# PURIFICATION AND PROPERTIES OF SPLEEN-TYPE PYRUVATE KINASE OF RATS

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Yosinobu NAGAO

CONTENTS

## III. RESULTS

### A) PYRUVATE KINASE ISOZYMES IN VARIOUS TISSUES OF RAT, AND INCREASE OF SPLEEN-TYPE PYRUVATE KINASE IN LIVER BY INJECTING CHROMATINS FROM SPLEEN AND RHODAMINE SARCOMA

B) RETENTION OF LIVER-TYPE PYRUVATE KINASE IN CULTURED RAT HEPATOMA CELLS, $MH_1C_1$ CELLS



C) PURIFICATION OF SPLEEN-TYPE AND MUSCLE TYPE PYRUVATE KINASE, AND THEIR DIFFERENTIAL PROPERTIES

# CURRICULUM VITAE

氏　　名　　長尾嘉信　（Nagao, Yosinobu）

生年月日　　１９４６年９月１０日

本籍地　　大阪市南区内安堂寺町通１丁目１８番地

現住所　　石川県河北郡内灘町字大学１丁目３番２４５号

## 略　　歴

１９７１年　３月　　鹿児島大学理学部化学科卒業

１９７１年　４月　　田辺製薬株式会社入社

１９７２年　２月　　同　退社

１９７２年１１月　　大阪大学蛋白質研究所酵素反応学門
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   〝クロマチン蛋白質のラット肝臓におけるピルビン酸キナーゼの等電点
   アイソザイムパターンに及ぼす影響〟
2. 宮崎香，長尾嘉信，松尾雄志，堀尾武一（１９７５）第３４回日本癌学会
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   による肝カタラーゼ活性への影響〟
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   第３６回日本癌学会
   〝ラット肝臓細胞核中に存在する３種類のＤＮａｓｅ　ｉｓｏｚｙｍｅに
   およぼすＲｈｏｄａｍｉｎｅ　ｓａｒｃｏｍａの影響〟
7. 萩原秀昭．宮崎香，長尾嘉信，中村敏一，堀尾武一（１９７７）第３６回
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   〝Ｒｈｏｄａｍｉｎｅ　ｓａｒｃｏｍａ細胞核内に存在する蛋白質分解酵素について〟
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〝担癌ラット肝臓における非ヒストン蛋白質のリン酸化の低下〟

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〝クロマチンに結合して存在するプロテアーゼの可溶化と精製について〟

15. 長尾嘉信，萩原秀昭，宮崎香，松尾雄志，堀尾武一，中村敏一，（１９７８）第５１回日本生化学会

〝ラット肝細胞核における非ヒストンタンパク質のリン酸化と担癌による変動〟

16. 長尾嘉信，萩原秀昭，宮崎香，松尾雄志，堀尾武一，（１９７９）第３８回日本癌学会

〝担癌ラット肝臓の細胞核蛋白質のin vivoにおけるリン酸化〟

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## INTRODUCTION

Greenstein described that some enzyme activities of the liver are affected when the animal bears tumor (1), and that many tumor cells show similar tumor-specific enzyme pattern, regardless of their derived mother cells (2). Since then, many workers studied of enzyme in cancer. With the glycolysis enzymes, deviation of aldolase isozymes was found by Schapira et al. (3) and Sugimura et al. (4), and that of hexokinase isozymes by Sugimura et al. (4) and Weinhouse (5), and that of pyruvate kinase isozymes by Tanaka et al. (6).

On the other hand, Nakahara and Fukuoka (7) extracted a substance having in vivo liver catalase-depressing activity from various kinds of tumor, and they called it " toxohormone". Since then, many workers carried out the purification and characterization of toxohormone (8-10).

We reported that the catalase activity in the liver was depressed, when nuclei or chromatin isolated from the tumor, Rhodamine sarcoma, is subcutaneously or intraperitoneally injected into normal animals (11-16). Matumoto et al. (17) purified from the chromatin of the tumor the non-histone protein effective for depression of liver catalase. It had a molecular weight of about 60,000 and an isoelectric point of about 5.0.

This paper mainly deals with the purification and properties of pyruvate kinase isozymes.

Tanaka et al. (6) found that in Yoshida ascites hepatoma has a pyruvate kinase isozyme, which is different from those present dominantly in normal liver. They (18) purified the liver-type

(L-type) pyruvate kinase, the major isozyme in the liver, and the muscle-type (M-type) enzyme, the major isozyme in the muscle. These two types are distinguishable electrophoretically,immunologically, kinetically, in reactivity to p-chloromercuribenzoate, in molecular weight, etc.

Suda et al. (19) reported that M-type pyruvate kinase increases in the liver of walker sarcoma-bearing rats. The increased isozyme was different from the L-type and M-type, called $M_2$-type. Tanaka et al. (6), using block zonal electrophoresis, found that four isozymes of pyruvate kinase is present in rat liver; one is identical with M-type isozyme in electrophoretic mobility. However, Susor and Rutter (20) claimed, using electrophoresis on a cellulose acetate membrane, that the M-type-like isozyme present in the liver is different from the M-type isozyme present in the muscle. this conclusion was supported by Jiméenez De Asúa et al. (21).

Imamura and Tanaka (22) succeeded in the purification of a new type of pyruvate kinase isozyme from Ehrlich ascites tumor of rats, designating it "$M_2$-type".

By isoelectric electrophoresis with Ampholine carrier-ampholytes, Hess et al. (23) found that pyruvate kinase of pig liver is distinguishable into at least two forms with respect to the mode of FDP-binding.

Nakamura et al. (24), by isoelectric electrophoresis, demonstrated that pyruvate kinase present in various tissues of the rat is separable into five pI-isozymes (different in pI value). They found that $M_2$-type (K-type) enzyme is the major isozyme in the

kidney as well as in Rhodamine sarcoma, and that it is also present in the liver, spleen, lung and erythrocytes. In addition, they observed that $M_2$-type enzyme increases in the liver of rats injected with chromatin prepared from the nuclei of the tumor cells. Ibsen et al. (25) reported that there are six pI-isozymes of K-type and at least three pI-isozymes of L-type. In addition, they (26) reported that K-type pyruvate kinase increases in the liver of mice injected with the extract from Ehrlich ascites tumor cells.

The findings in this report were: 1) Relationships among the various pI-isozymes of pyruvate kinase present in various tissues of rats were studied. We concluded that these pI-isozymes were fundamentally classified into the three types, L-type, M-type and S-type ($M_2$-type), in accordance with the Tanaka school (22). However, we call $M_2$-type spleen-type (S-type), because the spleen contained the isoenzyme in the highest amount among the normal tissues. 2) The spleen-type pyruvate kinase increased in the liver of rats injected with chromatin prepared from either Rhodamine sarcoma or the spleen, but not in those injected with chromatin prepared from the liver. The non-histone protein fraction prepared from Rhodamine sarcoma chromatin gave the same result as the chromatin prepared from the tumor. 3) Spleen-type and muscle-type pyruvate kinases were purified to a homogeneous purity. The purified enzymes were subjected to amino acid analysis and peptide mapping. We found that spleen-type and muscle-type were different in amino acid sequence, indicating that they were formed by different genes. 4) The FDP-binding and various cation-binding properties of spleen-type pyruvate kinase

were studied.

The isozyme pattern of pyruvate kinase is considered as a good marker for the differentiation or proliferation of the liver, because the increase of the spleen-type enzyme accompanied with the decrease of the liver-type enzyme has been reported in the regenerating and the fatal livers and during liver carcinogenesis (27, 28, 29). Furthermore, it was reported that liver-type pyruvate kinase is present in the fatal mouse liver culture in circumfusion system for two weeks (30) and in the primary culture of adult rat liver in at the resting phase (31), whereas an adult rat liver cell line synthesizes only spleen-type enzyme (32). However, we found that the pyruvate kinase isozyme present in a clonal strain of rat hepatoma cells ($MH_1C_1$) from Morris-hepatoma No.7795 is only of the L-type. This indicates that spleen-type pyruvate kinase is unsuitable as the marker of non-growing and normally differentiated hepatocytes.

Many workers consider that non-histone protein has important functions in the gene expression. In fact, the content and the species of non-histone proteins in nuclei are influenced by embryogenesis (33, 34), cell differentiation (35, 36), cell cycle (37, 38) and carcinogenesis (39, 40).

In the studies on nuclei of various tissues of rats, we (41) found that histone is essentially the same in species as well as in relative contents per mg DNA, while some of the species of non-histone protein are specific to the individual kinds of tissues, and that the contents of some species of non-histone protein decrease in

the liver of Rhodamine sarcoma-bearing rats.

## MATERIALS AND METHODS

### Animals and Tumor

Adult male albino rats of Donryu strain were used. Transplantation of Rhodamine sarcoma was carried out according to the method of Matuo et al. (11) with the modifications in which the tumor tissue was mixed with approximately 1 mg each of cephalosporin (Cefamezin) and streptomycin (dihydro-streptomycin sulfate) per g wet weight of tissue, and used without addition of Ringer's solution. About two weeks after the implantation, Rhodamine sarcoma was removed from rats and used for usual experiments. For the purification of pyruvate kinase, tissue was stored in a frozen state before use.

### Preparation of Pyruvate Kinase Extracts from Various Tissues

Normal and tumor-bearing rats were decapitated, and various organs and tumor were dissected out. Extracts from various tissues were prepared by the procedure of Nakamura et al. (24).

### Ammonium Sulfate Treatment of Pyruvate Kinase Extracts

Solid ammonium sulfate was added to various tissues or cells extracts to 70% saturation, followed by centrifugation at 20,000 x g for 20 min. The resulting precipitates were individually dissolved in 10 mM Tris-HCl buffer (pH7.5) containing 5 mM ethylenediamine tetraacetate (EDTA) and 10 mM β-mercaptoethanol. The solution thus obtained were dialyzed against the same buffer. This process is called "ammonium sulfate treatment".

Isoelectric Electrophoresis of Pyruvate Kinase

Pyruvate Kinase extracts with or without ammonium sulfate treatment were subjected to isoelectric electrophoresis with Ampholine carrier-ampholytes (pH 3.5-10) according to the method of Vesterberg and Svensson (42), using a 100 ml electrofocusing column. Otherwise, in the purification steps, a density gradient of form 50% sucrose at the bottom to 25% glycerol at the top was made in the electrofocusing column, since the enzyme was stabilized in the presence of glycerol.

Electrophoresis was carried out at 700V and 0-1°C for 48h. The eluate from the column was divided into 1 or 2 ml fractions and the resulting fractions were measured for pH, absorbance at 280 nm and pyruvate kinase activity. In some cases radioactivity was also measured.

Assay Method for Pyruvate Kinase Activity

The activity of pyruvate kinase was measured according to the method of Bücher and Pfleiderer (43). The standard reaction mixture comprised 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.5), 0.1 ml of 30 mM phosphoenolpyruvate, 50 µl of 40mM ADP, 0.1 ml of 1.5M 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 [L-lactate:$NAD^+$ oxidoreductase, EC 1.1.1.27] and water to make the total volume 1.0 ml. In some cases fructose 1,6-diphosphate was added to the standard reaction mixture. The reaction was started by adding trace volumes of pyruvate kinase solution, and the decrease of absorbance at 340 nm was measured at 24°C, using a Cary model 17 spectrophotometer. One unit of pyruvate

kinase was defined as the amount of enzyme that caused the oxidation of 1 μmole of NADH per min.

Determination of Phosphoenolpyruvate and Fructose 1,6-Diphosphate Concentrations

Phosphoenolpyruvate concentrations were determined by the method of assay for pyruvate kinase activity (see above). FDP concentrations were determined as follows; To 0.2 ml of appropriately diluted FDP solutions were added 0.5 ml of 0.2M Tris-HCl buffer (pH7.5), 50 μl of 0.1 M EDTA, 20 μl of 10 mM NADH and 0.2 ml of water. Ten μl of 80 unit/ml glycerol-3-phosphate dehydrogenase [sn-glycerol-3-phosphate: $NAD^+$ 2-oxidoreductase, EC 1.1.1.8] and 10 μl of 1,800 units/ml triosephosphate isomerase,[D-glyceraldehyde 3-phosphate ketolisomerase, EC 5.3.1.1] were then added in order to remove dehydroxyacetone phosphate and glyceraldehyde 3-phosphate from the reaction system. The reaction was started by adding 10 μl of 90 units/ml fructose diphosphate aldolase [fructose 1,6-diphosphate D-glyceraldehyde 3-phosphate-lyase, EC 4.1.2.13] and the amount of fructose 1,6-diphosphate present in the reaction mixture was estimated.

Preparation of Nuclei, Chromatin, DNA, Non-histone Protein, Histone and Histone-free Chromatin

Nuclei from Rhodamine sarcoma were isolated according to the method of Miyazaki et al. (15), which was a modification of the method of Higashi et al. (44).

Chromatins were isolated not only from Rhodamine sarcoma but also from spleen and liver according to the method of Clark et al. (45) and of Chalkley et al. (46) with some modifications. Details were

described by Nakamura et al. (24) and by Miyazaki et al. (15).

Separation of chromosomal protein and DNA from chromatin by molecular-sieve chromatography on a Sephadex G-200 column in 2M NaCl was carried out according to the method of Georgiev et al. (47), and then, protein-free DNA and non-histone protein fractions were prepared. Details were described by Miyazaki et al. (15).

Histone was extracted from chromatin with 0.1M $H_2SO_4$ and acid-insoluble fraction was saved as the histone-free chromatin, as described by Miyazaki et al. (15).

An aliquot of each preparation was injected into the subcutaneous region on the back of rats. As a control, 0.9% NaCl solution was injected. The injected rats were kept for 23 hr with free access to water and diet. In some cases, second or third injection was carried out at every 23 hr and rats were kept for farther 23 hr. They were then decapitated and the livers were dissected out and used for experiments.

Molecular-Sieve Chromatography on Sephadex G-200 Column

The determination of molecular weights was carried out according to the method of Andrews (48), using a Sephadex G-200 (1.5 x 90 cm) column (Pharmacia Fine Chemicals, Uppsala). Blue dextran (M.W. 2,000,000), rabbit muscle pyruvate kinase (M.W. 240,000), yeast alcohol dehydrogenase [EC 1.1.1.1] (M.W. 140,000), bovine serum albumin (M.W. 67,000) were used as molecular weight markers. The flow rate was adjusted to 7 ml/hr by a peristaltic pump, type 10,200 (LKB) Produkter AB, Stockholm-Bromma). The eluate was divided into 1 ml fractions.

SDS-Polyacrylamide Bel Disc Electrophoresis of Pyruvate Kinase

SDS-polyacrylamide gel disc electrophoresis was carried out accoding to the method of Weber and Osborn (49), using 7.5% polyacrylamide gel (5 x 90 mm).

Cells and Cell Culture

$MH_1C_1$ cells were purchased from the American Type Culture collection. BRL cells originally isolated by Coon (50) from normal adult rat liver, were generous gifts from Dr. Gordon Sato (University of California, San Diego). $MH_1C_1$ cells were cultured on 150-mm Falcon plates in Leibovitz's L-15/Ham's F-10 mixture (7:3) supplemented with 17% fetal calf serum. BRL cells were cultured on 100-mm Falcon plates in Dulbecco's modified Eagle's medium supplemented with 12.5% house serum and 2.5% fetal calf serum. Cultures were incubated at 37°C in a jumicified atmosphoere of 5% $CO_2$ and 95% air.

Preparation of Cell Extracts

The cells were harvested by trypsinizing subconfluent monolayer. Averages of 5 x $10^6$ BRL cell/100-mm plate and $10^7$ $MH_1C_1$ cell/150-mm plate were harvested. The trypsinized cells were centrifugally washed three times with cold saline solution containing phosphate buffer and suspended in 0.5 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM β-mercaptoethanol, 5 mM $MgCl_2$ and 1 mM EDTA. The cells were disrupted by freezing in liquid nitrogen and thawing at 37°C. This procedure was repeated three times. The cell lysate was centrifuged at 105,000 x g for 1 hr and the supernatant was used as the pyruvate kinase extract. In some cases, the extract was subjected to

"ammonium sulfate treatment" as described above.

Amino Acid Analysis

Dialyzed and then lyophilized samples (purified Spleen-type and muscle-type pyruvate kinase) were individually hydrolyzed in 6 N HCl in a sealed tube under vacuum at 110°C for 24 hr, 48 hr and 72 hr. After removal of HCl under vacuum, the sample was analyzed by a amino acid analyzer (model JLC-5AH, Japan Electron Optics Lab., Tokyo) according to the method of Moore and Stein (51).

Preparation of Reduced and S-Carboxymethylated Pyruvate Kinase and Its Peptide Mapping

Reduction and S-carboxymethylation of pyruvate kinase was carried out according to the method of Crestfield et al. (52). An aliquot of thus obtained sample was subjected to amino acid analysis to test the completion of the reaction and determine the content of half-cystine. To each 8 mg of reduced and S-carboxymethylated sample, 1.6 ml of 0.2 M ammonium bicarbonate buffer (pH 8.5) and 80 µg of trypsin (treated with L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone) were added, followed by incubation at 37°C. After 6 hr, additional 40 µg of trypsin was added, followed by further incubation for 6 hr. Resulting solution was lyophilized and thus obtained meterial was dissolved in 80 µl of 20% pyridine. Insoluble meterial was removed by centrifugation. The supernatant (2 µl containing 200 µg peptides) was subjected to chromatography on a cellulose thin layer (Merck 5716 plate 20 x 20 cm) in n-butanol/ pyridine/acetic acid/ water (15:10:3:12 by volume) for 6 hr at 24°C. The thin layer was dried

at 50-60°C for 15 min, allowed to stand at room temperature for 1 hr, and then moistened with pyridine/acetic acid/ water (1:10:89 by volume, pH 3.5). The second dimensional electrophoresis was carried out for 85 min at 700 V in a cold room (4°C). After resulting thin layer was dried at 100°C for 10 min.

Measurement of Radioactivity

The radioactivity of [$^{14}$C]FDP was measured within 5 hr after mixing 0.5 ml of a sample solution and 15 ml of Bray's solution in order to avoid chemical quenching, with a scintillation counter (model LS-250, Beckman Instruments, Inc., Fullerton).

Protein and DNA Content

Protein amount was determined by the method of Lowry et al. (53), using bovine serum albumin as a standard. DNA content was determined by the method of Schneider (54), using calf thymus DNA as a standard.

Reagents and Other Chemicals

Phosphenolpyruvate, fructose 1,6-diphosphate, molecular weight marker proteins, bovine serum albumin, ovalbumin, α-chymotrypsinogen A, horse myoglobin and cytochromec), dibutyryl cyclic AMP, ($Bt_2$cAMP), dibutyryl cyclic GMP ($Bt_2$cGMP), bovine pancreas insulin and glucagon were obtained from Sigma Chemical Co., St. Louis, Missouri. ADP, NADH, pig heart lactate dehydrogenase and yeast alcohol dehydrogenase were from Oriental Yeast Co., Ltd., Osaka. Blue dextran was from Pharmacia Fine Chemicals AB., Uppsala. Ampholine carrier-ampholytes were from LKB Produkter AB., Stockholm-Bromma. Rabbit muscle pyruvate kinase was from Boehringer Mannheim GmbH, Mannheim. Trypsin treated with L-(1-tosylamide-2-phenyl) ethyl chloromethyl ketone was from Worthington

Biochemical Corp., Freefold, New Jersey. For cell culture, trypsin was from Difco Lab., Michigan, and medium and serum were from Folw Lab. Inc., Inglewood, California. [$^{14}$C]FDP was from the Radiochemical Center, Amersham.

## RESULTS

### A) PYRUVATE KINASE ISOZYMES IN VARIOUS TISSUES OF RAT, AND INCREASE OF SPLEEN-TYPE PYRUVATE KINASE IN LIVER BY INJECTING CHROMATINS FROM SPLEEN AND RHODAMINE SARCOMA

#### 1. Isoelectric Electrophoresis of pI-Isozymes of Pyruvate Kinase Present in Various Tissues of Rats

When the extract from livers of normal rats was subjected to isoelectric electrophoresis, pI 5.4-, pI 5.6-, pI 6.2-, pI 7.4- and pI 7.8- isozymes were detectable (Fig.1). The pI 5.4-isozyme, if

Fig. 1

subjected again to isoelectric electrophoresis, was mostly converted into the pI 5.6- and pI 6.2-isozymes (Fig. 2). In repeated isoelect-

Fig. 2

ric electrophoresis, pI 5.6-isozyme converted into pI 6.2-isozyme, whereas pI 6.2-isozyme did not change. When liver extract which had been treated with ammonium sulfate was subjected to isoelectric electrophoresis, pI 5.4- and pI 5.6-isozymes were hardly detectable, and pI 6.2-isozyme increased significantly and pI 4.9-isozyme was newly formed, whereas pI 7.4- and pI 7.8-isozymes were hardly affected (Fig.1). The resulting pI 4.9-isozymes was labile, and it was not further examined; it seems likely that this isozyme was an

artifact generated by the ammonium sulfate treatment.

Hess et al. (23) found with isoelectric electrophoresis method, that two types of liver pyruvate kinase of pig can be distinguishable on the basis of FDP binding; pI 5.3-isozyme binds FDP and pI 6.1 is FDP-free form.

When the liver pI 6.2-isozyme fractions obtained by isoelectric electrophoresis were mixed with FDP (0.1 mM at final concentration) and subjected to isoelectric electrophoresis, pI 5.4- and pI 5.6-isozymes were formed with the ratio of approximately 2:1.

These rusults suggest that liver-type pyruvate kinase posseses two kinds of sites capalbe of binding with FDP; liver-type isoenzyme has a pI value of 6.2 when free of FDP, and when one kind of site ( the 1st site) binds with FDP, the pI value shifts from 6.2 to 5.6, and when both kinds of sites (1st and 2nd sites) bind with FDP, the pI value shifts further to 5.4.

When the extract from spleen of normal rats was subjected to isoelectric electrophoresis, pI 6.2-, pI 6.6-, pI 7.4- and pI 7.8-isozyme were detected (Fig. 3). In this experiment, pI 6.2-isozyme

Fig. 3

was dominant form. However, in some spleen extracts, pI 6.6-isozyme was dominant form. The resulting pI 6.2-isozyme, if it was again subjected to isoelectric electrophoresis, mostly converted into pI 6.6-isozyme (Fig. 4). The pI 6.6- and 7.8-isozymes were not affected by repeated

Fig. 4

isoelectric electrophoresis. When spleen extract which had been treated with ammonium sulfate was subjected to isoelectric electrophoresis, pI 6.2- and pI 6.6-isozymes did not appear, and then pI 7.8-isozyme content increased significantly (Fig. 3).

