THE FUNCTIONAL ROLE OF RED AND WHITE MUSCLE IN THE EEL, *ANGUILLA ROSTRATA*: AN ULTRASTRUCTURAL AND ENZYME STUDY

by William C. Hulbert

A thesis submitted to the School of Graduate Studies in partial fulfilment of the requirements for the Ph.D. degree in Biology

UNIVERSITY OF OTTAWA
OTTAWA, CANADA, 1976

BIOGRAPHY

Name: William C. Hulbert
Date of Birth: May 9,1946
Place of Birth: Spokane Washington, U.S.A.

Education
B.A. (Biology): 1972, Western Washington State College
M.Sc. (Biology): 1973, Western Washington State College
Ph.D. (Biology): 1976, University of Ottawa

Honors and Scholarships
ANL undergraduate research fellowship: 1972 Argonne National Laboratory, Chicago Ill.
Magna Cum Laude: 1972 Western Washington State College
National Research Council of Canada post-graduate scholarship: 1975,1976
National Research Council of Canada post-doctoral fellowship: 1976

Publications
Hulbert,W.C. and T.W.Moon (1975) Tissue ultrastructure and alterations as a result of applied hydrostatic pressure in two marine teleosts. Comp.Biochem.Physiol. 52B:117-126.

Moon,T.W. and W.C.Hulbert (1975) The ultrastructure of the mantle musculature of the squid, Symplectoteuthis oualaniensis. Comp.Biochem.Physiol. 52B:145-149.

Hulbert,W.C., D.E.Schneider, and T.W.Moon (1976) Temperature and salinity adaptation in the purple shore crab, Hemigrapsus nudus: an in vitro physiological study using excised gills. Mar.Biol.36(3):217-222.

Hulbert,W.C., D.E.Schneider, and T.W.Moon (1976) Temperature and salinity adaptation in the purple shore crab, Hemigrapsus nudus: an in vitro metabolic flux study using excised gills. Mar.Biol.36(3):223-232.

Hulbert,W.C., M.K.Komourdjian, T.W.Moon, and J.C.Fenwick (1976) The fine structure of the developing spore of Myxidium zelandicum from the eel, Anguilla rostrata. Can.J.Zool. in press.

Komourdjian,M.K., W.C.Hulbert, J.C.Fenwick, and T.W.Moon (1976) First reported occurrence of Myxidium zelandicum from the North American eel, Anguilla rostrata. Can.J. Zool. in press.

Moon,T.W., W.C.Hulbert, T.Mustafa, and D.F.Mettrick (1976) A study of LDH and MDH in adult Hymenolepis diminuta (Cestoda). Comp.Biochem.Physiol. in press.

Moon,T.W., T.Mustafa, W.C.Hulbert, R.B.Podesta, and D.F Mettrick (1976) The phosphoenol-pyruvate branch point in adult Hymenolepis diminuta (Cestoda): A study of pyruvate kinase and phosphoenol-pyruvate carboxykinase. J.Exp.Biol. in press.

Podesta,R.B., T.Mustara, T.W Moon, W.C.Hulbert, and D.F.Mettrick (1976) Anaerobes in an aerobic environment: role of CO_2 in energy metabolism of Hymenolepis diminuta. Int.Parasit.Cong. Brussels, in press.

Presented Papers

Hulbert,W.C. and T.W Moon (1974) Properties of red and white muscle LDH from the eel, Anguilla rostrata. Can.Soc. Zool. Univ. of New Brunswick, June 2-5,1974.

Hulbert,W.C. and T.W.Moon (1975) A comparative study of pyruvate kinase from red and white muscle and liver from the eel, Anguilla rostrata. Can.Soc.Zool. Univ. of Guelph. June 8-11,1975.

ABSTRACT

Considerable controversy has evolved around a purported 'organ' or 'metabolic' role for fish red muscle. This role involves the anabolism of metabolic end products from other tissues of the fish, especially the white muscle. The literature is extensive in documenting both a contractile and an organ function for red muscle. One problem from which these studies suffer is that the results from one fish species is not necessarily applicable to others, nor is there any reason to assume that the red muscle in some fishes will necessarily function as an organ. The American eel, Anguilla rostrata, was chosen to analyze this problem since it offers the unique opportunity to capitalize upon a natural state where the function of red muscle, as a recycling organ, may be significant; i.e., during a non-feeding, seaward migration during which all energy for maintenance, contraction and gonadal maturation must arise from stored body reserves.

Three experimental approaches were utilized in these investigations: an analysis of the structural properties of red and white muscle and liver from feeding, immature yellow eels and non-feeding, mature bronze eels; an analysis of the lactate oxidative capacity of the red and white muscle and liver; and, an analysis of the regulatory characteristics of pyruvate kinase (PK) from red and white muscle and liver. The results suggest that, 1) there is a marked alteration in the structural properties of red muscle

and liver commensurate with maturation while no change could be detected in white muscle; 2) the red muscle and liver lactate dehydrogenase (LDH) catalyze an equilibrium reaction while the white muscle LDH was primarily poised for pyruvate reduction; 3) the white muscle PK in the presence of FDP, was a non-regulatory enzyme of the type observed for mammalian M1-PK; 4) both white muscle PK and LDH exhibited coincident H^+ ion insensitivity, thus ensuring unimpeded flow of carbons from phosphoenol pyruvate to lactate during an anaerobic challange; 5) the regulatory characteristics of red muscle PK would permit it to participate in both a glycolytic and a gluconeogenic role in this tissue; 6) liver PK was not responsive to the classic mammalian L-PK modulators, suggesting the metabolic role of eel liver diverges from the classical description of this tissue; and 7) the enzyme phosphoenol pyruvate carboxykinase was detected only in eel red muscle and liver.

These data are consistent with the red muscle-organ hypothesis orginally proposed by Braekken. Therefore, during the seaward migration of the American eel, A. rostrata, it is suggested that the red muscle may serve an ancillary metabolic or 'liver'-like function by recycling metabolic end products generated either by the white muscle during muscular contraction or by the metabolism of other body tissues.

RÉSUMÉ

De nombreuses controverses ont surgi à propos d'un prétendu rôle d'organe ou métabolique pour le muscle rouge chez le poisson. Ce rôle implique l'anabolisme des produits terminals provenent du métabolisme des autres tissus du poisson, spécialement le muscle blanc. La littérature est intensive en documents à propos de la fonction de contraction et d'organe pour le muscle rouge. Un des problèmes que ces recherches on ; c'est que les résultats obtenus d'une espèce de poisson n'est pas nécessairement applicable aux autres espèces, aussi il n'y a aucune raison d'assumer que le muscle rouge, chez certains poissons fonctionne necessairement comme un organe l'anguille americaine, Anguilla rostrata, fut choisi pour analyser ce problème car il offre l'opportunité unique d'augmenter l'état naturel où la fonction du muscle rouge, comme or ane de recyclage, peut-être significative; c.a.d., durant le jeûne durant la migration en eau salée, toute l'énergie utilisée pour le maintien, la contraction musculaire et la maturation des gonads doit provenir des réserves emmagasinées dans le corps.

Trois approches experimentales ont été utilisées dans ces investigations; une analyse des propriétés structurales du muscle rouge, du muscle blanc et du foie a été faite avec l'anguille jaune non mature nourrie et à jeûne et avec l'anguille bronzée mature; une analyse de la capacité d'oxidation du lactate a été faite avec le muscle blanc,

le muscle rouge et le foie et, une analyse des charactéristiques régulatoires de la pyruvate kinase (PK) a été faite avec le muscle rouge, le muscle blanc et le foie. Les resultats suggèrent, 1) qu'il y a une altération marquée dans les propriétés structurales du muscle rouge et du foie parallèle avec la maturation alors qu'il n'y a acucun changement détectable avec le muscle blanc; 2) l'enzyme 'lactate dehydrogenase' du muscle rouge et du foie catalyse une reaction d'équilibre alors que le meme enzyme dans le muscle blanc n'est fait que pours rèdvine le pyruvate; 3) l'enzyme 'pyruvate kinase' du muscle blanc en presence du fructose 1,6-diphosphate, était un enzyme non-regulatoire comme l'enzyme M_1-PK observée chez les mammifères; 4) les deux enzymes PK et LDH ont montré une insensibilite à l'ion H^+, assurant donc uniquement un flot de carbones à parter du phosphoenol pyruvate au lactate durant une periode anaérobique; 5) les charactéristiques régulatoires de l'enzyme PK du muscle rouge lui permettrait de participer dans les deux rôles de ce tissus, sot la glycolyse et la gluconèogénèse; 6) l'enzyme PK du foie ne repondait pas aux inhibiteurs et stimulateurs comme le classique enzyme PK du foie des mammifères; et, 7) l'enzyme phosphoenol pyruvate carboxykinase fut détecté seulement dans le foie et le muscle rouge de l'anguille.

Ces résultats sont constants avec l'hypothèse du muscle rouge comme organe métabolique originalement proposée par Braekkan. Donc durant la migration en eau salée de l'anguille americaine, A. rostrata, il est suggéré que le muscle rouge

pourrait avoir une fonction métabolique ou une fonction ressemblant au foie pour le recyclage des produits terminals provement sot du muscle blanc durant la contraction musculaire ou du metabolisme des autres tissues de corps.

ABBREVIATIONS

FDPase	Fructose diphosphatase (EC 3.1.3.11 D-Fructose-1, 6-diphosphate 1-phosphohydrolase)
G-6-Pase	Glucose-6-phosphatase (EC 3.1.3.9 D-Glucose-6-phosphate phosphohydrolase)
HK	Hexokinase (EC 2.7.1.1 ATP:D-Hexose 6-phosphotransferase)
LDH	Lactic dehydrogenase (EC 1.1.1.27 L-Lactate:NAD oxidoreductase)
MDH	Malate dehydrogenase (EC 1.1.1.37 L-Malate :NAD oxidoreductase)
PC	Pyruvate carboxylase (EC 6.4.1.1 Pyruvate:carbon-dioxide ligase (ADP))
PEP CK	Phosphoenol-pyruvate carboxykinase (EC 4.1.1.32 GTP: oxaloacetate carboxylyase (transphosphorylating))
PFK	Phosphofructokinase (EC 2.7.1.11 ATP:D-Fructose-6-phosphate 1-phosphotransferase)
PK	Pyruvate kinase (EC 2.4.1.1 ATP:Pyruvate phosphotransferase)
SDH	Sucinnic dehydrogenase (EC 1.3.99.1 Succinate:(acceptor) oxidoreductase)
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
DMP	Dimethyl amino methylphenol
EDTA	Ethylene diamenetetracetic acid, Na^+ salt
FDP	Fructose-1,6-diphosphate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
IDP	Inosine diphosphate
ITP	Inosine triphosphate
NAD	Nicotinamide adenine dinucleotide, oxidized form

NADH	Nicotinamide adenine dinucleotide, reduced form
NBT	Nitro blue tetrazolium
OsO_4	Osmium tetroxide
PEP	Phosphoenol-pyruvate
PMS	Phenazine methosulfate
TRIS	Tris (hydroxymethyl) aminomethane

TABLE OF CONTENTS

		Page
I.	INTRODUCTION	1
	A. Red and White Muscle: General Considerations	2
	B. Red Muscle-Organ Hypothesis: Affirmative Evidence	6
	C. Red Muscle-Organ Hypothesis: Contrary Evidence	12
	D. The Eel: Anguilla rostrata	17
	E. Statement of the Problem: Metabolic Considerations	20
II.	MATERIALS AND METHODS	
	A. The Animal	25
	B. General Enzyme Procedures	25
	C. Specific Enzyme Procedures	
	1. Lactate Dehydrogenase	27
	a. Assay	27
	b. Electrophoresis	28
	c. Isoelectricfocusing	28
	2. Pyruvate Kinase	29
	a. Assay	30
	b. Electrophoresis	31
	c. Isoelectricfocusing	31
	D. Structural Studies	31
	1. Electron Microscopy	32
	2. Light Microscopy	33
	3. Cryoscopy and Histochemistry	34
	a. Succinic Dehydrogenase	35

b. Lactic Dehydrogenase 36

c. Myosin ATPase 36

III. MORPHOLOGY, HISTOCHEMISTRY AND ULTRASTRUCTURE 38

A. Introduction 38

B. Results 43

C. Discussion 63

IV. LACTIC DEHYDROGENASE 70

A. Introduction 71

B. Results 75

C. Discussion 90

V. PYRUVATE KINASE - CHARACTERISTICS 95

A. Introduction 96

B. Results 100

C. Discussion 127

VI. PYRUVATE KINASE - REGULATION 140

A. Introduction 141

B. Results 147

C. Discussion 155

VII. GENERAL DISCUSSION 170

VIII. APPENDIX 182

IX. REFERENCES 192

LIST OF FIGURES

Chapter III

Page

1. Red and white muscle distribution 44
2. Red and white muscle distribution 45
3. Cross section of eel 46
4. Myomere line drawing 48
5. 'Super cone' arrangement 49
6. Cross section of red-white interface 51
7. Cross section of red-white interface 51
8. Cross section of red muscle fibers 51
9. Myosin ATPase demonstration 53
10. SDH demonstration 53
11. LDH demonstration 53
12. LDH demonstration 53
13. Longitudinal section of mature eel red muscle ... 56
14. Longitudinal section of immature eel red muscle . 56
15. Longitudinal section of mature eel white muscle . 56
16. Red-white cross section from mature eel 56
17. Red muscle from mature eel 58
18. White muscle from mature eel 59
19. Red muscle from immature eel 61

Chapter IV

1. Electrophoretogram of eel tissue LDHs 76
2. Red muscle LDH separation by isoelectricfocusing . 77
3. White muscle LDH separation by isoelectric-focusing 78

4. Liver LDH separation by isoelectricfocusing 79
5. Effect of pH on RM-, WM-LDH DL-lactate saturation curves 81
6. NAD^+ saturation curves for WM-, RM-LDH 83
7. Effect of pH on lactate oxidation 86

Chapter V

1. Effect of EDTA in homogenizing and assay buffers on eel RM-PK 101
2. Effects of buffers and ions on eel white muscle PK 102
3. Electrophoretogram of eel WM-, RM-, and Lv-PKs 104
4. Isoelectricfocusing of eel RM-PK 105
5. Isoelectricfocusing of eel WM-PK 106
6. Effect of pH on eel Wm-, RM-, Lv-PKs 108
7. Arrhenius plots of eel Lv-PK 110
8. Arrhenius plots of eel WM-PK 111
9. Arrhenius plots of eel RM-PK 112
10. Temperature and pH effects on $K_{m(PEP)}$ for WM-, RM-, Lv-PKs 113
11. Modulator effects on WM-PK PEP kinetics 116
12. Modulator effects on RM-PK PEP kinetics 117
13. Modulator effects on Lv-PK PEP kinetics 118
14. Modulation of WM-PK Mg^{++} titration curve 121
15. Modulation of RM-PK Mg^{++} titration curve 122
16. Modulation of Lv-PK Mg^{++} titration curve 123

Chapter VII

1. Micrograph of liver tissue from immature feeding eel 172

2. Micrograph of liver tissue from mature migrating eel 172
3. Micrograph of liver tissue from mature migrating eel 173

LIST OF TABLES

Chapter IV

Page

1. $K_{m(DL\text{-}lac)}$ mM for eel tissue LDHs 82
2. $K_{m(NAD^+)}$ mM for eel tissue LDHs 84
3. PR/LO ratios for eel tissue LDHs 88

Chapter V

1. $K_{m(PEP)}$ modulation of WM-, RM- and Lv-PKs 115

Chapter VI

1. Eel WM-, RM-, Lv-PKs specific activity 148
2. Eel WM-, RM-, Lv-PKs $K_{m(PEP)}$ 149
3. Eel WM-, RM-, Lv-PKs V_{max}/K_m 150

Chapter VIII

1. Demonstration of PEP CK activity in eel tissues .. 188

AKNOWLEDGMENT

This is perhaps the most difficult part of the entire thesis to write; expecially when one acknowledges the high quality of supervision which I have received during the last three years, as they say, 'it (the words) don't come easy' (Ringo Starr, I believe). However, it is important to blow a horn periodically, and I feel that without the confidence and guidance of my supervisor, Dr. T.W.Moon, I wouldn't have accomplished as much as appears in this thesis. While it is important to give guidance, it is also important to allow a curious mind to challenge seemingly 'non thesis' oriented problems to develop, if nothing else, an appreciation for spontaneous research and the amount of time required to adequately pursue these extraneous problems; I am thankful for being allowed to pursue my curiosities, while at the same time progressing towards the academic ideal of excellence in a chosen field. I probably could have accomplished much more thesis research had I been restricted specifically to problems oriented within the thesis topic, however, I would have missed a whole world of knowledge and excitement had I not looked at the different problems which I did. For the opportunity to explore, I am grateful.

During my past three years, I have developed an appreciation for the 'endocrine'systems, as applicable to metabolic control, and to the development and stimulation of this awakening, I am deeply indebted to Dr.J.C.Fenwick.

Perhaps the most significant contribution, which does not really appear any where else except here (and which few people read), is the support, moral, financial, and typing fingers, which have been generously donated by my wife, Charlotte. Without her continued support, the pursuit of the elucive Ph. D. degree would have been much more painful than it was. Words cannot express the indebtedness which I feel.

I

INTRODUCTION

INTRODUCTION

Red and White Muscle: General Considerations

On the basis of color, fish striated skeletal muscle can be macroscopically differentiated into two basic groups, red and white. The red fibers owe their color to both the presence of a fine capillary net which surrounds each muscle fiber, the presence of myoglobin and to a minor degree, the presence of mitochondria and cytochromes. The white fibers, on the other hand, are relatively devoid of these elements and thus the red pigmentation (see Love, 1970, for extensive documentation).

In comparing the morphological and biochemical characteristics of fish muscle with mammalian muscle types, it is evident that several characteristics are shared in common. For example, the red muscle in both groups of animals, in contrast to white muscle, is characterized by: low water content, high intra and extracellular lipid deposits (note: lipids are deposited only extracellulary in white muscle), larger glycogen stores (only in fish red muscle), high lipase activities, large concentrations of mitochondria, oxidative enzymes and cytochromes, higher oxygen consumption, large concentrations of the vitamin-B complexes, longer sarcomere length, large concentrations of connective tissue and capillaries, lower concentrations of creatine phosphate, and lower concentrations and activities of the anaerobic glycolytic enzymes (see for extensive reviews, Bone, 1966;

Love, 1970; Bilinski, 1974).

In contrast to this extensive list of common traits shared by fish red and mammalian red muscle, and fish white and mammalian white muscle, there are also some striking differences. For example, although fish red muscle demonstrates electrophysical properties similar to that observed for mammalian red (or slow twitch) and amphibian red (or tonic) muscles (Takeuchi, 1959; Andersen et al., 1963; Hidaka and Toida, 1969), fish red muscle also possesses a highly organized transverse tubular system, prominent M-line and regular (as opposed to zig-zag) Z-lines (Kilarski, 1967; Nishihara, 1967; Nakajima, 1969; Nag, 1972; Patterson and Goldspink, 1972; Korneliussen and Nicolaysen, 1975). These features are not observed in mammalian or amphibian red muscle (Peachey and Huxley, 1962; Hess, 1966; Peachey, 1966). Thus, fish red muscle is structurally similar to mammalian fast or twitch muscle but electrophysically behaves more like mammalian slow twitch muscle.

Interestingly, while the structural and electrophysical properties of fish white muscle are generally similar to that observed for mammalian white muscle (see for example, Kilarski, 1967; Nishihara, 1967), the mode of innervation for the two muscle types is quite different. Mammalian fast muscle is characterized by single axonal innervation with 'en plaque' or single plate nerve endings (see for discussion, Hess, 1970). Fish white muscle, on the other hand, receives either single (Bone, 1966) or polyaxonal innervation

(Takeuchi, 1959) with 'en grappe' or multiple knob nerve endings. In this respect, fish white muscle innervation is more similar to that observed for insect muscle (Castillo et al., 1953) or crustacean muscle (Fatt and Katz, 1953) than to mammalian or amphibian white muscle. Another important difference between fish and mammalian innervation (but consistent with observations on amphibian slow muscle) is the apparent lack of infoldings of the post-junctional sarcomeric membrane in fish white muscle (Bergman, 1967; Nakajima, 1969). Innervation of fish and mammalian muscle is characterized by the 'en grappe' multiple end plate terminals which are distributed throughout the length of the fibers and arise from more than one axonal trunk (Bone, 1966; Nakajima, 1969). However, unlike mammalian red muscle, but consistent with amphibian slow muscle (Page, 1966), the post-junctional sarcomeric membrane of fish red muscle lacks infoldings (Nakajima, 1969).

While there are some quantitative differences in morphology and innervation patterns between red and white muscles from fish and mammals, there is general agreement that the mode of energy production for contraction in the 'homologous' fish and mammalian muscles is similar. That is, red muscle generates energy by the oxidation of fats, while white muscle anaerobically degrades carbohydrates (Love, 1970; Bilinski, 1974). Because many characteristics are shared in common between mammalian and fish red and white muscles, the functional roles of the mammalian muscles have

been liberally extended to apply for the respective fish muscles; however, the validity of this functional extention must be questioned in light of the quantitative differences which exist between fish and mammalian muscle types. Thus, the fish muscle controversy does not relate to morphological, biochemical or electrophysical observations per se, but rather to the expression of these differences in determining the physiological role of the two fish muscles in light of other muscle systems.

Red Muscle-Organ Hypothesis : Affirmative Evidence

The controversy over the physiological role of fish red and white muscle was initially sparked by Braekkan (1956). He observed the concentrations of the vitamin-B complexes in the red muscle and liver of the tunny, mackerel and halibut to be comparable and significantly higher than observed for white muscle. In this same communication, Braekkan reported that the red muscle of mackerel, herring and halibut contained 27-29% fat by weight which was significantly higher than the white muscle values. From these observations, he proposed that "the red muscle is able to carry out similar synthetic processes as those in the liver" (Braekkan, 1956, p.747), that "the anatomical situation of red muscle prevents it taking part in the main muscular work" (Ibid, p.747), and further, that due to the high fat content, the red muscle was unsuited for strenous or continuous activity. Thus, the red muscle-organ hypothesis

was formulated.

In 1959, Braekkan extended his observations relating the vitamin-B complex concentrations in red muscle and liver to include the porbeagle and Atlantic salmon. By choosing these species, he was able to report on fish which use their liver mainly for fat storage (cod, porbeagle and tunny) and fish which use their red muscle for fat storage (Atlantic herring, Atlantic salmon, mackerel). Significantly, he observed that the vitamin-red muscle-liver relationship was independent of lipid storage, and thus a degree of continuity was afforded to his hypothesis. In further arguments (Braekkan, 1959), he pointed out the disparity in creatine concentrations between the red and white muscle with the latter having significantly higher concentrations, and further, that the pH drop and lactic acid increase following death from struggling was only observed in the white muscle. Therefore, the white muscle was more suited for physical activity than the red muscle. Indeed, considerable support was given to Braekkan's hypothesis when Tsuchiya and Kunii (1960) confirmed his observations on lactic acid concentrations in white muscle and supported his red muscle-organ theory.

Further support for Braekkan's hypothesis has come mainly from the laboratory of Wittenberger. In a series of publications, he has provided evidence that the physiological significance of an ancillary metabolic role for red muscle is far greater than its proposed contractile role. For

example, when pieces of red and white carp muscle are incubated in a Ringers solution, both muscle types lose equivalent amounts of glycogen to the medium; however, when they are separated, the white muscle lost 22% more glycogen than when they were naturally joined (Wittenberger,1973). While lactate concentrations increased approximately 44% in the red muscle (as compared to the control) when both red and white muscle pieces are naturally joined, lactate only increased slightly when the pieces were separated (Wittenberger, 1973). Electrical stimulation of carp red and white muscle, at levels to elicit a 'moderate effect' demonstrated that white muscle glycogen and pyruvate did not change but lactate concentrations and oxygen consumption of red muscle did increase (Wittenberger and Diaciuc, 1965). With the electrical stimulation of the carp muscle to exhaustion, white muscle glycogen and pyruvate decreased and lactate concentration increased without changes in oxygen consumption; red muscle, on the other hand, demonstrated decreased glycogen and greatly increased lactate concentrations (Wittenberger and Diaciuc, 1965). Phloridzin, a glucose transport inhibitor, depressed the drop in glycogen from the carp red muscle when both red and white muscle were incubated naturally attached (as compared to when the muscle types were separated) (Wittenberger et al.,1975). Insulin decreased the loss of glycogen from carp white muscle when the white and red muscle was incubated naturally attached as compared to isolated incubation of the white muscle

(Wittenberger et al., 1975). Incubation of the carp red and white muscle in a glucose medium resulted in an increase in glycogen and lactate concentration in red muscle only (Wittenberger et al.,1975). Addition of adrenalin to carp red muscle elicited an enhanced loss of glycogen and increased lactate concentration in the red muscle only (Wittenberger et al.,1975). The turnover rate of glycogen for mackerel red muscle was found to be 41-times greater than white muscle and 37-fold greater than liver (Wittenberger, 1972). For the pelagic fish, Harengula homeralis, red muscle contains 12-14 times the amount of liver glycogen, and is 6-fold greater in weight than the liver (Wittenberger et al.,1969). Wittenberger's basic hypothesis is that coupled with the differential sensitivity of the red and white muscle to adrenalin and insulin, the demonstration of a direct transfer of lactate from the white to the red muscle and a concomitant transfer of glucose from the red to the white muscle, that the red muscle functions both as the storage site for carbohydrates and the burning site for catabolites produced in the white muscle.

Other evidence which supports Braekkan's red muscle-organ hypothesis was reported by Pritchard et al. (1971). They examined alterations in glycogen, lactic acid and fat concentrations in the red and white muscle, and liver glycogen and lactic acid concentrations of the jack mackerel forced to swim at various speeds. Their results suggest that in all cases, the total failure to swim was related

only to the total depletion of glycogen in white muscle, and that glycogen changes in red muscle of these fish were similar to that observed in the liver and were not correlated with a failure to swim. Reduction in the glycogen level of the red muscle and liver were, however, correlated with extended periods of swimming. Fat content decreased only in the red muscle after extended swimming at subthreshold speeds (i.e., below the 50% endurance threshold), and both muscle types exhibited high lactate levels which did not appear to be correlated with fatigue at any swimming speed. Due to the high lactate levels and glycogen depletion in the white muscle of exhausted fish, and the similar patterns of glycogen depletion in red muscle and liver regardless of swimming speed, Pritchard et al. proposed that the white muscle was the primary locomotory organ about the threshold for sustained speed and that the red muscle, similar to the liver, may provide nutrients to the white muscle under these conditions. It is important to note that while Wittenberger and associates consider the ancillary metabolic function the predominant role for fish muscle, Pritchard et al. suggest that it may act like the liver at swimming speeds about the threshold for sustained swimming, but at speeds below this, the red muscle provides a locomotory function.

In addition to the evidence of Wittenberger, Pritchard and others, Robinson and Mead (1973) and Smit et al. (1971) support the ancillary metabolic role hypothesis for red muscle. Interestingly, the evidence provided by these

authors is markedly different from that discussed by the above authors. Firstly, Robinson and Mead (1973) working with the rainbow trout confirmed the early work of Bilinski (1963; 1969) on the relative ability of the red and white muscles to oxidize ^{14}C-labelled fatty acids; however, more importantly, they demonstrated that the red muscle was also more active in the synthesis of depot triglyceride (TG) from free fatty acids (FFA) than was the white muscle. For example, while the TG content in white muscle was 50% less than red muscle (mg/g tissue), the incorporation, and/or synthetic rate, was only 5% of that observed for the red muscle. They further observed that, regardless of the nutritional state of the fish, in the red muscle there was a dynamic balance between the FFA, TG and cholesterol-ester pools. In the white muscle, this control was not observed. Thus, during starvation, the TG deposits of white muscle were mobilized preferentially to the other lipid deposits and the ratios between the various lipid compartments were in a constant state of change. From these observations they suggest that a tissue which is more concerned with metabolic processes as compared to contractile processes, will demonstrate tighter regulation over its biochemical pools. Thus, the red muscle in rainbow trout may function primarily in a metabolic capacity rather than a contractile one.

Smit et al. (1971) have determined the oxygen consumption of goldfish over a wide range of swimming speeds with the aim of correlating O_2 consumption of the fish with the

relative propulsive contribution by the red and white muscles. They observed that, since the O_2 consumption of the red muscle is higher than the white (see also Gordon 1968, 1972), and there is a finite limit to the ventilatory rate, extraction efficiency and O_2 carrying capacity of the blood, the maintenance of large portions of the body musculature as red muscle is not 'technically' or physiologically feasible (the argument refers specifically to fishes such as the goldfish or salmonids where the contractile versus metabolic role for red muscle is in contest). Thus, the portion of the body myotome which is red, or oxidative muscle, is kept to a minimum to reduce demands on the O_2 consumption capacity of the fish. They note further, that "this goal would be reached even more effectively if there were no red fibers at all, (thus) it must be assumed that the red fibers certainly have some other function, possibly resembling that of the liver" (Smit et al., 1971, p.26). It is significant to note that, both Robinson and Mead and Smit et al., support the suggestion of Pritchard et al. (1971), that the red muscle is capable of contributing to the mechanics of locomotion. Thus, only Wittenberger and Braekkan feel that the red muscle is not used as a contractile body.

In summary then, the major support for Braekkan's red muscle-organ hypothesis comes from data which suggests that: the red muscle, similar to the mammalian liver, is sensitive to adrenalin and insulin and possesses tight regulation over its carbohydrate and lipid pools, participates in a carbo-

hydrate for lactate exchange, and due to the physiological constraints posed by large quantities of red muscle on the oxygen consumption capacity of the fish, must necessarily function in a manner other than, or in conjunction with, contraction.

Red Muscle-Organ Hypothesis: Contrary Evidence

The opposing theory to the red muscle-organ hypothesis is that the red muscle is a continuously contracting tissue, similar to the heart, and generates the slow undulating movements which are characteristic of slow cruising speeds (see Bone, 1966; Love, 1970; Bilinski, 1974). As early as 1959, Boddeke et al. pointed out that the pectoral fin muscles which are continuously used (depending upon the fish) are highly vascularized and exemplify typical red muscle. Thus, contrary to the argument of Braekkan (1956), they suggested that red muscle could indeed be utilized for normal work. Boddeke et al. (1959) further suggested that, due to its anaerobic nature, the white muscle demonstrated characteristics of mammalian sprint or fast muscle, and that the red muscle, which is oxidative in nature, had the characteristics of stayer (or endurance) muscle (like heart muscle, for example). Thus, based on the relative content of red and white muscle, Boddeke et al. (1959) proposed that fishes could be grouped into four distinct catagories: sprinters (eg., pike), sneakers (eg., eel), crawlers (eg., bream) and stayers (eg., salmon). Their arguments were based

on the observation that fish such as the pike were characterized by a body musculature composed almost entirely of white fibers, while at the other extreme, the stayers possessed a well developed narrow fibered musculature about the lateral line in addition to deep mosaic musculature (mixed narrow-red, and broad-white, fibers).

Interestingly, Boddeke et al. considered the European eel, Anguilla anguilla, characteristic of a sneaker since it possessed neither the characteristics of a typical sprinter or stayer (see Chapter 3). Boddeke et al. proposed that as the relative proportion of the red muscle increases, the staying power concomitantly increased and this is reflected in the swimming behavior and general habitat preference of a particular fish.

George (1962) and George and Bokawala (1964) compared the histochemical characteristics of the red muscle from bats and pigeons to red muscle from the mackerel and roho carp and came to a conclusion similar to Boddeke et al. (1959). Thus, as a result of the histochemically demonstrated oxidative potential and high fat content of red muscle from the pigeon, bat and fish, it was concluded that all three red muscle types respired mainly aerobically and utilized fats for fueling continuous contractile activity. The white muscle, on the other hand, was histochemically anaerobic in nature and utilized carbohydrates for fuel and thus appeared best suited for sprint activity (see review by Drummond and Black, 1960; Drummond, 1971).

The histochemical observations made by George and his associates, on the oxidative potential of red muscle, were substantiated with tracer experiments by Bilinski (1963, 1969). He demonstrated an ability of the lateral dark muscle of rainbow trout to oxidize labelled FFA to $^{14}CO_2$ to be from 9-fold (for hexonate) to 56-fold (for octanoate) greater than the white muscle.

While these results provided indirect support for the heart-like function of red muscle, perhaps the most conclusive evidence for a continuous contractile function was afforded by the electrophysiological studies of Bone (1966). In a classic paper on the two basic types of myotomal musculature in elasmobranchs (Bone actually identified four different fiber types), Bone demonstrated that the red and white muscles represented two totally separate locomotion systems and thus, there could be no doubt as to the potential for red muscle to support muscular contractions contributing to swimming movements. In short, he found that action potentials were propagated from the red muscle (of a spinal dogfish) only during slow undulating movement; and further, that at these swimming speeds there were no spikes elicited from the deep white musculature. As the swimming speed increased however, spike propagation from the white muscle became apparent. Importantly, Bone (1966) correlated these electrophysiological responses to alterations in metabolite levels in the two muscle types. For example, 1) glycogen decreased in the white muscle only after exhaustive move-

ment, 2) fat content of the red muscle decreased only after prolonged sustained slow swimming, and 3) the glycogen content of the white fibers was unaltered during sustained slow cruising. These basic observations correlating the biochemical and electrophysiological parameters have provided strong evidence for a continuously contracting role for red muscle. Bone's work has been extensively supported by Rayner and Keenan (1967) working with the skipjack tuna, Roberts (1969) working with the dogfish, Greer-Walker (1971) working with cod, Greer-Walker and Pull (1973) working with the coalfish, and Hudson (1973) working with the rainbow trout.

Several workers attempting to correlate slow and fast muscle function with a corresponding ultrastructural architecture, support the hypothesis that red muscle is well suited for continuous movements and white muscle for bursts: Buttkus (1963) working with lingcod; Nishihara (1967) working with the snakefish; Nakajima (1969) working with the snakefish; Nag (1972) working with the rainbow trout; and Patterson and Goldspink (1972) working with cod. Interestingly, at least one group of investigators (Patterson and Goldspink, 1972) suggests that the preponderance of mitochondria in fish red muscle is seemingly in excess of that required to sustain lipid oxidation for the generation of contraction energy. Thus, in addition to lipid oxidation, the red muscle may also function in another specific oxidative capacity. However, as Smit et al. (1971) point out, just because a tissue has the potential to oxidize a specific

substrate does not necessarily mean that oxidation of that substrate is the main function of the tissue. For example, the heart readily oxidizes lactate as a fuel, however, that is certainly not the main function of the heart.

Although most workers accept the role of red muscle in providing slow cruising swimming, an increasing number also suggest that the red muscle is probably active during maximal swimming speeds:Pritchard et al. (1971) working with the jack mackerel; Smit et al. (1971) working with the goldfish; Johnston and Goldspink (1973) working with the cod; and further, that the white muscle is also active during slow cruising:Greer-Walker (1971) working with cod and coalfish; Smit et al. (1971) working with the goldfish; Johnston and Goldspink (1973) working with the cod; and Robinson and Mead (1973) working with the rainbow trout. Thus, to this date, a definitive evaluation of the contribution of red muscle as a metabolic organ as opposed to its contractile role, and the contribution of white muscle to sustained cruising is unclear.

The most important evidence which argues against the metabolic role for red muscle is the demonstration that fish red muscle is fully capable of supporting swimming movements and that spike activity has been recorded during slow cruising. The metabolite level studies can be interpreted using both metabolic and contractile role hypothesis.

Perhaps the major problem of the studies mentioned above is the great diversity of species examined with their

various activity levels and habitat preferences and thus, the lack of correlative evidence which might exist between these species. For example, there is no reason, a priori, to assume that the functional role of red muscle from one fish (a non-migrating fish, for example) will be identical or even similar to that of another (a migrating fish, for example), particularly since there is documentation for a dual role of fish red muscle (contractile and metabolic). Also the evidence presented thus far has resulted from either electrophysiological, histophysiological or metabolite studies with little attention directed to the regulation of metabolic pathways. This study was initiated to provide evidence at the level of enzyme regulation, concurrent with ultrastructural and histochemical evidence, for an ancilliary role for red muscle in the American eel, Anguilla rostrata.

The Eel: Anguilla rostrata

The life history, body musculature and economic value of the European eel, Anguilla anguilla, have been well documented which makes the American eel, A. rostrata, an excellent experimental tool. According to the work of Bertin (1956), Eales (1968) and Sinha and Jones (1975), it is generally accepted that both the European and American eels breed in the general proximity of the Sargasso Sea. Note: the exact breeding grounds within this area of the two eels is still in dispute; see Bertin (1956), Vladykov (1964) and Sinha and Jones (1975). Following fertilization,

the larvae develop into surface dwelling leptocephali which drift passively with the currents towards the coast of North America. Approximately one year after hatching, they arrive at the continental shelf of North America and begin to metamorphose first into the intermediate stage, the glass eel, and finally to completion as an elver in fresh water. The transition of the glass eel into an elver is marked by several significant morphological changes. For example, pigmentation develops, the mouth parts reorganize to allow active pursuit feeding (glass eels are planktonic filter feeders), the digestive tract and anus are repositioned, and importantly, the general morphology of the elver takes on the shape of the adult eel (glass eels are leaf-like) (see Sinha and Jones, 1975).

From the time of hatching to when the elvers begin the upstream freshwater migration, approximately 2-3 years has elapsed. Although it is generally accepted that only the females migrate up stream and the males remain to populate the estuarine areas, this aspect of the life history of the American eel is still unsettled (Sinha and Jones, 1975). The eels which do migrate upstream remain in freshwater for at least 5-10 years. During this time the eels are a characteristic yellow color but undergo a change in pigmentation to silver for the European eel, or bronze for the American eel (Vladykov, 1955) with the onset of preparation for the seaward migration. Other dramatic morphological and physiological changes which occur at this time in A. anguilla

are: an increase in fat deposits, a thickening of the skin, changes in the osmoregulatory capacity, increases in the number of chloride cells in the gills, development of the gonads, increased endocrine activity, cessation of food consumption, resorption of the digestive tract, increased quantities of red muscle and alterations in the activities of several oxidative and glycolytic enzymes from both red and white muscle (Bostrom and Johansson, 1972; Dave et al., 1974; Lewander et al., 1974); see also extensive reviews by Bertin (1956), Eales (1968) and Sinha and Jones (1975).

Since the eels cease feeding upon commencement of the seaward migration, the entire migration run is conducted using only stored energy for locomotion as well as gonadal maturation. After spawning, it is presumed that the eels die at sea since no adults have ever been captured in the Sargasso Sea area, nor have any adult eels ever been reported returning to the North American coast (Sinha and Jones, 1975). Therefore, the eel lends itself to studies on fish metabolism since they are in a natural state of nutritional deprivation during the migration period.

The second important consideration in choosing the eel as the experimental animal is that the body musculature is clearly divided into two relatively homogenous populations of red and white fibers. Thus, pure preparations of each muscle type can be obtained for enzymatic analysis. Further, as indicated above, there is a marked increase in the amount of red muscle concurrent with the seaward migration

suggesting that the red muscle may play a more important role in migration than at any other time during the life cycle of the eel.

At the onset, the third reason for choosing the eel as the experimental animal seems rather unrelated to the other two; namely, the economic value of the European eel, A. anguilla. However, because of the established eel fishery in Europe, a voluminous amount of data has been accumulated which thus provides an excellent base for comparison of results.

Statement of the Problem: Metabolic Considerations

From the above, it is evident that the eel provides a unique opportunity to examine the potential of a metabolic role in contrast to a contractile role for red muscle. Since the eel does not feed during the migration, the energy for gonadal maturation and muscular contraction must be generated from stored reserves; thus, it would be predicted that if red muscle does possess an ancillary metabolic role, this may be expressed as a capacity to recycle products of white muscle metabolism (eg., lactate). Further, if the hypothesis of Wittenberger and coworkers and Pritchard et al. (1971) are applicable here, then the ultimate fate of the metabolites produced in the white muscle could be glucose, or some other reserve material, produced and stored in the red muscle. Using lactate as an example, glycogen would be the assumed product.

In mammals, the conversion of lactate to glucose is thought to occur predominately in the liver and to a lesser extent in the kidney, although recent evidence may alter this belief (Hermansen, personal communication). Further, this conversion involves, in part, the by-passing of three thermodynamically irreversible glycolytic enzymes: HK[1], PFK, and PK (Krebs,1954). Two of these enzymatic reactions involve single enzyme steps: HK is by-passed by G-6-Pase and PFK is by-passed by FDPase. To by-pass the third irreversible enzyme PK, however, the concerted catalysis by three enzymes is required: LDH, PC and PEP CK (see Krebs,1954). Thus lactate is oxidized first to pyruvate (by LDH), which is carboxylated to form oxaloacetate (by PC), a key metabolite which is subsequently decarboxylated and phosphorylated to form PEP (by PEP CK). The PEP thus formed is the carbon substrate for PK. Therefore, if PK is not inhibited during the conversion of glucogenic substrates to glucose, then a futile cycle, at the level of PEP, will result (Scrutton and Utter,1968: Sols, 1968; Katz and Rognstad, 1976). On the other hand, PK is an important glycolytic enzyme and during periods of glycolytic demand must be active to allow for the ultimate generation of lactate, and/or oxidizable substrates for the Krebs cycle. It is apparent then, that the reciprocal regulation of PK would establish the strategy for metabolic control at this branch point. In conjunction with an examination of PK, an evaluation of the capacity for the red and

[1]See list of abbreviations, p.

white muscle LDHs to support lactate oxidation would also be instructive.

If the red muscle does indeed function in an ancillary metabolic capacity, then the following hypotheses can be formulated and tested: first, that the regulatory properties of the PK from red muscle be similar to that observed for mammalian liver, a known gluconeogenic organ; second, that the regulatory properties of eel white muscle be functionally similar to that observed for mammalian white skeletal muscle; third, that the presence of either PC or PEPCK be demonstrated in red muscle and absent from white; fourth, that the red muscle demonstrate an ability to oxidize lactate in concert with conditions which support PK inhibition; fifth, that the white muscle LDH function predominately as a pyruvate reductase.

Another experimental approach to the question of a metabolic role for eel red muscle is to examine the ultrastructural and histochemical properties of the two muscles from both yellow and bronze eels. For example, if the red muscle has a diminished contractile role during migration, then alterations of the basic contractile apparatus might be evident. Thus, one could predict that the red fibers may undergo fatty infiltration to the point that the contracting myofibrils are actually wedged apart. This would effectively decrease the physiological cross section of the fibers and thus result in diminished contractile ability. Further, the histochemical results should collaborate the data obtained

from enzymic studies.

If an analysis of the data satifies the above predictions, then it would be possible to suggest that the red muscle from the American eel, A. rostrata, in the bronze phase, possesses the potential to function according to Braekkan's organ hypothesis (1956). The work described in this thesis presents support evidence for this concept.

II

MATERIALS AND METHODS

MATERIALS AND METHODS

I. Enzyme Studies

A. The Animal

The American eels, Anguilla rostrata, used for the enzyme studies were maturing females netted from the St. Lawrence River at Quebec City during their seaward migration. They were in the bronze phase (Vladykov, 1955) which is comparable to the silver phase of the European eel Anguilla anguilla (Sinha and Jones, 1975). The animals weighed in excess of one kilogram and were in excess of 60 cm in length. Approximately 500 eels were maintained in a large holding tank (6 X 10 X 1.5 feet) which was supplied with free flowing, fresh, dechlorinated Ottawa city tap water. During this period in their life cycle, the eels do not naturally feed and would not respond to feeding attempts in the laboratory. Therefore, all enzyme data presented is from animals in the same relative state of nutritional deficit. Only healthy eels with large reserves of stored fat were used for the study.

B. General Enzyme Procedures

Both Pyruvate Kinase (PK) and Lactic Dehydrogenase (LDH) were prepared from pooled frozen tissue of at least five animals. This procedure was adopted since 1) no kinetic differences between the enzymes prepared from frozen and fresh tissue could be detected and 2) the thin (less than 2.5 mm) superficial red muscle could be obtained free of

contamination from the underlying white musculature. The eels were decapitated, exsangurated and rapidly frozen at -20°C until use. Once frozen, the eel was skinned, and the underlying red muscle scraped from the entire body with a cartilage knife. The white muscle was obtained in the region epaxial to the anus.

The frozen tissues were weighed and a volume of ice-cold 100 mM Tris-HCl, pH 7.4, containing 2 mM EDTA (for LDH) or 100 mM Tris-Maleate, pH 7.4, containing 2 mM EDTA (for PK) was added to give a 50% (w/v) final homogenate concentration. Following homogenization in an iced Sorval Omni-Mixer operating at full speed for 1½ mins, the homogenate was centrifuged for 20 mins at 29000Xg in a Sorval RC-2B refrigerated centrifuge at 0°C. The resultant high speed supernatant was then filtered through two layers of Kim-Wipes to remove fat and then used directly, as in the case of LDH, or partially purified as in the case for PK.

Enzyme activities were recorded as alterations in the oxidation or reduction state of NADH at 340 mm with a Gilford 2100 recording spectrophotometer. Cuvette temperatures were controlled by coupling the jacketed cuvette holder to a Haake refrigerated water bath. Cuvette temperatures were monitored with a Yellow Springs Instrument Telethermometer and did not vary by more than 0.1°C from the desired temperature. Enzyme activity is defined as µmols NADH/min/mg protein, at the appropriate temperature and conditions of assay. Protein concentration was estimated

either by the method of Lowry et al. (1951) or Layne (1957). Bovine serum albumin was used as a standard and the two methods did not vary by more than ±0.005 mg/ml. All biochemicals were obtained from Sigma Chemical Company, St. Louis, Missouri; all other chemicals were of the highest purity possible and were obtained from local distributors.

C. Specific Enzyme Procedures

Lactate Dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27)

Assay

Initial attempts to purify eel muscle and liver LDH were unsuccessful since the enzyme(s) was found to be extremely sensitive to dialysis. No other procedures were attempted since the enzyme was found to be stable to freezing and thawing for at least two months. Eel tissue LDHs were assayed in both the forward (pyruvate to lactate) and reverse (lactate to pyruvate) directions. All assays were done in duplicate, and were initiated with the addition of 20 µl of enzyme containing between 6.5 and 50 µg protein, depending upon tissue activities. The final cuvette volume of 0.5 ml contained various volumes of 100 mM Tris-HCl buffer (see figure legends for pH) and either 0.35 mM NADH and various concentrations of pyruvate, or 0.45 mM NAD^+ and various concentrations of DL-Lactate. The reactions exhibited first order kinetics and were linear for at least 5 mins.

