論文目録

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主論文

Effect of injecting of chromatins from Rhodamine sarcoma into rats on pI-isozymes of liver pyruvate kinase

(ローダミンサルコーマのクロマチンによるラット肝臓のピルビン酸キナーゼアイソザイムの変動について)

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EFFECT OF INJECTING OF CHROMATINS FROM RHODAMINE SARCOMA INTO RATS ON pI-ISOZYMES OF LIVER PYRUVATE KINASE

ADDENDUM : PURIFICATION AND PROPERTIES OF M_2-TYPE PYRUVATE KINASE FROM RHODAMINE SARCOMA

by

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SUMMARY

The pyruvate kinase present in extracts from various tissues of rats has been separated into five pI-isozymes by isoelectric fractionation, the isoelectric points (pI) of which are 5.4, 6.1, 6.5, 7.4 and 7.8. Extracts from skeletal muscle, heart muscle and brain contain only pI 7.4-isozyme, whereas extracts from liver, spleen, lung, erythrocyte, kidney and Rhodamine sarcoma contain three to four different pI-isozymes. Of the pI-isozymes present in an extract from each kind of tissue, the pI-isozyme highest in relative activity is pI 5.4-isozyme for liver, pI 6.5-isozyme for spleen, lung and erythrocyte, and pI 7.8-isozyme for kidney and Rhodamine sarcoma. The kinetic characteristics of the five pI-isozymes are different from one another. The growth of the tumor on the back of rats brings about a significant increase of the activity of pyruvate kinase in the livers; the pI 6.1-isozyme increases, whereas the other pI-isozymes are hardly influenced. When a preparation of chromatins from Rhodamine sarcoma has been injected into rats, the activity of pyruvate kinase in the livers increased to a significant extent; approximately 60 % of the increased activity is due to the pI 7.8-isozyme, the remainder due to the pI 6.1-isozyme.

INTRODUCTION

A multiplicity in type of pyruvate kinase was first reported by Tanaka et al.[19], who have shown, using rats, that most of the pyruvate kinase present in livers is of type L, whereas all the enzyme present in the rat muscles is of type M; these two types are distinguishable electrophoretically, immunologically and kinetically.

In the present study, extracts from various tissues of rats and from Rhodamine sarcoma grown in rats were subjected to isoelectric fractionation

with Ampholine carrier-ampholytes with attempts to examine the kinds and the relative activities of pI-isozymes of pyruvate kinase. Livers from Rhodamine sarcoma-bearing rats, and those from the rats that had been injected with chromatins prepared from the tumor were also examined with respect of the pI-isozymes.

METHODS AND MATERIALS

Animals and Tumor Adult male albino rats of the Donryu strain were fed on an ordinary laboratory diet (Oriental Yeast Co., Ltd., Osaka) with unlimited supply of water, and those weighing around 150 g were used for experiments. In some cases, rats were fed on the high carbohydrate diet, which was composed of 62 % glucose, 31 % casein, 1 % the vitamin mixture (see below), 4 % the salt mixture (see below), 0.01 % choline chloride and 2 % cellulose powder.[19] Transplantation of Rhodamine sarcoma on the back of rats was carried out as described previously.[12] Normal and tumor-bearing rats were decapitated, and then the various organs and the tumor were dissected out. The tumor tissues that had been freed of necrotic parts were used as tumor samples. Livers were homogenized in an equal volume (v/w) of 0.1 M Tris-HCl buffer (pH 7.5) containing 5 mM ethylenediamine tetraacetate and 10 mM β-mercaptoethanol with the use of a Potter-Elvejhem homogenizer. Other tissues were treated in the same manner as livers, except that the volumes of the buffer were varied. Nine volumes (v/w) of the buffer was used for skeletal muscles and Rhodamine sarcoma, and four volumes (v/w) of the buffer for the other tissues. The homogenates were centrifuged at 105,000 g for 1 hr. The resulting supernatants are called "extracts", and were used for analysis by isoelectric fractionation and for the activity

assay of pyruvate kinase. All the procedures were carried out in a cold room (approximately 4°).

Activity Assay of Pyruvate Kinase The activity of pyruvate kinase was measured according to the method of Bücher and Pfleiderer.[2] The standard reaction mixture was composed of 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.5), 0.1 ml of 20 mM phosphoenolpyruvate, 0.1 ml of 20 mM ADP, 0.1 ml of 1.5 M KCl, 50 µl of 0.2 M $MgCl_2$, 50 µl of 5 mM NADH, 10 µl of 150-200 units/ml of lactate dehydrogenase (D-lactate:NAD^+ oxidoreductase, EC 1.1.1.28) and water to make the total volume 1.0 ml. The reaction was started by adding 10 or 20 µl of an enzyme sample to the standard reaction mixture, and the decrease of absorbancy at 340 nm was measured at 24°. One unit of pyruvate kinase was defined as the amount of the enzyme that caused the oxidation of 1 µmole of NADH per min.

Activity Assay of Catalase The catalase activities of liver homogenates were measured in the presence of 0.3 % Triton X-100 according to the method described previously.[12]

Preparation of Chromatins from Rhodamine sarcoma and Injection of the Chromatins into Rats Chromatins were prepared from Rhodamine sarcoma according to the method of Clark et al.[4] and Chalkley et al.[3] with some modifications. All the preparation procedures were carried out 0-5°. Rhodamine sarcoma tissues at the frozen state were blended in 9 volumes (v/w) of 5 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 3 mM $CaCl_2$ with the use of a Waring blendor, and then filtered through a stainless steel net (hole size, 1 x 1 mm) to remove fibrous parts. The filtrate was centrifuged at 1,200 g for 20 min. The resulting precipitate was centrifugally washed twice in each with 9 volumes (v/w) of 0.1 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, and then twice in each with

9 volumes (v/w) of 0.1 M Tris-HCl buffer (pH 7.5) containing 20 mM ethlene-diamine tetraacetate and 80 mM NaCl (pH 7.5). The washed precipitate was suspended in 9 volumes (v/w) of water, and then sonicated at 10 kc for 5 min, followed by centrifugation at 12,000 g for 30 min. The resulting supernatant was supplemented with such an amount of NaCl that the concentration of the salt would be 0.15 M. The mixture was stirred for 90 min, followed by centrifugation at 12,000 g for 30 min. The resulting precipitate was suspended in 9 volumes (v/w) of water. The suspension thus obtained was used as a preparation of chromatins. The chromatin preparation was analyzed to contain 5.00 mg/ml of DNA, 0.35 mg/ml of RNA and 15.5 mg/ml of protein. It (2.0 ml) was injected into the subcutaneous region on the back of each rat. For the control, 2.0 ml of NaCl solution was injected. The injected rats were kept for 23 hr with unlimited supply of water and diet. They were then decapitated, and their livers were dissected out and used.

Determination of Contents of Protein, DNA and RNA The content of protein was determined by the method of Lowry et al.[11] with the use of human serum albumin as the standard. The content of DNA was determined by the diphenylamine reaction of Schneider[16], and the content of RNA by the orcinol reaction[16], with the use of herring sperm DNA and yeast RNA as the standards, respectively.

Isoelectric Fractionation with Ampholine-carrier Ampholytes The isoelectric fractionation on an electrofocusing column of pyruvate kinase present in extracts from various tissues of rats were carried out according to the method of Vesterberg and Svensson[20]; the procedure used was the same as that described previously.[14, 15]

Others Sodium phosphoenolpyruvate and ADP were purchased from Sigma Chemical Co., St. Louis, Missouri. Lactate dehydrogenase and NADH were commercial products of Boehringer Mannheim GmbH, Mannheim and Oriental Yeast Co., Ltd., Osaka, respectively. The salt mixture, the vitamin mixture, casein, choline chloride

and cellulose powder used for the high carbohydrate diet were obtained from Tanabe Pharmaceutical Co., Ltd., Osaka.

RESULTS

Survey of pI-Isozymes of Pyruvate Kinase Present in Various Tissues of Rat

When extracts from various tissues and Rhodamine sarcoma of rats were subjected to isoelectric fractionation, the pyruvate kinase present was separated into five fractions, which were distinctly different in isoelectric points (pI) and easily separable from one another. The enzymes present in these fractions are called "pI-isozymes".[14,15] Typical results obtained with extracts from liver, spleen, muscle and kidney of a normal rat and with an extract from liver of a tumor-bearing rat are shown in Fig. 1. The enzyme was remarkably

Fig. 1

stable against the isoelectric fractionation; the yields in all the cases were as high as 100 %. The pI-activity curve with the extract from normal rat liver has four peaks corresponding to pI 5.4-, pI 6.1-, pI 7.4- and pI 7.8-isozyme. Of the four, pI 5.4-isozyme is highest in relative activity. Although the same four pI-isozymes are present in the extract from normal rat kidney, pI 7.8-isozyme is highest in relative activity with the kidney extract. The enzyme present in the spleen extract also is separated into four pI-isozymes; instead of pI 5.4-isozyme is present pI 6.5-isozyme, highest in relative activity. Only pI 7.4-isozyme is seen with the extract from skeletal muscle. Essentially the same results were obtained with male rats of the Sprague-Dawley strain and the Wistar strain; the pI-activity curves remarkably resembled those with the rats used in this series of experiments.

Normal rats fed on the high carbohydrate diet for 72 hr were approximately three times as high in the activity of liver pyruvate kinase as normal rats fed on the ordinary labolatory diet. To the contrary, the activity was approximately one-half when rats had been starved for 24 hr. Of the four pI-isozymes present in extracts from normal rat livers, only the pI 5.4-isozyme was found to be significantly influenced in activity by the dietary conditions (Fig. 2).

Fig. 2

The relative activities of the pI-isozymes of pyruvate kinase present in extracts from various tissues and Rhodamine sarcoma of rats, and the yields in isoelectric fractionation are summarized in Table I. The enzyme was remarkably

Table I

stable in isoelectric fractionation; the yields in all the cases are as high as 100 %. The sucrose used for making the density gradient in the electrofocusing column, probably, were of advantage to the stabilization of the enzyme. In extracts from tissues of normal rats, lung and erythrocyte, besides spleen, show the highest relative activity of pI 6.5-isozyme. As is in the extracts from skeletal muscle, other isozyme than pI 7.4-isozyme is seen neither in the extract from heart muscle nor in the extract from brain. The extract from Rhodamine sarcoma has pI 6.1-isozyme, pI 7.4-isozyme and pI 7.8-isozyme; of these three, the pI 7.8-isozyme is highest in relative activity. Of the extracts from various tissues of rats so far examined, the extract from skeletal muscle showed the highest specific activity of the pyruvate kinase.

