REGULATORY ENZYMES OF CARBOHYDRATE METABOLISM IN GIANT SCALLOP PLACOPECTEN MAGELLANICUS (GMELIN)

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REGULATORY ENZYMES OF CARBOHYDRATE METABOLISM

IN THE GIANT SCALLOP

SYNOPSIS

It would appear from the relative activities of key glycolytic enzymes, that succinate is the main end product of glycolysis in both the mantle and the adductor muscle of the giant scallop. The pathway of succinate formation appears to be similar to that postulated for other invertebrates. All the enzymes but one catalyzing the formation of succinate in the mantle and the adductor muscle were found in the cytosol. The enzyme catalyzing the reduction of fumarate was found in the mitochondrial fraction.

The enzymes of the Bücher Shuttle were found in the adductor muscle. Thus two mechanisms are readily available in the adductor muscle for the oxidation of cytoplasmic NADH, viz. the malate dehydrogenase system and the Bücher Shuttle.

The high activities of phosphoenolpyruvic carboxykinase and fructose diphosphatase are compatible with active glycogen synthesis in the adductor muscle, and it appears the dicarboxylic acid shuttle is the mechanism by which the pyruvate kinase step is bypassed, as pyruvate carboxylase is absent.

The effects of known metabolic regulators of other systems were studied on several of the carbohydrate enzymes of the adductor muscle. Many showed similar properties to mammalian enzymes, although some interesting differences were found.

Studies on the thermal properties of the enzymes showed that the Km was influenced by temperature and that the effect of some metabolic regulators were temperature-dependent. It would appear temperature may be an important parameter in metabolic control in the scallop.

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CONTENTS

ABBREVIATIONS

The following abbreviations have been used in this thesis: KCl, potassium chloride; EDTA, (ethylenedinitrilo) tetraacetate, disodium salt; NAD, nicotinamide adenine nucleotide; NADH, reduced nicotinamide adenine nucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PEP, phosphoenolpyruvate; FDP, fructose1,6-phosphate; tris, tris(hydroxymethyl)aminomethane; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate, ITP, inosine triphosphate; CTP, cytosine triphosphate; GTP, guanosine triphosphate.

INTRODUCTION

1. Occurrence of Glycolysis in Invertebrates

It is generally accepted that the degradation of glucose to pyruvate in many organisms is by way of the Embden-Meyerhof sequence of reactions. There is evidence for believing that most of the individual reactions of the Embden-Meyerhof scheme are operative in many invertebrates. Bueding[1] has made it clear, however, that even though glucose is degraded through the same reactions into pyruvate, the disposition of pyruvate varies substantially from one organism to another.

Insects are the most intensively studied invertebrates and all the work reported on glucose degradation in insects as well as other aspects of carbohydrate metabolism has been the subject of a recent review by Chefurka[2]. After insects, the parasitic helminths have been the subject of much research into the nature of their metabolism. The breakdown of glycogen into glucose and the subsequent glycolytic reactions have been investigated in *Hymenolepis diminuta*[3], *Ascaris lumbricoides*,[4,5] *Echinococcus granulosus*,[6-8] *Trichinella spiralis*[9,10] and others. In all of these helminths it was apparent that the glycolytic pathway is in operation, except that little or no lactate could be measured when glycogen was broken down. In lieu, a variety of other

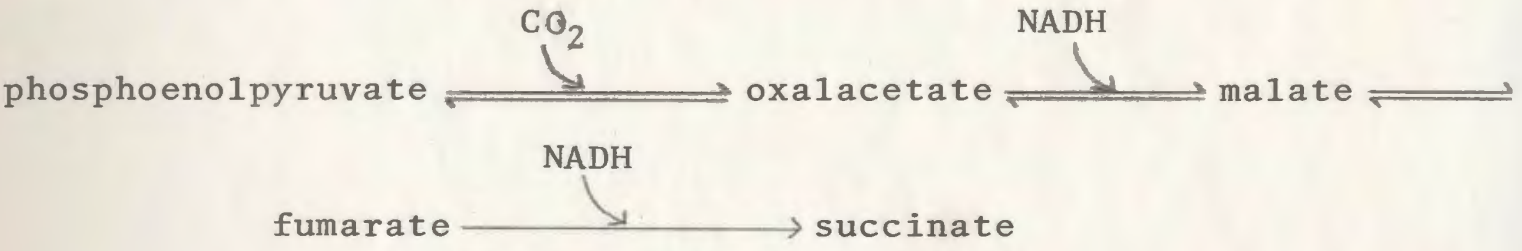
products were formed, among which succinate and fatty acids were identified[10-12].

The anaerobic disappearance of glycogen without a concomitant lactate formation is not limited to helminths. This situation has been known to occur in oyster muscle[13] and in freshwater snails[14]. The formation of succinate in invertebrates was believed to be the result of carboxylation reactions such as carboxylation of pyruvate, as suggested by Saz and Virdine[15], or carboxylation of propionate to yield succinate[16,17]. The latter surmise was based on the fact that CO_2 is incorporated very rapidly into succinate in a variety of marine invertebrates[16]. The formation of succinate as the end product of a primary carboxylation reaction became untenable when organisms known to form succinate by CO_2 fixation were found to lack propionyl CoA carboxylase activity[18].

Molluscs have a very active phosphoenolpyruvate carboxykinase. In one mollusc, Rangia cuneata, which is one of the richest sources of this enzyme among invertebrates[18], the activity of the enzyme was found to be on the average ten times higher than in chicken liver. A rapid carboxylation of phosphoenolpyruvate could readily account for the rapid formation of succinate from CO_2 in the marine organisms without involing a carboxylation of pyruvate or the necessity of its formation. Further studies on Rangia cuneata revealed that it produced from glucose large amounts of succinate and very little lactate[19]. It has an active pyruvate kinase, but the lactate dehydrogenase activity

was found to be extremely low.

Simpson and Awapara[19], on the basis of balance studies and isotope distribution, concluded that glucose is degraded in this mollusc, and perhaps in others, to phosphoenolpyruvate with much of the phosphoenolpyruvate being diverted to succinate formation by reacting with CO_2 at a rapid rate. Only a small amount of succinate is formed from the carboxylation of pyruvate as the malic enzyme is only present in small amounts and pyruvate carboxylase is absent. These workers have postulated that succinate is formed by the following sequence of reactions:



Chen and Awapara[20] found, that in the mantle of Rangia, all but one of the enzymes catalyzing the overall flow of carbon from glucose to succinate are localized in the cytosol; the single exception, the enzyme catalyzing the reduction of fumarate, is localized in the mitochondria. In some molluscs lactate dehydrogenase has been shown to be absent, and instead, octopine dehydrogenase is present[21]. Octopine dehydrogenase catalyzes the production of octopine from pyruvate. It must be pointed out that even in the molluscs that do contain octopine dehydrogenase, succinate is still the main end product of glucose

degradation. It seems that the tissues of species which exhibit octopine dehydrogenase activity are deprived of lactate dehydrogenase and reciprocally the presence of the last enzyme excludes the first one[22].

2. Oxidation of Cytoplasmic NADH

A feature of aerobic carbohydrate metabolism is that the NADH generated in the glyceraldehyde phosphate dehydrogenase reaction in the cytoplasm is re-oxidized at the expense of oxygen in the mitochondrion. Either the NADH itself must be able to penetrate to the respiratory chain or else reducing equivalents from the NADH must be transferred to the mitochondrial electron transport system by some special mechanism. Lehninger[23] found the respiratory chain of isolated liver mitochondria is almost inaccessible to NADH from the external medium, and a similar inaccessibility has been observed with kidney[24], tumour[25], and insect flight muscle[26]. From experiments in which [14C] nicotinic acid was injected into rats, Purvis and Lowenstein [27] deduced that the intra- and extra-mitochondrial pyridine nucleotides of liver do not undergo rapid equilibration, and they calculated that NADH enters liver mitochondria *in vivo* at a speed quite inadequate to account for the respiratory rate of the tissue. It is now accepted that virtually all mitochondria are impermeable to NADH both *in vivo* and *in vitro*[28,29]. It seems, therefore, that reducing equivalents must be transferred from the cytoplasm

to the respiratory chain by some means other than the transport of NADH.

The oxidation of cytoplasmic NADH is essential for the maintenance of glycolysis and the mechanisms involved seem to vary from tissue to tissue. In mammalian liver and muscle, lactate dehydrogenase provides the chief system by which NADH is oxidized in the cytoplasm. Other known mechanisms by which NADH can be oxidized in liver are by the malate/oxalacetate and malate/citrate shuttles, and by peroxisomes. In yeast alcohol dehydrogenase provides the mechanism. In insect flight muscle the Bücher shuttle provides the mechanism for the oxidation of cytoplasmic NADH[30-32].

The enzyme responsible for this oxidation is the cytochrome-linked flavoprotein α-glycerophosphate dehydrogenase. But flight muscle contains another α-glycerophosphate dehydrogenase, situated in the cytoplasm. This soluble enzyme performs the reversible oxidation of α-glycerophosphate to dihydroxyacetone phosphate by transferring electrons to and from NAD. The presence of two enzymes apparently performing the same function in two different compartments of the cell provided the clue to the significance of this shuttle in flight muscle. It had been known for some time that mitochondria were unable to oxidize NADH added externally in solution. But for every molecule of glucose consumed in the cell, one molecule of NADH is formed in the cytoplasm by the action of the enzyme glyceraldehyde-3-phosphate dehydrogenase, and unless this coenzyme

is immediately reoxidized by the transfer of electrons to another acceptor, energy production must cease. In mammalian muscle, pyruvate acts as the electron acceptor, being reduced to lactate by the action of lactate dehydrogenase. In insect flight muscle, however, lactate dehydrogenase has a very low activity, and its function is taken over by the soluble α-glycerophosphate dehydrogenase. Thus, dihydroxyacetone phosphate accepts electrons from NADH and is reduced to α-glycerophosphate, while NAD is restored for participation in the main Embden-Meyerhof pathway. The α-glycerophosphate so formed diffuses readily into the mitochondria, where it is reoxidized by the powerful α-glycerophosphate dehydrogenase present there, transferring electrons through the cytochromes to oxygen. The dihydroxyacetone formed in this reaction diffuses out of the mitochondria to complete the cycle, and is then ready to accept electrons once more. Thus the two α-glycerophosphate dehydrogenases and their substrates act as a powerful catalytic cycle whereby reducing equivalents derived initially from glyceraldehyde-3-phosphate are fed into the mitochondrial electron transport system, thus making possible the rapid and complete oxidation of glucose to carbon dioxide and water within the muscle cell.

Duplication of another enzyme system also occurs in flight muscle. Malic dehydrogenase is present as an NAD-linked enzyme in the cytoplasm, and is also found linked to the cytochromes in the mitochondria. The two malic dehydrogenases and

their substrates could thus set up a catalytic cycle similar to the α-glycerophosphate cycle, and it is possible that such a system is important in some tissues, but present evidence suggests that the malate system is of only minor importance in the oxidation of extramitochondrial NADH in flight muscle, because of the overwhelming activity of the α-glycerophosphate cycle.

In the mantle of the clam Rangia, Stokes and Awapara[33] have postulated that malic dehydrogenase is the enzyme system responsible for the oxidation of cytoplasmic NADH. Little work has been done on this in other marine invertebrates.

3. Gluconeogenesis in Invertebrates

Gluconeogenesis has been studied very little in invertebrates. In the clam Rangia, glucose is formed from pyruvate by a reversal of the degradative reactions of glucose[34]. Glucose synthesized from pyruvate-2-C^{14} has an isotope distribution that corresponds to the expected distribution if formed by a reversal of the degradative reactions and also if formed by the more indirect pathway involving the formation of phosphoenolpyruvate, via carboxylation of pyruvate to yield oxalacetate. The relatively high activity of phosphoenolpyruvate carboxykinase makes the indirect pathway appear feasible[18].

It is of interest to mention the high content of

glycogen known in some marine invertebrates. Rangia, for example, has 2-5 per cent glycogen (wet weight). The adductor muscle of the giant scallop has been shown to contain 3 per cent glycogen (wet weight)[35]. In addition the adductor muscle has considerable amounts of uridine diphosphate glucose suggesting active glycogenesis. This is in contrast to vertebrate muscle.

4. Enzymatic Regulation of Glycolysis and Gluconeogenesis

Glycolysis from glucose is a sequence of two pathways: the glucose phosphorylation pathway and the common glycolytic pathway. Complete oxidation of glucose involves an additional pathway: the Krebs cycle. Each of these pathways is feedback controlled at either its first step or its first irreversible step, by end product inhibition or some other allosteric effect, closely linked to changes in the respective end product. This arrangement allows a flexible regulation of glycolysis that can very rapidly adapt its rate to the energetic and biosynthetic requirements of the cell in a wide variety of conditions. This general pattern allows for some variations, both in the steps controlled and the controlling metabolites, in different organisms and in different cells within organisms.

Gluconeogenesis is closely intermingled with glycolysis. The enzymatic basis of the potential switch-over involves antagonistic enzymes at the level of the key

physiologically irreversible reactions. To make possible an efficient switching from one pathway (direction) to the other there is need, besides the regulatory mechanisms intrinsic to glycolysis, of control mechanisms specifically related to the potential switch-over.

A. Phosphofructokinase as a Regulatory Enzyme

Cori[36] pinpointed phosphofructokinase as a crucial control enzyme on the basis of his observation that large swings in the hexose monophosphate concentration in muscle were not accompanied by corresponding changes in the glycolytic end product, lactic acid. Lardy and Parks[37] noted the possibility for regulation in the fact that the enzyme was inhibited by ATP. Cyclic AMP was found to activate the enzyme from the liver fluke[38], the mammalian heart, and skeletal muscle[39,40]. Activation of the last two enzymes was also produced by AMP. These activators overcome the inhibitory effect of ATP[41].

B. Pyruvate Kinase as a Regulatory Enzyme

Hess, Haeckel and Brand[42] have reported that yeast pyruvate kinase is stimulated by fructose 6-phosphate. Enzyme preparations from rat liver[43,44], mouse liver[45], and rat adipose tissue[46] are similarly affected by fructose 6-phosphate. Rat skeletal-muscle pyruvate kinase is unaffected by fructose 6-phosphate[43] and rat liver has been shown to contain a pyruvate kinase with similar properties[47]. Pyruvate kinase preparations

from some sources have been found to be activated by other metabolites as well as fructose 6-phosphate. Thus the enzyme of E. coli is also activated by AMP[48] and that from loach embryo by cyclic AMP[49].

C. Lactate Dehydrogenase as a Regulatory Enzyme

Fritz[50] has shown that the Krebs cycle intermediates can activate lactate dehydrogenase from rabbit skeletal muscle but not from rabbit heart muscle.

D. Malate Dehydrogenases as Regulatory Enzymes

Cennamo, Montecuccoli and König[51] have shown that pig mitochondrial malate dehydrogenase, unlike the supernatant enzyme, is inhibited by citrate and fumarate. They have speculated on a possible regulatory role for this peculiar inhibition. Both enzymic forms are supposed to participate in lipogenesis and gluconeogenesis[52,53]. As far as lipogenesis is concerned, it could be suggested that the inhibition of mitochondrial malate dehydrogenase by citrate can prevent the transformation of oxalacetate (formed from pyruvate inside the mitochondria) into malate, a reaction favoured by the equilibrium position. Oxalacetate could then be utilized more efficiently for the synthesis of citrate, which can leak out of the mitochondria and be used as a source of acetyl CoA for the synthesis of fatty acids. The newly formed oxalacetate in this reaction would then produce pyruvate, via the supernatant malate dehydrogenase and by the malic enzyme. In these reactions NADH is transformed into NADPH, needed for the synthesis of

fatty acids.

Kuramitsu[54] has shown that ATP, ADP and AMP inhibit the malate dehydrogenase of pig heart, E. coli, and lemon.

E. Fructose Diphosphatase as a Regulatory Enzyme

In all animal systems considered so far, fructose diphosphatase is inhibited by AMP[55].

F. Phosphoenolpyruvate Carboxykinase as a Regulatory Enzyme

Exton and Park[56] have reported the regulation of phosphoenolpyruvate carboxykinase by cyclic AMP.

G. Malic Enzyme as a Regulatory Enzyme

The malic enzymes from rat liver[57] and E. coli[58] were inhibited by fructose 6-phosphate and 6-phosphogluconate. The bacterial system was also regulated by various nucleotides acetyl CoA and oxalacetate.

5. Temperature and Regulation of Enzyme Activity in Poikilotherms

The effect of temperature on the catalytic and regulatory properties of poikilothermic enzymes is not well understood. The sharp and frequently differential effects of temperature on these properties in mammalian and bacterial systems are often of such magnitude as to appear incompatible with poikilothermic existence. Enzymes of poikilothermic organisms are uniquely adapted to promote highly stable rates

of catalysis over the range of temperatures normally encountered by the organism in its habitat[59,60]. The basis of this temperature-independence of enzymic reaction rates at physiological substrate concentrations is as follows: for all enzymes of poikilothermic organisms examined, decreases in the assay temperature promote concomitant decreases in the Km for substrate. This temperature-Km effect is observed over most of the range of temperatures normally experienced by the organism, although sharp increases in Km are frequently noted at temperatures near the lower extreme of the organism's habitat temperature. In terms of enzyme regulation theory[61], decreases in temperature through the organism's physiological range can be said to act analogously to positive modulators of enzymes.

Behrisch[62], and Behrisch and Hochachka[63] have studied the effects of temperature on the regulatory functions of some poikilothermic enzymes. It was found that the AMP inhibition of rainbow-trout fructose diphosphatase is highly temperature-dependent[63], but the AMP inhibition of the migrating salmon enzyme is temperature-independent[62].

No work has yet been reported on the effect of temperature on the catalytic and regulatory properties of marine molluscs.