Polyacrylamide Disc-Gel Electrophoresis

Polyacrylamide electrophoresis was carried out using a modification of the technique by Davis (1964) on 5% gels.. Approximately 50 to 300 mg of protein was applied to 8mm X 9cm gels, and run at 6.5 mA per gel for 2 hrs using the standard Tris-glycine, pH 8.5, buffer system (5°C). The gels were stained in the dark at 5°C to reduce background and full development of the bands was usually observed within 2 hrs. Each gel was stained in 2.5 ml of a stock staining mixture which contained: 2 gm 80% DL-Lactate (liquid), 22 mg NAD^+, 22 mg NBT and a few grains of PMS in a total volume of 40 mls of 100 mM Tris-HCl pH 8.0.

Isoelectricfocusing

Isoelectricfocusing of the muscle and liver LDHs was accomplished using the granulated gel technique described by Radola (1969). The gel was prepared by swelling 7.5 gms G-75/40 Sephadex in 100 mls distilled deionized water containing 2.5 mls of LKB pH 5 to 8 ampholines. The bed preparation, which was boiled under vaccum, was spread on a 20 X 20 cm glass plate and gave a final thickness, after partial dehydration, of approximately 1½ mm. The standard 0.2 M H_2SO_4 and 0.4 M ethylene diamine buffers were used. The samples were prepared by concentrating the filtered high speed supernatant in crystalline sucrose (i.e., placing the supernatant in a dialysis tubing and then suspending the bag in a beaker of crystalline sucrose-10 mls supernatant to 175 g sucrose) to give a final protein concentration after

dilution of 10 mg/ml. Approximately 70 µl of this preparation was then applied to the gel bed, 7 cm from the anode, with the aid of a cover slip. The samples were focused at 350 volts for 4 hrs and then 800 volts for another 4 hrs. All three LDHs were focused concurrently and the experiments were conducted in duplicate. Evaluation of the focused samples was accomplished by removing the Sephadex bed in 0.35 cm width and adding 0.5 ml distilled deionized water to extract the enzyme proteins. The pH of each fraction was determined with a Radiometer BMS3 MK2 Bloodmicro system and the enzymic activity determined as described above.

Pyruvate Kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40)

Preparation

Following the basic extraction prodedures outlined above, the resultant high speed supernatant was brought to 40% $(NH_4)_2SO_4$ with the addition of solid $(NH_4)_2SO_4$. After stirring in the cold for 2 hrs, the precipitated protein was removed by centrifugation (15 mins at 1200 Xg) and the resultant supernatant raised to 70% saturation with the addition of solid $(NH_4)_2SO_4$. Following stirring in the cold for 2 hrs, the salt precipitated PK was collected by centrifugation (15 mins at 1200 Xg) and resuspended in a minimal volume of 100 mM Tris-Maleate buffer, pH 7.4 containing 2 mM EDTA. This procedure resulted in a 7-fold enzyme purification, and a preparation which was stable for at

least 6 months at -25°C. Before assaying, the enzyme preparation was exhaustively dialyzed against 100 mM Tris-Maleate buffer, pH 7.4, containing 2 mM EDTA (1:1000 dilution) for 12 hrs and then centrifuged for 15 mins at 12000 Xg.

Assay

Eel red and white muscle and liver PKs were assayed using a modification of the coupled method of Bucher and Pfleiderer (1955). The standard (optimal activity) assay which couples the stoichiometric oxidation of PEP to NADH via the LDH reaction, contained: 8 mM Mg^{++}, 40 mM K^{+}, 4 mM ADP, 0.35 mM NADH, 1-4 units dialyzed Sigma LDH, and various concentrations of PEP. The total cuvette volume was 0.5 ml and the standard buffer was 100 mM Tris-Maleate, at various pHs (see figure legends). The reaction was initiated with the addition of 20 µl of enzyme (approximately 4 to 5 µg protein depending on tissue activities), and there was no activity in the absence of any one of the following: PEP, ADP, NADH, and Mg^{++}. It should be noted that although the PK enzyme used in these studies required 40 mM K^{+} for maximal activity, there was always slight activity in the absence of added K^{+}. The largest source of K^{+} contamination was found to be the $MgCl_2$ and Tris buffer solutions as determined using Atomic Absorption analysis. Levels were found to approach 10 mM in the assay cocktail without any added K^{+}.

Electrophoresis

Polyacrylamide Disc-Gel Electrophoresis

Polyacrylamide electrophoresis was carried out as described above for LDH. Identification of the PK bands was accomplished using a modification of the Sussor and Rutter (1968) technique. The staining mixture contained (in a final volume of 40 mls) 100 mM Tris-HCl buffer, pH 6.0, 0.15 gm KCL, 0.08 gm $MgCl_2$, 0.09 gm ADP, 0.10 gm PEP, 0.008 gm NADH, approximately 500 units Sigma LDH and 0.002 gm FDP. The presence of PK was determined by examining the gels for lack of fluorescence at 340 nm, using a hand-held light source. Control gels were stained in the absence of PEP and produced no banding pattern.

Isoelectricfocusing

The procedure followed here was identical to that described above for LDH. Each fraction was assayed for PK with 2 mM PEP in standard reaction mixture indicated above.

II. Structural Studies

The light and electron microscopy was conducted on eels collected at Cornwall, Ontario, St. Andrews, New Brunswick and Quebec City, Quebec. The eels from Quebec City were held in the laboratory and under the conditions described above until they were sacrificed. The eels from Cornwall and Semore Pond (an estuary) at St. Andrews were sampled immediately after capture and the tissues fixed in ice-cold glutaraldehyde on the site. The fixed tissues were then

transported back to the laboratory, in an ice box, for further processing.

A. Fixation Procedure for Electron Microscopy

After decapitation and pithing, the skin of the eel was rapidly separated from the underlying musculature to expose the superficial red muscle. Pure pieces of red muscle were obtained by lifting the red muscle from the underlying white musculature with a dull probe wedged between adjacent myosepta and then cutting free the lifted muscle segment. Care was taken to prevent excessive stretching of the muscle fibers. Homogenous preparations of the underlying white muscle could then be easily obtained using a sharp scalpel. All muscle pieces were first placed in iced 4% glutaraldehyde, diced into pieces approximately 1½ mm^3 and transferred into vials containing fresh 4% glutaraldehyde. Following 2½ hrs primary fixation at 5°C, the tissues were washed with 100 mM Na-phosphate buffer, pH 7.4, and post fixed in 1½% OsO_4 in 100 mM Na-phosphate buffer, pH 7.4 for 2 hrs, the tissues were then dehydrated in a graded ethanol series (30%, 50%, 70%, 90%, 100%). Preparation for epoxy infiltration was initiated by processing the muscle pieces in 1:1 propylene oxide: ethanol, and then 100% propylene oxide. Infiltration with Epon 812 was accomplished according to the following regime: 2:1 propylene oxide: Epon for 2 hrs; 1:2 propylene oxide: Epon for 2 hrs and 100% Epon for 2 hrs. The Epon was mixed according to Luft (1961) and the A and B resins were combined in equal parts. Polymerization was facilitated

with 0.17 ml DMP to 10 ml of the mixed Epon.

The infiltrated tissue pieces were positioned in size 00 gelatin capsules, which were filled with Epon resin and then incubated in the following manner: 35°C for 8 hrs, 45°C for 8 hrs, and 60°C for 10 hrs. Trimming of the excess plastic surrounding the embedded specimen was facilitated with the use of a Hulbert block holder (unpublished design) and a OC¼-30° camfermill bit with attached Severance H110 handle. The embedded tissue was sectioned with a Porter-Blum MT-1 ultra microtome fitted with glass knives. Only silver-gold sections were kept for EM viewing. The thin sections were picked up on naked copper grids, stained with uranyl acetate (Watson, 1958) (5% in 50% ETOH) and lead citrate (Reynolds, 1963) (1.33 g $Pb(NO_3)_2$, 1.76 g Na-citrate, 42 ml distilled water, 8 ml 1 N NaOH) and examined with an AEI 800 transmission EM. Thick sections (approximately 1 micron) were stained with 5% toluidine blue, pH 10, for light microscopy viewing.

B. Fixation Procedures for Light Microscopy

The muscle examined by LM was fixed in either 4% glutaraldehyde or Bouin with acetic acid (750 ml saturated Picric acid, 250 ml 40% formalin, 50 ml Glacial Acetic acid) and embedded in Epon 812 as above or Tissuemat (Fisher) parafin. Fixation in gluaraldehyde reduced the muscle processing time since prolonged washes in 70% ETOH to remove the picric acid from the Bouin fixed fixation was not necessary. Further, the glutaraldehyde fixed material stained

more readily with the 1:1 methylene blue (0.5%): azure II (1%) and 1:1 ponceau (1%): orange G (2%) in 7% acetic acid than did the Bouin fixed muscle. The protocol following 4 hrs fixation in glutaraldehyde (at 5°C), or 7 hrs fixation in Bouin (at 5°C) and extended washing in 70% ETOH, was identical. The tissue was processed through the standard series of 30%, 50%, 70%, 90%, 100% ETOH, 1:1 ETOH-Xylene, 100% Xylene, 1:1 Xylene: parafin, and 2 changes of 100% parafin, using an American Optical Histokinette automated tissue processor.

The parafin embedded muscle was sectioned at 8 microns, and after fixation to the glass slides with albumin, and removal of the parafin, was stained with 1:1 methylene blue (0.5%): azure II (1%) for 5 mins and followed by 1:1 ponceau (1%): orange G (2%) for 20 secs. Interestingly, hydration of the larger muscle pieces after facing the parafin block, allowed sections to be cut in the 4-6 micron range.

C. Cryoscopy and Histochemical Procedures

General

Muscle pieces excised for histochemical analysis were first immersed in a bath of iso-pentane for 10 secs at -40°C and then placed directly in liquid nitrogen for at least 5 mins. Removal of the skin was facilitated by making a small incision parallel to the ventral fin and then prying the skin free after the liquid nitrogen stage. Eel muscle was particularly difficult to prepare for histochemical examination as the muscle was extremely sensitive to temp-

erature alterations. Indeed, without first infiltrating the tissue with iso-pentane, the muscle was riddled with holes and unsuitable for examination. Further, the standard freezing block on the Aymes Cryostat II could not be used to prepare muscle blocks since the tissue embedding material was at room temperature and thus warmed the frozen muscle and produced artifacts. To overcome this problem, the muscle pieces were placed directly onto wooden blocks covered with 7% Tachganth Gum and the entire block immersed first into the iso-pentane and then into the liquid nitrogen. The samples were then placed in the bottom of the cryostat and allowed to warm up to -25°C before sectioning. The tissue was sectioned at 6 microns, picked up on glass slides and allowed to dry 2-3 mins before processing specifically for either LDH, myosin ATPase, or SDH.

Succinic Dehydrogenase (SDH) Staining

SDH was demonstrated using the method of Nachlas et al. (1957). The slides were incubated for 10 hrs (at 22°C) in a substrate medium which contained 0.05 M Na-phosphate buffer, pH 7.6, 0.05 M Na-succinate and 1 mg/ml Nitro Blue Tetrazolium in a final volume of 50 mls. The slides were then washed in a saline (4% NaCl), fixed in 10% formal-saline for 10 mins, washed in 10% ETOH for 10 mins, rinsed in distilled water and mounted in Farrant's Medium (50 g Gum arabic, 50 ml distilled water, 50 ml glycerin and 1 g Arsenic trioxide). The site of enzyme activity was evidenced by blue-purple diformazan deposits. Control slides were processed in the

absence of Na-succinate and produced no staining.

LDH Staining

The presence of LDH was demonstrated using the method of Hess et al. (1958). The slides were incubated in a substrate solution containing 2 g liquid DL-Lactate, 0.022 g NAD^+, 0.22 g Nitro Blue Tetrazolium, and 0.1 M Na cyanide in 50 mls 0.06 M Na-phosphate buffer. The slides were then rinsed in saline, fixed for 10 mins in 10% formal-saline, washed with 10% ETOH for 10 mins and mounted in Ferrant's Medium. The sites of enzyme activity were evidenced by the characteristic blue-purple diformazan deposits. Control slides were incubated in the absence of DL-lactate and produced no diformazan formation. A time course substrate incubation procedure was adopted to allow qualitative assessment of the relative enzyme activities of red and white muscle. Thus, slides were removed at 30 min intervals from the substrate solution and then processed as above.

Myosin ATPase Staining

Myosin ATPase was demonstrated using the method of Niles et al (1964). The slides were incubated in a substrate solution which contained: 0.02 M Na-barbiturate, 0.18 M $CaCL_2$, 76 mg ATP, 30 mg 2,4-Dinitrophenol in a total volume of 50 mls. The slides were incubated at 22°C under the time course regime described above; however, due to the high ATPase activity, the slides were removed at 5 minute intervals. Following incubation, the slides were washed in three changes of 1% $CoCl_2$ for 2 mins each. The slides were then

washed for 5 mins in running distilled water and developed in 1% ammonium sulfide for 1 min. The slides were subsequently washed in distilled water and mounted in Ferrant's Medium. The presence of enzyme activity was evidenced by a black precipitate. Control slides were incubated in the absence of ATP and produced no staining patterns.

III

MORPHOLOGY, HISTOCHEMISTRY AND ULTRASTRUCTURE

INTRODUCTION

The correlations between cellular architecture and physiological function are now well documented for a number of systems. For example, ion transport tissue is characterized by an association of mitochondria and endoplasmic reticulum to form 'ion pumps' (Diamond and Bossert, 1967; Hochachka and Somero, 1973; Sackin and Boulpaep, 1975); hormone synthesizing and releasing tissue is characterized by specific cytoplasmic granules, extensive golgi and endoplasmic reticulum (Kobayshi, 1969). Likewise, mammalian slow and fast twitch muscle are characterized by specific structural arrangments (Gauthier, 1970) which seem to correlate well with their histochemistry (Hess and Pilar, 1963; Edgerton and Simpson, 1969) and physiological function (Peachey, 1968; Burke et al., 1971; Close, 1972). The establishment of these correlations for fish red and white muscle does not, however, appear as fortuitous.

As indicated in the Introduction, although the basic appearance of the contractile apparatus in fish red muscle is consistent with mammalian slow muscle, there are some significant structural differences. Thus, fish slow muscle possess a distinct M-line, a well developed sarcoplasmic reticulum, regular extensions of the transverse tubule system to form triads, with the dilated cisternae of the sarcoplasmic reticulum, regular and well defined Z-lines, and continuity of Z-lines between opposite sides of the

fiber (Kilarski, 1967; Nishihara, 1967; Nakajima, 1969; Nag, 1972; Patterson and Goldspink, 1972). While these structural characteristics are more commonly associated with mammalian fast or twitch muscle (see Hess, 1970), their presence in fish red muscle may be an expression of an alteration in the mechanism for tension generation in this muscle compared to mammalian slow fibers. Mammalian slow muscle contracts without shortening sarcomere length (Awan and Goldspink, 1970; Goldspink et al., 1970a, b). Swimming movements, however, require isotonic contractions (shortening of sarcomere length) for the generation of the propulsive wave form (Johnston et al., 1972) and fish red or slow muscle is involved in the production of swimming movements as previously noted (Bone, 1966; Greer-Walker and Pull, 1973). Thus, the inconsistencies in the structural characteristics between fish and mammalian slow muscle, and the similarity between fish slow and mammalian fast muscle, may simply be related to the generation of tension isotonically as opposed to isometrically.

A comparison of the basic structural characteristics common to mammalian fast twitch muscle with fish white muscle, suggests that, apart from differences in innervation, the ultrastructure of the two muscle types is basically identical. In fish white muscle, the neuromuscular junction is characterized by the 'en grappe' type endings observed for mammalian fast muscle (Hess, 1965; Page, 1965). Thus, fish white muscle receives both monoaxonal (Andersen et al.,

1963, hagfish) and polyaxonal innervation (Takeuchi, 1959) which terminated in multiple ending sites. Further, the post junctional sarcolemae membrane lacks infoldings. Therefore, innervation in most fish white muscle is similar to that observed for amphibian and mammalian slow muscle. Innervation of fish red muscle is also by the 'en grappe' type, but a larger number of motor terminals are distributed throughout the length of the fibers (Takeuchi, 1959; Nakajima, 1967; Nishihara, 1967).

The evidence suggests that while the correlation between the morphological, histochemical and physiological characteristics of the mammalian slow and fast musculature is well established, these correlations for fish slow and fast muscles are not as apparent. Therefore, fish muscle function may not be identical to that commonly accepted for the 'homologous' mammalian muscles.

Patterson and Goldspink (1972) working with the coalfish, and Nag (1972) working with rainbow trout, have recently reassessed the structure-function problem of fish red and white muscle. They were, however, unable to add to the existing arguments of their functional roles. To date, no one (to the authors knowledge) has attempted to evaluate this problem using the eel as the experimental animal; and to capitalize upon the documented changes in red muscle content (Lewander et al., 1974) and enzyme pattern (Bostrom and Johansson, 1972) concurrent with migration.

Basically, although the structural architecture of red

muscle is markedly different from mammalian red or slow muscle, the structural characteristics must be, none the less, related to its physiological function. Thus, if there are alterations in the physiological role of red muscle during migration, as suggested by the data of Lewander et al., (1974) and Bostrom and Johansson (1972) for the European eel, then an expression of this change should be manifest in the structural machinery.

This communication reports the ultrastructural and histochemical characteristics of muscle from both immature and mature American eels, Anguilla rostrata. The results indicate that maturation is accompanied by alterations in the structure of the red muscle and that these changes are absent in white. It is suggested that the contractile role of red muscle may be altered following maturation, and that the white muscle may provide contractile forces at swimming speeds other than sprinting.

RESULTS

General Characteristics and Morphology

The distribution and myotomal pattern of red and white muscle in the migrating American eel, A. rostrata, are presented in Figs. 1 and 2. The two muscle types appear to be distributed independently of each other, with the greatest concentration of red muscle at body segment #12 and white muscle at body segment #16. Therefore, the peak concentration of red and white muscle are approximately 10 cm apart. Also, the red muscle maintains approximately the same thickness throughout the abdominal region while the white muscle falls off sharply (Fig. 2). These observations suggest that the importance of each muscle type in generating swimming movements varies throughout the longitudinal axis of the fish as noted by Greer-Walker (1970). Little if any myotomal red muscle is found on young, up-river migrating eels (i.e., less than 30 cm in length).

Examination of an enlargement of body segment #16 reveals several characteristics of eel muscle (Fig. 3.). The red musculature, except for the invagination at the lateral line, is limited to a thin band distal to the subcutaneous fat layer. Thus, eel muscle does not possess the mosaic, or mixed, red-white pattern characteristic of salmonids (Nag, 1972). The perimysium of the red muscle inserts into the perichondrium of the axial skeleton as well as into the adjacent perimysium of the white musculature. This arrange-

Fig. 1. The distribution of red and white muscle in a mature migrating bronze eel. Each body segment is 2.5 cm in length.

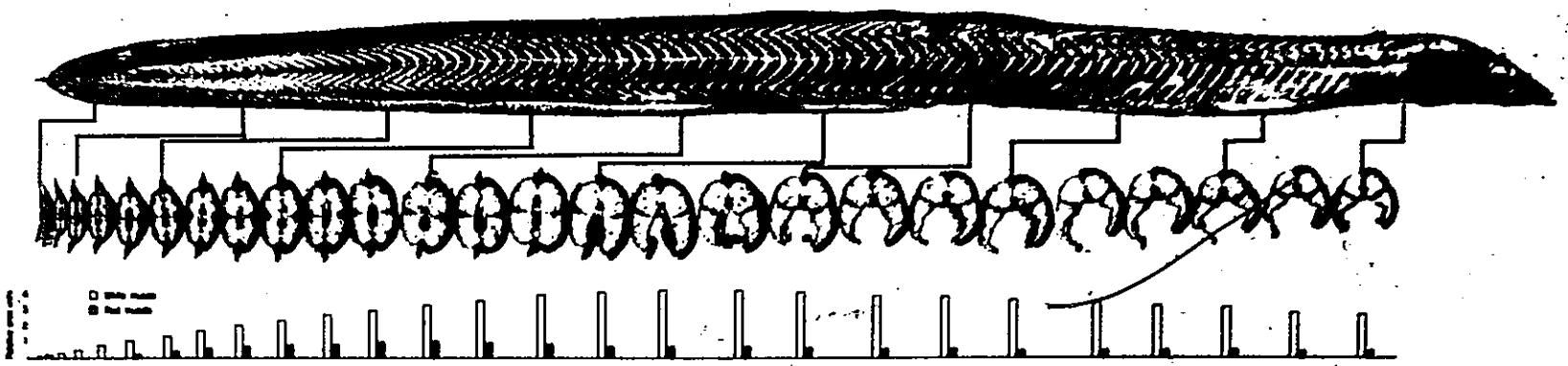


Fig. 2. Graphic presentation of the histogram presented in Fig. 1. Arbitrary length units are the 27-2.54 cm sections noted in Fig. 1.

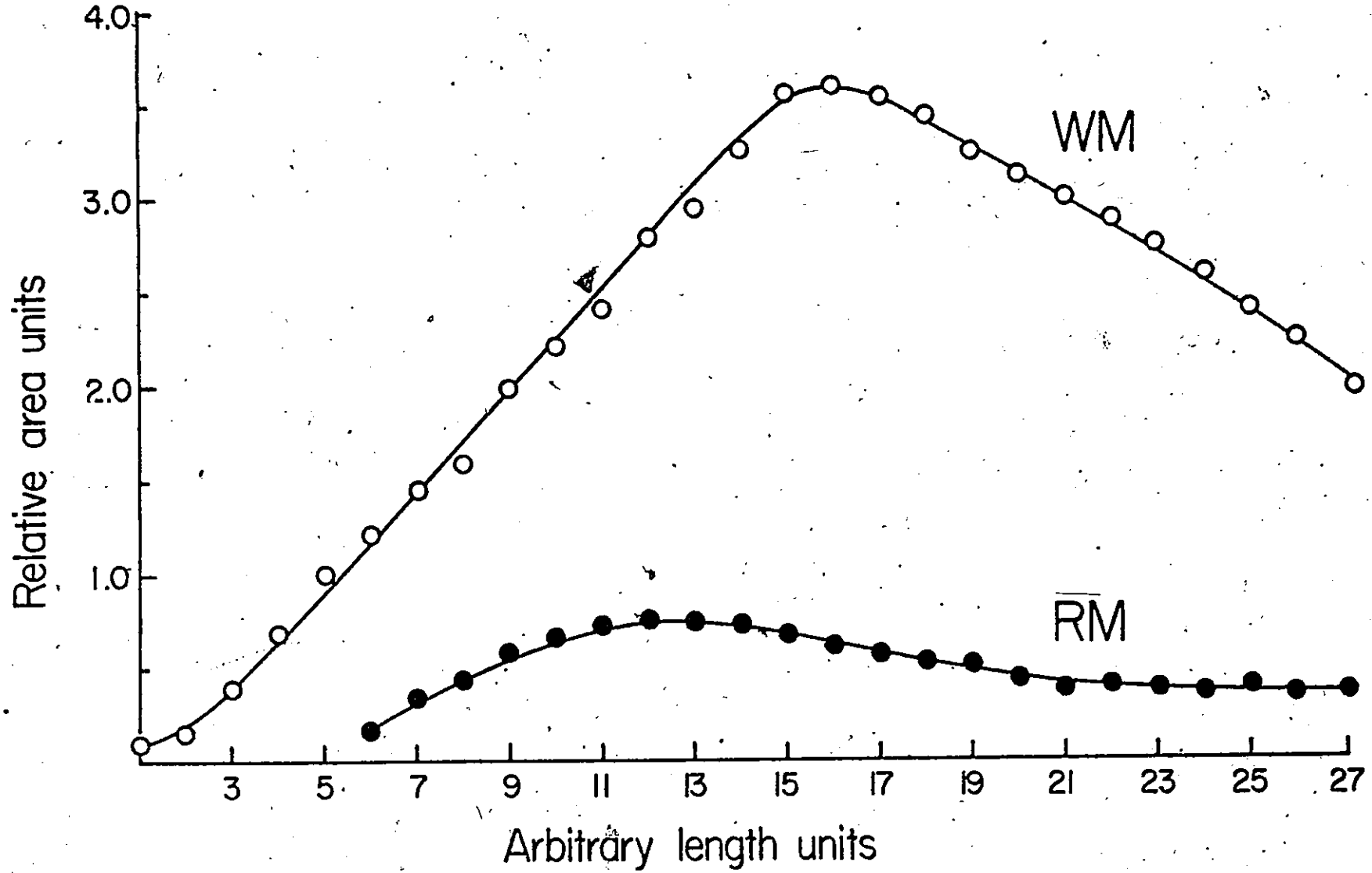
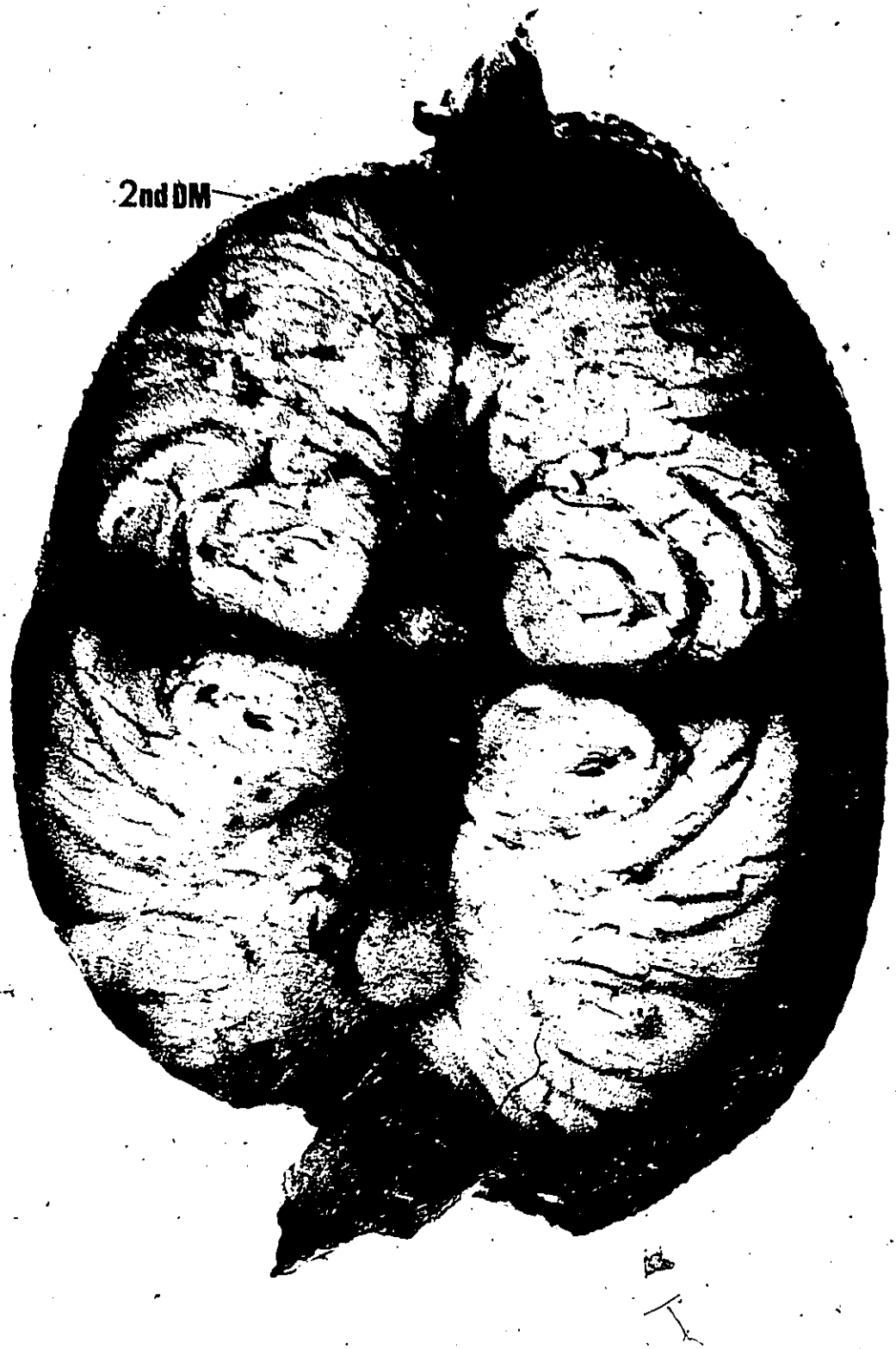

4.0
3.0
2.0
1.0
Relative area units
WM
RM
3
5
7
9
11
13
15
17
19
21
23
25
27
Arbitrary length units

Fig. 3. Enlargement of body segment #16 (see Fig. 1). Note the homogenous deep white musculature, and the relative amounts of red and white muscle. A distinct subcutaneous fat layer separating the red muscle and skin is also observed. 2nd DM, second dorsal myotome.


2nd DM

ment presents the characteristic series of 'loops' described by Alexander (1969) and Willemse (1972) for other fish species. Note that the symmetry of the perimysium is conserved about two axes; a vertical axis through the dorsal and ventral fins, and a horizontal axis through the lateral line.

Fig. 3 further underscores the relative proportion of the body musculature which is red compared to white. For this specimen, red muscle comprised 15.8% of the total body musculature, a value higher than that reported by Greer-Walker and Pull (1975) for A. anguilla (8.8%); however, it is probable that specimen was not in the migrating phase.

The myomeres of A. rostrata are arranged in a manner similar to that observed for dogfish (Nursal, 1955; Alexander, 1969; Willemse, 1972). This pattern consists (Fig. 4) of a series of three cones with alternate apecies pointing anteriorly. In addition to these directional cones on each myomere, the musculature of the eel is also organized into a series of anteriorly pointing cones which are laterally-medially oriented and composed of the loops prominent in body cross sections (Fig. 3, 5). By following the striped loop in the successive sections in Fig. 5, this pattern is evident. Interestingly, the number of these 'super cones' appears to depend upon the size of the eel. For example, only two cones were evident in a 30-35 cm size eel, while in a 60-75 cm eel, four cones were observed.

In addition to this complex organization of the individual

Fig. 4. Each myomere of the eel is organized into a 'W' shape with a medial anteriorly pointing cone (2A) and alternate posteriorly pointing cones (1P, 3P). The dorsal (DI) and ventral insertions (VI) are directed anteriorly.

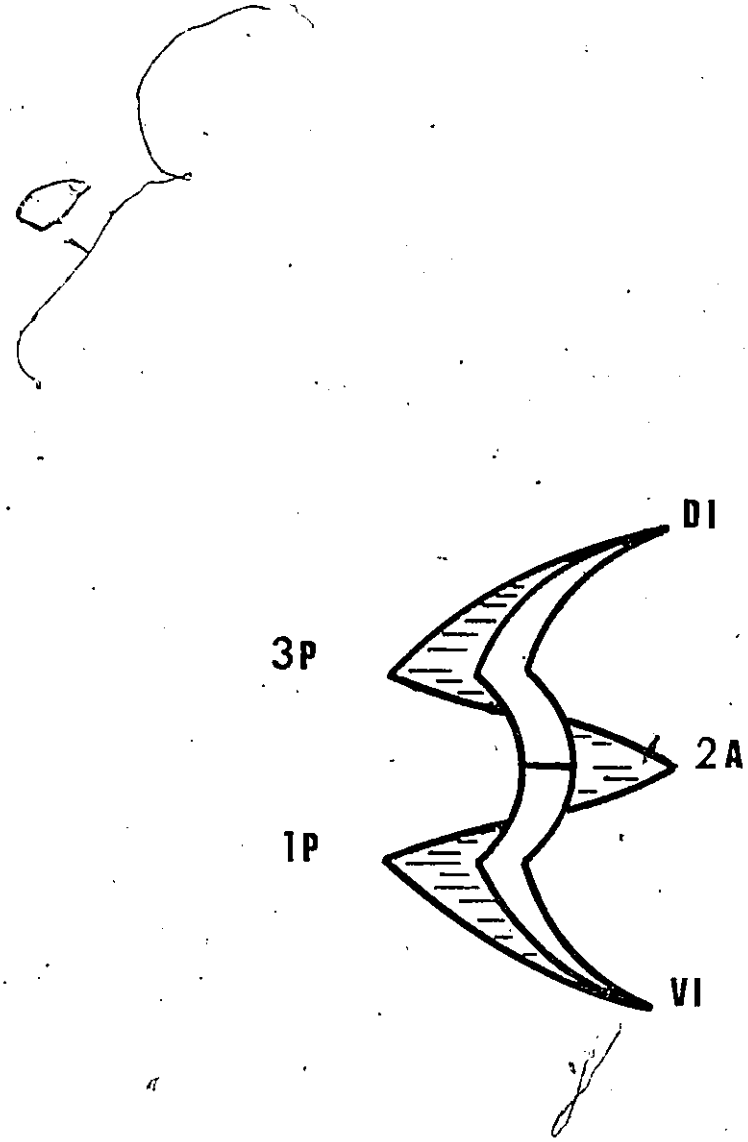
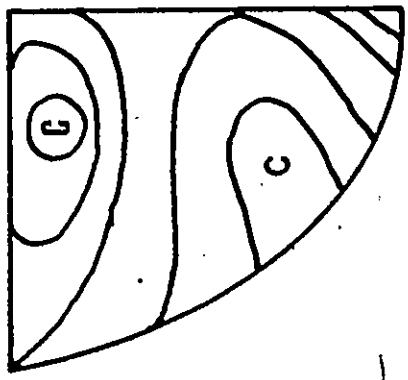
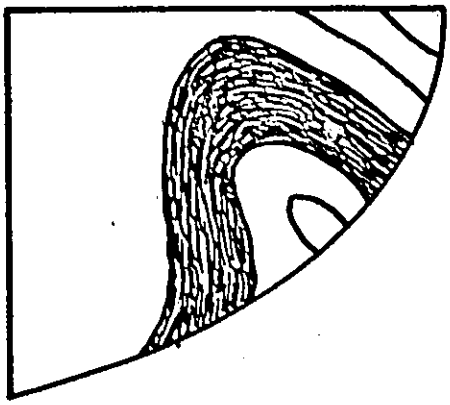
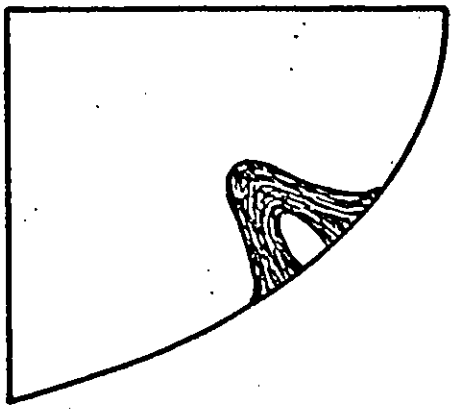
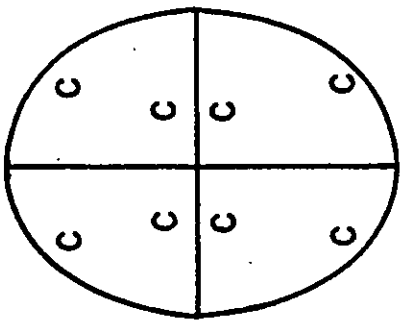

DI
3P
2A
1P
VI

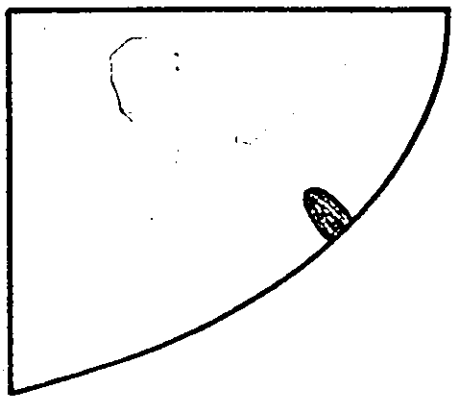
Fig. 5. Since the musculature of the eel is symmetrical about two axis, only one quarter is diagrammed. The line drawings suggest that the sucessive invaginations are extensions of a 'super-cone' structure which is oriented distal-proximal, anteriorly-posteriorly. The number of these cones is observed to vary with body length.


G
C






C
C
C
C
C
C
C
C



myomeres, perimycium and muscular loops, the individual muscle cells and myofibrils are arranged in a helical manner (see Willemse, 1959, 1972; Alexander, 1969). The period of the helix for the muscle cells was estimated to be in excess of 500 microns and approximately 200 microns for the myofibrils. Further 5 to 7 myofibrils twist together to form a structure analogous to a series of braided ropes twisting about each other within a larger structure which was itself twisting, but with a longer period. This pattern can be seen in Fig. 15. The importance of this helical nature of fish muscle was recently reviewed by Willemse (1972).

Light Microscopy and Histology

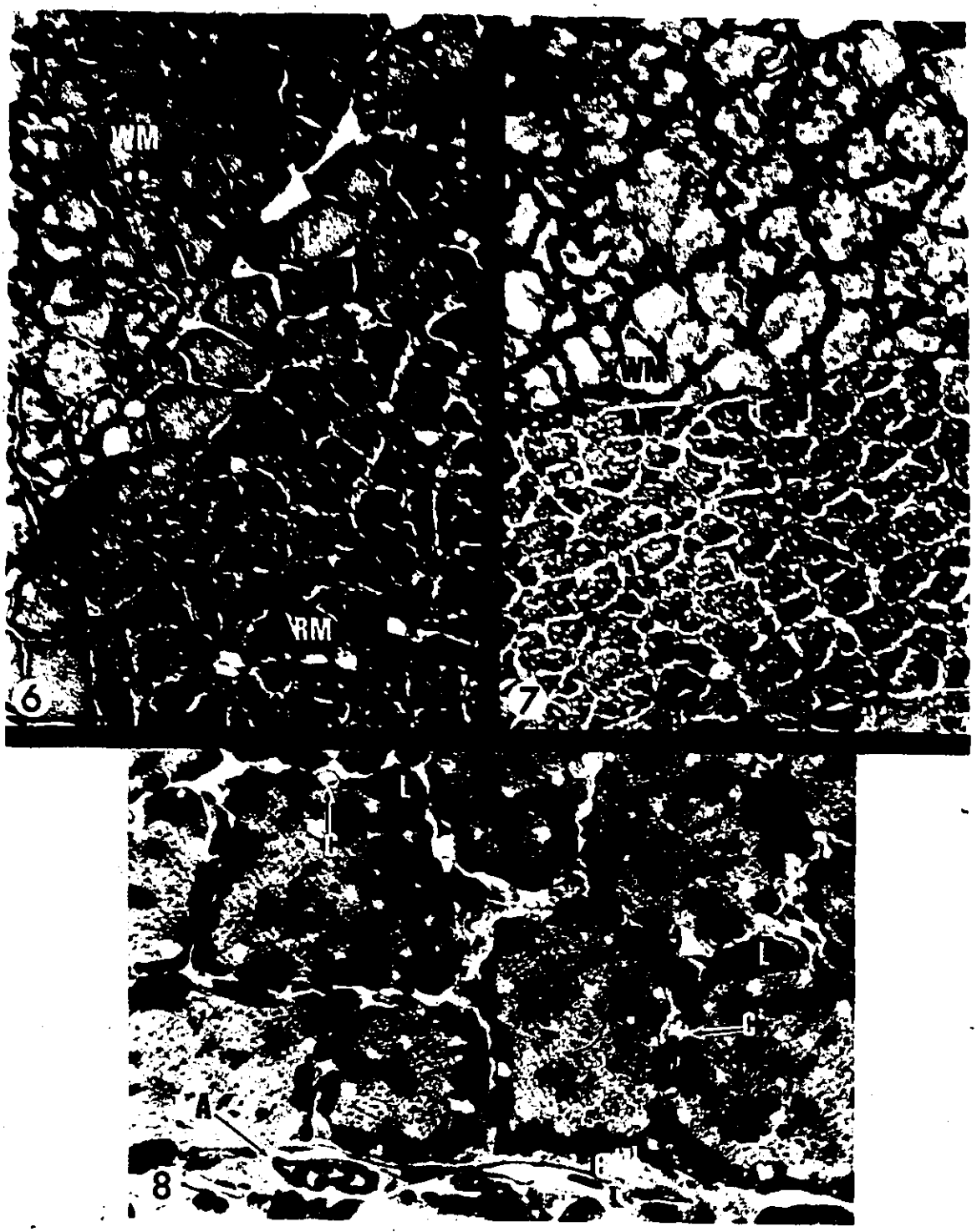
Figs. 6, 7 and 8 document the disparity in vascularity, fiber size and lipid content of the red and white muscles (see review by Love, 1970). Also, the presence of an intermediate fiber type, previously unreported for the eel, specifically localized to that part of the red muscle myotome which invaginates at the lateral line, is demonstrated in Fig. 6. Fig. 7, which was taken of the second ventral myomere (refer to Fig. 3), does not show the presence of this fiber type since it is not at the lateral line.

The highly developed vascular system of red muscle is clearly seen in Fig. 8. Consistent with the observations of Bone (1966) on the dogfish, the main axons arising from the dorsal nerve cord extend to the myotome through the myosepta as noted by the arrow on this figure.

Fig. 6. Cross section of red-white muscle interface of eel myotomal muscle where the red muscle (RM) invaginates at the lateral line. The darkly staining dots between the red muscle fiber are red blood cells, while the peripheral staining dots of both the red and white fibers (WM) are nuclei. The intermediate fibers are clearly evident as a band between the two muscle types (IF). Hematoxyln-eosin stained cryostat sections. X. 288.

Fig. 7. Cross section of the interface between the red (RM) and white (WM) muscles of the eel at the second ventral myotome (refer to arrow on Fig. 3). Note the marked difference in the fiber diameter of the two respective muscles and the lack of intermediate fibers (Fig. 6). X. 288.

Fig. 8. An enlarged cross sectional view of red muscle directly adjacent to a myosepta. The tissue was fixed with O_sO_4 and stained with 5% toluidine blue, pH 10, giving the lipids (L) a dark stain against the lighter myofibrils. Numerous capillaries (C) are seen between the muscle fibers and account for the large number of red blood cells observed in Figs. 6 and 7. A large axon (A) is noted buried in the myosepta (see arrow). X. 288


WM
RM
6
WM
7
L
C
L
C
A
8

Consistent with previous reports (see Love, 1970), the red fibers of the eel were significantly smaller than the white, and contain a single size class as compared to two size classes for the white fibers (Fig. 7). The diameter of the red fibers varied from 9.4 to 23.6 microns (937 fibers counted), while the white fibers ranged from 10 to 37.8 microns for the small size class, and 37.8 to 66 microns for the large size class (1859 fibers counted). The small class fibers exceeded the large fibers by approximately 25%. The intermediate fibers were similar to the large size class white fibers (Fig. 6).

Eel red and white muscle from both immature yellow and mature bronze specimens, was examined histochemically for the relative activities of SDH, LDH, and myosin ATPase. In all specimens examined (6 immature and 4 mature), the red muscle had consistently lower activities of ATPase (Fig. 9) but significantly higher activities of SDH (Fig. 10) and LDH (Fig. 11) and larger lipid deposits (Fig. 8), than the white muscle. These results are confirmed by those of George (1961), Nag (1972), Patterson et al., (1975).

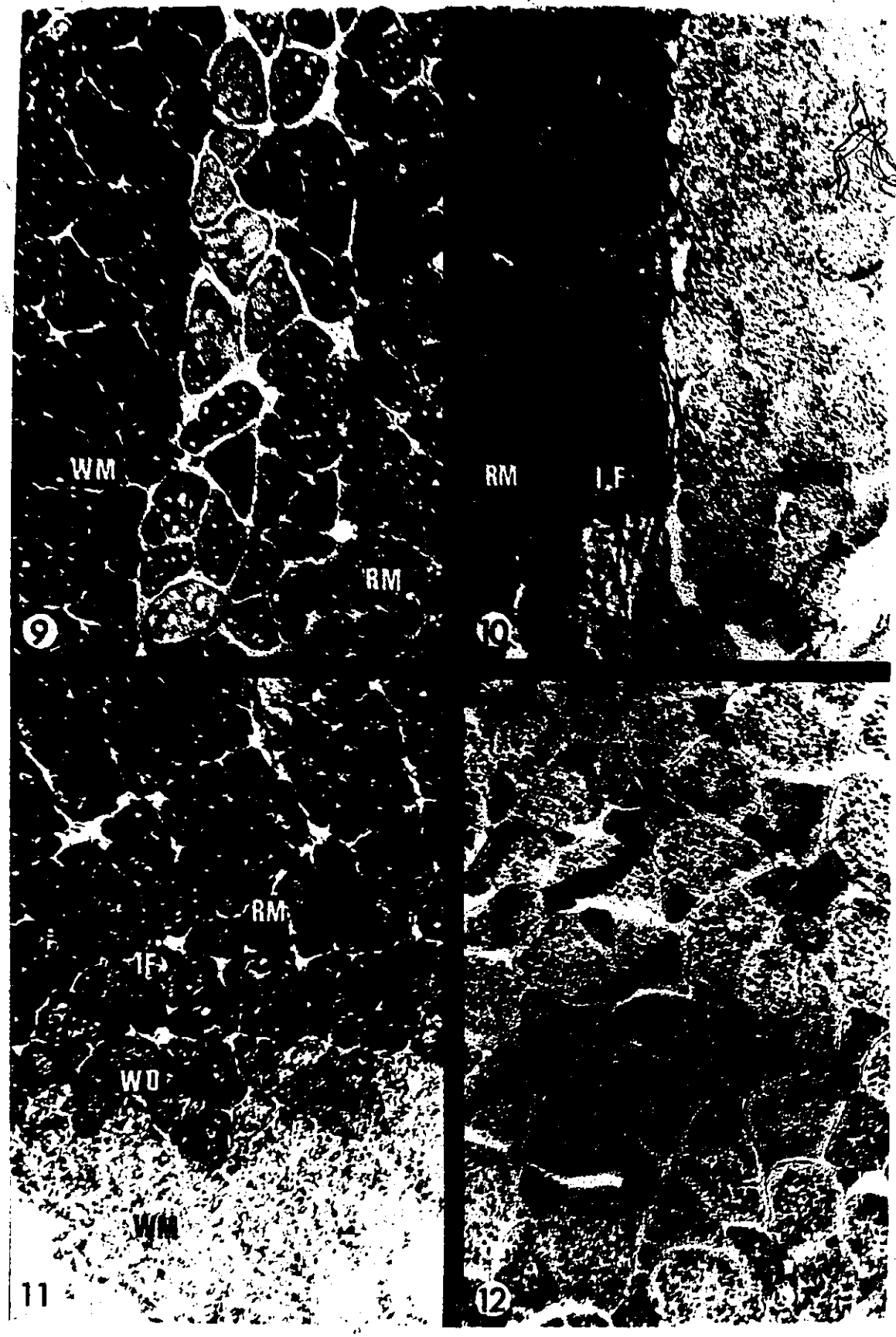
Due to the larger number of mitochondria in red muscle (see Nag, 1972; Patterson and Goldspink, 1972), higher levels of the mitochondrially associated SDH are expected (Fig. 10). For LDH (Fig. 11), however, Brostrom and Johansson (1972) working with A. anguilla, clearly demonstrated higher activity levels in white muscle than red (see also Chapter 4).

