PURIFICATION AND CHARACTERIZATION OF M2-TYPE PYRUVATE KINASE ISOZYME FROM HUMAN MENINGIOMA

A DOCTOR OF PHILOSOPHY THESIS

in

Biochemistry

Middle East Technical University

By

Ali Awsat MELLATI

June, 1991

Approval of the Graduate School of Natural and Applied Sciences

for Prof.Dr.O.Alpay ANKARA
Director

I certify that I have read this thesis and that in my oppinion is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

Prof.Dr.Emel ARINÇ
Chairman of the Department

We certify that we have read this thesis and that in our opinion is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Prof.Dr.Ufuk GÜNDÜZ
Supervisor

Assoc.Prof.Dr.Meral YÜCEL
Co-supervisor

Examining Committee in Charge:

Prof.Dr.Ufuk GÜNDÜZ

Prof.Dr.Emel ARINÇ

Prof.Dr.Wayne E.CRISS (Chairman)

Assoc.Prof.Dr.Meral YÜCEL

Assoc.Prof.Dr.Kamer KILIÇ

PURIFICATION AND CHARACTERIZATION OF M2-TYPE PYRUVATE KINASE ISOZYME FROM HUMAN MENINGIOMA

MELLATI, Ali Awsat
Institute of Natural and Applied Sciences
Department of Biochemistry, Ph. D. Thesis
Supervisor : Prof. Dr. Ufuk GUNDUZ
Cosupervisor : Assoc. Prof. Dr. Meral YUCEL
108 Pages, June, 1991

ABSTRACT

M2 - type pyruvate kinase was purified from human meningioma by ammonium sulfate fractionation, CM-cellulose chromatography and affinity chromatography. The purified enzyme had a final specific activity of 33.4 units/mg protein. The subunit molecular weight, determined by SDS-polyacrylamide gel electrophoresis, was 63,000±2000 daltons. Upon cellulose acetate paper electrophoresis, the purified enzyme gave a single band,

while crude extracts gave two wide bands corresponding to pyruvate kinase isozymes. The pI value of the purified enzyme was 6.9.

With phosphoenolpyruvate(PEP) as substrate, the purified enzyme showed sigmoidal kinetics, while in the presence of 0.6mM fructose 1,6 diphosphate as modulator gave a hyperbolic saturation curve with a Km value of 0.53mM. The enzyme was maximally activated by 0.6mM fructose 1,6 diphosphate and a Ka value of 0.15mM was obtained. The enzyme in the presence of 0.6mM F-1,6-DP expressed normal Michaelis-Menten kinetics with respect to ADP with a Km value of 0.58mM. L-Alanine noncompetitively inhibited the purified enzyme with respect to the substrates, phosphoenolpyruvate and ADP. The inhibitory effects of alanine was partially reversed by fructose 1,6 diphosphate. The purified enzyme was slightly susceptible to ATP inhibition.

Key words : M2-Type pyruvate kinase, Meningioma,
Brain tumor, Enzyme kinetics, Izosymes.

Science Code : 401.01.05

İNSAN BEYİN TÜMÖRLERİNDEN MENİNJİOMADA M2-TİP PİRUVAT KİNAZ İZOZİMİNİN SAFLAŞTIRILMASI ve TANIMLANMASI

MELLATİ, Ali Awsat
Fen Bilimleri Enstitüsü
Biokimya Bölümü Doktora Tezi

Danışman : Prof.Dr. Ufuk GÜNDÜZ
Yardımcı Danışman : Doç.Dr. Meral YÜCEL

Haziran 1991, 108 sayfa

ÖZET

M2-Tip piruvat kinaz (PK) insan meninjiomasından amonyum sülfat çökertmesi, CM-selüloz ve afinite kromotografisi kullanılarak saflaştırılmıştır. Saflaştırılan enzimin özgül aktivitesi 33,4 ünite/mg proteindir. Altbirim moleküler ağırlığı SDS-poliakrilamit jel elektroforezi kullanılarak 63.000±2000 D olarak bulunmuştur.

Saflaştırılmamış enzimin selüloz asetat elektroforezinde piruvat kinaz izozimlerine karşılık gelen iki geniş bant vermesine karşın, saflaştırıldıktan sonra tek bant verdiği görülmüştür. Saf enzimin pI değeri 6,9 olarak bulunmuştur.

Saf enzim fosfoenolpiruvat substratı için sigmoidal kinetik göstermektedir. 0,6 mM fruktoz 1,6 difosfat (F-1,6-DP) varlığında ise hiberbolik şekle dönüşmektedir ve Km değeri 0,53mM olarak bulunmuştur. Enzim en yüksek aktivitesine 0,6mM fruktoz 1,6 difosfat varlığında ulaşmış olup 0,15 mM değerinde bir Ka elde edilmiştir. 0,6 mM F-1,6-DP varlığında ADP için Km değeri 0,58 mM olup hiberbolik kinetik göstermektedir. Her iki substrat içinde (fosfoenolpiruvat ve ADP) L-alanin saf enzimi nonkompetetif

olarak inhibe etmektedir. L-alaninin inhibisyon etkisi F-1,6-DP tarafından bir miktar kaldırılmaktadır. Saf enzimin ATP inhibisyonuna düşük seviyede duyarlı olduğu görülmüştür.

Anahtar Sözcükler: M2-Tip piruvat kinaz, Menenjiom, Beyin tumoru, Enzim kinetiği, Izozim.

Anabilim Dalı Sayısal Kodu : 401.01.05

ACKNOWLEDGEMENTS

I would like to express my deep appreciation to Prof. Dr. Ufuk GUNDÜZ for her supervision and valuable suggestions throughout the course of this study.

I am deeply grateful to Assoc. Prof. Dr. Meral YÜCEL for her encouragement and valuable comments of this thesis.

I wish to thank Prof. Dr. Inci ÖZER for her suggestion about the analysis of kinetic data.

I would also like to express my appreciation to Prof.Dr. Wayne E.CRISS for his very helpful comments and professional corrections of english grammer of the thesis.

I would also like thank to my friends who helped me in the preparation of this thesis.

I am very grateful to Dr.Nur ALTINÖR for kindly supplying the human meningioma samples used in this study.

TABLE OF CONTENTS

Page

Page

LIST OF TABLES

Page

Table

LIST OF FIGURES

Page

Figure

ABBREVIATIONS

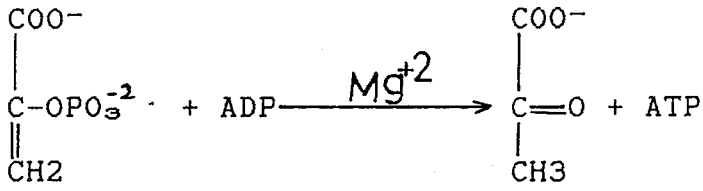
A	: Absorbance
ADP	: Adenosine-5'-diphosphate
ATP	: Adenosine-5'-triphosphate
CM-Cellulose	: Carboxymethylcellulose
F-1,6-DP	: Fructose-1,6-diphosphate
MOPS	: 3-(N-Morpholina) propanesulfonic acid
PAGE	: Polyacrylamide gel electrophoresis
PEP	: Phosphoenolpyruvate
PK	: Pyruvate Kinase
S	: Substrate
SDS	: Sodium dodecyl sulphate
Tris	: Tris (hydroxymethyl) aminomethane

CHAPTER I

INTRODUCTION

Tumor cells differ in many aspects from their normal counterparts. These differences are determined by many specific properties of tumor cells; i.e., their high proliferative capacity, and their ability to invade other tissues and to metastasize to other organs. One of the differences involves isozyme changes which includes enzymes involved in the turn over of membrane glycoproteins, enzymes regulating glycolysis (49,62,81) and nucleotide-converting enzymes. These changes often imply a shift in the isozyme patterns and the re-expression of other isozymes, which are normally only expressed during early developmental stages and regenerative processes (22,37). Changes in isozyme patterns are frequently used as markers in both embryonic and neoplastic tissues (78,85,109). One of the enzymes which show such variations in isozyme patterns is pyruvate kinase.

Pyruvate kinase (PK) (ATP: Pyruvate phosphotransferase, E.C. 2.7.1.40) is an important enzyme of the glycolytic pathway. It catalyzes the phosphorylation of ADP to ATP in the presence of phosphoenolpyruvate (PEP) and Mg^{2+}.



Phosphoenolpyruvate Pyruvate

The reaction is essentially irreversible and proceeds with a $\Delta G^{\circ} = -7.5$ kcal/mole. Pyruvate kinase, by controlling the rates of this reaction, is ultimately involved in the regulation of glycolysis and gluconeogenesis.

The normal developmental pattern of pyruvate kinase is reversed in tumors. For instance, the characteristic adult enzymes have been reported to be replaced by the fetal enzymes (21,22).

After the discovery of a shift of pyruvate kinase isozymes in hepatomas to a more fetal type (35), similar changes have been reported in brain tumor by Tolle et al (95).

I.1. Isozymes of Pyruvate Kinase

In mammalian tissues four distinct isozymes of pyruvate kinase have been identified, L, M1, M2 and R(7,39,88). These isozymes differ in their immunological and electrophoretic properties. Their distribution in tissue is specific. They also show different regulatory properties with allosteric effectors such as certain

amino acids and fructose 1,6 diphosphate. These are very important compounds for the coordination of glycolysis and gluconeogenesis (1,28,41,44,62,87).

The L-type isozyme is found in hepatocytes, in the small intestine and as the minor isozyme in the renal cortex (43,88,97). The M1-type isozyme is present in skeletal muscle as the only component, and in brain and cardiac muscle as the major component (13,94,97). The L-type is present in liver as the major component and in kidney as a minor component. Thus the M1 and L-types are present only in a few tissues (39,97). In erythrocytes, a specific R-type pyruvate kinase has been isolated (12,57) which is closely similar to L-type in immunological and genetic propertis, and amino acid composition (77,96). Pyruvate kinase in erythrocytes differs electrophoretically, immunologically and in amino acid composition from the M1, and M2-type (39). The R-type has a higher isoelectric point (12,13) and may posses a minor additional structure (12,77).

The M2-type isozyme, also frequently named K type (18,62,84,101) or pyruvate kinase class A (3,23), is widely distributed in high glycolytic tissues such as: liver, kidney, brain, heart, spleen, lung, adipose tissue, thymus, testis, ovary and tumor tissue (88,97). The electrophoretic patterns of the pyruvate kinase isozymes from rat tissues change frequently during

differentiation and dedifferentiation. The M2-type is predominant in fetal muscle, brain and liver. During differentiation from the fetus to the adult, the M2-type disappears and the M1-type becomes predominant in skeletal muscle and brain, the L-type increases and becomes predominant in liver(67). Thus it has been suggested (3,39) that the M2-type pyruvate kinase should be regarded as the prototype, while the M1 and L-types are the differentiated forms, since quantities of types M1 and L increase during the differentiation of the tissues.

The presence of hybrid isozymes has been demonstrated in many mammalian tissues, especially those from various rat organs (18,35,87) and tumor tissues (61,62,99).The tetrameric hybrids are composed of various combinations of type M1 and type M2 subunits. Five forms may be expected (78,85,98,108) when both M2 and M1 subunits are present, $(M2)_4$, $(M2)_3(M1)_1$, $(M2)_2(M1)_2$, $(M2)_1(M1)_3$ and $(M1)_4$. In fetal tissues, all five possible forms of pyruvate kinase are present as $(M2)_2(M1)_2$ which is the predominat form. In adult muscle tissue only $(M1)_4$ is present. Tumors are characterized by a profound shift to the M2-type; whereas the $(M1)_4$ type is not expressed at all (85).

The L, M1 and M2 type isozymes have distinct chemical

and physical properties. The L-type isozyme is relatievly more sensitive to inhibition by ATP, but less sensitive to inhibition to alanine and phenylalanine. It shows cooperative kinetics with phosphoenolpyruvate (21,38,41). Fructose 1,6 diphosphate allosterically activates the L-type isozyme. The M1 and M2 isozymes are both structurally and immunologically closely related (75,107), and share a common antigenic reactivity (34). However, they display different kinetic properties with respect to phosphoenolpyruvate. M1-type isozyme displays Michaels-Menten kinetics and is not activated by fructose 1,6 diphosphate (3,38,44); also it is not influenced by the amino acids, alanine and cysteine. M2-type pyruvate kinase, on the other hand was observed to display allosteric kinetics and to be activated by fructose 1,6 diphosphate (79). It is strongly, inhibited by the amino acids, alanine, phenylalanine and L-cysteine (3,28,101). Both isozymes (M1 and M2) showed hyperbolic kinetics with ADP. All the isozymes require K^+ and Mg^{2+} ions for their activity and are inhibited by ATP (3,38). Of the three pyruvate kinase isozyme found in mammals, only the liver or L-type isozymes exhibit responses to hormonal and dietary conditions(5,60,90).

I.2. Pyruvate Kinase In Tumors

Tanaka et al (1965) were the first to show

modifications of pyruvate kinase isozymes in cancer(90). They showed that in ascites hepatoma AH 130, most of the enzyme was M-type. This was confirmed by comparing pyruvate kinase in rat hepatoma with normal rat liver (21,78). They separated two forms of the enzyme by DEAE-cellulose chromatography, form I and form II. Form I is the predominate enzyme of poorly differentiated tumors. Schapira and Gregori (1971) showed that the pyruvate kinase isozyme from a poorly differentiated hepatoma (Reuber H. 178) resembles the placental pyruvate kinase from the point of view of its electrophoretic migration and kinetic behavior in the presence of fructose 1,6 diphosphate (78).

Imamura and Tanaka (39), using polyacrylamide gel electrophoresis, found that the major form in the AH 130 ascites hepatoma was identical with the minor (M) form in normal liver tissue. Walker and Potter (104), using starch gel electrophoresis, found an increased level of the minor M form in the preneoplastic liver, in the hyperplastic liver nodules, and in the hepatomas that developed after feeding of the carcinogen, 3'-methyl-4-dimethylaminoazobenzene, to rats. Taylor et al (93) reported that the poorly differentiated Morris hepatoma 3924 A had a pyruvate kinase that differed kineticaly from the rat liver isozyme. Farron et al (22) found that

rhabdomyosarcoma N 5104, which is carried in Fisher rats, had a pyruvate kinase pattern that was abnormal; its electrophoretic migration toward the anode on cellulose acetate strips was faster, and was similar to that of fetal muscle pyruvate kinase. Fetal and cancer tissue enzymes had the same electrophoretic mobilities as the M-type pyruvate kinase of adult and fetal liver tissues (78).

A switch toward the M2 type pyruvate kinase has been observed by many investigators who studied the kinetics and electrophoretic and immunological patterns of pyruvate kinase from different tissue sources, including the hepatoma, Ehrlich ascites, and other tumor tissues.

It was reported that in brain tumors (38,95) M1-type, which is present in normal tissue, is replaced by M2-type. During tumor development in the liver, a marked decrease in L-type pyruvate kinase and an increase in the M2-type pyruvate kinase have been described, particularly in the poorly differentiated hepatocellular carcinomas (15,50). In the non-cancerous portions of the liver of cancer-bearing animals, the L-type decreases, and the M2-type increases and becomes predominant with increased growth of the cancer. The isozyme pattern finally changes to that of cancer cells where the M2-type is the only component(39).

Tolle et al (95) found spontaneous tumors from rats which have been charaterized by a correlation between the amount of M2-type and the state of malignancy. Making use of the allosteric inhibition of the M2-type PK by L-alanine (62,101), a systematic study on the relative amount of the M2-type in different brain malignancies, as well as with differentiated glioma, i.e astrocytomas, oligodendrogliomas and ependymomas, was completed. In one histological study(62), it was suggested that the amount of inhibition of M2-type pyruvate kinase could be used to determine the grade of malignancy.

In general, from the studies on pyruvate kinase from tumors of different animal species, especially dogs, rats, chickens, human and Ehrlich ascite tumor cells, it is becoming increasingly evident that neoplasia is associated with profound alterations in isozyme patterns. The M2-type isozyme is predominant in various experimental tumors (22,72,83). This is in agreement with the suggestion that biochemical changes in tumors are not entirely random, the change is often toward the composition found in appropriate immature tissues (17,22,84). Probably M2-type isozyme is the prototype of pyruvate kinase isozyme in all mammal tissues (42,84).

Studies on the M2-type pyruvate kinase isozyme in tumors using tissue and cell cultures from different

animal species are now in progress and correlations between isozyme changes and the grades of malignancy and comparison with histological diagnosis is improving.

I. 3. Isozyme Expression

There is strong genetic evidence that the R and L-type pyruvate kinase isozymes are determined by the same structural gene but this gene does not code for the M1 and M2-type isozymes (35,37,68).

The M1 and M2 pyruvate kinases differ from the L and R-types by their immunological properties, amino acid composition and peptide finger print pattern (34,46,59,77,79). This suggests that genes of M1 and M2 types are different from L and R type isozymes. L and R type isozymes are encoded by the same gene but translated from different mRNAs (59).In various different mammals the M1 and M2 isozymes have been shown to be similar to each other immunologically and in amino acid composition except in humans, where the amino acid compositions of the M1 and M2 isozymes to be so different that they concluded that these two isozymes must be the products of separate genes (33,56,68). It has been demonstrated (64,65,89) that the differences in molecular weights and electrophoretic properties of the M1 and M2 isozymes result from difference in mRNA's coding for these two

isozyme types. These studies indicate that the M1 and M2 isozymes are either the products of two closely related genes or the products of a single gene processed into two distinct mature mRNAs. The M1 and M2 isozymes from several mammalian species were compared in their reactivity toward anti-pyruvate kinase monoclonal antibodies, using one and two-dimentional peptide mapping studies (31). The findings support the conclusion that the two isozymes are the products of a single gene (31,64). Amino acid analysis indicates that the difference is probably due to differential mRNA splicing. Other investigators explain that the difference between M1 and M2 isozymes could possibly be due to proteolytic cleavage of M1 subunits to give the smaller M2 molecule (65). There is a growing conviction that a common phenotypic expression of neoplasia may be an abnormality of genetic expression manifested by misprogramming of protein synthesis (110).

These four seemingly noninterconvertible forms of the enzyme exist in variant forms and may hybridize with each other.This has led to some confusion. Work is now in progress to establish whether the isozyme in tumor cells is generated by the differential splicing of mRNA (59,64), is coded by different genes (35,37) or it is the result of a proteolytic cleavage of a preformed enzyme (65,110).

I.4. Clinical point of View

I.4.1 M2-Type pyruvate kinase as a Marker in Cancer

Biochemical research in cancer was initiated some 65 years ago, with Warbug's discovery of the high aerobic glycolysis of tumor cells (78). An explanation, in molecular terms, for the uncontrolled growth and invasiveness that characterize cancer is yet not available. Evidence for isozyme alterations have been indicated by some laboratories that worked on rat hepatoma. Hepatomas in the rat lose to varying degrees of those isozymes that characterize the normal hepatocytes, which are involved in specific hepatic functions. As dedifferentiation and tumor progression occur, these liver "marker" isozymes disappear and are replaced by other isozymes which are low or absent in the well differentiated cell of origin (14,108,109).

The expression of oncodevelopmental markers by cancer cells is a well-established phenomenon. This includes the production of markers in both developmental and neoplastic tissues. Changes in isozyme patterns are frequently used as a marker. One of these markers is pyruvate kinase isozymes. A shift of the M1 to M2-type isozyme was correlated with the degree of malignancy (26,85,101,102). The tumors are characterized by a profound shift to M2-type where as M1-type is not

expressed at all(85). The isozyme shift of PK towards the M2-type was considered as an oncodevelopment marker for striated muscle cells and their malignant counterparts(85). A difference in isozyme composition of PK is found among the morphologically less differentiated tumors and tumors metastazised to lung; in the less differentiated ones $(M2)_4$ and $(M2)_3(M1)_1$ are predominant and no $(M2)_1(M1)_3$ and $(M1)_4$ is present, whereas in the metastazised tumor $(M2)_3(M1)_1$ and $(M2)_2(M1)_2$ are predominant with little and $(M2)_1(M1)_3$ (see table 1).

Well differentiated tumors grow slowly, have low glycolysis and a moderate to high respiration; where as the poorly differentiated tumors grow rapidly, have high rate of glycolysis, and low respiration (11). The tumor growth rate is positively correlated with the overall rate of aerobic glycolysis and PK activity. In myelodisplastic syndromes (92), elevated pyruvate kinase activity, with the persistence of the M2-type pyruvate kinase isozyme, was used as a diagnostic test for this disease (16). However liver neoplastic nodules induced by 25 weeks dietary thioacetamide adminstration demonstrated a clear decrease in pyruvate kinase activity can also used as a marker (50).

Amino acid inhibition of pyruvate kinase is used for

Table 1.: Isozyme composition of pyruvate kinase in normal, fetal and adult muscle, Rhabdomyosarcomas and the metastasis of a Rhabdomyosarcoma (85).

