Pyruvate Kinase of *Escherichia coli*

by

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ABSTRACT

Partial purification of pyruvate kinase from Escherichia coli was carried out, and the kinetics of the enzyme were investigated. However, later studies indicated that the enzyme preparation used was a mixture of two species or types of pyruvate kinase, although the purification procedure tended to isolate only one. Initial investigation showed that this one species was activated by AMP, had a subunit (M.W. 50,000) structure, and a K_m for PEP of 0.1 mM at pH 6.3. The allosteric nature of this enzyme depended strongly on the assay pH. At pH 6.3, the enzyme's optimum, the velocity plots yielded were very nearly hyperbolic. This enzyme showed instability in the absence of dithiothreitol and the subunits would dissociate. Incubation with dithiothreitol would cause aggregation of the subunit and a reappearance of activity. The second species of pyruvate kinase was activated by FDP, had a subunit (M.W. 35,500) structure, and a K_m for PEP of 3.5 mM. However, FDP activation, caused the second species to operate in the same physiological range as the first.

A G200-sephadex column elution profile indicated that there is a possibility that the two species formed "hybrid" enzymes of mixed subunits.

ABBREVIATIONS

The following abbreviations are used throughout this thesis:

Adenosine 3',5'-cyclic monophosphate	cyclic AMP
Adenosine 5'-diphosphate	ADP
Adenosine 5'-triphosphate	ATP
Adenosine 5'-monophosphate	AMP
Diethylaminoethyl	DEAE
Ethylenediaminetetraacetic acid	EDTA
Fructose 1,6-diphosphate	FDP
Glucose-6-phosphate	G-6-P
N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid	HEPES
Lactate dehydrogenase type III	LDH
2-(N-morpholino) ethanesulfonic acid	MES
Nicotinamide adenine dinucleotide, reduced	NADH
Nicotinamide adenine dinucleotide phosphate	NADP
Phosphoenol pyruvate acid	PEP
Tris(hydroxymethyl)aminomethane	Tris

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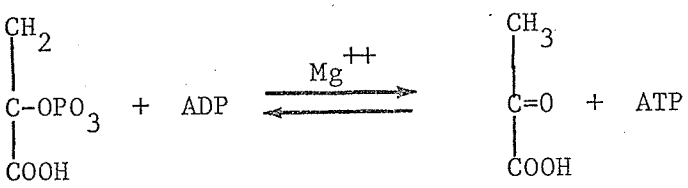
INTRODUCTION

In 1934, the Embden-Meyerhof pathway for the metabolism of glucose was completely resolved by the discovery of an intermediate, phosphoenol-pyruvate (PEP) by Lohmann and Meyerhof (1934) and by Parnas et al (1934). This compound is now known to be a substrate for the enzyme pyruvate kinase (EC2.7.1.40) which catalyses the following reaction:



PEP Pyruvate

The enzyme found in animal systems requires K+ for full activity (Boyer et al 1942, 1943).

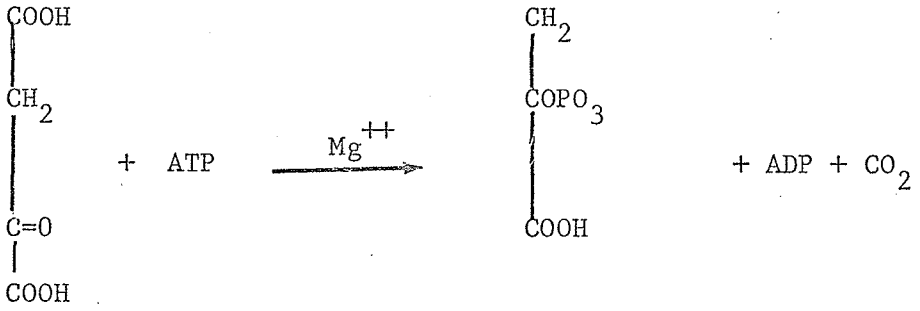
Earlier studies with pyruvate kinase were misleading in that the cell extracts used contained adenylate kinase, and led to the belief that pyruvate kinase caused the conversion of AMP to ATP. Such findings were reported by Lehmann (1935), Lutwak-Mann and Mann (1935), and by Needham and Van Heyningen (1935).

The discovery of adenylate kinase in 1943 by Colowick and Kalckar led to the understanding of the identify of pyruvate kinases's substrates as accepted today.

In 1945, Lardy and Ziegler demonstrated the reverse reaction which is the conversion of pyruvate and ATP to PEP and ADP. Further equilibrium studies by Meyerhof and Oesper (1949), McQuate and Utter (1959), and by Krimsky (1959), showed that the reaction was very much favoured towards the formation of pyruvate, and that it could be considered to be essentially irreversible.

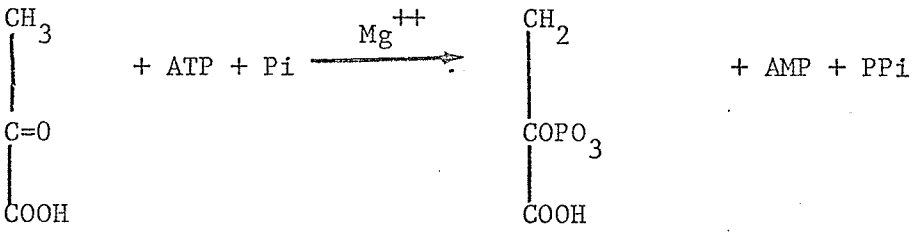
The discovery of anaplerotic pathways within cells of different organisms, have given additional weight to the view that pyruvate kinase is physiologically irreversible. Some anaplerotic pathways give alternative routes for the formation of PEP from pyruvate or intermediates of the tricarboxylic acid cycle, and thus allowing for the reversal of the Embden-Meyerhof pathway during gluconeogenesis. These anaplerotic pathways are more acceptable as routes to PEP synthesis than the reversal of pyruvate kinase in the light of thermodynamic considerations. The physiological importance and the history of the discovery of these anaplerotic pathways are brought to prominence in a review by Kornberg (1966).

Specifically, in Escherchia coli, two enzymes are known to form PEP in order to facilitate gluconeogenesis. These are phosphoenolpyruvate carboxykinase (EC4.1.1.32).



Oxaloacetate PEP

and PEP synthase



Pyruvate PEP

However, because pyruvate kinase has an equilibrium constant which favors pyruvate formation, the PEP formed by these anaplerotic enzymes, would be immediately converted to pyruvate, if there was not some form of control of the pyruvate kinase activity.

In animals the problem of control is solved in part by having different locations for different cellular functions. They are able to compartmentalize many metabolic functions within mitochondria, or they have developed specialized cells, with specialized enzyme compositions.

Pyruvatekinase was first isolated in crystalline form from rat muscle by Negelein during the early 1940's (Bucher and Pfleiderer, 1955). Since that time many crystalline preparations have been obtained from muscle tissues of different animals (Boyer et al, 1962). Muscles of animals are tissues which are not concerned with gluconeogenesis, but are only concerned metabolically, with the production of energy. The pyruvatekinase of muscle has been found to have no controlling features except product inhibition, notably by ATP. This enzyme is designated PyK M by Tanaka et al (1965 and 1967a). In the gluconeogenic tissue of the liver, Tanaka et al 1965 and 1967a) have found another pyruvate kinase (PyKL), which is modulated in its activity by FDP. This modulation by FDP has also been found in unicellular organisms in which cell differentiation is not possible, and in the case of bacteria, where compartmentalization does not exist. Yeast pyruvate kinase has been shown by Hess et al (1966) to be modulated by FDP, and Escherichia coli pyruvate kinase has been shown to be modulated by both FDP and AMP (Maeba and Sanwal, 1968). A report by Malcovati and Kornberg (1969), confirms the modulation of pyruvate kinase from Escherichia coli by FDP, but disagrees with the modulation by AMP. They instead tentatively propose the existance of two pyruvatekinases which are similar

to PyKM and PyKL found in animals.

In the last few years, much work has been reported on PyKL. Reports by Tanaka et al (1967b), Carminatti et al (1969), and Rozengurt et al (1969), consider the behaviour of PyKL in the presence of 1 mM dithiothreitol. They essentially showed an enzyme which yielded a sigmoidal curve when initial velocities were plotted against PEP concentrations. The sigmoidicity was dependent upon pH (Carminatti et al), and could be almost converted to a hyperbolic plot, at the pH optimum. FDP caused activation to produce a hyperbolic plot.

In contrast reports by Taylor and Bailey (1967), and Bailey et al (1968), showed very different kinetic properties for PyKL. These enzyme preparations required preincubation with PEP before the results would match those reported by the forementioned workers. Without preincubation, the initial velocity plot against PEP concentration was peculiar in shape, and did not show regular allosteric behaviour. The only major difference in the enzyme preparations between the two groups of reports was that Taylor and Bailey did not use dithiothreitol. This same difference occurs, along with different pHs for assay system, between the work of Maeba and Sanwal (1968) and Malcovati and Kornberg (1969). It would be pertinent to point out, that Maeba and Sanwal present some evidence to indicate that dithiothreitol was responsible for protein molecular weight changes which were correlated with loss of activity of pyruvate kinase.

Bailey et al (1968) have proposed two forms of PyKL, one which obeys, Michaelis-Menten kinetics LA, and another which is allosteric, LB. The idea that there are two forms of PyKL is supported by Susor and Rutter (1968), who postulate a FDP sensitive and FDP nonsensitive form of PyKL. Tanaka (1967b) also obtained a FDP nonsensitive PyKL.

Rozengurt et al (1969) interpret their results on the basis of the Monod, Wyman and Changeux (1965) model (MWC model), and postulate that pH can cause a conversion of the R state to the T state. Bailey et al (1968) also use the MWC model for interpretation, but it should be noted that they can also be interpreted by the theory of negative cooperativity proposed by Levitzki and Koshland (1969) as an extension of the model for allosterism proposed by Koshland et al (1966).

Tanaka et al (1965) in his initial paper, showed that PyKL from rat liver could be resolved into three separate protein peaks by zone electrophoresis. He called these three portions L_1, L_2, L_3. The crystalline enzyme studied by his group was solely type L_1. Thus the possibility that there are a number of different forms or completely different enzymes of PyKL appears to be well indicated.

In view of the behaviour of animal enzymes, it seems possible that more than one form of pyruvate kinase exists in Escherichia coli and the conflicting evidence of Maeba and Sanwal (1968) and of Malcovati and Kornberg (1969) could have its origins in the multiplicity of enzymes and their control characteristics.

The aim of the work that follows was to study the kinetics and control parameters of pyruvate kinase from Escherichia coli and to determine the molecular weight of the enzyme or enzymes.

MATERIALS AND METHODS

Organism

The organism used in this investigation was Escherichia coli K12 strain F3000.

Growth of Organism

The organism Escherichia coli K12 was maintained on slants of a medium (M.L.A.) with the following composition: 10g Bacto tryptone, 5 g yeast extract, 10 g NaCl, 1 ml IN NaOH, 11 g agar, added to one litre of distilled water. Starter cultures of the organism were grown in 5 ml of the above medium without agar and with a 0.5% glucose supplement which was autoclaved separately. The 5 ml aliquots were contained in 25 x 150 mm test tubes which were placed for 24 hrs on a slanted rack in a New Brunswick reciprocal shaker water bath, maintained at 37°C.

The medium (minimal salt A) used for growth of Escherichia coli K12 in larger quantities had the following composition: 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1.5 g $(NH_4)_2SO_4$, 0.05 g $MgCl_2$ SO_4, 0.5% glycerol added to one litre of distilled water. In one experiment 0.4% glucose, which was autoclaved separately, was used in place of the glycerol.

The 5 ml starter cultures were used as inoculums for 50 ml aliquots in 250 ml erlenmeyer flasks. After 24 hrs on a rotary shaker at 37°C, these 50 ml aliquots were used as inoculums for one litre amounts in 2800 ml Fernbach flasks, which were also placed on a rotary shaker for 24 hrs at 37°C. These one litre cultures were used as inoculums for 15 litre carbouys, aerated by sparges, and grown for 36 hrs at 37°C.

The cultures were checked for contamination by gram staining and by streaking M.L.A. agar plate with the cultures and placing drops

bacteriophage suspension on the streak. The resulting clear plaque indicated a pure culture as T2 bacteriophage is specific for Escherichia coli.

Harvesting and Storage of Cells

Cells from the carbouys were harvested by a Sharples steam-driven centrifuge operated at 50,000 r.p.m. The packed cells were weighed and washed in 0.05 M Tris-HCl pH 7.5 buffer with 1 mM EDTA. The suspension was then centrifuged in a refrigerated Sorvall RC2B centrifuge at 16000 g for 15 mins. The pellets of cells were re-suspended in a volume (ml) equal to the weight (mg) of the cells. The resulting homogenous suspension was stored in 100 ml amounts at $-30^{o}C$.

Purification of Pyruvate Kinase

The frozen 100 ml amounts of the cell suspension, containing approximately 50 g of cells, were thawed in a refrigerator, $4^{o}C$, overnight, and then stirred into an homogenous suspension with the addition of 50 ml of the forementioned buffer. Dithiothreitol was added to this suspension to a concentration of 1 mM. All purification procedures were carried out at $4^{o}C$, or in an ice bucket and the buffer used was always 0.05 M Tris HCl pH 7.5 with 1 mM EDTA and 1 mM dithiothreitol unless specifically indicated to the contrary.

Two 75 ml aliquots were then sonicated in a 10 Kc/sec Raytheon ultrasonic oscillator for 1/2 hr each to break open the cells. The combined sonicated suspensions were then centrifuged at 48,000 g for 15 min in a refrigerated Sorvall KC2B centrifuge, and the resulting pellets were discarded. These above conditions for centrifugation were used throughout the purification procedure unless specifically

mentioned to the contrary.

To the supernatant volume, V, V/5 ml of a 2% protamine sulphate solution was added, and the resulting solution was stirred for 15 min. The solution was then centrifuged for 10 min, and the pellets were discarded.

Ammonium sulphate was slowly added to the supernatant to 0.3 saturation, and the solution was stirred for 15 min, followed by centrifugation for 10 min. The pellets were discarded. More $(NH_4)_2SO_4$ was added until the supernatant reached 0.55 saturation. This solution was stirred for 1/2 hr, and then centrifuged for 20 min. The resulting pellet was resuspended in the volume of which was half the volume of the last supernatant.

With 10% acetic acid the pH of the suspension was lowered to 5.5, stirred for 5 min, and then centrifuged for 10 min. The pellets were discarded. The solution was then further adjusted by dropwise addition of 10% acetic acid to pH 5.1, and immediately centrifuged for 10 min. The pellets were discarded and the supernatant was adjusted to pH 7.0 by the addition of IN NaOH.

The protein was precipitated out of solution by addition of $(NH_4)_2SO_4$ to 0.75 saturation and stirred for 1/2 hr. The solution was centrifuged for 20 min and the pellets were resuspended in 5 to 10 ml of the buffer. This suspension was dialysed for 6 hrs against one litre of the same buffer.

A DEAE - cellulose column (2.5 x 45 cm) was equilibrated with 500 ml of the buffer which had 2 mM dithiothreitol. The dialysed suspension was then placed on top of the column, and was eluted with a 1000 ml ionic gradient of 0 to 0.3 M KCl, 10 ml fractions were collected, and those fractions showing pyruvate kinase

activity greater than 0.5 units/ml were combined. The protein was then precipitated out of solution by the addition of $(NH_4)_2SO_4$ to 0.75 saturation, and stirred for 1/2 hr. The solution was centrifuged for 20 mins and the pellets were resuspended in 2-3 ml of the buffer with 10 mM dithiothreitol. The suspension was then dialysed against 500 ml of the same buffer for 6 hrs.

An A50 DEAE - sephadex column (2.5 x 45 cm) was equilibrated with 500 ml of buffer with 10 mM dithiothreitol and 0.1 M KCl. The dialysed suspension was introduced to the column, and eluted by a 500 ml ionic gradient from 0.1 to 0.4 M KCl. Three ml fractions were collected, and those showing pyruvate kinase activity greater than 0.25 units/ml were combined. KCl was added to this combined eluant until the concentration was approximately 0.5 M.

After a protein determination by the method of Lowry, alumina C_γ suspended in the buffer, was added to the eluant such that there was 1 mg solid alumina C_γ to 1 mg of protein. The resulting suspension was stirred for 15 mins and then centrifuged at 12000 g for 10 mins. The enzyme pyruvate kinase was then eluted from the gel by three washings, each stirred for 30 mins and then centrifuged as above, with 20 ml of 0.05 M MES pH 6.5 with 0.1 M EDTA and 10 mM dithiothreitol. The protein was then precipitated from the solution by the addition of $(NH_4)_2SO_4$ to 0.75 saturation and stirred for 30 mins. After centrifugation for 20 mins, the pellets were resuspended in 2 mls of 0.05 M MES pH buffer with 10 mM dithiothreitol, and dialysed against 500 ml of the same buffer overnight.

This preparation was then dispensed into test tubes in 0.5 ml aliquots and stored at $-30^{o}C$. These were used for kinetic experiments after being resuspended in 9.5 ml of the 0.05 MES pH 6.5

buffer with 10 mM dithiothreitol.

Further purification could be obtained by passing the protein suspension through a G200 sephadex column (2.5 x 100 cm). This column was equilibrated with the buffer mentioned above. 1.5 ml fractions were collected and those fractions showing pyruvate kinase activity greater than 0.25 units/ml were combined, and the protein precipitated by the addition of $(NH_4)_2SO_4$ to 0.75 saturation with 1/2 hr stirring. After centrifugation for 20 mins the pellets were resuspended in 2 mls of the MES buffer, and dialysed overnight against 500 ml of the same buffer.

Measurement of Pyruvate Kinase Activity

1. For purification steps the following assay system was used: 0.2 mM PEP, 1.25 mM ADP, 10 mM $MgCl_2$, 0.15 mM NADH, 50 µg LDH, and 0.03 M HEPES pH 7.0 buffer to bring the total volume to 3 ml. The reaction was started by the addition of pyruvate kinase into the 3 ml cuvette with 1 cm light path. The oxidation of NADH at 20°C was followed at 340 mµ using an Unicam SP500 spectrophotometer connected with a SP22 recorder.

2. For all kinetic experiments except pyruvate inhibition, the assay system described above was used. When PEP was required to be saturating, 4.0 mM concentratinn was used. Kinetic reactions were measured by a Gilford model 2000 optical density converter connected to a Beckman D.U. monochromator and a 25 cm self-balancing nerve recorded with a multiple chart drive.

3. For pyruvate inhibition of pyruvate kinase activity the following assay was used; 4.0 mM PEP, 1.25 mM ADP, 10 mM $MgCl_2$, 1 mg Hexokinase, 30 mM glucose, 0.2 mM NADP, 27 µg G-6-P dehydrogenase with 0.03 M HEPES pH 7.0 buffer to bring the volume to 3 ml. The reduction of NADP was followed at 340 mµ on the same spectrophotometer as above. PEP and ADP were varied as required.

4. For reactivation experiments the assay used was an in 1. except that the PEP concentration was 2 mM and the measurement of activity was carried out on the Gilford assembly.

Definition of Activity

1 unit of pyruvate kinase activity ≡ the oxidation of 1 mole NADH min/ml, for the assay employing NADH and LDH.

1 unit of pyruvate kinase activity ≡ the reduction of 1 μmole NADP/min/ml, for the assay employing NADP, G-6-P dehydrogenase, hexokinase, and glucose.

Specific activity ≡ units/min/mg protein

Protein Determinations

Protein concentration was determined by the Folin-Ciocalteau reagent (Lowry et al, 1951) using crystalline serum albumin as the standard.

Preparation of DEAE - cellulose and A50 DEAE-sephadex

DEAE-cellulose was allowed to stand overnight in IN NaOH, after several hours of stirring. The liquid was then decanted and fresh IN NaOH wasaadded. This was stirred for 1/2 hr, and after standing for approximately 1 hr the liquid was again decanted. The DEAE-cellulose was then repeatedly washed in distilled water until the pH was less than 8.0, after which DEAE-cellulose was washed by a large volume of 0.05 M Tris-HCl pH 7.5 buffer with 1 mM EDTA. This was decanted and the DEAE-cellulose was resuspended in a small volume of the same buffer and stored at $4^{o}C$ until needed.