Fig. 9. The differential staining of myosin ATPase (see Materials and Methods) for eel muscle types. Note the homogenously dark staining white fibers (WM) compared to the lesser stained intermediate fibers (IF) and red muscle fibers (RM). X. 300.

Fig. 10. The differential staining of SDH (see Materials and Methods) for eel red (RM) and white muscle (WM) taken from the interface area of the second dorsal myotome (refer to Fig. 3). The large intermediate fibers (IF) are clearly evident within the red myotome. The residual staining of the (WM) musculature could not be differentiated from controls. X. 275.

Fig. 11. The histochemical localization of eel muscle LDH. The red muscle (RM) stains the darkest due to its high oxidative capacity, followed by the intermediate fibers (IF) and the glycolytic white fibers (WM). Section taken as in Fig. 12. X. 275.

Fig. 12. Histochemical localization of WM-LDH from the eel (see Materials and Methods). Note the concentration of activity in the small compared to the large fibers. X. 288.


WM
RM
9
RM
I.F
10
RM
IF
WD
WM
11
12

The histochemical stain for LDH is based on the formation of a diformazan salt of tetrazolium blue which precipitates upon oxidation of the dye. This occurs when the LDH reaction is driven in the direction of pyruvate formation. Therefore, the histochemical stain gives a measure of oxidative rather than glycolytic potential, as reported by Brostrom and Johansson (1972) and Chapter 4 (this thesis). Thus, the histochemical results are consistent with the SDH results which demonstrate that the red muscle has a higher oxidative capacity than white muscle, and a higher conversion rate of lactate to pyruvate (see Chapter 4).

The intermediate fibers stain lighter than both red and white muscle for myosin ATPase (Fig. 9), lighter than red but darker than white muscle for SDH (Fig. 10) and slightly lighter than red muscle but darker than white muscle for LDH (Fig. 11).

In addition to the red, white and intermediate fibers, a fourth fiber class was observed. These fibers have a limited oxidative capacity and lie within the white muscle perimycium boundary separating the red and white muscle myotomes. These fibers are specifically localized adjacent to the red muscle myotome which is inserting into the perichondrium of the skeleton. Thus, these fibers are only evident in the lateral line region (compare Figs. 10 and 11).

As indicated above, the deep white muscle was characterized by having two distinct fiber size classes; interestingly, both sizes stain equally for myosin ATPase and SDH

(note, this refers specifically to a lack of SDH activity, not its presence). For LDH, on the other hand, there appears to be a concentration of activity in the small class fibers as compared to the large fibers (Fig. 12).

Figs. 8 and 13 document the alterations in lipid content of the red muscle concurrent with the yellow-bronze transition for A. rostrata. The red muscle from an immature yellow eel (Fig. 14) demonstrated relatively few lipid droplets which have not yet coalesced to form a lipid sheath around the fibers, or to greatly infiltrate the fibers. Fig. 13, on the other hand, demonstrates the degree of fatty infiltration occurring with the transition to the bronze state, or at the point of the sea-ward migration. Indeed, the lipid volume, as measured in cross section, increased from approximately 1% to 50%. The major intracellular site of lipid deposition appears around the periphery of the fibers, although extensive intramyofibrillar deposits do occur (Fig. 13). The regularity of the A- and Z-lines and the conspicuous lack of intracellular lipid deposits in the white muscle is evident from Fig. 15. The extent of fatty infiltration for the red muscle is not limited to just disruption of the contractile apparatus regularity; indeed, from Fig. 16, it is evident that extensive deposition occurs within the perimycium and between bundles of red muscle fibers (see also Kilarski, 1960). The end result of this fatty "degenerative' process is the partitioning off of groups of red muscle cells. This condition was not as prevalent

Fig. 13. Longitudinal section of red muscle from a mature migrating eel. Note the periphery of the fibers are ensheathed in lipid and pockets of lipids have formed between the myofibrils. Stained with 5% toluidine blue, pH 10. X. 288.

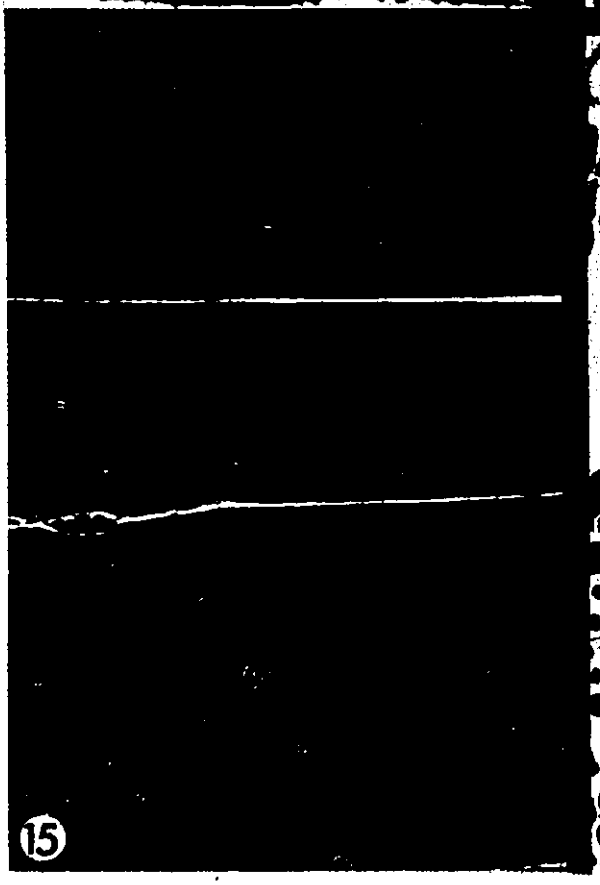
Fig. 14. Longitudinal section of red muscle from an immature yellow eel collected in an estuary in St. Andrews, New Brunswick. Note the few randomly distributed, darkly stained lipid droplets (LD). Stained with 5% toluidine blue, pH 10. X. 288.

Fig. 15. Longitudinal section of white muscle from a mature migrating eel. Note the total lack of intracellular lipids and a dirth of capillaries which is characteristic of the white muscle examined. The helical nature of the muscle can be observed upon close inspection. Stained with 5% toluidine blue, pH 10. X. 288.

Fig. 16. Cross section of red (RM) and white muscle (WM) demonstrating the degree of fatty deposition in a mature migrating bronze eel. The cryostat section was incubated in the substrate mixture for demonstrating myosin ATPase but was extensively over oxidized by ammonium sulfate to show the different fiber sizes. The lipid deposits (LD) appear as white voids. X. 440.


13


L
L


15


WM
L
L
BM
16

in the white muscle. Here the deposition occured predominately within the connective tissue separating the white muscle myotomes. These results are consistent with those of Kilarski (1960) who has reviewed the patterns of fat deposits in teleosts.

Ultrastructure: Red and White Muscle

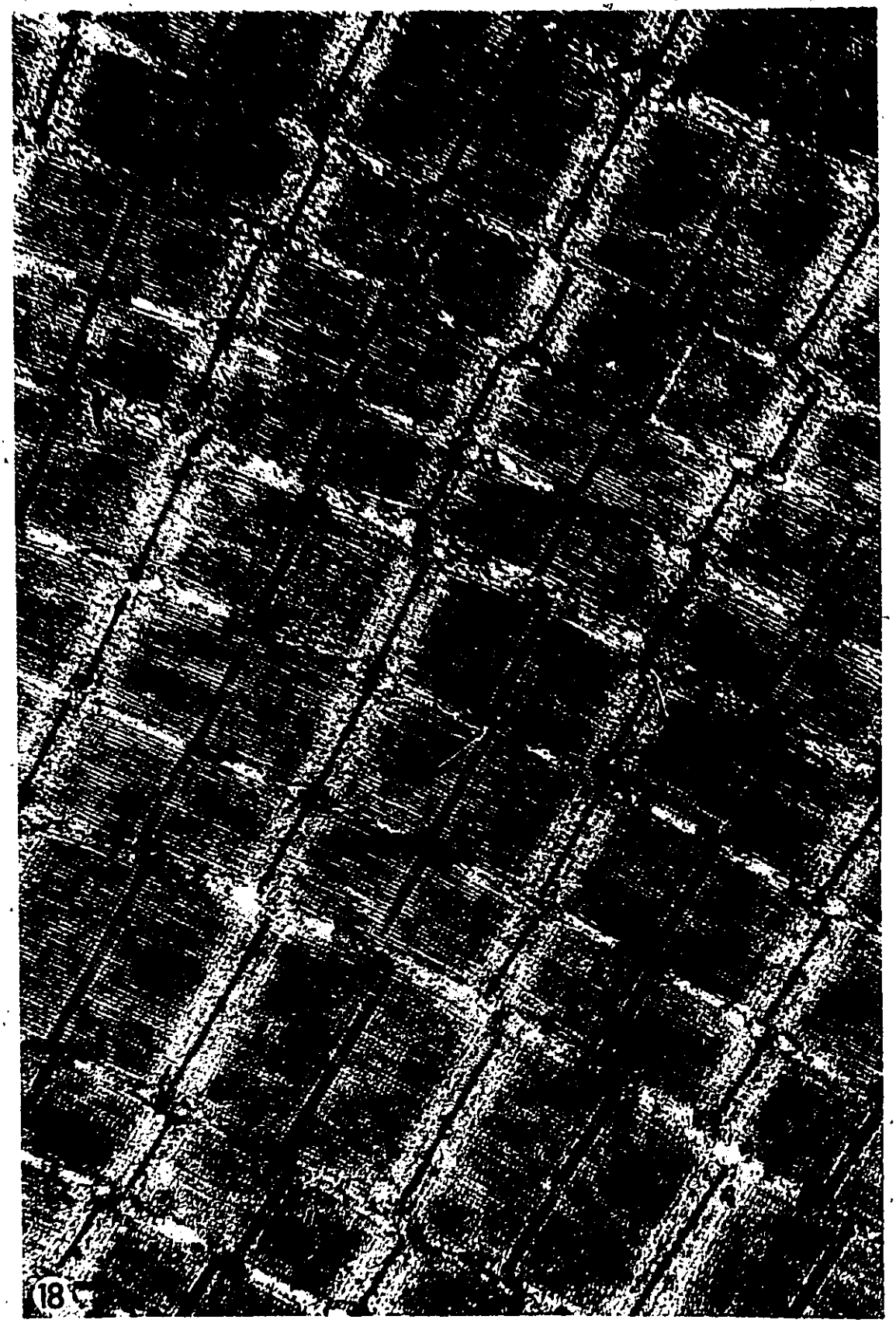
In addition to containing large intracellular deposits of lipids, eel red muscle, and fish red muscle in general, (see Love, 1970), also has larger deposits of glycogen than white muscle (Fig. 17). The extremely low concentrations of glycogen in eel white muscle could be due to a state of starvation (Fig. 18 is representative of bronze-eel white muscle); however, feeding, immature yellow eels also demonstrated low glycogen levels. In the case of the yellow feeding eels, low glycogen may reflect a 'panic' activity of the eels in the traps prior to sacrificing. Black et al. (1959) has demonstrated that only 2 mins. of strenous activity are needed to deplete the glycogen content of trout white muscle by 50%.

The general appearance of the myofibrillar organization (alternating actin and myosin) in both the red and white muscle (see Figs. 17 and 18) is consistent with that accepted for vertebrate striated muscle (see Huxley, 1966); however, there are some subtle differences between the structural architecture of the two muscle types. For example, while the Z-disc of the white muscle demonstrates the characteristic

Fig. 17. Electron micrograph of red muscle from a mature migrating eel. Note the increased fatty infiltration (L) and mitochondrial proliferation (M) as compared to Fig. 12. Two capillaries are enclosed within this field of view. EN. endothelial cell nucleus, CL. capillary lumen, TR. triad, SR. sarcoplasmic reticulum. X. 9700.


EN
M
17

Fig. 18. Electron micrograph of white muscle from a mature migrating eel. The triads (TR) are regular at each Z-disc (Z) and little glycogen is observed between the myofibrils. The M-line (M) is well developed and the alternating thick-myosin and thin-actin filaments are easily recognized. X. 23500.


18

'basket weave' pattern (Knappeis and Carlsen, 1962) observed for mammalian striated fast muscle (Fig 18). This pattern is obscured by the large concentrations of a Z-disc protein in the eel red muscle (Figs. 17 and 19).

The M-line is very prominent in both red and white muscle, as are the A- and I-bands. The ratio of actin to myosin is 8:1 and is within the range observed for most other vertebrate striated muscles (Prosser, 1973). Both muscle types have a well developed sarcoplasmic reticulum; however, the formation of triads with the transverse tubular system is more regular in white muscle than in the red, an observation previously reported by Nag (1972) for trout red and white muscle. Further, the triads are localized at the Z-disc for both muscles, rather than the A/I junction as reported for the red ocular fibers of the carp (Kilarski, 1967). Sarcomere length, which is an indication of contraction speed (Hess, 1970), for eel white fibers were found to be shorter than the red (1.8 as compared to 2.6 microns), but within the normal range reported for other fibers (Bone, 1966).

Evidence presented in Figs. 17 and 19 further documents the increase in lipid deposits in red muscle concurrent with the transition between immature yellow (Fig. 19) and mature bronze (Fig. 17) eels. Paralleling these changes, a proliferation in the mitochondrial numbers and the fiber volume they occupy can also be seen on these figures. Thus, the mitochondrial volume, as determined in cross sections, in-

Fig. 19. Electron micrograph of the red muscle from an immature yellow eel demonstrates the large concentration of glycogen (G), numerous mitochondria (M), and striking lack of intracellular lipids (L). This micrograph is taken from the same sample as presented in Fig. 9. The Z-discs (Z) are readily apparent and are thickened compared to the Z-disc in white muscle (Fig. 18). Both the (A) and (I) bands are prominent and the triads (TR) appear at the Z-disc (Z). Note the M-lines (M). MF. myofibril. X. 15400.


19

creased from approximately 10% to greater than 20% in the mature migrating eel (compare Figs. 17 and 19). Coupled with the increase in intracellular lipid deposits and mitochondrial numbers, there is a corresponding increase in the sarcoplasmic reticulum volume in the red muscle of the migrating eel. Importantly, no changes in the structural architecture of the eel white muscle, concurrent with migration were observed.

DISCUSSION

It is clear that significant differences exist in the cellular architecture between red and white muscle fibers of the eel, A. rostrata. These differences are consistent with observations reported for other fish red and white muscles. Thus, red fibers compared with white fibers have smaller fiber diameters, greater vascularity, intracellular lipid deposits, longer sarcomere length, and greater numbers of mitochondria. These results confirm and extend the works of Nag (1972) on rainbow trout, Nakajima (1969) on snakefish, Bergman (1964) on sea horses, Patterson and Goldspink (1972) on coalfish, Nishihara (1967) on carp, Bone (1966) on dogfish, Korneliussen and Nicolaysen (1973) on hagfish, Buttkus (1963) on lingcod, Franzini-Armstrong and Porter (1964) on black mollies, Bishop and Odense (1967) on cod, and Kilarski (1967) on tench, pond-loach, perch, guppy, little goby, and flounder. Histochemically the higher SDH and LDH activities (higher oxidative capacity) and lower ATPase activities (suggestive of slower contractile speed (Barany, 1967)) for red muscle, likewise support the results of George (1962) on mackerel, Munshi et al. (1975) on Bagarius bafarius, Johnston et al. (1975) on rainbow trout, Patterson et al. (1975) on glass fish, carp, coalfish, black mollies and grey mullet, and Nag and Nursall (1972) on rainbow trout.

With the definitive work of Bone (1966) and its sub-

sequent collaboration (e.g., Korneliussen and Nicolaysen, 1973), it is now generally accepted that fish musculature is divided into a series of intermediate fibers, in addition to the easily recognizable red and white. These fibers, lumped together as the pink fibers (Patterson et al., 1975), are spatially located between the red and white myotomes, and in the crucian carp, for example, are equal in the mass with the red muscle (Patterson et al. 1975). Bokdawala (1967) has suggested that these pink fibers are closely correlated with the staying capacity of the fish; i.e., fishes which swim continuously have a higher content of pink muscle. It is interesting in this respect that in the American eel, which is certainly a stayer during the latter part in its life cycle (see Sinha and Jones, 1976), the intermediate fibers are present only in a single, or double row (Fig. 10). Since these fibers are not delineated by a unique perimycium system, but rather are located within the red muscle myotome, it is possible that they could represent a differentiating red muscle area. This interpretation has been suggested by Patterson et al. (1975) for the strip of fibers from carp found in a similar spatial location; however, the fibers which they examined were smaller than even the red muscle fibers. Since these fibers in the eel fall within the large white muscle size class, and are found in both immature yellow and migrating bronze eels, interpretating them as differentiating seems unlikely.

The histochemical demonstration of lower SDH (Fig. 10)

and lipid levels in the eel intermediate fibers is consistent with observations on other fishes. Interestingly, the levels of myosin ATPase are markedly different than previously reported. For example, Johnston et al. (1975) differentiate intermediate fibers in the rainbow trout as having lower oxidative potential than the red but greater than the white, and levels of myosin ATPase activity comparable with white muscle. This is clearly not the case for the eel, where the intermediate fibers are truly intermediate in all the assays performed. Unlike these oxidative intermediate fibers, the fourth type of fibers in the eel, the oxidative white fibers, have similar activity levels for myosin ATPase as the deep white fibers, which demonstrate little oxidative capacity. Thus, further work is needed to ascertain the correlation between the structural/histochemical characteristics of the intermediate fibers and their physiological function.

Results on the relative mitochondrial volume (less than 1%) of eel white muscle, while contrasting with observations reported by Page (1965) for frog fast muscle, support the observations of Patterson and Goldspink (1972) on coalfish and Korneliussen and Nicolaysen (1975) on the hagfish. Indeed, the latter authors report that "one has to search through several fields of vision at a small magnification to find even a single one..." (Korneliussen and Nicolaysen, 1975, p. 276). In light of these observations, it is difficult to explain the rather substantial levels and increases in oxidative enzymes in the white musculature of

of the European eel following the yellow-silver transition (Bostrom and Johansson, 1972). Two explanations for this apparent discrepancy are possible. Firstly, cross contamination between red and white fibers is possible if separation is not done carefully; and secondly, the observed oxidative activity was due to the fourth fiber type (the white oxidative fibers) which lie within the perimycium adjacent to the red and white muscle interface at the lateral line. These fibers are more plentiful than the intermediate fibers indicated above, and thus could account for the portion of the oxidative activity they recorded.

Lewander et al. (1974) have reported an increase in red muscle volume with the yellow to silver transition. Coupled to this increase is an increase in lipid deposition and mitochondrial proliferation within the red fibers that ultimately leads to separation of the myofibrils. The increases in mitochondrial number observed in this study confirm the report by Bostrom and Johansson (1972) of increases in oxidative enzymes for the mature silver eel, A. anguilla. For example, cytochrome oxidase activities increased approximately 77% following the yellow-silver transition. Unfortunately, due to the small amount of red muscle in the immature yellow eels (Lewander et al., 1974), a quantitative estimate of lipid accumulation commensurate with maturation, has yet to be accomplished. There is no doubt, however, that the lipid content of the mature eel is greater than immature eels. McCance (1944) reported a 10-fold

increase in whole body fat content between immature yellow and mature silver A. anguilla eels.

The separation of myofibrils by lipids and mitochondria has been previously reported by Nag (1972) for rainbow trout red muscle. He suggested that the extensive infiltration resulted in separating the myofibrils from each other such that they extended throughout the length of the muscle cell ensheathed in lipid and mitochondria and appeared as independently contracting units. The structural alterations which occur with maturation in the eel red muscle appear as described by Nag and are suggestive of a possible modification of contractile capacity. While it is tempting to speculate on the physiological expression of this modification (i.e., whether contractile potential is actually diminished) electrophysiological experiments must be conducted to adequately analyze the condition to substantiate such a hypothesis.

Although these data do not prove a correlation exists between the observed structural changes and the proposed alteration in metabolic function for eel red muscle during migration, a structure-function pattern is emerging. For example, if eel white muscle is polyaxonally innervated as reported for most other fishes (see Introduction for references), then a graded response is possible. This suggestion was first proposed by Patterson and Goldspink (1972) to explain the contribution of white muscle at swimming speeds other than bursts or sprinting. The presence in the eel,

of a mixed white musculature (i.e., small and large fibers- not to be confused with mixed red and white fibers) is consistent with this hypothesis. Thus, it could be envisioned that the small fibers would contribute to locomotory movements at slow cruising speeds with large fibers firing at higher swimming speeds. In this regard, the comment by Greer-Walker (1970 p. 238) is appropriate "...it would be surprising if 83% of the musculature (of the cod) were reserved for escape reaction and only underwent passive contractions during sustained activity."

In summary, four basic fiber types have been identified from the musculature of the eel: red, intermediate, oxidative- and non-oxidative white. The structural and histochemical characteristics of the red, white and intermediate (with the exception of ATPase activity) fibers are consistent with observations from other fishes. The red musculature contains a single fiber size class, while the white muscle can be divided into two size classes. Concurrent with maturation, there was a marked increase in lipid and mitochondrial number in the red fibers with no apparent morphological change in the white muscle. The result of the fatty infiltration and increased mitochondrial number was to separate the myofibrils from each other such that they appeared to extend the length of the muscle cell as individual units. It is suggested that this marked disruption of red muscle integrity with maturation and the presence of two fiber classes in white muscle are consistent with the hypothesis

that red muscle has an altered functional role during migration and that white muscle may contribute to swimming at speeds other than bursts or sprinting.

IV

LACTIC DEHYDROGENASE

INTRODUCTION

The potential of fish red muscle to support a metabolite recycling function, in addition to a contractile role, has been the focal point of intensive research (see eg., Love, 1970; Bilinski,1974). Recent evidence suggests that this metabolic role involves the regulation of 1) lipid turnover (Robinson and Mead,1973), 2) utilization of amino acids for gluconeogenesis (Wittenberger and Giurgea,1973), 3) glycogen turnover (Wittenberger,1972) and 4) lactate recycling (Wittenberger and Diaciuc, 1965; Wittenberger,1973; Wittenberger et al.,1975). Thus, the proposed metabolite role of fish red muscle appears analogous to certain physiological functions of mammalian liver. In this respect, Braekkan's (1956) red muscle-liver hypothesis is clearly supported.

Wittenberger and co-workers have provided a major contribution towards developing and substantiating a hypothesis for lactic acid utilization by fish red muscle. However, to date they have not assessed the problem from an enzymatic point of view. A key enzyme which is intimately involved in the utilization of lactate by the red muscle is lactate dehydrogenase (LDH) (L-Lactate:NAD oxidoreductase, EC 1.1.1.27). This enzyme catalyzes the reversible oxidation of lactate to pyruvate, thus providing substrate for both the TCA cycle and gluconeogenesis, while maintaining the cytoplasmic redox state.

It is well established in birds and mammals that the five tetrameric isozymes of LDH arise from the random combination of the H and M subunits (i.e., H_4, H_3M_1, H_2M_2, H_1M_3, M_4) (see Bailey and Wilson, 1968; Kaplan and Everse, 1972; Everse and Kaplan, 1973; Markert et al., 1975). Although the H and M subunits are similar in molecular weight (approximately 35,000), their chemical, physical, immunological and catalytic properties, plus tissue distribution, are markedly different. The H_4 isozyme is the predominant form in the heart, (comprising 78% of the total LDH protein) a highly aerobic tissue. Importantly, this isozyme demonstrates high affinity for either substrate, pyruvate or lactate (low Km-values) but is strongly inhibited by both substrates. The M_4 isozyme, on the other hand, is found predominately in white skeletal muscle and is poised for the anaerobic production of energy and the maintenance of reducing potential for anaerobic glycolysis. Thus, it is weakly, if at all, inhibited by pyruvate or lactate and has a much lower affinity (higher Km-values) for these substrates than does the H_4 isozyme (Kaplan and Everse, 1972; Everse and Kaplan, 1973).

While the mammalian LDH exists in five discrete forms, fish LDHs are reported to vary in number from 1 to about 20 (Goldberg, 1965; Markert and Faulbert, 1965; Hochachka, 1966). Even though this wide disparity in isozyme numbers exists, similar physical and physiological characteristics have been reported for similarly charged (as indicated by

electrophoretic mobility) homologs from fish and mammalian tissues. For example, Bailey and Wilson (1968) have demonstrated that trout heart LDH, and purified chicken H_4 LDH, separated into a series of rapidly migrating anodal bands. The trout muscle extract, on the other hand, behaved like the purified chicken M_4 and did not migrate as rapidly as the heart isozymes. Further, the trout heart extracts and purified chicken H_4 LDH displayed similar resistance to thermal denaturation which was markedly different from the thermal characteristics of the muscle enzymes. Immunological tests revealed that the trout heart extract cross-reacted with the purified chicken H_4 isozyme but not with the purified chicken M_4 isozyme. The converse was also found to occur. Thus, even though the number of genes coding for fish LDH differs from mammals (see review by Markert et al., 1975), amd more isozymes are produced in fish, there is evidence for an analogous correlation between the anaerobic/aerobic nature of the fish tissue and the LDH complement. A notable exception to this generalization is the flounder heart which has a single isozyme, equilivant to the mammalian M_4 form (Lush et al., 1969; Markert and Holmes, 1969).

An indirect way to assess the anaerobic/aerobic capacity of a tissue, and thus the lactate oxidase (LO) potential, is to determine the H/M activity ratio (Kaplan and Goodfriend, 1964; Everse et al., 1970). Kaplan and associates (see Everse and Kaplan, 1973) report that tissues which have a primarily anaerobic metabolism will have a low H/M ratio

(due to the lack of pyruvate inhibition) relative to tissues which are aerobic in nature. Thus, values greater than 1 are not uncommon for heart LDHs while values less than 1 are characteristic of white skeletal muscle LDHs. Gesser and Poupa (1973) have reported that the H/M ratio for skeletal muscle of Anguilla anguilla, the European eel is 0.45, and therefore, possibly a highly anaerobic tissue.

A more direct assessment of the lactate oxidative potential of a tissue is to examine the kinetics of lactate oxidase (LO) in contrast to pyruvate reductase (PR) and determine the PR/LO ratio. Thus, a low $K_{m(lac)}$ coupled with a PR/LO ratio less than or approximating unity, would suggest an oxidative potential. Likewise, a high $K_{m(lac)}$ or low affinity for lactate, coupled with a PR/LO ratio greater than unity, would suggest that LDH functions primarily in pyruvate reduction. By comparing LDH kinetics and PR/LO ratios from tissues of known oxidative and gluconeogenic potential, a relative measure of the lactate conversion capacity can be estimated.

This communication reports the results of this approach in assessing the lactate oxidative potential of LDH from eel, A. rostrata, red and white muscle, heart, kidney, liver and gill. The results support the potential of a metabolic recycling role for eel red muscle and suggest that white muscle does not have this capacity.

RESULTS

In addition to the large variation in LDH isozyme number between the eel tissues, two important observations can be made from the polyacrylamide disc gel electrophoretograms presented in Fig. 1. First, the three muscle enzymes (red, white and heart) all possess an isozyme in common. Second, the red muscle, heart, kidney, gill and liver all share at least one isozyme with equal electrophoretic mobility. Thus, kinetic similarities between the three muscle LDHs and the red muscle-heart-organ LDHs may occur. To further characterize the red and white muscle and liver enzymes, homogenates from freshly sacrificed eels were prepared, concentrated and subjected to isoelectrofocusing; these experiments are presented in Figs. 2, 3 and 4.

In general, the number of activity peaks detected by isoelectrofocusing agrees well with the results obtained by polyacrylamide electrophoresis. The red muscle (Fig. 2) demonstrated three distinct peaks with apparent pI values of 5.2, 5.75 and 6.91, with the possibility of one additional peak buried in the 5.75 peak. The white muscle (Fig. 3), consistent with its disc gel electrophoretogram, had one major peak; however, as with the red muscle, the trailing edge of the pI 6.25 peak demonstrated a slight shoulder which might represent an additional form. The liver enzyme demonstrated three distinct, symmetrical peaks (Fig. 4) unlike the large smear seen with electrophoresis (Fig. 1).

The effects of changes in DL-lactate concentration at

Fig. 1. The marked variation in LDH isozyme number between the various tissues and organs of the eel, A. rostrata. The major activity band is shown as the dark solid line, minor bands are dotted. W, white muscle; H, heart; R, red muscle; K, kidney; G, gill; and L, liver.

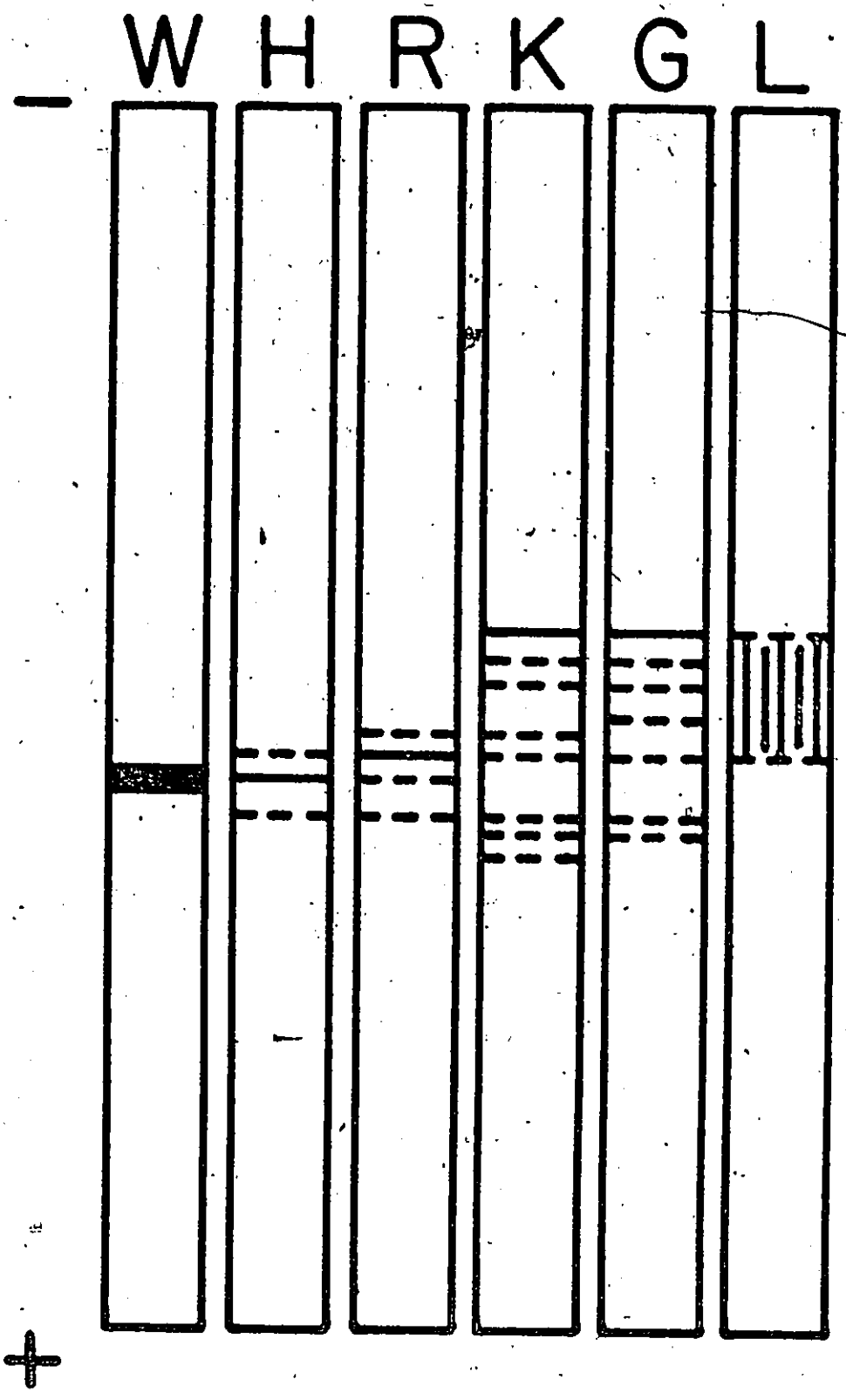

W
H
R
K
G
L
−
+

Fig. 2. The separation of eel red muscle LDH into at least three distinct bands by isoelectrofocusing. See Methods and Materials for isoelectrofocusing procedure. The standard reaction assay mixture contained, in a final volume of 0.5 ml, 0.35 mM NADH, 2 mM Pyr and 100 mM Tris-HCl buffer, pH 7.4, at 18°C.

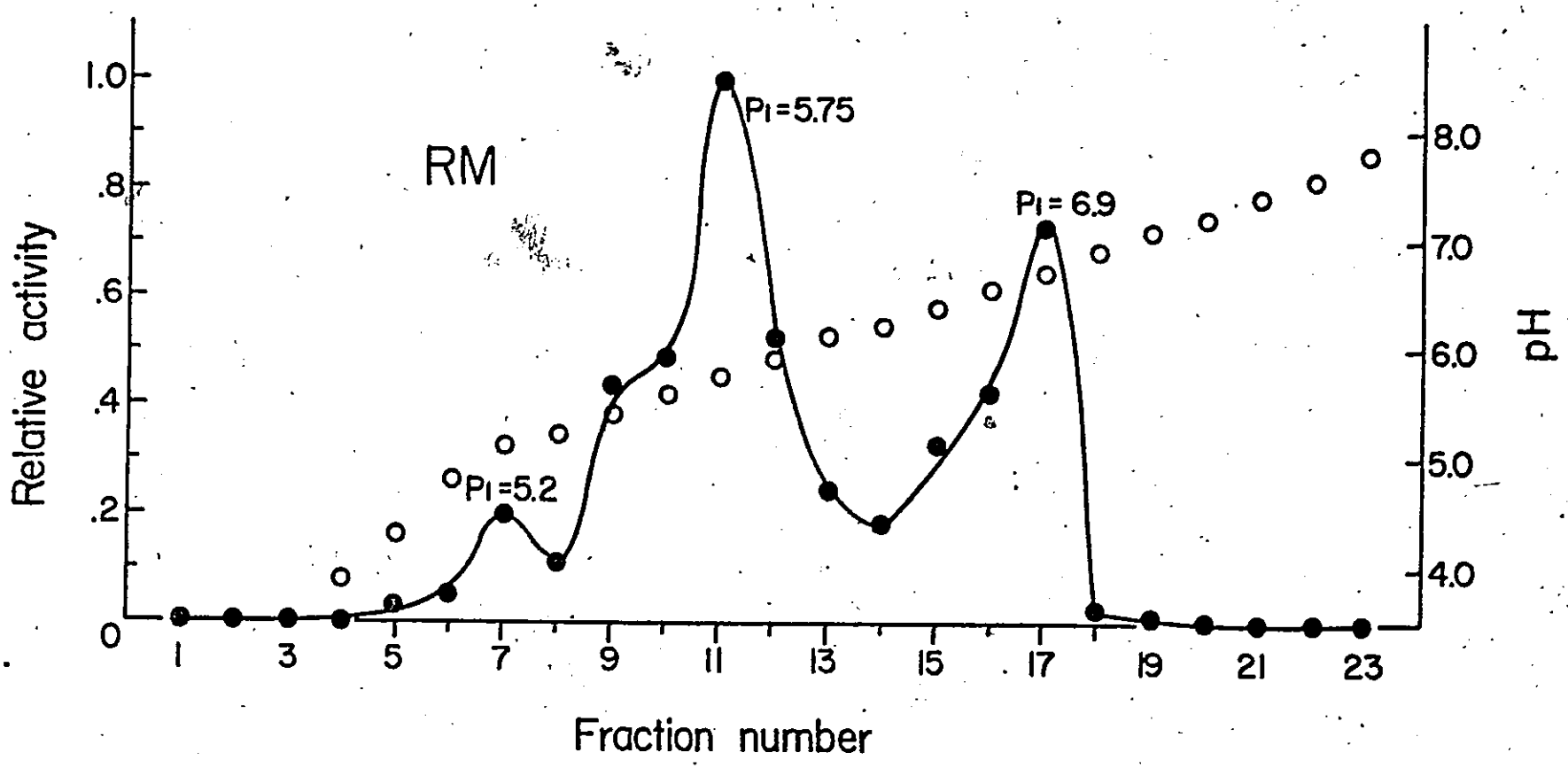

RM
Pi=5.75
Pi=6.9
Pi=5.2
Relative activity
pH
Fraction number
1.0
.8
.6
.4
.2
0
8.0
7.0
6.0
5.0
4.0
1
3
5
7
9
11
13
15
17
19
21
23

Fig. 3. The separation, by isoelectrofocusing, of eel white muscle LDH. Refer to Methods and Materials for isoelectrofocusing procedure. The standard reaction assay contained, in a final volume of 0.5 ml, 0.35 mM NADH, 2 mM Pyr and 100 mM Tris-HCl buffer, pH 7.4, at 18°C.

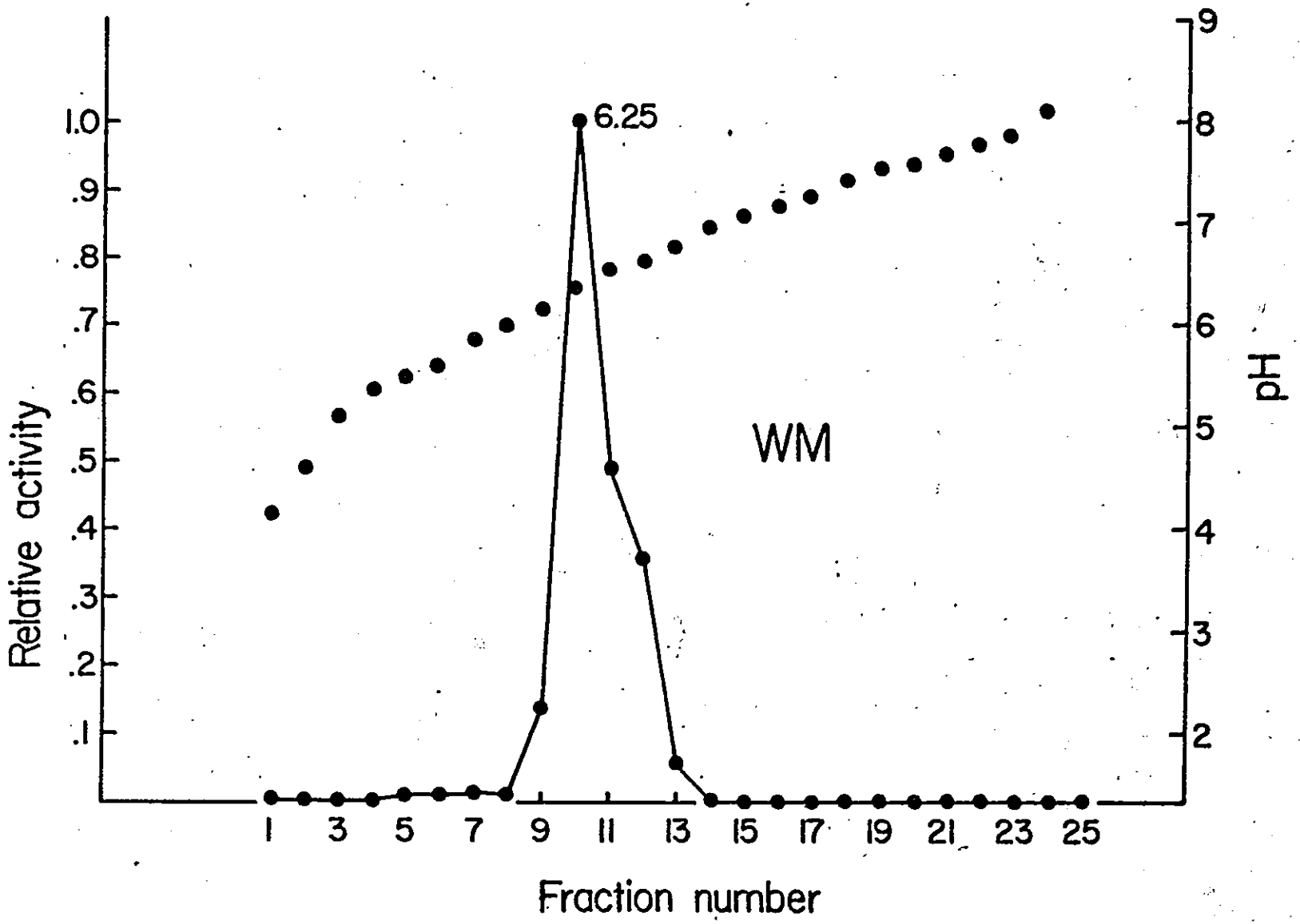
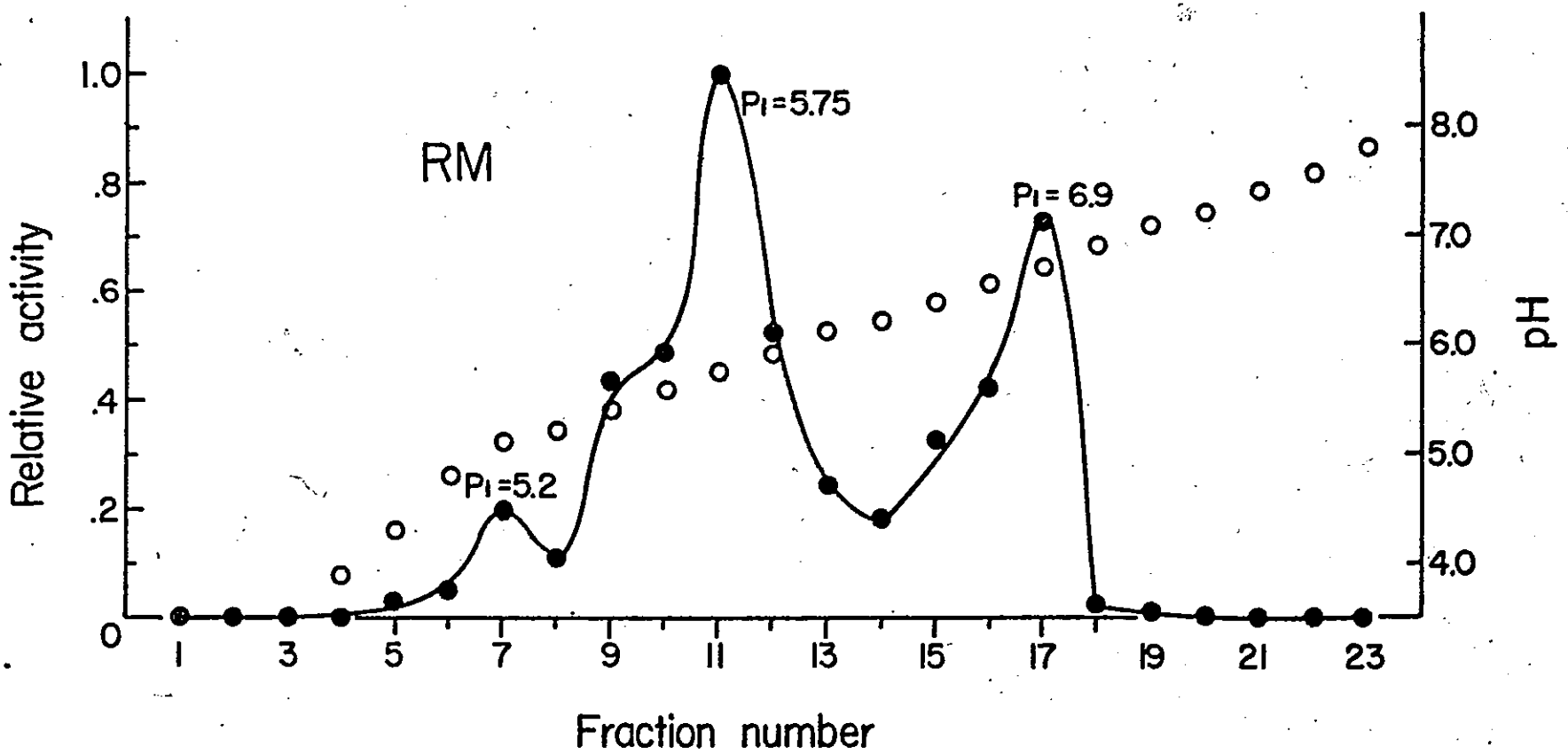

6.25
WM
Relative activity
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
pH
9
8
7
6
5
4
3
2
1 3 5 7 9 11 13 15 17 19 21 23 25
Fraction number

Fig. 4. The separation by isoelectrofocusing of eel liver LDH activity. Refer to Methods and Materials for the isoelectrofocusing procedure. The standard reaction mixture contained, in a final volume of 0.5 ml, 0.35 mM NADH, 2.0 mM Pyr and 100 mM Tris-HCl buffer, pH 7.4, at 18°C.


RM
Pi=5.75
Pi=6.9
Pi=5.2
Relative activity
pH
Fraction number
1.0
.8
.6
.4
.2
0
8.0
7.0
6.0
5.0
4.0
1
3
5
7
9
11
13
15
17
19
21
23

various pHs on LDH activities from red and white muscle is presented in Fig. 5. These data establish an alkaline activity optimum, major activity differences, and marked differences in DL-lactate affinity for the muscle enzymes.

Table 1 presents apparent $K_{m(lac)}$-values for the two muscle enzymes estimated from Fig. 5, with those of the other tissue LDHs examined. At the three pHs, the apparent $K_{m(lac)}$ for the red muscle enzyme is 2 to 10-fold lower than the white muscle enzyme, suggesting a higher lactate binding potential. These results for red muscle LDH are representative of the other tissue LDHs, with the exception of gill LDH at pH 6.5. Further, as pH increases, the apparent $K_{m(lac)}$ for eel white muscle LDH increases, in contrast to the decrease noted for the LDH of the other tissues. This result, coupled with an acidic pH optimum for pyruvate reduction, suggests that lactate is poorly oxidized by white muscle LDH.

The effect of NAD^+ concentrations on the oxidation of lactate (10 and 50 mM DL-lactate give identical results) by the red and white muscle LDHs is presented in Fig. 6. Both the maximum velocity and apparent affinity for NAD^+ vary greatly between the two muscle enzymes. Thus, at low concentrations of NAD^+ (eg., 0.2 mM), the velocity of the red muscle enzyme will be approximately two-fold greater than for the white muscle. Table 2 indicated that $K_{m(NAD^+)}$-values for red muscle LDH and the other tissues examined, compared to white muscle, differ by approximately 3-fold. Thus, at

Fig. 5. The effects of increasing DL-lactate concentrations, as a function of pH, on the activity of red and white muscle LDH. The standard reaction mixture contained, in a final volume of 0.5 ml, 1.0 mM NAD^+, 100 mM Tris-HCl buffer (pH indicated) and various concentrations of DL-lactate at 15°C.

