MODULATION OF PYRUVATE KINASE ACTIVITY IN THE RAT UTERUS

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CONTENTS

LIST OF ABBREVIATIONS

FDP; Fructose 1,6-diphosphate

ICD; Isocitrate dehydrogenase

HK; Hexokinase

PFK; Phosphofructokinase

PK; Pyruvate kinase

ATPase; Adenosine triphosphatase

NAD; Nicotinamide-adenine dinucleotide

NADH; Nicotinamide-adenine dinucleotide (reduced)

NADP; Nicotinamide-adenine dinucleotide phosphate

NADPH; Nicotinamide-adenine dinucleotide phosphate (reduced)

ADP; Adenosine diphosphate

ATP; Adenosine triphosphate

Cyclic AMP; Adenosine 3',5'-monophosphate

DNA; Deoxyribonucleic acid

L-Phe; L-Phenylalanine

PEP; Phosphoenolpyruvate

p-CMB; p-Chloromercuribenzoate

EDTA; Ethylenediamine tetraacetate

EGTA; Ethyleneglycol-bis(β-amino-ethyl ether)N,N'-tetraacetic acid

GDP; Guanosine diphosphate

GTP; Guanosine triphosphate

UDP; Uridine diphosphate

CDP; Cytosine diphosphate

TDP; Thymidine

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INTRODUCTION

The mammalian organism is constantly kept in a dynamic steady state through the delicate co-ordination of a series of metabolic reactions. One of the basic requirements in maintaining homeostatic equilibrium is the capacity of body tissues to alter the rate of metabolic reactions underlying various physiological processes. This is accomplished by supplying or cutting off the production or availability of metabolites and hormones. As a result of the ability to adapt itself to alterations in metabolic environment, an efficiently functioning organism is maintained in a homeostatic balance. At the molecular level, this ability to increase or decrease biochemical reaction rates in body tissues is entirely dependent upon the catalytic activities of a wide array of enzyme systems. A state of health is maintained as long as the development and operation of strategically located enzyme systems proceed smoothly and the various metabolic processes operate in perfect co-ordination.

Under normal physiological conditions, various enzyme activities exhibit a remarkable degree of constancy in face of a continuous turnover of numerous components of the enzyme forming system. The synthesis and degradation of enzymes is determined by genetic directives manifested through DNA-directed transfer of information to messenger RNA and the activity of template RNA on ribosomal sites. This constitutes a quantitative change in the activity of an enzyme and is due to effects exerted on the protein-synthesizing apparatus of the cell. Hormone action through induction of several important enzymes is now a well established phenomenon for a variety of mammalian tissues. A second

mechanism whereby cellular enzyme activity is altered involves qualitative changes in the catalytic properties of an enzyme. A number of specific metabolites have been demonstrated to interact with the enzyme molecule and produce positive or negative modulation of enzyme activity. The control of an enzyme through this mechanism is acute in nature since rapid changes are produced in its activity as a result of enzyme-modifier interactions. The ability of endogenous and foreign metabolites to acutely modify enzyme activity in mammalian tissues has been reviewed by Atkinson (1) and by Stadtman (2). Monod et al. (3) presented a highly specific model for general interpretations of protein-substrate-modifier interactions and suggested that certain modulators affect allosteric sites of regulatory enzymes.

Pyruvate kinase, which catalyzes the conversion of phosphoenolpyruvate and ADP into pyruvate and ATP is one of the important pacemaker enzymes in the glycolytic scheme of reactions in several mammalian tissues. Considerable work has been carried out on the hormonal and nutritional regulation of this enzyme in tissues such as the liver, prostate gland and seminal vesicles. Recently, studies have also been reported on the estrogenic and progestational control of the de novo synthesis of pyruvate kinase in the rat uterus (4). However, little or no attention has been focussed on examining the influence of specific metabolites which act as modulators of uterine pyruvate kinase activity under conditions of acute adaptation. The aim of the present study was to examine the modulation of pyruvate kinase activity by several metabolites in uteri of normal, mature rats. In particular, the effects of L-alanine, L-phenylalanine, copper, penicillamine, calcium, FDP, cyclic AMP and the

sulphydryl inhibitor, p-chloromercuribenzoate were investigated *in vitro* on the activity of uterine pyruvate kinase.

II. LITERATURE REVIEW

A. SOME IMPORTANT ENZYMES OF CARBOHYDRATE METABOLISM

The maintenance of glucose homeostasis is of fundamental importance for the existence of mammalian organisms since glucose constitutes one of the major fuels in day-to-day metabolic activities of many tissues and organs. Glucose is not only used during metabolism to generate biological energy in the form of ATP, but is converted into proteins, nucleic acids and lipids. The pentose phosphate pathway, which is involved in the direct oxidation of glucose, plays a significant role in its utilization toward the synthesis of nucleic acids and lipids. Liver and kidney are the two major tissues which are endowed with the capacity to form glucose from non-carbohydrate precursors. Glycogen, being the potential source of glucose, is stored mainly in liver and muscle tissue. Figure 1 illustrates the location of certain important key rate-limiting enzymes of carbohydrate metabolism involved in the processes of glycogenesis, glycogenolysis, glycolysis, gluconeogenesis as well as those in the phosphate shunt pathway. The phosphatases and the kinases of the glycogen phosphorylase and glycogen transferase systems have not been included in this figure. Glucose production depends on the functioning of the final common path of gluconeogenesis which encompasses the enzyme steps between lactate and glucose. From the point of view of function, direction of metabolic flow and regulatory significance, various enzymes between glucose and lactate can be divided into three groups (5).

(a) Key Gluconeogenic Enzymes

Glucose 6-phosphatase, fructose 1,6-diphosphatase, phosphoenolpyruvate

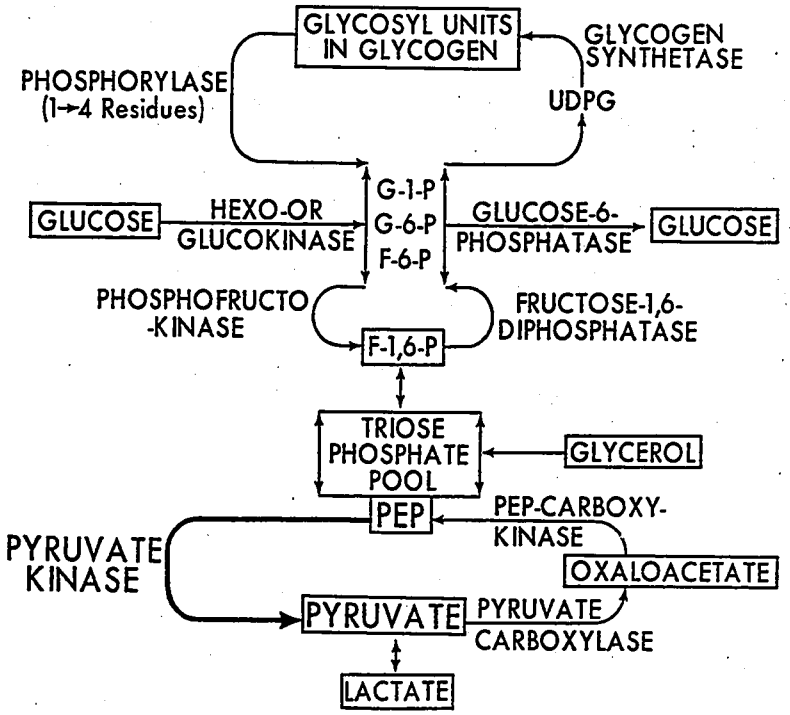


Fig. 1. Hexokinase (or glucokinase), phosphofructokinase and pyruvate kinase which play a key rate-limiting role in the glycolytic pathway are shown on the left. The four key enzymes (pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose 1,6-diphosphatase and glucose 6-phosphatase) of the gluconeogenic sequence are pictured on the right side of the figure. Also shown are the enzymes involved in the synthesis and breakdown of glycogen. The enzyme pyruvate kinase catalyzes the terminal reaction in the conversion of glucose to pyruvate via the pathway of glycolysis.

carboxykinase and pyruvate carboxylase exert a rate-limiting role in the production of glucose from lactate and other gluconeogenic precursors on the basis of the following properties. These enzymes (1) have low activities, (2) govern one-way reactions, (3) are involved in circumventing thermodynamic barriers and (4) are localized chiefly or exclusively in organs capable of gluconeogenesis.

(b) Key Glycolytic Enzymes

Glucokinase, phosphofructokinase and pyruvate kinase are involved in channeling glucose into lactate. They act in the opposite direction to that of the key gluconeogenic enzymes. These enzymes (1) have low activities, (2) govern one-way reactions, (3) are involved in ATP-utilizing or producing reactions and (4) show little organ specificity.

(c) Bifunctional Enzymes

Bifunctional enzymes act in the direction of both gluconeogenesis and glycolysis. The properties of bifunctional enzymes, phosphohexose isomerase, aldolase, triosephosphate isomerase, phosphoglyceric acid dehydrogenase, phosphoglycerokinase, phosphoglyceromutase, enolase and lactic dehydrogenase contrast sharply with those of the gluconeogenic enzymes. These enzymes (1) have high activity, (2) catalyze reversible reactions, (3) have no thermodynamic barriers involved in their reaction and (4) are present in almost all organs of the body.

B. MECHANISMS OF ENZYMATIC CONTROL

Although differentiation, growth and development have been far

better described morphologically than biochemically, a variety of chemical factors are known to bring about the differentiation and thus determine the specific functions of the mature organ. As each organ differentiates, new enzyme systems appear and existing ones are augmented or diminished. Similarly, acute and adaptive alterations generated in the metabolic processes by the action of hormones, diet, drugs and various other environmental stimuli are affected either through activation and inhibition or actual synthesis of enzymes located at strategic positions in the metabolic pathways. Which enzymes change when they appear, and what factors control their activities and their emergence are questions of great interest and complexity in molecular biology. Within the organism there exist mechanisms of regulatory control which are necessary because of the transitory nature of body constituents. Were it not for such control mechanisms, our cellular processes would be operating at maximal rates with the expenditure of much energy and efficiency. Hence, the basic instinct of survival demands the existence of regulatory controls whereby maximal growth can be achieved with a minimal expenditure of precursors. It is also evident that the complex processes of growth and differentiation have control mechanisms which are capable of co-ordinating the biosynthetic and degradative pathways of metabolism. These mechanisms fall into two general categories.

(a) Repression

This system of regulatory control was discovered in 1953 by Monod and Cohen-Basine (6) and Vogel (7) in bacteria and subsequently was termed "repression" by Vogel in 1957 (8). Repression may be defined as

the inhibition of the rate of enzyme synthesis by an end product of a series of metabolites or a close derivative thereof. This hypothesis describes the control of protein synthesis as a result of the action of several genes; a regulator gene which produces a repressor substance, and one or more structural genes which elaborate the molecular structure of the protein. The repressor acts by controlling either directly or indirectly the structural gene. Repression as a regulatory mechanism is ubiquitous in bacterial systems but has also been encountered in mammalian organisms. For example, Pitot et al. (9) observed that glucose and fructose had the ability to repress the synthesis of hepatic serine dehydratase and ornithine transaminase induced by caseine hydrolysate. Another good example in mammalian tissues is the repression of hepatic phosphoenolpyruvate carboxykinase by glucose (10).

(b) End Product Inhibition (Negative Feed-Back Inhibition)

In this mechanism, the end product inhibits one of the initial enzymes involved in that particular series of reactions (11). The important distinction is that in end product inhibition, there occurs an inhibition of enzymatic activity and not of enzyme synthesis. Although generalizations from studies on micro-organisms regarding inhibition of the first enzyme of a given sequence by its end product can be extended to certain routes of carbohydrate metabolism in vertebrate tissues, many major metabolic pathways require different types of regulatory mechanisms since the continued operation of these processes in highly differentiated multi-cellular organisms is essential to their existence.

(c) Control by Metabolic Effectors

The effectors acting at the catalytic site of an enzyme (substrates, products and their structural analogues) are known to function through "kinetic control" while the metabolic ligands which affect enzyme activity through conformational alterations by acting on a site other than the catalytic site are known to exert an "allosteric control" (12). Complex control mechanisms are known to be operative at various points of interaction between metabolic pathways (e.g. pyruvate metabolism). Energy link is provided through ATP: ADP (AMP) ratios in which ATP is an inhibitor of phosphofructokinase and isocitrate dehydrogenase and AMP and ADP, as activators of these important enzymes, thus rendering both pathways (glycolysis and Krebs cycle) subject to regulation by adenine nucleotides (13). The reciprocal regulator activates an enzyme in one pathway and at the same time inhibits the opposing enzyme in the antagonistic pathway. For example, reciprocal regulation is accomplished by AMP which activates phosphofructokinase but inhibits fructose 1,6-diphosphatase; also acetyl-CoA which activates pyruvate carboxylase but inhibits the activity of pyruvate kinase (14).

(d) Miscellaneous Factors

(i) Influence of changes in pH. Most isolated enzyme systems exhibit a well defined pH optimum for maximal activity. Studies on several enzymes whose catalytic activity is modulated by metabolic effectors suggest that these enzymes may be unusually sensitive to alterations in pH. Thus, the activity of DPN-linked isocitrate dehydrogenase

(15), activation of phosphofructokinase by AMP (16) and of pyruvate carboxylase by acetyl-CoA (17) are markedly affected by any pH changes within the physiological range. However, the precise significance of pH in enzyme activity control can not be assessed since the extent to which the intracellular pH changes, is presently unknown.

(ii) Reversible active-inactive conversion of enzymes. In this case, the structure of an enzyme is modified into the active form catalyzing the reaction. In carbohydrate metabolism, the two well documented examples of this type of regulatory mechanism involve the interconversion of the phospho- and dephospho-forms of glycogen phosphorylase and transferase systems by specific kinases and phosphatases (18-20). Since the activity of kinases is affected by cyclic AMP, a mechanism is provided by which hormones such as glucagon and epinephrine can affect glycogen metabolism in a variety of mammalian tissues (20).

