GENETIC AND METABOLIC REGULATION OF LIVER PYRUVATE KINASES

by

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A THESIS

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Abbreviations

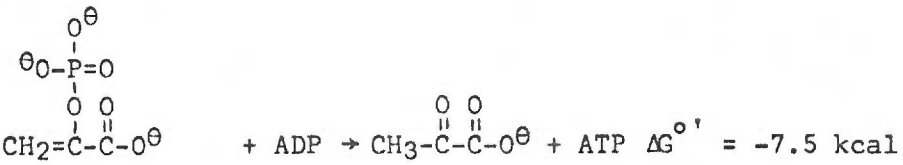
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
DEAE-cellulose	diethylaminoethyl cellulose
EDTA	(ethylenedinitrilo)-tetraacetate
FDP	fructose-1,6-diphosphate
G6P	glucose-6-phosphate
$K_{0.5s}$	concentration of substrate giving 50% maximum activity
K_m	apparent Michaelis constant
NADH	β-nicotinamide adenine dinucleotide (reduced form)
PEP	phosphoenolpyruvate
3PGA	3-phosphoglyceric acid
Tris	Tris(hydroxymethyl)-aminomethane

Introduction

To determine the significance of various metabolic control mechanisms, it is often necessary that information be gathered from a number of different species to provide insight into those features which are of wide prevalence. In examining just a single organism, it is unclear whether the control mechanisms in that animal are peculiar to it alone because of some special metabolic need or feature, or whether the mechanisms have a broader applicability and therefore are more fundamental. This thesis examines pyruvate kinase metabolism in two different features: 1) structural conservatism in evolution, and 2) metabolic mechanisms controlling the quantities of pyruvate kinase in tissue.

1. Pyruvate kinase

The enzyme pyruvate kinase (ATP: pyruvate phosphotransferase, EC. 2.7.1.40) catalyzes a key rate controlling step in the glycolytic pathway (1). The reaction catalyzed is the following:



PEP pyruvate

The enzyme has an absolute requirement for a divalent cation (Mg^{2+} or Mn^{2+}) and a monovalent cation (K^{+} or NH_4^{+}), except for the

pyruvate kinases of some procaryotes which do not require the monovalent cation (2). There is a high degree of specificity for the phosphate donor, but ADP may be substituted by other nucleotide diphosphates (3).

The regulation of pyruvate kinase has been reviewed by Seubert and Schoner (4), and the overall regulation of glycolysis and gluconeogenesis has been reviewed by Scrutton and Utter (5).

Several isozymes of pyruvate kinase have been described. Fellenberg et al. (6) in 1963 showed that different tissues of the rat have electrophoretically different types of pyruvate kinase. In 1965 Campos et al. (7) described kinetic differences between the human erythrocyte and leukocyte pyruvate kinases. Tanaka's group (8) first purified the major isozymes from rat liver and muscle, calling these the L- and M-types respectively. Bigley et al. (9) have shown that multiple types of isozymes exist in human tissues. A similar pattern in rat and other mammalian tissues was later described by Imamura and Tanaka (10). In 1972 Imamura et al. (11) characterized an isozyme present in minor amounts in rat liver which was immunologically similar to the muscle isozyme but electrophoretically distinct. The major and minor isozymes in liver and the muscle

isozyme are called PK-L, PK-M_2, and PK-M_1, respectively.[1] This nomenclature will be used in this thesis, with the addition of PK-R for the isozyme present in erythrocytes. The characteristics of the isozymes are summarized in Tables 1 and 2.

The isozyme PK-L exists as the major form in liver and the minor form in kidney (11). It has been shown that this isozyme exists only in the parenchymal cells of the tissues (27), and also that it exists in different conformational states, depending on various ligands and incubation conditions (28-30). Activity levels of PK-L have been shown to be responsive to the season (8), and dietary and hormonal fluctuations (31,32). The injection of insulin, glucagon, or epinephrine into the rat portal vein causes an immediate fluctuation of the level of PK-L which is not related to de novo synthesis (33,34). The activity levels of PK-L are reduced in animals which are fasting

[1]The nomenclature on the pyruvate kinases has been very confused, as each set of authors has applied a different system. Below is a table of the various nomenclatures in the literature. The recommendations of the IUPAC-IUB Commission correspond to the nomenclature used by Osterman et al. However, the system advanced by Tanaka's group is widely used in the literature.

Author	Notation			
Tanaka's group (8,10)	L-type	M_2-type		M_1-type
Bigley et al. (7,12)	PK-I	PK-II	PK-III	PK-III
Walker et al. (13,14)	Type I	Type III_A	Type III_B	Type II
Carbonell et al. (15,16)	Class L	Class A		Class M
Ibsen et al. (17,18)	Type L	Type K		Type M
Osterman et al. (19,20)	PK-1	PK-4		PK-3
Susor et al. (21,22)	PyK-B	PyK-C		PyK-A

Table 1. Summary of results on the three types of pyruvate kinases from rat tissues by Imamura et al. (11).

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	+	+	++

Table 2. Properties of the Human Pyruvate Kinase Isozymes[a]

	M_1	M_2	R
Specific Activity	382.5 units/mg	127 units/mg	77.4 units/mg
$K_{0.5S}^{Phosphoenolpyruvate}$	0.05 mM	0.25 mM	0.40 mM
$K_{0.5S}^{ADP}$	0.45 mM	0.30 mM	0.25 mM
Molecular Weight	240,700	206,700	225,400
Subunit Molecular Weight	61,000	50,500	60,000
NH_2-Terminal	Blocked	Blocked	Blocked
Immunological reactivity			
with anti M_1 antiserum	+	+	-
with anti R antiserum	-	-	+

[a]Data on the M_1 and M_2 isozymes are from Harkins (23) and the R isozyme from Chern et al. (24) and Peterson et al. (25). The $K_{0.5S}$ values were obtained at 25° and pH 7.2 using the pH stat assay for pyruvate kinase as described by Melchior (26).

or have been fed a high protein diet and are increased in animals which have been alloxan-treated or given a high carbohydrate diet.

The isozyme shows a sigmoidal velocity curve with respect to PEP, a Hill coefficient indicating positive cooperativity, and allosteric activation by FDP (35,36). Inhibitory factors include alanine and other amino acids (37), ATP (35), acetyl coenzyme A (38), succinyl coenzyme A (39), and fatty acids (40).

Kinetically, PK-R is very similar to PK-L (41,42). Based on electrophoretic mobilities and inactivation by antiserum against the rat M_2-isozyme, two groups of investigators have proposed that the human erythrocyte isozyme is a hybrid of the L- and M_2-isozymes (11,43,44). However, no conclusive proof of this has been presented and the relationship between the L-, M_2- and R-isozymes is still unclear.

The isozyme PK-M_2 is present in a number of rat (11) and human (9) tissues. It is normally found in the Kupffer cells of liver and kidney (14) and is the only isozyme in cultured rat liver cells (13). In addition, PK-M_2 is the predominant isozyme in rat and human hepatomas (11,45-51), regenerating liver (14,52,53), and fetal tissues (46,47).

Garnett et al. (52) have shown that parenchymal cells are capable of synthesizing PK-M_2 during liver regeneration following partial hepatectomy. However, since they found no hybrids of PK-L and PK-M_2 in the parenchymal cells, it appears that these cells are

capable of synthesizing PK-L or PK-M_2, but not both.

The kinetic properties of PK-M_2 are intermediate between the M_1 and L-isozymes (see Table 1). Like PK-L, PK-M_2has been shown to exist in different conformational states (54-56).

Table 3 summarizes the molecular weights and isoelectric points for the isozymes.

Pyruvate kinase, because of its role in the control of one of the most basic metabolic pathways, glycolysis, continues to be useful in examining many features of regulatory phenomena, kinetics and comparative biochemistry. Several different pyruvate kinases have been purified to homogeneity recently (8,11,23,24,41,58-60).

The basic physical and kinetic properties of the two liver isozymes have been described. The isozymes have similar molecular weights but differ significantly in their isoelectric points, kinetic properties and genetic controls. PK-M_2 is a constitutive isozyme, unresponsive to dietary or hormonal changes, whereas PK-L responds to both. The PK-L and PK-M_2 activities respond to different levels of several metabolic effectors, and the controlling features of each isozyme have very different characteristics.

Within the field of comparative biochemistry the liver presents an opportunity to examine the enzyme across species lines, but also an opportunity to examine comparative features of isozymes within an organism and even within a single tissue.

This thesis examines pyruvate kinase at these two levels

Table 3. Summary of Molecular Weights and Isoelectric Points for Liver and Erythrocyte Pyruvate Kinase Isozymes

Isozyme	Source	Molecular Weight Unit	Molecular Weight Subunit	Isoelectric Point
PK-L	human liver			7.0 (57)
	rat liver	208,300 (8)		5.5 (28)
	bovine liver	215,000 (58)	54,000 (58)	
	pig liver	202,000 (59)	50,000 (59)	
PK-R	human	225,400 (24)	60,000 (25)	6.3 and 6.8 (57)
PK-M_2	human liver			7.6 (57)
	human kidney	206,700 (23)	50,500 (23)	
	rat liver	216,000 (11)		
	rat kidney			6.4 (28)

References are in parentheses.

of genetic and metabolic regulation. In the first part, the degree of relatedness of pyruvate kinase extracted from the livers of several phylogenetically distant animals and also from micro-organisms is examined. This study basically discusses the question of evolutionary divergence of pyruvate kinase and the relative conservatism of structure which might have resulted from the evolution of one of the rate-controlling enzymes of the glycolytic pathway.

In the second part of this study the comparative processes which lead to the levels of isozymes within a single tissue - the rat liver - are examined. Certainly the overall metabolism within a tissue is related to the kinetics of the tissue enzymes, but the importance of the quantities of individual enzymes is also recognized. As will become clear later, the process of protein degradation is critical to enzyme levels, and therefore factors which might influence the degradation rates of pyruvate kinase isozymes also become important. Kinetic differences have been described earlier for the two isozymes of rat liver, and this thesis will examine the nature of several factors influencing the degradation rates of PK-L and PK-M_2 in a single tissue.

2. Immunological taxonomy

Taxonomy is derived from three interrelated concepts, whether it is the more classical taxonomy based on the external morphology in plants or skeletal structure in vertebrates, or the

more recent taxonomy which makes use of new information derived from the relatedness of proteins (61). The first concept is that natural systems must be based on the correlations of many characters, rather than single characters of overriding importance. The second is that the taxonomic system ought to be a reflection of evolutionary relationships, and the third concept is that all kinds of data are potentially useful, and therefore the system should be a synthesis of all the available evidence. In this context the newer taxonomy merely supplements the details developed by the classical taxonomist, but does not replace them. Rarely has the new taxonomy been at divergence with a system developed by classical methods. The relationship of methodology becomes clear in review materials containing the more classical approach (62) and those including newer methodologies (63,64).

In any attempt to classify, the taxonomists must remember the following five facts, among others (65):

i. Evolution always includes two sets of phenomena: the splitting (branching) of lineages, and the greater or lesser divergence of those lineages from each other;

ii. Different characters change at different rates and in different directions;

iii. There is no logical or <u>a priori</u> reason to base a classification scheme on any one characteristic;

iv. Similar selection pressures may produce phenotypic

similarity of independent and sometimes not even closely related lines; and

v. Since the phenotype of an organism is the product of the balanced interaction during development of the genotype, there are numerous limitations to the evolutionary potential.

Unless these are fully understood, there exists the danger of erroneous interpretations of similarities and an incorrect evaluation of characters.

The broad evolutionary outline for the vertebrates begins with the divergence between the procaryotes and eucaryotes at about one to two billion years ago based on fossil records (66) and on a calculated divergence estimated from 5S ribosomal RNA sequences (67). The divergence times of the major classes of vertebrates are shown in Figure 1.

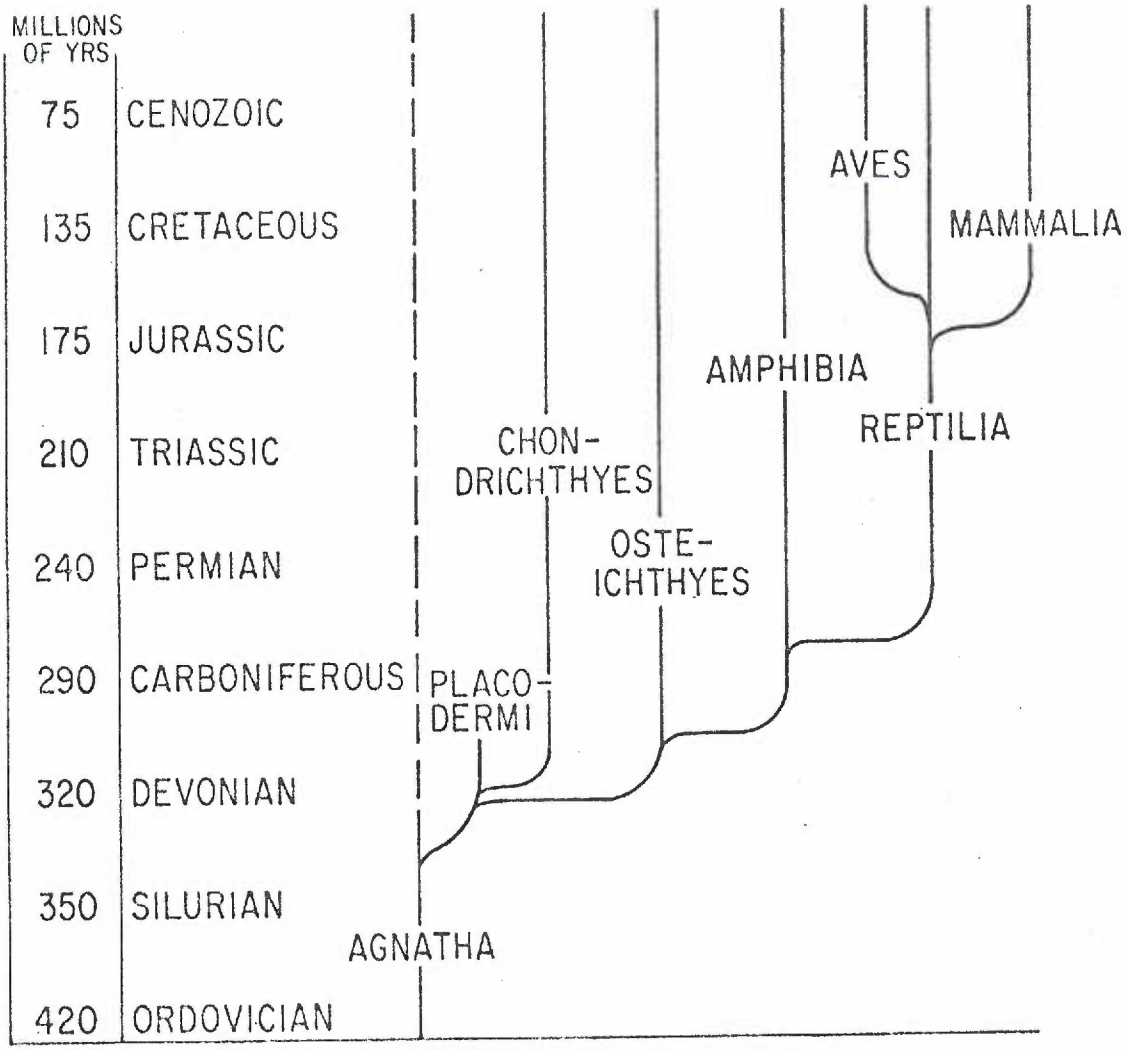
There are several different biochemical and biophysical methods for the development of phylogenetic relationships, depending on the nature of the available material (69,70). Each has been used in different studies.

i. Degree of interspecific hybridization of DNA (71).

ii. Number of differences in the peptides from enzymic digests of purified homologous proteins (72).

iii. Comparison by enzyme kinetics (73).

iv. Comparison by electrophoretic, chromatographic, or


MILLIONS OF YRS
75
CENOZOIC
135
CRETACEOUS
175
JURASSIC
210
TRIASSIC
240
PERMIAN
290
CARBONIFEROUS
320
DEVONIAN
350
SILURIAN
420
ORDOVICIAN
AGNATHA
PLACO-DERMI
CHON-DRICHTHYES
OSTE-ICHTHYES
AMPHIBIA
REPTILIA
AVES
MAMMALIA

other physical method (74).

v. Number of amino acid replacements between homologous proteins whose primary structure is known (70,75).

vi. Comparison of circular dichroic spectra (76).

vii. Comparison of amino acid compositions of homologous proteins (77).

viii. Comparison of immunological cross-reactivities of homologous proteins (78).

It is this last method which will be the focus of this section of the thesis.

Comparative serology can be said to have begun with Bordet (79) in 1899 when he injected rabbits with chicken serum and found that the resultant antiserum cross-reacted with dove serum. In 1904 Nuttall (80) reported experiments in which antiserum to whole human serum had been prepared. This antiserum gave a heavy precipitate with human serum, less precipitate with chimpanzee serum, and even less with monkey and prosimian serum. Even at this early date it was noted that the degree of cross-reactivity was proportional to the degree of systematic relationships between the organisms involved. This concept of quantitative phylogeny was refined and developed by Boyden and his colleagues (81,82).

Mutations find a direct expression in the determinants of the protein as a whole, as the determinant is based on the amino acid sequence and a change of a single amino acid may affect the determinant.

Praeger and Wilson (83) have suggested that a determinant might consist of about five amino acids and that it would take about two changes in the determinant to abolish cross-reactivity. From their studies they concluded that there is a correlation between the degree of immunological cross-reactivity and the degree of amino acid sequence similarity, with the limitation that proteins differing from each other by more than 40% in sequence show no cross-reactivity in the micro-complement fixation test. Protein evolution as a rule appears to be a surface phenomenon as, for instance, most of the amino acid replacements in bird lysozyme occur on the outside of the molecule (83). Therefore, immunological techniques can be used to detect most amino acid substitutions. It would also follow that the larger the molecule, and the slower the rate of evolution, the greater is the chance of detecting differences at the lower taxonomic levels (84).

Antisera have been prepared in rabbits against the chicken M- and H-isozymes of lactic dehydrogenase. In agreement with classical taxonomy, the micro-complement fixation test showed that the degree of cross-reactivity was (85):

birds > reptiles > amphibians > fish.

Similar results have been obtained by antibody inactivation studies, although the degree of enzyme inhibition was not always proportional to taxonomic distance as several orders of birds, which have evolved considerably further anatomically from reptiles than the

chicken, showed less inactivation with anti-chicken lactic dehydrogenase than did snakes (69).

The same order of cross-reactivities was shown in the microcomplement fixation tests using rabbit antiserum prepared against chicken muscle triosephosphate dehydrogenase, liver glutamic dehydrogenase, and muscle aldolase (85).

The very ancient divergence of amphibian and reptilian lines is indicated by the lack of cross-reactivity between antiserum against the plasma proteins of Rana temporaria and the plasma proteins of thirty-one species of reptiles, representing all orders (86). By the microcomplement fixation technique, indices of similarity for muscle lactic dehydrogenase suggested an amphibian-reptilian divergence 300 million years ago (86).

The evolutionary constancy of enolase has been suggested in studies where antiserum raised in chickens against mammalian muscle enolase cross-reacts with a wide range of vertebrate muscle enolases (87). In addition, the tissue distribution of the two basic types of enolase isozymes remains common throughout the same set of vertebrates. It is concluded that, because of similar molecular weights and kinetic parameters, the two isozymes of enolase arose from a common ancestral gene and that the divergence took place about 200-300 million years ago.

Therefore, antisera can be used to determine evolutionary change, as the determinants recognized by the antibodies are the

points where the variations occur. While there are some problems in interpretation of quantitative results, their value for the development of qualitative relationships has been demonstrated. To date, the results obtained with several proteins agree well with evolutionary relationships derived from classical taxonomy.

3. Protein turnover

The field has been very well covered in several recent reviews (88-94).

It is now well established that: 1) intracellular proteins exist in a state of dynamic equilibrium independent of cellular turnover, 2) turnover is extensive, 3) there is a heterogeneity of replacement rates, 4) the degradation of an enzyme, once synthesized, is random, and 5) there exist relationships between physical parameters of the protein and its rate of degradation (95).

In one of the first experiments demonstrating protein turnover in mammalian cells, Velick et al. (96,97) in 1954 injected ^{14}C-labeled amino acids into adult rabbits and subsequently determined the specific activities of the amino acids in a number of purified muscle proteins. Protein turnover in malignant cells was suggested in 1955 by the experiments of Greenlees and Le Page (98) using growing ascites tumor cells.

At the present time intracellular protein concentration change is formulated as a function of two processes, synthesis and degradation (99), which are related by the expression:

$$\frac{dC}{dt} = k_s - k_dC \qquad (1)$$

where C is the level of protein at any time t, k_s is the zero-order rate constant for synthesis and k_d is the first order rate constant for degradation. Under steady state conditions:

$$\frac{dC}{dt} = 0 \text{ and } k_s = k_dC$$

A change in protein concentration can be accomplished by an alteration of k_s or k_d and a new steady state concentration will be achieved according to equation 1.

Protein synthesis has been the subject of much investigation and the basic steps involved are now well established. The mechanisms of protein degradation are not well understood although Schimke (92) and others have shown that the degradation term in equation 1 plays an important role in establishing intracellular protein levels.

Protein turnover rates which are determined by k_s and k_d vary widely. The half-lives of rat liver enzymes have been found to vary from 11 minutes for ornithine decarboxylase (100) to as much as 18 days for NAD glycohydrolase (101). The same enzyme may have different degradation rates depending on its cellular location. δ-Aminolevulinate synthetase has a half-life of 20 minutes in the soluble fraction of rat liver but a half-life of 68 minutes in mitochondria (102). Degradation rates are also isozyme specific and

tissue specific (103).

Genetic, hormonal and dietary factors can influence the rate of synthesis, the rate of degradation, or both. Adrenocorticoid induction of tyrosine aminotransferase in rat liver increases the rate of synthesis (104). Diet alters the rate of synthesis of arginase (105). The rate of synthesis of catalase in liver is four times that of the enzyme present in kidney (106). In rat heart muscle under steady state conditions, the ratio of the rate constants for synthesis of isozymes 5 and 1 of lactic dehydrogenase is 1:17 (103).

The genetically determined trait of low catalase activity in a sub-strain of C57Bl mice occurs by an increase in the rate of degradation with no alteration in the rate of enzyme synthesis (107, 108). Intraperitoneal injection of tryptophan in the rat increases the liver tryptophan pyrrolase concentration by decreasing the degradation rate (109). Diet influences the degradation rate of acetyl coenzyme A carboxylase in the rat (110) and, in the rat muscle, the degradation rate constants for isozymes 5 and 1 of lactic dehydrogenase are in the ratio of 1:10 (103).

In some instances protein concentration changes are accomplished by alteration in both the rate of synthesis and the rate of degradation. When fasted rats are given a fat-free diet, there is an increase in the rate of synthesis and a decrease in the rate of degradation of liver acetyl coenzyme A carboxylase (110). The

opposite adjustment occurs for the enzyme serine dehydratase in rats after the administration of glucose (111). A reduction of both rate constants has been observed with rat liver catalase after feeding a diet containing cycasin, a potent carcinogen (112).

Several studies have indicated the importance of the protein's physical characteristics and conformation state as determinants in the protein's half-life. Conformational states will depend on interactions with a variety of ligands, including lipids, cofactors, metabolic intermediates, and other proteins. Litwack and Rosenfield (113) have shown a good correlation between the in vivo turnover of several pyridoxal-containing enzymes and their affinities for pyridoxal phosphate. The importance of dissociated proteins in the determination of half-lives is indicated by the studies of Tweto et al. (114), in which it was shown that the subunits of rat liver fatty acid synthetase have different degradation rates.

Studies have indicated a correlation between the protein subunit size and its in vivo half-life (115-117), with the proteins containing larger subunits having the shorter half-lives. Dice et al. (117) have performed a statistical analysis of this relationship using the published data for 33 liver proteins, which confirmed the earlier reports. Certain exceptions to this rule merely emphasize the importance of other factors.

One other factor is apparently the isoelectric point of the protein, as Dice and Goldberg (118) have found, using a double-label

technique for determining degradation rates, that acidic proteins have a higher degradation rate than the neutral or basic proteins. In a literature survey they found that 22 proteins for which data on rates of degradation and isoelectric points were available also confirmed this correlation. By calculating the partial correlation coefficients, it was possible to test statistically whether the influence of molecular weight on degradative rates can explain the observed relationship between half-life and isoelectric points or vice versa. They found that when the half-lives were held constant, the protein isoelectric point and subunit molecular weight were not related in the 22 proteins. This suggests that protein size and isoelectric point are separable factors influencing the degradation rates.

The lysosome has been implicated as the site of general protein degradation (119), and recently direct evidence has been published (120). Additionally, a correlation between the degradation rates of proteins in vivo and their susceptability to lysosomal proteases has been demonstrated (121).

Some studies have indicated that there are selective cytosolic proteases which are involved in the degradation of certain groups of proteins (122,123). In addition, evidence has been given for a relationship between the subunit molecular weight and protein degradation by protease(s) in the liver cytosolic fraction (124).

More generally, it has been shown that there is a correlation

between the protein half-life and its sensitivity to such proteolytic enzymes as pronase, trypsin, chymotrypsin, and subtilisn (116,125). Various ligands have been shown to protect specific proteins from the actions of the proteolytic enzymes (125).

Some studies on the sensitivity of pyruvate kinases to proteolytic enzymes have already been performed, and these served as preliminary material for establishing what ligands might be important in altering proteolytic sensitivity of the liver PK-L and PK-M_2. With pronase and trypsin, rat liver PK-L has been found to have increased sensitivity in the presence of FDP and decreased sensitivity in the presence of KCl and $MgCl_2$ (29). Neurospora pyruvate kinase has been found to be resistant to trypsin under all conditions tested, but sensitive to pronase with PEP, FDP, and ADP acting as protectors of pyruvate kinase activity, and ATP having no effect on the rate of pronase inactivation (126). With pronase, trypsin, and chymotrypsin, human erythrocyte pyruvate kinase has been found to be given some protection from proteolytic inactivation by PEP, FDP, ADP-Mg, and ATP-Mg (127).

There is evidence, therefore, that both synthesis and degradation are important in regulating observed enzyme levels and isozyme patterns, and that measurable factors of protein subunit molecular weight, protein isoelectric point, and protein sensitivity to proteolytic enzymes can be indicators of the protein degradation rate. There can be no justification for assuming that changes in

protein or enzyme levels are due to alterations in the process of synthesis alone. The lack of knowledge of the mechanisms of degradation can not detract from the potential contribution of the process. Until definitive evidence exists in any given case, the interpretation must be left open. In some instances, such as an unstable genetic variant of glucose-6-phosphate dehydrogenase (128), it is apparent that the rate of degradation is an inherent property of the molecule as synthesized. The specific control of degradation has the potential for cellular control of protein levels which could be as precise, accurate, and directed as any mechanism operating via protein synthesis.

The crux of any study of intracellular protein degradation is the methodology. No method has met universal acceptance and application. Indeed, in a recent study of catalase degradation, Poole et al. (129) found it necessary to compare data from five separate methods before reaching a tentative conclusion. The available methods have provided half-life estimates for lactic dehydrogenase of 3.5 and 16 days where there is no obvious factor to reconcile the discrepancy. Some methods give consistent results but are restricted in application to one protein (129), and others are valid only for the liver due to the requirement for a functional urea cycle (130,131). Administration of inhibitors of protein synthesis and detection of the subsequent loss of enzyme activity has been used to estimate degradation rate for a number of proteins

(99). The method is restricted to enzymes with relatively short half-lives and interpretation is complicated by evidence that protein degradation itself might depend on synthetic capacity (132).

The various methods which might be used in a study of the half-life of the pyruvate kinase isozymes in liver are discussed below with comments on their respective virtues and failings. The theoretical aspects of the methods are based on the treatment by Reiner (133), Koch (134), and Buchanan (135). The practical applications and limitations have been reviewed by Schimke (132).

The methods fall into two classes: 1) continuous radioactive labeling techniques, and 2) pulse radioactive labeling. Data analysis for the first is considerably simplified, but useful information can only be obtained for proteins with half-lives greater than 3-4 days. The major difficulty with the second is extensive reutilization of the isotope which tends to give a gross underestimate of the degradation rate and, thus, overestimates the half-life of the protein.

(1) Continuous labeling methods.

Several protocols involving continuous administration of a radiolabeled precursor have been developed to circumvent the problem of reutilization.

Swick et al. (136) have used the continuous incorporation of ^{14}C-carbonate from a rat diet. The carbonate is introduced into the guanidino group of arginine in the urea cycle in the liver and

thence into the liver proteins. The turnover is calculated from the expression:

$$A = U (1 - e^{-kt})$$

where A is the specific radioactivity of the guanidino-labeled arginine in the protein, U is the average specific radioactivity of the rat urinary urea formed during exposure to the radioactive carbonate after an initial 18 hour period and k is the degradation rate constant. The urea provides a measure of the intracellular metabolite labeling. The method gives a valid estimate of the degradation rate when the quantity of precursor is relatively small, when the precursor is rapidly renewed, and achieves its ultimate specific activity in a short time relative to turnover and when the specific activity of the precursor is unaffected by the reutilization of label.

Other labels have been used in continuous methods. However, with these, a time period of up to 24 hours is required before the ultimate specific activity of the intracellular precursor is reached. This fact restricts the method to proteins with half-lives greater than 3-4 days since proteins with short half-lives have a time course of incorporation into the protein identical to the incorporation into the precursor pool.

Schimke (105) has measured the half-life of arginase in rat liver by continuous addition of ^{14}C-lysine. From a plot of

ln (P_{max} - P) versus time, where P_{max} is the maximum specific activity of the protein, a value of the half-life was determined. The half-life obtained by this method agreed with the value obtained by use of guanidino-labeled arginine.

Fritz et al. (103,137) have used continuous administration of a uniformly labeled amino acid mixture in studies of lactic dehydrogenase. They give a comprehensive mathematical derivation for the method and describe the six simplifying assumptions upon which the derivation is based (137).

Under steady-state conditions with continuous administration of radioactive amino acids in the diet, the radioactivity in the intracellular free amino acid pool will rise and reach a constant maximum value. At this time, the specific radioactivity of the protein will have a value of P^*_o. As the experiment progresses, the specific radioactivity of the protein will continue to increase through P^* until it reaches a maximum value P^*_{max}. From the equation:[1]

$$\ln (P^*_{max} - P^*) = -k_d t + \ln (P^*_{max} - P^*_o)$$

a plot of ln (P^*_{max} - P^*) versus t will give a straight line with a negative slope equal to k_d.

The six necessary assumptions are:

i There exists an intracellular amino acid pool and an intracellular protein pool.

[1]This equation is a modification and correction of the one derived in reference 137.

ii A steady state protein concentration exists in vivo and $k_s = k_d C$ where synthesis is zero order and degradation first order.

iii Only amino acids from the amino acid pool are incorporated into proteins from the protein pool.

iv When protein is degraded, the products are always amino acids and they are always returned to the amino acid pool.

v The levels of protein and amino acids measured in the tissue extract are a true measure of the intracellular concentration of these substances.

vi The rates of intracellular amino acid and protein turnover are greater than the rates of cell turnover.

These assumptions are implicit in the other continuous label methods if not definitely stated. Various problems are encountered in the acceptance of several of these assumptions.

Assumption 1 is necessary to simplify the calculations, but studies by Righetti et al. (138) have suggested the possibility with leucine and phenylalanine that there is an amino acid compartment which does not equilibrate rapidly with the pool of amino acids used in protein synthesis.

A corollary of assumption 2 is that the animal is not growing, or rather that the tissue examined is not growing. Strictly speaking, this is not true with rats, and over the time period of

two to three weeks needed for the experiment, the tissue may in some cases grow quite significantly.

In regards to assumption 3, Hider et al. (139) have published experimental results which are consistent with a model whereby amino acids in the extracellular pool are incorporated directly into protein, and are inconsistent with a model involving an intracellular amino acid pool for the precursors of protein synthesis.

