The Purification and Characterization of Human M_1- and M_2-Pyruvate Kinase Isoenzymes

by

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A THESIS

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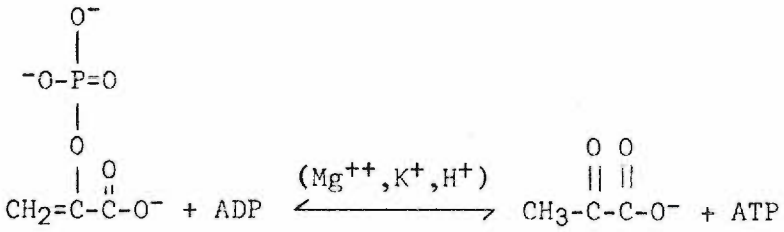
Abbreviations

Acetyl CoA	acetyl Coenzyme A
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
CM-cellulose	carboxymethyl cellulose
DEAE-cellulose	diethylaminoethyl cellulose
Dansyl-Cl	5'-dimethylamino-naphthalene sulfonyl chloride
dCDP	deoxycytosine diphosphate
EDTA	(ethylenedinitrilo)-tetraacetic acid
FDP	fructose-1,6-diphosphate
GDP	guanosine diphosphate
$K_{0.5s}$	concentration of substrate giving 50% maximum activity
K_{eq}	apparent equilibrium constant
K_i	apparent inhibition constant
K_m	apparent Michaelis constant
n	Hill coefficient
NADH	β-nicotinamide adenine dinucleotide (reduced form)
PEP	phosphoenolpyruvate
$S_{20,w}$	sedimentation coefficient in H_2O at 20°C
S_{obs}	observed sedimentation coefficient
SDS	sodium dodecyl sulfate
TLC	thin layer chromatography
Tris	Tris(hydroxymethyl)-aminomethane
V_{max}	maximum velocity

A. Introduction

Pyruvate kinase is a glycolytic enzyme which catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate (equation 1). The reaction

[1]



involves the transfer of a phosphoryl group from PEP to adenosine 5'-diphosphate (ADP), resulting in the formation of pyruvate and adenosine 5'-triphosphate (ATP). Both a divalent and a monovalent cation are required for activity. Pyruvate kinase has been designated by the Commission on Enzymes as ATP: pyruvate phosphotransferase EC 2.7.1.40 (1).

The reaction catalyzed by pyruvate kinase is essentially irreversible with an apparent equilibrium constant, defined in equation 2, reported as 2000 (2), 6500 (3), and 2200 (4). The discrepancies

$$[2] \qquad Keq = \frac{[ATP]\,[pyruvate]}{[ADP]\,[PEP]}$$

reflect in part differences in the conditions employed, the observation that the divalent cation Mg^{++} chelates nucleotides (5), and the evidence that the Mg-ADP complex functions as the true substrate in

the reaction (6).

Pyruvate kinase also catalyzes a "fluorokinase" (7) and an "hydroxylamine kinase" (8,9) reaction. In the former case fluoride replaces pyruvate and is converted to fluorophosphate in the presence of ATP, while in the latter reaction ATP and hydroxylamine are converted to ADP and phosphorylated hydroxylamine. Both reactions are stimulated by bicarbonate and require magnesium or zinc and potassium as cofactors for activity.

Studies on the substrate specificity of pyruvate kinase have shown that ADP may be substituted by a number of other nucleoside diphosphates. Reaction velocities in the presence of these analogs range from 0.3% for deoxycytosine diphosphate (dCDP) to 95% for guanosine diphosphate (GDP), compared to the natural substrate ADP (10). The results suggest that the active site of pyruvate kinase which binds the base portion of the nucleotide ADP does not have strict steric requirements. Using PEP analogs, a number of investigators (11-15) have shown that several such compounds combine with pyruvate kinase with binding constants similar to that of PEP. However, they act as poor substrates with a weak ability to donate a phosphoryl group. These experiments suggest a high degree of specificity for the phosphate donor at the PEP binding site of pyruvate kinase.

Pyruvate kinase requires both a monovalent (K^+ or NH_4^+) and a divalent cation (Mg^{++} or Mn^{++}) for catalytic activity. The

only exceptions have been in the case of the enzyme from some prokaryotes (16), where no such K^+ or NH_4^+ requirement is observed. Pyruvate kinase from eukaryotes was the first enzyme for which an absolute requirement for a monovalent cation was established (17,18). Studies with the enzyme from rabbit muscle have shown that K^+ gives the greatest activation, that NH_4^+, Rb^+, or Th^+ can replace K^+, but that Na^+, Li^+, or Cs^+ cannot replace K^+ (19-22). The studies suggest that the cation specificity may be determined by its hydrated ionic radius (21). The exact role of the monovalent cation in the catalytic mechanism of pyruvate kinase is uncertain. It has been proposed that monovalent cations function by inducing a conformational change in an enzyme resulting in a more active form (19,23). Lowenstein (24) suggested a more direct role for K^+ in transphosphorylation reactions, and this view has been supported by Suelter (25) who has shown that K^+ binds in or extremely close to the active site of rabbit muscle pyruvate kinase. More recently, Nowak and Mildvan (26) have provided evidence with the use of PEP analogs that K^+ plays a direct catalytic role by binding the carboxyl group of PEP at the active site.

Pyruvate kinase also requires a divalent cation, either Mg^{++} or Mn^{++}, for activity, though other ions may be substituted with less effectiveness (5,27). The function of the divalent cation in pyruvate kinase catalysis has been studied extensively, and the results suggest that the cation binds at the active site and forms

a bridge complex between the enzyme and the phosphoryl group undergoing transfer (26,28).

An exact model explaining the catalytic mechanism of pyruvate kinase has not been established. Boyer (5) has proposed an overlapping ATP-Phosphoenolpyruvate site model (Figure 1), in which the phosphoryl group of PEP occupies the same site as the terminal phosphate of the ATP product. The mechanism

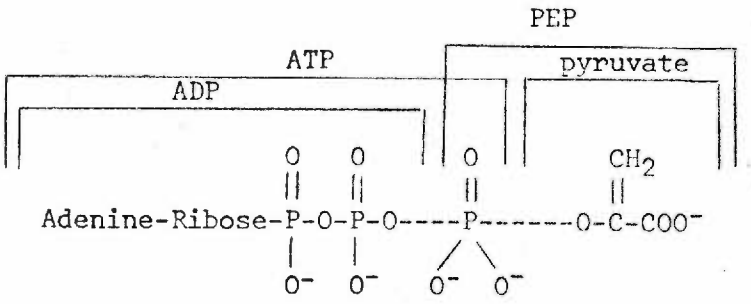


Figure 1

ATP-Phosphoenolpyruvate overlapping model for pyruvate kinase active site.

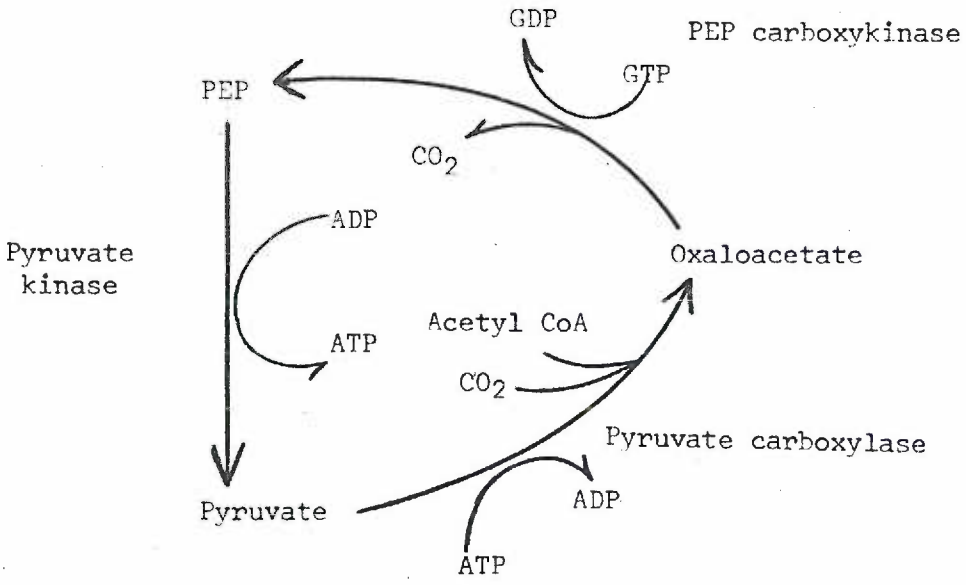
is thought to involve a direct transfer of the donor phosphoryl group (from PEP) to the acceptor (ADP). It is implicit in this proposal that the phosphoryl group undergoing transfer becomes covalently attached only to the oxygen atom of the acceptor, and there is evidence that no intermediate covalent enzyme complex with the phosphoryl group is formed (29,30). There is little known concerning the nature of the ligand binding at the active site with respect to either

substrates or cations. The lack of information in this area restricts a more definitive model of the catalytic mechanism of pyruvate kinase.

1. Pyruvate kinase isoenzymes

The regulation of pyruvate kinase activity is important because of its pivotal location in the glycolytic pathway. Hexokinase and phosphofructokinase, as well as pyruvate kinase, have been implicated as key regulatory enzymes in the control of glycolysis and the provision of energy to the cell (31-35). In tissues such as the liver and kidney, the glycolytic pathway can be reversed to synthesize glycogen in the process of gluconeogenesis. Because of their essentially irreversible nature, the key control enzymes are by-passed by special mechanisms during gluconeogenesis. At the level of pyruvate kinase, the reverse reaction is accomplished by the presence of the combined reactions of pyruvate carboxylase and PEP carboxykinase (equation 3).

[3]



The presence of these additional "by-pass" enzymes would result in the useless recycling of intermediates and a waste of high energy phosphate were it not for the regulation of pyruvate kinase and the other key enzymes accomplished by their unique kinetic and allosteric properties. The control of glycolysis and gluconeogenesis in mammalian systems has been reviewed by Scrutton et al. (36) and the regulation of pyruvate kinase examined in detail by Seubert and Schoner (37) and by Villar-Palasi and Larner (38).

Tanaka et al. (39), Weber et al. (40), and Krebs and Eggleston (41) observed that the level of pyruvate kinase in rat liver is under dietary and hormonal regulation, while the level of the enzyme in skeletal muscle is unaffected. Tanaka's group was able to separate electrophoretically (39), and subsequently purify and characterize two immunologically distinct forms of pyruvate kinase in the rat (42). The two forms were designated type L-(liver) and type M-(muscle) pyruvate kinase on the basis of their preponderance in those tissues. An earlier study by von Fellenberg et al. in 1963 (43), based upon the separation of pyruvate kinase on agar gel electrophoresis, described three different forms of the enzyme in rat organs. Imamura et al. (44,45) later confirmed that the M-type isozyme found in rat liver is distinct from that found in muscle and designated it as M_2-(liver type) pyruvate kinase, as opposed to M_1-(muscle type) pyruvate kinase. The occurrence of multimolecular forms of pyruvate kinase has also been observed in human tissue by Bigley et al. (46) who described three

electrophoretically distinct forms, and designated them PKI, PKII, and PKIII based upon their decreasing anodal mobility. In an attempt to avoid the confusion over nomenclature, the Subcommittee on Isozymes of the Standing Committee on Enzymes (47) recommended the designation of pyruvate kinase isozymes as PKI ("L" form of Tanaka), PKII ("M_2" form of Tanaka), and PKIII ("M_1" form of Tanaka). The system employed by Tanaka, however, has been used extensively in the literature and shall be maintained in this discussion, and clarified when possible confusion arises.

The properties of the various multimolecular forms of pyruvate kinase are complex. The isozymes which have been studied have distinct kinetic and physicochemical properties which appear to be a function of the metabolic state of the tissue or cell type from which the enzyme is examined. It has been shown that the isozymes exist as interconvertible forms (37) and many workers in the field have observed the formation of hybrids of pyruvate kinase isozymes (45, 48-51). Indeed as many as six isozymes of pyruvate kinase have been found in rat kidney extracts (52). However, from the present data it can be stated that the L-, M_1-, and M_2-isozymes occur as distinct non-interconvertible forms of pyruvate kinase in mammalian tissue (45,53,54). Table 1 summarizes and compares a number of properties of these three isozymes (43,55,56) from the data accumulated by Imamura et al. (44,45) on rat tissues.

Table 1. Summary of results on the three types of pyruvate kinases from rat tissues by Imamura et al. (45).

Type	M_1	M_2	L
Conditions when increased activity in liver observed		Regenerating liver fetal liver hepatoma	Insulin High carbohydrate diet
Distribution	Muscle, heart, brain	Kidney, spleen, lung, testis, ovary, stomach, adipose tissue, liver, small intestine, heart, brain, tumor	Liver, kidney
Molecular weight	250,000	216,000	208,000
Anti-M_1 Anti-M_2 Anti-L	Neutralized Neutralized Not neutralized	Neutralized Neutralized Not neutralized	Not neutralized Not neutralized Neutralized
S value	9.5	9.7	10.1
Hill coefficient for PEP	1.0 (Hyperbolic)	1.4-1.5 (Sigmoidal)	2.0 (Sigmoidal)
Km for PEP ($X10^{-4}$)	0.75	4.0	8.3
Activation by FDP	- (hyperbolic)	+ (hyperbolic)	+ (hyperbolic)
ATP inhibition	+	+	++

a. M_1-type pyruvate kinase

The M_1-isozyme of pyruvate kinase is believed to be confined to skeletal muscle, heart, and brain. The distribution of this form of the enzyme in primarily glycolytic tissues is consistent with its kinetic properties. The level of the isozyme is unaffected by either diet or hormones. The enzyme displays normal Michaelis-Menten kinetics with respect to both ADP and PEP with a Hill coefficient n=1, and is not affected by the potential feed-forward activator, fructose-1,6-diphosphate (FDP) (57). It has been observed that the M_1-isozyme is competitively inhibited by ATP (42, 57-59), and that this inhibition is reversed by Mg^{++} (45). The M_1-isozyme is highly active, as exhibited by a low Michaelis constant (Km) for PEP and a high specific activity (7). From these data it would appear that the enzyme would be well suited for tissues with a high glycolytic rate such as skeletal muscle, and in terms of the "energy charge" concept of Atkinson (60), the inhibition by ATP is reasonable. However, Purich and Fromm (61) have criticized this hypothesis of cellular regulation as applied to pyruvate kinase and the other key glycolytic enzymes, by pointing out that other factors such as the intracellular pH and levels of non-adenylate reaction products may play a more important regulatory role in vivo than the adenylate energy charge.

No clear concensus as to the kinetic mechanism of the M_1-isozyme of pyruvate kinase has been adopted. Reynard et al. (57) and Mildvan and Cohn (62), working on the rabbit muscle enzyme, have

concluded that the substrates bind randomly to the enzyme and that the enzyme-substrate complexes are in equilibrium with one another (equilibrium random order mechanism). Siebert et al. (63) found that the apparent Michaelis constant for PEP varies with ADP concentration, and along with Pon and Bondar (64) have argued that these results do not fit random order kinetics. Recent product inhibition studies by Ainsworth and Macfarlane (65) support a random order mechanism, and isotope exchange studies by Robinson and Rose (66) also support random order binding, but cast doubt on whether the enzyme follows rapid equilibrium kinetics.

Evidence that the kinetic properties of the M_1-isozyme cannot be interpreted by simple Michaelis-Menten kinetics was provided by Carminatti et al. (67) and by Vijavargiya et al. (68) who showed that L-phenylalanine allosterically inhibits the rabbit muscle enzyme. Kayne and Price (69) in confirming these results observed a conformational change detected by enhanced fluorescence upon L-phenylalanine binding. The implications of this inhibition have not been pursued thus far. Miller et al. (70) have examined the hypothesis that the brain damage associated with phenylketonuria may be related to the inhibition of pyruvate kinase by phenylalanine, but this study has been criticized (71) because such inhibition would be minimal at physiological pH. Kemp (71) has shown that physiological concentrations of creatine phosphate competitively inhibit M_1-pyruvate kinase and that the inhibition is more potent at high ATP levels. The

regulation of M_1-pyruvate kinase by creatine phosphate fits well with the observation that in resting muscle creatine phosphate concentrations are high enough to inhibit the enzyme, while muscle contraction leads to decreased levels of creatine phosphate, thus turning on pyruvate kinase (72,73).

b. L-type pyruvate kinase

The L-type isozyme of pyruvate kinase is a major form (90%) in liver and a minor form (30%) in kidney (74). It has been shown in rat liver (75-77) and in mouse liver (78), that the L-isozyme is present only in the parenchymal cells of these organs, while the M_2-isozyme is distributed in the non-parenchymal or Kuppfer cells. Support of a regulatory role for the L-isozyme in gluconeogenesis has been provided by Crisp and Pogson (78) who have shown that the parenchymal cell population of liver also contains the gluconeogenic enzymes glucose 6-phosphatase and fructose-1,6-diphosphatase.

The level of type L-pyruvate kinase in rats is subject to seasonal (42), dietary, and hormonal fluctuation (39-41). It has recently been shown that the fluctuation of L-pyruvate kinase in rat liver due to the injection of insulin, glucagon, or epinephrine into the portal vein is very rapid (within ten minutes), not due to de novo protein synthesis, and may be mediated by 3',5'-cyclic adenosine monophosphate (79-81). The level of the enzyme in rat livers is reduced in fasting animals, animals fed a high protein diet, or animals treated with alloxan. Under these conditions gluconeogenesis is stimulated in

the liver (36), the level of pyruvate carboxylase is increased (82), and thus by reducing pyruvate kinase levels, the conversion of pyruvate to PEP is favored without the futile recycling and waste of ATP. Injection of insulin restores the level of the type L-enzyme in alloxan diabetic rats. Animals fed a high carbohydrate diet also show marked increases in the liver of this form of pyruvate kinase. Such conditions would favor glycolysis and one might expect such increased enzyme levels to provide increased flux of metabolites through the glycolytic pathway.

Tanaka and co-workers have purified the L-type enzyme from rat liver and have shown it to be under allosteric control (42). Type L-pyruvate kinase exhibits a sigmoidal velocity curve with respect to PEP, a Hill coefficient indicating positive cooperativity, and allosteric activation by FDP (83,84). In addition the activation of this isozyme is pH dependent (85), and a number of other activators such as the monovalent cations K^+ or NH_4^+ (83) and sugar phosphates (49,50,86) have been observed to allosterically modulate type L-pyruvate kinase activity. This enzyme has also been reported to be allosterically inhibited by alanine and other amino acids (49,51), ATP (83), acetyl Coenzyme A (acetyl CoA) (87), and fatty acids (49,88). These observations have led a number of workers to propose that the allosteric model of Monod et al. (89) be applied to the L-type pyruvate kinase (83,85,90). The model of Monod et al. seeks to explain allosteric phenomena in terms of a pre-existing equilibrium between at

least two conformational states (R and T), each of which exhibits different affinities for both substrates and positive or negative affectors ("K" system of Monod). Indeed, interconvertible forms of L-type pyruvate kinase have been reported by Tanaka et al. (91), and a number of conditions such as preincubation with EDTA (84), FDP (84,92), or extraction or storage at low temperature (93) have been shown to initiate this interconversion. The two interconvertible forms differ in their kinetic properties and their response to allosteric activators. Recently Van Berkel et al. (94) and Seubert and Schoner (37) have suggested that the kinetic data are more correctly interpreted by the existence of more than two conformational states for L-type pyruvate kinase, and have proposed that sequential conformational changes take place according to the induced fit model proposed by Koshland (95) to explain cooperativity.

Because of the number and complexity of variables which can affect type L-pyruvate kinase, it is difficult to provide a clear picture of the regulation of this isozyme with respect to the changing needs of the cell. The problem has been discussed by Seubert and Schoner (37) and by Van Berkel (96). From these discussions it appears that the intracellular levels of ATP and alanine are sufficient to completely inhibit the enzyme's activity. FDP, which counteracts these negative effectors very well, may be a positive modulator of physiological significance. Sols and Marco (97), however, have proposed that because of FDP binding to other enzymes, this molecule

may not be as important as previously thought in affecting pyruvate kinase activity. Strong support for the role of intracellular pH and acetyl CoA in the regulation of the L-isozyme and the control of glycolysis and gluconeogenesis has been given by Utter et al. (87) and by Seufert et al. (98). Recently, Hjelmquist et al. (99) suggested that the activity of the L-type isozyme from pig liver may be regulated by phosphorylation-dephosphorylation. They were able to isolate and sequence a hexapeptide from a peptic digest of the enzyme in which a specific serine residue was labeled upon incubation with (^{32}P) ATP and a pig liver cyclic 3',5'-AMP-stimulated protein kinase.

Much study has been focused on the pyruvate kinase isozyme from human erythrocytes in order to understand the defect and disorders associated with the deficiency of the enzyme first described by Valentine et al. (100) in 1961. Work on erythrocyte pyruvate kinase deficiency has been concerned with electrophoretic, kinetic, and immunological studies of the mutant enzyme (55, 101-103), and with the possibility that the deficiency may be a secondary defect caused by the oxidation of pyruvate kinase thiol groups (104,105).

Initially it was thought that there was no difference between erythrocyte pyruvate kinase and type L-pyruvate kinase (46). Moreover, Bigley and Koler (106), upon examination of a liver biopsy from a patient with erythrocyte pyruvate kinase deficiency, found a decreased amount of L-type isozyme. This study has been confirmed by Miwa et al. (55) and by Imamura et al. (101). However, the same authors have

shown that the type L-isozyme is electrophoretically distinct from erythrocyte pyruvate kinase, which appears as two bands on electrophoresis (101,102,107). These two bands can be converted under special conditions to one band with a migration rate similar to type L-pyruvate kinase (101). It has been suggested that the erythrocyte enzyme may be a hybrid isozyme of the L- and M_2-type pyruvate kinase subunits (101). Kinetically, the normal erythrocyte pyruvate kinase enzyme is very similar to the allosteric type L-isozyme (108-110).

c. M_2-type pyruvate kinase

The M_2-isozyme of pyruvate kinase has been shown to be present in a number of rat (see Table 1) and human tissues (46). The M_2-isozyme is distributed in the Küpffer cell population of the kidney and liver (75,78). Though usually a minor form in liver, the M_2-isozyme is the only pyruvate kinase isozyme in rat liver cells in culture (111,112). In addition, the M_2-isozyme predominates in rat and human hepatomas (44, 113-119), regenerating liver (76,77,116,120) and in fetal tissue (44, 116-118, 121).

Kinetic studies on the M_2-isozyme (Table 1) from rat tissue show properties intermediate between the M_1- and L-isozymes. The enzyme exhibits a sigmoidal velocity curve with respect to PEP, a Km for PEP intermediate between the M_1- and L-isozymes, a Hill coefficient of 1.4-1.5, and activation by FDP. Studies on the M_2-isozyme from leucocytes (122), kidney (123), and liver (124) have led to the proposal that the allosteric model of Monod et al. (89) be

applied to explain the properties of this isozyme. Pogson (125,126) has found interconvertible forms of the M_2-isozyme in rat adipose tissue, the relative amounts of which can be varied depending upon the presence or absence of EDTA in the extraction buffer. Walker and Potter (112) have identified both of these forms and designated them as PK A and PK B, while Van Berkel et al. (127) have described their differing kinetic properties in detail. Recent studies (52,114,128) indicate that the PK A $\leftrightarrow$ PK B interconversion is a tetramer$\leftrightarrow$dimer equilibrium. The regulation of the M_2-type pyruvate kinase has been discussed in terms of the role of FDP in the PKA$\leftrightarrow$PKB interconversion by Farina et al. (114) and by Van Berkel et al. (129). Under conditions of high glycolysis, such as found in fetal, regenerating or malignant tissue, a build-up of FDP would convert the less active dimeric M_2-form to a non-allosteric highly active tetrameric form capable of meeting the higher energy requirements of these cell types.

2. Physicochemical properties

Pyruvate kinase has been isolated to varying degrees of homogeneity from a wide number of sources. More recently, the enzyme has been purified from human erythrocytes (108,130), bovine skeletal muscle and liver (131,48), Escherichia coli (132), Yoshida ascites hepatoma 130 cells (45), and pig liver (133).

a. Structure

Molecular weight studies of the purified preparations of pyruvate kinase indicate that it is a large molecule of approximately

200,000 daltons (Table 2). The rabbit muscle enzyme can be dissociated in urea or guanidine hydrochloride to yield monomers of molecular weight 57,000 (138,139). Cottam et al. (138) reported that the rabbit muscle enzyme can be dissociated at an intermediate concentration of 2M urea to yield a catalytically active dimer which sediments at 7.3S. A stable, active dimer intermediate has also been reported for the yeast enzyme (140). The dissociated species of both the yeast and rabbit muscle (141,142) pyruvate kinases have been reassociated to yield catalytically active enzymes. Recently, molecular weights of 150,000 to 200,000 and 105,000 to 125,000, respectively, have been determined for pyruvate kinase isolated from Novikoff hepatomas depending upon whether 4mM FDP is present or absent in the gel filtration elution buffer (114). The possibility and implications of a dimer$\leftrightarrow$tetramer equilibrium of pyruvate kinase subunits existing in vivo have been discussed above (127,129).

Pyruvate kinase has been crystallized from a number of sources (7,42,137,143,144). X-ray diffraction studies have been performed on the enzyme from rabbit muscle (145), pig and human muscle (146), cat muscle (137), and yeast (147). The progress of this work has been hampered by time-dependent deterioration of the crystals, but supporting evidence has been provided, at least for the cat and rabbit muscle enzymes, for a tetrameric structure composed of four highly similar subunits.

Table 2. Molecular weights reported for pyruvate kinase preparations.

Source	Molecular weight	Method
Rabbit muscle	237,000	sedimentation diffusion (134)
Saccharomyces carlsbergensis	200,000	gel filtration (21)
Saccharomyces cerevisiae	166,000	sedimentation diffusion (22)
Rat liver	250,000	sedimentation equilibrium (42)
Rat muscle	208,000	sedimentation equilibrium (42)
Ascites hepatoma	216,000	gel filtration (45)
Bovine liver	215,000	sedimentation equilibrium (48)
Bovine muscle	230,000	sedimentation equilibrium (131)
Eschericia coli	240,000	sedimentation equilibrium (132)
Human erythrocyte	225,000	sedimentation equilibrium (130)
	195,000	gel filtration (135)
	205,000	gel filtration (108)
Chicken muscle	211,000	sedimentation equilibrium (136)
Cat muscle	237,000	X-ray diffraction (137)

b. Composition

The amino acid composition for rabbit muscle (138), bovine muscle (131), Saccharomyces cerevisiae (22), Saccharomyces carlsbergensis (148), E. coli (132), and human erythrocyte pyruvate kinase (130) have been determined. The studies have revealed no covalently attached prosthetic groups or non-amino acid components. Noticeable are the low number of tyrosine, phenylalanine, and tryptophan residues present, which are reflected by the unusually low values for the extinction coefficient (22,131,138,144). No free N-terminal amino acid could be detected (130,132,138), and the work of Cottam et al. (138) on the rabbit muscle enzyme suggests that the N-terminus is acetylated. Valine has been determined as the C-terminal amino acid for the yeast enzyme (149). Peptide mapping of tryptic hydrolysates of the purified rabbit muscle enzyme have been performed (138), and reveal approximately one-fourth of the ninhydrin spots expected from total number of lysine and arginine residues obtained from the amino acid analyses. The results suggest the existence of four highly similar peptide chains in the native enzyme.

c. Active sites

Pyruvate kinase has been subjected to a number of chemical modifications in an attempt to elucidate possible reactive groups in the vicinity of the active site. Hollenberg et al. (150), using 2,4,6-trinitrobenzene-1-sulfonate to inactivate the rabbit muscle enzyme, presented evidence that lysyl epsilon-amino groups are involved in the

binding of ADP and are essential for catalytic activity. Johnson et al. (151), using pyridoxal 5-phosphate, reported a similar inactivation and suggested the formation of a Shiff base with two to four highly reactive epsilon-amino groups of lysine, though they made no inference as to their location. Evidence that sulfhydryl groups may also be essential for catalytic activity comes from inhibition by such sulfhydryl reagents as p-mercuribenzoate (42), iodoacetamide (152), and 5,5'-dithiobis-2-nitrobenzoate (153). The work of Flashner et al. (153) and Hollenberg et al. (150) suggest from the stoichiometry of reaction of the modifiers with the enzyme that there are four active sites per molecule, or one active site per subunit. In 1973 Flashner et al. (154) examined, by magnetic resonance techniques, the ability of the modified enzyme to bind divalent cations and substrates. Their results suggest that the sulfhydryl and lysine residues which are modified are both involved in the binding of nucleotides to the enzyme, and that the sulfhydryl group is closer to the divalent cation and PEP binding sites.

Studies performed to elucidate the number of PEP binding sites in pyruvate kinase have given values of 3.8 $\pm$ 0.3 for the rabbit muscle enzyme by equilibrium dialysis (20) and 4 for the bovine muscle enzyme by ultracentrifuge and gel filtration methods (132). Values of approximately 4 have also been reported for the number of divalent cation binding sites (155-157) by nuclear magnetic resonance and electron spin resonance techniques, and for the monovalent cation

binding sites by equilibrium dialysis (20).

3. Statement of thesis

It is apparent that each of the pyruvate kinase isoenzymes has distinct kinetic and regulatory properties, yet examination of the enzyme from several sources indicates a high degree of structural uniformity. The hybridization studies between pyruvate kinase isoenzymes of beef and chicken by Cardenas et al. (48,131) indicate that the subunits of these different isozymes have sufficient structural similarity to associate with one another once they have been dissociated. The immunological experiments of Imamura et al. (44) have shown that antibody prepared against rat muscle M_1-pyruvate kinase partially neutralizes rat M_2-pyruvate kinase, and indicate the presence of structurally similar determinant groups in the M_1- and M_2-isozymes. These studies, along with the observations that the M_2-isozyme is a predominant form in fetal and dedifferentiated tissue and that its properties are intermediate between the L- and M_1-isozymes, prompted Imamura et al. (45) to propose that the M_2-type pyruvate kinase represents the primordial pyruvate kinase from which the other isozymes have evolved. A mechanism for the evolution of highly complex enzyme systems by gene duplication has been presented by Ohno (158). From this discussion it seems reasonable to hypothesize that by gene duplication, followed by independent evolution, a series of pyruvate kinase isozymes having multiple loci have evolved which retain their basic structural features but have acquired divergent regulatory

mechanisms to accomodate the specialized functions of highly differentiated cell types.

The study of the pyruvate kinase isozymes has resulted in a mass of complex kinetic data. In order to obtain a clear understanding of the diverse functional characteristics of pyruvate kinase isozymes, a molecular framework must be established for their structural interrelationships. With the recent advances in the sequential analysis of proteins and the determination of their three-dimensional properties by X-ray crystallography, a structural approach to the understanding of enzyme function is entirely feasible. With respect to the human pyruvate kinase isozymes, such an effort initially requires the fractionation of these isozymes to a high degree of purity from limited amounts of starting material.

It is the purpose of this thesis to purify the human M_1- and M_2-type pyruvate kinase isoenzymes to homogeneity and determine a number of their structural and physical properties. The results will be integrated with the previous data on human pyruvate kinase isoenzymes and will provide the basis for a definitive characterization of the molecular interrelationships of these isozymes.

B. Experimental Procedure:

1. Materials

a. Tissue

Human psoas muscle was obtained at autopsy and frozen immediately at -16°C until use. Human kidney was obtained either at autopsy or through the Department of Urology at the University of Oregon Medical School when the kidneys were unsuitable for transplant. Outdated human blood was obtained from the American Red Cross.

b. Chemicals and reagents

The tricyclohexylamine salt of phosphoenolpyruvate (PEP), the sodium salt of adenosine 5'-diphosphate (ADP) grade 1 and fermentation grade, the reduced form of disodium β-nicotinamide adenine dinucleotide (NADH) grade III, the tetrasodium salt of D-fructose-1,6-diphosphate (FDP) grade II and Sigma grade, rabbit muscle lactic dehydrogenase (LDH) type II containing 1050 international units per milligram protein, bovine serum albumin, bovine trypsinogen and chymotrypsinogen, rabbit muscle aldolase and enolase, pepsin, and rabbit muscle pyruvate kinase type II were all purchased from Sigma Chemical Co., Saint Louis, Missouri. Ultra pure urea and special enzyme grade ammonium sulfate were obtained from Schwartz-Mann, Orangeburg, New York.

Sephadex G-25 medium, G-200, and Sepharose 6B were

purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. The preswollen, microgranular forms of carboxymethyl cellulose (Whatman CM52) and diethylaminoethyl cellulose (Whatman DE 52) were obtained from Reeve-Angel, Clifton, New Jersey. The dye, Cibacron blue F3GA, was obtained through the courtesy of the Dyestuffs and Chemicals Division of CIBA-Geigy, Ardsley, New York.

N,N,N',N'-tetramethylethylenediamine (TEMED), N,N'-methylene-bisacrylamide, and riboflavin were purchased from Eastman Organic Chemicals, Rochester, New York, acrylamide from Baker Chemical Co., Phillipsburg, New Jersey, and ammonium persulfate, sodium dodecyl-sulfate (SDS), and 2-mercaptoethanol from Matheson, Coleman, and Bell, Norwood, Ohio.

The amino acid standards for NH_2-terminal analysis were obtained from Calbiochem, La Jolla, California, and 5-dimethylamino-napthalene sulfonylchloride (Dansyl-Cl) from Sigma Chemical Co. Initially, #6061 Silica gel thin layer plates, from Eastman Kodak Co., Rochester, New York, were used as a support medium for thin layer chromatography (TLC). However, the method required 5 to 10 nmoles of purified protein, and the resolution of the separated dansyl amino acids was poor. Commercially prepared "Chen-Ching" polyamide layers from Gallard-Schlesinger Chem. Corp., Carle Place, New York, were finally chosen as the preferred support medium for several reasons: first, the method was sensitive to 0.1-1.0 nmoles

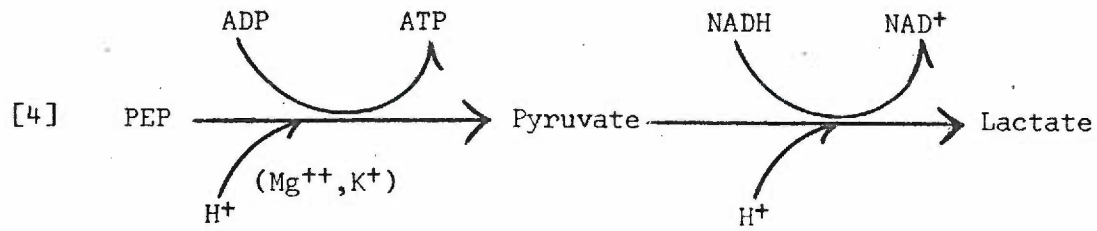
of sample; second, the plates are coated on both sides, allowing the positive identification of an unknown by a set of amino acid standards run simultaneously on the reverse side of the plate; third, the development time was under 30 minutes; finally, the method offered excellent resolution.

All other reagents and materials were of the best quality commercially available.

2. Methods

a. Pyruvate kinase assay

During the purification procedure, pyruvate kinase activity was detected by a coupled assay method developed by Bücher and Pfleiderer (159) and depicted in equation 4.



In the presence of PEP, ADP, Mg^{++}, K^{+}, NADH, and lactic dehydrogenase, pyruvate kinase activity was followed by the oxidation of NADH at 340 nm. The results were recorded for 10 minutes at 25°C on a Gilford model 2400 recording spectrophotometer in matched 1-cm path length

quartz cuvettes (Precision Cell, Inc., Hicksville, N.Y.). The standard reaction mixture had a final volume of 1 milliliter and contained 8mM triethanolamine-HCl buffer, pH 7.4, 8 mM magnesium sulfate, 75 mM potassium chloride, 0.4 mM ADP (grade 1), 0.128 mM NADH, 1.5 mM PEP, 10 units of lactic dehydrogenase, and the appropriate amount of pyruvate kinase. The reaction was initiated by the addition of the PEP solution and performed in duplicate. A unit of pyruvate kinase activity is defined as the amount of enzyme required to oxidize 1 μmole of NADH per minute, and the specific activity is expressed as units of activity per milligram of protein. The protein content was estimated during column chromatography by measuring the absorbance of the effluent fractions at 280 nm according to Layne (160) in a Beckman DB-GT spectrophotometer. This method is based upon the fact that tryptophan, tyrosine, and to a lesser extent, phenylalanine, absorb ultraviolet-light very strongly. This provides a rapid means of measuring the concentration of protein solutions. The disadvantage of the method was that it underestimated, by a factor of 1.5 to 2, the true protein content of the pure pyruvate kinase preparations because of the unusually low amounts of aromatic residues present in these isoenzymes (22,131,138,144). Therefore, when specific activities of the pyruvate kinase enzyme preparations were determined, and when other absorbing materials were present in the medium, protein was measured by the method of Lowry et al. (161) as modified by Oyama and Eagle (162), using bovine serum albumin as a standard.

The pH-stat assay for pyruvate kinase, which follows activity by the uptake of H^+ (equation 4), was used to determine the velocity profiles and kinetics of inhibition by Cibacron blue F3GA for the human M_1-, M_2-, and erythrocyte isozymes of pyruvate kinase. The assay was run at 25° and pH 7.2 on a Radiometer pH-stat. The reaction mixture had a final volume of 2.0 ml and contained 200 mM KCl, 8 mM $MgCl_2$, and varying amounts of either PEP or grade 1 ADP. For the inhibition studies, varying amounts of the dye, Cibacron blue F3GA, were included in the vessel at fixed concentrations of PEP and ADP. The reaction was initiated by the addition of 10 µl of pyruvate kinase to the vessel, and the activity was followed for 10 minutes by the uptake of 0.003 N HCl required to maintain the pH at 7.2.

b. Partial purification of human erythrocyte pyruvate kinase

The studies on human erythrocyte pyruvate kinase required a partial purification of the enzyme. Ten milliliters of either outdated blood or freshly drawn blood in heparin was applied under air pressure to a glass column (1.5x20 cm) containing white cotton yarn packed in 0.15 M KCl to a bed height of 10 cm. This procedure removes leucocytes (163), which adsorb to the yarn, and prevents the contamination of erythrocyte pyruvate kinase by the M_2-type pyruvate kinase isozyme present in the leucocytes (122). The red cells were washed three times with 0.15M KCl and then lysed with an equal volume of distilled water for 15 minutes. Three volumes of 0.15M KCl were

added and solid ammonium sulfate was added slowly with stirring at 25°C and pH 7.4 to 25% saturation (15.33 gms/100 ml). The solution was then centrifuged at 10,000xg in a Sorvall RC-2B centrifuge for 10 minutes, the precipitate discarded, and the supernatant taken to 40% saturation (8.91 gms/100 ml) with ammonium sulfate. The enzyme precipitates at this salt concentration and was stored at this stage at 4°C until used. For assays on the pH-stat the suspension was centrifuged and the precipitate was dissolved to the appropriate dilution in distilled water.