(iii) Isozymes. The existence of multiple molecular forms of enzymes is now a well known and a rather common phenomenon (21,22). In a single cell or tissue, there may be several forms of an enzyme, all of which carry out the same overall reaction, but have different physical and chemical properties. They may differ in their kinetic properties and in the rate of reaction with respect to different metabolic effectors (23). The existence of such variations in properties from one isoenzyme to another has provided another form of metabolic regulation. The presence of many isozymes in liver tissue has demonstrated that in many instances, this organ appears to have its own unique set of iso-

enzymes (24,25).

(iv) Enzyme binding to macromolecules. Relatively little is known about this form of regulatory mechanism. Its possible importance is demonstrated in the following example: glycogen is known to interact and stabilize phosphorylase b kinase (26) and phosphorylase a (27) and to inhibit glycogen synthetase phosphatase (19) with the end result being glycogenolysis and inhibition of glycogen synthesis. Since glycogen storage varies with the nutritional state of the animal (28) and since different glycogen species bind preferentially to phosphorylase and synthetase (29), formation of such complexes may allow glycogen to play an important role in the regulation of its own metabolism.

C. EVALUATION OF POTENTIAL CONTROL SITES

The search for enzymes participating in pathways of carbohydrate metabolism which are susceptible to regulation by one or more factors indicated the existence of many control sites. Although most of the work has been carried out on irreversible and low catalytic activity enzymes under *in vivo* conditions, it emerged that the reaction involved was rate-limiting for the particular overall metabolic pathway. The method most widely employed for detecting a rate-limiting step involved the application of the crossover theorem (30) to the studies in which the levels of various intermediates of a given pathway are measured during a transition period between two steady states. Another alternative for locating the rate-limiting step has been to study only those reactions which are far removed from equilibrium under *in vivo* conditions.

Such reactions are identified by measuring the mass action ratios under both *in vivo* and *in vitro* conditions. The regulatory significance of such reactions far from thermodynamic equilibrium *in vivo* is then determined by measuring the substrate levels under conditions of altered overall flux in the pathway concerned.

The difficulties involved in such studies are related to the possible existence of a heterogenous population of cells in a single tissue (e.g. brain in contrast to liver and kidney), multiple pools of intermediates (e.g. gluconeogenic as contrasted to glycolytic intermediates), and to the precise time intervals to be chosen which truly represent the transition phase. It may be mentioned that the use of kinetic properties of an isolated enzyme as a guideline for identification of regulatory factors, has also been used although this may sometimes be hazardous. First, the properties of an enzyme are, in most cases, inferred from studies performed at very low enzyme concentrations, while the intracellular enzyme concentrations may be much higher than those used for studies on isolated enzymes. Secondly, the intracellular environment is generally markedly different from *in vitro* assay conditions and thirdly, the procedures employed for isolation of enzymes may actually alter their properties causing changes such as loss of sensitivity to certain metabolic effectors.

D. HOMEOSTASIS AND GENERAL REGULATORY SYSTEMS

Homeostasis may be defined as a situation in which both internal and external factors are functioning harmoniously. External factors may be physical, ecological or nutritional, while the internal factors

consist of genetic, neural and hormonal systems. The vital factors controlling subcellular metabolic regulation include the autonomic nervous system and the hormonal and nutritional control of individual enzymes.

(a) Autonomic Nervous System

In considering regulatory mechanisms in multicellular organisms, the autonomic nervous system undoubtedly plays an important role. The hypothalamus holds a central position carrying out swift regulation through mediation of the sympathetic and parasympathetic components of the autonomic nervous system. Generally speaking, it could be stated that norepinephrine is the neurotransmitter at the sympathetic nerve endings with tyrosine hydroxylase (31,32) being the rate-limiting enzyme in its biosynthesis. The chemical agent elaborated by the parasympathetic system is acetylcholine; choline acetylase and acetylcholine esterase are the enzymes involved in its metabolism. It is well documented that the sympathetic and parasympathetic nerves act mostly in reciprocity on the respective target organs. Stimulation of the sympathetic nerve results in a very rapid response, while that of the parasympathetic evokes a relatively slow response. In keeping with these physiological aspects, Shimazu et al. (33,34) demonstrated that electrical stimulation of the splanchnic nerve (sympathetic) of rabbits (intact or pancreatectomized) caused a marked increase in glycogen phosphorylase and glucose 6-phosphatase activities as well as an increased blood glucose and decreased liver glycogen. These alterations occurred in liver within thirty seconds, while stimulation of the vagus nerve (parasympathetic)

had no effect on these enzymes. In point of fact, it counteracted the metabolic changes brought about by stimulation of the sympathetic nerve. The activity of glycogen synthetase was increased within 5 minutes after stimulation of the parasympathetic nerve. In contrast, stimulation of the sympathetic nerve produced no effect, although it effectively counteracted the changes produced by stimulation of the parasympathetic nerve. It was further observed that alterations brought about by sympathetic stimulation is due to activation (actinomycin-insensitive) of the inactive form while the recuperative process induced by parasympathetic stimulation was accompanied by enzyme synthesis de novo (actinomycin-sensitive). All of these studies indicate that the autonomic nervous system exerts an important role in enzyme regulating mechanisms in certain animal tissues.

(b) Hormonal and Dietary Control

During the last 15 years, a multitude of studies has shown that enzyme levels in animal tissue can be altered by a wide variety of nutritional and hormonal manipulations. The list of enzymes so affected is remarkably large and includes examples from every major metabolic pathway in one or more tissues. In the next few pages, an attempt has been made to summarize such changes, mainly with respect to enzymes of the glycolytic and gluconeogenic pathways in hepatic tissue. This tissue has been chosen by most investigators simply because of its ready availability and relatively homogenous nature with respect to cell-type population. One of the major physiological functions of liver is to modify ingested nutrients to maintain constancy of the milieu interieur. It is therefore not unexpected that liver enzyme levels fluctuate the

greatest in response to various stimuli in vivo. Sometimes it is extremely difficult to know whether the hormonal or nutritional stimulus acts directly on an enzyme system or is a consequence of secondary compensatory responses of the intact animal. In order to by-pass some of these complexities, use has also been made of isolated organs or cell culture systems by certain investigators.

(i) Hormonal regulation. Tryptophan pyrrolase was the first liver enzyme to increase with the administration of glucocorticoids (35) and subsequently also by tryptophan and its analogues (36). Feigelson and Greengard (37) suggested that the mechanisms involved in the induction of hepatic tryptophan pyrrolase by glucocortical hormones and by tryptophan were different. It was demonstrated that, while hydrocortisone increased the biosynthetic rate of this enzyme, treatment with tryptophan diminished the rate of enzyme degradation (38). Tyrosine transaminase was another important enzyme found to be increased by glucocorticoid treatment (39). Kenney and his group (40) later showed that both insulin and glucagon could also increase the rate of synthesis of tyrosine transaminase in rat liver. Chronic administration of glucocorticoids was found to induce the activity of hepatic glutamate-alanine transaminase (41,42). Glucocorticoid hormones were also observed to function as inducers of hepatic glucose 6-phosphatase, fructose 1,6-diphosphatase, phosphoenolpyruvate carboxykinase and pyruvate carboxylase (5), the four rate-limiting enzymes of the gluconeogenic pathway. Evidence has been presented that one and the same hormone can result in a reciprocal regulation of catabolic and anabolic enzymes. For example, growth hormone can induce 3-phosphoglycerate

dehydrogenase (catalysing serine synthesis) while at the same time suppressing the activity of serine dehydratase (catalysing serine degradation) (43). Conversely, hydrocortisone suppresses the biosynthesis of serine and at the same time can promote its catabolism (43). Similarly, Weber et al. (44) demonstrated that insulin acts in a dual capacity as an inducer of key glycolytic enzymes and as a suppressor of the biosynthesis of gluconeogenic enzymes in hepatic tissue.

Insulin acts as an inducer for glycogen synthetase (45) for the three strategic glycolytic enzymes (44), and for the key enzymes of fatty acid synthesis (46), thus resulting in an increase in glucose uptake, glycogenesis, glycolysis and lipogenesis. On the other hand, insulin acts as a suppressor of the four key gluconeogenic enzymes. Thus, insulin integrates hepatic metabolic function at the enzyme level resulting in increased metabolic utilization and storage of glucose and decreased production of this hexose. Insulin action is emphasized by its attacking at key metabolic points where metabolic flow direction is determined and by its acting antagonistically on the biosynthesis of opposing enzyme systems. In alloxan diabetes the activities of the four hepatic key gluconeogenic enzymes are markedly increased, whereas the opposing three glycolytic enzymes are decreased. Insulin brings enzyme activities to normal by simultaneously decreasing the biosynthesis of the gluconeogenic enzymes and inducing the biosynthesis of the glycolytic enzymes. When ratios of activities of the rate-limiting enzymes of gluconeogenesis and glycolysis were calculated roughly (glucose 6-phosphatase/glucokinase; fructose 1,6-diphosphatase/phosphofructokinase; phosphoenolpyruvate carboxykinase + pyruvate carboxylase/pyruvate kinase), it was found that

these ratios in the diabetic rat increased 30, 6 and 13-fold, respectively. Insulin returned these ratios to normal. Thus, insulin achieves its integrative function by a simultaneous but antagonistic action on opposing rate-limiting enzyme systems at the branching points of metabolism.

(ii) Dietary regulation. Considerable evidence has accumulated which indicates that a direct relationship exists between the activity of many enzymes and the nutritional state of the animal. Arginase, one of the enzymes involved in amino acid metabolism, was found to be increased when animals were maintained for 2 weeks on a high protein diet. The observed rise was due to increased enzyme synthesis rather than a decreased rate of its degradation (47). When rats were maintained on a protein-free diet for 5 days and then force-fed either casein hydrolysate or an equimolar mixture of eight essential amino acids, the enzyme serine dehydratase increased almost 100-fold (48-50). Simultaneous administration of glucose or fructose completely abolished the observed effects of amino acids on hepatic serine hydratase. The activity of acetyl CoA carboxylase, a rate-limiting enzyme in lipid biosynthesis, was found to be increased over 25-fold in animals maintained on a fat free diet, when compared to those fasted for several days (51). Using the combined immunologic and isotopic techniques, Majerus and Kilburn (52) observed that degradation of enzymes is accelerated in starved animals while enzyme synthesis is increased in animals fed a fat free diet.

Krebs and Eggleston (53) reported that the activity of hepatic pyruvate kinase decreased to about one third on changing from a standard diet to a low-carbohydrate diet. When the animals were kept on a high-

carbohydrate diet, pyruvate kinase activity rose more than three-fold of the control values. Hepatic glucokinase decreased to extremely low levels during starvation and reappeared on refeeding (54). The activity of high Km glucokinase also decreased markedly in livers of alloxan-diabetic rats; enzyme activity was restored to the normal range upon administration of insulin (55-57). Treatment with glucose was effective in provoking the reappearance of glucokinase in fasted rats while the low Km hexokinase remained essentially unaltered. Inhibition of the glucose or refeeding-induced restoration of glucokinase by several inhibitors of RNA and protein synthesis suggested that an actual de novo synthesis of the enzyme may have been involved.

E. GLYCOLYTIC CONTROL MECHANISMS

Since the maximal catalytic activity of the enzymes of glycolytic pathway exceeds the observed rates of glycolysis in several tissues examined (58), the control of the activities of various rate-limiting enzymes and the availability of substrates are probably the major factors contributing to regulation of the glycolytic flux. Comparison of the mass action ratios for the glycolytic enzymes in perfused rat heart (59) and guinea pig cerebral cortex slices (60) with equilibrium constants measured for the isolated enzymes under similar conditions, indicated that the activities of hexokinase, phosphofructokinase, pyruvate kinase and of glyceroaldehyde 3-phosphate dehydrogenase are far from equilibrium in vivo. The rate-limiting steps in the glycolytic pathway have also been defined by application of the cross-over theorem (30) to measurements of the levels of glycolytic intermediates before and after perturbation of the metabolic flux. Such pertubations have been induced by an

aerobic to anaerobic transition (increased flux) in yeast and mouse brain and by addition of acetate or fluoroacetate (decreased flux) in perfused rat hearts (61-65). In all cases, a cross-over was obtained between fructose 6-phosphate and fructose 1,6-diphosphate indicating that the glycolytic flux is controlled primarily at the level of phosphofructokinase. The increased flux observed during aerobic-anaerobic transition probably results from decreased levels of ATP and increased levels of fructose 1,6-diphosphate and AMP which cause activation of phosphofructokinase (66). On the other hand, the decreased flux observed after addition of acetate or fluoroacetate to perfused rat hearts may result from the accumulation of citrate (59,65,67,68) since citrate is a potent inhibitor of phosphofructokinase (69).

(a) Control of Glycolysis in Gluconeogenic Tissues

In both liver and kidney, the contributions of the pentose phosphate pathway and of glycerol to glucose 6-phosphate utilization complicate precise estimates of the glycolytic flux. However, the observed flux is considerably lower than the maximal catalytic activities of key glycolytic enzymes in these tissues. Although alterations in various glycolytic enzymes have been achieved by administration of hormones or changes in dietary status, regulation by substrate availability and metabolic effectors may also provide a primary control mechanism in these tissues. A cross-over between glucose 6-phosphate and fructose 1,6-diphosphate has been reported to occur in rat kidney slices when the glycolytic flux is either increased by anoxia and cyanide or decreased by short-chain fatty acids or ketone bodies (70). The increased flux

induced by anoxia or cyanide reduces ATP: AMP, which in turn causes marked activation of phosphofructokinase (69).

