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INTERACTIONS OF SMOOTH MUSCLE AND SKELETAL MUSCLE PYRUVATE KINASES WITH CREATINE KINASE

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ABSTRACT

INTERACTIONS OF SMOOTH MUSCLE AND SKELETAL MUSCLE PYRUVATE KINASES WITH CREATINE KINASE

By

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Physical and functional coupling of pyruvate kinase (PK) and creatine kinase (CK) has been previously noted with the skeletal enzymes. An isolation procedure was developed for PK from smooth muscle in order to investigate the coupling in this tissue. The coupling of PK with CK in smooth muscle was investigated in relation to the known coupling in the skeletal muscle.

Kinetic parameters of smooth muscle PK (SMPK) were determined to be similar to those of skeletal PK (SKPK). The Km's for smooth muscle PK were 214 uM ADP and 86 uM PEP. The Km's for skeletal muscle PK were 254 uM ADP and 40 uM PEP. The average Vmax for SKPK was 0.068 U/ug and for SMPK was 0.041 U/ug. Arrhenius plots were linear for both enzymes and the observed activation energy was 61,100 J/mole for SMPK and 56,400 J/mole for SKPK.

A new method for quantitative measuring of coupling between molecules was developed using capillary electrophoresis (CE). Using these methods it was possible to control the degree of coupling independently by either controlling the concentration of the molecules or the electric field. A CE method was developed for running PK and CK. Qualitative measurements of PK-CK coupling were achieved using the CE methods.

Procedures which had been previously used for measuring the coupling between the skeletal enzymes were used and adjusted to measure the coupling between the smooth muscle enzymes. These procedures involved the measurement of absorbance changes by

the enzymes and changes in their ethanol solubility. A new effect of CK to increase the activity of PK was discovered in the course of these experiments.

MMCK is the CK isoform found in skeletal muscle. Smooth muscle has been shown to contain mostly another isoform, BBCK, and some MMCK. The coupling experiments showed that SMPK does not couple with BBCK or MMCK, but that SKPK couples to both. These findings suggest that it may be the isoform of PK that determines PK-CK coupling in different tissues.

Since glycolytic fluxes in skeletal and smooth muscle are different, the differences in SMPK and SKPK coupling may reflect features of glycolytic flux control which are specific to smooth and skeletal muscle. Using the CE coupling methods the electric field required to uncouple molecules can be determined. The electric field required for dissociation is smaller than that found at distances (0-10 nm) from membranes which are several times larger than the diameter of the enzymes. This suggests that the location of the enzymes at specific sites within the cell may effect their coupling properties.

This dissertation is dedicated to the person who is dearest to me,

Kelly.

Your patience, your caring, your work, and most of all your love;
I will always remember when I think of these times.

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LIST OF ABBREVIATIONS

AA	ascorbic acid
AAP	ascorbic acid phosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BBCK	BB isoform of CK
C	degree Celcius
cAMP	cyclic adenosine triphosphate
CDTA	trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
CE	capillary electrophoresis
CK	creatine kinase
CoA	coenzyme A
DMSO	dimethyl sulfoxide
EOF	electroosmotic flow
F-actin	filamentous actin
FRAP	fluorescence recovery after photobleaching
g	force of gravity
kD	kilodalton
Km	Michaelis-Menten constant
LDH	lactate dehydrogenase
Mi-CK	mitochondrial CK
MMCK	MM isoform of CK
NAD+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NE	norepinephrine
NMR	nuclear magnetic resonance
PCr	phosphocreatine
PEP	phosphoenol pyruvate
PK	pyruvate kinase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SKPK	skeletal muscle pyruvate kinase
SMPK	smooth muscle pyruvate kinase
TCA	tricarboxilic acid
TEMED	N,N,N',N'-tetramethylenediamine
U	enzyme activity unit
UTP	uridine triphosphate
GTP	guanosine triphosphate
Vmax	maximum velocity constant of the Michaelis-Menten equation
w/v	weight per volume

INTRODUCTION

The transfer of ATP between SKPK and MMCK was discovered in 1989 using NMR saturation techniques (Dillon and Clark, 1990). Since then many types of functional and physical interaction have been discovered between these two enzymes. These interactions were detected by changes in migration rates on paper chromatography, in absorbance, in solubility, and in activity.

These interactions are important because they connect two areas of metabolism which are closely related: energy production from glycolysis, the PK reaction, and energy buffering, the CK reaction. The PK-CK interaction is the most direct connection between the two. These interactions are also important on a more general level because they are further evidence supporting the current trend in the modern view of metabolism. This modern view attributes greater importance to protein interactions than the classical view of metabolism.

These interactions were studied further in relation to the enzymes of smooth muscle. This was done because smooth muscle has lower energy fluxes than skeletal muscle, and its energy fluxes do no change as rapidly. Many of the features of the modern view of metabolism are best illustrated as ways to tightly control energy fluxes in tissues which have the ability to make rapid and large changes in these fluxes.

The PK-CK interaction is an interaction which can occur while the enzymes are free in solution. Capillary electrophoresis (CE) methods were developed in order to study such interactions. Both PK and CK are difficult to run through capillary tubes but in the course of these investigations a new method for measuring binding

of small molecules was developed. This method had advantages of low sample requirements and quantitative results. More importantly this method yielded new information about the binding of molecules in that the effect of an electric field on the association of free molecules could be determined. This spurred the development of new theories relating to the behavior of moleclues which bind to each other when they are in proximity to membranes.

These new findings are not limited to molecules smaller than proteins as it is possible to show the effect using antibodies. So far the quantitative method is limited by the adherence of the molecules to the capillary. This is a problem with kinases of intermediary metabolism and glycolytic enzymes in general. Despite these problems it was possible to show a qualitative effect with SKPK and MMCK. In addition, absorbance and activity techniques have been used to compare the smooth muscle and skeletal muscle pyruvate and creatine kinases.

BACKGROUND

The classical view of metabolism.

Principles of Michaelis-Menton kinetics.

The classical view of metabolism assumes that most enzymes are free in solution. Michaelian kinetics are the basis for the control of their activity. Although the concentrations of substrates and products are manipulated *in vitro* in order to determine the kinetic parameters of enzymes, namely the Kd and Vmax; the range of concentrations found in the cell is restricted. This is because the total enzyme activity in the cell is adjusted to meet the demands of the pathways to which they belong. If the enzyme is not regulated, the Km of the enzyme is expected to be close to the usual substrate concentration so that the enzyme is responsive to changes in substrate concentration. An enzyme with a hyperbolic concentration curve would also be responsive to changes in substrate concentration when this concentration was much lower than the Km, but there is no benefit to having such a Km. Enzymes which are Michaelian while free in dilute solutions but coupled because of their local cellular environment would tend to have Km's and Vmax's which are not adjusted to the cellular conditions.

Regulatory enzyme kinetics and similarities with the modern view concepts.

Several modifications of this basic pattern are able to increase the efficiency of enzymes: cooperativity, allosteric regulation, and covalent modification. Cooperativity is usually seen in multisubunit enzymes because permanently bound subunits have a direct link to each other. The interaction of freely soluble enzymes with each other is similar in that there is information transferred from one enzyme to another about the activity in that particular enzyme's pathway. In cooperative enzymes both parts are operating in the same metabolic pathway. When enzymes from different pathways interact there is the possibility of coordinated control between pathways. When freely soluble enzymes interact, there is a third possibility. If some metabolites are common between the two pathways, it is then possible to create a link between the two which can act as an additional control site. Like cooperativety this is a way to control metabolic pathways more precisely.

In a sense, cooperativaty is a form of allosteric regulation, while channeling of metabolites has the added feature of direct transfer of metabolites. In positive cooperativity the enzyme is turned into an on-off switch by the sigmoidal dependence of its rate on substrate concentration. Enzymes which channel metabolites in a hand-off manner while they are not permanently bound can have a similar property. In cases where there is no control of channeling, as in tryptophan synthase, there is one multifunctional enzyme with several activities and no intermediary is released. This type of channeling is an efficiency feature but it cannot be controlled. Conversely, separate enzymes which act in a hand-off manner can act as on-off switches if the coupling is itself controlled.

For cooperative enzymes low substrate concentrations cause the rate of catalysis to be low. Unlike enzymes where the rate has a hyperbolic relationship to the substrate concentration, the cooperative enzymes do not increase in rate quickly

as the substrate concentrations increase from these low levels. But around the substrate concentration which causes half maximal activity, the change in rate with respect to the change in substrate concentration--the second derivative of the substrate concentration with respect to time--reaches its highest point. In this case the enzyme may help maintain the substrate close to the substrate concentration at which it causes half maximal activity. This is especially useful for substrates which are used by several pathways but it cannot directly regulate which pathway uses the metabolite. Regulated channeling does have this added control feature.

Two of the roles that the modern view of metabolism assigns to protein interactions is the control of such branch points independently of this mechanism. Substrates that have two competing roles in that they are intermediates for several pathways are often regulators of key enzymes (Newsholme, 1981, p. 70). In a similar way, the interactions that these enzymes have with enzymes of other pathways may coordinate parallel pathways that have a common metabolite. The sigmoid behavior is a response to this problem: a 10% to 90% change in activity on a hyperbolic activity curve would require an 80 fold increase in substrate concentration while the same change in activity can be achieved with a 4 fold increase in substrate concentration by a sigmoid activity curve (Newsholme, 1981, p. 70-71). This makes it possible for the substrates to remain in the concentration range needed by various pathways while allowing them to perform their regulatory function. Channeling through coupled enzymes would also be useful in performing this dual role; channeling may have a different function in enzymes which operate far from equilibrium.

Allosteric regulation is also involved in the regulation of enzymes by molecules binding to sites other than the catalytic center. In this case activation by molecules earlier in the pathway or inhibition by molecules later in the pathway results in the control of the enzyme to match the availability of substrate or need of

product. A well studied example is the binding of fructose 2,6-bisphosphate to phosphofructokinase. Fructose 2,6-bisphosphate is formed from fructose 6-phosphate early in glycolysis. When it binds to phosphofructokinase it causes a conformational change and a resulting shift in the activity curve of phosphofructokinase with respect to fructose 6-phosphate. The curve becomes hyperbolic thus increasing the activity. These effectors can also work across pathways as coordinating signals. The best example, ATP, is used by almost every pathway and is also an allosteric effector in several pathways. It is allosteric on PK and phosphofructokinase in glycolysis, on isocitrate dehydrogenase in the TCA, on dihydroorotase in pyrimidine biosynthesis, on cholesterol monooxygenase in steroid biosynthesis, on myo-inositol oxygenase in inositol metabolism, and others. The skeletal PK is not effected in this way so channeling in this tissue is probably not related to whole cell ATP control.

The need for tight regulation and quick response times comes into evidence in several well characterized systems. Some pathways maintain elaborate series of enzymes whose function is to create a signal amplification, to balance the rate of competing pathways, or to decrease the response time of the pathway. A good example which acts in all three capacities is the signaling pathway involving epinephrine, cAMP, glycogen synthase, and glycogen phosphorylase. More evidence for the importance of tight regulation and quick response times comes from the existence of substrate cycles, sometimes called futile cycles. In these systems enzymes in opposing pathways catalyze reactions which reverse each other's effect on the metabolites in question while using up a form of energy such as ATP. The product of one reaction is the substrate for the next and the product of the second reaction is the substrate for the initial reaction. While one pathway is on there is a low level of activation in the opposing pathway continually using energy. An example is the continual phosphorylation and hydrolysis of fructose 6-phosphate.

Reciprocal allosteric control tends to make one pathway predominant but isotope labeling has shown that during gluconeogenesis fructose 6-phosphate is still phosphorylated (Katz and Rognstad, 1976). Such enzyme pairs may help amplify metabolic signals (Newsholme *et al.*, 1984) or decrease the response time of a switch from one metabolic state to another (Newsholme and Start, 1981, pp. 71-73). These two examples are important to the concept of channeling. They show that, although a model of the cell's metabolism can be designed without them, these phenomena are important enough in increasing efficiency that they exist despite their use of energy. In the same way, channeling is not necessary for a coherent model of cell metabolism but its existence is nevertheless present in some systems and should be included in models of cell metabolism.

The value of a mechanism like a futile cycle can be estimated by its energy expenditure at times when it is not in use. Regulated channeling can have the same effect without incurring the maintenance cost.

Multi-functional enzymes and enzyme complexes.

Multi-functional enzymes and stable enzymes complexes give further levels of control but do not alter the classical view significantly. The pyruvate dehydrogenase complex is a large complex made of 60 peptides and three different species of prosthetic groups. In four steps it catalyzes the conversion of pyruvate into acetyl-CoA and all intermediates are tightly bound to the enzyme complex. In such a case it would be impossible to regulate the individual subunits without effecting the rate of the entire complex. Clearly, multi-functional enzymes and enzyme complexes can be treated as single catalytic units. This type of channeling does not let intermediates accumulate in the bulk phase and, as such, it is part of an extended multi-step catalytic mechanism. Another type of channeling occurs in

tryptophan synthase. This enzyme catalyzes two sequential steps in the biosynthesis of tryptophan: cleavage of indole-3-glycerol phosphate--which yields the indole intermediate--and the condensation of indole with serine. During the steady state reaction, it has been impossible to trap the indole and this led to the conclusion that channeling was occurring (Demoss, 1962; Matchett, 1974). The crystal structure was found to contain a 25-Å hydrophobic tunnel connecting the two catalytic sites (Hyde *et al.*, 1988). In 1994 Schlichting *et al.* made a mutant tryptophan synthase with a bulkier amino acid in the tunnel. They showed transient formation of indole and obstruction of the channel by rapid chemical quench methods and x-ray chrystallography, thus confirming the suspected mechanism (Schlichting *et al.*, 1994). In this case it appears that the intermediate has to travel from one site to the next but is still not able to escape into the bulk phase; it is still impossible to regulate either the channeling or the individual steps.

Compartmentation within organelles.

Compartmentation within organelles establishes physical compartments in which classical metabolism operates. This modifies the classical view further since within a cell several pathways will have intermediates that are not available to each others' enzymes, also a putative function of channeling. Organelles also maintain environmental conditions or structural organization which are conducive to certain pathways. For example the mitochondrial matrix maintains a pH and calcium concentration far from the cytoplasm's. Also, the inner mitochondrial membrane is the structural basis for the coupling of oxidative processes in the electron transport system and for the phosphorylation of ADP by providing a hydrogen ion barrier. Finally, organelles help keep intermediates and their enzymes in the same area. The

organelles then are a way of sub-dividing classical metabolic pathways and providing the different structural and environmental necessities of these pathways.

It is interesting to note that functional compartmentation of reductive power has been achieved without organelles by the use of the NADH/NADPH pair. Glycolysis and biosynthesis are able to run at full speed concomitantly even though they are in the same physical compartment. Classical channeling may achieve the same result on a smaller scale but the linking of entire pathways in this manner is unlikely.

Channeling among free enzymes and localization of pathways within areas of a single compartment is intrinsically different from compartmentation in organelles in that it supplies only some of the functions of organelle compartmentation but is able to do it in a dynamic and regulated way: Changes in energy demand don't translocate the TCA cycle to the cytoplasm but phosphofructokinase binding to sub-cellular structures does correlate with changes in energy demand (Luther & Lee, 1986). Dynamic channeling would make it easier to coordinate the regulation of diverse pathways because regulatory enzymes could act as the coordinators while equilibrium enzymes would be affected by their location. Phosphofructokinase may be able to coordinate glycolysis in this way by altering its binding to subcellular structures and changing its activity (Marmillot *et al.*, 1992).

Experiments on isolated enzymes as basis for classical view; its inadequacy.

The classical view of metabolism comes from the approach of most studies. Enzymes are isolated from cells. Their activity is tested against various substrates and effectors. Metabolic pathways are reconstructed from the behavior of the individual enzymes. Finally, models of metabolism in cells are designed. As these models get more complicated it becomes harder to predict the effect of alterations on

aspects of metabolism. Interactions between pathways which are unsuspected are generally not tested and remain undiscovered until a perturbation *in vivo* did not yield the theoretically expected result. The metabolism of *Escherichia coli* is one of the most well characterized, yet overexpression of a few genes was found to effect the expression of many unlinked genes through unexpected alterations in metabolism (Liao *et al.*, 1994). This is the largest problem in the field of genetic engineering where modeling is now being extensively used (Bailey, 1991; Stephanopoulos and Vallino, 1991). These problems are not necessarily due to channeling, but they illustrate how our understanding of how pathways are integrated depends increasingly on empirical evidence as we view more pathways together. This is a prime concern to the field of metabolic engineering because its main concern is the result of altering individual pathways in a system which does contain all the pathways.

Certain phenomena such as metabolite oscillations are difficult to predict. They are not expected from the classical theory unless mathematical modeling is done. Modeling of substrate behavior in one dimensional (Ottaway and Mowbray, 1976, pp. 138-143) or two dimensional space may yield such information (Marmillot *et al.*, 1992), but modeling reactions in three dimensional space is much more difficult and even two dimensional results do not necessarily apply in three dimensions. Even when no new parameters arise the same values may yield different results in three dimensions. Adding a time parameter to non-steady state models renders many of these problems intractable. Sometimes anomalous experimental findings *in vivo* can help clarify the relationship between pathways. This is the case with oscillatory behavior. It is usually not present *in vivo* but can be seen under certain conditions or as oscillatory relaxations after perturbations and can bring new relationships to light (Kohen *et al.*, 1987). Sometimes temporal modeling gives oscillatory results. This has occurred with the pyruvate dehydrogenase

complex (Selvanov *et al.*, 1994) and phosphofructokinase (Termonia and Ross, 1981). Another such phenomenon is the existence of substrate gradients; these have been seen in experiments (Blaedel *et al.*, 1972; Thomas *et al.*, 1972). When modeled these results are generally sensitive to the parameters which are difficult to measure. This sensitivity makes these phenomena of possible use in understanding the regulatory roles of substrates.

PK and CK have simple roles according to the classical view. PK catalyzes the last step of glycolysis and is allosterically regulated. CK catalyzes the exchange of phosphate between ADP and creatine thus maintaining a readily available pool of phosphates for rephosphorylating ADP. But even without dynamic channeling, these roles take on new levels of complexity when we analyze them in terms of entire metabolic systems.

Classical view of PK.

PK is an important enzyme. It controls the flow of three carbon units arising from glycolysis to pyruvate. Pyruvate has several fates. It can be oxidatively decarboxylated to acetyl CoA to supply fuel to the TCA cycle. During anaerobic glycolysis it can be used to replenish the NAD^+ which was used earlier in glycolysis and the lactate can be released into the blood. In one step it can also be converted into alanine and from there to phenylalanine. More generally, glycolysis is the main flux channel during times of acute high energy use. It then produces ATP anaerobically. During prolonged high energy use it supplies the pyruvate which will be converted to acetyl CoA. Glycolysis is also connected to other pathways: It provides intermediates for the biosynthesis of several amino acids; through it's three connections with the pentose phosphate pathway it supplies the right balance of reductive power for biosyntheses and ribose units for purine and pyrimidine

nucleotide synthesis; via dihydroxyacetone phosphate it can yield glycerol 3-phosphate which enters lipid metabolism; it can supply intermediates to maintain the capacity of the TCA cycle via PEP to oxaloacetate; and it is connected to numerous peripheral pathways such as inositol metabolism via glucose 1-phosphate and erythrocyte regulation via 2,3-bisphosphoglycerate. With such a central role in metabolism there is hardly a time when glycolysis is not being closely regulated to perform various conflicting tasks. As an integral part of the ATP production by glycolysis and as a key regulatory enzyme of glycolytic outflow, PK is a critical enzyme.

PK is a tetrameric enzyme with molecular weight approximately 237 kD. There are several named PK isoforms: L in liver, M1 in skeletal muscle, M2 or A or K in visceral organs, fetal, and neoplastic tissues, and R in erythrocytes. Cardenas and Dyson (1973) have reported that the mammalian type L gives three electrophoretically distinguishible isozymes and that type L isozyme can form hybrids with type M from skeletal muscle. L-PK is the most regulated. It is allosterically activated by fructose 1,6-bisphosphate and inhibited by ATP and alanine. It also undergoes cAMP-dependent phosphorylation in response to gluconeogenic hormones. This phosphorylated PK is less active. M1-PK is the least regulated of the PK isozymes and is not regulated by phosphorylation. It is not allosterically affected by fructose 1,6-bisphosphate and is inhibited by phenylalanine more than alanine. Kurganov *et al.* (1985) list fructose 1,6-bisphosphate, citrate in low concentrations, and 6-phosphogluconate as activators; and acetyl CoA as an inhibitor. M2-PK is like L-PK kinetically but is not inhibited by alanine. It has also been shown to undergo phosphorylation in pancreatic islets but this does not appear to have an effect on its activity (MacDonald and Chang, 1985)--a phenomenon known as silent phosphorylation and that has been attributed to various functions including regulation of degradation rates. Baranowska and Baranowski (1977) have

reported that 25% of PK activity from human skeletal muscle can be isolated in an RNA-PK complex and that the RNA may have a regulatory function (1982) although changes in activity due to complexing also occurred with heterologous RNA. Oberfelder *et al.* (1984) showed that the phenylalanine binding causes a change in conformation in M1-PK which is 90% complete by the time 25% of the binding sites are taken up. This was taken as evidence that M1-PK exists in either of two conformational states which can be described by the Monod-Wyman-Changeux model first used to describe the oxyhemoglobin dissociation curve. Conformational change and silent phosphorilation may have implications for the diazymatic interaction or for the activity change which accompanies it.

The mechanism of the PK reaction is thought to be associative (Hasset et al., 1982). This is done in a way analogous to an S_N2 reaction with the nucleophile attacking the anti-bonding orbital of a carbon and forming a single transition state. In the case of phosphate transfer the PEP phosphate would form its transition state by coordinating bonds of three of its oxygens with PK in a plane, with the PEP bonded oxygen also stabilized by PK on one side of the plane, and forming a fifth bond with the ADP molecule on the other side of the plane (Hassett et al., 1982).

Classical view of CK and refinements from experimental results.

CK is also an important enzyme in that it helps maintain the energy charge during short periods of high ATP use. The reaction it catalyzes is near equilibrium under cellular conditions and it is therefore useful as a buffer. The change in ADP concentration due to cellular activity is fractionally much greater than the change in ATP concentration so that rapid rephosphorylation of ADP is able to maintain the ATP concentration near its original value. CK is generally found in tissues that have short and sudden energy demands which cannot be met by the adenylate kinase

reaction or the rephosphorylation of ATP during glycolysis. It is present in all muscle types, in brain, in photoreceptors, and in spermatozoa, but is absent in liver where the energy flux may be high but more continuous (Wallimann *et al.*, 1989). It is a dimer and there are two types of subunits: M, muscle, and B, brain. MM-CK is found in skeletal muscle and heart, BB-CK in the brain and heart, and both in smooth muscle. MB-CK is the predominant form in heart (Van der Veen and Willebrands, 1966) and small amounts have been found in smooth muscle (Clark, 1990). The M and B subunits are conserved; enzymatically active hybrid MB-CK has been obtained by combining subunits from different mammalian species (Perryman *et al.*, 1983). There is a mitochondrial CK (Mi-CK) which has no structural relationship to M-CK or B-CK. It is an integral membrane protein.

The modern view of metabolism, non-specific interactions.

Concentration and diffusion of proteins in the cellular environment.

The protein concentration inside cells is high. It has not been possible to attain the cellular concentrations of some proteins *in vitro*. Substrates generally diffuse 100 times faster than proteins in dilute solutions (Ottaway and Mowbray, 1976, p.137) and proteins diffuse about 10 to 100 times slower in cells than in dilution solutions (Dick, 1963). Ottaway and Mowbray (1976) note that regardless of whether or not the proteins are truly in solution, they act as stationary objects compared to the substrates. Some proteins appear to have different mobilities in different parts of the cell. This may have been the case with aldolase in Swiss 3T3 cells (Pagliaro, 1993).

With the application of fluorescence redistribution after photobleaching (FRAP) diffusion rates of proteins can be measured. This technique involves microinjection of trace amounts of a fluorescent protein into the cytoplasm of a cell. A small area of the cell is monitored and the fluorescent probe in that region is permanently bleached with a laser. The reappearance of fluorescent molecules is measured and a diffusional constant can be calculated. Using this method, Brass *et al.* (1986) showed that the diffusion coefficient of the maltose binding protein in *E. coli* was one thousandth of that measured in water. Using this method Pagliaro (1993) showed that the diffusion coefficient of aldolase--which was shown to bind actin *in vitro*--was higher in the perinuclear area where actin content is lower. This may be due to dynamic interactions on a time scale which cannot be resolved by FRAP. Cellular diffusion of macromolecules may not only be affected by the non-Newtonian properties of the medium; dynamic binding to sub-cellular structures may cause the cytoplasmic matrix to act as a warped diffusional field.

It is thought that the cellular protein concentration is so high that it has a significant effect by water exclusion on the substrate concentrations and on the ionic environment (Ottaway and Mowbray, 1976). Type II water is water whose rotation has been significantly impaired as measured in NMR experiments (Cooke and Kuntz, 1974). Type II water--which surrounds proteins and is structurally more organized by the generally anionic character of most proteins--makes up 20% of cellular water (Ottaway and Mowbray, 1976, p. 140). There is evidence that some cells are able to control this and that this may be one mechanism for cell motility. A high concentration of protein and their adjacent water molecules causes a high degree of organization of water. Not only does this alter the dielectric constant of water, but it makes it difficult to define on the scale of proteins since the electric field around the protein is not homogeneous. This makes it difficult to thermodynamically characterize protein interactions. For example, if the surfaces of

interacting proteins are known, it is still difficult to estimate the equilibrium constant for the interaction because the solvation of the protein is altered and conformational changes result in a transfer of energy to or from the internal structure of the protein. Conformational changes can also lead to changes in the trapped water content of proteins (Colombo *et al.*, 1992). The additional effect of changes in internal water molecules also makes thermodynamic calculations difficult if conformational changes occur.

Water exclusion and true substrate concentration, diffusion of substrates.

If water exclusion is important this could have an effect on substrate concentration. Red blood cell cytoplasm is about 35% w/w hemoglobin which leads to a non-ideal solution where activities of substrates are far from their concentrations (Creighton, 1984, p. 339). Thermodynamic equilibria in these conditions are different from those expected by measuring equilibrium constants in dilute solutions (Creighton, 1984, p. 339). Although the effect on proteins is greater than on substrates, slower diffusion rates of these substrates and higher effective concentrations may lead to an increased localization of substrates. Substrate concentrations *in vivo* are usually lower than their Km's (Brooks and Storey, 1991, p. 372). This may signify that activities are higher than expected in the cell or it may signify that the apparent activity--in terms of pathway fluxes--in the cell is lower because of specific enzyme-enzyme interactions which reduce diffusional distances or decrease the Km's in an allosteric manner. Also, calculations that show that waves of substrate concentration need cells whose size is at least the average size of mammalian cells (Marmillot *et al.*, 1992) may underestimate the effect of decreasing the diffusion rate.

Atkinson (1977) has noted that solvation capacity is a limiting factor and its optimization may be a driving evolutionary principle. Since most substrates have only one fate, channeling at intermediary steps would beneficial (Srere, 1987, p. 93). Although the concept of optimization of solvation capacity as an evolutionary driving force of leaky channeling has been criticized (Cornish-Bowden, 1991), tight channeling may still be used in the intermediate steps of a pathway. Even leaky channeling will be a target of optimization if it has the effect of matching substrate availability with the enzymes' various Km's.

Dynamic complexes in membranes.

It is interesting that the diffusion coefficients of proteins in membranes were found to be larger than expected. Glycophorin, for example, has about 50 times more mass than phospholipids tested but has a diffusion coefficient only half of the phospholipids' (Sackmann, 1983, p. 435, col. 2). This has been attributed to the weak dependence of lateral diffusion on the radius of the particle. The concept of channeling between freely diffusing enzymes in the mitochondrial membrane is well established. The search for super-complexes of glycolytic enzymes has not yielded good evidence and the occurrence of super-complexes in the inner mitochondrial membrane is not resolved. This membrane has conditions which would tend to favor complex formation: 50% of the surface is protein and 35% of the protein in the membrane is involved in a single pathway--the electron transport system. Hackenbrok (1981) has determined that the rate of diffusion of complexes in isolated membranes is fast enough to account for the activity of the enzymes. These experiments have been criticized because of the low viscosity of his system compared to that found in vivo (Srere, 1987).

The modern view of metabolism, specific interactions.

Channeling of substrates by enzymes in solution, the diazymatic mechanism.

