THE UNIVERSITY OF HULL

DEVELOPMENT AND PHYSIOLOGICAL CHARACTERISTICS OF AVIAN FAST AND SLOW CONTRACTING MUSCLES.

being a Thesis submitted for the Degree of

Doctor of Philosophy

in the University of Hull

by

GUDRUN ELISABETH MOORE, B.A. (YORK)

SEPTEMBER, 1982.

For my mother and father,

who have combined their love for

me with continuous support and

encouragement in all of my

educational endeavours.

"The man who welcomes and acts on criticism will prize it almost above friendship: the man who fights it out of concern to maintain his position is clinging to non-growth".

Magee in comment on
Popper's philosophy, (1979).

CONTENTS

CONTENTS CONTINUED

CONTENTS CONTINUED

TABLE OF TABLES

TABLE OF THE ABBREVIATIONS USED IN THIS THESIS

ALD	-	Anterior latissimus dorsi
PLD	-	Posterior latissimus dorsi
FG	-	Fast-glycolytic
SO	-	Slow-oxidative
FOG	-	Fast-oxidative-glycolytic
ST	-	Sarcotubular
SR	-	Sarcoplasmic reticulum
T-system	-	Transverse system
ATP	-	Adenosine triphosphate
a	-	a form of phosphorylase
b	-	b form of phosphorylase
Ca^{2+}	-	Calcium ions
Mg^{2+}	-	Magnesium ions
XB	-	Cross-bridge
ATPase	-	Adenosine triphosphatase
EDTA	-	Ethylene diaminetetraacetate
EGTA	-	Ethylene glycol bis-amino ethyl ether N, N' tetra acetate
T_3	-	3, 5, 3'-triiodothyronine
T_4	-	3, 5, 3', 5'-tetraiodothyronine
G1P	-	Glucose-mono-phosphate (disodium)
AMP	-	Adenosine-mono-phosphate (disodium)
TCA	-	Trichloroacetic acid
ADP	-	Adenosine di-phosphate
CP	-	Creatine phosphate
CPK	-	Creatine phospho-kinase
pCa	-	$-\log_{10}$ of the Calcium ion concentration
I.P.	-	Intra peritoneal
I.M.	-	Intra muscular
DTNB	-	5'-5'- dithiobis (2-nitro-benzoic acid)

(FG, SO, FOG: Pertaining to fibre types.)

CHAPTER 1

INTRODUCTION

Developmental cell biology has become an important field in science and there is an obvious need to expand this area as far as muscle research is concerned. The work involved using two very different chicken muscles as a developmental model. The muscles studied were the M. Anterior latissimus dorsi (ALD) and the M. Posterior latissimus dorsi (PLD).

These muscles are situated dorsally, close together, beneath the surface of the skin; the ALD lies attached to the backbone via an aponeurosis from the neural spines of the last cervical and first two thoracic vertebrae to the humerus on the posterior surface; the PLD is also attached atendinously to the neural spines of the ninth to eleventh thoracic vertebrae and inserts onto the medial surface of the proximal portion of the humerus via a long flat tendon passing under the ALD muscle. (Fig. 2(i) Page 10).

The fibres of the ALD are multi-innervated and do not respond to nerve stimuli with twitch characteristics. It is a tonic muscle whose normal function is holding the wing folded against the bird. This muscle contracts slowly and has an extremely low fatiguability. The fibres of the PLD are focally-innervated and will produce single twitches in response to a single shock. It is a phasic muscle whose normal function is a rapid "flipping back" of the wing against the back of the bird. This muscle contracts and fatigues rapidly. It does in fact, have one of the fastest twitch contraction times found among vertebrate muscles studied.

These two adjacent latissimus dorsi muscles provide an excellent model for the study of development ex ovo until early adulthood. Both muscles develop at approximately the same rate and their close proximity allows dual studies to be completed with great ease.

The techniques described in this thesis included histochemical, histological and biochemical analysis of these muscles both throughout normal development and with artificially imposed experimental constraints such as immobilisation and thyroidectomy.

Various histochemical stains were chosen to demonstrate not only contractile properties (Myofibrillar ATPase stain) but also metabolic properties (Phosphorylase stain). Histological stains were chosen to study the structural changes that occur during development (e.g. acetyl-choline-esterasestain is used to show up nerve endings on the muscle fibres). To complement the histochemical approach quantitatively, techniques were developed to look at the biochemical changes in the activities of two of the enzymes studied, one contractile, Mg^{2+} - activated myofibrillar ATPase and one metabolic, Phosphorylase.

Single fibre mechanics were used to give a better understanding of the actual triggering of contraction via calcium ions of the systems in the two greatly different muscles.

The association between contractile function and fibre-types within muscles has been of great interest for the last 25 years. The first, well known, histochemical typing of muscle fibres to show differences related to contractility was carried out by Padykula and Herman (1955 a), b)) on rat muscle and was devised to show the activity of adenosine triphosphatase. The technique was later modified by Guth and Samaha (1969) to relate more precisely the differences in the activity of fast and slow contracting fibres to the physiological contraction speed of the muscle as a whole. The adenosine triphosphatase examined was a myosin adenosine triphosphatase with stain intensity related to contractile activity. This work encouraged the combination of histochemical stains for both metabolic and contractile enzyme activities; in the study of changes in muscle throughout development; and in analysing various pathological conditions.

The rôle of the nerve in the development and differentiation of muscles and muscle fibre types has been an area of controversy for many years. The complex interaction of the nerve with muscle throughout development, has led to much theorising as to the exact degree of control each has on the other's development. Does the muscle fibre's differentiation depend totally on the innervation? That is to say, is it the electrochemical activity of the nerve or a trophic substance from within the axon that affects the changes seen in muscle fibre development?

These questions and others have caused a variety of experimental techniques to be devised. These have most often employed systems of

physiological constraint on the muscle such as tenotomy of experimental, synergistic or antagonistic muscles (Hikida, 1972; Hikida and Wang, 1981) ; or denervation of the experimental muscle (Hikida and Bock, 1972; Feng, Wangyan and Daxing, 1981; Gordon, Vrbová and Wilcock, 1981). Other experiments involve more complicated combinations, with for example total muscle removal and replacement after mincing in the original position or with the antagonist. (Gordon and Vrbová, 1975; Hikida, 1976). Cross-innervation of nerves originally innervating a predominantly fast muscle with that of a slow muscle and vice versa has led to some exciting changes. (Bárány and Close, 1971; Jimanová and Zelena, 1975; Buller, Eccles and Eccles, 1960 a) and b)). The experiments involve removing or changing either the nervous activity or the muscle's position or normal load to study the degree of control the nerve has over the muscle.

More recently another factor found to have influence on muscle development is the hormonal status of the animal. Hormonal control has always been considered to be less specific than neuronal, particularly with respect to muscle growth. However recent evidence is not consistent with this generalisation. For example some hormones appear to have very specific control over muscle development both in terms of general protein turnover (Goldberg, 1980) and in contractile and metabolic changes (Nwoye, Mommaerts, Simpson, Seraydarian and Marusich, 1982).

In this study the effects of immobilisation and thyroidectomy were chosen to look more closely at the above two problems, the nerve-muscle and the hormonal control, on muscle growth respectively. Immobilisation for various time periods through development with the muscles fixed in different positions gives information on the changes that occur when inactivity is imposed. Unlike many of the techniques devised to study the nerve-muscle controversy this leaves the nerve intact and causes very little stress to the

animal as no surgery or damage is incurred in the tissues.

The fixation of the muscle at varying positions (ie. shortened or lengthened) can yield information regarding the general growth patterns of muscles when stretched or relaxed abnormally. Results from this type of study could be of medical significance especially with the prolific use of plaster-casting to repair fractured bones. The thyroid hormones, thyroxine discovered by Kendall (1915) and triiodothyronine by Gross and Pitt-Rivers (1952) are located in the thyroid and are generally referred to as T_4 (3, 5, 3', 5' - tetraiodothyronine) and T_3 (3, 5, 3' - triiodothyronine) respectively. They are both derived from the amino acid tyrosine and their biological activities are dependent on the presence of iodine. The mechanism of action of the thyroid hormones at the tissue level is not known. It is clear however, that the iodothyronines T_4 and T_3 exert numerous effects on nearly every tissue of the body. Most of the effects appear to be separated into two types of process, firstly growth and differentiation and secondly energy metabolism. Reduction of thyroid hormone via thyroidectomy will have a drastic effect on these processes and monitoring these changes in muscle would give more detailed information on the mechanism of thyroid control.

Muscle research often appears split, between developmentalists taking a holistic approach and academic fundamentalists concentrating on the mechanics of the "acto-myosin interaction". This latter approach has evoked much interest, particularly from physicists who have designed precision apparatus to study the minute changes that occur within single fibres. The fibres chosen are for practical reasons of size or pure academic interest, obscure and often from animals phylogenetically distant, (e.g. barnacles, insects, frogs and rats).

It was felt necessary therefore to carry out some muscle mechanics experiments on single fibres from the ALD and PLD to characterise these muscles as they do seem rather different from those muscles previously studied. The relationship between maximum isometric tension and calcium concentration was determined for single fibres from this phasic-tonic experimental model. The understanding of the rôle of calcium in the triggering of muscle contraction is incomplete and this analysis meant that single fibres within the same species from the two different muscles could be directly compared. In this thesis, histochemistry, biochemistry and mechanics are combined in an attempt to elucidate the changes that occur during development of the fast,contracting PLD and slow,contracting ALD. Characterisation of the different fibre types within these muscles is also attempted. The combination of these techniques together with the single animal model should lead to a clearer understanding of muscle growth and show up inconsistencies between techniques.

CHAPTER 2

GENERAL DEVELOPMENTAL CHARACTERISTICS OF THE ALD AND PLD MUSCLES.

Introduction

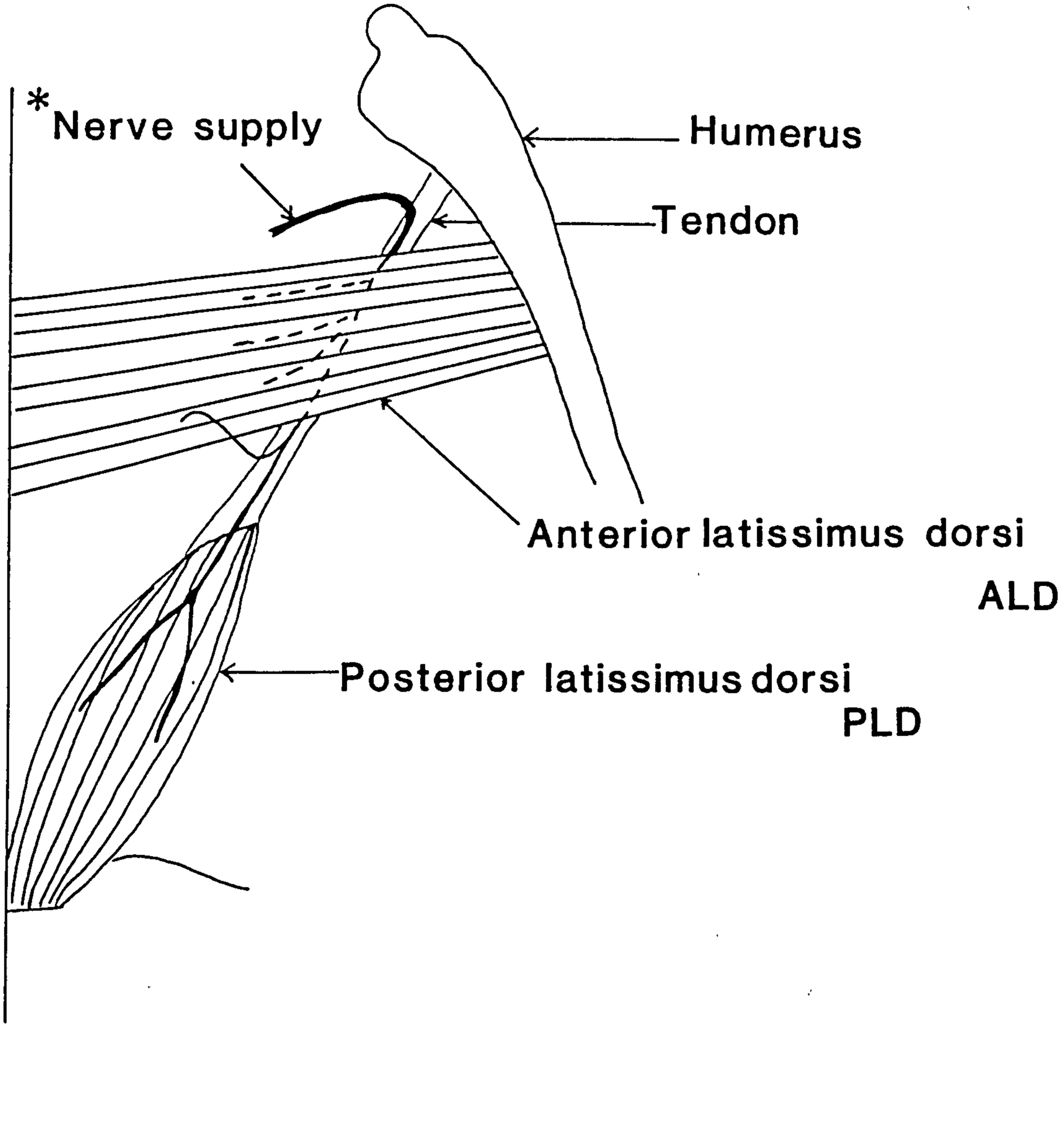
Essential to a developmental study of any tissue is a good understanding of the general growth characteristics of the animal as a whole. In the chicken, Gallus domesticus, the skeletal muscles studied, the ALD and PLD, are located in the back just beneath the skin. (Fig.2(i) Page10). Most skeletal muscles are phasic, exhibiting either fast or slow twitch characteristics in response to nervous input. Tonic muscles contract and relax slowly and are found in functional positions where postural isometric contractions are required. In this study comparison was made between a fast-phasic muscle, the PLD, and a slow tonic muscle, the ALD.

See overleaf for Figure and Figure Legend 2(i).

Figure 2(i)

A diagram to illustrate the anatomical positions of the Anterior latissimus dorsi (ALD) and Posterior latissimus dorsi (PLD) of the chicken. (Adapted from Ginsborg, 1960).

Vertebral Column



*Nerve supply to the ALD enters beneath the muscle

Fast-phasic muscles have usually been compared with slow-phasic muscles e.g. the extensor digitorum longus with the soleus. The avian latissimus dorsi muscles provide excellent models of tonic-phasic systems.

Ginsborg, (1960) has made some important general contractile distinctions between these two muscles. He showed that in response to direct stimulation, fibres of the ALD are able to conduct action potentials with a speed of conduction, for muscle action potential, between 0.41 and 0.70 m/sec. (six muscles with a temperature range of 28-34°C). The PLD, however, conducts at a rate of 2.3-2.8 m/sec. (four muscles, 31-36°C). Prolonged contractures in response to prolonged depolarisation were not generally exhibited by the PLD in contrast to the sustained contraction of the ALD. The innervation of these two muscles differs consistently and it is possible that the differing contractile response could be linked directly to this. The ALD shows multiply-innervated, "en-grappe", innervation whereas the PLD is focally-innervated, "en-plaque" (Hess, 1961). A table of the general characteristics and comparison of the ALD and PLD muscles is given on Pages 12-13.

Canfield (1971) has measured the mechanical properties and heat production of the ALD and PLD during tetanic contractions. The PLD reaches maximum tetanic tension 10 times faster and relaxes 8 times faster than the ALD. The ratio of heat rate to isometric tension (heat rate/ tension x length) for the PLD is 7-8 times larger than for the ALD. The ALD however, maintains substantial isometric tension for more than 2 minutes of stimulation. In the PLD tetanic tension falls after only 1 second. The tension-length curve of ALD has a pronounced plateau and is broader than that of the PLD. The normalised force-velocity relations for the two muscles are similar and can be fitted by Hill's equation with a value of a/Po = 0.15-0.16. An important point is that the maximum velocity of

TABLE 1

A COMPARISON OF THE GENERAL CHARACTERISTICS OF ALD AND PLD MUSCLES.

Anterior latissimus dorsi ALD	Posterior latissimus dorsi PLD	Reference General Review
	Structural Differences	
"Fibrillenstructur" (incomplete splitting of fibres)	"Felderstructur" (discrete and regularly shaped myofibrils)	Hess, 1961. Krüger & Günther, 1955.
"En grappe", multi-innervated (small diameter axons, low threshold) Endings 750-1000μ apart	"En-plaque", focally-innervated (large diameter axons, high threshold)	Ginsborg & Mackay, 1961; Hess, 1961; Atsumi, 1979.
Irregular and small amounts of T-system	Large amounts and well structured T-system	Hess, 1967; Page & Slater, 1965.
Low concentration of sarcoplasmic reticulum	High concentration of sarcoplasmic reticulum	Page, 1969; Ryan & Shafig, 1980.
	Contractile Differences	
Tonic	Phasic (twitch characteristics)	
Innervation by motoneurones with low frequency patterns of activity.	Innervation by motorneurones with an intense pattern of activity.	Ginsborg, 1960.
	Isometric Tension	
Tetanic		
3-4 secs to reach maximum isometric tension	0.3-0.6 secs to reach maximum isometric tension	Canfield, 1971.
$t_{\frac{1}{2}}$ 200msec - sustained 2 minutes	$t_{\frac{1}{2}}$ 25msec - sustained 1 sec	
Single Twitch		
500-600msec to peak tension	50-100msec to peak tension	
Maintenance of body posture - contracts slowly	Fast flipping back of wing - contracts rapidly	

Isometric Tension (Cont.)

Sustained low frequency type of activity	Short bursts of activity, followed by long periods of relaxation	

Differences in Energetics

Heat production during a twitch response = 0.29 ± 0.0 7 m cal/g (7 muscles)	Heat production during a twitch response = 0.93 ± 0.08 m cal/g (3 muscles)	
Therefore Ratio Heat Production 1 : 3		
Lower activation heat needed Ratio 1 : 20	Higher activation heat needed	Matsumoto, Hoekman and Abbott, 1973.
Total heat production tetanus = 3.0 m cal/g	Total heat production during a tetanus = 75.8 m cal/g	
(Peak tension level = 50g wt)	(Peak tension = 20g wt)	
Resting ATP level: 1 μ mole/g	Resting ATP level: 3 μ moles/g	Goldspink, Larson and Davies, 1970.
Isotonic work, low efficiency = 100g cm/μ mole of ATP/ gram muscle	-	
Isometric tension, high efficiency. After 60 seconds maintenance of tension = 540×10^{3} g sec/μ mole of ATP/ gram muscle.	-	Canfield, 1971.

Ratio of heat rate to isometric tension (heat rate/ tension x length)

1 : 7-8

unloaded shortening of the PLD is 4-5 times that of the ALD. Further mechanical differences between these two musoles and their single fibres are discussed in Chapter 6. These basic differences between the properties, contractile, structural and in energetics of the ALD and PLD (See Table 1), further support their use as a model for a slow-tonic, fast-phasic, comparison.

In this study immobilisation was employed in an attempt to separate the effects of nervous and muscle activity throughout development. Two positions of immobilisation were used, both imposing a certain but different degree of inactivity on the right hand set of muscles. The first position was termed the "resting-position" with the wing adducted, "rested", normally against the back. For the PLD muscle this is also a position at which the muscle is at optimum sarcomere length thus giving maximum isometric tension. (Shear and Goldspink, unpublished observations). The second position intended to hold the muscles in a more contracted state, termed the "shortened position"; the humerus being held parallel to the backbone. (Fig.2(ii), Page 18). The contralateral muscles were studied as a separate control group because immobilisation of the right hand side might affect the left. A complete group of non-immobilised animals acted as the controls in all the experimental comparisons.

Research using immobilisation as a physiological constraint has been used in rats (Booth, 1977; 1978), mice (Williams and Goldspink, 1973; 1978), as well as in chickens, (Shear, 1978;1981). Muscles from the wings in chickens have also been constrained with fixation in an extended position. (Holly, Barnett, Ashmore, Taylor and Molé, 1980). Various changes in the fibres, both structurally and in the activities of their

enzymes have been reported.

Williams and Goldspink studied the changes in longitudinal growth of the mouse soleus across development, the muscle being immobilised in both shortened and lengthened positions, (1973). They found that changing the functional length of the muscle by shortening or lengthening generally caused the muscle to lose or add sarcomeres in series respectively. Adult muscle thus has the capacity to attain optimum sarcomere length, when immobilised in the shortened or lengthened positions. (Williams and Goldspink, 1978). However, in the case of mice younger than $2\frac{1}{2}$ weeks, immobilisation in either position resulted in the sarcomere number being reduced in comparison with the controls. This suggested that over the first weeks of growth, contraction of the muscle is important for normal sarcomere addition. The importance of activity in the normal growth of immobilised young muscles compared to adults was of interest, particularly when it was decided to study the development ex ovo of the ALD and PLD, with imposed inactivity.

Booth, has shown using rat soleus and gastrocnemius muscles that atrophy of the muscles is one of the obvious changes when immobilisation is carried out in the shortened position. (1977; 1978). In addition he studied the time course of recovery of these muscles after 10 and 28 days immobilisation in this position. He found that by the 50th day after removal of the casts, wet weights, total amount of protein and total amount of citrate synthase had returned to control or normal values. In most previous studies adult animals have been used. In the present study the effects of immobilisation in a shortened position of the chick ALD and PLD were studied during development.

In this respect the study was similar to that of Shear, 1978, who immobilised the PLD muscle from chickens for various time periods over the

initial developmental period of 30 days. He used immobilisation in the resting position (the length which produced maximum isometric tension) to study the ultra-structural changes that occur. He found that immobilisation of the PLD resulted in a reduction of mean myofibril cross-sectional growth in chickens of all age groups. Once again these results indicate that in young animals the sarcomeres, in this case, of the fast-twitch fibres of the PLD must actively shorten to grow and develop normally. Shear has also shown that complete recovery is expected for the PLD muscle on removal of the cast, except in the case of immobilisation immediately after hatching. In this case there was only limited recovery and it is not known whether full recovery would return. No studies, as yet, have been conducted comparing the ALD and PLD with immobilisation.

Holly et al., 1980, have studied stretch-induced growth of chicken muscles without immobilisation. Stretch-induced growth using fixation of the wing in an extended position, for 6 weeks, showed hypertrophy in both the ALD and patagialis (PAT) muscles. Longitudinal growth was complete within the first week. Cross-sectional growth of the myofibres occurred over the next 5 weeks. This study compared the tonic ALD muscle with the twitch PAT muscle and is some of the only work reflecting changes in a tonic-twitch model with the same physiological constraint imposed on both muscles. Induced stretch caused the muscles to hypertrophy and adapt enzymatically but the responses were found to be dissimilar in twitch and tonic muscles. The hypertrophy caused by stretch-induced growth was expected to give changes opposite to those of shortened immobilisation i.e. atrophy.

In most of the research on this area the emphasis has been on functional length changes using comparative, opposing, immobilised positions,

e.g. lengthening against shortening. The work has used adult animals with few reports of changes that occur with the inactivity throughout development. It was therefore, interesting, to study the ALD and PLD with imposed inactivity in two different positions and to compare their changes across development. In this chapter the muscle length and weight changes are discussed for both control, experimental and contralateral groups. The histochemical and biochemical changes in enzymatic activities for the same groups are dealt with in later chapters.

