inhibited by AMP.

Recently a hexokinase has been purified from the starfish Asterias (18) it had a molecular weight of 50,000 , showed linear substrate kinetics and was strongly inhibited by glucose 6-phosphate.

Much of the information on the hexokinase reaction has come from the study of yeast hexokinase where the native enzyme exists in two isoenzyme forms 'A' and 'B' which differ in isoelectric point and in that form B has a 3-fold greater maximum velocity than form A (19). Yeast hexokinase has a subunit molecular weight of about 54,000 and under most conditions exists as a dimer of molecular weight 108,000 (20). Yeast hexokinase has been crystallised and the dimer found to bind 3 ATP molecules, one at each active site and one at an intersubunit site, presumed to be an allosteric site (21). However studies of ligand binding to the enzyme in free solution (22) reveal only two ATP binding sites in the dimer. Yeast hexokinase is not strongly inhibited by the reaction product glucose 6-phosphate.

Hexokinase does not have a central importance in the metabolism of plants but has beenidentified in a number of plant tissues. Two hexokinase isoenzymes of about 50,000 molecular weight (and smaller quantities of two hexokinases of 100,000 molecular weight) have been purified from wheatgerm (23). Neither form is potently inhibited by glucose 6-phosphate but one form shows mnemonical kinetics (24), the other form has a higher affinity for fructose than glucose (25).

The mammalian hexokinases

Much of the information in the following paragraph is from the reviews of Purich, Fromm and Rudolph (26) and Walker (27). Four mammalian hexokinase isoenzymes, types I to IV, have been classified on the basis of increasing electrophoretic mobility, type IV migrating the fastest (28). Types I,II and III all exhibit a low Km for glucose and a fairly broad substrate specificity and are given the Enzyme Commission number E.C.2.7.-1.1. Types I,II and III hexokinase have molecular weights of approximately 100,000 (28) and are potently inhibited by glucose 6-phosphate.

In contrast to types I and II hexokinase, which show linear substrate kinetics, type III hexokinase is inhibited by high levels of its substrate glucose. Of the mammalian hexokinase isoenzymes, type III is the least studied.

In contrast to the 'low Km' mammalian hexokinases type IV hexokinase (E.C.2.7.1.2) exhibits a high Km for glucose and is highly specific for this substrate hence its trivial name 'glucokinase'. Glucokinase is cooperatively activated by glucose and is an example of a mnemonical enzyme as is wheatgerm L_1 hexokinase (24). After purification, types I,II and IV hexokinase appear monomeric, however the type I enzyme undergoes glucose 6-phosphate enhanced dimerisation (29). Types I and II hexokinase have specific activities of around 80 units / mg (higher specific activities have been observed) , glucokinase can be purified to a specific activity of 150 units / mg.

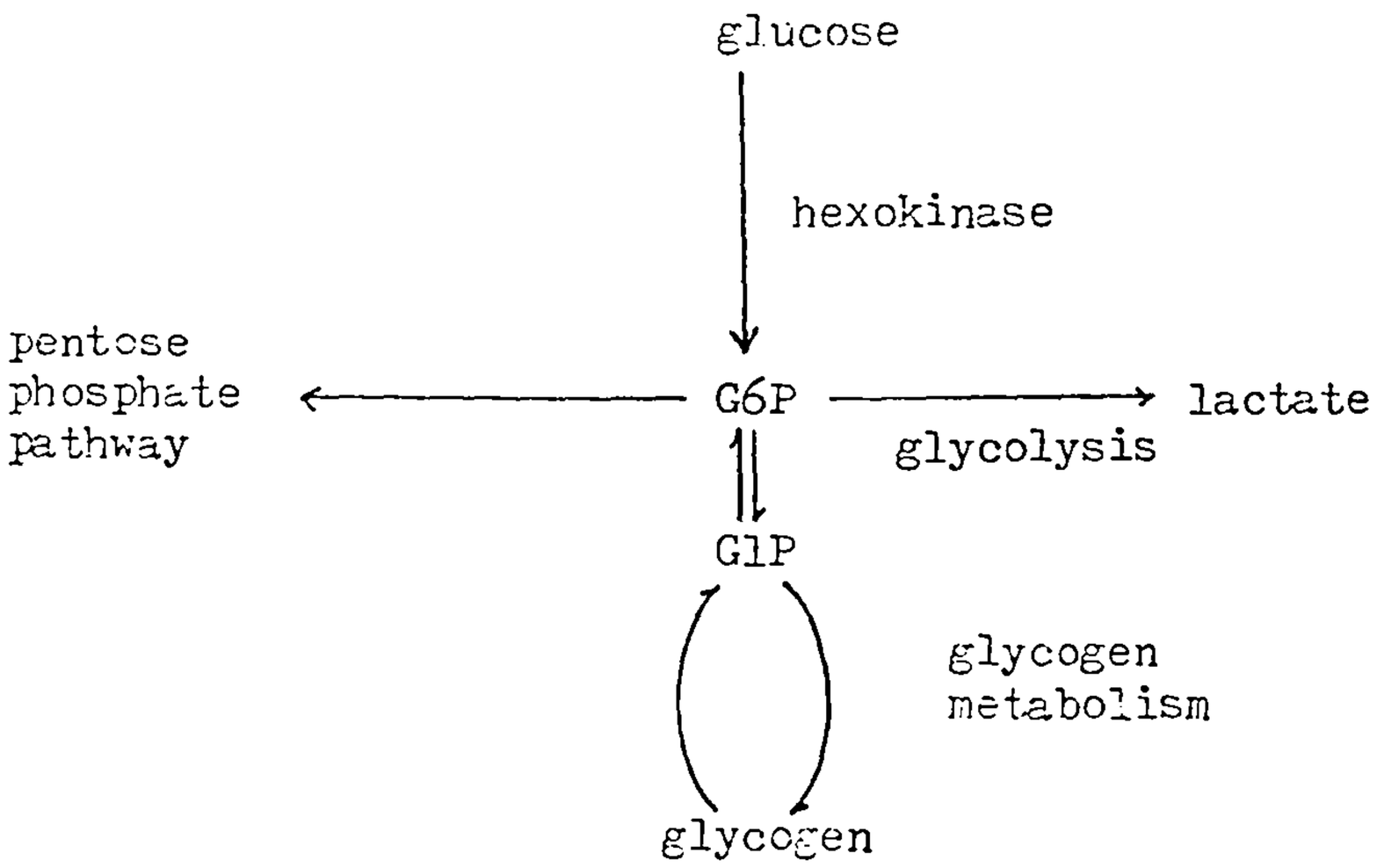
Apart from glucokinase, liver also contains a fructokinase (E.C.2.7.1.3) which phophorylates fructose at the 1 position and unlike the hexokinase isoenzymes has an absolute requirement for K^+ ions (30). Liver additionally contains a galactokinase (E.C.2.7.1.6.). Like glucokinase, galactokinase is an adaptive enzyme. Unlike glucokinase, galactokinase is inhibited by an excess of its substrate, galactose (31).

SECTION 1.4 THE ROLE OF HEXOKINASE IN THE CARBOHYDRATE METABOLISM OF MAMMALS.

Soon after the classification of the mammalian isoenzymes it became apparent that each form was associated with a particular class of tissue. The isoenzyme types differed in regulatory properties, each type playing a different role in carbohydrate metabolism. Glucose 6-phosphate is a potential substrate for glycolysis, glycogenesis and the pentose phosphate pathway and can be formed through gluconeogenesis or glycogenolysis as shown in Figure 1.1 .

Figure 1.1

A highly simplified scheme showing the central role of glucose 6-phosphate in mammalian carbohydrate metabolism.



G6P = glucose 6-phosphate

G1P = glucose 1-phosphate

Type 1 hexokinase

Type I hexokinase occurs in such tissues as brain, heart and the erythrocyte all of which depend wholly or largely on glucose as a substrate for glycolysis. Brain, which is absolutely dependent on glucose as a fuel, contains relatively high levels of hexokinase. Lowry and Passonneau (32) established that hexokinase was a major control point in glycolysis, working in concert with the enzyme phosphofructokinase. The glucose 6-phosphate inhibition of brain hexokinase is reversed by phosphate (33) a finding that is consistent with its role in the brain viz. high phosphate levels would signal a need for an increase in the rate of glycolysis which might otherwise be inhibited by glucose 6-phosphate. Like heart hexokinase the brain enzyme is associated with the outer mitochondrial membrane and the reasons for this association are not fully established. However, Knull et al. (34) observed that by making chicks hypoglycaemic, the level of hexokinase in the particulate fraction of brain increased and that this correlated with a decrease in the levels of several glycolytic intermediates including glucose 6-phosphate. The linkage of ATP production by mitochondrial oxidative phosphorylation to glycolysis could be a reason for the association of hexokinase with these organelles (35). Heart is not solely reliant upon glucose as a fuel and can metabolise a variety of substrates including fatty acids. Glycolysis in the heart is known to be controlled by glucose transport, hexokinase, phosphofructokinase and to a lesser extent the phosphorylase system (36). Additionally, the β-oxidation of fatty acids depresses glycolysis, most probably because a build-up of citrate inhibits phosphofructokinase thus allowing a build-up of hexose-phosphate and concomitant inhibition of hexokinase (36).

Type II hexokinase

Type II hexokinase is associated with insulin-sensitive tissues like skeletal muscle where it is present as a soluble enzyme in low levels. It is inhibited by phosphate and would probably be highly inhibited during muscular spasm when phosphate levels are high. Additionally, type II hexokinase is potently inhibited by glucose 1,6-bisphosphate (37). Type II hexokinase is probably involved in glycogen synthesis.

Glucokinase

Unlike the low Km hexokinases which which are probably saturated with glucose throughout its physiological range, glucokinase is never fully saturated with glucose and is cooperatively activated by this substrate explaining why the liver does not synthesize glycogen from blood glucose unless the concentration of the latter is well above its fasting level (38).

SECTION 1.5 THE REGULATION OF HEXOKINASE

The exact nature of the glucose 6-phosphate inhibition of hexokinase has long been a source of controversy which is discussed in detail by Solheim and Fromm (39). The arguement centres on whether or not the glucose 6-phosphate binding site overlaps the active site ie. whether glucose 6-phosphate displaces ATP by direct competition for the same binding site or by an allosteric effect. The profound effects of glucose 6-phosphate on the conformation of type I hexokinase and its strong inhibition of the enzyme rule out the possibility that glucose 6-phosphate is acting as anormal end product inhibitor as it appears to do in yeast hexokinase. Glucose 6-phosphate inhibits hexokinase competitively with respect to ATP and noncompetitively with respect to glucose. Also, the glucose 6-phosphate enhanced dimerisation of type I hexokinase can take place in the presence of 0.1 M glucose (29). Cassaza and Fromm (40) proposed that a shift in the position of the glucose moiety after phosphorylation was responsible for the

lack of competition between glucose and glucose 6-phosphate and that the glucose 6-phosphate site overlaps the active site. Recently Solheim and Fromm (39), studying the back reaction of hexokinase from bovine brain found glucose 6-phosphate to exhibit Michaelis-Menten kinetics as a substrate this being consistent with the view that G6P exerted its inhibitory effects on the forward reaction by binding at the active site. However, levels of glucose 6-phosphate over 60 μM inhibited the back reaction of hexokinase which suggests the existence of another binding site for the ligand.

If there were two glucose 6-phosphate binding sites on the low Km mammalian hexokinases, one a high affinity regulatory site and one a low affinity product binding site, then it is possible that the great difference in affinity between the two sites would make the low affinity site difficult to distinguish in kinetic or ligand-binding studies. A recently published ligand-binding study (41) identified two glucose 6-phosphate sites for rat brain hexokinase but has been strongly criticised on experimental grounds (39).

Hexose phosphates other than glucose 6-phosphate inhibit the low Km hexokinases ; mannose 6-phosphate is a poor inhibitor of type I hexokinase compared to glucose 6-phosphate although the free sugars are equally good substrates (42), facts that are consistent with the existence of a regulatory site for hexose phosphate whether or not it overlaps the active site. Glucose 1,6-bisphosphate inhibits both type I and type II hexokinase , inhibition of the type II isoenzyme is very potent and highly pH dependent (43).

Unlike brain hexokinase, the heart enzyme is little affected by phosphate at concentrations less than 10 mM whereas the glucose 6-phosphate inhibition of brain hexokinase is reversed by phosphate ; thus although

pig heart and pig brain hexokinases are both type I isoenzymes the difference in response to phosphate means that they can be regarded as different isoenzymes of type I hexokinase (44). Additionally, the low Km hexokinases are inhibited by ADP and AMP and part of the present thesis was devoted to a study of the effects of these inhibitors as well as hexose phosphates on the hexokinase reaction.

SECTION 1.6 THE EVOLUTION OF HEXOKINASE

From comparison of the regulatory properties of the low Km mammalian hexokinases with those of yeast hexokinase a theory for the evolution of the former was proposed (45,46) whereby the high molecular weight hexokinases evolved from a 50,000 molecular weight ancestor by partial gene duplication and fusion, the resulting enzyme having two active sites one of which was subsequently sacrificed for a regulatory site. The great similarity in the amino acid compositions of the low Km mammalian hexokinases and glucokinase has been taken to be consistent with the partial gene duplication theory (47). Recently however, a 50,000 molecular weight hexokinase that was strongly inhibited by low levels of glucose 6-phosphate was purified from starfish (18), a result that would not be predicted in the light of the above discussion.

Addendum

An addendum giving further explanation of the theory of partial gene duplication in the evolution of hexokinase is to be found at the back of this thesis. Additionally, it gives details of dye screening studies carried out on hexokinases from Asterias , rat skeletal muscle and pig heart.

CHAPTER 2

GENERAL MATERIALS AND METHODS

MATERIALS & METHODS

SECTION 2.1 REAGENTS

General reagents

Tris was purchased from Hopkin & Williams (Essex,UK). Potassium dihydrogen phosphate, ammonium sulphate, ammonium persulphate, magnesium chloride hexahydrate, hydrochloric acid, disodium EDTA phenol and glucose were all of analytical reagent grade and were purchased from either Fisons (Loughborough,UK) or British Drug Houses (Poole,UK). All other general reagents were from either Fisons or BDH and were of laboratory grade.

Electrophoresis

SDS 'specially pure' was from Fisons. Acrylamide 'specially pure for electrophoresis' was purchased from BDH. Nitro blue tetrazolium, phenazine methosulphate, Coomassie brilliant blue R and cyanogen bromide were purchased from Sigma (Poole,UK).

Chromatography

DEAE-cellulose DE-52, CM-cellulose CM-52 and phosphocellulose P-11 were supplied by Whatman (Maidstone,UK). DEAE-Sephadex A-50, Sephadex G-200 and Sepharose 6B were supplied by Pharmacia (Uppsala, Sweden). AcA 34 and AcA 44 were from LKB (France). Triazine dyes, a product of ICI, were a kind gift of Dr.P.D.G.Dean of this department.

Enzyme assays

Nucleotides were purchased from PL Biochemicals (Milwaukee,USA). Glucose used in assays was of analytical grade and was supplied by Fisons. Bakers' yeast glucose 6-phosphate dehydrogenase (E.C.-1.1.1.49) was either purified by the method desribed in this thesis (chapter 5) or the 'grade II' enzyme of Boehringer & Sons (Mannheim, FRG) was purchased.

Dialysis & concentration

Dialysis tubing (Visking tubing) was supplied by the Scientific Instrument Centre (London,UK). Minicon concentrators were from Amicon (Mass., USA).

SECTION 2.2 IONIC STRENGTH & BUFFERS

The ionic strength of a solution is defined as ;

$$I = \tfrac{1}{2} \Sigma M. z^2 \qquad (2.2.1)$$

the summation is for all ionic species in solution with molarities, M and charge, z. Debye and Hückel defined the activity, γ of an electrolyte as ;

$$\log_{10} \gamma = - \frac{A z^2 I^{\frac{1}{2}}}{1 + B I^{\frac{1}{2}}} \qquad (2.2.2)$$

where A depends on temperature, dielectric constant etc. and is equivalent to 0.5092 for an electrolyte in water at 25°C. B depends on ionic size and is close to 1.6 . Thus for the calculation of compositions for monoprotic buffers an equation relating activity and the Henderson-Hasselbach equation can be derived (48) ;

$$pH = pK + \log_{10} \frac{[A^-]}{[HA]} + \frac{0.5092 (2z-1) I^{\frac{1}{2}}}{1 + 1.6 I^{\frac{1}{2}}} \qquad (2.2.3)$$

where pK is the pK_a at infinite dilution, [HA] and $[A^-]$ are the concentrations of the acid and conjugate base respectively and z is the charge on the acid.

SECTION 2.3 ENZYME ASSAYS

Hexokinase assay

The routine assay was based on the procedure of Slein et al. (49). Each assay contained 50 µmol Tris- HCl pH 7.6 , 40 µmol glucose, 24 µmol Mg^{2+} , 10 µmol ATP, 100 nmol NADP and 0.2 unit of yeast glucose 6-phosphate dehydrogenase in 1.0 ml. The reaction was monitored

at 340 nm and 1 unit of hexokinase defined as the amount that catalyses the phosphorylation of 1 µmol glucose per min. at 30 °C. The molar extinction coefficient of NADPH was taken to be 6,220 (50).

Glucose 6-phosphate dehydrogenase assay

Each assay contained 50 µmol Tris-HCl pH 7.6 , 24 µmol Mg^{2+}, 1.2 µmol glucose 6-phosphate and 0.37 µmol NADP in a total volume of 1 ml. 1 unit of glucose 6-phosphate dehydrogenase catalyses the reduction of 1 µmol glucose 6-phosphate per min. at 30°C as monitored by the increase in absorption at 340 nm. Glucose 6-phosphate dehydrogenase is inhibited by sulphate ions thus all samples from ammonium sulphate precipitation were dialysed against assay buffer before assay.

Diaphorase assay

The method of Massey (51) was used. Each assay contained 50 µmol potassium ferricyanide and 200 µmol NADH in 1.0 ml of 150 mM sodium acetate buffer pH 4.8. Reduction of ferricyanide was measured at 420 nm. 1 unit of diaphorase corresponds to the reduction of 1 µmol of ferricyanide per min. at 30°C which is equivalent to an absorbance change of 1.0 per min.

SECTION 2.4 PROTEIN ESTIMATION

Routine measurement of protein in the absence of added NADP or ATP was performed by the method of Warburg and Christian (52) where :

$$\text{Protein (mg/ml)} = 1.55\ A_{280nm} - 0.76\ A_{260nm}$$

In the presence of exogenous nucleotide or where accurate specific activity measurements were necessary the microtannin method of Mejbaum-Katzenellenbogen and Dobryszycka (53) was used. The following solutions were prepared ;

Tannic acid : 2 ml of warmed phenol and 10 g tannic acid were dissolved in a solution of 2 M hydrochloric acid (98 ml) warmed to 80°C.

Gum arabic : 0.1 g gum arabic (acacia) was dissolved in 100ml warm

water.

Both reagents were filtered before use. Protein to be assayed was diluted in 0.9 % sodium chloride solution. 0.5 ml prewarmed tannin reagent was added to 0.5 ml of protein solution and the mixture incubated with occasional shaking in a water-bath at 30°C ; after 10 min 1 ml of the gum arabic soltion was added , the mixture quickly shaken and cooled under the tap. After standing at room temperature for 10 min the absorbance of the mixture at 650 nm was read. A calibaation curve from samples containing 0 to 50 µg bovine serum albumin was made. The absorbance of the calibration samples was stable for at least 12 hr but if left this long samples required remixing as some precipitation occurred. Polyethylene glycol and high levels of phosphate interfere with the microtannin assay.

SECTION 2.5 PROTEIN PRECIPITATION TECHNIQUES

Polyethylene glycol precipitation

Polyethylene glycol 6000 (PEG 6000) was used as a protein precipitant. Since PEG 6000 dissolves slowly and localised high concentrations of this substance might result from addition of the solid to a solution of protein, the polyethylene glycol was first dissolved in water. For routine precipitation a 30 % (by weight) solution of PEG 6000 was added to protein mixtures and details for making PEG 6000 solutions of known % composition are given in the appandix to this thesis.

The volume n (ml) of 30 % PEG 6000 required to give a final concentration of x % PEG is $30\,n / V_2$. As the final volume, V_2 (ml), of the protein solution equals n plus the initial volume V_1 ;

$$n = \frac{V_1}{(30 / x) - 1}$$

The PEG solution was slowly added to the protein solution over about 30 min with continuous stirring. The mixture was then allowed to stand for a further 30 min. PEG contains some acidic impurities and it is often necessary to re-adjust the pH with dilute KOH.

Ammonium sulphate fractionation

Solid ammonium sulphate was added into stirring solutions of protein over about 30 min and the resultant precipitate collected by centrifugation . The amount of ammonium sulphate required to make a solution of given % was read off a published nomogram (54).

SECTION 2.6 ION EXCHANGE CHROMATOGRAPHY

All ion echange resins were recycled and equilibrated according to manufacturers instructions. Equilibrated resins were poured into glass columns plugged with cotton-wool. Incoming buffer was applied at the top of the column through a pierced rubber bung and a layer of buffer of at least 2 cm depth was maintained over the resin to allow mixing of incoming salt gradients. Linear gradients were formed by gradual mixing of the contents of two reservoirs containing equilibration buffer with and without salt respectively. The gradient was drawn from the reservoir which initially contained no salt. To avoid uneven mixing of the gradient a small plug of glass wool was fitted into the bridge connecting the two reservoirs.

Cellulose based ion exchange resins could tolerate a high flow-rate (up to 100 ml/hr) but DEAE-Sephadex columns were run at no more than 30 ml/hr. At initially low ionic strengths, increasing salt concentration causes shrinkage of DEAE-Sephadex; this effect was minimised by equilibrating the resin in 0.1 I buffer. At this ionic strength subsequent addition of salt causes little further shrinkage.

SECTION 2.7 DYE-LIGAND CHROMATOGRAPHY

Synthesis of H-8BN-Sepharose 6B

A procedure similar to that recommended by Dean and Watson (55) was followed. Typical preparations contained a slurry of Sepharose 6B (1 part Sepharose to 2 parts water) to which was added sodium carbonate(20 mg/ml of Sepharose), sodium chloride (100 mg/ml of Sepharose and Procion Red H-8BN (7 mg/ml of Sepharose.) The mixture was either shaken for 40 hr at 45°C or for 3 days at 30°C. Unbound dye and salts were washed from the Sepharose with several volumes of 4 M urea followed by several volumes of 2 M sodium chloride. Before use the Sepharose was washed with at least 30 column volumes of equilibration buffer.

Determination of dye concentration

The amount of substitution of Sepharose with dye was determined after hydrolysis in 50 % acetic acid, using an $E^{1\%}_{550nm}$ of 315 as determined by Qadri (56).

Hydrolysis of H-8BN

The reactive chlorine atom of H-8BN was removed by hydrolysis as described by Moe and Piskiewicz (57). The hydrolysed dye was not purified before use.

Chromatography

H-8BN-Sepharose was poured into glass columns plugged with cotton-wool. The column was equilibrated with a large volume of equilibration buffer (approx 30 column volumes) and protein solutions applied at flow-rates of 20 to 60 ml/hr depending on column size. Before affinity elution unbound protein was washed from the column. After use dye columns were washed with 5 column volumes 4 M urea followed by the same amount of 2 M NaCl. Columns were stored in 0.02 % sodium azide solution at 4°C.

SECTION 2.8 GEL FILTRATION

Before pouring, gels were thoroughly degassed under a vacuum of approximately one atmoshere then introduced slowly into columns as slurries of about 1 part gel to about 4 parts buffer. Once poured gel filtration columns were stored at $4^{o}C$ in the presence of 0.02 % sodium azide. Before application protein samples were centrifuged for about 3 min at 10,000 g in a microcentrifuge, this removed particulate matter which would otherwise clog filters in the column or impair the performance of the gel.

SECTION 2.9 DIALYSIS & CONCENTRATION

Prior to use, dialysis tubing was soaked for 1 hour in 1 mM EDTA to remove heavy metal contaminants. Protein solutions were usually concentrated by vacuum dialysis in $\frac{1}{4}$" dialysis tubing. The tubing was fitted through a pierced rubber bung to a glass reservoir. This was inserted in a Buchner funnel which was then evacuated to a vacuum of about 1 atmosphere. Smaller volumes were concentrated in Minicon concentrators.

SECTION 2.10 POLYACRYLAMIDE GEL ELECTROPHORESIS

The method used was that of Davis (58).

The following solutions were made ;

Solution A : 36.3 g Tris, 48 ml 1 M HCl , 0.46 ml TEMED , 0.296 g disodium EDTA and 0.26 ml β-mercaptoethanol in 100 ml water

Solution B : 40 g acrylamide and 1.06 g bis acrylamide in 100 ml water, filtered and stored in the dark at $4^{o}C$.

Solution C : 0.14 g ammonium persulphate per 100 ml water, freshly made.

Electrode buffer : 28.8 g glycine and 60 g Tris per 1 of water.

Solutions A, B and water for either 7.5 % or 10 % gels were mixed

in the proportions listed in the table below ;

Gel composition :	7.5 %	10 %
solution A	1	1
solution B	1.5	2
water	1.5	1
solution C	4	4

The mixtures were degassed and an equal volume of solution C added. Gels were poured into stoppered electrophoresis tubes (7.5 x 0.5 cm) to a depth of approximately 6.5 cm. A few drops of distilled water were immediately layered onto the gel surface to flatten its meniscus. After setting (usually 40 min) gels were pre-electrophoresed for 30 min to remove persulphate ions.

40 to 200 µg protein was loaded onto each gel in a maximum volume of 100 µl. Protein solutions contained 10 µl of 0.01 % bromophenol blue and 20 to 50 µl glycerol. Electrophoresis was carried out at 3 mA per tube. The position of the bromophenol blue after electrophoresis was recorded.

Staining for protein

Gels were stained for 20 min at room temperature in solution of 1 % naphthalene black in 7 % acetic acid. Destaining was in 7 % acetic acid and was accelerated by warming to 60°C.

Staining for enzyme activity

Hexokinase activity on gels was detected by the method of Katzen et al (59). The stain contained 0.5 mM NADP, 10 mM $MgCl_2$, 5 mM ATP , 0.1 M glucose , 0.4 unit /ml yeast glucose 6-phosphate dehydrogenase , 400 µg /ml nitro blue tetrazolium and 40 µg/ml phenazine methosulphate in 0.1 I Tris-HCl pH 7.6 .

Glucose 6-phosphate dehydrogenase activity on gels was detected with

a stain of the same composition except omitting ATP, glucose and glucose 6-phosphate dehydrogenase. For both enzymes, staining was carried out in the dark at room temperature. Gels could be destained in a solution containing 5 % methanol and 7.5 % acetic acid.

SECTION 2.11 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

The method of Weber and Osborn (60) was used.

Preparation of gels ; the following solutions were made;

gel buffer , 0.1 M sodium phosphate pH 7.2, containing 7.8 g NaH_2PO_4. $2H_2O$, 38.6 g $Na_2HPO_4.7H_2O$ and 2 g sodium dodecylsulphate per litre.

acrylamide solution, 22.2 g acrylamide and 0.6 g N,N'-methylene bis-acrylamide in 100 ml water, filtered and stored in the dark at 4^oC.

1 % ammonium persulphate solution , freshly made.

Electrode buffer was gel buffer diluted 2-fold with water.

For 10 % polyacrylamide gels, 10 ml gel buffer was mixed with 9 ml of acrylamide solution and degassed. 1 ml ammonium persulphate solution and 30 µl TEMED were added to the degassed mixture which was then poured into stoppered glass tubes (7.5 x 0.5 cm). A small amount of water was layered onto the gel surface. After polymerisation gels were pre-electrophoresed to remove persulphate ions. 7.5 % polyacrylamide gels could also be made in this way using an appropriate dilution of acrylamide.

Preparation of protein samples

Protein solutions were adjusted to a concentration of 1 to 2 mg / ml in 0.1 M sodium phosphate buffer pH 7.2 containing 1 % sodium dodecylsulphate and 1 % β-mercaptoethanol. Samples were heated at 100^oC for 5 min then dialysed overnight at room temperature against the same buffer containing 1 % sodium dodecylsulphate and 1 % β-mercaptoethanol. 10 µl of 0.01 % bromophenol blue and 50 µl glycerol were added to the protein solution and a maximum volume of 100 µl containing

20 to 40 µg protein was applied to each gel. Electrophoresis was conducted at room temperature at a current of 6 mA per gel.

Staining SDS gels

Gels were stained overnight at room temperature in a solution of 0.1 % Coomassie Brilliant Blue R in 10 % trichloroacetic acid and 10 % sulphosalicylic acid. Gels were destained in a solution of 7.5 % acetic acid and 5 % methanol.

SECTION 2.12 ULTRACENTRIFUGATION

The diffusion of a solute particle in a solvent will depend on the size and shape of that particle , the characteristics of the solvent and also any interaction between the solute and the solvent. Being a function of translational freedom, diffusion is also temperature dependent. A definition of diffusion coefficient,D, and its relation to the frictional coefficient, f is

$$D = \frac{R\ T}{N\ f} \qquad (2.12.1)$$

where R is the universal gas constant, T is the absolute temperature and N is Avogadros' number. Under the influence of an applied centrifugal field sedimentation of the particle will occur. Sedimentation can be expressed in terms of the sedimentation coefficient, s which has the units S, Svedbergs (10^{-13} sec) ;

$$s = \frac{\text{velocity}}{\omega^2\ r} = \frac{dr/dt}{\omega^2\ r} = \frac{1\ d\ \ln\ r}{\omega^2\ d\ t} \qquad (2.12.2)$$

where ω is the angular velocity and r is the distance from the centre of rotation. Sedimentation is also dependent on the buoyancy of the particle in a given solvent through the factor $(1-\bar{V}\rho)$ where ρ is the density of the solvent and $\bar{V}$ is the partial specific volume of the solute. Sedimentation coefficient is related to the diffusion coefficient by the Svedberg equation ;

$$s = \frac{M\ (1-\bar{V}\rho)\ D}{R\ T} \qquad (2.12.3)$$

where M is the molecular weight of the solute. Thus from equations 2.12.2 and 2.12.3 ;

$$M = \frac{R\,T}{\omega^2(1-\bar{V}\rho)\,D} \cdot \frac{d\,\ln\,r}{d\,t} \qquad (2.12.4)$$

If the solute is a polyelectrolyte eg. a protein ,complications may arise due to the interaction of protein with counter-ions which will have far smaller S values than the protein; this is known as the primary charge effect. A secondary charge effect results from differences in S values between ions of opposite charge when large concentrations of supporting electrolyte are present. S values for the solute are usually corrected to standard conditions ie. 20°C with water as the solvent. Such a correction is made with data from International Critical Tables and the relationship;

$$S_{20,w} = S_{obs} \cdot \left(\frac{\eta\,t}{\eta_{20}}\right)\left(\frac{\eta_{sol}}{\eta_w}\right)\frac{(1-\bar{V}\rho_{20,w})}{(1-\bar{V}\rho_{t,sol})} \qquad (2.12.5)$$

where η is the viscosity at a given temperature. In sedimentation velocity experiments high applied centrifugal fields cause sedimentation of solutes towards the bottom of the cell and a continually moving boundary results which is visualised here by Schlieren optics. From a plot of log radial distance r versus time t, the sedimentation coefficient, S, is obtained from the slope (see equation 2.12.2) and is corrected to standard conditions.

At lower centrifugal forces however, sedimentation of the solute is opposed by diffusion until an equilibrium is reached whereby mass-transport towards the bottom of the cell is equal to that towards the meniscus. In practice, $\log_{10} y / r$ was plotted against r^2 where y is the height of the refractive index gradient and is proportional to dc/dr. Schlieren plates were interpreted with the equation ;

$$M_z = \frac{2\ R\ T\ 2.303}{\omega^2(1-\bar{V}\rho)} \cdot \frac{d\ \log_{10}\ y/r}{d\ r^2} \qquad (2.12.6)$$

The molecular weight for a polydisperse solute determined in the above manner is the z-average molecular weight, M_z , defined as

$$M_z = \frac{\Sigma\ c_i\ M_i^2}{\Sigma\ c_i\ M_i} \qquad (2.12.7)$$

and c_i is the concentration of the solute of molecular weight M_i .

Ultracentrifugation was carried out in a Beckman Spinco Model E analytical ultracentrifuge equipped with electronic speed control and temperature control (RTIC). All experiments were performed at 10°C in an An-D series rotor. Buffers used in ultracentrifugation contained 1 mM disodium EDTA and 5 mM β-mercaptoethanol. Glucose was also present in buffers when hexokinase was being studied. EDTA and β-mercaptoethanol levels were not taken into consideration when calculating the density and viscosity of buffers. $\bar{V}$ was taken to be 0.74 for all calculations of molecular weight. Photographic plates were measured by projection on a Nikon comparator.

SECTION 2.13 AMINO ACID ANALYSIS

0.6 mg protein was dialysed against water to remove buffer components and the dialysate made up to 0.5 ml with water. 0.5 ml of HCl (Analar grade) was added to the protein solution and the mixture sealed in vaccuo in a hydrolysis tube. Hydrolysis was at 106°C for 23 hr. After hydrdysis samples were freeze dried and amino acids dissolved in pH 2.2 citrate buffer for automatic analysis, based on ninhydrin reaction, in an LKB 4400 amino acid analyser.

CHAPTER 3

THE PURIFICATION OF HEXOKINASE FROM PIG HEART

SECTION 3.1 INTRODUCTION

Heart and brain hexokinases are particulate enzymes bound to the outer mitochondrial membrane (61, 62, 63) and solubilisation of these enzymes has been a major problem in the development of purification procedures. Paranjpe and Jagannathan (64) used freeze-thaw treatment and proteolytic digestion to solubilise hexokinase from a crude extract of ox heart. Schwartz and Basford (65) used proteolysis and detergents to liberate hexokinase from bovine brain prior to its purification. Easterby and O'Brien (46) circumvented the use of proteolytic enzymes or detergents by solubilising porcine brain hexokinase with high levels of Mg^{2+} and their purification forms the basis of the procedure used in the present study. Heart is a good source of type I hexokinase since it contains relatively high levels of this enzyme and comparatively much less lipid than the brain which makes extraction of the enzyme from heart easier.

Easterby and O'Brien (46) purified type I hexokinase by a procedure involving Mg^{2+} solubilisation, DEAE-cellulose chromatography, phosphocellulose chromatography and gel filtration. Two forms of the enzyme were resolved on phosphocellulose and a thorough study of their kinetics (42) showed them to be very similar. The reasons for the occurrence of sub-forms of porcine heart hexokinase are not clear and other tissues are known to contain multiple forms of a particular hexokinase isoenzyme (66, 67).

In the present study the established procedure for the purification of porcine heart hexokinase (46) was modified to avoid the use of phosphocellulose for reasons discussed in Chapter 6, (Section 6.2). Instead, affinity elution from the immobilised triazine dye Procion

Red H-8BN was used (68). One advantage of using dye-ligand chromatography in this case is that porcine heart hexokinase can be separated from physically similar diaphorase (lipoamide dehydrogenase) species which co-purify with hexokinase during ion exchange chromatography (69).

SECTION 3.2 MATERIALS AND METHODS

Fresh pig hearts were obtained from a local abbatoir and either used immediately or stored frozen at -20°C.

Purification of heart hexokinase.

All buffers contained 100 mM glucose and 5 mM β-mercaptoethanol.

Buffer 1, 0.2 I acetate pH 5.6, 0.2M $MgCl_2$;
13.6 g sodium acetate trihydrate, 0.257 ml acetic acid and 40.6 g $MgCl_2$ per litre.

Buffer 2, 0.08 I phosphate pH 8; 4.52 g K_2HPO_4, 0.29 g KH_2PO_4 and 0.372 g disodium EDTA per litre.

Buffer 3, 0.05 I Tris-HCl, pH 7.5; 7.4 g Tris and 0.372 g EDTA per litre titrated to pH 7.5 with HCl.

Extraction of enzyme

Two litres of ice cold water were poured through 500 g of minced heart in cheesecloth to remove blood. The tissue was homogenised in 800 ml buffer 1 for two mins. in an MSE Atomix and stood on ice for 45 mins. prior to centrifugation at 30,000 g for 30 mins. The supernatant was filtered through glass-wool to remove floating lipid and then titrated to pH 8 with 0.2 M KOH. The residue was re-extracted with 800 ml buffer 1 and the supernatant added to that from the first extraction and retitrated to pH 8.

Dialysis

The supernatant was dialysed overnight against 25 litres of buffer 2. The precipitate which formed was removed by centrifugation for 25 mins. at 30,000 g and the extract was dialysed for a further 24 hr against 20 litres buffer 2.

Chromatography on DEAE-cellulose, pH8

The dialysed extract was batch-adsorbed onto 900 ml DEAE—cellulose equilibrated in buffer 2 and unbound protein was removed by washing with 4 litres buffer 2 on a Büchner funnel. The exchanger was then poured into a 38 x 5.5 cm column, washed with a litre buffer 2 and developed with a 2 litre 0 to 0.4 M KCl linear gradient in buffer 2. Flow-rate was 80 ml / hr and 12.5 ml fractions were collected. See figure 3.1.

Affinity elution from H-8BN-Sepharose

Active fractions from DEAE-cellulose were pooled and dialysed overnight against buffer 3 before they were pumped onto a 6 x 4.5 cm column of H-8BN-Sepharose 6B equilibrated in buffer 3. Flow rate was 60 ml / hr. The column was thoroughly washed with buffer 3 to remove unbound protein ; hexokinase was eluted with 1.8 mM ATP in buffer 3. Fractions of 10 ml were collected (figure 3.2). Fractions 62 to 70 were pooled.

Gel filtration

The eluate from the previous stage was concentrated by vacuum dialysis against buffer 3 and applied to a column of Sephadex G-200 (100 x 125 cm) equilibrated in buffer 3. The column was developed at a flow-rate of 8 ml / hr and 4.5 ml fractions collected (figure 3.3). All active fractions were combined.

Storage of pig heart hexokinase.

The enzyme was concentrated by vacuum dialysis against 0.1 I Tris-HCl

Chromatography of heart hexokinase on DEAE-cellulose, pH 8. (see text for details)

Figure 3.1

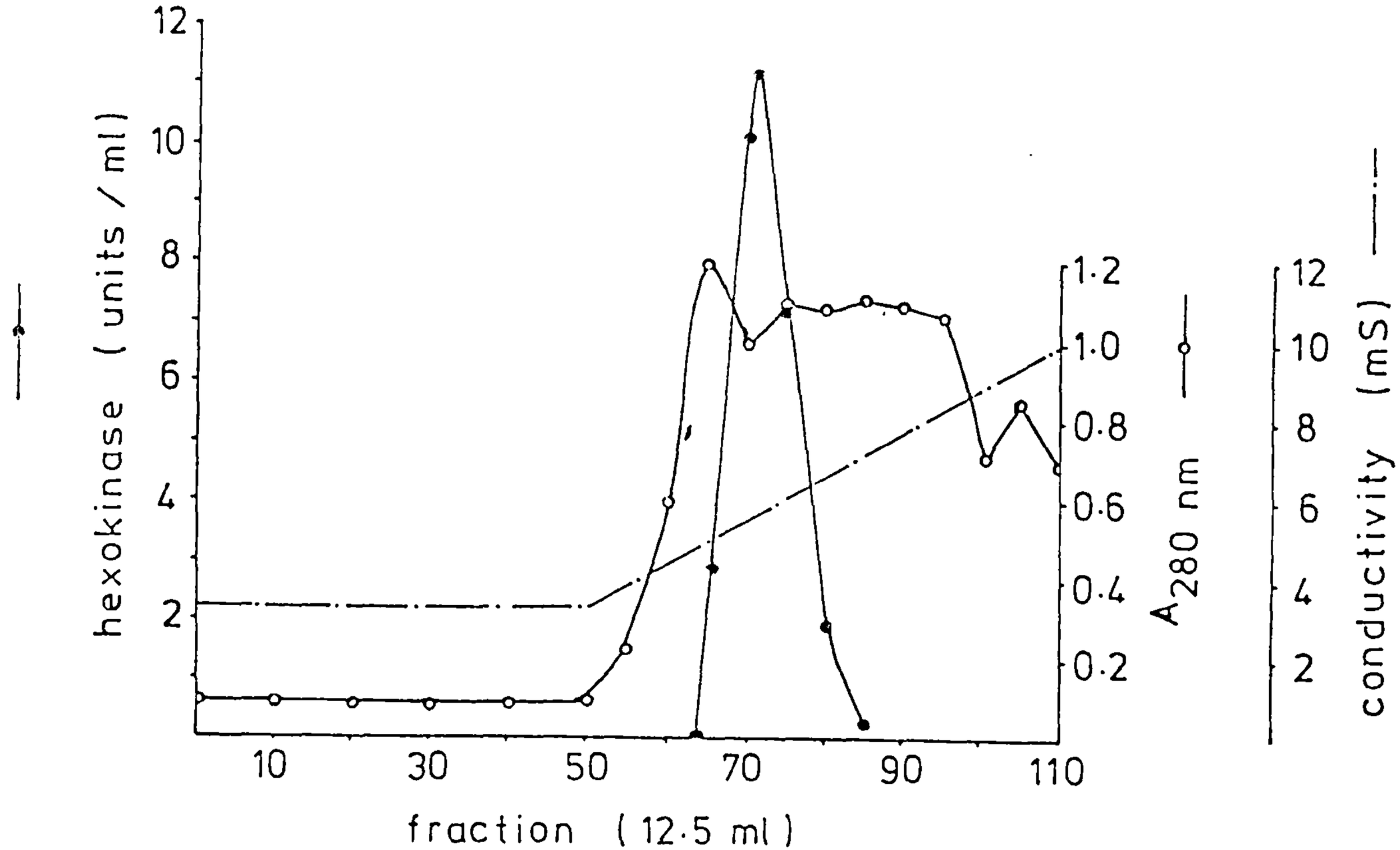
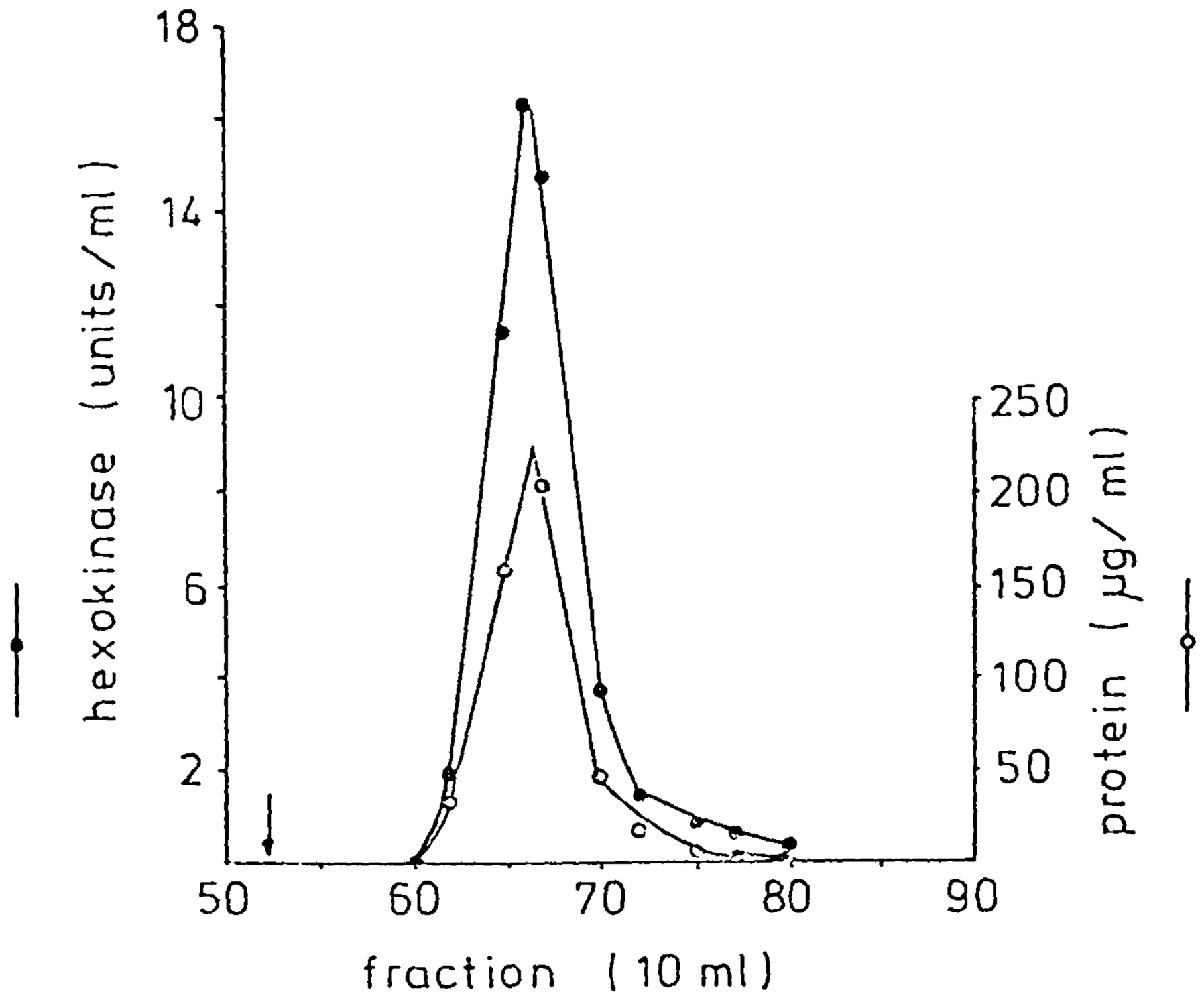


Figure 3.2

The chromatography of heart hexokinase on H-8BN-Sepharose 6B, pH 7.5.
Arrow marks the inclusion of 1.8 mM ATP in the column buffer. See text for details.



Note ; a considerable amount of protein did not bind the immobilised dye and was washed off the column before inclusion of ATP in the buffer.

Figure 3.3

The chromatography of heart hexokinase on Sephadex G-200.
See text for details.

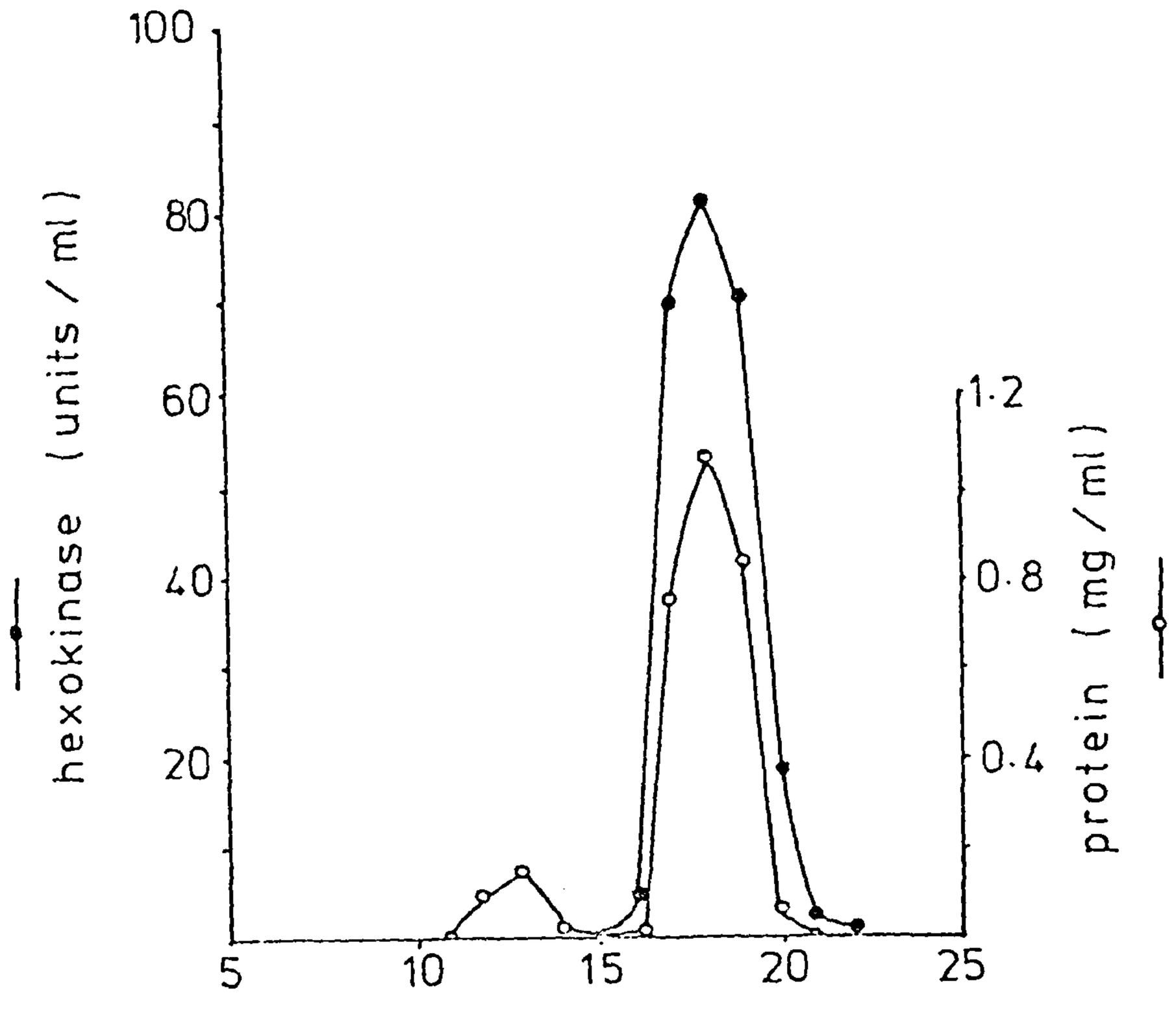


Table 3.1

THE PURIFICATION OF HEART HEXOKINASE

500 g heart were used.

Stage	Enzyme (units)	Protein (mg)	Specific activity (units /mg)	Purification (fold)	Yield (%)
Extract 1	1069	39,770	0.049	1	100
Extract 2	880				
Postdialysis	1553	14250	0.109	2.2	80
DEAE-cellulose	1120	243	4.6	93	57
H-8BN-Sepharose	990	14	71	1450	51
Sephadex G 200	775	9.2	84	1714	40

Figure 3.4

Gel electrophoresis of purified heart hexokinase

In both cases the origin of the gel is nearest the top of the figure and the position of the marker-dye bromophenol blue is indicated by bpb.

7.5 % polyacrylamide gel electrophoresis

The gel was loaded with approximately 20 µg protein and stained for protein.

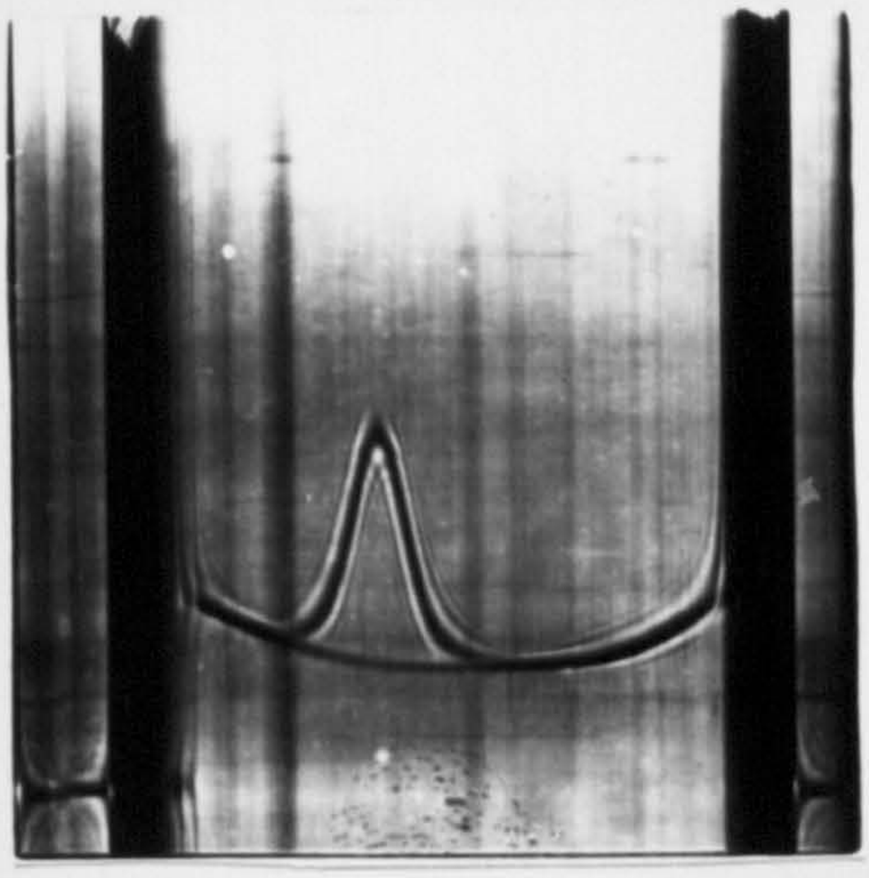
SDS polyacrylamide gel electrophoresis

The gel was loaded with approximately 30 µg protein.

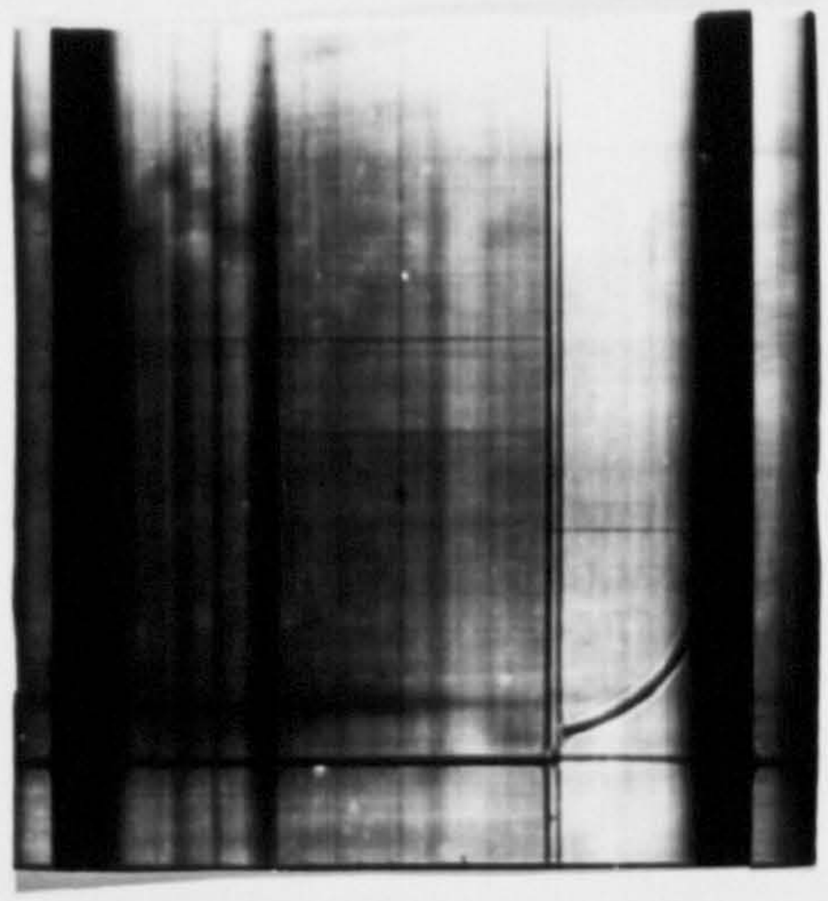
Figure 3.5

The ultracentrifugation of heart hexokinase in 0.1 I Tris-HCl, pH 8 containing 1 mM disodium EDTA, 5 mM β-mercaptoethanol and 100 mM glucose.

A.



B.



Photograph A shows the sedimentation velocity of purified heart hexokinase. The photograph was taken 90 min after reaching a speed of 60,000 rpm. The wire angle was 60°. Full details are given in figure 3.6.

Photograph B shows the sedimentation equilibrium of purified heart hexokinase, the wire angle was 60° and details are given in figure 3.7 .

Photograph C shows the sedimentation velocity of a sample of heart hexokinase after chromatography on H-8BN-Sepharose 6B. A high molecular weight contaminant, later removed by gel filtration, is clearly seen to the right of the much larger hexokinase Schlieren peak.

C.

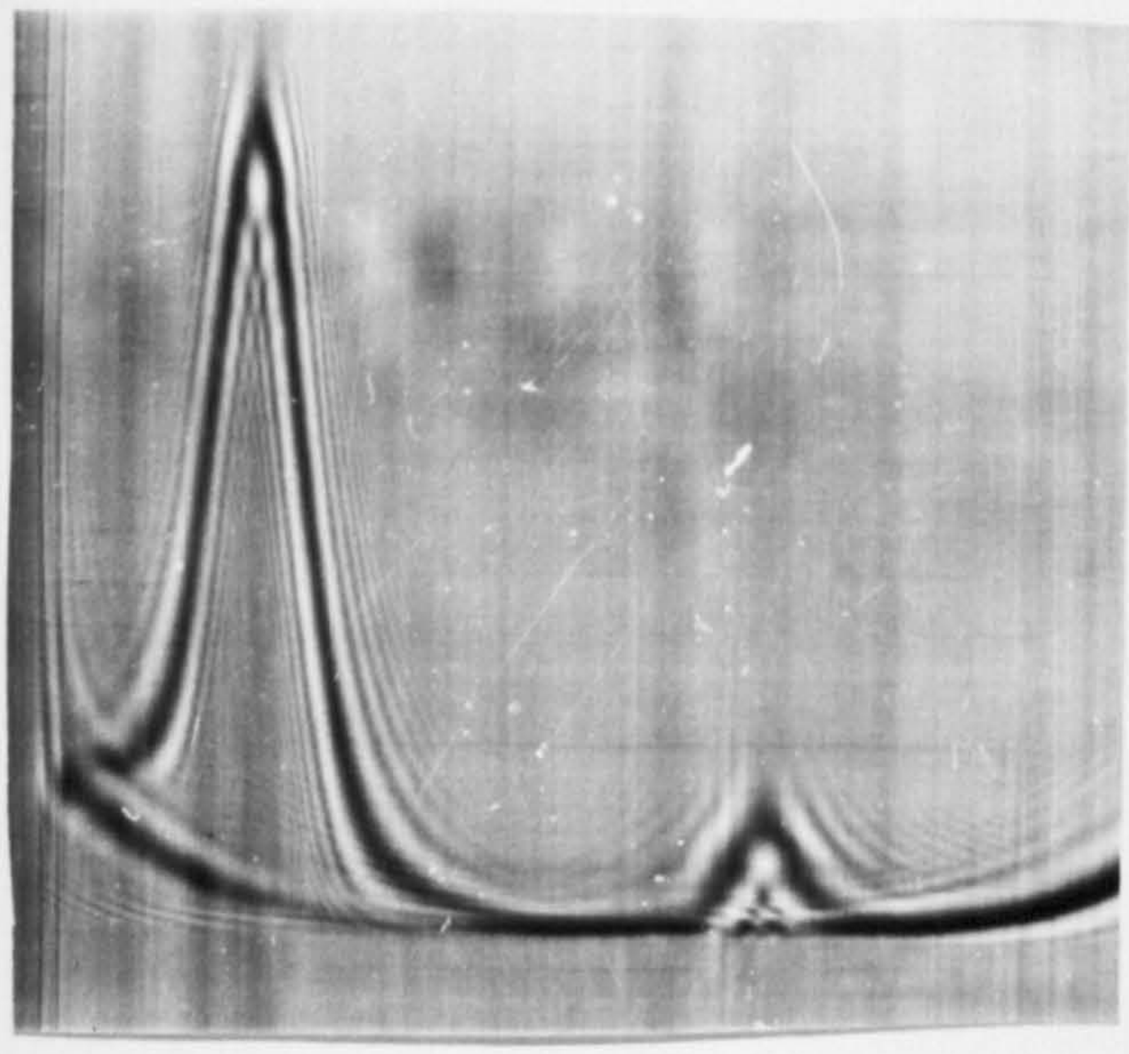
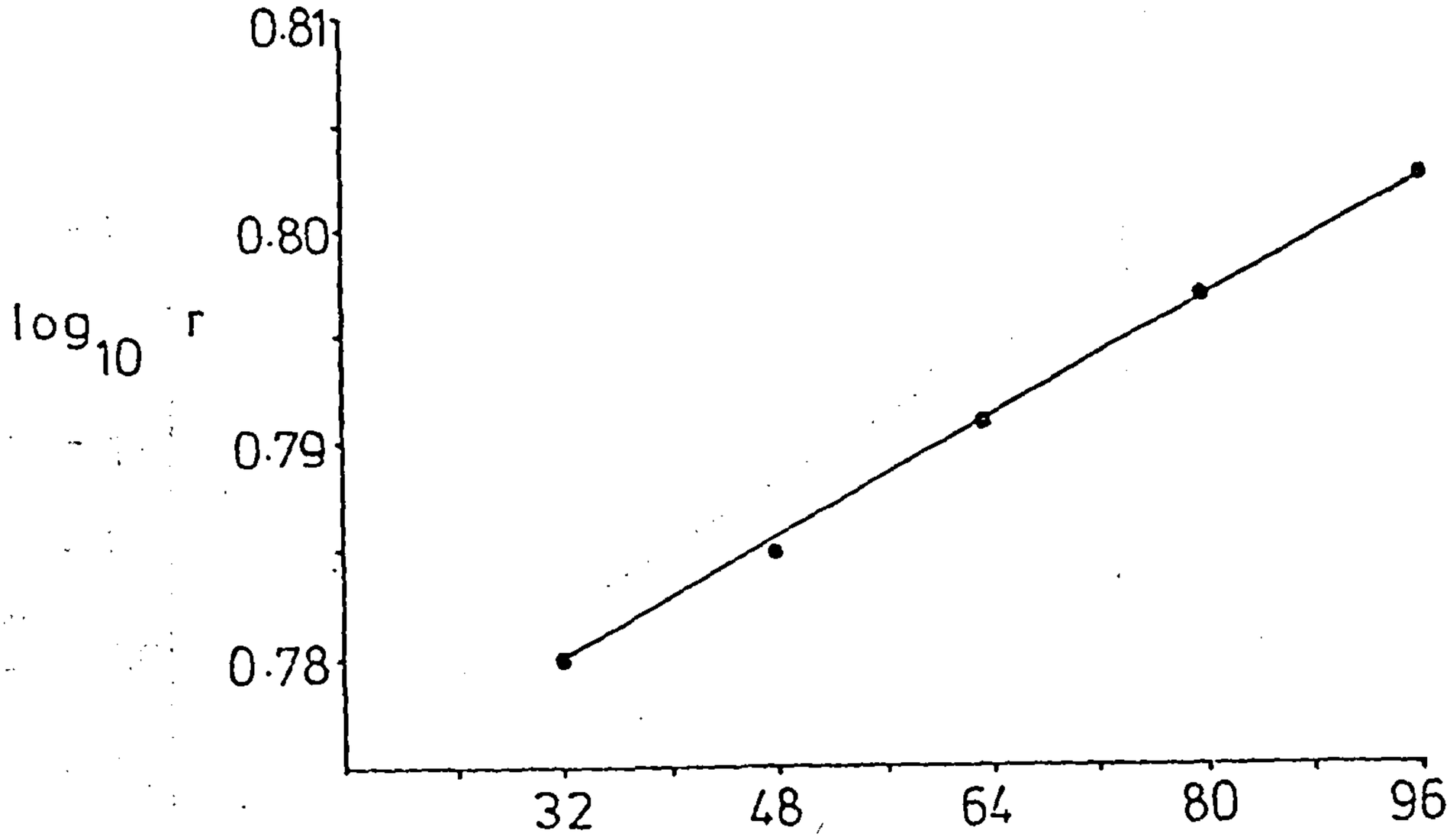


Figure 3.6

Heart hexokinase, determination of sedimentation coefficient.



Heart hexokinase in 0.1 I Tris-HCl pH 8.0 buffer containing 1 mM disodium EDTA, 5 mM β-mercaptoethanol and 100 mM glucose was centrifuged at 60,000 rpm at a temperature of 10°C . The protein concentration was 7.1 mg / ml . From linear regression of the data shown above an $S_{20,w}$ of 5.17 S was calculated.

pH 7.5 buffer (containing 14.7 g Tris, 0.372 g disodium EDTA, 18 g glucose and 0.35 ml β-mercaptoethanol per litre) and stored at 4°C.

SECTION 3.3 DISCUSSION

9.2 mg of hexokinase was purified from 500 g pig heart, the purification is summarised in Table 3.1 . The enzyme appeared homogeneous by criteria of analytical ultracentrifugation (Figure3.5) and electrophoreis (Figure 3.4) and had a specific activity of 84 units / mg . The purified enzyme did not contain diaphorase activity.

In order that the purification be most effective, hexokinase with a specific activity of at least 4 to 5 units / mg had to be applied to the immobilised dye column. If for some reason the specific activity of the enzyme from DEAE-cellulose was lower than this it was rechomatographed on DEAE-cellulose before application to the dye column. If enzyme of 2 units / mg was applied to the dye column the specific activity recovered was about 40 to 50 units / mg. It is also important to thoroughly wash unbound protein from the dye column before effecting affinity elution of hexokinase. The immobilised H-8BN column was reliable and gave very high recovery of enzyme, it could be used at high flow-rates (up to 60 ml / hr) and no evidence of time-dependent binding (70) of hexokinase to the dye column was observed. After elution from immobilised H-8BN, pig heart hexokinase was at least 90 % pure (as judged by gel scanning and analytical ultracentrifugation) although a heavy contaminant was observed (see Figure 3.4) and could be removed by gel-filtration.