Apart from the channeling of substrates within enzyme complexes, there may be many enzymes which channel in a hand-off manner, passing substrates by temporarily binding in solution. This effect would be important if regulation of local concentrations of metabolites occurs in a pathway. Brooks and Storey (1991, p. 373) have noted that direct transfer between glycolytic enzymes is unlikely because of the constraints imposed in lining up large multi-subunit enzymes. But as seen with cytochrome c in two dimensions this may not always be the case. Large molecules have a large number of electrostatic charges which may be involved in approaching each other with the correct alignment and this may offset the problem of their mass. Also the translational diffusion of proteins is probably slow enough compared to their rotational diffusion that when two proteins collide they encounter a larger area than the original collision area. Multi subunit proteins with identical subunits and binding sites facing out also have a larger percentage surface area available for binding at that site than the single subunits. Brooks and Storey also noted that these interactions would be limited to pairs of enzymes which use substrates that undergo cycles such as ATP and ADP or NADH and NAD^+. But all enzymes using these substrates still use other substrates which do not undergo cycles so their argument would limit channeling to a smaller subset where the cycling intermediates are tightly bound unless there is a channeling interaction. They also refute Srivastava and Bernhard's (1988) hypothesis that it is the large off-rate constants of the coupling reactions that make it difficult to see these complexes in dilute solutions by pointing out that the overall flux through the glycolytic pathway cannot be changed by increasing the number of interactions if the overall bound proportion of enzymes

does not change. These two arguments ignore allosteric behavior during protein-protein coupling--a central feature of coupling theory. Although there are not many pure cycling enzymes CK is an exception. CK catalyzes a reaction which acts as a buffering mechanism so it could easily be used with a single other enzyme species. Creatine would never have to leave CK if CK acted as a shuttle.

Channeling enzymes involved in unidirectional substrate flow could not come together productively more often than specified by the product release rate of the first enzyme. One way for channeling with free enzymes to be possible during unidirectional substrate flow is for the first enzyme to have a relatively tightly bound product. This would lengthen the time during which a successful encounter could occur with the second enzyme. If the change in free energy due to the binding of the enzymes was negative when the first enzyme is bound to its product, some of this energy could be transferred to the first enzyme. This could lead to a conformational change which would cause the release of the substrate. The substrate could travel through the inter-protein space to the second enzyme and the free energy drop of its binding there could cause this enzyme to alter its conformation to one less likely to bind the first enzyme. Catalysis would then cause the drop in free energy necessary for release of the final product from the second enzyme. Also the first enzyme would bind another substrate and catalyze its reaction and the free energy drop from that reaction would be conserved in the maintenance of the enzyme product complex. Here the enzymes are ready to go again. The free energy of catalysis of the first enzyme supplied the energy needed for the conformational change which made that enzyme more likely to bind to the second enzyme. This process may be considered an "energy transfer" scheme whereby the activation energy for each step is derived directly from the previous step instead of through a bulk thermal mechanism (Gaertner, 1978).

The "ambiquitous" model and localization of pathways.

Some enzymes bind to subcellular structures and have altered activity when bound. Such enzymes have been called "ambiquitous" (Wilson, 1980). The best example is phosphofructokinase which was shown to bind to F-actin and have an increase in activity of 34% (Luther & Lee, 1986). Other enzymes which have been shown to bind to F-actin are lactate dehydrogenase, phosphoglycerate kinase, and triosephosphateisomerase (Brooks and Storey, 1991, p. 362). Using FRAP, Pagliaro (1993) showed that aldolase had a 20% bound fraction in the perinuclear area of Swiss 3T3 cells while enolase had no bound fraction. This binding disappeared when 2-deoxyglucose was put in the supporting medium. Since this 2-deoxyglucose depletes glycolytic intermediates reaching aldolase, it implicates a dynamic interaction between binding to subcellular structures and catalysis.

The binding of enzymes to subcellular structures also provides a way to impart them with topographic information. This property, without which differential localization of pathways is impossible, is absent in totally free molecules. Localization of substrates may have any of a number of roles. Selective control of substrate availability to various pathways is the main role. The following have also been proposed: dynamic compartmentation of metabolites, signal transmission, efficiency, excitability, metabolic-shift efficacy, and spurious kinetic phenomena which are internally damped (Marmillot *et al.*, 1992, p. 12106, top col. 2).

Metabolic pathway requirements: Matching Km's and isozymes.

Since it may not be valid to average substrate concentrations across the cell, the Km's may yield additional information about particular pathways. One reason for channeling and localization of pathways may be to regulate substrate

concentrations to levels which are optimized for the particular pathway. This would explain the substrate concentrations being generally lower than the Km's (Brooks and Storey, 1991) because it would be necessary for enzymes to be less active when not interacting with a particular pathway. Also, different isoforms of one enzyme might be used to keep substrates tracked to one pathway as opposed to another competing pathway or to control pathways differentially depending on the metabolic status of the cell (Ureta, 1991). One set of experiments have yielded positive results. Cellular micro-injection of anti-hexokinase-C antibody inhibited ^{14}C-glucose incorporation into glycogen without stopping CO_2 output but ^{14}C-glucose-6-phosphate injection reversed the inhibition (Ureta, 1991). It may then be important that smooth muscle contains both MM-CK and BB-CK. And does smooth muscle contain several PK isozymes? Chicken liver has been shown to contain both L-PK and M2-PK (Eigenbrodt and Schoner, 1977) so it appears that there are tissues with several PK isozymes. Also, the silent phosphorylation of M2-PK may be a mechanism by which it is transferred from one pathway to another.

Glycolysis and the modern view of metabolism.

The classical view, glycolysis, and channeling.

Identifying non-equilibrium reactions may be done by measuring the maximal enzyme activity and the mass-action ratios (Newsholme and Start, 1981, p. 96). In analyzing glycolytic enzymes from various tissues it has been noted that the relative activities of hexokinase, phosphofructokinase, aldolase, and enolase are low and these enzymes are thus classified as non-equilibrium enzymes (Newsholme and Start, 1981). However only hexokinase and phosphofructokinase are non-

equilibrium enzymes by their mass-action ratios (Newsholme and Start, 1981). The high mass-action ratios do not suggest that channeling is occurring in these cases. Even if glycolysis operated via channeling in most cases, there are definite cases of uncoupling of specific energy production and energy use pathways, so channeling is not excluded. This was noted in Paul's (1983) work when removal of glucose from the medium didn't cause the contracture usually associated with inhibition of the sodium-potassium ATPase but did result in a decrease in lactate production. It is still difficult to tell when and where coupling is important. All pathways may be coupled and regulated according to demand, or some may be coupled and functioning at a high steady state while the overall uncoupled capacity is only being used at a low level. In the later case, either increased coupling or increased uncoupled flux could occur in times of stress. Enzymatic biochemical analyses *in vitro* tell us that almost all systems are able to function in the uncoupled state. The question of efficiency has been addressed by some in order to determine if the activities measured in these conditions can account for the rates measured *in vivo* and it has been concluded that many *in vitro* rates do not meet this requirement (Srere, 1987). Low relative activities of enzymes that are catalyzing equilibrium reactions suggest that channeling may be occurring. In this case channeling would function as a form of steady state flux enhancement and would maintain reduced transition times of metabolites (Melendez-Hevia and Montero, 1991).

Glycolysis and the ambiquitous model.

Much work has been done on the binding properties of glycolytic enzymes since the first associations with F-actin were studied in 1971. Brooks and Storey (1991) have taken the association constants of the enzymes with each other and with F-actin, and the activity changes reported for these enzymes from twenty-three

separate studies. From these values they designed a model of glycolysis. In this model, they found that a substantial proportion of the enzymes were bound to either F-actin or other enzymes. They also found that the long sought single glycolytic complex did not exist. They found that the binary enzyme complexes increased the flux through some enzymes but decreased it through others. And finally they found that the binding of phosphofructokinase to F-actin could play a significant role in regulating glycolytic flux.

It can be argued that slowly diffusing proteins cannot increase the flux of quickly diffusing metabolites through pathways. Since the rate of catalysis is generally slower than the diffusion rate it has even been argued that channeling within complexes is unnecessary. Several analyses have shown that channeling can in fact increase flux (Welch and Easterby, 1994; Smolen and Keizer, 1990). In the case of unequal distribution of enzymes, it is the spatial distribution of the substrate that is deemed important (Ottaway and Mowbray, 1976) and this was the explanation used for the increase in catalysis seen by Mosbach (Srere *et al.*, 1973) in his experiments with glycolytic enzymes bound to a solid support.

Glycolytic channeling and ambiquity *in vivo*.

Physical coupling was also correlated *in vivo* with functional changes in metabolism. In skeletal muscle, enzyme binding correlated with changes in glycolytic flux brought on by exercise (Clarke *et al.*, 1984; Brooks and Storey, 1988), and in cardiac muscle, binding occurred in response to ischemia (Choate *et al.*, 1985; Stephan *et al.*, 1986).

The most striking example of channeling in glycolysis is R. Paul's work on smooth muscle (1983). He recorded lactate production and oxygen consumption as measures of glycolysis and oxidative phosphorylation respectively. He was

investigating the unusual aerobic glycolysis known to occur in smooth muscle (Paul *et al.*, 1979). When he stopped Na^+-K^+ transport with ouabain, lactate production was inhibited. When he increased potassium influx, lactate production was increased. In both cases oxygen consumption was found to parallel changes in isometric force and ouabain had no affect on oxygen consumption even though isometric force was maintained. It appears that there is functional coupling between the Na^+-K^+ ATPase-pump with glycolysis on one hand; and coupling of oxidative phosphorylation with force development on the other. There have been other reports of coupling of glycolysis with membrane transport of sodium and potassium in erythrocytes (Solomon, 1978) and in ascites tumor cells (Racker, 1976), and of calcium in the sarcoplasmic reticulum (Entman *et al.*, 1976). In smooth muscle, glycolysis was associated with calcium uptake and both PK and CK were found in membrane fractions (Hardin, 1992).

METHODS

Urinary bladder smooth muscle PK isolation.

Introduction.

Two procedures were followed for isolating PK from smooth muscle. The PK used to determine the kinetic parameters was isolated using the first procedure. Subsequently a more rapid second procedure was developed. The PK used in the CE experiments and in the PK-CK interactions experiments was isolated using this second procedure.

Bulk methods for the first procedure.

Early bulk techniques where adapted from Eigenbrodt and Schoner, 1977, and altered in the course of developing a procedure specific for smooth muscle PK. Beef urinary bladders were obtained from Bain's Packing and Refrigeration at the time of slaughter and individually frozen in liquid nitrogen. When not mentioned otherwise, the isolation was done in a cold room at 4 to 7 C and the centrifugation was at 25,000 g for 30 minutes.

The bladders were collected at the time of slaughter. Each bladder was removed with scissors within five minutes of the kill. The fatty tissue and fascia were removed. The bladder was cut open and rinsed in starting buffer cooled with ice. The bladder was then packed in ice. The bladders were in ice for about five hours before the isolation was

started. The urothelium was first removed from the bladders with scissors and the bladders were cut into small strips. The bladder smooth muscle and urothelium were weighed separately and the smooth muscle was put in 1 ml/g-bladder of 100 mM Tris pH 7.25 and stirred for one hour at room temperature. The contents were then transferred to a blender, cooled to 4 C, and homogenized for a total of fifteen minutes. This was done in one minute intervals with the temperature monitored and cooling intervals with ice bags were used as necessary. The contents were then divided into batches and each batch done in sequence. Each batch was divided and centrifuged at 20,000 g for twenty minutes. The precipitate was tested for activity but no re-homogenization was done although as much as 15% of the activity remained in the precipitate. 100 ml of ethanol per 1000 ml of supernatant was added under stirring and the mixture incubated for two hours at 40 C. The denatured protein was removed by centrifugation. One ml of acetone pre-cooled to -20 C per 2 ml of supernatant was added dropwise with stirring. This was followed by centrifugation and lyophilization. The acetone step sometimes resulted in a large fraction of the PK being permanently denatured. Bringing up the precipitated protein in 100 mM imidazole, pH 7.6 yielded little activity even if the mixture was not centrifuged. If the loss of activity was greater than 85% the isolation was restarted.

Bulk methods for the second procedure.

In this case the bladders were put on ice and kept that way until homogenization. The homogenization was carried out as in the first procedure except that 100 mM imidazole pH 7.6 was used. It had been determined earlier that 15% PEG precipitated PK without precipitating most other proteins. Unfortunately most of the activity did not appear in the brought up precipitate. Early experiments with PEG also caused much of the PK brought back up to re-precipitate upon centrifugation. This second centrifugation was necessary to clear the solution before the chromatography steps. By bringing up the

first precipitate to between 2 and 6 mg/ml of total protein and by mixing for several hours at room temperature all the activity was retained.

Samples were taken from the supernatant of the first centrifugation. To each sample was added an equal volume of PEG at a particular % w/v and the samples were centrifuged. The precipitate was brought up with 100 mM imidazole, diammonium citrate, or Tris. The original 100 mM imidazole, pH 7.6, was found to give an increase in specific activity at a final nominal PEG concentration between 12% and 20% with the best increase in specific activity at 12% (figure 1). Tris used at several pH's above 8 and diammonium citrate at pH's 4.0, 4.5, and 5.0 caused much of the activity to remain in the precipitate.

A 30% PEG solution was added in equal volume to the supernatant from the first centrifugation. The PEG had an average molecular weight of 3,350. PEG with a molecular weight of 400 was found to be ineffectual up to a concentration of 60%. The post-PEG mixture was stirred for one hour at room temperature and then centrifuged. The precipitate was brought up using 100 mM imidazole, pH 7.6, and centrifuged a second time. The activity and total protein of the brought up precipitate and the supernatant to the second centrifugation were measured. Pre-cent and post-cent in figure 3 refer to the second post-PEG centrifugation. The supernatant of the second centrifugation was kept and was ready for the chromatography.

Only gel filtration and lyophilization was used to change the buffers for experiments which required a specific buffer because of the high losses of activity occurring during dialysis of small samples. Some of the lost activity could be recovered from the dialysis membranes with a 500 mM NaCl wash.

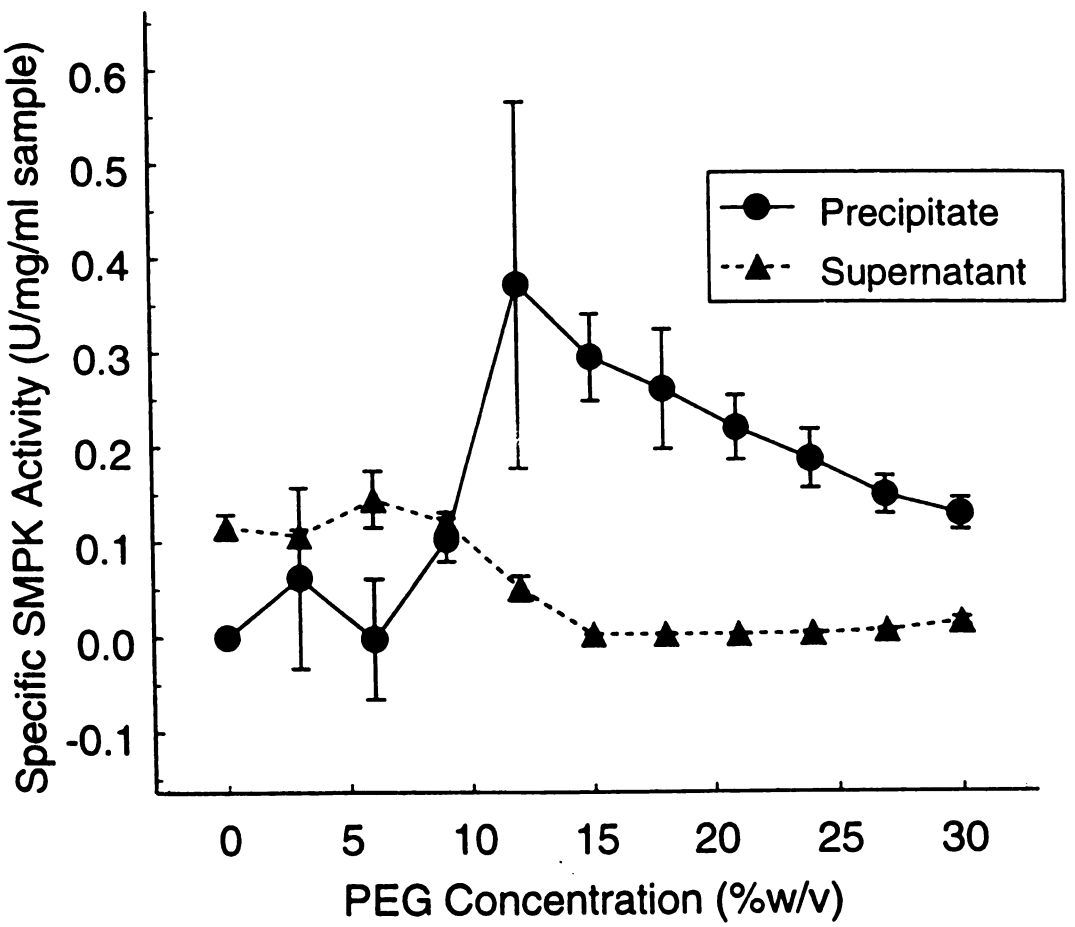

Figure 1. Effect of polyethylene glycol concentration on smooth muscle pyruvate kinase activity.

Preparation for chromatography for the first procedure.

The precipitate was re-dissolved in 400 ml of 50 mM Tris, pH 7.5, and stirred at room temperature for 3 hours. It was then incubated for 40 minutes in 50 C and centrifuged. 10 ml of 1 M $MgCl_2$ and 10 ml of diethyleneglycol was added per 100 ml of supernatant. This mixture was incubated for 2.5 hours at 45 C and centrifuged. The precipitate was then taken up in 100 ml of 10 mM magnesium citrate, pH 8.5, and stirred for 2 hours and centrifuged. The supernatant was then dialyzed for 12 hours against a solution of 5 mM CDTA, 10 mM $MgHPO_4$, 1 mM H_3PO_4, and 1 mM dithiothreotol with the pH maintained at 4.8 with 1 N H_3PO_4. The solution was then centrifuged and the precipitate stirred for 2 hours with 200 ml of 10 mM magnesium citrate, and 40 mM sucrose, pH 8.5. The solution was centrifuged and the supernatant kept. The turbidity in the solution was found to not interfere with the chromatography so no further bulk methods were used.

Column chromatography for the first procedure.

First a hydroxyapatite column was used. Column equilibration was done with 1 mM NaH_2PO_4, pH 6.8. The sample was prepared with the equilibration buffer and glycerol was added to a final concentration of 1%. The proteins were eluted with 5 mM $MgCl_2$, then a 1-500 mM NaH_2PO_4 gradient. PK was eluted with the gradient.

The isolation sample was then loaded onto a carboxy-methyl cation exchange column with loading buffer of 20 mM diammonium citrate, pH 4.8. PK was eluted using a salt gradient of 0-1 M NaCl in 20 mM diammonium citrate, pH 4.8.

Further purification was achieved using a Bio-Rad affi-gel blue gel column. The PK was loaded using 20 mM diammonium citrate, pH 4.8 and only bound partially to the column but one protein peak was found to not bind at all. This second peak eluted early.

This step resulted in very dilute solutions. It was possible to prevent this dilution by using a NaCl gradient (0-1M). After the sample was loaded, 5 ml were allowed to elute and then the gradient was started.

CM-cation exchange experiment (second procedure).

This experiment gave the conditions necessary for the CM-cation exchange chromatography to be consistent. The gel filtration step does not require a particular salt concentration or pH so the conditions determined to cause tight binding of PK to the CM groups were used during the gel filtration step. The gel filtration step tends to dilute the sample and it is placed before the CM step. Since the CM group binds tightly to PK under the appropriate conditions, the CM step can accommodate the large volumes of sample created by the gel filtration step and concentrate the PK upon elution.

Ten 1.5 ml centrifuge tubes were each filled with 200 ul of CM-agarose beads. These were washed repeatedly with 10 mM buffers at pH's evenly distributed between 4.0 and 8.5. When the pH of the collected wash no longer changed the tubes were filled with buffer to a total volume of 1 ml. Then 50 ul of isolation sample was added to each tube. The sample added was from a supernatant of the second centrifugation after the PEG precipitation of PK. Enough sample had to be added to be able to measure the activity of the sample after its dilution into the tube. Because the isolation sample had a higher concentration buffer (100 mM imidazole) the pH in the tubes changed. The new pH was estimated by mixing larger volumes of the appropriate buffers and measuring the new pH. The tubes were shaken and the beads were allowed to settle. The activity was measured and compared to the expected activity if there was no PK binding. Figure 2 shows the pH dependence of PK binding to the CM groups. To determine if the binding caused loss of activity, 20 ul of 1N NaOH was added to the pH 4.7 tube and the activity was re-measured and compared to the expected activity. No loss of activity was expected from

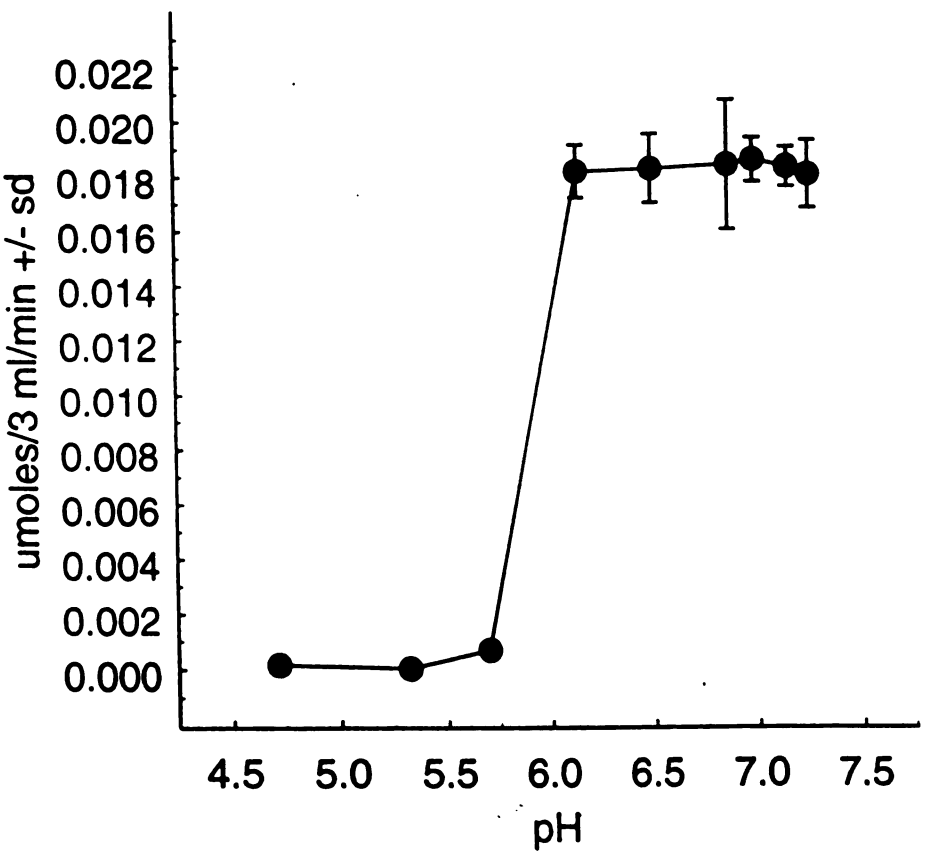

Figure 2. pH - dependence of smooth muscle pyruvate kinase binding to carboxy-methyl coated agarose beads.

adding 1N NaOH because it was added to the supernatant and was diluted before it reached the PK bound on the beads. Figure 3 shows the maintenance of activity in relation to the previous two centrifugations. The post-CM measurements were done after the sample was added to the beads. The first post-CM is for a tube that did not bind PK and shows that there is no loss of activity from the dilution in the new buffer, contact with the beads, or contact with the plastic walls of the tube at this dilution. The second post-CM, marked as post-pH, was the measurement from the pH 4.7 tube after the NaOH was added. It shows that all the activity was recovered.

Column chromatography for the second procedure.

The chromatography for the second procedure, with the exception of the affinity chromatography, was carried out at 25 C. The affinity chromatography was still carried out at 4 C. The post-PEG sample was put through an agarose gel filtration column. In figure 4 the PK can be seen to be at the head of the protein peak and the first three fractions are kept for their high specific activity. The buffer used for elution was 10 mM diammonium citrate, pH 5.3. The second peak in figure 4 was confirmed to be imidazole by running several fractions on the CE. On the CE runs the imidazole peak height matched the level of absorbance in the second peak in figure 4. The eluting PK was then loaded onto a CM-cation exchange column using the same buffer. After two column volumes of buffer have eluted a NaCl gradient is begun (0 to 1 M) and PK elutes at approximately 300 mM NaCl (figure 5).

The PK was then loaded onto a Bio-Rad affi-gel blue gel column using 10 mM diammonium citrate, pH 5.3 and bound partially to the column as in the first procedure but one protein peak was found to not bind at all (figure 6). This second peak eluted early. In this first large peak with no PK activity appears to have several peaks but this is an artifact caused by protein that accumulated on the cuvette walls. Washing the cuvette

tended to drop the absorbance which would then rise again. As in the first procedure, this step resulted in very dilute solutions. It was possible to prevent this dilution by using a NaCl gradient (0-1M). After the sample was loaded, 5 ml were allowed to elute and then the gradient was started. In earlier experiments, the PK peak was found to have a low specific activity shoulder when a higher pH was used. This shoulder disappeared when the gradient was added at the lower pH. A second run at a higher pH also showed a low specific activity shoulder and was used as the final step in the isolation (figure 7).

Analysis methods.

The SDS-PAGE gels showing the major proteins at several steps and comparing them to SKPK and MMCK are discussed in the results section. The gels were 20 cm wide. The resolving gel was about 14 cm tall and the stacking gel was about 2 cm tall. The procedures followed for polymerizing, loading, and running the gels were the Bio-Rad procedures for the Protein ii electrophoresis system. The following conditions were specific to the gels presented here. The polymerization was carried out exactly as the instructions recommended except for the last three gels. Because of the age of the chemicals, these were polymerized with 5% extra TEMED and ammonium persulfate. The thin gels were 0.8 mm thick and run at 13 mA per gel during stacking and 19 mA during resolving. The thick gels were 1.75 mm thick and run at 25 mA during stacking and 40 mA during resolving. All were run under constant current conditions. They were usually stained with coomassie brilliant blue. Some samples did not respond well to lyophilization. Especially when the sample was small, much of the protein adhered to the glass and was lost. After lyophilization, some buffers caused the proteins to clump and were also lost. In these situations, when less protein was seen on the gels than expected, it was possible to silver stain the gels after they had already been stained with coomassie brilliant blue.

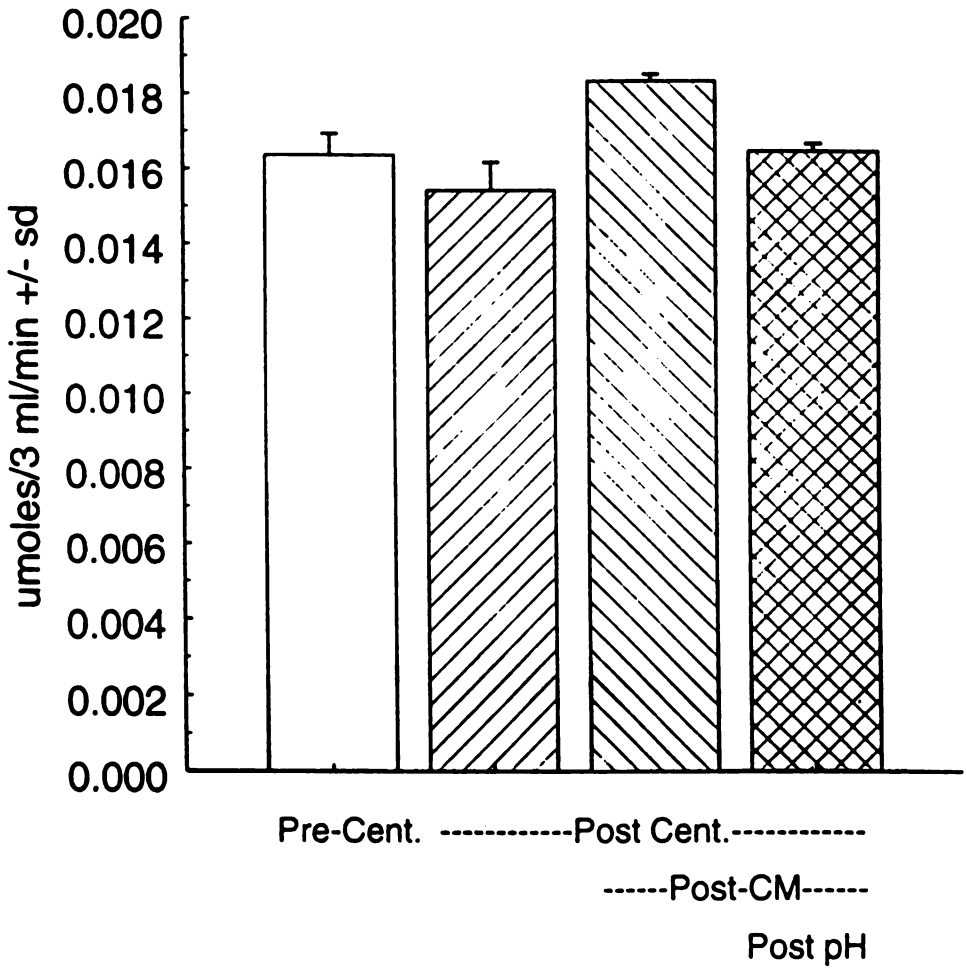

Figure 3. Non-effects of centrifugation, carboxy-methyl binding, and pH on smooth muscle PK activity during SMPK isolation.