Materials and Methods

Birds

Rhode Island Red cross Light Sussex, male chickens were used. Measurements of body weight, muscle wet weight and muscle length were taken at 3, 8, 15, 22, 29 and 51 days for the control groups. Immobilisation at 2 days age was carried out on the right-hand side in the resting position for periods of 6, 13 and 20 days. Immobilisation was also carried out in the shortened position for the same time periods with a further group with 7 days recovery after 20 days immobilisation.

Immobilisation was performed using yellow adhesive tape to hold the wings in the set resting and shortened positions. The tape was replaced every 2 days during the immobilisation period to allow for growth of the animal. (It was found to be important to use yellow tape as opposed to any other colour because if other colours were used the birds pecked off each others' tape).

In order to study numerous age groups the actual recordings of controls and experimental groups were staggered by 12 hours but this was considered to be well within any experimental error associated with the actual birth date of the birds. All the groups contained 12 animals.

See overleaf for Figure and Figure Legend 2(ii).

Figure 2(ii)

Plates to illustrate the method of immobilisation in the "resting" and shortened positions for the 3 day old chicken (a) and for the 8 day old chicken (b) and (c); (b) shows the tape fixing the right-hand wing in the "resting" position; (c) shows the tape fixing the right-hand wing in the shortened position.

a)

b)

c)

The length measurement was made when the wing of the bird was in its "resting position". The measurement for the ALD muscle was from its origin at the backbone to its insertion at the humerus as there is no tendon. The PLD was measured from its origin at the backbone to its insertion at the start of the tendon.

Results

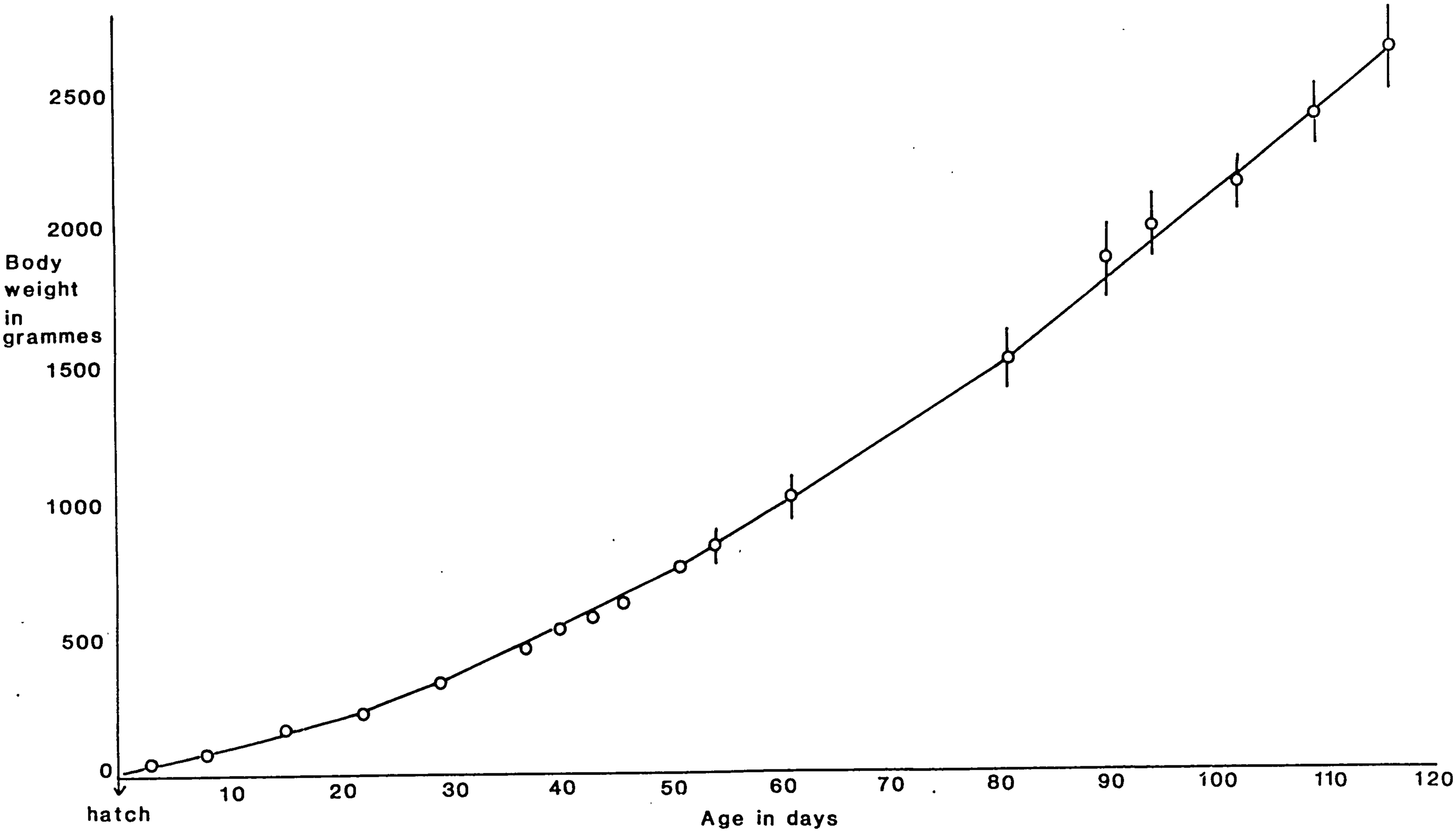
The growth curve for the body weight of the chickens with age, on Fig 2(iii) Page 20, shows that there was a gradual increase in body weights of the birds. Adulthood, which is assumed when the body weight levels off, had not been reached, even at 116 days of age. Statistical analysis indicated that the growth curve was curvilinear. During the first 50 days the rate of weight gain was slower than during the subsequent 100 days. The first 50 days were analysed more closely for ex ovo developmental changes. (See Appendix A, Page 164, for data and statistical tests for linearity and growth rates).

The wet weights and lengths of the ALD and PLD muscles are graphically represented in Fig 2(iv), Page 21. Analysis of variance tests (ANOVAS) were conducted on these data. Both the ALD and PLD increased in weight and length with age (Weights: ALD, $F(5, 66) = 523.69$, $p < 0.001$; PLD, $F(5, 66) = 670.35$, $p < 0.001$. Lengths: ALD, $F(5, 66) = 538.59$, $p < 0.001$; PLD, $F(5, 66) = 706.38$, $p < 0.001$). However an initial similarity in weight diverged as the weight of the PLD increased to be heavier than the ALD over the first 10 days. For both the ALD and PLD, weights and lengths were similar when a right versus left comparison was made. (See Appendix A Page 164, for data and statistical analysis).

See overleaf for Figure and Figure Legend 2(iii).

Figure 2(iii)

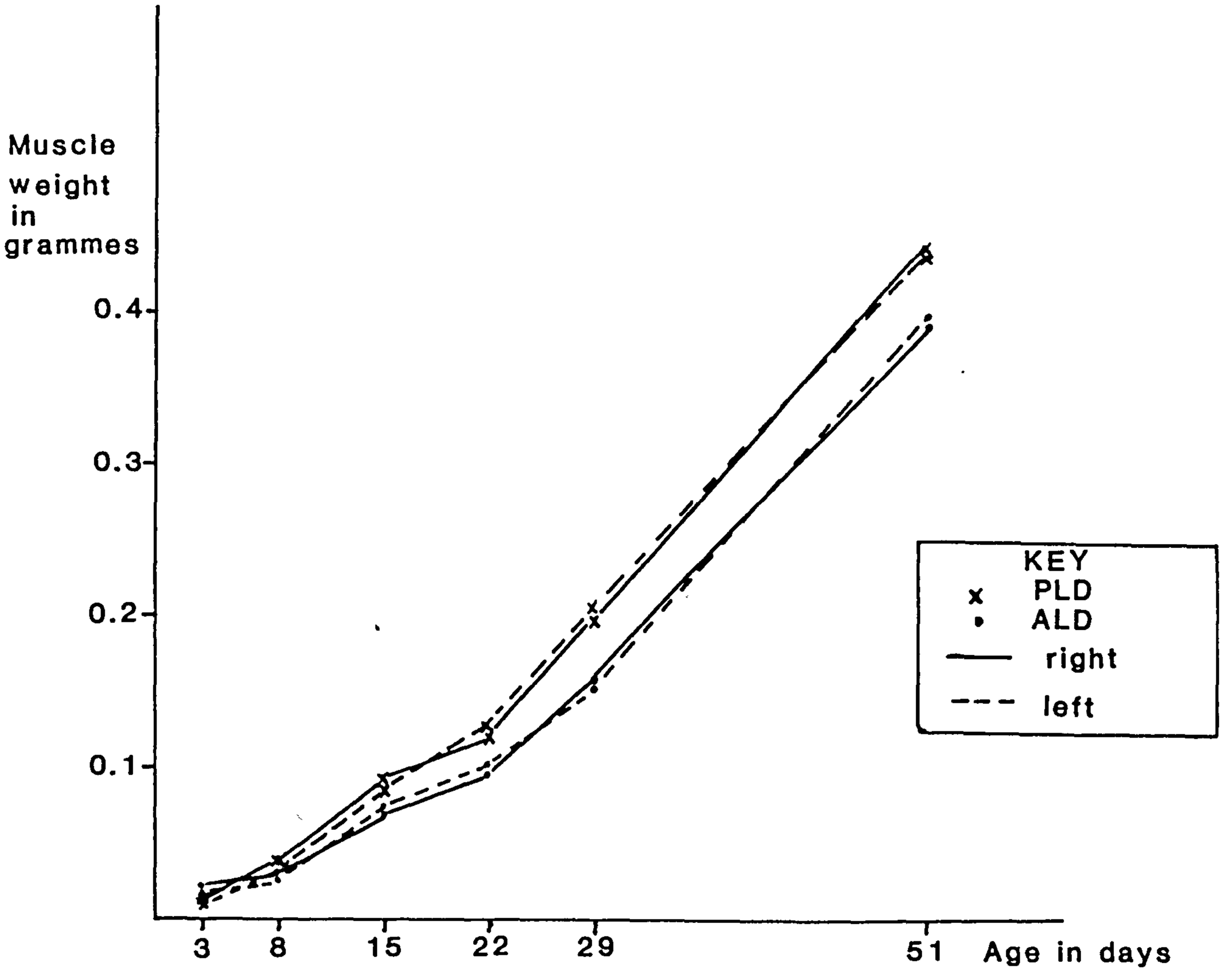
The growth curve for the body weight in grammes of the chickens with age in days.

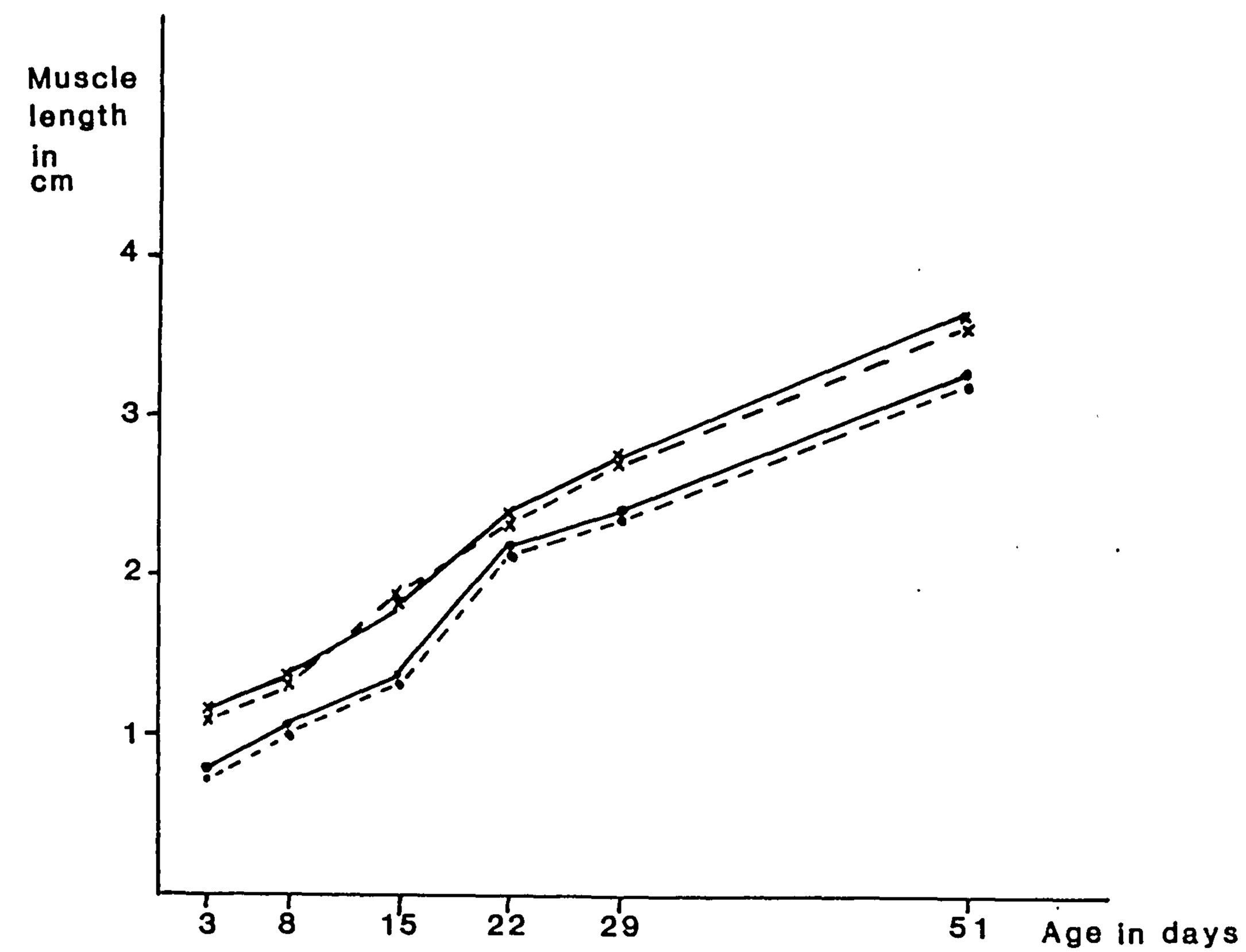

2500
2000
Body
weight
in
grammes
1500
1000
500
0
hatch
10
20
30
40
50
60
70
80
90
100
110
120
Age in days

See overleaf for Figure and Figure Legend 2(iv).

Figure 2(iv)

Graphs to show the increase in wet weight in grammes and length in cm of the ALD and PLD muscles from the control groups with age in days.


Muscle weight in grammes
0.4
0.3
0.2
0.1
KEY
PLD
ALD
right
left
3
8
15
22
29
51
Age in days


Muscle length in cm
4
3
2
1
3
8
15
22
29
51
Age in days

This similarity meant that from the controls both sides could be used separately as control groups to compare the right-handed experimental muscles and left-handed contralateral muscles for the two immobilised conditions.

Fig.2(v), Page 23 , shows the weight changes exhibited by the birds in the two immobilised conditions compared with the controls of the same age. A point of concern was that the birds may have been stressed in the immobilised conditions and thereby lost weight. In fact, the birds showed an increase in weight, particularly after 20 days of immobilisation, with immobilisation in the resting position associated with the greatest weight gain. (At 20 days immobilised shortened : $F(1, 66) = 16.726$, $p < 0.001$; At 20 days immobilised resting : $F(1, 66) = 72.38$, $p < 0.001$).

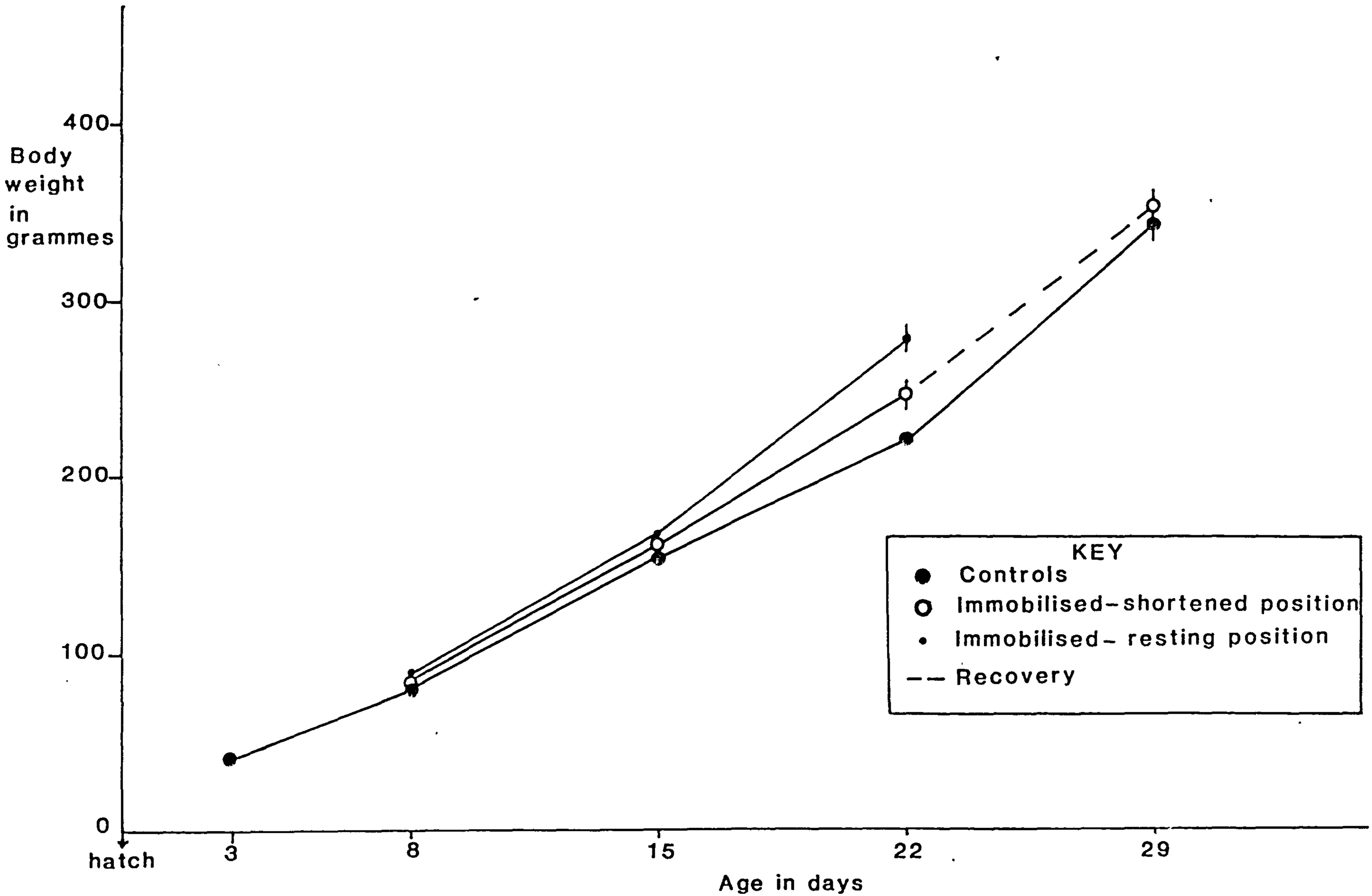
A comparison of the experimental muscles' weights and lengths with the controls' muscle for both conditions of immobilisation is given on Fig 2(vi),Page 24 . A 2-way ANOVA on these data showed no overall difference in weight when the ALD and PLD were compared with their respective controls. The ALD however, when immobilised for 20 days in the resting position showed a significant increase in weight ($F(1, 66) = 13.52$, $p < 0.001$). A reduction in length was found for the ALD after 20 days of immobilisation in both conditions of constraint. (Immobilised resting, $F(1, 66) = 12.30$, $p < 0.001$; immobilised shortened, $F(1, 66) = 25.19$, $p < 0.001$). Whereas, the PLD showed no significant length change under either immobilised condition.

A comparison of the contralateral muscles' weights and lengths with the control muscles' from both conditions of immobilisation is shown on Fig2(vii) Page 25. In this case there was a significant change in weight

See overleaf for Figure and Figure Legend 2(v).

Figure 2(v)

Graphs to show the weight changes exhibited by the chickens in the two immobilised conditions in comparison with the control chickens, with age in days.


400
Body weight in grammes
300
200
100
0
hatch
3
8
15
22
29
Age in days
KEY
Controls
Immobilised–shortened position
Immobilised– resting position
Recovery

See overleaf for Figure and Figure Legend 2(vii).

Figure 2(vi)

Graphs comparing the weight and lengths of the experimental ALD and PLD muscles (from the immobilised conditions) with the control muscles. All the muscles were taken from the right-hand side.

Immobilised in the shortened position

Muscle weight in g

0.2

0.1

Recovery

Muscle length in cm

3.0

2.0

1.0

Recovery

0 8 15 22 29

Immobilised in the resting position

Age in days

Key

Expems PLD △ ALD ○

Controls PLD △ ALD ○

Muscle weight in g

0.2

0.1

Muscle length in cm

3.0

2.0

1.0

0 8 15 22 29

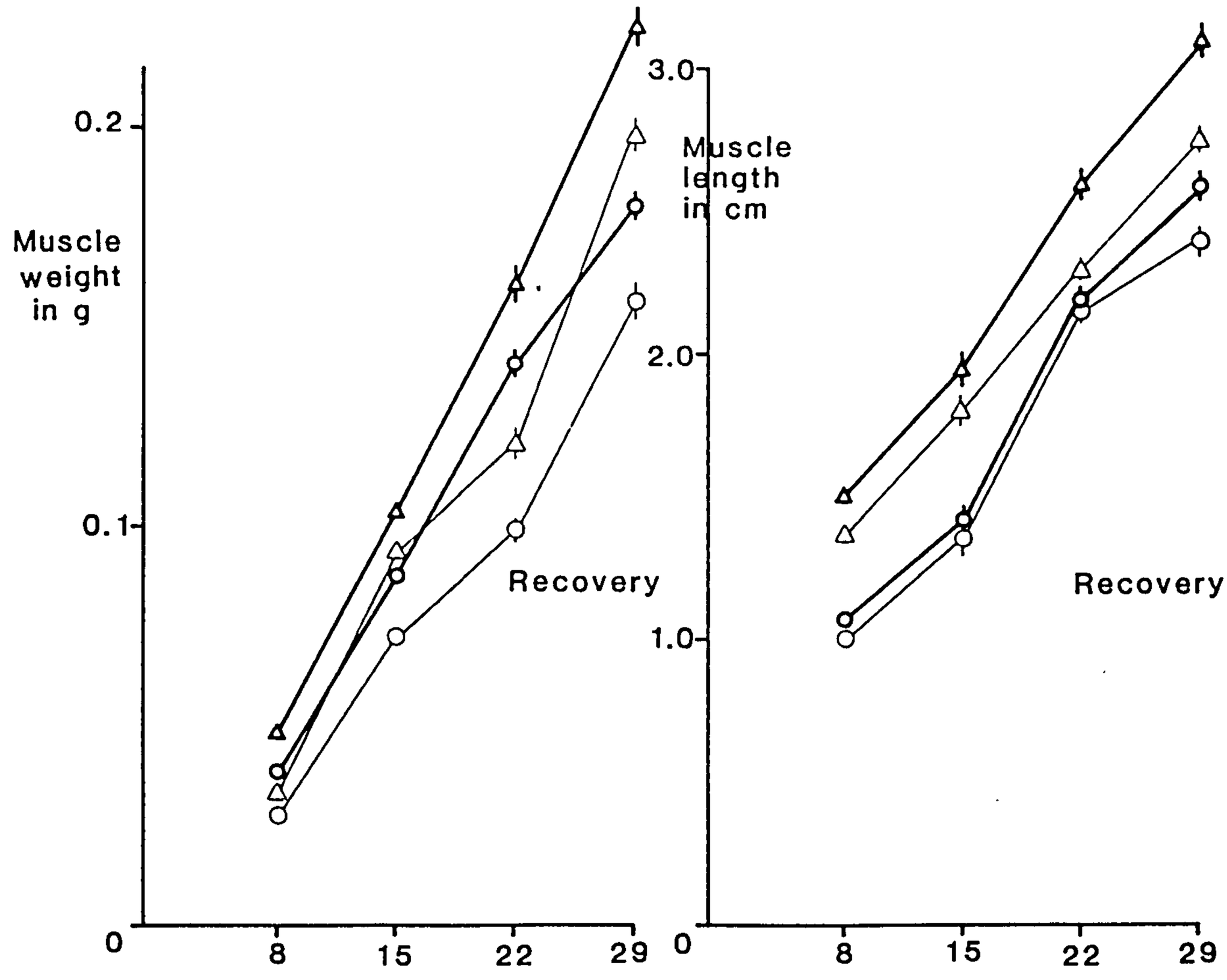
Age in days

See overleaf for Figure and Figure Legend 2(vii).