Wilson (71) investigated the interaction of purified rat brain hexokinase with immobilised Blue Dextran where , although the column had a very small capacity for hexokinase , it could be eluted with

10 mM ATP. The interaction of yeast hexokinase with immobilised triazine dyes has been the subject of several studies (72,73,74,75). Clonnis et al. (75) found Mg^{2+} to promote the binding of yeast hexokinase to immobilised Procion Green H-4G ; in Mg^{2+} (10 mM) containing buffers yeast hexokinase could be eluted from immobilised H-4G with ATP (10 mM), glucose (20 mM) or by omission of Mg^{2+} from the buffer. In the present study, pig heart hexokinase could be eluted from H-8BN-Sepharose with low levels of ATP (1 to 2 mM). The presence of 10 mM Mg^{2+} had little effect on chromatography and was not present in the buffer as this would have caused turnover of the enzyme. Glucose (100 mM) was present in all buffers throughout the purification but the heart hexokinase could bind immobilised H-8BN in its absence. Hydrolysed Procion Red H-8BN was found to inhibit coupling enzymes in the hexokinase assay thus a quantitative study of the inhibition of hexokinase by the free dye was not possible. A more detailed discussion of this and of the mechanism of affinity elution from H-8BN are given in Chapter 6 , Section 6.3 .

The amino acid composition of pig heart was very similar to that obtained for the same enzyme by Easterby and O'Brien (46) and is given in Chapter 10, Section 10.3 .

See Appendix II for a published account of this purification.

CHAPTER 4

THE PURIFICATION OF HEXOKINASE FROM THE STARFISH ASTERIAS RUBENS

SECTION 4.1 INTRODUCTION

Mochizuki and Hori (18) reported the purification of hexokinase from the pyloric caeca of the starfish Asterias amurensis. The enzyme had a molecular weight of 50,000 and was inhibited by low concentrations of its products MgADP and glucose 6-phosphate in common with the 'low K_m' mammalian hexokinases. This was an important finding as previously no 50,000 molecular weight hexokinase that was regulated by glucose 6-phosphate had been purified, and it raised questions about the validity of the theory of partial gene-duplication in the evolution of 100,000 molecular weight low K_m hexokinases (45, 46, 76) which is discussed in chapter 1.

The procedure used by Mochizuki and Hori (18) to purify hexokinase from A. amurensis is based on conventional techniques: ammonium sulphate fractionation, ion exchange and gel filtration. However application of the procedure to the purification of hexokinase from A. rubens was not entirely successful and the preparation had to be re-designed. A. rubens hexokinase did not bind cation-exchange resins or dye-ligand columns at usable pHs, so a procedure based on two anion exchange steps carried out at different pHs was devised.

SECTION 4.2. MATERIALS AND METHODS

Source and storage of Starfish.

Asterias rubens were purchased from Millport Marine Biological Station, Millport, Scotland. They were transported live in seawater and immediately on receipt were drugged by addition of Sandoz MS 222 to the sea water. Once stationary the Asterias were dissected and the caeca washed in ice-cold 0.02 I phosphate buffer, pH7 and, if necessary, stored as a slurry at -20°C.

Hydrophobic chromatography

Sepharose 6B-N-6-aminohexane was synthesised by the method recommended by Parikh et al. (77). Sepharose 6B (32.5 g) was activated with 1.5 g cyanogen bromide. Coupling of 6-aminchexane was achieved by adding the sepharose to 50 ml of 0.2M hexylamine pH10 in 6% ethanol and stirring for 16 hr at 4°C. Hydrophobic columns were equilibrated in 0.05I Tris-HCl, pH 7.5 buffer for use.

Protein determination

Polyethylene glycol 6000 was found to form an insoluble complex with tannic acid thus ruling out use of the microtannin turbidimetric method (53) in its presence. After PEG 6000 precipitation the protein content of the supernatant was estimated by the method of Warburg and Christian (52) described in chapter 2 and is therefore only an approximation owing to the turbidity of the solution and other limitations of this method.

Purification of A rubens hexokinase

Results of the purification are given in table 4.1

Buffers

Buffer 1 0.02 I phosphate, pH 7; 0.854 g K_2HPO_4 and 0.721 g KH_2PO_4 per litre

Buffer 2 0.04 I Tris-HCl, pH 8.5; 15.76 g Tris per litre titrated to pH 8.5

Buffer 3 0.1 I Acetate, pH 5.5; 13.6 g sodium acetate trihydrate titrated to pH 5.5 with acetic acid

Buffer 4 0.1 I Tris-HCl, pH 7.5 14.7 g Tris per litre titrated to pH 7.5 with HCl

All buffers additionally contained 1 mM Na_2 EDTA 5mM β-mercapto-ethanol and 50 mM glucose.

Extraction of hexokinase pH 7, 80 g starfish gut stored frozen as a

slurry in 0.02 I phosphate, pH 7 was extracted by homogenisation in 750 ml buffer 1 in an MSE Atomix. The homogenate was titrated to pH 7 by addition of 0.5 M KOH and centrifuged for 40 min at 28,000 g at 0°C. The supernatant was filtered through glass-wool to remove floating lipid and its volume measured.

PEG precipition pH 7 The supernatant was made 8% in PEG 6000 as described in chapter 2 (section 2.5). After stirring for 1 hour at 4°C the mixture was centrifuged as above. The supernatant was retained and titrated to pH 8.5 with 0.5 M KOH.

DEAE-cellulose chromatography pH 8.5

The enzyme was batch adsorbed onto c.600 ml DEAE cellulose equilibrated in buffer 2. After two washes each of 2 l buffer 2, the resin was poured into a column (36 x 4.7 cm) and washed with a further 2 l buffer 2 at 90 ml / hr before developing with a 2 l linear gradient of 0 to 0.3 M KCl in buffer 2. 10 ml fractions were collected at a flow-rate of 90 ml / hr. Fractions 75 to 90 (Figure 4.1) were titrated to pH 7 then concentrated by vacuum dialysis to approximately 15 ml, before dialysis against buffer 3.

DEAE Sephadex - A50 chromatography, pH 5.5

The hexokinase was pumped onto a 21 x 3 cm column of DEAE-sephadex A-50 equilibrated in buffer 3, at 30 ml / hr. The column was washed with 600 ml buffer 3 and developed with a 1.5 l linear gradient of 0.1 I to 0.6 I sodium acetate at 30 ml / hr. 8.5 ml fractions were collected. Fractions 38 to 49 (Fig. 4.2) were pooled and immediately titrated to pH 7.5 before concentration by vacuum dialysis against buffer 4. This stage is carried out as quickly as possible as the hexokinase is not particularly stable at pH 5.5.

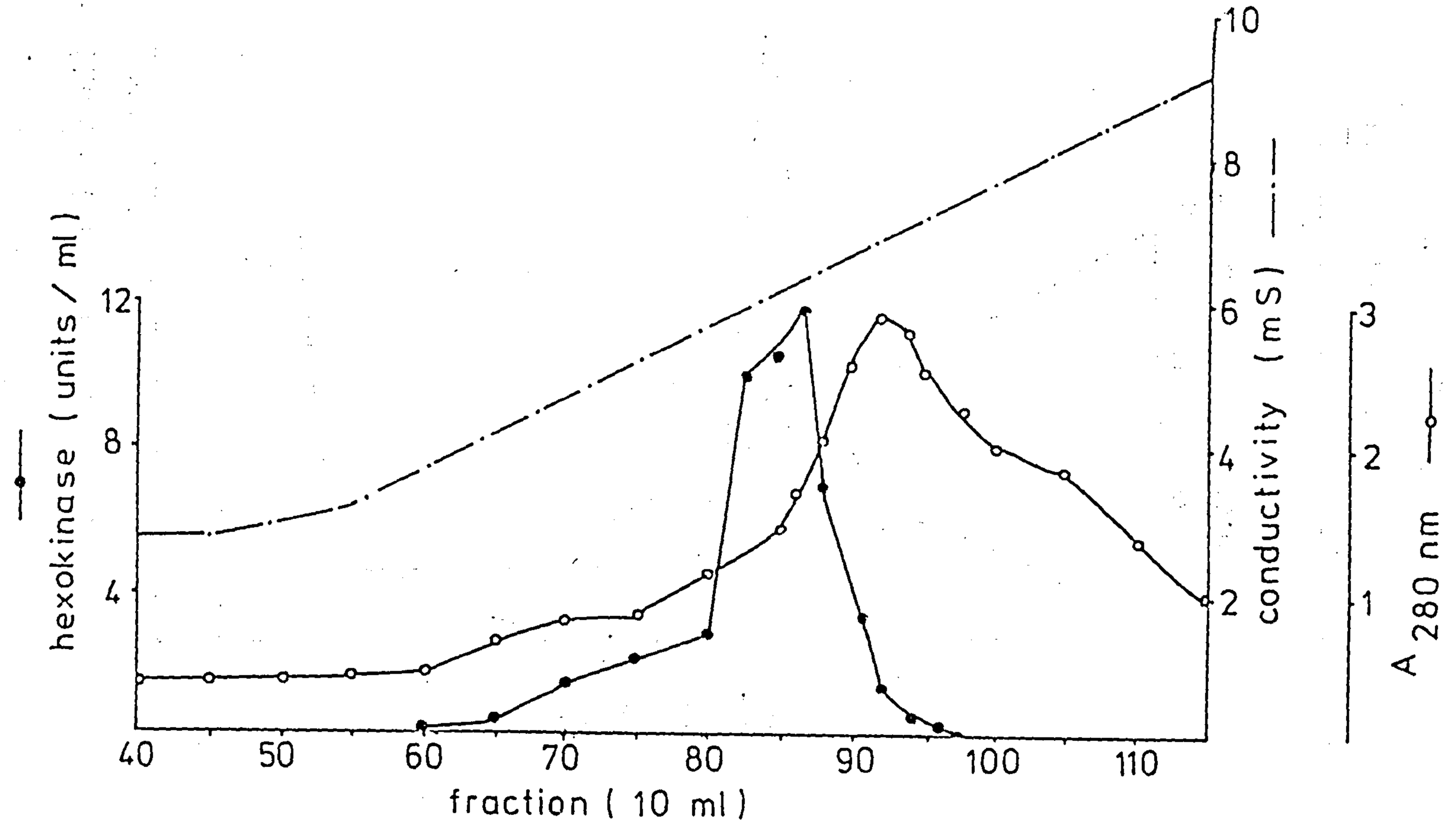
Gel filtration, pH 7.5

The concentrated enzyme in c. 2 ml buffer 4 was applied to an 8.5 x 2.5

Chromatography of Asterias hexokinase on DEAE-cellulose, pH 8.5.

Figure 4.1

Some hexokinase is seen to elute before the main peak of activity, most of this was however discarded and only fractions 75 to 90 were retained.



Chromatography of Asterias hexokinase on DEAE-Sephadex, pH 5.5

Figure 4.2

See text for details.

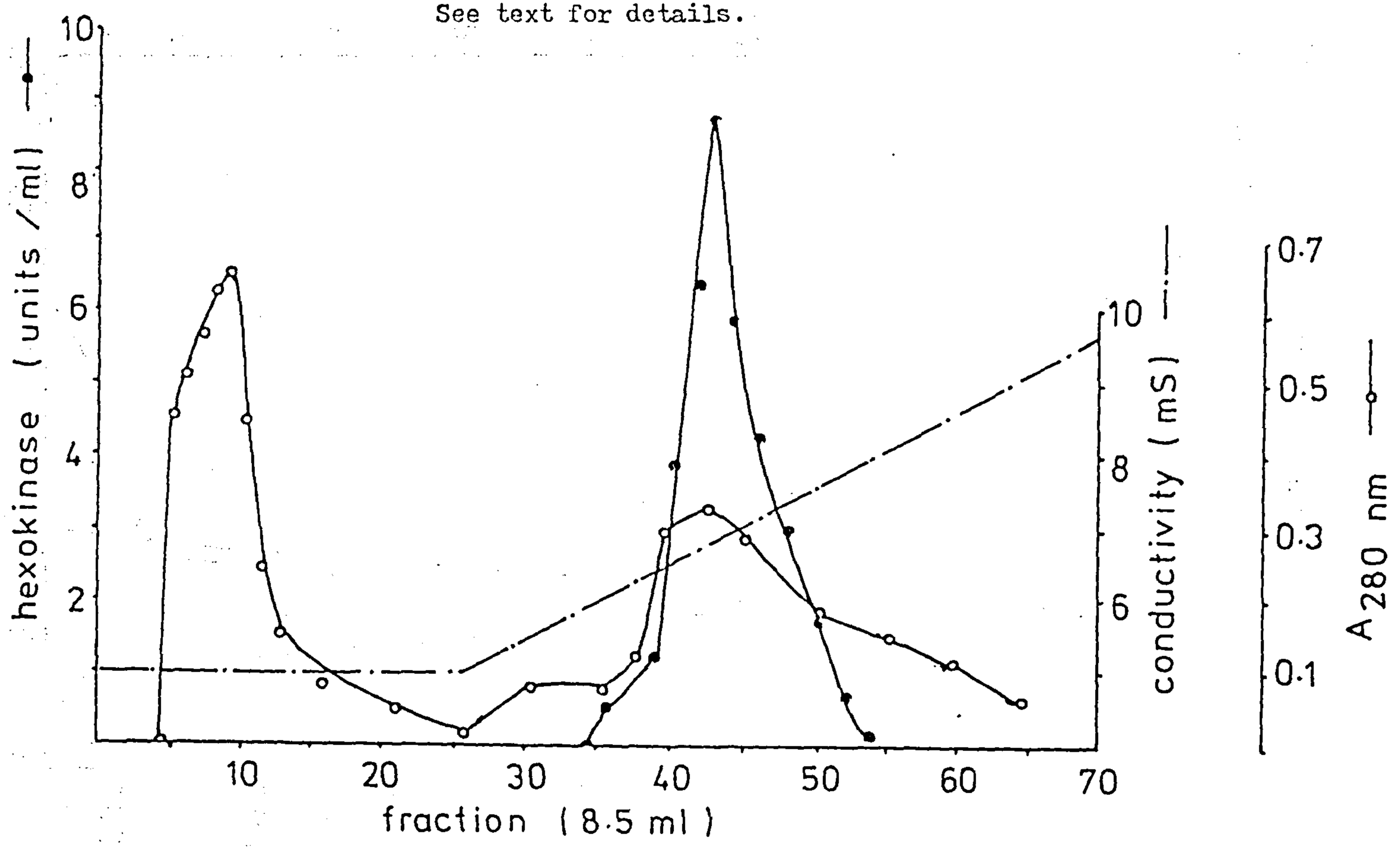


Figure 4.3

The chromatography of Asterias hexokinase on AcA 44, pH 7.5

Details are given in the text.

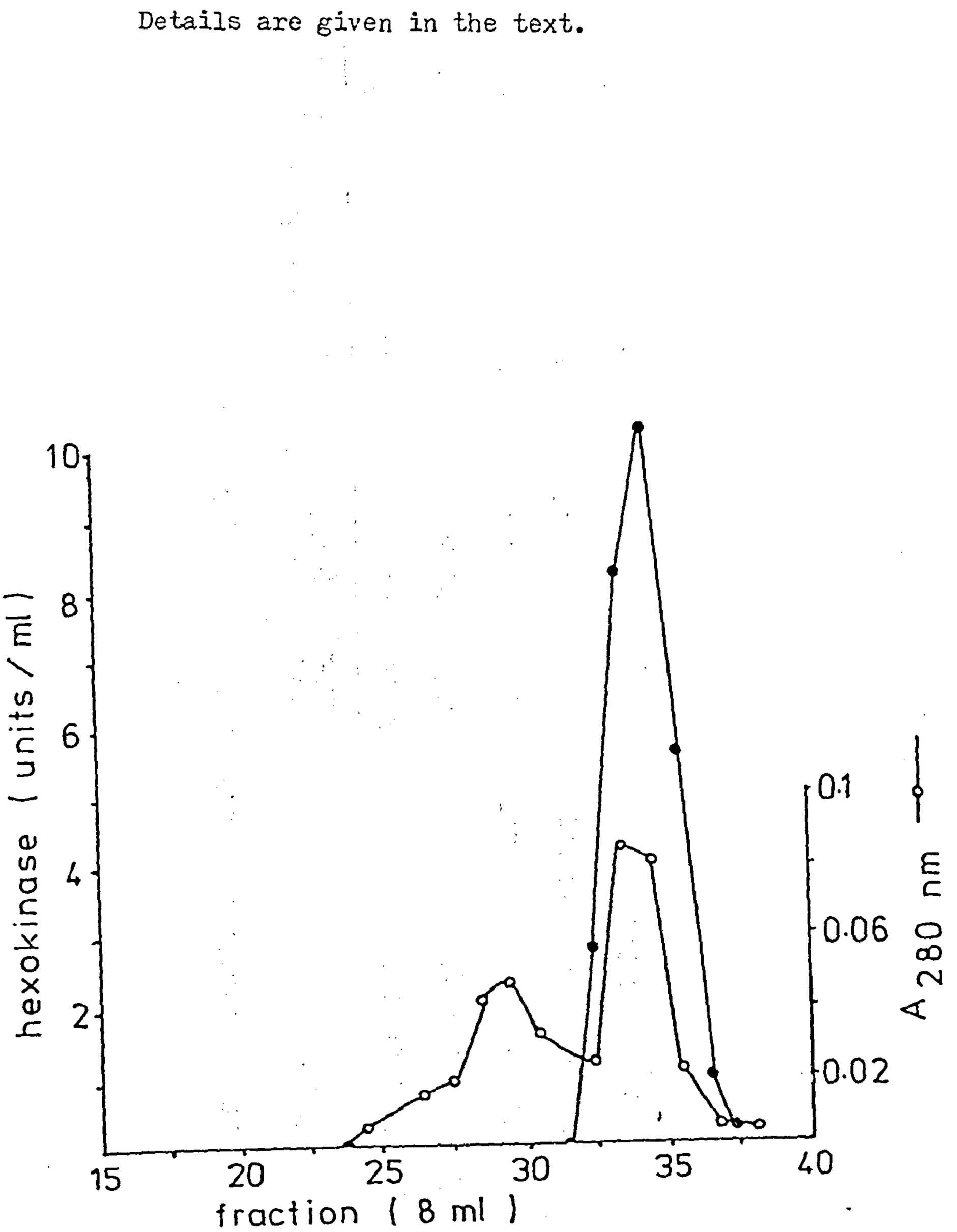


Table 4.1

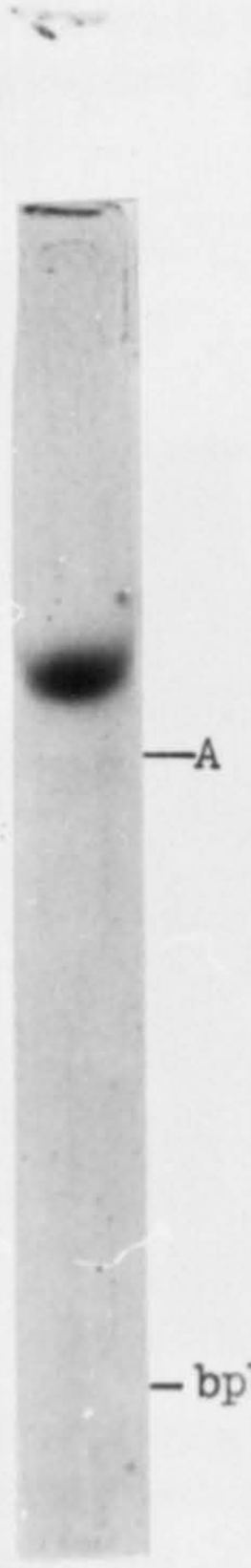
THE PURIFICATION OF HEXOKINASE FROM ASTERIAS RUBENS. (80 g gut used)

Stage	Enzyme (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Extract pH 7	1,470	4,740	0.31	1	100
PEG 6000, pH 7	1,260	2,470	0.51	1.6	86
DEAE-cellulose, pH 8.5	550	30.6	18	58	37
DEAE-Sephadex, pH 5.5	290	6.3	46	148	20
AcA 44, pH 7.5	240	2.1	112.5	363	16

Figure 4.4

Gel electrophoresis of Asterias hexokinase.

Polyacrylamide electrophoresis
A 10 % polyacrylamide gel loaded with approximately 35 µg purified Asterias hexokinase. The origin is at the top of the figure. The position of bromophenol blue marker is indicated by bpb. Heterogeneity in the sample is clearly seen. The gel was stained for protein.



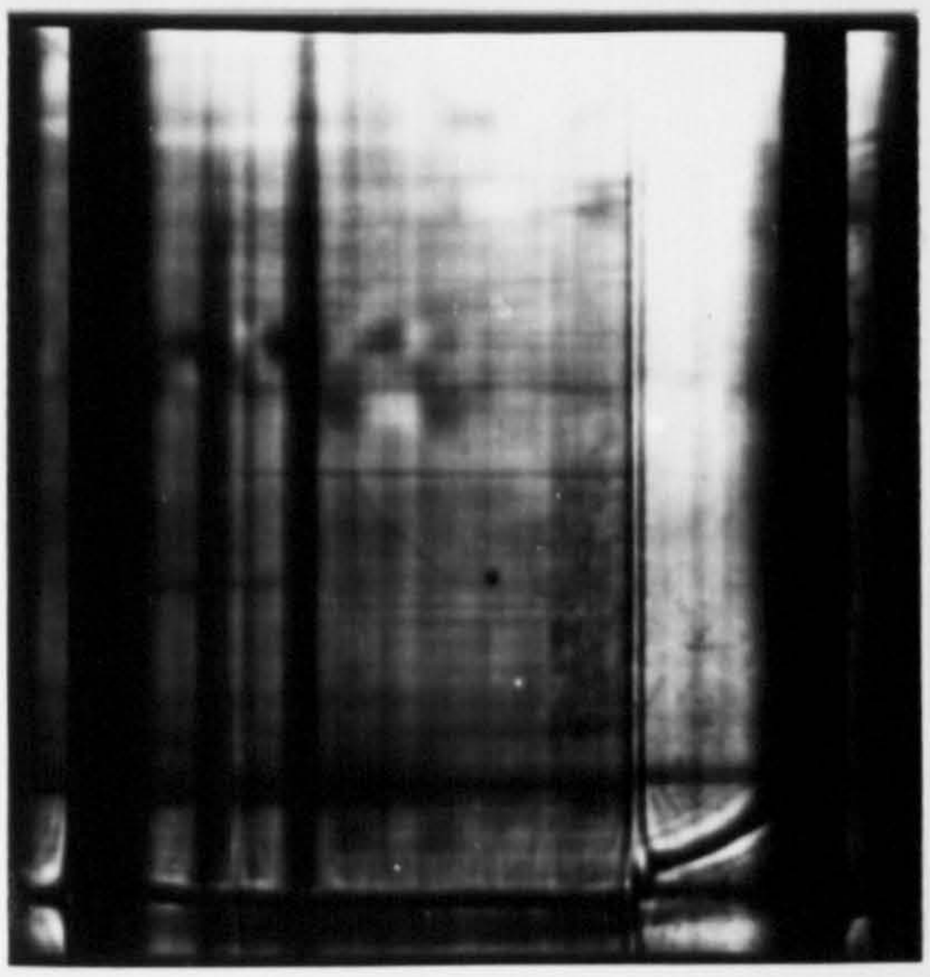
SDS polyacrylamide electrophoresis
The same sample applied to a 10 % SDS polyacrylamide gel. The origin is at the top of the figure and bromophenol blue indicated by bpb. The sample appears largely homogeneous, however a faint band is sometimes observed at the region marked A .

Figure 4.5

The ultracentrifugation of Asterias hexokinase

Buffer in both cases was 0.05 I Tris-HCl , pH 7.5 containing 1 mM disodium EDTA, 5 mM β-mercaptoethanol and 100 mM glucose.

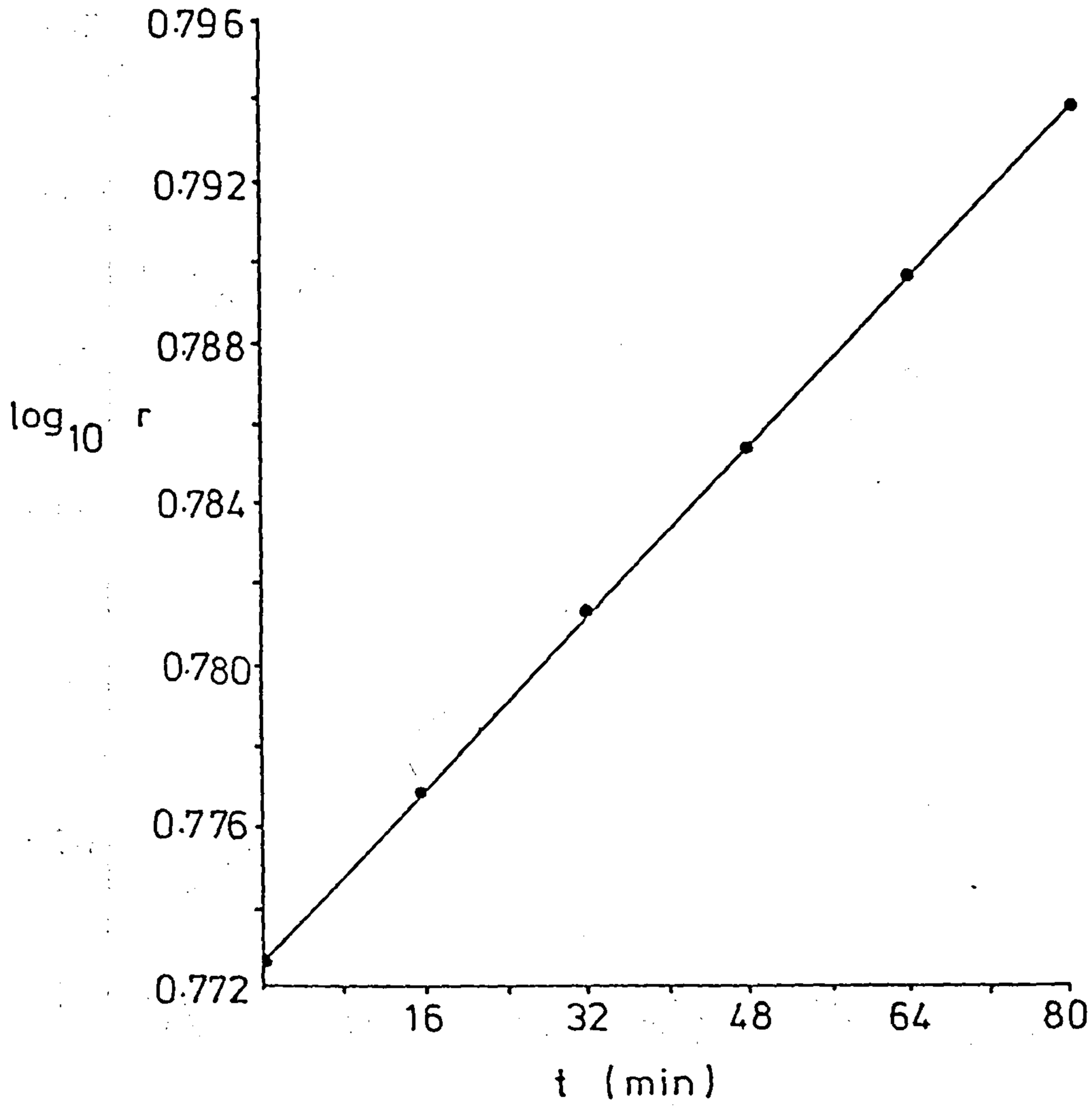
A The sedimentation velocity of purified Asterias hexokinase photographed at a wire angle of 60°. Details are given in figure 4.6 .



B The sedimentation equilibrium of purified Asterias hexokinase photographed at a wire angle of 60°, details are given in figure 4.7 .

Figure 4.6

Asterias hexokinase, determination of sedimentation coefficient by sedimentation velocity.

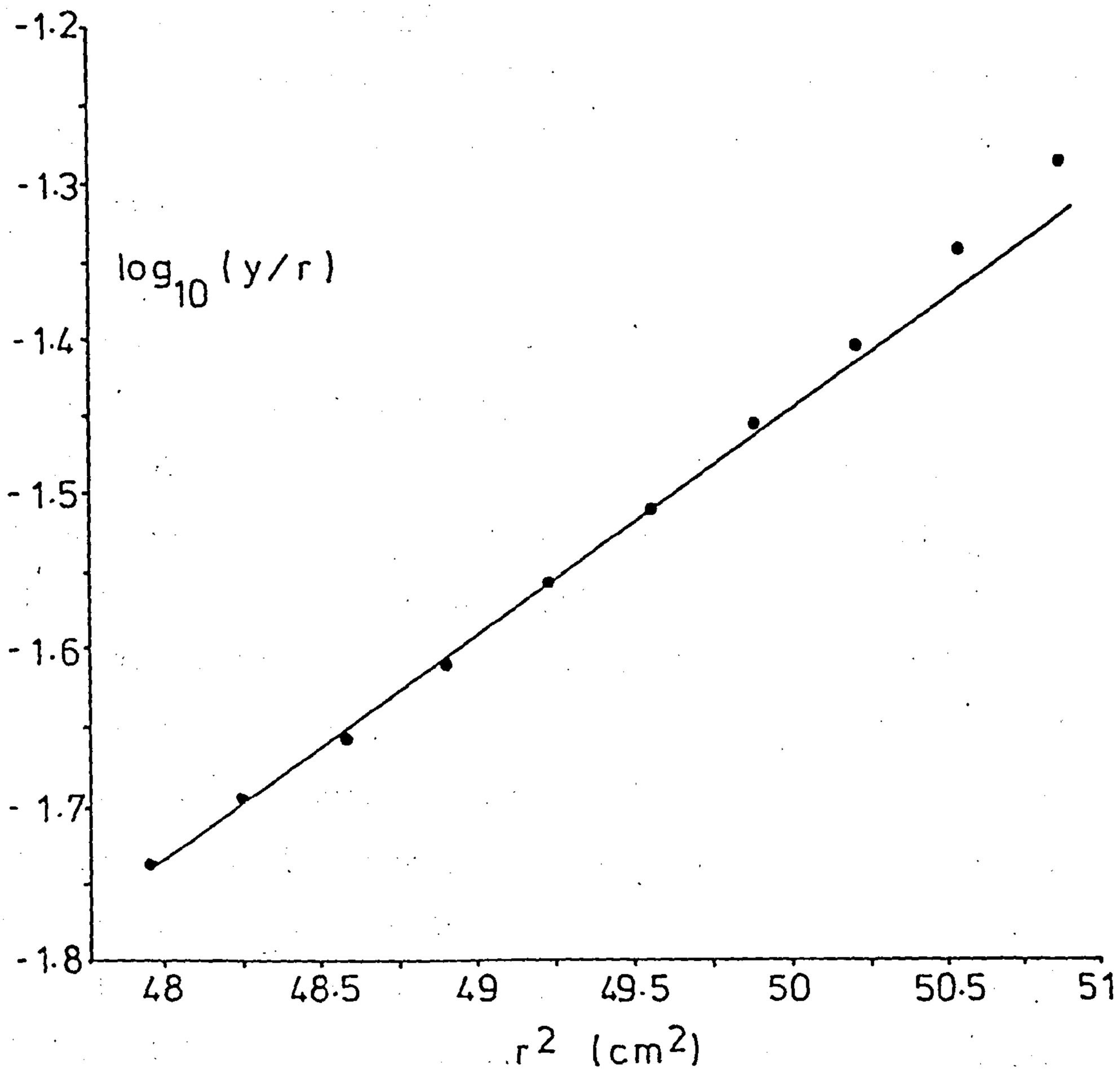


Buffer was 0.05I Tris-HCl, pH 7.5 containing 1 mM disodium EDTA, 5 mM β-mercaptoethanol and 100 mM glucose. The temperature was 10°C and the rotor speed 60,000 rpm. Protein concentration was 1.45 mg / ml. An $S_{20,w}$ of 3.59 S was calculated after linear regression analysis.

A similar experiment at a protein concentration of 5.1 mg / ml gave an $S_{20,w}$ of 3.36S. Extrapolation of the two values of sedimentation coefficient to infinite dilution gave an $S^{o}_{20,w}$ of 3,7 S.

Figure 4.7

Asterias hexokinase, determination of z-average molecular weight by sedimentation equilibrium.



Buffer was 0.05 I Tris-HCl, pH 7.5 containing 1 mM disodium EDTA, 5 mM β-mercaptoethanol and 100 mM glucose. Asterias hexokinase was centrifuged at a speed of 11,000 rpm and a temperature of 10°C . A z-average molecular weight of 51,000 was calculated by linear regression of the first 7 points on the above plot. Protein concentration was 1.45 mg / ml.

cm AcA 44 column and developed by pumping upwards at 20 ml / hr. 8 ml fractions were collected and fractions 32 to 36 (Figure 4.3) pooled.

Storage of Asterias hexokinase

Immediately after gel filtration hexokinase was concentrated by vacuum dialysis against buffer 4 and stored as a suspension in 3 M ammonium sulphate at 4°C.

SECTION 4.3 DISCUSSION

The hexokinase purified from Asterias rubens appeared homogeneous by analytical ultracentrifugation and SDS gel electrophoresis. From analytical ultracentrifugation data the hexokinase had an $S_{20,w}$ of 3.6 S and a z-average molecular weight of approximately 50,000 (figures 4.5 to 4.7). However PAGE revealed heterogeneity (Figure 4.4). The extent of heterogeneity in the purified hexokinase increased if the gut had been stored prior to use and addition of 3 mM phenyl methyl sulphonyl fluoride (a serine protease inhibitor) in the extraction buffer had little effect on the heterogeneity of the hexokinase. However heterogeneity may well be a result of proteolysis as the starfish gut is likely to contain protease. Mochizuki and Hori (18) did not report any heterogeneity in the hexokinase they purified from Asterias amurensis.

Another problem encountered with the gut was its high content of lipid. Gut extract applied to DEAE-cellulose shortened its working life; by using a polyethylene glycol precipitation stage prior to DEAE-cellulose chromatography quite a lot of protein, and presumably lipid, was removed and the working life of the cellulose thereby increased. Ammonium sulphate fractionation of crude gut extract proved ineffective as several phases resulted after centrifugation. Ammonium sulphate cuts

and retro-gradient chromatography also proved of little use later in the purification as the Asterias hexokinase behaved as an average protein in this respect. Thus polyethylene glycol precipitation proved a useful way of removing some protein from a crude extract of gut and, as the polyethylene glycol is uncharged, it does not increase the ionic strength of solutions and they can be applied directly to ion-exchange columns, in this case DEAE-cellulose. Polyethylene glycol washed off the cellulose with unbound protein. A. rubens hexokinase did not bind to phospho-cellulose or carboxy-methyl-cellulose at usable pH's, nor did it bind to any of a large number of dye-ligand columns screened at pH 7.5 and pH5 (Farmer, Pepper and Easterby unpublished results). The enzyme was however found to bind to the hydrophobic resin sepharose 6B-N-6-aminohexane (see materials and methods, section 4.2) and could be eluted in good yield with salt gradients (data not shown) However, hydrophobic chromatography was not used in the purification of Asterias hexokinase. Gel filtration was carried out on AcA 44 rather than AcA 34 as used by Mochizuki and Hori (18) as the former is better suited to the resolution of proteins of around 50,000 molecular weight.

Thus the purification is fairly simple, relying on conventional techniques. The overall recovery of hexokinase is however fairly low (16 % in the preparation described), and this is mainly due to loss of enzyme at the DEAE-cellulose chromatography stage where some activity is discarded,as it elutes before the major activity peak (see Figure 4.1). The fact that not all the hexokinase elutes as one peak may indicate charge heterogeneity in the enzyme.

CHAPTER 5

THE PURIFICATION OF YEAST GLUCOSE 6-PHOSPHATE DEHYDROGENASE FOR USE AS A COUPLING ENZYME IN THE HEXOKINASE ASSAY

SECTION 5.1 INTRODUCTION

The most widely used assay for hexokinase involves coupling it to yeast glucose 6-phosphate dehydrogenase (G6PDH). Yeast is an important commercial source of G6PDH and enzyme of high specific activity is expensive. Noltmann et al. (78) have published a purification procedure for this enzyme from bakers yeast but the procedure is very lengthy, requiring both silver and solvent precipitation stages, and multiple crystallisations of the enzyme. G6PDH has also been purified from the yeast Candida utilis (79) and has similar physical and chemical properties to the bakers yeast enzyme (78). Easterday and Easterday (72) were able to elute G6PDH and various other enzymes from a crude bakers yeast extract applied to immobilised cibacron blue using various dinucleotides and a pH change.

Whilst trying to study the effects of hydrolysed Procion red H-8BN (H-8BN-OH) on the free solution kinetics of pig heart hexokinase it was noted that the presence of the dye caused long transition times (80) in the assay indicating inhibition of the coupling enzyme, yeast G6PDH. Further study confirmed the strong inhibition of yeast G6PDH although no quantitative data were collected.

Commercial yeast G6PDH was found to bind H8BN Sepharose 6B and could be eluted with low concentrations of NADP. A purification procedure partly based on these results was developed in order to isolate G6PDH of high purity for use as a coupling enzyme in the hexokinase assay.

The purification makes use of ammonium sulphate fractionation, ion-exchange chromatography, affinity elution from a dye-ligand column and gel filtration.

I gratefully acknowledge the assistance of Mrs. K. Hall in developing this purification.

SECTION 5.2 MATERIALS AND METHODS

Drying and storage of yeast

Fresh bakers yeast was purchased from United Yeast Co., Haydock, Liverpool and dried on blotting paper for 6 days at room temperature before storage at -20°C.

Synthesis of H-8BN-Sepharose-6B

2 g of sodium chloride and 1.7 g sodium carbonate were added to a slurry of Sepharose 6B (100 ml) and water (40 ml). The mixture was shaken and 0.2 g H-8BN added. Coupling was achieved by shaking for 40 hr at 45°C. Before and after use the H-8BN-Sepharose column was thoroughly washed with 4 M urea followed by 2 M KCl.

Purification of glucose 6-phosphate dehydrogenase

Buffers

Buffer 1 0.2 M sodium phosphate, pH c. 8;

71.63 g Na_2HPO_4 12 H_2O per litre (not titrated)

Buffer 2 0.05 I phosphate, pH 8;

2.82 g K_2HPO_4 0.2 g KH_2PO_4 per litre

Buffer 3 0.05 I Tris - HCl, pH 7.5;

7.4 g Tris per litre titrated to pH 7.5 with HCl

Buffer 4 0.1 I Tris - HCl, pH 7.5;

14.66 g Tris per litre titrated to pH 7.5 with HCl

All buffers contained 1 mM EDTA and 5 mM β-mercaptoethanol. Buffers 3 and 4 additionally contained 5% glycerol.

Lysis of yeast

200 g dried yeast was added to 500 ml buffer 1 and incubated with occasional stirring for 3 hr at 37°C. Lysed cells were removed by certrifugation at 25,000 g for 50 minutes.

Ammonium sulphate precipitation

The supernatant from above (570 ml) was made 48% in ammonium sulphate by slow addition of 170 g (298 g / l) of the salt and after stirring for 30 min. was centrifuged at 25,000 g for 45 min. The precipitate was discarded and the supernatant (600 ml) made 67% in ammonium sulphate by addition of a further 88 g (148 g / l) of the salt and after 30 min. stirring and centrifugation were repeated. The resultant supernatant was discarded and the precipitate resuspended in a minimum volume (145 ml) of buffer 2. The suspension was dialysed for 36 hr against two changes of 4 l of buffer 2.

DEAE - cellulose chromatography pH 8·

The dialysed solution was pumped onto a DEAE cellulose column (22 x 3.8 cm) equilibrated in buffer 2. Flow-rate was 80 ml / hr. Unbound protein was removed by washing the column with 500 ml buffer 2. The column was developed with a 2 l linear gradient of 0 to 0.25 M KCl in buffer 2. 10 ml fractions were collected. Fractions 73 to 82 (Fig. 5.1) were retained and appeared yellow.

H-8BN - Sepharose chromatography pH 7.5

Active fractions from DEAE - cellulose were vacuum dialysed overnight against buffer. The concentrated enzyme (about 10 ml) was pumped onto a column of H-8BN-Sepharose 6B (9 x 2.3 cm) equilibrated in buffer 3 at a flow-rate of 20 ml /hr. The column was washed with buffer 3 until unbound protein was no longer eluted. Glucose 6-phosphate dehydrogenase was eluted by inclusion of 1 mM NADP in the buffer. 7.5 ml fractions were collected. Fractions 43 to 47 were pooled and appeared pale yellow. See figure 5.2.

AcA 34 chromatography pH 7.5.

Enzyme from H-8BN-Sepharose was concentrated by vacuum dialysis against

Figure 5.1

Chromatography of yeast glucose-6-phosphate dehydrogenase on DEAE-cellulose, pH 8

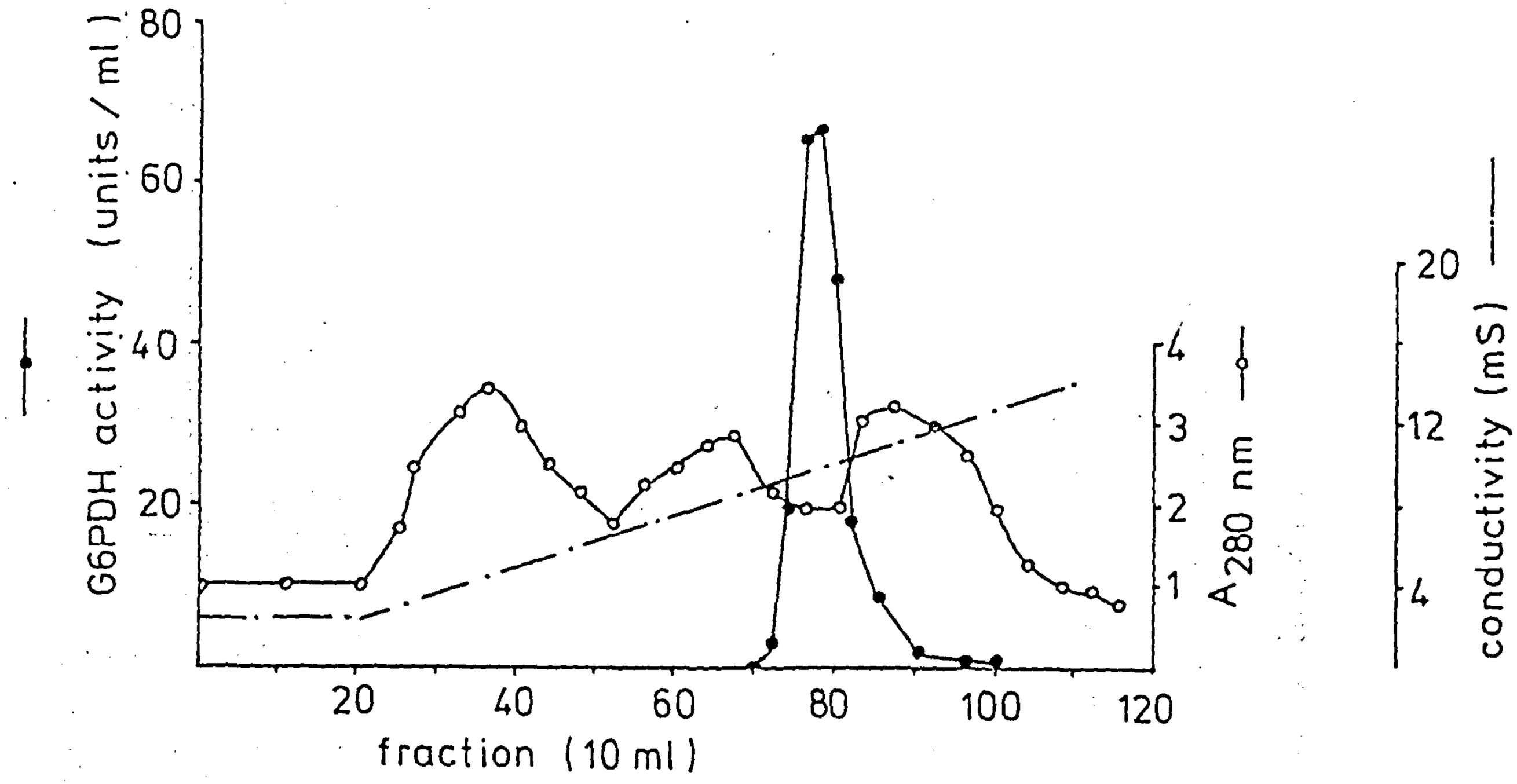


Figure 5.2

The chromatography of yeast glucose 6-phosphate dehydrogenase on H-8BN-Sepharose 6B, pH 7.5.

The arrow marks the inclusion of 1 mM NADP in the column buffer. Further details are given in the text.

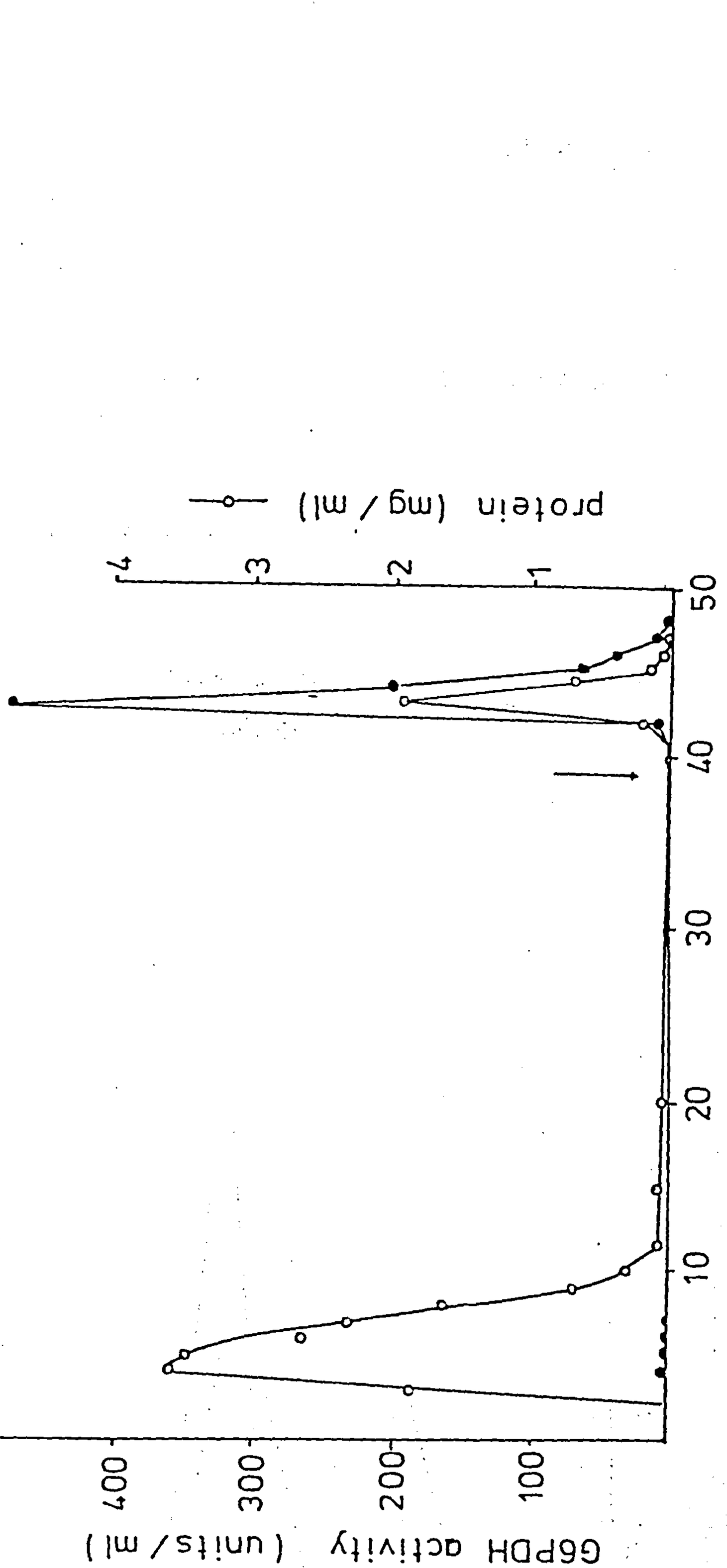


Figure 5.3

The chromatography of yeast glucose 6-phosphate dehydrogenase on AcA 34, pH 7.5.
The last protein peak to elute appears faintly yellow, details are given in the text.

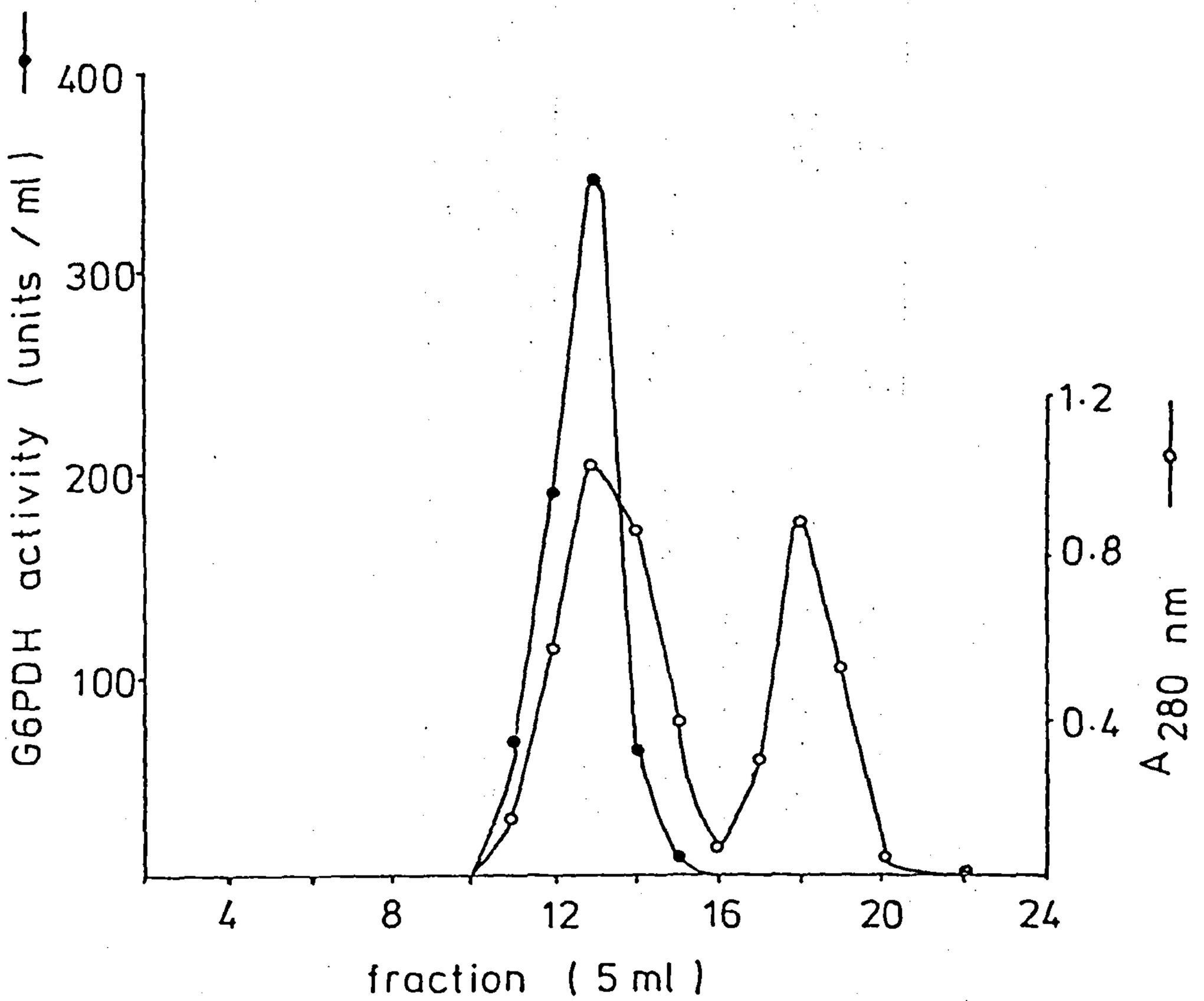


Table 5.1

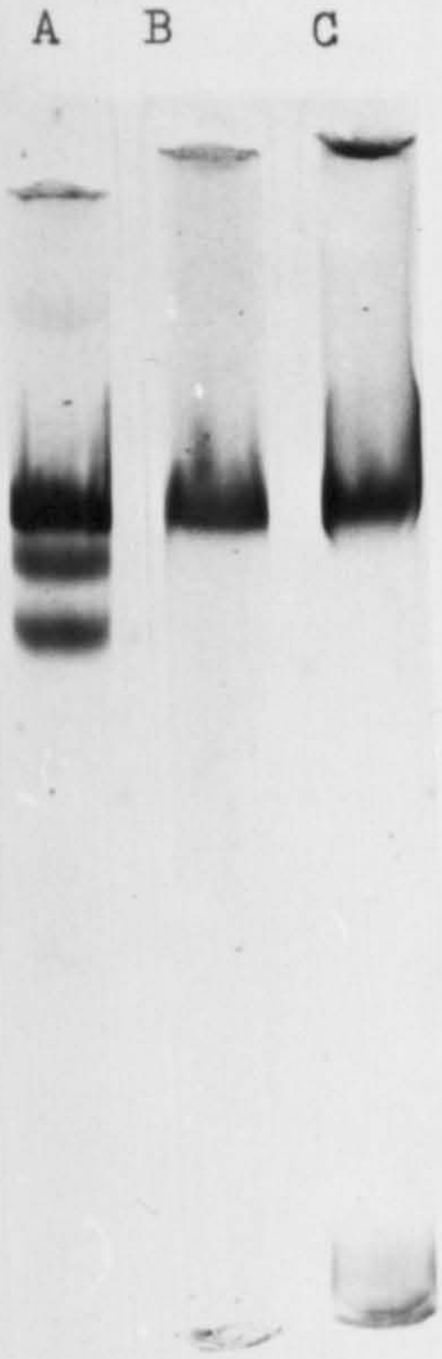
PURIFICATION OF YEAST GLUCOSE 6-PHOSPHATE DEHYDROGENASE (using 200 g freshly dried yeast)

Stage	Enzyme (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Lysate	5590	34,200	0.16	1	100
Amm. sulphate ppt.	6470	10,100	0.64	4	116
DEAE-cellulose, pH 8	4684	212	22	138	84
H-8BN-Sepharose, pH 7.5	4851	31.7	153	956	87
AcA 34, pH 7.5	2962	8	370	2308	53

Figure 5.4 a

Gel electrophoresis of yeast glucose 6-phosphate dehydrogenase.

In all cases the origin of the gel is nearest the top of the figure and the position of the marker-dye bromophenol blue is indicated by bpb.



7.5 % polyacrylamide gel electrophoresis

Gel A ; G6PDH after chromatography on H-8BN-Sepharose 6B . Stained for protein.
Gel B ; after gel filtration, stained for protein.
Gel C ; G6PDH after gel filtration and stained for G6PDH activity.

SDS gel electrophoresis of G6PDH , gel D.

The gel was 10 % acrylamide and was loaded with approximately 40 µg protein from the peak fraction of G6PDH activity after gel filtration (fraction 13 in Figure 5.3).

Figure 5.4 b

Gel electophoresis of yeast glucose 6-phosphate dehydrogenase contd.

After chromatography on H-8BN-Sepharose yeast G6PDH is further purified by gel filtration on AcA 34. A representative gel filtration profile is given in Figure 5.3 and it is seen that two protein peaks elute, the leading one containing G6PDH activity.

Gel E is run on the leading edge of the first pritein peak to elute in gel filtration (fraction 12 in Figure 5.3), gel F on the trailing edge of the first protein peak to elute in gel filtration (fraction 14 in Figure 5.3) and gel G was loaded from a mixture of equal volumes of fraction 12 and 14 in Figure 5.3 , showing the relative proportions of G6PDH (band 1) and contaminant protein (band 2).

All gels are 7.5 % polyacrylamide and are stained for protein, the origin of the gels is nearest the top of the figure.

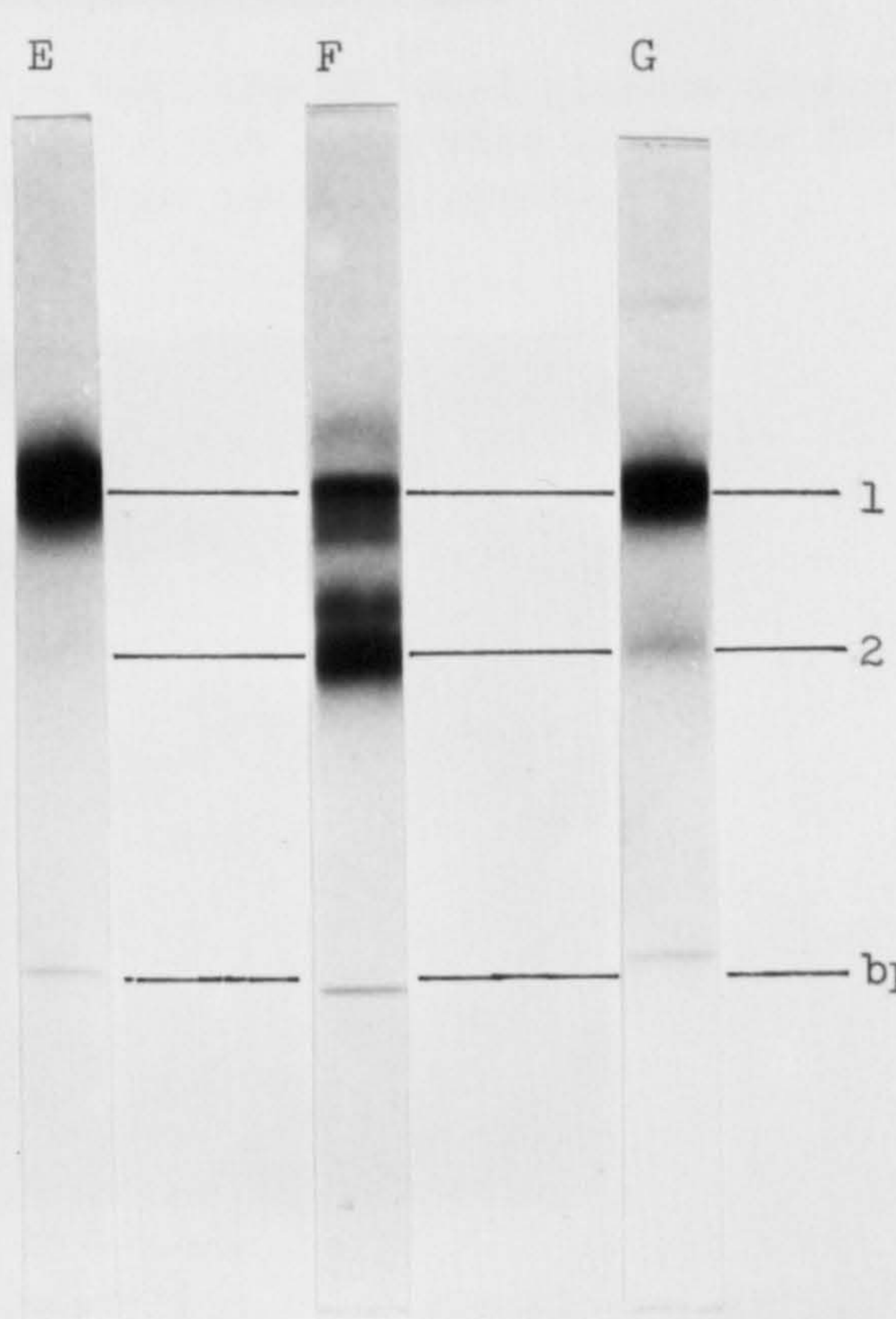
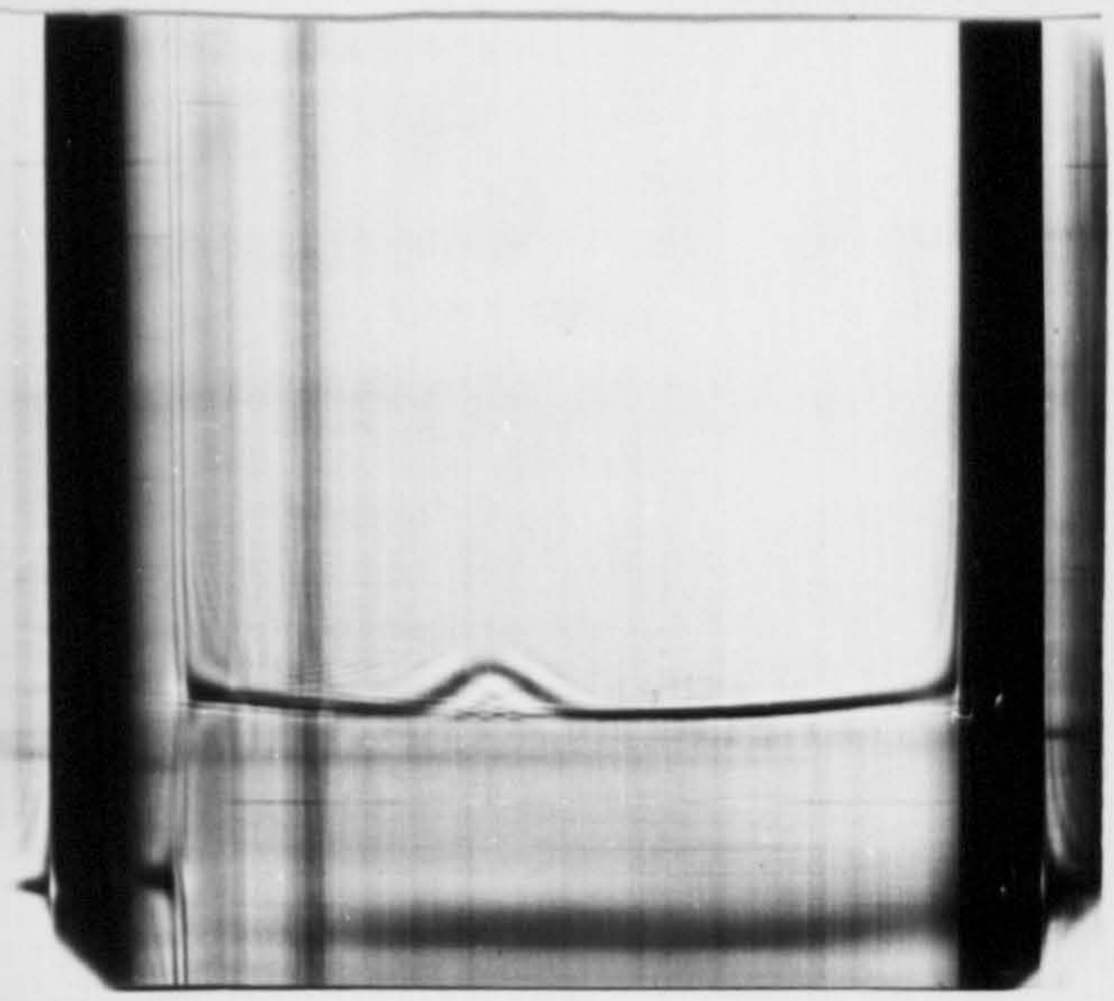
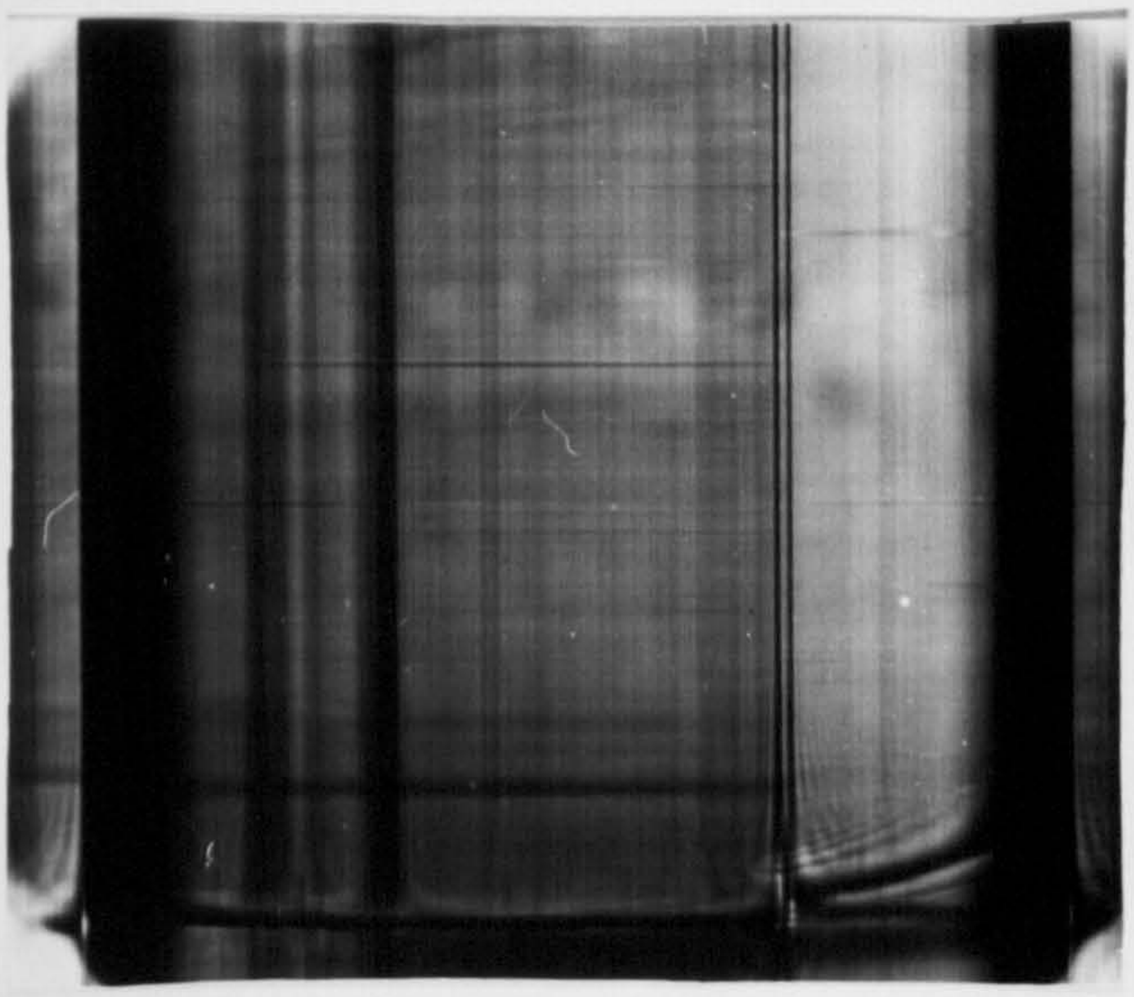


Figure 5.5

The ultracentrifugation of yeast glucose 6-phosphate dehydrogenase . Buffer was 0.1 I Tris-HCl, pH 7.5 containing 1 mM disodium EDTA and 5 mM β-mercaptoethanol.