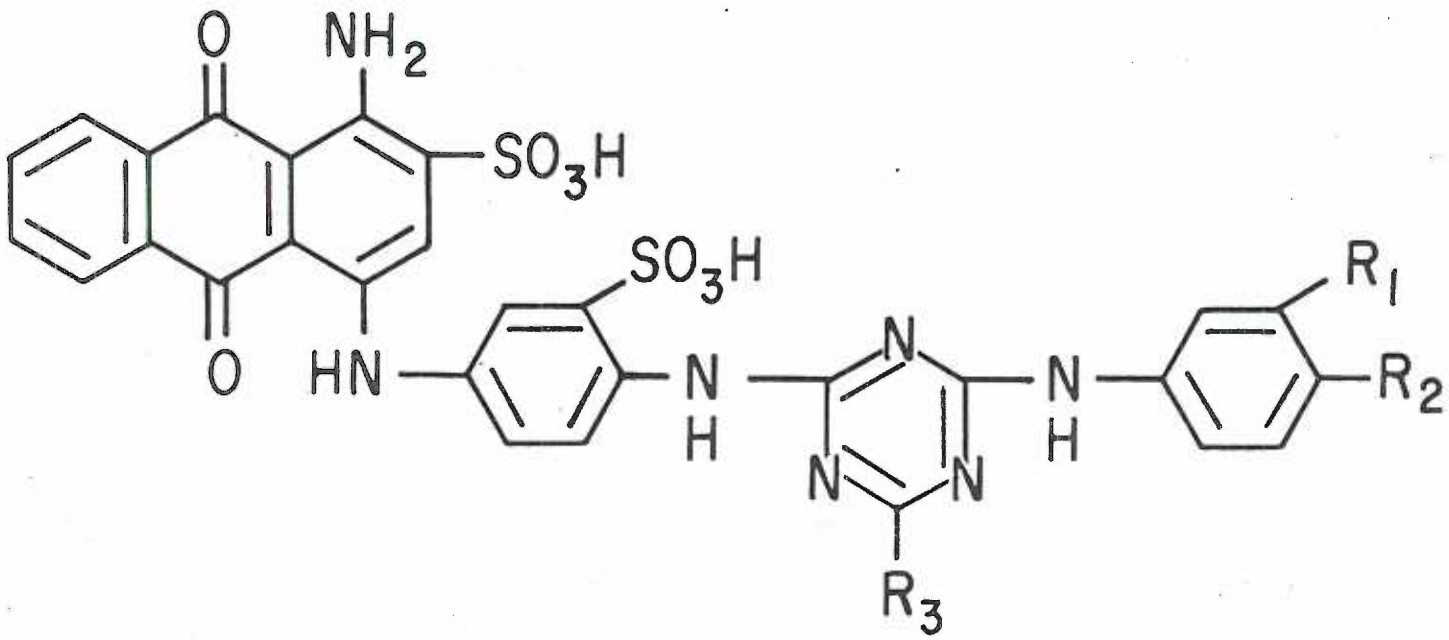
c. Preparation of Cibacron blue F3GA-Sepharose 6B resin for affinity chromatography

Cibacron blue F3GA has a 4-phenylamino-1-aminoanthroquinone structure (Figure 2). Blue Dextran 2000 is composed of this dye molecule covalently coupled to Dextran 2000 by means of cyanuric chloride. Upon obtaining a sample of the dye from CIBA-Geigy, its identification as Cibacron blue F3GA was checked by comparing its visible spectrum with Blue Dextran 2000 on a Cary 15 scanning spectrophotometer (Figure 3). Both compounds were dissolved in distilled water to approximately equal concentrations and scanned from 320 nm to 700 nm in matched 1-cm path length quartz cuvettes against a water blank.

Following the results of Easterday and Easterday (164) on the coupling of Cibacron blue F3GA to different resins, Sepharose 6B was chosen as the most suitable on the basis of its flow properties,

Figure 2.

Structural formula of Cibacron blue F3GA.



R_1 = H or SO_3H

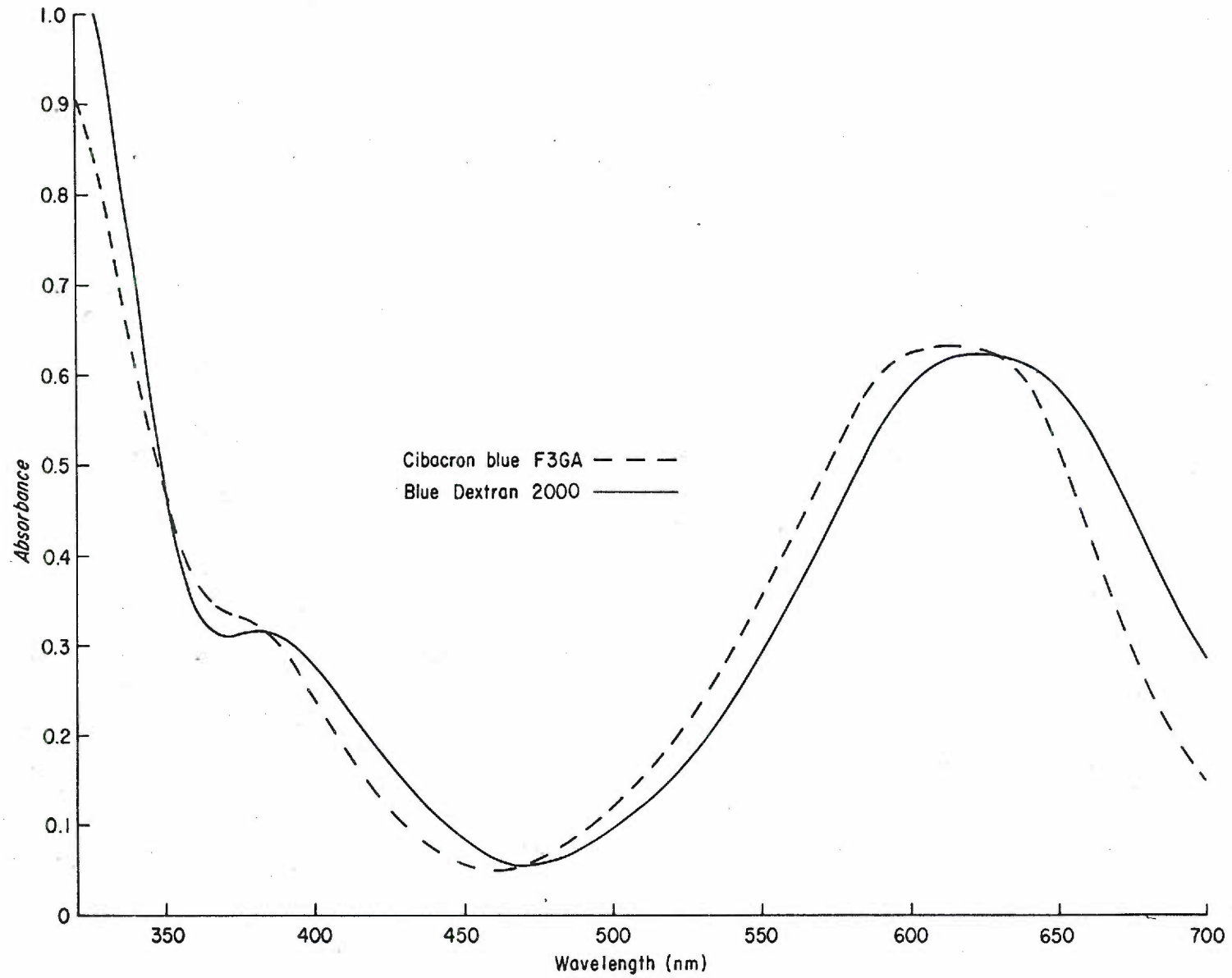
R_2 = SO_3H or H

R_3 = O-Dextran 2000 = Blue Dextran 2000

R_3 = Cl = Cibacron blue F3GA

Figure 3.

Visible spectrum of Blue Dextran 2000 and Cibacron blue F3GA. Both compounds were dissolved in distilled water to equal concentrations and scanned from 320 nm to 700 nm in matched 1 ml quartz cuvettes against a water blank.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
Cibacron blue F3GA
Blue Dextran 2000
350
400
450
500
550
600
650
700
Wavelength (nm)

narrow elution profiles, and small bed volume changes with changing ionic strength. Before coupling, Sepharose 6B was cross-linked with epichlorohydrin and desulphated by alkaline hydrolysis according to the method of Porath et al. (165). Cross-linking greatly improves the flow properties of the resin and alkaline hydrolysis removes sulfate groups on the resin which cause the non-specific binding of proteins. Two hundred milliliters of pre-swollen Sepharose 6B were mixed at 25°C with 200 ml of 1M NaOH containing 5 ml of epichlorohydrin and 1 g sodium borohydride ($NaBH_4$). The mixture was stirred for 1 hour at 60°C, and then filtered on a Buchner funnel with hot distilled water until the pH was 7.0. The gel was taken to 500 ml in distilled water and then 500 ml of 2M NaOH and 2.5 g $NaBH_4$ were added. The mixture was heated to 120°C for 1 hour in an autoclave. The Sepharose resin was then washed in a Buchner funnel with 1M NaOH containing 0.5% $NaBH_4$, first with 1.5 liters of hot solution at 70°C, then with 1.5 liters of cold solution at 4°C. The resin was transferred quickly to a beaker containing finely crushed ice and the pH was adjusted to 4.0 with 1M glacial acetic acid. The gel was then washed on a Buchner funnel with 1 liter of hot distilled water and finally with ice water.

The dye, Cibacron blue F3GA, was coupled to the Sepharose 6B resin by the method of Böhme et al. (166), in which Cibacron blue F3GA-O-Sepharose is produced by the nucleophilic attack of the hydroxyl oxygens of agarose upon the triazerine chlorine

group of the dye molecule (Figure 2), at alkaline pH. The washed, cross-linked, and desulphated resin was taken up to a volume of 300 ml in distilled water and 2 gms of the dye in 50 ml distilled water were added dropwise with stirring at 60°C. After stirring for 30 minutes, 45 gms of NaCl were added and the stirring was continued for 1 hour at 60°C. The temperature was increased to 80°C, 4 gms of Na_2CO_3 were added, and stirring was continued for 2 hours. After coupling the Sepharose resin was washed in a Buchner funnel with 1 liter of 0.02M sodium acetate buffer pH 4.5 in 2M NaCl, 1 liter of 0.02M sodium bicarbonate buffer pH 9.0 in 2M NaCl, and 2 liters of distilled water, or until the filtrate was colorless. The amount of dye coupled to the Sepharose resin was determined by pooling the filtrate and measuring its absorbance at 615 nm against a set of dye standards diluted from a 40 mg/ml stock solution (Figure 4). The molecular weight of the dye was calculated as 773 gms per mole based upon its structural formula (Figure 2). The weight of Sepharose was determined by allowing the resin to settle for 24 hours, measuring its packed volume, and multiplying this value by an agarose concentration of 6% (167). The results of the coupling of Cibacron blue F3GA to Sepharose 6B are shown in Table 3, and indicate a high degree of substitution of the dye to the Sepharose gel. The coupled Sepharose resin proved extremely stable and was stored at 4°C in distilled water containing 0.02% sodium azide to prevent bacterial contamination. Regeneration of the Sepharose involved washing with 3M KCl in

Figure 4.

Standard curve for Cibacron blue F3GA. A stock solution (40 mg/ml) of Cibacron blue F3GA in distilled water was diluted to the appropriate concentrations, and the absorbance was read at 615 nm. Each point is the average reading obtained from duplicate samples.

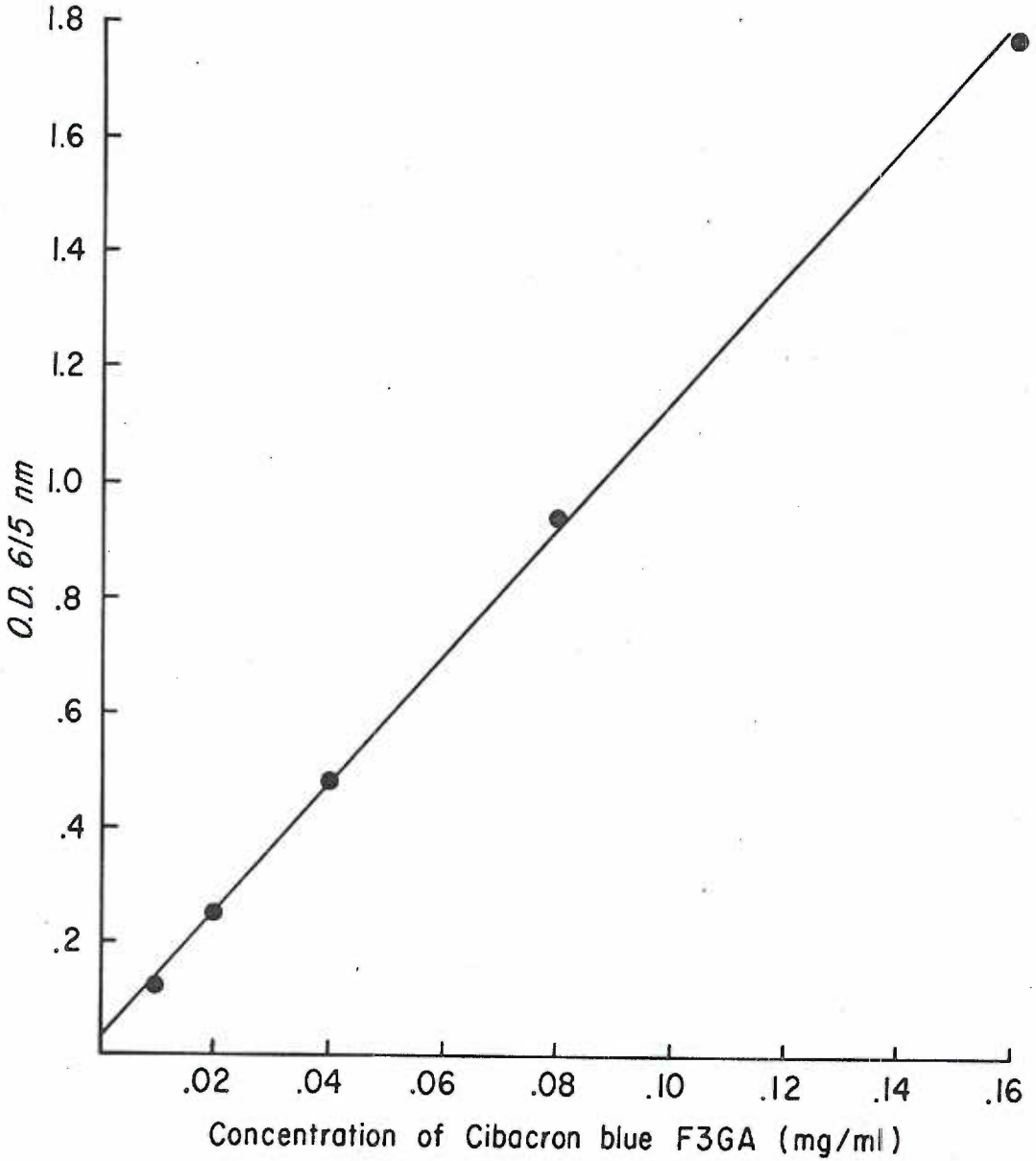

1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
O.D. 615 nm
.02
.04
.06
.08
.10
.12
.14
.16
Concentration of Cibacron blue F3GA (mg/ml)

Table 3. Statistics on coupling of Cibacron blue F3GA to Sepharose 6B (see "Methods" for coupling procedure)

1. Amount of Cibacron blue F3GA added to resin = 2.0 gms

2. Filtrate
   a) volume = 3750 ml
   b) O.D. 615 nm (1/10 dln) = 0.265 O.D.
   c) concentration [a]F3GA in filtrate = 0.22 mg/ml
   d) amount F3GA not coupled = 785 mg

3. Amount of F3GA coupled to resin = 1.215 gms = 61% binding

4. Coupled Sepharose 6B resin
   a. packed volume = 200 ml
   b. weight agarose = 12 gms

5. Dye substitution = 1.215 gms F3GA/12 gms resin

   = 7.86 µM F3GA bound/ml resin
   = 131 µM F3GA bound/gm resin

[a]The dye, Cibacron blue F3GA, is abbreviated as "F3GA"

distilled water, followed by the washing procedure outlined above.

d. Criteria of homogeneity and characterization studies

A number of methods were employed to show that the human M_1- and M_2-pyruvate kinase isozyme preparations were homogeneous. The results of one method, taken by itself, provided evidence of purity only with respect to the inherent limitations of the method. For example, the presence of a single band obtained from polyacrylamide disc gel electrophoresis provided evidence that the sample was homogeneous with respect to size and charge. However, the reliability of this evidence depended upon the running pH of the electrophoresis system, the percent of cross-linking of the polyacrylamide gel, the sensitivity of the staining procedure, and the possibility that the results merely reflected coincidental properties of multiple components. Therefore, the purified pyruvate kinase preparations were examined for purity not only by disc gel electrophoresis, but also by disc gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), ultracentrifugation, NH_2-terminal analysis, and, in the case of the human M_1-isozyme, by immunological methods. The final purity of the enzyme preparations was determined by considering the results of all of these criteria.

Polyacrylamide gel electrophoresis

To determine the homogeneity of the purified enzyme preparations, polyacrylamide disc gel electrophoresis was performed with a Buchler disc gel apparatus according to the method provided

by Buchler Instruments (168). The anionic system at a running pH of 9.3 was adopted with a 7.5% polyacrylamide running gel and a 2.5% polyacrylamide spacer gel. To prevent the aggregation of proteins by the oxidation of thiol groups, 0.1% 2-mercaptoethanol was added as a reducing agent to the upper and lower buffers. To stabilize the M_2-pyruvate kinase isozyme 0.1 mM FDP (grade II) was also included in the buffers, along with 0.4 mM FDP in the gel buffers. Samples were dialyzed for 2 hours against upper buffer, and then 20 µl of glycerol, to increase sample density, and 30 µl of tracking dye, 0.001% Bromphenol blue, were added to 100 µl of each sample. Before sample application the gels were pre-run at 1.25 ma per gel for 15 minutes to remove any residual ammonium persulfate which might cause oxidation of the sample. The samples were then carefully added to the gels with a micro-pipette and electrophoresis was performed at 2.5 ma per gel until the tracking dye had reached the bottom of the gel. The gels were removed, stained in 0.1% Amido Schwartz in 7% glacial acetic acid, and de-stained and stored in 7% glacial acetic acid. The migration of pyruvate kinase in the gels could be followed by running duplicate samples and following the above staining and destaining procedure with one gel. The other gel was sliced into 4 mm sections immediately after electrophoresis, incubated in 500 µl of upper gel buffer for 30 minutes, and then assayed for pyruvate kinase activity.

Disc gel electrophoresis in the presence of 0.1% sodium

dodecyl sulfate (SDS) and 0.1% 2-mercaptoethanol was performed according to Weber and Osborn (169). SDS is an anionic detergent which has been shown to bind to proteins and confer upon them a negatively charged surface. Oligomeric proteins dissociate into their constituent subunits under these conditions and the migration of these subunits in polyacrylamide gel electrophoresis in the presence of SDS is dependent upon their molecular weight. Purified samples of human pyruvate kinase M_1- and M_2-isozymes and protein standards were dialyzed for 2 hours against incubation buffer consisting of 0.01M sodium phosphate buffer, pH 7.0, 1% SDS, and 1% 2-mercaptoethanol, and then incubated for 1 hour at 37°C. The samples were applied to 10% polyacrylamide gels containing 0.1% SDS and were run for 4 hours at 8 ma per gel. Following the prescribed staining and destaining procedure (169), the mobilities of the protein standards relative to the tracking dye were plotted against the logarithm of their known subunit molecular weights. The subunit molecular weights for the pyruvate kinase isoenzymes were obtained by interpolation of the straight line obtained from the protein standards.

Horizontal thin layer polyacrylamide electrophoresis was carried out at pH 8.2 by a modification of the method of Imamura and Tanaka (44). The gel buffer contained 10 mM Tris-HCl, pH 8.2, 5 mM $MgSO_4$, 0.5 mM FDP (grade II), and 10 mM 2-mercaptoethanol. Samples were dialyzed for 2 hours against gel buffer, and 5 µl

aliquots were put into the slots and covered with liquified vaseline. Electrophoresis was performed for 3-1/2 hours at 440 volts and 4°C. After the run, pyruvate kinase activity was visualized by a modification of the overlay method of Susor and Rutter (186). The reaction mixture contained 0.17M Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 3 mM KCl, 0.5 mM PEP, 0.8 mM ADP (grade 1), 0.2 mM NADH, 4.8 units of lactic dehydrogenase, and 5 mg of Noble agar per milliliter. Pyruvate kinase activity appeared as dark bands on a fluorescent background when illuminated with ultraviolet light. The results were recorded by Polaroid photography.

Ultracentrifugation

To determine the homogeneity and the sedimentation coefficients of the purified proteins, sedimentation velocity studies were performed using a Spinco model E analytical ultracentrifuge equipped with Schlieren optics and run at a speed of 59,780 rpm. During the run, the protein sediments down the cell and a migrating concentration gradient is formed. This region of change in concentration with distance is proportional to the change in refractive index with distance, and when viewed through Schlieren optics, the gradient appears as a migrating peak. A pure preparation will be observed to sediment as a single symmetrical peak. The sedimentation coefficient is determined by measuring the migration of the peak from the center of the rotor with time. The partial specific volume used in the calculations was obtained from the amino acid

composition of the enzyme by the method of Cohn and Edsall (170). The sedimentation coefficient was expressed as the $s_{20,w}$, or sedimentation coefficient in water at 20°C. Before the run the samples were dialyzed for 24 hours against the sample buffer, 0.1M Miller's buffer pH 7.2 (171), with or without 0.1 mM FDP. The viscosity and density of the sample buffer for the M_1-isozyme had been determined previously in the laboratory of Dr. D. Rigas, University of Oregon Medical School. For the M_2-isozyme, 0.1 mM FDP (Sigma grade) was added to the buffer, and new density and viscosity measurements were made.

Sedimentation equilibrium studies were performed to provide additional evidence for the homogeneity of the purified enzymes and to obtain an accurate determination of their molecular weight. These experiments were performed according to the low speed method as described by Chervenka (172). The model E ultracentrifuge was equipped with Rayleigh interference optics and run at 8225 rpm. The samples were prepared in the same manner as for the sedimentation velocity studies. During the run, equilibrium was reached within 72 hours, at which time a photograph of the interference pattern was taken. The deflection of the fringes from the meniscus toward the bottom of the cell was measured at several points and the difference between these values was defined as ΔY. A linear plot of log ΔY versus r^2 (r = the distance from the rotor center to the point where ΔY was measured) indicated a homogeneous solute.

The slope of the line was obtained by the least squares method from which the molecular weight of the enzyme was calculated according to DiCamelli et al. (173).

Immunodiffusion

Rabbit antiserum against purified human erythrocyte pyruvate kinase was prepared by Dr. C. J. Chern, in this laboratory, according to the method of Chern et al. (130). Using a similar procedure antisera against purified human muscle pyruvate kinase and against an impure preparation of the M_1-isozyme were prepared.[1] A complex of the enzyme preparations with bovine serum albumin was formed according to Plescia et al. (174), mixed with an equal volume of complete Freund's adjuvant, and injected subcutaneously into the rabbits. Antisera, obtained from ear bleedings of the rabbits, were prepared according to the method of Campbell et al. (175). By immunodiffusion techniques, the purity of the M_1-isozyme preparation was determined. Also the immunoreactivity of the human M_1- and M_2-pyruvate kinase isozymes was tested by immunodiffusion against antisera prepared against human M_1-pyruvate kinase and antisera prepared against human erythrocyte pyruvate kinase.

[1]Antiserum prepared against an impure preparation of human M_1-pyruvate kinase was prepared using a muscle pyruvate kinase extract fractionated by Dr. Robert Bigley, Division of Medical Genetics, Univ. of Oregon Medical School (see "Appendix" for method).

NH_2-terminal analysis

The purified enzymes were analyzed for their NH_2-terminal amino acid(s) using 1-dimethylaminonaphthalene-5-sulfonyl chloride, (Dansyl-Cl), which forms a highly fluorescent sulfonamide upon reacting with the free amino groups of proteins. In addition to the NH_2-terminus and the epsilon amino group of lysine, Dansyl-Cl also reacts with the imidazole group of histidine, the sulfhydryl group of cysteine, and the phenolic hydroxyl group of tyrosine. Ten nmoles of histidine, tyrosine, lysine, and aspartic acid were dansylated according to Woods and Wang (176). Ten nmoles of bovine serum albumin and the purified preparations of the M_1- and M_2-pyruvate kinase isozymes were dansylated in the presence of 8M urea by the method of Gros and Laboyesse (177). The presence of urea insured the complete unfolding of the polypeptide chains and exposure of the NH_2-terminal region. After dansylation the proteins were hydrolyzed under a vacuum in 6N HCl for 12 hours at 110°C. The acid was removed on a Buchler rotary evaporator and the samples were examined by two-dimensional thin layer chromatography on 5 x 5 cm polyamide sheets coated on both sides. The dansylated protein samples were dissolved in 10 µl of acetone-glacial acetic acid (3:2) and applied to one side of the plate with a 1 µl micropipette in a spot 1 mm in diameter at the corner of a 1 x 1 cm border. On the reverse side of the plate a mixture of the dansylated amino acid standards was applied in a similar fashion. The plates were developed

in 150 ml beakers sealed with Parafilm^{R} with water-90% formic acid (100:1.5) in the first dimension, and benzene-glacial acetic acid (9:1) in the second dimension. The separated dansyl amino acids were visualized by ultraviolet irradiation, and identified by comparison with the migration of the dansylated standards on the reverse side of the plates.

Amino acid analysis

Samples of the purified enzyme were dialyzed for 24 hours against distilled water to remove excess ammonium sulfate, and an equal volume of concentrated HCl was added. The samples were divided into three equal portions and hydrolyzed in sealed evacuated ampoules at 110°C for 24, 48, and 72 hours. The acid was removed on a Buchler rotary evaporator and the amino acid content of the hydrolysates was determined in duplicate on a Beckman 120C amino acid analyzer (178). Half-cystine was determined as cysteic acid and methionine as methionine sulfone following performic acid oxidation according to Moore (179). Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton (180). The values for serine and threonine were obtained by extrapolation to zero time, and the values of valine and isoleucine at 72 hours were used. A mixture of 0.03 μmoles of amino acid standards was run before each of the samples was analyzed in order to calibrate the machine. The chromatogram peak areas were calculated by manual integration, whereby the net height of the peak in optical density units was multiplied by

the width at half the height. The width at half the height was determined by counting the printed dots on the chromatogram that occurred above the half-height line, using interpolation if necessary. By comparing the peak areas of the sample chromatograms with those of the 0.03 μmolar amino acid standards, the number of μmoles of each residue was determined. The number of residues per mole of enzyme was calculated on the basis of the molecular weight of the enzyme obtained from the sedimentation equilibrium studies.

C. Results

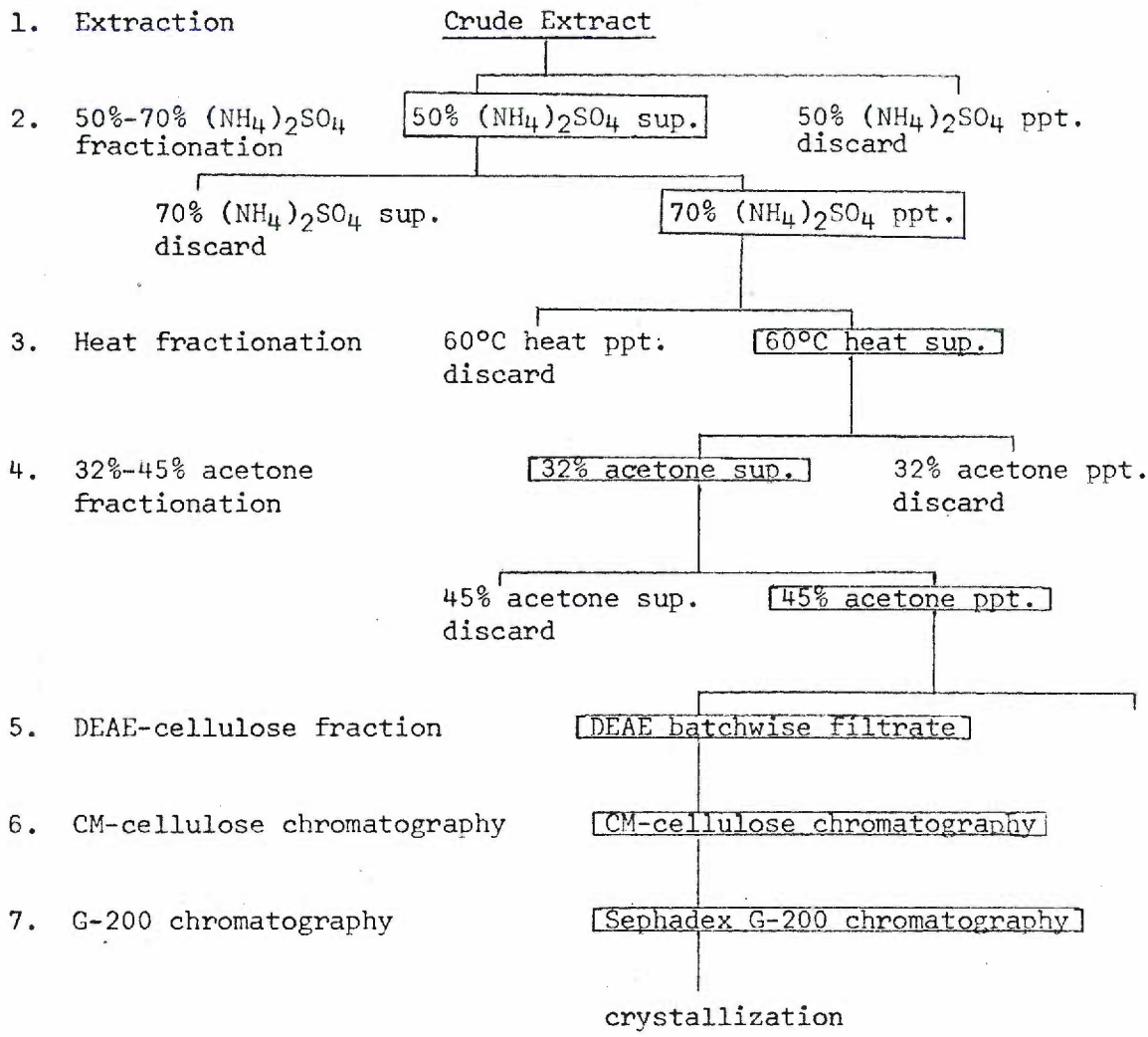
1. Purification of human M_1-type pyruvate kinase

In 1944 Kubowitz and Ott (144) published a procedure for the isolation of pyruvate kinase from human muscle. The procedure involved the extraction of human thigh muscle with distilled water, 67% acetone precipitation, fractional precipitation with nucleic acid, heat fractionation at 60°C, the removal of nucleic acid by protamine sulfate, 56.5% acetone precipitation, crystallization at pH 5.1, washing the crystals with 22.5% saturated ammonium sulfate in cold distilled water, and recrystallization. Though the preparation could be easily crystallized, the authors did not report any attempt to determine the purity of the enzyme preparation. When examined in this laboratory, this procedure did not result in a homogeneous enzyme product as determined by disc gel electrophoresis. The procedure which was finally adopted involved the application of additional steps to remove the contamination (Table 4). All the steps of purification were carried out either in the cold room at 4°C or with the enzyme solution on ice. The preparation could be stored between the fractionation steps at 4°C as a 75% saturated ammonium sulfate suspension without any significant loss of activity.

a. Steps 1-4: extraction of M_1-pyruvate kinase

Frozen human psoas muscle, obtained at autopsy, was thawed and the adipose and connective tissue were removed. Five hundred grams of the tissue were ground and distilled water was

Table 4. Fractionation scheme for human M_1-pyruvate kinase (see "Results" for details)



The enclosed fractions are those which contained the M_1-pyruvate kinase isozyme.

added to a volume equal to twice the weight of the muscle. The extract was stirred for 2 hours at 4°C, and then centrifuged for 30 minutes at 10,000 xg in a Sorvall RC-2B centrifuge equipped with a GSA-3 rotor. The supernatant was filtered through two layers of cheesecloth, placed in an ice bath, and solid ammonium sulfate was added slowly with stirring to 50% saturation following the nomogram of Dawson et al. (181). The pH of the solution was maintained at 7.0 by the addition of 1 N NaOH. After all the salt had been added, the solution was stirred for 15 minutes, centrifuged for 15 minutes at 10,000 xg, and the precipitate was discarded. The supernatant was placed in an ice bath and taken to 70% ammonium sulfate saturation by the same procedure as above, and then centrifuged.

The 70% ammonium sulfate precipitate was dissolved in 50 mM triethanolamine-hydrochloric acid buffer pH 7.0 with 0.1% 2-mercaptoethanol to a protein concentration of 20 mg/ml (determined by the absorbance at 280 nm), and heated to 60°C on a hot plate with stirring for 10 minutes. The solution was then rapidly cooled to 4°C in an ice bath, centrifuged for 15 minutes at 10,000 xg, and the precipitate discarded. The supernatant was placed in an ice bath. Cold acetone, at -10°C, was added dropwise with stirring to 32% by volume. After 15 minutes, the solution was centrifuged, the precipitate discarded, and the 32% acetone supernatant was taken to 45% acetone by volume in the same manner.

b. Step 5: DEAE-cellulose batchwise fractionation of M_1-pyruvate kinase

The 45% acetone solution of step 4 was centrifuged and the precipitate was dissolved in 25 ml of 3 mM potassium phosphate buffer pH 7.4 containing 0.1% 2-mercaptoethanol. The sample was dialyzed against the same buffer until the pH and conductivity were identical to the dialysis buffer. During this time 50 gms of DEAE-cellulose (Whatman DE52) were equilibrated with dialysis buffer. The cellulose was titrated to pH 7.4 and resuspended several times to remove fines. After full equilibration had been reached, the dialyzed sample was mixed with the slurry and stirred for 30 minutes at 4°C. The mixture was filtered on a Buchner funnel and washed with 300 ml of the 3 mM potassium phosphate buffer. The filtrate was pooled, assayed for protein and pyruvate kinase, and stored at 4°C as a 75% ammonium sulfate suspension.

c. Step 6: CM-cellulose chromatography of M_1-pyruvate kinase

The 75% ammonium sulfate suspension from the previous step was centrifuged, and the precipitate was dissolved in 20 ml of CM-cellulose column buffer, consisting of 50 mM potassium phosphate, pH 5.5, with 0.1% 2-mercaptoethanol. The sample was desalted on a 2.5 x 100 cm Sephadex G-25 column equilibrated with CM-cellulose column buffer, and applied to a 1.5 x 30 cm column of CM-cellulose (Whatman CM52) packed to a bed height of 20 cm and fully equilibrated

with column buffer. The column was washed with 25 ml of column buffer at a flow rate of 35 ml per hour, and then the enzyme was eluted by the application of a linear gradient, consisting of 100 ml of 50 mM potassium phosphate pH 5.5 with 0.1% 2-mercaptoethanol and 100 ml of 200 mM potassium phosphate pH 5.5 with 0.1% 2-mercaptoethanol. Five milliliter fractions were collected and assayed for protein and pyruvate kinase (Figure 5). The potassium phosphate gradient was measured at 25°C with a Radiometer model CDM2d conductivity meter. Fractions 26 through 30 were pooled, assayed, and concentrated to a volume of 2 ml by vacuum dialysis.

d. Step 7: Sephadex G-200 chromatography of M_1-pyruvate kinase

The 2 ml sample from the previous step was applied to a 1.5 x 100 cm column of Sephadex G-200 equilibrated with 50 mM triethanolamine-HCl buffer pH 7.4 with 0.1% 2-mercaptoethanol. The column was developed by gravity flow with a hydrostatic pressure of 12 cm. Fractions of 2.5 ml volume were collected and assayed for protein and enzyme activity (Figure 6). Fractions 24-30 were pooled, assayed, and stored at 4°C as a 75% ammonium sulfate suspension. This preparation was used for the subsequent characterization studies.

e. Crystallization of M_1-pyruvate kinase

Crystals of human M_1-pyruvate kinase were grown by dissolving a portion of the enzyme preparation from the previous

Figure 5

CM-cellulose chromatography of human M_1-pyruvate kinase. The enzyme product of step 5 was desalted, equilibrated with 50 mM potassium phosphate, pH 5.5, with 0.1% 2-mercaptoethanol, and applied at a flow rate of 35 ml per hour to a 1.5 x 30 cm CM-cellulose column (bed height = 20 cm). The column was washed with 25 ml column buffer and then developed with a linear potassium phosphate gradient, consisting of 100 ml of column buffer at 50 mM potassium phosphate and 100 ml of column buffer at 200 mM potassium phosphate. Five milliliter fractions were collected and assayed for pyruvate kinase (O----O), optical density at 280 nm (●——●), and conductivity (▲——▲).