Walter (140) has proposed that protein degradation does not proceed as far as the free amino acids, but that the energy in the peptide bond may be conserved in the formation of an amino acid derivative which could be utilized for protein synthesis, and so there is a question about assumption 4.

(2) Pulse label methods

A single injection of a radiolabeled amino acid, usually leucine, is given to the experimental animal and the subsequent loss of label from the protein of interest observed (141). Tritiated water has also been used as a pulse label (142). The equation used to determine the degradation rate is:

$$\frac{dP}{dt} = -k_d P \qquad (2)$$

where P is the specific radioactivity of the protein in the tissue, and k_d is the rate constant of degradation.

For the technique to be successful, the labeled amino acid

must achieve maximum specific activity rapidly in the protein, disappear rapidly from the free amino acid pool, and not be re-utilized upon protein degradation. Problems have been encountered in the first two requirements, but isotope reutilization has caused the greatest difficulty in data interpretation.

Gan and Jeffay (143) have studied amino acid reutilization. They continually infused labeled amino acids into rats and found that the specific radioactivity of the liver amino acid pool increased to a maximum value which was less than the specific activity of the amino acids in the plasma. If the intracellular protein degradation is responsible for 50% of the liver amino acid pool, the discrepancy is explained. Righetti et al. (138) have determined in HeLa cells that the reutilization of leucine, lysine, and phenylalanine was 81%, 86%, and 84%, respectively. It has also been shown that ^{3}H-leucine is still present in the free liver pool as long as 10 days after injection (144). This degree of reutilization would cause significant underestimation of protein degradation by the pulse label method and direct comparison of this method with others (129) confirms this error.

Swick (130) introduced the use of guanidino-labeled arginine in the study of liver proteins to circumvent the reutilization problem. Intracellular arginine in the liver is rapidly degraded in the urea cycle, and therefore the reutilization of the guanidino label is small, but measurable. Even the low degree of reutilization

has been considered a source of some error (129).

Millward (145,146) has used ^{14}C-carbonate as a pulse label in the study of skeletal muscle proteins. The carbonate is incorporated into aspartic and glutamic acids by exchange in the tricarboxylic acid cycle and into arginine in the urea cycle in liver. Reutilization of this label is considered small, presumably due to the continued participation of the amino acids in the two cycles. It was originally thought necessary to isolate these amino acids because of the presumed reincorporation and reutilization of other amino acids radiolabeled by the introduction of ^{14}C-carbonate throughout the integrated pathways of amino acid metabolism. Ip et al. (147) isolated the arginine because of the concern for labeling of the carboxyl groups of several amino acids including arginine by the ^{14}C-carbonate.

However, in a later study Swick and Ip (148) concluded that this is not necessary as all amino acids significantly labeled by ^{14}C-carbonate are likely to have a low probability of reutilization. They therefore concluded that ^{14}C-carbonate is the label of choice for the study of hepatic protein turnover because: 1) it is relatively inexpensive, 2) it is available with high specific activity, 3) the k_d values obtained are better (higher) than those observed with guanidino-labeled arginine, even when the arginine is not isolated, and 4) it is unnecessary to perform elaborate manipulations of data to correct for reutilization of

labeled amino acids in order to obtain more valid estimates of protein half-lives.

A double label pulse method has been developed by Arias et al. (149). The animal is first injected with a ^{14}C-labeled amino acid and then just before sacrifice reinjected with the same amino acid containing a ^{3}H-label. The ratio of ^{3}H to ^{14}C gives a relative rate of turnover but not an actual value for k_d. This method requires the additional assumption that the rates of synthesis of the protein are the same at the times of the two injections.

Glass and Doyle (115) have modified the method to provide estimates of k_d. This is accomplished by determining the $^{3}H/^{14}C$ ratio for a number of intracellular components for which the k_d value has been determined by the guanidino-arginine method. A calibration curve is then constructed and the k_d value of the protein of interest determined from its experimental $^{3}H/^{14}C$ ratio. The method will be as accurate as the values used in the construction of the calibration curve.

Poole (144) has considered other limitations of the pulse label method and concludes that the method will only yield significant figures for proteins with half-lives of three days or less. Where the half-life is greater than this, the kinetics of loss of isotope are such that actual differences will fall within the range of experimental error. He suggests conditions whereby the

experimental design can be optimized for proteins with longer half-lives, and the data presented by Glass and Doyle (115) indicates that, under optimal conditions, their method is effective for proteins with half-lives up to 5-6 days.

The importance of protein degradation as a metabolic method for controlling the level of enzymes has been well established. The degradation rates have been shown to be responsive in several systems to various influences including genetic, hormonal, dietary, seasonal factors and the levels of metabolic intermediates. Even the physical structure and amino acid composition of proteins has been shown to have a role. The interactions and relative importance of each of these factors varies with each protein so that each must be studied in the system of interest.

For determining the best method of measuring half-lives of the pyruvate kinase isozymes in this study, a preliminary half-life for the rat liver pyruvate kinase isozymes was estimated as 2.5 days (k_d = 0.28) using published subunit molecular weights for the isozymes (see Table 3) and the studies correlating the molecular weight and degradation rate (115,116). On this basis the methods using pulse-labeling appeared the most appropriate for use, and after examining the available literature summarized above, it was decided that the use of a single pulse of ^{14}C-carbonate was the best method available for determining the degradation rates of rat liver proteins.

4. Statement of Thesis

The interaction of genetic and metabolic features in determining the total metabolic flux of intermediates through enzymes is well known. The levels of an enzyme can be controlled by the processes of synthesis and degradation, but, without metabolic control through certain effector molecules, the opportunities for immediate and fine control of enzyme activity would not be available. In addition, gene duplication and subsequent mutations provide an opportunity for the development of multiple isozymes responsive to different conditions present in various tissues and cells of species. With these interactions, cellular metabolism can be responsive to the particular metabolic needs of the diverse cell types.

To examine these interactions requires considerable information about molecular structure, kinetics, and evolution. It is necessary to examine the various isozymes which are present in several different species in order to examine which features are important in particular circumstances and therefore which features are necessary for general purposes.

Two liver isozymes of pyruvate kinase have been described in rat and man, and they have been found to respond to different effector molecules. However, it was uncertain whether certain structural characteristics of the two isozymes were necessary for the metabolic activities of these two isozymes in livers of different

species, or whether the structures of pyruvate kinase isozymes found in rat and man had developed particular features for the environment of those two species. If livers from different species have a similar environment and metabolism, they might be expected to have similar structures to accomodate similar requirements.

Only one isozyme had been described in chicken liver (150-152), and this had been identified as M_2-like (150). This left it uncertain, when combined with the information available for several mammals, whether one or multiple isozymes were necessary in liver metabolism. Because of the different kinetic features of PK-L and PK-M_2, the absence of one isozyme would be expected to have an impact on the available modes of regulatory control of glycolysis in liver. The absence of an isozyme which has been identified in other species as responsive to diet and hormonal control, PK-L, would suggest that it had developed later in evolution to accomodate the specialized metabolic needs of the livers of a higher class of vertebrates - the mammals. However this hypothesis was uncertain because five pyruvate kinase isozymes had been described in frog liver supernatant (153).

If an enzyme is found to have changed little over evolutionary time, this would suggest strong evolutionary pressure on the maintenance of that structure for the enzyme to play its necessary role in tissue or cell metabolism. The first part of this thesis provides a frame for asking questions about the metabolic

need for particular forms of pyruvate kinase isozymes. Using available species from several phylogenetically distant classes of organisms, this thesis examines whether multiple isozymes are necessary for liver metabolism, whether there are certain structural characteristics which have been preserved during evolution, whether multiple isozymes, if found, bear structural relationships to the PK-L and PK-M_2 isozymes found in human and rat, and whether there is general agreement between the evolutionary history found in pyruvate kinase structures and the history developed by classical methods of taxonomy.

To answer these questions, electrophoretic and immunologic techniques were used to examine the degree of conservatism in pyruvate kinase from different species. Antibodies prepared against the human erythrocyte isozyme, the human muscle isozyme, and the bovine muscle isozyme were used as probes for structural similarities.

The second part of this thesis focuses on the characteristics of pyruvate kinase isozymes found in a single species, and indeed within a single tissue, in order to examine comparative features of metabolic regulation of enzyme levels. The liver of man and rat contains both PK-L and PK-M_2 and therefore provides an opportunity to examine differences within a single tissue. As discussed in the introduction, the levels of isozymes are controlled by two processes: synthesis and degradation. In this part of the thesis

the role of degradation in the metabolic control of these two isozymes was examined. For rat liver the degradation rate constants were determined, and then several physical and chemical characteristics of the two isozymes were examined to look for a basis for the observed degradation rates. These factors included the subunit molecular weights, isoelectric points, and sensitivities to proteolytic enzymes.

Experimental Procedure

1. Chemicals and reagents

The tricyclohexylamine salt of phosphoenolpyruvate (PEP), the sodium salt of adenosine 5'-diphosphate (ADP) grade 1, the disodium salt of adenosine 5'-triphosphate (ATP) Sigma grade, adenosine 5'-monophosphoric acid (AMP) Sigma 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 type II, and bovine serum albumin grade V were all purchased from Sigma Chemical Co., Saint Louis, Missouri. Special enzyme grade ammonium sulfate was obtained from Schwarz-Mann, Orangeburg, New York. Aquasol-2 and ^{14}C-Na_2CO_3, 10-20 mcuries/mM, were obtained from the New England Nuclear Corporation, Boston, Massachusetts.

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

Lyophilized trypsin, 194 units/mg, and lyophilized α-chymotrypsin, 55 units/mg,were purchased from Worthington Biochemical Corp., Freehold, New Jersey. Pronase, grade B, was obtained from Calbiochem, Los Angeles, California.

Sephadex G-25 medium and G-200-120 were obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey. The preswollen,

microgranular form of diethylaminoethyl cellulose (Whatman DE 52), and phosphocellulose (Whatman P11) were obtained from Reeve-Angel, Clifton, New Jersey.

The Cibacron blue F3GA-Sepharose 6B was a gift of Dr. Richard N. Harkins, presently at the Department of Biochemistry, Marischal College, University of Aberdeen, Aberdeen, Scotland.

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

2. Methods

a. Pyruvate kinase assay

Pyruvate kinase activity was determined by the coupled assay method developed by Bucher and Pfleiderer (154). The activity was followed by the oxidation of NADH at 340 nm for 5-10 min at 25° on a Gilford model 2400 recording spectrophotometer in matched 1 cm path length quartz cuvettes (Hellma Cells, Inc., Jamaica, New York). The standard reaction mixture had a final volume of 1 ml and contained 100 mM triethanolamine-HCl buffer, pH 7.4, 8 mM $MgSO_4$, 75 mM KCl, 0.4 mM ADP, 0.128 mM NADH, 1.5 mM PEP, 10-20 units of lactic dehydrogenase, and the appropriate amount of pyruvate kinase. In addition, for the measurement of yeast pyruvate kinase activity, the reaction mixture contained 1 mM FDP, and for the measurement of activity in Escherichia coli, the reaction mixture contained 1 mM FDP, or 1 mM AMP, or both. 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.

b. Protein concentration

The protein concentration was estimated during column chromatography by measuring the absorbance of the effluent fractions at 280 nm in a Beckman DB-GT spectrophotometer.

When specific activities of the pyruvate kinase preparations were determined, the protein was measured by the method of Lowry et al. (155) as modified by Oyama and Eagle (156), using bovine serum albumin as a standard.

c. Polyacrylamide gel electrophoresis

Polyacrylamide disc gel electrophoresis was performed with a Buchler disc gel apparatus according to the method provided by Buchler Instruments. The anionic system with a running pH of 9.3 was used with a 7.5% polyacrylamide running gel and a 2.5% polyacrylamide stacking gel. Samples were dialyzed for two hours against the upper buffer and then a small amount of sucrose, to increase sample density, was added to each sample. Tracking dye, 0.001% bromphenol blue, was added to the upper buffer reservoir, and then electrophoresis was performed at 2.5 ma per gel until one hour after the tracking dye had reached the bottom of the gel. The gels were removed, stained in 0.1% Amido Schwarz in 7% glacial acetic acid, and then destained and stored in 7% glacial acetic acid.

Horizontal thin layer polyacrylamide gel electrophoresis was performed at pH 8.2 by a modification of the method of Imamura and Tanaka (10). 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 two hours against the gel buffer, and 10 µl was put into the gel slots and covered with liquified vaseline. Electrophoresis was performed at 4° for 2.5 hours at 400 volts or 16.5 hours at 150 volts. Pyruvate kinase activity was visualized by a modification of the overlay method of Susor and Rutter (22). The reaction mixture contained 0.17 M Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 0.9 mM ADP, 3 mM PEP, 50 mM KCl, 0.2 mM NADH, 1 mM FDP, 1 unit/ml lactic dehydrogenase and 5 mg/ml Noble agar. Pyruvate kinase activity appears as dark bands on a fluorescent background when illuminated with ultraviolet light. The results were recorded by Polaroid photography.

Thin layer polyacrylamide electrophoresis was also performed on samples of pyruvate kinase mixed with different antibodies. Equal volumes of the dialyzed pyruvate kinase, 2 units/ml, and either an antibody solution or gel buffer were premixed before aliquots were placed into the gel slots. The procedure was then as described above.

d. Preparation of homogenates

Livers were obtained fresh from the following species: Bos taurus (bovine), Gallus gallus (chicken), Chrysemys picta

(painted turtle), Didelphis marsupialis virginianus (opossum), Salmo gairdneri (steelhead trout), Rana pipiens (frog), and Ratus ratus (rat). A liver was obtained at autopsy from Homo sapiens (human). These were stored frozen until use.

Ten grams of each tissue were homogenized in 20 ml of 20 mM Tris-HCl, pH 7.5 and 0.2 mM PEP. The homogenate was centrifuged at 20,000 x g for 30 min, and the supernatant decanted for use in later experiments.

Pyruvate kinase was prepared from yeast using Fleishmann's dry yeast by a modification of the method of Hunsley and Suelter (157). About 21 g of dry yeast was rehydrated with 20 ml distilled, deionized water and then 15 ml of toluene and 16.5 ml of cold 20 mM Tris-HCl, pH 7.5 and 0.2 mM PEP were added and the mixture was shaken. This was left overnight at 4° and then centrifuged at 20,000 x g for 30 min. The middle aqueous layer was removed and recentrifuged. The middle aqueous layer of this was then used in later experiments.

Escherichia coli Sear were prepared with the growth medium described by Waygood and Sanwall (158) from the stock culture collection of the Department of Microbiology and Immunology, University of Oregon Health Sciences Center. After 18 hours at 37° the cells from two liters of medium were harvested by centrifugation, and then sonicated for a total of 3 min with a Bronwill Biosonik after adding 30 ml of cold 20 mM Tris-HCl, pH 7.5, 0.2 mM

PEP and 10 mM 2-mercaptoethanol to resuspend the pellets.

e. Preparation of crude liver PK-L, PK-M_2, and crude PK-R

When impure PK-L or PK-M_2 was needed, it was prepared from human or rat liver supernatants by ammonium sulfate precipitation. PK-L and PK-M_2 were precipitated in the 20-40% and 50-75% fractions, respectively. PK-R was prepared from washed human erythrocytes as described previously (159).

f. Separation of the two pyruvate kinase isozymes in E. coli

The DEAE column method of Malcovati and Kornberg (160) was used to separate the two pyruvate kinase isozymes of E. coli. The buffer was 2.5 mM NaH_2PO_4, 2.5 mM KH_2PO_4, pH 7.5, 1 mM K_2EDTA and 10 mM 2-mercaptoethanol. The DEAE column was 35 x 1.5 cm, with the flow rate of 40 ml/hr. Fractions were collected every six minutes.

g. Antibody inactivation

The preparations in rabbits of the antibodies against pyruvate kinase have been described earlier for the human erythrocyte (24), bovine muscle (60), and human muscle (23).

For inactivation studies, the antibody was serially diluted with 100 mM triethanolamine-HCl, pH 7.4. Equal volumes of enzyme solutions (0.26 units/ml for the L-isozyme, the erythrocyte isozyme, and the tissue homogenates, and 0.46 units/ml for the M_2- and M_1-isozymes) in the same buffer were then added to each dilution. The activity differences of the L-, M_2- and M_1-, and erythrocyte isozymes were calculated to give equal molar concentrations of the isozymes,

assuming that the human isozymes have the same activity ratios as the rat isozymes (8,11,44). After incubation for 5 min at 37°, a sample was removed to determine enzyme activity. Controls used isozyme incubated with buffer in place of antibody. Control globulin preparations from non-pyruvate kinase-immunized rabbits have no inhibitory activity in this assay.

h. Immunodiffusion

Standard gel immunodiffusion techniques (161) were used to determine the immunoreactivity of various pyruvate kinase preparations with different antisera. These tests were performed by Dr. Marvin Rittenberg, Department of Microbiology and Immunology, University of Oregon Health Sciences Center.

i. Stability tests on partially purified human liver PK-L

Human liver PK-L was purified through the G-200 step (see the "Results" section) and redissolved in 18 mM potassium phosphate buffer to a final concentration of 0.25 units/ml. In one series of experiments the buffer pH was varied from 5.0 to 7.2. In a second set of experiments the enzyme was redissolved in 18 mM potassium phosphate, pH 7, 5 mM 2-mercaptoethanol, and also one of the following sets of additives: 1) 1 mM FDP, 2) 10 mM alanine plus 5 mM ATP, 3) 5 mM $MgCl_2$, 100 mM KCl, and 1 mM EDTA, 4) 0.5 mM ADP and 0.5 mM PEP, 5) 0.5 M sucrose, and 6) 20% glycerol. Aliquots in both sets of experiments were then incubated at 5° or 25°, and at various times, small aliquots were removed for activity

measurements.

j. Sedimentation equilibrium

Sedimentation equilibrium was performed in the laboratory of Dr. Demetrios Rigas, Department of Biochemistry, University of Oregon Health Sciences Center, on the purified human liver PK-L according to the low speed method as described by Chervenka (162) using a Spinco model E analytical ultracentrifuge equipped with Rayleigh interference optics and run at 5220 rpm. Before the run the sample was dialyzed for 24 hours against 0.1 M Miller's buffer, pH 7.2 (163) with 0.1 mM FDP. The buffer density has been previously determined (23). Equilibrium was reached within 72 hours, and a photograph of the interference pattern was taken at that time. 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) indicates a homogeneous solute. The slope of the line was obtained by the least squares method from which the molecular weight of the liver enzyme was calculated according to the method of DiCamelli et al. (164), assuming a partial specific volume of 0.739, which is that of human PK-R (24).

k. Inactivation by proteolytic enzymes

Human liver PK-L and PK-M_2 were prepared by a 20-35% acetone fractionation performed in the cold room with acetone at

-10°, followed by 20-40% and 50-75% $(NH_4)_2SO_4$ fractionations to yield PK-L and PK-M_2, respectively. The partially purified human muscle PK-M_1 had been previously prepared by Harkins (23).

In the first set of experiments the enzymes were adjusted to 0.35 units/ml in 100 mM triethanolamine-HCl, pH 7.4 and the OD_{280} of each solution was adjusted to the same value with bovine serum albumin. The proteolytic enzymes trypsin, chymotrypsin, and pronase were dissolved in the same buffer to a concentration of 40 µgm/ml. At time 0, equal volumes of the isozyme and one of the proteolytic enzymes were mixed, an aliquot immediately removed and the pyruvate kinase activity measured for the control value. The solutions were incubated at 28°, and aliquots were removed at various times to determine pyruvate kinase activity.

In the second set of experiments, the enzymes were adjusted to 0.5 units/ml in 100 mM triethanolamine-HCl, pH 7.4. Pronase was dissolved in the same buffer to a concentration of 50 µgm/ml. Solutions were made of the following additives: 1) PEP, 2) 3PGA, 3) ADP, 4) ADP plus $MgCl_2$, 5) ATP, 6) ATP plus $MgCl_2$, 7) $MgCl_2$, 8) sodium citrate, 9) L-alanine, 10) L-phenylalanine, and 11) G6P. The isozymes were preincubated for 10 minutes with the additives, and at time 0 either the buffer alone for the control sample or the buffer containing the pronase was added. The final isozyme concentration was 0.25 units/ml, the final pronase concentration was 20 µgm/ml, and the final concentrations of all additives was 1 mM. The

samples were incubated at room temperature, and at various times an aliquot was removed from both the control sample without pronase and the sample with pronase for the determination of pyruvate kinase activity.

1. Half-lives of rat liver PK-L and PK-M_2

Female Buffalo rats, weighing 140-150 g were used. The radiolabel was ^{14}C-Na_2CO_3, 10-20 mcuries/mM, from New England Nuclear. On day 0, 5.0 mcuries of radiolabel were dissolved in 6.3 ml of isotonic saline. Each rat was then injected i.p. with 0.5 ml.

At the same time each day on days 2, 3, 4, and 7, three rats were killed by decapitation, the livers were excised and homogenized in 20 ml of extraction buffer which was 20 mM Tris, pH 7.4, 0.2 mM PEP and 10 mM 2-mercaptoethanol. The homogenates were centrifuged at 20,000 x g for 30 minutes, and PK-L and PK-M_2 were prepared from the supernatants by 20-40% and 50-75% $(NH_4)_2SO_4$ cuts, respectively.

From the same set of rats, it was desired to determine the half-lives of PK-L, PK-M_2 and the whole liver supernatants. The specific activities of the PK-L, PK-M_2 and the supernatant was determined by immunoprecipation of the two pyruvate kinase isozymes with appropriate antibodies, and by using known quantities of liver supernatant. The details of the immunoprecipitation experiments are described below.

On day 8, the ammonium sulfate precipitates were redissolved in 1.5 ml of extraction buffer and dialyzed for two hours against the same buffer. For the PK-L samples: to 0.065 ml aliquots of the pyruvate kinase samples was added either 0.080 ml anti-PK-R antibody or 0.16 ml normal rabbit IgG immunoglobulin. (The amounts of anti-PK-R and normal immunoglobulin present equivalent amounts of protein.) This was incubated for 15 min at 37°, and then 2.56 ml of sheep anti-rabbit IgG (for preparation, see Appendix 1) was added. This was incubated for 30 min at 37° and then for 24 hours at 5°.

For the PK-M_2 samples: to 0.310 ml aliquots of the pyruvate kinase samples was added either 0.040 ml anti-M_1 antibody or 0.040 ml normal rabbit serum. This was incubated for 15 min at 37° and then 0.640 ml of sheep anti-rabbit IgG was added. This was incubated for 30 min at 37° and then for 24 hours at 5°.

The quantities of pyruvate kinase used was about 1 unit for the two isozymes. The quantities of anti-pyruvate kinase, and sheep anti-rabbit IgG had been previously determined by Dr. Marvin Rittenberg at the University of Oregon Health Sciences Center by quantitative precipitation (161) as providing the maximum precipitation for this quantity of pyruvate kinase.

After incubation each immunoprecipitate was washed three times with cold isotonic saline, and then the precipitates were dissolved in 0.2 ml of 0.3 N NaOH, transferred to a scintillation vial, and the precipitate tubes were washed once with 0.2 ml of 0.3

N NaOH, which was also transferred to the scintillation vial. This was neutralized with 0.4 ml of 0.3 N HCl. To each vial was added 10 ml Aquasol-2 and the vials were shaken vigorously.

An aliquot of each rat liver supernatant was added to scintillation vials containing 0.4 ml of 0.3 N NaOH and 0.4 ml of 0.3 N HCl. Then 10 ml Aquasol-2 was added to each vial. Control vials were prepared using a similar amount of water instead of rat liver supernatant.

The vials were counted in a Packard Tri-Carb liquid scintillation spectrometer for 50 minutes in order to reduce the counting error to less than 5%. Counting efficiency was 80%.

The protein concentration of each pyruvate kinase sample and supernatant was determined by the modified method of Lowry et al. (156) and the radioactivities were expressed, after subtracting the control radioactivities, as cpm/mg protein.

Equation 2, which was discussed earlier, is:

$$\frac{dP}{dt} = -k_d P$$

where P is the specific radioactivity and k_d is the rate constant of degradation. Upon integration, this yields:

$$P_t = P_o e^{-k_d t}$$

where P_t is the specific radioactivity at time t, and P_o is the initial radioactivity. The value k_d is the slope of the line

calculated from a least squares determination of ln P_t versus t. The half-life, $t_{1/2}$ is calculated from the formula:

$$t_{1/2} = \frac{\ln 2}{k_d}$$

The standard error of the mean for k_d was calculated from the formula provided in Ezekiel and Fox (165).

Results

1. Purification of human liver L-type pyruvate kinase

In 1973 Cardenas and Dyson (58) published a paper describing the purification of bovine liver L-type pyruvate kinase. This procedure, with several modifications, was adopted for the purification of the human isozyme.

In early purification attempts, human liver PK-L was purified through the G-200 step (see later purification procedure) and then assayed under various conditions to determine its stability at different pH's, temperatures and the effects of various additives.

Figure 2 shows that PK-L has maximum stability in the pH range of 6.4 to 6.8. The buffer was 18 mM potassium phosphate. The incubation times at 5° and 25° were 3 and 2 hours, respectively.

Figure 3 shows the results when PK-L was incubated in 18 mM potassium phosphate, pH 7, and 5 mM 2-mercaptoethanol at 5°, with various compounds added to separate incubation solutions. Under the various conditions tested, the addition of 0.5 mM ADP and 0.5 mM PEP gave the greatest protection to the pyruvate kinase activity. The others, in order of their protecting ability, were:

20% glycerol ≅ 5 mM $MgCl_2$ + 100 mM KCl + 1 mM EDTA > 1 mM FDP ≅ 0.5 M sucrose > 10 mM L-alanine + 5 mM ATP > none.

Figure 4 shows the results when PK-L was incubated in 18 mM potassium phosphate, pH 7, and 5 mM 2-mercaptoethanol at 25°,

Figure 2

pH Stability of human liver PK-L. The incubation times at 5° and 25° were three and two hours respectively. The buffer was 18 mM potassium phosphate.

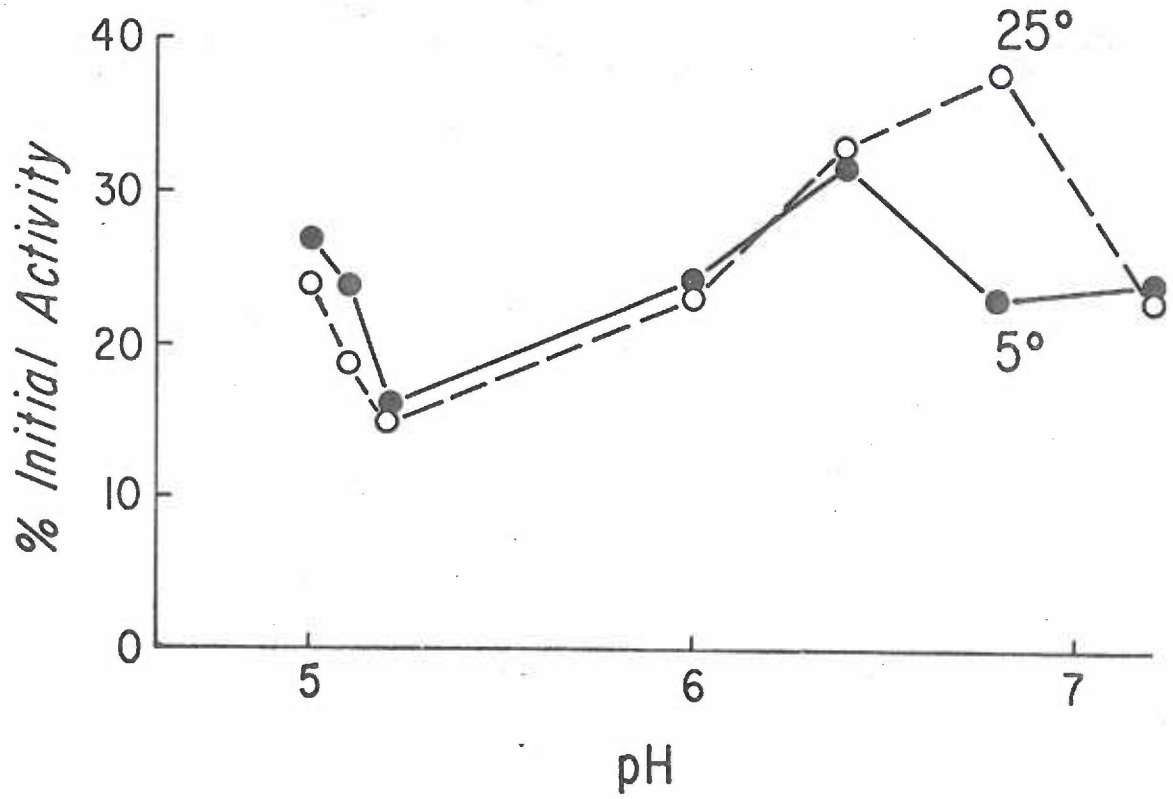

40
30
20
10
0
% Initial Activity
25°
5°
5
6
7
pH

Figure 3

Effects of various compounds on the stability of human liver PK-L at 5°. The buffer was 18 mM potassium phosphate and 5 mM 2-mercaptoethanol, pH 7. Additional compounds in the buffer: None (X); 1 mM FDP (O); 10 mM L-alanine and 5 mM ATP (▲); 5 mM $MgCl_2$, 100 mM KCl and 1 mM K_2EDTA (Δ); 0.5 M sucrose (■); 20% glycerol (●); 0.5 mM ADP and 0.5 mM PEP (□).