Kinetic properties of the five pI-isozyme of pyruvate kinase are summarized in Table II. Of the fractions of one pI-isozyme obtained by isoelectric fractio-

Table II

nation of an extract from each kind of tissue, the fraction showing the highest activity was used as a sample of the pI-isozyme. The same pI-isozyme was prepared from various kinds of tissues. For example, enzyme samples used for pI 6.1-isozyme, pI 7.4-isozyme and pI 7.8-isozyme were obtained from kidney. Although different kinds of tissues were used for preparation of each of the five pI-isozymes, no difference in the kinetic properties has been found among various sources of the respective pI-isozymes. The Km values for ADP are 0.1-0.4 mM for all the five pI-isozymes. With pI 5.4-isozyme and pI 7.4-isozyme, the Km values for phosphoenolpyruvate are 60 μM with the Hill constant (n) = 1.0; the reaction rates are accelerated by fructose 1,6-diphosphate at various concentrations of phosphoenolpyruvate. With the other pI-isozymes, the curves for initial rate versus phosphoenolpyruvate concentration were of sigmoid shapes; with pI 6.1-isozyme, pI 6.5-isozyme and pI 7.8-isozyme, the n values are 1.3, 1.8 and 1.4, respectively, and the "apparent" Km values for phosphoenolpyruvate (the substrate concentrations for half the rates at sufficient concentrations of the substrate) are 1-2 mM, 0.2 mM and 1 mM, respectively. In the presence of phosphoenolpyruvate of 0.1 mM, which is lower than the Km values, the activities of pI 6.1-isozyme and pI 6.5-isozyme were 27 times and 12 times stimulated by adding 0.1 mM fructose 1,6-diphosphate, respectively. The activity of pI 7.8-isozyme is hardly stimulated by fructose 1,6-diphosphate, in spite of that the curve for initial rate versus phosphoenolpyruvate concentration was of a sigmoid shape and the "apparent" Km value for phosphoenolpyruvate is as high as 1 mM.

In a good accordance with the description by Hess and Kutzbach[6], pI 5.4-isozyme and pI 6.1-isozyme were convertible in a reversible manner. When all the pyruvate kinase present in extracts from livers of normal rats was precipitated by 70 %-saturated ammonium sulfate at pH 7 without addition of fructose 1,6-

diphosphate and the resulting precipitate was then subjected to isoelectric fractionation, the amount of pI 6.1-isozyme increased; the increase was in parallel with the decrease in amount of the pI 5.4-isozyme that had been obtained before the treatment. In the opposite way, pI 5.4-isozyme was obtained by electrofocusing of a mixture of pI 6.1-isozyme and fructose 1,6-diphosphate. It is conceivable, therefore, that pI 5.4-isozyme is the pI 6.1-isozyme that has been associated with fructose 1,6-diphosphate.

Effect of Injection of Chromatins Prepared from Rhodamine sarcoma and Growth of the Tumor on Isozyme Pattern of Liver Pyruvate Kinase

When Rhodamine sarcoma grew on the back of rats, the activity of pyruvate kinase of the livers increased to a significant extent. In the average of five experiments, the activity increased to 165 % (Table III). In the isozyme pattern,

Table III

the pI 6.1-isozyme increases markedly, wherea the other pI-isozymes are hardly influenced (see also Fig. 1). More that 70 % of the increased activity is due to the increase of the pI 6.1-isozyme, the remainder due to the increase of the pI 5.4-isozyme.

When chromatins prepared from Rhodamine sarcoma were injected into rats, the activity of pyruvate kinase of the livers increased to the same extent as when the tumor grew on the back of rats (Table III). In the pI-isozymes patterns, there are, however, marked differences between the injected and the tumor-bearing rats. In the livers of the injected rats, pI 7.4-isozyme and pI 7.8-isozyme increase by two times and by three times, respectively, whereas both the pI-isozymes are not influenced in the livers of the tumor-bearing rats. The only similarity between the injected and tumor-bearing rats is that pI 6.1-

isozyme increases in both cases.

In chromatin-injected rats, the catalase activity of the livers decreased to a significant extent, in a good accordance with the findings with the use of mice[10] (Table IV). The catalase activity of the tumor tissues was negligible.

Table IV

DISCUSSION

Tanaka et al.[19] have succeeded in preapration of crystalline, pure pyruvate kinase from skeletal muscles and livers of rats, and demonstrated that the antiserum against the muscle enzyme neutralizes the activity of the muscle enzyme, but not the activity of the liver enzyme, and vice versa. On the basis of the findings, they proposed to classify the enzyme into type M and type L. The enzyme of the type M displays Michaelis-Menten kinetics with respect to phosphoenolpyruvate. On the other hand, the enzyme of the type L displays sigmoid kinetics with respect to phosphoenolpyruvate, allosteric activation by fructose 1,6-diphosphate and inhibition by ATP. Suda et al.[17] have shown that the enzyme of the type M increased in livers of rats bearing Walker's sarcoma. Susor and Rutter[18] claimed that in electrophoresis on a cellulose acetate membrane, the pyruvate kinase of the type M present in rat liver was different from the enzyme of the type M present in rat skeletal muscle; their claim was recently suppoeted by the kinetic study of Jiménez de Asúa et al.[9]. In the present study, it was found that by isoelectric fractionation, the pyruvate kinase present in various tissues of rats was separable into five isozymes, the pI values of which were 5.4, 6.1, 6.5, 7.4 and 7.8. The relative activities of these pI-isozymes in various tissues were also measured. Skeletal muscle, heart muscle and brain possessed only pI 7.4-isozyme, the kinetic

properties of which were essentially the same as those of the enzyme of the type M characterized by Tanaka et al.[19]. This pI 7.4-isozyme is, therefore, doubtless to be of the type M. Criss[5] also carried out isoelectric fractionation of an extract from skeletal muscle of rats, and reported that the pyruvate kinase present was of pI 6.75. At the present time, the difference between his and our data on the pI value is not explainable; in the present study, the pI values of isozymes were accurate within 0.1 in several times-repeated experiments with the use of rats of three different strains. The pI 7.8-isozyme was most abundant in kidney, of the tissues of normal rats tested. It is feasible that pI 7.8-isozyme is of the type M_2 classified by Imamura et al.[8], who have shown that the activity of this isozyme is neutralized by the antiserum against the type M enzyme. In livers of normal rats fed on an ordinary laboratory diet, the activity of pI 7.8-isozyme was 15 % of the total activity of all the four pI-isozymes present, three times as high as the activity of pI 7.4-isozyme. In comparision to the tissues of rats examined, Rhodamine sarcoma showed a relatively high activity of pyruvate kinase, approximately 80 % of which was due to pI 7.8-isozyme; the activity of the isozyme per mg protein in the tumor is highest, four times as high as that in kidney. Nakamura et al. (to be published) have recently succeeded in preparation of crystalline, pure pI 7.8-isozyme from Rhodamine sarcoma.

The existence of pI 6.5-isozyme of pyruvate kinase was first observed in the present study. Spleen of normal rats possessed four pI-isozymes; 78 % of the total activity of all the pI-isozymes was due to the activity of pI 6.5-isozyme. Of the pI-isozymes present in the extracts from various tissues of normal rats tested, the pI 6.5-isozyme present in the extract from spleen was highest both in relative activity and in specific activity (U/mg protein);

thus, it is called the type S enzyme tentatively. The tissues other than spleen, lung and erythrocyte did not possess the type S enzyme.

It is doudtless that pI 6.1-isozyme of pyruvate kinase, when so tightly bound with fructose 1,6-diphosphate that the bound fructose 1,6-diphosphate would not be dissociated from the enzyme molecule by the electrofocusing procedure for isoelectric fractionation, is converted into pI 5.4-isozyme. The kinetic properties of pI 6.1-isozyme were essentially the same as those of the type L enzyme[19]; thus, it may be appropriate to classify pI 5.4-isozyme into the type L'. In fresh extracts from livers, almost all the type L' seen after isoelectric fractionation is, perhaps, present at the form of the type L, at which the binding between the enzyme molecule and the fructose 1,6-diphosphate under the intracellular conditions is not so tight as described above; the kinetic behaviors of pyruvate kinase, when assayed with fresh preparations of extracts from livers of normal rats, were essentially the same as the sum of those of each of the pI-isozymes, if calculation was made under the assumption that the type L' obtained by isoelectric fractionation had been present as the type L in the extracts. The conversion of the type L into the type L' during the period of electrofocusing of the extracts may take place as follows: In liver cells, the concentration of fructose 1,6-diphosphate is approximately 0.5 mM (Hosoi et al., to be published), but the enzyme molecules of the type L are not bound with fructose 1,6-diphosphate tightly because of the sufficiently high ionic strength of the salts present. When an extract from the cells is subjected to isoelectric fractionation, the salts migrate apart from the zone of the enzyme molecules gradually during the period of electrofocusing. When the ionic strength of the zone is lowered to an appropriate extent, the enzyme molecules are bound tightly with the fructose 1,6-diphosphate molecules that have still remained at a lower

concentration in the zone. It seems conceivable, therefore, that the type L' is the artifact, and that in the cells of tissues, the isozyme molecules of the type L are associated with fructose 1,6-diphosphate such weakly that the extent of the association depends on the concentration of fructose 1,6-diphosphate in a reversible manner. The ratio in activity of the type L' enzyme to the type L enzyme obtained by isoelectric fractionation of an cell extract, possibly, depends mainly on the concentration of fructose 1,6-diphosphate present in the cells; the higher the concentration, the higher the ratio.

The pyruvate kinase present in livers of normal rats was separable into type L', type L, type M and type M_2. By dietary conditions of rats, the type L' and the type L were influenced to a significant extent, but not the other types. The activities of the type L' enzyme and the type L enzyme, and the ratio in activity of the type L' enzyme to the type L enzyme increased when rats had been fed on a high carbohydrate diet, whereas they decreased, when rats had been starved. Dietary conditions of rats, doubtless, change the concentrations of various metabolites in the liver cells. It is probable, therefore, that the increased and decreased concentrations of phosphoenolpyruvate in liver cells are responsible for the increase and decrease in amount of the type L enzyme in the cells, respectively, and that the increased and decreased concentrations of fructose 1,6-diphosphate in the extracts are responsible for the increase and decrease in the ratio in activity of the type L' isozyme to the type L isozyme, respectively.

When chromatins prepared from Rhodamine sarcoma were injected into normal rats, the activity of liver catalase decreased to 76 %, whereas the activity of liver pyruvate kinase increased to 154 %. Approximately 40 % and 60 % of

the increased activity of the enzyme were due to the increase of the activity of the type L enzyme and the type M_2 enzyme, respectively. In Rhodamine sarcoma-bearing rats, the activity of liver pyruvate kinase was 160 % of that in normal rats. Approximately 80 % and 20 % of the increased activity of the enzyme were due to the increase on the activity of the type L enzyme and the type L' enzyme, respectively. On the other hand, in Rhodamine sarcoma tissues, approximately 80 % of the activity of pyruvate kinase was due to the activity of the type M_2 enzyme. One of the possible explanation for this may be as follows. Either injection into rats of chromatins prepared from Rhodamine sarcoma or bearing of Rhodamine sarcoma on the back of rats bring about the increase in concentration of phosphoenolpyruvate, but not fructose 1,6-diphosphate, in the livers, so that the amount of the type L enzyme increased, leaving the amount of the type L' enzyme unchanged.