(b) Estradiol Action on Uterine Glycolytic Enzymes

Hormonal regulation of the functional differentiation of accessory sex organs entails an increase in the synthesis of many proteins including those enzymes which govern secretory activity, cellular maintenance and physiological function. Evidence indicates that estrogenic hormones regulate uterine glucose metabolism by exerting their effects on receptor sites at the source of enzyme formation to turn on or off the biosynthesis of certain enzymes involved in carbohydrate metabolism (71,72). The activities of hexokinase (73), phosphofructokinase (74) and pyruvate kinase (4) decreased markedly on castration and returned to the normal range following estrogen treatment. Investigations with estriol, estrone, diethylstilbestrol and estradiol-17β indicate that the enzymatic responses to these estrogens parallel their known physiological potencies with estradiol-17β being the most potent inducer of these enzymes. While a single injection of estradiol-17β (10.0 μg/100 g) by the intramuscular route produced significant increases in enzyme activities at 4 hours, greater increases as early as 2 hours were observed after intraperitoneal or intravenous administration of this hormone. The increases in uterine enzyme activities were dose-dependent. The estradiol-induced enhancement of uterine glycolytic enzymes was blocked effectively by actinomycin, puromycin, cycloheximide, 5-fluorouracil and ethionine, indicating that these enzymatic responses involve stimulation of both RNA and protein synthesis. Testosterone and progesterone were also capable of antagonizing

the estradiol-induced increases in the activities of uterine hexokinase, phosphofructokinase and pyruvate kinase; these observations emphasize the importance of hormonal interactions in the regulation of uterine metabolism. The estrogen-stimulated changes in uterine enzymes showed positive correlation with the enhanced production of lactate and α-glycerophosphate by uterine supernatants (75,76). The results obtained are consonant with the suggestion that estrogenic hormones induce simultaneously the de novo synthesis of all three of the key rate-limiting glycolytic enzymes to amplify the process of glycolysis in uterine tissue (72).

F. ROLE OF PYRUVATE KINASE IN GLYCOLYSIS (ATP-pyruvate-phosphotransferase).

The enzyme pyruvate kinase plays an important role in the overall control of uterine glycolysis. This enzyme catalyses the formation of pyruvate and ATP from phosphoenolpyruvate and ADP. For all practical purposes, the reaction is irreversible with a high negative value for the free energy change (F^{o}, = -5.72). The exergonic nature of pyruvate kinase (77) and other glycolytic enzyme reactions led Sir Hans Krebs (78) to postulate that the carbohydrate synthesis by direct reversal of glycolysis was impossible under physiological conditions. The product of pyruvate kinase catalyzed reaction (pyruvate) occupies a central position in carbohydrate metabolism as shown in Figure 2.

In glucose forming tissues such as liver and kidney, the process of gluconeogenesis requires not only active pyruvate carboxylase and phosphoenolpyruvate carboxykinase, but also simultaneous inhibition of pyruvate kinase which has a rather high turnover of activity. An unimpeded pyruvate kinase activity would result not only in impaired glucose formation, but also in a futile cycle resulting in the wasteful

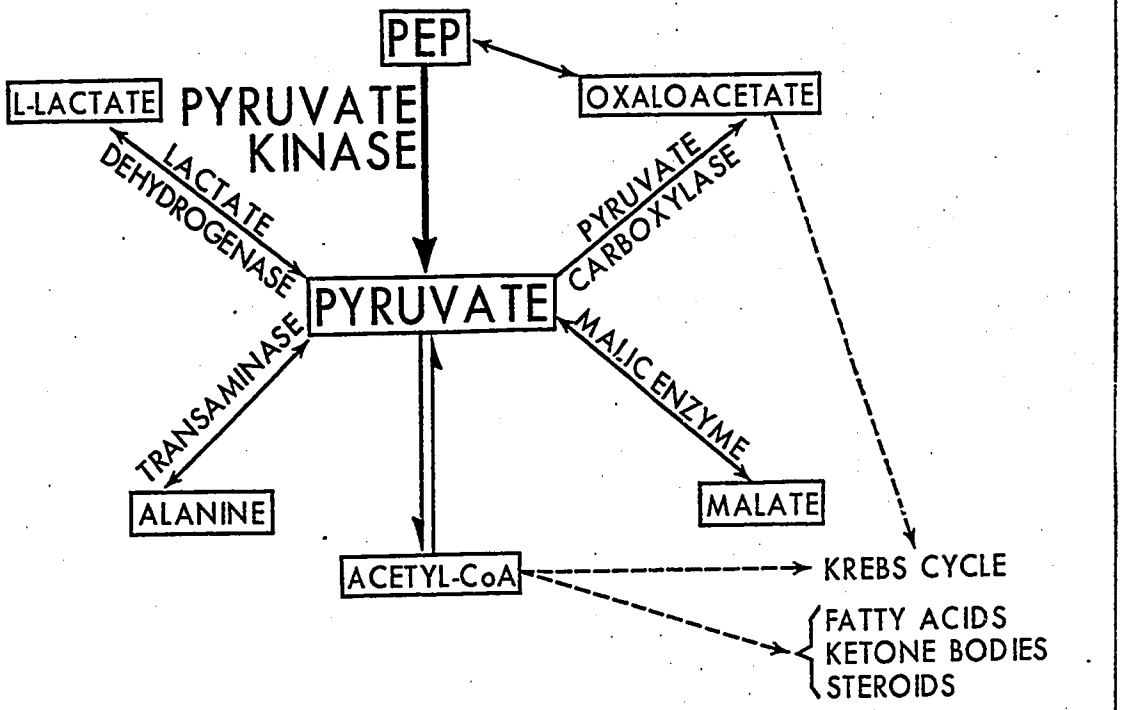


Fig. 2. Bold face letters indicate the conversion of PEP to pyruvate catalyzed by the enzyme pyruvate kinase. Pyruvate may be converted to malate (malic enzyme), lactate (lactate dehydrogenase), alanine (transaminase) and to oxaloacetate (pyruvate carboxylase). Pyruvate is also metabolized to acetyl-CoA which is then channeled into the Krebs cycle and the formation of fatty acids, ketone bodies and steroids.

breakdown of ATP. This enzyme is also of great interest to clinicians since a genetically determined deficiency of erythrocyte pyruvate kinase results in a severe hemolytic anemia (79) and also because of the easy vulnerability of pyruvate kinase in phenylketonuric fetuses leading to severe metabolic damage and dysfunction in the neonatal brain (80). A severe deficiency in the important ATP generating reaction in glycolysis catalyzed by pyruvate kinase, and the consequent interference with the ability to oxidize NADH at the following lactic dehydrogenase step presumably imposes a metabolic handicap leading to premature erythrocyte destruction in the body, hemolytic anemia, and the attendant clinical manifestations of the disease. In addition, the possible relevance of pyruvate kinase in Wilson's disease (81,82), characterized by accumulation of abnormal amounts of copper, has further served to focus attention on the metabolic potential of pyruvate kinase in brain and other tissues.

(a) Regulation of Hepatic Pyruvate Kinase Activity

Krebs and Eggleston (53) were the first to report marked alterations in pyruvate kinase activity of liver without any concurrent changes in the kidney cortex enzyme when starved rats were fed a high carbohydrate diet. These investigators suggested that whereas low pyruvate kinase activity (starvation or low carbohydrate diet) favours gluconeogenesis, high activity of the enzyme favours the degradation of carbohydrate and its conversion into fat. Pyruvate kinase activity could therefore play a key role in the switch-over from glycogen storage to fat storage. Weber et al. (44) reported that the overall activity of hepatic pyruvate kinase was markedly reduced in alloxan-diabetic animals which returned to the normal range on insulin treatment. The fact that

hepatic pyruvate kinase, but not the skeletal muscle enzyme, was influenced by dietary (53) and hormonal manipulations (83) suggested that the enzyme in these two tissues might be different. Indeed, subjecting the crude liver extracts to further purification, two types of pyruvate kinase have been identified by electrophoretic mobility and immunological procedures. It was demonstrated that the major component, designated as type-L, was the one which increased upon administration of insulin or a high carbohydrate diet. In contrast, the type-M enzyme, which resembled pyruvate kinase from skeletal muscle, showed only little or no change in response to hormonal or dietary treatment. Furthermore, in both regenerating liver and hepatoma cells, most or all of the pyruvate kinase activity was of the M-type (83).

The type-M enzyme which remains relatively unchanged by dietary modifications and increases in regenerating liver and certain hepatomas, appears to be related to the degree of differentiation of the organ. Since the total hepatic pyruvate kinase activity was found to be increased during the weaning period, Middleton and Walker (84) examined the nature of the various forms of hepatic pyruvate kinase at different stages of development. It was observed that the M-type enzyme which is neither stimulated by fructose 1,6-diphosphate nor inhibited by copper, exhibited high activity in the fetal liver but decreased during late gestation. The activity of type-M pyruvate kinase fell to a low value after birth and remained constant throughout the developing period. In contrast, the activity of L-type enzyme was low in early fetal and neonatal livers but increased markedly at the onset of weaning. While searching for regulatory mechanisms which selectively control the activity of both

types of pyruvate kinase, Passeron et al. (85) found that the L-type enzyme (precipitated by 20-45% ammonium sulfate) was strongly inhibited by copper; almost complete inhibition being achieved with copper ion concentration of 0.006 mM. The effect of other metal ions was also examined on the activity of pyruvate kinase type-L. Only zinc (at a concentration of 0.066 mM) produced an inhibition of 70%, whereas calcium, barium, manganese, iron, cadmium and lead ions showed no appreciable effects. It was further observed that the inhibition by copper of L-type pyruvate kinase was completely "reverted" by fructose 1,6-diphosphate. Copper inhibition of L-type pyruvate kinase activity also was "reversible" by ethyleneglycol bis (aminoethylether) tetra-acetic acid and other chelating agents like ethyleneamine tetraacetate and 8-hydroxyquinoline. However, fructose 1,6-diphosphate proved by far the most effective in reversing the inhibition produced by copper. It is of interest that both fructose 1,6-diphosphate and various chelating agents by themselves exerted activating effects (20-100%) on the L-type enzyme activity when measured in presence of low concentrations of the substrate. However, copper, fructose 1,6-diphosphate and chelating agents failed to exert any such effects on pyruvate kinase type-M. Following preincubation at 37°C, the type-L enzyme declined in sensitivity to inhibition by copper while the activation effect of fructose 1,6-diphosphate in the absence of the inhibitor was markedly increased. Since copper concentrations in liver of adult mammals are well over the range (86,87) which produce inhibition of the L-form of pyruvate kinase "in vitro," this type of the enzyme may normally remain inhibited in liver so that its activity "in vivo" could be controlled by small variations in fructose

1,6-diphosphate which acts selectively on the L-form of hepatic pyruvate kinase.

Bailey, Stirpe and Taylor (88) also investigated the effects of the interaction between copper ions and fructose 1,6-diphosphate on L-type pyruvate kinase in hepatic tissue. These workers observed that the activity curves for the L-type enzyme had two components; one saturated at a low concentration of phosphoenolpyruvate (Km = 0.1 mM) and conforming th Michaelis-Menten kinetics (L_a-form) while the other exhibiting sigmoidal kinetics with a half maximal activity at about 1 mM (L_b-form) concentration of the substrate. The L_a (low Km form) form was not affected by fructose 1,6-diphosphate while the type L_b (high Km form) was allosterically stimulated by fructose 1,6-diphosphate. Preincubation of the liver extract at 25°C for 10 minutes converted L_a into L_b form causing a marked increase in co-operativity with respect to both phosphoenolpyruvate and fructose 1,6-diphosphate. Although these authors found a similar inhibitory effect of copper on the L-type enzyme as that reported by Passeron et al. (85), some points merit further consideration. Passeron and his co-workers (85) reported reversal of copper ion inhibition by fructose 1,6-diphosphate and EDTA for which Bailey and his collaborators found little evidence. The latter authors made distinction between protection and reversal and preferred to call the antagonizing action of fructose 1,6-diphosphate, EDTA and other chelating agents as protection since they were able to prevent inhibition only when added before, but not if after copper had already been added to the incubation medium. Also, the substrate phosphoenolpyruvate at higher concentrations was found to be effective in protecting the enzyme inhibition by copper. The observed

effects of phosphoenolpyruvate and fructose 1,6-diphosphate were accounted for by their ability either to chelate copper or alter the conformation of the enzyme rendering the sites less accessible for copper ions. These authors also confirmed the findings that high carbohydrate diet containing fructose can increase pyruvate kinase activity only in liver but not in kidney, brain, heart and spleen, which is in accord with the observations that only the L-type activity is influenced by diet and that only liver may contain L-type enzyme (85). While studying the influence of pH, it was observed that acidity was detrimental to the enzyme activity irrespective of the concentration of the substrate (phosphoenolpyruvate) or the activator (fructose 1,6-diphosphate) used. In the pH range of 7 to 8, L-type enzyme activity was inactivated, particularly at low substrate concentrations regardless of whether fructose 1,6-diphosphate was present or not.

(i) Effects of nucleotides. Weber et al. (14) examined the influence of various nucleotides on pyruvate kinase activity in liver and hepatoma cells. The nucleotide, ADP, participates in pyruvate kinase reaction as an acceptor. Among the diphospho nucleotides (ADP, GDP, UDP, IDP, CDP and TDP) tested, ADP was found to have maximum affinity for hepatic and hepatoma pyruvate kinase. In contrast, all triphosphates examined proved to be inhibitory, ATP being the most potent followed by GTP. The inhibitory effects exerted by GTP may be of particular interest since GTP is an activator as well as a cofactor in the reaction catalyzed by phosphoenolpyruvate carboxykinase which opposes the reaction catalyzed by pyruvate kinase. This type of reciprocal inhibition which plays an

important role in metabolic regulations has also been implied in the actions of AMP and acetyl-CoA; AMP being an inhibitor of fructose 1,6-diphosphatase and an activator for phosphofructokinase while acetyl CoA being an activator for pyruvate carboxylase and an inhibitor for pyruvate kinase. Thymidine triphosphate, which is a feedback inhibitor of several important enzymes of nucleic acid biosynthesis, was also observed to be a potent inhibitor for pyruvate kinase with an apparent K_i of 0.39 M for liver and 0.33 M for hepatoma pyruvate kinase when assayed in the presence of low levels of ADP (14).