The spleen pI 7.8-isozyme fractions obtained by isoelectric electrophoresis were mixed with FDP (0.1 mM at final concentration), and followed by subjecting to isoelectric electrophoresis (Fig. 5).

Fig. 5

It was found that pI 7.8-isozyme converted into pI 6.2- and pI 6.6-isozymes. In some experiment, only pI 6.6-isozyme was formed. The extent of conversion increased with increasing concentrations of FDP. In addition, pI 7.8-isozyme was mostly converted into pI 6.2-isozyme when 1 mM FDP was added to the whole of the solution placed in the electrofocusing column.

These results suggest that spleen-type pyruvate kinase posseses two kinds of sites capable of binding with FDP; spleen-type isoenzyme has a pI value of 7.8 when free of FDP, and when one kind of site ( the 1st site) bind with FDP, the pI value shifts from 7.8 to 6.6 when both kinds of sites (1st and 2nd sites) bind with FDP, the pI vlaue shifts further to 6.2.

When the extract from skeletal muscle of normal rats was subjected to isoelectric electrophoresis, only pI 7.4-isozyme was detectable

(Fig. 6). The muscle pI 7.4-isozyme was not influenced by repeated

Fig. 6

isoelectric electrophoresis before and after ammonium sulfate treatment (Fig. 6) or with and without addition of FDP.

These suggest that muscle-type isoenzyme can not bind with FDP.

Nakamura et al. (24) previously reported that extract from kidney, lung and erythrocytes of normal rat contain pI 6.2- and/or pI 6.6-isozymes with pI 7.8-isozyme. When these extracts were subjected to ammonium sulfate treatment and then to isoelectric electrophoresis, most of the pI 6.2-isozyme in the kidney extract and all of the pI 6.6-isozyme in the lung and erythrocyte extracts were converted into pI 7.8-isozymes. In addition, even if after ammonium sulfate treatment, pI 6.2-isozyme existed in the extracts of kidney and erythrocyte indicating these extracts contain the liver pI 6.2-isozyme. These results were summarized in Table I.

When the extract from Rhodamine sarcoma was analyzed by isoelectric electrophoresis, pI 6.2-, pI 7.4- and pI 7.8-isozyme were detectable (Fig. 7) and when ammonium sulfate treated extract was

Fig. 7

subjected to isoelectric electrophoresis, pI 6.2-isozyme disappeared and then pI 7.8-isozyme content increased (Fig. 7 and Table I). With

Table I

respect to the behavior in isoelectric electrophoresis, pI 6.2-isozyme was identical to spleen pI 6.2-isozyme, and pI 7.4-isozyme was identical to muscle pI 7.4-isozyme.

2. Kinetical Differences in pI-Isozyme of pyruvate Kinase of Rats

Various pI-isozymes were obtained from extracts of livers, spleens, skeletal muscles and Rhodamine sarcoma of rats by isoelectric electrophoresis before and after ammonium sulfate treatment. Using the pI-isozymes thus obtained, the effects of various concentrations of phosphoenolpyruvate on activities in the presence and absence of 0.1 mM FDP were measured. The reaction was started by adding a small volume of enzyme sample to the standard reaction mixture. In some cases, FDP was added to the standard reaction mixture before adding the enzyme.

Using liver pI 6.2-isozyme, the kinetical curve for initial rate versus phosphoenolpyruvate concentration was sigmoidal (n = 2.0; Km = 1.5 mM) if FDP was absent in the standard reaction mixture (Fig. 8). While, in the presence of FDP, the activity was signifi-

Fig. 8

cantly stimulated and kinetical curve showed hyperbolic (n = 1.0; Km = 0.1 mM). The use of liver pI 5.4- and pI 5.6-isozymes gave practically the same results. This indicates that these isozymes liberated

its bound FDP when FDP was not added to the reaction mixture, and coverted into pI 6.2-isozyme, and that resulting pI 6.2-isozyme could bind with FDP stimulating the activity, if FDP was provided.

Using spleen pI 6.2-isozyme, the kinetical curve for initial rate versus phosphoenolpyruvate concentration was hyperbolic (n = 1.0; Km = 0.2 mM) if FDP was not added in the reaction mixture (Fig.9). In the presence of FDP, the activity was significantly

Fig. 9

stimulated when the concentration of phosphoenolpyruvate was lower than 1 mM (n = 1.0; km = 0.06 mM). The use of spleen pI 6.6-isozyme, and Rhodamine sarcoma pI 6.2- and pI 6.6-isozymes gave practically the same results. Using spleen pI 7.8-isozyme, the kinetical curve showed sigmoidal in the absence of FDP (n = 1.7; Km = 0.7 mM. The activity was only slightly stimulated in the presence of FDP (n = 1.7; Km = 0.5 mM). Practically the same results were obtained with Rhodamine sarcoma pI 7.8-isozyme. It is conceivable that when FDP was not added in the reaction mixture spleen pI 6.2-isozyme liberated a part of its bound FDP and was thus converted into pI 6.6-isozyme, and that when FDP was added to the reaction mixture, the resulting pI 6.6-isozyme could bind with FDP, stimulating the activity, and that spleen pI 7.8-isozyme could not bind FDP even FDP was provided in this assay condition.

Using muscle pI 7.4-isozyme, the activity was not affected by FDP and kinetical curve showed hyperbolic in the absence of FDP (n =

1.0; Km = 0.06 mM) (Fig. 10).

Fig. 10

These results were summarized in Table II.

Table II

3. Pyruvate Kinase pI-Isozymes in Livers of Rhodamine sarcoma-bearing Rats

Nakamura et al. (24) found, using the isoelectric electrophoresis, when Rhodamine sarcoma is transplanted into rats, the total activity of pyruvate kinase increases in liver; pI 6.2-isozyme content increases, whereas other pI-isozymes are little influenced. Their results were reproduced (Fig. 11). When the

Fig. 11

extract from liver of Rhodamine sarcoma-bearing rat was subjected to ammonium sulfate treatment and then to isoelectric electrophoresis, pI 7.8-isozyme content increased to remarkably more extent than when normal liver extract was used (Fig. 11 and Fig. 1). These date indicate that increase of the pyruvate kinase activity of tumor-bearing rats was mostly due to the increase of spleen-type isozyme (pI 6.2- or pI 7.8-isozyme).

4. Effect of Injection of Nuclei from Rhodamine sarcoma on pI-isozyme Pattern of Pyruvate Kinase in Livers

The nuclei prepared from Rhodamine sarcoma was injected into the subcutaneous region on the back of rats. The liver extract from these rats was subjected to ammonium sulfate treatment, and followed by subjecting isoelectric electrophoresis, The increase of pI 7.8-isozyme content showed dose dependency (Table III); at single injec-

Table III

tion of maximum dose tested, 3.3-fold increase was observed. In addition, at daily injection for 3 days pI 7.8-isozyme content increased 4 times. These observations support the previous report by Nakamura et al. (24) They used the chromatin preparations from Rhodamine sarcoma instead of nuclei.

5. Effect of Injection of Chromatins Prepared from Rhodamine sarcoma, Spleen and Liver into Rats, on pI-Isozyme Pattern of Pyruvate Kinase in Livers

The extract from livers of rat injected with chromatin prepared from Rhodamine sarcoma was subjected to ammonium sulfate treatment and then to isoelectric electrophoresis. As reported by Nakamura et al. (24), the pI 7.8-isozyme content increased to 2.3 times the levels in extract from livers of normal rats (Table III). The same kind of experiment was carried out with chromatins prepared from spleens and livers of normal rats. It was found that when spleen

chromatin was injected into rats, and the liver extract was subjected to ammonium sulfate treatment, the pI 7.8-isozyme content in the livers increased appreciably. The injection of liver chromatin did not affect the pI-isozyme pattern (Table III).

6. Localization of Spleen-Type-Isoenzyme-Increasing Substance in Chromatin

Prepared from Rhodamine sarcoma

The chromatin preparation from Rhodamine sarcoma was divided into the four fractions, histone-free chromatin, histone, non-histone protein and DNA. Each of resulting fractions was injected into rats, and the liver extract was subjected to ammonium sulfate treatment and followed by subjecting to isoelectric electrophoresis (Table III). When histone-free chromatin was injected, pI 7.8-isozyme increased to approximately two time the level of control rats, and this increase was similar to that of when whole chromatin was injected. When non-histone protein fraction was injected, pI 7.8-isozyme increased appreciably. Whereas histone and DNA fractions had no effect on the increase of pI 7.8-isozyme. These suggest that pI 7.8-isozyme-increasing activity localized in non-histone protein fraction of Rhodamine sarcoma.

B) RETENTION OF LIVER-TYPE PYRUVATE KINASE IN CULTURED RAT HEPATOMA CELLS, $MH_1C_1$ CELLS

1. Effects of Phosphoenolpyruvate Concentrations and FDP on Pyruvate Kinase Activities in BRL and $MH_1C_1$ Cell Extracts

The specific activity of pyruvate kinase in the extract of BRL cells was about 20 times that in the extract of $MH_1C_1$ cells (Table IV), and about 5 times that in the extract of normal liver tissue (24). The growth rates of BRL cells and $MH_1C_1$ cells under the present conditions, as represented by doubling times, were about 16 hr and 24 hr, respectively (data not shown).

In the kinetic studies, pyruvate kinase activity in the extract of $MH_1C_1$ cells was stimulated significantly by FDP (Fig. 12); the Km

Fig. 12

values for phosphoenolpyruvate in the presence and absence of FDP were 0.13 mM and 1.5 mM, respectively. On the other hand, the activity in the extract of BRL cells was only slightly stimulated by FDP; the Km values for phosphoenolpyruvate in the presence and absence of FDP were 0.50 mM and 0.55 mM, respectively.

As described already, liver-type isoenzyme can bind with FDP stimulatin the activity, whereas spleen-type isozyme can not bind with FDP in the reaction mixture of conventional activity assay. on the bases of these FDP-binding properties and the km values for phosphoenolpyruvate, it was suggested that the pyruvate kinase isozymes in the extract of $MH_1C_1$ cells and BRL cells were mainly liver-type and spleen-type, respectively.

## 2. Isoelectric Electrophoresis of Pyruvate Kinase Isozymes from BRL and $MH_1C_1$ Cells

When the extract of BRL cells was subjected to isoelectric electrophoresis, pyruvate kinase activity was separated into two fractions: pI 7.8-isozyme as the main component and pI 7.4-isozyme as the minor component (Fig. 13). Treatment with ammonium sulfate did not influence

Fig. 13

the pI-isozyme pattern (data not shown). These results show that BRL cells contain spleen-type isoenzyme as a main component and muscle-type isoenzyme as a minor component. Although BRL cells retain some liver-specific functions (60), they are not fully differentiated with regard to synthesis of pyruvate kinase isozymes. Similar deviation of expression of pyruvate kinase isozyme in cultured liver cells was reported previously (32). On isoelectric electrophoresis, an extract of $MH_1C_1$ cells gave two fractions of pyruvate kinase activity, pI 5.4-isozyme and pI 6.2-isozyme (Fig. 14); there was no detectable activity at pI 7

Fig. 14

to pI 8. As described already, the pI 5.4-isozyme is liver-type isoenzyme associated with FDP at two kinds of binding sites, whereas the pI 6.2-isozyme is liver-type isoenzyme free from bound FDP or spleen-type isoenzyme associated with FDP at two kinds of binding sites. On ammonium sulfate treatment of the extract, the content of the pI 6.2-isozyme increased with decrease in that of pI-5.4 isozyme (Fig.

15). As described already for rat liver (Fig. 1), pI 4.9-isozyme also

Fig. 15

appeared. Again, no isozymes with pI values of 7.4 and 7.8 were detectable. These results indicate that $MH_1C_1$ cells, which are a clonal strain, contain only liver-type pyruvate kinase. Expression of liver-type pyruvate kinase in $MH_1C_1$ cells is stable because the cells were established before 1969 (55) and this phenotype has not changed during culture for at least the last two years.

3. Effects of Insulin, Glucagon, $Bt_2$cAMP and $Bt_2$cGMP on Liver-Type Pyruvate Kinase Activities in $MH_1C_1$ Cells

It is known that the activity of liver-type pyruvate kinase is controlled by hormones (56,57) and diets (18,24). We tested the effects on liver-type pyruvate kinase activity in cultured $MH_1C_1$ cells of insulin, glucagon, $Bt_2$cAMP and $Bt_2$cGMP by adding these compounds to the medium. Insulin has no effects on stimulation of pyruvate kinase activity at any concentrations tested (0.1-3 μg/ml) for 5 min to 48 hr. Glucagon (1 μg/ml), $Bt_2$cAMP ($10^{-6}$ and $10^{-4}$M) and $Bt_2$cGMP ($10^{-6}$ and $10^{-4}$ M) has little effects. These results were summarized in Table V. Thus,

Table V

it seems like that the systems for transmission of signals of hormones to expression of enzyme activity are probably disconnected in $MH_1C_1$

cells.

## C) PURIFICATION OF SPLEEN-TYPE AND MUSCLE-TYPE PYRUVATE KINASE, AND THEIR DIFFERENTIAL PROPERTIES

### 1. Purification and Crystallization of Spleen-Type Pyruvate Kinase from Rhodamine sarcoma of Rats

Frozen tissue of Rhodamine sarcoma (5 Kg in wet weight) was put in 5 volumes of 10 mM Tris-HCl buffer containing 10 mM β-mercaptoethanol, 5 mM $MgCl_2$, and 1 mM EDTA (pH 7.5), and then homogenized in a Waring blender (Model CB-2-10, Eberbach Corporation, Michigan) at the medium speed for 1 min. The buffer used is called "Tris-MME buffer." The homogenate was centrifuged at 14,000 x g for 20 min. The supernatant is hereafter called "extract."

The extract (30 liters) was subjected to ammonium sulfate fractionation. The 40 to 70% saturated ammonium sulfate precipitate was dissolved in Tris-MME buffer, and then passed through a Sephadex G-25 column (the bed volume=the sample volume x 4) equilibrated with Tris-MME buffer. The desalted protein solution is called "40-70% sat. $AmSO_4$ fraction."

The 40-70% sat. $AmSO_4$ fraction (4 liters) was passed through a DEAE-cellulose column (16 x 30 cm) equilibrated with Tris-MME buffer. To the protein fraction that was not absorbed on the column (passed fraction), ammonium sulfate was added up to 70% saturation. The resulting precipitate was dissolved in small volume of 5 mM potassium citrate buffer containing 25%(v/v) glycerol and 10 mM β-mercaptoethanol

(pH 6.2) and then dialyzed against an excess volume of the same buffer. The dialyzed solution in called "DEAE-cellulose passed fraction."

The DEAE-cellulose passed fraction (500 ml) was passed through a CM-cellulose column (6.5 x 100 cm) equilibrated with 5 mM potassium citrate buffer containing 25%(v/v) glycerol and 10 mM β-mercaptoethanol (pH 6.2) (Fig. 16). The charged column was then eluted with a linear

Fig. 16

concentration gradient formed of 7 liters of 5 mM potassium citrate buffer(pH 6.2) and 7 liters of 30 mM potassium citrate buffer(pH 6.2), both of which contained 25%(v/v) glycerol and 10 mM β-mercaptoethanol. The eluate was divided into 250-ml fractions. The fractions showing higher specific activities of pyruvate kinase than 15 units/$A_{280nm}$ were mixed. The resulting mixture (2.3 liters) was concentrated to approximately 1 liter by means of membrane filtration and dialyzed against an excess volume of 70 mM Tris-HCl buffer containing 25%(v/v) glycerol and 10 mM β-mercaptoethanol(p 7.5) ("Tris-GM buffer"). The dialyzed solution is called "CM cellulose eluate."

The CM-cellulose eluate was passed through a P-cellulose column (6.5 x 65 cm) equilibrated with Tris-GM buffer (Fig. 17). The charged

Fig. 17

column was washed with three-fold bed volumes of Tris-GM buffer, and then eluted with Tris-GM buffer containing 25 mM potassium phosphate.

The resulting eluate was divided into 40-ml fractions. The fractions showing higher specific activities of pyruvate kinase than 74 units/ $A_{280nm}$ were mixed. The resulting mixture (400 ml) was concentrated to approximately 50 ml by means of membrane filtration. The concentrated solution is called "P-cellulose eluate."

The P-cellulose eluate was divided into portions containing 4,000-5,000 units of pyruvate kinase, and the portions were individually subjected to isoelectric electrophoresis with Ampholine carrier-ampholytes (Fig. 18). Pyruvate kinase (FDP-free) was

Fig. 18

collected into fractions, the pH values of which were centered at 7.8 ("isoelectric separation: 1st"). These fractions containing the pI 7.8-isozyme of the enzyme were preincubated with 1 mM FDP for a sufficient length of time (see below) and then subjected to a second isoelectric electrophoresis. Pyruvate kinase (FDP bound with the 1st site) was collected into fractions, the pH values of which were centered at 6.6 ("isoelectric separation: 2nd(+FDP)"). The enzyme in these fractions is called "pI 6.6-isozyme". The results of the purification are summarized in Table VI.

Table VI

The spleen-type pyruvate kinase preparations purified by the 2nd isoelectric separation were subjected to crystallization. To the enzyme

solution (approximately 600 units/ml) (30 ml) were added a one-twentieth volume of 0.2M Tris-HCl buffer containing 25% glycerol (pH 7.5) and a one-hundredth volume of 0.1 M FDP. The resulting solution was supplemented with solid $(NH_4)_2SO_4$ up to 50% saturation, adjusting the pH to approximately 7 with $NH_4OH$. The enzyme solution thus obtained was concentrated to approximately 2 ml by means of membrane filtration. In this step the enzyme solution became slightly turbid. To the concentrated enzyme solution, solid $(NH_4)_2SO_4$ was added up to 70% saturation. When the resulting enzyme solution was allowed to stand overnight, the turbidity increased so that a silky stream was detectable upon shaking of the solution. Small needle-shaped crystals were seen under a microscope (Fig. 19).

Fig. 19

All the procedures described above were carried out at 4°C. The purity was not appreciably improved after crystallization.

Crystales of the enzyme were collected by centrifugation and dialyzed against an excess volume of water for 2 days, during which the water was renewed 4 times. The dialyzed enzyme was lyophilized and then dried at 60°C under vacuum in a $P_2O_5$ desiccator. It was determined that 1 mg/ml of the enzyme showed $A_{280nm}=0.47$ in $H_2O$ with the cuvette of light pass 1 cm. Absorbance spectrum of spleen-type pyruvate kinase in 0.1 N NaOH was also measured. (Fig. 20)

Fig. 20

2. Effect of Preincubation with FDP on Activity of Spleen-Type Pyruvate Kinase

As described above, spleen-type pyruvate kinase is hardly stimulated by FDP when the initial rate is measured by adding the pI 7.8-isozyme (FDP-free). On the other hand, the activity of pI 7.8-isozyme was stimulated by FDP to a significant extent, when 20 µl of the enzyme which was preincubated with 0.1 mM FDP in 10 mM Tris-HCl buffer (pH 7.5) containing 25% glycerol was added to 1.0 ml of the standard reaction mixture for activity assay containing 0.1 mM phosphoenolpyruvate and 0.15 M KCl (final concentration of FDP=2 µM (Fig. 21). On the basis of kinetical behavior, it was clear that pI 7.8-isozyme were mostly coverted into pI 6.2-isozyme by preincubation with FDP at low ionic strength (10 mM Tris-HCl buffer without KCl). The conversion from the pI 6.6-isozyme into the pI 6.2-isozyme by further binding with FDP presumably took place at high ionic strength.

3. Effect of Preincubation Time with FDP on Activity of spleen-type Pyruvate Kinase

In this study, the pI 7.8-isozyme was dissolved in 10 mM Tris-HCl buffer containing 25%(v/v) glycerol and 1 mM FDP (pH 7.5). The enzyme solution thus obtained was preincubated at room temperature for various lengths of time, and the enzyme activity was measured by adding the preincubated enzyme solution (10µl) to the standard reaction mixture for activity assay which contained 0.1 mM phosphoenolpyruvate. With increasing preincubation time, the initial rate gradually increased; it took approximately 20 min to reach the maximum ("+FDP" in Fig. 22).

When the pI 7.8-isozyme was preincubated in the standard reaction mixture containing 0.1 mM phosphoenolpyruvate and 1 mM FDP but free of ADP, and when the enzyme activity was measured by adding the preincubated enzyme solution (10μl) to the standard reaction mixture for activity assay which contained 0.1 mM phosphoenolpyruvate but not FDP, the maximum stimulation of enzyme activity increased to a remarkably higher extent ("+RM-ADP+FDP"). When the pI 7.8-isoenzyme was preincubated in the standard reaction mixture containing 1 mM FDP but free of phosphoenolpyruvate, and when the enzyme activity was measured by adding the preincubated enzyme solution (10μl) to the standard reaction mixture for activity assay which contained 0.1 mM phosphoenolpyruvate, almost the same result as above was obtained ("+RM-PEP+FDP"). On the other hand, a slight but appreciable stimulation of the enzyme activity was observed when the pI 7.8-isoenzyme was preincubated in the standard reaction mixture free of ADP or phosphoenolpyruvate ("+RM-ADP" and "+RM-PEP").

The standard reaction mixture used for the preincubation contained 50 mM Tris-HCl, 0.15 M KCl, 10 mM $MgCl_2$, etc. Therefore, these results suggest that the FDP-binding rate to the 1st site of spleen-type isoenzyme was remarkably slow regaredless of the ionic strength in preincubation. In addition, the fact that the pI 7.8-isozyme was activated to a much higher extent by preincubation in the standard reaction mixture containing 1 mM FDP than by preincubation in 10 mM Tris-HCl buffer containing 1 mM FDP, suggests that the enzyme was additionally activated by the factor present in the reaction mixture; the activation reached the maximum within 10 min. This factor was the

monovalent cations present in the reaction mixture (See below).