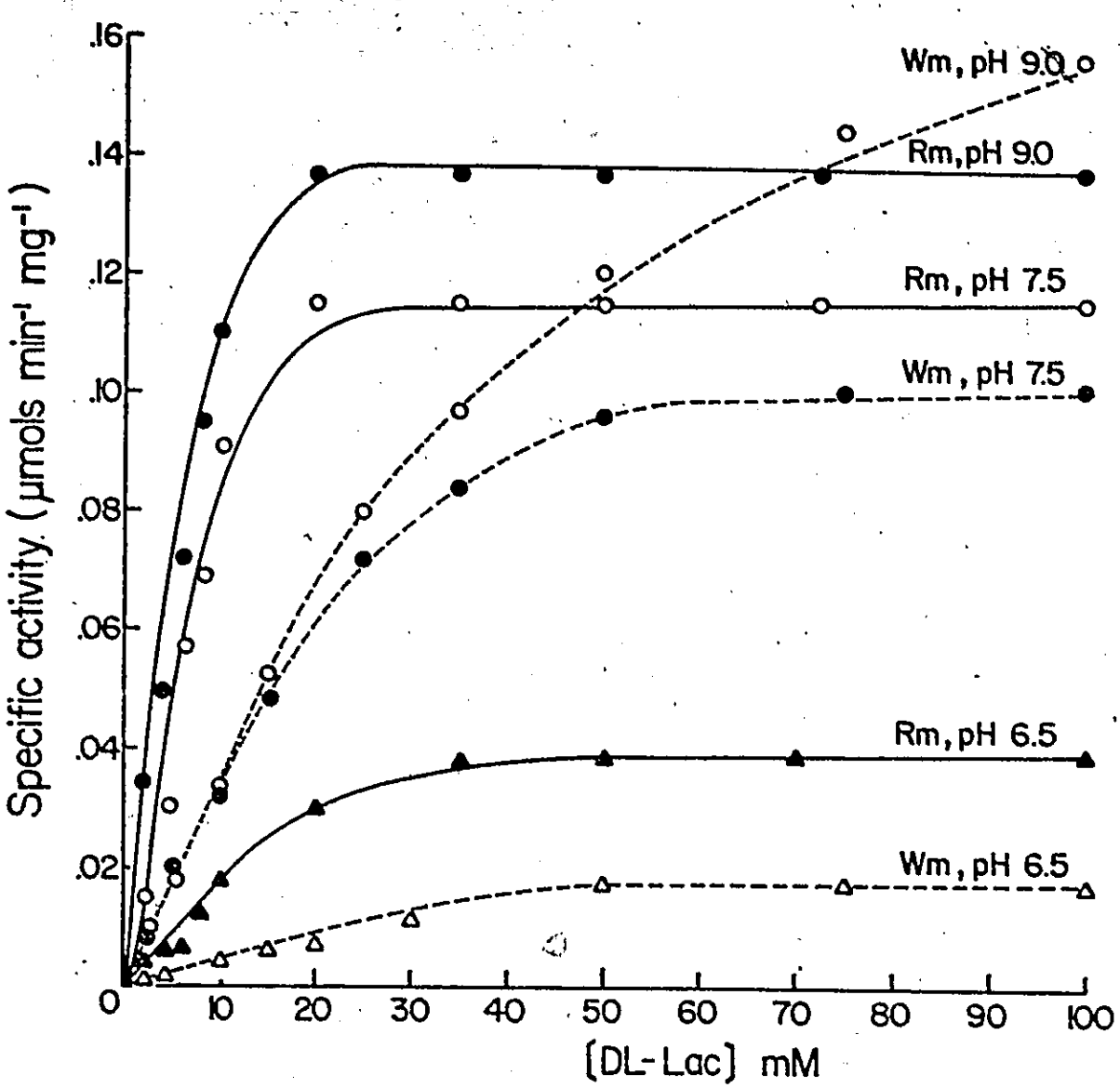

Specific activity. (μmols min⁻¹ mg⁻¹)
.16
.14
.12
.10
.08
.06
.04
.02
0
10
20
30
40
50
60
70
80
90
100
[DL-Lac] mM
Wm, pH 9.0
Rm, pH 9.0
Rm, pH 7.5
Wm, pH 7.5
Rm, pH 6.5
Wm, pH 6.5

Table 1. Values of the apparent $K_{m(lac)}$ for eel tissue LDHs as a function of pH. The enzymes were assayed at 15°C in a reaction mixture which contained, in a final volume of 0.5 ml, 1.0 mM NAD^+, 100 mM Tris-HCl buffer (pHs indicated) and various concentrations of DL-Lactate. $K_{m(lac)}$ was calculated from Lineweaver-Burke plots, of data similar to that in Fig. 5. RM, red muscle; WM, white muscle; H, heart; K, kidney; L, liver; G, gill.

	$K_{m(DL\text{-}lactate)}$ mM		
	pH 6.5	pH 7.5	pH 9.0
RM	11.0	6.5	5.0
WM	23.0	23.1	50.0
H	10.0	10.0	5.0
K	7.5	10.0	3.5
L	8.1	13.3	10.1
G	20.2	6.0	4.2

Fig. 6. The effect of NAD^+ concentrations at either 10 or 50 mM DL-lactate, on the activity of eel red and white muscle LDH. The standard reaction mixture contained at (15°C), in a final volume of 0.5 ml, either 10 or 50 mM DL-lactate, 100 mM Tris-HCl buffer, pH 7.4, and various concentrations of NAD^+.

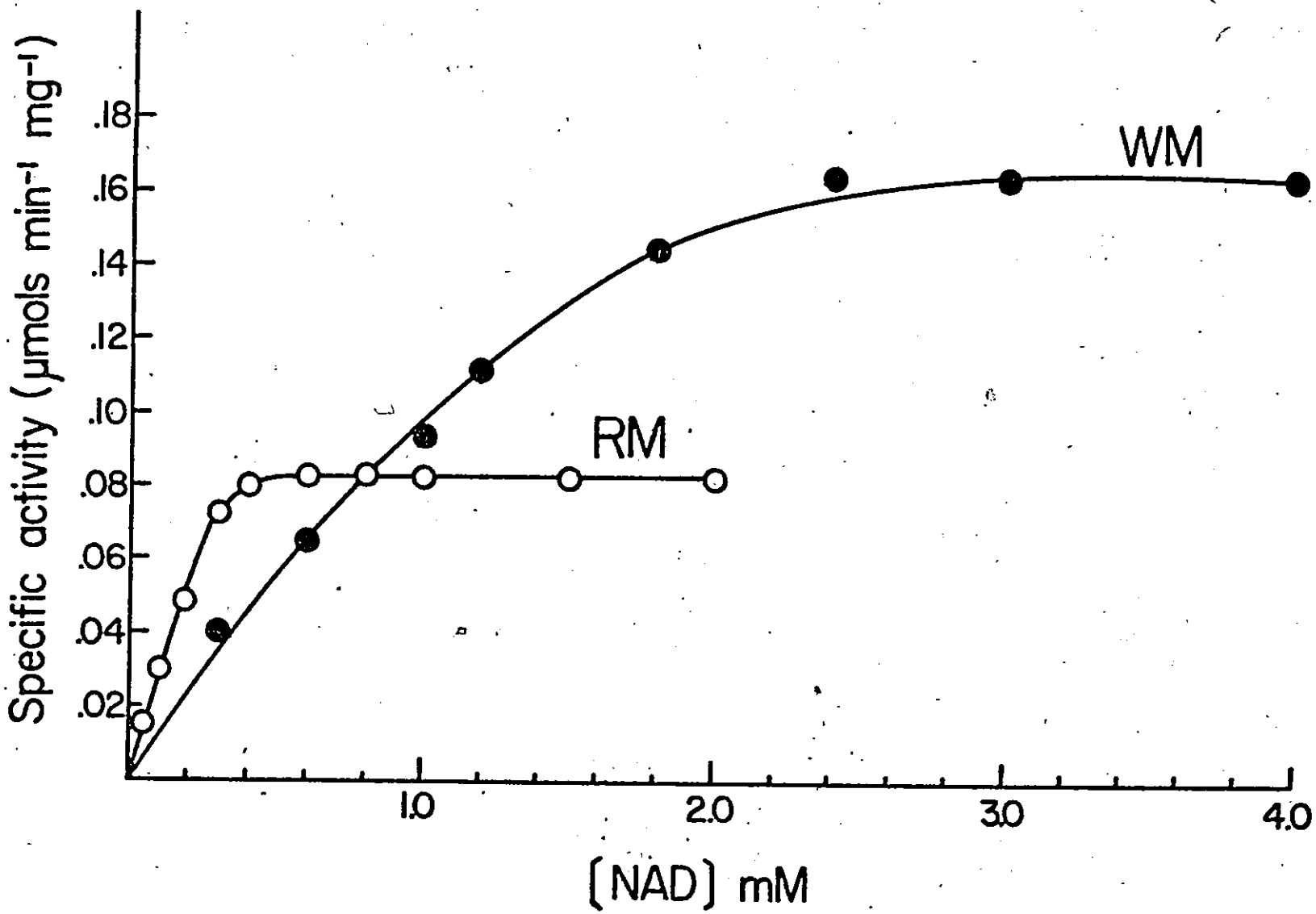

Specific activity (μmols min⁻¹ mg⁻¹)
.18
.16
.14
.12
.10
.08
.06
.04
.02
WM
RM
1.0
2.0
3.0
4.0
[NAD] mM

Table 2. Values of the apparent $K_{m(NAD)}$ for eel tissue LDSs. The $K_{m(NAD)}$ values were determined from Lineweaver-Burke plots. The enzymes were assayed in a reaction mixture which contained, in a final volume of 0.5 ml, various concentration of NAD^+, either 10 or 50 mM DL-lactate, and 100 mM Tris-HCl buffer, pH 7.4, at 15°C.

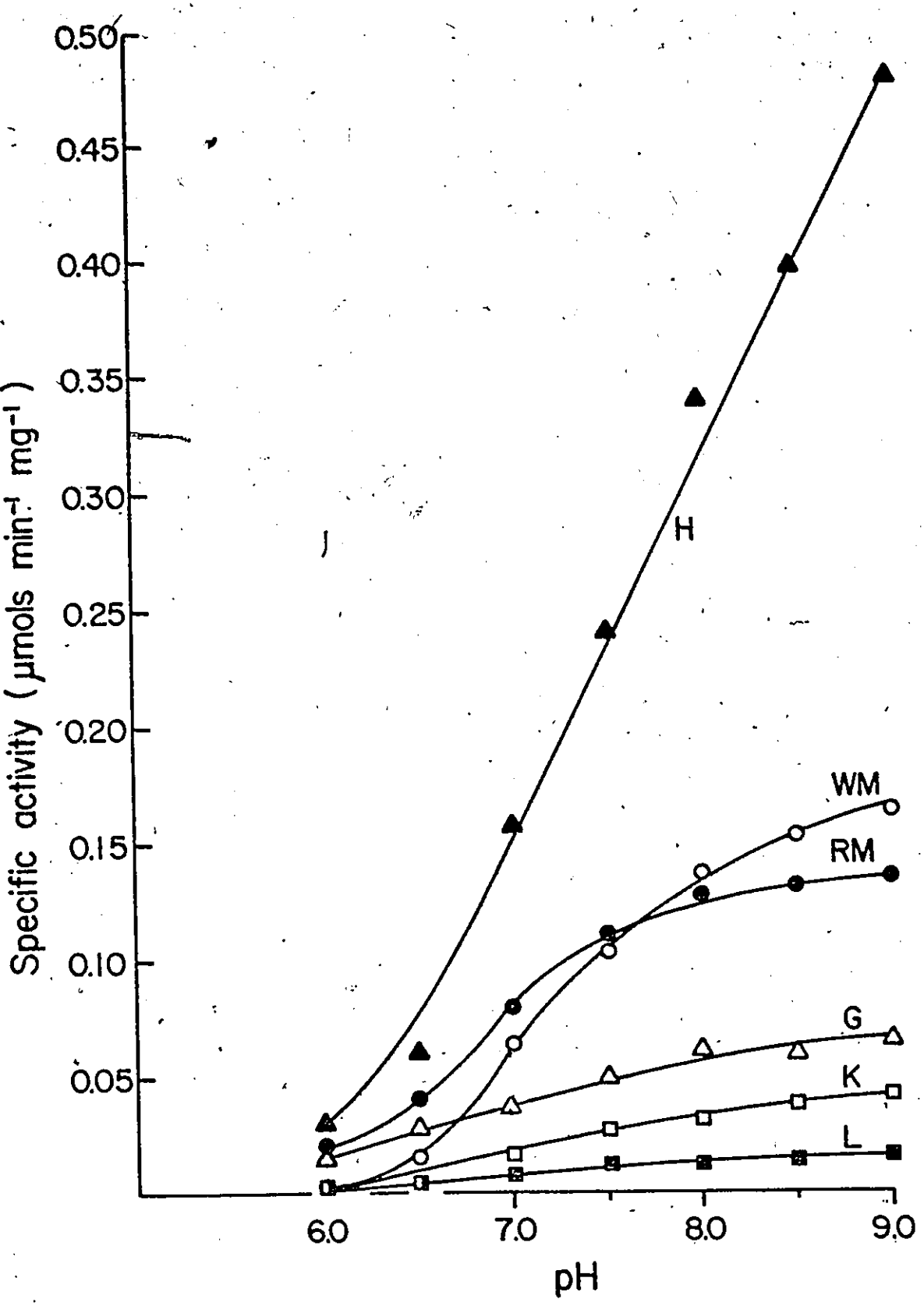
Tissue	$K_{m(NAD)}$ mM
K	0.20
RM	0.25
Lv	0.25
G	0.25
H	0.35
WM	0.90

values of DL-lactate between 10 and 50 mM, and low (presumably physiological) levels of NAD^+, lactate oxidation is favored in red muscle compared to white muscle. This interpretation is based on the assumption that the pH of both the red and white muscles will be approximately 7.5, and that lactate equilibrates between the muscle types.

The effect of pH on the oxidation of lactate by LDH from red, white and heart muscles, kidney, gill and liver is presented in Fig. 7. All tissue LDHs demonstrate alkaline pH optima and can be divided into three groups based on the rate of lactate oxidation at alkaline pH: heart LDH, red and white muscle LDH, and gill, kidney and liver LDH. The heart enzyme, in addition to being the most sensitive to pH (20-fold increase between pH 6.0 and 9.0), also demonstrated the highest activity at all pHs examined. The two skeletal muscle enzymes responded similarly except that the white muscle enzyme was more severely inhibited below pH 7.5 than was the red muscle enzyme. Indeed, at pH 6.0, the activity of the white muscle was negligible compared to the red muscle enzyme. At lower DL-lactate concentrations (see Fig. 5), this difference is further exaggerated. With strenuous exercise in rainbow trout, Black et al. (1959) reported lactate levels in the range of 10 to 14 mM; under these conditions, lactate oxidation in RM exceeds that in WM by at least 3-fold, depending upon pH (Fig. 5).

Further, Fig. 7 suggests the third activity group, consisting of gill, kidney and liver LDHs, are relatively in-

Fig. 7. The effects of pH on lactate oxidation by eel red (RM), white (WM) and heart (H) muscle and kidney (K), gill (G) and liver (L) LDHs. The assays were conducted at 15°C in a reaction mixture which contained, in a final volume of 0.5 ml, 1.0 mM NAD^+, 50 mM DL-lactate and 100 mM Tris-HCl buffer at the pHs indicated.


0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
Specific activity (μmols min⁻¹ mg⁻¹)
H
WM
RM
G
K
L
6.0
7.0
8.0
9.0
pH

sensitive to pH alterations, at least with respect to lactate oxidation. Note the relative oxidative capacity under these conditions of red muscle LDH as compared to the liver and kidney, which in mammals are known to have high gluconeogenic capacities. For example, at pH 7.5, the red muscle lactate oxidation by LDH is 5-fold greater than kidney and 10-fold greater than liver.

Activities of eel tissue LDHs in the lactate to pyruvate direction depend critically upon pH conditions, but the poise of the enzyme will depend upon the ratio of pyruvate reduction to lactate oxidation (PR/LO). Table 3, indicated that all tissue LDHs, with the exception of the white muscle enzyme, catalyze an equilibrium reaction (PR/LO ratios of approximately 1.0) where the direction of catalysis will depend, at least partially, on the respective levels of pyruvate and lactate. Consistent with the pH optima for PR and LO, the PR/LO ratio drops in all tissues as pH increases.

Values of PR/LO for white muscle LDH, especially at low pH, suggest this enzyme is poised for lactate accumulation, which is consistent not only with the anaerobic nature of vertebrate white muscle, but also the pH optimum of PEP conversion to pyruvate by PK (Chapter 4). Thus, during glycolytic demand in the white muscle, both the enzymes PK and LDH are poised for the production of lactate. For the other tissue and organ LDHs examined, the conversion of pyruvate to lactate, or vice versa, appears independent of

Table 3. The ratio of pyruvate reductase and lactate oxidase of eel tissue LDHs measured at both K_m and saturating (V_{max}) levels of substrate. The assay medium for the pyruvate reductase reaction contained, in a final volume of 0.5 ml, either 0.5, 0.2 or 2.0 mM Pyr (refer to the Table) and 0.35 mM NADH with 100 mM Tris-HCl at the pHs indicated. The assay medium for the lactate oxidase reaction contained, in a final volume of 0.5 ml, either 2, 50, or 30 mM DL-Lactate 1.0 mM NAD^+, (for WM-LDH) or 0.45 mM NAD^+ for the other LDHs, and 100 mM Tris-HCl at the pHs indicated.

	PR/LO Ratios			
Tissue	pH 6.5	pH 7.5	pH 9.0	substrate Pyr/lac(mM)
W	2.24	1.98	0.20	.5/5
W	95.6	10.70	1.40	2/50
R	0.92	1.30	0.02	.2/5
R	3.83	2.1	0.53	2/30
H	5.55	1.9	0.06	.2/5
H	10.3	2.64	1.15	2/30
K	4.9	3.35	0.32	.2/5
K	2.57	2.77	1.16	2/30
G	2.58	2.64	0.19	.2/5
G	2.96	0.86	0.38	2/30

pH but dependent upon the levels of the two substrates.

DISCUSSION

The similarities in the electrophoretic mobilities of the eel tissue LDHs (Fig. 1) predict that homologies in kinetic behavior may exist between the red, white and heart muscle enzymes, and also between the red muscle, heart, liver, kidney, and gill enzymes. The data presented support this prediction. Interestingly, the two groups of enzymes possess significantly different characteristics; that is, the three muscle enzymes have higher catalytic potentials and greater sensitivity to H^+ ions than the other enzymes (Fig. 7). On the other hand, the red muscle, heart, liver, kidney and gill LDHs share similar affinities for the co-factor NAD^+ (Table 2), the substrate lactate (Table 1, Fig. 5) and PR/LO ratios (Table 3); the white muscle enzyme is entirely different. Indeed, these characteristics may be physiologically more important than a high catalytic potential as seen for WM-LDH (see below).

The low apparent $K_{m(lac)}$ observed for the red muscle-organ group is similar to that reported for the mammalian H_4 isozyme (Everse and Kaplan, 1973); likewise, the high apparent $K_{m(lac)}$ or low affinity observed for the white muscle enzyme is similar to that observed for the mammalian M_4 isozyme (Everse and Kaplan, 1973). It is important to note that the response of the eel red muscle-organ LDHs is not entirely homologous with the mammalian H_4 isozyme, or for that matter, other fish H-type LDHs (see Hochachka, 1965). These differences relate principally to substrate inhibition.

Thus, while no inhibition by pyruvate (up to 6 mM) was observed for the eel muscle or organ LDHs, Kaplan and Everse (1973) report that the mammalian H_4 isozyme is inhibited at 2 mM pyruvate and Hochachka (1965) demonstrated that goldfish muscle LDH was inhibited by 3 mM pyruvate. Similarly, no lactate inhibition was noted up to 100 mM DL-lactate. Thus, the similarities between the eel and mammalian LDHs arise from their relative affinities for lactate and pyruvate.

As a result of the low affinity of the white muscle enzyme for NAD^+ (Table 2) and lactate (Table 1), and the marked increase in the PR/LO ratio with decreasing pH (Table 3), eel white muscle LDH appears to be poised primarily for the reduction of pyruvate and subsequent production of lactic acid and NAD^+. These results correlate well with those of Driedzic and Hochachka (1975), which demonstrated that carp white muscle does not have the capacity to support amino acid fermentation (an oxidative process) and depends entirely on anaerobic glycogenolysis for the production of energy for muscle contraction. Similarly, Pritchard et al. (1971) found that failure to swim by jack mackerel at any speed was associated with the almost complete depletion of glycogen from the white muscle. Thus, the physiological role of fish white muscle would appear to require that LDH function as a pyruvate reductase and that the lactate produced from glycolysis be oxidized by another tissue or organ. This hypothesis is in contrast to that forwarded by Hermansen (1976) for human muscle. Human skeletal muscle, according

to Hermansen, is capable of lactate oxidation and only 10% of the lactate produced in the muscle is actually converted to glucose/glycogen in the liver by the Cori cycle (the remainder is oxidized by the muscle). Significantly, human leg muscle is a mixed red/white muscle, and localizing lactate oxidation to any particular muscle type would be impossible in these experiments; whether red or white muscle does the oxidation is unknown. In the eel, the deep white muscle appears homogeneous in regard to its lack of oxidative potential, or rather its anaerobic capacity (see Chapter 3). Thus, the muscle fiber composition is markedly different between the eel and human skeletal muscle, but it is obvious that in the American eel, the tissues which maintain a capacity to oxidize lactate, over a wide range of H^+ ion concentrations, are red muscle, heart, liver, kidney and gill, the latter three showing low capacities.

Although the evidence demonstrates that lactate oxidation by the red muscle-organ group can occur, the data do not give any clues as to the fate of pyruvate which is produced from lactate oxidation. At least two possibilities exist: oxidation to CO_2, water and energy via the TCA cycle-oxidative phosphorylation systems; and/or, recycling to glucose/glycogen. The first fate is possible in any oxidative tissue, while the second can occur only in tissues with gluconeogenic capacities. In mammals, a prerequisite for gluconeogenesis is a regulatory pyruvate kinase (PK) to eliminate carbon and energy cycling (see Scrutton and Utter, 1968; Sols, 1968); thus both liver and kidney are gluconeo-

genic, converting substrates such as amino acids and lactate to glucose/glycogen. Eel liver does not have a regulatory PK, which is found in both white and red muscle (see Chapter 4 and 5). Since white muscle LDH is poised principally for lactate production not oxidation, gluconeogenesis from lactate in this tissue is not likely. Thus, it is tempting to speculate upon a lactate recycling role for red muscle.

Wittenberger and associates have championed this hypothesis, and for the European carp, present convincing evidence. For example, the incubation of excised pieces of red and white muscle resulted in an increase in lactic acid in red muscle which was greater than could be explained by the catabolism of stored glycogen. Concurrently, the free glucose concentration decreased significantly in the white muscle. Thus they interpretated these results as indicating transfer of lactic acid from the white to the red muscle (Wittenberger et al., 1975); but more importantly, they have demonstrated the reciprocal transfer of glucose from the red muscle to the white. Again, incubating isolated red and white muscle pieces, Wittenberger (1968) found that the white muscle pieces lost more glycogen when separated from the red muscle, than when they were naturally joined. Thus, Wittenberger proposed that the decrease in glycogen in isolated white muscle pieces, as opposed to intact red and white muscle pieces, is due to the disrupted glucose transfer from the red to the white muscle which requires the white muscle to catabolize more of its stored glycogen.

Pritchard et al. (1971), using a different experimental design, came to the same basic conclusion. After excercising jack mackerel at various swimming speeds, they examined the levels of specific metabolites in red and white muscle, and liver; their results suggested that, at certain swimming speeds, the glycogen present in both the red muscle and liver provided the energy for contraction of the white muscle.

The results presented above suggest that, consistent with our understanding of the anaerobic nature of fish white muscle, the eel white muscle probably does not have the capacity to oxidize lactate. On the other hand, the red muscle, heart, kidney, liver and gill have all demonstrated a potential to convert lactate to pyruvate. These observations can be intreperted as supporting the red muscle-organ, or metabolite recycling, hypothesis of Braekkan (1956) as later expanded by Wittenberger and associates (eg., Pritchard et al.,1971; Robinson and Mead, 1973; Wittenberger et al., 1975).

V

PYRUVATE KINASE-CHARACTERISTICS

INTRODUCTION

Since the early work of Tanaka et al. (1967) and Susor and Rutter (1968), there has been a wealth of information reported which suggests that the glycolytic enzyme pyruvate kinase (EC 2.7.1.40; ATP:pyruvate phosphotransferase) (PK), exists in three distinct isozymic forms (see reviews by Schubert and Schoner, 1971; Kayne, 1973). Tanaka and associates have designated these forms as M_1, M_2 and L (Imamura et al., 1972; Imamura and Tanaka, 1972) and their nomenclature will be adopted for this discussion.

Type L-PK is the major component found in rat liver and a minor component in kidney (Tanaka et al., 1967; Van Berkel et al., 1973). This isozyme displays cooperative kinetics in the presence of substrate, PEP, and is modulated by the positive effector FDP and the negative effectors, ATP and L-alanine (see above reviews for extensive documentation). Type L-PK conforms to the allosteric model of Monod et al. (1965) and exists in two interconvertible forms, each with markedly different sensitivities to the positive and negative effectors indicated above (Bailey et al., 1968; Imamura et al., 1972). The physiological role of this allosteric PK appears to be closely correlated to conditions which affect glycolytic and gluconeogenic flux. For example, the activity of the enzyme is sensitive to specific hormones (insulin, for example) and different nutritional states of the organism (Tanaka et al., 1967). Thus, activity levels

increase in concert with high or increased carbohydrate metabolism and decrease during low carbohydrate utilization.

The M_1-PK is the only form found in skeletal muscle, and is the major component of mammalian brain and heart PK (Tanaka et al., 1967). This skeletal muscle enzyme is non-regulatory (Llorente et al., 1970), although Carminatti et al. (1971) have reported allosteric inhibition by phenylalanine. The isozyme displays classic Michaelis-Menton kinetics with respect to PEP and is unresponsive to FDP, ATP and L-alanine (see review by Boyer, 1959).

Type M_2-PK is widely distributed in most adult and fetal tissues and is the predominant form in kidney and the minor component in liver (Susor and Rutter, 1968; Carbonell et al., 1973). The isozyme exhibits cooperative kinetics with PEP, and allosteric interactions with the heterotrophic effector FDP, and the homotrophic effectors, ATP and L-alanine (see eg., Jimenez et al., 1971). It is important to note, however, that although both the L-and M_2-PKs share similar qualitative responses to these effectors, there are significant quantitative differences in their respective responses (Imamura et al., 1972). The M_2 isozyme is also reported to exist in two interconvertible forms, each with different sensitivities to the allosteric modulators (Pogson, 1968a,b; Van Berkel et al, 1973). Although some cross reactivity between the M_1 and M_2-PKs has been demonstrated (Tanaka et al., 1967), recent evidence of Imamura et al. (1972) suggests these observations are in error and immunological cross-reactivity

does not occur between the M_1 and M_2 forms. No cross-reactivity between the L- and M_1-or M_2-PKs has ever been observed (Tanaka et al., 1967; Imamura et al., 1972).

Besides being sensitive to physiological alterations, variations in the preparation and treatment of PK have led to reports of different isozymic, or transitional forms of the enzyme, each with altered regulatory properties (see eg., Pogson, 1968a,b,). Factors contributing to the reported differences between these forms include: 1) the presence or absence of EDTA in either the homogenizing or assay buffers, or both; 2) whether Tris or Phosphate buffers were used; 3) the degree of enzyme purity; 4) the method of enzyme storage; 5) the addition or deletion of allosteric modulators during a pre-incubation period; and, 6) exposure to heat or cold before assaying (see Schubert and Schoner, 1971).

In several cases, the explanation for the observed alteration in regulatory and kinetic properties of PK with specific treatments has been based upon the allosteric model of enzyme regulation by Monod et al. (1965). For example, Bailey et al. (1968) demonstrated that the reversible transition from sigmoidal to hyperbolic kinetics for L-PK was a function of FDP. Their data suggested that the equilibrium between the R and T conformational states of PK was displaced towards the R form in the presence of FDP and the T form in its absence. The results of Hess and Kutzbach (1971) support the interpretation of Bailey et al. (1968) that the

markedly different kinetic properties of L-PK, obtained by altering incubation media, resulted from a modification in the conformational state of a single protein, and not the presence of multiple proteins. In this definitive experiment, Hess and Kutzbach demonstrated that the pre-incubation of L-PK with FDP resulted in altering its isoelectric point from 6.1 to 5.3, and that by manipulating the FDP concentration, they could change the relative amounts of the 6.1 and 5.3 forms. Although strong evidence has been presented which demonstrates that some of the anomalous kinetic responses exhibited by PK are due to alterations in the R-T equilibrium, not all the varied results can be explained in this manner (Schubert and Schoner, 1971).

This communication examines the effects of buffers, temperature, pH and various modulators on the kinetic response of PK isolated from white muscle (WM), red muscle (RM) and liver (Lv) of the American eel, Anguilla rostrata. The results suggest that 1) the three tissue PKs exhibit markedly different H^+ ion sensitivities, V_{max} activities, temperature- H^+ ion interactions with enzyme-substrate affinity, and interactions with potential modulatory ligands. Interestingly, only the eel muscle PKs were responsive to the modulators, ATP, L-alanine, FDP and H^+ ions; thus, in terms of ligand sensitivity, the WM- and RM-PKs response is more like that observed for mammalian L-PK, while eel Lv-PK behaves more like mammalian M_1-PK.

RESULTS

1. Effects of Buffers on Eel PK

The kinetics of eel PK depends both upon the homogenizing and assay buffers (Figs. 1 and 2). Red muscle (RM) homogenized in buffer plus EDTA exhibits a 2.5-fold increase in maximal catalytic activity (V_{max}) of PK and a 5-fold decrease in the $K_{m(PEP)}$, when compared to eliminating EDTA from the medium (Fig. 1). The $K_{m(PEP)}$, but not V_{max}, effect can be reversed if the EDTA-free preparation is incubated or assayed in the presence of EDTA. A similar trend was observed for WM-PK.

Assaying WM-PK in a Na-phosphate buffer, pH 7.0, results in sigmoidal kinetics (n_H = 2.0) as compared to hyperbolic kinetics (n_H = 1.0) in the presence of K-phosphate buffer, pH 7.0, or Tris-maleate (n_H = 1.2) buffer, pH 7.0 (Fig. 2). A similar result was noted for RM-PK. This apparent homotrophic cooperativity with Na-phosphate is correlated with Na^+ ions since 1) K-phosphate buffer (pH 7.0) exhibits hyperbolic kinetics and 2) Na-phosphate buffer added to Tris-maleate gives sigmoidal kinetics (n_H =1.9). Phosphate, per se, results in an increased V_{max} and a decreased $K_{m(PEP)}$ which cannot be solely attributed to increased levels of K^+ (the obligatory monovalent cation) since the enzyme is saturated at levels of 30-40 mM K^+ which were used in these assays (Fig. 2).

Fig. 1. The effects of EDTA in the homogenizing and assay buffers on RM-PK kinetics. Symbols are: empty circles, homogenized with 2 mM EDTA present in buffer; filled circles, homogenized without EDTA in buffer; filled triangles, homogenized without EDTA in the buffer but with 2 mM EDTA added to the assay cuvette; filled boxes, homogenized without EDTA in the buffer but incubated for 1 hr or 12 hr with EDTA before assay. Refer to Materials and Methods for standard assay cocktail.

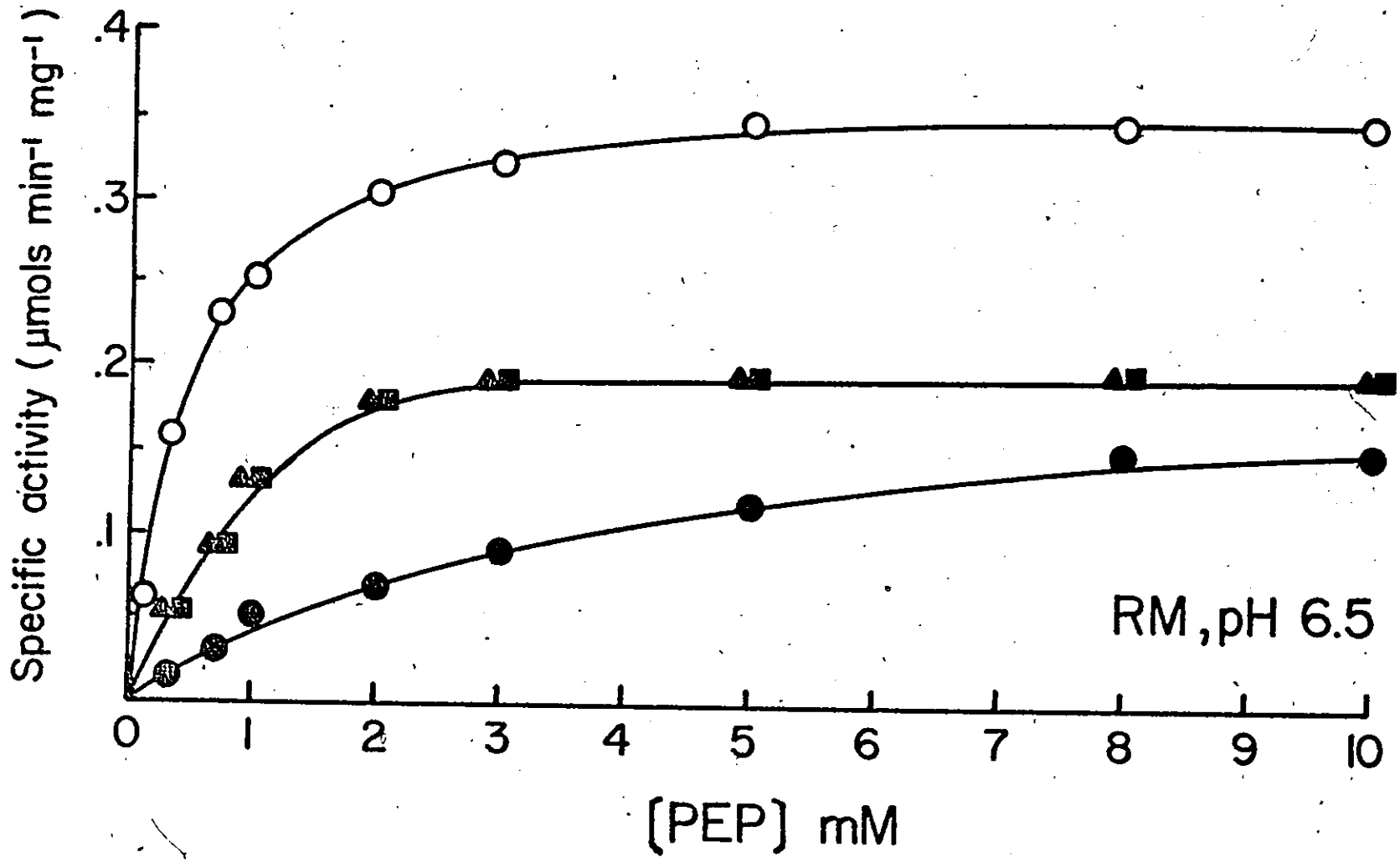
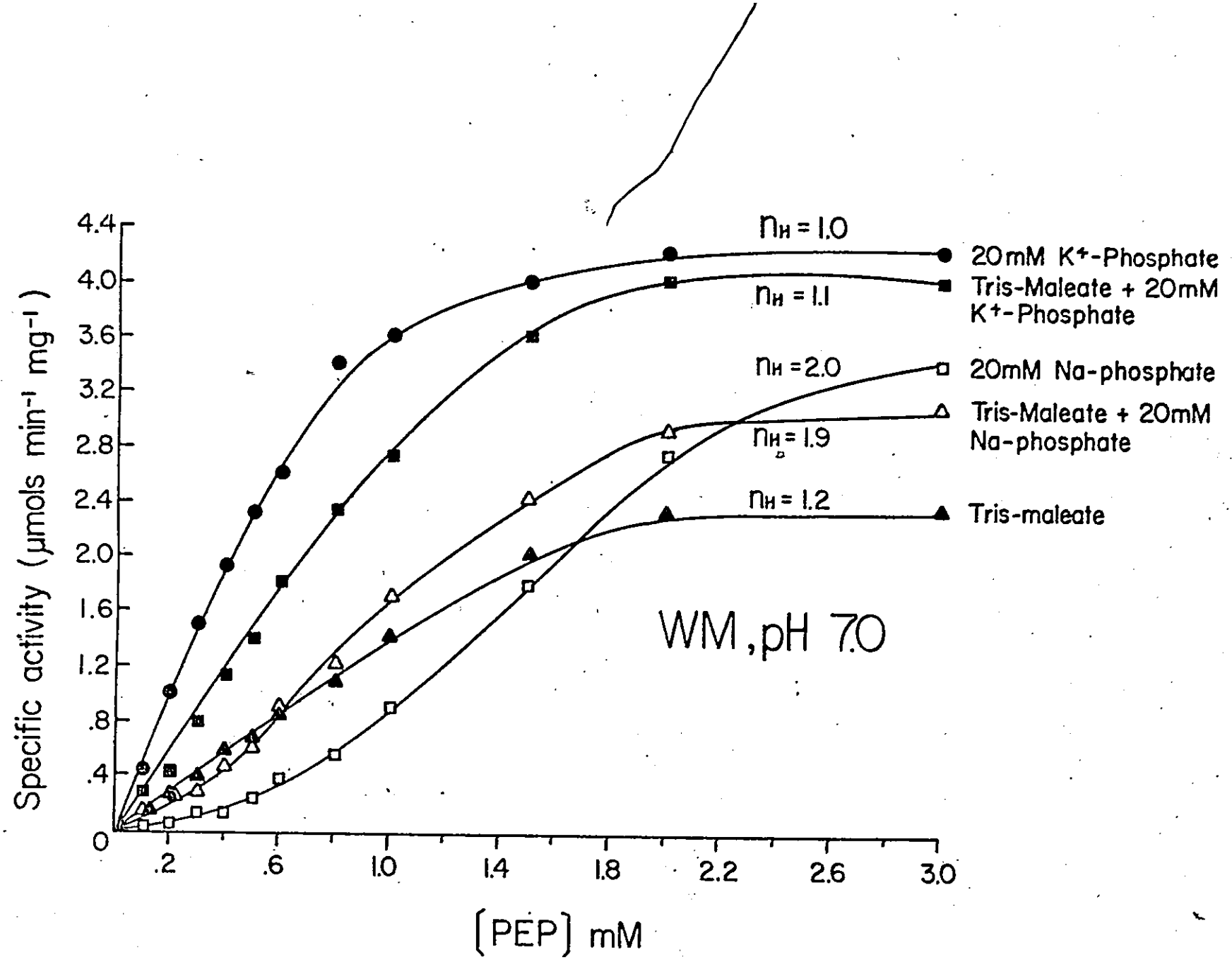

Specific activity (μmols min⁻¹ mg⁻¹)
.4
.3
.2
.1
0
1
2
3
4
5
6
7
8
9
10
[PEP] mM
RM, pH 6.5

Fig. 2. The effects of the buffers, Tris-Maleate and Phosphate, and cations, K^+ and Na^+ on the cooperativity of eel WM-PK with PEP. The PK enzyme was assayed at 20°C in the standard assay cocktail according to the procedure outlined in Materials and Methods.


Specific activity (μmols min⁻¹ mg⁻¹)
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
.8
.4
0
.2
.6
1.0
1.4
1.8
2.2
2.6
3.0
[PEP] mM
nH = 1.0
nH = 1.1
nH = 2.0
nH = 1.9
nH = 1.2
20mM K+-Phosphate
Tris-Maleate + 20mM K+-Phosphate
20mM Na-phosphate
Tris-Maleate + 20mM Na-phosphate
Tris-maleate
WM, pH 7.0

2. Electrophoresis of Eel PKs

Polyacrylamide disc gel electrophoresis indicated that RM- and WM-PKs share similar electrophoretic mobilities while being significantly different from the liver (Lv) enzyme (Fig. 3). Unfortunately, this electrophoretic procedure is inadequate to resolve possible microheterogeneity between RM- and WM-PKs.

3. Isoelectricfocusing of Eel PK

While both the muscle enzymes were separated by electricfocusing (Figs. 4 and 5), eel Lv-PK could not be recovered from the gel slab. Isoelectricfocusing of fresh muscle homogenates resulted in two distinct activity peaks for each muscle type. The two peaks for the RM were observed at a pI of 5.2 and 5.9 with the activity distributed approximately 40 and 60%, respectively (Fig. 4). For WM-PK, however, the two pI values were 5.85 and 6.75, with almost 100% of the activity in the 5.85 fraction (Fig. 5). In both cases, pre-incubation of the fresh homogenates with 1 mM FDP resulted in the disappearance of the more basic peak (5.9 for RM and 6.75 for WM) with the persistence of the acidic one (5.2 for RM and 5.85 for WM). Further, for both muscles, the basic peak was found by in vitro assay to be insensitive to FDP, ATP or L-alanine. The acidic peak, on the other hand, was activated by FDP and inhibited by ATP and L-alanine. Following ammonium sulfate fractionation (40-70%), only one activity peak was observed for both muscle enzymes (5.45 for RM and 5.55 for WM), consistent with the results of

Fig. 3. The electrophoretic mobilities of partially purified PK from eel WM (on left), Lv (centre) and RM (on right) on polyacrylamide. Refer to Materials and Methods for experimental and staining procedure.

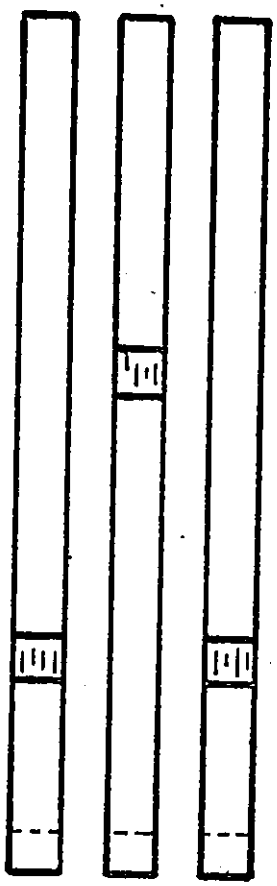


Fig. 4. Isoelectrofocusing of eel RM-PK. Solid line, fresh muscle homogenate; dotted line, partially purified homogenate (40-70% $(NH_4)_2SO_4$). Incubation of the fresh homogenate in the presence of 1 mM FDP resulted in the absence of the pI 5.9 peak, but the persistence of the pI 5.2 peak. Circles, pH gradient across the slab. See Materials and Methods for experimental procedure and assay conditions.