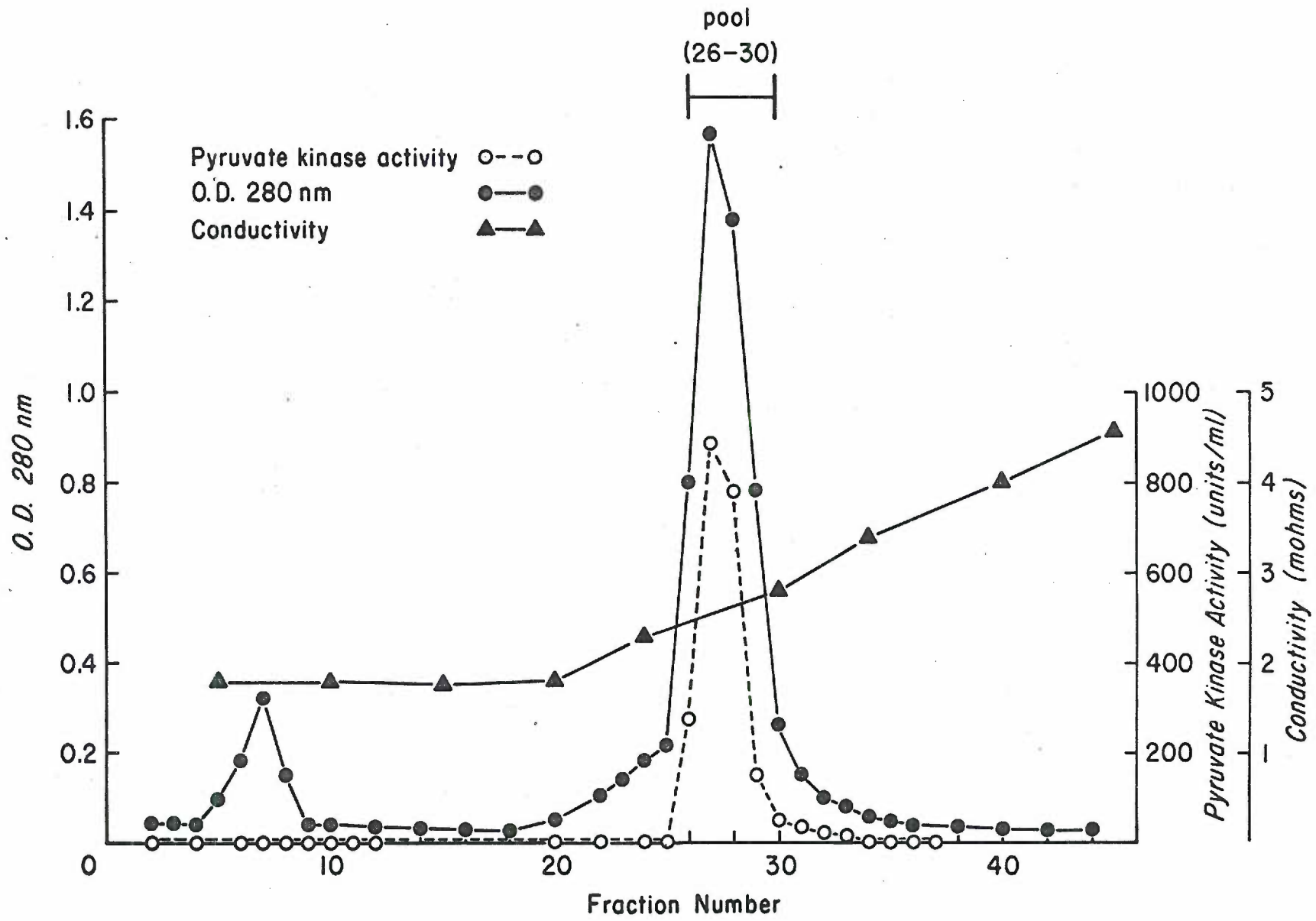
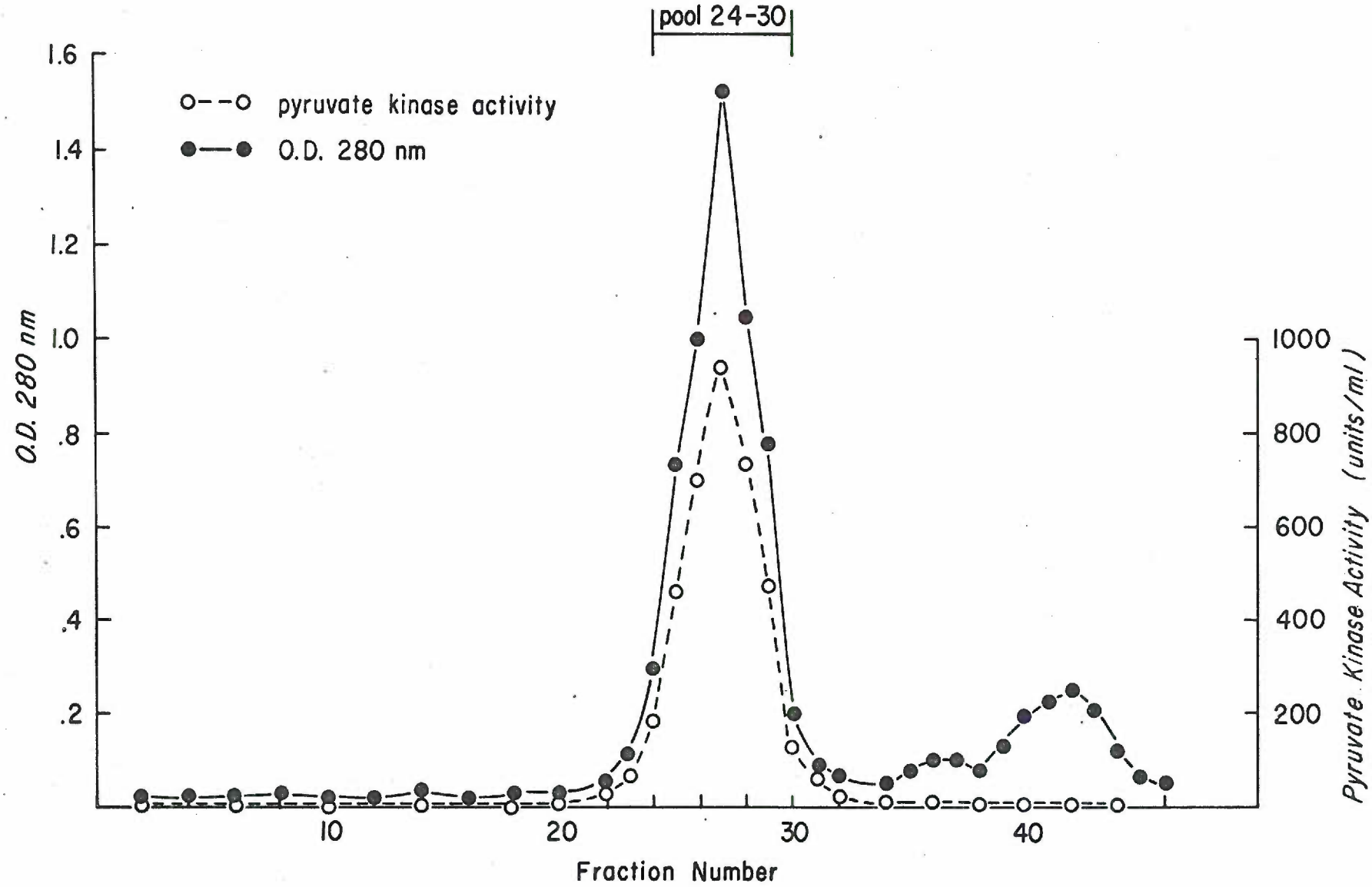

pool
(26–30)
Pyruvate kinase activity
O.D. 280 nm
Conductivity
O. D. 280 nm
Pyruvate Kinase Activity (units/ml)
Conductivity (mohms)
Fraction Number
0
10
20
30
40
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
200
400
600
800
1000
1
2
3
4
5

Figure 6

Sephadex G-200 chromatography of human M_1-pyruvate kinase. The product from step 6 of the purification was concentrated by vacuum dialysis and applied at a flow rate of 3 ml per hour to a 1.5 x 100 cm column of Sephadex G-200, equilibrated with 50 mM triethanalamine-HCl, pH 7.4., with 0.1% 2-mercaptoethanol. Fractions of 2.5 ml were assayed for pyruvate kinase (O----O) and for absorbance at 280 nm (●——●).


pool 24-30
pyruvate kinase activity
O.D. 280 nm
O.D. 280 nm
Pyruvate Kinase Activity (units/ml)
Fraction Number
1.6
1.4
1.2
1.0
.8
.6
.4
.2
1000
800
600
400
200
10
20
30
40

purification step to a concentration of 10 mg/ml (measured by Lowry-phenol method). The pH was adjusted to 5.1 with 1M sodium acetate buffer, pH 4.0, and ammonium sulfate was added slowly to 50% saturation. The solution was placed in a wetted dialysis bag and suspended at 4°C. Crystallization occurred within 12 hours. A photomicrograph of the crystalline suspension was taken at a 787-fold magnification (Figure 7), under which the crystals appeared orthorhombic in nature.

f. Summary of M_1-pyruvate kinase purification

The overall purification for the human M_1-pyruvate kinase isozyme is summarized in Table 5. The final product had a specific activity of 382.5 units per mg protein and represented a 294-fold purification with an over-all yield of 10.8%.

g. Homogeneity and characterization studies of human M_1-pyruvate kinase

Disc gel electrophoresis

Disc gel polyacrylamide electrophoresis was performed on the purified human M_1-pyruvate kinase preparation from step 7. The results are shown in Figure 8. Gel 1 shows the components present in the crude extract, step 1. Gel 2 shows the results of purification after step 4, the 32%-45% acetone fractionation. Steps 1, 2, 3, and 4 were adopted from the purification of Kubowitz and Ott (144), after which the enzyme preparation could be crystallized. However, the results of gel 2 show two strong bands along with other

Figure 7

Photomicrograph of human M_1-pyruvate kinase crystals. Crystals were grown (see "Results" for method), placed on a microscope slide with a Pasteur pipette, and photographed at a magnification of 787.5.

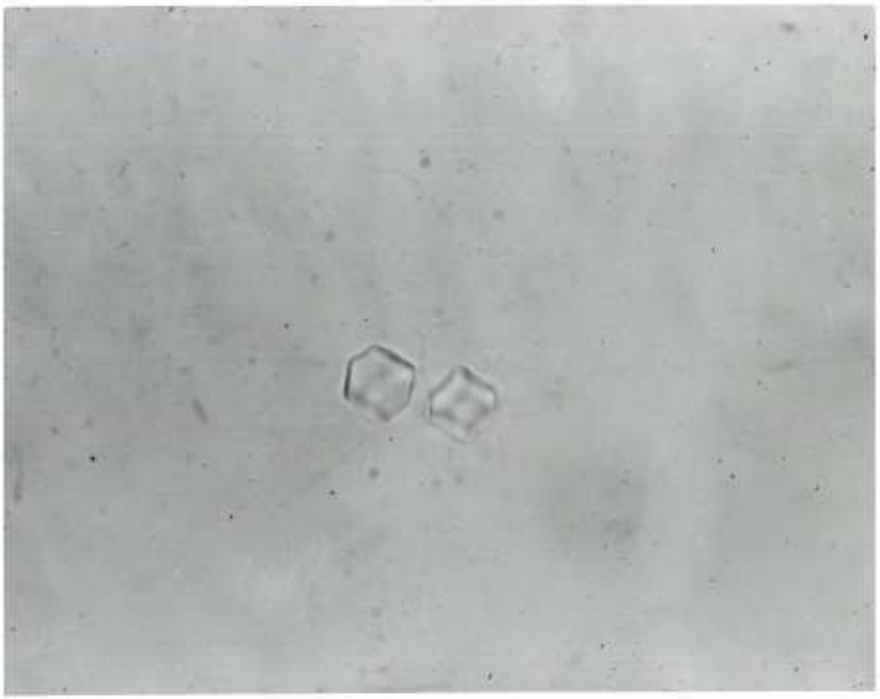


Table 5. Purification of human M_1-pyruvate kinase

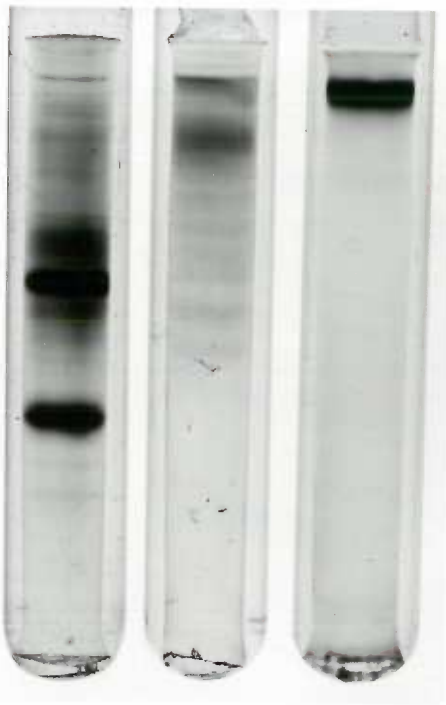
Purification Step	Total Units[a]	Yield (%)	Total Protein (mg)	Specific Activity (units/mg protein)[b]	Purification
1. Crude extract	83,615	100	64,312	1.3	1
2. 50-70% Ammonium sulfate precipitate	51,261	61.3	13,780	3.7	2.8
3. 60°C heat supernatant	44,670	53.4	3667	12.2	9.4
4. 32-45% acetone precipitate	27,521	32.9	398	69.2	53.2
5. DEAE-cellulose, batchwise	13,517	16.2	87	155.4	119.5
6. CM-cellulose chromatography	10,538	12.6	35.3	298.5	229.6
7. G-200 chromatography	9065	10.8	23.7	382.5	294.2

[a]A unit of pyruvate kinase activity is defined as the amount of enzyme required to oxidize 1 μmole NADH per minute under the conditions for the coupled-assay conditions given under "Methods".

[b]Specific activity is defined as units per mg protein, where protein is measured by the modified Lowry-phenol method of Oyama and Eagle (162).

Figure 8

Polyacrylamide disc gel electrophoresis of human M_1-pyruvate kinase preparations. Gel 1, a crude extract of M_1-pyruvate kinase from step 1; gel 2, the product of step 4; gel 3, the product of step 7.



1 2 3

minor contamination. Therefore, further purification procedures (steps 5,6,7) were employed to remove the residual contaminants. Gel 3 shows the product of step 7, and indicates, by the single major protein band, that the enzyme preparation is homogeneous with respect to this method. Duplicate gels of the preparation from step 7 were run, and one was sliced (see "Methods") to determine whether the single band seen in Figure 8, gel 3, had pyruvate kinase activity. The results are shown in Figure 9 and confirm the identity of the band as pyruvate kinase.

SDS gel electrophoresis

To determine the subunit molecular weight, and to provide additional evidence of homogeneity, polyacrylamide disc gel electrophoresis was performed on the M_1-pyruvate kinase preparation in the presence of 0.1% SDS. Figure 10 shows a photograph of the results of SDS gel electrophoresis on the M_1-isozyme and on four protein standards which were run simultaneously. From left to right the gels are: gel 1, bovine serum albumin; gel 2, human M_1-pyruvate kinase; gel 3, aldolase; gel 4, chymotrypsinogen; gel 5, ribonuclease. Gel 2, human M_1-pyruvate kinase, contains a single band and indicates that the preparation is homogeneous, and that the subunits of the M_1-isoenzyme have extremely similar or identical molecular weights. The subunit molecular weight of the M_1-pyruvate kinase isoenzyme was estimated by plotting the mobility of the protein standards versus the logarithm of their subunit molecular weights (Figure 11). Each standard

Figure 9

Disc gel electrophoresis and assay of purified human M_1-pyruvate kinase. Disc gel electrophoresis was performed in duplicate on the product of step 7. Immediately after electrophoresis, one gel was stained (photo), and the other was sliced into 4 mm sections, incubated, and assayed for pyruvate kinase (see "Methods"). Activity is expressed as units/ml.

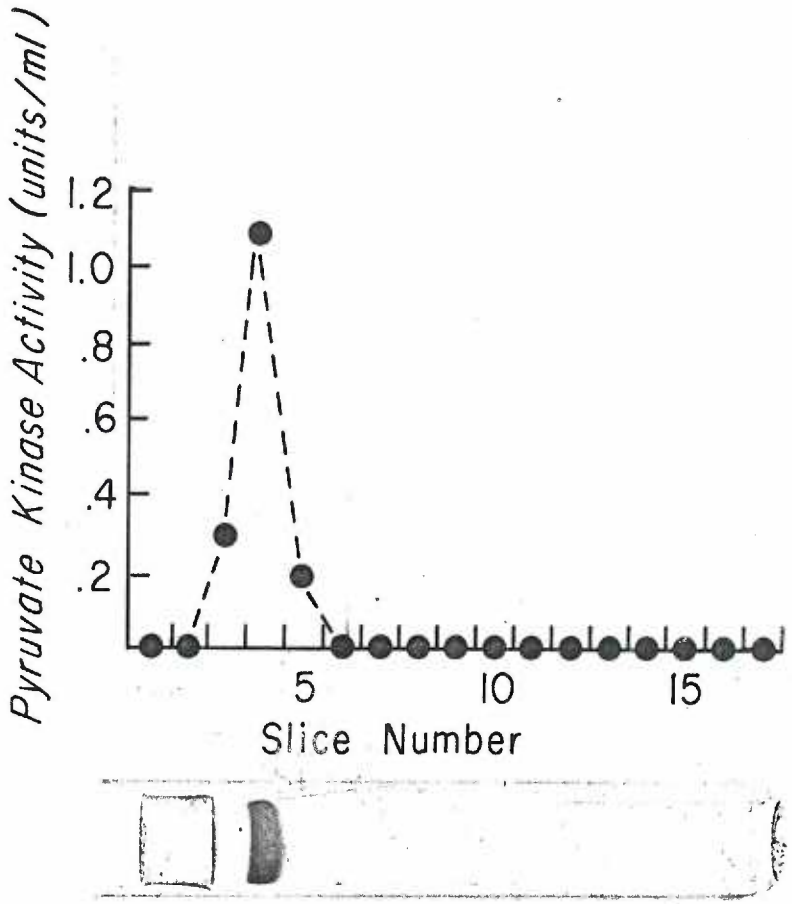
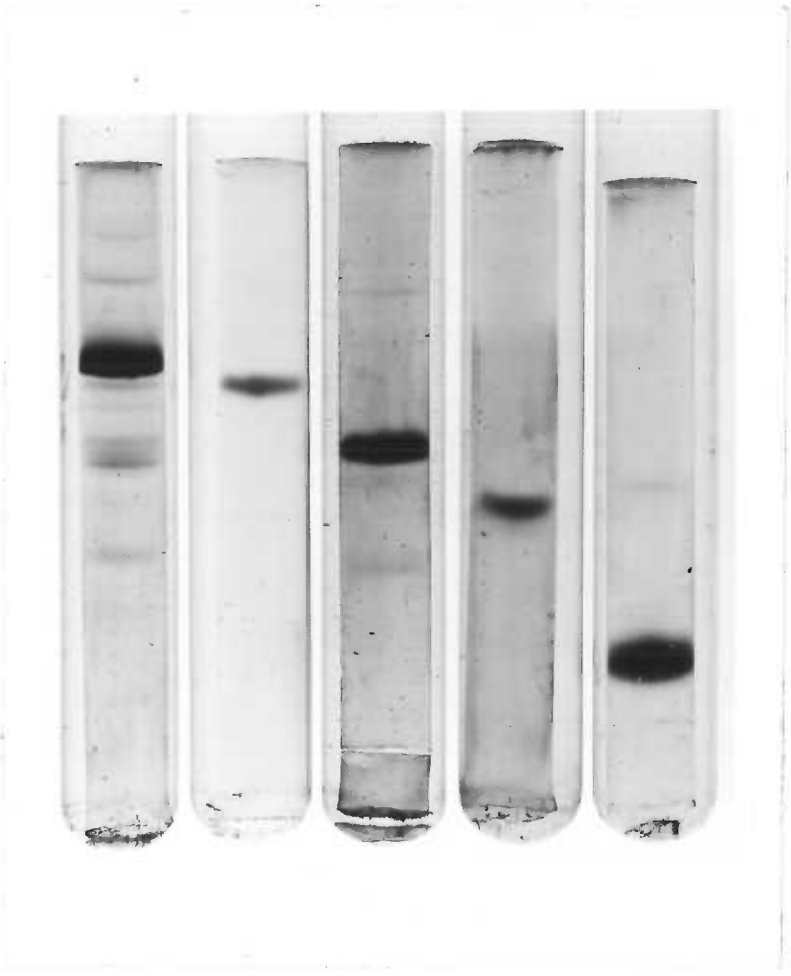

Pyruvate Kinase Activity (units/ml)
1.2
1.0
.8
.6
.4
.2
5
10
15
Slice Number

Figure 10

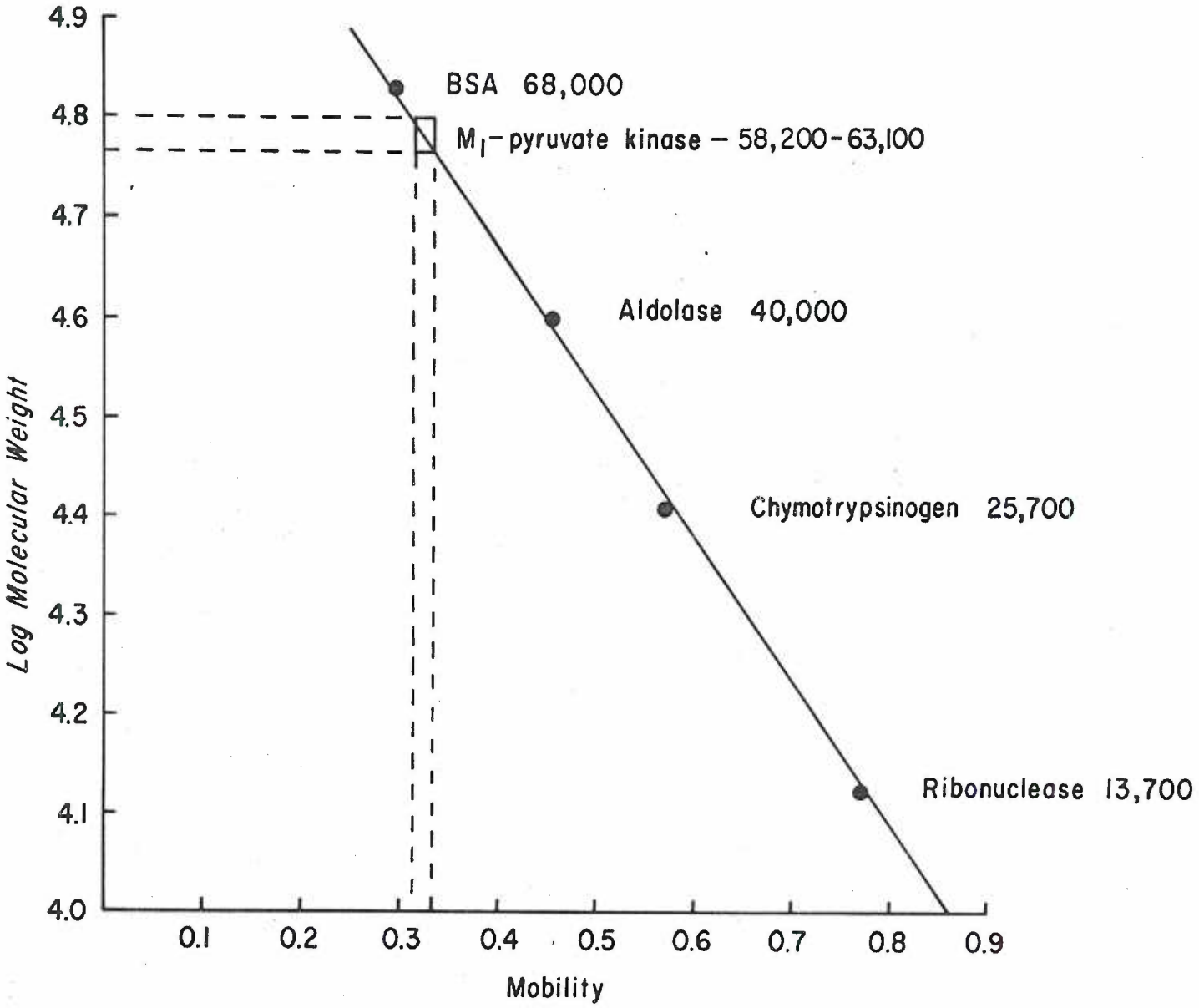
SDS gel electrophoresis of human M_1-pyruvate kinase and protein standards. Gel 1, bovine serum albumin; gel 2, M_1-pyruvate kinase; gel 3, aldolase; gel 4, chymotrypsinogen; gel 5, ribonuclease.



1 2 3 4 5

Figure 11

Subunit molecular weight determination of human M_1-pyruvate kinase. The mobility of the protein standards relative to the tracking dye from the SDS gel electrophoresis run is plotted versus the log of their subunit molecular weights. Each point is the average value for duplicate gels. The line was drawn by the method of least squares.


4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4.0
Log Molecular Weight
BSA 68,000
M1 – pyruvate kinase – 58,200-63,100
Aldolase 40,000
Chymotrypsinogen 25,700
Ribonuclease 13,700
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Mobility

was run in duplicate, and the points represent an average mobility. A line was drawn through the points by the method of least squares. Two gels containing the M_1-isozyme were run and they exhibited mobilities of 0.315 and 0.330 relative to the tracking dye. By interpolation of the line through the protein standards, this corresponds to an average subunit molecular weight of 61,000 ± 2,000.

Sedimentation velocity

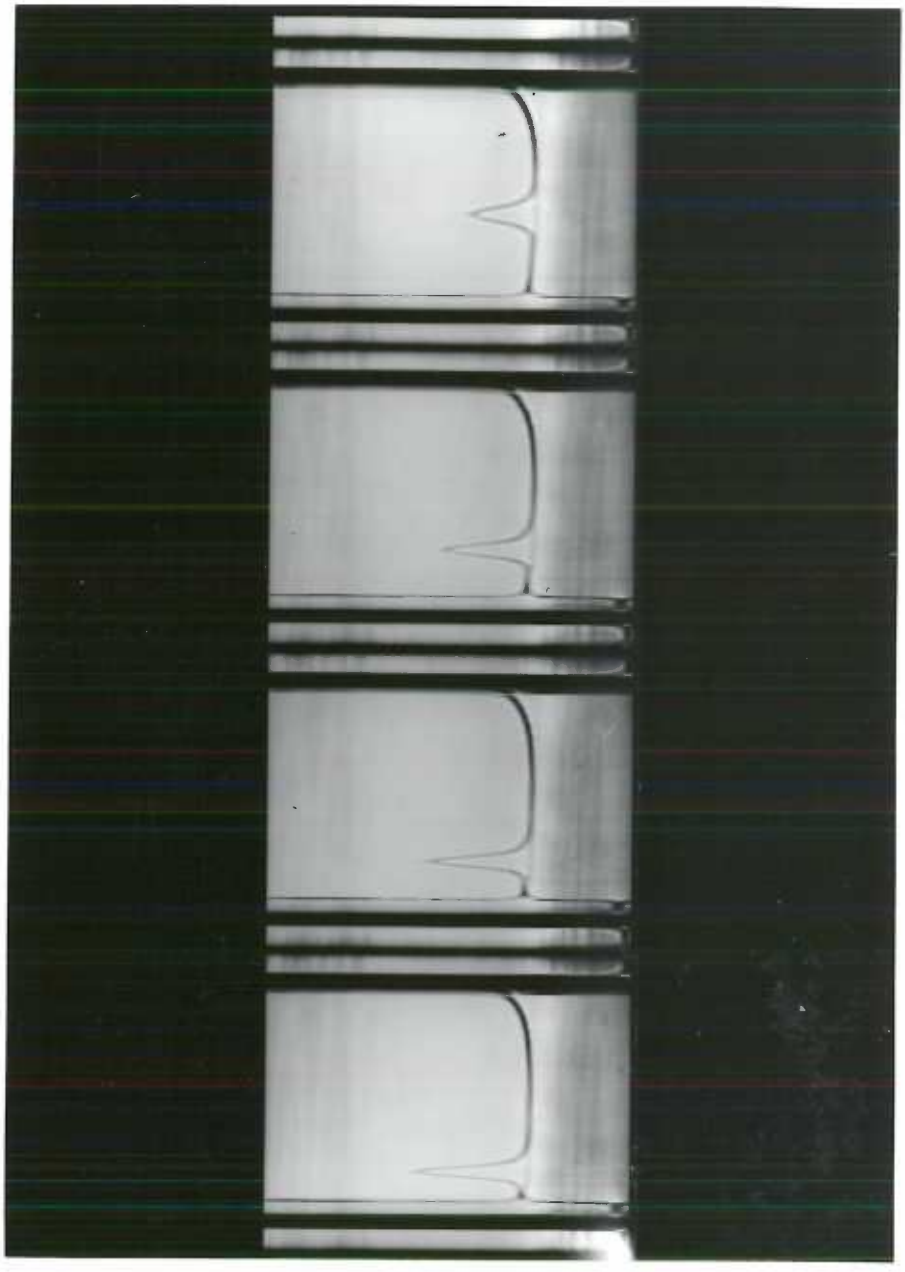
A sedimentation velocity experiment was performed on the purified human M_1-pyruvate kinase preparation from step 7. A 4.46 mg/ml sample (determined by the Lowry-phenol method) of the enzyme was dialyzed against 0.1M Miller's buffer, pH 7.2 for 24 hours before the run. Figure 12 is a photograph of the results taken through phase plate Schlieren optics. The results show a single symmetrical peak, consistent with a homogeneous preparation, sedimenting to the bottom of the cell with time. The photographs were taken at 0, 4, 8, and 24 minutes. By measuring the migration of the peak with time, an observed sedimentation coefficient (s_{obs}) of 9.77Swas obtained. The $s_{20,w}$ (sedimentation coefficient in water at 20°C) was calculated by equation 5, where ($\eta/\eta o$) = intrinsic viscosity, $\rho_{20,w}$ = density of

$$[5] \qquad s_{20,w} = (s_{obs})\cdot(\eta/\eta o)\cdot\left(\frac{1-(\bar{\nu}\rho_{20,w})}{1-(\bar{\nu}\rho_{20,s})}\right)$$

water at 20°C, $\rho_{20,s}$ = density of sample buffer at 20°C, and $\bar{\nu}$ = partial specific volume of the sample. A value of 1.01580 was calculated for $\eta/\eta o$, 0.99820 gm/cc for $\rho_{20,w}$, and 1.00279 gm/cc for

Figure 12

Sedimentation boundary photograph of human M_1-pyruvate kinase through Schlieren optics. The product of step 7, at a concentration of 4.46 mg/ml, was dialyzed for 24 hours against 0.1M Miller's buffer, pH 7.2 before the run. The photos were taken as the boundary moved from left to right at 0, 4, 8, and 24 minutes after reaching speed.



$\rho_{20,s}$.[2] The partial specific volume, $\bar{\nu}$, for the M_1-pyruvate kinase isozyme was calculated from the amino acid composition (170). Using the formula in equation 6,

$$[6] \quad \bar{\nu}_{protein} = \frac{\sum_i \bar{\nu}_i \omega_i}{\sum_i \omega_i}$$

where $\bar{\nu}$ protein = partial specific volume of the protein

$\bar{\nu}_i$ = partial specific volume of amino acid i

ω_i = percent weight of amino acid i in protein

$\sum_i \omega_i = 100\%$

a partial specific volume of 0.730 cc/gm was obtained. With these values a $s_{20,w}$ of 10.04S was calculated for human M_1-pyruvate kinase.

Sedimentation equilibrium

The molecular weight of the purified enzyme was determined by sedimentation equilibrium analysis. The same sample and buffer solvent from the sedimentation velocity experiment was used. A picture was taken at 72 hours after the run had begun and after equilibrium had been reached. A photograph of the interference

[2]The values for $\eta/\eta o$, $\rho_{20,w}$, and $\rho_{20,s}$ were determined by Dr. C. Rigas of the Department of Biochemistry, University of Oregon Medical School.

pattern through Rayleigh interference optics was taken and ΔY and r^2 were calculated by measuring the deflection of the fringes. Figure 13 shows the sedimentation equilibrium plot of log ΔY versus r^2 for the human M_1-pyruvate kinase isozyme. The linear plot indicates a homogeneous solute with respect to molecular weight. The line drawn through the points was calculated by the method of least squares, and had a correlation coefficient of 0.99954, a slope $\left(\frac{d \log \Delta Y}{d r^2}\right)$ of 0.39597, and a Y-intercept of -20.39544. Using equation 7, according

$$[7] \quad \bar{M}_{apparent} = \frac{d \log \Delta Y}{d r^2} \quad \frac{4.606RT}{(1-\bar{\nu}\rho_{20,s})\omega^2} = 240{,}700 \pm 450$$

where T = 293.16°K

ω = angular velocity

to Di Camelli et al. (173), a molecular weight of 240,700 ± 450 was obtained. The values for $\bar{\nu}$ and for $\rho_{20,s}$ were the same as those employed for the sedimentation velocity experiment.

Immunodiffusion

The final enzyme product from Step 7 was tested by immunodiffusion with antiserum prepared against purified human erythrocyte pyruvate kinase, against purified human M_1-pyruvate kinase, and against an impure preparation of human M_1-pyruvate kinase (see "Appendix"). The results are shown in the photograph of Figure 14 (see legend). The single precipitin line shown between well 5, containing the purified human M_1-pyruvate kinase preparation from Step

Figure 13

Sedimentation equilibrium plot of log ΔY versus r^2 for human M_1-pyruvate kinase. The same sample from the sedimentation velocity run was used for the sedimentation equilibrium analysis. The line through the points was determined by the method of least squares, and has a correlation coefficient = 0.99954, slope = 0.39597, and Y-intercept = -20.39544.

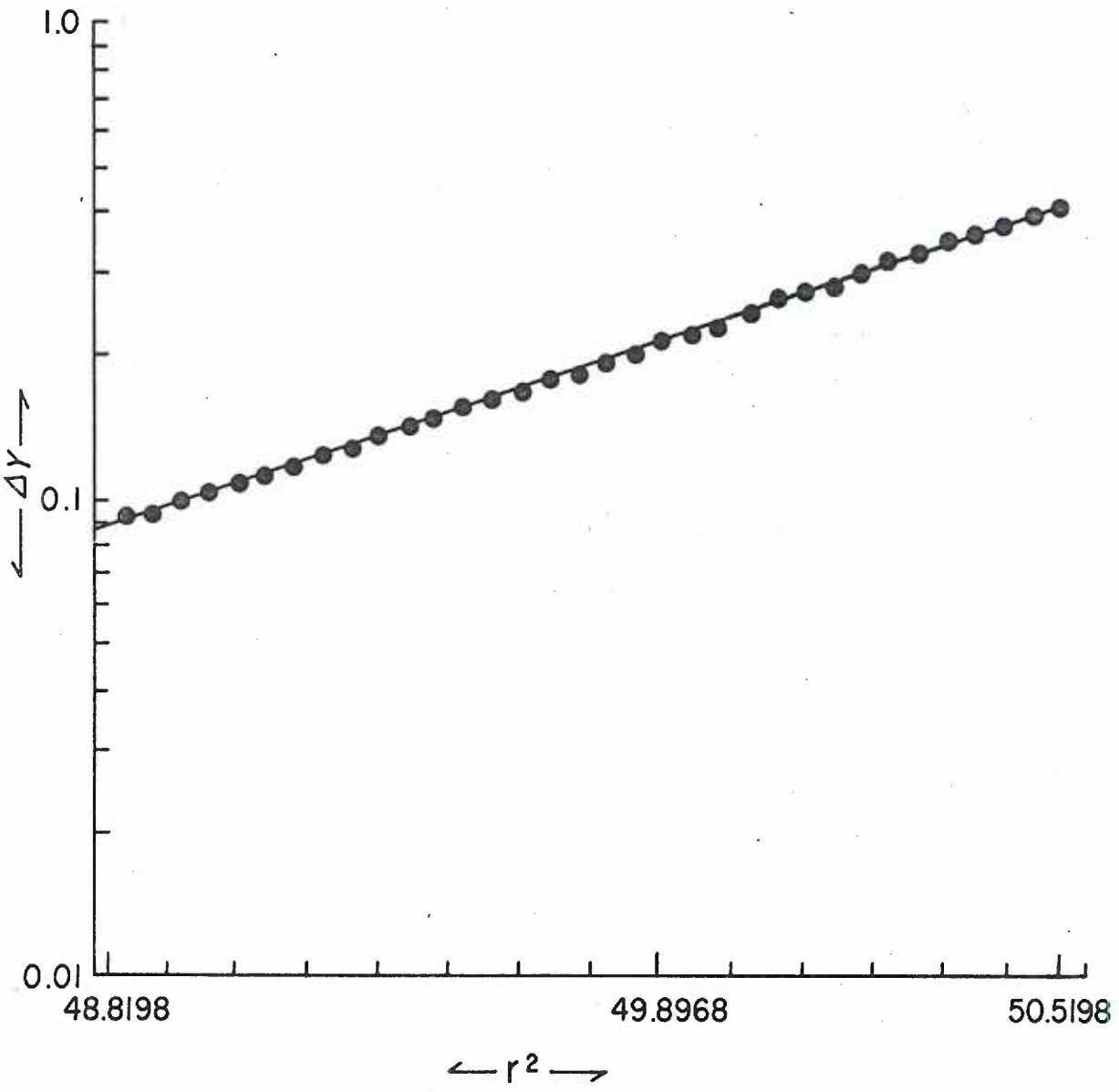
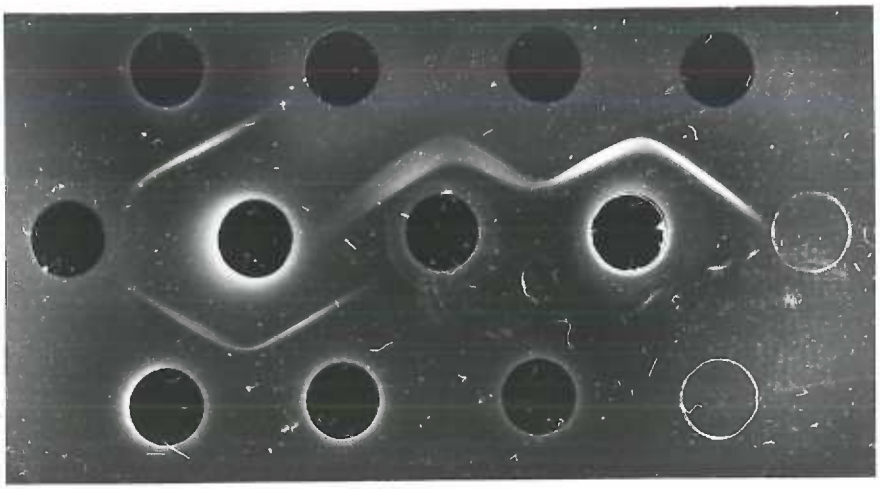
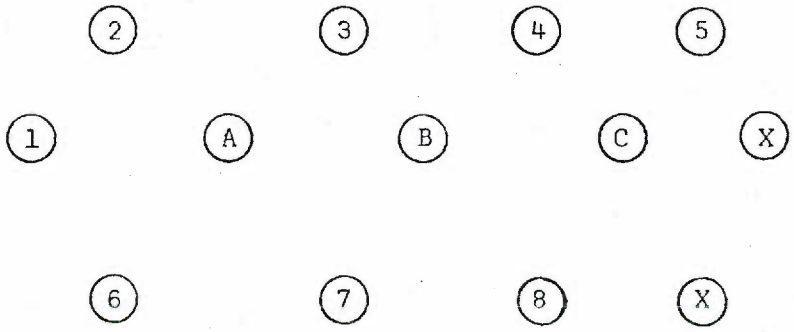

1.0
0.1
0.01
← Δy →
48.8198
49.8968
50.5198
← r² →

Figure 14

Immunodiffusion of purified human M_1-pyruvate kinase. The center well contained: A, purified human erythrocyte pyruvate kinase antiserum; B, impure human M_1-pyruvate kinase antiserum; C, purified human M_1-pyruvate kinase (from step 7) antiserum. The outer wells contained: 1 and 2, purified human erythrocyte pyruvate kinase; 3 and 5, purified human M_1-pyruvate kinase (from step 7); 4, an impure preparation of human M_1-pyruvate kinase (from step 4); 6 and 7, an impure preparation of rabbit erythrocyte pyruvate kinase; 8, an impure preparation of human erythrocyte pyruvate kinase. The remaining wells were empty.