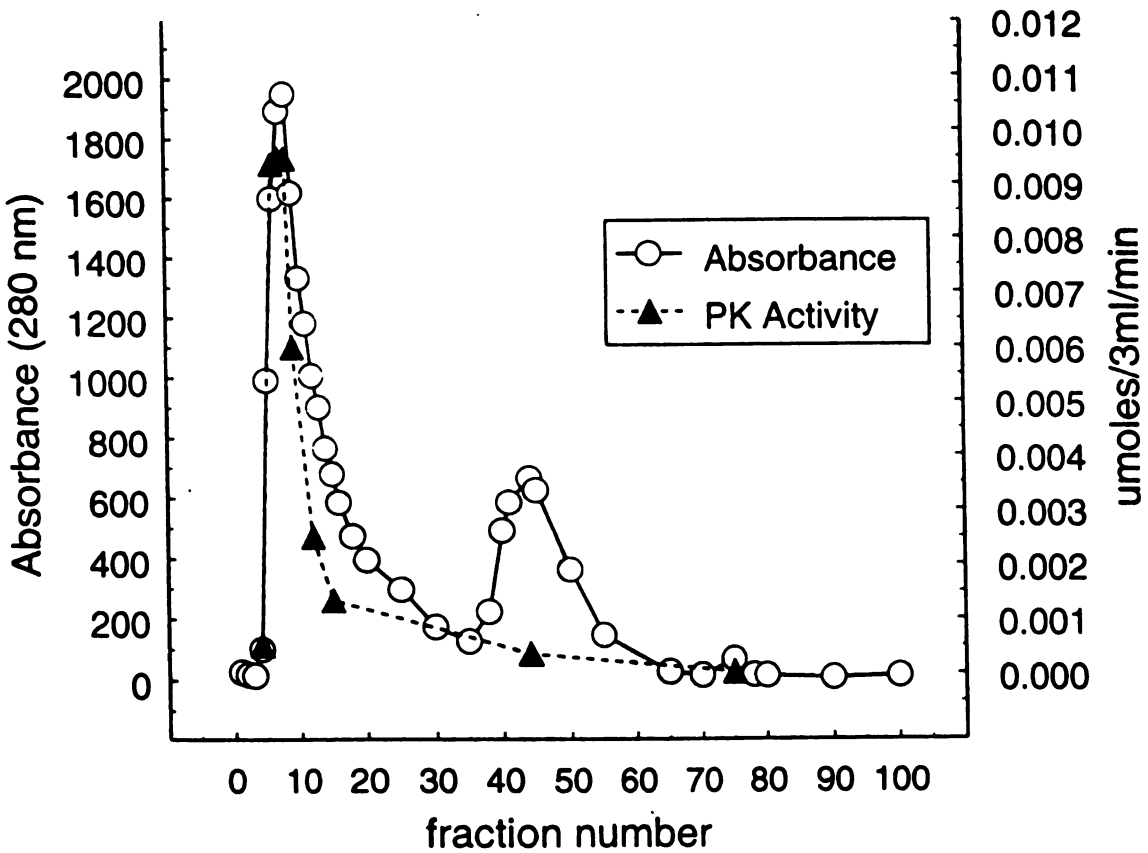

Figure 4. 280 nm Absorbance and smooth muscle PK fractions during agarose bead gel filtration.

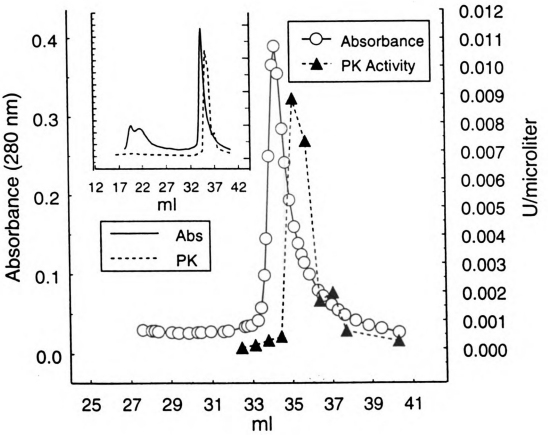

Figure 5. 280 nm Absorbance and smooth muscle PK fractions during elution from carboxy-methyl agarose ion exchange column.

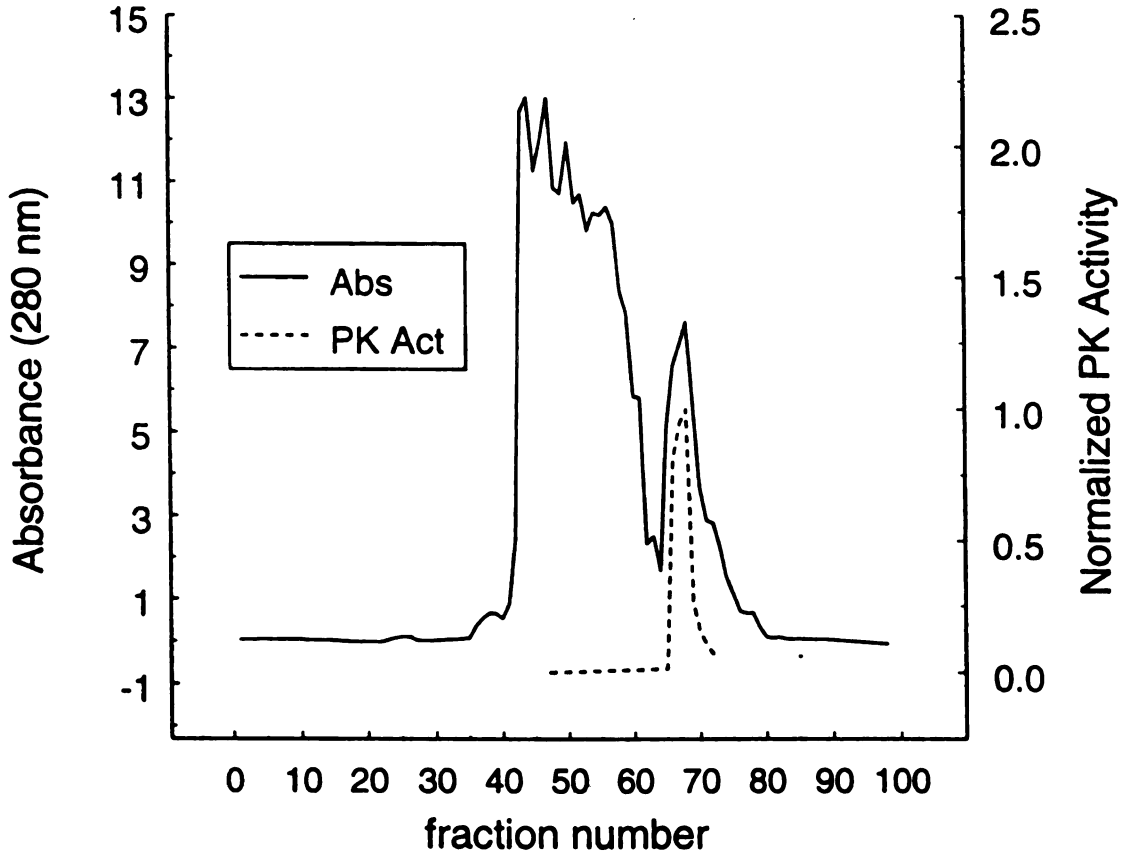

Figure 6. 280 nm Absorbance and smooth muscle PK fractions during elution from Blue Dye - agarose column at pH 6.3.

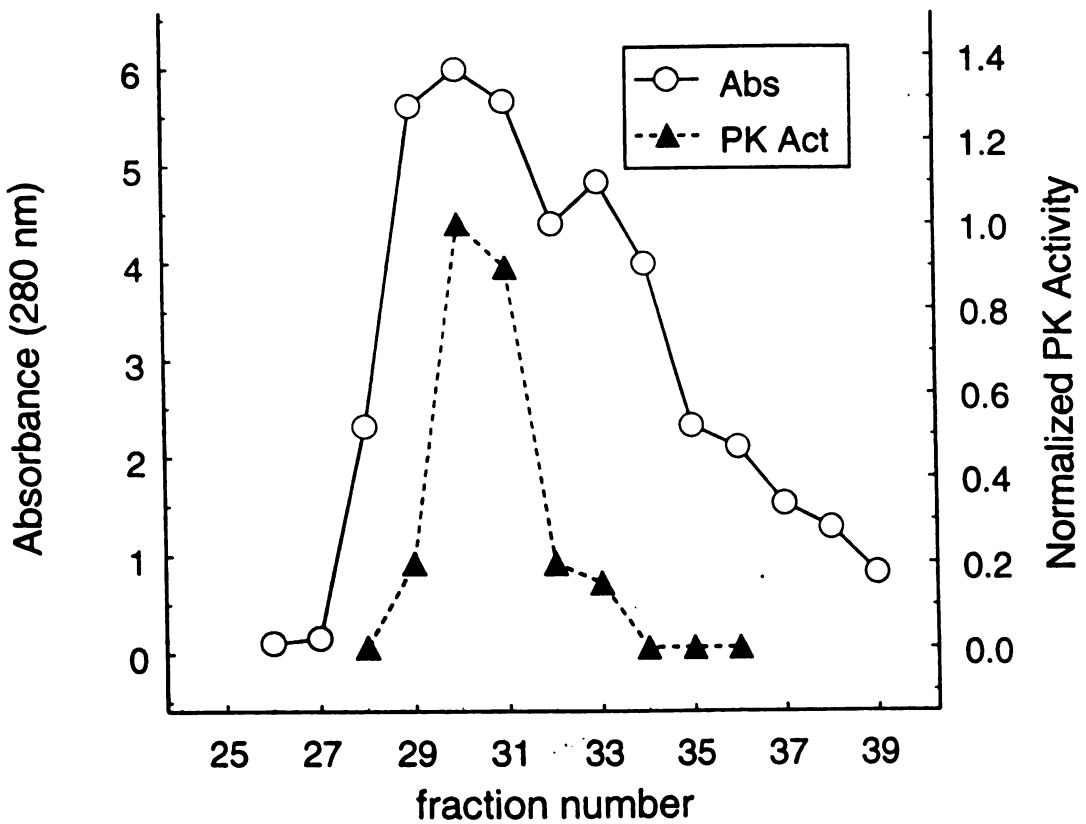

Figure 7. 280 nm Absorbance and smooth muscle PK fractions during elution from Blue Dye - agarose column at pH 7.5.

During the bulk procedures the total protein was measured using the Bio-Rad Bradford and Dc assays. When imidazole was used as the sample buffer during a Dc assay it caused a white turbidity to appear in the tubes. The absorbance increase due to the imidazole when no protein was present was determined. It was found that subtraction of this expected absorbance from the total absorbance of a Dc assay of PK in an imidazole buffer did not yield the absorbance due to the PK alone. For this reason the Dc assay was not used in steps where PK was in imidazole. During the chromatography the total protein was determined using the absorbance at 280 nm and the extinction coefficient of PK (5.4 for 1% solution, 1cm path length). The absorbance at 280 was also used to standardize the protein solutions from skeletal muscle.

Several PK assays were used. All assay were done using 50 mM imidazole pH 7.6, 240 uM NADH, 1.5 mM ADP, 1.5 mM PEP, 120 mM KCl, 62 mM $MgSO_4$, 0.0135 U LDH. For the purpose of comparing the activity to literature values, the enzyme assay was done at 37 C in 3 ml. For the purpose of comparing activity values within an experiment, the assays were done at 25 C in 3 or 1 ml. The activity was measured by following the decrease in absorbance at 340 nm over time as NADH was oxidized. A unit is defined as one u-mole of PEP dephosphorylated per minute in the 3 ml assay. For the 1 ml assays the activity was converted to u-moles/min/3 ml so that the activities would be comparable. The substrate solutions were run on the CE and their quality could be monitored by observing the change in the ratio of the several peaks. For example, the imidazole peak never changed and ran at the EOF. The NADH peak could be compared to the imidazole peak and to the NAD+ peak. Also two small peaks appeared next to the NADH peak and may be inhibitors which are mentioned in the Sigma Chemical Co. information.

All the enzymes apart from the SMPK and all the substrates for enzyme assays and CE standardization with one exception were bought from Sigma Chemical Co. Phenylalanine was bought from Aldrich Chemical Co.

General CE methods used in all sections.

The conditions for all the runs are as follows unless stated otherwise. The capillary had a diameter of 100 um, an overall length of 98 cm, and a length to the window of 66 cm . The background buffer was 25 mM sodium borate, pH 9, for experiments analyzing metabolites from bladders and assay substrates. The injection time was 2 s for the metabolites and 5 s for the proteins. The absorbance range measured was zero to 0.05 and the auto-zero was always done at or below the absorbance range used. The rise time for the absorbance measurement was 3.2 s. The wavelength was 195 nm. The runs were carried out at a constant voltage of 20.0 kV. The current was monitored and was approximately 90 uA. The current was proportionally lower in those experiments where the voltage was reduced to change the electric field.

Bladder metabolites.

Rabbit bladder method.

Figure 8 shows a typical electropherogram of rabbit bladder metabolites. The peaks were identified by spiking the samples with a known amount of one of the metabolites. The peaks identified using this method were creatine, NAD+, lactate, UDP-glucose, NADH, PCr, ADP, ATP, GTP, and UTP. The vehicle shows the effect of the solution that the metabolites are extracted in and the background buffer in the capillary on the absorbance. This includes a buffer peak which runs close to the electro-osmotic flow position and a late peak also seen upon injection with no sample. Figure 9 shows capillary electropherograms spiked with NAD+, UDP-glucose, PCr, GTP, and NADH.

The rabbit metabolite capillary electropherograms were reproducible enough to give quantitative results without calculating the ratio of the metabolite peak to a standard peak. When a standard peak is used it is placed in the sample buffer. Some buffers such as imidazole can be used as a standard peak at most wavelengths. DMSO is a useful standard peak in that it can be controlled independently of the buffer and runs at the EOF position thus making it possible to accurately calculate the electrophoretic mobility of any molecule in the run. Figure 10 shows the peak heights for many of the distinct peaks in figure 9 and their standard deviations. Figure 11 shows that the standard deviation was a constant fraction of mean peak height, about 0.10, and that it was not related to the peak height. Figure 12 shows that the technique was accurate enough to measure differences in bladder metabolites between two rabbits. Four of the peaks marked with an asterisk were significantly different in the two rabbits, while the vast majority were not different.

When doing the CE runs the condition of the capillary may be effected by the sample injection or the prior runs and so a procedure must be established which maintains the runs identical. One effect is shown in figure 13. Note that as the injection duration increases the migration times also increase. At the high injection durations the expected peak height drops. The expected peak height in figure 13 was made the same for each run by dropping the sensitivity as the amount injected was increased, yet the measured peak height decreased noticeably in the last run. This effect is not statistically significant until a 20 sec injection is used (figure 14).

The effect of the previous runs is complex. Sample injection tends to de-condition the capillary wall and thus effect EOF. Figure 15 shows this effect. The abscissa positions eight peaks according to their position during a run with a 2 sec. control injection and following the conditioning of the capillary with 0.1 N NaOH. The ordinate marks the ratio of the migration time of a 2 sec. injection done immediately after a run with and injection time of either 2, 20, or 50 sec. to the 2 sec. control injections. This clearly shows that the run times of compounds with a low overall velocity are much

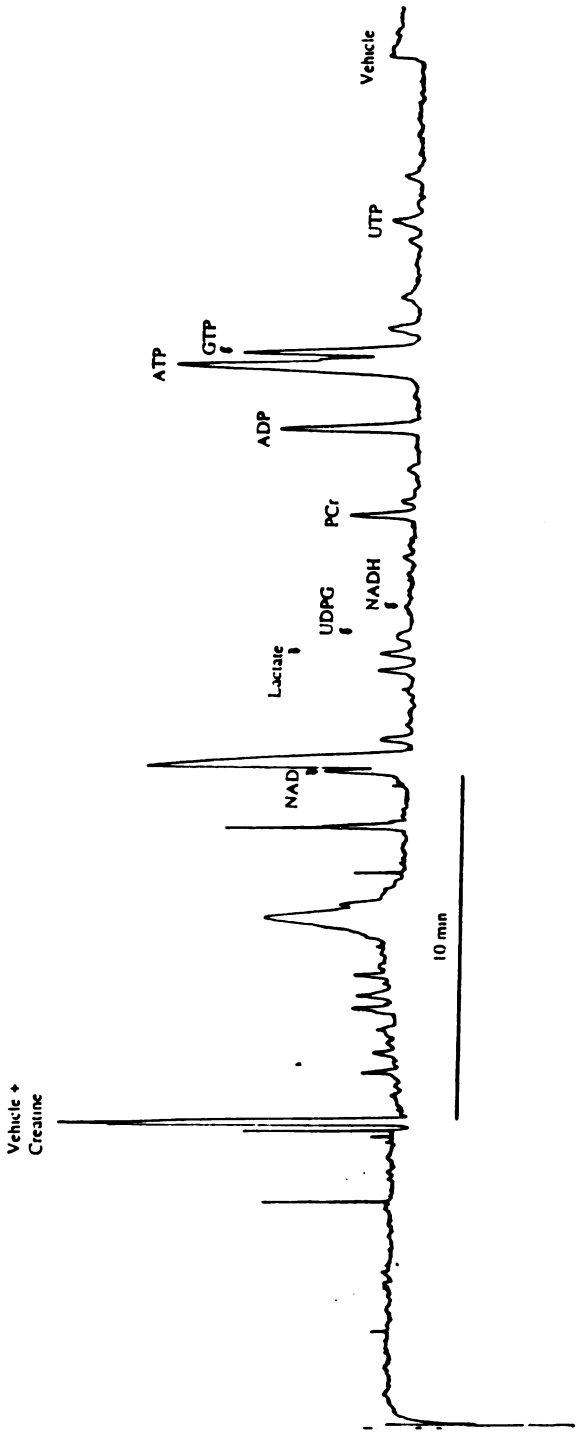

Figure 8. Capillary electropherogram of rabbit bladder metabolites in borate buffer.

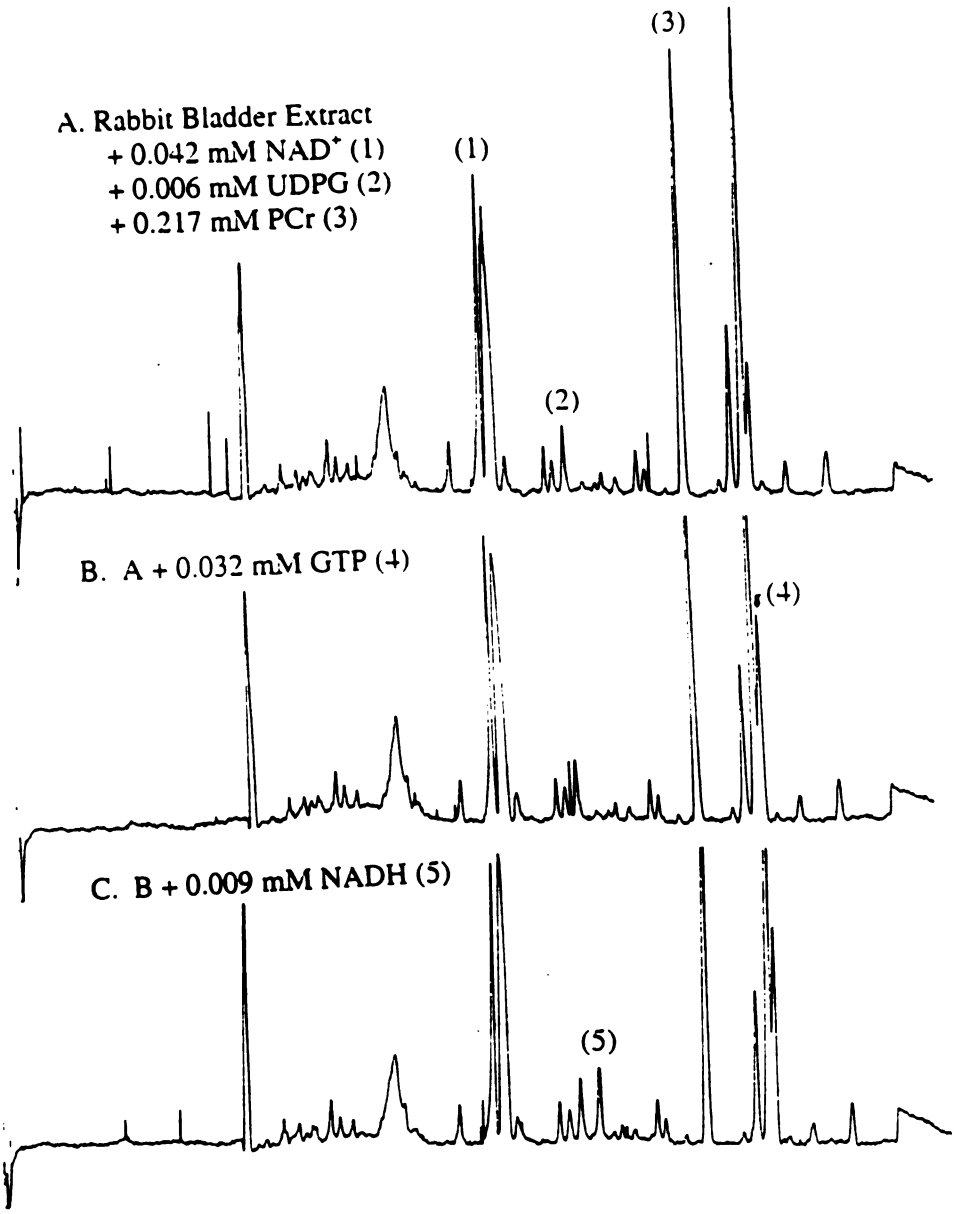

Figure 9. Capillary electropherograms of rabbit bladder metabolites with additions of NAD^+, UDP-glucose, PCr, GTP, and NADH.

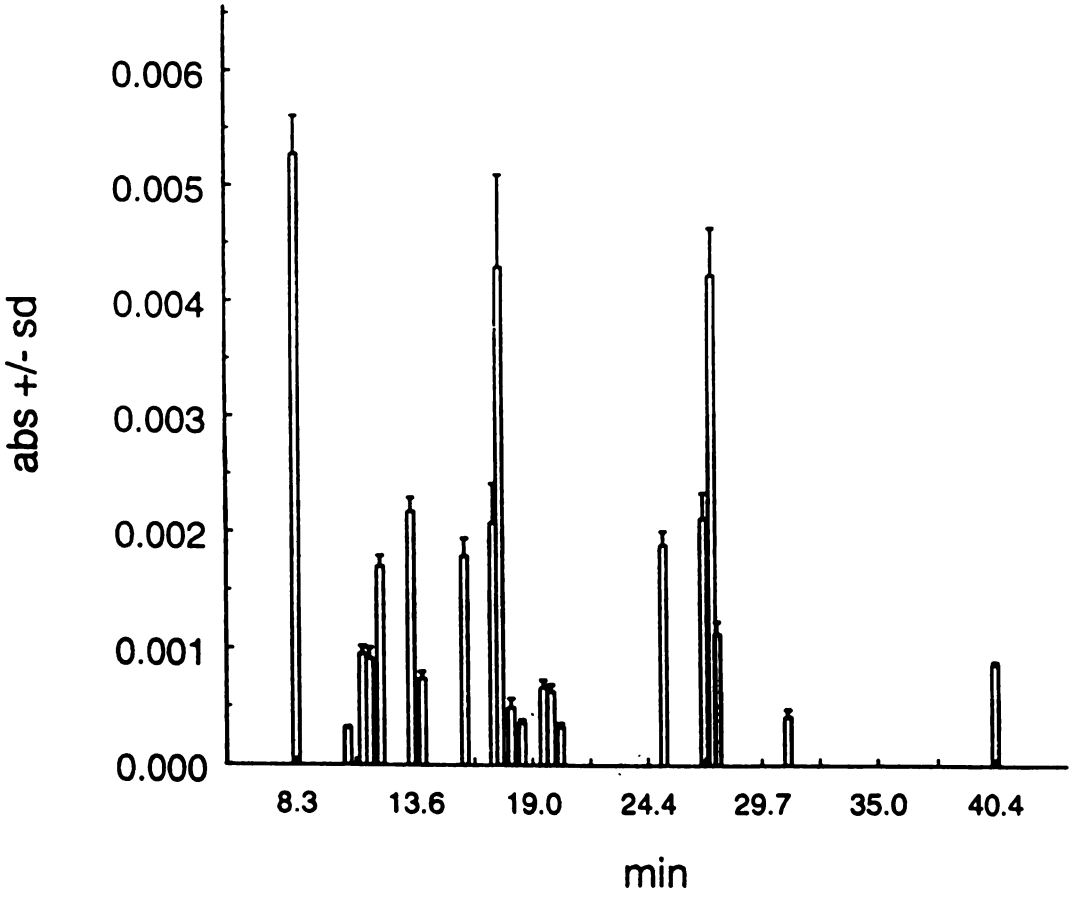

Figure 10. Reproducibility of the same rabbit bladder extract capillary electropherograms.

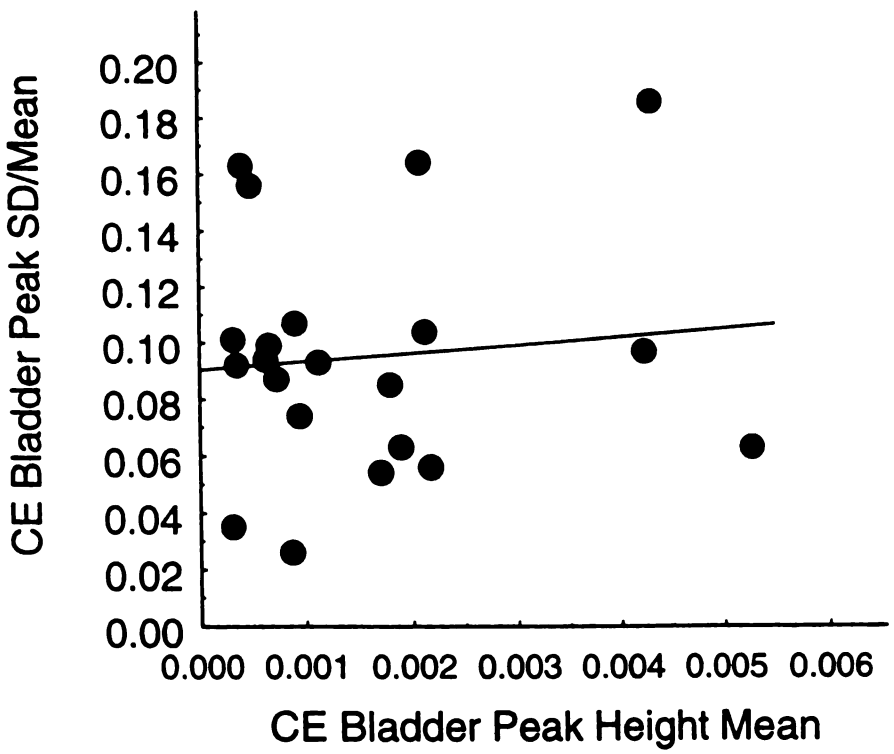

Figure 11. Non-effect of CE peak height on peak height variability.

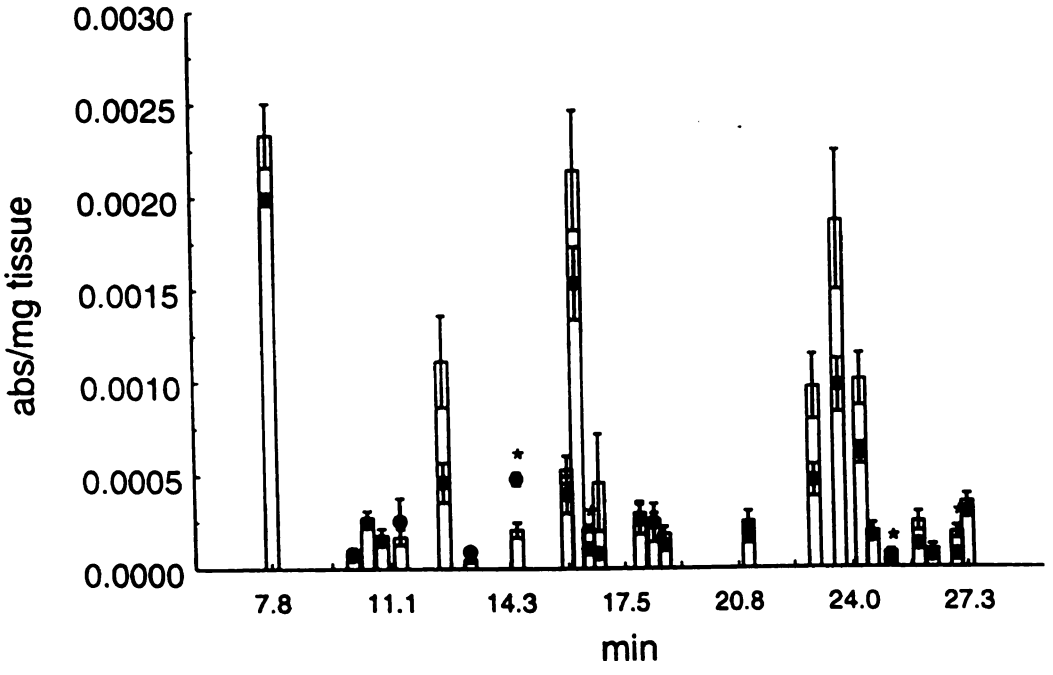

Figure 12. Comparisons of rabbit bladders metabolites from two different rabbit using capillarly electrophoresis.