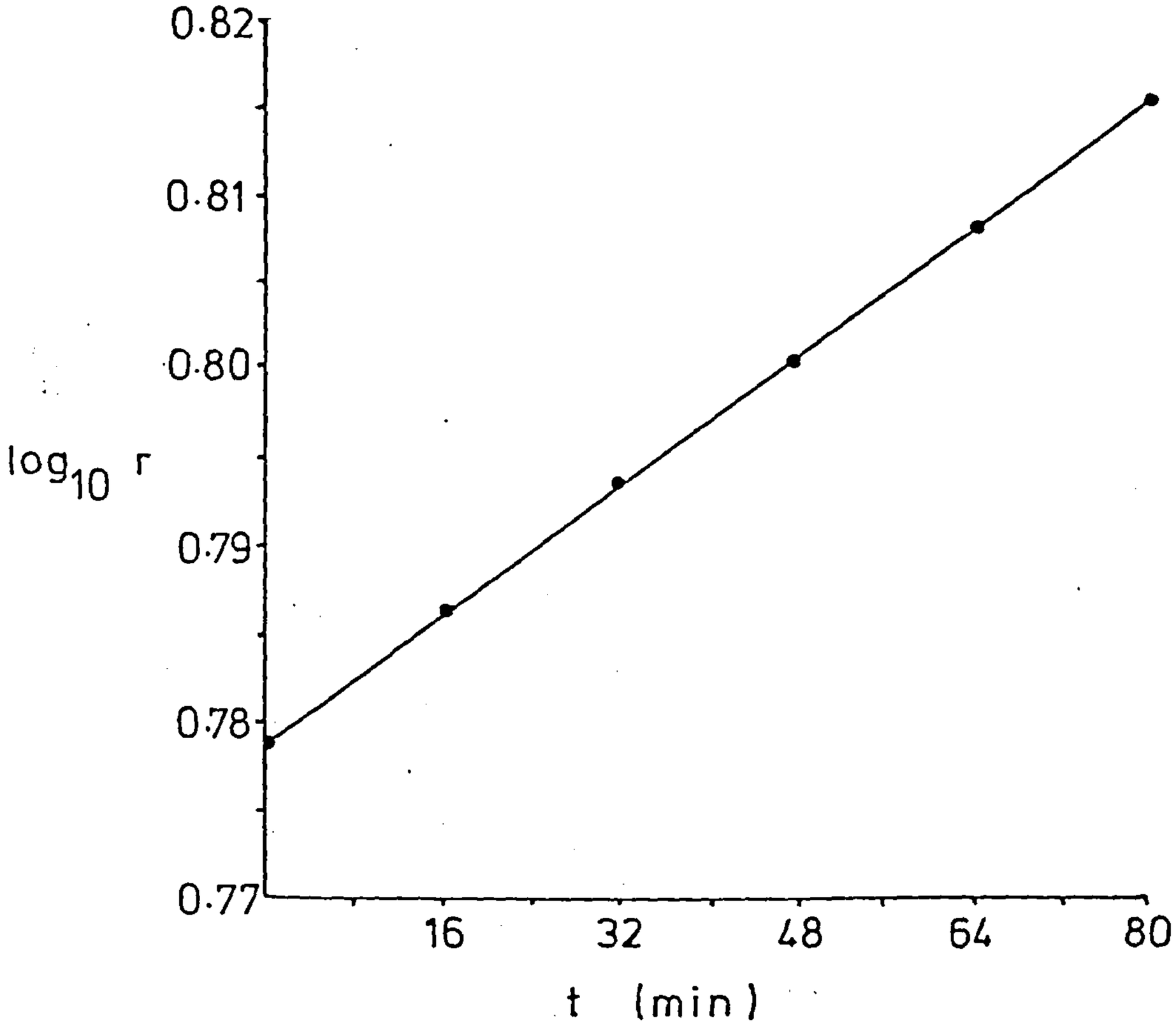
A The sedimentation velocity of yeast glucose 6-phosphate dehydrogenase centrifuged at 60,000 rpm . The wire angle was 60°. The photograph was taken 90 min after reaching speed.



B Sedimentaton equilibrium. The protein concentration was 0.99 mg / ml and details of this experiment are given in figure 5.7 .

Figure 5.6

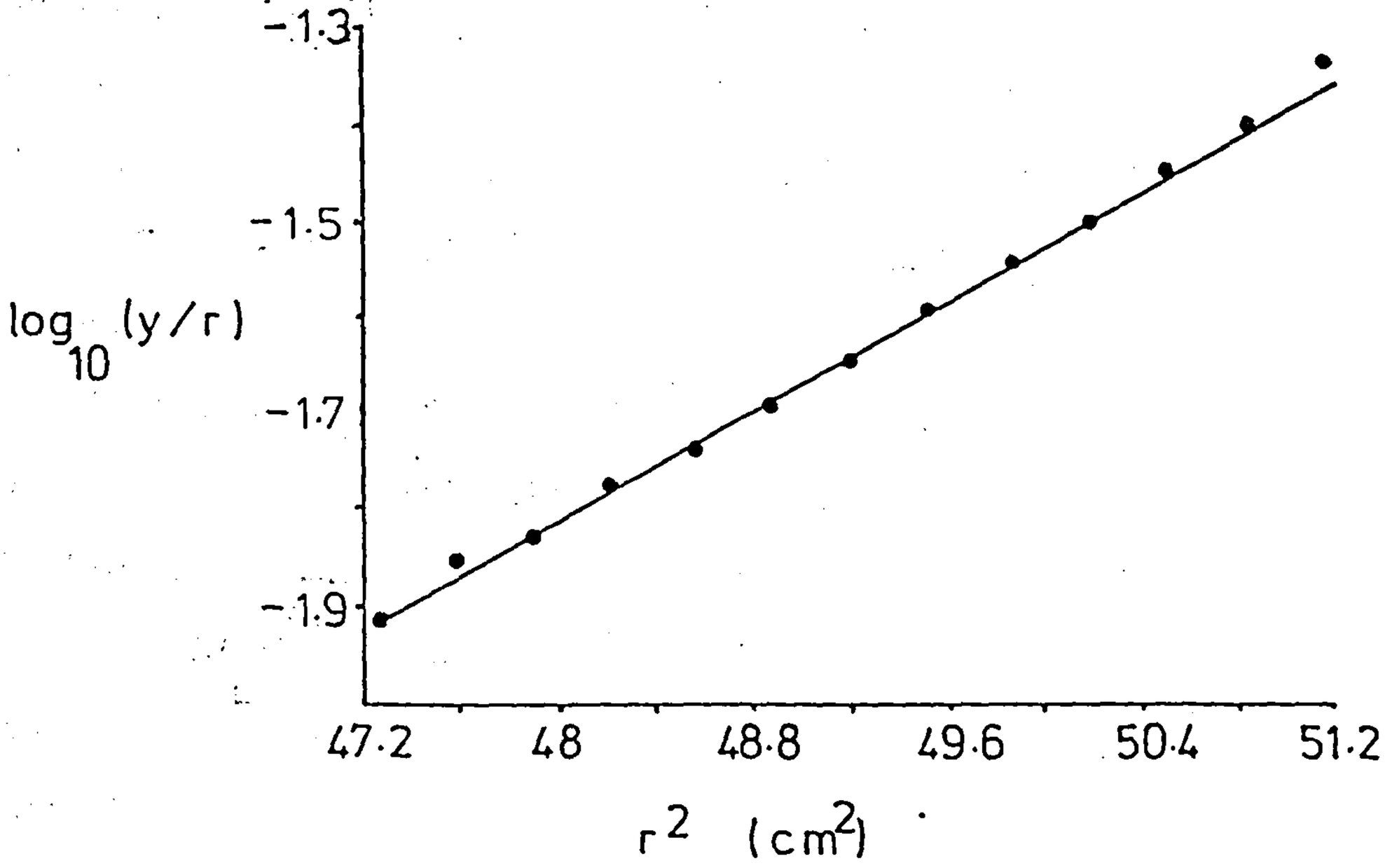
Yeast glucose 6-phosphate dehydrogenase,,sedimentation coefficient.



A plot of log radial distance versus time. Rotor speed was 60,000 rpm and temperature was 10°C. The buffer was 0.1 I Tris-HCl, pH 7.5 and contained 1 mM disodium EDTA and 5 mM β-mercaptoethanol. Protein concentration was 0.99 mg / ml. From linear regression and correction to standard conditions an $S_{20,w}$ value of 5.98 S was obtained.

Figure 5.7

Yeast glucose 6-phosphate dehydrogenase, z-average molecular weight.



Protein concentration was 0.99 mg / ml , temperature 10°C and rotor speed 7,200 rpm . Buffer was the same as that used in determination of sedimentation coefficient. A z-average molecular weight of 105,500 was calculated.

buffer 4 to approximately 1.5 ml and pumped onto a 97 x 1.4 cm AcA 34 column at 6 ml / hr. 5 ml fractions were collected. Fractions 11 to 13 (Fig. 5.3) were pooled and appeared colourless. Details of the purification are given in table 5.1.

DISCUSSION

In the purification illustrated, 8 mg of glucose 6-phosphate dehydrogenase was purified from 200 g dried yeast. The enzyme was judged homogeneous by SDS polyacrylamide electrophoresis and analytical ultracentrifugation which gave an $S_{20,w}$ of 5.98 S and a z-average molecular weight of 105,500.(figures 5.4 to 5.7). The initial stages of the purification are straight forward and a large degree of purification is obtained by chromatography on DEAE-cellulose. The success of dye-ligand chromatography of yeast G6PDH depends on the size of the H8BN-Sepharose column used and the degree of substitution with the dye. Too small a column will not bind all the enzyme applied and elution of G6PDH from too large a column is costly in NADP as elution is no longer 'frontal' but is retarded. The size limits of the column are however not critical and are easily found by experimentation. One drawback of this part of the purification is that it requires relatively large amounts of NADP to effect elution, however the resultant purification, excellent reproducibility and very high recovery of G6PDH justify the use of NADP as an eluting ligand. After affinity elution from H-8BN-Sepharose the pooled fractions containing G6PDH appeared slightly yellow indicating the presence of contaminating proteins. Polyacrylamide gels run on these samples showed the presence of at least three proteins (figure 5.4). The contaminants are removed by gel filtration where two major protein peaks are seen. The first

peak to elute contains G6PDH and some other protein (see Fig 5.4) and is not entirely coincident with the peak of enzyme activity. For this reason fractions containing G6PDH which appear after the fraction of peak enzyme activity were discarded. Gel filtration should be carried out immediately after dye-ligand chromatography as the enzyme appears to be susceptible to proteolysis at this stage. If left at $4^{o}C$ for a few days, more protein bands appear on polyacrylamide gels run on the trailing edge of the first protein peak to elute on gel filtration. The protein peak eluting well after that containing the G6PDH appeared faintly yellow and had a UV absorption spectrum similar to that of nucleoproteins (81). It contained no diaphorase activity. Inclusion of 5% glycerol in buffers 3 and 4 significantly improved the yield of G6PDH.

The purified enzyme had a specific activity of 370 u / mg (table 5.1) and appeared largely homogeneous. Noltmann et al (78) reported a final specific activity of 668 u / mg for their preparation of bakers' yeast G6PDH. This difference is hard to explain as enzyme from the present purification was invariably more than 94% pure as judged by polyacrylamide gel electrophoresis. A solution containing 2 units per ml of yeast G6PDH from this purification contained no detectable hexokinase activity and was considered suitable for use as a coupling enzyme.

CHAPTER 6

DISCUSSION OF THE APPLICATION AND MECHANISM OF TECHNIQUES IN PROTEIN PURIFICATION

SECTION 6.1 PROTEIN PRECIPITATION

The use of two non-specific protein precipitants, ammonium sulphate and polyethylene glycol 6000 (PEG 6000) is described in this thesis. Ammonium sulphate is probably the most widely used protein precipitant and is the salt of choice not only because it is cheap, readily soluble and innocuous (82) but also because SO_4^{2-} is one of the most effective protein precipitants in the Hofmeister Series, choice of cation having less effect on precipitation. It is thought that protein is precipitated by ammonium sulphate because the salt competes with protein for water of solvation, thereby decreasing the proteins solubility. The solubility, s of a protein in ammonium sulphate is given by the Cohn equation,

$$\log s = X - \alpha \cdot C$$

where C is the concentration of the salt. X and α are constants. Less is understood of the mechanism of polyethylene glycol precipitation and it is an infrequently used technique. Foster et al. (83) found the behaviour of PEG as a precipitant similar to that of ammonium sulphate but unlike salting-out, PEG precipitation is somewhat dependent on protein concentration and molecular weight; average enzymes being precipitated by a 6 to 12% concentration of PEG 6000 and viruses between 2 to 4% of the same precipitant. Honig and Kula (84) have shown that smaller proteins are precipitated by larger polyethylene glycols and that smaller molecular weight polyethylene glycols tend to make more specific precipitants. However larger polyethylene glycols eg. PEG 6000 tend to precipitate proteins more sharply than small polyethylene glycols. Jukes (85) has proposed that polyethylene glycol in solution sterically excludes protein from the solvent and thus effects precipitation. Ammonium sulphate tends to precipitate proteins fairly sharply, over a narrow range of the salt. For this reason, and due to the rapid solution of ammonium sulphate precipitated

protein in fresh buffer, the salt is ideal for use in multiple precipitation steps ('cuts') as was used for yeast glucose 6-phosphate dehydrogenase in this thesis. Alternatively it can be used for retrogradient chromatography on unsubstituted agarose (86). The precipitation profiles of various glycolytic enzymes by PEG 6000 were investigated by Kopperschlager and Johansson (87) and found to differ considerably. Asterias rubens hexokinase was precipitated over a fairly wide range of PEG 6000 (between 8 to about 15% at pH 7.0) and difficulty in resuspending hexokinase in good recovery ruled out the use of multiple precipitations in this case. Instead, one precipitation was used to remove some contaminant protein. Other workers have incubated protein with polyethylene glycol for up to 2 days to achieve full precipitation of protein, however in the Asterias purification, enough precipitation of protein was achieved in 1 hr to make use of the technique desirable in this case. One advantage of polyethylene glycol over ammonium sulphate is that it does not increase the ionic strength of solutions and they can thus be applied directly to ion-exchange resins whereby polyethylene glycol washes off with unbound protein; additional polyethylene glycol is cheap, readily available and low in heavy metal contaminants.

SECTION 6.2 ION-EXCHANGE CHROMATOGRAPHY

Ion-exchange chromatography is a well established and widely used technique. Ion-exchange resins have a high capacity for protein and commonly give large purifications from crude protein preparations, thus making useful early stages in protein purification.

Generally, proteins bind anion-exchange resins at pH's above their pI's, and cation-exchangers at pH's below their pI's, however unevenly distributed protein surface charge may cause type I and type II hexokinase to bind phosphocellulose at pH's above their pI's (46,88). The properties and uses of cellulose based ion exchange resins has been

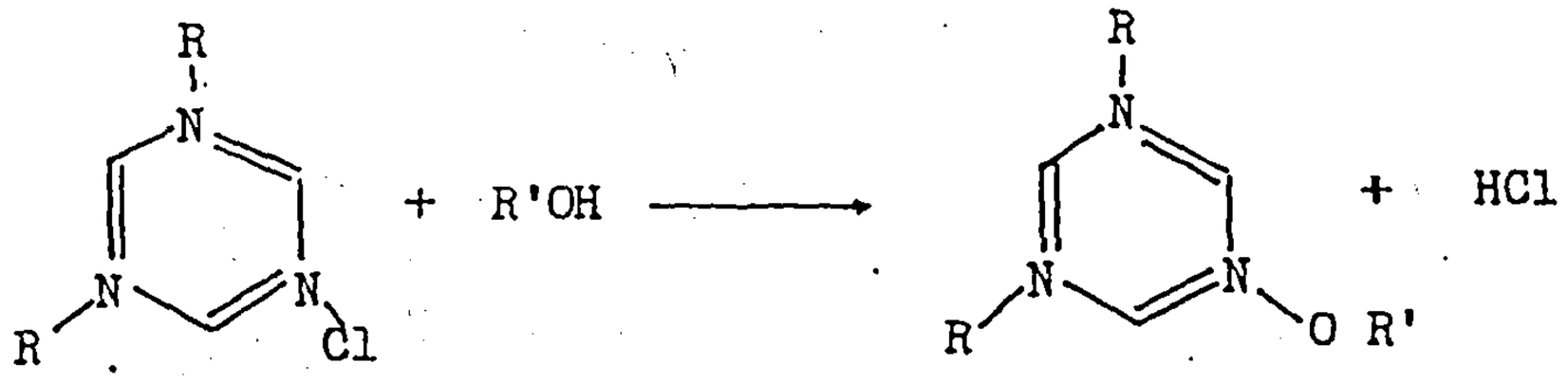
reviewed by Peterson (89). A low and even distribution of charge groups (1.0 meq / g, Whatman technical data) on DEAE-cellulose DE-52 allows mild recovery conditions. DEAE-Sephadex A-50, an agarose based ion-exchange resin has a higher charge density (3.5 ± 0.5 meq / g, Pharmacia technical data) and behaves in a slightly different manner to DEAE-cellulose DE-52. Although some further purification of a crude protein is usually achieved by rechromatography of the sample on DEAE-cellulose, a larger purification can be obtained by using DEAE-Sephadex as a second chromatographic step after DEAE-cellulose. Holroyde and Trayer (47) achieved a further 5-fold purification of muscle type II hexokinase in this manner. In the present study Asterias rubens hexokinase is subject to consecutive DEAE-cellulose and DEAE-Sephadex chromatography at pH 8.5 and pH 5.5 respectively, with a $2\frac{1}{2}$ - fold gain in purity from the second step.

The use of phosphocellulose has been avoided where possible as the commercial resin is of variable quality and pKs of the multiple phosphoryl groups are sensitive to ionic strength changes ie. increasing ionic strength depresses the pK thereby lowering the pH of the column and thus making a protein bind tighter (see equation 2.2.3, chapter 2)

SECTION 6.3 DYE-LIGAND CHROMATOGRAPHY

Triazine dyes are water-soluble polyaromatic compounds based on substituted cyanuric chloride. In dichlorotriazine dyes one chloride from cyanuric chloride is replaced by a non-labile chromophore whereas in monochlorotriazine dyes cyanuric chloride is substituted with both a chromophore and a second non-labile group, often aminobenzene sulphonate. The chromophore can be based on anthraquinone, phthalocyanine or azo-compounds (90). Dichlorotriazine dyes

are more reactive than monochlorotriazine dyes but under alkaline conditions both types react readily with polysaccharide supports (R' OH) as hydroxyl groups replace labile chlorine atoms by nucleophilic attack:



The structures of several triazine dyes including the widely used Cibacron Blue F-3GA and Procion Red HE-3B are given in figure 6.1. The structure of the dye H-8BN used in the present study is not available.

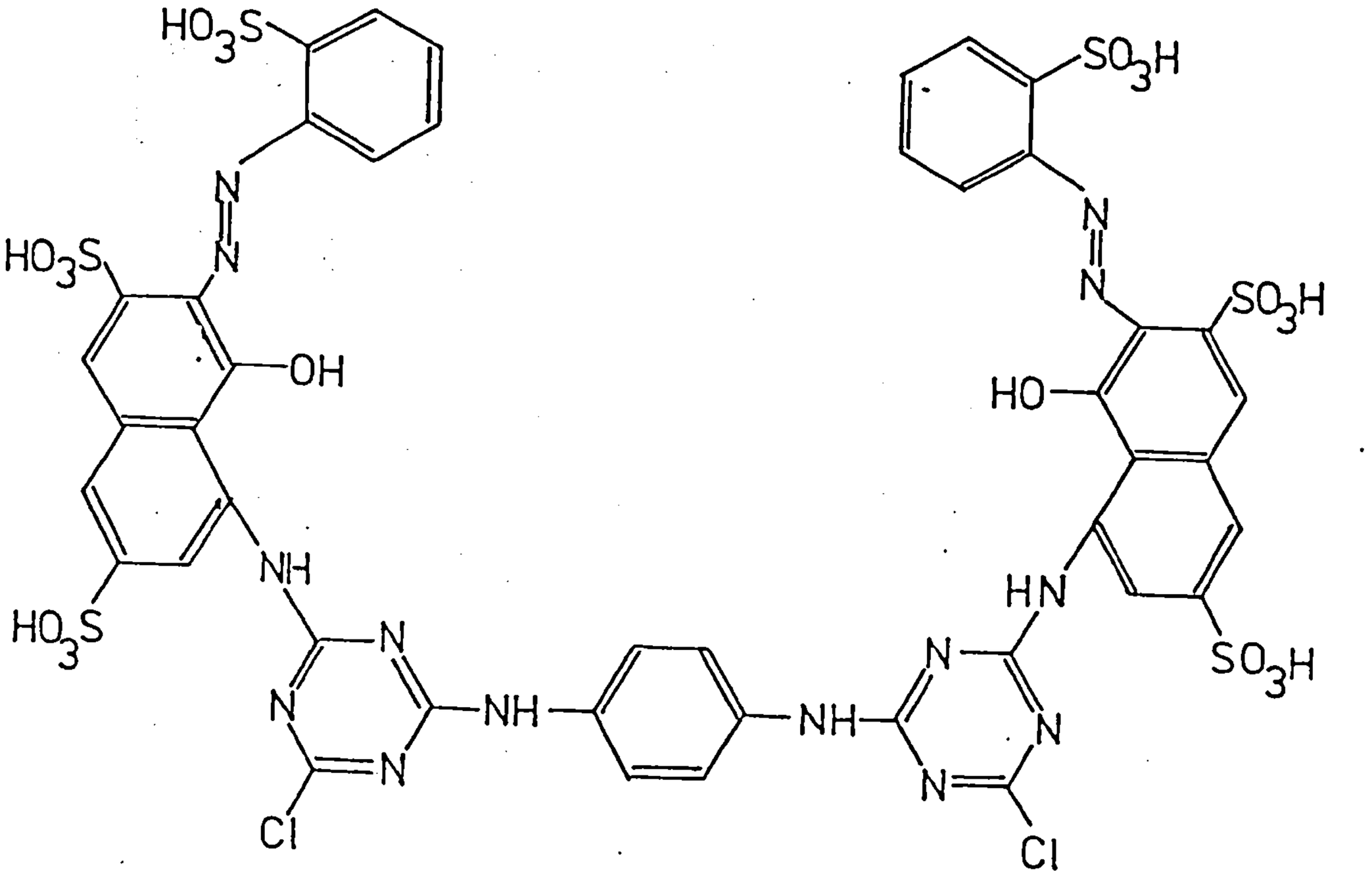
Dye-ligand columns have capacities for protein 10 to 100 times higher than immobilised nucleotide columns (91) but a lower capacity than ion-exchange media thus dye-ligand chromatography is ideally used after an ion exchange stage in the purification of a protein. A wide variety of proteins bind immobilised triazine dyes and early claims that the dyes were nucleotide analogues binding only proteins possessing the dinucleotide fold are now largely discounted (92). Much non-specific binding of protein can occur and in order to improve the degree of purification attainable affinity elution from the dye columns is becoming increasingly popular. Salt elution has also been used to recover protein from dye-ligand columns although the purification achieved in this way is often less than that from affinity elution. Recently Robinson et al. (93) have suggested a systematic approach to salt elution from dye-ligand columns, choice of conditions having previously been largely empirical.

Immobilised dyes are good supports for affinity elution as the dyes, being negatively charged, are unlikely to bind substrates or effectors (which are normally negatively charged themselves) and binding of the

Figure 6.1

THE STRUCTURES OF SOME REPRESENTATIVE TRIAZINE DYES.

Procion red HE-3B



Procion yellow H-A.

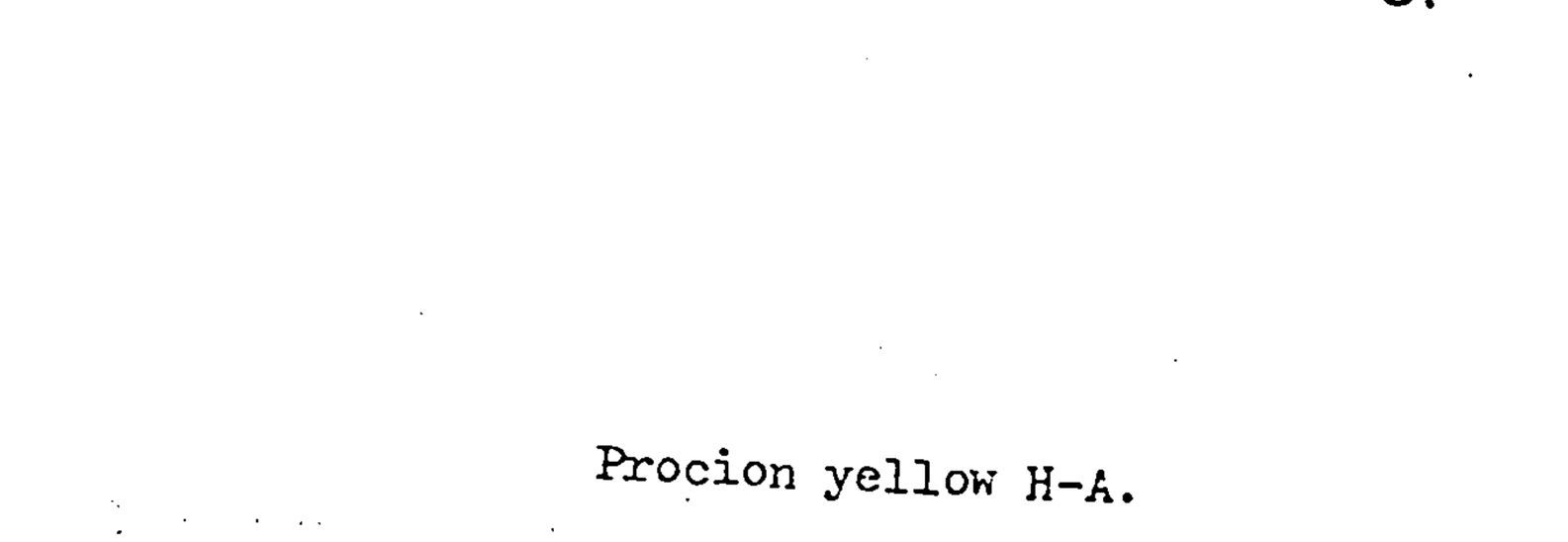
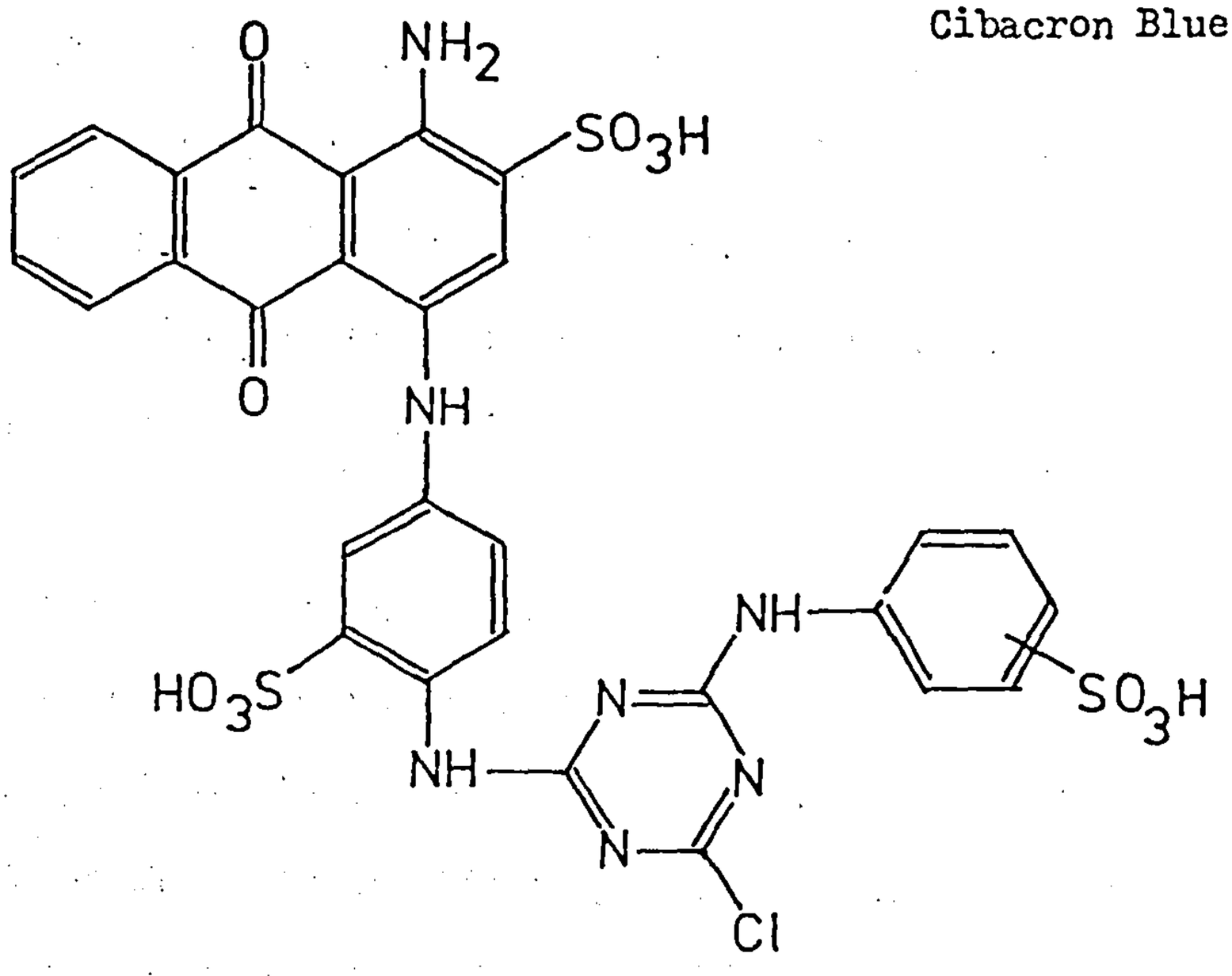
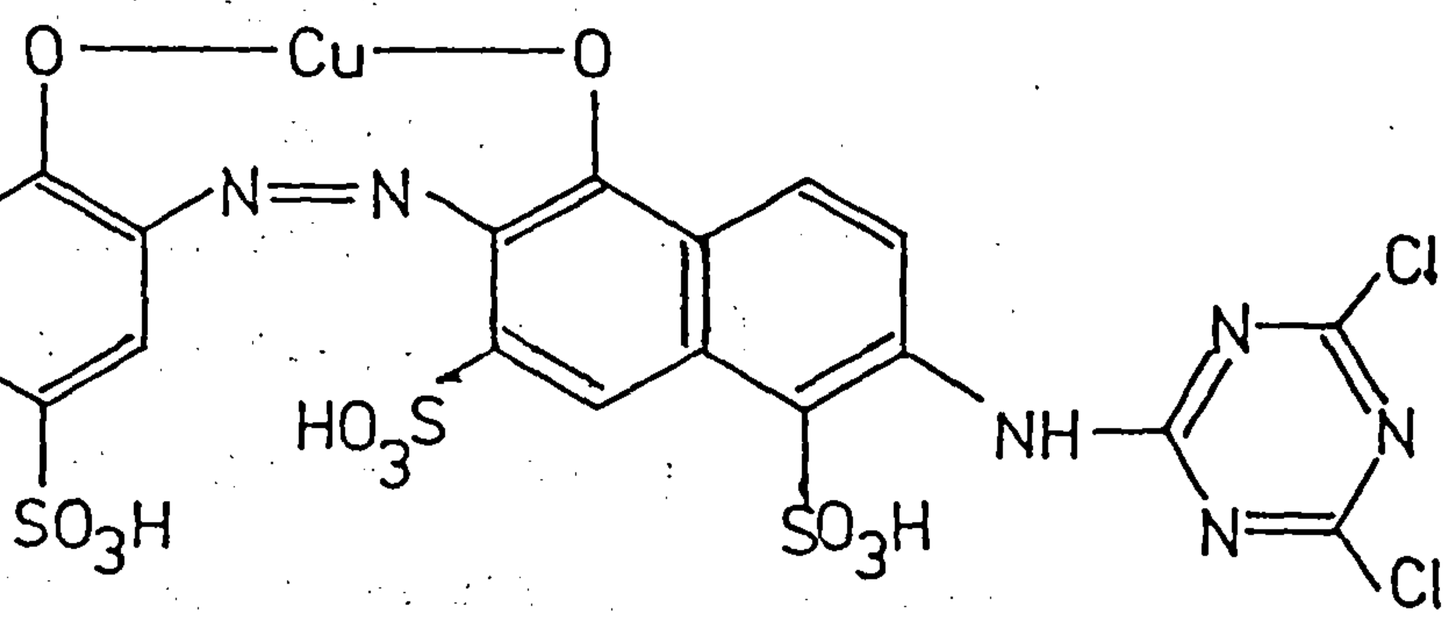


Figure 6.1 contd.

Cibacron Blue F3G-A



Procion Rubine MX-B



protein to the column is often weak enough to allow such elution.

In order to select the best dye for a particular purification it is necessary to carry out screening to establish the efficiency of binding and elution of a given protein from a number of immobilised dyes. However such data for porcine heart hexokinase and yeast glucose 6-phosphate dehydrogenase are not given as screening was not carried out under uniform conditions. In retrospect, screening although tedious is unavoidable if the best dye is to be chosen.

The use of free solution kinetics to select those dyes that interact with an enzyme is possible except, as in the present study, when the dye inhibits the coupling enzyme. The hydrolysed dye H-8BN-OH was found to inhibit pig heart hexokinase weakly but was a much stronger inhibitor of the coupling enzymes yeast glucose 6-phosphate dehydrogenase and rabbit muscle pyruvate kinase. This ruled out the possibility of kinetic studies of the binding of H-8BN-OH to hexokinase as impracticaly high levels of both NADP and glucose 6-phosphate dehydrogenase would have been necessary to assay hexokinase in the presence of the dye. Other groups also using yeast glucose 6-phosphate dehydrogenase as a coupling enzyme in the hexokinase assay have not reported this effect with other free dyes (71,94). Qualitatively, the degree of inhibition of heart hexokinase and yeast glucose 6-phosphate dehydrogenase by H-8BN-OH did however seem to be related to the ease with which the enzymes could be eluted from the immobilised dye with their respective ligands. In the purifications described heart hexokinase was eluted with 1.8 mM ATP (3x the Km for MgATP) whereas the dehydrogenase was eluted with 1 mM NADP (c. 50 x its Km (95)), less NADP did not elute this enzyme sharply and large volumes of NADP solution were necessary

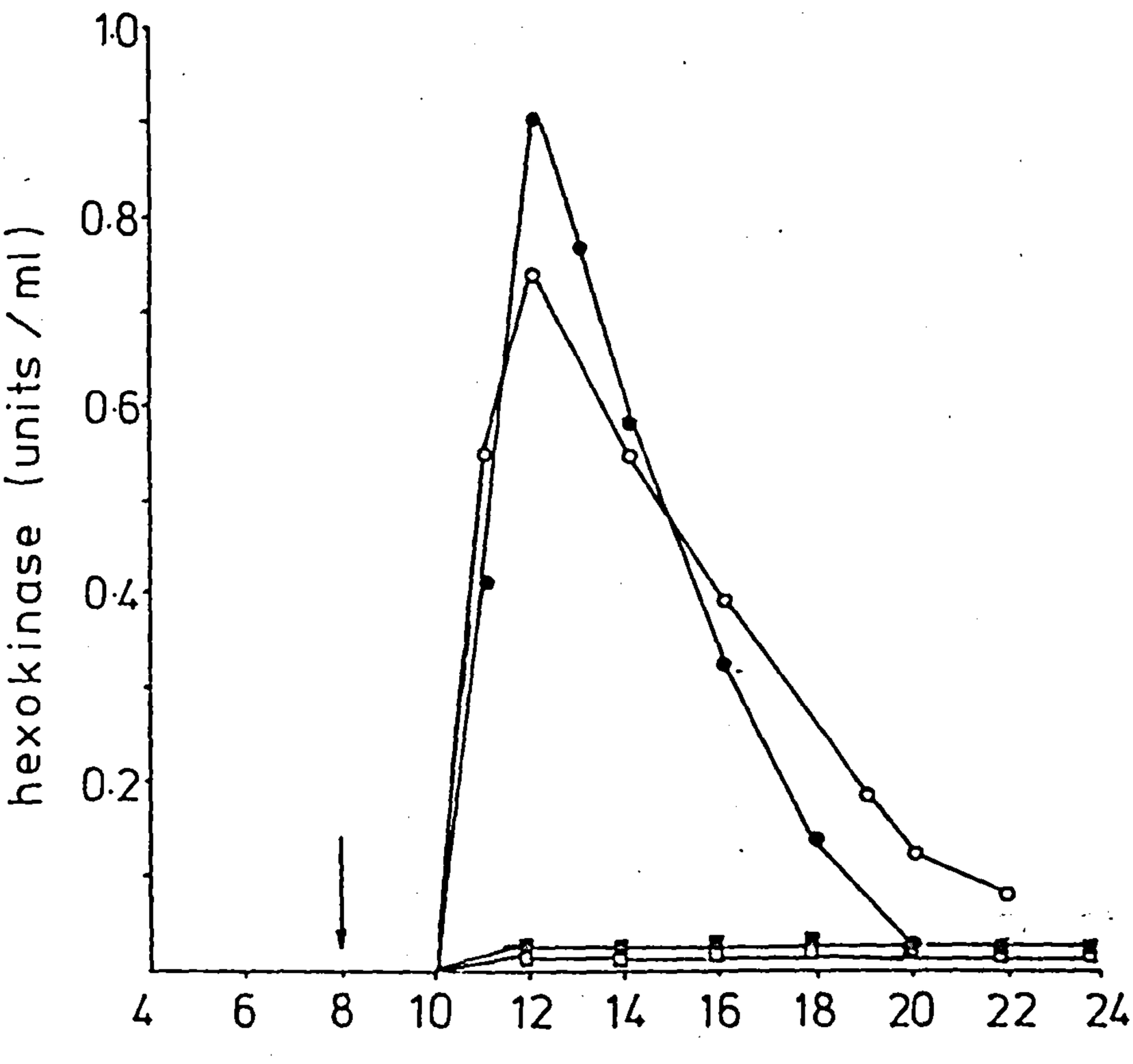
to achieve good recoveries of activity. The yeast glucose 6-phosphate dehydrogenase purification could be improved by selecting a dye which allowed elution of the enzyme with far lower levels of NADP which is an expensive ligand. Alternatively , it might be possible to tailor buffer conditions to allow weaker binding of the dehydrogenase for example by increasing pH (55). The glucose 6-phosphate dehydrogenase purification was however reliable and efficient.

The effects of a number of ligands on the elution of heart hexokinase fron H-8BN-Sepharose 6B were investigated , the results are displayed in figure 6.2 . Columns holding homogeneous hexokinase were pulsed with various ligands at concentrations approximately 1.5 times their respective Km and Ki values. Glucose 6-phosphate and glucose 1,6-bisphosphate did not effect more than very slight leakage of hexokinase from the column (and there is often a very low level of background leakage of enzyme from small triazine dye columns) whereas AMP and ATP both caused sharp elution of the enzyme. Since glucose 1,6-bisphosphate is more highly charged than AMP the elution is not through a charge specific effect as was implicated by Qadri and Easterby (96) for the elution of type II hexokinase from the ion-exchange resin phosphocellulose. It thus seems possible that in both the case of porcine heart hexokinase and yeast glucose 6-phosphate dehydrogenase the dye is acting as a nucleotide analogue and that nucleotide competes with immobilised dye to effect elution of the enzyme. Recently , Biellman et al. showed in an X-ray study that Cibacron Blue F-3GA bound the nucleotide site of crystallised horse liver alcohol dehydrogenase (97). Similar conclusions for other dehydrogenases and kinases have been drawn on the basis of free solution kinetics(71,98) and affinity labelling with the unhydrolysed dye(99).

Figure 6.2

THE EFFECTS OF LIGANDS ON THE ELUTION OF HEXOKINASE FROM H-8BN-SEPHAROSE 6B.

Fifteen units of enzyme were applied to a 2 x 4-cm column equilibratedwith 0.05 I Tris-HCl, pH 7.5, containing 1 mM EDTA, 100 mM glucose and 5 mM β-mercaptoethanol. Flow-rate was 30 ml/h. Elution was effected by the inclusion of the following ligands in the buffer from the arrow onwards:
1.5 mM ATP (•)
7.8 mM AMP (○)
66 µM or 2 mM glucose 6-phosphate (■)
150 µM glucose 1,6-bisphophate (□).



Note ; low levels of ADP also elute heart hexokinase from immobilised H-8BN.

CHAPTER 7

THE STEADY STATE KINETICS OF HEXOKINASE :

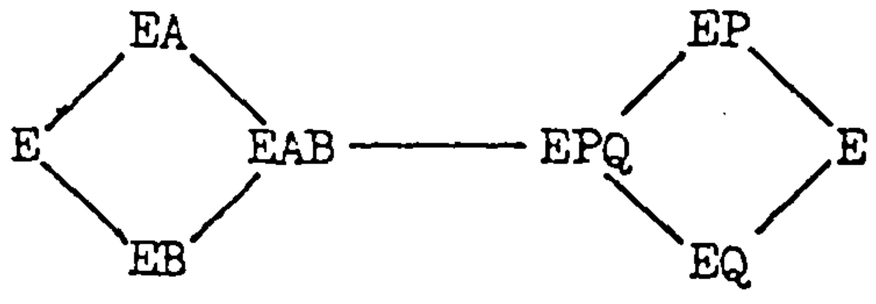
MATERIALS AND METHODS

SECTION 7.1 INTRODUCTION

Steady-state kinetics is a well established and powerful technique for the study of enzyme action and has the advantages that a working enzyme is studied in a continuous and fully characterised assay.

In the present study the substrate kinetics of heart and Asterias hexokinase were investigated and a study of the single inhibitor kinetics then made. Finally, to establish the interrelationship of inhibitor binding sites ,'double inhibitor kinetics'were carried out. Easterby and Qadri (100) used this technique to study skeletal muscle type II hexokinase and the equations presented are based on those of Qadri (88) and are more generalised and more fully developed than those published by other workers (101 op.cit) in that substrate binding is taken into account.

The equations are developed for a rapid equilibrium random mechanism for hexokinase;



where A and B represent the substrates $MgATP^{2-}$ and glucose and P and Q represent the products glucose 6-phosphate and $MgADP^{-}$ respectively.

Assay conditions for hexokinase from both heart and Asterias were kept as similar as possible so the results from both enzymes were readily comparable. The kinetic studies aimed to identify regulatory ligands, particularly those of physiological importance , and to clarify the nature of their interaction with respect to the substrates MgATP and glucose. Double inhibitor experiments would provide information on the interrelationship of inhibitor binding sites.

Hexokinases can be inhibited by a variety of ligands including G6P, AMP, ADP and phosphate. The nature of G6P inhibition of hexokinase was discussed in Chapter 1, Section 1.5 ; G6P is known to be a potent inhibitor of the 100,000 molecular weight, low Km hexokinases but does not inhibit the 50,000 molecular weight hexokinases from,for example, yeast or liver. The theory of partial gene duplication in the evolution of hexokinase was proposed on the basis of these facts, only the high molecular weight hexokinases having a regulatory site for G6P (Chapter 1, Section 1.6). On this basis one would also expect to find only one nucleotide binding site in the 50,000 molecular weight hexokinases but possibly two nucleotide binding sites in the high molecular weight hexokinases.

The mechanism of regulation of hexokinase by G6P and other ligands like nucleotides can only be understood when the nature of binding is ascertained and when the number of binding sites for each ligand is known. In order that the mechanism of regulation of hexokinase in the presence of a number of ligands be known the interrelationship of binding sites must be established.

Thus a steady state kinetic study of both heart and Asterias hexokinases was undertaken to establish the nature of regulation of both enzymes and to test the theory of partial gene duplication in the evolution of hexokinase.

SECTION 7.2 MATERIALS AND METHODS

Reagents

All reagents used were of the highest purity available and were purchased from either Fisons (Loughborough,UK) or BDH (Poole,UK).

Coupling enzymes

Yeast glucose 6-phosphate dehydrogenase (E.C.1.1.149) was either purified as described in chapter 5 of this thesis or purchased from Boehringer and Sons (Mannheim,FRG) as the 'grade II' preparation of 140 units/mg. Rabbit muscle pyruvate kinase (E.C.2.7.1.40) was purified in this laboratory by Mrs.K.Hall following the procedure of Tietz and Ochoa (102). Rabbit muscle lactate dehydrogenase (E.C.1.1.1.27) was purchased from Boehringer and Sons.

Nucleotides

ATP, NADP and NADH were from PL Biochemicals (Milwaukee,USA). All other nucleotides were from Boehringer and Sons.

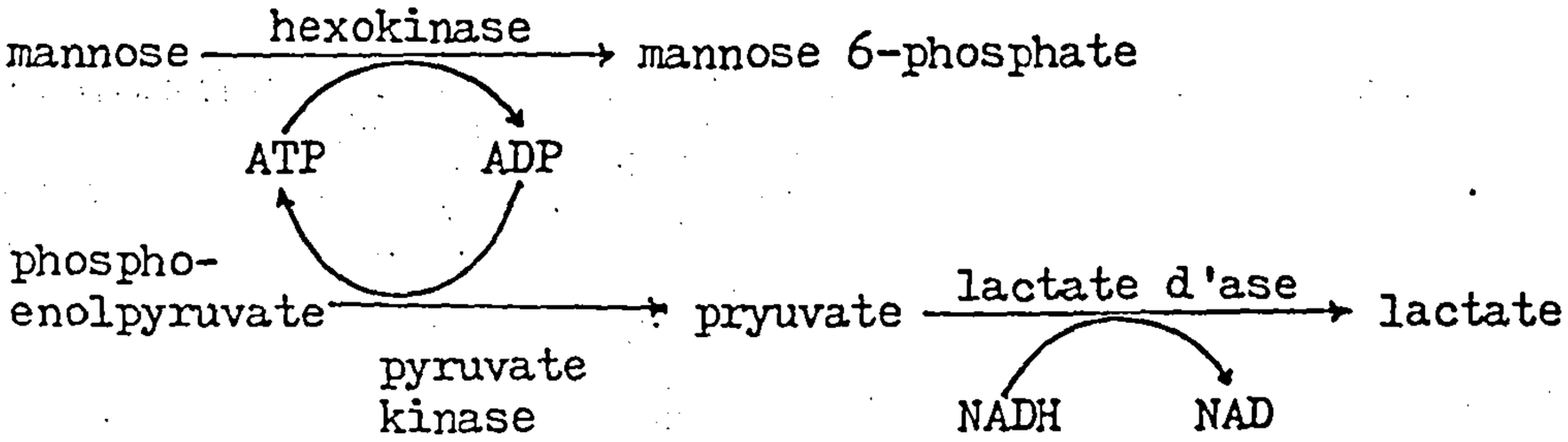
Sugars

Glucose of analytical grade was purchased from Fisons and mannose from BDH.

Assays

The glucose 6-phosphate dehydrogenase linked assay of hexokinase described earlier (chapter 2, section 2.3) was used for kinetic measurements when glucose was a substrate for hexokinase. When glucose 6-phosphate inhibition of hexokinase was studied mannose was used as the substrate for hexokinase and ADP production was coupled to pyruvate kinase and lactate dehydrogenase (25). The reaction was

monitored by a decrease in absoption at 340 nm due to NADH oxidation;



Each assay contained 60 µmol Tris-HCl, pH 7.5 , 1.5 µmol phosphoenol-pyruvate, 50 µmol KCl , 0.2 µmol NADH , 7.5 units pyruvate kinase and 4.5 units lactate dehydrogenase in a total volume of 1.0 ml. Both coupling enzymes were dialysed against assay buffer for 2 hr to remove ammonium sulphate. Great care was taken to use freshly purchased NADH; once partially decomposed NADH caused a lag in the assay which was exacerbated by addition of further NADH.

Assay solutions

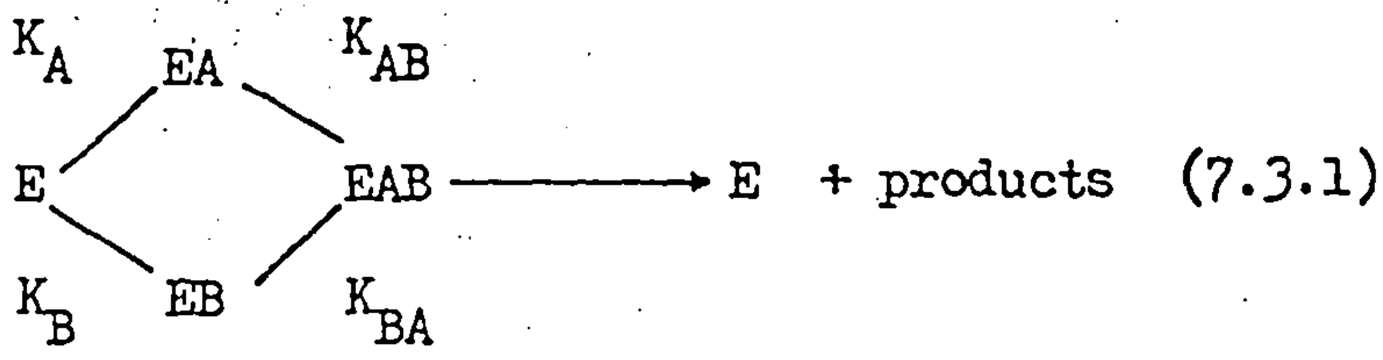
Hexose substrate , hexose phosphates and AMP were made up in assay buffer. ADP and ATP were dissolved in assay buffer and carefully titrated to pH 7.5 with 0.5 M KOH. For phosphate inhibition studies pH 7.5 potassium phosphate buffers of requisite molarity were made. When variation of substrate or inhibitor levels introduced a wide variation in the ionic strength of the assay (ie. greater than 0.004 I) solutions of KCl in assay buffer were added to maintain constant ionic strength.

$MgATP^{2-}$ and $MgADP^{-}$ levels

The level of Mg^{2+} in the assay was usually maintained at 2 mM above the total of ATP and ADP such that levels of free nucleotide would be negligilbly small for the purpose of kinetic analysis (103).

SECTION 7.3 SUBSTRATE KINETICS

For the rapid equilibrium random addition of substrate to hexokinase where E is hexokinase , A is ATP and B is glucose the interconversion of complexes is ;



(7.3.1)

K values are the dissociation constant for each complex. The total amount of enzyme in the presence of substrates A and B is ;

$$[E_T] = [E] + [EA] + [EB] + [EAB] \qquad (7.3.2)$$

$$\text{and } [EA] = \frac{[E]\,[A]}{K_A} \qquad (7.3.3)$$

$$[EB] = \frac{[E]\,[B]}{K_B} \qquad (7.3.4)$$

$$[EAB] = \frac{[EA]\,[B]}{K_{AB}} = \frac{[E]\,[A]\,[B]}{K_A . K_{AB}} \qquad (7.3.5)$$

$$\text{thus, } [E_T] = [E]\left(1 + \frac{[A]}{K_A} + \frac{[B]}{K_B} + \frac{[A]\,[B]}{K_A . K_{AB}}\right) \qquad (7.3.6)$$

the ratio of maximum velocity V, to actual velocity v, is equal to the ratio $[E_T] / [EAB]$, thus dividing equation 7.3.6 by $[EAB] = \frac{[A]\,[B]}{K_A . K_{AB}}$ and rearranging ;

$$\frac{1}{v} = \frac{1}{V}\left(\frac{K_A . K_{AB}}{[A]\,[B]} + \frac{K_{AB}}{[B]} + \frac{K_A . K_{AB}}{K_B\,[A]} + 1\right) \qquad (7.3.7)$$

For a random order equilibrium mechanism as shown in 7.3.1 , $K_A.K_{AB} = K_B.K_{BA}$ so that equation 7.3.7 may be modified ;

$$\frac{1}{v} = \frac{1}{V}\left(\frac{K_A.K_{AB}}{[A][B]} + \frac{K_{AB}}{[B]} + \frac{K_{BA}}{[A]} + 1\right) \qquad (7.3.8)$$

For practical purposes this equation can be rearranged to the Hanes form, [substrate]/ v versus [substrate];

$$\frac{[A]}{v} = \frac{1}{V}\left(\left(K_{BA} + \frac{K_A.K_{AB}}{[B]}\right) + [A].\left(1 + \frac{K_{AB}}{[B]}\right)\right) \qquad (7.3.9)$$

At a constant [B] , a plot of [A] / v versus [A] will have an ordinate intercept of ;

$$\frac{1}{V}\left(K_{BA} + \frac{K_A.K_{AB}}{[B]}\right) \qquad (7.3.10)$$

an abscissa intercept of ;

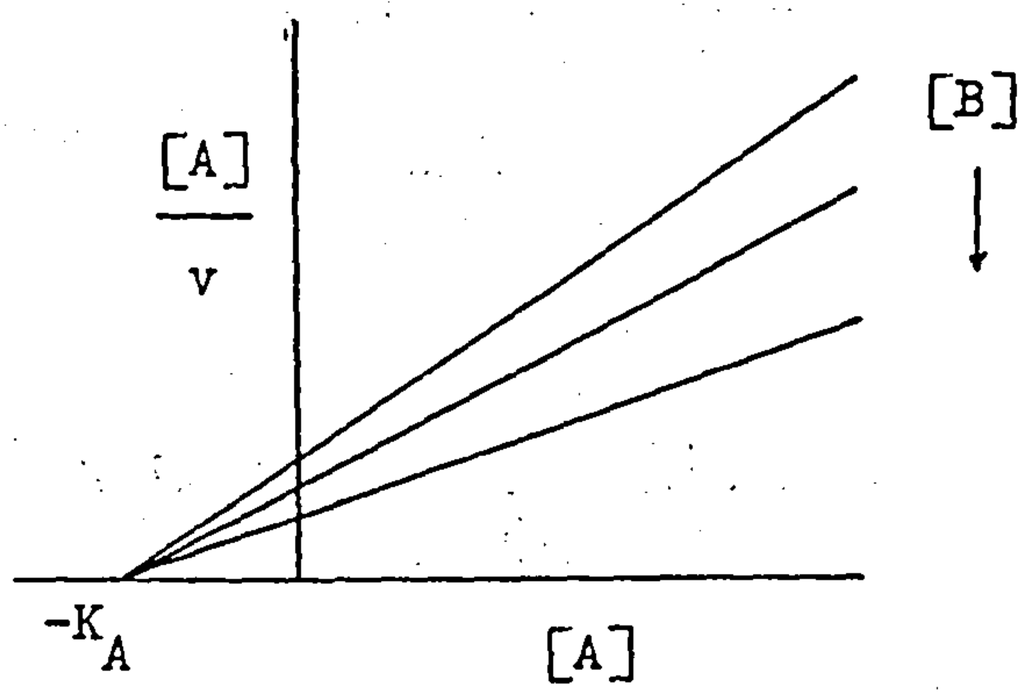
$$-\frac{K_{BA} + \frac{K_A.K_{AB}}{[B]}}{1 + \frac{K_{AB}}{[B]}} \qquad (7.3.11)$$

and a slope of ;

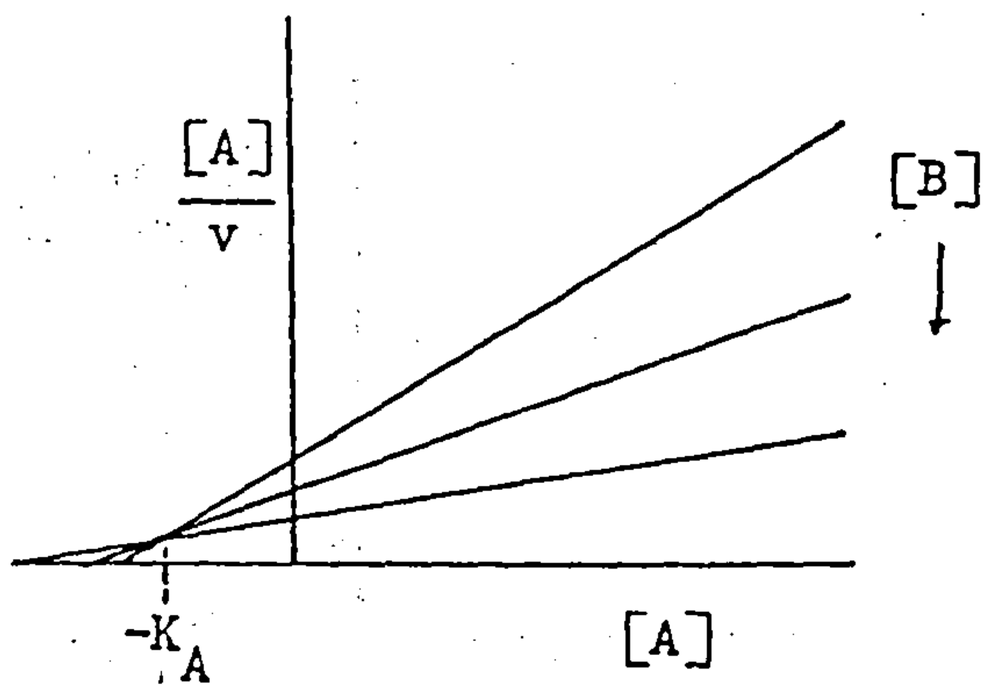
$$\frac{1}{V}\left(1 + \frac{K_{AB}}{[B]}\right) \qquad (7.3.12)$$

At various concentrations of B, a family of [A] / v versus [A] lines would intersect at $-K_A = [A]$. However the abscissa intercept value depends on whether the binding of one substrate affects the binding of the other. There are three possible cases ;

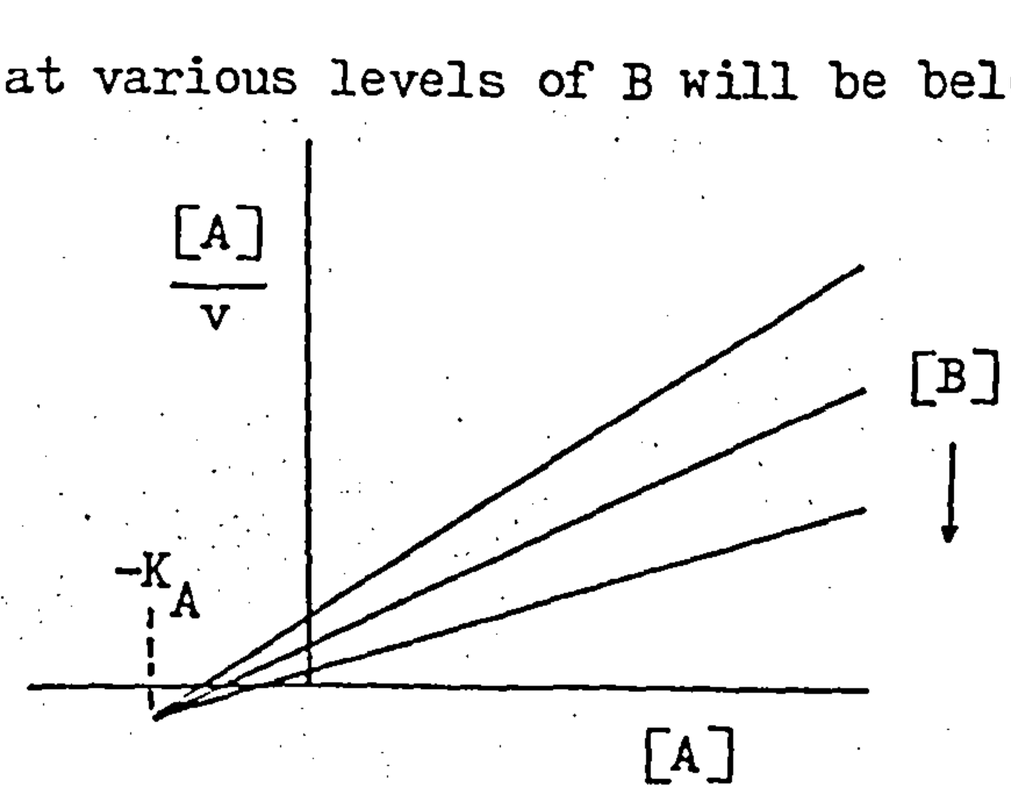
a). If $K_A = K_{BA}$ (and thus from the relationship $K_A . K_{AB} = K_B . K_{BA}$, $K_B = K_{AB}$) the two substrates bind the enzyme independently not affecting one anothers affinity for the enzyme. The abscissa intercept is then equal to $-K_A$ and a family of [A] / v versus [A] lines at various [B] intercept on the abscissa at [A] = $-K_A$



b). When $K_A > K_{BA}$, the common intercept of the [A] / v versus [A] lines will be above the abscissa ;



c). When $K_A < K_{BA}$,that is when B decreases the affinity of A for the enzyme, the common intercept of a series of [A] / v versus [A] lines at various levels of B will be below the abscissa;



Secondary plots for substrate kinetics

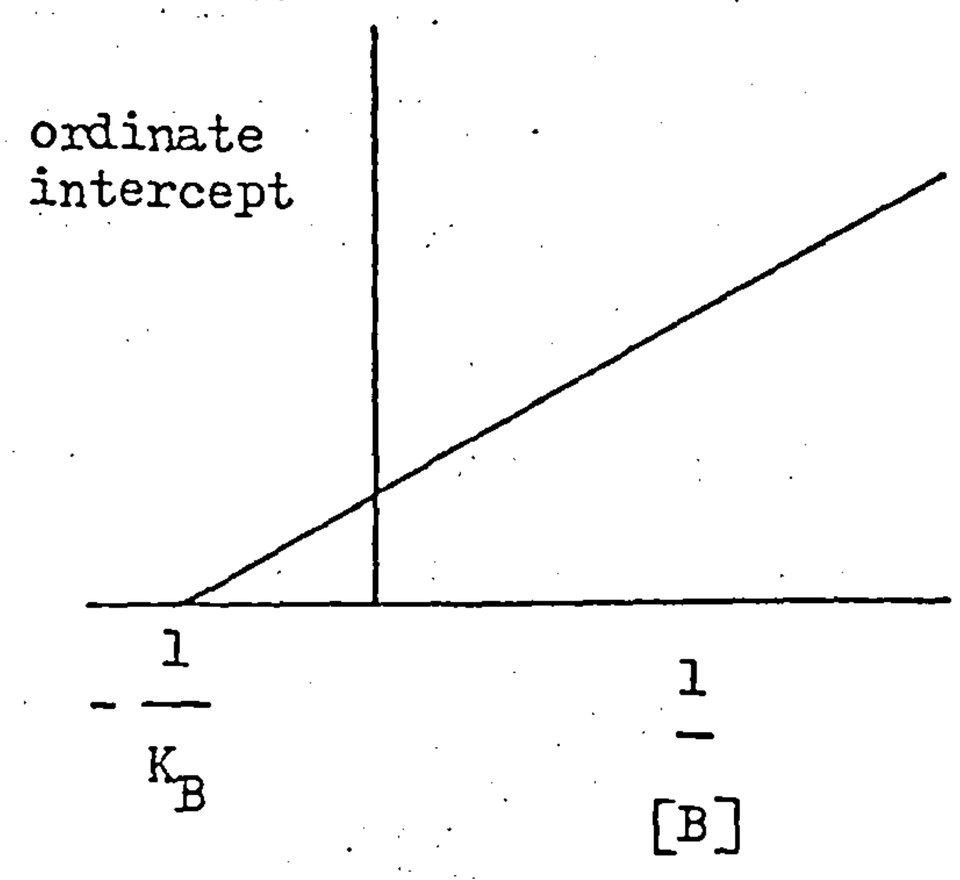
Values for K_B and for K_{AB} can be obtained by plotting 1 / [B] versus the ordinate intercept or the slope respectively of the lines from a plot of [A] / v versus [A].

