

GLUCONEOGENIC ADAPTATIONS IN  
CANCER MAGISTER

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## ABSTRACT

The periodic requirement for a new chitinous exoskeleton imposes a large biosynthetic load on the metabolism of crustaceans, with the hypodermis facing the brunt of the load. Since the freshly molted animal is highly susceptible to predation, the mechanisms for efficient gluconeogenesis in support of chitin synthesis are of definite survival value to the organism. Measurements of enzyme activities in the hypodermis, gill and muscle of Cancer magister indicate that the hypodermis and muscle undergo considerable metabolic changes during the molt cycle. Freshmolt hypodermis shows elevated specific activities of both the gluconeogenic and the glycolytic enzymes, while freshmolt muscle shows decreased levels of the glycolytic and increased levels of the gluconeogenic enzymes. Hypodermis maintains a highly gluconeogenic orientation throughout the molt cycle.

Phosphoglycerate kinase is considered to be one of the bifunctional enzymes in the glycolytic pathway, but the kinetic characteristics of the previously studied enzymes are ill suited for function in a gluconeogenic system. Since the intermolt and freshmolt muscle and hypodermis present a variety of metabolic poises (i. e. ranging from highly gluconeogenic to highly glycolytic), I studied the control of phosphoglycerate kinase in these tissues. I found that the intermolt muscle enzyme shows kinetics much like those of the mammalian muscle and the yeast enzyme, with a high sensitivity to MgADP/ADP inhibition ( $MgADP/K_i = 1.3 \times 10^{-5}M$ ) and a relatively low affinity for ATP as a

substrate ( $K_m = 2.03 \times 10^{-4} M$ ). By contrast, the freshmolt hypodermal enzyme shows a considerably decreased sensitivity to MgADP/ADP inhibition ( $MgADP K_i = 2 \times 10^{-4} M$ ) and a considerably increased affinity for ATP ( $K_m = 6.8 \times 10^{-5} M$ ). The freshmolt muscle enzyme also shares these changed affinities. The intermolt hypodermal phosphoglycerate kinase shows the decreased sensitivity to MgADP/ADP inhibition but shares the ATP affinity of the intermolt muscle enzyme. The kinetic characteristics of the freshmolt hypodermal and muscle enzymes reduce the susceptibility of the enzymes to inhibition by MgADP and facilitate the reversal of the reaction for gluconeogenesis.

The control of pyruvate kinase is integral to the control of both glycolysis and gluconeogenesis. In glycolysis, it forms the second major control site; in gluconeogenesis, it is one of the prime determinants of the rate of gluconeogenesis from lactate and amino acids. Muscle and hypodermal pyruvate kinases from Cancer magister are distinct proteins, on the basis of isoelectric points, kinetic characteristics, and thermal denaturation behavior. In contrast to the phosphoglycerate kinase system, there are no pronounced differences between freshmolt and intermolt forms. Muscle pyruvate kinase is activated by FDP, inhibited by MgATP, arginine phosphate, Mg2citrate, tryptophan and is also sensitive to some inhibition by alanine,  $\alpha$ -glycerolphosphate, Mg-malate and  $\alpha$ -ketoglutarate. The muscle enzyme has a high affinity for PEP ( $K_m = 0.1 \text{ mM}$ ) and the addition of  $0.05 \text{ mM}$  FDP drops the PEP  $K_m$  to  $0.05 \text{ mM}$ . In comparison with other muscle pyruvate kinases, the enzyme is quite sensitive to MgATP inhibition ( $K_i = 1.8 \text{ mM}$ ) and shows FDP

reversal of the inhibition. Arginine phosphate inhibition is competitive with ADP, and is not reversed by FDP. The reversal of the reaction accounts for only 0.5% of the forward reaction. Although high levels of ATP and arginine phosphate strongly inhibit the reaction, the inhibition is not sufficient to allow net flux through the low levels of the bypass enzymes present in the muscle of freshmolt animals. Thus, muscle pyruvate kinase has kinetic characteristics which suit it for function in the control of glycolysis, but do not allow gluconeogenic flux past the reaction locus.

In contrast, hypodermal pyruvate kinase is a complex protein capable of making large transitions between high activity during oxidation of carbohydrate substrates and virtually no activity during gluconeogenesis from lactate and amino acids. Hypodermal pyruvate kinase exists in two conformational states, one a high affinity state (PK I) and the other a low affinity state (PK II). PK I has a  $K_m$  for PEP of 0.1 mM and a  $K_a$  for FDP of  $1.3 \times 10^{-5}M$ . PK II has a  $K_m$  for PEP of 0.55 mM and a  $K_a$  for FDP of  $9 \times 10^{-8}M$ . For both forms, FDP facilitates the binding of PEP. Both forms are sensitive to MgATP inhibition and show FDP reversal of the inhibition. PK II is more sensitive to inhibition by alanine, serine, and Mg2citrate. For PK II, FDP alters the inhibition due to these compounds, changing the interactions between these inhibitors and both PEP and ADP. Incubation of PK II with 0.05 mM FDP produces PK I. Prolonged dialysis of PK I leads to an enzyme with the characteristics of PK II. The levels of FDP associated with PK I are higher than the levels associated with PK II.

During gluconeogenesis, the FDP levels in the cell are low. This would shift the equilibrium between the two forms towards PK II. Since physiological levels of PEP, ADP, ATP, alanine, and serine limit PK II activity to less than 0.5% of maximal, considerable flux through the phosphoenolpyruvate carboxykinase and pyruvate carboxylase bypass would be feasible. The rise in FDP levels during inhibition of gluconeogenesis would shift the equilibrium in favor of PK I. This shift would immediately raise pyruvate kinase activity from less than 0.5% to around 50% of maximal activity. This, coupled with the other changes in metabolite levels during an inhibition of gluconeogenesis, would lead to a marked activation of pyruvate kinase activity. These conformational states allow rapid changes in flux through the reaction, and thus would allow flexible and responsive regulation of this important glycolytic and gluconeogenic control site. Thus, both the phosphoglycerate kinase and pyruvate kinase present in the hypodermis of Cancer magister have special characteristics which facilitate efficient gluconeogenesis.

To elucidate the possible importance of ions in regulating the activity of the above enzymes, I measured the levels of sodium, potassium, magnesium, and calcium in the muscle and hypodermis of intermolt and freshmolt animals. I found that the extracellular space of the hypodermis is considerably higher than that of the muscle (45% versus 12%), but that there was no variation between intermolt and freshmolt tissue extracellular space. While freshmolt muscle sodium concentrations were significantly higher than intermolt sodium concentrations, none

of the other ions showed significant differences between molt cycle stages. However, there were significant differences between the ionic concentrations in hypodermis and muscle. Hypodermis showed higher calcium levels and lower potassium levels than muscle in both freshmolt and intermolt animals. Although ionic changes do not play a role in differential regulation of enzyme activity during the molt cycle, the ionic concentrations present in these tissues are such that the ions could set guidelines for the activity of phosphoglycerate kinase, pyruvate kinase, phosphofructokinase, and fructose diphosphatase in these tissues.

## TABLE OF CONTENTS

INTRODUCTION .....	1
Molt Cycle Physiology .....	6
Intermediary Metabolism in Crustaceans .....	16
Central Questions in this study .....	30
GENERAL MATERIALS AND METHODS	
Experimental Animals .....	38
Reagents .....	39
Preparation of Enzymes .....	39
Starch Gels and Isoelectrofocusing .....	40
Kinetic Analysis .....	41
PART I. Changing Enzyme Activities in the Hypodermis, Gill, and Muscle of <u>Cancer magister</u> during the Molt Cycle	
INTRODUCTION .....	43
MATERIALS AND METHODS .....	43
Preparation of the Homogenate for Measurement of Enzyme Activities .....	44
Enzyme Assays .....	44
RESULTS AND DISCUSSION .....	47
PART II. Control of Hypodermal Phosphoglycerate Kinase in Intermolt and Freshmolt <u>Cancer magister</u>	
INTRODUCTION .....	54
METHODS	
Preparation of Phosphoglycerate Kinase .....	57
Metabolite Measurements .....	58

Enzyme Assays .....	59
---------------------	----

## RESULTS

Specific Activities .....	59
Different Forms of Phosphoglycerate Kinase .....	61
Kinetic Properties .....	65
Mass Action Ratios .....	81
DISCUSSION .....	85

## PART III. Muscle Pyruvate Kinase

INTRODUCTION .....	93
--------------------	----

## METHODS

Preparation of Pyruvate Kinase .....	96
Enzyme Activities .....	97

## RESULTS

Electrophoresis and Isoelectrofocusing .....	98
Catalytic and Regulatory Properties .....	99
Substrate Affinities .....	100
Activators .....	102
Inhibitors .....	102
DISCUSSION .....	118

## PART IV Hypodermal Pyruvate Kinase

INTRODUCTION .....	122
--------------------	-----

METHODS .....	123
---------------	-----

Measurement of Bound Fructose 1,6 Diphosphate .....	124
Enzyme Assays .....	124

## RESULTS

Electrophoresis and Electrofocusing .....	124
Catalytic and Regulatory Characteristics .....	125
Substrate Affinities .....	128
Activators .....	131
Inhibitors .....	133
DISCUSSION .....	153

PART V. Cationic Composition of Intermolt and  
Freshmolt Hypodermis and Muscle

INTRODUCTION .....	160
--------------------	-----

## METHODS

Collection and Maintenance of Animals .....	162
Tissue Preparation for Ion Measurements .....	163
Determination of Ion Concentrations .....	164
Determination of Extracellular Space .....	164

## RESULTS AND DISCUSSION

Extracellular Space Determination .....	165
Ionic Composition of the Hemolymph .....	167
Ionic Composition of Muscle and Hypodermis .....	169

GENERAL DISCUSSION AND SUMMARY .....	178
--------------------------------------	-----

Hypodermal Gluconeogenesis .....	179
Muscle Gluconeogenesis .....	180
Molt Cycle Variations in Enzyme Levels .....	182
Control of Glycolysis and Gluconeogenesis in the Hypodermis .....	183
Integration of Gluconeogenesis and Lipid Oxidation ....	186

Inhibition of Gluconeogenesis and Activation of Glycolysis .....	188
Phosphoglycerate Kinase in Glucconeogenic Tissues .....	192
Regulatory Properties of Pyruvate Kinase from <u>Cancer</u> <u>magister</u> .....	193
REFERENCES .....	197

## LIST OF FIGURES

Figure 1. Variation in specific activity of phosphoglycerate kinase throughout the molt cycle in muscle and hypodermis. ....	60
Figure 2. A diagrammatic sketch of a starch gel electrophoretogram showing zones of phosphoglycerate kinase activity extracted from muscle and hypodermis of <u>Cancer magister</u> . ....	64
Figure 3. Michaelis constants for ATP and 3-P-glycerate at varying levels of cosubstrate and activator. ....	68
Figure 4. ADP inhibition of intermolt muscle phosphoglycerate kinase, effect of increasing magnesium concentration. ....	72
Figure 5. Intermolt muscle phosphoglycerate kinase, ADP and MgADP inhibition patterns. ....	74
Figure 6. Molting hypodermal phosphoglycerate kinase, ADP and MgADP inhibition patterns ....	78
Figure 7. Effect of MgATP and FDP on PEP affinity of <u>Cancer magister</u> muscle pyruvate kinase. ....	103
Figure 8. MgATP inhibition, effect on the affinity of muscle pyruvate kinase for PEP. ....	104
Figure 9. MgATP inhibition, effect on ADP affinity of muscle pyruvate kinase. ....	105
Figure 10. Arginine phosphate inhibition, effect on the ADP affinity of <u>Cancer magister</u> muscle pyruvate kinase. ...	107
Figure 11. Arginine phosphate inhibition, effect on the PEP affinity of the pyruvate kinase from <u>Cancer magister</u> muscle. ....	108
Figure 12. Dixon plot of tryptophan inhibition of the muscle pyruvate kinase from <u>Cancer magister</u> . ....	110
Figure 13. Dixon plot of Mg-malate inhibition of the muscle pyruvate kinase from <u>Cancer magister</u> . ....	111
Figure 14. Dixon plot of Mg-citrate inhibition of the muscle pyruvate kinase from <u>Cancer magister</u> . ....	113
Figure 15. Thermal denaturation of <u>Cancer magister</u> muscle pyruvate kinase. ....	117
Figure 16. Phosphoenolpyruvate affinity of hypodermal PK I. ....	129

Figure 17. Phosphoenolpyruvate affinity of hypodermal PK II. ....	130
Figure 18. Influence of FDP on the phosphoenolpyruvate affinity of PK I and PK II from the hypodermis of <u>Cancer magister</u> . ....	132
Figure 19. MgATP inhibition of PK I, influence of MgATP on PEP affinity and the reversal of MgATP inhibition. ....	134
Figure 20. MgATP inhibition of phosphoenolpyruvate binding by PK II and the reversal of this inhibition by FDP. ....	135
Figure 21. Alanine inhibition of PK I and PK II and the influence of FDP on the inhibition pattern. ....	138
Figure 22. Serine inhibition of PK II and the influence of FDP upon the inhibition pattern. ....	139
Figure 23a. Mg <sub>2</sub> citrate inhibition of PK II, inhibition versus ADP and influence of FDP upon the inhibition pattern versus ADP and PEP. ....	141
Figure 23b. Mg <sub>2</sub> citrate inhibition of PK II, inhibition versus PEP. ....	142
Figure 24. Conversion of PK II into PK I by incubation with 0.05 mM FDP. ....	147
Figure 25. Thermal denaturation of <u>Cancer magister</u> hypodermal pyruvate kinase I. ....	150
Figure 26. Thermal denaturation of <u>Cancer magister</u> hypodermal pyruvate kinase II. ....	151
Figure 27. A summary metabolic map of the control of carbohydrate metabolism in the crustacean hypodermis. ....	156

## LIST OF TABLES

Table 1. Specific activities of various enzymes in tissues of freshmolt and intermolt <u>Cancer magister</u> . ....	48
Table 2. ATP and 3-P-glycerate affinities of phosphoglycerate kinase from freshmolt and intermolt tissues of <u>Cancer magister</u> . ....	62
Table 3. Michaelis constants for activating cations of freshmolt and intermolt phcsphoglycerate kinases. ....	67
Table 4. Michaelis constants for ATP and MgATP . ....	70
Table 5. Ki values for ADP and MgADP inhibition of phosphoglycerate kinase from freshmolt and intermolt muscle and hypodermis. ....	73
Table 6. Ki values for AMP, 2,3 DPG and CaCl <sub>2</sub> inhibition of phosphoglycerate kinase from freshmolt and intermolt muscle and hypodermis. ....	82
Table 7. Phosphoglycerate kinase mass action ratios from intermolt and freshmolt muscle and hypodermis. ....	83
Table 8. Nucleotide diphosphate acceptability for the muscle pyruvate kinase in <u>Cancer magister</u> . ....	101
Table 9. Effectiveness of activators and inhibitors of muscle pyruvate kinase from <u>Cancer magister</u> . ....	114
Table 10. Reversal of the muscle pyruvate kinase from <u>Cancer magister</u> . ....	116
Table 11. Effectiveness of various inhibitors of hypodermal PK I. ....	143
Table 12. Effectiveness of various inhibitors of hypodermal PK II. ....	144
Table 13. Effect of FDP, ATP and alanine on the interconversions of hypodermal PK I and PK II from <u>Cancer magister</u> . ....	148
Table 14. Extracellular space in muscle and hypodermis of <u>Cancer magister</u> . ....	166
Table 15. Hemolymph ion concentrations in intermolt and freshmolt <u>Cancer magister</u> . ....	168
Table 16. Freshmolt and intermolt muscle ion concentrations. ....	170
Table 17. Freshmolt and intermolt hypodermal ion concentrations. ....	171

Table 18. Comparison of muscle and hypodermal ion concentrations. ....	173
--	-----

Table 19. Comparison of kinetic characteristics of muscle and hypodermal pyruvate kinases from <u>Cancer</u> <u>magister</u> . ....	194
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## INTRODUCTION

To grow, crustaceans must periodically shed their exoskeleton, expand in volume while their exoskeleton is soft, and then quickly harden the "soft shell". This rapid thickening and hardening requires both efficient gluconeogenesis to support chitin synthesis, and rapid calcification of the newly deposited lamellae of chitin. While ecdysis is the most dramatic event in the process of crustacean growth, it is only one phase of a complex series of physiological and behavioral changes which constitute the crustacean molt cycle. The hypodermis, lying directly beneath the exoskeleton, is most directly associated with this large biosynthetic load, but other crustacean tissues are also profoundly affected by the molt cycle.

Several major changes occur during the molt cycle. During intermolt, the animal is actively feeding and deposits considerable lipid and glycogen. Lipid is stored primarily in the hepatopancreas (Passano, 1960), while glycogen deposits occur in muscle, hypodermis, hepatopancreas, and blood cells (Hohnke, 1971; Travis, 1955a; Renaud, 1949; Johnston & Davies, 1972; Dall, 1975). Hormonal signals initiate the premolt period: one of the early changes is the separation of the hypodermis from the exoskeleton. After some initial resorption of the organic (and in freshwater crustaceans, the inorganic) components of the exoskeleton, the new cuticle starts growing under the old. New gill filaments form inside the old, and the leg muscles decrease to 60% of their intermolt mass (Skinner, 1966). During late premolt, wholesale resorption of chitin from

the old cuticle occurs (Speck & Urich, 1971). Just prior to molt, the animal begins to increase its volume, resorbs much of the chitin from the molting suture (Travis, 1955b), splits the suture and begins to pull out of the old shell. Generally, the animal seeks a sheltered place and ceases feeding shortly before molt. Once the animal has withdrawn from the old cuticle, rapid thickening and hardening of the new cuticle occurs. This is critical as the soft, immobile animal is highly susceptible to predation. Chitin synthesis is maximal and rapid calcium deposition occurs until the cuticle is hardened (Stevenson, 1972; Adelung, 1971; Travis, 1955a; Digby, 1966). Once the exoskeleton is relatively firm, the animal resumes feeding, and tissue growth within the increased space begins (Passano, 1960).

The duration of the molt cycle is a function of a number of physiological and environmental variables, and different species respond to these variables differently (Bliss & Boyer, 1964; Passano, 1960; Adelung, 1971). Generally, the crab molts when it has grown a sufficient quantity, i.e. a given percentage of its freshmolt mass. Thus, animals of greater size take longer to complete the cycle (Passano, 1960; Adelung, 1971). Loss of legs also serves to stimulate molting. If more than five of eight walking legs are removed, the intermolt duration is reduced by 50% (Skinner & Graham, 1972; Adelung, 1971). The presence of larger members of the same species in the same tank will cause an increase in intermolt duration, while the presence of an equal size member of the species does not affect the intermolt duration in Carcinus maenas (Adelung, 1971). Gecarcinus lateralis also shows inhibition of proecdysial growth

in the presence of another crab, as well as under high temperature and light conditions. On the other hand, darkness, moderate temperature, solitude, and a source of water all favor proecdysial growth in this land crab. Gecarcinus lateralis also has the capability to delay ecdysis once the initial portions of premolt have occurred (Bliss & Boyer, 1964).

The first extensive formulation of the cyclic pattern of variations which constitutes the molt cycle was made by Drach (1939), in his studies of Carcinus maenas and other brachyurans. His classification scheme assigns morphological conditions, primarily involving changes in cuticle structure, to each molt cycle stage. The crustacean cuticle is composed of four major layers: the epicuticle which contains a high proportion of lipoprotein, the exocuticle which contains protein, chitin, crosslinks, and calcium, the endocuticle which contains protein, chitin and calcium, and the underlying membranous layer composed of chitin and protein alone. In stage A1, the animal is freshly molted, while in stage A2 some mineralization of the soft cuticle has begun. During stage B1 endocuticle secretion begins; and in stage B2 the chelae are hard, active endocuticle formation is occurring, and tissue growth begins. During the various "C" stages the animal is hard; during C1 and C2 the main tissue growth occurs, in C3 the completion of the exoskeleton by the formation of the membranous layer occurs. In C4 the classic "intermolt" condition prevails, with considerable accumulation of organic reserves. The stages from D1 through D4 constitute the premolt period; D0 has no morphological correlates, but it marks the initiation of premolt by hormonal changes. D1 is

characterized by both the separation of the hypodermis from the old exoskeleton and the formation of an epicuticle. During D2 the formation of the exocuticle begins, in D3 the major portion of resorption occurs, and in D4 the ecdysial sutures open. Stage E is ecdysis, the actual act of molting (Drach, 1939). While the definition of these stages has been modified slightly to facilitate staging in certain organisms (Passano, 1960; Stevenson, 1972), the basic pattern has been found to apply to the development of most crustaceans (Passano, 1960). Drach's staging criteria were used throughout the present study.

Numerous studies of the molt cycle have followed Drach's pioneering work. These subsequent investigations have led to a considerable clarification of the physiology of molting. Furthermore, the many studies of patterns of carbon flux help elucidate the metabolic control mechanisms utilized by crustaceans. In the following pages, I will attempt to summarize the current picture of crustacean metabolism and its varying functions throughout the molt cycle. The studies which form the body of this thesis concern metabolic changes in the hypodermis and muscle of Cancer magister as the animal passes through the molt cycle. Specifically, the work focuses upon the enzymatic control mechanisms which facilitate gluconeogenesis, the synthesis of sugars from non-carbohydrate precursors, in the hypodermis and their comparison with control mechanisms present in the predominantly glycolytic muscle.

The two enzymes which I examined were phosphoglycerate kinase and pyruvate kinase. Both enzymes present interesting

loci in the control of glycolysis and gluconeogenesis. Phosphoglycerate kinase is one of the glycolytic enzymes thought to function during the reversal of glycolysis for gluconeogenesis. However, the kinetics of previously examined phosphoglycerate kinases strongly favor glycolytic function. To elucidate how reversal of the reaction is facilitated for gluconeogenesis, I compared the regulatory characteristics of muscle and hypodermal phosphoglycerate kinases. During gluconeogenesis, pyruvate kinase activity must be curtailed to allow flux past the reaction, while during carbohydrate oxidation, pyruvate kinase is central in the regulation of glycolytic flux. A comparison of pyruvate kinase in hypodermis and muscle should clarify how these distinct control requirements are fulfilled. Fructose diphosphatase, the other major gluconeogenic control site, has been studied in the hypodermis, gill, and muscle of Paralithodes camtchatica (Hochachka, 1972; Behrisch, 1971, 1972; Behrisch & Johnson, 1974). A close examination of the regulatory properties of phosphoglycerate kinase and pyruvate kinase should allow the formulation of a metabolic control theory describing the regulation of hypodermal gluconeogenesis. Comparison of the enzymes from muscle and hypodermis will indicate to what extent separate isozymes have evolved to answer the gluconeogenic requirements of the hypodermis, as well as clarifying the control mechanisms of muscle metabolism.

## Molt Cycle Physiology

The major organs of the crustacean body undergo considerable variations in size and composition as the animal goes through the molt cycle. Particularly marked changes are shown by the hypodermis, the hepatopancreas, and the muscle. The hypodermis is an active biosynthetic tissue which undergoes major alterations in size according to the extent of its biosynthetic activity. The hepatopancreas is both a digestive and a storage organ, which shows considerable shifts in the levels of organic reserves. While the role of the changes shown by muscle is not completely clarified, they are still remarkable.

Hypodermis. Upon initiation of premolt, the hypodermis increases in thickness and extent (Travis, 1955a), shows an increase in the rate of protein synthesis (Skinner, 1968; McWhinnie *et al.*, 1972; Stevenson, 1972; Humphreys & Stevenson, 1973), and shows an increase in oxygen consumption (Skinner, 1958; Skinner, 1962). The protein synthesis is directed towards both the enlargement of the hypodermis and the eventual deposition of protein in the new exoskeleton. The increased oxygen consumption may account for the increase in whole animal oxygen consumption found during premolt (Bulnheim, 1974; Lewis & Haefner, 1976). During the first part of premolt, the hypodermis secretes fluid, enzymes, and possibly cells into the space between itself and the old exoskeleton. These enzymes are involved in the resorption of chitin and protein from the old exoskeleton (Travis, 1955a; Dennell, 1960). The hypodermis is

capable of chitin synthesis throughout the molt cycle (Hornung & Stevenson, 1971; Stevenson, 1972); however, the rate increases markedly during stage D2 of premolt, and reaches its maximum during stages A and B directly following ecdysis (Stevenson, 1972). During premolt, large quantities of glycogen and some lipid are deposited in the hypodermis (Travis, 1955a; Renaud, 1949). These reserves are present immediately after the molt, but then decline during secretion of the new exoskeleton (Renaud, 1949; Travis, 1957). The connective tissue which lies directly beneath the hypodermis is supplied with numerous reserve cells, which probably originate from blood cells. These cells undergo considerable variations both in number and in amount of stored materials, during the postmolt period (Travis, 1957; Skinner, 1962). These cells are sometimes full of glycoprotein, mucopolysaccharides, or lipoprotein. They may also contain deposits of calcium and phosphate (Sewell, 1955; Travis, 1957; Skinner, 1962). Large quantities of inorganic material, primarily calcium and phosphate, also move through the hypodermis to the new exoskeleton during postmolt. Both the preexuvial layers and the freshly deposited lamellae of chitin are calcified after the molt. After the synthesis and calcification of the exoskeleton is complete, the hypodermis decreases in size and only performs a low rate of chitin synthesis (Travis, 1955a; Skinner, 1962; Stevenson, 1972).

Hepatopancreas. The hepatopancreas of intermolt crustaceans generally shows high levels of glycogen and lipids (Renaud, 1949; Travis, 1955a; Heath & Barnes, 1970). In Carcinus maenas, the levels of fatty acids are ten fold those of

glycogen (Heath & Barnes, 1970). In Cancer pagurus, lipids also form a major part of the metabolic stores (Renaud, 1949). The hepatopancreas is composed of a variety of cell types, including absorptive, secretory, and reserve cells. During intermolt and early premolt, fat and glycogen are abundant in both the absorptive and the secretory cells. In late premolt, the reserve cells increase in number and are found to contain mucopolysaccharide, lipid, and sometimes deposits of calcium. In freshwater forms, calcium storage begins early in premolt with the formation of gastroliths, while in marine crustaceans calcium and phosphate storage occurs only in late premolt (McWhinnie et al., 1972; Chen & Lehninger, 1973; Glynn, 1968; Travis, 1955a; Renaud, 1949). In Panulirus argus latrillei, glycogen levels in the hepatopancreas are at their peak during the first day after ecdysis, and then progressively decline until the seventh day, by which time they have disappeared (Travis, 1955a). Lipid levels decline as well but not to the same extent as the glycogen levels. The reserve cells show cyclic variations, which, if taken in relation to those shown by the reserve cells in the hypodermis (see above), could indicate that these cells are moving from the hepatopancreas to the hypodermis, and supplying the hypodermis with substrate for chitin synthesis (Travis, 1955a; Travis, 1957). Calcium levels in the hepatopancreas drop markedly so that a week after the molt, no "calcospherites" are visible in the tissue (Travis, 1957). Renaud (1949) has calculated that 70 % of the protein nitrogen present in the stage D1 hepatopancreas is utilized during the succeeding molt. Similar cyclic variations in

hepatopancreatic stores have been found to occur in Cancer pagurus (Renaud, 1949), Carcinus maenas (Heath & Barnes, 1970), and Panulirus argus latrille (Travis, 1955a; Travis, 1957). However, such storage cycles do not necessarily hold for all crustaceans. Carcinus maenas have been observed to molt with virtually no metabolic stores (Heath & Barnes, 1970). In Orconectes limosus, the metabolic stores present in the animal do not significantly augment the maintenance metabolism or the active biosynthetic output during ecdysis. In these animals, carbon from resorbed acetylglucosamine provides the sole support for both chitin synthesis and energy metabolism during the late premolt and early postmolt period (Speck & Urich, 1971).

Muscle. The changes incurred by crustacean muscle as a function of the molt cycle may contribute to the above described cycles of stored compounds in the hypodermis and hepatopancreas. However, no information is available to either support or refute this concept. The changes in muscle have been thoroughly described in only one animal, Gecarcinus lateralis (Skinner, 1966). During premolt, the muscles present in the legs of these animals decrease to 60% of their intermolt mass. The ratio of protein to wet weight stays constant, thus, the change is not due to a change in tissue hydration. The amount of DNA in the whole muscle stays constant, indicating that there is a drop in the amount of protein present per cell (Skinner, 1966). This decrease in muscle mass may facilitate the withdrawal of the leg through the narrow joints in the old exoskeleton. Reformation of the muscle did not occur until between 6 and 20 days postmolt (Skinner, 1966). Although Skinner's (1966) study is the only

systematic study of this topic, early researchers (Hoet & Kerridge, 1926) found similar changes in the overall characteristics of muscle from intermolt and freshmolt animals. Furthermore, it is an established fact among lobster fishermen that the muscle of freshly molted animals is quite different from that in intermolt animals. The lipoprotein cells, which are known to invade the muscle during premolt (Sewell, 1955), may aide in the destruction of muscle protein. According to a recent study, the protein lost from the muscle accounts for the increased levels of hemolymph protein which occur just before molt (Mantel et al., 1975). The muscle protein could also contribute to the increased levels of tissue free amino acids found during the late premolt period (Duchateau et al., 1959; McWhinnie et al., 1972; Speck & Urich, 1971).

Hemolymph. The other tissue which undergoes a fair number of changes is the hemolymph. During premolt, the whole blood is characterized by high levels of amino acids, protein, calcium, phosphate, glucose, and fats (Travis, 1955b; Florkin, 1960; Adelung, 1971). In Gecarcinus lateralis, the blood osmotic concentration drops from 1000 mOsm before molt to 875 mOsm at ecdysis (Mantel et al., 1975). Presumably, the premolt increase in blood osmolarity facilitates the uptake of water during ecdysis and late premolt (Adelung, 1971). Changes in the ionic composition of the serum also occur. Specifically, postmolt sodium, potassium, magnesium, and chloride concentrations are considerably lower than the premolt values, for Carcinus maenas (Robertson, 1960). Changes in the magnesium and calcium concentration were found for Homarus vulgaris by Glynn (1968)

and Carcinus maenas by Adelung (1971). In the latter study, pronounced variations in the potassium concentration were not found, and sodium concentrations were found to vary in a less systematic fashion than in Robertson's (1960) study. The absolute number of blood cells circulating at molt is 2 to 5 times higher than during intermolt (Maynard, 1960). There are two types of blood cells in crustaceans, one of which has been found to contain a large glycogen and acid polysaccharide store, as well as the enzyme glucose-6-phosphatase (Johnston *et al.*, 1973). The carbohydrates contained by these cells represent approximately 50% of the total available carbohydrate in Carcinus maenas (Johnston *et al.*, 1973). By contrast, Dall (1975), found only 5.5% of available carbohydrate in the blood cells of Panulirus longipes. The whole blood contains a variety of circulating carbohydrates, including glucose, trehalose, maltose, fructose, fucose, glucosamine, glucose-6-phosphate, galactose, mannose, maltotriose, various oligosaccharides, and in Panulirus longipes, two distinct glycoproteins (Dall, 1975; Telford, 1968; Schwoch, 1972; Parvarthy, 1970; Johnston & Davies, 1972). With the exception of glucose, no clear variation of any of these components with the molt cycle has been found (Jeniaux, 1971). Even the variation due to glucose is somewhat erratic (Adelung, 1971). However, glucose concentrations generally decrease after molt (Adelung, 1971; Dall, 1975).

Hormonal Control Mechanisms. Hormonal control mechanisms are undoubtedly of underlying importance in the control of the molt cycle. Considerable work has been done on hormonal control

mechanisms in Crustacea, and a relatively clear picture of the control of molting has now emerged. Two major endocrine glands are involved in the control of the molt cycle, the sinus gland-medulla terminalis X organ complex and the Y organ. The sinus gland and the medulla terminalis X organ are located in the eyestalk; they contain and release, among other hormones, a molt inhibiting hormone. Thus, eyestalk ablation usually results in precocious ecdysis. The major target organ of this molt inhibiting hormone is probably the Y organ. This organ shows histological changes which are well correlated with the molt cycle (Gabe, 1953). Furthermore, removal of these glands leads to a permanent inhibition of molting in intermolt animals (Echalier, 1954). The Y organ releases a molt accelerating hormone or hormones (Fingerman, 1973). The Y organ may secrete a prohormone which is then converted to an active form in various tissues throughout the body (Fingerman, 1974). The hormones involved are steroids similar to ecdysone: crustecdysone is 20-hydroxyecdysone and callinecdysone A and B are inoksterone and makisterone respectively (Fingerman, 1973). The hormonal content of Carcinus maenas and Callinectes sapidus has been measured at intervals throughout the molt cycle (Adelung, 1971; Faux et al., 1969). In Carcinus maenas, the titre of 20-hydroxyecdysone passes through several distinct maxima during premolt, with the major peak and fall occurring shortly before molt (Adelung, 1971). Orconectes limosus also showed a steep drop in 20-hydroxyecdysone just before molt (Keller & Adelung, 1970). In Callinectes sapidus, a rising titre of hormones was found as well, but in this organism

several hormones were measured, callinecdysone A, 20-hydroxyecdysone, and callinecdysone B. Early in premolt, only callinecdysone A was present. It continued to increase in concentration and later in premolt was joined by low levels of 20-hydroxyecdysone. After ecdysis, the latter hormone was present in 70 fold higher levels than before molt, in contrast to the situation in Carcinus maenas. Callinecdysone B was also present in low levels during postmolt (Faux et al., 1969). Thus, a complex interplay of different ecdysones may occur, with peaks of hormone content initiating certain aspects of development. Adelung (1971) correlated the peaks in 20-hydroxyecdysone with peaks in hemolymph protein and amino acid concentration, suggesting that the steroid stimulated protein synthesis. Ecdysone stimulation of protein synthesis is indicated in some insect systems (Karlson & Sekeris, 1962). The evidence is not as perfected for crustacean systems, but the circumstantial evidence is quite good. Increases in rRNA parallel the increase in protein synthesis in Gecarcinus lateralis (Skinner, 1968). Actinomycin D inhibition of RNA synthesis prevents the premolt initiation of chitin synthesis in the crayfish (Stevenson & Tung, 1971). Slight increases in the rate of amino acid incorporation were found upon ecdysone stimulation of the hypodermis of Orconectes virilis (McWhinnie et al., 1972). In Orconectes obscurus and Orconectes sanborni the rate of protein synthesis increases during premolt showing two maxima (Stevenson, 1972) which correspond with the maxima of 20-hydroxyecdysone shown during premolt in Carcinus maenas (Adelung, 1971). In Orconectes limosus, peaks of RNA synthesis

follow the peaks in 20-hydroxyecdysone with a certain delay (Keller & Adelung, 1970).

In summary, the initiation of premolt comes about through a reduction in the levels of a molt inhibiting hormone secreted by the medulla terminalis X organ. This then releases the Y organ from inhibition and allows it to release the appropriate molt accelerating hormone or prohormone. This hormone then activates the systems of protein synthesis, primarily in the tegumental glands and the hypodermis (Keller & Adelung, 1970). The increasing titres of hormone as the animal passes through premolt probably play an important role in initiating certain physiological activities. However, the question as to what causes the initial reduction of molt inhibiting hormone needs consideration. In some careful studies of Carcinus maenas, Adelung (1971) showed that reduction of molt inhibiting hormone was probably dependent upon the animal reaching a certain degree of growth. A shortening of the intermolt period, i.e. a precocious reduction in the levels of molt inhibiting hormone, occurred only after removal of more than five of the eight walking legs. All the factors which inhibit premolt initiation, seem to do so via an inhibition of growth. The animals consume less food during unfavorable environmental conditions, in the presence of a larger member of the same species, or in cold temperatures (Adelung, 1971). Further proof of these interacting control mechanisms comes from the fact that different size animals, which would ordinarily take different lengths of time to molt, molt with equal rapidity after eyestalk ablation. Furthermore, animals from different nutritional

regimes also molt with the same frequency after eyestalk ablation (Adelung, 1971). However, the exact physical mechanism whereby adequate growth is translated into the cessation of secretion of molt inhibiting hormone is not known.

The molt cycle is not the only system which is under hormonal control in crustaceans. Besides directing the pattern of reproductive events (Fingerman, 1973), hormones are involved with the control of salt and water balance (Heit & Fingerman, 1975) and with control of respiration during temperature acclimation (Silverthorn, 1975). Furthermore, hormones also control certain aspects of carbohydrate metabolism. The sinus gland contains a hyperglycemic peptide hormone. Injection of eyestalk extract in Cancer magister and Hemigrapsus nudus caused an increase in blood maltose levels (Ramamurthi et al., 1968). Destalking these animals leads to an increase in muscle glycogen synthetase levels and a concomitant decrease in total phosphorylase. The expected increase in glycogen deposition was not found, instead, an increase in turnover was found (Ramamurthi et al., 1968). Keller (1969) found that the hyperglycemic peptide hormone of Orconectes limosus was cross reactive with closely related species but not with a variety of other crustaceans. Telford (1975) found that this peptide induced increased muscle glycogen phosphorylase activity and that muscle contributed the bulk of the glucose to hyperglycemia.

## Intermediary Metabolism in Crustaceans

Within this framework of major physiological alterations during the molt cycle, the control mechanisms and basic patterns of intermediary metabolism in crustaceans assume additional interest. Not only would the growing premolt hypodermis serve as a good system for the study of hormonal induction of enzyme synthesis, but the muscle would serve as a good system within which to consider the changes which occur during tissue breakdown. Furthermore, and of central interest to this study, the gluconeogenic and biosynthetic mechanisms utilized by the hypodermis during the initial postmolt period are critical for the survival of the soft, highly vulnerable animal. Since these mechanisms have been placed under evolutionary selective pressures, they should serve as a good system for the examination of efficient systems for gluconeogenesis and chitin biosynthesis.

Reaction Pathways. Most previous studies of crustacean metabolism have centered around identification of major reaction pathways. Despite early doubts about the presence of the glycolytic pathway in crustacean tissues (Scheer & Scheer, 1951), the presence of glycolytic and Krebs cycle enzymes is now well established (Vatsis & Schatzlein, 1972; Keller, 1965; Boulton & Huggins, 1970). There is considerable variation in the levels of glycolytic and Krebs cycle enzymes in different tissues. Muscle is primarily glycolytic, while heart, gills, and antennal gland show high levels of Krebs cycle enzymes, hexokinase, and malic enzyme (Keller, 1965). Hypodermis showed

relatively low enzyme activities in these starved intermolt animals, however, it evidenced an ample capacity for oxidation of carbohydrate substrates (Keller, 1965). Keller's (1965) study of Cambarus affinis metabolism is the only study in which enzyme levels in a variety of organs within the same organism are compared. He also compared the oxidation of a variety of substrates by these organs. In correspondence with studies of lobster muscle metabolism (Kermack et al., 1954), Keller (1965) found only minimal oxygen uptake by muscle. However, succinate, glutamate,  $\alpha$ -glycerolphosphate, glucose, and glucose-6-phosphate were oxidized by homogenates of heart, antennal gland, gonads, gills, intestine, and hepatopancreas. Succinate was the most efficiently oxidized substrate (Keller, 1965).

Succinate dehydrogenase from both the muscle and gill of Pachygrapsus crassipes differs from the enzyme in other systems in that it utilizes NAD and NADP as electron acceptors (Vatsis & Schatzlein, 1972). Aconitase, fumarase, isocitrate dehydrogenase, and succinate dehydrogenase were found both in the mitochondria and in the cytosol by these workers. Only the NADP linked isocitrate dehydrogenase was found in muscle and gill (Vatsis & Schatzlein, 1972). While citrate synthase and the NADP linked isocitrate dehydrogenase were found in all the crustacean muscles examined by Alp and coworkers (1976), the NAD linked isocitrate dehydrogenase was found only in Portunus puber and Limulus polyphemus. These two animals showed considerably higher levels of muscle citrate synthase and the NADP linked isocitrate dehydrogenase than the other crustaceans examined (Alp et al., 1976). Only the NADP linked isocitrate dehydro-

genase was found in the the nervous tissue of Homarus vulgaris (Sugden & Newsholme, 1975a).

Muscle Metabolism. Crustacean muscle metabolism has received considerable attention, with the result of considerable clarification of its metabolic control mechanisms. Boulton and Huggins (1970) measured the majority of the glycolytic enzymes in the muscle of Carcinus maenas, Cancer pagurus, and several other crustaceans. As was found by Keller (1965), hexokinase had the lowest specific activity in all the muscles where it could be measured. Glyceraldehyde-3-phosphate dehydrogenase levels were three fold higher than those of lactate dehydrogenase, in contrast to the situation in most mammalian tissues in which the two are present in equal levels (Boulton & Huggins, 1970). This finding, in combination with measurements of relatively high levels of  $\alpha$ -glycerol phosphate dehydrogenase in Cambarus affinis and Cambarus bartoni muscle (Keller, 1965; Popham & Dandy, 1976), implies that redox regulation in these muscles may proceed according to a different pattern than in mammalian muscle. The dehydrogenases may also be directly affected by hormonal controls, since Tilgner and Lesicki (1976) have found that eyestalk removal leads to changes in the kinetics of muscle lactate dehydrogenase. These changes were not due to the synthesis of a new isozyme of lactate dehydrogenase.

Both glycogen phosphorylase and phosphofructokinase are present in five fold higher levels than hexokinase in the muscle of Carcinus maenas (Crabtree & Newsholme, 1972). Muscle

phosphorylase and glycogen synthetase levels vary with the molt cycle (Hohnke, 1971). Opie and Newsholme (1967) measured fructose diphosphatase in the pincer muscle of Maia squinado and found 0.48 units/gram/minute at 25 °C. If similar levels are found in Carcinus maenas, the ratio of phosphofructokinase to fructose diphosphatase would lie around 10. At such low values of this ratio, considerable cycling can occur around this reaction locus. While this cycling is wasteful, it provides the potential of considerable amplification of net glycolytic or gluconeogenic flux. AMP is the most efficient regulatory signal at this reaction locus since it leads to net changes in flux far greater than its actual changes in concentrations. This occurs since it activates phosphofructokinase while inhibiting fructose diphosphatase (Newsholme & Start, 1973). Changes in the levels of inorganic phosphate and ammonium ion (Sugden & Newsholme, 1975b) as well as changes in the levels of inhibitors of fructose diphosphatase would also lead to net changes in glycolytic flux. High degrees of cycling are characteristic of predominantly glycolytic tissues (Newsholme & Start, 1973). Studies of glycolytic control in Paralithodes camtchatica muscle indicated hexokinase, phosphofructokinase, and pyruvate kinase as the important control sites (Hochachka et al., 1970). Thus, in these characteristics, this highly glycolytic crustacean muscle is similar to vertebrate white muscle.

Phosphofructokinase has been examined in muscle of Paralithodes camtchatica (Freed, 1971) and of Homarus vulgaris (Sugden & Newsholme, 1975b). The king crab muscle enzyme shows

kinetics similar to previously examined phosphofructokinases, including inhibition by high levels of ATP, activation by AMP, and accentuation of ATP inhibition by citrate (Freed, 1971). The lobster muscle enzyme showed activation by ammonium ion which was increased synergistically by the presence of inorganic phosphate. Concentrations of  $K^+$  over 50 mM caused inhibition of the lobster muscle enzyme (Sugden & Newsholme, 1975b). Apart from a study of temperature effects upon king crab pyruvate kinase (Somero, 1969), the regulatory properties of this enzyme have not been examined in crustacean tissues. Behrisch (1972; 1976) in studies on Paralithodes camtchatica muscle, has found high levels of the gluconeogenic enzymes, fructose diphosphatase, phosphoenolpyruvate carboxykinase, and pyruvate carboxylase. He has also found isotopic evidence for glucose synthesis from alanine, lactate, and pyruvate (Behrisch, 1976). The presence of this gluconeogenic capacity markedly differentiates Paralithodes camtchatica muscle from other muscles.