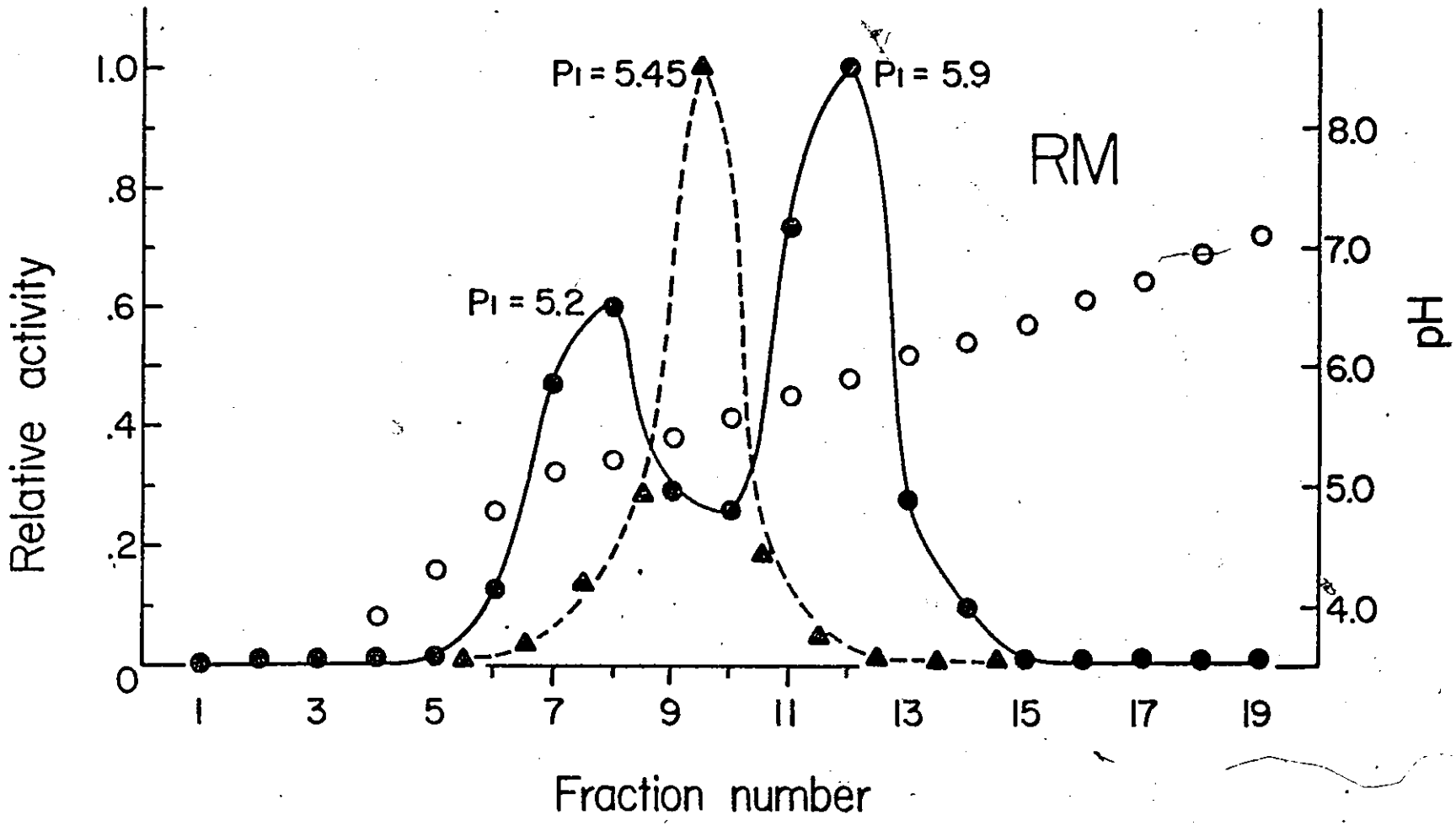
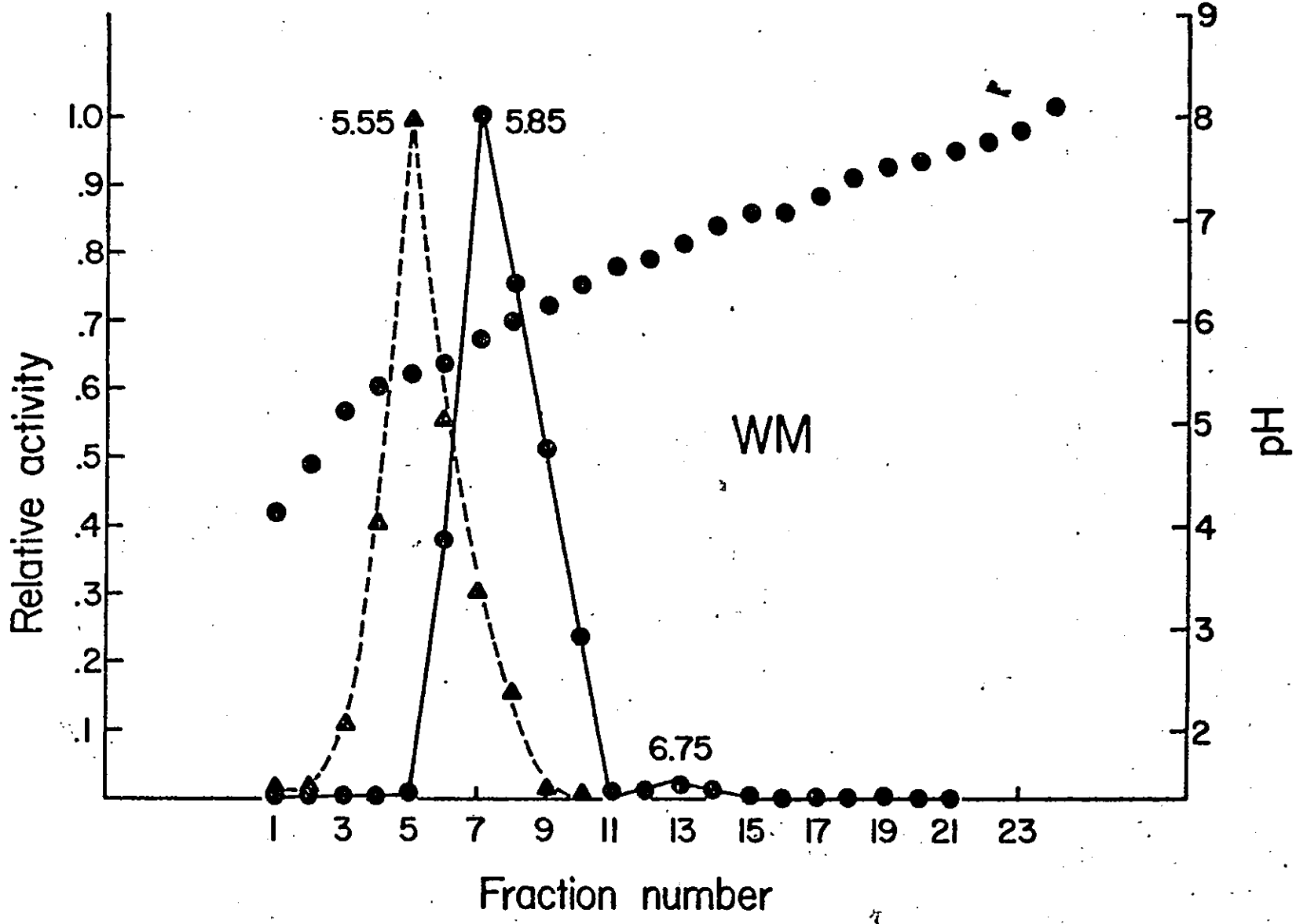

Relative activity
1.0
.8
.6
.4
.2
0
1
3
5
7
9
11
13
15
17
19
Fraction number
pH
8.0
7.0
6.0
5.0
4.0
RM
PI = 5.45
PI = 5.9
PI = 5.2

Fig. 5. Isoelectricfocusing of eel WM-PK. Solid line, fresh homogenate; dotted line, partially purified homogenate (40-70% $(NH_4)_2SO_4$). Incubating the fresh homogenate in the presence of 1 mM FDP resulted in the disappearance of the pI 6.75 peak, but the persistence of the pI 5.85 peak. Closed circles, pH gradient across slab. Refer to Materials and Methods for experimental procedure and assay conditions.


Relative activity
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
5.55
5.85
6.75
WM
pH
9
8
7
6
5
4
3
2
1
3
5
7
9
11
13
15
17
19
21
23
Fraction number

polyacrylamide electrophoresis, where both muscle PKs migrated similarly.

4. Effects of pH on Eel PKs

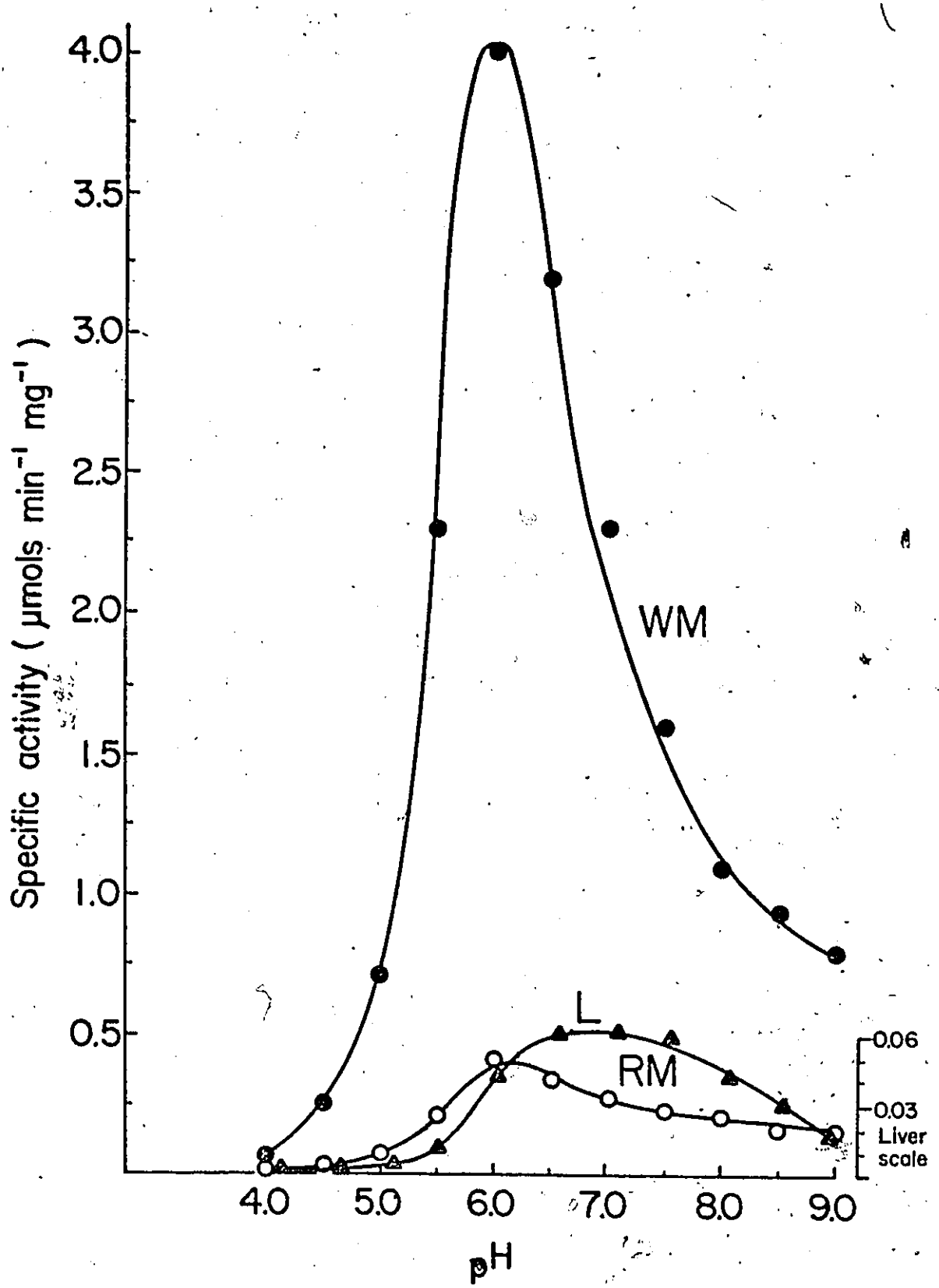
From data presented in Fig. 6, it is evident that while the pH optima for the three PK enzymes are similar (approximately 6.0 for RM- and WM-, and 7.0 for Lv-PK), the overall catalytic capacity and H^+ ion sensitivity of WM- is far greater than RM- or Lv-PK. For example, at pH 8.5, 7.5 and 6.5, WM-PK activity is 4-, 5- and 9-fold greater than RM-PK, and 35-, 40- and 50-fold greater than Lv-PK (Fig. 6). Similarly, RM-PK activity was greater than Lv-PK; however, the difference here was approximately 7-fold less (over the same pH range) than that observed between WM- and Lv-PK (Fig. 6).

Decreasing pH from 8.5 to 6.5 resulted in a 3-fold activation of WM-, a 1.4-fold activation of RM- and a 2-fold increase in Lv-PK activities (Fig. 6). Due to the differential nature of the H^+ ion interaction with each enzyme, however, 66% of this increase for WM- and 80% for Lv-PK occurred between pH 7.5 and 6.5 while the RM-enzyme activity increased approximately the same between pH 8.5 and 7.5 and 7.5 and 6.5.

5. Effects of Temperature on Eel PKs

When the effects of temperature on enzyme activity at saturating or V_{max} concentrations of substrates are analyzed by Arrhenius plots, an indication of enzyme thermal sensitivity can be obtained (see Hochachka and Somero, 1973).

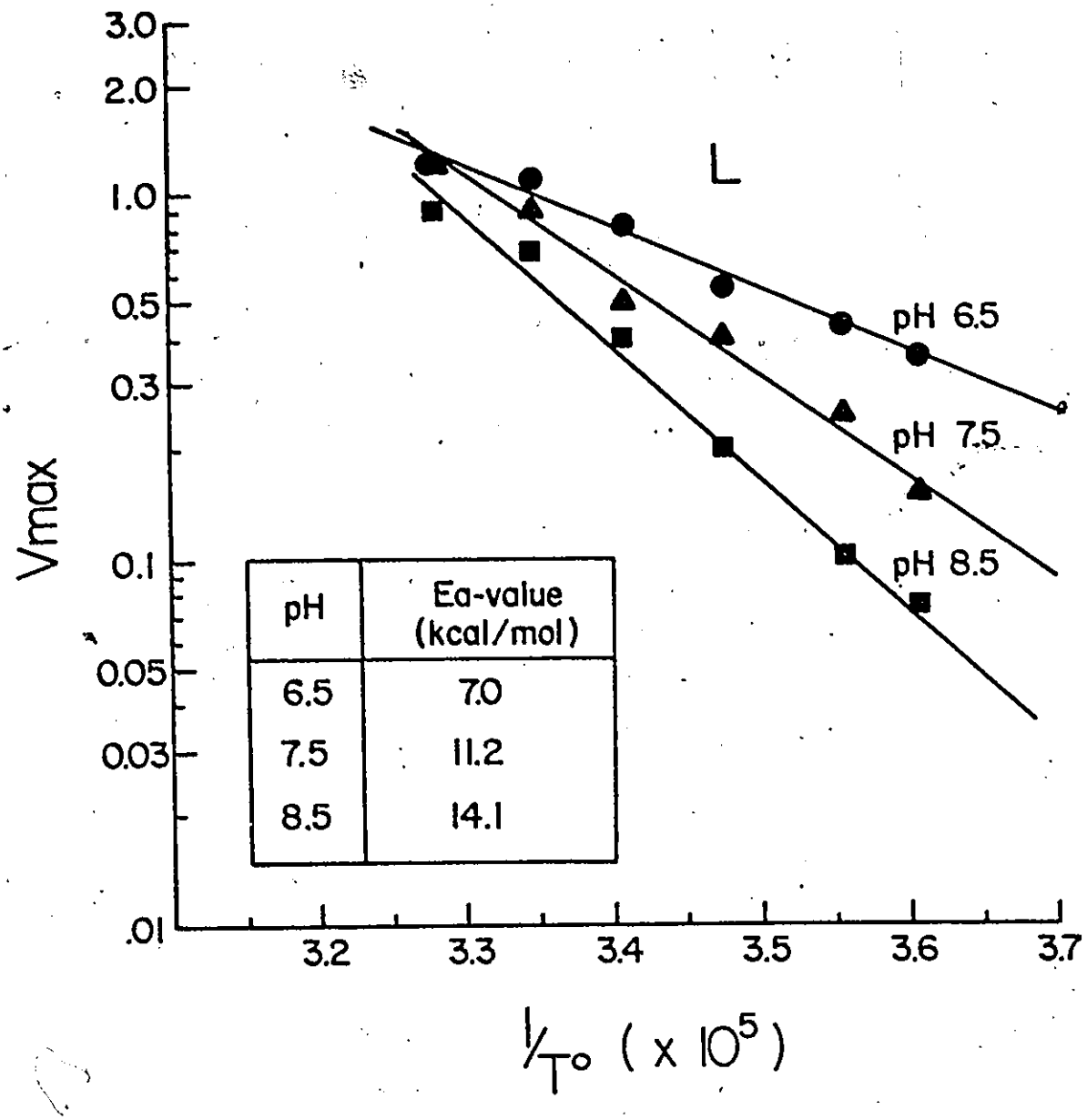
Fig. 6. The effects of pH on the three eel PKs. The enzymes were assayed in 100 mM Tris-Maleate buffer, 20°C, in the presence of 2 mM PEP and the standard assay cocktail (see Materials and Methods).


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Specific activity (μmols min⁻¹ mg⁻¹)
WM
L
RM
0.06
0.03
Liver scale
4.0
5.0
6.0
7.0
8.0
9.0
pH

As seen in Figs. 7, 8 and 9, the Ea, or activation energy, increases as the pH increases from 6.5 to 8.5 in both Lv- (Fig. 7) and WM-PKs (Fig. 8), but remain relatively constant in the RM-enzyme (Fig. 9). Major quantitative differences exist between Ea-values with Lv-PK generally exhibiting the lowest values (Fig. 7) and RM-PK the highest (Fig. 9). The WM-enzyme is more complex, exhibiting a high value at pH 8.5, but relatively low values at the other pHs (Fig. 8).

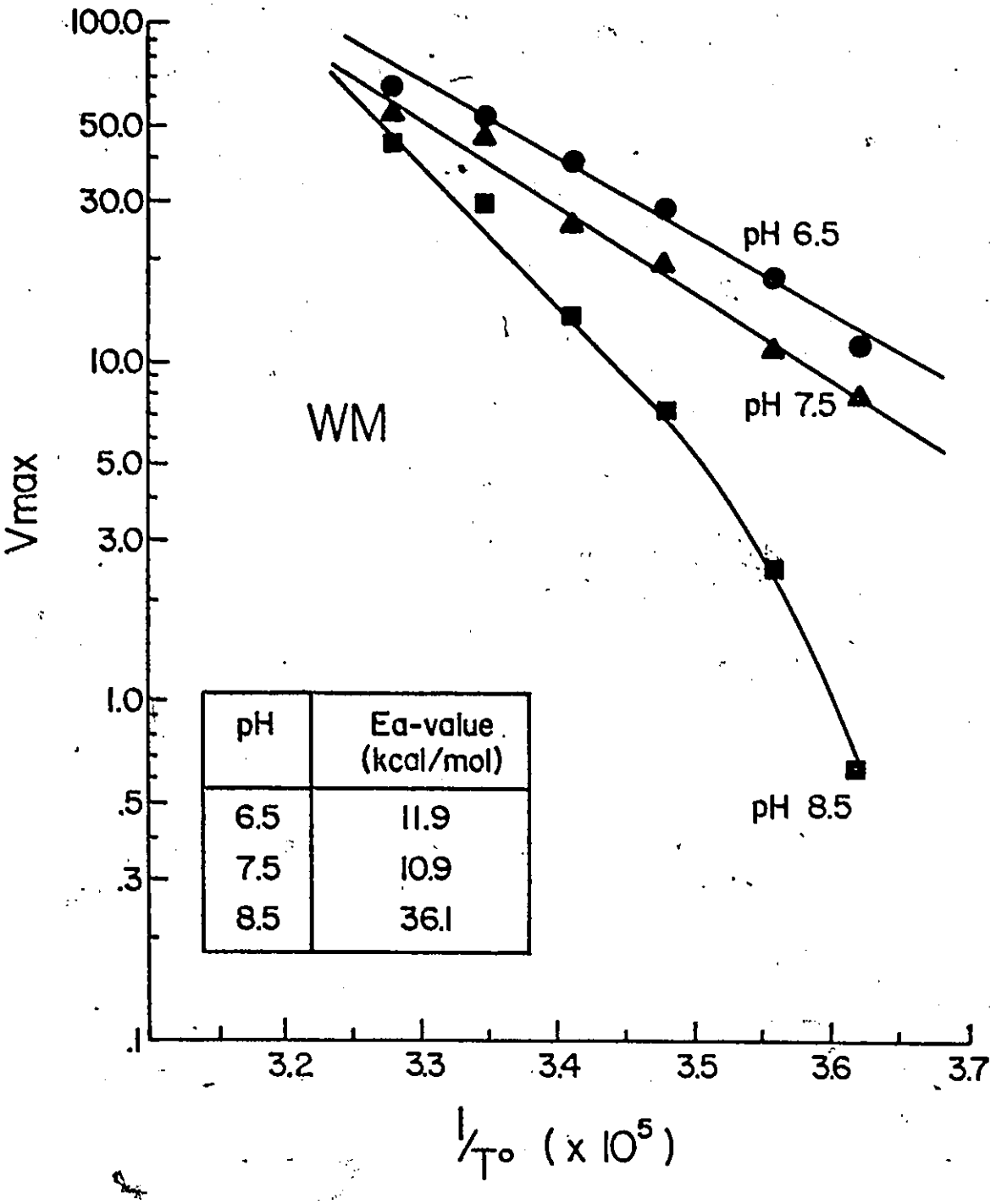
Temperature and pH may also interact to modify the binding of PEP to PK, as estimated by changes in the $K_{m(PEP)}$. In general, the smallest variation in $K_{m(PEP)}$ as a function of temperature occurs at the pH optima of the three enzymes, while the largest variation (5- to 16-fold) is observed at pH 8.5 (Fig. 10). The $K_{m(PEP)}$ of WM-PK exhibits an inverse response to temperature at pH 8.5 and 7.5 (8- and 6-fold increase with temperature decreases from 33° to 3.5°C) while being essentially independent of thermal change at pH 6.5. The affinity of the Lv-enzyme is positively correlated with temperature change at pH 8.5 (a 5-fold decrease as temperature decreases from 33° to 3.5°C), but varies little with temperature at pH 7.5 and 6.5 (2-fold variation over the same temperature range). With the exception of pH 8.5, where the $K_{m(PEP)}$ increases 3-fold below 8.5°C, the RM-enzyme follows a pattern similar to that of Lv-PK. It should also be noted from Fig. 10, that even though the $K_{m(PEP)}$ for RM- and Lv-PKs demonstrate characteristic pH-temperature responses, both are 2-to 10-fold lower at the higher pH and

Fig. 7. The effect of temperature as a function of pH (Tris-Maleate buffer) on the V_{max} of eel Lv-PK. Refer to Materials and Methods for assay conditions.



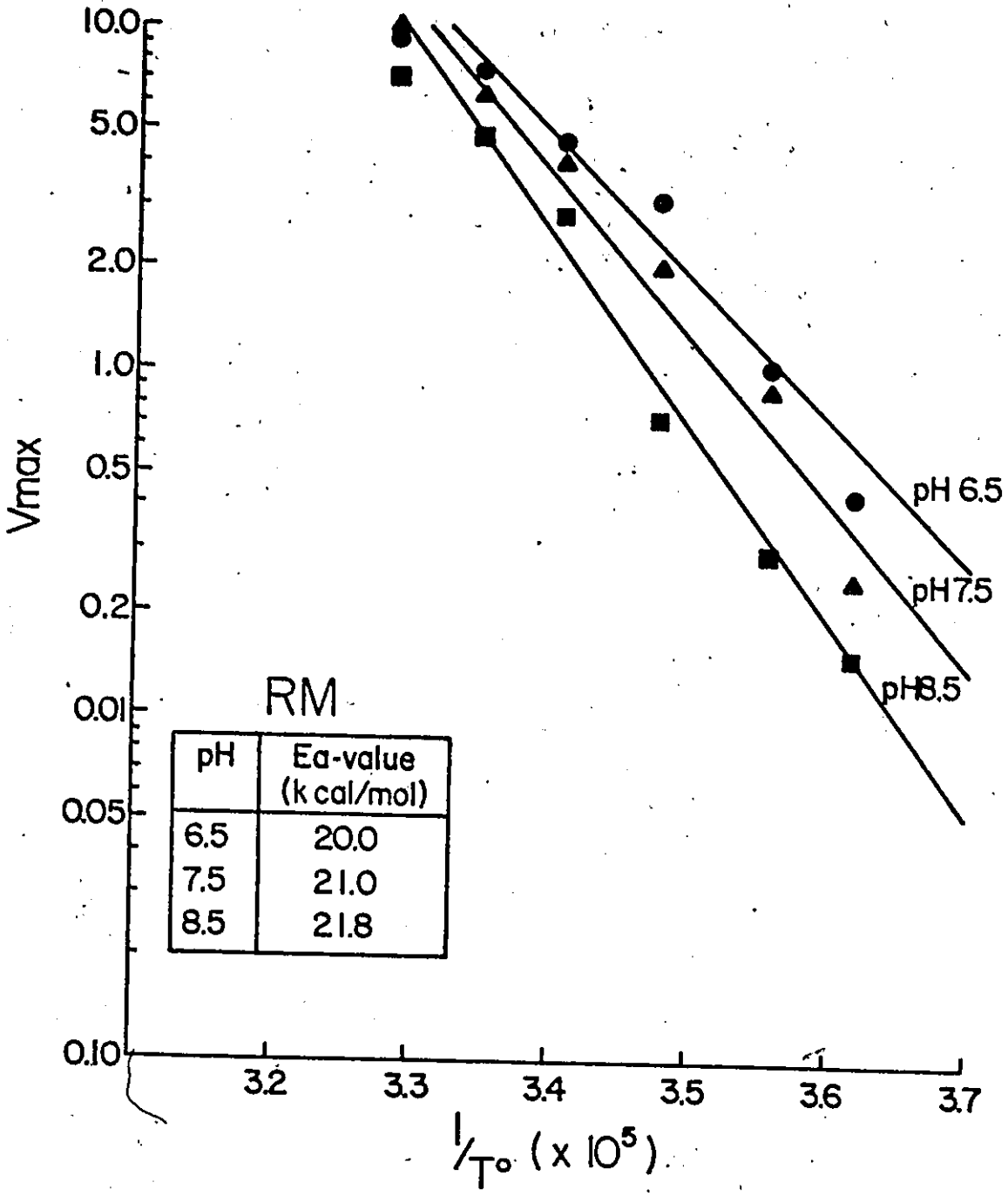
pH	Ea-value (kcal/mol)
6.5	7.0
7.5	11.2
8.5	14.1

Fig. 8. The effect of temperature and pH (Tris-Maleate buffer) on the V_{max} of eel WM-PK. The Ea value at pH 8.5 was calculated from a slope determined by a least square regression. Refer to Materials and Methods for conditions of assay.



pH	Ea-value (kcal/mol)
6.5	11.9
7.5	10.9
8.5	36.1

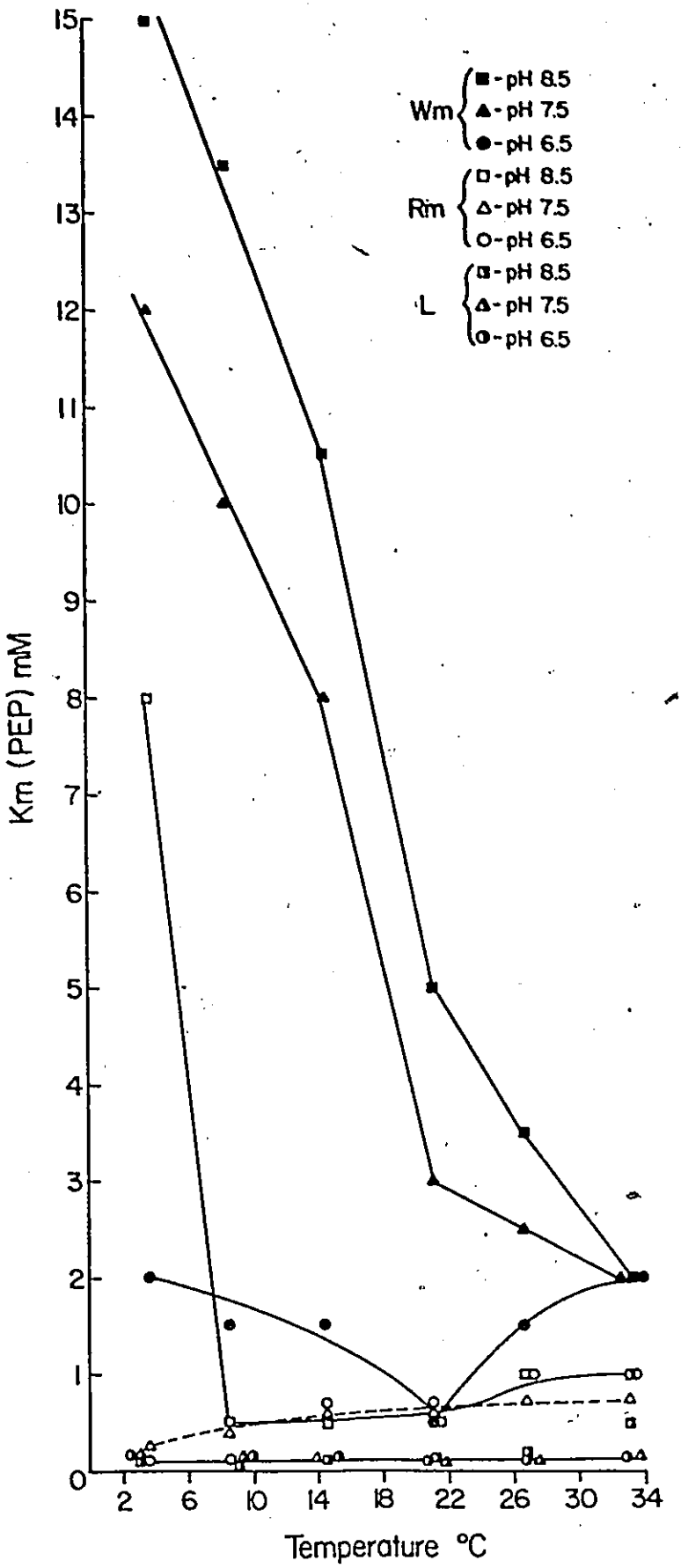
Fig. 9. The effect of temperature and pH (Tris-Maleate buffer) on the V_{max} of eel RM-PK. The Ea values were calculated from slopes determined by a least square regression. The regression coefficients for the three slopes were not observed to be less than 0.985. Refer to Materials and Methods for conditions of assay.



RM

pH	Ea-value (k cal/mol)
6.5	20.0
7.5	21.0
8.5	21.8

Fig. 10. The effect of temperature on the apparent $K_{m(PEP)}$ for the eel RM, WM and Lv-PKs. The enzymes were assayed in the standard assay cocktail and the $K_{m(PEP)}$ values were estimated from Lineweaver-Burke plots. Refer to materials and methods for conditions of the assay.


Km (PEP) mM
Temperature °C
Wm
pH 8.5
pH 7.5
pH 6.5
Rm
pH 8.5
pH 7.5
pH 6.5
L
pH 8.5
pH 7.5
pH 6.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
2
6
10
14
18
22
26
30
34

lower temperature ranges.

It is evident from Figs. 7, 8, 9 and 10, that the pH-temperature interaction is quantitatively different under saturating (V_{max}) and non-saturating (K_m) substrate conditions. This is particularly evident for the RM-enzyme, where the temperature sensitivity of V_{max} remains constant (approximately 21.0 kcal/mol^{-1}) while the $K_{m(PEP)}$ varies up to 16-fold as a function of pH (pH 8.5, below 8.5°C).

6. Effects of Metabolites on Eel PKs

a. FDP

The apparent $K_{a(FDP)}$ for RM- and WM-PKs was 0.15 µM and 0.10 µM, respectively. These low activation constants are in contrast to the lack of interaction observed for the Lv-PK enzyme (Table 1). Further, although both WM- (Fig. 11) and RM-PKs (Fig. 12) demonstrated mixed activation in the presence of FDP, (increased V_{max} and decreased $K_{m(PEP)}$), the quantitative response by the two muscle enzymes was quite different. For example, the V_{max} of both muscle PKs was increased by the same increment of activity such that in the presence or absence of FDP, WM-PK activity is approximately 5-fold greater than that described by RM-PK. However, the $K_{m(PEP)}$ for WM-PK (Fig. 11, Table 1) was more sensitive to FDP decreasing by 15-fold while the RM-enzyme (Fig. 12, Table 1) decreased only 6-fold. As a result of this sensitivity difference, the WM-$K_{m(PEP)}$ is 5-fold lower than the RM-$K_{m(PEP)}$ in the presence of FDP but 2-fold lower in the absence of FDP.

Table 1. The effects of various ligands on the enzyme-substrate affinity and the apparant activator and inhibitor constants for PK from eel white muscle, red muscle and liver. Assay conditions are: 3 mM ADP, 0.45 mM NADH, 40 mM K^+, 8 mM Mg^{++}, excess dialyzed sigma LDH and various concentrations of the modulators. Activities were determined using Tris-maleate buffer, pH 7.5 at 21°C. Values were estimated from double reciprocal plots.

	WM-PK	RM-PK	Lv-PK
$K_{m(PEP)}$[a]	3.0	6.0	0.11
+ATP	3.0	3.5	0.20
+L-Ala	3.5	3.5	0.11
+FDP	0.2	1.0	0.11
+FDP + ATP	0.3	1.0	0.20
+FDP + L-Ala	0.2	1.0	0.11
$K_{a(FDP)}$[b]	0.1	0.15	----
$K_{i(ATP)}$[a]	3.0	3.0	4.0
$K_{i(Ala)}$[a]	4.0	1.0	----
$K_{i(EDTA)}$[a]	2.5	3.5	4.0

a = values given in mM
b = values given in μM

Fig. 11. The effects of the positive effector FDP, and the two negative effectors, ATP and L-alanine on the heterotrophic interactions between eel WM-PK and the substrate, PEP. The enzyme was assayed at 20°C in the standard reaction cocktail. Refer to Materials and Methods for details.

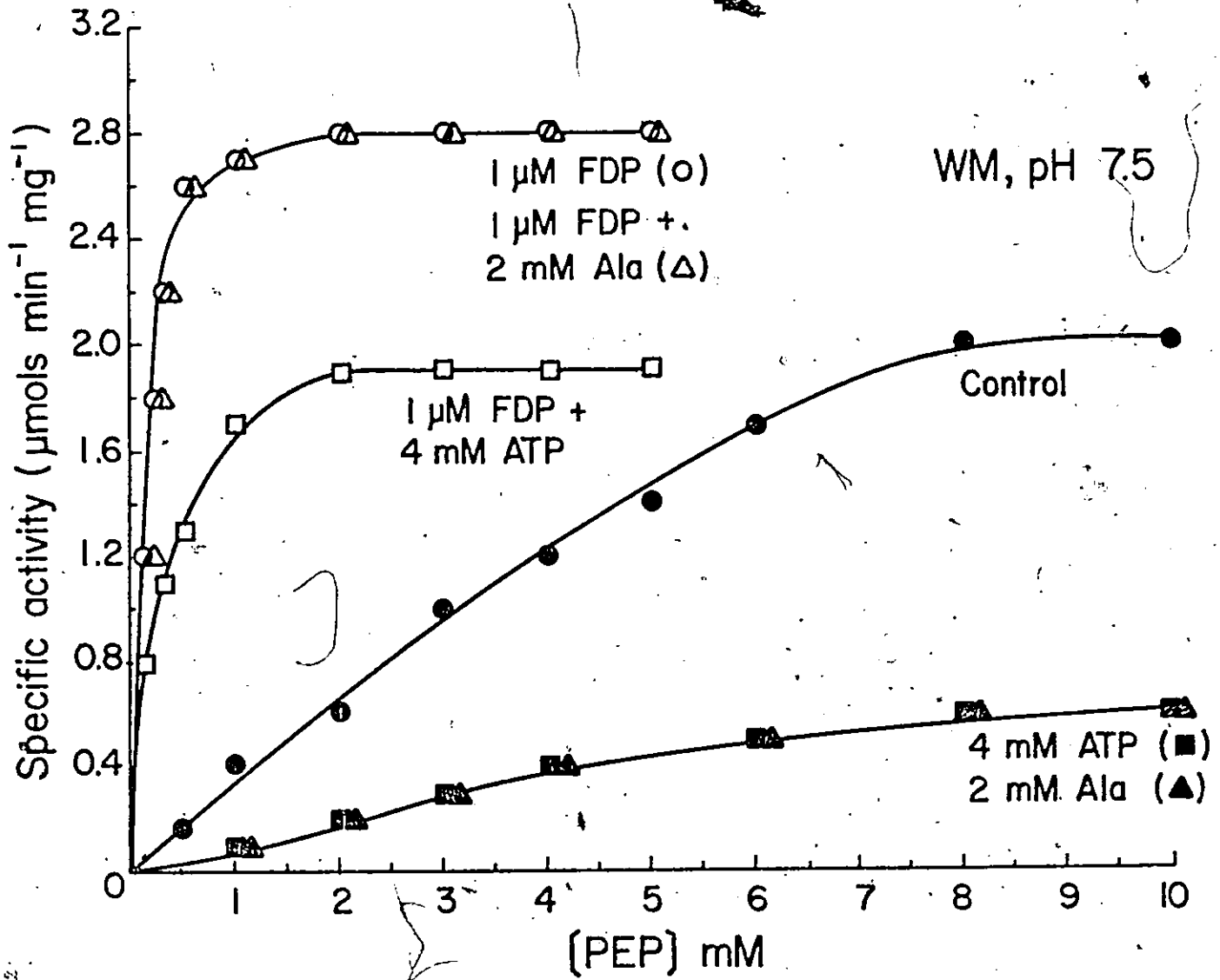

Specific activity (μmols min⁻¹ mg⁻¹)
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
1 μM FDP (o)
1 μM FDP +
2 mM Ala (Δ)
WM, pH 7.5
1 μM FDP +
4 mM ATP
Control
4 mM ATP (■)
2 mM Ala (▲)
1
2
3
4
5
6
7
8
9
10
[PEP] mM

Fig. 12. The effects of the positive effector, FDP, and the two negative effectors, ATP and L-alanine, on the heterotrophic interactions of eel RM-PK with the substrate, PEP. The enzyme was assayed at 20°C in the standard reaction cocktail. Refer to Materials and Methods for details.

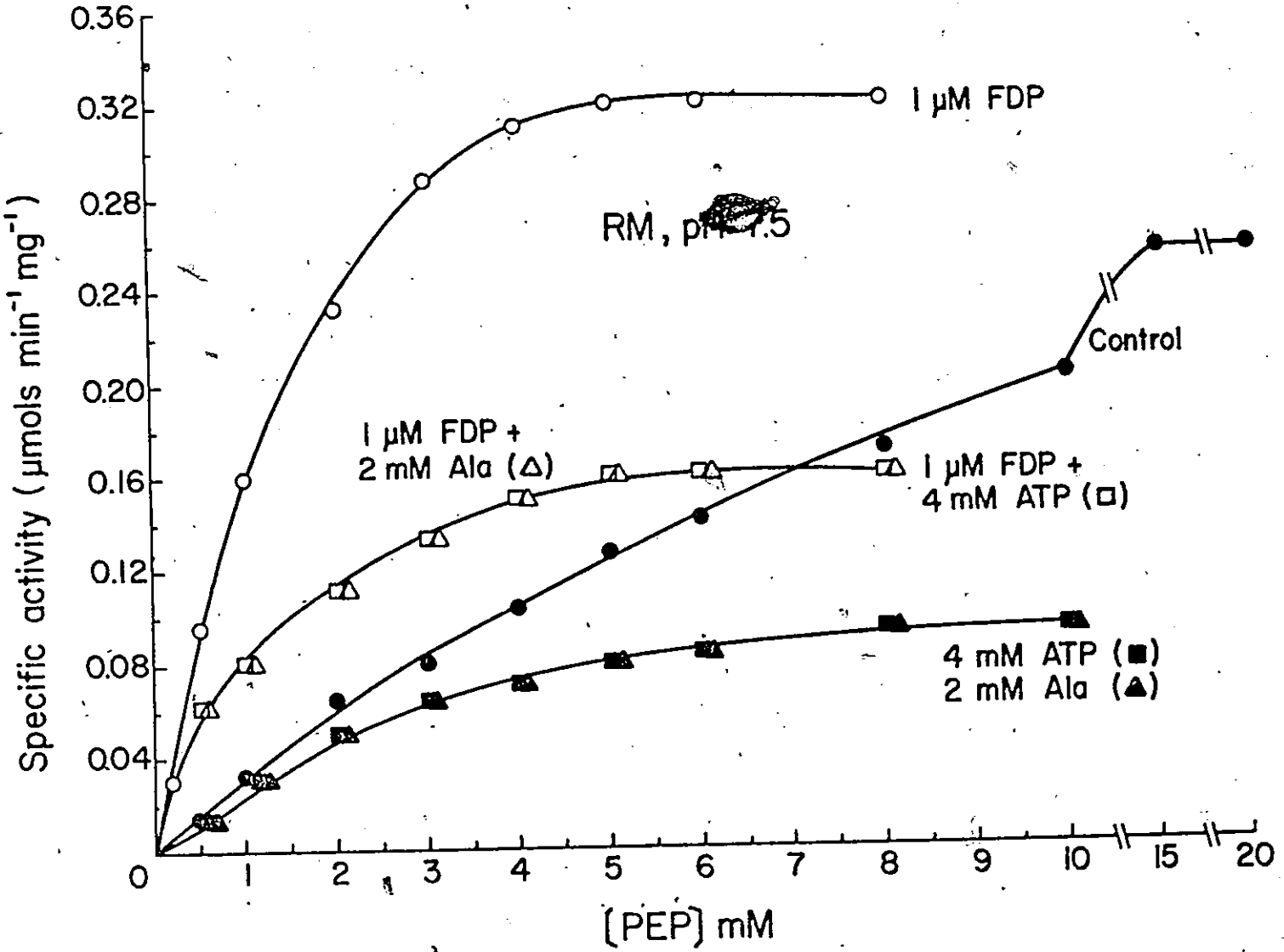
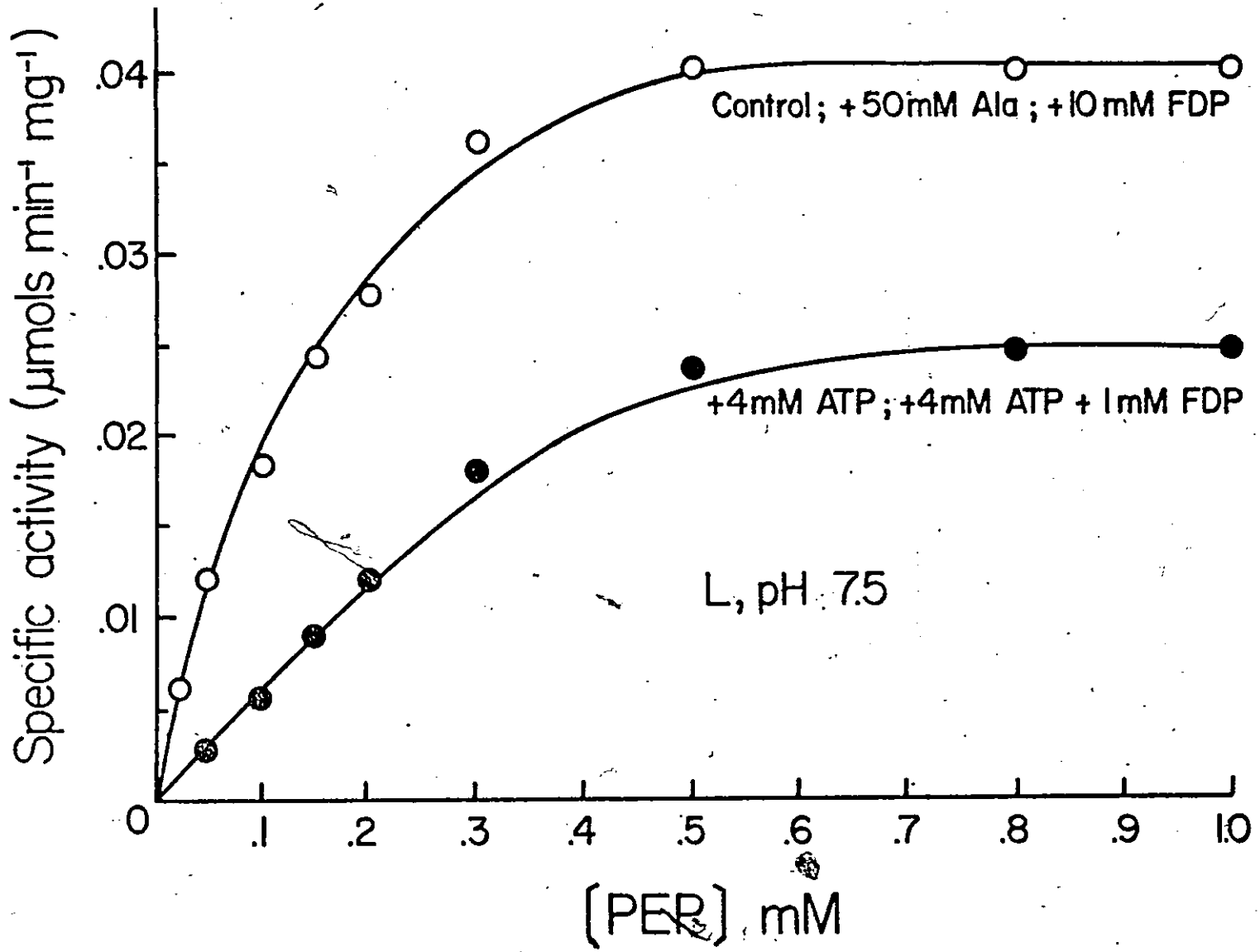

Specific activity (μmols min⁻¹ mg⁻¹)
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
1
2
3
4
5
6
7
8
9
10
15
20
[PEP] mM
1 μM FDP
RM, pH 7.5
Control
1 μM FDP +
2 mM Ala (△)
1 μM FDP +
4 mM ATP (□)
4 mM ATP (■)
2 mM Ala (▲)

Fig. 13. The effects of the positive effector, FDP, and the two negative effectors, ATP and L-alanine, on the heterotropic interactions of eel Lv-PK with the substrate, PEP. The enzyme was assayed at 20°C in the standard reaction cocktail. Refer to Materials and Methods for details.


Specific activity (μmols min⁻¹ mg⁻¹)
.04
.03
.02
.01
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
[PEP] mM
Control; +50mM Ala; +10mM FDP
+4mM ATP; +4mM ATP + 1mM FDP
L, pH 7.5

b. ATP and L-Alanine

All three eel enzymes were inhibited by ATP with an apparent $K_{i(ATP)}$ of 3 mM for RM- and WM-PK and 4 mM for Lv-PK (Table 1). The WM-PK V_{max} was the most sensitive to ATP (4.2-fold inhibition, Fig. 11) followed by RM-PK (2.6-fold inhibition, Fig. 12) and Lv-PK (1.3-fold inhibition, Fig. 13). While ATP was without effect on the WM-$K_{m(PEP)}$ (Table 1), the RM-$K_{m(PEP)}$ was decreased approximately 2-fold and Lv-PK $K_{m(PEP)}$ increased approximately 2-fold (Table 1). In contrast to these results (Table 1), Lv-PK (Fig. 13) was unaffected by L-alanine, RM-PK was modulated in an identical manner by both L-alanine and ATP (Fig. 12), and WM-PK was not inhibited as markedly by L-alanine as by ATP (3-fold as compared to 4.2-fold, Fig. 11). Therefore, both muscle enzymes demonstrated qualitatively similar behaviors in the presence of L-alanine and ATP, although the ability to FDP to reverse these effects is not similar.

c. FDP Plus ATP and L-Alanine

Since the role of an inhibitor may be diminished in the presence of low concentrations of a positive effector, the ability of FDP to reverse the inhibitory action of ATP and L-alanine on the three tissue enzymes was examined (Figs. 11, 12 and 13; Table 1). For WM-PK, FDP in the presence of L-alanine and ATP, decreased $K_{m(PEP)}$ approximately 15-fold to values obtained with just FDP alone (from 3.0 mM to 0.2 mM PEP) (Table 1). In RM, although FDP decreased the $K_{m(PEP)}$, this decrease was only 3.5-fold (from 3.5 mM to

1.0 mM PEP). The response of the two muscle enzymes at the level of V_{max} was markedly different from the response observed at $K_{m(PEP)}$. For WM-PK, FDP reversed ATP inhibition back to control V_{max} levels rather than activities consistent with FDP activation (Fig. 11). On the other hand, FDP did reverse the L-alanine inhibition and elevated V_{max} to the FDP activated level (Fig. 11). With RM-PK, the activity levels of the FDP plus ATP and FDP plus L-alanine were identical and were 1.6-fold below controls and 2-fold below the FDP activated V_{max} values. As indicated above, FDP was without affect on Lv-PK.

d. Modulation by EDTA

It has recently been proposed that the inhibition of PK by ATP involves chelation of Mg^{++}, the obligatory divalent cation, rather than a classical allosteric property (Wood, 1968; Van Berkel, 1974). The chelation hypothesis was examined (Figs. 14, 15 and 16) by comparing the inhibition resulting from the addition of ATP to that with EDTA, a known Mg^{++} chelator. The apparent $K_{i(EDTA)}$ for the RM-, WM-, and Lv-PKs was 3.5, 2.5 and 4 mM, respectively (Table 1), and thus similar to the 50% inhibition values for ATP. For WM- (Fig. 14) and RM-PKs (Fig. 15), the addition of equal concentrations of ATP or EDTA (4 mM) increased the $K_{m(Mg^{++})}$ from 4 to 12 mM Mg^{++}. The Lv-enzyme (Fig. 16) on the other hand, responded differently to these effectors; 4 mM ATP increased the $K_{m(Mg^{++})}$ 2-fold (from 3 to 6 mM Mg), while 4 mM EDTA increased the $K_{m(Mg^{++})}$ by 4.3-fold to 13 mM Mg^{++}.