A50 DEAE-sephadex was prepared by a similar method except that 0.5 N NaOH was used, and the buffer used to suspend it in, contained 0.1 M KCl.

Preparation of G-200 Sephadex

G-200 sephadex was allowed to swell in a large volume of distilled water for 3 to 5 days, the distilled water being changed often. The G-200 sephadex was equilibrated with the buffer to be used after the column was packed.

Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis was carried out according to the method of Davis (1964), using a Canalco-Disc Electrophoresis Trial Kit supplied by Canal Industrial Corporation. Samples of 100 to 200 µg protein were mixed with an equal volume of 40% sucrose to a maximum total volume of 0.4 ml, and were layered on the spacer gel. After the run, the columns were stained with 2% Cromassie Blue for 1-1/2 hrs, then washed with 7% acetic acid, followed by 12 hrs of destaining in 7% acetic acid.

Heat Inactivation

Ten λ of the concentrated 150 fold purified pyruvate kinase was added to 1 ml of 0.05 M HEPES pH 7.0 buffer and heated at 41°C and 50°C for 5 and 10 min periods. The samples were immediately cooled by introduction of the test tubes into melting ice. Fifty λ aliquots were used for the assays. The assay mixture employed 0.2 mM PEP, whilst other reagents were saturating. Assays were also performed with 5.0 mM FDP and 1 mM AMP.

Microsomal Inactivation of Pyruvate Kinase

Microsomes were obtained by the method of Goldberger et al (1963). Equal volumes of 150-fold purified pyruvate kinase, and the microsomal preparation were added together, or one volume of pyruvate kinase to two volumes of the microsomal preparation. Assays used 2 mM PEP.

Reactivation Experiments

The 150 fold purified enzyme was dialyzed against 0.05 M HEPES pH 7.0 buffer for 48 hrs. The buffer used was changed three times over this

period. The approximately 3 μg of enzyme were added to 1 ml of the HEPES buffer, a sulphydryl reducing compound, and any ligand whose effect was to be tested. Fifty λ of this solution was sufficient for assay purposes, using 2 mM PEP.

Initial reactivation studies were carried out without ligands and 1 mM dithiothreitol, 10 mM dithiothreitol, 10 mM β-mercaptoethanol, 100 mM β-mercaptoethanol, 1mM glutathione (reduced), 10 mM glutathione (reduced), 1 mM lipoic acid (reduced), 0.1 mM coenzyme A and 1 mM Coenzyme A.

Reactivation experiments were carried out with 2 mM or 10 mM dithiothreitol. Ligands used were at saturating concentration where known, except PEP which was at 2 mM. Concentrations of NAD and NADH were 1 mM and FDP was 5 mM.

Molecular Weight Studies

The pyruvate kinase used in these studies was obtained from cells grown in glucose. The purification procedure was carried out as far as the DEAE-cellulose column elution. Assays were carried out in the presence of 1 mM FDP and 0.2 mM PEP during purification, and lead to a substantial difference in the elution pattern from DEAE-cellulose column as compared to the normal purification.

A G200-sephadex column (45 x 100 cm) was equilibrated with 0.05 M HEPES pH 7.0. Pyruvate kinase dialysed for 48 hrs was applied without dithiothreitol present. In a second run non-dialysed enzyme was applied in the presence of 5 mM dithiothreitol.

The column was calibrated by use of proteins of known molecular weight: Catalase (MW 225,000), Hexokinase (MW 96,000), Haemoglobin (MW68,000) and Cytochrome C_3 (MW 13,000). The assay systems employed were:

1. For Catalase: pH 7.0, 0.01 M sodium phosphate buffer and 0.1M H_2O_2 whose breakdown was followed at 230 mμ.

2. Hexokinase: 30 mM glucose, 0.2 mM NADP, 27 μg G-6-P dehydrogenase, 10 mM ATP in 0.05 M HEPES pH 7.0 buffer. NADP reduction was followed at 340 mμ.

3. Haemoglobin: O.D. was measured at 410 mμ.

4. Cytochrome C_3: O.D. was measured at 500 mμ.

5. Pyruvate kinase: saturating reagents as previously described except PEP was at 0.2 mM. Separate assays were carried out with 1 mM FDP and 1 mM AMP present as well as without any effectors.

The method and analysis of the results was after the method of Andrews (1963).

RESULTS

Growth Medium

Escherichia coli K12 was grown in 1 litre amounts of minimal salts broth with the carbon supplements shown in Table I. After sonication and centrifugation, it was established that extracts obtained from cells grown on 0.5% glycerol had the highest specific activity for pyruvate kinase. This carbon supplement was, therefore, used for growing cells in larger quantities.

The high specific activity obtained from cells grown in 1 litre amounts was not obtained from cells grown in 0.5% glycerol in carbouy amounts. The specific activity of such cells can be seen in Table II.

Purification

Pyruvate kinase was routinely purified 100 to 150 times as shown in Table II. With the inclusion of a G-200 sephadex column step the enzyme was purified 200 to 250 times, but only with the loss of the majority of the enzyme. The purest preparation obtained showed 5 bands on polyacrylamide gel after disc electrophoresis.

Other purification methods were attempted. Ethanol precipitation between 30% and 50% duplicated the ammonium sulphate fractionation and would give no further purification. Calcium phosphate gel would give a small amount of purification, but various attempts using different concentrations of Tris-HCl buffer, ammonium sulphate, and EDTA did not yield sufficient recovery to make its use worthwhile. Hydroxylapatite mixed with 30% cellulose, was used in a column after the G-200 sephadex step However, an ionic gradient up to 2 M KCl failed to elute any enzyme from the column. Pyruvate kinase proved to be heat labile, when heat precipi-

tation was tried. Reverse ammonium sulphate precipitated was tried however, pyruvate kinase would lose activity very significantly when exposed to high ammonium sulphate concentration for some time. The activity was regained after precipitation out of ammonium sulphate solution. This feature made the determination of the exact precipitation and dissolving levels difficult. Some of the pyruvate kinase was found to respond in 30% ammonium sulphate, but with no activity apparent, whilst the bulk of the enzyme required 20% ammonium sulphate for resuspension. This solution would show some activity, but more was apparent upon precipitation from the ammonium sulphate solution. This result can be explained by the subunit structure of pyruvate kinase to be shown later.

The ability of pyruvate kinase to survive was dependent upon two factors. The first was dithiothreitol, whose concentration had to be increased with increasing purity of the enzyme. The second was protein concentration. If the final preparation of the enzyme after the alumina Cγ step was frozen as a diluted (i.e. 10 ml suspension), the enzyme was destroyed. As a 0.5 ml aliquot it would survive with full activity for up to one month. Longer storage was not attempted. The enzyme would lose about 20% of its activity per day when left at $4^{o}C$. This was apparent when prolonged dialysis was attempted, and was a major factor along with dilution of the protein in the poor recovery obtained from the slow running G-200 sephadex column. The purification of the enzyme as shown in Table II was completed in three days to the end of the alumina Cγ step. This meant that 50% of the enzyme would have been lost before any purification steps could be taken into consideration. For examples of prolonged dialysis see Table III.

Kinetic Results

These results were obtained using 150 fold purified pyruvate kinase in solution containing 10 mM dithiothreitol. Evidence presented later indicates that pyruvate kinase under such conditions would have been in a tetrameric, AMP-activated form. Whether or not all of the enzyme present was in a tetrameric form is uncertain.

(a) Saturation Values.

The saturating values employed for the substrates PEP, ADP and $MgCl_2$ were 4.0 mM, 1.25 mM and 10 mM respectively.

(b) Enzyme Concentration

Inspection of Fig. 1 shows the relationship between protein concentration and pyruvate kinase activity. For all the kinetic experiments 50 λ and 100λ aliquots were used. Variation in the maximum velocity rates from one experiment to another depended upon how long the enzyme preparation had been diluted, and unfrozen.

(c) Buffer Effects

For the determination of the optimum pH various buffers were used. Pyruvate kinase showed no activity in 0.05 M sodium phosphate (Sorensens) buffer and none in 0.05 M citrate-phosphate buffer. A fine precipitate appeared upon the addition of the enzyme.

At pH values greater than 7.5, both 0.05 M HEPES and 0.05 M Bicine buffers showed comparable activities, whilst 0.05 M Tris-HCl showed approximately half of the activity found with HEPES. For acid pH values 0.05 M MES and 0.05 M sodium cacodylate had comparable activities.

For the determination of pH curve the following buffers were used:

pH 5.0 ⟶ pH 7.0	0.05 M MES
pH 7.0 ⟶ pH 8.5	0.05 M HEPES
pH 8.5 ⟶ pH 9.5	0.05 M Bicine

(d) pH Optimum

Pyruvate kinase showed optimum activity at pH 6.2 - 6.3, which was not in agreement with a previous report (Maeba, 1968). The optimum did not shift with varying values of PEP, and was the same when $MnCl_2$ replaced $MgCl_2$. The shape of the curve for $MnCl_2$ was slightly different showing better relative activity at alkaline pH (Fig. 2). There was no activity at pH 5.0.

(e) Variation of Kinetic Properties with pH

The allosteric nature of pyruvate kinase was markedly changed by pH. At pH 6.3, the activity of pyruvate kinase plotted against PEP concentration yielded a near hyperbolic curve. As the pH changed away from the optimum the allosteric nature became more pronounced. This facet is illustrated by Hill plots in Fig. 3.

The following kinetic results were obtained from experiments carried out at pH 7.0 unless specifically mentioned to the contrary.

(f) Substrate Kinetics

From initial velocity studies PEP yielded sigmoidal plots (Fig. 4) with an approximate Km value of 0.22 mM at pH 7.0. At pH 6.3, the Km value was approximately 0.12 mM. The pyruvate kinase activity in the presence of saturating PEP, but variable ADP yielded Michaelis-Menten hyperbolic plots (Fig. 5). At saturating PEP (4.0 mM), the K_{ADP} value is 0.1 mM. The double reciprocal plots, Fig. 6 and Fig. 7, show the relationship between pyruvate kinase activity and $MgCl_2$ concentration. When both PEP and ADP are saturating Km for Mg is 1.55 mM.

$MnCl_2$ could replace $MgCl_2$, but the results obtained were quantitatively different. The approximate Km for PEP was 0.02 mM. In Fig. 8 it can be seen that $MnCl_2$ and $MgCl_2$ appear to be competative with each other. $MnCl_2$ appeared to be "preferred" by the enzyme, as the shape of the curve obtained when the Mg^{++}: Mn^{++} ratio is 1:1, was very close to the curve obtained when only $MnCl_2$ was present. When using $MnCl_2$ the activity of pyruvate kinase was higher at low PEP concentrations but the Vmax was only one third of the Vmax for $MgCl_2$. Thus $MnCl_2$ appears to be an inhibitor of pyruvate kinase's potential activity. When PEP and ADP were saturating, a double reciprocal plot (Fig. 9) showed that the Km for $MnCl_2$ is 2.5 mM, although at $MnCl_2$ concentration less than 0.5 mM, the line does not remain linear.

(g) Product Inhibition

High levels of pyruvate acid were found necessary for inhibition of pyruvate kinase activity. When PEP is saturating, levels of pyruvate acid 5 mM, 10 mM and 20 mM were required to produce Fig. 10. When ADP was saturating 20 mM pyruvate acid would produce only 25% inhibition when the PEP concentration was 0.2 mM. The affect of 5 mM and 10 mM pyruvate acid was correspondingly less.

When ADP was saturating, 2 mM ATP would produce 50% inhibition at 0.2 mM PEP concentration (Fig. 11). However, when PEP was saturating, higher concentrations of ATP were required to produce significant inhibition. It was found that commercial preparations of ATP contained sufficient ADP to produce great inaccuracies when low ADP concentrations were used in the reaction mixture. As any data obtained would be of dubious worth, no plots are presented.

(h) Activation of Pyruvate Kinase

Pyruvate kinase as reported by Maeba (1968) was activated by FDP and

AMP. The apparent conflicting evidence by Malcovati and Kornberg (1969) can be explained by consideration of pH and enzyme species present.

AMP was shown to convert the sigmoidal activity of pyruvatekinase to a regular hyperbola at 1 mM concentration (Fig. 12). Fig. 13 shows that the Km for PEP is 0.11 mM in the presence of 1 mM AMP. These results were obtained at pH 7.0. At pH 6.3 the allosteric nature of pyruvate kinase was practically eliminated, and AMP activation was slight. The approximate Km for pyruvate kinase at pH 6.3 for PEP was 0.12 mM, whilst the Km in the presence of AMP was 0.11 mM.

FDP activation (Fig. 14) did not produce, at any pH, hyperbolic plots, even with 10 mM concentration. The activation did not cause a very great change in the n number obtained from Hill plots (Fig. 15).

When $MnCl_2$ replaced $MgCl_2$ in the assay mixture, similar qualitative results were observed.

Heat Inactivation

Attempts to desensitize pyruvatekinase by heat failed, as the enzymes activity disappeared as quickly as did the loss of activation (Table IV).

Dissociation of PyruvateKinase by Dialysis

An earlier report by Maeba (1968) had indicated that upon dialysis pyruvatekinase lost its activity and its molecular weight was reduced by half. This indicated a subunit structure for the enzyme.

Prolonged dialysis for 48 hrs during this investigation failed to remove completely the pyruvatekinase activity, but did reduce it to approximately 5% of the activity before dialysis.

Reassociation of PyruvateKinase by Dialysis

Incubation of the dialysed pyruvatekinase showed a slow reactivation

by β-mercaptoethanol and dithiothreitol (Fig. 16). Coenzyme A and reduced glutathione failed to reactivate pyruvate kinase.

Incubation of pyruvate kinase with 2 mM and 10 mM dithiothreitol with various legands added, showed that only 1.25 mM ADP and 10 mM $MgCl_2$ together produced any significant effect upon the rate of reactivation. After 4 hrs, the pyruvate kinase activity in the above ADP, $MgCl_2$ and dithiothreitol mixture was only 50% of the activity of the control with only dithiothreitol. Other ligands tried on their own or in combinations with each other were FDP, AMP, ATP, Cyclic AMP, PEP, Pyruvate acid, NADH, NAD. None of these produced any significant effect. Neither FDP nor AMP produced an activation, but instead a slight inhibition.

Microsomal Thiol-Disulphide Interchange Enzyme

Microsomes obtained by the method of Goldberger et al (1963) were added to pyruvate kinase and incubated at $4^{o}C$. The results in Fig. 17 show that only partial inactivation was achieved and that this inactivation would gradually disappear with time.

A50 DEAE-Sephadex Elution Profiles

During the course of purification it was shown that the slow running A50 DEAE-sephadex column required the presence of 10 mM dithiothreitol to stabilize the enzyme. Initially 2mM dithiothreitol had been used, and the elution pattern shown in Fig. 18 was found. If there was no dithiothreitol present, the pattern of elution was as in Fig. 19, and with 10 mM dithiothreitol the pattern followed Fig. 20. The faster flowing DEAE-cellulose column showed the elution pattern as in Fig. 21 in the presence of 2 mM

dithiothreitol. Comparison of Fig. 18, Fig. 19 and Fig. 20 shows that there appears to be present two enzyme species which are dependent upon the concentration of dithiothreitol.

Kinetics of Dialysed Enzyme

These kinetic results were obtained at pH 6.3 and when there was apparently a non-homogeneous enzyme system operating. Fig. 22 and Fig. 23 are the same results, but different scales. The initial activity in the absence of FDP was thought to be due to the presence of the tetrameric species which is activated by AMP. The activity in the presence of 1 mM FDP was practically hyperbolic and similar to the activity of the tetrameric species at pH 6.3. However, the curve was not any more sigmoidal at pH 7.5, which was unlike the tetrameric species, activated by AMP.

After the enzyme suspension was used to obtain the above results, it was incubated for 12 hrs with 20 mM dithiothreitol at $4^{o}C$. Kinetic results with these incubated suspension are shown in Fig. 24 and Fig. 25. It is interesting to note that the Vmax has increased by the amount that the Vmax for the initial activity without effectors increases (i.e. Velocity at 2 mM PEP). When the cell suspension was assay immediately after sonication a similar curve to Fig. 24 is obtained (remembering that adenylate kinase would be present).

Determination of Protomer Number and 'L' Number

In a recent paper by Horn and Bornig (1969), it was shown that by assumption of the Monod, Wyman, and Changeux (1965) model for allosteric enzymes, that it was possible to determine the 'L' number and the 'n' number of an enzyme by plotting the following equation:

$$\text{Log} \left(\frac{\alpha}{Y_S} - \alpha - 1\right) = \text{Log } L' - (N - 1) \log (1 + \alpha)$$

as $\log \left(\frac{\alpha}{Y_S} - \alpha - 1\right)$ against $\log (1 + \alpha)$

where $x = \frac{\text{Substrate concentration}}{\text{Microscopic dissociation constant of the substrate}}$

$Y_S = V/Vmax$

N = protomer number

$L' = L (1 + \beta)^N / (1 + \gamma)^N$

$\beta = \frac{\text{Inhibitor concentration}}{K_I}$

$\gamma = \frac{\text{Activator concentration}}{K_A}$

The resulting plots would give an intercept $\text{Log } (1 + \alpha) = \frac{\text{Log } L'}{(N-1)}$

and a gradient $= -(N-1)$.

Using results obtained here, Fig. 26 shows the plots using the above equation. For A, pyruvate kinase is in 10 mM dithiothreitol, there are noaactivators or inhibitors ($\therefore L = L'$) and the pH is 7.0. The plots would indicate a tetramer is present.

Graph is plotted by using data from graph 22, corrected for residual "tetrameric activity".

The microscopic dissociation constant was considered to be 0.11 mM

for both cases shown as this is the value for AMP activation of the tetrameric form, and also the value that FDP activation approaches with the dialysed enzyme.

The occurence of the number N=5 is strange, but using data from Malcovati and Kornberg (1969), the result is the same.

Molecular Weight Studies

The results of the G200 sephadex column runs were unusual. Pyruvate kinase activity was found the whole length of the column eluant when the column was run with 5 mM dithiothreitol. Assays carried out in the presence of FDP and AMP produced a number of peaks of activity. The peak with greatest activity (10x that of any other) was an FDP activated enzyme with an approximate molecular weight of 140,000. An AMP-activator peak found at approximately 100,000, molecular weight has no FDP activation. Activity without effectors was low when associated with FDP activated peaks, but relatively high when associated with AMP activated peaks. This is in agreement with kinetic results mentioned earlier. In the presence of no dithiothreitol, the only active species found had a molecular weight of 35,500. Maeba and Sanwal (1968) had reported a M.W. species of 50,000. Although this was not found, results of the column in the presence of 5 mM dithiothreitol suggest strongly that this subunit should exist. Table VI shows the approximate molecular weight obtained. There was too much interference from different species to give accurate results. Fig. 27 shows the calibration of the G200 sephadex column.