From 7.3.10 a plot of 1 / [B] versus ordinate intercept will have an ordinate intercept of; $\frac{K_{BA}}{V}$ (7.3.13)

an abscissa intercept of $-\frac{K_{BA}}{K_A.K_{AB}} = \frac{1}{K_B}$ (7.3.14)

and a slope of $\frac{K_A.K_{AB}}{V}$ (7.3.15)

and will have the form ;

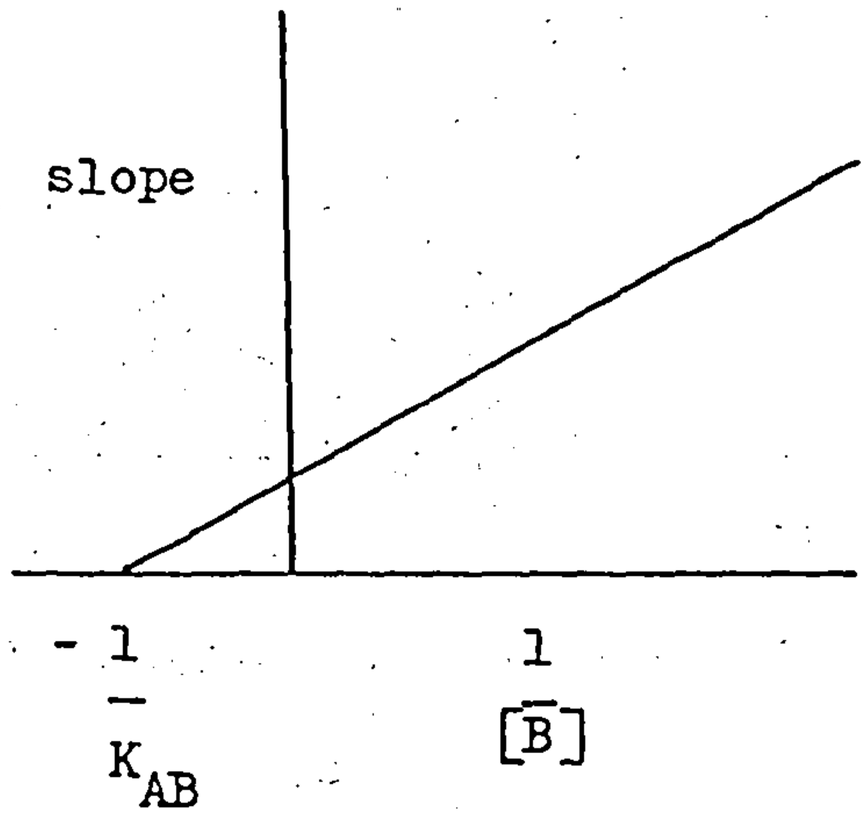


a secondary plot of slope versus 1 / [B] has an ordinate intercept equal to $\frac{1}{V}$, (7.3.16)

an abscissa intercept of $-\frac{1}{K_{AB}}$ (7.3.17)

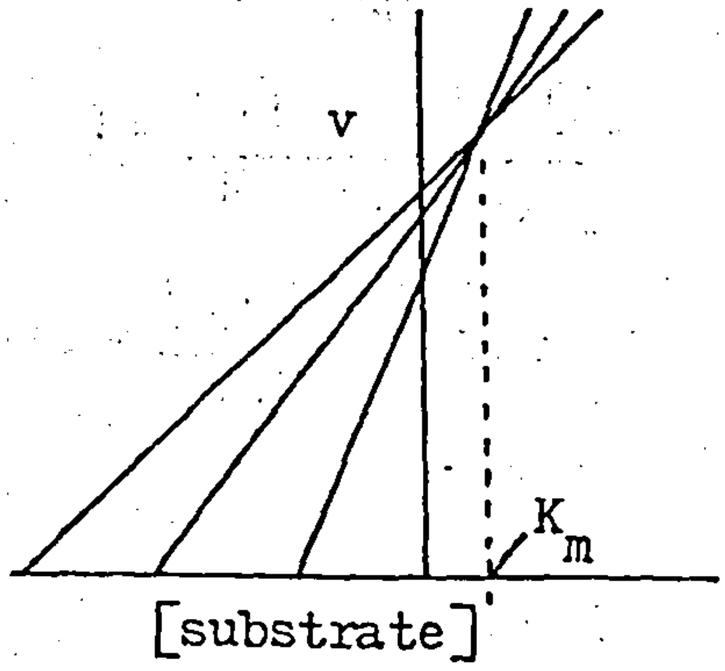
and a slope of $\frac{K_{AB}}{V}$ (7.3.18)

and has the form



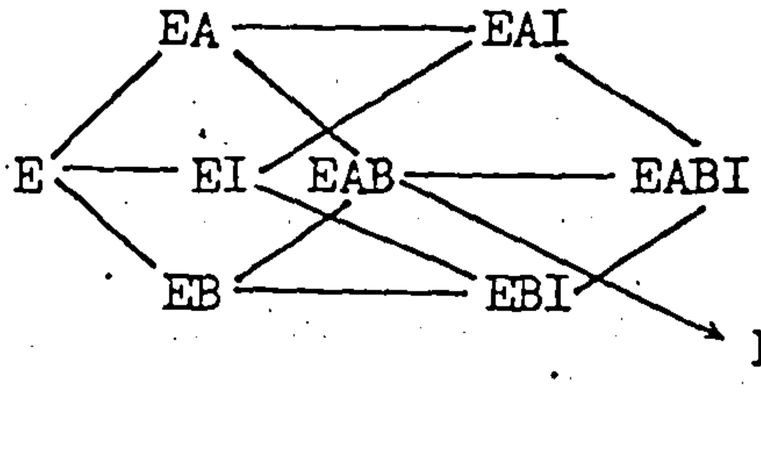
The direct linear plot

Some substrate kinetic results presented herein are analysed by the direct linear plot of Eisenthal and Cornish-Bowden (104) whereby experimental values of velocity are marked on the ordinate and corresponding values of substrate concentration marked on the abscissa. Pairs of values for each rate measurement are then joined by lines which exceed the ordinate. The median value of the intersection of a number of these lines gives the Km.



SECTION 7.4 SINGLE INHIBITOR KINETICS

There are seven possible enzyme-ligand complexes for an enzyme in the presence of two substrates , A and B and an inhibitor, I when the enzyme obeys a rapid equilibrium,random order sequential mechanism ;



(7.4.1)

the total concentration of such an enzyme in the presence of these ligands is

$$[E_T] = [E] + [EA] + [EB] + [EI] + [EAI] + [EBI] + [EABI] + [EAB] \quad (7.4.2)$$

the concentration of each species related to its dissociation constant is ;

$$[EA] = \frac{[E]\,[A]}{K_A} \quad (7.4.3)$$

$$[EB] = \frac{[E]\,[B]}{K_B} \quad (7.4.4)$$

$$[EI] = \frac{[E]\,[I]}{K_i} \quad (7.4.5)$$

$$[EAI] = \frac{[E]\,[A]\,[I]}{K_A \cdot K_{Ai}} \quad (7.4.6)$$

$$[EBI] = \frac{[E]\,[B]\,[I]}{K_B \cdot K_{Bi}} \quad (7.4.7)$$

$$[EABI] = \frac{[E]\,[A]\,[B]\,[I]}{K_A \cdot K_{AB} \cdot K_{ABi}} \quad (7.4.8)$$

$$[EAB] = \frac{[E]\,[A]\,[B]}{K_A \cdot K_{AB}} \quad (7.4.9)$$

thus

$$[E_T] = [E]\left(1 + \frac{[A]}{K_A} + \frac{[B]}{K_B} + \frac{[I]}{K_i} + \frac{[A][I]}{K_A.K_{Ai}} + \frac{[B][I]}{K_B.K_{Bi}} + \frac{[A][B][I]}{K_A.K_{AB}.K_{ABI}} + \frac{[A][B]}{K_A.K_{AB}}\right) \qquad (7.4.10)$$

since the ratio of maximum velocity to actual velocity, V/v is equal to the ratio of total enzyme to the ternary complex, $[E_T]/[EAB]$, equation 7.4.10 can be multiplied by $\frac{K_A.K_{AB}}{[A][B]}$;

$$\frac{V}{v} = \frac{K_A.K_{AB}}{[A][B]} + \frac{K_{AB}}{[B]} + \frac{K_A.K_{AB}}{[A]K_B} + \frac{K_A.K_{AB}[I]}{[A][B]K_i} + \frac{K_{AB}[I]}{[B]K_{Ai}} + \frac{K_A.K_{AB}[I]}{[A]K_B.K_{Bi}} + \frac{[I]}{K_{ABi}} + 1 \qquad (7.4.11)$$

to obtain the Hanes form this equation can be multiplied by [A] and rearranged ;

$$\frac{[A]}{v} = \frac{1}{V}\left(\frac{K_A.K_{AB}}{K_B} + \frac{K_A.K_{AB}[I]}{[B]K_i} + \frac{K_A.K_{AB}[I]}{K_B.K_{Bi}} + \frac{K_A.K_{AB}}{[B]}\right) + \frac{[A]}{V}\left(1 + \frac{K_{AB}}{[B]} + \frac{K_{AB}[I]}{K_{Ai}[B]} + \frac{1}{K_{ABi}}\right) \qquad (7.4.12)$$

Primary plots for the interpretation of data from single inhibitor studies

Data from single inhibitor studies are first displayed on a Hanes plot according to equation 7.4.12 . (As noted in Section 7.1 , substrate B is maintained at its Km in these experiments.)

A plot of [A] / v versus [A] will thus have an abscissa intercept of ;

$$-\frac{\frac{K_A.K_{AB}}{[B]} + \left(1 + \frac{[I]}{K_i}\right) + \frac{K_A.K_{AB}}{K_B}\left(1 + \frac{[I]}{K_{Bi}}\right)}{\left(1 + \frac{[I]}{K_{ABi}}\right) + \frac{K_{AB}}{[B]}\left(1 + \frac{[I]}{K_{Ai}}\right)} \qquad (7.4.13)$$

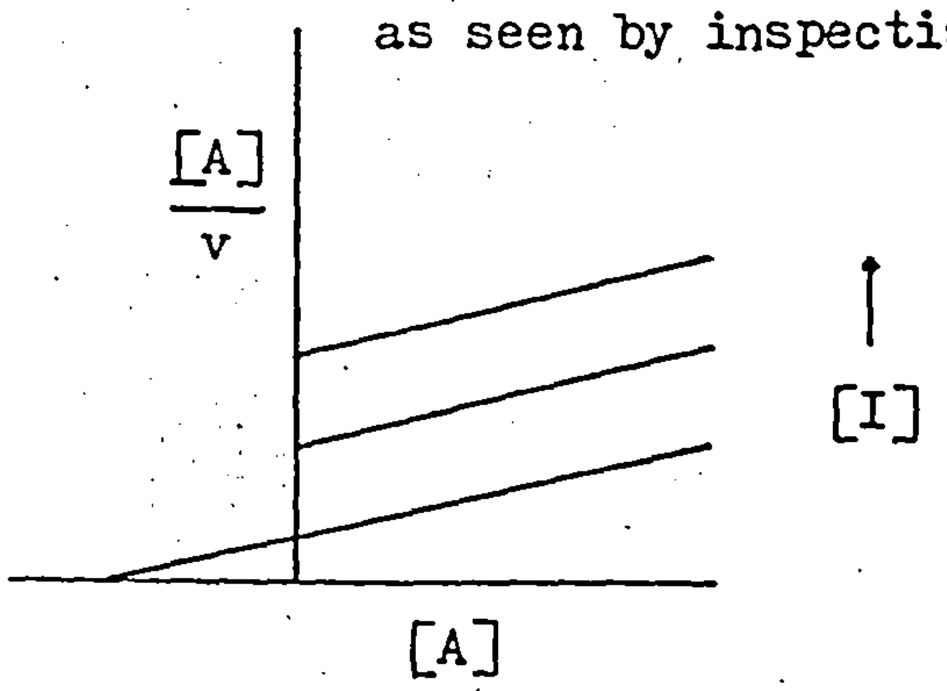
such lines would intersect (when [I] is varied) at ;

$$[A] = -\frac{\frac{K_A.K_{AB}}{K_i[B]} + \frac{K_A.K_{AB}}{K_{Bi}.K_B}}{\frac{K_{AB}}{K_{Ai}[B]} + \frac{1}{K_{ABi}}} \qquad (7.4.14)$$

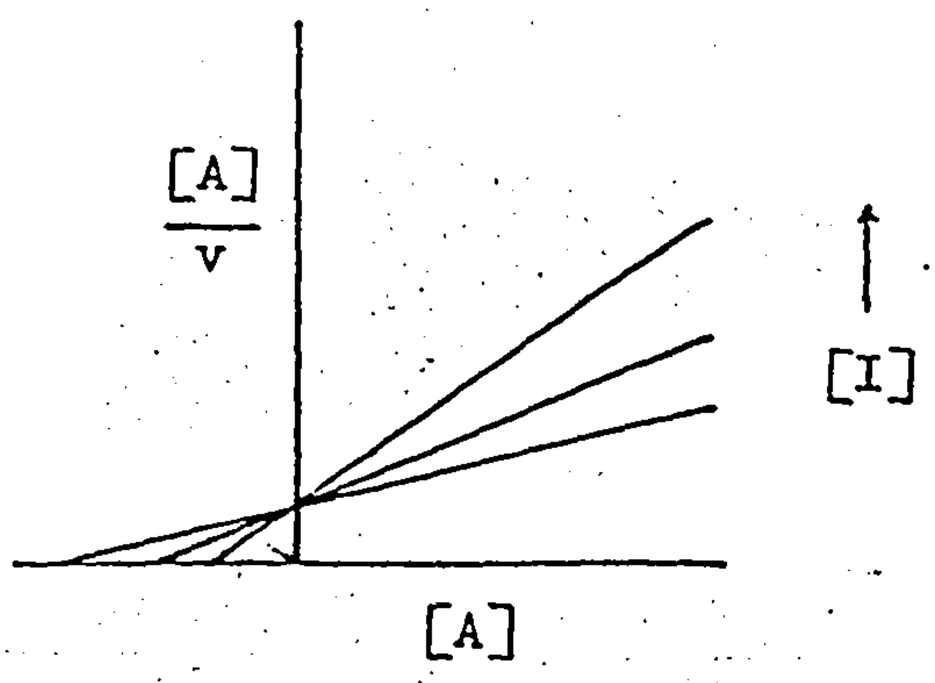
Interpretation of primary plots for single inhibitor experiments

When the interaction between A and I is ;

a). Competitive $K_{Ai} = \infty$ and $K_{ABi} = \infty$ lines are parallel. as seen by inspection of equation 7.4.14 .



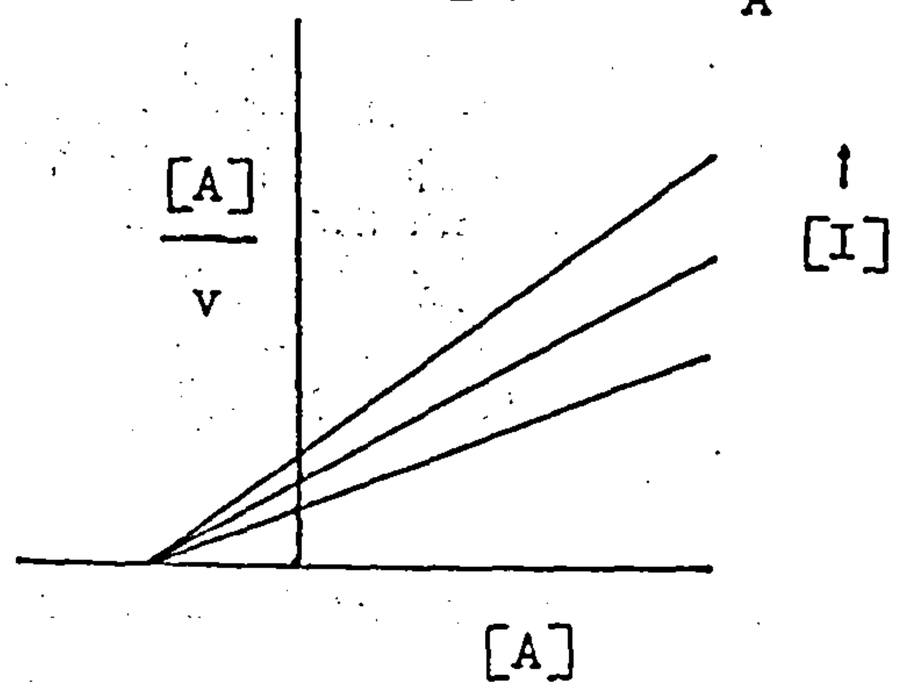
b). Uncompetitive $K_i = \infty$ and $K_{Bi} = \infty$. Lines will intersect on the ordinate where [A]= 0 .



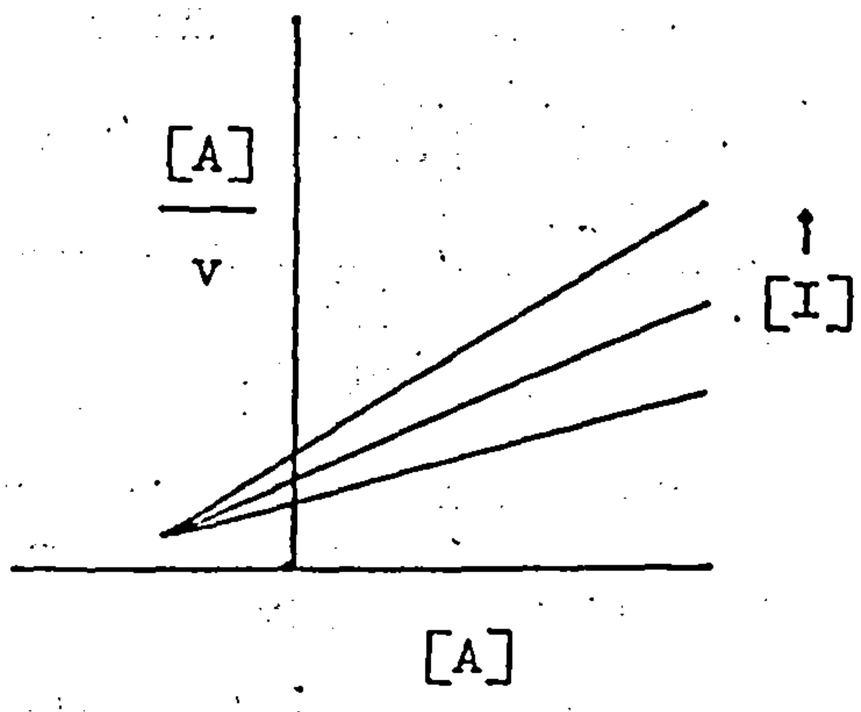
Interpretation of primary plots for single inhibitor experiments

contd.

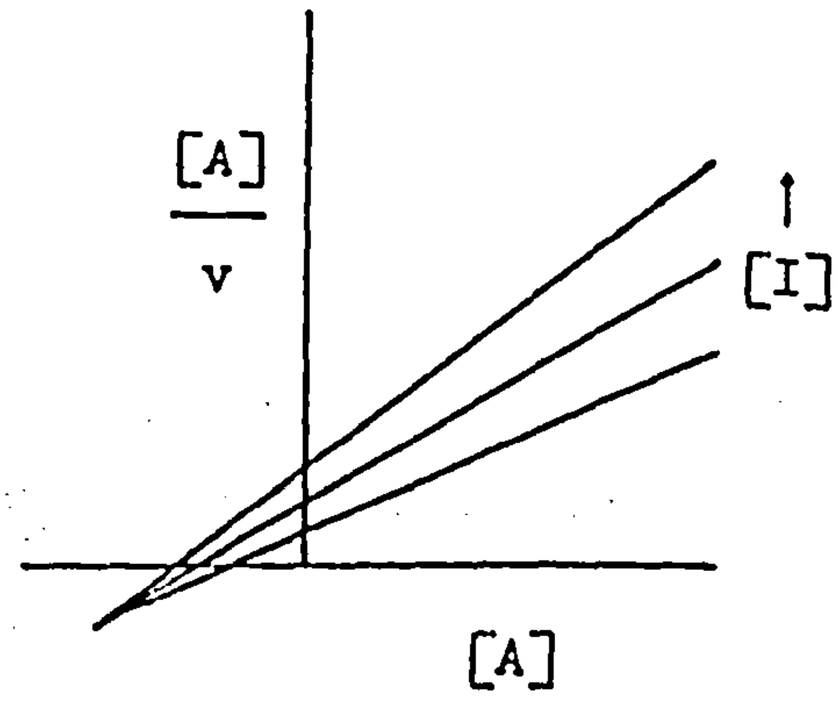
c). Noncompetitive $K_i = K_{Ai}$ and $K_{Bi} = K_{ABi}$. Here the intersection point is given by equation 7.4.14. However in the case of hexokinase where $K_{AB} = K_B$ the intersection is then at $[A] = -K_A$.



d). Mixed $K_i \neq K_{Ai}$ and $K_{Bi} \neq K_{ABi}$. The lines will intersect and the intersection point is given by equation 7.4.14 . The lines will intersect above the abscissa if $K_i > K_{Ai}$ and below the abscissa if $K_i < K_{Ai}$.



$K_i > K_{Ai}$



$K_i < K_{Ai}$

Secondary plots for single inhibitor experiments

Plotting ordinate intercept from a primary plot against [I].

By rearranging the ordinate intercept component of equation 7.4.12

thus ;

$$[I].\frac{1}{V}\left(\frac{K_A.K_{AB}}{[B]\,K_i}+\frac{K_A.K_{AB}}{K_B.K_{Bi}}\right)+\frac{1}{V}\left(\frac{K_A.K_{AB}}{[B]}+\frac{K_A.K_{AB}}{K_B}\right) \quad (7.4.15)$$

aplot of ordinate intercept versus [I] is seen to have an abscissa intercept of ;

$$[I]=-\frac{\frac{K_{AB}}{[B]}+\frac{K_{AB}}{K_B}}{\frac{K_{AB}}{[B]\,K_i}+\frac{K_{AB}}{K_B.K_{Bi}}} \quad (7.4.16)$$

Similarly a plot of the slope of lines from the primary plot against [I] will have an abscissa intercept of ;

$$[I]=-\frac{[B]}{\frac{1}{K_{ABi}}+\frac{K_{AB}}{[B]\,K_{Ai}}} \quad (7.4.17)$$

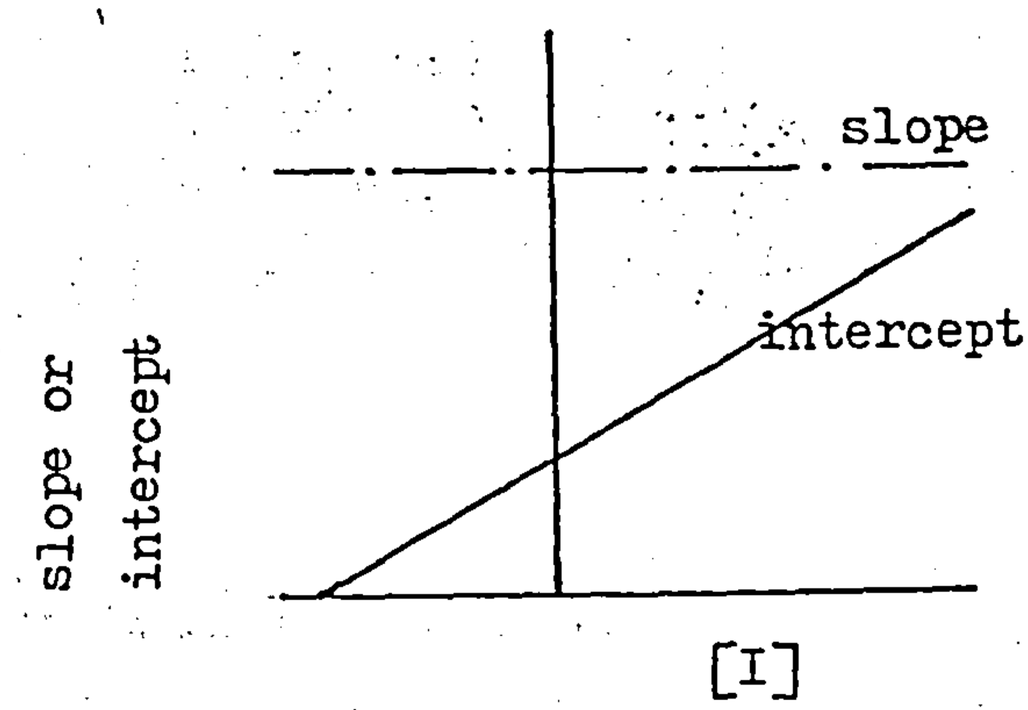
The secondary plots possible for each case of interaction between I and A are given below, where ;

—·——·— represents the secondary line of slope values from the primary plot.

and ———————— represents the secondary line of ordinate intercept values from the primary plot.

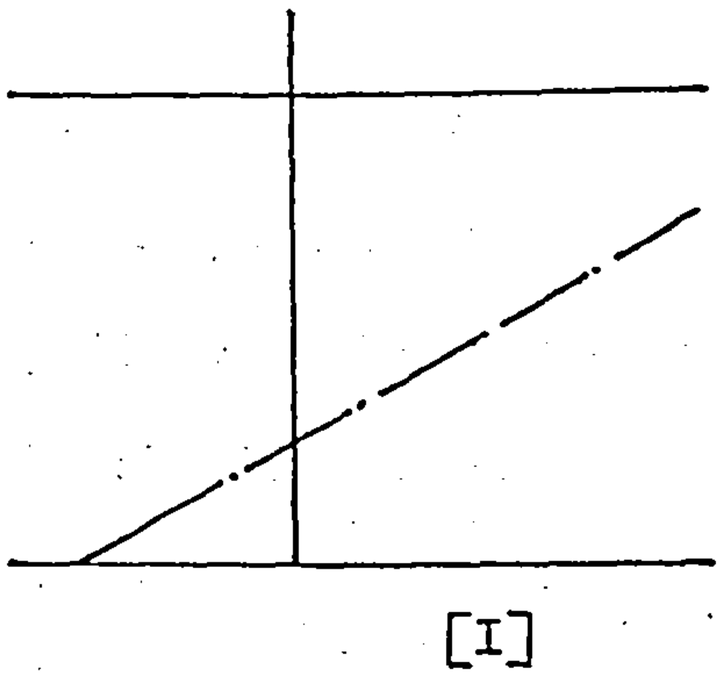
When the interaction of I and A is ;

a). Competitive — $K_{Ai}=\infty$ and $K_{ABi}=\infty$. The secondary plot has the form



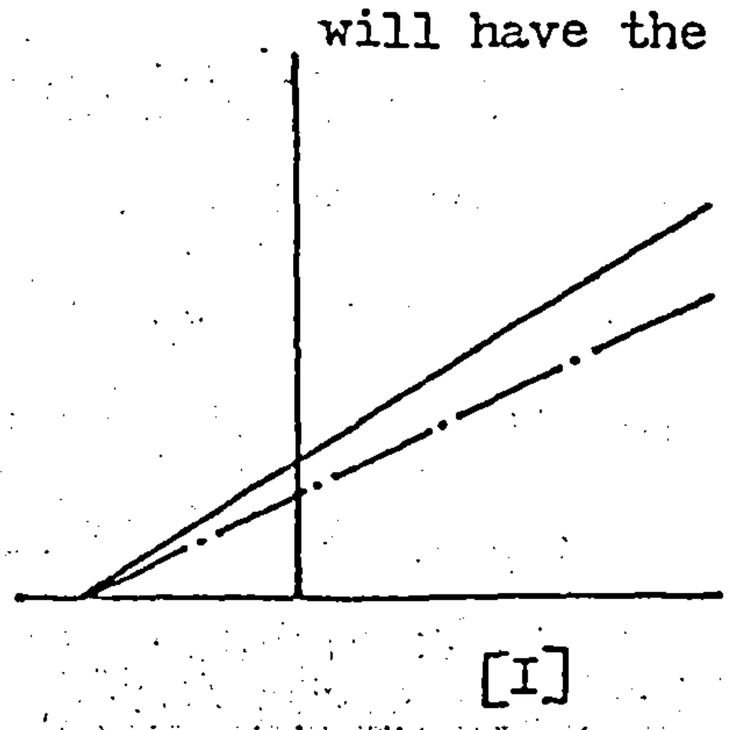
the abscissa intercept of the slope line, when I competes with A, is $-\infty$ and the abscissa intercept of the ordinate line has a value equal to $-K_i$ when I is noncompetitive with respect to B.

b). <u>Uncompetitive</u> $K_i = \infty$ and $K_{Bi} = \infty$. The secondary plot has the form



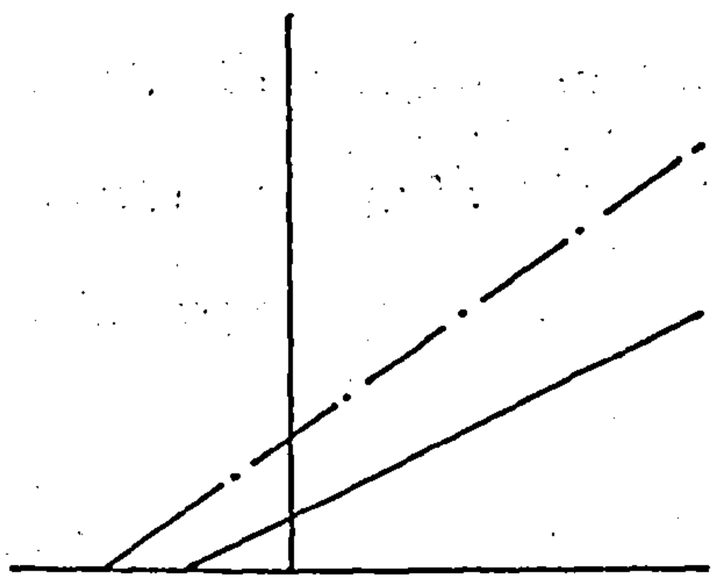
the ordinate line does not intersect the abscissa whilst the slope line intersects the abscissa at $-K_{Ai}$ assuming that $K_{Ai} = K_{Bi}$.

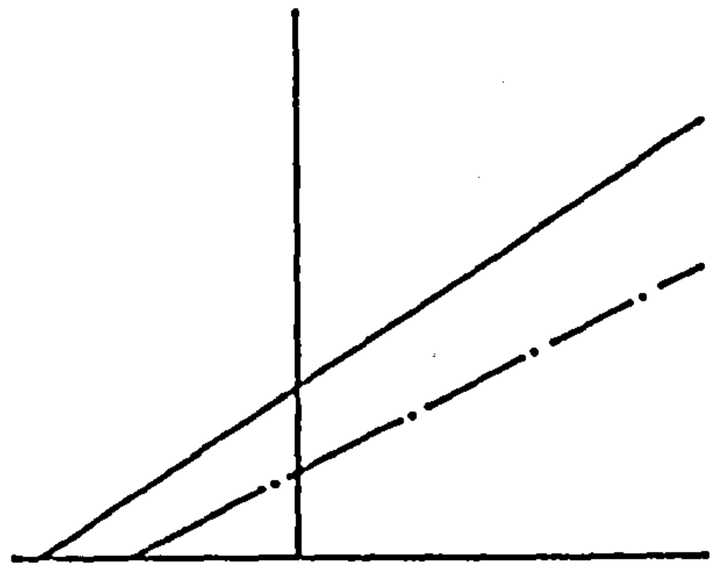
c). <u>Noncompetitive</u> $K_i = K_{Ai}$ and $K_{Bi} = K_{ABi}$. The secondary plot will have the form ;



both lines intersect on the abscissa and will do so at the same point if , as in hexokinase, $K_{AB} = K_B$. Moreover if I is competitive with B ($K_{Bi} = \infty$, $K_{ABi} = \infty$) the intersection points equals $K_i \left(1 + \frac{[B]}{K_B} \right)$.

d). Mixed ; $K_i \neq K_{Ai}$ and $K_{Bi} \neq K_{ABi}$





Both the ordinate line and the slope line will intersect the abscissa but at different points . The slope line will intersect at $-K_{Ai}$ if I is noncompetitive with respect to B (ie. $K_i = K_{Bi}$) or will intersect the abscissa at $-K_{Ai} \left(1 + \frac{[B]}{K_B} \right)$ if I is competitive with respect to B, ($K_{Bi} = \infty$, $K_{ABi} = \infty$)

The ordinate line will intersect the abscissa at $- K_i$ if I is noncompetitive with respect to B or at $- K_i \left(1 + \frac{[B]}{K_B} \right)$ if I is competitive with respect to B.

The interpretation of single inhibitor experiments.

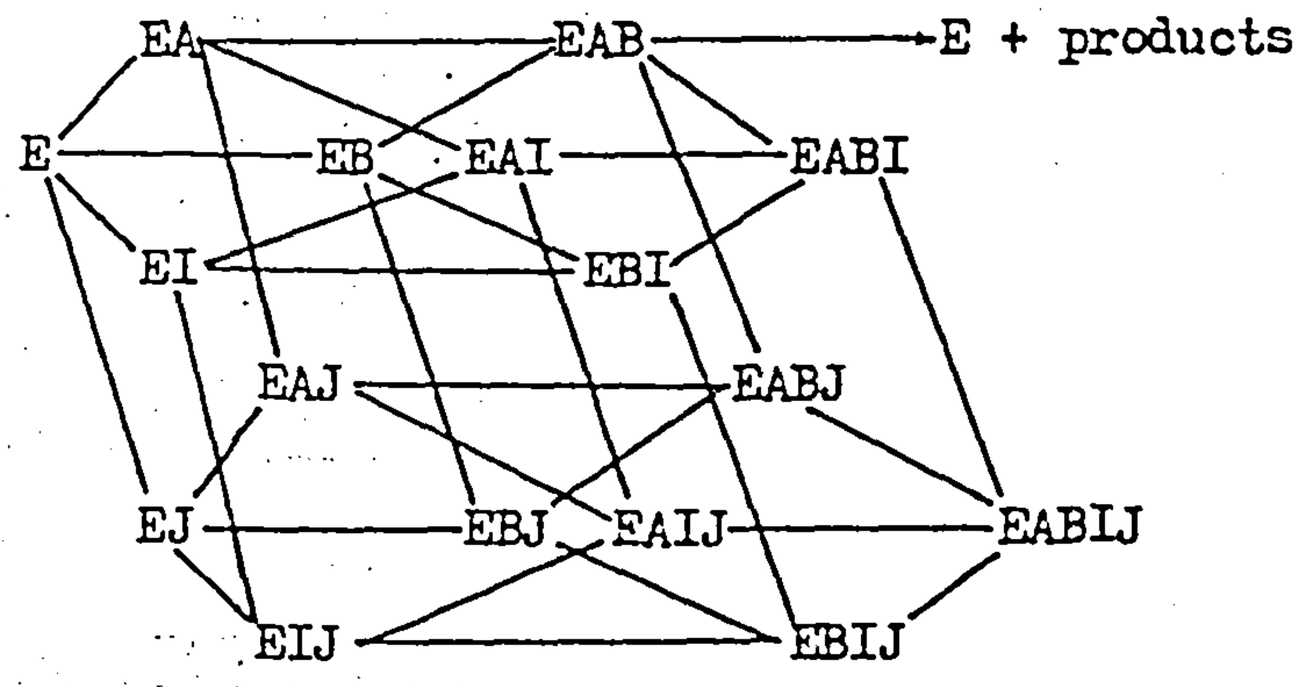
These studies show the quantitative importance of given enzyme-ligand complexes but not preferred routes for their formation. When there is competition between two ligands it is not possible to deduce whether both ligands compete for the same binding site or through

causing a conformational change in the protein.

SECTION 7.5 DOUBLE INHIBITOR KINETICS

Single inhibitor kinetics help to establish the relationship between substrate binding sites and inhibitor binding sites. However many enzymes, for example hexokinase, are inhibited by a variety of ligands and it is important to establish how the binding of one inhibitor will affect the binding of another. This allows an insight into how an enzyme would be regulated in the presence of a number of inhibitory ligands as it might be in the cell.

For an enzyme obeying a sequential mechanism of the rapid equilibrium random type and having two substrates, A and B and two inhibitory ligands I and J the following complexes could be formed ;



(7.5.1)

There are 15 possible enzyme-ligand complexes and the total amount of enzyme, $[E_T]$ is the sum of all possible complexes ;

$$[E_T] = [E] + [EA] + [EB] + [EI] + [EJ] + [AB] + [EAI] + [EAJ] + [EBI] + [EBJ] + [EIJ] + [EABI] + [EAIJ] + [EAIJ] + [EBIJ] + [EABJ] + [EABIJ] \qquad (7.5.2)$$

The concentration of each species can be represented in terms of the respective dissociation constant,(K) ;

$$[EA] = \frac{[E]\,[A]}{K_A} \qquad (7.5.3)$$

$$[EB] = \frac{[E]\,[B]}{K_B} \qquad (7.5.4)$$

$$[EI] = \frac{[E]\,[I]}{K_i} \qquad (7.5.5)$$

$$[EJ] = \frac{[E]\,[J]}{K_j} \qquad (7.5.6)$$

$$[EAB] = \frac{[E]\,[A]\,[B]}{K_A \cdot K_{AB}} \qquad (7.5.7)$$

$$[EAI] = \frac{[E]\,[A]\,[I]}{K_A \cdot K_{Ai}} \qquad (7.5.8)$$

$$[EAJ] = \frac{[E]\,[A]\,[J]}{K_A \cdot K_{Aj}} \qquad (7.5.9)$$

$$[EBI] = \frac{[E]\,[B]\,[I]}{K_B \cdot K_{Bi}} \qquad (7.5.10)$$

$$[EBJ] = \frac{[E]\,[B]\,[J]}{K_B \cdot K_{Bj}} \qquad (7.5.11)$$

$$[EIJ] = \frac{[E]\,[I]\,[J]}{K_i \cdot K_{ij}} \qquad (7.5.12)$$

$$[EABI] = \frac{[E]\,[A]\,[B]\,[I]}{K_A \cdot K_{Ai} \cdot K_{AiB}} \qquad (7.5.13)$$

$$[EALJ] = \frac{[E]\,[A]\,[I]\,[J]}{K_A \cdot K_{Ai} \cdot K_{Aij}} \qquad (7.5.14)$$

$$[EBLJ] = \frac{[E]\,[B]\,[I]\,[J]}{K_i \cdot K_{ij} \cdot K_{ijB}} \qquad (7.5.15)$$

$$[EABJ] = \frac{[E][A][B][J]}{K_A . K_{Aj} . K_{AjB}} \qquad (7.5.16)$$

$$[EABIJ] = \frac{[E][A][B][I][J]}{K_A . K_{Ai} . K_{Aij} . K_{AijB}} \qquad (7.5.17)$$

Equation 7.5.2 can be divided by [EAB] since the ratio of the actual velocity to the maximum velocity, v/V is equal to the ratio of $[EAB]/[E_T]$.

Hence,

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_A . K_{AB} . [J]}{K_j . K_{jB} . [A]} + \frac{K_{AB}[J]}{K_{Aj} . K_{AjB}} + \frac{K_{AB}[J]}{K_{Aj}[B]} + \frac{[J] K_A . K_{AB}}{[A][B] K_j}\right.$$

$$+ \frac{K_A . K_{AB}}{[A][B]} + \frac{K_{AB}}{[B]} + \left.\frac{K_A . K_{AB}}{K_B [A]}\right) + [I]\left(\frac{K_{AB}}{K_{Ai} . K_{AiB}} + \frac{[J] K_{AB}}{K_{Ai} . K_{Aij}[B]}\right.$$

$$+ \frac{[J] K_{AB}}{K_{Ai} . K_{Aij} . K_{AijB}} + \frac{K_A . K_{AB}}{K_i . K_{iB}[A]} + \frac{K_{AB}}{K_{Ai}[B]} + \left.\frac{K_A . K_{AB}}{[A][B] K_i}\right.$$

$$\left. + \frac{[J] K_A . K_{AB}}{K_i . K_{ij} . [A][B]} + \frac{[J] K_A . K_{AB}}{K_i . K_{ij} . K_{ijB}[A]}\right) \qquad (7.5.18)$$

This equation can be simplified if both I and J are noncompetitive with respect to B (ie. $K_{AB} = K_{AiB} = K_{AjB} = K_{AijB}$) and if the enzyme is fully saturated with substrate B in which case terms in $K_{AB}/[B]$ can be neglected ;

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_A . K_{AB}}{[A] K_B} + \frac{[J] K_A . K_{AB}}{K_j . K_{jB}[A]} + \frac{[J]}{K_{Aj}}\right) +$$

$$\frac{[I]}{V}\left(\frac{1}{K_{Ai}} + \frac{[J]}{K_{Ai} . K_{Aij}} + \frac{K_A . K_{AB}}{K_i . K_{iB}[A]} + \frac{[J] K_A . K_{AB}}{K_i . K_{ij} . K_{ijB}[A]}\right)$$

(7.5.19)

A plot of 1 / v versus [I] will , from equation 7.5.19,have an abscissa intercept of

$$-\frac{1+\frac{K_A.K_{AB}}{[A]\,K_B}+\frac{K_A.K_{AB}\,[J]}{K_j.K_{jB}\,[A]}+\frac{[J]}{K_{Aj}}}{\frac{1}{K_{Ai}}+\frac{[J]}{K_{Ai}.K_{Aij}}+\frac{K_A.K_{AB}}{K_i.K_{iB}[A]}+\frac{[J]\,K_A.K_{AB}}{K_i.K_{ij}.K_{ijB}[A]}} \qquad (7.5.20)$$

This equation simplifies for hexokinase where the dissociation of B from the enzyme is unaffected by A ie. $K_{AB}= K_B= K_{jB}= K_{iB}= K_{ijB}$

thus the abscissa intercept is now equal to ;

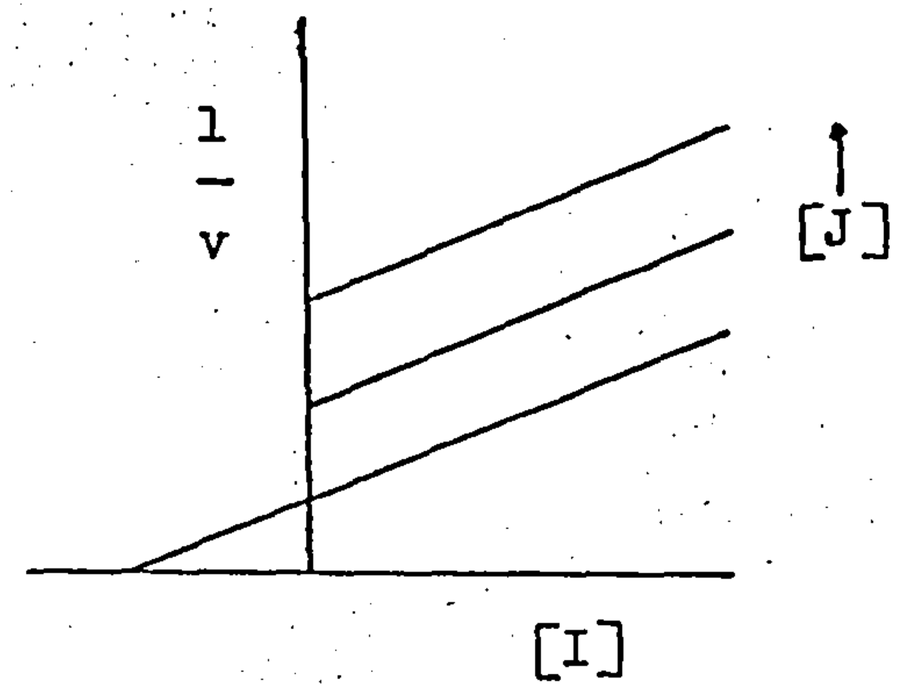
$$-\frac{1+\frac{K_A}{[A]}+\frac{K_A\,[J]}{[A]\,K_j}+\frac{[J]}{K_{Aj}}}{\frac{1}{K_{Ai}}+\frac{[J]}{K_{Ai}.K_{Aij}}+\frac{K_A}{K_i\,[A]}+\frac{[J]\,K_A}{K_i.K_{ij}[A]}} \qquad (7.5.21)$$

and a series of lines from a plot of 1/ v versus [I] , each line from a different [J] will have a common intersection point at ;

$$[I] = -\frac{\frac{K_A}{[A]\,K_j}+\frac{1}{K_{Aj}}}{\frac{1}{K_{Ai}.K_{Aij}}+\frac{K_A}{K_i.K_{ij}[A]}} \qquad (7.5.22)$$

Interpretation of 1 / v versus [I] plots

a). When J is competitive with I irrespective of whether or not I competes with A . The lines do not intersect ;



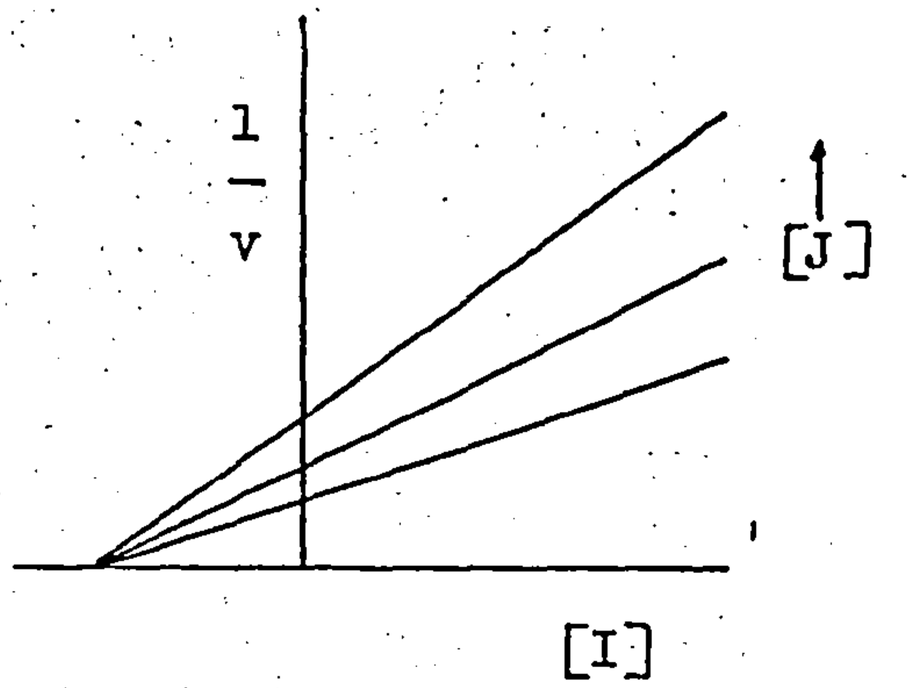
b). <u>When Jis noncompetitive with I but competitive with A.</u>

($K_j = K_{ij}$, $K_{Aj} = \infty$ and $K_{Aij} = \infty$) the abscissa intercept of a plot of 1 / v versus [I] is then at ;

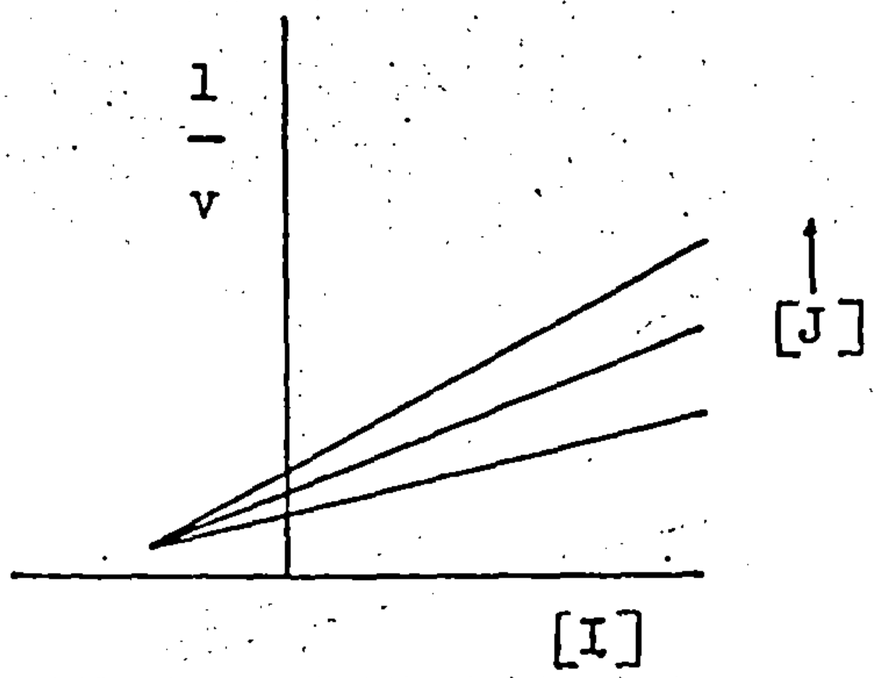
$$[I] = - \frac{1 + \frac{K_A}{[A]} + \frac{[J]\,K_A}{K_j\,[A]}}{\frac{1}{K_{Ai}} + \frac{K_A}{K_i[A]} + \frac{K_A\,[J]}{K_i\,[A]\,K_j}} \qquad (7.5.23)$$

The lines can intersect on , above or below the abscissa

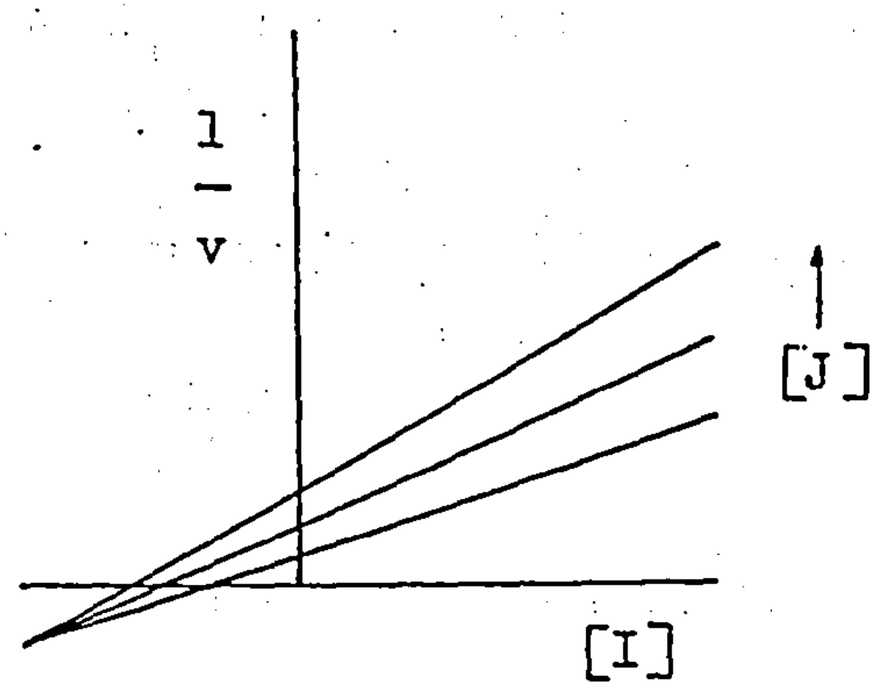
i). when I is noncompetitive with A , $K_i = K_{Ai}$, the common intersection point is on the abscissa at $[I] = -K_i$



ii). if $K_i \geq K_{Ai}$ the intersection is above the abscissa ;



iii). if $K_i < K_{Ai}$ the intersection point of a family of lines is below the abscissa ;



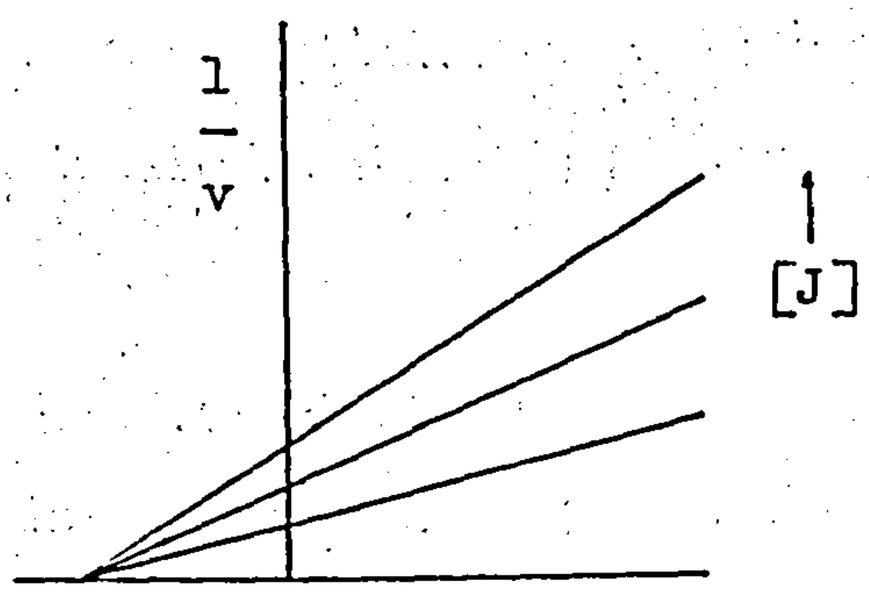
c). <u>When J is noncompetitive with respect to both I and A</u>

$K_j = K_{ij} = K_{Aj} = K_{Aij}$. From equation 7.5.22 a family of lines will intersect on the abscissa at ;

$$[I] = - \frac{\frac{K_A}{[A]} + 1}{\frac{1}{K_{Ai}} + \frac{K_A}{K_i [A]}} \qquad (7.5.24)$$

whatever the nature of the interaction of I with respect to A .

Plots of 1 / v versus [I] in this case will have the form ;



However the abscissa intercept value will depend on the interrelationship of the binding of I and A such that ;

i). if I is noncompetitive with respect to A the abscissa intercept will be at $-K_i$ since $K_i = K_{Ai}$ (see equation 7.5.24).

ii). if I is competitive with A. ie. $K_{Ai} = \infty$ the abscissa intercept is at ; $$-K_i \left(1 + \frac{[A]}{K_A} \right) .$$

iii). if I is mixed with respect to A (ie $K_i = K_{Ai}$) then the abscissa intercept is at ;

$$[I] = - \frac{K_A . K_{Ai} . K_i + K_{Ai} . K_i [A]}{K_A . K_{Ai} + K_i [A]}$$

The interpretation of double inhibitor experiments

Having carried out double inhibitor experiments it is important to check that they are consistent with results previously obtained in single inhibitor studies. One way of testing this is to compare the abscissa intercept value from a line recorded in the absence of J with the known K_i (of I) previously established by single inhibitor experiments. From inspection of equation 7.5.24 . This interpretation is only valid when substrate A is at its' Km and substrate B saturating.

i). When I is noncompetitive with respect to A, ($K_i = K_{Ai}$), the abscissa intercept of a plot of 1 / v versus [I] is at [I] = $-K_i$.

ii).When I is competitive with respect to A, ($K_{Ai} = \infty$) the abscissa intercept is at [I] = $-2K_i$.

iii). When I is uncompetitive with respect to A , ($K_i = \infty$) and the abscissa intercept of a plot of 1 / v versus [I] is at [I]= $-2K_i$.

iv). When I is mixed with respect to A ($K_i = K_{Ai}$) the abscissa intercept is somewhere between [I] = $-K_i$ and [I] = $-2K_i$.

Secondary plots for double inhibitor experiments

A plot of the slope of each line from the 1 / v versus [I] plot against [I] can be used to identify convergence of lines from the primary plot.

As with single inhibitor experiments it is not possible to deduce whether competitive interaction between two ligands is due to direct competition for one binding site or through an allosteric effect , however if enough experiments can be performed it should be possible to resolve this question in some cases.

SECTION 7.6 EXPERIMENTAL APPROACH USED IN KINETIC STUDIES OF HEXOKINASE

Initially Km values for glucose and ATP were found as inhibition studies depend on a knowledge of these parameters. In the case of glucose, which stabilises hexokinase, the Km was found to be quite low (50 μM) for both enzymes studied. This prevented the use of glucose levels well below the Km as a). glucose in the assay was depleted too quickly and b). hexokinase was unstable at the assay temperature of 30°C.

Single inhibitor studies were carried out under similar conditions to substrate kinetics however one substrate was maintained at its Km whilst the level of the other was varied as was the level of the inhibitory ligand in question.

In order that equations for double inhibitor kinetics be valid, glucose was always saturating and a level of 11 mM was maintained in the assay. The other substrate, ATP, was maintained at its Km whilst the levels of the two inhibitory ligands were varied.

Definition of inhibition constants

As discussed in Section 7.4 , two values of inhibition constant were obtained by plotting data from the primary plots (in single inhibitor studies) against [I]. Slope plotted against [I] gave the value K_i' which is the dissociation constant of inhibitor from the enzyme-substrate (EA) complex. Ordinate intercept (from the primary plot) plotted against [I] gave the value K_i which is the dissociation constant of inhibitor from the free enzyme.

The lines on both primary and secondary plots were drawn after unweighted linear regression of the data. Inhibitor dissociation constants were obtained in this manner from secondary plots.

CHAPTER 8

THE KINETICS OF HEART HEXOKINASE : RESULTS

THE KINETICS OF HEART HEXOKINASE

SECTION 8.1 RESULTS

The average Km values for MgATP and glucose were 0.7 and 0.05 mM respectively and thus these substrate levels were used in single inhibitor studies. In double inhibitor experiments MgATP was maintained at 0.7 mM and glucose at 11 mM. The data from substrate and single inhibitor kinetics are presented in table 8.1 and that from double inhbitor studies in table 8.2. The assignment of inhibition type is from a consideration of both primary and secondary plots for each experiment.

Phosphate was found to be such a weak inhibitor of pig heart hexokinase (Ki $\approx$ 25 mM) that it was not included in the list of inhibitory ligands investigated. Both AMP andMgADP inhibited heart hexokinase (figures 8.4 to 8.7), each being competitive with respect to MgATP. The inhibition of heart hexokinase by fructose 6-phosphate was qualitatively similar to inhibition by glucose 6-phosphate and thus the former was used as a glucose 6-phosphate analogue in double inhibitor studies. These results are discussed more fully in chapter 10.

Estimated Ki values for glucose 6-phosphate were found to vary more than those of other inhibitors although the reason for this is not clear. Thus the difference in Ki values when ATP and mannose were used as varied substrates (see Table8.1) is not viewed as the result of differing inhibitory behaviour of glucose6-phosphate.

The results, based on representative plots, are given in table 8.1 .

Table 8.1

The substrate and single inhibitor kinetics of heart hexokinase.

A represents MgATP, G glucose, M mannose, G6P glucose 6-phosphate, and F6P fructose 6-phosphate. All values are expressed in mM. The values for Km are averages from several preparations of enzyme. Inhibition types are represented by C, competitive; N, noncompetitive; Ki and Ki' are defined in Chapter 7, Section 7.6 .

varied substrate	inhibitor	mM Km	K_i	K_i'	inhibition type	figure
A		0.7				8.1
G		0.05				8.2
M		0.05				8.3
A	MgADP		2.4	∞	C	8.4
G	MgADP		4.0	2.4	N	8.5
A	AMP		2.2	∞	C	8.6
G	AMP		14.0	4.4	N	8.7
A	G6P		0.0032	∞	C	8.8
M	G6P		0.018	0.016	N	8.9
A	F6P		0.2	∞	C	8.10
G	F6P		4.1	0.52	N	8.11

Table 8.2

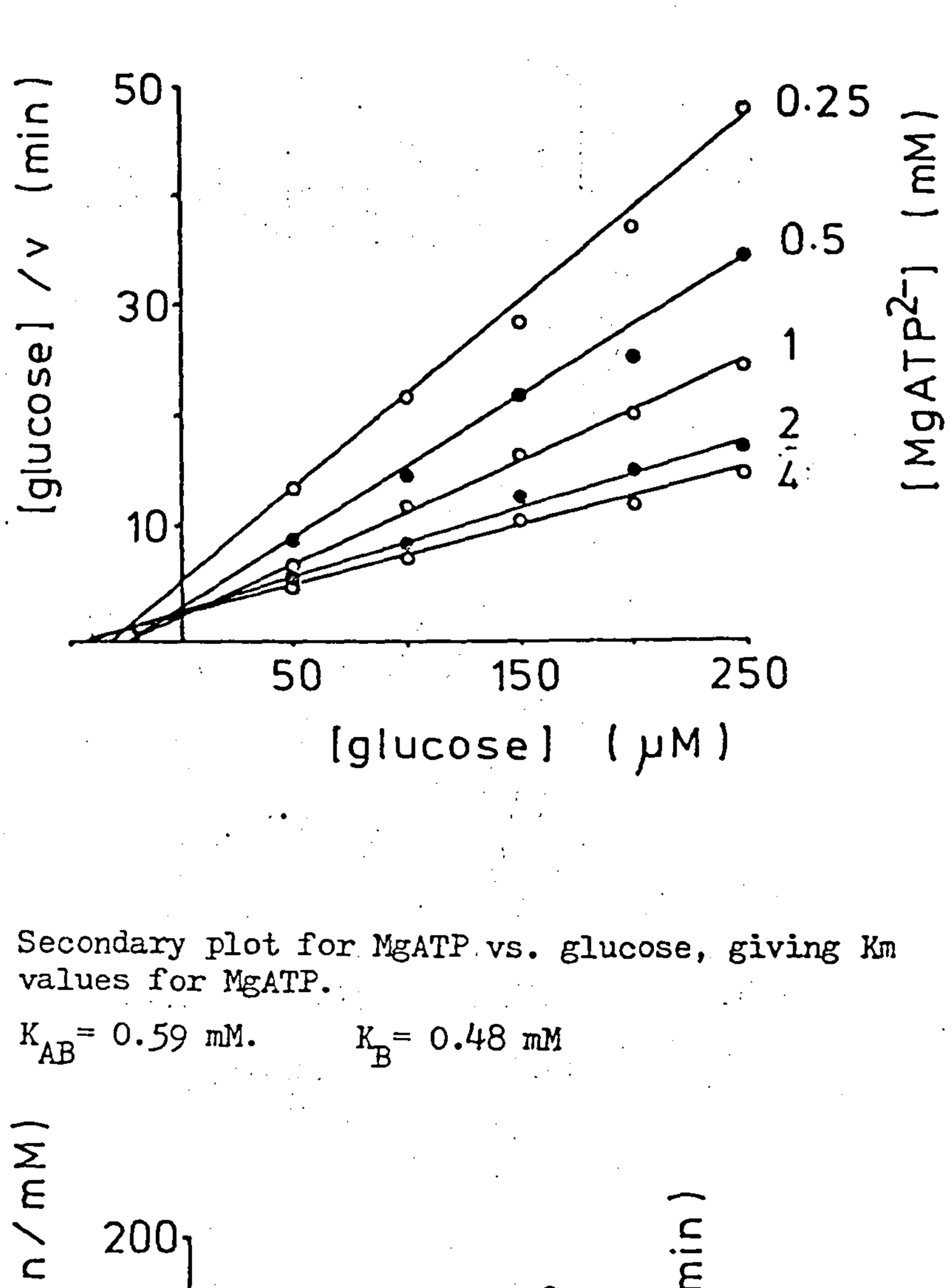
Double inhibitor kinetics of heart hexokinase.

inhibitor I	inhibitor J	interaction type	figure
MgADP	F6P	C	8.12
MgADP	AMP	C	8.13
AMP	F6P	C	8.14

Figure 8.1

MgATP vs. glucose Heart hexokinase.

Mg^{2+} was constant at 5mM.



Secondary plot for MgATP vs. glucose, giving Km values for MgATP.

K_{AB}= 0.59 mM. K_B= 0.48 mM

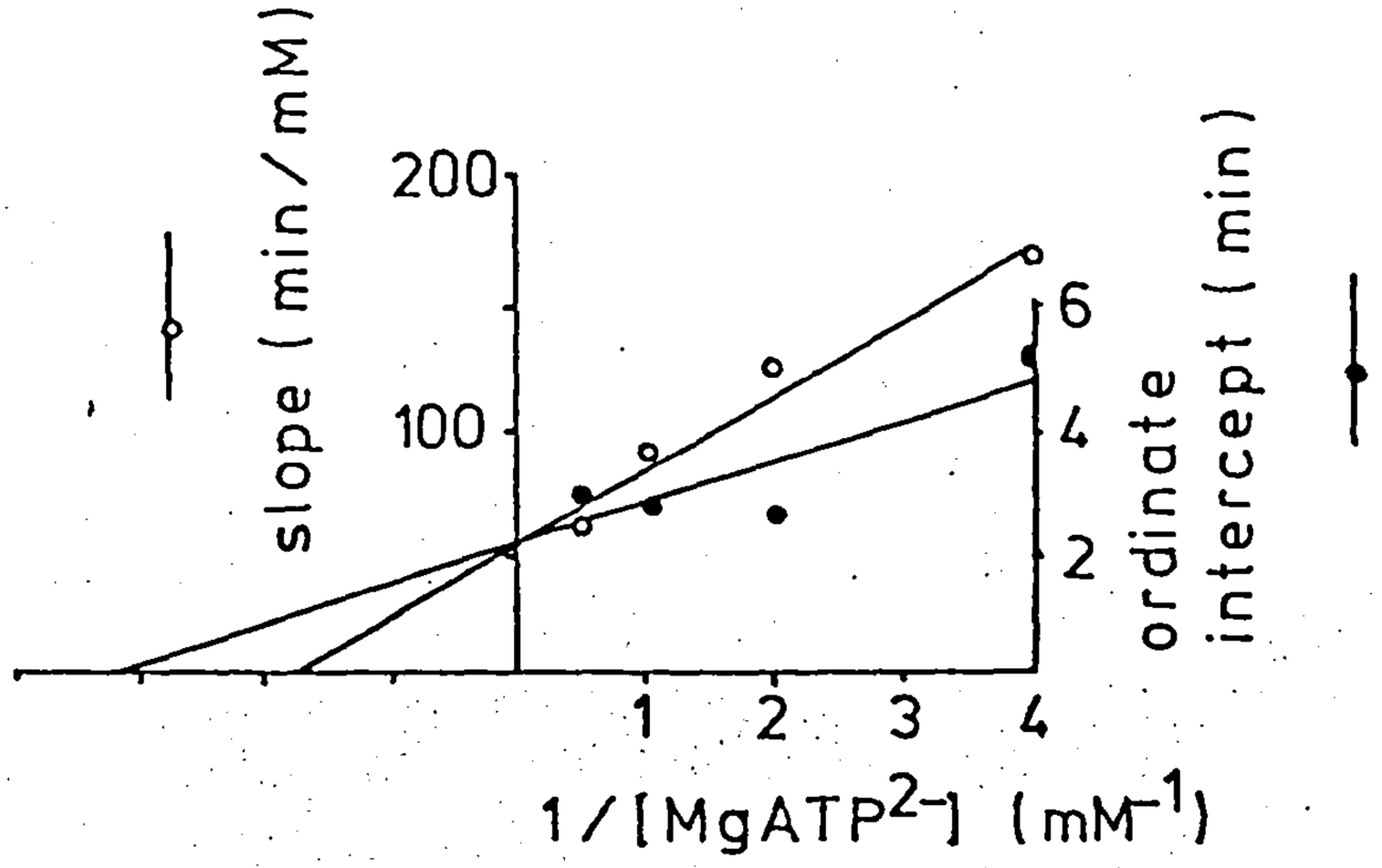


Figure 8.2

Km for glucose. Heart hexokinase.
Mg^{2+} was held at 5 mM and MgATP at 0.7 mM
Km for glucose was 0.05 mM.