Fig. 14. The effects of EDTA and FDP, and their interactions, on the Mg^{++} titration curve for WM-PK. The enzyme was assayed in the presence of 1.0 mM PEP, Tris-Maleate buffer, pH 7.5, 20°C, and the standard assay cocktail.

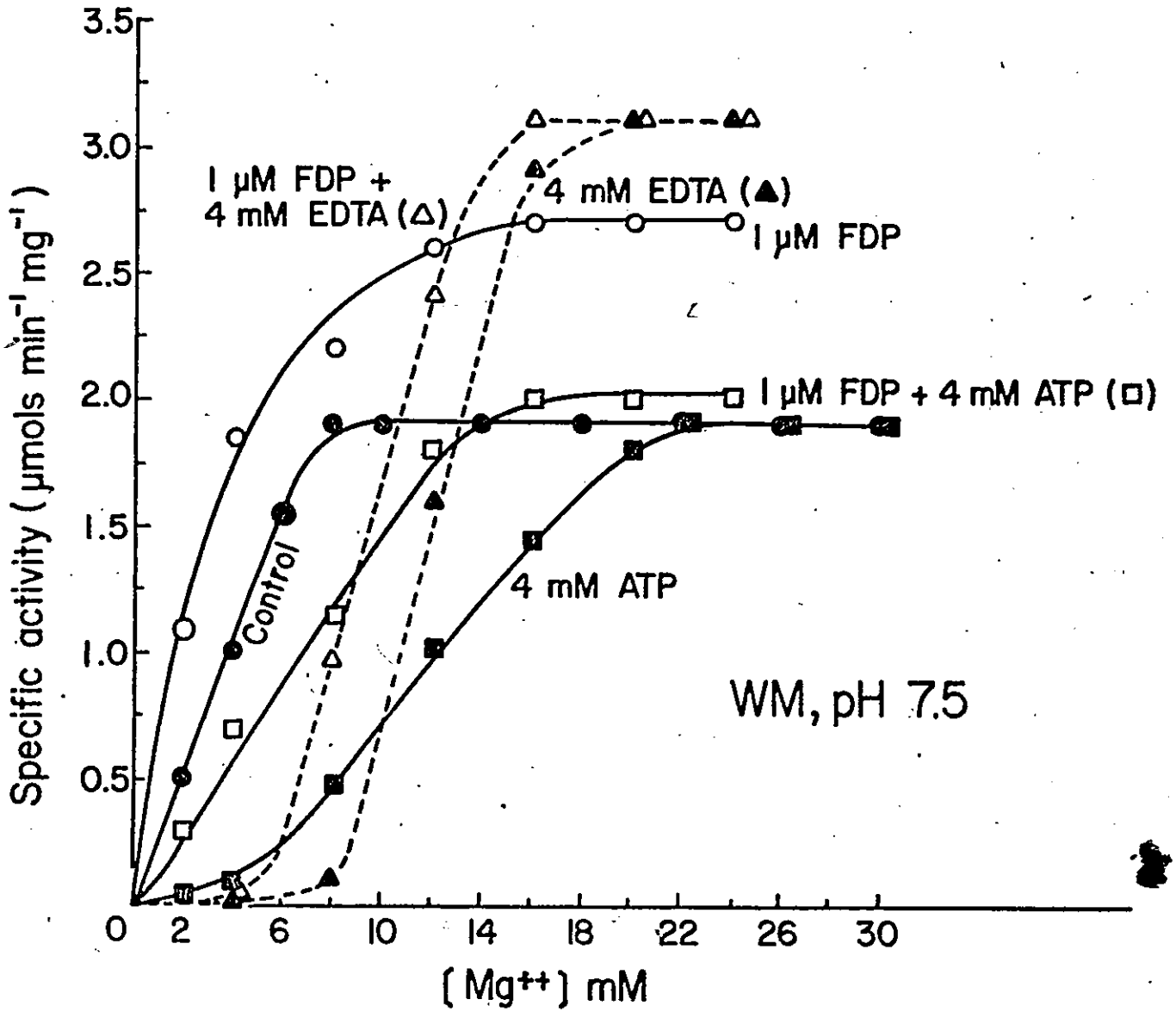

Specific activity (μmols min⁻¹ mg⁻¹)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
2
6
10
14
18
22
26
30
[Mg++] mM
1 μM FDP + 4 mM EDTA (△)
4 mM EDTA (▲)
1 μM FDP
1 μM FDP + 4 mM ATP (□)
Control
4 mM ATP
WM, pH 7.5

Fig. 15. The effects of EDTA and FDP and ATP, and their interactions, on the Mg^{++} saturation curve for RM-PK. The enzyme was assayed in the presence of 1.0 mM PEP, Tris-Maleate buffer, pH 7.5, 20°C and the standard assay cocktail.

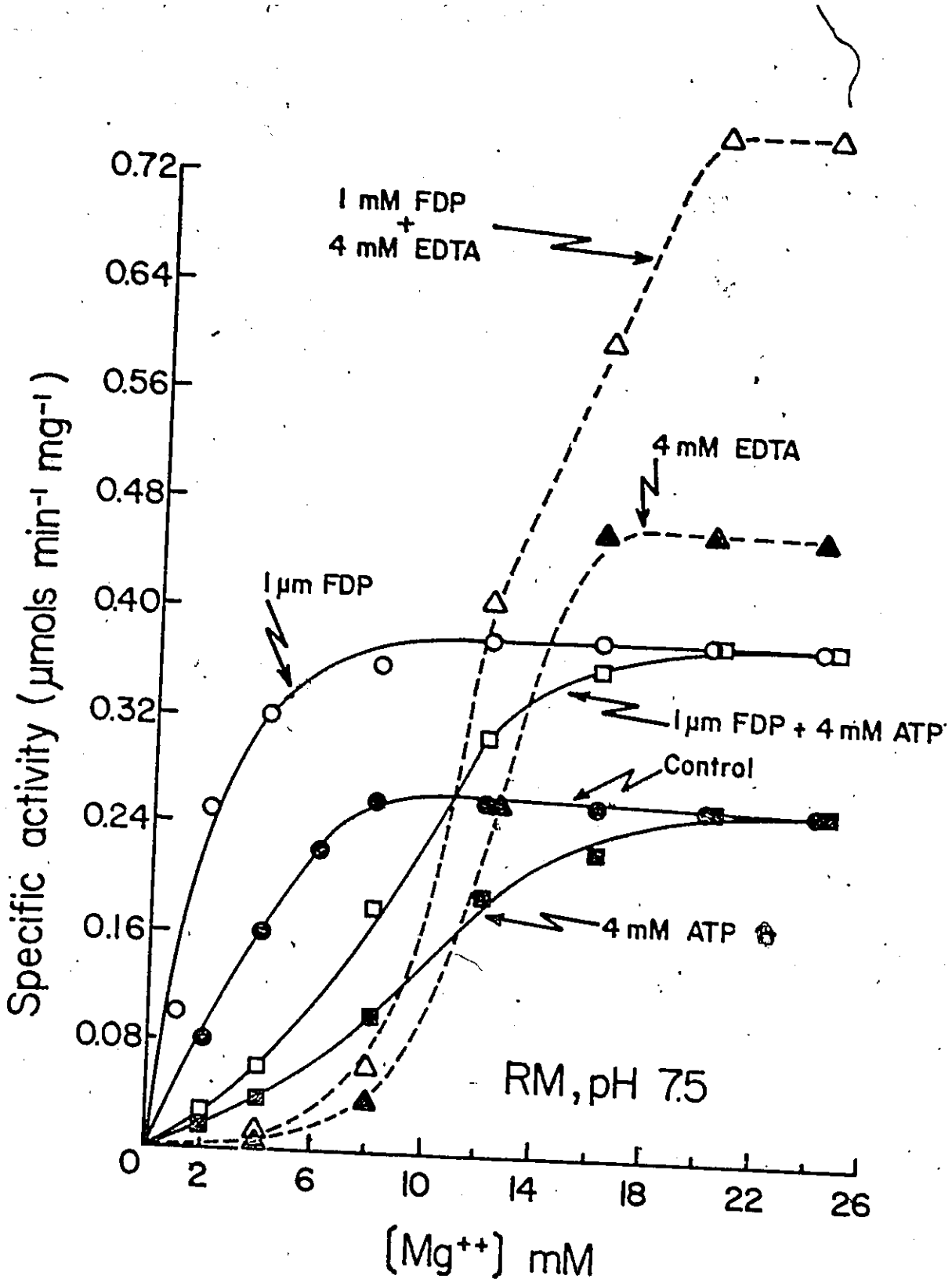
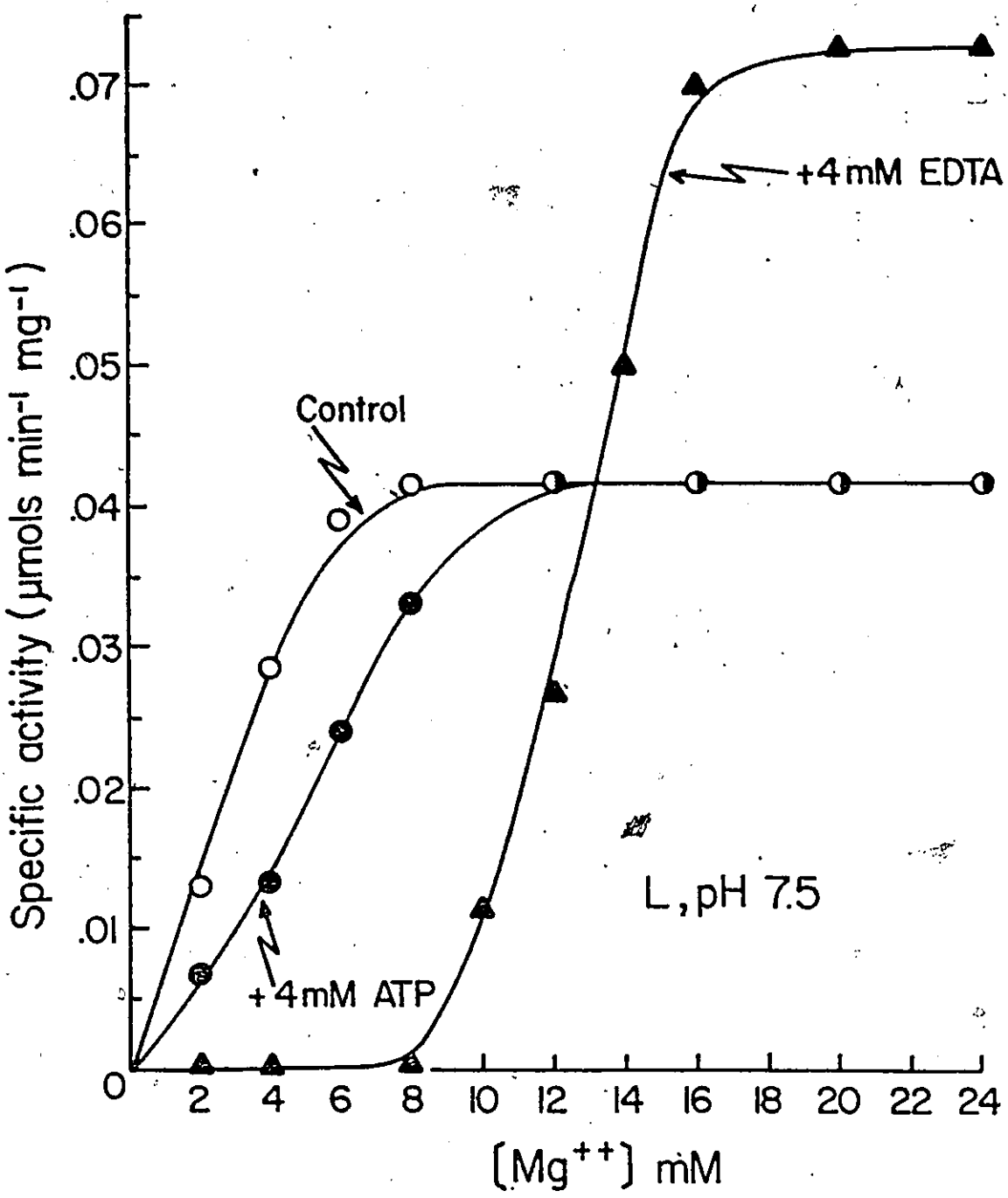

0.72
0.64
0.56
0.48
0.40
0.32
0.24
0.16
0.08
0
Specific activity (μmols min⁻¹ mg⁻¹)
1 mM FDP
+
4 mM EDTA
4 mM EDTA
1 μm FDP
1 μm FDP + 4 mM ATP
Control
4 mM ATP
RM, pH 7.5
2
6
10
14
18
22
26
[Mg⁺⁺] mM

Fig. 16. The effects of EDTA and ATP on the Mg^{++} titration curve for eel Lv-PK. The enzyme was assayed in the presence of 1.0 mM PEP, Tris-Maleate buffer, pH 7.5, 20°C and with the standard assay cocktail.


Specific activity (μmols min⁻¹ mg⁻¹)
.07
.06
.05
.04
.03
.02
.01
0
2
4
6
8
10
12
14
16
18
20
22
24
[Mg⁺⁺] mM
+4mM EDTA
Control
+4mM ATP
L, pH 7.5

The addition of ATP in 2 mM increments, over the range 2 to 6 mM, increased the $K_{m(Mg)}^{++}$ by consistent 4 mM multiples. Also, ATP did not effect the Lv-enzyme at 2 mM and only increased $K_{m(Mg)}^{++}$ by 2.5-fold between 4 and 6 mM ATP. In all three tissues examined, however, the inhibition of the $K_{m(Mg)}^{++}$ by ATP could be reversed to control levels by simply increasing the Mg^{++} concentrations (Figs. 14, 15 and 16). This characteristic (i.e., the ability of Mg^{++} to reverse ATP inhibition) generated an interesting family of curves which have a common V_{max} but incrementally increasing $K_{m(Mg)}^{++}$s.

The effects of FDP were examined to determine if FDP could alter the interaction of ATP with the substrate-Mg^{++} complex and thus exert a direct regulatory effect at this level (Figs. 14, 15 and 16). For both WM- and RM-PKs, FDP activated V_{max} 1.5-fold at saturating concentrations of Mg^{++}. In the presence of ATP, FDP addition to WM-PK did not alter V_{max} while in RM-PK, under similar conditions, FDP activated V_{max} 1.5-fold above that observed for either ATP alone or the control conditions. For WM-PK (Fig. 14), FDP decreased $K_{m(Mg)}^{++}$ 1.8-fold below that observed for just ATP alone, while in RM-PK, FDP decreased $K_{m(Mg)}^{++}$ by 1.3-fold. Further to the role of ATP, EDTA appears to act in a manner dissimilar to ATP. While both modulators did increase $K_{m(Mg)}^{++}$, EDTA also activated V_{max} significantly above that observed for controls. This response to EDTA was identical for all three tissue PKs examined (i.e., a 1.8-fold increase in V_{max} with the addition of 4 mM EDTA) and is probably a direct result

of the activation effects of the molecule demonstrated in Fig. 1. FDP reversed the increased $K_{m(Mg^{++})}$ in the presence of EDTA for the WM-enzyme, but was without effect for RM-PK (Fig. 15). An interesting difference was noted at V_{max}; in WM-PK, FDP in the presence of EDTA does not activate V_{max} above that obtained with just EDTA alone, but in RM-PK, the separate activations resulting from FDP and EDTA are additive (1.5-fold activation by each) and the V_{max} is activated a total of 3-fold above the control.

Thus, ATP inhibition can only be partly explained by the chelation hypothesis (Wood, 1968; Van Berkel, 1974). Interestingly, FDP, in addition to modulating $K_{m(PEP)}$, also affects the affinity of the PK enzymes for the substrate-Mg^{++} complex. This decrease in the $K_{m(Mg^{++})}$ resulting from FDP addition may partially explain the reversal of ATP inhibition observed at physiological levels of Mg^{++}.

DISCUSSION

These data suggest that although some of the kinetic and regulatory properties of eel PKs are similar to data reported by other investigators (eg., Schubert and Schoner, 1971), the American eel maintains yet another unique PK complement. Previous studies (Bailey et al., 1968; Pogson, 1968a,b) have reported that the presence of EDTA in the homogenizing buffer results in a transition from a less to a more reactive PK species; however, even though EDTA activates the eel PKs, there is no modification of the hyperbolic kinetics with respect to PEP (Fig.1) as seen with mammalian L-PK. For most PKs, the transition from sigmoidal to hyperbolic kinetics is mediated by alterations in the charge balance of the individual amino acid residues which results in modifications of the protein secondary structure (Helical nature) (Kapoor and Tronsgaard, 1972). For eel PKs, this transition depends upon the presence of Na^+, not EDTA (Fig. 2). Phosphate exerts considerable influence at both the $K_{m(PEP)}$ (marked decrease) and V_{max} (significant increase) levels of PEP. Recent findings (Moon et al., 1976) substantiates this phosphate effect; but contrasts that reported for ox muscle PK (Focant and Watts, 1973).

Flory et al. (1974) have suggested that Tris buffers are non-physiological and that Phosphate buffers should be used for the analysis of PK regulatory properties. However, the cationic Tris buffers were used in these studies for

three reasons: 1) Tris may more nearly mimic the cellular interaction between charged amino acids and PK (Kapoor, 1975); 2) since these are migrating, non-feeding eels, amino acid flux may represent a potential mode of metabolic regulation; and, 3) phosphate, per se, did not alter the n_H-value, or the degree of interaction between the enzyme and PEP (Fig. 2). Therefore, a Tris-maleate buffer system was used in all kinetic measurements of eel PKs.

The specific activity of eel WM-PK, 1.6 µmols $min^{-1}mg^{-1}$, at pH 7.5, is comparable (after adjustment for differences in assay temperature) to that observed by Borgmann and Moon (1976) for normothermic and hibernator bat muscle PK (1.44 and 1.6, respectively), by Scrutton and Utter (1968) for mammalian M_1-type PK (1.6), and by Bostrom and Johansson (1972) for white muscle PK from the European eel, Anguilla anguilla (2.64). Further, the specific activity of eel Lv-PK (0.04 at pH 7.5) is comparable to that observed by Carminatti et al. (1968) for mammalian L-PK (0.05) and Van Berkel (1974) for mammalian M_2-PK (0.05), but is less than observed by Scrutton and Utter (1968) for mammalian L-PK (0.1) and Borgmann and Moon (1976) for normothermic and hibernator bat liver PK (0.12 and 0.2, respectively). Eel RM-PK specific activity (0.32 at pH 7.5) lies between the values observed for eel WM- and Lv-PK and intermediate between values observed by Johnston (1975) for carp red muscle (0.037) and Bostrom and Johansson (1972) for the European eel red muscle (0.93).

The pH optima are similar for both muscle enzymes, approximately pH 6.0, but significantly lower than reported for other vertebrates muscle PKs, including fish (rainbow trout, pH 7.0-7.5; Moon et al., 1971), mammalian M_1-PK (pH 7.5; Imamura et al., 1972) and the oyster, a facultative anaerobe (pH 8.5; Mustafa and Hochachka, 1971). The pH optima for eel Lv-PK is very broad (pH 6.5-7.5) and thus dissimilar to the sharp optima at pH 7.0 reported for mammalian L-PK (Carminatti et al., 1968).

The results demonstrating that eel WM-PK is more active than RM-PK (Fig. 6) (approximately 5-fold) are consistent with those of Bostrom and Johansson (1972) working with the European eel. No comparable results on eel Lv-PK are available; however, Cardenas et al. (1975) reported no difference in V_{max} of purified (crystalline) bovine M_1, M_2 and L-PK. On the other hand, Scrutton and Utter (1968) found that the activity of purified (crystalline) rat M_1-PK was 15.6-fold greater than purified (crystalline) rat L-PK. Thus, although the eel enzymes were only partially purified, their activity patterns followed the trend observed for rat muscle and liver PKs.

The differences in the pH responses of the three eel PKs suggest that pH may play an important role in modulating the glycolytic potential of the different tissues. In particular, the WM-PK response is sharp as compared to the rather broad curves of RM- and Lv-PKs (Fig. 6). In tissues where the anaerobic production of energy predominates, the feed-forward

activation of PK by increasing H^+ ions produced during muscle contraction, would insure maximum glycolytic flux through PK by preventing a possible bottle-neck at this level (Hochachka and Somero, 1973). Alternatively, if the production of cellular energy is predominately aerobic, as is the case for fish red muscle (Drummond and Black, 1960), pH changes may be of minor importance.

In contrast to specific activities, apparent enzyme-substrate affinity parameter ($K_{m(PEP)}$) varied substantially from the literature values, with the exception of eel Lv-PK (0.11 mM PEP). The $K_{m(PEP)}$ for both the RM- (6.0 mM PEP) and WM-PKs (3.0 mM PEP) was an order of magnitude greater than reported in the literature. For example, the $K_{m(PEP)}$ values for other fish PKs range from 0.05 mM PEP for the Antarctic Trematomus bernacchii (Somero and Hochachka, 1968), 0.26 mM PEP for carp red muscle (Johnston, 1975), 0.6 mM PEP for sturgeon white muscle (Randall and Anderson, 1975) and 0.9 mM PEP for rainbow trout skeletal muscle (Moon et al., 1971). Further, although the $K_{m(PEP)}$ for eel muscle PKs is higher than Lv-PK, the mammalian M_1- and L-PKs demonstrate an opposite effect (compare 0.075 mM PEP for M_1-PK with 0.84 mM PEP for L-PK; Tanaka et al., 1967). The $K_{m(PEP)}$ for the mammalian M_2-PK is comparable to that observed for the M_1 isozyme (0.06 mM PEP; Van Berkel, 1974). Thus, while the apparent $K_{m(PEP)}$ for eel Lv-PK is markedly lower (approximately 8-fold) than observed for the mammalian L-PK, the $K_{m(PEP)}$ for eel WM- and RM-PKs is 40-and 80-fold greater,

respectively, than reported for mammalian M_1-PK. These data suggest that eel muscle PKs are more like the mammalian L-PK while the eel Lv-PK enzyme is more similar to mammalian muscle PK.

The effect of temperature on $K_{m(PEP)}$ (Fig. 10) and V_{max} (Figs. 7, 8 and 9) as a function of pH differ markedly from data for other fish species. Somero and Hochachka (1968) found that, at pH 7.4, white muscle PK from both rainbow trout and T. bernacchii, demonstrated $K_{m(PEP)}$-temperature plots which described a 'U' or 'J' shape with minima at approximately the acclimation or environmental temperature. For A. rostrata, WM-, RM-, and Lv-PKs this is not the case. Eel WM-PK, at pH 6.5, exhibits a temperature-insensitive $K_{m(PEP)}$; however, at pH 8.5, $K_{m(PEP)}$ increases approximately 7-fold as temperature decreases from 33 to 3°C (Fig. 10). These results suggest an extensive disruption of the hydrophobic interactions at the PEP binding site at pH 8.5 (see Hochachka et al., 1976). Thus, a high H^+ ion concentration may reduce the hydrophobic contribution of substrate binding, resulting in the observed $K_{m(PEP)}$-temperature independent pattern at pH 6.5. Somero (1976) has termed this response a 'coupled compensatory change'. Obviously, low temperature and high pH would reduce the glycolytic potential of white muscle.

In contrast, RM- and Lv-PKs demonstrate less pH sensitivity of the $K_{m(PEP)}$-temperature response compared to the WM-enzyme (Fig. 10). The $K_{m(PEP)}$ for Lv-PK, at all pHs

assayed, was thermally independent even though Ea-values rise with pH (Fig. 7). Red muscle PK is similar except for an aberrant point at 3°C and pH 8.5 where a large, unexplainable rise occurs. At all other assayed pHs, $K_{m(PEP)}$ decreased linearly with a temperature decrease, suggestive of an ionic and/or hydrogen bond interaction at the enzyme active site (Hochachka and Somero, 1973). Thus, even though RM- and WM-PKs appear electrophoretically similar (Fig. 3), their thermal responses are very different. In the absence of a marked H^+ ion effect, RM- and Lv-PKs maintain glycolytic potential during alteration in the physiological state and thus may exceed WM-PK activity under specific conditions.

As indicated in the Introduction, FDP is postulated to mediate a shift in the R-T equilibrium state of mammalian L-PK resulting in a change in the degree of cooperativity (i.e., as evidenced by shifts from sigmoidal to hyperbolic kinetics, and vice versa) between the enzyme and its substrate, PEP (Bailey et al., 1968; Rozengurt et al., 1969; Irving and Williams, 1973). This shift is demonstrated by an alteration of the isoelectric point from a basic to a more acidic value (eg., pH 6.1 to 5.3; Hess and Kutzbach, 1971); since FDP does not bind to M_1-PK, there is no alteration in the isoelectric point of this isozyme (Ibsen et al., 1976). Interestingly, chicken L-PK responds to FDP with a basic rather than acidic shift in the apparent isoelectric point.

The response by eel muscle PKs (Figs. 1, 4 and 5) is

similar to that described by Hess and Kutzbach (1971) for mammalian L-PK, suggesting a similarity in this physical property between eel muscle and mammalian L-PKs. Thus, although two distinct peaks of PK activity were present in fresh muscle preparations (one sensitive to FDP, ATP and L-alanine, and the other not), upon the addition of FDP and subsequent isoelectricfocusing, only the modulator-sensitive (a more acidic peak) peak remained. Therefore, these two are in equilibrium with each other, rather than two discrete isozymes. The relative distribution of activity between the two forms of eel RM- and WM-PKs varies greatly, and may reflect endogenous levels of FDP. For RM-PK (Fig. 4), the activity peaks are distributed similarly to that observed for mammalian L-PK by Hess and Kutzbach (1971) with approximately 60% being in the modulator insensitive form; for WM-PK (Fig. 5) this value is only 1%.

Both muscle PKs responded similarly to ammonium sulfate fractionation producing a single, unique form. These results collaborate the evidence obtained with polyacrylamide disc-gel electrophoresis which also produced a single banding pattern for each muscle PK. This alteration in the isoelectric point, with purification procedures, has been reported by Ibsen et al. (1976) for various tissue and organism PKs. Although an apparent conformational change occurred in the two muscle PKs with partial purification, as evidenced by an altered isoelectric point, this alteration probably involved only the exposure of different amino

acid residues which do not participate in the active or allosteric sites of the enzyme. This interpretation is supported by a comparative analysis of the modulator response of the salt fractionated and modulator sensitive (produced from isoelectricfocusing) enzymes. In both cases, no differences in the kinetic response to FDP, ATP or L-alanine could be detected between the differently prepared enzymes.

The modulation of PK by FDP (Figs. 11 and 12) is a common characteristic of fish muscle (eg., Somero and Hochachka, 1968; Johnston, 1975), bivalve mollusc muscle (Mustafa and Hochachka, 1971) and mammalian L-PK (Tanaka et al., 1967), but not for mammalian M_1-PK (Boyer, 1959). Interestingly, the apparent $K_{a(FDP)}$ for the eel RM- (0.15 μM) and WM-PKs (0.1 μM) is lower than reported for any other PK enzyme (to the authors knowledge), and thus suggestive of a much higher affinity by the eel muscle PKs for FDP. Values of $K_{a(FDP)}$ reported by Van Berkel (1974) for M_2-PK (1 μM), Hess et al. (1966) for yeast PK (0.15 mM), Llorent et al. (1970) for L-PK (0.5 μM) Somero and Hochachka (1968) for rainbow trout muscle PK (2 μM) and Moon and Borgmann (1976) for normothermic bat liver PK (0.5 μM) all represent saturating levels for the two eel PK enzymes. Only the data reported by Moon and Borgmann (1976) for PK from hibernating bat liver are similar (0.2 μM).

The presence in eel red and white muscle of a FDP-sensitive PK with isoelectricfocusing characteristics similar to mammalian L-PK suggests that greater similarities

in structure and function may exist between the eel muscle PKs and mammalian L-PK, than between the eel and mammalian skeletal muscle PKs. However, a significant difference exists between the response of mammalian L-PK and eel muscle PKs to activation by FDP. Consistent with the observations of Somero and Hochachka (1968) on rainbow trout muscle PK, Johnston (1975) on carp red muscle and Mustafa et al. (1971) on rattail skeletal muscle PK, FDP decreased the $K_{m(PEP)}$ and increased V_{max} values for both RM- and WM-PKs. For mammalian L-PK, FDP alters the homotrophic cooperativity with PEP to a heterotrophic effect, but only modulates $K_{m(PEP)}$, not V_{max}. The lack of an FDP-Lv-PK effect in eel liver (Fig. 13) and other fishes (Somero and Hochachka, 1968) is in sharp contrast to mammalian L-PKs (Schubert and Schoner, 1971; Carbonell et al., 1973). This apparent desensitization of eel Lv-PK to FDP could result if the enzyme was saturated with endogenous FDP. Since FDP concentrations increase in liver homogenates upon standing (Hess and Kutzbach, 1971; Irving and Williams, 1973) the subsequent kinetics could represent the 'R' conformation of a L-type PK, or a FDP-insensitive enzyme (Schubert and Schoner, 1971). However, incubation with EDTA and ATP, two agents which mediate the R-T equilibrium (eg., Bailey et al., 1968), failed to resensitize the enzyme to FDP (Fig. 16). This unique response by a liver PK warrants further examination.

The apparent $K_{i(ATP)}$ for the three eel enzymes (Table 1) is similar to that reported by Moon et al. (1971) for rain-

bow trout (3. mM), by Mustafa et al. (1971) for the rattail (4.0 mM), and slightly higher than reported by Imamura et al. (1972) for mammalian M_2-PK (2.5 mM). The $K_{i(ATP)}$ for mammalian L-PK is 0.15 mM (Imamura et al., 1972) and is thus significantly lower than observed for eel Lv-PK. Although eel Lv-PK was inhibited by ATP, the inhibition could simply be reversed by increasing the Mg^{++} concentrations (Fig. 16), which is not the case for the mammalian liver enzyme (Imamura et al., 1972). Imamura and associates reported a $K_{i(ATP)}$ for mammalian M_1-PK of 3.0 mM (a value identical to the eel muscle PKs); however, since 10 mM Mg^{++} completely reversed the inhibition, simple chelation of Mg^{++} by ATP was suggested and not true allosteric inhibition as demonstrated for mammalian L-PK.

Eel Lv-PK is not inhibited by L-alanine (Fig. 13) and thus responds comparably to mammalian M_1-PK (Imamura et al., 1972). The eel muscle PKs (Figs. 11 and 12), however, are inhibited by L-alanine, a response only noted for mammalian L- and M_2-PKs (Imamura et al., 1972). Imamura et al. (1972) reported a $K_{i(ala)}$ of 1 mM for mammalian L-PK which is identical to that found for eel RM-PK, but contrasts with the 4.0 mM value determined for eel WM-PK. Further, the levels of L-alanine required for 50% inhibition of eel RM- and WM-PKs are significantly higher than observed by Imamura et al. (1972) for mammalian M_2-PK (0.6 mM), Storey and Hochachka (1974) for turtle heart PK (0.09 mM), and Johnston (1975) for carp red muscle (0.17 mM).

The inhibitory effects of 4 mM ATP and 2 mM L-alanine on PEP saturation kinetics of WM- and RM-PKs are identical (Figs. 11 and 12); however, the magnitude of inhibition is quite different. Red muscle PK demonstrated a decrease in both V_{max} and $K_{m(PEP)}$ such that activities at low (and presumably physiological) concentrations of PEP, are not markedly altered (Fig. 12). This is in sharp contrast to a marked decrease in V_{max} but no change in $K_{m(PEP)}$ for the WM-PK; thus, activity levels are reduced at low substrate levels (Fig. 11).

While the separate effects of the inhibitors ATP and L-alanine are kinetically interesting, it is obvious from Figs. 11 and 12 that activity levels for both muscle enzymes are low at physiological levels of PEP (PEP levels in rat liver are reported to vary between 0.024 and 0.59 mM; Williamson, 1966) regardless of the presence or absence of the inhibitors. Indeed, Van Berkel (1974) has suggested that the regulation of M_2-PK from rat liver will depend upon fluctuations in the concentrations of the positive effector FDP, rather than alterations in levels of the negative modulators ATP and L-alanine, the effects of which are characteristically overridden by FDP (Schubert and Schoner, 1971). A similar situation is apparent in these results.

For mammalian L-PK, FDP reverses ATP and L-alanine inhibition returning V_{max} and $K_{m(PEP)}$ values to control levels (Tanaka et al., 1967). For eel muscle PKs, very low $K_{a(FDP)}$ values permit levels of only 0.2 μM FDP (or 5-fold

less than required for rat L-PK; Llorent et al., 1970) to reverse ATP and L-alanine inhibition. As noted in Figs. 11 and 12, the two muscle PKs respond differentially to the interaction of FDP with ATP and L-alanine. Indeed, the response observed for WM-PK, where FDP reversed ATP inhibition to control values but reversed L-alanine inhibition to FDP activated values, is identical to that reported by Johnston (1975) for carp red muscle PK. The response of the eel RM-PK to FDP with ATP was similar to that reported by Moon et al. (1971) for the rainbow trout and sea bass. In all three cases, ATP depressed V_{max} below control levels even with the addition of FDP, but activities at low PEP concentrations were still greatly activated since $K_{m(PEP)}$ was decreased. Since the addition of μM quantities of FDP reverses, and in the case of WM-PK overrides, the inhibitory action of ATP and L-alanine, the physiological significance of inhibition by these two modulators must be questioned. In the absence of FDP, however, these two modulators could significantly depress PK activity (see Chapter 6).

Regardless of the physiological significance of ATP inhibition of eel PKs, it is, nevertheless, of interest to establish the underlying mechanism; i.e., true allosteric inhibition, or simple Mg^{++} chelation. Indeed, Wood (1968), Van Berkel(1974) and Imamura et al.(1972) have suggested the latter hypothesis is probably the case for the M_1 and M_2-PKs. From data presented in Figs.11,12 and 13, it is evident that all three eel enzymes were inhibited by ATP;

however, in all cases, this inhibition could be totally reversed by increasing the Mg^{++} concentration. In effect, ATP increases the apparent $K_{m(Mg^{++})}$ thereby decreasing PK activity at all PEP concentrations (Figs.14,15 and 16). If ATP was simply chelating Mg^{++}, the substitution of EDTA, a known Mg^{++} chelator, should mimic the kinetic response to ATP. The initial lag in the kinetic curves of Figs.14,15 and 16, indicates that EDTA reduces free Mg^{++} levels below that necessary to activate PK. Above 8 mM Mg^{++}, adequate free Mg^{++} is available and PK activity increases. Only for Lv-PK (Fig.16) are the ATP and EDTA activity patterns parallel suggesting a strict chelatin phenomenon. For both WM- and RM-PKs (Figs.14 and 15), the slope of the EDTA curve is greater than that for ATP suggesting more than simple chelation is involved. Interestingly, in all cases EDTA activates eel PKs above the control value at high Mg^{++} concentrations, an observation also noted by Van Berkel(1974). Thus, the nature of ATP inhibition of eel muscle PKs is complex resulting in a homotrophic interaction between this metabolite and the PK-Mg^{++}-PEP complex.

In summary, the lack of a regulatory PK enzyme in eel liver is suggestive of either a marked divergence in gluconeogenic regulation (as compared to mammals), or the hint that eel liver is not the major gluconeogenic organ during migration and that this function may be fulfilled by another tissue. While it is reasonable to expect a regulatory PK in tissue concerned with glycolytic and gluconeogenic regu-

lation, the presence of a modulator sensitive PK in both eel muscles is difficult to understand. For the red muscle, however, its presence is consistent with the red muscle-organ hypothesis of Braekkan (1956); for white muscle, on the other hand, its presence is an enigma. Indeed, the very presence of a modulator-sensitive PK in white skeletal muscle is extremely puzzling, a point noted by Schubert and Schoner(1971). However, the extremely high activity of WM-PK (most regulatory enzymes are not characterized by high catalytic rates, Hochachka and Somero,1973),and high affinities for PEP and FDP suggest that WM-PK regulation may not be effected in a manner analogous to red muscle. Further, it is possible that the in vivo regulation of these muscle PKs will involve an H^+ ion interaction component which may modify the kinetic responses observed above. Chapter 6 is an analysis of these interactions.

VI

PYRUVATE KINASE-REGULATION

INTRODUCTION

Chapter 5 demonstrated that the eel PK enzymes exhibited regulatory properties at variance with the homologous mammalian muscle and liver PKs. Indeed, the data suggested that the metabolite interactions and kinetic characteristics of the eel RM- and WM-PKs are more similar to that observed for mammalian L-PK than M_1-PK, and the eel Lv-PK displayed interactions and characteristics more in common with mammalian M_1-PK rather than L-PK. Thus, the question is posed, do these marked differences provide any clues concerning a functional role for eel tissue PK?

A regulatory PK has been correlated with gluconeogenesis in mammals (eg., Sols, 1968) and more recently with facultative anaerobiosis in bivalve molluscs (Mustafa and Hochachka, 1971; Hochachka and Mustafa, 1972; Hochachka and Somero, 1973). In facultative anaerobes, the physiological significance of metabolite modulation of PK is analogous to that observed during gluconeogenesis; that is, reciprocal inhibition and activation of PK depending upon the metabolic demands of the cell. The metabolic signals which mediate PK activity and the aerobic-anaerobic transition in these organisms are, in part, altering levels of H^+ ions, L-alanine, ATP and FDP. For example, as the oxygen tension decreases in the oyster, there is a concommitant increase in the H^+ ion and CO_2 concentrations (Randall, 1974). This increase in H^+ ions inhibits PK and phosphoenol pyruvate

carboxykinase (PEP CK), the enzyme which competes for PEP with PK and is active during anoxia (Mustafa and Hochachka, 1971). Importantly, and in contrast to mammalian L-PK where H^+ ions act as positive modulators (eg., Rozengurt et al., 1969), the increase in H^+ ions potentiates and thus amplifies the inhibition of PK by both ATP and L-alanine, thereby insuring an inhibited PK enzyme while PEP CK is active (see discussion by Hochachka and Somero, 1973). Although such a switch has beencriticized for its simplicity (de Zwaan and Wijsman, 1976) it does serve as a testable hypothesis.

Recently Johnston (1975) has demonstrated that the regulatory properties of carp red muscle PK are similar to that described by Hochachka associates for PK from facultative anaerobes. Since it is known that the European carp does experience reduced ozygen tensions during the winter months when the lakes are frozen over (Hlazka, 1958), the correlation of a regulatory PK in the carp red muscle with a capacity to support anaerobiosis, may be more than fortuitous; however, such a correlation has not been demonstrated for the American eel, Anguilla rostrata, although it is known to burrow in the mud of river bottoms (Bertin, 1956).

As indicated in Chapter 1, the importance of tight metabolite regulation over mammalian L-PK is due to the disparity in enzyme activity between PK and the two gluconeogenic enzymes pyruvate carboxylase (PC) and PEP CK (compare 50 μmols/min/g wet weight for PK with 6.7 for both PC and PEP CK (Scrutton and Utter, 1968). Thus, during gluco-

neogenesis, PK has the potential to reduce the PEP formed from oxaloacetate, thereby creating a futile carbon cycle through PEP. Even though it has been suggested that sufficient levels of ATP and L-alanine are present to totally inhibit mammalian L-PK during gluconeogenesis (Llorent et al., 1970; Schoner et al., 1970; Schubert and Schoner, 1970), the available experimental evidence does not support this hypothesis (see Katz and Rognstad, 1976). For example, isotope exchange labelling experiments by Rognstad et al. (1970), Freidman et al. (1970, 1971) and Rognstad and Katz (1972) demonstrate that even in the presence of 0.1 mM cAMP in the rat liver perfusate, the recycling of lactate through the PEP branch point reaches 13% (i.e., 13% of the PEP formed from lactate through oxaloacetate is reduced to pyruvate). Since lactate contributes only approximately 25% of the carbon skeletons for gluconeogenesis with the remainder derived from amino acids (Hems et al., 1966), the total extent of cycling through PEP may be considerably greater. At the onset, this degree of futile substrate cycling during gluconeogenesis appears contrary to the function of the anabolic sequence; however, PEP cycling may be analogous to that observed by Newsholme and co-workers for the PFK-FDPase reactions (see discussion in Newsholme and Start, 1973). They postulate that the high opposing enzyme activities maintain a higher degree of sensitivity to extremely small alterations in modulator concentrations. For example, if the activities of both FDPase and PFK change by 10%, then

the overall net change in flux will be 20%.

The Newsholme hypothesis becomes more attractive since only 48 hrs of starvation are needed to depress L-PK activity by 60% (Tanaka et al., 1967), and that both PEP CK and PC are positively influenced by the glucocorticoids (Scrutton and Utter, 1968). Under conditions of nutritional deprivation it is possible that the activities of PK and PEP CK-PC will be approximately equal. This condition of equivalent enzyme activities of the opposing enzymes, is one of the theoretical constraints placed upon the substrate cycling hypothesis by Newsholme and associates. Such a regulatory strategy (i.e., substrate cycling) may be of physiological importance during periods of extreme nutritional duress; however, a mechanism of PK regulation which will allow the synthesis and degradation of glucose under normal 'ad libitum' feeding conditions must exist.

As a result of the physiological significance of gluconeogenesis and the implications of PEP cycling, an extensive amount of research has focused on elucidating the in vivo regulation of PK. This research has produced no less than five basic hypotheses which attempt to correlate the kinetic response of the enzyme with a purported metabolic role. These five regulatory strategies can be grouped into two basic categories: regulation through alteration of the native protein and regulation through interactions with low molecular weight ligands.

The first category can be further subdivided into

1) regulation through subunit aggregation (eg., equilibrium displacement between dimeric and tetrameric states (Hofmann et al., 1975), or between trimeric, tetrameric and pentameric configurations (Ibsen et al., 1971)), 2) phosphorylation-dephosphorylation (i.e., phosphorylation by a protein kinase which is activated by cAMP and thus depresses PK activity during gluconeogenesis (Llungstrom et al., 1974; Titanji et al., 1976)) and 3) oxidation-reduction of sulfhydral bonds (SH) critical to the steric configuration of the active site (i.e., oxidation of the SH bonds depresses PK activity and also decreases enzyme sensitivity to modulators (Van Berkel, et al., 1973)). For each of these hypotheses, the data presented are extremely convincing, but, their application to the in vivo condition remains speculative.

The second group of strategies, regulation through interactions with low molecular weight ligands, can also be subdivided. One group of investigators (eg., Llorent et al., 1970; Carbonelle et al., 1973) favor the inhibitory effects of ATP and L-alanine to depress PK activity during gluconeogenesis. Another group (eg., Schoner et al., 1970; Florey et al., 1974; Van Berkel, 1974) suggests that FDP activation is physiologically more important than the proposed ATP and L-alanine inhibition. Indeed, Van Berkel (1974) has proposed that FDP will ultimately determine L-PK activity since the levels of ATP remain relatively constant during glycolysis and gluconeogenesis and L-alanine levels actually decrease during gluconeogenesis due to an increased turnover rate.

While some investigators disagree as to the relative regulatory roles exerted by ATP, L-alanine and FDP, the evidence for FDP as a physiological activator of L-PK is well supported. Therefore, although it is apparent that the kinetic results in the literature can be interpreted according to a myriad of regulatory strategies, the actual physiological regulation of L-PK during gluconeogenesis remains very much in doubt.

The American eel, A. rostrata, undergoes a non-feeding migration at which time gonadal maturation also occurs (Bertin, 1956). Thus, a reciprocal regulation of muscle PK can be envisioned as being of particular importance due to its association with the metabolic pathways involved in carbon recycling and the generation of energy for contraction. Since both eel muscle PKs did respond to the modulators ATP, L-alanine and FDP, but eel Lv-PK did not (Chapter 5), it is important to establish whether these ligand interactions could be correlated with specific functional roles for eel tissue PKs. In particular, a correlation between the eel WM- and RM-PKs and the mammalian M_1- and L-PKs, respectively, would be suggestive of a glycolytic or gluconeogenic function. The results suggest that, under certain conditions, such a correlation between the mammalian and eel enzymes does exist. The role of the L-PK in contributing to either glycolysis or gluconeogenesis is not clear. The data are also analyzed according to the two state allosteric enzyme model of Monod et al. (1965), and a model based on metabolite interactions is proposed.

RESULTS

A. H^+ ions

The effects of H^+ ions on V_{max}, $K_{m(PEP)}$ and V_{max}/K_m values for PK from eel red and white muscles and liver are presented in Tables 1, 2 and 3, respectively. All PKs responded similarly to an increase in H^+ ion concentration with increased maximal activities; however, the WM-PK was most sensitive, followed by Lv-PK and RM-PK (compare 2.9-fold increase with a decreased pH from 8.5 to 6.5 for WM-PK, with a 2-fold for Lv-PK and 1.4-fold for RM-PK) (Table 1). The effects of increasing H^+ ions on $K_{m(PEP)}$ for the two muscle PKs was also similar (decreased $K_{m(PEP)}$ with increased H^+ ions), but the $K_{m(PEP)}$ for Lv-PK was insensitive to H^+ ion modulation between pHs 8.5 and 6.5. Although the $K_{m(PEP)}$ for both muscle PKs behaved similarly, the $K_{m(PEP)}$ for RM-PK was more sensitive to H^+ ion modulation than WM-PK (12-fold decrease for RM-PK as compared to 8-fold decrease for WM-PK with decreasing pH from 8.5 to 6.5) (Table 2). Consistent with the V_{max} data, all tissue PKs demonstrated increased V_{max}/K_m values with increasing H^+ ion concentration. WM-PK was, however, the most sensitive to H^+ ion modulation at this level followed by RM- and Lv-PKs (Table 3).

B. FDP Modulation

The effects of FDP on WM- and RM-PK V_{max} are markedly dependent upon the H^+ ion concentration; eel Lv-PK was unaffected by FDP, but at low ($K_{m(PEP)}$) and high (V_{max})

Table 1. Anguilla rostrata. The effects of the various modulators on maximal (V_{max}) activity of WM-, RM- and Lv-PK as a function of pH at 21°C. Assay conditions are: 3 mM ATP, 0.45 mM NADH, 40 mM K^+, 8 mM Mg^{++}, excess dialyzed Sigma LDH and 4 mM ATP, 2 mM ALA, and 0.001 mM FDP where applicable.