Tissue	Isozyme composition (%)				
	K_4	K_3M	K_2M_2	KM_3	M_4*
Fetal skeletal muscle (19 Weeks)	11	27	37	18	8
Fetal skeletal muscle (23 Weeks)	11	18	36	23	13
Adult skeletal muscle	--	--	--	--	100
Rhabdomyosarcoma (No 1) (Less differentiated)	57	38	5	--	--
Rhabdomyosarcoma (No 2) (Metastasis)	28	34	26	12	--
Rhabdomyosarcoma (No 3) (Less differentiated)	49	44	7	--	--

*) $K_4 = (M2)_4$, $K_3M = (M2)_3(M1)_1$, $K_2M_2 = (M2)_2(M1)_2$, $KM_3 = (M2)_1(M1)_3$, $M_4 = (M1)_4$

the diagnosis of neoplastic disorders. By alanine inhibition of the M2-type a systematic study has been carried out (72,100,101,102) on the relative presence of the M2-type in different brain malignancies. The inhibition of pyruvate kinase activity was measured in 51 gliomas with different grades of malignancy (62). It was confirmed that glioma benign tumors have a low level of inhibition (less than 50%) and that the more malignant the tumor, the higher the level of inhibition (more than 75%). However, when grade II and III astrocytomas and grade II and III oligodendrogliomas were analyzed, their level of inhibition was found to be variable. Grade II showed low and moderate levels of inhibition and grade III moderate and high levels. When the survival times of patients with brain tumors were compared with both the histological diagnosis and pyruvate kinase inhibition, the prediction of the survival time on the basis of low and high levels of inhibition correlated well with the histological diagnosis (62). Electrophoresis indicated the presence of mainly $(M2)_4$-type and the hybrid $(M2)_3(M1)_1$, which is in agreement with the alanine inhibition (101).

It was found (28) that a decrease in electrophoretic mobility of pyruvate kinase isozymes, and an increase of the immobilization and tumoriogenic properties,

respectively, in human urethelial cell lines was characterized by different grades of transformation in vitro. The utilization of pyruvate kinase specific isozymes kinetic propertis (inhibition by amino acids) as markers for benign and malignant tumors requires further investigation.

I. 4.2. Deficiency of Pyruvate Kinase

Many cases have been reported (40,61,82) since the original description of hemolytic anemia was associated with hereditary deficiency of erythrocyte pyruvate kinase. So far, more than 300 pyruvate kinase variant associated with chronic nonspherocytic hemolytic anemia have been described (82).

Acquired pyruvate kinase deficiency has been reported in various hemolytic disorders, such as acute leukemia, chronic myelogenous leukemia, malignant lymphoma, and myelodysplastic syndrome (4,51,103,111). Determination of red cell enzyme activities of patients with hemolytic anemia revealed that the red cell pyruvate kinase activity was remarkably low, being 15.1% of normal controls (53).

Table II. The molecular weight of M1,M2 and L-type pyruvate kinase from different species.

Animal	Tissue	Method of Estimation	Native MW	Subunit MW	Isozyme form
Chicken	Embryo Cells	SDS-PAGE(20)	240,000	60,000	M2
Rat	Ascites Hepatoma	Sephadex G-200 (41)	216,000	54,000	M2
Rat	Lung	SDS-PAGE(79)	224,000	64,000	M2
Human	Kidney	Sedimentation Equilibrium(34)	206,000	52,000	M2
Bovine	Skeletal muscle	Sedimentation Equilibrium(9)	230,000	57,000	M1
Rat	Liver	Sephadex G-200(41)	250,000	62,000	L
Bovine	Brain	SDS-PAGE(94)	229,000	57,000	M1
Rabbit	Muscle	Sephadex G-200(13)	230,000	57,000	M1
Human	Liver	Gel filtration(58)	240,000	60,000	L

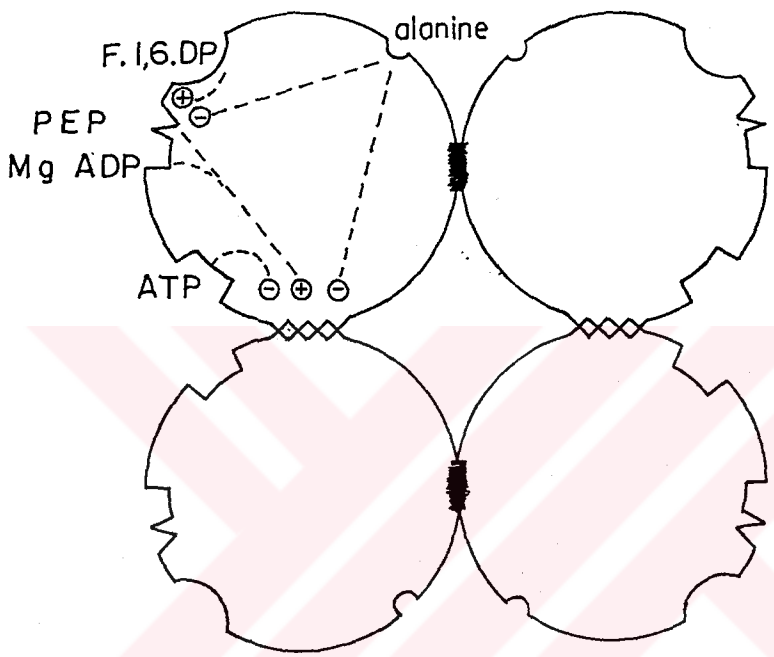


Figure 1 : Tentative molecular model of M2-type pyruvate kinase (24).

I.5. The Physicochemical Properties of M2-Type Pyruvate Kinase

I.5.1. Molecular Parameters and Subunits

The molecular weight of M2-type pyruvate kinase has been determined from many mammalian sources by different methods (20,34,41,79,83,84). It appears to be a homotetramer (73,79), and its molecular weight seems to be quite similar to the other isozyme forms of pyruvate kinase (table II). The four subunits of the M2-type isozyme are identical and its tentative molecular model is given in Figure 1.

I.5.2. Amino Acid Composition and pI

Table III (2) gives the amino acid composition of pyruvate kinase from several tissue of the dog, chicken and rat.(19,32,76,79).

The values for the tumor M2 isozyme are, in general, within the range reported for other pyruvate kinase isozymes. The lower isoelectric point of the M2 isozyme from dog tumor as compared to the dog lung isozyme is reflected by an increased amount of glutamate in the tumor enzyme (table III). It has been shown that most pyruvate kinase isozymes of the M2-type are closely related (79). On the basis of amino acid composition M2

is more similar to M1 than L-type pyruvate kinase (32).

Human pyruvate kinase isozymes (34) have blocked or labile NH_2-terminal amino acids. Similar results have been reported for all other pyruvate kinase enzymes from other animal species (30,33).

Table IV shows pI values of M2-type pyruvate kinase from different sources with and without fructose 1,6 diphosphate. The enzyme complexed with fructose 1,6 diphosphate showed a little lower ionization point (36).

The pI values of the M2 isozymes vary a little depending on the species and tissues which are evaluated. Moreover, the isoelectric point of the M2 isozyme from dog tumor is a little lower than dog lung isozyme (2), which is the result of an increase in the amount of glutamate in the tumor enzyme.

I.5.3. Stability of The M2-type Pyruvate Kinase

I.5.3.1. pH and Temperature Effect

The activity of M2-type pyruvate kinase isozyme varied considerably with pH (2,36,38). The optimum pH is between 7 to 7.8. M2-type pyruvate kinase isozyme shows maximum activity at pH 7.0 in the absence, and at pH 7.6 in the presence of fructose 1,6 diphosphate (3,83,84). At pH levels below 7.0 the M2-type enzyme appeared to

Table III. Comparison between amino acid analysis of pyruvate kinase isoenzymes, isolated from different tissues of dog, chicken and rat (2).

	Type M1			Type M2					
	Muscle			Lung			Lung Tumor		
	Dog	Chicken	Rat	Dog	Chicken	Rat	Dog	Chicken	Rat
Lys	33	38	39	26	28	35	27	40	39
His	13	19	12	16	21	22	13	14	13
Arg	34	32	33	27	28	27	23	33	32
Asp	51	49	49	50	59	50	51	53	49
Thr	26	24	23	25	27	25	28	29	27
Ser	26	25	27	35	33	30	50	40	27
Glu	69	49	51	75	63	56	87	47	52
Gly	40	42	41	47	51	50	53	53	42
Ala	56	57	61	72	54	48	46	56	60
Val	48	43	46	40	41	37	36	43	48
Ile	33	33	36	29	29	26	22	24	48
Leu	42	37	45	38	48	43	37	47	43
Tyr	10	7	10	9	15	13	13	16	10
Phe	18	16	16	16	21	19	17	18	16
Met	16	18	18	10	11	9	11	14	16
	515	492	507	515	529	490	514	527	513

Table IV- Isoelectric points (pI) of M1 and M2 type pyruvate kinases (with and without F-1,6-DP) from different organs of dog, rat and mouse.

Pyruvate kinases Isozyme	Animal source	Tissue	Isoelectric Point (pI).
Without F-1,6-DP			
M2	Dog	Lung(2)	7.73
M2	Dog	Lung tumor(2)	7.31
M2	Rat	Kidney Cortex(3)	6.4
M2	Rat	Lung(19)	5.8
M2	Mouse	Spleen(36)	6.6
M1	Dog	Muscle(2)	8.86
With F-1,6-DP			
M_2	Mouse	Spleen(36)	6.2
M1	Mouse	Spleen(36)	7.2

follow Michaelis-Menten type of enzyme kinetics with respect to phosphoenolpyruvate (3). However, at pH 7.5 and 8.0 this was not the case, and a straight line was not obtained in a double reciprocal plot.

The optimum temperature of pyruvate kinase varies between 25°C and 35°C. The pyruvate kinase activity has been measured at this range of temperature by most of the workers. The thermostability of leukocyte M2-type pyruvate kinase was studied at 50°C (46). In this study the activity was decreased 56% within 60 minutes.

I.5.3.2. Preservation of Pyruvate Kinase

The purified pyruvate kinase enzyme is usually preserved at -10°C to 4°C in different solvents (ammonium sulfate, glycerol, ethylene glycol) by most laboratories. When bovine brain enzyme (M1-type) was kept in 20% V/V glycerol at -10°C (94) the loss of activity was no more than 4.5% in a month. Upon storage of the L-type enzyme in 30% glycerol at -20°C for 3 days in the absence of fructose 1,6 diphosphate, PK dissociated into dimers, and in the presence of 0.1 mM F-1,6-DP, it behaved as a tetramer (34,45). The effect of organic solvents on the kinetics of M2-type pyruvate kinase from hepatoma was studied (41). It was activated by low concentrations of the solvents (25% ethylene glycol, 20% glycerol), and

inactivated by higher concentration of them. In the presence of 25% V/V ethylene glycol, no further activation of the enzyme was observed by addition of fructose 1,6 diphosphate.

I.6. Kinetic Characterization and Influence of Allosteric Effectors

I.6.1. Kinetic Properties

The kinetic properties of M2-type pyruvate kinase isozymes which have been isolated from different sources are slightly similar. The enzyme shows a sigmoid saturation curve with respect to phosphoenolpyruvate (2,17,24,46,79). A plot of M2-pyruvate kinase activity against PEP concentration is similar to that of L-pyruvate kinase (sigmoidal behavior); however, the cooperative interaction of M2-pyruvate kinase with phosphoenolpyruvate is lower than that of L-type enzyme (38,41). Similar kinetic behaviors (sigmoidal saturation curve, poor cooperativity, Hill coefficient near one for both enzymes) of M2-type pyruvate kinases from human leuckocyte and erythrocytes have been observed (46). Considerable kinetic differences were found between the M2-type pyruvate kinase from lung and dog tumors, with respect to their half maximal saturation with

phosphoenolpyruvate and their response to allosteric activators and inhibitors (2). An approximate Km value (101) for the substrate, phosphoenolpyruvate, with pyruvate kinase from brain tumor meningioma is about 2 mM. This is higher than the Km of M2-type isozyme from other tissue sources (2,3,46,79). M2-type enzyme shows hyperbolic kinetics with respect to MgADP (10,24). The reactivity of M2-type pyruvate kinase from different sources toward dinucleotides decrease in the following sequence; ADP>GDP>UDP>CDP. M2-type isozyme is inhibited by ATP. A greater effect of ATP on keeping the enzyme in the sigmoidal kinetic form, than the other nucleotide triphosphates(GTP,UTP,CTP) have been observed (24,38,41) The inhibition of M2-type pyruvate kinase by ATP is partially reversed by Mg^{2+} ion (3,41).

I.6.2. Effect of Fructose 1,6 Diphosphate and Amino Acids on M2 -Type Pyruvate Kinase

Kinetic properties of pyruvate kinase activity from fetal animal thigh muscle, determined as a function of phosphoenolpyruvate concentration in the absence and presence of fructose 1,6 diphosphate (86). The sigmoidal velocity curves of this enzyme became hyperbolic when fructose 1,6 diphosphate was added. The properties of M2-type pyruvate kinase from rat lung (79) was studied. The

cooperativity indicated by the Hill coefficient decreased upon the addition of 0.2 mM fructose 1,6 diphosphate. Similarly, the Km value for phosphoenolpyruvate changed from 0.26 to 0.056 mM. M2-type pyruvate kinase from leukocytes and red blood cells, were studied, in both cases Km value for phosphoenolpyruvate decreased upon the addition of fructose 1,6 diphosphate (46). When pyruvate kinase from Yoshida ascite hepatoma 130 cells, was incubated with $2x10^{-4}$ M fructose 1,6 diphosphate at 37°C, the phosphoenol pyruvate saturation curve was transformed to a monophasic, normal Michaelis-Menten type. When the fructose 1,6 diphosphate was removed, the phosphoenolpyruvate saturation curve of this enzyme became sigmoidal (41).The M2-type pyruvate kinase from normal and tumor dog lung activated by fructose 1,6 diphosphate allosterically with a Ka value of 0.03 mM and 0.02 mM respectively (2).

Some workers suggested that fructose 1,6 diphosphate did not activate the pyruvate kinase from Ehrlich ascites tumor cells in the absence of inhibitory amino acids, but reversed the inhibition by alanine and phenylalanine with an apparent Ka of 0.003 mM (23,83).

Alanine and phenylalanine inhibit M2-type pyruvate kinase from different sources (23,24,39,62,83,84,101). It

was found that alanine inhibits pyruvate kinase from human fetal brain and from the brain of a newborn more than adult brain tissue (101). Phenyalanine and alanine inhibited both placental and tumor pyruvate kinase (84). While serine activated the isozyme from both of the tissues. Serine is an activator for M2-type pyruvate kinase (2,84) and L-cysteine is an inhibitor for this enzyme from different sources (28,47,55).

L-alanine acts as negative allosteric effector on the enzyme from ascites tumor cells (83). The allosteric effect of alanine which causes a dissociation of the tumor pyruvate kinase (Ehrlich ascites) into two half molecules can be reversed by the positive effector, fructose 1,6 diphosphate (Fig. 2).

ATP, alanine and phenylalanine are important effectors. They dissociate the class A (or M2 type) pyruvate kinase from Ehrlich Ascites tumor cells into dimers. After incubation with fructose 1,6 diphosphate the enzyme is converted into the kinetic form which gave a hyperbolic saturation curve for phosphoenolpyruvate and high affinity for this substrate (24). Based on this property, a tentative molecular model was suggested (24)for the enzyme from Ehrlich ascites tumor cells (figure 3).

The model assumes two main conformations of a single

type of subunit and the degrees of association of the protein. The dimers would be inactive or markedly less active than the tetramers. The actual validity of this model for the pyruvate kinase of Ehrlich ascites tumor cells and its presumptive applicability to M2 type pyruvate kinase from other tissues that may contain related but different isozymes, require further investigations.

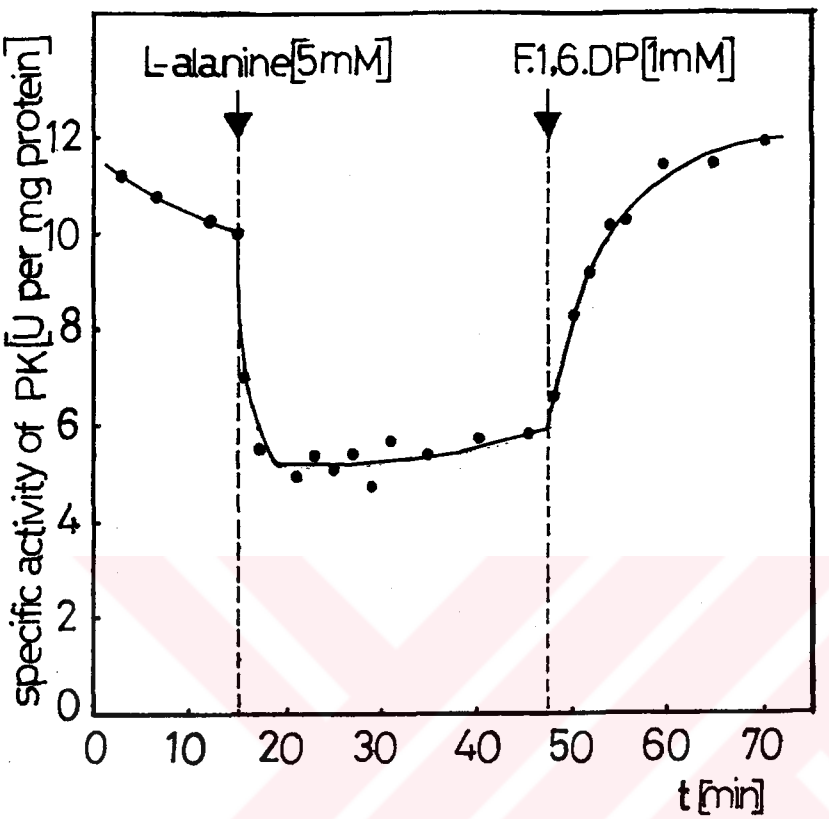


Figure 2 : Inhibition and reactivation of pyruvate kinase by L-alanine and fructose 1,6 diphosphate. Reproduced from Sparman, J., and Schulz, J.,(83).

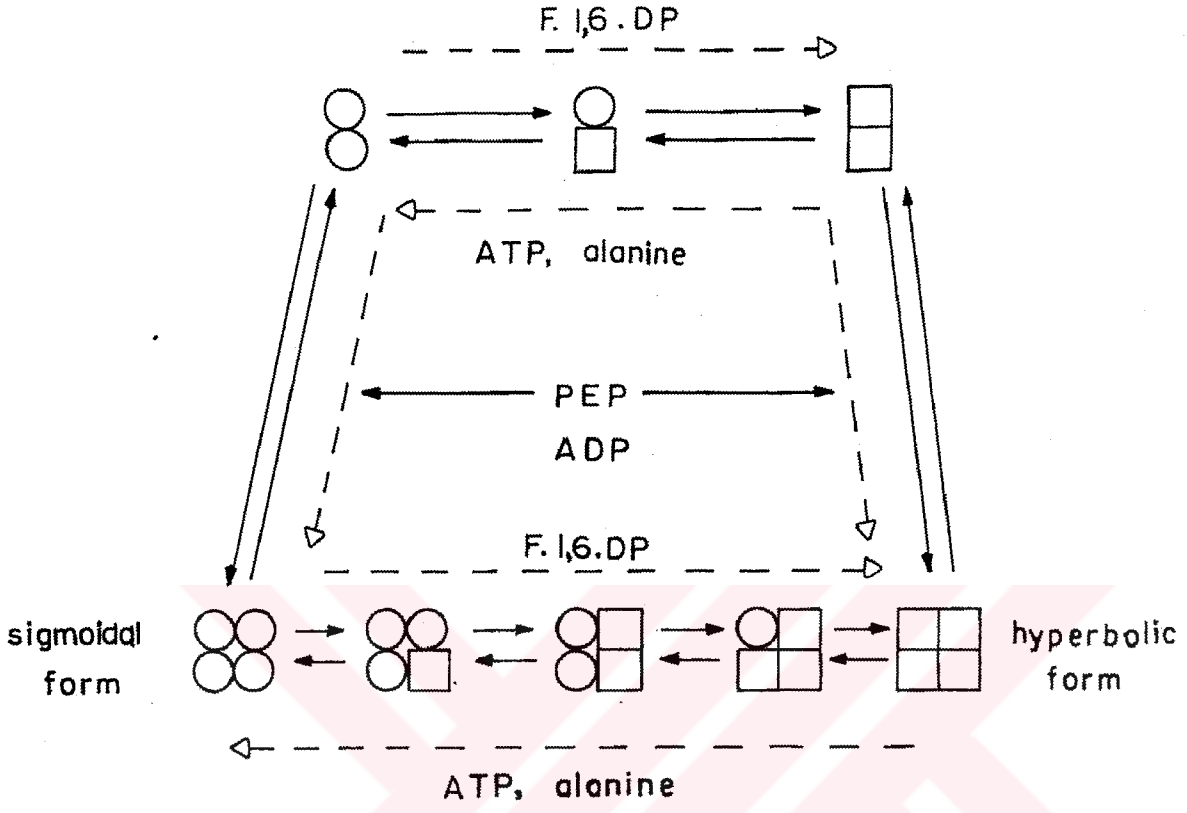


Figure 3 : Two main conformations of a single type of subunit, two degrees of association of the protein, dimers and tetramers. PEP and ADP associate the enzyme to a tetramer. F-1,6-DP and alanine change the conformation toward active and inactive forms respectively (24).

I.6.3. The Effect of Cations

It has been reported that Mg^{2+}, K^{+}, Mn^{2+} ions are activators and Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+} are inhibitors of M2-type pyruvate kinases from different sources (3,24 ,38,41,75,79). Both the pig muscle and kidney pyruvate kinase were found to be inactive in the absence of potassium or magnesium ions, maximal activity was obtained at 50 mM potassium chloride (3). It was found that Mg^{2+} at high concentrations inhibited the M2-type pyruvate kinase (38,74,79), however, some other investigators claimed that even high concentration of Mg^{2+} (30 mM) did not inhibit the enzyme (71,94). It was indicated that Mg^{2+} protected the enzyme from the inhibitory effect of ATP, by complexing with it (25,43). The exact function of these ions in the overall catalytic mechanism is still unknown.