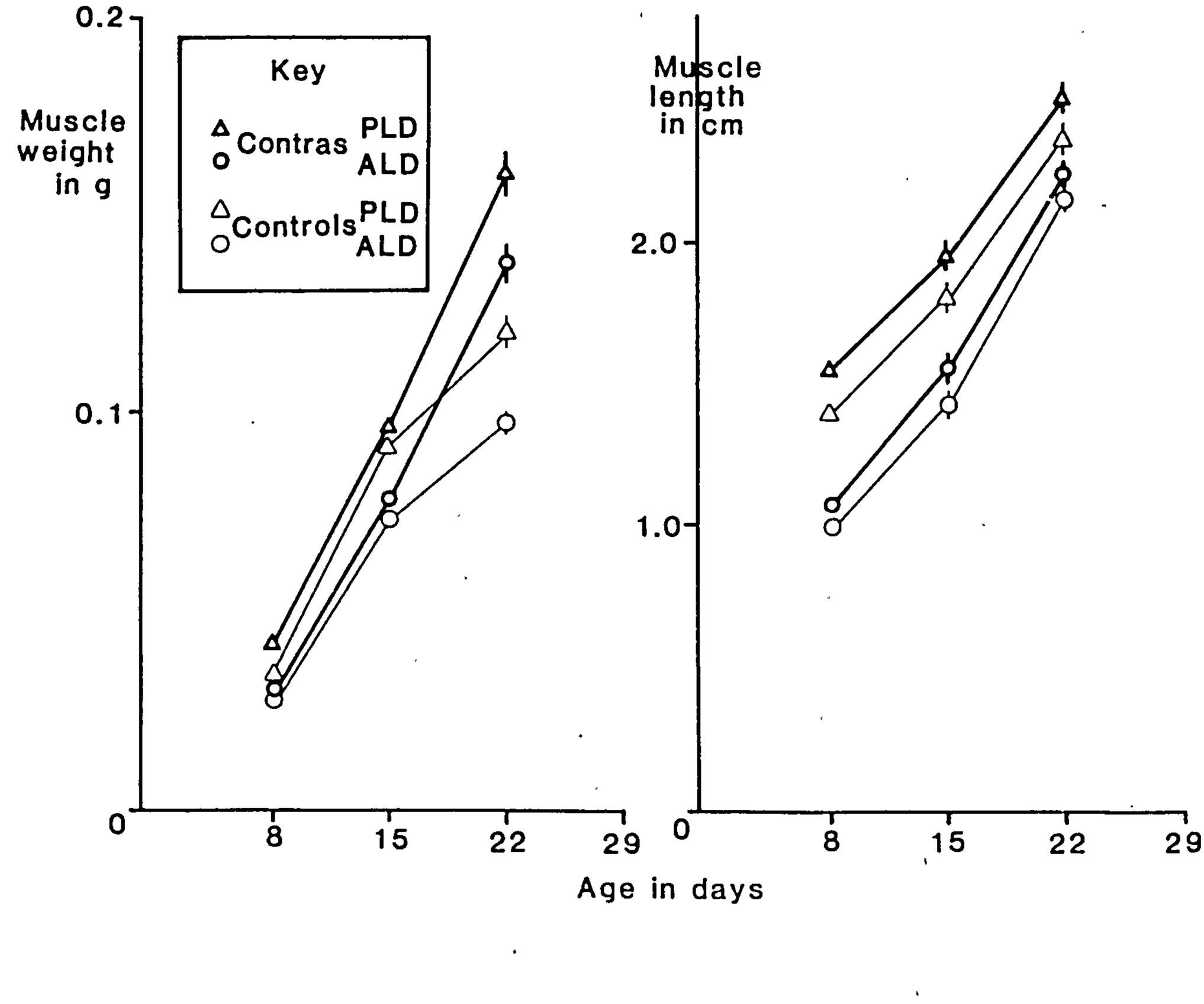
Figure 2(vii)

Graphs comparing the weight and lengths of the contralateral ALD and PLD muscles (from the immobilised conditions) with the control muscles. All the muscles were taken from the left-hand side.

Immobilised in the shortened position



Immobilised in the resting position



and length. The contralateral muscles increased in weight in both conditions of immobilisation. The contralateral ALD showed a weight increase after 20 days with immobilisation in the resting position ($F(1, 66) = 12.48$, $p < 0.001$; At 20 days $F(1,66) = 95.81$, $p < 0.001$). The contralateral PLD showed a weight increase after 20 days with immobilisation in the resting position ($F(1, 66) = 89.48$, $p < 0.001$); and after 20 days with immobilisation in the shortened position ($F(1, 66) = 73.25$, $p < 0.001$). With regard to length, the contralateral ALD, showed no increase while the contralateral PLD was longer after all three time periods of immobilisation in the shortened position; (At 6 days, $F(1, 66) = 11.677$, $p < 0.001$; At 13 days, $F(1, 66) = 12.56$, $p < 0.001$; At 20 days, $F(1, 66) = 49.76$, $p < 0.001$) and at the last two periods of immobilisation in the resting position. (At 13 days, $F(1, 66) = 7.0$, $p < 0.01$; At 20 days, $F(1, 66) = 7.86$, $p < 0.01$).

The period of recovery for 7 days after 20 days of immobilisation in the shortened position caused the experimental ALD to return to above normal levels in length. ($F(1, 22) = 12.774$, $p < 0.01$). Removal of the restraining tapes also caused the experimental PLD to become longer than the controls. ($F(1, 22) = 30.216$, $p < 0.001$). Both the experimental ALD and PLD were similar to the controls in weight after recovery.

With recovery the contralateral ALD and PLD, from the immobilisation in the shortened position, continued to show heavier and longer muscles in comparison to their controls. (Weights: ALD: $F(1, 22) = 10.24$, $p < 0.01$; PLD: $F(1, 22) = 7.811$, $p <> 0.05$-0.01. Lengths: ALD: $F(1, 22) = 17.055$, $p < 0.001$; PLD: $F(1, 22) = 36.27$, $p < 0.001$). After 7 days recovery the overall body weights were found to be similar or just significantly higher than the body weights of the control birds ($F(1, 22) = 4.517$, $p < 0.05$).

Conclusions

The results show that over the first 50 days all the birds increased in weight. Hence, constraint through immobilisation did not result in weight loss.

The control animals showed no difference between left and right sides with respect to weight and length measurements for the ALD and PLD muscles.

With immobilisation in both the shortened and resting conditions after 20 days the ALD decreased in length; this was most marked with immobilisation in the shortened condition. The ALD also showed a small but significant increase in weight after immobilisation in the resting position for 20 days. The PLD however did not change in weight or length in either condition after any time period of immobilisation.

The contralaterals showed hypertrophy, weight and length gain and this clearly indicates that their use as a control group under these experimental conditions was inappropriate.

Allowing one week recovery for the group immobilised in a shortened position for 20 days retains the weight of the experimentals at control values. The shortened length of the ALD increases to above control values. The PLD also, shows an increase in length to above control values after freedom from the cast.

On recovery for 7 days, the contralaterals continue to show values for length and weight above their controls.

Discussion

An appreciation of the adaptive growth of muscles to match their

anatomical locations and functional requirements is of great importance to the understanding of the development of whole animals. In this study immobilisation has been employed to prevent the normal isotonic contractile activity of the ALD and PLD, over the first 22 days of growth. Studies have already been conducted on the effects of immobilisation of the PLD in the resting position. (Shear, 1977; 1978). However, no studies have been conducted comparing the ALD and PLD with this immobilised position or one with imposed shortening.

In this chapter the overall changes in weight and length of these two muscles, both in normal and immobilised growth, have been analysed. From these measurements alone, the changes that occur as a result of immobilisation have not been completely elucidated. Immobilisation produced very little change in wet weight or length, for either the ALD or PLD, in either immobilised condition.

It was anticipated that both muscles would lose weight in both conditions. In this respect the PLD appeared to be less affected than the ALD. It is suggested that although the overall wet weights may not have changed, the internal ratio of muscle fibre to connective tissue may have altered. Shear, (1978), found a great reduction in muscle fibre size with all periods of immobilisation of the PLD, ranging from 3 to 27 days. He also noted a pronounced proliferation of perimysial connective tissue, which accounted for his less dramatic loss in total dry weight. It may however be significant that many of his comparisons were made with the contralateral muscles. In the present study these have been shown to hypertrophy. The use, therefore of the contralateral control may have led to artifactual evidence of atrophy due to immobilisation. Booth, (1978) reported significant atrophy of the gastrocnemius muscle after 10 days ($P < 0.01$) and after 28 days ($P < 0.001$) after hindlimb

immobilisation. The atrophy exhibited by the gastrocnemius under these immobilised conditions has not been shown for the ALD or PLD for immobilisation in this study. It is however difficult to compare different animals and muscles directly. Also, the rats used for Booth's studies were adults which may show different characteristics to growing young animals in respect to atrophy with immobilisation.

It was also anticipated that the ALD and PLD would become reduced in length in the shortened conditions. The ALD however did show a significant shortening after 20 days of inactivity in the resting and shortened position. Even in this case however a more drastic effect on length was expected for both ALD and PLD muscles. The PLD shows no length change and this might be accounted for by a tendon adaptation in length leaving the muscle to grow in length normally. The PLD has a long tendon, in contrast to the ALD which has none. The PLD may have adapted to it's imposed shortening through some different staggering of the fibres within its structure. Measurement of sarcomere size between the experimentals and controls would elucidate this theory. The sarcomeres may have not adapted to the new functional state.

Williams and Goldspink, (1978) found that young muscle immobilised in either the shortened or lengthened positions, showed an overall length reduction compared with the controls. They suggested that this could be due to an increase in tendon length or that the distance between origin and insertion is reduced.

One of the most important points from this study was the obvious hypertrophy of the contralateral muscles. The positions of immobilisation cause these muscles, in some way, to perhaps exercise more, to balance the complete inactivity of the immobilised experimental muscles.

This hypertrophy could be in some way analogous to exercise induced or synergistic tenotomy,hypertrophy. (Reitsma, 1969; Rowe and Goldspink, 1968). The differences exhibited by the contralaterals stress the need for a separate control group against which to evaluate any changes.

CHAPTER 3

HISTOCHEMICAL AND HISTOLOGICAL CHARACTERISTICS OF THE ALD AND PLD THROUGH NORMAL DEVELOPMENT AND WITH IMMOBILISATION

Introduction

In the second chapter the development of the complete ALD and PLD in relation to the chicken's growth were discussed. It was felt that the next stage was to study the growth processes at the cellular level. The cell or cellular unit of muscle is the muscle fibre, which contains the contractile proteins, actin and myosin. These are linearly arranged to slide past each other. The sliding is believed to be produced by cross-bridges and is associated with the utilisation of ATP. Muscle fibres also contain the metabolites and organelles necessary for ATP production. Structurally the muscle fibre consists of myofibrils, sarcoplasm, sarcoplasmic reticulum, T-system, mitochondria and nuclei ensheathed by the outer membrane, the sarcolemma. These components form a highly organised system, the function

of which is the production of mechanical force. However some muscle fibres are adapted to produce force slowly and economically whilst others are adapted for rapid and powerful contractions. Therefore one finds a considerable diversity in structure and metabolism between fibres of different muscles and different animals. (For an excellent review of muscle structure, function, energetics and diversity refer to McNeil Alexander and Goldspink, 1977).

A fully developed muscle may contain large numbers of fibres ranging from 500 to 500,000 with a diameter size range of 20-150μ M. Groups of fibres are innervated by a single motoneurone and are termed motor units. Each motor unit has been shown to contain fibres with uniform metabolic and contractile enzymes. (Edström and Kugelberg, 1968; Nemeth, Pette and Vrbová, 1980). Utilisation of combinations of histochemical stains has led to characterisation of fibres in relation to their contractile and metabolic differences (Table 2, Page 33 , gives a summary of nomenclature and stains in the literature).

The fibres in most mammals may be divided into three and even four basic types. (Ariano, Armstrong and Edgerton, 1973). These fibre types show contractile speeds from fast-twitch to slow-twitch and in conjunction with these functional differences exhibit related metabolic differences. A fast-twitch fibre needs a rapid, readily available energy supply and therefore shows high concentrations of glycolytic enzymes e.g. phosphorylase. This fibre type is termed "fast-glycolytic" (FG). A fast-twitch fibre that shows intermediate contractile speed will need both enzymes for rapid production of energy via the glycolytic chain and mitochondrial enzymes for a more efficient oxidative back up. These fibres are termed "fast-oxidative-glycolytic" (FOG). A slow-twitch fibre contracts slowly, retains tension for longer than the other two fibres and has a high

TABLE 2

COMPARISON OF FIBRE TYPE NOMENCLATURE IN PHASIC AND TONIC MUSCLE ON THE BASIS OF HISTOCHEMICAL ENZYMOLOGICAL DIFFERENCES FOUND IN THE LITERATURE.

Classifications	Avian and Mammalian Phasic Muscle Fibres			Avian Tonic Muscle Fibres	
FIBRE TYPES :	1	2	3	4	5
(1) Ogata & Mori (1958;1964)	White	Medium	Red	-	-
(2) Peter et al. (1972)	FG	SO	FOG	-	-
(3) Burke et al. (1973)	FF	S	FR	-	-
(4) Stein & Padykula (1962)	A	B	C	-	-
(5) Romanul (1964)	I	III	II	-	-
(6) Engel (1962)	II	I	II	-	-
(7) Padykula & Gauthier (1967)	White	Inter-mediate	Red	-	-
(8) Barnard et al. (1971)	Fast Twitch White	Slow Twitch Inter-mediate	Fast Twitch Red	-	-
(9) Ashmore & Doerr (1971)	αW	βR	αR	β'	α'
(10) Brooke & Kaiser (1974)	IIB	I	IIA (and IIC)	-	-
(11) Koenig & Fardeau (1973)	F_2A	F_1	F_2B	F	F
(12) Khan (1976)	II White	I Red	II Red	I Red A	I Red B
(13) Barnard E.A.et al. (1982)	IIB	I	IIA	IIIA	IIIB
Histochemical Properties					
Succinate dehydrogenase activity (4) and Lee (1971)	Even Network	Even Network	Predominantly subsarcolemmal	Even Network	Even Network
Oxidative enzyme activities (4,1,5 and 13)	Low	High or Inter-mediate	Intermediate or High	Intermediate	
Mitochondrial ATPase Gauthier (1969)	Low	Inter-mediate	High	-	-
Glycolytic activity (5)	High	Low	Intermediate	-	-

Mammalian

avian

TABLE 2 (CONTINUED)

FIBRE TYPES :	Phasic Muscle Fibres			Tonic Muscle Fibres	
	1	2	3	4	5
Myofibrillar ATPase at pH 9.4 (6,13)	High	Low	High	High	High
pH sensitivity of Myofibrillar ATPase (10 and others)	Acid Labile	Alkali Labile	Acid Labile	Acid and Alkali Stable	
	Alkali Stable	Acid Stable	Alkali Stable	Most Labile in both	Least Labile in both
Phosphorylase (13)	High to Intermediate	Low	High to Intermediate	Low Most Active	Intermediate Least Active

activity of all oxidative or mitochondrial enzymes to provide energy on a more continuous basis. These fibres are termed "slow-oxidative" (SO).

In the present study the Myosin ATPase stain was used in conjunction with stains for metabolic enzymes, phosphorylase or succinate dehydrogenase to cross correlate the fibre types (Peter, Barnard, Edgerton, Gillespie and Stempel, 1972; Edjtehadi and Lewis, 1979). With the standard Myosin ATPase stain using an alkaline preincubation, fast contracting fibres stain intensely and slow contracting fibres show no or very little activity. The fast contracting fibres also stain intensely for enzymes from the glycolytic pathway (e.g. phosphorylase) whereas the slow fibres show less activity. However the slow fibres do stain intensely for mitochondrial enzymes (e.g. succinate dehydrogenase), in contrast with the fast-twitch fibres which show much lower activities.

Histochemical staining of fibres from avian muscle has been carried out by several research groups. (Ashmore, Kikuchi and Doerr, 1978; Toutant, Toutant, Renaud and LeDouarin, 1979; Butler and Cosmos, 1981). Most of the research has been based on the ALD and PLD with some analysis of other larger mixed muscles; (e.g. the pigeon, serratus superficialis metapatagialis, Hikida, 1973; the chick, Biventer cervicis, Toutant, Rouand and LeDouarin, 1981).

The basis of the Myosin ATPase stain is that a preincubation at an acid pH will preferentially denature the myofibrils of the fast-contracting fibres . This staining pattern is reversed by preincubation at an alkaline pH; the reciprocity of the stain is important and in an analysis of a new species or muscle, reciprocity must not be assumed.

The purpose of this work using histochemical methods to show the activities of the myofibrillar adenosine triphosphate enzyme (Myosin

ATPase), phosphorylase and succinate dehydrogenase was to characterise the muscle fibres of the ALD and PLD throughout development and in changed activity such as that which resulted from immobilisation.

As well as characterising the fibres from a metabolic and contractile point of view it was considered necessary to study the structural differences between the muscles using a more histological approach. Fibres from all types of muscle, both phasic and tonic, have been shown to be initially polyinnervated. In the case of phasic muscles the elimination of the polyneuronal condition occurs during late embryological development. (Bagust, Lewis and Westerman, 1973; Brown, Cotter, Hudlická and Vrbová, 1976). Tonic muscle fibres on the other hand maintain the polyinnervated condition throughout development.

To compare the end-plate density a stain for acetylcholinesterase activity is used (Toop, 1976). Acetylcholinesterase is the enzyme that breaks down the neurotransmitter acetylcholine which is specifically allocated at the synaptic junction. This stain very readily showed differences between the singly-innervated PLD with the multi-innervated ALD across ex ovo development.

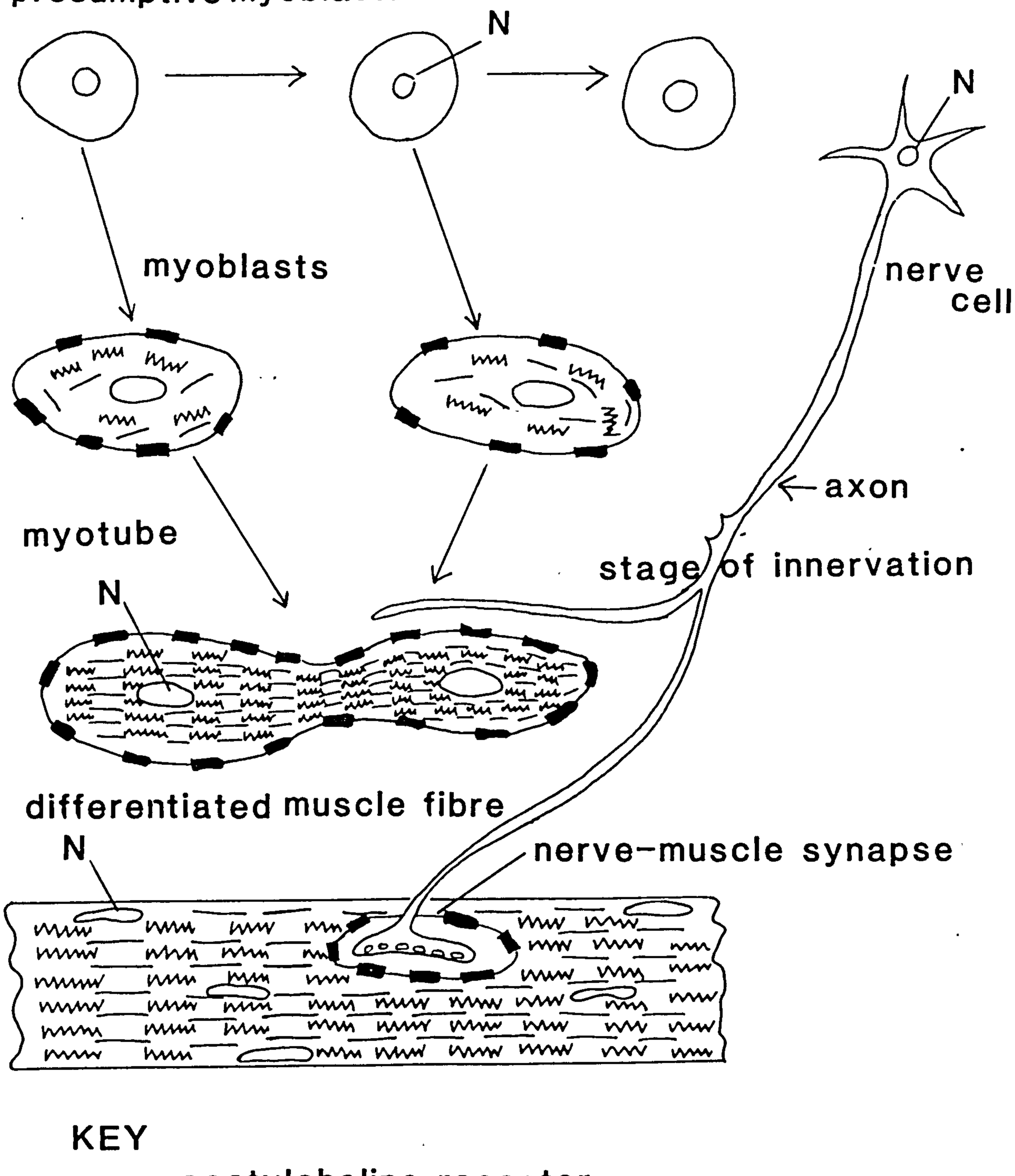
In embryonic development of muscle, the initial muscle cell, or myoblast, has its nucleus at the centre. This eventually migrates to the edge of the myofibrils during the final stages of fibre development. (Fig.3(i) Page 37). A standard histological stain (Haematoxylin and eosin) was employed to show up the position of the nuclei in relation to the cytoplasm. In the present analysis this is used to give an indication of fibre maturity at post ovo stages.