4. Effect of FDP Concentration in Preincubation on Activity of Spleen-Type Pyruvate Kinase

The pI 7.8-isozyme of spleen-type pyruvate kinase was preincubated with various concentrations of FDP in 10 mM Tris-HCl buffer (pH 7.5) containing 25%(v/v) glycerol for 30 min, which is sufficient for the maximum stimulation (See Fig. 23). An aliquot of

Fig. 23

the preincubated enzyme solution was added to a 50-fold volume of the standard reaction mixture for activity assay, which contained 0.1 mM phosphoenolpyruvate and 0.1 mM FDP, and the initial rate for activity was then measured. With increasing concentration of FDP in preincubation, the rate increased (ε in Fig. 23). The Km value for FDP in preincubation was approximately $3 \times 10^{-7}$M. On the other hand, the pI 7.8-isozyme of spleen-type pyruvate kinase was preincubated in the same manner as described above, but the preincubated enzyme solution was added to the standard reaction mixture for activity assay, which contained 0.1 mM phosphoenolpyruvate but not FDP. With increasing concentrations of FDP in preincubation, the rate increased by two steps; the Km values for FDP in preincubation were approximately $3 \times 10^{-7}$M and $2 \times 10^{-5}$M (η in Fig. 23). Together with the fact that the one kind of FDP-binding sites (1st site), if once bound with FDP, dose not release the FDP in isoelectric electrophoresis (pI 6.6-isozyme in Fig.

4 and Fig. 5) whereas the other kind of FDP -binding sites (2nd site) is able to bind with FDP provided that the 1st site had been bound with FDP and free FDP was present in the medium (Fig. 9), these results indicate that 3 x $10^{-7}$M was the Km value for FDP binding to the 1st site and 2 x $10^{-5}$M was the apparent Km value for FDP binding to the 2nd site. In the latter case, the enzyme solution incubated with FDP was 50-fold diluted with the standard reaction mixture for activity assay, which did not contain FDP; thus the concentrations of FDP actually present in activity assay should be as those indicated by the dotted line in the figure. Therefore, it is conceivable that the Km value for FDP binding to the 2nd site in approximately 4 x $10^{-7}$M.

5. Effect of Various Kinds of Salt on Activity of Spleen-Type Pyruvate Kinase

The pI 7.8-isozyme of spleen-type pyruvate kinase was preincubated with various concentrations of NaCl or KCl in 10 mM Tris-HCl buffer (pH 7.5) containing 25%(v/v) glycerol for 10 min. The activity was then measured by adding the preincubated enzyme solution to the standard reaction mixture for activity assay, which contained 0.15 M KCl and 3 mM or 0.1 mM phosphoenolpyruvate. The initial rate for activity increas ed with increasing concentrations of the salts. NaCl and KCl gave essentially the same results; the maximum rate was obtained at 0.3 M with either 3 mM or 0.1 mM phosphoenolpyruvate (Fig. 24). LiCl, RbCl,

Fig. 24

$NH_4Cl$, $(NH_4)_2SO_4$, KI, $KHCO_3$ and Tris-HCl also gave the same results as NaCl and KCl.

The pI 7.8-isozyme was preincubated with 0.3 M NaCl in the presence and absence of 0.1 mM FDP. The activity was then measured by adding the preincubated enzyme solution to the standard reaction mixture for activity assay, in which the phosphoenolpyruvate concentration was varied (Fig. 25). When the enzyme was preincubated with 0.3 M NaCl, the $V_{max}$ value was raised by 30-40%, and the Km value for phosphoenolpyruvate shifted from 0.7 mM in the absence of FDP and from 0.1 mM to 0.04 mM in the presence of FDP. LiCl, KCl, RbCl, $NH_4Cl$, $(NH_4)_2SO_4$, KI, $KHCO_3$, and Tris-HCl gave the same results as NaCl. It may be noteworthy that the concentration of the salts for preincubation (0.3 M) was 50-fold diluted in the reaction mixture for activity assay; thus, their concentration in the reaction mixture (6 mM) is negligibly low in comparison to the concentration of KCl in the reaction mixture (0.15 M). In addition, the pI 7.8-isozyme was preincubated with various kinds of divalent cation salts (0.3 M) such as $MgCl_2$, $SrCl_2$, $Cacl_2$ and $MnCl_2$ in the absence of FDP. The activity was then measured by adding the preincubated enzyme solution to the standard reaction mixture for activity assay, in which the phosphoenolpyruvate concentration was varied. By preincubation with $MgCl_2$, the $V_{max}$ value was hardly changed, whereas the Km value for phosphoenolpyruvate shifted from 0.7 mM to 0.3 mM. By preincubation with $SrCl_2$, $CaCl_2$ and $MnCl_2$, the $V_{max}$ value was lowered by 23%, 55%, and 93%, respectively, and the Km value for phosphoenolpyruvate dropped to 0.3 mM by preincubation with $SrCl_2$ and $CaCl_2$ (data not shown).

The pI 7.8-isozyme was preincubated for 10 min with various kinds of salt (0.3 M), and the activity was then measured by adding the preincubated enzyme solution to the standard reaction mixture for activity assay, which contained various concentrations of KCl or NaCl besides 3 mM phosphoenolpyruvate. The activity in the presence of 3 mM phosphoenolpyruvate (nearly equal to $V_{max}$) was significantly raised when the enzyme had been preincubated with LiCl, NaCl, KCl, RbCl and Tris-HCl (0.3 M at preincubation and 1.5 mM in the reaction mixture for activity assay), regardless of the kinds of salt contained in the reaction mixture for activity assay (0.15 M KCl or NaCl) (Fig. 26). All the salts tested stimulated the activity to a similar extent.

Fig. 26

The pI 7.8-isozyme was added to the standard reaction mixture which contained various concentrations of several salts instead of 0.15 M KCl, for activity assay. When the activity was then measured, the initial rate for activity was greatly stimulated by KCl, RbCl and $NH_4Cl$, this being in good accordance with the findings by Boyer (58) (Fig. 27). Maximum stimulation was obtained at 0.1 M KCl, 0.1 M RbCl

Fig. 27

and 0.03M $NH_4Cl$. Such stimulation was not observed with the other salts including NaCl, LiCl and Tris-HCl. The relative activities of spleen-type pyruvate kinase under various assay conditions are summarized in

Table VII.

Table VII

6. Purification of Muscle-Type Pyruvate Kinase from Skeletal Muscle of Rats

Frozen skeletal muscle of rats (1 Kg was homogenized with 2 liters of 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM β-mercaptoethanol, 5 mM $MgCl_2$ and 1 mM EDTA (Tris-MME buffer) by waring blender (model CB-2-10, Eberbach Corp., Michigan) at the medium speed for 2 min, followed by centrifugation at 14,000 x g for 20 min. Resulting supernatant (2 liters) ("extract") was supplemented with ammonium sulfate up to 50% saturation, followed by centrifugation. Resulting supernatant was further supplemented with ammonium sulfate up to 70% saturation. Resulting precipitate centrifugally collected was dissolved in Tris-MME buffer and dialyzed against the same buffer ("50-70% sat. $AmSO_4$"). Precipitate formed during dialysis was removed by centrifugation. Resulting supernatant was heated at 60°C for 30 min, followed by centrifugation. The supernatant thus obtained ("heat-treatment") was passed through a DEAE-cellulose column (2.6 x 28 cm) equilibrated with Tris-MME buffer. The fraction that was not adsorbed ("DEAE-cellulose non-adsorbed") was dialyzed against 5 mM potassium citrate buffer (pH 5.5) and charged on the CM-cellulose column (6.8 x 33 cm) equilibrated with the same buffer. Pyruvate kinase was eluted with a linear concentration gradient formed with 2 liters of 5 mM potassium citrate buffer (pH 5.5) containing 0.1 M KCl and the 2 liters of the same buffer containing 0.4 M KCl. the fractions showing specific activities higher

than 240 $U/A_{280nm}$ were combined. The mixture (1.4 liter) was concentrated to approximately 200 ml by Diaflow filtration apparatus (model 402 with PM30 filter membrane, Amicon Corp., Lexington), followed by dialysis against 70 mM Tris-HCl buffer (pH 7.5) containing 25%(v/v) glycerol and 10 mM β-mercaptoethanol (Tris-GM buffer) ("CM-cellulose eluate"). The CM-cellulose eluate was applied on a P-cellulose column (6.8 x 42 cm) equilibrated with 70 mM Tris-GM buffer and eluted with the same buffer containing 10 mM ATP. Resulting eluate ("P-cellulose eluate") was concentrated to approximately 50 ml as described above, followed by dialysis against 10 mM Tris-GM buffer. A part of the resulting sample containing 10,000 units of pyruvate kinase was subjected to isoelectric electrophoresis with 2% Ampholine carrier-ampholytes (pH 3.5-10). The fractions of specific activities higher than 430 $U/A_{280nm}$ were combined ("isoelectric electrophoresis").

Summary of the purification procedures was shown in Table VIII.

Table VIII

7. pH- and Heat-Stabilities of Spleen-Type and Muscle-Type Pyruvate Kinase

Heat-stabilities of spleen-type and muscle-type pyruvate kinase were tested using "40-70% sat. $AmSO_4$" fraction and "50-70% sat. $AmSO_4$" fraction respectively (Fig. 28A and B). When enzymes were

Fig. 28

treated at 60°C for various lengths of time, 80% of muscle-type pyruvate kinase activity was retained even after heat treatment for 50 min, whereas spleen-type pyruvate kinase activity completely disappeared within 10 min. Addition of glycerol and FDP has no effect on heat-stability of spleen-type pyruvate kinase (data not shown). During this heat-treatment, the specific activity of muscle-type pyruvate kinase was increased about five fold.

pH- stabilities of spleen-type and muscle-type pyruvate kinase were also tested with the non-adsorbed fractions from DEAE-cellulose column chromatography. In the absence of glycerol, spleen-type pyruvate kinase was unstable and the activity was decreased to the half level of the original activity within four hours at pH 7.0-8.0 (data not shown). Whereas, in the presence of 25% glycerol, spleen-type pyruvate kinase was stable at pH 5.0-9.0 for at least three days (at 4°C) (Fig. 29A).

Fig. 29(A,B)

Relatively to spleen-type, in the absence of glycerol, muscle-type pyruvate kinase was stable and approximately 70% of the enzyme activity was retained at 4°C after three days at pH 7.0-9.0. In the presence of 25% glycerol, muscle-type activity was nearly 100% retained at any pH tested (pH 4.0-9.0) for three days (Fig. 29B).

8. SDS-Polyacrylamide Gel Electrophoresis of Spleen-Type and Muscle-Type Pyruvate Kinase

Spleen-type and muscle-type pyruvate kinase from final purifica-

tion procedures ( "isoelectric separation; 2nd(+FDP)" and "isoelectric electrophoresis" respectively) were dialyzed against distilled water sufficiently and lyophilized. Thus obtained samples were subjected to SDS-polyacrylamide gel electrophoresis, in which bovine serum albumin (M.W. 67,000), ovalbumin (M.W. 45,000), α-chymotrypsinogen A (M.W. 25,700),horse myoglobin (M.W. 17,600) and beef heart cytochrome c (M.W. 12,000) were used as markers. In SDS-polyacrylamide gel electrophoresis, both of spleen-type and muscle-type pyruvate kinase formed a single band correspond to a molecular weight of 60.000 ± 5,000, showing the same migration patterns (Fig. 30).

Fig. 30

9. Molecular-Sieve Chromatography of Spleen-Type and Muscle-type Pyruvate Kinase

The molecular weight of spleen-type pyruvate kinase was reported to be 216,000 by Imamura et al. (22), 178,000, 140,000 and 116,000 by Ibsen et al. (59) Hofmann et al. (60) and Ibsen et al. (25) described that this isoenzyme exists at a dimer-tetramer equilibrium and the presence of FDP favors the tetrameric state. In this study, the molecular weights of spleen-type and muscle-type pyruvate kinase were estimated at various concentrations of enzymes by molecular-sieve chromatography on Sephadex G-200 column.

The pI 7.8-isozyme of spleen-type pyruvate kinase (FDP-free form) was preincubated with 1 mM FDP for sufficient lengths of time, placed on the bottom of a Sephadex G-200 column equilibrated with 50 mM

Tris-HCl buffer containing 0.15 M KCl, 10 mM $MgCl_2$ and 1 mM FDP (pH 7.5)("Tris-KMF buffer") and then developed upward with the Tris-KMF buffer. Glycerol was not added to the developing buffer, because the chromatographic profile was not reproducible. With either 30 units/ml to 150 units/ml of the enzyme charged to the column, the activity was eluted at practically the same elution volume forming a nearly symmetrical peak corresponding to a molecular weight of 240,000 $\pm$ 10,000. This indicate that the spleen-type pyruvate kinase, when bound with FDP, existed as a tetramer composed of subunits having a molecular weight of approximately 60,000. On the other hand, activity was eluted, forming a peak having a shoulder on the lower molecular weight side, when 30 units/ml of the pI 7.8-isozyme without preincubation with FDP was charged to the column equilibrated with the buffer containing no FDP ("Tris-KM buffer", pH 7.5) and then developed with Tris-KM buffer. The peak and the shoulder correspond to molecular weights of 100,000 $\pm$ 10,000, and 60,000 $\pm$ 10,000, respectively. This suggests that at 30 units/ml, the enzyme existed as a mixture of dimer and monomer. The results of molecular-sieve chromatography of spleen-type pyruvate kinase under various conditions are summarized in (Fig. 31A). Studies

Fig. 31A

by analytical centrifugation were fruitless, because the monomer-dimer-tetramer equilibrium occurred at such low protein concentrations (0.16-0.79 mg/ml) that the analysis was not effective.

In molecular-sieve chromatography of muscle-type pyruvate kinase

on a Sephadex G-200 column in the presence of 1 mM FDP (with the use of "Tris-KMF buffer"), the activity was eluted at approximately 240,000 daltons. The elution position was unchanged not only by the concentrations of enzyme but also by the depletion of FDP. These indicate that although muscle-type as well as spleen-type was comprised of four identical or similar subunits, the binding of FDP favored the formation of tetrameric structure with spleen-type, but not with muscle-type. The results of molecular-sieve chromatography of muscle-type pyruvate kinase under various conditions are summarized in (Fig. 31B).

Fig. 31B

10. Isoelectric Electrophoresis of Spleen-Type and Muscle-Type Pyruvate Kinase Preincubated with [$^{14}$C]FDP

In order to estimate the amount of FDP bound with spleen-type pyruvate kinase, the pI 7.8-isozyme of spleen-type pyruvate kinase ("isoelectric separation: 1st") was preincubated with 1 mM [$^{14}$C]FDP for a sufficient length of time, and then subjected to isoelectric electrophoresis, in which free FDP could be removed from the enzyme zone (Fig. 32A). As described already, the pI value shifted from 7.8 to 6.6 (if in

Fig. 32(A.B)

the presence of free FDP, the pI 6.6-isozyme instantly binds with FDP to form the pI 6.2-isozyme). In addition, the radioactivity and $A_{280nm}$ were also focused in the pH 6.6 fraction. In two experiments, it was

estimated that in the pI 6.6-isozyme, 2.1 and 2.4 mol of FDP were bound per mol of the tetramer, on the basis of M.W.=240,000 and $E^{1\%}_{280nm}$=4.7. It is predictable that on the basis of the pI value, the pI 6.2-isozyme is bound with 4 mol of FDP per mol of tetramer.

When the muscle-type pyruvate kinase ("P-cellulose eluate") was preincubated with 1 mM FDP and subjected to isoelectric electrophoresis, the activity and the radioactivity were separately focused at approximately pH 7.4 and anode fraction, respectively (Fig. 32B). As described already, the pI value of the muscle-type was not influenced by preincubation with FDP. These results suggest that muscle-type has not an ability to bind FDP and that FDP-binding properties are remarkably different between muscle-type and spleen-type pyruvate kinase.

## 11. Comparison of Amino Acid Compositions of Spleen-Type and Muscle-Type Pyruvate Kinase

Crystallized spleen-type sample and muscle-type sample from final purification procedure ("isoelectric electrophoresis") were dialyzed against water sufficiently and lyophilized. Thus obtained samples were used for amino acid analysis and peptide mapping.

The amino acid compositions of spleen-type and muscle-type are presented in Table IX. The number of amino acid residues was calculated

Table IX

on the basis of the subunit molecular weight of 59,000 daltons with isoenzymes. The data were in a good accordance with those of rabbit

muscle (61) and human muscle (62). The amino acid compositions of spleen-type and muscle-type were significantly similar to each other. The total numbers of dicarboxylic amino acid residues (aspartate and glutamate) and those of basic amino acid residues (lysine, histidine and arginine) are 100 and 83 with spleen-type, and 102 and 80 with muscle-type, respectively. This accords with the facts that at the FDP-free form, both isoenzymes were slightly alkaline, the pI value of muscle-type was lower than that of spleen-type.

12. Differences in Peptide Maps of Spleen-Type and Muscle-Type Pyruvate Kinase

Tryptic peptide maps of spleen-type and muscle-type pyruvate kinase were shown in (Fig. 33). Approximately 66 and 65 spots of

Fig. 33

peptides were detectable on each tryptic peptide maps of spleen-type and muscle-type, respectively. These numbers were almost coincident with those calculated on the basis that each of these isoenzymes is composed of four identical subunits; 72 and 69 ninhydrin-positive spots for spleen-type and for muscle-type, respectively. As predicted from amino acid analysis, the peptide maps of both isoenzymes were highly similar. Of the spots in the peptide maps, 61 spots were common for both isoenzymes; 5 spots were specific to spleen-type and 4 spots were specific to muscle-type. These results indicate that spleen-type and muscle-type were the products of different genes.

## DISCUSSION

Multiple forms of pyruvate kinase have been observed by many workers (18, 20, 22-25).

In this report, it was concluded that pyruvate kinase in various tissues of rats, if they were free of FDP could be classified into three types of isoenzymes, liver-type (L-type), spleen-type (S-type) and muscle-type (M-type). This conclusion agrees with that of Tanaka et al. (18, 22), who designated the spleen-type as $M_2$-type on the basis of cross reaction of the antibody for the muscle-type isoenzyme with the spleen-type isoenzyme. However, we designated it to spleen-type on the basis of the tissue which contains the isoenzyme most abundantly.

The liver-type isoenzyme had a pI value of 6.2 when it was free of FDP (liver-type pI 6.2-isozyme). This isoenzyme was present in kidney and erythrocyte besides liver. The molecule of liver-type isoenzyme probably possesses two kinds of FDP-binding sites. When one kind of site (the 1st site) bind with FDP, the pI value of the isoenzyme shifts from 6.2 to 5.6 (liver-type pI 5.6-isozyme). When both kinds of sites (1st and 2 nd sites) bind with FDP, the pI value shifts further to 5.4 (liver-type pI 5.4-isozyme). On the kinetical behavior, liver-type pI 5.4-isozyme and 5.6-isozyme were indistinguishable (Table II). It seems likely that the binding with FDP at the 1st and 2nd sites occurs almost simultaneously and rapidly (even if it is the fact that the 2nd site can not bind with FDP unless the 1st site already carries the FDP) in the reaction mixture of conventional activity assay which contains 0.15M KCl and where the ionic strength is approximately 0.16 (high ionic strength), if provided excess concentration of FDP. Therefore, if

FDP was provided enough, all the pI-isozymes of liver-type employed in activity assay covert into pI 5.4-isozyme. Whereas, if FDP was not provided, all the pI-isozymes of liver-type employed in activity assay convert into pI 6.2-isozymes liberating its bound FDP in the reaction mixture (high ionic strength). However, on the isoelectric electrophoresis (at low ionic strength), pI 5.4 and pI 5.6 isoenzymes were distinguishable. For example, liver-type pI 6.2-isozyme was converted into pI 5.4-isozyme and then into pI 5.6-isozyme on repeated isoelectric electrophoresis (Fig. 2). It seems likely that the two kinds of sites are not equivalent with respect to the ability to bind with FDP.

The muscle-type isoenzyme was unable to bind with FDP even by preincubation at low ionic strength (muscle-type pI 7.4-isozyme). This was confirmed with purified muscle-type isoenzyme from skeletal muscle. The result indicate that no radioactivities of [$^{14}$C]FDP was incorporated into muscle-type isoenzyme (Fig. 32B). This isoenzyme was abundant in skeletal muscle, brain and heart muscle, and a detectable amount was present in all other tissues tested. The subunit molecular weight of muscle-type isoenzyme was estimated to 59,000 by amino acid analysis, and it composed of four identical subunits in good agreement with the report by others (73). Their tetramer structure was not affected by enzyme concentrations and FDP, unlike the spleen-type isoenzyme as described below.

The spleen-type isoenzyme had a pI value of 7.8 when free of FDP (spleen-type pI 7.8-isozyme). This isozyme was present in spleen, lung, kidney, liver and erythrocyte besides Rhodamine sarcoma, but was scarce

in skeletal muscle, heart muscle or brain. The spleen-type isoenzyme possesses two kinds of FDP-binding sites which are different in their mode of FDP-binding. On the spleen-type isoenzyme, unlike the liver-type pI 6.2-isozyme, the FDP-binding rate to the 1st site was remarkably slow (Fig. 22) regardless of the ionic strength. Therefore, the activity of spleen-type pI 7.8-isozyme was hardly stimulated by FDP in a conventional assay in a limited time (Fig. 9), whereas when the FDP once bound to the 1st site, the pI value of spleen-type isoenzyme shifts from 7.8 to 6.6, and the FDP once bound at 1st site was not released in isoelectric electrophoresis and in the reaction mixture of activity assay. In the pI 6.6-isozyme, it was estimated that 2 mol of FDP were bound per mol of tetramer (Fig. 32A). On the contrary, the 2nd site could rapidly bind with FDP provided that the 1st site had been bound with FDP and free FDP was present in the medium, and then, the pI value shifts from 6.6 to 6.2. On the basis of pI value, the spleen-type pI 6.2-isozyme is supposedly bound with 4 mol of FDP tetramer. It was estimated that the Km values for FDP-binding to the 1st and 2nd sites were nearly the same; 3-4 x $10^{-7}$M (Fig. 23). The molecular weight of spleen-type isoenzyme was estimated to 59,000 by amino acid analysis. It could exist as monomer, dimer and tetramer, and all of which were enzymically active; low concentrations of the enzyme favored the formation of the monomer, whereas high concentrations favored the formation of the tetramer. However, when FDP was present in the medium, all the enzyme existed as the tetramer even at lowest concentration tested (0.16 mg/ml). Therefore, in a conventional activity assay (at very low concentrations of enzyme), it seems likely that pI

7.8-isoenzyme exist as the monomer and pI 6.6-isozyme exists as the dimer, and pI 6.2-isozyme exists as the tetramer. By the finding of Boyer (58), it is known that pyruvate kinase requires $Mg^{2+}$or $Mn^{2+}$ as a divalent cation and $K^+$, $NH_4^+$ or $Rb^+$ as a monovalent cation. The activity of spleen-type pyruvate kinase was also stimulated by $K^+$, $NH_4^+$ of $Rb^+$ by instant manner in the presence of $Mg^{2+}$ in agree with the finding of Boyer. Such stimulation was not observed with $Na^+$, $Li^+$, $Cs^+$, $Sr^{2+}$, $Ca^{2+}$ and $Tris^+$ (Fig. 27). On the other hand, the activity was significantly stimulated by time dependent manner by $Na^+$, $Li^+$, and $Tris^+$ as well as by $K^+$, $NH_4^+$ and $Rb^+$, when the enzyme was preincubated with one of these cations before activity assay. This later stimulation was not observed by $Mg^{2+}$, $Sr^{2+}$, $Ca^{2+}$ or $Mn^{2+}$. Therefore, it seems likely that the enzyme had two different sites capable of binding various monovalent cations; one kind of site could bind at slow rate with all the cations tested, whereas the other kind of site could bind at fast rate with $K^+$, $NH_4^+$ and $Rb^+$ but not with the others; the binding of monovalent cations to either site raised the Vmax value, the extent thus raised being additive.