I.7. Hormonal and Dietary Condition

The M2-type pyruvate kinase in liver (Yoshida ascites hepatoma 130 cells) is apparently not under hormonal or nutritional control (41). The M2-type pyruvate kinase from rat liver showed only a slight response to change in the diet and hormones (91) and the activity of the M2-type isozyme from rat small intestine did not fluctuate following hormonal and dietary changes (67).

In carbohydrate deficient rats, refeeding carbohydrate the rat within 24 hours and increased its activity to twice the normal level after 72 hours. Little or no change occured in M2-type pyruvate kinase (21). Of the four isozymes of pyruvate kinase found in mammals, only the liver or L-type isozyme exhibits major changes in the amount of enzyme present in response to hormonal and dietary fluctuations (67,70,88,90); while the specific activity of the M2-type isozyme in liver remains almost constant. However it was suggested by other workers that gluconeogenesis in rat kidney, which contains a high amount of pyruvate kinase type M2, is regulated by hormones, glucagon and epinephrine (80).

Tyrosine phosphorylation of M2-type pyruvate kinase from chicken embryo cells by Rous sarcoma virus (RSV) leads to a reduction in the affinity of pyruvate kinase for the substrate phosphoenolpyruvate (73). Cyclic adenosine3':5'- monophosphate(cAMP)-independent protein kinase phosphorylates and inactivates the M2-type pyruvate kinase from human brain tumor (106) and chicken embryo cells (72), and pyruvate kinase M2-type, from rat epidymal fat pads, is phosphorylated by a cAMP-independent protein kinase (79).

In several experiments with perfused human liver or in vitro, the short term regulation of M2-type pyruvate

kinase by insulin was demonstrated (25) with physicochemical and immunochemical methods.The Michaelis-Menten constant for PEP was found to be decreased in M2-type pyruvate kinase of epidymal fat pats from rats exposed to insulin (79). By some workers it was suggested that tumor pyruvate kinase resembled muscle enzyme with regard to independency from hormones and dietary conditions (78).

Some of the findings (25,63,72,73,79,80,106) indicate, not only a regulatory role of hormones on the activity of M2-type pyruvate kinase, but also suggest that the enzyme may be regulated by a cAMP-independent pyruvate kinase in general. However others (21,41,67,78,91) suggest that M2-type pyruvate kinases from rat liver, rat intestine and tumors did not fluctuate upon dietary and hormonal effects.

I.8. Aim of This Study

Several studies have been reported in the literature, using crude preparations or homogenates, or using partially purified pyruvate kinases of human brain tumors (62,99,101,106). However, there are very few kinetic studies. It has been reported by several authors that the dominant tumor isozyme is M2-type (21,95,99,101). This isozyme shift in brain cancer from M1 to M2-type

pyruvate kinase is used as a tumor marker.

The aim of the present study was to purify M2-type pyruvate kinase from human meningioma, and to study the electrophoretic and kinetic properties of the enzyme. The effects of L-alanine and fructose 1,6 diphosphate as negative and positive effectors were studied and a mechanism of the action of these two effectors was suggested.

CHAPTER II

MATERIALS AND METHODS

II .1 Materials

Reactive Blue-4-Agarose, Carboxymethyl-cellulose, 6-amino-n-caproic acid, Adenosine-5'-triphosphate (disodium salt),phosphoenolpyruvate (monopotasium salt), pharmalytes (pH 5-8) and (pH 3-10), L-alanine, DL-dithiothreitol (DL - DTT), MOPS were purchased from Sigma Chemical Company. Adenosine-5'-diphosphate (disodium salt), NADH (disodium salt), lactate-dehydrogenase (rabbit muscle), fructose 1,6 diphosphate (trisodium salt) were obtained from Boehringer (Mannheim,FRG). Common chemicals and acrylamide, N;N'-methylene-bis-acrylamide came from Sigma Chemical Company. All other chemicals were standard reagent grade.

Brain tumors (meningioma) were obtained from SSK DISKAPI Hospital and kindly supplied by Dr. Nur Altinors.

II.2. Methods

II.2.1 Purification of M2-type Pyruvate Kinase

All steps of purification were carried out in the cold room at 4°C. M2-type pyruvate kinase was purified according to earlier methods (2,33) with some

modifications. A piece (45 g) of the tumor tissue, fibroblastic meningioma which was a left parietal lobe from 48 years old female was taken from surgery and delivered into the laboratory in ice. It was washed free from blood in 100 mM Tris-HCl buffer (pH 7.0) at room temperature, and the extraction was carried immediately (in few of the previous purifications the tissue was frozen and kept at -20°C until use).

STEP 1 : Extraction

Washed tissue (42 g) was homogenized in a Waring blender in a 250ml Tris-HCl buffer (pH 7.0) containing 1mM EDTA and 20mM $MgCl_2$ at 4°C for 1.5 minutes.

The homogenate was centrifuged (Sorvall RC2-B,SS 34 Rotor) at 10,000xg for 15 minutes. The supernatant was ultracentrifuged (Beckman ultracentrifuge, Spinco 12658) at 108,000g for one hour. Then the supernatant was filtered through two layers of cheesecloth.

STEP 2 :Ammonium sulfate Fractionation

The supernatant from ultracentrifugation (140ml) was brought to 40% saturation by addition of 31.6g solid $(NH_4)_2SO_4$ and centrifuged at 15,000xg for 15 minutes. Then 33.5 g solid $(NH_4)_2SO_4$ was added to the supernatant

to give 80% saturation and pyruvate kinase was precipitated by centrifugation at 15,000xg for 15 minutes. The precipitate was suspended in 20 ml H_2O and stirred for 1 hour in the cold room.

STEP 3 :CM-Cellulose Chromatography

The enzyme solution (22ml) was desalted on a Sephadex G25 column (35x2.5cm) and then applied to a 22x1.8 cm column of CM-cellulose (Whatman CM52) which was equilibrated with 50 mM potassium phosphate buffer (pH 5.5) containing 1mM 6-amminocaproic acid, 1mM 2-mercaptoethanol and 5 mM $MgCl_2$.

The column was washed with 100 ml of the above buffer. The enzyme was eluted from the column with a linear KCl gradient (0.0 to 0.4 M) in 200 ml of the same buffer. Elution was performed at a flow rate of 40 ml/h. Pyruvate kinase containing fractions (eluted at 0.25-0.3M KCl gradient) were combined (35 ml) and dialyzed overnight against 1 liter of 20 mM Tris-HCl buffer (pH 7.0) which contained 1mM 2-mercaptoethanol, 1 mM 6-aminocaproic acid, and 5mM $MgCl_2$.

STEP 4 :Chromatography on Blue 4-Agarose

The dialysed pyruvate kinase (37ml) was applied onto a Blue 4-Agarose column (1.5x8cm) previously equilibrated with dialysis buffer. The column was washed with 400ml

of dialysis buffer. Pyruvate kinase was then eluted by applying a gradient of KCl from 0.0 to 500mM, in 100ml of the above buffer which was supplemented with 0.4mM fructose 1,6 diphosphate.

The enzyme thus purified was stored in 25% ethylene glycol (41) or in 2M ammonium sulfate with or without 0.2 mM fructose 1,6 diphosphate at 4°C.

II.2.2 Protein Determination

Protein concentration was measured by the method of Lowry et al (54), using bovine serum albumin as a standard. During the purification steps, protein was assayed spectrophotometrically at 280 nm.

II.2.3 Determination of Enzymatic Activity

Two methods were used for assay of pyruvate kinase. During the purification procedures the enzyme activity was assayed according to the method of Kimberg and Yielding(48) by measurement of pyruvate formed from phosphoenolpyruvate in the presence of ADP with 2,4-dinitrophenylhydrazine as a color reagent.

Throughout the purification steps and in the kinetic experiments, the activity of pyruvate kinase was determined at 32°C by the rate of NADH oxidation in a

reaction coupled with lactate dehydrogenase at 340nm (6), using a Spectronic 21 (Bausch and Lomb) equipped with a recorder. The entire mixture was preincubated at 32°C for three minutes and the reaction was started by addion of 10 µl enzyme (which was diluted two folds with 5 mM MOPS-KOH buffer, pH 7.0). The temperature of cell compartment was controlled with a thermostated circulator.

The reaction mixture contained in 0.6ml : 25 mM Tris-HCl buffer (pH7.2), 0.21mM NADH, 1.5 units of lactate dehydrogenase, 10mM 2-mercaptoethanol, 5mM $MgCl_2$, 0.6 mM fructose 1,6 diphosphate, various concentrations of ADP (0.1-8.0mM) and various concentrations of phosphoenol-pyruvate (0.05-5.0mM), 1 to 3 mM of ATP, and L-alanine(0-5mM).

One unit of pyruvate kinase activity was defined as the amount of enzyme converting one µmole of phosphoenolpyruvate per minute at 32°C and specific activity is given as enzyme units/mg of protein.

II.2.4 Cellulose Acetate Paper Electrophoresis

Cellulose acetate electrophoresis and detection of pyruvate kinase activity was done according to the procedure of Cardenase and Dyson(8) with some modifications.

Separation of pyruvate kinase isozymes on cellulose

acetate paper (Sartorius GmbH 3400 Gottingen West Germany,7x7cm) was carried out for 4 hours at 85 volts (12volts per cm) at room temperature using the 0.02M Tris-HCl buffer (pH 7.2) which contained 0.5M sucrose, 1mM EDTA and 10mM 2-mercaptoethanol. Bands of pyruvate kinase activity were visualized by pressing the cellulose acetate strip against an agar film containing components of lactate dehydrogenase coupled assay. Exposure to ultraviolet light revealed a strong fluorescence by NADH except in regions where pyruvate kinase coupled to lactate dehydrogenase converted NADH to NAD^+.

The agar film was prepared as follows; a) The coupled enzyme assay mixture, 2mM ADP, 2mM phosphoenolpyruvate, 1mM NADH, 0.1 M $MgCl_2$,0.1M KCl, 10 units per ml of lactate dehydrogenase dissolved in 2ml of 0.05M imidazole-HCl (pH 7.2). b) 100 mg agarose which was dissolved in 8 ml of 0.05 M imidazole-HCl (pH 7.2) and boiled until agarose was melted. After lowering the temperature of agarose solution to 50°C, the solutions of a and b were mixed and applied to clean glass plates (8x8 cm) .

Sample solutions (3 µl) from different fractionation steps, (homogenate, supernatant of ultracentrifugation and elution from affinity column chromatography) were

applied on cellulose acetate paper respectively. Electrophoresis was carried out in a LKB electrophoresis apparatus for 4 hours until bromophenol blue reached to the bottom of the paper. After electrophoresis, the cellulose acetate paper was removed from apparatus by carefully cutting it. Then, the cellulose acetate paper was pressed against an agar reagent assay plate and allowed to stay at 30°C for half an hour .

The reaction was followed visually by observing changes in intensity of fluorescence of the pyridine nucleotide upon illumination with 340 nm light (short range UV lamp was used). After bands appeared, the patterns were photographed by Bioblock Scientific UV Trait (BP 11) using a 667 polaroid film.

II.2.5 SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out essentially according to Weber and Osborn (105) and SDS-Polyacrylamide gels were prepared according to the method of Laemli (52).

The molecular weight of purified pyruvate kinase was determined by using 12.5% SDS-Polyacrylamide slab gel electrophoresis. The separatory buffer contained 1.5 M Tris-HCl (pH 8.8) with 0.4 percent SDS and stacking buffer was prepared by 0.5 M Tris-HCl (pH 6.8) with 0.4

percent SDS. Gels were prepared from a stock solution of 30 %W/V of acrylamide and 0.8 %W/V of N,N'-methylene-bis-acrylamide. For 30ml of 12.5 % gel, 12.5ml of acrylamide :N,N'methylene bis acrylamide solution (30 : 0.8) and 7.5ml of separatory buffer were mixed, then 10ml distilled water was added and the gel was polymerized by the addition of 0.15ml ammonium per sulfate (10%) and 30µl tetramethylethylene diamine (TEMED). After mixing the solution, 13.5cm of running gel was prepared in slab and 2cm of 4.7 percent of stacking gel was layerd on top. The stacking gel(4.7%) was prepared by mixing 1.25 ml of acrylamide: N,N'-methylene-bis-acrylamide (30 :0.8), 0.94ml of stacking buffer, 5.16ml of dH_2O and polymerized by the addition of 75µl 10% ammonium per sulfate and 6µl TEMED.

Standard proteins were dissolved separately in water (1 mg/ml) and 10µl from each were mixed with 40µl sample buffer.40µl of sample buffer was also added to each of the enzyme sample solutions. Sample buffer (pH 6.8) contained 5% SDS, 80mM Tris-HCl, 10% v/v glycerol and 0.001% bromophenol blue. The proteins were completely dissociated by immersing the samples for 2 min in boiling water. Then 20µl of each sample were applied to gel. Running gel buffer (pH 8.3), contained 0.25 M Tris-HCl,

0.192M glycine and 0.1 percent SDS.

Electrophoresis was carried out at room temperature at a constant current of 20 mA. When the dye front (bromophenol blue) reached to the bottom of the gel (about 10 hours), the gel was removed from slab and stained with 0.2% Coomasie Brillant Blue in methanol-glacial acetic acid-distilled water (40:7:53)for 3 hours. Destaining was carried out using a methanol-acetic acid-water mixture (50:75:850).

Molecular weights were determined by comparing the mobilities with a standard molecular weight curve, prepared by using Bovine serum albumin (MW 67,000), Egg albumin (MW 45,000), Trypsin (MW 23,300), ß-Lactoglobulin(MW 18,400) and Egg white Lysozome (MW 14,300).

II.2.6 Isoelectrofocusing

Isoelectrofocusing was carried out according to O'Farrell (66) in 5% ampholyte (pH 3-10 , 5-8) at room temperature for 10 hours at 400 volts and 8 hours at 800 volts.

"UKS" solubilization buffer was prepared as follows; 5.7g urea was dissoved in 2ml of water (vigorously stirred by magnetic stirrer). 50mg of dipotassium

carbonate (K_2CO_3), 1.25ml of 10% sodiumdodecylsulfate (SDS), 50mg dithiothreitol (DTT), 60µl Triton X-100, 200µl ampholyte (pH 3-10) was added to urea solution and the volume of solution completed to 4ml by distilled water. The solution was strirred for half an hour.

To prepare 6ml of isoelectrofocusing gel solution: 4g urea was dissolved in 3ml H_2O, 33mg N,N'-methylene-bis-acrylamide was added and stirred until all the urea was dissolved.Then, to this solution, 1.89 ml acrylamide (30%), 0.48 ml ampholyte (pH 5-7), 0.12ml ampholyte (pH 3-10), and 0.45ml Triton X-100 were added then the volume was brought to 6ml by distilled water. For polymerization of this gel 25µl ammonium per sulfate (10%) and 12µl TEMED (N,N,N',N - tetramethylethylene diamine) were added.

Isoelectrofocusing gels were made in glass tubes (110x1.6mm inside diameter) sealed at the bottom with parafilm. Immediately after addition of TEMED, the gel solution was loaded into the four gel tubes. To avoid trapped bubbles, the tubes were filled from the bottom by a syringe. The tubes were filled until 1.5cm from the top. After one hour the gel was overlayed with 1:4 diluted "UKS" solubilization buffer. The tube gels were kept at room temperature overnight. Then, the parafilm was removed from the bottom of each tube, and the tube

gels were placed in a standard tube gel electrophoresis chamber (before placing the gel tube in chamber lower reservoir was filled with 0.01M H_3PO_4). The solubilization buffer was removed from the surface of the gel and 30µl purified enzyme sample solution, which was diluted with "UKS" solubilization buffer (2µg enzyme) was loaded by micropipette on each gel tube. The enzyme sample solution were overlayed with 1:4 diluted solubilization buffer. The upper reservoir was filled with 0.02M NaOH (before addition to the reservoir, NaOH solution was extensively degassed to remove CO_2). The gels were run at 400 volts for 10 hours and then at 800 volts for 8 hours. After this period the power was turned off and tubes were removed from chamber.

The gels were removed from the tubes, by pressurized water from a syringe. The pH profile, was determined by slicing the gel to 0.5cm pieces and extracting in water. The gel pieces were placed in small tubes and 2ml dH_2O was added. The tubes were closed and left for one hour. After that each tube was vortex mixed for 30 seconds and the pH of the solutions were measured.

Other gels, after removing from tubes, were placed in an erlemayer flask. 50ml of 5% TCA (trichloroacetic acid) was added. This was shaken slowly in an incubator at 30°C

(every few hours, the solution was discarded and new solution of 5% TCA was added). This washing was continued until the ampholytes were completely leached out. The gels were then stained by 0.2% Coomassie Brillant Blue in ethanol-glacial acetic acid-water(40:7:53) for one hours, then destained by ethanol-acetic acid-water mixture (50:75:850) for 48 hours. Gel scanning was done by a CAMAG densitometer. For determinig the position of the protein band, the gel was compared with the pH profile of the sliced gel .

CHAPTER III

RESULTS

III.1 Purification of M2-type Isozyme of Pyruvate Kinase

Purification steps of human brain tumor (meningioma) M2-type pyruvate kinase are summarized in Table V. The degree of purification was 49.3 fold. The yield was 6.5% with a specific activity of 33.4 units/mg of protein.

In Figure 4 the CM-cellulose chromatography profile is given. The enzyme activity was assayed in each fraction and the resolution of M1 and M2 isozymes was achieved. The first isozyme was eluted at 0.0 to 0.1 M KCl gradient and the activity was independent of fructose 1,6 diphosphate,mainly corresponding to the M1-type isozyme. The second peak eluting at 0.2-0.3 M KCl gradient was the M2-type pyruvate kinase. Using CM-cellulose chromatography M2-type isozyme was purified 3.18 fold with %30.5 yield.

Further purification of the enzyme was achieved by affinity chromatography on Blue 4-Agarose column, (Fig.5). The majority of activity was obtained between 0.1 and 0.25 M KCl gradient. Enzyme in the pooled fractions (number 280 to 300) gave a specific activity of 33.4 U/mg protein, and the degree of purification was 49.3 fold.

Table V.: Purification of M2-Type pyruvate kinase from human brain tumor (meningioma).

Purification Steps	Volume (ml)	Total Enzyme Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purfication Factor
10,000xg Supernatant	170	1,003	1,482	0.677	100	1
108,000xg Supernatant	140	994	725.9	1.37	99	2.02
Amm. Sulfate Fractionation (40-80%)	22	374.3	441	0.85	37.3	1.25
CM-Cellulose Column Chromatography	35	305.9	142.34	2.15	30.5	3.18
Affinity Chromatography on Blue 4-Agarose	27	65.1	1.95	33.4	6.5	49.3
Three Fractions With Highest Activity	4.5	24.75	0.22	112.5	2.46	166.2

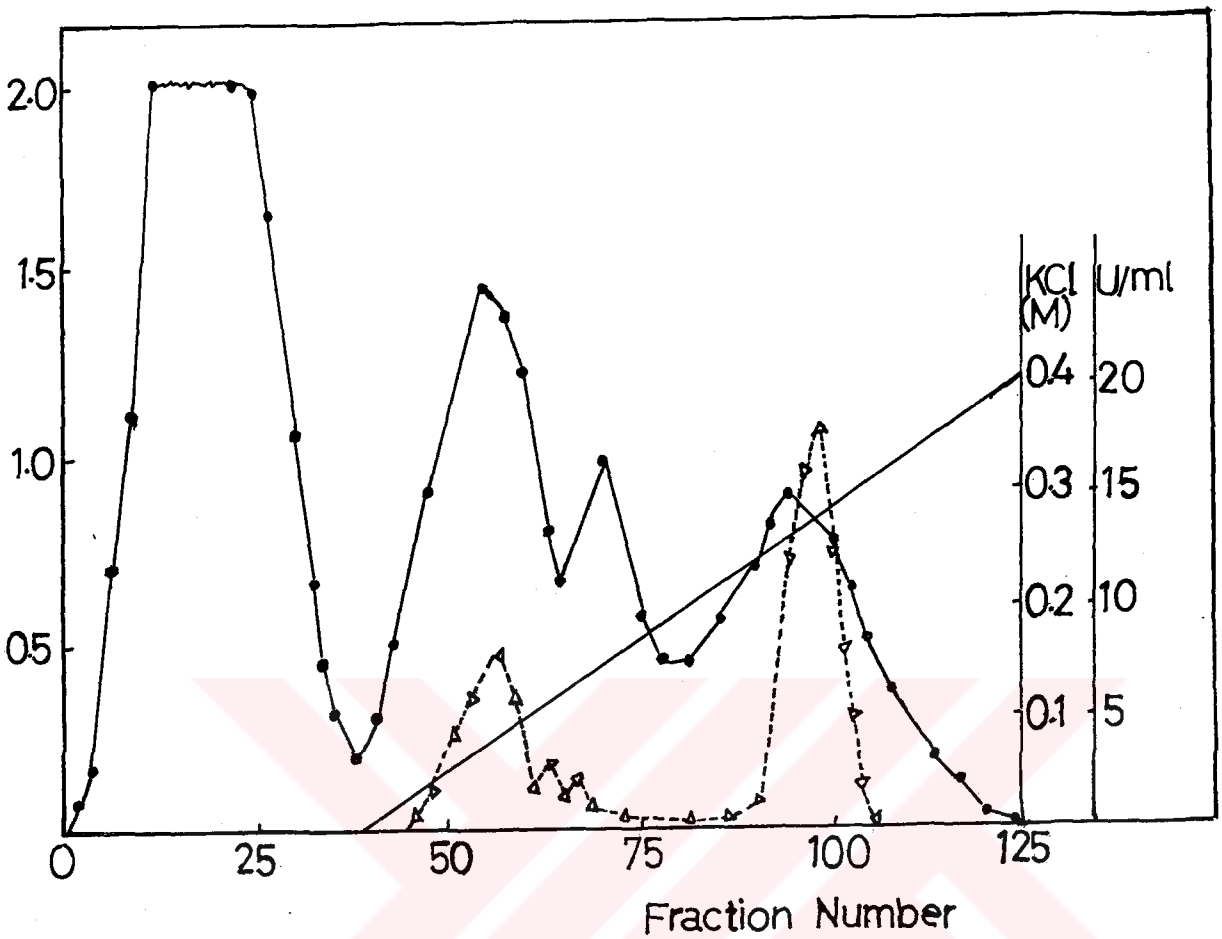


Figure 4.: CM-Cellulose column chromatography

Column dimensions: 22 x 1.8 cm
Flow rate : 40 ml/hr
Fraction volume : 2.5 ml
Washing buffer : 50 mM potassium phosphate buffer (pH 5.5), 1 mM 6- aminocaproic acid, 1mM 2-mercaptoethanol, 5 mM $MgCl_2$.