Characterisation of the fibre types in mammalian muscle has been extensively researched and the types of fibres found have been clearly

See overleaf for Figure and Figure Legend 3(i).

Figure 3(i)

A diagram to illustrate the embryonic development of a muscle fibre from a presumptive myoblast showing the initiation of nervous contact. (Adapted from P.3, Vrbova, Gordon and Jones, 1978).


presumptive myoblasts
N
myoblasts
nerve cell
axon
myotube
N
stage of innervation
differentiated muscle fibre
N
nerve-muscle synapse
KEY
acetylcholine receptor
actin
myosin
:contractile proteins
N nucleus

defined. In avian muscle, particularly with reference to tonic muscles, the characterisation is incomplete and this study hoped to clarify further any differences between muscles and fibre types.

Materials and Methods

Birds

N.B. Chemicals, all analar grade, with suppliers are listed in the Appendix B, Page 192.

1. Preparation of muscle sections

The ALD and PLD muscles from both sides of 6 chickens (Rhode Island Red x Light Sussex) in each age group were dissected, cut in half transversely, set on cork blocks using tissue-tek, frozen in precooled isopentane and stored at -180°C in liquid nitrogen (Fig 3(ii), Page 39). The blocks were sectioned using a Bright cryostat microtome at -20°C. Every tenth section each of 10 μ thickness is melted onto a slide in sets of 6-8 sections/slide. These are kept at -20°C until the histochemistry or histology is carried out, always within 6 months. It is advisable, however, to stain for succinate dehydrogenase in the first week to demonstrate full activity of this enzyme.

2. Histochemical methods

(A) Myosin, Actomyosin or Myofibrillar Adenosine Triphosphatase (Myosin ATPase)

(Tunell and Hart, 1977, with modifications by P.W. Watt and G.E. Moore, Muscle Research Unit, Hull University).

The Ca^{2+} - activated Myosin ATPase reaction is carried out with an incubation medium at 37°C, pH 9.4 and employs preincubations with many

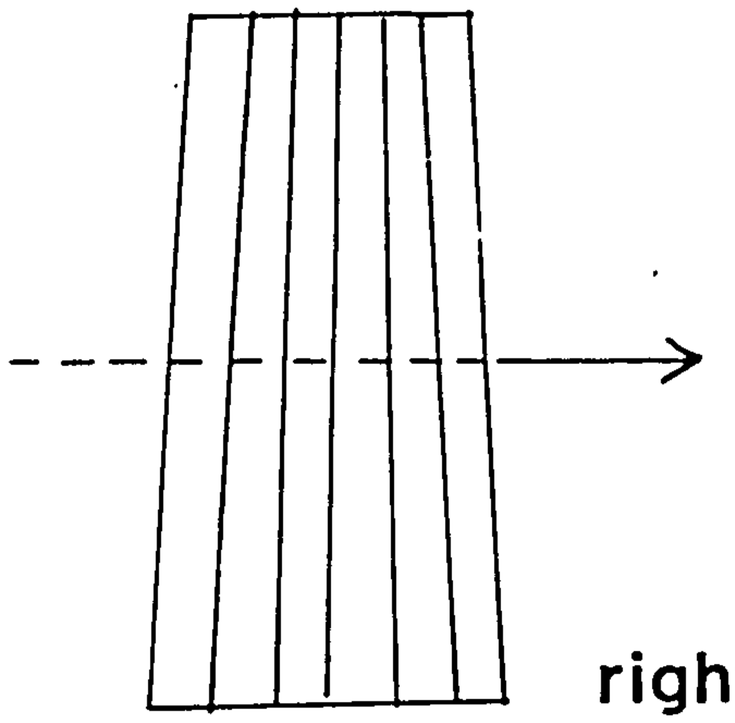
See overleaf for Figure and Figure Legend 3(ii).

Figure 3(ii)

A diagram to illustrate the method for mounting and freezing muscles in preparation for sectioning and staining histochemically.

cut transversely

through the belly of the muscle

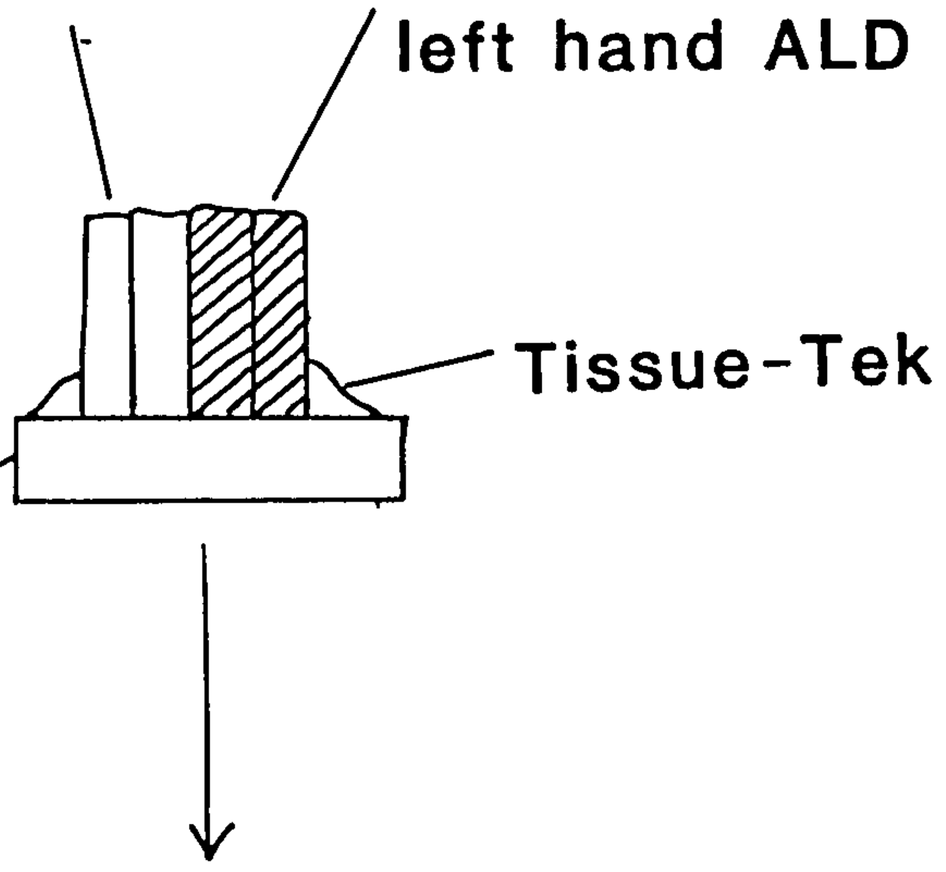


ALD

freshly dissected

right hand ALD

left hand ALD



frozen in isopentane precooled in

liquid nitrogen

stored in liquid nitrogen until sectioned

modifications. The preincubation media is intended to allow differentiation between activities of the Myosin ATPases from different fibres. The incubation media then activates the remaining active fibres with a release of phosphate forming calcium phosphate. The sections are then reacted with aqueous cobalt chloride to give cobalt phosphate and on addition to aqueous ammonium sulphide produce the dark salt of cobalt sulphide. This stains up active fibres light brown to black with inactive fibres remaining colourless.

Reagents

(i) * Preincubation medium

Buffered mix of 0.2M Succinic Acid, 0.2M Sodium Hydroxide

pH 4.35 (staining the ALD sections)

0.2M NaOH	14.0 ml
0.2M Succinic Acid	25.0 ml

Diluted to 100ml with distilled water

pH 4.6 (staining the PLD sections)

0.2M NaOH	20.0 ml
0.2M Succinic Acid	25.0 ml

Diluted to 100ml with distilled water

(ii) * Incubation medium

0.1M Histidine-HCl	4.19 g (for a stable alkaline pH)
0.1M $CaCl_2 2H_2O$	2.94 g

Dissolve in 120ml of distilled water and warm to 37°C

Add 0.005M ATP, 0.304g Adjust pH to 9.4

Dilute to 200ml, warm to 37°C and recheck pH 9.4.

* Solutions that should be freshly made.

(iii) 2% Cobaltous chloride solution

$Co\ Cl_2\ 6H_2O$ in 200ml 7.32 g

(iv) 1% Calcium chloride solution

$CaCl_2\ 2H_2O$ in 1 litre 13.0 g

(v) * 2% Ammonium sulphide solution

4ml (lab stock 100%) to 200ml distilled water

(must be kept within a fume cupboard)

Procedure

1) Dry sections at room temperature for 15 minutes.
2) Preincubate at room temperature for 15 minutes.
3) Incubate at 37°C for 35 minutes.
4) Wash in 1% $CaCl_2 2H_2O$ for 1 minute, 4 times.
5) Immerse in 2% $CoCl_2 6H_2O$ for 3 minutes.
6) Wash in distilled water for 1 minute, twice (to remove adsorbed cobalt)
7) Immerse in 2% NH_4S for 1 minute (sections darken almost immediately)
8) Immerse in running tap water for 3-5 minutes.
9) Dehydrate through ascending alcohols, clear in xylene and mount in DPX.

The stain is permanent and slides can be stored at room temperature.

Note:

Due to complications with obtaining reciprocal staining using ALD and PLD sections many preincubation pH's were tested.

For alkaline pH's buffers Histidine-HCl (as in the incubation)

* Solutions that should be freshly made.

were used.

For acid pH's buffers the succinic acid-sodium hydroxide mix was used.

(B) Succinate Dehydrogenase

(Stein and Padykula, 1962, with modifications, from Nachlas, Tsou, De Souza, Cheng and Seligman, 1957).

The activity of succinate dehydrogenase is related to the aerobic capacity of the muscle fibres. Succinate dehydrogenase is a key enzyme in the Kreb's cycle and is located in the mitochondrial cristae. It is a readily soluble iron flavoprotein and catalyses the reversible oxidation of succinic acid to fumarate. The enzyme activates the hydrogen ions from succinic acid and transfers them to the cytochrome system to produce ATP.

In this histochemical stain the transfer is to the tetrazolium salt. Demonstration of succinate dehydrogenase activity is achieved by incubation of unfixed frozen section with succinate in the presence of a tetrazolium salt in a buffered medium. Enzyme activity produces a deposition of a formazan. The rate of formazan production is proportional to the activity of the enzyme, providing adequate concentrations of substrate and tetrazolium salt are present. The staining should result in a classical mitochondrial portrayal and should reveal characteristic differences in mitochondrial morphology in different cell types. The enzyme has been widely studied in skeletal muscle, cardiac muscle and also in organs with experimentally induced cancer. (Barka and Anderson, 1963).

Reagents

(i) * Phosphate buffer pH 7.6

a) 0.2M Na_2HPO_4 5.68 g in 200 ml distilled water

* Solutions that should be freshly made.

b) 0.2M $NaH_2PO_4.2H_2O$ 1.94 g in 50 ml distilled water

Add 26ml of b) to 174ml of a). Adjust pH by addition of b) dropwise until pH 7.6. (Optimum pH for activity of succinate dehydrogenase).

(ii) * Sodium succinate

0.2M sodium succinate 10.8 g in 200 ml distilled water

Add 200ml of (ii) to 200ml of (i)

(iii) * Incubation medium

Take 100ml of above combination dilute with 100ml distilled water and add 100mg Nitroblue tetrazolium (NBT).

(iv) 0.9% Saline

Add 3.6 g $NaCl_2$ to 400 ml distilled water

(2 months maximum storage)

(v) Formol saline

40 ml of 40% formalin added to 360 ml of 0.9% saline.

Procedure

1) Dry sections at room temperature for 15 minutes.
2) Incubate in solution (iii) at 37°C for 30 minutes.
3) Wash in saline for 1 minute, twice.
4) Fix in formol saline for 10 minutes.
5) Rinse in 15% alcohol for 5 minutes.
6) Mount in glycerin jelly.

* Solutions that should be freshly made.

The stain is permanent and slides can now be stored at room temperature.

(C) Phosphorylase

(Dubowitz and Brooke, 1973, p. 32, after Eranko and Palkama, 1961, and Takeuchi and Kuriaki, 1955).

Phosphorylase, a glycolytic enzyme is located in high concentrations in the cytoplasm of anaerobic tissues. In vivo it degrades glycogen by breaking -1, 4-glucosidic linkages. In vitro it will also catalyse the reversible reaction of glycogen to glucose-1-phosphate. Histochemical demonstration of the activity of phosphorylase depends on the synthesis of polysaccharide chains from glucose-1-phosphate, The length of these chains is proportional to the activity of phosphorylase present. High activity would be demonstrated by a staining with iodine of deep blue giving chains with 30-35 glycosyl units, to degrees of less activity; light blue 20-30 units; reddish shades, 8-12 units; yellow-white, 4-6 units. (Swanson, 1948). Synthesis of branched polysaccharides of glycogen or amylopectin requires an additional enzyme, branching enzyme or amylo-1, 4-6 transglucosidase (Cori and Cori, 1943). Newly formed branching polysaccharides stain violet or brownish-purple and any residual glycogen colour reddish-brown with iodine. The distribution of the branching enzyme in animal tissues is similar to that of phosphorylase. Inhibition of this enzyme can be effected using methanol in the incubation media.

A more detailed account of the complex biochemistry of phosphorylase is given in Chapter 4 in preparation of discussion of the enzyme's assay activity.

Reagents

(i) * Incubation medium pH 5.7

0.2M Acetate buffer 100 ml

AMP (Adenosine monophosphate, sodium salt) 10 mg

: activates inactive phosphorylase b enzyme and active phosphorylase a, to give total phosphorylase activity.

G-1-P (Glucose-1-phosphate, disodium salt) 50 mg

Glycogen 1 mg

: primes the reaction if present in small amounts

0.02M Sodium fluoride 1.25 ml

: prevents reconversion of active phosphorylase a to "inactive" b via the enzyme phosphorylase phosphatase.

Soluble insulin (40iu/ml) 1 drop

: enhances phosphorylase activity, Barka and Anderson (1963)

Addition to 6.25 ml of distilled water.

(ii) Lugols iodine

Iodine 1 g

Potassium iodide 2 g

Distilled water 100 ml

Procedure

1) Dry sections at room temperature for 15 minutes.

2) Incubate at 37°C for 40 minutes.

3) Rinse in distilled water.

4) Wash in Lugols iodine (diluted 1:10) for 10 minutes.

5) Mount in glycerin jelly.

* Solutions that should be freshly made.

The stain is not permanent but if Lugol's iodine is added to the glycerin jelly on mounting the stain will be visible for a maximum of 2 weeks. (Photographs were taken the following day).

3. Histological methods

(A) Haematoxylin and Eosin

A common histological stain using two dyes to stain and counterstain. The blue-purple colour of haematoxylin adheres to the nuclei rendering them blue and when this is counterstained with red eosin the nuclei remain blue after the final wash with the cytoplasm staining red-pink. This enables the position of the nuclei to be picked out easily from the pink surrounding tissues.

Reagents

(i) Delafields haematoxylin

Haematoxylin	8 g
Ethanol	250 ml
dissolve and filter -	
Ammonium alum	16 g
Distilled water	800 ml

Mix the above two solutions and add 200 ml glycerol.

Expose to daylight to ripen for 6 weeks or add 0.4 g potassium permanganate in 10 ml distilled eater.

(ii) 5% TCA

Add 5 g of Tricarboxylic acid to 100 ml distilled water.

(iii) 1% Eosin

Add 1 g of Eosin to 100 ml distilled water.

Procedure

1) Dry sections at room temperature for 15 minutes.

2) Fix in 5% TCA aqueous. (Prevents proteins from dissolving out)

3) Stain in Delafield's Haematoxylin for 10 minutes.

4) Immerse in running tap water for 2 minutes.

5) Counter-stain in 1% Eosin for 2 minutes.

6) Dehydrate in ascending alcohols and clear in xylene. Mount in DPX.

The nuclei should stain blue to black and the cytoplasm is pink. The stain is permanent and the slides can be stored at room temperature.

(B) Acetylcholinesterase stain (AChE)

(Adaptation of a method by Toop, 1976)

To demonstrate the number, morphology and position of end-plates in these muscles a stain to show synapses is used where the activity of acetylcholinesterase is exhibited. This method incorporates the traditional staining for neuromuscular junctions using silver with that of the stain for activity of acetylcholinesterase. The sections are incubated for acetylcholinesterase and then in aqueous silver nitrate with final development in 1% quinol. Axons and subneural apparatus stain dark brown to black in contrast to less well stained muscle fibres and nuclei.

Reagents

(i) Acetylcholinesterase incubation medium

Stock solution

$CuSO_4 5H_2O$	0.3 g
Maleic acid	1.75g
Glycine	0.375g

$MgCl_2 6H_2O$	1.0 g
IN NaOH	30 ml
20-25% Na_2SO_4	170 ml

* Before use add 20mg acetylthiocholine iodide dissolved in 0.1ml of distilled water to 10ml stock. Adjust the pH to 5.5 with IN HCl.

(ii) * 0.5% Potassium ferrocyanide

0.5g $K_3Fe(CN)_6$ to 100ml distilled water

(iii) * Formol saline - buffered to pH 7.0

Add 40ml 40% formaldehyde to 360ml 0.9% NaCl.

(iv) * 20% Silver nitrate aqueous

20g of $AgNO_3$ plus 0.1g $CuSO_4 5H_2O$ to 100ml distilled water.

(a small amount of $CaCO_3$ can be placed in the staining jar).

(v) Developer

1g quinol

5g Na_2SO_3

100ml distilled water

(vi) 5% sodium thiosulphate

5g of sodium thiosulphate in 100ml distilled water.

Procedure

1) Dry sections for 15 minutes at room temperature.

* Solutions that should be freshly made.

2) Incubate in AChE medium for 12 minutes at 37°C.

3) Rinse in distilled water, three times.

4) Place in fresh 0.5% $K_3Fe(CN)_6$ for 10 minutes at room temperature.

5) Rinse in distilled water, three times.

6) Fix in buffered formol saline for 30 minutes at room temperature.

7) Rinse in distilled water, for 10 minutes.

8) Incubate in 20% aqueous $AgNO_3$ containing 0.1% $CuSO_4 5H_2O$ for 20 minutes at 37°C.

9) Rinse in distilled water.

10) Develop in developer at room temperature. Two baths of developer should be used, the first for only 10 seconds.

11) Rinse in distilled water, three times.

12) Fix in 5% sodium thiosulphate for 1-2 minutes.

13) Rinse in distilled water, three times.

14) Dehydrate in ascending alcohols, clear in xylene, and mount in DPX.

The subneural apparatus should stain black; axons dark brown to black; muscle fibres golden brown and nuclei unstained to pale brown. The stain is permanent and the slides can be stored at room temperature.

Results

The histochemical and histological results are shown in Figures 3(ii) to 3(vi).

Fig3(ii), Page50, shows the variation in staining of ALD and PLD fibres for Myosin ATPase at serial preincubation pH's using serial sections from a separate control group at 54 days age. From this it can be seen how the

See overleaf for Figure and Figure Legend 3(iii).

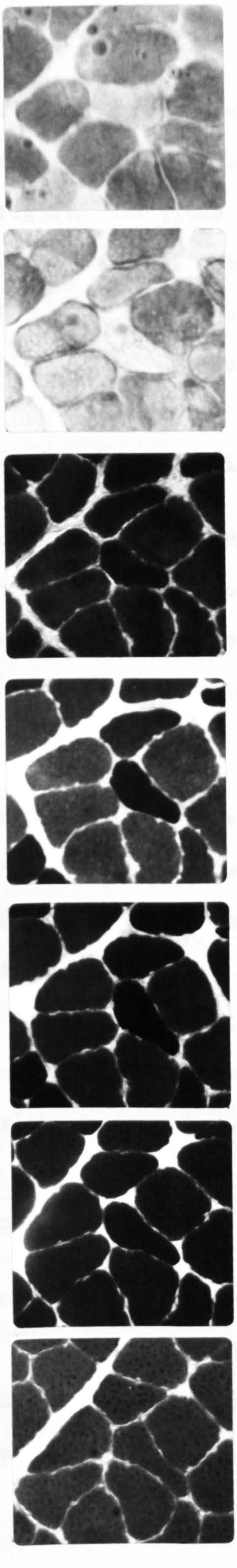
Figure 3(iii)

A series of plates illustrating the variation in staining of the ALD and PLD fibres for Myosin ATPase at serial preincubation pH's using serial sections from a control group of chickens at 54 days of age.

ALD

Preincubation pH

PLD



3.8 No stain

4.0 No stain

4.2

4.4

4.6

4.8

Fibre types most distinquish -able between pH 4.2-4.6

Fibre types most distinquish -able

Magnification

200µm

10.4 Alkaline preincubation

(Guth and Samaha ,1969)

staining intensities of ALD and PLD fibres changedacross these pH's.

The comparison of the stains for Myosin ATPase and Phosphorylase for ALD and PLD using sections from a control group at 24 days age are shown in Fig 3(iv). The group was chosen for its central position in the 50 day growth period. Photographs of the succinic dehydrogenase stain for ALD and PLD are shown at the bottom of this page. After this initial analysis it was decided to use only Myosin ATPase and Phosphorylase in analysis of the control and experimental groups as the succinate dehydrogenase stain did not show up different fibre types within or between muscles.

Staining for Myosin ATPase and Phosphorylase for selected control and experimental groups (in immobilised conditions) is shown on Fig 3(v), Page 53. The figure shows clearly the development of the fibres and the changes in the fibre types across development and with experimentally imposed immobilisation.

Histological demonstration for nuclei and cytoplasm for the early control groups of 3 and 8 days using the Haematoxylin and Eosin stain is shown on Fig 3(vi), Page 54. These can be directly compared with staining for acetylcholinesterase giving the nerve endings on the cross-sections. Also displayed on Fig3(vi) are further photographs showing the innervation across selected control and experimental groups for both ALD and PLD using the same acetylcholinesterase staining procedure.

Conclusions

The histochemical activities of Myosin ATPase and Phosphorylase proved to be the most useful methods in this study of chicken fibres in the ALD and PLD muscles. In retrospect a further aerobic enzyme marker should perhaps have been used for a more complete analysis. However the results

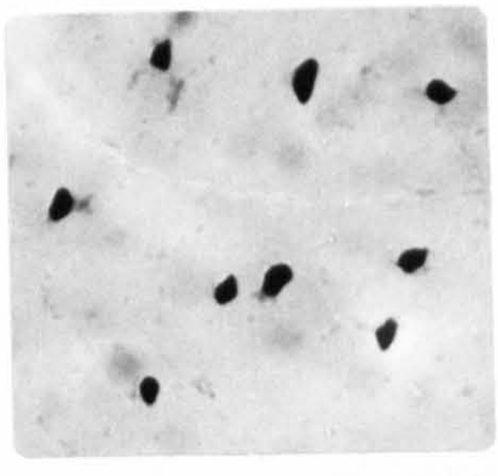
See overleaf for Figure and Figure Legend 3(iv).