TABLE I

Carbon Source	Pyruvate Kinase Activity	Protein	Specific Activity
	μ moles NADH/min/ml	mg/ml	units/mg
0.5% glycerol	2.58	36	0.0715
1% glycerol	2.08	75	0.0277
1% succinate	1.84	78	0.0236
0.5% glucose	2.26	64	0.0353
1% glucose	2.49	61	0.0408

TABLE II

PYRUVATE KINASE PURIFICATION

	Step	Volume ml	Total Protein mg	Total Units	Specific Activity	% Yield	Purity
1.	Sonication and centrifugation	140	6440	140	0.0217	-	-
2.	2% Protamine SO_4	160	3200	160	0.0500	100%	2.3
3.	0.3 sat. $(NH_4)_2SO_4$	165	2150	130	0.0600	92%	2.8
4.	0.55 aat. $(NH_4)_2SO_4$	80	1020	110	0.1085	80%	5.0
5.	pH 5.1 precipitation	80	510	105	0.2100	75%	10.0
6.	D.E.A.E. cellulose column eluant	120	165	77	0.8800	55%	40.0
7.	A50-D.E.A.E. sephadex eluant	60	98	56	1.520	40%	70.0
8.	Alumina C_γ eluant precipitated and dialysed	60	28	42	3.26	30%	150.0
9.	G-200 eluant	30	9.3	14	4.34	10%	200.0

TABLE III

Purification Step	Initial Activity units/ml	Final Activity units/ml	Hours	% Loss per 24 hrs.
Dialysis after $(NH_4)_2SO_4$ fractionation	4.2	2.74	48	20%
Dialysis after D.E.A.E.-cellulose column and $(NH_4)_2SO_4$ precipitation	3.55	1.53	60	30%

TABLE IV

HEAT INACTIVATION

	% of Control Activity			
	41°C/5 mins	41°C/10 mins	50°C/5 mins	50°C/10 mins
Normal Assay 0.2 mM PEP	75	58	7	0
+ 5 mM FDP	82.5	70	21	0
+ 1 mM AMP	92	83.5	32	0

TABLE V

G-200 SEPHADEX. CALIBRATED MOLECULAR WEIGHT COLUMN

APPROXIMATE MOLECULAR WEIGHTS OF PEAKS OF PYRUVATE KINASE ACTIVITY

Assayed without Effectors	Assay with 1 mM FDP	Assayed with 1 mM AMP
170,000	140,000	150,000 to 180,000
100,000	70,000 to 85,000	
	35,000	100,000

Fig. 1. The activity of pyruvate kinase in relation to the protein concentration of the 150 fold purified fraction. The assay system used 0.2 mm PEP and the other substrates were saturating. The line (- - -) indicates a possible linear relationship and the line (———) shows the actual relationship.

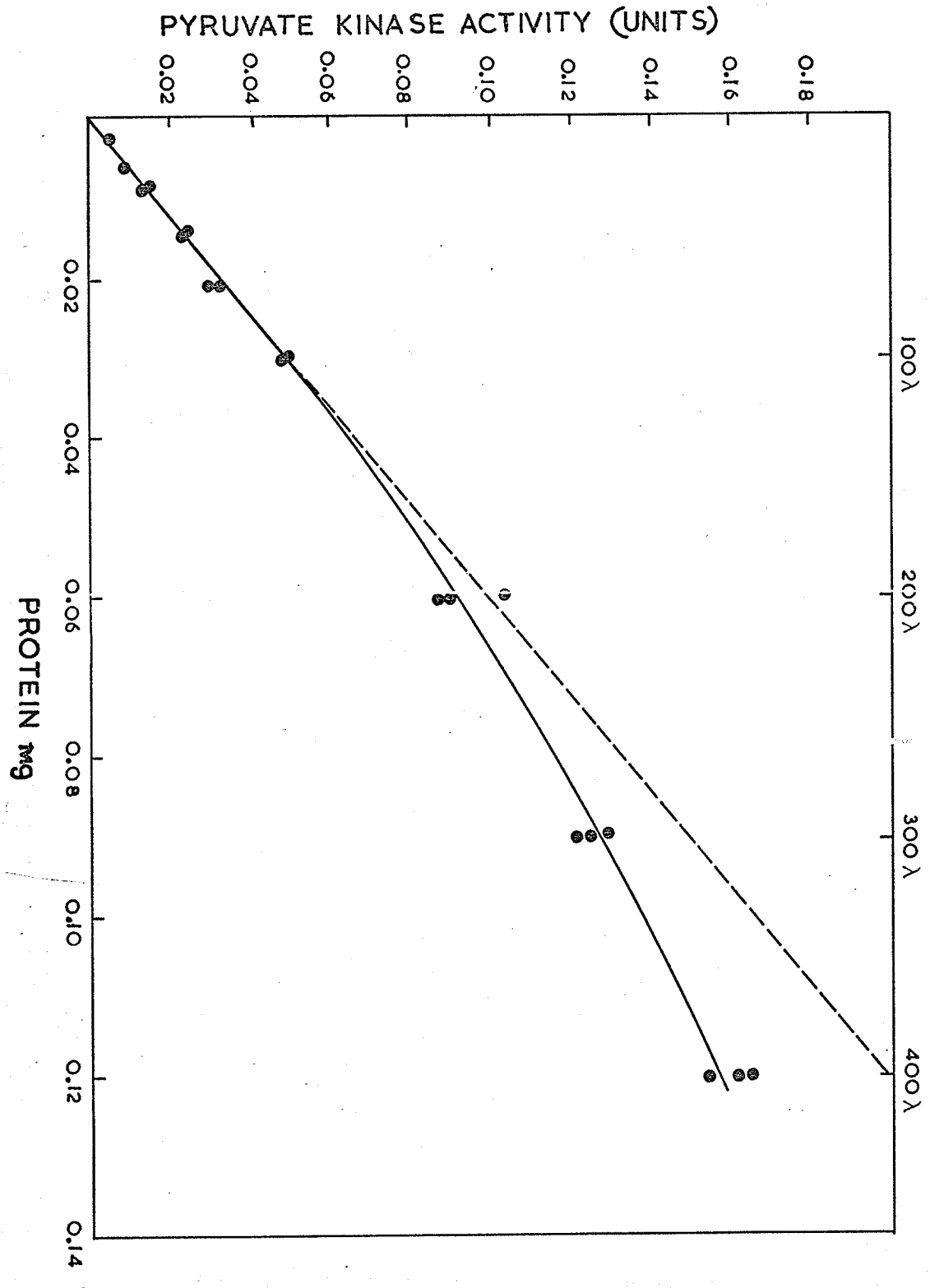

PYRUVATE KINASE ACTIVITY (UNITS)
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
100 λ
200 λ
300 λ
400 λ
PROTEIN mg
0.02
0.04
0.06
0.08
0.10
0.12
0.14

Fig. 2. Pyruvate kinase activity is shown as a function of pH at various PEP concentrations. The optimum pH is 6.2-6.3. The curve marked $MnCl_2$ was obtained using 0.2 mM PEP and 10 mM $MnCl_2$ which replaced $MgCl_2$ in the assay mixture.

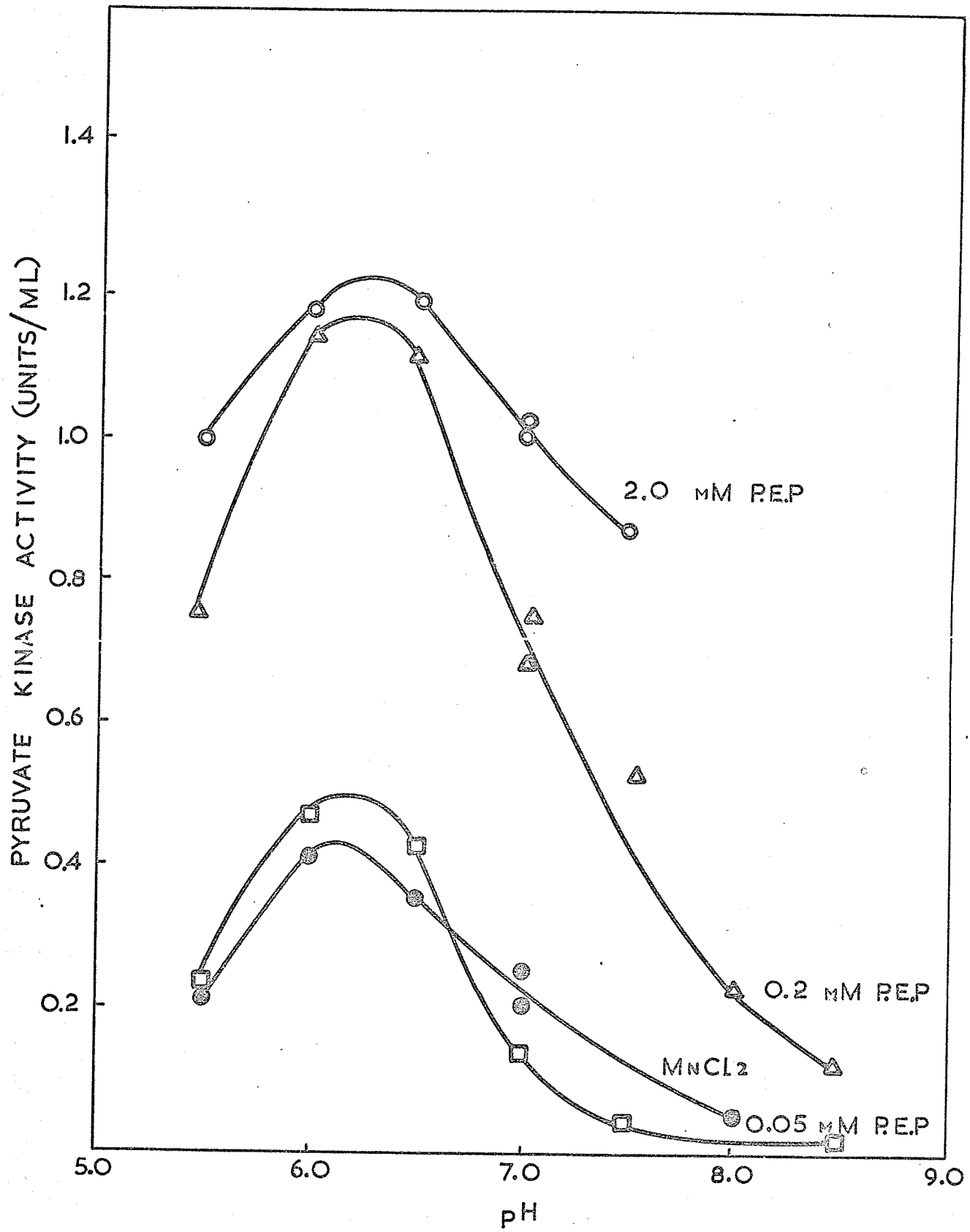

PYRUVATE KINASE ACTIVITY (UNITS/ML)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
5.0
6.0
7.0
8.0
9.0
pH
2.0 mM P.E.P
0.2 mM P.E.P
MnCl2
0.05 mM P.E.P

Fig. 3. Hill plots for pyruvate kinase activity are shown at various pH values. At pH 6.0, (●—●—●) the N value is 1.12, pH 6.3 (○—○—○), the optimum pH has an N value of 1.04. Increasing pH values yield higher N values: pH 6.8 (Δ—Δ—Δ), n=1.52; pH 7.0 from Fig 15, N=2.00; pH 7.5 (x—x—x) N=2.43.

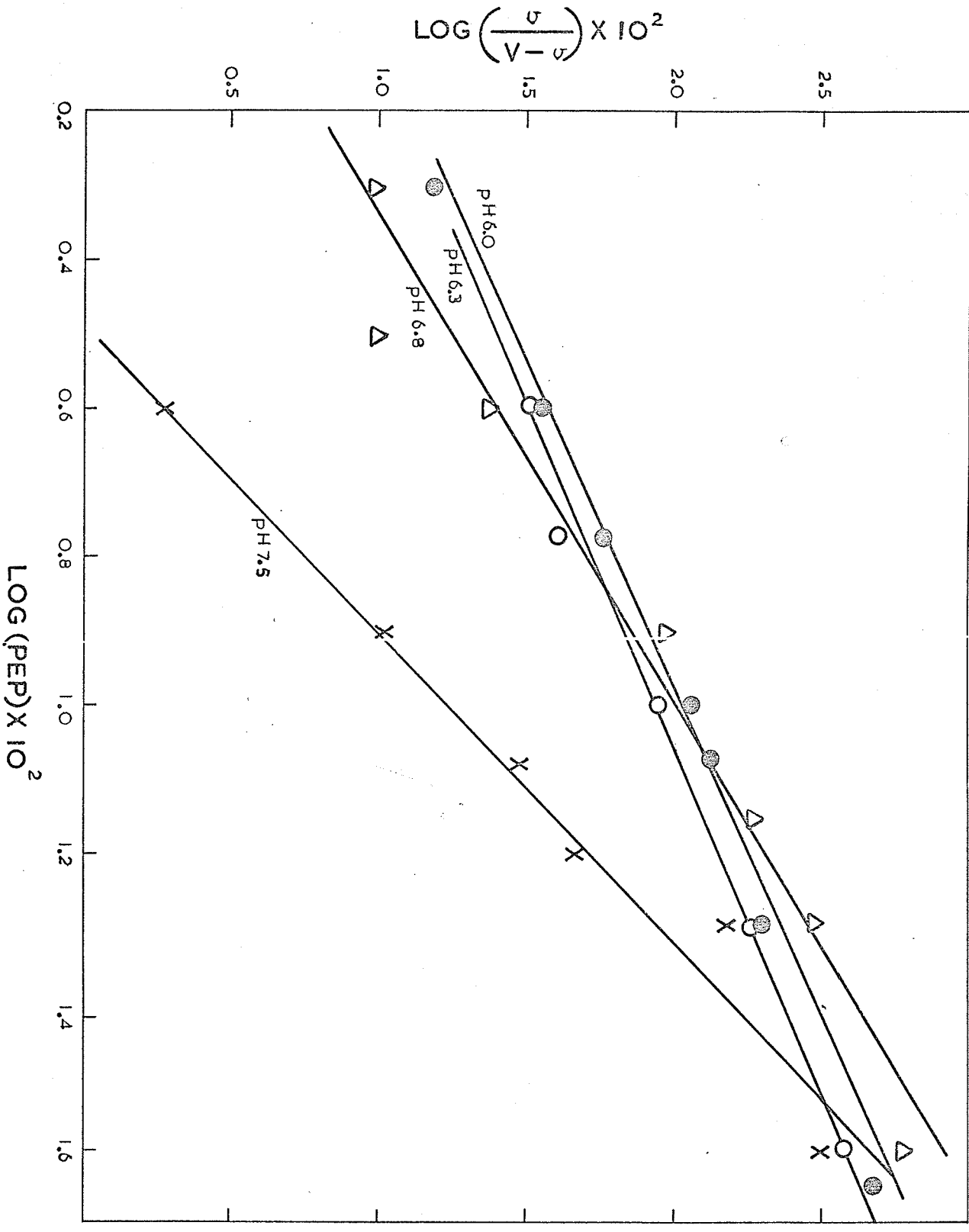

LOG (PEP) X 10²
LOG (v / (V − v)) X 10²
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0.5
1.0
1.5
2.0
2.5
pH 6.0
pH 6.3
pH 6.8
pH 7.5

Fig. 4 Pyruvate kinase activity is shown to be allosteric for PEP. The curves were obtained at various ADP concentrations. These concentrations are: 1.25 mM, 0.625 mM, 0.3125 mM, and 0.156 mM; which correspond to curves 1,2,3 and 4 respectively.

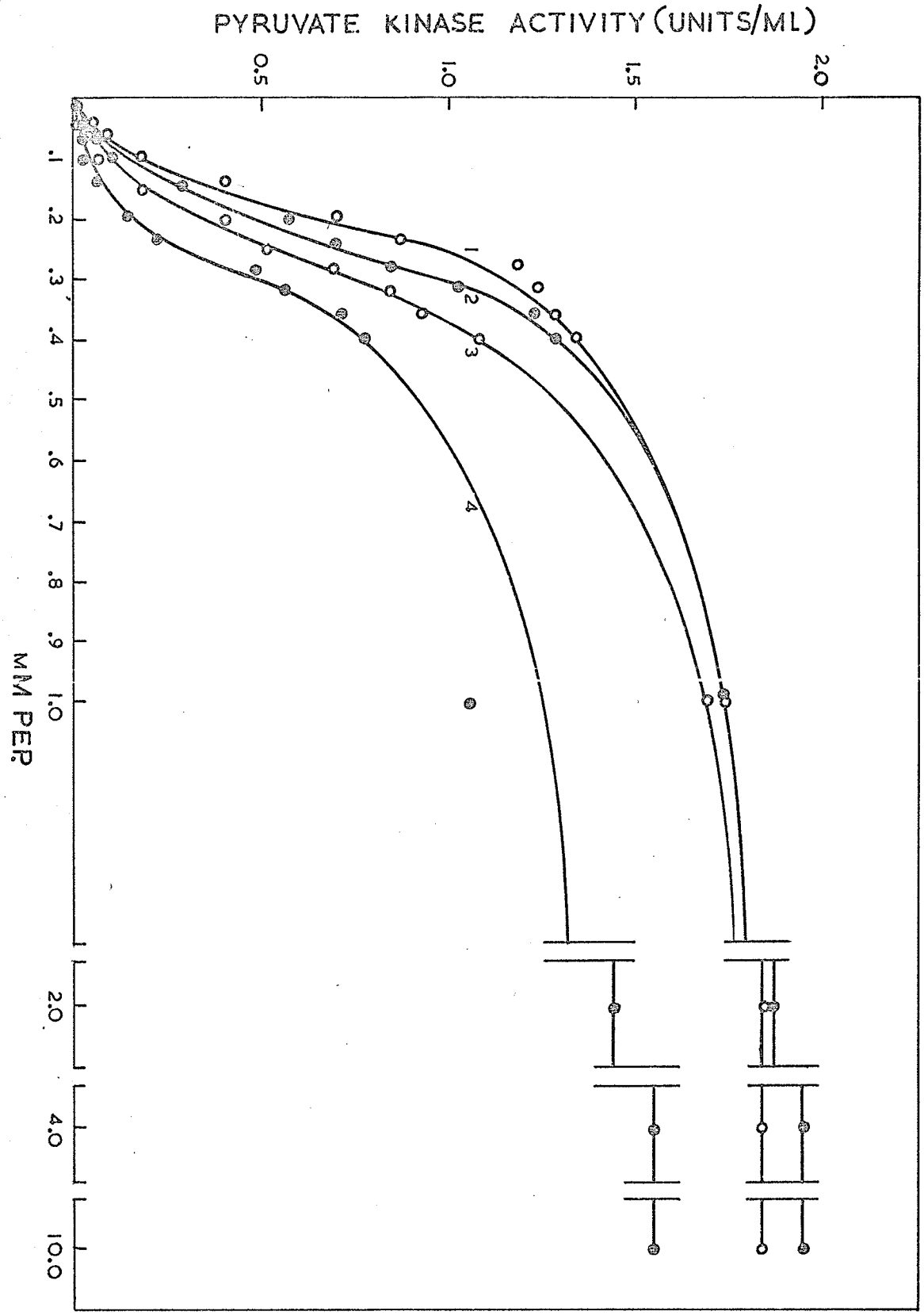

PYRUVATE KINASE ACTIVITY (UNITS/ML)
0.5
1.0
1.5
2.0
MM PEP
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
2.0
4.0
10.0
1
2
3
4

Fig. 5. Michaelis-Menten hyperbolic plots are obtained at various fixed concentration of PEP, and varying ADP.