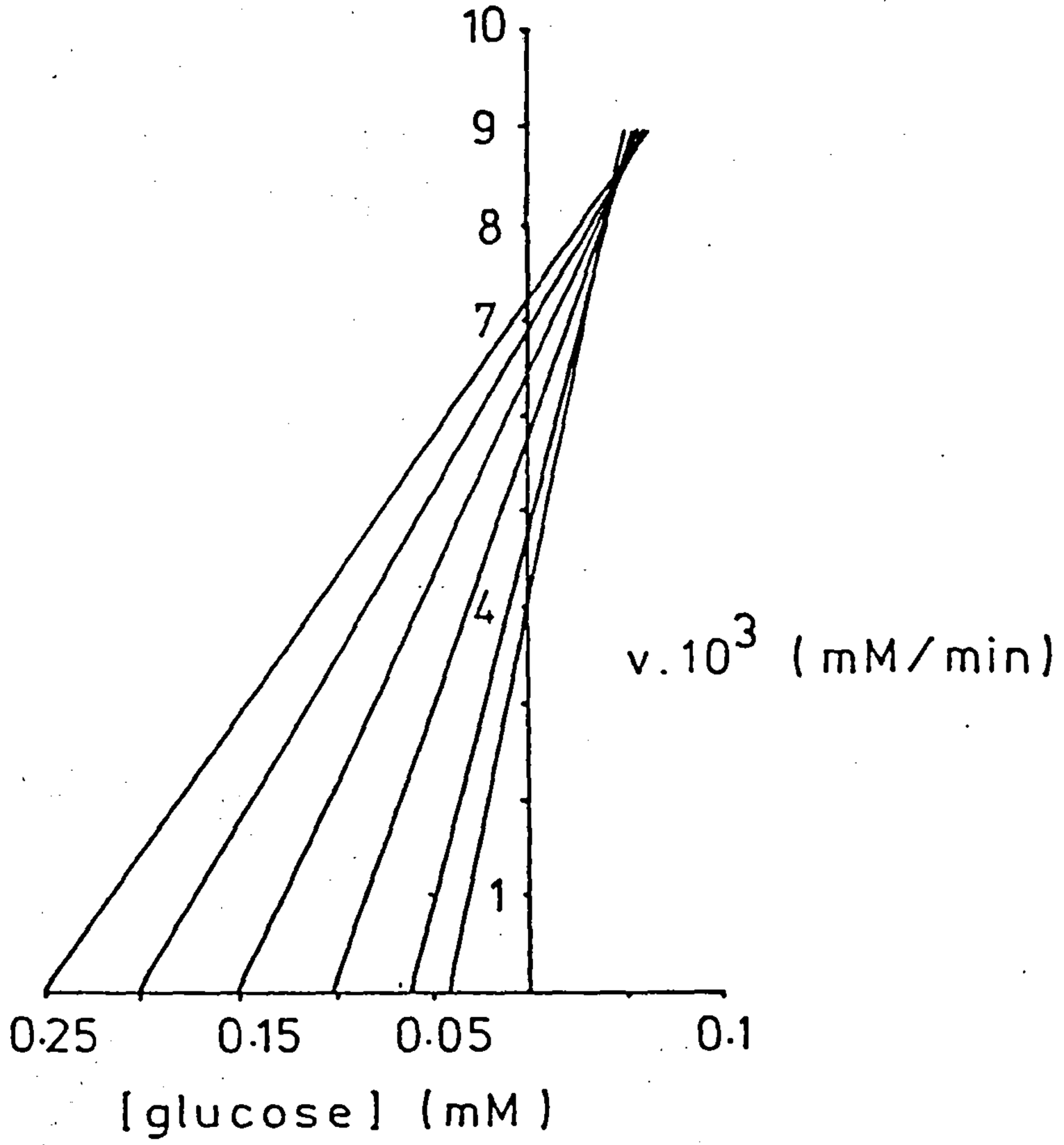


Figure 8.3

Km for mannose. Heart hexokinase.
Mg^{2+} was held at 5 mM.
Km for mannose was 0.05 mM.

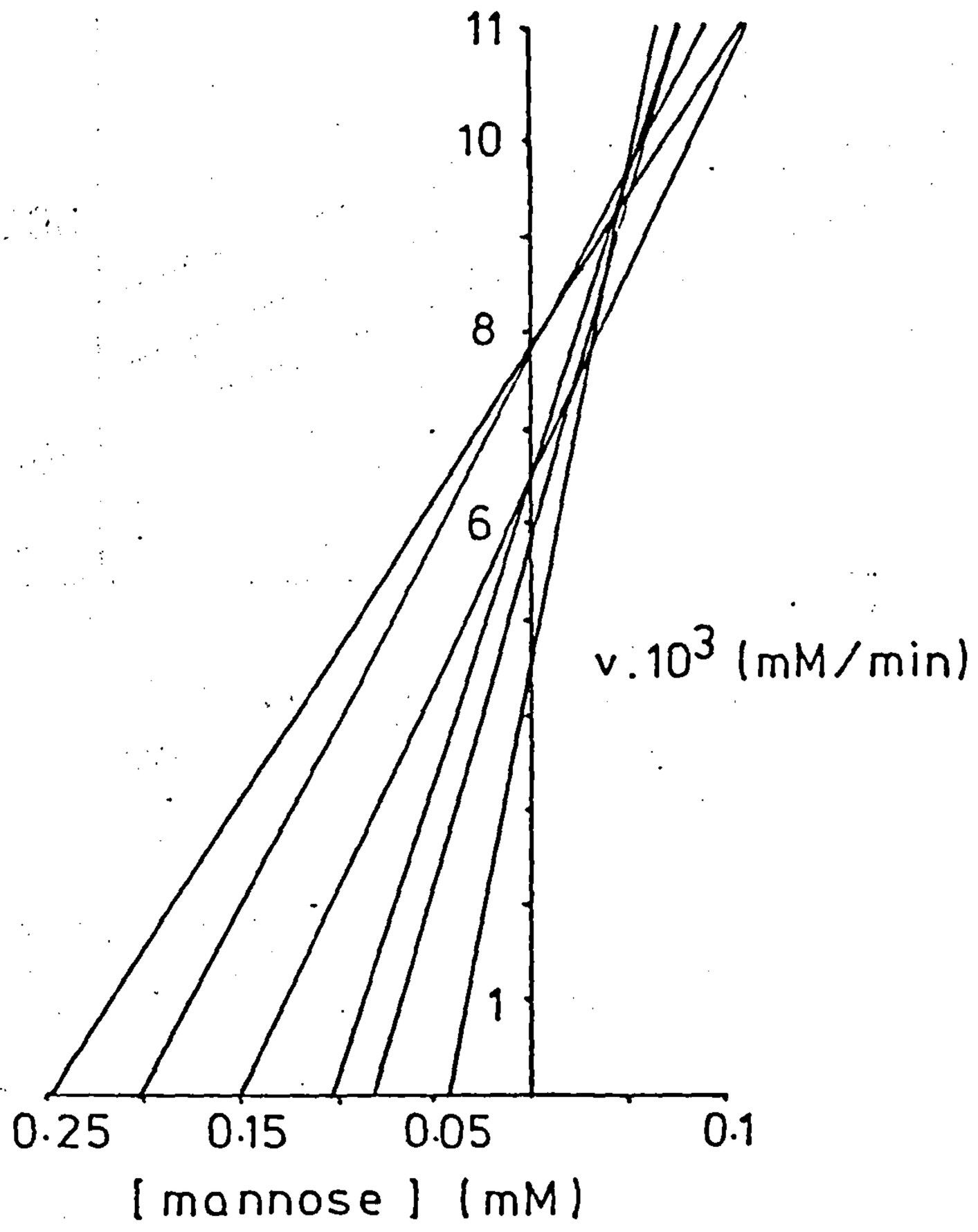
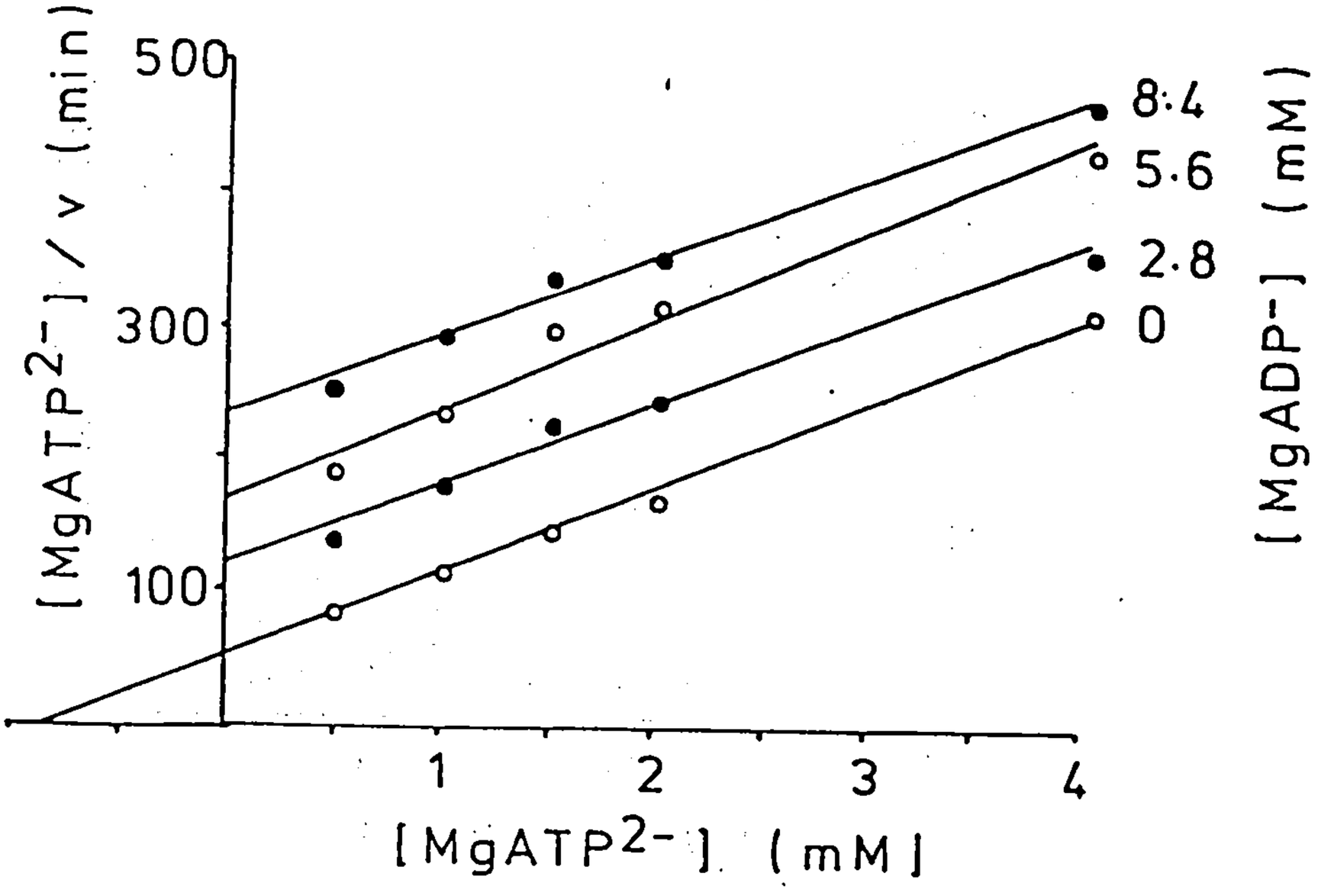


Figure 8.4

MgADP vs. MgATP Heart hexokinase.
Mg^{2+} was 2 mM above the concentration of nucleotide, glucose was 0.05 mM.



Secondary plot for MgATP vs. MgADP.
K_i = 2.4 mM K_i' = ∞

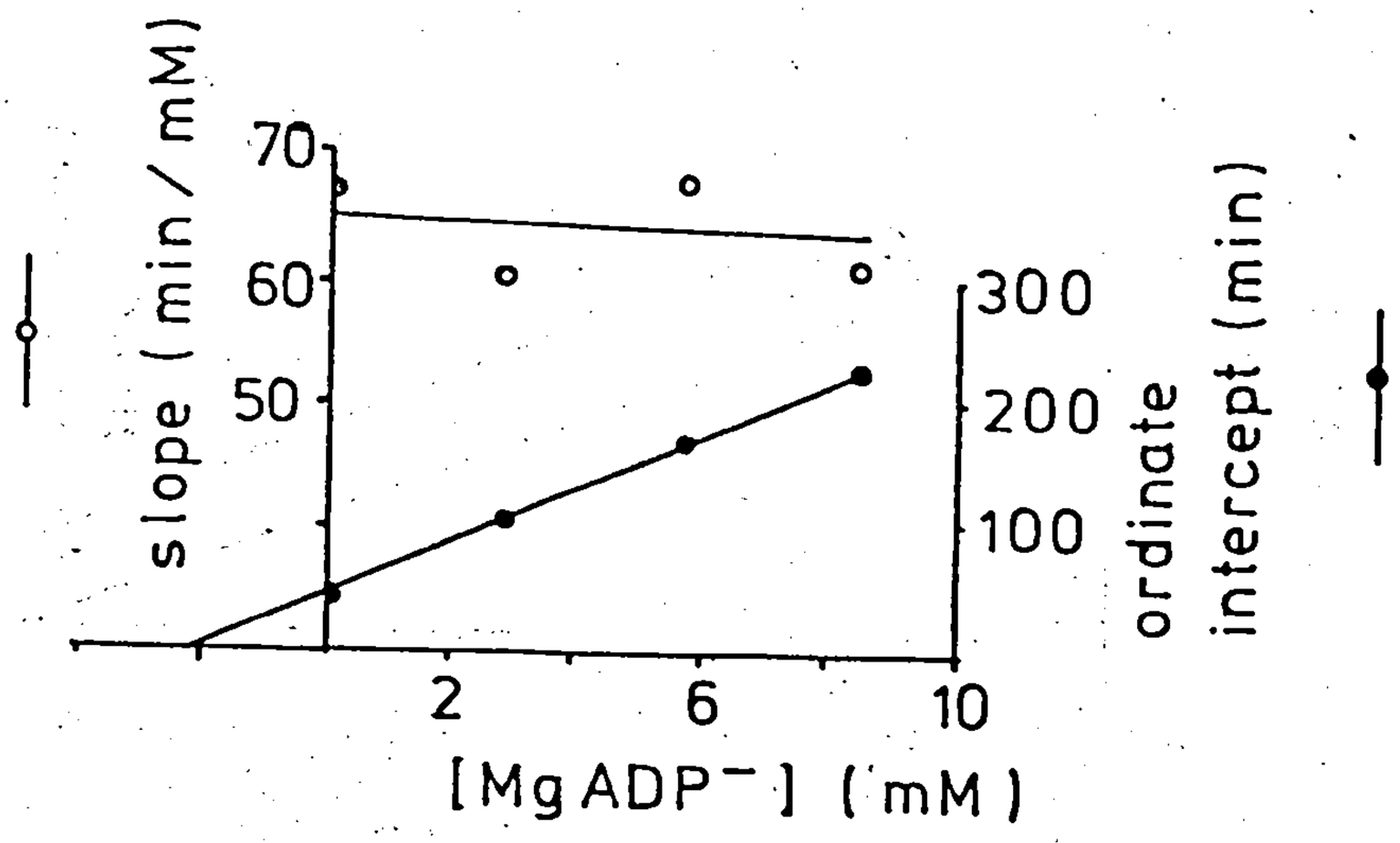
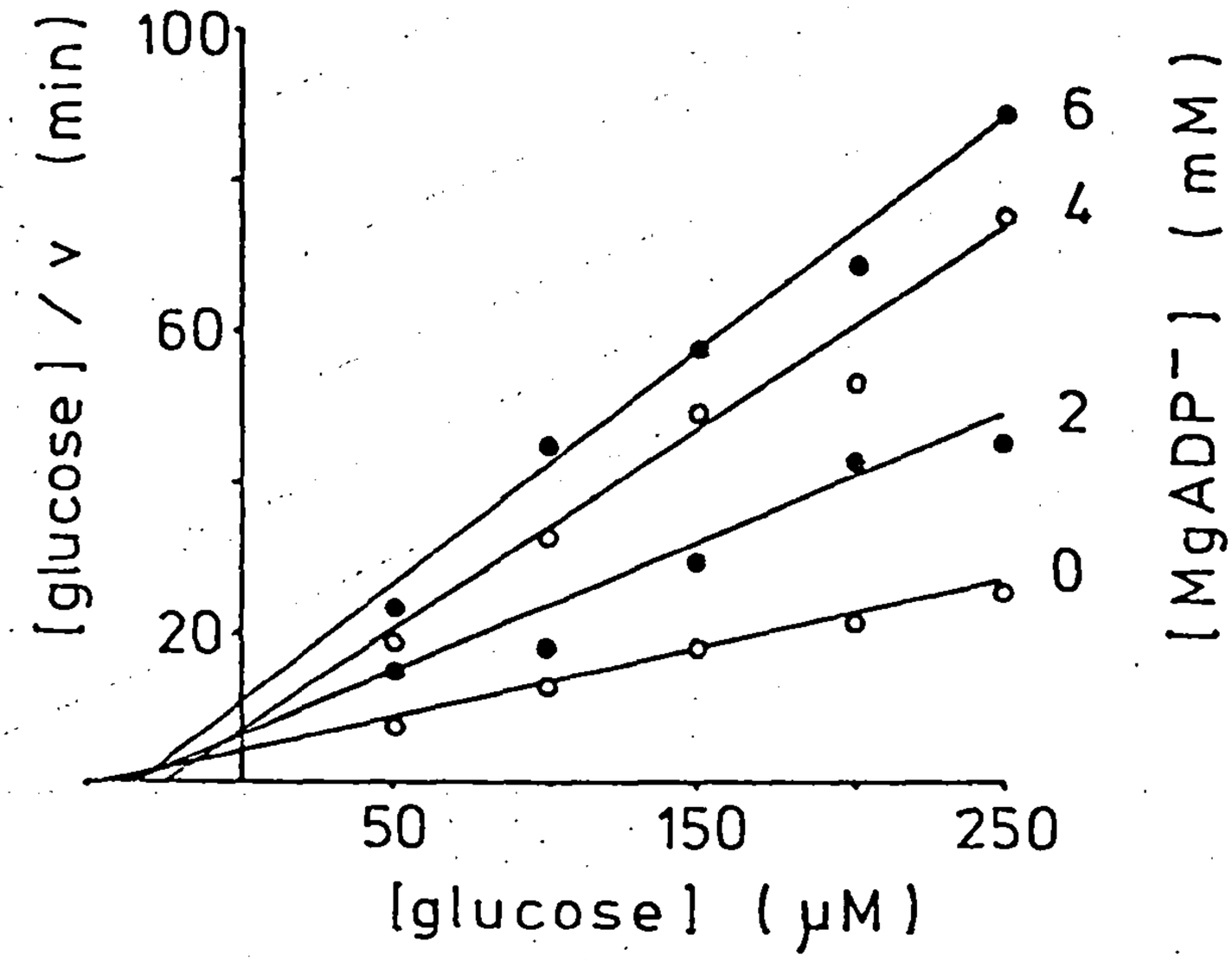


Figure 8.5

MgADP vs. glucose Heart hexokinase.

Mg^{2+} was 2 mM above the concentration of nucleotide, glucose was 0.05 mM.



Secondary plot for MgADP vs. glucose

K_i = 4 mM. K'_i = 2.4 mM

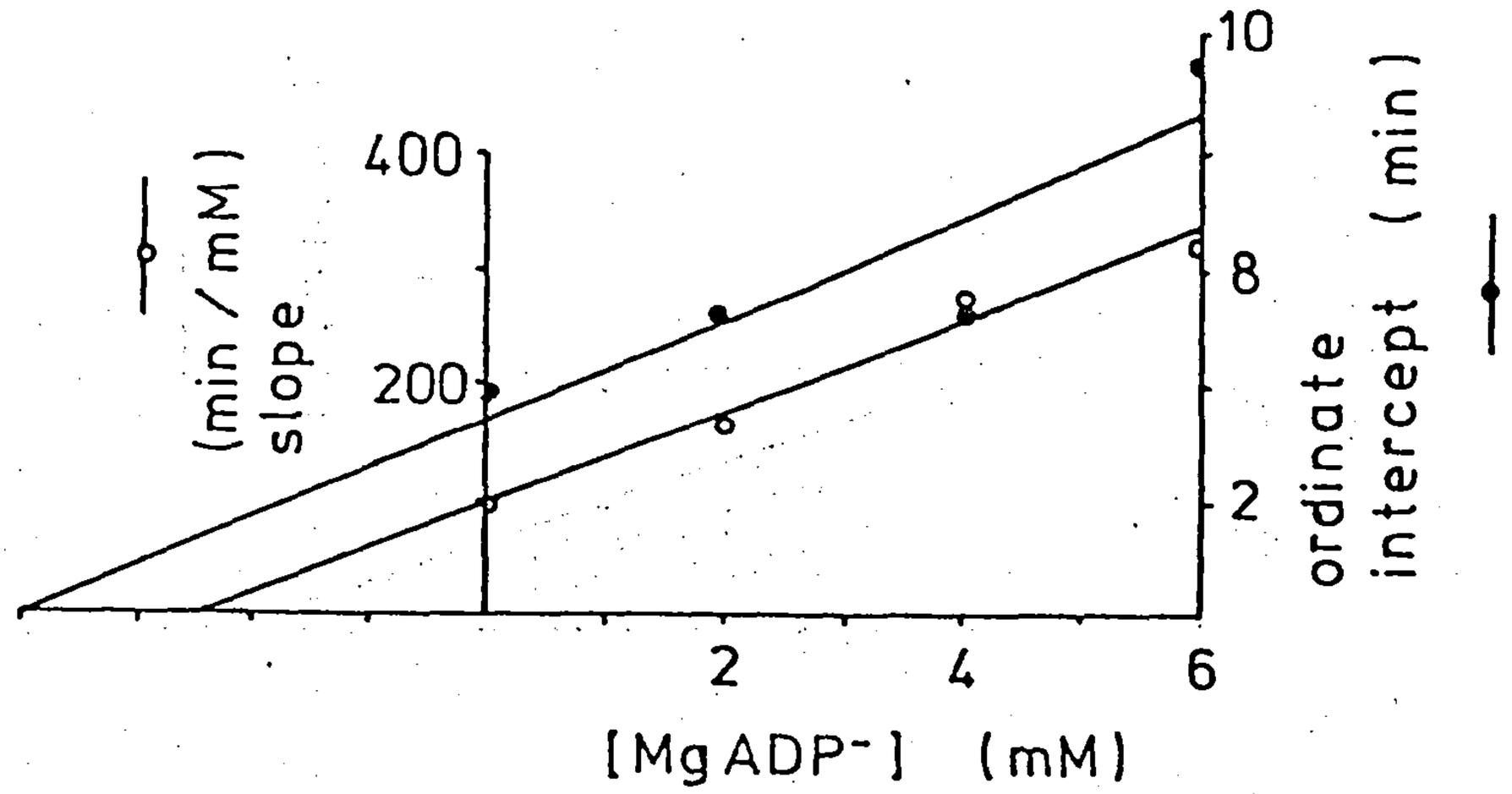
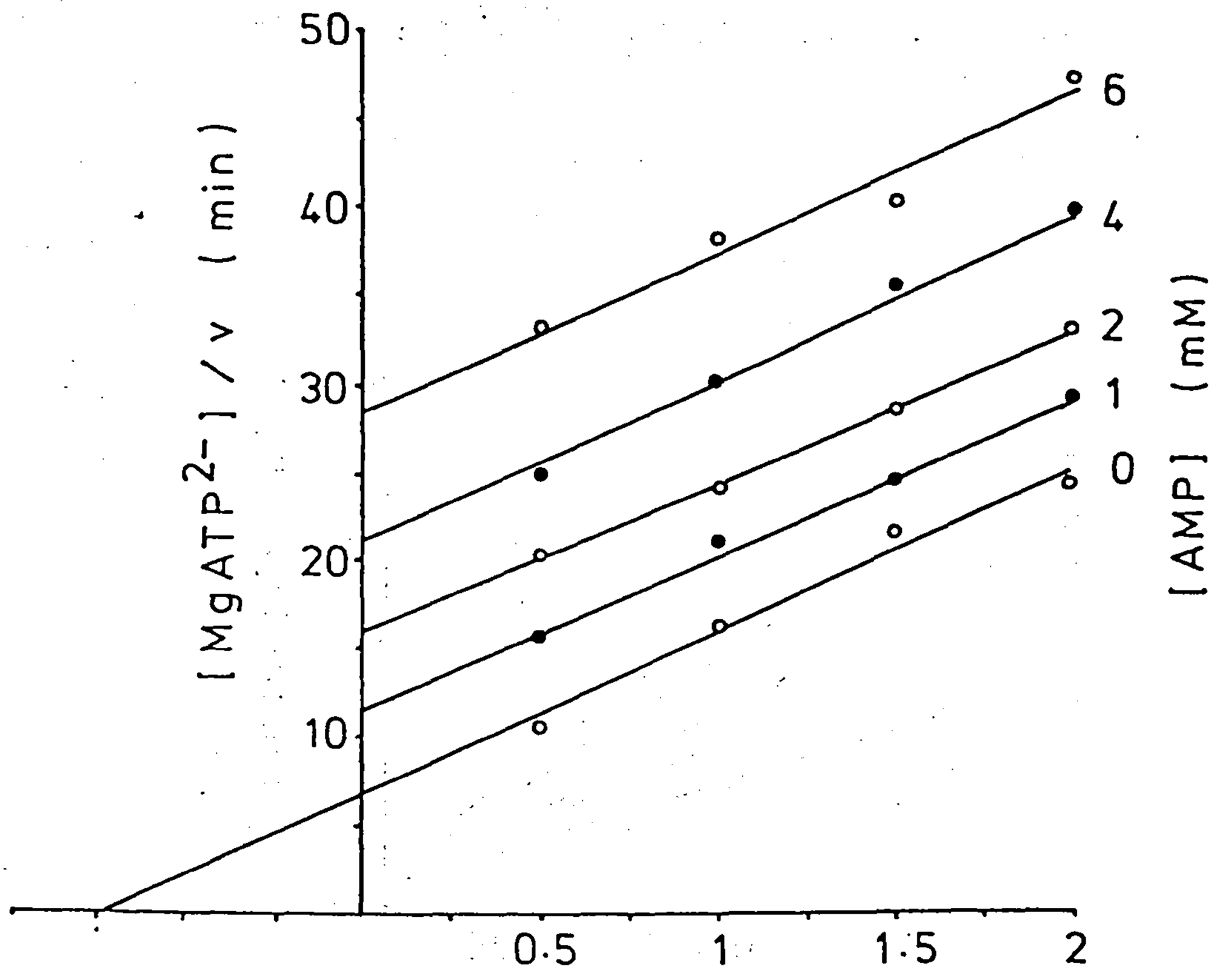


Figure 8.6

AMP vs. MgATP. Heart hexokinase.
Mg^{2+} was held at 7 mM, glucose was 0.05 mM.



Secondary plot for AMP vs. MgATP.
K_i = 2.2 mM. K_i' = ∞

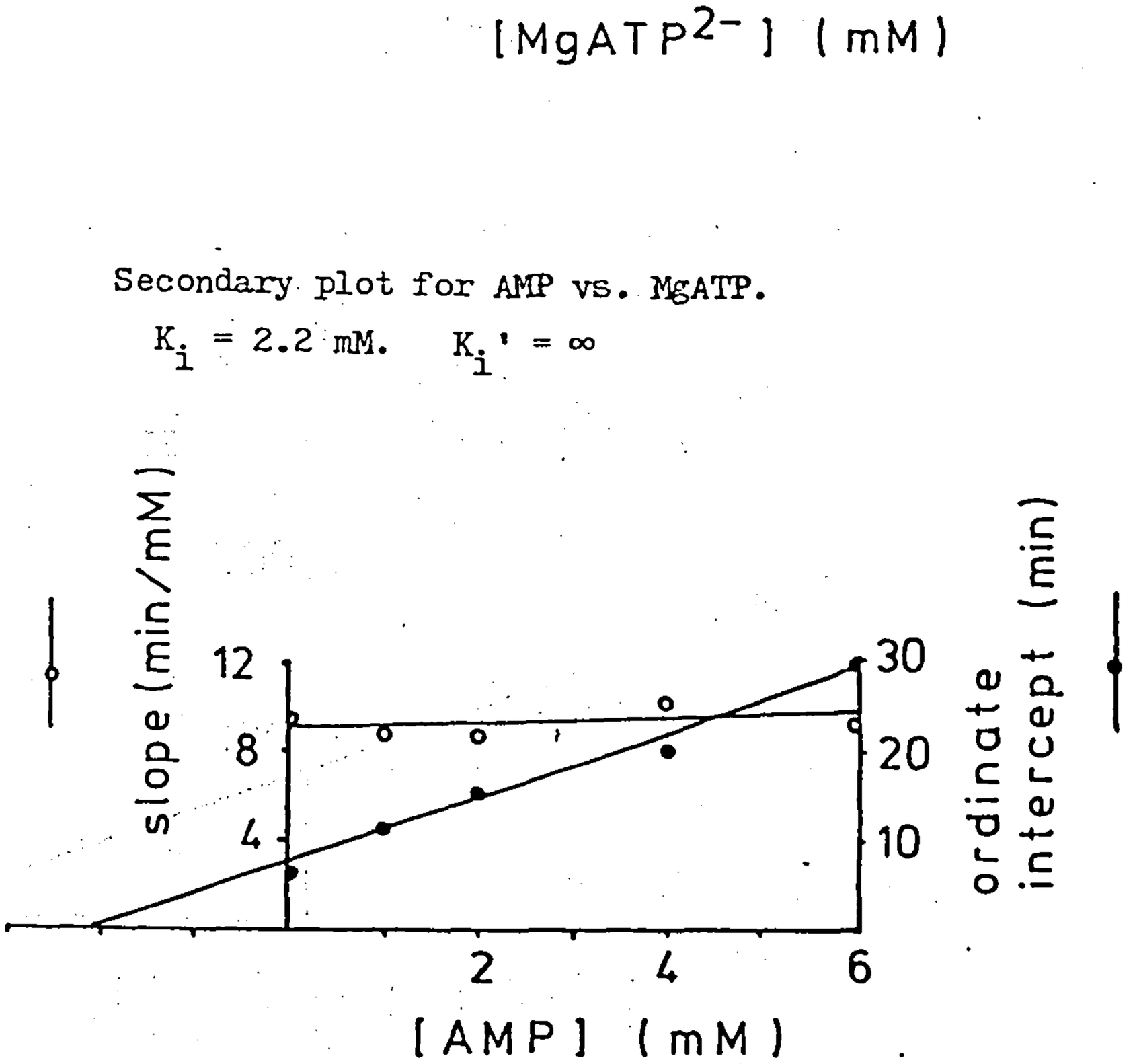
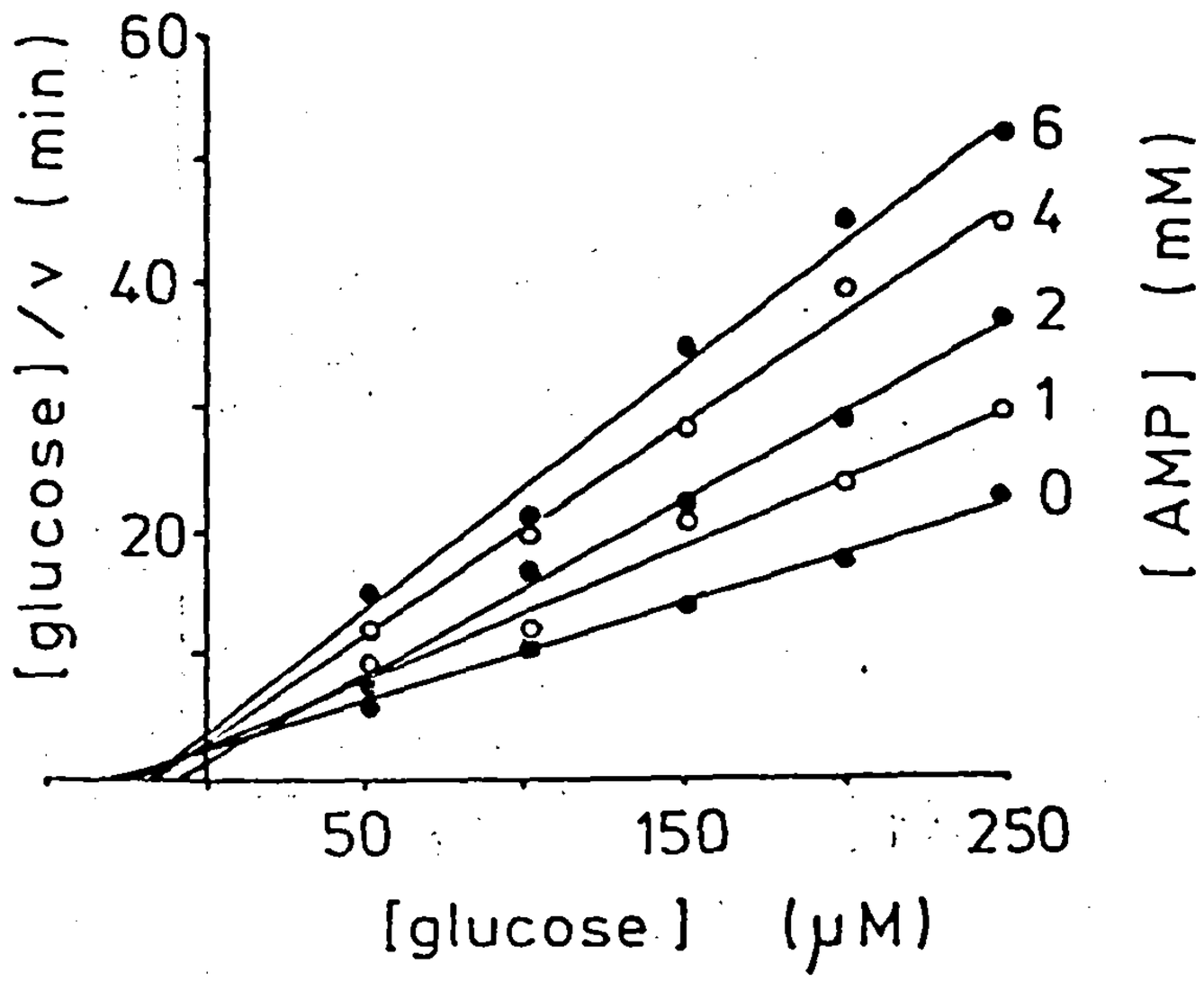
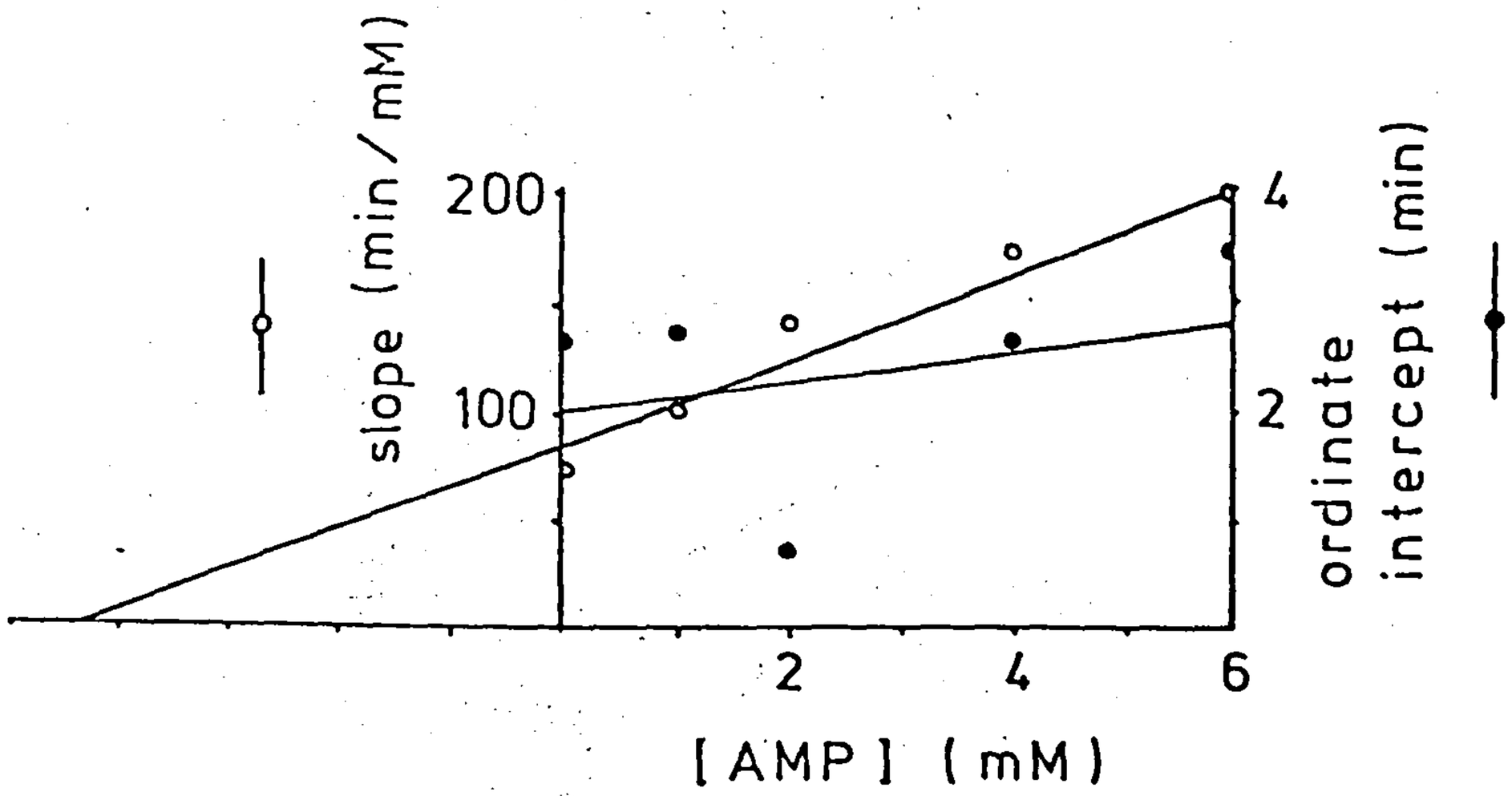


Figure 8.7

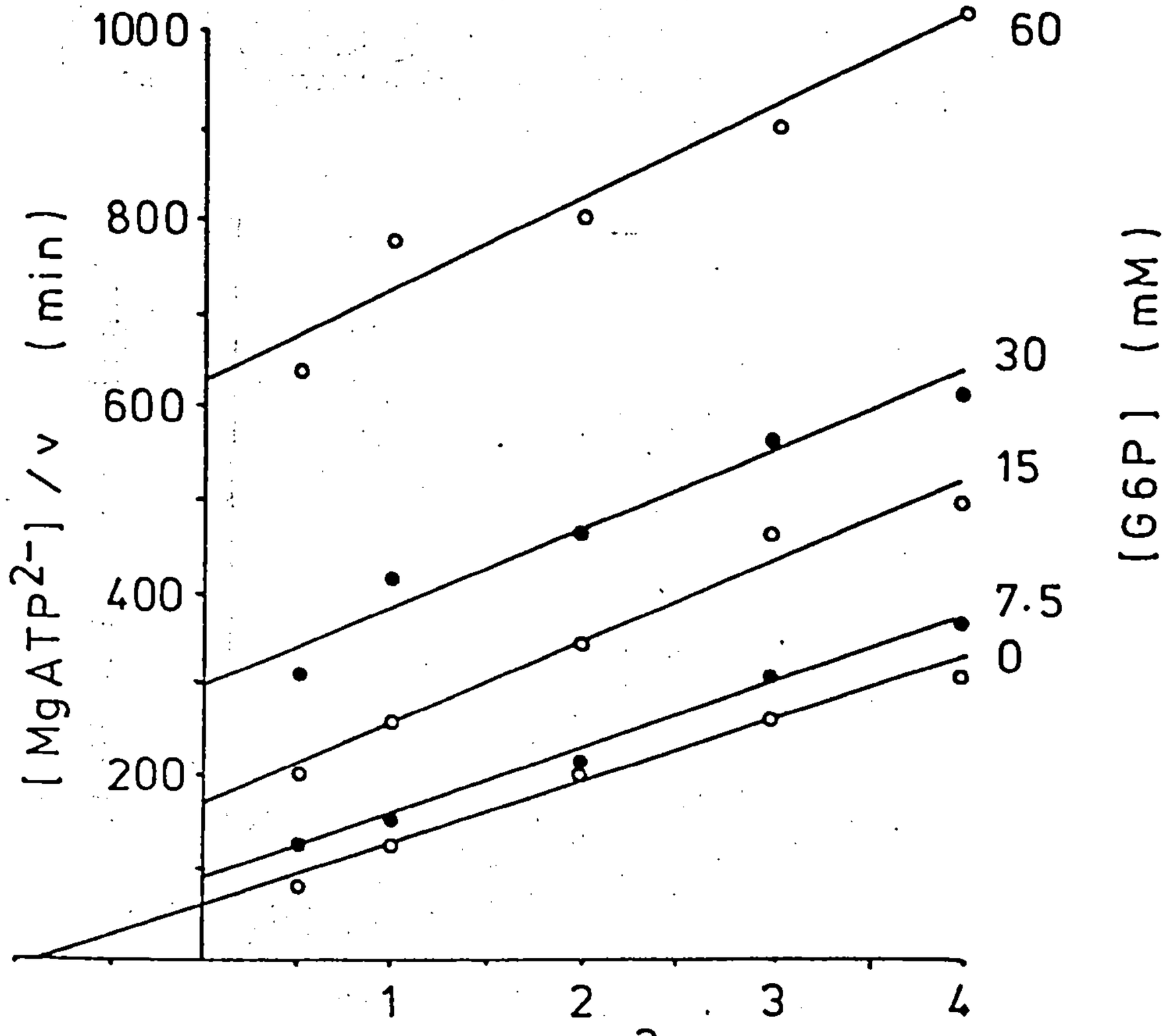
AMP vs. glucose. Heart hexokinase
Mg^{2+} was held at 7 mM, MgATP was held at 0.7 mM.



Secondary plot for AMP vs. glucose.
K_i = 14 mM. K_i' = 4.4 mM.



Glucose 6-phosphate vs. MgATP. Heart hexokinase. Figure 8.8
Mannose was used as a substrate at a concentration of 0.06 mM. Mg^{2+} was held at 5 mM. The pyruvate kinase - lactate dehydrogenase linked assay was used.



Secondary plot for glucose 6-phosphate vs. MgATP.
K_i= 3.2 uM. K_i'= ∞.

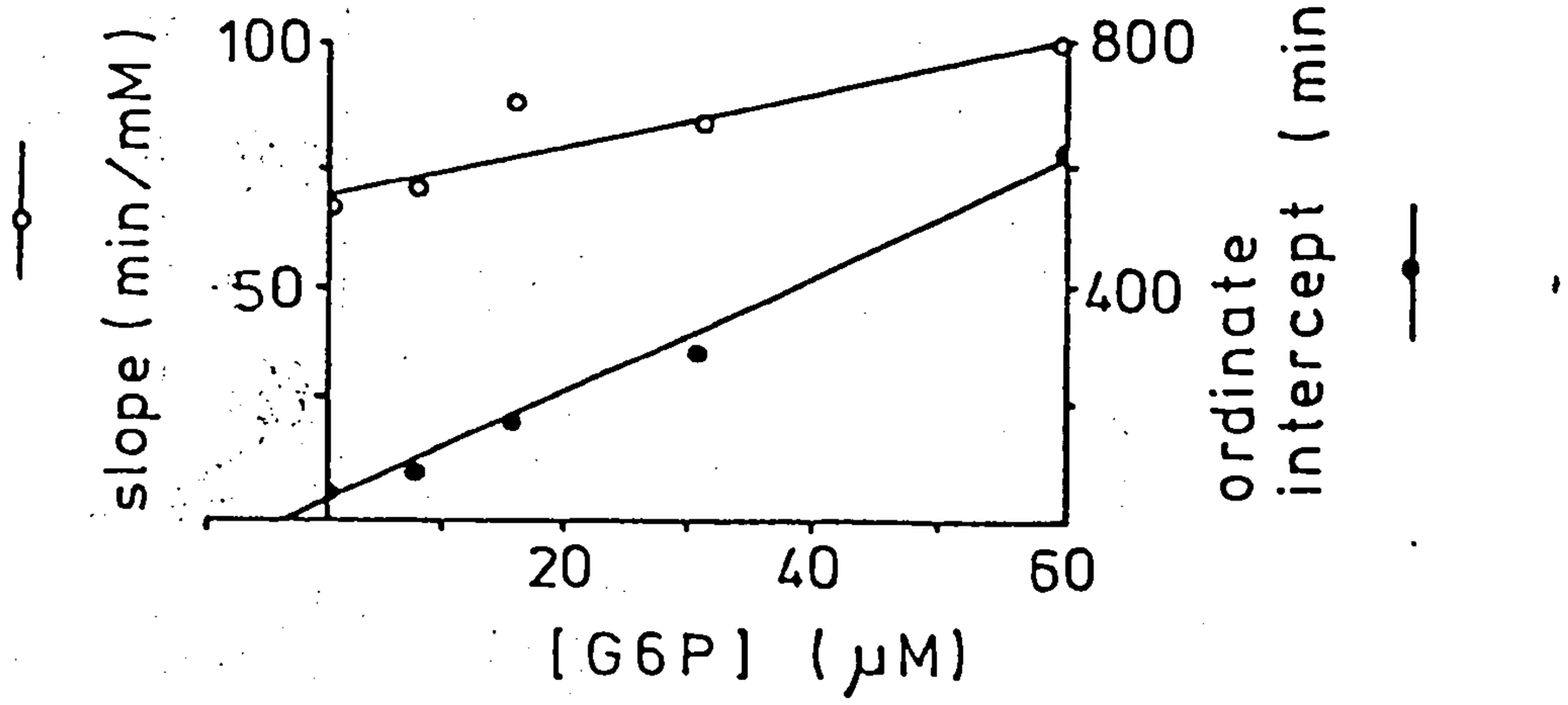
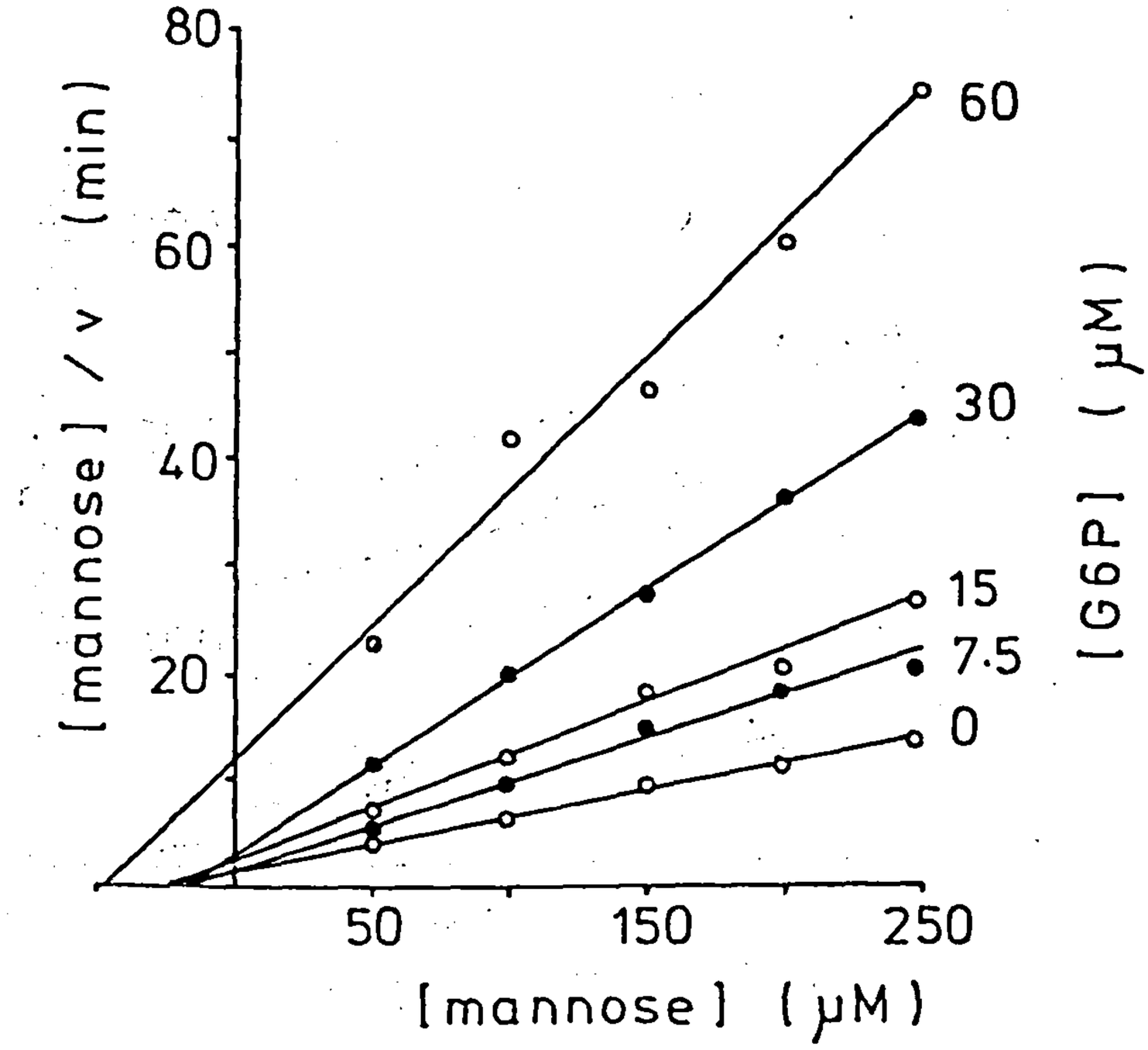


Figure 8.9

Glucose 6-phosphate vs. mannose. Heart hexokinase. Mg^{2+} was held at 5 mM. MgATP was 0.7 mM. The pyruvate kinase - lactate dehydrogenase linked assay was used.



Secondary plot for glucose 6-phosphate vs. mannose. K_i = 18 uM. K_i = 16 uM.

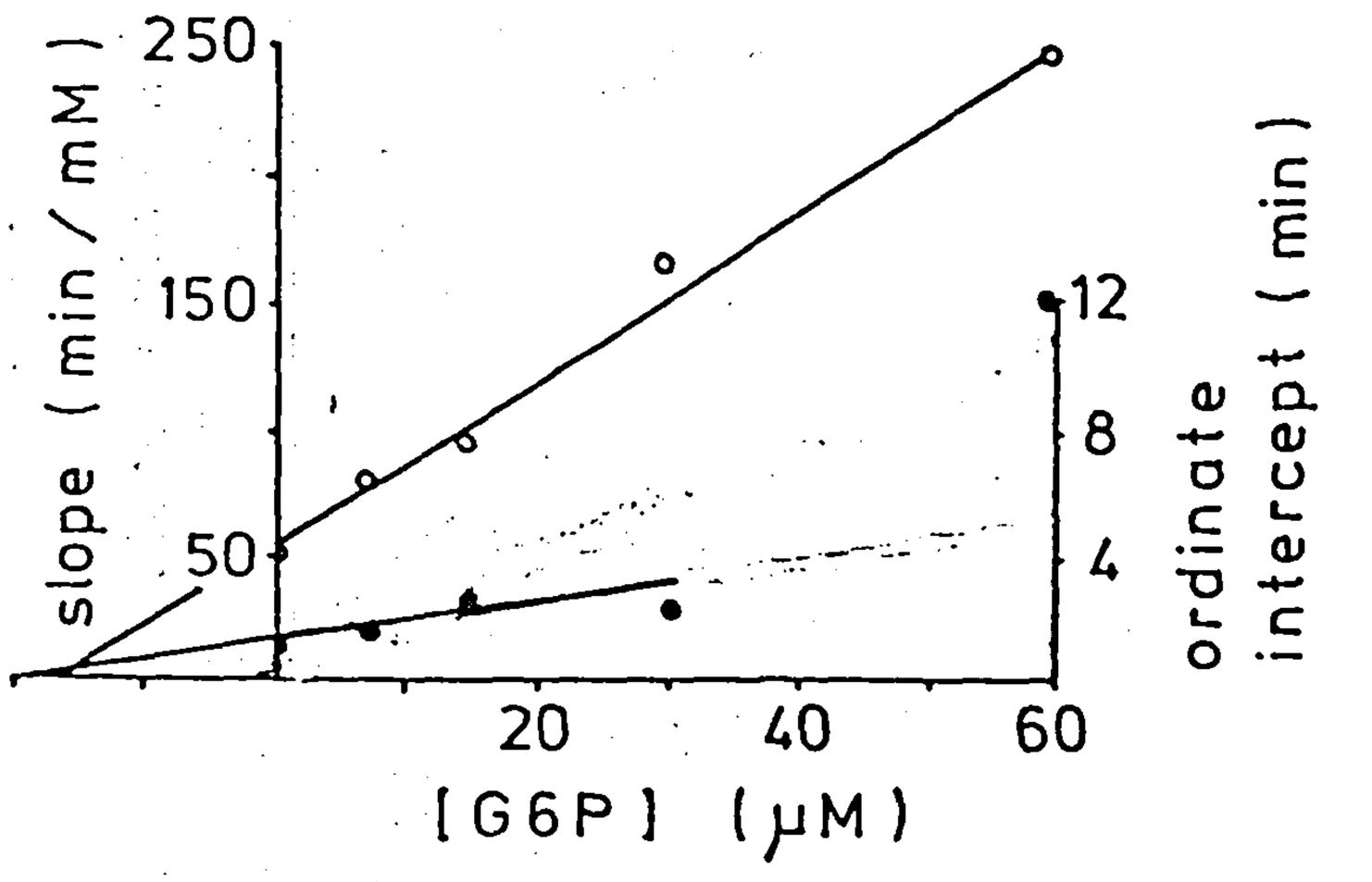
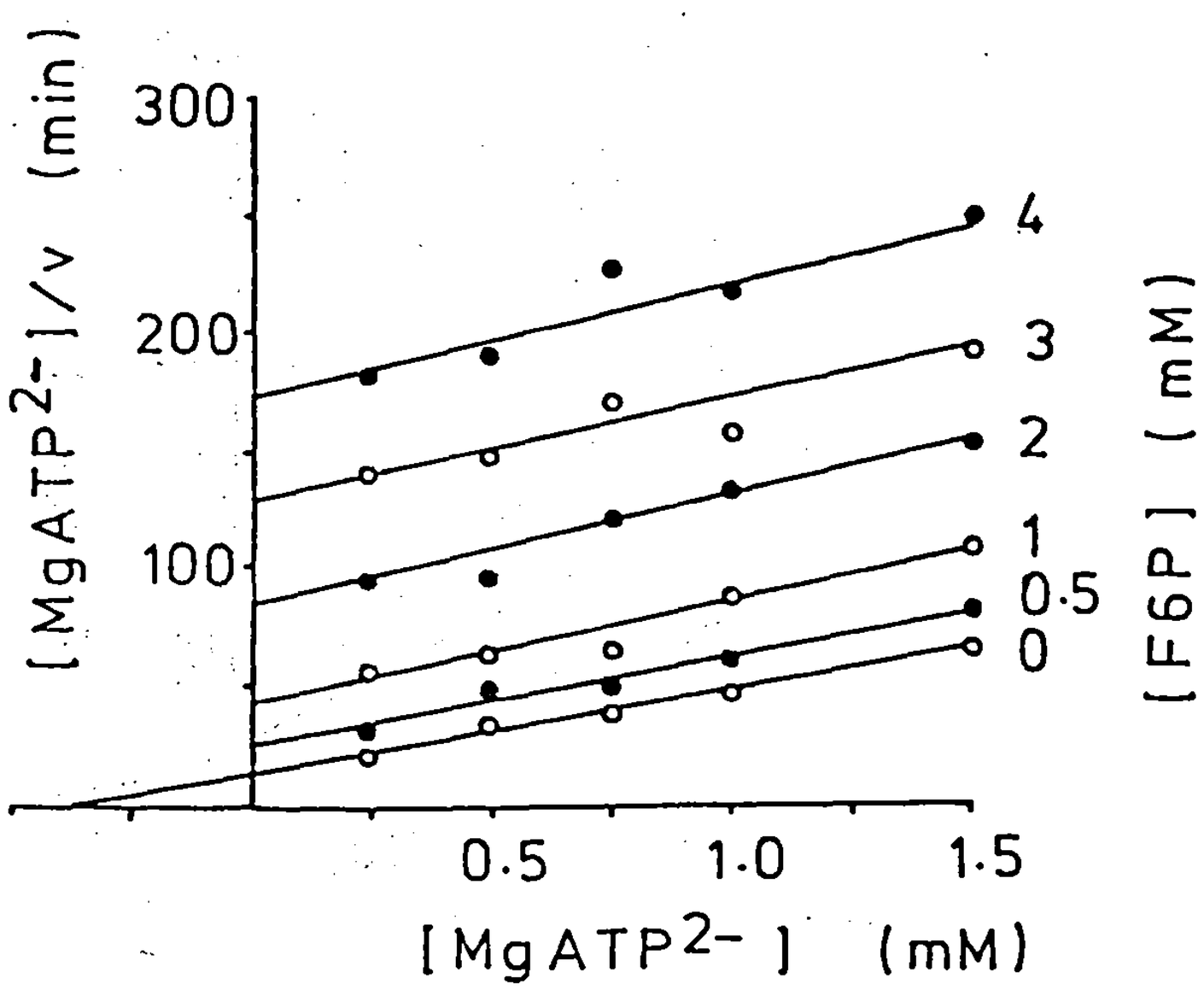


Figure 8.10

Fructose 6-phosphate vs. MgATP. Heart hexokinase.
Mg^{2+} was held at 5 mM, glucose was at 0.05 mM.



Secondary plot for fructose 6-phosphate vs. MgATP
K_i = 0.2 mM. K_i' = ∞

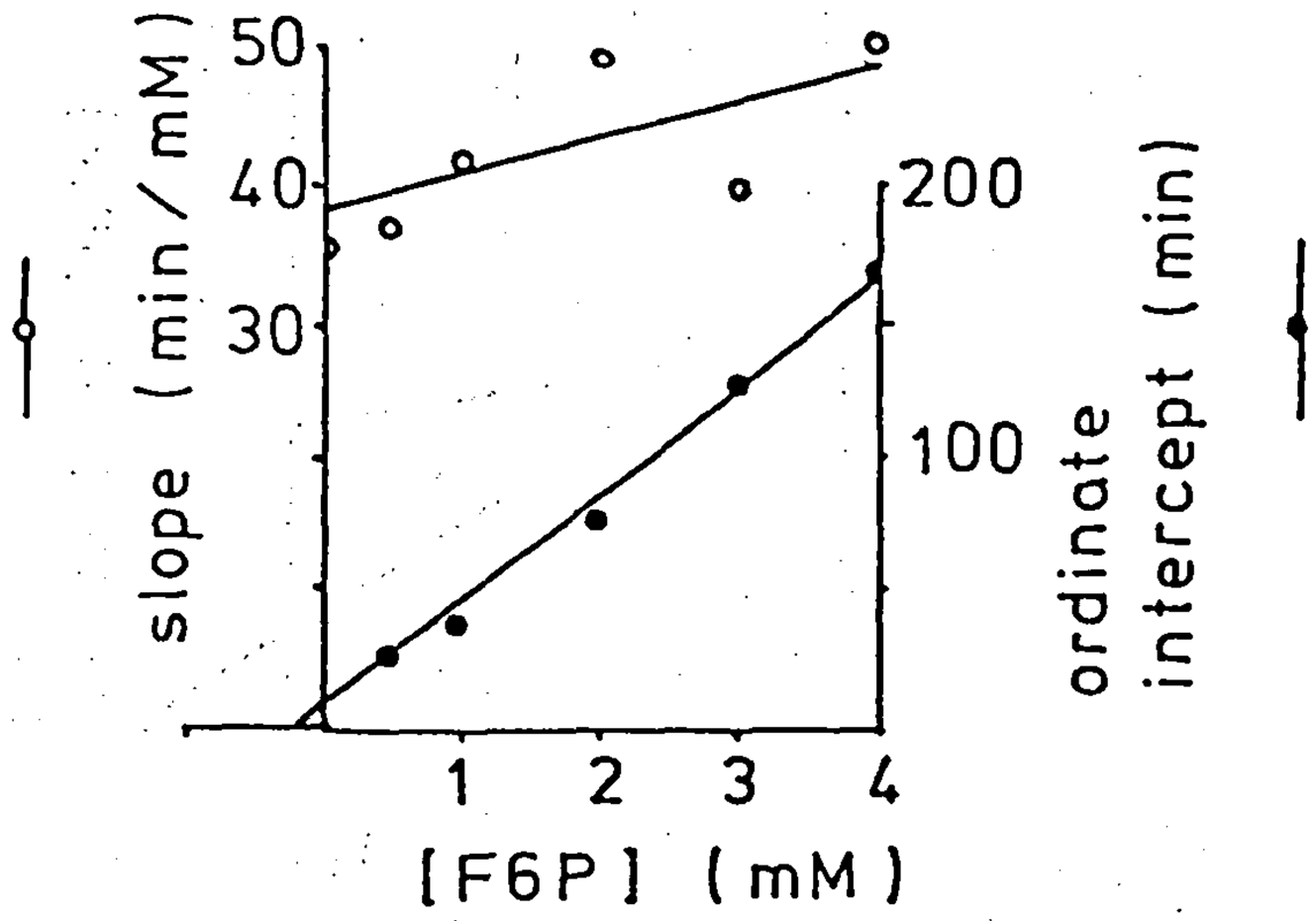
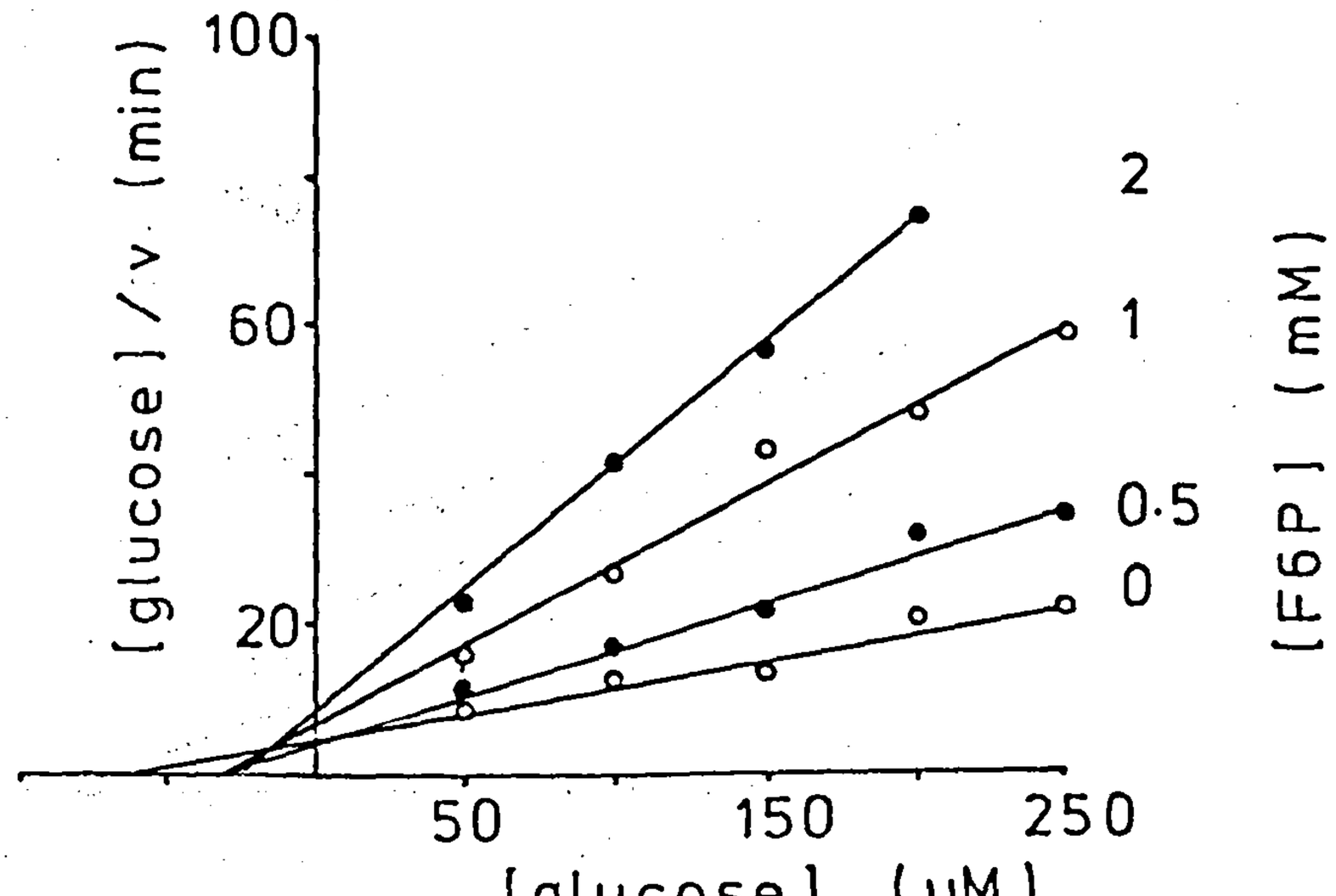


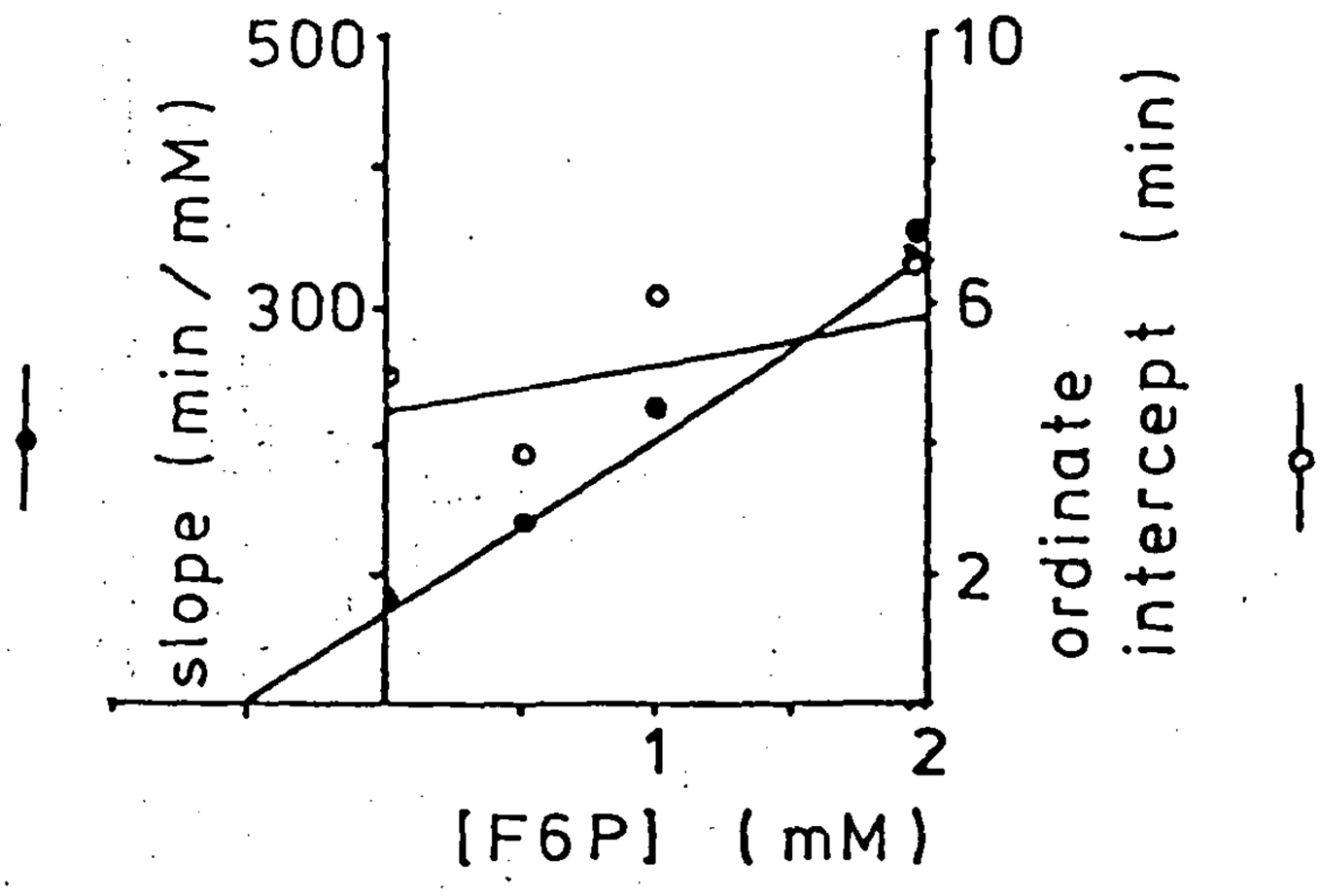
Figure 8.11

Fructose 6- phosphate vs. glucose. Heart hexokinase. Mg^{2+} was held at 5 mM and MgATP at 0.7 mM.