●——● : Absorbance at 280 nm

△·····△ : Pyruvate kinase activity (U/ml)

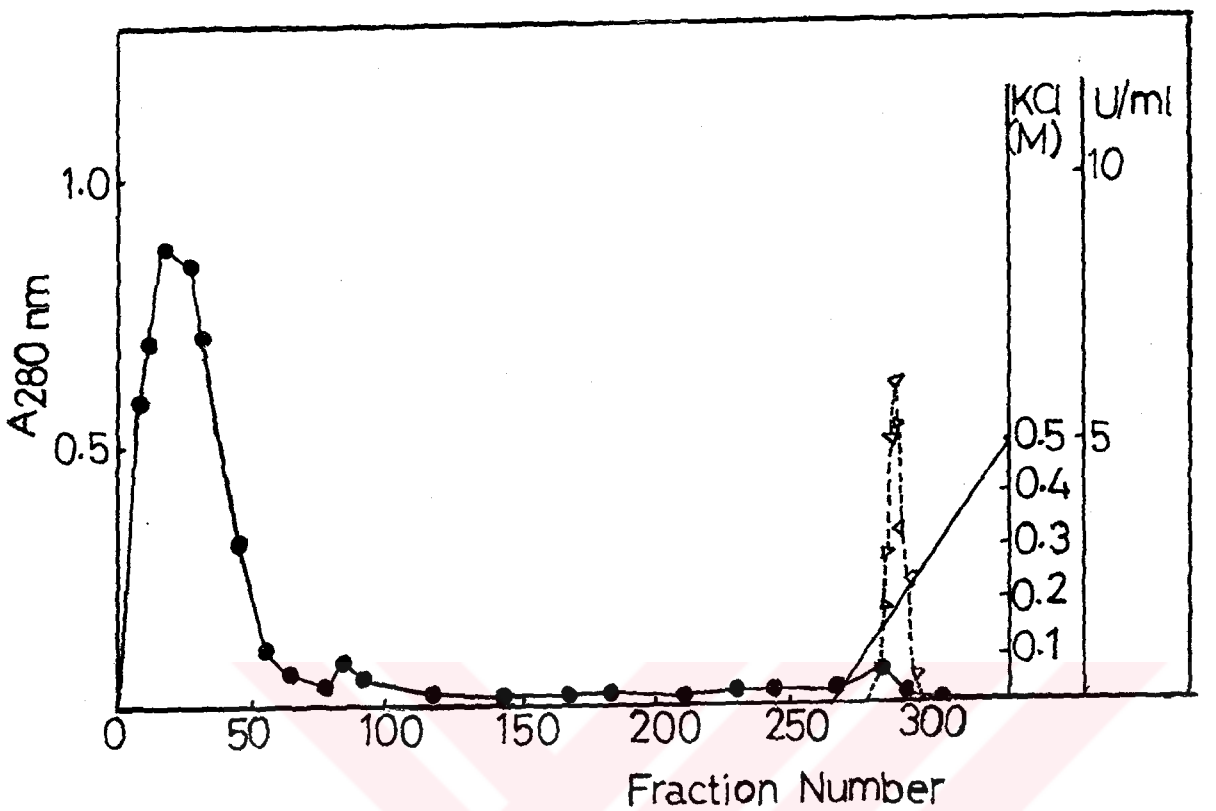


Figure 5 : Blue 4- Agarose column chromatography

Column Dimensions: 1.5 x 8 cm
Flow rate : 20 ml/h
Fraction Volume : 1.5 ml
Washing Buffer : 20mM Tris-HCl(pH 7.0), 1 mM 2-mercapto-ethanol, 1 mM 6-aminocaproic acid, 5 mM $MgCl_2$.
Gradient : 0.0 to 500 mM KCl in above buffer plus 0.4 mM F-1,6-DP
●——● : Absorbance at 280 nm
Δ- - - -Δ : Pyruvate kinase activity (U/ml)

III.2 Electrophoretic Characterization

The electrophoretic behavior of the purified pyruvate kinase from human meningioma was investigated in different systems: SDS-polyacrylamide gel electrophoresis (Fig.6,Fig.7), cellulose acetate paper electrophoresis (Fig.8), and isoelectrofocusing in polyacrylamide gel (Fig.9).

The purity of the enzyme was tested by electrophoresis in 12.5% SDS-polyacrylamide gels (Fig.6). The single band which was obtained with the purified isozyme suggests that the subunits are very similar or identical in molecular weight. The MW value of the enzyme monomers were calculated by interpolation of the best fit line through the points plotted for the mobilities of the standard proteins under the same conditions (Fig.7). The corresponding molecular weight was found to be 63,000 $\pm$ 2000 daltons.

Using cellulose acetate paper electrophoresis, isozymes (M1 and M2) were separated from each other (Fig.8). Two wide bands were observed when crude extracts were applied, while only a single band was observed by applying the purified enzyme onto the cellulose acetate paper.

The isoelectric point of the enzyme was determined by isoelectrofocusing (using ampholytes pH 3-10, pH 5-8). A single band was observed which was focused at pH 6.9 (Fig.9).

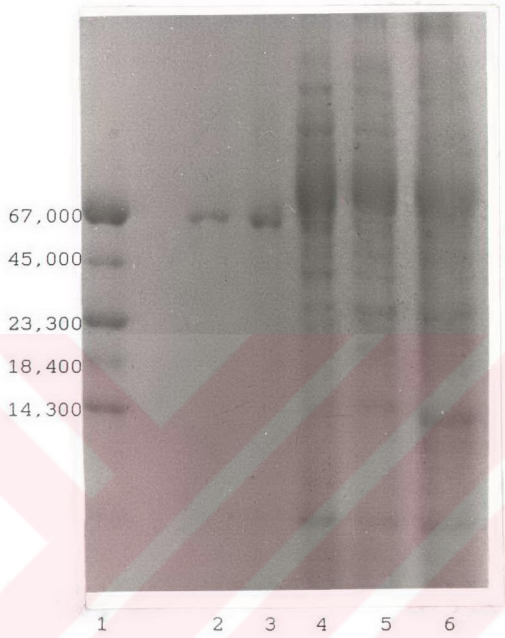


Figure 6.: SDS-polyacrylamide slab gel electrophoresis of purified and crude pyruvate kinases from human meningioma; 1) standard proteins (Bovine serum albumin MW 67,000, Egg albumin MW 45,000, Trypsin MW 23,300, ß-lactoglobulin MW 18,400 and Egg lysozome MW 14,300), 2) PK eluted from affinity column, 3) PK eluted from CM-cellulose column, 4) enzyme after ammonium sulfate fractionation, 5)supernatant of ultracentrifugation, 6) homogenate.

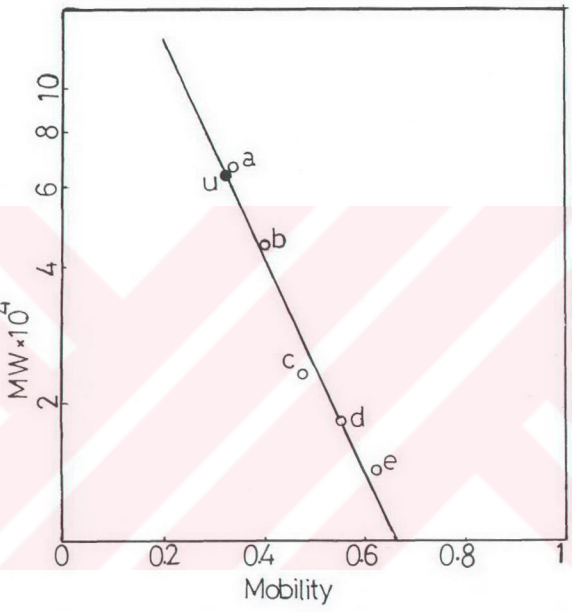


Figure 7 : Subunit molecular weight determination of purified pyruvate kinase by SDS-polyacrylamide gel electrophoresis a: Bovine serum albumin b: Egg albumin c: Trypsin d: ß-lactoglobulin e: Egg lysozome u: Purified pyruvate kinase.

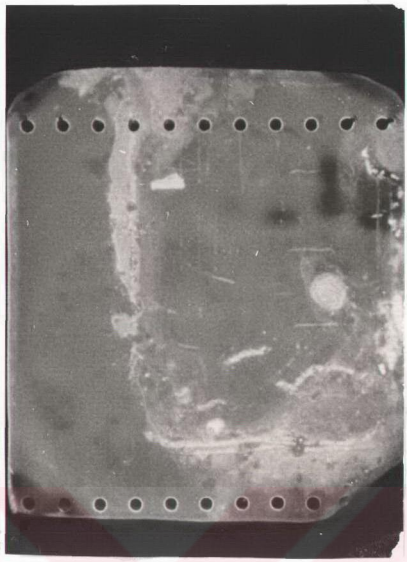
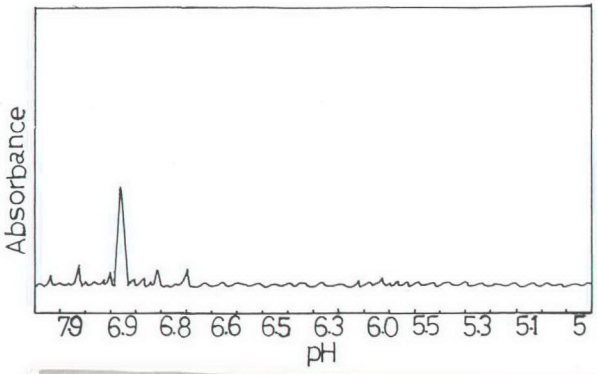


Figure 8.: Electrophoresis of purified and crude human meningioma pyruvate kinase on cellulose acetate paper ; 1) M2-type enzyme eluted from affinity column 2) Isozymes from supernatant of ultracentrifugation 3) Isozymes from homogenate.



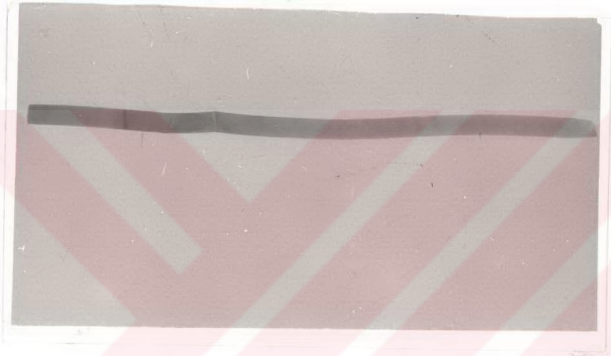


Figure 9.: Isoelectrofocusing of purified pyruvate kinase from human brain meningioma, single band was observed. The upper figure shows the result of gel scaning by densitometer, the position of purified enzyme was observed as a sharp single peak.

III. 3 Kinetic Properties of M2-type Pyruvate Kinase

III. 3.1 Influences of Substrate Concentration on Activity of M2-type Pyruvate Kinase

III. 3.1.1 Phosphoenolpyruvate Hydrolysis in the Absence and Presence of Two Different Concentrations of Fructose 1,6 Diphosphate

The influence of the substrate phosphoenolpyruvate on the reaction rate of purified meningioma enzyme in the absence and presence of 0.3mM and 0.6mM fructose 1,6 diphosphate was investigated. As seen in Figure 10 and Figure 11, the rate response curve was sigmoid in the absence and presence of 0.3mM fructose 1,6 diphosphate with an apparent Km values of 1.8mM and 0.65mM, respectively. The kinetic response to PEP concentration in the presence of 0.6mM fructose 1,6 diphosphate was normalized to a hyperbolic form in the Michaelis-Menten plotting. Fructose 1,6 diphospate in 0.6 mM concentration increases the affinity of the enzyme for phosphoenol-pyruvate. At this concentration of fructose 1,6 diphosphate, the concentration of PEP required for half maximal velocity (Km) was 0.53 ± 0.02 mM and V_{max} was 7.3 mM/min (Table VI). The Hill plots of the saturation data are presented in Figure 12. In the absence and

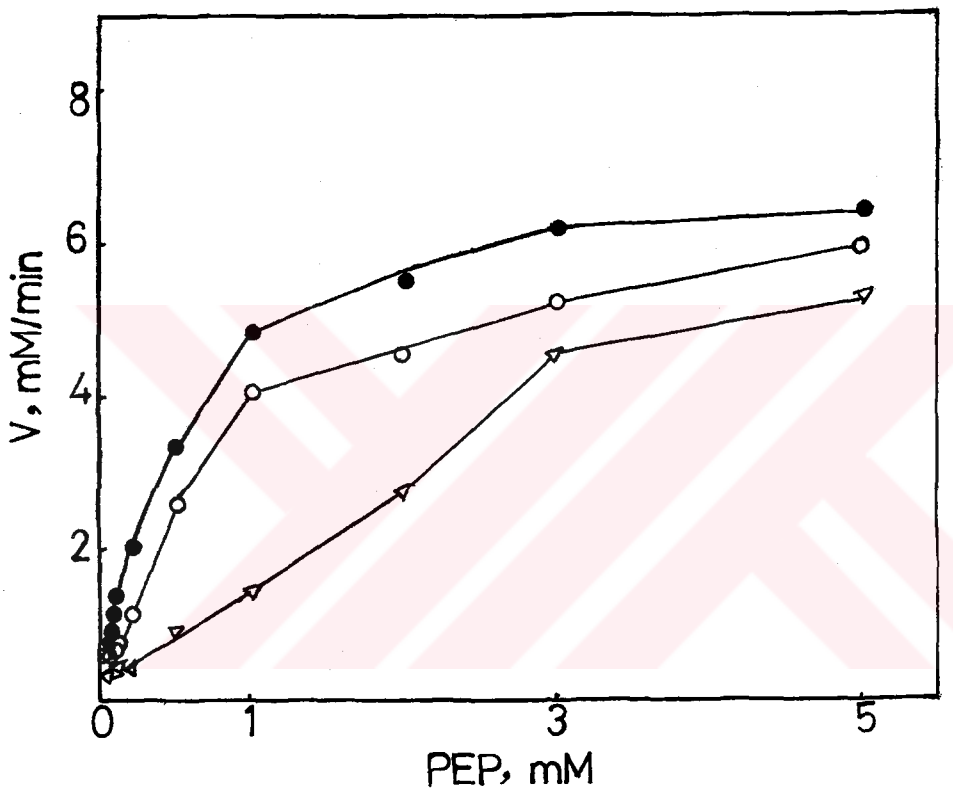


Figure 10 : Michaelis Menten plots for purified enzyme in the absence (Δ——Δ) and presence of 0.3 mM (o——o) and 0.6 mM (●——●) Fructose 1,6 diphosphate. Enzyme was fresh, [ADP]: 2mM.

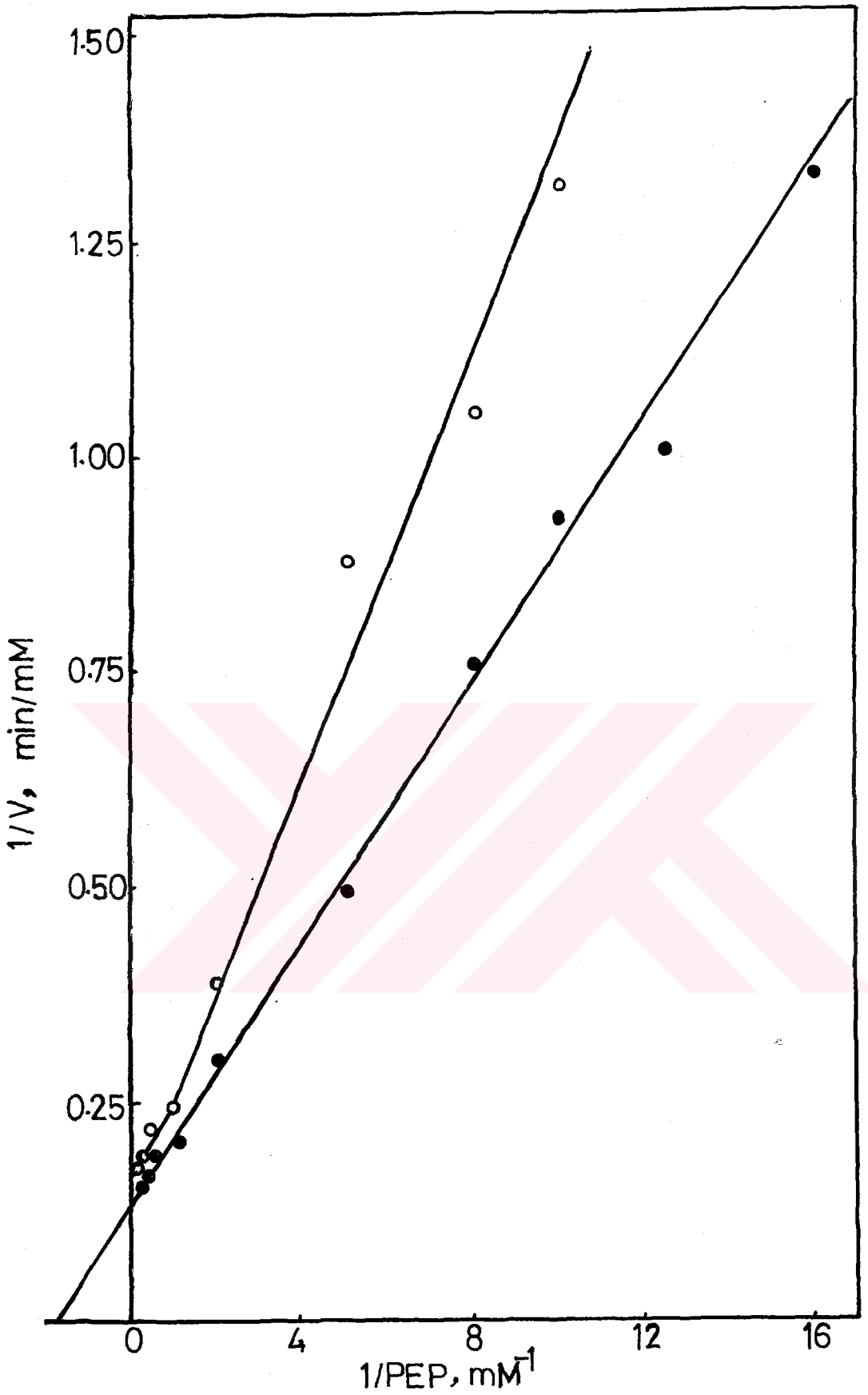


Figure 11.: Lineweaver Burk plot of purified pyruvate kinase isozyme, in the presence of 0.3 mM (o——o) and 0.6 mM (●——●) fructose 1,6 diphosphate. [ADP]: 2mM

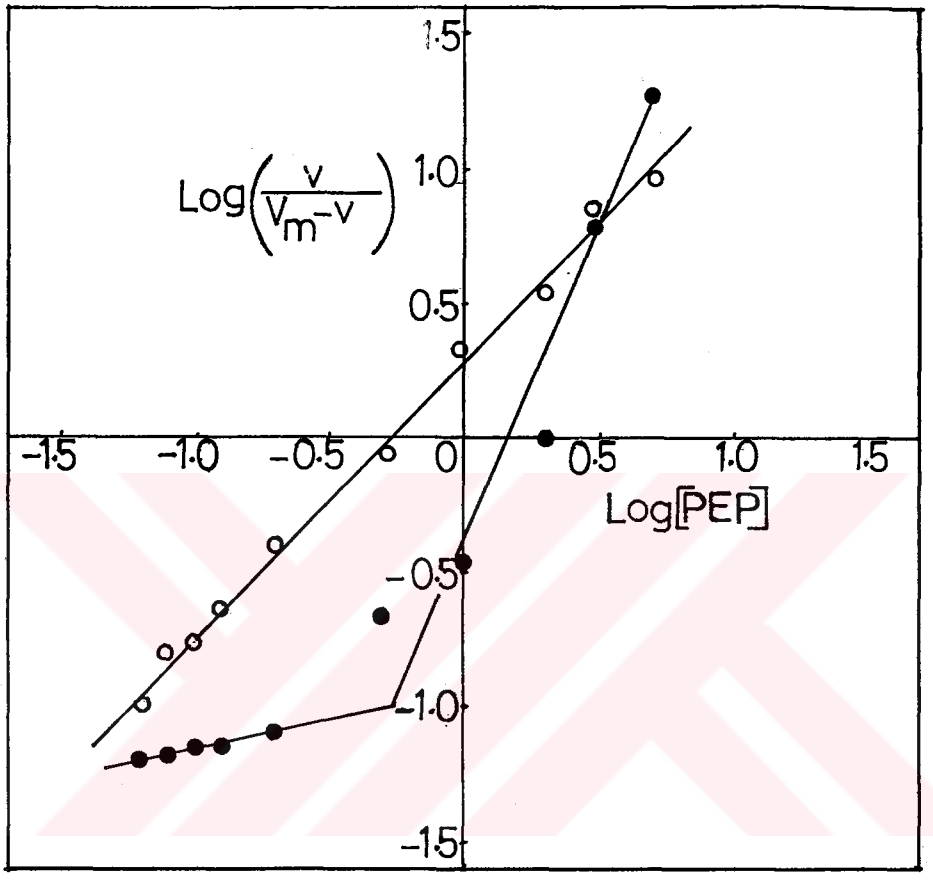


Figure 12.: Hill plot for purified pyruvate kinase in the absence (●——●) and presence of 0.6 mM (o——o) fructose 1,6 diphosphate.