Immunological studies by Imamura et al. (22) suggest that liver-type is different from muscle-type and spleen-type, whereas spleen-type and muscle-type cross-reacted each other. However, there are little data concerning the relationship between spleen-type and muscle-type pyruvate kinase. The results presented here indicate that spleen-type and muscle-type pyruvate kinase were the different molecules in amino acid compositions and in peptide maps, although they were very similar on its respects, suggesting they were the products from distinct genes.

In Rhodamine sarcoma-bearing rats, the content of spleen-type pyruvate kinase increased in the livers to remarkable extent (Fig. 11 and Table III). In addition, when nuclei or chromatin prepared from Rhodamine sarcoma was injected into rats, spleen-type isoenzyme content increased in their liver to an extent similar to that in Rhodamine sarcoma-bearing rats, in accordance with the finding by Nakamura et al. (24). When chromatin prepared from spleen of rats was injected into rats, the isoenzyme content increased in their liver to an appreciable extent, although the extent was less than in the case with sarcoma chromatin. Furthermore, when histone-free chromatin or non-histone protein fraction prepared from Rhodamine sarcoma was injected into rats, spleen-type isoenzyme increased in their liver, whereas histone and DNA fraction had no effect on the increase of this isoenzyme. On the other hand, it has been reported that in vivo liver catalase-depressing substance is a non-histone protein having a pI value of 5.1 and a molecular weight of approximately 60,000, and which is bound with chromatin in the muscle nuclei and the Rhodamine sarcoma nuclei (14, 15, 17). These results suggest that the factors controlling the gene expression for catalase and pyruvate kinase are non-histone proteins. Concerning with the factor which causes the increase of spleen-type isoenzyme in the liver of host animal, similar observations have reported in the liver of parabiotic twin of tumor-bearing animal (19), and in liver perfused with blood from tumor-bearing animal (63) by Suda et al., and in the liver of animal injected with the blood of tumor-bearing animal (26) by Ibsen et al. These observations suggest that the factor can be released from tumor cells into circulatory system.

However, at present time, it is not clear whether the factor acts in the parenchymal cells of the liver with derepressing the gene of spleen-type isoenzyme or acts in non-parenchymal cells of the liver with stimulating the synthesis of spleen-type isoenzyme. The present data suggest that the factor could exist in tissue in which spleen-type isoenzyme was expressed abundantly. This supports that the factor itself is a regulator of gene expression of spleen-type pyruvate kinase. Thus, the elucidation of the properties of this factor will depend on its purification and development of an *in vitro* assay system.

Farina *et al.* (29), using the transplantable Morris hepatoma demonstrated that one highly differentiated hepatoma (9618A) has pyruvate kinase isozyme pattern similar to that of liver, and that other highly and well differentiated hepatoma had much lower activity of liver-type isoenzyme than normal liver with a preponderance of spleen-type isozyme, whereas the poorly differentiated hepatoma had little liver type isoenzyme and showed a extremely high activity of spleen-type isoenzyme. On the other hand, increase of spleen-type isoenzyme with decrease in liver-type isoenzyme has been observed in regenerating liver (27, 29), fetal liver (27, 28, 29) and during liver carcinogenesis (27). From these observations, isozyme pattern of pyruvate kinase has been considered as a good marker of liver differentiation or proliferation (27). In fact, it has proved difficult to establish a line of fully differentiated mature liver cells in culture (64, 65), and a adult rat liver cell line synthesized only spleen-type isoenzyme (32). However, in the present study it was found that a line of Morris hepatoma cell from Buffalo rat, $MH_1C_1$, contained only liver-

type pyruvate kinase, whereas a line of normal liver cells, BRL, from rat had the spleen-type isoenzyme mainly and the muscle-type isoenzyme in small amount. This finding that growing hepatoma cells in culture synthesize only liver-type isoenzyme suggests that the expression of liver-type isoenzyme is not suitable as a marker of non-growing and normally differentiated hepatocytes. $MH_1C_1$ line was established by Richardson (55) in 1969 from Morris hepatoma No.7795. These cells have retained some liver-specific function, including secretion of serum albumin and response of tyrosine transferase (EC 2.6.1.6) activity to hydrocortisone. The expression of liver-type isoenzyme is stable because this phenotype has not changed during the culture for at least the last two years. It seems likely that this hepatoma cells defect the system for transmission of signals of hormones to control the activity of liver-type isoenzyme since insulin, glucagon, $Bt_2$cAMP and $Bt_2$cGMP had no effects on activity of liver-type isoenzyme. Because, $MH_1C_1$ line is a cell line that express the liver-type isoenzyme repressing the spleen-type isoenzyme, it will be expected that this cell line is available in an <u>in vitro</u> assay system for the factor which causes the increase of spleen-type isoenzyme in the liver, as described above. This problem is under investigation.

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Table I. Amounts of pI-isozymes in extracts of various tissues after conversion into their fructose 1,6-diphosphate-free forms by ammonium sulfate treatment

| Tissues | Specific activities (units/g protein) | | |
|---|---|---|---|
| | pI 6.2-isozyme (Liver-type) | pI 7.4-isozyme (Muscle-type) | pI 7.8-isozyme (Spleen-type) |
| Liver | 190 | 14 | 36 |
| Rhodamine sarcoma | 0 | 140 | 1260 |
| Spleen | 0 | 73 | 537 |
| Lung | 0 | 86 | 524 |
| Kidney | 50 | 67 | 443 |
| Erythrocytes | 3 | 1 | 17 |
| Skeletal muscle | 0 | 8,900 | 0 |
| Heart muscle | 0 | 950 | 0 |
| Brain | 0 | 1,900 | 0 |

Table II. Properties of pI-isozyme of pyruvate kinase from various tissues of rats.

| Origin of pI-isozyme | Liver | | | | | | spleen | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Type of pI-isozyme | Liver-type | | | | | | Spleen-type | | | | | |
| pI-Isozyme used for assay[a] | 5.4 | | 5.6 | | 6.2 | | 6.2 | | 6.6 | | 7.8 | |
| pI-Isozyme assayed[a] | 6.2 | 5.4 | 6.2 | 5.4 | 6.2 | 5.4 | 6.6 | 6.2 | 6.6 | 6.2 | 7.8 | 7.8 |
| Kind of site binding FDP in presence of 0.15M KCl | 1st&2nd | | 1st&2nd | | 1st&2nd | | 1st | 1st&2nd | 1st | 1st&2nd | | |
| Addition of 0.1 mM FDP to reaction mixture for activity assay | - | + | - | + | - | + | - | + | - | + | - | + |
| Km for PEP (mM) | 1.5 | 0.1 | 1.5 | 0.1 | 1.5 | 0.1 | 0.2 | 0.06 | 0.2 | 0.06 | 0.7 | 0.5[c] |
| Hill constant for PEP[b] | 2 | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1.7 | 1.7 |
| Stimulation by FDP | + | | + | | + | | + | | + | | ± | |

| Origin of pI-isozyme | Skeletal muscle | | Rhodamine sarcoma | | | |
|---|---|---|---|---|---|---|
| Type of pI-isozyme | Muscle-type | | Spleen-type ($M_2$-type) | | | |
| pI-isozyme used for assay[a] | 7.4 | | 6.2 | | 6.6 | | 7.8 | |
| pI-isozyme assayed[a] | 7.4 | 7.4 | 6.6 | 6.2 | 6.6 | 6.2 | 7.8 | 7.8 |
| Kind of site binding FDP in presence of 0.15M KCl | | | 1st | 1st&2nd | 1st | 1st&2nd | | |
| Addition of 0.1 mM FDP to raction mixture for activity assay | - | + | - | + | - | + | - | + |
| Km for PEP (mM) | 0.06 | 0.06 | 0.2 | 0.06 | 0.2 | 0.06 | 0.7 | 0.5[c] |
| Hill constant for PEP[b] | 1 | 1 | 1 | 1 | 1 | 1 | 1.7 | 1.7 |
| Stimulation by FDP | - | | + | | + | | ± | |

[a]Since the ionic strength of the reaction mixture for activity assay was approximately 0.16, the fructose 1,6-diphosphate (FDP) bound with liver-type pI 5.4-isozyme and spleen-type pI 6.2-isozyme was partially dissociated in the reaction mixture when sugar phoaphate was not added externally; thus, the pI-isozyme assayed was deduced on the basis of the kinetic results.

[b]Phosphoenolpyruvate. [c]Due to slight stimulation by FDP.

Table III. Localization of the factor which causes the increase of spleen-type pyruvate kinase in the liver by its injection into rats.

| Rats | Dose Protein[a] or DNA[b] ( mg ) | Injection time | Specific activities (units/g protein) | | |
|---|---|---|---|---|---|
| | | | Liver-type | Muscle-type | Spleen-type |
| Normal | | | 190 | 14 | 36 |
| Rh. sarcoma-bearing | | | 155 | 20 | 155 |
| Injected with | | | | | |
| 0.15M NaCl | | 1 | 188 | 14 | 33 |
| Rh. sarcoma nuclei | 14[a] | 1 | 182 | 17 | 76 |
| " | 28[a] | 1 | 174 | 19 | 90 |
| " | 56[a] | 1 | 136 | 20 | 120 |
| " | 20[a] | 1 | 181 | 20 | 81 |
| " | 20[a] | 2 | 165 | 19 | 98 |
| " | 20[a] | 3 | 160 | 19 | 146 |
| Rh. sarcoma chromatin | 30[a] | 1 | 199 | 24 | 83 |
| Rh. sarcoma histone-free chromatine | 30[a] | 1 | 202 | 21 | 75 |
| Rh. sarcoma non-histone protein | 30[a] | 1 | 210 | 20 | 61 |
| Rh. sarcoma histone | 30[a] | 1 | 190 | 15 | 38 |
| Rh. sarcoma DNA | 30[b] | 1 | 193 | 15 | 39 |
| Spleen chromatin | 30[a] | 1 | 184 | 17 | 69 |
| Liver chromatin | 30[a] | 1 | 198 | 15 | 35 |

Table IV. Pyruvate kinase activity of cultured liver and hapatoma cells

| Cells | Activity[1] | |
|---|---|---|
| | mU/mg protein[2] | mU/$10^7$ cells[2] |
| BRL | 1,100 ± 200 | 710 ± 160 |
| $MH_1C_1$ | 55 ± 6 | 43 ± 16 |

1) Activity was measured in the presence of 3 mM phosphoenolpyruvate with ($MH_1C_1$ cells) or without (BRL cells) 1 mM FDP.

2) Values are means ± S.D. for 6 samples.

Table V. Effects of insulin, glucagon, $Bt_2$cAMP and $Bt_2$cGMP on activities of pyruvate kinase in $MH_1C_1$ cell extracts

| Additions | Concentration | Treated time | Specific activity (mU/mg protein) |
|---|---|---|---|
| None | | | 53 |
| Insulin | 0.1 µg/ml | 24 hr | 53 |
| | 0.3 µg/ml | 24 hr | 50 |
| | 1 µg/ml | 5-30 min | 53 |
| | " | 24 hr | 51 |
| | " | 48 hr | 51 |
| | 3 µg/ml | 24 hr | 54 |
| Glucagon | 1 µg/ml | 0.5-1 hr | 53 |
| $Bt_2$cAMP | $10^{-6}$ M | 5-30 min | 49 |
| | $10^{-4}$ M | 5-30 min | 41 |
| $Bt_2$ cGMP | $10^{-6}$ M | 5-30 min | 54 |
| | $10^{-4}$ M | 5-30 min | 46 |

Table VI. Summary for purification of spleen-type (type $M_2$) pyruvate kinase from Rhodamine sarcoma of rats.

| Steps | Total activity[a] (units) | Total protein ($A_{280nm}$) | Specific activity[a] (units/$A_{280nm}$) | Purification | Yield (%) |
|---|---|---|---|---|---|
| Extract | 241,000 (361,000)[b] | 1,080,000 | 0.223 (0.334)[b] | (1.00) | 100 |
| 40-70% sat.$(NH_4)_2SO_4$ fraction | 221,000 | 256,000 | 0.863 | 3.87 | 92 |
| DEAE-Cellulose passed fraction | 118,000 | 12,500 | 9.44 | 42.3 | 49 |
| CM-Cellulose eluate | 84,700 | 1,880 | 45.1 | 202 | 35 |
| P-Cellulose eluate | 64,100 | 336 | 191 | 857 | 27 |
| Isoelectric separation: | | | | | |
| 1st | 41,700 (62,500)[b] | 106 | 393 (590)[b] | 1,760 | 17 |
| 2nd(+FDP) | 33,400 (50,200)[b] | 82.5 | 405 (608)[b] | 1,820 | 14 |

[a]The enzyme was properly diluted with 10 mM Tris-HCl buffer containing 25% (v/v) glycerol (pH 7.5), and the activity was measured by adding the diluted enzyme solution to the standard reaction mixture containing 0.15M KCl. [b]The enzyme was preincubated in 10 mM Tris-HCl buffer containing 25% (v/v) glycerol and 0.3M NaCl (pH 7.5), and the activity was measured by adding the preincubated enzyme solution to the standared reaction mixture containing 0.1M KCl.

Table V.

The growing cultures (2-5 x $10^6$ cells/150 mm plate) were replaced in the Dulbecco's modified Eagles's medium supplemented with 2% FCS, and then, after 48 hr, cells were harvested. The cells were treated with indicated concentrations on hormones for indicated time before harvesting the cells.

Table VII. Relative activities of spleen-type pyruvate kinase under various assay conditions.

| Reagents added in preincubation | | Value of pI of isozyme assayed | Relative activities of spleen-type pyruvate kinase (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.1 mM phosphoenolpyruvate | | | | 3 mM phosphoenolpyruvate | | | |
| Salt | FDP | | $NH_4Cl$ | RbCl | KCl | | $NH_4Cl$ | RbCl | KCl | |
| 0.3 M | 1 mM | | 30 mM | 100mM | 100 mM | 150mM | 30 mM | 100 mM | 100 mM | 150 mM |
| - | - | 7.8[a] | 10 | 5.0 | 6.0 | 5.0 | 75 | 20 | 110 | (100)[a] |
| - | - | 6.6[b] | 35 | 14 | 30 | 26 | 75 | 67 | 110 | 100 |
| - | + | 6.2[c] | 48 | 41 | 55 | 50 | 75 | 67 | 110 | 100 |
| NaCl | - | 7.8[a] | 56 | 10 | 38 | 25 | 110 | 77 | 150 | 140 |
| NaCl | - | 6.6[b] | 74 | 16 | 45 | 40 | 110 | 100 | 150 | 140 |
| NaCl | + | 6.2[c] | 90 | 80 | 110 | 98 | 110 | 100 | 150 | 140 |

[a]The pI 7.8-isozyme was dissolved in 10 mM Tris-HCl buffer containing 25% (v/v) glycerol (pH 7.5), and the activity was then measured in the standard reaction mixture, which contained 0.1 mM or 3 mM phsphoenolpyruvate and various salts as indicated. The specific activity measured in the presence of 3 mM phsphoenolpyruvate and 0.15M KCl (405 units/$A_{280nm}$=190 units/mg protein) was taken as 100%. [b]The pI 6.6-isozyme was preincubated at 24°C for 10 min with and without 0.3M NaCl in 10 mM Tris-HCl buffer containing 25% (v/v) glycerol (pH 7.5), and the activities were then measured in the same manner as for (a). [c] The pI 7.8-isozyme was preincubated at 24°C for 30 min with 1 mM FDP in the presence and absence of 0.3M NaCl in 10 mM Tris-HCl buffer conatining 25% (v/v) glycerol (pH 7.5), and the activities were then measured in the same manner as for (a).

Table VIII. Summary for purification of muscle-type pyruvate kinase from muscle of rats.

| Steps | Total activity ( units) | Total protein ($A_{280nm}$) | Specific activity ( units/$A_{280nm}$) | Purification | Yield (%) |
|---|---|---|---|---|---|
| Extract | 217,000 | 45,000 | 4.82 | (1.00) | 100 |
| 50-7% sat. $(NH_4)_2SO_4$ fraction | 187,000 | 13,000 | 14.4 | 2.99 | 86 |
| Heat-treatment | 159,000 | 2,190 | 72.6 | 15.1 | 73 |
| DEAE-Cellulose passed fraction | 96,000 | 1,020 | 94.1 | 19.5 | 44 |
| CM-Cellulose eluate | 88,400 | 231 | 383 | 79.5 | 41 |
| P-Cellulose eluate by ATP* | 61,000 | 124 | 492 | 102 | 28 |
| Isoelectric separation | 42,600 | 86.4 | 493 | 102 | 20 |

* $A_{280nm}$ was measured after dialysis

Table IX A. Amino acid composition of spleen-type pyruvate kinase from rat Rhodamine sarcoma

| Amino acids | Molar ratio relative to phenylalanine | | | | Moles per 59,000 g protein |
|---|---|---|---|---|---|
| | 24 hrs | 48 hrs | 72hrs | Corrected[a] | |
| Aspartate | 3.26 | 3.30 | 3.22 | 3.26 | 49 |
| Threonine | 1.60 | 1.63 | 1.52 | 1.64 | 25 |
| Serine | 1.56 | 1.45 | 1.23 | 1.76 | 26 |
| Glutamate | 3.36 | 3.42 | 3.35 | 3.38 | 51 |
| Proline | 1.57 | 1.60 | 1.53 | 1.57 | 24 |
| Glycine | 2.82 | 2.74 | 2.74 | 2.77 | 42 |
| Alanine | 3.98 | 3.90 | 3.91 | 3.93 | 59 |
| Half-chstine[c] | 0.667 | | | | 10 |
| Valine | 2.96 | 3.02 | 3.28 | 3.28 | 49 |
| Methionine | 1.11 | 1.03 | 1.01 | 1.05 | 16 |
| Isoelucine | 2.44 | 2.44 | 2.47 | 2.47 | 37 |
| Leucine | 2.68 | 2.68 | 2.67 | 2.68 | 40 |
| Tyrosine | 0.670 | 0.687 | 0.670 | 0.676 | 10 |
| Phenylalanine | 1.00 | 1.00 | 1.00 | 1.00 | 15 |
| Tryptophan[d] | | | | | 5 |
| Lysine | 2.52 | 2.58 | 2.60 | 2.57 | 39 |
| Histidine | 0.751 | 0.787 | 0.774 | 0.771 | 12 |
| Arginine | 2.03 | 2.14 | 2.13 | 2.10 | 32 |

Table IX B. Amino acid composition of muscle-type pyruvate kinase from rat muscle

| Amino acids | Molar ratio relative to phenylalanine | | | | Moles per 59,000g |
|---|---|---|---|---|---|
| | 24 hrs | 48 hrs | 72hrs | Corrected[a] | |
| Aspartate | 3.33 | 3.34 | 3.35 | 3.34 | 50 |
| Threonine | 1.53 | 1.30 | 1.29 | 1.67 | 25 |
| Serine | 1.49 | 0.978 | 0.959 | 1.75 | 26 |
| Glutamate | 3.40 | 3.44 | 3.52 | 3.45 | 52 |
| Proline | 1.50 | 1.50 | 1.54 | 1.51 | 23 |
| Glycine | 2.70 | 2.75 | 2.70 | 2.72 | 41 |
| Alanine | 4.02 | 4.06 | 4.11 | 4.06 | 61 |
| Half-cystine[c] | 0.638 | | | | 10 |
| Valine | 3.05 | 3.09 | 3.01 | 3.01 | 45 |
| Methionine | 1.20 | 1.22 | 1.20 | 1.21 | 18 |
| Isoeleucine | 2.30 | 2.31 | 2.26 | 2.26 | 34 |
| Leucine | 2.85 | 2.89 | 2.82 | 2.85 | 43 |
| Tyrosine | 0.686 | 0.700 | 0.695 | 0.694 | 10 |
| Phenylalanine | 1.00 | 1.00 | 1.00 | 1.00 | 15 |
| Tryptophen[d] | | | | | 5 |
| Lysine | 2.43 | 2.49 | 2.44 | 2.45 | 37 |
| Histidine | 0.795 | 0.810 | 0.783 | 0.796 | 12 |
| Arginine | 2.06 | 2.09 | 2.07 | 2.07 | 31 |

Footnote for Table IX

[a]The values for threonine and serine were obtained by extrapolation to zero time. For valine and isoleucine, the values at 72 hours were taken. For other amino acids, average values were taken.

[b]The number of phenylalanine was taken as 15 residues.

[c]Determined as S-carboxymethylcysteine.

[d]Determimed spectrophotometrically by the method of Bencze and Schmid (66).

Fig. 1. Effect of ammonium sulfate treatment on isozyme pattern of pyruvate kinase from normal rat liver. A portion (1.5 ml) of the extract from nomal rat liver was subjected to isoelectric separation with Ampholine carrier ampholytes of pI from 3 to 10,at a final concentration of 1% (w/v). After electrofocusing, the eluate was divided into 1-ml fractions. The pH and the pyruvate kinase activity (U/ml) of each fraction were measured. In a parallel experiment, another portion of the same extract was subjected to ammonium sulfate treatment by the procedure described in the text. The resulting precipitate was dissolved, and the solution thus obtained was subjected to isoelectric separation. ○, Without ammonium sulfate treatment; ●, with ammonium sulfate treatment.