CHAPTER 1

Glycolysis and Gluconeogenesis in the Giant Scallop, *Placopecten magellanicus* (Gmelin)

METHODS AND MATERIALS

Methods

Preparation of Subcellular Fractions

A. Subcellular fractions of the adductor muscle of the Giant Scallop

The scallops were obtained from Salmonier, Newfoundland. The excised muscles were stored at -20° for up to 3-5 weeks. All preparative operations were carried out at 0-2°.

The adductor muscle was fractionated by a modification of the method of Ernster and Nordenbrand[64]. Care was taken to exclude the small, yellowish, "catch" muscle attached to the adductor. The excised muscle was immersed into ice-cold 0.15M KCl and the tissue cut with scissors into small pieces, and then rinsed with several portions of 0.15M KCl. Cutting was continued until a fine mince was obtained. The minced-tissue was rinsed with Chappell-Perry medium and suspended in about one volume of the same medium. Sucrose was not used since it favoured gel formation. Homogenization was carried out by nine strokes up and down with a Teflon Potter homogenizer. The homogenate was diluted with Chappell-Perry medium to obtain a final ratio of 15 g muscle in 90 ml of Chappell-Perry medium, and then filtered through nylon.

The homogenate was centrifuged at 650 g for 10 minutes to give supernatant I and sediment (nuclei-myofibril fraction). The sediment was suspended by gentle homogenization

in a Teflon Potter homogenizer in half the original volume of Chappell-Perry medium and again centrifuged at 650 g for 10 minutes giving supernatant Ia and the nuclei-myofibril fraction.

Supernatants I and Ia were combined and centrifuged for 10 minutes at 8000 g giving supernatant II and the mitochondrial fraction. The latter was suspended by gentle homogenization in 20 ml Chappell-Perry medium and centrifuged for 10 minutes at 8000 g giving the washed mitochondrial fraction and supernatant IIa. The washing of the mitochondrial fraction was repeated twice, spinning at 8000 and 8500 g respectively. The brown-coloured mitochondrial fraction was then taken up in 10 ml of 0.15M KCl. The supernatants II, IIa, IIb and IIc were combined and centrifuged at 105,000 g for 70 minutes giving the microsomal fraction and the particulate-free supernatant.

B. Subcellular fractions of the mantle of the Giant Scallop

The mantles were fractionated generally according to the procedure of Chen and Awapara[20]. The excised mantles from several scallops were washed several times with cold 0.02M phosphate buffer, pH 7.4; then they were blotted and weighed. About 1 g of tissue was suspended in 4 ml of 0.4M sucrose-0.01M phosphate buffer, pH 7.4 and homogenized by six strokes up and down with a Teflon Potter homogenizer. The cell suspension was centrifuged at 3000 g for 10 minutes. The residue was washed with a small volume of the sucrose-phosphate solution;

the washing was added to the supernatant portion while the washed residue was discarded. The supernatant was centrifuged at 33,000 g for 1 hr. This gave the mitochondrial fraction which was washed twice with the sucrose-phosphate solution. The supernatants were combined and centrifuged at 105,000 g for 70 minutes giving the microsomal fraction and the particulate-free supernatant.

C. Subcellular fractions of a rat liver

An adult rat was killed by a blow to the head and the liver removed. The liver was washed several times with cold sucrose and cut up into small pieces. Homogenization was carried out in 0.3M sucrose-0.002M EDTA using a Teflon-Potter homogenizer. The homogenate was diluted with sucrose to obtain a final ratio of 15g liver tissue in 90 ml of sucrose. The nuclei-free supernatant was sonicated and then centrifuged at 105,000g for 70 minutes, and the resultant supernatant collected according to the method of Sedgwick and Hübscher[65].

D. Subcellular fraction of rat skeletal muscle

5g of rat leg skeletal muscle were treated similarly to the scallop adductor muscle. The nuclei-myofibril free supernatant was sonicated, centrifuged at 105,000 g for 70 minutes and the resultant supernatant collected.

Enzyme Assays

Lactate Dehydrogenase [EC 1.1.1.27] was assayed by a modification of the method of Kornberg[66]. The reaction mixture in a total volume of 3.0 ml contained: 0.25 M phosphate buffer, pH 7.2, 0.1 mM NADH, 0.01M pyruvate and enzyme extract. The activity was measured by following the disappearance of NADH in a Unicam SP500 spectrophotometer coupled to a Beckman chart recorder.

Pyruvate Kinase [EC 2.7.1.40] was assayed by a modification of the method of Bücher and Pfleiderer[67]. The reaction was coupled with lactate dehydrogenase to give oxidation of NADH which was followed continuously by measuring the change in E_{340} spectrophotometrically. The reaction mixture contained: 0.25M phosphate buffer, pH 7.5; KCl, 70 mM; NADH, 0.15 mM; ADP, 1.0 mM; $MgSO_4$, 8.0 mM; lactate dehydrogenase, 10 units. The reaction was carried out at 10^{o}.

Malic Dehydrogenase [EC 1.1.1.37] was assayed by a modification of the method of Ochoa[68]. The reaction mixture contained in 3.0 ml: 0.25 phosphate buffer, pH 7.4; 0.15 mM NADH; 0.7 mM oxalacetate and enzyme extract. The reaction, carried out at 10^{o}, was measured by following the oxidation of NADH spectrophotometrically at 340 mμ.

Phosphoenolpyruvate Carboxykinase [EC 4.1.1.31] was assayed by a modification of the procedure of Utter et al[69].

Oxalacetate formation from phosphoenolpyruvate and $NaHCo_3$ was determined by measuring the oxidation of NADH in the presence of malate dehydrogenase at 10°. The reaction mixture contained in 3.0 ml: 0.25 M phosphate buffer, pH 7.0, $MnCl_2$, 5 mM; $NaHCo_3$, 20 mM; ITP, 2 mM; NADH, 0.12 mM; malate dehydrogenase, 4 units; and enzyme extract.

Succinate Dehydrogenase [EC 1.3.99.1] was assayed by the method of Pennington[70] using 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride as the electron acceptor. A slight reduction of this dye occurred in the absence of succinate and this was allowed for in the determination.

Glucose 6-phosphatase [EC 3.1.3.9] was assayed according to the method of Hübscher and West[71].

6-Phosphogluconate Dehydrogenase [EC 1.1.1.44] was was assayed according to the method of Glock and McLean[72] by following spectrophotometrically the rate of reduction of NADH at 340 mμ.

Octopine Dehydrogenase was assayed according to the method of Regnouf and Van Thoai[73].

Pyruvate Carboxylase [EC 6.4.1.1] was assayed by a modification of the method of Utter and Keech[74]. The reaction mixture contained in 3 ml: Tris-HCl buffer, pH 7.6, 50 mM; sodium pyruvate, 5 mM; $NaHCo_3$, 5 mM; $MgCl_2$, 3 mM; ATP, 1 mM; KCl, 33 mM; NADH, 0.1 mM; malate dehydroganase, 1 unit; and

enzyme extract. The reaction was followed spectrophotometrically by following the oxidation of NADH at 340 mμ.

Malic Enzyme [EC 1.1.1.39] was assayed according to the method of Ochoa[75].

Succinate Reductase [EC 1.3.99.1] was assayed according to the method of Rahat et al[76].

Glycerolphosphate Dehydrogenase [EC 1.1.99.5] and [EC 1.1.1.8] was assayed according to the method of Chappell[77].

Fumarase [EC 4.2.1.2] was assayed according to the method of Massey[78].

Fructose-1,6-Diphosphatase [EC 3.1.3.1] was assayed by a modification of the method of Pontremoli[79]. The reaction contained 0.2M glycine buffer, pH 9.0; $MgCl_2$, 0.01M; NADP 0.02M; glucose 6-phosphate isomerase, 4 units; glucose 6-phosphate dehydrogenase, 3 units; and enzyme extract. The reaction was followed by measuring the reduction of NADP at 340 mμ.

Unless otherwise stated all enzymic reactions were carried out at 10^{o}.

Protein was determined using the biuret method of Hübscher, West and Brindley[80].

Materials

All chemicals were obtained from the Sigma Chemical Co., St. Louis.

RESULTS

Comparative levels of glycolytic and gluconeogenic enzymes

In Table 1 the activities of key glycolytic and gluconeogenic enzymes in the mantle and adductor muscle of the scallop are shown and their activities compared with those of rat liver and skeletal muscle. One of the most outstanding features of the Table is the enormous difference in lactic dehydrogenase activities in the scallop tissues and in the rat tissues. Malic dehydrogenase levels do not form any particular pattern. The levels of pyruvate kinase and phosphoenolpyruvic carboxykinase are quite similar in the mantle and adductor muscle. It is of interest to note that pyruvate carboxylase activity was not detected in either of the scallop tissues, as was octopine dehydrogenase. The levels of the gluconeogenic enzymes phosphoenolpyruvic carboxykinase and fructose 1,6,diphosphatase in the mantle and adductor muscle are relatively high and this is compatible with active glycogen synthesis. The enzyme assays of the scallop tissues and of the rat skeletal muscle were carried out on nuclei-myofibril free homogenates and that of the rat liver on a nuclei-free homogenate. All homogenates were sonicated prior to assaying, centrifuged at 105,000 g for 70 minutes, and the assays carried out on the supernatant. The scallop enzymes were assayed at 10^{o} and the rat enzymes at 23^{o}.

TABLE 1

COMPARATIVE LEVELS OF SOME GLYCOLYTIC AND GLUCONEOGENIC ENZYMES

Tissue	LDH*	Pyruvate Kinase*	MDH*	Pyruvate Carboxylase*	PEP Carboxykinase*	Malic Enzyme**	FDPase**	ODH*
Scallop Muscle	0.025*** ± 0.003	0.038 ± 0.004	3.48 ± 0.10	-	0.049 ± 0.003	0.021 ± 0.004	0.040 ± 0.005	-
Scallop Mantle	0.022 ±0.003	0.031 ± 0.002	2.91 ± 0.08	-	0.037 ± 0.002	0.017 ± 0.003	0.034 ± 0.006	-
Rat Muscle	1.11 ± 0.04	0.141 ± 0.005	3.14 ± 0.07	-	0.0007 ± 0.0002	0.006 ± 0.002	0.008 ± 0.003	
Rat Liver	1.32 ± 0.05	0.023 ± 0.003	3.23 ± 0.11	0.047 ± 0.003	0.008 ± 0.002	0.043 0.004	0.052 0.004	

The enzymes were assayed in sonicated homogenates as described.

* µmoles NADH oxidised/mg protein/min.
** µmoles NADP reduced/mg protein/min.
***Figures represent the average value of three samples.

LDH = Lactic Dehydrogenase, MDH = Malic Dehydrogenase, PEP carboxykinase = Phosphoenolpyruvic carboxykinase, FDPase = Fructose 1,6 Diphosphatase, ODH = Octopine Dehydrogenase.

Subcellular distribution of phosphoenolpyruvic carboxykinase, malic dehydrogenase, fumarase and fumarate reductase in the mantle and adductor muscle of the scallop

Tables 2 and 3 show the subcellular distribution of these enzymes in the mantle and the adductor muscle. The assay of typical marker enzymes were employed for the identification of the subcellular structures isolated. The enzyme markers used for the identification of the mitochondrial, microsomal and particulate-free supernatant fractions were succinate dehydrogenase, glucose-6-phosphatase and 6-phosphogluconate dehydrogenase respectively. Both in the mantle, and adductor muscle of the scallop, phosphoenolpyruvic carboxykinase and fumarase are shown to be cytoplasmic enzymes. Malic dehydrogenase is distributed between the cytoplasmic and mitochondrial fractions, while fumarate reductase appears to be mitochondrial.

Presence of Bücher Shuttle Enzymes in the Adductor Muscle

The activities of the mitochondrial and supernatant forms of glycerol-3-phosphate dehydrogenase are shown in Table 4. The activity of the Bücher shuttle enzymes in the scallop muscle is quite comparable with the activity in insect flight muscle and a sharp contrast with the activity in rat tissues[87].

TABLE 2

SUBCELLULAR DISTRIBUTION OF PEP CARBOXYKINASE, MALIC DEHYDROGENASE, FUMARASE AND FUMARATE REDUCTASE IN THE MANTLE OF THE GIANT SCALLOP

	Fraction		
	Mitochondrial	Microsomal	Soluble
Succinate Dehydrogenase:			
% of total recovered	74* ± 1.5	3 ± 0.4	23 ± 1.6
Specific activity	1.12 ± 0.02	0.04 ± 0.003	0.21 ± 0.02
Glucose-6-phosphatase:			
% of total recovered	8 ± 1	77 ± 2	15 ± 1.5
Specific activity	0.31 ± 0.06	1.98 ± 0.6	0.04 ± 0.03
6 Phospho gluconate dehydrogenase:			
% of total recovered	-	6 ± 0.03	94 ± 1.4
Specific activity		0.03 ± 0.02	0.49 ± 0.5
PEP carboxykinase:			
% of total recovered	-	4 ± 2	96 ± 2
Specific activity		0.04 ± 0.02	0.49 ± 0.5
MDH:			
% of total recovered	43 ± 3	2 ± 5	55 ± 3
Specific activity	0.96 ± 0.07	0.06 ±0.003	0.38 ± 0.04
Fumarase:			
% of total recovered	-	5 ± 1.2	95 ± 1.2
Specific activity		0.03 ± 0.01	0.48 ± 0.08
Fumarate Reductase:			
% of total recovered	73 ± 1.6	5 ± 0.4	22 ± 1.2
Specific activity	1.8 ± 0.4	0.02 ± 0.01	0.14 ± 0.3

* Figures represent the average of three preparations.

TABLE 3

SUBCELLULAR DISTRIBUTION OF PEP CARBOXYKINASE, MALIC DEHYDROGENASE, FUMARASE AND FUMARATE REDUCTASE IN THE ADDUCTOR MUSCLE OF THE GIANT SCALLOP

	Fraction		
	Mitochondrial	Microsomal	Soluble
Succinic dehydrogenase:			
% of total recovered	73* ± 1.8	5 ± 0.4	22 ± 1.9
Specific activity	2.36 ± 0.51	0.03 ± 0.02	0.38 ± 0.09
Glucose-6-phosphatase:			
% of total recovered	11 ± 2	74 ± 3	15 ± 2.5
Specific activity	0.08 ± 0.11	0.52 ± 0.13	0.09 ± 0.06
6-Phospho gluconate dehydrogenase:			
% of total recovered	-	5.5 ± 2.5	94.5 ± 3.4
Specific activity	-	0.05 ± 0.04	0.93 ± 0.19
PEP carboxykinase:			
% of total recovered	-	6 ± 1.5	94 ± 3.4
Specific activity	-	0.02 ± 0.01	0.93 ± 0.19
MDH:			
% of total recovered	39 ± 2.5	2 ± 0.5	59 ± 3
Specific activity	1.26 ± 0.18	0.03 ± 0.02	2.01 ± 0.46
Fumarase:			
% of total recovered	-	4 ± 1	96 ± 2.5
Specific activity	-	0.07 ± 0.02	0.62 ± 0.21
Fumarate Reductase:			
% of total recovered	71 ± 1.5	7 ± 1	22 ± 2
Specific activity	2.47 ± 0.66	0.06 ± 0.03	0.26 ± 0.09

* Figures represent the average of three preparations.

(The malic enzyme was also found to be a soluble enzyme in both muscle and mantle.)

TABLE 4

ACTIVITY OF GLYCEROLPHOSPHATE DEHYDROGENASE

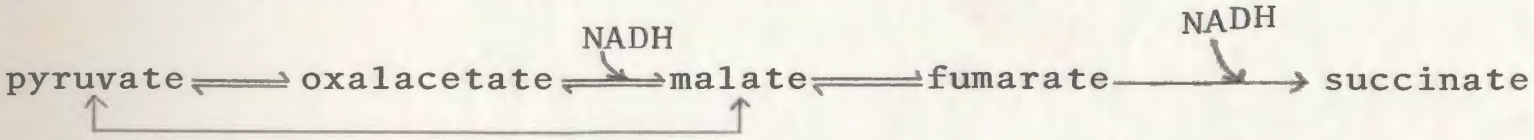
Mitochondrial Enzyme	Supernatant Enzyme
0.60 *	0.65 **
± 0.04	± 0.05

* μmoles of O_2 uptake/min/mg protein

** μmoles of NADH oxidized/min/mg protein

DISCUSSION

Most investigators concerned with the metabolism of invertebrates agree that succinate production under anaerobic conditions replaces lactate production as a means of oxidizing NADH produced during the oxidoreduction step in glycolysis[18,19,33]. From the enzyme activities in Table 1 one could postulate that both in the mantle and adductor muscle of scallop, the end product of glycolysis is not lactate but that it is succinate. The production of succinate has been postulated[15] to begin with the carboxylation of pyruvate yielding oxalacetate and thus providing the cell with a reducible substrate; conversion of oxalacetate to fumarate via malate yields a second reducible substrate:



Thus in this sequence of reactions NADH could conceivably be oxidised to maintain glycolysis in a continuous fashion. It is also possible that phosphoenolpyruvate instead of pyruvate is carboxylated. This is understandable when the levels of phosphoenolpyruvate carboxykinase and the malic enzyme are compared in scallop mantle and muscle. Simpson and Awapara[19] failed to find malic enzyme activity in some marine molluscs they examined, thus they deduced that phosphoenolpyruvate was carboxylated. In others, as in the scallop, both enzymes were found to be present, although in all cases the activity of

phosphoenolpyruvic carboxykinase was found to be greater than the malic enzyme. Knowing which is the preferred pathway adds little to the significance of the production of succinate. It may be pointed out that both in scallop mantle and muscle, it would appear that such conditions do favour oxalacetate formation from phosphoenol pyruvate in spite of the competing high pyruvate kinase activities.