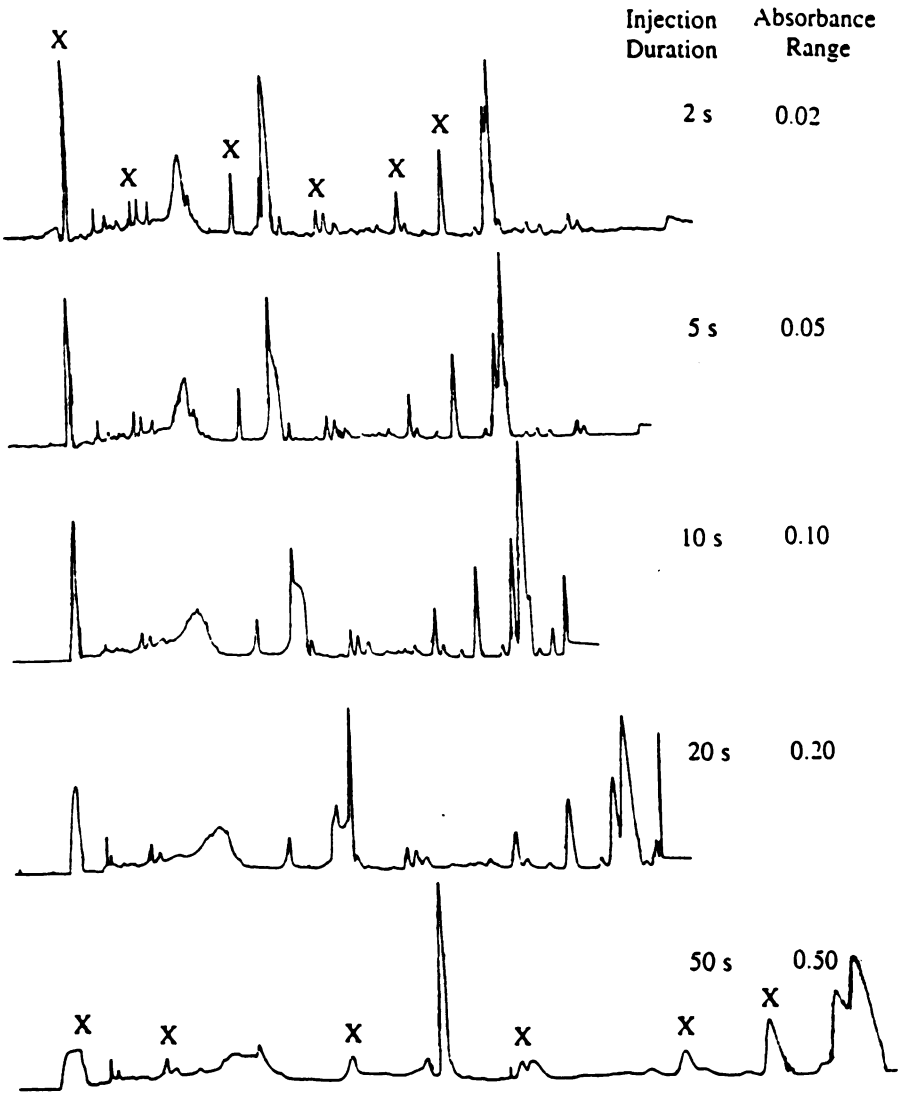

Figure 13. Variation in capillary electropherograms of rabbit bladder metabolites with injection duration (and volume).

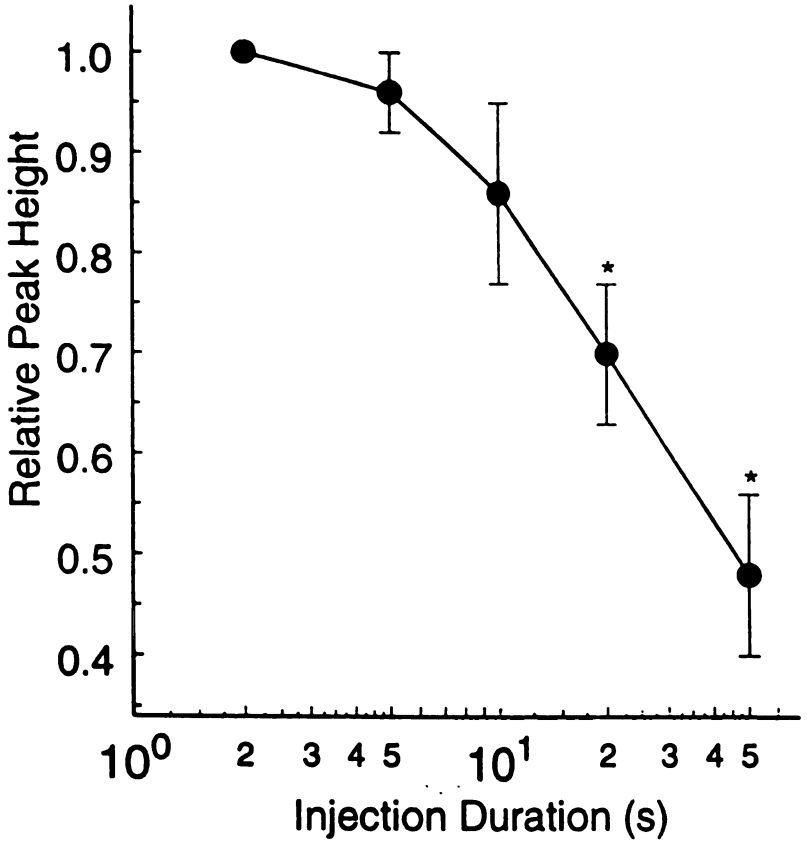

Figure 14. Change in selected rabbit bladder metabolite peak heights as a function of injection duration.

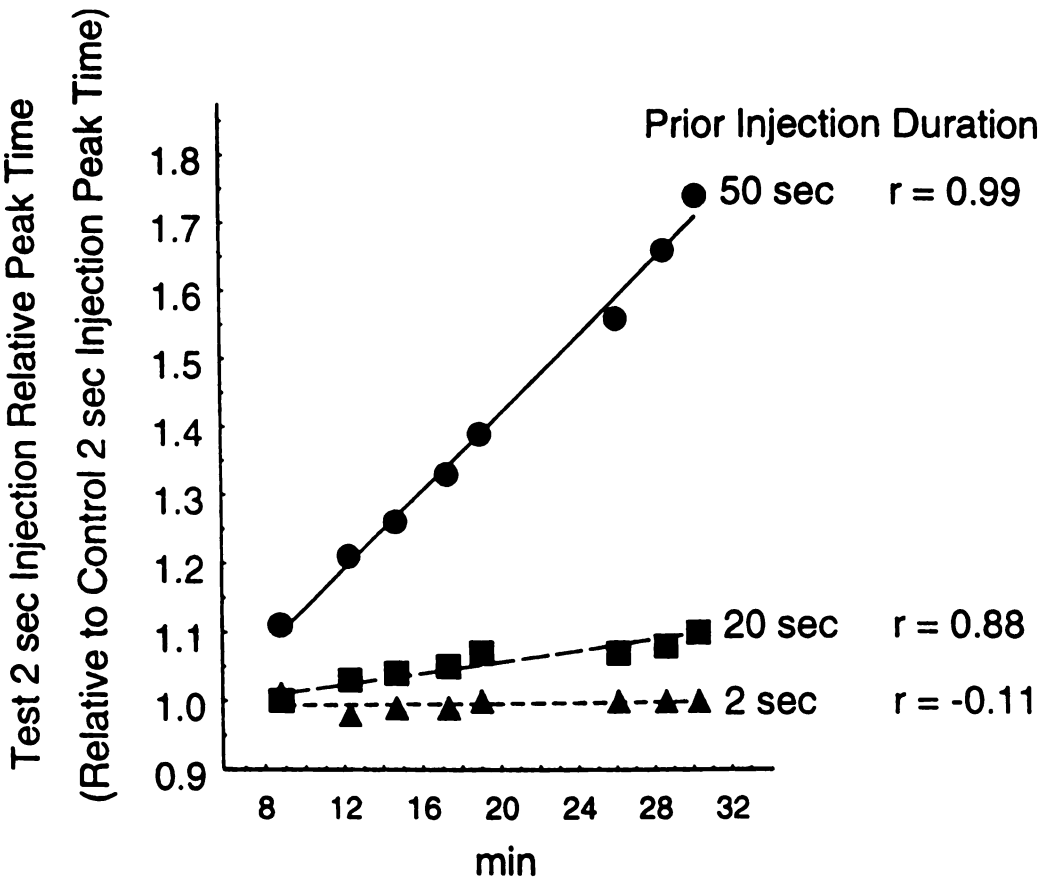

Figure 15. Influence of prior capillary electrophoresis injection duration on a 2-sec test injection of rabbit bladder metabolites.

more effected. These compounds tend to be acidic and therefore are attracted to the positive end of the capillary. This end is also the sample injection end. During the run the background buffer moves toward the negative end of the capillary. These acidic compound show up later because, although they are carried by the background buffer, they are retarded by the electric field acting on their negative charge. Injecting 0.1 N NaOH returned the runs to their normal, higher speed. The acidic compounds appear to remove Na+ from the capillary wall. The Na+ was replenished by the NaOH rinse. During the protein experiments, it was necessary to rinse the capillary with NaOH before every run.

Kinetics.

The assays for measuring Km's were the 3 ml assays done at 37 C. For each substrate concentration three assays were simultaneously run. The substrate being altered, either PEP or ADP, was prepared in solutions so that the volume added to the assay was the same as with a normal activity assay, 100 ul.

For the measurement of the activation energy the time course for the heating of the thermal cuvette holder was first determined for the highest temperature. The SMPK assays were continued until a change in the activity was observed while an assay was running. The buffer was prepared separately for each assay using the temperature dependence of the pH of an imidazole buffer and all the runs were done in one sitting to minimize the effect of a change in observed activity due to a change in the PK solution. The absorbance spectrum of SKPK at 30, 42, 47, and 71 C was used to determine the temperature range for the Ea experiment. Figure 16 shows the spectrum at the first three temperatures. When the assays with SKPK were done the activity did not show a decrease in slope at 44 C. The SMPK assays were prepared for the range up to 75 C. The

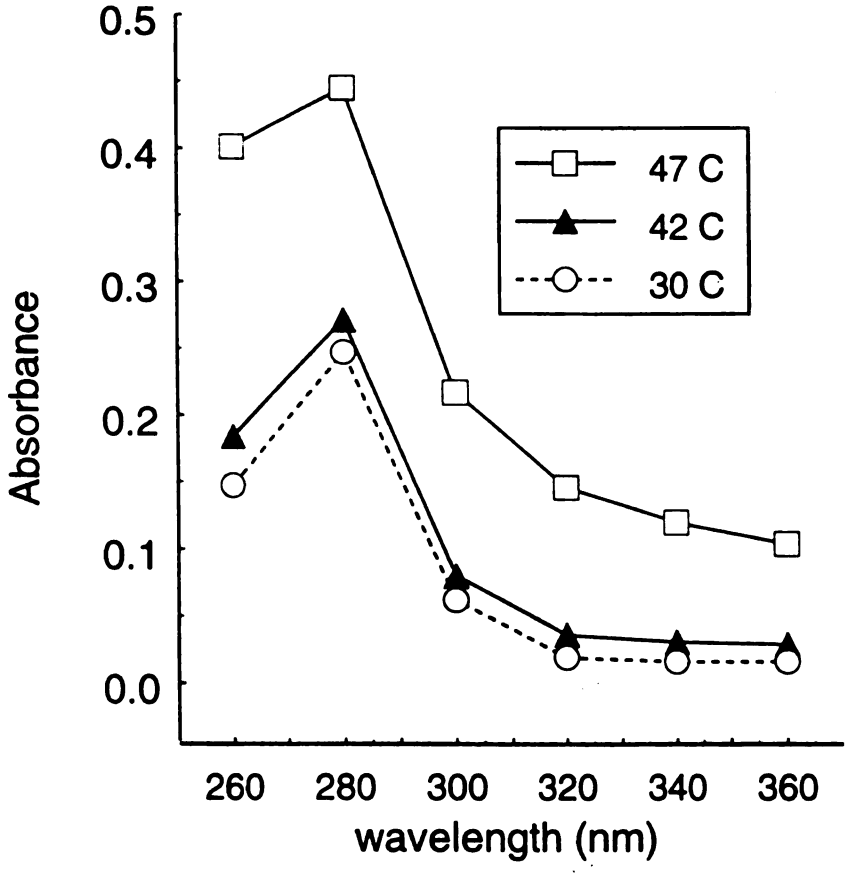

Figure 16. Temperature dependence of skeletal muscle pyruvate kinase absorbance.

substrates were all added without any enzymes and the cuvette was allowed to heat. The final temperature measured during this heating step was the one used in the analysis. Then LDH was added and a baseline activity was determined for that temperature. The baselines were subtracted from the total activity measured. At the highest temperature saturating pyruvate was added to determine the extent of LDH denaturation. The absorbance had decreased to its final value before the spectrophotometer door was closed showing that LDH was not limiting at this high temperature.

Tests with fructose 1,6-bisphosphate and phenylalanine were done using the 1 ml assays at 25 C. The final concentrations were 300 uM for fructose 1,6-bisphosphate and 900 uM for phenylalanine.

CE coupling.

CE methods specific to the coupling experiments.

Norepinephrine (NE) and AA (ascorbic acid) ran widely separated in a borate buffer at pH 7.4 and 9.4. A complex peak appeared at pH 9.4 and ran along with the NE peak at pH 7.4 (figure 17). In the case of NE-AA binding at pH 7.4, both free NE and the complex were in the same peak. A decrease in the observed NE peak could be seen as the AA concentration was dropped. This was the technique later used for the qualitative detection of binding in the kinases. At pH 9.4 the NE and complex peaks were separated and reciprocal changes in the peaks were clearly seen as the AA concentration was dropped. The ratio of the complex to the total NE present was dependent on both the concentration of the AA and the electric field (figure 18). The data points at each electric field were linearized (figure 19), and the parameters of the equation were used to calculate the curves on figure 18. From the linearized plot a Ke could be

calculated (open triangle, figure 19). The Ke represents a dissociation constant of molecules in an particular electric field. When the logarithm of the Ke's is plotted as a function of the electric field a linear relation was seen and an extrapolation to zero electric field gave a dissociation constant, (figure 20). In the case of the NE/AA the dissociation constant was 74 uM AA when NE was 1 mM.

Using the linearization did not depend on using 1 mM NE as it also worked at 0.7 and 0.03 mM NE (figure 21). Using the points from figure 21, the dependence of NE-AA binding on NE concentration was also confirmed (figure 22). This technique was used to detect binding of NE to several other chemicals. Figure 23 shows the effect the difference in binding of AA, AAP, and glucose. Other amines were also detected to bind AA in a similar way (figure 24). Epinephrine had the best binding, Ke 7.9 mM, and tyrosine the worst binding, Ke 27.4 mM, at an electric field of 179 V/cm. Serotonin did not bind to NE. Morphine also bound to NE (figure 25) and a Ke at zero electric field similar to that found for AA could be calculated (figure 26).

PK-CK CE interaction methods.

Angiotensin II antibodies were also shown to run the CE unit and so the technique is appropriate for the investigation of binding in larger molecules. Separate peaks were found for free antibody and antibody bound to Angiotensin II. The kinases were run under conditions that were strictly limited because of the need to prevent adherence to the capillary wall and the need to maintain EOF. For experiments on the coupling of enzymes the buffer was 20 mM Na/PO4, pH 6.25, made up by mixing 20 mM NaH2PO4 and 20 mM Na2HPO4 in order to maintain a low ionic strength. Both CK and PK CE peak heights had a strong pH dependence. PK's pH dependence was the strictest and there was only a small peak at pH 6.3. The experiments were done at 6.3 because it is at this pH that the absorbance effect was found to occur.

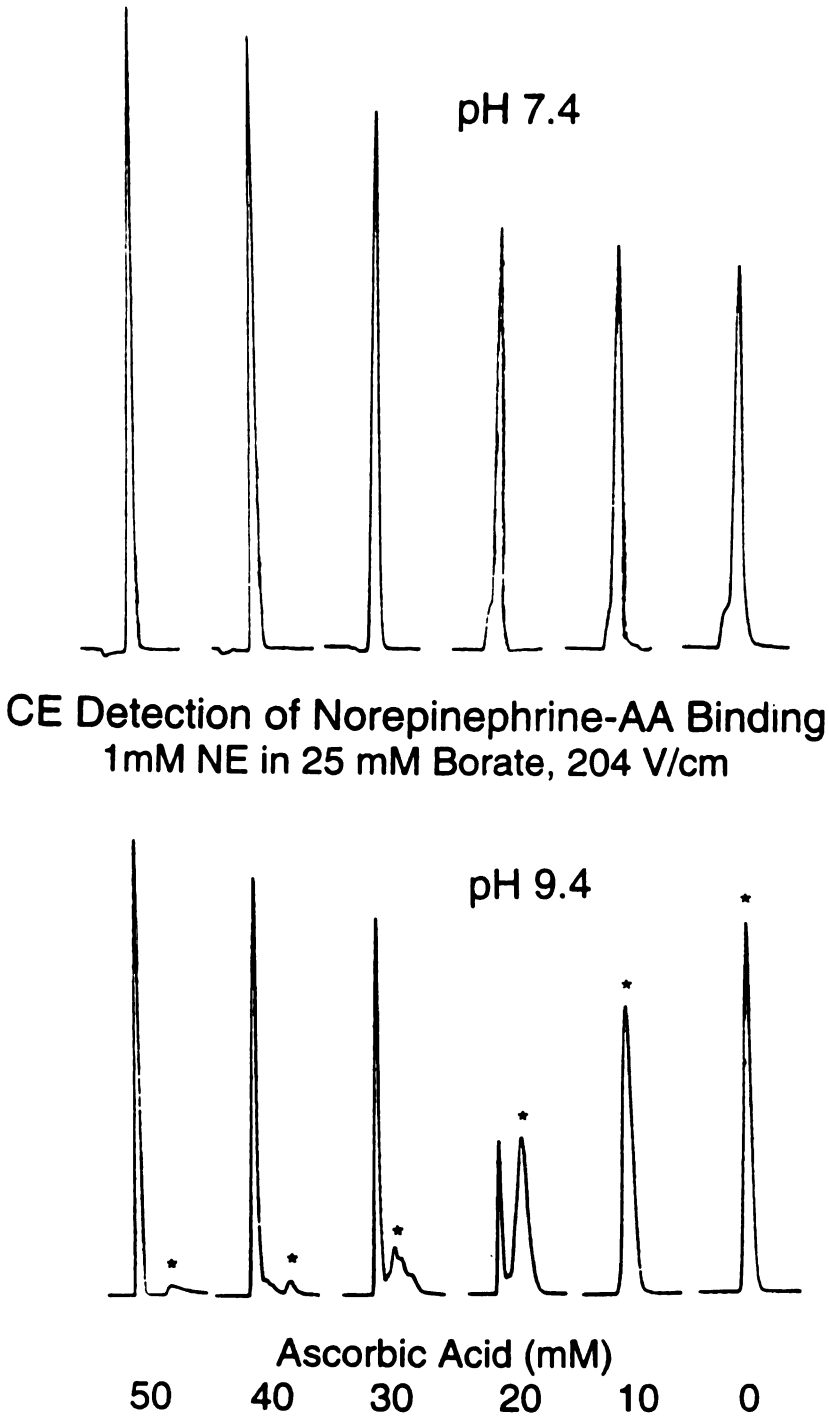

Figure 17. Changes in capillary electropherograms of norepinephrine during ascorbic acid additions in borate buffer at pH 7.4 and 9.4.

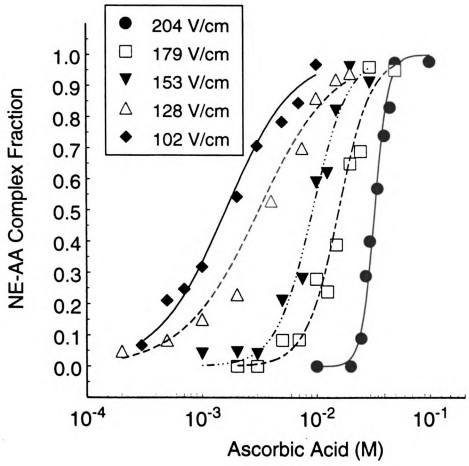

Figure 18. Electric field dependence of norepinephrine-ascorbic acid binding.

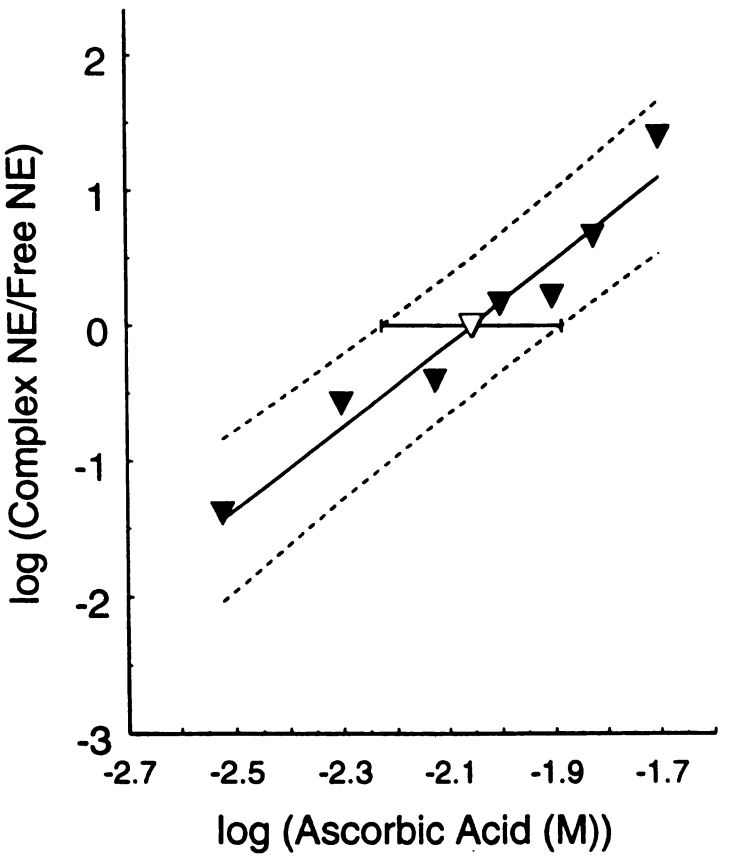

Figure 19. Linearization of NE-AA binding and determination of the binding constant and 95% confidence interval in CE experiments.

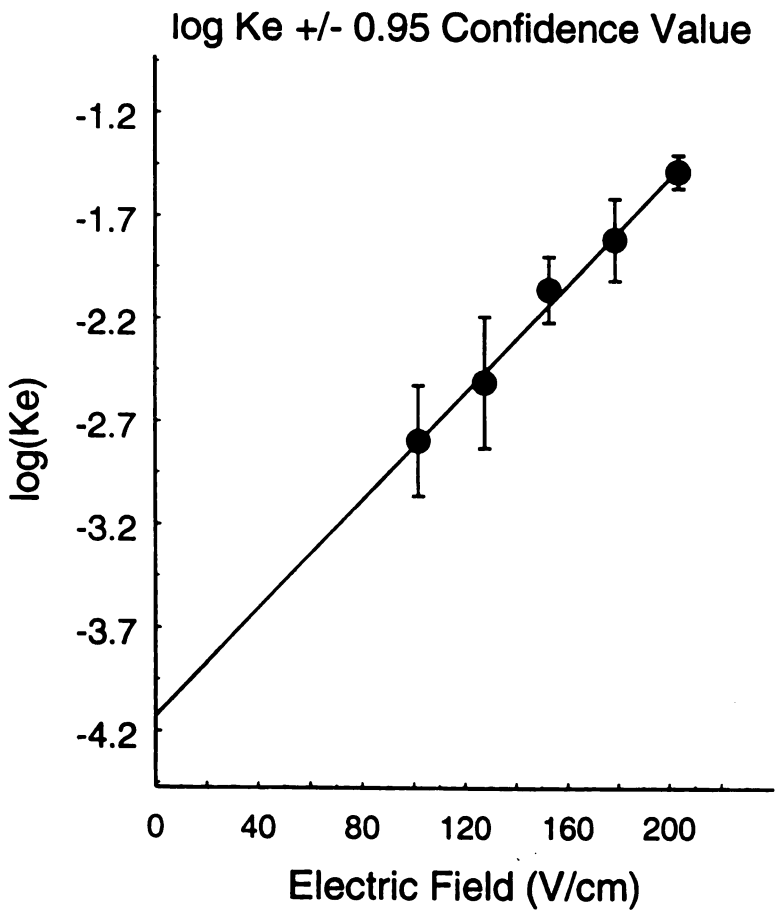

Figure 20. Extrapolation of The NE-AA binding constant to zero electric field from the electric field dependence of NE-AA binding.

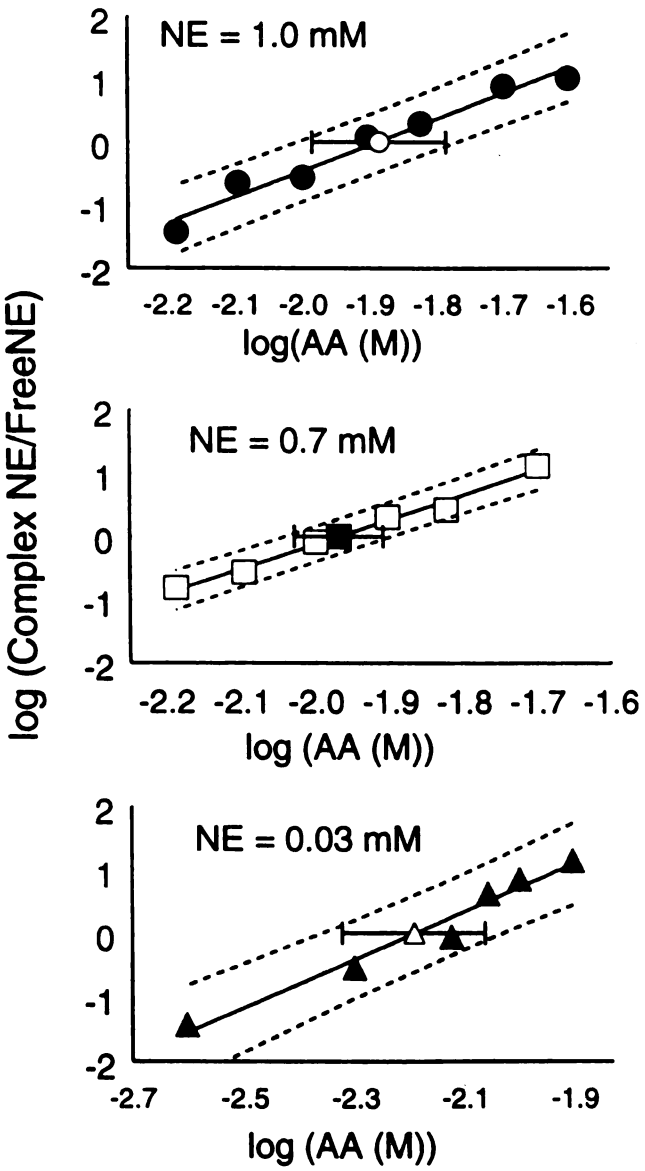

Figure 21. Linearizations of NE-AA binding at different NE concentrations.