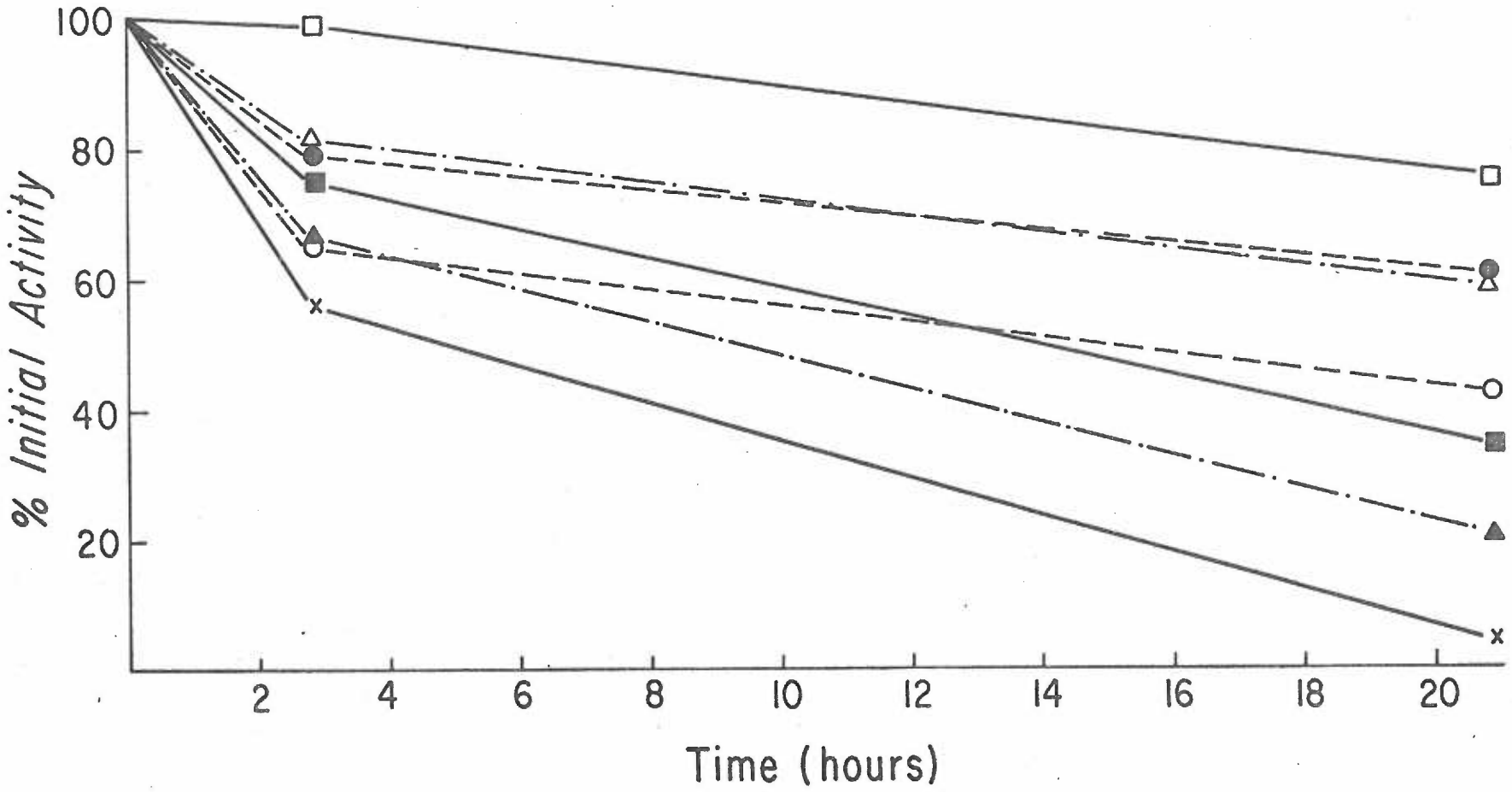
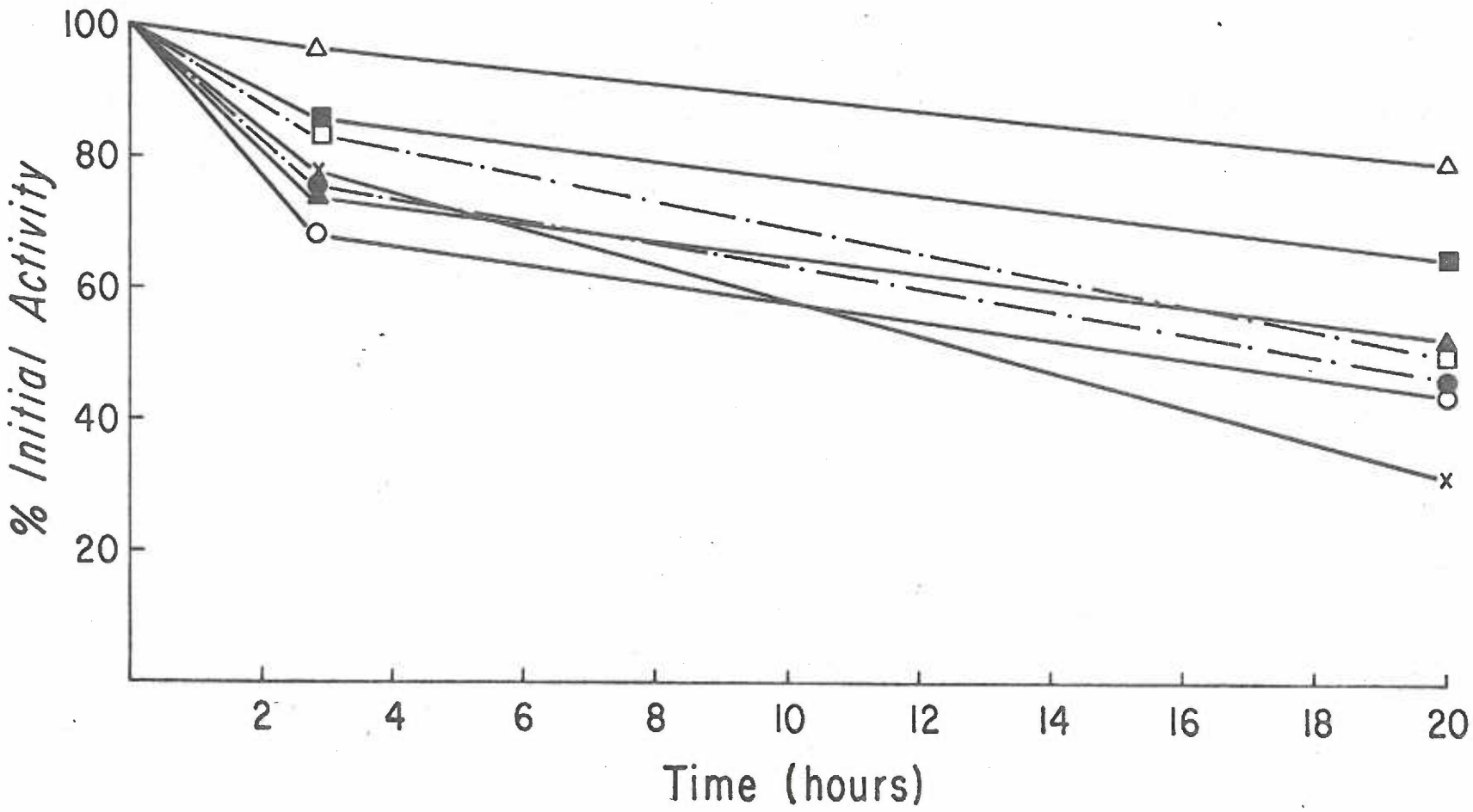

100
80
60
40
20
% Initial Activity
2
4
6
8
10
12
14
16
18
20
Time (hours)

Figure 4

Effects of various compounds on the stability of human liver PK-L at 25°. The buffer was 18 mM potassium phosphate and 5 mM 2-mercaptoethanol, pH 7. Additional compounds in the buffer: None (X); 1 mM FDP (O); 10 mM L-alanine and 5 mM ATP (▲); 5 mM $MgCl_2$, 100 mM KCl and 1 mM K_2EDTA (Δ); 0.5 M sucrose (□); 20% glycerol (●); 0.5 mM ADP and 0.5 mM PEP (■).


100
80
60
40
20
% Initial Activity
2
4
6
8
10
12
14
16
18
20
Time (hours)

with various compounds added to separate incubation solutions. Under these conditions the addition of 5 mM $MgCl_2$ + 100 mM KCl + 1 mM EDTA gave the greatest protection. The others, in order of their protecting ability were:

0.5 mM ADP + 0.5 mM PEP > 1 mM FDP ≅ 0.5 M sucrose ≅ 20% glycerol ≅ 10 mM L-alanine + 5 mM ATP > none.

From these stability experiments it was concluded that the addition of PEP and 2-mercaptoethanol would provide the best protection during the period between each purification step. Therefore, 0.2 mM PEP and 10 mM 2-mercaptoethanol were adopted in the purification procedure as necessary additions between each step if they were not already present. These compounds and the particular concentrations were used because of availability limitations.

a. Step 1: Extraction

Human livers were obtained at autopsy and either used immediately or frozen at -16° until use. Livers weighing 1400-1600 g were homogenized in a Waring blender with 2 volumes (2800-3200 ml) of 20 mM Tris-HCl, pH 7.5, and 0.2 mM PEP. Debris was removed by centrifugation at 10,000 x g for 30 min at 4° in a Sorvall RC-2B centrifuge. The supernatant was decanted and filtered through cheesecloth.

b. Step 2: Acetone fractionation

Acetone (precooled to -20°) was added to a final concentration of 20% (v/v). The resulting precipitate was removed by

centrifugation at 10,000 x g for 20 min at -10°. Additional acetone was added to a final concentration of 35% (v/v) and the second precipitate was collected as above. This precipitate was dissolved in one-half volume (700-800 ml) of 20 mM Tris, pH 7.5, 0.2 mM PEP and 10 mM 2-mercaptoethanol and gently stirred overnight at 4° to remove the acetone.

c. Step 3: pH 5 ammonium sulfate fractionation

The pH of the redissolved precipitate from step 2 was adjusted to 5.0 with 6 N HCl, and then solid ammonium sulfate was slowly added with stirring to a final concentration of 20%. The resulting precipitate was removed by centrifugation at 10,000 x g for 20 min. To the supernatant was added solid ammonium sulfate to a concentration of 35%, and this precipitate was collected by centrifugation as described above.

d. Step 4: pH 7 ammonium sulfate fractionation

The precipitate from step 3 was dissolved in one-fourth volume (350-400 ml) of 20 mM Tris-HCl, pH 7.5, 0.2 mM PEP, and 10 mM 2-mercaptoethanol, and the pH was adjusted to 7.0 with 3 N KOH. The protein fractionating between 20% and 35% ammonium sulfate was collected as described in step 3, with the pH held at 7.0 throughout this step.

e. Step 5: DEAE cellulose batch

The precipitate from step 4 was dissolved in a minimum amount of 10 mM KH_2PO_4, 0.5 M sucrose, and 10 mM 2-mercaptoethanol,

adjusted to pH 7.1 with 4 N KOH. This sample was desalted at 4° on a Sephadex G-25 column (5 x 100 cm) equilibrated against the same buffer.

DEAE cellulose (Whatman DE52) had been previously equilibrated at room temperature against the same buffer. As the final step of preparation, the DEAE slurry was filtered on a Buchner funnel until cracks formed in the cake. A 250 g portion of the cake was weighed, and this was added to the pooled pyruvate kinase fractions from the G25 column after the pool had been warmed to room temperature. The slurry was stirred for 20 min. Only 10% of pyruvate kinase activity remained in the supernatant after adding the DEAE.

The DEAE slurry was filtered on a Buchner funnel and washed with 250 ml of the buffer described above. The DEAE cake was resuspended in 250 ml of the buffer and 15 ml of the same buffer containing 100 mg KCl/ml was added. The slurry was stirred for 20 min, then filtered on a Buchner funnel and the cake was washed with 250 ml of the buffer containing KCl.

The filtrate was pooled, PEP was added to 0.2 mM and the solution was stored at 4° as a 70% ammonium sulfate suspension.

f. Step 6: Sephadex G-200 gel filtration

A column (2.5 x 100 cm) of G-200 equilibrated against 20 mM sodium acetate and 10 mM 2-mercaptoethanol was set-up for reverse flow at 4°. The flow rate was controlled at 18 ml/hr with a Gilson

Minipuls II pump.

The ammonium sulfate suspension from step 5 was centrifuged at 10,000 x g for 10 min, and the precipitate was dissolved in a minimum amount of column buffer. The sample was applied to the column and fractions were collected every 8 min. Fractions were assayed for pyruvate kinase activity and for optical density at 280 nm (Figure 5). The peak fractions of pyruvate kinase activity were pooled, then sucrose was added to a concentration of 0.5 M and the pH was adjusted to 5.2. This was cooled to 4° and immediately used in step 7.

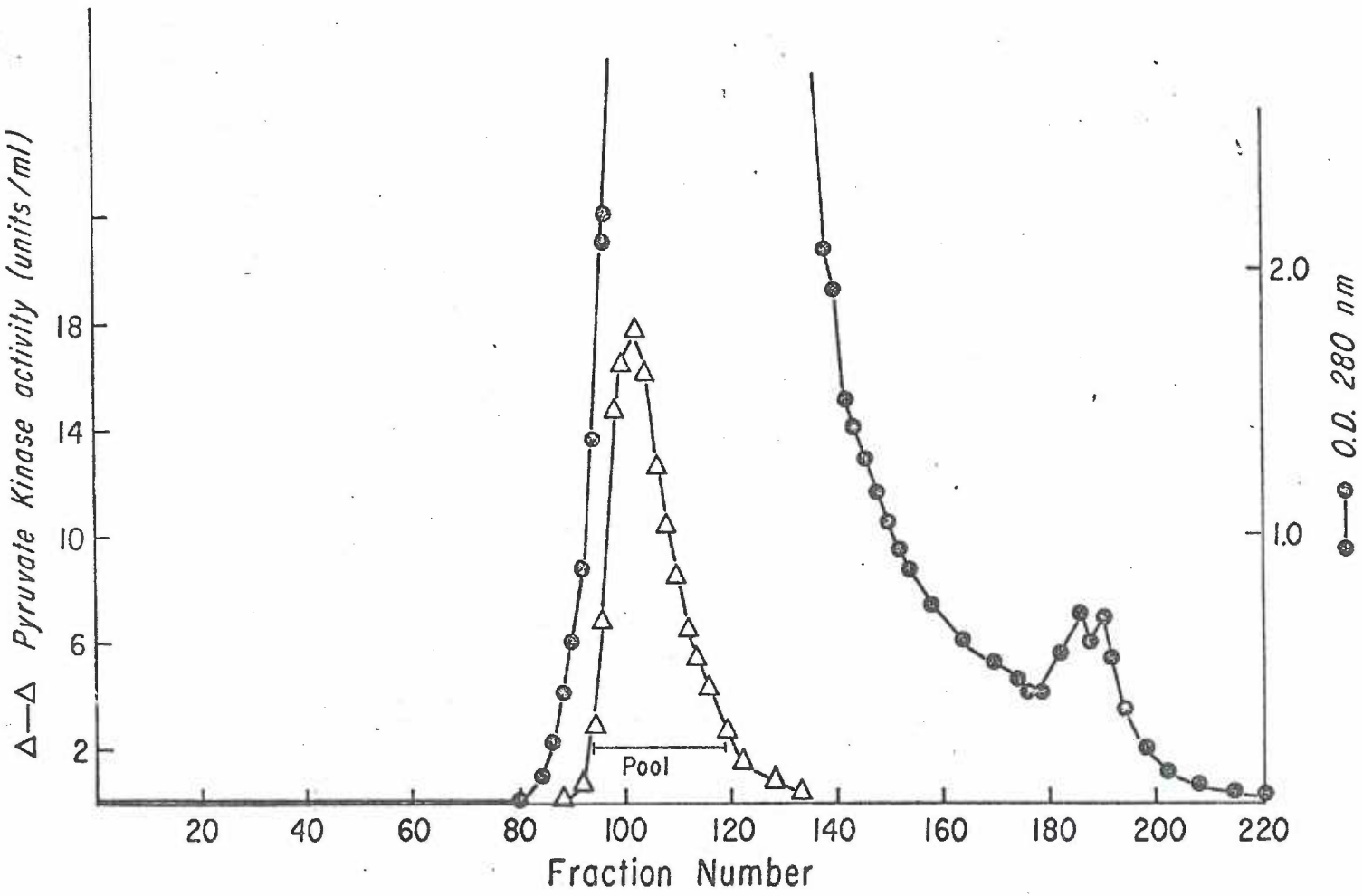
g. Step 7: Phosphocellulose chromatography

The phosphocellulose (P11) was prepared by several washings with 1 N NaOH until the color had been removed, one wash with 1 N HCl, and finally several washings with distilled, deionized water. This material was used to prepare a column (2.5 x 40 cm) after equilibration with 20 mM sodium acetate, pH 5.2, 0.5 M sucrose, and 10 mM 2-mercaptoethanol. This column was run at a temperature of 4° and a flow rate of 40 ml/hr.

The sample from step 6 was applied to the column, with fractions collected every 10 min. After the sample was on the column the column was washed for 3 hrs with the column buffer. The column was then eluted with a gradient formed using five chambers of a Buchler nine-chambered gradient maker: chamber 1 contained 400 of the column buffer, and chambers 2-5 each contained 400 ml of

Figure 5

Sephadex G-200 chromatography of human liver PK-L. Each fraction contained 2.5 ml.


Δ—Δ Pyruvate Kinase activity (units/ml)
18
14
10
6
2
2.0
1.0
O.D. 280 nm
Pool
20
40
60
80
100
120
140
160
180
200
220
Fraction Number

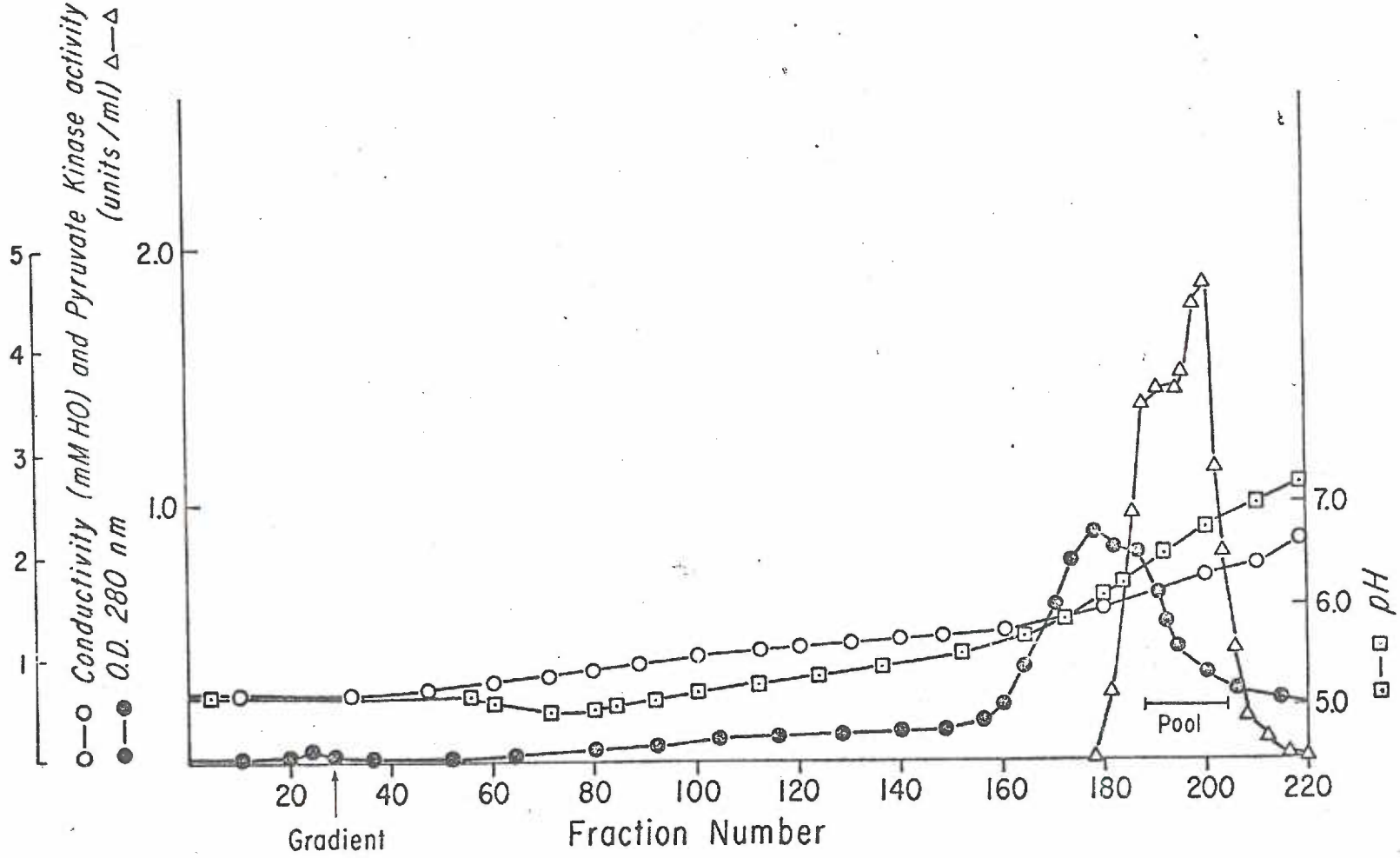
50 mM KH_2PO_4, 0.5 M sucrose, and 10 mM 2-mercaptoethanol, adjusted to pH 7.5 with 4 N KOH. The fractions were assayed for pyruvate kinase, for optical density at 280 nm, for conductivity at 25° with a Radiometer CDM 2d conductivity meter and for pH at 25° with a Radiometer pH meter 26 (Figure 6). The peak fractions with pyruvate kinase activity were pooled, PEP was added to a concentration of 0.2 mM, and the sample was stored at 4° as an 80% ammonium sulfate suspension.

h. Step 8: Cibacron blue F3GA-Sepharose 6B affinity chromatography

The ammonium sulfate suspension from step 7 was centrifuged at 10,000 x g for 10 min, and the precipitate was dissolved in a buffer modified from that used by Staal et al. (41): 5 mM KH_2PO_4, 5 mM $MgSO_4$, 10 mM 2-mercaptoethanol and 0.5 M sucrose, adjusted to pH 6.8 with 1 N KOH. This sample was desalted at room temperature on a Sephadex G-25 column (2.5 x 40 cm) equilibrated against the same buffer. Flow rate was maintained at 120 ml/hr with the Gilson pump. The pyruvate kinase fractions were pooled and applied to a column (1.6 x 7 cm) of Cibacron blue F3GA-Sepharose 6B previously equilibrated with the same buffer at room temperature. The column flow rate was 40 ml/hr and fractions were collected every 6 min. The column was washed for 30 min with column buffer and then eluted with a KCl gradient consisting of 200 ml of the column buffer and 200 ml of column buffer containing 1 M KCl. The fractions were

Figure 6

Phosphocellulose chromatography of human liver PK-L. Each fraction contained 6.7 ml.


Conductivity (mM HO) and Pyruvate Kinase activity (units/ml) Δ—Δ
O.D. 280 nm
pH
Pool
Gradient
Fraction Number
5
4
3
2
1
2.0
1.0
7.0
6.0
5.0
20
40
60
80
100
120
140
160
180
200
220

assayed for pyruvate kinase, for optical density at 280 nm, and for conductivity at 25° (Figure 7). The peak fractions of pyruvate kinase activity were pooled, PEP was added to 0.2 mM, and the sample was stored at 4° as an 80% ammonium sulfate suspension.

i. Summary of PK-L purification

Table 4 shows the yield and specific activities for the purification of human liver L-type, from 1460 g of starting material. The final product had a specific activity of 56.4 units/mg as assayed in the standard assay conditions, and represented an overall yield of 1.7% of the total liver PK activity.

j. Characterization studies of purified human liver PK-L

Disc gel electrophoresis

Polyacrylamide disc gel electrophoresis was performed on the purification product during several steps of the procedure. The results are shown in Figure 8. The major protein band indicates that the final product is nearly homogeneous with respect to this method.

Sedimentation equilibrium

The interference pattern photograph of the sample after equilibrium had been reached was measured, and a plot of log ΔY versus r^2 was made using overlapping intervals (Figure 9). The line drawn through the plot by the method of least squares had a correlation coefficient of 0.984, a slope of 0.2314, and a Y-intercept of -11.876. Using $\bar{\nu} = 0.739$, which is the partial specific volume of human PK-R (24), and $\rho_{20,s} = 1.002612$ for the solvent

Figure 7

Cibacron blue F3GA-Sepharose 6B affinity chromatography of human liver PK-L. Each fraction contained 4.0 ml.

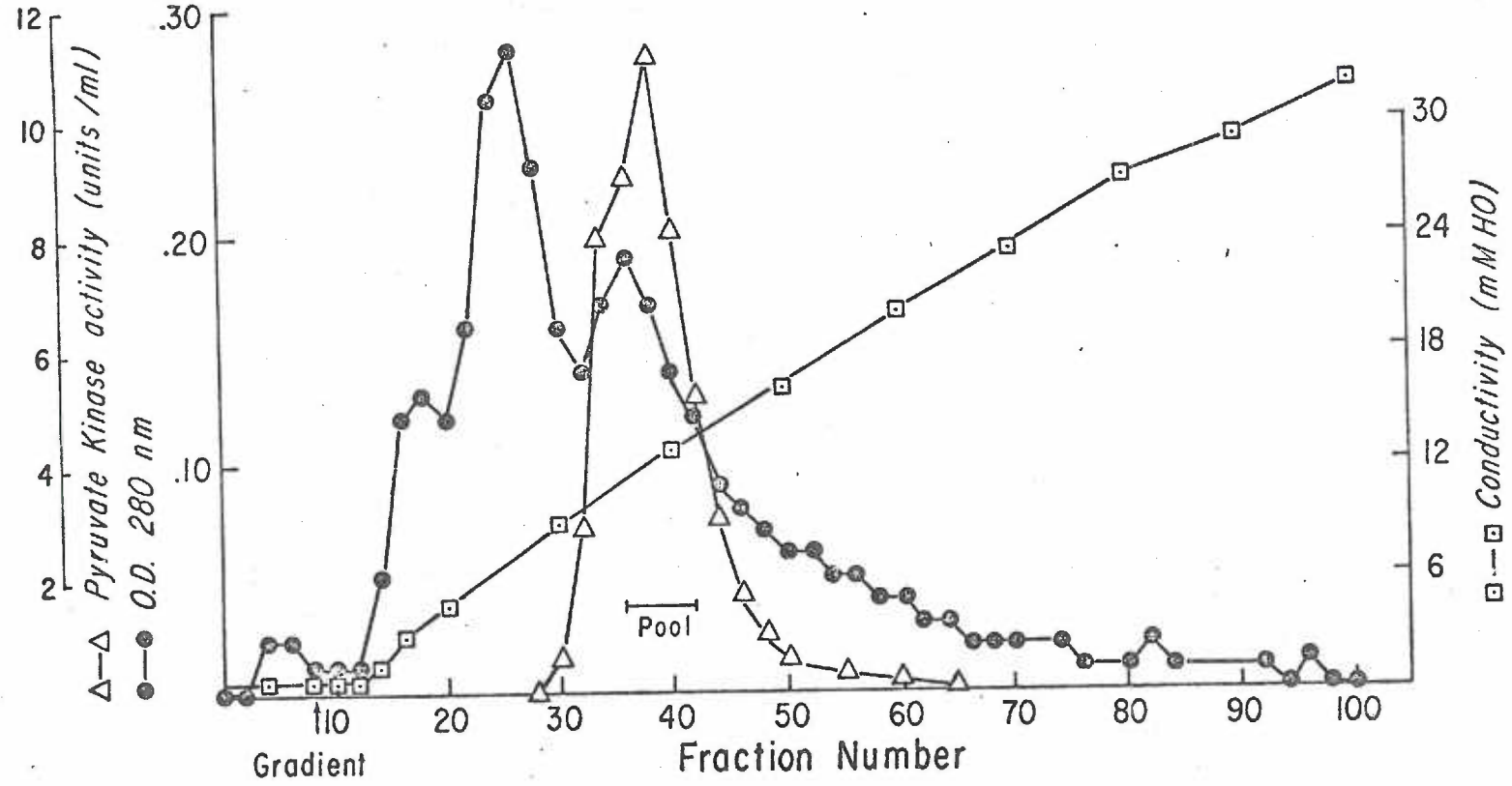
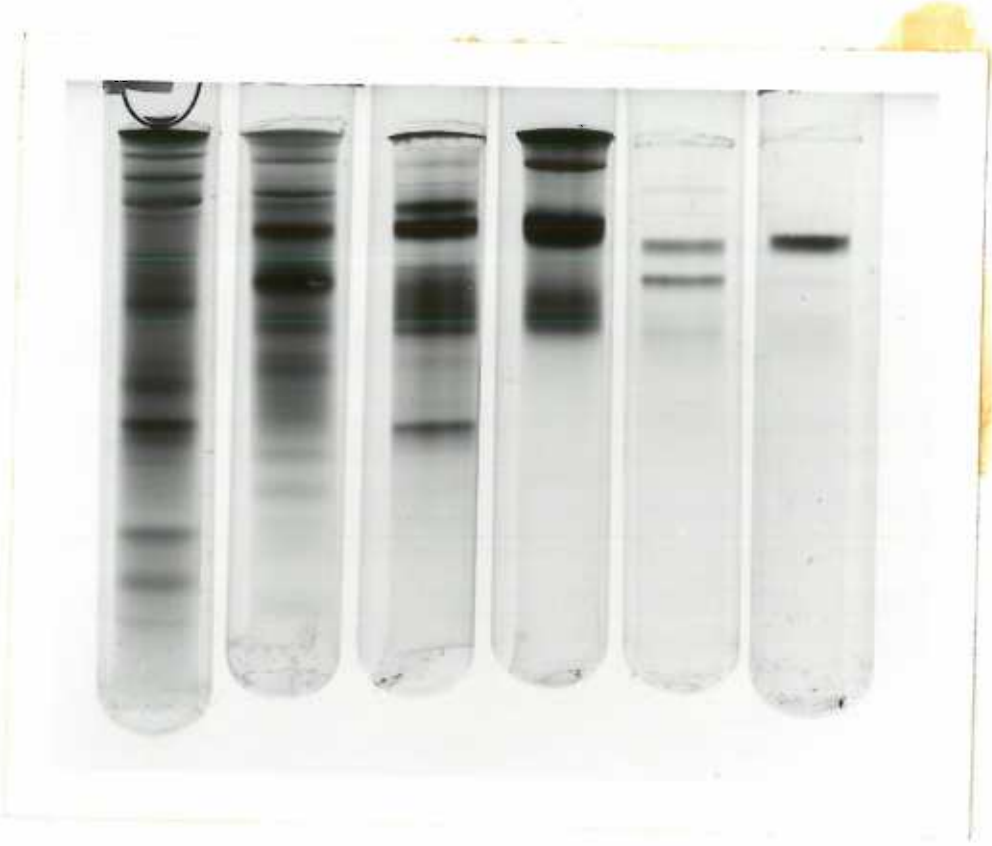

12
10
8
6
4
2
Pyruvate Kinase activity (units /ml)
△—△
.30
.20
.10
O.D. 280 nm
●—●
30
24
18
12
6
Conductivity (mM HO)
□—□
Pool
10
20
30
40
50
60
70
80
90
100
Gradient
Fraction Number

Table 4. Purification of human liver PK-L

Purification Step	Total Units	Total Protein (mg)	Specific Activity (units/mg protein)	Yield (%)	Purification
1. Crude extract	11,803	74,700	0.158	100	1
2. 20-35% Acetone fractionation	5,023	33,900	0.148	43	0.94
3. 20-35% pH 5 Ammonium sulfate fractionation	2,080	9,110	0.228	18	1.4
4. 20-35% pH 7 Ammonium sulfate fractionation	1,375	4,330	0.318	12	2.0
5. DEAE batch	1,212	567	2.14	10	14
6. Sephadex G-200 chromatography	660	226	2.92	5.6	18
7. Phosphocellulose chromatography	367	24.8	14.8	3.1	94
8. F3GA Affinity chromatography	206	3.65	56.4	1.7	360

Figure 8

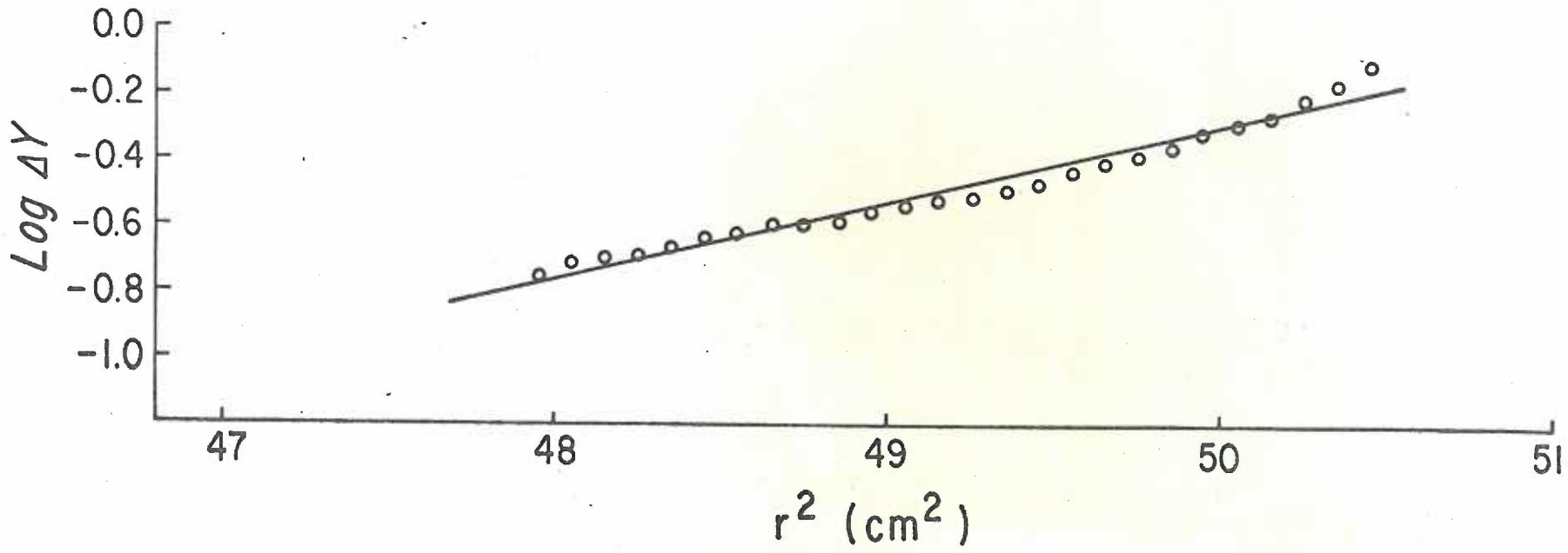
Polyacrylamide disc gel electrophoresis of human liver PK-L preparations. 1, Step 2, acetone fractionation; 2, Step 4, pH 7 ammonium sulfate fractionation; 3, Step 5, DEAE batch; 4, Step 6, Sephadex G-200 chromatography; 5, Step 7, phosphocellulose chromatography; 6, Step 8, Cibacron blue F3GA-Sepharose affinity chromatography.