Table VI : Effect of different concentration of Fructose 1,6 Diphosphate on Km and Vmax.

Concentrations of F-1,6-DP	Km(mM)	Vmax(mM/min)
Without	1.8	4.8
0.3 mM	0.65±0,02	6.0
0.6 mM	0.53±0.02	7.3

presence of 0.6 mM fructose 1,6 diposphate, the Hill coefficient were found to be 2.3 and approximately 1, respectively.

III. 3.1.2 Influence of Different ADP concentrations on the Reaction Rate of Tumor Enzyme at Constant Phosphoenolpyruvate concentration.

The activity of the purified tumor enzyme as a function of the second substrate, ADP, was tested as shown in Figure 13. The double reciprocal plot gave a straight line which indicates that the enzyme showed hyperbolic relationship to this substrate. The Km value for ADP was determined to be 0.58 mM at 2mM PEP concentration.

III.3.2 Influence of Effectors on Purified Pyruvate Kinase

III.3.2.1 Effect of Fructose 1,6 Diphosphate Concentration on Enzyme Activity

Figure 14A shows the effect of increasing the concentration of fructose 1,6 diphosphate at 2mM phosphoenolpyruvate.The rate response curve is in hyperbolic form. A modified Lineweaver Burk plot (Fig. 14B) of these data gave a Ka value of 0.15mM for F-1,6-DP, which was somewhat higher than the values reported for M2-type pyruvate kinase from other sources (2,3,24).

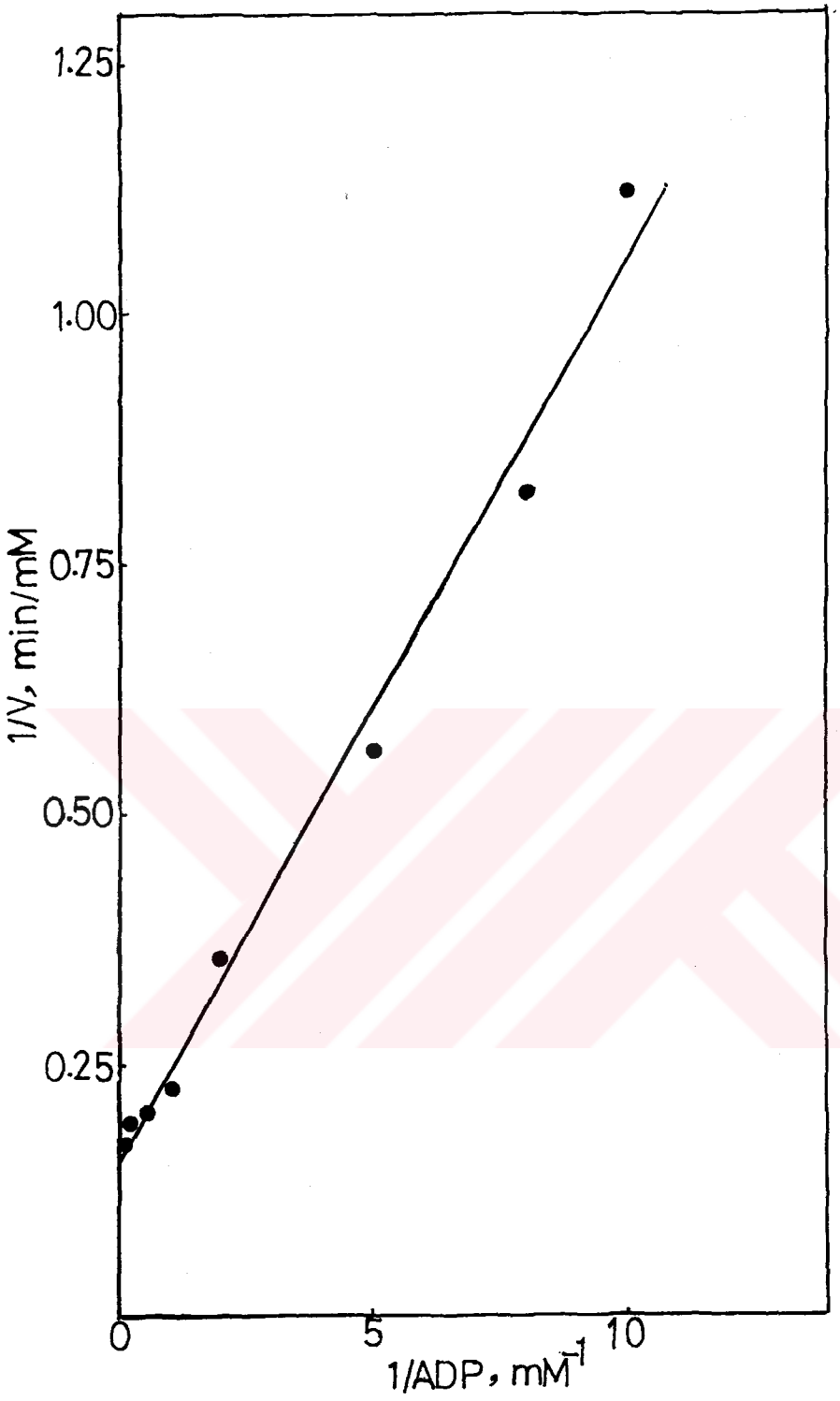


Figure 13.: Lineweaver-Burk plot for ADP as substrate, [PEP]: 2 mM, [F-1,6-DP]: 0.6 mM.

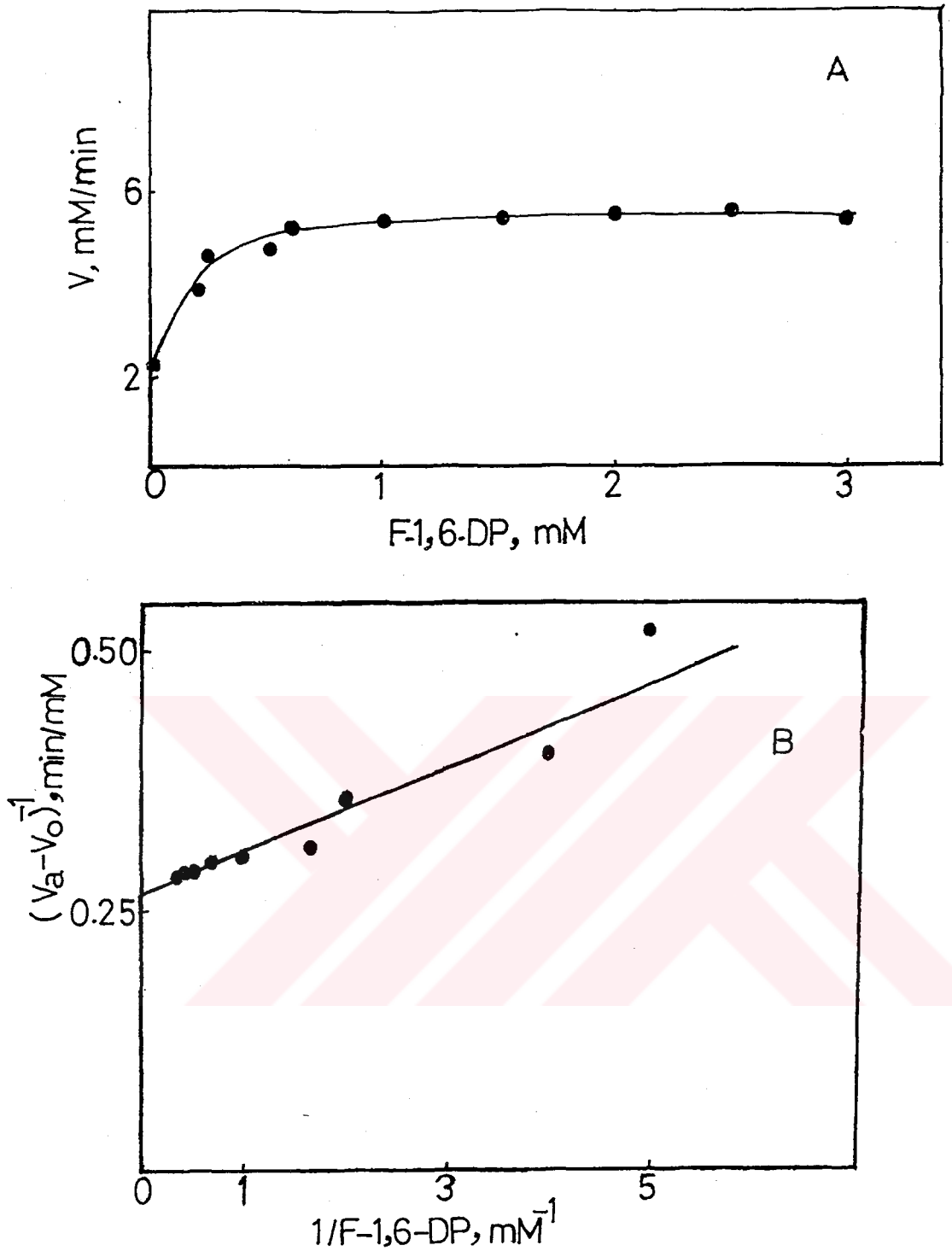


Figure 14 : A) Effect of increasing the concentration of F-1,6-DP on enzyme activity B) Modified Lineweaver-Burk plot for increasing the concentration of F-1,6-DP. , [PEP]: 2 mM, [ADP]:2mM.V_o,velocity in the absence of F-1,6-DP;V_a,velocity with F-1,6-DP.

III.3.2.2 Inhibition of Purified Pyruvate Kinase Activity by Different Concentrations of L-Alanine

Enzyme activity was assayed at varying concentrations (0.0-5.0mM) of L-alanine with 2mM PEP as substrate. The observed inhibition by this allosteric effector in the absence and presence of 0.6mM F-1,6-DP is illustrated in Figure 15. At a concentration of 1mM alanine, without F-1,6-DP, inhibition was found to be 54% and reached by 86% at 5mM alanine(Fig.15). It can be concluded that tumor enzyme is effectively inhibited by increasing the concentration of L-alanine. However in the presence of 0.6mM F-1,6-DP the inhibition at 1mM and 5mM alanine concentrations was 14% and 72% respectively(Fig.15). Fructose 1,6 diphosphate was partly effective in preventing the inhibition of the enzyme activity by L-alanine. The Dixon plot (Fig. 16) for high concentration range of alanine (0.5—4 mM) at 2mM PEP and in the presence of 0.6mM F-1,6-DP showed an upward curvature consistent with cooperativity for the binding of alanine to tumor pyruvate kinase.

III.3.2.3 Effect of L-Alanine on Phosphoenolpyruvate Hydrolysis

The purified enzyme was assayed in the presence of 0.5 and 1 mM alanine at different concentrations of PEP as substrate with 0.6mM F-1,6-DP. The Michaelis-Menten curve was shown in Figure 17. The kinetic behavior was

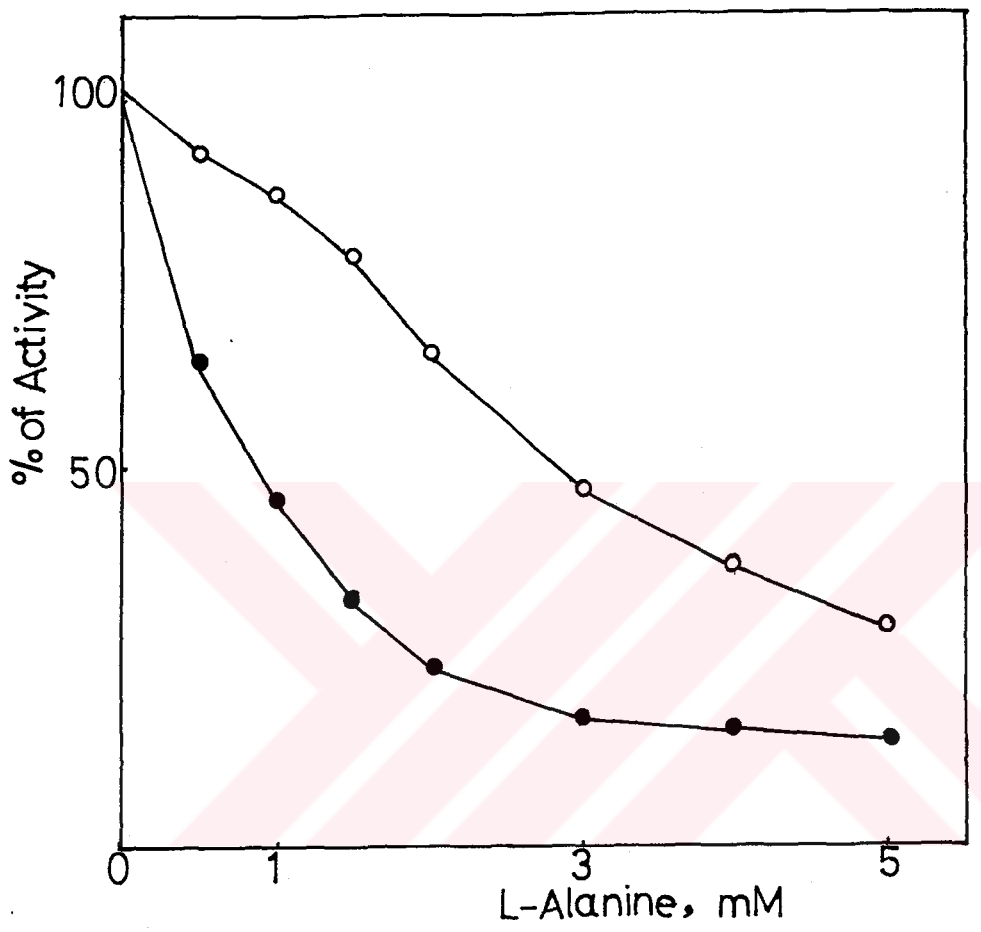


Figure 15 : Effect of alanine at different concentrations on the activity of purified pyruvate kinase from human meningioma. In the absence (●—●) and presence (o—o) of 0.6mM fructose 1,6 diphosphate. [PEP]: 2mM. [ADP]: 2mM

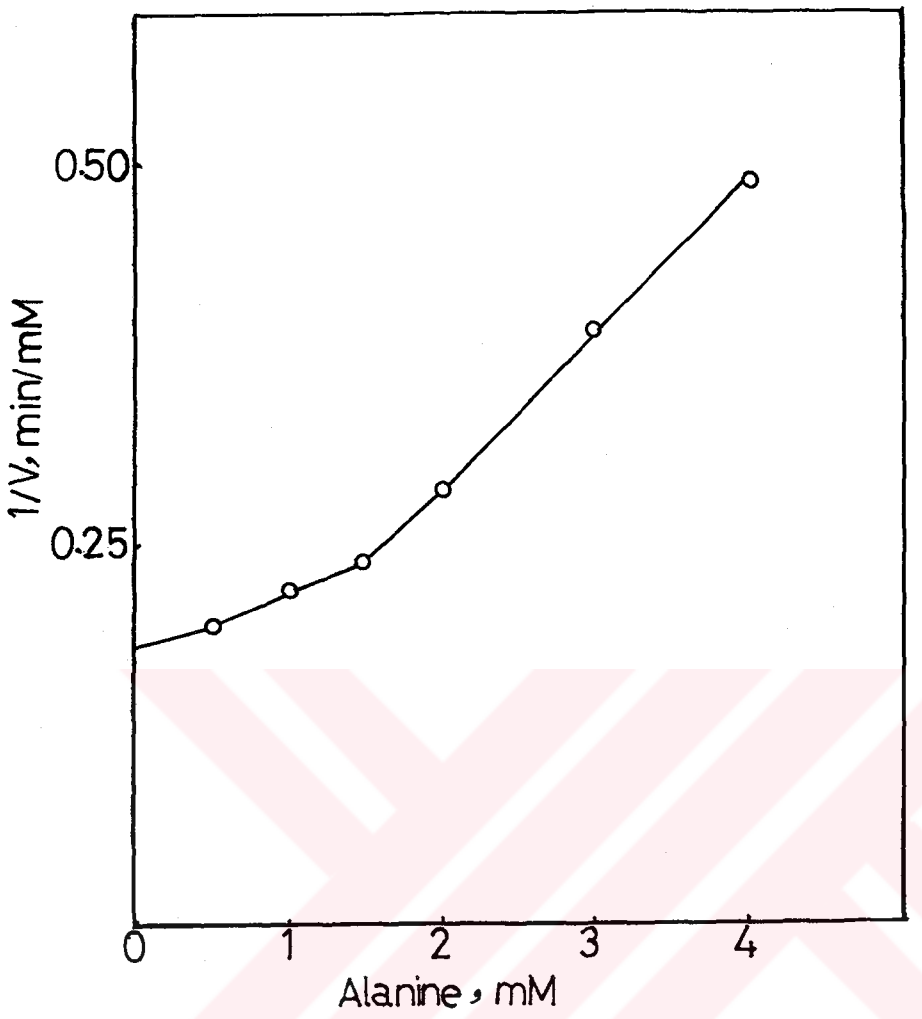


Figure 16 : Dixon plot for alanine, in the presence of 0.6 mM F-1,6-DP. [PEP]: 2 mM, [ADP]: 2mM

hyperbolic in the absence and presence of 0.5mM alanine, whereas slightly sigmoidal kinetics was observed in the presence of 1mM alanine. The double reciprocal plot of this assay for control (without alanine) and in the presence of 0.5mM alanine was presented in Figure 18. With 0.5mM alanine the maximal velocity decreased and the intersection point of two straight lines was not on the V^{-1} axis. Probably the binding sites of alanine are different from that of the substrate PEP. The Dixon plot for different concentrations of L-alanine (0.0-1.5mM) at two different concentration of PEP is shown in Figure 19. By increasing the alanine concentration at 2mM PEP a straight line while at 1mM PEP an upward curvature were obtained. It is indicated that inhibitory effect of alanine was dependent on the PEP concenration. The apparent Ki value in the presence of 0.6mM F-1,6-DP was found to be approximately 2.5mM.