Another interesting observation from Table 1 is the correlation between the relatively high phosphoenolpyruvate carboxykinase and fructose 1,6 diphosphatase activity and the extremely high content of glycogen in the scallop. The adductor muscle of the scallop contains 3% glycogen (wet weight)[35]. Such high levels of glycogen are not usually known in the muscle of vertebrates. The source of glycogen in the adductor muscle of the scallop is not known, but if it is formed from nonhexose precursors one could speculate on the role of these two glycogenic enzymes. If glycogen is formed from nonhexose precursors these precursors could well be the diatoms on which the scallop feeds. Hiltz and Dyer[35] have suggested that the terminal step in the synthesis of glycogen in the adductor muscle may involve UDPG-glycogen glucosyltransferase.

In vertebrates, it has become clear that formation of phosphoenolpyruvate from four-carbon dicarboxylic acids leads to a pathway for glycogen formation that bypasses the

pyruvate kinase step. In vertebrate liver and kidney pyruvate carboxylase is the enzyme most active in the production of oxalacetate from pyruvate for glycogen synthesis[81]. In scallop mantle and muscle, however, pyruvate carboxylase is shown to be absent. Similar results have been shown with the mantles of other invertebrates[18]. It is evident, therefore, that glycogen formation in molluscs must follow some other pathway. The conversion of four-carbon dicarboxylic acids to glycogen could be understood in terms of the reverse reactions and without the need for a pyruvate kinase. From the marked differences in the activities of phosphoenolpyruvate carboxykinase and fructose 1,6 diphosphatase in the adductor muscle of the scallop and the skeletal muscle of the rat in conjunction with their known difference in glycogen content, it would appear that glycogen formation in the adductor muscle is quite active and follows a similar route as postulated for the mantles of other marine invertebrates[18].

As phosphoenolpyruvate carboxy kinase is known to be involved in glucose degradation as well as thought to be involved in glycogen formation, it was decided to investigate the intracellular localization of this enzyme as well as that of malic dehydrogenase, fumarase and fumarate reductase, the other enzymes involved in the formation of succinate. In vertebrates the intracellular distribution of phosphoenolpyruvate carboxykinase varies with tissues and with species[82].

In rat liver it is largely cytoplasmic, in guinea-pig liver it is divided between mitochondria and cytoplasm roughly in the proportion 2:1, and in rabbit and pigeon liver it is mitochondrial. It is also a mitochondrial enzyme in rat brain. The explanation for this variation is unknown.

The localization of phosphoenol-pyruvate carboxykinase in the cytoplasm of both the mantle and the adductor muscle of the scallop is not unique amongst the invertebrates. Prescott and Campbell[83] found this enzyme in the soluble fraction in Hymenolysis diminuta, and Prichard and Schofield[84] found it in the soluble fraction of Fasciola hepatica. Chen and Awapara[20] also found it in the soluble fraction of the mantle of the clam Rangia cuneata. This is the first report of its location in the mantle and the adductor muscle of the scallop.

The presence of both a large amount of malic dehydrogenase and of fumarase in the cytoplasm is not too surprising in view of earlier observations made by Ward and Schofield[85], in parasitic helminths and by Chen and Awapara[20] in Rangia mantle. There is a definite advantage as far as the scallop is concerned in having malic dehydrogenase in the cytoplasm; it can take the role of lactic dehydrogenase which in this organism is at too low a level to maintain anaerobic glycolysis. The scallop possesses a high activity of phosphoenolpyruvate carboxykinase allowing the organism to obtain a substrate reducible by NADH instead of pyruvate which is reducible to a lesser extent. The reduction of oxalacetate can proceed at a

rapid rate in the scallop since the level of malic dehydrogenase is quite high.

In the scallop, the production of succinate will be dictated in the main by (i) the relatively high rate of phosphoenolpyruvate carboxylation, (ii) the relatively low level of lactic dehydrogenase and (iii) the relatively high level of malic dehydrogenase. Under most circumstances the rate-limiting step in anaerobic glycolysis is the oxidation of NADH by one or other of the end-products. From the recorded observations one can infer that the oxidation of NADH must be catalysed by lactic dehydrogenase or malic dehydrogenase. It is, therefore, the levels of one of these two enzymes which will determine the rate of anaerobic glycolysis and the nature of the final products. It would be difficult to envisage an organism that lacks both enzymes and is still capable of anaerobic glycolysis, unless NADH is oxidized in some other way. Octopine formation is one possibility, but in the scallop this is ruled out owing to the absence of octopine dehydrogenase. The presence of lactic dehydrogenase and the absence of octopine dehydrogenase in the scallop follows the pattern observed in other molluscs by Regnouf and Van Thoai[21] whose work concluded that the two enzymes seem to be mutually exclusive.

As has been pointed out by other investigators, one must also consider the availability of the two reducible substrates, pyruvate and oxalacetate. It would be difficult to

establish their steady-state concentration by measuring the activity of two enzymes, as for example, pyruvate kinase and phosphoenol pyruvate carboxykinase. Bueding and Saz[86] attempted to do this in three species of parasitic worms and concluded that the ratio of the two enzymes determines the nature of the end-products. In Ascaris muscle phosphoenol pyruvate carboxykinase is twenty times more active than pyruvate kinase and according to them this correlates with the fact that Ascaris produces mainly succinate. It is, however, interesting that in Ascaris the concentration of pyruvate is half that of phosphoenolpyruvate and in H. diminuta, which also produces succinate, pyruvate and phosphoenolpyruvate are present in equal amounts. It would appear there is no limitation in the amount of reducible substrates. The limitation is on the activity of lactic dehydrogenase which is quite low in these two worms. In Ascaris lactic dehydrogenase activity is nearly one-tenth of the activity measured in Schistosoma mansoni which produces mainly lactate. It would appear, therefore, that succinate formation vis-a-vis lactate formation is better explained on the basis of the relative activities of the malic and lactic dehydrogenases. According to the determination of Bueding and Saz[86] the ratio of malic to lactic dehydrogenase is 37 in Ascaris and 0.25 in S. mansoni. The first produces mainly succinate, the second mainly lactate.

The same rule can be applied to a number of molluscs that are known to produce succinate. Simpson and Awapara[19] have shown that for example in Rangia, pyruvate kinase and phosphoenolpyruvate carboxykinase are equally active, but Rangia produces mostly succinate. In Rangia, however, malic dehydrogenase activity is a hundred times greater than lactic dehydrogenase. From Table 1 it can be seen that both in the mantle and adductor muscle of the scallop, malic dehydrogenase activity is about 130-140 times greater than lactic dehydrogenase activity. From this and also from the fact that the other necessary enzymes are present, it can be postulated that in both the mantle and the adductor muscle of the scallop, the end product of anaerobic glycolysis is succinate.

The search for the Bücher shuttle enzymes was carried out because the scallop adductor muscle showed similarity in activity of lactic dehydrogenase with the flight muscle of insects, and because it is well documented that this shuttle is thought to be the main mechanism by which insect flight muscle oxidizes cytoplasmic NADH[30-32]. This is the first report of the presence of this shuttle in a marine invertebrate muscle. In this mechanism, the glycerolphosphate dehydrogenase in the cell sap catalyses the reduction of dihydroxyacetone phosphate by NADH and the resulting glycerolphosphate passes into the mitochondria where it is oxidised back to dihydroxyacetone phosphate by the FAD-glycerolphosphate dehydrogenase and the respiratory chain. As in insect flight muscle, there is a

duplication of enzyme systems that can oxidize cytoplasmic NADH, viz. malic dehydrogenase and the Bücher shuttle. Which of the two systems is the more important in the scallop muscle is debatable, but if the two muscles were directly compared, it would be postulated that the Bücher shuttle is the more important, as it is in the insect flight muscle. The presence of this shuttle in the adductor muscle could well explain the strength of the muscle. As lactate production is very low no oxygen debt would build up, and the only products of glucose catabolism would be carbon dioxide and water. This is in sharp contrast to mammalian muscle where an oxygen debt is quickly built up due to the accumulation of lactate.

A few final comments are worthy of note. The activities of the enzymes assayed remained relatively constant in muscles that were stored at -20° for up to 5 weeks. This finding is consistent with the work of Hiltz and Dyer[35] who have recently demonstrated a relatively slow overall postmortem catabolic rate in scallop muscle. All the work reported in this thesis was carried out on mature scallops and no attempt was made to correlate enzyme activities with sexual differences or seasonal variations.

METHODS AND MATERIALS

Methods

All enzyme studies were carried out on particulate-free supernatant and mitochondria of the giant scallop adductor muscle, prepared as described in Chapter 1.

Assay of Enzymes

Phosphofructokinase (E.C.2.7.1.11). This was assayed as described by Underwood and Newsholme (1965)[88]. The reaction mixture contained in 3.0 ml; Tris-HCl buffer, pH 7.6 400 µmoles; $MgCl_2$, 10 µmoles; KCl, 600 µmoles; ATP, 6.0 µmoles; KCN, 1.0 µmole; glucosephosphate isomerase, 2 units; aldolase, 0.5 units; triosephosphate isomerase, 12 units; glycerol phosphate dehydrogenase, 0.2 units; NADH, 0.4 µmole; and glucose 6-phosphate 5.0 µmoles. The reaction was followed by measuring the oxidation of NADH spectrophotometrically at 340 mµ and at 10^{o}.

All other enzymes were assayed as described in Chapter 1.

Materials

ATP, ADP, AMP, CTP, UTP, ITP, fructose diphosphate, phosphoenolpyruvate, glucose 6-phosphate, glucose 1-phosphate, 6-phosphogluconate, pyruvate, aspartate, glutamate, succinate, fumarate, malate, oxalacetate, citrate, isocitrate, lactate dehydrogenase, phosphoglucose isomerase, pyruvate kinase, α-glycerophosphate dehydrogenase, triose phosphate isomerase and aldolase were obtained from the Sigma Chemical Company.

RESULTS

Properties of Adductor Muscle Phosphofructokinase

Effect of Fructose 6-Phosphate concentration on Phosphofructokinase (PFK) activity

Figure 1 shows the effect of fructose 6-phosphate concentration on the velocity of the enzyme reaction. In the presence of 1 mM ATP the curve is sigmoidal and half-maximal velocity is obtained at approximately 3 mM fructose 6-phosphate. With 2 mM ATP the curve is displaced to the right, showing that ATP inhibition may be explained in part at least by an increased Km of the enzyme for fructose 6-phosphate.

Effect of ATP concentration on PFK activity

The effect of ATP concentration on the activity of the adductor muscle PFK is shown in Figure 2. The curves show that activity increases rapidly at very low ATP concentrations, whereas higher concentrations inhibit. Inhibition by ATP is less at high fructose 6-phosphate concentration. AMP relieves the ATP inhibition.

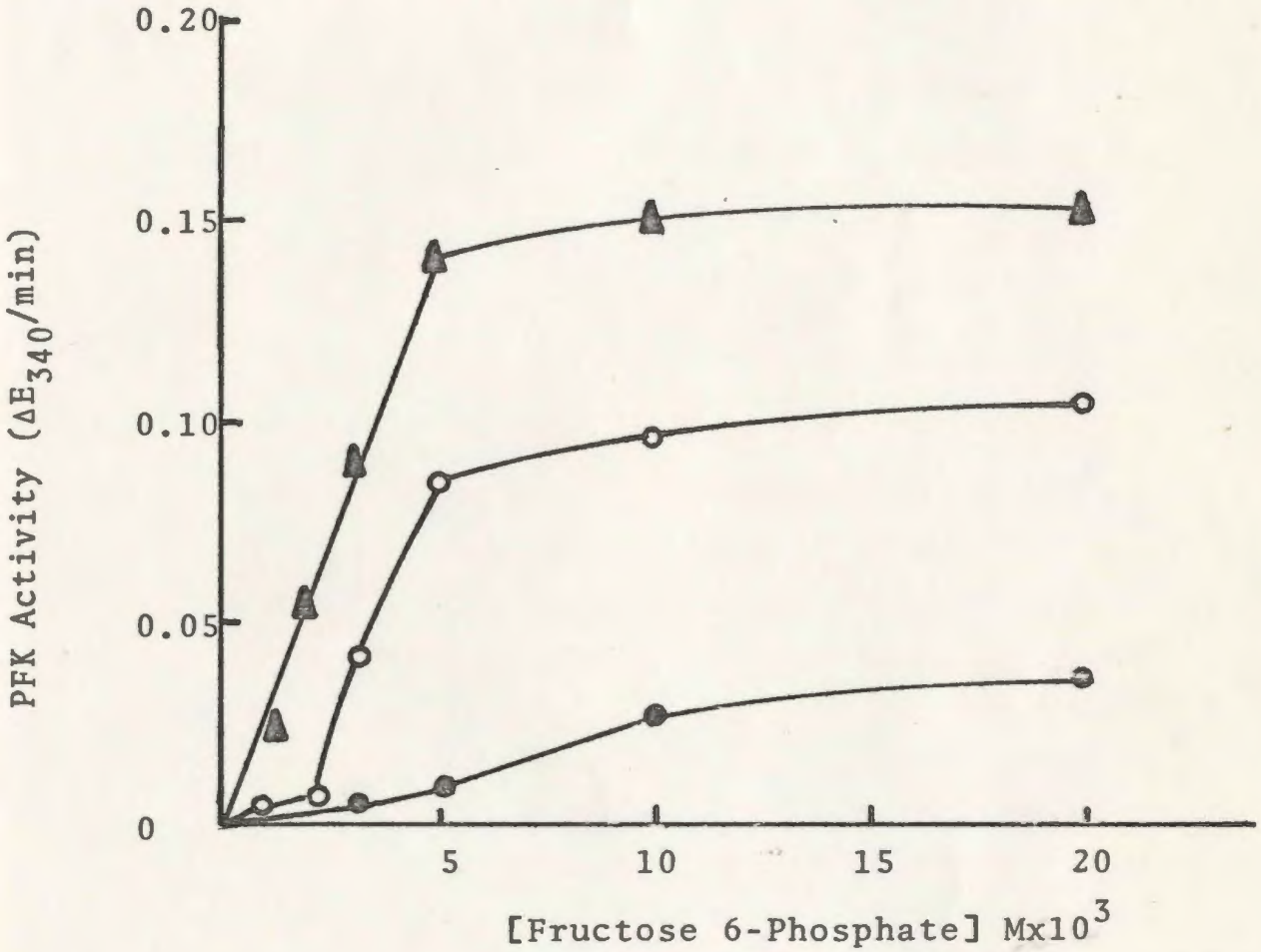
Effect of AMP concentration on PFK activity

Figure 1 shows the effect of 2 mM AMP on the fructose-6-phosphate activity curve of the enzyme in the presence of 1 mM ATP. AMP converts the sigmoidal curve into a hyperbolic curve, lowering the half-maximal velocity for the muscle enzyme. Figure 2 shows that 2 mM AMP relieves inhibition of ATP and also

Figure 1

Effect of Fructose 6-Phosphate on Adductor Muscle PFK Activity

The reaction mixture contained 0.2 mg protein/ml of adductor muscle supernatant.



2mM ATP

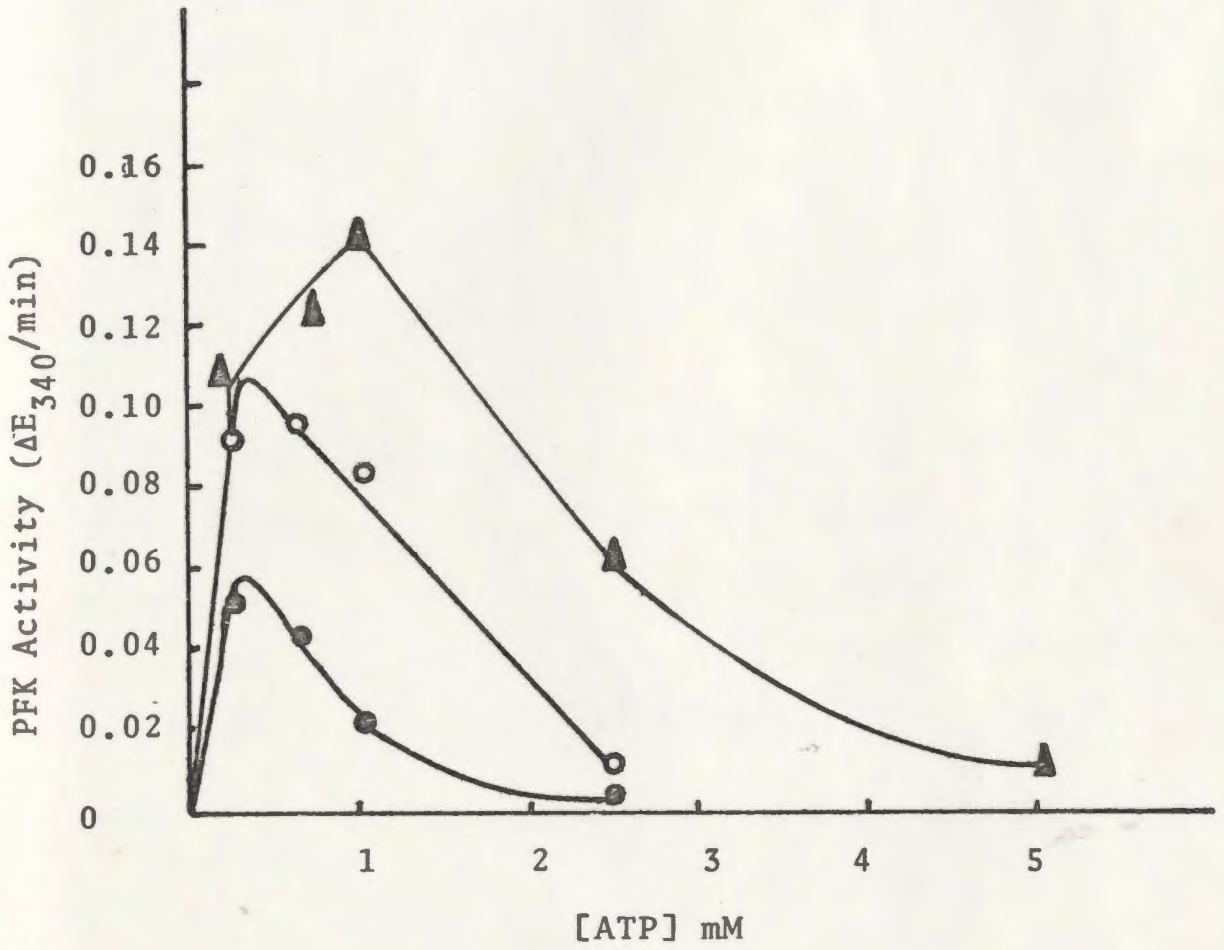
1mM ATP

1mM ATP, plus 2mM AMP (added to the reaction mixture after the initial rate had been measured)

Figure 2

Inhibition of Adductor Muscle PFK activity by ATP

The reaction mixture contained 0.2 mg protein/ml of adductor muscle supernatant.