2
3
4
5
1
A
B
C
X
6
7
8
X

7, and well C, containing purified human M_1-pyruvate kinase antiserum, indicates that the purified M_1-enzyme product is homogeneous. The more diffuse single line between well 3, purified human M_1-pyruvate kinase, and well B, antiserum prepared from an impure preparation of pyruvate kinase, indicates that there is no antigen other than pyruvate kinase present in the pure human M_1-isozyme preparation which cross-reacts with antibody against the muscle extract. The reverse of this experiment is shown between well 4, a crude preparation of human M_1-pyruvate kinase (from Step 4), and wells B and C, the impure M_1-antiserum and purified M_1-antiserum, respectively. The presence of a diffuse precipitin line between wells 4 and B is not immediately understandable. Since well B contained antisera prepared against an impure preparation of human M_1-pyruvate kinase, and well 4 contained a crude preparation of human M_1-pyruvate kinase, one would expect to observe a number of precipitin lines. However, the fact that the lines between wells 4 and B, 4 and C, and 5 and C converge smoothly, suggests strongly that the precipitation is due to the formation of a human M_1-pyruvate kinase antibody-antigen complex. The presence of a single diffuse line between wells 4 and B is most likely due to the inequality of the antiserum concentration in well B and antigen concentration in well 4.

The precipitin line shown between wells 2 and A and between 1 and A (faint) are due to the complex formed between purified human erythrocyte pyruvate kinase[3] (wells 1 and 2) and antiserum prepared against purified human erythrocyte pyruvate kinase. Wells 6 and 7 contained an impure preparation of rabbit erythrocyte pyruvate kinase. As shown by the line formed between wells 6 and A, and between wells 7 and A, rabbit erythrocyte pyruvate kinase cross-reacts with antiserum prepared against purified human erythrocyte pyruvate kinase. There is a faint spurring of the lines seen between wells 1 and 6, indicating only partial identity between the rabbit enzyme and the antiserum in well A.

Well 3 contained purified human M_1-pyruvate kinase. The fact that no precipitin line was formed between wells 3 and A indicates that the human M_1-isozyme has no determinant groups in common with the human erythrocyte pyruvate kinase isozyme. Well 8 contained an impure human erythrocyte pyruvate kinase preparation. No precipitin line is observed between this well and well C, containing purified human M_1-pyruvate kinase antiserum. These results provide additional evidence to support the previous suggestion that the human M_1- and erythrocyte pyruvate kinase isozymes share no common determinants which can be detected by the antiserum used in these experiments.

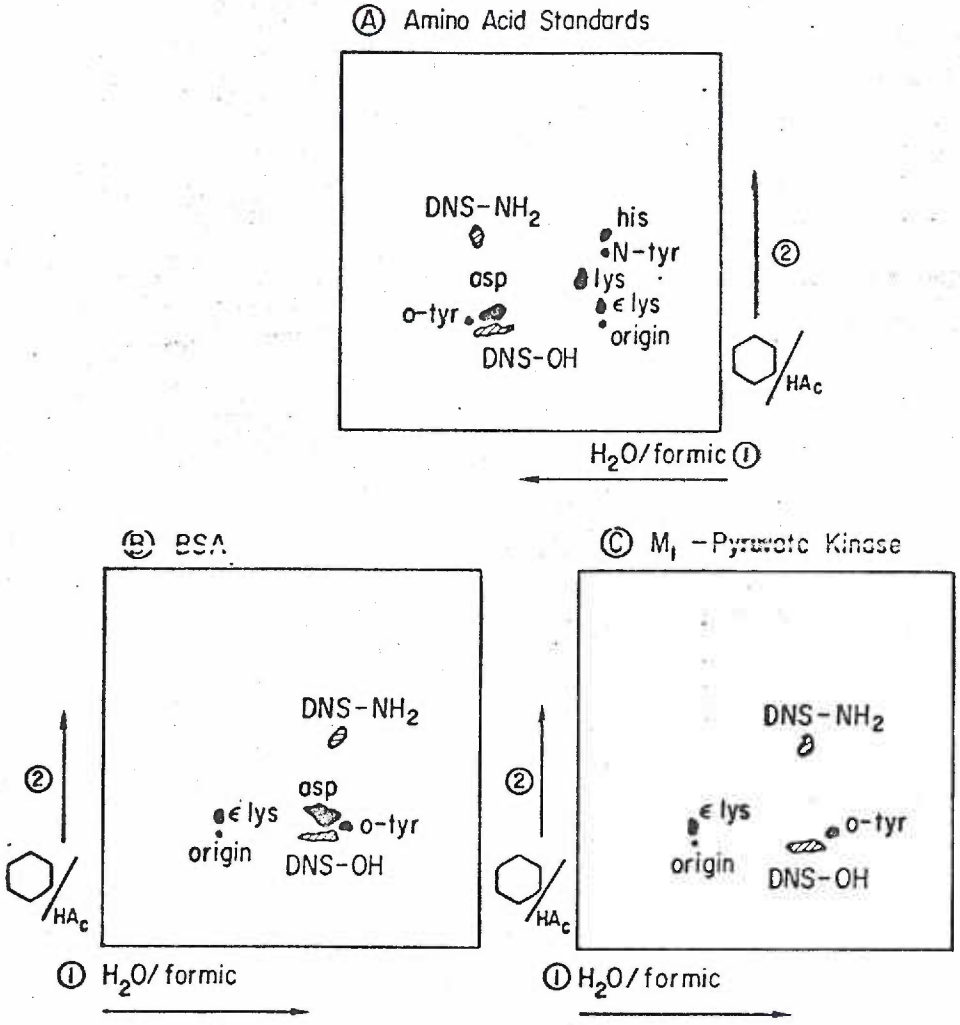
[3]Human erythrocyte pyruvate kinase was purified by Dr. C. Chern by the method of Chern et al. (130).

NH_2-terminal analysis

NH_2-terminal analysis of the purified human M_1-pyruvate kinase preparation from step 7 was performed by thin layer chromatography of the dansylated protein hydrolysate. Ten nmoles of bovine serum albumin were dansylated and hydrolyzed, and 1 nmole was chromatographed to check the method. The results are shown in Figure 15, B. Dansylated-aspartic acid, -histidine, -lysine, and -tyrosine were run as dansyl-amino acid standards on the reverse side of the thin layer plate, and the results are also shown in Figure 15, A. These standards were chosen because dansyl chloride reacts with ε-aminolysyl, sulfhydryl, imidazole, and phenolic hydroxyl groups as well as with the NH_2-terminus of a protein. However, the dansylated derivitives of the sulfhydryl and imidazole compounds are labile in acid and are broken down completely under the conditions used to hydrolyze the labeled proteins. Figure 15, B shows that the NH_2-terminus of bovine serum albumin, aspartic acid, is clearly resolved by this method. The only other derivatives observed, other than dansyl-aspartic acid, were the two by-products of the reaction, DNS-NH_2(1 dimethylaminonapthalene-5-sulfonamide) and DNS-OH(1 dimethylaminonapthalene-5-sulfonic acid), and DNS-ε-amino lysine and DNS-O-tyrosine. The results of thin layer chromatography of 1 nmole of dansylated human M_1-pyruvate kinase are shown in C, Figure 15. Other than the expected dansylated derivatives of amino acid side chains, and the by-products of the reaction, no

Figure 15

Thin layer chromatography of dansylated human M_1-pyruvate kinase. The samples were prepared and chromatographed (see "Methods") with H_2O-90% formic acid (100:1.5) in the first dimension, and benzene-glacial acetic acid (9:1) in the second dimension. A, 0.5 nmoles each of dansyl-aspartic acid, -histidine, -lysine, and -tyrosine; B, 1 nmole dansylated and hydrolyzed bovine serum albumin; C, 1 nmole purified human M_1-pyruvate kinase (from step 7).


Ⓐ Amino Acid Standards
DNS-NH2
his
N-tyr
asp
lys
o-tyr
ε lys
origin
DNS-OH
②
HAc
H2O/formic ①
Ⓑ BSA
DNS-NH2
asp
ε lys
o-tyr
origin
DNS-OH
② HAc
① H2O/formic
Ⓒ M1 - Pyruvate Kinase
DNS-NH2
ε lys
o-tyr
origin
DNS-OH
② HAc
① H2O/formic

other spot was observed. This result suggests that the NH_2-terminus of the M_1-isozyme is either proline or tryptophan, which are extensively degraded during acid hydrolysis and therefore not observed by thin layer chromatography, or that the NH_2-terminal group of human M_1-pyruvate kinase is blocked. These results do show that there are no contaminating proteins in the preparation which have free NH_2-terminal amino acids detected by this method.

Amino acid composition

The results of the amino acid analyses of the purified human M_1-pyruvate kinase isozyme are shown in Table 6. The final number of residues are expressed as residues per mole of protein, where 1 mole = 240,700 gms. The value for the molecular weight was obtained from the sedimentation equilibrium analysis. No non-amino acid constituents, such as carbohydrate moieties, were detected by this method. Noticeable in the composition are the low amounts of tyrosine, phenylalanine, and tryptophan. This was reflected during the purification procedure as the discrepancy between calculating the protein content by the Lowry-phenol method and by the absorbance at 280 nm. Since the absorbance at 280 nm is based upon the number of tyrosine and tryptophan residues in the medium, the protein content was unusually low when measured in this manner.

Table 6. Amino acid composition of human M_1-pyruvate kinase

Amino acid	24 hr	48 hr	72 hr	Average value[a]	Residues[b]
	μmoles/mg protein[c]				
Lysine	0.603	0.605	0.605	0.604	145
Histidine	0.218	0.210	0.215	0.214	52
Ammonia	0.753	0.758	0.760	0.747	180
Arginine	0.503	0.501	0.493	0.499	120
Aspartic acid	0.822	0.826	0.823	0.824	199
Threonine	0.449	0.431	0.411	0.465	111
Serine	0.481	0.426	0.406	0.497	120
Glutamic acid	0.984	0.967	0.996	0.983	237
Proline	0.369	0.354	0.379	0.368	87
Glycine	0.685	0.680	0.676	0.681	165
Alanine	0.898	0.917	0.907	0.908	218
Half-cystine[d]				0.251	61
Valine	0.748	0.765	0.767	0.767	185
Methionine[e]				0.323	78
Isoleucine	0.563	0.571	0.574	0.574	138
Leucine	0.669	0.671	0.664	0.667	161
Tyrosine	0.157	0.159	0.158	0.158	38
Phenylalanine	0.289	0.299	0.298	0.296	71
Tryptophan[f]				0.049	12

[a]The values for threonine, serine, and ammonia were obtained by extrapolation to zero time. The 72-hour values for valine and isoleucine were used.

[b]Calculated for a molecular weight of 240,700 obtained by sedimentation equilibrium analyses.

[c]The value presented is the average of duplicate analyses performed at each time interval.

[d]Determined as cysteic acid following performic acid oxidation (179).

[e]Determined as methionine sulfone following performic acid oxidation (179).

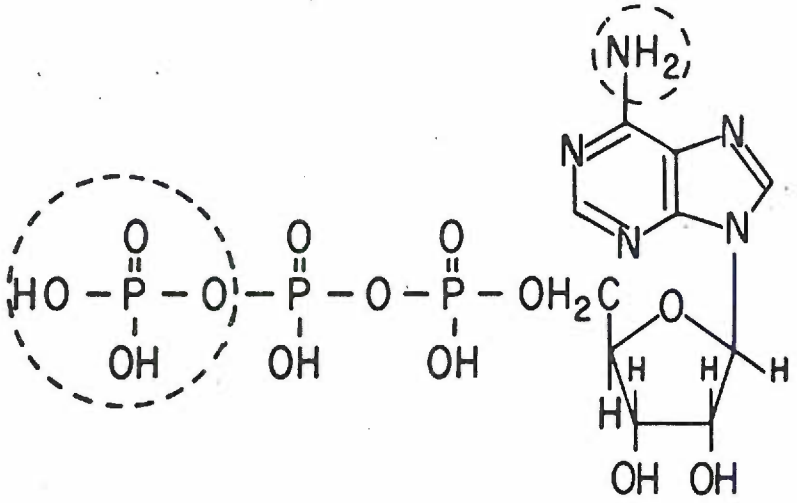
[f]Determined spectrophotometrically by the method of Goodwin and Morton (180).

2. Studies on the binding of Cibacron blue F3GA to human pyruvate kinase isoenzymes

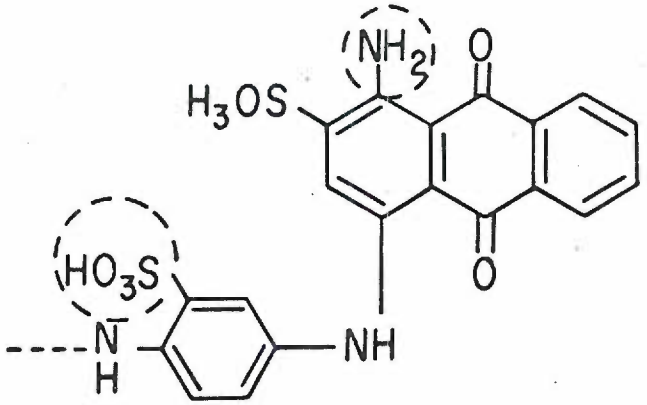
The chromophoric group found in Blue Dextran 2000, Cibacron blue F3GA, is able to bind tightly to kinases and dehydrogenases (164). Several workers have taken advantage of this binding as a means of purification for lactic dehydrogenase (183), phosphofructokinase (166), and yeast pyruvate kinase (184). Böhme et al. (166) have suggested that the affinity of Cibacron blue F3GA for these nucleotide binding enzymes may be due to the structural similarity of the dye to the adenine mononucleotide, ATP. Figure 16 shows the proposed similarities of the two molecules, in which amino group as well as the sulphonic acid residue in the 2'-position of the phenylenediamine ring of Cibacron blue F3GA have a structural arrangement resembling that of the amino and γ-phosphate groups within the ATP molecule. However, this hypothesis has not been fully substantiated. Therefore, a study was undertaken to examine more closely the affinity of Cibacron blue F3GA for human pyruvate kinase isoenzymes for two reasons: first, in an attempt to understand the nature of the binding phenomenon with respect to its effect upon enzyme activity; and second, to determine whether the M_2-pyruvate kinase isoenzyme could be purified by an affinity column to which the dye molecule had been attached.

Figure 16

Structural similarity of adenine nucleotide and portion of Cibacron blue F3GA. As proposed by Böhme et al. (166), the amino group as well as the γ-phosphate group of ATP (a) have a structural arrangement resembling the amino group of the anthroquinone and the sulfonic acid residue in the 2'-position of the phenylenediamine ring of Cibacron blue F3GA (b). Only a portion of the Cibacron blue F3GA structure is shown. The reader is referred to Figure 2 for a complete structure.



a) ATP



b) Proposed reactive portion
Cibacron blue F3GA

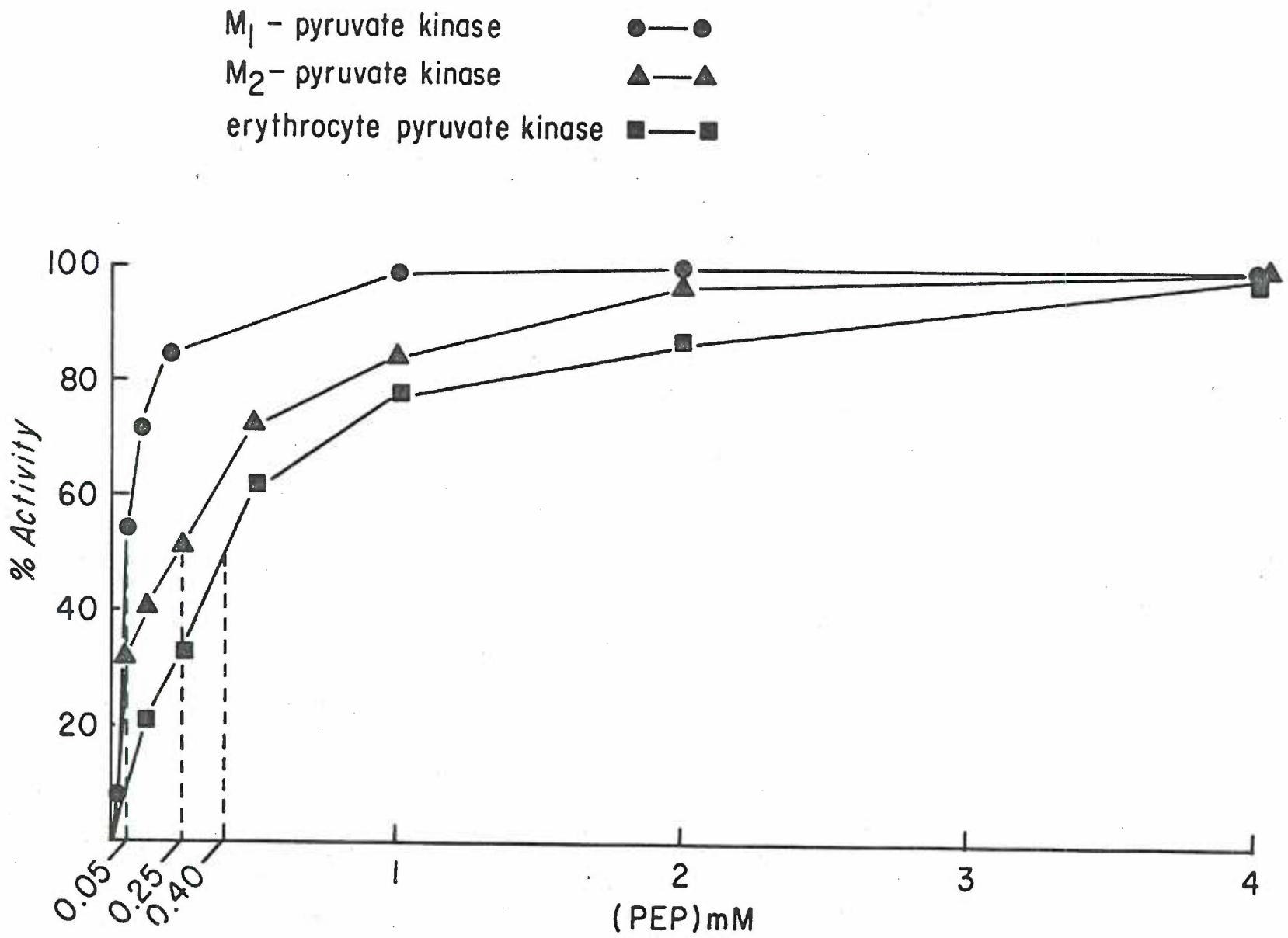
a. Velocity profiles for human M_1-, M_2-, and erythrocyte pyruvate kinase isoenzymes

In order to illustrate the kinetic differences between the human pyruvate kinase isoenzymes and to establish the assay conditions for the inhibition studies, the velocity profiles for the human M_1-, M_2-, and erythrocyte pyruvate kinase isoenzymes were determined in duplicate using the pH-stat assay at pH 7.2, 25°C (see "Methods"). The pH-stat method was preferred over the spectrophotometric assay for pyruvate kinase because of the intense absorption of Cibacron blue F3GA at 340 nm. The samples studied were the purified M_1-isozyme, a partially purified human erythrocyte isozyme (see "Methods"), and a 50% ammonium sulfate supernatant from a human kidney extract. The reaction profiles for human M_1-, M_2-, and erythrocyte pyruvate kinase isozymes with varying PEP concentration are shown in Figure 17. For each isozyme the rate of the reaction was plotted as the percent of maximum velocity versus PEP concentration at a fixed ADP concentration of 4 mM. The concentration of PEP giving 50% maximum velocity was expressed as $K_{0.5s}$, an indication of the affinity of the substrate for the enzyme. The M_1-isozyme has a hyperbolic reaction profile with a $K_{0.5s}$ equal to 0.05 mM PEP. The erythrocyte isozyme shows a sigmoidal velocity curve with $K_{0.5s}$ equal to 0.40 mM PEP. The M_2-isozyme has an intermediate progress curve with $K_{0.5s}$ equal to 0.25 mM PEP.

Similar plots of reaction velocity were performed for

Figure 17

Velocity profiles for human M_1-, M_2-, and erythrocyte pyruvate kinase isozymes versus PEP concentration. Holding the ADP concentration constant at 4 mM, KCl = 200 mM, and $MgCl_2$ = 8 mM, a pH-stat assay was used at pH 7.2, 25°C, to measure the velocity profiles of the human M_1- (●——●), M_2- (▲——▲), and erythrocyte (■——■) pyruvate kinase isozymes. Each point is expressed as a percent of maximum activity and is the average of duplicate runs. $K_{0.5s}$ values were determined by the concentration of PEP giving 50% maximum activity.


M1 - pyruvate kinase
M2 - pyruvate kinase
erythrocyte pyruvate kinase
% Activity
100
80
60
40
20
0.05
0.25
0.40
1
2
3
4
(PEP) mM

the three isozymes while varying the ADP concentration and keeping the PEP concentration fixed at 4 mM. The results are shown in Figure 18, and a summary of the $K_{0.5s}$ values obtained from the two experiments is shown in Table 7. An inverse relationship is observed between the $K_{0.5s}$ (PEP) and $K_{0.5s}$ (ADP) values for the M_1-, M_2-, and erythrocyte isozymes. The $K_{0.5s}$ (PEP) is lowest (0.05 mM) for M_1-pyruvate kinase, and increases to 0.25 mM for type M_2-, and to 0.40 mM for the erythrocyte isozyme. On the other hand, the $K_{0.5s}$ (ADP) decreases progressively for 0.45 mM for the M_1-isozyme to 0.25 mM for the erythrocyte isozyme. The results suggest that under the conditions used, there exists an inverse relationship between the affinities of the two substrates, ADP and PEP, for the human M_1-, M_2-, and erythrocyte pyruvate kinase isozymes, respectively.

b. Inhibition studies of the human M_1-, M_2-, and erythrocyte pyruvate kinase isoenzymes by Cibacron blue F3GA

Using the same conditions which were established for determining the velocity profiles of the M_1-, M_2-, and erythrocyte pyruvate kinase isoenzymes, the effect of Cibacron blue F3GA on pyruvate kinase activity was tested for each isozyme. The inhibition of enzyme activity by the dye was determined as the apparent inhibition constant (K_i) by plotting the reciprocal of the velocity versus Cibacron blue F3GA concentration according to the method of Dixon (185), where K_i is defined as the reciprocal of the

Figure 18

Velocity profiles for human M_1-, M_2-, and erythrocyte pyruvate kinase isozymes versus ADP concentration. The plot was determined in an analogous fashion as previously described (see "Results" or legend to Fig. 17), except that the PEP concentration was held constant at 4 mM.

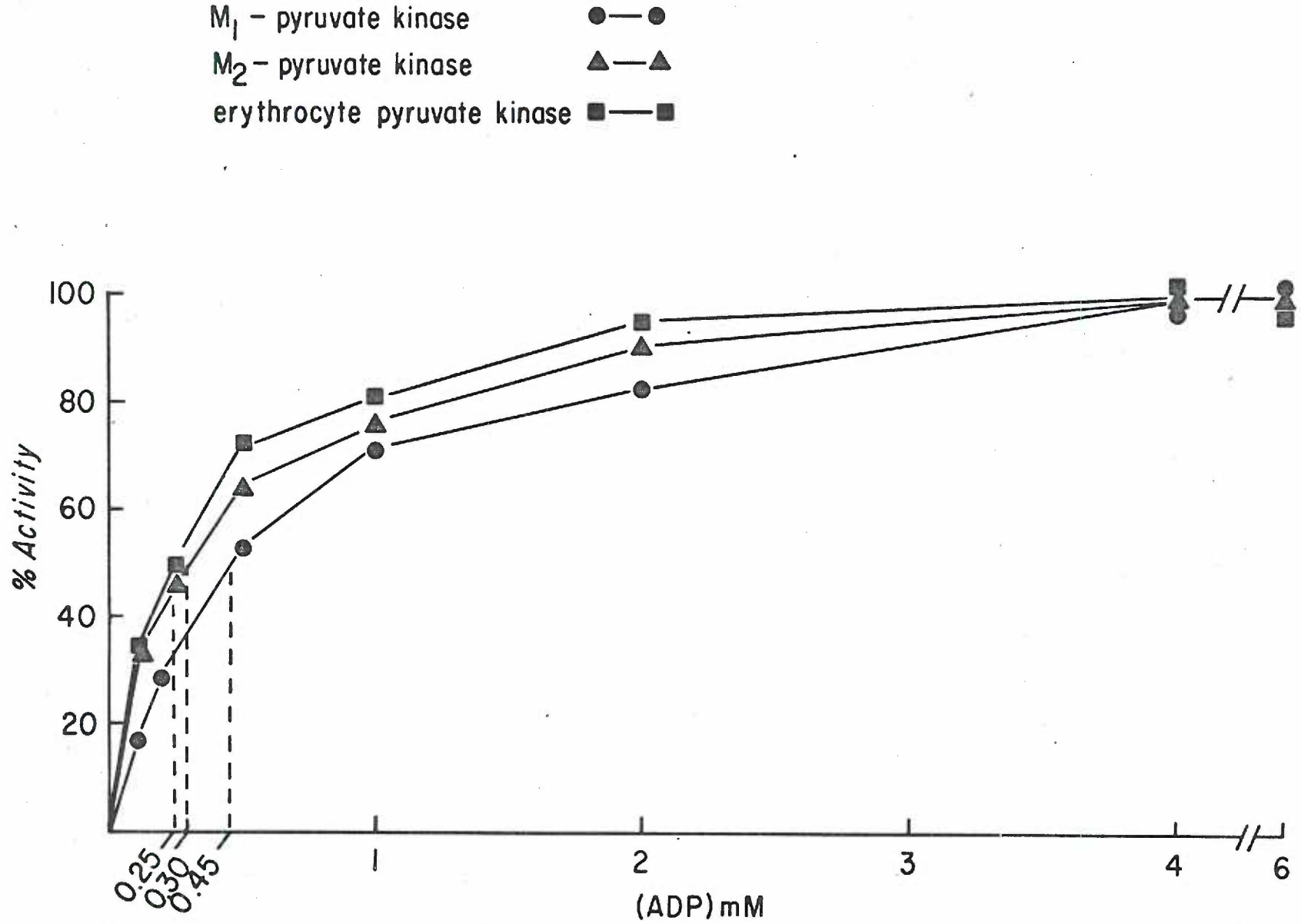

M1 – pyruvate kinase
M2 – pyruvate kinase
erythrocyte pyruvate kinase
% Activity
100
80
60
40
20
0.25
0.30
0.45
1
2
3
4
6
(ADP) mM

Table 7. Summary of $K_{0.5s}$ (PEP) and $K_{0.5s}$ (ADP) for human M_1-, M_2-, and erythrocyte pyruvate kinase isozymes.

	$K_{0.5s}$ (mM PEP)	$K_{0.5s}$ (mM ADP)
M_1-pyruvate kinase	0.05	0.45
M_2-pyruvate kinase	0.25	0.30
erythrocyte pyruvate kinase	0.40	0.25

$K_{0.5s}$ was measured from the velocity profiles, using a pH-stat at 25°C, pH 7.2, 200 mM KCl, and 8 mM $MgCl_2$.

enzyme-inhibitor affinity.

The dye was dissolved in distilled water at the appropriate concentrations based upon a molecular weight of 773 gms per mole (Figure 2). The dye was incubated in the reaction vessel at pH 7.2, 25°C with all the other substrates, and the reaction was initiated by the addition of 10 µl of enzyme. The isozyme preparations which were used were prepared in the same manner as for the velocity profile studies. The results of the inhibition of the M_1-isozyme are shown in Figures 19 and 20. In Figure 19 the ADP concentration was held constant at 2 mM and the inhibition of enzyme activity was plotted versus dye concentration at both 2 mM PEP and 4 mM PEP. Lines were drawn through the points by the method of least squares. The two lines intersect at the abscissa, $(-K_i)$, and indicate that for the M_1-isoenzyme the inhibition by Cibacron blue F3GA is non-competitive with respect to PEP with a K_i equal to 0.11 mM Cibacron blue F3GA. Figure 20 shows the results of the inhibition of M_1-pyruvate kinase at a fixed concentration of 4 mM PEP. The two lines drawn through the points plotted for inhibition at 1 mM ADP and 2 mM ADP intersect above the abscissa at a dye concentration of -0.01 mM, and, in the case of competitive inhibition, at an ordinate value corresponding to the reciprocal of the maximum velocity of the reaction $(1/V_{max})$. The results of Figure 20 show that the inhibition of human M_1-pyruvate kinase by Cibacron blue F3GA is competitive with respect to ADP with a K_i value equal to 0.01 mM.

Figure 19

Dixon plot of the inhibition of human M_1-pyruvate kinase by Cibacron blue F3GA: K_i determination at fixed ADP concentration = 2 mM. Using a purified preparation of human M_1-pyruvate kinase, the pH-stat assay at pH 7.2, 25°C (see "Methods") was used to plot the reciprocal of the velocity versus varying concentrations of Cibacron blue F3GA at 2 mM PEP (●——●) and at 4 mM PEP (▲——▲). Each point is the average of duplicate runs, and lines were drawn through the points by the method of least squares.

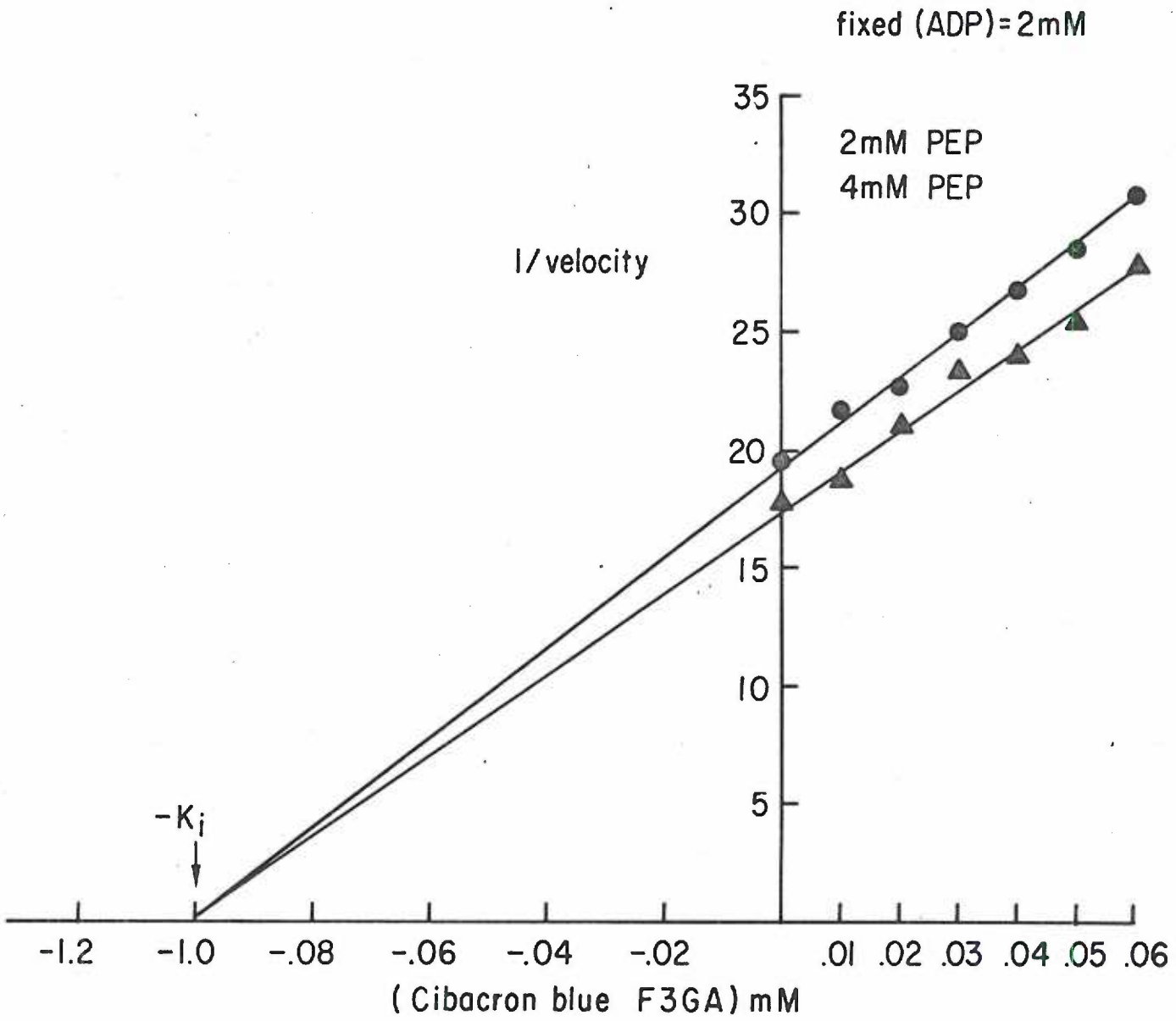
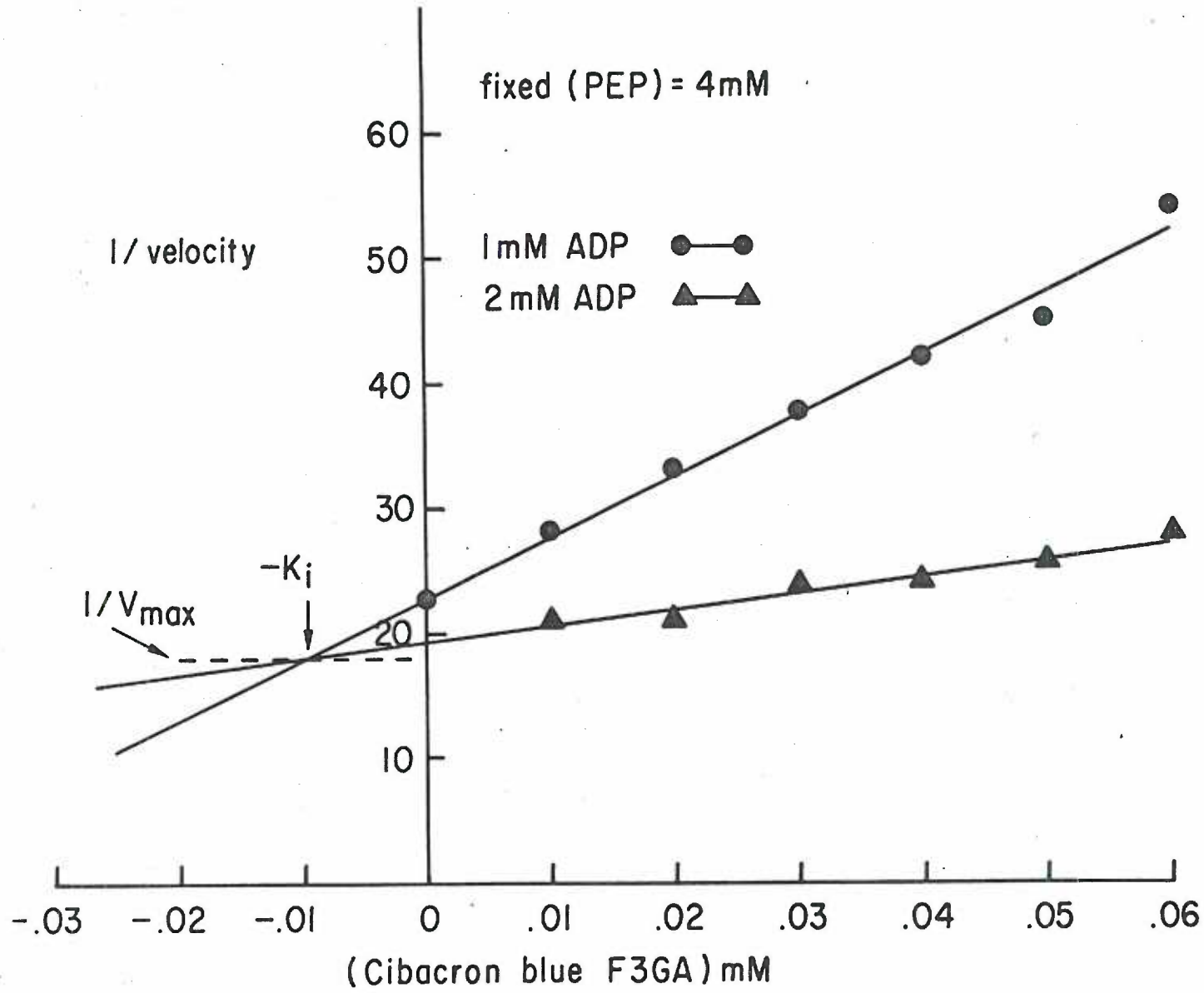

fixed (ADP)=2mM
2mM PEP
4mM PEP
1/velocity
35
30
25
20
15
10
5
-Ki
-1.2
-1.0
-.08
-.06
-.04
-.02
.01
.02
.03
.04
.05
.06
(Cibacron blue F3GA) mM

Figure 20

Dixon plot of the inhibition of human M_1-pyruvate kinase by Cibacron blue F3GA: K_i determination at fixed PEP concentration = 4 mM. The conditions of the assay and the sample were the same as for the previous plot (see "Methods" or legend to Fig. 19). The velocities were determined by varying the concentration of inhibitor, Cibacron blue F3GA, at 1 mM ADP (●——●) and at 2 mM ADP (▲——▲). The points are the average value of duplicate runs, and the lines were drawn by the method of least squares. The value of K_i was determined by the intersection of the two straight lines.


fixed (PEP) = 4mM
1/ velocity
1mM ADP
2mM ADP
60
50
40
30
20
10
$-K_i$
$1/V_{max}$
-.03
-.02
-.01
0
.01
.02
.03
.04
.05
.06
(Cibacron blue F3GA) mM

The results for the inhibition of M_2-pyruvate kinase by Cibacron blue F3GA are shown in Figures 21 and 22. The inhibition plots are non-linear at high dye concentrations and low substrate concentrations, and indicate a more complex mixed-type kinetics of inhibition. A straight line could be drawn, however, through the initial points, and indicate to a first approximation that the inhibition is non-competitive with PEP and competitive with ADP. The same sorts of results were obtained for the human erythrocyte isozyme (Figures 23 and 24). At low substrate levels of either ADP or PEP, a non-linear relationship between the inhibitor concentration and reciprocal of the velocity was observed at high levels of inhibitor. However, the initial plot of the curves was linear and indicates that the inhibition is competitive with respect to ADP, and non-competitive with respect to PEP. A summary of the results of the inhibition experiments and the velocity curves is presented in Table 8. The inhibition studies indicate that the dye is a competitive inhibitor of the human pyruvate kinase isozymes with respect to ADP. The K_i values for the competitive inhibition by ADP are lower by a factor of 10 than for the K_i (PEP) values, and suggest a very high affinity of the dye molecules for the ADP binding site. This is also reflected by the fact that the erythrocyte isoenzyme, having the lowest value for the $K_{0.5s}$ for ADP among the isozymes, also exhibits the greatest inhibition by Cibacron blue F3GA, with a K_i = 0.0065 mM. Moreover, there is an inverse relationship between

Figure 21

Dixon plot of the inhibition of human M_2-pyruvate kinase by Cibacron blue F3GA: K_i determination at fixed ADP concentration = 2 mM. A 50%-65% $(NH_4)_2SO_4$ precipitate of a human kidney extract was used as a sample for the M_2-isozyme. The reciprocal of the velocity is plotted versus inhibitor concentration at 0.5 mM PEP (●——●) and 2.0 mM PEP (▲——▲). K_i was determined in a manner previously described (legend Fig. 19) using the first five points of the 0.5 mM PEP plot and all the points of the 2.0 mM PEP plot.