(ii) Effects of free fatty acids, acetyl CoA, NADH and alanine.

A rise in the levels of free fatty acids and acetyl-CoA in liver has been reported in many metabolic states such as starvation, diabetes and on glucagon or cortisol treatment. These circumstances which are associated with a predominance of gluconeogenesis over glycolysis, suggested a role for these various metabolites in controlling the activities of glycolytic enzymes. Studies undertaken by Weber et al. (89) demonstrated that free fatty acids exerted inhibitory action on hepatic pyruvate kinase, which was dependent upon the dose of free fatty acid and the length of preincubation. The effective concentration of laurate, myristate and octanoate, which inhibited pyruvate kinase activity, was within the physiological range of their levels reported to be present in the plasma of diabetic rats and humans (90-92). Addition of acetyl-CoA was also found to produce inhibition of hepatic pyruvate kinase in a dose-dependent manner. In addition, NADH which increases during lactate to pyruvate conversion, inhibited non-competitively the activity of hepatic pyruvate kinase,

the inhibition being more pronounced upon preincubation. It is of interest that the effective concentrations of NADH to inhibit pyruvate kinase under in vitro conditions were considerably higher than those found in liver under the conditions of increased gluconeogenesis. Seubert and co-workers (93) found that alanine was also capable of producing marked inhibition of liver pyruvate kinase. L-Alanine increased the Km for phosphoenolpyruvate and protected the enzyme from thermal inactivation in both liver and hepatoma cells. D-Alanine, on the other hand, neither inhibited the activity of hepatic pyruvate kinase nor protected the enzyme from thermal inactivation (94).

(b) Muscle and Brain Pyruvate Kinase

It became apparent from the studies of Bailey et al. (88) and of Tanaka et al. (83) that muscle pyruvate kinase is not adaptive to diet or hormonal stimuli. The muscle enzyme, when subjected to starch block zone electrophoresis, was observed to yield a single peak in contrast to 4 bands seen for liver extracts. The muscle enzyme (like the hepatic M-type) exhibited normal Michaelis Menten kinetics towards phosphoenolpyruvate and the Km value for this substrate was noted to be around 8.7×10^{-5}M. In contrast to hepatic L-type pyruvate kinase, the skeletal muscle enzyme was totally insensitive to feed forward control by fructose 1,6-diphosphate and was only slightly affected by ATP and copper. Weber et al. (94) demonstrated that L-alanine which was a competitive inhibitor of hepatic pyruvate kinase, failed to affect the muscle enzyme. In addition, muscle pyruvate kinase was shown to be one order of magnitude less sensitive than the hepatic L-type to the non-

competitive inhibition produced by acetyl-CoA (89).

In contrast to hepatic pyruvate kinase, the enzyme activity in rat and human brain was markedly inhibited by L-phenylalanine with an apparent K_i of 8.5 mM. Unlike pyruvate kinase in liver, L-alanine failed to produce any appreciable effect on the brain enzyme (14). While fructose 1,6-diphosphate stimulated pyruvate kinase in liver, it exerted no significant effect on enzyme activity from the brain (82). Schwark et al. (82) also noted that the inhibition of brain pyruvate kinase by Cu^{++} and L-phenylalanine could be prevented and reversed by L-alanine. Although the cerebro-cortical enzyme was resistant to thermal inactivation at 37°C, incubation of the enzyme preparation at 55°C for 60 minutes resulted in approximately 60% inhibition of its activity. Preincubation with L-alanine provided partial protection against thermal inactivation of the enzyme. Although L-alanine exerted no effect on the non-competitive inhibition of pyruvate kinase produced by calcium, such inhibition was prevented and reversed by EDTA. The sulphydryl inhibitor, p-chloromercuribenzoate, produced a dose-dependent inhibition of cerebro-cortical pyruvate kinase, whereas addition of penicillamine resulted in a slight activation of the enzyme. Although inhibition of pyruvate kinase by p-chloromercuribenzoate was unaffected by L-alanine, penicillamine effectively prevented and reversed the inhibition produced by p-chloromercuribenzoate.

(c) Pyruvate Kinase in Adipose Tissue, Prostate and Seminal Vesicles

Tanaka et al. (95) reported that rat adipose tissue contained the fructose 1,6-diphosphate insensitive type-M enzyme. This was later

confirmed by Pogson (96) who also demonstrated that in presence of EDTA, the enzyme could be converted to a fructose 1,6-diphosphate-sensitive form which showed similar electrophoretic pattern as the liver L-type enzyme. Recently, Vijayvargiya et al. (97) studied the modulation of pyruvate kinase activity by several metabolites in the prostate gland and seminal vesicles of normal mature rats, and concluded that the enzyme in these male sexual tissues might be similar to the M-type isozyme. Whereas direct addition of fructose 1,6-diphosphate to the reaction mixture exerted no effect on pyruvate kinase activity, slight activation of the prostatic and seminal vesicular enzyme was produced by L-alanine. Pyruvate kinase activity in both accessory sexual tissues was rapidly inactivated by incubation of the supernatant fluids at 37°C; this activation was prevented by L-alanine. Whereas L-phenylalanine was found to be a competitive inhibitor of prostatic and vesicular pyruvate kinase, L-alanine was capable of preventing and reversing the inhibition produced by L-phenylalanine. Pyruvate kinase activity in both tissues was also inhibited by Cu^{2+} in concentrations as low as 0.005 mM; 50% inhibition of the enzyme was produced by 0.2 mM Cu^{2+}. Addition of L-alanine prevented as well as reversed the inhibition produced by low concentrations of this cation. Although the precise mechanism by which L-alanine modulates pyruvate kinase activity in the prostate and seminal vesicles was not fully elucidated, its ability to activate and protect the enzyme against Cu^{2+} and L-phenylalanine inhibition was considered to be consistent with an allosteric regulatory mechanism. The sulphydryl inhibitor, p-CMB produced a dose-dependent inhibition of prostatic and vesicular pyruvate kinase whereas addition of penicillamine resulted in a slight activation

of the enzyme. In addition, penicillamine effectively reversed the inhibition produced by p-CMB as well as by Cu^{2+}. The thermal inactivation of pyruvate kinase activity in these male sexual tissues was also prevented effectively when the supernatant fluids were preincubated in the presence of penicillamine (97).

Although considerable work has been carried out on the modulation of pyruvate kinase activity in brain, liver, skeletal muscle, adipose tissue, prostate gland and seminal vesicles, little or no attention has been paid to the control of this enzyme by specific metabolites in uterine tissue. In the present investigation, an attempt has been made to identify certain factors which are capable of affecting the in vitro activity of this key rate-limiting glycolytic enzyme in uteri of normal, mature rats.

III. MATERIALS AND METHODS

A. ANIMALS

Mature female rats of the Wistar strain weighing approximately 180-200 g were obtained locally from Biobreeding Laboratories of Canada Ltd., Ottawa, Ontario. Animals housed in individual cages and maintained on Master Laboratory Chow and free access to water were used throughout the course of this study.

B. SAMPLE PREPARATION

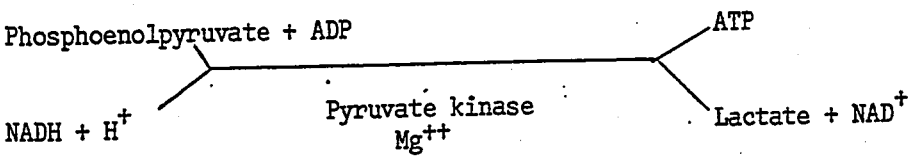
Rats were stunned, killed by decapitation and exsanguinated. The uteri were rapidly removed, dissected free of all adhering fat and connective tissue, weighed on a Roller-Smith torsion balance (Federal Pacific Electric Co., Newark, N.J.) and placed immediately in beakers immersed in crushed ice. In each case, uteri were pooled from 3-4 rats and finely minced with a pair of scissors. The tissue minces were weighed and 5% homogenates prepared in 0.15 M KCl of pH 7.4. Homogenization was carried out in a prechilled Potter-Elvehjem homogenizer (Fisher Scientific Co., Fairlawn, N.J.), fitted with a teflon plastic pestle turning at 700 r.p.m. for a period of exactly 90 seconds. The homogenate was then spun for 30 minutes at 100,000 X g at 5^{o}C in a refrigerated I.E.C. model B-60 centrifuge (International Equipment Co., Needham Heights, Mass.). The supernatant fluids were carefully decanted in glass vials which were kept immersed in crushed ice during the duration of each experiment. This preparation was always prepared fresh for studying the effects of various metabolic effectors on uterine pyruvate kinase activity. The

supernatant fluid was further diluted (1:1) with double glass distilled water and 0.1 ml aliquot of this diluted preparation was used for assaying enzymic activity.

C. DETERMINATION OF PYRUVATE KINASE ACTIVITY

(a) Principle

The activity of pyruvate kinase which catalyzes the conversion of phosphoenolpyruvate and ADP into pyruvate and ATP was determined using a modified procedure of Weber et al. (14) by assaying the disappearance of NADH in a system coupled with lactate dehydrogenase as shown below.



Since the determination of pyruvate kinase activity involved a compound assay, the coupling enzyme (lactate dehydrogenase) was added to the assay mixture in excess prior to the addition of supernatant fluid. The change in optical density due to the conversion of NADH to NAD was recorded at 340 mμ in a constant recording Unicam spectrophotometer model SP 800 (Unicam Instruments, Ltd., Cambridge, England) which was thermostated to run at 37°C.

(b) Preparation and the Source of Various Reagents

All reagents were of the purest grade available. They were always prepared fresh in glass distilled water just prior to the enzyme assay.

NADH (0.216 mM): Diphosphopyridine nucleotide reduced form, disodium salt (Boehringer). 25 mg of NADH was weighed on a Sartorius balance (Fisher Scientific Co.), dissolved in 10 ml of distilled water and the pH adjusted to 7.4 by adding approximately 2 drops of 1N NaOH.

LDH (10 mg protein/ml) Lactic dehydrogenase (Sigma). 0.1 ml aliquot of stock LDH solution was diluted to 1.0 ml with distilled water.

ADP (0.02 M) Adenosine diphosphate, sodium salt (Sigma). 100 mg of ADP was weighed and dissolved in about 5 ml of distilled water. This was then transferred into a 10 ml volumetric flask and made up to the volume.

PEP (0.016 M) Phosphoenolpyruvate, tricyclohexylamine salt (Sigma). 80 mg of PEP was weighed and dissolved in about 5 ml of distilled water in a beaker. The solution was then transferred quantitatively into a 10 ml volumetric flask and made up the volume with distilled water.

$MgSO_4$ and KCl - Magnesium sulphate (0.125 M) and Potassium chloride (0.5 M) (both from Fisher Scientific). 0.92 g of potassium chloride and 0.77 g of $MgSO_4 \cdot 7H_2O$ were weighed in separate beakers and dissolved in a few ml of distilled water. The two solutions were then mixed, transferred to a volumetric flask and made up the volume to 25 ml with distilled water.

Tris buffer (0.5 M), tris (hydroxymethyl) amino methane (Sigma). 3.0275 g of tris was weighed using a Mettler balance (Fisher Scientific) and dissolved in about 25 ml of distilled water. The pH was adjusted to pH 7.4 by slowly adding 1N HCl. The solution was then made up to a final volume of 50 ml in a volumetric flask.

(c) Pyruvate Kinase Assay

The standard reaction mixture (final volume 3.0 ml) contained the following components in the given order of addition: tris buffer (pH 7.4), 125 µmoles; $MgSO_4$, 18.75 µmoles; KCl, 75 µmoles; phosphoenolpyruvate, 16 µmoles; ADP, 4 µmoles; NADH, 0.65 µmole; lactate dehydrogenase, 0.1 mg protein. The reaction was initiated by addition of an appropriate dilution of the supernatant fluid and changes in optical density were measured against a blank which contained no phosphoenolpyruvate. Unless stated otherwise, freshly prepared supernatant fluids were used in the case of each experiment. Preliminary experiments were carried out to establish linear kinetics with regard to time and amount of the tissue. Pyruvate kinase activity was therefore assayed under strictly linear kinetic conditions. Changes in optical density were recorded for a period of at least 5 minutes (the reaction was linear up to 10-12 minutes); the final pH and temperature were checked at the termination of each assay. In several instances, data are presented from a typical experiment although they were replicated in other experiments. In other cases, data were subjected to statistical evaluation (by Student's t test) and significant differences are indicated between the various means calculated as p values. No statistical significance is indicated when the p value was >0.05. The activity of uterine pyruvate kinase was calculated as micromoles of substrate metabolized per hour per g of tissue at 37°C as described by Schwark et al. (82).

IV. RESULTS

A. EFFECT OF FRUCTOSE 1,6-DIPHOSPHATE ON UTERINE PYRUVATE KINASE

Since fructose 1,6-diphosphate is known to stimulate pyruvate kinase in liver and yeast (85,99), the influence of fructose 1,6-diphosphate on the activity of this enzyme in the uterus was investigated. The changes in enzyme activity following the addition of various amounts of fructose 1,6-diphosphate (0.5-37 mM) are recorded in Table I. It can be seen that the direct addition of increasing concentrations of fructose 1,6-diphosphate to the reaction mixture produced no significant effect on pyruvate kinase activity of the uterus. Enzyme activity remained completely unaltered even in the presence of very high concentration (37 mM) of fructose 1,6-diphosphate. The results indicate that pyruvate kinase in the uterus might resemble the enzyme in skeletal muscle, kidney, heart, brain, leucocytes, bovine hypophysis and Lactobacillus fermenti at least with respect to its insensitivity to fructose 1,6-diphosphate stimulation.