1 2 3 4 5 6

Figure 9

Sedimentation equilibrium plot of log ΔY versus r^2 for human liver PK-L.


0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Log ΔY
47
48
49
50
51
r² (cm²)

buffer as determined previously (23), a molecular weight of 335,600 ± 2400 was calculated from the following formula (164):

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

where T = 293.16° K, and ω = the angular velocity.

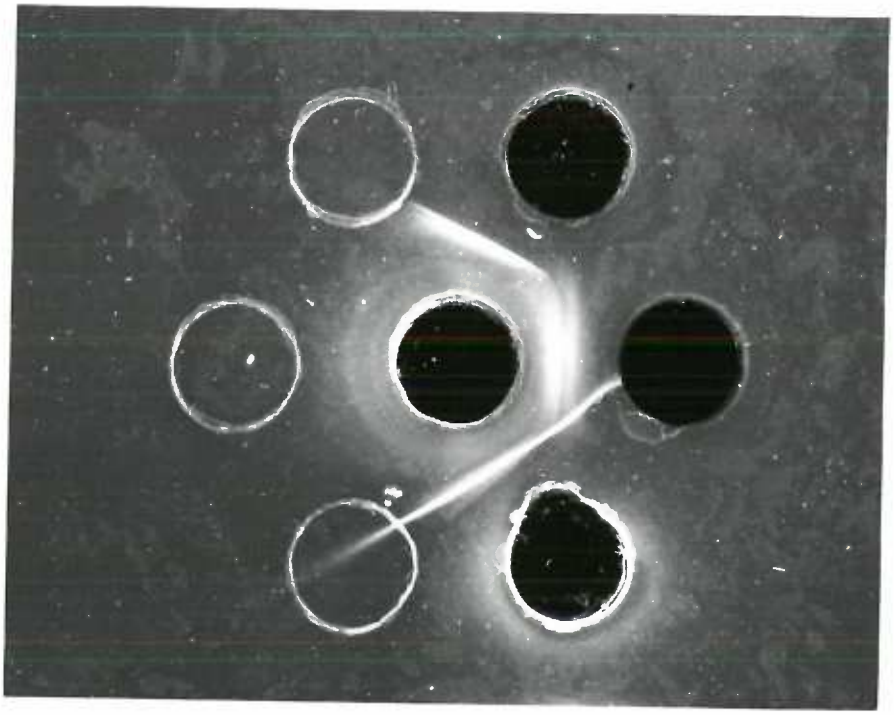
Immunodiffusion

Figure 10 shows the results of an immunodiffusion test using the anti-human PK-L prepared as described in the appendix. There is a heavy precipitin line and a much fainter line between the antiserum and the crude PK-L. This suggests that there may have been a minor contaminant in the purified PK-L used to prepare the antiserum.

The presence of multiple precipitin lines between the anti-PK-L and one sample of purified PK-L is suggestive of polymerization or pyruvate kinase degradative products. The intersection of the lines of the two samples of purified pyruvate kinase and one of the two samples with the crude supernatant indicate that the antisera is recognizing the same determinants in all three samples, except that the spur formation of the crude supernatant indicates that it has additional determinants not present in the purified enzymes. This may be related to the different conformational states

Figure 10

Gel immunodiffusion of preparations of human liver PK-L. 1, 3 and 6, empty; 2, purified PK-L, 19.5 units/ml; 4, rabbit anti-human PK-L; 5, purified PK-L, 124 units/ml; 7, crude PK-L, 10.5 units/ml.



① ②

③ ④ ⑤

⑥ ⑦

which PK-L can assume.

2. Immunological properties of pyruvate kinases from diverse species

Anti-human PK-M_1 significantly inhibits PK-M_2 and PK-M_1 from humans, but has no effect on PK-L and PK-R (Figure 11). Anti-human PK-R significantly inhibits PK-L and PK-R, but has no effect on PK-M_2 (Figure 12). The interactions of anti-human PK-M_1 with PK-M_1 and PK-M_2 and the lack of interaction with PK-L and the interactions of anti-human PK-R with PK-L and PK-R but the absence of interaction with PK-M_2 are also found in gel immunodiffusion experiments (Figure 13).

a. The relationship of the human erythrocyte isozyme to the human liver isozymes

To test the hypothesis proposed by Tanaka's group (11,43) and Whittel et al. (44) that the human PK-R is a hybrid of the L- and M_2- isozymes, several studies were performed using these three isozymes and the two antibodies, anti-human PK-M_1 and anti-human PK-R. This study in large part has been previously published (166).

Crude human liver PK-L and PK-M_2 and crude human PK-R were prepared as previously described (see "Methods"), and the human PK-M_1 was a gift from Dr. Richard N. Harkins.

The four isozymes were checked for contamination with the other isozymes by thin-layer polyacrylamide gel electrophoresis. Figure 14 shows that each preparation gave only the distinct band typical for the respective system. Human PK-R gives two bands in

Figure 11

Antibody inactivation of human pyruvate kinase isozymes by anti-human PK-M_1.

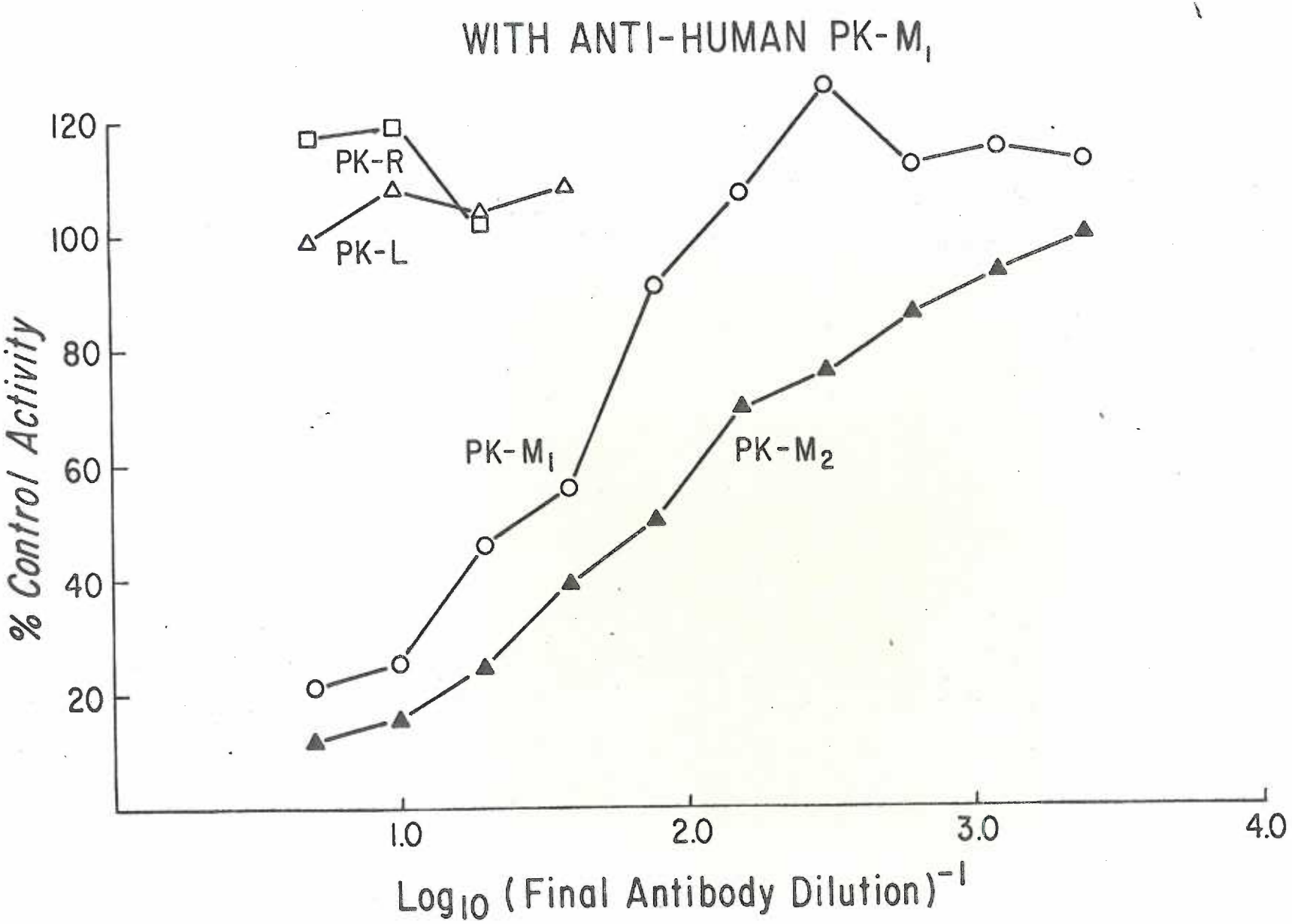

WITH ANTI-HUMAN PK-M1
120
100
80
60
40
20
% Control Activity
PK-R
PK-L
PK-M1
PK-M2
1.0
2.0
3.0
4.0
Log10 (Final Antibody Dilution)-1

Figure 12

Antibody inactivation of human pyruvate kinase isozymes by anti-human PK-R.

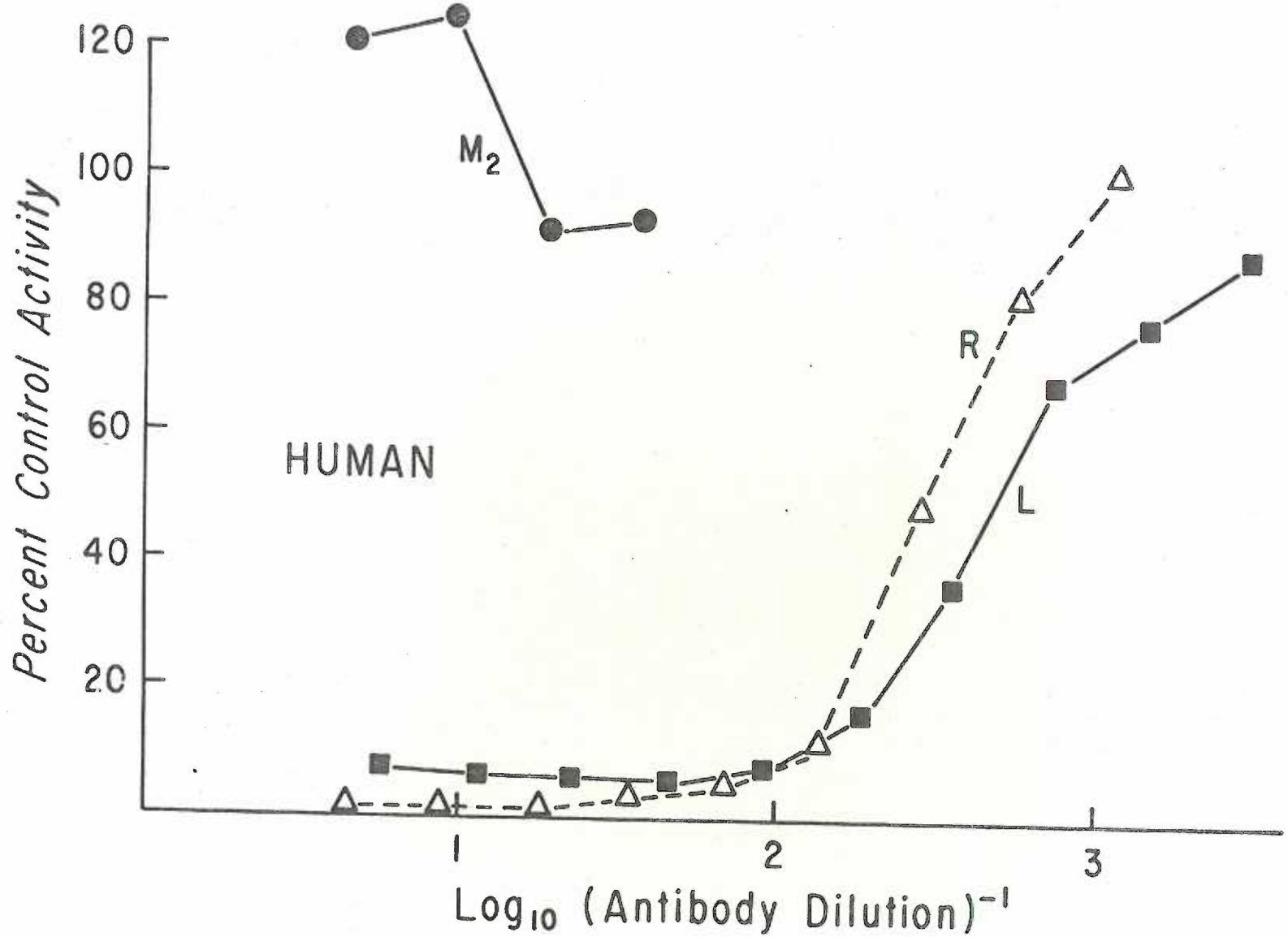
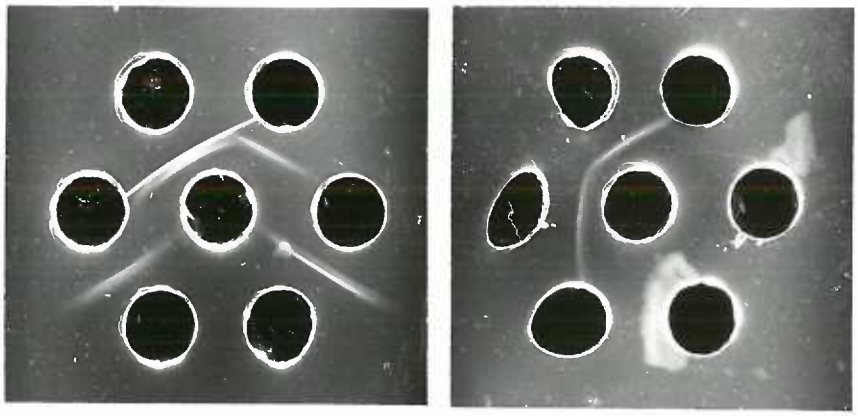

120
100
80
60
40
20
Percent Control Activity
M2
HUMAN
R
L
1
2
3
Log10 (Antibody Dilution)-1

Figure 13

Gel immunodiffusion analysis of human and rat pyruvate kinase isozymes. A. Human isozymes: 1, PK-R, 10.6 units/ml; 2, PK-L, 2.0 units/ml; 3, liver PK-M_2, 0.68 units/ml; 4, anti-human PK-R; 5, PK-M_1, 6.9 units/ml; 6 and 7, anti-human PK-M_1. B. Rat isozymes: 1, liver PK-M_2, 3.1 units/ml; 3, PK-L, 30.4 units/ml; 4, anti-human PK-R; other wells empty. Note particularly the precipitin line between anti-PK-R and the rat PK-M_2.



① ② ① ②

③ ④ ⑤ ③ ④ ⑤

⑥ ⑦ ⑥ ⑦

A B

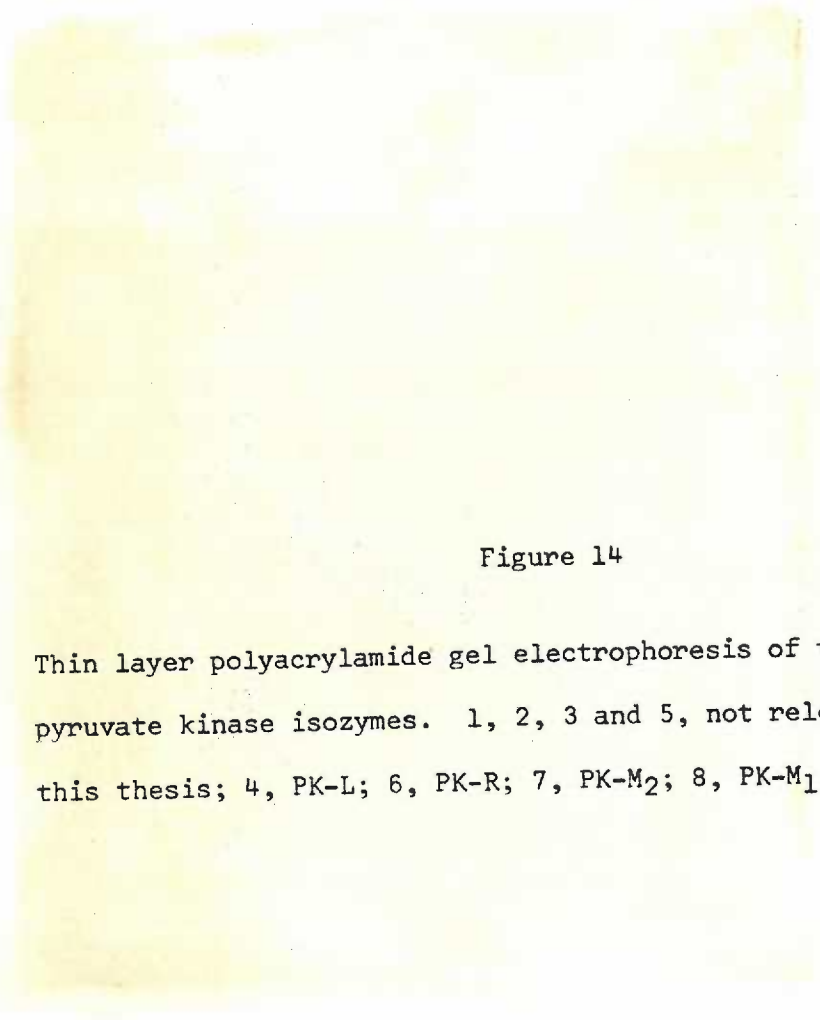


Figure 14

Thin layer polyacrylamide gel electrophoresis of the human pyruvate kinase isozymes. 1, 2, 3 and 5, not relevant to this thesis; 4, PK-L; 6, PK-R; 7, PK-M_2; 8, PK-M_1.

+

← origin

-

1 2 3 4 5 6 7 8

this electrophoretic system (167).

The rat pyruvate kinase isozymes were inactivated by anti-human PK-R and anti-human PK-M_1 in a pattern similar to that described earlier for the human isozymes.

In order to examine interactions with determinants other than those related to the active sites, electrophoresis of the isozymes in the presence and absence of antibodies was performed. These results are shown in Figure 15, and once again indicate that anti-human PK-R interacts only with the R- and L-isozymes while anti-human PK-M_1 interacts only with PK-M_2 and PK-M_1.

In a further test immunodiffusion was used to determine the interactions of the isozymes with the antisera. As shown in Figure 13, the anti-PK-R cross-reacts with human PK-L but not with the human PK-M_2 or PK-M_1 isozymes, while anti-PK-M_1 cross-reacts only with PK-M_2.

The cross-reaction between human PK-R and the human PK-L is complicated by the second precipitin band in the PK-R preparation. This second inner band is partially reactive with but not identical to PK-L as indicated by the spur extending beyond the inner PK-R band and in turn intersecting the major precipitin band. The latter interaction is also one of only partial identity indicating that the anti-PK-R antibody detects determinants not found in the PK-L.

In immunodiffusion studies with rat isozymes, it was found that the anti-human PK-R does cross-react with rat PK-L and PK-M_2,

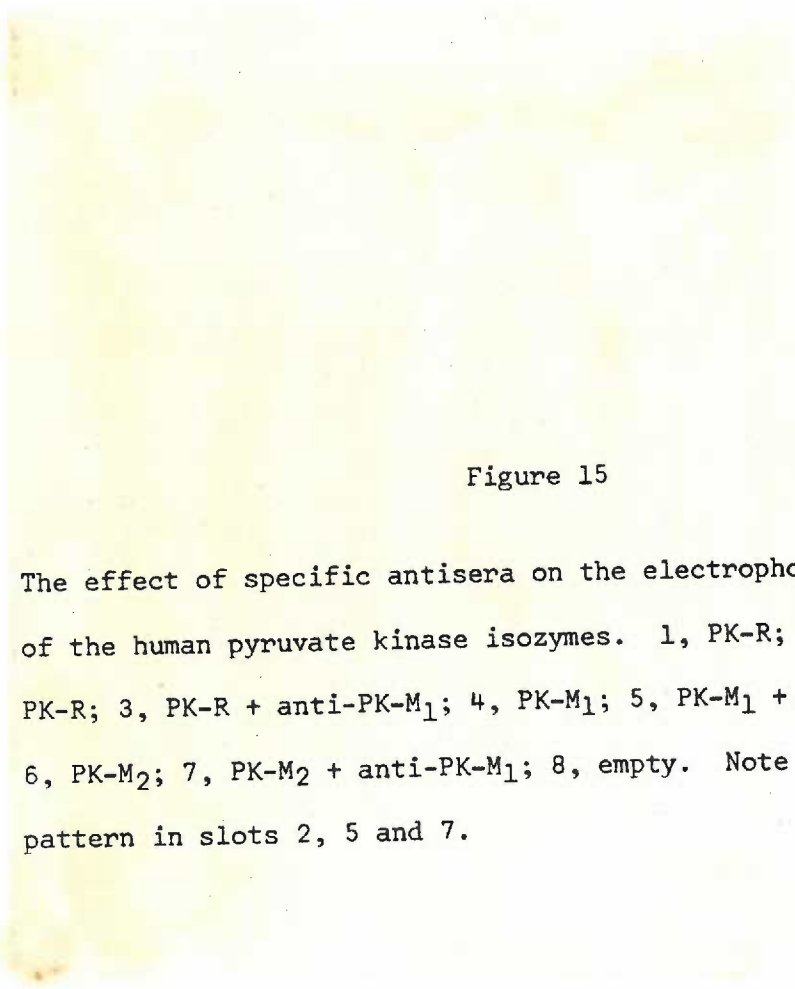


Figure 15

The effect of specific antisera on the electrophoretic mobility of the human pyruvate kinase isozymes. 1, PK-R; 2, PK-R + anti-PK-R; 3, PK-R + anti-PK-M_1; 4, PK-M_1; 5, PK-M_1 + anti-PK-M_1; 6, PK-M_2; 7, PK-M_2 + anti-PK-M_1; 8, empty. Note the altered pattern in slots 2, 5 and 7.


+
origin
-
1
2
3
4
5
6
7
8

as shown in Figure 13, and with rat PK-R as reported earlier (168), indicating shared antigenic determinants.

In one further test of the relatedness of PK-R with PK-L and PK-M_2, a naturally occurring hybrid of the L- and M_2-isozymes found in human kidney and partially purified by Dr. Robert Bigley, University of Oregon Health Sciences Center (for method, see Appendix 2), was tested in the systems used above. This isozyme migrates to a position half-way between PK-L and PK-M_2 on horizontal gel electrophoresis, pH 8.2. The results are shown in Figure 16 for the antibody inactivation test and Figure 17 for gel immunodiffusion. Both of these experiments show that this hybrid of PK-L and PK-M_2 does interact with both anti-PK-R and anti-PK-M_1 antibodies. Also, the isozyme shows lines of identity with human PK-L against anti-PK-R and with human PK-M_2 against anti-PK-M_1 (Figure 17).

b. Liver and cell supernatants

As the distinctive characteristics of anti-PK-R and anti-PK-M_1 have been demonstrated, they were used along with anti-bovine muscle pyruvate kinase to test the immunoreactivity of pyruvate kinases present in liver or cell extracts from several species. These tests included: 1) antibody inactivation, 2) gel immunodiffusion, and 3) gel electrophoresis of samples combined with antibodies. Cross-reaction with anti-PK-R is assumed to indicate the presence of determinants similar to PK-R or PK-L, and cross-reaction with anti-PK-M_1 is assumed to indicate the presence of determinants

Figure 16

Antibody inactivation of a human kidney pyruvate kinase isozyme.

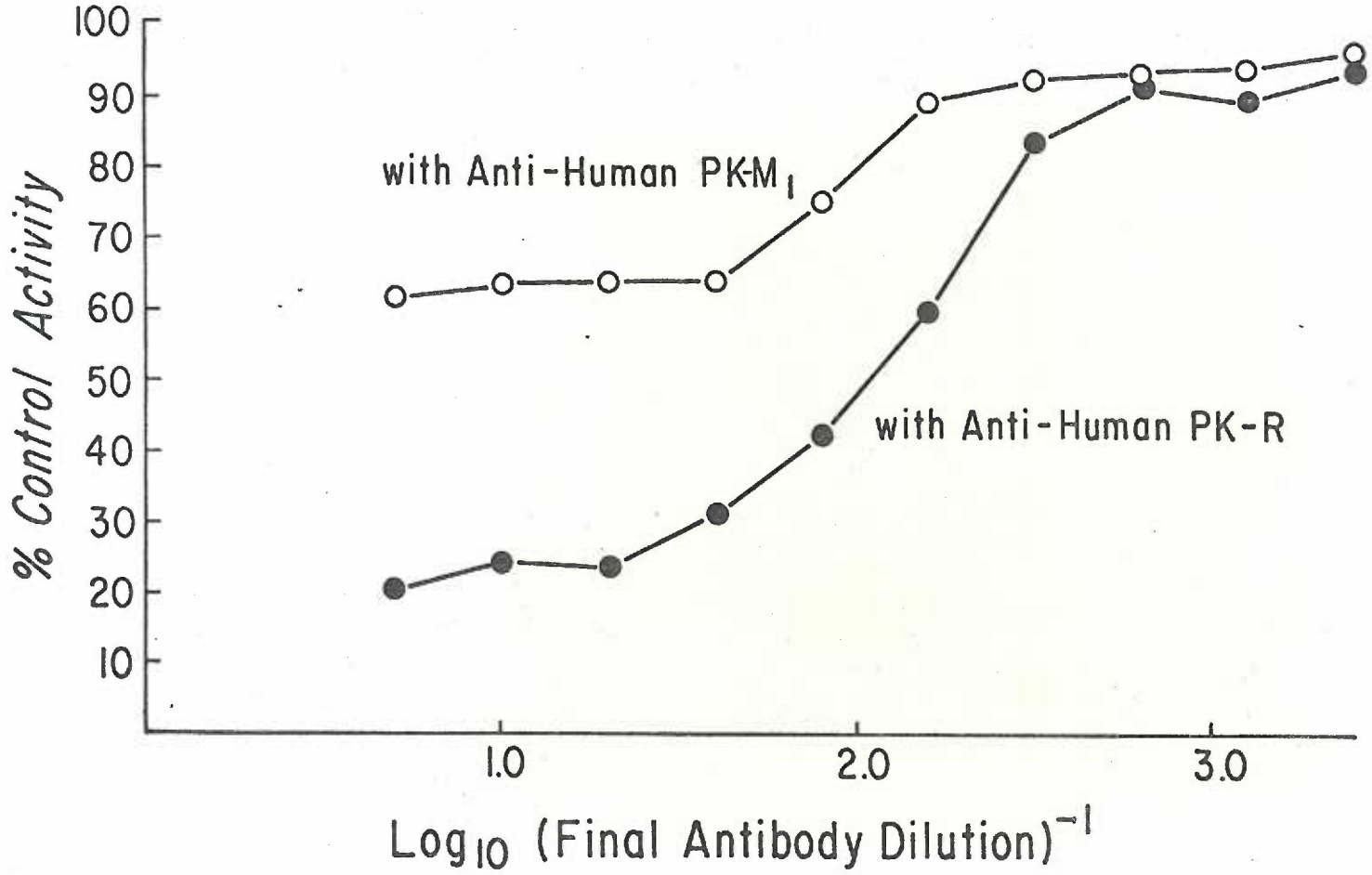
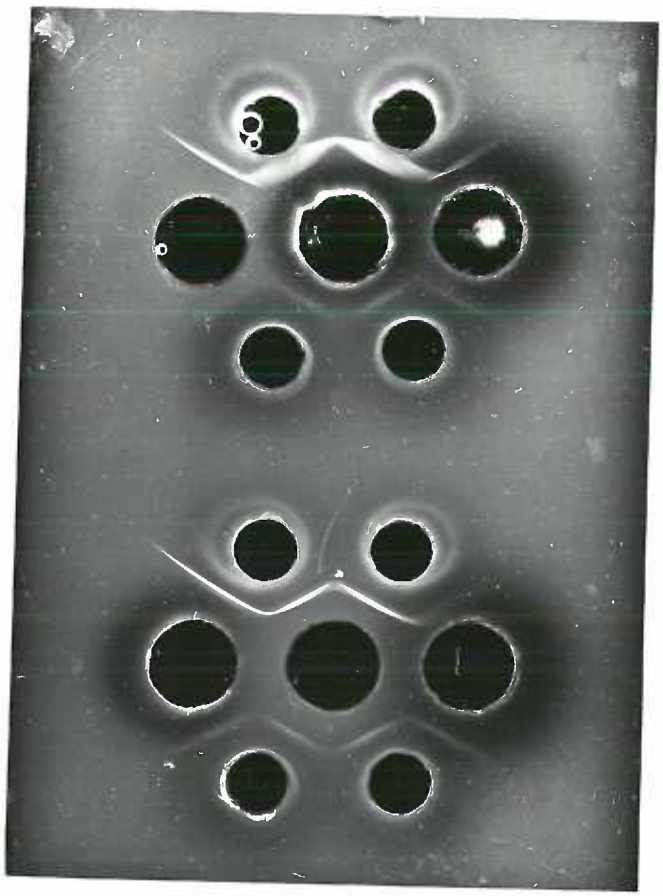

100
90
80
70
60
50
40
30
20
10
% Control Activity
with Anti-Human PK-M1
with Anti-Human PK-R
1.0
2.0
3.0
Log10 (Final Antibody Dilution)-1

Figure 17

Gel immunodiffusion analysis of a human kidney pyruvate kinase isozyme. 1, 2, 8 and 9, anti-human PK-R; 3 and 11, kidney isozyme, 3.72 units/ml; 4 and 10, human PK-L, 7.27 units/ml; 5 and 12, human PK-M_2, 2.7 units/ml; 6, 7, 13 and 14, anti-human PK-M_1. Note the lines of identity between the kidney isozyme and the two liver isozymes.



① ②

③ ④ ⑤

⑥ ⑦

⑧ ⑨

⑩ ⑪ ⑫

⑬ ⑭

similar to PK-M_1 or PK-M_2.

The presence of multiple isozymes of pyruvate kinase in the extracts presents some problems in interpreting the results of the antibody inactivation experiments. A method for dealing with this problem is discussed in a footnote to Table 5. In the antibody inactivation results, values of the ordinate greater than 100% at high antibody concentrations merely indicate the importance of high protein concentrations to the stability of the pyruvate kinase, as buffer, not normal serum, was used in the control samples.

1. human liver

The human liver supernatant shows significant inactivation with anti-PK-R (Figure 18), a lesser inactivation with anti-PK-M_1 (Figure 19), and almost no inactivation by anti-bovine muscle pyruvate kinase (Figure 20). Gel immunodiffusion showed that the supernatant reacted with anti-PK-R only (not shown). Gel electrophoresis (Figure 21) showed that the faster moving isozyme reacted with anti-PK-R and the slower isozyme with anti-PK-M_1. Earlier experiments have shown that these two isozymes are PK-L and PK-M_2 respectively. At least two minor pyruvate kinase isozymes are present between PK-L and PK-M_2 in the gel electrophoresis. These bands were lost when either anti-PK-L or anti-PK-M_1 was added, indicating that these isozymes react with either antibody and are therefore hybrids of the L- and M_2-isozymes.