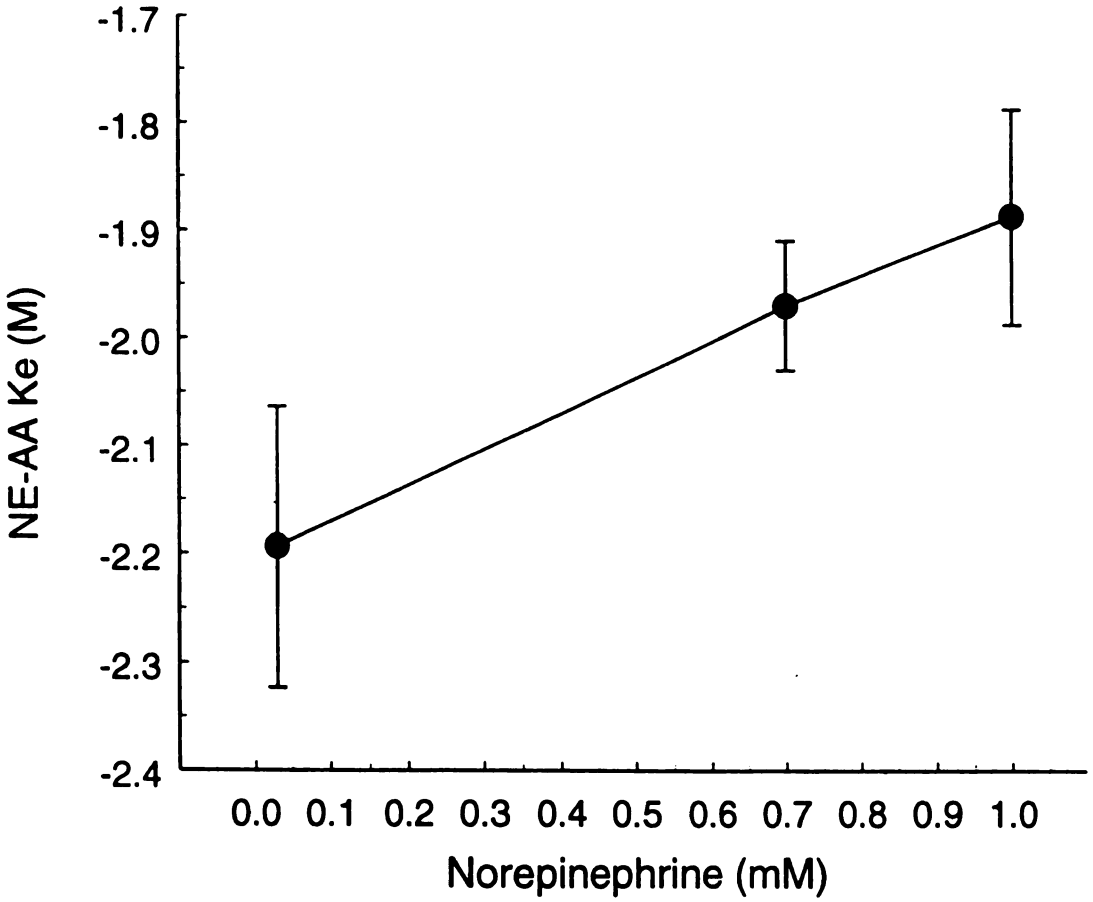

Figure 22. Concentration dependent change (fugacity) of NE-AA binding in an electric field.

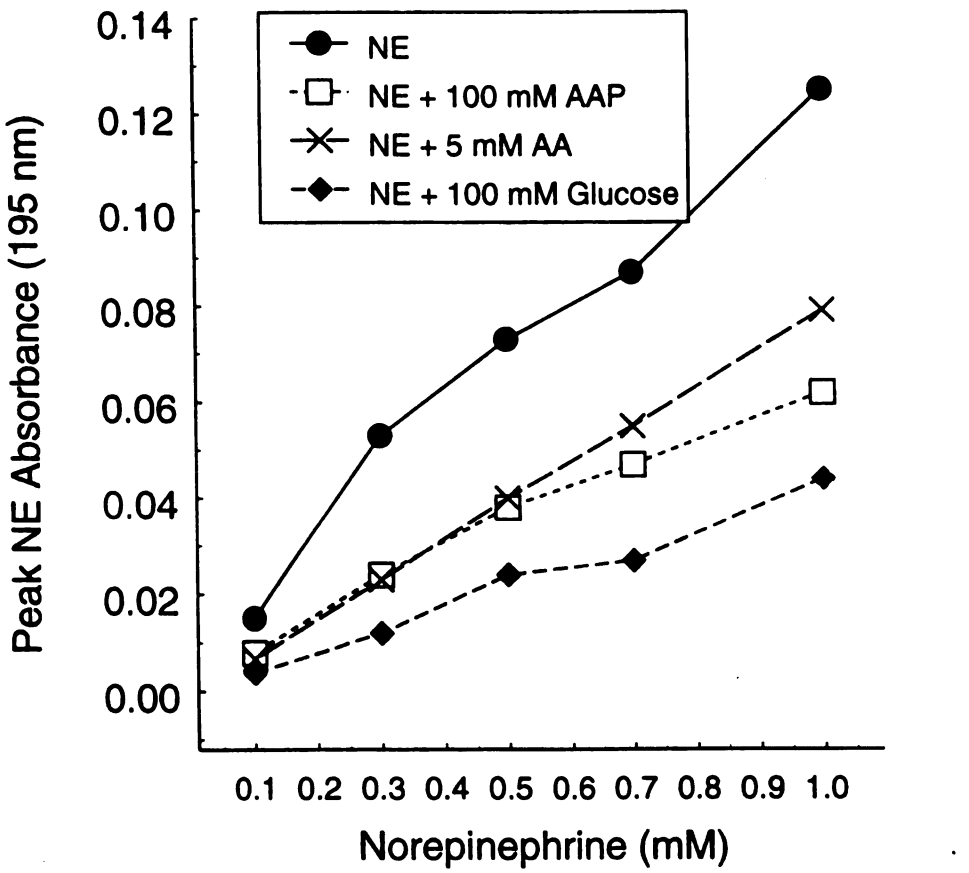

Figure 23. Effect of ascorbic acid - related compounds on CE binding to norepinephrine.

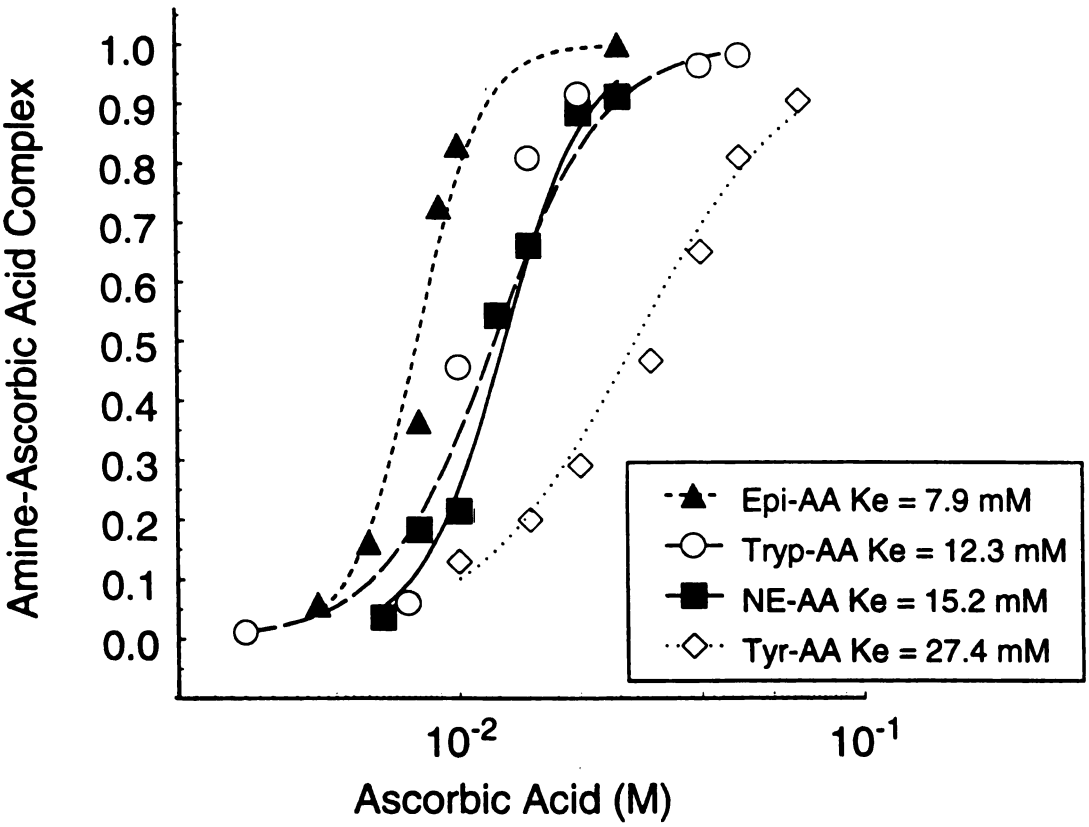

Figure 24. Effect of norepinephrine - related compounds on CE binding to ascorbic acid.

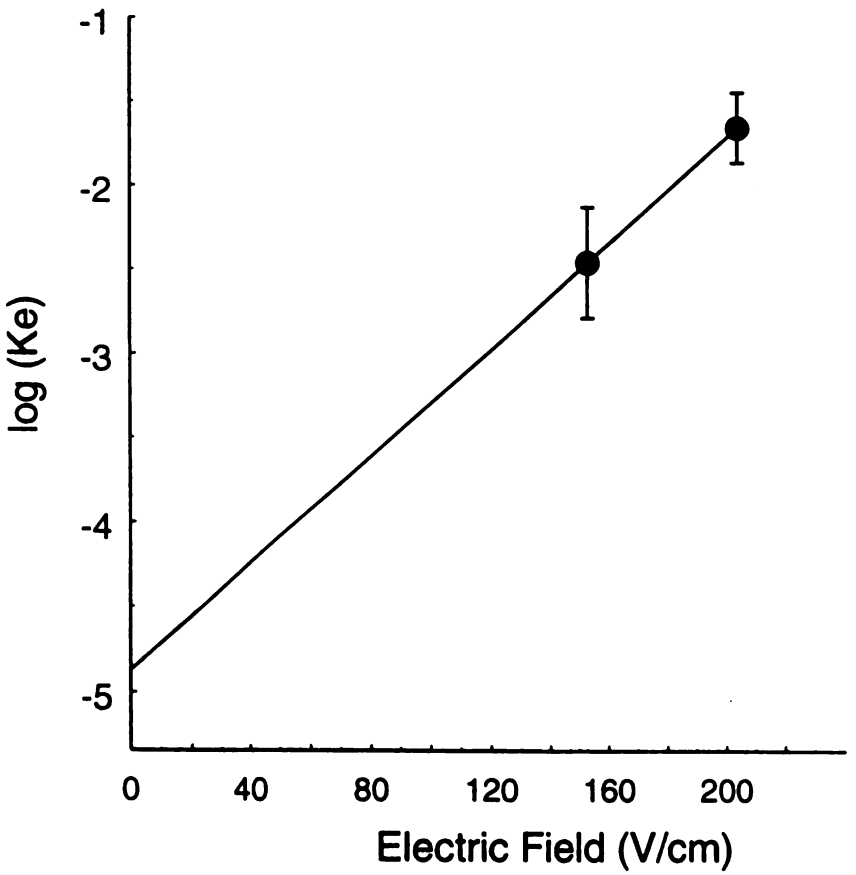

Figure 25. Extrapolation of NE-morphine binding to zero electric field from electric filed dependence of NE-morphine binding.

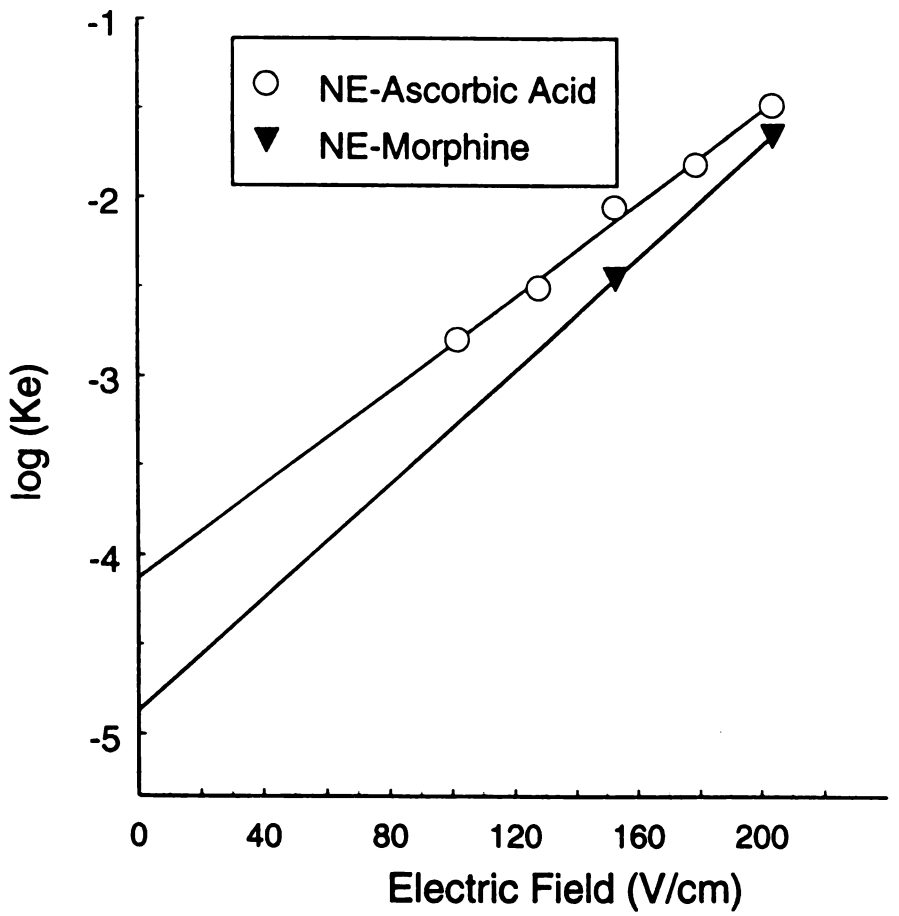

Figure 26. Comparison of NE-AA and NE-morphine extrapolations of CE determined binding constants.

PK-CK interactions.

Sample preparations.

SMPK from the isolation and SKPK made up in an identical buffer were tested against MMCK made up in a buffer that would bring the pH close to 6.3 when mixed in know volumes. When required, the SMPK was filtered into a new buffer and lyophilized. For the absorbance methods the SMPK was in diammonium citrate pH 5.3. When mixed in a 10:9 ratio with CK in 20 mM Na/PO4, pH 6.7, the pH came to 6.25. SKPK was again made up in the same solution as the SMPK.

For the absorbance measurements the enzymes were mixed at 25 C and the solutions were allowed to develop for at least one hour. The development of the solutions in a cuvette at 25 C was followed at 340 nm over an hour (figure 27). When the solution was mixed with a plastic rod the solution only showed a slight drop in absorbance. The absorbance did not rise much for the twenty minutes following this point. A second mixing dropped the absorbance again to the same level. The choice of 340 nm was made in order to compare the experiments to previous ones and because the greatest effect on absorbance was known to occur in two ranges, around 230 to 250 nm and around 310 to 500 nm (figure 28). At the high wavelength end of the spectrum the effect is more difficult to see because of the low absorbance of the enzymes.

The ethanol solubility experiments were conducted by adding an equal volume of a water-ethanol solution to a solution of PK or PK+CK. The solutions were mixed and allowed to stand for 10 minutes. They were then centrifuged at 10,000 g for twenty minutes. The activity in the supernatant was then measured. This method of quantitating the amount of PK in solution was chosen because its sensitivity required the least amount of protein.

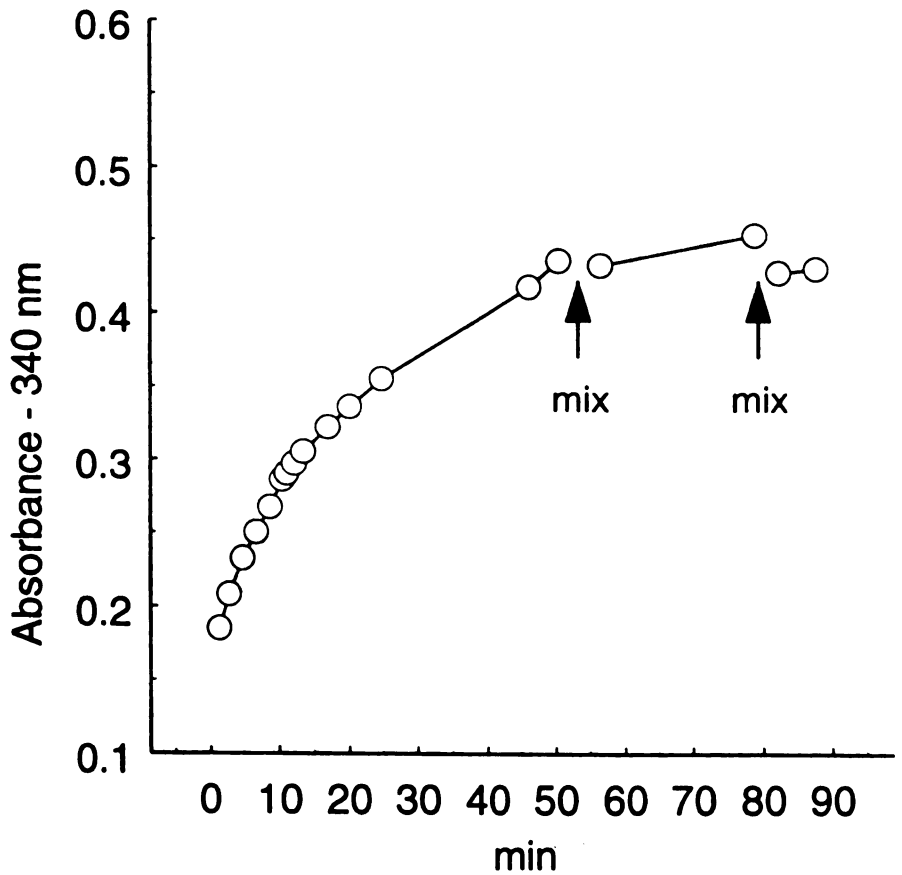

Figure 27. Temporal development of absorbance change of combining MM creatine kinase and skeletal muscle pyruvate kinase.

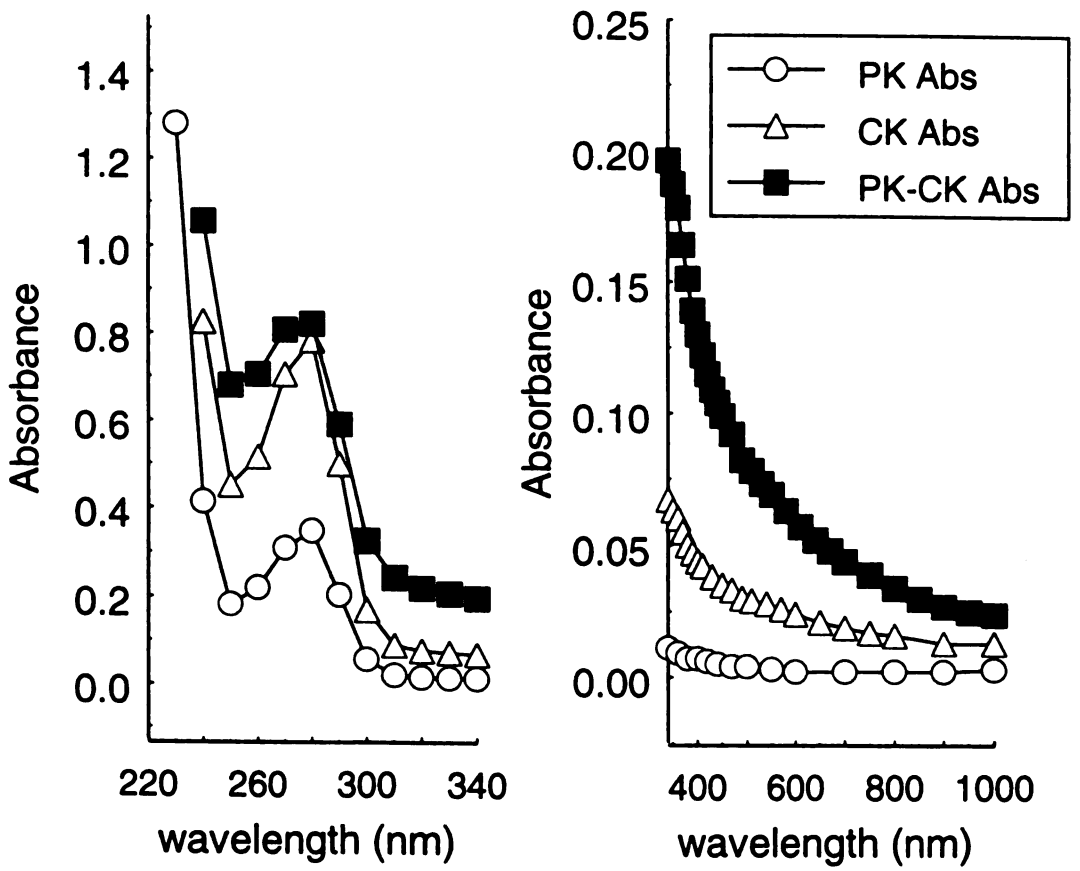

Figure 28. Spectrums of skeletal PK alone, MMCK alone, and a 0.5-0.5 mix of SKPK and MMCK.

The activity measurements were done with several PK and CK concentrations with 0% ethanol. The lowest PK concentration in the original solution was 144 ug/ml while the highest was 2 mg/ml. It was thus discovered that CK had an effect on PK activity that did not require the low Mg++ assays previously used (Dillon, et. al, 1995). These assays were repeated in two ways. First the procedure was repeated with neither a centrifugation nor a development step. Second, an assay was started with PK alone and CK was added halfway through the assay.

RESULTS

Isolation of smooth muscle pyruvate kinase.

PEG (polyethyleneglycol) with an average molecular weight of 3,350 was able to precipitate PK to the exclusion of other proteins. The brought up precipitate showed little loss of activity with a six to eight fold increase in specific activity.

Each step in the second procedure chromatography yielded better than a 5 fold increase in specific activity except for the gel-filtration and the second affinity step. Both of these were still required. The gel filtration was required to prepare the post-PEG sample for the CM chromatography and the blue gel because no other step yielded an increase specific activity at that point in the isolation. From 200 g of bladder, the urothelium was removed and not weighed during this isolation. At the start there was 82.5 g of protein and 6430 U of activity. The fold purification during the best two steps was 7.52 for the PEG step and 5.72 for the CM chromatography. The gel filtration gave the poorest recovery: 48%. The final specific activity was 294 U/mg at 37 C and a total of 1400 U were collected. The last chromatography step was run in 5 steps and the eluent collected had a concentration of 0.160 mg/ml before lyophilization. The SDS-PAGE (figure 29) revealed that after the CM step there was one major protein contaminant with a high molecular weight. At this stage the isolated PK band was still very faint when only coomassie staining was used. The SDS-PAGE showed that when 1.25 mm thick gel was overloaded the sample did contain two minor bands other than PK. These bands only showed up when the gel was silver stained. SKPK from Sigma Chemical Co. also

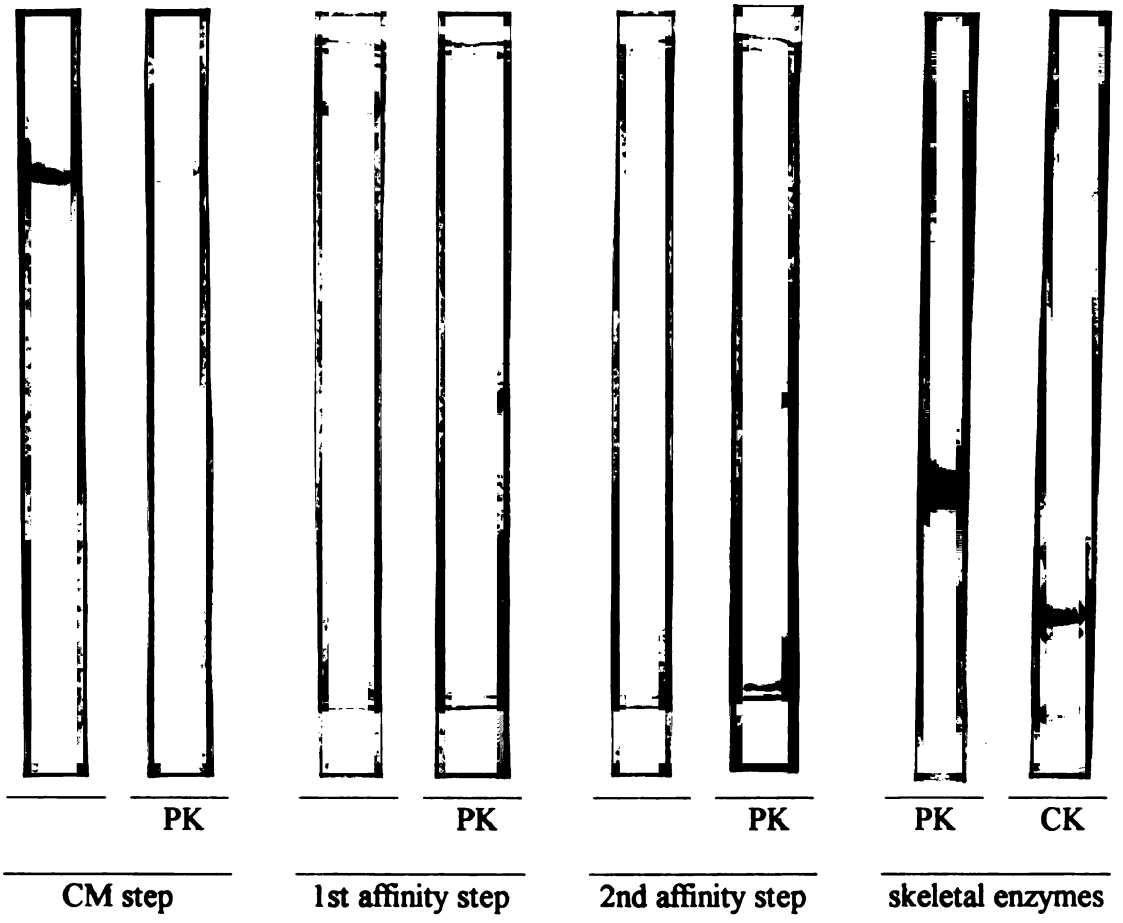

Figure 29. SDS gels of smooth muscle and skeletal muscle pyruvate kinase.

showed extra bands. The isolation of SMPK produced purification similar to that of commercial SKPK.

Each bulk step in the first and second procedures was tested on the homogenate supernatant and every time the CK activity was separated from the PK activity. It was not possible to co-isolate the proteins as a complex. The final isolated PK showed no CK activity.

The first isolation technique was also tried using porcine tissue up to but not including the affinity chromatography. The results were similar in every case except that the ammonium sulfate gave an increase in specific activity only when it was used as the first step in the porcine isolation. In the bovine isolation it was not successful.

Bladder smooth muscle metabolites run on the CE made it possible to assess the effect of the period spent on ice (figure 30). The peaks that were identified by using the technique developed with the rabbit bladders are numbered. In figure 31 the level of the different metabolites is given for five samples taken from five different bladders. Although the PCr was low and the ADP was high, there were still large triphosphate peaks and the lactate level remained low.

Kinetic properties of skeletal and smooth muscle pyruvate kinases.

The Lineweaver-Burk plots for ADP were linear as expected (figures 32 and 33) and the Km's were similar: 254 uM for SKPK and 214 uM for SMPK. When plotted on the same graph by using the specific activity they appeared similar (figure 34). Both samples were lyophilized and gave low specific activities calculated from the Vmax. Figures 35, 36, and 37 show the same graphs for PEP. The Km's are 40 uM for SKPK and 86 uM for SMPK. When phenylalanie, ATP, and fructose 1,6-bisphosphate were

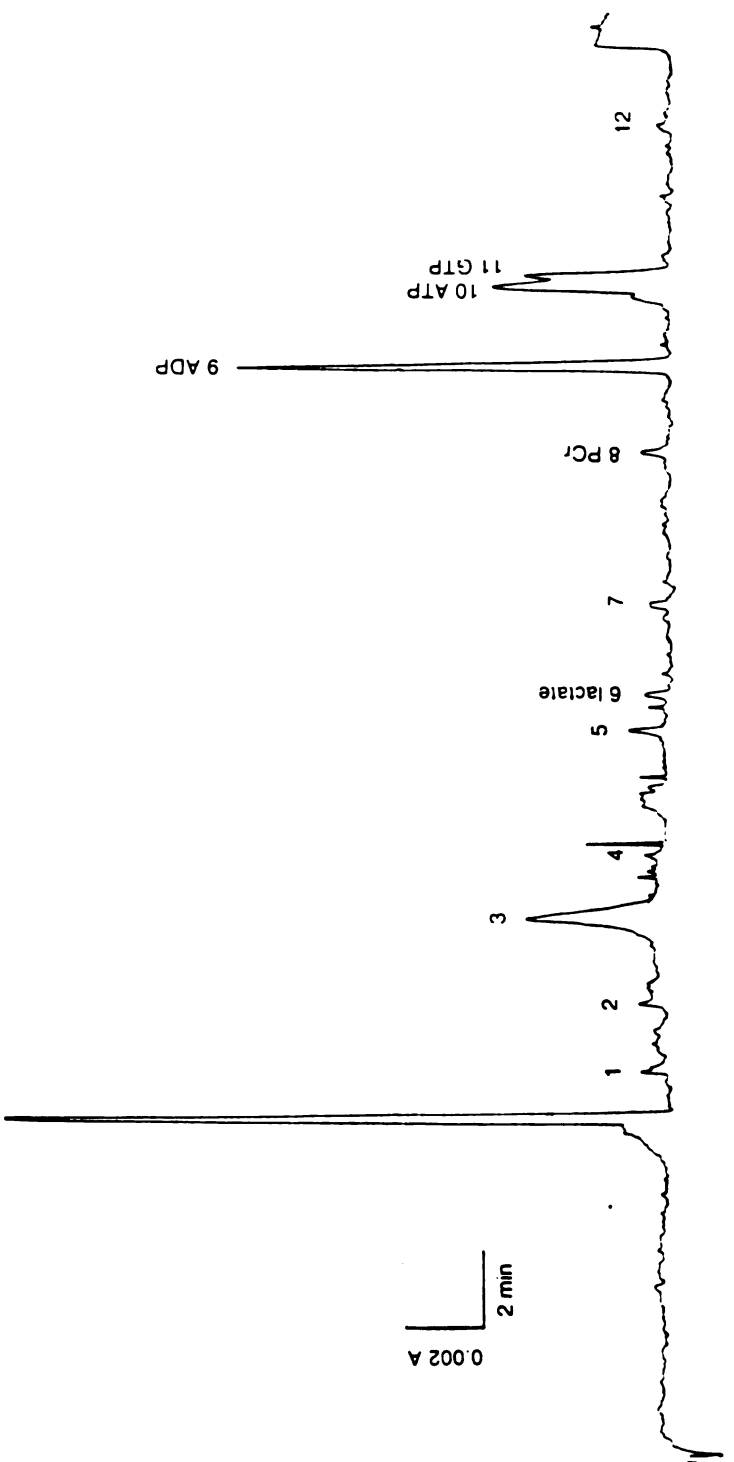

Figure 30. Capillary electropherogram of beef bladder extract.