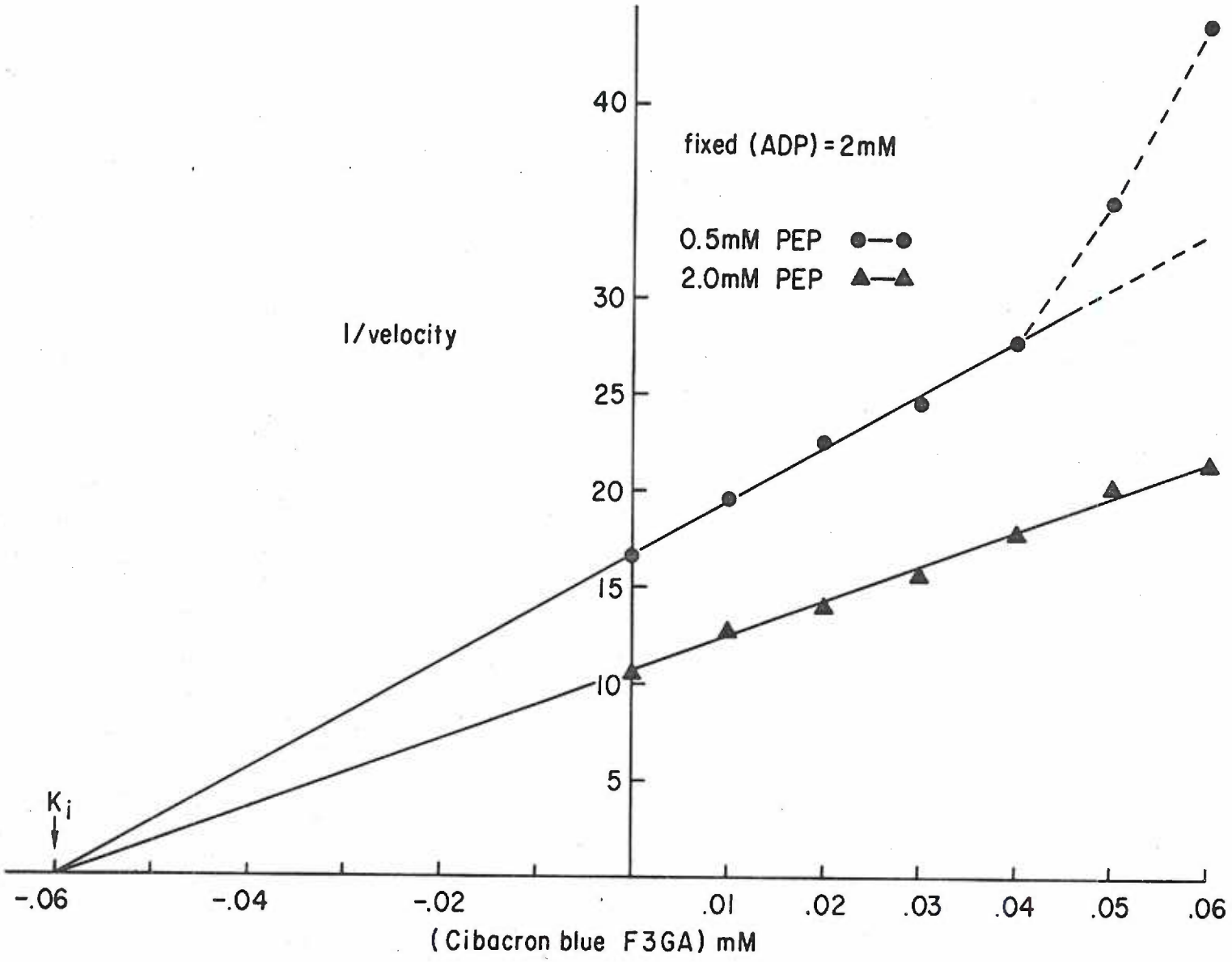

40
30
25
20
15
10
5
fixed (ADP) = 2mM
0.5mM PEP
2.0mM PEP
1/velocity
K_i
-.06
-.04
-.02
.01
.02
.03
.04
.05
.06
(Cibacron blue F3GA) mM

Figure 22

Dixon plot of the inhibition of human M_2-pyruvate kinase by Cibacron blue F3GA: K_i determination at fixed PEP concentration = 2 mM. The sample and conditions were the same as described (legend Fig. 21). The reciprocal of the velocity is plotted versus inhibitor concentration at 0.5 mM ADP (●——●) and at 2.0 mM ADP (▲——▲). Lines were drawn by the method of least squares using the first five points of the 0.5 mM ADP plot and all the points of the 2.0 mM ADP plot.

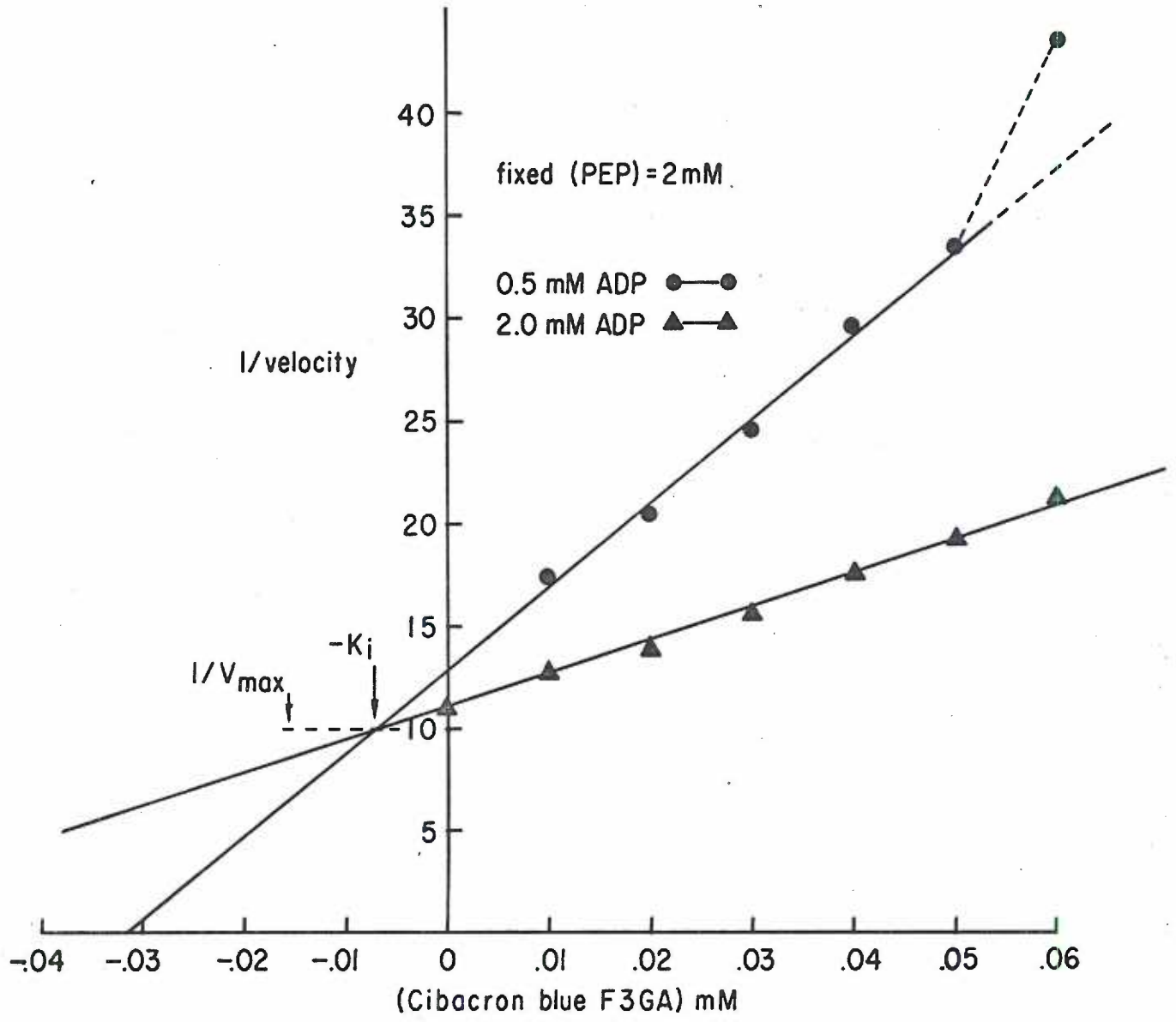

fixed (PEP) = 2mM
0.5 mM ADP
2.0 mM ADP
1/velocity
40
35
30
25
20
15
10
5
-Ki
1/Vmax
-.04
-.03
-.02
-.01
0
.01
.02
.03
.04
.05
.06
(Cibacron blue F3GA) mM

Figure 23

Dixon plot of the inhibition of human erythrocyte pyruvate kinase by Cibacron blue F3GA: K_i determination at fixed ADP concentration = 2 mM. An impure preparation of human erythrocyte pyruvate kinase (see "Methods") was used as a sample. The reciprocal of the velocity is plotted versus inhibitor concentration at 0.5 mM PEP (●——●) and at 2.0 mM PEP (▲——▲). K_i was determined as described (legend Fig. 19), using the first four points of the 0.5 mM PEP plot, and all the points of the 2.0 mM PEP plot.

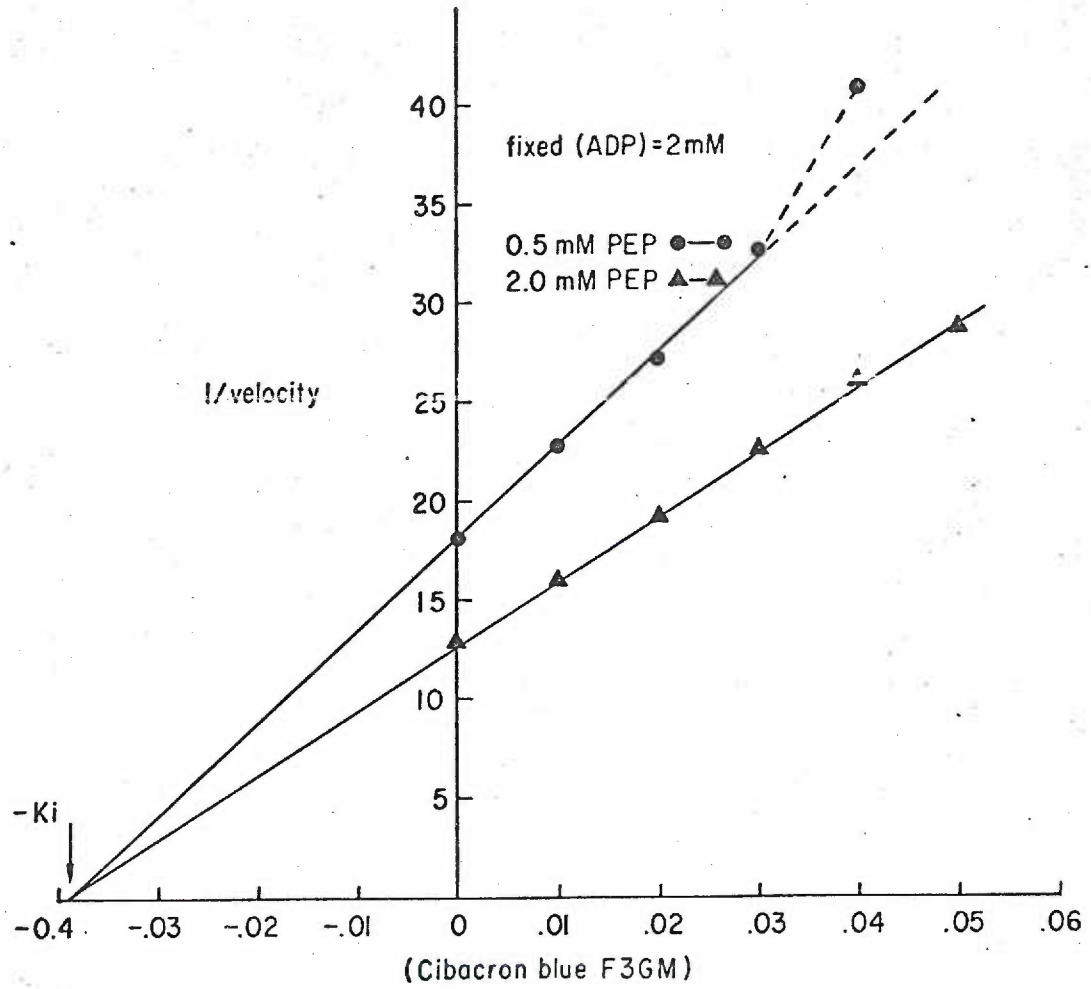

40
35
30
25
20
15
10
5
fixed (ADP)=2mM
0.5 mM PEP
2.0 mM PEP
1/velocity
-Ki
-0.4
-.03
-.02
-.01
0
.01
.02
.03
.04
.05
.06
(Cibacron blue F3GM)

Figure 24

Dixon plot of the inhibition of human erythrocyte pyruvate kinase by Cibacron blue F3GA: K_i determination at fixed PEP concentration = 2 mM. The same sample was used as for the previous plot (legend Fig. 23), and the reciprocal of the velocity is plotted versus inhibitor concentration at 0.5 mM ADP (●——●) and 2.0 mM ADP (▲——▲). K_i was determined using the first five points of the 0.5 mM ADP plot and all the points of the 2.0 mM ADP plot.

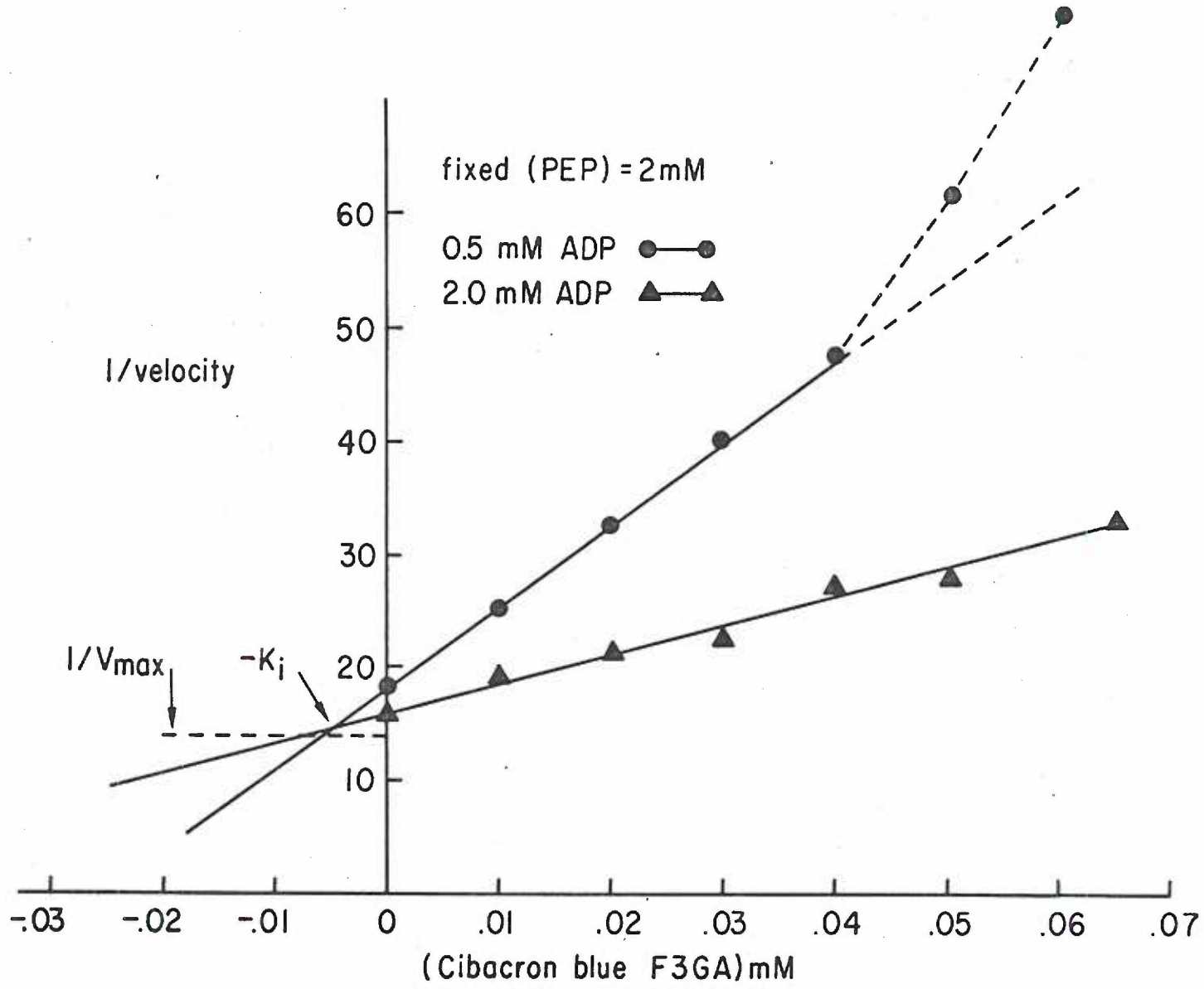

fixed (PEP) = 2mM
0.5 mM ADP
2.0 mM ADP
1/velocity
60
50
40
30
20
10
1/Vmax
-Ki
-.03
-.02
-.01
0
.01
.02
.03
.04
.05
.06
.07
(Cibacron blue F3GA) mM

Table 8. Summary of the velocity profiles and the inhibition by Cibacron blue F3GA of human M_1-, M_2-, and erythrocyte pyruvate kinase isoenzymes.[a]

	PEP		ADP	
Isozyme[b]	$K_{0.5s}$[a] (mM)	Inhibition by[a] Cibacron blue F3GA K_i (mM)	$K_{0.5s}$ (mM)	Inhibition by Cibacron blue F3GA K_i (mM)
M_1-	0.05	0.11 (non-competitive)	0.45	0.01 (competitive)
M_2-	0.25	0.06 (non-competitive-mixed)	0.30	0.0075 (competitive-mixed)
erythrocyte	0.40	0.04 (non-competitive-mixed)	0.25	0.0065 (competitive-mixed)

[a]Both $K_{0.5s}$ and K_i were measured by a pH-stat assay at 25°C, pH 7.2, 200 mM KCl, and 8 mM $MgCl_2$. K_i was determined according to the method of Dixon (185).

[b]The preparation of the pyruvate kinase isozymes is described in the text.

the $K_{0.5s}$ (PEP) values and the K_i (PEP) values for the M_1-, M_2-, and erythrocyte isozymes, respectively, suggesting that the affinity of the dye is independent of affinity of PEP for the isozymes.

In summary, the conclusions from these studies are that the dye molecule, Cibacron blue F3GA, is a strong inhibitor of the human pyruvate kinase isoenzymes, and that the affinity is directed toward the ADP binding sites of these isoenzymes. In addition, the studies show that because of the high affinity for these isoenzymes, the dye molecule may serve as a powerful tool for their fractionation by means of affinity chromatography.

3. Purification of M_2-pyruvate kinase

The studies on pyruvate kinase isozymes indicate that the M_2-isozyme is a predominant form in kidney. Therefore, this tissue was chosen for the extraction and purification of the human M_2-isozyme. The fractionation scheme for human M_2-pyruvate kinase is shown in Table 9. All steps were carried out at 4°C or with the enzyme preparation in an ice bath.

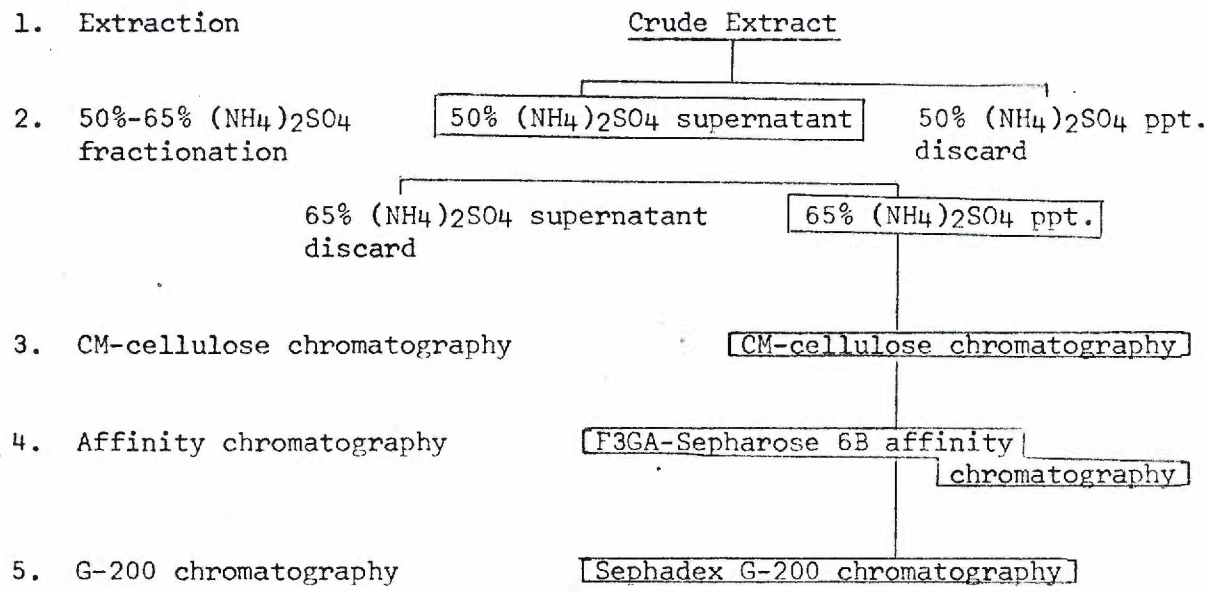
a. Steps 1-2: extraction of M_2-pyruvate kinase

Frozen human kidneys were thawed and the adipose tissue was removed. The kidneys were sectioned into small pieces and mixed with two volumes of extraction buffer, which contained 25 mM Tris-HCl pH 7.0, 10 mM $MgSO_4$, 1 mM EDTA, and 0.1% 2-mercaptoethanol. The mixture was homogenized for 2 minutes in a Waring blender, stirred for 1 hour at 4°C, and then centrifuged at 10,000 xg for 30 minutes in a Sorvall RC-2B refrigerated centrifuge equipped with a GSA-3 rotor. The supernatant was filtered through two layers of cheese-cloth and ammonium sulfate was added according to the procedure described for the human M_1-pyruvate kinase isozyme. The M_2-isozyme precipitated in the 50% to 65% ammonium sulfate range.

b. Stability studies of M_2-pyruvate kinase

During the initial fractionation of M_2-pyruvate kinase, the yields obtained were extremely low. It was found that these results were due to the instability of the enzyme, which was sensitive to both temperature and the presence or absence of FDP.

Table 9. Fractionation scheme for human M_2-pyruvate kinase



The enclosed fractions are those which contained the M_2-pyruvate kinase isozyme.

Figure 25 shows the sensitivity of M_2-pyruvate kinase to temperature. The enzyme was extracted according to the above procedure and the 65% ammonium sulfate precipitate was dissolved in 25 mM Tris-HCl pH 7.5. The sample was divided into three portions, incubated at 4°C, 25°C, and 37°C, and assayed at 30 minute intervals for 4 hours. Under these conditions the enzyme lost 50% of its initial activity in 2-1/2 hours at either 4°C or 25°C. The enzyme was more stable at 37°C, retaining 70% of its original activity after 2-1/2 hours. Various metabolites were added to the incubation medium to see if they might stabilize M_2-pyruvate kinase. Figure 26 shows the results of these studies. At 4°C, 1 mM ADP or 1 mM PEP were ineffective in stabilizing the enzyme. However, when 0.1 mM FDP (Sigma grade) was added to the incubation medium, the enzyme retained 92% of its original activity after 4 hours. Therefore 0.1 mM FDP (Sigma grade) was included in the initial extraction buffer and in all the subsequent steps of purification for the M_2-pyruvate kinase isozyme.

c. Step 3: CM-cellulose chromatography of M_2-pyruvate kinase

The 65% ammonium sulfate precipitate from step 2 was centrifuged and dissolved in the column buffer for CM-cellulose chromatography, which consisted of 33 mM potassium maleate, pH 5.5, 2 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM FDP. The sample was

Figure 25

Temperature stability of human M_2-pyruvate kinase. The sample, a 50%-65% $(NH_4)_2SO_4$ precipitate of human kidney (step 2), was diluted to the proper concentration, divided into three portions, and incubated at 37°C (▲—▲), 25°C (■—■), and 4°C (●—●) in 25 mM Tris-HCl, pH 7.5. The enzyme was assayed at 1/2 hour intervals for 4 hours by a coupled-assay method (see "Methods" for conditions). Each point is the average of duplicate assays and is expressed as the percent of remaining activity with time, where time 0 = 100%.

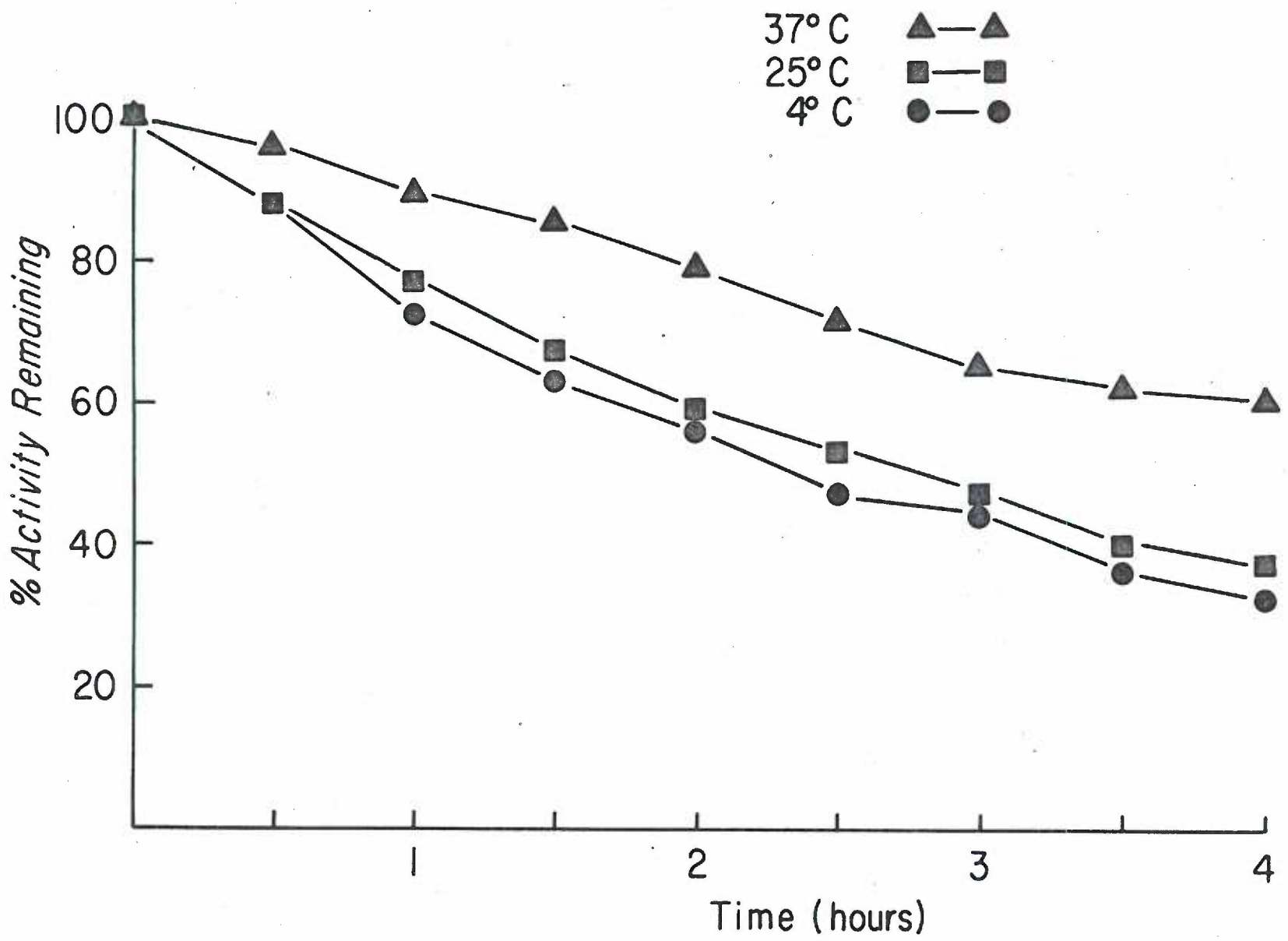
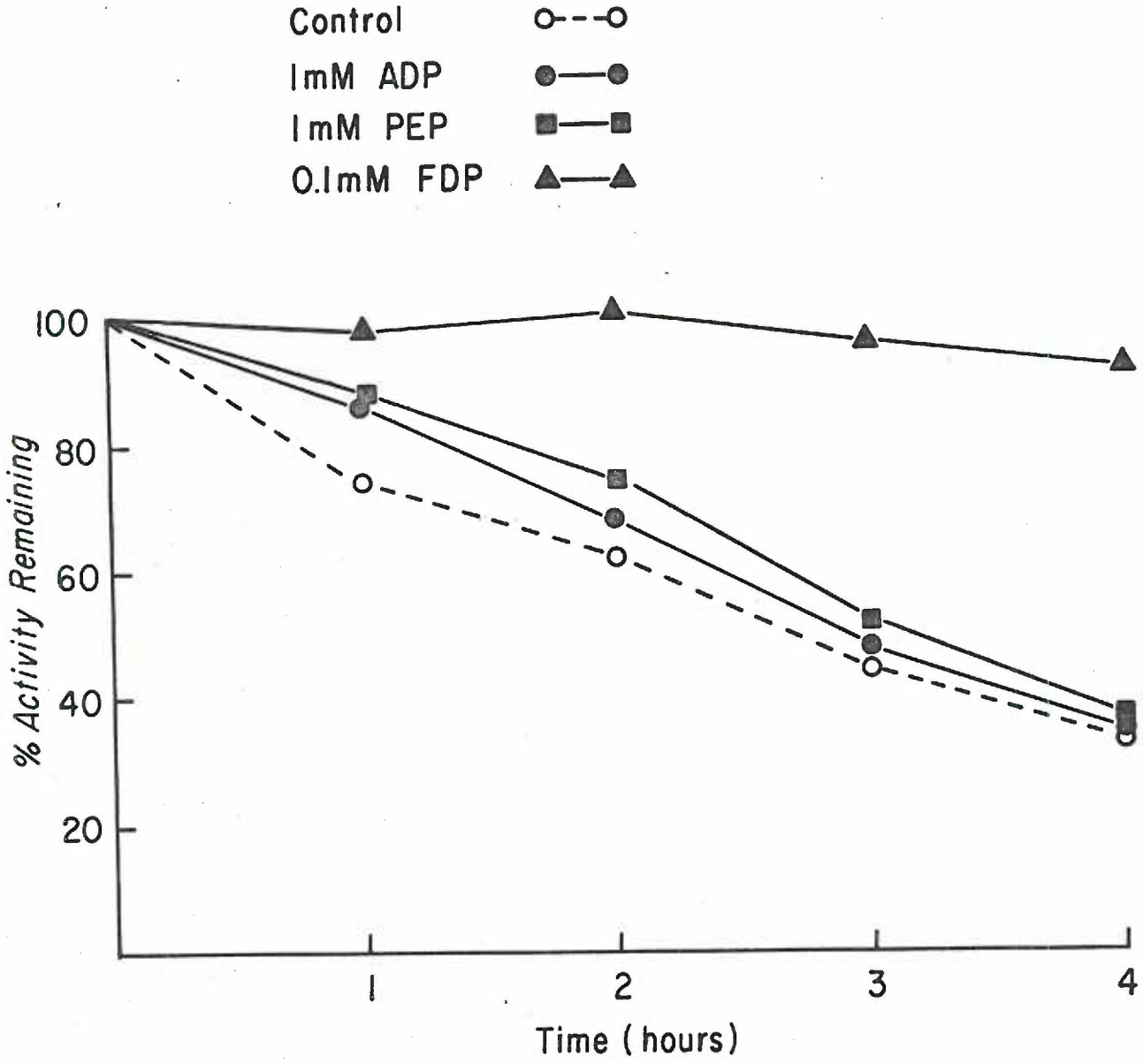

37° C
25° C
4° C
100
80
60
40
20
% Activity Remaining
1
2
3
4
Time (hours)

Figure 26

Stability of human M_2-pyruvate kinase in the presence of substrates and effectors. The sample, a 50%-65% $(NH_4)_2SO_4$ precipitate of a human kidney extract (step 2), was diluted to the proper concentration with 25 mM Tris-HCl, pH 7.5, and incubated at 4°C in the presence of the following effectors: control (O——O); 1 mM ADP (●—●); 1 mM PEP (▩—▩); 0.1 mM FDP (▲—▲). The samples were assayed by a coupled-assay method (see "Methods" for conditions) at 1 hour intervals for 4 hours. Each point is the average of duplicate assays and is expressed as a percent of the remaining activity with time, where time 0 = 100%.


Control
1mM ADP
1mM PEP
0.1mM FDP
100
80
60
40
20
% Activity Remaining
1
2
3
4
Time (hours)

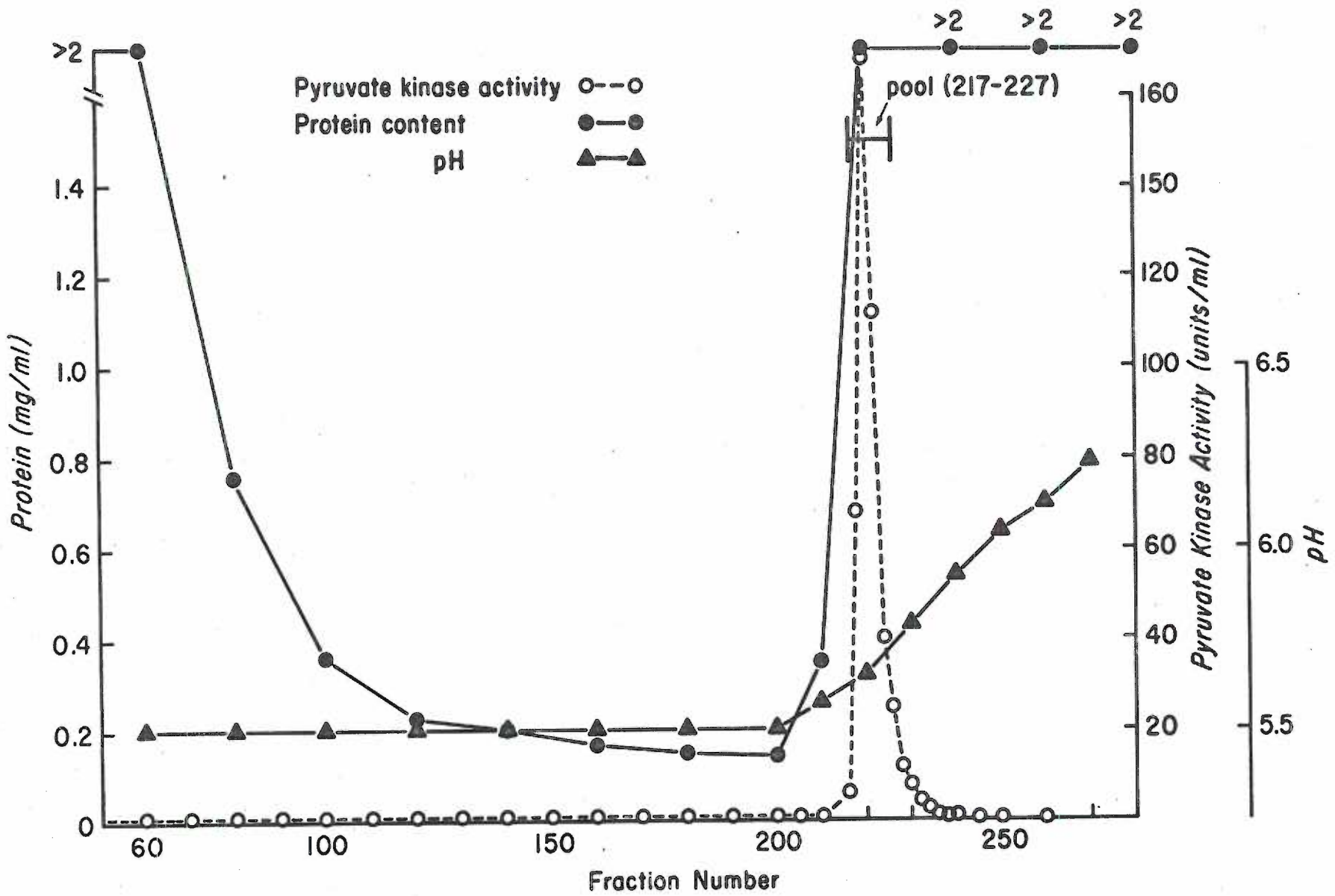
desalted on a 5 x 100 cm Sephadex G-25 column equilibrated with CM-column buffer, and then applied to a 2.5 x 40 cm column of CM-cellulose packed to a bed height of 35 cm. The column was washed with 1 liter of CM-column buffer at a flow rate of 100 ml per hour using a Sigmamotor peristaltic pump. When the protein content of the effluent fractions was below 200 µg/ml, a linear pH gradient was applied. The gradient buffers consisted of 500 ml of the CM-column buffer, pH 5.5, and 500 ml of the same buffer titrated to pH 7.0 with 2M KOH. Ten milliliter fractions were collected and assayed for protein by the Lowry-phenol method (due to the strong ultraviolet absorbance of maleic acid), and for pyruvate kinase activity (Figure 27). The pH gradient was monitored at 4°C with a Radiometer model 26 pH meter. Fractions 217-227 were pooled, assayed, and stored at 4°C as a 75% ammonium sulfate suspension.

d. Cibacron blue F3GA-Sepharose 6B affinity chromatography of rabbit muscle pyruvate kinase

To test the ability of the coupled Sepharose 6B resin (see "Methods") to bind pyruvate kinase in an affinity column, a modified procedure of Easterday and Easterday (164) was adopted using rabbit muscle pyruvate kinase. At 4°C, the Cibacron blue F3GA-Sepharose 6B resin was poured into a 2.5 x 40 cm column to a bed height of 20 cm and equilibrated thoroughly with starting column buffer. The buffer consisted of 20 mM Tris-HCl pH 8.5, 5 mM $MgCl_2$, $2x10^{-3}$ mM 2-mercaptoethanol, 0.4 mM EDTA, and 5 mM KCl. Eight hundred

Figure 27

CM-cellulose chromatography of human M_2-pyruvate kinase. The product of step 2 was prepared and equilibrated with the CM-column buffer, 33 mM potassium maleate, pH 5.5, 2 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM FDP. The sample was applied to a 2.5 x 40 cm column (bed height = 35 cm) of CM-cellulose at a flow rate of 100 ml/hour, using a peristaltic pump. The column was washed with CM-column buffer until the effluent fractions contained 200 µg/ml protein or less, and then a linear pH gradient was applied with 500 ml of starting CM-column buffer, pH 5.5, and 500 ml of starting column buffer titrated to pH 7.0 with 2M KOH. Ten milliliter fractions were assayed for pyruvate kinase (O----O), protein by the Lowry-phenol method (●—●), and for pH at 4°C (▲—▲).