2. rat liver

Figure 18

Antibody inactivation of the liver supernatants from human, rat, bovine and opossum by anti-human PK-R.

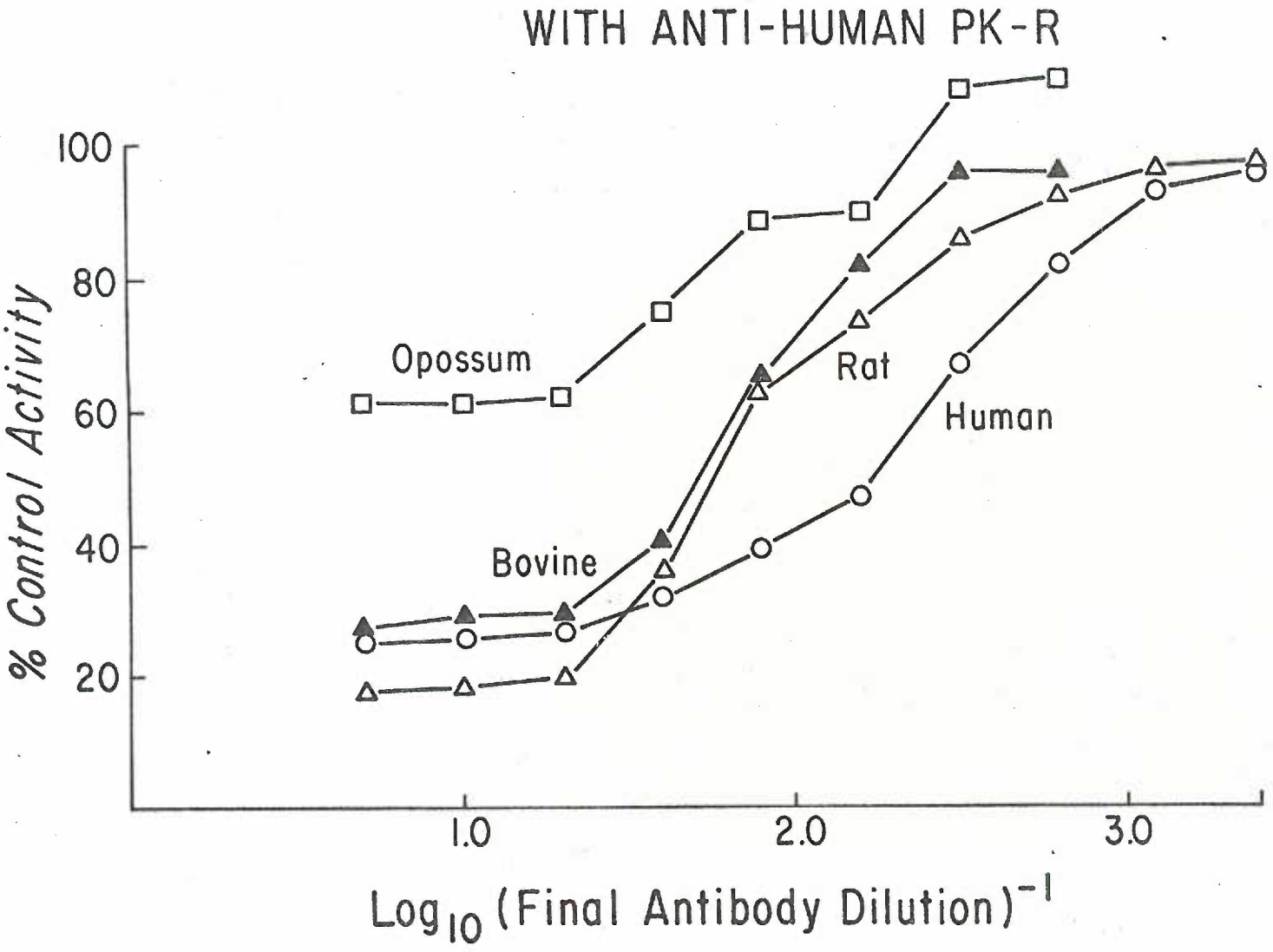

WITH ANTI-HUMAN PK-R
100
80
60
40
20
% Control Activity
Opossum
Rat
Human
Bovine
1.0
2.0
3.0
Log10 (Final Antibody Dilution)-1

Figure 19

Antibody inactivation of the liver supernatants from human, rat, bovine and opossum by anti-human PK-M_1.

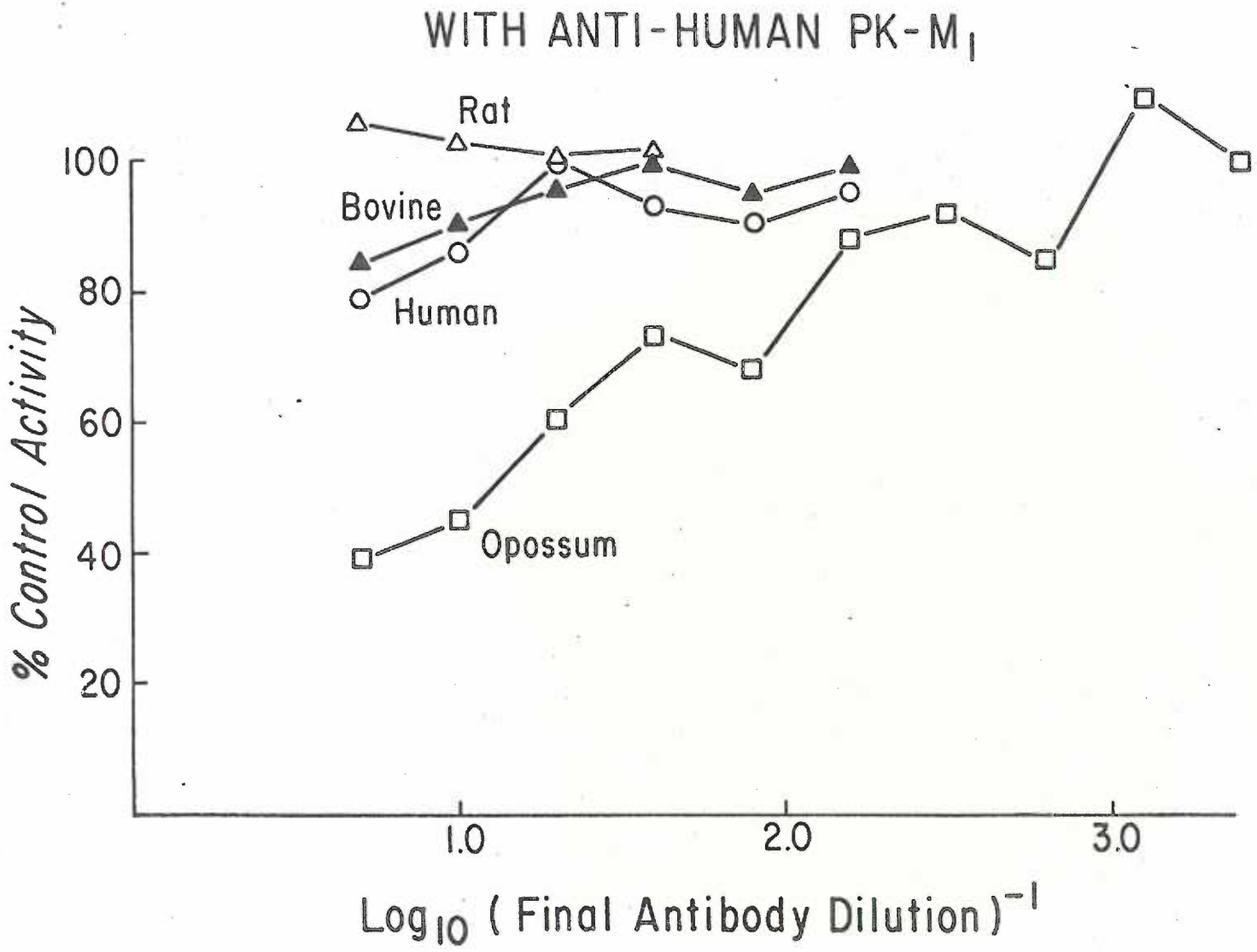

WITH ANTI-HUMAN PK-M1
Rat
Bovine
Human
Opossum
100
80
60
40
20
% Control Activity
1.0
2.0
3.0
Log10 (Final Antibody Dilution)-1

Figure 20

Antibody inactivation of the liver supernatants from human, rat, bovine and opossum by anti-bovine muscle pyruvate kinase.

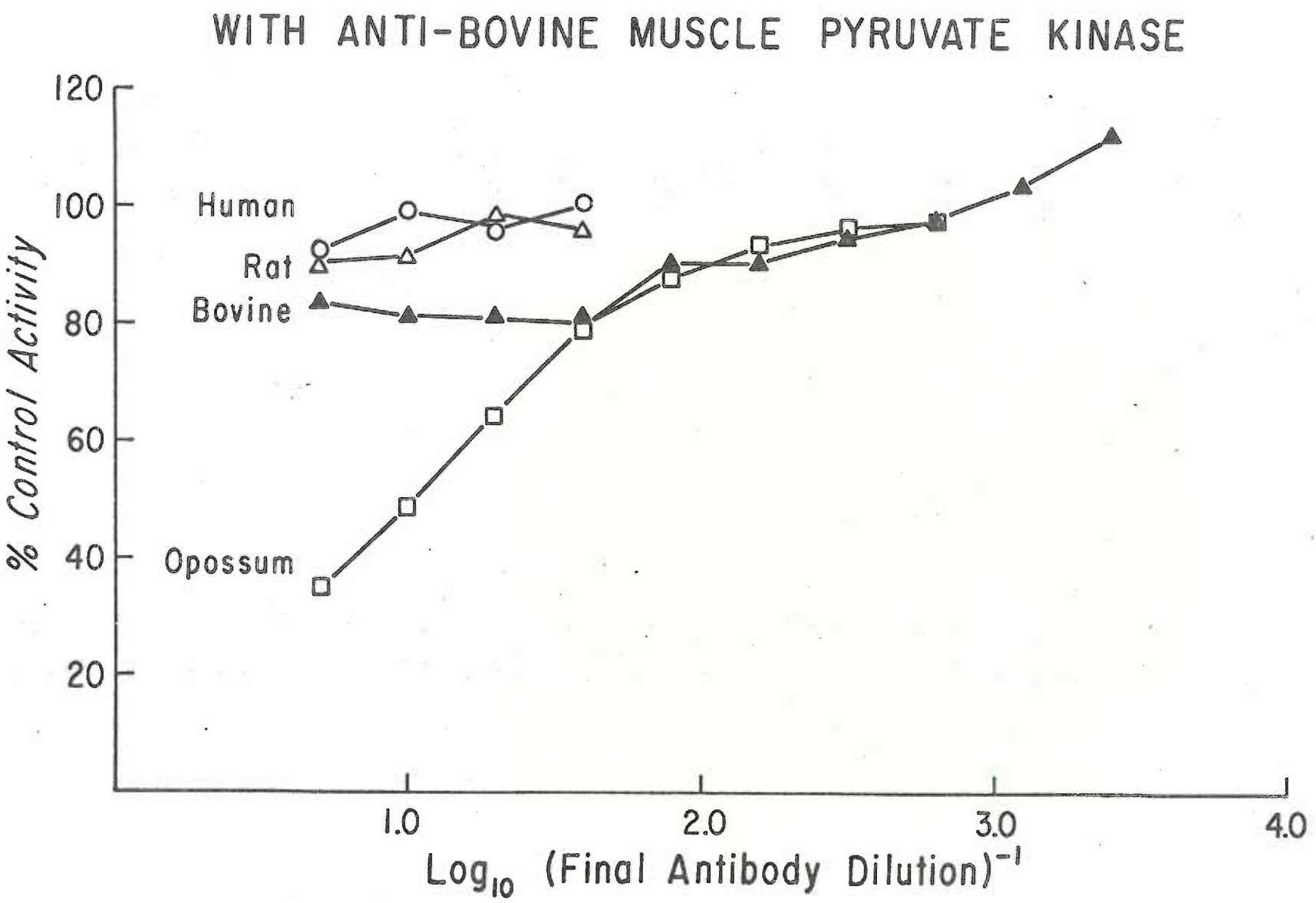
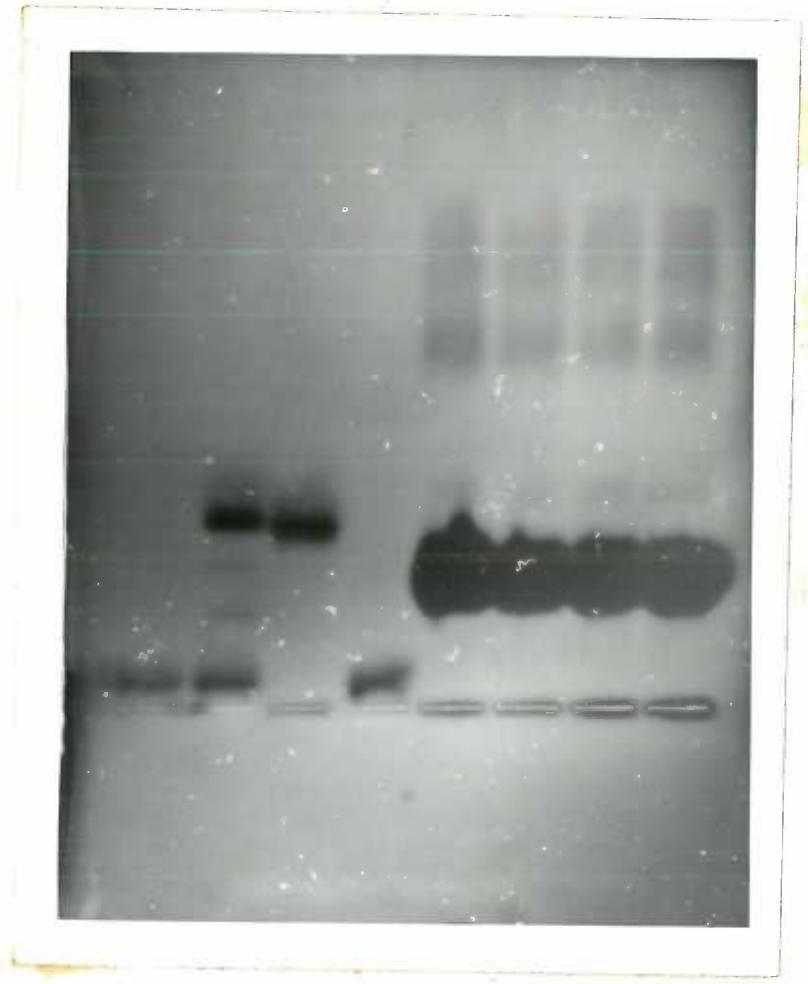

WITH ANTI-BOVINE MUSCLE PYRUVATE KINASE
% Control Activity
120
100
80
60
40
20
Human
Rat
Bovine
Opossum
1.0
2.0
3.0
4.0
Log10 (Final Antibody Dilution)-1

Figure 21

The effect of specific antisera on the electrophoretic mobility of pyruvate kinase isozymes in human liver and yeast supernatants. Electrophoresis was performed for 2.5 hours at 400 v. 1 and 4, human liver + anti-PK-R; 2, human liver; 3, human liver + anti-PK-M_1; 5, yeast; 6, yeast + anti-PK-M_1; 7 and 8, yeast + anti-PK-R. The yeast supernatant was unaffected by the antisera, but the isozymes in human liver supernatant were altered by either of the antisera or both.


+
← origin
-
1 2 3 4 5 6 7 8

The supernatant pyruvate kinase activity was significantly reduced by anti-PK-R (Figure 18), but not by either anti-PK-M_1 (Figure 19) or anti-bovine muscle (Figure 20). Gel immunodiffusion confirmed that the rat liver supernatant reacted with anti-PK-R (not shown) and also anti-PK-M_1 (not shown). Gel electrophoresis (Figure 22) showed that there was only a single isozyme in the supernatant and that the activity was eliminated by anti-PK-R, but not by anti-PK-M_1.

3. bovine liver

The liver supernatant pyruvate kinase activity was significantly reduced by anti-PK-R (Figure 18), and to a lesser extent by anti-PK-M_1 (Figure 19) and by anti-bovine muscle (Figure 20). Gel immunodiffusion indicated no cross-reaction between the supernatant and any of the three antibodies (not shown). Gel electrophoresis (Figure 23) showed two major isozymes, with the faster migrating isozyme eliminated by anti-PK-R, and the slower by anti-PK-M_1.

4. opossum liver

The opossum liver supernatant was moderately inactivated by anti-PK-R (Figure 18), by anti-PK-M_1 (Figure 19), and by anti-bovine muscle (Figure 20). Gel immunodiffusion showed that the supernatant cross-reacted with anti-PK-R and anti-PK-M_1, but not anti-bovine muscle (Figure 24). Gel electrophoresis (Figure 25) showed two major isozymes present in the supernatant, and a minor component migrating slightly behind the faster major isozyme. The

Figure 22

The effect of specific antisera on the electrophoretic mobility of pyruvate kinase isozymes in rat liver and yeast supernatants. Electrophoresis was performed for 12 hours at 150 v. 1 and 4, rat liver + anti-PK-R; 2, rat liver; 3, rat liver + anti-PK-M_1; 5, yeast; 6, yeast + anti-PK-M_1; 7 and 8, yeast + anti-PK-R. In this rat liver supernatant only the anti-PK-R affected the migration.

+

← origin

-

1 2 3 4 5 6 7 8

Figure 23

The effect of specific antisera on the electrophoretic mobility of pyruvate kinase isozymes in bovine liver supernatant. Electrophoresis was performed for 12 hours at 150 v. 1, beef liver; 2, beef liver + anti-PK-M_1; 3, beef liver + anti-PK-R; 4-8, not relevant to this thesis. The anti-PK-M_1 affected the most cathodal isozyme of the bovine supernatant and the anti-PK-R affected the most anodal component.

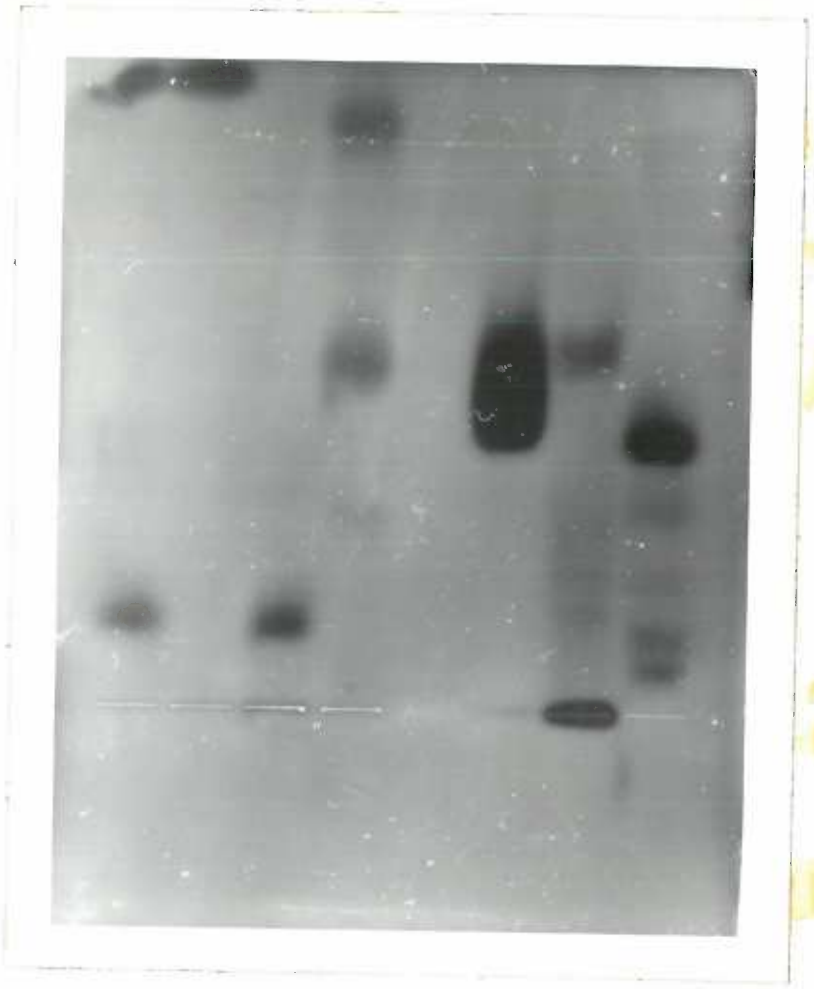
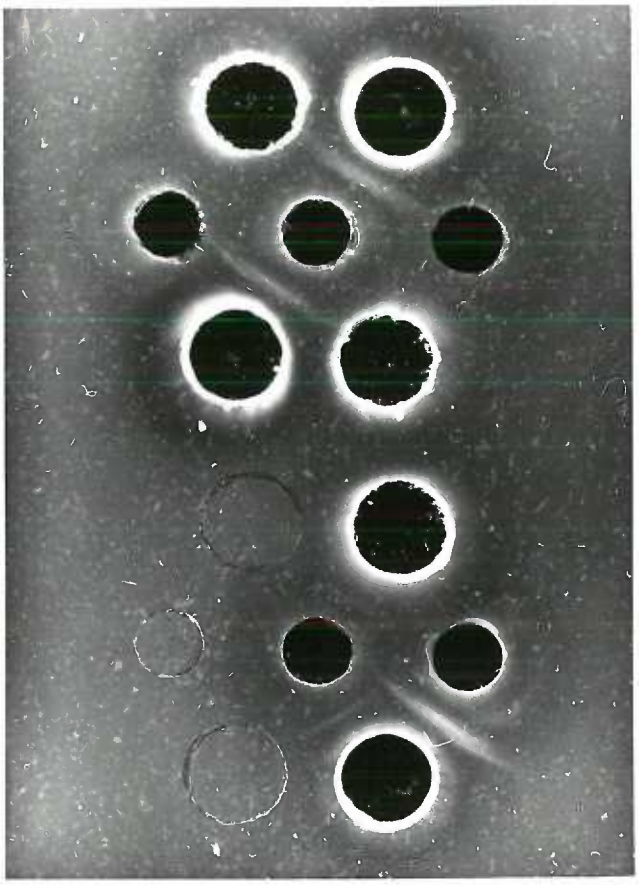

+
origin
-
1
2
3
4
5
6
7
8

Figure 24

Gel immunodiffusion analysis of the pyruvate kinases in opossum and turtle liver supernatants. 1, 7 and 9, turtle liver, 5.4 units/ml; 2, 6 and 14, opossum liver, 3.6 units/ml; 3, anti-bovine muscle pyruvate kinase; 4 and 12, anti-PK-R; 5 and 11, anti-PK-M_1; other wells were empty. Note the precipitin lines between opossum liver supernatant and anti-PK-R (2, 4 and 6), and opossum liver supernatant and anti-PK-M_1 (11 and 14).



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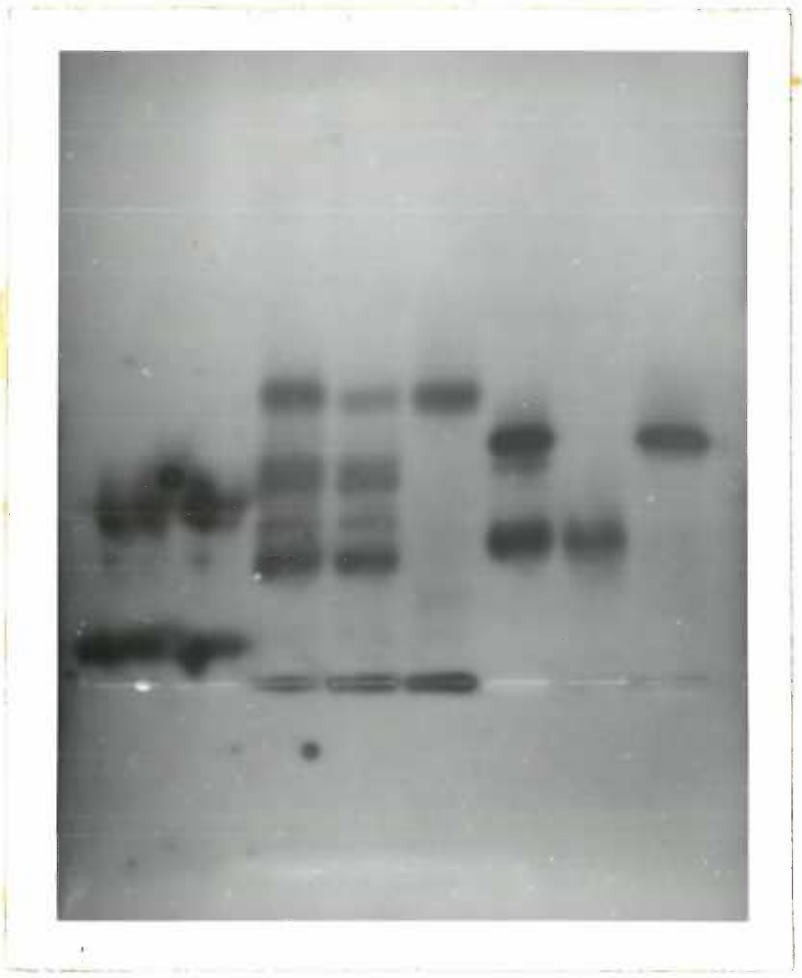
⑬ ⑭

Figure 25

The effect of specific antisera on the electrophoretic mobility of pyruvate kinase isozymes in opossum and turtle liver supernatants. Electrophoresis was performed for 2.5 hours at 400 v. 1 and 2, not relevant to this thesis; 3, turtle liver; 4, turtle liver + anti-PK-R; 5, turtle liver + anti-PK-M_1; 6, opossum liver; 7, opossum liver + anti-PK-R; 8, opossum liver + anti-PK-M_1. In slot 4 the staining of the most anodal isozyme appears to be reduced by the addition of anti-PK-R. The three slower migrating isozymes of turtle liver were eliminated by anti-PK-M_1 (slot 5). The most cathodal isozyme of the opossum liver supernatant was eliminated by anti-PK-R, and the other major isozyme by anti-PK-M_1.


+
← origin
-
1 2 3 4 5 6 7 8

fast major and the minor isozymes were eliminated by anti-PK-R, and the slower isozyme by anti-PK-M_1.

5. chicken liver

Anti-PK-R and anti-PK-M_1 did not inactivate chicken liver supernatant (Figures 26 and 27), but anti-bovine muscle did (Figure 28). Immunodiffusion gave no cross-reactions with any of the three antibodies (not shown). Gel electrophoresis showed only one pyruvate kinase isozyme in chicken liver (Figure 29), and the migration of this isozyme was unaffected by either anti-PK-R or anti-PK-M_1.

6. frog liver

Anti-PK-R did not inactivate frog liver supernatant (Figure 26), but the supernatant was moderately inactivated by anti-PK-M_1 (Figure 27) and by anti-bovine muscle (Figure 28). Gel immunodiffusion gave no cross-reactions with any of the three antibodies (not shown). Gel electrophoresis indicated only one pyruvate kinase isozyme (Figure 29), which was unaffected by anti-PK-R, but the staining intensity of the sample with anti-PK-M_1 was slightly reduced as compared with the control sample containing no antibody.

7. turtle liver

Turtle liver supernatant showed only minor inactivation by anti-PK-L (Figure 26), and no inactivation by either anti-PK-M_1 (Figure 27), or anti-bovine muscle (Figure 28). Immunodiffusion showed no cross-reactions with any of the three antibodies (Figure

Figure 26

Antibody inactivation of the liver supernatants from chicken, frog, turtle and steelhead trout by anti-human PK-R.

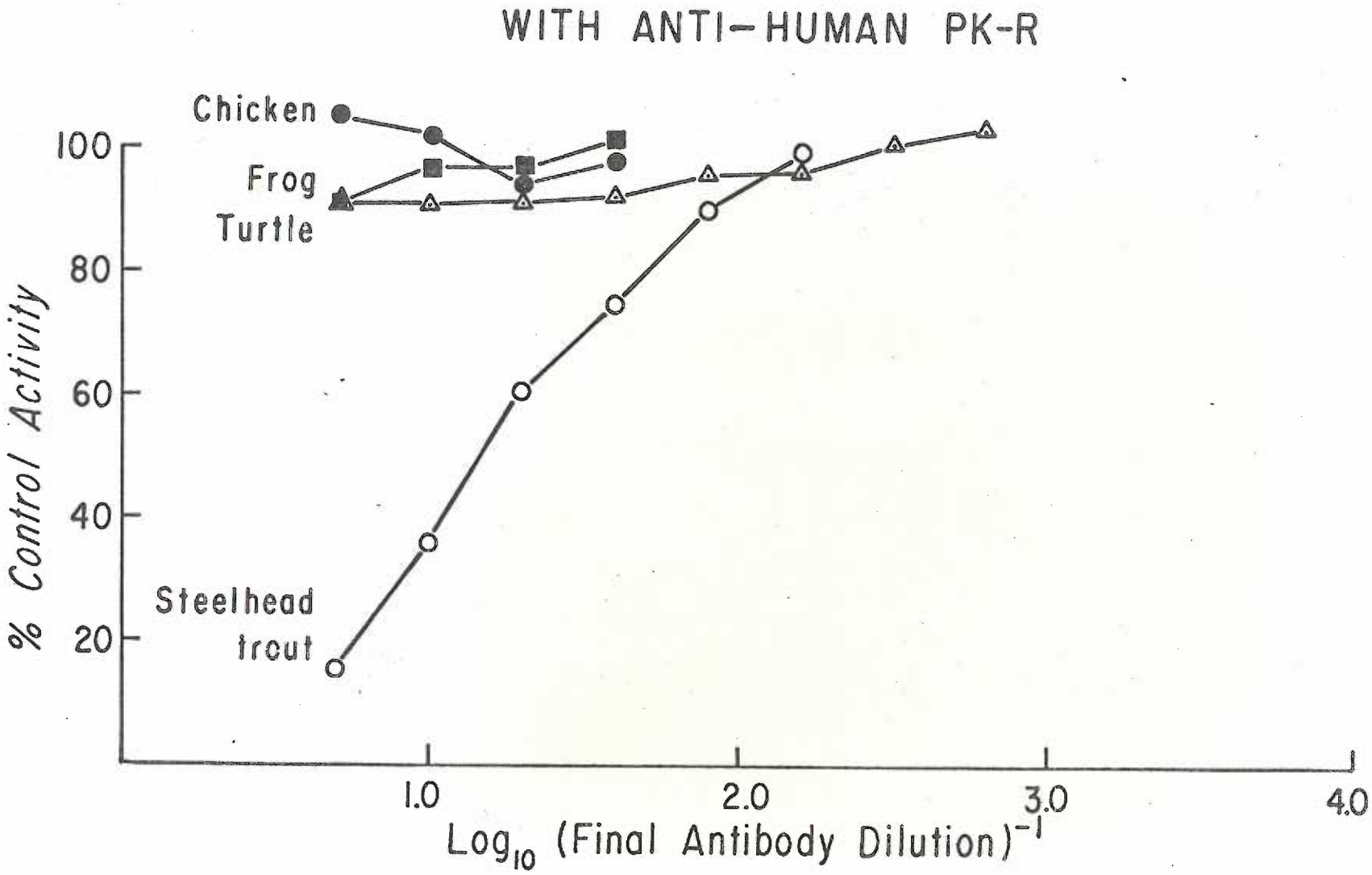

WITH ANTI-HUMAN PK-R
Chicken
Frog
Turtle
Steelhead trout
100
80
60
40
20
% Control Activity
1.0
2.0
3.0
4.0
Log10 (Final Antibody Dilution)-1

Figure 27

Antibody inactivation of the liver supernatants from chicken, frog, turtle and steelhead trout by anti-human PK-M_1.