Figure 3(iv)

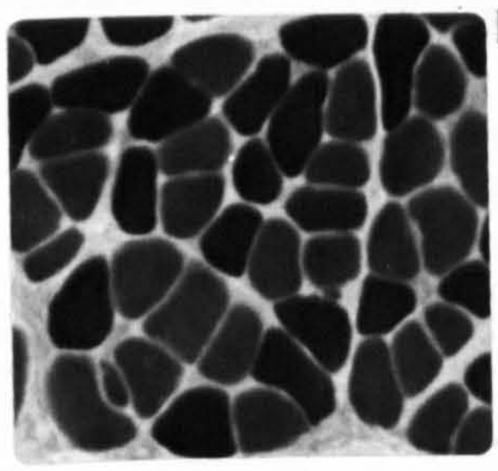
A series of plates illustrating the comparison of the stains for Myosin ATPase and Phosphorylase for the ALD and PLD using serial sections from a control group for chickens at 24 days of age. Plates illustrating the stain for succinate dehydrogenase for the ALD and PLD are shown at the bottom of the figure.

ALD 24 days Age

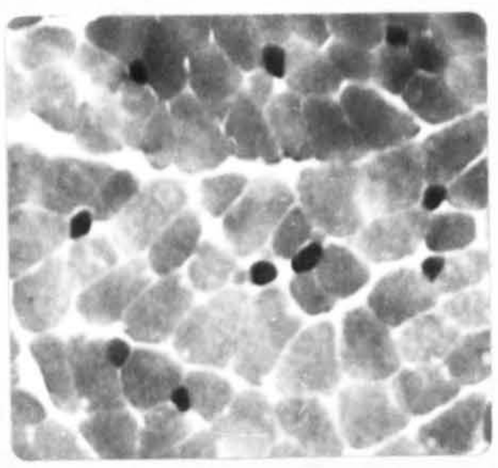
PLD 24 days Age



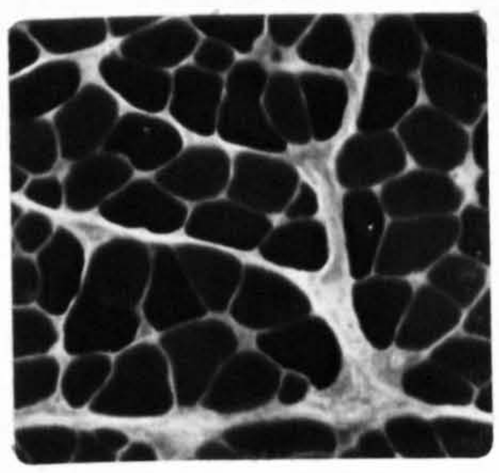
Myosin ATPase
preincubation
pH 4.3



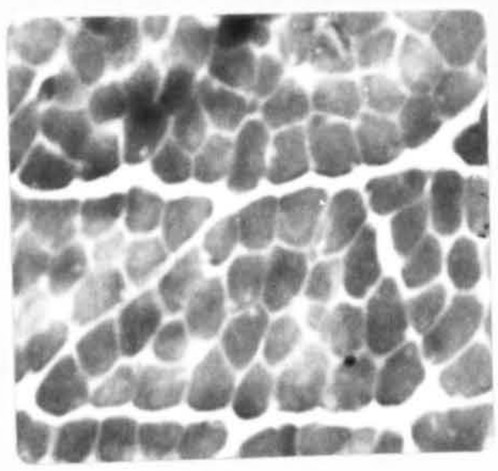
Myosin ATPase
preincubation
pH 4.3



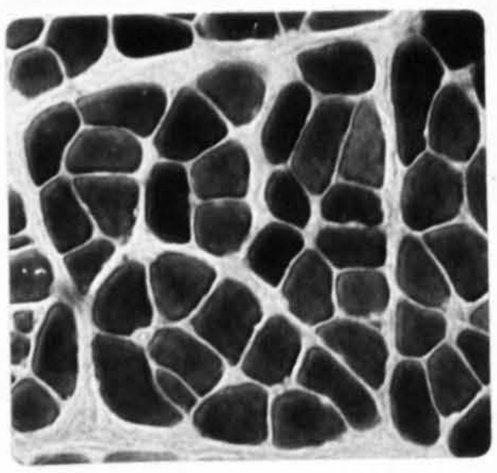
pH 4.6



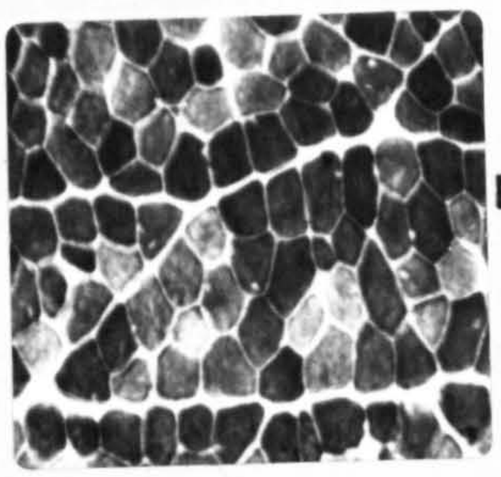
pH 4.6



pH 4.6

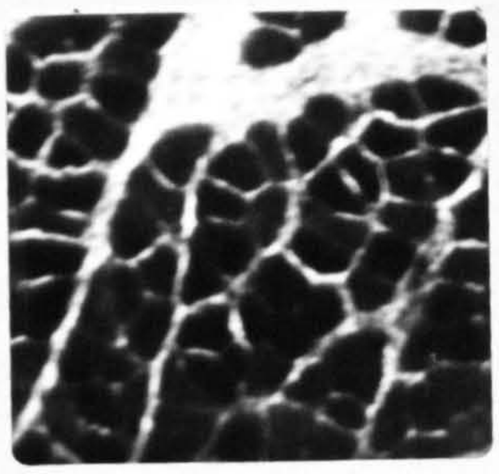


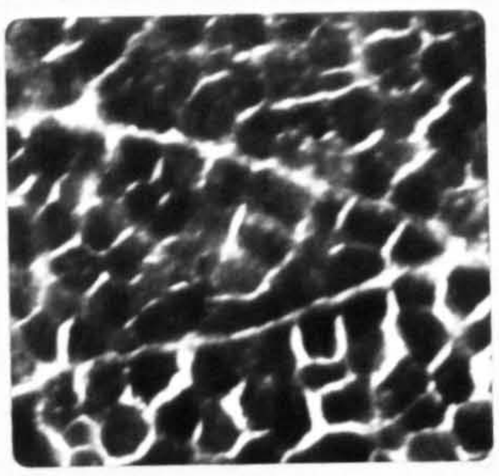
Phosphorylase



Phosphorylase

Succinate dehydrogenase





No types observed- staining intensity equivalent in both muscles

Magnification ⊢——⊣ 200μm

See overleaf for Figure and Figure Legend 3(v).

Figure 3(v)

Plates illustrating the staining for Myosin ATPase and Phosphorylase for selected control and experimental groups for the ALD and PLD.

PLD

ALD

Myosin ATPase preincubation pH 4.6

Phosphorylase

Myosin ATPase preincubation pH 4.3

Phosphorylase

Controls

Age days

3

8

29

51

Immobilised in the resting position for 6 days

8

Immobilised in the shortened position for 6 days

8

Magnification

200 µm

See overleaf for Figure and Figure Legend 3(vi).

Figure 3(vi)

Plates illustrating the histological staining for the nuclei and cytoplasm for the ALD and PLD in early control groups at 3 and 8 days age. Additional plates also display the staining of the nerve endings for the ALD and PLD in selected control and experimental groups; with the use of the acetylcholinesterase stain.

Controls

Age days

PLD

ALD

3

8

Haematoxylin & Eosin

Acetylcholin-esterase

Haematoxylin & Eosin

Acetylcholin-esterase

15

(22 days old)

51

longitudinal section

Immobilised in the resting position for 6 days

8

Immobilised in the shortened position for 6 days

8

Magnification

200µm

from the one that was used proved to be rather disappointing (Fig 3(iv), Page 52). The overall conclusions for the fibre-typing for the ALD and PLD muscles using these histochemical methods is given in Table 3a),3b), Page 56.

The reversal of the Myosin ATPase stain at acid and alkaline pH was not acheived as can be seen from Fig 3(ii) Page 50. The ALD gave two distinct fibre types after a preincubation pH between 4.2 and 4.6. At and below pH 4.0 preincubation, the ALD shows little Myosin ATPase activity although the staining that appears is confined more often to fibres that showed less activity at the preincubation between pH 4.2 and 4.6. At the more commonly used preincubation of pH 10.4 the fibres cannot be distinguished. The PLD shows little or no staining of fibre types until a preincubation of pH 4.3 and above is employed. The fibres do appear to be fairly uniform in activity although at a preincubation of pH 4.6 several types appear. It is thought that at this pH the balance of lability or stability of the fibres is transient. A direct comparison with mammalian phasic muscle fibres would classify the PLD as a uniform fast-twitch muscle. However the PLD muscle at 24 days, does show a small percentage of fibres scattered across the muscle that have high Myosin ATPase activity at all preincubation pH. (Fig 3(iv), Page 52). These fibres are not as frequent in the adult muscle and hence could be considered to be an embryonic fibre.

A comparison of the staining showing Myosin ATPase and Phosphorylase activities for the ALD and PLD, Fig 3(iv), Page 52, showed that the staining for one enzyme in a group of fibres is not cross-correlated with a stain for another. In the ALD two types of fibre are present as far as the Myosin ATPase is concerned and two for Phosphorylase. In most muscles the fibres that are most active (darkest) for Myosin ATPase are also most active (darkest) for Phosphorylase. Phosphorylase portrays the PLD as containing

TABLE 3a)

SUMMARY OF THE HISTOCHEMICAL DATA FOR THE FIBRES IN THE ALD AND PLD MUSCLES.

		PLD	ALD
Myosin ATPase			
pH of preincubation	3.8	No staining	2 types - less stain in dark fibres at pH 4.3
		Few dark fibres	
	4.3	No staining	2 types < dark, grey
	4.6	3-4 types	2 types < dark, grey
	4.8	All stained black	All stained black
Phosphorylase		3-4 types purple to pale blue few golden	2 types - brown, light brown, few golden.
			brown ≡ dark (Myosin ATPase)
			light brown ≡ grey (Myosin ATPase)
Succinate Dehydrogenase		All fibres darkly stained	All fibres darkly stained

TABLE 3b)

CLASSIFICATION OF FIBRES IN ALD AND PLD

BASED ON THE HISTOCHEMICAL FINDINGS.

PLD	ALD
Uniform with Myosin ATPase. (some embryonic)	2 types Equivalent to α' and β' of Ashmore and Doerr 1978; both stains give the 2 types.
3-4 types with phosphorylase.	
Highly anaerobic.	Less anaerobic.
Aerobic.	Aerobic.

two or even three metabolic types of fibre. However these are not suitable when Myosin ATPase alone is used as a marker. The stain used for the aerobic enzyme, succinate dehydrogenase, shows a uniform staining for both ALD and PLD, Fig 3(iv), Page 52.

Throughout development the ALD shows two fibre types with Myosin ATPase. These were just visible at 3 days post-hatching, Fig 3(v), Page 53. Two fibre types were also shown using Phosphorylase, the staining colouration showing brown or golden fibres, implying that the chains of glycosyl units formed are between 12 and 4 units in length. With immobilisation in both the resting and shortened positions the two types are still present in both Myosin ATPase and Phosphorylase staining although the intensity of stained had dropped dramatically. The examples shown are for the first week of immobilisation, very little or no stain was exhibited for the longer time periods.

The PLD shows a uniform staining through development using Myosin ATPase with a few darker smaller fibres in the younger age groups. Clear differences in fibre types are shown using Phosphorylase with the staining colouration varying from dark purple to pale blue and even a golden colour in some fibres. The colouration shown indicates glycosyl chains of between 35 and 4 units and demonstrates the higher activity of Phosphorylase enzyme in this fast-phasic anaerobic muscle in comparison with the slow-tonic aerobic ALD. With immobilisation both Myosin ATPase and Phosphorylase activity is reduced. Phosphorylase gives the most dramatic change with no staining of fibres occuring after 2 weeks or longer immobilisation, in both the shortened and resting positions. It was this dramatic reduction in the histochemical activity of these enzymes that led to further direct biochemical assays of these enzymes across development and immobilisation (See Chapter 4).

Histological examination of the ALD and PLD for nerve endings and for the position of the nuclei shows some interesting differences. Transverse and longitudinal sections of the ALD show the high number of nerves and end-plates found in this multi-innervated muscle; Fig 3(vi),Page 54. In contrast the PLD shows only a few end-plates per cross-section usually localised in one area of the muscle. Using Haematoxylin and Eosin the nuclei in the 3 and 8 day old ALD and PLD appear localised at the edges of the muscle fibres indicating fibre maturity. However, the stain for acetylcholinesterase picked up nuclei also and for conditions with immobilisation the nuclei appear in higher concentration than in the controls. This could be as a result of the fibres atrophy with immobilisation, and hence, the normal number of nuclei appearing in a higher concentration.

Discussion

Histochemistry has elucidated many developmental and pathological problems and in this study has been employed as a preliminary step in the analysis of changes during development and with immobilisation of the ALD and PLD.

Clarification of the fibre types within tonic muscles has been a subject of interest to several research groups. Ashmore *et al.* (1978) divided the tonic fibres into two types, α' and β'; after acid preincubation pH 4.35 for Myosin ATPase. β' types show the greater activity after this preincubation. These two types despite non-reciprocity have also been verified by other groups, (Khan, 1976; Ovalle, 1978; Asiedu and Shafig, 1972; Butler and Cosmos, 1981). The PLD however has always been considered to be a uniformly fast-contracting fibred muscle. This analysis shows that different fibre types do occur with the Myosin ATPase stain but at a very selective preincuba-

tion pH and the staining differences could be merely a transient lability-stability between fibres. There is a group, however, of small fibres with high activity of Myosin ATPase at all preincubation pH's exhibited during the younger age groups. Embryonic muscle fibres have been shown to stain darkly with non-reciprocity for Myosin ATPase, (Guth and Samaha, 1972) and it is thought that these could be embryonic fibres which are slow in changing to adult types.

The activity of Phosphorylase shows striking differences between the PLD and ALD. These differences also observed by Barnard, Lyles and Pizzey (1982) and Nene and Chinoy (1965) show up the distinctly high glycolytic capacity of the PLD across development in comparison with the generally lower activity in the ALD. The PLD when stained for Phosphorylase apparently presents many metabolic types of fibre with a chequer-board colouration of purple, pale blue to white. Use of this enzyme as a marker shows clearly that two fibres which show an overall similar contractile activity may show different metabolic activities or related different glycogen stores.

Alternatively, it could be that the subtle differences in the myosins of the PLD fibres cannot be distinguished by the standard Myosin ATPase histochemical stain. The ALD shows lower activity for phosphorylase with the production of glycosyl chains of fewer units giving brown or yellow colouration.

With immobilisation in both the resting and the shortened positions the activities for both Myosin ATPase and Phosphorylase are reduced dramatically. It is difficult to assess how the fibre types vary, if at all, but immobilisation for periods of 1 week and longer reduce both the contractile and metabolic activity of these two mucles considerably.

Staining the end-plates for acetylcholinesterase activity demonstrates the difference in end-plate number in the ALD and PLD. This stain has been used for ALD and PLD in embryonic and adult age groups by other researchers. (Hess, 1961; Vrbová et al., 1978; E.A. Barnard et al., 1982). The ALD shows many end-plates per cross-section. The PLD shows only a few end-plates and these are localised to one section of the muscle. These few fibres could be multi-innervated fibres as reported by Hess, 1961 who found a few fibres from the PLD with "en grappe" endings. The endings of adjacent muscle fibres occuring at about the same level. Otherwise it must be assumed that PLD fibres are focally-innervated with one end-plate per fibre (Hess, 1961).

With immobilisation the number of nerve endings appeared normal with a high concentration for the ALD and a low concentration for the PLD.

The reason for the apparent increase in nuclei number in the immobilised muscles is not known but the atrophic state that these fibres exhibit, particularly the ALD could be related to this. There may be an increase in connective tissue and some of the nuclei could belong to this tissue.

CHAPTER 4

BIOCHEMICAL ANALYSIS OF THE ACTIVITIES OF Mg^{2+}-ACTIVATED MYOFIBRILLAR ATPase AND PHOSPHORYLASE IN THE ALD AND PLD MUSCLES IN NORMAL AND IMMOBILISED DEVELOPMENT.

Introduction

The preceding two chapters were concerned with the general growth and the histochemical differences of developing ALD and PLD muscles. As a sequence of this work the changes in two enzymes, Mg^{2+}-activated myofibrillar ATPase and Phosphorylase, were examined in a quantitative manner. Enzymatic assays were modified for use with chicken muscle. The changes in the activity of these enzymes was followed in the ALD and PLD with normal development and with periods of immobilisation, in both the resting and shortened positions.

The main contractile proteins of muscle are actin and myosin. It is the interaction of the myosin, using its myosin "head" (termed a cross-bridge), with the actin that is believed to result in force development and length changes of the sarcomere and therefore overall contraction (Gordon, Huxley and Julian, 1966). This interaction utilises ATP as the immediate energy supply (Davies, 1964). Isolated myosin has been found to cleave ATP, with a catalytic site activity of about 0.04 mols. s^{-1} (Eisenberg and Moos, 1968), which is low in comparison with the catalytic site activity of actin and myosin combined, which is about 10 mols. s^{-1} (Eisenberg and Moos, 1970). A review of the kinetics of muscle contraction, with more details of the mechanism of cross-bridge cycles is given by White and Thorson (1975).

Although, studies on isolated contractile proteins, and analysis of the various enzyme kinetics they portray can give useful information about muscle contraction; it is difficult to relate these activities to the in vivo dynamic state of the muscle. In this comparative, developmental study, therefore, Mg^{2+}-activated myofibrillar ATPase activity was assayed instead of purified myosin or actomyosin, ATPase, activity. The normal substrate for ATP hydrolysis in solution is Mg-ATP (Lymn and Taylor, 1970). This substrate was used to activate the myofibrils, in which the contractile proteins, actin and myosin, should be unaltered structurally or biochemically. This system would be expected to approximate to the in vivo condition more accurately than would have been the case if isolated proteins had been used.

Bárány, (1967) studied the ATPase activity of myosin in various muscles in which the overall speed of muscle contraction had been determined. He found that a positive relationship existed between the

speed of shortening and the ATPase activity of myosin. The use of various activators, actin and Mg^{2+}, or Ca^{2+} or Ethylenediaminetetraacetate (EDTA) all showed proportionality, but of different ratios. Although the activity of Ca^{2+}-activated myofibrillar ATPase is used as a measure of contractile activity the ionic media of in vivo muscle shows Mg^{2+} in excess of Ca^{2+}. In this in vivo situation, Ca^{2+}-activated myofibrillar ATPase has been found to be depressed (Bendall, 1961). More physiological significance, therefore, is shown by the ATPase activated by actin or Mg^{2+}, than Ca^{2+} or EDTA. See Table 4, Page 65 for a summary of Bárány's findings.

Reasons and Hikida, (1973) have directly compared the actomyosin triphosphatase (AM-ATPase) activity of adult ALD and PLD muscles. They found only 1.4 times this enzyme's activity in the fast-contracting PLD than in the slow-contracting ALD. This is lower than would be expected from the relatively high speed of contraction of the PLD compared with the very slow-contracting ALD (Canfield, 1971; Page, 1969). This discrepancy is discussed with respect to the results obtained in this study for the Mg^{2+}-activated myofibrillar ATPase activities of the ALD and PLD muscles across development and with immobilisation.

Changes in the activity of the enzyme phosphorylase were examined to give some indication of the metabolic changes during development and with immobilisation, in ALD and PLD muscles. This is a predominant enzyme in the degradation of glycogen and its level has found to alter in various myopathies (Dubowitz and Pearse, 1961; Tassoni, Mantel and Harman, 1964; Leonard, 1957).

TABLE 4

RELATIONSHIP BETWEEN MAXIMAL SPEED OF SHORTENING (Vo)

AND ATPase ACTIVITY OF MYOSIN OF VARIOUS MUSCLES.

(Adapted from a table by Barany, 1967).

Muscle	Temp °C	Vo muscle lengths /sec	ATPase activity in presence of: Actin	ATPase activity in presence of: Ca^{2+}	References for Vo
			µmoles Pi/g/sec		
Mouse *extensor digitorum longus* (EDL)	35-36	24.0	30.03	21.81	*Close, (1965)
Rat EDL	35-36	17.2	27.95	20.64	
Mouse *soleus*	35-36	12.8	14.38	14.93	
Rat *soleus*	35-36	7.2	10.74	9.62	
Cat *soleus*	36	4.2	7.50	7.37	
Frog *sartorius*	22	10	16.91	11.55	Hanson & Lowy, (1960)
Frog *sartorius*	0	2	2.04	3.03	Ritchie & Wilkie, (1956)
Mytilus posterior adductor	14	0.1	0.15	0.17	Abbott & Lowy, (1953)
Pecten striated adductor	14	3	4.03	3.14	Hanson & Lowy, (1960)
Human elbow *flexor*	37	6	11.38	9.89	
Dogfish *coraco-hyoid*	0	2	1.83	2.77	Ritchie & Wilkie, (1956)
Rabbit uterus	37	0.2	0.23	0.47	Hanson & Lowy, (1960)
Tortoise *ilio-fibularis*	20	0.4	2.02	0.88	
Tortoise *ilio-fibularis*	0	0.1	0.13	0.20	Ritchie & Wilkie, (1956)
Rat diaphragm	37	11	15.32	13.56	Hanson & Lowy, (1960)

* Vo calculated from data of Close, (1965) by dividing maximum speed of shortening/sarcomere by the sarcomere length, 2.5µ .

The function of glycogen in muscle is as an energy store from which glucose can be rapidly formed when contraction is required. Skeletal muscle can be simply divided into two metabolic types, aerobic, slow and anaerobic, fast, muscle.

An aerobic, slow, muscle, for example the ALD muscle in chickens, has a good blood supply, many mitochondria and a high capacity for aerobic oxidation of glucose or fatty acids. In these types of muscle the supply of these substrates and oxygen via the blood should be sufficient for the energy needed for moderate but sustained mechanical activity. If the energy demands of the contraction exceeds the aerobic capacity, when oxygen becomes limited, extra energy can still be supplied via glycolysis. In this situation glycogen is anaerobically degraded to lactate. The anaerobic rate of ATP production is less than 10% of the aerobic rate and this extra ATP generation can only be maintained for a reasonably short time because lactate builds up and the glycogen reserves are soon depleted.

An anaerobic, fast muscle, for example the PLD muscle of chickens, contracts rapidly but only for short periods and its energy production and metabolism via glycolysis, is adapted to this rôle. Glycogen stores are greater in anaerobic fast muscle than in aerobic slow muscle and the phosphorylase activity is also higher reflecting this functional difference.

Skeletal muscle phosphorylase or α-glucan phosphorylase (Enzyme Commission No. 2.4.1.1.) exists in two forms termed a and b (Cori and Green, 1943). Phosphorylase a shows 60-70% of its maximal activity in the absence of adenosine-5'-monophosphate (AMP) whereas maximal activity phosphorylase b requires the presence of AMP (0.001M concentration,

pH 6.6). In a solution containing both a and b, measurement of enzymatic activity in the presence and absence of AMP can be used to calculate the relative proportions of the two enzymes and total activity.

Purification of mammalian muscle phosphorylase was first demonstrated by Cori and Cori, 1936. The work of the Coris' and colleagues have shown clearly the moleuclar relationship between the various phosphorylase forms. Phosphorylase b has a molecular weight of 242,000 half that of phosphorylase a, (495,000) (Keller and Cori, 1953).