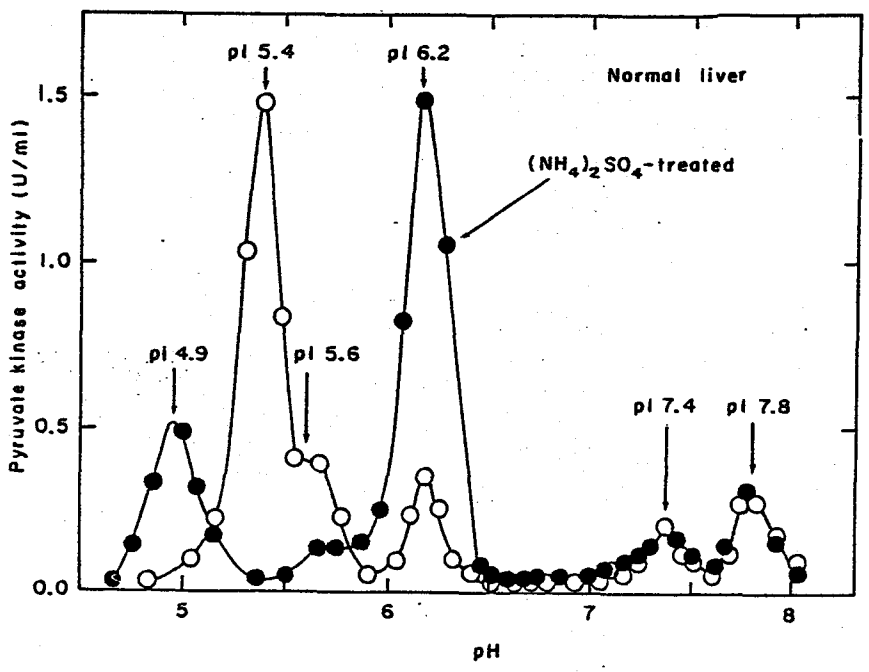


Fig. 2. Repeated isoelectric separation of pI 5.4-isozyme and pI 6.2-isozyme obtained by isoelectric separation of extracts from normal rat liver. The fractions for pI 5.4-isozyme and pI 6.2-isozyme obtained in the experiment in Fig. 1 were each subjected to isoelectric separation. Other experimental conditions were the same as in Fig. 1. ○, Re-electrofocusing of liver pI 5.4-isozyme; △, re-electrofocusing of liver pI-6.2-isozyme. Re-electrofocusing of the pI 6.2-isozyme obtained from the isoelectric separation of extracts with and without ammonium sulfate treatment gave essentially the same results.

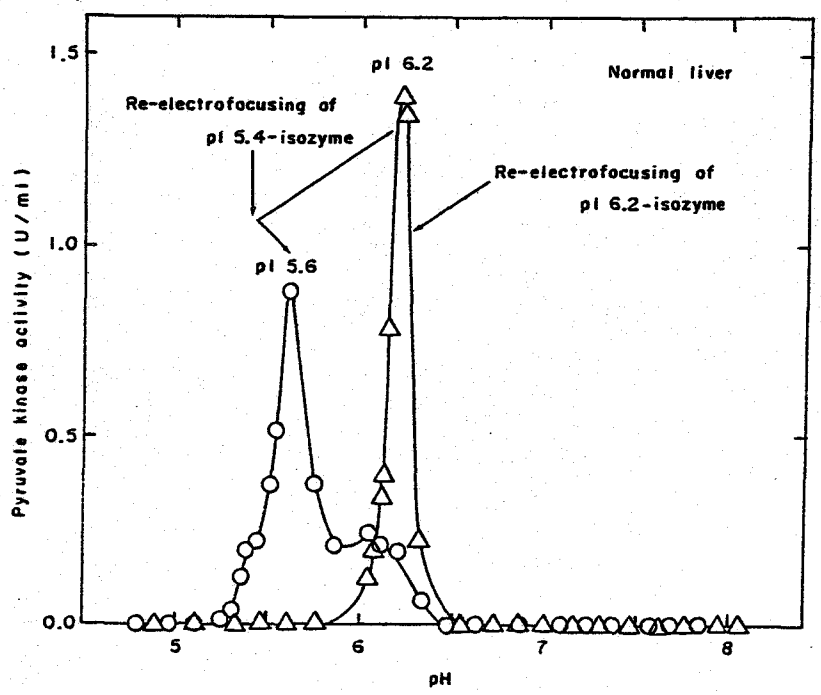


Fig. 3. Effect of ammonium sulfate treatment on isozyme pattern of pyruvate kinase from normal rat spleen. Experimental conditions were the same as in Fig. 1, except that extracts from normal rat spleen were used. ○, Without ammonium sulfate treatment; △, with ammonium sulfate treatment.

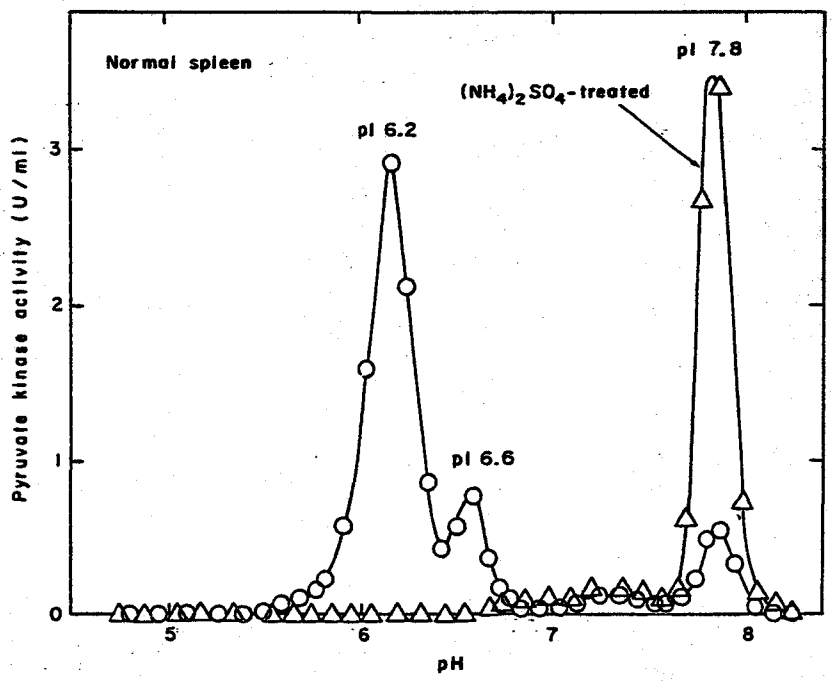


Fig. 4. Repeated isoelectric separations of pI 6.2-isozyme and pI 7.8-isozyme obtained by isoelectric separations of extract from normal rat spleen. The fractions for pI 6.2-isozyme and pI 7.8- isozyme obtained in the experiment in Fig. 3 were each subjected to isoelectric separation. Other experimental conditions were the same as in Fig. 1. ○, Re-electrofocusing of spleen pI 6.2-isozyme; △, re-electrofocusing of spleen pI 7.8-isozyme.

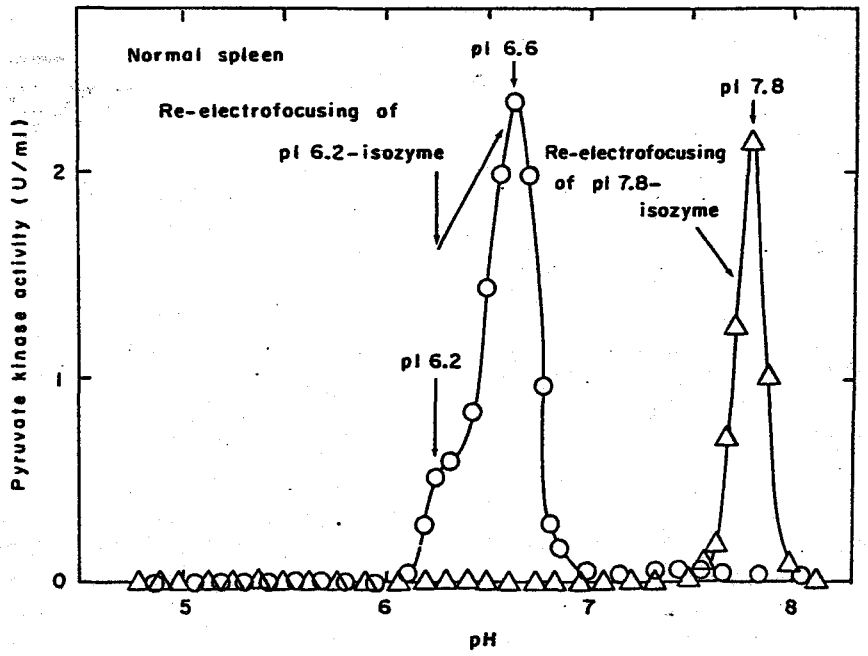


Fig. 5. Conversion of spleen pI 7.8-isozyme to pI 6.6 and pI 6.2-isozymes by binding with fructose 1, 6-diphosphate. The experimental conditions are described in the text. ○, re-electrofocusing of spleen pI 7.8-isozyme; △, re-electrofocusing of a mixture of 5ml of spleen pI 7.8-isozyme and 5ml of 0.2mM fructose 1,6-diphosphate; □, re-electrofocusing of spleen pI 7.8-isozyme in the electrofocusing column, in the presence of 1 mM fructose 1,6-diphosphate.

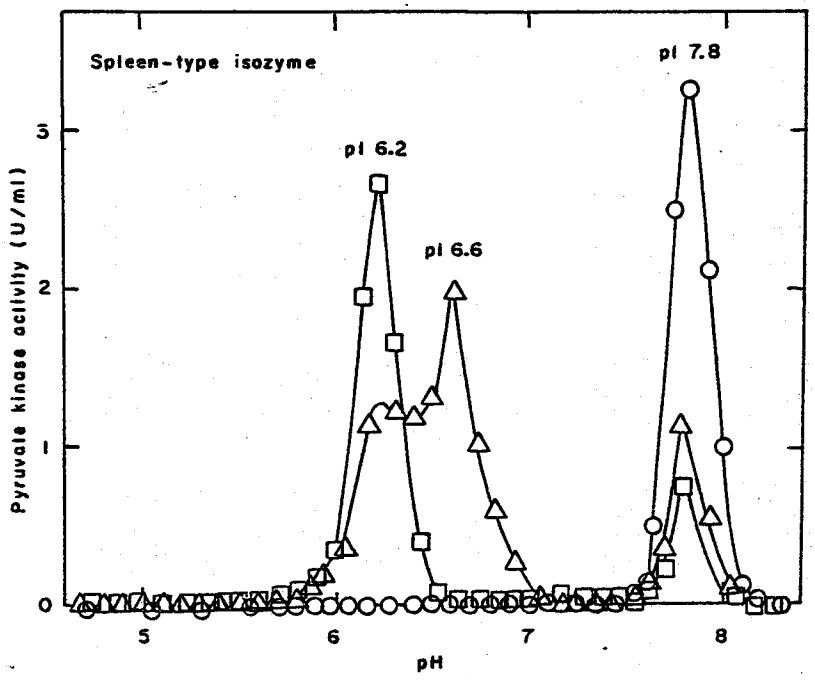


Fig. 6. Effect of ammonium sulfate treatment of on isozyme pattern of pyurvate kinase from normal rat muscle. ○, Without ammonium sulfate treatment; ●, with ammonium sulfate treatment.

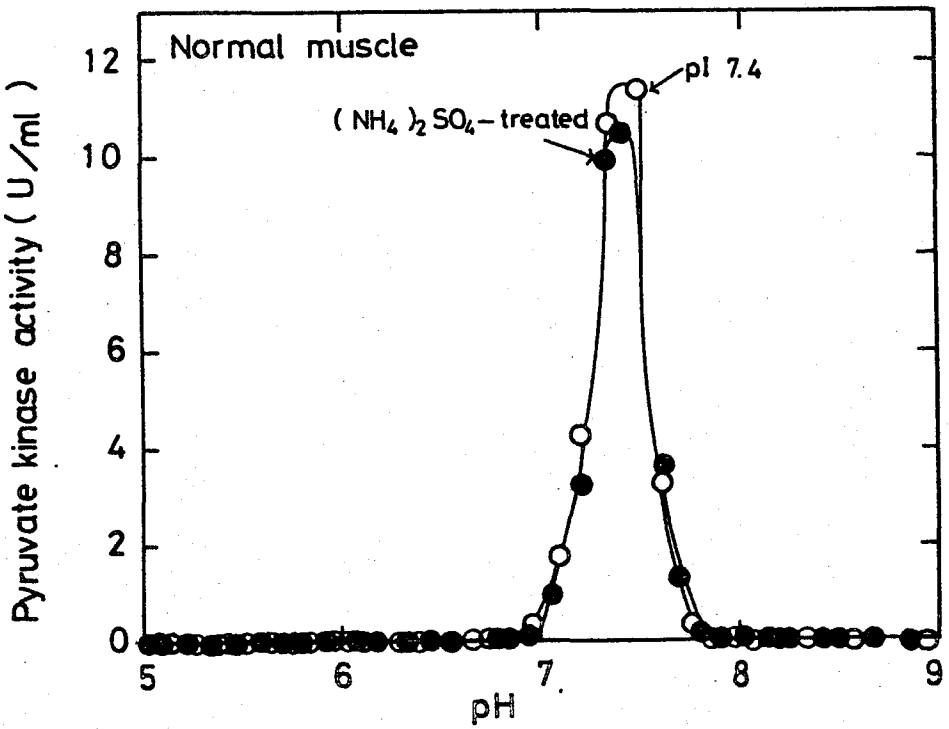


Fig. 7. Effect of ammonium sulfate treatment on isozyme pattern of pyurvate kinase from Rhodamine sarcoma. ○, Without ammonium sulfate treatment; ●, with ammonium sulfate treatment.

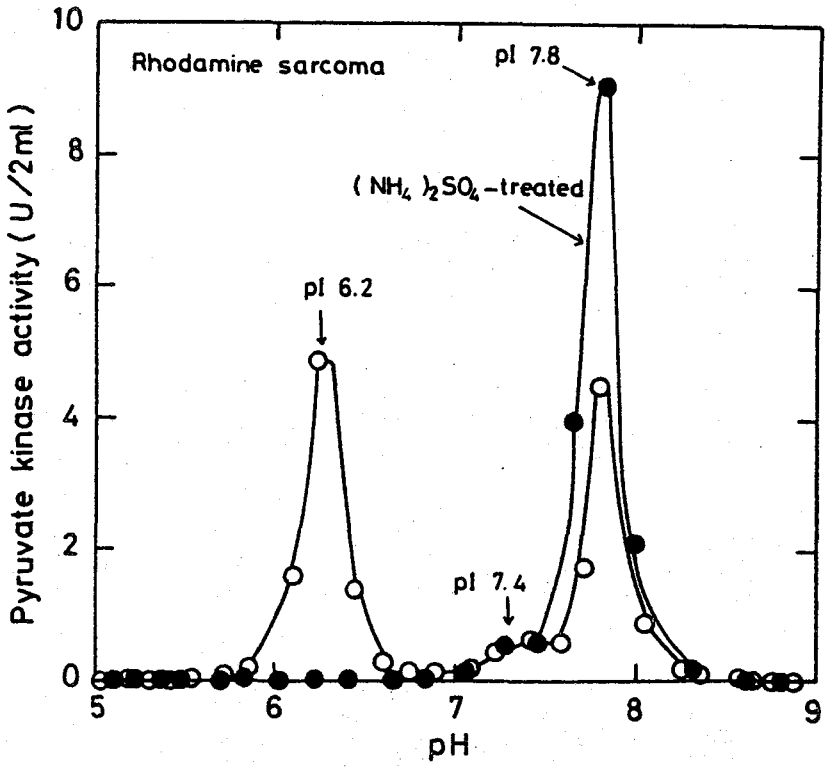


Fig. 8. Effect of phosphoenolpyruvate concentrations on activity of liver pI 6.2-isozyme. Liver pI 6.2-isozyme was prepared by isoelectric electrophoresis of ammonium sulfate treated liver extract. The activities were measured in the presence (●) and absence (○) of 0.1mM FDP. The activities in the presence of 5 mM phosphoenolpyruvate were taken as 100%. The use of liver pI 5.4- and pI 5.6-isozyme gave essentially the same results as the use of liver pI 6.2-isozyme.

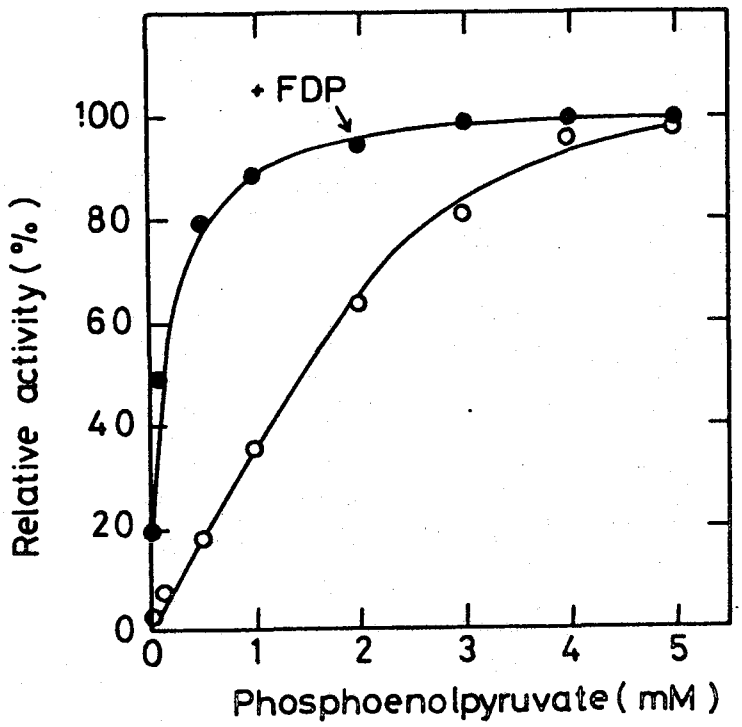


Fig. 9. Effect of phosphoenolpyruvate concentration on activity of spleen pI 7.8- and pI 6.2-isozymes. Spleen pI 7.8-isozyme was prepared by isoelectric separation of extracts from normal rat spleens. Spleen pI 6.2- and pI 6.6-isozymes were prepared by isoelectric separation of spleen pI 7.8-isozyme mixed with fructose 1,6-diphosphate according to the method in Fig. 5. The activities were measured in the presence and absence of 0.1 mM fructose 1,6-diphosphate (FDP). Other experimental conditions are described in the text. The activities of the various pI-isozymes in the presence of 5 mM phosphoenolpyurvate were taken as 100%. The use of spleen pI 6.6-isozyme gave essentially the same results as the use of spleen pI 6.2-isozyme.

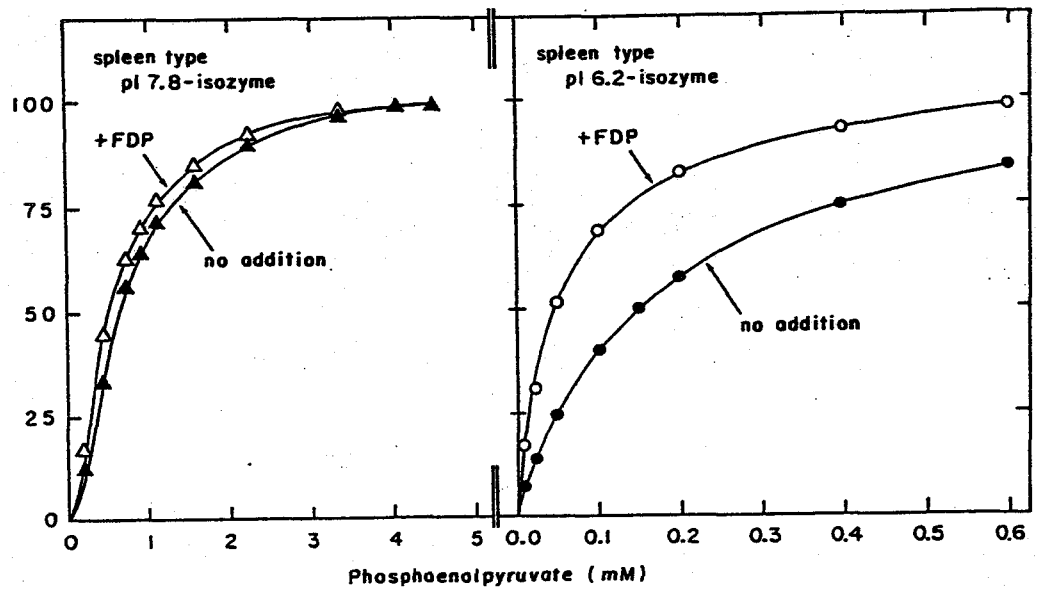


Fig. 10. Effect of phosphoenolpyruvate concentrations on activity of muscle pI 7.4-isozyme. The activities were measured in the presence (●) and absence (○) of 0.1 mM FDP.

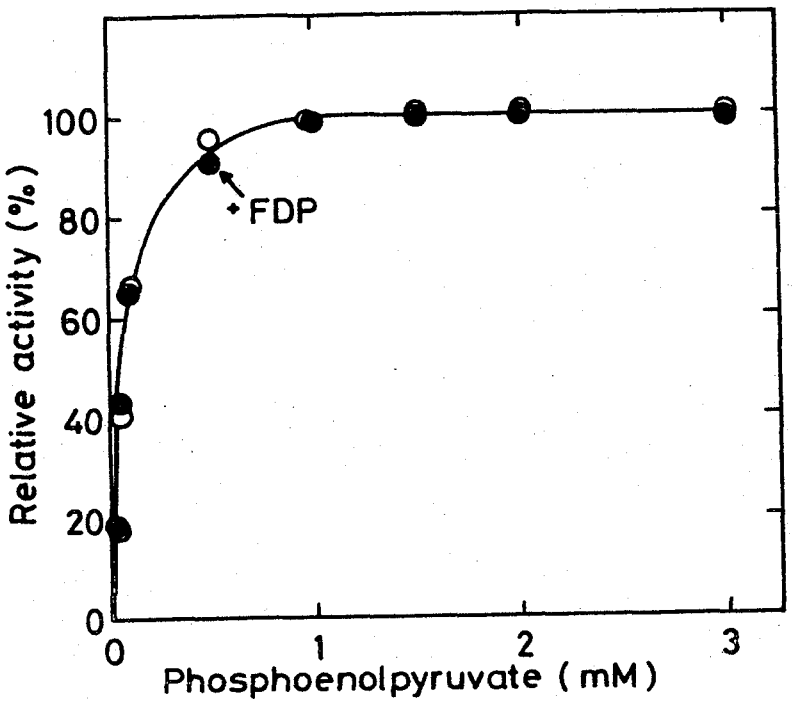


Fig. 11. Effect of ammonium sulfate treatment on isozyme pattern of pyruavte kinase from Rhodamine sarcoma-bearing rat liver. Experimental conditions were the same as in Fig.1, Except that extracts from Rhodamine sarcoma-bearing rat livers were used. ○, Without ammonium sulfate treatment; ●, with ammonium sulfate treatment.