2 mM Fructose 6-Phosphate

5 mM Fructose 6-Phosphate

5 mM Fructose 6-phosphate, plus 2 mM AMP (added to the reaction mixture after the initial rate had been measured)

activates the enzyme at non-inhibitory ATP concentrations. Thus AMP appears to act by relieving inhibition of PFK by ATP and by lowering the Km with respect to fructose-6-phosphate at both inhibitory and non-inhibitory concentrations of ATP. Figure 3 gives the AMP activation curve for PFK in the presence of 1 mM-ATP and 2 mM fructose-6-phosphate. In these results the activity without AMP has been defined as zero.

Effect of FDP and Pi on PFK activity

The effects of these compounds on the adductor muscle are shown in Table 5. The values are calculated assuming the addition of no effectors to be 100% activity.

Pi at 1 mM activates the enzyme in the presence of 1 mM ATP. FDP at 1 mM inhibits the enzyme. This effect was studied in more detail and the results are shown in Figure 4. Figure 4 shows that the inhibition increases with increased FDP concentration and that this inhibition in increased by increasing the ATP concentration.

Effect of 3'5'-(cyclic)-AMP on PFK activity

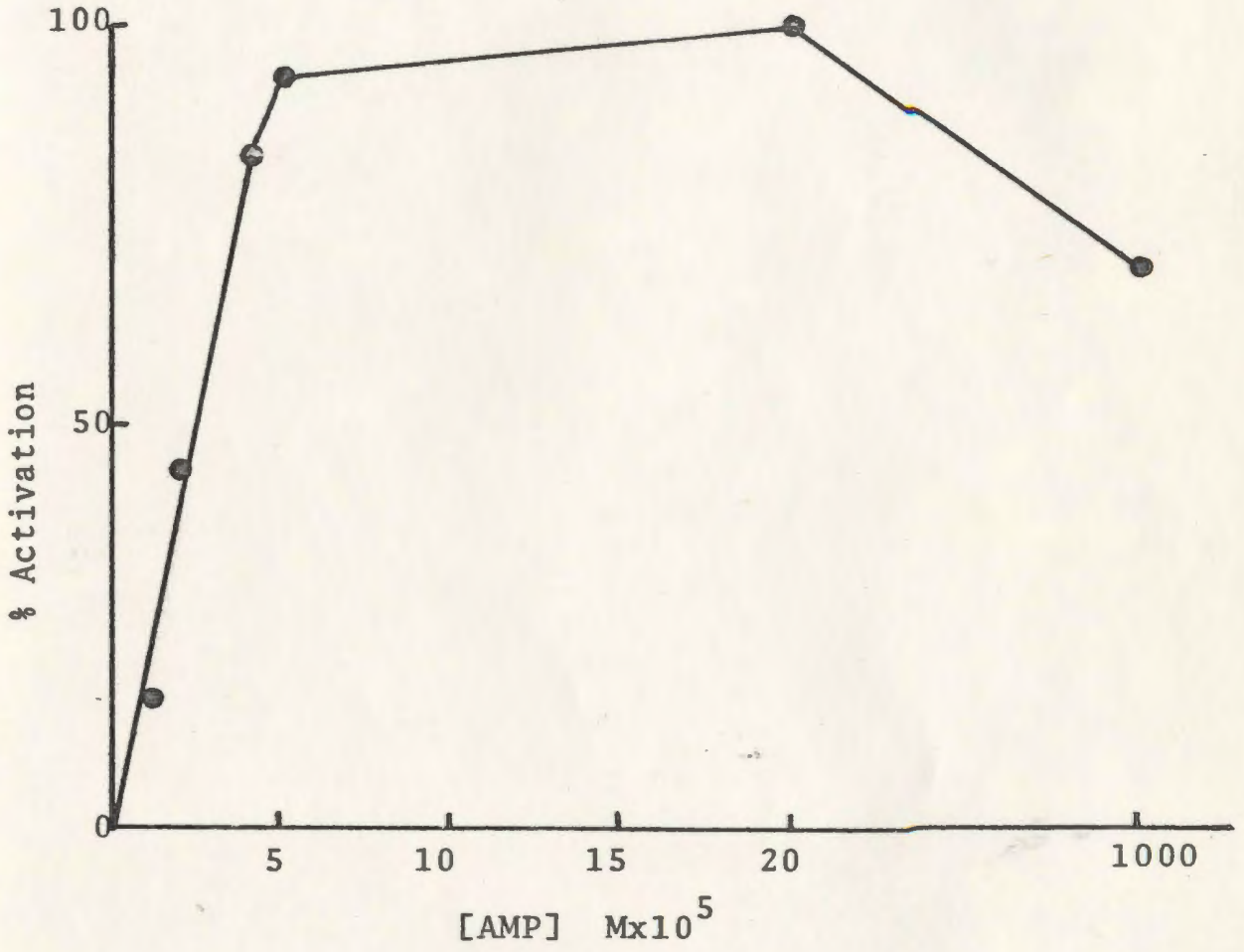
Table 5 shows that 0.5 mM 3'5'-(cyclic)-AMP activates the PFK at 1 mM ATP, but has no effect at 0.1 mM ATP, suggesting that 3'5'-(cyclic)-AMP activates by relieving ATP inhibition.

Effect of citrate and PEP on PFK activity

Citrate has been shown to inhibit PFK from mammalian[88-91] and plant sources[92,93] and PEP has been shown

Figure 3

Stimulation of Adductor Muscle PFK activity by AMP



The reaction mixture contained 0.2 mg protein/ml of adductor muscle supernatant.

TABLE 5

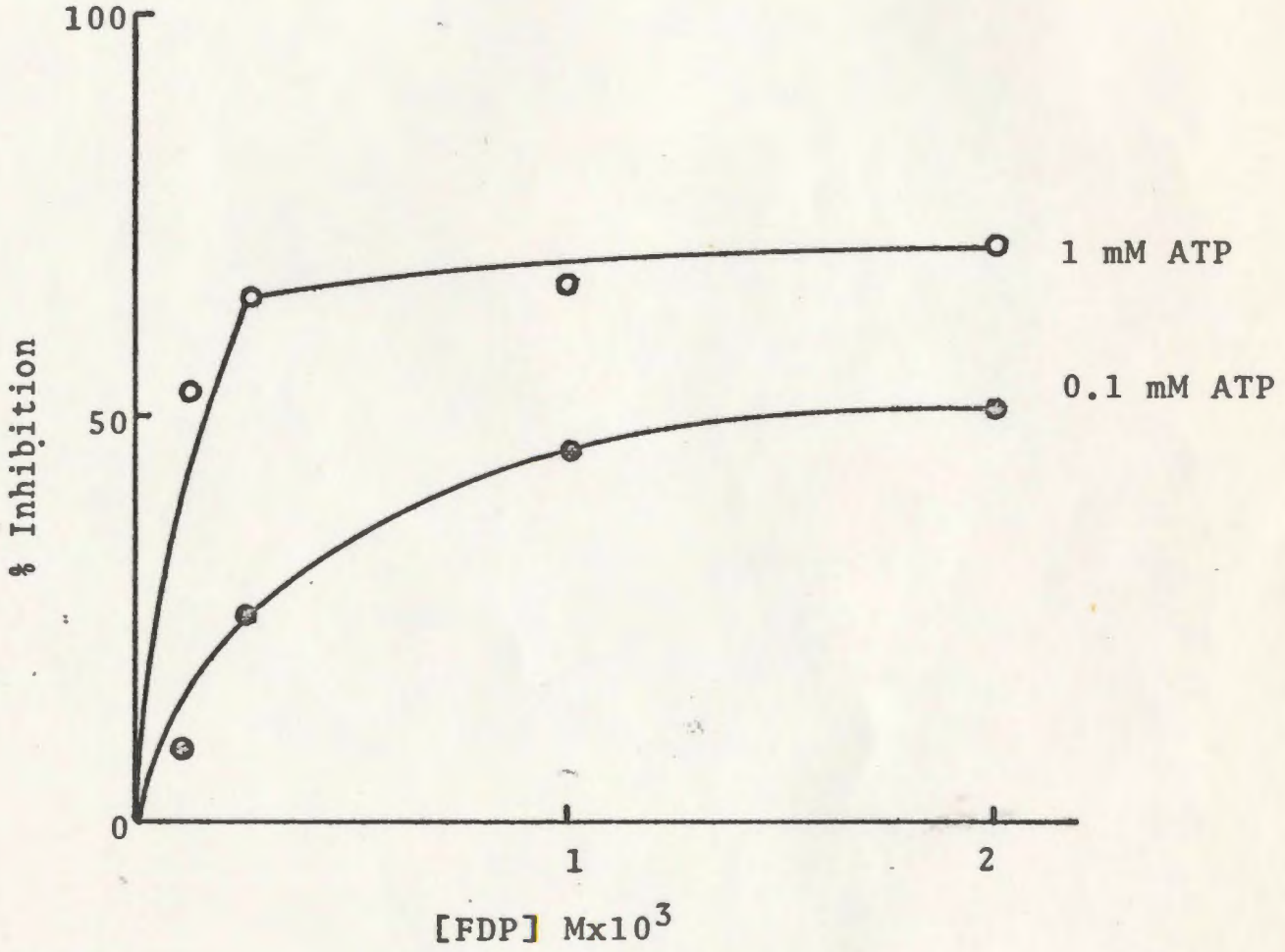
ACTION OF EFFECTORS AND PRODUCTS ON ADDUCTOR MUSCLE PFK ACTIVITY

[ATP] (mM)	Addition	% Activity
1.0	2 mM-AMP	180
1.0	0.5 mM 3'5' cyclic AMP	145
1.0	1 mM-Pi	143
1.0	1 mM FDP	54
0.1	0.5 mM 3'5' cyclic AMP	100

The reaction mixture contained 0.2 mg protein/ml of adductor muscle supernatant.

Figure 4

Inhibition of Adductor Muscle PFK activity by FDP



The reaction mixture contained 0.2 mg protein/ml of adductor muscle supernatant.

to inhibit E. coli[94,95], plant[92,93], and skeletal muscle PFK[39]. PEP and citrate at 1 mM have no effect on the adductor muscle enzyme.

Properties of Adductor Muscle Pyruvate Kinase

Effect of FDP on Pyruvate Kinase activity

The stimulation of the adductor muscle enzyme by FDP depends not only on the FDP concentration but also on the PEP concentration and on pH. The interrelationship between FDP and PEP is illustrated by showing the influence of FDP on the PEP concentration-activity curve (Figure 5). In the absence of FDP the curve for the initial velocity plotted against PEP concentration is sigmoidal and half-maximal velocity is attained at 0.2 mM PEP. In the presence of high concentrations of FDP (0.1 mM) the response to PEP concentrations is transformed to give a normal Michaelis-Menten curve, the Km of which is 0.05 mM, i.e. one-quarter of that in the absence of FDP.

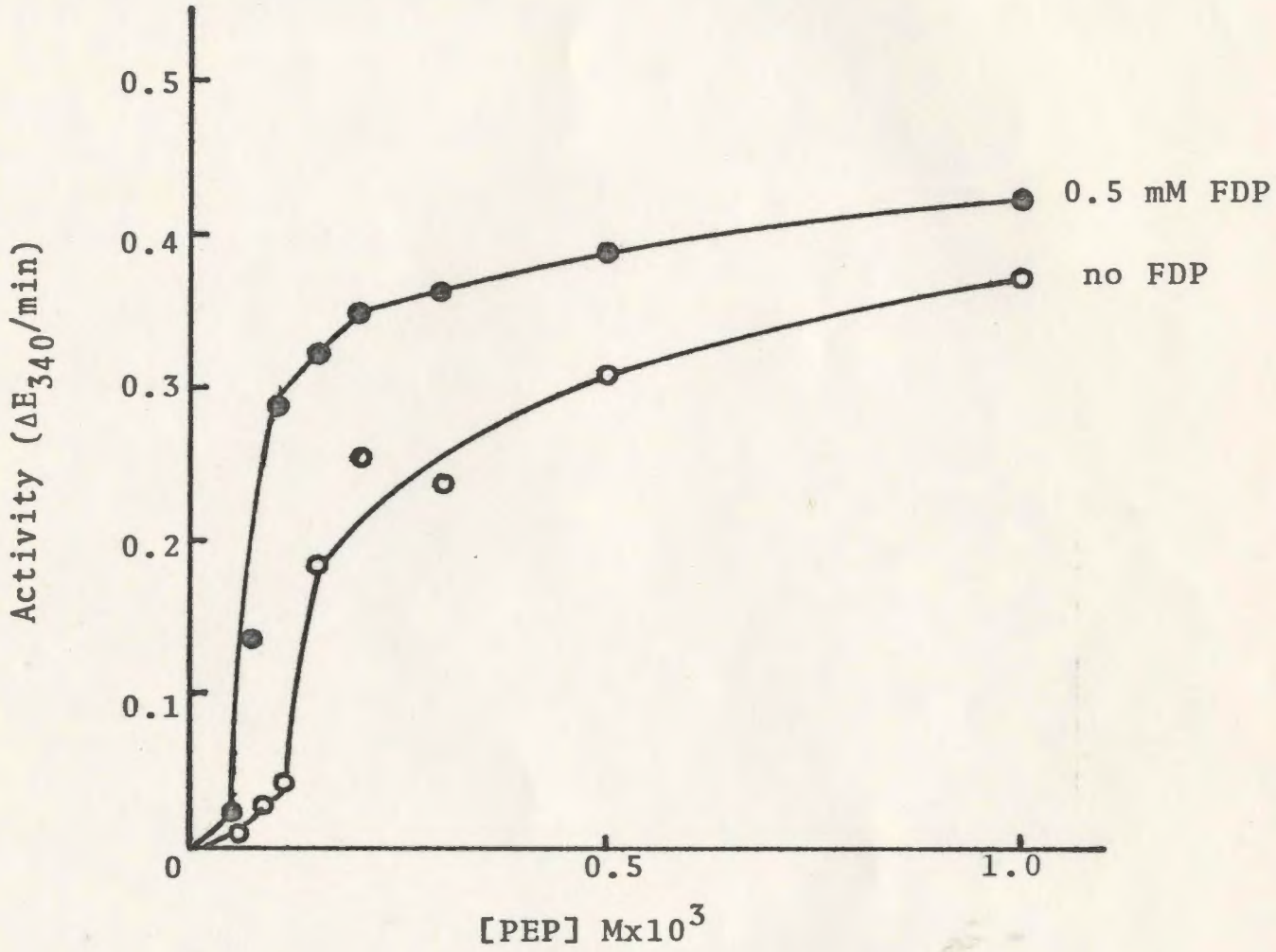
The low concentration of FDP capable of stimulating the enzyme activity is demonstrated in Figure 6, which shows the activities of the enzyme in the presence of 0.1 mM PEP and various amounts of FDP. The activation values are calculated assuming that 0.5 mM FDP gives 100% activation.