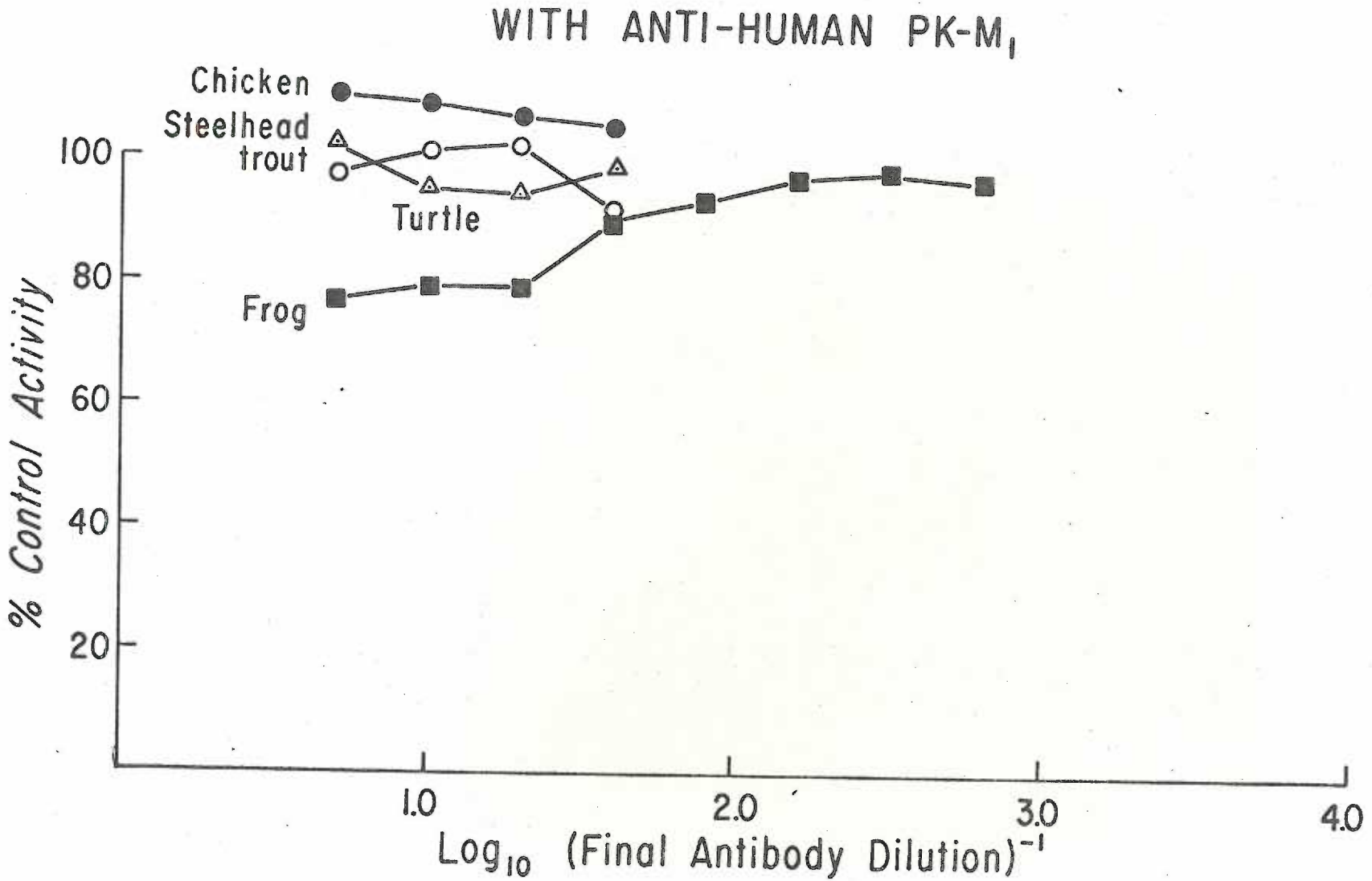

WITH ANTI-HUMAN PK-M1
Chicken
Steelhead trout
Turtle
Frog
100
80
60
40
20
% Control Activity
1.0
2.0
3.0
4.0
Log10 (Final Antibody Dilution)-1

Figure 28

Antibody inactivation of the liver supernatants from chicken, frog, turtle and steelhead trout by anti-bovine muscle pyruvate kinase.

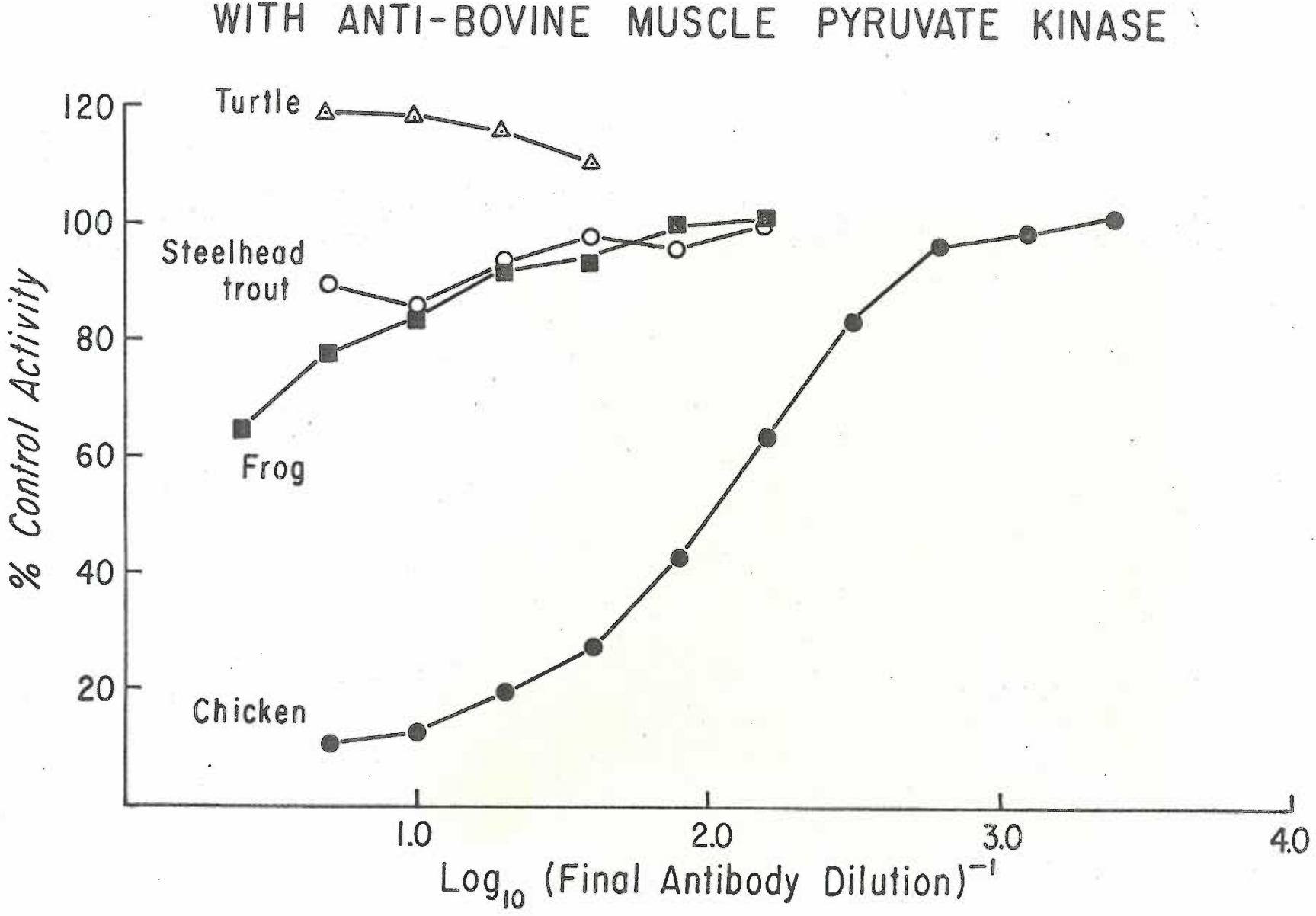
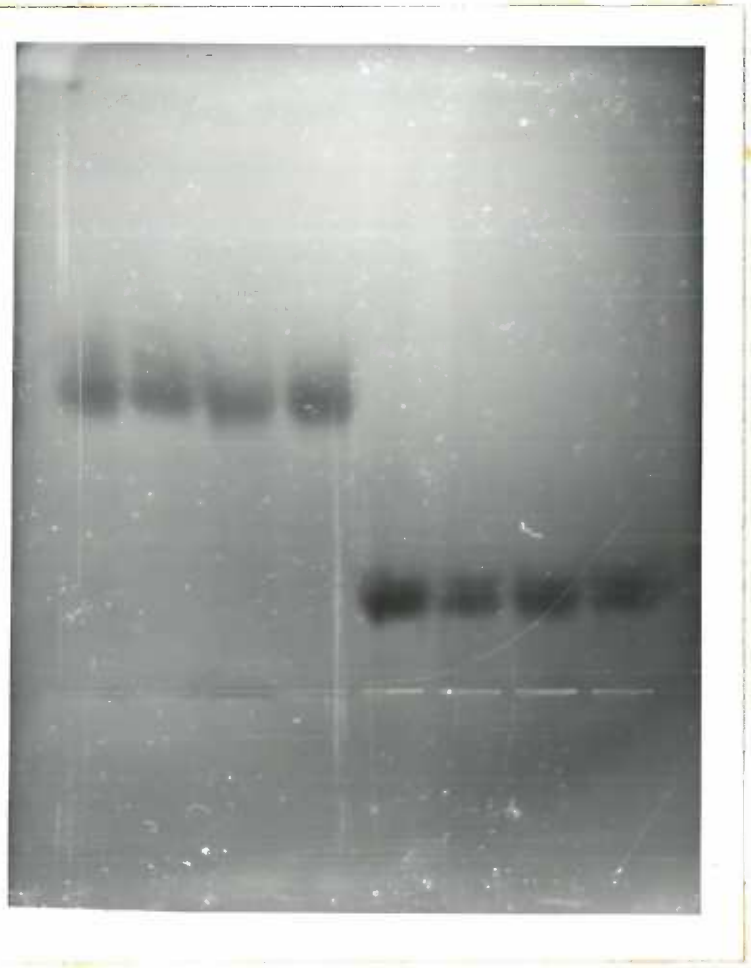

WITH ANTI-BOVINE MUSCLE PYRUVATE KINASE
Turtle
Steelhead trout
Frog
Chicken
120
100
80
60
40
20
% Control Activity
1.0
2.0
3.0
4.0
Log10 (Final Antibody Dilution)−1

Figure 29

The effect of specific antisera on the electrophoretic mobility of pyruvate kinase isozymes in chicken and frog liver supernatants. Electrophoresis was performed for 12 hours at 150 v. 1 and 4, frog liver + anti-PK-R; 2, frog liver; 3, frog liver + anti-PK-M_1; 5, chicken liver; 6, chicken liver + anti-PK-M_1; 7 and 8, chicken liver + anti-PK-R.


+
← origin
-
1 2 3 4 5 6 7 8

24). Gel electrophoresis showed five pyruvate kinase isozymes (Figure 25) with the staining of the fastest migrating isozyme reduced in the sample containing anti-PK-R as compared to the control sample. The addition of anti-PK-M_1 to one sample caused the elimination of the three intermediate isozymes in the gel sample.

8. steelhead trout liver

Steelhead trout liver supernatant was inactivated by anti-PK-R (Figure 26), only slightly inactivated by anti-bovine muscle (Figure 27), and was not inactivated by anti-PK-M_1 (Figure 28). Gel immunodiffusion showed that the supernatant cross-reacted only with anti-PK-R (Figure 30). Gel electrophoresis presented only one isozyme (Figure 31), and the staining intensity of the samples with either anti-PK-R or anti-PK-M_1 appeared to be slightly reduced when compared to the control sample.

9. yeast supernatant

Yeast pyruvate kinase was not inactivated by anti-PK-R, anti-PK-M_1, or anti-bovine muscle (Figure 32). This lack of interaction was confirmed by gel immunodiffusion (not shown), and by gel electrophoresis (Figures 21 and 22), which showed principally one isozyme which was unaffected by the addition of either anti-PK-R or anti-PK-M_1.

10. E. coli supernatant

This supernatant showed no inactivation by anti-bovine

Figure 30

Gel immunodiffusion analysis of the pyruvate kinase in steelhead trout liver and E. coli supernatants. 1, 7, 9 and 13, anti-PK-M_1; 2, 6, 8 and 14, anti-PK-R; 3 and 10, human PK-M_1, 32 units/ml; 4, steelhead trout liver, 3.86 units/ml; 5 and 12, human PK-R, 4.5 units/ml; 11, E. coli supernatant, 2.52 units/ml. Note the lines of identity between the E. coli supernatant, anti-PK-R and anti-PK-M_1. With steelhead trout liver supernatant, there was a precipitin line only with anti-PK-R.

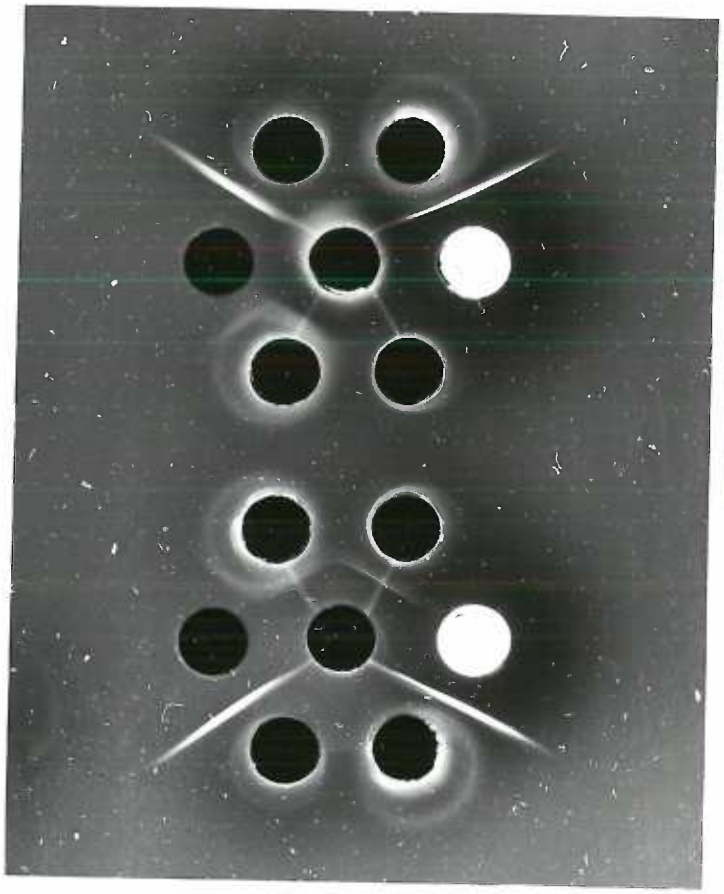


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Figure 31

The effect of specific antisera on the electrophoretic mobility of pyruvate kinase isozymes in steelhead trout liver and E. coli supernatants. Electrophoresis was performed for 3 hours at 400 v. The agar gel overlay for pyruvate kinase activity visualization for the trout liver supernatant samples was as described in the "Methods" section, but the overlay for the E. coli supernatant contained also 1 mM AMP. 1, E. coli supernatant; 2, E. coli supernatant + anti-PK-M_1; 3 and 4, E. coli supernatant + anti-PK-R; 5, no sample; 6, steelhead trout liver; 7, steelhead trout liver + anti-PK-M_1; 8, steelhead trout liver + anti-PK-R. Note the slight decrease in staining intensity of the more cathodal isozyme in slot 2 and the presence of some pyruvate kinase activity at the origin in comparison to the control slot of E. coli supernatant (slot 1).

+

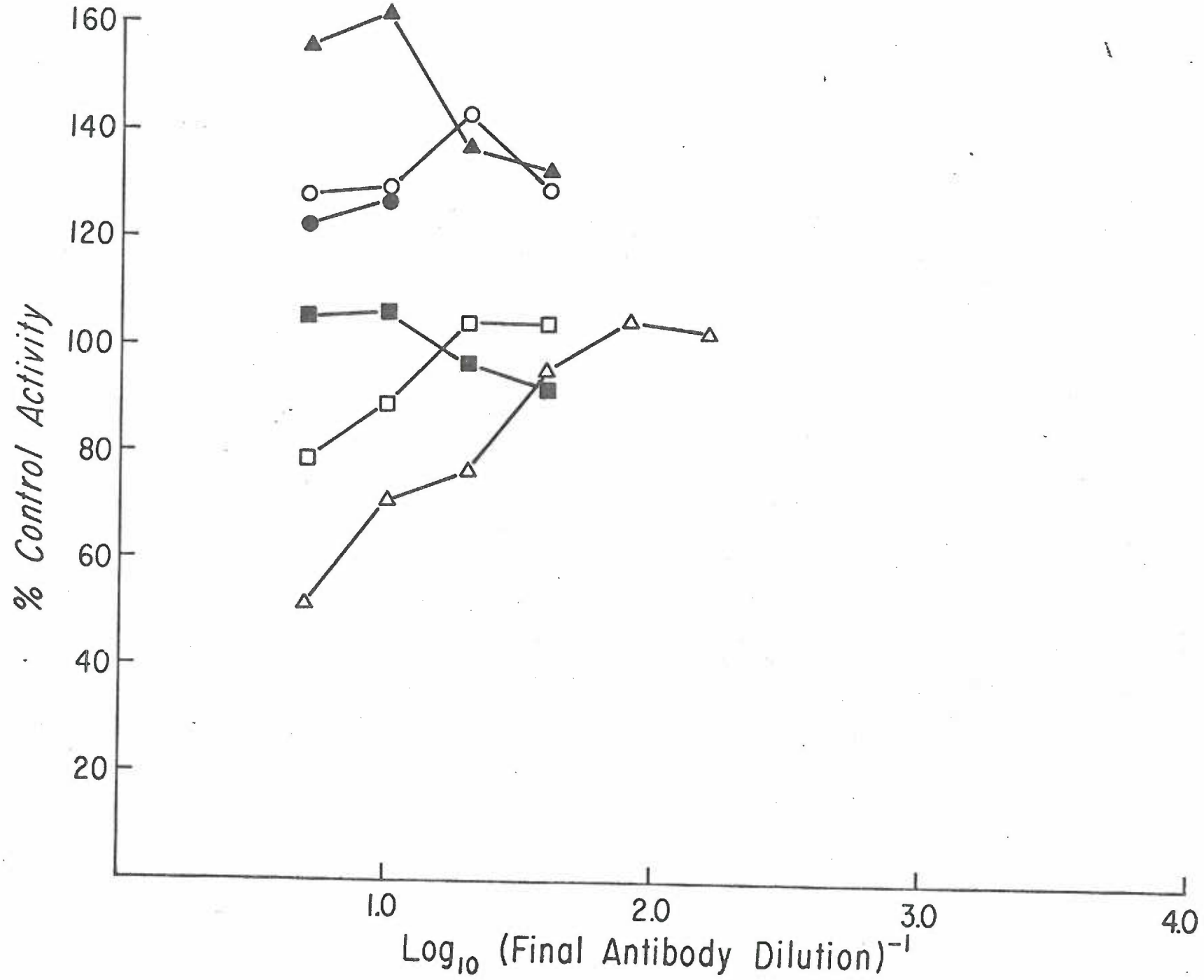
← origin

-

1 2 3 4 5 6 7 8

Figure 32

Antibody inactivation of the yeast and E. coli supernatants by anti-human PK-R, anti-human PK-M_1 and anti-bovine muscle pyruvate kinase. The standard pyruvate kinase assay mix contained also: 1 mM FDP for the yeast supernatant; and 1 mM FDP and 1 mM AMP for the E. coli supernatant. Yeast supernatant + anti-PK-M_1 (▲); yeast supernatant + anti-PK-R (O); yeast supernatant + anti-bovine muscle pyruvate kinase (●); E. coli supernatant + anti-PK-M_1 (Δ); E. coli supernatant + anti-PK-R (□); E. coli supernatant + anti-bovine muscle pyruvate kinase (■).


160
140
120
100
80
60
40
20
% Control Activity
1.0
2.0
3.0
4.0
Log10 (Final Antibody Dilution)-1

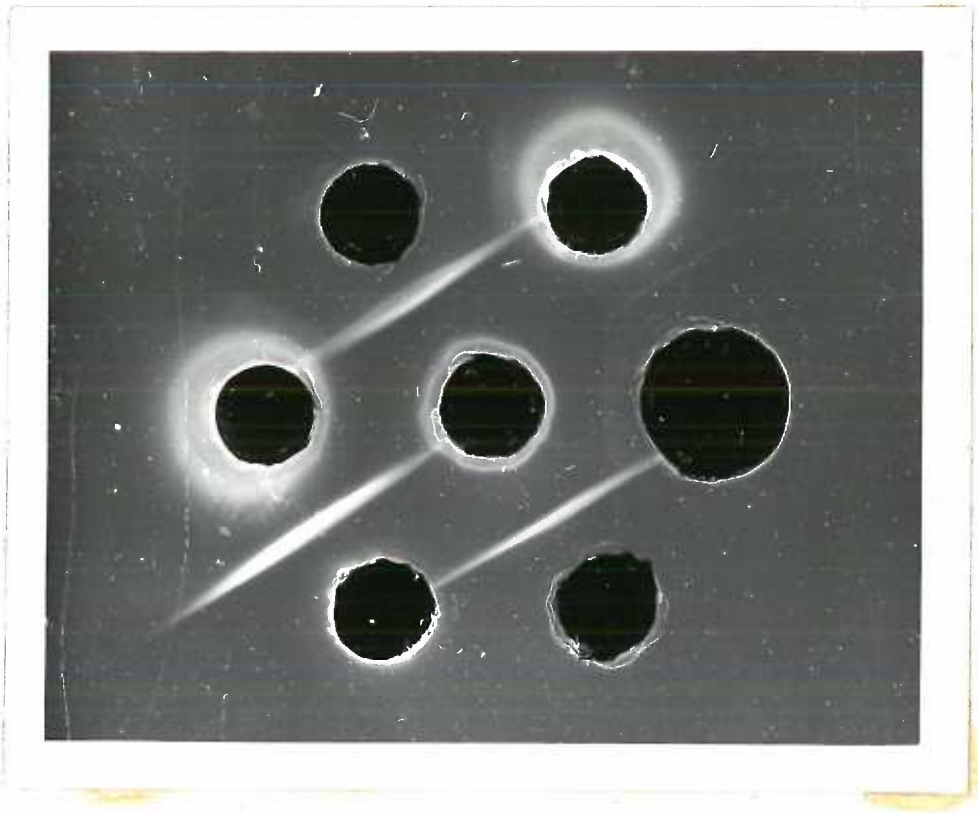
muscle, and moderate inactivation by both anti-PK-R and anti-PK-M_1 (Figure 32). Gel immunodiffusion showed cross-reactions with anti-PK-R, anti-PK-M_1 (Figure 33), and anti-bovine muscle pyruvate kinase (Figure 34). In addition, the supernatant showed lines of identity between anti-PK-M_1 and anti-bovine muscle pyruvate kinase (Figure 34), and lines of identity between anti-PK-R and anti-PK-M_1 (Figure 30). Figure 34 shows a spur of partial identity with human PK-M_1 against anti-PK-M_1, indicating that the human PK-M_1 has some determinants recognized by the anti-PK-M_1 which are not present in the isozymes of E. coli.

Gel electrophoresis indicated the presence of two pyruvate kinase isozymes in E. coli supernatant (Figure 31). The staining intensity of the slower migrating component in the sample containing anti-PK-M_1 appeared to be somewhat lighter than the control sample containing no antibody.

The two isozymes were separated by DEAE-cellulose chromatography as described in the "Methods" section. The fractions were assayed for pyruvate kinase activity, conductivity, and optical density at 280 nm (Figure 35). The maximum pyruvate kinase activity fractions in the peaks were used in antibody inactivation studies, and also for gel electrophoresis. Electrophoresis of these two peaks showed that Peak I moved further than Peak II at pH 8.2 (Figure 36). The antibody inactivation studies showed that Peak I was not inactivated by anti-PK-R, anti-PK-M_1 or anti-bovine muscle

Figure 33

Gel immunodiffusion analysis of E. coli supernatant with anti-human PK-R and anti-human PK-M_1. 1 and 7, human PK-M_1, 32 units/ml; 2 and 3, anti-PK-R; 4, anti-PK-M_1; 5, E. coli supernatant, 0.04 units/ml; 6, human PK-R, 3.0 units/ml. The precipitin lines between wells 2 and 5, and 4 and 5 are faint, but they show a line of identity between E. coli supernatant and the two antisera.



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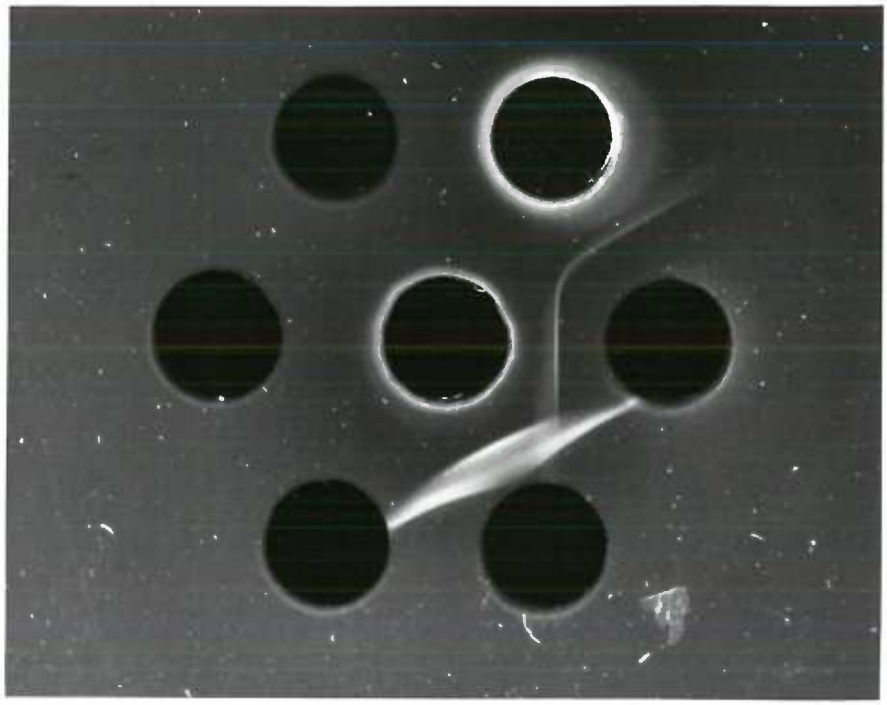
③ ④ ⑤

⑥ ⑦

Figure 34

Gel immunodiffusion analysis of E. coli supernatant with anti-bovine muscle pyruvate kinase and anti-human PK-M_1. 1, 3 and 6, empty; 2, anti-bovine muscle pyruvate kinase; 4, anti-PK-M_1; 5, E. coli supernatant, 2.52 units/ml; 7, human PK-M_1, 32 units/ml. There is a line of identity between the E. coli supernatant and the two antisera. The spur formation in the PK-M_1 indicates that it contains determinants not present in the E. coli supernatant.



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③ ④ ⑤

⑥ ⑦

Figure 35

DEAE-cellulose chromatography of E. coli pyruvate kinase isozymes. The pyruvate kinase assay mix was the standard mix described in the "Methods" section and contained neither AMP nor FDP. Each fraction contained 4.0 ml.

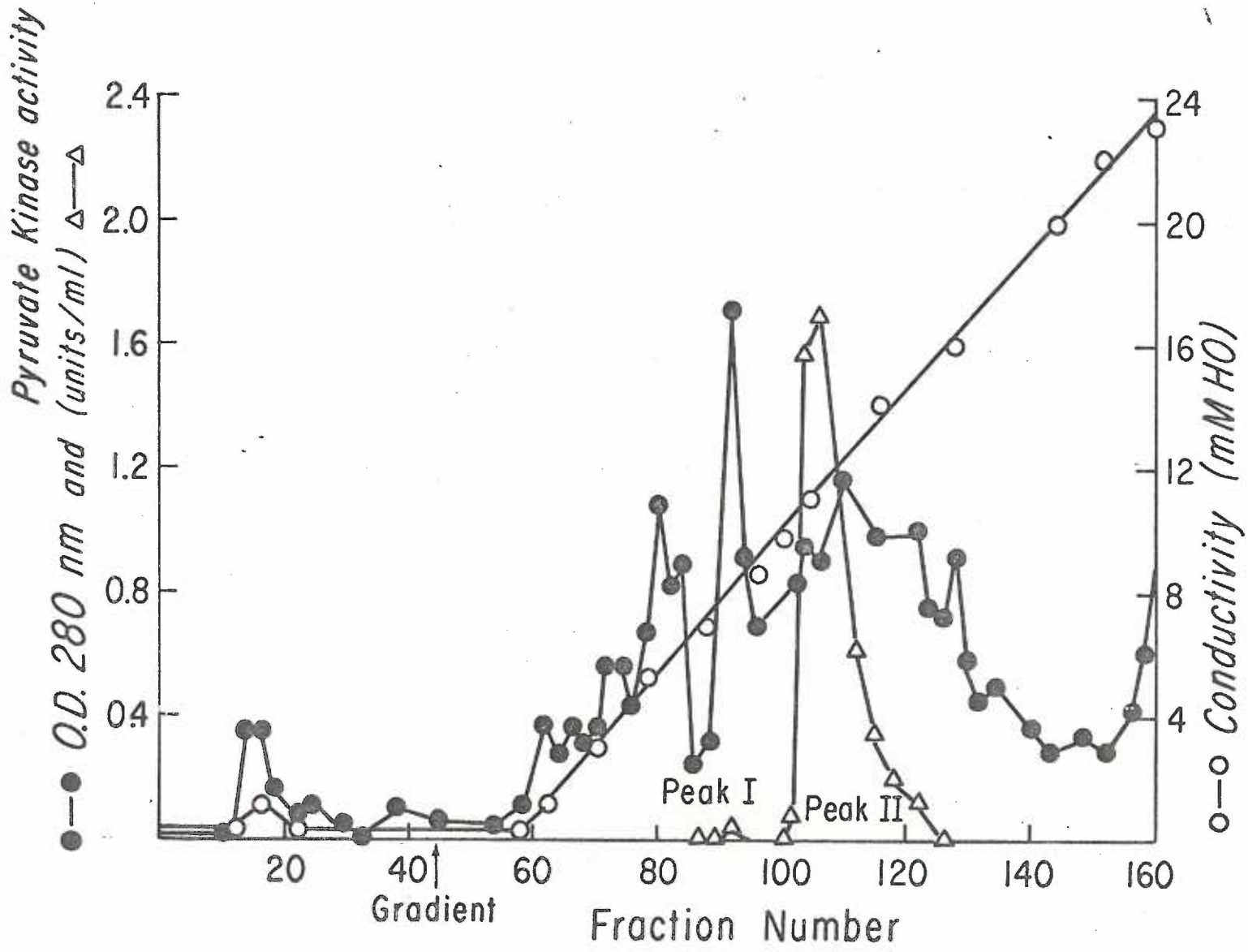

Pyruvate Kinase activity
●—● O.D. 280 nm and (units/ml) △—△
o—o Conductivity (mM HO)
2.4
2.0
1.6
1.2
0.8
0.4
24
20
16
12
8
4
Peak I
Peak II
20
40
60
80
100
120
140
160
Gradient
Fraction Number

Figure 36

Thin layer polyacrylamide gel electrophoresis of the E. coli pyruvate kinase isozymes separated by DEAE-cellulose chromatography. Electrophoresis was performed for three hours at 400 v. The agar gel overlay to visualize pyruvate kinase activity contained 1 mM FDP and 1 mM AMP. 1, 3, 5 and 7, peak I; 2, 4, 6, 8, peak II.

+

← origin

-

1 2 3 4 5 6 7 8

pyruvate kinase (Figure 37), whereas Peak II was at least partially inactivated by anti-PK-R and by anti-PK-M_1, but not by anti-bovine muscle pyruvate kinase (Figure 38).