It is probable that the high relative activity of the type M_2 enzyme in the tumor tissue and the increase in activity of the isozyme in the livers of rats injected with the chromatins were invoked by the same cause. Matuo *et al.*[13] solubilized and purified the liver catalse-*in vivo*-depressing substance [toxohormone(liver catalase)] from Rhodamine sarcoma tissues, and Kannan *et al.*[10] demonstrated that the depressing substance is located in the chromatins of the nuclei. Recently, Nishikawa *et al.* (to be published) have succeeded in a complete purification of the depressing substance; it is a neutral protein (pI value = 7) free of nucleic acids. There is the possibility that the chromatins of Rhodamine sarcoma possess a regulator functional in "switch-on" of the biosynthesis of type M_2 enzyme. Perhaps, the regulator is scarcely liberated *in vivo* from the tumor cells, different from the case of the liver catalase-*in vivo*-depressing substance.

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Table I. Relative Activities of pI-Isozymes of Pyruvate Kinase Present in Various Tissues of Rats

Rats	Diets	Tissues	Specific activities in extracts	Recoveries in isoelectric fractionation	Relative activities of pI-isozymes				
					pI 5.4	pI 6.1	pI 6.5	pI 7.4	pI 7.8
			(U/mg protein)	(%)	(%)	(%)	(%)	(%)	(%)
Normal	Ordinary	Liver	0.24	90	65	14	0	6	15
,,	,,	Spleen	0.61	91	0	5	78	12	5
,,	,,	Lung	0.61	103	0	0	51	14	35
,,	,,	Erythrocyte	0.02	104	11	9	62	3	15
,,	,,	Skeletal muscle	8.9	95	0	0	0	100	0
,,	,,	Heart muscle	0.95	87	0	0	0	100	0
,,	,,	Brain	1.9	95	0	0	0	100	0
,,	,,	kidney	0.56	115	7	32	0	12	49
,,	High carbohydrate	Liver	0.70	70	85	9	0	2	6
Rh.sarcoma-bearing	Ordinary	Liver	0.40	96	37	45	0	5	13
,,	,,	Rh.sarcoma	1.4	90	0	13	0	10	77
Normal	,,	Liver of rat injected with Rh.sarcoma chromatins	0.33	85	40	22	0	9	29
					L'	L	S	M	M_2 (or K)[a]
					Types of enzyme				

a) This type is most abundant in kidney, but not present in muscles at all.

Table II. Kinetic Properties of Five Types of Pyruvate Kinase of Rat

The experimental conditions were the same as those described in the text, except that in the reaction mixture for the activity assay, various concentrations of phosphoenolpyruvate and ADP were added for determination of the Km values for phosphoenolpyruvate and ADP, respectively, and various concentrations of phosphoenolpyruvate and 0.1 mM fructose 1,6-diphosphate were added for measurement of the activation by fructose 1,6-diphosphate. Hill constants (n) for phosphoenolpyruvate were calculated on the basis of curves of activity vs. phosphoenolpyruvate concentration according to the method of Atkinson et al.[1,7)]

Table II. Kinetic Properties of Five pI-Isozymes of Pyruvate Kinase of Rats

	Pyruvate kinase pI-isozymes				
	pI 5.4	pI 6.1	pI 6.5	pI 7.4	pI 7.8
Km values for phosphoenolpyruvate	0.06 mM	1 — 2 mM	0.2 mM	0.06 mM	1 mM
Km values for ADP	0.2 mM	0.3 mM	0.2 mM	0.4 mM	0.1 mM
Hill constants for phosphoenolpyruvate	1.0	1.3	1.8	1.0	1.4
Activation by 0.1 mM fructose 1,6-diphosphate	-	+++	+++	-	- a)
Sources for pI-isozymes used	Liver: Normal rat Rh.sarcoma bearing rat Rat injected with Rh.sarcoma-chromatins	Liver: Rh.sarcoma-bearing rat Rat injected with Rh.sarcoma-chromatins Kidney Rh.sarcoma	Spleen Lung Erythrocyte	Skeletal muscle Heart muscle Brain Kidney	Kidney Rh.sarcoma Spleen Liver of rat injected with Rh.sarcoma-chromatins

a) The inhibitory effect of alanine was in part neutralized by adding fructose 1,6-diphosphate.

Table III. Comparison in pI-Isozyme Pattern of Pyruvate Kinase among Liver of Normal Rat, Liver of Rhodamine sarcoma-bearing Rat, Liver of Normal Rat Injected with Rhodamine sarcoma Chromatins, and Rhodamine sarcoma Tissue

Tissues of rats	Number of animals tested	Specific activities of pyruvate kinase in extracts	Amounts of pyruvate kinase subjected to isoelectric fractionation	Amounts obtained by isoelectric fractionation				
				pI 5.4-isozyme	pI 6.1-isozyme	pI 6.5-isozyme	pI 7.4-isozyme	pI 7.8-isozyme
		(U/mg protein)	(U)	(U)	(U)	(U)	(U)	(U)
Liver of normal rat	5	0.23 ± 0.01	6.5 ± 2.4	4.3 ± 0.3	0.9 ± 0.2	0	0.4 ± 0.1	0.9 ± 0.3
Liver of Rh.sarcoma-bearing rat	5 a)	0.35 ± 0.01	10.4 ± 3.9	5.6 ± 1.1	4.1 ± 0.9	0	0.4 ± 0.1	0.9 ± 0.3
Liver of normal rat injected with Rh. sarcoma chromatins	4	0.33 ± 0.01	10.0 ± 3.0	4.0 ± 0.3	2.2 ± 0.1	0	0.9 ± 0.2	2.9 ± 0.1
Rhodamine sarcoma	2	1.40 ± 0.20	21.5 ± 5.2	0	2.6 ± 0.3	0	1.9 ± 0.2	14.9 ± 4.6

a) When rats were decapitated, the ratios in weight of the tumor to the whole body were from 7 % to 13 %. No regularity was seen between the increases of the ratio and the specific activity. One experiment was carried out with the liver of a rat of the ratio = 20 % ; the specific activity of pyruvate kinase was approximately one-half that of normal rat liver; pI 5.4-isozyme was not detectable, pI 6.1-isozyme increased by approximately two times, and neither pI 7.4-isozyme nor pI 7.8-isozyme were changed.

Table IV. Effect of Injection into Rats of Chromatins Prepared from Rhodamine sarcoma Tissue on Catalase Activity of Livers.

Tissues	Number of animals tested	Catalase activities of tissues ($K_{obs.} \times 10^2$)
Liver from normal rat	4	3.03 ± 0.43
Liver from normal rat injected with Rh. sarcoma-chromatins	4	2.29 ± 0.27
Rhodamine sarcoma	4	0

Fig. 1. Isoelectric fractionation of pyruvate kinase present in extracts from various tissues of rats

Extracts of various tissues (1 ml each) were individually subjected to isoelectric fractionation. Ampholine-carrier ampholyte (LKB-Produkter, AB., Stockholm-Bromma) of pI from 3 to 10 were used at a final concentration of 1 % (w/v). The electrolysis was carried out for 42 hr at 0-1° in a 110-ml electrofocusing column, and then, the column content was eluted. The eluate was divided into 1-ml fractions. The pH and the activity of pyruvate kinase (U/ml) were measured of each fraction. Open circles, liver of normal rat; closed circles, liver of Rhodamine sarcoma-bearing rat (tumor/body = 13 %): open squares, spleen of normal rat; open triangles, skeletal muscle of normal rat; closed triangles, kidney of normal rat.