III.3.2.4 Dependence of Enzyme Activity on the Alanine Concentration at Different Concentrations of ADP

Dixon plot, L-alanine concentrations (0.0-1.5 mM) versus V^{-1} with three different concentrations of ADP (0.5,1,2mM) and at a fixed concentration of PEP (2mM) is presented in Figure 20. A family of straight lines that intersect on the abscissa was seen,which

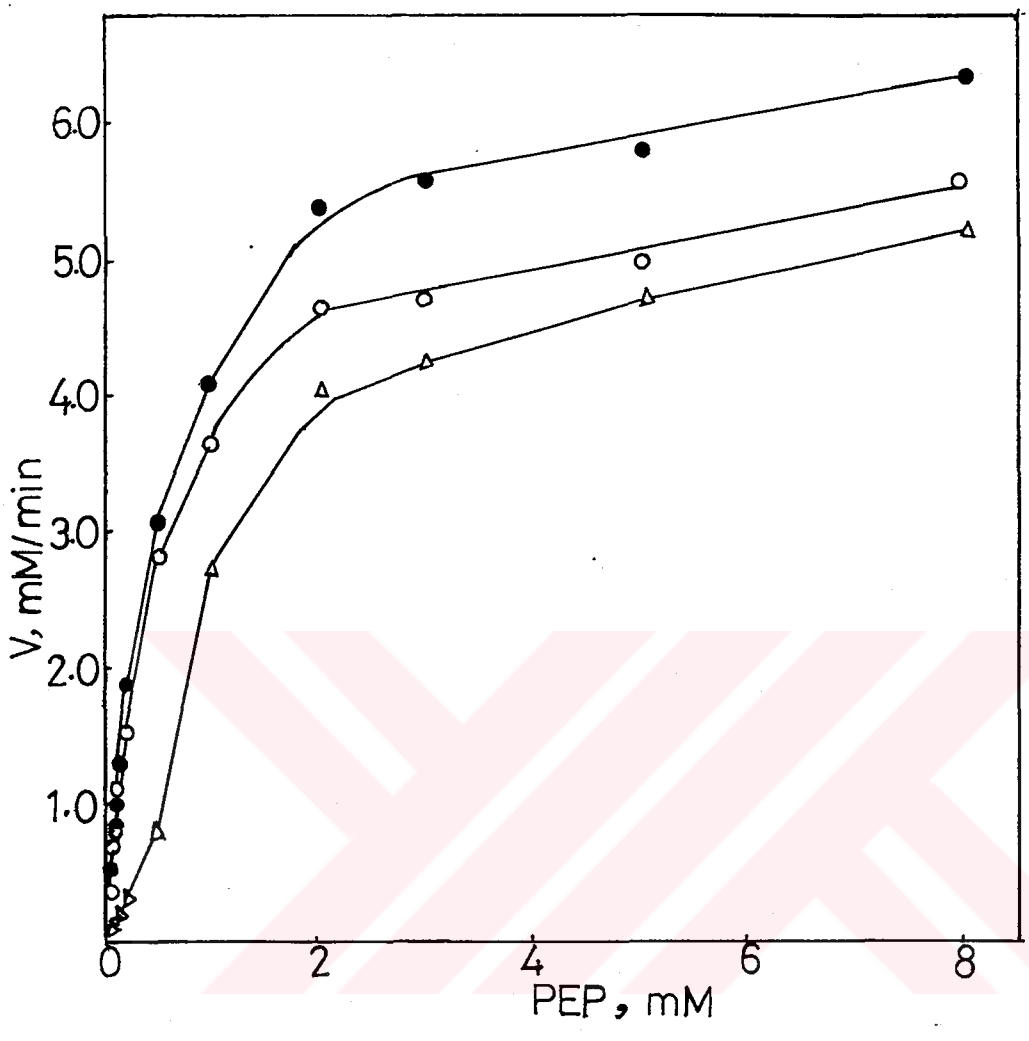


Figure 17 : Activity of purified pyruvate kinase from human meningioma as a function of the concentration of phosphoenolpyruvate, in the absence (●—●) and presence of 0.5 mM (○——○) and 1 mM (△—△) alanine. [F-1,6-DP]: 0.6 mM, [ADP]: 2mM

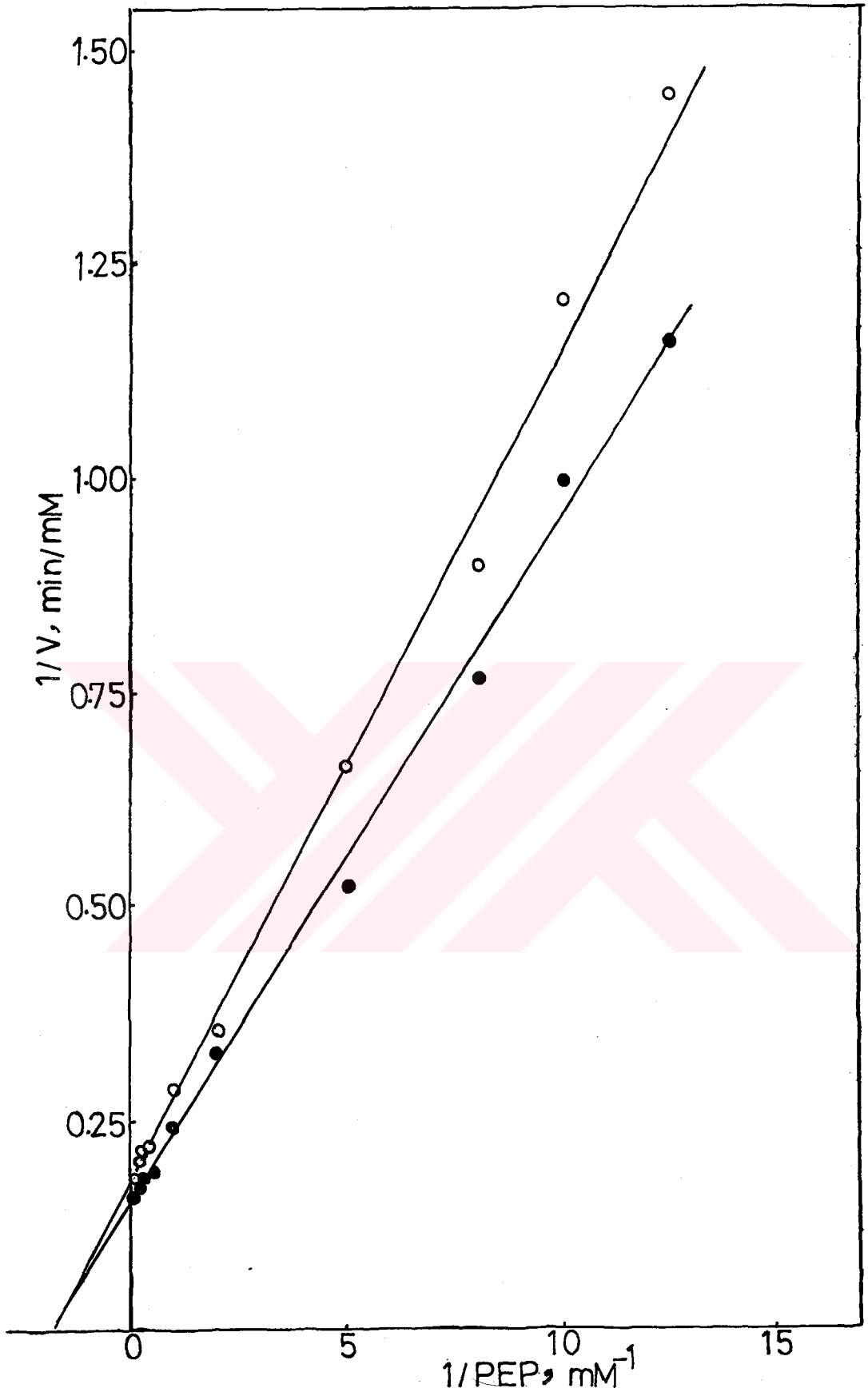


Figure 18 : Double reciprocal plot of initial velocity versus PEP concentrations, in the absence (●——●) and presence (o——o) of 0.5 mM alanine. [ADP]: 2mM, [F-1,6-DP]: 0.6 mM.

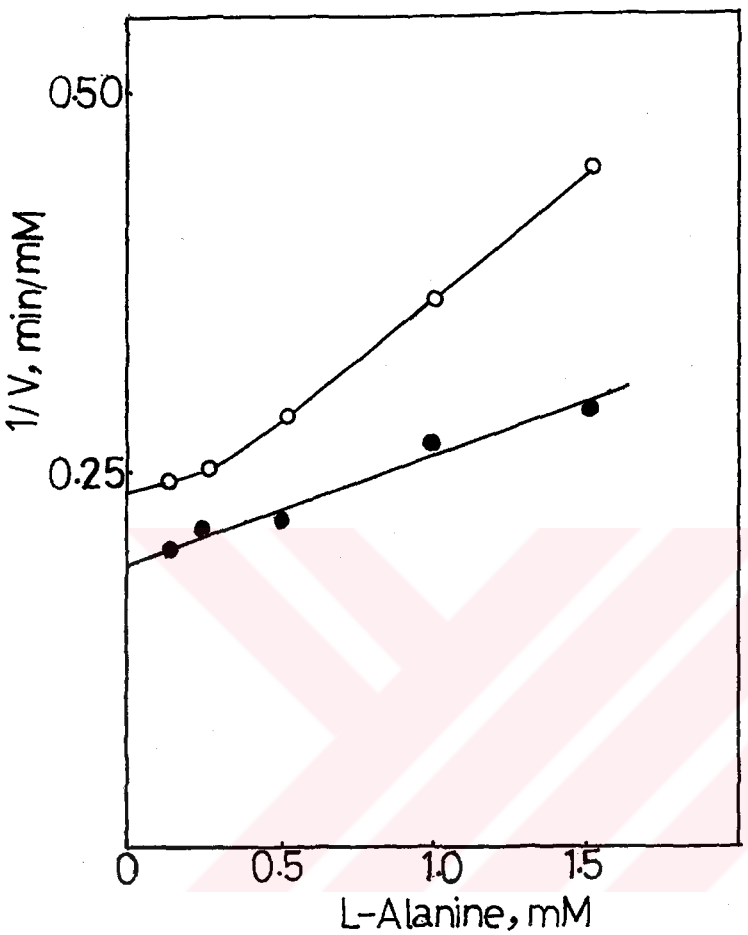


Figure 19 : Dixon plot for alanine inhibition of purified pyruvate kinase at 1 mM (o——o) and 2 mM (●——●) concentrations of phosphoenolpyruvate. [F-1,6-DP]: 0.6 mM, [ADP]: 2mM

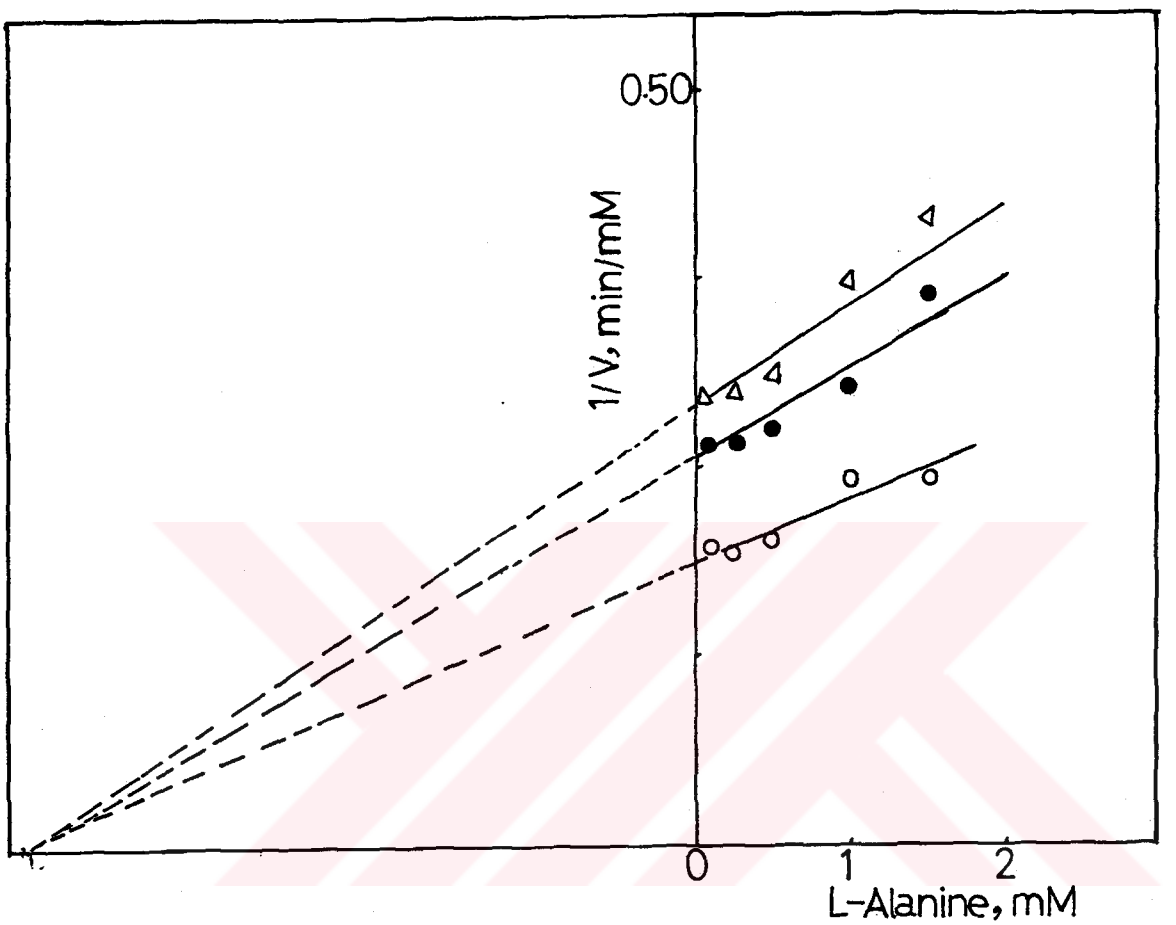


Figure 20 : Dixon plot for the dependence of purified meningioma pyruvate kinase activity on L-alanine at various concentrations of ADP, Δ——Δ (0.5 mM), ●——● (1 mM), ○——○ (2 mM). [PEP]: 2mM, [F-1,6-DP]: 0.6mM

indicated that the enzyme was noncompetitively inhibited by alanine which depended upon the concentration of ADP. As the concentration of ADP decreased, inhibition was more effective. The Km of the enzyme was unchanged. However, the V_{max} was decreased. The Ki for the alanine inhibition of enzyme with regard to changing the concentration of ADP in the presence of 0.6mM F-1,6-DP, was found to be 4.6mM.

III.3.2.5 Simultaneous Presence of L-Alanine and Fructose 1,6 Diphosphate

The kinetic behavior of purified meningioma enzyme with respect to two different fixed concentrations of alanine(0.5 and 1 mM) at various concentrations of F-1,6-DP and fixed concentration of PEP (2mM) as substrate was studied. A double reciprocal plot is shown in Figure 21. Fructose 1,6 diphosphate activated the enzyme in a hyperbolic manner with a Ka value of 0.15mM. However this Ka value was increased to 0.36mM by 0.5mM alanine. At low levels of F-1,6-DP (0.2-0.3mM) the inhibition by 1mM alanine was more effective and slighty upward curvature was obtained. The intersection point of three lines on the ordinate (V^{-1}) is different. From the slope of the above plots and different intersection points on the ordinate, it was suggested that alanine acts as

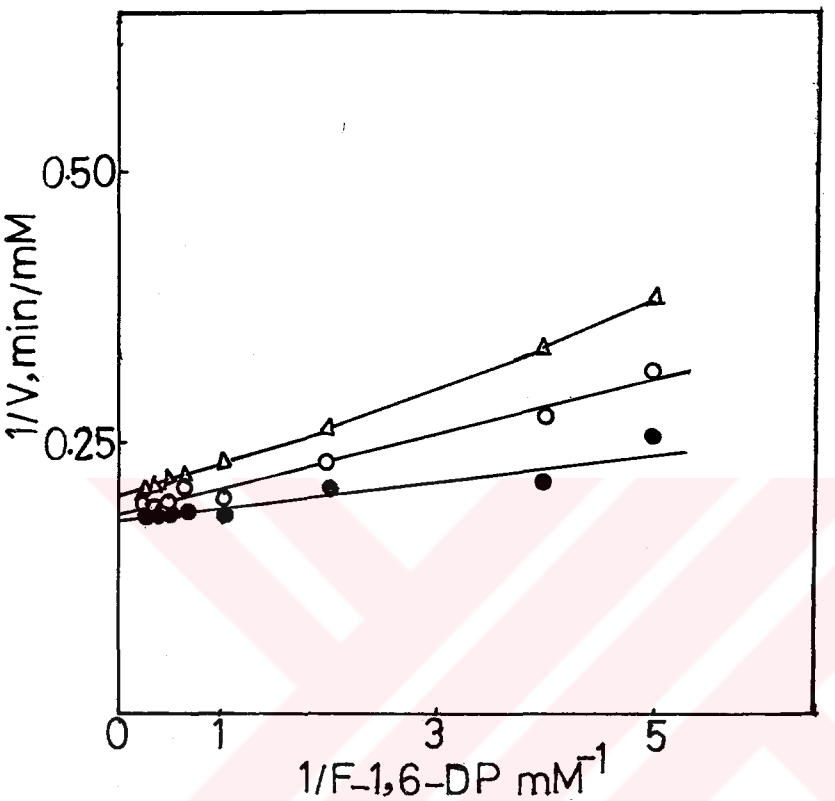


Figure 21 : Double reciprocal plot of velocity versus [F-1,6-DP] , in the absence (●——●) and presence of 0.5 (○——○) and 1 (△——△) mM L-alanine concentration. [PEP]: 2mM, [ADP]: 2mM.

negative allosteric effector for the purified enzyme and this inhibitory effect was partially removed by increasing the concentration of F-1,6-DP.

III.3.2.6 Inhibition by ATP

The influence of two fixed concentrations of ATP (1 and 3 mM) on enzyme activity at different concentrations of PEP, and 2mM fixed concentration of ADP in the presence of 0.6mM F-1,6-DP was studied. The Lineweaver-Burk plot of the data (Fig.22) gave a family of straight lines that intersect on the 1/V axis. In the presence of 3mM ATP,the Km (PEP) of purified meningioma enzyme was increased 1.2 fold from 0.54 to 0.67mM. The enzyme was competetively inhibited by ATP, which is in conflict with results that are reported from other laboratories for human placenta and tumor (84).

III.3.3 Effect of Mg^{+2} Concentration

Enzyme activity was assayed at various concentrations of $MgCl_2$. Figure 23 shows the effect of increasing Mg^{+2} concentration on the reaction velocity of M2-type pyruvate kinase under fixed substrate concentration,

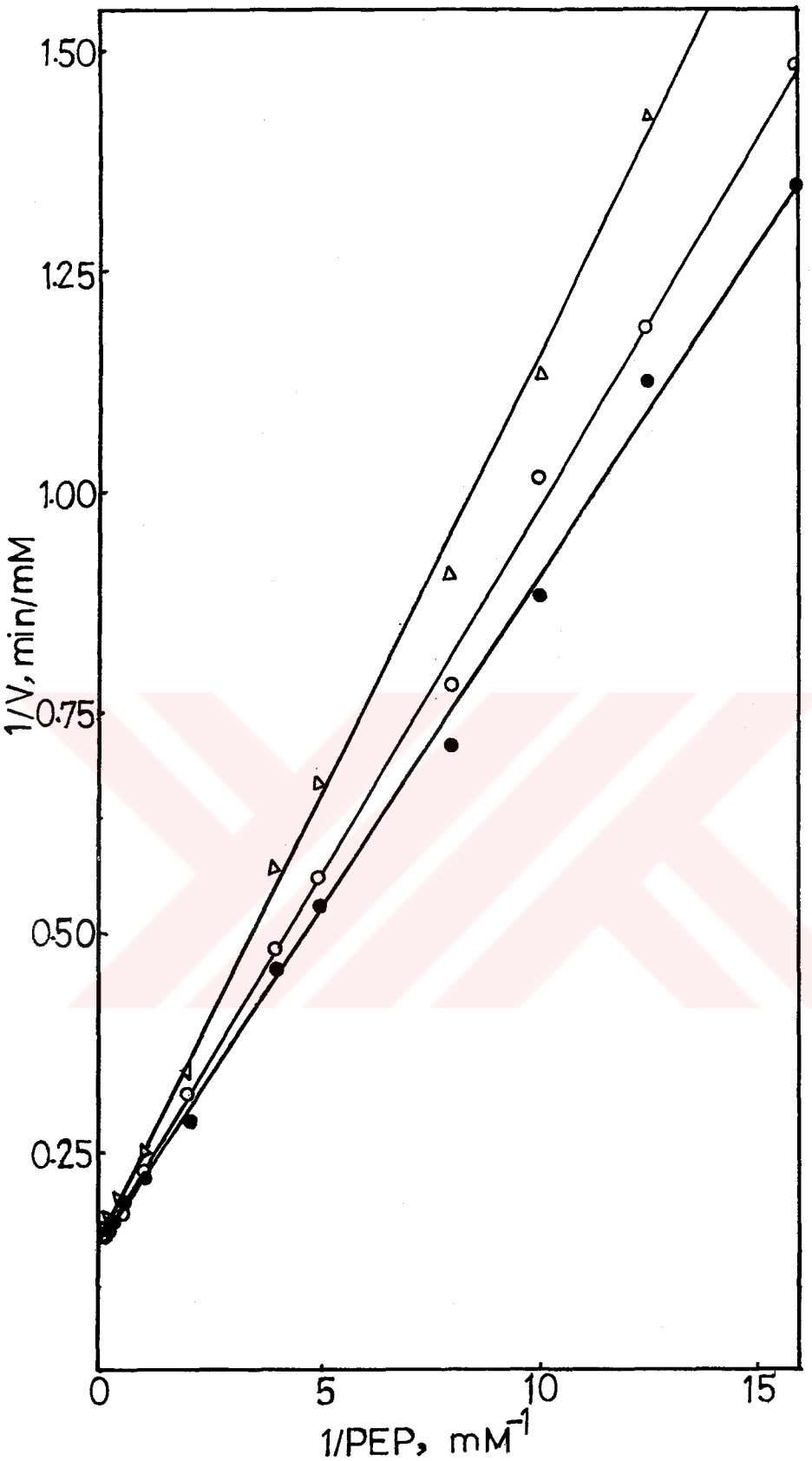


Figure 22.: Double reciprocal plots for the ATP-inhibited enzyme, without ATP(●——●), with 1 mM ATP(○——○), with 3 mM ATP(△——△). [ADP]:2mM, [F-1,6-DP]:0.6mM

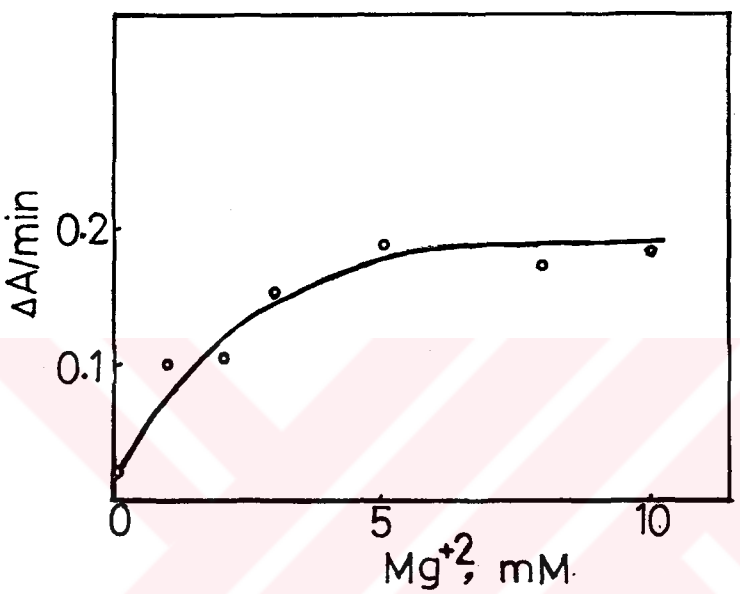


Figure 23 : Effect of Mg^{2+} concentration on purified enzyme activity from human meningioma. [PEP]: 2mM, [ADP]: 2mM, [F-1,6-DP]: 0.6mM.