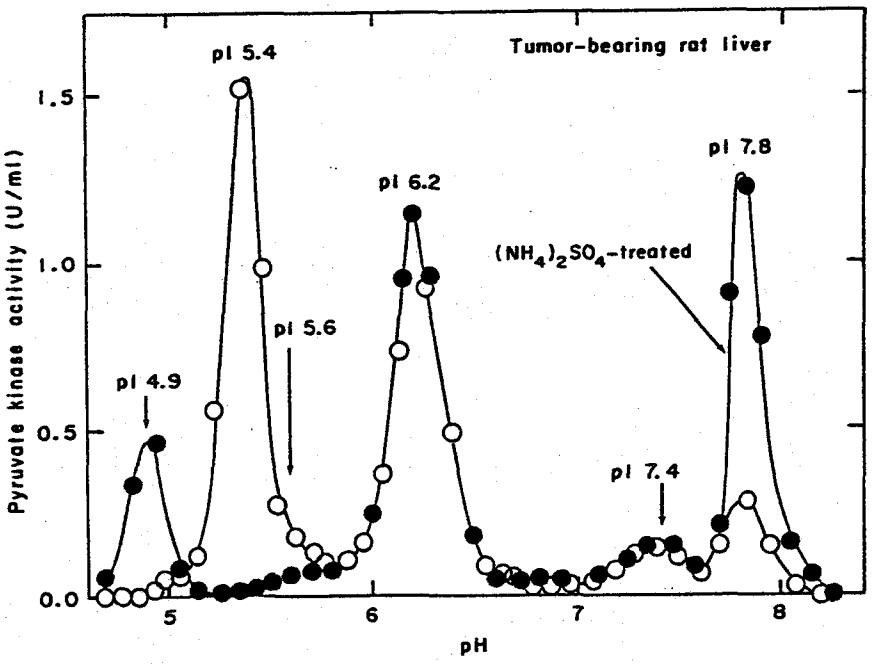


Fig. 12. Effect of phosphoenolpyruvate concentration on pyruvate kinase activities in extracts of $MH_1C_1$ cells and BRL cells. Activities were measured in the presence (●) and absence (○) of 1 mM FDP. Activities of pyurvate kinase in the presence of 3 mM phosphoenolpyruvate and 1 mM FDP in each sample were taken as 100%; 100% activity corresponded to 1.4 U/ml for $MH_1C_1$ cells and 30 U/ml for BRL cells. With the extract of $MH_1C_1$ cells, pyruvate kinase activity in the presence of 5 mM phosphoenolpyruvate and the absence of FDP was about 100%.

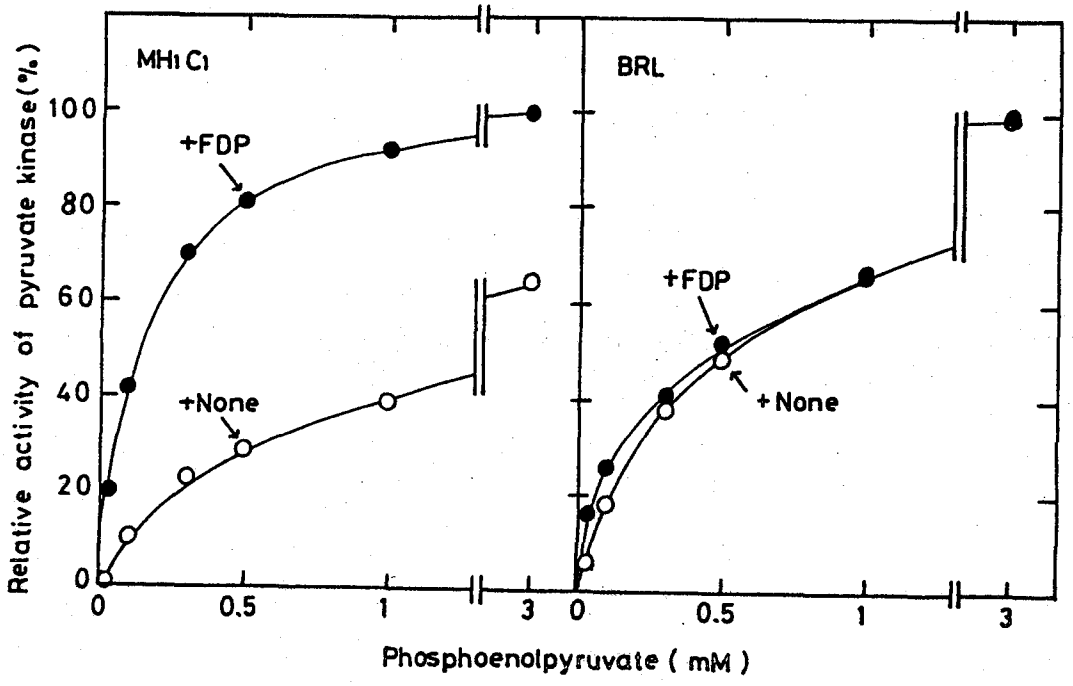


Fig. 13. Isoelectric separation of pyurvate kinase in an extract of BRL cells. Experimental conditions were as described in the text. Extract (0.50 ml, 1.6 U) from 1.6 x $10^7$ BRL cells was subjected to isoelectric focusing. The yield of activity after fractionation was 71 %.

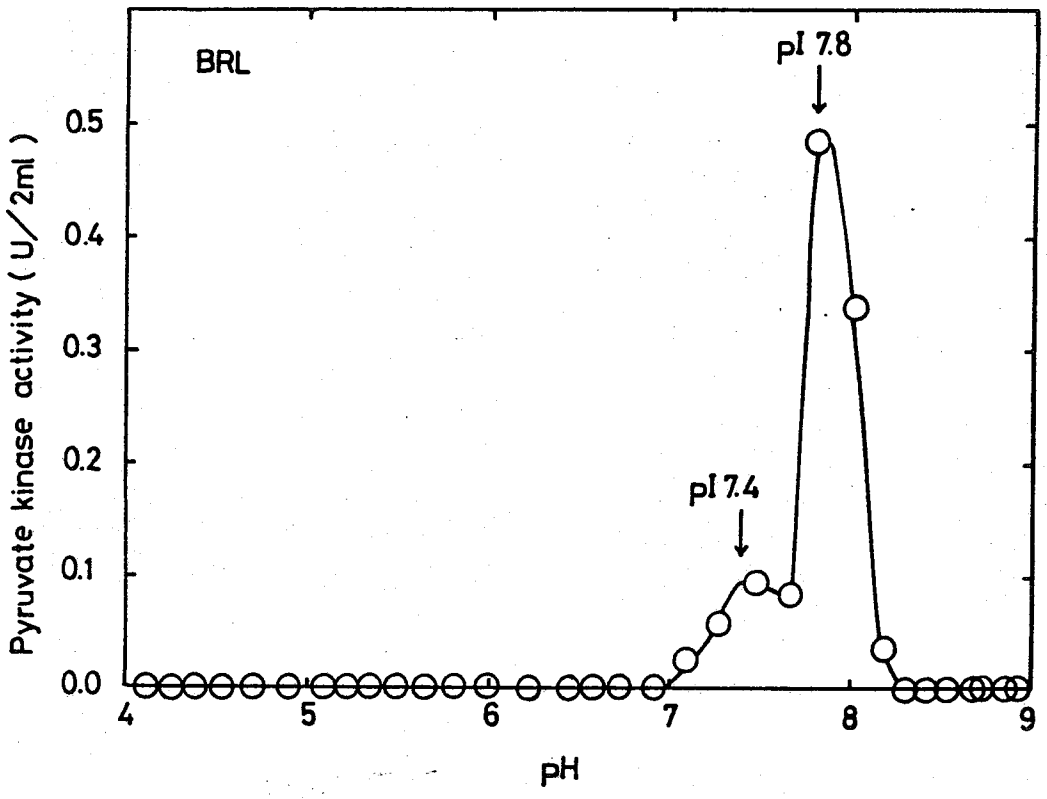


Fig. 14. Isoelectric separation of pyurvate kinase in an extract of $MH_1C_1$ cells. Experimental conditions were as described in the text. Extract (1.7 ml, 0.49 U) from 9.2 x $10^7$ $MH_1C_1$ cells was subjected to isoelectric focusing. The yield of activity after fractionation was 85 %.

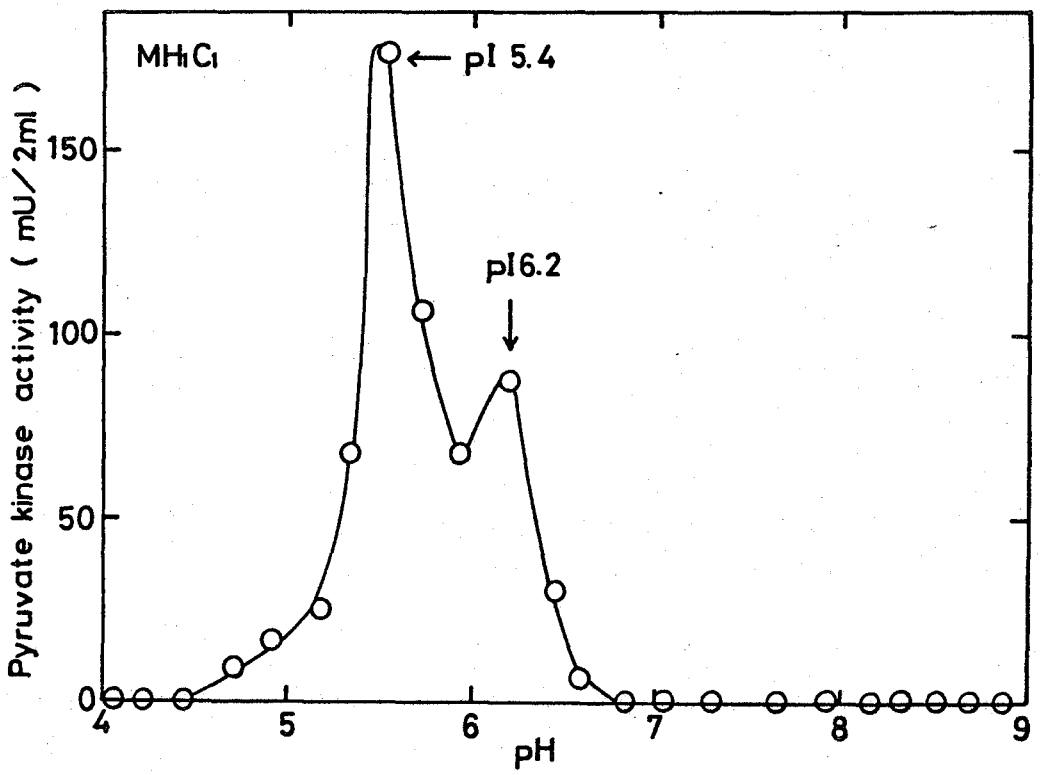


Fig. 15. Effect of ammonium sulfate treatment on the isozyme pattern of pyurate kinase from $MH_1C_1$ cells. An extract of 2.0 x $10^8$ $MH_1C_1$ cells was treated with ammonium sulfate (7.0 ml, 0.49 U) and then subjected to isoelectric focusing. Fractions of 1 ml of eluate were collected. Other experimental conditions were as described in the text. The yield of activity on fractionation was 107 %.

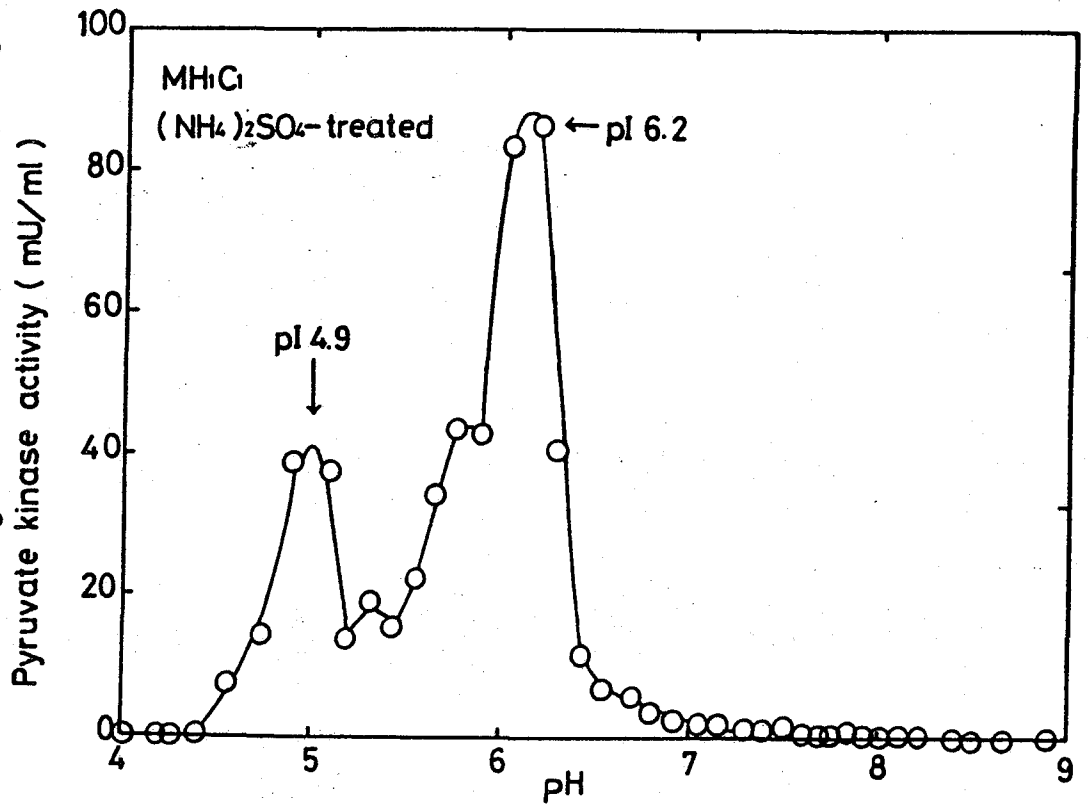


Fig. 16. Chromatography of spleen-type pyruvate kinase from Rhodamine sarcoma on CM-cellulose column. Experimental conditions were described in the text.

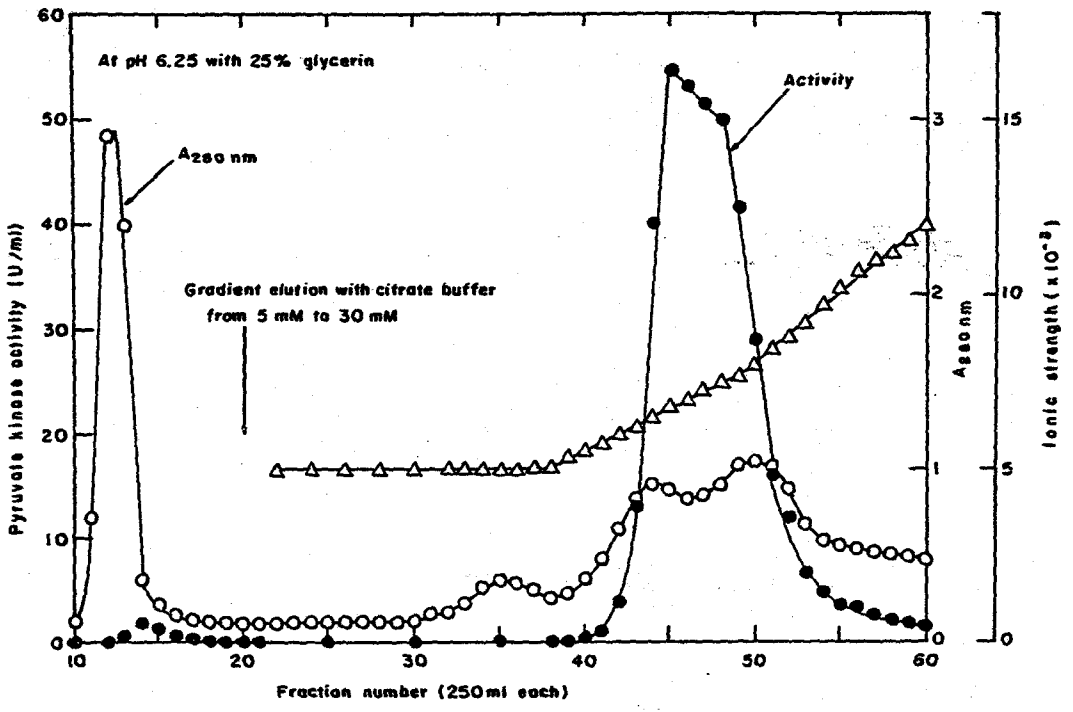


Fig. 17. Chromatography of spleen-type pyruavte kinase from Rhodamine sarcoma on P-cellulose column.

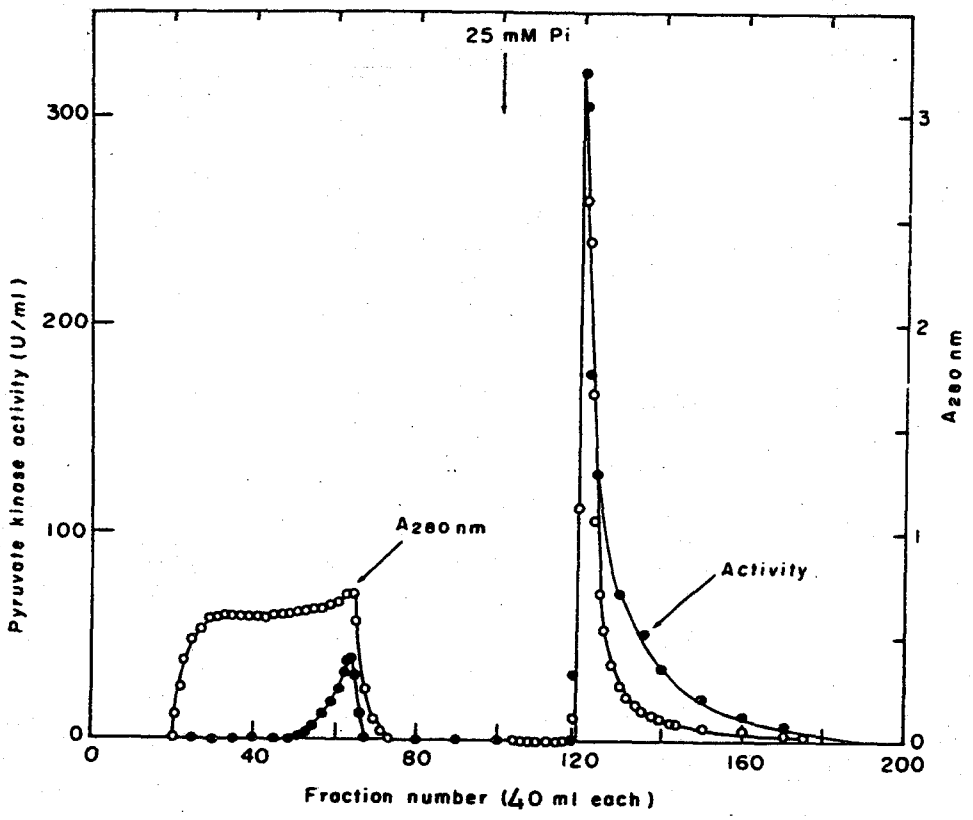


Fig. 18. 1st and 2nd isoelectric separations of spleen-type pyruvate kinsae from Rhodamine sarcoma. The eluate form the P-cellulose column (4 ml containing 4,000-5,000 units of pyruvate kinase) was subjected to the 1st isoelectric separation with Ampholine carrier-ampholytes of pI values from 3.5 to 10 at a final concentration of 2% (w/v). The total recovery of activity which centered at PH 7.8 was 90 to 98%. The fraction having a specific activity higher than 350 units/$A_{280nm}$ were collected (recovery, 60 to 70%) and used for the 2nd isoelectric separation. The fractions of the pI 7.8-isozyme collected by the 1st isoelectric separation (4,000-5,000 units) were preincubated with 1 mM FDP at 24°C for 30 min and then subjected to the 2nd isoelectric separation by the same method as for the 1st isoelectric separation. The total recovery of spleen-type pyurvate kinase activity which centered at pH 6.6 was nearly 100%. The fractions having a specific activity higher than 400 units/$A_{280nm}$ were collected (recovery, 75 to 85%). △&▲, 1st isoelectric separation; ○&●, 2nd isoelectric separation.

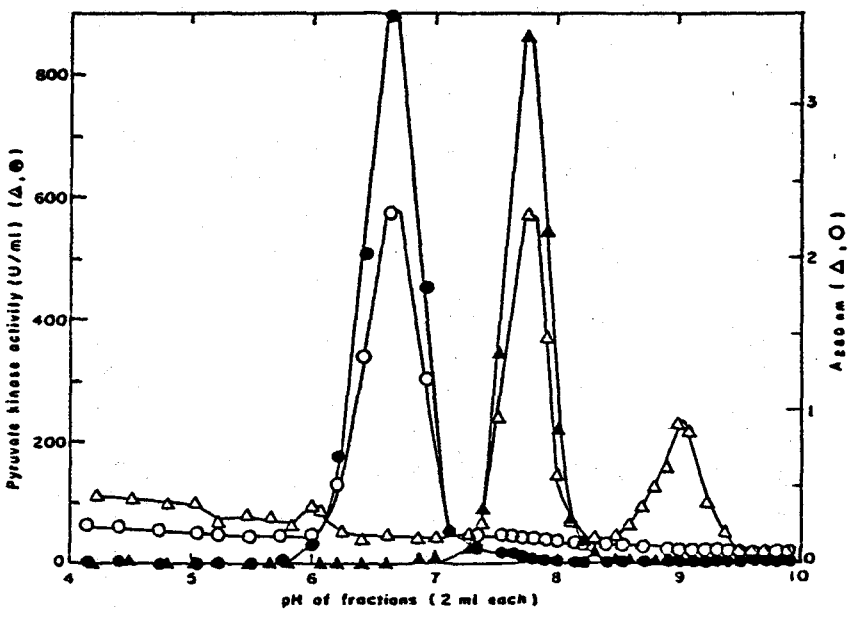


Fig. 19. Crystals of spleen-type pyruvate kinase. The crystals were photographed on 35 x 35-nm film at a magnification of 160, and the final magnification is 900.