Effects of compounds other than FDP on adductor muscle pyruvate kinase activity

Table 6 shows the effect of various metabolites, two of which (AMP and 3'5'-(cyclic)-AMP) are known to stimulate pyruvate kinase from other sources[48,49], on the pyruvate kinase activity of the adductor muscle. None of the

Figure 5

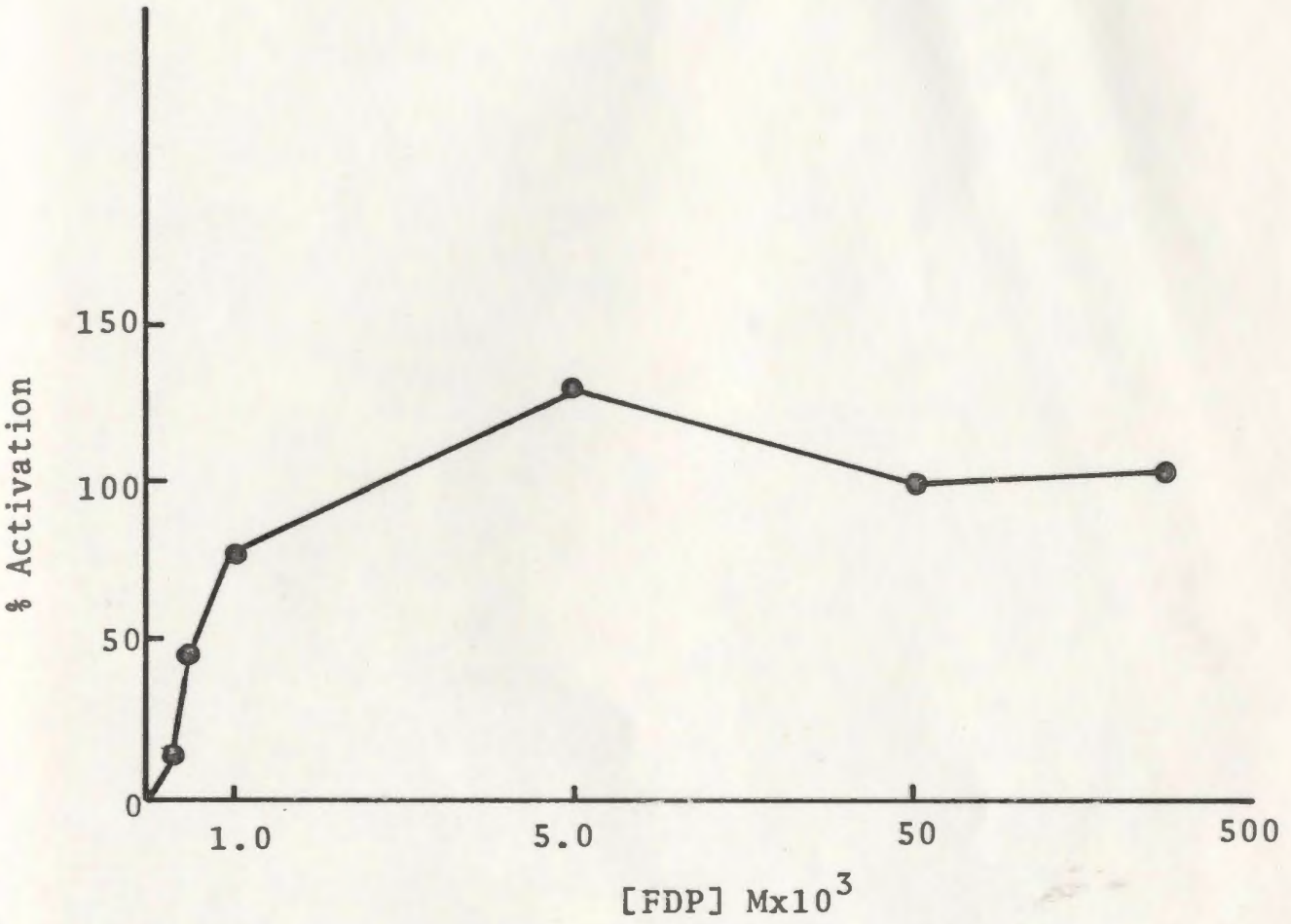
Influence of FDP on the relationship between pyruvate kinase activity and PEP concentration



The reaction mixture contained 0.15 mg protein/ml of adductor muscle supernatant.

Figure 6

Activation of Pyruvate Kinase by FDP



The reaction mixture contained 0.15 mg protein/ml of adductor muscle supernatant.

TABLE 6

EFFECT OF VARIOUS METABOLITES ON ADDUCTOR MUSCLE PYRUVATE KINASE

Pyruvate kinase was assayed in the presence of 1 mM of the various metabolites.

Additive	Reaction Rate (%)
None	100
Glucose 6-phosphate	104
Glucose 1-phosphate	95
6-Phosphogluconate	105
Glyceraldehyde 3-phosphate	87
AMP	97
Cyclic AMP	93

The reaction mixture contained 0.15 mg protein/ml of adductor muscle supernatant.

metabolites, tested at 1 mM, had any effect on the enzyme activity. Similar results were obtained with the metabolites at 0.1 mM.

Effect of ADP concentration on adductor muscle Pyruvate Kinase activity

The effect of ADP concentration on the activity of the enzyme in the presence and absence of FDP (0.5 mM) is shown in Figure 7. The enzyme requires 1 mM ADP for maximal activity and is inhibited by high concentrations of ADP.

Inhibition of Pyruvate Kinase activity by ATP and the effect of FDP

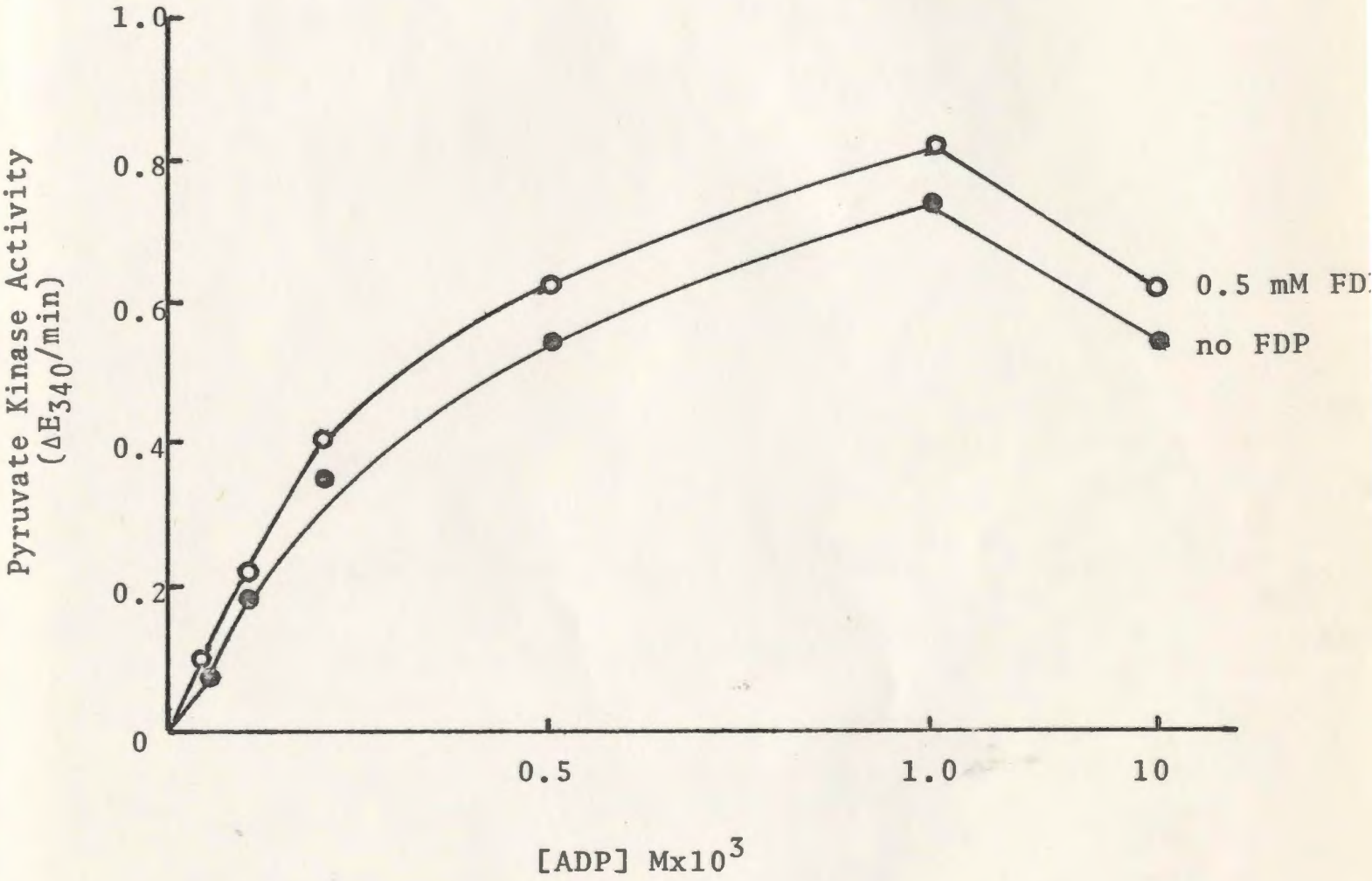
The inhibition of the adductor muscle enzyme by ATP is shown in Figure 8. The half-maximal inhibition is caused by about 0.5 mM ATP. FDP has little effect on the inhibited muscle enzyme.

Effect of pH and FDP on adductor muscle pyruvate kinase activity

Figure 9 shows the effect of pH on the activity of the adductor muscle enzyme with tris buffer in the reaction mixture. The pH-activity curve in the absence of FDP shows a sharp peak at about pH 7.2 and a rapid decline as the pH of the reaction mixture is raised further. In the presence of FDP, however, the activity of the enzyme is relatively constant over the range pH 7.5-9.0.

Figure 7

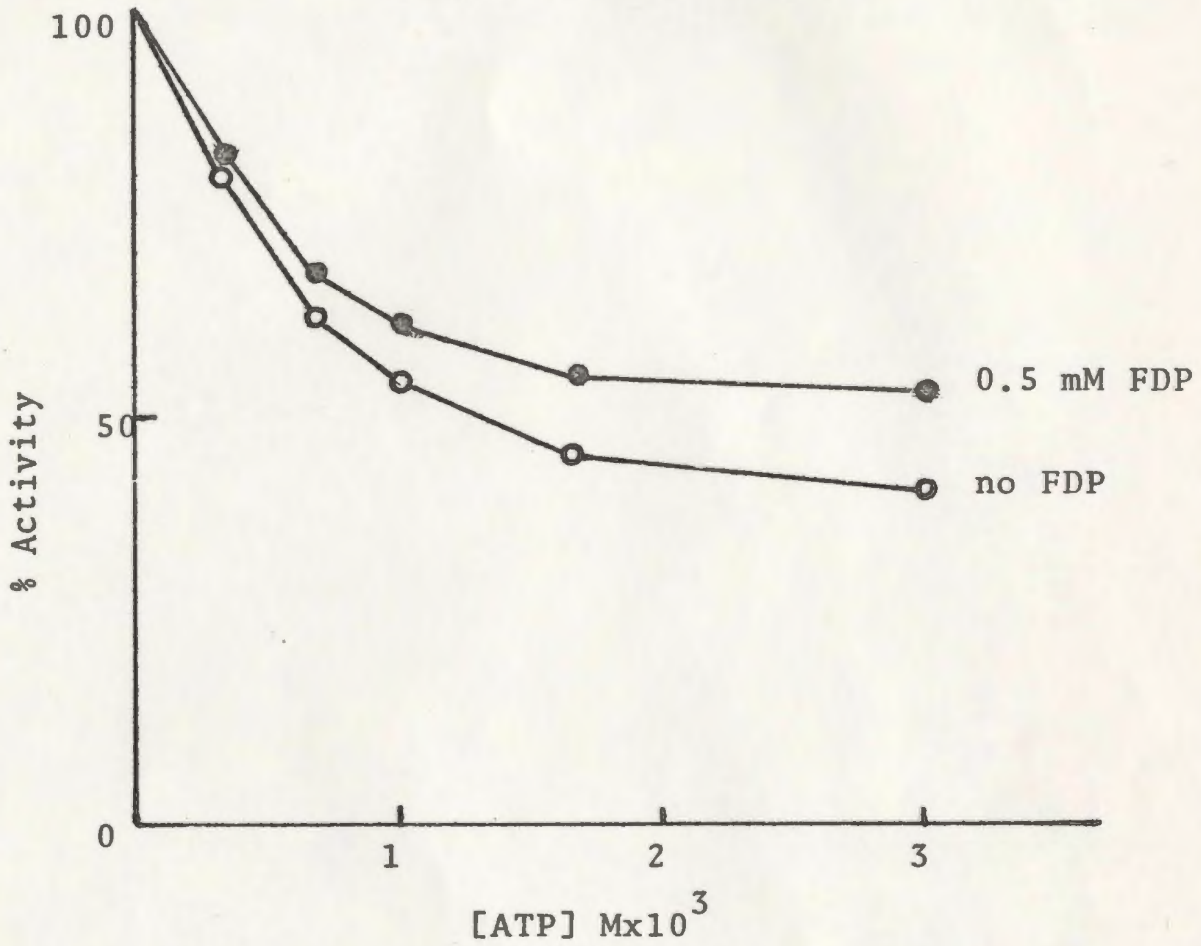
Effect of FDP on the Relationship between Pyruvate Kinase Activity and ADP Concentration



The reaction mixture contained 0.15 mg protein/ml of adductor muscle supernatant.

Figure 8

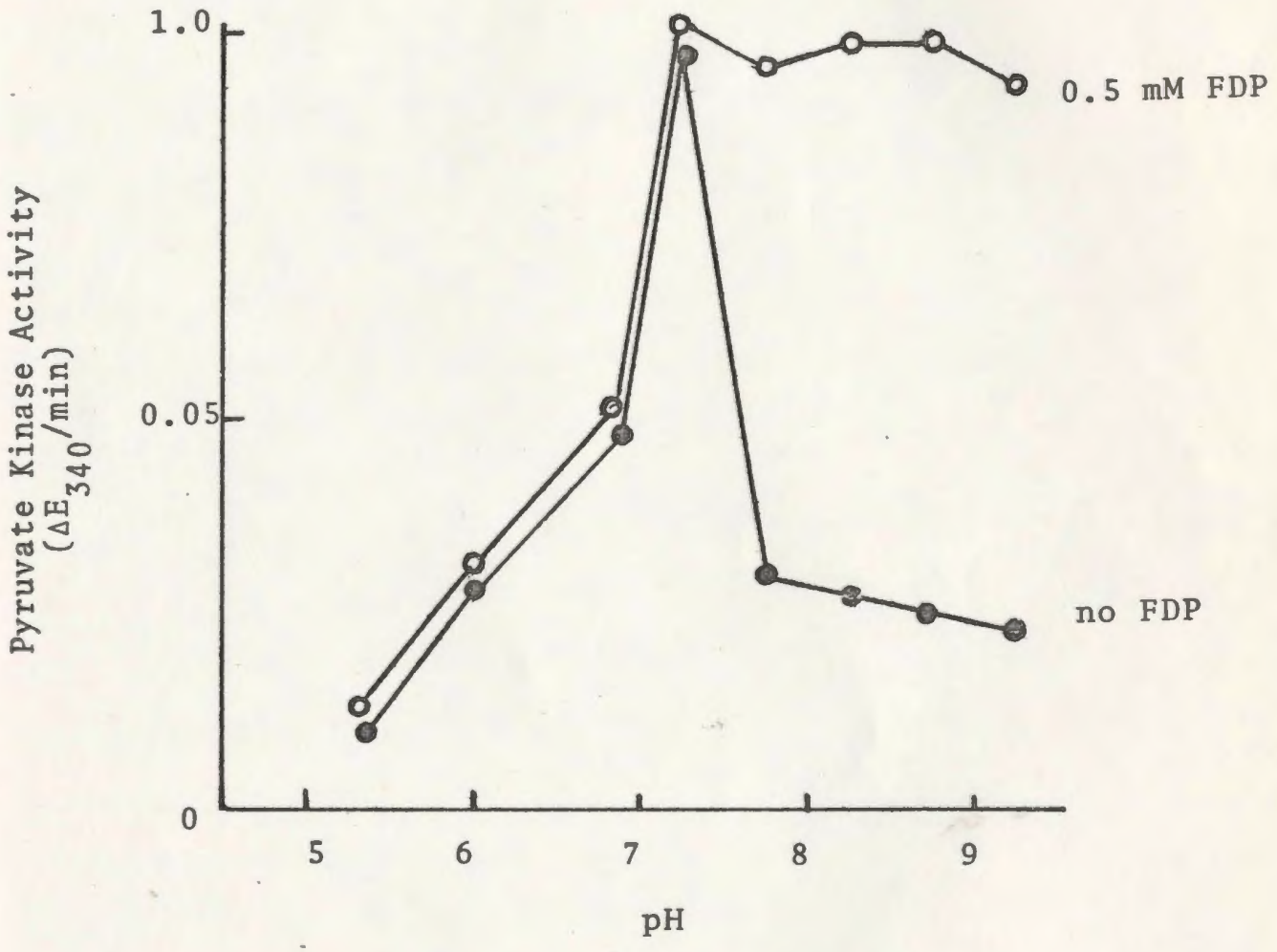
Inhibition of pyruvate kinase activity by ATP and the effect of FDP



The reaction mixture contained 0.15 mg protein/ml of adductor muscle supernatant.

Figure 9

Effect of FDP on the relationship between pyruvate kinase activity and pH



The reaction mixture contained 0.15 mg protein/ml of adductor muscle supernatant.

Properties of Adductor Muscle Lactic Dehydrogenase

Effects of Aspartate and Glutamate on Lactic Dehydrogenase

Table 7 shows the effects of aspartate and glutamate on adductor muscle lactic dehydrogenase. In contrast to the mammalian enzyme[50] these metabolites decrease the activity of the enzyme.

Effects of Purine and Pyrimidine Nucleotides on Lactic Dehydrogenase

ATP, ADP, AMP, GTP, CTP, UTP and ITP at concentrations of 1 mM had no effect on lactic dehydrogenase. No effect of these nucleotides has been reported on lactic dehydrogenases from other sources.

Effects of Krebs Cycle intermediates on lactic dehydrogenase

In contrast to mammalian lactic dehydrogenase[50], the activity of the adductor muscle enzyme was decreased with the effect of Krebs Cycle intermediates as shown in Table 8. Succinate and isocitrate were shown to exhibit competitive inhibition with respect to pyruvate as shown in Figure 10. It is possible the other intermediates also exhibit competitive inhibition.

TABLE 7

THE EFFECT OF ASPARTATE AND GLUTAMATE ON ADDUCTOR MUSCLE LACTIC DEHYDROGENASE

[Metabolite] (mM)	% Activity	
	Aspartate	Glutamate
0	100	100
0.1	90	93
0.2	78	80
0.3	72	70
0.4	67	64
0.5	60	57

The reaction mixture contained 0.2 mg protein/ml of adductor muscle supernatant.