	Specific Activity (umols min^{-1} mg^{-1})								
pH	8.5			7.5			6.5		
Tissue	WM	RM	Lv	WM	RM	Lv	WM	RM	Lv
Control	1.12	0.26	0.032	1.60	0.32	0.040	3.20	0.36	0.064
+FDP	1.44	0.30	0.032	2.24	0.40	0.040	3.20	0.36	0.064
+ATP	0.38	0.08	0.024	0.38	0.12	0.032	1.60	0.14	0.040
+ALA	0.38	0.08	0.032	0.51	0.12	0.040	2.80	0.14	0.064
FDP+ATP	1.12	0.10	0.024	1.47	0.20	0.032	1.60	0.12	0.040
FDP+ALA	1.44	0.14	0.032	2.11	0.20	0.040	2.80	0.15	0.064

Table 2. Anguilla rostrata. The effects of the various modulators on $K_{m(PEP)}$ from WM-, RM- and Lv-PK, as a function of pH at 21°C. Units are in mM PEP. Assay conditions are: 3 mM ADP, 0.45 mM NADH, 40 mM K^{+}, 8 mM Mg^{++}, excess dialyzed Sigma LDH and 4 mM ATP, 2 mM ALA, and 0.001 mM PEP where applicable.

$K_{m(PEP)}$ mM

pH	8.5			7.5			6.5		
Tissue	WM	RM	Lv	WM	RM	Lv	WM	RM	Lv
Control	5.5	5.0	0.1	3.0	6.0	0.11	0.45	0.70	0.09
+FDP	0.2	1.5	0.1	0.2	1.0	0.11	0.10	0.15	0.09
+ATP	8.0	3.5	0.12	3.0	3.5	0.20	0.60	0.90	0.11
+ALA	8.0	3.5	0.1	3.5	3.5	0.11	0.60	0.90	0.09
FDP+ATP	0.4	2.0	0.12	0.3	1.0	0.20	0.20	0.10	0.11
FDP+ALA	0.2	2.0	0.1	0.2	1.0	0.11	0.20	0.20	0.09

Table 3. Anguilla rostrata. $V_{max}/K_{m(PEP)}$ ratios for RM-, WM- and Lv-PK were determined at 21°C and at pHs 8.5, 7.5, and 6.5. Assay conditions are: 3.0 mM ADP, 0.45 mM NADH, 40 mM K^+, 8 mM Mg^{++}, excess dialyzed Sigma LDH, and 4 mM ATP, 2 mM ALA, 0.001 mM FDP where applicable.

V_{max}/K_m Modulation

pH	8.5			7.5			6.5		
Tissue	WM	RM	Lv	WM	RM	Lv	WM	RM	Lv
Control	0.20	0.052	0.32	0.53	0.053	0.36	7.1	0.51	0.71
+FDP	7.20	0.200	0.32	11.20	0.40	0.36	32.0	2.40	0.71
+ATP	0.05	0.022	0.20	0.13	0.03	0.16	2.7	0.16	0.36
+ALA	0.05	0.022	0.32	0.15	0.03	0.36	4.7	0.16	0.71
FDP+ATP	2.80	0.050	0.20	4.90	0.20	0.16	8.0	1.20	0.36
FDP+ALA	7.20	0.070	0.32	10.55	0.20	0.36	14.0	0.75	0.71

levels of PEP. For both muscle PKs, FDP only affected V_{max} at the basic pHs, (above pH 6.5), a condition observed for mammalian L-PK (Rozengurt et al., 1969). Importantly, in addition to being influenced by H^+ ions, FDP activated V_{max} values above that observed for the control (Table 1). Modulation of $K_{m(PEP)}$ by FDP was also observed to be responsive to pH for RM-PK, but not for WM- or Lv-PKs. Thus, in the presence of 1 μM FDP, the $K_{m(PEP)}$ for eel WM-PK was stabilized at approximatley 0.2 mM PEP, over the pH range 8.5 to 6.5; red muscle, on the other hand, exhibited a decreased $K_{m(PEP)}$ in the presence of FDP as compared to the control, but remained responsive to alterations in H^+ ions. For example, the $K_{m(PEP)}$ for RM-PK sequentially decreased as the pH was decreased from 8.5 to 6.5, with or without FDP. Since FDP activated V_{max} and decreased $K_{m(PEP)}$, the V_{max}/K_m values for the two muscle enzymes increased significantly in the presence of FDP, and this response was further modulated by H^+ ions (Table 3). The most striking increases were observed for WM-PK due to the effect of FDP on the $K_{m(PEP)}$.

C. ATP and L-alanine Modulation

Similar to the mammalian L-PK (Rozengurt et al., 1969), increasing H^+ ions partially reversed the inhibitory effects of ATP and L-alanine (Table 1). For RM-PK, both negative modulators inhibited V_{max} identically at all pHs examined; for WM-PK, however, although both ATP and L-alanine inhibited V_{max} equally at pH 8.5, at pH 6.5, ATP was a much stronger inhibitor (50% of control activity as compared to 88%).

Again, eel Lv-PK was unaffected by either ATP or L-alanine. The small inhibition observed for Lv-PK by ATP is due to Mg^{++} chelation (see Chapter 5). A significant difference in the response of the two muscle PKs to ATP and L-alanine on the $K_{m(PEP)}$ was observed (Table 2). For WM-PK, like the condition for mammalian L-PK (Rozengurt et al., 1969), the presence of ATP and L-alanine increased the $K_{m(PEP)}$; for RM-PK, a marked decrease at pHs 8.5 (1.5-fold) and 7.5 (2-fold) was noted (Table 2). The resultant depressive effects of ATP and L-alanine are evident in examining the V_{max}/K_m values in Table 3. At all pHs examined, the ratios are depressed below the control values suggesting that catalytic rates at low substrate concentrations are significantly decreased. Importantly, the ratios are responsive to increasing H^+ ions and the values did increase with decreasing pH.

D. Interaction Between FDP, ATP and L-Alanine

The most meaningful physiological comparisons of the effects of ATP and L-alanine on tissue PKs are in the presence of FDP. For the eel muscle PKs, since the effects of FDP on V_{max} are limited to basic pHs, the ability of FDP to override ATP and L-alanine inhibition was also pH-dependent (Table 1). Thus, for WM-PK at pHs 8.5 and 7.5, the V_{max}-value for FDP plus ATP are equivalent to control values while FDP plus L-alanine activities are equivalent to FDP activated values. At pH 6.5, however, FDP is not able to override any of the inhibition by ATP and L-alanine. In contrast, the V_{max}-values of RM-PK in the presence of FDP and ATP or L-alanine

were depressed below control values at all pHs examined. As with WM-PK, ATP was also a more potent inhibitor of RM-PK.

The effects of FDP on ATP and L-alanine modulation of enzyme substrate affinity was significantly different for the two muscle PKs (Table 2). For WM-PK, the presence of FDP negated the inhibitory action of ATP and L-alanine and returned the $K_{m(PEP)}$ values to that observed with FDP alone (approximately 2.0 mM PEP) and this response was independent of pH. For RM-PK, however, while FDP with ATP or L-alanine did decrease the $K_{m(PEP)}$ to values obtained with FDP alone, the H^+ ion interaction was still present and thus as the pH decreased from 8.5 to 6.5, the $K_{m(PEP)}$ sequentially decreased. Thus, FDP freed the WM-PK $K_{m(PEP)}$ from modulation by H^+ ions, ATP and L-alanine; this response was not observed for RM-PK. The net effect of this modulatory action on $K_{m(PEP)}$ for WM-PK is readily apparent (Table 3). Here, the presence of FDP significantly increased the V_{max}/K_m ratio over that observed in the presence of ATP and L-alanine; further, this effect was H^+ ion sensitive such that as the pH was decreased the values were increased even more (compare 2.8 at pH 8.5 with 8.0 at pH 6.5 for ATP). As above, ATP was a much stronger effector of the V_{max}/K_m-values, depressing activities greater than L-alanine plus FDP at low substrate concentrations. For RM-PK, FDP also increased the V_{max}/K_m-values as compared to values determined with ATP and L-alanine present and this effect was enhanced by increasing H^+ ion concentrations. Importantly, with the addition of 1 μM FDP to both

muscle PKs, the catalytic rates in the presence of ATP and L-alanine was actually greater than control levels; eg., at pH 7.5, V_{max}/K_m-values for RM-PK with FDP plus ATP or L-alanine are approximately 4-fold greater than the control.

It is apparent that the response of the three eel enzymes to FDP, H^+ ions, ATP and L-alanine is different from that observed for the homologous mammalian PK enzymes, and also quantitatively different between the eel PKs. However, while the eel Lv-PK did not behave similarly to mammalian L-PK, its response was analogous to that observed for mammalian M_1-PK (Imamura et al., 1972). Further, under certain conditions (i.e., the presence of FDP) the eel WM-PK also behaved similarly to mammalian M_1-PK, in that H^+ ions, ATP and L-alanine fail to modulate the enzyme. The RM-PK, on the other hand, responds more like mammalian L-PK in that FDP, H^+ ions, ATP and L-alanine all exert an effect under conditions where none is observed for WM-PK.

DISCUSSION

These results and those presented in Chapter 5, clearly demonstrate that the PK isolated from red and white muscles and liver of the American eel, A. rostrata, represent yet another PK enzymatic system with unique regulatory characteristics. Although the PK from eel muscle, facultative anaerobes (Mustafa and Hochachka, 1971; de Zwann and Wijsman, 1976), diving mammals (Storey and Hochachka, 1974a), turtle heart (Storey and Hochachka, 1974b) and mammalian liver (eg., Rozengurt et al., 1969) are all modulated by H^+ ions, ATP, L-alanine and FDP, there are significant quantitative differences between the responses of PK from the eel and these other sources; however, a common regulatory strategy is emerging for these ligand sensitive PKs.

A. Eel Liver PK

The lack of a regulatory PK from eel liver is of interest in light of the mammalian enzyme which has been implicated in the transition between glycolysis and gluconeogenesis. Even the slight ATP inhibition of eel Lv-PK noted in Chapter 5, could be attributed solely to Mg^{++} chelation. A fish liver FDP-insensitive PK was reported by Somero and Hochachka (1968) for rainbow trout, but they offered no explanations for its presence. The maintenance of a non-regulatory PK in eel liver of the type observed for mammalian M_1-PK (Tanaka et al., 1967; Imamura et al., 1972) and frog heart muscle PK (Flanders et al., 1971) suggests three possibilities, First,

eel Lv-PK responds to effectors other than those examined, implying that the metabolic signals for gluconeogenesis and glycolysis are different from those in mammalian liver; second, that the liver in the eel is not a major gluconeogenic organ during migration, and third, that eel Lv-PK has an extremely high affinity for FDP and is saturated with this ligand. While the first two possibilities are tenable, the third is difficult to accept in light of the enzyme treatment (extensive dialysis) and the experiments with EDTA and ATP, two modulators which mediate the desensitization of the enzyme in mammals to FDP (Schubert and Schoner, 1971) (see Chapter 5). A further difference between the eel and mammalian enzymes is that the specific activity of the former is approximately 10-fold less than the latter (Scrutton and Utter, 1968).

In addition to a significantly higher specific activity, the mammalian L-PK has a higher initial catalytic rate as indicated by the V_{max}/K_m-values (the ratios were calculated from data presented in Scrutton and Utter, 1968). This ratio for mammalian L-PK is approximately 7-fold greater, at pH 7.5, than observed for the eel Lv-PK (Table 3). Thus, in terms of catalytic rate at both low (below K_m) and high (V_{max}) levels of substrate, the mammalian L-PK is a more efficient enzyme. The apparent $K_{m(PEP)}$ for the eel Lv-PK is, however, 8-fold lower than observed for mammalian L-PK (Rozengurt et al, 1969; Imamura et al., 1972), but within the range reported for mammalian M_1-PK (Imamura et al., 1972).

Unlike the mammalian enzyme, the $K_{m(PEP)}$ for eel Lv-PK is independent of H^+ ion concentrations between pH 6.5 and 8.5, an observation which has only been demonstrated for oyster adductor muscle (Mustafa and Hochachka, 1971) and eel WM-PK (Table 2) in the presence of FDP.

Assuming the eel liver enzyme is non-regulatory, its physiological significance is not immediately apparent. Unpublished ultrastructural observations of eel liver tissue indicate that a high degree of fatty infiltration and hyperplasia occurs with maturation (see Chapter 7). These data, coupled with the enzymatic results indicate that the regulation of carbohydrate and lipid metabolism in the mature migrating eel may be considerably more complex than previously accepted for other fishes and mammals. Although a correlation between the enzymatic results and a physiological role of the liver in nutritionally deprived migrating eels is of considerable interest, further studies are required to realize this goal.

B. Eel Muscle PKs

Johnston (1975) demonstrated that red muscle PK from the European carp was modulated by ATP, L-alanine and FDP in a manner similar to that described by Hochachka and associates for facultative anaerobes (see Hochachka and Somero, 1973). Further, Johnston (1975) proposed on the basis of the carp's habitat, that these modulatory interactions could be explained in a manner analogous to facultative anaerobes. Although our results indicate the existence of modulator-sensitive PKs in

red and white muscles, the effector interactions more closely resemble the response of the mammalian rather than the facultative anaerobe PKs. This statement is based primarily on the interaction between H^+ ions and PEP binding in the presence of the modulators ATP and L-alanine. In facultative anaerobes (Mustafa and Hochachka, 1971) and carp red muscle (Johnston, 1975), H^+ ions potentiate inhibition by ATP and L-alanine, whereas for eel muscle (Table 2) and mammalian L-PKs (Rozengurt et al., 1969), H^+ ions reverse ATP and L-alanine inhibition. This marked difference in H^+ ion-ligand interactions may reflect the nature of the metabolic end products accumulated during anaerobiosis by these organisms; mammals and presumably eels utilize LDH for redox balance and produce lactic acid, while facultative anaerobes utilize MDH (malate dehydrogenase) and produce the weaker acids, succinate and propionate.

For facultative anaerobes, the key to metabolic end-product accumulation during anaerobiosis is the competition between PK and PEP CK for the common substrate PEP. In these organisms, H^+ ions and L-alanine inhibit PK during anoxia and energy is generated through substrate level phosphorylations by carboxylating PEP with PEP CK to oxaloacetate and driving the Krebs cycle backwards (Hochachka and Somero, 1973). Since mammals, and presumably the eels (see appendix) do not carboxylate PEP in this manner, PK must remain functional even if cellular pH changes occur due to lactic acid accumulation. The effects of H^+ ions on the regulatory properties

of PK in these two groups of animals, therefore, is coupled directly to their unique physiology.

Interestingly, the resting oxygen tension of deep white epaxial muscle from the eel has been reported to be as low as 1-4 mm Hg (Jankowsky, 1966) although venous blood levels ranged from 20-50 mm Hg; this muscle value is at least an order of magnitude less than the 40 mm Hg reported for the oyster (Randall, 1974) and the intestinal parasite, Hymenolepis diminuta (Befus and Podesta, 1976), both of which are classified as facultative anaerobes. Even though the cellular environment would appear to favor a PK enzyme with regulatory characteristics in common with the facultative anaerobes, this does not appear to be the case in the eel. Therefore, any correlation between the PKs of eel muscle and carp red muscle would be premature in the absence of further studies. This infers, and the evidence suggests, that the regulatory characteristicsof eel WM- and RM-PKs may be more closely aligned with those of the mammalian enzymes.

For mammalian L- and M_2-PK, H^+ ions significantly alter PEP binding at both K_m and V_{max} levels of substrate. However, although there is no dispute that a decrease in assay pH below 7.0 results in a transition from sigmoidal to hyperbolic kinetics with an activation in V_{max} (Rozengurt et al., 1969; Schoner et al., 1970; Van Berkel et al., 1974), it has been questioned whether the pH will actually decrease below 7.0 in the intact mammalian liver for an alteration

in H^+ ions to act as an effective regulator (Llorent et al., 1970; Carbonell et al., 1973). Unfortunately, little data are available on in vivo liver pH or possible compartmentalization of H^+ ion concentrations.

H^+ ions also affect the heterotrophic cooperativity between FDP and PEP for mammalian L- and M_2-PKs (eg., Flory et al., 1974). Thus, after the transition from sigmoidal to hyperbolic kinetics occurs, FDP has no further activating effect. Therefore, the greatest effect of FDP on mammalian L-PK is observed above pH 7.0. Carminatti et al. (1968), for example, found that L-PK was activated (at K_m levels of substrate) 1.8-and 2.6-fold at pH 7.4 and 8.2, respectively. Although the eel RM- and WM-PKs exhibit hyperbolic kinetics with respect to PEP over the pH range 6.5 to 8.5. FDP is a mixed activator exerting its greatest effect at the more basic pHs, as reported for the mammalian L-PK (Tables 1 and 2). Wieker and Hess (1971) have proposed that this equivalent kinetic response to H^+ ions and FDP is a result of their converting the enzyme into an identical conformation with respect to PEP binding. This H^+ ion-FDP interaction is not, however, identical for the two eel muscle PKs (Tables 1 and 2). Consistent with the observations of Mustafa and Hochachka (1972) on oyster adductor muscle PK, the presence of FDP frees eel WM-PK from H^+ ion modulation, at least between pH 8.5 and 6.5. A similar pH decrease results in the $K_{m(PEP)}$ for eel RM-PK decreasing by the same increment regardless of the presence or absence of FDP. Therefore, once the WM-

enzyme is activated by either FDP or H^+ ions an optimal conformation for catalysis exists; however, for the RM-PK, H^+ ions and FDP act synergistically by enhancing the active conformation.

Another indication of the similarity between the effects of H^+ ions and FDP, is that the presence of either activator negates the inhibitory effects of ATP and L-alanine (Tables 1 and 2). Although quantitatively different, both eel muscle PKs and mammalian L-PK (Rozengurt et al., 1969) demonstrate this response. Both eel WM- and RM-PKs were inhibited approximately 70% by ATP and L-alanine at pH 8.5; however, decreasing the pH to 6.5 resulted in reversing L-alanine and ATP inhibition to 88% and 50% of control values, respectively, for WM-PK, while the inhibition was only reversed to approximately 40% of control values for both inhibitors for RM-PK

Another important property of mammalian L- and M_2-PK is the interaction between the heterotrophic ligand, FDP, and the allosteric inhibitors, ATP and L-alanine. Several investigators (eg., Llorent et al., 1970; Schubert and Schoner, 1971; Van Berkel, 1974) have suggested that it is this effect of FDP overriding ATP and L-alanine inhibition which constitutes the most significant in vivo regulatory signal for liver PK. Both ATP and L-alanine inhibit the mammalian enzymes competitively with PEP (Imamura et al., 1972; Van Berkel et al., 1973); FDP, a heterotrophic ligand of the 'K' allosteric type (Monod et al., 1965), reverses

the homotrophic cooperativity observed in the presence of the inhibitors ATP and L-alanine. The literature on mammalian L- and M_2-PKs, however, is not clear with regards to the extent of FDP reversal of this inhibition. Llorent et al. (1970) and Imamura et al. (1972), for example, have reported that 100 μM FDP overrides ATP and L-alanine inhibition and returns the activity to control levels. Rozengurt et al. (1969) on the other hand, found that 5 mM ATP inhibition was reversed by 2 μM and 100 μM FDP to only 52 and 60%, respectively.

One of the problems when comparing the modulation by FDP, ATP and L-alanine of the mammalian and eel muscle PKs, is that, as indicated in Chapter 5, FDP acts as a mixed heterotrophic effector of fish muscle PKs, affecting both K_m and V_{max} (Somero and Hochachka, 1968; Johnston, 1975), but only acts as a 'K' type effector (Monod et al., 1965) for mammalian regulatory PKs, affecting only K_m. Thus, FDP reversal of ATP and L-alanine inhibition of mammalian PKs refers specifically to activity levels at $K_{m(PEP)}$, whereas the picture is more complicated for the fish muscle enzymes.

The response of eel RM-PK at V_{max} levels of substrate (Table 1) is identical to that described above by Rozengurt et al. (1969) for mammalian L-PK; i.e., FDP does not reverse ATP or L-alanine inhibition even at 1 μM. Eel WM-PK, on the other hand, responds more like the data reported by Llorent et al. (1970); 1 μM FDP reversed ATP and L-alanine inhibition to near control levels depending upon pH. The ability of FDP

to override the inhibitory effects of ATP and L-alanine in variable, depending upon the fish species. Moon et al. (1971) reported that 0.1 mM FDP reverses ATP inhibition to 75% of control for sea bass muscle PK, to 90% of control in rainbow trout, and to 100% for leatherjacket muscle PK. Thus, sensitivity at V_{max} must be related in some manner to differences in enzyme structure, particularly at the FDP binding site, between species

Although these data characterize the respective enzyme-ligand interactions at high substrate levels, the effects at $K_{m(PEP)}$ are physiologically more significant (Hochachka and Somero, 1973). If we examine the $K_{m(PEP)}$ (Table 2), the data demonstrate that for WM-PK, the presence of FDP, with or without ATP and L-alanine, reduces the apparent enzyme-substrate affinity to approximately 0.2 mM PEP over the pH range 8.5 to 6.5. Thus, FDP is the most important regulatory parameter, making the $K_{m(PEP)}$ insensitive to all other modulators, be these H^+ ions, ATP or L-alanine. For eel RM-PK, 1 µM FDP negates the effects of the inhibitors ATP and L-alanine on the $K_{m(PEP)}$ at each pH examined, but FDP does not free the RM-PK from the H^+ ion interaction. Therefore, unlike the WM-PK, the RM-enzyme responds to H^+ ions even in the presence of other modulators. Thus, the activating effects of FDP and H^+ ions are the most distinctive differences between the WM- and RM-PKs, and strongly suggest that the eel WM-PK is analogous to mammalian M_1-PK and eel RM-PK is more typical of mammalian L-PK.

C. A Model for Eel Muscle PK

The kinetic responses of regulatory PKs to H^+ ions, ATP, L-alanine and FDP have been explained by many investigators according to the two-state allosteric model of Monod et al. (1965) (eg., Rozengurt et al, 1969). This model is adequate to explain the allosteric responses of the two regulatory mammalian PKs, L and M_2; however, for the two eel muscle PKs, another dimension must be considered. There are three basic problems in fitting the eel muscle PKs to the Monod model: first, there is no homotrophic cooperativity with respect to PEP in the absence of Na^+ ions; second, ATP and L-alanine do not result in homotrophic cooperative kinetics; and third, FDP is a mixed heterotrophic effector. The eel muscle PKs are true allosteric enzymes, however, since only one allosteric effector need demonstrate cooperative kinetics according to the Monod model, and for the eel enzyme, the presence of Na^+ ions result in sigmoidal PEP kinetics (Fig. 2, Chapter 5).

According to the Monod model, an allosteric protein can exist in two different conformational states, R and T. Using PK as an example, two allosteric systems are possible (Monod et al., 1965): a 'K' system where the R and T states have different affinities of PEP, but the modulators FDP, H^+ ions, L-alanine and ATP alter this affinity while not affecting the maximum catalytic activity of the state; a 'V' system where the two states have the same affinity for PEP and the modulators alter the catalytic activity, but not

$K_{m(PEP)}$. In both systems, positive effectors bind preferentially to the R conformation, which would be the highest substrate affinity state of the 'K' system or the highest catalytic state of a 'V' system. Negative effectors preferentially bind to the T conformation and in a 'K' system this would be the low substrate affinity state and the low activity state of a 'V' system. Another important aspect of the model is that the positive heterotrophic effectors (eg., FDP) in the presence of a negative homotrophic ligand, alter the degree of substrate cooperativity resulting in hyperbolic rather than sigmoidal kinetics.

The results presented above and in Chapter 5 suggest the eel muscle enzymes can be explained in part according to a simple two state system based on the sensitivity of the PK conformation to general ligand interaction rather than specific ligand binding as dictated by the Monod model. Electricfocusing data (Chapter 5) demonstrated the presence of two enzymatic forms for both muscle PKs, and that the more acidic form in each case was sensitive to FDP, ATP and L-alanine while the more basic peak was insensitive to these effectors. Further, incubation of the enzyme mixture with FDP before electricfocusing produced only the ligand sensitive enzyme peak; this result suggests that the equilibrium between the insensitive and sensitive forms is shifted to the sensitive forms, as in the Monod model. Although the sensitive form in the crude homogenate maintains an isoelectric point below the partially purified enzymes, these

two forms are essentially identical based upon their ligand interactions. A diagrammatic representation of a possible model, similar to that proposed by Van Berkel (1974) for rat liver M_2-PK, is presented in Fig. 1. Here, the ligand-insensitive form is designated as C_I and the ligand-sensitive (prepared in the presence of FDP or partially purified with $(NH_4)_2SO_4$) is designated C_{SL}. The C_{SL} form is characterized by giving Michaelis-Menton kinetics with respect to PEP, except in the presence of Na^+ ions when sigmoidal kinetics are observed (C). This part of the schematic ($C \rightleftharpoons C_{SL}$) can be interpreted according to Monod et al. (1965); where the presence of an effector (Na^+ ions) transforms the hyperbolic kinetics into cooperative kinetics with respect to PEP. The C_{SL} form is further characterized by having a low affinity for the substrate, PEP, and also a low catalytic rate in the presence of ATP and L-alanine. In the presence of FDP or increasing H^+ ion concentration, this low affinity, low activity form is converted into a high substrate affinity (low $K_{m(PEP)}$) and high catalytic activity form with Michaelis-Menton kinetics. Importantly, and consistent with the Monod model, FDP was able to reverse (though to different extents for the two inhibitors) the inhibition by ATP and L-alanine, as would be expected if C_{SL} and C_{SH} were in equilibrium.

Although not complete, this model could be used to design experiments to further characterize the eel regulatory PKs. It represents a model consistent, in part, with the

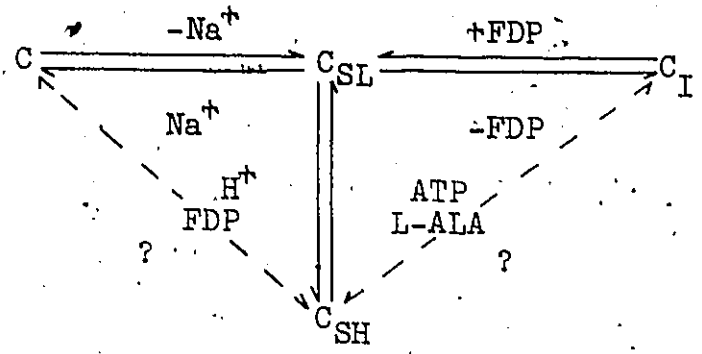


Fig. 1. A schematic of a model which may explain the kinetic behavior observed for the eel muscle PKs. C_I - insensitive conformation detectable in crude extracts as the basic isoelectric peak of the two peaks present (see Chapter 5). It is insensitive to FDP, ATP and L-alanine, but upon incubation with FDP is converted to C_{SL}. C_{SL} - sensitive, low substrate affinity configuration. The acidic form present upon electricfocusing crude homogenate, and the form persisting throughout partial purification. It is sensitive to FDP, ATP, and L-alanine, and is the predominate form in the absence of FDP. This form is characterized by low catalytic activity, low substrate affinity and Michaelis-Menton kinetics. C - conformation present with addition of Na^+ ions. It is the only form which gave sigmoidal kinetics with respect to the substrate PEP. C_{SH} - sensitive, high substrate affinity conformation. The form present with the addition of FDP and H^+ ions, and it has the highest activity and lowest $K_{m(PEP)}$ of the forms, and also gives Michaelis-Menton kinetics.

Monod-base mammalian models, but is not a true 'K' or 'V' system (eg., mixed ligand interactions support this interpretation). In fact, subtle differences must be incorporated in the model to predict accurately the behavior of the two eel muscle PKs.

D. Summary

These results suggest that under certain conditions, the $K_{m(PEP)}$ of eel WM-PK will be freed of all constraints imposed by inhibitory ligands and H^+ ion alteration. Under identical conditions, the $K_{m(PEP)}$ for eel RM-PK is modulated by the combined interaction of FDP, ATP, L-alanine and H^+ ions. Further, FDP reverses the inhibitory effects of ATP and L-alanine on V_{max} for WM-PK to a much greater extent than observed for the eel RM-PK. In effect, due to the marked difference in the effects of micromolar quantities of FDP on the two muscle enzymes, the eel WM-PK behaves analogously to the mammalian M_1-PK and the eel RM-PK to the mammalian L-PK. In contrast, the properties of eel Lv-PK indicate that glycolytic regulation and the gluconeogenic role of the liver in a migrating eel is probably much different from the picture normally accepted for mammalian and other fish livers. As a result of the differences in ligand sensitivity, the heterotrophic effects of FDP, and the Michaelis-Menton kinetics, the eel muscle PKs cannot be completely described by the general allosteric model of Monod et al. (1965). Thus, a model was postulated which may account for this altered behavior.

VII

GENERAL DISCUSSION

DISCUSSION

Five hypotheses were raised in the Introduction, which if confirmed, would be supportive evidence for Braekkan's (1956) red muscle-organ theory as applied to the American eel, A. rostrata, in the bronze phase. It is the aim of this final discussion to correlate and evaluate the results of the various experimental approaches used in this thesis in light of these hypotheses.

The first hypothesis was whether the structural properties of the red muscle were altered as a function of maturation but not white muscle. From results presented in Chapter 3, there can be little doubt that an alteration in the red muscle morphology does occur commensurate with maturation; however, the full extent of the physiological changes which may accompany the structural changes are unknown. No detectable change in the structural properties of the white muscle was observed.

It is tempting to speculate on the possible physiological changes accompanying these structural modifications in red muscle. If the contractile role of red muscle does diminish, as might be suggested from the data, and the metabolic recycling role increases during migration, then it is possible that a reflection of this altered physiological role might be observed in the eel liver. Possibly, the liver may become a more specialized biosynthesic site; for example, if the red muscle functioned to regulate whole body carbohydrate levels, the liver may function more in controlling

the lipid pools. As with red muscle, an altered role for liver may be suggested by a change in liver morphology between the liver of immature yellow and mature bronze eels. The results of an examination of the structural properties of liver tissue from immature and mature eels is presented in Figs. 1, 2 and 3. Fig. 1 is a representative section of liver from an immature yellow eel, while both Figs. 2 and 3 are from mature bronze eels. Two important observations can be made from these figures: first, the fine structure of the liver from the immature yellow eel is similar to mammalian liver (the main site of gluconeogenesis) but is significantly different from that observed for the mature bronze eels; and second, there is a significant degree of fatty infiltration, nuclear degeneration, and loss in structural definition in the liver from the mature bronze eel. Fig. 3 is a low magnification light micrograph which illustrates the overall degree of fatty deposition in this liver. These striking results suggest that an alteration in the function of the liver may have occurred. Indeed, the mature eel liver appears to be more of a lipid depot rather than a site of biosynthesis and recycling.

Interestingly, during short term starvation, liver glycogen in fish with red muscle (eg., European eel; Larsson and Lewander, 1973) and without (eg., northern pike; Ince and Thorpe, 1976) is mobilized to the same degree (83% decrease following 3-months starvation for both fish); however, it is only the fish with red muscle which can

Fig. 1. Electron micrograph of liver tissue from an immature feeding yellow eel. N. nucleus, L. lipid, M. mitochondria, LY. lyosome, G. glycogen, BC. bile canaliculus. X. 10500.

Fig. 2. Electron micrograph of liver tissue from mature migrating bronze eel. N. nucleus, L. lipid. X. 10500.

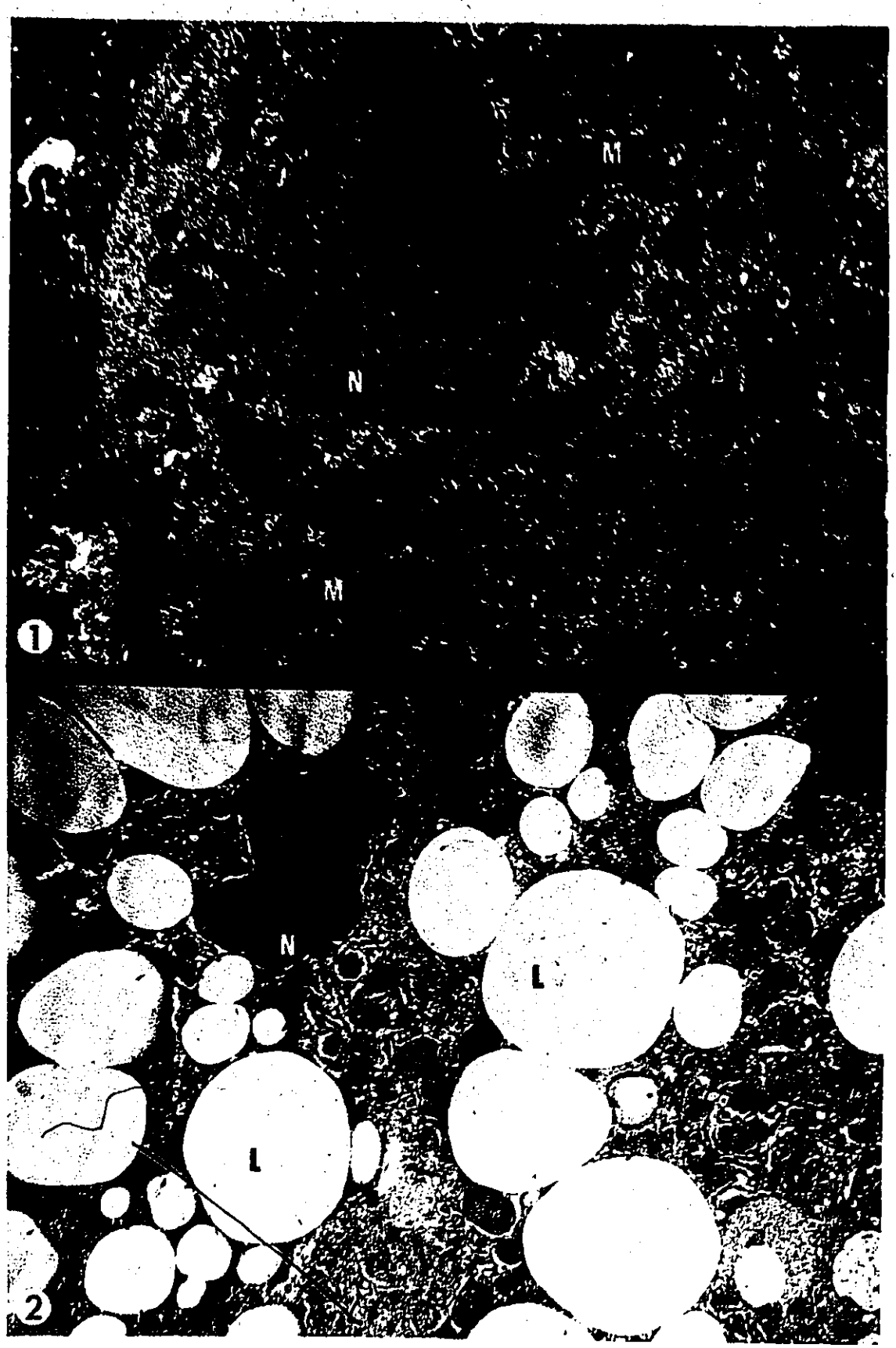
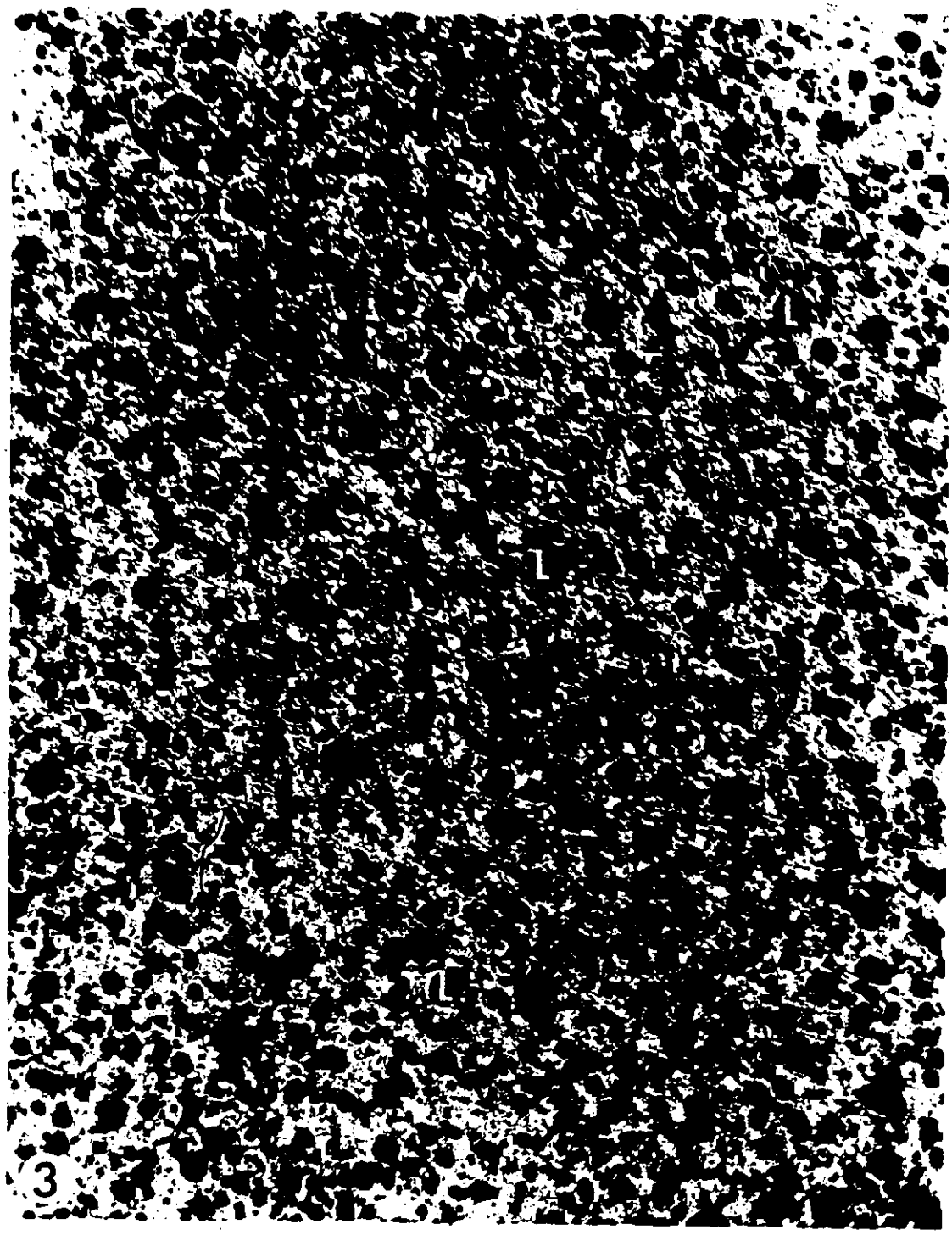

M
N
M
1
N
L
L
2

Fig. 3. Light micrograph of liver tissue from mature migrating bronze eel. L. lipid. X. 420.


1
1
1
3

maintain plasma glucose levels (15% decrease for the European eel following 164 days starvation (Dave et al., 1975), compared to 40% decrease for northern pike following 120 days starvation (Ince and Thorpe, 1976)) and muscle glycogen levels (no decrease for the Eurpoean eel following 164 days starvation (Dave et al., 1975), compared to 55% decrease for the northern pike following 120 days starvation (Ince and Thorpe, 1976)). Thus, consistent with the proposals of Wittenberger and co-workers (see Introduction), the altered structural appearance of the bronze eel red muscle may be associated with an increased role in regulating body carbohydrate levels. Albeit, this is speculative there is little doubt, however, that both the red muscle and liver in the bronze eels examined underwent marked structural changes which are suggestive of an alteration in their physiological roles. Further work is proceeding at this moment to describe the physiological role of the liver in immature and mature bronze eels.

The second hypothesis was whether white muscle LDH was primarily geared for the production of pyruvate and that red muscle LDH catalyzed an equilibrium reaction, the direction of catalysis depending upon the levels of pyruvate or lactate. Data presented in Chapter 4 indicate marked variations in the potential for lactate oxidation by the two muscle enzymes. At pH values which have been reported in fish white muscle (below 7.5; Love, 1970), the potential for eel white muscle LDH to oxidize lactate is virtually absent,

based on the high PR/LO ratios. On the other hand, the oxidative capacity (PR/LO ratios equal to 1.0) of red muscle appears independent of pH and thus it may function as an equilibrium reaction over the pH range 6.5 to 9.0. Further, the $K_{m(lac)}$ and $K_{m(NAD)}$ for the red muscle enzyme were significantly lower than WM-LDH. These data suggest different physiological functions for the two muscle enzymes and specifically implicate the red muscle in the metabolism of lactic acid, which is again at least indirect support of the red muscle-organ hypothesis.

The third and fourth hypotheses were related to the properties of eel tissue PK. This glycolytic enzyme has been extensively studied in mammals and other organisms, and its regulatory properties have been directly linked to a specific tissue metabolite pattern. Thus, a regulatory PK (eg., mammalian L- and M2-PK) would permit control over PEP metabolism and a gluconeogenic potential, while a non-regulatory PK (eg., mammalian M1-PK) would insure maximal oxidation and energy production. Data presented in Chapters 5 and 6 indicate that the PKs from the eel WM- and Lv-PKs are kinetically analogous to mammalian M1-PK and eel RM-PK is kinetically analogous to mammalian L-PK. Thus, the question is raised, what does this observation indicate about the metabolism of these eel tissues?

A possible overview of metabolism in the red muscle during migration must include both a possible metabolic recycling role as well as a contractile role. The fuel for

contractile activity will most likely arise from lipid oxidation (George, 1962). Thus, the ATP generated in the Krebs cycle and electron transport chain would result in increased concentration of some of the Krebs cycle intermediates, especially citrate. Citrate inhibits phosphofructokinase in both fish and mammals (Hochachka and Somero, 1973) and thus FDP levels would tend to be low. Under these conditions, the red muscle PK enzyme would be responsive to the endogenous levels of H^+ ions, ATP and L-alanine.

Red muscle is extensively vascularized and due to its small mass is probably in equilibrium with or slightly lower than, the bathing fluid. Black et al.,(1959) have demonstrated that rainbow trout blood pH does not decrease below 7.25 following strenuous excercise; if an analogous response is found for the eel, the potential role for modulation of red muscle PK by H^+ ions would appear minimal, even during swimming. However, since the RM-PK remains sensitive to H^+ ions, even in the presence of FDP, the capacity of H^+ ions to regulate this enzyme cannot be totally discounted (see below).

In mammals, cessation of excercise is accompanied by a release of large quantities of L-alanine from the muscle into the blood stream, and an L-alanine-glucose cycle between the muscle and liver has been proposed (Felig, 1973). There are no comparative data on fish plasma levels of L-alanine following excercise. However, it is well documented that many euryhaline poikilotherms, including the lamprey

(Cholette et al, 1970), increase and decrease the intracellular free amino acid pool in concert with increasing and decreasing external osmolarity; one of the most common amino acids utilized to alter the pool is alanine (eg., Schoffeniels and Gilles, 1970). Thus, during migration, cellular levels of L-alanine may increase as a result of either increased muscular contraction or in response to the osmotic chall of salt water. Under these conditions, and with possible changes in ATP, the activity of eel RM-PK will be mostly responsive to fluctuations in FDP levels, a situation proposed by Van Berkel (1974) for mammalian L-PK.

The proposal has been made that the red muscle in jack mackerel (Pritchard et al., 1971) and the goldfish (Smit et al., 1971) is utilized in conjunction with the white muscle during burst or sprint swimming; this theory may also apply to the migrating eel. The relative large quantities of red muscle glycogen, the modulation by H^+ ions and the FDP removal of ATP and L-alanine modulation of the $K_{m(PEP)}$ for RM-PK, support this contention. Thus, during periods of burst swimming, glycogen would be preferentially degraded; FDP concentrations would rise and PK activities would be keyed to the fall in H^+ ions resulting from increased lactic acid production. Further, the increased FDP would remove the red muscle enzyme from ATP and L-alanine inhibition during these anaerobic periods. Therefore, the RM-PK would be poised for the production of pyruvate eliminating a possible bottle-neck at the level of PEP in glycolysis

during burst swimming.

During migration, eel red muscle would be 1) obtaining contractile energy from lipid oxidation and generating glycerol, a potential gluconeogenic substrate; 2) faced with lactic acid generated from white muscle, another gluconeogenic substrate; and 3) faced with increased concentrations of L-alanine resulting from ionoregulation or muscular contraction; L-alanine is also a gluconeogenic substrate. The end result would be the marked inhibition of PK which would facilitate the conversion of the above substrates to carbohydrate. However, during periods of glycolytic demand, levels of FDP would increase and essentially free RM-PK from ATP and L-alanine modulation. Thus, the regulatory characteristics of eel RM-PK appear well suited to the physiological role required for both gluconeogenesis and glycolysis, a condition analogous to the mammalian L-PK.

Eel white muscle PK, although responsive to the modulators FDP, H^+ ions, ATP and L-alanine, in vivo is probably saturated with FDP due to its high affinity for this modulator. Thus, under these conditions, the ligand interactions with H^+ ions, ATP and L-alanine are removed. These responses would be advantageous if the mode of energy production is glycolytic, and the intracellular pH subject to variation. Thus, the maintenance of a PK enzyme which is insensitive to H^+ ions and other modulators, insures maximal glycolytic flux and prevents a bottle-neck at the level of PEP. Further, the pH optima for white muscle LDH (pyruvate

to lactate conversion) is also pH independent between 5.5 and 8.0. Both enzymes, therefore, are poised for the rapid conversion of PEP to lactic acid, a situation analogous to mammalian M1-PK. This conclusion may be naive however, since the maintenance of a regulatory enzyme for a non-regulatory purpose has not been previously observed. More work is necessary to further evaluate the in vivo function of this enzyme.

The lack of a regulatory PK enzyme in eel liver suggests that the functional role of the liver has been altered with maturation as compared to mammalian liver. Thus, the enzyme behaves as if it was geared for maintenance of glycolytic flux rather than participating in biosynthesis processes. The altered cellular structure supports this proposal and certainly suggests that other organs may take over the classic anabolic role of the liver in the bronze eel.

Therefore, data presented in Chapters 5 and 6 give supportive evidence that eel RM-PK responds to metabolic demands in a manner like that observed for mammalian L-PK, and that eel WM-PK, though responding to various modulators, in vivo may be kinetically analogous to mammalian M1-PK. The PK enzyme from eel liver was also observed to respond like mammalian M1-PK. These data give further, more direct, support for Braekkan's (1956) red muscle-organ hypothesis.