Pathways of Carbohydrate Metabolism. The contributions of the different pathways of carbohydrate metabolism have been considered in the crustacean gill (Hochachka et al., 1970; Thabrew et al., 1971), in the hepatopancreas (Hochachka et al., 1962; Puyear, 1967), in the intact intermolt crayfish (Puyear et al., 1965), and in various tissue of Orconectes limosus (Lang, 1971). Puyear and coworkers (1965) found that glycolysis, the pentose phosphate shunt, the glucuronic acid pathway, and the Krebs cycle all play important roles in the metabolism of intermolt Pacifastacus leniusculus. Studies by Hochachka et al.

(1962) indicate the presence of the pentose phosphate shunt, gluconeogenesis, glycolysis, and Krebs cycle activity in lobster hepatopancreas. Evidence for gluconeogenesis comes from incorporation of radioactive carbon from acetate into glycogen. Puyear (1967) demonstrated the presence of the glucuronic acid pathway in the hepatopancreas of Callinectes sapidus. In gills of both Paralithodes camtchatica and Carcinus maenas, the contribution of the pentose phosphate shunt is high relative to glycolysis (Hochachka *et al.*, 1970; Thabrew *et al.*, 1971). Both groups also found that the Krebs cycle made a major contribution to respiration, in agreement with Keller (1965) and Thabrew and coworkers (1973). In Paralithodes camtchatica gill, the levels of phosphofructokinase are considerably lower than those of hexokinase (Hochachka *et al.*, 1970), while in Carcinus maenas gill the two are present in equal levels (Thabrew *et al.*, 1971). In Carcinus maenas gill, fructose diphosphatase and phosphofructokinase are present in equal levels as well. Such high levels of fructose diphosphatase bring a high gluconeogenic potential. In accordance with this, isolated gills perform high rates of gluconeogenesis, particularly in sodium depleted sea water. The rates under these conditions approach those of rat liver slices (Thabrew *et al.*, 1971). During premolt, the gills have to synthesize new chitinous coverings for the individual gill filaments. Gluconeogenesis will thus provide the hexose units needed for chitin synthesis.

Mitochondrial Metabolism. Most studies of crab mitochondria have been done with hepatopancreas mitochondria. The initial work done by Beechey (1961), Munday and Thompson

(1962), and Munday and Munn (1962) showed that the mitochondria were similar to mammalian mitochondria. Beechey (1961) found flavoproteins and cytochromes in the characteristic ratios present in other animals, and found redox changes which accompanied the phosphorylation of ADP to ATP. Munday and Thompson (1962) found oxidation of the various Krebs cycle intermediates and sparking of pyruvate oxidation by addition of trace levels of fumarate. Furthermore, these mitochondria contained various Krebs cycle enzymes as well as aspartate and alanine aminotransferases. Munday and Munn (1962) found that hepatopancreas mitochondria were able to oxidize octanoate to carbon dioxide without the addition of Krebs cycle intermediates or carbohydrate precursors. However, the addition of trace amounts of succinate led to a sparking of fat oxidation. Chen and Lehninger (1973) prepared hepatopancreas mitochondria in the presence of bovine serum albumin, and were able to obtain a high degree of respiratory control. The addition of both pyruvate and proline stimulated respiration, while the addition of either compound alone did not. As with mammalian mitochondria, dicarboxylate, tricarboxylate,  $\alpha$ -ketoglutarate, and pyruvate carriers are present. External NADH,  $\beta$ -OHbutyrate, and  $\alpha$ -glycerolphosphate are not oxidized by these mitochondria (Chen & Lehninger, 1973). By contrast, Keller (1965) was able to demonstrate some  $\alpha$ -glycerolphosphate stimulation of oxygen uptake by Cambarus affinis hepatopancreas homogenates. Both Chen and Lehninger (1973) and Munday and Thompson (1962) found that high levels of phosphate and magnesium were needed for full stimulation of oxygen uptake by these mitochondria. Chen and

Lehninger (1973) also noted the high phosphate and calcium content of these mitochondria. In further studies, Chen et al. (1974) were able to demonstrate a marked stimulation of respiration by calcium as well as the concomitant accumulation of high levels of calcium and phosphate. Calcium uptake took precedence over phosphorylation of ADP for respiratory energy. Another novel characteristic of these Callinectes sapidus hepatopancreas mitochondria is a marked stimulation of ATP formation by the addition of arginine. The arginine kinase through which this effect is mediated is located outside the atractyloside barrier. Thus, the transfer of the terminal phosphate group of ATP onto arginine does not occur directly in the matrix. Arginine kinase is usually thought to be restricted to muscle and nervous tissue where it functions in the accumulation and breakdown of arginine phosphate.

Lipid Metabolism. Lipid metabolism in crustaceans has not been the object of much study, although lipids represent a major portion of the metabolic stores throughout the molt cycle. During the beginning of premolt the lipid content of the hepatopancreas increases (Renaud, 1949; Bollenbacher et al., 1972). In some crustaceans, such as the isopod Porcellio laevis, the lipids represent the major molt cycle reserve (Alikhan, 1972). The digestive juice of the hepatopancreas contains a high level of surfactants which facilitate lipid absorption (Vonk, 1960). Lipids are also involved with vitellogenesis; a new hemolymph lipoprotein appears in females during this period of rapid incorporation of lipid into the ovary (Allen, 1972). Free fatty acids are the major form in

which lipid is transported through the hemolymph (Speck & Urich, 1970; Allen, 1972). The oxidation of lipids was demonstrated by Munday and Munn (1962) in their studies of hepatopancreas mitochondria. Labelled carbon from acetate is quickly converted to amino acids, Krebs cycle intermediates, and eventually to sugar phosphates in Carcinus maenas (Huggins, 1966), in Astacus astacus and Astacus leptodactylus (Zandee, 1966a; Zandee, 1966b; van Marrewyk & Zandee, 1975), in Palaemon serratus (Cowey & Forster, 1971) and in Artemia salina (Huggins, 1969). Carbon from palmitate is preferentially converted into amino acids in Orconectes limosus (Speck & Urich, 1969a). There is some evidence that fats are the preferential substrates during starvation in both Orconectes limosus and Carcinus maenas (Speck & Urich, 1969b; Heath & Barnes, 1970). However, another study of the effect of starvation upon the metabolic reserves of Carcinus maenas indicates that starvation leads to no change in the level of carbohydrates or lipids in the hepatopancreas or gill (Marsden et al., 1973). The control of the metabolic pathways involved with lipid synthesis and degradation has not been studied in crustaceans.

Amino Acid Metabolism. Amino acids play an important role in osmoregulation, as energy sources, as well as acting as the substrates for protein synthesis. The enzymatic reactions involved in their biosynthesis and utilization have received considerable attention. Zandee (1966a, 1966b) has published some studies on Astacus astacus indicating that the following amino acids were non-essential: glycine, alanine, serine, aspartate, glutamate (glutamine), tyrosine, proline, OH-proline,

and arginine. No label from U  $^{14}\text{C}$  glucose or 1  $^{14}\text{C}$  acetate was incorporated into valine, leucine, isoleucine, threonine, lysine, histidine, phenylalanine, cysteine, or tryptophan. Similar results were found in more recent experiments with Astacus leptodactylus (van Marrewyk & Zandee, 1975) with Artemia salina (Huggins, 1969), with Palaemon serratus (Cowey & Forster, 1971), with Carcinus maenas (Huggins, 1966), with Panaeus aztecus (Shewbart et al., 1972), and with Uca pugilator (Claybrook, 1976). Amino acids seem to enjoy a central role in energy metabolism since they are highly labelled by carbon from glucose and from acetate. The synthesis of the non-essential amino acids can be explained on the basis of standard enzymatic reactions as both aspartate aminotransferase and alanine aminotransferase are present in high levels in various crustacean tissues. A variety of other transaminations have also been demonstrated in gill, muscle and hepatopancreas of Carcinus maenas (Chaplin et al., 1967). Gilles and Schoffeniels (1964) found anomalous labelling patterns in their experiments on amino acid formation by lobster nerve cord. They concluded that some pyruvate carbon must enter the Krebs cycle by way of oxaloacetate or malate, and also that some pyruvate is eventually generated from Krebs cycle intermediates such as malate. Malic enzyme is present in various crab tissues (Keller, 1965), thus, the suggestions made by Gilles and Schoffeniels (1964) may in fact be correct. The marked changes in the levels of tissue free amino acids during the molt cycle of Carcinus maenas and Callinectes sapidus nauplii are largely due to changes in the concentrations of the non-essential amino

acids, glutamate, alanine, glycine, and proline (Duchateau et al., 1959; Tucker & Costlow, 1975).

Metabolic Flux Patterns during the Molt Cycle.— The most comprehensive studies of crustacean metabolism and physiology have been carried out on the crayfish Orconectes limosus by Urich and coworkers. These studies center upon the changing patterns of carbon flux during the molt cycle and the metabolic fate of carbon from different foodstuffs and resorbed chitin. Particular emphasis was placed upon carbon flux during the passage of compounds from one tissue to another. These studies show that ingested or resorbed compounds are subject to extensive interconversions, and that amino acids and lactate are of central importance in the energy metabolism of crustaceans. The picture of carbon flux which emerges from the histological studies of Renaud (1949) and Travis (1955a; 1957) implies that glycogen and fat provide the bulk of the carbon for chitin synthesis. In animals, the net synthesis of carbohydrate from fat is impossible. By contrast, glucose units from glycogen could well be used to support chitin synthesis. The early histological studies also imply that carbon from the resorbed chitin is quickly converted to glycogen stored within the hypodermis and the hepatopancreas. The studies of Urich and coworkers show that this picture of carbon flux is a considerable over-simplification. Considerable redistribution of carbon from blood glucose or resorbed acetylglucosamine into sugars, organic acids, amino acids, polysaccharides, proteins, and lipids occurs (Herz-Hübner & Urich, 1973). Between 20 and 40% of carbon from hemolymph glucose and palmitate is initially

converted into amino acids. In freshmolt animals, carbon from amino acids is subsequently found both in chitin and in respiratory carbon dioxide. In both intermolt and freshmolt animals, amino acids are oxidized more readily than glucose or palmitate (Speck & Urich, 1969a). Thus, amino acids play a central role in energy metabolism, and gluconeogenesis from amino acids and organic acids plays a critical role as a source of hexose units for chitin synthesis.

Orconectes limosus lacks appreciable metabolic stores, and during the molt covers all of its energetic and biosynthetic needs with resorbed acetylglucosamine (Speck & Urich, 1971). In this respect it differs from crustaceans which utilize stored glycogen, lipids and proteins as well as resorbed acetylglucosamine to support their activity during molt (Renaud, 1949; Travis, 1955a; Travis, 1957; Heath & Barnes, 1970). However, the degree to which decapod crustaceans accumulate stores varies considerably (Heath & Barnes, 1970). Therefore, the utilization of resorbed acetylglucosamine represents a basic feature of molting physiology which is utilized to differing degrees depending upon the elaboration of metabolic stores. The pathway to and from chitin joins glycolysis at the level of fructose-6-phosphate. Acetylglucosamine resorbed from the old exoskeleton must be deacetylated before it can be reconverted into chitin (Wolter, 1968), but then it forms the preferential substrate for premolt chitin synthesis (Speck et al., 1972; Stevenson, 1972). Only 20% of the resorbed acetylglucosamine remains unchanged ten minutes after resorption by the hypodermis. The remainder is converted largely into various

organic and amino acids as well as to chitin, protein, and oligosaccharide (Speck & Urich, 1972). Some carbon from resorbed acetylglucosamine accumulates in the abdominal muscle and is not released or redistributed until a considerable period after the molt (Speck & Urich, 1972). Carbon from acetylglucosamine accumulates in the hypodermis before the molt. After the molt the organic acids, sugars, proteins and polysaccharides into which it was converted are preferentially utilized for chitin synthesis (Speck & Urich, 1972; Herz-Hübner & Urich, 1973). The means by which this preferential utilization is achieved are not clear.

Glucose is not used for chitin synthesis until after molt, presumably due to an inhibition at the level of glutamine: fructose-6-phosphate amidotransferase (E.C.2.6.1.16) (Speck et al., 1972). This inhibition may facilitate efficient resorption and distribution of carbon from the old exoskeleton into the general metabolic pools. After the molt, however, chitin synthesis from glucose is rapid (Hornung & Stevenson, 1971; Stevenson, 1972; Speck et al., 1972). Carbon from resorbed acetylglucosamine and hemolymph glucose is rapidly distributed amongst amino acids, glycolytic and Krebs cycle intermediates, chitin, protein, polysaccharides, and CO<sub>2</sub> (Speck & Urich, 1972; Herz-Hübner & Urich, 1973). Three hours after injection of label, 33% of hemolymph glucose carbon is incorporated into proteins, while 55% is present in the form of intermediary compounds. In comparison, 18% of the acetylglucosamine carbon is in protein, 44% in intermediary compounds (of which amino acids constitute between 15 and 30%) and 24% is in chitin (Herz-

Hübner & Urich, 1973). During absorption of glucose from foodstuffs, only 9% of the glucose leaves the digestive system unchanged. Other sugars, organic acids, and amino acids constitute one third each of the total material transferred from the hepatopancreas and gut to the hemolymph (Urich *et al.*, 1973). A comparison of the utilization of glucose, glutamate, and palmitate by Orconectes limosus shows that both glucose and glutamate are quickly metabolized. Glucose carbon is quickly incorporated into amino acids, while glutamate carbon is more quickly oxidized to CO<sub>2</sub> than carbon from either glucose or palmitate (Speck & Urich, 1969). Lactate accounts for 57% of the substance transferred between hemolymph and body tissues (Herz-Hübner *et al.*, 1973). In Cambarus robustus, blood lactate levels equal blood glucose levels (Telford, 1975), in Orconectes limosus they exceed the blood glucose levels (Andrews, 1967), while in Homarus americanus they are somewhat lower (Stewart *et al.*, 1966). Thus, amino acids and lactate are of central importance in crustacean energy metabolism. Since hemolymph and hepatopancreatic glucose as well as resorbed acetylglucosamine show preferential conversion to amino acids and lactate; chitin synthesis from both stored glucose and resorbed acetylglucosamine will involve gluconeogenesis.

#### Central Questions in this Study

Gluconeogenesis in crustaceans normally supports glycogen synthesis, synthesis of blood glucose, trehalose, maltose, and

mannose as well as chitin synthesis. This diversity of endproducts increases the complexity of the metabolic control requirements. Since amino acids and lactate are of central importance in crustacean energy metabolism (Herz-Hübner *et al.*, 1973) these compounds logically form major substrates for gluconeogenesis. Gluconeogenesis occurs in a variety of crustacean tissues including gill (Thabrew *et al.*, 1971), hypodermis (Herz-Hübner & Urich, 1973), hepatopancreas (Hochachka *et al.*, 1962) and possibly blood (Johnston *et al.*, 1972) and muscle (Behrisch, 1976). Since hypodermal gluconeogenesis supports chitin synthesis during the critical hardening of the freshly molted animal, the hypodermis is the tissue of choice within which to study gluconeogenic control mechanisms. Gluconeogenesis has two well defined control sites in vertebrate tissues. One lies at the reversal of the pyruvate kinase reaction, i.e. the phosphoenolpyruvate carboxykinase and pyruvate carboxylase reactions, and the other at the reversal of the phosphofructokinase reaction by fructose diphosphatase (Scrutton & Utter, 1968). Both of these control sites occur in apposition to glycolytic reactions which are characterized by large free energy changes, are functionally irreversible in the cell, and constitute major glycolytic control sites (Scrutton & Utter, 1968; Newsholme & Start, 1973; Rolleston, 1972).

Another glycolytic reaction which is characterized by a large free energy change is the phosphoglycerate kinase reaction. The equilibrium of the reaction strongly favors ATP and 3-phosphoglycerate formation (Krietsch & Bücher, 1970; Rose & Warms, 1970). In the cell, the reaction is considered to be

freely reversible for gluconeogenesis, and thus to catalyze a near equilibrium reaction. Evidence of a crossover between glyceraldehyde-3-phosphate and 3-phosphoglycerate is usually interpreted as rate limitation at the dehydrogenase reaction (Williamson, 1965; Rose & Warms, 1970; Rolleston, 1972). Precise elucidation of the *in vivo* role of phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase has been hampered by the difficulty in measuring their common substrate 1,3 diphosphoglycerate. The kinetic properties of the enzyme have been examined in yeast and rabbit muscle. The enzyme from these sources shows a high affinity for 1,3 diphosphoglycerate ( $K_m = 2 \mu M$ ) and a 1000 fold lower affinity for 3-phosphoglycerate ( $K_m =$  approximately 2 mM) (Krietsch & Bücher, 1970). For both the yeast and the rabbit muscle enzyme, the  $K_i$  for MgADP as an inhibitor is lower than its  $K_m$  value as the glycolytic substrate (Larsson-Raznikiewicz & Arvidsson, 1970; Scopes, 1973). Thus, both kinetic and thermodynamic barriers have to be overcome to achieve net gluconeogenic flux. In contrast to the situation in glycolytic tissues where the exact role of phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase is not clear (Williamson, 1965; Parker & Hoffman, 1967; Newsholme & Start, 1973; Tornheim & Lowenstein, 1975), the phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase system is at equilibrium under a variety of metabolic conditions in the rat liver (Veech *et al.*, 1970; Stubbs *et al.*, 1972). While some careful kinetic studies of the liver glyceraldehyde-3-phosphate dehydrogenase have elucidated the means whereby this reaction is reversed under gluconeogenic conditions (Smith & Velick, 1972),

no investigations of kinetic mechanisms which might facilitate the reversal of the phosphoglycerate kinase reaction for gluconeogenesis have been made. Since crustacean hypodermis and muscle show a variety of metabolic poises during the course of the molt cycle, they provide an excellent biological situation within which the control mechanisms of this reaction can be examined, under both gluconeogenic and glycolytic conditions.

Of the two well defined control sites in gluconeogenesis, the fructose diphosphatase reaction (FDPase) has been well characterized in hypodermis, gill, and muscle of Paralithodes camtchatica (Hochachka, 1972; Behrisch, 1972; Behrisch & Johnson, 1974; Behrisch, 1975). The hypodermal FDPase is present in two forms (I and II). FDPase II is the predominant form present in the gill. This form is highly sensitive to feedback inhibition by ribose-5-phosphate, UDP-acetylglucosamine, and UDP-glucose. Low levels of UDP-acetylglucosamine activate the binding of fructose-1,6 diphosphate by FDPase II. Both forms are sensitive to AMP inhibition (Hochachka, 1972). Both tissues have a relatively complex carbohydrate metabolism. The hypodermis synthesizes large quantities of both chitin and glycogen, while the gill's primary polysaccharide product is chitin. The pentose shunt is active in both gill and hypodermis (Thabrew et al., 1971; Hochachka et al., 1970; Keller, 1965). While gluconeogenic flux has not been measured in the hypodermis, glucose production by the gill reaches rates equivalent to those attained by rat liver slices (Thabrew et al., 1971). In the gill of Carcinus maenas, phosphofructokinase and fructose diphosphatase are in a 1:1

ratio (Thabrew *et al.*, 1971). A similar situation seems to prevail in the gill of *Paralithodes camtchatica* (Hochachka, 1972; Hochachka *et al.*, 1970). The presence of high levels of the closely regulated FDPase II, in combination with low levels of FDPase I, will control futile cycling and channel carbon flux into the appropriate synthetic pathway. A similar, but more flexible situation occurs in the hypodermis which has higher levels of FDPase I. The relative levels of phosphofructokinase and fructose diphosphatase in the hypodermis are not known; thus this aspect of control cannot be discussed. One form of fructose diphosphatase occurs in muscle; and while its kinetics are similar to those of FDPase II, it is not sensitive to inhibition by UDP-glucose, and is activated by phosphoenolpyruvate (Behrisch, 1972; Behrisch & Johnson, 1974). A comparison of the regulatory properties of these crustacean FDPases with those of mammalian FDPases reveals a considerably greater complexity of the regulation of this gluconeogenic enzyme in crustacean tissues.

The second major gluconeogenic control site is central to the metabolism of these animals, since it represents both a critical step in the conversion of amino acids and small organic acids into sugars, and an important rate limiting step in glycolysis. The two "sides" of this control site consist of pyruvate kinase in the glycolytic span and phosphoenolpyruvate carboxykinase and pyruvate carboxylase in the gluconeogenic span (Scrutton & Utter, 1968). While the presence of pyruvate kinase in crustacean tissues is well documented, the presence of the gluconeogenic enzymes has not been clearly established (Munday &

Poat, 1970). Furthermore, the overall control of this reaction locus has not been examined. In vertebrate tissues, phosphoenolpyruvate carboxykinase and pyruvate carboxylase levels are low in comparison with those of pyruvate kinase (Scrutton & Utter, 1968; Newsholme & Start, 1973). Thus, a critical element in the control of this reaction locus is the control of pyruvate kinase. The premolt and postmolt hypodermis shows high rates of chitin synthesis and a marked increase in oxygen consumption (Skinner, 1962; McWhinnie *et al.*, 1972), and carbohydrate oxidation (Herz-Hubner & Urich, 1973). Thus, flux through the pyruvate kinase reaction must be subject to flexible yet close control. Studies of the control of muscle metabolism in Paralithodes camtchatica indicate that pyruvate kinase is an important control site in glycolysis (Hochachka *et al.*, 1971). However no studies of the regulatory properties of this enzyme in muscle or hypodermis have been performed. Somero (1969) found temperature dependent interconversions of the muscle enzyme from Paralithodes camtchatica, but did not examine the further regulation of the enzyme in his study. Thus, to elucidate how this important control site is regulated in both gluconeogenic and glycolytic tissues, a comparison of pyruvate kinase in muscle and hypodermis was undertaken.

Metabolic control can be exerted on various levels, one is via control of enzyme levels, another is via kinetic mechanisms which control the activity of the enzyme in the cell. These two means obviously interact in determining the metabolic potential of a tissue. Crustacean tissues undergo considerable changes as the organism passes through the molt cycle. The high levels of

protein synthesis occurring during early premolt in the hypodermis (Stevenson, 1972; McWhinnie et al., 1972), and the considerable breakdown of protein in premolt muscle (Skinner, 1966) certainly raise the possibility of changes in enzyme levels in these two tissues during the molt cycle. Furthermore, the nature of metabolic control in the tissues might change as the levels of enzymes are altered. To elucidate the degree to which such metabolic changes occur in the muscle and hypodermis of Cancer magister, the levels of several key glycolytic and gluconeogenic enzymes were measured in intermolt and freshmolt animals. Hypodermal enzymes generally show increased specific activity in freshmolt animals. The overall poise remains strongly gluconeogenic in both freshmolt and intermolt animals. Intermolt muscle shows a considerably stronger glycolytic orientation than freshmolt muscle.

To assess the importance and nature of kinetic controls, I chose to examine the catalytic and regulatory properties of the phosphoglycerate kinase and pyruvate kinase reaction in the muscle and hypodermis of intermolt and freshmolt animals. While the mechanisms for the reversal of phosphoglycerate kinase and the inhibition of pyruvate kinase are of critical importance for effective gluconeogenesis in the hypodermis, the activation of both of these reactions in the glycolytic direction is essential for both the hypodermis and the highly glycolytic muscle. An examination and comparison of the control mechanisms shown by these enzymes in freshmolt and intermolt muscle and hypodermis should elucidate the means by which these control requirements have been fulfilled in Cancer magister. Several solutions

seemed possible: 1) the evolution of different tissue forms of the enzymes each with regulatory characteristics suited for the tissue's metabolism, 2) the evolution of forms which are synthesized during different portions of the molt cycle in response to the changing needs of the tissues, or 3) the evolution of a single highly sophisticated protein species whose kinetic regulation makes its function under a variety of metabolic circumstances feasible.

In examining the properties of pyruvate kinases and phosphoglycerate kinases from these animals, I found tissue specific forms of pyruvate kinase and molt cycle related forms of phosphoglycerate kinase. The freshmolt forms of phosphoglycerate kinase show an increased affinity for ATP and a decreased sensitivity to MgADP inhibition compared to the intermolt muscle enzyme. Under physiological concentrations of substrates and inhibitors, these kinetic alterations would serve to facilitate the reversal of the reaction for gluconeogenesis. Muscle and hypodermal pyruvate kinases show no molt cycle correlated changes, but the two tissues have distinct isozymes. The muscle enzyme is inhibited by ATP, arginine phosphate and Mg<sub>2</sub>citrate, and is activated by FDP. The hypodermal enzyme exists in two interconvertible states, PK I and PK II. PK I shows a high affinity for PEP and a low affinity for FDP. By contrast, PK II shows a low affinity for PEP, a high affinity for FDP and an increased sensitivity to inhibition by amino acids and Mg<sub>2</sub>citrate. Interconversion of the two forms can be achieved by the addition or removal of FDP. Shifting the enzyme into the low affinity conformation would lead to a drastic limitation of

enzyme activity under physiological concentrations of substrates and inhibitors, thus allowing gluconeogenic flux through the enzymatic bypass. These two conformational states allow sensitive regulation of flux through this major control site in both gluconeogenesis and glycolysis. Thus, the crustacean hypodermis has evolved enzymatic adaptations which facilitate gluconeogenic carbon flux.

## GENERAL MATERIALS AND METHODS

Experimental Animals

Freshmolt and intermolt Cancer magister were collected by SCUBA diving courtesy of John Himmelman, at Jericho Pier, Vancouver, B.C. Freshmolt animals were used within a day of collection. Intermolt animals were either used immediately or maintained for at least one week in the refrigerated, recirculating sea water system to allow acclimation to the new conditions. Both male and female animals were used in this study. Mature female Cancer magister molt before mating, at which time they are being held and protected by a hard shelled male. Mating occurs most often between April and September, so female molting occurs in this period. Males molt asynchronously with the females. Thus, it is possible to find molting animals throughout the year. Molting males are particularly common in the late fall and early winter, while molting females are common in the spring and summer months. Molt cycle stages were determined according to the criteria of Drach (1939). Animals were fed canned salmon or freshly collected mussels twice a week.

In preliminary experiments, I tried to stimulate molting by a variety of methods. The various treatments included: maintaining the animals in warmer water (10 °C) in individual containers, maintaining the animals in the dark under both cold and warm conditions in private containers, eyestalk ablation, and crustecdysone injection (3 µg/gm body weight). None of

these treatments proved successful. Eyestalk ablation led primarily to death, while molting in the other conditions was not increased over molting in the "communal" tanks. Thus, it was decided to rely upon field collections of molting animals.

### Reagents

All reagents were the highest grade available, most were from Sigma Chemicals (St. Louis, Missouri). Coupling enzymes such as beef heart lactate dehydrogenase and malate dehydrogenase, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, triose-phosphate isomerase, and  $\alpha$ -glycerolphosphate dehydrogenase, as well as citrate synthase, phosphoglycerate kinase, glutamate dehydrogenase, aldolase, phosphoglucosyl isomerase, and glucose-6-phosphate dehydrogenase were from Sigma as well. Peanut phosphoenolpyruvate carboxylase was purified according to Dyson *et al.*, (1975) and Lane *et al.*, (1967). Hydroxylapatite, cellulose phosphate and DEAE Sephadex ion exchangers were from Sigma and Pharmacia respectively. Ion exchangers were pretreated according to manufacturer's instructions. Starch was from Connaught Laboratories (Toronto) and ampholines were from LKB (Uppsala, Sweden).

### Preparation of Enzymes

Since the purification procedures are of direct importance to the discussion of each enzyme, the methods utilized in preparing each enzyme will be presented together with the results. Generally all crabs were "anaesthetized" by cooling in

a freezer until only slow movements came in response to a stimulus. The animals were then sacrificed by cutting away the dorsal surface of the carapace. Care was taken to destroy the cerebral ganglia in the process. The dissection of the tissues from the animal took place on ice. The hypodermis, the epithelium directly underlying the exoskeleton, was simply peeled off of the dorsal carapace and the outer covers of the branchial chambers. Muscle was removed from the walking legs and the chelipeds and freed of adhering hypodermis. All subsequent operations were at 4 °C or on ice. Spectrophotometric assays of enzyme activity took place on a Unicam SP1800 recording ultraviolet spectrophotometer, most often by monitoring the change in absorbance due to the oxidation or reduction of NAD(H) at 340 angstroms.

#### Starch Gels and Isoelectrofocusing

Thirteen percent starch gels were prepared using Tris-citrate or Tris-citrate-KCl buffer systems with various combinations of gel and tank buffer pH and ionic strength. The most effective system for separation of phosphoglycerate kinase was 35.6 gm Tris and 9.5 gm citrate per liter (pH = 7.5), while the most effective system for electrophoresis of pyruvate kinase was 37.8 gm Tris, 9.5 gm citrate, and 7.45 gm KCl per liter (pH = 8.5) as tank buffer. In both cases, the gel buffer was a 1:20 dilution of the tank buffer. After the gels had cooled to 4 °C centrifuged, dialyzed tissue extracts were coated onto filter paper wicks and inserted into the gel. To ensure sufficient activity, two or more wicks were often inserted into

the gel. A prechilled plexiglass block was laid across the gel. Gels were run at 150–200 volts (current 25–45 mAmps) for varying lengths of time (6–18 hours). Both phosphoglycerate kinase and pyruvate kinase were stained by the negative method described by Scopes (1968). Developing the stain with phenazine methasulphate and nitroblue tetrazolium, gave only partially satisfactory results. Instead, the stain was monitored with an ultraviolet light. NADH appears yellow under ultraviolet light, while NAD appears dark purple. Thus, the appearance of enzyme activity can be visualized. The relative migration velocities were measured and the positions recorded as the stains became visible.

Isoelectrofocusing was carried out according to the method of Häglund (1967). Enzyme preparations were dialyzed against 1% glycine, pH 7.0 in preparation for electrofocusing. As these crustacean enzymes, particularly the hypodermal forms, were relatively unstable only 500 mvolts were used during electrofocusing. The enzymes were electrofocused only until the current stabilized at a low level, since longer electrofocusing times led to considerable loss of enzyme activity.

### Kinetic Analyses

Kinetic parameters were determined by several means. The primary method utilized for the determination of  $K_m$  values was the Lineweaver–Burk,  $1/V$  versus  $1/S$  plot.  $K_i$  values for various inhibitors were determined from both the Lineweaver–Burk plots and Dixon plots. Slope and intercept replots derived from the

Lineweaver-Burk plots were used to determine the pattern of inhibition. The methods described by Webb (1963) were used to evaluate complex inhibition patterns. Since the determination of reaction rates from the lines given by the recording spectrophotometer involves utilizing a visual "best fit" method, it was felt that computer drawn lines would still be based upon this possible experimental bias. Thus, lines were fitted to the points, via a visual "best fit" method. In most cases, the best fit was obvious. Kinetic determinations were performed on a minimum of two different enzyme preparations, and in many cases, were performed between 5 and 10 times. Repeat determinations indicate that  $K_m$  determinations are subject to approximately a 5% error. Measurements of kinetic effects due to various cations utilized cations which were not corrected for possible changes in the degree of hydration between different experiments. Protein concentrations were determined by measuring the absorption of the solution at 260 and 280 nanometers. These values were then used to arrive at the protein concentration in the solution by using the method of Lane (1957).

## Part I.

Changing Enzyme Activities in the Hypodermis,  
Gill, and Muscle of Cancer magister during the Molt Cycle.

## INTRODUCTION

Since crustacean tissues undergo pronounced structural and physiological changes during the molt cycle, the organization of metabolism in the tissues may be subjected to changes in overall orientation as well. To elucidate whether any marked changes in the organization of carbohydrate metabolism occur during the molt cycle, I measured the levels of a variety of glycolytic and gluconeogenic enzymes in both intermolt and freshmolt muscle, hypodermis and gill. Phosphoenolpyruvate carboxykinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, and lactate dehydrogenase were chosen as representative glycolytic enzymes. The gluconeogenic enzymes were fructose diphosphatase, phosphoenolpyruvate carboxykinase, and pyruvate carboxylase. The levels of glutamine: fructose-6-phosphate amidotransferase, the first enzyme in the pathway to chitin, were also determined in these tissues.

## MATERIALS AND METHODS

The glycolytic enzymes and fructose diphosphatase were measured in the high-speed supernatant remaining after centrifugation at 27,000 g for twenty minutes. Phosphoenol-

pyruvate carboxykinase, pyruvate carboxylase, phosphofructokinase, and glutamine: fructose-6-phosphate amidotransferase were measured in both the high speed supernatant and the whole homogenate.

#### Preparation of the Homogenate for Measurement of Enzyme Activities

Anaesthetized crabs were cut open, the gills, several grams of hypodermis and several grams of leg muscle were quickly dissected out, blotted, and weighed. Muscle was homogenized in three volumes of cold 50 mM imidazole-Cl, pH 7.0, while gill and hypodermis were homogenized in two volumes. For preparation of the high speed supernatant, a Sorval Omnimixer was set at top speed and run for 2 minutes. For preparation of the whole homogenate, a teflon homogenizer was used and the tissue was homogenized with 20-30 strokes. In these experiments, 4 volumes of buffer were used with muscle and three volumes with gill and hypodermis. The final volume of the homogenate was noted for both methods of preparation. The supernatants or homogenates were used directly for measurement of the enzyme activity.

#### Enzyme Assays

Enzyme levels in the various tissues were determined by standard spectrophotometric coupled enzyme assays. Optimal conditions were determined for each enzyme from each tissue, and the maximal velocity was used in calculating enzyme levels. Each value represents the mean of the values found for at least

three different animals, except in the case of the gill values. The activity measurements do not include the effect of metabolite activators. The assay temperature in all cases was 20 °C. The concentrations of reagents for each enzyme assay are given below.

Phosphofructokinase: 0.1 M Tris-HCl buffer, pH 8.0, 0.5 mM ATP, 1 mM fructose-6-phosphate, 5 mM MgCl<sub>2</sub>, 100 mM KCl, 0.2 mM NADH, excess aldolase, triose phosphate isomerase, and  $\alpha$ -glycerolphosphate dehydrogenase.

Glyceraldehyde-3-phosphate dehydrogenase: 0.1 M Tris buffer, pH 7.5, 75 mM KCl, 1 mM ATP, 4 mM MgCl<sub>2</sub>, 2 mM 3-phosphoglycerate, 0.3 mM NADH, excess phosphoglycerate kinase. The reaction was initiated by the addition of 3-phosphoglycerate. This assay method was chosen as the purity and stability of the d-l glyceraldehyde-3-phosphate was low. Furthermore, the comparison of the maximal gluconeogenic reaction rates of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase was of interest.

Phosphoglycerate kinase: 50 mM imidazole-Cl buffer, pH 7.0, 4 mM MgCl<sub>2</sub>, 0.2 mM NADH, 1 mM ATP, 2 mM 3-phosphoglycerate and excess glyceraldehyde-3-phosphate dehydrogenase.

Pyruvate kinase: 50 mM imidazole-Cl buffer, pH 7.0, 4 mM MgCl<sub>2</sub>, 0.2 mM NADH, 75 mM KCl, 2 mM ADP, 2 mM phosphoenolpyruvate, excess lactate dehydrogenase.

Lactate dehydrogenase: 0.1 M Tris-HCl buffer, pH 7.5, 0.3 mM NADH, 3 mM pyruvate.

Fructose diphosphatase: 50 mM Tris-HCl buffer, pH 7.5, 5 mM MgCl<sub>2</sub>, 0.1 mM NADP, 0.01 mM to 0.03 mM fructose 1,6 phosphate,

excess phosphohexose isomerase and glucose-6-phosphate dehydrogenase.

Glutamine: fructose-6-phosphate amidotransferase: 0.1 mM Tris-HCl buffer, pH 7.6, 5 mM glutamine, 3 mM fructose-6-phosphate, 0.3 mM NAD, excess glutamate dehydrogenase. In this assay, which measured the evolution of glutamate, it was essential to use the ammonium ion free glutamate dehydrogenase and to check that the glutamine used in the assay was free of glutamate.

Pyruvate carboxylase: Two methods were used to measure the activity of this enzyme.

1) 0.1 M Tris-HCl buffer, pH 8.4, 10 mM pyruvate, 3 mM ATP, 20 mM KHCO<sub>3</sub>, 0.1 mM acetyl CoA, 8 mM MgCl<sub>2</sub>, 0.2 mM NADH, excess malate dehydrogenase.

2) 0.05 M Tris-HCl buffer, pH 7.5, 0.2 mM DTNB (5,5'-dithiobis-2-nitrobenzoic acid), 10 mM MgSO<sub>4</sub>, 2.5 mM ATP, 0.2 mM acetyl CoA, 100 mM KHCO<sub>3</sub>, 10 mM pyruvate, excess citrate synthase. The higher extinction coefficient of DTNB versus NADH makes this assay more sensitive to the production of oxaloacetate. This reaction is monitored at 412 angstroms.

Phosphoenolpyruvate carboxykinase: 0.1 mM Tris-maleate buffer, pH 6.0, 100 mM NaHCO<sub>3</sub>, 2 mM phosphoenolpyruvate, 2 mM GDP, IDP, or CDP, 1 mM MgCl<sub>2</sub>, 0.2 mM NADH, excess malate dehydrogenase. Phosphoenolpyruvate carboxykinase activity is determined from the increase in E<sub>340</sub> due to the addition of HCO<sub>3</sub> to the complete assay mixture.

## RESULTS AND DISCUSSION

The results of the enzyme activity measurements are shown in Table 1. The determination of several of these enzyme activities required additional controls and experiments to ensure proper measurement of enzyme activity. The measurement of pyruvate carboxylase and phosphoenolpyruvate carboxykinase requires particularly careful controls for interfering enzyme activities. The first assay method for pyruvate carboxylase requires a control for endogenous lactate dehydrogenase, and NADH oxidase. The second method requires a control for endogenous hydrolysis of acetyl CoA, as well as careful regulation of the levels of sulfhydryl groups present in the assay. The assay for phosphoenolpyruvate carboxykinase also requires precautions: sodium bicarbonate must be used to minimize activation of pyruvate kinase and the coupling enzyme must be dialyzed to remove  $\text{NH}_4^+$ , an activating cation for pyruvate kinase. Obviously the important control for this assay is pyruvate kinase activity. A control for sodium activation of pyruvate kinase must be included.

The two methods of measuring pyruvate carboxylase activity yielded equivalent results. Both pyruvate carboxylase and phosphoenolpyruvate carboxykinase activities are associated with the particulate fraction. Thus, measurement of their levels in the whole homogenates yielded considerably higher values than measurements in the supernatant. This corresponds with the mitochondrial location of pyruvate carboxylase and the partially

Table 1

## Specific Activities of Various Enzymes in the Muscle, Gill and

Hypodermis of Cancer magister

Specific activities are the mean of the values found for three or four different animals, except in the case of the gill values where usually only one animal was measured. Comparisons of freshmolt and intermolt enzyme activities were made using Student's T test. The results of the comparison are indicated as follows: (\*) significant at  $P=0.05$ , (\*\*) significant at  $P=0.01$ , and (N.S.) not significant. Enzyme activities are expressed in umoles/min gm wet weight at  $20^{\circ}\text{C}$ , — indicates that the enzyme was not measured in the tissue, 0 indicates that the enzyme was not detectable in the tissue.

Enzyme	Intermolt			Freshmolt		
	Hypodermis	Gill	Muscle	Hypodermis	Gill	Muscle
FDPase	0.66	0.44	0.33	1.2 **	0.56 N.S.	0.484**
PFK	0.182	0.241	5.81	0.375**	0.156	2.122**
F6PGAT	0.12	0.12	0	0.15*	0.135	0.0025
LDH	4.06	1.205	18.87	7.5*	2.04	19.64 N.S.
G3PDH	5.0	—	43.0	14.5**	—	12.5**
PGK	3.615	3.278	13.02	9.585**	4.34	8.36**
PK	6.3	7.7	61.7	15.4**	16.2	51.5 N.S.
PEPCK	0.2	0.08	0	0.5**	0.12	0.105
pyruvate carboxylase	0.48	—	0.05	1.1**	—	0.1065**

mitochondrial location of phosphoenolpyruvate carboxykinase in mammalian tissues (Saggerson & Evans, 1975). Measurements of phosphoenolpyruvate carboxykinase in various rat tissues indicate that the gluconeogenic reaction shows a higher specific activity than the "downhill" reaction. However, the ratio of activities varies between tissues (Pogson & Smith, 1975) and varies considerably from ratios obtained with the purified mitochondrial enzyme (Chang *et al.*, 1966). Thus, no conclusion about the actual relationship of the measured "downhill" reaction and the gluconeogenic reaction can be made.

Since phosphofructokinase has been reported to be associated with the precipitate after high speed centrifugation (Opie & Newsholme, 1967), its levels in the whole homogenate were compared with the levels found in the supernatant. No difference was found. In measurements of fructose diphosphatase activity, endogenous AMP could have inhibited the enzyme. To check this possibility, an "AMP trap" consisting of 0.2 mM ATP and excess myokinase was added to the assay system. This would convert the AMP to ADP and thus deinhibit the enzyme. No change in activity was found upon the addition of this trap. Presumably endogenous AMP levels were low during extraction.

A consideration of the enzyme activities shown in Table 1 makes it readily apparent that considerable changes occur in levels of key enzymes between freshmolt and intermolt in both muscle and hypodermis. A consideration of enzyme levels in the intermolt and freshmolt gill shows that they do not undergo as marked variations as enzyme levels in the muscle or hypodermis.

Generally, the gill shows a high gluconeogenic capacity. Intermolt muscle generally showed high levels of all enzymes except the gluconeogenic enzymes, fructose diphosphatase and pyruvate carboxylase. Phosphoenolpyruvate carboxykinase was not present in intermolt muscle. Freshmolt muscle showed considerably reduced levels of phosphofructokinase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase. By contrast, the levels of fructose diphosphatase and pyruvate carboxylase were higher than in intermolt muscle. Phosphoenolpyruvate carboxykinase was present in equal levels with pyruvate carboxylase in freshmolt muscle. Thus, the glycolytic potential of freshmolt muscle is considerably reduced and the presence of the various gluconeogenic enzymes makes the reversal of glycolysis for the production of sugar or chitin precursors at least feasible.

All hypodermal enzymes are present in higher concentrations in freshmolt than in intermolt hypodermis. This is indicative of the general increase in protein synthesis during premolt (Adelung & Keller, 1970; Stevenson, 1972) as well as the increase in the amount of protein/DNA during premolt (Stevenson, 1972). Both hypodermal and gill enzyme levels are considerably lower than those in the muscle. The large contribution of extracellular space to the wet weight of the tissues (see Part V) will partially account for this difference. The low ratio of phosphofructokinase/fructose diphosphatase indicates that this reaction locus is strongly poised towards gluconeogenesis. Pyruvate kinase, pyruvate carboxylase, and phosphoenolpyruvate carboxykinase all show the same percentage increase between

intermolt and freshmolt tissues. Thus, pyruvate kinase remains approximately 20 fold the level of phosphoenolpyruvate carboxykinase throughout the molt cycle, giving this control site a strongly glycolytic poise. Although the activity of phosphoenolpyruvate carboxykinase may be somewhat higher when measured in the gluconeogenic direction, the two fold increase for the enzyme in most tissues (Pogson & Smith, 1975) would not alter the overall poise appreciably. Thus, pyruvate kinase must be subjected to considerable regulation for gluconeogenic flux to occur, and its activity is a prime determinant of flux through the reaction locus.

As was found by Boulton and Huggins (1970), glyceraldehyde-3-phosphate dehydrogenase levels were considerably higher than lactate dehydrogenase levels in muscle. However, the levels of glyceraldehyde-3-phosphate dehydrogenase found by Boulton and Huggins (1970) were considerably higher than the levels I found in Cancer magister. In this study, phosphoglycerate kinase levels are lower than glyceraldehyde-3-phosphate dehydrogenase levels in all tissues. The levels of fructose diphosphatase and pyruvate carboxylase found in the muscle of Cancer magister are similar to those found by Opie and Newsholme (1967) for Carcinus maenas and by Crabtree et al. (1972) in Cancer pagurus. Phosphoenolpyruvate carboxykinase was not detected by Crabtree et al. (1972) in Cancer pagurus muscle, but most probably the animals were in intermolt. In the present study, phosphoenolpyruvate carboxykinase was detected in only freshmolt muscle. The values of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and fructose diphosphatase found by Behrisch (1976) in

the muscle of Paralithodes camtchatica are considerably higher than the levels found in Cancer pagurus (Crabtree et al., 1972) in Carcinus maenas (Opie & Newsholme, 1967) or in Cancer magister in the present study.