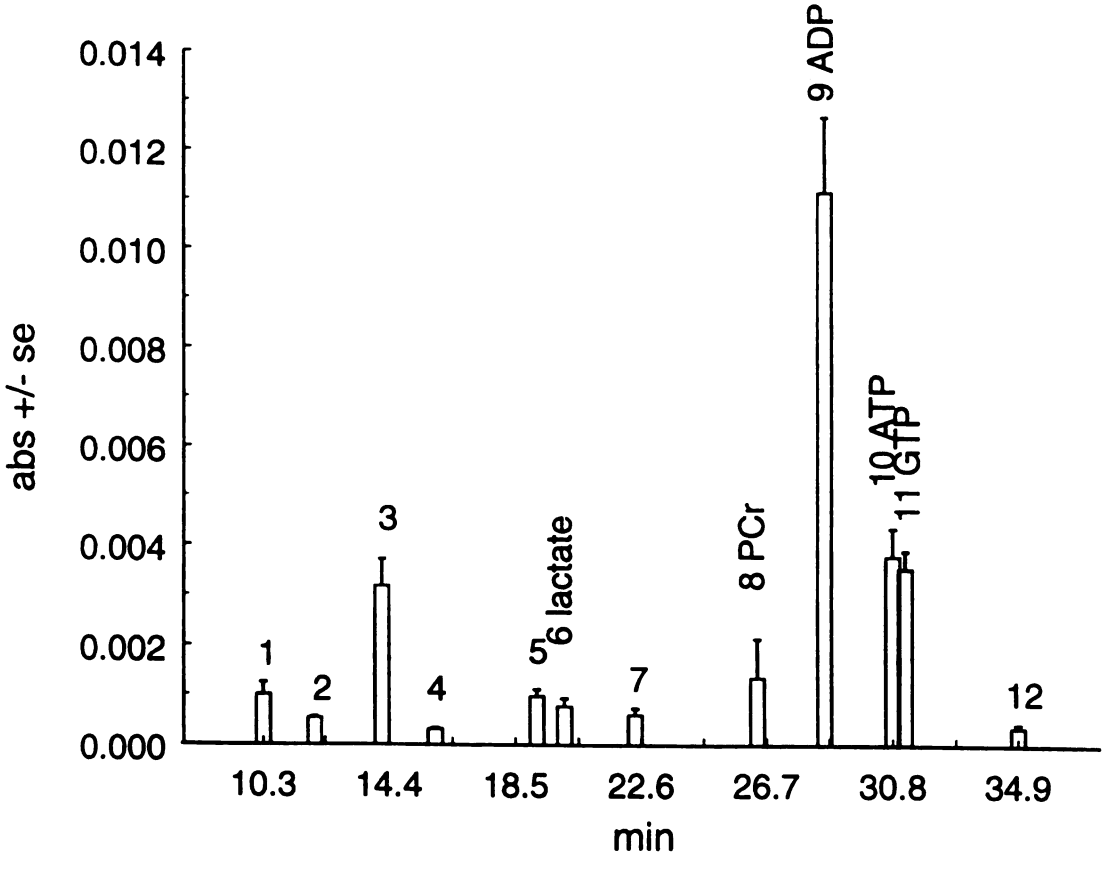

Figure 31. CE-measured beef bladder metabolites from five bladders used for smooth muscle PK isolation.

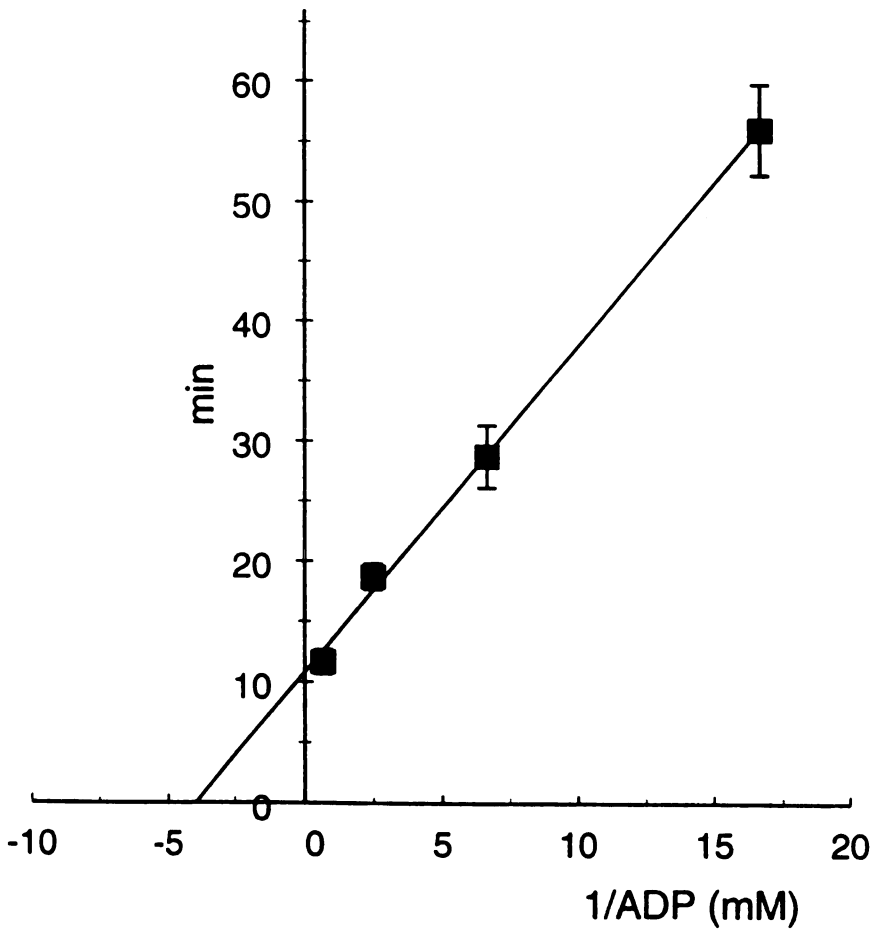

Figure 32. Lineweaver-Burk plots of ADP dependence of skeletal muscle PK.

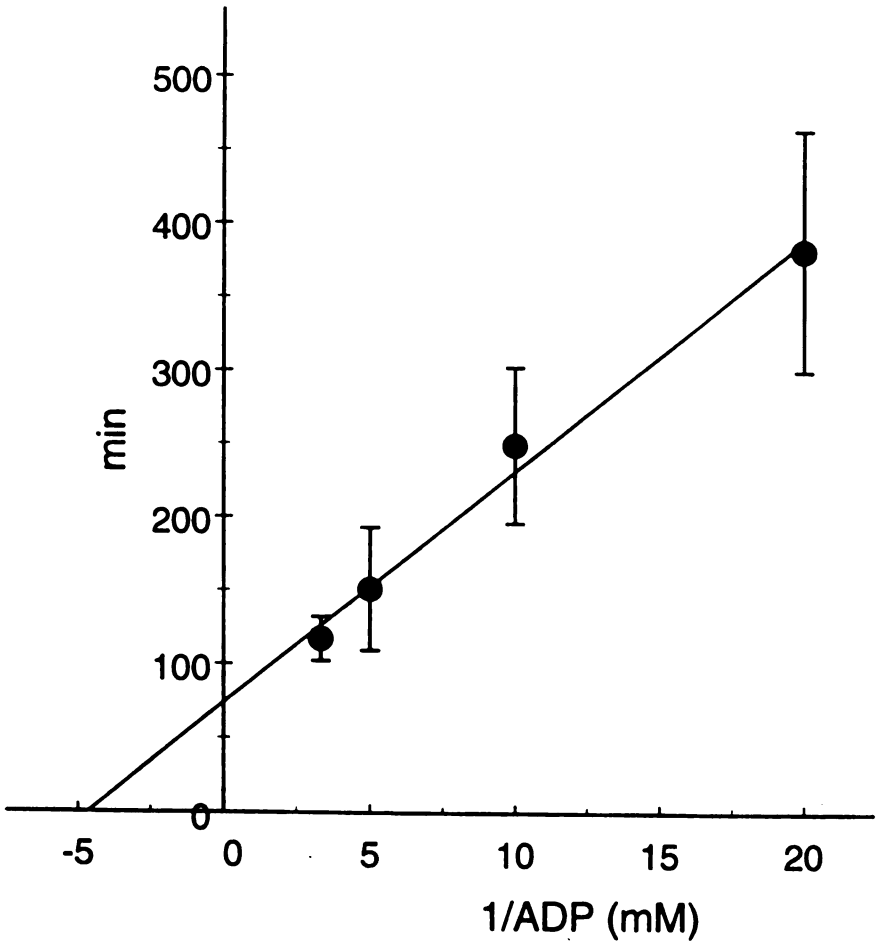

Figure 33. Lineweaver-Burk plots of ADP dependence of smooth muscle PK.

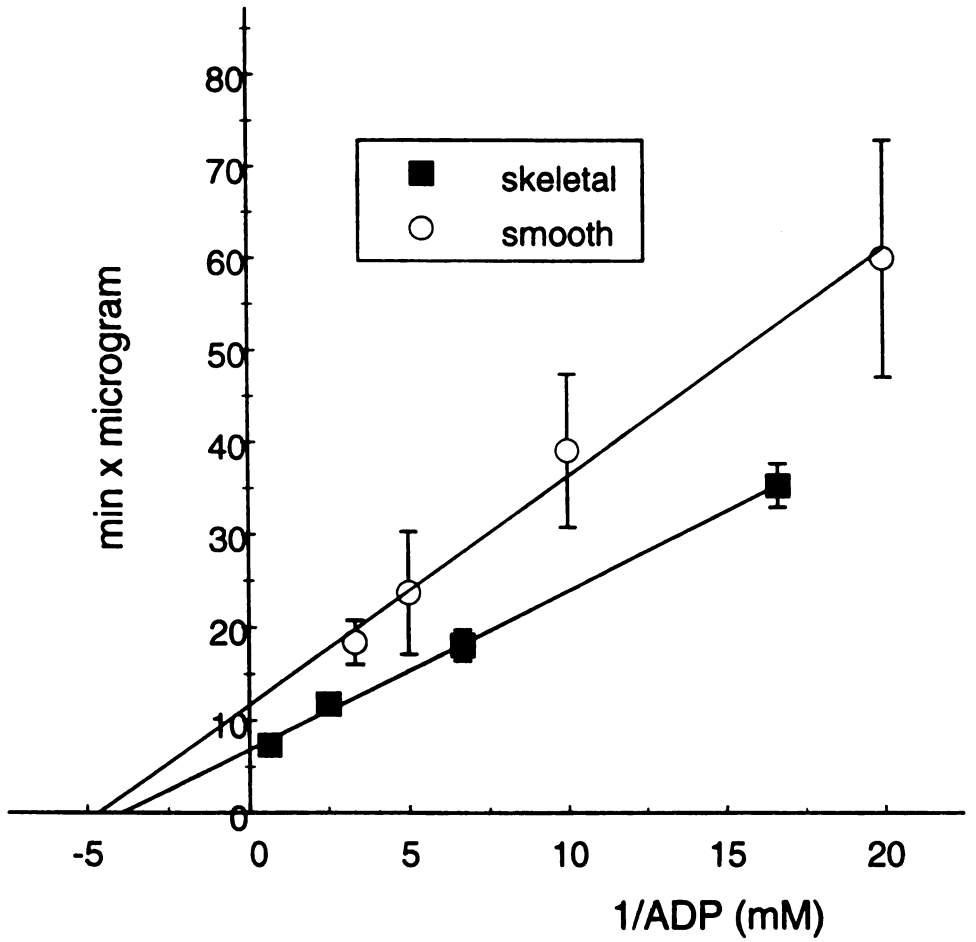

Figure 34. Comparison of the ADP-dependent kinetics of SKPK and SMPK.

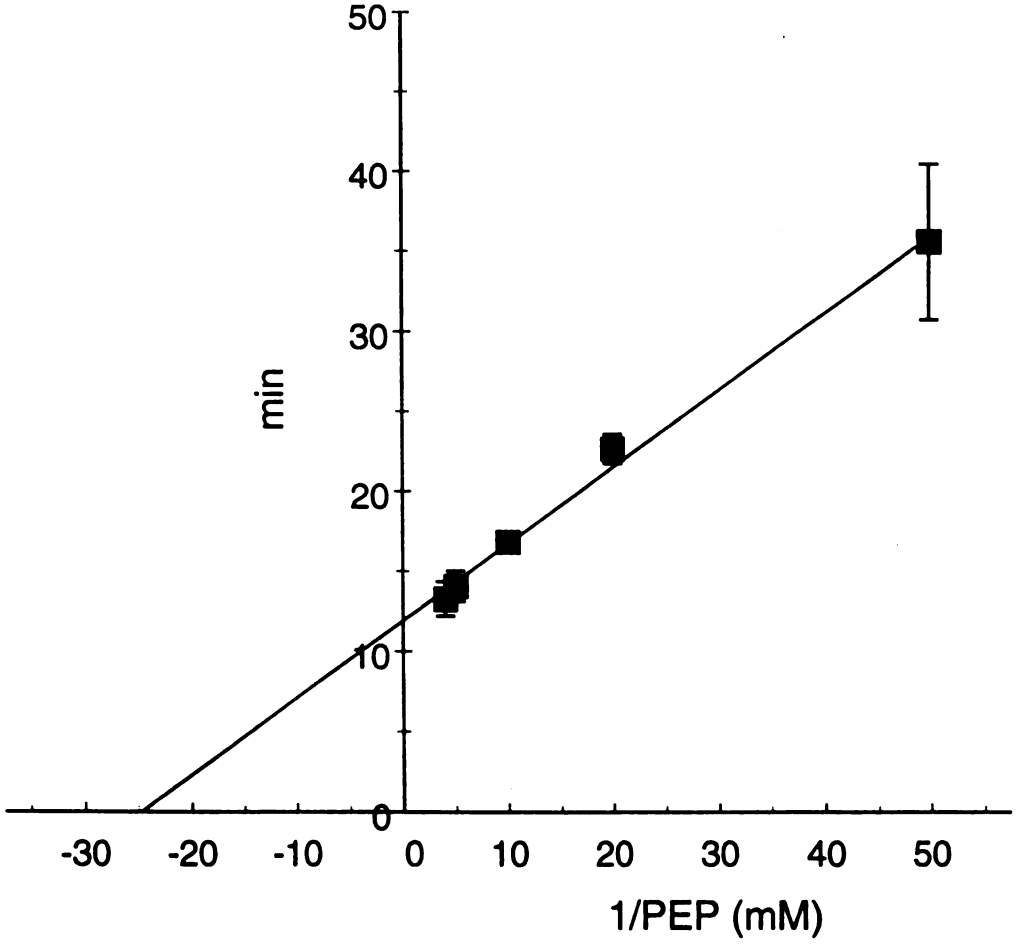

Figure 35. Lineweaver-Burk plots of PEP dependence of skeletal muscle PK.

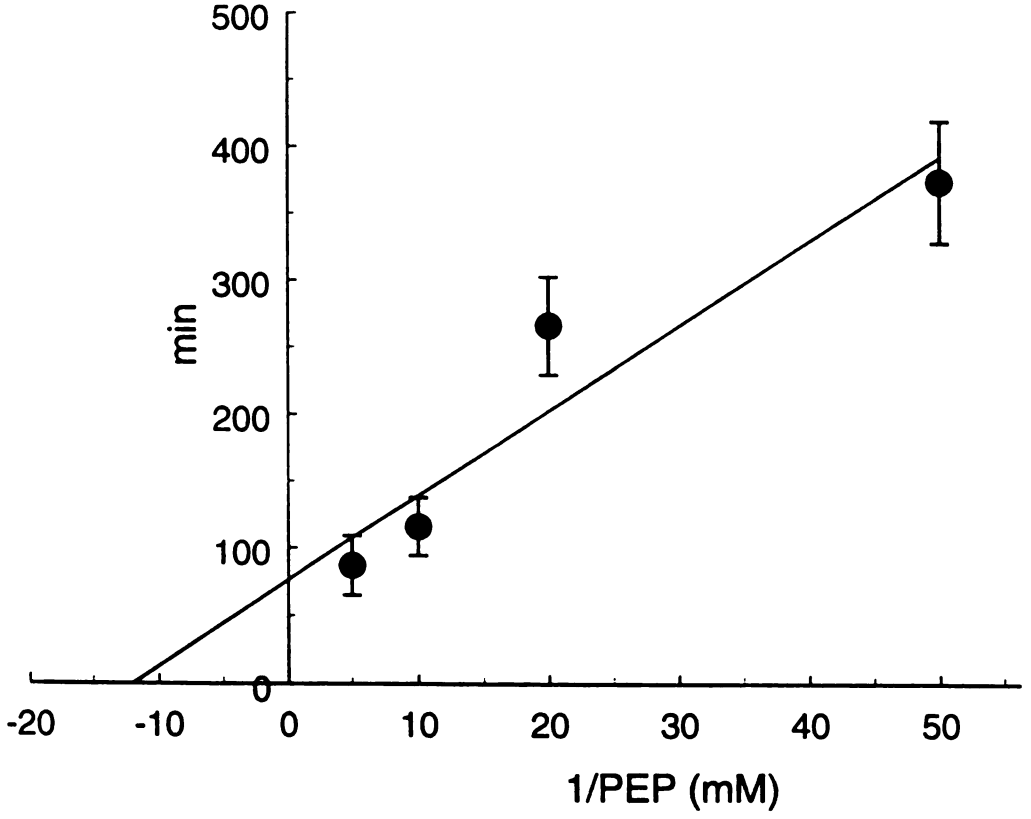

Figure 36. Lineweaver-Burk plots of PEP dependence of smooth muscle PK.

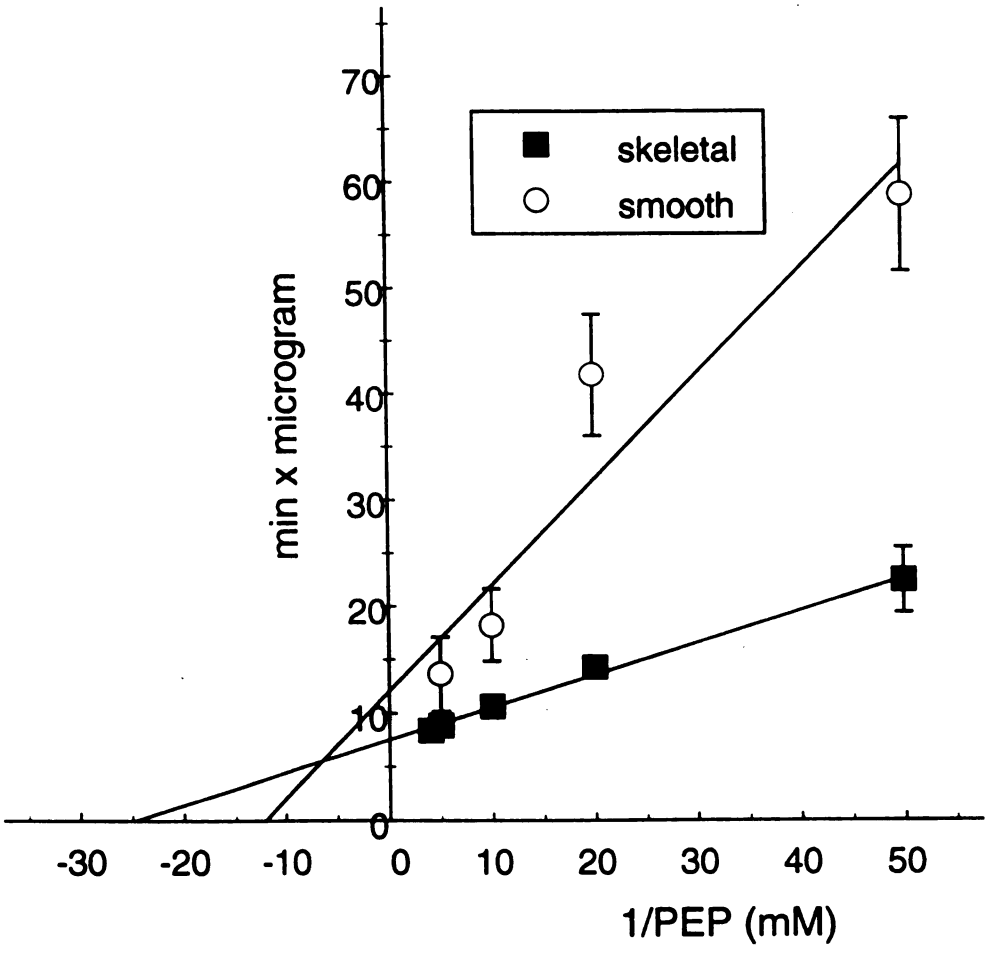

Figure 37. Comparison of the PEP-dependent kinetics of SKPK and SMPK.

tested as effectors of peak SMPK activity no effect was found. The Vmax values in U/ug determined using ADP (0.071 for SKPK, 0.042 for SMPK) were similar to those determined using PEP (0.064 of SKPK, 0.040 for SMPK).

The temperature dependence of the activity of SMPK and SKPK is shown in figure 38. For SMPK a decrease in the expected activity was first seen at 55 C but the slopes of the individual assays did not appear non-linear until 60 C was reached. The Arrhenius plots (figure 39) of both kinases were similar with neither one showing a prominent bend at the non-denaturing temperatures. The activation energies calculated from the least squares regression line were 56,400 for SKPK and 61,100 J/mole for SMPK.

CE coupling experiments.

The absorbance relation at 195 nm produced a decrease in absorbance when SKPK was added to MMCK, as opposed to the increased absorbance seen at 340 nm (figure 40). The SKPK was strongly effected by the MMCK in concentrations greater than 1.0 mg/ml. The SMPK absorbance was not strongly effected by the presence of MMCK. When CE is used on MMCK the peak area is not linearly related to the concentration of CK (figure 41) as it is for metabolites. Yet an effect similar to the NE-AA effect can still be seen when MMCK is run in combination with SKPK (figure 42). As expected from the measurements of absorbance at 195 without using CE (figure 40), the combined MMCK/SKPK peak was lower than expected from the individual absorbances. When this experiment was done using a constant SKPK concentration, an effect on the combined peak could be seen (figure 43). The ratio of the peaks including the complexes and the peaks of CK run alone did not approach 1.0 (as it would if there

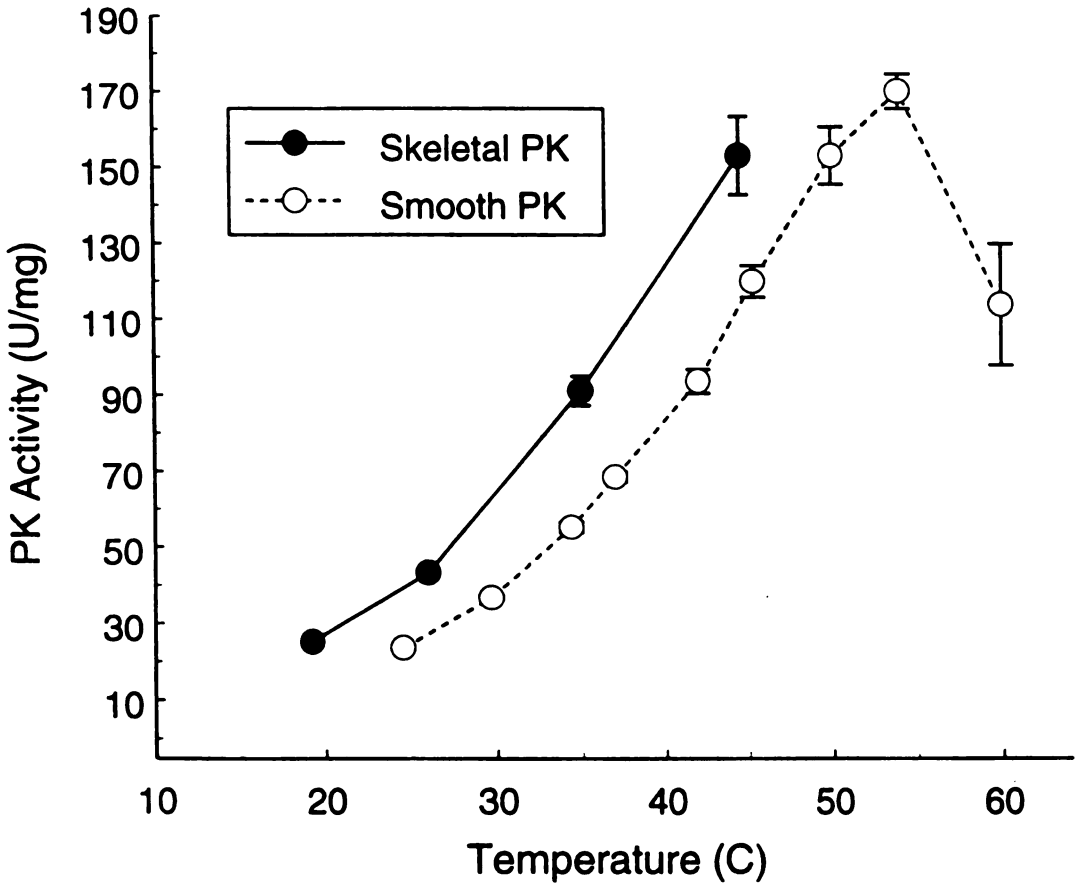

Figure 38. Temperature dependence of SKPK and SMPK activity.

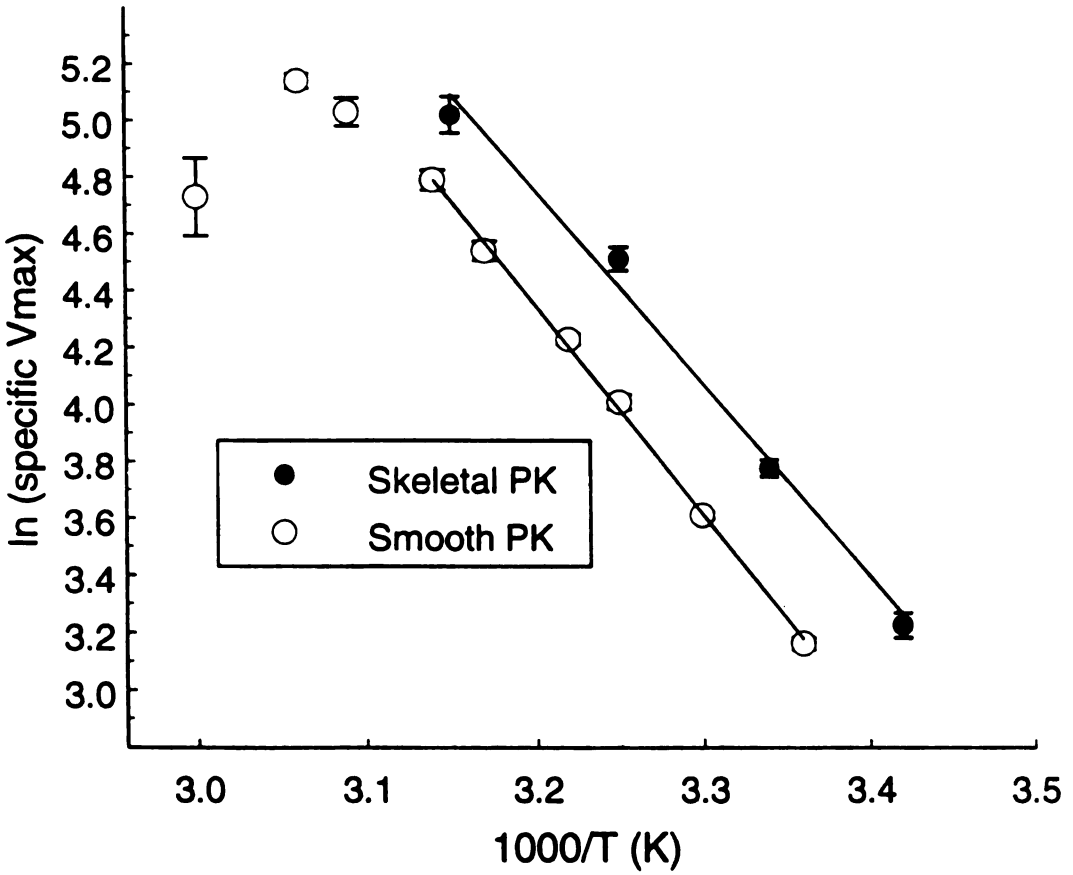

Figure 39. Activation energy determination of SKPK and SMPK.