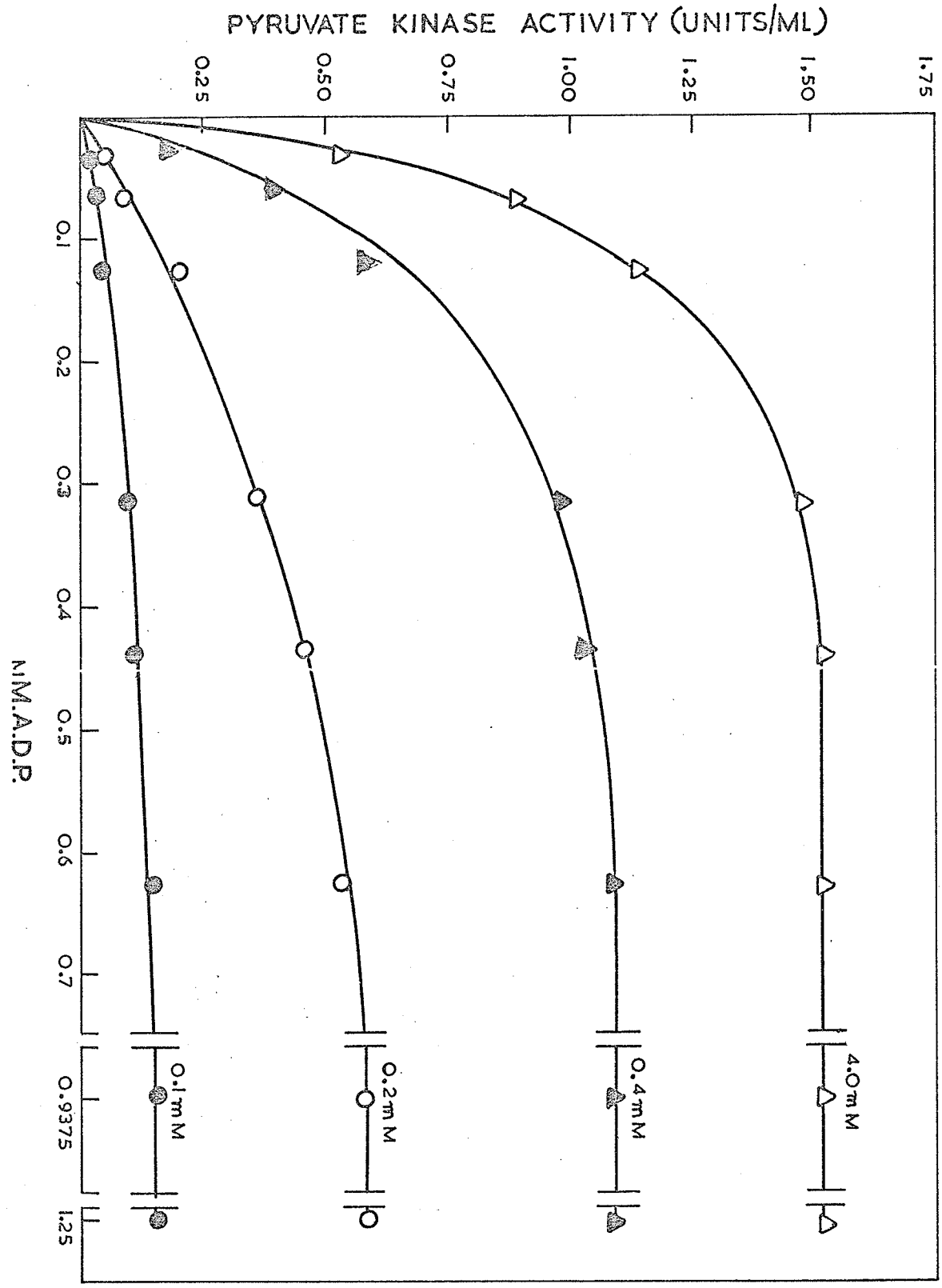

PYRUVATE KINASE ACTIVITY (UNITS/ML)
0.25
0.50
0.75
1.00
1.25
1.50
1.75
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.9375
1.25
MM.A.D.P.
4.0 mM
0.4 mM
0.2 mM
0.1 mM

Fig. 6. ADP is saturating and PEP is at various fixed concentration as indicated. This double reciprocal plot shows a peculiar relationship between pyruvate kinase activity and $MgCl_2$ concentration under the above conditions. At saturating PEP the Km for $MgCl_2$ is 1.55 mM.

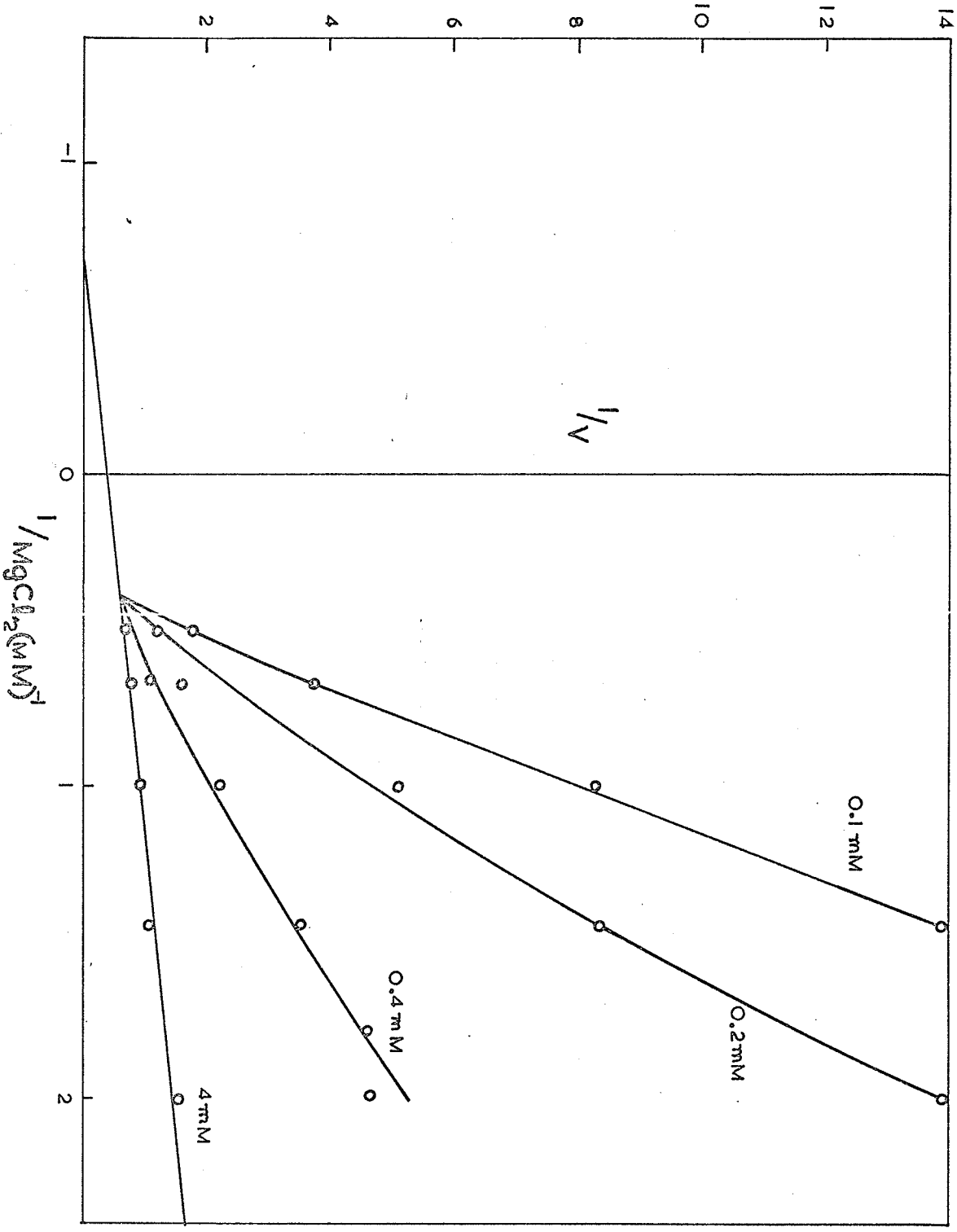

1/V
1/MgCl2(mM)-1
0.1 mM
0.2mM
0.4 mM
4 mM

Fig. 7. PEP is saturating and ADP is used at fixed concentrations as indicated. These are double reciprocal plots of pyruvate kinase activity against $MgCl_2$ concentration.

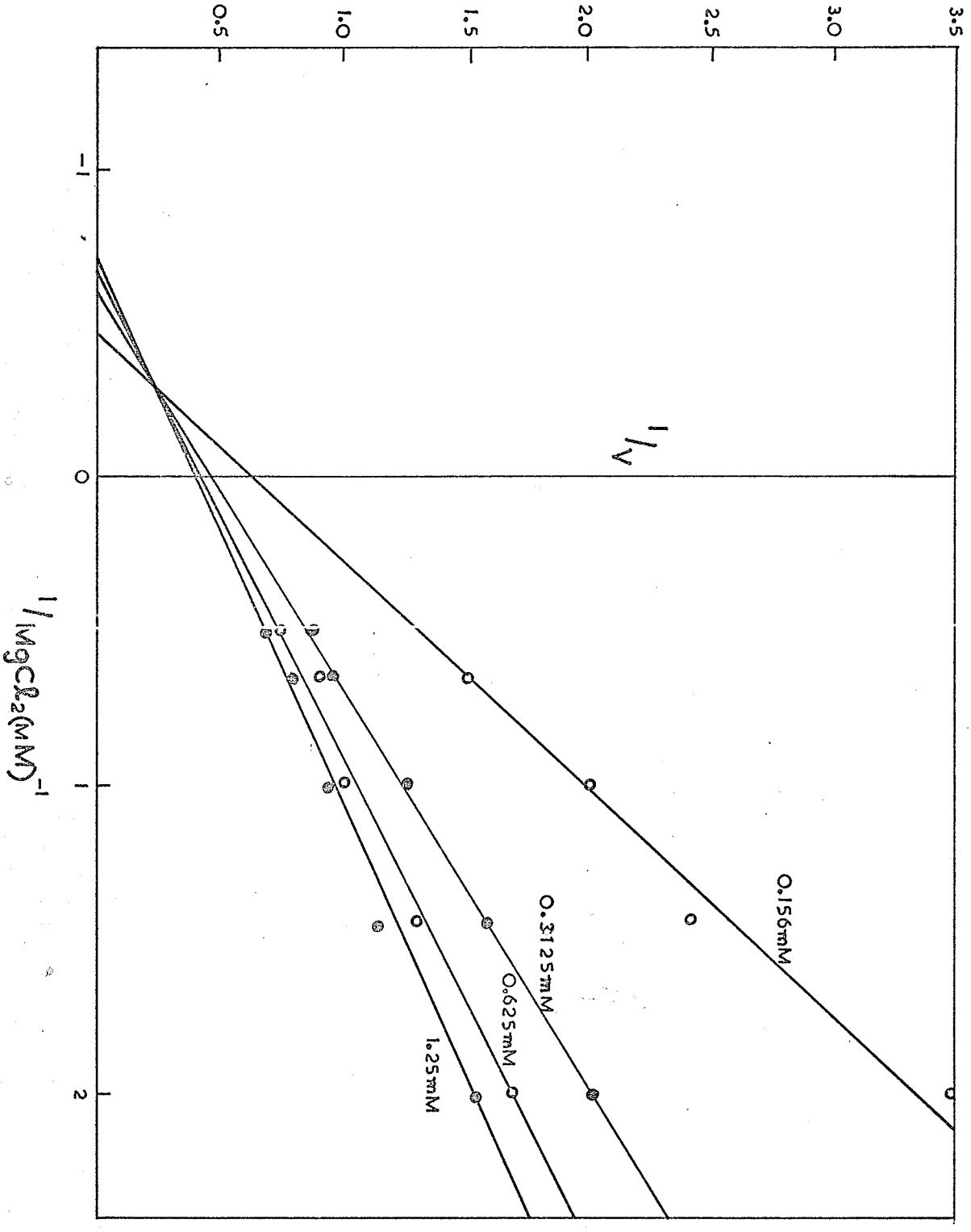

3.5
3.0
2.5
2.0
1.5
1.0
0.5
1/v
-1
0
1
2
1/MgCl2(mM)-1
0.156mM
0.3125mM
0.625mM
1.25mM

Fig. 8. Pyruvatekinase activity is shown with variable PEP concentration at fixed concentrations of $MnCl_2$ and $MgCl_2$: (●—●—●) is 10 mM $MgCl_2$; (0—0—0) is 10 mM $MgCl_2$ and 2 mM $MnCl_2$; (Δ—Δ—Δ) is 10 mM $MnCl_2$; (X—X—X) is 5 mM $MgCl_2$ and 5 mM $MnCl_2$.

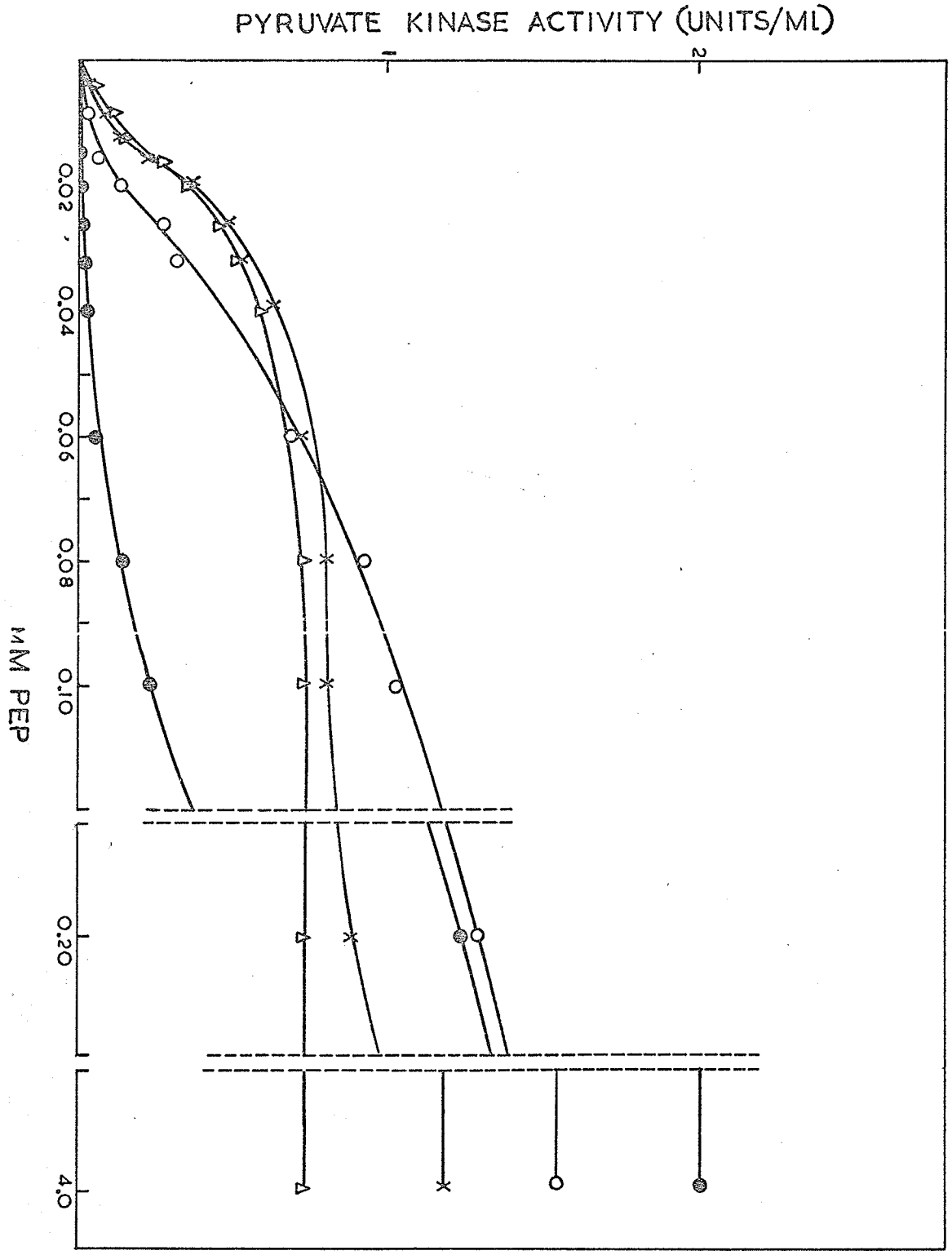

PYRUVATE KINASE ACTIVITY (UNITS/ML)
1
2
0.02
0.04
0.06
0.08
0.10
0.20
4.0
MM PEP

Fig. 9. PEP and ADP are saturating. The double reciprocal plot for pyruvate kinase activity against $MnCl_2$ concentration yields a plot which is linear for $MnCl_2$ concentrations from 4.0 mM to 0.5 mM, but is non-linear for lower concentrations of $MnCl_2$. A Km of 2.5 mM is extrapolated.

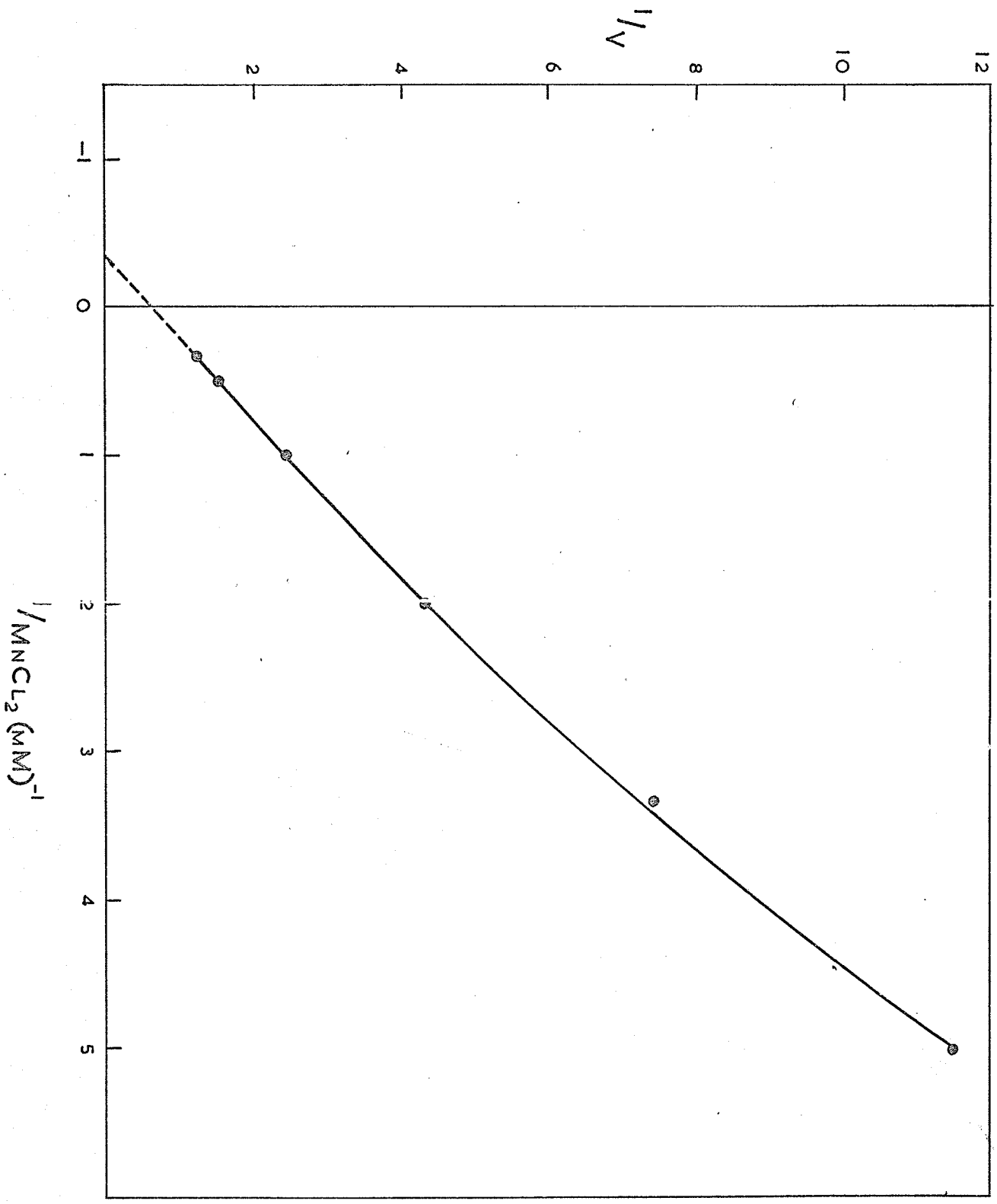

1/V
2
4
6
8
10
12
-1
0
1
2
3
4
5
1/MnCl2 (mM)-1

Fig. 10. PEP was saturating, and ADP was varied. The double reciprocal plot shows pyruvate kinase activity against ADP concentration at the following levels of pyruvate acid: (Δ—Δ—Δ) 5 mM, (0—0—0) 10 mM, (◎—◎—◎) 20 mM. The inhibition appears to be uncompetative.