[PEP]: 2 mM. The rate of the reaction increased with increasing Mg^{2+} ion concentrations from 0.0 to 5.0 mM. A similar effect was reported for M2_type pyruvate kinase from kidney cortex (38) however, an inhibitory effect of Mg^{2+} on the M2_type enzyme from rat lung was observed (79).

III.3.4 Stability

The stability of the purified enzyme upon storage was investigated. The enzyme solution was suspended in a) 2 M ammonium sulfate b) 2M ammonium sulfate containing 0.2 mM F-1,6-DP, and c) in 25% ethylene glycol.

The solutions were kept in three different eppendorf tubes at 4°C. The enzyme activity was measured each week for 3 months. The activity was measured by hydrolysis of 2 mM PEP as substrate in the presence of 0.6 mM F-1,6-DP as described in Material and Methods. The remaining percentage of activity versus period of preservation (up to 87 days) is shown in Figure 24.

The enzyme which was suspended in 2 M ammonium sulfate without F-1,6-DP was highly unstable and lost up to 75% of its original activity after 87 days at 4°C. However, the enzyme in the presence of 0.2 mM F-1,6-DP under the same conditions was more stable and lost only 15% of its activity over the same period of time. In the

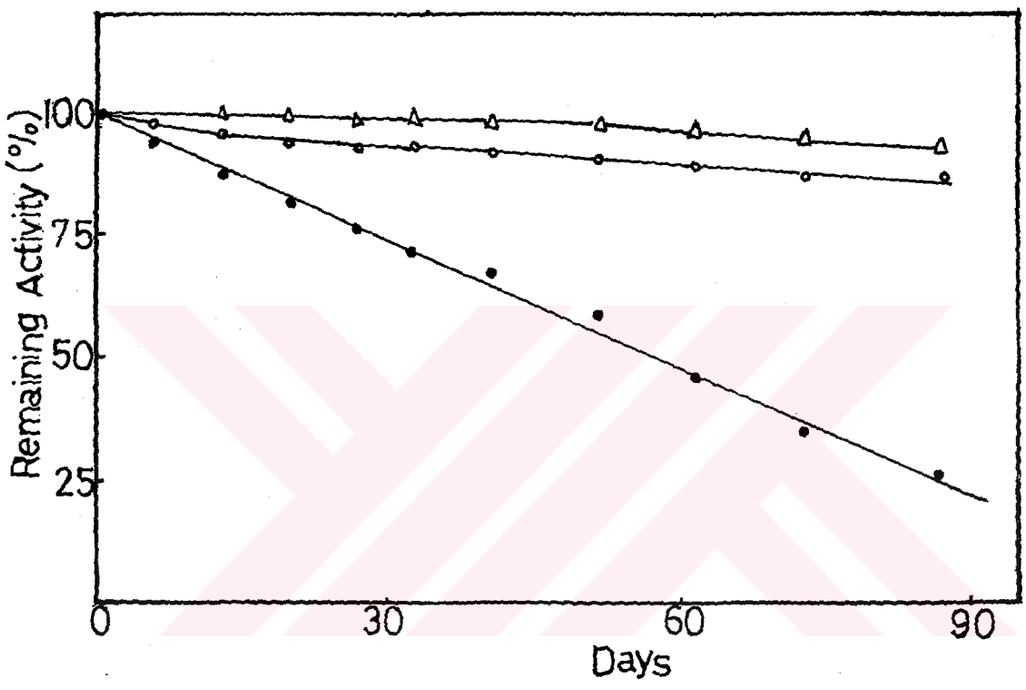


Figure 24 : Stability of purified pyruvate kinase enzyme from human meningioma upon storage. Enzyme was preserved in 25% ethylene glycol (Δ—Δ), ammonium sulfate suspension without (●——●) and with (o——o) 0.2 mM [F-1,6-DP], respectively.

presence of 25% ethylene glycol enzyme was also more stable. In this storage condition the enzyme lost only 10% of its original activity by the end of approximately 3 months.

CHAPTER IV

DISCUSSION

It has become evident recently that human brain cancer is associated with many alterations of isozyme patterns. In brain cancer the M1-type pyruvate kinase isozyme, that characterize the normal brain is lost; and as dedifferentiation and tumor progression occurs, the M2 isozyme which is low or absent in the highly differentiated cells appears (62,95,99). There is a marked resemblance of this pattern with that of placenta (84), where the M2-type is predominant and M1-type isozyme is absent.

IV.1. Molecular Characterization of M2-Type Pyruvate Kinase

In this study, the M2-type isozyme was purified to homogenity from human meningioma by using ion exchange and affinity column chromatography. The purified preparation of human meningioma pyruvate kinase was obtained with a specific activity of 33.4 units/mg protein. The enzyme appeared to be homogenous upon SDS-polyacrylamide gel electrophoresis where a single band of 63,000$\pm$2000 dalton was observed.

For the purified enzyme, a pI value of 6.9 was obtained. This value is lower than what was reported

(2,20) for dog lung tumor enzyme (pI 7.31) and M2-type isozyme from chicken liver (pI 8.3). Also the pI of the meningioma enzyme was higher than the pI values reported (79) for M2-type enzymes from rat lung (pI 5.6) and pig kidney (pI 5.8). Thus, it was concluded that purified isozyme from meningioma is not identical to the M2-type isozymes from other sources which supported the assumption that there are primary structural differences between different isozymes.

M2-type isozyme from different type of tumors were migrated faster toward the anode on cellulose acetate chromatography compared to the M1-type isozyme (62,99). A similar result for the purified tumor pyruvate kinase from meningioma was obtained (Fig.8). Meningioma and malignant gliomas are characterized by the low level of M1-type pyruvate kinase, while M2-type and the hybrid $(M2)_3(M1)_1$ are predominant (101). It has been proposed (22) that rapidly growing hepatomas produce only type $(M2)_4$ but those slowly growing tumors of liver or muscle retained the potential to produce other types as well. Meningiomas are slowly growing and malignant glioma are rapidly growing tumor types.

The crude enzyme gave two wide bands (Fig.8) on the cellulose acetate paper electrophoresis which indicated the existence of isozymes M1 and M2 in meningioma. The

separation of these two isozymes was achieved by CM-cellulose column chromatography (Fig.4).

IV.2. Kinetic Properties of Purified Pyruvate Kinase

The purified enzyme, on Michaelis-Menten plots, showed sigmoidal kinetics with phosphoenolpyruvate as substrate in the absence of fructose 1,6 diphosphate (Fig.10). This sigmoidal kinetics shows positive homotropic cooperativity of the enzyme for this substrate which was confirmed by an apparent Hill coefficient of n=2.3 (Fig. 12). The same kind of kinetic behavior was observed for M2-type enzyme from Yoshida ascite hepatoma by Imammura and Tanaka (41), and for the enzyme from kidney cortex by Ibsen and Trippet (38). In the presence of 0.6 mM fructose 1,6 diphosphate, the sigmoidal plot was converted to a hyperbolic form. The positive cooperativity indicated by Hill coefficient n=2.3 (in the absence of F-1,6-DP) decreased by addition of F-1,6-DP to n=1.03. In the presence of 0.6mM F-1,6-DP a Km value of 0.53 was determined. The kinetics of the enzyme for ADP as substrate was also hyperbolic with a Km value of 0.58mM (Fig.13). The high Km values indicated that the affinity of the enzyme toward substrates was lower than which was observed by enzymes from other sources (79,86).

Significant differences exist in the Lineweaver-Burk

plot analysis for phosphoenolpyruvate hydrolysis by this enzyme and biphasic lines that was obtained for tumor enzyme from meningioma by other workers (101). They suggested that the break in their curve was due to the presence of different hybrids of pyruvate kinase with different affinities for the substrate phosphoenol-pyruvate. However, in our results, no evidence of a break (biphasic form) was observed, which indicated that the purified M2-type enzyme was not contaminated with other isozymes.

IV.3. Regulation of the Enzyme Activity by Effectors Fructose 1,6 Diphosphate and L-Alanine

The results of Figure 14 indicates that by increasing F-1,6-DP concentration, the rate of hydrolysis of phosphoenolpyruvate increased. However the enzyme was maximally activated by 0.6mM F-1,6-DP and further activation of the enzyme was not observed by the addition of F-1,6-DP. The obtained Ka value of 0.15mM for F-1,6-DP was slightly higher than reported from other laboratories (2,71).

The results of the experiments related to the effect of F-1,6-DP on the enzyme activity, indicated that this effector acts as a sensitive modulator on the enzyme

activity, by gradually decreasing the sigmoidicity in V versus [S] curve, with phosphoenolpyruvate. The affinity of the enzyme towards PEP increased (Km decreased) by increasing the concentration of F-1,6-DP.

L-Alanine appears to be an effective inhibitor for purified pyruvate kinase from human meningoima(section III.3.2.2). A similar result, has been reported previously for the enzyme from human brain tumor (62,101).However, their data showed that the inhibitory effect by alanine was higher than what we observed. At a concentration of 1mM and 4mM alanine, 88% and 91% inhibition have been reported(101), respectively. The alanine inhibition indicates the presence of the M2 isozyme, and from a clinical point of view, the determination of the percent of alanine inhibition may be a valuable tool assisting in tumor diagnosis.

The results of the inhibitory effect of a high concentration of alanine on the enzyme (Fig. 16) indicates positive cooperativity for the binding of alanine to the enzyme. L-Alanine noncompetitively inhibited the meningioma pyruvate kinase when phosphoenolpyruvate and ADP were the two substrates (Fig.18,Fig.20). This implies that the binding site for alanine is different from both of these substrates.

A sigmoidal curve was observed in a Michealis-Menten plots (V versus S as a function of phosphoneolpyruvate) at 1mM alanine (Fig.17). On the other hand, a line with an upward curvature was obtained at a fixed phosphoenolpyruvate concentration (1mM) and different concentrations of alanine (Fig.19). These results indicated that alanine acts as allosteric inhibitor on tumor enzyme and its allosteric inhibition is more pronounced at high alanine and low phosphoenolpyruvate concentrations.

Figures 15 and 21 give further information of the simultaneous effect of fructose 1,6 diphosphate as positive and L-alanine as negative allosteric effectors on meningioma pyruvate kinase. The binding of fructose 1,6 diphosphate resulted in a decreased apparent affinity of the enzyme by alanine. Figure 21 demonstrated that 0.5mM alanine increased the Ka of fructose 1,6 diphosphate to 0.36mM. Further increases of alanine to 1mM decreased the Vmax and a slightly upward curvature was obtained. In general, it was concluded that fructose 1,6 diphosphate and alanine are positive and negative allosteric effectors, respectively of human meningioma enzyme; while the negative allosteric effect by alanine was partially reversed by fructose 1,6 diphosphate. This was slightly different from the results that fructose 1,6

diphosphate can relieve completely the inhibition produced by alanine on the enzyme from Ehrlich ascites tumor cells (83) and the enzyme from rat kidney cortex (38). By simultaneous addition of fructose 1,6 diphosphate and alanine, it was suggested that effector mediated control of ascites tumor pyruvate kinase activity is caused by a dimer-tetramer equilibrium (83).

IV.4. Effect of ATP

In this study, it was observed that ATP slightly inhibited the M2-type enzyme from human meningioma. It has also been reported that M2-type pyruvate kinase from different nontumor sources was inhibited by ATP (3,38,71). However this effector did not effect on pyruvate kinases from tumors and human placenta (21,84), and Ehrlich ascites tumor cells (26,29). The lack of total inhibition of glycolysis by ATP in partially permeabilized Ehrlich ascites cells was clearly traced to the partial sensitivity or nonsensitivity of the pyruvate kinase to allosteric inhibition by ATP. The existence of a second site of glycolytic control was suggested (26,27) for pyruvate kinase, which might be partially operative in tumor cells. The result is the high aerobic glycolysis, and low Pasteur effect.

IV.5. Conclusion

If we compare the results of above experiments with the reports of other laboratories that studied the M2-type enzyme from different biological sources including kidney cortex, Ehrlich ascites tumor cells, hepatoma and meningioma, it can be concluded that the purified enzyme from human meningioma has M2-type pyruvate kinase characteristics.

The kinetic parameters and results have confirmed a cooperative kinetics for purified human meningioma pyruvate kinase which is characteristic of the M2-type isozyme. By this way M2 is distinguished from M1-type isozyme which is insensitive to the allosteric effectors, fructose 1,6 diphosphate and L-alanine (21,38,41,94). The kinetic characteristics also show that the purified enzyme is a regulatory enzyme since it is controlled by allosteric effectors. Although some kinetic studies such as Km determination and alanine inhibition were carried out previously on crude pyruvate kinases of human tumors (62,84,101), the results must be interpreted with caution because of the experimental difficulties involved in performing kinetic studies on crude enzymes. In this study a purified M2-type isozyme with high specific activity was obtained. Although, the enzyme seemed pure according to kinetic and electrophoretic studies, more sensitive methods such as monoclonal antibodies may be

used to detect any possible isozyme contamination (107).

In the adult brain, the M1-type isozyme is predominant. However, in meningiomas, the amount of M1 type is considerably low, whereas M2 type is predominant (101). Therefore it was concluded that meningiomas are associated with a shift to M2-type pyruvate kinase. Ibsen (35) has pointed out several possibilities, which may contribute to the observed alteration of the isozyme pattern. One of these possibilities includes derepression of a regulatory gene, which is involved in the regulation of the synthesis of M2 isozymes. The validity of this mechanism for meningiomas was also suggested (101).

The amino acid compositions of the M1 and M2 isozymes from human skeletal muscle and kidney were found to be very different. Therefore, it was concluded that these two isozymes must be the products of separate genes(34). Recently, however, Noguchi et al (64,65) and others (89) determined the complete nucleotide sequences of both M1 and M2 -type pyruvate kinases from rat by sequencing cDNAs. The derived amino acid sequence turned out to be identical except a region of 45 residues. Even within this region the M1 and M2 sequences show a high degree of homology. It was suggested that the two isozyme are derived from the same gene (64,89). However it was also found that M1 and M2-type isozymes are translated from

different mRNAs. After transcription of the gene into a primary mRNA, this mRNA is in turn spliced into two mRNAs, one coding for the M1 subunit and the other for the M2 subunit. Since the expression of the M1 and M2 types are developmentally regulated, the process of alternative RNA splicing might be involved in this regulation.

As a conclusion, the determined kinetic parameters of the isozyme might be helpful for the confirmation of a diagnosis in brain tumors. For further understanding of the genetic expression, more information including amino acid analysis of pyruvate kinases from normal brain and brain tumor tissues are necessary.

LIST OF REFERENCES

1. Barwell, C.J., and Hess, B., 1971, " Regulation of Pyruvate Kinase During Gluconeogensis in Saccharomyces Cerevisiae " FEBS Letters, Vol.9, No 1, pp. 1-4.

2. Becker, K.J., Geyer, H., Eingenbrodt, E., and Schoner, W., 1986, " Purification of Pyruvate Kinase Isozymes Type M_1 and M_2 from Dog (Canis Familaris) and Comparison of Their Properties with Those from Chicken and Rat. " Comparative Biochemistry and Physiology Vol.83B, No 4, pp. 823-829

3. Berglund, L., and Humble, E., 1979, " Kinetic Properties of Pig Pyruvate Kinase Type A from Kidney and Type M from Muscle " Archieves of Biochemistry and Biophysics, Vol.195, No 2, pp.347-361

4. Boivin, P., Galand, C., Hakin, J., Kahn, A., 1975, " Acquired Erythroenzymeopathies in Blood Disorders : Study of 200 cases " Brithish Journal of Haematology, Vol.31, pp.531-543

5. Bonnie, C., Miller and Cottom, G., 1987, " Hormonal Regulation of L-type Pyruvate Kinase in Rat Liver Cell in Culture " Archieves of Biochemistry and Biophysics, Vol.259, No 1, pp.66-78

6. Bucher, T., and Pfleiderer, G., 1955, " Pyruvate Kinase From Muscle " Methods in Enzymology, Vol.1, pp.435-440

7. Carbonell, J., Feliu, J.E., Marcro, R., and Sals, A., 1973, " Pyruvate Kinase-Classes of Regulatory Isozyme in Mammalian Tissues. " European Journal of Biochemistry, Vol.37, pp.148-156

8. Cardenas, J.M., Dyson, R. D., 1973 " Bovine Pyruvate Kinase " Journal of Biochemistry, Vol.248, No 20, pp.6938-6944

9. Cardenas, J.M., Dyson, R. D., and Strandholm, J. J., 1973, " Bovine Pyruvate Kinase " Journal of Biochemistry, Vol.248, No 20, pp.6931-6937

10. Carvajal, N., Gonzaloz, R., and Kessi, E., 1990, " Aspartate Activation of Pyruvate Kinase from The Kidney of Concholepas Concholepas (Gastropoda : Muricidae), Comparative Biochemistry and Physiology, Vol.95 B, No 1, pp.85-89

11. Chai-ho, Lo, Vincent,J., Cristofalo, Morris, H.P., and Weinhouse, S., 1968, "Studies on Respiration and Glycolysis in Transplanted Hepatic Tumors of The Rat " Cancer Research, Vol.28, No 4, pp.1-10

12. Chern, J.C., Rittenberg, M.B., and Block, J.A., 1972, " Purification of Human Erythrocyte Pyruvate Kinase " Journal of Biological Chemistry, Vol.247, pp.7173-7180

13. Cottam, G.L., Halenberg, P.F., and Coon, M.J., 1969 " Subunit Structure of Rabbit Muscle Pyruvate Kinase " Journal of Biological Chemistry, Vol.244, pp.1481-1486

14. Criss, W.E., 1971, " A Review of Isozyme in Cancer " Cancer Research, Vol.31, pp.1523-1542

15. Criss, W.E., 1969, " A New Pyruvate Kinase Isozyme In Hepatoma " Biochemical and Biophysical Research Communication Vol.35, No 6, pp.901-905

16. Duthei, G.G., Arthur, J.R., Simpson, S.P., Nical, F., 1988, " Plasma Pyruvate Kinase Activity Versus Creatine Kinase Activity as an Indicator of the Porcine Stress Syndrome " American Journal of Veterinary Research, Vol.49, No 4, pp.508-510

17. Dworkin, M.B., Segil, N., and Dworkin, E., Rastl, 1987, " Pyruvate Kinase Isozymes in Oocyte and Embryos from The Frog Xenopus Laevis " Comparative Biochemistry and Physiology Vol.88B, No 3, pp.743-749

18. Dyson,R.D., Cardenas, J.M., Richardes, T.C., and Garnett, M.E., 1977 " Pyruvate Kinase Isozymes in Cells Isolated From Fetal and Regenerating Rat Liver " Biochimica et Biophysica Acta, Vol.481, pp.115-126

19. Eigenbrodt, E., Leib, s.,Kramer, W., Friis, R.R., and Schoner, W.,1983, "Structural and Kinetic Differences Between the M_2-type Pyruvate Kinase from Lung and Various Tumors " Biomed. Biochim. Acta, Vol.42, pp.278-282

20. Eigenbrodt, E., and Schoner, W., 1979 " Modification of Pyruvate Kinase Activity by Proteins in Chicken Liver" Physiological Chemistry, Vol.360, pp.1243-1252

21. Farina, F., Shatton, J., Morris, H.P., and Weinhouse, S., 1974, "Isozymes of Pyruvate Kinase in Liver and Hepatomas of the Rat " Cancer Research, Vol.34, pp.1439-1446

22. Farron, F., Howard, H., Hsu, T., and Knox, W.E., 1972, " Fetal Type Isozyme in Hepatic and Nonhepatic Rat Tumors " Cancer Research, Vol.32, pp.302-306

23. Feliu, J.E., 1975, " Interconvertible Forms of Class A Pyruvate Kinase From Ehrlich Ascites Tumor cells " FEBS Letters, Vol.50, No3, pp.334-338

24. Feliu, J.E., and Sols,A., 1976, " Interconversion Phenomena Between Two Kinetic Forms of Class A Pyruvate Kinase From Ehrlich Ascites Tumor Cells " Molecular and Cellular Biochemistry Vol.13, No 1, pp.31-44

25. Gali, P., Clavsse, S., Fofana, L., Hartmann, L., 1985, " Physicochemical and Immunochemical Proofs of Short-Term Insulin Regulation of Hepatic M_2-Type Pyruvate Kinase " Enzyme, Vol.33 pp.49-56

26. Gosalvez, M., Garcia-Suarez, S., and Lopez-Alarcan , L.,1978 "Metabolic Control of Glycolysis in Normal and Tumor Permeablized Cells " Cancer Research,

Vol.38,pp.142-148

27. Gosalvez, M., Lopez-Alarcon, L., Grcia-Surez, S., Montalvo,A., Weinhouse, S., 1975, " Stimulation of Tumor Cell Respiratin By Inhibitors of Pyruvate Kinase " European Journal of Biochemistry, Vol.55, pp.315-321

28. Guminska, M., Stachurska, M.B., Christensen, B., Tromhalt, V., Kieler, J., Radzikowski,Cz, and Dus, D., 1989 " Pyruvate Kinase Inhibited By L-Cysteine as a Marker of Tumorigenic Human Urothelial Cell Lines " Experienta, Vol.45, pp.571-574

29. Guminska, M., Stachuska, M.B., and Ignacak, J., 1988, " Pyruvate Kinase Isozymes in Chromatin Extracts of Ehrlich Ascites Tumor, Morris Hepatoma 7777 and Normal Mouse and Rat Livers " Biochimica et Biophysica Acta, Vol.966, pp.207-213

30. Hall, G.R., Kohl, E.A., and Cottam, G.L., 1978, " The Subunit Structure of Rat Liver Pyruvate Kinase " Biochem. Biophys. Res. Commun., Vol.80, pp.586-592

31. Hance,A.J., Lee, J, and Feitelson, M., 1982 " The M_1 and M_2 Isozymes of Pyruvate Kinase are The Products of The Same Gene " Biochemical and Biophysical Research Communications, Vol.106, No 2, pp. 492-499

32. Harada, K., Saheki, S., Wada, K., and Tanaka, T., 1978, " Purification of Four Pyruvate Kinase Isozymes of Rats by Affinity Elution Chromatogrphy " Biochimica et

Biophysica Acta, Vol.524, pp.327-339

33. Harkins, R.N., Black, J.A., and Marin, B., Henberg, R., 1977 " Purification and Characterization of Human Muscle Pyruvate Kinase " Canadian Journal of Biochemistry, Vol.55, pp.301-307

34. Harkins, R.N., Black, J.A., and Rittenberg, M.B., 1977, " M_2-Type Isozyme of Pyruvate Kinase from Human Kidney as the Product of a Separate Gene: Its Purification and Characterization " Biochemistry, Vol.16 No 17, pp.3831-3837

35. Ibsen, K.H., 1977, " Interrelationship and Functions of the Pyruvate Kinase Isozymes and Their Variant Forms, A Review" Cancer Research, Vol.37, pp.341-353.