TABLE 8

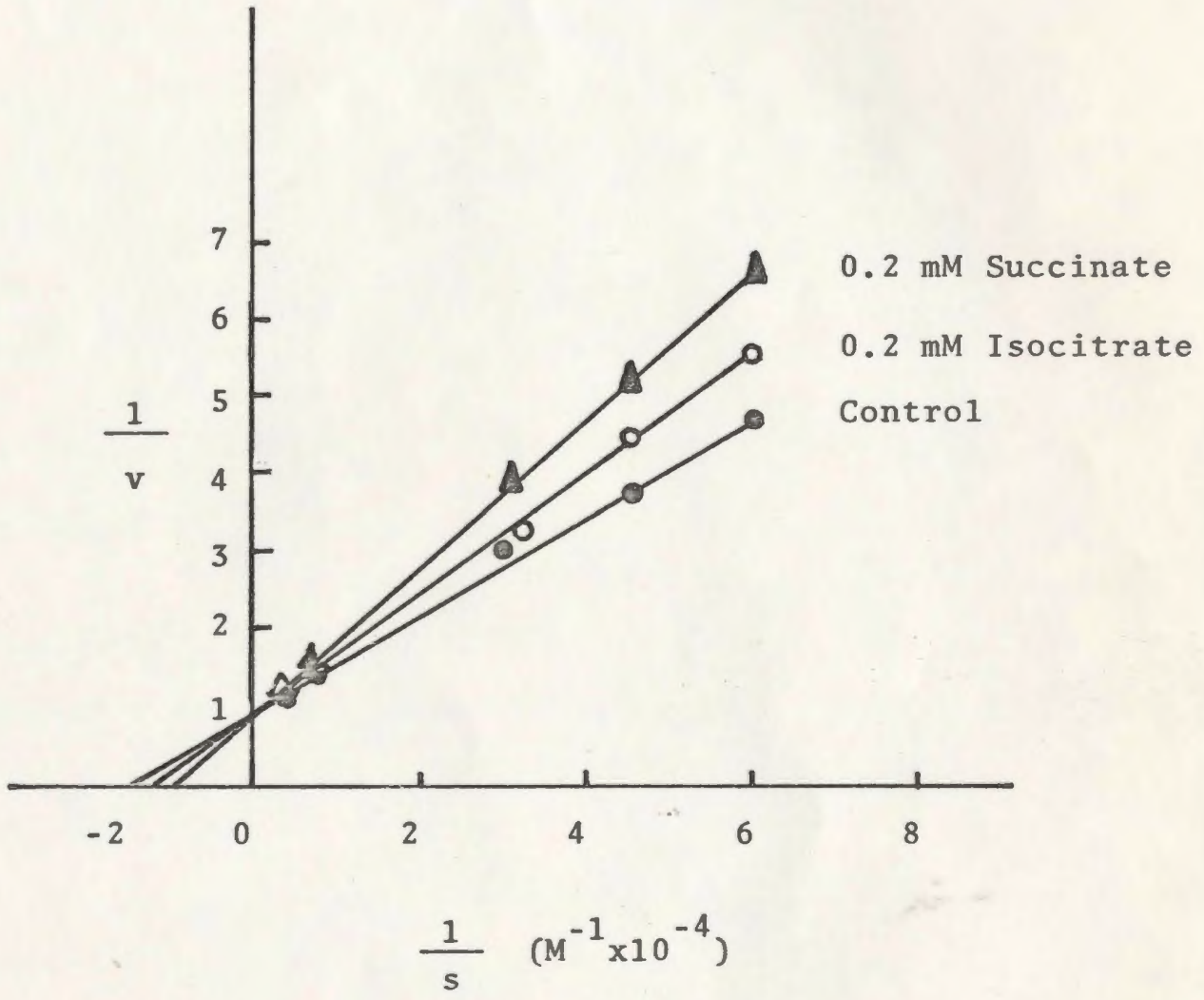
EFFECT OF KREBS CYCLE INTERMEDIATES ON LACTIC DEHYDROGENASE

[Metabolite] (mM)	% Activity					
	Citrate	Isocitrate	α-Ketoclutarate	Succinate	Fumarate	Malate
0	100	100	100	100	100	100
0.1	96	93	96	93	93	93
0.2	86	90	80	83	78	78
0.3	78	80	72	78	67	72
0.4	67	75	67	67	62	64
0.5	60	67	57	62	55	57

The reaction mixture contained 0.2 mg protein/ml of adductor muscle supernatant.

Figure 10

Effect of Isocitrate and Succinate on Km of Lactic Dehydrogenase



Properties of Adductor Muscle Malate Dehydrogenase

Effect of citrate and fumarate on Mitochondrial and Supernatant Malate Dehydrogenase

The effect of citrate and fumarate on the adductor muscle malate dehydrogenases is shown in Table 9. As has been reported from studies on pig heart malate dehydrogenases[51], the mitochondrial enzyme is affected by both metabolites, whereas the supernatant enzyme is not. With the mitochondrial enzyme, the inhibition by citrate was found to be competitive with NADH.

Substrate Inhibition of Mitochondrial and Supernatant Malate Dehydrogenase

As is the case with other malate dehydrogenases[96-99], the adductor muscle mitochondrial malate dehydrogenase is more susceptible to inhibition by high concentrations of oxalacetate than the adductor muscle supernatant malate dehydrogenase (Figure 11).

Using malate as substrate, inhibition of the adductor muscle enzymes was observed only at high concentrations of malate. A similar case has been reported for tuna malate dehydrogenases[99]. Inhibition of the adductor muscle supernatant malate dehydrogenase is slightly greater than inhibition of the mitochondrial enzyme in this reaction, representative values being 40 and 55% inhibition, respectively, at a malate concentration of 0.5 M.

TABLE 9

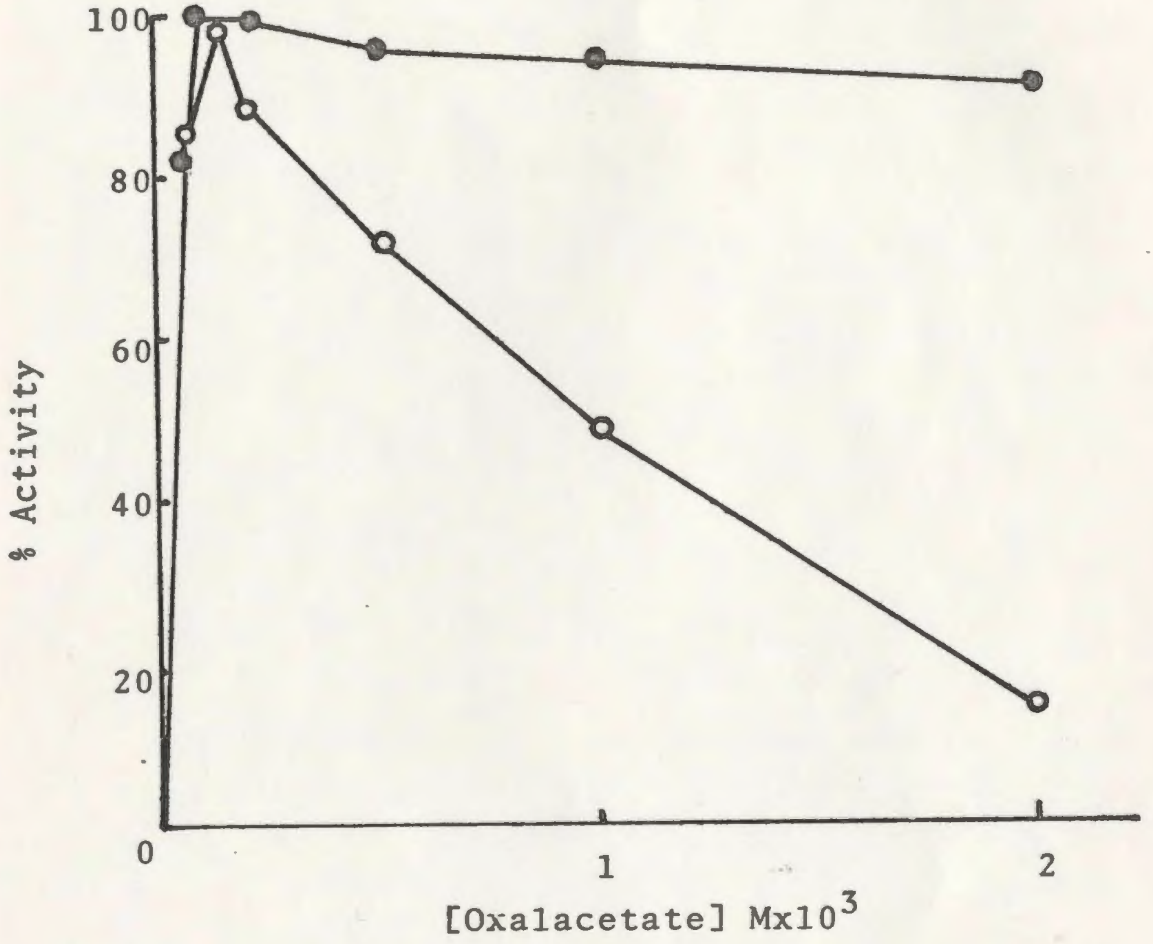
THE EFFECT OF CITRATE AND FUMARATE ON ADDUCTOR MUSCLE MALATE DEHYDROGENASES

Additions	Malate Dehydrogenase Activity (%)	
	Mitochondrial	Supernatant
None	100	100
0.5 mM citrate	78	100
1 mM citrate	65	100
2 mM citrate	24	96
0.5 mM fumarate	60	100
1 mM fumarate	44	100
2 mM fumarate	31	93

The mitochondrial fraction contained 1.5 mg protein/ml and the supernatant fraction 0.2 mg protein/ml.

Figure 11

The Effect of [Oxalacetate] on the Activity of Mitochondrial and Supernatant Malate Dehydrogenase



Mitochondrial MDH

Supernatant MDH

The mitochondrial fraction contained 1.5 mg protein/ml and the supernatant fraction 0.2 mg protein/ml.

The effects of Nucleotides on the Malate Dehydrogenases

Tables 10 and 11 show the effect of ATP, ADP, and AMP on mitochondrial and supernatant malate dehydrogenase. With both enzymes, increasing concentration of nucleotide gives rise to increased inhibition. At a concentration of 3 mM, ATP and ADP inhibition of the mitochondrial enzyme is less than that of the supernatant enzyme. The inhibition produced by ATP was found to be competitive with NADH. The inhibition of malate dehydrogenases by adenine nucleotides in the adductor muscle is similar to that found in pig heart, E. coli and lemon[54].

TABLE 10

THE EFFECT OF ADENINE NUCLEOTIDES ON MITOCHONDRIAL MALATE DEHYDROGENASE

[Nucleotide] (mM)	% Activity		
	ATP	ADP	AMP
0	100	100	100
0.66	93	90	87
1.0	64	70	72
1.66	44	49	55
3.0	31	29	36

The reaction mixture contained 1.5 mg protein/ml of adductor muscle mitochondria.

TABLE 11

THE EFFECT OF ADENINE NUCLEOTIDES ON SUPERNATANT MALATE DEHYDROGENASE

[Nucleotide] (mM)	% Activity		
	ATP	ADP	AMP
0	100	100	100
0.66	87	90	93
1.0	75	72	67
1.66	60	55	46
3.0	49	44	32

The reaction mixture contained 0.2 mg protein/ml of adductor muscle supernatant.

Properties of Adductor Muscle Malic Enzyme

Effect of FDP on Malic Enzyme

Figure 12 shows the effect of FDP on the malic enzyme activity of the adductor muscle. The inhibitory effect of FDP increases with increasing concentration. A Lineweaver-Burk plot indicates that FDP is a competitive inhibitor for malate altering the apparent Km of the substrate as shown in Figure 13.

Effect of 6-Phosphogluconate on Malic Enzyme

The effect of 6-Phosphogluconate on the malic enzyme is shown in Table 12.

As with FDP, increased concentration of effector results in increased inhibition. Figure 14 indicates that 6-Phosphogluconate is a competitive inhibitor for malate.

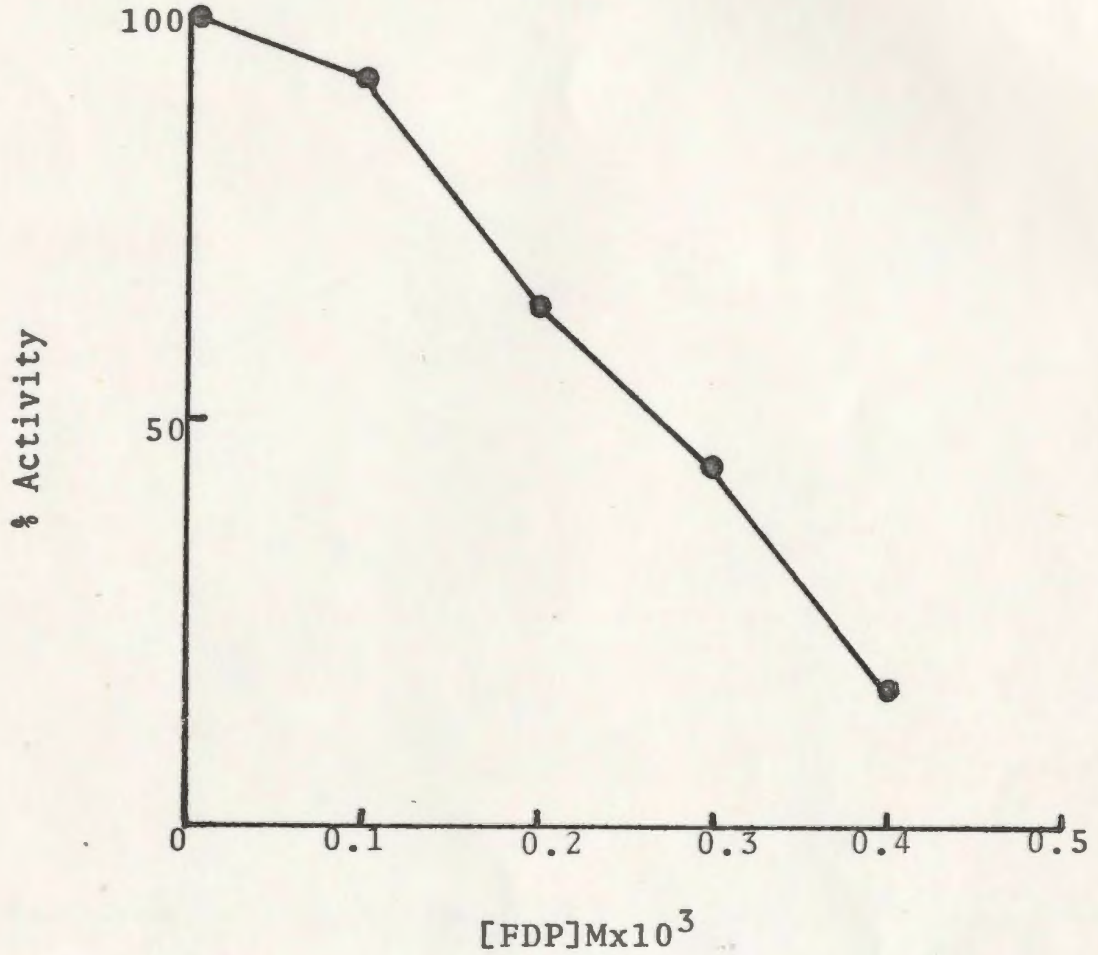
Effect of Inhibition of Nucleotides

The effect of the nucleotides CTP, GTP, ITP, AMP, ADP and ATP were examined on the malic enzyme. Only ATP was an effective inhibitor. The effect of ATP is shown in Table 13.

In Figure 15 the ATP inhibition on the adductor muscle malic enzyme is shown to be competitive with respect to malate.

Figure 12

Effect of Fructose Diphosphate on Malic Enzyme



The malic enzyme was assayed in particulate-free supernatant containing 0.15 mg protein/ml. The results are calculated such that activity in absence of FDP is 100%.

Figure 13

Effect of Fructose Diphosphate on Km of Malic Enzyme

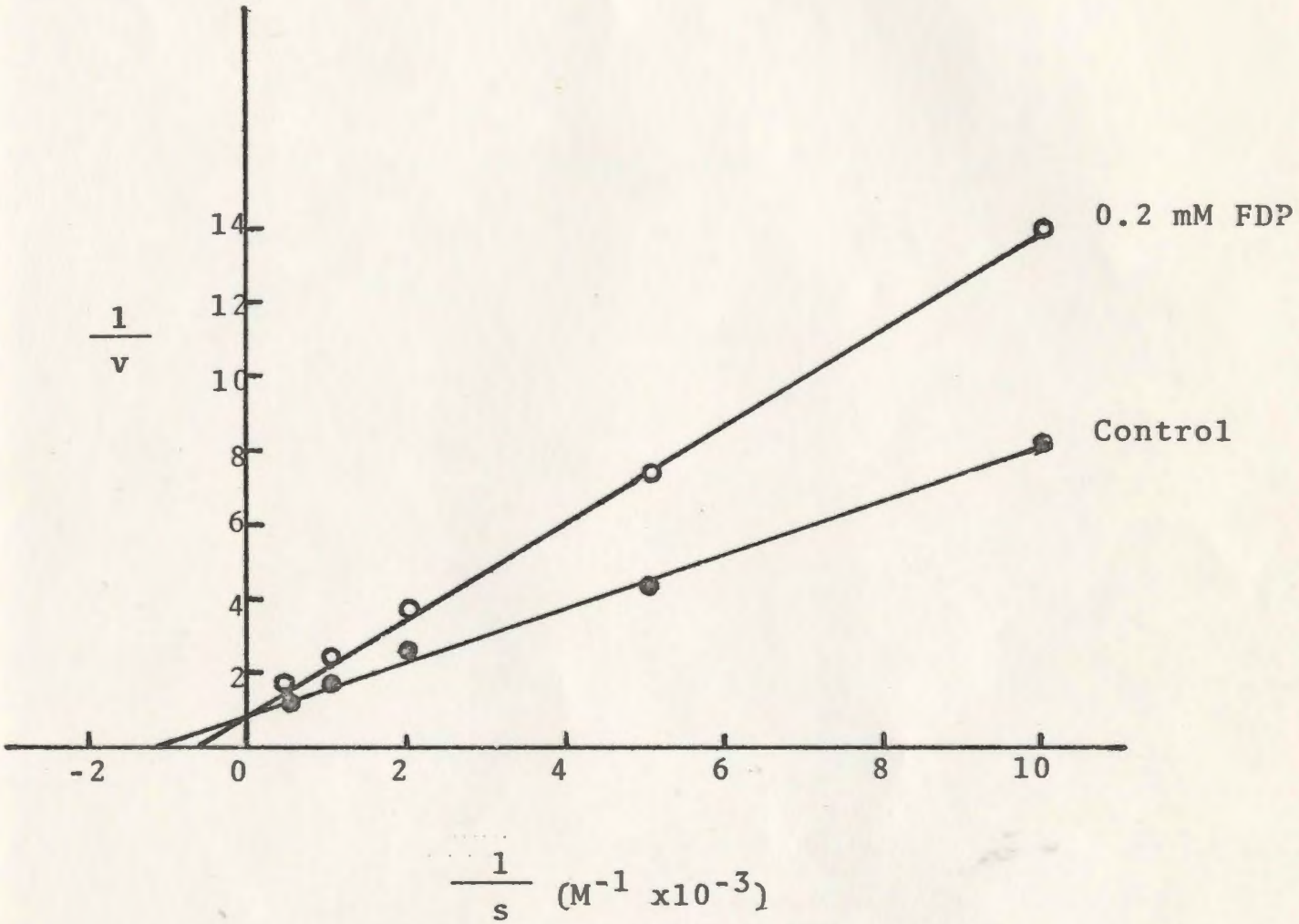


TABLE 12

EFFECT OF 6-PHOSPHOGLUCONATE ON MALIC ENZYME

[6-Phosphogluconate] (mM)	% Activity
0	100
0.1	90
0.2	70
0.3	57
0.4	30

The reaction mixture contained 0.15 mg protein/ml of adductor muscle supernatant.