B. EFFECTS OF L-ALANINE

As L-alanine has been shown to inhibit the activity of hepatic pyruvate kinase (93,94) and to exert slight or no effect on cerebrocortical, prostatic and vesicular enzyme (82,97), the influence of this amino acid was studied on pyruvate kinase activity of the uterus. Results presented in Table II show that addition of increasing concentrations of L-alanine (1.25-25.0 mM) directly to the assay system failed to exert any significant effect on this uterine enzyme. The inability of L-alanine

TABLE I

EFFECT OF FRUCTOSE 1,6-DIPHOSPHATE ON UTERINE PYRUVATE KINASE ACTIVITY

Increasing concentrations of fructose 1,6-diphosphate were added to the reaction mixture just prior to the addition of the supernatant fluid. Enzyme activity is expressed as micromoles of substrate metabolized per g of tissue per hour. Means ± S.E.M. represent 4 values in each group. All values were statistically insignificantly different from controls. Results are also given in percentages taking the control values as 100%.

FRUCTOSE 1,6-DIPHOSPHATE (mM)	ENZYME ACTIVITY (μmoles/g/hr)	PERCENT OF CONTROL
None (control)	3585±122	100
0.5	3488±64	96
1.0	3394±95	93
2.0	3626±98	102
4.0	3506±239	97
10.0	3269±158	88
25.0	3452±102	95
37.0	3688±114	104

TABLE II

EFFECT OF L-ALANINE ON UTERINE PYRUVATE KINASE ACTIVITY

Increasing concentrations of L-alanine were added to the reaction mixture just prior to the addition of the supernatant fluid. Enzyme activity is expressed as micromoles of substrate metabolized per g of tissue per hour. Means ± S.E.M. represent 4 values in each group. All values were statistically insignificantly different from controls. Results are also given in percentages taking the control values as 100%.

L-ALANINE (mM)	ENZYME ACTIVITY (µmoles/g/hr)	PERCENT OF CONTROL
None (control)	3284±235	100
1.25	3377±212	102
2.5	3238±286	99
5.0	3215±198	99
12.5	3007±255	92
25.0	3115±227	93

to affect pyruvate kinase activity of the uterus appears to be in contrast to the activation of prostatic and vesicular enzyme (36% and 20%, respectively) seen in presence of 25 mM L-alanine.

C. EFFECT OF L-PHENYLALANINE

Previous investigations have demonstrated that the related amino acid, L-phenylalanine is a competitive inhibitor of pyruvate kinase in brain (14,82) and accessory sexual tissues of male rats (97). The influence of varying amounts of L-phenylalanine on uterine pyruvate kinase activity was studied since this amino acid is known to accumulate in blood and tissues of phenylketonuric individuals (80). The results in Figure 3 show that addition of L-phenylalanine to the assay system produced a dose-dependent inhibition of uterine pyruvate kinase. When assayed in the presence of 2 µmoles of phosphoenolpyruvate, a definite inhibition of enzyme activity could be achieved with concentrations as low as 0.5 mM whereas maximal inhibition (80-95%) was observed with 15-20 mM L-phenylalanine. The degree of enzyme inhibition decreased slightly as the concentration of phosphoenolpyruvate was increased in the assay system. The Lineweaver-Burk plots obtained for pyruvate kinase activity in the presence and absence of L-phenylalanine extrapolated to the same point on the ordinate suggesting that L-phenylalanine acts as a competitive inhibitor of this enzyme in the rat uterus. The apparent K_i for L-phenylalanine calculated from these data was found to be 1.34×10^{-4} M (Fig. 4).

Recently, Vijayvargiya et al. (97) demonstrated that L-alanine could readily protect the prostatic and vesicular enzyme against the

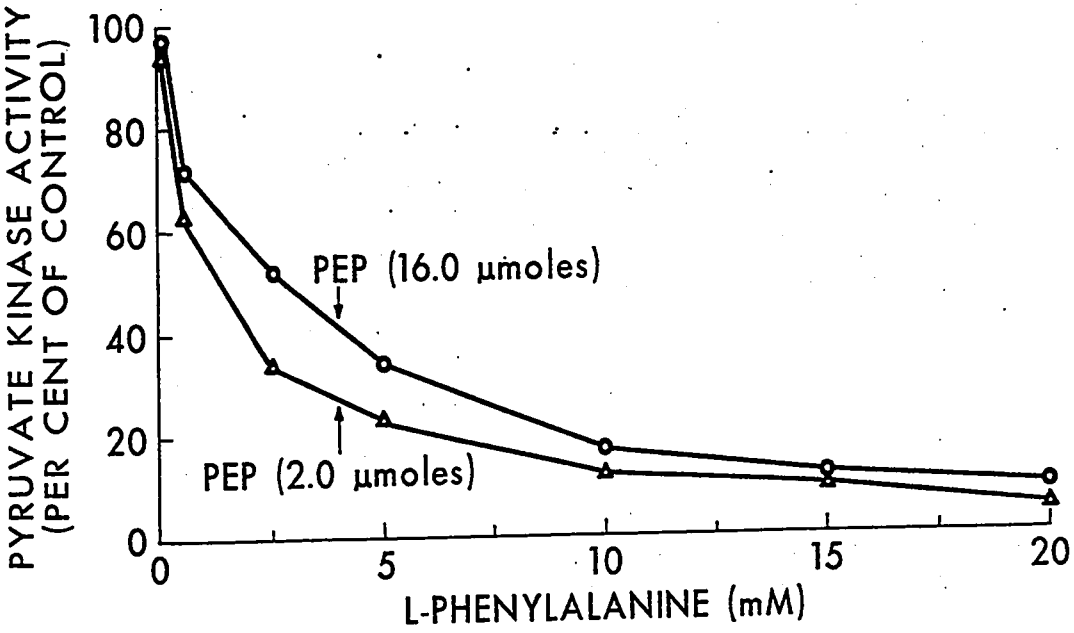


Fig. 3. Activity of pyruvate kinase in rat uterus as a function of the concentration of L-phenylalanine. Enzyme activity was measured in the presence of two different concentrations of the substrate, phosphoenolpyruvate (PEP). Pyruvate kinase activity is expressed as percentage of the control value taken as 100%.

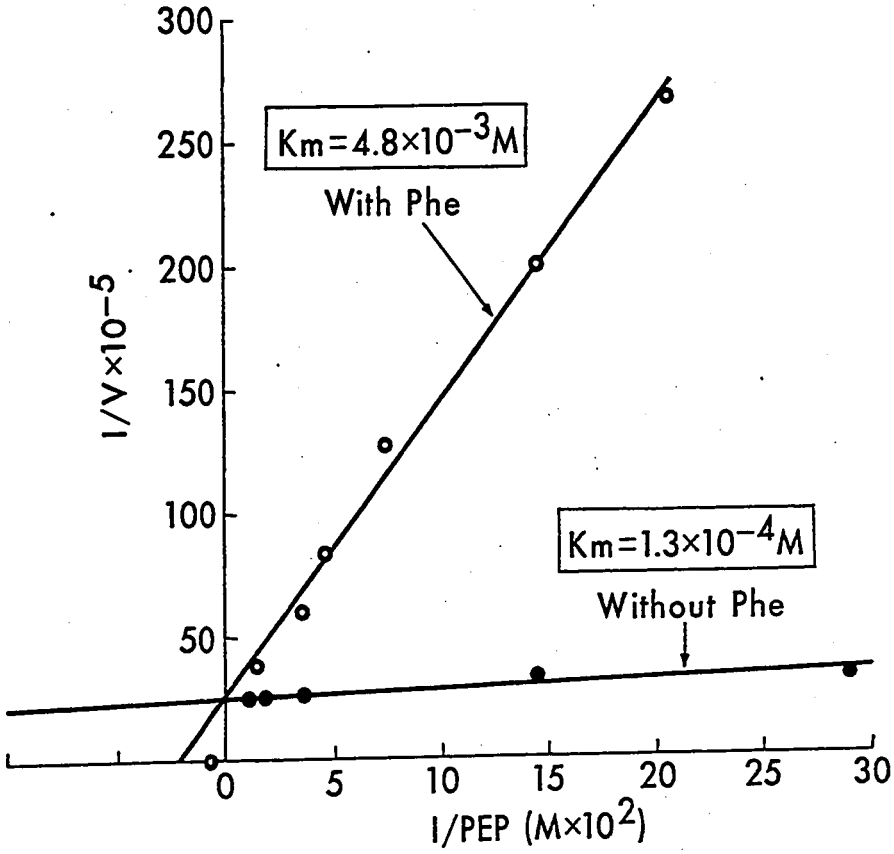


Fig. 4. Lineweaver-Burk plot illustrating competitive inhibition of uterine pyruvate by L-phenylalanine (Phe). The final concentration of L-phenylalanine in the reaction mixture was 5.0 mM.

TABLE III

PROTECTIVE ACTION OF L-ALANINE AGAINST L-PHENYLALANINE INHIBITION OF UTERINE PYRUVATE KINASE

L-Phenylalanine (5.0 mM) and L-alanine (5.0 or 12.5 mM) were added to the reaction mixture just prior to the addition of the supernatant fluid. Enzyme activity is expressed as micromoles of substrate metabolized per g of tissue per hour. Results are also given in percentages taking the control values as 100%.

ADDITIONS	ENZYME ACTIVITY (μmoles/g/hr)	PERCENT OF CONTROL
None (control)	3516	100
L-Alanine (5.0 mM)	3318	92
L-Alanine (12.5 mM)	3450	94
L-Phenylalanine (5.0 mM)	1110	32
L-Phenylalanine plus L-Alanine (5.0 mM)	3017	85
L-Phenylalanine plus L-Alanine (12.5 mM)	3261	93

inhibitory effects of L-phenylalanine. The question whether L-alanine could also offer protection to uterine pyruvate kinase against the inhibition produced by L-phenylalanine was therefore investigated. The results presented in Table III show that addition of L-phenylalanine (5.0 mM) alone resulted in 68% inhibition of uterine pyruvate kinase. However, when L-alanine (5.0 mM) was added simultaneously with L-phenylalanine, the extent of enzyme inhibition was reduced to 15%. When the concentration of L-alanine was raised to 12.5 mM, pyruvate kinase inhibition was almost completely prevented and its activity remained within the control range. Data indicate that in analogy to earlier findings for several other tissues, L-alanine is fully capable of protecting uterine pyruvate kinase against the inhibitory effects of L-phenylalanine.

D. EFFECTS OF COPPER

Since pyruvate kinase activity in male accessory sex organs (97) is strongly inhibited by copper, it was of interest to investigate the influence of this cation on the uterine enzyme. Data presented in Figure 5 show that when pyruvate kinase was estimated in presence of 16 micromoles of phosphoenolpyruvate, a definite inhibition could be observed with concentrations of copper as low as 0.015 mM. Whereas 50% inhibition of uterine pyruvate kinase activity was produced by 0.12 mM copper, almost complete inhibition was noted with 1.0 mM copper. The degree of enzyme inhibition became somewhat more pronounced when pyruvate kinase activity was assayed in presence of a lower concentration of the substrate (1.0 mM). In this circumstance, complete inhibition of uterine enzyme activity was produced with 0.5 mM copper. Figure 6 shows reciprocal

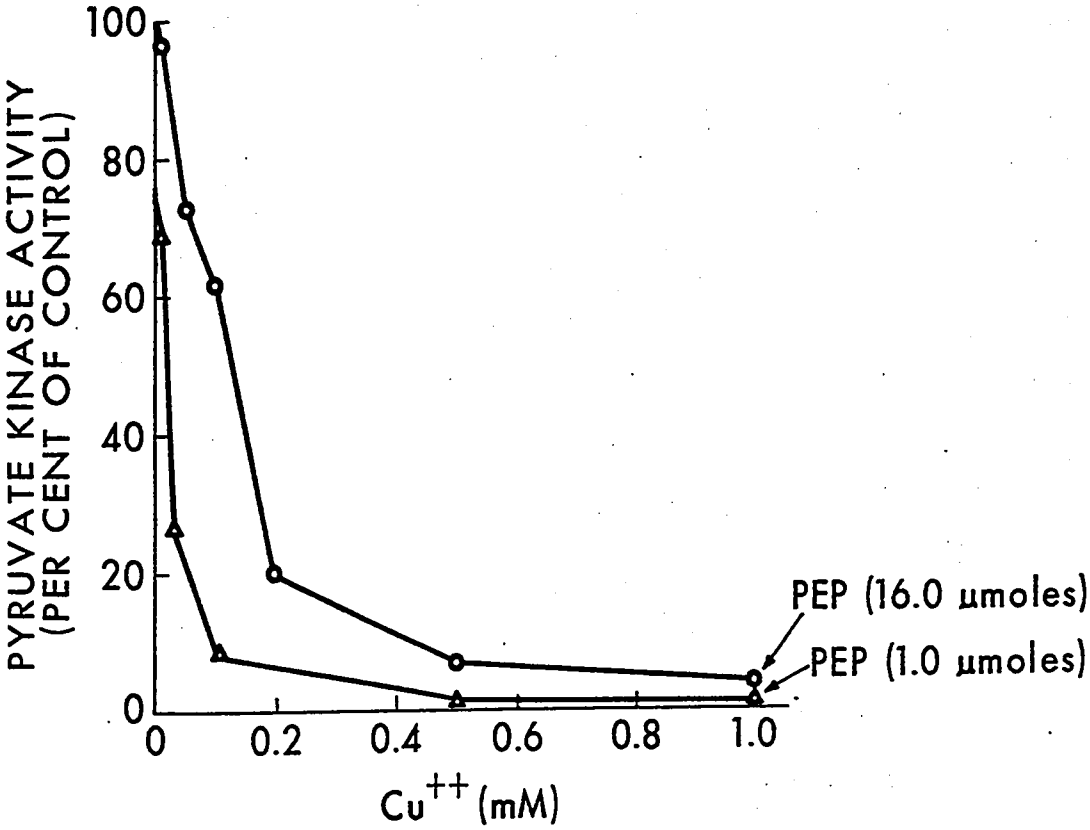


Fig. 5. Activity of pyruvate kinase in the rat uterus as a function of the concentration of copper. Enzyme activity was measured in the presence of two different concentrations of the substrate, phosphoenolpyruvate (PEP). Pyruvate kinase activity is expressed as percentage of the control value taken as 100%.