Fig. 20. Absorbance spectrum of spleen-type pyruvate kinase purified from Rhodamine sarcoma. The spectrum was measured at concentration of 0.88 mg of spleen-type pyruvate kinase per 1.0 ml of 0.1 N NaOH.

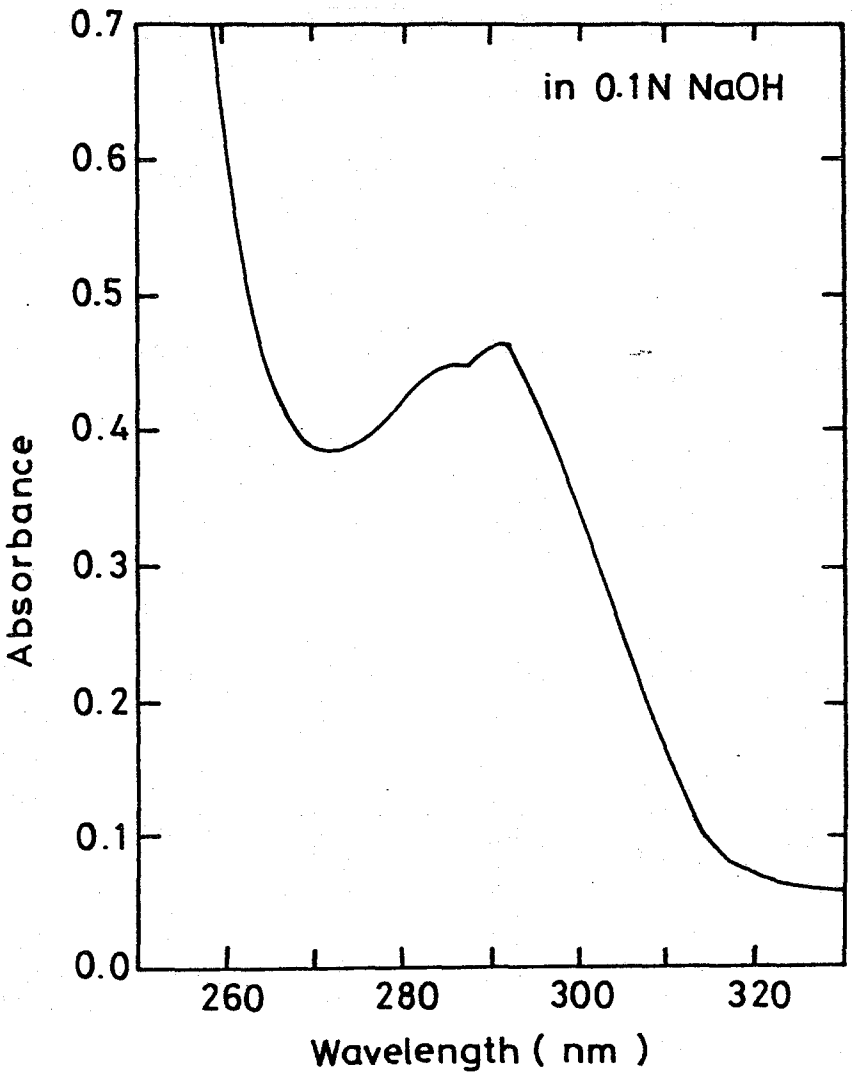


Fig. 21. Effect of KCl concentration on activities of spleen-type pI 7.8-isozyme with and without preincubation with fructose 1,6-diphosphate. Spleen pI 7.8-isozyme prepared by isoelectric separation of extracts from normal rat spleens or Rhodamine sarcoma was used. The reaction mixture was as described in the text, except that the KCl concentration was varied. In some cases, 1 mM fructose 1,6-diphsophate was added to the reaction mixture. In other cases, the isozyme in 10 mM Tris-HCl buffer (pH 7,5) with 25% glycerol was preincubated with 0.1 mM fructose 1,6-diphosphate for a few minutes, and then used for activity assay. △, Activity of spleen pI 7.8-isozyme in a reaction mixture containing no FDP; ▲, activity of the enzyme in a reaction mixture containing 1 mM FDP; ○, activity of the preincubated enzyme in a reaction mixture containing no FDP; ●, activity of the preincubated enzyme in a reaction mixture containing 1 mM FDP. In the presence of 3 mM phosphoenolpyruvate, spleen pI 7.8-isozyme showed practically the same activity, regardless of preincubation with FDP and addition of FDP(■).

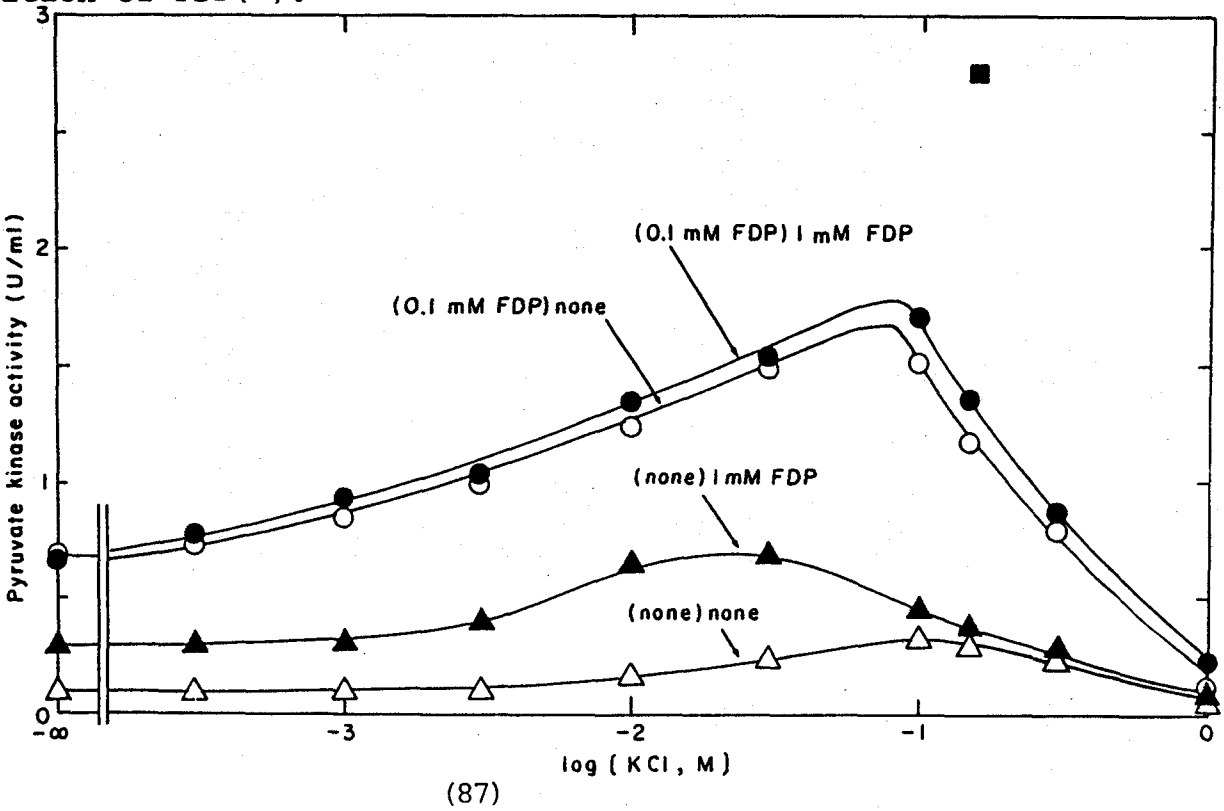


Fig. 22. Effect of preincubation with FDP on activity of spleen-type pyruvate kinase. The pI 7.8-isozyme obtained by the 1st isoelectric separation was preincubated with and without 1 mM FDP at 24°C for various lengths of time under the conditions described below. The activities of the preincubated enzyme solutions were assayed by adding 10 µl of the preincubated enzyme solutions to 1.0 ml of the standard reaction mixtures, in which the phosphenolpyruvate concentration was fixed at 0.1 mM, but not at 3 mM. —□— & --□--, The pI 7.8-isozyme was added to 10 mM Tris-HCl buffer (pH 7.5) containing 25% (v/v) glycerol with(—□—) and without (--□--) 1 mM FDP, and preincubated. —●— & --○--, The pI 7.8-isozyme was added to the standard reaction mixture containing 0.1 mM phosphoenolpyruvate and 25% (v/v) glycerol with (—●—) and without (--○--) 1mM FDP, in which ADP, as the substate, was omitted, and preincubated. —▲— & --△--, The pI 7.8-isozyme was added to the standard reaction mixture containing 25% (v/v) glycerol with (—▲—) and without (--▽--) 1 mM FDP, in which phosphoenolpyruvate, as the substate, was omitted, and preincubated. (+RM-PEP-ADP) gave essentially the same result as (+RM-PEP) and (+RM-ADP), and (+RM-PEP-ADP+FDP) the same as (+RM-PEP+FDP) and (+RM-ADP+FDP)

Fig. 22.

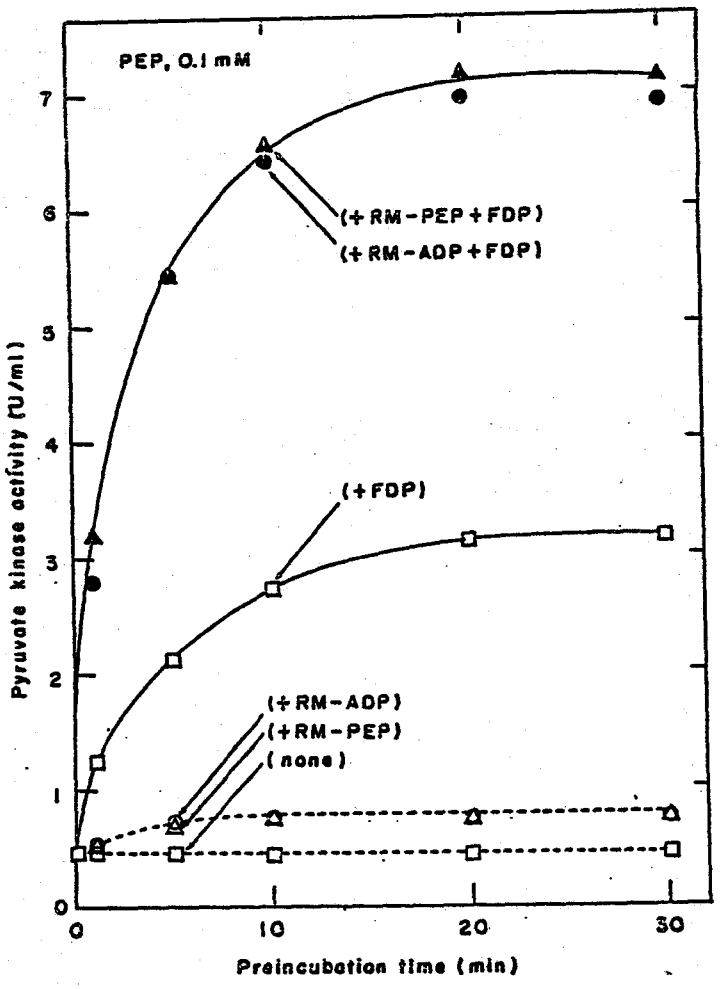

PEP, 0.1 mM
(+ RM-PEP + FDP)
(+ RM-ADP + FDP)
(+FDP)
(+RM-ADP)
(+RM-PEP)
(none)
Pyruvate kinase activity (U/ml)
Preincubation time (min)
0
1
2
3
4
5
6
7
0
10
20
30

Fig. 23. Effect of FDP concentration in preincubation on spleen-type pI 7.8-isozyme activity. An aliquot (10 µl) containing 4.5 units of the pI 7.8-isozyme obtained by the 1st isoelectric separation was added to 1.0 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 25% (v/v) glycerol and various concentrations of FDP, and preincubated at 24°C for 30 min. After preincubation, the enzyme activities were assayed by adding 20 µl of the preincubated enzyme solution to 1.0 ml of the standard reaction mixtures with and without 1 mM FDP, in which the phosphoenolpyruvate concentration was fixed at 0.1 mM. —o—, Assayed in the standard reaction mixture with 1 mM FDP; —Δ—, assayed in the standard reaction mixture without FDP; ----, described in the text. The results were reproducible in experiments done in triplicate.

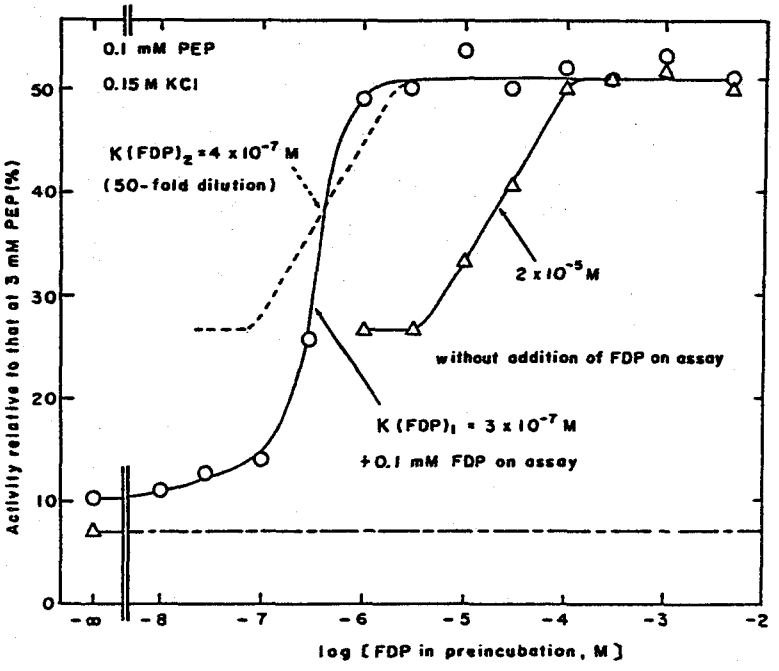


Fig. 24. Effect of preincubation of spleen-type pyruvate kinase with NaCl and KCl on its activity. An aliquot (50 µl) containing 20 units of the pI 7.8-isozyme obtained by the 1st isoelectric separation was dissolved in 1 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 25% (v/v) glycerol and various concentrations of either NaCl or KCl, and then preincubated at 24°C for 10min. After preincubation, the enzyme activities were assayed by adding an aliquot (10 µl when assayed at 0.1 mM phosphoenolpyruvate, and 2 µl when assayed at 3 mM phosphoenol-pyruvate) of each preincubated enzyme solution to the standard reaction mixture containing either 3 mM or 0.1 mM phosphoenolpyruvate. ○ & ●, Preincubated with KCl, and assayed at 0.1 mM and 3 mM phosphoenol-pyurvate, respectively; △ & ▲, preincubated with NaCl, and assayed at 0.1 mM and 3 mM phosphoenolpyruvate, respectively.

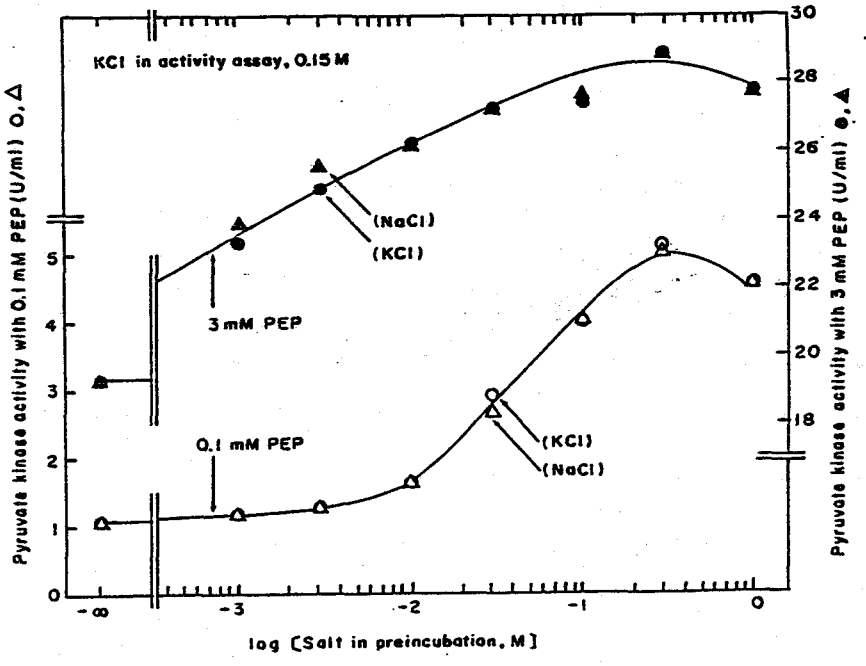


Fig. 25. Effect of phosphoenolpyruvate concentration on activity of spleen-type pyruvate kinase preincubated with NaCl and FDP. An aliquot (10 µl) containing 3.5 units of the pI 7.8-isozyme obtained by the 1st isoelectric separation was dissolved in 1 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 25% (v/v) glycerol, to which 0.3 M NaCl and/or 0.1 mM FDP was added. The resulting enzyme solutions were preincubated at 24°C for 30 min. The enzyme activity of each preincubated enzyme solution was assayed by adding 20 µl of the preincubated enzyme solution to 1.0 ml of the standard reaction mixture containing various concentrations of phosphoenolpyruvate. ○, Non-preincubated; ●, preincubated with 0.1 mM FDP; △, preincubated with 0.3 M NaCl; ▲, preincubated with 0.3 M NaCl plus 0.1 mM FDP.

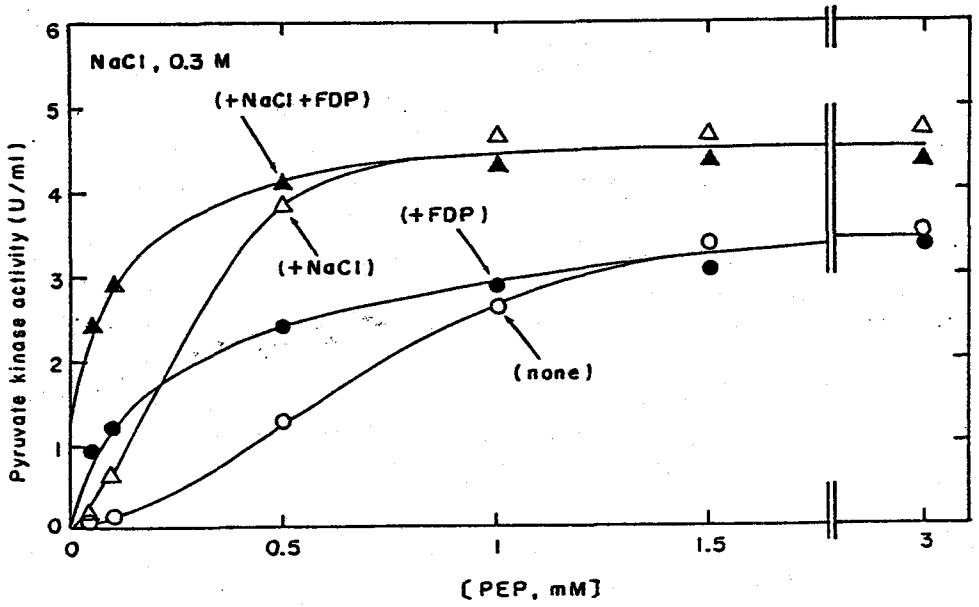


Fig. 26. Effect of preincubation of spleen-type Pyruvate kinase with various salts on its activity in presence of KCl or NaCl. An aliquot (20 µl) containing 9.5 units of the pI 7.8-isozyme obtained by the 1st isoelectric separation was dissolved in 1.0 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 25% (v/v) glycerol and 0.3 M of various salts (LiCl, NaCl, KCl, RbCl, or Tris-HCl), and preincubated at 24°C for 10 min. The enzyme activity of each preincubated enzyme solution was assayed by adding 5 µl of the preincubated enzyme solution to 1.0 ml of the standard reaction mixture containing various concentrations of either KCl or NaCl besides 3 mM phosphoenolpyruvate. ●, Non-preincubated and assayed in the presence of KCl; ▲, non-preincubated and assayed in the presence of NaCl; (□,△,○,▽,x)—KCl, preincubated with 0.3 M of LiCl, NaCl, KCl, RbCl, and Tris-HCl, respectively, and assayed in the presence of KCl; (□,△,○,▽,x)—NaCl, preincubated with 0.3 M of LiCl, NaCl, KCl, RbCl, and Tris-HCl, respectively, and assayed in the presence of NaCl. When the enzyme was preincubated with other salts such as $NH_4Cl$, $(NH_4)_2SO_4$, KI, or $KHCO_3$, essetially the same results were obtained.

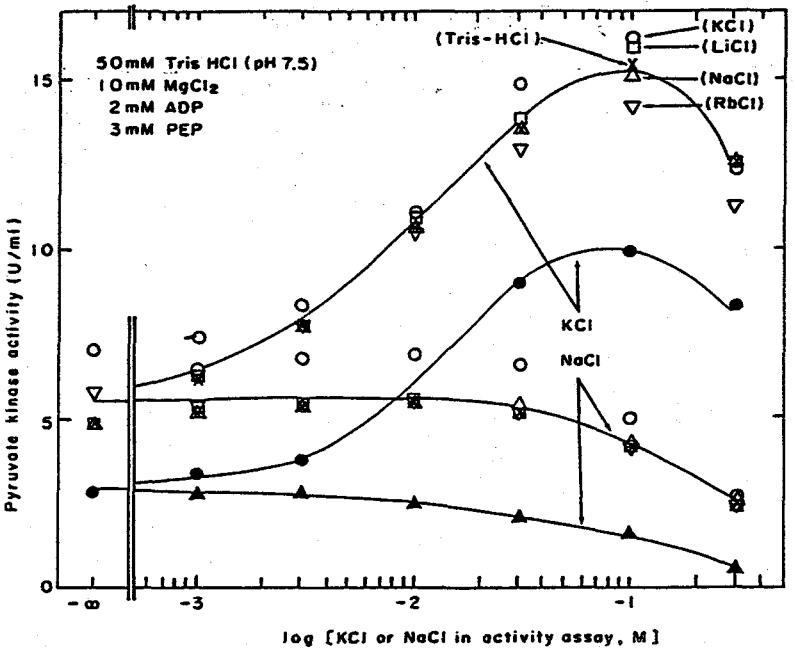


Fig. 27. Effect of various salts added on activity assay of spleen-type pyruvate kinase. An aliquot (40 µl) containing 13 units of the pI 7.8-isozyme obtained by the 1st isoelectric separation was dissolved in 1.0 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 25% (v/v) glycerol and 0.3 M NaCl, and preincubated at 24°C for 10 min. The activity was assayed by adding 5 µl of the preincubated enzyme solution to 1.0 ml of the standard reaction mixture, which contained various concentrations of saveral salts instead of 0.15 M KCl as indicated. ○, KCl or Tris-HCl; --△--, RbCl or LiCl; ■, $NH_4Cl$;--□--,CsCl; ●, NaCl; —□—, $SrCl_2$; —△—, $CaCl_2$.