The results of the antibody inactivation experiments for all of the samples of pyruvate kinase are summarized in Table 5.

3. Protein half-lives and proteolytic sensitivities of PK-L and PK-M_2

The half-lives of rat liver supernatant, PK-L and PK-M_2 were determined in three different experiments, although only in experiment 2 was a half-life for PK-M_2 obtained. The decay in radioactivity for the supernatant, PK-L and PK-M_2 is shown in Figure 39 for experiment 2. The results are summarized in Table 6. The average degradation rate constants, in $days^{-1}$, were 0.242 ± 0.064, 0.382 ± 0.115, and 0.277 ± 0.075 for the supernatant, PK-L and PK-M_2 respectively. These correspond to half-lives, in days, of 2.86, 1.81, and 2.50.

In the first set of experiments designed to examine the proteolytic sensitivities of the human L-, M_2- and M_1-isozymes, the enzyme activities were adjusted to the same value, the protein concentrations were adjusted with BSA to the same optical density at 280 nm, and the same concentration of the proteolytic enzymes chymotrypsin, trypsin, and pronase was used. The loss of enzyme activity with time for the three isozymes is shown in Figure 40 for chymotrypsin, Figure 41 for trypsin, and Figure 42 for pronase.

Figure 37

Antibody inactivation of the E. coli pyruvate kinase Peak I from the DEAE-cellulose chromatography. The pyruvate kinase assay mix contained 1 mM FDP in addition to the components of the standard mix. Anti-PK-M_1 (O); anti-PK-R (⊡); anti-bovine muscle pyruvate kinase (●).

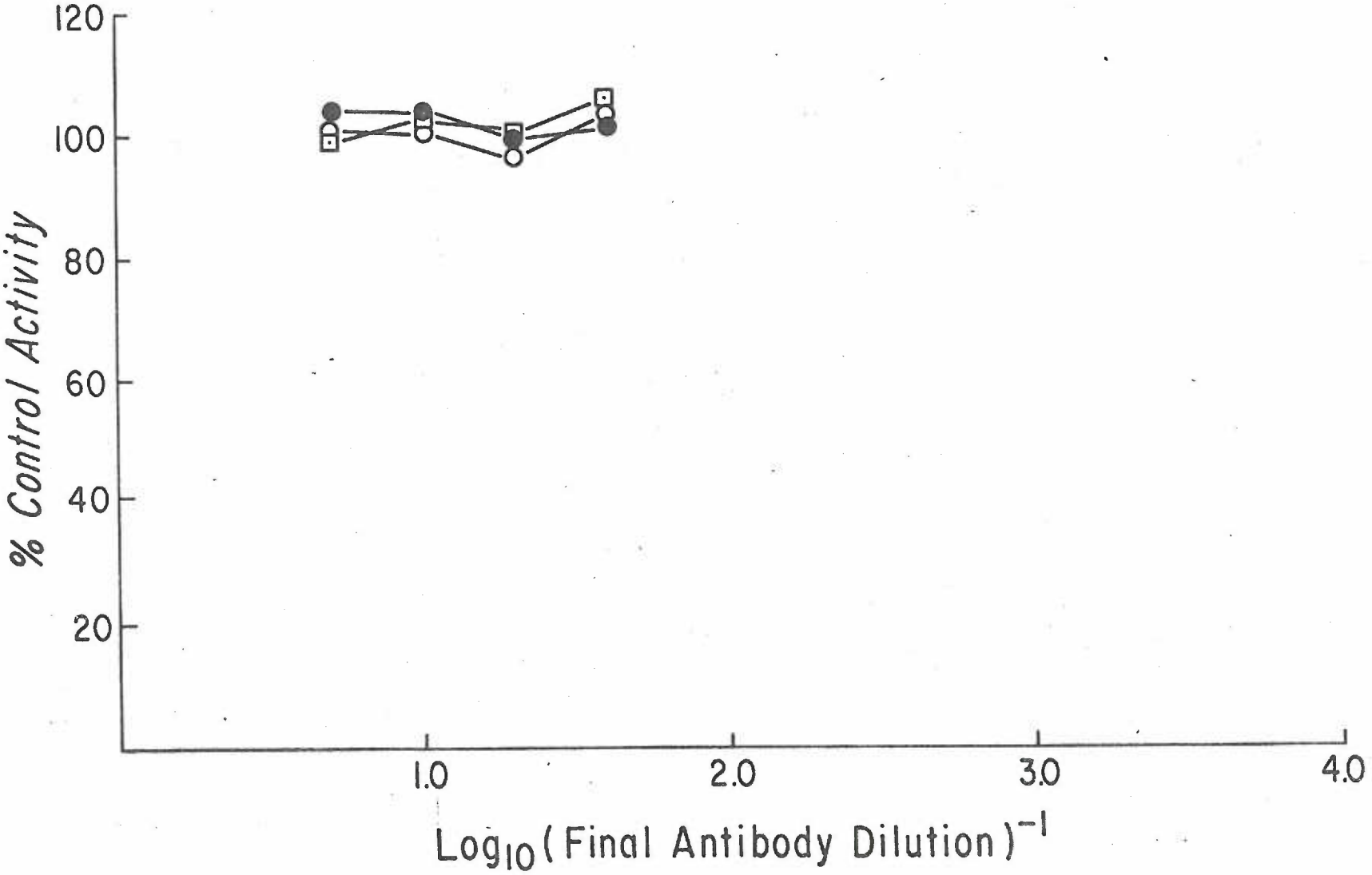

120
100
80
60
40
20
% Control Activity
1.0
2.0
3.0
4.0
Log10 (Final Antibody Dilution)−1

Figure 38

Antibody inactivation of the E. coli pyruvate kinase Peak II from the DEAE-cellulose chromatography. The pyruvate kinase assay mix contained 1 mM AMP in addition to the components of the standard mix. Anti-PK-M_1 (O); anti-PK-R (⊡); anti-bovine muscle pyruvate kinase (●).

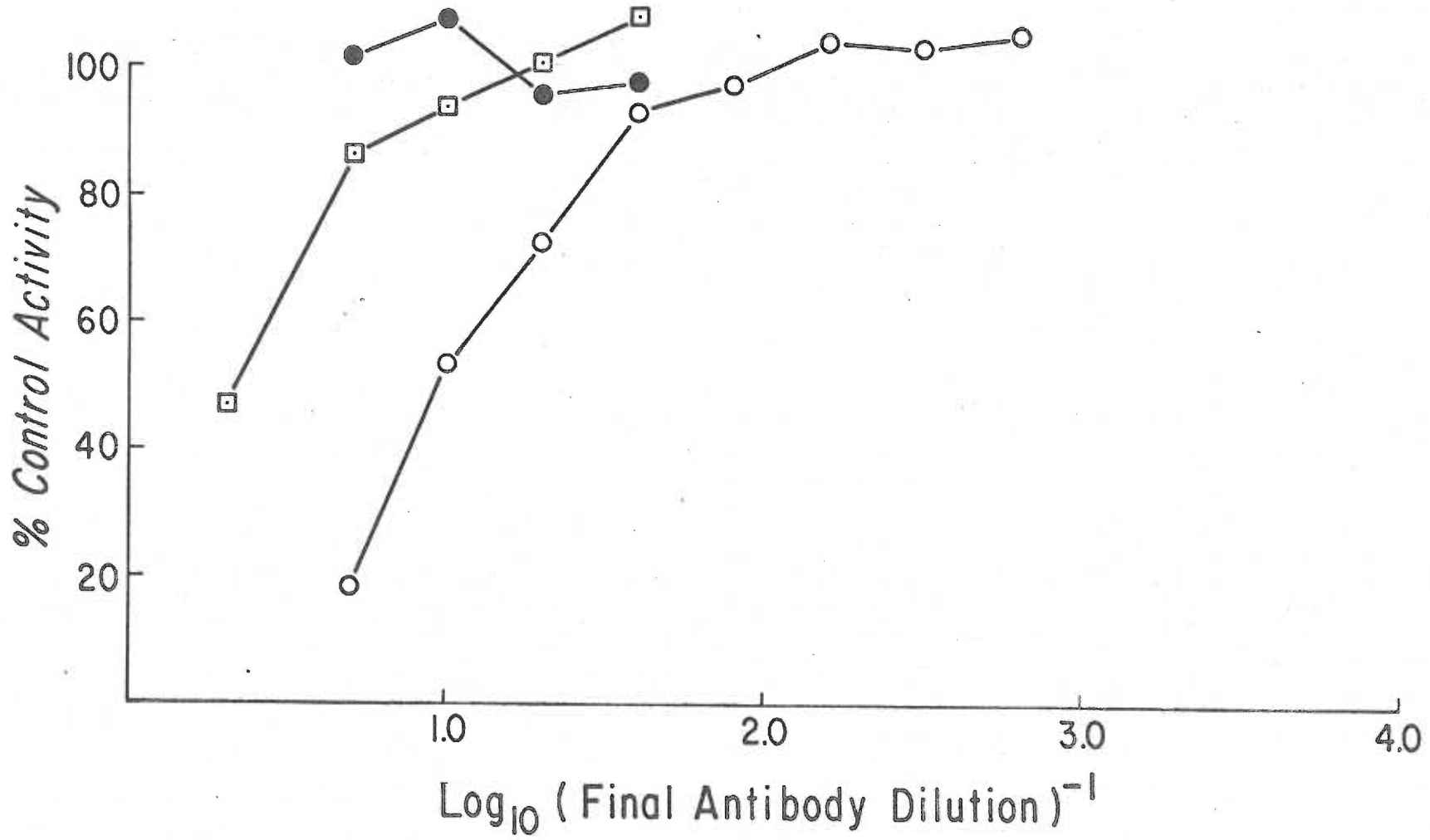

100
80
60
40
20
% Control Activity
1.0
2.0
3.0
4.0
Log10 (Final Antibody Dilution)-1

Table 5. Summary of antibody inactivation of pyruvate kinases in crude extracts from various species

enzyme source	anti-PK-R activity[a]	anti-PK-R 50% inhibit[b]	anti-PK-M$_1$ activity[a]	anti-PK-M$_1$ 50% inhibit[b]	anti-bovine muscle pyruvate kinase activity[a]	anti-bovine muscle pyruvate kinase 50% inhibit[b]
Mammalia						
human liver	++++	2.2	+	1.0	-	
rat liver	++++	1.8	-		-	
bovine liver	++++	1.9	+	1.1	+	2.6
opossum liver	++	2.2	+++	2.0	+++	1.3
Aves						
chicken liver	-		-		++++	2.1
Amphibia						
frog liver	-		+	1.5	+	1.0
Reptilia						
turtle liver	-		-		-	
Pisces						
steelhead liver	++++	1.3	-		+	1.4
Microorganisms						
yeast	-		-		-	
E. coli	+	1.0	++	1.3	-	
Peak I	-		-		-	
Peak II	++	0.8	++++	1.1	-	

[a]The symbols have the following meaning, relative to %-control activity at a value $\log_{10}(\text{final antibody dilution})^{-1} = 0.7$:

-	>90%
+	70-90%
++	50-70%
+++	30-50%
++++	0-20%

[b]If the supernatants had contained a single isozyme, the antibody dilution which inhibited an enzyme 50% could easily be defined as that point where the activity reaches 50% of the control value. However, most of the extracts contained multiple isozymes, and the ratios of these isozymes varied with the species. To obviate these problems a plot of "%-control activity" vs. "$\log_{10}$ (final antibody dilution)$^{-1}$" is made. The "50% inhibit" value is then defined as the value of the abscissa corresponding to the ordinate value halfway between the ordinate values at the highest and lowest antibody dilutions tested. Large "50% inhibit" values indicate that the antibody was highly effective in inactivating the pyruvate kinase and that the pyruvate kinase has a high degree of similarity with the isozyme used to prepare the antibody.

Figure 39

The decay in radioactivity for the rat liver supernatant, PK-L and PK-M_2. The values plotted are the mean ± S.D. of the observed specific radioactivities in experiment 2. The lines were calculated by a least squares determination using the individual values.

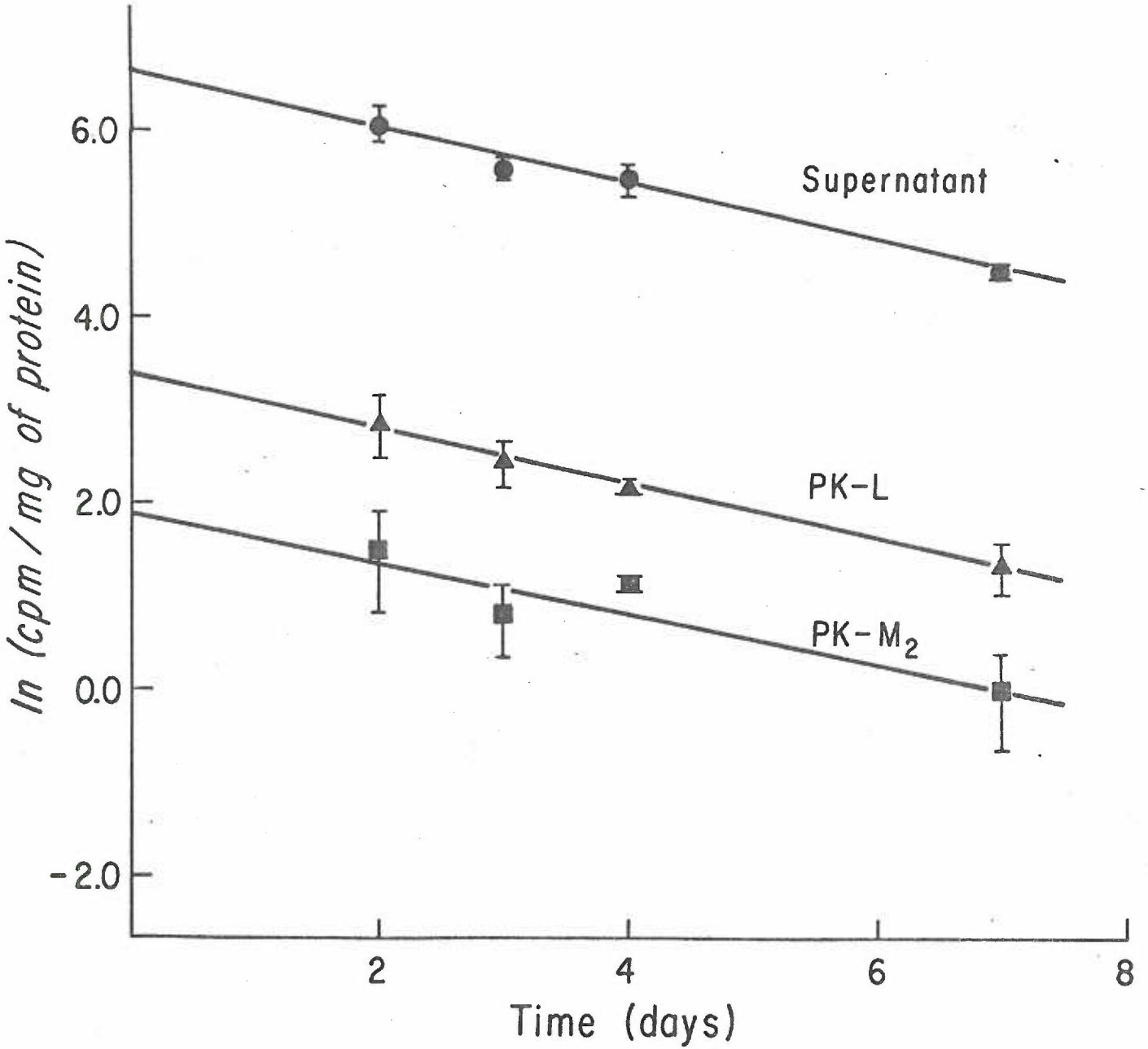

6.0
4.0
2.0
0.0
-2.0
ln (cpm / mg of protein)
Supernatant
PK-L
PK-M2
2
4
6
8
Time (days)

Table 6. Apparent degradation rate constants and half-lives for rat liver PK-L, PK-M_2, and rat liver supernatant (The values of k_d are ± S.E. of the slope)

protein	experiment	k_d ($days^{-1}$)	$t_{1/2}$ (days)
supernate	1	0.201 ± 0.050	3.45
	2	0.300 ± 0.022	2.31
	3	0.224 ± 0.033	3.09
	average	0.242 ± 0.064	2.86
PK-L	1	0.352 ± 0.099	1.97
	2	0.298 ± 0.035	2.33
	3	0.497 ± 0.048	1.41
	average	0.382 ± 0.115	1.81
PK-M_2	2	0.277 ± 0.075	2.50

Figure 40

Proteolytic inactivation of human PK-L, PK-M_2 and PK-M_1 by chymotrypsin.

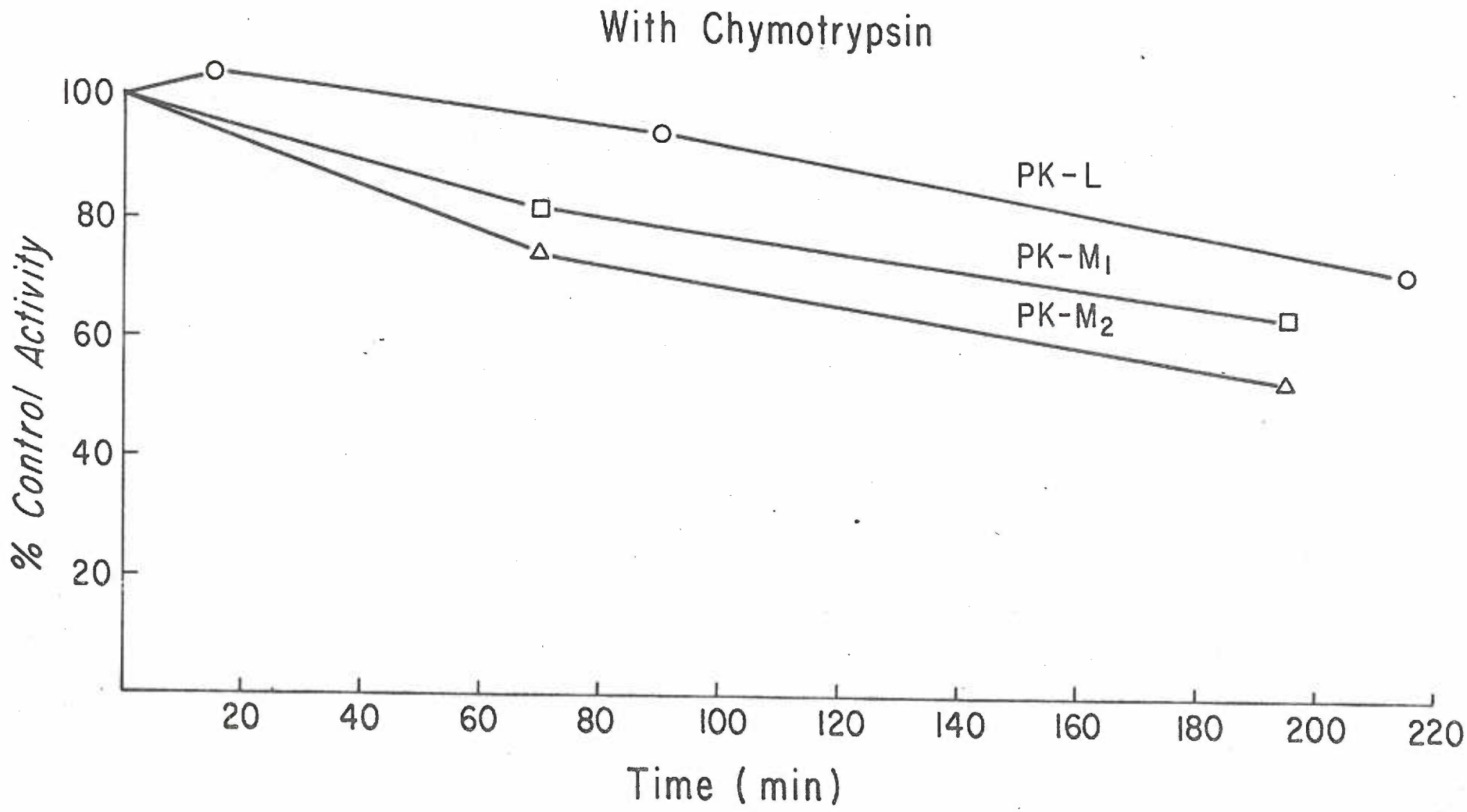

With Chymotrypsin
% Control Activity
100
80
60
40
20
PK-L
PK-M1
PK-M2
20
40
60
80
100
120
140
160
180
200
220
Time (min)

Figure 41

Proteolytic inactivation of human PK-L, PK-M_2 and PK-M_1 by trypsin.

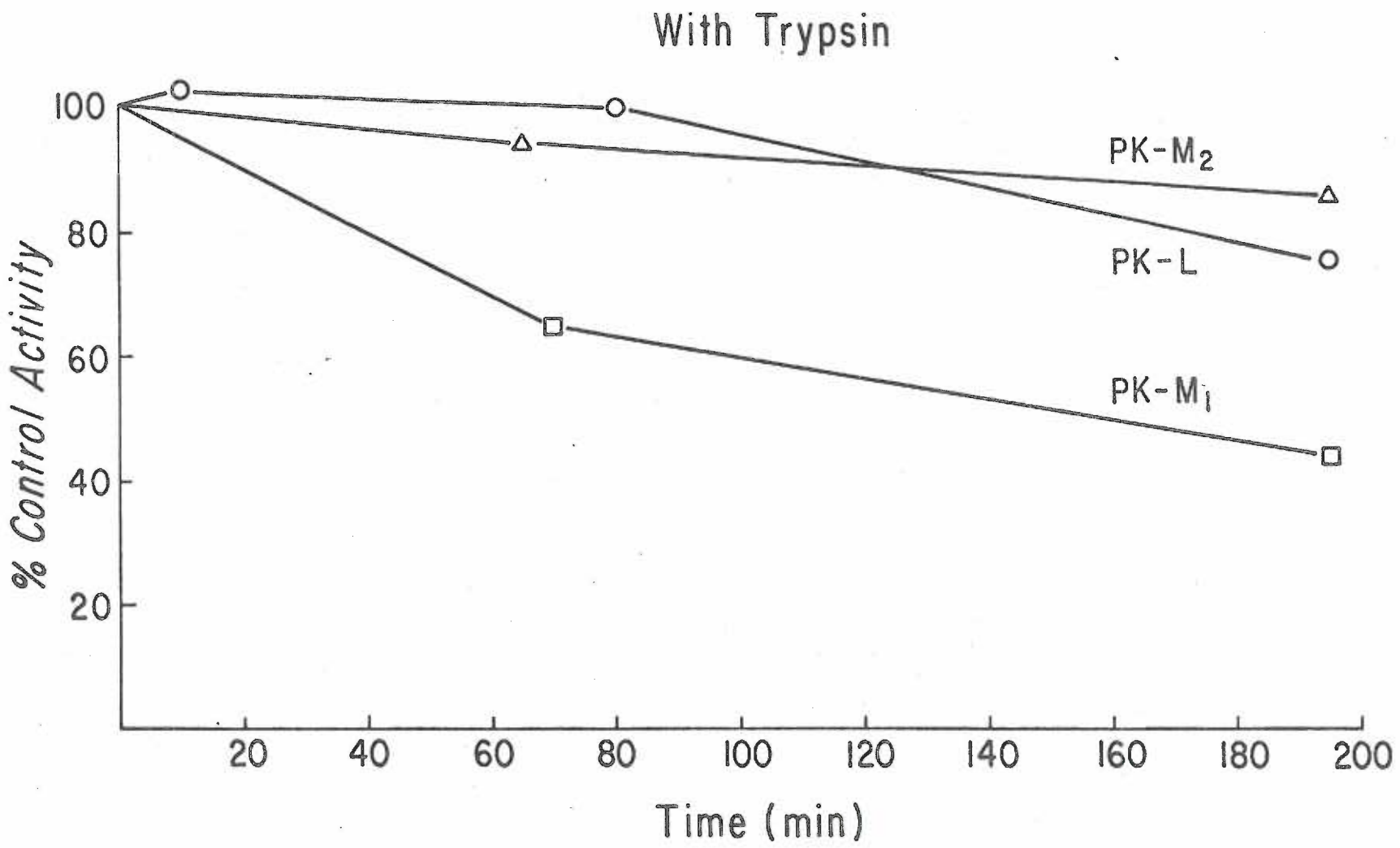
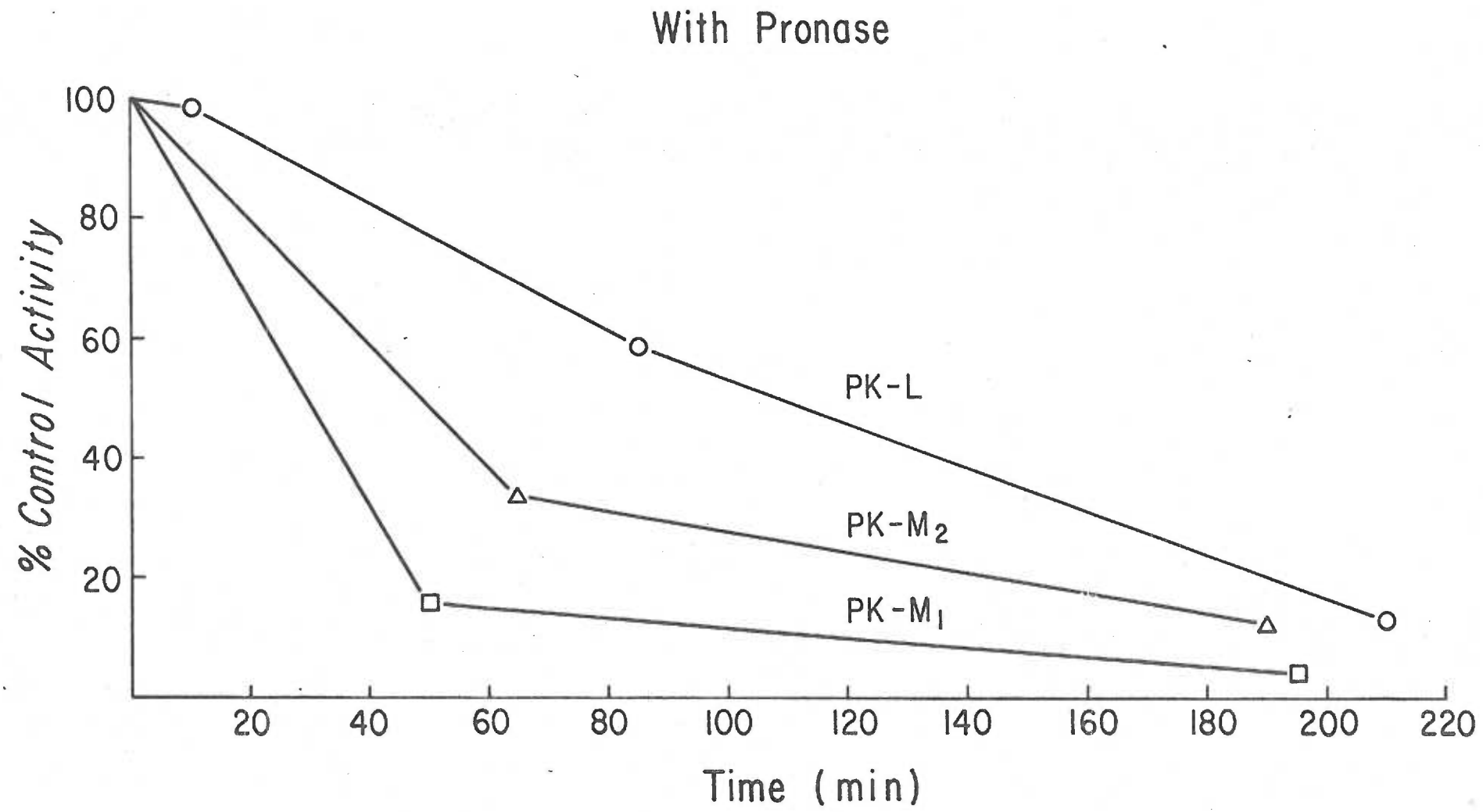

With Trypsin
100
80
60
40
20
% Control Activity
PK-M2
PK-L
PK-M1
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 42

Proteolytic inactivation of human PK-L, PK-M_2 and PK-M_1 by pronase.


With Pronase
% Control Activity
100
80
60
40
20
PK-L
PK-M2
PK-M1
20
40
60
80
100
120
140
160
180
200
220
Time (min)

They show that the degree of sensitivity with chymotrypsin is PK-M_2 > PK-M_1 > PK-L, with trypsin is PK-M_1 > PK-M_2 ≅ PK-L, and with pronase is PK-M_1 > PK-M_2 > PK-L. With regards to the liver isozymes, PK-M_2 generally showed greater sensitivy to proteolytic attack than did PK-L.

Table 7 shows the results of the proteolytic inactivation of human PK-L, PK-M_2 and PK-M_1 by pronase in the presence of several compounds at 1 mM concentrations. These results are summarized in Table 8 where the effects of the various compounds are grouped into three categories: 1) those which protect the enzyme from inactivation, defined as additives which result in activity more than 5 percentage points above the "none" level, 2) those which sensitize the enzyme to inactivation, defined as those additives which result in activity less than 5 percentage points below the "none" level, and 3) those which apparently have no effect on the degree of sensitivity to pronase.

Table 7. Proteolytic inactivation of human PK-L, PK-M_2 and PK-M_1 by pronase

	Isozyme		
	PK-L	PK-M_2	PK-M_1
incubation time (hrs)	2.5	2.0	.67
additive			
none	48.8	65.1	56.8
PEP	108	106	96.8
3PGA	44.4	59.8	56.6
ADP	51.5	56.3	65.8
ATP	67.8	60.2	58.4
$MgCl_2$	57.4	65.0	78.2
ADP + $MgCl_2$	56.2	60.5	83.0
ATP + $MgCl_2$	75.6	62.7	77.8
FDP	86.2	108	75.2
G6P	32.4	58.0	57.2
citrate	60.8	57.8	59.6
L-alanine	58.6	81.1	28.8
L-phenylalanine	55.7	54.8	16.6

Values are expressed as percent of controls without pronase.

Table 8. Summary of proteolytic sensitivities of human PK-L, PK-M_2 and PK-M_1 to pronase

isozyme	category	relative degree of effect
PK-L	protector	PEP > FDP > ATP + $MgCl_2$ > ATP > $MgCl_2$ ≅ citrate ≅ ADP + $MgCl_2$ ≅ L-alanine ≅ phenylalanine
	sensitizer	G6P
	neutral	ADP, 3PGA
PK-M_2	protector	PEP ≅ FDP > L-alanine
	sensitizer	L-phenylalanine ≅ ADP ≅ 3PGA ≅ G6P ≅ citrate
	neutral	ATP + $MgCl_2$, ADP + $MgCl_2$, ATP, $MgCl_2$
PK-M_1	protector	PEP > ADP + $MgCl_2$ > ATP + $MgCl_2$ ≅ FDP ≅ $MgCl_2$ > ADP
	sensitizer	L-phenylalanine > L-alanine
	neutral	citrate, G6P, ATP, 3PGA

Discussion

By acrylamide gel electrophoresis, sedimentation equilibrium, and gel immunodiffusion, the human liver PK-L was nearly homogeneous. The molecular weight of 335,600 determined by sedimentation equilibrium is considerably higher than the molecular weights of other isozymes of pyruvate kinase previously reported (see Table 3), which generally have been in the range of 200,000 to 250,000. It is possible that this molecular weight of 335,600 represents a weight average of polymers of the pyruvate kinase present during the sedimentation equilibrium run, as a detailed examination of the plot of log ΔY vs. r^2 revealed the possibility that there may be multiple values of molecular weight present. There was a slight upward trend from that expected in a straight line near the higher values of r^2. This interpretation of polymerization would be consistent with the gel immunodiffusion results, which indicated two or possibly more species of pyruvate kinase present.