Generally, the enzyme levels measured in Cancer magister indicate that intermolt muscle metabolism presents a highly glycolytic orientation, while freshmolt muscle shows a considerably reduced glycolytic potential and an increased gluconeogenic capacity. Intermolt and freshmolt hypodermis both show a relatively high gluconeogenic capacity. Freshmolt hypodermis shows higher specific activities, diagnostic of the high rate of chitin synthesis during this period. While the enzyme levels are generally lower than those in mammalian tissues, the relationships between enzyme levels are similar. Thus, in both the mammalian liver and the crustacean hypodermis, fructose diphosphatase levels are 3-4 fold those of phosphofructokinase. Pyruvate kinase levels in both tissues are approximately 15 fold higher than those of the enzymatic bypass. In contrast to mammalian systems, the levels of phosphoglycerate kinase are considerably lower than those of glyceraldehyde-3-phosphate dehydrogenase. Furthermore, pyruvate kinase levels are higher than those of phosphoglycerate kinase in both hypodermis and muscle. As in mammalian muscle, there is a ten fold difference between phosphofructokinase and pyruvate kinase levels in intermolt muscle. The ratio of phosphofructokinase to fructose diphosphatase in intermolt muscle is 18. This low value is characteristic of muscles which experience large changes in glycolytic rate (Newsholme & Start, 1973). Freshmolt

muscle shows an even lower value for this ratio. This coupled with the increased levels of pyruvate carboxylase and phosphoenolpyruvate carboxykinase lends a higher gluconeogenic capacity to this tissue.

## Part II.

## Control of Hypodermal and Muscle

Phosphoglycerate Kinase in  
Intermolt and Freshmolt Cancer magister

## INTRODUCTION

The role of phosphoglycerate kinase (E.C.2.7.2.3.) in the regulation of glycolysis and gluconeogenesis is open to some question. In some situations, it is considered a possible glycolytic control site, while in most it is considered to catalyze a near-equilibrium reaction. A reversal of the reaction is meant to occur during gluconeogenesis, but the kinetic characteristics of previously studied phosphoglycerate kinases do not favor the reversal. A comparison of the regulatory properties of the enzyme from the muscle and hypodermis could elucidate whether different catalytic modifications are utilized by the enzyme from a gluconeogenic and a glycolytic tissue. Since the hypodermis and muscle of Cancer magister undergo considerable variation in the degree of gluconeogenic poise with the molt cycle, the properties of phosphoglycerate kinase from the muscle and hypodermis of freshmolt and intermolt animals were also compared.

In most mammalian tissues, phosphoglycerate kinase is present in high levels along with glyceraldehyde-3-phosphate dehydrogenase (Newsholme & Start, 1973). In crustacean tissues by

contrast, phosphoglycerate kinase levels are considerably lower than those of both glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase. Thus, in terms of absolute enzyme activities, phosphoglycerate kinase may be a rate limiting enzyme. The difficulty in elucidating the exact role of phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase in the cell lies in the measurement of their shared intermediate, 1,3 diphosphoglycerate. Thus, the reactions are generally evaluated on the basis of a combined equilibrium constant. In glycolytic systems, an early and transient rate limitation is often found between glyceraldehyde-3-phosphate and 3-phosphoglycerate. In mammalian heart, brain, and erythrocytes this was interpreted as an effect due to glyceraldehyde-3-phosphate dehydrogenase (Williamson, 1965; Rolleston & Newsholme, 1967; Rose & Warms, 1970; Rolleston, 1972). However, other studies with the electric organ from the electric eel, and with mammalian erythrocytes, implicate phosphoglycerate kinase as a rate limiting step (Parker & Hoffman, 1967; Maitra *et al.*, 1964; Segal *et al.*, 1975).

Although there is some question about the exact routes of carbon flux during gluconeogenesis (Veneziale *et al.*, 1970), there is good evidence for the reversal of both the phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase reactions. Inadequate supplies of NADH can cause glyceraldehyde-3-phosphate dehydrogenase to be a rate limiting step during gluconeogenesis from alanine (Williamson *et al.*, 1969). However, the mammalian enzyme shows cooperative interactions between the binding of NAD, 1,3 diphosphoglycerate, and NADH,

which facilitate the reversal of the reaction for gluconeogenesis (Smith & Velick, 1972). Thus, under energy saturating conditions when the NAD/NADH ratio is high, the reverse reaction is not subject to product inhibition by NAD. In the liver, the phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase reaction system was found to be near equilibrium under a variety of metabolic conditions, providing a means whereby the cytoplasmic redox balance can be coordinated with the phosphorylation potential (Veech *et al.*, 1970; Stubbs *et al.*, 1972).

While the means by which glyceraldehyde-3-phosphate dehydrogenase is reversed during gluconeogenesis have been elucidated, the catalytic and regulatory properties of phosphoglycerate kinase from gluconeogenic tissues have received little attention. Previous kinetic studies of phosphoglycerate kinase have utilized the yeast and rabbit muscle enzyme (Scopes, 1973). Both enzymes show kinetics which strongly favor the glycolytic reaction. In these enzymes, a high sensitivity to MgADP as an inhibitor of the gluconeogenic reaction is coupled with a 1000 fold greater affinity for 1,3 diphosphoglycerate over 3-phosphoglycerate. In the cell, 3-phosphoglycerate concentrations are considered to be approximately ten fold higher than 1,3 diphosphoglycerate concentrations. The  $K_m$  for MgADP as a substrate is higher than the  $K_i$  for MgADP as an inhibitor. Thus, the gluconeogenic reaction would be kinetically limited at virtually all MgADP levels. This kinetic regulation is appropriate for a glycolytic tissue, but problematical for a gluconeogenic one. Thus, it seems reasonable to assume that the

regulatory properties of the enzyme from the gluconeogenically poised hypodermis should differ from those in the glycolytically poised muscle.

## METHODS

### Preparation of Phosphoglycerate Kinase

Hypodermis and muscle were quickly dissected out of anaesthetized crabs, rinsed in cold extraction buffer, blotted dry and weighed. Tissues were homogenized in 3 volumes of 50 mM imidazole-Cl buffer, pH 7.0, for one minute. The homogenate was centrifuged at 27,000 g for twenty minutes. Solid ammonium sulphate was added with constant stirring over 30 minutes, bringing the supernatant to 65% saturation. The mixture was centrifuged (12,000 g for 10 minutes) and solid ammonium sulphate slowly added to bring the supernatant to 75%. The resulting precipitate was collected by centrifugation, and then redissolved in a small quantity of extraction buffer. The enzyme extract, usually around 5 ml, was dialyzed over a 4 hour period against two changes of 3 liters each of 50 mM imidazole-Cl buffer, pH 7.0, with 3 mM EDTA. In studies of freshmolt and intermolt hypodermal phosphoglycerate kinases, this preparation was used directly for kinetic studies, as no major contaminants which would interfere with the various experiments could be shown. Also the hypodermal forms were largely inactivated by the steps involved in the further purification. Both muscle preparations, particularly the freshmolt muscle, contained large amounts of myokinase. Thus,

further purification was needed.

Ten grams of DEAE cellulose were pretreated according to manufacturer's directions, and equilibrated with 10 mM imidazole-HCl, 3 mM EDTA, 1 mM mercaptoethanol, pH 7.5. The enzyme extract was dialyzed against the same buffer, diluted until approximately the same ionic strength as the buffer, and then applied onto the column. The application of a KCl gradient (0-300 mM) led to the distinct separation of phosphoglycerate kinase from myokinase. Both this phosphoglycerate kinase preparation and the coupling enzyme were dialyzed against 50 mM imidazole-Cl, 3 mM EDTA, pH 7.0, and used for kinetic studies.

#### Metabolite Measurements

One leg was amputated from the experimental animal, 4 aliquots, of approximately 300 mg each, of both muscle and hypodermis were then quickly dissected out and dropped into liquid nitrogen. Since the tissue samples were small, I did not use the freeze clamping method. Since the exoskeleton needed to be opened, the procedure took between 15 and 30 seconds. Amputation could not be achieved without some resistance, thus, the measurements do not reflect resting values.

Neutralized perchlorate extracts of these frozen tissue samples were made according to the method of Williamson and Corkey (1969). The neutralized extracts were stored at -20 °C. Metabolite assays were performed shortly after the extraction, with the assay for the unstable 1,3 diphosphoglycerate performed directly after tissue extraction. Every effort was made to

perform all manipulations quickly and to maintain the extracts near 0 °C before the enzyme assay. All assays were performed in duplicate following spectrophotometric methods given by Bergmeyer (1965).

### Enzyme Assays

The enzyme was assayed according to Bücher (1947): 1,3 di-phosphoglycerate formation is coupled to glyceraldehyde-3-phosphate dehydrogenase, and the phosphoglycerate kinase activity is measured by the decrease in E340 due to NADH oxidation. All standard assays included 3-phosphoglycerate, ATP, NADH, MgCl<sub>2</sub>, and excess coupling enzyme in the concentrations shown in the figure legends. Imidazole-Cl buffers were used throughout the kinetic studies. Saturating conditions for the enzymes were: 1 mM ATP, 2 mM 3-phosphoglycerate, 4 mM MgCl<sub>2</sub> for the intermolt forms, 0.6 mM ATP, and 2 mM 3-phosphoglycerate for the freshmolt forms.

## RESULTS

### Specific Activities

In both muscle and hypodermis, phosphoglycerate kinase levels show considerable variation with the molt cycle (Figure 1). In the hypodermis, levels are high at molt (7-8 units/gm fresh weight), fall gradually to around 3 units/gm fresh weight during intermolt, and rise again sometime in premolt. Muscle shows the opposite trend, being low at molt and high during intermolt. There is a paucity of premolt data, as Cancer

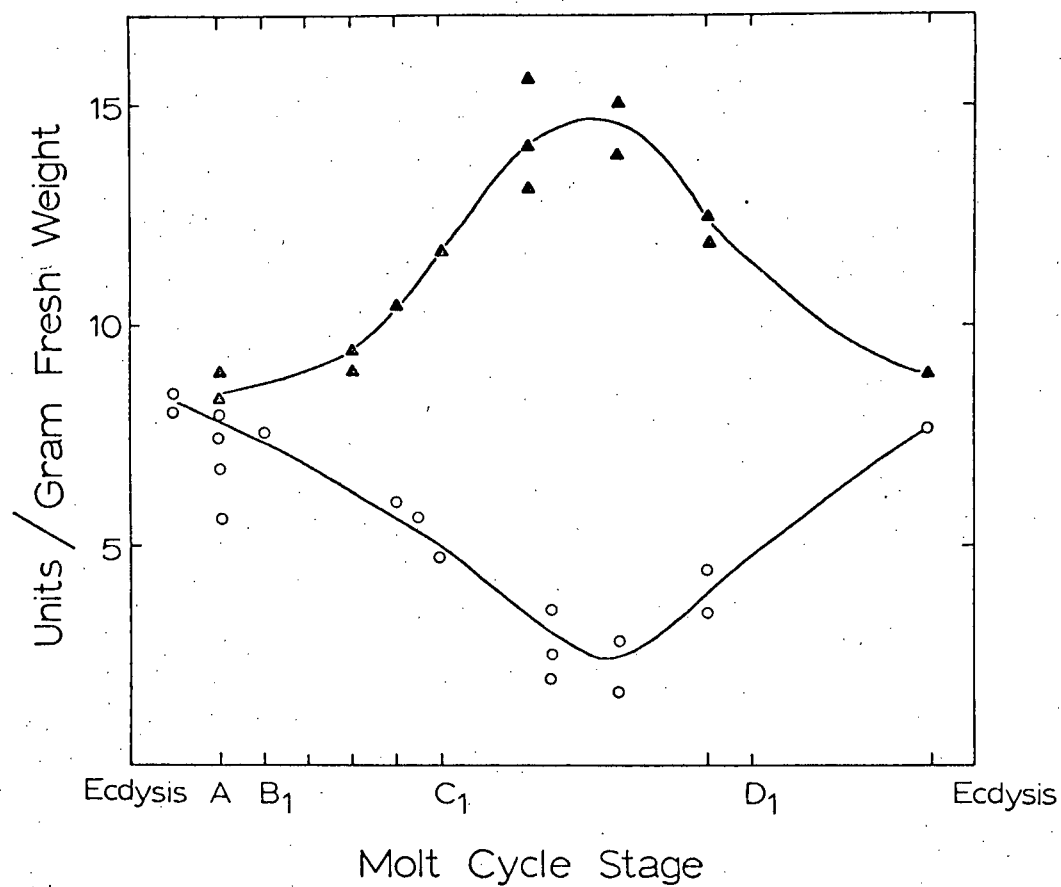


Figure 1. Variation in specific activity levels of phosphoglycerate kinase throughout the molt cycle in muscle and hypodermis. Assay conditions are described in Materials and Methods, (▲) muscle and (○) hypodermis, units are expressed in umoles product produced per minute.

magister does not readily go into premolt in captivity. No attempt was made to control for effects of size, sex, or season on enzyme levels, although little variation due to these factors was observed. These specific activities reflect the changing tissue roles between the highly mobile feeding intermolt animal and the more sedentary, but actively synthesizing molting animal, and indicate that at least in part, phosphoglycerate kinase function is regulated by control of enzyme levels.

#### Different Forms of Phosphoglycerate Kinase

The Michaelis constants for the two substrates of the enzymes from molting and intermolt muscle and hypodermis are shown in Table 2. Two sets of comparisons were made for both the 3-phosphoglycerate and the ATP affinities: the first looked for tissue differences and the second for molt cycle related differences. In intermolt animals, there is no significant difference ( $P>0.05$ ) between muscle and hypodermal phosphoglycerate kinases in terms of either their 3-phosphoglycerate or ATP affinity. In molting animals, there is no significant difference ( $P>0.05$ ) between the ATP affinities of the muscle and hypodermal forms, but there is a highly significant difference between their 3-phosphoglycerate affinities ( $P<0.001$ ), even though the magnitude of the difference is small and probably not of physiological importance. Thus, the 3-phosphoglycerate sites of the muscle and hypodermal enzymes behave differently in response to the molt cycle, indicating some difference between these tissue forms.

Table 2

## ATP and 3-P-glycerate affinities

Michelis constants, here expressed in millimoles/litre were determined at saturating concentrations of the cosubstrate and activating magnesium cation (see Experimental procedures). Values shown are the means with their standard deviations, the number in parentheses indicates the number of different animals used for the determinations.

	Intermolt muscle	Intermolt Hypodermis	Molting Muscle	Molting Hypodermis
PGA $K_m$	0.273 $\pm$ 0.057 (8)	0.271 $\pm$ 0.054 (9)	0.218 $\pm$ 0.042 (6)	0.320 $\pm$ 0.036 (9)
ATP $K_m$	0.203 $\pm$ 0.045 (7)	0.174 $\pm$ 0.063(12)	0.071 $\pm$ 0.009 (8)	0.068 $\pm$ 0.014(13)

Comparisons between intermolt and molting phosphoglycerate kinases reveal several marked differences. Intermolt and molting hypodermal phosphoglycerate kinases differ significantly in both their ATP and 3-phosphoglycerate affinities ( $P < 0.01$ ). The ATP affinities of molting and intermolt muscle phosphoglycerate kinases are also significantly different ( $P < 0.01$ ), but their 3-phosphoglycerate affinities do not differ significantly ( $P > 0.05$ ). Thus, on the basis of substrate affinities, there is good evidence for changes of the phosphoglycerate kinase forms with the molt cycle as well as for changes between tissue forms of the enzyme. While the changes in 3-phosphoglycerate affinities are probably not of physiological significance, the changes in ATP affinities are large and may be of regulatory importance.

Starch gel electrophoresis and isoelectrofocusing were carried out to see if there was an isoenzymic basis for these different substrate affinities. In gel electrophoresis, both muscle and hypodermal phosphoglycerate kinases show a molting variant with faster anodal migration than the intermolt form (Figure 2). Hypodermal and muscle forms of the enzyme show different velocities. The pI values as determined by isoelectrofocusing were 5.4 for the intermolt muscle, 5.1 for the molting muscle, 5.3 for the intermolt hypodermis, and 5.0 for the molting hypodermis. The relative magnitudes of the pI values confirm the results from the starch gels, since the lower the pI, the greater the anodal mobility at pH 7.5, given no great size difference. However, the pI values for the two molting enzymes are quite close as are those of the two

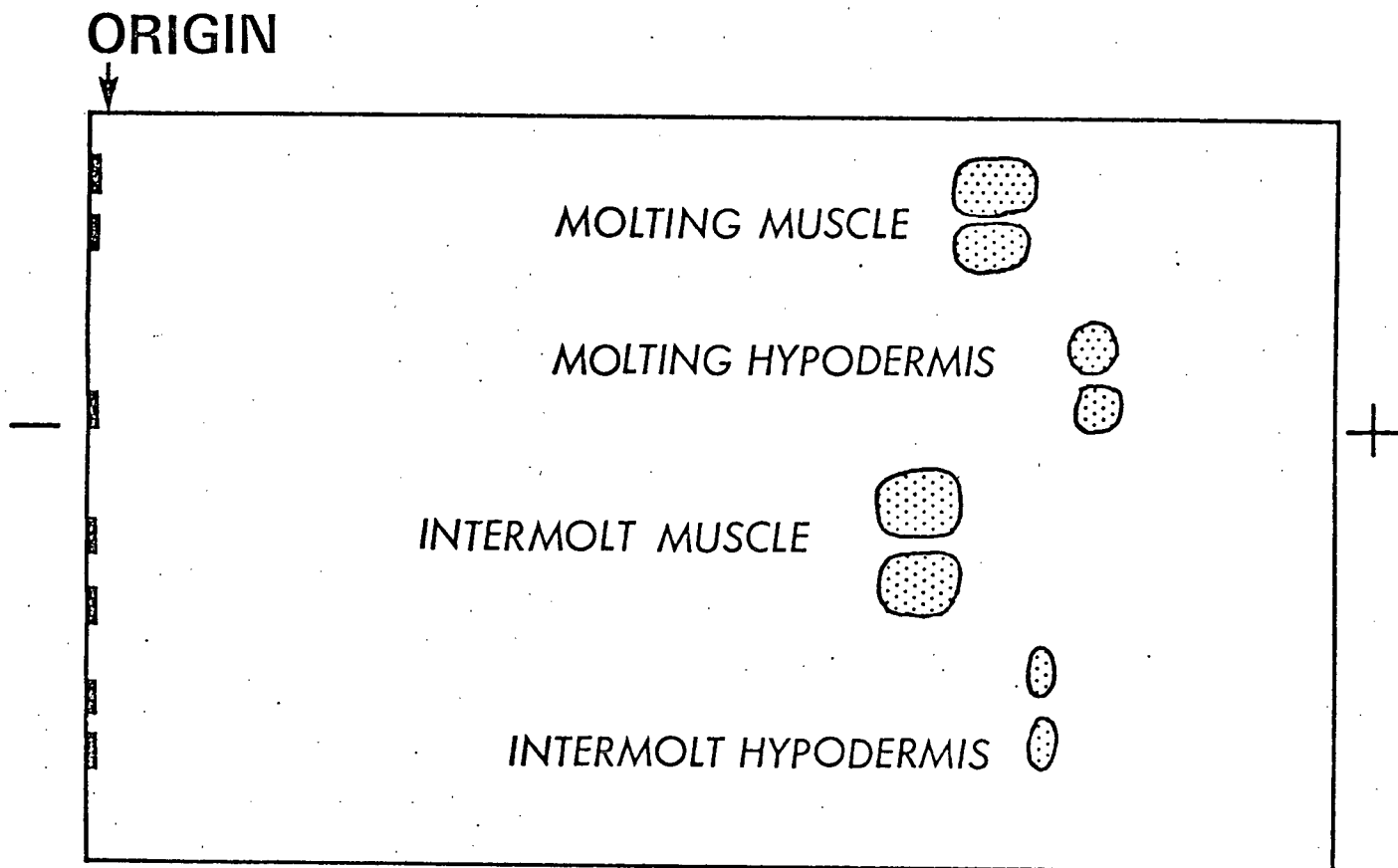


Figure 2. A diagrammatic sketch of a starch gel electrophoretogram showing zones of phosphoglycerate kinase activity extracted from muscle and hypodermis of Cancer magister. The gel was run at 250 mvolts, 25 mamps using a Tris-citrate, pH 7.0, buffer system. Duration of run was 30 hours.

intermolt forms. The instability of the hypodermal forms prevented me from dealing with more purified proteins, or from doing exhaustive isoelectrofocusing (48 hours or longer). Thus, the small differences between 5.0 and 5.1, as well as those between 5.3 and 5.4, cannot be taken as clear indication that muscle and hypodermis have distinct phosphoglycerate kinases at molt and during intermolt. These different pI values could be a reflection of differing ratios of two gene products, four different gene products, or one protein undergoing varying degrees of modification in these tissues during the molt cycle. In none of the tissues was there any evidence, during electrofocusing or column chromatography, of more than one phosphoglycerate kinase form. The mobility differences indicate that at least two enzyme species are present in the four crab tissues, one present in freshmolt and the other in intermolt tissues.

### Kinetic Properties

To see how phosphoglycerate kinase is regulated under varying metabolic requirements, as well as whether any differences in catalytic characteristics accompanied the substrate and mobility differences, an analysis of the kinetics of the phosphoglycerate kinases from the four tissues was undertaken. Both hypodermal phosphoglycerate kinases show a broad pH optimum with maximal activity between pH 6.0 and 8.0. The two muscle forms show a similar response, but with maximal activity between pH 6.5 and 7.5. While divalent cations (particularly  $Mg^{++}$  and  $Mn^{++}$ ) provide a pronounced and

stoichiometric activation of the reaction, it is difficult to demonstrate an absolute cation requirement with these enzymes. These all show a residual enzyme activity in the absence of added magnesium. Whether this is due to bound magnesium, to a lack of an absolute cation requirement, or to incomplete dialysis, could not be clearly demonstrated, as prolonged dialysis (8 hours) tended to inactivate crab phosphoglycerate kinases, particularly the hypodermal forms. Generally, the variations in the Michaelis constants for  $MgCl_2$  and  $MnCl_2$  (Table 3) parallel those of the ATP  $K_m$ .

Initial kinetic studies showed that the 3-phosphoglycerate and ATP affinities are independent of each other, and of the  $MgCl_2$  concentration. With respect to magnesium, this was true if the total magnesium was held constant as the ATP or 3-phosphoglycerate were varied. Magnesium effects up to 6 mM were checked. Representative curves are shown in Figure 3, with the same basic results holding for all four enzymes. In studies where magnesium and ATP concentrations were varied simultaneously, the Michaelis constant for MgATP was higher than the corresponding  $K_m$  in studies where the total magnesium was held constant (this effect was larger in the muscle than in the hypodermal forms) (Table 4).

Both NaCl and KCl strongly activate these crustacean phosphoglycerate kinases leading to as much as 150% increase in their activity over that in the presence of saturating magnesium. The Michaelis constants (Table 3) are fairly high indicating that the stimulation is probably due to a change in

Table 3

### Michelis constants for activating cations

$K_m$  values were determined at saturating levels of the substrates and  $MgCl_2$  (except in the determination of the  $MgCl_2$   $K_m$ ) and indicate the concentration of cation required for 50% of the maximal activity in the presence of that cation. The values are expressed in millimolar units.

$V_{max}$  values are expressed as % of the maximal activity in the presence of saturating magnesium levels.

	Intermolt		Molting	
	Muscle	Hypodermis	Muscle	Hypodermis
$MgCl_2$ $K_m$	0.252	0.193	0.09	0.077
$MgCl_2$ $V_{max}$	100%	100%	100%	100%
$MnCl_2$ $K_m$	0.164	0.165	0.152	0.058
$MnCl_2$ $V_{max}$	88%	95%	75%	95%
$NaCl$ $K_m$	100.0	90.0	30.0	19.0
$NaCl$ $V_{max}$	216%	250%	215%	242%
$KCl$ $K_m$	85.0	80.0	65.0	80.0
$KCl$ $V_{max}$	188.5%	230%	220%	184%

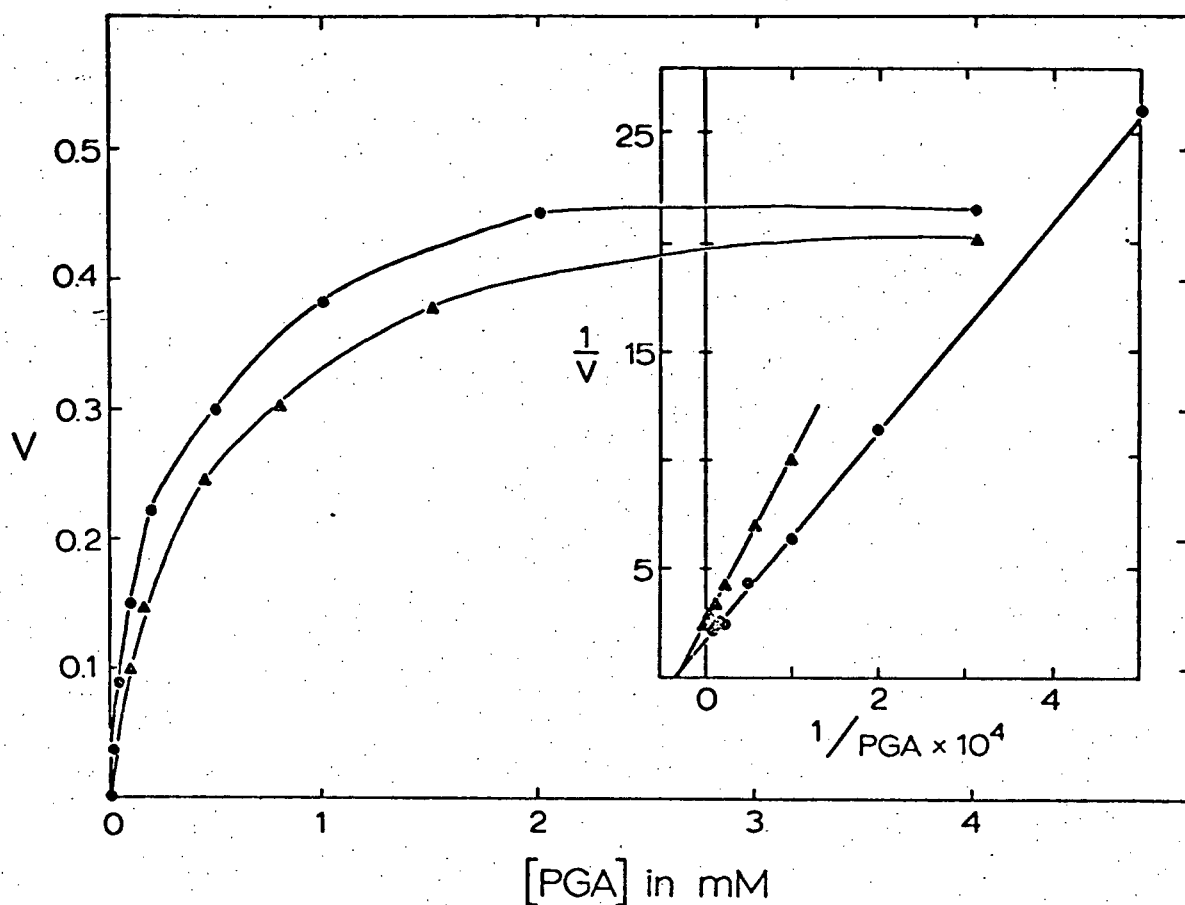


Figure 3 (a).

Michelis constants for ATP and 3-P-glycerate at varying levels of cosubstrate and activator. (a) 3-P-glycerate (PGA) affinity of the molting hypodermal phosphoglycerate kinase: in the presence of 1 mM ATP (•), in the presence of  $3.5 \times 10^{-4}$  M ATP (▲). (b) ATP affinity of the molting muscle phosphoglycerate kinase: in the presence of 4 mM 3-P-glycerate (○, △), in the presence of  $4 \times 10^{-4}$  M 3-P-glycerate (•, ▲). (c) ATP affinity of intermolt muscle enzyme in the presence of 2 mM  $\text{MgCl}_2$  total (△), in the presence of  $6 \times 10^{-4}$  M  $\text{MgCl}_2$ , total (○), and in the presence of 6 mM  $\text{MgCl}_2$  (◻).

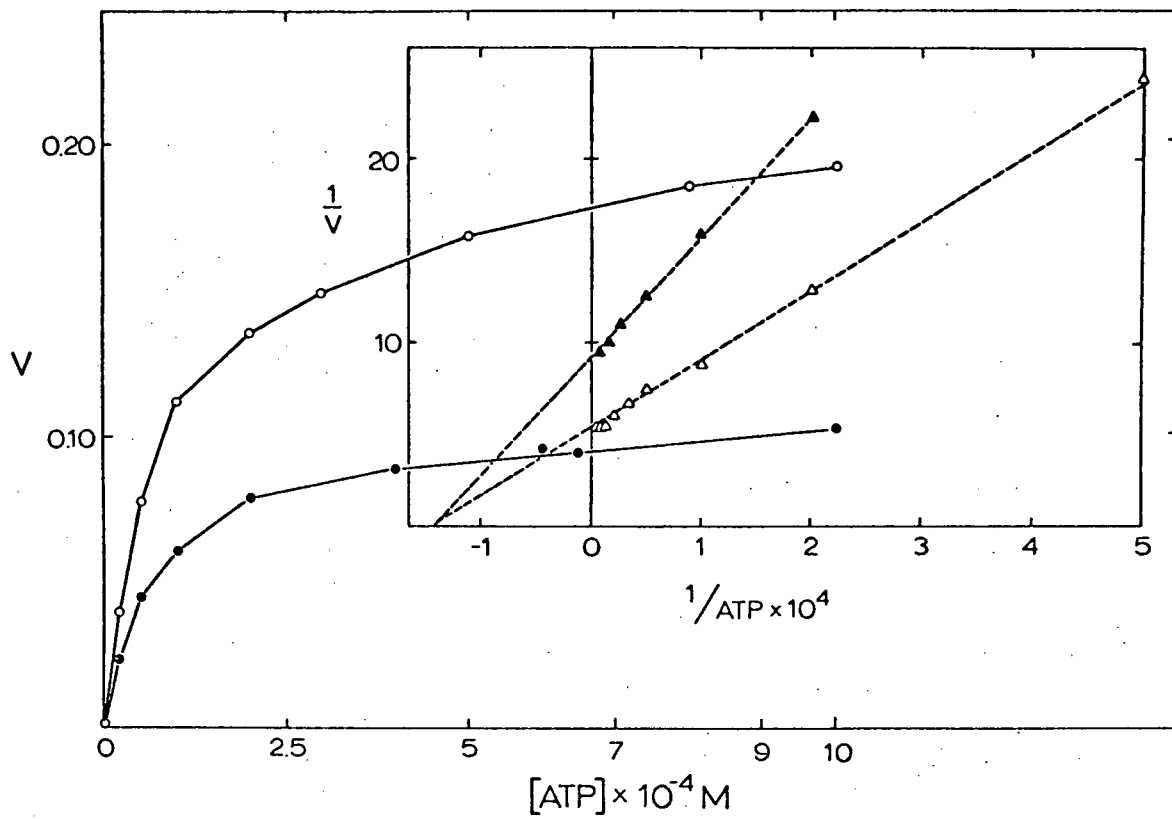


Figure 3b.

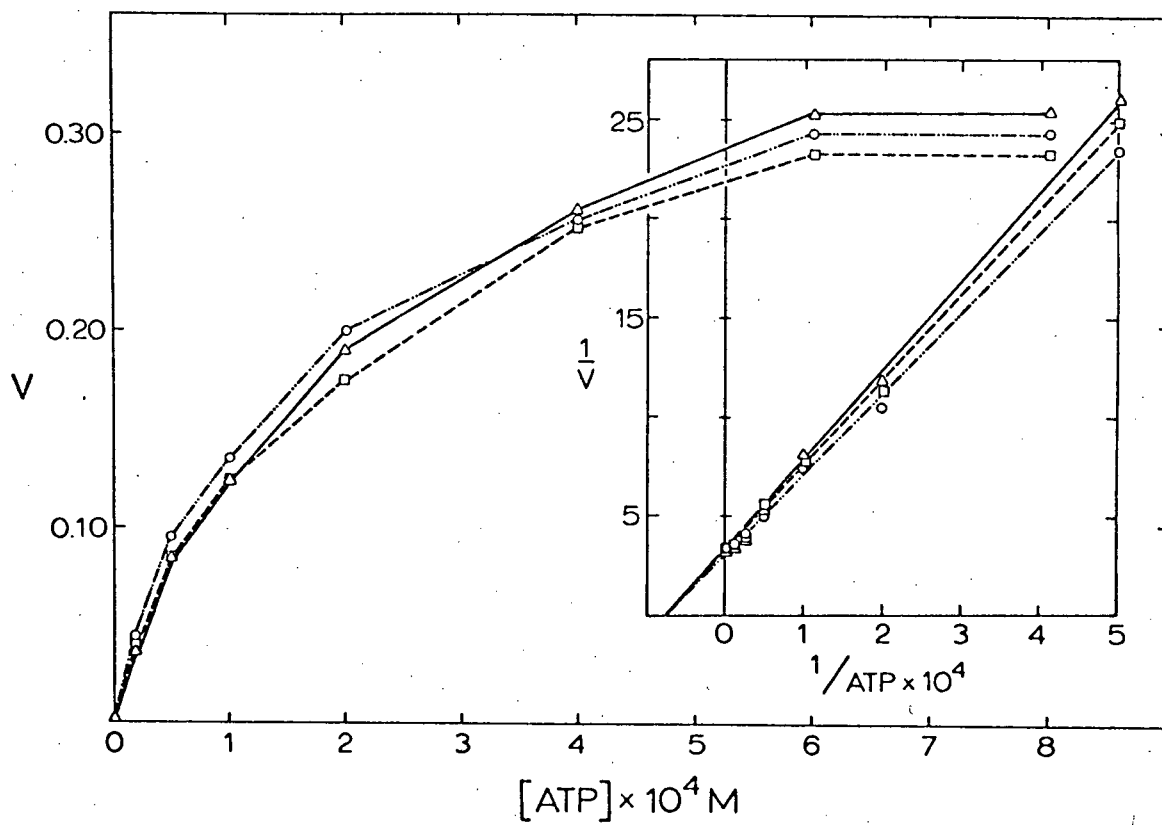


Figure 3c.

Table 4

### Michelis Constants for ATP and MgATP

The Michelis constants for ATP were determined in studies where the total  $\text{MgCl}_2$  was maintained at a fixed total greater than the total ATP. The constants for MgATP were determined by simultaneously varying  $\text{MgCl}_2$  and ATP in a 1:1 ratio. All values in millimolar units.

	Intermolt Muscle	Intermolt Hypodermis	Molting Muscle	Molting Hypodermis
ATP $K_m$	0.16	0.118	0.071	0.067
MgATP $K_m$	0.226	0.142	0.178	0.087

the solvent structure rather than to a specific binding to the enzyme. NaCl and KCl do not affect the substrate affinities except in the case of the molting muscle enzyme where they both raise the substrate  $K_m$  values. The 3-phosphoglycerate  $K_m$  changes from 0.245 mM to 0.29 mM and the ATP  $K_m$  rises from 0.085 to 0.11 mM.

A wide range of compounds was tested for their possible effects on these phosphoglycerate kinases. They included glycolytic and Krebs cycle intermediates, important amino acids, nucleotides, and coenzymes. Only very close substrate and product analogues as well as the adenylate product were inhibitors. The difficulty of 1,3 diphosphoglycerate synthesis, and its instability made its use impractical. The product, ADP, is the most potent inhibitor, but AMP, 2,3 diphosphoglycerate, and  $\text{CaCl}_2$  also exercise inhibitory effects on the reaction.

The inhibition by ADP was strongly influenced by magnesium levels (Figure 4). At saturating levels of ATP and 3-phosphoglycerate, increasing magnesium levels increase the effectiveness of the ADP inhibition. This is true for the enzyme in all four tissues. Both MgADP and ADP acted as inhibitors, but the  $K_i$  of MgADP was considerably lower than that for ADP. The patterns are summarized in Table 5, and some representative curves from the intermolt muscle and molting hypodermal phosphoglycerate kinases are shown in Figures 5 and 6. ADP and MgADP affect the binding of both 3-phosphoglycerate and ATP.

ADP inhibition follows the same basic pattern in all four

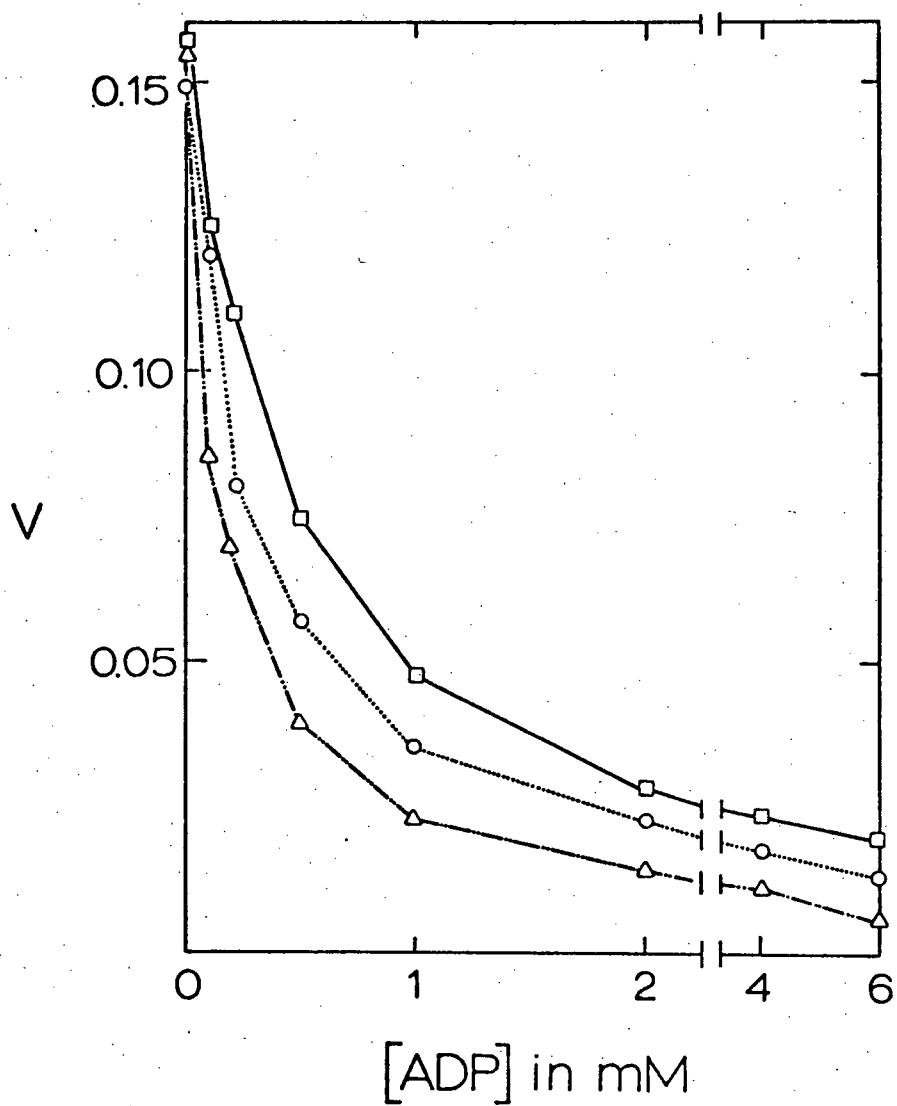


Figure 4.

ADP inhibition of intermolt muscle phosphoglycerate kinase, effect of increasing magnesium ion concentration. Assay conditions are described in Materials,  $6 \times 10^{-4}$  M  $\text{MgCl}_2$  ( $\square$ ), 2 mM  $\text{MgCl}_2$  ( $\circ$ ), and 6 mM  $\text{MgCl}_2$  ( $\triangle$ ).

Table 5

$K_i$  values for ADP and MgADP inhibition.

$K_i$  values were estimated from Lineweaver-Burk (L-B) and Dixon plots. Assay conditions are as described in Materials and Methods, with the varying total magnesium concentrations as shown. All  $K_i$  values are in millimolar units.

Total MgCl <sub>2</sub>	Intermolt Muscle		Molting Muscle		Intermolt Hypodermis		Molting Hypodermis	
	L-B	Dixon	L-B	Dixon	L-B	Dixon	L-B	Dixon
$K_i$ , ADP vs PGA Mg=ATP		.13	.23	.27	.39	.42	.230	.33
$K_i$ , MgADP vs PGA 2 mM	.012		.12	.13	.19			.18, .20
$K_i$ , ADP vs MgATP Mg=ATP	.06	.08		.40	.50	.60	.40	.50
$K_i$ , MgADP vs MgATP 2 mM	.013		.2009	.20	.19	.20	.20	.23
" " " " 4 mM		.05		.10		.16		.145, .15
" " " " 6 mM		.045			.11			

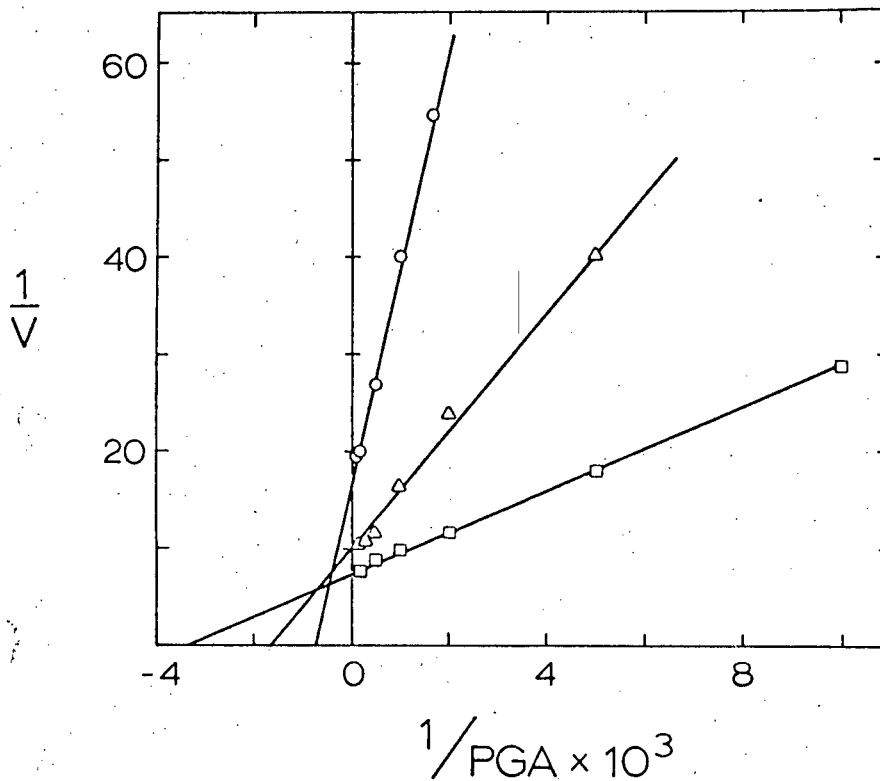


Figure 5a.