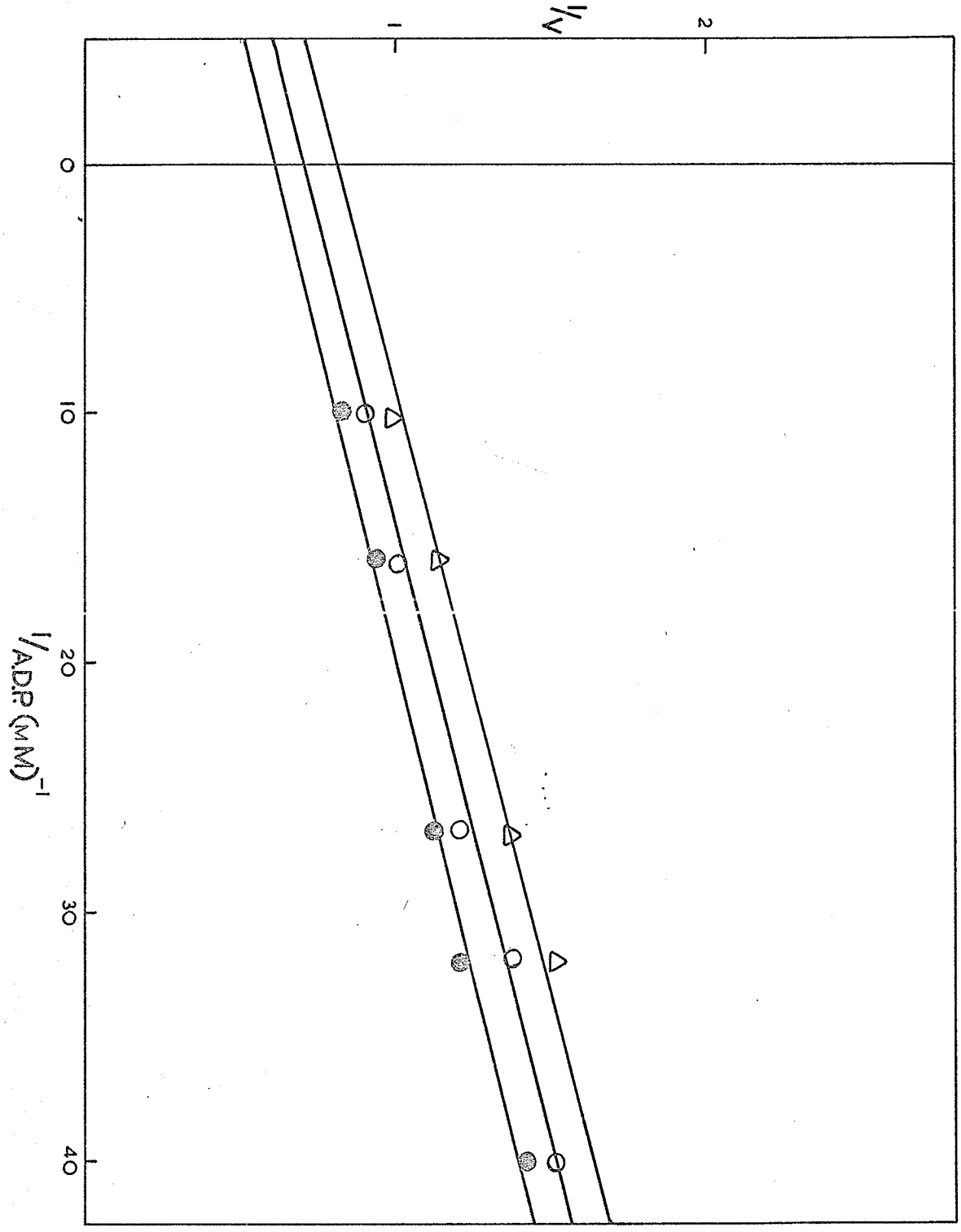

1/v
1
2
0
10
20
30
40
1/A.D.P. (mM)⁻¹

Fig. 11. ADP was saturating and PEP was varied at the indicated fixed concentrations of ATP.

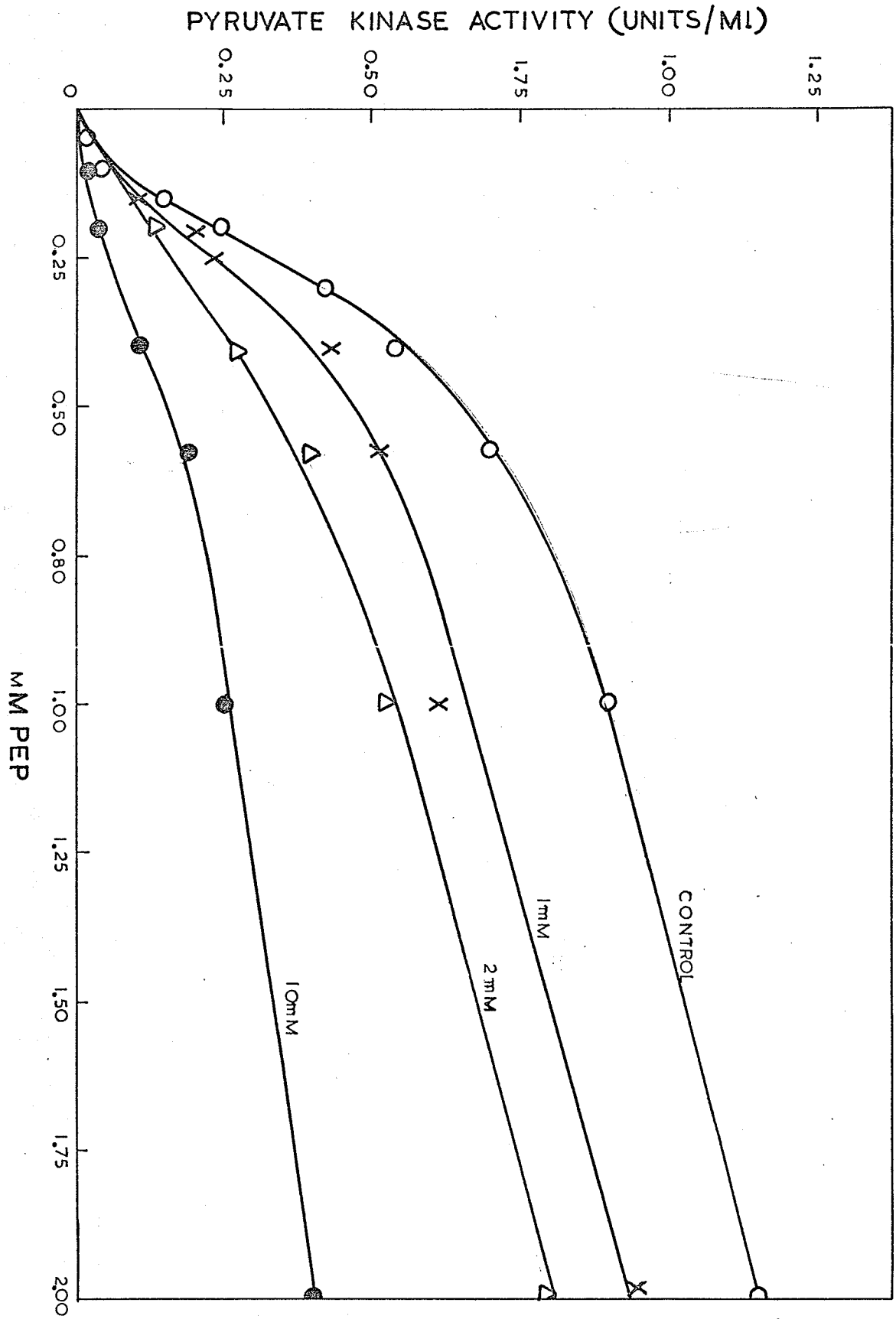

PYRUVATE KINASE ACTIVITY (UNITS/ML)
0
0.25
0.50
1.75
1.00
1.25
0.25
0.50
0.80
1.00
1.25
1.50
1.75
2.00
MM PEP
CONTROL
1mM
2mM
10mM

Fig. 12. Double reciprocal plots of pyruvate kinase activity against PEP concentration at fixed AMP concentrations of 0, 0.2 mM, 0.5 mM and 1 mM (1,2,3, and 4 respectively), show the activation caused by AMP.

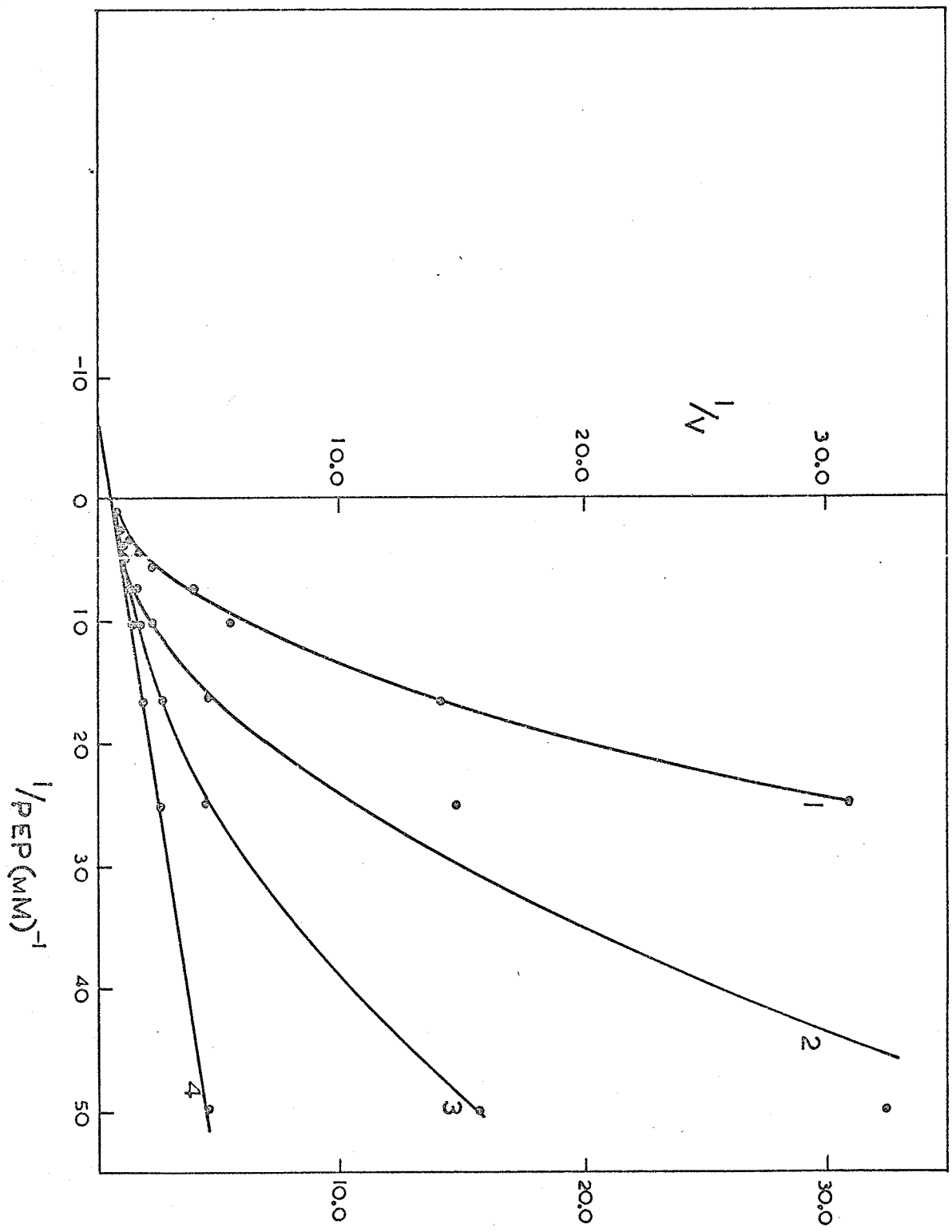

1/V
10.0
20.0
30.0
-10
0
10
20
30
40
50
1/PEP (mM)⁻¹
1
2
3
4

Fig. 13. As in Fig. 12, the Km for PEP is shown to be 0.11 mM in the presence of 1 mM AMP.

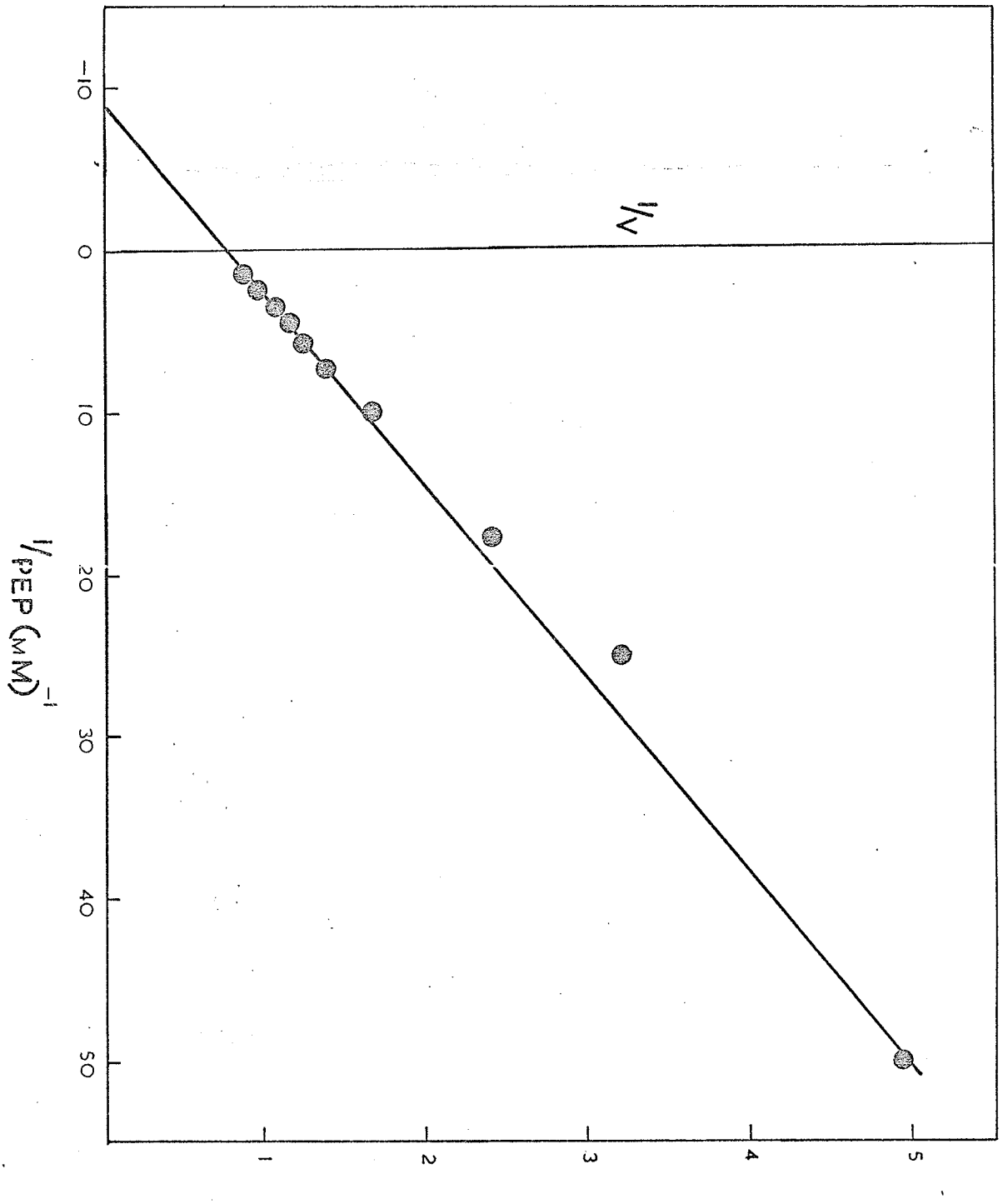

1/V
1/PEP (mM)⁻¹
-10
0
10
20
30
40
50
1
2
3
4
5

Fig. 14. Double reciprocal plot showing FDP activation of pyruvate kinase. (●—●—●) no FDP; (▲—▲—▲) 0.5 mM FDP; (Δ—Δ—Δ) 1 mM FDP, (X—X—X) 2 mM FDP; (o—o—o) 10 mM FDP.

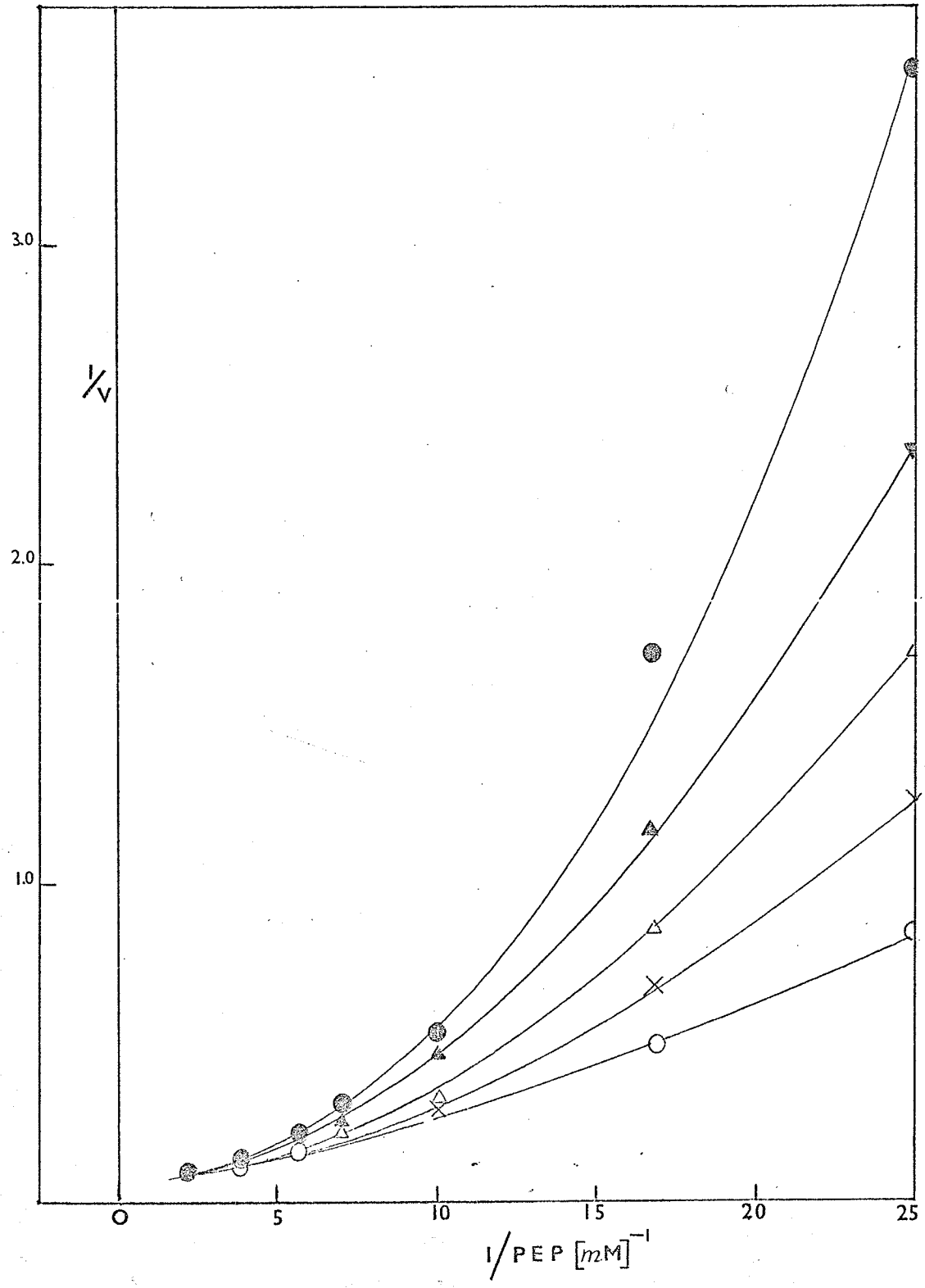

3.0
2.0
1.0
1/V
0
5
10
15
20
25
1/PEP [mM]⁻¹

Fig. 15. Hill plot of the pyruvate kinase activity at various concentrations of FDP: (X—X—X) no FDP; (⊛—⊛—⊛) 1 mM FDP; (0—0—0) 2 mM FDP; (Δ—Δ—Δ) 5 mM FDP. The values for N are 2.00, 1.88, 1.88 and 1.80 respectively.