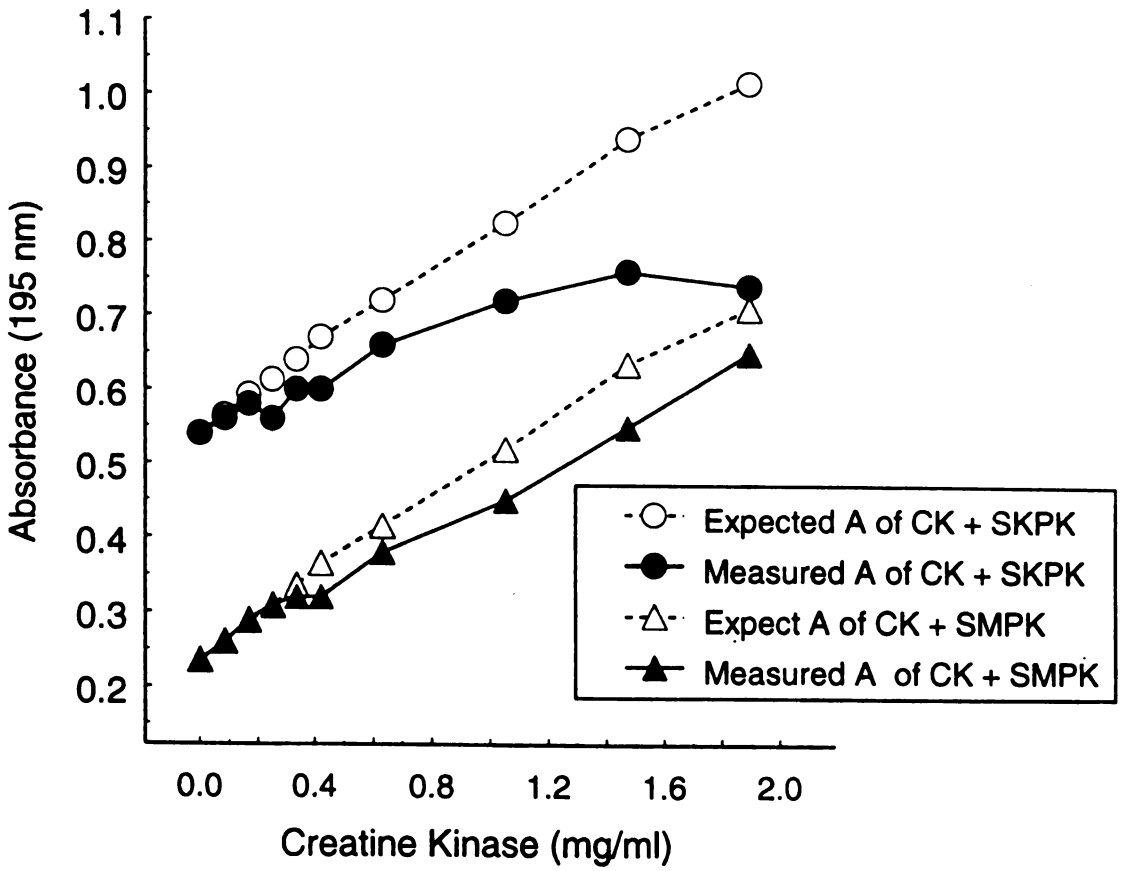

Figure 40. 195 nm measurement of the expected absorbance of solutions containing both PK and CK from the absorbance of individual solutions containing SKPK, SMPK or MMCK.

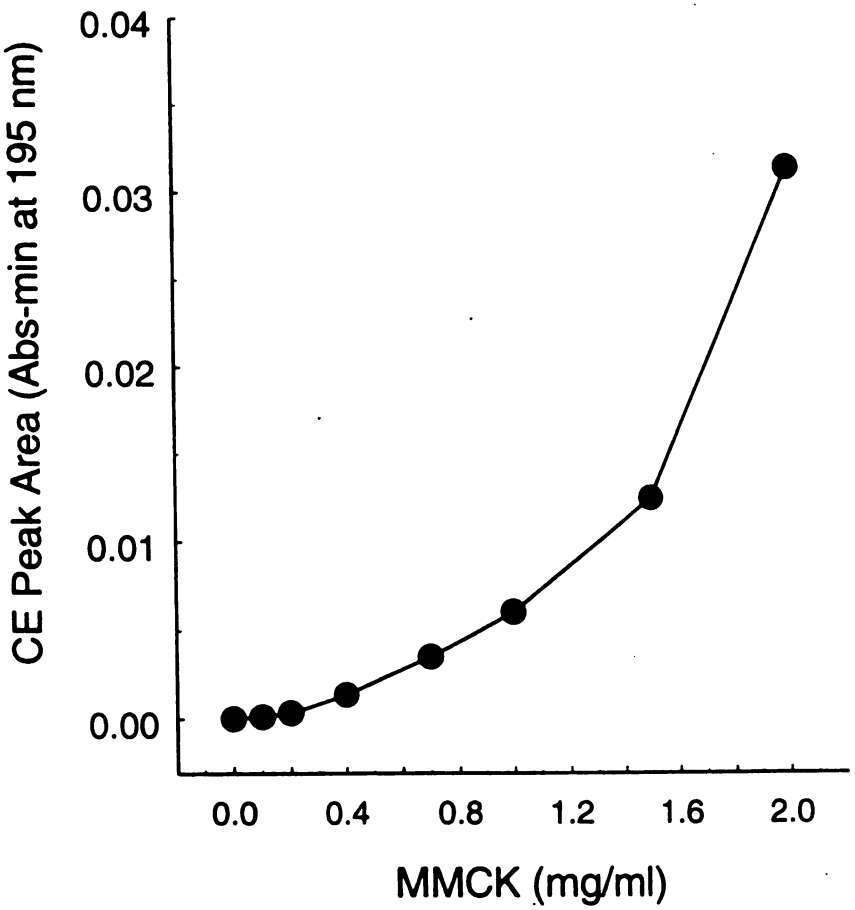

Figure 41. Concentration dependence of the peak areas of capillary electropherograms of solutions of MM creatine kinase.

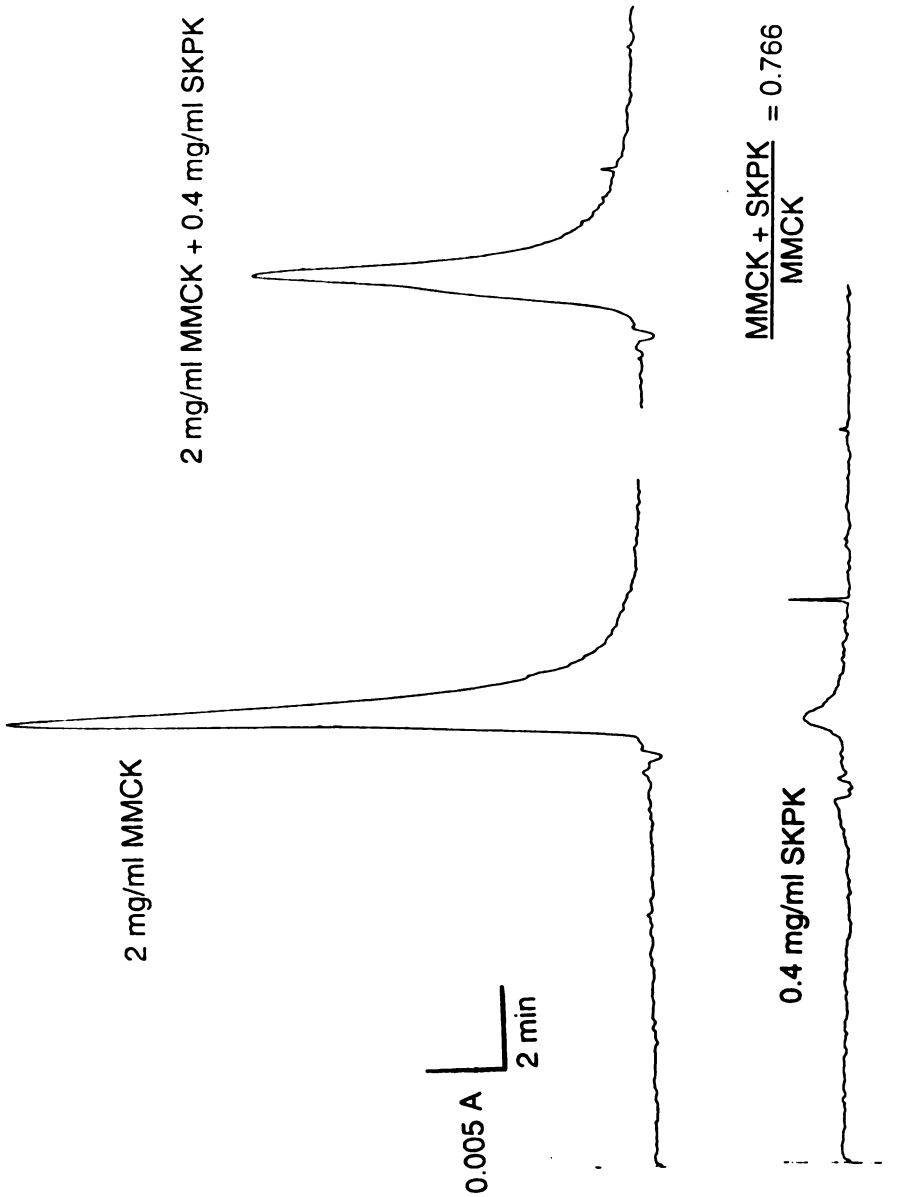

Figure 42. Capillary electropherograms of MMCK, SKPK, and their combination.

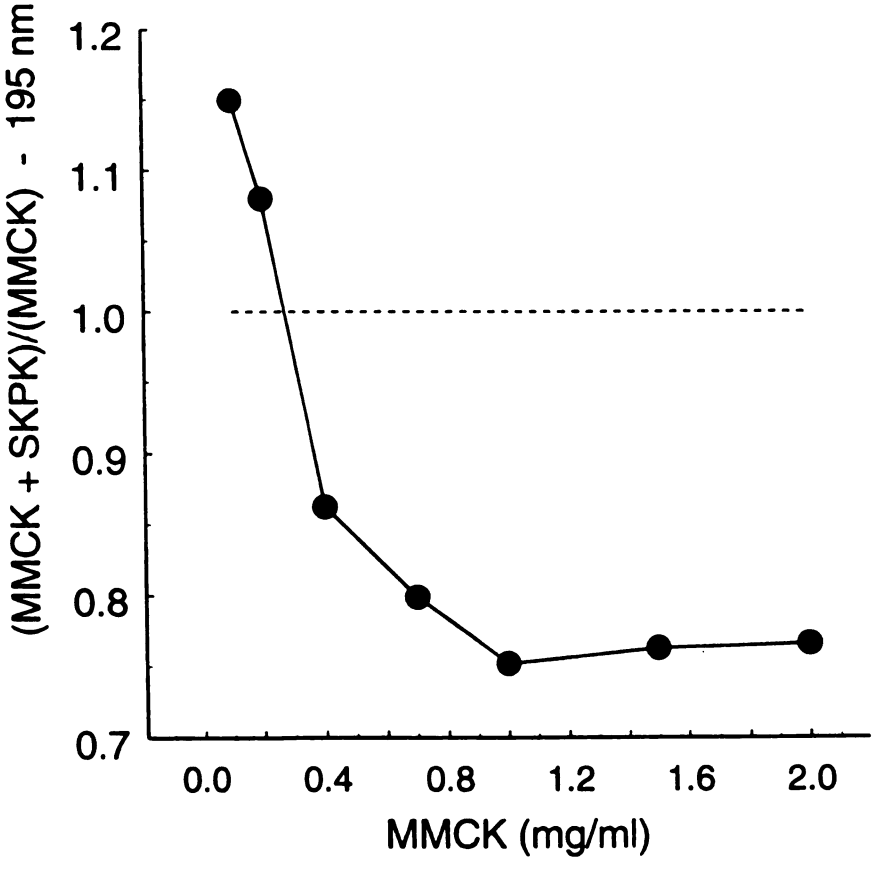

Figure 43. Ratio of the CE absorbance of MMCK + SKPK to the absorbance of MMCK as the creatine kinase concentration increases at 204 V/cm.

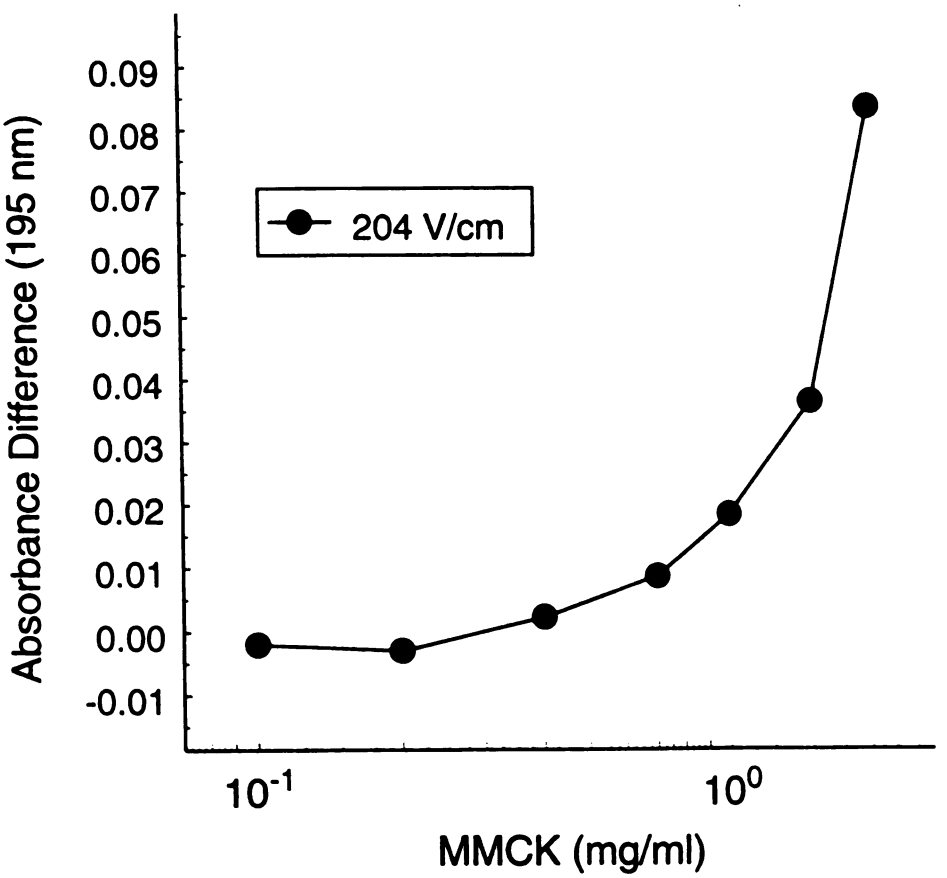

Figure 44. The CE absorbance difference between MMCK and MMCK + SKPK as the MMCK concentration increases at 204 V/cm.

were no interactions) as the MMCK concentration was increased. Figure 44 shows the difference between the MMCK peak and the combined peak. As the MMCK concentration increases, the difference between the peaks gets larger.

PK-CK interactions.

The absorbance of PK-CK interaction was measured at 340 nm by matching the most prominent species in the two tissues (figure 45). MMCK and SKPK showed a marked increase in absorbance above that expected for the addition of the two solutions. The BBCK and SMPK combination did not show this relationship. When the effect of BBCK was tested on SMPK solubility and activity in ethanol it was found to have no effect (figure 46). The same relationship was shown when testing SMPK versus MMCK (not shown). When the same experiment was done with SKPK and MMCK a new effect was discovered. A marked increase in activity of SKPK was seen and it persisted up to at least 26 % ethanol (figure 47). Without ethanol there is a larger relative effect at lower SKPK concentrations than at higher SKPK concentrations (figure 48). The SMPK, at a concentration between the two SKPK measurements, was not effected by either BBCK or MMCK. But the effect was seen when SKPK was combined with BBCK. When the data are plotted using specific activities (figure 49), the SKPK concentrations both higher and lower than those used for the SMPK tests still show a marked effect of MMCK.

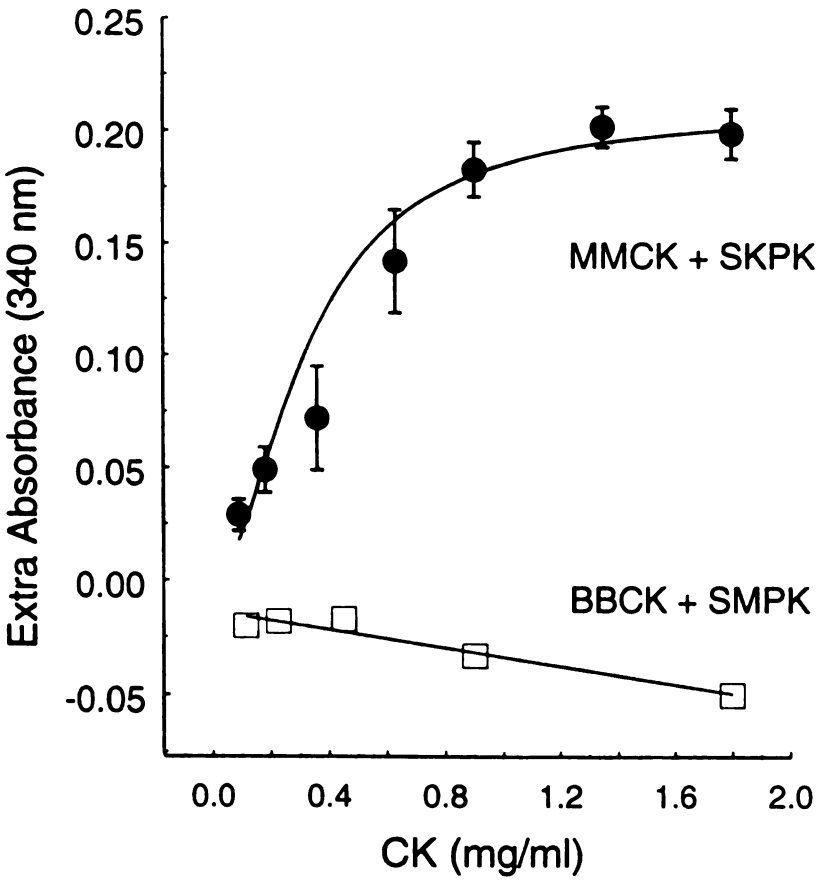

Figure 45. Comparison of the 340 nm absorbance differences produced by combinations of MMCK + SKPK and BBCK + SMPK.

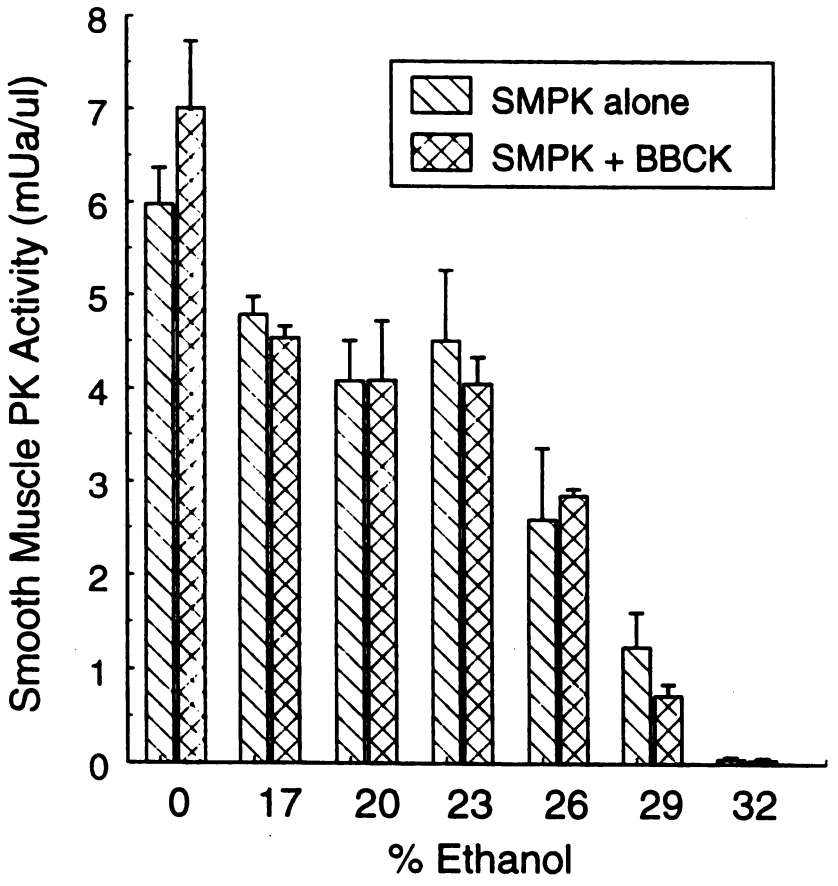

Figure 46. Effect of BBCK and ethanol on SMPK activity.

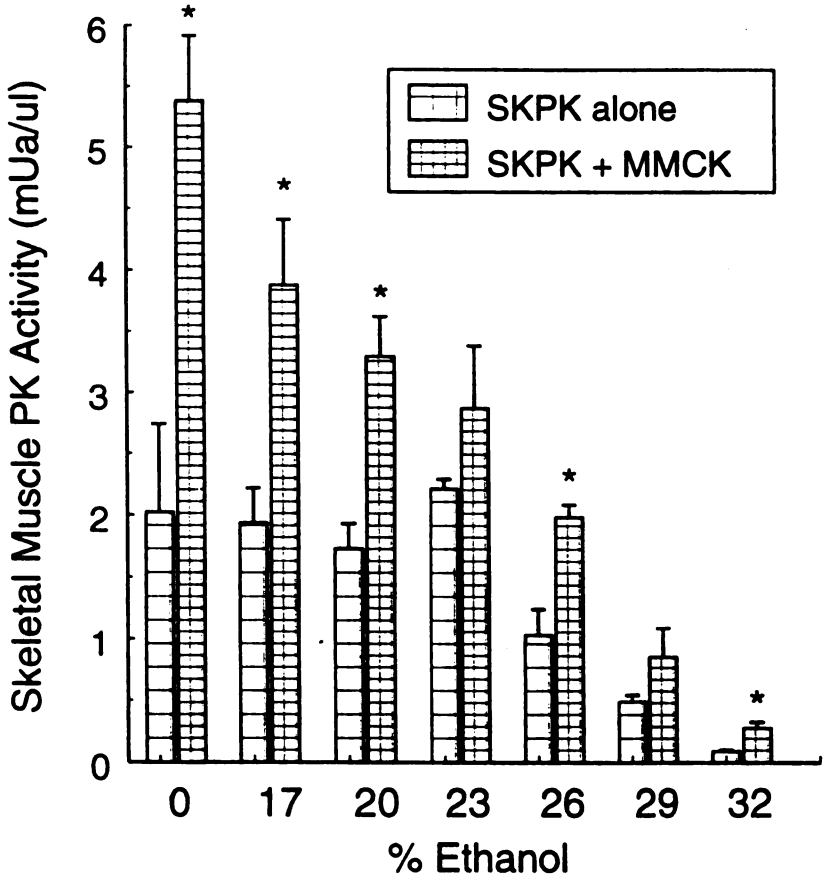

Figure 47. Effect of MMCK and ethanol on SKPK activity.

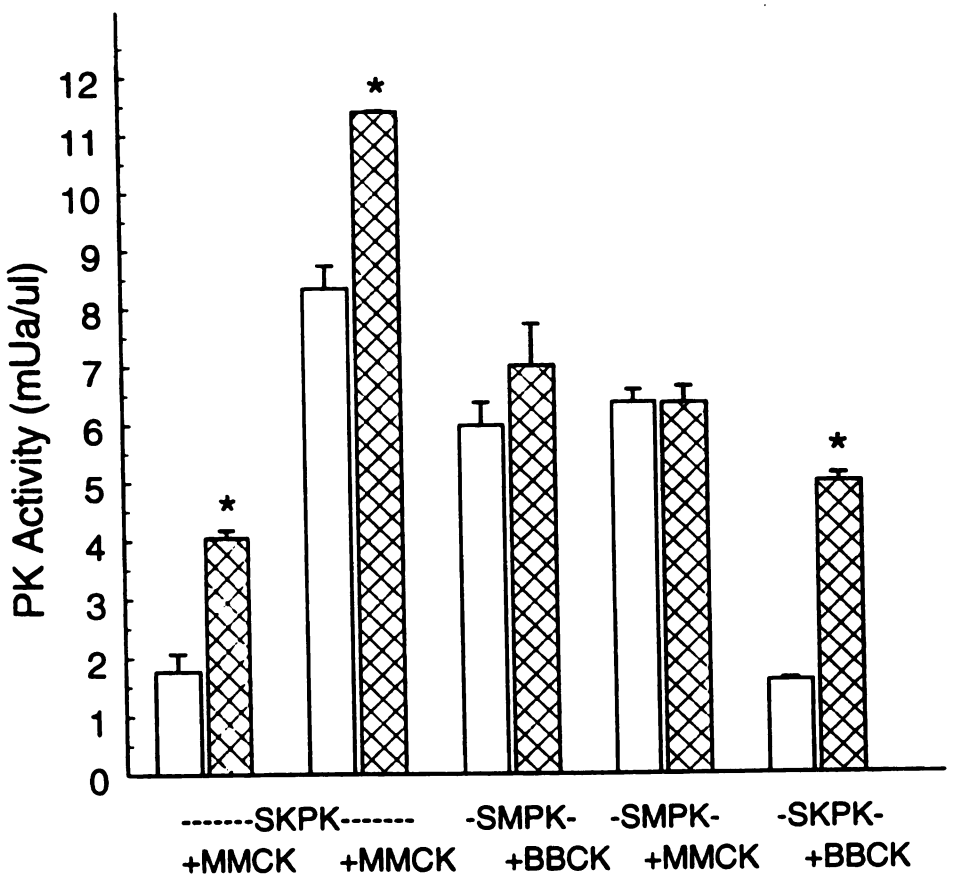

Figure 48. Effect of both MMCK and BBCK on the activity of SKPK and SMPK.

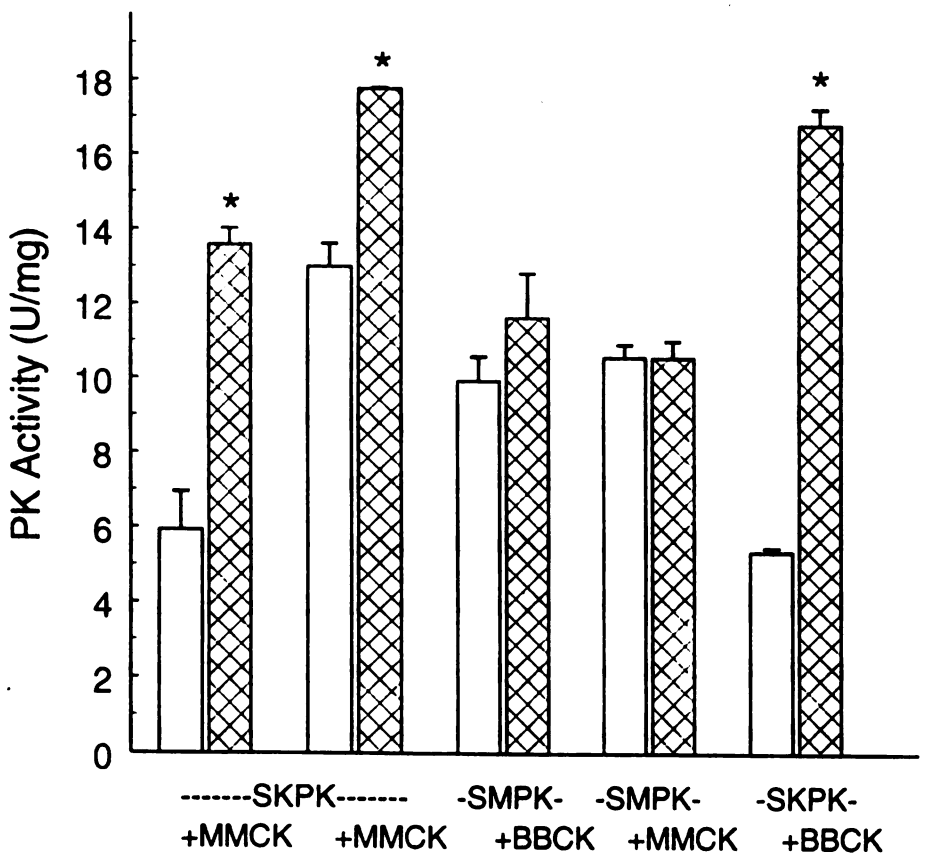

Figure 49. Effect of both MMCK and BBCK on the activity of SKPK and SMPK per unit of PK.