Intermolt muscle phosphoglycerate kinase, ADP and MgADP inhibition patterns. (a) 3-P-glycerate affinity in presence of 2 mM  $\text{MgCl}_2$  and no ADP ( $\square$ ), 2 mM  $\text{MgCl}_2$  and  $2 \times 10^{-5}\text{M}$  ADP, 70% of which is MgADP ( $\Delta$ ), and 2 mM  $\text{MgCl}_2$  and  $1 \times 10^{-4}\text{M}$  ADP, 60% of which is MgADP ( $\odot$ ). (b) MgATP affinity in presence of no ADP ( $\odot$ ), of  $2 \times 10^{-5}\text{M}$  ADP ( $\square$ ), of  $1 \times 10^{-4}\text{M}$  ADP ( $\Delta$ ). (c) ATP affinity in presence of 2 mM  $\text{MgCl}_2$  and no ADP ( $\bullet$ ), of 2 mM  $\text{MgCl}_2$  and  $2 \times 10^{-5}\text{M}$  ADP (75% of the ADP present as MgADP) ( $\Delta$ ), of 2 mM  $\text{MgCl}_2$  and  $1 \times 10^{-4}\text{M}$  ADP (75% ADP as MgADP) ( $\circ$ ). (d) Dixon plot of ADP inhibition in the presence of 4 mM  $\text{MgCl}_2$ , 4 mM ATP, 5 mM 3-P-glycerate ( $\circ$ ), 4 mM  $\text{MgCl}_2$ , 4 mM ATP,  $5 \times 10^{-4}\text{M}$  3-P-glycerate ( $\Delta$ ),  $5 \times 10^{-4}\text{M}$   $\text{MgCl}_2$ ,  $5 \times 10^{-4}\text{M}$  ATP, and 4 mM 3-P-glycerate ( $\blacktriangle$ ), 4 mM  $\text{MgCl}_2$ ,  $4 \times 10^{-4}\text{M}$  ATP, 5 mM 3-P-glycerate ( $\square$ ), and 6 mM  $\text{MgCl}_2$ ,  $5 \times 10^{-4}\text{M}$  ATP, 5 mM 3-P-glycerate ( $\bullet$ ).

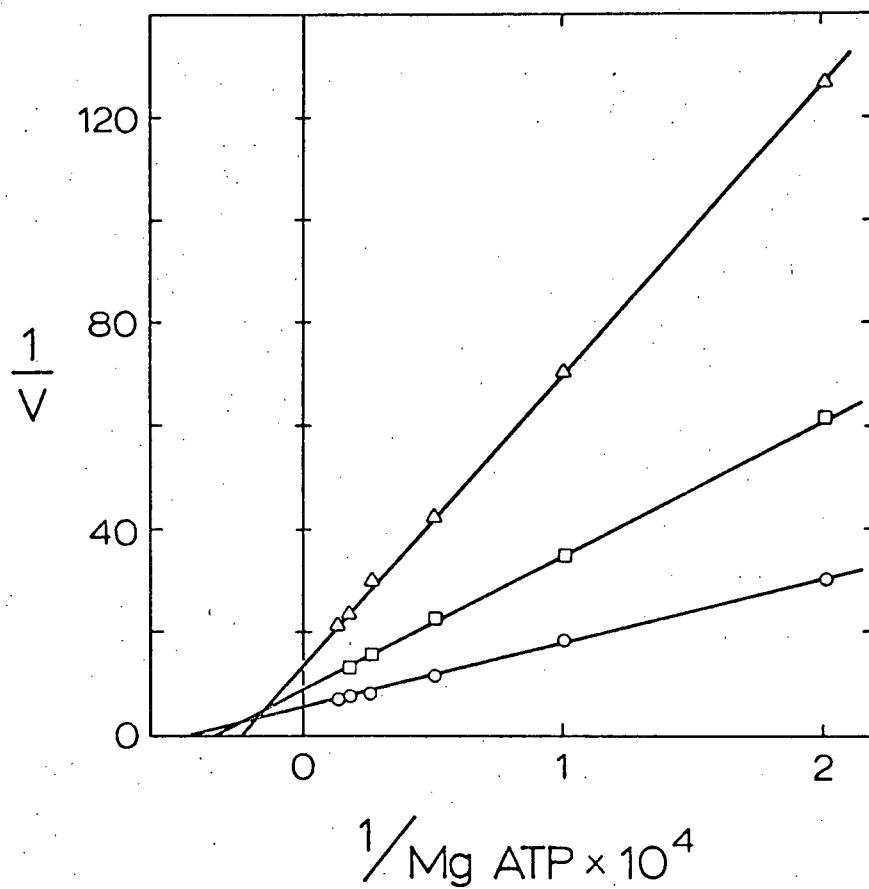


Figure 5b.

MgATP affinity and the effect of ADP inhibition, intermolt muscle phosphoglycerate kinase. Symbols explained in the legend to Figure 5a.

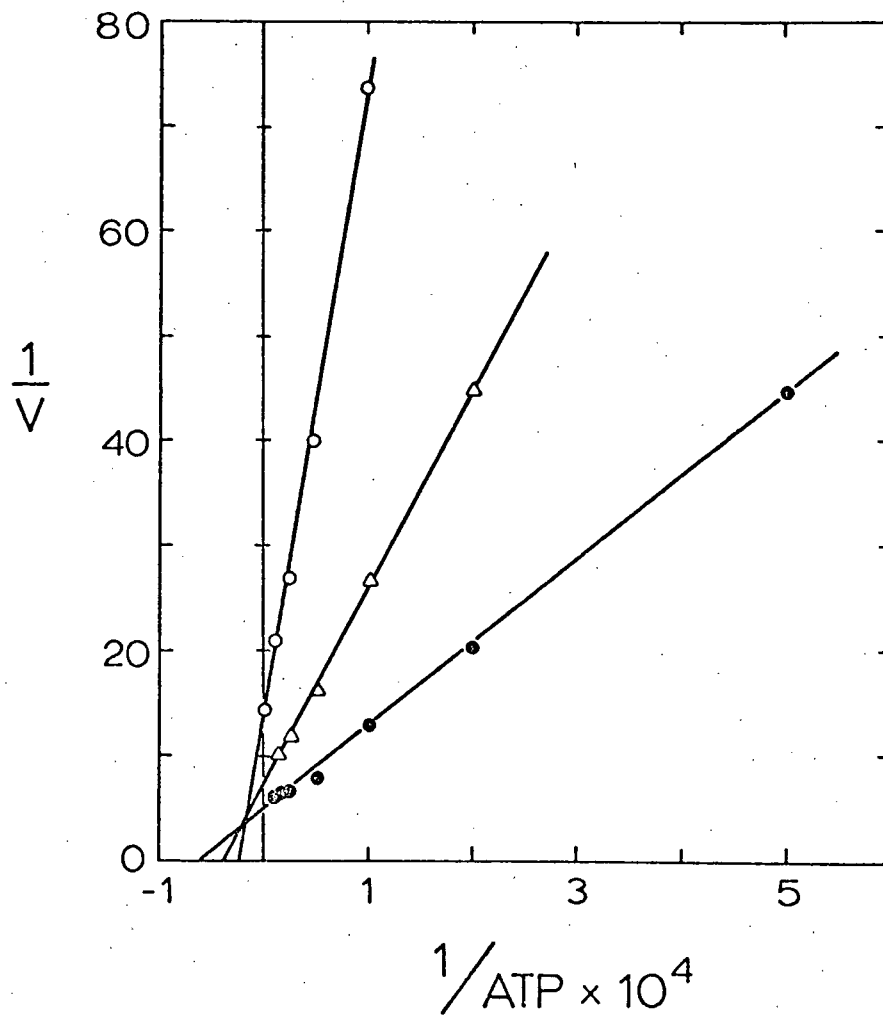


Figure 5c.

ATP affinity and the effect of MgADP upon the intermolt muscle phosphoglycerate kinase. Symbols explained in the legend to figure 5a.

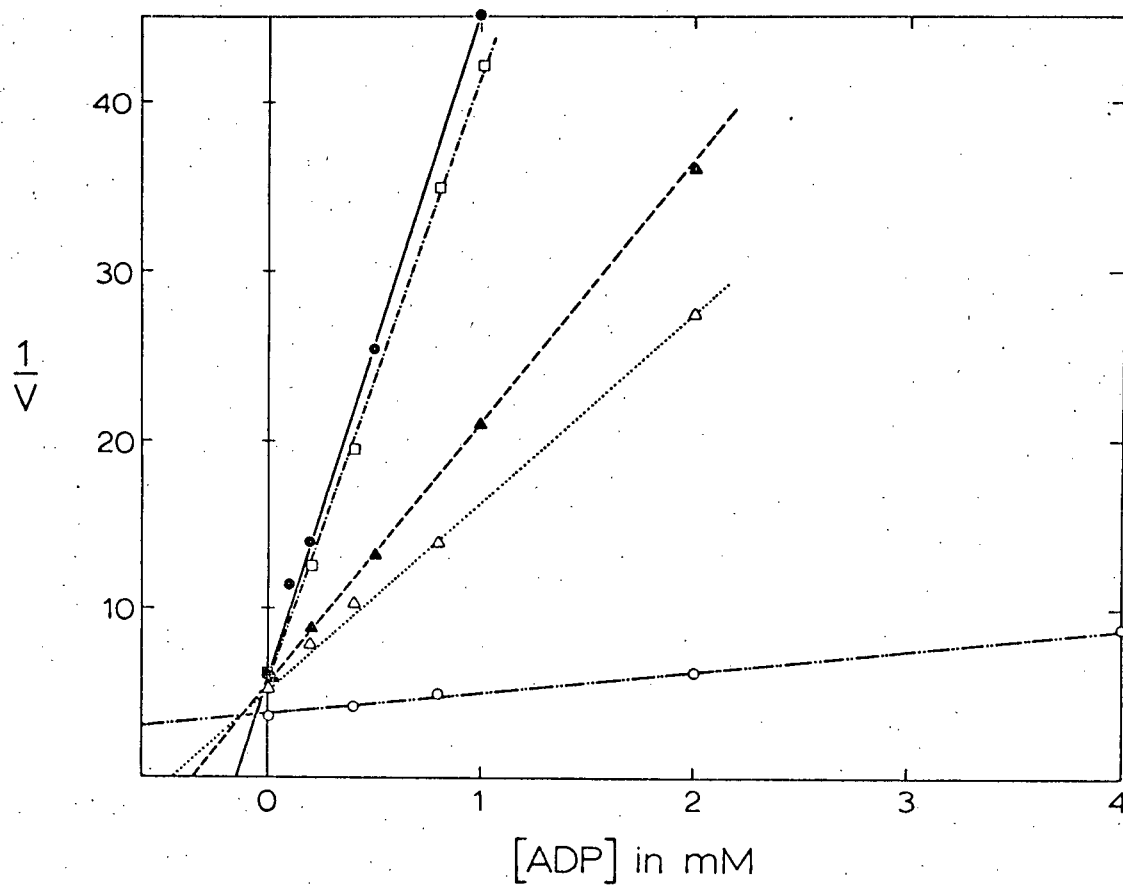


Figure 5d.

ADP inhibition of intermolt muscle phosphoglycerate kinase.

Symbols given in the legend to Figure 5a.

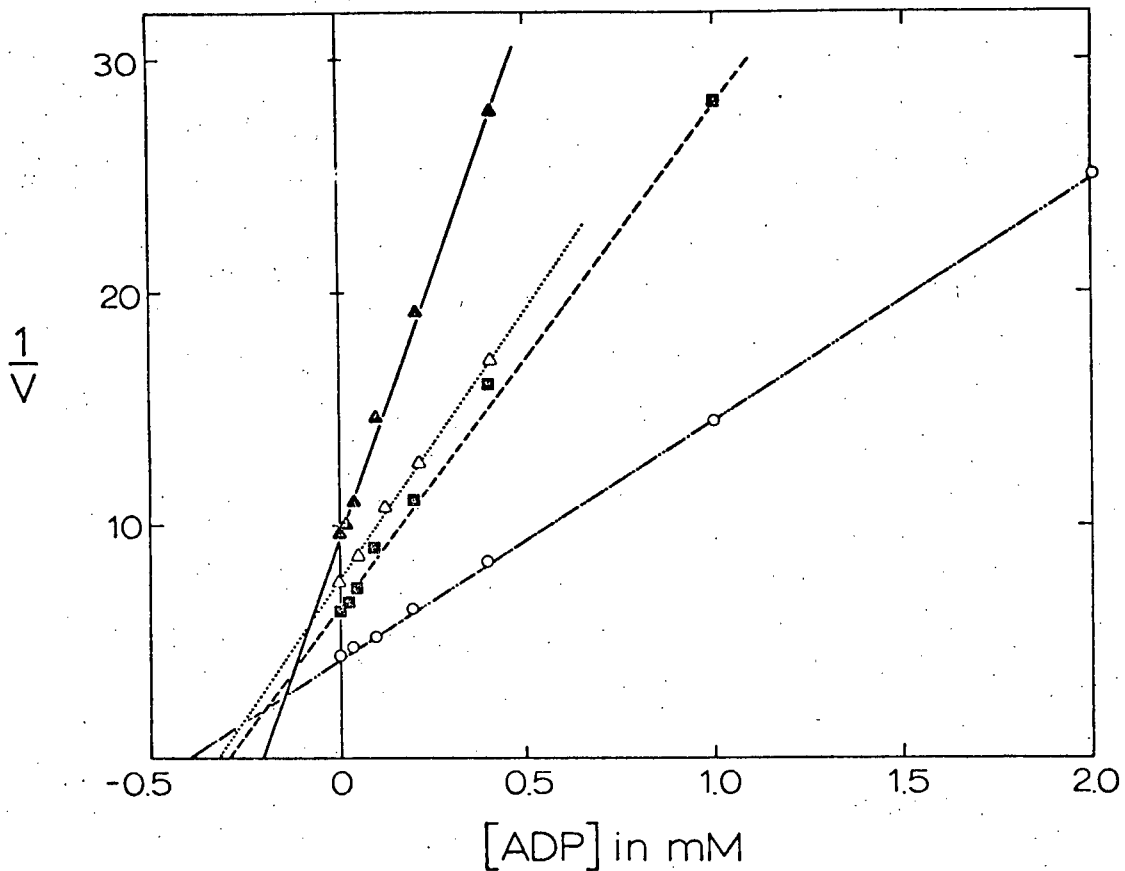


Figure 6 (a).

Molting hypodermal phosphoglycerate kinase, ADP and MgADP inhibition patterns. (a) Dixon plot of ADP inhibition in presence of 4 mM  $\text{MgCl}_2$ , 4 mM 3-P-glycerate, 1 mM ATP (O), of 4 mM  $\text{MgCl}_2$ ,  $4 \times 10^{-4}$  M 3-P-glycerate, 1 mM ATP (■), of 4 mM  $\text{MgCl}_2$ , 4 mM 3-P-glycerate, and  $1 \times 10^{-4}$  M ATP (Δ), and 4 mM 3-P-glycerate, 1 mM  $\text{MgCl}_2$ ,  $1 \times 10^{-4}$  M ATP (▲). (b) Dixon plot of ADP inhibition in presence of 1 mM  $\text{MgCl}_2$ , 1 mM ATP, 4 mM 3-P-glycerate (O), of 1 mM  $\text{MgCl}_2$ , 1 mM ATP,  $4 \times 10^{-4}$  M 3-P-glycerate (■) and of  $3.4 \times 10^{-4}$  M MgATP and 4 mM 3-P-glycerate (▲).

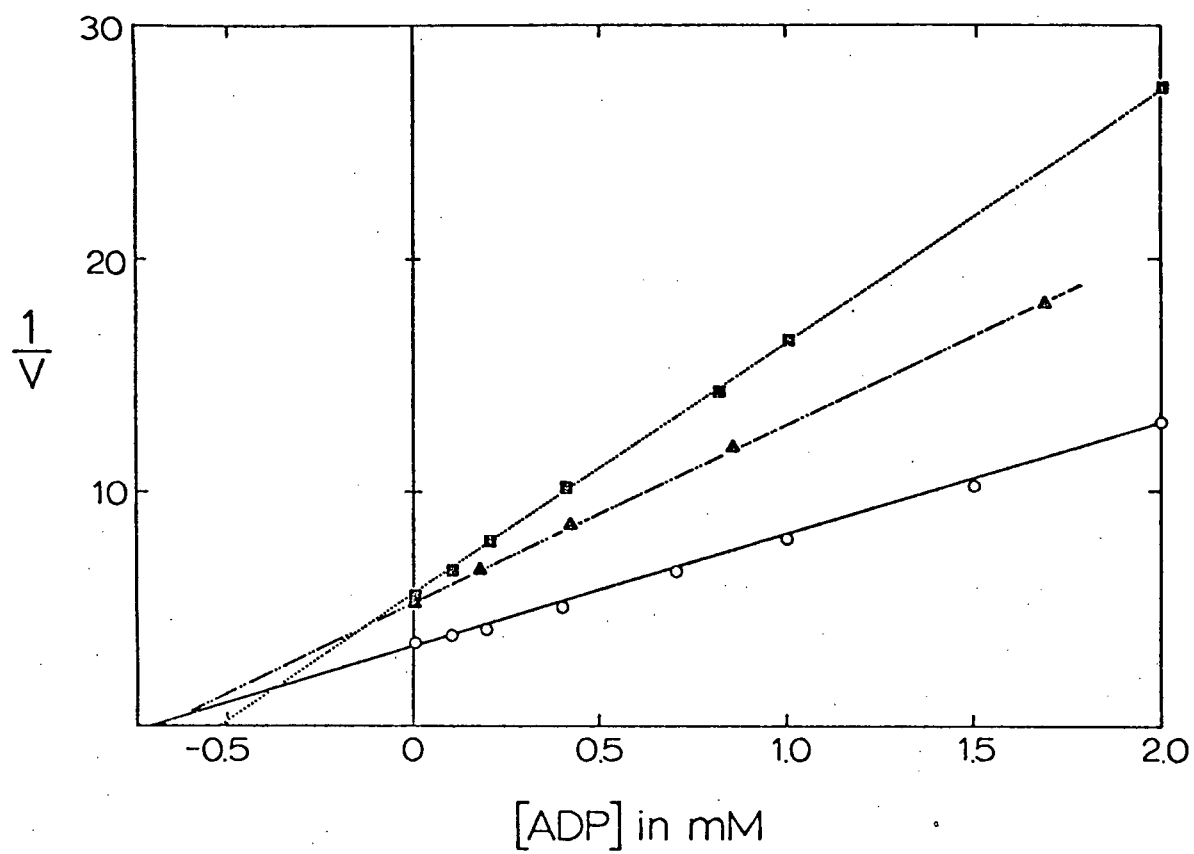


Figure 6b.

enzymes and a description of the intermolt muscle ADP/MgADP response will illustrate the form of the inhibition for the others. At low magnesium, when essentially all the ADP is uncomplexed, ADP is a mixed competitive inhibitor with both ATP and 3-phosphoglycerate, with  $K_i$  values of  $8 \times 10^{-5}$  M and  $1.3 \times 10^{-4}$  M respectively. As the levels of magnesium are increased, thereby increasing the proportion of ADP present as MgADP, the  $K_i$  values drop sharply as the inhibition with respect to 3-phosphoglycerate ( $K_i = 1.2 \times 10^{-5}$  M) becomes more competitive, and that versus ATP ( $K_i = 1.3 \times 10^{-5}$  M) becomes completely competitive.

While the ADP inhibition follows the same basic pattern in the three other phosphoglycerate kinases, the order of magnitude of the inhibition constants differs markedly. The molting muscle and hypodermal enzymes as well as the intermolt hypodermal enzyme show a  $K_i$  value of  $2 \times 10^{-4}$  M for the completely competitive MgADP versus ATP inhibition. This value is ten times that for the intermolt muscle enzyme. The MgADP  $K_i$  versus 3-phosphoglycerate is raised to  $1.8 \times 10^{-4}$  M from  $1.2 \times 10^{-5}$  M, another ten fold change. The inhibition constants for ADP are also increased, though not as markedly (Table 5). While the interactions between MgADP, ATP, and 3-phosphoglycerate are much the same for these three enzymes, the intermolt hypodermal enzyme is least sensitive to ADP inhibition. All three enzymes are an order of magnitude less sensitive to inhibition by ADP or MgADP than the intermolt muscle enzyme.

The other competitive interactions are summarized in Table

6. In all four forms, AMP is inhibitory only with ATP. For molting muscle, intermolt and molting hypodermis, the  $K_i$  values are much the same, 2.15-2.5 mM. The intermolt muscle enzyme differs, having a  $K_i$  for AMP of 7.6 mM. In all four enzymes, 2,3-diphosphoglycerate is competitive with both ATP and 3-phosphoglycerate. The  $K_i$  versus 3-phosphoglycerate is much the same for all forms, and ranges from 1.93 to 2.3 mM. Intermolt muscle and hypodermis as well as molting muscle phosphoglycerate kinases showed a 2,3 diphosphoglycerate versus ATP  $K_i$  of 4.3 to 5.0 mM, while the molting hypodermal enzyme showed a  $K_i$  versus ATP of 16 mM.  $\text{CaCl}_2$  inhibition is competitive with 3-phosphoglycerate, probably due to the formation of an unreactive  $\text{Ca}^{++}$ -3-phosphoglycerate complex. The  $K_i$  for the interaction of calcium with 3-phosphoglycerate is an order of magnitude lower than the  $K_i$  for its interaction with magnesium or ATP. In contrast to the other enzymes, the intermolt muscle enzyme shows a strong, non-linear response to calcium inhibition versus magnesium, but does not show much interaction between calcium and 3-phosphoglycerate.

### Mass Action Ratios

The differing ATP affinities and ADP/MgADP sensitivities of these various phosphoglycerate kinases may affect the poise of the reaction system in vivo. In order to check this possibility, I measured the levels of ATP, 3-phosphoglycerate, ADP, and 1,3 diphosphoglycerate in muscle and hypodermis of freshmolt and intermolt animals (Table 7). As mentioned in the methods, these values do not represent resting values, since

Table 6

$K_i$  values for AMP, 2,3 DPG and  $\text{CaCl}_2$

$K_i$  values were determined from Dixon plots. Assay conditions are described in Materials and Methods.  $K_i$  values are expressed in millimolar units.

	Intermolt Muscle	Molting Muscle	Intermolt Hypodermis	Molting Hypodermis
$K_i$ , AMP vs ATP	7.6	2.15	2.5	2.3
$K_i$ , 2,3 DPG vs P6A	2.0	2.0	2.5	1.93
$K_i$ , 2,3 DPG vs ATP	4.3	5.0	4.5	16.0
$K_i$ , $\text{CaCl}_2$ vs P6A	35.0	2.7	2.3	3.0
$K_i$ , $\text{CaCl}_2$ vs ATP	28.0	24.5	42.0	13.6
$K_i$ , $\text{CaCl}_2$ vs $\text{MgCl}_2$	Parabolic	24.0	43.0	13.6

Table 7

## Mass Action Ratios from Intermolt and Freshmolt Tissues.

Each value represents the mean of at least three tissue samples from each animal. Metabolite measurements in each sample were made in duplicate. Metabolite concentrations expressed as uMoles/gm fresh weight.

$$\bar{\gamma} = \frac{\text{ATP} \times 3\text{-phosphoglycerate}}{\text{ADP} \times 1,3 \text{ diphosphoglycerate}} \cdot \bar{\gamma}^* \text{ has been calculated using}$$

resting ATP/ADP ratios from Beis and Newsholme (1975).

Tissue	Animal number	ATP	ADP	3-phospho-glycerate	1,3 diphospho-glycerate	$\bar{\gamma}$	$\bar{\gamma}^*$
Freshmolt muscle	1	1.69	.449	.233	.0697	12.62	25.2
—	2	1.68	.204	.103	.045	18.8	18.8
—	3	3.14	.383	.158	.0726	17.8	17.8
Freshmolt hypodermis	1	2.33	.835	.2827	.0304	26.03	52.06
—	2	1.794	.381	.2369	.0467	23.87	47.7
—	3	3.279	.428	.278	.033	64.64	64.64
Intermolt muscle	4	1.986	.837	.1364	.0134	24.17	74
—	5	3.196	.831	.1571	.0277	21.81	43.62
—	6	1.939	.531	.1542	.0603	9.34	18.7
Intermolt hypodermis	4	3.04	.535	.3848	.0427	51.27	75.42
—	5	2.298	.4933	.219	.033	31.05	61.1
—	6	1.36	.322	.174	.0365	20.22	40.44

amputation of legs could not be achieved without some resistance by these large and mobile animals. The mass action ratios ( $\gamma = \text{ATP} \cdot 3\text{PGA} / 1,3\text{-DPG} \cdot \text{ADP}$ ) derived from the concentrations were smaller than the equilibrium value of 1200-2000 (Rose & Warms, 1970; Krietsch & Bucher, 1970). If the values are recalculated using the ATP:ADP ratios characteristic of resting muscle (Beis & Newsholme, 1975), they become somewhat higher and closer to the equilibrium value (Table 7). However, in any one animal, the muscle ratios are always smaller than the hypodermal ratios. Generally, these mass action ratios are near the borderline between equilibrium and non-equilibrium reactions ( $\gamma/K_{eq} = 0.05$ ) defined by Rolleston (1972), with the muscle values lying further from equilibrium. Thus, none of these enzymes fall definitely within the rate limiting category occupied by pyruvate kinase and phosphofructokinase.

All the tissue samples contained measurable, but low values of 1,3 diphosphoglycerate. The levels were near the lower limit of spectrophotometric sensitivity, so the percentage accuracy is low. The levels of 3-phosphoglycerate were also low. For the mass action ratios to approach equilibrium, the levels of 1,3 diphosphoglycerate would need to be 50-100 fold lower, or the levels of 3-phosphoglycerate considerably higher. The ratios from the muscle of intermolt crabs show considerable variation, making precise conclusions of the *in vivo* role of the enzyme difficult. While neither the muscle nor the hypodermal enzymes show a mass action ratio far removed from equilibrium, generally, the hypodermal enzymes show mass action ratios which lie nearer to the equilibrium constant.

## DISCUSSION

One concept of considerable metabolic significance emerges from these data. Hypodermal phosphoglycerate kinase is kinetically modified for gluconeogenic function in distinct contrast to the intermolt muscle enzyme. The previously studied phosphoglycerate kinases were from yeast and muscle, and their kinetics, which are similar to those of the intermolt muscle enzyme from Cancer magister, seem to curtail reversal of the reaction. These enzymes have a higher affinity for MgADP as an inhibitor than as a substrate (Krietsch & Bücher, 1970; Scopes, 1973). Simultaneously, their affinities for ATP and 3-phosphoglycerate are an order of magnitude lower than their affinity for MgADP as an inhibitor. Physiological levels of ATP, ADP, and MgCl<sub>2</sub> are well above their respective K<sub>m</sub> and K<sub>i</sub> values, while 3-phosphoglycerate levels are somewhat lower than its K<sub>m</sub> value. By interacting competitively with the binding of both MgATP and 3-phosphoglycerate, physiological levels of MgADP would strongly limit the gluconeogenic reaction.

The kinetics of phosphoglycerate kinases from gluconeogenic tissues provide a strong contrast to those of the intermolt muscle enzyme. Intermolt hypodermis, which sustains a continual basal rate of chitin synthesis, and the molting hypodermis which must cope with a large gluconeogenic load, both show phosphoglycerate kinases considerably modified for gluconeogenic function. The intermolt hypodermal form shares the substrate affinities of the "glycolytic" enzyme, but has considerably increased K<sub>i</sub> values for MgADP inhibition versus both substrates.

Thus, the effectiveness of product inhibition is greatly reduced. The molting hypodermal enzyme has both a low sensitivity to product inhibition, as well as a significantly increased affinity for ATP. Thus, in this tissue where metabolic flexibility, i.e. easy reversal of this reaction, is mandatory, the glycolytic orientation of the kinetics of the intermolt muscle phosphoglycerate kinase has been overcome in two ways. First there is a strong increase in ATP affinity, and second a marked decrease in sensitivity to ADP and MgADP inhibition.

In most studies of glycolytic control mechanisms, phosphoglycerate kinase has been considered to catalyze a near equilibrium, non-rate limiting reaction. The mass action ratios show that in Cancer magister muscle, the gluconeogenic reaction accounts for between 3 and 8% of the glycolytic reaction, if one accepts an equilibrium constant between 1000 and 1500 (Rose & Warms, 1970). Thus, the reaction lies on the borderline between equilibrium and non-equilibrium reactions defined by Rolleston (1972) as  $1/K_{eq} < 0.05$ . Given no other changes, increased levels of ADP and 1,3 diphosphoglycerate shift the reaction away from equilibrium and favor the glycolytic reaction by a mass action effect. Increased levels of ADP, as would occur during the onset of muscle contraction, would further curtail reversal by a kinetic effect. The levels of 1,3 diphosphoglycerate found in crustacean tissues are in contrast to the low levels in rat heart (Williamson, 1965) and brain (Lowry *et al.*, 1964; Rolleston & Newsholme, 1967). However, Rose and Warms (1970), in a study of erythrocyte glycolysis, have shown that under

certain conditions 1,3 diphosphoglycerate levels reach  $110 \mu\text{M}$ , leading to a substantial drop in the mass action ratio. Yeast and ascites tumor cells have been shown to have  $5\text{--}20 \mu\text{M}$  1,3 diphosphoglycerate (Hess, 1968). Recently, Edington and coworkers (1972) have reported 1,3 diphosphoglycerate levels up to  $1 \text{ mM}$  in rat muscle. In the present study, all samples had measurable 1,3 diphosphoglycerate concentrations. All individuals show higher mass action ratios in the hypodermis than in the muscle, indicating that the hypodermal system lies closer to equilibrium. However, since the mass action ratios were subject to considerable variation between animals, these differences may not be completely meaningful. Furthermore, the precise position of the equilibrium under physiological conditions is unclear, since Rose and Warms (1970) and Leadbetter and coworkers (1976) have shown that the  $K_{eq}$  varies with the  $\text{Mg}^{++}$  level present in the system. In any case, in both muscle and hypodermis, phosphoglycerate kinase is relatively near equilibrium compared to the major rate limiting enzymes, phosphofructokinase and pyruvate kinase (Newsholme & Start, 1973). Since the enzymes involved in a possible bypass of the reaction could not be found in hypodermis (Storey, 1972) and since the hypodermal reaction system lies relatively close to equilibrium, the logical conclusion is that phosphoglycerate kinase is reversed during gluconeogenesis. While some phosphoglycerate kinase reversal may occur in the intermolt muscle, the kinetic modifications found in the enzyme present in the intermolt and freshmolt hypodermis as well as those found for the freshmolt muscle enzyme would considerably facilitate reversal of the reaction.

The kinetics of the molting muscle enzyme are rather intriguing in view of the potential for gluconeogenesis in freshmolt muscle. Fructose diphosphatase is present in muscle of freshmolt Cancer magister in 25% of the levels of phosphofructokinase (Table 1). High levels of fructose diphosphatase have been found in the leg muscle of the arctic king crab and the arctic tanner crab (Behrisch, 1971, 1972; Behrisch & Johnson, 1974). Behrisch found these muscle enzymes to share the complex regulatory properties of the gill and hypodermal enzymes. Furthermore, isotopic evidence for gluconeogenesis from lactate, alanine, and pyruvate has been found in the muscle of Paralithodes camtchatica (Behrisch, 1976). The low levels of pyruvate carboxylase and phosphoenolpyruvate carboxykinase in the muscle of molting Cancer magister make gluconeogenesis at least feasible. The increased kinetic reversibility of the molting muscle's phosphoglycerate kinase is reasonable in this context. However, the mass action ratio data indicate no difference between the poise of freshmolt and intermolt muscle phosphoglycerate kinases.

The inhibition patterns of these crustacean phosphoglycerate kinases are qualitatively similar to those reported for the yeast enzyme (Larsson-Raznikiewicz & Arvidsson, 1971). Increasing magnesium increases the effectiveness of ADP inhibition, by lowering the  $K_i$  values for this nucleotide, and increasing the competitiveness of its interaction with ATP. These crustacean enzymes differ from the yeast enzyme in that increasing magnesium also increases the competitiveness of the

interaction between ADP and 3-phosphoglycerate . In all tissue forms, AMP is competitive only with ATP, again as in the yeast enzyme. The inhibition is probably of little physiological significance, as the  $K_i$  values are in the millimolar range.

A comparison of these crustacean phosphoglycerate kinases shows that the forms which have similar AMP affinities also share the  $K_i$  values for MgADP inhibition versus ATP. The two hypodermal and the molting muscle enzymes show much the same MgADP versus ATP  $K_i$  (see Table 5), and their AMP affinities all lie near 2.3 mM. The intermolt muscle phosphoglycerate kinase shows markedly different values for both of these constants: the AMP  $K_i$  is 7.6 mM, a three fold increase over the other forms, and the MgADP  $K_i$  is  $5 \times 10^{-5}$  M, a four fold decrease. These variations in inhibitor affinities are independent of the ATP affinities, since the two hypodermal forms show significantly different ATP affinities, but the same MgADP versus ATP  $K_i$  values. ADP must bind to a site which overlaps with the 3-phosphoglycerate and the ATP sites. The affinity for adenylate inhibitors depends upon variations in this site. Increasing magnesium concentrations increase the interaction of ADP with both the 3-phosphoglycerate and the ATP sites. These data support the notion of two nucleotide binding sites for these phosphoglycerate kinases. The bulk of the literature of phosphoglycerate kinase supports this binding pattern, on the basis of initial velocity studies (Larsson-Raznikiewicz, 1964, 1967), of essential sulfhydryl groups (Krietsch & Bucher, 1970), and on the basis of product inhibition studies (Larsson-Raznikiewicz & Arvidsson, 1971). However, recent spectroscopic evidence from

the yeast enzyme (Roustan *et al.*, 1973) indicates only one nucleotide binding site on the phosphoglycerate kinase active site.

In contrast with the findings of Krietsch and Bücher (1970) in their study of the rabbit muscle enzyme, but in agreement with Bojanovski *et al.* (1974) and Ponce *et al.* (1971), 2,3-diphosphoglycerate is a competitive inhibitor of these phosphoglycerate kinases. The inhibition is more potent versus 3-phosphoglycerate than ATP. Its interaction with both sites indicates that it may behave like 1,3-diphosphoglycerate, the actual glycolytic substrate. In this sense, the crustacean enzymes must be more flexible or have a rather different shape at the active site than the mammalian muscle enzyme.

These crustacean enzymes show a marked stimulation by NaCl and KCl, and some inhibition by calcium. Calcium inhibition was also found for the erythrocyte enzyme (Okonkwo *et al.*, 1973). The NaCl and KCl effects are probably due to changes in the solvent structure surrounding the enzyme, rather than to their direct participation in the catalysis. The  $K_m$  values for the KCl activation lie within the intracellular ion concentrations. Even though crustacean tissues have higher intracellular sodium than do mammalian tissues (Prosser, 1973), the intermolt enzymes'  $K_m$  values for NaCl activation are well above this range. The considerably lower NaCl  $K_m$  shown by the molting enzymes may simply result in additional stimulation of the reaction, or it may signify a different catalytic mechanism. Larsson-Raznikiewicz and Arvidsson (1971) include 0.25 M NaCl in

all their assays, perhaps for its activating effect.

Unfortunately, due to problems in stabilizing and further purifying these crustacean phosphoglycerate kinases, I was not able to work out the exact molecular changes which accompany the kinetic differences between the tissues. The results do not facilitate the choice between the various possible alternatives: four gene products, two proteins in varying ratios, or one gene product subjected to varying degrees of modification in the cell. However, they do establish that there are at least two kinetically and electrophoretically distinct forms in these crustaceans. This stands in agreement with work done on the bovine liver enzyme showing it to be distinct from the bovine muscle enzyme, on electrophoretic and molecular grounds as well as in overall orientation of kinetics (Bojanovski *et al.*, 1974). Comparisons of mammalian muscle, erythrocyte, and yeast enzymes show distinct kinetic and immunological differences (Okonkwo *et al.*, 1973). These results all contrast with the observation of a lack of kinetic, electrophoretic, or immunological differences between phosphoglycerate kinases of rat liver, muscle, and heart (Fritz & White, 1974). In Cancer magister, there are tissue and molt cycle related differences in the amounts and the kinetic properties of the phosphoglycerate kinases present. Within this enzyme system, two basic alterations of the reaction kinetics suffice to transform the glycolytically poised enzyme into another gluconeogenically adapted form. An increase in the ATP affinity with a concomitant decrease in the sensitivity to ADP and MgADP product inhibition make the reversal of the reaction more feasible. The other affinity differences between the forms

probably arise as a consequence of these major changes. Similar alterations of ADP and ATP affinities will probably be found in phosphoglycerate kinases in other gluconeogenic systems.

## Part III

## Muscle Pyruvate Kinase

## INTRODUCTION

Crustacean muscle undergoes considerable changes with the molt cycle. It is a highly glycolytic tissue during the intermolt portion of the cycle (Hochachka et al., 1971), and undoubtedly maintains a high capacity for glycolytic generation of ATP throughout the premolt and postmolt periods. With the onset of premolt, the muscle mass decreases markedly, dropping to 60% of the intermolt mass (Skinner, 1966). In Cancer magister, the levels of glycolytic enzymes drop, and the levels of gluconeogenic enzymes rise (Table 1). Thus, the glycolytic potential of the tissue is considerably reduced, leading to the possibility of gluconeogenesis in the muscle of molting Cancer magister. Phosphoglycerate kinase from freshmolt muscle shows kinetic modifications which favor gluconeogenesis, in contrast to the enzyme from intermolt muscle. This lends further credence to the possibility of gluconeogenesis in freshmolt muscle. The fructose diphosphatase present in Paralithodes camtchatica muscle has complex control characteristics which are suited for function in a gluconeogenic system as well as in a highly glycolytic system (Behrisch, 1972; Behrisch & Johnson, 1974). Phosphofructokinase from Paralithodes camtchatica muscle is inhibited by high levels of ATP and activated by low levels of AMP (Freed, 1971). These characteristics would allow the

inhibition of the enzyme during periods of gluconeogenesis, and then strong activation of the enzyme for glycolysis. Thus, the enzymatic machinery which has been examined in crustacean muscle has control characteristics which are compatible with some gluconeogenesis in freshmolt muscle.

The control of pyruvate kinase (E.C. 2.7.1.40) is important under various metabolic conditions. During glycolysis, it is a major rate limiting and thus regulatory site (Beis & Newsholme, 1975; Williamson, 1965). During gluconeogenesis, when the pyruvate kinase reaction is reversed by an enzymatic bypass (Scrutton & Utter, 1968), its downhill function must be efficiently curtailed. In mammals, these rather different control requirements are met by three distinct pyruvate kinase forms. The muscle enzyme (M type) is unaffected by FDP, shows a high affinity for its substrates, and a low sensitivity to ATP inhibition (Kayne, 1973; Tanaka *et al.*, 1967). The muscle enzyme is also sensitive to creatine phosphate inhibition (Kemp, 1973). The liver enzyme (L type) shows a strong sensitivity to glucose 1,6 diphosphate and FDP activation, to inhibition by ATP, alanine, citrate, and other metabolites, and low affinity for its substrate phosphoenolpyruvate (PEP). In contrast to the muscle enzyme, the liver enzyme shows sigmoidal kinetics (Van Berkel *et al.*, 1974; Cardenas & Dyson, 1973; Tanaka *et al.*, 1967). The third form of mammalian pyruvate kinase, present in blood cells, kidney, adipose tissue, and fetal tissue is distinguished as the K or M2 type. This form displays intermediate kinetics, combining FDP activation and sensitivity to inhibitors with hyperbolic

kinetics (Cardenas et al., 1975; Van Berkel et al., 1973).

The pyruvate kinases in tissues of lower vertebrates and invertebrates face much the same glycolytic and gluconeogenic control requirements as those in mammalian tissues, but the system of enzymes evolved to deal with these needs has not been elucidated. Most of the available information concerns muscle enzymes, many of which show allosteric regulatory properties. Generally, these enzymes are regulated by an interplay between inhibition by ATP, citrate, alanine, and various other metabolites, and activation by FDP. This pattern holds true for the enzyme from Rana pipiens heart, Coryphenoides muscle, sturgeon muscle, turtle heart, oyster adductor, Mytilus edulis adductor, rainbow trout, Trematomus, and carp muscle. (Flanders, 1971; Mustafa et al., 1971; Randall & Anderson, 1975; Storey & Hochachka, 1974; Mustafa & Hochachka, 1971; de Zwaan & Holwerda, 1972; Somero & Hochachka, 1968; Johnston, 1975). Exceptions to the above are the muscle enzymes from Rana pipiens, desert locust, Schistocerca gregaria, octopus, shrimp, and squid which do not show FDP activation (Flanders et al., 1971; Bailey & Walker, 1969; McManus, 1975; Guderley et al., 1976a; Guderley et al., 1976b; Storey & Hochachka, 1975).

This study of Cancer magister muscle pyruvate kinase had two major goals; 1) to work out the regulation of the enzyme in intermolt muscle, and 2) to determine whether the enzyme and/or the pattern of regulation changes as the muscle changes in preparation for molt. This information would elucidate both the means by which pyruvate kinase can be regulated during

glycolysis as well as the feasibility of gluconeogenic flux past this reaction locus.

## METHODS

### Preparation of Pyruvate Kinase

Muscle was quickly dissected out of anaesthetized crabs, freed of any adhering tissue, blotted dry, and weighed. The tissue was then homogenized in four volumes of 50 mM imidazole-HCl, pH 7.0. The homogenate was centrifuged at 27,000 g for twenty minutes. Solid ammonium sulphate was added with constant stirring to bring the supernatant to 45% saturation. This mixture was centrifuged at 12,000 g for 10 minutes, then solid ammonium sulphate was gradually added, with constant stirring, to bring the supernatant to 60% saturation. The mixture was centrifuged as above, the supernatant discarded, and the pellet redissolved in a small volume of 50 mM imidazole-HCl, 3 mM, pH 7.0. In studies where the properties of the unpurified enzyme were examined, approximately 4 ml of this preparation were dialyzed against two changes of 2 liters each of 50 mM imidazole-HCl, 3 mM EDTA, pH 7.0, for a total period of 3 hours.

When further purification was desired, it was achieved via column chromatography on cellulose phosphate or on DEAE Sephadex. Cellulose phosphate was pretreated according to manufacturer's instructions, and then equilibrated in 5 mM phosphate, 1 mM mercaptoethanol, pH 7.5. The DEAE Sephadex was allowed to swell in 50 mM imidazole-HCl, 3 mM EDTA, 4 mM MgCl<sub>2</sub>,

pH 7.5, and then equilibrated in the same buffer. Depending on the amount of tissue utilized, either 2.5 x 15 cm or 2.5 x 30 cm columns were used. The enzyme extract was prepared for column chromatography by dialysis against 2 changes of 2 liters each of the appropriate column buffer. After approximately three hours of dialysis, the enzyme preparation was applied to the column, followed with 50 ml of the column buffer. Then a gradient (0-300 mM KCl in the column buffer) was applied to the column. For the small column, 300 ml of gradient were used, for the larger column, 500 ml were used. The cellulose phosphate columns were eluted at 35 ml/hour, while the DEAE Sephadex columns were eluted at 20 ml/hour. The 1.5 ml fractions were checked for enzyme activity, protein, and conductivity. When either of these columns was used, a purification of 800 to 1000-fold was attained, leading to a specific activity of 100 units/mg protein. The peak fractions were concentrated by ultrafiltration in an Amicon cell.

### Enzyme Assays

Pyruvate kinase activity was measured according to the method of Bücher and Pfeleiderer (1955) which couples pyruvate formation to the decrease in optical density at 340 angstroms through the lactate dehydrogenase reaction. All standard assays included PEP, ADP, KCl, MgCl<sub>2</sub>, NADH, and excess lactate dehydrogenase in the concentrations shown in the figure legends. Kinetic studies were done at 20 °C, using imidazole buffers.

Pyruvate kinase activity in the direction of phosphoenol-

pyruvate formation was measured according to Dyson et al. (1975). Standard assays included ATP, MgCl<sub>2</sub>, KCl, pyruvate, KHC0<sub>3</sub>, NADH, excess malate dehydrogenase, and excess peanut phosphoenolpyruvate carboxylase. The activity due to the reversal of pyruvate kinase was determined by subtracting the two controls, one without pyruvate kinase and one without PEP carboxylase. These studies were done at 20 °C using imidazole-HCl buffered to pH 7.5. Considerable care was taken during measurements of the reversal of pyruvate kinase to remove all traces of lactate dehydrogenase from the cuvettes.