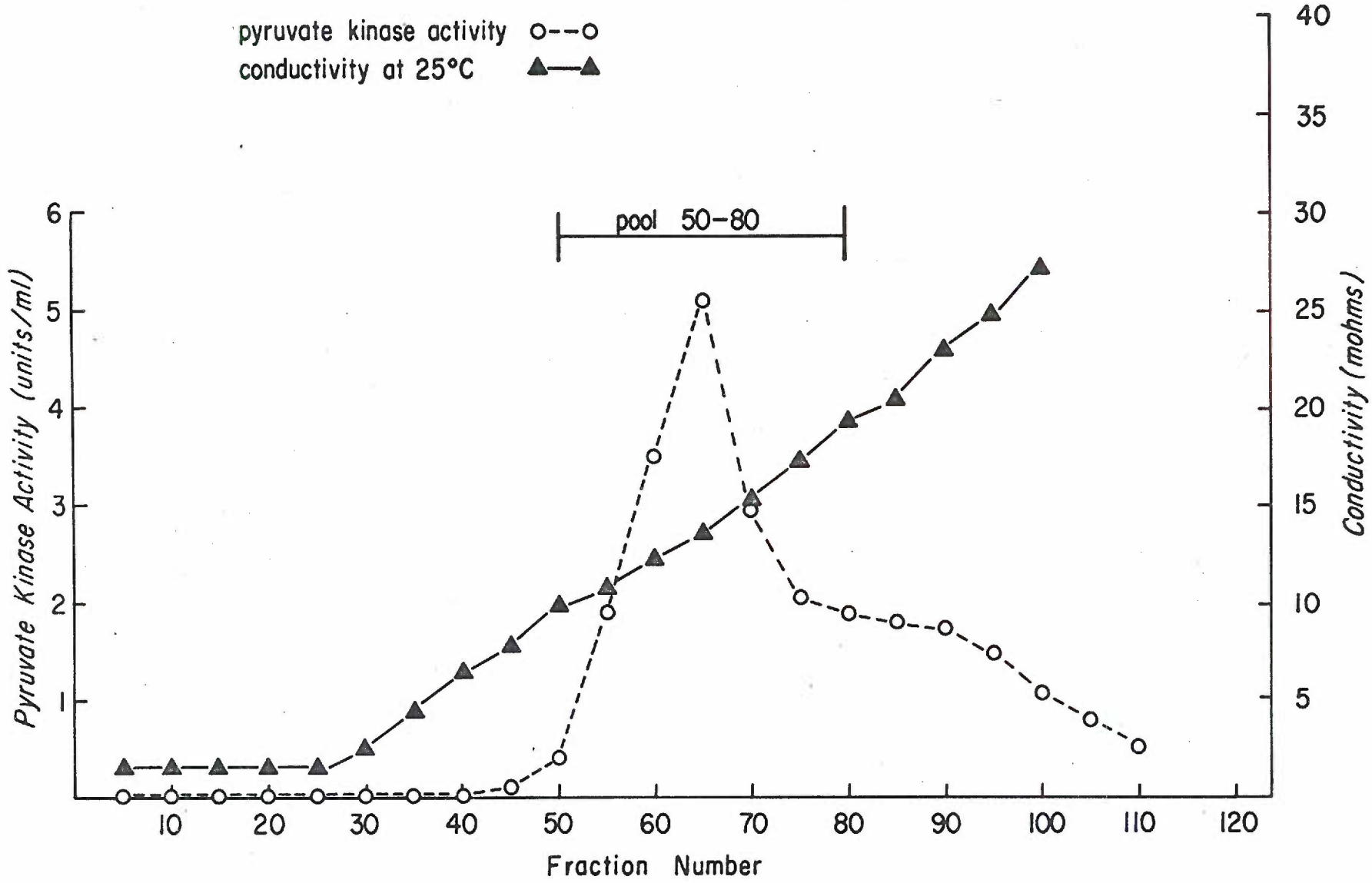

Pyruvate kinase activity
Protein content
pH
pool (217-227)
>2
>2
>2
>2
Protein (mg/ml)
Pyruvate Kinase Activity (units/ml)
pH
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
20
40
60
80
100
120
150
160
5.5
6.0
6.5
60
100
150
200
250
Fraction Number

units of rabbit muscle pyruvate kinase in a volume of 2 ml were desalted on a 2.5 x 100 cm Sephadex G-25 column equilibrated with starting buffer, and applied to the affinity column at a flow rate of 60 ml per hour. The column was washed with 200 ml of starting column buffer, and then a linear KCl gradient from 5 mM KCl to 750 mM KCl was applied. The gradient was developed from 500 ml of starting buffer with 5 mM KCl and 500 ml of starting buffer with 750 mM KCl. Fractions of 10 ml were collected and assayed for pyruvate kinase activity and for conductivity at 25°C with a Radiometer model CDM2d conductivity meter (Figure 28). The enzyme was eluted off in a 95% yield at a conductivity of 7.5 mohms. This corresponded to a KCl concentration of approximately 140 mM.

The results of the inhibition studies of pyruvate kinase by Cibacron blue F3GA indicated that the inhibition is competitive with the substrate ADP. Therefore ADP was a reasonable choice for the specific elution of pyruvate kinase off the affinity column. From the previous KCl gradient elution of the rabbit muscle enzyme from the affinity column, a 100 mM KCl starting buffer was chosen as the salt concentration at which the enzyme would adhere to the column, and yet be able to be specifically eluted off by 10 mM ADP. The F3GA-Sepharose 6B column was washed (see "Methods") and re-equilibrated with a starting buffer of the same composition as before, except that 100 mM KCl was included. Fractions 50 through 80 from the previous affinity column (Figure 28) were pooled and concentrated

Figure 28

Affinity chromatography of rabbit muscle pyruvate kinase: KCl gradient elution. Eight hundred units of pure rabbit muscle pyruvate kinase were desalted and equilibrated with starting buffer, containing 20 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, $2x10^{-3}$ mM 2-mercaptoethanol, 0.4 mM EDTA, and 5 mM KCl. The sample was applied to a 2.5 x 40 cm column (bed height = 20 cm) of Cibacron blue F3GA-Sepharose 6B at a flow rate of 60 ml/hr. The column was washed with 200 ml of starting buffer and then a linear KCl gradient was applied with 500 ml of starting column buffer at 5 mM KCl and 500 ml of starting column buffer at 750 mM KCl. Ten milliliter effluent fractions were assayed for pyruvate kinase (O----O) and for conductivity at 25°C (▲—▲).


pyruvate kinase activity
conductivity at 25°C
pool 50-80
Pyruvate Kinase Activity (units/ml)
Conductivity (mohms)
Fraction Number
1
2
3
4
5
6
5
10
15
20
25
30
35
40
10
20
30
40
50
60
70
80
90
100
110
120

to a volume of 20 ml. The sample was equilibrated to the proper conductivity by Sephadex G-25 column chromatography and applied to the F3GA-Sepharose 6B column at a flow rate of 60 ml per hour. The enzyme adhered to the F3GA-Sepharose and the column was washed with 300 ml of the starting buffer with 100 mM KCl. The column was then washed with 250 ml of starting buffer containing 100 mM KCl and 10 mM ADP (fermentation grade). Ten milliliter fractions were assayed for pyruvate kinase activity and conductivity (Figure 29). The enzyme was eluted in a narrow peak containing 95% of the original activity.

e. Step 4: Cibacron blue F3GA-Sepharose 6B affinity chromatography of M_2-pyruvate kinase

A procedure analagous to the one described above for rabbit muscle pyruvate kinase was used to further fractionate human M_2-pyruvate kinase. One of the complications which arose in applying affinity chromatography to the purification of this isoenzyme is the presence of multiple pyruvate kinase activities in the human kidney. Horizontal thin layer polyacrylamide electrophoresis was used to monitor the fractionation of the M_2-isozyme. Figure 30 shows the electrophoretic pattern for different fractions of human kidney and liver pyruvate kinase isoenzymes. Slot 1, a crude extract of human kidney, shows a strong M_2-pyruvate kinase band near the origin and three other faint bands of activity distributed toward the anode. Slot 2, a 50% ammonium sulfate supernatant, shows a strong band of

Figure 29

Affinity chromatography of rabbit muscle pyruvate kinase: ADP elution. Fractions 50-80 from the previous affinity column (Fig. 28) were pooled, concentrated, and re-equilibrated with the same affinity column starting buffer (see "Results"), except that the KCl concentration was 100 mM. The sample was applied to a 2.5 x 40 cm column (bed height = 20 cm) of Cibacron blue F3GA-Sepharose 6B equilibrated with starting buffer. The column was washed with 300 ml of starting buffer at a flow rate of 60 ml/hr, and then with 250 ml of starting buffer containing 10 mM ADP. Ten milliliter effluent fractions were assayed for pyruvate kinase (O----O) and for conductivity at 25°C (▲—▲).

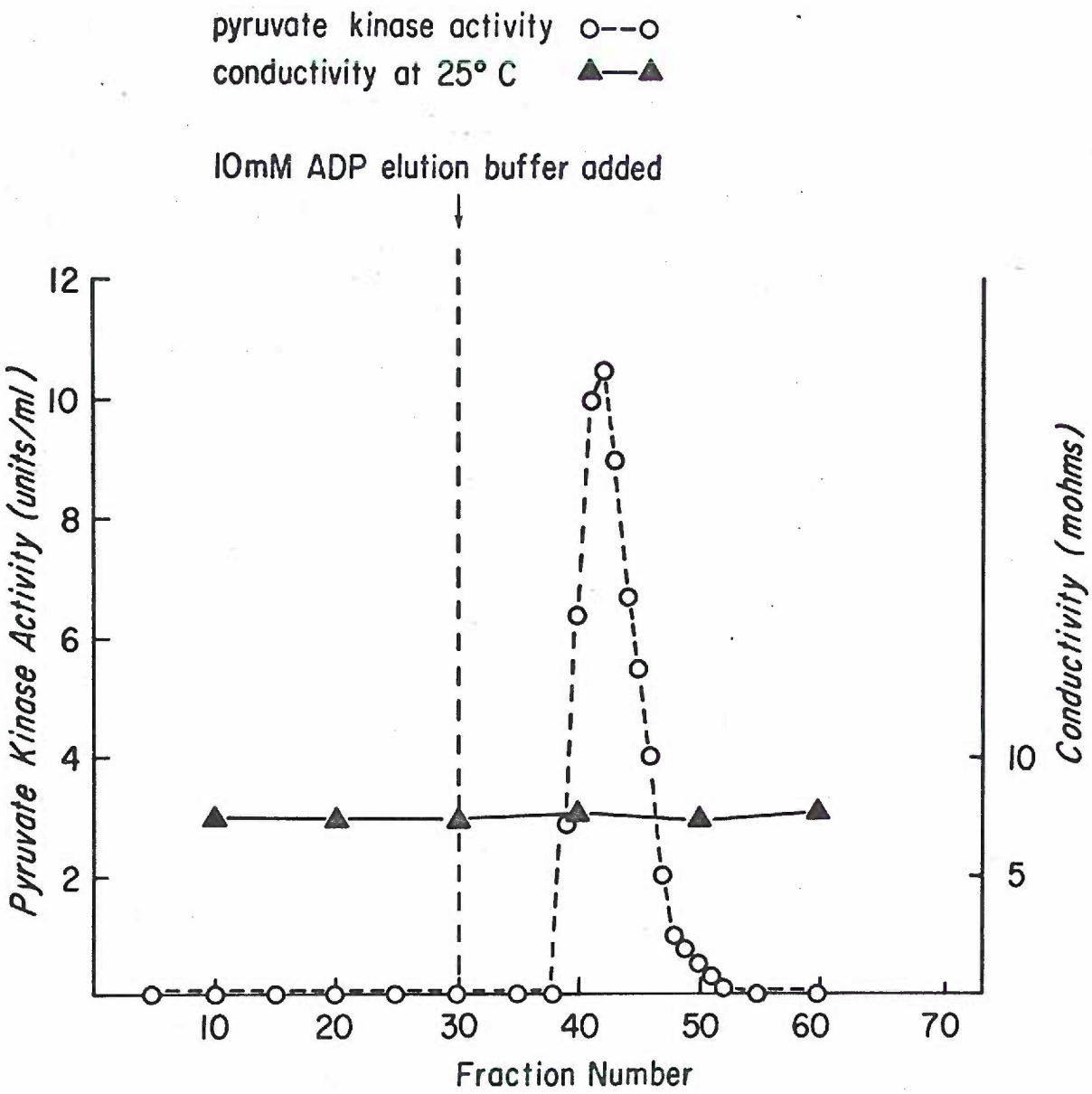
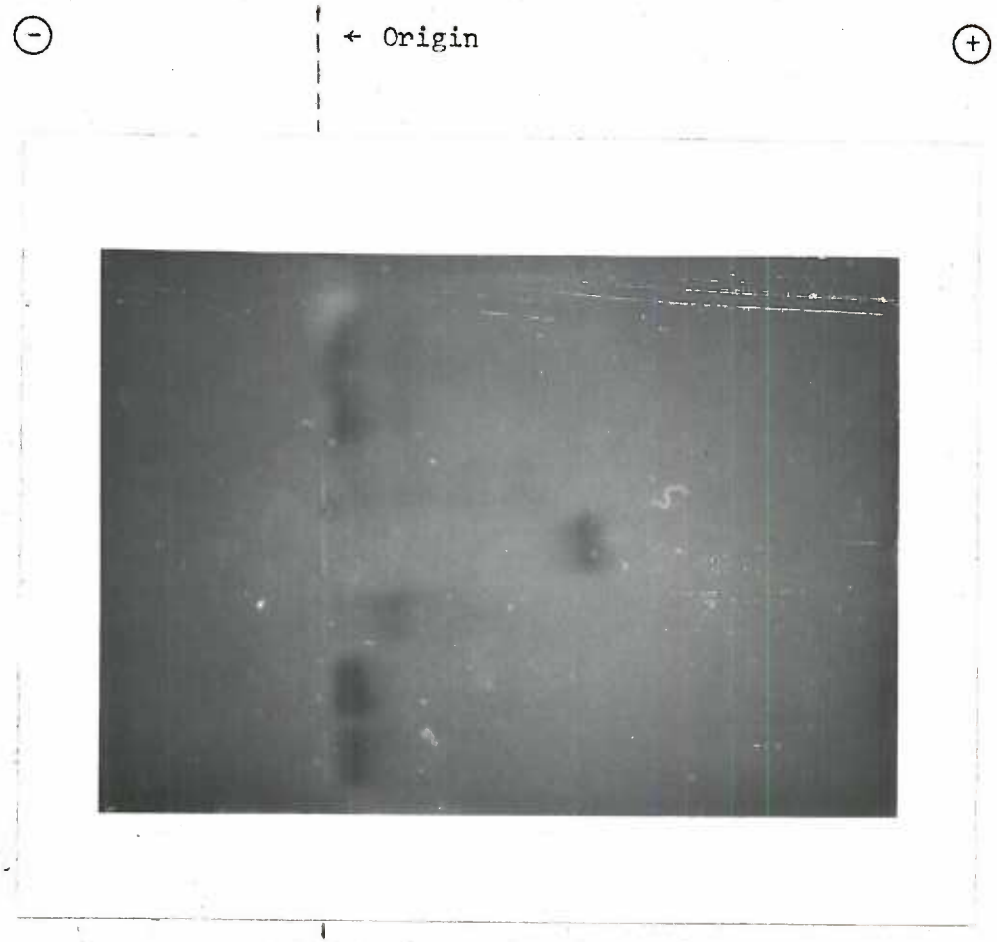

pyruvate kinase activity
conductivity at 25° C
10mM ADP elution buffer added
Pyruvate Kinase Activity (units/ml)
Conductivity (mohms)
12
10
8
6
4
2
10
5
10
20
30
40
50
60
70
Fraction Number

Figure 30

Horizontal thin layer polyacrylamide gel electrophoresis of human kidney and liver pyruvate kinase preparations. Sample preparation, electrophoresis, and visualization of pyruvate kinase activity was carried out as described (see "Methods"). The samples were: slot 1, a human kidney extract (step 1); slot 2, a 50% $(NH_4)_2SO_4$ supernatant of a human kidney extract; slot 3, a 20%-40% $(NH_4)_2SO_4$ precipitate of a human kidney extract; slot 4, a 20%-40% $(NH_4)_2SO_4$ precipitate of a human liver extract; slot 5, Peak A, from pool 140-155 (see Fig. 31); slot 6, Peaks B and C, from pool 171-240 (see Fig. 31); slot 7, Peak 2, from pool 150-160 (see Fig. 32).

slot number

1. Human kidney extract
2. 50% $(NH_4)_2SO_4$ kidney sup.
3. 20%-40% $(NH_4)_2SO_4$ kidney ppt.
4. 20%-40% $(NH_4)_2SO_4$ liver ppt.
5. Peak A
6. Peaks B and C
7. Peak 2



of M_2-pyruvate kinase near the origin in addition to some remaining bands of activity which were not removed by ammonium sulfate fractionation. Slot 3 is a 20% to 40% ammonium sulfate precipitate of human kidney which shows three bands of pyruvate kinase activity in addition to the M_2-band. Slot 4 is a 20% to 40% ammonium sulfate precipitate from human liver, and reveals a single strong band of the L-isozyme midway between the origin and the anode. The results indicate that at least three other forms of human kidney pyruvate kinase, in addition to the M_2-isozyme, are resolved by this method of electrophoresis. Furthermore, the results show that a 50% to 65% ammonium sulfate fractionation of a human kidney extract is not a sufficient means of separating the M_2-isozyme from these other forms of pyruvate kinase.

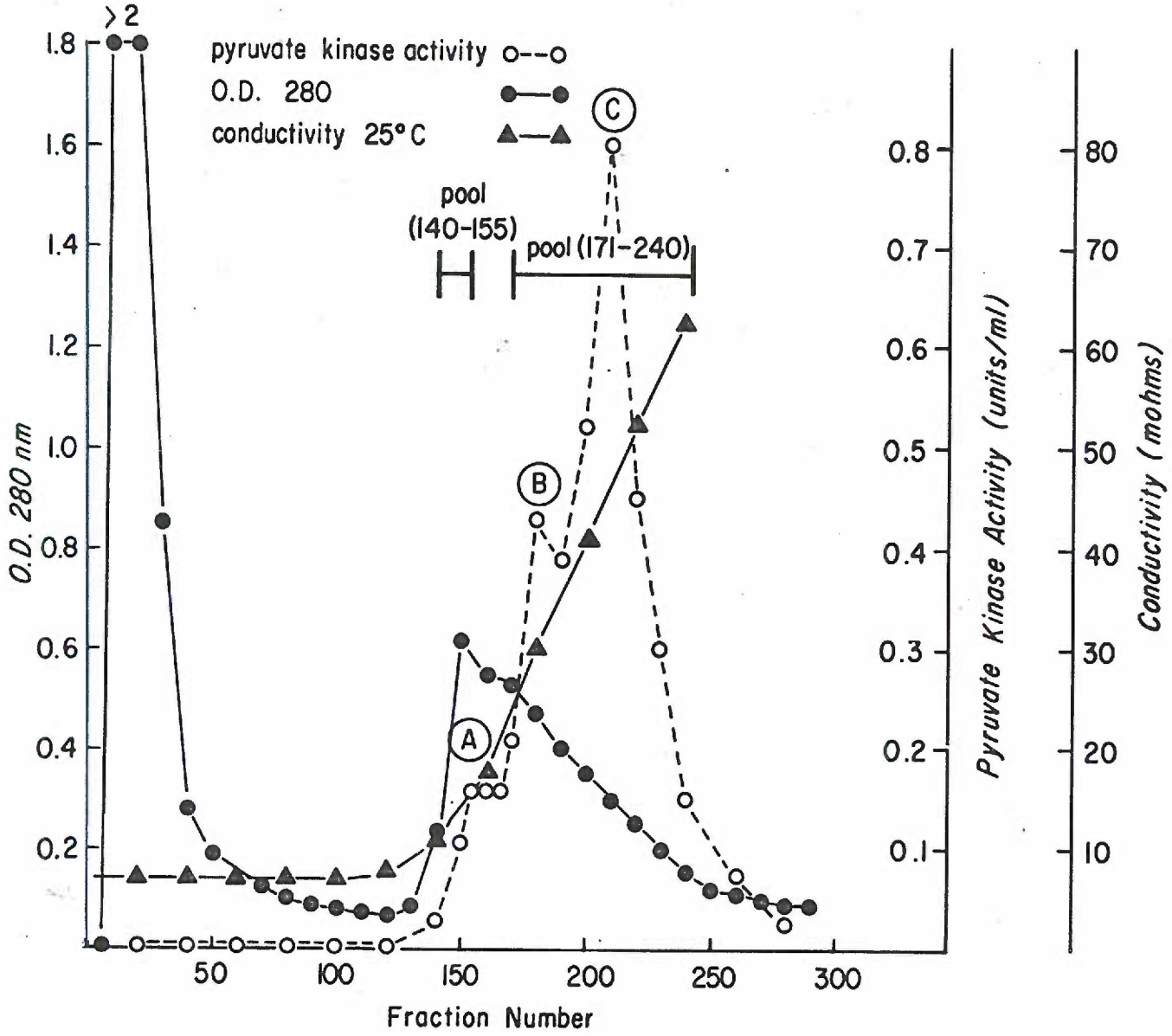
In an attempt to remove the residual forms of pyruvate kinase from the human kidney M_2-pyruvate kinase preparation, a F3GA-Sepharose 6B affinity column was prepared. The affinity column used for the experiments with rabbit muscle pyruvate kinase was regenerated, and then equilibrated with the same starting column buffer with 0.1 M KCl, except that 0.1 mM FDP was included to stabilize the M_2-isozyme. A sample of a 50% ammonium sulfate supernatant from human kidney was desalted and applied to the affinity column at a flow rate of 60 ml per hour. The column was washed with 1 liter of starting column buffer or until the absorbance at 280 nm was below 0.1 optical density. The column was then developed with a linear KCl gradient,

consisting of 1 liter of starting buffer at 0.1 M KCl and 1 liter of starting buffer at 2 M KCl. Ten milliliter fractions were collected and assayed for enzyme activity, protein absorbance, and conductivity (Figure 31). The elution profile shows three uncompletely resolved peaks of pyruvate kinase activity, designated as A, B, and C. Fractions 140 through 155 (peak A) and fractions 171-240 (peaks B and C) were pooled, concentrated, and analyzed by thin layer electrophoresis. The results are shown in slot 5 (peak A) and slot 6 (peaks B and C) in Figure 30. Peak A contains two bands of activity which correspond to two of the bands observed in the 20% to 40% ammonium sulfate precipitate of human kidney (slot 3). Slot 6, containing aliquots from peaks B and C, reveals one distinct band corresponding to type M_2-pyruvate kinase. The results show that the affinity column is an effective means of fractionating the M_2-isozyme from the other forms of pyruvate kinase in human kidney.

Inspection of the elution profile for the previous affinity column (Figure 31) indicated that the M_2-isozyme (peaks B and C) was resolved at a conductivity of approximately 22 mhos (measured at 25°C). This corresponds to a salt concentration of a little more than 0.3 M KCl. In order to verify that the M_2-isozyme could be fractionated from the other forms of pyruvate kinase isozymes at 0.3 M KCl, another F3GA-Sepharose 6B column was run under the same conditions as the previous column, except that the starting buffer and equilibration steps contained 0.3 M KCl. A sample,

Figure 31

Affinity chromatography of human kidney pyruvate kinase: 0.1M KCl to 2M KCl gradient elution. A 50% $(NH_4)_2SO_4$ supernatant of a human kidney extract (step 1) was concentrated, desalted and equilibrated with starting column buffer, 20 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, $2x10^{-3}$ mM 2-mercaptoethanol, 0.4 mM EDTA, 0.1 mM FDP and 0.1M KCl. The sample was applied to a Cibacron blue F3GA-Sepharose 6B column in the same manner as described (see "Results"). The column was washed with 1 liter of starting buffer, and then a linear KCl gradient was applied with 1 liter of starting buffer at 0.1M KCl and 1 liter of starting buffer at 2M KCl. Ten milliliter fractions were assayed for pyruvate kinase (O----O), optical density 280 nm (●—●), and for conductivity at 25°C (▲—▲). The three unresolved peaks of pyruvate kinase activity are designated as A, B, and C.

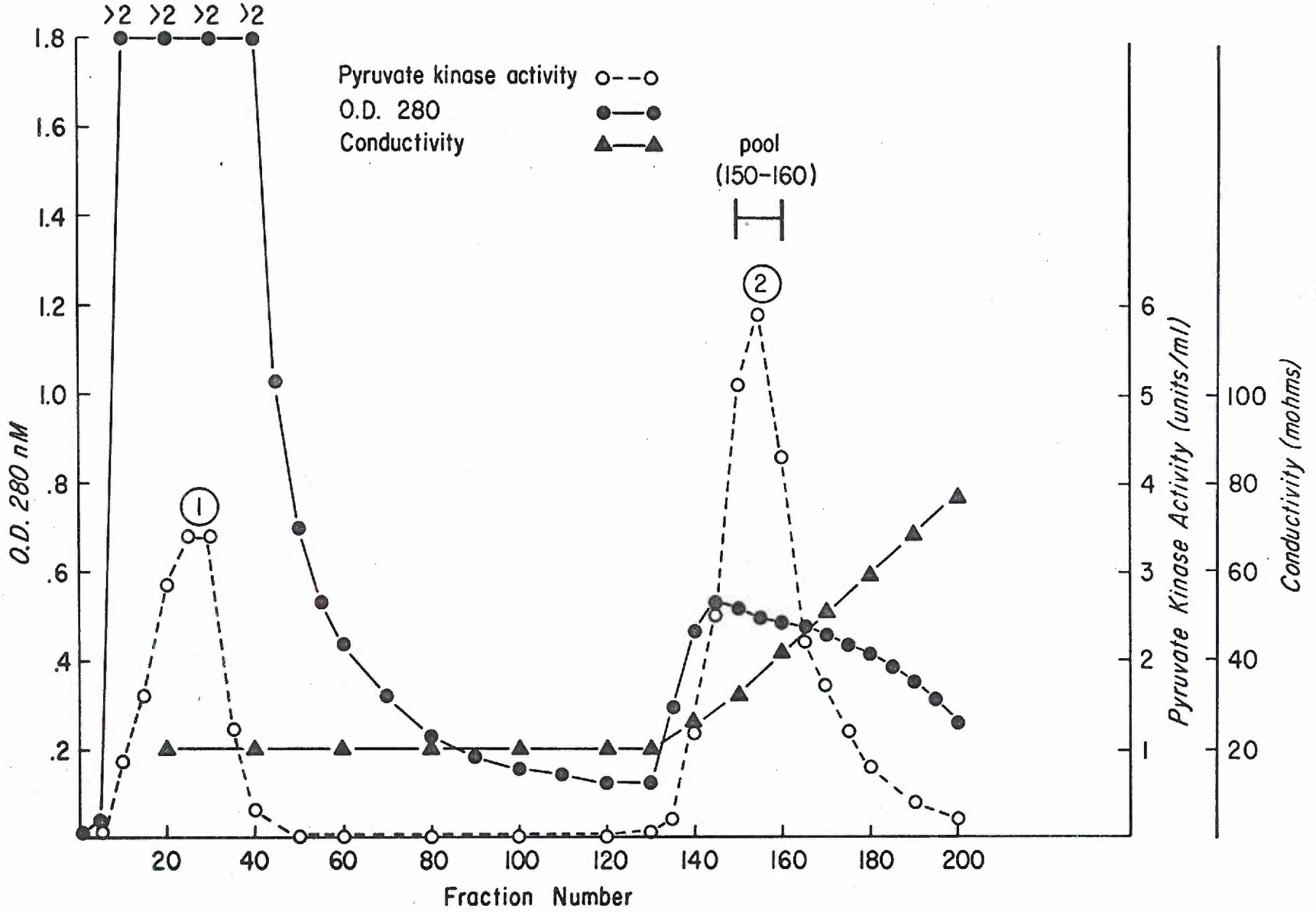

pyruvate kinase activity
O.D. 280
conductivity 25° C
pool (140-155)
pool (171-240)
A
B
C
O.D. 280 nm
Pyruvate Kinase Activity (units/ml)
Conductivity (mohms)
Fraction Number
>2
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
80
70
60
50
40
30
20
10
50
100
150
200
250
300

prepared in the same manner as for the previous column, was applied to the column, washed with starting buffer, and then a linear gradient, consisting of 500 ml of starting buffer with 0.3 M KCl and 500 ml of starting buffer with 2 M KCl was applied. The elution profile is shown in Figure 32, and reveals that two peaks of pyruvate kinase activity were resolved. Peak 1 did not adhere to the column at 0.3 M KCl and came off in the void volume. Peak 2 was bound to the column at 0.3 M KCl and was resolved at the beginning of the KCl gradient. Fractions 150 through 160 of peak 2 were pooled, concentrated, and examined on thin layer electrophoresis. The results are shown in slot 7, Figure 30, and confirm that the enzyme recovered as peak 2 was the M_2-pyruvate kinase isozyme.

The results of the previous two affinity chromatography columns, performed on human kidney pyruvate kinase preparations, established the conditions for the separation of the M_2-isozyme from the other forms of pyruvate kinase. In a manner analagous to the procedure for rabbit muscle pyruvate kinase, the conditions were also established for the specific elution by ADP of the M_2-isozyme from the affinity column. Though the M_2-isozyme could be fractionated by KCl gradient elution, ADP elution was chosen because of a much narrower elution profile and a higher yield. The Cibacron blue F3GA-Sepharose 6B affinity column was washed and equilibrated with starting buffer, consisting of 20 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 0.4 mM EDTA, $2x10^{-3}$ mM 2-mercaptoethanol, 0.1 mM FDP, and 0.3 M KCl. The

Figure 32

Affinity chromatography of human kidney pyruvate kinase: 0.3M KCl to 2M KCl gradient elution. The sample preparation and column procedure were the same as previously described (see Fig. 31 and "Results"), except that the starting column buffer contained 0.3M KCl, and the linear KCl gradient consisted of 500 ml of starting buffer at 0.3M KCl and 500 ml of starting buffer at 2M KCl. The pyruvate kinase peak resolved in the void volume is designated as peak 1, and the peak resolved during the gradient is designated as peak 2.


>2 >2 >2 >2
Pyruvate kinase activity
O.D. 280
Conductivity
pool
(150-160)
1
2
O.D. 280 nM
Pyruvate Kinase Activity (units/ml)
Conductivity (mohms)
Fraction Number
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
6
5
4
3
2
1
100
80
60
40
20
20
40
60
80
100
120
140
160
180
200

sample was the M_2-pyruvate kinase preparation, stored as a 75% ammonium sulfate suspension, from the CM-cellulose chromatography purification step (Step 3, Table 9). The sample was centrifuged, desalted on a 2.5 x 100 cm Sephadex G-25 column equilibrated with starting buffer, and applied to the affinity column at a flow rate of 100 ml per hour. The column was washed with starting buffer until the protein absorbance at 280 nm was 0.2 optical density or less, and then eluted with 300 ml of starting buffer containing 10 mM ADP (fermentation grade). Ten milliliter fractions were assayed for enzyme activity and protein (Figure 33) and fractions 88 through 93 were pooled, assayed, and stored as a 75% ammonium sulfate suspension.

f. Step 5: Sephadex G-200 chromatography of M_2-pyruvate kinase

The 75% ammonium sulfate suspension from the preceding affinity chromatography step was centrifuged, and the precipitate was dissolved in 25 mM Tris-HCl, pH 7.0, 2 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM FDP (Sigma grade). The sample was applied to a 1.5 x 100 cm column of Sephadex G-200 equilibrated with the sample buffer. Effluent fractions of 3.0 ml were assayed for protein at 280 nm and for pyruvate kinase activity (Figure 34). The specific activity was calculated by the number of units per mg protein, based upon the optical density at 280 nm. Those fractions (23 through 26) having the highest specific activity, approximately 200 units per

Figure 33

Affinity chromatography of human M_2-pyruvate kinase: ADP elution. The enzyme product from step 3 of the purification procedure was concentrated and equilibrated with affinity column starting buffer, containing 20 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, $2x10^{-3}$ mM 2-mercaptoethanol, 0.4 mM EDTA, 0.1 mM FDP, and 0.3M KCl. The sample was applied to a 2.5 x 40 cm column (bed height = 20 cm) of Cibacron blue F3GA-Sepharose 6B at a flow rate of 100 ml/hr. The column was washed with starting buffer until the effluent fractions had an absorbance at 280 nm of 0.2 or less, and then the column was washed with 300 ml of starting buffer containing 10 mM ADP. Ten milliliter fractions were assayed for pyruvate kinase (C----C) and for absorbance at 280 nm (●—●).

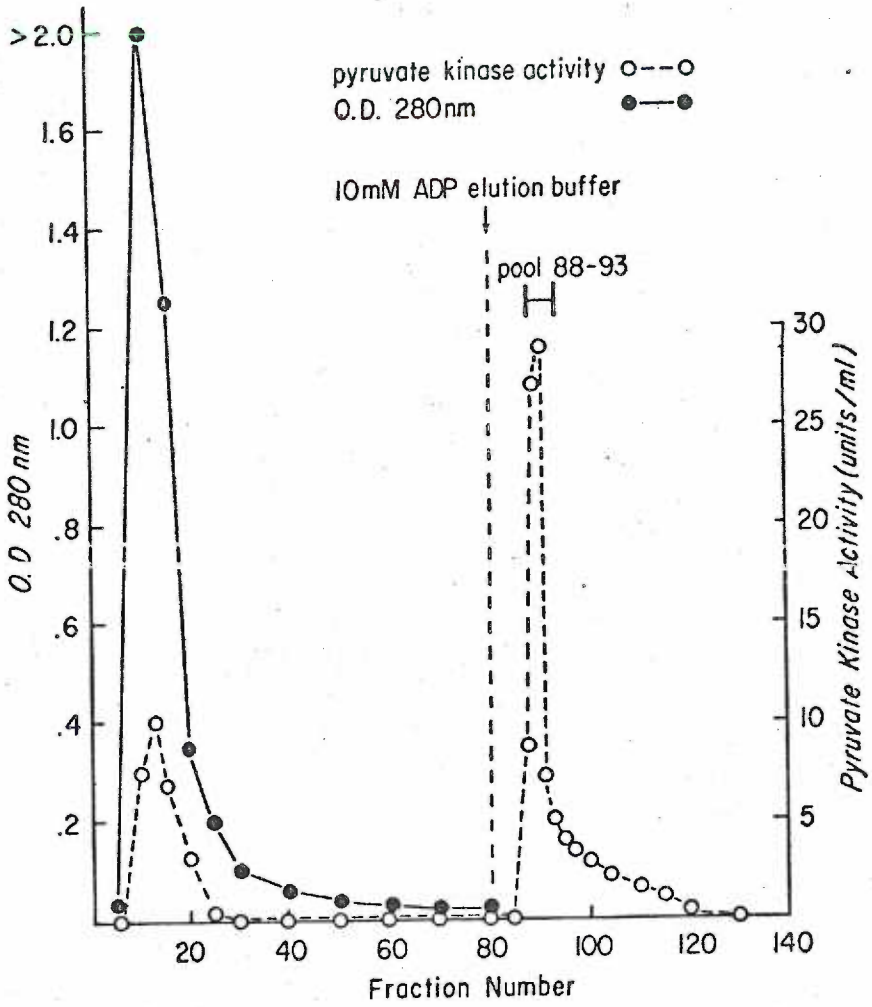
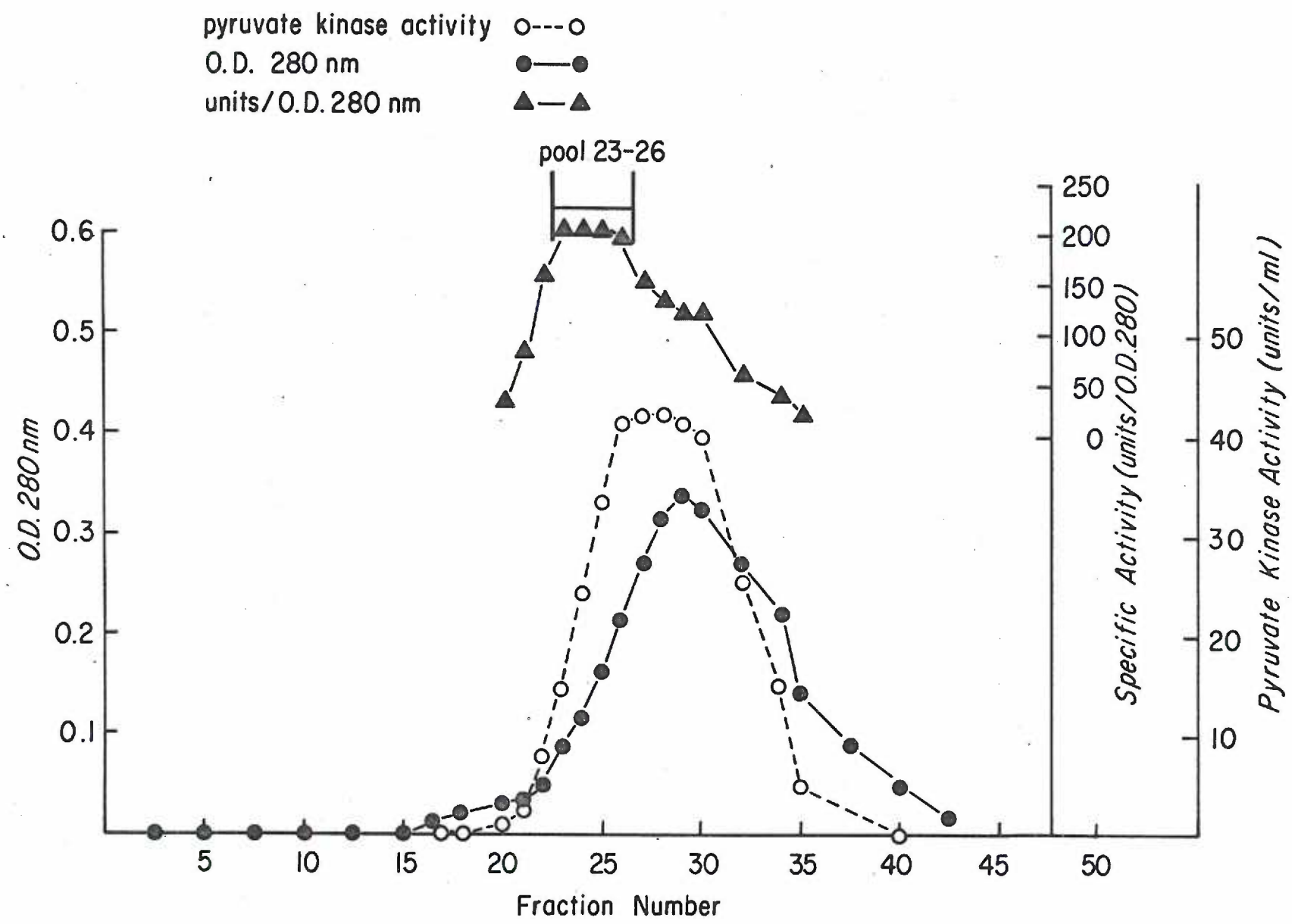

pyruvate kinase activity
O.D. 280nm
10mM ADP elution buffer
pool 88-93
O.D 280 nm
Pyruvate Kinase Activity (units/ml)
Fraction Number
>2.0
1.6
1.4
1.2
1.0
.8
.6
.4
.2
30
25
20
15
10
5
20
40
60
80
100
120
140

Figure 34

Sephadex G-200 chromatography of human M_2-pyruvate kinase. The enzyme product from step 4 (pool 88-93, Fig. 33) was concentrated by 75% $(NH_4)_2SO_4$ precipitation, dissolved in 25 mM Tris-HCl, pH 7.0, 2 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM FDP, and applied to a 1.5 x 100 cm column of Sephadex G-200. Fractions of 3.0 ml were assayed for pyruvate kinase (O---O) and for absorbance at 280 nm (●—●). The specific activity is expressed as units of pyruvate kinase/O.D. 280 nm (▲—▲).


pyruvate kinase activity
O.D. 280 nm
units/O.D. 280 nm
pool 23-26
O.D. 280 nm
Specific Activity (units/O.D.280)
Pyruvate Kinase Activity (units/ml)
Fraction Number
0.6
0.5
0.4
0.3
0.2
0.1
250
200
150
100
50
0
50
40
30
20
10
5
10
15
20
25
30
35
40
45
50

optical density unit at 280 nm, were pooled, assayed, and stored as a 75% ammonium sulfate suspension. This material was used for the characterization studies of the M_2-pyruvate kinase isozyme. Fractions 27 through 30 were rechromatographed on the Sephadex G-200 column by the same procedure. Those fractions having a specific activity of 200 units per optical density unit at 280 nm which were eluted from this column were pooled, assayed, and stored with the material from the first Sephadex G-200 column.

g. Summary of M_2-pyruvate kinase purification

Table 10 shows the yield and specific activities for the purification of M_2-pyruvate kinase, from 550 gms of starting material. The final product had a specific activity of 127.0 units per mg protein in the presence of 0.1 mM FDP, and represented an over-all yield of 3.7%.

h. Homogeneity and characterization studies of human M_2-pyruvate kinase

Disc gel electrophoresis

Polyacrylamide disc gel electrophoresis was performed in the presence of 0.1 mM FDP on the human M_2-pyruvate kinase preparation during certain steps of the fractionation procedure. The results are shown in Figure 35. Gel 1 contains the product of step 2, the 50% to 65% ammonium sulfate fractionation. Gel 2 contains the product of step 3, CM-cellulose chromatography. Gel 3 shows the results of affinity chromatography, step 4. Gel 4 shows a single major band and is the

Table 10. Purification of human M_2-pyruvate kinase

Purification Step	Total Units[a]	Yield (%)	Total Protein (mg)	Specific Activity (units/mg protein)[b]	Purification
1. Crude extract	23,150	100	85,740	0.27	1
2. 50-65% Ammonium sulfate precipitate	17,370	75	37,760	0.46	1.7
3. CM-cellulose chromatography	7,646	33	294	26.0	96.3
4. F3GA Affinity chromatography: ADP elution	1,325	5.7	12.9	103.0	381.5
5. Sephadex G-200 chromatography	849	3.7	6.7	127.0	470

[a]A unit of pyruvate kinase activity is defined as the amount of enzyme required to oxidize 1 µmole NADH per minute under the conditions for the coupled-assay conditions given under "Methods".