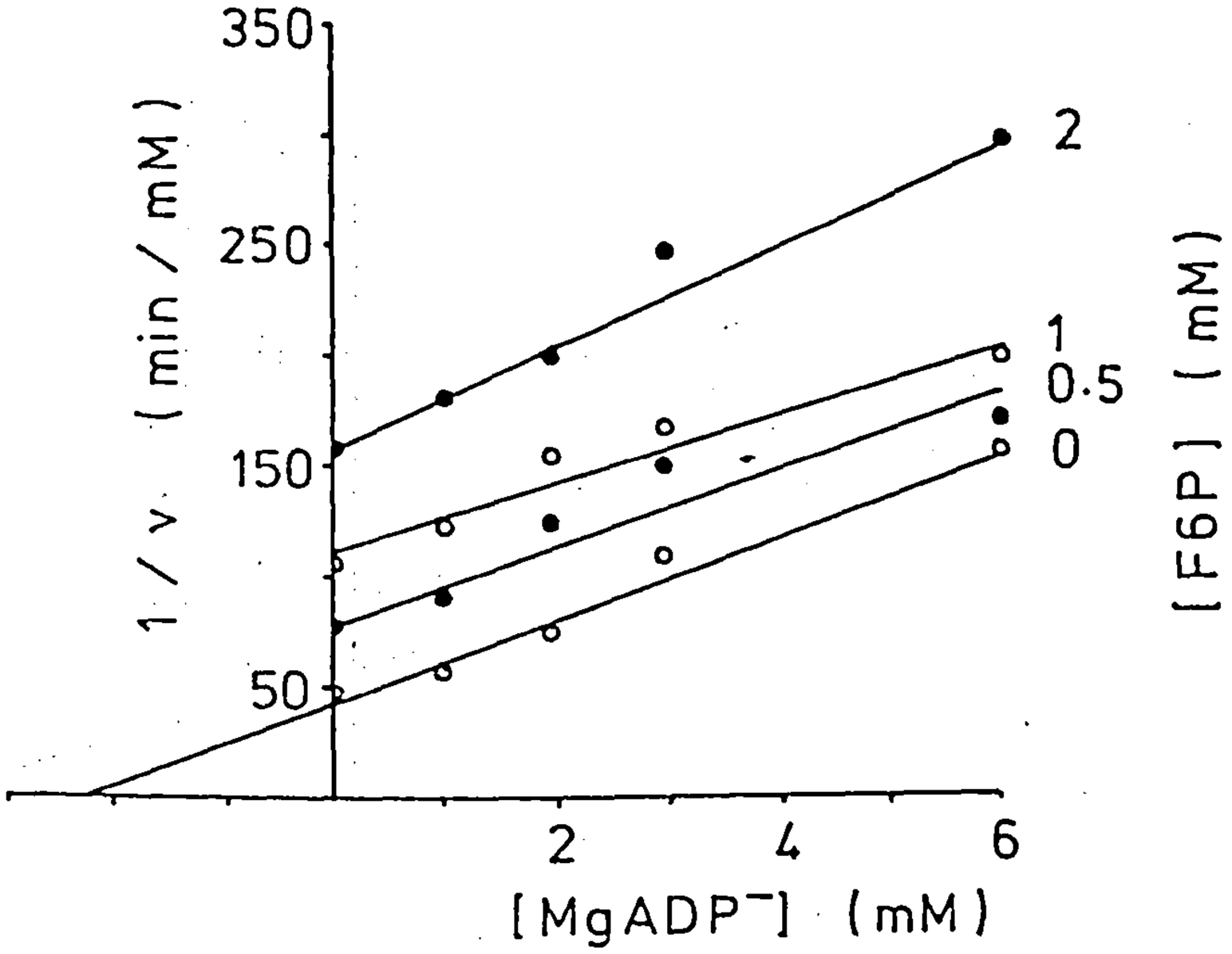
Secondary plot for fructose 6-phosphate vs. glucose

K_i = 4.1 mM. K_i' = 0.52 mM.

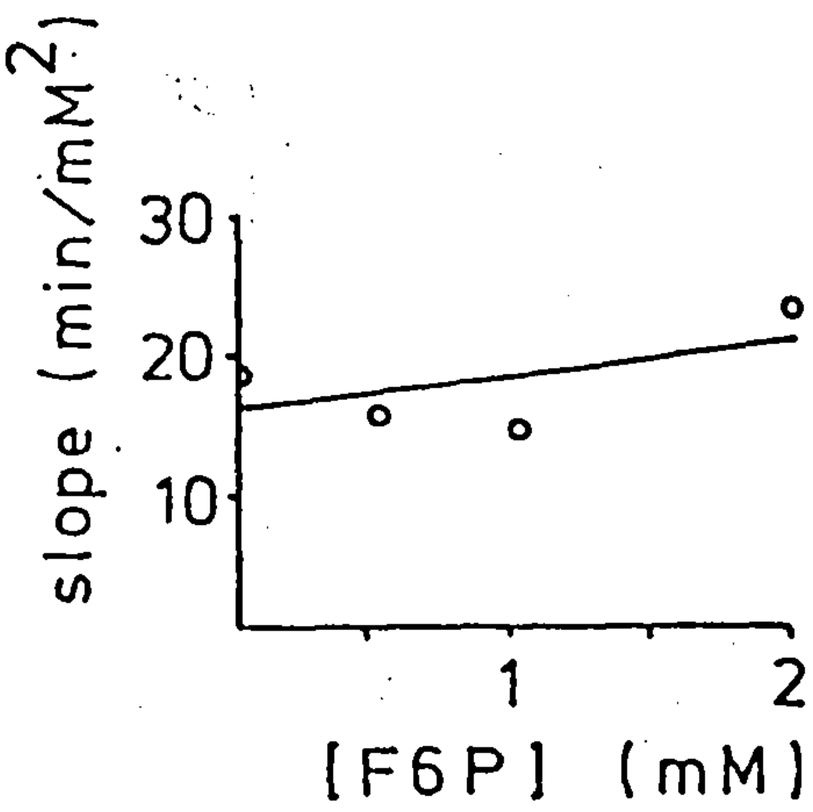


Double inhibitor studies. Heart hexokinase. Figure 8.12

MgADP vs. fructose 6-phosphate.
Mg^{2+} was held 2 mM above the nucleotide concentration, glucose was at 11 mM and MgATP at 0.7 mM.

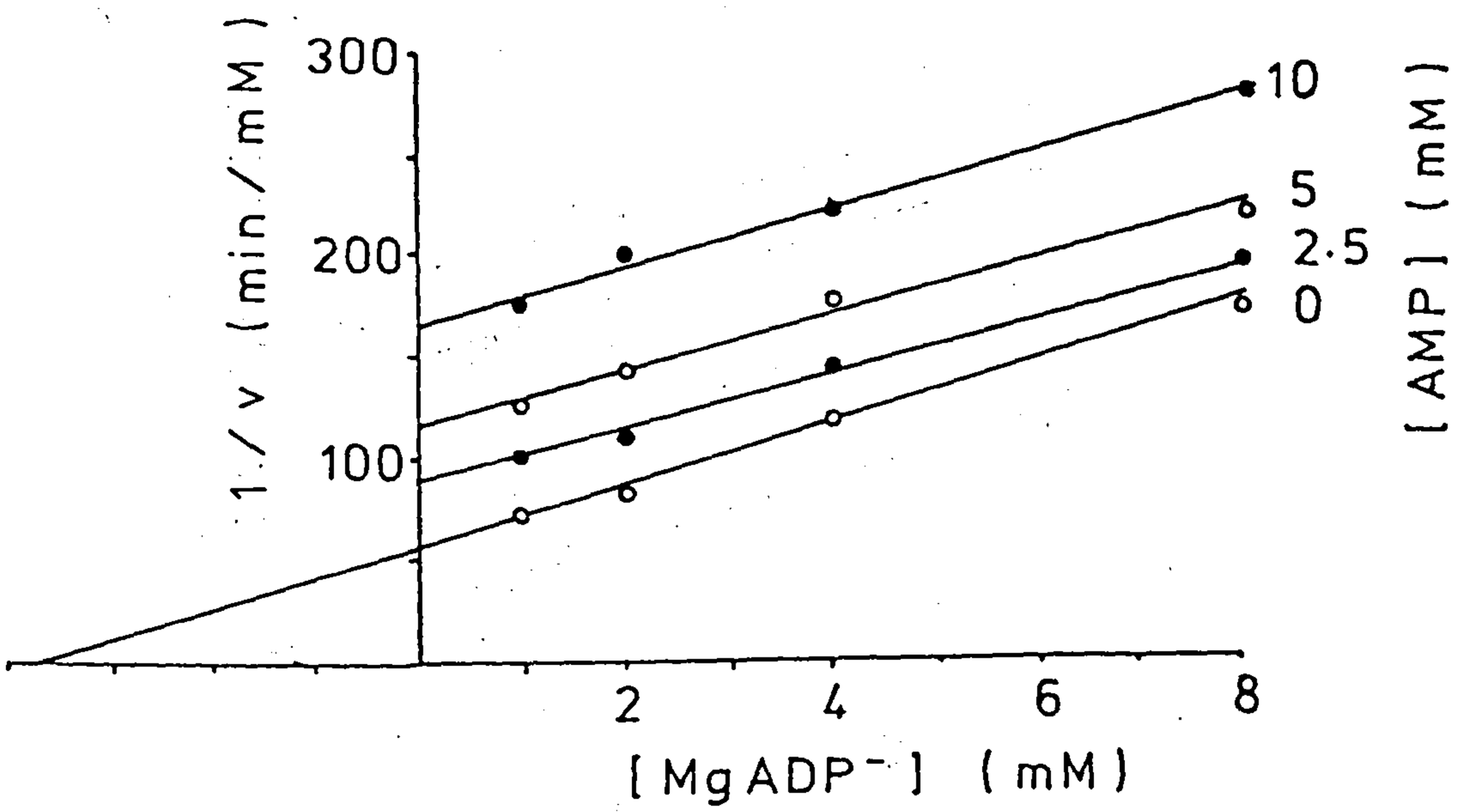


Secondary plot for MgADP vs. fructose 6-phosphate.

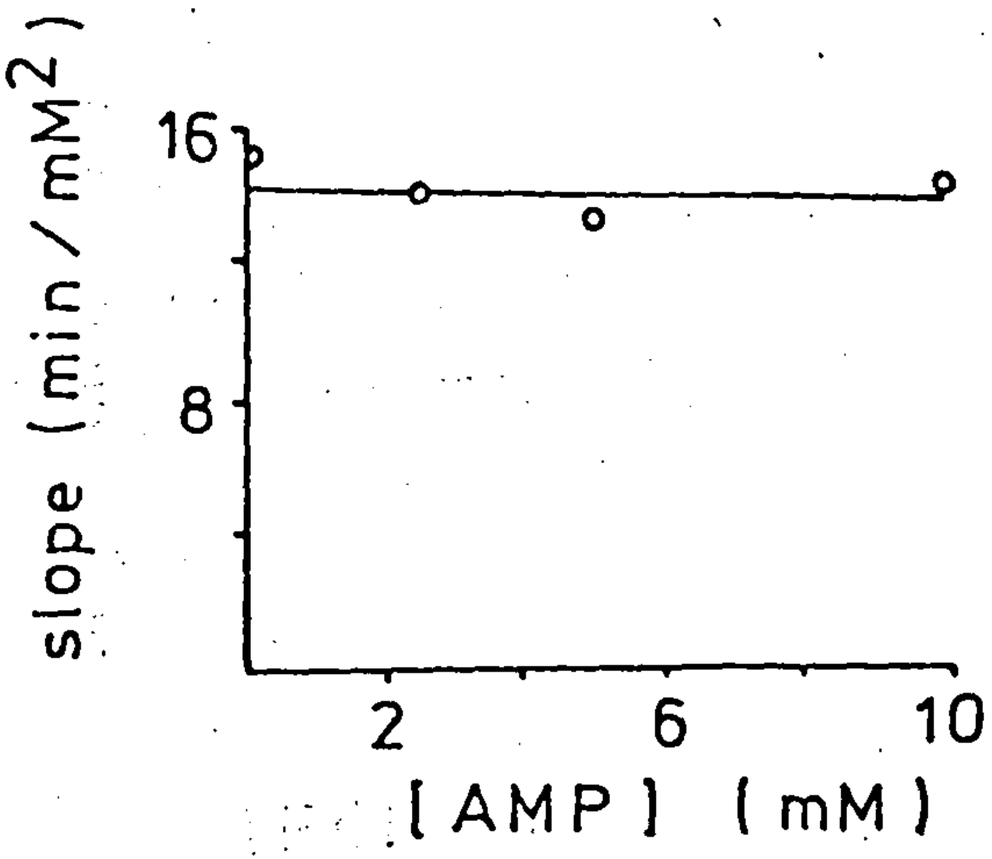


Double inhibitor studies. Heart hexokinase Figure 8.13

MgADP vs. AMP.
Mg^{2+} was 2 mM above the total nucleotide concentration, glucose was at 11 mM and MgATP at 0.7 mM.



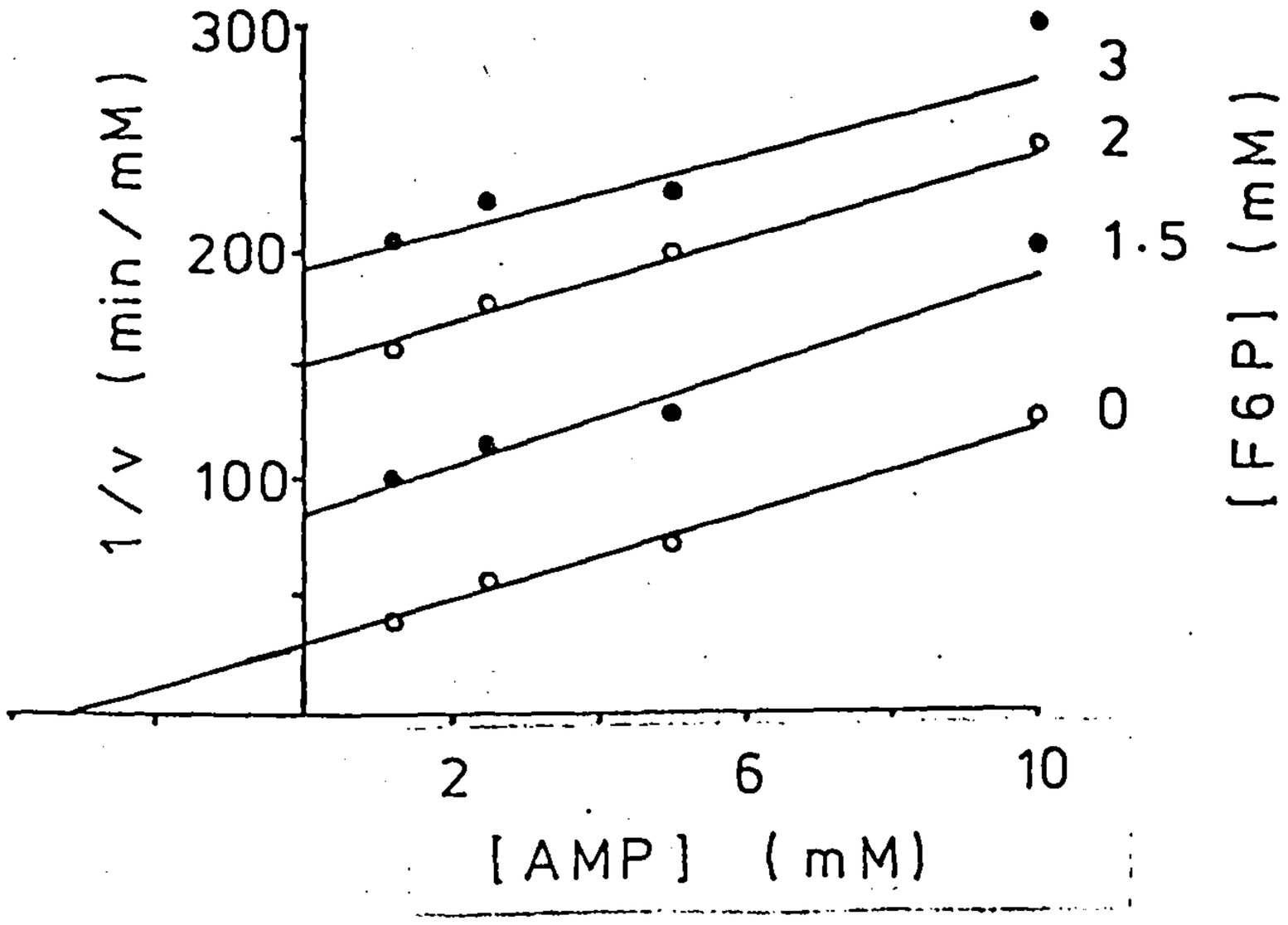
Secondary plot for MgADP vs. AMP.



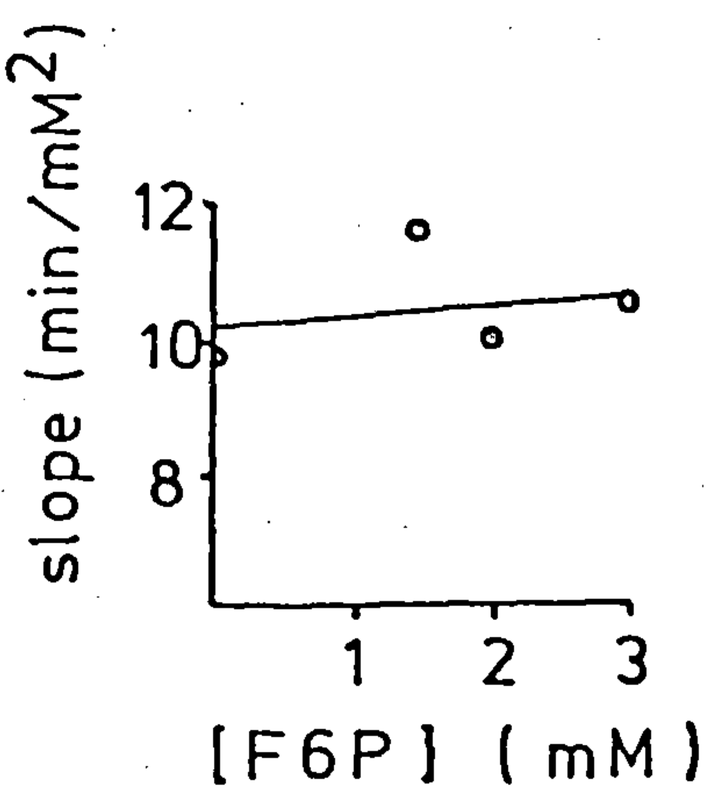
Double inhibitor studies. Heart hexokinase. Figure 8.14

AMP vs. fructose 6-phosphate.

Mg^{2+} was held at 5 mM, glucose at 11 mM and MgATP at 0.7 mM.



Secondary plot for AMP vs. fructose 6-phosphate.



CHAPTER 9

THE KINETICS OF *ASTERIAS* HEXOKINASE : RESULTS

THE KINETICS OF ASTERIAS HEXOKINASE

SECTION 9.1 RESULTS

The maximum activity of Asterias hexokinase was unchanged between pH 7 and pH 8.5 and, like other hexokinases, required Mg^{2+} (figure 9.1) and was therefore assayed in the same way as was heart hexokinase.

The average Km's for MgATP and glucose were 0.14 mM and 0.05 mM respectively and these levels of substrate were used in single inhibitor studies. In double inhibitor experiments MgATP was maintained at 0.14 mM and glucose at 11 mM.

The results for kinetic studies are given in tables 9.1 and 9.2 . Asterias rubens hexokinase showed linear substrate kinetics and is inhibited by a variety of ligands including MgADP (figures 9.5 and 9.6), AMP (figures 9.7 and 9.8), phosphate (figures 9.9 and 9.10) and also glucose 6-phosphate (figures 9.11 and 9.12) and fructose 6-phosphate (figures 9.13 and 9.14). In double inhibitor studies MgADP, AMP and fructose 6-phosphate all competed for binding sites on the enzyme. These results are discussed in more detail in chapter 10.

Table 9.1

Substrate and single inhibitor kinetics for Asterias hexokinase.

A represents MgATP, G glucose, M mannose, Pi phosphate, G6P glucose-6-phosphate and F6P fructose 6-phosphate. All values are expressed in mM. Inhibition types are represented by C, competitive; N, non-competitive and U, uncompetitive.

varied substrate	inhibitor	mM Km	mM K_i	mM K_i'	inhibition type	figure
A		0.14				9.2
G		0.05				9.3
M		0.06				9.4
A	MgADP		1.10	0.9	N	9.5
G	MgADP		1.03	1.11	N	9.6
A	AMP		1.15	∞	C	9.7
G	AMP		∞	1.33	U	9.8
A	Pi		4.73	∞	C	9.9
G	Pi		6.46	7.66	N	9.10
A	G6P		0.055	∞	C	9.11
M	G6P		0.09	0.28	N	9.12
A	F6P		2.30	∞	C	9.13
G	F6P		1.56	2.18	N	9.14

Table 9.2

Double inhibitor kinetics of heart hexokinase.

inhibitor I	inhibitor J	interaction type	figure
MgADP	F6P	C	9.15
MgADP	AMP	C	9.16
AMP	F6P	C	9.17

Figure 9.1

The effect of Mg^{2+} on the reaction catalysed by Asterias hexokinase.

Purified Asterias hexokinase was assayed in a 0.05 I, pH 7.5 Tris-HCl buffer containing 11 mM glucose and 0.1 mM ATP. Mg^{2+} was varied between 0 and 20 mM. In the presence of no added Mg^{2+} there is a residual activity which is not affected by the addition of 1 mM EGTA but is abolished in the presence of 1 mM EDTA.

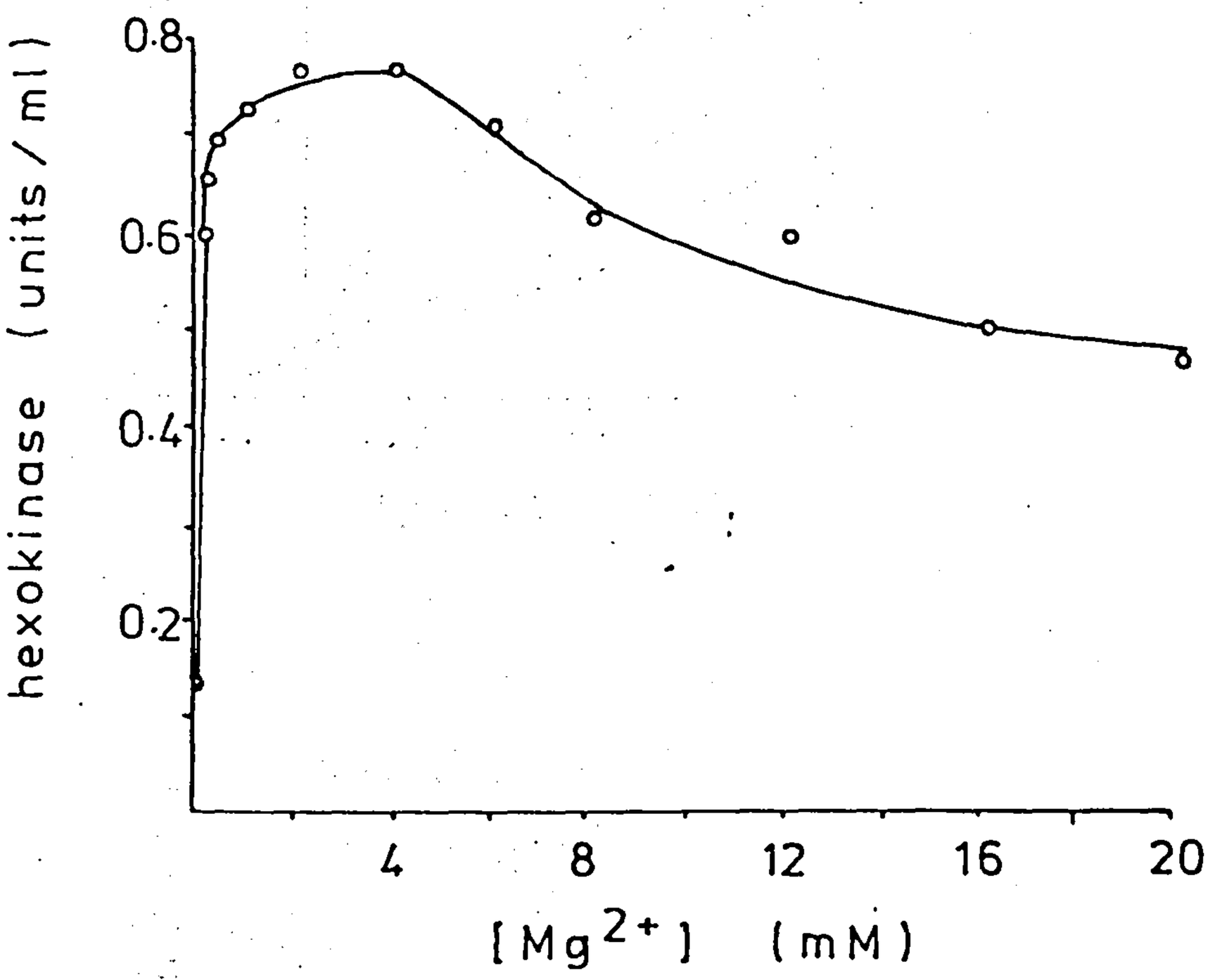
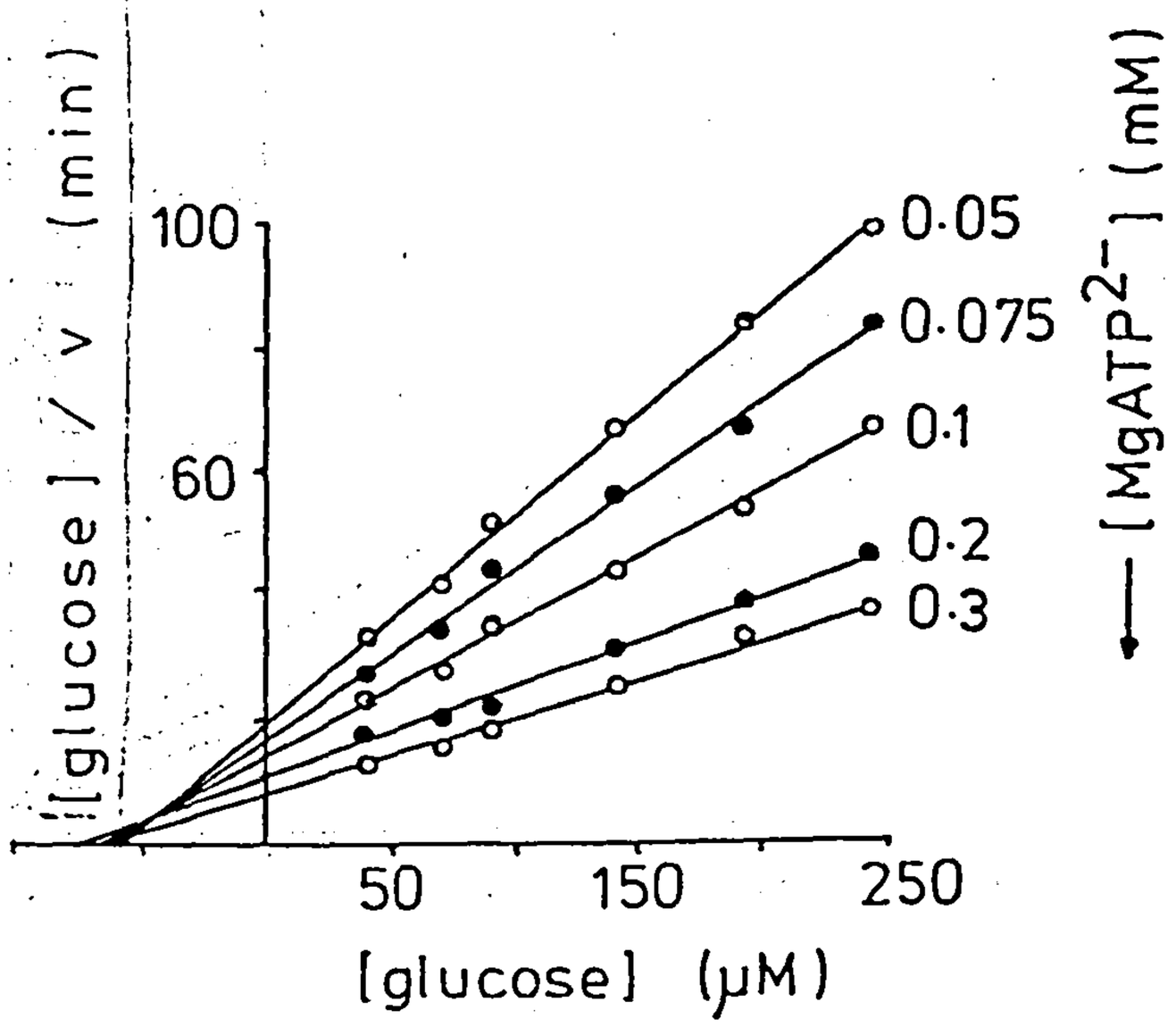


Figure 9.2

MgATP vs. glucose. Asterias hexokinase.
Mg^{2+} concentration was 5 mM.



Secondary plot for MgATP vs. glucose.
K_{AB} = 0.14 mM. K_B = 0.13 mM.

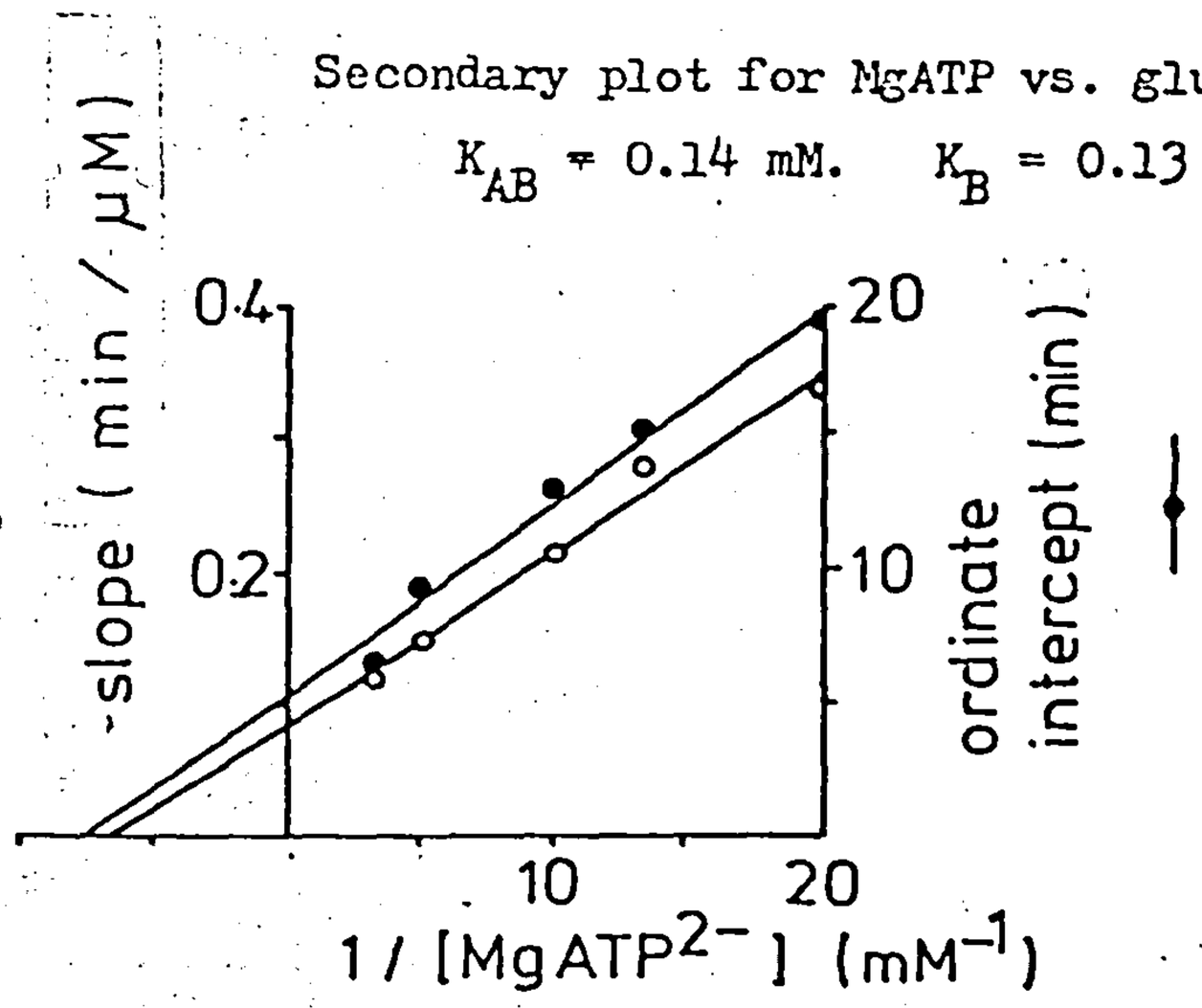
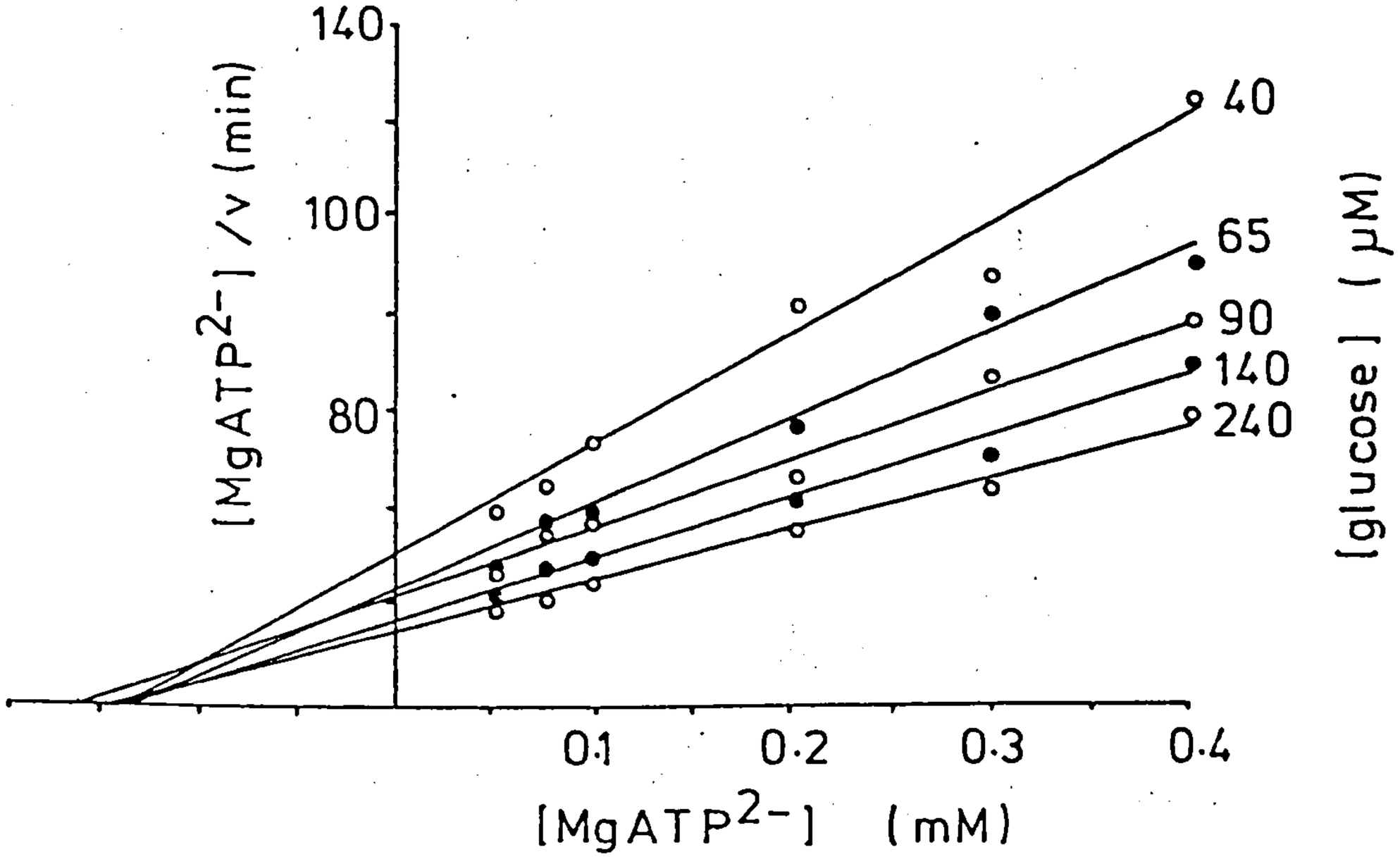


Figure 9.3

glucose vs. $MgATP^{2-}$ — *Asterias* hexokinase

Mg^{2+} concentration was 5 mM.



Secondary plot for glucose vs. MgATP

K_{AB} = 67 uM, K_B = 52 uM

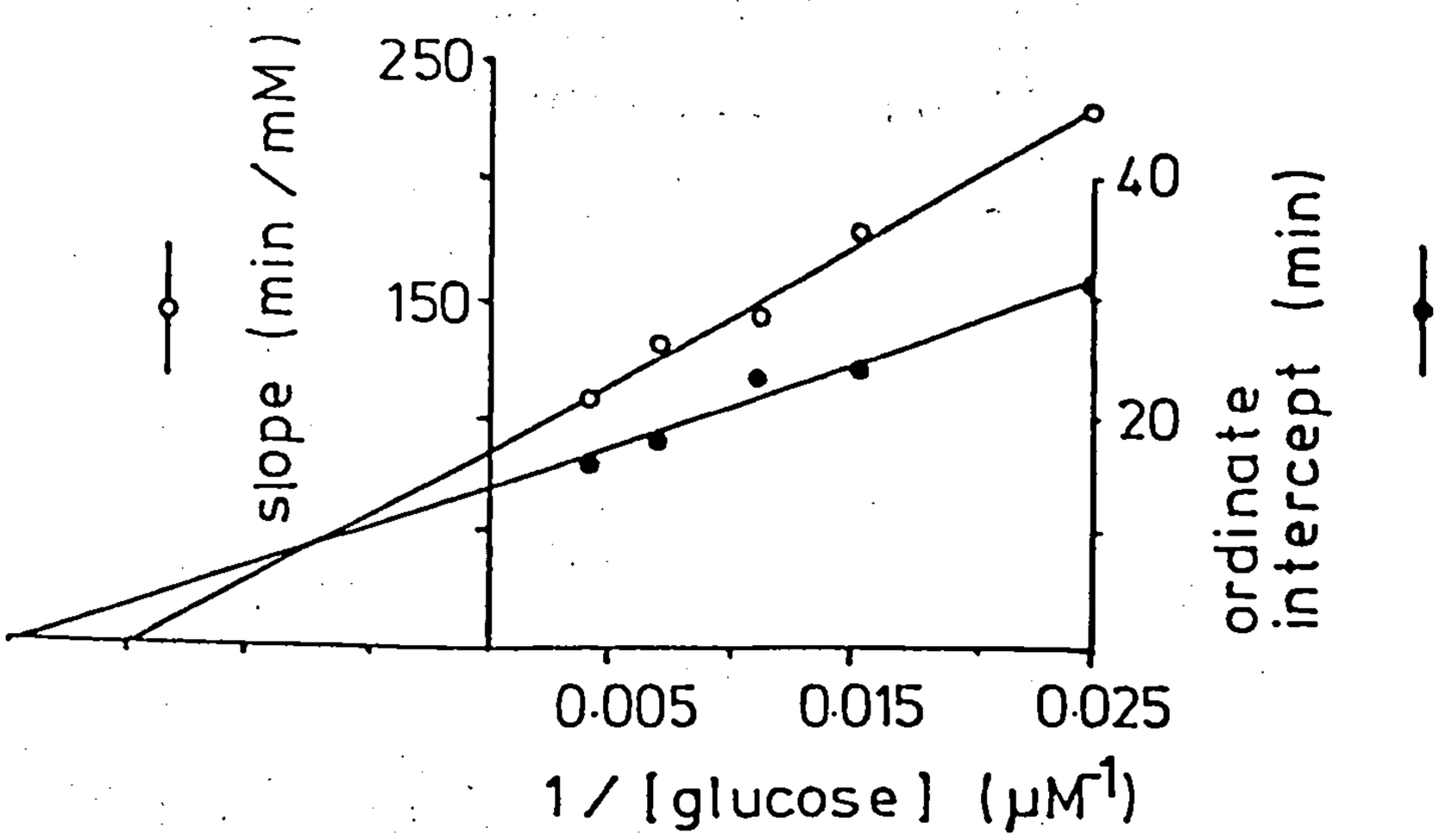


Figure 9.4

Km for mannose. Asterias hexokinase.
Mg^{2+} was held at 5 mM.MgATP was 0.14 mM.

Km was 0.05 mM.

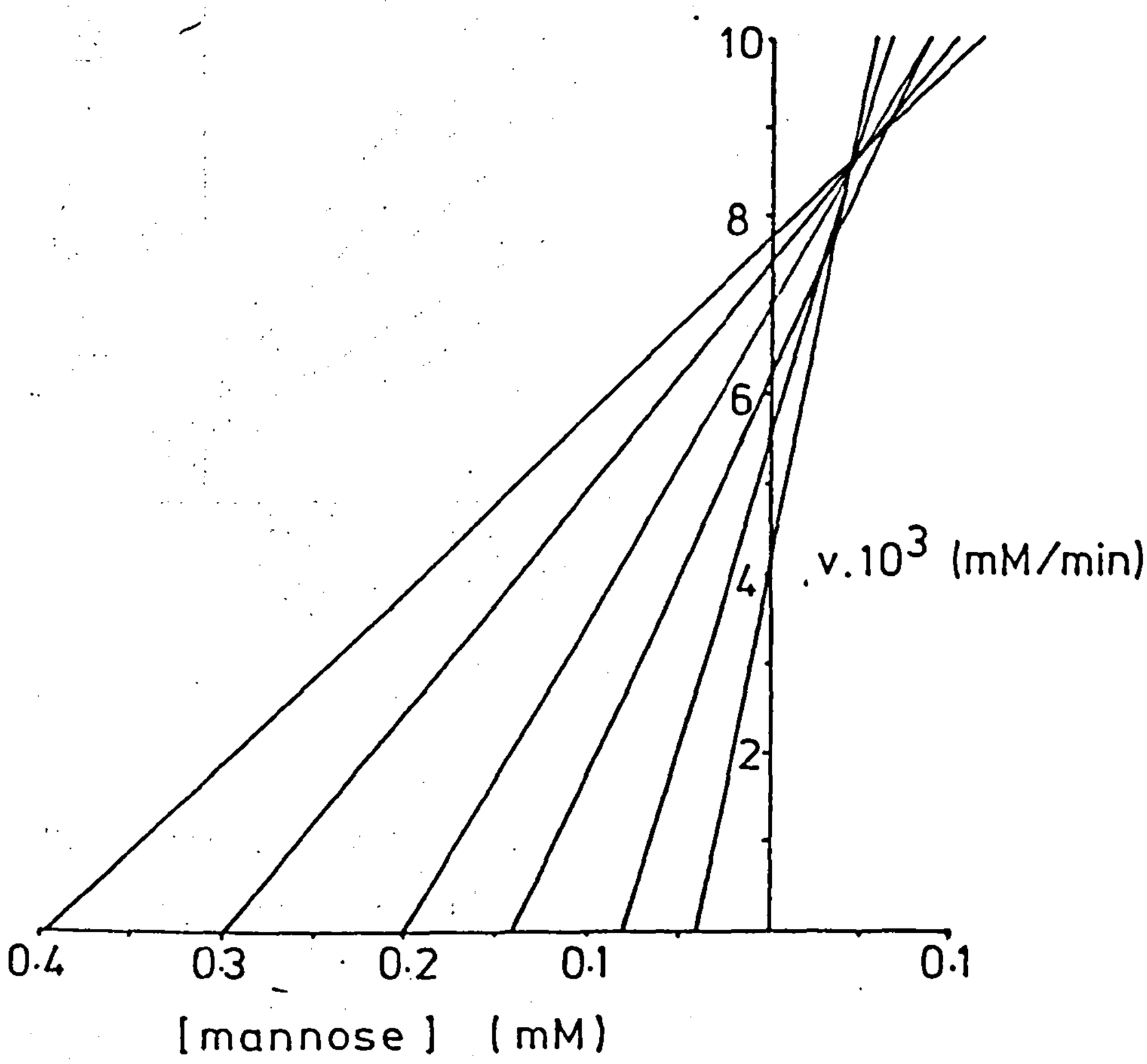
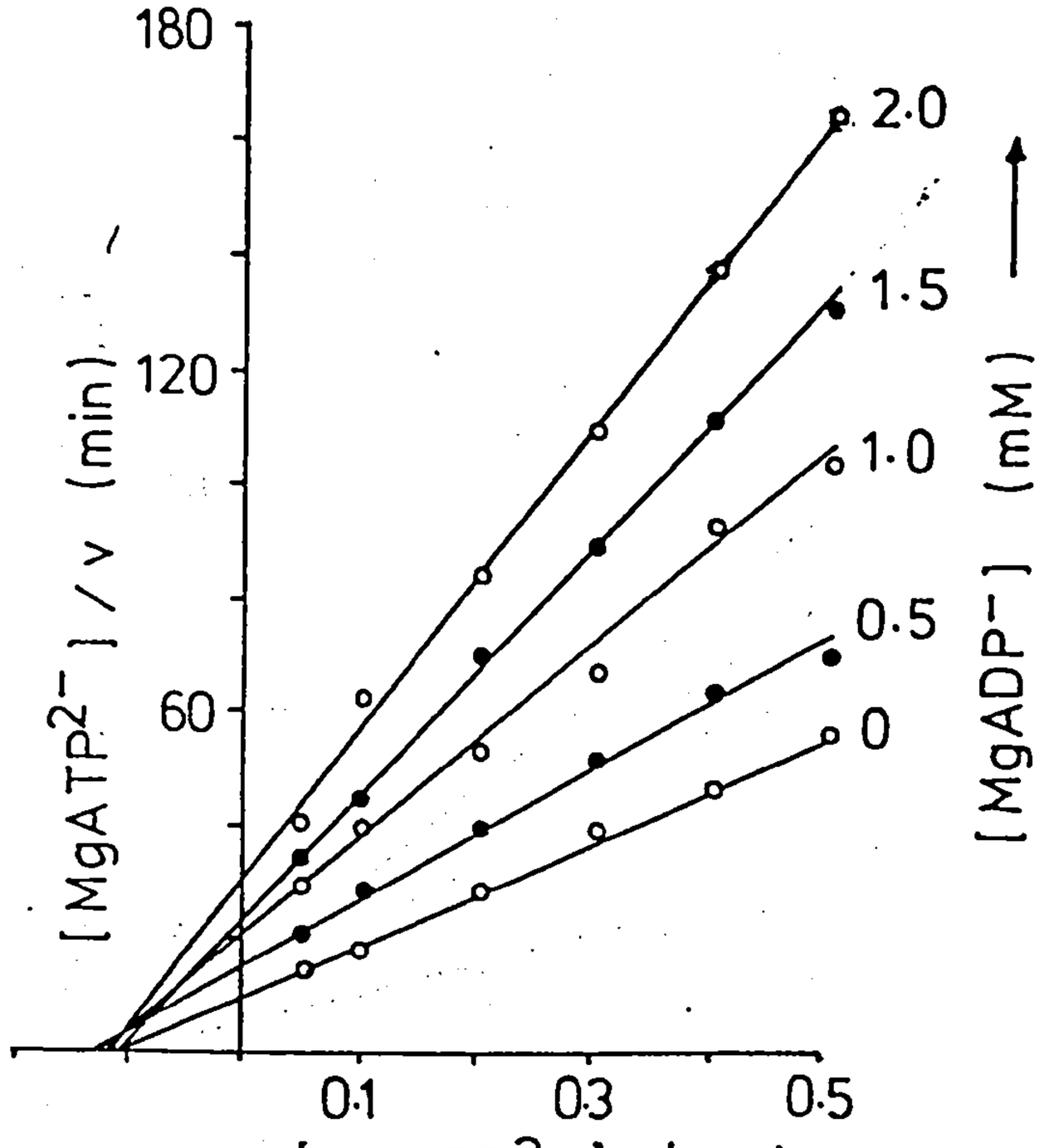


Figure 9.5

MgATP vs. MgADP. Asterias hexokinase.
Mg^{2+} concentration was held at 5 mM, glucose was at 0.06 mM



Secondary plot for MgATP vs. MgADP.

K_i = 1.1 mM. K_i' = 0.9 mM.

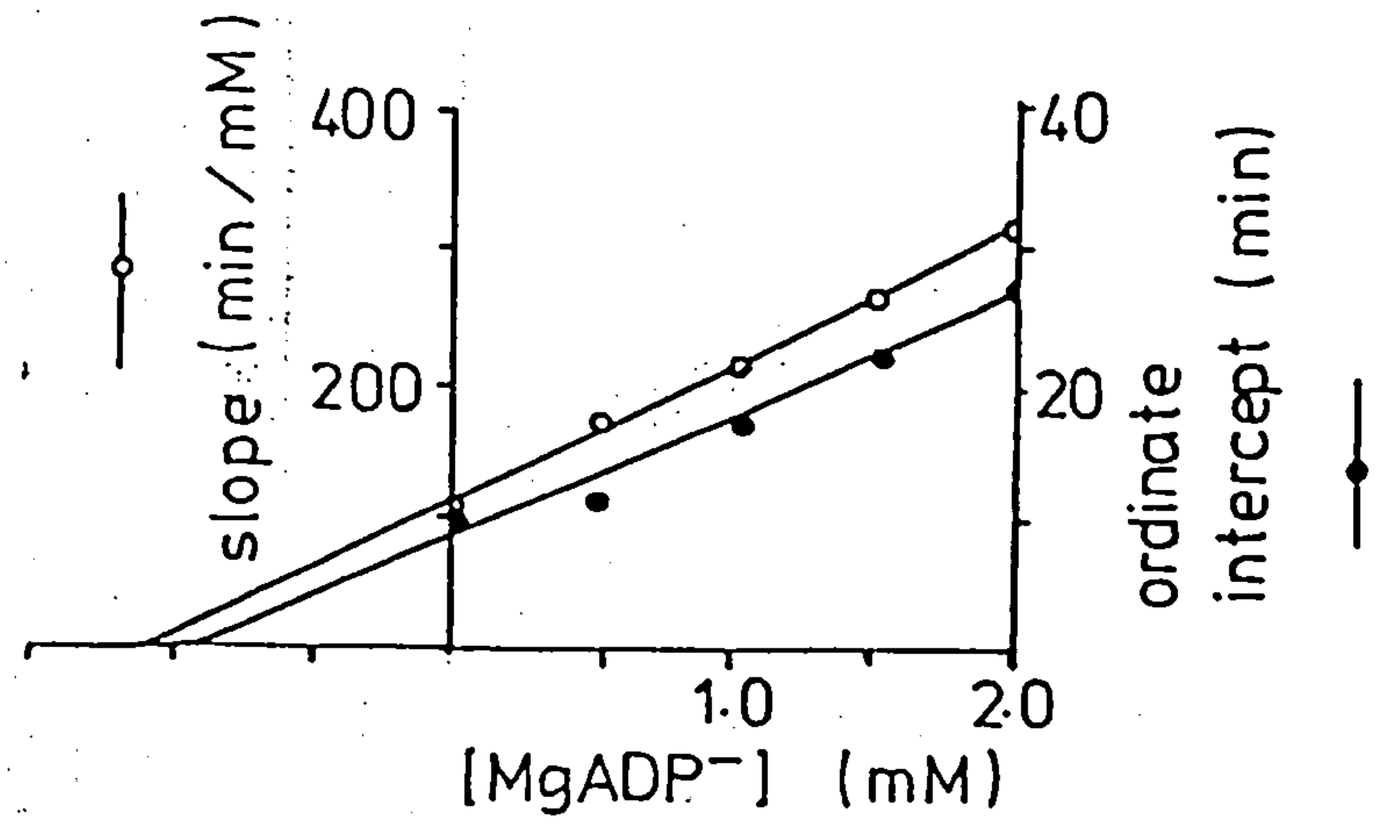
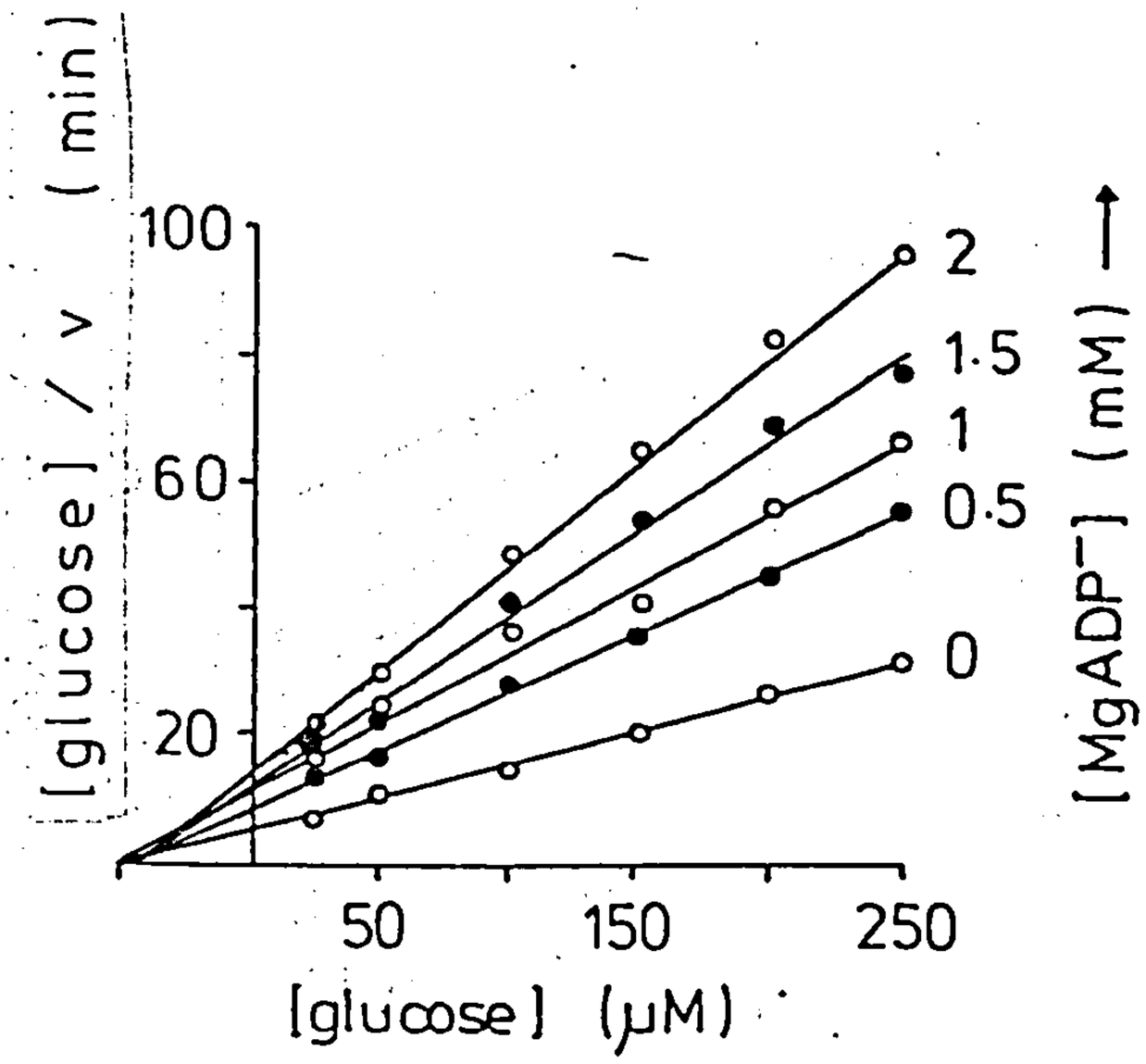


Figure 9.6

MgADP vs. glucose. Asterias hexokinase.
Mg^{2+} concentration was 5 mM. MgATP concentration was 0.14 mM.



Secondary plot for MgADP vs. glucose.
K_i = 1.03 mM. K_i' = 1.11

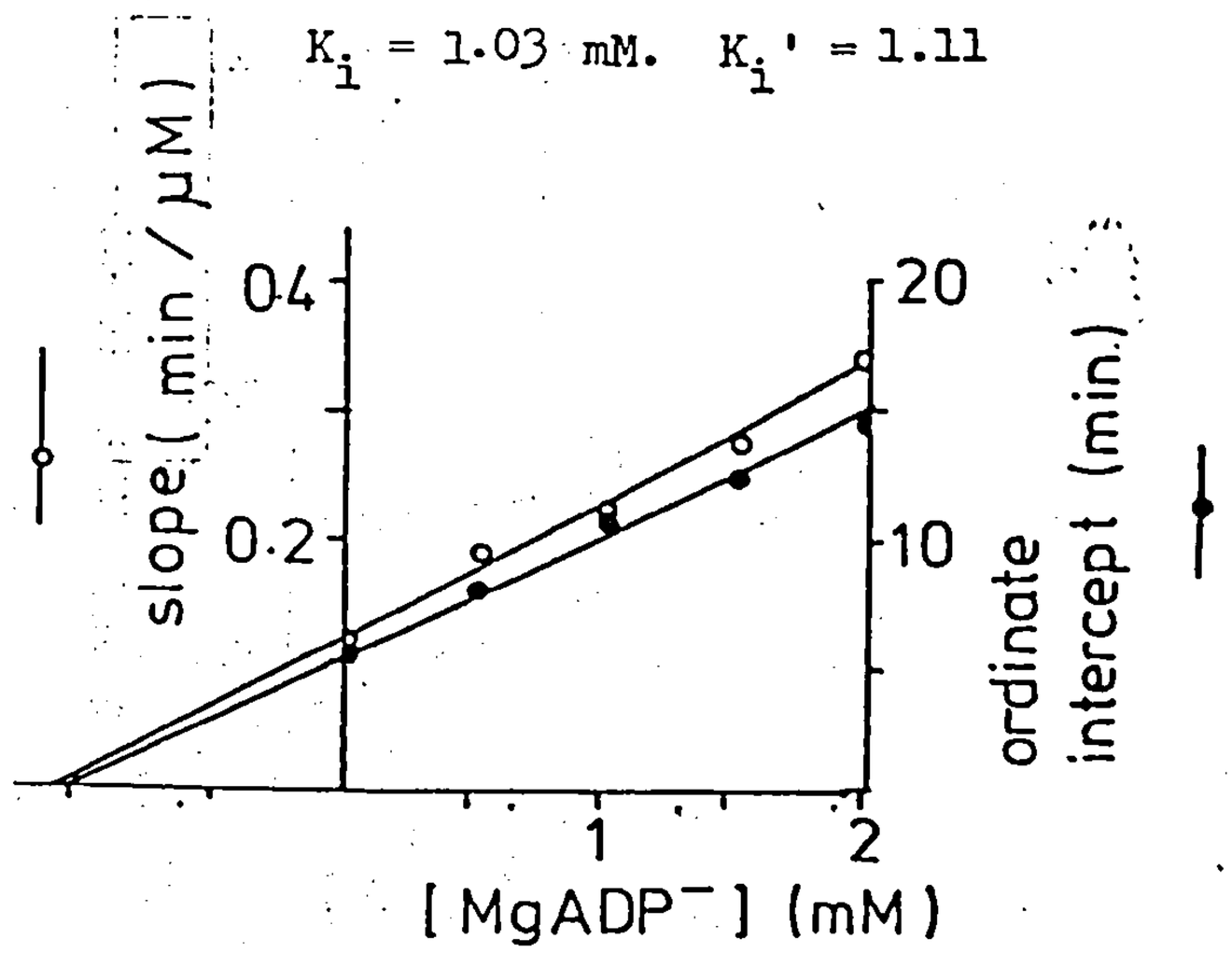
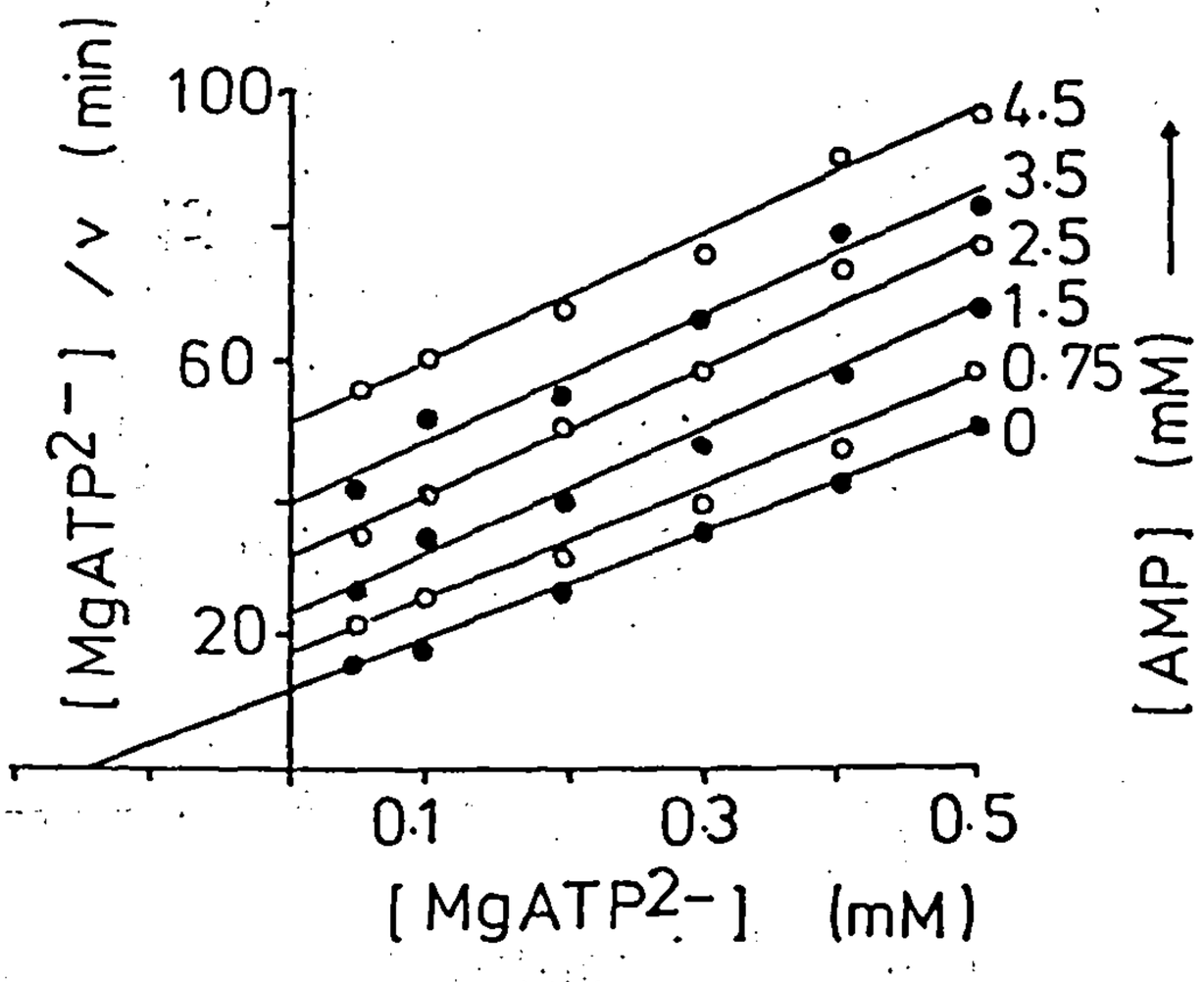


Figure 9.7

AMP vs. MgATP. Asterias hexokinase.
Mg^{2+} concentration was constant at 6 mM, glucose was at 0.05 mM.