## RESULTS

### Electrophoresis and Isoelectrofocusing

Extensive electrophoretic comparison of the intermolt muscle pyruvate kinase with the enzyme from the freshmolt muscle revealed no consistent differences. Of forty gels, five showed a slightly faster migration for the freshmolt muscle enzyme and two showed a slightly faster migration for the intermolt muscle enzyme. Thus, in the majority of cases, the molting and intermolt muscle pyruvate kinase showed identical migration. Several different migration systems were used to test out the various possibilities completely. Since no kinetic differences (see below) were found even with those enzymes which showed slightly different migration patterns, it is probable that these slight differences represent allelic differences which do not affect the catalytic characteristics of the muscle enzyme. The experimental animals were a random sample of the natural

population and some phenotypic variation between individuals is not surprising.

Isoelectrofocusing also failed to reveal differences between the intermolt and the freshmolt muscle enzymes. After the 45 to 60% ammonium sulphate fractionation, each enzyme was focused on a 3-10 pH gradient as well as a 5-8 pH gradient. In all cases, a pI of  $6.4 \pm 0.05$  was found. Thus, I concluded that the pyruvate kinases in intermolt and freshmolt muscle had much the same overall charge and were probably the same protein.

### Catalytic and Regulatory Properties

Kinetic analyses were carried out with ammonium sulphate fractions and purified pyruvate kinases from both freshmolt and intermolt muscle. Since the results showed close agreement between the various preparations, only one set of information will be presented. The close agreement of the kinetic results supported the conclusion that only one form of pyruvate kinase is present in the muscle throughout the molt cycle.

In common with other pyruvate kinases, the crustacean muscle enzyme required both a monovalent and a divalent cation for activity (Kayne, 1973). Both  $K^+$  and  $NH_4^+$  were good activators. The enzyme had a higher affinity for  $NH_4^+$  ( $K_a = 5$  mM) than for  $K^+$  ( $K_a = 15$  mM), but showed a two-fold higher  $V_{max}$  with  $K^+$  than with  $NH_4^+$ . Similarly, the enzyme showed a higher affinity for  $Mn^{++}$  ( $K_a = 0.15$  mM) than for  $Mg^{++}$  ( $K_a = 0.6$  mM), but showed a lower maximal velocity with  $Mn^{++}$  (50% of that with  $Mg^{++}$ ).  $Na^+$  was inhibitory in the presence of

optimal  $K^+$ , but produced a slight activation in the absence of  $K^+$  ( $K_a = 60$  mM). Calcium inhibited the reaction and showed a competitive interaction with both  $Mg^{++}$  ( $K_i = 0.1$  mM) and ADP ( $K_i = 0.7$  mM). Calcium was less effective as an inhibitor versus  $Mn^{++}$  ( $K_i = 0.66$  mM) than  $Mg^{++}$ . This decrease in sensitivity was not as pronounced as that shown by pyruvate kinase from Mytilus edulis adductor (de Zwaan et al., 1975). The pH optimum for Cancer magister muscle pyruvate kinase was pH 7.0.

### Substrate Affinities

Substrate saturation curves for both PEP and ADP followed Michaelis-Menten behavior. The affinity for PEP was high, with  $K_m$  values ranging from  $7 \times 10^{-5}$  M to  $1.1 \times 10^{-4}$  M. The only difference between molting and intermolt pyruvate kinases lay in this parameter, the molting muscle enzyme usually showed a higher affinity for PEP ( $K_m$  values between  $8 \times 10^{-5}$  M and  $1.0 \times 10^{-4}$  M) than the intermolt muscle enzyme ( $K_m$  values between  $9.0 \times 10^{-5}$  M and  $1.1 \times 10^{-4}$  M). However, there was considerable overlay in these  $K_m$  values. The ADP affinities showed much less variation; they ranged from 0.23 mM to 0.29 mM, for both the intermolt and the freshmolt enzyme. Substrate affinities were measured in the presence of 4 mM  $MgCl_2$  and 75 mM KCl in 50 mM imidazole-cl, pH 7.0. The affinity for each substrate was independent of variations in the levels of cosubstrate, indicating a random binding mechanism. UDP, IDP, and GDP all served as nucleotide substrates, but with substantially reduced effectiveness compared to ADP (Table 8).

TABLE 8

## Nucleotide diphosphate acceptability.

<u>Nucleotide diphosphate</u>	<u>K<sub>m</sub></u>	<u>V<sub>max</sub></u>
ADP	0.23 mM	100%
GDP	0.8 mM	63%
IDP	1.0 mM	50%
UDP	1.5 mM	55%

### Activators

In common with many other invertebrate muscle pyruvate kinases, the enzyme was activated by low levels of fructose 1,6 diphosphate ( $K_a = 2 \times 10^{-5}$  M). The major effect of FDP was to facilitate the binding of PEP, 0.05 mM FDP reduced the PEP  $K_m$  from  $8.5 \times 10^{-5}$  M to  $4.5 \times 10^{-5}$  M. FDP also reversed the inhibition caused by MgATP, probably via its effect on the enzyme's affinity for PEP (Figure 7). In freshly prepared homogenates, and in ammonium sulphate fractions, glucosamine-6-phosphate, UDP-acetylglucosamine and FDP were equally good activators. However, with the purified enzyme, only FDP remained a strong activator, while glucosamine-6-phosphate and UDPAG showed a decreased capacity to reverse ATP inhibition (see Table 9, p. 114).

### Inhibitors

As mentioned above, MgATP is an inhibitor of this crustacean muscle enzyme. MgATP showed the same effectiveness as an inhibitor of the purified and unpurified enzymes. In comparison with previously studied muscle pyruvate kinases, this muscle enzyme is quite sensitive to MgATP inhibition. The inhibition versus PEP was competitive with a  $K_i$  of 1.8 mM (Figure 8) while that versus ADP was non-competitive and showed a  $K_i$  of 5 mM (Figure 9). The enzyme showed much lower sensitivity to the other nucleotide triphosphates (MgUTP, MgITP, and MgGTP) than to MgATP. Arginine phosphate plays an important role as the storage phosphagen in crustacean muscles (Beis &

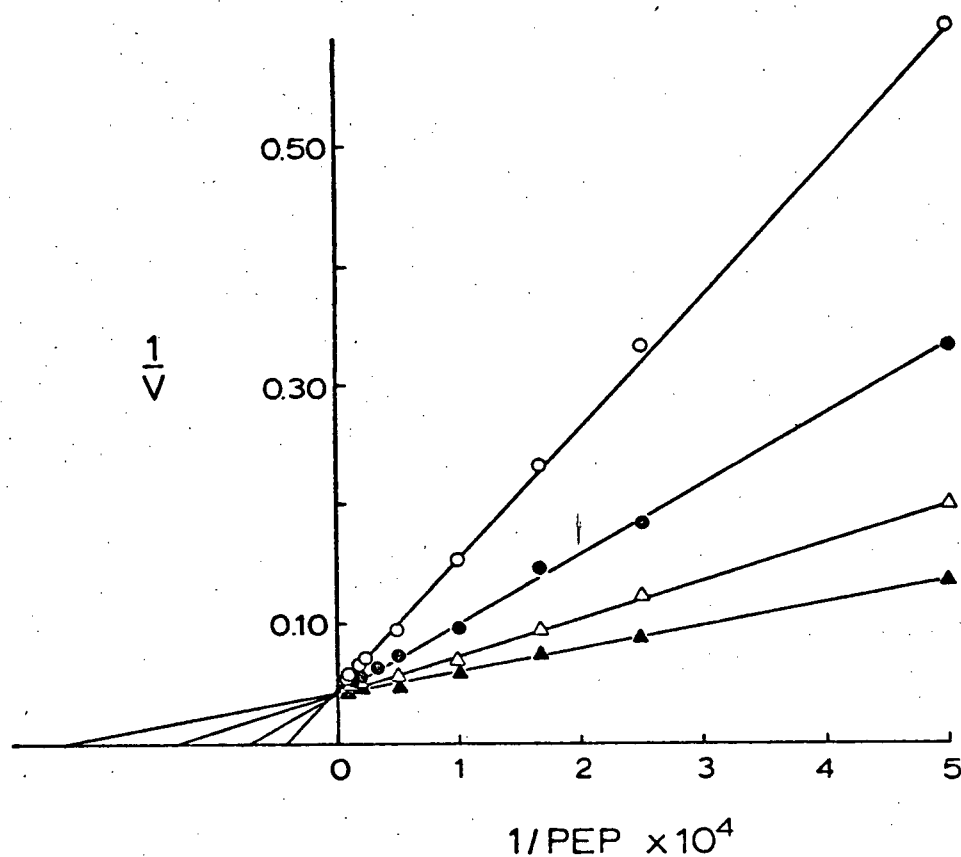


Fig. 7. Effect of MgATP and FDP on PEP affinity of *C. magister* muscle pyruvate kinase. All experiments were performed in 50 mM imidazole-Cl, pH 7.0 with 75 mM KCl, 5 mM MgCl<sub>2</sub>, 1.2 mM ADP, 0.1 mM NADH, and excess lactate dehydrogenase. (O) in the presence of 2 mM MgATP; (●) in the presence of 2 mM MgATP and 0.5 mM FDP; (Δ) control; (▲) in the presence of 0.5 mM FDP.

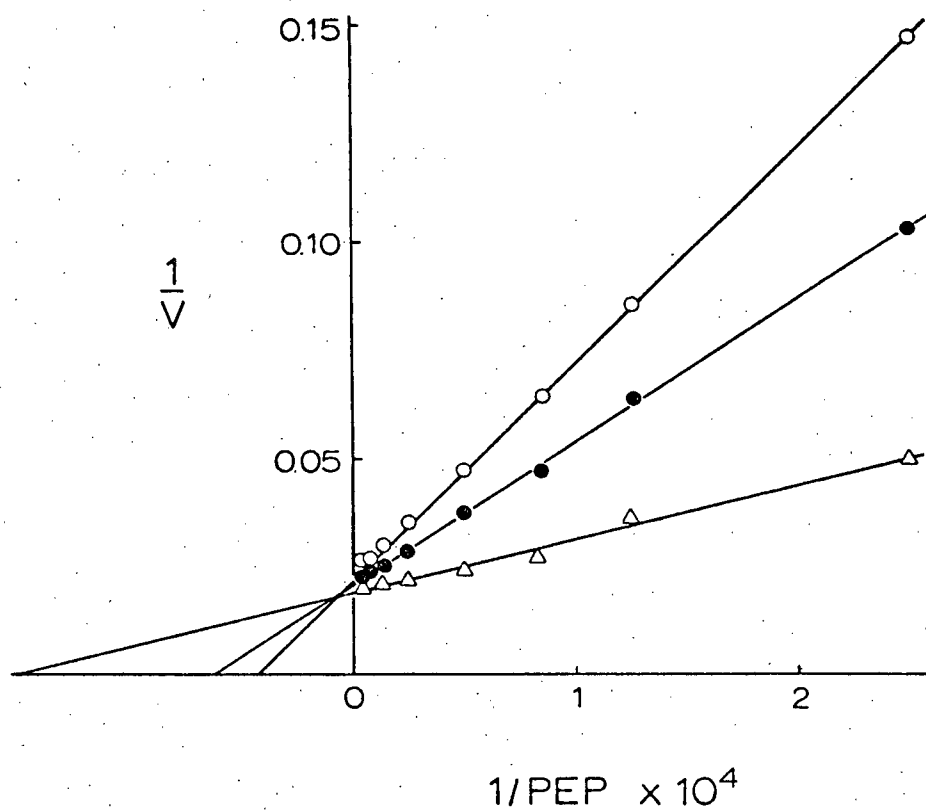


Fig. 8. MgATP inhibition, effect on PEP affinity. Experimental conditions were as given in the legend for Figure 7. Treatments are as follows: (O) 5 mM MgATP; (●) 2.5 mM MgATP; ( $\Delta$ ) control.

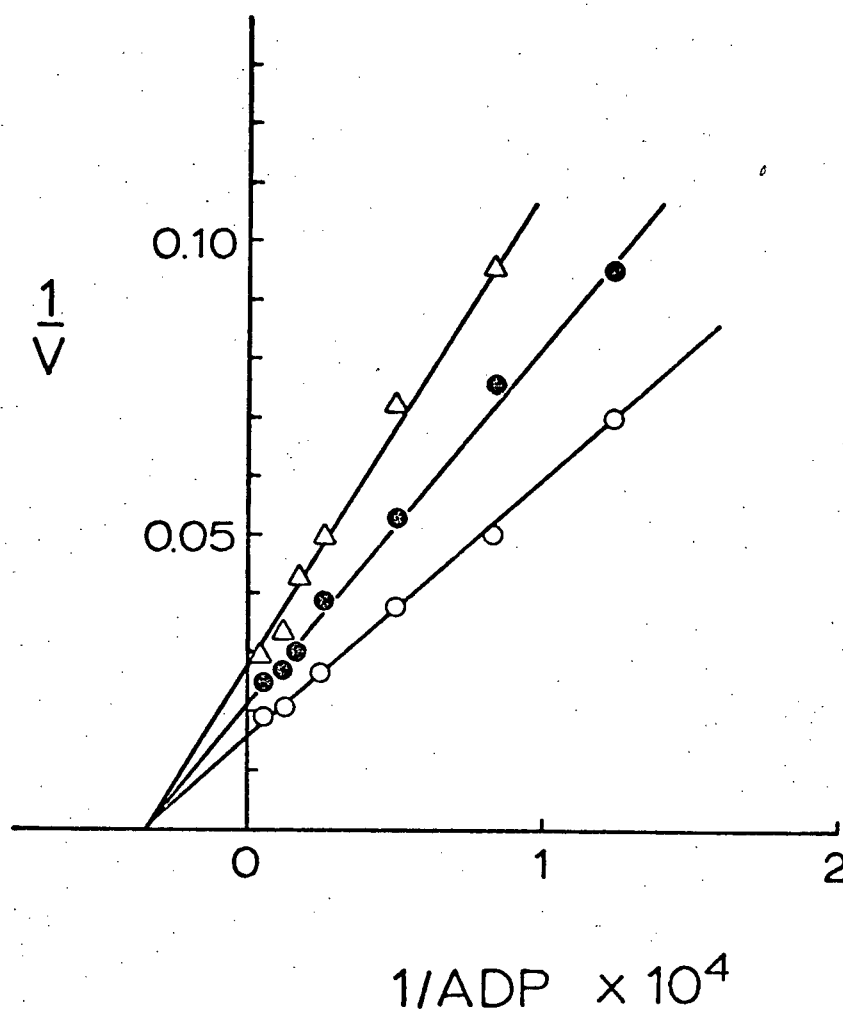


Fig. 9. MgATP inhibition, effect on ADP affinity. Experimental conditions are as in Figure 7 except that PEP was held constant at 2 mM and ADP was varied. ( $\Delta$ ) 5 mM MgATP; ( $\bullet$ ) 2.5 mM MgATP; (O) control.

Newsholme, 1975), so I checked for its effect on the pyruvate kinase reaction. Physiological levels of arginine phosphate substantially reduced enzyme activity. Arginine phosphate was competitive with the enzyme for ADP ( $K_i = 2.8$  mM) (Figure 10), as well as partially hindering the binding of PEP ( $K_i = 11$  mM) (Figure 11). While FDP reversed the inhibition due to ATP, it did not reverse that due to arginine phosphate. Arginine phosphate inhibition was not due to ATP formation by arginine phosphokinase.

A variety of metabolites, including amino acids, glycolytic and Krebs cycle intermediates have been implicated in the control of pyruvate kinase from various tissues. In order to see which, if any, intracellular compounds could further modulate the activity of this muscle enzyme, the following compounds were tested at the levels shown in brackets: taurine (1-5 mM), arginine (1-5 mM), alanine (2, 5, 10, and 20 mM), phenylalanine (1, 3, and 20 mM), glutamate (5 mM), proline (5 mM), valine (5 mM), asparagine (5 mM), aspartate (5 mM), leucine (10 mM), histidine (10 mM), lysine (10 mM), cysteine (10 mM), glycine (10 mM), glutamine (10 mM), methionine (10 mM), isoleucine (10 mM), threonine (10 mM), serine (10 mM and 20 mM), 3-PGA (1 mM), AMP (1-4 mM), tryptophan (1-5 mM), acetyl CoA ( $1 \times 10^{-4}$  M), fructose-6-phosphate (0.5 mM), glucose-6-phosphate (1 mM), NADH (0.05-0.2 mM), cyclic AMP (0.05 mM), Mg<sup>2+</sup>citrate (1-5 mM), Mg-malate (1-5 mM),  $\alpha$ -ketoglutarate (1-5 mM),  $\alpha$ -glycerolphosphate (1-5 mM), oxaloacetate (0.01-0.6 mM), 2,3 diphosphoglycerate (1 mM), and CoA (0.1-0.8 mM). These tests were run both at saturating and  $K_m$  levels of substrates, and

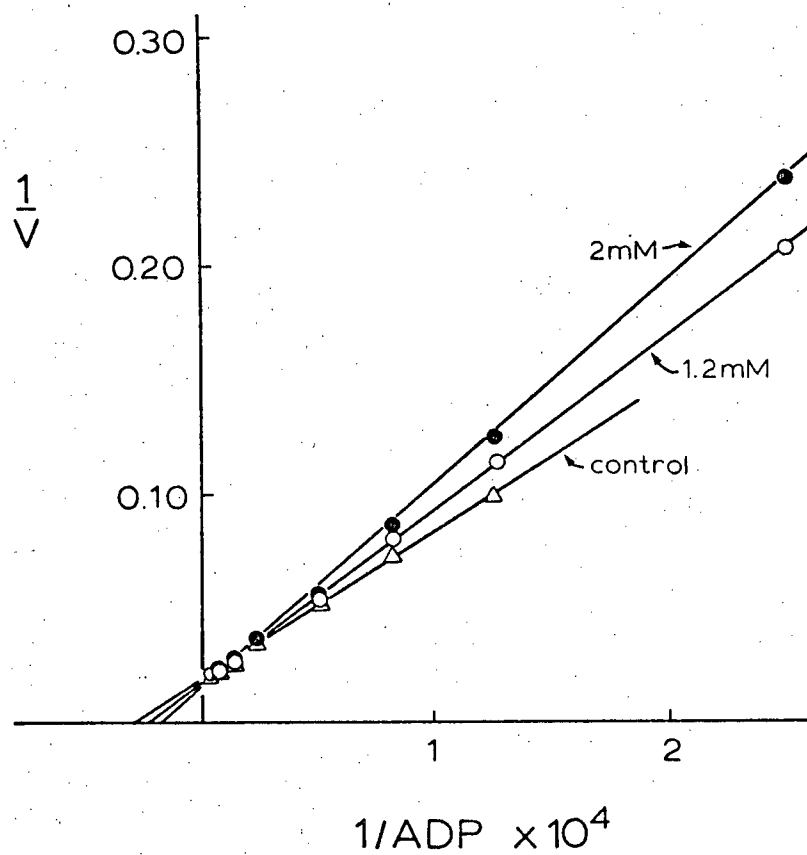


Fig. 10. Arginine phosphate inhibition, effect on ADP affinity. Experimental conditions are as in Figure 9. (●) 2 mM arginine phosphate; (○) 1.2 mM arginine phosphate; and (Δ) control.

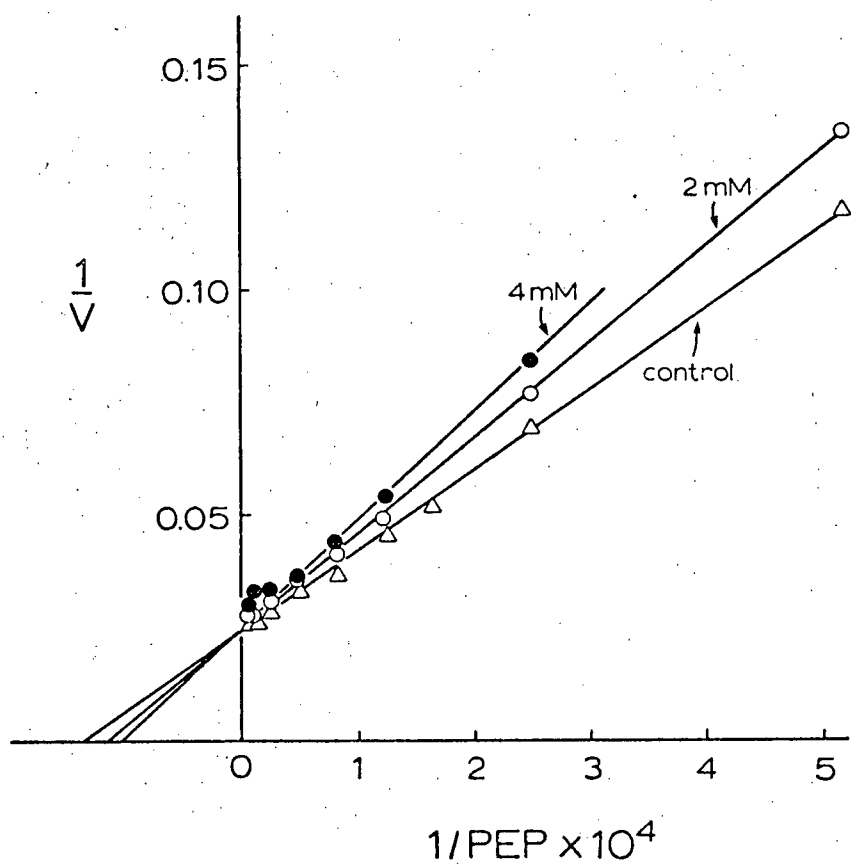


Fig. 11. Arginine phosphate inhibition, effect on PEP affinity. The experimental conditions are those given in Figure 7. ( $\bullet$ ) 4 mM arginine phosphate; ( $\circ$ ) 2 mM arginine phosphate; and ( $\Delta$ ) control.

were carried out with purified enzymes. Alanine, valine, serine, tryptophan, Mg<sub>2</sub>citrate, Mg-malate,  $\alpha$ -ketoglutarate, and  $\alpha$ -glycerolphosphate affected enzyme activity, although none of these was as potent as MgATP or arginine phosphate. Alanine and serine both showed mixed competitive inhibition versus PEP, with  $K_i$  values around 30 mM. Valine demonstrated only a weak, non-competitive inhibition. Tryptophan was competitive with ADP (Figure 12) and showed a  $K_i$  of 1.6 mM.  $\alpha$ -glycerolphosphate showed competitive inhibition with both ADP and PEP, however, the  $K_i$  values were well outside the physiological range for this compound ( $K_i$  versus PEP = 20 mM;  $K_i$  versus ADP = 40 mM).

The inhibition due to the tricarboxylic acid cycle intermediates was more complex than that due to any of the above inhibitors. Malate, tested as Mg-malate to avoid chelation effects, showed competitive inhibition versus both substrates ( $K_i$  versus PEP = 5 mM;  $K_i$  versus ADP = 17 mM). Kinetic plots for malate inhibition were linear, except in the region of high malate and low substrate concentrations (Figure 13). Inhibition by  $\alpha$ -ketoglutarate showed similar kinetics but its effectiveness was lower ( $K_i$  versus PEP of 8 mM;  $K_i$  versus ADP of 15 mM). For neither malate nor  $\alpha$ -ketoglutarate was there any indication in the kinetics that the inhibitor was complexing with the substrates. With citrate as an inhibitor, the deviations from linearity became more pronounced. Both Mg-citrate and Mg<sub>2</sub>citrate were tested as inhibitors. The kinetics of Mg-citrate inhibition indicated that the inhibitor was complexing with both ADP and PEP. In this case, the inhibition showed both curved Lineweaver-Burk plots and curved

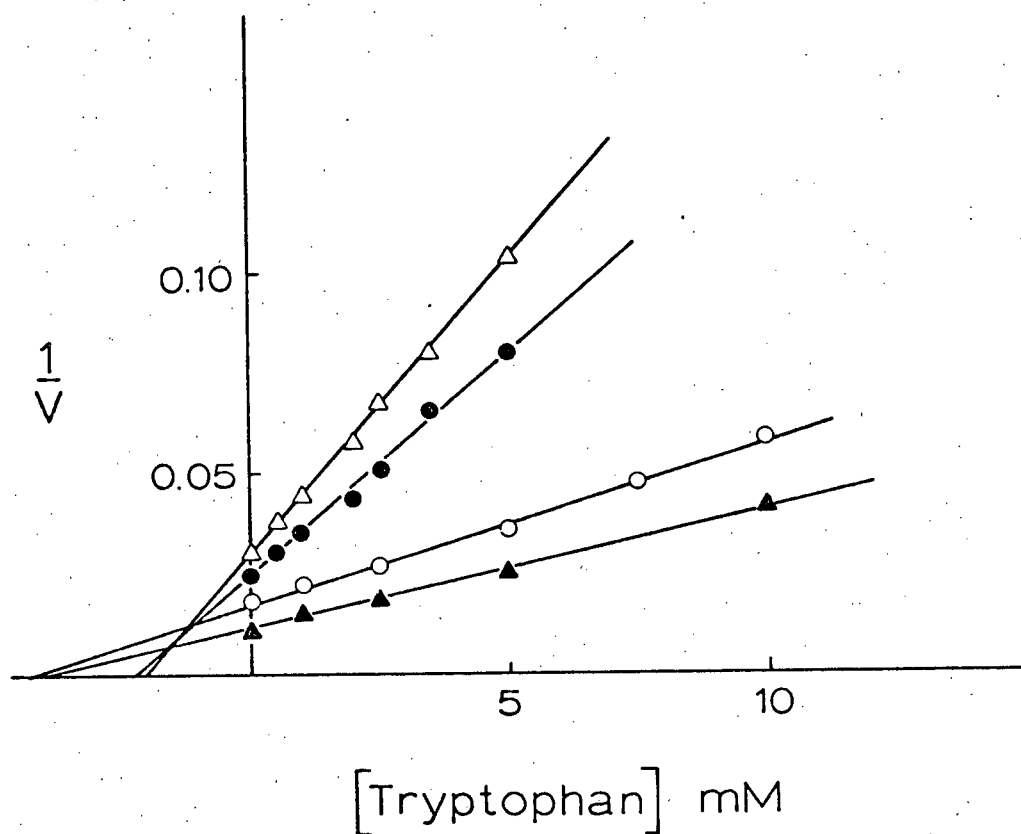


Fig. 12. Dixon plot of tryptophan inhibition. All experiments were performed at 20°C in 50 mM imidazole-Cl, pH 7.0, 75 mM KCl, 0.12 mM NADH, 4 mM MgCl<sub>2</sub>, and excess lactate dehydrogenase. ( $\Delta$ ) 0.12 mM ADP, 1.0 mM PEP; ( $\bullet$ ) 0.2 mM ADP, 1.0 mM PEP; ( $\circ$ ) 1.2 mM ADP, 0.1 mM PEP; and ( $\blacktriangle$ ) 1.2 mM ADP and 1.0 mM PEP.

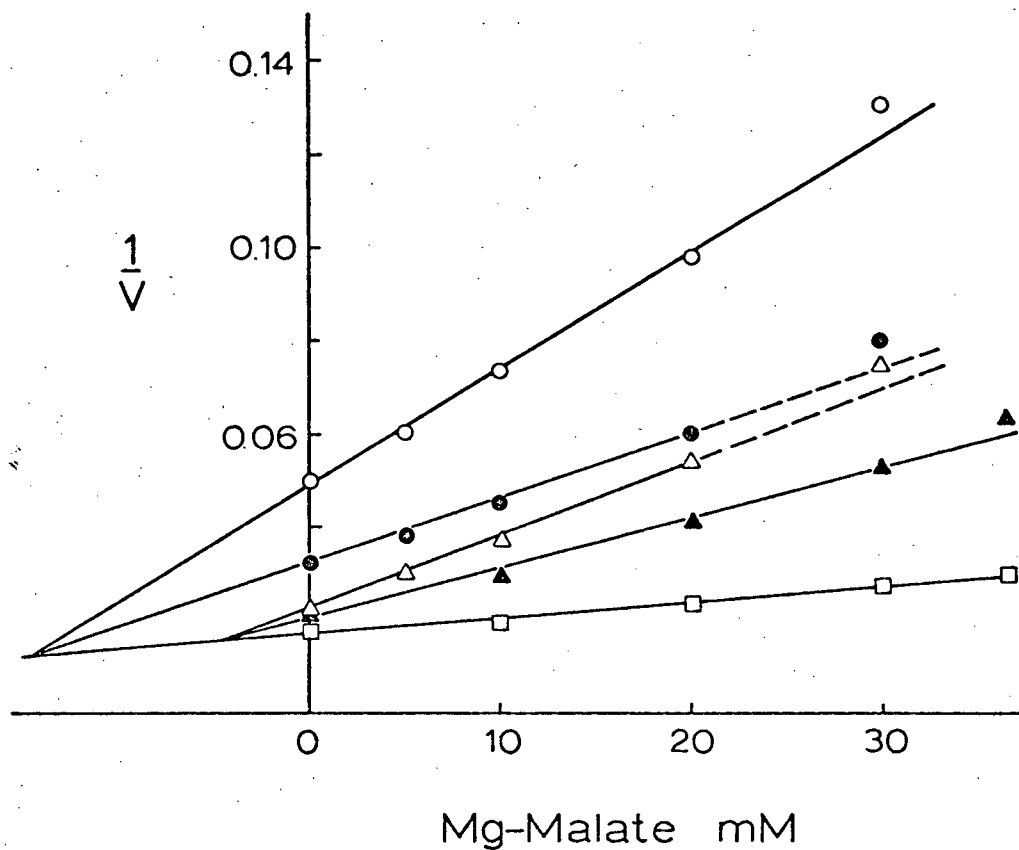


Fig. 13. Dixon plot of Mg-malate inhibition. Experimental conditions are given in the legend to Figure 12, (O) 2 mM PEP and 0.12 mM ADP; (●) 2 mM PEP and 0.2 mM ADP; (Δ) 0.12 mM PEP and 2 mM ADP; (▲) 0.2 mM PEP and 2 mM ADP; and (□) 2 mM PEP and 2 mM ADP.

Dixon plots. Mg<sub>2</sub>citrate kinetics did not produce curved Lineweaver-Burk plots, but Dixon plots of Mg<sub>2</sub>citrate inhibition versus PEP still showed curved lines (Figure 14). According to Webb (1963), this is an indication that more than one molecule of citrate is affecting PEP binding. Inhibition versus ADP indicated simple linear kinetics with a  $K_i$  of 27 mM. A gauge of Mg<sub>2</sub>citrate's effectiveness as an inhibitor lies in these figures: 5 mM Mg<sub>2</sub>citrate causes 23% inhibition, 20 mM Mg<sub>2</sub>citrate causes 50% inhibition.

Table 9 allows a comparison of the effectiveness of these various inhibitors, added singly or in concert, on the activity of the purified enzyme at  $K_m$  levels of both substrates. Since, in this case, the  $K_m$  values are reasonable approximations of physiological levels of both PEP and ADP, this allows evaluation of the activity of the enzyme *in vivo*. From this table it can be seen that the inhibition due to ATP was additive with that caused by arginine phosphate. Furthermore, the inhibition due to the Krebs cycle intermediates was additive with that due to MgATP and arginine phosphate. However, the effective levels of citrate, malate, and  $\alpha$ -ketoglutarate were well above those to be expected in the cytoplasm.

Recent work with rabbit muscle pyruvate kinase indicates that the reversal of the reaction is feasible in the tissue (Dyson *et al.*, 1975; Giles *et al.*, 1975), and that this reversal may account for previously measured rates of glycogen synthesis from lactate in frog and rabbit muscle (Bendall & Taylor, 1970). There is some evidence that crustacean muscle has an increased

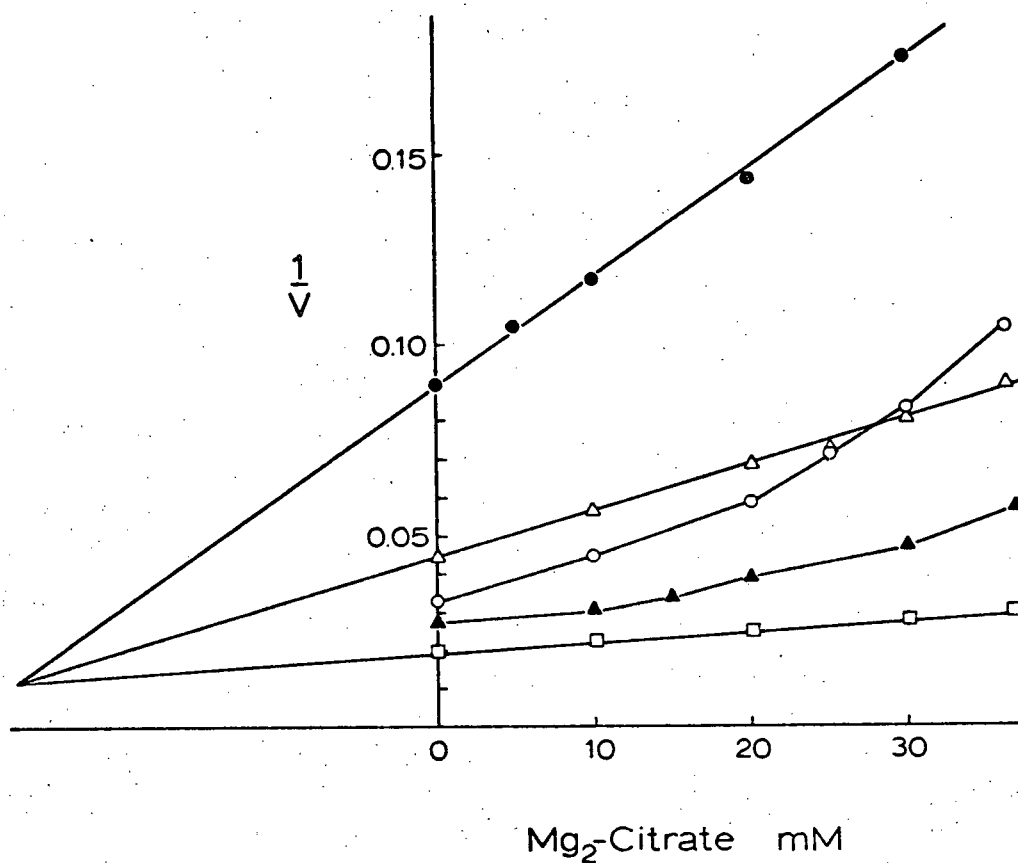


Fig. 14. Dixon plot of  $Mg_2$ citrate inhibition. Experimental conditions are given in the legend to Figure 12. (●) 2 mM PEP and 0.12 mM ADP; (△) 2 mM PEP and 0.2 mM ADP; (○) 0.12 mM PEP and 2 mM ADP; (▲) 0.2 mM PEP and 2 mM ADP; (□) 2 mM PEP and 2 mM ADP.

TABLE 9

## Effectiveness of activators and inhibitors.

All experiments except the last two were done with  $2 \times 10^{-4}$  M PEP,  $2 \times 10^{-4}$  M ADP, 75 mM KCl,  $1 \times 10^{-4}$  M NADH, 4 mM  $\text{MgCl}_2$ , with excess lactate dehydrogenase (Sigma) in 50 mM imidazole-Cl, pH 7.0. The last two were the same except for a PEP concentration of  $4 \times 10^{-5}$  M and an ADP concentration of 2 mM. Activity was measured as  $\Delta\text{OD}_{340}/\text{min}$ . All values are the mean of three determinations.

<u>Treatment</u>	<u>% Control</u>
Control	100
5 mM MgATP	47
5 mM MgATP + 0.05 mM FDP	58
5 mM MgUTP	94
5 mM arginine phosphate	72
5 mM arginine phosphate + 0.05 mM FDP	72
10 mM arginine phosphate	57
5 mM $\text{Mg}_2$ citrate	78
5 mM $\text{Mg}_2$ citrate + 0.05 mM FDP	78
5 mM $\alpha$ -ketoglutarate	80
5 mM Mg-malate	80
1 mM tryptophan	73
5 mM $\alpha$ -glycerol-phosphate	88
5 mM MgATP + 5 mM arginine phosphate	27
5 mM MgATP + 5 mM $\text{Mg}_2$ citrate	35
5 mM MgATP + 5 mM ketoglutarate	33
5 mM MgATP + 5 mM ketoglutarate + 5 mM $\text{Mg}_2$ citrate	27
5 mM MgATP + 5 mM ketoglutarate + 5 mM $\text{Mg}_2$ citrate + 5 mM arginine phosphate	18
$5 \times 10^{-5}$ M acetylCoA	102
$1 \times 10^{-4}$ M UDP-acetyl glucosamine	116
$5 \times 10^{-5}$ M FDP	130

gluconeogenic potential compared to other invertebrate and vertebrate muscle. However, the kinetic characteristics of this muscle pyruvate kinase would not permit appreciable flux through the low levels of the bypass enzymes. An effective reversal of the reaction might significantly augment gluconeogenic flux. Measurements of the reverse reaction (results shown in Table 10) indicate that at saturating pyruvate (10 mM) and at saturating MgATP (2 mM), the maximal velocity of the reaction did not rise above 0.5% of the maximal velocity of the forward reaction.

Recent work with rat liver pyruvate kinase showed that alanine and ATP, which are both allosteric inhibitors, stabilize the enzyme against thermal denaturation (Van Berkel *et al.*, 1975). Since the isoelectric point and the electrophoretic mobility of this crustacean muscle enzyme are more like those of the mammalian liver enzyme than the other mammalian forms, and since a variety of metabolites affect the activity of the enzyme, I characterized the thermal denaturation behavior of the purified enzyme (Figure 15). In contrast to the behavior of the mammalian liver enzyme, ATP accelerated the denaturation of the enzyme. This occurred when the enzyme was incubated with ATP alone, as well as with ATP and possible protectants like ADP, PEP, and FDP. Close examination of the denaturation curves indicates that in the first 15 minutes, the presence of PEP, ADP, and FDP in combination, prevented the accelerated denaturation caused by ATP. However, after 20 minutes, this combination followed the same pattern as all the other ATP containing incubations. Since alanine is only a weak inhibitor, I did not test its effect on the denaturation.

TABLE 10

## Pyruvate kinase reversal.

The velocity of the reverse reaction is expressed in  $\Delta OD_{340}/20$  minutes. The  $V_{\max(\text{forward})}$  for the identical quantity of enzyme protein used in the reversal experiments was  $8.0 \Delta OD_{340}/\text{minute}$ . All values are the mean of at least three determinations, and were the difference between the reaction rate in the presence of all substrates, pyruvate kinase, PEP carboxylase and malate dehydrogenase and the sum of the rate shown by the two controls. The controls were (1) substrates and pyruvate kinase alone as well as (2) substrates and coupling enzymes (PEP carboxylase and malate dehydrogenase). All reaction mixtures contained 75 mM KCl except where indicated.

	<u><math>V_b</math></u>	<u><math>V_b/V_{\max(\text{forward})}</math></u>
2 mM pyruvate 2 mM MgATP	0.6	0.00375
4 mM MgATP 2 mM pyruvate	0.5	0.0031
2 mM MgATP 10 mM pyruvate	0.8	0.005
4 mM MgATP 10 mM pyruvate	0.6	0.00375
2 mM MgATP 10 mM pyruvate - KCl	0.02	0.000125
2 mM ATP 10 mM pyruvate - Mg	0.03	0.0001875

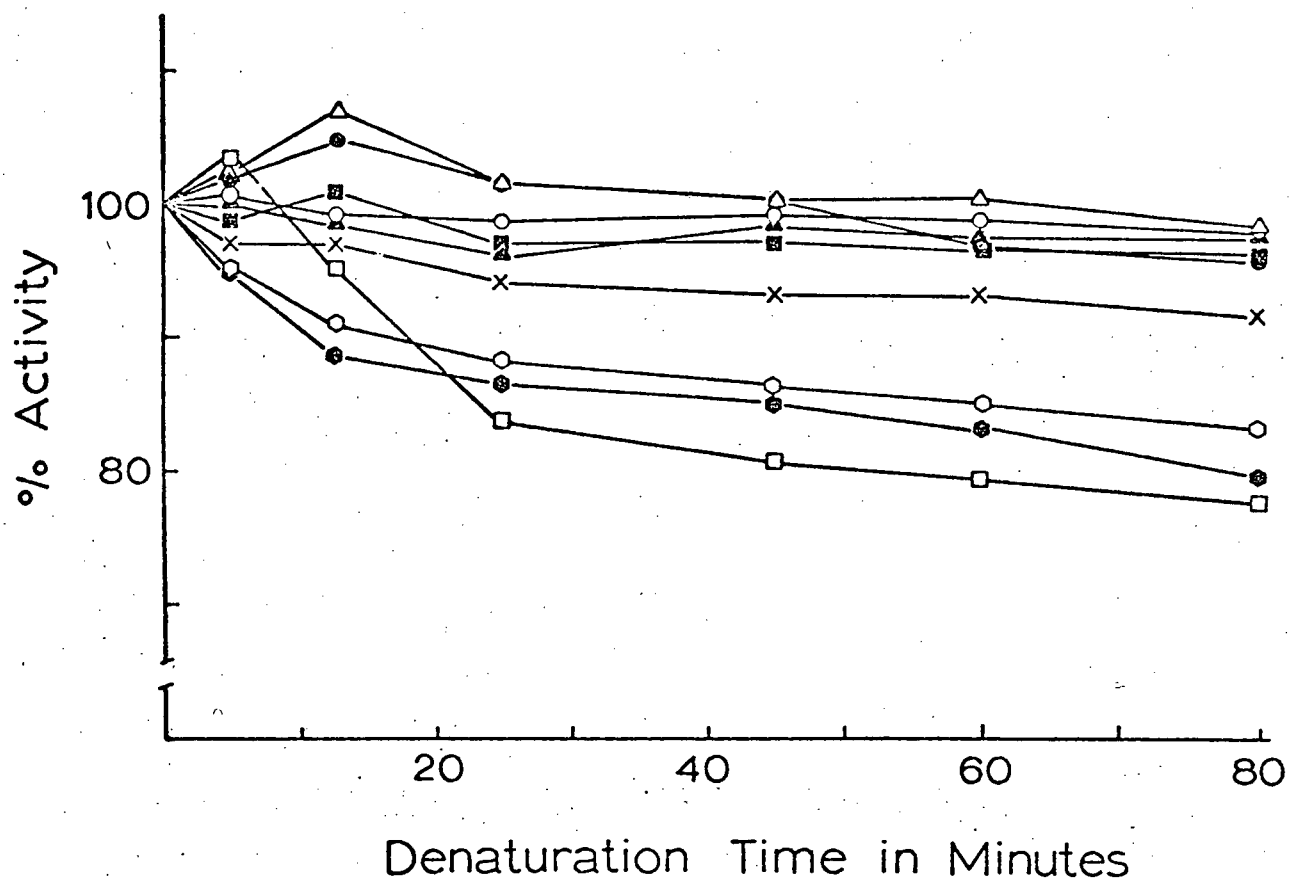


Figure 15 Thermal denaturation of C. magister muscle pyruvate kinase.

Experiments were run at 40°C under the indicated incubation conditions. Enzyme activity was measured under saturating conditions of substrates and cofactors. (●) control; (○) 4 mM MgCl<sub>2</sub> and 4 mM ATP; (Δ) 0.1 mM FDP; (◐) 0.1 mM FDP, 4 mM ATP, and 4 mM MgCl<sub>2</sub>; (■) 2 mM PEP; (○) 2 mM ADP and 2 mM PEP; (▲) 2 mM ADP, 2 mM PEP, and 0.1 mM FDP; (□) 2 mM ADP, 2 mM PEP, 0.1 mM FDP, 4 mM MgCl<sub>2</sub>, and 4 mM ATP; (X) 75 mM KCl.