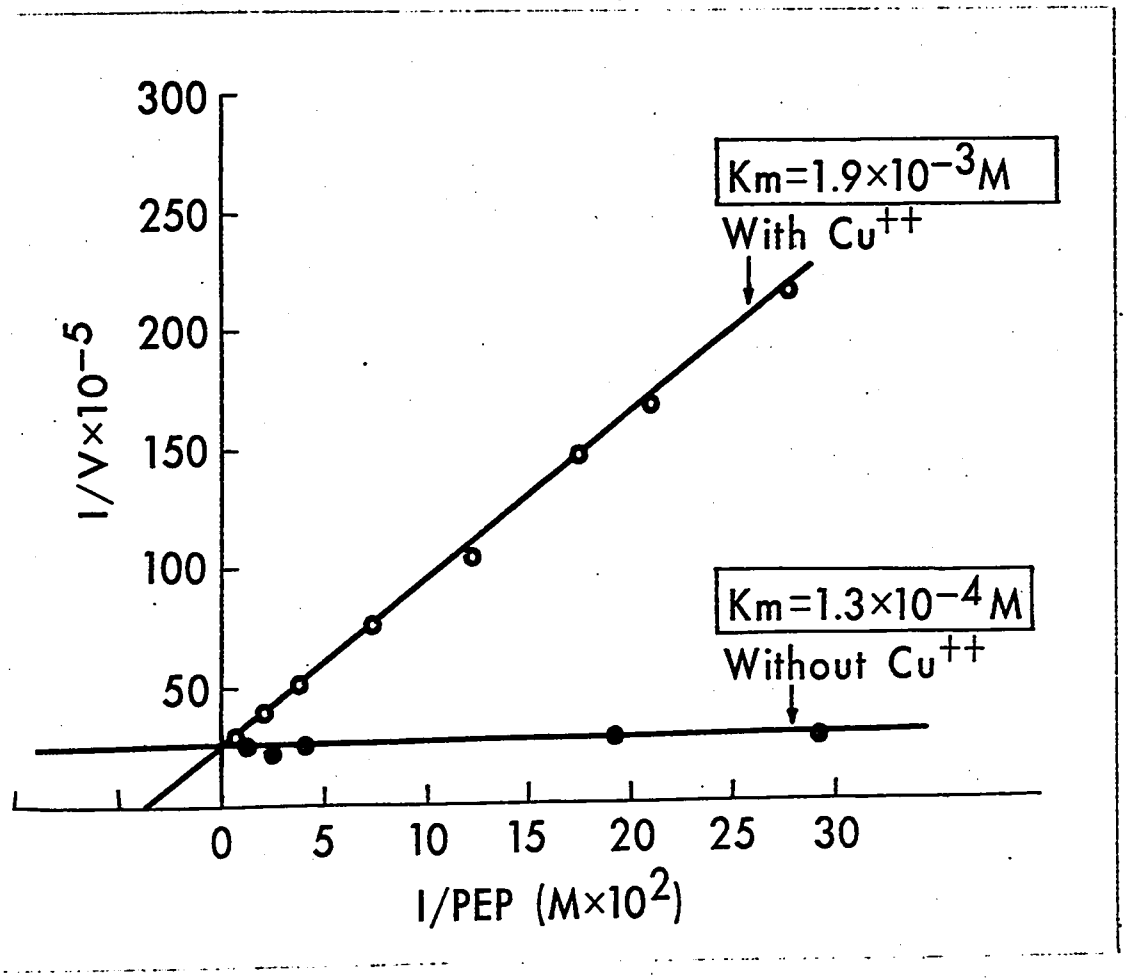


Fig. 6. Lineweaver-Burk plot illustrating competitive inhibition of uterine pyruvate kinase by copper. The final concentration of copper in the reaction mixture was 0.05 mM.

plots of velocity against substrate concentrations (Lineweaver-Burk plots) in the absence and presence of copper (0.05 mM). Increase in the concentration of phosphoenolpyruvate readily protected the enzyme against copper inhibition. The apparent K_i for copper ions calculated from these data was found to be 1.1×10^{-4} M. The results demonstrate that inhibition of uterine pyruvate kinase activity by copper is of the competitive type.

Since L-alanine was able to offer protection against the inhibition of uterine pyruvate kinase by L-phenylalanine, the effect of L-alanine on copper-induced inhibition of the enzyme was investigated. Results presented in Table IV demonstrate that addition of 0.05 mM copper to the reaction mixture inhibited uterine pyruvate kinase activity by 44%. The simultaneous addition of L-alanine (5.0 mM) and copper partially protected the enzyme against the inhibitory effects of this cation. When the concentration of L-alanine was raised to 12.5 mM, complete protection of the uterine pyruvate kinase was achieved against copper inhibition.

E. INFLUENCE OF PENICILLAMINE

Since penicillamine is known to chelate copper and is used therapeutically in the treatment of Wilson's disease (100), the ability of this compound to protect uterine enzyme activity against copper-induced inhibition was investigated. The changes in uterine pyruvate kinase following the addition of varying amounts of penicillamine to the assay system are shown in Table V. Penicillamine, by itself, produced activation of the uterine pyruvate kinase; enzyme activity increased by 22% in presence of 2.0 mM penicillamine and maximal stimulation (48%) was

TABLE IV

EFFECT OF L-ALANINE ON THE INHIBITION OF UTERINE PYRUVATE KINASE PRODUCED BY Cu^{++}

$CuSO_4$ (0.05 mM) and L-alanine (5.0 mM or 12.5 mM) were added to the reaction mixture immediately prior to the addition of the supernatant fluid. Enzyme activity is expressed as micromoles of substrate metabolized per g of tissue per hour. Data are also given in percentages taking the control values as 100%.

ADDITIONS	ENZYME ACTIVITY (µmoles/g/hr)	PERCENT OF CONTROL
None (control)	3238	100
Cu^{++} (0.05 mM)	2150	66
L-Alanine (5.0 mM)	3256	101
L-Alanine (12.5 mM)	3007	92
Cu^{++} plus L-Alanine (5.0 mM)	2769	85
Cu^{++} plus L-Alanine (12.5 mM)	2920	90

TABLE V

EFFECT OF VARYING CONCENTRATIONS OF PENICILLAMINE ON THE ACTIVITY OF UTERINE PYRUVATE KINASE

Penicillamine was added to the reaction mixture just prior to the addition of the supernatant fluid. Means ± S.E.M. represent 4 values in each group. Enzyme activity is expressed as micromoles of substrate metabolized per g of tissue per hour. Data are also given in percentages taking the control values as 100%.

PENICILLAMINE (mM)	ENZYME ACTIVITY (µmoles/g/hr)	PERCENT OF CONTROL
None (control)	3562±134	100
0.5	3556±211	99
1.0	4025±287	112
1.5	4103±301	115
2.0	4355±130	122*
4.0	5278±178	148*

*Statistically significant difference when compared to the control values ($p = <0.05$).

seen following the addition of 4.0 mM concentration of this compound.

Results presented in Table VI show the protective action of penicillamine against the copper-induced inhibition of uterine pyruvate kinase. When equimolar concentrations of copper and penicillamine (0.05 mM) were present in the assay mixture, no protection of pyruvate kinase could be seen against copper inhibition. However, an increase in the concentration of penicillamine (2.0 mM) resulted in complete protection against the inhibitory effects of copper on pyruvate kinase activity of this tissue.

F. EFFECTS OF CALCIUM

Schwark et al. (97) demonstrated that the activity of cerebrocortical pyruvate kinase was inhibited by calcium whereas the addition of EDTA resulted in an activation of the brain enzyme. Takagaki (101) also showed that calcium markedly inhibited pyruvate kinase activity of the guinea pig cerebrum. Results presented in Figure 7 demonstrate changes in uterine pyruvate kinase activity following the addition of varying concentrations of calcium. In analogy to the brain enzyme, addition of calcium to the assay system produced a marked inhibition of pyruvate kinase activity in uterus. A definite inhibition could be seen with 0.1 mM calcium and increasing its concentration to 2.0 mM resulted in 75% inhibition of the uterine enzyme. Reciprocal plots of velocity against substrate concentration (Lineweaver-Burk plots) indicated that calcium produced a non-competitive inhibition of uterine pyruvate kinase with an apparent K_i of 7.3×10^{-4}M (Fig. 8).

The protective action of EDTA against the inhibition of pyruvate kinase activity by calcium was also studied and the results are shown

TABLE VI

PROTECTIVE ACTION OF PENICILLAMINE AGAINST Cu^{++} INHIBITION OF PYRUVATE KINASE

$CuSO_4$ (0.05 mM) and varying concentrations of penicillamine were added to the reaction mixture just prior to the addition of the uterine supernatant fluid. Enzyme activity is expressed in terms of micromoles of substrate metabolized per hour per g weight of the tissue. Data are also given in percentages taking the control values as 100%.

ADDITIONS	ENZYME ACTIVITY (µmoles/g/hr)	PERCENT OF CONTROL
None (control)	3562	100
Cu^{++} (0.05 mM)	2592	73
Cu^{++} plus Penicillamine (0.05 mM)	2681	75
Cu^{++} plus Penicillamine (1.0 mM)	2813	78
Cu^{++} plus Penicillamine (2.0 mM)	4209	118

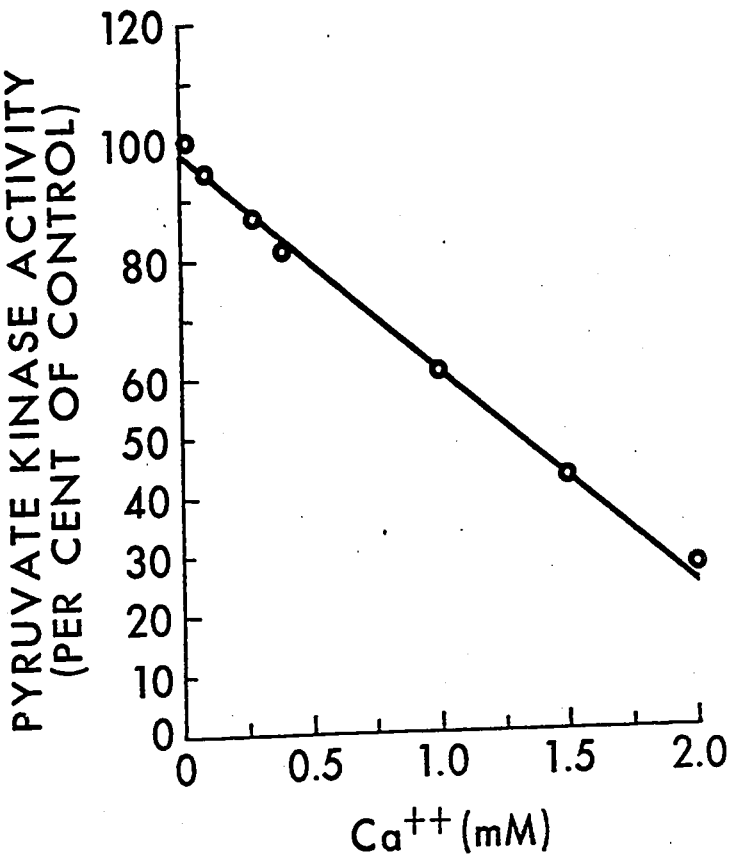


Fig. 7. Effect of varying concentrations of calcium on uterine pyruvate kinase activity. Enzyme activity is expressed as percentage of the control value taken as 100%.