Fig. 1

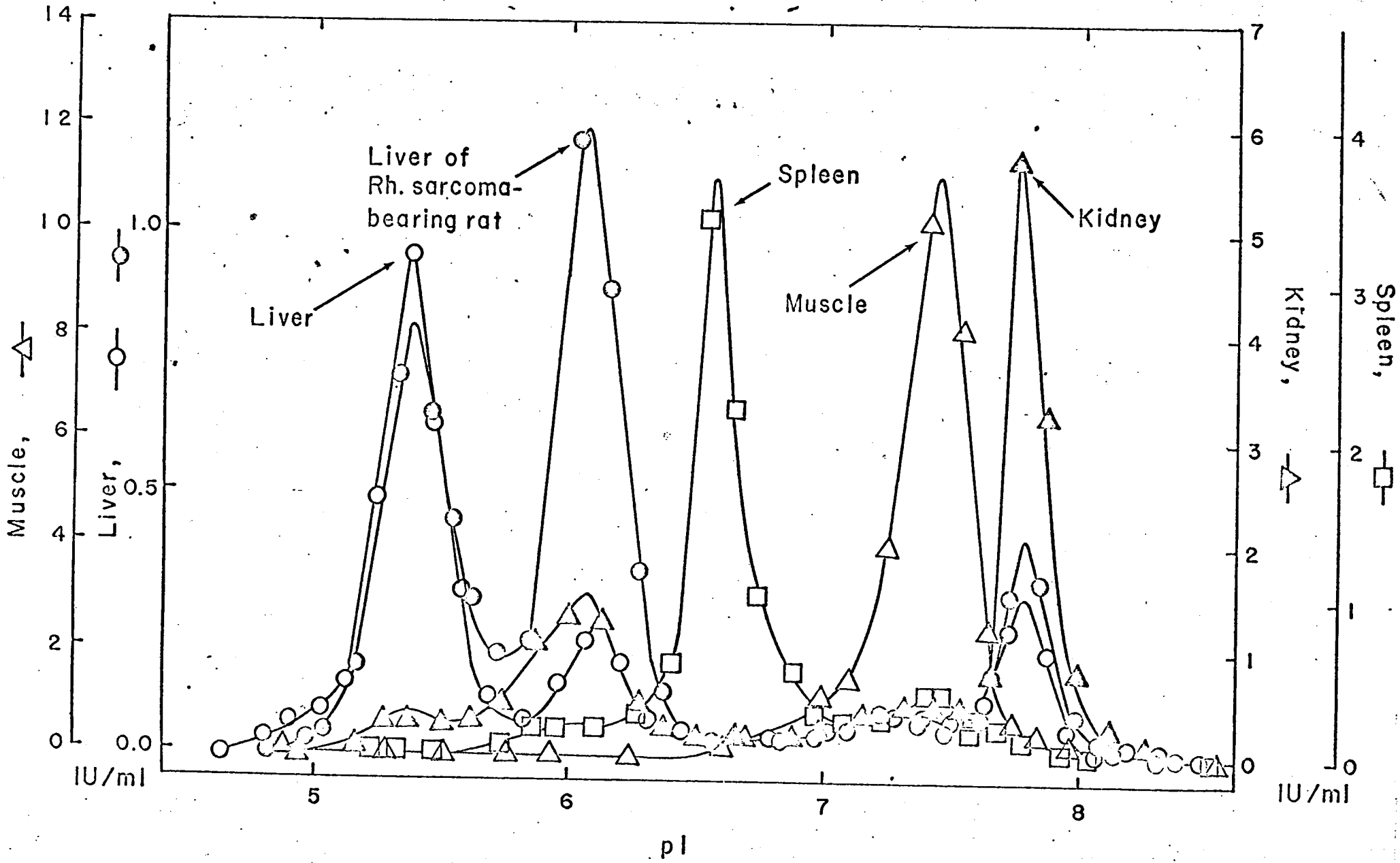
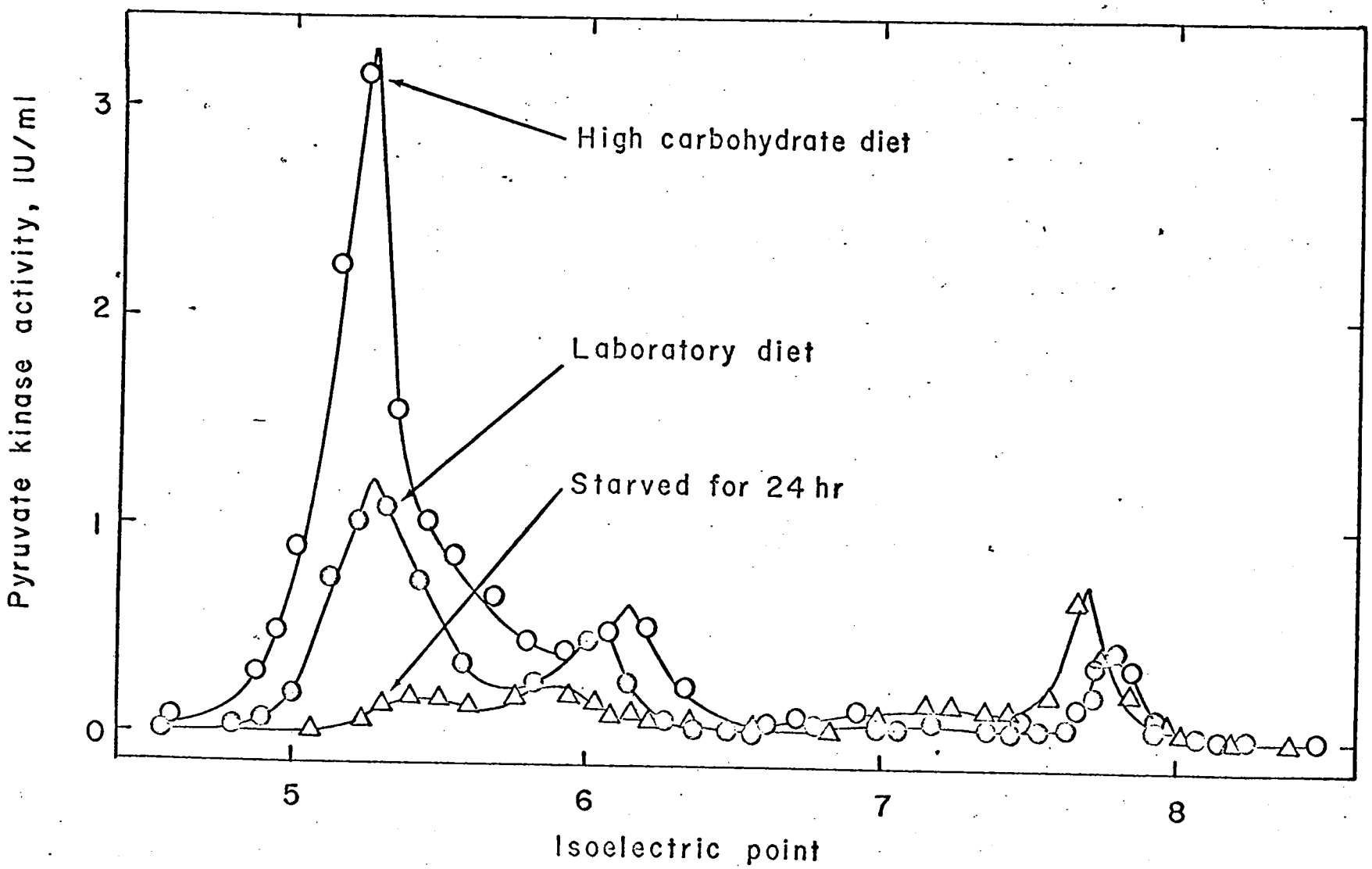

Liver of
Rh. sarcoma-
bearing rat
Liver
Spleen
Muscle
Kidney
Muscle,
Liver,
Kidney,
Spleen,
IU/ml
IU/ml
pI
14
12
10
8
6
4
2
0
1.0
0.5
0.0
7
6
5
4
3
2
1
0
4
3
2
1
0
5
6
7
8

Fig. 2. Effect of high carbohydrate diet and starvation on isozyme pattern of pyruvate kinase in rat liver

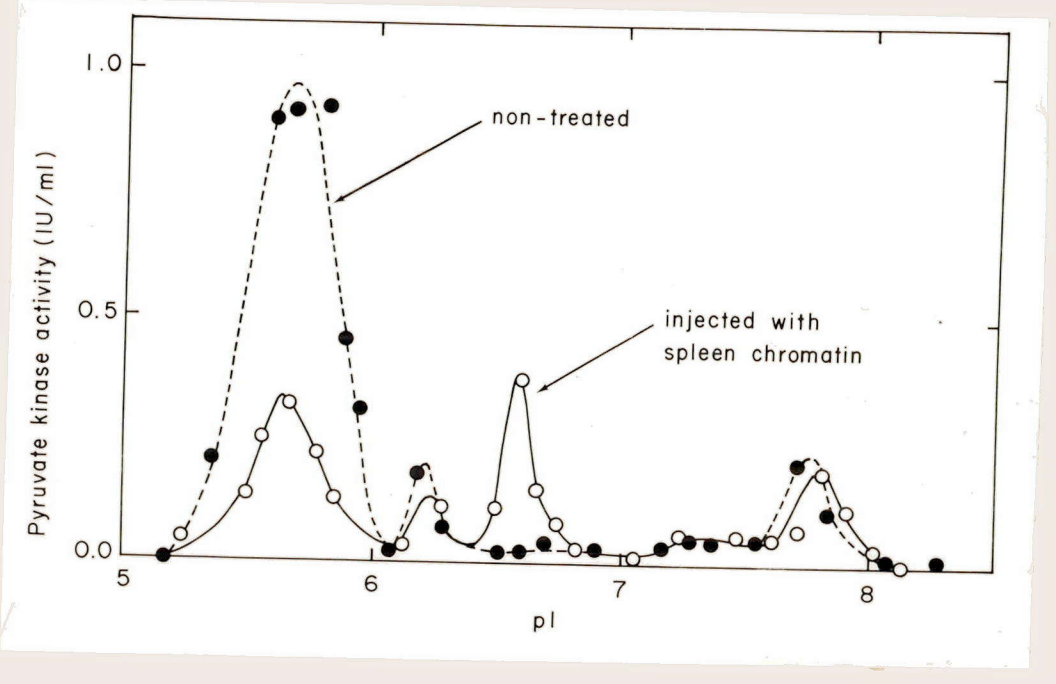
Open circles, rat fed on high carbohydrate diet; closed circles, rat fed on laboratory diet; open triangles, rat starved for 24 hr.

Fig. 2


Pyruvate kinase activity, IU/ml
3
2
1
0
High carbohydrate diet
Laboratory diet
Starved for 24 hr
5
6
7
8
Isoelectric point

ADDENDUM

Fig. 3 Effect of injection of spleen chromatins on isozyme pattern of pyruvate kinase in liver of normal rat



Chromatins were prepared from spleen of normal rat according to the method of Dingman and Sporn with some modifications. The chromatin preparation was analyzed to contain 3.9 mg/ml of DNA and 9.0 mg/ml of protein. It (2.5 ml) was injected into the subcutaneous region on the back of each rat. For the control, 2.5 ml of NaCl solution was injected. The injected rats were kept for 24 hr with unlimited supply of water and diet. They were then decapitated, and their livers were dissected out and used.

Dingman, C., Sporn, M., J. Biol. Chem., 239, 3483 (1964)

PURIFICATION AND PROPERTIES OF M_2-TYPE PYRUVATE KINASE FROM RHODAMINE SARCOMA

by

Tosikazu NAKAMURA

It was previously reported [11] that the pyruvate kinase present in extracts from various tissues of rats is easily separable into five pI-isozymes by means of isoelectric fractionation; their isoelectric points (pI) are 5.4, 6.1, 6.5, 7.4 and 7.8, and their kinetic characteristics are different from one another.

Nakamura et al. [11] have reported that when a preparation of chromatins from Rhodamine sarcoma is injected into rats, the activity of pyruvate kinase in the livers increases to a significant extent; approximately 60% of the increased activity is due to the pI 7.8-isozyme, the remainder due to the pI 6.1-isozyme.

The present paper deals with studies on the purification and properties of M_2-type (pI 7.8-isozyme) pyruvate kinase from Rhodamine sarcoma.

Purification of M_2-Type Pyruvate Kinase from Rhodamine sarcoma

Rhodamine sarcoma tissues at frozen state (400 g in wet weight) were homogenized in 6 volumes (v/w) of 0.25 M sucrose solution containing 1 mM EDTA and 10 mM β-mercaptoethanol with the use of a Waring blendor. The homogenate was centrifuged at 9,000g for 30 min. The resulting supernatant is called "extract".

The extract was supplemented with 277 g/l of ammonium sulfate (45% in saturation) and allowed to stand for half an hour, followed by centrifugation at 9,000g for 20 min. The resulting supernatant was further supplemented with 472 g/l of ammonium sulfate (70% in saturation) and allowed to stand for an hour, followed by centrifugation. The resulting precipitate was dissolved in

100 ml of 0.25 M sucrose solution containing 1 mM EDTA and 10 mM β-mercaptoethanol, and the solution was dialyzed against an excess volume of 0.25 M sucrose solution containing 1 mM EDTA and 10 mM β-mercaptoethanol.

The dialyzed solution ($A_{280\ nm}$/ml = 96) was passed through a column of phosphocellulose (diameter, 5 cm; length, 50 cm), previously equilibrated with 0.25 M sucrose solution containing 1 mM EDTA and 10 mM β-mercaptoethanol. The charged column was washed with 1 liter of 0.25 M sucrose solution containing 1 mM EDTA and 10 mM β-mercaptoethanol. Most of the enzyme activity was by-passed.

The by-passed solution ($A_{280\ nm}$/ml = 9.75) was supplemented with such a volume of 0.5 M Tris-HCl buffer (pH 7.5) that the buffer concentration of the resulting mixture would be 20 mM. The mixture was then passed through a column of DEAE Sephadex A-50 (diameter, 5.0 cm; length, 50 cm), previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA and 10 mM β-mercaptoethanol. All the enzyme was adsorbed on the column. The charged column was washed with 1 liter of the buffer used for the equilibration. Nearly all the enzyme was then eluted with 1 liter of 20 mM Tris-HCl buffer (pH 7.5) containing 0.3 M KCl, 0.25 M sucrose, 1 mM EDTA and 10 mM β-mercaptoethanol (Fig. 1).