DISCUSSION

Smooth muscle PK isolation.

The isolation is unusual in that one of the most successful isolation steps was one of the bulk methods. The final PK isolated had a specific activity close to that of SKPK. Also, the SKPK had a few minor contaminants when run on SDS-PAGE. Considering that the effects of CK on SKPK were clear, it is unlikely that the lack of observed effect with SMPK was due to interference by contaminants. For this to be the case the contaminant would need to have a specific effect. The failure of a CK-PK co-isolation was not surprising since the coupling is a transient one. It was even less surprising when it was discovered that CK did not have the effects on SMPK as it did on SKPK.

There was a possibility of tissue degradation when the bladders were kept on ice. This was especially true since the entire batch of bladders had to be put through the first step of the isolation as soon as the tissue was brought to the laboratory. The capillary electropherograms showed that the tissue was still in good shape after the time spent on ice. The high tri-phosphates especially showed that the tissue had not used up its energy reserves. The low lactate levels reflect low use of glycolysis. Most of the energy used was derived from the stored PCr pool. The ability to keep the tissue on ice for an extended period of time was useful since freezing in liquid nitrogen caused some loss of activity. Also of importance was the fact that no broad raised area appeared in the electropherograms; figure 30 shows a level baseline. Such a broad peak would have been a sign of proteolysis as seen in previous experiments using trypsin.

Properties of SKPK and SMPK compared.

The two enzymes appear similar except that the SMPK has a lower specific activity. As seen in figure 48, where the activities of the two SKPK experiments bracketed the activity used in the SMPK experiment, this lower specific activity of the isolation sample cannot explain the difference in the activity effect of CK on the SKPK as opposed to SMPK. All the other parameters were similar for SMPK and SKPK. The use of lyophilized sample made the SKPK specific activity calculated from the Vmax low. The same relationship was seen in the Vmax calculated from the Lineweaver-Burk plots as from the specific activity of SKPK and SMPK measured with saturating conditions during assays at 37 C. The SMPK Km for ADP of 214 uM is high compared with the resting ADP in smooth muscle, which has been measured at 30 uM (Fisher and Dillon, 1988). Under severe conditions the ADP has been shown to reach 120 uM (Fisher and Dillon, 1988). Increases in ADP therefore should significantly increase flux through SMPK. As mentioned in the background though, changes in glycolytic flux have been observed which can not be explained by changes in the substrate concentrations for PK. In such cases, the increase in PK activity is assumed to come from another effect. There is then a dissociation between the effects of changes in substrate concentration *in vitro* and the effects of changes in substrate concentration *in vivo*. Channeling of metabolites has been suggested as an explanation for these mismatches but the lack of interactions between SMPK and CK suggests that this does not involve CK. The lack of effect of phenylalanine, ATP, and fructose 1,6-bisphosphate on the activity of SMPK under saturating substrate conditions suggests that any effect of these could only be on the Km of ADP or PEP. The effect on the SMPK Km would not be comparable to an effect in SKPK because of the transfer of metabolites between SKPK and MMCK in skeletal tissue.

The Activation energies for the two enzymes are similar at 56.4 and 61.1 kJ/mole. They are also comparable to other enzymes. It is interesting that there was a large change in the absorbance spectrum of PK (figure 16) when it was heated to temperatures at which the activity measurements did not show any denaturation (figure 38). The absorbance measurements were all done using a single buffer so there may be a pH effect due to the changes in temperature.

CE coupling experiments.

The original idea for measuring weak interactions was to measure the diffusion coefficients of molecules in capillary tubes in the presence or absence of other molecules. The new technique developed is more efficient for several reasons. A model for the change in shape of the peak does not have to be produced since a empirical linearization was possible. The experiments can be run as normal CE runs instead of allowing the molecule to diffuse overnight. And most importantly a new parameter can be measured, the Ke. This dissociation constant measured in a particular electric field is not just used to extrapolate the dissociation constant in zero electric field. It can be used to estimate the effect of naturally occurring electric fields on biomolecules. The electric field generated by a membrane's surface charges can be calculated as a function of the distance from the membrane in an aqueous solution (figure 50). Using the Ke's determined for NE-AA dissociation and the distance dependence of the membrane's electric field, it is possible to plot the Ke as a function of the distance from the membrane (figure 51). Putting both graphs together (figure 52) it can be seen that the NE-AA complex is expected to dissociate completely before it reaches the membrane. The experiments using NE and AA led to the theory that, for a hormone that binds to another molecule, the concentration of free hormone increases close to their binding sites on membranes

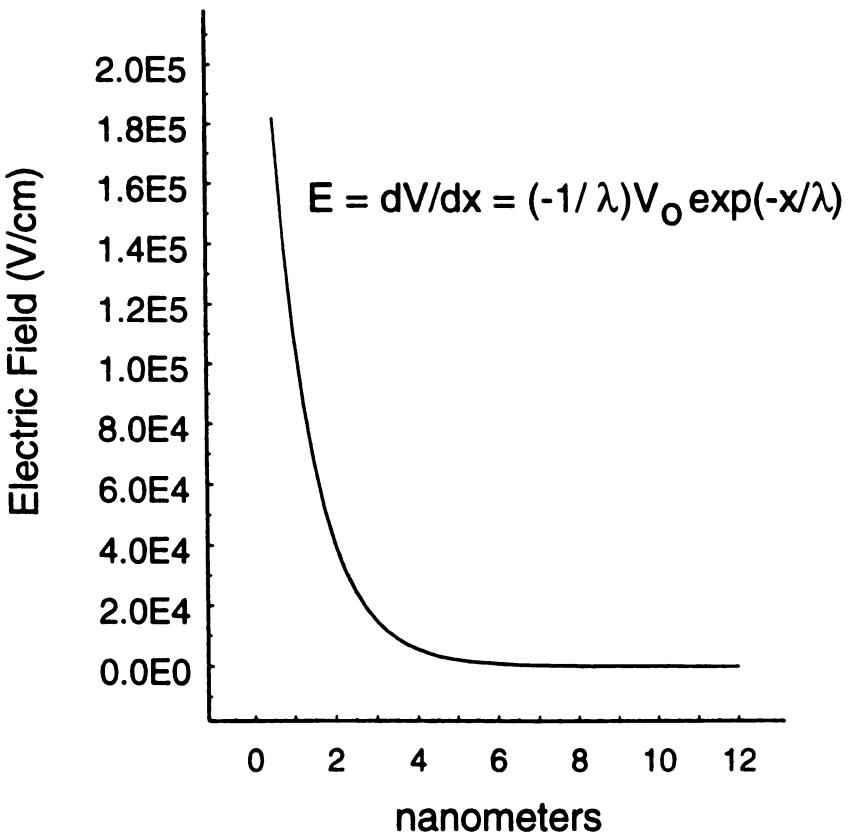

Figure 50. Estimation of the electric field generated by a cell membrane.

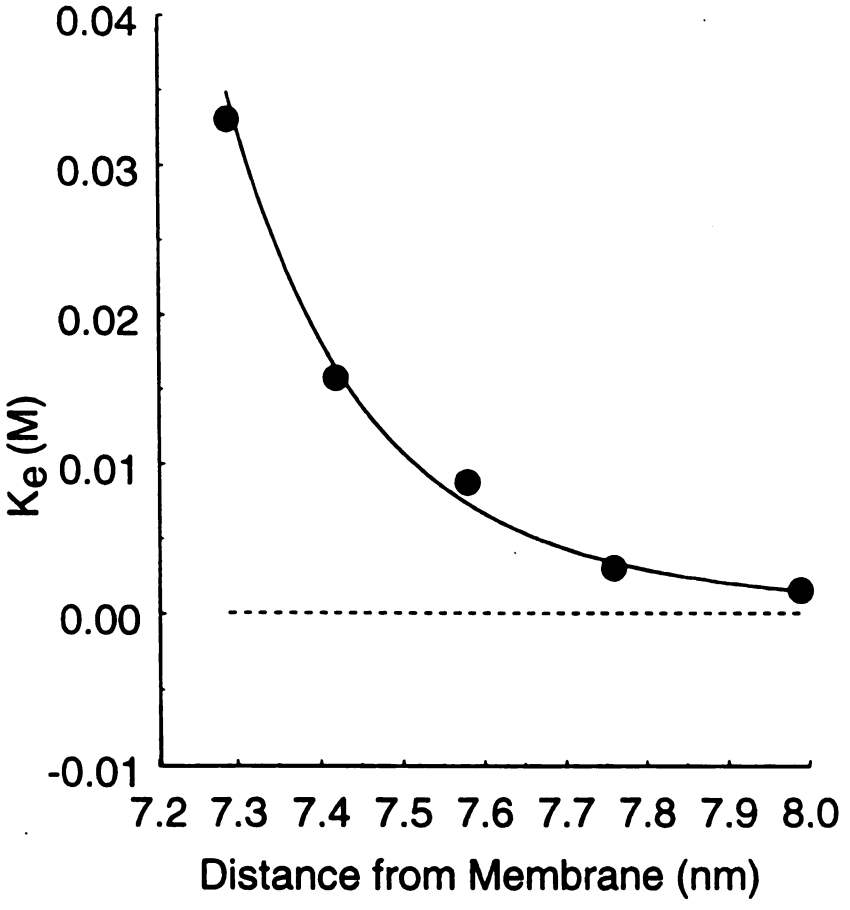

Figure 51. Estimation of the change in NE-AA dissociation constant at different distances from a cell membrane.

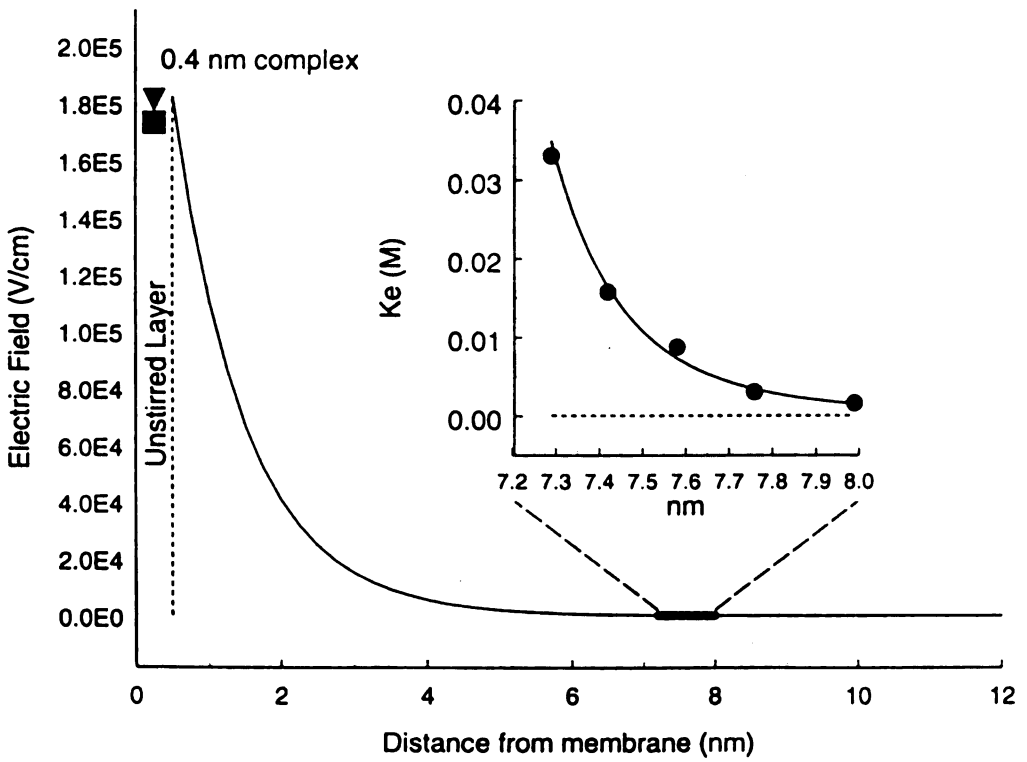

Figure 52. Experimental range of Ke measurements of NE-AA dissociation relative to the electric field generated by a cell membrane.

because of the electric field generated by the surface groups on the membrane. This method was applied to SKPK-MMCK binding and gave qualitative evidence that the binding occurs. This suggests that the same effects may be seen on the inside of membranes (where a smaller but similar electric field exists) with interacting proteins as on the outside of membranes with hormones.

The failure to show, with the PK-CK interaction, a quantitative relationship similar to that shown for the smaller molecules was due to several factors. First the conditions under which PK and CK run through a capillary are restricted by the need to prevent adherence to the capillary walls. Second, the concentration dependence of the CE peak area, as measured using CK, is non-linear. This may also be due to adherence. The size of the molecules is not in itself a problem because the angiotensin II antibodies showed two peaks, a free and complexed peak.

Specific properties of pyruvate kinase and odd characteristics.

Problems in the current understanding of PK.

PK has several odd characteristics. The mass-action ratio for PK suggests that its reaction is far from equilibrium while its maximal catalytic activity suggests otherwise (Newsholme and Start, 1981). It is thought that mammalian muscle PK is usually inhibited because of its exceedingly high activity (Newsholme and Start, 1981). *In vivo* ATP and the low concentration of PEP compared to PK's Km may cause this inhibition (Newsholme and Start, 1981). Yet the concentrations of these substrates do not change enough in cells to make a noticeable difference in PK activity. In a case where glycolytic flux is increased 100-fold there was no observed change in PEP concentration (Sacktor and Wormser-Shavit, 1966). The increase in

activity in this system can not be the result of decreased ATP or increased PEP concentration.

The diazymatic PK and CK reaction.

In previous research PK and CK were shown to bind and to exchange ATP formed from their respective reactions without ATP being released into the bulk medium; this was named a diazymatic reaction (Dillon and Clark, 1990; Dillon *et al.*, 1995). During NMR experiments, Hseih and Balaban (1988) may have inadvertently measured this ATP exchange *in vivo* when they saturated in the position where PEP would be. Since the transit time of the phosphate at the position where saturation is tacking place is always going to be longer than the time it takes to damp it, it is the flux of the metabolite through a point being saturated which is important in transferring a damping signal to another metabolite. Although PEP is not seen, it is still being damped and if the flux is high through the diazymatic reaction the damped signal will accumulate in the phosphocreatine pool. When Balaban saturated the position where PEP is located, there was a decrease in the ratio of the phosphocreatine to γATP peak compared to the experiment during which there was no PEP saturation.

Only a few facts about the mechanism of the diazymatic reaction can be deduced: the rate of exchange was measured with the NMR; the PK reaction, which is associative with respect to PEP hydrolysis , precludes the possibility of directly transferring the phosphate from PEP to creatine bound on CK; and it is also not possible to isolate phosphate exchange between PK and CK without ATP in the solution since there is no other intermediate for the PEP phosphate.

Creatine kinase and channeling.

Specifics of CK and channeling.

CK is better understood than PK. One of the most interesting areas of research in CK is the phosphoryl-creatine circuit. An original creatine phosphate shuttle hypothesis was proposed by Bessman and Geiger in 1981. The phosphoryl-creatine circuit is a modified version of the phosphocreatine-shuttle hypothesis of Wallimann and Eppenberger (1983). Antibodies against M-CK have been used to localize CK in chicken skeletal muscle. This enzyme thought to be free in solution was actually found on the M-line and I-band but not on the Z-line. The solubilization of the plasma membrane and washing of the tissue prior to fixation caused the I-band CK to disappear but had no effect on the M-band (Wallimann et al., 1989) This M-band bound CK amounted to 3 to 7% of the total CK. In general the phosphocreatine circuit postulates that the sources of ATP generation are connected to ATP usage sites via phosphocreatine. High energy phosphates from the mitochondria are translocated to the cytoplasm by Mi-CK in the form of phosphocreatine. This is supported by the close association of Mi-CK with the ADP-ATP translocase at points of contact between the inner and outer mitochondrial membranes. The hypothesis also supposes glycolysis as an entry point for ATP into the circuit. If this is true, diazymatic interaction of PK and CK may be involved.

Significance of CK's properties.

The functions of the circuit which have been proposed are energy buffering, energy transport and channeling, and regulation of local ATP levels. One interesting observation is that B-CK has two isoforms and may be phosphorylated as

demonstrated with two-dimensional gel electrophoresis (Walliman *et al.*, 1989, p. 168). This fits well with the concept that isoforms of an enzyme may have different affinities for other enzymes or subcellular structures and thus may differentially distribute their kinetic properties to various pathways (Ureta, 1991).

Although many tissues work well without CK, its importance is probably greater at high workloads. There is considerable evidence for this and in many of these cases CK is also associated with subcellular structures. Training for long distance running and the resultant adaptation of fast-twitch to slow-twitch fibers occurs at the same time as a decrease in total muscle CK and a increase in Mi-CK (Wallimann *et al.*, 1989, p. 171). This was used to support the concept of a dual function for the phosphoryl-creatine circuit as an energy buffering system in acute energy needs and as an energy channeling system in prolonged energy needs. There has also been positive correlation between oxygen consumption and CK reaction rate by 13-P NMR, biochemical measurements, and model calculations (Wallimann *et al.*, 1989, p. 171).

Smooth and skeletal muscle PK-CK interactions

The absorbance measurements at 340 and 195 nm both showed an effect of CK on SKPK. The MMCK is the CK isoform found in skeletal muscle. Smooth muscle contains mostly BBCK but it also contains a small amount of MMCK. The fact that there was no effect of either BBCK, tested at 340 nm, or MMCK, tested at 195 nm, on SMPK was the first strong evidence that SMPK acted differently from SKPK. The measurements at 195 nm were only possible by injecting the samples into the CE unit. The spectrum measured down to 220 nm using the spectrophotometer always showed a positive change in absorbance upon interaction of the proteins. This change was not as great at 220 nm but was never negative. The negative effect seen at 195 nm also showed

a difference between the SMPK and the SKPK. This effect at 195nm in combination with the changes in the strength of the positive effect seen at higher wavelengths suggests that there may be some specificity to this interaction. In the spectrum measurements on figure 28 the relative difference between the CK and PK curves decreases as they approach 220 nm. If the absorbance of the interacting species continues to changes in the same direction as the wavelength decreases, this could cause two coupled molecules to have less absorbance than the free molecules. The steepness of the curves at this position makes it impossible to accurately compare them from the two or three points on the rising portion of each curve.

The ethanol solubility experiments worked well for SMPK and showed no effect of either MMCK or BBCK. Since no effect of CK on SMPK was seen at 0% ethanol it is assumed that the SMPK activity accurately measured the amount of SMPK in the solution. It is interesting to note that earlier ethanol experiments using SDS-PAGE to quantify the amount of SMPK remaining in solution needed more ethanol to decrease the level of PK in solution. This may be due to differences in protein concentration or in buffer, or it may reflect an effect of ethanol to decrease the activity of PK. It is also interesting to note that the SKPK required higher ethanol to start to decrease in activity (figure 47) than the SMPK (figure 46). The effects seen in the SKPK experiment are complex. There are two effects which cannot be separated. First there is the effect of MMCK to increase the activity of SKPK. Since the SKPK+MMCK 23% sample (figure 47) has as more activity than the SKPK 0% sample, the activity effect of MMCK must still be occurring at this level of ethanol. At greater than 23% ethanol, when SKPK starts to decline, it is not possible to determine what fraction of the increase in activity is due to a direct activity effect as opposed to a solubility effect.

BBCK and SKPK are not found in the same tissue. Despite this, the strong effect of BBCK on SKPK activity suggests an important role for the type of PK found in a tissue. It may be that it is the PK isozyme found in a tissue which determines whether it

will couple to CK. In this case the property that makes it possible for CK to couple with SKPK is found on all CK isozymes. This also suggests that there are surface structures on CK isozymes which are identical in all the isozymes and which are complementary to structures on the SKPK isozyme but not complementary to structures on the SMPK isozyme.

The energy fluxes in skeletal muscle can be characterized as changing rapidly and to extreme values compared to other tissues. Smooth muscle has many of the same characteristics as skeletal muscle because of their similar function but smooth muscle never experiences the changes in energy demand occurring in skeletal muscle. The possibility that a channeling mechanism between the energy producing pathway (PK) and energy buffering pathway (CK) exists in skeletal muscle but not in smooth muscle is significant. It suggests that, in the case of the PK-CK interaction, the effects emphasized by the modern view of metabolism are part of the design of the metabolic pathway that makes it specific to the needs of the tissue. Because of the much greater glycolytic flux in skeletal muscle than in smooth muscle, PK-CK coupling may play a central role in skeletal muscle energy metabolism.

A general modern view of the cell.

When thinking of the cell as a gel of continually interacting proteins and of a number of these interactions leading to functional coupling, is there a problem with aggregation due to weak interactions? Even if the interactions are weak, if the concentration of molecules is high enough, one would expect that the number of interactions would lead to a substantial bound fraction at all times. This would tend to cause aggregation which implies precipitation. It doesn't seem possible that, even if two proteins help solvate each other, a large number of proteins interacting in this

way would cause a concomitant increase in solvation. On the contrary, one would assume that an optimum is quickly reached and that further interactions would be counterproductive. In cells, the largest multi-enzyme complexes in solution are ribosomes and even their "soluble" population may be bound to the sub-cellular lattice. Past a certain molecular weight molecules are difficult to keep in solution.

In the case of glycolytic enzymes such as PK, large multi-enzyme complexes have been suggested (Kurganov *et al.*, 1985) because of the number of interactions found between many of theses enzymes. These structures have not been seen in electron micrographs of muscle tissue where they were proposed. The interactions observed may therefore be due to sites which act transiently. The problem of precipitation would be especially important in complexes which did not act transiently if the proteins were globular.

The problem of precipitation is avoided in structures such as the sub-cellular lattice and the contractile apparatus by forming molecules which have a long axis and whose interactions define a minimum distance. Research has found that, in cells which need to form a gel phase for the purpose of locomotion, the gel phase is created by long actin filaments bound together by an actin binding protein which has angled binding sites so that the filaments must cross at ninety degree angles in order to bind to each other (Stossel, 1994). As viewed in electron micrographs, there is an orthogonal network of strands. In other words, the cell has maintained a minimum distance between the gel forming molecules so that there is solution space between them. Although these molecules have, in effect, precipitated, they have done it in a specific way which has spared other proteins.

It is known that water molecules, sometimes large networks of water molecules, are trapped in locations within folded proteins and isolated from the bulk solvent (Creighton, 1984, p. 243). Similar networks may form as proteins approach and their highly ordered hydration spheres become intermeshed. Just as in the inside

of proteins, these water molecules may be an integral part of the ionic interactions. They may be important during weak interactions to keep the proteins from aggregating. Also the concept of complimentary is useful in that, if many of these interactions are through specific sites but that there is a large number of types of sites and a limited number of sites per molecule, then some molecules will be complimentary only to certain other molecules. Once these molecules have bound, the sites on these molecules will be taken up and they will not bind further and aggregate.

The number of weak non-specific interactions between molecules and the concentration of these molecules may be optimized quantities. Different cell types may have different optima and different modes of operation of individual cells may change the optimum, leading to the regulation of these parameters. For example, a macrophage which starts to migrate in response to a chemotactic stimulus may need to change these parameters in order to facilitate cytoplasmic streaming. Even local areas within cells are apt to maintain these parameters differentially.

It is also interesting that many of the enzymes thought to associate for functional reasons tend to associate with sub-cellular structures. This may be a way of forcing these dynamic interactions to occur on supports which cause them to be separated in space. This would also mean that the need for cellular support space may be an driving evolutionary principle.

The relativity of specific weak interactions.

Is there a problem with specific weak interactions as opposed to non-specific interactions? Non-specific interactions are weak. If an interaction is strong then it will be specific. The words weak and non-specific are almost defined in terms of

each other. The term weak is relative and intermediate between non-specific and strong. Although experimentally these interactions require high protein concentrations and low ionic strengths, these are ways to bring the solutions closer to the cellular conditions (Brooks and Storey, 1991). In the case of PK and CK low ionic strength is not a necessity.

It is still possible for weak interactions to lead to specific functional coupling. Functional coupling will only occur through the exchange of metabolites which are specific to the binding sites of the enzymes and allows the proteins to come together with the alignment which will optimize transfer. It is easy to see that, when non-specific interactions between two defined surfaces--one on each protein--occur, the degrees of freedom will be restricted by the approaching electrostatic charges. Electrostatic interactions have the potential for acting at greater distances than other types of interactions. The other interactions; hydrophobic, Van der Walls, and steric; in combination with these electrostatic interactions also have all the effect of reducing the degrees of freedom of motion of the proteins when they are adjacent. These may not be strong enough to cause a strong interaction, but if the proteins come in contact they may be forced into a relative orientation which favors a functional interaction. This is the essence of non-specific interactions. It is not possible for weak interactions to bring proteins together in dilute solutions and force functional coupling to occur. But in more concentrated solutions where interactions will occur, the weak interactions can cause the correct orientation of the proteins when their appropriate surfaces collide. A protein with a large dipole moment, like cytochrome c, could use these interactions from further away. Such dipoles caused by α-helices have been shown to aid in the binding of substrates (Hol *et al.*, 1978). Although the substrates which bind are generally in hydrophobic clefts which help to shield them from a solvent reaction field, protein interactions have the advantage that

both species can develop these large dipole moments, and allosteric interactions may help release metabolites.

It may not be necessary for enzymes to be tightly bound for their substrates to be exchanged. It may simply be necessary for enzymes to be close enough to each other compared to other enzymes for the diffusional distance of metabolites to be reduced and for channeling to take place. This possibility has been used to explain the increase in activity seen in sequential enzymes which Mosbach had bound to a solid support(Ottaway and Mowbray, 1976, p. 141), Ottaway and Mowbray have modeled this possibility and found it to be plausible(Ottaway and Mowbray, 1976), and "Bernhard and Srivastava... ...argued that 'relative thermodynamic stability of protein-protein complexes has no bearing on the channeling phenomena'"(p. 193, Ovádi, 1991).

The mechanism by which complementarity yields specificity in weak interactions may also be important. This is especially true if these transient interactions have allosteric effects which increase the rate of product release. Both steady state fluxes and channeling may be increased by this mechanism.

Current protein interactions research.

Most of the current research in protein interactions does not involve enzymes which act in a hand-off manner. The research on interactions between subunits of proteins is extensive. Interactions between separate molecules are also being investigated. For example the interactions between endophilin and amphiphysin (Micheva et al., 1997) are being investigated in a manner than emphasizes the involvment of particular protein domains. New techniques include methods of studying protein interactions which can also be applied to enzymes that hand off

substrates. Such techniques include "high-throughput screening strategies" (Kay and Paul, 1996).

The increased speed of desk-top computers has resulted in an increase in modeling. The most extensively used method is known as Brownian Dynamics (Northrup, 1998). In this approach, two proteins of know structure are used. One protein is assigned a central position surrounded by a sphere whose diameter is assigned by the modeler. The second protein is placed at an initial position on the sphere. The algorithm takes the structures of the proteins and the electrical fields that they generate in determining the movements of the second protein. When the program is started, the second protein is allowed to take a random walk. Several criteria can be set to test if the proteins have bound. The simplest test is the setting of an arbitrary distance between the two proteins. The program stops when either of two conditions is met--the two proteins have bound (as defined by the programmer), or the second protein has left the sphere. By running the program repeatedly, a probability of docking can be determined and compared to that of other proteins. Also of interest is the relative orientation of the two proteins at the time of docking.

SUMMARY

The interactions of SKPK and SMPK with MMCK and BBCK were investigated. An isolation procedure was developed for SMPK and it was found to have properties similar to SKPK.

In the course of the investigations on interacting molecules, a CE technique was developed to measure binding between molecules. This technique made it possible to quantitatively measure the effect of the electric field on the dissociation of molecules that bind. This technique can be used to estimate the effect of the electric field of biomembranes on molecules that bind to each other.

It was determined that SMPK does not interact with CK isozymes found in smooth muscle the way SKPK interacts with MMCK in skeletal muscle. It was also found that it may be the PK isozyme that determines whether the interaction with CK will occur.

The modern view of metabolism emphasizes mechanisms which require interactions between enzymes in the control of metabolism. It is suggested that the PK-CK interaction is such a mechanism, that it is tissue specific, and that it is specifically adapted to the particular needs of skeletal muscle.

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