## DISCUSSION

Although crustacean muscle goes through rather marked metabolic changes as the animal goes through the molt cycle, these changes are not reflected by changes in the isozyme of pyruvate kinase present in the tissue. The pyruvate kinase which is found in the muscle of Cancer magister has complex regulatory properties which would allow careful modulation of its activity under a variety of metabolic conditions. Although it is subject to allosteric inhibition, it does not show cooperativity of substrate binding. Its high affinity for substrates, high sensitivity to inhibition, as well as its activation by FDP, make the kinetic characteristics of the enzyme similar to those of the mammalian M2 or K type pyruvate kinase (Van Berkel et al., 1973a). However, this muscle enzyme showed no indication of the EDTA,  $Mg^{++}$ , or FDP induced changes in conformation which have been found for mammalian M2 enzyme (Pogson, 1968; Van Berkel, 1974) and is more sensitive to  $MgATP$  inhibition than the mammalian M2 enzyme (VanBerkel, 1974).

In vivo this muscle enzyme is probably regulated primarily by ATP, arginine phosphate, and FDP levels. In crab and lobster muscle, resting ATP levels are between 4 and 6 mM (Freed, 1971; Beis & Newsholme, 1975). Arginine phosphate levels as high as 33 mM have been measured in lobster muscle (Beis & Newsholme, 1975). These values are considerably above the respective  $K_i$  values for the two inhibitors. Considering that PEP levels are probably below the  $K_m$  value, and that ADP levels are near the  $K_m$  value (Beis & Newsholme, 1975), these high levels of ATP and

arginine phosphate would lead to a strong inhibition of the enzyme in resting muscle. If any gluconeogenesis were to occur in muscle, it would logically occur when the muscle is at rest. As the muscle is activated and contraction begins, ATP is hydrolyzed to ADP and inorganic phosphate. The ADP is then reconverted to ATP by the action of arginine phosphokinase and arginine phosphate levels drop. Such a drop in arginine phosphate would serve to deinhibit the pyruvate kinase reaction. Further deinhibition would come through FDP activation. This would serve as a feed-forward mechanism whereby increased levels of an early glycolytic intermediate serve to increase flux through a later rate limiting glycolytic reaction. Since FDP is one of the major activators of phosphofructokinase (Tornheim & Lowenstein, 1975), this mechanism serves to integrate flux through the two portions of glycolysis. Further integration is achieved through adenylate coupling, and inhibition by high levels of the storage phosphagen and ATP.

Citrate, malate, and  $\alpha$ -ketoglutarate may play some regulatory role in vivo. However, the effective levels of these compounds are unlikely to occur in this mainly glycolytic tissue (Hochachka et al., 1971; Freed, 1971; Williamson, 1965; Williamson et al., 1969). Any effects which would occur would have the general effect of moderating pyruvate kinase activity at times of high Krebs cycle flux. This would prevent an overshoot of pyruvate and eventually acetyl CoA formation. Similarly, an effect by  $\alpha$ -glycerolphosphate would occur very rarely, and then only in situations of extreme anaerobiosis when the tissue has lost the capacity to balance redox through the

lactate dehydrogenase reaction. In this context, the oxidation of  $\alpha$ -glycerolphosphate by tissue homogenates from various organs of Cambarus affinis is of interest (Keller, 1965). However, there was no indication of utilization of this substrate by muscle, since muscle homogenates showed no measurable oxygen uptake (Keller, 1965). Tryptophan levels in crustacean muscle are low (Schoffeniels & Gilles, 1970), and there is no evidence of fluctuations which are correlated with energy metabolism. Its efficiency as an inhibitor is probably based on a structural similarity with ADP. The mixed competitive inhibition which both alanine and serine evidence toward PEP is probably of small physiological significance since large fluctuations would be needed for an effect.

The reversal of the muscle pyruvate kinase from Cancer magister amounted to less than 0.5% of the forward reaction at saturating levels of pyruvate (10 mM) and ATP (2 mM). ATP levels lie between 4 and 6 mM in resting muscle (Beis & Newsholme, 1975), and at these concentrations ATP slightly inhibited the reversal of the reaction. Pyruvate levels rarely rise above 0.1 mM in either crustacean or mammalian muscle (Hochachka et al., 1971; Freed, 1971; Williamson, 1965), and thus approach only 1% of the levels needed for saturation. Therefore it is unlikely that the reversal approaches more than 0.005% of the forward reaction rate. The rabbit muscle enzyme showed a reverse reaction with similar optimal conditions which amounted to 5% of the forward rate (Dyson et al., 1975; Giles et al., 1975). Since the levels of pyruvate kinase in mammalian muscle are high, pyruvate kinase reversal may account for the

gluconeogenesis which has been observed in rabbit muscle (Bendall & Taylor, 1970). However, it seems unlikely that gluconeogenesis occurs via this route in crustacean muscle. Intermolt muscle in Cancer magister lacks a functional enzymatic bypass for the pyruvate kinase reaction. While freshmolt muscle has both pyruvate carboxylase and phosphoenolpyruvate carboxykinase (Table 1), the levels are low compared to pyruvate kinase levels. Pyruvate carboxylase exhibits optimal activity at 2 mM pyruvate (Ashman et al., 1972); hence its function is feasible under physiological conditions. However, for any net gluconeogenic carbon flux to occur, the pyruvate kinase reaction must be inhibited to less than 0.2% of its maximal activity. Since 5 mM ATP and 5 mM arginine phosphate only inhibit the enzyme by 73%, gluconeogenic flux through this reaction locus is unlikely.

## Part IV.

## Hypodermal Pyruvate Kinase

## INTRODUCTION

As outlined before, the hypodermis is most closely associated with the production of the new exoskeleton. During the early postmolt period, the hypodermis carries out considerable gluconeogenesis for chitin synthesis. High rates of carbohydrate oxidation support this biosynthetic activity (Speck & Urich, 1971; Speck et al., 1972). For any gluconeogenesis to occur, pyruvate kinase must be effectively inhibited; however, during carbohydrate utilization, it forms a glycolytic control site. Thus, the control of pyruvate kinase is central to hypodermal metabolism. The freshmolt hypodermis shows both a considerable increase in pyruvate kinase activity (Table 1) and a marked increase in oxygen consumption (Skinner, 1962; McWhinnie et al., 1972; Bulnheim, 1974). In crayfish, all the carbon utilized for chitin synthesis and energy metabolism during the late premolt and early postmolt period is derived from hexose units resorbed from the old exoskeleton (Speck & Urich, 1971). In larger marine crustaceans, such as Cancer magister, the increased oxygen consumption during late premolt and early postmolt is not solely due to the utilization of carbohydrates, since lipids, and possibly proteins may be mobilized at this time as well (Renaud, 1949; Travis, 1955a; Heath & Barnes, 1970). However, carbohydrates form a major

substrate during this period, and thus downhill flux through the pyruvate kinase reaction is mandatory. During this period chitin synthesis from amino acids and intermediary compounds, such as lactate, is maximal as well (Herz-Hübner & Urich, 1973; Herz-Hübner et al., 1973). Thus, the metabolic control requirements faced by the pyruvate kinase reaction are stringent since it must be able to switch from virtually totally inhibited during periods of gluconeogenesis to high rates of catalysis within short periods of time. Since understanding of this control site is mandatory for a description of both gluconeogenic and glycolytic control mechanisms in the hypodermis, I studied the regulatory properties of the pyruvate kinase. Since the hypodermis undergoes marked physiological changes as a function of the molt cycle (Skinner, 1962), a comparison of the properties of the freshmolt and intermolt hypodermal enzymes was carried out as well.

#### METHODS

Preparation of hypodermal pyruvate kinase followed the methods described in Part III for the muscle enzyme. Initial homogenization proceeded in two rather than four volumes. Column chromatography on DEAE Sephadex and cellulose phosphate led to approximately 100 fold purification, with specific activity of 50 units/mg protein from the cellulose phosphate column.

### Measurement of Bound Fructose 1,6 Diphosphate

The method used was essentially that of Bergmeyer (1965). Two ml of enzyme from the peak tubes of the DEAE Sephadex and the cellulose phosphate columns were calibrated for enzyme activity, placed in boiling water for 2 minutes, cooled in ice, and centrifuged for 10 minutes at 27,000 g. The supernatant was used directly for the assay of fructose diphosphate, via the aldolase, triose phosphate isomerase and  $\alpha$ -glycerolphosphate dehydrogenase reactions. The concentration of NADH in the cuvette was 0.08 mM. This method of measuring fructose diphosphate yields two molecules of  $\alpha$ -glycerol phosphate and NAD per fructose diphosphate, thus all  $\Delta OD_{340}$  values were divided by 2 to calculate the original concentration of fructose diphosphate.

### Enzyme Assays

Pyruvate kinase activity was measured following procedures given in Part III.

## RESULTS

### Electrophoresis and Electrofocusing

Extensive electrophoretic comparison of intermolt and freshly molted hypodermal pyruvate kinases revealed no consistent differences. Most often their migration velocities were the same, but in 5 out of 25 experiments, the intermolt hypodermal enzyme showed slightly faster migration. In one

case, the freshmolt hypodermal enzyme was faster. Electrophoretic comparison of the intermolt and freshmolt muscle and hypodermal pyruvate kinases showed only slight migrational differences with the muscle enzyme showing somewhat faster anodal migration. Several different buffer systems were used to test out the possibilities completely, but under no circumstances could pronounced differences between molt stages be demonstrated. In all cases pyruvate kinase activity was confined to one band.

Electrofocusing also failed to reveal any differences between the freshmolt and the intermolt hypodermal pyruvate kinases. Freshmolt hypodermal pyruvate kinase showed an isoelectric point of 6.1, whereas intermolt hypodermal pyruvate kinase showed an isoelectric point of 6.005. Both intermolt and freshmolt pyruvate kinases focused in sharp, symmetrical peaks. Thus, I concluded that the pyruvate kinases present in intermolt and freshmolt hypodermis have the same net charge and are probably the same protein. The muscle enzymes, in contrast, show isoelectric points of 6.4, indicating some charge difference.

### Catalytic and Regulatory Characteristics

In initial experiments, it was found that the presence of EDTA and magnesium during the preparation and storage of the enzyme had profound effects upon the catalytic characteristics of the enzyme. In the absence of EDTA, the enzyme showed a lowered affinity for its substrate, phosphoenolpyruvate, and was

more sensitive to activation by fructose diphosphate. The presence of EDTA prevented these changes in PEP and FDP affinities. With Carcinus maenas hepatopancreas pyruvate kinase, EDTA was found to mimic the kinetic effects of FDP (Giles et al., 1976). In contrast, EDTA promotes the transition of both the mammalian M2 and L type pyruvate kinases into a low affinity state (Bailey et al., 1968; Badwey & Westhead, 1975; Pogson, 1968; Van Berkel, 1974). The kinetics of the muscle enzyme from Cancer magister were not affected by the presence or absence of EDTA. In further experiments, I sought to determine whether these EDTA promoted effects were artifacts or conformational changes of potential regulatory significance. Further purification indicated that pyruvate kinase from intermolt and freshmolt hypodermis could exist in two functional forms. Purification on DEAE Sephadex stabilized the enzyme in a high affinity form which I will call PK I. In all work with this form of the enzyme I included both 3 mM EDTA and 4 mM  $MgCl_2$ , since  $Mg^{++}$  has important effects upon some pyruvate kinases (Van Berkel, 1974). Purification of the enzyme upon cellulose-phosphate promoted the transition of the enzyme into another functional form with a lowered PEP affinity (PK II). The stability and close similarity of the two forms in all but a few kinetic criteria, as well as the lack of information pointing to separate enzymes within the hypodermis, led me to assume that these are interconvertible forms of the same protein. Furthermore, incubation of PK II with fructose diphosphate (see below) produced an enzyme with characteristics of PK I, while extensive dialysis of PK I in the absence of EDTA

and  $Mg^{++}$  led to an enzyme with a lowered affinity for PEP and an increased sensitivity to fructose diphosphate. Muscle pyruvate kinase did not show these effects. These results support the conclusion that these effects are not artifacts, but represent interconvertible forms of the enzyme which may be of regulatory significance in vivo. Therefore, the kinetic behavior of both PK I and PK II was completely characterized in further studies.

All the kinetic and purification experiments were performed on both intermolt and freshmolt hypodermal pyruvate kinases. Since the results were the same for both tissues only one set of results will be presented. The close agreement of the kinetic results supports the conclusion that different forms of pyruvate kinase are not synthesized as the hypodermis changes during the molt cycle. The levels of pyruvate kinase in the hypodermis rise as the animal goes from intermolt into premolt (Table 1). Thus, control at this reaction locus is partly vested in changing enzyme levels.

In common with other pyruvate kinases, the hypodermal enzyme required both a monovalent and a divalent cation for activity. Both forms showed a higher affinity for  $NH_4^+$  ( $K_m = 6.0$  mM) than for  $K^+$  ( $K_m = 10$  mM) but the  $V_{max}$  with  $NH_4^+$  as the activating cation was only 38% of that with  $K^+$  as the activating cation. At optimal  $K^+$  concentrations,  $Na^+$  inhibited catalysis, showing a  $K_i$  versus PEP of 40 mM and a  $K_i$  versus  $K^+$  of 85 mM. At approximately  $K_m$  levels of  $K^+$ ,  $Na^+$  provided a slight activation. Both  $Mn^{++}$  and  $Mg^{++}$  could act as the activating divalent cation for both PK I and PK II.  $Mn^{++}$  showed a lower  $K_m$

value (0.15 mM) than did  $Mg^{++}$  (0.6 mM). However, the  $V_{max}$  with  $Mn^{++}$  was only 39% of that with  $Mg^{++}$ .  $Ca^{++}$  was a competitive inhibitor versus both of these divalent cations, and showed a tenfold higher effectiveness versus  $Mg^{++}$  ( $K_i = 0.075$  mM) than versus  $Mn^{++}$  ( $K_i = 0.6$  mM). Both the hypodermal PK I and PK II show a broad pH optimum from pH 6.5 to pH 8.0.

### Substrate Affinities

For both PK I and PK II, substrate saturation curves for phosphoenolpyruvate and ADP followed Michaelis-Menten behaviour. PK I showed a high affinity for phosphoenolpyruvate with  $K_m$  values ranging between 0.09 mM and 0.18 mM (Figure 16). In this characteristic there was some variation between intermolt and freshmolt pyruvate kinases; the intermolt enzyme generally had  $K_m$  values between 0.1 mM and 0.18 mM, while the freshmolt enzyme had  $K_m$  values between 0.09 mM and 0.11 mM. Similar variations in these phosphoenolpyruvate  $K_m$  values were found for the muscle enzyme from these animals. For PK I, PEP affinity decreased as the pH was changed from pH 7.0. Affinity curves were hyperbolic at pH values of 6.5, 7.0, and 7.5, in contrast to the mammalian M2 and L type enzymes (Van Berkel *et al.*, 1973a; Seubert & Schoner, 1971). PK II showed a much lower affinity for phosphoenolpyruvate (Figure 17).  $K_m$  values ranged from 0.4-0.8 mM with no particular variation between intermolt and freshmolt preparations. By contrast to the PEP  $K_m$  values, the ADP  $K_m$  values were similar for all preparations. Both PK I and PK II showed  $K_m$  values for ADP that ranged from 0.2-0.3 mM. In contrast with recent findings with crab hepatopancreas

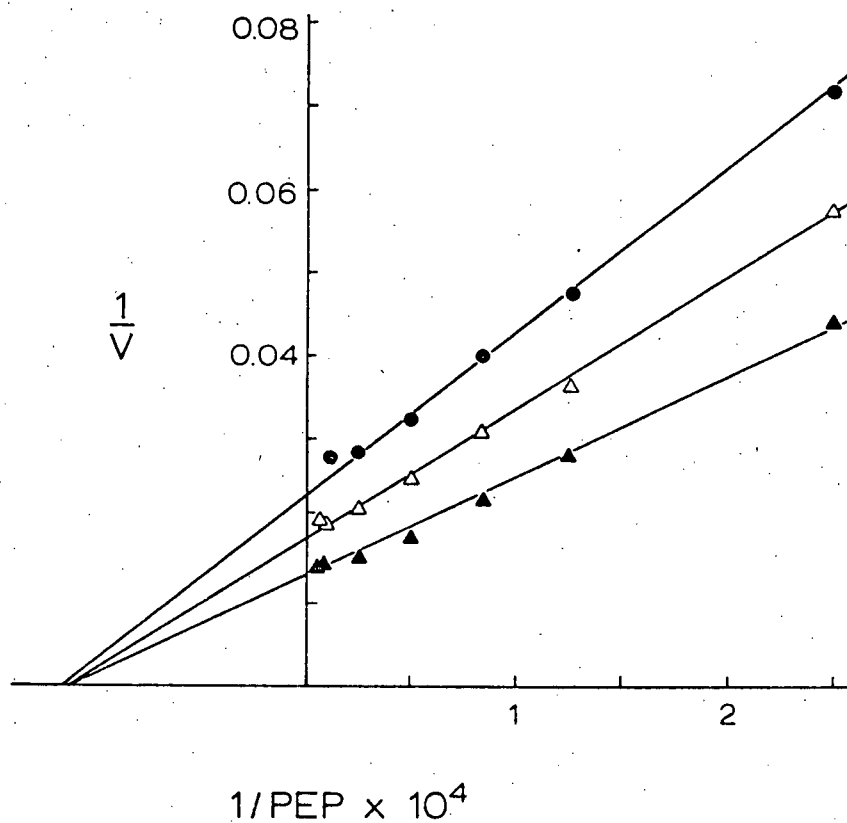


Fig. 16. Phosphoenolpyruvate affinity of PK I. Assay conditions were as follows: 50 mM imidazole-Cl, pH 7.0, 75 mM KCl, 4 mM  $MgCl_2$ , 0.1 mM NADH, PEP and ADP as shown. (▲) 2 mM ADP, (Δ)  $4 \times 10^{-4}$  M ADP, (●)  $2 \times 10^{-4}$  M ADP.

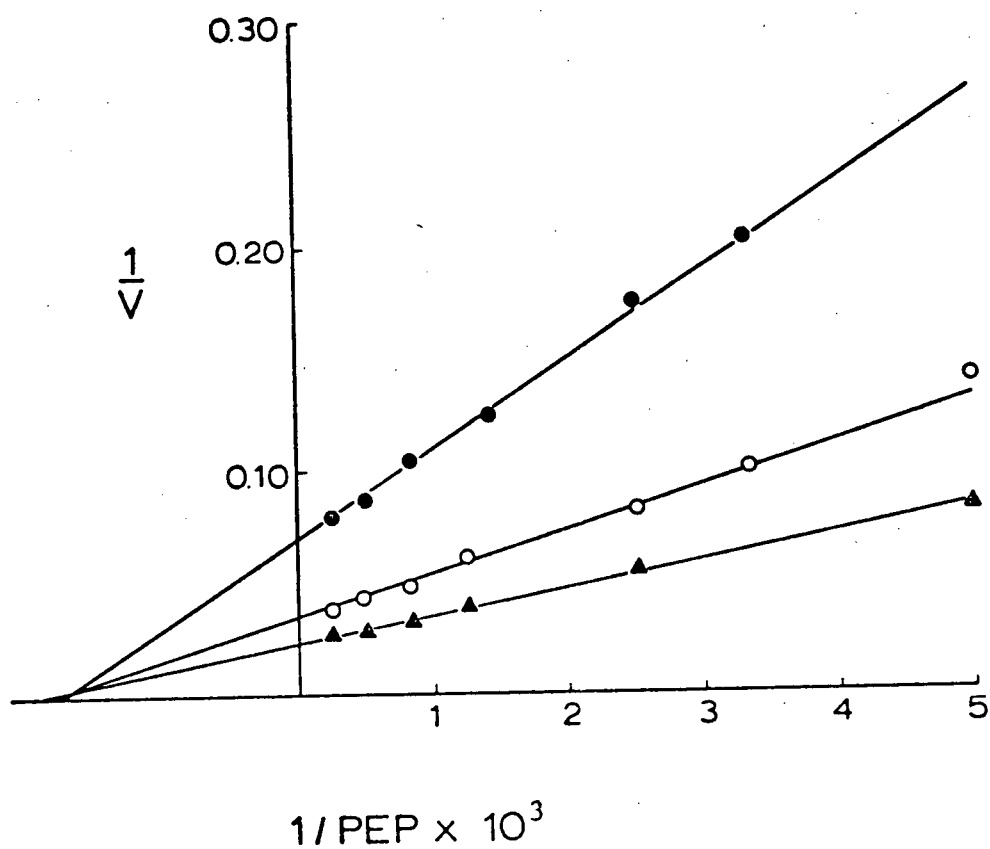


Fig. 17. Phosphoenolpyruvate affinity of PK II. Assay conditions as in the figure legend to Figure 16. (▲) 2 mM ADP, (○) 0.8 mM ADP, (●) 0.2 mM ADP.

pyruvate kinase (Giles *et al.*, 1976), the affinity for each substrate was independent of variation in the concentrations of cosubstrate, indicating a random binding mechanism. UDP, GDP, and IDP all served as nucleotide substrates, but with substantially lower affinities than ADP;  $K_m$  values were 1.5 mM for UDP, 1.2 mM for IDP, and 0.9 mM for GDP.

### Activators

Both pyruvate kinase I and pyruvate kinase II were strongly activated by low levels of fructose diphosphate (FDP). The prime effect of fructose diphosphate was to facilitate the binding of PEP. For PK II, the addition of 0.05 mM FDP reduced the PEP  $K_m$  from 0.7 to 0.15 mM (Figure 18). These levels of FDP also reversed MgATP inhibition for both forms. Pyruvate kinase I showed a  $K_a$  for FDP of  $1.3 \times 10^{-5}M$ , while pyruvate kinase II showed a much increased affinity for FDP with  $K_a$  value of  $9 \times 10^{-8}M$ . PK II thus binds FDP 100 times as strongly as PK I. In crude homogenates or ammonium sulphate fractions, UDP-acetylglucosamine (UDPAG), FDP and glucosamine-6-phosphate were equally good activators. These activators all reversed the inhibition due to MgATP. With the purified enzymes, FDP remained a potent activator, while UDPAG and glucosamine-6-phosphate produced only 25% of the activation caused by FDP.

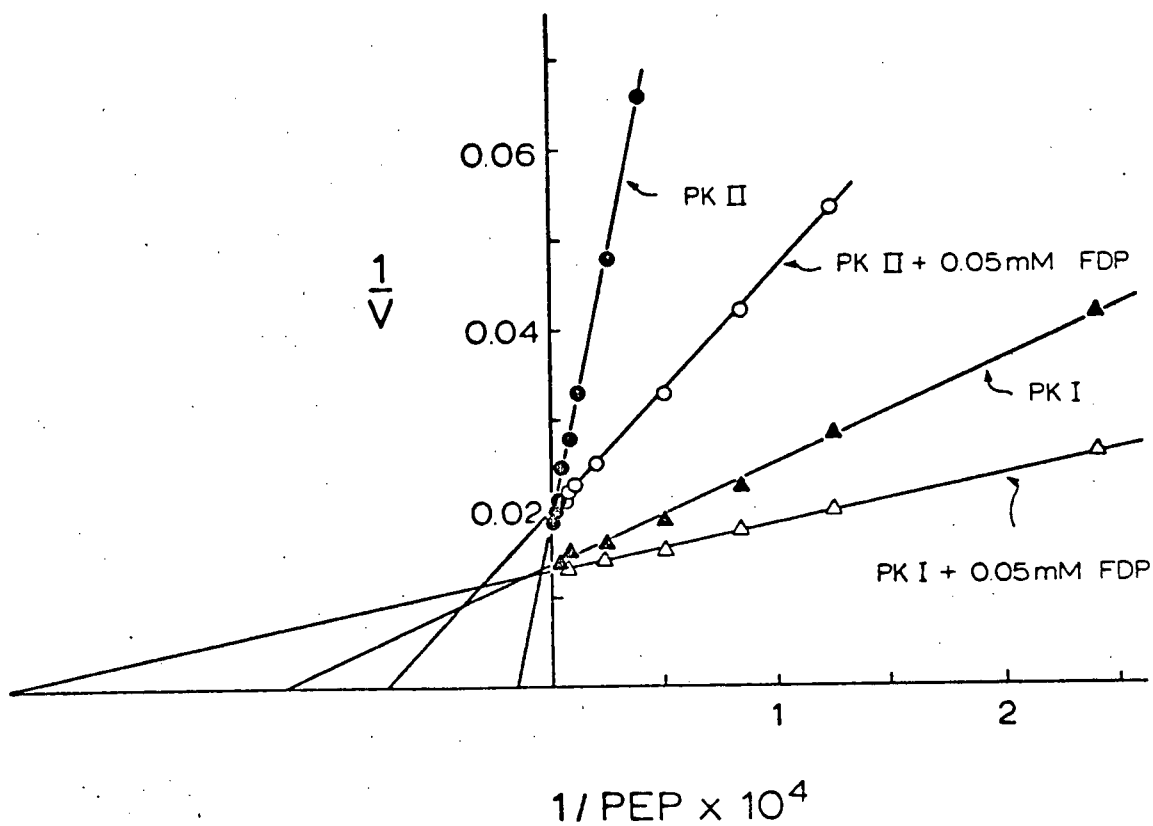


Figure 18. Influence of FDP on the phosphoenolpyruvate affinity of PK I and PK II. Assay conditions are as given in the legend to Figure 15. ADP concentration was 2 mM in all experiments.

## Inhibitors

Both PK I and PK II were affected by a variety of inhibitors, with PK II generally showing a greater sensitivity to inhibition, certainly at physiological levels of substrates and inhibitors. One of the prime inhibitors for both PK I and PK II was MgATP. MgATP  $K_i$  values versus PEP ranged from 0.95-1.8 mM, with the intermolt enzymes showing values at the lower portion of the range and the freshmolt enzymes lying at the upper end. Both purified and unpurified enzymes showed the same response to MgATP. MgATP inhibition was mixed competitive versus PEP (Figure 19) as well as ADP ( $K_i$  values of 2 mM). For pyruvate kinase II, MgATP was a competitive inhibitor versus PEP ( $K_i = 1.2$  mM) (Figure 20) and a noncompetitive inhibitor versus ADP. For both forms FDP reversed the effect of MgATP on PEP binding (Figures 19 & 20) but did not affect the inhibition versus ADP. The other nucleotide triphosphates (MgUTP, MgITP, and MgGTP) produced only a slight inhibition of these hypodermal pyruvate kinase forms. Arginine phosphate was an inhibitor of both PK I and PK II. For PK I the inhibition was competitive with both substrates, and showed  $K_i$  of 7.0 mM versus ADP and a  $K_i$  of 7.7 mM versus PEP. Arginine phosphate inhibition was competitive versus PEP ( $K_i = 6$  mM) and uncompetitive versus ADP for PK II. For both forms the inhibition could be partially reversed by the addition of 0.05 mM FDP. The arginine phosphate inhibition was not due to ATP formation by arginine phosphokinase.

A variety of metabolites, including amino acids, Krebs

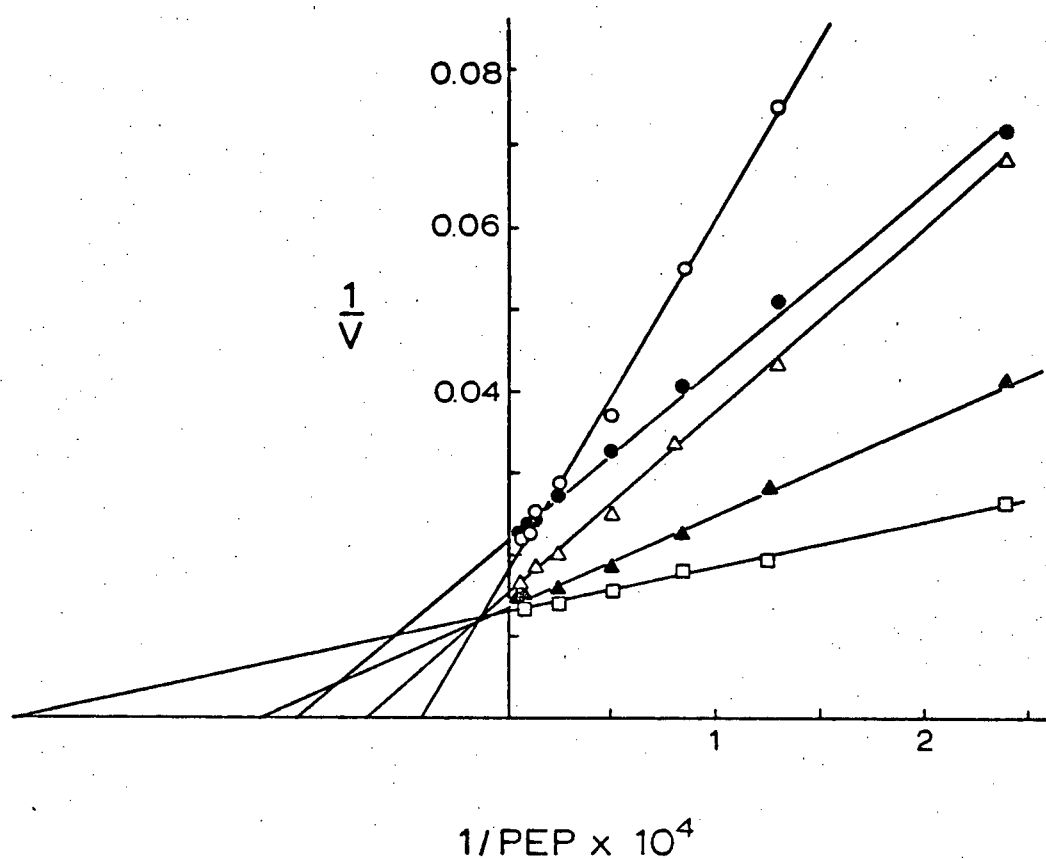
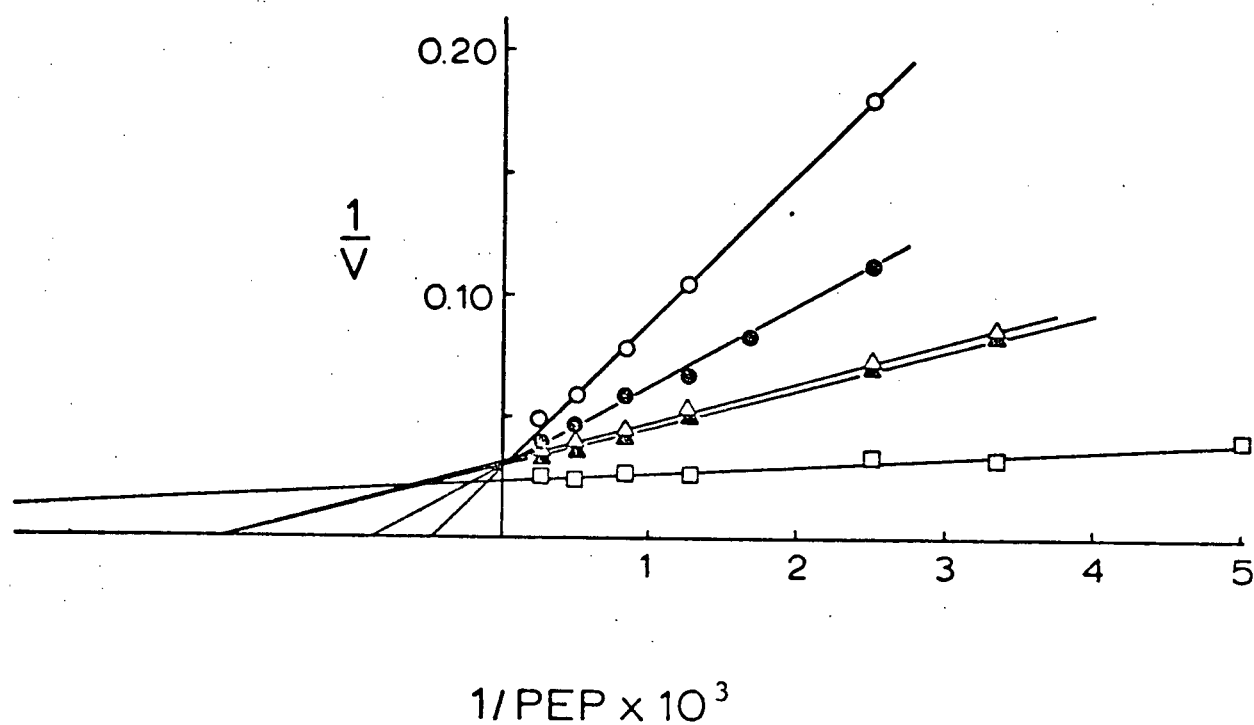


Fig. 19. MgATP inhibition of PK I, influence of MgATP on PEP affinity and reversal of MgATP inhibition by FDP. Assay conditions are as in the legend to Figure 16. (▲) 2 mM ADP, no MgATP, (Δ) 2 mM ADP, 2.5 mM MgATP, (○) 2 mM ADP, 5 mM MgATP, (□) 2 mM ADP, no MgATP, 0.01 mM FDP, (●) 2 mM ADP, 5 mM MgATP, 0.01 mM FDP.



**Fig. 20.** MgATP inhibition of phosphoenolpyruvate binding by PK II and the reversal of this inhibition by FDP. Assay conditions as in the legend to Figure 16, symbols as follows: ( $\Delta$ ) 2 mM ADP, no MgATP, ( $\bullet$ ) 2 mM ADP, 2 mM MgATP, ( $\circ$ ) 2 mM ADP, 4 mM MgATP, ( $\square$ ) 2 mM ADP, no MgATP, 0.05 mM FDP, ( $\blacktriangle$ ) 2 mM ADP, 4 mM MgATP, 0.05 mM FDP.

cycle and glycolytic intermediates have been implicated in the control of pyruvate kinase from various tissues. To see which, if any, intracellular compounds could further modulate the activity of the hypodermal forms of pyruvate kinase, the following compounds were tested at levels shown in brackets: taurine (1-5 mM), arginine (1-5 mM), alanine (2, 5, 10, and 20 mM), phenylalanine (1, 3, and 20 mM), glutamate (5 mM), proline (5 mM), valine (5 mM), asparagine (5 mM), aspartate (5 mM), leucine (10 mM), histidine (10 mM), lysine (10 mM), cysteine (10 mM), glycine (10 mM), glutamine (10 mM), methionine (10 mM), isoleucine (10 mM), threonine (10 mM), serine (10 and 20 mM), 3-P-glycerate (1 mM), AMP (1-4 mM), tryptophan (1-5 mM), acetyl CoA ( $1 \times 10^{-4}$  M), fructose diphosphate (0.5 mM), glucose-6-phosphate (1 mM), NADH (0.05-0.2 mM), cyclic AMP (0.05 mM), Mg<sub>2</sub>citrate (1-5 mM), Mg-malate (1-5 mM),  $\alpha$ -ketoglutarate (1-5 mM),  $\alpha$ -glycerol phosphate (1-5 mM), oxaloacetate (0.01-0.6 mM), 2,3 diphosphoglycerate (1 mM), and CoA (0.1-0.8 mM). These tests were run both at saturating and K<sub>m</sub> levels of substrates for both the freshmolt and intermolt PK I and PK II, and were carried out with purified enzymes. I found that alanine, serine, tryptophan, Mg<sub>2</sub>citrate, Mg-malate,  $\alpha$ -ketoglutarate, and  $\alpha$ -glycerol phosphate affected both PK I and PK II, although none of these was as potent an inhibitor as MgATP. PK II was more affected by the inhibitors than PK I.

Alanine, serine, and valine were weak inhibitors for PK I. All three were not completely competitive with either PEP or ADP, and the addition of more than a particular level of the amino acid did not lead to a further inhibition. The K<sub>i</sub> for

alanine inhibition versus PEP was 30 mM (Figure 21), the  $K_i$  for serine inhibition 35 mM, and that for valine inhibition 45 mM. FDP did not affect the inhibition caused by these amino acids. Pyruvate kinase II showed no response to valine, but showed a more marked response to serine and alanine than PK I. Serine was a mixed competitive inhibitor versus both PEP and ADP, with  $K_i$  values of 10.5 mM and 36 mM, respectively. The addition of 0.01 mM FDP partially reversed the inhibition, raising the  $K_i$  value for the serine versus PEP inhibition to 19 mM and changing the inhibition pattern versus ADP to uncompetitive (Figure 22). The  $K_i$  for alanine inhibition versus PEP was 7 mM and the addition of 0.01 mM FDP raised this  $K_i$  value to 26 mM (Figure 21). The  $K_i$  for alanine inhibition versus ADP was 26 mM. For PK II, inhibition due to serine and alanine was additive (Table 12, p. 144). Tryptophan was a competitive inhibitor versus ADP for both PK I and PK II ( $K_i$  values of 1.8 and 2.5 mM, respectively). Tryptophan inhibition was not reversed by FDP.

Mg<sub>2</sub>citrate,  $\alpha$ -ketoglutarate,  $\alpha$ -glycerol phosphate, and Mg-malate affect both PK I and PK II, but particularly for the last three, the effective levels were unphysiological (Williamson et al., 1969). Since the levels of malate,  $\alpha$ -ketoglutarate, and  $\alpha$ -glycerol phosphate which cause 50% inhibition are the same for both PK I and PK II, I only investigated the mode of inhibition for PK I. Mg<sub>2</sub>citrate affects the two forms differently, so I examined the mode of inhibition for both.  $\alpha$ -glycerol phosphate showed linear inhibition kinetics, and was mixed competitive versus both substrates with a  $K_i$  of 24 mM versus a PEP and a  $K_i$  of 29 mM

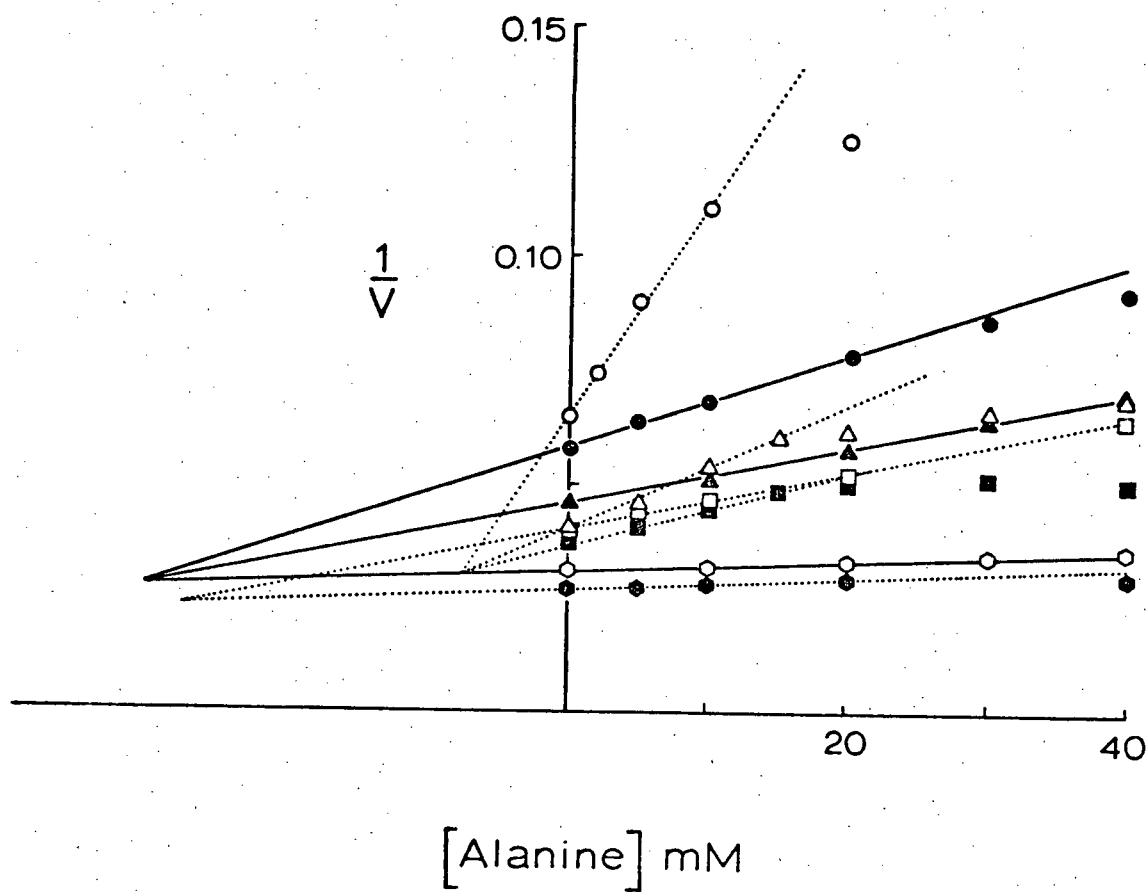


Fig. 21. Alanine inhibition of PK I and PK II and the influence of FDP on the inhibition pattern. Assay conditions as in the legend to Figure 16, ADP concentration was held constant at 2 mM.

— PK I, ..... PK II, (■) 2 mM PEP, (Δ) 1.2 mM PEP, (○) 0.4 mM PEP, (●) 2 mM PEP, 0.01 mM FDP, (□) 0.4 mM PEP, 0.01 mM FDP, (◇) 2 mM PEP, (▲) 0.2 mM PEP, (◐) 0.12 mM PEP.

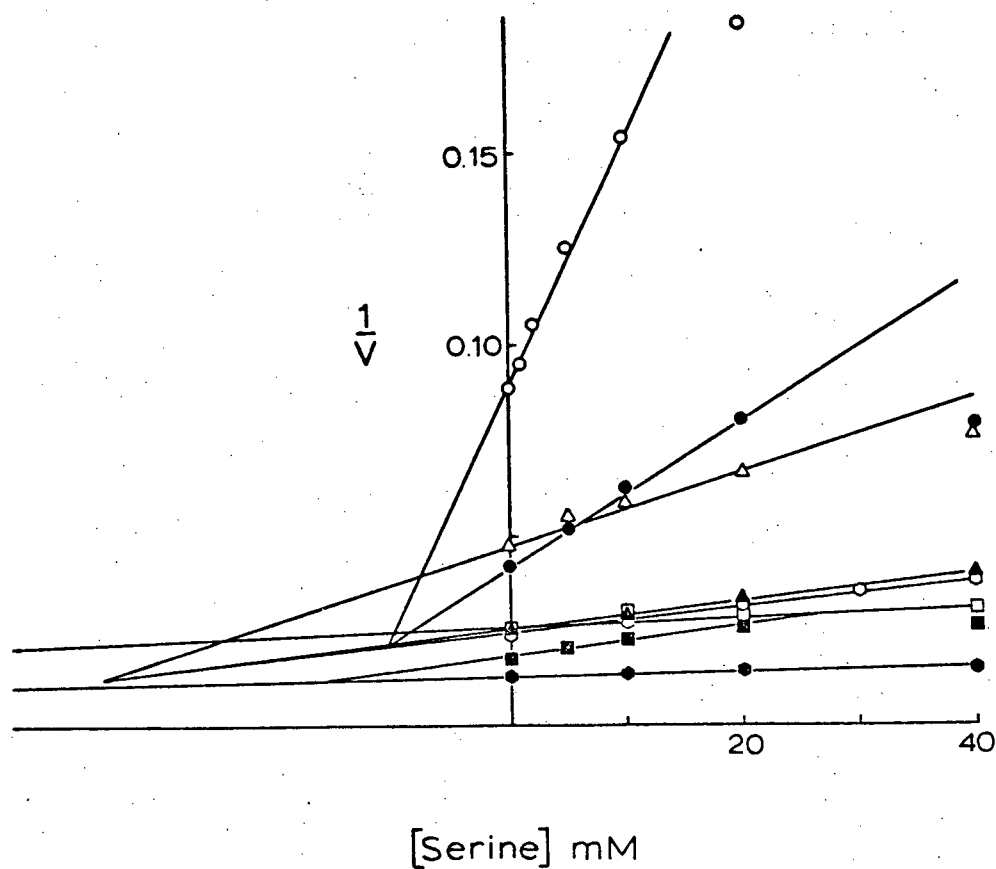


Fig. 22. Serine inhibition of PK II and the influence of FDP upon the inhibition pattern. Assay conditions as in the legend to Figure 16. (O) 0.4 mM PEP, 2 mM ADP, (●) 0.8 mM PEP, 2 mM ADP, (◇) 2 mM PEP, 2 mM ADP, (▲) 2 mM PEP, 0.4 mM ADP, (⊠) 2 mM PEP, 2 mM ADP, 0.01 mM FDP, (■) 2 mM ADP, 0.4 mM PEP, 0.01 mM FDP, (□) 2 mM PEP, 0.2 mM ADP, 0.01 mM FDP.

versus ADP.  $\alpha$ -Ketoglutarate showed a 8 mM  $K_i$  in the interaction with PEP and a 20 mM  $K_i$  versus ADP. Mg-malate inhibition showed linear inhibition versus ADP with a  $K_i$  of 20 mM, but the inhibition versus PEP showed curved Dixon plots. Lineweaver-Burk replots of the data were linear, indicating that in the inhibition versus PEP, Mg-malate is interacting at more than one site (Webb, 1963). For PK I, Mg<sub>2</sub>citrate showed curved Dixon plots versus both PEP and ADP. In both cases, Lineweaver-Burk replots of the data were linear, again indicating interaction at more than one site. By contrast, PK II showed linear kinetics for the interaction of Mg<sub>2</sub>citrate with both PEP and ADP ( $K_i$  values of 7.5 and 8.0 mM). In the presence of 0.01 mM FDP the  $K_i$  versus PEP rose to 10 mM and the inhibition versus ADP became uncompetitive (Figure 23a & b). At 5 mM levels, Mg<sub>2</sub>citrate inhibited PK I by 43% and PK II by 52%.