An important key to the suggestion that red muscle is poised predominately for the production of carbohydrates is

the demonstration of an active PEP CK enzyme (see Appendix 1), as implied by the fifth hypothesis. This enzyme, together with pyruvate carboxylase, form the concerted by-pass of PK and allow the production of PEP from lactate, L-alanine and other precursors which generate oxaloacetate. Using the most sensitive assay available, no PEP CK activity could be detected in eel white muscle. The activity of RM- and Lv-PEP CK, at the level of substrates used, was comparable to that found for the PEP CK from the tapeworm, H. diminuta, a faculative anaerobe with known high levels of PEP CK activity (Moon et al., 1976). The experiment is only qualitative, but it further supports the potential of red muscle to serve a recycling role.

Two important aspects of metabolism have been severely neglected in this discussion. The first is the integration of amino acid transamination to the energy conditions of the two muscle types. This problem is currently being explored and will be the subject of a further thesis. It might be predicted, however, that the levels of glutamate-pyruvate transaminase would increase, coupled with increased L-alanine concentrations, and that glutamate-oxaloacetate transaminase levels would decrease in red muscle. This condition was recently reported for the European carp (Wittenberger and Giurgea, 1973) under nutritional conditions designed to experimentally exploit the metabolic recycling role of red muscle. The second, and indeed more significant, aspect of metabolic control is the role of hormones. There can be

little doubt that the overall metabolic processes, both anabolic and catabolic, will be under the influence of the glucocorticoids and catecholamines; however, the literature dealing with hormonal regulation of fish metabolism, and in particular the effects of hormones on specific enzymatic reactions, is meager and inconsistent. Thus, this important aspect of metabolic control will have to await further experimentation.

In summary, these results are consistent with the red muscle-organ hypothesis of Braekkan (1956). Therefore, for a migrating American eel, A. rostrata, the red muscle appears to have the potential to act in an ancillary metabolic recycling manner possibly substituting for a liver which has a changed physiological role. This capacity for recycling is not shared by the white muscle, but this tissue may serve as the source for recyclable substrates for the red muscle.

VIII

APPENDIX 1

INTRODUCTION

It is now well established that, in mammals, the enzyme phosphoenol-pyruvate carboxykinase (EC 4.1.1.32; PEP CK) is unique to the gluconeogenic pathway and catalyzes the decarboxylation of oxaloacetate (Utter and Kurahashi, 1954; Bandurski and Lipmann, 1956; Nordlie and Lardy, 1963; Chang and Lane, 1966). The physiological significance of the PEP CK reaction is that together with pyruvate carboxylase it forms a bypass of the thermodynamically unfavorable reversal of pyruvate kinase (PK) (Krebs, 1954). Dyson et al. (1975), however, have recently demonstrated that the mammalian skeletal muscle M1-PK reaction is reversible and proposed it could play a role in supporting gluconeogenesis in muscle.

The PEP CK catalyzed reaction is the following:

$$OXA + \begin{matrix}ITP\\or\\GTP\end{matrix} \underset{\text{Mn or Mg}}{\rightleftharpoons} PEP + CO_2 + \begin{matrix}IDP\\or\\GDP\end{matrix}$$

Although the favored direction of PEP CK in mammals is OXA decarboxylation (Utter and Kurahashi, 1954), in facultative anaerobes such as bivalve molluscs (Hochachka and Mustafa, 1972) and intestinal parasites (Moon et al., 1976), PEP carboxylation is preferred. In these organisms, the enzyme is not gluconeogenic, but serves to couple anaerobic glycolysis to a portion of the Krebs cycle to enhance energy production from carbohydrates.

An examination of PEP CK activity was undertaken in the red and white muscle and liver of mature, bronze American eels, Anguilla rostrata, to assess the potential for gluco-

neogenesis in the respective tissues. The results suggest that both the red muscle and liver possess active PEP CK enzymes, but that this enzyme was undetectable in the white muscle.

MATERIALS AND METHODS

American eels, Anguilla rostrata, were collected from the St. Lawrence River at Quebec City, Quebec, during the seaward migratory phase of their life cycle. They were transported to the laboratory and maintained in tanks of free flowing dechlorinated Ottawa city tapwater at 15±3°C. Eels were decapitated and the liver, red muscle and white muscle rapidly removed and homogenized in five volumes (W/V) of ice cold Tris-HCl buffer, pH 7.4, for one minute at full speed in an iced Sorval Omnimixer. Following homogenization, the suspensions were centrifuged at 35,000x g for 25 mins in a refrigerated Sorval RC-2B centrifuge at 3°C. The resultant highspeed supernatants were diluted with Tris-HCl buffer, pH 7.4, to a final protein concentration of 10 mg/ml and used directly for the isotope assay.

All biochemicals were purchased from Sigma Chemical Company; other chemicals were purchased from local distributors and were of the highest purity available. The $KH^{14}CO_3$ was obtained from New England Nuclear.

The PEP CK activity was detected using a modification of the oxaloacetate-$H^{14}CO_3$ exchange assay of Chang and Lane (1966). In addition to catalyzing a reversible decarboxylation of oxaloacetate, PEP CK also catalyzes an ITP or GTP and Mn^{++} or Mg^{++}-dependent exchange between HCO_3^- and oxaloacetate (Utter and Kurahashi, 1954). This reaction is the most sensitive assay for PEP CK activity and can be

conducted using either crude homogenates or purified preparations without any detectable alteration in exchange rates (Chang and Lane, 1966).

The reaction mixture contained 0.1 mM oxaloacetate, 1 mM GTP or ITP, 8.0 mM Mg^{++} or 0.5 mM Mn^{++} and 0.015 mM $KH^{14}CO_3$ (specific activity, 7 mc/m mole), 0.5 ml of tissue homogenate (10 mg/ml) highspeed supernatent and Tris-HCl buffer, pH 7.4, to give a final volume of 1.0 ml. The reaction was initiated with the addition of oxaloacetate and allowed to proceed for 15 mins at 21°C. Radioactive oxaloacetate was reduced to malate with the addition of 0.3 mM NADH and the reaction was terminated 5 mins later with the addition of 0.5 ml 4 N HCl. The experiments were conducted in triplicate using a total of six animals. Aliquots of the reaction mixture were counted in duplicate and the results are given as CPM $\pm$2 sigma confidence intervals, corrected for background, quenching and efficiency. The data are expressed as CPM because a quantitative analysis of the reaction rate as a function of concentration of the added substrated was not conducted. The experiments were only designed to assess the presence or absence of the enzyme.

To insure reproducibility of results, the aliquots were counted after sitting in the cold for 48 hrs. This procedure was adopted to allow the non-incorporated, acid precipitated $H^{14}CO_3^-$ to diffuse out of solution. A time

course count revealed that 48 hrs were required to stabilize the number of counts.

Table 1. The effects of different nucleotides and divalent ions on PEP CK activity from red and white muscle and liver tissue from the American eel, A. rostrata. Two comparative values are also presented. See Materials and Methods for explanation of data presentation.

Assay Conditions	RM	WM	Lv
0 mM oxaloacetate 1.0 mM GTP 0.5 mM Mn	0	0	0
0.1 mM oxaloacetate 0 mM GTP 0.5 mM Mn	0	0	0
0.1 mM oxaloacetate 1.0 mM GTP 0.5 mM Mn	2985 ± 109[a] 4684 ± 140[b] 5239 ± 149[c]	0	7320 ± 175[a] 5968 ± 153[b]
0.1 mM oxaloacetate 1.0 mM GTP 8.0 mM Mg	2576 ± 103	0	3378 ± 123
0.1 mM oxaloacetate 1.0 mM ITP 8.0 mM Mg	3842 ± 130	0	4670 ± 139
0.1 mM oxaloacetate 1.0 mM ITP 0.5 mM Mn	4427 ± 132	0	5718 ± 154

a = values from single immature yellow eel

b = values from mature bronze eels (6)

c = values from homogenate of Hymenolepis diminuta

RESULTS AND DISCUSSION

The results presented in Table 1 suggest that both the eel red muscle and liver possess an active PEP CK and that this enzyme is either lacking in eel white muscle, or the conditions of the assay are inadequate to detect its presence. Further, although only one animal was examined, PEP CK activities tend to be higher in liver and lower in red muscle of the immature yellow eel than the mature bronze eel. The activity levels of eel red muscle and liver PEP CK were also found to be comparable to that obtained from H. Himinuta, an intestinal parasite known to maintain high levels of this enzyme for anaerobic metabolism (Moon et al., 1976).

The eel enzyme demonstrates specific ion requirements (Mn^{++} preferred to Mg^{++}) but, either guanosine or inosine trinucleotides can provide the nucleotide co-substrate. These results are consistent with those of Chang and Lane (1966) for PEP CK isolated from pig liver.

The presence of PEP CK in white muscle from chicken, rabbit, frog, and pigeon has been reported by Opie and Newsholme (1967); in fact, white muscle activities exceeded those in red muscle for these organisms. Opie and Newsholme (1967) suggested that the presence of High PEP CK and fructosediphosphatase (FDPase) activities in white muscle may reflect an emphasis on the utilization of the malate-oxaloacetate and glycerophosphate-dihydroxyacetone phosphate cycles for the maintenance of cellular redox balance. They

further suggested that the presence of PEP CK and FDPase would permit gluconeogenesis from those precursors producing oxaloacetate and malate; however, this is difficult to accept in light of the presence of a non-regulatory PK in most skeletal muscles (eg., Tanaka et al., 1967). The results reported here clearly do not support the presence of an active PEP CK in eel white muscle. These results are, however, consistent with the red muscle-organ hypothesis of Braekkan (1956). Thus, for the migrating American eel, A. rostrata, the red muscle may have an ancillary function involving the recycling of metabolic end products generated during muscular contraction, presumably from the white muscle. An active PEP CK would be a prerequisite to allow funneling of the various precursors through the gluconeogenic pathway. Also, this is the first report (to the authors knowledge) of a PEP CK in fish tissues.

IX

REFERENCES

REFERENCES

Alexander,R.McN. (1969) the orientation of muscle fibers in the myomeres of fishes. J.mar.biol.Ass.U.K. 49:263-290.

Andersen,P., J.K.S.Jansen, and Y.Loyning (1963) Slow and fast fibers in the Atlantic hagfish. Acta.Physiol.Scand. 57:167-179.

Anderson,P.J. and R.F.Randall (1975) Comparison of the subunit and primary structures of the pyruvate kinases from rabbit and sturgeon muscles. BiochemJ.145:575-579.

Awan,M.Z. and G.Goldspink (1972) Energetics of the development and maintenance of isometric tension by mammalian fast and slow muscle. J.Mechanochem.Cell.Motility.1:97-108.

Bailey,E., F.Stirpe, and C.G.Taylor (1968) Regulation of rat liver pyruvate kinase: The effect of preincubation, pH, copper ions, fructose 1,6-diphosphate and dietary changes on enzyme activity. Biochem.J.108:427-436.

Bailey,G.S. and A.C.Wilson (1968) Homologies between isoenzymes of fishes and those of higher vertebrates. J. Biol.Chem.243(22):5843-5853.

Bandurski,R.S. and F.Lipmann (1956) Studies on an oxaloacetic carboxylase from liver mitochondria. J.Biol.Chem.219: 741-752.

Barany,M. (1967) ATPase activity of myosin correlated with speed of muscle shortening. J.Gen.Physiol.50:197-216.

Befus,A.D. and R.B.Podesta (1976) Problems associated with the gastrointestinal lumen as a habitat for metazoan parasites. In: Ecological Aspects of Parasitology, C.R. Kennedy, ed., North Holland, Amsterdam, in press.

Bergman,R.A. (1964) The structure of the dorsal fin musculature of the marine teleosts, Hippocampus hudsonius and H. zosterae. Bull.John Hopkins Hosp.114:325-343.

Bergman,R.A. (1967) Motor nerve endings of twitch muscle fibers in Hippocampus hudsonius. J.Cell.Biol.32:751-757.

Bertin,L. (1956) Eels-A biological study. Cleaver-Hume Press, London,pp.192.

Bilinski,E. (1963) Utilization of lipids by fish I. Fatty acid oxidation by tissue slices from dark and white muscle of rainbow trout (Salmo gairdneri). Can.J. Biochem.Physiol.41:107-112.

Bilinski,E. (1969) Lipid catabolism in fish muscle, In: Fish in Research, O.W.Neuhaus and J.E.Halver, eds., Academic Press, New York,pp.135-151.

Bilinski,E. (1974) Biochemical aspects of fish swimming. In: Biochemical Perspectives in Marine Biology.Vol.1,D.C. Malins and J.R.Sargent,eds., Academic Press, New York, pp.239-288.

Bishop,C.M. and P.H.Odense (1967) The ultrastructure of the white striated myotomal muscle of the cod, Gadus morphus. J.Fish.Res.Bd.Can.24:2549-2553.

Black,E.C., W.G.Chiv, F.D.Forbes, and A.Hanslip (1959) Changes in pH, carbonate and lactate of the blood of yearling kamloops trout, Salmo gairdnerii, during and following severe muscular activity. J.Fish.Res.Bd.Can.16(4):391-402.

Blazka,P. (1958) The anaerobic metabolism of fish. Physiol. Zool.31:117-128.

Boddeke,R., E.J.Slijper, and A.Van der Stelt (1959) Histological characteristics of the body musculature of fishes in connection with their mode of life. Proc.Ser.C.Bio. Med.Sci.42:576-587.

Bokdawala,F.D. (1967) A histochemical study of fat in the red and white muscle fibres of fish skeletal muscle. J. Anim.Morph.Physiol.14:231-241.

Bone,Q. (1966) On the function of the two types of myotomal muscle fibre in elasmobranch fish. J.mar.biol.Ass.U.K. 46:321-349.

Borgmann,A.I. and T.W.Moon (1976) Enzymes of the normothermic and hibernating bat,Myotis lucifugus: Temperature as a modulator of pyruvate kinase.J.Comp.Physiol.107:185-199.

Bostrom,Stig-L., and R.G.Johansson (1972) Enzyme activity patterns in white and red muscle of the eel (Anguilla anguilla) at different developmental stages.Comp.Biochem. Physiol.42B:533-542.

Boyer,P.D. (1959) Pyruvate kinase. In: The Enzymes, Vol.VI, P.D.Boyer,H.Lardy, and K.Myrback,eds., Academic Press, New York,pp.95-114.

Braekkan,O.R. (1956) Function of red muscle in fish.Nature 178:747-748.

Braekkan,O.R. (1959) A comparative study of vitamins in the trunk muscles of fishes. Fisk.Dir.Skr.Serio.Tek.Under. 3(8):1-42.

Bucher,T. and G.Pfleiderer (1955) In: Methods in Enzymology Vol.1., S.P.Colowick and N.O.Kaplan,eds., Academic Press, New York,pp.435-436.

Burke,R.E., D.N.Levine, F.E.Zajac, P.Tsairis, and W.K.Engel (1971) Mammalian motor units: Physiological-histochemical correlation in three types in cat gastrocnemius. Science. 174:709-711.

Buttkus,H. (1963) Red and white muscle of fish in relation to rigor mortis. J.Fish.Res.Bd.Can.20(1):45-58.

Carbonell,J., J.E.Feliu, R.Marco, and A.sols (1973) Pyruvate kinase, classes of regulatory isoenzymes in mammalian tissues. Eur.J.Biochem.37:148-156.

Cardenas,J.M., J.J.Stranholm, and J.M.Miller (1975) Bovine pyruvate kinase. Biochem.14:4041-4045.

Carminatti,H. L.Jimenez de Asua, E.Recondo, S.Passeron, and E.Rozengurt (1968) Some kinetic properties of liver pyruvate kinase (type L). J.Biol.Chem.243(11):3051-3056.

Carminatti,H. L.Jimenez de Asua, B.Leiderman, and E.Rozengurt (1971) Allosteric properties of skeletal muscle pyruvate kinase. J.Biol.Chem.246(23):7284-7288.

del Castillo,J., G.Hoyle, and X.Machne (1953) Neuromuscular transmission in a locust. J.Physiol.121:539-547.

Chang,H.-C. and M.D.Lane (1966) The enzymatic carboxylation of phosphoenol pyruvate. II. Purification and properties of liver mitochondrial phosphoenolpyruvate carboxykinase. J.Biol.Chem.241(10):2413-2420.

Cholette,C. A.Gagnon, and P.Germain (1970) Isomotic adaptation in Myxiene glutinse L. I. Variations of some parameters and role of amino acid pool in muscle cells. Comp. Biochem.Physiol.33:333-346.

Close,R. (1972) Dynamic properties of mammalian skeletal muscle. Physiol.Rev.52:129-170.

Dave,G. M.-L.Johansson, A.Larsson, K.Lewander, and V.Lidman (1974) Metabolic and hematological studies on the yellow and silver phases of the European eel, Anguilla anguilla. Comp.Biochem.Physiol.47B:583-591.

Dave,G. M.-L.Johansson, A.Larsson, K.Lewander, and V.Lidman (1975) Metabolic and hematological effects of starvation in the European eel, Anguilla anguilla L. I.Carbohydrate, lipid, protein and inorganic ion metabolism. Comp.Biochem. Physiol.52A:423-430.

Davis,D.J. (1964) Disc electrophoresis II. Method and application to human serum proteins. Ann.N.Y.Acad.Sci.121: 404-427.

Diamond,J.M. and W.H.Bossert (1967) Standing-gradient osmotic flow: A mechanism to coupling of water and solute transport in epithelia. J.Gen.Physiol.50:2061-2073.

Driedzic,W.R. and P.W.Hochachka (1975) The unanswered question of high anaerobic capabilities of carp white muscle. Can.J.Zool.53(6):706-712.

Drummond,G.I. (1971) Microenvironment and enzyme function: Control of energy metabolism during muscle work. Am. Zool.11(1):83-97.

Drummond,G.I. and E.C.Black (1960) Comparative physiology: fuels of muscle metabolism. Ann.Rev.Physiol.22:169-190.

Dyson,R.D., J.M.Cardenas, and R.J.Barsotti (1975) The reversibility of skeletal muscle pyruvate kinase and an assessment of its capacity to support glyconeogenesis. J.Biol.Chem.250(9):3316-3321.

Eales,J.G. (1968) The eel fisheries of eastern Canada. J. Fish.Res.Bd.Can.Bull.166.pp.77.

Edgerton,V.R. and D.R.Simpson (1969) The intermediate muscle fiber of rats and guinea pigs. J.Histochem.Cytochem.17: 828-837.

Everse,J., R.L.Berger, and N.O.Kaplan (1970) Physiological concentrations of lactate dehydrogenases and substrate inhibition. Science.168:1236-1238.

Everse,J. and N.O.Kaplan (1973) LDH:Structure and function Adv.Enzymol.37:61-133.

Fatt,P. and B.Katz (1953) Distributed 'end-plate potentials' of crustacean muscle fibres. J.Exp.Biol.30:433-439.

Felip,P.(1973) The glucose-alanine cycle. Metab.22(2):179-207.

Flanders,L.E., J.R.Bamburg, and H.J.Sallach (1971) Pyruvate kinase isozymes in adult tissue and eggs of Rana Pipiens II. Physical and kinetic studies of purified skeletal and heart muscle pyruvate kinases. Biochim.Biophys.Acta. 242:566-579.

Flory,W. B.D.Peczon, R.E.Koeppe, and H.O.Spivey (1974) Kinetic properties of rat liver pyruvate kinase at cellular concentrations of enzyme, substrates and modifiers. Biochem.J.141:127-131.

Franzini-Armstrong,C. and K.R.Porter (1964) Sarcolemmal invaginations constituting the T system in fish muscle fibers. J.Cell.Biol.22:675-695.

Friedman,B., E.H.Goodman, H.L.Saunders, V.Kostos, and S. Weinhouse (1970) Estimation of pyruvate recycling during gluconeogenesis in perfused rat liver. Fed.Proc. Fed.Amer.Soc.Exp.Biol.29:676.

Friedman,B., E.H.Goodman, H.L.Saunders, V.Kostos, and S. Weinhouse (1971) An estimation of pyruvate recycling during gluconeogenesis in the perfused rat liver. Arch. Biochem.Biophys.143:566-578.

Gauthier,G.F. (1970) The ultrastructure of the three fiber types in mammalian skeletal muscle. In: The Physiology and Biochemistry of Muscle as a Food. Vol.2., E.J.Brisky, R.G.Cassens, and B.B.Marsh, eds., University of Wisconsin Press, Madison,pp.103-157.

George,J.C. (1962) A histophysiological study of the red and white muscles of the mackerel.Am.Midl.Nat.68:487-494.

George,J.C. and F.D.Bokdawala (1964) Cellular organization and fat utilization in fish muscle. J.Anim.Morph.Physiol. 11:124-132.

Gesser,H. and O.Poupa (1973) Tha lactate dehydrogenase system in the heart and skeletal muscle of fish: A comparative study. Comp.Biochem.Physiol.46B:683-690.

Goldberg,E. (1965) Lactate dehydrogenases in trout: Evidence for a third subunit. Science.148:391-392.

Goldspink,G. R.E.Larson, and R.E.Davies (1970a) Thermodynamic efficiency and physiological characteristics of chick anterior latissimus-dorsi muscle. Z.Vergl.Physiol.66: 379-388.

Goldspink,G., R.E.Larson, and R.E.Davies (1970b) The immediate energy supply and the cost of maintenance of isometric tension for different muscles in the hamster. Z.Vergl. Physiol.66:389-397.

Gordon,M.S. (1968) Oxygen consumption of red and white muscles from tunafishes. Science.159:87-9-.

Gordon,M.S. (1972) Comparative studies on the metabolism of shallow-water and deep-sea marine fishes.II Red-muscle metabolism in shallow-water fishes. Mar.Biol. 15:246-250.

Greer-Walker,M. (1970) Growth and development of the skeletal muscle fibres of the cod (Gadus morhua L.). J.Cons. Perm.Int.Explor.Mer.33:228-244.

Greer-Walker,M. (1971) Effect of starvation and exercise on the skeletal muscle fibers of the cod (Gadus morhua L.) and the coalfish (Gadus virens L.) respectively. J.Cons.Perm. Int.Explor.Mer.33:421-426.

Greer-Walker,M. and G.Pull (1973) Skeletal muscle function and sustained swimming speeds in the coalfish Gadus virens L. Comp.Biochem.Physiol.44A:495-501.

Greer-Walker,M. and G.Pull (1975) A survey of red and white muscle in marine fish. J.Fish.Biol.7:295-300.

Hems,R., B.D.Ross, M.N.Berry, and H.A.Krebs (1966) Gluconeogenesis in the perfused rat liver. Biochem.J.101:284-292.

Hermansen (1976) Personal communication

Hess,A. (1965) The sarcoplasmic reticulum, the T system, and the motor terminals of slow and twitch muscle fibers in the garter snake. J.Cell.Biol.26:467-476.

Hess,A. (1966) The fine structure of the striated muscle fibres and their nerve terminals in the avian iris: Morphological 'twitch-slow' fibres. Anat.Rec.154:357-369.

Hess,A. (1970) Vertebrate slow fibers. Physiol.Rev.50:40-62.

Hess,A. and G.Pilar (1963) Slow fibres in the extraocular muscles of the cat.J.Physiol.169:780-787.

Hess,B., R.Haeckel, and K.Brand (1966) FDP-activation of yeast pyruvate kinase. Biochem.Biophys.Res.Comm.24(6): 824-831.

Hess,B. and C.Kutzbach (1971) Identification of two types of liver pyruvate kinase. Z.Physiol.Chem.352:453-458.

Hess,R., D.G.Scarpelli, and A.G.E.Pearse (1958) Cytochemical localization of pyridine nucleotide-linked dehydrogenases. Nature.181:1531-1532.

Hidaka,T. and N.Toida (1969) Biophysical and mechanical properties of red and white muscle fibres in fish.J.Physiol. 201:49-59.

Hochachka,P.W. (1965) Isoenzymes in metabolic adaptation of a poikilotherm: Subunit relationships in lactic dehydrogenases of goldfish. Arch.Biochem.Biophys.111:96-103.

Hochachka,P.W. (1966) Lactate dehydrogenases in poikilotherms: Definition of a complex isozyme system. Comp.Biochem. Physiol.18:261-269.

Hochachka,P.W. and T.Mustafa (1972) Enzyme mechanisms and pathways in invertebrate faculative anaerobiosis. Science. 176:1056-1060.

Hochachka,P.W. and G.N.Somero (1973) Strategies of Biochemical Adaptation. W.B.Saunders company, Philadelphia,pp.358.

Hochachka,P.W., C.Norberg, J.Baldwin, and J.H.A.Fields (1976) Enthalpy-entropy compensation of oxamate binding by homologous lactate dehydrogenases. Nature.260:648-650.

Hofmann,E., B.I.Kurganov, W.Schellengerger, J.Schulz, G.Sparmamn, K.-W.Wenzel, and G.Zimmermann (1975) Association-dissociation behavior of erythrocyte phosphofructokinase and tumor pyruvate kinase. In:Advances in Enzyme Regulation.Vol.13. G.Weber, ed., Academic Press, New York,pp.247-277.

Hudson,R.C.L (1973) On the function of the white muscles in teleosts at intermediate swimming speeds. J.Exp.Biol. 58:509-522.

Huxley,H.E. (1966) The fine structure of striated muscle and its functional significance. In: The Harvey Lectures 1964-65. Academic Press, New York,pp.85-118.

Ibsen,K.H., K.W.Schiller, and T.A.Haas (1971) Interconvertible kinetic and physical forms of human erythrocyte pyruvate kinase. J.Biol.Chem.246:1233-1240.

Ibsen,K.H., L.Murray, and S.W.Marles (1976) Electrofocusing and kinetic studies of adult and embryonic chicken pyruvate kinases. Biochem.15(5):1064-1073.

Imamura,K and T.Tanaka (1972) Multimolecular forms of pyruvate kinase. J.Biochem.71:1043-1057.

Imamura,K., K.Taniuchi, and T.Tanaka (1972) Multimolecular forms of pyruvate kinase II. Purification of M_2-type pyruvate kinase from Yoshida Ascites Hepatoma 130 cells and comparative studies on the enzymological and immunological properties of the three types of pyruvate kinases L,M_1 and M_2. J.Biochem.72:1001-1015.

Ince,B.W. and A.Thorpe (1976) The effects of starvation and force-feeding on the metabolism of the northern pike, Esox lucius L. J.Fish.Biol.8:79-88.

Irving,M.G. and F.J.Williams (1973) Studies on the interaction between rabbit liver pyruvate kinase and its allosteric effector fructose 1,6-diphosphate. Biochem.J.131:303-313.

Jankowsky,H.-D. (1966) The effect of adaptation temperature on the metabolic level of the eel Anguilla vulgaris L. Helgolander Wiss.Meersunters.13:402-407.

Jedryczkowski,W. and Z.Fitscher (1973) Preliminary report on the metabolism of the silver eel (Anguilla anguilla L.) Pol.Arch.Hydrobiol.20(3):507-516.

Jimenez de Asua,L., E.Rozengurt, J.J.Devalle, and H.Carminatti (1971) Some kinetic differences between the M isozymes of pyruvate kinase from liver and muscle. Biochem.Biophya. Acta.235:326-334.

Johnston,I.A. (1975) Pyruvate kinase from the red skeletal musculature of the carp (Carassius carassius L.) Biochem. Biophys.Res.Comm.63(1):115-120.

Johnston,I.A., N.Frearson, and G.Goldspink (1972) Myofibrillar ATPase activities of red and white myotomal muscles of marine fish. Experientia.28:713-714.

Johnston,I.A. and G.Goldspink (1973) A study of the swimming performance of the crucian carp (Carassius carassius L.) in relation to the effects of exercise and recovery on biochemical changes in the myotomal muscle and liver.J. Fish.Biol.5:249-260.

Johnston,I.A., P.S.Ward, and G.Goldspink (1975) Studies on the swimming musculature of the rainbow trout I.Fiber types. J.Fish.Biol.7:451-458.

Kaplan,N.O. and J.Everse (1972) Regulatory characteristics of LDHs. In: Advances in Enzyme Regulation,Vol.10, G. Weber, ed., Pergamon Press, Oxford,pp.323-336.

Kaplan,N.O. and T.L.Goodfriend (1964) The role of the two types of lactic dehydrogenases. In: Advances in Enzyme Regulation,Vol.2,G.Weber, ed., Pergamon Press, Oxford, pp.203-212.

Kapoor,M (1975) Subunit structure and some properties of pyruvate kinase of Neurospora. Can.J.Biochem.53(2): 109-119.

Kapoor,M. and T.M.Tronsgaard (1972) Conformational changes of Neurospora pyruvate kinase induced by tris buffer. F.E.B.S.Lett.23(3):349-351.

Katz,J. and R.Rognstad (1976) Futile cycles in the metabolism of glucose. In: Current Topics in Cellular Regulation, Vol.10, B.L.Horecker and E.R.Stadtman, eds., Academic Press, New York,pp.237-289.

Kayne,F.J. (1973) Pyruvate kinase. In: The Enzymes,Vol.8. P.D.Boyer, ed., Academic Press,New York,pp.353-382.

Kayne,F.J. and C.H.Suelter (1968) The temperature-dependent conformational transitions of pyruvate kinase.Biochem. 7:1678-1684.

Kilarski,W. (1960) Fat in some teleost. Acta.Biol.Crac.Zool. 3:23-57.

Kilarski,W. (1967) The fine structure of striated muscles in teleosts. Zeit.Zellforsch.79:562-580.

Knappeis,G.G. and F.Carlsen (1962) The ultrastructure of the Z disc in skeletal muscle. J.Cell.Biol.13:323-337.

Kobzyshi,K. (1969) Light and electromicroscopic studies on the pancreatic acinar and islet cells in Xenopus laevis. Gunma J.Med.Sci.42:60-103.

Korneliussen,H. and K.Nicolaysen (1975) Distribution and dimension of the T-system in different muscle fiber types in the atlantic hagfish (Myxine glutinosa,L.) Cell.Tiss. Res.157:1-16.

Krebs,H.A. (1954) Considerations on the pathways of synthesis in living matter. Bull.John Hokpins Hosp.95:19-33.

Larsson,A. and K.Lewander (1973) Metabolic effects of starvation in the eel, Anguilla anguilla L. Comp.Biochem. Physiol. 44A:367-374.

Layne,F.C. (1957) Spectrophotometric and turbidimetric methods for measuring proteins. In: Methods in Enzymology,Vol.III. S.P.Colowick and N.O.Kaplan, eds., Academic Press, New York,pp.451-454.

Lewander,K., G.Dave, M.-L.Johansson, A.Larsson, and V.Lindman (1974). Metabolic and hematological studies on the yellow and silver phases of the European eel, Anguilla anguilla L. I.Carbohydrate, lipid, protein, and inorganic ion metabolism. Comp.Biochem.Physiol.47B:571-581.

Llorent,P., R.Marco, and A.Sols (1970) Regulation of liver pyruvate kinase and the phosphoenol-pyruvate crossroads. Eur.J.Biochem.13:45-54.

Llungstrom,O., G.Hjelmquist, and L.Engstrom (1974) Phosphorylation of purified rat liver pyruvate kinase by cyclic 3', 5'-AMP-stimulated protein kinase. Biochim.Biophys. Acta.358:289-298.

Love,R.M. (1970) The Chemical Biology of Fishes. Academic Press, New York.pp.547.

Lowry,D.H., N.J.Rosebrough, A.L.Farr, and R.J.Randall (1951) Protein measurement with the folin phenol reagent. J.Biol. Chem.193:265-270.

Luft,J.H. (1961) Improvements in epoxy resin embedding methods. J.Biophys.Biochem.Cytol.9:409-414.

Lush,I.E., C.B.Cowey, and D.Knox (1969) The lactate dehydrogenase isozymes of twelve species of flat fish (Heterosomata). J.Exp.Zool.171:105-118.

Lush,I.E. (1970) Lactate dehydrogenase isozymes and their genetic variation in coalfish (Gadus virens) and cod (Gadus morpha). Comp.Biochem.Physiol.32:23-32.

Markert,C.L. and I.Faulber (1965) Lactate dehydrogenase isozyme patterns of fish. J.Exp.Zool.159:319-332.

Markert,C.L. and R.S.Holmes (1969) Lactate dehydrogenase isozymes of the flat fish Pleuronectifornes: kinetic, molecular and immunological analysis.J.Exp.Zool.171:85-104.

Markert,C.L., J.B.Shaklee, and G.S.Whitt (1975) Evolution of a gene. Science.189:102-114.

McCance,R.A. (1944) The chemistry of growth and food value of the common eel (Anguilla anguilla (L.)). Biochem.J. 38:474-479.

Monod,J., J.Wyman, and J.P.Changeux (1965) On the nature of allosteric transitions: A plausible model. J.Mol.Biol. 12:88-118.

Moon,T.W. and A.I.Borgmann (1976) Enzymes of the normothermic and hibernating bat, Myotis lucifugus: Metabolites as modulators of pyruvate kinase. J.Comp.Physiol.107: 201-210.

Moon,T.W., T.Mustafa, and P.W.Hochachka (1971) The adaptation of enzymes to pressure II. A comparison of muscle pyruvate kinases from surface and mid water fishes with the homologous enzyme from an offshore benthic species. Am. Zool.11:491-502.

Moon,T.W., T.Mustafa, W.C.Hulbert, R.B.Podesta, and D.F.Mettrick (1976) The phosphoenol-pyruvate branchpoint in adult Hymenolepis diminuta (Cestoda): A study of the pyruvate kinase and phosphoenol-pyruvate carboxykinase. J.Exp.Biol. in press.

Munshi,J.S.D., J.Ouha, and A.K.Mittal (1975) Succinic dehydrogenase activity of the respiratory muscles of a fresh water teleost, Bagarius bagarius (Ham.) (Sisoridae, Pisces). Acta.Anat.92:534-559.

Mustafa,T. and P.W.Hochachka (1971) Catalytic and regulatory properties of pyruvate kinases in tissues of a marine bivalve. J.Biol.Chem.246(10):3196-3203.

Mustafa,T., T.W.Moon, and P.W Hochachka (1971) Effects of pressure and temperature on the catalytic and regulatory properties of muscle pyruvate kinase from an offshore benthic fish. Am.Zool.11:451-466.

Nachlas,M.M., K.C.Tsuo, E.de Souza, C.S.Cheng, and A.M. Seligman (1957) Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazone. J.Histochem.Cytochem.5:420-424.

Nag,A.C. (1972) Ultrastructure and adenosine triphosphatase activity of red and white muscle fibers of the caudal region of a fish, Salmo gairdnerii. J.Cell.Biol.55:42-57.

Nag,A.C. and J.R.Nursall (1972) Histogenesis of white and red muscle fibres of the trunk muscle of a fish, Salmo gairdnerii. Cytobios.6:227-247.

Nakajima,Y.(1969) Fine structure of red and white muscle fibers and their neuromuscular junctions in the snake fish (Ophiocephalus argus). Tiss.Cell.Res.1(2):229-246.

Newsholme,E.A. and C.Start (1973) Regulation in Metabolism. John Wiley and Sons, London.pp.349.

Niles,N.R., J.Chayen, G.J.Cunningham, and L.Bittensky (1964) Cytological localization of Ca^{++} activated ATPase. J. Histochem.Cytochem.12:740-743.

Nishihara,H. (1967) Studies on the fine structure of red and white fin muscles of the fish (Carassius auratus). Arch.Histol.Jap.28(4):425-447.

Nordlie,R.C. and H.A.Lardy (1963) Mammalian liver phosphoenolpyruvate carboxykinase activities. J.Biol.Chem. 238(7):2259-2263.

Nursall,J.R. (1955) The lateral musculature and the swimming of fish. Proc.Zool.Soc.Lond.126:127-143.

Opie,L.H. and E.A.Newsholme (1967) The activities of fructose 1,6-diphosphatase,phosphofructokinase and phosphenolpyruvate carboxykinase in white muscle and red muscle. Biochem.J. 103:391-399.

Page,S.G. (1965) A comparison of the fine structures of frog slow and twitch muscle fibers. J.Cell.Biol.26:477-483.

Page,S.G. (1966) Intrafusal muscle fibres in the frog. Int. Micro.5:101-104.

Patterson,S. and G.Goldspink (1972) The fine structure of red and white myotomal muscle fibres of the coalfish (Gadus virens). Z.Zellforsch.133:463-473.

Patterson,S., I.A.Johnston, and G.Goldspink (1975) A histochemical study of the lateral muscles of five teleost species. J.Fish.Biol.7:159-166.

Peachey,L.D. (1968) Muscle. Ann.Rev.Physiol.30:410-429.

Peachey,L.D. (1966) Fine structure of two fiber types in cat extraocular muscles. J.Cell.Biol.31:84A.

Peachey,L.D. and A.F.Huxley (1962) Structural identification of twitch and slow striated muscle fibers of the frog. J.Cell.Biol.13:177-185.

Pogson,C.I. (1968a) Two interconvertible forms of pyruvate kinase in adipose tissue. Biochem.Biophys.Res.Comm. 30(3):297-302.

Pogson,C.I. (1968b) Adipose tissue pyruvate kinase properties and interconversion of two active forms. Biochem.J.110: 67-77.

Pritchard,A.W., J.R.Hunter, and R.Lasker (1971) The relation between exercise and biochemical changes in red and white muscle and liver in the jack mackerel, Trachurus symmetricus. Fish.Bull.69(2):379-386.

Prosser,C.L. (1973) Muscles. In: Comparative Animal Physiology C.L.Prosser, ed., W.B.Saunders Company,Philadelphia., pp.719-780.

Radola,B.J. (1969) The use of isoelectricfocusing in the separation of proteins. Biochem.Biophys.Acta.184:335-338.

Randall,D. (1974) The role of ventilation and CO_2 in pH regulation. In: Proceedings of the Canadian Society of Zoologists, M.D.B.Burt, ed. University of New Brunswick, Fredericton, Canada.pp.89-94.

Randall,R.F. and P.J.Anderson (1975) Purification and properties of the pyruvate kinase of sturgeon muscle. Biochem. J.145:569-573.

Rayner,M.D. and M.J.Keenan (1967) Role of red and white muscles in the swimming of the skipjack tuna. Nature.214: 392-393.

Reynolds,E.S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J.Biophys. Biochem.Cytol.10:208-212.

Roberts,B.L. (1969) The co-ordination of the rhythmical movements of dogfish. J.mar.biol.Ass.U.K.49:357-425.

Robinson,J.S. and J.F.Mead (1973) Lipid absorption and deposition in rainbow trout (Salmo gairdnerii). Can.J.Biochem. 51(7):1050-1058.

Rognstad,R., J.Genovese, and J.Katz (1970) Determination of reversibility in the gluconeogenic pathway. Fed.Proc.Fed. Amer.Soc.Exp.Biol.29:674.

Rognstad,R. and J.Katz (1972) Gluconeogenesis in the kidney cortex: Quantitative estimation of carbon flow. J.Biol. Chem.247:6047-6054.

Rozengurt,E., L.Jimenez de Asua, and H.Carminatti (1969) Some kinetic properties of liver pyruvate kinase (type L) II. Effect of pH on its allosteric behavior. J.Biol. Chem.244(2):3142-3147.

Sackin,H. and E.L.Boulpaep (1975) Models for coupling of salt and water transport.J.Gen.Physiol.66:671-733.

Schoffeniels,E. and R.Gilles (1970) Osmoregulation in aquatic arthropods. In: Chemical Zoology,Vol.5. M.Florikin and B.T.Sheer, eds., Academic Press, New York,pp.255-282.

Schoner,W., V.Haag, and W.Seubert (1970) Regulation of carbohydrate metabolism by cortisol independent of the de novo synthesis of enzymes in rat liver. Z.Physiol.Chem. 351:1071-1088.

Schubert,W. and W.Schoner (1971) The regulation of pyruvate kinase. In: Current Topics in Cellular Regulation, Vol.3., B.L.Horecker and E.R.Stadtman, eds., Academic Press, New York,pp.237-267.

Scrutton,M.C. and M.F.Utter (1968) The regulation of glycolsis and gluconeogenesis in animal tissues. Ann.Rev.Biochem. 37:249-302.

Sinha,V.R.P. and J.W.Jones (1975) The European Freshwater Eel. Liverpool, University Press, Liverpool.pp.146.

Smit,H., J.M.Amelink-Koutstall, J.Viyverberg, and J.C.Von Vaupelklein (1971) Oxygen consumption and efficiency of swimming goldfish. Comp.Biochem.Physiol.29A:1-28.

Sols,A. (1968) Regulation of carbohydrate metabolism. In: Carbohydrate Metabolism and its Disorders. Vol.1., F.Dickens, P.J.Randle, and W.J.Whelan, eds., Academic Press, New York,pp.53-87.

Somero,G.N. (1976) The role of isozymes in adaptation to varying temperatures. In: Isozymes, Physiological Function, Vol.2., C.L.Markert, ed., Academic Press, New York,pp.221-234.

Somero,G.N. and P.W.Hochachka (1968) The effect of temperature on catalytic and regulatory functions of pyruvate kinases of the rainbow trout and the Antarctic fish Trematomus bernacchii. Biochem.J.110:395-400.

Storey,K.B. and P.W.Hochachka (1974a) Glycolytic enzymes in muscle of the pacific dolphin: role of pyruvate kinase in aerobic-anaerobic transition during diving. Comp. Biochem.Physiol.49B:119-128.

Storey,K.B. and P.W.Hochachka (1974b) Enzymes of energy metabolism in a vertebrate facultative anaerobe, Pseudemys scripta:turtle heart pyruvate kinase. J.Biol.Chem.249(5): 1423-1427.

Susor,W.A. and W.J.Rutter (1968) Some distinctive properties of pyruvate kinase purified from rat liver. Biochem. Biophys.Res.Comm.30:14-22.

Takeuchi,A. (1959) Neuromuscular transmission of fish skeletal muscles investigated with intracellular microelectrode. J.Cell.Comp.Physiol.54:211-221.

Tanaka,T., Y.Harano, F.Sue, and H.Morimura (1967) Crystalization characterization and metabolic regulation of two types of pyruvate kinase isolated from rat tissues. J.Biochem. 62(1):71-91.

Titanji,V.P.K., O.Zetterquist, and L.Engstrom (1976) Regulation of rat liver pyruvate kinase by phosphorylation-dephosphorylation reactions, catalyzed by cyclic-AMP dependent protein kinases and a histone phosphatase. Biochem,Biophys.Acta.422:98-108.

Tsuchiya,Y. and K.Kunii (1960) Studies on the influence of treatments immediately after catching upon the quality of fish flesh-V. Determination of lactic acid. Bull. Jap.Soc.Scient.Fish.26:284-288.

Utter,M.F. and K.Kurahashi (1954) Purification of oxaloacetic carboxylase from chicken liver. J.Biol.Chem.207:787-802.

Vladykov,V.D. (1955) Eel, fishes of Quebec. Dept. of Fisheries Quebec.6:1-12.

Vladykov,V.D. (1964) Quest for the true breeding areas of the American eel (A.rostrata LeSneur). J.Fish.Res.Bd.Can. 21:1523-1530.

Van Berkel,Th.J.C. (1974) Some kinetic properties of M-type pyruvate kinase from rat liver at physiological Mg^{++} concentration. Biochim.Biophys.Acta.370:140-152.

Van Berkel,Th.J.C., J.F.Koster, and W.C.Hulsmann (1973) Some kinetic properties of the allosteric M-type pyruvate kinase from rat liver: Influence of pH and the nature of amino acid inhibition. Biochim.Biophys.Acta.321:171-180.

Van Berkel,Th.J.C., J.F.Koster, and W.C.Hulsmann (1973b) Two interconvertible forms of L-type pyruvate kinase from rat liver. Biochim.Biophys.Acta.293:118-124.

Watson,M.L. (1958) Staining of tissue sections for electron microscopy with heavy salts. J.Biophys.Biochem.Cytol. 4:475-485.

Weiker,H.-J. and B.Hess (1971) Allosteric interactions of yeast pyruvate kinase as a function of pH. Biochem.10(7): 1243-1248.

Willemse,J.J. (1959) The way in which the flexures of the body are caused by muscular contractions. Proc.Kon.Ned.Akad. Wet.C.62:589-593.

Willemse,J.J. (1972) Arrangment of connective tissue fibers in the musculus lateralis of the spiny dogfish, Squalus acanthias L. (Chondrichthyes). Z.Morph.Tiere.72:231-244.

Williamson,J.R. (1966) Mechanisms for the stimulation in vivo of hepatic gluconeogenesis by glucagon. Biochem.J.101: 11c-14c.

Wittenberger,C. (1972) The glycogen turnover rate in mackerel muscles. Mar.Biol.16:279-280.

Wittenberger,C. (1973) Metabolic interaction between isolated white and red carp muscles. Rev.Roum.Biol.18(1):71-76.

Wittenberger,C. D.Coprean, and L.Morar (1975) Studies on the carbohydrate metabolism of the lateral muscles in carp (influence of Phloridzin, insulin and adrenaline). J. Comp.Physiol.101:161-172.

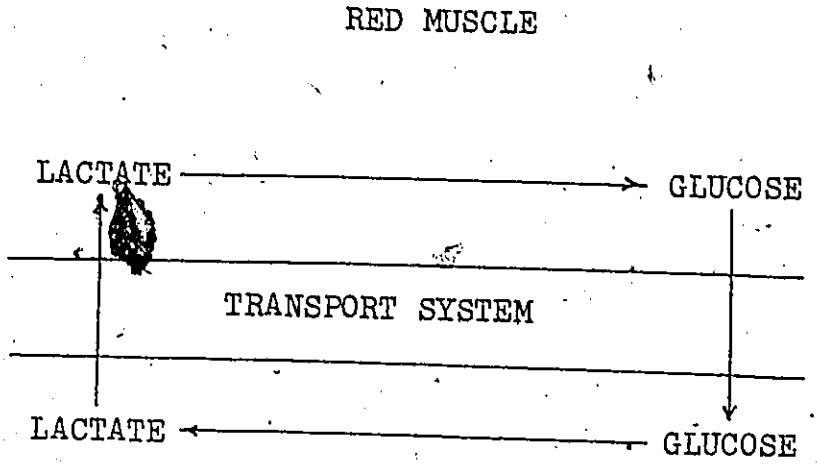
Wittenberger,C., A.Coro, G.Suarez, and N.Portilla (1969) Composition and bioelectrical activity of the lateral muscles in Harengula humeralis. Mar.Biol.3:24-27.

Wittenberger,C. and I.A.Diaciuc (1965) Effort metabolism of lateral muscles in carp. J.Fish.Res.Bd.Can.22(6): 1397-1406.

Wittenberger,C. and R.Giurgea (1973) Transaminase activities in muscles and liver of the carp.Rev.Roum.Biol.Zool. 18(6):441-444.

Wood,T. (1968) Mechanism of adenylate inhibition of pyruvate kinase. Biochem.Biophys.Res.Comm.31:779-783.

de Zwann,A. and T.C.M.Wijsman (1976) Anaerobic metabolism in bivalvia (Mollusca). Characteristics of anaerobic metabolism. Comp.Biochem.Physiol.54(3B):313-324.



Postulated metabolite recycling between red and white muscle. See text for details.