[b]Specific activity is defined as units per mg protein, where protein is measured by the modified Lowry-phenol method of Oyama and Eagle (162).

Figure 35

Polyacrylamide disc gel electrophoresis of human M_2-pyruvate kinase preparations. Electrophoresis was performed under the conditions described in the text (see Methods), with the addition of 0.1 mM FDP to the upper and lower gel buffers and to the gel. Gel 1, product of step 2; gel 2, product of step 3; gel 3, product of step 4; gel 4, product of step 5 (spacer gel is missing).

1 2 3 4

final product of step 5, Sephadex G-200 chromatography. The results indicate that the final product is homogeneous with respect to this method. The major band observed in gel 4 of Figure 35 was tested for pyruvate kinase activity in the presence of 0.1 mM FDP by slicing a duplicate gel immediately after electrophoresis into 4 mm slices, and assaying the slices subsequent to buffer elution. The results are shown in Figure 36 and reveal a single peak of pyruvate kinase activity, corresponding to the single protein band.

The inclusion of 0.1 mM FDP was shown to stabilize the human M_2-isoenzyme. Without the addition of this component to the purification buffers, the yields fell drastically. Figure 37 shows the product of purification from step 4 when 0.1 mM FDP was not a constituent of the fractionation buffers. The gel was run in duplicate and sliced and assayed for pyruvate kinase activity. The result shows a strong protein band having activity which was migrated over half the distance of the gel. Comparison with Figure 36 reveals that the presence or absence of 0.1 mM FDP results in a vast difference in migration rate toward the anode. In addition, since approximately 40 µg of protein were applied to each gel, the relative specific activities for the M_2-isozyme in the presence and absence of 0.1 mM FDP could be calculated from inspection of the assay of the gel slices in Figures 36 and 37. The results show that after electrophoresis the activity of the M_2-isozyme in the absence of 0.1 mM FDP is approximately 30% of the activity of the isozyme in the

Figure 36

Polyacrylamide disc gel electrophoresis of purified human M_2-pyruvate kinase in the presence of 0.1 mM FDP. The enzyme product from step 5 was run in duplicate. Immediately after electrophoresis, one gel was stained (photo), and the other gel was sliced into 4 mm sections, incubated, and assayed for pyruvate kinase (see "Methods"). Activity is expressed as units/ml. The spacer gel in the photo is missing.

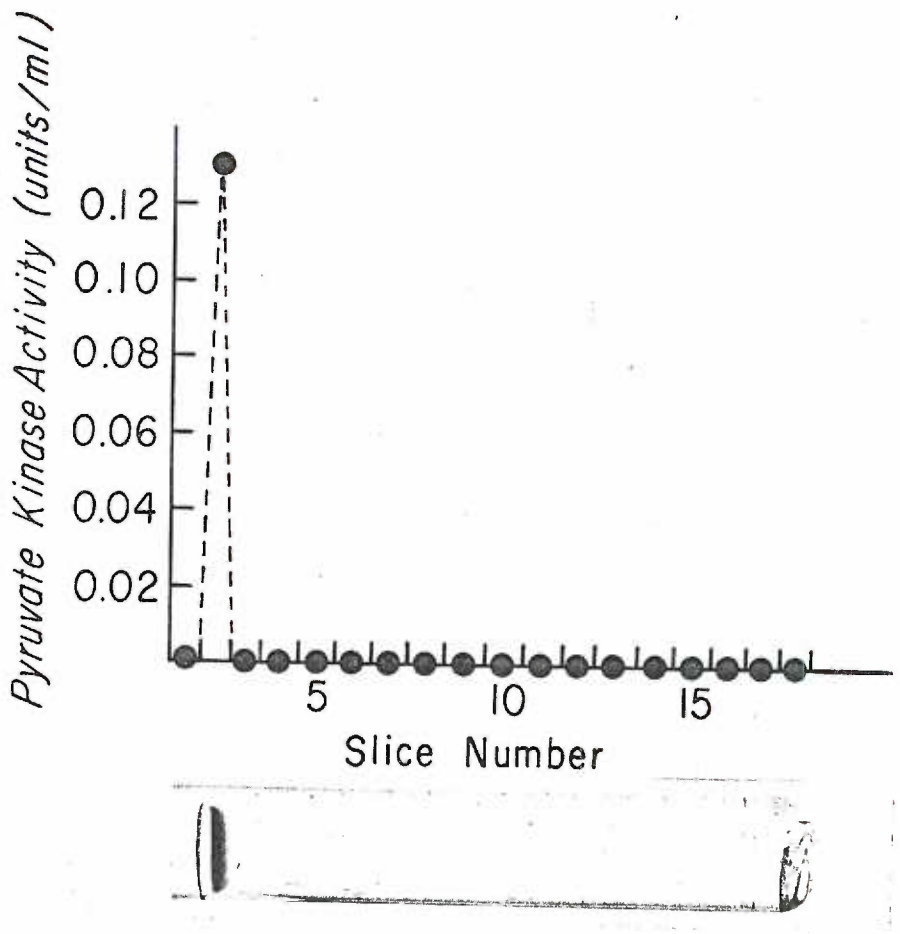
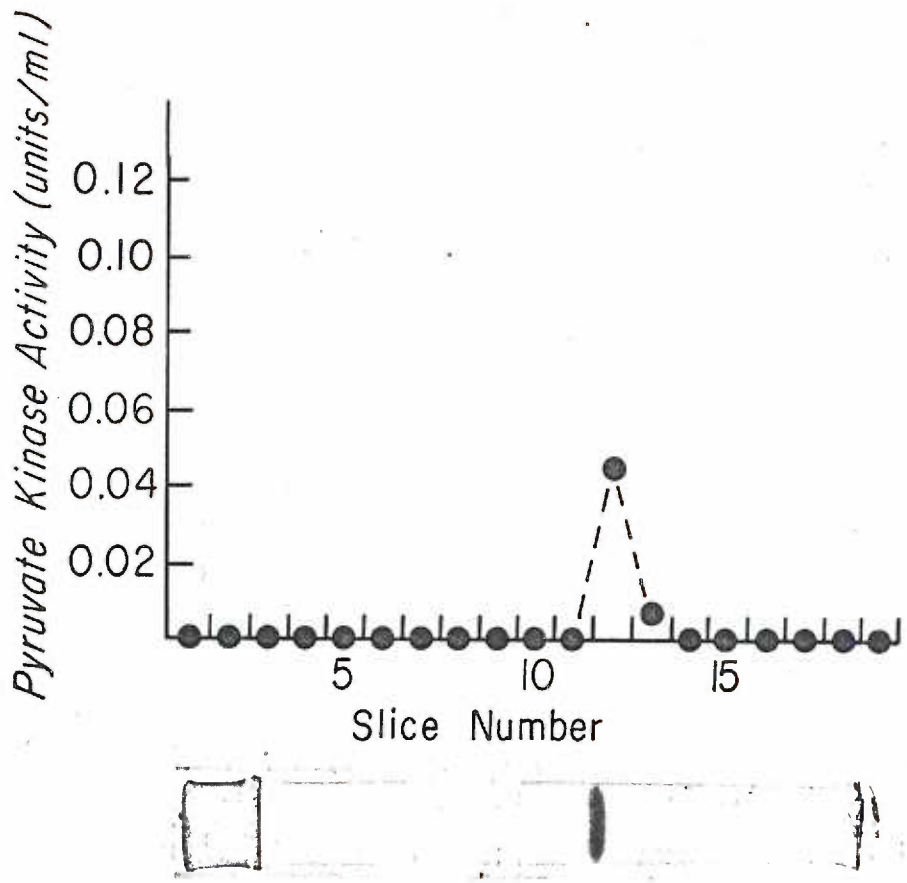

Pyruvate Kinase Activity (units/ml)
0.12
0.10
0.08
0.06
0.04
0.02
5
10
15
Slice Number

Figure 37

Polyacrylamide disc gel electrophoresis of human M_2-pyruvate kinase in the absence of 0.1 mM FDP. The enzyme product of step 4 prepared in the absence of 0.1 mM FDP was subjected in duplicate to disc gel electrophoresis run without 0.1 mM FDP. Immediately after electrophoresis one gel was stained (photo), and the other was sliced into 4 mm sections, incubated, and assayed for pyruvate kinase. Activity is expressed as units/ml.


Pyruvate Kinase Activity (units/ml)
0.12
0.10
0.08
0.06
0.04
0.02
5
10
15
Slice Number

presence of 0.1 mM FDP (0.046 u/ml/$_{0.130}$ u/ml). The polyacrylamide technique employed here separates molecules on the basis of charge and size. The significantly greater mobility of the enzyme in the absence of 0.1 mM FDP suggests that a major alteration of the molecule has occurred in either charge or size, which has resulted in a great decrease in activity.

SDS gel electrophoresis

The purified human M_2-pyruvate kinase preparation from step 5 was analyzed by disc gel electrophoresis in the presence of 0.1% SDS. This gel together with a set of gels containing protein standards is shown in Figure 38. Gel 2 contains the human M_2-isozyme, and reveals a single band, indicating homogeneity of the preparation and homogeneity of the M_2-subunits with respect to molecular weight. The calculation of the subunit molecular weight is shown in Figure 39, where the logarithm of the subunit molecular weights of the protein standards is plotted versus their mobility. Each standard was run in duplicate, and the points represent an average mobility. Two gels containing the M_2-isozyme were run and they exhibited mobilities of 0.325 and 0.350 with respect to the dye fronts. The average subunit weight for the human M_2-isozyme was calculated as 50,500 ± 2500 by interpolation of the best fit line through the points plotted for the standards.

Sedimentation velocity

A 2.65 mg/ml sample (determined by Lowry-phenol method)

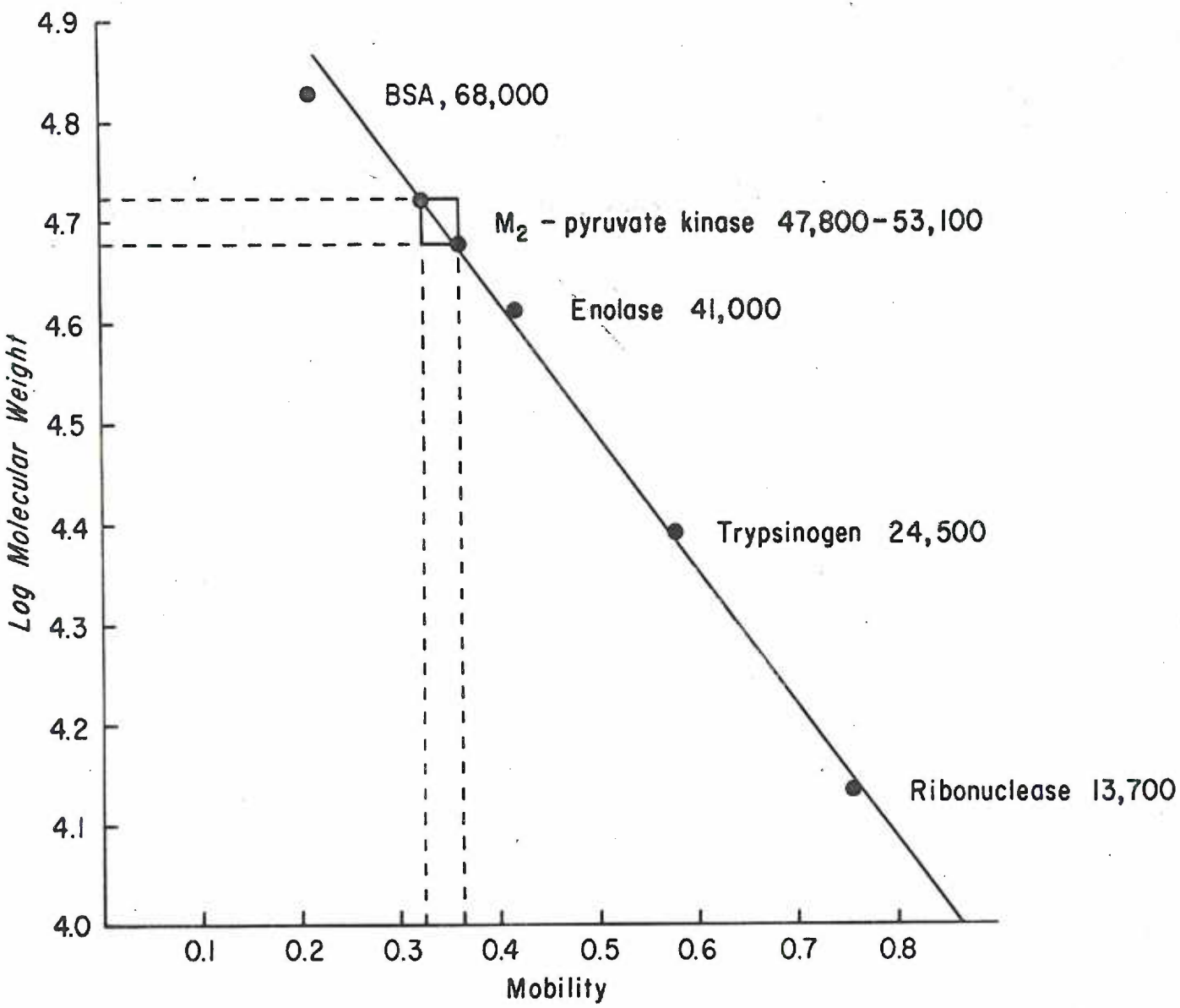
Figure 38

SDS gel electrophoresis of purified human M_2-pyruvate kinase and protein standards. Gel 1, bovine serum albumin; gel 2, purified M_2-pyruvate kinase (product of step 5); gel 3, enolase; gel 4, trypsinogen; gel 5, ribonuclease.

1 2 3 4 5

Figure 39

Subunit molecular weight determination of human M_2-pyruvate kinase. The mobility of the protein standards relative to the tracking dye from the SDS gel electrophoresis run is plotted versus the log of their subunit molecular weights. Each point is the average value for duplicate gels. The line was drawn through the points by the method of least squares.


4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4.0
Log Molecular Weight
BSA, 68,000
M2 - pyruvate kinase 47,800-53,100
Enolase 41,000
Trypsinogen 24,500
Ribonuclease 13,700
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Mobility

of the purified human M_2-isozyme from step 5 was dialyzed for 24 hours against 0.1M Miller's buffer, pH 7.2, with 0.1 mM FDP. Figure 40 is a photograph of the results taken through phase plate Schlieren optics. The results show a single symmetrical peak sedimenting with time, and indicate a homogeneous preparation. An observed sedimentation coefficient (s_{obs}) of 9.03S was obtained by calculating the migration of the apex of the peak with time. The $s_{20,w}$ was determined in the same manner as for the human M_1-pyruvate kinase isozyme [eq 5], except that additional values for the intrinsic viscosity and density of the solvent buffer had to be calculated because of the addition of 0.1 mM FDP. A value of 1.01296 was determined for $\eta/\eta o$ and 1.00261 for $\rho_{20,s}$.[4] The partial specific volume, $\bar{v}$, for the human M_2-pyruvate kinase isoenzyme was determined as 0.716 cc/gm, from the amino acid composition in the same manner as for the human M_1-isozyme [eq 6]. With these values a $s_{20,w}$ of 9.25S was obtained for the purified human M_2-pyruvate kinase isozyme.

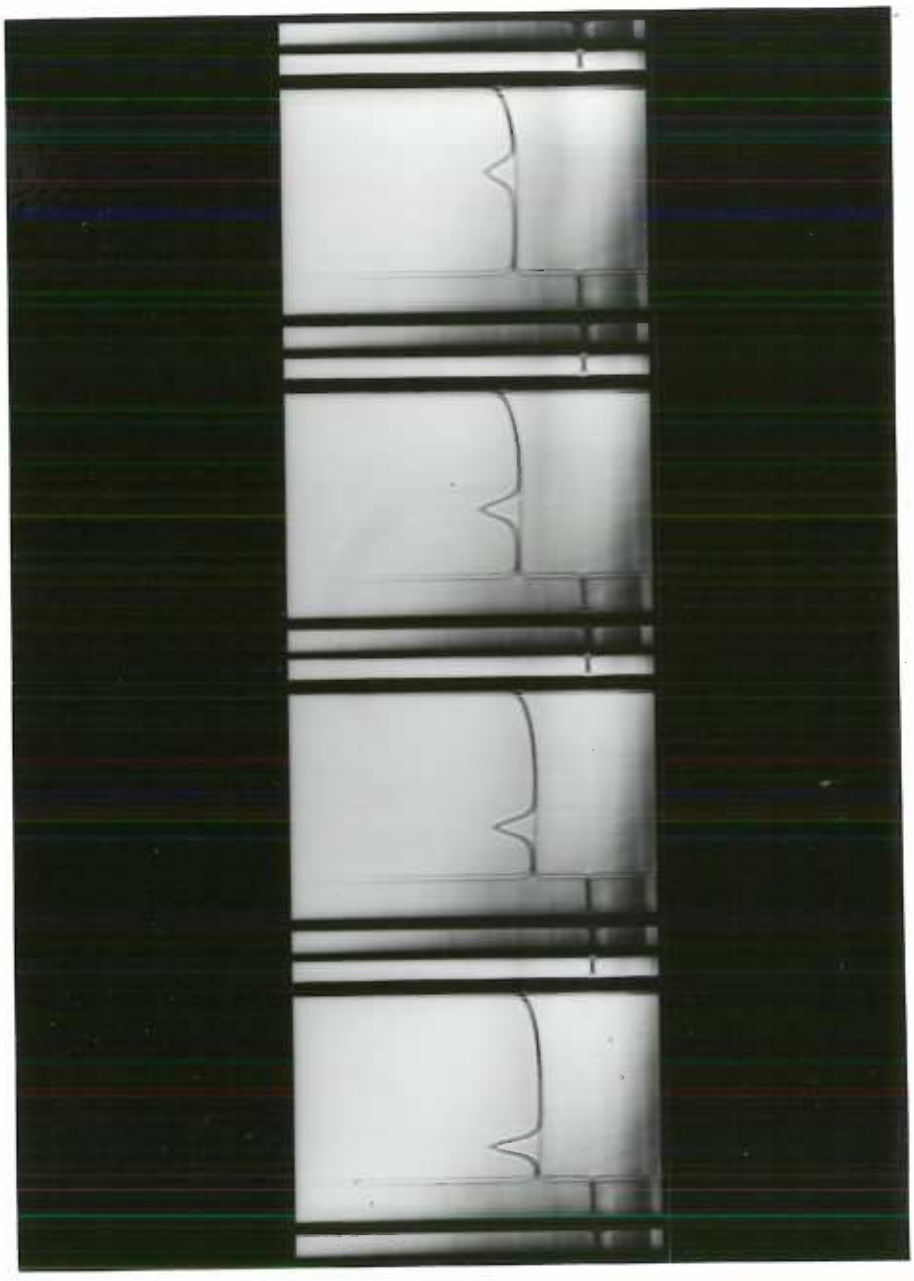
Sedimentation equilibrium

The same sample and buffer solvent used for the sedimentation velocity run was used to determine the molecular weight of the purified human M_2-pyruvate kinase isozyme by sedimentation equilibrium

[4]The values for $\eta/\eta o$ and $\rho_{20,s}$ for 0.1M Miller's buffer pH 7.2, containing 0.1 mM FDP were kindly determined by Dr. C. Rigas, Department of Biochemistry, University of Oregon Medical School.

Figure 40

Sedimentation boundary photograph of human M_2-pyruvate kinase through Schlieren optics. The product of step 5, at a concentration of 2.65 mg/ml, was dialyzed for 24 hours against 0.1M Miller's buffer, pH 7.2, containing 0.1 mM FDP before the run. The photos were taken as the boundary moved from left to right at 0, 8, 16, and 32 minutes after reaching speed.



analyses. By measuring the interference pattern photograph taken of the sample after equilibrium had been reached, a plot of log ΔY versus r^2 was made (Figure 41). The line drawn through the plot by the method of least squares had a correlation coefficient = 0.97826, a slope $\frac{d \log \Delta Y}{d\, r^2}$ = 0.35292, and a Y-intercept = -19.96373. By the same method used for the M_1-isozyme [eq 7], and using the values determined for $\bar{\nu}$ and $\rho_{20,s}$ for the solvent buffer with 0.1 mM FDP, a molecular weight of 206,700 ± 600 was obtained for human M_2-pyruvate kinase.

Immunodiffusion

The results of the immunodiffusion studies for human kidney M_2-pyruvate kinase are shown in Figure 42. The outer wells 1, 2, 3 and 4 contained antiserum prepared against purified human M_1-pyruvate kinase, while the center wells 5 and 7, and 6, contained human kidney M_2- and human muscle M_1-pyruvate kinase, respectively. The results show a cross-reaction between the M_2-isozyme and anti M_1-pyruvate kinase. Furthermore the precipitin line forms a complete line of identity, with no spurring, with the precipitin line between the M_1-isozyme and the anti M_1-pyruvate kinase. These results show that the human M_2-pyruvate kinase isozyme is immunologically indistinguishable from the human M_1-isozyme by this method, and that the M_2-isozyme contains all the immunological determinants of the M_1-isozyme. The shadowing observed between wells 7 and 3 and between wells 6 and 3 is due to the excess of antigen present in wells 6 and 7. The faint precipitin lines observed between wells 5

Figure 41

Sedimentation equilibrium plot of log ΔY versus r^2 for human M_2-pyruvate kinase. The same sample and sample buffer from the sedimentation velocity run was used for the sedimentation equilibrium analysis. The line through the points was determined by the method of least squares, and has a correlation coefficient = 0.97826, slope = 0.35292, and Y-intercept = -19.96373.

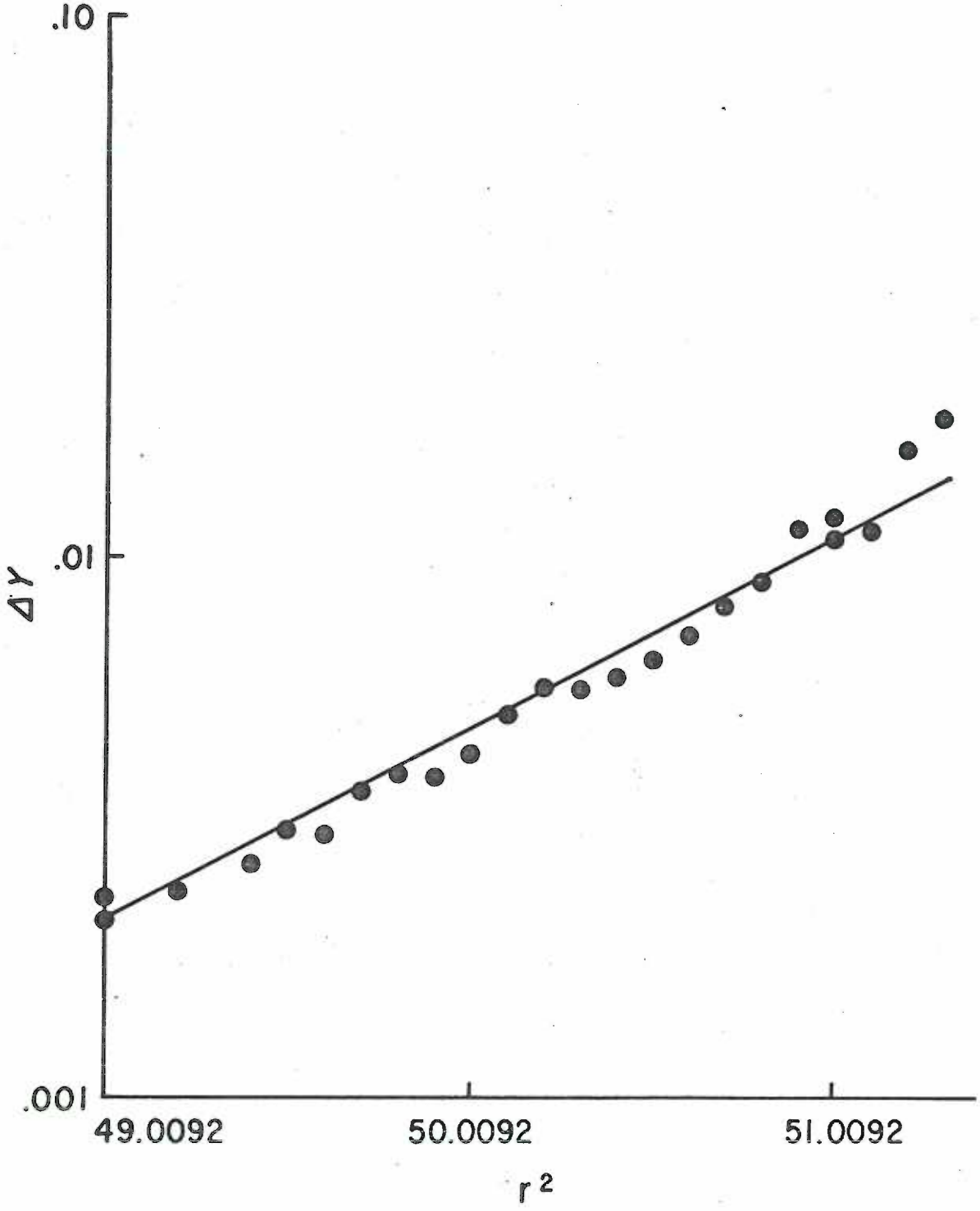
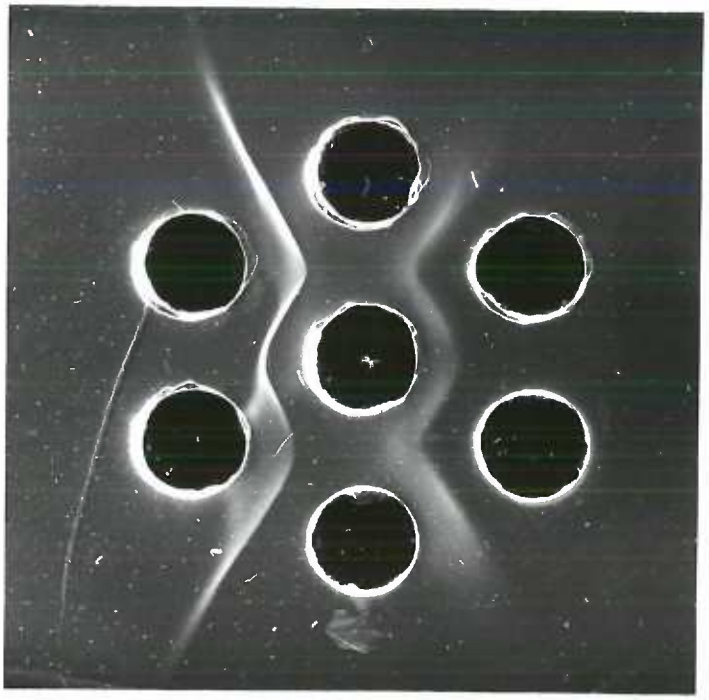

.10
.01
.001
ΔY
49.0092
50.0092
51.0092
r²

Figure 42

Immunodiffusion of human M_2-pyruvate kinase. Wells 1, 2, 3 and 4 contained antiserum prepared against purified human muscle M_1-pyruvate kinase. Each well contains antiserum obtained from different rabbits. Wells 5 and 7 contain human kidney M_2-pyruvate kinase from step 3 of the purification procedure. Well 6 contains purified human M_1-pyruvate kinase.



5

1 2

6

3 4

7

and 2, 6 and 2, 6 and 4, and between 4 and 7, are due to the low concentration of antibody present in wells 2 and 4.

NH_2-terminal analysis

Ten nmoles of purified M_2-pyruvate kinase were dansylated and examined for an NH_2-terminus in the same manner as for the M_1-isozyme. Figure 43 shows the results of chromatographing 1 nmole of the hydrolyzed, dansylated enzyme on a polyamide sheet. No dansylated amino acid was observed to separate out, other than the usual reaction products of dansylation, DNS-NH_2, DNS-OH, DNS-O-tyrosine, and DNS-ε-lysine. The results suggest that either the NH_2-terminus of this enzyme is blocked, or that the NH_2-terminus is proline or tryptophan, since the dansylated derivatives of these amino acids are destroyed by acid hydrolysis. The results also indicate that there are no contaminating proteins present in the M_2-preparation whose NH_2-terminus is detected by this method.

Amino acid composition

Table 11 shows the results of the amino acid analyses for human M_2-pyruvate kinase. The results are expressed as residues per mole of protein, where 1 mole = 206,700 gm, based upon the molecular weight obtained from ultracentrifuge analyses. There is an unusually low number of aromatic residues, which was expected from the discrepancy in measuring protein content by the Lowry-phenol method and by O.D. 280 nm.

Figure 43

Thin layer chromatography of dansylated human M_2-pyruvate kinase. One nmole of purified human M_2-pyruvate kinase (from step 5) was dansylated and chromatographed (see "Methods"). The chromatogram was developed with H_2O-90% formic acid (100:1.5) in the first direction and benzene-glacial acetic acid (9:1) in the second direction.

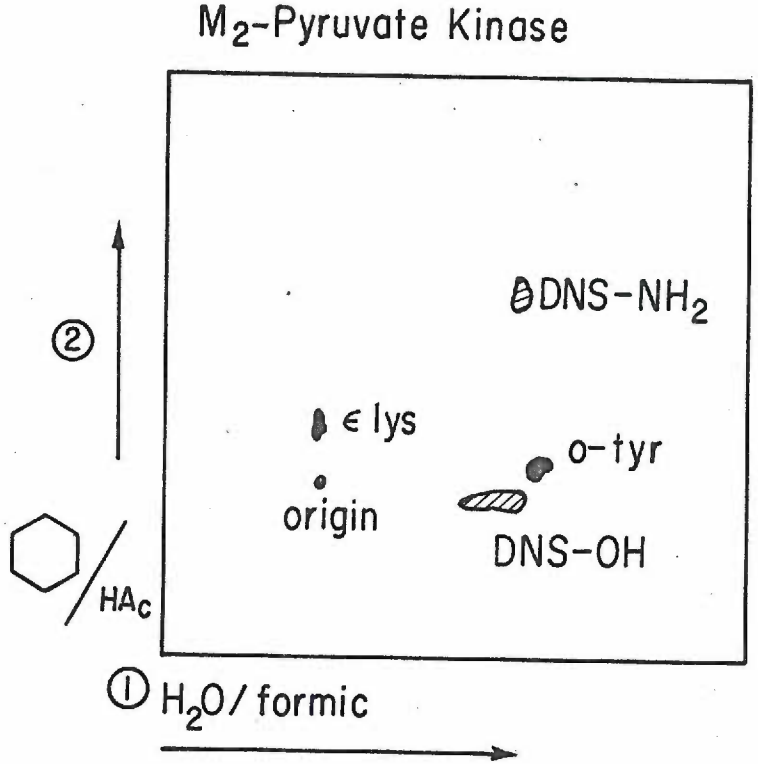

M2-Pyruvate Kinase
DNS-NH2
ε lys
o-tyr
origin
DNS-OH
②
HAc
① H2O/ formic

Table 11. Amino acid composition of human M_2-pyruvate kinase

Amino acid	24 hr	48 hr	72 hr	Average value[a]	Residues[b]
	µmoles/mg protein[c]				
Lysine	0.551	0.549	0.549	0.550	114
Histidine	0.211	0.210	0.205	0.208	43
Ammonia	1.015	1.029	1.044	1.001	207
Arginine	0.503	0.507	0.505	0.504	104
Aspartic acid	0.818	0.823	0.822	0.821	170
Threonine	0.465	0.460	0.441	0.474	98
Serine	0.525	0.516	0.499	0.537	111
Glutamic acid	0.848	0.853	0.869	0.857	177
Proline	0.432	0.435	0.439	0.436	90
Glycine	0.493	0.489	0.497	0.493	102
Alanine	0.829	0.816	0.807	0.818	169
Half-cystine[d]				0.242	50
Valine	0.713	0.721	0.722	0.722	149
Methionine[e]				0.469	97
Isoleucine	0.576	0.587	0.590	0.590	122
Leucine	0.621	0.636	0.656	0.638	132
Tyrosine	0.148	0.142	0.145	0.145	30
Phenylalanine	0.280	0.281	0.283	0.282	58
Tryptophan[f]				0.049	10

[a]The values for threonine, serine, and ammonia were obtained by extrapolation to zero time. The 72-hour values for valine and isoleucine were used.

[b]Calculated for a molecular weight of 206,700 obtained by sedimentation equilibrium analyses.

[c]The value presented is the average of duplicate analyses performed at each time interval.

[d]Determined as cysteic acid following performic acid oxidation (179).

[e]Determined as methionine sulfone following performic acid oxidation (179).

[f]Determined spectrophotometrically by the method of Goodman and Morton (180).

D. Discussion

1. Procedure for the purification of human muscle M_1-pyruvate kinase

a. Methodology

The procedure presented here for the purification of human M_1-pyruvate kinase involved the application of a number of additional steps to the method reported by Kubowitz and Ott (144). Though the enzyme preparation could be crystallized after the 32% to 45% acetone fractionation step, several bands were observed when the crystals were dissolved and examined by disc gel electrophoresis. To remove the contamination, two ion-exchange steps (steps 5 and 6) and a gel filtration step (step 7) were employed.

The same situation has been shown to occur with rabbit muscle pyruvate kinase prepared by the method of Tietz and Ochoa (7). Bondar and Pon (187) reported that rabbit muscle pyruvate kinase, prepared according to this method, gave a single symmetrical peak during sedimentation velocity ultracentrifugation, formed large and uniformly shaped crystals, but gave four bands on disc gel electrophoresis. By CM-Sephadex column chromatography, they were able to remove the contaminating bands, and obtain an enzyme preparation having a 19-fold increase in specific activity which gave a single band on disc gel electrophoresis.

b. Criteria of homogeneity

Because of the limitations inherent in the techniques

used to judge the purity of an enzyme preparation, the purified human M_1-isozyme of pyruvate kinase was examined for homogeneity by several criteria. Taken together, the results of disc gel electrophoresis in the absence and in the presence of SDS, sedimentation velocity and equilibrium analyses, NH_2-terminal analysis, and immunodiffusion studies show that the M_1-pyruvate kinase preparation is homogeneous.

2. Procedure for the purification of human kidney M_2-pyruvate kinase

a. Methodology

There were three fundamental problems which were encountered in attempting to establish a method of purification for the human M_2-pyruvate kinase isozyme: first, it was found that the human M_2-isozyme is very unstable, and therefore it was advantageous to attempt to stabilize the enzyme and also to devise a rapid purification procedure; second, since there was a limited availability of tissue, it was necessary that the purification method permit the fractionation of a workable amount of enzyme product from a small amount of starting material; third, the presence of several bands of pyruvate kinase activity in human kidney extracts, which were observed by thin layer gel electrophoresis, required a method of purification which was selective for the M_2-isozyme.

The addition of FDP to the purification media was a very effective means of stabilizing the M_2-isozyme. By including 0.1 mM FDP in all the buffers used during the purification procedure,

and by storing the enzyme preparation between steps as a 75% $(NH_4)_2SO_4$ suspension at 4°C, no significant loss of enzyme activity due to instability was observed.

The application of affinity chromatography as a purification step for the M_2-isozyme enabled the isolation of a pure enzyme product within three days. Furthermore, the high selectivity of the affinity resin for pyruvate kinase eliminated the necessity for a large number of purification steps which would result in a decreased yield. Approximately 7 mg of purified human M_2-pyruvate kinase could be obtained from 550 gms of starting material. Because of the differential affinity of the coupled Cibacron blue F3GA-Sepharose 6B resin for the different forms of pyruvate kinase found in human kidney, the M_2-isozyme could be selectively fractionated by adjusting the salt concentration of the column buffer.