Early work of the Coris' indicated the importance of Sulphydryl groups in the maintenance of activity and solubility of muscle phosphorylase. The presence of cysteine in the isolation media enhanced the phosphorylase storage life and activity. This gave further support to the concept that phosphorylase is an "SH-enzyme", i.e., an enzyme whose activity depends on the integrity of one or more sulphydryl groups.

Phosphorylase b can be converted to phosphorylase a by an additional enzyme "phosphorylase b kinase" which adds two phosphate groups to each molecule of phosphorylase b (Fischer and Krebs, 1955 (a)).

$$2\ \text{Phosphorylase b} + 4\text{ATP} \xrightarrow{\text{phosphorylase b kinase}} \text{Phosphorylase a} + 4\text{ADP}$$

Phosphorylase b kinase, exhibits little or no activity at pH 7.0 or below and is activated by ATP in the presence of Mg^{2+} ions. It can be inhibited to some degree by EDTA (Krebs, Graves and Fischer, 1959).

An enzyme has also been found which catalyses the conversion of phosphorylase a to phosphorylase b (Cori and Green, 1943). This enzyme is termed the "PR" enzyme or phosphorylase phosphatase.

$$\text{Phosphorylase } \underline{a} + 4H_2O \xrightarrow{\text{phosphorylase phosphatase (PR enzyme)}} 2 \text{ phosphorylase } \underline{b} + Pi$$

This enzyme can be strongly inhibited by AMP and Fluoride (F^-) ions (Keller and Cori, 1955).

Addition of fluoride ions and EDTA prevented little interconversion of the two enzymes and made direct analysis of the proportions of the two enzymes more accurate. The balance of the a and b forms in different physiological conditions is discussed in more detail with respect to the results obtained in this study.

Totally activated glycogen phosphorylase, therefore, catalyses this reversible reaction:

$$\text{Glycogen} + nH_3PO_4 \rightleftharpoons n \text{ (glucose-1-phosphate)}$$
$$\text{(G-1-P)}$$

The equilibrium is pH-dependent, with greater acidity favouring a larger production of glycogen and phosphate. The reaction assay in vitro follows this pattern:

$$n \text{ (glucose-1-phosphate)} + \text{(glycogen)}m \longrightarrow \text{(glycogen) } m+n+ nH_3PO_4$$

where (glycogen)m represents the primer of chain length m which becomes extended to a chain length of m+n. The effect of the phosphorylase

enzyme, therefore, is the transfer of glucose residues from G-1-P to the glycogen primer carbohydrate chain. Phosphorylase activity has been found to be predominantly in the supernatant fraction of crude tissue preparations (Dixon and Webb, 1964).

The overall activity of phosphorylase is believed to give a direct indication of the anaerobic capacity of the muscle (Krebs and Fischer, 1955 (b); Cosmos, 1966). The study of this catabolic enzyme in conjunction with a contractile enzyme such as Mg^{2+}-myofibrillar ATPase will lead to a better understanding of the changes that occur during normal muscle development; and how these normal growth patterns are altered with inactivity.

Materials and Methods

Birds

Rhode Island Red cross Light Sussex, male chickens were used (the same strain and suppliers as those used for the histochemical and general growth analyses in Chapters 2 and 3).

The ALD and PLD muscles were used to measure the enzyme activities of Mg^{2+}-myofibrillar ATPase and Phosphorylase. These activities were measured in the above muscles from control groups at 3, 8, 11, 15, 18, 22, 29, 36, 44 and 51 days of age.

Experimental groups using immobilisation in the resting and shortened positions were sacrificed and the muscles analysed at 6, 8, 15 and 22 days after immobilisation for 4, 6, 13 and 20 days from 2 days age. One group, immobilised in the shortened position for 20 days was left to recover for 7 days and sacrificed at 29 days of age.

Enough animals were used for each group to give between 0.75 and 2.0 grammes of muscle, when pooled. Therefore the younger age groups contained 40 animals; the oldest age groups containing a minimum of 5 animals. The contralaterals from the immobilised groups were considered separately.

Preparation of myofibrils

The chickens were killed with an overdose of pentobarbitone sodium (SAGATAL) to prevent spasmodic movements on death that could cause a depletion of phosphorylase a giving an abnormal rise in phosphorylase b (Cori and Green, 1943; Cori, 1945).

The ALD and PLD muscles were quickly dissected out, chopped finely and immersed in ice-cold 0.1M kCl; 5mM tris-HCl pH 7.5; 1mM dithiothreitol (dithiothreitol protects the sulphydryl groups, Cleland, 1964). All subsequent operations were carried out at 0°C. The chopped muscle was homogenised for 2 x 30 seconds with a pre-cooled Polytron blender, allowing the homogenate to cool in ice between homogenisations.

The suspension was then centrifuged at 1000g for 15 minutes; the supernatant was stored on ice. This supernatant has been shown to contain most of the phosphorylase activity (Dixon and Webb, 1964). The residue was then resuspended in the same solution and the procedure repeated twice more to wash out catheptic enzymes and any sarcoplasmic reticulum fragments which possess high ATPase activity (Ulbrecht, 1962). The myofibrils were then prepared according to the method of Perry and Grey (1956). The residue was resuspended and centrifuged for 30 seconds at 400g; the supernatant was then centrifuged for 15 minutes at 600g. The residue was then resuspended and the above procedure of

differential centrifugation repeated to give a well washed myofibril suspension.

Protein determination

The Biuret reaction (Gornall, Bardawill and David, 1949) was standardised with BSA and the E_{550}, measured after 15 minutes, was used to determine the protein concentration in the myofibrillar suspensions and initial supernatants (See AppendixC,Page194 for the standard curve). This protocol is suitable for estimation of protein concentration in the range of $1mgml^{-1}$ to $20mgml^{-1}$.

Mg^{2+}-myofibrillar ATPase activity

Mg^{2+}-myofibrillar ATPase activity was measured according to the method of Perry and Grey (1956) modified by Penney (1978). 1ml of solution containing 25mM tris-HCl, 0.2mM $CaCl_2$ and 1mg protein (myofibrils) at pH 8.0 was incubated at 22°C (room temperature) for 5 minutes. The reaction was initiated by addition of 1.5mM ATP in 3mM $MgCl_2$ and terminated by addition of an equal volume of 10% (w/v) Tricarboxylic acid (TCA) to the reaction mixture. Precipitated protein was removed by centrifugation at 3000g for 5 minutes. The supernatant fraction was then assayed for inorganic phosphate. Control incubations contained myofibrils in suspension buffer without ATP or $MgCl_2$. The incubations were carried out in triplicate whenever possible.

Phosphorylase activity

(adaptation of a method by Illingworth and Cori, 1953).

After protein determination the supernatant was diluted with 0.06M

cysteine HCl; 0.07M Na-glycerophosphate buffer at pH 6.6 to give a final protein concentration of $1mgml^{-1}$. The buffer also contained 20mM NaF which inhibits the "PR" enzyme and 1mM EDTA which inhibits the kinase that converts phosphorylase b to the a form.

Total phosphorylase activity (a and b) was measured in a reaction mixture of 0.8ml containing 0.037M Glucose-1-phosphate; 0.001M Adenosine-mono-phosphate; 1% Glycogen (w/v); $1mgml^{-1}$ protein.

Triplicate assays were carried out at 30°C for 30 minutes at pH 6.6 and were terminated with 5 mls of 10% perchloric acid. Aliquots of the supernatant of the reaction were then assayed for inorganic phosphate.

Phosphorylase a activity, was measured in the same manner as described above but the 5' Adenosine-mono-phosphate was omitted.

Control incubations contained the whole supernatant without glucose-1-phosphate or 5' Adenosine-mono-phosphate. Controls were also incubated with substrate only, to observe any endogenous release of contaminating phosphate.

Inorganic phosphate assay

(Rockstein and Herron, 1951).

Inorganic phosphate was measured using 0.5ml aliquots of the terminated reaction supernatant. This was added to 0.5ml of fresh ferrous sulphate solution and 0.5ml of ammonium molybdate solution with 1ml of distilled water. After 10 minutes the E_{700} was measured; standardized with oven dried KH_2PO_4. This method for the assay of phosphate is sensitive between 0-2 μ MPi ml^{-1} (See AppendixC,Page194, for the standard curve).

Preliminary assays for establishing the optimum assay conditions for Mg^{2+}-myofibrillar ATPase and Phosphorylase pH Optimum

Optimum pH of the incubation was found to be pH 8.0 for Mg^{2+}-myofibrillar ATPase and pH 6.6 for phosphorylase for both ALD and PLD. See Figs. 4(i)&4(ii), Pages 74 & 75, for graphical illustrations.

The establishment of the optimum pH was especially important for phosphorylase to minimise any remaining phosphorylase b kinase enzyme activity. Activity of this enzyme is reduced at pH 7.0 and under. It is clear from Fig. 4(ii), Page 75, that any conversion of phosphorylase b to a had already been completed before the assay was carried out. The balance of the two enzymes was assumed to be reflective of the situation on sacrifice of the birds.

Linearity of enzyme activity with time

The period of incubation for the Mg^{2+}-myofibrillar ATPase enzyme was carried out for 5 minutes. See Fig. 4(iii), Page 76, for the graphical illustration of linearity of activity at 5 minutes for both muscles.

The period of incubation for phosphorylase enzyme was set at 30 minutes. See Fig. 4 (iv), Page 77, for the graphical illustration of linearity of both enzymes, a and a+b with time.

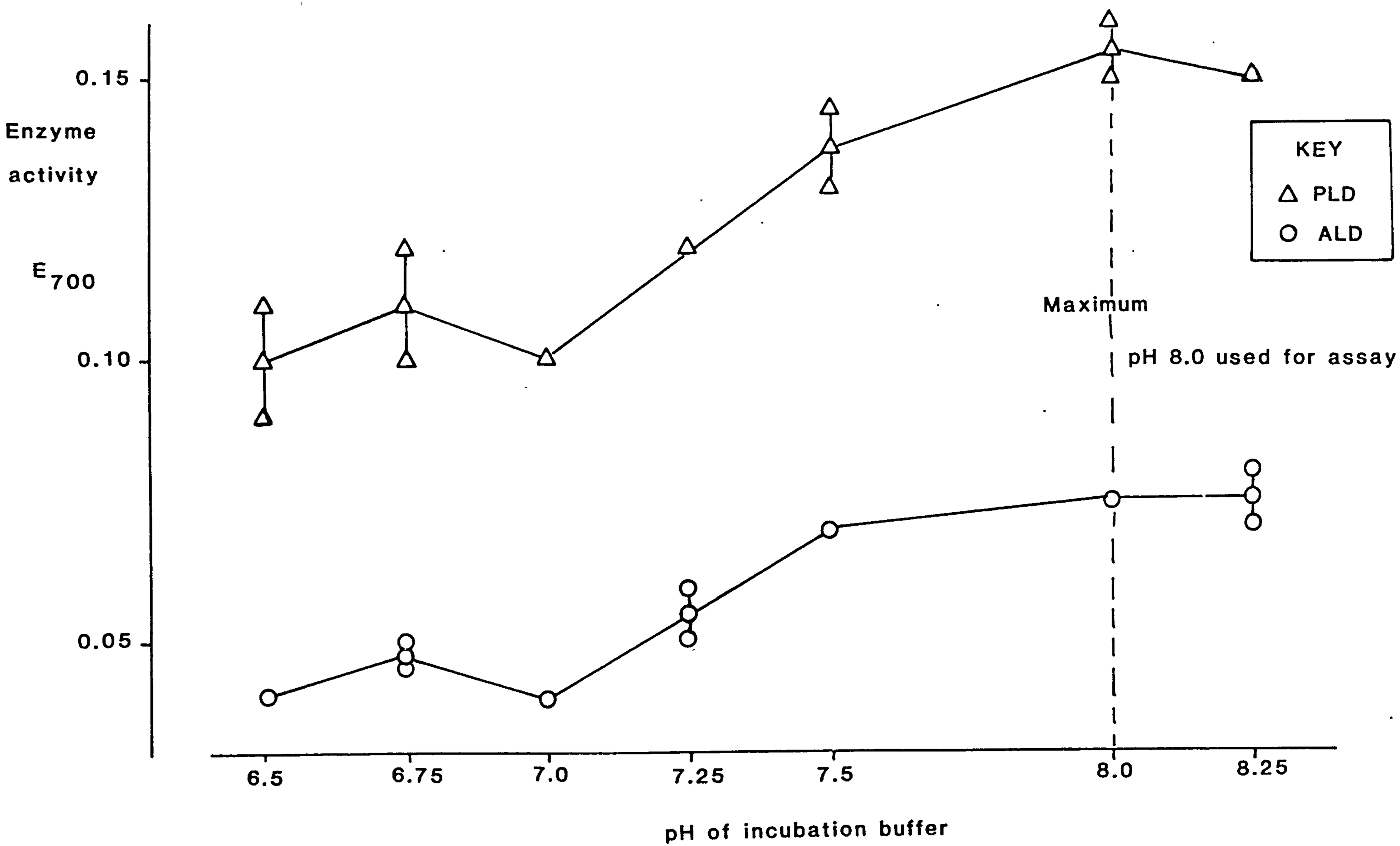
Linearity of enzyme activity with temperature

Both enzymes were incubated for the set experimental time periods at 3 different temperatures; 10°C, 22°C (room temperature) and 30°C for phosphorylase and 10°C, 20°C and 37°C for Mg^{2+}-myofibrillar ATPase (See Figs. 4(v) & 4(vi), Pages 78 & 79). Phosphorylase was incubated at 30°C

See overleaf for Figure and Figure Legend 4(i).

Figure 4(i)

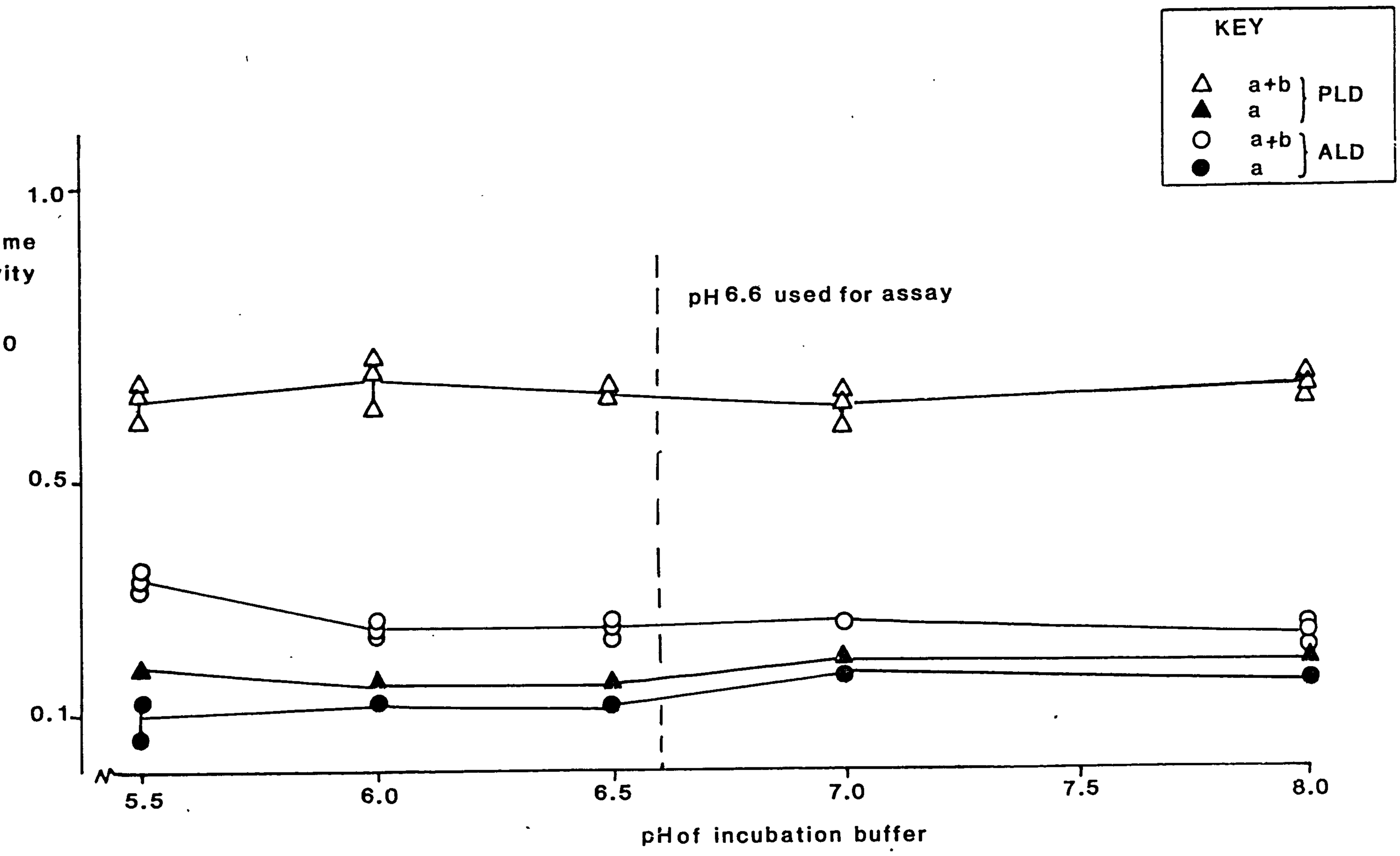
Mg^{2+}-activated myofibrillar ATPase activity against the pH of the incubation buffer for the ALD and PLD muscles; incubated at 22°C for 5 minutes.


0.15
Enzyme activity
E_{700}
0.10
0.05
KEY
PLD
ALD
Maximum
pH 8.0 used for assay
6.5
6.75
7.0
7.25
7.5
8.0
8.25
pH of incubation buffer

See overleaf for Figure and Figure Legend 4(ii).

Figure 4(ii)

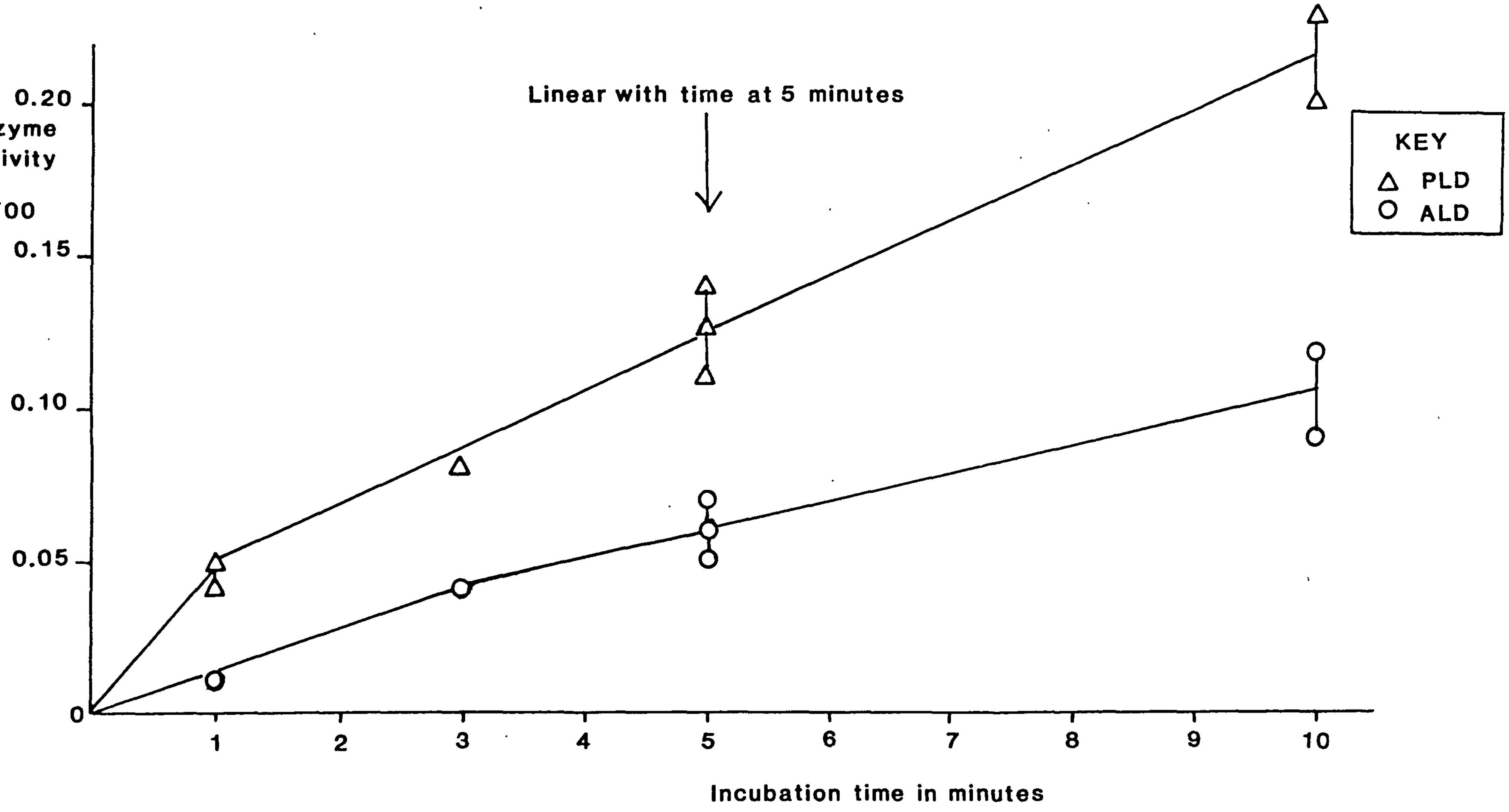
Phosphorylase a and (a + b) activity against the pH of the incubation buffer for the ALD and PLD muscles; incubated at 30°C for 30 minutes.


KEY
a+b
a
PLD
a+b
a
ALD
1.0
0.5
0.1
Enzyme activity
E700
pH 6.6 used for assay
5.5
6.0
6.5
7.0
7.5
8.0
pH of incubation buffer

See overleaf for Figure and Figure Legend 4(iii).