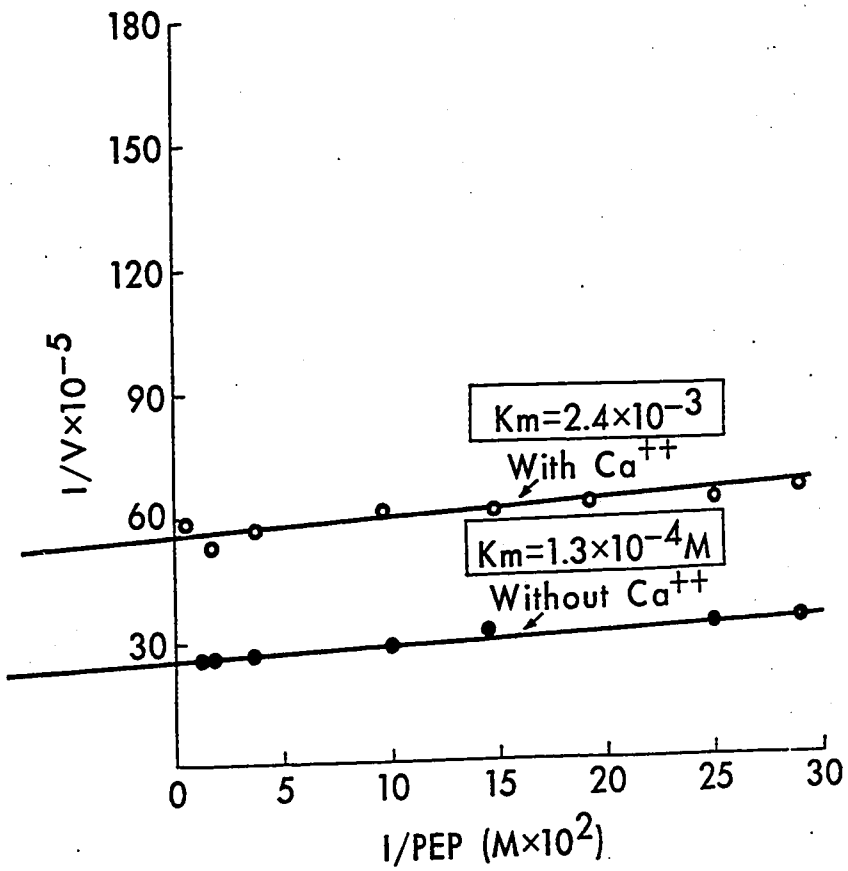


Fig. 8. Lineweaver-Burk plot illustrating non-competitive inhibition of uterine pyruvate kinase by calcium. The final concentration of calcium in the reaction mixture was 1.0 mM.

in Table VII. Addition of a low concentration of EDTA (0.5 mM) to the assay medium failed to exert any measurable effect on enzyme activity. However, increasing the concentration of this chelating agent to 2.0 mM produced 21% inhibition of uterine pyruvate kinase. Table VII also shows that addition of calcium (1.0 mM) alone resulted in a 37% inhibition of the uterine enzyme. It is of interest that when EDTA (0.5 mM) was added simultaneously with calcium, the inhibition was reduced to 11%. Addition of a higher concentration of EDTA (2.0 mM) further reduced the inhibition of uterine pyruvate kinase seen with the calcium ion.

G. EFFECT OF Mg^{++} ON THE INHIBITION PRODUCED BY EDTA

The assay of pyruvate kinase requires magnesium as a co-factor (102,103). Since magnesium is known to be chelated by EDTA (104), the possibility whether high concentrations of EDTA might have inhibited uterine pyruvate kinase by chelating magnesium ions was investigated. Results presented in Table VIII show that addition of EDTA alone resulted in 34% inhibition of pyruvate kinase which was reduced to 13% upon simultaneous addition of 5.0 mM magnesium. A further reduction in inhibitory effects of EDTA on uterine enzyme activity was observed when the concentration of magnesium was raised to 10 mM. Results indicate that indeed, increase in the amount of magnesium present in the assay mixture can completely overcome the inhibition produced by EDTA.

H. EFFECTS OF CYCLIC AMP

Since administration of cyclic AMP to ovariectomized and immature rats produced significant stimulation of uterine pyruvate kinase and other

TABLE VII

PROTECTIVE ACTION OF EDTA ON THE INHIBITION OF UTERINE PYRUVATE KINASE BY CALCIUM

$CaCl_2$ (1.0 mM) and EDTA (0.5 mM or 2.0 mM) were added to the reaction mixture just prior to the addition of the supernatant fluid. Enzyme activity is expressed as micromoles of substrate metabolized per g of tissue per hour. Data are also given in percentages taking the control values as 100%.

ADDITIONS	ENZYME ACTIVITY (μmoles/g/hr)	PERCENT OF CONTROL
None (control)	3516	100
Ca^{++} (1.0 mM)	2217	63
EDTA (0.5 mM)	3446	98
Ca^{++} plus EDTA (0.5 mM)	3146	89
EDTA (2.0 mM)	2775	79
Ca^{++} plus EDTA (2.0 mM)	3261	92

TABLE VIII

EFFECT OF Mg^{++} ON EDTA-INDUCED INHIBITION OF UTERINE PYRUVATE KINASE ACTIVITY

Disodium EDTA (5.0 mM) and $MgSO_4$ (5.0 or 10.0 mM) were added to the assay medium just prior to the addition of the supernatant fluid. Enzyme activity is expressed as micromoles of substrate metabolized per g of tissue per hour. Data are also given in percentages taking the control values as 100%.

ADDITIONS	PYRUVATE KINASE ACTIVITY (μmoles/g/hr)	PERCENT OF CONTROL
None (control)	3516	100
EDTA (5.0 mM)	2313	66
EDTA (5.0 mM) + Magnesium (5.0 mM)	3053	87
EDTA (5.0 mM) + Magnesium (10.0 mM)	3308	94

key glycolytic enzymes, the influence of direct addition of this nucleotide to the reaction mixture was investigated on pyruvate kinase activity of the uterus. Varying concentrations of cyclic AMP (0.5-10.0 mM) were added to the reaction mixture just prior to the addition of uterine supernatant fluid and the results are shown in Table IX. In contrast to the *in vivo* effects of cyclic AMP, this nucleotide when added *in vitro*, failed to exert any appreciable effect on the uterine enzyme. The presence of even very high concentrations of cyclic AMP (10.0 mM) did not produce any measurable stimulation of uterine pyruvate kinase activity.

I. INFLUENCE OF p-CHLOROMERCURIBENZOATE AND PENICILLAMINE

Both the L- and the M-type isozymes of pyruvate kinase are known to be inhibited by the sulphydryl inhibitor, p-chloromercuribenzoate (95). Recently, Schwark *et al.* (82) also demonstrated the inhibitory effects of p-chloromercuribenzoate on the cerebrocortical enzyme. In order to study the possible involvement of sulphydryl groups in the control of uterine pyruvate kinase, the effect of p-chloromercuribenzoate was investigated. The results in Figure 9 show that addition of p-chloromercuribenzoate to the reaction mixture resulted in a marked inhibition of uterine pyruvate kinase activity. The observed effect was dose-dependent and a nearly complete inhibition of the enzyme was produced when p-chloromercuribenzoate was present in a concentration of 0.01 mM. Since penicillamine is known to be a potent sulphydryl donor, the ability of this compound to prevent the observed inhibition of pyruvate kinase activity by p-chloromercuribenzoate was also examined. Data presented in Table X show that addition of p-chloromercuribenzoate (0.002 mM)

TABLE IX

EFFECTS OF VARYING CONCENTRATIONS OF CYCLIC AMP ON THE ACTIVITY OF UTERINE PYRUVATE KINASE

Varying amounts of cyclic AMP were added to the reaction mixture just prior to the addition of the supernatant fluid. Enzyme activity is expressed as micromoles of substrate metabolized per g of tissue per hour. Means ± S.E.M. represent 4 values in each group. All values were statistically insignificantly different from controls. Data are also given in percentages taking the control values as 100%.

CYCLIC AMP (mM)	ENZYME ACTIVITY (μmoles/g/hr)	PERCENT OF CONTROL
None (control)	2845±223	100
0.5	2868±269	101
1.0	2845±242	100
5.0	2845±261	100
10.0	2868±210	101

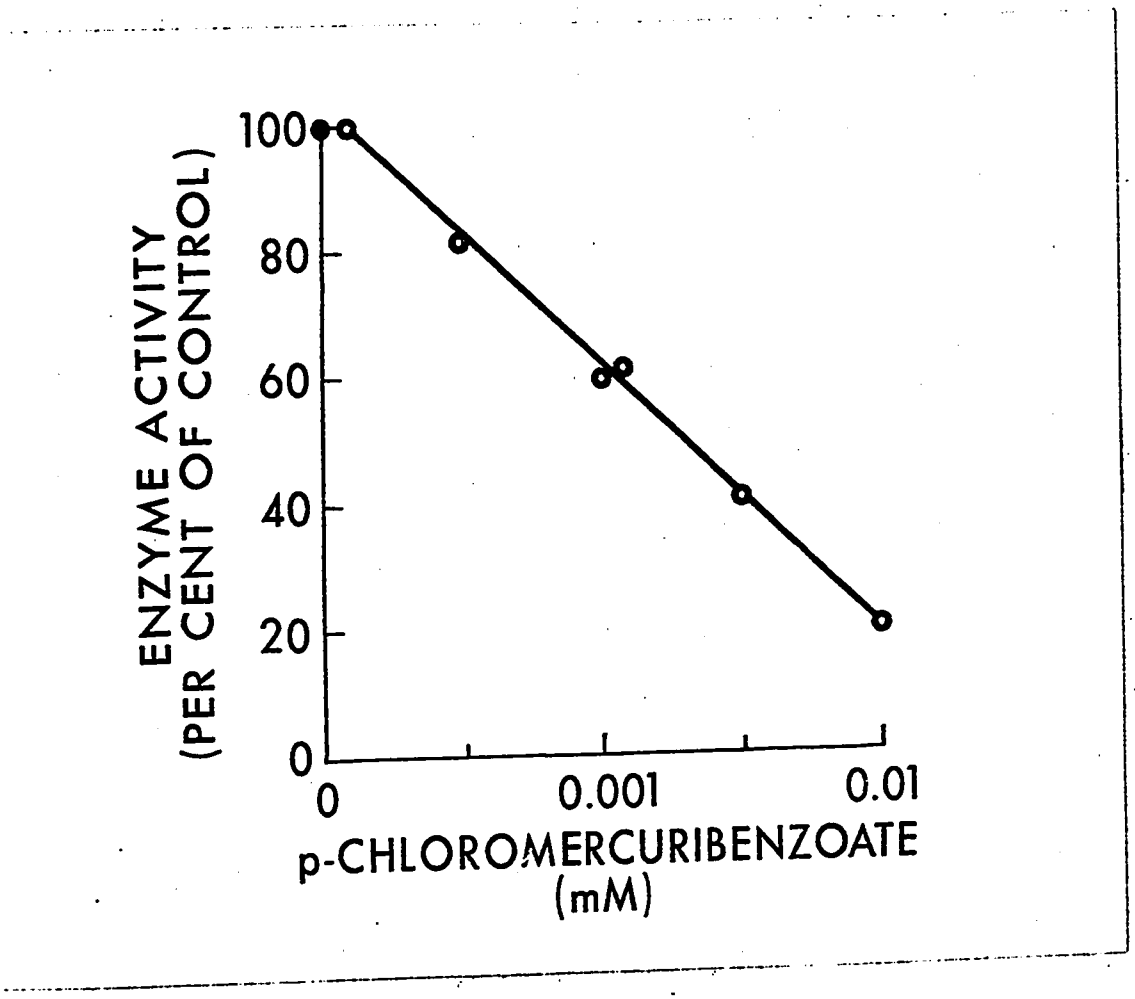


Fig. 9. Influence of varying concentrations of p-chloromercuribenzoate on uterine pyruvate kinase activity. Enzyme activity is expressed as percentage of the control value taken as 100%.

TABLE X

PROTECTIVE ACTION OF PENICILLAMINE AGAINST PYRUVATE KINASE INHIBITION BY p-CMB

p-CMB (0.002 mM) and penicillamine (2.0 mM) were added to the reaction mixture just prior to the addition of the supernatant fluid. Enzyme activity is expressed as micromoles of substrate metabolized per g of tissue per hour. Data are also given in percentages taking the control values as 100%.

ADDITIONS	ENZYME ACTIVITY (µmoles/g/hr)	PERCENT OF CONTROL
None (control)	3238	100
p-CMB (0.002 mM)	2059	63
Penicillamine (2.0 mM)	4136	121
p-CMB (0.002 mM) + Penicillamine (2.0 mM)	3493	107

inhibited enzyme activity by 37%. However, when penicillamine (2.0 mM) was added simultaneously with p-chloromercuribenzoate, inhibition of the enzyme was completely prevented and the values were restored to the control range. These results indicate that p-chloromercuribenzoate inhibits uterine pyruvate kinase activity by inactivating the sulphydryl groups and that penicillamine may protect against this inhibition by providing free sulphydryl groups to the enzyme.

V. DISCUSSION

Pyruvate kinase catalyzes a terminal reaction in the glycolytic sequence and has been shown to play an important role in the regulation of overall glycolysis and gluconeogenesis (5,95). This enzyme has been shown to exist as isoenzymes in a variety of animal tissues. Koler et al. (105) were the first to report molecular differences between pyruvate kinase extracted from human red blood cells of pyruvate kinase-deficient and normal patients. Their studies demonstrated that the two enzymes differed in their chemochromatographic, electrophoretic and kinetic properties and suggested that a distinct genetic locus might control the synthesis of each isoenzyme. Tanaka et al. (95) subsequently investigated the tissue distribution, physiological function and properties of pyruvate kinase isoenzymes from various rat tissues. The two major isoenzymes reported, type-L or liver pyruvate kinase and type-M or muscle pyruvate kinase differed in their electrophoretic, immunochemical and kinetic properties. Type-M or muscle pyruvate kinase displayed normal Michaelis-Menton kinetics with phosphoenolpyruvate whereas type-L or liver pyruvate kinase exhibited sigmoid kinetics with this substrate. Results of the present study show that pyruvate kinase of the rat uterus does not display sigmoidal kinetics but conforms to the normal Michaelis-Menton kinetics like the type-M or skeletal muscle enzyme.

A. EFFECTS OF FRUCTOSE 1,6-DIPHOSPHATE AND L-ALANINE

The glycolytic intermediate, fructose 1,6-diphosphate has been shown to activate the type-L enzyme (106) as well as pyruvate kinase in

yeast (99) and adipose tissue (96). In addition, Bailey et al. (88) reported that fructose 1,6-diphosphate could change the sigmoidal relationship between substrate concentration and the velocity for L-type enzyme into a normal hyperbolic function. These authors also speculated that fructose 1,6-diphosphate might exert an important regulatory influence of pyruvate kinase in vivo since the hepatic levels of this substrate are known to fluctuate with the nutritional state of the animal. The presently observed inability of fructose 1,6-diphosphate to stimulate pyruvate kinase from uterine supernatants indicates that the uterine enzyme is not subject to the feed-forward control by fructose 1,6-diphosphate. Vijayvargiya et al. (97) reported complete failure of fructose 1,6-diphosphate to activate the prostatic and vesicular pyruvate kinase even after preincubation for 60 minutes. It would seem that pyruvate kinase activity of smooth muscles such as the accessory sex organs of male and female rats is insensitive to stimulation by fructose 1,6-diphosphate which is also the case for the M-type isoenzyme (106).