Tables 11 and 12 allow an analysis of the effectiveness of the various modulators added singly or in concert, in controlling the activity of these two pyruvate kinase forms. The tables show that for both PK I and PK II, MgATP, arginine phosphate, and Mg<sub>2</sub>citrate inhibition was additive. Amino acid inhibition was additive with that due to MgATP and arginine phosphate, but this inhibition was meaningful only for PK II. The combination of 5 mM MgATP and 5 mM Mg<sub>2</sub>citrate inhibited PK II by 99.5%, while the same combination only inhibited PK I by 63%. FDP was also far more effective as an activator for PK II than for PK I.

In comparison with PK I, PK II showed a decreased affinity

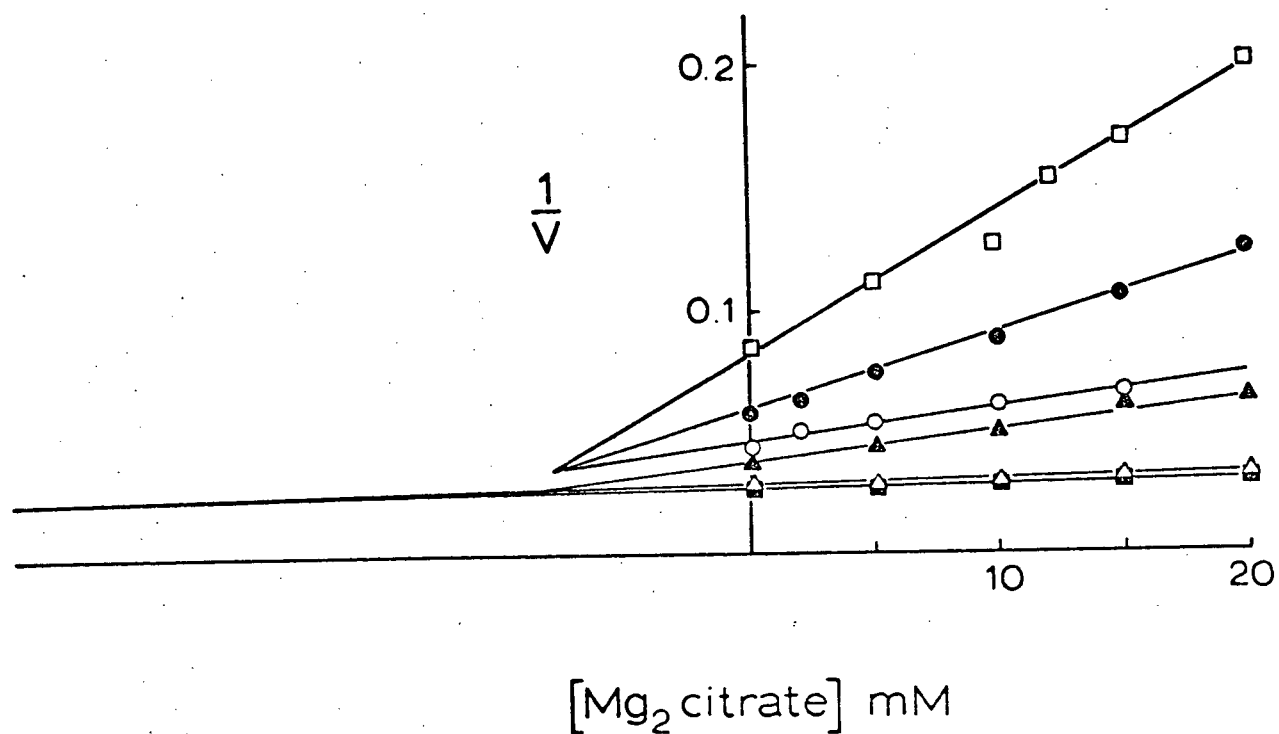


Fig. 23a.  $\text{Mg}_2$  citrate inhibition of PK II, inhibition versus ADP and influence of FDP upon the inhibition pattern versus ADP and PEP. Assay conditions are as in the legend to Figure 16 (O) 2 mM ADP, 2 mM PEP, ( $\square$ ) 2 mM PEP, 0.2 mM ADP, ( $\bullet$ ) 2 mM PEP, 0.8 mM ADP, ( $\blacksquare$ ) 2 mM PEP, 2 mM ADP, 0.01 mM FDP, ( $\triangle$ ) 0.8 mM ADP, 2 mM PEP, 0.01 mM FDP, ( $\blacktriangle$ ) 2 mM ADP, 0.4 mM PEP, 0.01 mM FDP.

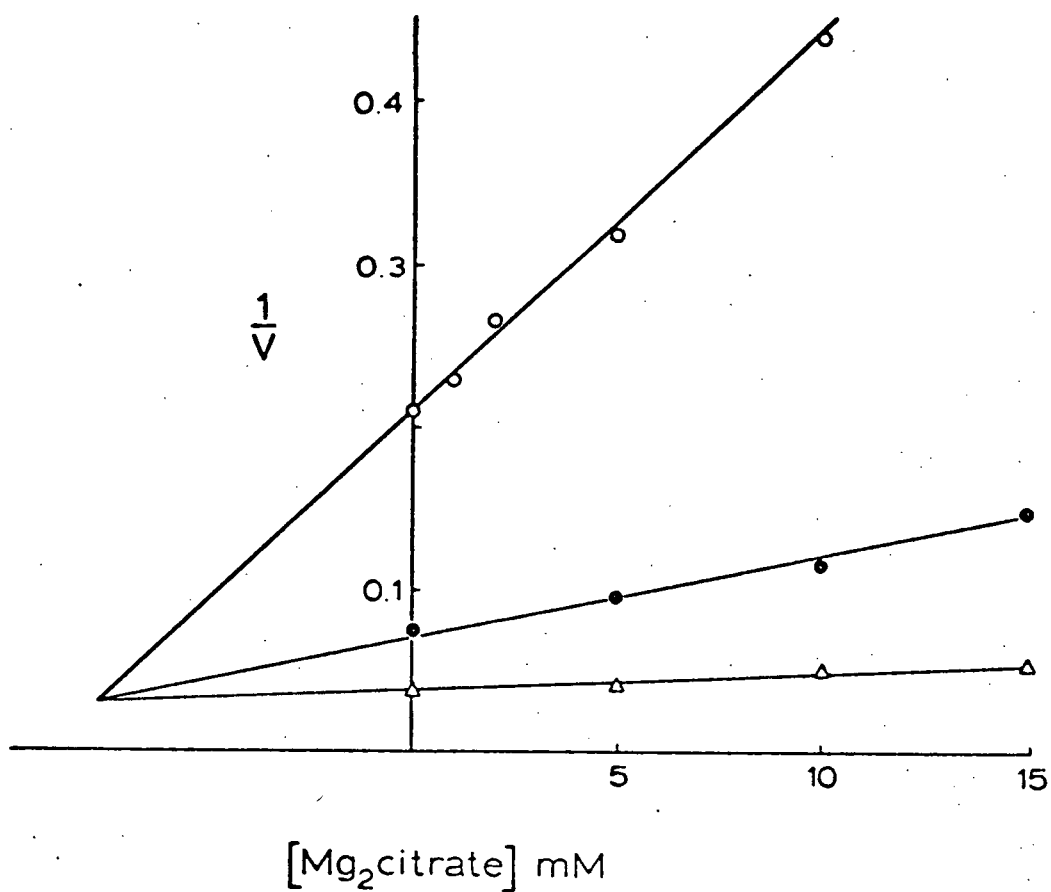


Figure 23b.  $\text{Mg}_2\text{citrate}$  inhibition of PK II, inhibition versus PEP. Assay conditions as in the legend to Figure 16 ADP concentration constant at 2 mM. (O) 0.4 mM PEP, (●) 0.8 mM PEP, ( $\Delta$ ) 4 mM PEP.

TABLE 11

Effectiveness of various inhibitors of hypodermal pyruvate kinase I.

Enzyme activity was measured at 0.2 mM PEP, 0.2 mM ADP, 4 mM  $\text{MgCl}_2$ , 75 mM KCl, 0.1 mM NADH, and excess LDH in 50 mM imidazole-HCl, pH 7.0. Enzyme activity is expressed as percent of control activity. If FDP was added to the treatment, the concentration was 0.05 mM.

<u>Treatment</u>	% Control Activity	
	<u>Plain</u>	<u>+ FDP</u>
5 mM MgATP	50%	-
5 mM arginine phosphate	68%	85%
5 mM $\text{Mg}_2\text{citrate}$	67%	-
5 mM $\alpha$ -ketoglutarate	82%	-
5 mM MgATP + 5 mM arginine phosphate	35%	46%
5 mM $\alpha$ -ketoglutarate + 5 mM arginine phosphate	57%	68%
5 mM arginine phosphate + 5 mM MgATP + 5 mM $\text{Mg}_2\text{citrate}$ + 5 mM $\alpha$ -ketoglutarate	30%	42%
5 mM MgATP + 5 mM $\text{Mg}_2\text{citrate}$ + 5 mM $\alpha$ -ketoglutarate	41%	49%
5 mM $\text{Mg}_2\text{citrate}$ + 5 mM $\alpha$ -ketoglutarate	54%	61%
5 mM MgATP + 5 mM $\alpha$ -ketoglutarate	43%	-
5 mM MgATP + 5 mM $\text{Mg}_2\text{citrate}$	35%	48%

TABLE 12

Effectiveness of various inhibitors of hypodermal pyruvate kinase II.

Conditions of the experiments were the same as those given in the legend for Table 11, with the exception that when FDP was added, the final concentration was 0.01 mM.

<u>Treatment</u>	% Control Activity	
	<u>Plain</u>	<u>+ FDP</u>
5 mM MgATP	18%	35%
5 mM arginine phosphate	64%	-
20 mM serine	69%	-
20 mM alanine	78%	-
5 mM Mg <sub>2</sub> citrate	50%	120%
10 mM $\alpha$ -ketoglutarate	75%	-
5 mM arginine phosphate + 5 mM MgATP	14%	-
5 mM MgATP + 5 mM Mg <sub>2</sub> citrate	0.5%	34%
5 mM MgATP + 20 mM alanine	12%	20%
20 mM alanine + 20 mM serine	57%	85%
20 mM alanine + 20 mM serine + 5 mM MgATP	0.9%	-
5 mM arginine phosphate + 5 mM MgATP + 20 mM alanine	14%	25%
2.5 mM tryptophan + 5 mM MgATP	13%	-
5 mM arginine phosphate + 5 mM Mg <sub>2</sub> citrate	45%	116%
0.01 mM FDP	180%	-
0.01 mM UDP-acetylglucosamine	116%	-

for PEP and a considerably increased affinity for FDP as an activator. There was no electrophoretic or isoelectrofocusing evidence for different proteins corresponding to PK I and PK II in the hypodermis. Since the two forms were similar, but could be reproducibly separated into two stable forms which displayed this reciprocal relationship between substrate and activator affinities, I felt they could represent two interconvertible forms of the same protein. For this interconversion to be of physiological significance, my purification techniques must in some way have mimicked the method by which these forms are interconverted within the cell. Phosphorylation, binding of  $Mg^{++}$  or of  $Ca^{++}$ , oxidation of sulphydryl groups, and binding and release of FDP have all been implicated in interconversions of either the L type or the M2 type of mammalian pyruvate kinase (Hess & Kutzbach, 1971; Van Berkel *et al.*, 1973b; Van Berkel, 1974; Ljungstrom *et al.*, 1974; Flikweert *et al.*, 1975). Since the two enzymes already possess a binding site for FDP, and since the affinity of this site varies strongly between the two forms, mediation of the transition by changes in the amount of FDP associated with the enzyme seemed the simplest mechanism. Since FDP is not directly involved with the catalysis, it seemed feasible that FDP molecules remain bound to the enzyme for relatively long durations. Such long term binding of the activator could stabilize the protein in a more active conformation.

To evaluate this hypothesis, I tested the effect of incubation with FDP upon PK II. A thirty minute incubation with 10  $\mu M$  FDP was sufficient to convert the low affinity form into

the high affinity PK I (Figure 24). 0.5  $\mu$ M FDP did not affect a complete conversion, while MgATP in combination with 10  $\mu$ M FDP prevented the full conversion of PK II into PK I (Table 13). After the various incubations, the enzyme remained sensitive to FDP activation of PEP binding (Figure 24), thus supporting the concept of interconvertible enzyme forms. The results of this experiment indicate that kinetic experiments involving the addition of FDP to PK II are actually monitoring a mixture of PK II and PK I, or some intermediate form. The "two stage activation", ie. the initial shift from a low to a high affinity form, and the further activation by FDP, supports the existence of two conformational states.

A further evaluation of the hypothesis of FDP mediated conversion came from measurements of the amount of FDP which occurred with PK I and PK II. I found that both PK I and PK II had measurable amounts of FDP bound to or closely associated with them. Since I was not working with homogeneous proteins, I could not express the values as moles FDP/mole enzyme, but I was able to find that per unit of enzyme activity, PK I was associated with 0.0014 moles FDP and PK II with 0.0003 moles FDP. While there was some variation in the values between different experiments, for any one animal, the FDP content of PK I and PK II were always in approximately a 4:1 ratio. It was also interesting to find that the muscle enzyme from Cancer magister contained bound FDP, but in more variable amounts. Control experiments using column effluent of equal protein concentration, but no pyruvate kinase activity, showed no FDP.

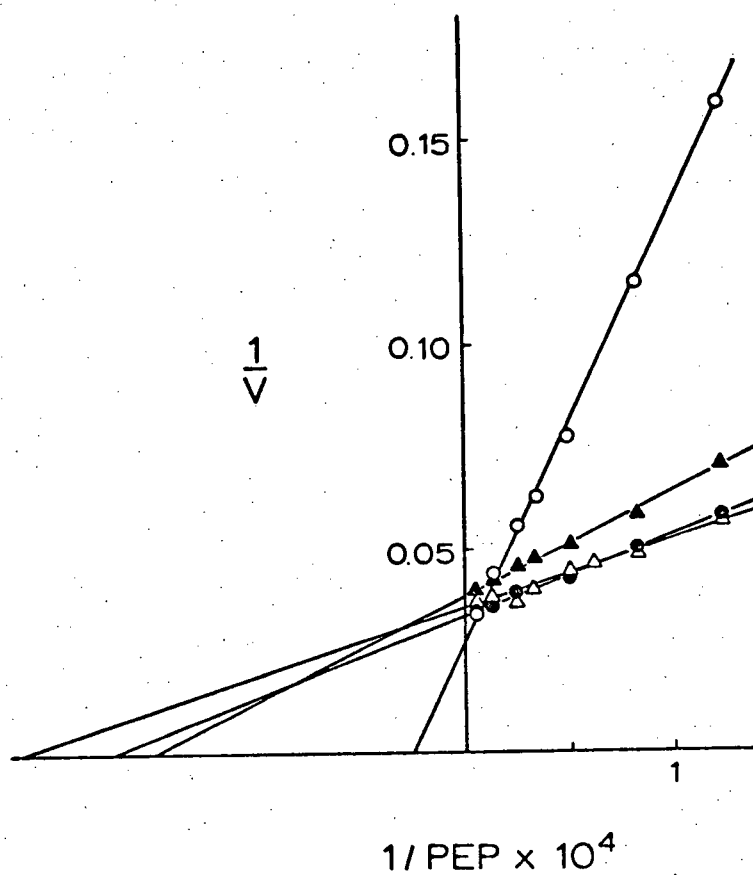


Fig. 24. Conversion of PK II into PK I by incubation with 0.05 mM FDP. Filled symbols show the PEP affinity curves measured in the presence of 0.05 mM FDP, while the open symbols show the affinity curves in the absence of FDP. Circles represent the enzyme before the thirty-minute incubation with 0.05 mM FDP, while triangles show the enzyme after incubation.

TABLE 13

Effect of FDP, ATP and alanine on the interconversions of PK I and PK II.

The percentage of PK I and PK II in each experiment were determined from the reaction rate at 0.5 mM PEP. For PK I the rate at this concentration of

substrate was 95% of  $V_{\max}$ , while for PK II the rate was only 50% of  $V_{\max}$ .

$V_{\max}$  for PK I, PK II, and the incubated enzyme were determined from Lineweaver-

Burk plots. The following equation was used in the calculations:

$$\% \text{ PK I} = \frac{\text{observed rate} - \text{rate with PK II}}{\text{rate with PK I} - \text{rate with PK II}}, \quad \text{the } \% \text{ PK II} = 100\% - \% \text{ PK I}$$

<u>Treatment</u>	<u>% PK II</u>
no incubation	93%
30 minutes incubation with 50 $\mu\text{M}$ FDP	15.5%
2 minutes incubation with 50 $\mu\text{M}$ FDP	25%
no incubation	75%
30 minutes incubation with 10 $\mu\text{M}$ FDP	13%
30 minutes incubation with 0.5 $\mu\text{M}$ FDP	56%
30 minutes incubation with 5 mM MgATP, 20 mM alanine, and 10 $\mu\text{M}$ FDP	23%

Recent work with the rabbit muscle pyruvate kinase indicates that the reversal of the reaction is feasible in the tissue (Dyson *et al.*, 1975; Giles *et al.*, 1975). Since the levels of pyruvate kinase in the freshmolt hypodermis are quite high compared to those of pyruvate carboxylase and phosphoenolpyruvate carboxykinase (Table 1), a high rate of reversal would be one way of facilitating gluconeogenesis. I found, however, that the rate of the reverse reaction was the same for PK I and PK II and at  $V_{max}$  consisted of 0.4% of the  $V_{max}$  of the forward reaction.

Since the thermal denaturation behavior of proteins can provide one means by which different proteins can be distinguished, I characterized the effect of prolonged incubation at 40 °C on both PK I and PK II and compared this with the response shown by the muscle enzyme (Figure 15). The two hypodermal enzymes were considerably more affected by the incubations than the muscle enzyme (compare Figure 15 with Figures 25 and 26). After 80 minutes at 40 °C, 95% of the original enzyme activity remained in the muscle preparation, while PK I had 75% and PK II only 45% of the original activity. For all enzymes, ATP accelerated denaturation. However, the muscle enzyme retained 80% of its activity after 80 minutes incubation with 4 mM MgATP, while both PK I and PK II retained only 25% of the initial activity. A comparison of the denaturation behavior of PK I and PK II indicates that PK II is generally more prone towards denaturation. None of the protective treatments succeeded in maintaining PK II activity above 60% after 80 minutes denaturation time, while the presence

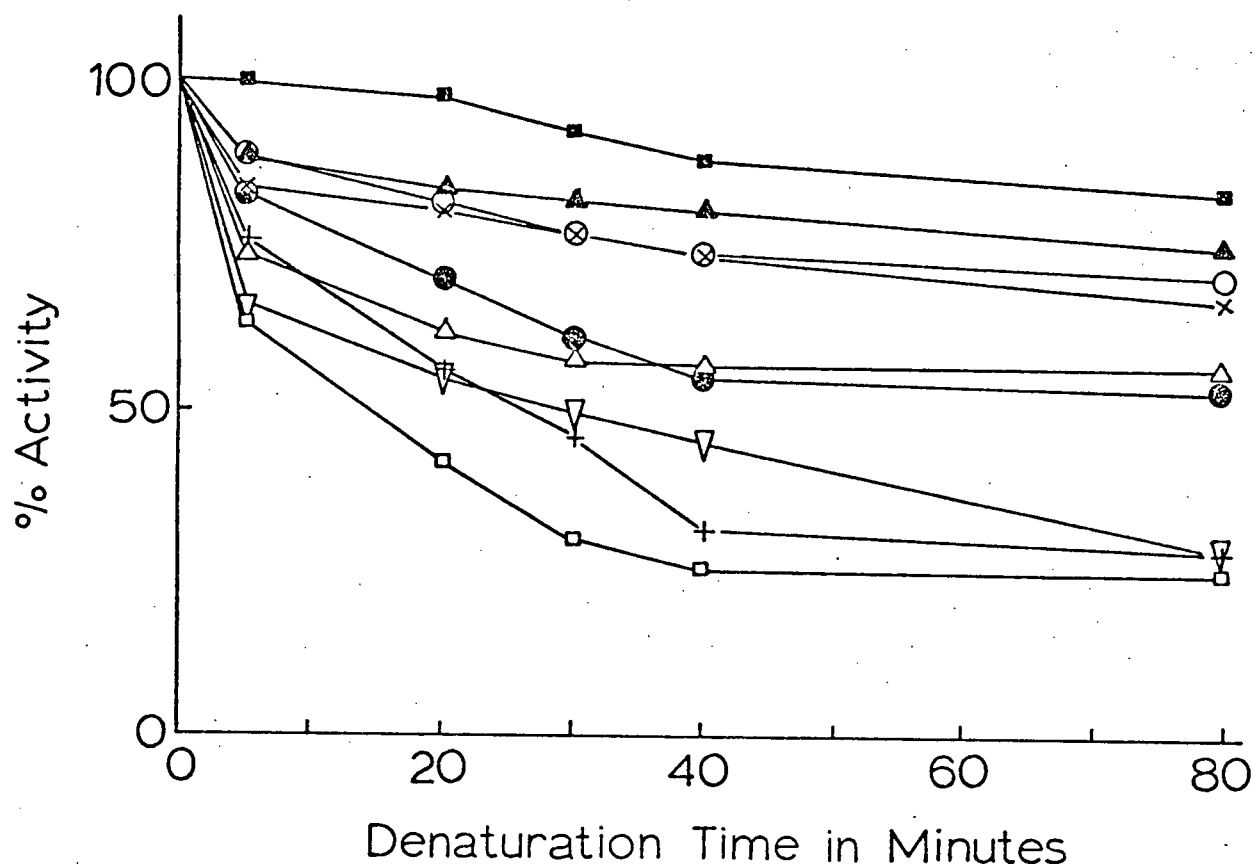


Figure 25. Thermal denaturation of Cancer magister hypodermal pyruvate kinase I. Experiments were run at 40°C under the indicated incubation conditions. Enzyme activity was measured under saturating conditions of substrates and cofactors. (▲) control; (□) 4 mM MgATP; (x) 0.1 mM FDP; (▽) 0.1 mM FDP, 4 mM ATP, and 4 mM MgCl<sub>2</sub>; (+) 2 mM PEP; (o) 2 mM ADP and 2 mM PEP; (⊠) 2 mM ADP, 2 mM PEP, and 0.1 mM FDP; (⊙) 2 mM ADP, 2 mM PEP, 0.1 mM FDP, and 4 mM MgATP; (△) 75 mM KCl.

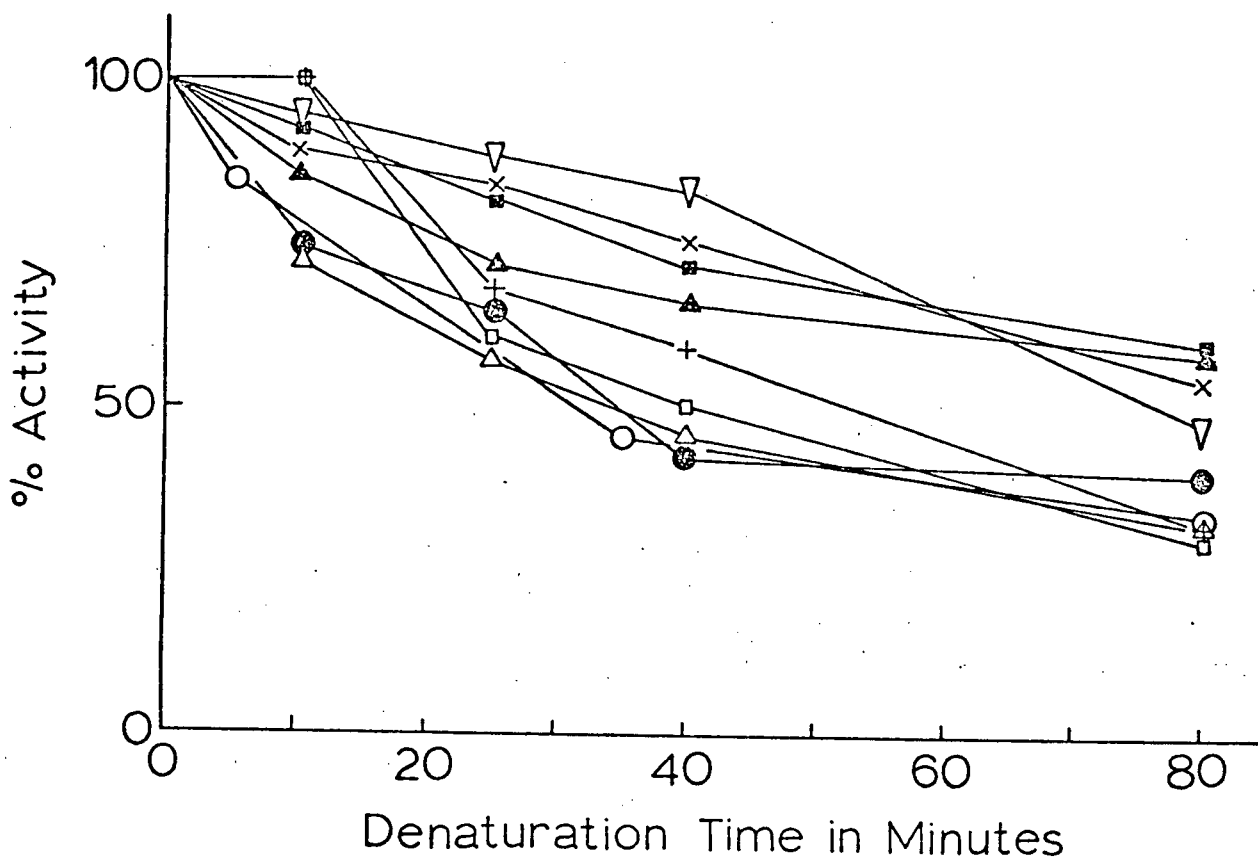


Figure 26. Thermal denaturation of Cancer magister hypodermal pyruvate kinase II. Experiments were run at 40°C under the indicated incubation conditions. Enzyme activity was measured under saturating conditions of substrates and cofactors. (▽) control; (□) 4 mM MgATP; (■) 0.1 mM FDP; (+) 0.1 mM FDP, 4 mM MgATP; (⊙) 2 mM PEP; (x) 2 mM ADP and 2 mM PEP; (▲) 2 mM ADP, 2 mM PEP, and 0.1 mM FDP; (o) 2 mM ADP, 2 mM PEP, 0.1 mM FDP and, 4 mM MgATP; (Δ) 75 mM KCl.

of 2 mM ADP + 2 mM PEP + 0.1 mM FDP maintains PK I at 85% of the original activity. Both PK I and PK II show accelerated denaturation in the presence of 2 mM PEP, while 2 mM PEP and 2 mM ADP afford some protection. Neither PEP nor PEP + ADP affect the denaturation of the muscle enzyme.

Considerable differences seem to exist between the denaturation patterns of the muscle enzyme and the two hypodermal forms. However, the overall denaturation patterns of PK I and PK II are quite similar. A difference between the two forms comes in the slight degree of protection afforded by 0.1 mM FDP to PK II. This may reflect the decreased levels of FDP bound to PK II. The higher degree of denaturation shown by PK II may indicate that this form is in a less stable conformational state than PK I. One effect which I did not examine experimentally involved the difference in specific activity of the different enzyme preparations. The protein concentration in the three experiments was the same, but the specific activity of the pyruvate kinase was not. Thus, the possibility of increased self-association may partially explain the thermal tolerance of the muscle enzyme. However, the central conclusion which emerges from these denaturation experiments is that the muscle pyruvate kinase is different from the two hypodermal enzymes.

## DISCUSSION

In freshmolt hypodermis, the pyruvate kinase branchpoint faces two urgent requirements: (1) for gluconeogenesis from amino acids and lactate, the pyruvate kinase reaction must be inhibited, and (2) pyruvate kinase must be able to shift from low to high activity to support high rates of carbohydrate oxidation during this period (Herz-Hübner & Ulrich, 1973). Hypodermal pyruvate kinase activities are considerably higher than those of the enzymatic bypass, so fluctuations in pyruvate kinase rates are probably the prime determinant of flux through this branchpoint. Hypodermal pyruvate kinase appears to be a complex allosteric protein capable of making the required transitions in activity. Hypodermal pyruvate kinase was found to exist in two forms, one which can be considered to be a high affinity form (PK I), and the other a low affinity form (PK II). In vitro interconversion by FDP as well as the different levels of FDP associated with the two forms indicated that the in vivo interconversion between the two is probably mediated by FDP. There are quite a number of precedents for interconvertible pyruvate kinases, the best established being the FDP mediated conversion of mammalian L type pyruvate kinase from a sigmoidal low affinity state to a hyperbolic high affinity state (Hess & Kutzbach, 1971; Ibsen et al., 1975). Phosphorylation of the L type enzyme leads to an enzyme with lowered PEP affinity (Ljungstrom et al., 1974). While ATP induces a dissociation of ground squirrel liver pyruvate kinase into inactive dimers, FDP mediates the reassociation of these dimers into tetramers (Behrisch, 1974). The L type enzyme also undergoes

interconversions under the influence of oxidized and reduced glutathione. The oxidized enzyme shows a markedly reduced affinity for both FDP and PEP. This effect is reversed by the addition of reduced glutathione (Van Berkel *et al.*, 1973b). The M2 or K type pyruvate kinase has also been found to undergo several types of transitions. Pogson (1968) found that EDTA transformed the enzyme from a high to a low affinity state. These interconvertible forms have been shown to exist *in vivo* (Walker & Potter, 1973; Feliu *et al.*, 1975). Van Berkel (1974) found that this effect was due to the removal of  $Mg^{++}$  from the vicinity of the enzyme.  $Ca^{++}$  has also been found to produce interconversions of the M2 enzyme (Flikweert *et al.*, 1975), as has FDP (Van Berkel, 1974). An alanine mediated inactive dimer to active tetramer transition, and a magnesium mediated dimer-tetramer transition have been found for the mammalian M2 enzyme (Schulz *et al.*, 1975; Ibsen *et al.*, 1975). With the crustacean hypodermal pyruvate kinase, I found no activation due to the reduced mercaptoethanol used in the purification, and in agreement with Giles *et al.*, (1976), I found that the responses to EDTA were opposite to those shown by the mammalian enzymes.

The major functional distinction between the two hypodermal pyruvate kinase forms is their disparate PEP affinity. In the various vertebrate and invertebrate tissues where PEP levels have been measured, its levels rarely reach or exceed 0.2 mM (Exton & Park, 1969; Beis & Newsholme, 1975). Therefore, PK II, with its  $K_m$  for PEP of 0.55 mM, would be functioning at approximately 15% of  $V_{max}$ , while PK I, with its PEP  $K_m$  of 0.1 mM, would be functioning at approximately 80% of  $V_{max}$ . This

reckoning assumes an absence of modulators. ATP levels in the hypodermis lie between 2 and 3 mM (Table 7). Such levels would drop pyruvate kinase II to approximately 2% of maximal activity, while reducing pyruvate kinase I to between 50 and 60% of its  $V_{max}$ . Marine crustaceans have high intracellular levels of amino acids with alanine at approximately 20 mM and serine at 5 mM (Schoffeniels & Giles, 1970). While PK I activity is relatively unaffected by these levels, the presence of both of these compounds would serve to further inhibit PK II to less than 0.2% of maximal activity, as would any accumulation of  $Mg_2$ citrate (Figure 27). Thus, pyruvate kinase activity can essentially be turned off, allowing gluconeogenic flux past the reaction.

Reversal of pyruvate kinase inhibition can be brought about by an increase in the levels of FDP. During gluconeogenesis, increasing FDP levels would indicate some inhibition at the FDPase reaction. In king crabs, hypodermal FDPase exists in two forms, one which is highly sensitive to feedback inhibition by compounds such as UDP-acetylglucosamine and UDP-glucose (Hochachka 1972). Whenever these compounds accumulate due to a slowdown of chitin or glycogen synthesis, this FDPase would be inhibited, leading to an accumulation of FDP. The second form of FDPase is also subject to some feedback inhibition by high levels of UDP-acetylglucosamine (Hochachka, 1972). During extreme inhibition of chitin synthesis, this FDPase would be limited as well. While FDP is the most potent activator of both PK I and PK II, UDP-acetylglucosamine and glucosamine-6-phosphate are also activators. The accumulation of FDP would do



two things, initially an activation of PK II and then as the numbers of FDP molecules bound to the enzyme increased, PK II would change conformation, forming PK I. The high affinity of PK II for FDP would facilitate the transition. PK I would then funnel carbon into Krebs cycle, leading to both a drop in FDP and an increase in ATP levels. An extension of this model predicts that PK I would remain in this conformation until a sufficient drop in FDP levels would favour dissociation rather than binding. The decreased affinity for FDP could be significant in this context. Theoretically, other modulators could affect the rate of this transition, since alanine and MgATP partially prevent the FDP induced interconversion of the two forms (Table 13). However, I was not able to find another ligand capable of converting PK I into PK II. Thus, the binding and release of FDP seems the prime vehicle for conversion of the two forms.

The degree to which PK II can be inhibited would allow considerable flux through the pyruvate carboxylase and phosphoenolpyruvate carboxykinase bypass. The hypodermal levels of these enzymes are considerably higher in freshmolt than in intermolt animals (Table 1). In freshly molted animals, phosphoenolpyruvate carboxykinase, the rate limiting enzyme in the bypass (Scrutton & Utter, 1968; Exton & Park, 1969), has risen to 4% of the pyruvate kinase activity. This is tenfold higher than the maximal rate of pyruvate kinase reversal. Furthermore, the maximal velocity for PEP carboxykinase measured in the gluconeogenic direction may be higher (Pogson & Smith, 1975). Since, at physiological levels of PEP, ADP, ATP, alanine

and serine, PK II shows less than 0.5% of its maximal velocity, net flux through the enzymatic bypass is feasible. Some experiments in mammalian systems indicate that alternate routes of gluconeogenic flux past the pyruvate kinase reaction may exist (Veneziale et al., 1970). While I have no information as to their existence in crustacean hypodermis, the inhibition of pyruvate kinase would also be necessary with any alternative route which entered below the phosphoglycerate kinase reaction.

The regulatory properties of this pyruvate kinase are distinct from those of previously described pyruvate kinases in that FDP mediates a transition between two forms, each of which is sensitive to FDP activation. The mammalian L type shows a FDP mediated transition between two forms, but the activated form is not subject to further activation (Seubert & Schoner, 1971). The M2 type shows a high and a low affinity form with  $Mg^{++}$  and FDP promoting a slow transition between the forms. Of the two forms, only the high affinity form is sensitive to FDP activation (Van Berkel, 1974). A dimer-tetramer transition may be involved in this activation (Ibsen et al., 1975). A further distinction of the hypodermal pyruvate kinase is the lack of cooperativity of the substrate or modulator binding. This is in contrast to the enzyme from the hepatopancreas of Carcinus maenas (Giles et al., 1976) and other liver type enzymes from vertebrates and invertebrates (Kayne, 1973). Generally, the pyruvate kinases which show cooperativity of substrate binding show a decrease in cooperativity and an increase in affinity as the pH is decreased. This is taken to indicate the involvement of an ionizable group in the regulation of the enzyme (Seubert &

Schoner, 1971). In contrast, hypodermal pyruvate kinase showed a decrease in affinity for PEP as the pH was either increased or decreased from pH 7.0. As in both the L and M2 mammalian enzymes, FDP strongly affected PEP binding by both PK I and PK II. However, FDP activation of PK II showed another interesting feature; with both serine and  $Mg^{2+}$  citrate inhibition, addition of FDP not only relieved the effect on PEP binding, but also changed the pattern of inhibition versus ADP. In both cases, the inhibition versus ADP became uncompetitive. Thus, the regulatory effects of FDP extend to both substrate binding sites. Of all the inhibitors, only arginine phosphate is of questionable significance, since its presence in the tissue has not been demonstrated. The presence of arginine phosphokinase in hepatopancreas mitochondria (Chen & Lehninger, 1973) indicates that the enzyme and the phosphagen may have a wider distribution than previously considered. However, the effect exerted by arginine phosphate may simply testify to an evolutionary relationship between the hypodermal and muscle enzymes, since in many ways, the kinetics of PK I are similar to those of the muscle enzyme (see discussion). However, the muscle enzyme exists only in one functional form and exhibits rather different thermal denaturation behavior than either PK I or PK II from the hypodermis.

## Part V

## Cationic Composition of Intermolt and Freshmolt

## Hypodermis and Muscle

## INTRODUCTION

The enzymes which have been examined in this study are all affected by the four major intracellular cations, sodium, potassium, magnesium, and calcium. The phosphoglycerate kinases were particularly sensitive to the level of magnesium, since high levels of magnesium markedly increase the efficiency of ADP inhibition. Both the pyruvate kinases and the phosphoglycerate kinases display either a complete or a partial divalent cation requirement. Potassium is required for pyruvate kinase activity and strongly activates phosphoglycerate kinase activity. Calcium is a strong inhibitor of all of the enzymes examined. Sodium is an activator of the phosphoglycerate kinases and an inhibitor of the pyruvate kinases. The  $K_m$  and  $K_i$  values for these ionic effects are within or near the range of previously determined ion concentrations in crustacean muscle. While various studies have been made of muscle ion concentrations (Shaw, 1955; Dunham & Gainer, 1968; Lang & Gainer, 1969a), I was not able to find any measurements for hypodermal ion concentrations. Furthermore, no values exist for the intracellular concentrations in muscle of Cancer magister. Thus, in order to better evaluate the significance of the ionic effects upon these pyruvate kinases and phosphoglycerate

kinases, I measured the ionic composition of hypodermis and muscle from Cancer magister. Intracellular ion concentrations were determined by measuring the total tissue ion content and then correcting this for the ionic contribution of the extracellular space. Finally, the ionic compositions were expressed in terms of the concentration of the ion within the portion of the tissue occupied by the cells. Thus, extracellular space values for both muscle and hypodermis, hemolymph ion concentrations, and tissue ion contents needed to be determined.

During the molt cycle, the ionic composition of crustacean hemolymph undergoes considerable variation. Generally, the ions rise in concentration before ecdysis and then gradually drop during the postmolt period. In the marine crab, Carcinus maenas, calcium shows the most marked variation, increasing by 30% between intermolt and premolt, and then dropping 30% below the intermolt value in postmolt. Sodium and potassium show increases of approximately 10% (Robertson, 1960). The freshwater crayfish, Orconectes limosus, shows more marked premolt increases in  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{K}^+$  and maintains elevated  $\text{Ca}^{++}$  levels during postmolt (Andrews, 1967). While considerable information concerning crustacean body fluid composition and its variation with respect to the molt cycle (Robertson, 1960; Andrews, 1967; Glynn, 1968), salinity (Engelhardt, 1970; Alspach, 1972; Schmidt-Nielsen, 1975; Hunter & Rudy, 1975), and season (Dehnelt & Carefoot, 1965; Colvocoresses et al., 1974) is available, tissue analyses are much less common. Most studies of tissue ionic composition have concerned muscle and nerves

(Shaw, 1955; Dunham & Gainer, 1968; Lang & Gainer, 1969a; Schmidt-Nielsen, 1975) and have been concerned with the properties of excitable membranes, volume regulation, and the response to changing external salinities. No studies have specifically considered molt cycle correlated changes in ionic composition. Since hemolymph ion concentrations change with the molt cycle, tissue ion concentrations may fluctuate as well. Any major shifts in ionic composition would affect a variety of cellular control functions, would shift the equilibria of metal ion-metabolite complex formation (Cumme *et al.*, 1973), and certainly would affect the function of numerous enzymes. As explained above, phosphoglycerate kinases and pyruvate kinases from hypodermis and muscle of Cancer magister are affected by the four major cations. Furthermore, phosphofructokinase from lobster muscle and fructose diphosphatase from king crab hypodermis are also sensitive to the levels of  $K^+$  and  $Mg^{++}$  (Hochachka, 1972; Sugden & Newsholme, 1975b). Since these four enzymes are involved in the control of both glycolysis and gluconeogenesis, changes in ion levels could be of regulatory significance. To evaluate the regulatory potential of such changes, I measured the ion concentrations in muscle and hypodermis of both intermolt and freshmolt Cancer magister.

## METHODS

### Collection and Maintenance of Animals

Adult Cancer magister were collected by SCUBA diving at Jericho Pier, Vancouver, B. C. Both male and female animals

were collected. The animals were maintained in a refrigerated recirculating salt water system. Water for this system comes from an intake 6 m below mean low water spring tides, at First Narrows in the Vancouver Harbour. The salinity of the system was about  $27.3 \pm 1\text{‰}$  throughout the period of study. The ionic concentrations of local seawater corresponding to this salinity are  $\text{Na}^{++}$  370 mM,  $\text{K}^{+}$  8.2 mM,  $\text{Ca}^{++}$  8.05 mM, and  $\text{Mg}^{++}$  42 mM (Engelhardt, 1970). The temperature of the salt water system was maintained at 5-7 °C throughout the study. All animals were acclimated to the new conditions for at least one week. Only freshly molted animals and intermolt animals were used for this study, since these were the stages from which enzyme preparations had been made.

#### Tissue Preparation for Ion Measurements

Preliminary experiments indicated that the following method yielded the most consistent results of various possible ways of sample preparation. Individual crab legs were amputated, cut open, and used for preparation of 3-5 samples, of approximately 0.4 g, of both muscle and hypodermis. Each sample was quickly dipped into 0.4 M sucrose (less than 3 seconds submersion), blotted between two pieces of No. 1 filter paper, and weighed. Samples were weighed, ashed, and measured in glass scintillation vials. These vials were found not to contribute sodium or potassium to the samples. The samples were ashed at 450 °C (24 hours), cooled, and then taken up in the appropriate swamp. Blood samples were taken by inserting a hypodermic syringe into the arthrodial membrane and withdrawing approximately a 0.5 ml

sample. This was then emptied onto a clean petri plate. A calibrated Lang-Levy pipette was then used to deliver 50  $\mu$ l of blood into the sample vials. These blood samples were then simply diluted with the appropriate swamp. Distilled water was the swamp used for the measurement of sodium, 500 parts per million  $\text{Na}^+$  the swamp used for potassium, 1.5% EDTA the swamp used for magnesium, and 0.5% lanthanum chloride (as  $\text{La}^+$ ) the swamp used for calcium measurement.