The resulting eluate ($A_{280\ nm}$/ml = 1.25) was supplemented with 350 g/l of ammonium sulfate (55% in saturation) and allowed to stand for half an hour, followed by centrifugation at 15,000g for 20 min. The resulting supernatant was further supplement with 472 g/l of ammonium sulfate (70% in saturation) and allowed to stand for 1 hr, followed by centrifugation. The resulting precipitate was dissolved in 5 ml of 0.25 M sucrose containing 1 mM EDTA and 10 mM β-mercapto-ethanol, and the solution was dialyzed for overnight against an excess volume of 0.25 M sucrose solution containing 1 mM EDTA and 10 mM β-mercaptoethanol.

The dialyzed solution was centrifuged at 15,000g for 20 min to remove the precipitate formed during the period of dialysis.

The resulting clear supernatant ($A_{280\ nm}$/ml = 35) was subjected to isoelectric fractionation with Ampholine carrier ampholytes of the range of pH from 6 to 8 (Fig. 2). The enzyme was in most part collected in fractions, the pH values of which were centered to 7.8. Of the fractions, those showing relatively high specific activities of pyruvate kinase (No. 36 to No. 39 in Fig. 2) were mixed, and the mixture was concentrated to 3 ml with the use of a collodion bag under a reduced pressure.

The concentrated solution (3 ml) ($A_{280\ nm}$/ml = 0.86) was passed through a column of Sephadex G-200 (diameter, 2.5 cm; length, 90 cm) previously equilibrated with 0.05 M tris-HCl buffer (pH 7.5) containing 0.15 M KCl, 1 mM EDTA and 10 mM β-mercaptoethanol. The column was then developed with the buffer at a flow rate of 24 ml/hr, and the eluate was divided into 5-ml fractions. The fractions were measured of protein content and pyruvate kinase activity (Fig. 3). Fractions from No. 42 to No. 50 were mixed, and the mixture was concentrated into 1 ml with the use of a collodion bag under a reduced pressure.

All the procedures were carried out in a cold room (approximately 4°)

Yield, Specific Activity and Purity of Purified Preparation of M_2-Type Pyruvate Kinase

The yields and specific activities of preparation of pyruvate kinase obtained at the preparation procedure described above are summarized in Table I. The purest preparation of M_2-type pyruvate kinase was approximately 2,500 times as high in specific activity as the extract, and catalyzed the conversion of 250 μmoles of phosphoenolpyruvate into pyruvate/$A_{280\ nm}$/min. When it was subjected to polyacrylamide gel electrophoresis, only a single band due to pyruvate kinase

protein was detectable (Fig. 4). When it was subjected to isoelectric fractionation with Ampholine carrier ampholytes, the activity of pyruvate kinase was all electrofocused in one group of fractions, the pH values of which were centered at 7.8. When it was subjected to Biuret reaction, it was determined that one unit of $A_{280\ nm}$ is equivalent to 0.67 mg of the protein. The aqueous solution of pure pyruvate kinase was colorless; the absorbance spectrum of the enzyme had a maximum at 280 nm.

Molecular Weight of Purified Pyruvate Kinase

A pure preparation of pyruvate kinase and the marker proteins were subjected to molecular-sieve fractionation on a Sephadex G-200 column in order to determine the molecular weight of the enzyme (Fig. 5). It was found that the molecular weight of M_2-type pyruvate kinase was approximately 112,000, which was approximately one half of the molecular weight of M_1-type pyruvate kinase (Fig. 6).

The molecular weight of the dissociated M_2-type pyruvate kinase was determined to be approximately 58,000 by means of polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate. This indicates that M_2-type pyruvate kinase is present as a dimmer (Fig. 7).

Effect of pH on Activity of Purified M_2-Type and M_1-Type Pyruvate Kinase

As shown in Fig. 8, M_2-type pyruvate kinase is most active around pH 6.6, whereas M_1-type pyruvate kinase around pH 7.0.

Effect of Cations on Activity of Purified M_2-Type Pyruvate Kinase

M_2-type pyruvate kinase shows an sbsolute requirement of divalent cation for exhibition of the activity. The effect of Mg^{2+}concentration on enzyme activity is given in Fig. 9. The enzyme displays a sigmoidal kinetics toward Mg^{2+}; the concentrations of Mg^{2+} required for exhibition of 50% of the maximum rate

are 2.2 mM and 0.8 mM in the presence of 0.5 mM and 5.0 mM phosphoenolpyruvate, respectively. The Hill coefficient value is 2.2 in the presence of 0.5 mM phosphoenolpyruvate and 1.7 in the presence of 5.0 mM the substrate. Mg^{2+} can be replaced by Mn^{2+}, but not by Ca^{2+}. The kinetic behavior of M_2-type pyruvate kinase toward Mn^{2+} in the presence of 0.5 mM and 5.0 mM phosphoenolpyruvate was significantly different from that for Mg^{2+} (Fig. 10). It seems likely that the Km value for the substrate in the presence of Mn^{2+} is lower than that in the presence of Mg^{2+}.

Effect of Phosphoenolpyruvate Concentrations on Activity of Purified M_2-Type Pyruvate Kinase

It was found that the pI 7.8-isozyme (M_2-type) of pyruvate kinase purified from Rhodamine sarcoma by isoelectric fractionation was hardly stimulated by fructose 1,6-diphosphate, in spite of that the curve for initial rate versus phosphoenolpyruvate concentration was of a sigmoid shape (n = 1.4); the "apparent" Km value for phosphoenolpyruvate (the substrate concentration for half the maximum rate) was as high as 1 mM.[11)]

The effect of phosphoenolpyruvate on activity of purified M_2-type pyruvate kinase in presence and absence of 0.1 mM fructose 1,6-diphosphate is shown in Fig. 11.

The curves for initial rate versus phosphoenolpyruvate concentration is of a sigmoid shape and "apparent" Km value for phosphoenolpyruvate is 0.6 mM with Hill constant (n) = 1.5 both in the presence and absence of 0.1 mM fructose 1,6-diphosphate (Fig. 12).

Effect of Fructose 1,6-diphosphate Concentrations on Activity of Purified M_2-Type Pyruvate Kinase

Fig. 13 shows plots of the effect by fructose 1,6-diphosphate as a function of the concentration of fructose 1,6-diphosphate in the presence of either 0.5 mM or 5.0 mM phosphoenolpyruvate. Fructose 1,6-diphosphate shows no effect on the activity of M_2-type pyruvate kinase in the same manner as on the activity of M_1-type pyruvate kinase.

Effect of ATP Concentrations Activity of Purified M_2-Type Pyruvate Kinase

It was reported by Tanaka et al.[13] that the concentration of ATP required for 50%-inhibition of the activity of L-type pyruvate kinase was 0.15 mM, which was approximately 1/20 of that of M_1-type enzyme (3.5 mM) and that the inhibition of the activity of L-type pyruvate kinase by ATP was neutralized by adding fructose 1,6-diphosphate. Curves of enzyme activity as a function of ATP-Mg concentration in the presence and absence of 0.1 mM fructose 1,6-diphosphate are shown in Fig.14. In the presence of either 0.5 mM or 5.0 mM phosphoenolpyruvate, 3 mM ATP depressed the activity of M_2-type pyruvate kinase to 50%. The neutralization of the ATP inhibition by fructose 1,6-diphosphate, which has been observed for L-type enzyme, is not observed for M_2-type enzyme. The inhibition by ATP-Mg of M_2-type pyruvate kinase is almost the same as that of M_1-type enzyme.

Effect of L-Alanine Concentrations on Activity of Purified M_2-Type Pyruvate Kinase

Jiménez de Asúa et al.[8] have shown that M-type pyruvate kinase from skeletal muscle (M_1-type) is inhibited by L-phenylalanine, but not by L-alanine, whereas that from liver (M_2-type) either by L-phenylalanine or by L-alanine.

Curves of the activity of M_2-type pyruvate kinase as a function of L-alanine are shown in Fig. 15. The shape of the inhibition curves is of the Michaelis-Menten type. In the presence of 0.5 mM and 5.0 mM phosphoenolpyruvate, 0.1 mM

and 0.7 mM L-alanine, respectively, give 50%-inhibition. The extent of the inhibition by L-alanine reach a maximum at 3 mM L-alanine; the maximum extent is significantly with 0.5 mM than with 5.0 mM phosphoenolpyruvate. The type of inhibition of M_2-type pyruvate kinase by L-alanine is of the type of the partial competing inhibition, which have been proposed by Gerhart and Pardee[6] on the basis of their studies on aspartate transcarbamylase; the L-alanine inhibition may be caused by the binding of L-alanine with an allosteric site.

The addition of fructose 1,6-diphosphate greatly lowers both the maximum extent of the inhibition by L-alanine and the concentration of L-alanine required for the maximum extent of the inhibition. This suggests that L-alanine and fructose 1,6-diphosphate binds with a common binding site.

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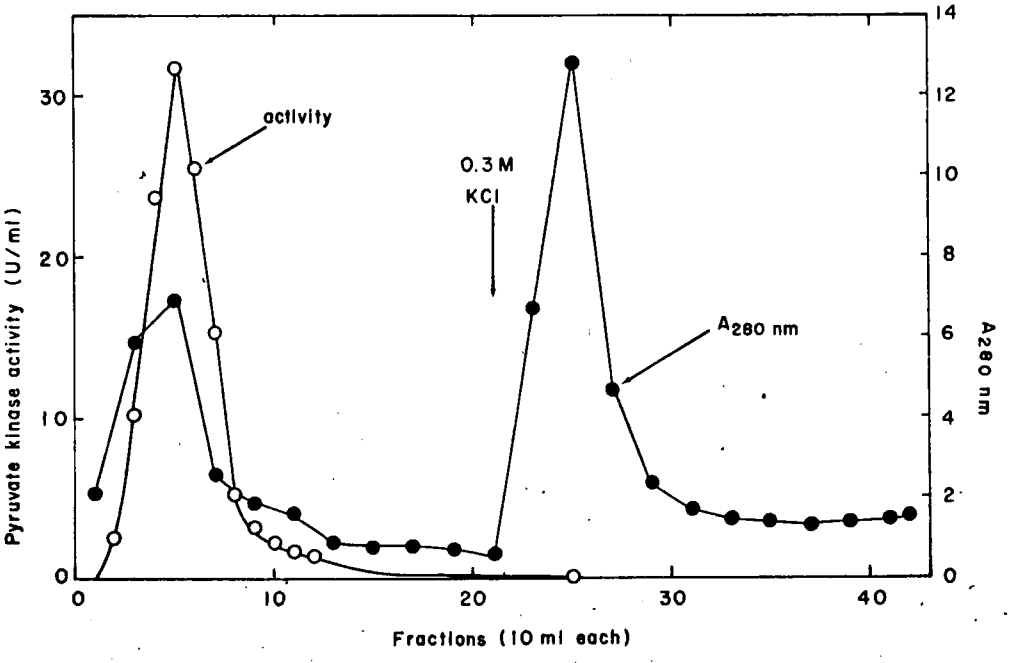
Table I Purification of M_2-Type Pyruvate Kinase from Rhodamine sarcoma