Vijayvargiya et al. (97) demonstrated that addition of L-alanine produced slight activation of pyruvate kinase activity of the prostate gland and seminal vesicles of normal, mature rats. A definite activating effect of L-alanine was observed at 5 mM concentration whereas maximal activation of 36% in the prostate and 20% in seminal vesicles was produced by 25 mM L-alanine. Schwark et al. (82) demonstrated that the presence of L-alanine in the reaction mixture exerted little or no effect on pyruvate kinase activity of the rat cerebral cortex. Our results show that concentrations of L-alanine as high as 25 mM failed to produce any significant change in the activity of the uterine enzyme. Weber et al.

(94) demonstrated that L-alanine acts as a competitive inhibitor of the L-type pyruvate kinase in hepatic tissue. The physiological significance of L-alanine inhibition of L-type hepatic pyruvate kinase becomes evident when one realizes that this amino acid not only accumulates under gluconeogenic conditions, but is an important precursor in glucose synthesis from non-carbohydrate sources (107). Since uterus possesses little or no capacity for active gluconeogenesis and is considered chiefly to be a glycolytic organ, such inhibition of pyruvate kinase by L-alanine would probably be of little consequence to the control of overall glycolysis in this tissue.

B. INHIBITION BY L-PHENYLALANINE: PROTECTION BY L-ALANINE

The present study also demonstrates that in contrast to L-alanine, addition of L-phenylalanine produced a competitive inhibition of uterine pyruvate kinase. During phenylketonuria, there is large accumulation of L-phenylalanine (and phenylpyruvate) due to a decrease in the activity of hepatic phenylalanine hydroxylase (108). Studies on the action of L-phenylalanine on pyruvate kinase from rat and adult human brain revealed that L-phenylalanine competitively inhibited the enzyme; the inhibition being reversible upon increasing the concentration of phosphoenolpyruvate (80). In contrast, the hepatic enzyme proved insensitive to the action of L-phenylalanine (80). It is of interest that the concentration of L-phenylalanine which inhibited brain pyruvate kinase were comparable to those found in the plasma of phenylketonuric patients (80). Schwark et al. (82) confirmed these observations and demonstrated that this amino acid produced a dose-dependent inhibition of pyruvate kinase activity in

rat cerebral cortex. Weber (80) suggested that accumulation of L-phenylalanine in phenylketonuric individuals may curtail brain glycolysis and thus interfere with vital cellular processes such as lipid biosynthesis. Indeed, O'Brien and Ibbot (109) reported marked alterations of lipid metabolism in the brains of phenylketonuric patients. Interference with glycolysis could also decrease production of ATP and other nucleotide triphosphates which play important roles in the biosynthesis of proteins and nucleic acids (80). In addition, Weber (80) suggested that extremely low levels of pyruvate kinase present in the neonatal brain may increase the vulnerability of this tissue at birth to the inhibitory effects of L-phenylalanine. Recently, Vijayvargiya et al. (97) showed that L-phenylalanine was also a competitive inhibitor of prostatic and vesicular pyruvate kinase and that L-alanine was capable of protecting the enzyme against the inhibitory effects of L-phenylalanine. Results of the present study show that the inhibition of uterine pyruvate kinase by L-phenylalanine could also be prevented by L-alanine. It is of interest that L-alanine was also shown to completely protect pyruvate kinase activity against thermal inactivation in liver and prostate although it provided only partial protection against heat inactivation of the brain enzyme. Whereas the precise mechanism by which L-alanine modulated pyruvate kinase in the cerebral cortex and the male accessory sex organs is unknown, it has been suggested that an allosteric regulatory mechanism might be involved (97).

C. INHIBITION OF UTERINE PYRUVATE KINASE BY COPPER: PROTECTION BY L-ALANINE

Results presented in this study demonstrate that copper is a

competitive inhibitor of uterine pyruvate kinase and that this inhibition also can be prevented by L-alanine. A similar inhibition by copper and its prevention by L-alanine has been reported previously for the cerebrocortical enzyme (82). It is possible that L-alanine may cause some conformational change in the enzyme protein rendering the site of attack by copper less accessible (85). It was Passeron et al. (85) who first demonstrated that L-type pyruvate kinase was strongly inhibited by copper whereas the M-type enzyme was only slightly affected. Furthermore, the inhibition by copper was reverted by fructose 1,6-diphosphate and chelating agents such as EGTA, EDTA and 8-hydroxyquinoline. It may be noted that the concentration of copper required to produce complete inhibition of the uterine pyruvate kinase is approximately 20 times greater than that reported by Passeron et al. (85) for the L-type enzyme. The physiological significance of the observed negative modulation of uterine pyruvate kinase by copper remains obscure at present since the concentration of this cation in uterine cells is unknown.

Several neurological disorders are known to be associated with accumulations of abnormal quantities of metabolites in body fluids and tissues of the affected individual. However, little is known about the cellular events that are disrupted by toxic levels of the metabolite. It has been suggested that the inhibition of brain pyruvate kinase by copper might have some relevance to Wilson's disease which is characterized by accumulation of large quantities of copper in liver as well as the central nervous system (81). Data on the concentration of copper in normal and pathological tissues are needed before any direct correlation can be made between the observed inhibition of pyruvate kinase by

copper, in brain, prostate, liver and uterus and the clinical condition. Incidentally, the amount of total copper in the liver of several adult mammals has been shown to vary between 0.06 and 0.12 pmoles/g wet weight of the tissue (86,87). These figures appear to be well above the range which produce maximum inhibition of the L-type pyruvate kinase *in vitro* (85).

D. EFFECTS OF CYCLIC AMP, CALCIUM AND EDTA

The influence of various concentrations of cyclic AMP, a presumed mediator in the mechanism of action of biogenic amines and numerous hormones and cell regulating substances was also examined on the activity of uterine pyruvate kinase. Monsoor (12) demonstrated that phosphofructokinase activity was allosterically activated by cyclic AMP in liver fluke and sheep heart. Moreover, Milman and Yurowitzki (110) found that cyclic AMP produced activation of pyruvate kinase from fish embryo. Our results show that cyclic AMP, when added *in vitro*, failed to exert any appreciable effect on pyruvate kinase activity of the rat uterus. The presence of even very high concentrations of cyclic AMP did not produce any measurable activation or inhibition of the uterine enzyme. These results are in contrast to the ability of *in vivo* cyclic AMP to produce marked stimulation of pyruvate kinase activity in uteri of both ovariectomized and immature rats (72).

Takagaki (101) demonstrated that calcium produced marked inhibition of pyruvate kinase from the guinea pig cerebrum. Later, Schwark *et al.* (82) reported that calcium inhibited rat cerebrocortical pyruvate kinase and that the inhibition was of the non-competitive type. Our results

on calcium inhibition of the uterine enzyme and its prevention by EDTA are similar to those reported for the cerebral cortex (82). Mildvan and Cohn (111) have shown that the inhibition of pyruvate kinase by calcium may involve a competition with magnesium, one of the co-factors of this enzyme. The observed prevention of the calcium-induced inhibition of uterine pyruvate kinase by EDTA may well involve removal of calcium from the assay system by chelation (82).

E. ROLE OF SULPHYDRYL GROUPS: EFFECTS OF p-CHLOROMERCURIBENZOATE AND PENICILLAMINE

The activity of several enzymes is known to be dependent upon the maintenance of unaltered sulphydryl residues within the enzyme structure (97). Heavy metals such as copper, arsenic and mercury can inactivate the sulphydryl enzymes and in many cases, the inhibition can be prevented by thiol compounds like cysteine, glutathione and Cleland's reagent (97, 111). Both cysteine and glutathione have been shown to reverse p-chloromercuribenzoate inhibition of hexokinase in brain and of lactate dehydrogenase in cardiac tissue (112,113). Our results on the inhibition of p-chloromercuribenzoate are in line with the suggestion that sulphydryl groups are important to the maintenance of pyruvate kinase activity in the rat uterus. The inhibition of the uterine enzyme by p-chloromercuribenzoate was prevented by penicillamine which is known to act both as a donor of sulphydryl groups and a chelator of heavy metals. Although the mechanism by which penicillamine prevents p-chloromercuribenzoate inhibition of uterine pyruvate kinase is presently unknown, two possibilities may be considered. Since penicillamine is a sulphydryl donor,

it may reverse the p-chloromercuribenzoate inhibition of uterine pyruvate kinase by simply making more sulphydryl groups available to the enzyme. Secondly, penicillamine can chelate heavy metals and may thus prevent the the observed p-chloromercuribenzoate inhibition by removing Hg^{++} ions bound to sulphydryl groups on the enzyme molecule (82). Further work is clearly necessary to determine which, if any, of these mechanisms is involved in the observed effects of penicillamine on uterine pyruvate kinase. It is of interest that a similar protection of p-chloromercuribenzoate inhibition by penicillamine has been reported previously by other investigators (82,97) for pyruvate kinase activity in the cerebral cortex and the male accessory sex organs of the rat.

VI. SUMMARY AND CONCLUSIONS

Evidence indicates that the living organisms maintain dynamic homeostasis among various metabolic steps through the influence of hormones and certain specific metabolites which act as modifiers of enzyme action. Regulatory mechanisms operating at the level of enzyme biosynthesis and enzyme activity are believed to co-exist and co-operate in the control of an enzyme system. Pyruvate kinase is one of the important key, rate-limiting enzymes involved in the process of glucose utilization *via* the Embden-Meyerhof pathway of glycolysis in a variety of mammalian tissues. This enzyme is also of interest to clinicians since a genetically determined deficiency of erythrocyte pyruvate kinase has been shown to result in a severe haemolytic anemia accompanied by premature destruction of erythrocytes and reticulocytes. Considerable work has been carried out on the regulation of pyruvate kinase activity by several metabolites and hormones in liver, kidney, prostate gland and seminal vesicles, etc. Recently, the ability of estrogenic substances and of cyclic 3',5'-adenosine monophosphate to stimulate pyruvate kinase activity *in vivo* was demonstrated in uteri of estrogen-deprived animals. However, little or no attention has been paid to investigating the influence of various metabolites which act as modulators of uterine pyruvate kinase activity under conditions of acute adaptation. The purpose of the present study was to examine the *in vitro* modulation of pyruvate kinase by certain cations and specific metabolites in the uterus of the normal, mature rat.

When uterine pyruvate kinase activity was assayed under optimal conditions of substrate and co-factor requirements, the activity corresponded to 3585±122 micromoles of substrate metabolized per g wet weight of the tissue per hour at 37°C. Addition of various concentrations of fructose 1,6-diphosphate directly to the assay medium failed to produce any significant stimulation of uterine pyruvate kinase. This was in contrast to the known stimulatory action of this glycolytic intermediate on the liver and yeast enzyme. Similarly, L-alanine which inhibits hepatic pyruvate kinase, failed to produce any significant effect on the uterine enzyme. In contrast, the related amino acid, L-phenylalanine, produced a dose-dependent inhibition of uterine pyruvate kinase; the inhibition being less pronounced when the concentration of phosphoenolpyruvate was increased in the assay system. The inhibition was of the competitive type and when L-alanine was added simultaneously with L-phenylalanine, the observed inhibition of pyruvate kinase was completely prevented. Pyruvate kinase activity in the uterus was also inhibited by copper in concentrations as low as 0.015 mM although 50% inhibition of the enzyme was produced by 0.012 mM copper. Copper also produced a competitive type inhibition of the uterine enzyme with apparent K_i being 1.1×10^{-4}M. Addition of L-alanine simultaneously with copper offered complete protection against the inhibition of uterine pyruvate kinase by this cation.

Penicillamine, a known chelator of copper, by itself, produced a slight activation of uterine pyruvate kinase. When equimolar concentrations of copper and penicillamine were added to the reaction mixture, no

protection of pyruvate kinase could be demonstrated against copper inhibition. However, an increase in the concentration of penicillamine to 2 mM resulted in almost complete protection against the inhibitory effects of copper. Similar to the action on brain enzyme, addition of calcium produced a marked inhibition of uterine pyruvate kinase. The degree of inhibition was related to the amount of calcium present in the assay system. Lineweaver-Burk plots indicated that calcium produced a non-competitive inhibition of the uterine enzyme with an apparent K_i around 7.3×10^{-4}M. The observed inhibition produced by calcium was prevented by ethylenediamine tetraacetate (EDTA). The slight inhibitory effect seen in the presence of high concentrations of EDTA (2 mM) could be overcome by increasing the concentration of magnesium.

Whereas cyclic 3',5'-adenosine monophosphate failed to exert any appreciable effect on the uterine enzyme, addition of the sulphydryl inhibitor, p-chloromercuribenzoate produced a dose-dependent inhibition of uterine pyruvate kinase activity. The inhibition was almost complete when p-chloromercuribenzoate was present in a concentration of 0.01 mM. When penicillamine was added simultaneously with p-chloromercuribenzoate, the inhibition of pyruvate kinase was prevented indicating that whereas p-chloromercuribenzoate inhibited the uterine enzyme by inactivating sulphydryl groups, penicillamine protected against this inhibition by providing free sulphydryl groups to the enzyme. The results presented in this dissertation suggest that pyruvate kinase activity in the uterus may be subject to several types of positive and negative modulating influences exerted by cations and specific metabolites. The kinetic characteristics of uterine pyruvate kinase seem to resemble those of the M-type

isozyme believed to be also present in tissues such as brain, heart, kidney, leucocytes, spleen, fat pads and testes.

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