### Determination of Ion Concentrations

Tissue ionic content was determined by flame photometry of the samples. Sodium was measured by its emission at 589.5 m $\mu$ , potassium by its emission at 766.8 m $\mu$ , magnesium by its absorbance at 285.8 m $\mu$ , and calcium by its absorbance at 423.5 m $\mu$ . A Techtron AA 120 Atomic Absorption Spectrophotometer was used in these studies with an air-acetylene flame. Once tissue and blood ion contents were determined, the intracellular ionic concentrations were determined by correcting the tissue ion content for the ions due to the extracellular space. This proceeded according to the following calculation:

$$\text{Intracellular ion concentration} = \frac{\text{Tissue Ion Content} - \text{Ions in ECS}}{\text{Tissue grams} - \text{ECS grams}}$$

### Determination of Extracellular Space

Three intermolt and three freshmolt animals were injected with  $^{14}\text{C}$  inulin (dose approximately 1  $\mu$ curie per 10 g body weight). At hourly intervals, blood samples were taken and analyzed for radioactivity. When the  $^{14}\text{C}$  inulin in the blood

had equilibrated, 1 leg per individual was amputated below the main joint to the body, so that the animal would not need to be sacrificed. Six hours later further samples were taken. Immediately after sampling, the legs were cut open, and 3 semi-uniform samples of both muscle and hypodermis dissected out. The samples were quickly dipped into isosmotic sucrose, thoroughly blotted between two pieces of No. 1 filter paper to remove adhering surface blood, and then weighed. Four drops of 10% KOH were added to the vials and allowed to digest overnight. The digested samples were neutralized with  $H_2SO_4$ , and counted in 10 ml of Aquasol or Scintiverse. Blood samples taken just before the tissue samples were counted simultaneously and used to determine the amount of extracellular space within the respective tissues. Separate extracellular space determinations were not made for all the tissues used in this study; rather, the means for freshmolt and intermolt muscle and hypodermis were used to calculate the intracellular ionic concentrations in the respective tissues.

## RESULTS AND DISCUSSION

### Extracellular Space Determination

Table 14 shows the results of the extracellular space determinations for muscle and hypodermis. The results show clearly that there is a large difference in extracellular space between the muscle and the hypodermis, but that the difference between intermolt and freshmolt tissues is small. The muscle extracellular space values are similar to those found by

Table 14 Extracellular Space in Muscle and Hypodermis.

Measurements were made as described in Materials and Methods. Each measurement represents the mean of four determinations on one individual animal.

		<u>Mean</u>
Intermolt muscle	14.16%	
	10.94%	12.04%
	12.04%	
Intermolt hypodermis	39.62%	
	41.40%	43.64%
	49.94%	
Freshmolt hypodermis	41.90%	
	48.62%	45.27%
	45.30%	
Freshmolt muscle	11.16%	
	13.61%	12.34%
	12.44%	

Robertson (1961) for the Norway lobster, higher than the 9% value found by Dunham & Gainer (1968) for Homarus americanus, lower than those for Callinectes sapidus (Lang & Gainer, 1969b), and somewhat higher than those found by Alspach (1972) for Cancer magister in high salinities. Alspach (1972) indicated that the muscle extracellular space increased as the animals adapted to dilute salinities. The lack of change in extracellular space with the transition from intermolt to freshmolt indicates that the uptake of fluid during the molt does not affect the volume of extracellular fluid associated with a tissue.

#### Ionic Composition of the Hemolymph

Table 15 shows the mean values for hemolymph cation concentrations of intermolt and freshmolt animals. The volume corrected mean reflects the calibration of the Lang-Levy pipette. There was no significant difference between intermolt and freshmolt  $K^+$  and  $Mg^{++}$  concentrations. There was a significant difference between calcium and sodium concentrations in intermolt and freshmolt hemolymph, with sodium being higher and calcium lower in freshmolt hemolymph. The low calcium level may reflect increased calcium mobilization during the initial calcification period. Robertson (1960) found increased sodium values during late premolt and just after molt, in parallel with the results of this study. When these ion concentrations are compared to those of the medium (see Methods in this section), magnesium stands out as the major ion whose concentration is controlled. Cancer magister strongly hyporegulates magnesium

Table 15 Hemolymph Ion Concentrations.

Each mean value represents the mean of the ion concentrations in as many individual animals as is indicated under "N". The volume corrected mean expresses these ion concentrations in terms of the actual volume transferred by the Lang-Levy pipettes used for sample measurement. P expresses the results of Student's t test comparison of molting and intermolt ion concentrations. V.C. mean indicates volume corrected mean. P = 0.05 was the level of significance.

	Intermolt				Freshmolt				
	<u>Mean</u>	<u>Stan. Dev.</u>	<u>N</u>	<u>V.C. Mean</u>	<u>Mean</u>	<u>Stan. Dev.</u>	<u>N</u>	<u>V.C. Mean</u>	<u>P</u>
K <sup>+</sup>	8.15 mM	±1.13	9	8.57 mM	8.19 mM	±0.68	8	8.62 mM	N.S.
Na <sup>+</sup>	346.5 mM	±23.5	9	364.7 mM	376.85 mM	±16.7	8	396.7 mM	=0.01
Mg <sup>++</sup>	14.53 mM	±2.36	9	15.29 mM	15.03 mM	±0.67	8	15.82 mM	N.S.
Ca <sup>++</sup>	9.35 mM	±0.89	10	9.84 mM	6.72 mM	±0.3	8	7.07 mM	=.000001

concentrations (14 mM in the blood versus 42 mM in the medium). Intermolt calcium levels are very similar to those in the medium while freshmolt levels were somewhat lower. Hyporegulation of magnesium and hyperregulation of calcium was shown by Engelhardt (1970) for Cancer magister from local waters, by Hunter and Rudy (1975) for Cancer magister from Oregon, as well as by Colvocoresses et al., (1974) for Callinectes sapidus.

### Ionic Composition of Muscle and Hypodermis

Table 16 shows the intermolt and freshmolt muscle ion concentrations. For the sample size examined, only sodium levels showed a significant difference ( $P=0.05$ ) between freshmolt and intermolt muscle. The higher sodium levels in freshmolt muscle correspond to the increase in hemolymph sodium in freshmolt animals. The calcium, magnesium and potassium concentrations were similar for intermolt and freshmolt muscle.

Table 17 shows the intermolt and freshmolt hypodermal ion concentrations. The mean values for freshmolt and intermolt hypodermis were similar for each ion, although there was considerable variation in the samples, particularly for intermolt calcium and potassium values. For each ion the null hypothesis that the cation concentrations in freshmolt and intermolt hypodermis were the same was accepted ( $P>0.05$ ). The variability of the samples may be due to a lack of homogeneity within the tissues, or to small variations in the amount of extracellular space in the tissues. The latter would be a particular problem in the measurement and calculation of sodium

Table 16 Freshmolt and Intermolt Muscle Ion Concentrations.

For each experimental animal a mean ion concentration was determined from three to four separate samples. The mean value presented in this table represents the mean of these means. All values have been corrected for the ionic contribution of the extracellular space in the tissue according to the formula in Materials and Methods.  $P = 0.05$  was the significance level utilized.

	Intermolt			Freshmolt			<u>P</u>
	<u>Mean</u>	<u>Stan. Dev.</u>	<u>N</u>	<u>Mean</u>	<u>Stan. Dev.</u>	<u>N</u>	
Na <sup>+</sup>	45.32 mM	±22.7	5	89.81 mM	±4.77	4	=.045
K <sup>+</sup>	112.70 mM	±10.67	4	114.00 mM	±19.8	4	N.S.
Ca <sup>++</sup>	2.79 mM	±1.40	5	2.87 mM	±1.43	4	N.S.
Mg <sup>++</sup>	12.23 mM	±2.74	4	13.43 mM	±2.1	4	N.S.

Table 17 Freshmolt and Intermolt Hypodermal Ion Concentrations.

For each experimental animal, a mean ion concentration was determined from three to four tissue samples. The mean value presented in this table represents the mean of these means. All values have been corrected for the ionic contribution of the extracellular space.  $P = 0.05$  was chosen as the level of significance.

	<u>Mean</u>	<u>Intermolt</u> <u>Stan. Dev.</u>	<u>N</u>		<u>Mean</u>	<u>Freshmolt</u> <u>Stan. Dev.</u>	<u>N</u>	<u>P</u>
Na <sup>+</sup>	49.51 mM	±22.7	4		52.4 mM	±19.77	3	N.S.
K <sup>+</sup>	74.7 mM	±19.7	5		70.81 mM	±7.64	4	N.S.
Ca <sup>++</sup>	16.78 mM	±11.88	5		16.07 mM	±4.86	4	N.S.
Mg <sup>++</sup>	19.33 mM	±4.23	4		15.90 mM	±3.02	4	N.S.

concentrations, since sodium levels in the hemolymph are 5-6 fold those in the hypodermis. In contrast to the muscle, hypodermis shows no increase in intracellular sodium between intermolt and freshmolt animals.

Comparison of hypodermal and muscle ion concentrations from both intermolt and freshmolt animals reveals several marked differences (Table 18). In both molting and intermolt tissues, calcium levels are significantly higher ( $P=0.05$ ) in the hypodermis than in muscle. Potassium is significantly higher in intermolt muscle than intermolt hypodermis, and while the difference is not significant for molting tissues, it is still large. Neither magnesium nor sodium show a significant difference between muscle and hypodermis. There appears to be a difference in sodium levels of molting tissues, but a larger sample size would be necessary to confirm this (not significant at  $P=0.05$ ). The levels of potassium and sodium are more similar in the hypodermis than in muscle. Calcium provides a greater ionic contribution in hypodermis than in muscle. Magnesium levels are approximately the same as calcium levels in hypodermis, while in muscle, magnesium is present in four-fold higher concentrations than calcium. Thus, the relationships between the major cations show notable differences in muscle and hypodermis.

A comparison of these muscle ion concentrations with ion concentrations in other crustaceans shows that Cancer magister muscle has a lower potassium level than found in Homarus americanus and Homarus vulgaris (Dunham & Gainer, 1968).

Table 18 Comparison of Muscle and Hypodermal Ion Concentrations.

Means are taken from the other tables. Although  $P = 0.05$  is the chosen level of significance,  $P$  values which lie near this level are also presented.

INTERMOLT							
	Muscle			Hypodermal			<u>P</u>
	<u>Mean</u>	<u>Stan. Dev.</u>	<u>N</u>	<u>Mean</u>	<u>Stan. Dev.</u>	<u>N</u>	
Na <sup>+</sup>	42.32 mM	±22.7	5	49.51 mM	±22.7	4	N.S.
K <sup>+</sup>	112.7 mM	±10.67	4	74.7 mM	±19.7	5	=0.014
Ca <sup>++</sup>	2.79 mM	±1.40	5	16.78 mM	±11.88	5	=0.05
Mg <sup>++</sup>	12.23 mM	±2.74	4	19.33 mM	±4.23	4	N.S.

FRESHMOLT							
	Muscle			Hypodermal			<u>P</u>
	<u>Mean</u>	<u>Stan. Dev.</u>	<u>N</u>	<u>Mean</u>	<u>Stan. Dev.</u>	<u>N</u>	
Na <sup>+</sup>	89.82 mM	±4.77	4	52.4 mM	±19.77	3	=0.08
K <sup>+</sup>	114.0 mM	±19.8	4	70.8 mM	±7.64	4	=.06
Ca <sup>++</sup>	2.87 mM	±1.43	4	16.08 mM	±4.86	4	=.013
Mg <sup>++</sup>	13.43 mM	±2.1	4	15.91 mM	±3.02	4	N.S.

However  $K^+$  levels are similar to those in Carcinus muscle (Shaw, 1955). The sodium values are in the same range or lower than most previously published results (Robertson, 1961; Dunham & Gainer, 1968; Mackay & Prosser, 1970). The calcium concentration found in Cancer magister muscle was considerably lower than that in king crab muscle (Mackay & Prosser, 1970) or Carcinus muscle (Shaw, 1955). By contrast, magnesium levels were higher than those of king crabs, and lower than those of Carcinus maenas. The concentrations of both magnesium and calcium in Carcinus maenas hemolymph (Shaw, 1955), and magnesium in king crab hemolymph (Mackay & Prosser, 1970), were considerably higher than the corresponding concentrations in the hemolymph of Cancer magister. This may account for the difference in muscle magnesium and calcium levels. No values for ion concentrations in hypodermis of other crustaceans are available for comparison. However, in other animals, when intracellular sodium is high, the intracellular potassium concentration is low (Schmidt-Nielsen, 1975). This pattern holds true for hypodermis. The total sum of these four cations in both muscle and hypodermis is similar to the values found in other organisms (Mackay & Prosser, 1970; Schmidt-Nielsen, 1975).

When one compares the cation concentrations in the two tissues (Tables 16 & 17, or 18) with the cation concentrations in the hemolymph, several conclusions can be made. Both hypodermis and muscle accumulate potassium and extrude sodium. The concentrations of magnesium in the two tissues are only slightly different from the concentrations in the hemolymph. The muscle maintains slightly lower magnesium concentrations,

while the hypodermis has slightly elevated levels. The tissues regulate their calcium concentrations in opposite ways; the hypodermis seems to accumulate calcium, while the muscle seems to exclude calcium. In the king crab, muscle maintains only slightly lower levels of calcium than the hemolymph, while the magnesium concentration is considerably lower than the hemolymph concentration (Mackay & Prosser, 1970). In Carcinus maenas muscle, magnesium and calcium levels are regulated in much the same way as in Cancer magister (Shaw, 1955). The higher levels of calcium in the hypodermis are probably related to calcification and repair of the exoskeleton.

Although these concentration measurements do not represent the actual activities of the ions in the cells, they do present guidelines as to the concentrations which the enzymes encounter in the cell. Thus, one can say that the competitive inhibition between  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  will be of small importance in muscle, where  $\text{Mg}^{++}$  levels are 4-fold higher than calcium levels. In the hypodermis, however,  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$  concentrations are approximately the same. Thus, unless there is significant compartmentalization and binding of these divalent cations, competitive inhibition by  $\text{Ca}^{++}$  of  $\text{Mg}^{++}$  activation may play a significant role in regulating the activity of such enzymes as pyruvate kinase and phosphoglycerate kinase. Pyruvate kinase is particularly sensitive to calcium inhibition, with a  $K_i$  versus magnesium of  $7.5 \times 10^{-5} \text{M}$ . For phosphoglycerate kinase,  $\text{Ca}^{++}$  inhibition is most effective versus the binding of 3-phosphoglycerate,  $K_i = 2 \text{mM}$ , while the inhibition versus  $\text{Mg}^{++}$  shows a  $K_i$  value of 20 mM. Of the two enzymes, pyruvate kinase is thus

more susceptible to inhibition by calcium. Pyruvate kinase must be inhibited for gluconeogenesis to occur while phosphoglycerate kinase is one of the functional enzymes in the gluconeogenic pathway. Thus, in the context of gluconeogenesis, these differential sensitivities to calcium inhibition make functional sense. There is no significant difference between calcium levels in intermolt and freshmolt hypodermis, so differential calcium inhibition during the various parts of the molt cycle does not come into play in the regulation of these enzymes.

Potassium levels in all tissues are high enough to fully activate pyruvate kinase ( $K_a = 15$  mM). Since the  $K_i$  for sodium inhibition versus phosphoenolpyruvate binding is 40 mM, and the  $K_i$  versus  $K^+$  is 85 mM, sodium inhibition will probably be of little significance. Potassium and sodium both activate phosphoglycerate kinase. For intermolt tissues, the  $K_a$  values for sodium activation are 100 mM, well above intracellular sodium levels. In molting tissues, sodium levels are higher than in intermolt tissues. The  $K_a$  values for sodium activation in molting tissues drop to 30 mM and 19 mM for muscle and hypodermis, respectively. Thus, sodium activation in molting tissues is more feasible. Potassium  $K_a$  values do not fluctuate much throughout the molt cycle, remaining around 80 mM for both hypodermal and muscle phosphoglycerate kinase. In muscle, potassium levels are approximately 110 mM, while in hypodermis, potassium levels are lower, near 70 mM. Thus, activation of phosphoglycerate kinase by potassium would be appreciable, but would not fluctuate between intermolt and freshmolt. While the data on the effects of these four cations on crustacean phospho-

fructokinase are not available, magnesium is required for its catalytic activity and potassium activates between 0 and 50 mM and inhibits between 100 and 200 mM (Sugden & Newsholme, 1975b). Depending upon the degree of association of  $K^+$  with tissue proteins,  $K^+$  lies in the inhibitory concentration range within the muscle cells, and on the borderline of this inhibitory range in the hypodermis.  $K^+$ ,  $Na^+$ , and  $Mg^{++}$  all have profound effects upon the activity of hypodermal fructose diphosphatase (Hochachka, 1972). Sodium is inhibitory at all concentrations tested,  $Mg^{++}$  or  $Mn^{++}$  is required for catalysis, and  $K^+$  activates at levels up to 100 mM.  $K^+$  also accentuates the AMP inhibition of the enzyme, while  $Mg^{++}$  reverses this inhibition. The ionic concentrations which affect enzyme activity are within the intracellular concentration range. Therefore, the interactions between  $K^+$ ,  $Mg^{++}$ , AMP, and FDP provide an important element in the control of hypodermal FDPases (Hochachka, 1972). Thus, even though these cations probably do not play a role in quick changes of enzyme activity, their concentrations could partially define the limits of catalytic activity.

## GENERAL DISCUSSION AND SUMMARY

These studies of phosphoglycerate kinase and pyruvate kinase from Cancer magister have led to several important conclusions concerning both the regulatory properties of the enzymes from muscle and hypodermis as well as the possible enzymatic adaptations which facilitate efficient gluconeogenesis and glycolysis in the same tissue. This study has also provided some insight into the changes in metabolic organization which occur in the muscle and hypodermis during the molt cycle, as well as allowing evaluation of the potential for gluconeogenesis in the muscle of Cancer magister. To conclude this study, the major implications of these findings will be discussed, along with questions leading to further experimentation.

The requirement for efficient gluconeogenesis in crustaceans arises from the periodic shedding of the old exoskeleton and the subsequent synthesis of a new larger chitinous exoskeleton. In most crustaceans, the synthesis of the new exoskeleton is supported by resorbed material, as well as by endogenous stores (Renaud, 1949; Heath & Barnes, 1970; Speck et al., 1971). Experiments with Orconectes limosus indicate that the resorbed material undergoes initial conversion into chitin, amino acids, and lactate. The latter two compounds are subsequently utilized for both chitin synthesis and energy provision (Herz-Hübner et al., 1973). Similar conversions into amino acids and lactate occur with hemolymph glucose and ingested glucose in Orconectes limosus (Speck & Urich, 1969; Urich et al., 1973; Herz-Hübner & Urich, 1973) and in other

crustaceans (Huggins, 1966; Huggins, 1969; Shewbart *et al.*, 1972). Thus, the metabolic routes for the conversion of amino acids and lactate into polysaccharides are emphasized during the biosynthesis of the chitinous exoskeleton. Utilization of endogenous stores would augment these basic metabolic flux patterns.

### Hypodermal Gluconeogenesis

Measurement of enzyme activities indicates that gluconeogenesis in crustaceans can follow the same pathway utilized in vertebrates (Scrutton & Utter, 1968). Thus, pyruvate carboxylase and fructose diphosphatase are present in all tissues, while phosphoenolpyruvate carboxykinase is present in all tissues except intermolt muscle. The effective functioning of these bypass enzymes is partially dependent upon the activity of the corresponding glycolytic reaction. In both the freshmolt and intermolt hypodermis, fructose diphosphatase levels are three fold higher than those of phosphofructokinase. Thus, net gluconeogenic flux through this reaction locus is feasible. By contrast, pyruvate kinase levels are approximately 20 fold higher than those of the bypass enzymes. Gluconeogenic flux through this reaction locus necessitates effective inhibition of pyruvate kinase. At physiological levels of PEP, ADP, ATP, alanine, and serine, the low affinity form of hypodermal pyruvate kinase (PK II) shows less than 0.2% of its maximal activity. Thus, net flux through the pyruvate carboxylase, phosphoenolpyruvate carboxykinase bypass is possible. The maximal gluconeogenic flux through the bypass is approximately

30% of the maximal FDPase activity. As mentioned before, the values for PEPCK may be low, since the enzyme was not measured in the gluconeogenic direction (Chang *et al.*, 1966; Pogson & Smith, 1975). The other possible "bottleneck" in the gluconeogenic pathway occurs at the phosphoglycerate kinase reaction. Phosphoglycerate kinase levels, measured in the gluconeogenic direction, are considerably higher than those of PEPCK, pyruvate carboxylase, and FDPase. Furthermore, both the hypodermal and the freshmolt muscle enzymes show kinetic modifications which facilitate the reversal of the reaction for gluconeogenesis. The presence of glutamine: fructose-6-phosphate amidotransferase in the hypodermis and in freshmolt muscle indicates that the fructose-6-phosphate generated by the FDPase reaction can be directed towards chitin synthesis.

### Muscle Gluconeogenesis

The various enzymes in the gluconeogenic sequence are present in freshmolt muscle. However, the ratios of phosphofructokinase to FDPase and of pyruvate kinase to phosphoenolpyruvate carboxykinase and pyruvate carboxylase are considerably higher than in the hypodermis. The control mechanisms of Cancer magister muscle phosphofructokinase are not known exactly. However, extrapolation from the control properties of lobster and king crab phosphofructokinases indicates that at physiological fructose-6-phosphate levels, high levels of ATP and arginine phosphate would cause more than 75% inhibition (Freed, 1971; Sugden & Newsholme, 1975b). This would allow net gluconeogenic flux through the reaction locus. Pyruvate kinase

is present at nearly 500 fold the levels of its bypass enzymes. Unlike the hypodermal pyruvate kinase, the muscle enzyme does not exist in interconvertible high and low affinity states. Thus, the kinetic characteristics of muscle pyruvate kinase make it highly unlikely that any combination of inhibitors could reduce enzyme activity to less than 0.2% of  $V_{max}$ . In contrast to rabbit muscle pyruvate kinase, where the reverse reaction accounts for 5% of the glycolytic reaction (Dyson et al., 1975), the reverse reaction of the enzyme from Cancer magister muscle accounted for only 0.5% of the forward reaction. Maximal reversal occurred at 10 mM pyruvate, a highly unphysiological concentration. Although the kinetic modifications of freshmolt muscle phosphoglycerate kinase would facilitate gluconeogenesis, the characteristics of the pyruvate kinase locus are such as to prevent net gluconeogenic flux from lactate, pyruvate, or alanine. This conclusion contrasts with the findings of Behrisch (1976) who reports both high levels of the bypass enzymes and high rates of gluconeogenesis from lactate and pyruvate in Paralithodes camtchatica muscle (Behrisch, 1976). The levels of FDPase, PEPCK, and pyruvate carboxylase found by Behrisch (1972, 1976) are considerably higher than those found in the present study or those found by Opie and Newsholme (1967) and by Crabtree et al. (1972) in the muscle of various invertebrates including Carcinus maenas and Maia squinado. Unless there is significant compartmentalization of enzyme activities, which somehow succeeds in separating the phosphoenolpyruvate used by pyruvate kinase as a substrate from the phosphoenolpyruvate produced by phosphoenolpyruvate carboxy-

kinase, net gluconeogenic flux through this reaction locus is not likely to occur in Cancer magister muscle.

#### Molt Cycle Variations in Enzyme Levels

Both hypodermal and muscle enzyme activities were found to change during the molt cycle. Enzyme levels in the hypodermis generally increased during the transition from intermolt to molt. This is diagnostic of the increase in metabolic activity of the hypodermis during premolt and postmolt, as well as the increase in amount of protein per DNA during premolt (Stevenson, 1972). While muscle enzyme activities generally decrease during the transition from intermolt to freshmolt, some enzymes, such as FDPase, PEPCK and pyruvate carboxylase are present in increased levels in the muscle of freshly molted animals. As discussed in the previous paragraph, this increased gluconeogenic potential is probably not utilized for synthesis of polysaccharides from amino acids and lactate. Other gluconeogenic precursors such as glycerol may be utilized. In this context, some of the observations of Speck and Urich (1972) on Orconectes limosus are of interest. These workers found that while considerable carbon from resorbed acetylglucosamine was incorporated into various compounds in the muscle, the distribution of labelled carbon in these compounds did not change if one compared the muscle of late premolt, early postmolt and partially hardened animals. While some of the carbon from acetylglucosamine was converted into polysaccharides in the muscle, the bulk of the label stayed in the fraction with the intermediary compounds, including amino acids, small organic

acids, and sugars. In other studies (Herz-Hübner & Urich, 1973), it was found that only a small proportion of acetylglucosamine is released from the resorbing tissues unchanged. Thus, label which entered the muscle was in the form of small intermediary compounds. These studies indicate that in these crayfish, muscle plays a relatively quiescent role during the biosynthesis of the exoskeleton during premolt and postmolt. However, this situation may be somewhat altered in animals with large, long appendages, where the muscle forms the bulk of the tissue in the vicinity of the hypodermis and the nascent exoskeleton. While this is an interesting concept, the present study does not provide supportive evidence.

#### Control of Glycolysis and Gluconeogenesis in the Hypodermis

While hypodermal enzyme activities indicate that the tissue is poised towards gluconeogenesis, they also show that it maintains an ample glycolytic capacity. The information gleaned from this study, coupled with previous work on the control properties of the hypodermal FDPase from Paralithodes cam-  
chatica (Hochachka, 1972), allows the formulation of a metabolic control theory describing the means by which hypodermal metabolism switches from gluconeogenesis to glycolysis. During gluconeogenesis from lactate or amino acids such as alanine, glutamate and aspartate, the levels of ATP and the overall energy charge are high. The high levels of ATP and the high energy charge would combine to inhibit pyruvate kinase and presumably phosphofructokinase. Since hypodermal levels of phosphofructokinase lie below those of fructose diphosphatase,

only partial inhibition of phosphofructokinase is necessary to achieve appreciable gluconeogenic flux. The high energy charge would serve to activate both FDPases present in the hypodermis, since high energy charge indicates low levels of AMP, a potent inhibitor of these and other FDPases (Hochachka, 1972). The low levels of UDP-acetylglucosamine which would occur during the initiation of chitin synthesis activate FDPase II by decreasing the  $K_m$  for fructose 1,6 diphosphate (Hochachka, 1972). This activation of FDPase would lead to a decrease in levels of fructose 1,6 diphosphate. This decrease in FDP would shift the equilibrium between hypodermal PK I and PK II in favor of the low affinity form, PK II, which is more susceptible to inhibition by amino acids and citrate (Figure 27, p. 156). In addition, PK II has such a low affinity for phosphoenolpyruvate that its glycolytic function would be limited in the presence of physiological PEP concentrations. Thus, the shift in pyruvate kinase enzymes from the high affinity to the low affinity conformation would effectively inhibit pyruvate kinase and allow flux through the enzymatic bypass. The exact means whereby flux through this bypass is activated are not known. In the mammalian liver, high levels of ATP favor both the conversion of pyruvate to oxaloacetate and the conversion of oxaloacetate to phosphoenolpyruvate (Newsholme & Start, 1973; Garber & Salganicoff, 1973). Cycling around this reaction locus may provide costly, but precise regulation of net gluconeogenic flux (Newsholme & Start, 1973), or it may be utilized to support the transfer of reducing units, for purposes other than gluconeogenesis, from the mitochondria to the cytoplasm (Meijer

& Williamson, 1974).

High levels of ATP and low levels of ADP, characteristic of a high energy charge, also favor the gluconeogenic reversal of phosphoglycerate kinase. The enzyme present in the hypodermis of the freshly molted animal shows a high affinity for ATP compared to the intermolt muscle enzyme. Furthermore, the sensitivity to ADP and MgADP inhibition is considerably reduced in the hypodermal forms. These two kinetic modifications combine to facilitate the reversal of the reaction. Removal of 1,3 diphosphoglycerate by the glyceraldehyde-3-phosphate dehydrogenase, would also favor reversal of the phosphoglycerate kinase reaction by a mass action effect. Mammalian glyceraldehyde-3-phosphate dehydrogenase shows kinetic modifications which facilitate the reductive dephosphorylation during the high NAD/NADH ratios characteristic of energy saturating conditions (Smith & Velick, 1972). Since similar kinetics are shown by both the mammalian liver and muscle enzymes, and since crustacean muscle and mammalian muscle glyceraldehyde-3-phosphate dehydrogenases are much the same (Trentham, 1971; De Vijlder *et al.*, 1969; Harrigan & Trentham, 1974), similar kinetic modifications probably hold for the hypodermal enzyme. Thus, at low levels of NADH and 1,3 diphosphoglycerate, NAD stimulates the binding of these substrates at one subunit, while itself binding at the other three. At high levels of NADH and 1,3 diphosphoglycerate, NAD competes for the binding site, but at low levels, it promotes the binding of 1,3 diphosphoglycerate. NAD binding at the acylated site is restricted, while NADH binding is favored. Thus, in the face of the high

NAD/NADH ratio which accompanies energy saturating conditions, the high affinity of the dehydrogenase for NAD does not inhibit the gluconeogenic reversal of the reaction, but instead facilitates it (Smith & Velick, 1972). ATP and creatine phosphate inhibit the glycolytic reaction of mammalian muscle glyceraldehyde-3-phosphate dehydrogenase (Oguchi *et al.*, 1973). These compounds may possibly facilitate the reverse reaction, thus stimulating gluconeogenic flux through both phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase.

#### Integration of Gluconeogenesis and Lipid Oxidation

This model of gluconeogenic control mechanisms in the crustacean hypodermis has not touched upon a number of additional regulatory interactions. These deal primarily with the effects of lipid oxidation on gluconeogenesis. In species with an appreciable cytosolic phosphoenolpyruvate carboxykinase activity, lipid oxidation during periods of gluconeogenesis has a stimulatory effect (Williamson *et al.* 1969; Cornell *et al.*, 1974). This effect is thought to arise from the high levels of citrate and acetyl CoA which result from lipid utilization. There is evidence for similar effects during gluconeogenesis in crustaceans. Citrate is a potent inhibitor of hypodermal PK II, and presumably of phosphofructokinase. As is true for other pyruvate carboxylases (Ashman *et al.*, 1972), the enzyme present in Cancer magister tissues shows a dependence upon acetyl CoA for catalytic activity. Full stimulation of activity came at 0.05 mM acetyl CoA. High levels of acetyl CoA would inhibit pyruvate dehydrogenase, leading to an accumulation of pyruvate

(Guder & Wieland, 1974). Increased levels of pyruvate would then act in conjunction with the high levels of acetyl CoA to activate pyruvate carboxylase. Increased formation of oxaloacetate would then facilitate PEP formation from oxaloacetate by PEPCK.

By contrast, in species which have higher levels of phosphoenolpyruvate carboxykinase in the mitochondria than in the cytoplasm, lipid oxidation inhibits gluconeogenesis (Söling *et al.*, 1973; Jomain-Baum & Hanson, 1975). This inhibitory effect is directly related to a decrease in the mitochondrial NAD/NADH ratio, which leads to a marked decrease in the rate of phosphoenolpyruvate formation. Increased levels of ammonium chloride (1 mM) reverse this inhibition and lead to an increase in the NAD/NADH ratio, via the glutamate dehydrogenase reaction. During perfusion of isolated liver, further increases in ammonium ion (2 mM) did not relieve the inhibition until their addition was terminated. These high levels of ammonium ion led to considerable increases in the levels of both glutamate and aspartate. Aspartate was derived from oxaloacetate, thus reducing the availability of this compound for PEP formation (Jomain-Baum & Hanson, 1975). Thus, the NAD/NADH ratio plays a central role in modulating the rate of mitochondrial PEP formation. However, competition between aspartate aminotransferase and phosphoenolpyruvate carboxykinase will also strongly affect the rate of PEP formation. The separate regulation of the cytoplasmic and the mitochondrial NAD/NADH ratios may explain the lack of inhibition of cytoplasmic PEP formation during fatty acid oxidation. In Cancer magister,

phosphoenolpyruvate carboxykinase activity is present in both the mitochondria and the cytoplasm, but its exact distribution between the two compartments has not been determined. Thus, no conclusions can be drawn about the net effects of lipid oxidation on hypodermal gluconeogenesis. However, the effects of citrate upon PK II and acetyl CoA upon pyruvate carboxylase shown in the present study could well be of physiological significance.

#### Inhibition of Gluconeogenesis and Activation of Glycolysis

Inhibition of gluconeogenesis can occur under various metabolic conditions including the depletion of ATP supplies or the production of adequate quantities of polysaccharide. Information regarding adequate synthetic activity would probably be hormonal and would have its initial effect upon glycogen synthetase or chitin synthetase function. The inhibition due to lack of ATP would occur particularly frequently if carbohydrate formed the major substrate for energy provision as well as the substrate for biosynthetic activity. Since carbohydrate is one of the major substrates utilized for energy provision during premolt and postmolt in the hypodermis (Speck & Urich, 1971; Herz-Hübner & Urich, 1973), cyclic variations in glycolytic and gluconeogenic flux can be expected. When ATP levels decline, ADP and AMP levels rise. As indicated above, AMP is a potent inhibitor of most FDPases. Furthermore, it is a strong activator of phosphofructokinases. Thus, the effect of increases in AMP is amplified, since it affects the two sides of the phosphofructokinase-fructose diphosphatase cycle in opposite

ways (Newsholme & Start, 1973). A paucity of ATP would lead to an accumulation of carbohydrate precursors, including fructose-6-phosphate and either UDP-acetylglucosamine or UDP-glucose (depending upon the polysaccharide currently being synthesized). An accumulation of fructose-6-phosphate would cause an activation of phosphofructokinase, while UDP-acetylglucosamine and UDP-glucose both inhibit hypodermal FDPase II (Hochachka, 1972).

The inhibition of gluconeogenesis due to decreased levels of ATP would also affect the second control site, by deinhibiting the pyruvate kinase reaction. The increased levels of FDP, which would occur after the inhibition of fructose diphosphatase, would activate pyruvate kinase in two ways. First, FDP would increase the catalytic rate by activating the binding of PEP and reversing the inhibition due to ATP and amino acids. Furthermore, the increased levels of FDP would promote the conversion of the low affinity PK II into the high affinity form, PK I (Figure 27, p. 156). This conversion would lead to a considerable increase in glycolytic flux through this reaction locus. Increased levels of ADP would also activate glycolytic flux, while inhibiting both the pyruvate carboxylase and the PEP carboxykinase reactions (Newsholme & Start, 1973; Garber & Salganicoff, 1973).

When gluconeogenesis is inhibited due to an effect upon chitin synthetase or glycogen synthetase, the regulatory effects would be expressed at several loci as well. The initial effect, in the case of an inhibition of chitin synthetase, would be an

accumulation of UDP-acetylglucosamine. This would lead to an inhibition of both glutamine: fructose-6-phosphate amidotransferase and fructose diphosphatase (Winterburn & Phelps, 1971a; Hochachka, 1972). While glutamine: fructose-6-phosphate amidotransferase has not been examined in crustaceans, its properties have been characterized in rat liver, locust hypodermis, and Drosophila salivary glands (Winterburn & Phelps, 1971a; Winterburn & Phelps, 1971b; Kress & Enghofer, 1975; Surholt, 1975). The enzyme from all sources is subject to feedback inhibition by UDP-acetylglucosamine, a mechanism which effectively prevents further accumulation of the compound. Both FDPase I and FDPase II from king crab hypodermis are sensitive to inhibition by this metabolite. FDPase II is subject to 80% inhibition by 1 mM UDP-acetylglucosamine while FDPase I is inhibited by 50% by 2.5 mM levels of this compound (Hochachka, 1972). Thus, partial inhibition of flux through the FDPase reaction can be achieved by presumably moderate levels of UDP-acetylglucosamine. Besides inhibiting FDPase, UDP-acetylglucosamine activates pyruvate kinase. This activation occurs via a facilitation of substrate binding and a reversal of ATP inhibition. If glycogen synthesis is activated during inhibition of chitin synthesis, FDPase I would continue to provide fructose-6-phosphate and maintain low levels of fructose 1,6 diphosphate. These would keep pyruvate kinase in its low affinity conformation. Thus, in the absence of complete inhibition of both FDPase I and FDPase II, high levels of UDP-acetylglucosamine would only create a small increase in pyruvate kinase activity. The sensitivity of both FDPase II and pyruvate

kinase to the endproducts of chitin synthesis distributes control over the two rate limiting steps in the pathway, and allows selective channeling of fructose-6-phosphate into glycogen, chitin or energy metabolism. During premolt and postmolt, when chitin is the primary product of the hypodermis, FDPase II levels may increase, allowing greater regulatory specificity.

Thus, the enzymes in the crustacean hypodermis have a number of specific modifications which allow a high degree of control over the two major "bottlenecks" in glycolysis and gluconeogenesis. Specific characteristics which are unique to the crustacean hypodermis are the multiple forms of fructose diphosphatase and the high and low affinity forms of pyruvate kinase. A further distinctly gluconeogenic modification occurs in the molt cycle specific variants of phosphoglycerate kinase. A comparison of the control mechanisms present in the hypodermis with those present in the muscle, indicates that these control characteristics must have evolved in answer to the critical need for efficient gluconeogenesis during the initial postmolt period. A number of questions need to be answered before the exact picture of control of gluconeogenesis and glycolysis in the hypodermis can be definitively explained. Percentage changes in the forms of FDPase present in the different portions of the molt cycle, and the exact regulation of phosphofructokinase, pyruvate carboxylase, and phosphoenolpyruvate carboxykinase remain to be established. Furthermore, questions of in vivo flux patterns as well as the influence of hormonal regulation also need to be answered. However, this study has

provided considerable elucidation of available gluconeogenic and glycolytic control mechanisms in both the hypodermis and muscle of Cancer magister.

### Phosphoglycerate Kinase in Gluconeogenic Tissues

The regulatory properties of the phosphoglycerate kinases investigated in this study may elucidate the means by which the reversal of the reaction for gluconeogenesis is facilitated in other organisms. The kinetic modifications of freshmolt muscle and hypodermal phosphoglycerate kinases have considerable potential for overcoming the MgADP inhibition of the gluconeogenic reaction. The combination of increased affinity for ATP and decreased sensitivity to ADP and MgADP inhibition may provide the means for the easy reversal of phosphoglycerate kinase in other gluconeogenic tissues. The mass action ratios show that the reaction catalyzed by phosphoglycerate kinase is relatively near equilibrium in all the tissues examined. Evidently the activity of the enzyme is high enough in both hypodermis and muscle to bring the reaction close to equilibrium. Certainly the mass action ratios for phosphofructokinase and pyruvate kinase are orders of magnitude further removed from equilibrium than those of the phosphoglycerate kinase reaction. However, qualitative differences indicate that the reaction is further from equilibrium in muscle than in hypodermis. The kinetic characteristics of the hypodermal and freshmolt muscle enzymes would facilitate the reversal of the reaction, in contrast to the properties of the intermolt muscle enzyme.

### Regulatory Properties of Pyruvate Kinase from Cancer magister

The regulatory properties of pyruvate kinase from muscle and hypodermis (Table 19) are interesting both in relation to other pyruvate kinases and as examples of protein adaptation for specific metabolic purposes. Muscle pyruvate kinase is distinct from the enzyme in other muscles in that it is sensitive to activation by UDP-acetylglucosamine and glucosamine-6-phosphate. This effect may well be specific to crustaceans and reflects the influence of chitin metabolism. Arginine phosphate inhibition probably occurs in all animals which utilize arginine phosphate as their storage phosphagen, and has been found for pyruvate kinase from octopus (Guderley *et al.*, 1975) and shrimp (Guderley *et al.*, 1976). Although the muscle enzyme shows allosteric effects, i.e. activation by FDP, inhibition by citrate and arginine phosphate, it does not show sigmoidal kinetics. Various other pyruvate kinases show sigmoidal kinetics at high pH values (Seubert & Schoner, 1971), in the presence of  $Mg^{++}$  rather than  $Mn^{++}$  (de Zwaan *et al.*, 1975), or show sigmoidal kinetics which are converted to hyperbolic kinetics by the addition of FDP (Kayne, 1973). The presence of measurable levels of FDP associated with the enzyme indicates that this lack of sigmoidal kinetics may be due to bound FDP which somehow prevents cooperative interactions between subunits. Further examination of this aspect of pyruvate kinase regulation would be of considerable interest.

The similarity of the regulatory properties of muscle pyruvate kinase and hypodermal PK I (Table 19) makes it tempting

Table 19

Comparison of Muscle and Hypodermal Pyruvate Kinases

	Muscle PK	Hypodermal PK I	Hypodermal PK II
$K_m$ PEP	0.1 mM	0.1 mM	0.55mM
$K_m$ ADP	0.2 mM	0.2 mM	0.2 mM
$K_a$ FDP	$2 \times 10^{-5} M$	$1.3 \times 10^{-5} M$	$9 \times 10^{-8} M$
$K_i$ MgATP	1.8 mM (vs PEP)	1.2 mM (vs PEP)	1.2 mM (vs PEP)
$K_i$ arginine phosphate	2.8 mM (vs ADP)	7.0 mM (vs ADP)	7.0 mM (vs ADP)
$K_i$ tryptophan	1.6 mM	2.0 mM	2.0 mM
$K_i$ alanine	30 mM	30 mM	7.0 mM
$K_i$ alanine (with FDP)	30 mM	30 mM	26 mM
$K_i$ serine	30 mM	35 mM	10.5 mM
$K_i$ serine (with FDP)	30 mM	35 mM	26 mM
$K_i$ Mg <sub>2</sub> citrate	complex two site interaction	complex two site interaction	7.5 mM
$K_i$ CaCl <sub>2</sub>	0.1 mM (vs Mg <sup>++</sup> )	.075 mM (vs Mg <sup>++</sup> )	.075 mM (vs Mg <sup>++</sup> )
	0.66 mM (vs Mn <sup>++</sup> )	.6 mM (vs Mn <sup>++</sup> )	.6 mM (vs Mn <sup>++</sup> )
$K_a$ K <sup>+</sup>	15 mM	10 mM	10 mM
$K_a$ NH <sub>4</sub> <sup>+</sup>	5 mM	6 mM	6 mM
$K_a$ Mg <sup>++</sup>	0.6 mM	0.6 mM	0.6 mM
$K_a$ Mn <sup>++</sup>	0.15 mM	0.1 mM	0.1 mM
pI	6.4	6.1	6.1

to speculate that the hypodermal pyruvate kinase evolved from the muscle enzyme. However, the two enzymes are definitely different proteins, since the muscle enzyme does not show interconversions between a high and a low affinity state. The difference between the hypodermal and the muscle pyruvate kinase may be due to a considerably higher affinity of the muscle enzyme for FDP or to a few amino acid substitutions which facilitate conformational changes at the PEP binding site of hypodermal pyruvate kinase. Since the apparent FDP affinity of the muscle enzyme was not high, the former alternative seems unlikely. Hypodermal pyruvate kinase is distinct from other previously examined pyruvate kinases in that FDP promotes a conversion between two forms, each of which is sensitive to further activation of substrate binding. The exact mechanism of this interconversion is not clear, but the increased levels of FDP associated with the high affinity form indicate that the interconversions may involve long term binding of FDP to the protein. Long term binding has been indicated for the mammalian L and M2 forms (Hess & Kutzbach, 1971; Seubert & Schoner, 1971; Ibsen *et al.*, 1975). Subunit cooperativity does not seem to be involved in the regulation of the hypodermal enzyme. A comparison of the hypodermal enzyme with the enzyme from the hepatopancreas might elucidate structural differences which underlie the lack of subunit interactions on the part of the hypodermal enzyme. An examination of the enzyme from the hepatopancreas for similar stable high and low affinity states would ascertain whether subunit interactions and transitions between high and low affinity conformations represent alternate

forms of adaptation for gluconeogenic function. Also, closer examination of possible structural differences between the muscle and hypodermal enzymes, and of the in vivo significance of the two affinity states would be of great interest.

In conclusion, the results of this study further substantiate the hypothesis that the crustacean hypodermis, faced with a critical need for efficient gluconeogenesis, would have evolved special enzymatic adaptations to answer this need.

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