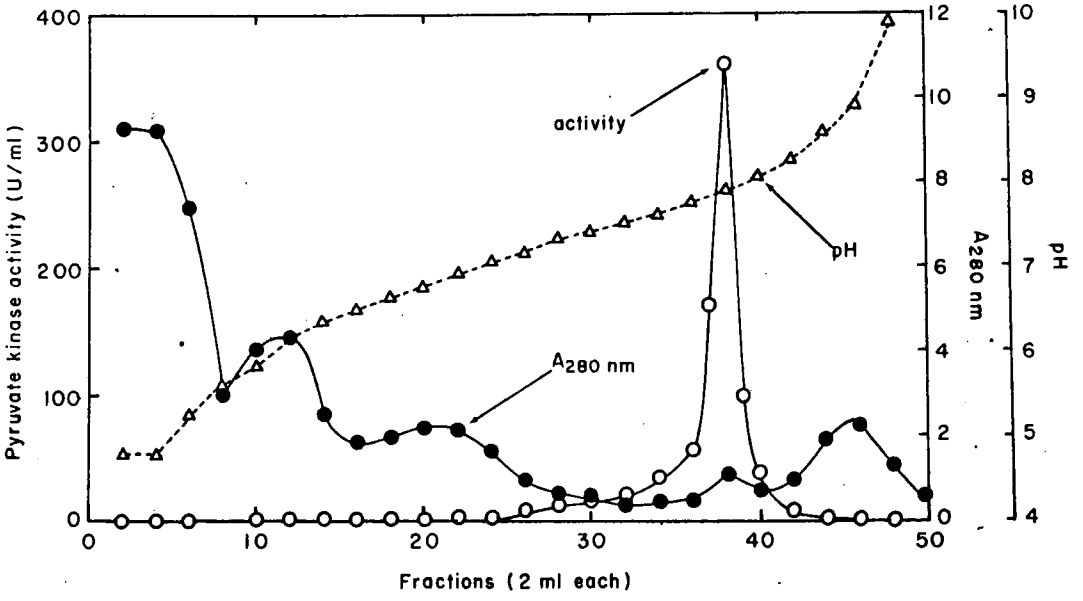
Procedures	Volume (ml)	Concentration (units/ml)	Total units	Protein (A_{280nm}/ml)	Specific activity (units/A_{280nm})	Yield (%)	Purification
Extract	2,000	4.40	8,890	43.5	0.100	100	1
45-70% ammonium sulfate fractionation	146	38.1	5,560	96.0	0.397	63	4
P-cellulose	450	9.69	4,360	9.75	0.994	49	10
DEAE-sephadex A-50	900	4.68	4,210	1.25	3.75	47	38
55-70% ammonium sulfate fractionation	15.4	242	3,720	35.0	6.91	42	69
Electrofocusing by Ampholine-carrier ampholyte	28.5	96.5	2,750	0.857	113	31	1,130
Sephadex G-200	47.0	47.0	2,210	0.188	250	25	2,500

Fig. 1 Chromatography on DEAE-Sephadex A-50 column of M_2-type pyruvate kinase from Rhodamine sarcoma



The activity of pyruvate kinase was measured according to the method of Bücher and Pfleiderer.[3] The standard reaction mixture was composed of 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.5), 0.1 ml of 20 mM phosphoenolpyruvate, 0.1 ml of 20 mM ADP, 0.1 ml of 1.5 M KCl, 50 µl of 0.2 M $MgCl_2$, 50 µl of 5 mM NADH, 10 µl of 150-200 units/ml of lactate dehydrogenase (D-lactate:NAD^+ oxidoreductase, EC 1.1.1.28) and water to make the total volume 1.0 ml. The reaction was started by adding 10 or 20 µl of enzyme sample to the standard reaction mixture, and the decrease of absorbancy at 340 nm was measured at 24°. One unit of pyruvate kinase was difined as the amount of the enzyme that caused the oxidation of 1 µmole of NADH per min.

Fig. 2 Isoelectric fractionation of M_2-type pyruvate kinase



Isoelectric fractionation on an electrofocusing column of M_2-type pyruvate kinase was carried out according to the method of Vesterverg and Svensson [14]; the procedure used was the same as that described previously.[9, 10, 11]

Fig. 3 Molecular sieve fractionation on Sephadex G-200 column of M_2-type pyruvate kinase

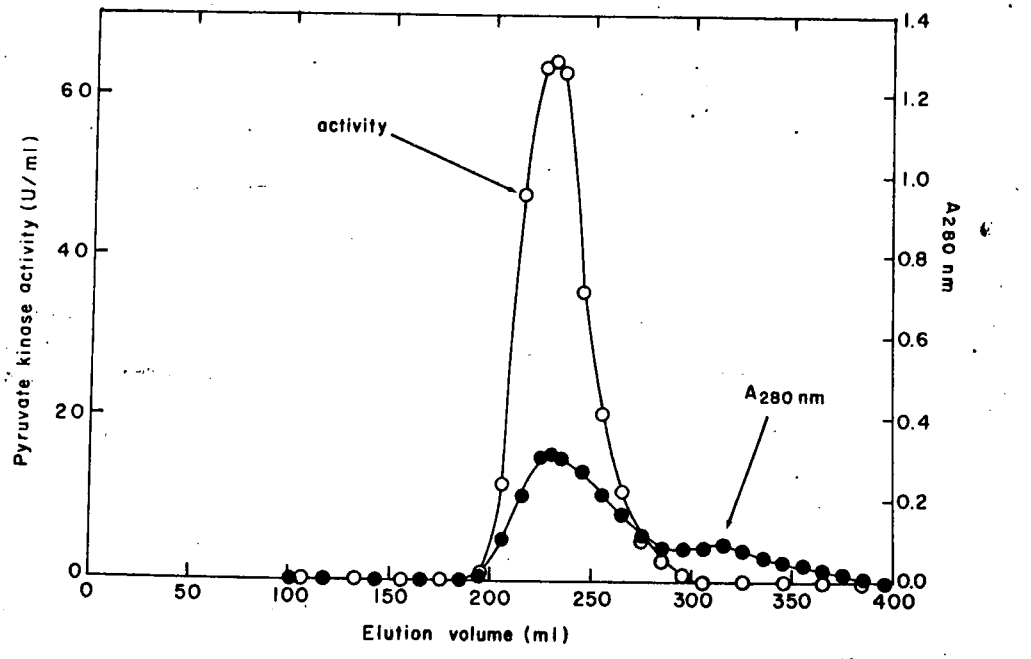
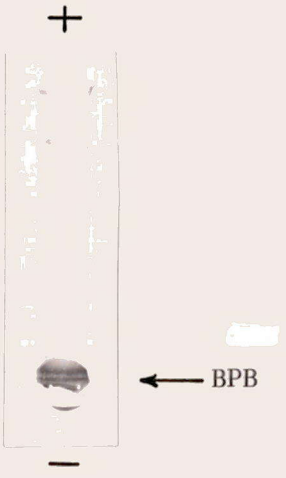
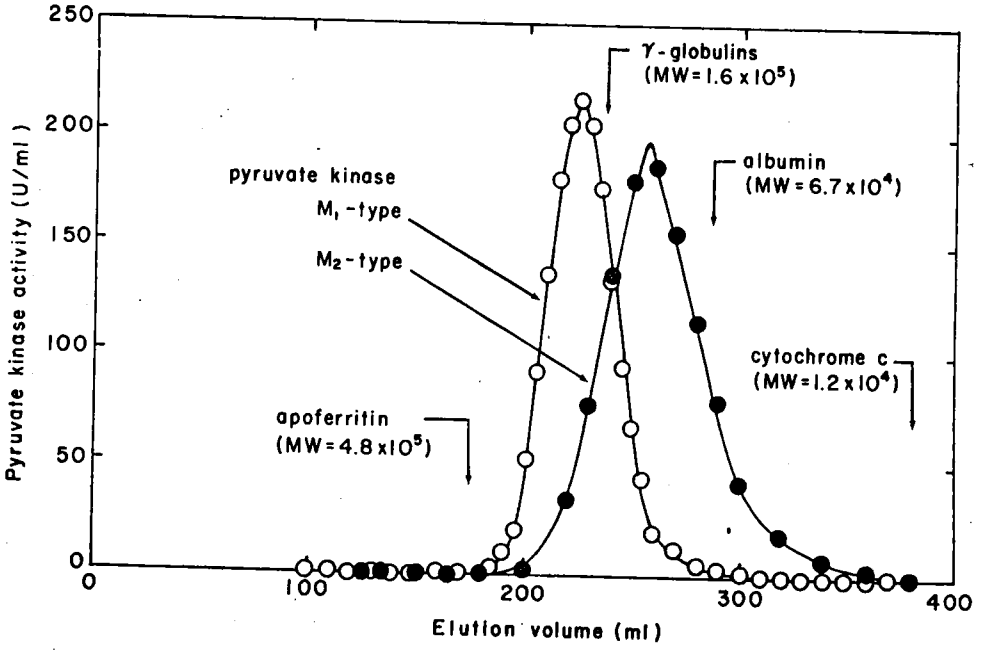


Fig. 4 Polyacrylamide gel electrophoresis of purified M_2-type pyruvate kinase



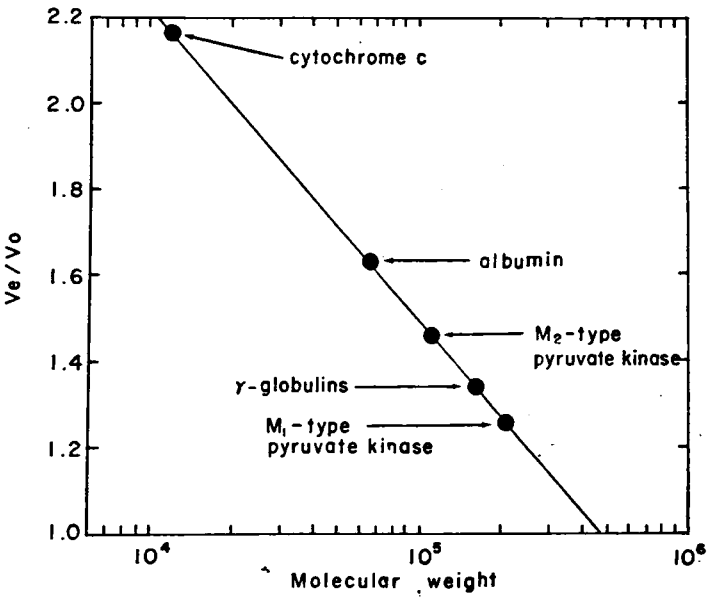
Electrophoresis on a column of polyacrylamide gel was carried out according to the method of Ornstein[12] and Davis[5]. The column had on inner diameter 5 mm and a length of 80 mm. In the column, 0.4 ml of the large-pore gel (2.5%) was layered over 1.5 ml of the small-pore gel (7.5%). A total of 10 μg of M_2-type pyruvate kinase in sucrose dense solution was applied on the gel. Electrode buffer carefully layered on top of sample to fill the column. The electrophoresis was carried out in 0.8 M Tris-HCl buffer (pH 8.9) at 3 mM per column and at 4° for 2.5 hr. The polyacrylamide gel was then taken out of the column, and immersed in a 12.5% trichloroacetic acid solution for 1 hr and successively in a staining solution for 1 hr. The staining solution was prepared freshly by a 20-fold dilution with 12.5% trichloroacetic acid of the stock solution, 1% Coomssie Brilliant Blue (Colab Laboratories Inc.,Chicago), as described by Chrambach et al.[4]. The resulting gel was then transferred in a pan containing a 12.5% trichloroacetic acid solution and allowed to stand for two days. The staining gel was examined in the purity of the enzyme preparation applied, and photographed.