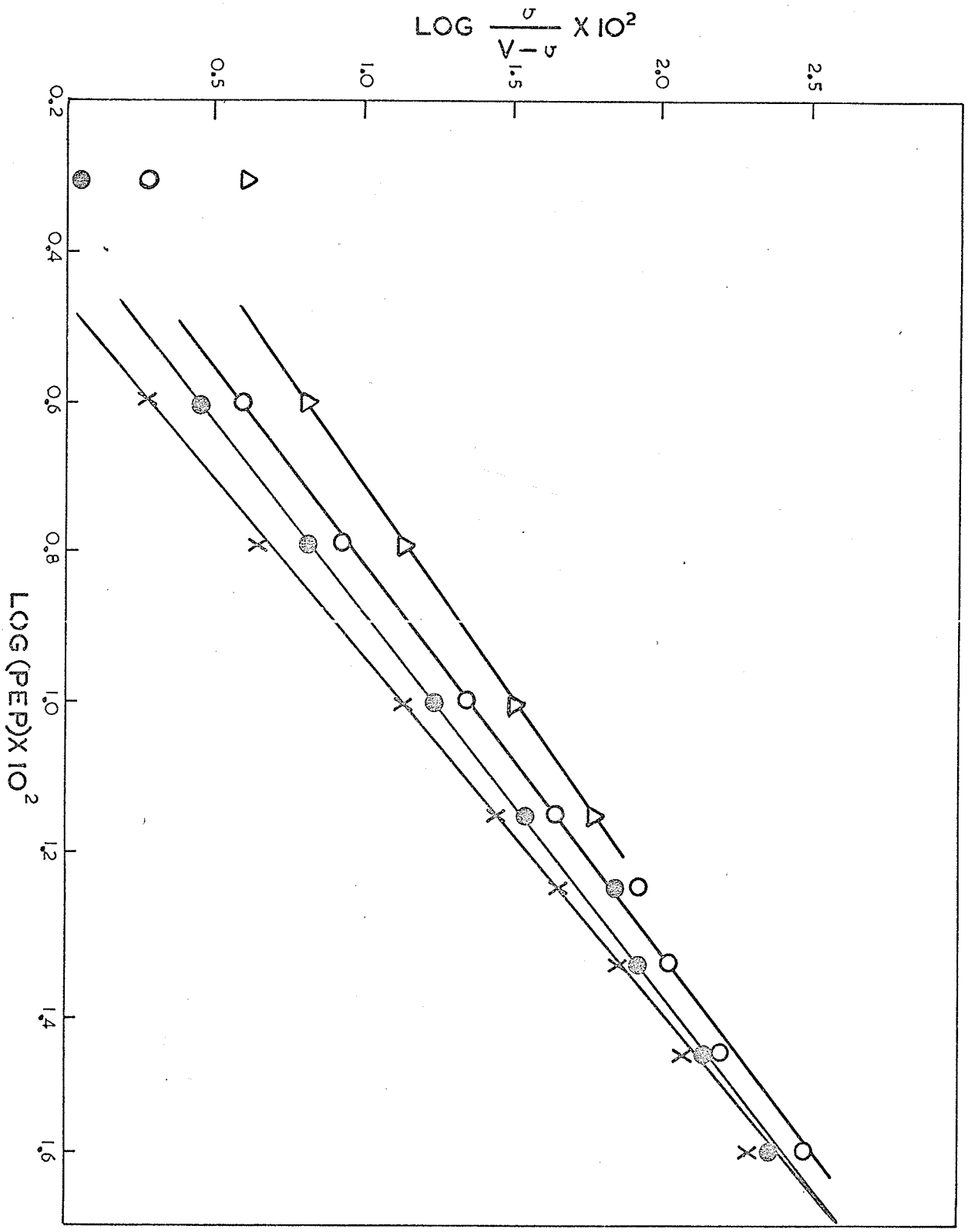

LOG (PEP) X 10²
LOG v / (V − v) X 10²
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0.5
1.0
1.5
2.0
2.5

Fig. 16. Reactivation of 48 hr dialysed pyruvatekinase with (0—0—0) 100 mM mercaptoethanol; (▲—▲—▲) 10 mM dithiothreitol; (Δ—Δ—Δ) 10 mM mercaptoethanol; (X—X—X) 1mM dithiothreitol; (◉—◉—◉) control. The incubation was at 4°C and in 0.05 M HEPES pH 7.0 buffer. The assay mixture used 2.0 mM PEP.

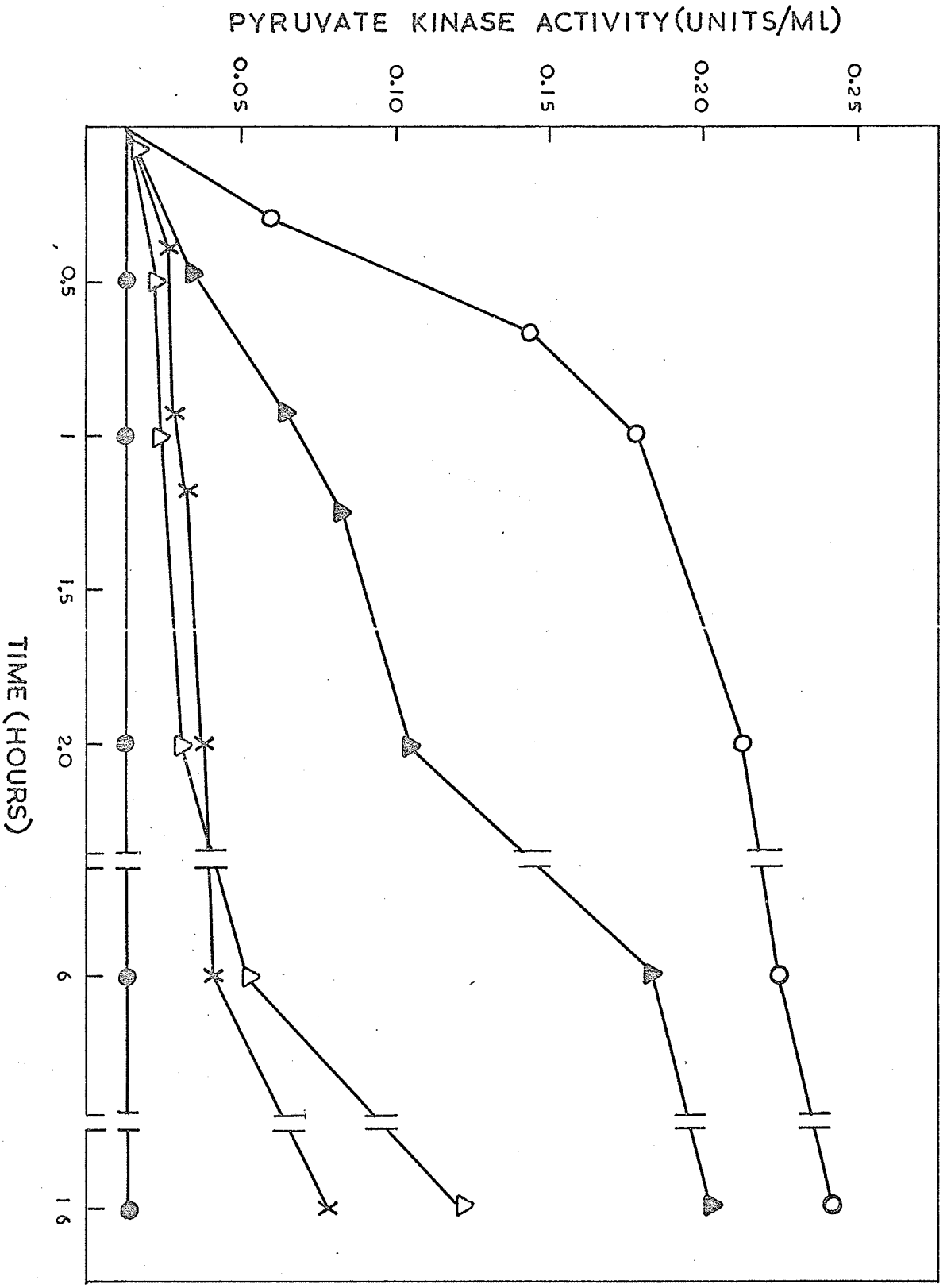

PYRUVATE KINASE ACTIVITY (UNITS/ML)
0.05
0.10
0.15
0.20
0.25
TIME (HOURS)
0.5
1
1.5
2.0
6
16

Fig. 17. Microsomal preparation containing the microsomal sulphide exchange enzyme was added to pyruvate kinase. The figure shows the partial inactivation of pyruvate kinase, followed by a gradual reactivation. The inactivation producing 50% inactivation, employed twice as much microsomal suspension as in the other result.

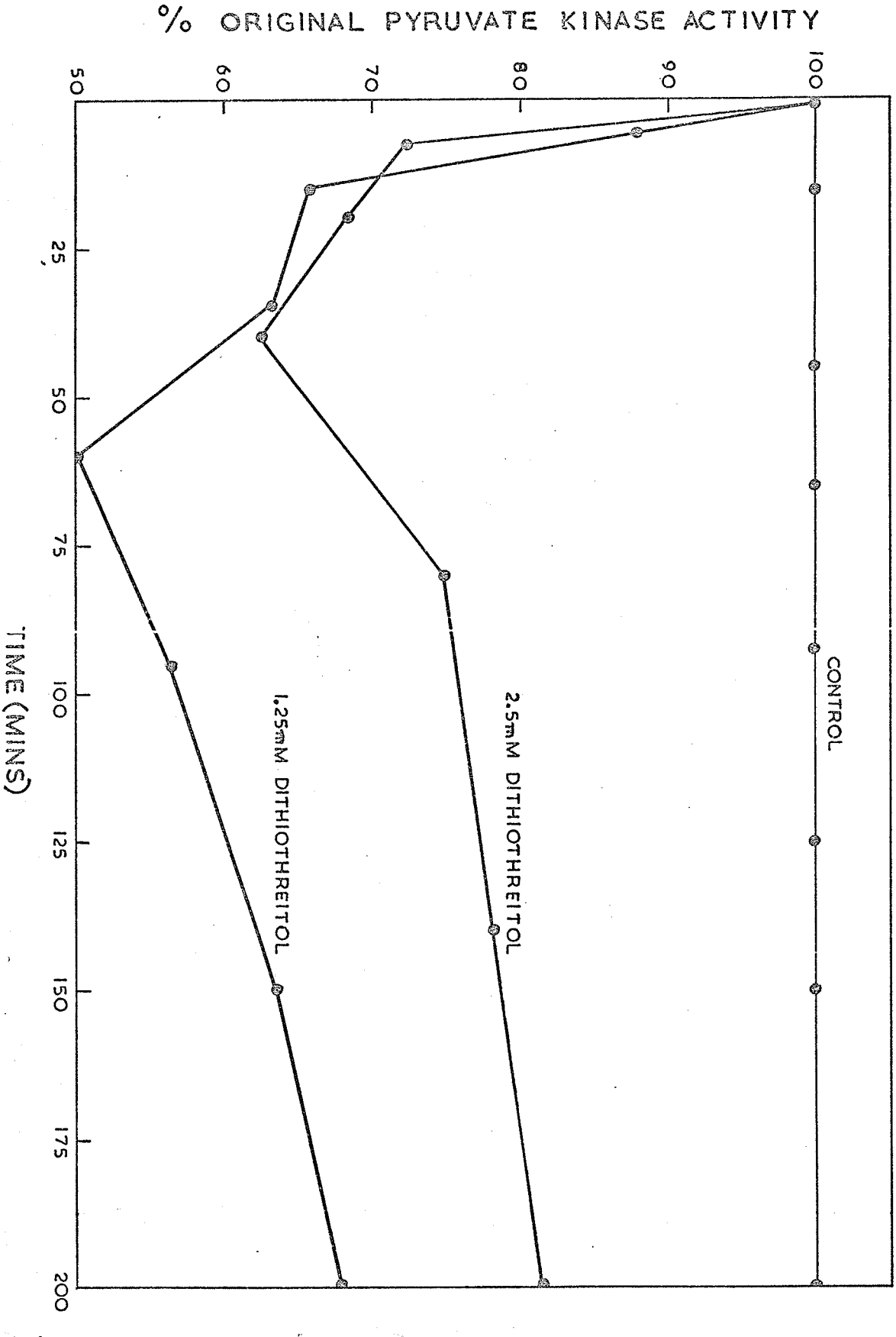

% ORIGINAL PYRUVATE KINASE ACTIVITY
50
60
70
80
90
100
TIME (MINS)
25
50
75
100
125
150
175
200
CONTROL
2.5mM DITHIOTHREITOL
1.25mM DITHIOTHREITOL

Fig. 18. A50 DEAE-sephadex elution pattern with 2 mM dithiothreitol showing two enzyme species. (●—●—●) normal assay employing 0.2 mM PEP; (0—0—0) assay employing 0.2 mM PEP and 10 mM dithiothreitol. Note the non-symmetrical activation by dithiothreitol. The dashed line is protein concentration of the eluant.

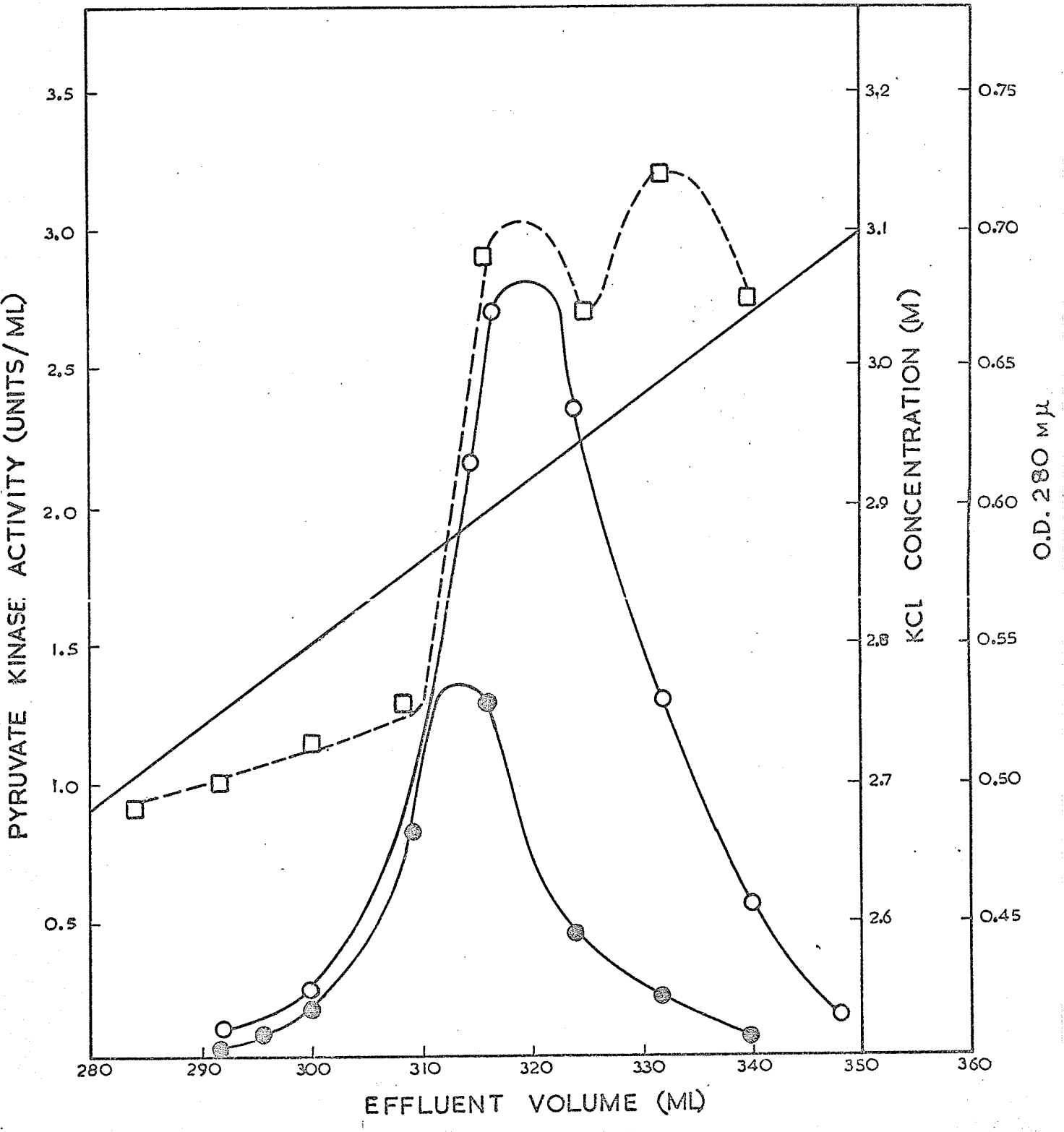

PYRUVATE KINASE ACTIVITY (UNITS/ML)
KCL CONCENTRATION (M)
O.D. 280 mμ
EFFLUENT VOLUME (ML)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
3.2
3.1
3.0
2.9
2.8
2.7
2.6
0.75
0.70
0.65
0.60
0.55
0.50
0.45
280
290
300
310
320
330
340
350
360

Fig. 19. A50 DEAE-sephadex elution pattern with no dithiothreitol showing one enzyme species. (●—●—●) assay with 0.2 mM PEP; (0—0—0) assay with 0.2 mM PEP and 1.0 mM dithiothreitol. The enzyme is symmetrically activated. The dashed line is protein concentration of the eluant.

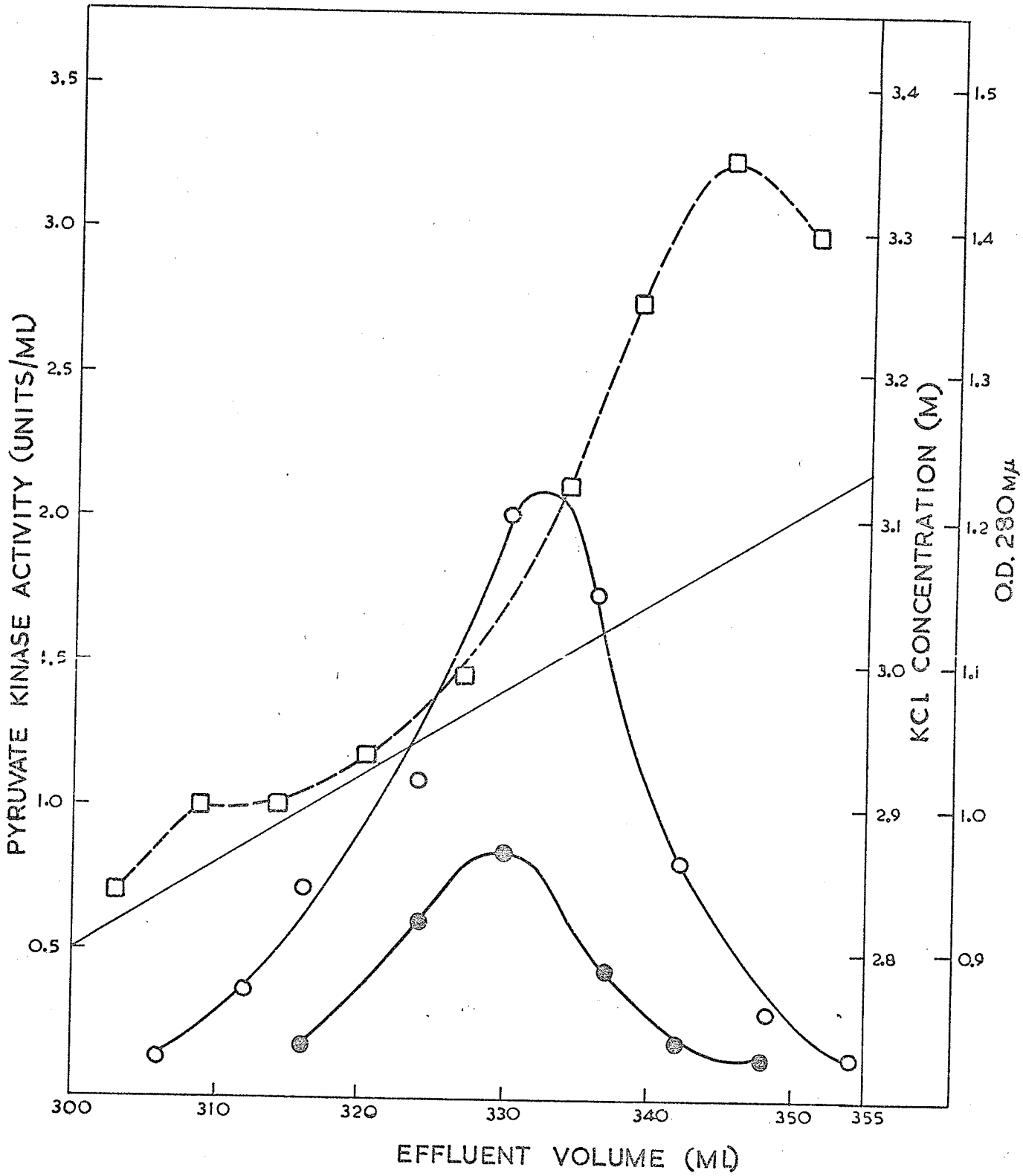

PYRUVATE KINASE ACTIVITY (UNITS/ML)
KCL CONCENTRATION (M)
O.D. 280 Mμ
EFFLUENT VOLUME (ML)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
3.4
3.3
3.2
3.1
3.0
2.9
2.8
1.5
1.4
1.3
1.2
1.1
1.0
0.9
300
310
320
330
340
350
355

Fig. 20. A50 DEAE-sephadex elution pattern with 10 mM dithiothreitol showing one enzyme species assayed at 0.2 mM PEP. No additional activity was found when the assay included 10 mM dithiothreitol. The dashed line is protein concentration of the eluant.