The affinity column exhibited excellent flow properties and could be re-used several times merely by washing and re-equilibration. One disadvantage of the affinity resin concerned the elution of the pyruvate kinase isozymes from the column. Though KCl gradient elution was effective in releasing pyruvate kinase from the complex formed with the resin, and also in fractionating the M_2-isozyme from the other forms of pyruvate kinase in human kidney extracts, the elution profiles were very broad, covering up to five bed volumes. This same phenomenon was observed when gradient elution was attempted with ADP. To overcome the broad elution profiles, it

became necessary to employ a stepwise elution with 10 mM ADP.

b. Criteria of homogeneity

As with the human M_1-isozyme, the homogeneity of the human M_2-pyruvate kinase isozyme was determined by several methods. These included: a single protein band on disc gel electrophoresis in the absence and in the presence of SDS; a single migrating peak observed during sedimentation velocity analysis; a linear log ΔY versus r^2 plot obtained from sedimentation equilibrium analysis; and, the absence of an NH_2-terminal dansyl derivative observed by thin layer chromatography. Taken together, these results showed that the M_2-pyruvate kinase preparation was homogeneous.

3. Properties of human pyruvate kinase isozymes

a. The number of human pyruvate kinase isozymes.

It has generally been assumed that there are at least three non-interconvertible forms of pyruvate kinase in mammalian tissues. In 1968, Bigley et al. (46) examined human tissue extracts by horizontal starch gel electrophoresis and found three electrophoretically distinct forms of pyruvate kinase. This observation has been confirmed in this laboratory by purifying to homogeneity three distinct human pyruvate kinase isoenzymes. The human erythrocyte isozyme has been purified and characterized by Dr. C. J. Chern (130), and the purification and characterization of the human M_1- and M_2-isozymes are described in this text.

There are some studies which indicate that there may be

more than three distinct forms of human pyruvate kinase. The work of Bigley et al. (46) showed that there was no electrophoretic difference between the isozyme band observed from human liver extracts and the band observed from human erythrocytes. However, using polyacrylamide thin layer electrophoresis, Nakashima et al. (102) observed two bands of human erythrocyte pyruvate kinase activity, neither of which had the same mobility as the L-type pyruvate kinase isozyme from human liver. The two human erythrocyte pyruvate kinase bands could be converted, under special conditions, to a single band which was electrophoretically indistinguishable from the human L-type pyruvate kinase (long standing at 4°C, repeated freezing and thawing, and addition of liver extract). When extracts of human kidney are examined by polyacrylamide thin layer electrophoresis, the results presented in Figure 30 show that three bands of pyruvate kinase are resolved, in addition to the M_2-isozymes. Previous studies, using this same method, have detected only the L- and M_2-isozymes in both rat (44) and human tissues (101). It is possible that one or more of these multiple pyruvate kinase bands represents a distinct pyruvate kinase isozyme, not hitherto observed in mammalian tissues. On the other hand, it is possible that these additional activities represent different forms of the human M_1-, M_2-, and erythrocyte pyruvate kinase isozymes, which have been well established as distinct isozymes. This latter possibility seems to be the more plausible alternative in view of

the experiments which have shown: first, that mammalian pyruvate kinase isozymes can exist in more than one conformational state (52,91), depending upon the temperature (93) or the presence or absence of certain chemicals in the extraction buffers (84,92); second, that mammalian pyruvate kinase isozymes can be hybridized *in vitro* (48). In particular, there is electrophoretic evidence which suggests that the human M_1- and erythrocyte pyruvate kinase isozymes can form hybrids *in vitro* (personal communication).[5]

b. Kinetic studies

In mammalian tissues the multiple forms of pyruvate kinase have been shown to have different kinetic properties. Imamura et al. (45) have shown that the M_2-isozyme from rat Yoshida ascites hepatoma 130 cells exhibits kinetics which are intermediate between the hyperbolic rat muscle M_1-pyruvate kinase isozyme and the allosteric rat liver L-pyruvate kinase isozyme. The results of the studies presented here show that the human kidney M_2-pyruvate kinase isozyme displays kinetics which are intermediate between the hyperbolic velocity profile of the human M_1-isozyme and the allosteric velocity profile of the human erythrocyte isozyme. The

[5]The observation by electrophoretic methods of hybrid isozymes between the human M_1- and erythrocyte pyruvate kinase isozymes is the result of work performed by Dr. R. Bigley, Division of Medical Genetics, University of Oregon Medical School.

previous kinetic studies by Bigley et al. (46,188) on the human pyruvate kinase isozymes support the kinetic properties presented here for the human M_1- and erythrocyte isozymes. However, the data gathered by Bigley and co-workers on the M_2-isozyme was from an enzyme sample prepared by a 38% to 45% $(NH_4)_2SO_4$ fractionation of a human kidney extract, while the kinetic results presented here on the human M_2-isozyme were gathered from an enzyme sample prepared by a 50% to 75% $(NH_4)_2SO_4$ fractionation of a human kidney extract. A comparison of the two sets of kinetic data on the human M_2-isozyme is precluded since each sample was prepared from different $(NH_4)_2SO_4$ fractions, since evidence is presented here that there is more than one form of pyruvate kinase in human kidney, and since recent work in this laboratory has shown that the two samples are electrophoretically distinguishable by the same method.[6]

The results of the inhibition studies of the human M_1-, M_2-, and erythrocyte pyruvate kinases by Cibacron blue F3GA suggests that the dye binds very tightly at the ADP binding site(s) of these enzymes. The Dixon plots show that for each of these isozymes the

[6]Recent work in this laboratory, by David Lincoln, graduate student, Department of Biochemistry, University of Oregon Medical School, has shown that the M_2-isozyme, prepared according to the method of Bigley et al. (188), is electrophoretically distinct from the M_2-isozyme, prepared according to the procedure presented here. Electrophoresis was performed on both preparations by the thin layer polyacrylamide method described under "Methods".

dye is a competitive inhibitor with respect to ADP, and a non-competitive inhibitor with respect to PEP. Evidence which complements these results is provided by the observation that the K_i (ADP) and K_i (PEP) values which were calculated for the M_1-, M_2-, and erythrocyte isozymes, respectively, follow the order of the $K_{0.5s}$ (ADP) values determined for the three isozymes. As an example, among the three isozymes which were examined, the erythrocyte isozyme exhibited the highest apparent affinity for the substrate ADP ($K_{0.5s}$ = 0.25 mM), and the lowest apparent affinity for the other substrate, PEP ($K_{0.5s}$ = 0.40 mM). Upon examination of the Dixon plots for the three isozymes, the erythrocyte enzyme displayed the highest affinity for the inhibitor with respect to both ADP (K_i = 0.0065 mM) and PEP (K_i = 0.04 mM).

The Dixon plots which were calculated from the inhibition studies on the M_2- and erythrocyte isozymes (Figs. 21-24) show a concave upward curve at low substrate concentration with increasing inhibitor concentration. Though the nature of this non-linearity in the Dixon plots is not known, the results are consistent with a mixed-type of inhibition in which the inhibitor binds to more than one form of the enzyme, or in which the inhibitor binds at more than one site on the enzyme (189). The first explanation is in agreement with the current thoughts concerning the allosteric properties of the human erythrocyte isozyme. The cooperative kinetics of this isozyme have been explained by the presence of at least two

conformational states which are in equilibrium, according to the model of Monod et al. (89). Though the kinetics of the human M_2-isozyme have not been examined in detail, Walker and Potter (112), Farina et al. (114), and Van Berkel et al. (127,129) have proposed that the M_2-isozyme from rat adipose tissue exists *in vivo* as two interconvertible forms. The second explanation for the non-linear Dixon plots observed for the human M_2- and erythrocyte isozymes suggests that the inhibitor binds at more than one site on these isozymes. Evidence for this interpretation depends upon the nature of the binding between Cibacron blue F3GA and the M_2- and erythrocyte isozymes. The results presented here show that the inhibition is competitive with respect to ADP, and suggest that the inhibitor binds at the ADP binding site(s) of these isozymes. However, there is a lack of information regarding the number of ADP binding sites on the human pyruvate kinase isozymes. Therefore, if the inhibitor is binding at an ADP binding site, it is not possible to state, at this time, whether this second explanation is true or not.

The results suggesting that Cibacron blue F3GA binds at the ADP site(s) of human pyruvate kinase isozymes is supported by the work of Böhme et al. (166), and more recently, by the studies of Thompson et al. (190). Both groups have proposed that the specificity of the dye, Cibacron blue F3GA, for nucleotide binding enzymes resides in the structural similarity of the dye to adenine nucleotides. Thompson and co-workers (190) have studied the

inhibition kinetics of lactic dehydrogenase and phosphoglycerate kinase by Blue Dextran 2000, a complex formed between Cibacron blue F3GA and dextran. They have shown that the inhibition of lactic dehydrogenase with the dye-complex is competitive with respect to NADH ($K_i = 10^{-10}M$), and the inhibition of phosphoglycerate kinase with the dye-complex is competitive with respect to ATP ($K_i = 2x10^{-6}M$). These results, and studies on the Blue Dextran 2000 complexes with a wide range of other nucleotide binding proteins, have led these workers to propose that the dye is specific for a super-secondary structure within these proteins called the "dinucleotide fold." If this proposal is valid, then the high affinity of the dye for human pyruvate kinase isozymes suggests that these enzymes also possess a "dinucleotide fold" or a remnant of such a structure.

c. Molecular interrelationships

Table 12 shows the physical properties of the human M_1-, M_2-, and erythrocyte pyruvate kinase isozymes. In agreement with the studies on pyruvate kinases from other species, the human pyruvate kinase isozymes all have molecular weights of around 200,000 to 240,000 daltons. The similarity in size among the three human pyruvate kinase isozymes is shown also by their similar sedimentation coefficients. However, molecular weights of 100,000 (128) and 126,000 (121) have been reported for the M_2-isozymes from rat and human tissues under certain conditions. Sparman et al. (128) derived the molecular weights for the M_2-pyruvate kinase isozyme from ascites

Table 12. Summary of results on three types of pyruvate kinases from human tissues

Type	M_1	M_2	erythrocyte
Molecular weights	240,700	206,700	225,400[a]
Subunits	4 (identical)	4 (identical ?)	4 [b] (non-identical)
Subunit molecular weight (approx.)	61,000	50,500	60,000[b]
$s_{20,w}$	10.04	9.25	9.79[a]
NH_2-terminal	blocked	blocked	blocked[a]
Anti M_1[c]	+++	+++	-
Anti RBC[d]	-		+++

[a]The results concerning the molecular weight, $s_{20,w}$, and NH_2-terminal analysis for the human erythrocyte isozyme are from Chern et al. (130).

[b]The results concerning the number of subunits and subunit molecular weights for the human erythrocyte isozyme are from Peterson et al. (191).

[c]+++ indicates full cross-reaction on gel diffusion, + indicates partial cross-reaction, - indicates no cross-reaction.

[d]Immunodiffusion studies have not been performed to test the cross-reaction between human M_2-isozyme and anti human erythrocyte pyruvate kinase. Antibody inactivation studies using this antiserum fail to show any neutralization of human M_2-pyruvate kinase activity (see "Discussion").

tumor cells by sucrose density centrifugation, and obtained values of 100,000 daltons in the presence of ADP or absence of FDP, and 220,000 in the presence of FDP. Spellman et al. (121), working with a purified preparation of human M_2-pyruvate kinase from placenta, obtained an apparent molecular weight by Sephadex G-200 gel filtration of 126,000 in the absence of FDP. The results presented here show that the human M_2-isozyme is very unstable in the absence of FDP, and that the mobility of the M_2-isozyme during disc gel polyacrylamide gel electrophoresis in the absence of FDP is greatly increased over that of the M_2-isozyme run in the presence of FDP. These observations show that FDP exerts a major effect upon the M_2-isozyme, and provide additional evidence that FDP is important in modulating two interconvertible forms of the human M_2-isozyme, which are very different in molecular weight.

The subunit molecular weights, calculated by SDS gel electrophoresis, indicate that the M_1-, and M_2-isozymes are tetramers consisting of four subunits of 61,000 and 50,500 daltons, respectively. Since only one band was observed on the SDS gels for each isozyme, this suggests that the subunits of each isozyme are identical in molecular weight. However, it cannot be concluded from this work alone that the subunits are the same in composition, as well as in size. Recently, workers in this laboratory have examined the subunit structures of the purified human M_1- and erythrocyte pyruvate kinase isozymes by both SDS gel electrophoresis and by two

dimensional fingerprinting of their cyanogen bromide peptides (191). The M_1-isozyme exhibited a single band on the SDS gel, and the fingerprint of the cyanogen bromide peptides of the M_1-isozyme revealed a total of 21 ninhydrin spots. From the amino acid composition (Table 6), there are 78 methionine residues per mole of enzyme (240,700 gm/mole), and therefore 21 peptides would be expected if the subunits are identical. The human M_1-isozyme is therefore a tetramer containing four identical subunits. However, the human erythrocyte isozyme, purified by the method of Chern et al. (130), gave two distinct bands on SDS gel electrophoresis, each of which had a molecular weight of approximately 60,000. The fingerprint of the cyanogen bromide peptides of the erythrocyte isozyme separated 22 ninhydrin positive spots. The amino acid composition of the human erythrocyte isozyme (130) shows that there are 46 methionine residues per mole of enzyme (225,000 gms/mole). Therefore, if the erythrocyte isozyme contained identical subunits, one would expect to observe 13 cyanogen bromide peptides, whereas if there are two types of subunits, one would expect to observe 25 peptides on the fingerprint. The close agreement between the 22 peptides observed and the 25 expected for two types of subunits, and the two bands observed on SDS gel electrophoresis suggests that the human erythrocyte isozyme is a tetramer containing two pairs of non-identical subunits.

In addition to the similarity in size and in tetrameric

structure of the human M_1-, M_2-, and erythrocyte pyruvate kinase isozymes, each isozyme is apparently blocked at the NH_2-terminus. Studies which have attempted to identify the NH_2-terminal amino acid for rabbit muscle pyruvate kinase (138) and for the enzyme from E. coli (132) have also failed. Cottam et al. (138) found that purified rabbit muscle pyruvate kinase contains about four N-acetyl groups per mole of enzyme, and suggested that the NH_2-terminal residues of the four peptide chains are acetylated. No studies have been attempted in this laboratory to elucidate the nature of the blocking groups for the human M_1-, M_2-, or erythrocyte pyruvate kinase isozymes.

The results of the immunodiffusion studies, obtained by testing the cross-reaction of the purified human M_1-, M_2-, and erythrocyte isozymes against antiserum prepared from the purified human M_1-isozyme and antiserum prepared from the purified human erythrocyte isozyme, are summarized in Table 12. The fact that the erythrocyte isozyme failed to cross-react with anti-M_1-pyruvate kinase, and that the M_1-isozyme failed to cross-react with anti-erythrocyte pyruvate kinase (Figure 14) shows that the two isozymes are immunologically distinguishable, and share no immunological determinants with one another. Using the human M_2-isozyme (Figure 42), it was observed that there was a cross-reaction with the anti-M_1-pyruvate kinase, which shows that the human M_2-isozyme shares the same immunological determinants as the human M_1-isozyme. The

results of this work have been substantiated in this laboratory by antibody inactivation studies, in which it has been shown that the human M_1-pyruvate kinase antiserum completely neutralizes both human M_1- and human M_2-pyruvate kinase activity, but has no effect upon human erythrocyte pyruvate kinase activity. On the other hand, human erythrocyte pyruvate kinase antiserum completely neutralizes human erythrocyte pyruvate kinase, but has no effect upon the activity of either the human M_1- or M_2-pyruvate kinase isozymes.[7] These studies show that the human M_2- and erythrocyte isozymes are completely distinguishable by this immunological method.

The results of the studies which have been presented here show that there is a relationship among the human M_1-, M_2-, and erythrocyte pyruvate kinase isozymes with respect to their physical properties. In particular, it has been shown that the human M_1- and M_2-isozymes are homologous with respect to the immunological methods which have been employed, and that this homology is absent between either the human M_1- or M_2- and human erythrocyte isozymes. Ideally the elucidation of the apparent molecular interrelationships underlying the immunological homology which exists between the human

[7]The antibody inactivation studies were performed in this laboratory by David Lincoln, graduate student, Department of Biochemistry, University of Oregon Medical School. The antibody preparations were prepared according to the procedure outlines in "Methods".

M_1- and M_2-isozymes would be provided by comparing the primary and tertiary structures of these isozymes. Furthermore, the comparison of the primary sequences of the human pyruvate kinase isozymes with the pyruvate kinases from other species would provide a valuable insight into how mammalian isoenzymes have evolved to acquire their divergent regulatory functions in highly specialized cells.

At this time, none of the sequences of the pyruvate kinases has been determined. However, methods have been developed by which amino acid compositions can be correlated with the similarity or dissimilarity of sequences among a class of proteins. The reasoning behind this approach is that if two proteins are similar in amino acid sequence, this similarity will be reflected in the amino acid compositions. Furthermore, very dissimilar primary structures would be expected to have dissimilar amino acid compositions. Dedman et al. (192) have proposed a quantitative method by which the amino acid compositions of homologous proteins from different animal species can be correlated to the amino acid sequences and phylogenetic relationships. These workers have developed a statistical method from which the Composition Coefficient (CC), defined in equation 8,

$$[8] \quad \text{Composition Coefficient (CC)} = \frac{\sum(XY)-[(\sum X)(\sum Y)/n]}{\left\{[\sum(X)^2-(\sum X)^2/n][\sum(Y)^2-(\sum Y)^2/n]\right\}^{1/2}}$$

where X and Y = mole fractions of corresponding amino acids in the pair of compositions being compared

n = total number of comparisons in the pair of compositions being compared (i.e., usually 18 amino acids)

measures the degree of similarity between two protein amino acid compositions. When the linear correlation between two amino acid compositions is high, the CC value approaches 1.0; when little correlation exists, a value of 0.0 is approached. The limitations of this method have been examined by calculating the CC values for all the possible pairs of known cytochrome c sequences from 41 species, and plotting these 820 values against the number of linear amino acid differences in each pair examined [see "Appendix" for a listing of the cytochrome c species and references (193-198) from which the sequences were obtained].[8] The results are shown in Figure 44. Figure 45 shows the same results plotted as the mean number of amino acid differences ± 1 standard deviation versus the Composition Coefficient, and indicate that a linear correlation exists between the mean number of amino acid differences and the CC

[8]The CC values were determined according to Dedman et al. (192), using a computer analysis program written by Dr. John Black, Department of Biochemistry and Division of Medical Genetics, University of Oregon Medical School.

Figure 44

Plot of Composition Coefficient (CC) versus amino acid differences for cytochrome c. All possible pairs of 41 known cytochrome c sequences (see Appendix for list) were examined for the number of amino acid differences between each pair. The values were plotted against the Composition coefficient (CC) of each pair, calculated according to the method of Dedman et al. (192).

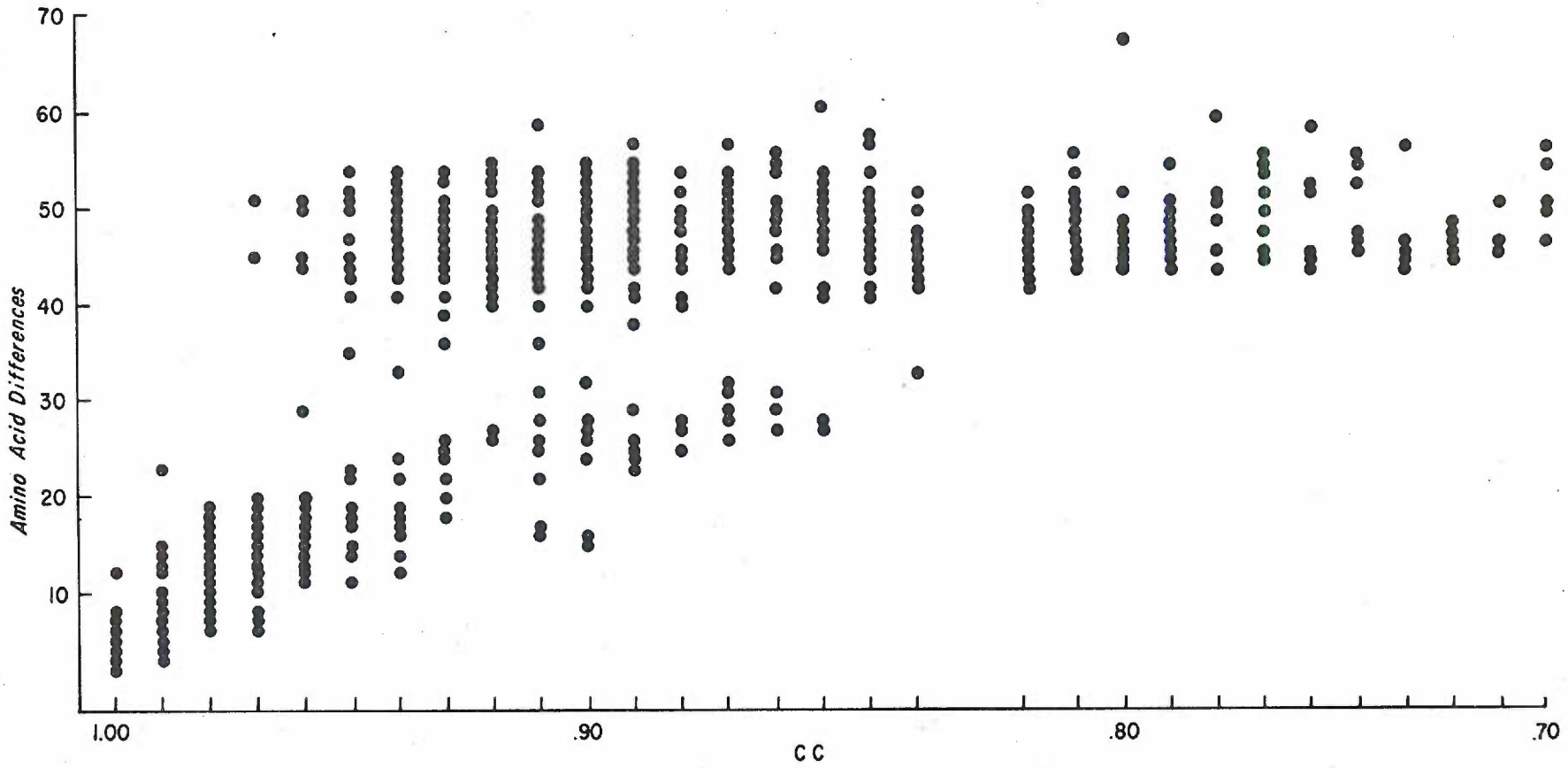
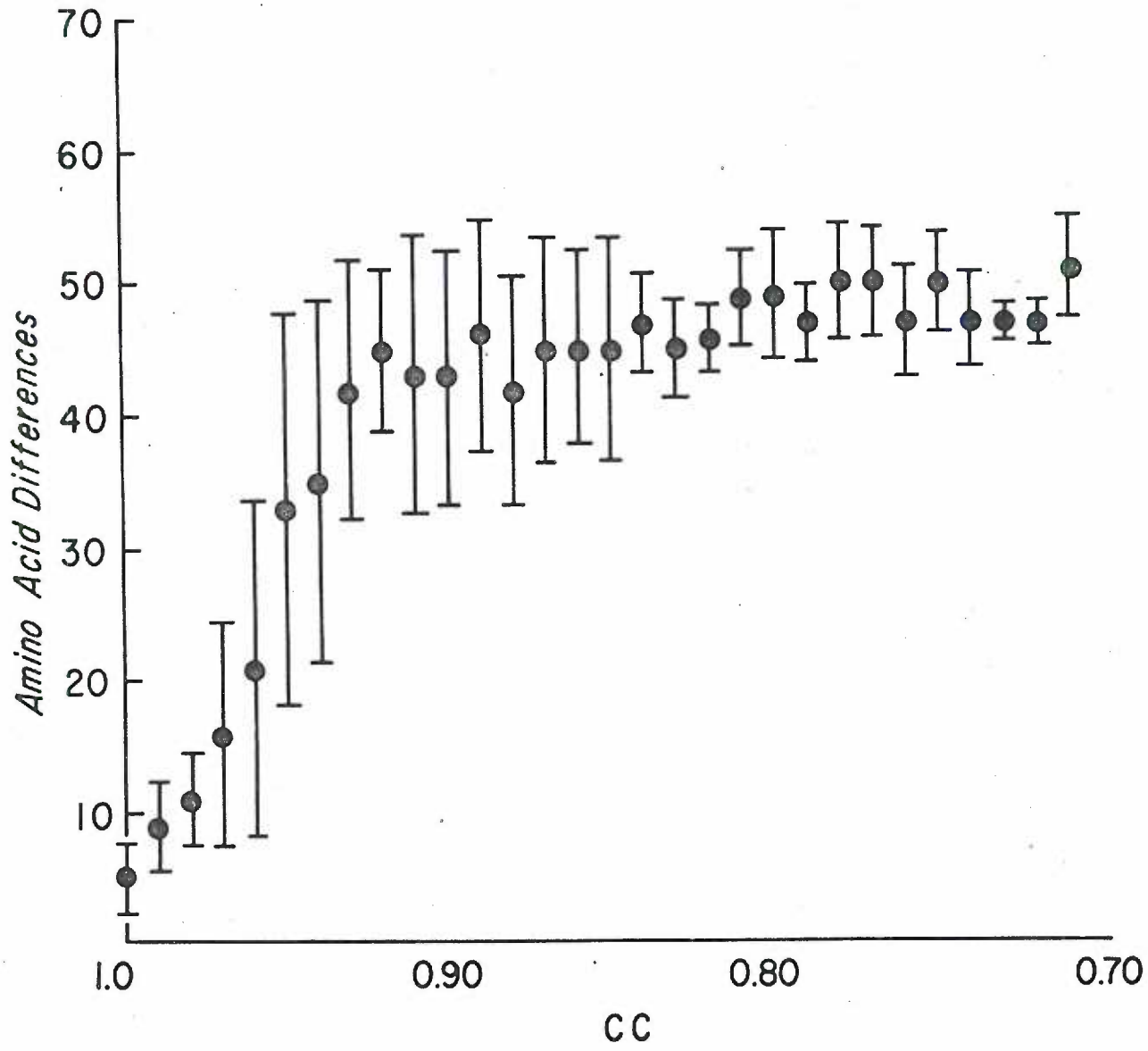

70
60
50
40
30
20
10
Amino Acid Differences
1.00
.90
.80
.70
C C

Figure 45

Plot of Composition Coefficient (CC) versus the mean number of amino acid differences ± 1 standard deviation for cytochrome c. The data from Figure 44 was used to calculate the mean values ± 1 S.D. versus C.C.


70
60
50
40
30
20
10
Amino Acid Differences
1.0
0.90
0.80
0.70
CC

values for the sequences examined down to a CC value of 0.96. Beyond this value a plateau region of scattered points is approached wherein the correlation between amino acid sequence and composition vanishes. The results of this study using known cytochrome c sequences show that the CC value is a reliable statistical method, within certain limitations, for estimating the degree of similarity between the amino acid sequences of proteins.

The method for determining the Composition Coefficient has been applied to all the pyruvate kinases whose amino acid compositions are known, and the results are shown in Table 13. Only those values for the CC above 0.96 are considered to be reliable. This analysis shows that the human M_1- and M_2-pyruvate kinase isozymes, having a CC value of 0.96, share a considerable amount of homology with respect to their amino acid compositions. Unfortunately, the limitations of this method preclude a discussion of the relationship of the human erythrocyte isozyme to either the M_1- or M_2-isozymes. However, the results presented here, along with the immunological studies, show that the human M_1- and M_2- isozymes share a greater degree of homology with each other than with the human erythrocyte isozyme.

Further inspection of Table 13 shows that the M_1-isozyme has an amino acid composition which is more similar to the compositions of M_1-isozymes from other species (beef and sturgeon), than to the composition of the human M_2-isozyme. Similar results have also

Table 13. Quantitative comparison of pyruvate kinases by Composition Coefficient (CC).

	human rbc	human M_1	rabbit M_1	beef M_1	sturgeon M_1	human M_2	E. coli	S. cerevisciae
human M_1	0.89							
rabbit M_1[a]	0.92	[0.97]						
beef M_1[b]	0.90	[0.99]	[1.00]					
sturgeon M_1[c]	0.90	[0.98]	[0.97]	[0.98]				
human M_2	0.85	[0.97]	0.95	[0.96]	0.94			
E. coli[d]	0.78	[0.96]	0.91	0.95	0.94	0.93		
S. cervisciae[e]	0.62	0.77	0.75	0.79	0.81	0.77	0.86	
S. carlsbergensis[f]	0.73	0.88	0.86	0.89	0.89	0.87	0.94	0.95

Values were calculated from the data of:
[a]Cottam et al. (138)
[b]Cardenas et al. (131)
[c]Anderson et al. (199)
[d]Waygood and Sanwal (132)
[e]Hunsley and Suelter (22)
[f]Bischafberger et al. (148)
[g]Chern et al. (130)

Note: CC values below 0.960 are considered to be unreliable. The compositions were normalized to 1000 amino acid residues before determining CC values.

been observed by comparing the isoenzymes of aldolase (200), and suggest that the homologous isozyme forms from various species have greater similarity than heterologous isozymes within the same animal. Figure 46 illustrates the homology which appears to exist among the pyruvate kinase isozymes, based upon the CC determinations.

Table 14 shows the results of CC values which were determined for known hemoglobin sequences from several of the same species for which the pyruvate kinases were examined. The only comparison of significance is that between the α-chains of human and bovine hemoglobin. In comparing pyruvate kinases from the same species, the number of significant CC values is much greater. These results suggest that the pyruvate kinases from these species are more related than are the hemoglobins, and that their primary structures have been conserved to a greater degree during evolution. Dickerson and Geis (201) have discussed the conservative evolutionary rate of cytochrome c proteins in terms of the functional importance of this macromolecule in meeting the needs of aerobic organisms. The results presented here show that the pyruvate kinases from different species have maintained a high degree of relatedness with respect to their amino acid compositions, and emphasize the key regulatory role which this enzyme plays in the provision of energy to the cell.

Figure 46

The homology between pyruvate kinase isozymes. The distances between each of the pyruvate kinases has been estimated from the CC values in Table 14.

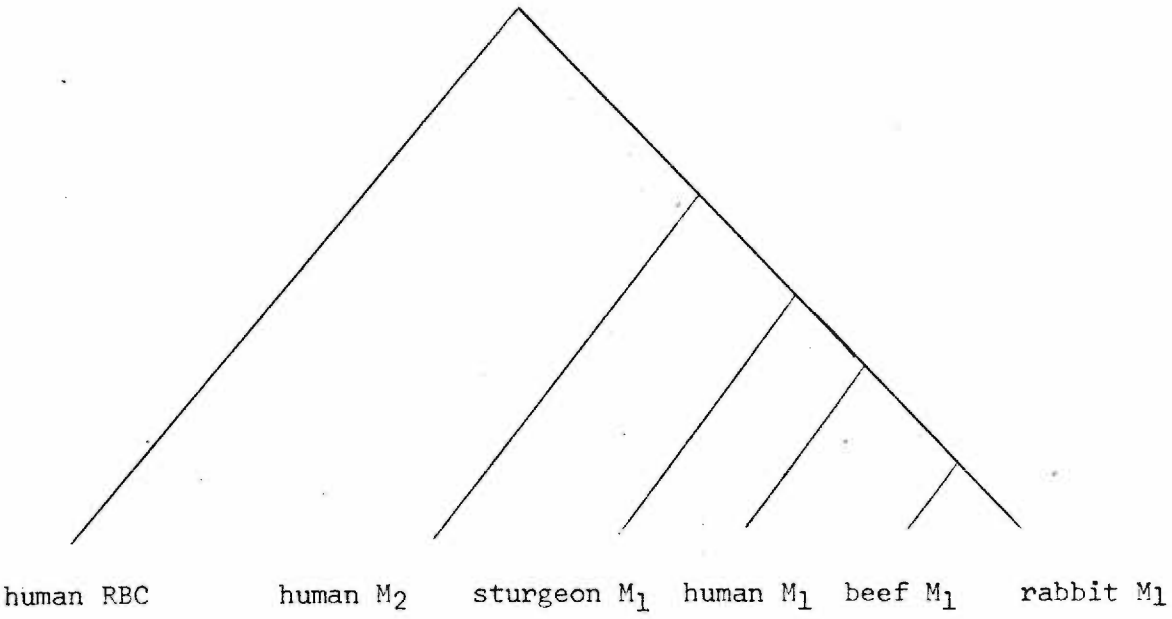

human RBC
human M_2
sturgeon M_1
human M_1
beef M_1
rabbit M_1

Table 14. Quantitative comparison of hemoglobin chains by Composition Coefficient.

α-human	α-human			
α-rabbit	0.92	α-rabbit		
α-bovine	0.96	0.91	α-bovine	
α-carp	0.82	0.77	0.78	α-carp
β-human	0.85	0.86	0.82	0.76

Note: The amino acid compositions used in this table were obtained from the published sequences reported in Dayhoff (196).

E. Summary and Conclusions

Human M_1-pyruvate kinase has been crystallized and purified to homogeneity from psoas muscle. The final enzyme preparation had a specific activity of 382 units per mg protein and represented a 294-fold purification over the crude enzyme extract. Homogeneity of the enzyme preparation was determined by polyacrylamide disc gel electrophoresis in the absence and in the presence of sodium dodecyl sulfate (SDS), by sedimentation velocity and equilibrium analyses, by NH_2-terminal analysis, and by immunodiffusion methods. Characterization studies of the purified human M_1-pyruvate kinase isozyme show that the enzyme is a tetramer of 240,700 ± 450 daltons with a sedimentation coefficient ($S_{20,w}$) of 10.04. The results of SDS gel electrophoresis and peptide mapping show that the four subunits of the human M_1-isozyme are identical. NH_2-terminal analysis indicates that the NH_2-terminus of the peptide chains of the human M_1-isozyme are blocked.

Human kidney M_2-pyruvate kinase has been purified to homogeneity by means of affinity chromatography. The purification procedure involved the application of an affinity chromatography step which utilized the ability of the dye, Cibacron blue F3GA, to bind tightly to human pyruvate kinase isozymes. Kinetic studies show that the dye is a strong inhibitor of the human M_1-, M_2-, and erythrocyte pyruvate kinases, and indicate that the dye is competitive with the ADP binding site(s) of these isozymes. During the

purification procedure it was observed that the human M_2-isozyme is unstable in the absence of fructose-1,6-diphosphate (FDP). The results of polyacrylamide disc gel electrophoresis of the M_2-isozyme, run in the presence and in the absence of FDP, suggest that the instability of the isozyme may be the result of a major change in molecular weight. The final enzyme product had a specific activity of 127 units per mg protein and represented a 470-fold purification over the crude enzyme extract. Homogeneity of the enzyme preparation was determined by polyacrylamide disc gel electrophoresis in the absence and in the presence of SDS, by sedimentation velocity and equilibrium analyses, and by NH_2-terminal analysis. Characterization studies of the purified human M_2-pyruvate kinase isozyme show that the enzyme is a tetramer of 206,700 ± 600 daltons with a sedimentation coefficient ($S_{20,w}$) of 9.25. The results of SDS gel electrophoresis indicate that the enzyme consists of four subunits of identical molecular weight. NH_2-terminal analysis indicates that the NH_2-terminus of the peptide chains of the human M_2-isozyme are blocked.

Comparison of the properties of the human M_1-, M_2-, and erythrocyte pyruvate kinases shows that the three isozymes are similar with respect to their molecular weights, number of subunits, sedimentation coefficients, and with respect to the absence of a detectable NH_2-terminus. Using antiserum prepared against purified human M_1-pyruvate kinase and antiserum prepared against purified

human erythrocyte pyruvate kinase, immunodiffusion studies show that the human M_1-isozyme is immunologically indistinguishable from the M_2-isozyme, yet immunologically distinct from the erythrocyte isozyme. The apparent homology between the human M_1- and M_2-isozymes has been examined more closely by comparing their amino acid compositions, and the results indicate that the two isozymes are also closely related with respect to their primary structures. Hopefully, a full understanding of the molecular interrelationships observed during this work will be obtained by an analysis of the primary structures of these isozymes.

F. Appendix

1. Preparation of antiserum against an impure preparation of human muscle M_1-pyruvate kinase.

Human psoas muscle was ground, weighed, and two volumes of distilled water were added. The extract was stirred for 2 hours, centrifuged, and the supernatant was taken to 45% $(NH_4)_2SO_4$ saturation. The suspension was centrifuged and the supernatant was then taken to 60% $(NH_4)_2SO_4$ saturation. The suspension was centrifuged and the precipitate was dissolved in distilled water and taken through a second 45% to 60% $(NH_4)_2SO_4$ fractionation. The precipitate from this second $(NH_4)_2SO_4$ fractionation was dissolved in distilled water and dialyzed exhaustively against distilled water containing 0.01M EDTA. The preparation was then heated for 1 hour at 40°C, centrifuged, and the supernatant was dialyzed for 24 hours against buffer for column chromatography, consisting of 0.05M potassium maleate buffer, pH 5.0, containing 5 mM $MgSO_4$, 1 mM EDTA, and 1 mM 2-mercaptoethanol. The preparation was then applied to a column of cellulose phosphate (Whatman P70, Reeve Angel, Clifton, New Jersey), fully equilibrated with column buffer. The column was washed and then a linear pH gradient was applied, which consisted of column buffer at pH 5.0 and the same buffer at pH 7.1. Those fractions containing pyruvate kinase were pooled and then taken to 65% $(NH_4)_2SO_4$. The 65% $(NH_4)_2SO_4$ precipitate was then centrifuged and taken up in distilled water to a specific activity of approximately

150 units per mg protein. This preparation was used to prepare antiserum against impure M_1-pyruvate kinase.

2. List of cytochrome c sequences used to evaluate the Composition Coefficient (Figure 44).

		Reference
1.	Humicola lanuginosa	193
2.	Rust fungus ustilago spaerogena	194
3.	Mung bean	193
4.	Pumpkin	195
5.	Buckwheat	"
6.	Cauliflower	"
7.	Abutilon	"
8.	Cotton	"
9.	Horse and donkey	196
10.	Pig, bovine, and sheep	"
11.	California gray whale	"
12.	Rabbit	"
13.	Gray kangaroo	"
14.	Chicken and turkey	"
15.	King penguin	"
16.	Bonito	"
17.	Puget Sound dogfish	"
18.	Pacific lamprey	"
19.	Fruit fly	"
20.	Silkworm moth	"
21.	Screw-worm fly	"
22.	Tobacco horn worm moth	"
23.	Wheat	"
24.	Debaryomyceskloeckeri	"
25.	Baker's yeast	"
26.	Neurospora crassa	"
27.	Elephant seal	193
28.	Camelus dromedarius	"
29.	Crithidia oncopelti	195
30.	Candida krusei	196
31.	Castor bean	193
32.	Sesame seed	"
33.	Sunflower seed	"
34.	Tomato	195
35.	Spinach	193
36.	Snapping turtle heart	"
37.	Potato	197
38.	Nasturtium	198
39.	Acer (box-elder)	"
40.	Elder	"
41.	Parsnip	"

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