Fig. 5 Molecular-sieve fractionation on Sephadex G-200 column of M_1-type and M_2-type pyruvate kinase



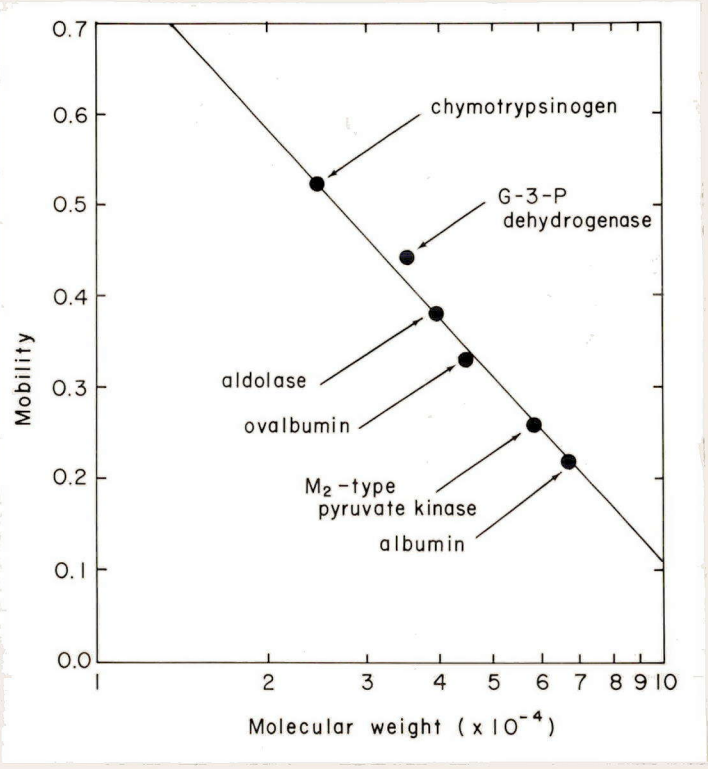
Samples (3 ml) were passed upward through a Sephadex G-200 column equipped with an Upward-Flow adaptor (Pharmacia Fine Chemicals, Uppsala) which was previously equilibrated by 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M KCl, 10 mM β-mercaptoethanol and 1 mM EDTA. The charged column was developed with the same buffer; the flow rates was adjusted at 24 ml/hr by a Peristalitic pump, type 10,200 (LKB-Produkter AB., Stockholm-Broma). The eluate from the column was fractionated into 5 ml aliquots by a fraction collector.

Fig. 6 Plotting of Ve/Vo against log[molecular weight] in molecular-sieve fractionation on Sephadex G-200 column of M_1-type and M_2-type pyruvate kinases and marker proteins



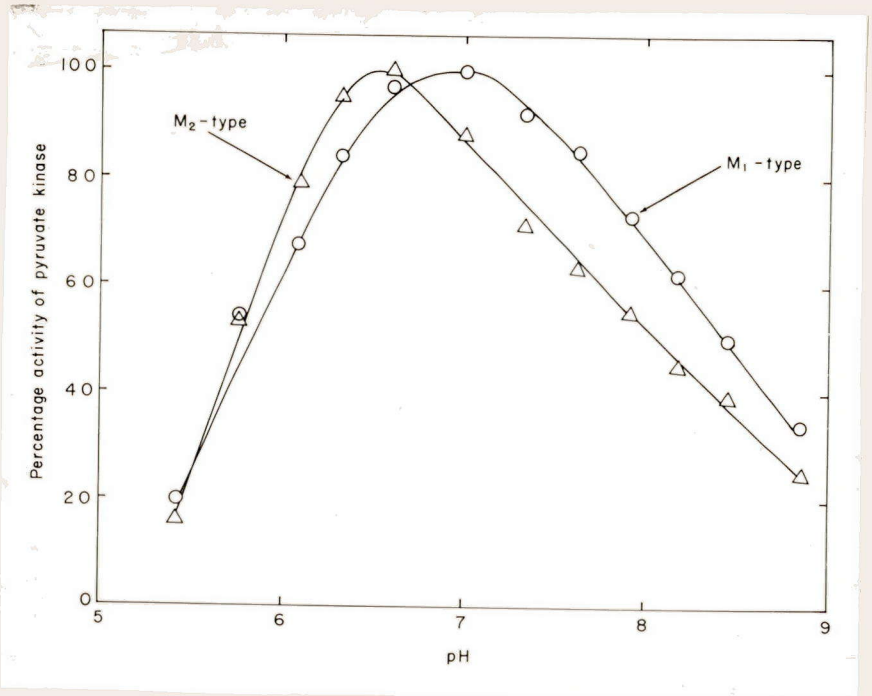
Of the markers used, apoferritin (mol. wt. = 480,000) was eluted at a volume (Ve) of 175 ml, γ-globulins (mol. wt. = 160,000) at Ve of 235 ml, albumin (mol. wt. = 67,000) at Ve of 285 ml and cytochrome c (mol. wt. = 12,000) at Ve of 380 ml. On the basis of these values, molecular weight of M_2-type pyruvate kinase was calculated approximately according to the description of Andrews.[1)]

Fig. 7 Polyacrylamide-gel electrophoresis of purified M_2-type pyruvate kinase and marker proteins in presence of sodium dodecyl sulfate



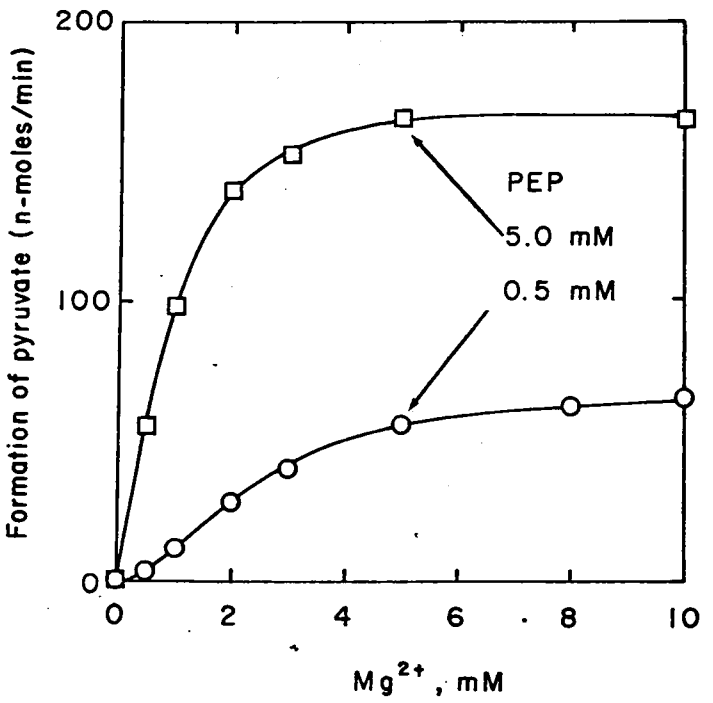
Acrylamide-gel electrophoresis in presence of sodium dodecyl sulfate and the pretreatment used for the runs was carried out by the method of Weber and Osborn.[15] Albumin, ovalbumin, aldolase, glyceraldehyde-3-phosphate dehydrogenase and chymotrypsinogen were used as the reference markers for the molecular weight calibration. A total of 10 μg of M_2-type pyruvate kinase was used for electrophoresis.

Fig. 8 Effect of pH on activities of purified M_1-type and M_2-type pyruvate kinase



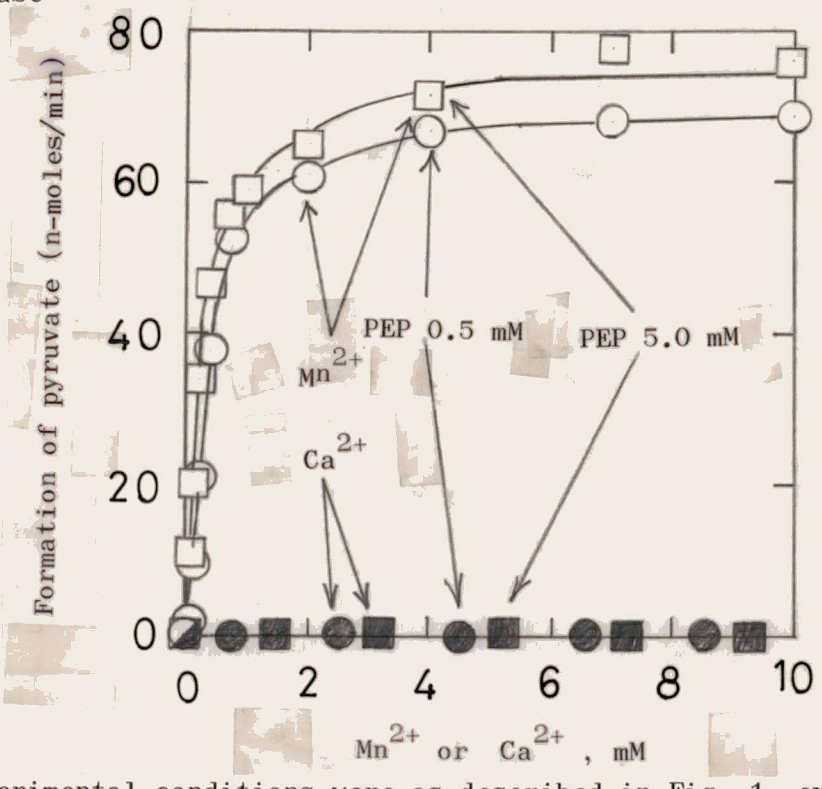
The experimental conditions were as described in Fig. 1, excepted that a buffer composed of 3,3-dimethylglutarate, Tris and 2-amino-2-methylpropane-1,2-diol was used and reaction mixtures were adjusted to various pH values.