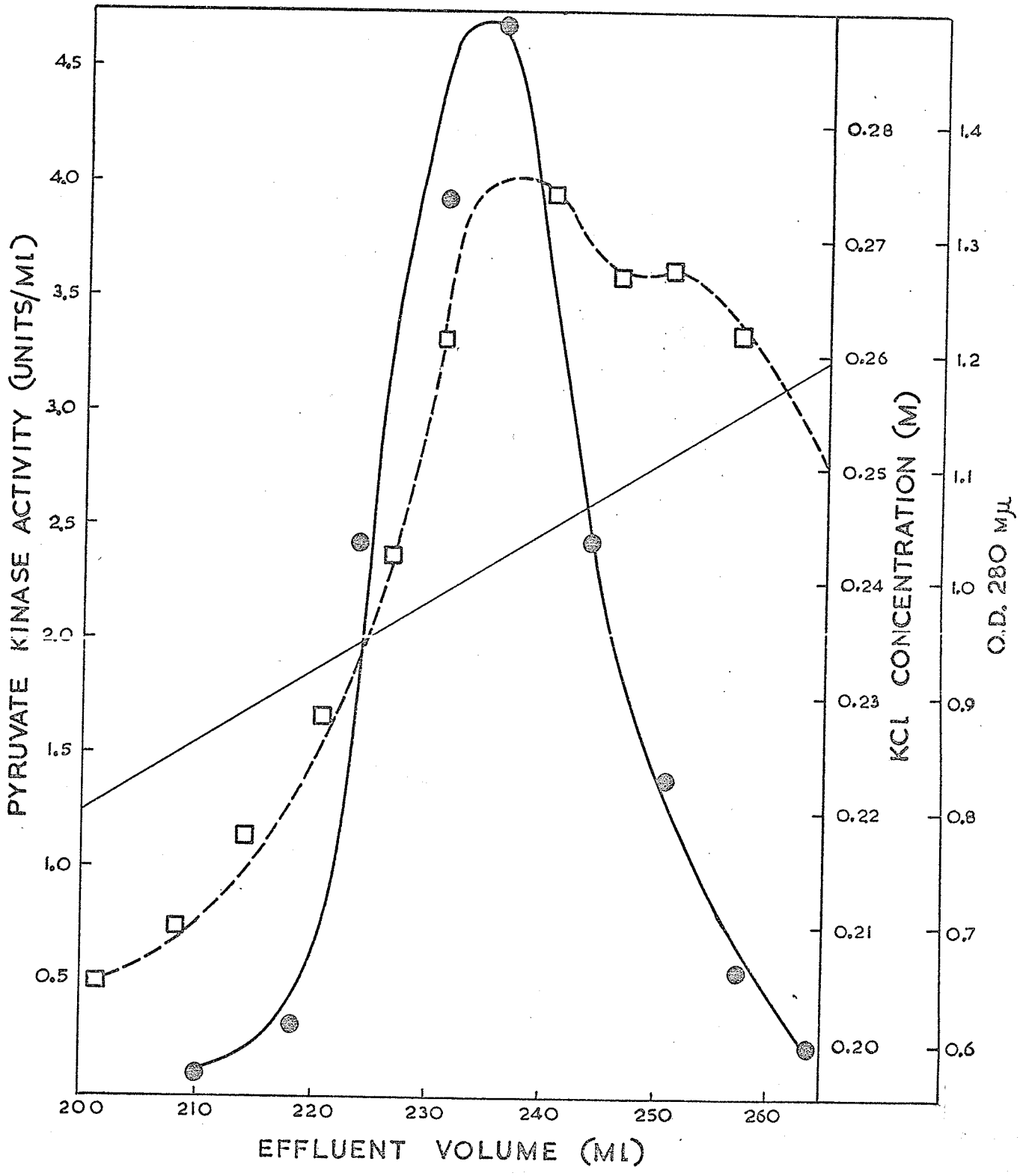

PYRUVATE KINASE ACTIVITY (UNITS/ML)
KCl CONCENTRATION (M)
O.D. 280 mμ
EFFLUENT VOLUME (ML)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.28
0.27
0.26
0.25
0.24
0.23
0.22
0.21
0.20
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
200
210
220
230
240
250
260

Fig. 21. DEAE cellulose elution pattern.

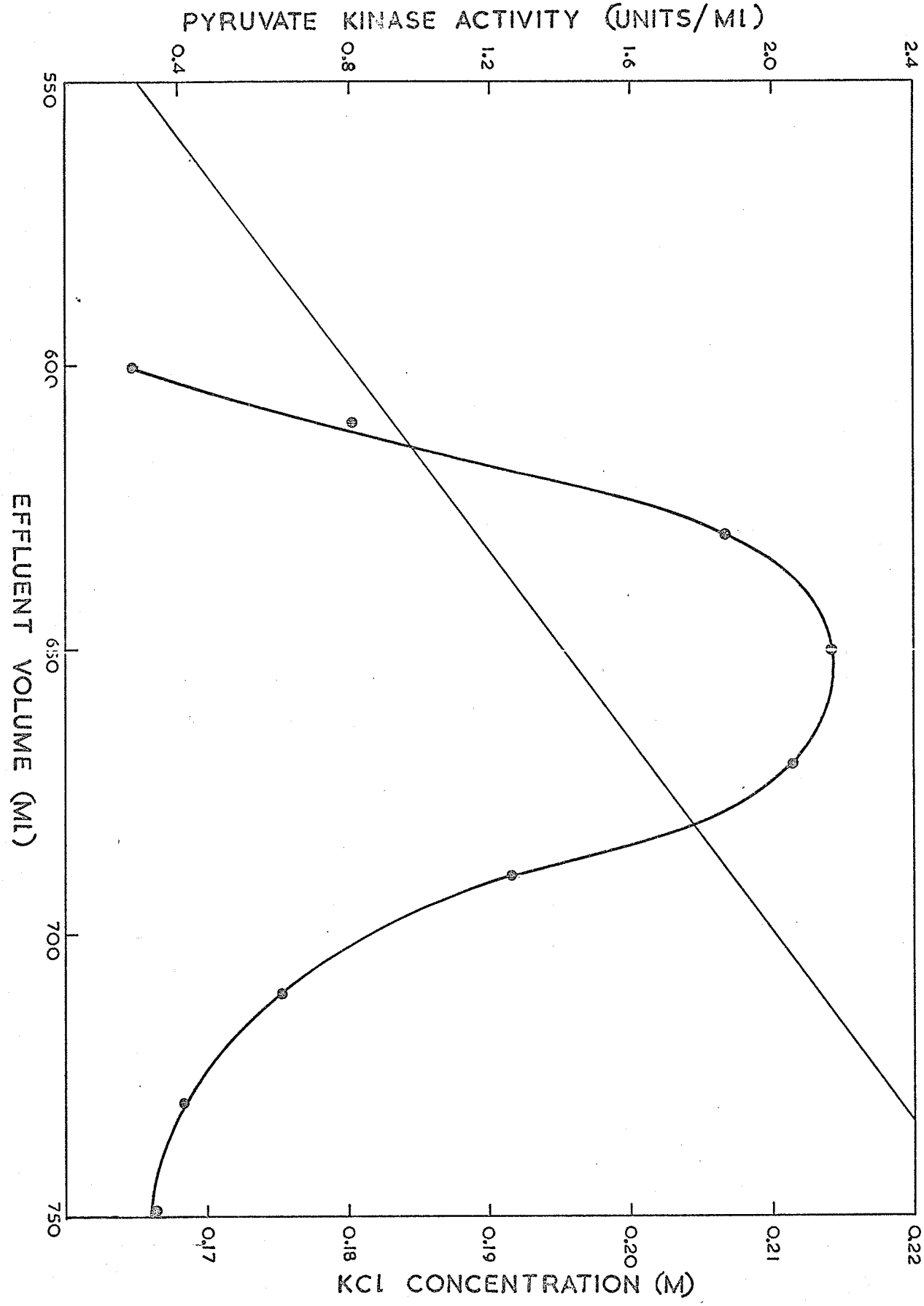

PYRUVATE KINASE ACTIVITY (UNITS/ML)
0.4
0.8
1.2
1.6
2.0
2.4
550
600
650
700
750
EFFLUENT VOLUME (ML)
0.17
0.18
0.19
0.20
0.21
0.22
KCl CONCENTRATION (M)

Fig. 22. Kinetics of pyruvate kinase after 48 hrs dialysis. (O—O—O) assayed with 1 mM FDP; (Δ—Δ—Δ) assayed with 10 mM dithiothreitol; (X—X—X) assayed with 0.5 mM AMP; (●—●—●) assayed with no effectors. The buffer was 0.05 M MES pH 6.3.

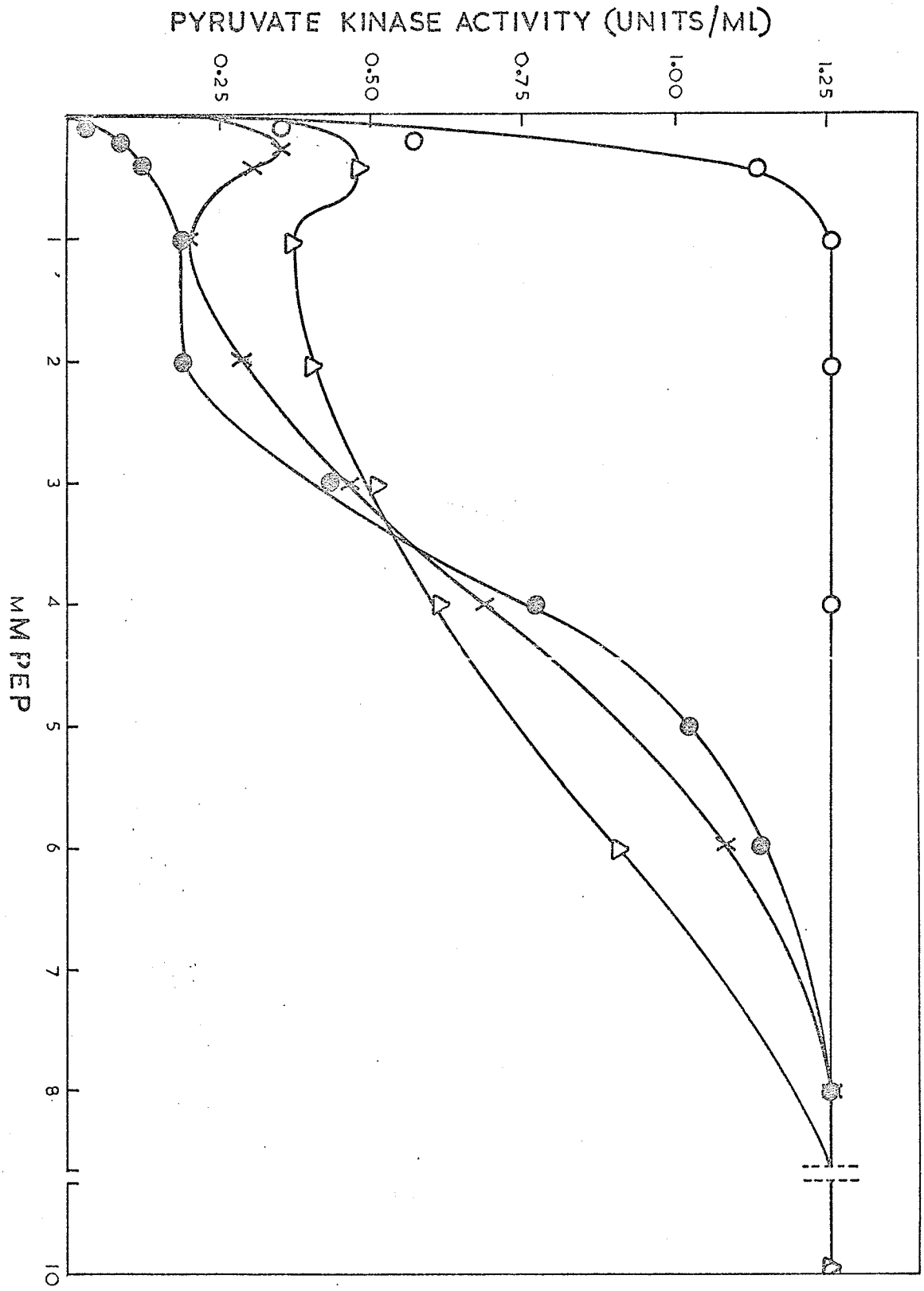

PYRUVATE KINASE ACTIVITY (UNITS/ML)
0.25
0.50
0.75
1.00
1.25
MM PEP
1
2
3
4
5
6
7
8
10

Fig. 23. Expanded scale of initial part of Fig. 22.

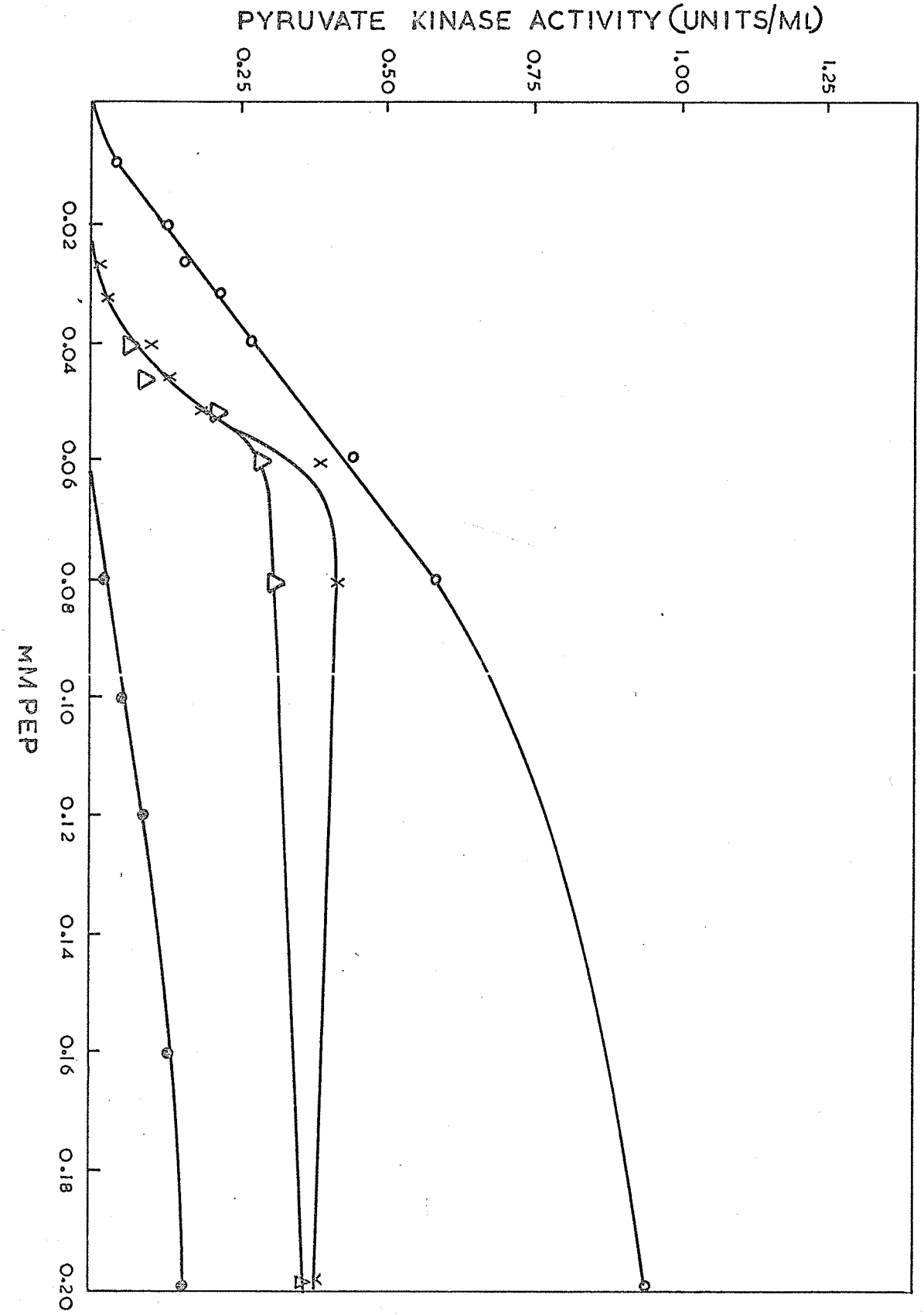

PYRUVATE KINASE ACTIVITY (UNITS/ML)
0.25
0.50
0.75
1.00
1.25
MM PEP
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20

Fig. 24. Kinetics of pyruvatekinase suspension after 48 hrs dialysis and 12 hrs incubation with 20 mM dithiothreitol. (0—0—0) assayed with 1 mM FDP (X—X—X) assayed with 0.5 mM AMP (●—●—●) assayed with no effectors. Note that the increase in Vmax over that in Fig. 22 is the same as the increase in the curve with no effectors.