Secondary plot for AMP vs. MgATP.

$K_i = 1.15$ mM $K_i' = \infty$

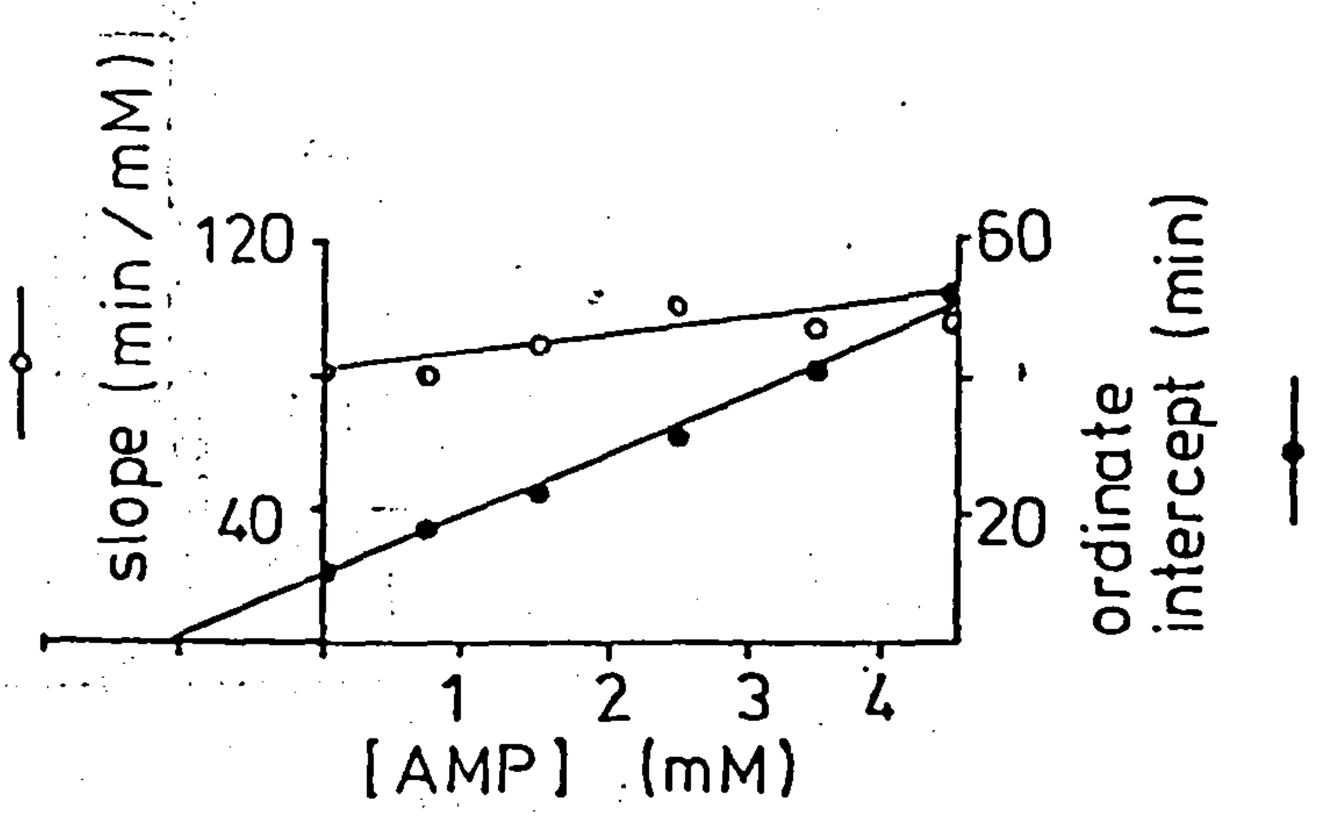
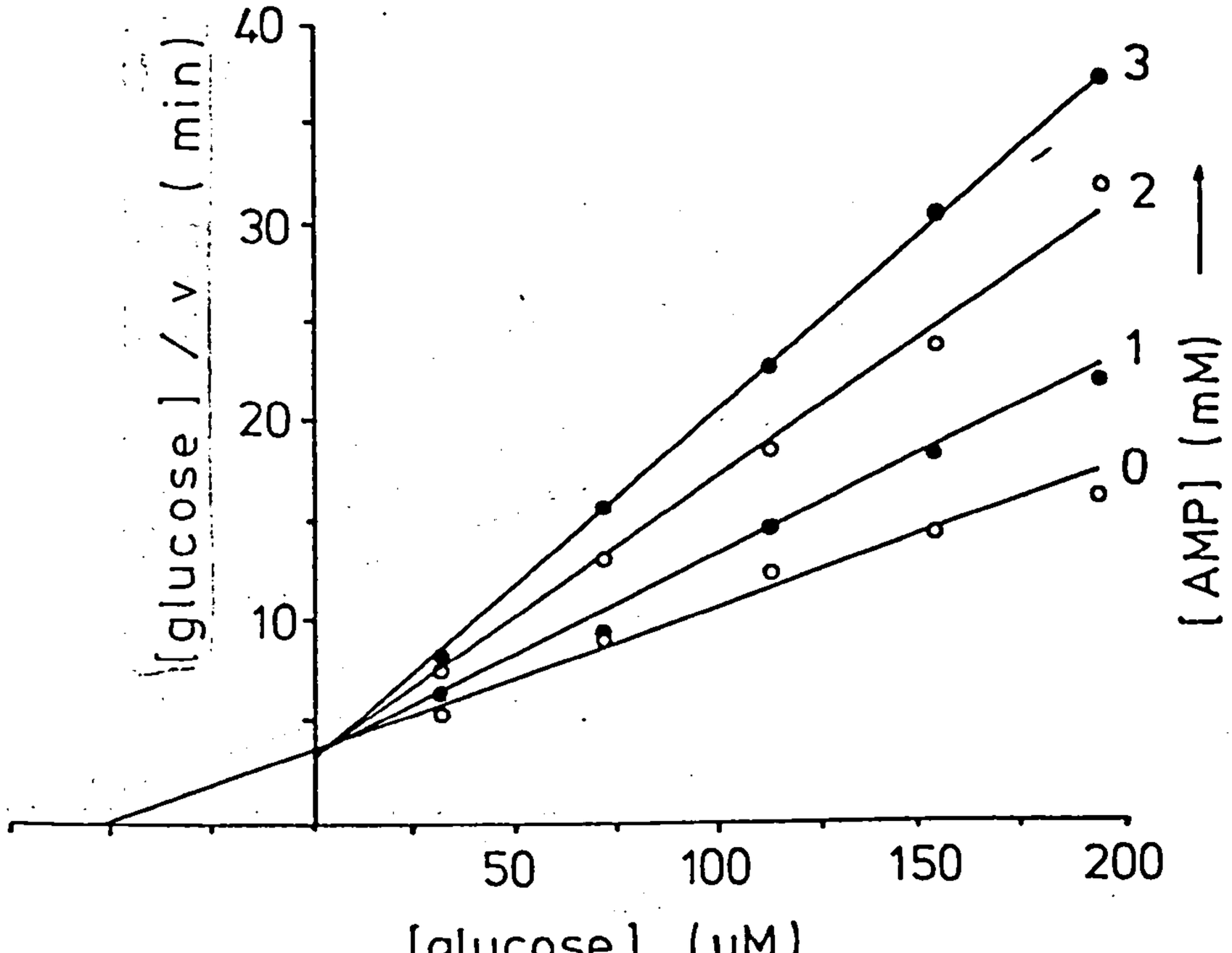


Figure 9.8

AMP vs. glucose. Asterias hexokinase
Mg^{2+} concentration was 5 mM, MgATPwas 0.15 mM.



Secondary plot for AMP vs. glucose.

$K_i = \infty$ $K'_i = 1.33$ mM.

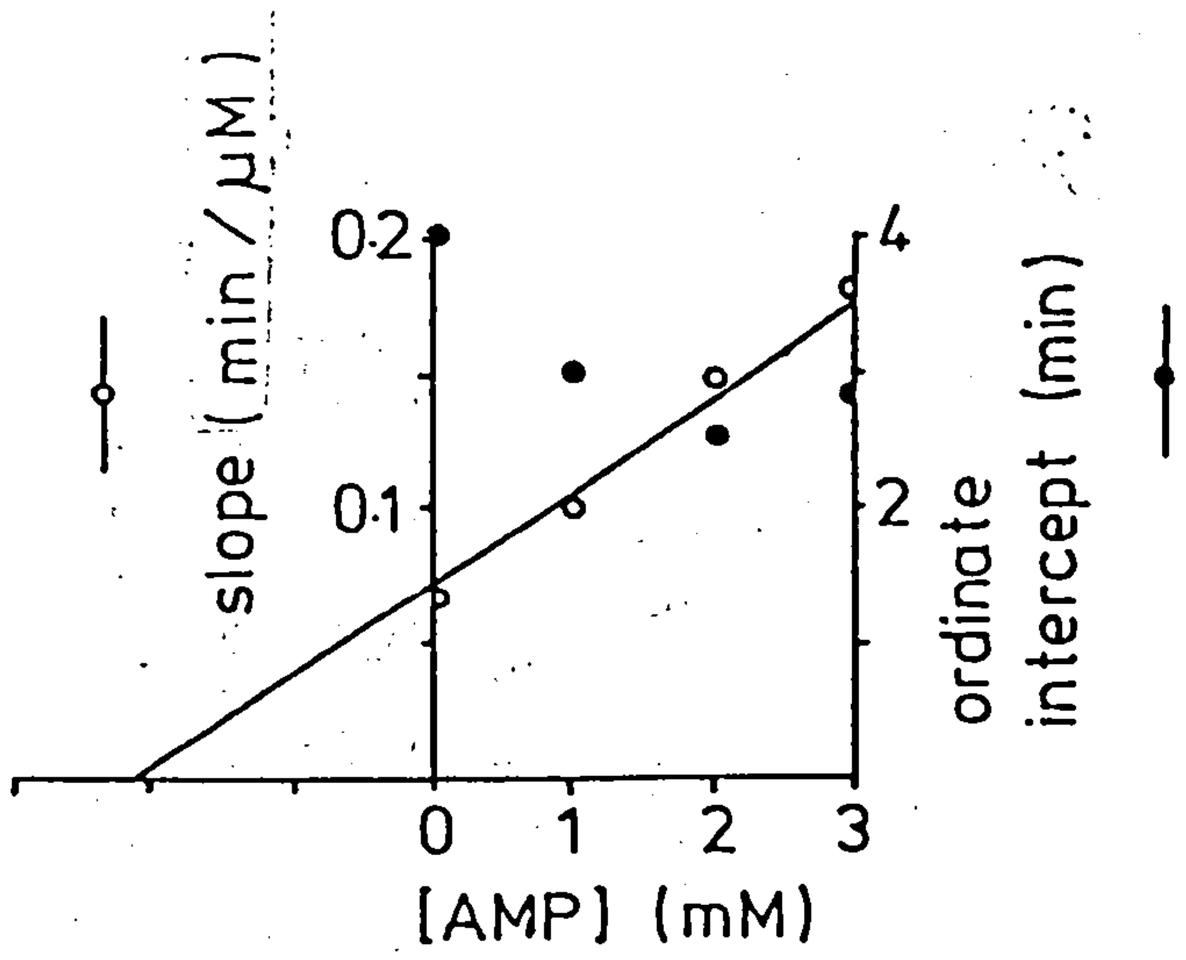
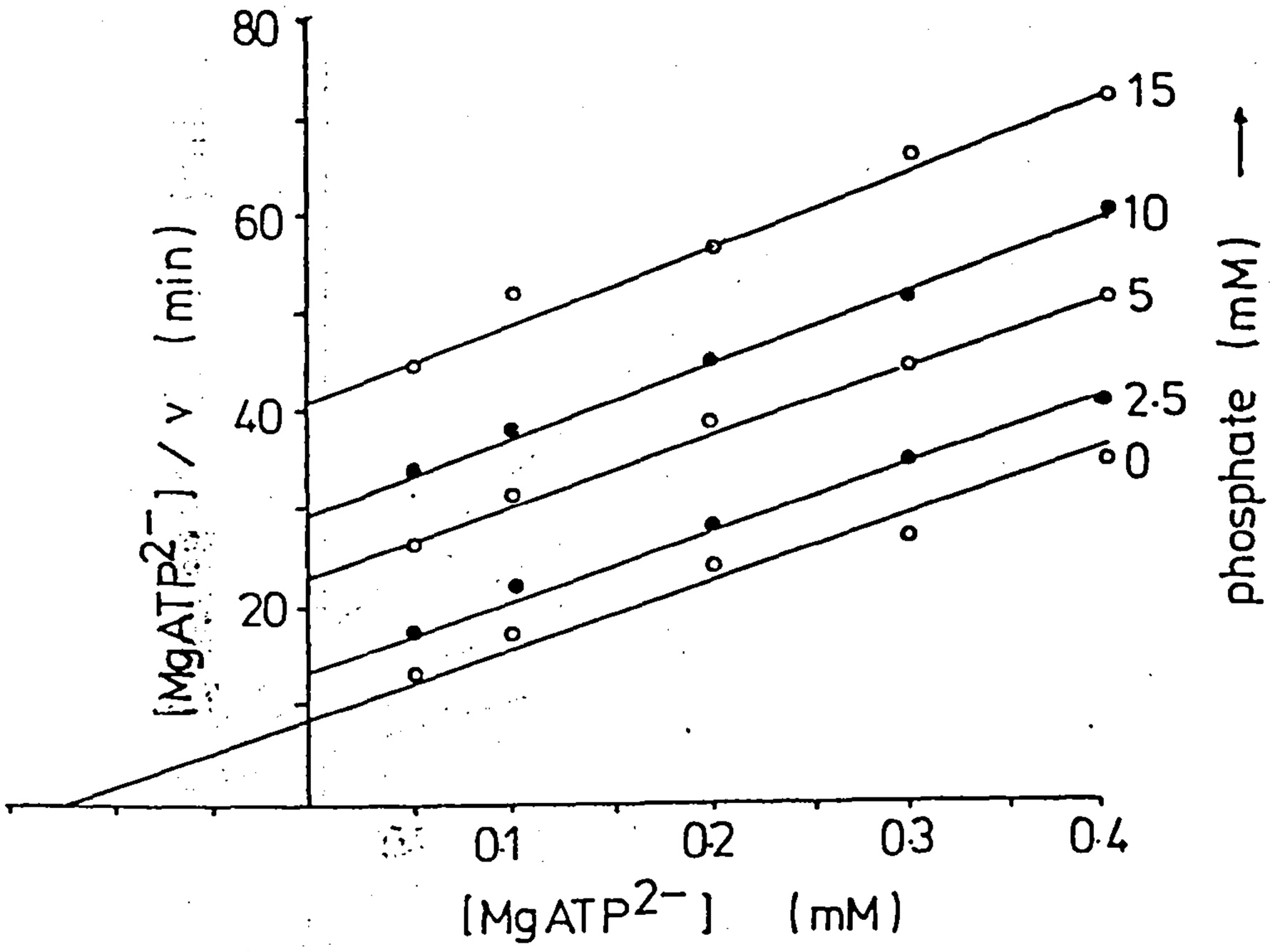


Figure 9.9

Phosphate vs. MgATP. Asterias hexokinase.
Mg^{2+} concentration was held at 6 mM, glucose was at 0.05 mM.



Secondary plot for phosphate vs. MgATP.
K_i = 4.73 mM. $K_i' = \infty$

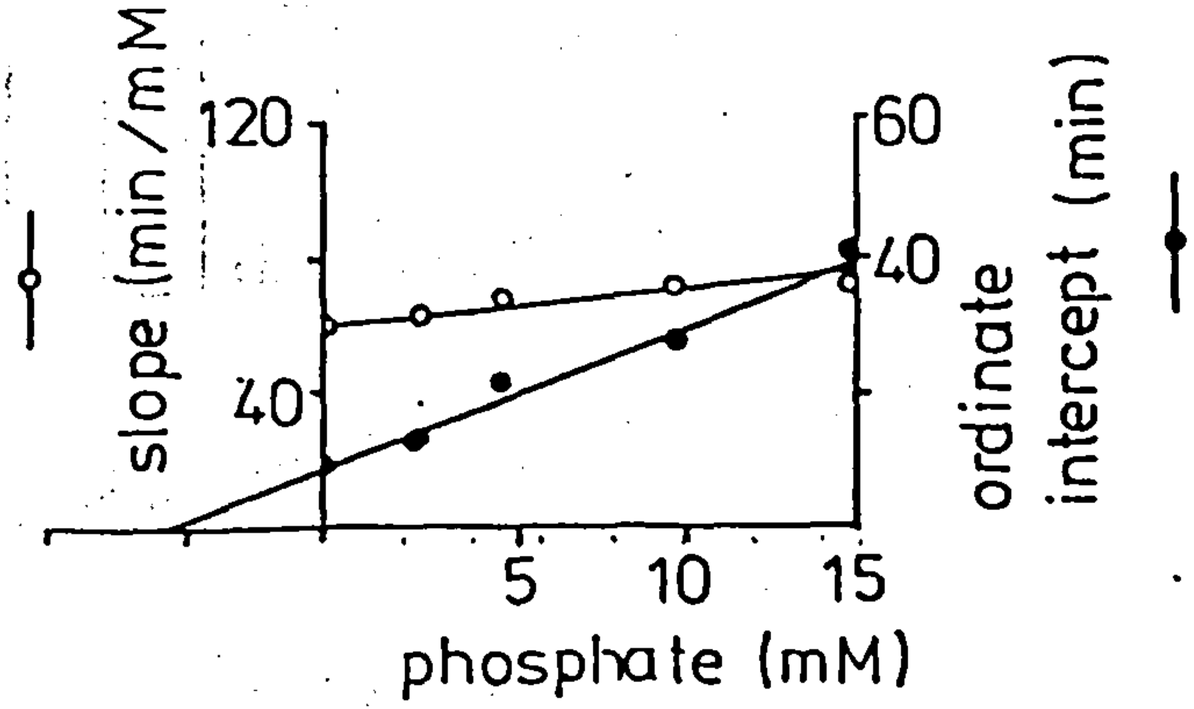
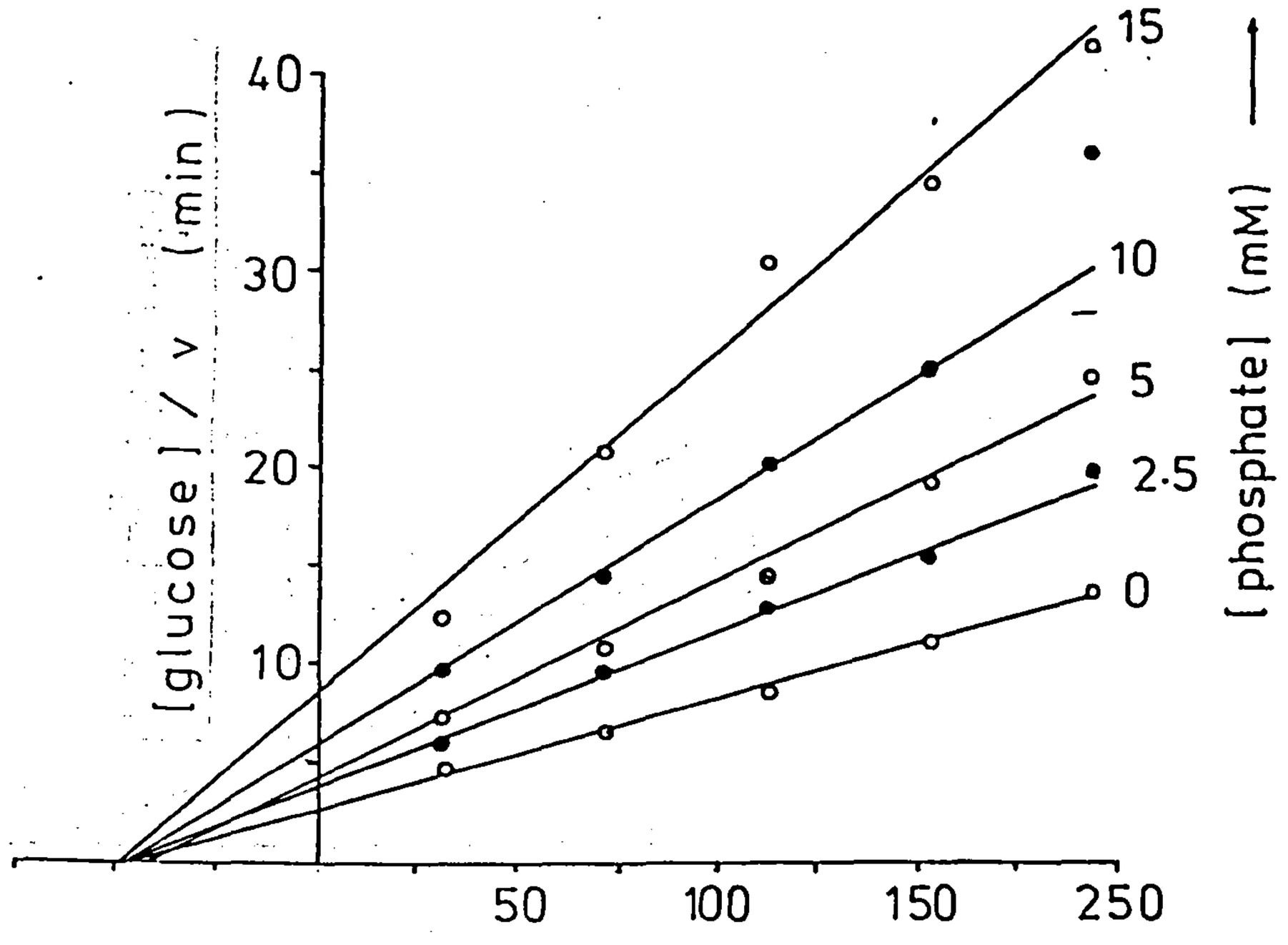


Figure 9.10

Phosphate vs. glucose. Asterias hexokinase.
Mg^{2+} was 6 mM, MgATP was 0.125 mM.



Secondary plot for phosphate vs. glucose.
K_i = 6.46 mM. K_i' = 7.66 mM.

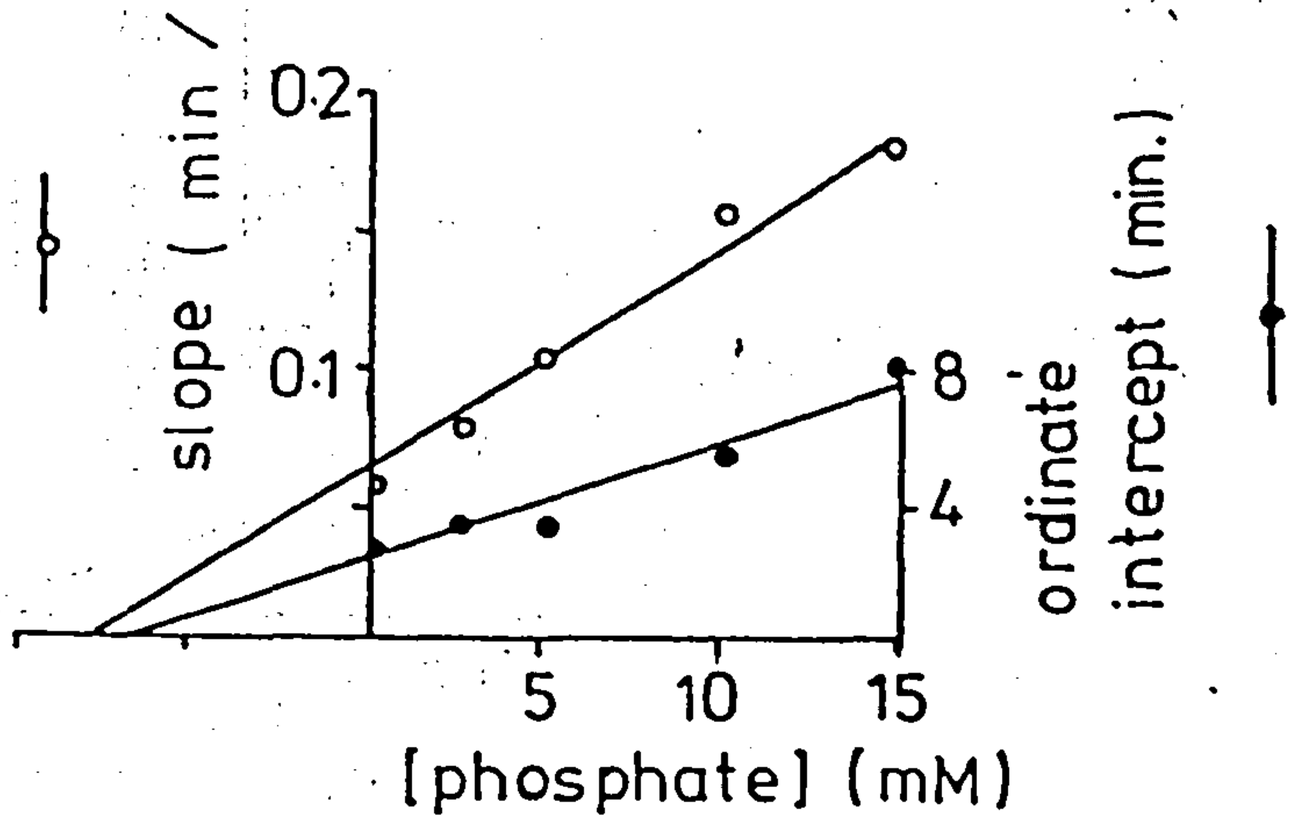
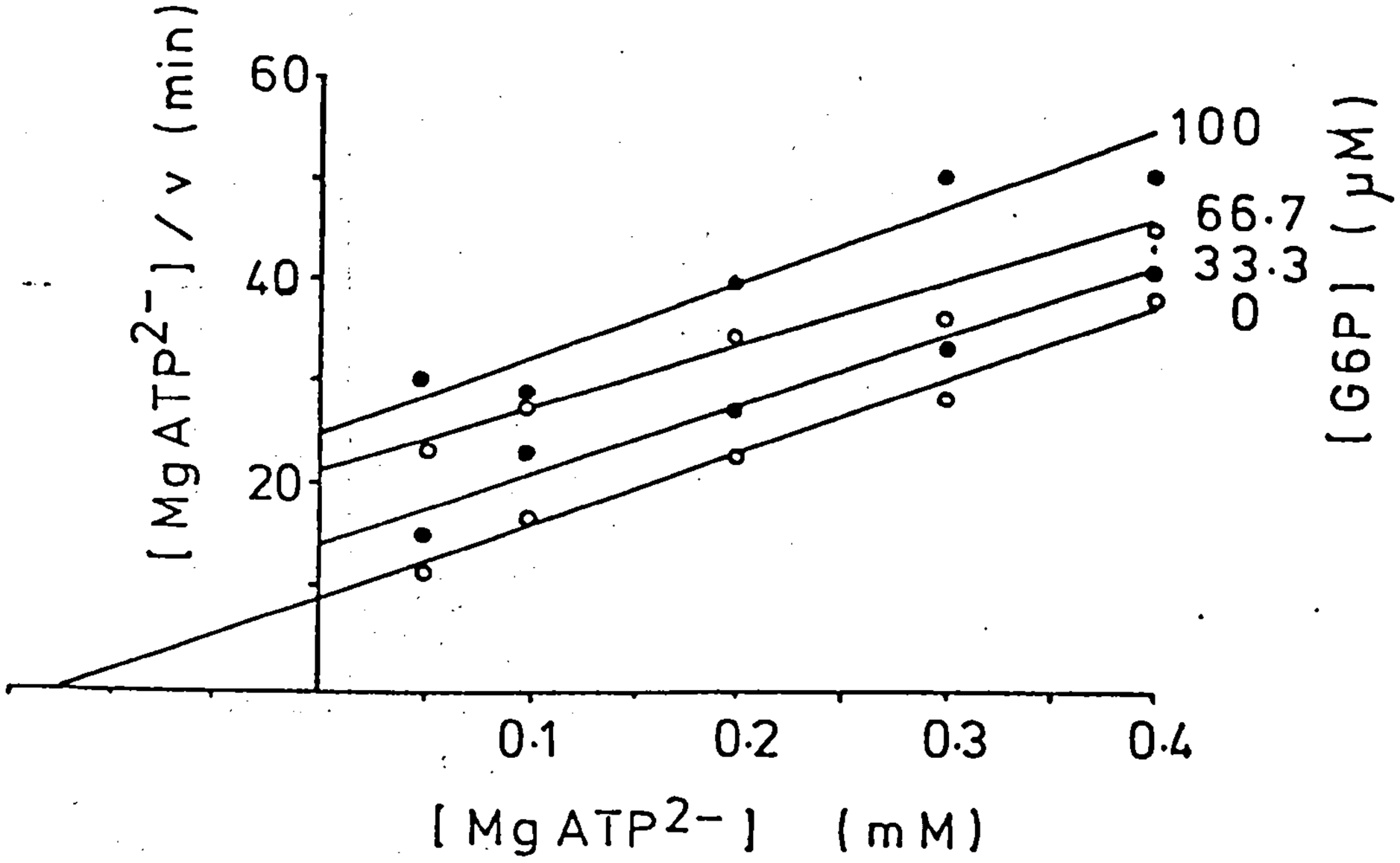


Figure 9.11

Glucose 6-phosphate vs. MgATP. Asterias hexokinase. Mg^{2+} concentration was 5 mM, mannose concentration was 0.05 mM. The pyruvate kinase - lactate dehydrogenase linked assay was used.



Secondary plot for glucose 6-phosphate vs. MgATP.

$K_i = 0.055$ mM. $K_i' = \infty$

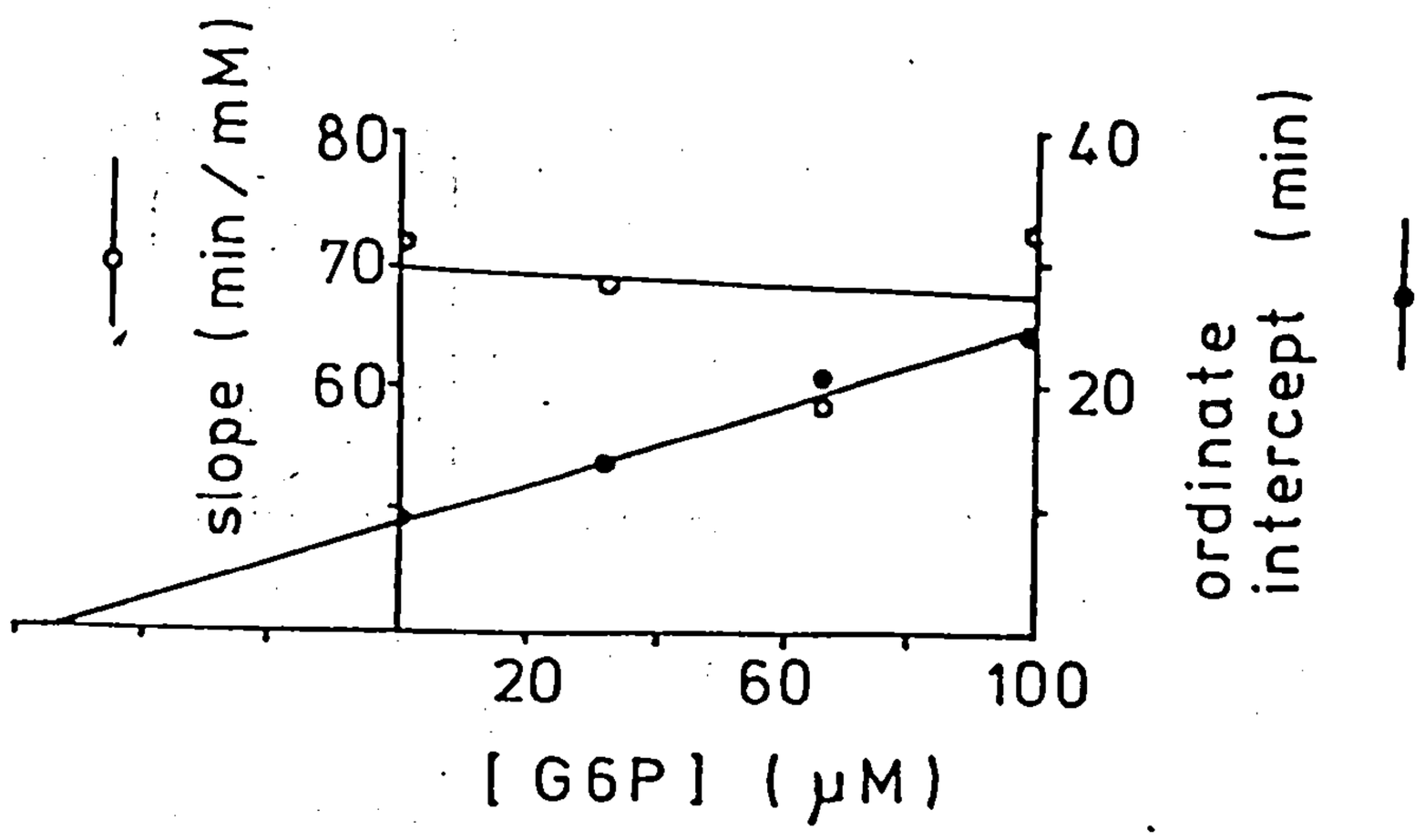
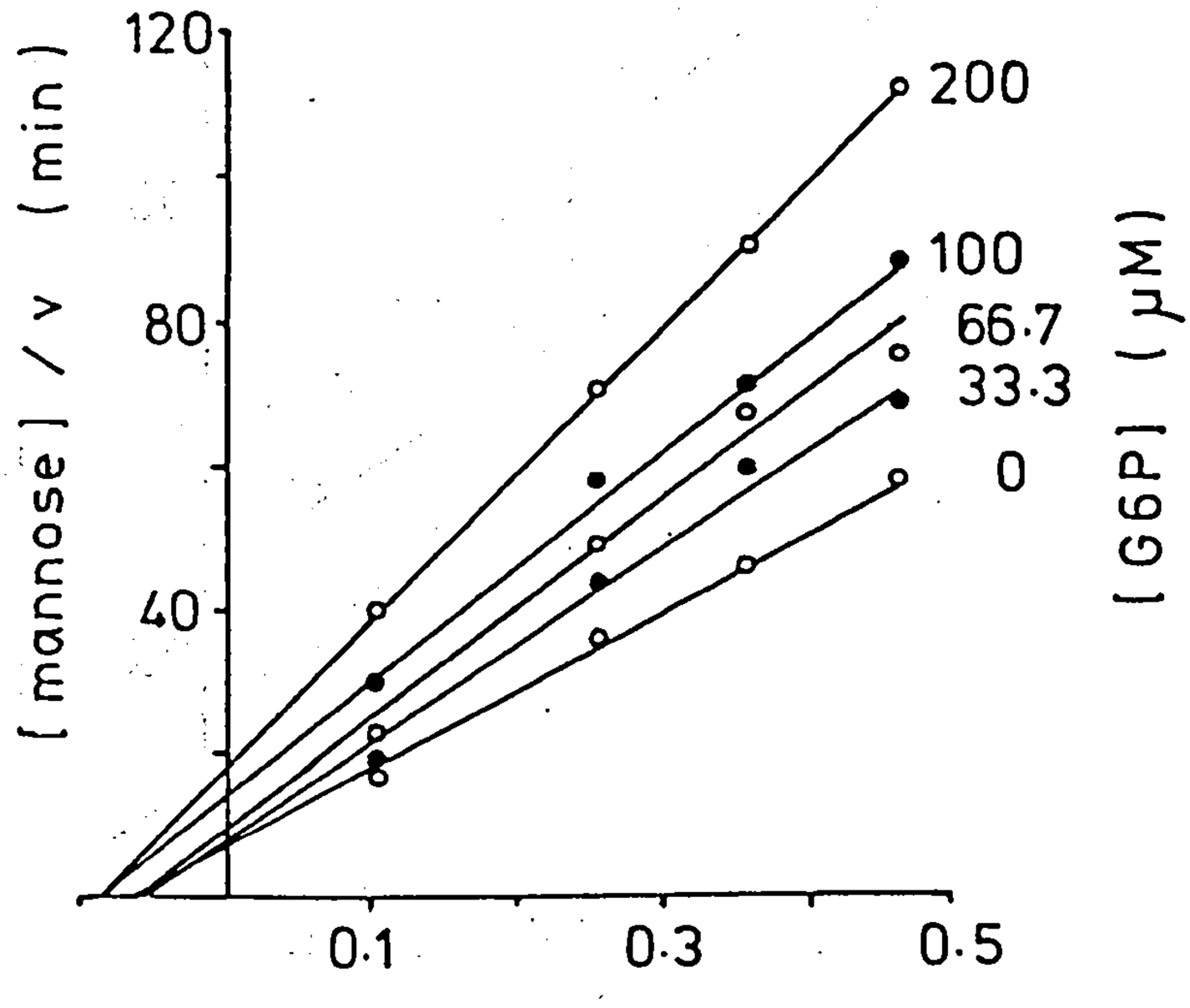


Figure 9.12

Glucose 6-phosphate vs. mannose. Asterias hexokinase. Mg^{2+} was held at 5 mM, and MgATP at 0.14 mM. The pyruvate kinase - lactate dehydrogenase assay was used.



Secondary plot for glucose 6-phosphate vs. mannose. K_i = 0.09 mM. K_i' = 0.28 mM.

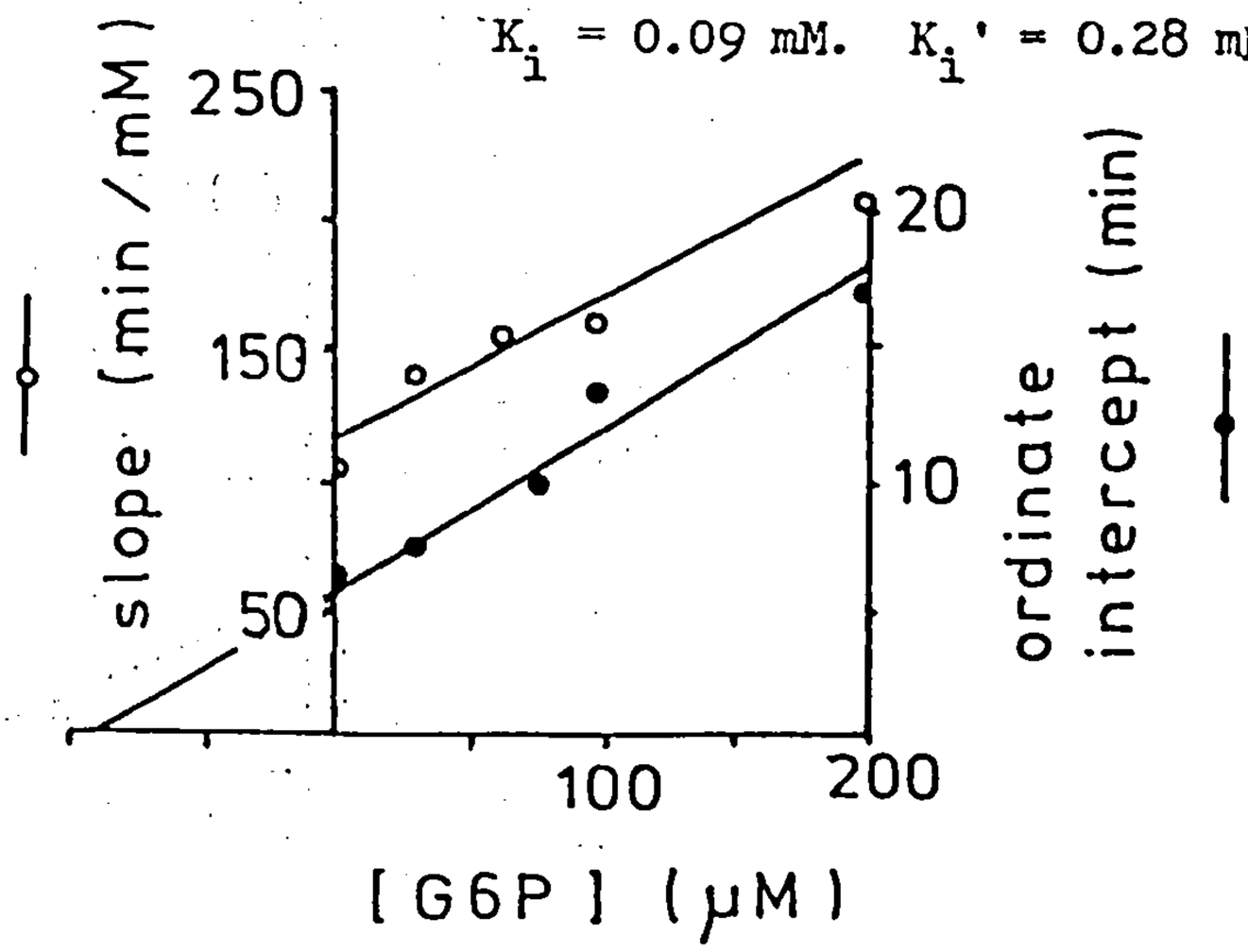
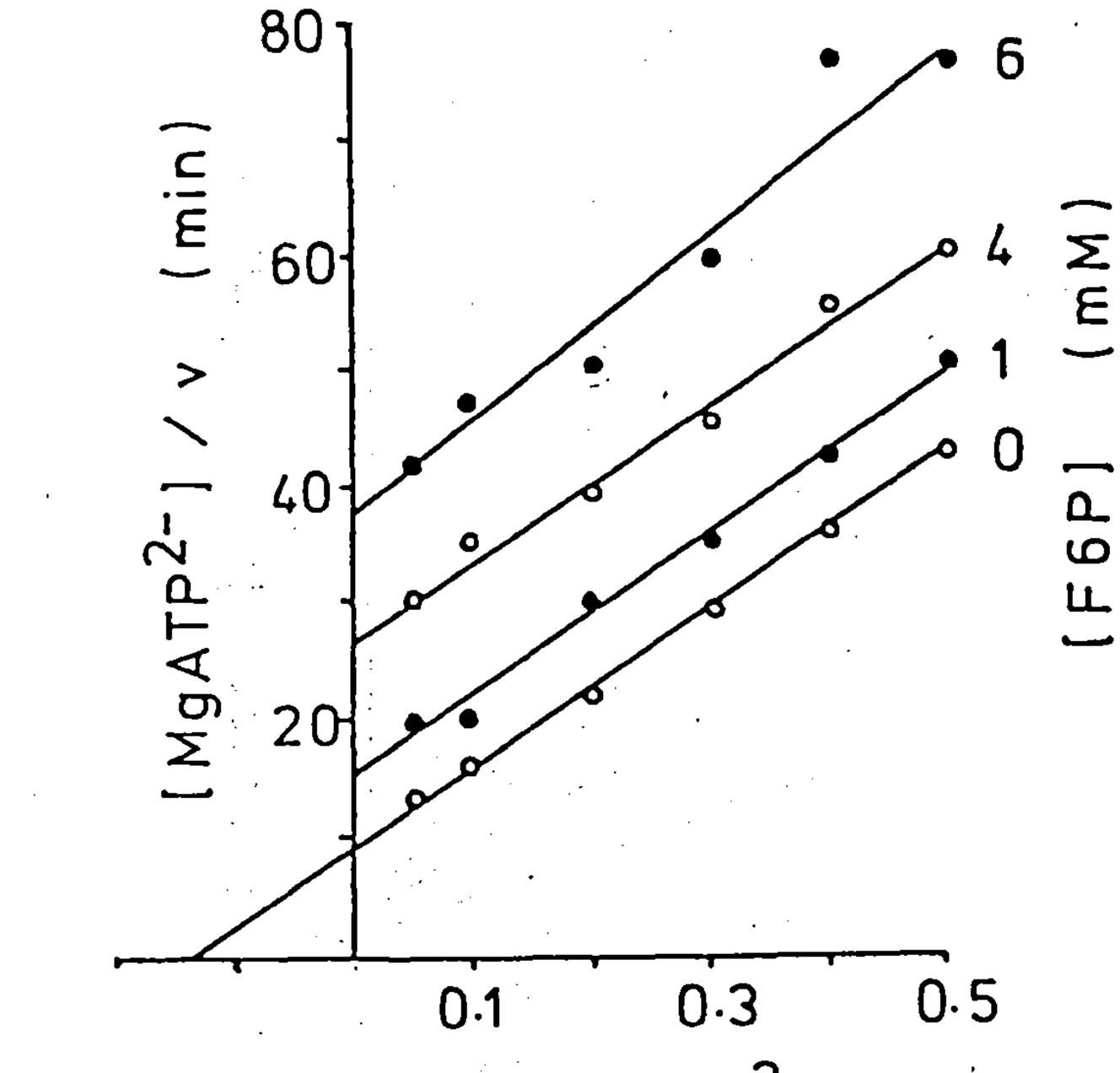


Figure 9.13

Fructose 6-phosphate vs. MgATP. Asterias hexokinase. Glucose was maintained at 0.05 mM and Mg^{2+} at 5 mM.



Secondary plot for fructose 6-phosphate vs. MgATP.

K_i = 2.3 mM. K_i' = ∞

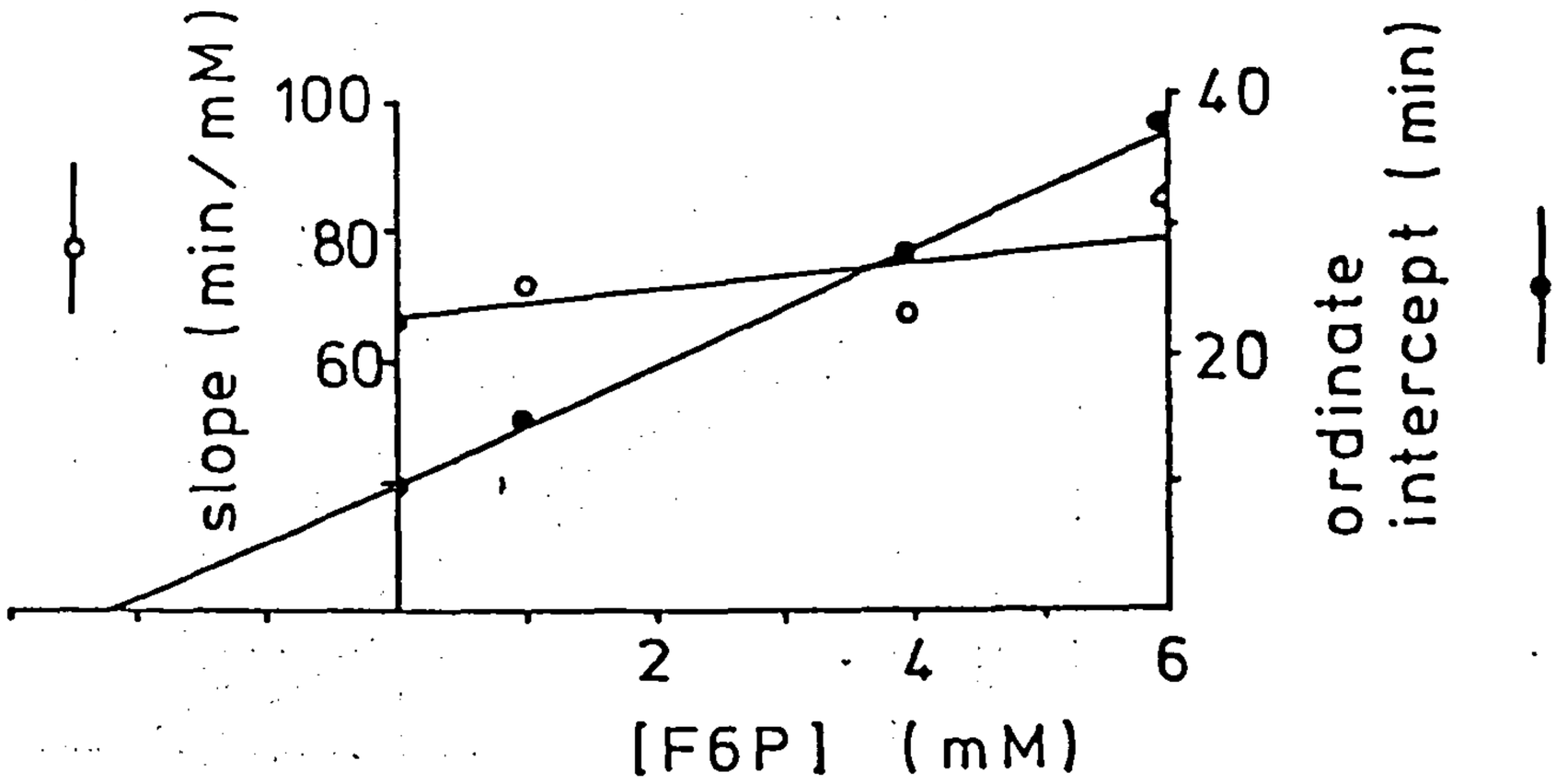
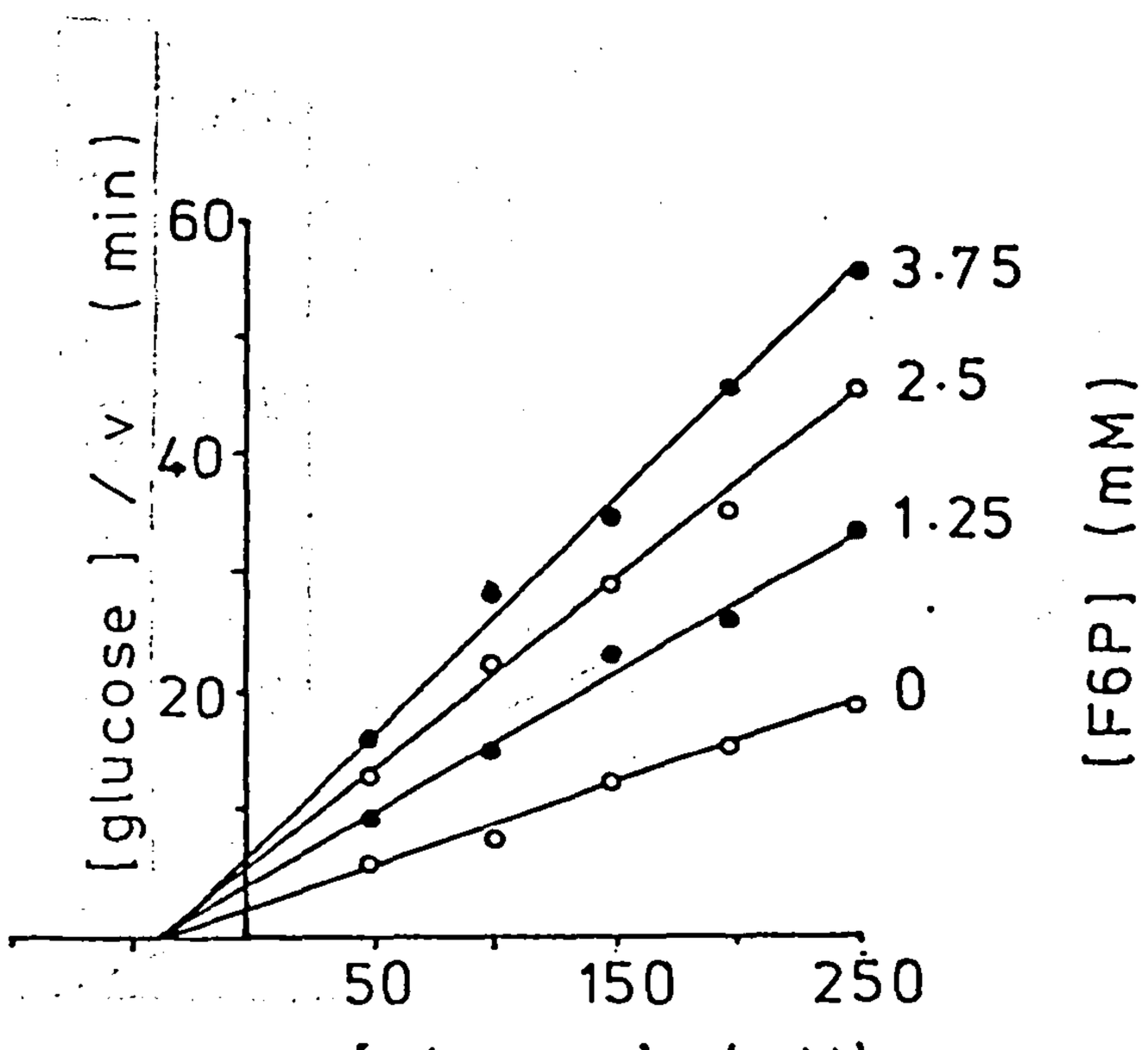
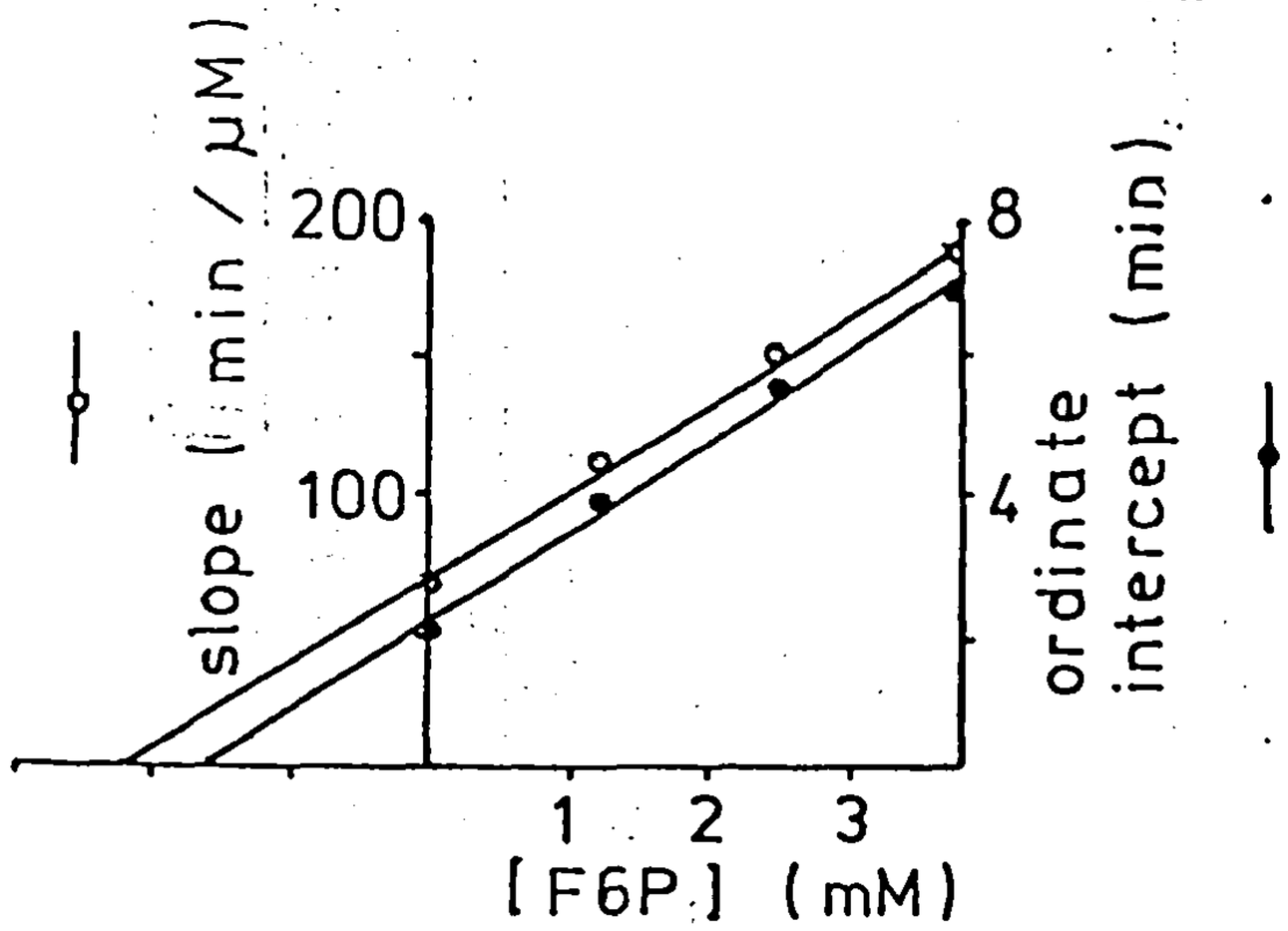


Figure 9.14

Fructose 6-phosphate vs. glucose. Asterias hexokinase. Mg^{2+} was 5 mM and MgATP 0.14 mM.



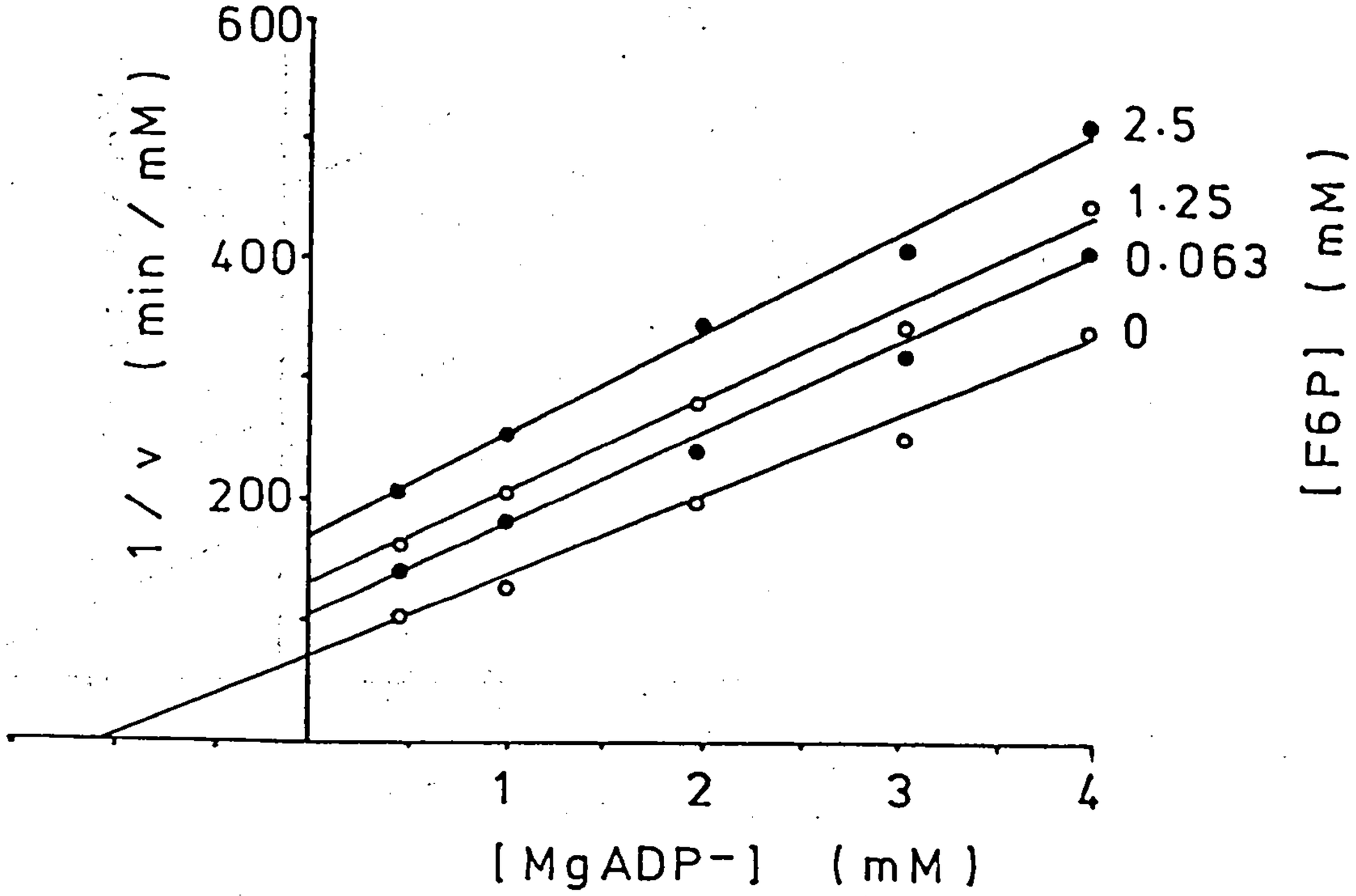
Secondary plot for fructose 6-phosphate vs. glucose. K_i = 1.56 mM. K_i' = 2.18 mM.



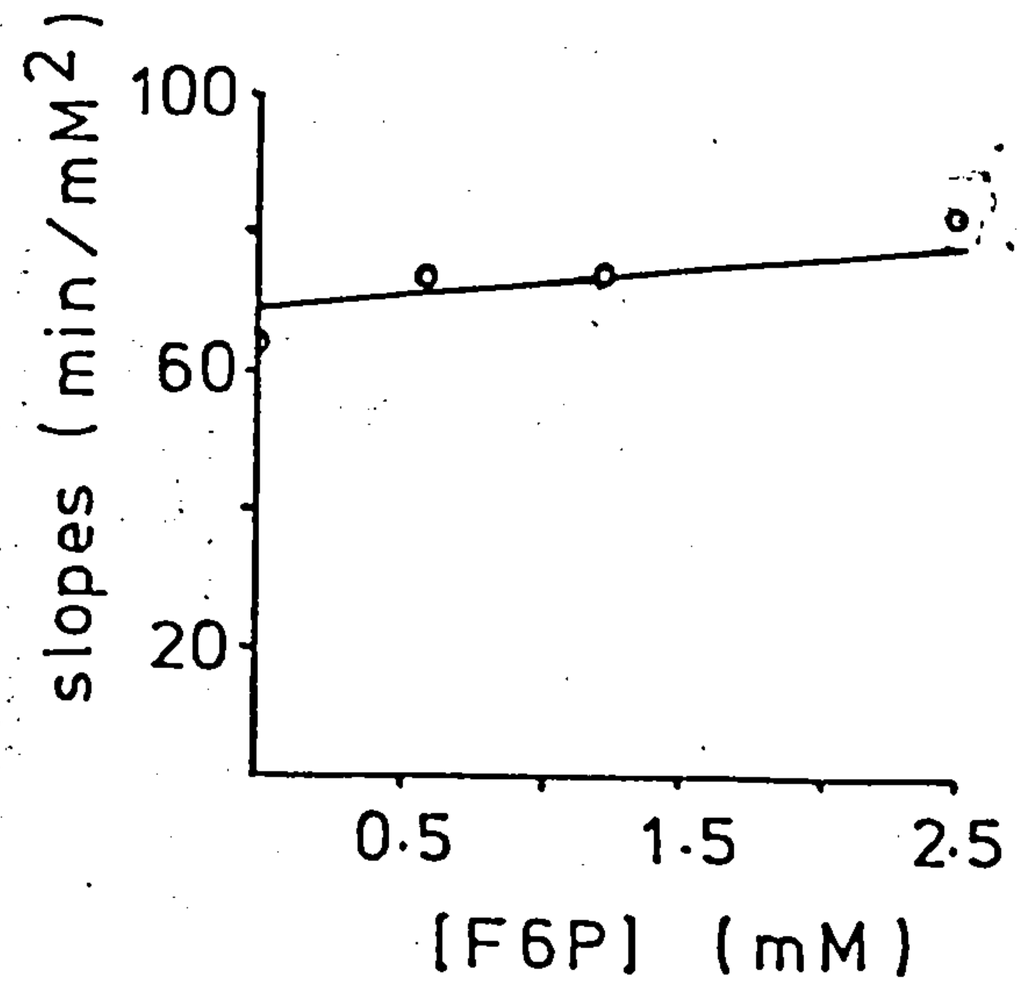
Double inhibitor studies. Asterias hexokinase. Figure 9.15

MgADP vs. fructose 6-phosphate.

Mg^{2+} concentration was 5 mM, glucose at 11 mM and MgATP at 0.14 mM.