Fig. 9 Effect of Mg^{2+} concentrations on activity of M_2-type pyruvate kinase



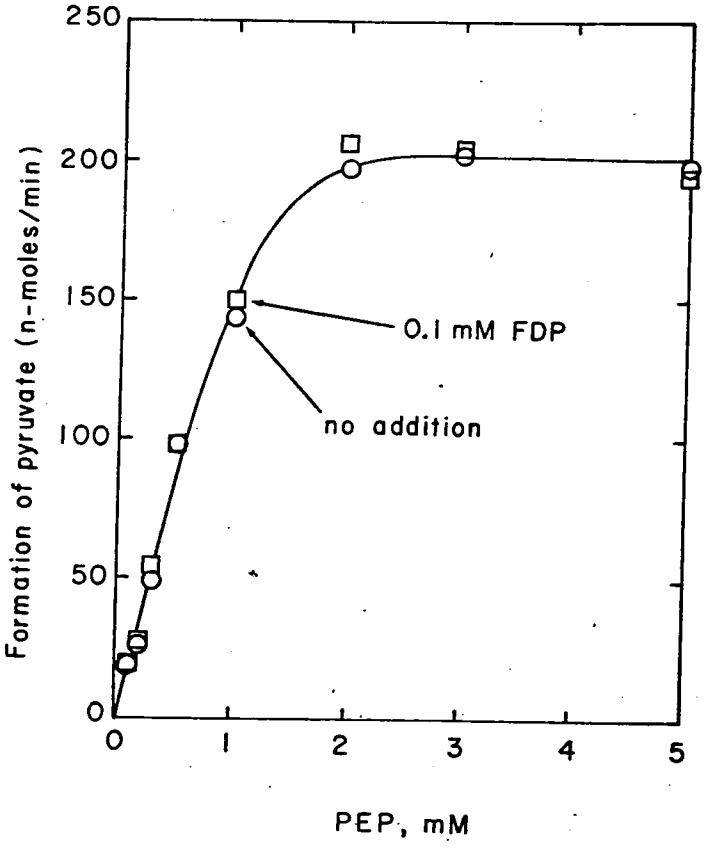
The experimental conditions were as described in Fig. 1, except that the concentration of phosphoenolpyruvate was fixed at 0.5 mM or 5.0 mM and various concentrations of $MgCl_2$ were added to reaction mixture.

Fig. 10 Effects of Mn^{2+} and Ca^{2+} concentrations on activity of M_2-type pyruvate kinase



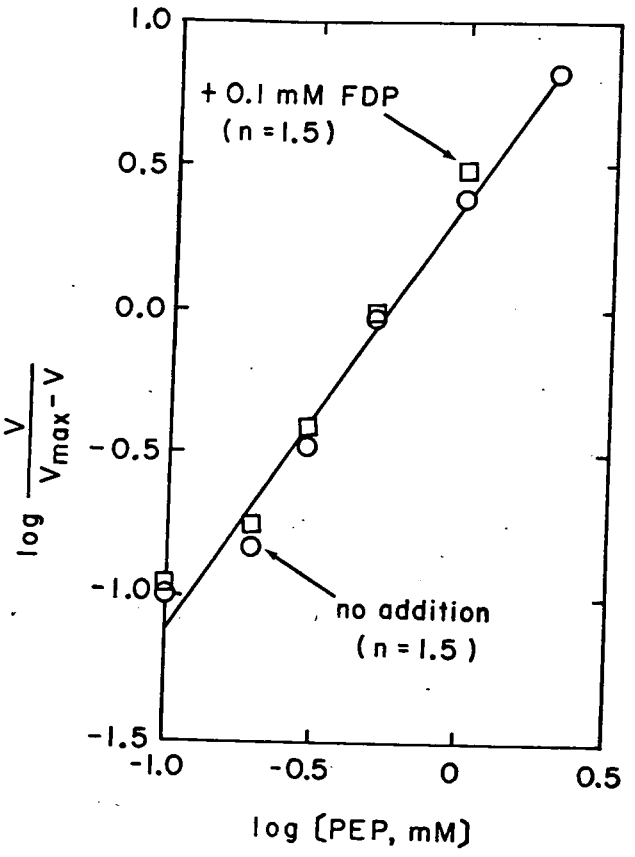
The experimental conditions were as described in Fig. 1, except that the concentration of phosphoenolpyruvate was fixed at 0.5 mM or 5.0 mM and various concentrations of $MnCl_2$ or $CaCl_2$ were added to reaction mixture.

Fig. 11 Effect of PEP concentrations on activity of M_2-type pyruvate kinase in presence and absence of FDP



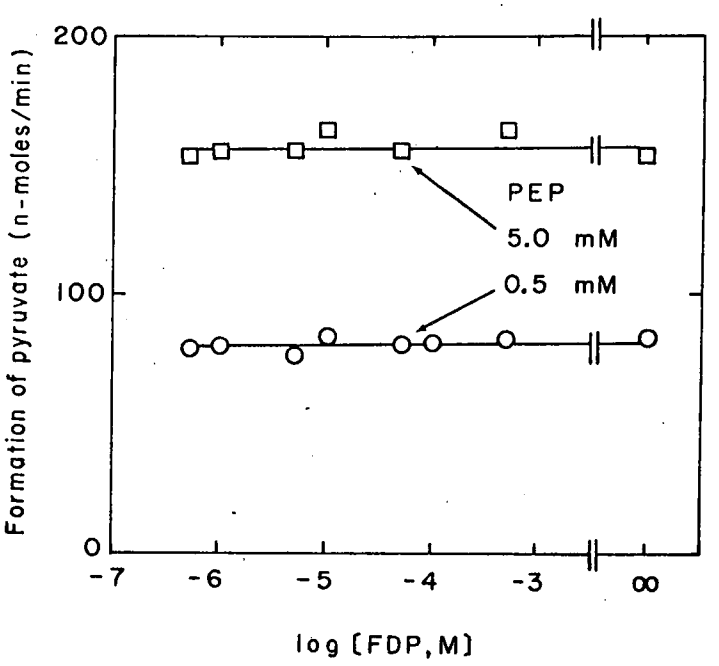
The experimental conditions were the same as those described in Fig. 1, except that in the reaction mixture for the activity assay, the concentration of ADP was fixed at 2 mM, whereas the concentration of phosphoenolpyruvate was varied and various concentrations of phosphoenolpyruvate and 0.1 mM fructose 1,6-diphosphate were added for measurement of the activation by fructose 1,6-diphosphate.

Fig. 12 Hill's plotting of activity of M_2-type pyruvate kinase as function of PEP concentration



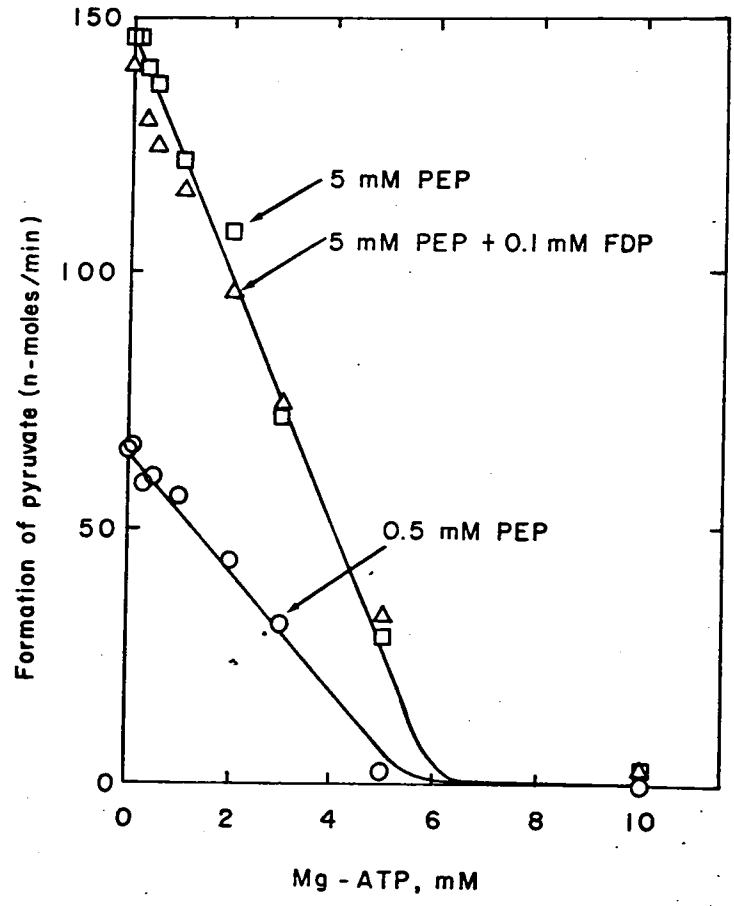
Hill constants (n) for phosphoenolpyruvate were calculated on the basis of curves of activity vs. phosphoenolpyruvate concentrations according to the method of Atkison et al..[2)]

Fig. 13 Effect of FDP concentrations on activity of M_2-type pyruvate kinase



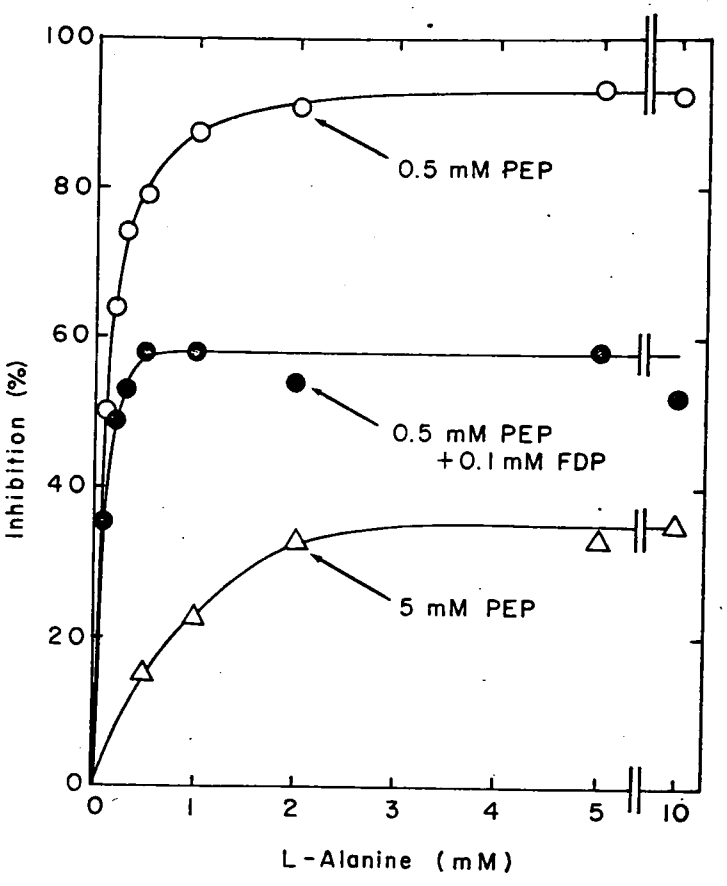
The experimental conditions were as described in Fig. 1, except that the concentration of phosphoenolpyruvate was fixed at 0.5 mM or 5.0 mM and various concentrations of fructose 1,6-diphosphate were added to reaction mixture.

Fig. 14 Effect of ATP concentrations on activity of M_2-type pyruvate kinase



The experimental conditions were as described in Fig. 1, except that the concentration of phosphoenolpyruvate was fixed at 0.5 mM or 5.0 mM and various concentrations of Mg-ATP were added to reaction mixture in presence and absence of 0.1 mM fructose 1,6-diphosphate.

Fig. 15 Inhibition on activity of M_2-type pyruvate kinase by L-alanine



The experimental conditions were as described in Fig. 1, except that the concentration of phosphoenolpyruvate was fixed at 0.5 mM or 5.0 mM and various concentrations of L-alanine were added to reaction mixture in presence and absence of 0.1 mM fructose 1,6-diphosphate.