TABLE 13

INHIBITION OF MALIC ENZYME BY ATP

[ATP] (mM)	% Activity
0	100
0.33	90
0.66	77
1.0	71
1.66	66
3.0	43

The reqction mixture contained 0.15 mg protein/ml of adductor muscle supernatant.

Figure 14

Effect of 6-Phosphogluconate on Km of Malic Enzyme

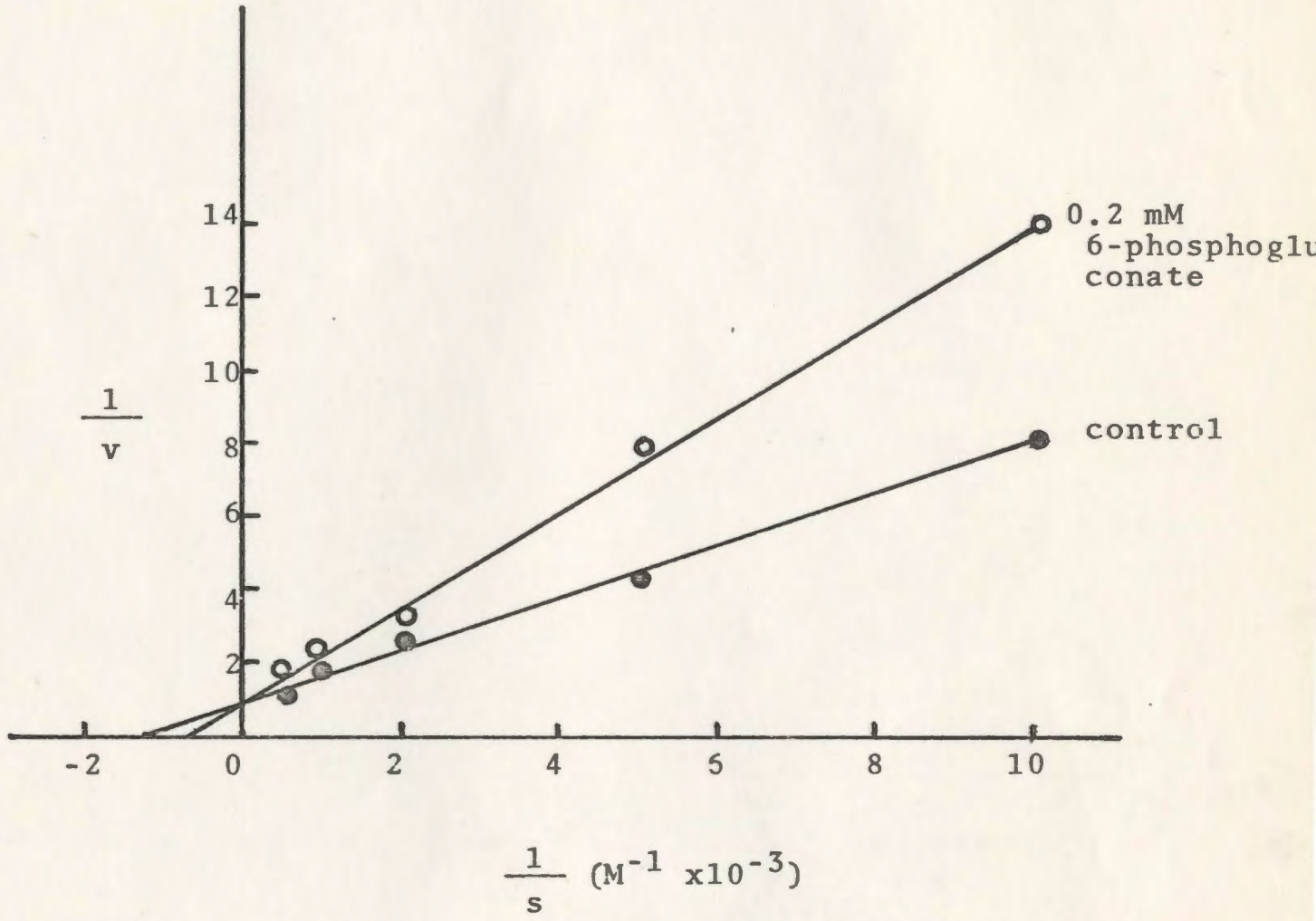
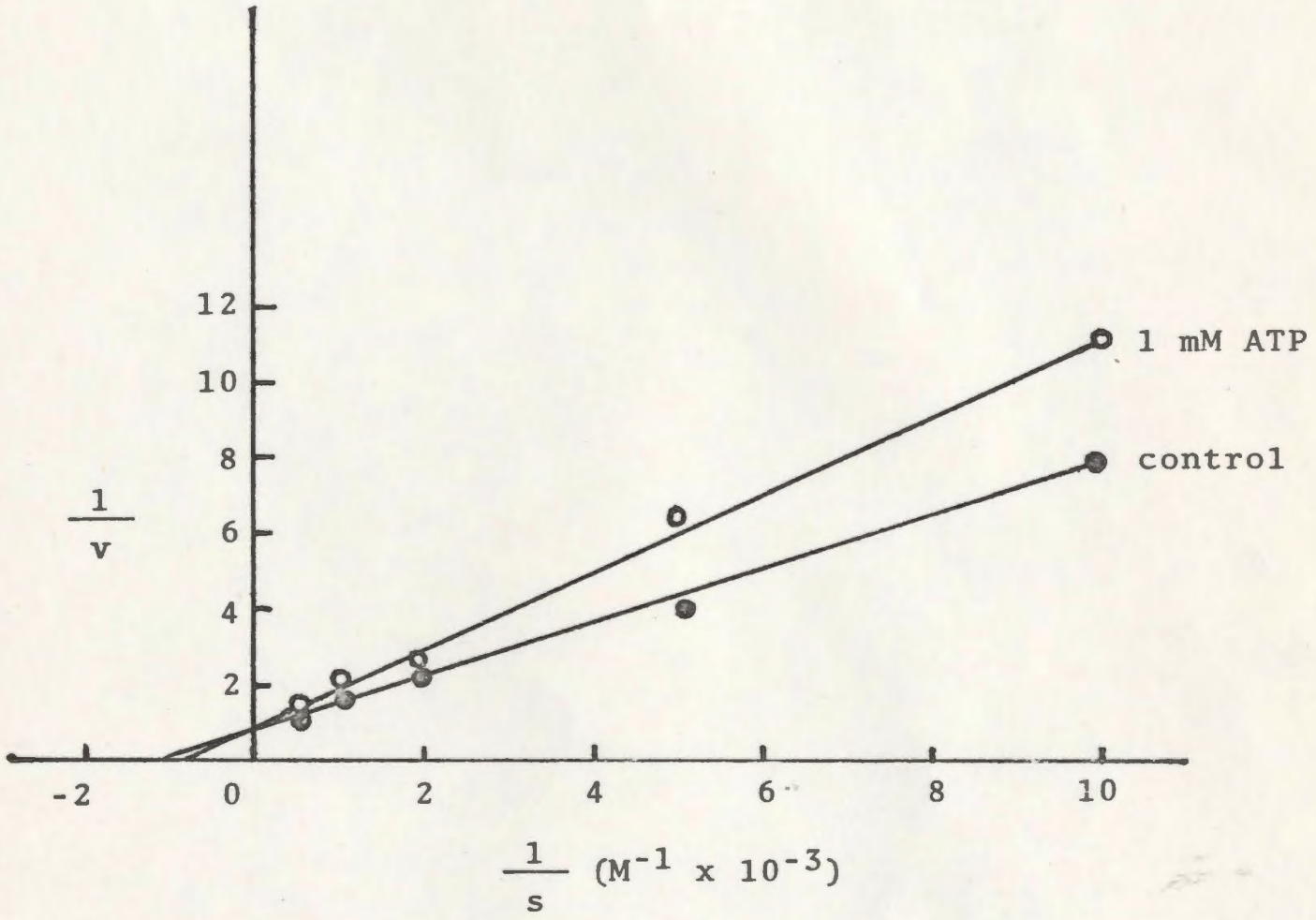


Figure 15

Effect of ATP on Km of Malic Enzyme



Effect of Acetyl CoA and Oxalacetate on Malic Enzyme

It has been shown that both acetyl CoA and oxalacetate act as effectors on malic enzyme in bacterial systems[58]. On the adductor muscle malic enzyme at both 0.1 and 1 mM concentration, neither metabolite had an effect on the enzyme.

Properties of Adductor Muscle Fructose 1,6,Diphosphatase

Search for a metabolic activator for FDPase

A search was made for metabolic intermediates that would activate the adductor muscle FDPase. Considerable interest centered on phosphoenolpyruvate and ATP, since the substrate of FDPase, FDP, is a positive modular of pyruvate kinase and ATP is a negative modulator for phosphofructokinase in the adductor muscle. The ability of phosphoenolpyruvate to act as a positive modulator towards FDPase would have made possible the realization of a coupling of two control sites in glycolysis and gluconeogenesis. Likewise, ATP activation of FDPase would couple with ATP inhibition of phosphofructokinase. However, the enzyme was not affected by either phosphoenolpyruvate or ATP, or by any other of the following metabolites tested: glucose-6-phosphate, pyruvate, lactate, oxalacetate, malate, citrate, succinate, ADP and NADH.

Properties of Adductor Muscle Phosphoenolpyruvic Carboxykinase

Effects of Nucleotides on PEP carboxykinase

The effects of AMP, ADP, ATP, UTP, and CTP were studied on the enzyme. None of these nucleotides had an effect and no such effect has as yet been reported from other sources.

DISCUSSION

A detailed comparison of the regulatory enzymes of scallop adductor muscle with those of the various systems that have been studied is difficult because the work has been carried out on preparations of different degrees of purity, and various buffers and pH values have been used in the assay systems. Some general observations can, however, be made.

All the phosphofructokinases studied show a sigmoidal fructose 6-phosphate activation curve, typical of many regulatory enzymes. The concentration of fructose 6-phosphate for half-maximal activity varies from 0.028 mM in guinea-pig heart (Mansour)[100] to 5 mM in the liver fluke (Mansour and Mansour)[39].

The value of 1 mM for scallop adductor muscle falls within this range. All the enzymes show a similar ATP activity pattern, which is dependent on fructose 6-phosphate concentrations. The optimum ATP concentration varies from about 0.02 mM in ox heart (Frenkel)[90] and rat adipose tissue (Denton and Rangle)[101], to more than 1 mM in E. coli (Atkinson and Walton)[102] and liver fluke (Mansour and Mansour)[39]; the corresponding value for scallop adductor is about 0.25 mM. Above this optimum concentration, ATP becomes inhibitory to all the enzymes except that of E. coli (Blangy et al)[95].

The scallop adductor muscle enzyme resembles the mammalian muscle enzyme in that it is activated by AMP, 3'5'-(cyclic)-AMP and Pi. It is, however, inhibited by FDP, in this respect resembling the yeast enzyme (Betz and Moore)[103]. The role of 3'5'-(cyclic)-AMP in invertebrates metabolism is not yet clear. Phosphoenolpyruvate[39] and citrate[88-91] inhibit mammalian muscle, but have no effect on scallop muscle enzyme.

It has been shown that FDP can activate pyruvate kinase of rat liver and adipose tissue, but not skeletal-muscle enzyme,[43,44,46]. In the scallop adductor muscle, however, FDP did have a positive effect. The pyruvate kinase of the adductor muscle resembles the rat liver enzyme further in that only FDP appears to have a stimulating effect whereas bacterial[48] and fish[49] pyruvate kinase enzymes are activated by AMP and 3'5'-(cyclic)-AMP respectively. The concentration (1μM) of FDP required for half-maximal stimulation of the adductor muscle enzyme is very similar to that for the rat liver enzyme[43] and about one-hundredth of that required to activate the yeast enzyme[42]. As with the rat liver enzyme[44], ADP at high concentrations (2 mM and above) inhibits the enzyme activity. The presence of FDP has little effect on the inhibition.

The adductor muscle pyruvate kinase enzyme is inhibited by ATP with half-maximal inhibition occurring at

about 0.5 mM. The concentration of ATP required is higher than that required for half-maximal inhibition of the rat liver enzyme (0.16 mM) but lower than that required for half-maximal inhibition of the rat muscle enzyme (3 mM)[44].

The question arises whether the ATP and other nucleotides used in this in vitro study are in the physiological range occurring in the adductor muscle. The data in the literature indicate that this is the case (Hiltz and Dyer)[35].

Glutamate and aspartate, and the Krebs Cycle intermediates decrease the activity of scallop adductor muscle lactate dehydrogenase, whereas in mammalian skeletal muscle[50], these metabolites activate the enzyme. Activation of lactate dehydrogenase is advantageous in mammalian skeletal muscle, as it results in less pyruvate being available for conversion to acetyl coenzyme A and more NAD being available to spark glycolysis through the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase. Thus, when the Krebs Cycle substrates act to reduce the concentration of a substance which is one of their precursors, they are in effect controlling their own concentrations, a simple case of feedback control by an end product of a synthetic pathway. In mammals, the conversion of pyruvate to lactate is the chief means by which cytoplasmic NADH is oxidized. In the scallop muscle however, the activity of lactate dehydrogenase is low and lactate is not the end product of anaerobic

glycolysis. In addition, cytoplasmic NADH can be oxidized by cytoplasmic malate dehydrogenase and by the Bücher shuttle. It therefore would be of no advantage to the adductor muscle to accumulate lactate, a compound which it may not be able to metabolize at any appreciable rate. In fact, by decreasing the lactate dehydrogenase activity, it is possibly ensuring a channelling system away from lactate and on to succinate, and, on the way, oxidizing NADH at the malate dehydrogenase level.

The malate dehydrogenases of scallop muscle resemble those from vertebrate sources in a number of ways. Inhibition of mitochondrial malate dehydrogenase by oxalacetate has also been observed in chicken[98], tuna[99], beef and pig[96,97] mitochondrial malate dehydrogenases. The effects of citrate and fumarate on the scallop malate dehydrogenases are similar to that reported for pig heart[51], and the effects of the adenine nucleotides similar to that reported for pig heart, E. coli and lemon[54].

The behaviour of the scallop muscle malic enzyme in the presence of fructose diphosphate and 6-phosphogluconate resembles the malic enzyme from rat liver[57] and bacterial systems[58]. In contrast to the bacterial enzyme, the only nucleotide which will inhibit scallop malic enzyme is ATP, while acetyl CoA and oxalacetate have no effect on it.

The effects on both phosphoenolpyruvic carboxykinase[56] and fructose 1,6 diphosphatase[55] are similar to that of other systems studied.

CHAPTER 3

Thermal Studies on Adductor Muscle Enzymes

METHODS AND MATERIALS

All enzyme studies were carried out on particulate-free supernatant which was prepared as described in Chapter 1.

Enzyme Assays

Pyruvate Kinase, LDH, FDPase and PEP carboxykinase were assayed as described in Chapter 1.

Materials

All chemicals were obtained from the Sigma Chemical Co.