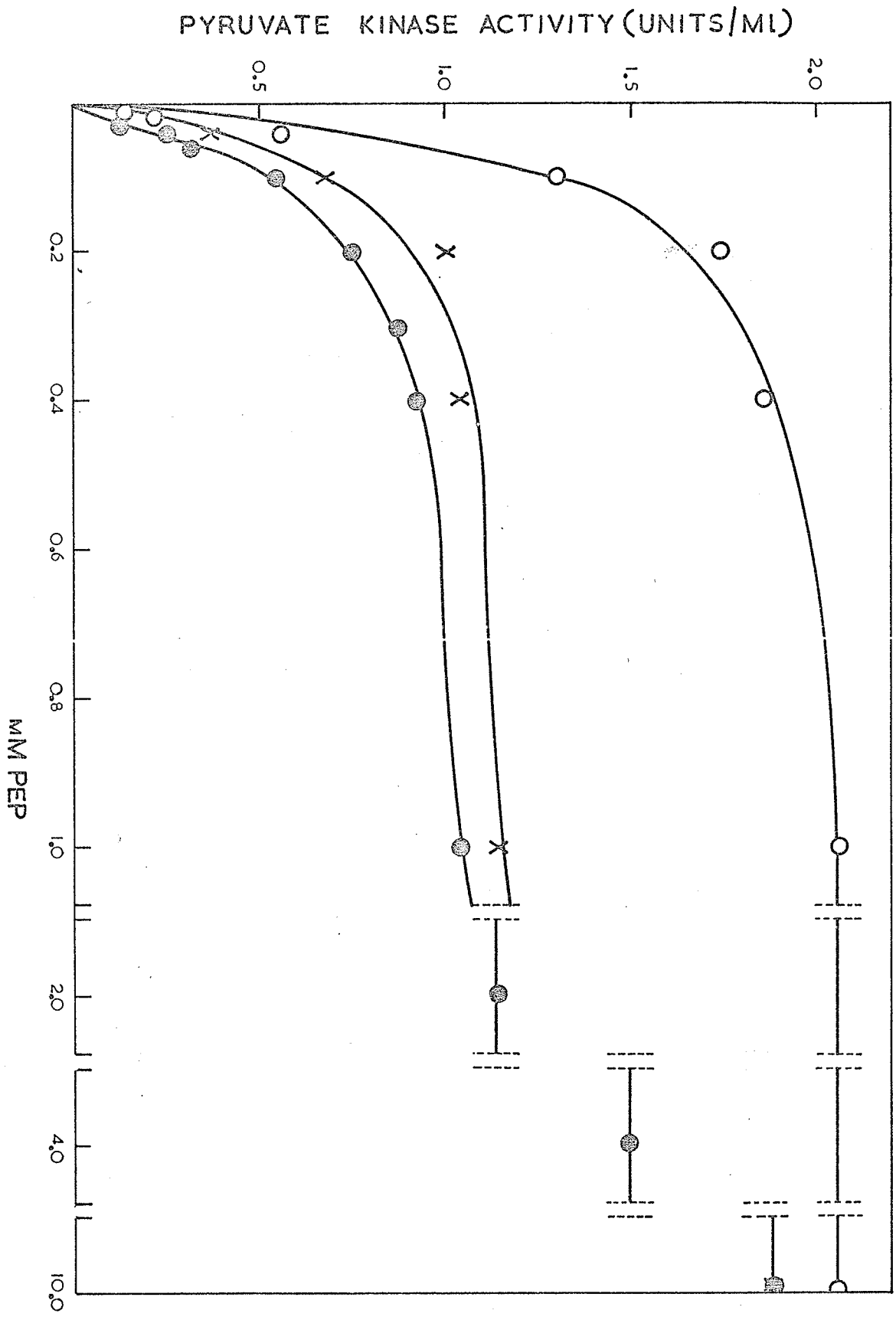

PYRUVATE KINASE ACTIVITY (UNITS/ML)
0.5
1.0
1.5
2.0
MM PEP
0.2
0.4
0.6
0.8
1.0
2.0
4.0
10.0

Fig. 25. As Fig. 24 except with expanded scale.

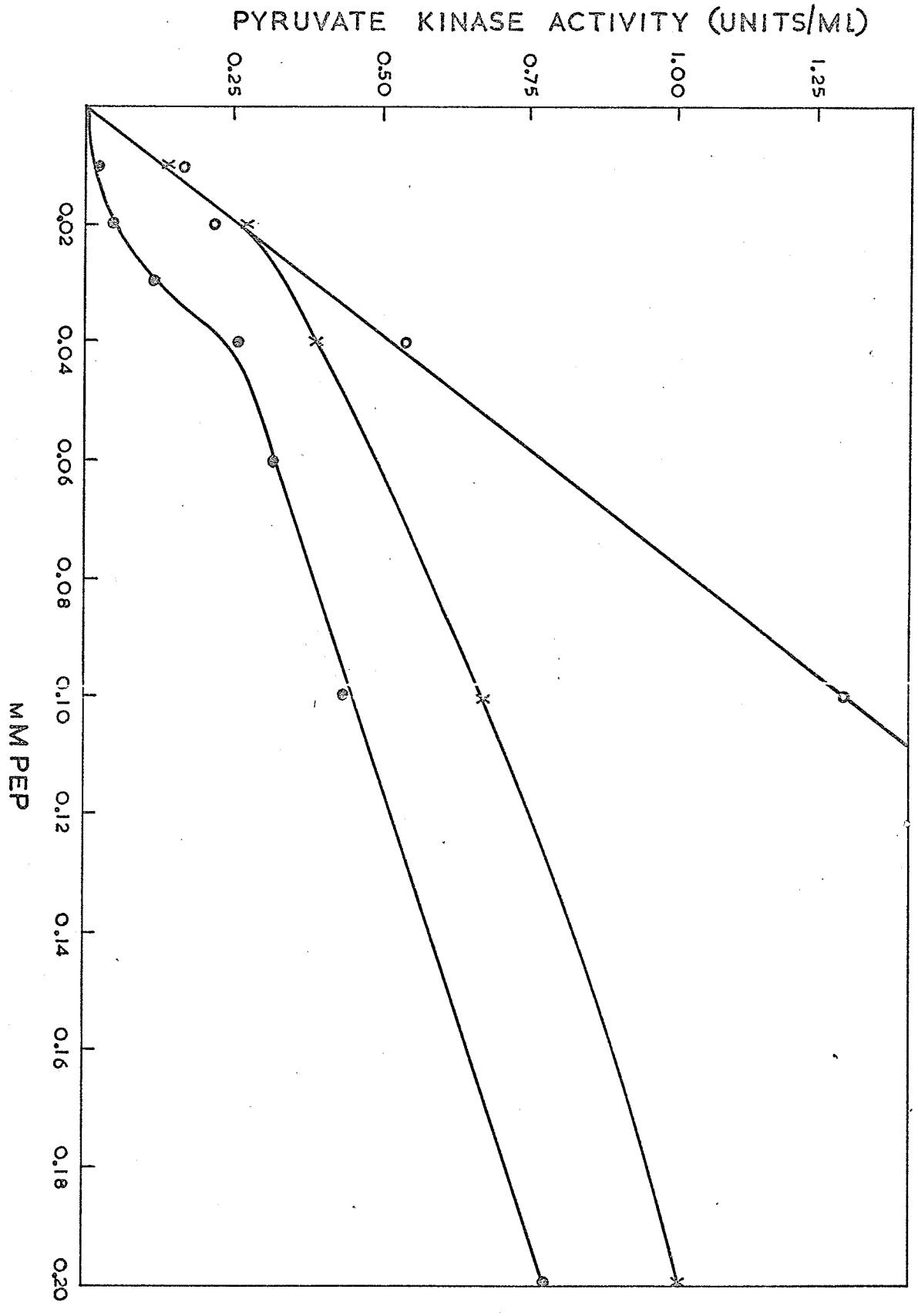

PYRUVATE KINASE ACTIVITY (UNITS/ML)
0.25
0.50
0.75
1.00
1.25
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
MM PEP

Fig. 26. These two plots using the method of Horn and Bornig (1969) give the N and L numbers for pyruvatekinase.

A. Pyruvate kinase is in the presence of 10 mM dithiothreitol, there are no activations or inhibitors therefore L = L', and the pH is 7.0. The plot indicates a tetramer.

B. Using data from graph 22, corrected for residual 'tetrameric activity', with no activation or inhibitors, therefore L = L', and the pH is 6.3. The plot gives N=5.

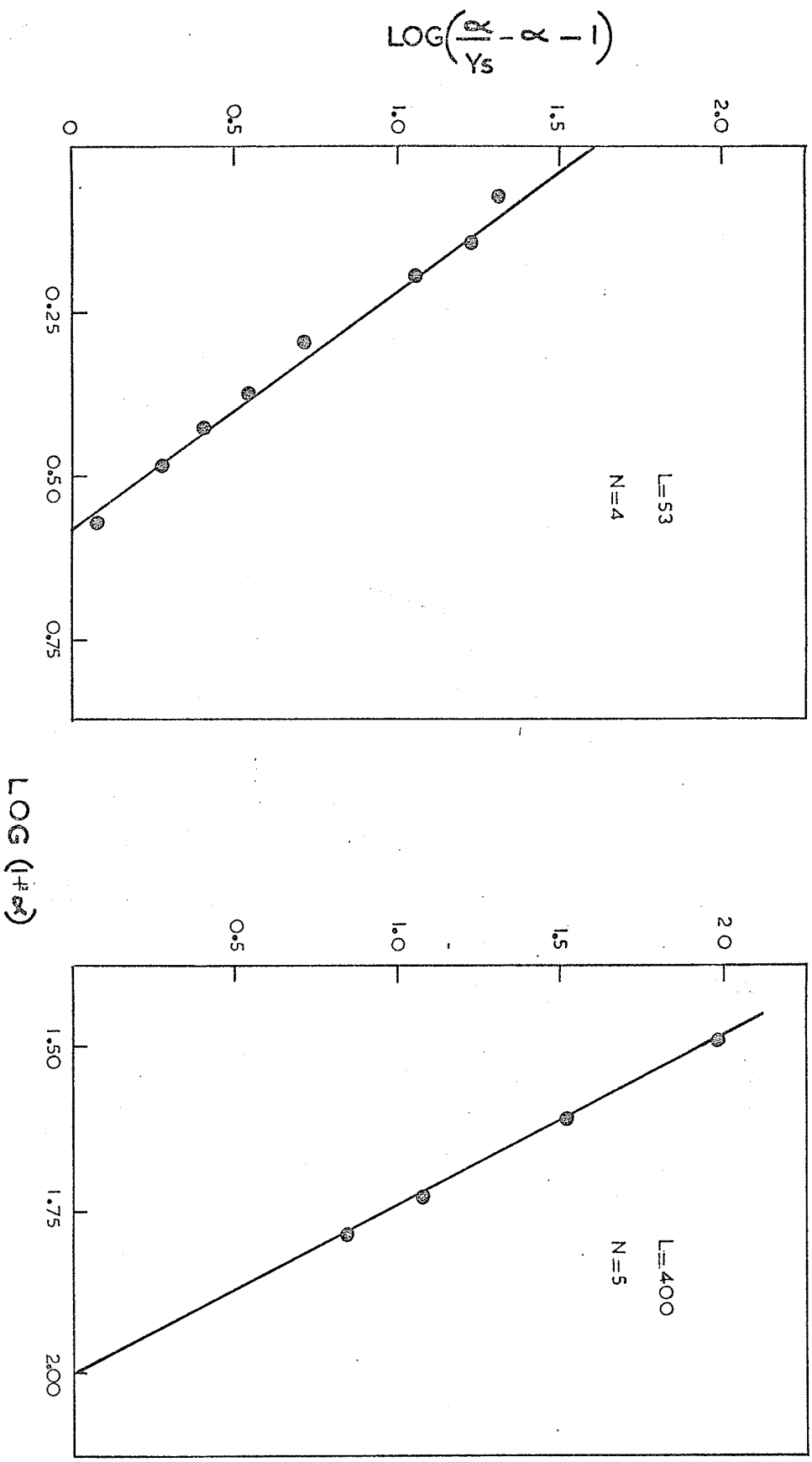
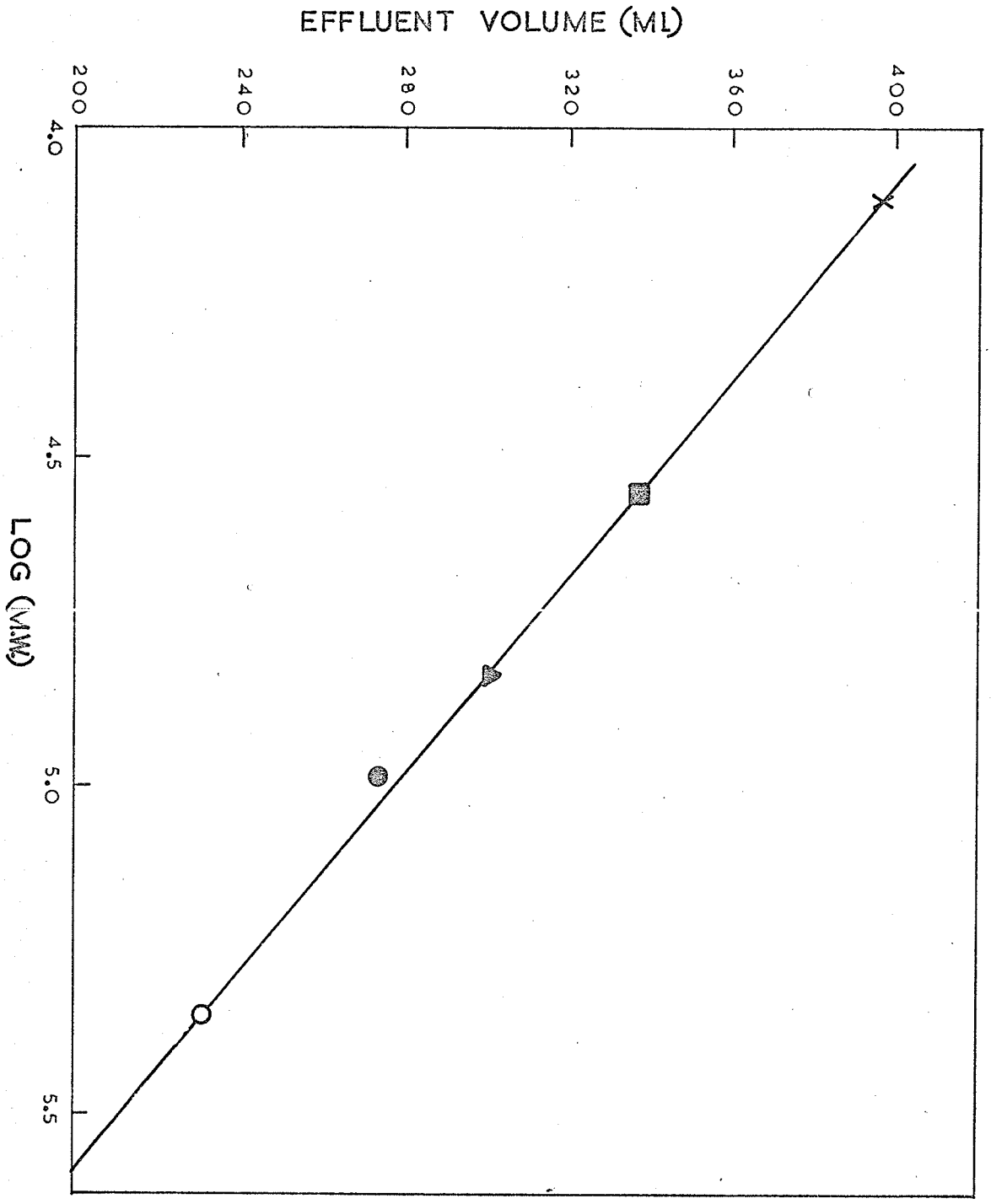

A
$\log\left(\frac{\alpha}{Y_S} - \alpha - 1\right)$
0
0.5
1.0
1.5
2.0
0.25
0.50
0.75
L=53
N=4
B
$\log(1+\alpha)$
0.5
1.0
1.5
2.0
1.50
1.75
2.00
L=400
N=5

Fig. 27. G-200 sephadex column calibration with proteins of known molecular weight: Cytochrome C_3 (13,000) X; Haemoglobin (68,000) ▲ ; Hexokinase (96,000) ● ; Catalase (225,000) 0. ■ is the molecular weight species of pyruvate kinase eluted from the column run without dithiothreitol.


EFFLUENT VOLUME (ML)
200
240
280
320
360
400
LOG (M.W.)
4.0
4.5
5.0
5.5

DISCUSSION

Pyruvate kinase has been shown to be controlled by two ligands AMP and FDP. This is in agreement with a previous report by Maeba and Sanwal (1968). In 1969, Malcovati and Kornberg reported that there were two pyruvate kinases in Escherichia coli, one being allosteric, and activated by FDP, and the other showing normal Michaelis-Menten kinetics. Neither was reported to be activated by AMP. However, it should be noted that the assay medium employed by Malcovati and Kornberg had a pH of 6.5, which is very close to the optimum pH of 6.3 reported here. At this pH allosteric behaviour occurs only below a level of 0.05 mM PEP, and this is the lower limit reported by Malcovati and Kornberg. If the assays had been carried out at pH 7.0 or higher, the allosteric nature would have become more obvious.

The initial M.W. studies carried out here indicate that there are two dissimilar protein subunits which when aggregated are responsible for pyruvate kinase activity. One has a molecular weight of approximately 50,000 and when aggregated is activated by AMP. The second has a molecular weight of approximately 35,500 and is activated by FDP irrespective of whether it is aggregated or dissociated. For simplicity we will call the former PyKamp and the latter PyKfdp.

PyKamp has a Km for PEP close to 0.1 mM at pH 6.3 and a Km of 0.22 mM at pH 7.0. AMP activates the enzyme significantly only of pH values higher than 6.3. Irrespective of the pH value, however, when the enzyme is fully activated by AMP, PEP yields a Km of 0.1 mM. Also, the enzyme is inhibited by ATP fairly effectively. In comparison with PyKamp for which velocity becomes independent of substrate at 1 mM PEP,

the PyKfdp has very little activity at 1 mM PEP in the absence of FDP. Its Km for PEP at pH 6.3 is approximately 3.5 mM. Because the purification procedure used to isolate pyruvate kinase employed 0.2 mM PEP, it is in retrospect assumed that the kinetic results reported here would be basically that of PyKamp. In fact, FDP and AMP activation had been followed through the purification procedure, and FDP activation decreased sharply, and even led to the isolation on a G200 sephadex column of a pyruvate kinase which was not activated by FDP on one occasion. PyKamp kinetics as reported here show remarkable similarity to the kinetics of PyKL isolated by both Tanaka et al (1967) and Rozengurt et al (1969), except that this animal enzyme is activated by FDP.

Since there are indications (see Results) that PyKamp requires the presence of dithiothreitol during purification, and loses activity in its absence, it seems possible that the kinetics of enzyme preparations retained after dialysis in the absence of dithiothreitol would primarily be due to the presence of the surviving PyKfdp, although there is no certainty that hybrid forms (containing 35,000 and 50,000 M.W. subunits) do not exist. It should be remembered that Tanaka et al (1965) reported that there are three PyKLs isolated electrophoretically from his preparations.

The results by Malcovati and Kornberg (1969) show that the relative amounts of PyKamp and PyKfdp are controlled by the carbon source on which the cells are grown. By observation of the relative amounts found in glucose grown cells and in glycerol grown cells used in this investigation, one would tend to agree with the above finding.

However, Malcovati and Kornberg (1968) showed two distinct types unlike the many M.W. species found eluted from G200 sephadex column. Whether these mixed subunit aggregations found actually exist in vivo is a matter for further investigation.

The initial velocity curves obtained from crude extracts, and from the "dialysed enzyme" are due to the addition of the activaties of two or maybe more species of pyruvate kinase. This fact has interesting theoretical ramifications as the curves are similar to those which Levitzki and Koshland (1969) would consider to be due to "negative cooperativity". If one considers that both species of pyruvate kinase have a mechanism in accord with the Monod, Wyman and Changeux (1965) model for allosterism, then it is clear that the addition of their activities would produce "negativly cooperative " curves. Simulation of this theoretical condition is being studied by means of an IBM 1620 computer and initial, but unreported results are encouraging. If hybrid species of the two dissimilar subunits could be obtained, it would be interesting to see whether these kinetics would yield the so-called "negativly cooperative" curves.

Considering the simplest case that the two species PyKamp and PyKfdp exist as two separate and distinct forms, the physiological implications are of great interest. At first, ignoring the interrelationships with anaplerotic pathways, it can be seen that in the case where there is no FDP in the system, then only PyKamp would be operational. If ATP was high, then this enzyme would be reasonably effectively inhibited, and not activated by AMP which would be low (Krebs, 1964 and Atkinson, 1965). However, at low energy levels within the cell, the enzyme would have high activity at low PEP concentrations due to AMP activation and then allow the TCA cycle to function continually. The FDP activation of PyKfdp, which can be considered almost inactive without FDP when compared to PyKamp, would allow for rapid turnover of PEP when the cells were grown on glucose. This would have the effect of enhancing the irreversibility of pyruvate kinase, and thus creating the theromodynamic pull on the Embden-

Meyerhof pathway. As there has not at present been any studies carried out with ATP inhibition, no conclusion can be made as to the enzymes inhibition. However, it would seem likely that ATP should be a good inhibitor of PyKfdp activity.

The ability of FDP to activate PEP carboxylase (Maeba and Sanwal, 1969) assume that a sufficient supply of oxaloacetate would be available for efficient operation of the TCA cycle, when cells are grown on glucose. In the cases where growth occurs on other substrates (e.g. acetate), a balance between acetyl CoA activated PEP carboxylase and PyKamp would control the operation of the TCA cycle.

In conclusion, there would appear to be two pyruvate kinases in Escherichia coli, each allosteric, each comprised of subunits but of different molecular weights 35,500 and 50,000 and activated by FDP and AMP respectively. The possibility of hybrids or isoenzymes is also indicated.

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