In the purification of human PK-R, Chern et al. (24) reported a small contaminant in the final product which they believed to be due to aggregation of the major component of pyruvate kinase. The presence of FDP in the buffer used for the sedimentation equilibrium may have been a contributing factor to the polymerization, or the phenomenon may be concentration dependent. Unfortunately, the small amount of purified PK-L available precluded further studies.

The stabilizing influence of hydrophobic bonds on pyruvate

kinase was suggested by the results of the temperature stability studies. Comparing Figures 3 and 4, it appears that the enzyme is more stable at 25° than at 5°, which is consistent with hydrophobic bond stabilization. In addition, 20% glycerol was found to be an effective stabilizer of activity at 5°, whereas its effect at 25° was very much reduced. Glycerol is considered to stabilize hydrophobic bonding by providing an altered environment. In contrast, Figure 2 suggests that PK-L is more stable at 5° than at 25°. The difference between these experiments was the presence of 2-mercaptoethanol in the buffer for Figures 3 and 4.

Based upon the allosteric model of Monod et al. (169), Rozengurt et al. (170) developed a two-state model for PK-L to describe the various allosteric interactions. The model predicts the existence of two unique conformational states. The R state is induced by the activators FDP, H^+ ions, K^+ ions and high PEP concentrations. The T state is stabilized by the inhibitors ATP and alanine. Recent evidence has shown that FDP and K^+ ions induce a different conformational state rather than a unique state (29), and also that alanine and ATP induce a different T conformation probably by binding to different sites (30,171). These results suggest that sequential conformational changes are involved in the allosteric transitions of this enzyme.

All of the compounds tested in the temperature stability tests described in this thesis gave added protection to pyruvate

kinase activity when compared to no addition. Particularly at 5°, those compounds described to induce the R state -- FDP, K^+ ions and PEP -- gave a higher degree of protection than those described to induce the T state -- ATP and alanine.

This laboratory now has purified the four major isozymes of pyruvate kinase in humans: PK-L (this thesis), PK-R (24), PK-M_1 and PK-M_2 (23). As mentioned earlier, based on electrophoretic mobilities and inactivation by antiserum against the rat PK-M_2 isozyme, it has been proposed that the human R-isozyme is a hybrid of the L- and M_2-isozymes (11,43,44). This hypothesis was tested in several experiments using anti-PK-R and anti-PK-M_1 prepared from isozymes purified in this laboratory from human cells.

The results of the antibody inactivation, gel immunodiffusion and gel electrophoresis with antibodies are all inconsistent with the hypothesis. In all experiments the anti-PK-R only interacted with the L- and R-isozymes from human, whereas the anti-PK-M_1 interacted only with the M_1- and M_2-isozymes. With rat isozymes, the anti-human PK-R inactivated the rat L- and R-isozymes, and the anti-human PK-M_1 inactivated the rat M_1- and M_2-isozymes. However, gel immunodiffusion showed a cross-reaction between the anti-PK-R and the rat L-, R-, and M_2-isozymes, indicating shared antigenic determinants. Thus, antisera prepared against rat isozymes could be misleading if used to interpret the subunit relationships among the human isozymes of pyruvate kinase.

It could be argued that the proposed interaction of the L- and M_2-subunits in forming the R-isozyme alters the conformation of the M_2-subunits to such an extent that "native M_2" antigenic determinants are not recognized by antibodies against the M_2-subunits present in the R-isozyme. This would appear to be unlikely since one naturally occurring hybrid of the L- and M_2-isozymes present in human kidney is partially inactivated by both anti-PK-R and anti-PK-M_1 sera and both antisera cross-react with this hybrid. Therefore, the hypothesis that human PK-R is a hybrid of PK-L and PK-M_2 is no longer tenable. PK-R therefore must either be a separate gene product, or the result of post-translational modification of one of the other isozymes.

The immunological properties of PK-L and PK-R are very similar. In addition, the freezing of human PK-R solution (43), the incubation of red cell hemolysate with liver extract (172), or an acute case of hepatitis (172) produced a form of erythrocyte pyruvate kinase which migrated on gel electrophoresis to a position similar to human liver PK-L. The liver PK-L in cases of erythrocyte pyruvate kinase deficiency where it has been tested has often been altered either in the amount or electrophoretic mobility (43,167, 172), providing further evidence for a close relationship of these two isozymes. It is still unclear whether they are the result of separate genes, or of some post-translational event.

The pyruvate kinases of several evolutionary distant

species were examined with immunological techniques. As probes for structural similarities, anti-human PK-R was used to detect PK-L determinants, and anti-human PK-M_1 and anti-bovine muscle pyruvate kinase were used to detect different muscle isozyme determinants.

Earlier studies had not resolved the question of the multiplicity of isozymes in liver. They have indicated that the chicken liver has only one isozyme (150-152), that turtle liver pyruvate kinase in whole supernatants has kinetic properties like the L- and M_2-isozymes (173,174), that frog liver supernatant has five isozymes composed of subunits from the two major isozymes which have L- and M-like characteristics (153), that E. coli has two isozymes of which one is constitutive and the other responsive to changes in the carbon source in the media (160,175), and that yeast has one isozyme of pyruvate kinase (157).

The electrophoresis studies performed in this thesis largely substantiates these earlier reports, and extends the general rule of multiple pyruvate kinase isozymes in animal livers. All of the mammals studied -- human, rat, bovine, and opossum -- had at least two forms of pyruvate kinase in the liver supernatants. The fact that a second rat isozyme was not found in electrophoresis in this thesis is probably just a variation in the relative proportions of the two isozymes, as two isozymes have been consistently identified in rat liver in previous studies (10,11,14). Two major isozymes of pyruvate kinase have also been identified in kangaroo, pig, sheep,

rabbit, guinea pig, and mouse (44).

The chicken does have only one pyruvate kinase isozyme in its liver, as evidenced by the electrophoretic pattern described in this thesis, and by earlier studies (150-152). While Schloen et al. (153) described five isozymes in the frog liver by electrophoresis, there was one isozyme which predominated, and this is probably the isozyme detected in the electrophoresis performed for this thesis. Once again, differences may appear because of the relative proportions of the isozymes within liver supernatants and the adjustment of samples to give a constant total pyruvate kinase activity.

The electrophoretic pattern of the turtle isozymes indicated multiple species, which is consistent with the kinetic studies performed earlier (173,174). The steelhead trout had only one isozyme in the liver supernatant as detected by electrophoresis, as did the yeast, but the E. coli had two isozymes.

The electrophoresis results also suggest that in the case of multiple isozymes of pyruvate kinase, the L-like isozyme is more acidic than the M_2-like isozyme, particularly among mammals. In electrophoresis at pH 8.2, the isozyme with the greatest electrophoretic mobility reacts with the anti-PK-R, and the slower isozyme reacts with the anti-PK-M_1. This feature seems to have been preserved throughout a certain portion of evolution, as it is found in the four mammals studied and turtle liver.

There are several problems which affect the interpretation of serological data, particularly attempts at quantitative interpretation. The specificity of the antibody depends on several factors, but particularly the multiplicity of distinct determinants against which the antibodies are formed and the heterogeneity of antibodies to each determinant with respect to binding affinity. Other factors affecting relative specificity are: 1) differences in the response of individual animals to immunization, 2) the time length of the immunization schedule, and 3) the phylogenetic relationship of animals producing the antibody and the species of the antigen donor.

Antigens used in phylogenetic correspondence studies must be in a similar state of preservation and purity. For the antibody inactivation studies it was assumed that the pyruvate kinase specific activities of the isozymes in the supernatants were the same, as they were adjusted on the basis of activity and not on the weight of pyruvate kinase present in the supernatants. This assumption is unlikely to be correct, but probably does not alter the general conclusions made in this thesis from the experimental results.

The immunochemical correspondence is considered to be a reflection of the similarity of the cross-reacting antigen to the reference antigen. In phylogenetic terms the comparison of protein homologs is considered to be an examination of the structural

similarities retained within the homologous proteins during evolution from a common ancestor. A decrease in the rate of evolutionary modification of antigen of any species within a group after its divergence from another species will result in a greater resemblance to the common ancestral molecule. This will result in a higher immunochemical correspondence to the reference antigen than expected based on the preliminary assumption that the correspondence reflected the relative length of evolutionary time lapse from the divergence (176).

Enzyme inhibition is probably not as satisfactory a method of measuring phylogenetic relationships as certain precipitin reactions such as the quantitative or complement fixation methods. This is because the mechanism of the inhibition is not clear as it may depend on a single determinant at or near the catalytic site, or it may be the result of interactions with several determinants over the enzyme surface.

However, this thesis does not depend on a single method for examining the phylogenetic relationships. Three methods were employed: 1) antibody inactivation, 2) gel immunodiffusion, and 3) gel electrophoresis of samples mixed with antibodies to examine which isozymes had alterations in their electrophoretic mobilities. All three methods did not always lead to the same conclusion as to which supernatants were affected by the two antibodies used, as some differences were noted between the antibody inactivation

results and gel immunodiffusion. These variances are most likely the result of differences in the sensitivities of these two methods, as greater levels of pyruvate kinase activity have been generally noted to be a requirement of the immunodiffusion method as compared to the antibody inactivation method. It is quite possible that the immunodiffusion gels were not sensitive enough to some low levels of pyruvate kinase isozyme present in some of the supernatants.

The immunological data in this thesis are in general agreement with earlier phylogenetic studies performed using more classical taxonomic techniques. The time of evolutionary divergence from man is generally indicated in Table 5, where those species further from man in evolutionary time are placed further down in the table. With increasing distance from man, the amount of activity remaining after incubation with antibodies and also the general decrease in the values of "50% inhibit" is generally consistent with the increasing time of divergence from man. It would have been desirable to use antibodies prepared from pyruvate kinases in other species, as suggested by Praeger and Wilson (83), but the large amount of tissue required for the purification of pyruvate kinase precluded these additional studies.

With the mutations which have occurred during evolution, some of the determinants have been lost. It has not been shown that the human isozymes have undergone similar losses in determinants since only anti-human pyruvate kinases have been used to probe for

such variations, but it is more reasonable to believe that it is not possible to detect the loss rather than assuming that the human isozymes carry all of the ancestral determinants.

To partially obviate this problem, rabbit anti-bovine muscle pyruvate kinase was also used, with results which differed somewhat from those produced by the anti-human PK-M_1. Whereas the anti-human PK-M_1 did not affect the chicken, turtle or steelhead liver supernatants in antibody inactivation experiments, anti-bovine muscle pyruvate kinase did. The chicken muscle pyruvate kinase is also inactivated by anti-bovine muscle pyruvate kinase.[1] This suggests that some of the ancestral determinants have been lost from the human isozymes.

There are certain interesting exceptions to the general relationship between immunological cross-reactivity of the pyruvate kinases and evolutionary distance from humans. The steelhead trout was significantly inhibited by the anti-PK-R and to a lesser extent by the anti-bovine muscle pyruvate kinase, whereas this reactivity to anti-PK-R was not found in the chicken, frog, or turtle livers. These latter three species are closer to humans in evolutionary time than is the trout, and therefore might have been expected to have determinants closer to the human L-isozyme than the trout.

The most unexpected finding in the research performed for

[1]Black, J. A., unpublished results.

this thesis was the immunoreactivity of one of the pyruvate kinase isozymes from E. coli. The second isozyme separated by DEAE-cellulose chromatography showed sensitivity to both of the anti-human antibodies used in this thesis. The E. coli supernatant showed lines of identity by gel immunodiffusion with anti-PK-R and anti-PK-M_1. By electrophoresis it was shown that only the second isozyme of pyruvate kinase from the DEAE-cellulose column is affected by the anti-human pyruvate kinase antibodies. It was shown earlier that the first isozyme from the DEAE-cellulose column is responsive to FDP (158,160) and the media carbon source (160), whereas the second isozyme is responsive to AMP and other organic monophosphates (177) and is apparently constitutive as the quantity is unaffected by media conditions (160).

Cohen et al. (178) have proposed that the central role of the glycolytic pathway in the maintenance of the mature red cell has restricted the evolutionary process acting upon erythrocyte glycolytic enzymes. While the liver has more metabolic pathways for ATP production, the apparent conservatism of enolase evolution described earlier (87,179), and of pyruvate kinase described in this thesis would argue that the same statement is valid in liver metabolism. It is interesting to note that it is apparently the constitutive isozyme in E. coli which contains determinants which cross-react with both the anti-PK-R and the anti-PK-M_1. It might be expected that a constitutive isozyme would bear greater

evolutionary pressure against mutations because any alterations in the activity of this enzyme might have severe implications for the organisms. The basic necessity of the glycolytic pathway would also place severe restrictions on metabolically acceptable mutations.

The immunology of cytochrome c (180) and lysozyme (83) from different species suggests that immunological cross-reactivity disappears when sequences differ by about 30-40%. This suggests that the amino acid sequence of the second isozyme of E. coli contains a high degree of sequence homology with both the R- and M_1-isozymes from humans. There are two possible explanations for this observation: 1) the mutation rate of the E. coli isozyme has been very slow, and that while PK-R and PK-M_1 have evolved to gene products sufficiently different so that they do not cross-react, they still contain some of the early ancestral determinants present in the E. coli isozyme, or 2) the E. coli isozyme has shown evolutionary convergence toward a structure with determinants similar to human R- and M_1-isozymes. There is presently insufficient evidence to distinguish the two processes.

The results presented in Table 5 suggest that the gene duplication which led to the divergence of PK-L and PK-M_2 took place before the divergence of the amphibian and mammalian classes, or about 300 million years ago. Because of the few number of species in each vertebrate class in this study, it is not clear whether this conclusion would be altered by the addition of

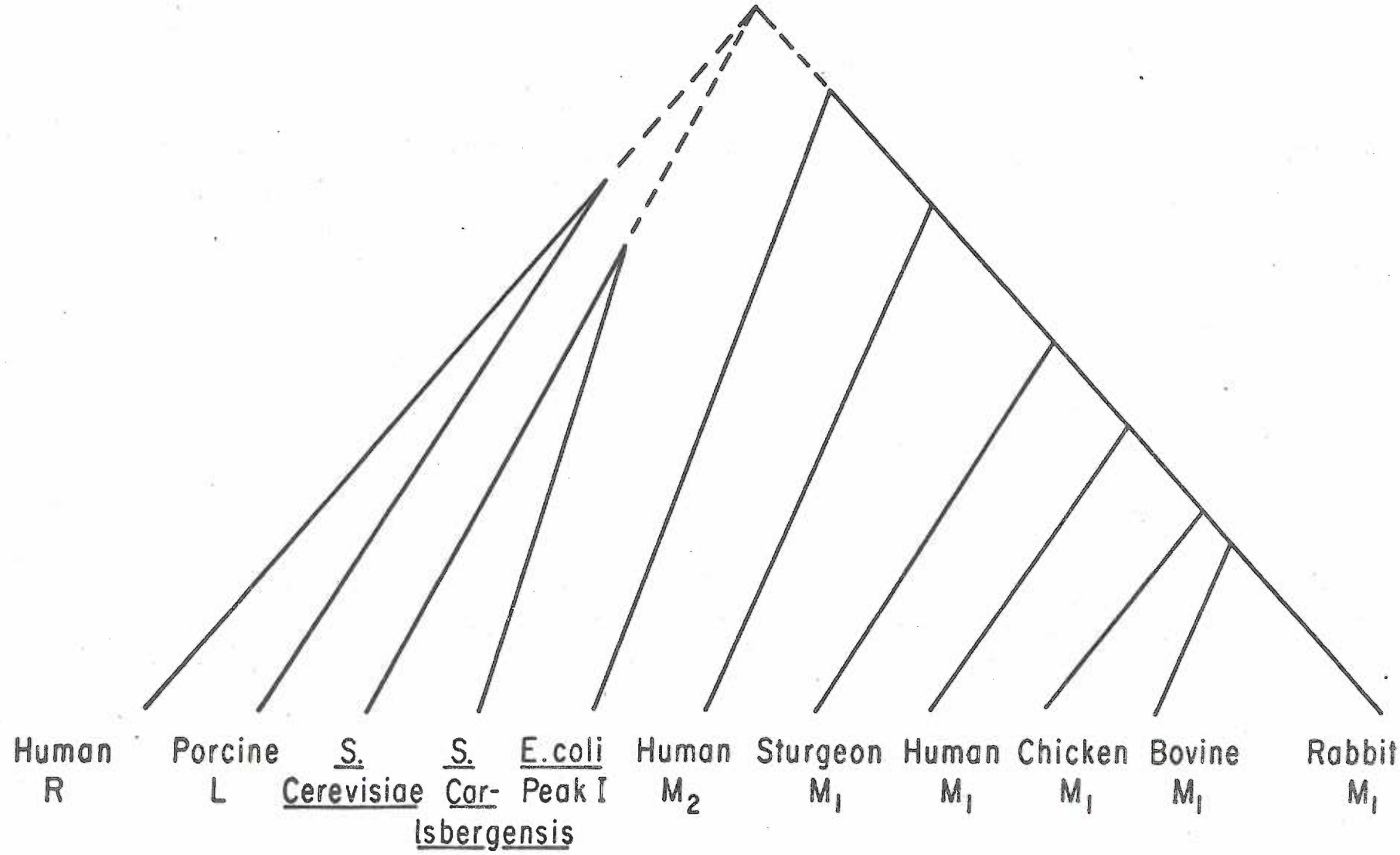
different species. Schloen et al. (153) presented evidence of a frog liver isozyme of pyruvate kinase which is immunologically distinct from the frog skeletal muscle isozyme, and also a frog liver isozyme which cross-reacts with the frog skeletal muscle isozyme, a situation which is analogous to that in the mammals studied. The gene duplication which led to the separation of PK-L and PK-M_2 could possibly have occurred as early as the divergence of the fish or procaryotes from the mammalian class. The evidence is not clear. Cardenas et al. (181) have suggested that the divergence of the M_2- and M_1-isozymes occurred prior to the divergence of the avian and mammalian classes, or more than 175 million years ago.

Unfortunately, the amino acid sequences are not available for any isozyme of pyruvate kinase, as more detail of the evolutionary relationships between the isozymes could be derived from a comparison of the primary and tertiary structures of these isozymes. However, the amino acid compositions of some of these isozymes are available, and Dedman et al. (77) have proposed a quantitative method whereby the amino acid compositions of homologous proteins from different species can be correlated to phylogenetic relationships. From this method, Black and Harkins (182) have developed a figure showing the homology for several of the pyruvate kinase isozymes (Figure 43).

It is interesting to note that human PK-M_1 is apparently

Figure 43

The homology between pyruvate kinase isozymes (182).


Human R
Porcine L
S. Cerevisiae
S. Carlsbergensis
E.coli Peak I
Human M2
Sturgeon M1
Human M1
Chicken M1
Bovine M1
Rabbit M1

more homologous to other M_1-isozymes in species such as sturgeon, chicken, bovine and rabbit than it is to human PK-M_2. This suggests that homologous isozymes from various species are more related than heterologous isozymes within the same specie as judged by amino acid compositions. This, in conjunction with the immunological data discussed earlier, demonstrates once again the relatedness of isozymes performing similar roles in the same differentiated tissues from different species, and suggests a high degree of structure conservatism. This strong evolutionary pressure on isozymes in the same metabolic position in differentiated tissue emphasizes the key role that pyruvate kinase plays in the glycolytic pathway.

Also in Figure 43, it can be seen that the gene duplication which led to the divergence of the L- and R-isozymes from the M-isozymes occurred in the distant evolutionary past as the data at this time are not capable of separating the divergence of yeast from the other species and the divergence of the L- and R-isozymes from the other species.

This conservatism in structure leads to an apparently constant relationship in subunit molecular weight and isoelectric points for the two liver pyruvate kinase isozymes. In both the rat and the human the PK-M_2 has a lower molecular weight and a greater isoelectric point as compared to the PK-L (see Table 3). In the immunological studies described in this thesis, it was

found that the anti-PK-R tended to cross-react with the faster migrating isozyme on electrophoresis at pH 8.2, whereas the anti-PK-M_1 cross-reacted with the slower isozyme. This further substantiates the relationship that the L-like isozyme is more acidic than the M_2-like isozyme.

These two factors, the molecular weight and the isoelectric point, have been identified as factors which correlate with the half-life of proteins. Those proteins with low subunit size have long half-lives. It has been proposed that the rate of degradation is based on an overall chance of being "hit" by a protease producing the initial rate-limiting cleavage with subsequent unfolding and rapid degradation, and because of their greater number of bonds, larger subunits are seen as presenting a greater chance of being hit (95). Four reasons have been proposed as to why acidic proteins may degrade faster: 1) acidic proteins are more sensitive to the cell's proteolytic enzymes, 2) native conformations of acidic polypeptides are inherently less stable, 3) crucial proteases may preferentially hydrolyze polypeptides at acidic residues or proteins of a certain charge, and 4) acidic proteins may preferentially accumulate in cellular regions where degradation takes place (118).

The difference in the half-lives of the two pyruvate kinase isozymes from rat liver was not statistically significant (α=0.10). Therefore, the two isozymes are not in agreement with the general rule correlating half-life and isoelectric point. PK-L has the more acidic isoelectric point as compared with PK-M_2 and therefore would

have been expected to have the shorter half-life. However, there is general agreement with the earlier prediction in this thesis that the half-lives of the isozymes would be 2.5 days.

It has also been noted that there is a correlation between the protein half-life and protein sensitivity to proteolytic enzymes. Those proteins most sensitive to proteolytic enzymes have the shortest half-life. With chymotrypsin and pronase, human liver PK-L was less sensitive than PK-M_2, and with trypsin, the two isozymes had about the same sensitivity. Since no statistically significant difference was found between the half-lives of the rat liver isozymes, the data on the isozyme proteolytic sensitivities are not in agreement with the general rule.

On the other hand, it could be that the conditions of the incubation influenced the relative relationships of the two isozymes to proteolytic enzymes. As was shown in Table 7, the degree of proteolytic sensitivity of these two isozymes is quite dependent on presence or absence of various compounds. For instance, glucose-6-phosphate increased the sensitivity of human PK-L to pronase, whereas it had less effect on PK-M_2. L-phenylalanine apparently had little effect on the sensitivity of PK-L, but it increased the sensitivity of PK-M_2. L-alanine apparently protected PK-M_2, whereas it had less effect on PK-L. Phosphoenolpyruvate protected both isozymes from

proteolysis by pronase. These results suggest that the half-life of pyruvate kinase may be the result of a complex interaction of numerous metabolic intermediates.

Alanine has been recently described as causing a reversible, temperature-dependent partial inactivation of PK-M_2 which is evidently reversible by the addition of FDP (55,56). This inactivation is apparently a transition to a sub-active dimer from the active tetramer, and the transition takes several minutes. The tetramer is favored in the absence of alanine by PEP, ADP, and FDP. In the presence of alanine, only FDP reverses the transition at relatively low levels, although high levels of PEP can favor the formation of the tetramer in the presence of alanine (56). It appears from Table 8 that both the dimer and tetramer can be protected by the appropriate ligand since alanine, PEP and FDP protected the enzyme from proteolytic inactivation by pronase.

Weber et al. (183) have shown that phenylalanine inhibits pyruvate kinase in human and rat brain. Because of the high levels of phenylalanine which are found in patients with phenylketonuria, it was suggested that the brain damage found in these patients is related to the inhibition of pyruvate kinase by phenylalanine (184). The results in Table 7 indicate that phenylalanine causes an increase in the proteolytic sensitivity of PK-M_1 and therefore the high level of phenylalanine found in cases of phenylketonuria may not only inhibit the PK-M_1 present but may also cause a decrease in the

quantity of PK-M_1 present by increasing its sensitivity to proteolytic degradation. When high levels of phenylalanine were induced in a rat, the changes in the brain glycolytic intermediates were consistent with pyruvate kinase inhibition in vivo, but there was no decrease in the levels of ATP or creatine phosphate. This suggests that there are other compensatory modes for the maintenance of ATP (184). While this suggests that the brain damage in patients with phenylketonuria is not related to the reduced activity of pyruvate kinase, it leaves unclear whether the quantity of brain pyruvate kinase is reduced in these cases.

Because of the importance of the glycolytic pathway, all of the enzymes in the pathway have been purified from some source, and several of the half-lives of these enzymes have been measured. Table 9 lists the enzymes in the pathway with their reported molecular weights, numbers of subunits, predicted half-lives as based on studies correlating half-life with subunit size, and the reported half-lives.

The predicted half-lives and reported half-lives are not in agreement in four instances: glucokinase, phosphofructokinase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase. The exact reasons for this are not clear, although these could just be cases where the general correlation is not applicable, perhaps because of the importance of other factors. The methodologies for determining the half-lives could also be a problem as different methods can

Table 9. The molecular weights and half-lives for enzymes in the glycolytic pathway

enzyme	source	Molecular weight	subunits	Predicted half-life (days)[a]	Reported rat liver half-life (days)	method
glucokinase	rat liver	50,000(185)	1	3	0.46(194)	decay of enzyme activity
					0.50-0.67(195)	decay of enzyme activity
					1.25(196)	decay of induced activity
hexokinase	rat liver	100,000(185)	1	<1		
phosphoglucoisomerase	rabbit muscle	132,000(186)	2	2		
phosphofructokinase	rabbit muscle	360,000(187)	4	<1	7(197)	double label
aldolase	rat liver	160,000(188)	4	5-6	2.8(198)	pulse label corrected by ^{14}C-guanido arginine
glyceraldehyde-3-phosphate dehydrogenase	rat liver	120,000(189)	4	7-8	3.1(198)	pulse label corrected by ^{14}C-guanido arginine
phosphoglycerate kinase	rabbit muscle	45,000 - 48,000(190)	1	3-4		
phosphoglyceromutase	rat liver	54,000(191)	2	~10		
enolase	rat liver	82,000 - 100,000(192)	2	~4		
pyruvate kinase	rat liver					
type L		208,000(8)	4	2.5	1.8	pulse label - ^{14}C-carbonate
					1.25(117)	enzyme induction
type M_2		216,000(11)	4	2.5	2.5	pulse label - ^{14}C-carbonate
lactic dehydrogenase	rat liver	150,000(193)	4	5-6	19(103)	continuous labeling
					16(137)	continuous labeling
					6-7(115)	double label
					3.5(198)	pulse label corrected by ^{14}C-guanido arginine
					4.1(199)	average of double label and pulse injection of ^{3}H-leucine

[a]Based on figures in references 115 and 116.

References are in parentheses.

produce different results, as is the case for lactic dehydrogenase. If the half-life of phosphofructokinase is less than one day as predicted, an unreliable half-life would have resulted from the use of the double-label method because the label lingers in the cell rather than acting as a short pulse injection (115).

In those cases where the molecular weight of the rabbit muscle enzyme was used in predicting the half-life, the liver enzyme might have a different molecular weight which would alter the prediction for the enzyme half-life. It should also be noted that the same liver enzyme may have different half-lives depending on the species (200).

Glucokinase, phosphofructokinase, and pyruvate kinase have been identified as the rate-controlling enzymes in glycolysis (1). It is interesting to note that these enzymes have among the lowest half-lives, which would be anticipated if rapid changes in the levels of these enzymes were needed to respond to changing metabolic conditions. Those enzymes with the shortest half-lives could vary their concentrations in the shortest period of time since the steady state concentrations will be more sensitive to small changes in k_s, the rate constant for synthesis, or k_d, the rate constant for degradation.

Summary and Conclusions

Human PK-L has been purified to homogeneity from liver. The final enzyme preparation had a specific activity of 56.4 units/mg protein and represented a 360-fold purification over the crude liver enzyme. The results of the sedimentation equilibrium indicated that the purified enzyme had a molecular weight of 335,600 ± 2400 daltons. The stability of human liver PK-L was notably enhanced by the presence of reagents such as 2-mercaptoethanol, PEP and KCl. It appears that the PK-L is stabilized by hydrophobic bonding.

By immunologic techniques, human erythrocyte pyruvate kinase was found not to be a hybrid of the human L- and M_2-isozymes as had been earlier proposed. Thus, it appears that the R-isozyme is the result of a separate gene product, or that PK-L and PK-R are related by a post-translational modification.

Electrophoresis studies of the supernatants from the livers of species representing the various classes of vertebrates has provided additional evidence that the presence of two isozymes of pyruvate kinase in liver is characteristic of liver metabolism. In addition, it appears to be generally true based on immunological studies that the more acidic isozyme has R- or L-like structure, whereas the more basic isozyme has M_1- or M_2-like structure. As anticipated, it was also found that the degree of recognition of the

different pyruvate kinase isozymes by antibodies prepared against human PK-R and PK-M_1 decreased with increasing evolutionary distance from humans.

A very interesting exception to this last general conclusion was found in the isozymes of pyruvate kinase from E. coli. The second isozyme from the DEAE-cellulose chromatography of the E. coli supernatant showed a line of identity with anti-human PK-R and anti-human PK-M_1, indicating that the antisera were recognizing the same determinants on the E. coli isozyme. Because the divergence of eucaryotes and procaryotes occurred 1-2 billion years ago, this suggests an extreme conservatism in the evolution of pyruvate kinase isozymes, or the convergence of the E. coli isozyme and PK-R and PK-M_1 in humans.

The degradation rate constants for rat liver PK-L and PK-M_2 were found to be 0.382 ± 0.115 and 0.277 ± 0.075 $days^{-1}$, respectively. These correspond to half-lives of 1.8 and 2.5 days. In additional experiments using the human liver isozymes, it was found that PK-M_2 tended to be more sensitive to proteolytic enzymes than PK-L, which does not agree with earlier studies which suggested that there was a negative correlation between the sensitivity to proteolytic enzymes and half-lives. However, in further experiments, it was determined that the degree of proteolytic sensitivity of these

two isozymes was quite dependent on the presence of several metabolic intermediates. This suggests that the half-life of pyruvate kinase may be the result of a complex interaction of numerous metabolic intermediates.

Appendices

1. Preparation of sheep anti-rabbit IgG and rabbit anti-human PK-L

The sheep anti-rabbit IgG serum was a gift from Dr. Marilyn Baltz at the University of Oregon Health Sciences Center and was prepared by injecting 2 mg of DEAE-Sephadex purified rabbit IgG in complete Freund's adjuvant intramuscular into an adult sheep, injecting an additional 2 mg two weeks later, and then by bleeding two weeks after the second injection.

The rabbit anti-human PK-L serum was prepared by Dr. Marvin Rittenberg from purified liver PK-L prepared as described in this thesis. The PK-L in complete Freund's adjuvant was injected into several locations: 0.25 mg was injected into each of the rabbit's rear footpads, 1 mg was injected intramuscular, and 0.5 mg was injected sub-cutaneously. The rabbit was bled one month later.

2. Partial purification of one human kidney pyruvate kinase isozyme

This method was developed by Dr. Robert Bigley at the University of Oregon Health Sciences Center.

To a minced human kidney is added two volumes of cold, distilled, deionized water. The mixture is homogenized in a Waring blender at low speed for 1 min and stirred for 2.5 hrs at 4°. The homogenate is centrifuged at 20,000 x g for 1 hour at 4°, and the supernatant is used in the following steps. All further centrifugation steps are at 20,000 x g for 10 min at 4°.

Solid ammonium sulfate is added to the supernatant to a final concentration of 38%, and the solution is stirred on ice for 30 min. The solution is then centrifuged and the precipitate discarded. Solid ammonium sulfate is added to the supernatant to a final concentration of 45%, and the solution is stirred on ice for 30 min. After centrifuging the precipitate is dissolved in one-half volume of 0.15 M KCl and an equal volume of 0.02 M K_2EDTA. This solution is heated for 10 min at 45° with constant rotation, and then centrifuged. The precipitate is discarded and to the supernatant is added solid ammonium sulfate to a final concentration of 45% while stirring on ice. The suspension is centrifuged and the precipitate is dissolved in one-half volume of ice-cold 0.15 M KCl. The solution is brought to 38% ammonium sulfate by the addition of ice-cold 90% ammonium sulfate. The suspension is centrifuged and to the supernatant is added ice-cold 90% ammonium sulfate to a final concentration of 45%.

After centrifugation the precipitate contains a single pyruvate kinase isozyme with an electrophoretic mobility half-way between PK-L and PK-M_2.

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