36. Ibsen, K,H., Chiu, R.H.C., Park, H.R., Dannis, A., Ray, S.S., Garratt, K.N., and Mueller, M.K., 1981 "Purification and Properties of Mouse Pyruvate Kinase K and M and of a Modified K subunit " Biochemistry, Vol.20, pp.1497-1506

37. Ibsen, K.H, Fischman, W.H. 1979, " Developmental Gene Expression in Cancer " Biochimica et Biophysica Acta Vol.560, pp.243-280

38. Ibsen, K.H., and Trippet,P., 1973 " A Comparison of Kinetic Parameters Obtained With Three Major Non-interconvertible Isozymes of Rat Pyruvate Kinase

"Archieves of Biochemisty and Biophysics, Vol.156, pp.730-744

39. Imammura, K., Tanaka, T., 1972, " Multimolecular Forms of Pyruvate Kinase From Rat and Other Mammalian Tissues " Journal of Biochemistry, Vol.71,pp.1043-1051

40. Imammura, K., Tanaka, T., Nishima, T., Nakashima, K., and Miwa, S., 1973 " Studies on Pyruvate Kinase Defficiency " Journal of Biochemistry, Vol.74, pp.1165-1175

41. Imammura, K., Taniuchi, K., and Tanaka, T., 1972 "Multimolecular Forms of Pyruvate Kinase II. Purification of M_2-type Pyruvate Kinase from Yoshida Ascites Hepatoma 130 cells and Comparative Studies on the Enzymological and Immunological Properties of the Three Types of Pyruvate Kinases, L, M_1 and M_2 " Journal of Biochemistry, Vol.72, pp.1001-1015

42. Inaba, M., and Maede, Y., 1989 "Inherited Persistence of Immature Type Pyruvate Kinase and Hexokinase Isozymes in Dog Erythrocytes". Comparative Biochemistry and Physiology, Vol. 92B, No 1, pp.151-156

43. Jimenez De Asua, L., Rozengurt, E., and Carminatt, H., 1971, "Two Different Forms of Pyruvate Kinase in Rat Kidney Cortex", FEBS Letters, Vol.14, No 1, pp.22-24

44. Jimenez De Asua, L., Rozengurt, E., Davalle, J.J., and Carminatti, H., 1970, "Some Kinetic Differences Between the Isozymes of Pyruvate Kinase from Liver and Muscle" Biochimica et Biophysica Acta, Vol.235, pp.326-334

45. Kayrin, L., and Ozer, I., 1986, "The Effect of Storage On the Kinetic Propertis of Sheep Hepatic Pyruvate Kinase" Biochemical Medicine and Metabolic Biology, Vol.35, pp.50-58

46. Kechemir, D., Audit, J.M., Rosa, R., 1989, "Comparative Study of Human M_2-type Pyruvate Kinases Isolated from Human Leukocytes and Erythrocytes of a Patient with Red Cell Pyruvate Kinase Hyperactivity" Enzyme Vol.41, pp.121-130

47. Kedryna, T., Gumiska, M., and Marchut, E., 1990 " Pyruvate Kinase from Cytosolic Fractions of the Ehrlich ascites Tumor, Normal Mouse Liver and Skeletal Muscle " Biochimica et Biophysica Acta, Vol. 1039, pp. 130 - 133

48. Kimberg, D.V., Yielding, K.L., 1962, "Pyruvate Kinase, Structural and Functional Change, Induced by Diethylstillbestrol and Certain Steroid Hormones" Journal of Biological Chemistry, Vol.237, No 10, pp.3233-3240

49. Kit, S., 1976, "Thymidine Kinase, DNA Synthesis and Cancer", Molecular Cell Biochemistry, Vol.11, pp.161-182

50. Klimek, F., Moore, M.A., Scheider, E., and Bannach, P., 1988, "Histochemical and Microbiochemical Demonstration of Reduced Pyruvate Kinase Activity in Thioacetamide-Induced Neoplastic Nodules of Rat liver," Histochemistry, Vol.90, pp.37-42

51. Labor, R., Stavljenic, A., Jusnfhodzic, L., 1984, " Red Cell Pyruvate Kinase In Acute Leukemia " Enzyme, Vol.32, pp.178-183

52. Laemli, U.K., 1970, "Cleavage of Structure Proteins During the Assembly of Head of Bacteriophage T4" Nature, Vol.227, pp.680-685

53. Leki, R., Miwa, S., Fujii, H., Kudoh, S., Kimura, H., Takaku, F., 1990, "Patient with Pyruvate Kinase Deficiency Developed Acute Myelogenous Leukemia" American Journal of Hematology, Vol.34, pp.64-68

54. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.G., 1951, "Protein Measurement With the Folin Phenol Reagent" Journal of Biological Chemistry, Vol.193, pp.265-275

55. Marchut, E., Guminska, M., Kedryna, T., Radzikawski, Cz., and Kusnierczyk, H., 1987, "A Pyruvate Kinase Variant in Different Mouse Transplanted Tumors" Experienta, Vol.44, pp.25-27

56. Marie, J., Kahn, A., and Boivin, P., 1976, "Pyruvate Kinase Isozymes in Man" Human Genetic, Vol.31, pp.35-45

57. Marie, J., Kohn, A., and Boivin, P., 1977, "Human Erythrocyte Pyruvate Kinase Purification and Evidence for its Antigenic Identity with L-type Enzyme" Biochimica et Biophysica Acta, Vol.481, pp.96-104

58. Marie, J., Kahn, A., and Boivin, F., 1976, "L-type Pyruvate Kinase from Human Liver. Purification by Double Affinity Elution, Electrofocusing and Immunological Studies", Biochimica et Biophysica Acta, Vol.438, pp.393-406

59. Marie, J., Simon, M.P., Dreyfus, J.C., Kahn, A., 1981, "One Gene, but Two Messenger RNAs Encoded Liver L and Red Cell L' Pyruvate Kinase Subunits" Nature Vol.292, pp.70-71

60. Miller, R.C., and Cottam, G.L., 1987, "Hormonal Regulation of L-type Pyruvate Kinase in Rat Liver Cells in Culture" Archieves of Biochemistry and Biophysics, Vol.259, No 1, pp.66-78

61. Miwa, S., Nakashima, K., Ariyoshi, K., Shinotiara, K., Oda, E., Tanaka, T., 1975, "Four New Pyruvate Kinase Variants and a Classical Pyruvate Kinase Deficiency" Brithish Journal of Haematology Vol.29, pp.157-169

62. Mostert, H.W., de Both, N.J., Rhijrsburger, E.H., Mackay, W.M., Van den Berge, J.H., and Stefanko, S.Z., 1986, "Pyruvate Kinase Inhibition in the Diagnosis of

Gliomas with an Intermediate Degree of Malignancy" Acta Neuropathol. Vol.70, pp.296-301

63. Ngo, J.L., and Ibsen, K.H., 1985, "Regulation of Pyruvate Kinase Expression in Differentiating Culture Lines" Federation Proceedings, Vol.19, No 3979. pp. 1398

64. Noguchi, T., Inoue, H., Tanaka, T., 1986, "The M_1 and M_2 type Isozymes of Rat Pyruvate Kinase are Produced from Same Gene by Alternative RNA Splicing" Journal of Biological Chemistry, Vol.261, No 29, pp.13807-13812

65. Noguchi, T., Tanaka, T., 1982, "The M_1 and M_2 Subunit of Rat Pyruvate Kinase are Encoded by Different Messenger RNAs" Journal of Biological Chemistry, Vol.257, No 3, pp.1110-1113

66. O'Farrell, Patrick H., 1975, "High Resolution Two-Dimensional Electrophoresis of Proteins" Journal of Biological Chemistry, Vol.250, No 10, pp.4007-4021

67. Ogier, H., Munnich, A., Lonnet, S., Vaulont, S., Reach, G., and Kahn, A., 1987, "Dietary and Hormonal Regulation of L-type Pyruvate Kinase Gene Expression in Rat Small Intestine" European Journal of Biochemistry, Vol.166, pp.365-370

68. Peters, J., and Andrews, S.J.,1984" The Pk-3 Gene Determines Both the Heart,M_1, and the Kidney, M_2, Pyrvute Kinase Isozymes in the Mouse;And a Simple Electrophoretic Method for separating phosphoglucomutase-3"

Biochemical Genetics, Vol.22, Nos, 11/12, pp. 1047-1063

69. Pinilla, M., Jimeno, P., Moreno, M., and Luque, J., 1990, "Fractionation in Two-Phase Systems of Red Cells During Rat Development: Changes in Pyruvate Kinase and Bisphosphoglycerate Mutase Activities in Relation to Red Cell Switching" Molecular and Cellular Biochemistry, Vol.94, pp.37-44

70. Pitter, R.A., and Fain, J.N., 1988, "Insulin Regulation of Pyruvate Kinase Activity in Cultured Rat Hepatocytes, in the Presence of Vasopressin, Ionophore A23187 or 4B-Phorbol 12ß Myristase 12 -Acetate" Biochemical Journal, Vol.257, pp.717-721

71. Pogson, C.I., 1968, "Adipose-Tissue Pyruvate Kinase" Biochemical Journal, Vol.110, pp.67-77

72. Presek, P., Glossmann, H., Eigenbrodt, E., Schoner, W., Rubsman, H., Friis, R.R., Bauer, H., 1980, "Similarities Between a Phosphoprotein (pp60src)-Associated Protein Kinase of Rous Sarcoma Virus and a Cyclic Adenosine 3':5' Monophosphate-Independent Protein Kinase that Phosphorylates Pyruvate Kinase Type M_2 " Cancer Research, Vol.40, pp.1733-1741

73. Presek, P., Reinacher, M., and Eigenbrodt, E., 1988, " Pyruvate Kinase Type M_2 is Phosphorylated at Tyrosine Residues in Cells Transformed by Rous Sarcoma Virus" FEBS, Vol.242, No 1, pp.194-198

74. Rosa, C.D., Rosa, R., Radrigues, E., Ocampos, D., De Vuono, L., Bacila, M., 1987, "Metabolic Activity of the Stickers Lymphosarcoma" International Journal of Biochemistry, Vol.19, No 4, pp.329-336

75. Saheki,S., Saheki, K., and Tanaka, T., 1982, "Peptide Structures of Pyruvate Kinase Isozymes" Biochimica et Biophysica Acta, Vol.704, pp.484-493

76. Saheki,S., Saheki, K., and Tanaka, T., 1981, " Peptide Structures of Pyruvate Kinase Isozymes. 2. Origins of Type M_1 and M_2 Isozymes Suggested from Species Variations in Their Peptide Maps" Biochimica et Biophysica Acta, Vol.704, pp.494-502

77. Saheki,S., Saheki, K., and Tanaka, T., 1978, "Peptide Mapping by Limited Proteolysis of Four Pyruvate Kinase Isozymes" FEBS Letters, Vol.93, No 1, pp.25-28

78. Schapira, F., 1973, "Isozymes and Cancer" Advance in Cancer Research, Vol.18, pp.77-153

79. Schering, B., Eigenbrodt, E., Linder, D., and Schoner, W., 1982, "Purification and Properties of Pyruvate Kinase Type M_2 from Rat Lung" Biochimica et Biophysica Acta, Vol.717, pp.337-347

80. Schering, B., Reinacher, M., and Shoner, W., 1986, "Localization and Role of Pyruvate Kinase Isozymes in the Regulation of Carbohydrate Metabolism and Pyruvate Recycling in Rat Kidney Cortex" Biochemica et Biophysica

Acta, Vol.881, pp.62-71

81. Schwartz, M., 1978, "Biological Markers of Neoplasia" Enzymes and Cancer, Vol.2, pp.103

82. Shinohara, K., Miwa, S., Nakashima, K., Oda, E., Kgoka, T., Tugino, G., 1976, "A New Pyruvate Kinase Variant Demonstrated by Partial Purification and Condensation" American Journal of Human Genetic, Vol.28, pp.474-481

83. Sparmann, G., Schulz, C., and Hofmann, E., 1973, "Effects of L-Alanine and Fructose 1,6 Diphosphate on Pyruvate Kinase from Ehrlich Ascites Tumor Cells" FEBS Letters, Vol.36, No 3, pp.305-308

84. Spellman, C.M., and Fottrell, P.F., 1973, "Similarities Between Pyruvate Kinase from Human Placenta and Tumors" FEBS Letters, Vol.37, No 2, pp.281-284

85. Staal, G.E.J., Rijksen, G., Brigit, A., Van Orischot, and Roholl, P.J.M., 1989, "Characterization of Pyruvate Kinase from Human Rhabdomyosarcoma in Relation to Immunohistochemical and Morphological Criteria" Cancer, Vol.63, pp.479-483

86. Strandholm, J.J., Cardenas, J.M., and Dyson, R.D., 1975, "Pyruvate kinase Isozymes in Adult and Fetal Tissues of Chicken" Biochemistry, Vol.14, No 10, pp.2242-2246

87. Strandholm, J.J. , Dyson, R.D. , and Cardenas , J.M. , 1976, " Bovine Pyruvate Kinase Isozymes and Hybrid

Isozymes Electrophoretic Studies and Tissue Distribution " Archieves of Biochemistry and Biophysic, Vol.173, pp.125-131

88. Susor, W.A., and Rutter, W.J., 1968, "Some Distinctive Properties of Pyruvate Kinase Purified from Rat Liver" Biochemical and Biophysical Research Communication Vol.30, pp.14-20

89. Takenaka, M., Noguchi, T., Inoue, H., Yamada, K., Matsuda, T., and Tanaka, T.,1989," Rat Pyruvate Kinase M Gene " Journal of Biological Chemistry, Vol.264, No.4, pp.2363 - 2367

90. Tanaka, T., Harano, Y., Morimura, H., and Mori, R., 1965, "Evidence for the Presence of Two Types of Pyruvate Kinase in Rat Liver" Biochemical and Biophysical Research Communication, Vol.21, No 1, pp.55-60

91. Tanaka, T., Harano, Y., Sue, F., and Morimura, H., 1967, "Crystalization, Characterization and Metabolic Regulation of Two Types of Pyruvate Kinase Isolated from Rat Tissues" Journal of Biochemistry, Vol.62, No 1, pp.71-90

92. Tani, K., Fuji, H., Takahashi, K., Kado,H., Takaku, S.A.F., Miwa, S., 1989, "Erythrocyte Enzyme Activities in Myelodysplastic Syndromes:Elevated Pyruvate Kinase Activities" American Journal of Hematalogy,Vol.30, pp.97-103

93. Taylor, C.B., Morris, H.P., and Weber, G., 1969, "A Comparison of the Properties of Pyruvate Kinase from Hepatoma 3924-A, Normal Liver and Muscle" Life Science, Vol.8, pp.635-644

94. Terlecki, G., 1989, "Purification and Properties of Pyruvate Kinase Type M_1 from Bovine Brain" International Journal of Biochemistry, Vol.21, No 9, pp.1053-1060

95. Tolle, S.W., Dyson, R.D., Newburgh, R.W., Cardenas, J.M., 1976, "Pyruvate Kinase Isozymes in Neurons, Glia, Neuroblastoma, and Glioblastoma" Journal of Neucrohemistry, Vol.27, pp.1355-1360

96. Van Berkel, J.C., and Koster, J.F., 1973, "M_2-Type Pyruvate Kinase of Leucocyte: An Allosteric Enzyme" Biochimica at Biophysica Acta, Vol.293, pp.134-139

97. Van Berkel, J.C., Koster, J.F., and Halsmann, W.C., 1972, "Distribution of L- and M-Type Pyruvate Kinase Between Parenchymal and Kupffer Cells of Rat Liver" Biochimica et Biophysica Acta, Vol.276, pp.425-429

98. Van Erp, H.E., Roholl, P.J.M., Rijksen, G., Sprengers, E.D., Van Veelen, C.W.M., Staal, G.E.J., 1988, "Production and Characterization of Monoclonal Antibodies Against Human Type K Pyruvate Kinase" European Journal of Cell Biology, Vol.47, pp.388-394

99. Van Veelen, C.W.M., Rijksen, G., and Staal, G.C.J., 1988, "Discrimination Between Neuronal and Glial Cell Tumors by Pyruvate Kinase Electrophoresis" Acta Neurochemistry, Vol.91, pp.126-129

100. Van Veelen, C.W.M., Rijksen, G., Vlug, A.M.C., Staal, G.E.J., 1981, "Correlation Between Alanine Inhibition of Pyruvate Kinase and Composition of K-M Hybirds" Clinicia Chimica Acta, Vol.110, pp.113-120

101. Van Veelen, C.W.M., Verbiest, H., Annie, M.C.Y., Rijksen, G., and Staal, G.E.J., 1978, "Isozymes of Pyruvate Kinase from Human Brain Meningiomas and Malignant Gliomas" Cancer Research, Vol.38,pp.4681-4687

102. Van Veelen, C.W.M., Verbiest, H., Zulch, K., Van Ketal, Ba, Van der Vlist, M.J.M., Vlug, A.M.C., Rijksen, G., Staal, G.E.J., 1979, "L- Alanine Inhibition of Pyruvate Kinase from Tumors of the Human Central Nervous System" Cancer Research, Vol.39, pp.4263-4269

103. Vives Carrons, J.L., Pujade, M.A., Sierra, J., Ribera, J.M., 1987, "Characteristic of Red Cell Pyruvate Kinase (PK) and Pyrimidine-5'-nucleotidase (P5N) Abnormalities in Acute Leukemia and Chronic lymphoid Disease with Leukemic Expression" British Journal of Haematology, Vol.66, pp.173-177

104. Walker, P.R., and Potter, V.R., 1972, "Isozyme Studies on Adult Regenerating, Precancerous and Developing Liver in Relation to Findings in Hepatomas"

Advanced Enzyme Regulation, Vol.10, pp.339-364

105. Weber, K., and Osborn, M., 1969, "The Reliability of Molecular Weight Determinations by Dodecylsulfate-polyacrylamide Gel Electrophorsis" Journal of Biological Chemistry, Vol.244, pp.4406-4412

106. Weernink, P.A.O., Rijksen, G.,Heijden, M.C.M.,and Staal, G.E.J., 1990" Phosphorylation of Pyruvate Kinase type K in Human Gliomas by a Cyclic Adenosine 5'- Monophosphate - independent Protein Kinase " Cancer Research, Vol.50, pp.4604 - 4610

107. Weernink, P.A.O., Rijksen, G., and Staal, G.G.J., 1988, "Production of a Specific Antibody Against Pyruvate Kinase Type M_2 Using a Synthetic Peptide" FEBS Letters, Vol.236, No 2, pp.391-395

108. Weinhouse, S., 1972, "Glycolysis, Respiration, and Anamolous Gene Expression in Experimental Hepatomas" Cancer Research, Vol.32, pp.2007-2016

109. Weinhouse, S., 1973, "Metabolism and Isozyme Alteration in Experimental Hepatomas" Federation Proceedings, Vol.32, pp.2162-2167

110. Weinhouse, S., Gasalvez, M., Shatton, J.B., and Morris, H.P., 1976, "Isozyme Composition, Gene Regulation and Metabolism of Experimental Hepatomas" Control Mechanism in Cancer; Ravin Press, New York, pp.303-315

111. Zanella, A., Colombo, M.B., Miniero, R., Perron, L., Mellon, T., and Sirchia, G., 1988, "Erythrocyte Pyruvate Kinase Deficiency:11 New Cases" Brithish Journal of Haematology, Vol.69, pp.399-404

VITA

The author was born in Bijar, Iran, on April, 1958. He received his BSc. in chemistry from Shiraz university in 1980. He obtained his MSc. degree in Biochemistry from Middle East Technical university, Turkey, in 1987.

Since 1986 has been working as a research assistant at Biochemistry Depatment.