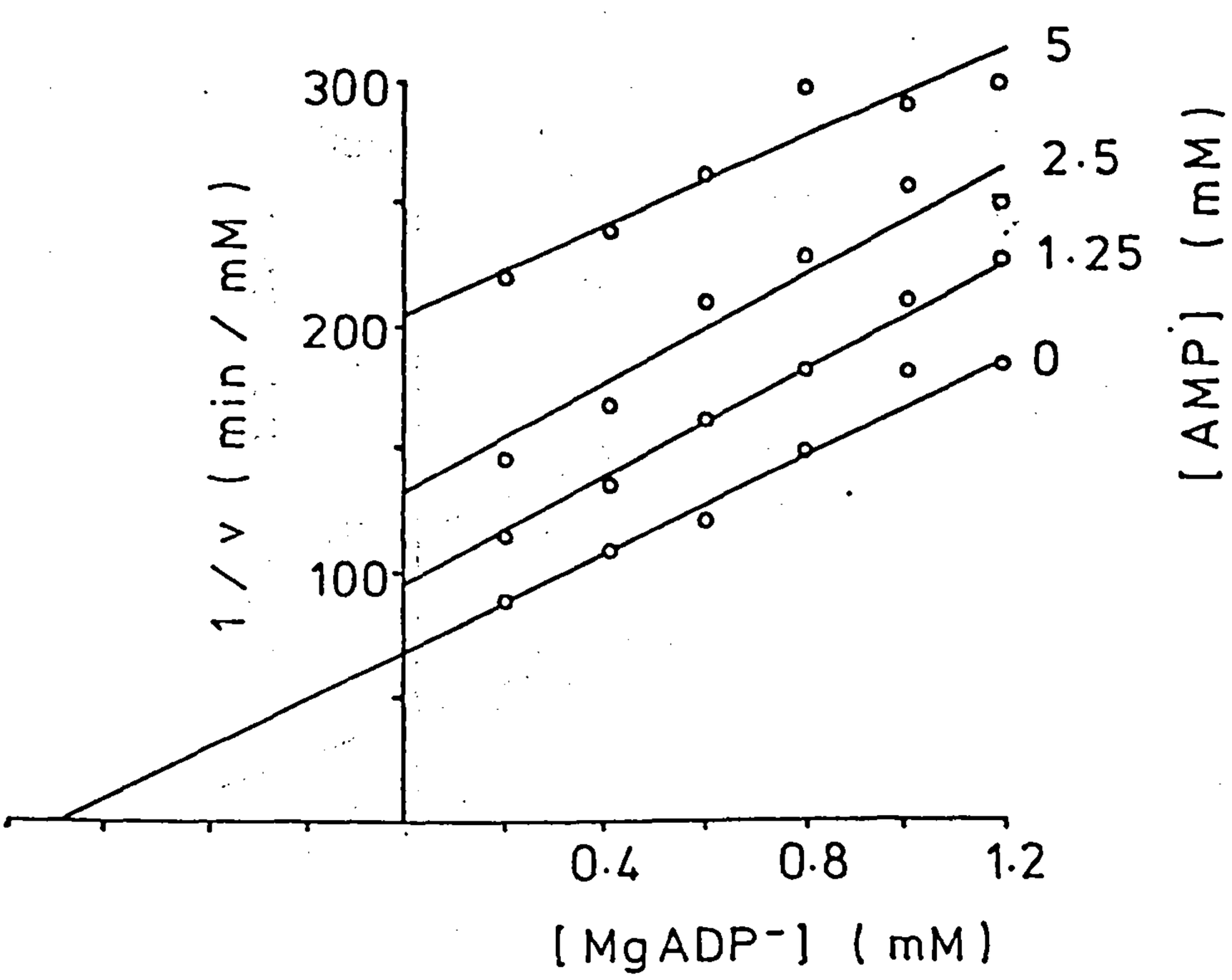


Secondary plot for MgADP vs. fructose 6-phosphate.

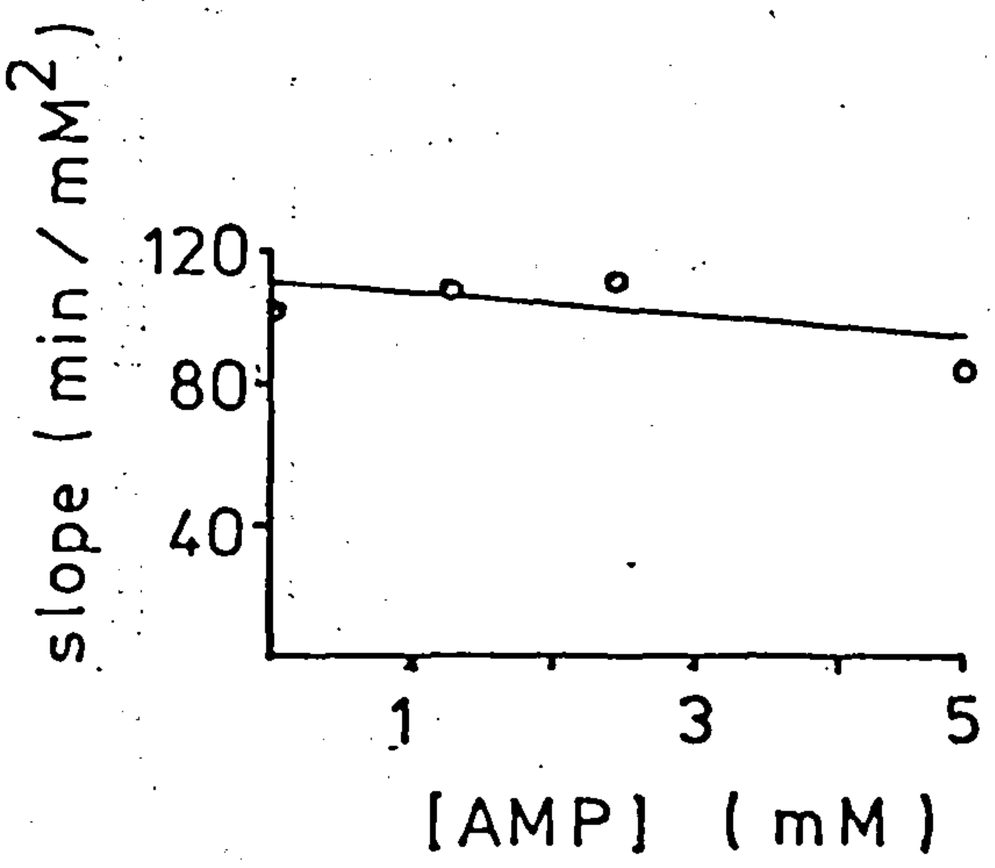


Double inhibitor studies. Asterias hexokinase. Figure 9.16

MgADP vs. AMP.
Mg^{2+} concentration was 2 mM above the total nucleotide concentration, glucose was at 11 mM and MgATP at 0.15 mM.

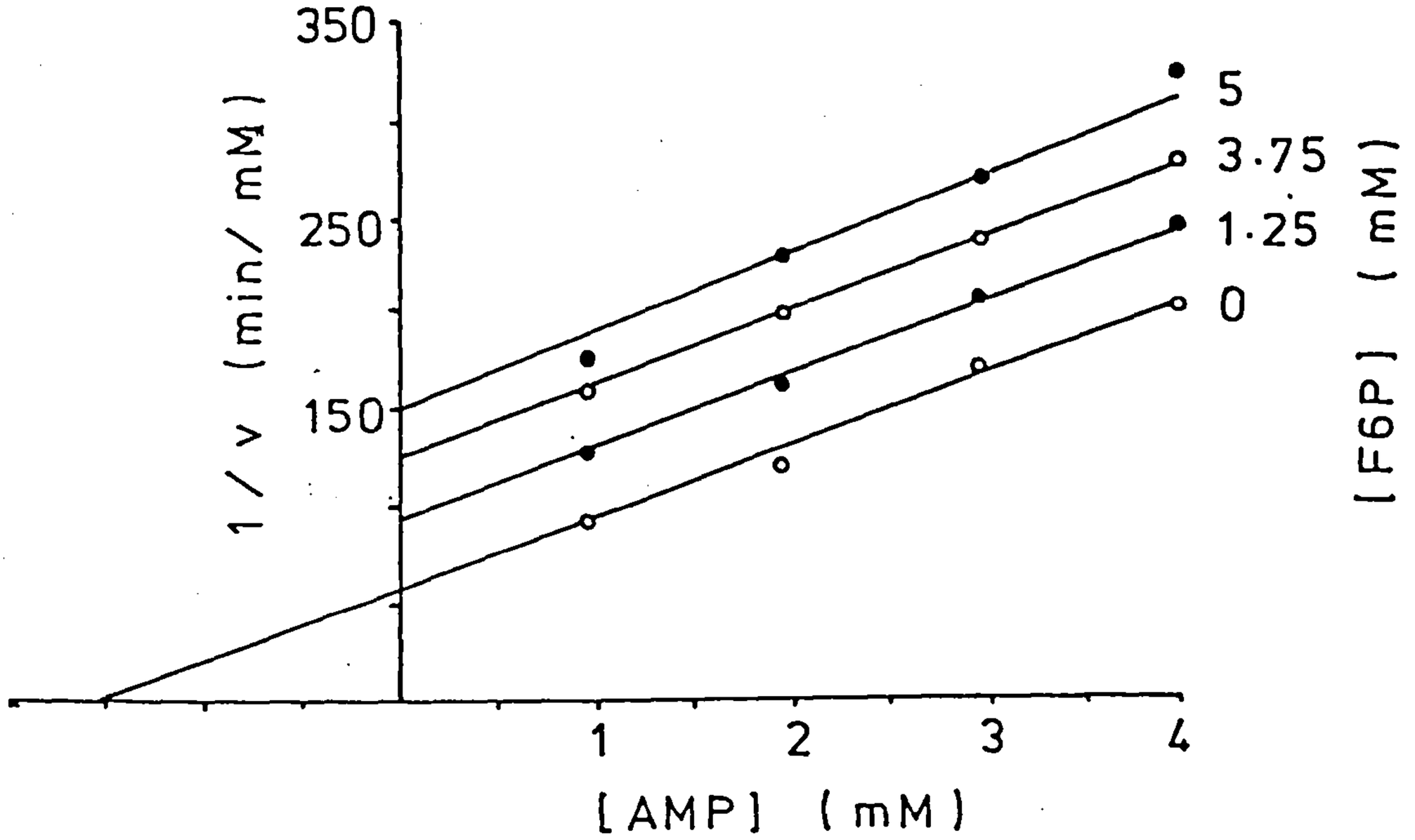


Secondary plot for MgADP vs. AMP.

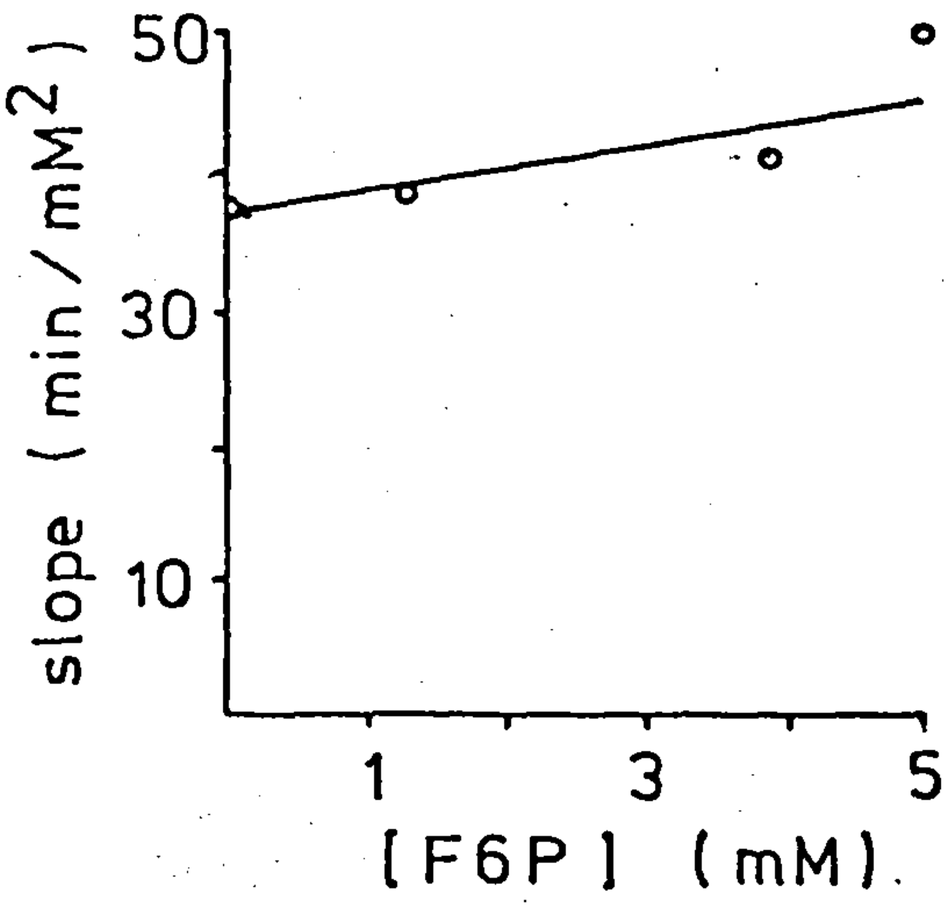


Double inhibitor studies. Asterias hexokinase. Figure 9.17

AMP vs. fructose 6-phosphate.
Mg^{2+} was constant at 5 mM, glucose was 11 mM and MgATP was 0.14 mM.



Secondary plot for AMP vs. fructose 6-phosphate.



CHAPTER 10

GENERAL DISCUSSION

In this discussion the properties of heart and Asterias hexokinases have been treated comparitively in the context of what is known of hexokinases from other sources.

Since Asterias hexokinase was approximately half as large as heart hexokinase and yet was reported to have comparable regulatory properties (18) it was hoped that it might make a simpler subject for a detailed study of hexokinase regulation and might help lead towards an understanding of the regulation of the heart enzyme and thus other mammalian isoenzymes. Furthermore it was of interest to compare the properties of Asterias hexokinase to those of hexokinases of similar molecular weight but very different regulatory behaviour.

The results obtained for the kinetics of heart hexokinase in this study are largely consistent with those of Easterby and O'Brien (46), Vowles (69) and Lambert (105); thus purification by dye-ligand chromatography did not alter the kinetic behavior of this enzyme.

Amongst the low Km mammalian hexokinases the pig heart isoenzyme is unique in that ADP is a competitive inhibitor with respect to ATP. ADP is a noncompetitive inhibitor (with respect to ATP) of other type I and type II hexokinases including ox heart hexokinase (64). Also, ADP inhibition (with respect to ATP) of both glucokinase (106) and wheatgerm L_{II} hexokinase (25) is mixed.

The rat skeletal muscle hexokinase is inhibited by phosphate and G6P, although in the brain isoenzyme, phosphate relieves glucose 6-phosphate inhibition (107). In contrast, pig heart hexokinase is very weakly inhibited by phosphate and it is questionable whether this ligand plays an important part in the regulation of this enzyme. However, despite differences in its regulation by ADP and phosphate, pig heart hexokinase is kinetically similar to type I isoenzymes from other sources , showing linear substrate kinetics and binding substrates and inhibitory ligands with a similar affinity to other type I enzymes studied.

Hexokinase purified from Asterias rubens was broadly comparable to the low Km hexokinases from mammals, especially type II hexokinase from skeletal muscle, since it was inhibited by ADP and phosphate in a similar way. Indeed the overall similarity was such that it seems reasonable to assume that the mechanism and regulation of both Asterias hexokinase and the low Km mammalian hexokinases are basically the same.

Competition between two ligands may result from either competition for the same binding site or through an allosteric effect, and a limitation of steady-state kinetic studies is their failure to distinguish between these two possibilities. This means that competition between ADP and ATP, in the case of pig heart hexokinase, does not exclude the possibility of separate binding sites for both ligands. Similarly, AMP was shown to compete with ATP. Double inhibitor studies showed that AMP also competed with ADP, thus it is likely that all three nucleotides bind to the same site on the enzyme. A similar interpretation is not as straight-forward for Asterias hexokinase because, although ADP is a noncompetitive inhibitor with respect to ATP, AMP competes with both ATP and ADP.

For some double inhibitor experiments, fructose 6-phosphate (F6P) was used as a glucose 6-phosphate (G6P) analogue. It may be however that F6P is not the best choice of G6P analogue ; Easterby (29) has shown that neither mannose 6-phosphate (M6P) or F6P (both at concentrations up to 10 mM) would cause dimerisation of pig heart hexokinase although glucose 1,6-bisphosphate ($G16P_2$), at a concentration of 200 μM was shown to cause a small but detectable dimerisation of the enzyme. (These experiments were carried-out in the ultracentrifuge in pH 8 Tris buffer, since then Lambert has shown that the inhibition of pig heart hexokinase by $G16P_2$ is pH dependent and the Ki approaches infinity as pH is increased towards pH 8 (105). It is likely then, that at a lower pH (eg. pH 7.5) $G16P_2$ would cause more dimerisation of the enzyme and would be a good G6P analogue for double inhibitor studies.) The major disadvantage of using $G16P_2$ would be its expense.

Other approaches for double inhibitor studies

Double inhibitor studies need not be carried out by means of steady-state kinetics. Other approaches have already been used, for example Vowles (69) studied the effects of various ligands on the G6P promoted dimerisation of hexokinase from pig heart. In one experiment ADP (10 mM) was shown to have no effect on G6P (0.1mM) promoted dimerisation, a result that would indicate a noncompetitive interaction between the two ligands.

The back reaction of hexokinase

The ligands ADP and G6P, which inhibit the forward reaction of hexokinase can be used as substrates in the back reaction. The kinetics that these ligands show in the back reaction provide information on the mechanism and regulation of the enzyme. In a recent study by Solheim and Fromm (39) the back reaction of bovine brain hexokinase showed Michaelis-Menten kinetics, neither substrate influencing the others binding. These data indicated that product release from the forward reaction would be random and that G6P exerted its inhibitory effects at the active site of hexokinase. At concentrations of G6P above 60μM, the back reaction was however inhibited, suggesting the existence of a second G6P binding site on the hexokinase.

Inactivation studies

Another approach to studying the interrelationship of the binding of two inhibitory ligands is through inactivation studies. Mammalian hexokinases have been inactivated by, for example, chymotrypsin (108), glutaraldehyde (108), diethylpyrocarbonate (69) and iodate (105) as well as a variety of affinity labels. Such studies have usually been concerned with the effects of single ligands on the rate of inactivation

for example, G6P was found to be very effective in protecting rat brain hexokinase from glutaraldehyde inactivation, F6P and M6P being less effective (108). This approach might also be useful in studying pairs of inhibitory ligands.

SECTION 10.2 MODELS OF THE REGULATION OF HEXOKINASE

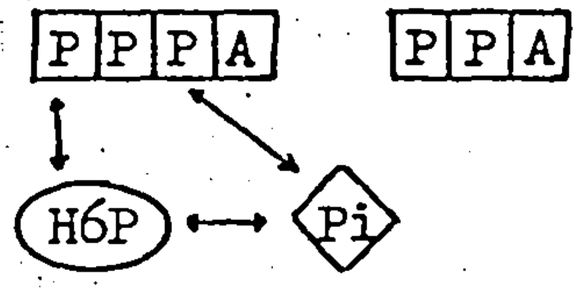
Anumber of models for the regulation of hexokinase have been proposed although confusion over the nature of G6P inhibition has hindered the development of models that can be tested experimentally. Wilson (109) developed a model for the regulation of rat brain hexokinase based on the effects of phosphate; the enzyme was thought to exist in two coformations, only one having a high affinity for phosphate. The model was later refined on the basis of the effects of other ligands on chymotryptic and glutaraldehyde inactivation of the enzyme (107). The refined model is however too complex to test easily and rests partly on the poor assumption (considering G6P promoteed dimerisation) that the hexokinase-glucose complex is similar to that of the hexokinase-glucose-G6P complex.

More acceptable models have been proposed on the basis of product inhibition ; the general ideas of active-site overlap in G6P binding, which come chiefly from Fromm's laboratory (and are discussed in Chapter 1) have been extended by Easterby and Qadri (100) in tne light of double inhibitor studies on skeletal muscle type II hexokinase. Qadri (88) found that hexose 6- phosphate was a competitive ligand with respect to ATP but not ADP and AMP. A model for the regulation of type II hexokinase is given in Figure 10.1 . The model proposed for type II hexokinase does not distinguish between allosteric or active site overlap binding of G6P.

The interaction of ligand binding sites on hexokinase Figure 10.1

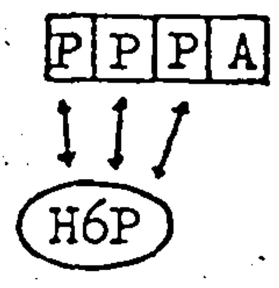
In all cases G represents glucose, H6P is hexose 6-phosphate , PPPA is ATP, PPA is ADP and Pi is phosphate. Arrows represent conformational changes which result in competition between ligands.

a). Type II hexokinase from skeletal muscle (from Easterby & Qadri (100))



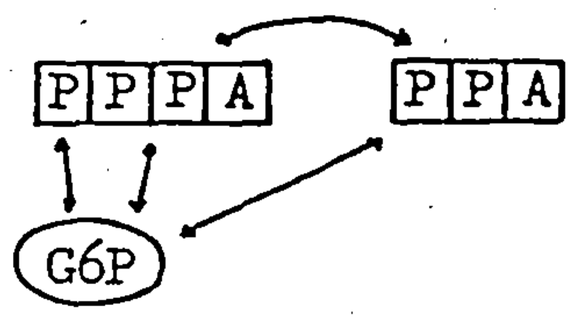
ADP does not compete with any other ligand. H6P competes with ATP but not AMP which itself competes with ATP. Pi is competitive with all ligands except ADP and glucose. The phosphate of H6P may or may not overlap the ATP terminal phosphate.

b). Type I hexokinase from pig heart



All nucleotides compete for binding to the enzyme and hexose 6-phosphate competes with all nucleotides. These observations make the possibility of ATP γ phosphate site overlap binding of H6P unlikely and a distinct site for H6P is proposed . G6P is known to have profound effects on the conformation of pig heart hexokinase (29).

c). Asterias hexokinase



ATP and ADP do not compete with one another but AMP competes with both these ligands. H6P competes with all nucleotides. As for heart hexokinase the simplest explanation is that H6P occupies an allosteric site and causes a conformational change that displaces other ligands. The fact that AMP displaces both ADP and ATP may suggest that nucleotide binding sites interact allosterically.

SECTION 10.3 COMPARISON OF HEXOKINASE FROM DIFFERENT SOURCES

It is interesting to consider the the relationship between the kinetic mechanism of an enzyme and its regulation, ie. whether its kinetic mechanism influences or determines its regulation. Both glucokinase and the low Km mammalian hexokinases appear basically similar in mechanism; in the former there is a prefered order of substrate binding and product release, however random addition of substrates (similar to that seen in the low Km hexokinases) is possible under certain conditions (6). The major differences in the regulation of various hexokinases are the affinity for glucose and for G6P and these could well be quantitative differences rather than qualitative differences. An overall similarity in mechanism,and to some extent physical structure is apparent in many hexokinases that have been closely studied. Despite the existence of a partial gene duplication theory for the evolution of 100,000 molecular weight hexokinases , there is no substantial evidence for the existence of more than one G6P binding site on such an enzyme and Asterias hexokinase, although half the molecular weight of the low Km mammalian hexokinases appears to be regulated in an almost identical way.

Comparison of amino acid composition

The striking similarity in the amino acid compositions of liver glucokinase and skeletal muscle type II hexokinase has been taken as consistent with the partial gene duplication theory in the evolution of the latter enzyme (47). Since heart and Asterias hexokinases appeared so similar in their kinetic properties it was interesting to compare their amino acid compositions.

The comparison index chosen was that of Cornish-Bowden (110). Providing proteins to be compared are either of equal length or that one protein

is approximately double the length of the other then the index termed $S\Delta_n$ can be easily applied. In this case Asterias hexokinase is considered to be half the molecular weight of pig heart hexokinase and the S_n index effectively compares the Asterias hexokinase composition with half of the heart hexokinase composition.

For a protein A (in this case heart hexokinase), n_{iA} is the number of the ith amino acid residue and N_A is the total number of amino acid residues in the protein. Protein B in this case is Asterias hexokinase. $S\Delta_n$ can be defined as ;

$$S\Delta_n = \tfrac{1}{2} N_B^2 \Sigma \left(\frac{n_{iA}}{N_A} - \frac{n_{iB}}{N_B} \right)^2$$

When $S\Delta_n$ is less than $0.42N_B$ significant relationship in terms of sequence similarity is predicted. If $S\Delta_n$ falls between 0.42 and 0.93 N_B then the test is inconclusive (111). Data for the comparison of heart and Asterias hexokinases are shown in Table 10.1 and from this table a relationship between the two compositions is apparent even when n_{iA} and n_{iB} are compared directly. From the calculation, $S\Delta_n = 117$ and $0.42N_B = 158$.

Thus the amino acid compositions of pig heart hexokinase and Asterias hexokinase are very similar except in respect to glutamic acid. Interestingly, in the comparison of the amino acid compositions of type II hexokinase and glucokinase (47) glutamic acid levels differed more than those of other amino acids.

The amino acid compositions of wheatgerm L_{II} hexokinase, yeast hexokinase and pig heart hexokinase have been compared by Higgins (112) and found to show significant similarity. This relationship in amino acid composition may indicate evolution from a common ancestor.

Table 10.1

COMPARISON OF THE AMINO ACID COMPOSITIONS FOR PIG HEART AND ASTERIAS RUBENS HEXOKINASE.

The two amino acid compositions are compared by the $S\Delta_n$ index of Cornish-Bowden as described in the text. The table shows the number of each amino acid residue, n_{iA} and n_{iB}, in protein A (heart hexokinase) and protein B (Asterias hexokinase) respectively. Protein A is composed of a total of N_A residues and protein B of N_B residues.

AMINO ACID	A : heart hexokinase n_{iA}	$100n_{iA}/N_A$	B : Asterias hexokinase n_{iB}	$100n_{iB}/N_B$	$10^4\left(\frac{n_{iA}}{N_A}-\frac{n_{iB}}{N_B}\right)$
Asp	74	9.69	36	9.55	0.0196
Thr	49	6.41	27	7.16	0.5625
Ser	49	6.41	26	6.90	0.2401
Glu	97	12.70	36	9.55	9.9225
Pro	28	3.66	18	4.77	1.2321
Gly	65	8.51	32	8.49	0.0004
Ala	63	8.25	33	8.75	0.2500
Val	45	5.89	22	5.84	0.0025
Met	20	2.62	13	3.45	0.6889
Ile	44	5.76	16	4.24	2.3104
Leu	67	8.77	34	9.02	0.0625
Tyr	19	2.49	10	2.65	0.0256
Phe	33	4.32	19	5.04	0.5184
His	17	2.23	6	1.59	0.4096
Lys	48	6.28	25	6.63	0.1225
Arg	46	6.02	24	6.37	0.1225
totals	764(=N_A)		377(=N_B)		16.49 @

@ from this total $S\Delta_n$ is calculated. $S\Delta_n = 16.5\ (N_A^2 / 20{,}000) = 117$.
When $S\Delta_n$ is less than $0.42\ N_B$ (= 158) the compositions are related.

Note : The amino acid compositions for both hexokinases are from the average of two hydrolyses. The number of each amino acid residue, in both heart and Asterias hexokinase was calculated from experimental amino acid analysis (Chapter 2, Section 2.13) assuming molecular weights of 100,000 and 50,000 respectively. Rigorously determined molecular weight for heart hexokinase is nearer 97,000 (46) and the molecular weight of Asterias hexokinase found to be about 51,000 (Chapter 4, Figure 4.7). The calculation of $S\Delta_n$ is facilitated by assuming Asterias hexokinase is half the molecular weight of heart hexokinase.

Indeed the similarity of heart and Asterias hexokinases in terms of both regulation and amino acid composition suggests that they had a common ancestor and that the larger heart hexokinase evolved by partial gene duplication from an ancestor similar to Asterias hexokinase. However, the fact that the 50,000 molecular weight Asterias hexokinase is regulated in a very similar manner to heart hexokinase shows that it is not necessary to postulate gene duplication to account for the existence of a regulatory site for G6P. The absence of any firm evidence that there is more than one G6P binding site on any hexokinase suggests that the effects of this ligand result from binding at a single site.

The question remains, does G6P exert its regulatory effects at the active site by direct competition with ATP or at an allosteric site distinct from the active site? The profound conformational changes induced by G6P in the pig heart hexokinase suggest that the ligand could act in an allosteric manner. The models given in Figure 10.1 are however not inconsistent with the proposal that G6P binds at the active site, overlapping the terminal phosphate of ATP (40). Indeed, the G6P binding site could be close to but not necessarily coincident with the active site (ie.not necessarily overlapping the ATP terminal phosphate site). In the cases of Asterias and heart hexokinases G6P may well act through a combination of effects displacing AMP and ADP through effecting a conformational change and possibly displacing ATP by direct competition. Further experiments on the mechanism of product release from the low Km hexokinases would be valuable in the understanding of the regulatory behaviour of G6P.

It is not apparent why 100,000 molecular weight hexokinases have evolved when a 50,000 molecular weight species appears to have almost identical regulatory properties.

SECTION 10.4 THE REGULATION OF GLYCOLYSIS IN THE HEART

Glycolytic flux in perfused heart is accelerated by anoxia or glucose plus insulin and is inhibited by diabetes and by the oxidation of fatty acids or ketones which are the prefered glycolytic fuels in rat heart. (113,114). Phosphofructokinase has been identified as a major control point in cardiac glycolysis as are glucose transport and hexokinase (36). The relative activities of each cardiac glycolytic enzyme are given in Table 10.2 . Both the levels of phosphofructokinase and hexokinase are relatively low, hexokinase activity being lower than any other enzyme. Metabolite levels in cardiac tissue in the presence and absence of insulin in the perfusion buffer are given in table 10.3 .

Fatty acid oxidation causes an increase in cardiac citrate levels, citrate inhibits phosphofructokinase thus allowing accumulation of hexose phosphates which in turn inhibit hexokinase. ATP levels change relatively little when well oxygenated heart is made anoxic, but the levels of both AMP and phosphate rise, probably relieving the inhibitory effects of ATP on phosphofructokinase. Thus phosphofructokinase activity is enhanced during anoxia and depressed during fatty acid oxidation.

When glycolytic rate is depressed, for example by perfusion of the heart with fluoroacetate (117), the level of hexose monophosphates increases. Similarly, hearts from alloxan-diabetic rats have depressed rates of glucose uptake and glycolysis, and have higher levels of G6P than hearts from normal rats (118). Addition of 1 mM α-bromostearate to the prefusion medium (α-bromostearate inhibits fatty acid oxidation) of diabetic rat hearts causes an increase in glucose uptake and glycolysis and a decrease in G6P levels which return to those of the normal heart (118). Again, these effects are thought to operate primarily through the control of phosphofructokinase by citrate, however increased G6P levels would tend to inhibit hexokinase, and possibly affect its association with the

Table 10.2

The relative activities of glycolytic enzymes in the heart

From Newsholme and Start (115), based on an activity of 7 µmol / min /g for hexokinase.

hexokinase	1
phosphoglucoisomerase	9
phosphofructokinase	2
aldolase	4
triosephosphate isomerase	83
glyceraldehyde 3-phosphate dehydrogenase	19
phosphoglycerate kinase	10
phosphoglycerate mutase	3
enolase	2
pyruvate kinase	21
lactate dehydrogenase	55

Metabolite levels in perfused, working rat hearts. From Opie et al. (116).

Hearts were perfused in a buffer containing 11.1 mM glucose in the presence and absence of insulin, after 15 min hearts were freeze clamped and metabolite levels were subsequently measured. Under these conditions glycogen breakdown is negligible (114).

	glycolytic flux µmol glucose / min / g .	intracellular glucose (µmol/ml)	nmol / g				µmol / g			
			G6P	F6P	$F16P_2$	citrate	ATP	ADP	AMP	creatine phosphate
no insulin	3.12	n.d @	113	29	23	181	3.56	1.08	0.11	3.0
+ insulin	5.61	3.9	304	68	37	433	3.49	0.9	0.12	3.79

G6P represents glucose 6-phosphate; F6P, fructose 6-phosphate; $F16P_2$, fructose 1,6-bisphosphate. n.d, not detectable.

@ cardiac glucose would have been depleted after perfusion.

Table 10.3

mitochondrion (34).

An estimate of the <u>in vivo</u> activity of hexokinase can be made from data in Table 10.3 by assuming that hexokinase in rat heart has similar regulatory properties to that in pig heart . Although the calculation is an approximation and rests on this assumption,it serves as an interesting example of the use of equations derived for double inhibitor studies.

Looking at Table 10.3 , for the case of heart perfused in the presence of insulin , the inhibitory ligands F6P and AMP are present in low levels relative to their Ki's but G6P levels are high (300 μM) relative to its Ki value of around 20 μM. ADP may also be in sufficient concentration to influence the activity of hexokinase. Product inhibition studies showed that G6P, ADP and ATP competed with one another and were noncompetitive with respect to glucose. Also, from Table 10.3, glucose would be saturating as its intracellular concentration is at least 3.9 mM.

Equation 7.5.19 from Chapter 7 can thus be used to predict the velocity of hexokinase in the presence of G6P and ADP or any other pair of inhibitory ligands provided they are all noncompetitive with respect to glucose.

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_A.K_{AB}}{[A]\,K_B} + \frac{[J]\,K_A.K_{AB}}{K_j.K_{jB}\,[A]} + \frac{[J]}{K_{Aj}}\right) +$$

$$\frac{[I]}{V}\left(\frac{1}{K_{Ai}} + \frac{[J]}{K_{Ai}.K_{Aij}} + \frac{K_A.K_{AB}}{K_i.K_{iB}[A]} + \frac{[J]\,K_A.K_{AB}}{K_i.K_{ij}.K_{ijB}[A]}\right) \qquad (7.5.19)$$

Equation 7.5.19 can be simplified if B (glucose) is noncompetitive with respect to both I (G6P) and J (ADP) (that is $K_{AB}=K_{iB}=K_{jB}$) and if I and J compete with one another ($K_{ij}=\infty$, $K_{iA}=\infty$, $K_{Aj}=\infty$, $K_{Aij}=\infty$) in which case terms in K_{ij}, K_{iA}, K_{Aj} and K_{Aij} are lost. The simplified equation is ;

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_A}{[A]}\left(1 + \frac{[J]}{K_j} + \frac{[I]}{K_i}\right)\right) \qquad (10.4.1)$$

and so

$$\frac{v}{V} = \frac{[A]}{[A] + K_A\left(1 + \frac{[I]}{K_i} + \frac{[J]}{K_j}\right)} \qquad (10.4.2)$$

Taking the Km for ATP as 0.7 mM and Ki values of 20 µM and 2 mM for G6P and ADP respectively, the metabolite levels in Table 10.3 would yield a v/V value of 0.2 . Thus hexokinase should be 80 % inhibited and V from Table 10.3 is 5.61 µmol glucose / min / g ie. the predicted hexokinase activity only accounts for the phosphorylation of 1.1 µmol glucose / min / g.

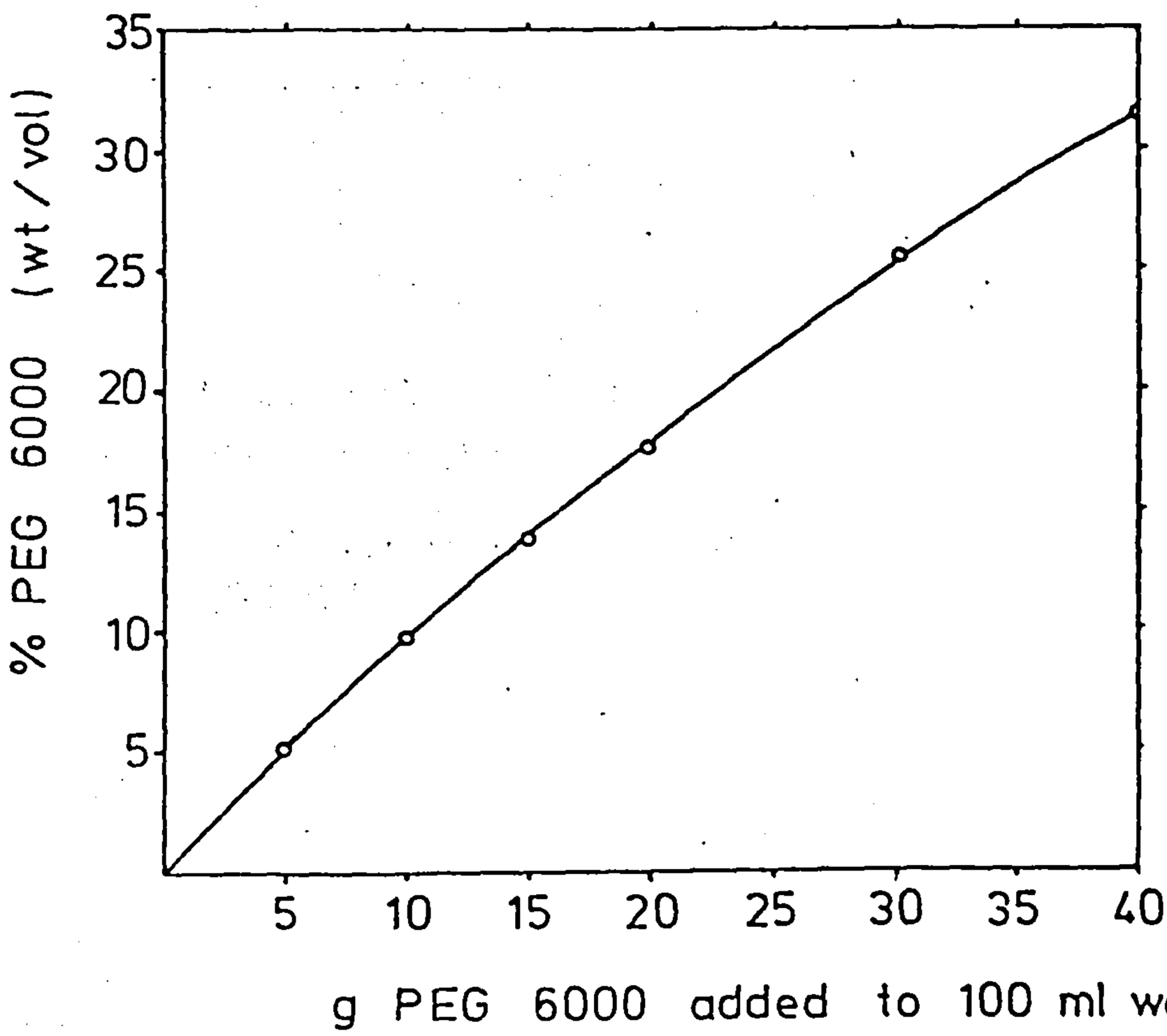
The above calculation rests on the assumption that metabolites are evenly distributed within the cardiac muscle cells. By rearranging equation 10.3.2 it can be calculated, using a hexokinase level of 7 units per g rat heart (119), that an ATP concentration of about 46 mM would be required to account for the glycolytic flux seen in Table 10.3 .

Thus the predicted hexokinase activity is too low to account for the rate of glycolytic flux in the perfused heart, an increased hexokinase activity might be possible if there were locally high concentrations of ATP and in this context the proposal that mitochondrially bound hexokinase might have preferential access to ATP (120, 35) is interesting.

APPENDICES

APPENDIX I

POLYETHYLENE GLYCOL 6000 SOLUTIONS OF KNOWN % COMPOSITION.



Increments of polyethylene glycol 6000 were weighed and dissolved in water at room temperature and the volume measured.

APPENDIX II

'Purification of Heart Hexokinase by Dye-Ligand Chromatography'

Farmer,E.E. and Easterby,J.S. Anal. Biochem. 123 373-377

ANALYTICAL BIOCHEMISTRY 123, 373–377 (1982)

Purification of Heart Hexokinase by Dye–Ligand Chromatography

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Received January 11, 1982

Heart hexokinase (EC 2.7.1.1) has been purified by a procedure that uses affinity elution from a column of red triazine dye, H-8BN, immobilized to Sepharose 6B. Homogeneous protein (9 mg) is obtained from 500 g heart in 40% yield. The mechanism of affinity elution from the dye is discussed.

Hexokinase occurs in animal tissues in four isozymic forms; the most abundant is the type 1 enzyme. This isozyme has previously been purified from brain (1,2), pig heart (3), and human heart (4) by both conventional nonspecific methods and by procedures involving varying degrees of selective elution from subcellular particles. The most effective procedure for the purification of heart hexokinase has involved the use of ion-exchange resins including phosphocellulose (3). The latter exchanger is often tedious to recycle and equilibrate and the commercial product is of variable quality.

Recently, advances have been made in the use of matrices containing triazine dyes for the chromatography of dehydrogenases and kinases. Early claims that these dyes behaved exclusively as nucleotide analogs now seem to be unfounded (5), and their ability to bind proteins probably results from a mixture of hydrophobic and ionic interactions. Nevertheless, the molecules tend to be flat, fairly apolar, and contain a number of negative charge groups which may provide a fortuitous and not entirely specific affinity for nucleotide binding sites. Their advantages over classical ion-exchangers and affinity ligands are that they are cheap, easily coupled to matrices, and many varieties are available allowing selection of dye with the appropriate affinity.

In the present paper, we report an improved procedure for the purification of heart hexokinase that combines the advantages of selective binding of enzymes by careful choice of dye with affinity elution by nucleotide substrates or inhibitors. It also overcomes previous problems of separation of enzyme from physically similar diaphorase species.

MATERIALS AND METHODS

Reagents

Fresh pig hearts were obtained from a local slaughterhouse and stored frozen. Nucleotides were purchased from P-L Biochemicals (Milwaukee, Wisc.). Hexose phosphates and glucose-6-phosphate dehydrogenase were from Boehringer-Mannheim (Mannheim, West Germany). Analytical grade potassium phosphates and glucose were from Fisons (Loughborough, England). All other reagents were obtained from British Drug Houses (Poole, England) and were of Analar grade. Preswollen DE-52 was purchased from Whatman (Maidstone, England) and recycled according to manufacturers' instructions. Sephadex G-200 and Sepharose 6B were from Pharmacia (Uppsala, Sweden). The monochlorotriazine dye Procion brilliant red H-8BN was a product of I.C.I. and a gift from Dr. P. D. G. Dean.

0003-2697/82/100373-05$02.00/0

Synthesis of the Triazine Dye Column

The triazine dye H-8BN was coupled to Sepharose 6B using the procedure recommended by Dean and Watson (6) using 150 g wet Sepharose 6B and 1 g H-8BN. The amount of substitution was estimated after hydrolysis in 50% acetic acid using an $E^{1\%}_{550\,nm}$ of 315 as determined by Qadri (7) and was found to be 1 mg/ml Sepharose. After use the bound protein was eluted from the dye column by washing with 4 M urea followed by 2 M KCl. The column was stored at 4°C in the presence of 0.02% sodium azide.

Enzyme Assays

Hexokinase was assayed with a Gilford 240 spectrophotometer by coupling to glucose-6-phosphate dehydrogenase (EC 1.1.1.49) as described previously (3). A unit of enzyme is defined as the amount of enzyme catalyzing the phosphorylation of 1 μmol glucose/min at 30°C. Diaphorase assay consisted of the reduction of potassium ferricyanide by NADH (8). The assay contained 50 μmol potassium ferricyanide and 200 μmol NADH in 1 ml of 150 mM sodium acetate, pH 4.8. Reduction of ferricyanide was measured at 420 nm. One unit of enzyme corresponds to the reduction of 1 μmol ferricyanide/min at 30°C, which is equivalent to an absorbance change of 1.0 unit/min.

Protein Estimation

Protein was measured by the microtannin turbidimetric method of Mejbaum-Katzenellenbogen and Dobryszycka (9).

Electrophoresis

Polyacrylamide gel electrophoresis was performed according to Grossman and Potter (10). Sodium dodecyl sulfate-gel electrophoresis was performed according to Weber and Osborn (11). Protein was stained with naphthalene black.

Kinetic Analysis

The interaction of H-8BN with hexokinase was studied by steady-state kinetic analysis. To eliminate the possibility of covalent modification of hexokinase or glucose-6-phosphate dehydrogenase, the reactive chlorine atom of H-8BN was removed by hydrolysis (12). The hydrolyzed dye (referred to as H-8BN-OH) was redissolved in 0.05 *I* Tris–HCl, pH 7.6, for use. Assay conditions were similar to those used in the standard assay.

Purification of Hexokinase

Buffers. All buffers contained 100 mM glucose and 5 mM β-mercaptoethanol. Buffer A: 0.2 *I* acetate, pH 5.6, 0.2 M $MgCl_2$; 13.6 g sodium acetate trihydrate, 0.257 ml acetic acid, and 40.6 g $MgCl_2$ per liter. Buffer B: 0.08 *I* phosphate, pH 8; 4.52 g K_2HPO_4, 0.29 g KH_2PO_4, and 0.372 g disodium EDTA per liter. Buffer C: 0.05 *I* Tris–HCl, pH 7.5; 7.4 g Tris and 0.372 g EDTA per liter titrated to pH 7.5 with HCl.

Extraction of enzyme. Two liters of ice-cold water was poured through 500 g of minced heart in cheesecloth to remove blood. The tissue was homogenized in 800 ml buffer A for 2 min in an MSE Atomix and stood on ice for 45 min prior to centrifugation at 30,000*g* for 30 min. The residue was reextracted with 800 ml buffer A, and the supernatants were combined and slowly titrated to pH 8 with 0.2 M KOH.

Dialysis. The supernatant was dialyzed overnight against 25 liters of buffer B. The precipitate formed was removed by centrifugation for 25 min at 30,000*g*, and the extract was dialyzed for a further 24 h against 20 liters buffer B.

Chromatography on DEAE-cellulose. The dialyzed extract was batch-adsorbed onto 900 ml DEAE-cellulose equilibrated in buffer

B, and unbound protein was removed by washing with 4 liters of buffer B on a Büchner funnel. The exchanger was then poured into a 38 × 5.5-cm column, washed with a liter of buffer, and developed with a 2-liter 0–0.4 M KCl linear gradient in buffer B. Flow rate was 80 ml/h and 12.5-ml fractions were collected.

Affinity elution from H-8BN–Sepharose. Active fractions from DEAE-cellulose were combined and dialyzed overnight against 5 liters buffer C before they were pumped onto a 6 × 4.5-cm column of H-8BN–Sepharose 6B equilibrated in buffer C. Flow rate was 60 ml/h. The column was thoroughly washed with buffer C to remove unbound protein, and hexokinase was eluted with 1.8 mM ATP in buffer C. Fractions (10 ml) were collected (Fig. 1). Mg-ATP was not used to elute hexokinase, as this would have resulted in turnover of the enzyme. Fractions 62–70 were pooled.

Gel filtration. The eluate from the previous stage was concentrated by vacuum dialysis against buffer C and applied to a column of Sephadex G-200 (100 × 1.25 cm) equilibrated in buffer C. The column was developed at a flow rate of 8 ml/h and 4.5-ml fractions were collected. All active fractions were pooled.

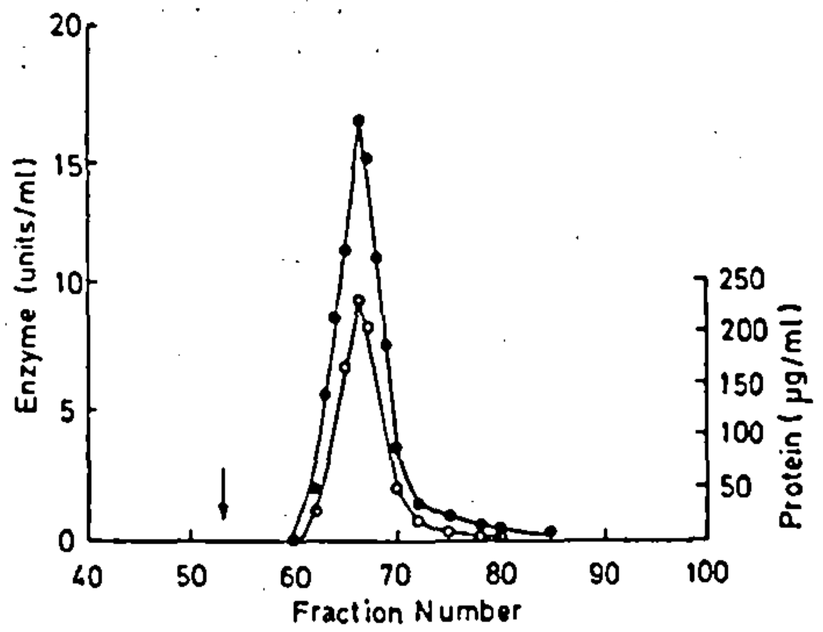

FIG. 1. Affinity elution of heart hexokinase from H-8BN-Sepharose. Buffer was 0.05 *I* Tris-HCl, pH 7.5, containing 1 mM EDTA, 100 mM glucose, and 5 mM β-mercaptoethanol. Flow rate was 60 ml/h and 10-ml fractions were collected. Protein (○) and enzyme (●) are shown. The enzyme was eluted by inclusion of 1.8 mM ATP in the buffer from the fraction indicated by the arrow.

TABLE 1

PURIFICATION OF HEART HEXOKINASE

Stage	Enzyme (units)	Protein (mg)	Specific activity (U/mg)
Extract 1	1096	39,770	0.049
Extract 2	880		
Postdialysis	1553	14,250	0.109
DEAE-cellulose	1120	243	4.6
H-8BN-Sepharose	990	14	71
Sephadex G-200	775	9.2	84

Note. 500 g of heart were used.

RESULTS AND DISCUSSION

The results of a typical preparation of hexokinase are shown in Table 1. The enzyme was largely homogeneous after H-8BN–Sepharose chromatography, but gel filtration was included to remove aggregated material. The final enzyme was homogeneous by criteria of polyacrylamide gel electrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and analytical ultracentrifugation.

The purification of heart hexokinase using the immobilized triazine dye H-8BN offers several advantages over previously published methods (3,4). After initial extraction, the enzyme is maintained at a pH above 7, which improves stability. There is no background leakage of enzyme from the H-8BN–Sepharose column and recovery is high. The use of ATP as the eluting ligand means that affinity elution is not costly, and it is reproducible because the conditions required are not critical. Moreover, elution with ATP selects for kinetic competence of the enzyme. One problem encountered in previous purifications of hexokinase from heart was the co-purification of physically similar diaphorase species (lipoamide dehydrogenases), which are abundant in this mitochondrion-

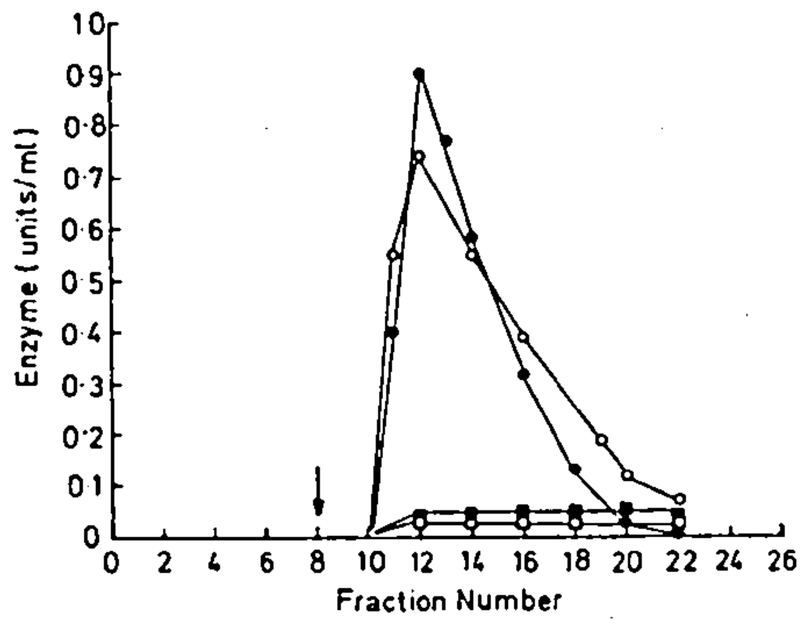

FIG. 2. The effects of ligands on the elution of hexokinase from H-8BN–Sepharose. Fifteen units of enzyme were applied to a 2 × 4-cm column equilibrated with 0.05 *I* Tris–HCl, pH 7.5, containing mM EDTA, 100 mM glucose, and 5 mM β-mercaptoethanol. Flow rate was 30 ml/h. Enzyme was eluted by inclusion of 1.5 mM ATP (●), 7.8 mM AMP (○), 66 μM glucose 6-phosphate, 2 mM glucose 6-phosphate (■), or 150 μM glucose 1,6-bisphosphate (□) in the buffer from the arrow onward.

rich tissue. This problem has been overcome here by the use of affinity elution. For the most successful purification, however, it is advisable that the enzyme eluted from DEAE-cellulose have a specific activity of at least 4 to 5 units/mg before application to the dye column. Previous reports have suggested the existence of multiple forms of type 1 hexokinase (3,13), but no evidence of this was seen in the present purification.

The effect of various ligands on the elution of heart hexokinase from H-8BN–Sepharose is shown in Fig. 2. Glucose 6-phosphate and glucose 1,6-bisphosphate cause only very slight leakage of hexokinase from the column, even at concentrations far in excess of their K_i values. By contrast, AMP, ADP, and ATP eluted the enzyme effectively at 1.5 times their respective K_i and K_m values. No hexokinase was eluted by KCl at an ionic strength equivalent to that of the ligand under study. The charge on the eluting ligand does not seem to be the overriding factor determining elution from the dye column in this study. In contrast, Qadri and Easterby (14) implicated charge-specific effects (15,16) in the affinity elution of muscle hexokinase from an ion-exchange resin.

Free-solution kinetic studies of the inhibition of hexokinase by H-8BN–OH were undertaken to establish the nature of the interaction. Although inhibition was observed, this was far weaker than the inhibition of yeast hexokinase by triazine dyes observed by Wilson (17). Furthermore, H-8BN–OH was found to be a potent inhibitor of yeast glucose-6-phosphate dehydrogenase and rabbit muscle pyruvate kinase, making the use of coupled assays very difficult. The unhydrolyzed dye did not inactivate hexokinase after incubation for 1 h at room temperature at a concentration of 100 μg/ml. It may be concluded that, although the dye does inhibit hexokinase, the interaction is weak and this may facilitate the use of affinity elution procedures. KCl gradients would also elute the enzyme from the dye column, but 10% ethylene glycol was ineffective. Interaction of the dye with the enzyme does not therefore seem to be purely hydrophobic. Dye columns are a useful addition to the procedures of ion-exhange and affinity chromatography, but nonspecific interactions may occur between dye and proteins (5), and the purification achieved must be largely due to elution with specific ligands.

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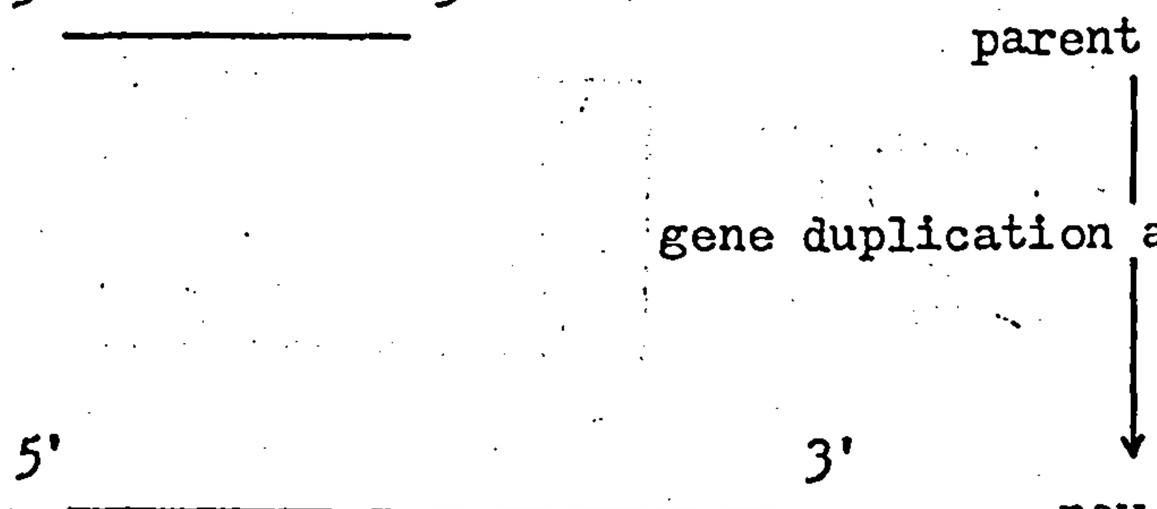
Addendum

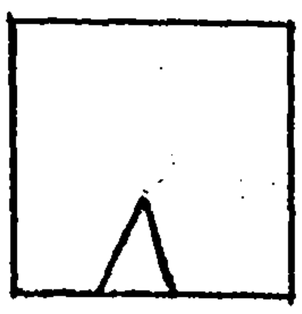
Partial gene duplication would result if two copies of the same gene arose and became fused in such a way that both retained their original polarity and that the fusion caused no disruption in the reading frame of codons throughout the gene. Such a fused gene could either be present *per se* in the genome or result through the exision of introns through transcriptional processing. It is envisaged that the new gene would code for a protein of somewhat less than twice the size of the parent protein and that the new protein would be a single polypeptide.

The process of partial gene duplication for the evolution of the 100,000 molecular weight hexokinases is illustrated below , after gene fusion an intermediate hexokinase form , having two active sites and a molecular weight of 100,000 would result. One of the active sites would then become a regulatory site independently of the process of gene duplication. For the genes;



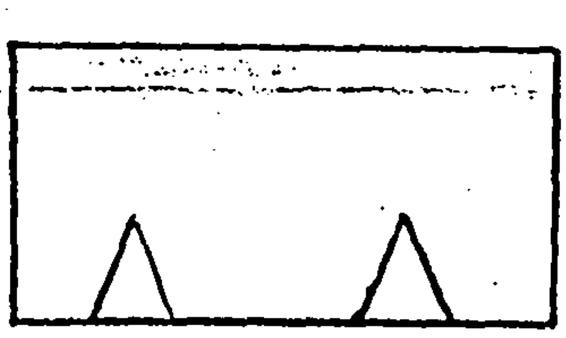
Partial gene duplication and fusion in the case of the 100,000 molecular weight hexokinases.

The blocks represent hexokinase , open triangles are active sites and the closed triangle is a regulatory site ;

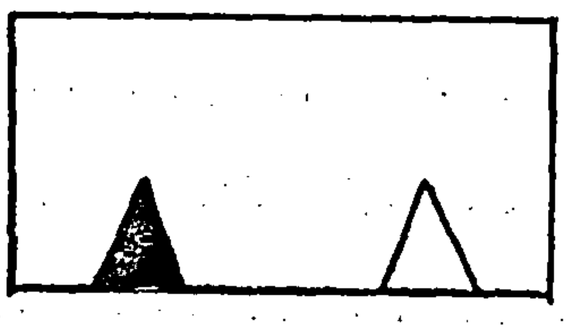


50,000 molecular weight ancestor hexokinase possessing a single active site viz. wheatgerm or yeast hexokinase type

gene duplication and fusion



100,000 molecular weight hexokinase possessing two active sites



100,000 molecular weight hexokinase with one active site and one regulatory site as for the 'low Km' mammalian hexokinases.

In 1973 Colowick (1) pointed out that although hexokinase from different sources showed different regulatory properties the kinetic mechanisma of these enzymes were basically similar. Since then alot of new information on the hexokinases has been gathered and a

new mechanism (mnemonical kinetics) has been identified in wheatgerm hexokinase (2) and also in liver glucokinase (3) an enzyme of similar molecular weight. Colowicks' remarks nevertheless remain valid implying a close relationship between the hexokinases , especially those of 50,000 molecular weight. Similarity is also evident in the amino acid compositions of hexokinases from a variety of sources , and irrespective of molecular weight.

Thus until the publication of the properties of hexokinase from the starfish Asterias (4) it appeared that there were two classes of hexokinase, those not inhibited by the product glucose 6-phosphate (of molecular weight 50,000) and those strongly inhibited by glucose 6-phosphate (of 100,000 molecular weight) . Asterias hexokinase however had a molecular weight of 50,000 and yet was strongly inhibited by glucose 6-phosphate.

It was an aim of this project to purify and characterise hexokinase from Asterias and to compare both its regulatory properties and its amino acid composition with those of hexokinase from pig heart which has already been studied in some detail. In order that a good comparison of these two hexokinases be made, the regulatory properties of the enzymes were investigated in some detail and inhibitors of posible physiological importance identified.

Initially, substrate kinetic experiments were undertaken and from these it appeared safe to make the rapid-equilibrium assumption, as applied to many other hexokinases , such that the ternary complex EAB is in equilibrium with the complex EPQ where E is enzyme , Aand B are substrates and P and Q are products .

Thus in the case of ADP , which appears to act as a normal product inhibitor of hexokinase, any noncompetitive interaction with respect to ATP is taken to indicate a separate site for ADP rather than the binding of ADP to an enzyme form not in equilibrium with that enzyme form binding ATP. This arguement does not apply to glucose 6-phosphate, the other product of the hexokinase -catalysed reaction, as for both pig heart and Asterias hexokinase this ligand does not behave as a normal end-product inhibitor .

Triazine dye screening studies

During the development of the enzyme purifications described in this thesis a number of immobilised triazine dyes were screened in the hope that they might provide a useful way of isolating the enzyme in question.

Initially, screening studies were carried out on type II hexokinase from skeletal muscle and through this work a dye that allowed the purification of pig heart typeI hexokinase was discovered. A large number of immobilised dyes were screened in the case of Asterias hexokinase although none proved useful in its purification.

Screening was carried out at 4^{o} C ; small volumes of buffer containing a known amount of enzyme were applied to small columns of immobilised dye which were allowed to run slowly, under gravity, and were washed with buffer before being developed with a pulse of salt solution or with a solution of ligand which might bring off bound enzyme.

Type II hexokinase from rat skeletal muscle.

Enzyme was partially purified by DEAE-cellulose chromatography after Qadri and Easterby (5) and had a specific activity of around 0.15 U / mg. Columns were 0.5 ml Sepharose 6B immobilised dye and were equilibrated with 0.02 I Tris-HCl buffer, pH 7.6 containing 5 mM β-mercaptoethanol , 100 mM glucose and 9 mM magnesium chloride. Magnesium was added to the buffers as at the time it was thought that it might promote the binding of kinases to triazine dyes (6). A number of dyes were screened and the results shown below. In all cases

bound enzyme was recoverable from the column by washing with 1 M KCl although there was a substantial loss of activity in the case of Procion Red H-8BN. In the table below + indicates poor binding and ++ indicates good binding ;

Procion Blue	H5R	++
	MXG	++
	MX2G	+
Procion Red	H8BN	++
	HE3B	++
Procion Orange	HER	++
Procion Green	HE4BD	++
Procion Brown	MX5BR	+
	H4RD	++
Cibacron Blue	3GA	+

Dyes that bound the enzyme most tightly and allowed best recovery were HE4BD, MXG, HER, H4RD and HE3B. These immobilised dyes were then screened under a variety of conditions . Little purification was achieved by salt elution although HE3B and HER allowed about a 2 fold purification when enzyme was eluted with KCl gradients. Attempts to elute enzyme from the dyes with ATP were not successful.

Type I hexokinase from pig heart

Whilst screening type II skeletal muscle hexokinase a crude preparation of the pig heart type I enzyme was applied to several dye columns. Fortuitously, the pig heart enzyme was found to bind Procion Red H8BN and could be recovered in good yield by ATP elution.

Crude pig heart hexokinase of approximately 0.2 U /mg was dialysed against 0.05 I maleic acid buffer, pH 7 containing 5 mM β-mercapto-ethanol, 100 mM glucose and 1 mM disodium EDTA. Enzyme was applied to 1 ml columns of dye immobilised to Sepharose 6B and equilibrated in the above buffer. Cibacron Blue 3GA and Procion Red HE3B failed to bind the heart hexokinase under these conditions . H8BN bound the enzyme and was then screened under a variety of different conditions before a large column of immobilised H8BN was synthesised.

Hexokinase from Asterias rubens

A series of 0.8 ml Matrex®-immobilised dye columns were equilibrated in 0.05 I Tris-HCl buffer, pH 7.5, containing 50 mM glucose, 1 mM disodium EDTA and 5 mM β-mercaptoethanol. Crude Asterias hexokinase of about 0.2 U/mg was dialysed against the above buffer and about 2 units applied to each column. Columns were washed with equilibration buffer and then with 1 M KCl in equilibration buffer.

In all cases no significant proportion of enzyme bound the dye column and the screening was repeated at pH 5.5 (in 0.1 I sodium acetate buffer containing 5 mM β-mercaptoethanol, 50 mM glucose and 1mM disodium EDTA. Again no significant quantity of hexokinase was bound. The addition of magnesium to the buffers was not investigated. This work was carried out with the help of Mr S.J.Pepper and a list of dyes screened is given below;

Procion dyes used in screening studies

Blue	HB	Green	HE4BD
	MX3G		H4G
	HGR		
	SP3R	Orange	MXG
	MX7RX		H2G
	H5R		HER
	P3BN		
	MXG	Brown	H2R
	MX2G		HGR
	HBRD		MXGRN
	MX4GD		H5BR
			H5R
			MX5BR
Red	H8BN		H4RD
	MX8B		
	H3B	Rubine	MXB
	MX5B		
	HE3B	Scarlet	MXG
	HE7B		HRN
	MX2B		
	MXG	Navy	MXRB
	P3BN		H4R
Yellow	MX6G	Turquoise	H5G
	H5G		MXG
	HE3G		H7G
	HA		
	H3R	Olive	H7G
	MXGR		MX3G
	MX3R		
	MX4R		
	M4G		
	MX4G		
	HE4R		
	MXR		

Cibacron dyes used in screening studies

Blue	3GA
	BRP
Olive	GP
Brilliant Green	T3GB
	4GA
Violet	2RP
Yellow	3GP
Black	BGA

Note : all dyes were immobilised on Matrex®, a product of Amicon, and were a gift from Dr. P.D.G.Dean.

References for the addendum

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