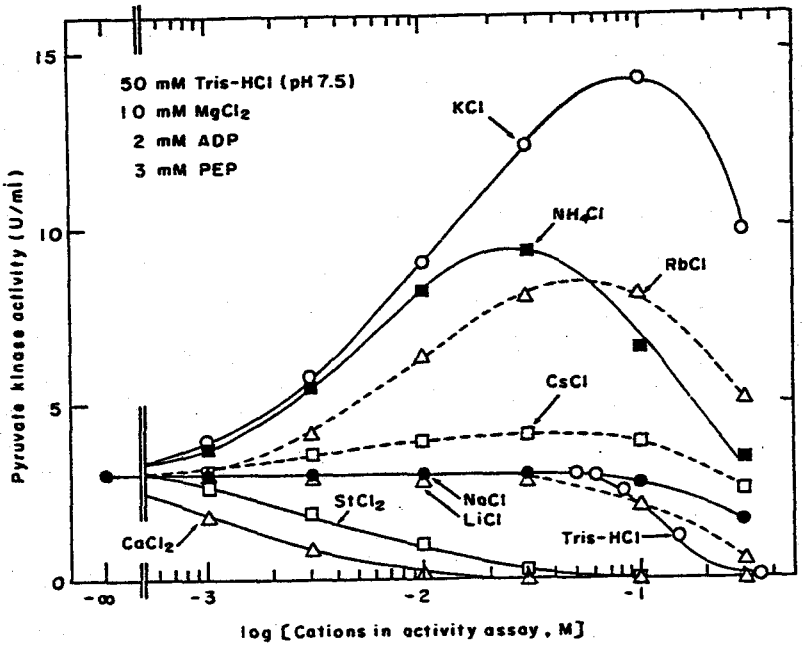


Fig. 28. Effect of heat treatment on activities of spleen-type and muscle-type pyruvate kinase. 1 ml of each isoenzyme solution was heat treated at 60°C for various lengths of time indicated. Resulting precipitate was removed by centrifugation, and the supernatant was used for activity assay and measurement of $A_{280\ nm}$. A, With the use of spleen-type ; B, with the use of muscle-type; ○, activity; △, $A_{280\ nm}$. 100% activity and 100% $A_{280\ nm}$ correspond to 55 U/ml and $A_{280\ nm}$=64, respectively for spleen-type, and those for muscle-type are 375 U/ml and $A_{280\ nm}$=26.

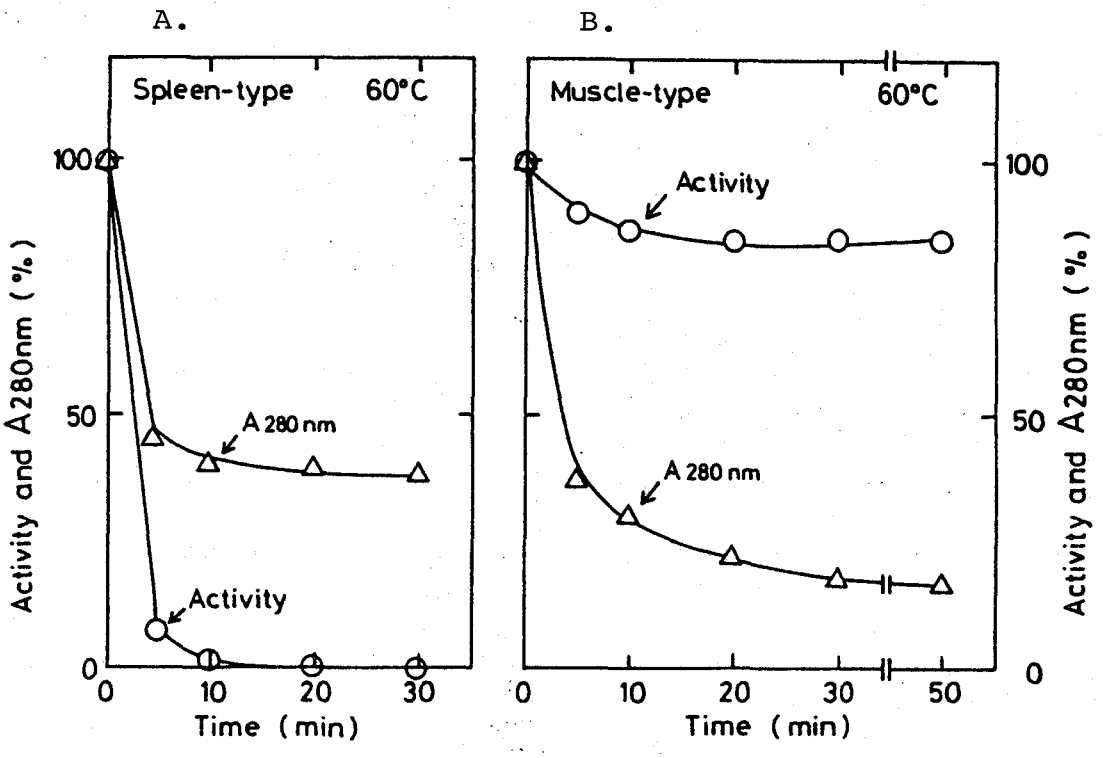


Fig. 29. Effect of glycerol on stabilites of spleen-type and muscle-type pyruvate kinase. Each isoenzyme was dissolved in the buffer solutions which contain various concentrations of glycerol and had indicated pH valued. The used buffer solutions were 10 mM K-citrate buffer for pH 4.5-6.0 and 10 mM Tris-HCl buffer for pH 7.0-9.0. Resulting solutions were stood at 4°C for 3 days, and then activities were measured. ●, The activities at 0 time without addition of glycerol; ▲, without addition of glycerol (0%); □, addition of 10% glycerol (10%); △, addition to 25% glycerol (25%); ○, addition of 50% glycerol (50%); A, with the use of spleen-type; B, with the use of muscle-type.

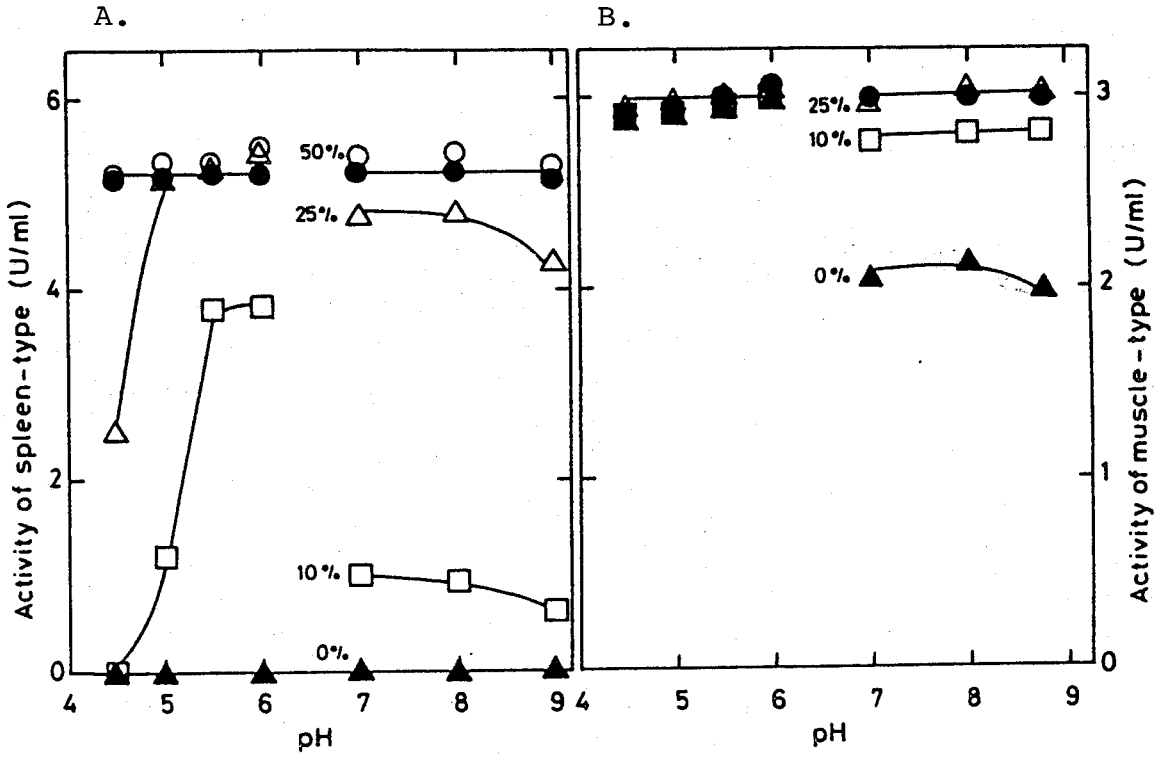


Fig. 30. SDS-polyacrylamide gel disc electrophoresis of purified spleen-type and muscle-type pyruvate kinase. Approximately 50 μg of spleen-type and 100 μg of muscle-type were analyzed.

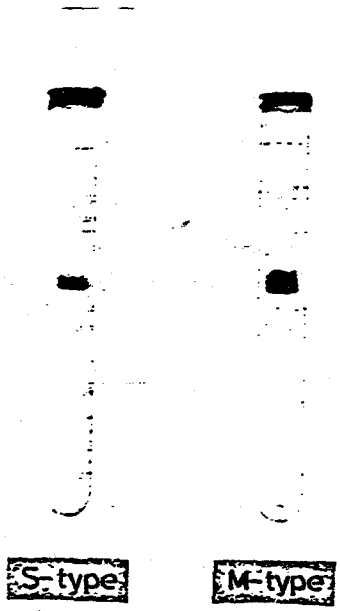
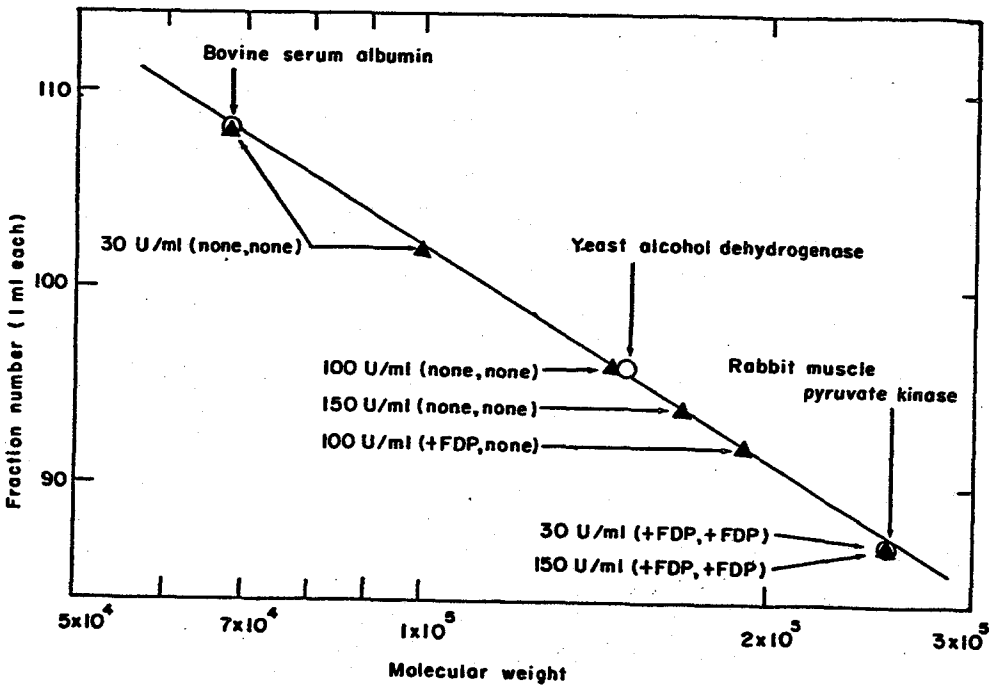


Fig.31. Summary of molecular-sieve chromatography of spleen-type and muscle-type pyruvate kinase on Sephadex G-200 column. A, Spleen-type; B, muscle-type. ○, Molecular weight marker proteins; bovine serum albumin (M.W. 67,000), yeast alcohol dehydrogenase (M.W. 140,000), and rabbit muscle pyruvate kinase (M.W. 240,000). ▲, Elution volume and apparent molecular weights of pyuravte kinase under indicated conditions. The values (U/ml) show the enzyme concentrations applied to the column. The symbol on the left in parenthesis, "none" or "+FDP", shows wether the enzyme was preincubated with 1 mM FDP (+FDP) or not (none), and symbol on the right in perenthesis,"none" or "+FDP", shows wether the buffer solutions for equilibration and the elution of the column contained 1 mM FDP (+FDP) or not (none).

Fig. 31

A


Bovine serum albumin
110
100
90
Fraction number (1 ml each)
30 U/ml (none,none)
Yeast alcohol dehydrogenase
100 U/ml (none,none)
150 U/ml (none,none)
100 U/ml (+FDP,none)
Rabbit muscle
pyruvate kinase
30 U/ml (+FDP,+FDP)
150 U/ml (+FDP,+FDP)
5x10^4
7x10^4
1x10^5
2x10^5
3x10^5
Molecular weight

B

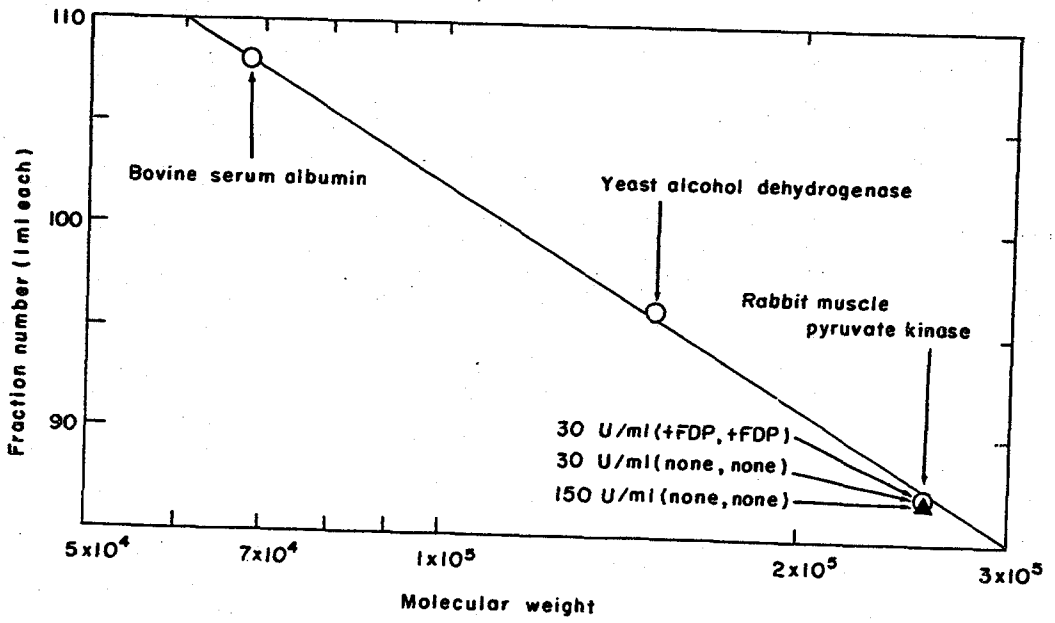
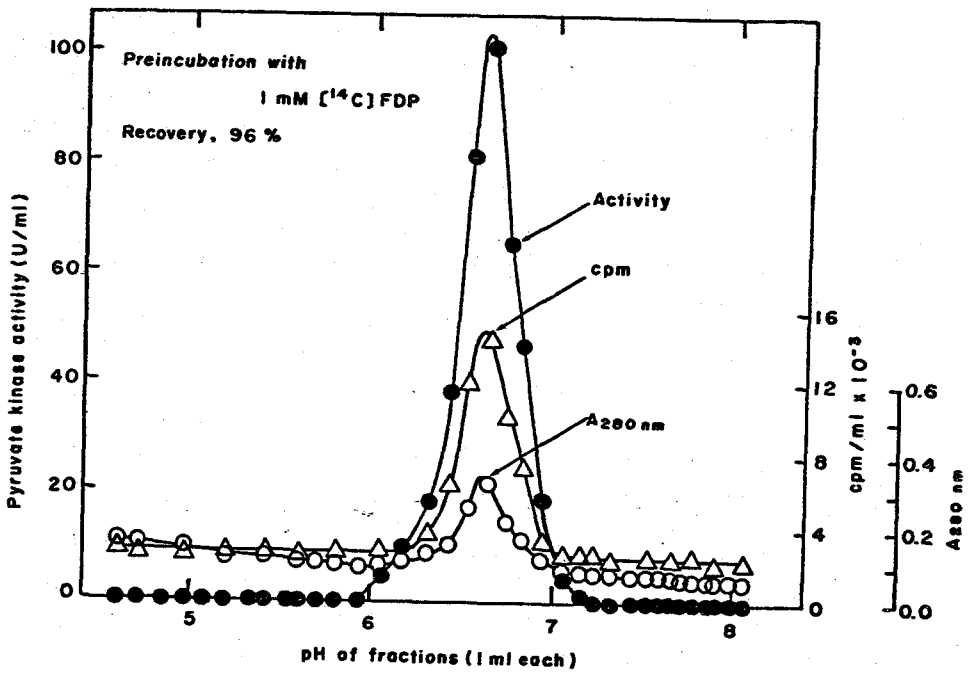

110
100
90
Fraction number (1 ml each)
Bovine serum albumin
Yeast alcohol dehydrogenase
Rabbit muscle
pyruvate kinase
30 U/ml (+FDP,+FDP)
30 U/ml (none, none)
150 U/ml (none, none)
5x10^4
7x10^4
1x10^5
2x10^5
3x10^5
Molecular weight

Fig. 32. Isoelectric electrophoresis of spleen-type and muscle-type pyurvate kinase preincubated with [$^{14}$C]FDP. A, Spleen-type pI 7.8-isozyme obtained by 1st isoelectric separation (2 ml containing 400 units) was incubated with 2 ml (4 μCi) of 2 mM [$^{14}$C]FDP (1 Ci/mol) in 10 mM Tris-HCl buffer (pH 7.5) containing 25%(v/v) glycerol, at 24°C for 30 min, and then subjected to isoelectric electrophoresis; B, muscle-type sample (P-cellulose eluate) was dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 25% glycerol. An aliquot (2 ml containing 1000 units) was treated with [$^{14}$C]FDP as the same manner as described above.

Fig. 32

A


Preincubation with
1 mM [14C] FDP
Recovery, 96 %
Activity
cpm
A280 nm
Pyruvate kinase activity (U/ml)
cpm/ml x 10-3
A280 nm
pH of fractions (1 ml each)

B

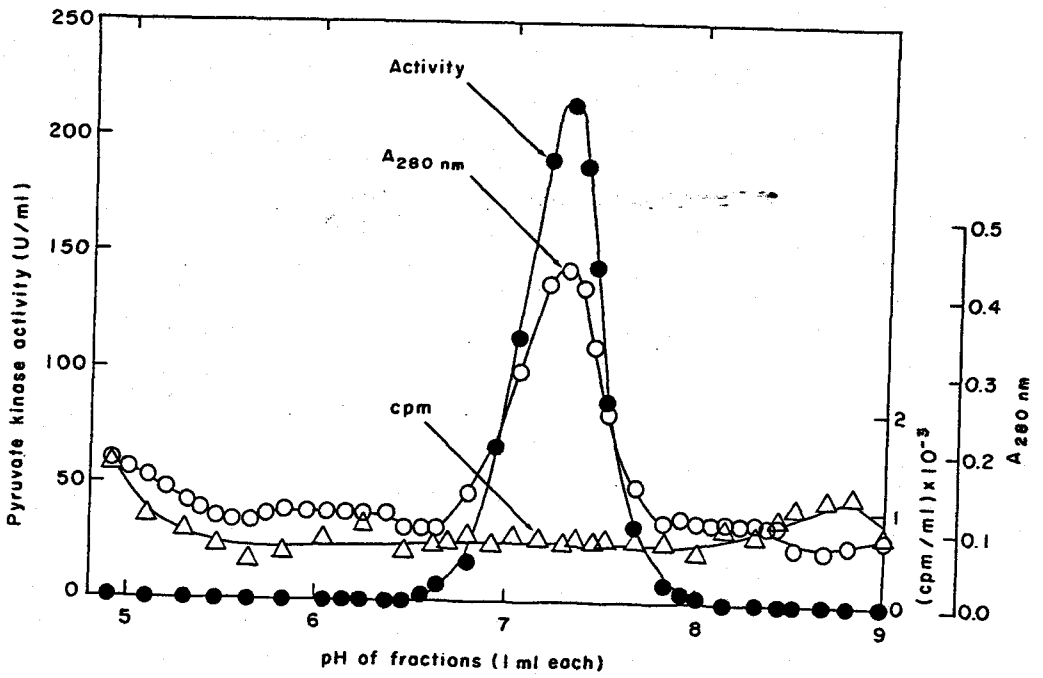

Activity
A280 nm
cpm
Pyruvate kinase activity (U/ml)
(cpm/ml) x 10-3
A280 nm
pH of fractions (1 ml each)

Fig. 33. Peptide maps of spleen-type and muscle-type pyruvate kinase. Of the ninhydrin-positive spots, less-dense ones are shown by dotted circles, and these different between both types by dlinded circles. Experimental conditions were described in the text. A, Muscle-type; B, Spleen-type.