Figure 4(iii)

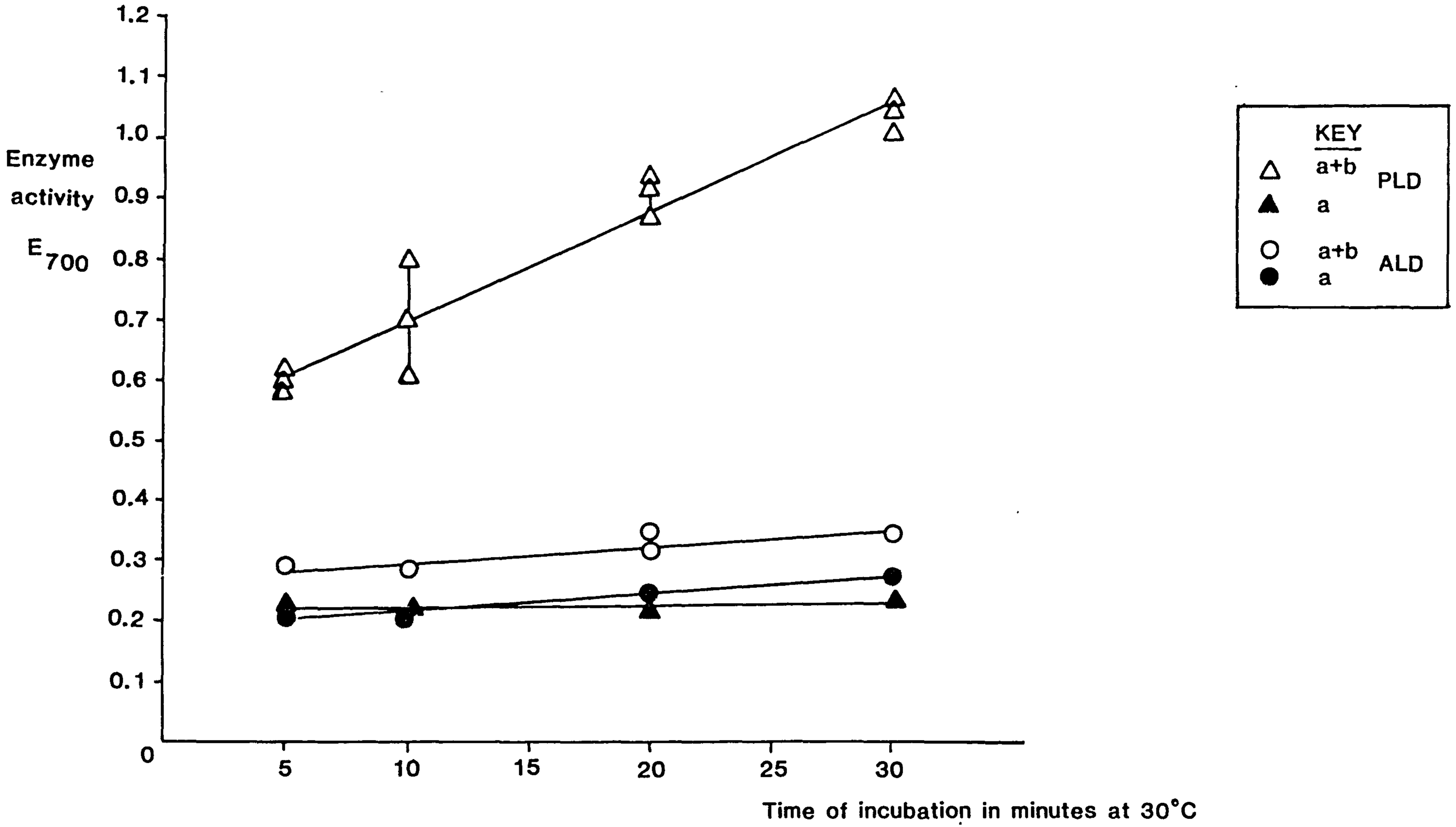
Mg^{2+}-activated myofibrillar ATPase activity against the time of reaction for the ALD and PLD muscles; incubated at 22°C, pH 8.0.


Linear with time at 5 minutes
KEY
PLD
ALD
0.20
Enzyme activity
E_{700}
0.15
0.10
0.05
0
1
2
3
4
5
6
7
8
9
10
Incubation time in minutes

See overleaf for Figure and Figure Legend 4(iv).

Figure 4(iv)

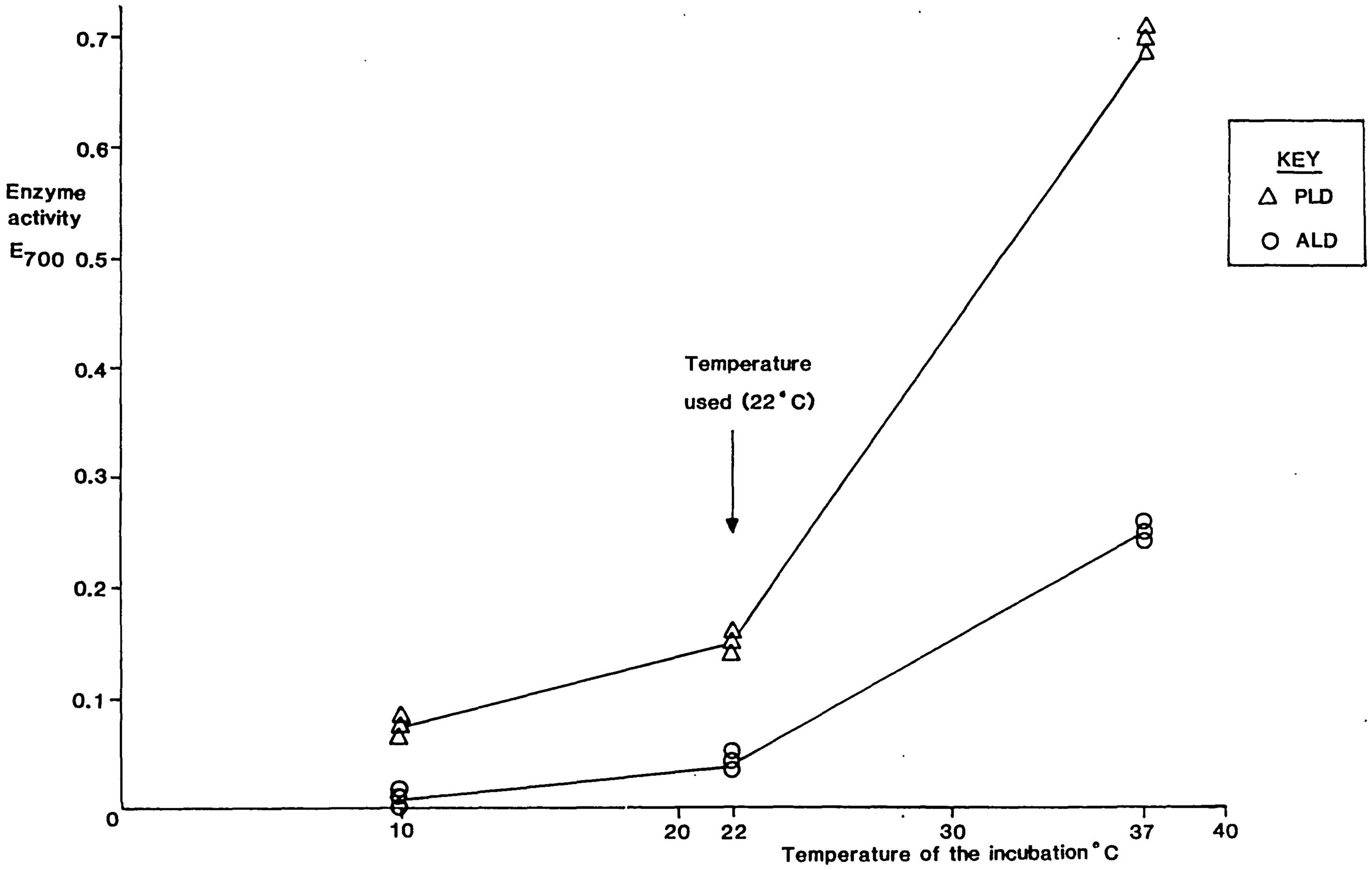
Phosphorylase a and (a + b) activity against the time of reaction for the ALD and PLD muscles; incubated at 30°C, pH 6.6.


Enzyme activity E_{700}
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
30
Time of incubation in minutes at 30°C
KEY
a+b
a
PLD
a+b
a
ALD

See overleaf for Figure and Figure Legend 4(v).

Figure 4(v)

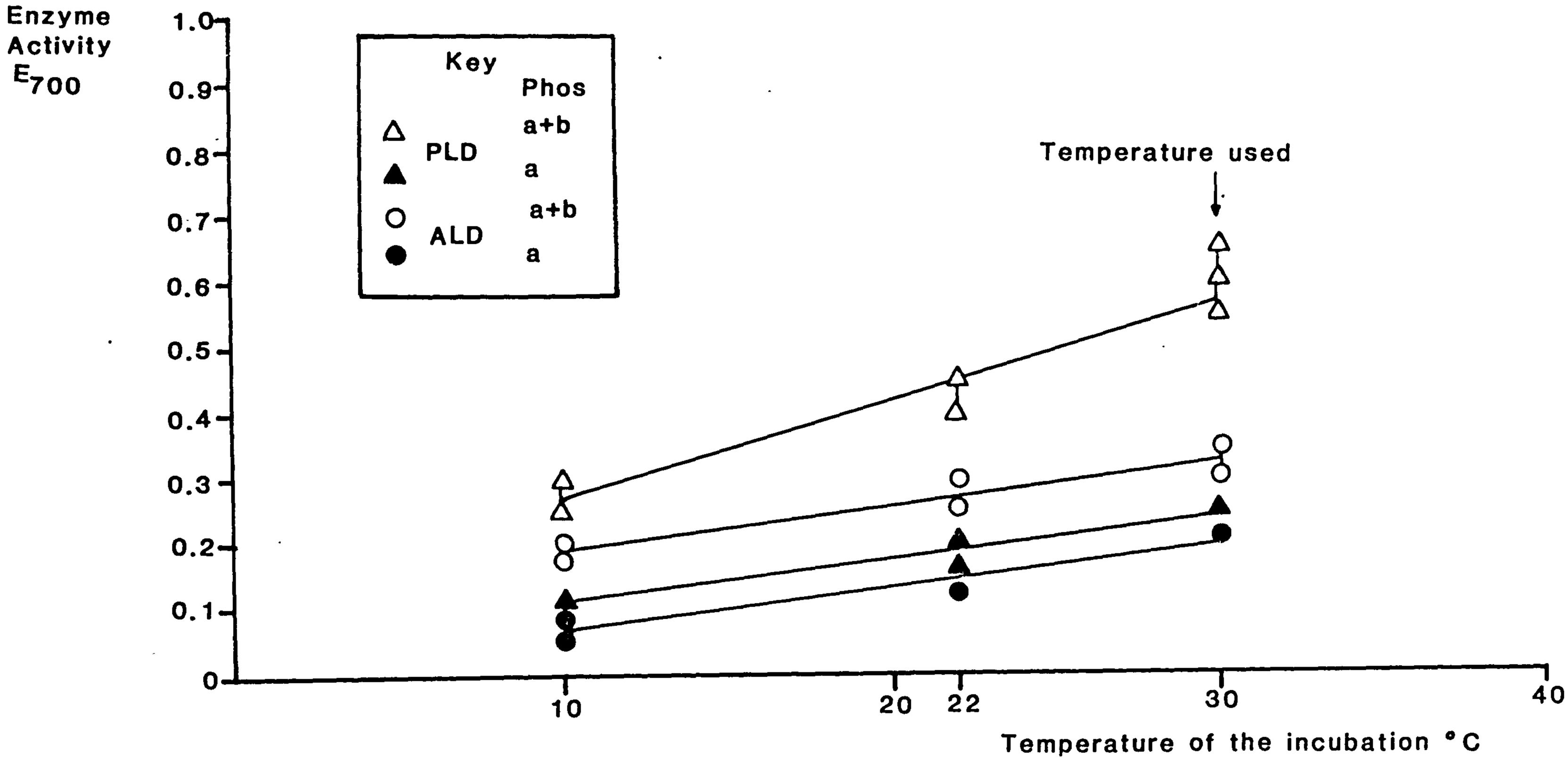
Mg^{2+}-activated myofibrillar ATPase activity during a 5 minute incubation period at pH 8.0 for the ALD and PLD muscles against temperature.


0.7
0.6
Enzyme activity
E_{700} 0.5
0.4
0.3
0.2
0.1
0
KEY
△ PLD
○ ALD
Temperature used (22° C)
10
20
22
30
37
40
Temperature of the incubation °C

See overleaf for Figure and Figure Legend 4(vi).

Figure 4(vi)

Phosphorylase a and (a + b) activity during a 30 minute incubation period at pH 6.6 for the ALD and PLD muscles against temperature.


Enzyme Activity E_{700}
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Key
Phos
PLD
a+b
a
ALD
a+b
a
Temperature used
10
20
22
30
40
Temperature of the incubation °C

for all further assays. This temperature was chosen as it gave a high concentration of liberated phosphate for measurement and it is the temperature suggested by the adapted method (Illingworth and Cori, 1953).

22°C or room temperature was chosen for Mg^{2+}-myofibrillar ATPase for its direct comparison with the findings of Reasons and Hikida, 1973, who used 22°C also.

Substrate availability

Assays for Mg^{2+}-myofibrillar ATPase and Phosphorylase were performed with varying substrate concentrations to ensure that the substrate was not limiting the enzymatic reactions (See Figs.4(vii) & 4(viii),Pages 81 & 82).

These preliminary assays were carried out using the enzyme extracts from muscles of birds 50 days and over; triplicate assays were performed wherever possible.

Units of enzyme activity

1. Mg^{2+}-myofibrillar ATPase activity

The optical density at 700nm was converted to mols. of ATP split/mol. myosin/second using the conversion for phosphate (See AppendixC,Page194), dilution factor, and the following assumptions for the molecular weight of myosin:

μMPi/mg protein/minute ⟶ mols. ATP split/mol. myosin/second.

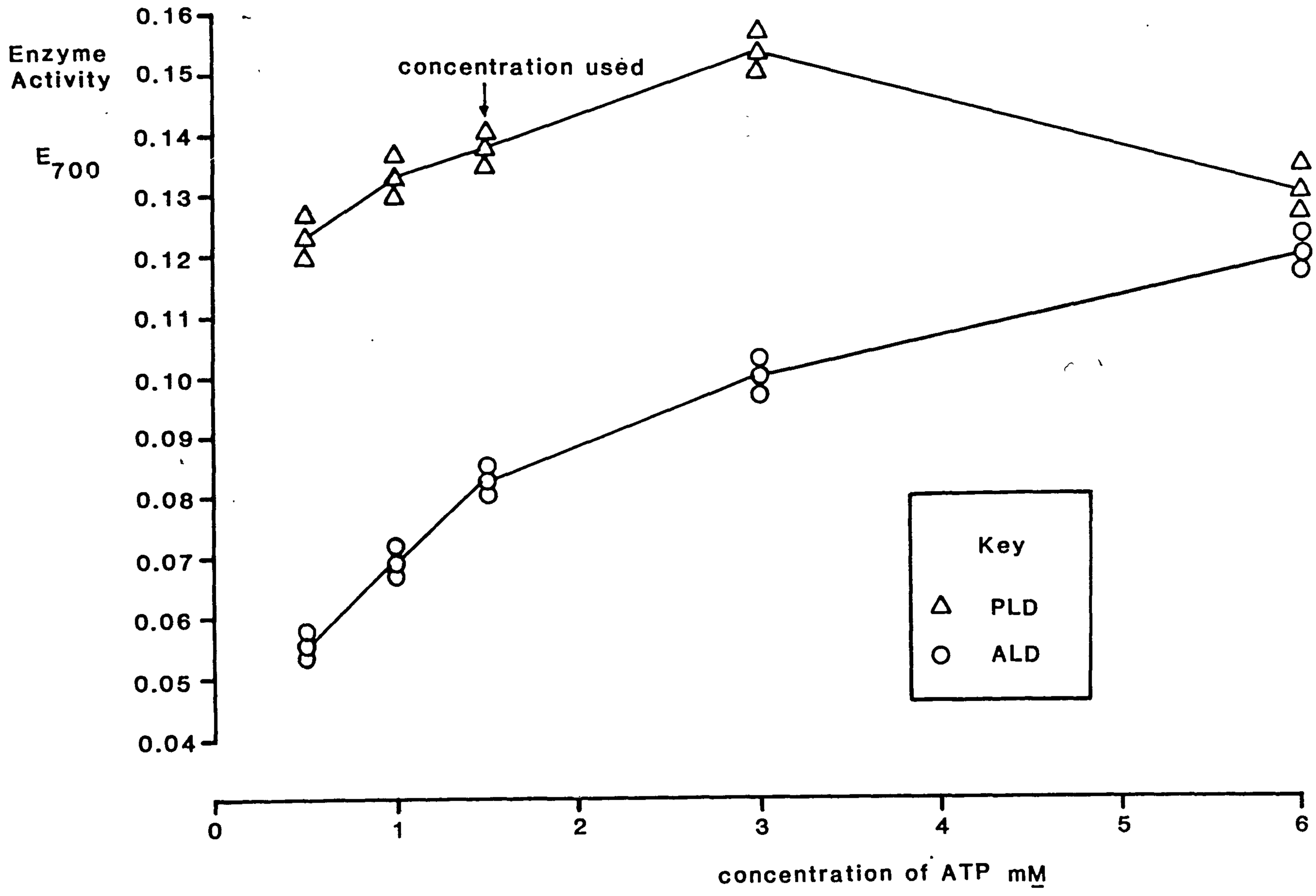
Molecular weight of myosin = 240,000 (Lowey, Slayter, Weeds and Baker, 1969; Godfrey and Harrington, 1970).

Myosin = 54% of the myofibril (Bendall, 1969).

See overleaf for Figure and Figure Legend 4(vii).

Figure 4(vii)

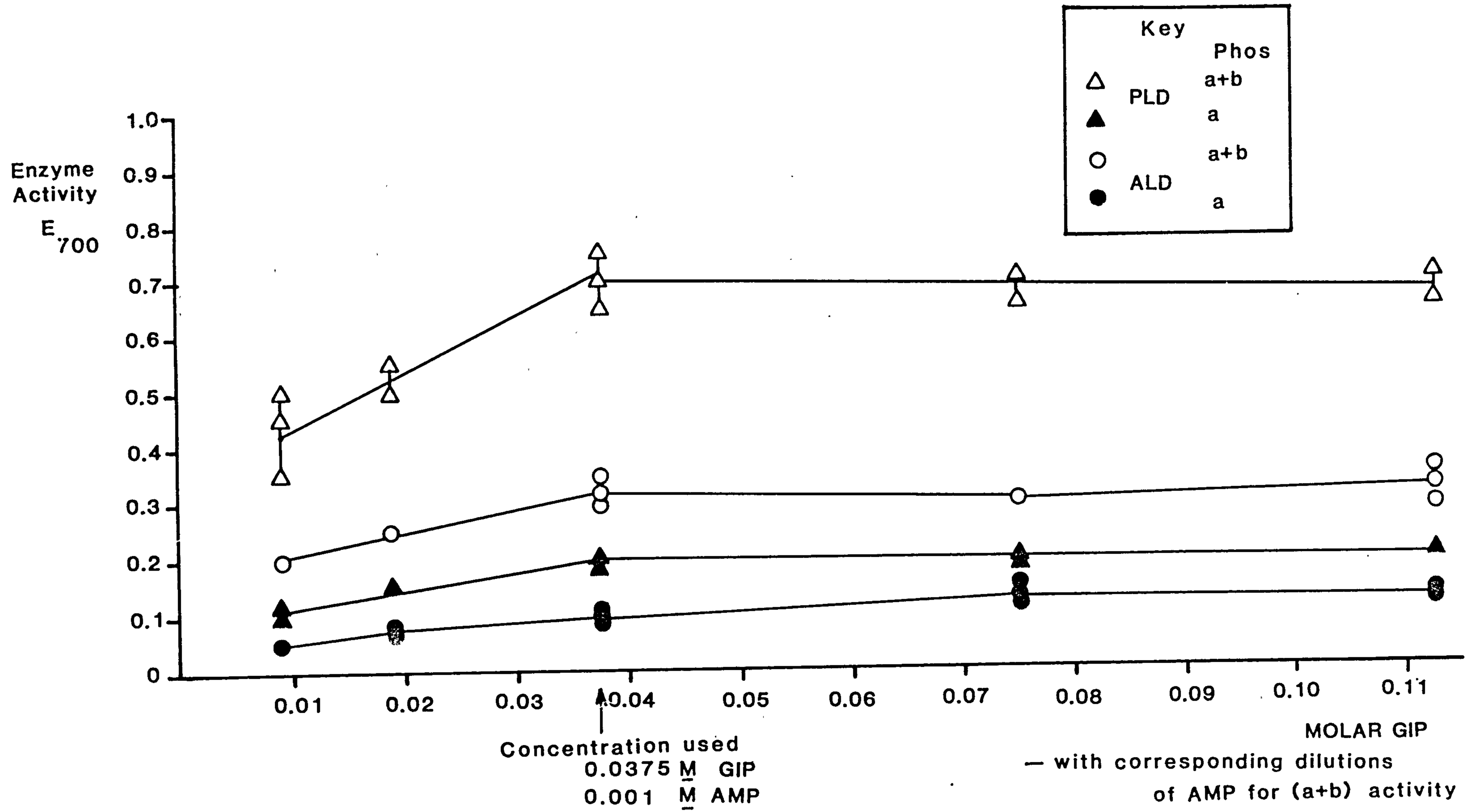
Mg^{2+}-activated myofibrillar ATPase activity against substrate availability for the ALD and PLD muscles; incubated at 22°C; pH 8.0 for 5 minutes.


Enzyme Activity
E_{700}
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
concentration used
Key
PLD
ALD
0
1
2
3
4
5
6
concentration of ATP mM

See overleaf for Figure and Figure Legeng 4(viii).

Figure 4(viii)

Phosphorylase *a* and (*a* + *b*) activity against substrate availability for the ALD and PLD muscles; incubated at 30°C, pH 6.6 for 30 minutes.


Key
Phos
PLD
a+b
a
ALD
a+b
a
Enzyme Activity
E 700
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
0.11
Concentration used
0.0375 M GIP
0.001 M AMP
MOLAR GIP
— with corresponding dilutions
of AMP for (a+b) activity

1mg protein = 0.54mg myosin.

1M myosin = 240,000g

$$0.54 \text{ mg myosin} = \frac{1}{240{,}000} \times 0.00054 = 0.00225\ \mu M$$

1 μMPi/mg protein/minute = 1 μMATP split/0.0025 μM myosin/60 seconds

= 7.4 mols. ATP split/mol. myosin/second

Conversion equation

$$\frac{\Delta OD \times 1.8 \text{ (phosphate conversion factor} \times 4 \text{ (dilution factor)} \times 7.4}{\text{Time (5 minutes)} \times \text{(Protein)}}$$

= mols. ATP split/mol. myosin/second.

2. Phosphorylase activity

The optical density at 700nm was converted to μMPi/mg protein/minute using the conversion factor for phosphate (1.8) times the dilution factor (11.6) divided by the time (30 minutes).