RESULTS

Effect of Temperature on the Activity of Adductor Muscle Enzymes

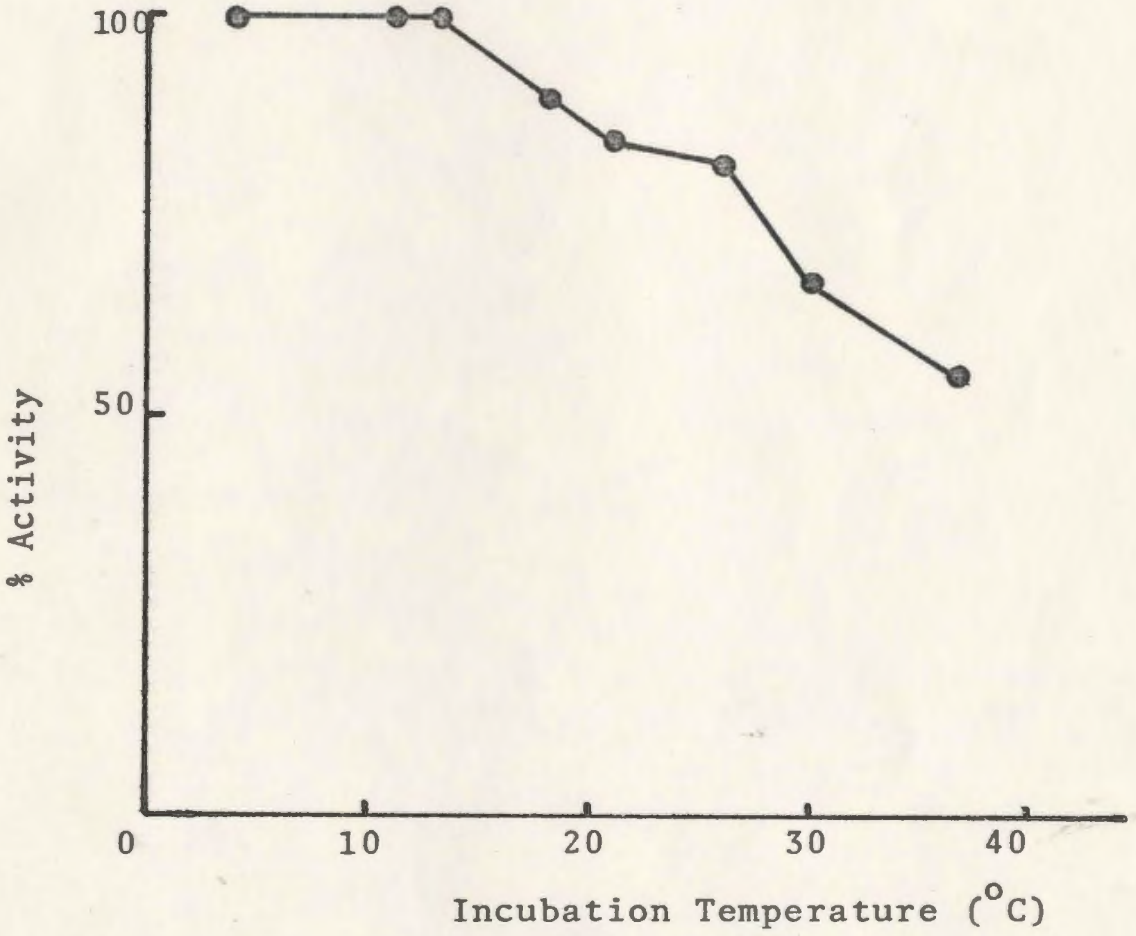
Figure 16 shows the effect of incubation of enzyme solution for 20 minutes on the activity of pyruvate kinase. It is interesting to note that the enzyme activity remains constant between 4^{o} and 13^{o}, the approximate limits of the scallop's environmental temperature. Above this temperature, there is a steady decrease in activity. A similar effect was found with phosphofructokinase, lactate dehydrogenase, malic enzyme, phosphoenolpyruvic carboxykinase and fructose 1,6 diphosphatase. This indicates the scallop's enzymes are remarkably thermolabile at temperatures above their environmental temperatures.

Effect of Temperature on Km

Figure 17 shows the effect of temperature on the Michaelis constants of pyruvate kinase and lactic dehydrogenase. The effect of temperature on the Michaelis Constants of both enzymes is fundamentally similar. Above a critical temperature the Km increases with increasing temperature. At temperatures below the critical range, the Km again tends to increase. The thermal optimum, defined operationally as the temperature range through which Km values are at a minimum, fall well within the physiological temperature range for the

Figure 16

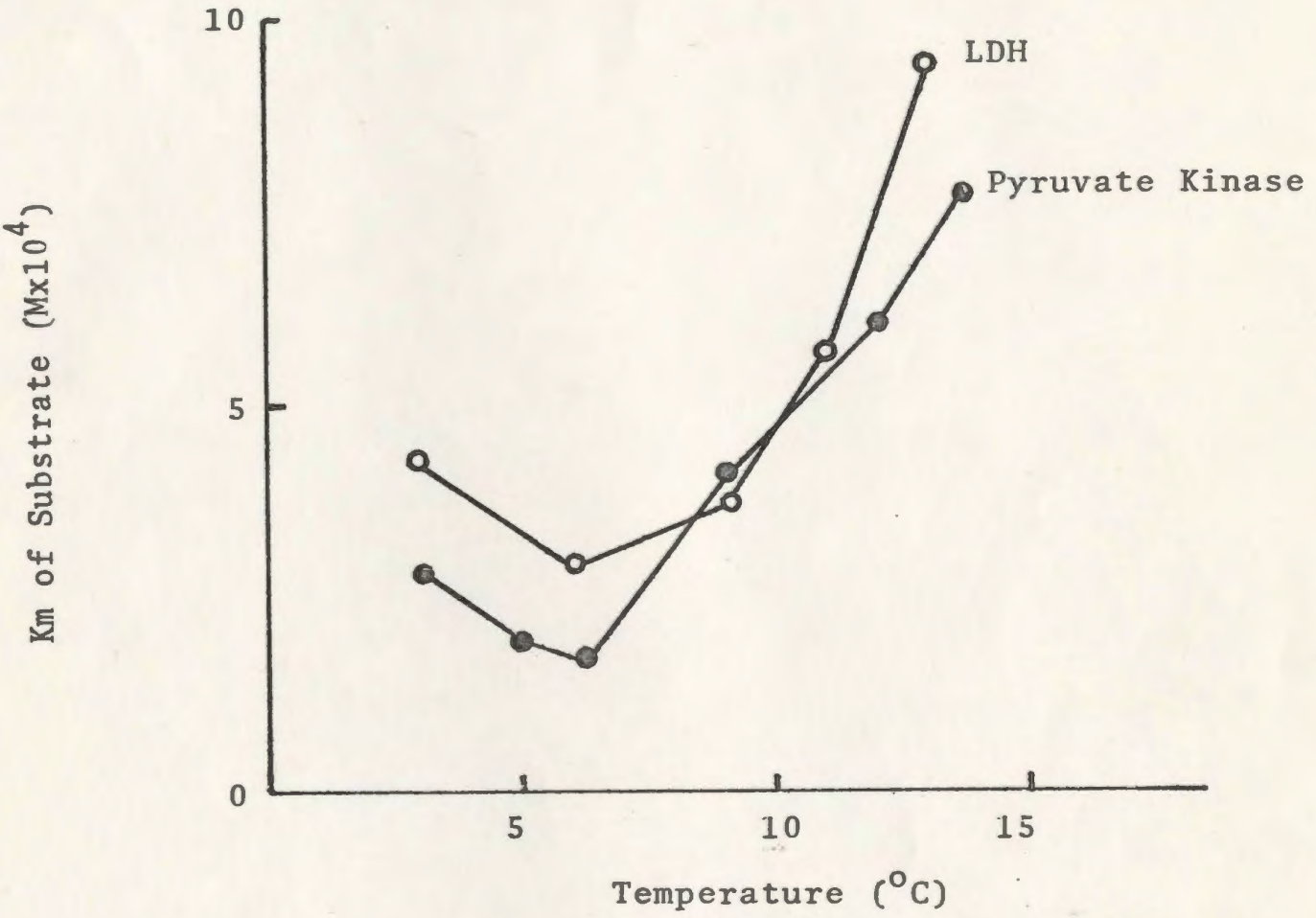
Effect of Temperature on the Activity of Pyruvate Kinase after 20 Minutes Incubation



The reaction mixture contained 0.2 mg protein/ml of adductor muscle supernatant.

Table 17

The Effect of Temperature on the Km of Lactic Dehydrogenase and of Pyruvate Kinase



Lactic Dehydrogenase and Pyruvate Kinase were assayed in particulate-free supernatant containing 0.2 mg protein/ml.

scallop and hence it would appear to be of adaptive significance. Hochachka and Somero[59,60] have obtained similar patterns with vertebrate poikilotherms.

Effect of Temperature on the FDPase Inhibition by AMP

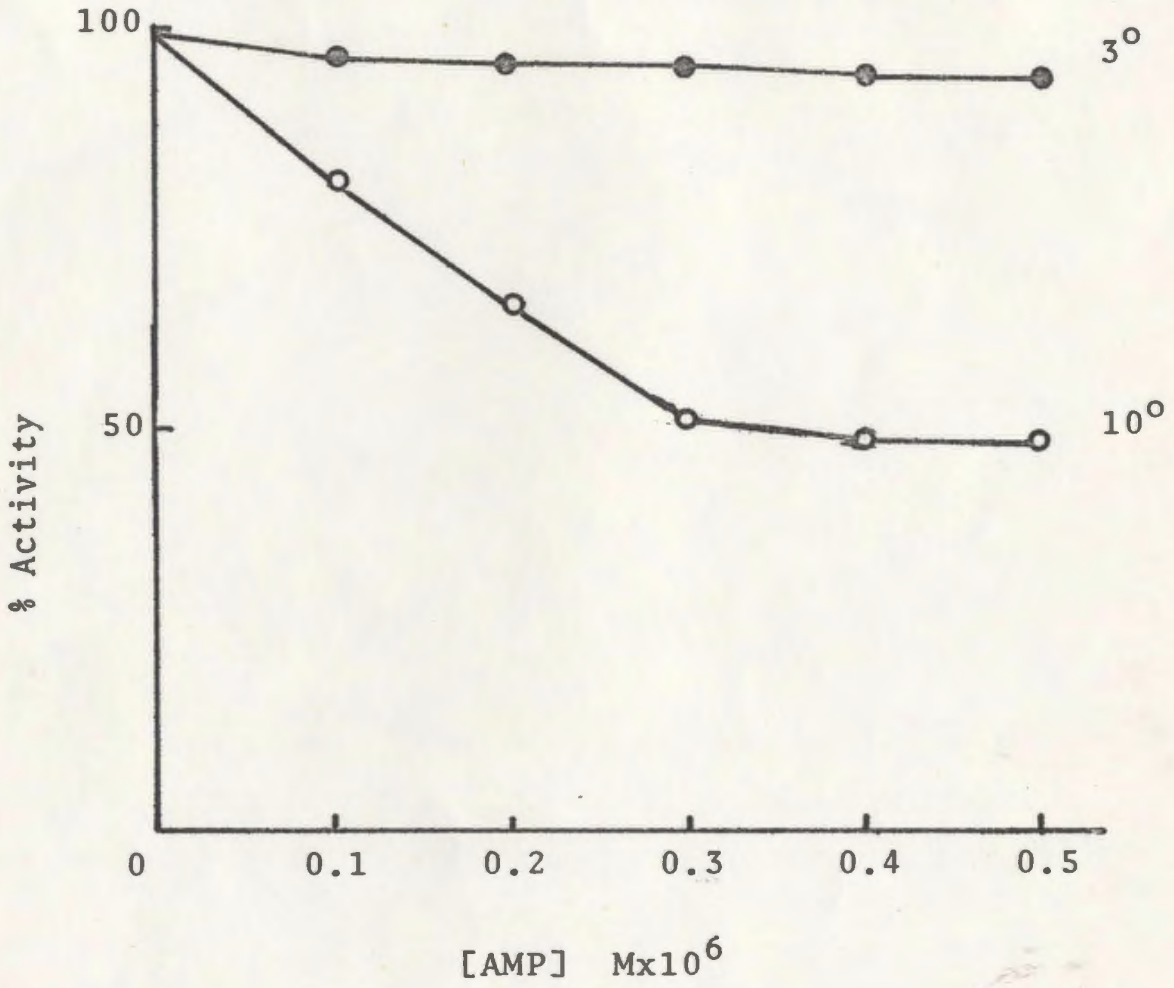
The effect of temperature on the inhibition of FDPase by AMP is shown in Figure 18. At 3°, which is taken as the lower environmental temperature, the inhibition at 0.5 μm is 20% of what it is at 10°. The AMP did not effect the thermal stability of the enzyme.

Effect of Temperature on the Activation of PEP Carboxykinase by Cyclic AMP

In the case of PEP carboxykinase the activation at a cyclic AMP concentration of 0.3 μm was six times as much at 3° as at 10° (Figure 19).

Figure 18

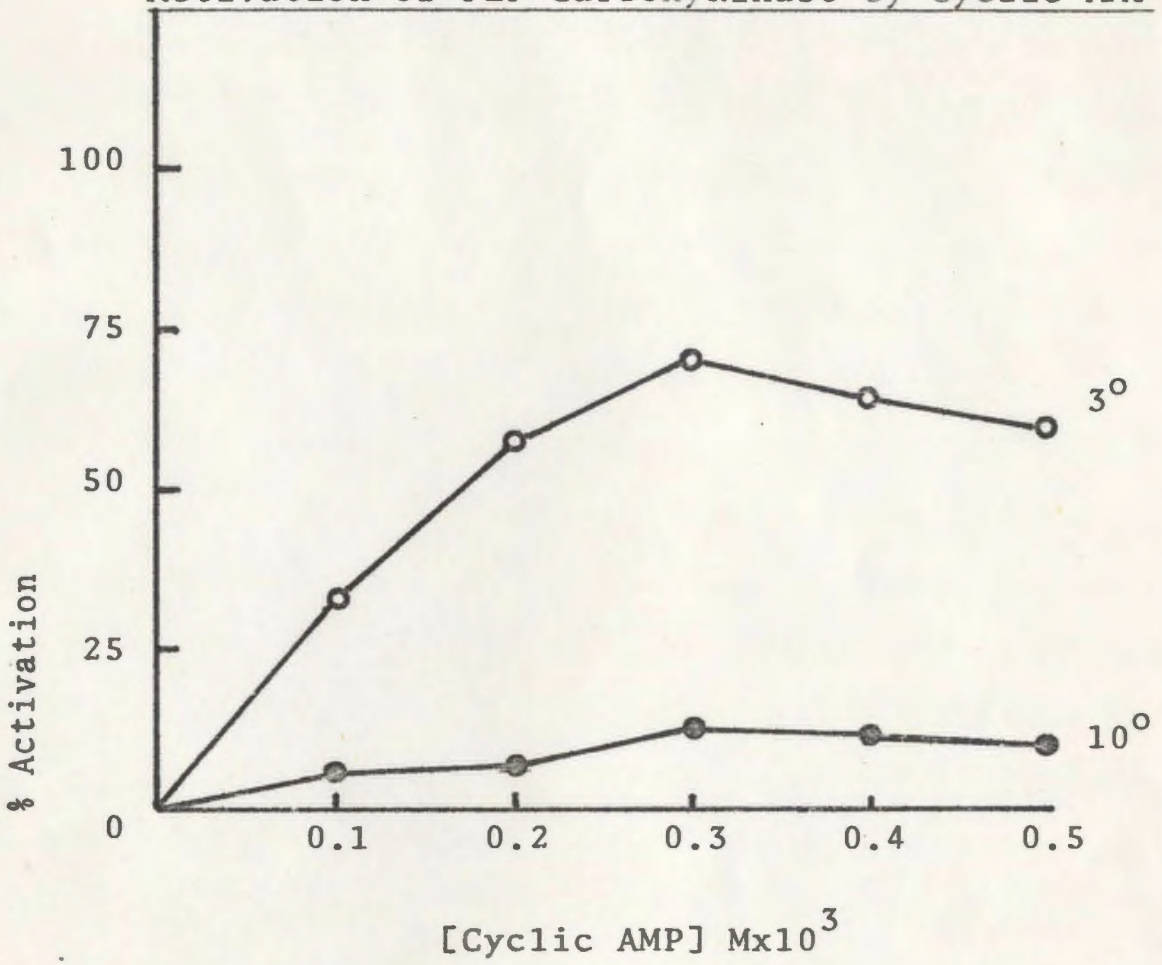
Inhibition of FDPase by AMP



The enzyme was assayed in particulate-free supernatant containing 0.2 mg protein/ml.

Figure 19

Activation of PEP Carboxykinase by Cyclic AMP



The enzyme was assayed in particulate-free supernatant containing 0.2 mg protein/ml.

DISCUSSION

This is the first report of thermal studies on the enzymes of scallops. The heat inactivation of the scallop enzymes at temperatures above its normal environment is an interesting phenomenon when contrasted with similar effects on warm-blooded animals. Vertebrate poikilotherms that have been studied have shown similar properties.

The variation in the Km of an enzyme due to temperature seems to be peculiar to poikilothermic enzymes. Hochachka and Somero[59,60] have shown that the Km pattern of trout, tuna, an Antarctic fish, and the South American lungfish exhibit similar behaviour over the limits of their environmental temperature. In each case the minimum Km occurs at a temperature which closely coincides with the ambient environmental temperature. This property could well be of importance to the metabolic control of the scallop during temperature changes in its environment as its provides a mechanism by which a given reaction velocity can be held relatively independent of temperature, at least under conditions of limiting substrate. It is possible, that other enzymes of the scallop, and indeed of other molluscs, may show similar effects.

The temperature-dependence of the cyclic AMP activation of PEP carboxykinase and the AMP inhibition of

FDPase could also be an interesting mechanism of metabolic control in the adductor muscle. It has been established that in molluscs, gluconeogenesis is favoured during low-temperature acclimatization. This would explain why at 3° the inhibition of FDPase by AMP is slight and why cyclic AMP can activate PEP carboxykinase. FDPase and PEP carboxykinase are two important gluconeogenic enzymes in the scallop. Behrisch and Hochachka[63] have shown that the AMP inhibition of FDPase is also temperature-dependent in a vertebrate poikilotherm, while Taketa and Pogell [104] have shown similar properties with rat liver FDPase. It is thus possible that in the scallop temperature may play an important role in metabolic regulation.

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