Results

1. Mg^{2+}-myofibrillar ATPase activity

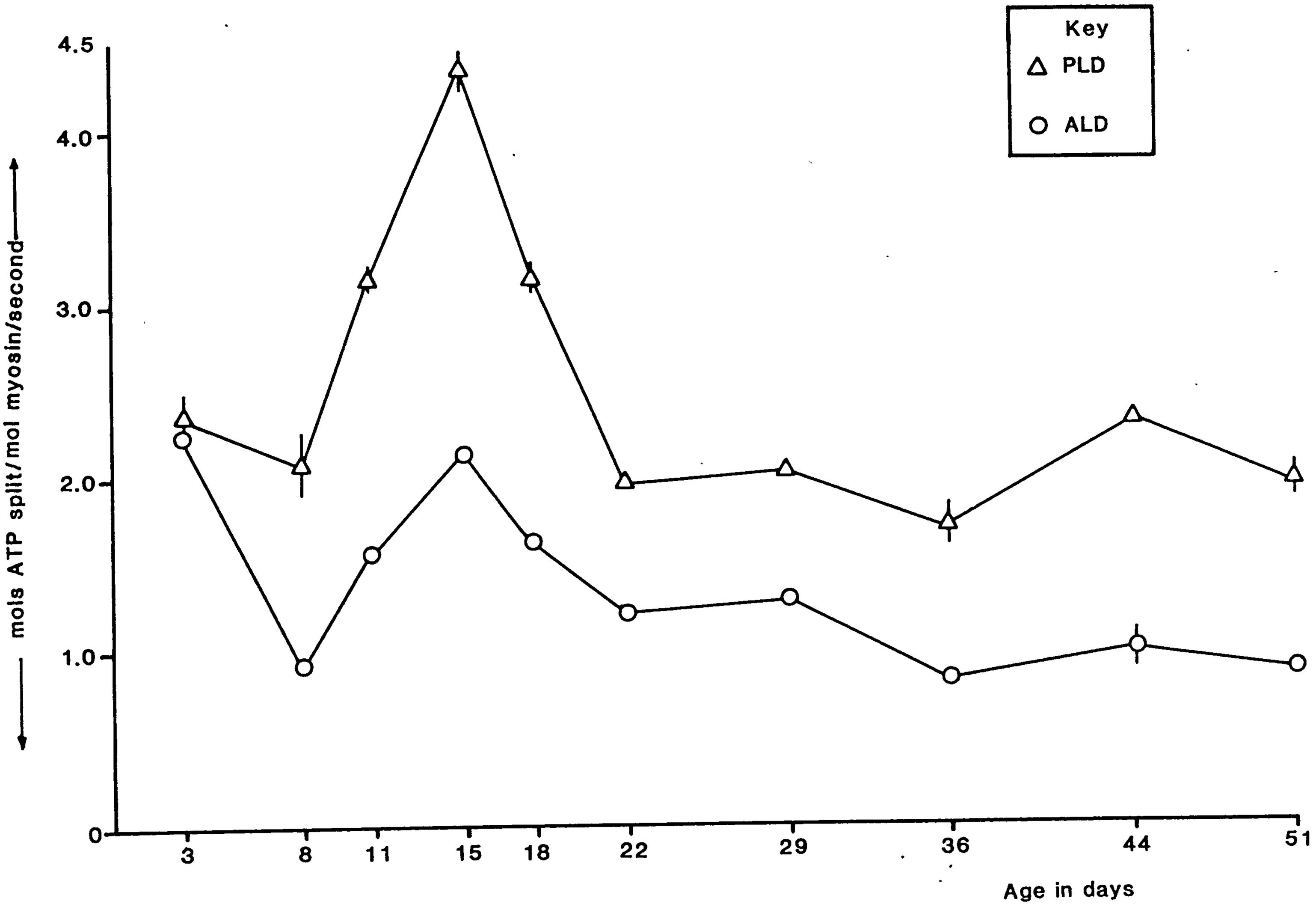
The results from the activity of Mg^{2+}-myofibrillar ATPase throughout development and with immobilisation are described; for the control muscles on Fig. 4(ix) Page 84 ; for the experimental muscles on Fig. 4(x), Page 85 ; and for the contralateral muscles on Fig. 4(xi), Page 86 .

See overleaf for Figure and Figure Legend 4(ix).

Figure 4(ix)

Mg^{2+}-activated myofibrillar ATPase activity for the control ALD and PLD muscles during the first 51 days, ex ovo; incubated at room temperature 22°C, pH 8.0.

(Standard errors are only for the technique).


Key
PLD
ALD
4.5
4.0
3.0
2.0
1.0
0
mols ATP split/mol myosin/second
3
8
11
15
18
22
29
36
44
51
Age in days

See overleaf for Figure and Figure Legend 4(x).

Figure 4(x)

Mg^{2+}-activated myofibrillar ATPase activity for the experimental ALD and PLD muscles in comparison with the controls.

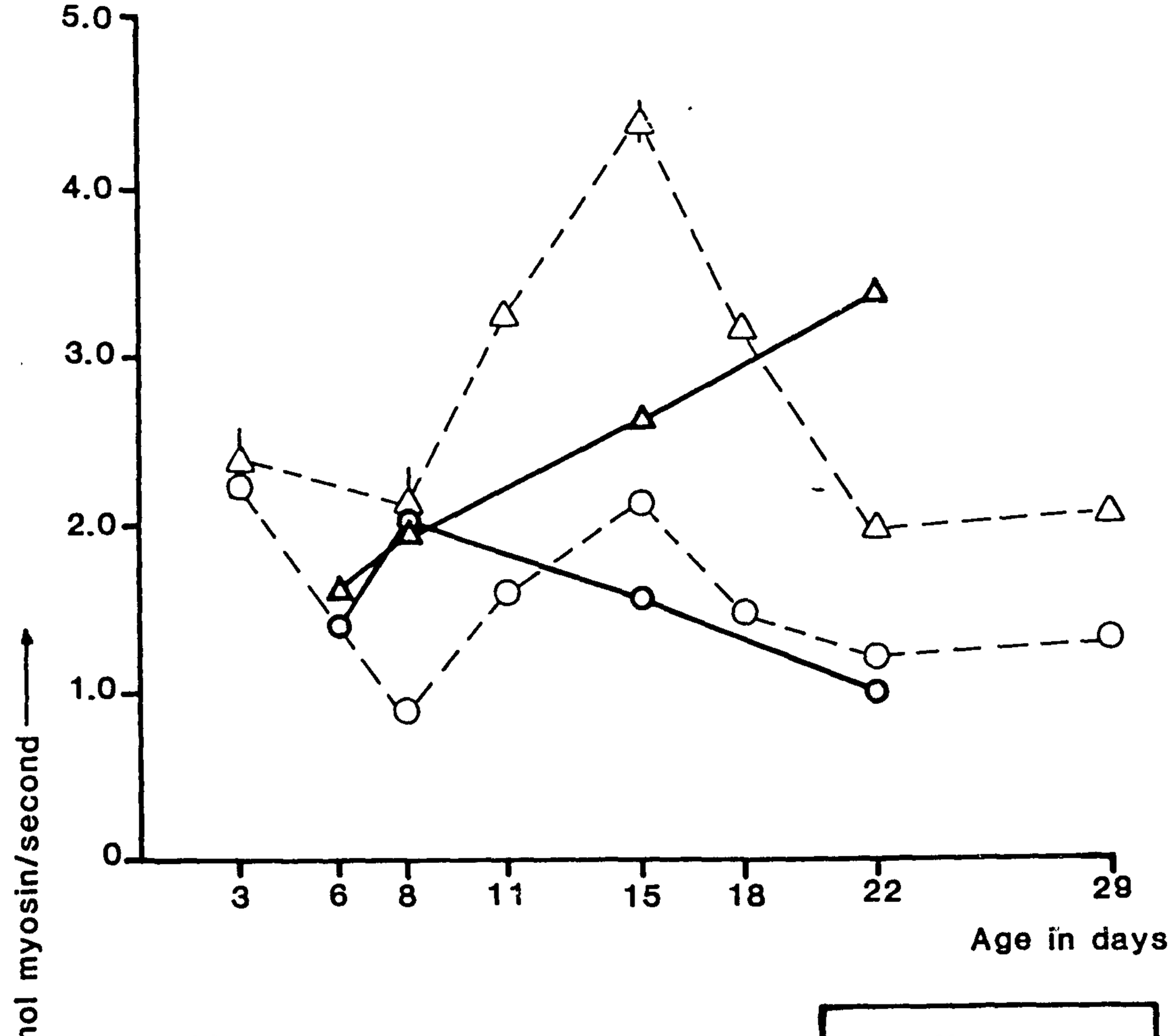
(Incubated at room temperature, (22°C), pH 8.0).

a) Experimentals immobilised in the resting position

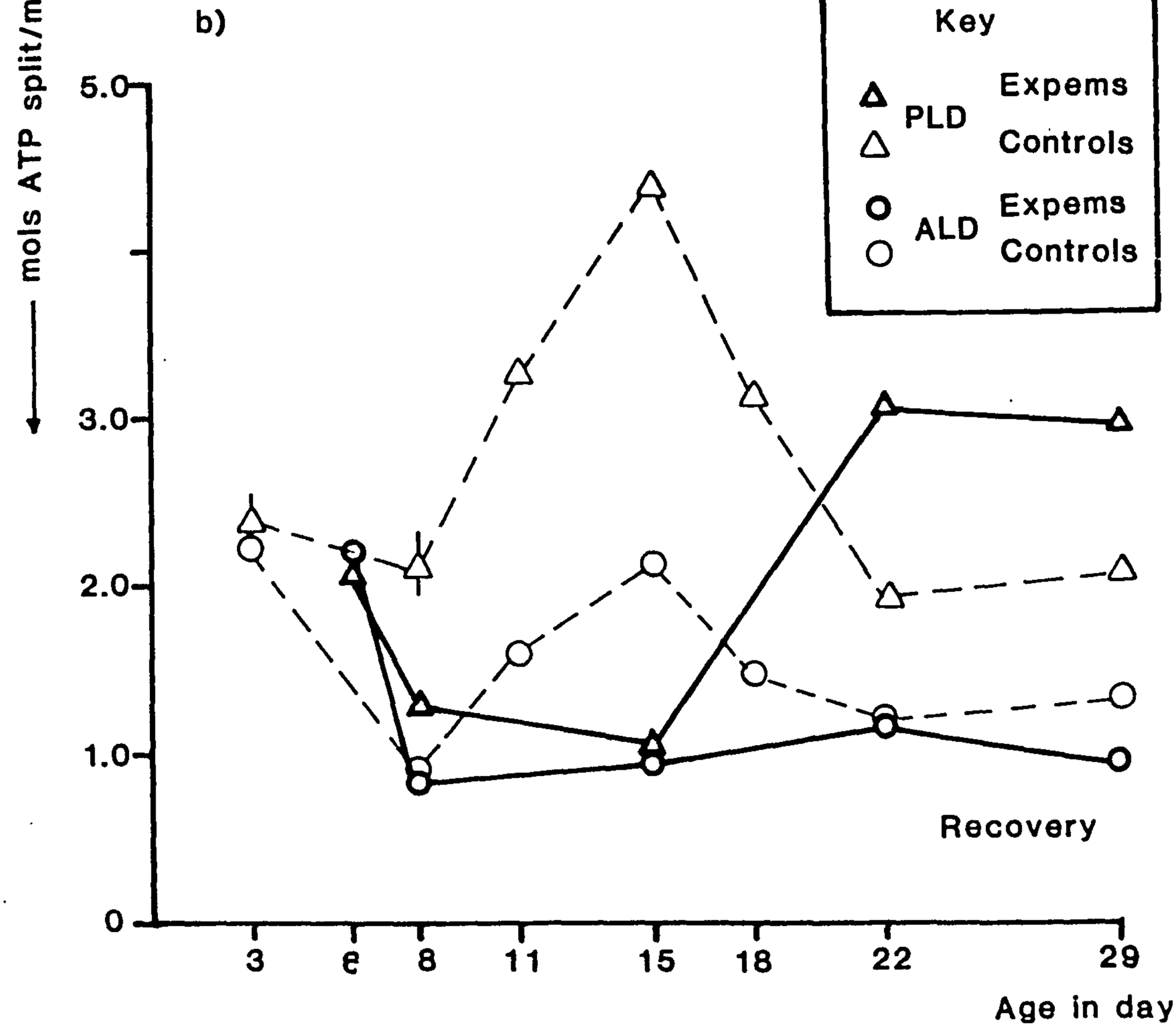
b) Experimentals immobilised in the shortened position

(Standard errors are only for the technique)

a)


5.0
4.0
3.0
2.0
1.0
0
mols ATP split/myosin/second
3
6
8
11
15
18
22
29
Age in days

b)


Key
PLD
Expems
Controls
ALD
Expems
Controls
5.0
3.0
2.0
1.0
0
mols ATP split/mol
3
6
8
11
15
18
22
29
Recovery
Age in days

See overleaf for Figure and Figure Legend 4(xi).

Figure 4(xi)

Mg^{2+}-activated myofibrillar ATPase activity for the contralateral ALD and PLD muscles in comparison with the controls.

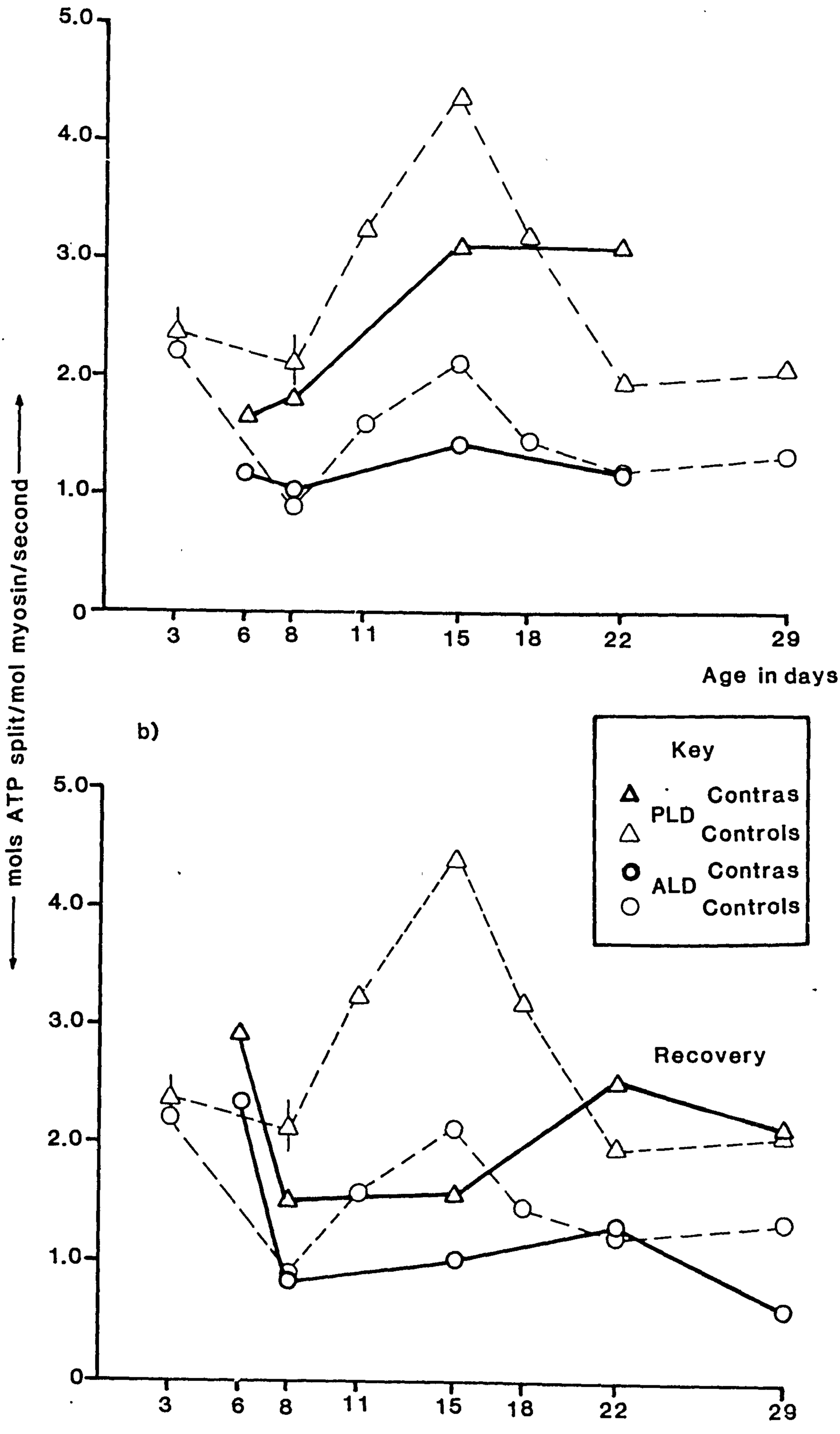
(Incubated at room temperature, (22°C), pH 8.0).

a) Contralaterals from immobilisation in the resting position

b) Contralaterals from immobilisation in the shortened position

(Standard errors are only for the technique)

a)


5.0
4.0
3.0
2.0
1.0
0
3
6
8
11
15
18
22
29
Age in days
— mols ATP split/mol myosin/second —
b)
Key
PLD Contras
PLD Controls
ALD Contras
ALD Controls
Recovery

From Fig. 4(ix), Page 84 , it can be seen that both the ALD and PLD showed a similar activity at 3 days of age and this diverged in the first week of life with the PLD remaining high in activity at 2.0 mols. ATP split/mol. myosin/s. with the ALD's activity reduced from 2.0 to 1.0 mols. ATP split/mol. myosin/s. Between 10 and 20 days of age both the ALD and PLD showed a peak in activity reaching · 4.0 mols. ATP split/mol. myosin/s. for the PLD at 15 days and back up to 2.0 for the ALD. From 20 days onwards the activity of the PLD remained around 2.0 mols. ATP split/mol. myosin/s. with the ALD dropping from just over 1.0 to just over a half or 0.5 mols. ATP split/mol. myosin/s. by 50 days of age. The end result at 51 days showed the PLD's Mg^{2+}-myofibrillar ATPase activity at 4 times that of the ALD. The pattern of this enzyme's changes can be divided into 3 phases.

1. An initial similarity of activity at 2.0 mols. ATP split/ mol. myosin/s. for both muscles.

2. A peak of activity for both muscles with a top value of 4.0 mols. ATP split/ mol. myosin/s. for the PLD muscle and 2.0 mols. ATP split/mol. myosin/s. for the ALD muscle at 15 days.

3. A maintenance of a steady level of 2.0 mols. ATP split/mol. myosin/s. for the PLD muscle from 20 days onwards. A steady drop from 1.0 mols. ATP split/mol. myosin/s. for the ALD muscle to 0.5 mols. ATP split/mol. myosin/s. by 51 days.

The activity peak at 15 days was confirmed with extra assays performed at 11 and 18 days of age. These activities, very convincingly, fell on each side of the peaks in both muscles, indicating that this really was a feature of development and not just a spurious result.

Fig. 4(x), Page 85, shows the changes in Mg^{2+}-activated myofibrillar ATPase and PLD muscles with immobilisation in the resting position. After 4 days of immobilisation the activities for both muscles were similar with the PLD just above 1.5 mols. ATP split/mol. myosin/s. and the ALD just below. After 6 days the activities of both muscles increased to 2.0 mols. ATP split/mol. myosin/s. The two longer time periods of immobilisation showed an increase for the PLD muscle to just over 3.0 mols. ATP split/mol. myosin/s. by 20 days of immobilisation. The ALD however showed a decrease in activity to a value just below 1.0 mols. ATP split/mol. myosin/s. after 20 days of immobilisation. When immobilisation for 20 days was applied in the resting position the peak in activity exhibited by the controls appeared to have been displaced for the PLD from 15 days to 22 days of age. The peak exhibited by the ALD in the controls, although much less pronounced than the PLD was not apparent when immobilisation was applied in the resting position.

Immobilisation in the shortened position showed an overall decrease for the PLD muscle to 1.0 mols. ATP split/mol. myosin/s. at 15 days after 13 days of inactivity (See lower graph, Fig. 4(x), Page 85). Immobilisation for 20 days in this shortened position showed a final increase to 3.0 mols. ATP split/mol. myosin/s. with little change in activity exhibited after 7 days of recovery.

Both muscles showed a similar activity after 4 days of immobilisation in the shortened position at 2.0 mols. ATP split/mol. myosin/s. which was similar to that of the control muscles at 3 days age.

The ALD, for the longer time periods of immobilisation in the shortened position showed a decrease in activity remaining around 1.0 mols. ATP split/mol. myosin/s. Once again the peak in activity shown by the

control muscles at 15 days seems to be displaced for the PLD to 22 days of age and was not observable for the ALD.

Fig. 4(xi), Page 86 , compares the activities of the contralateral muscles for the two immobilised positions with the controls.

The contralaterals from the groups immobilised in the resting position showed values similar to those of the controls although the activity was reduced slightly for both muscles and all age groups.

The contralaterals from the groups immobilised in the shortened position showed high activities for both muscles after 4 days of immobilisation in comparison with all other results for muscles at this age group. This high activity becomes reduced to lower values after 6 and 13 days of immobilisation for both muscles. There is some recovery to more normal values after 20 days of immobilisation. The overall pattern for the contralaterals from immobilisation in the shortened position with time reflects that of the experimentals in an interesting manner.

The following table condenses the results for groups at 8, 15 and 22 days of age to give a clearer picture of the changes in absolute values during the peak activity period.

Mg^{2+}-MYOFIBRILLAR ATPase ACTIVITY AT 22°C pH 8.0

IN MOLS ATP SPLIT/MOL. MYOSIN/SECOND

		8 Days	CHANGE	15 Days	CHANGE	22 Days	Age CHANGE
ALD							
	CONTROLS	0.924 0.04		2.130 0.11		1.208 0.04	
IMMOBILISED RESTING	EXPERIMENTALS	2.060 0.07	⇉	1.279*	↓↓	0.995 0.04	↓
	CONTRALATERALS	1.060*	--	1.492*	↓↓	1.273*	--
IMMOBILISED SHORTENED	EXPERIMENTALS	0.853 0.11	--	0.995 0.09	↓↓	1.279 0.06	--
	CONTRALATERALS	0.924 0.13	--	1.066*	↓↓	1.279*	--
PLD							
	CONTROLS	2.096 0.22		4.369 0.11		1.989 0.04	
IMMOBILISED RESTING	EXPERIMENTALS	1.954 0.04	--	2.629 0.15	⇉	3.374 0.09	⇉
	CONTRALATERALS	1.812 0.10	↓	3.126 0.07	⇉	3.126 0.07	⇉
IMMOBILISED SHORTENED	EXPERIMENTALS	1.385*	⇉	1.030 0.04	⇉	3.090*	⇉
	CONTRALATERALS	1.527 0.04	↓	1.532 0.09	⇉	2.522 0.09	↑

Standard errors are for the triplicate assays and give an indication of technique accuracy only.

→ change less than 0.5 of an ATP

⇉ change greater than 0.5 of an ATP

-- difference less than 0.1 of an ATP

* standard error for the technique = zero

2. Phosphorylase activity

The results for the activity of phosphorylase a and phosphorylase (a+b) throughout development and with immobilisation are shown; for the control muscles on Fig. 4(xii), Page 92 ; for the experimental muscles on Fig. 4(xiii), Page 93 ; and for the contralateral muscles on Fig. 4(xiv), Page 94 . The lower two lines on each graph are for phosphorylase a alone. Phosphorylase a+b activity will be referred to as the total phosphorylase activity.

From Fig. 4(xii), Page 92 , the graphs show the changes in phosphorylase activity for the ALD and PLD during normal development for the first 51 days ex ovo. At 3 days of age the total phosphorylase activity for the ALD was half that of the PLD, approximately 0.25 and 0.5 μ MPi/mg protein /minute respectively. The amount of phosphorylase a activity was the same for both muscles and low, approximately at 0.05 μ MPi/mg protein/ minute. Over the next 12 days total phosphorylase activity increased for both muscles to a peak at 15 days of 0.73 and 1.11 μ MPi/mg protein/ minute for ALD and PLD respectively. This level then dropped for the PLD to a value of approximately 0.75 μ MPi/mg protein/minute at 51 days of age. The level of 0.73 for the ALD remained high until 36 days of age when it steadily dropped to a value of 0.29 μ MPi/mg protein/minute at 51 days of age. At the end point of the study, 51 days, the PLD showed a 3 times higher total activity of phosphorylase than the ALD. Phosphorylase a activity followed a similar pattern to the total activity but did not exhibit a peak at 15 days. The activities were always higher for the PLD than the ALD at each age studied.

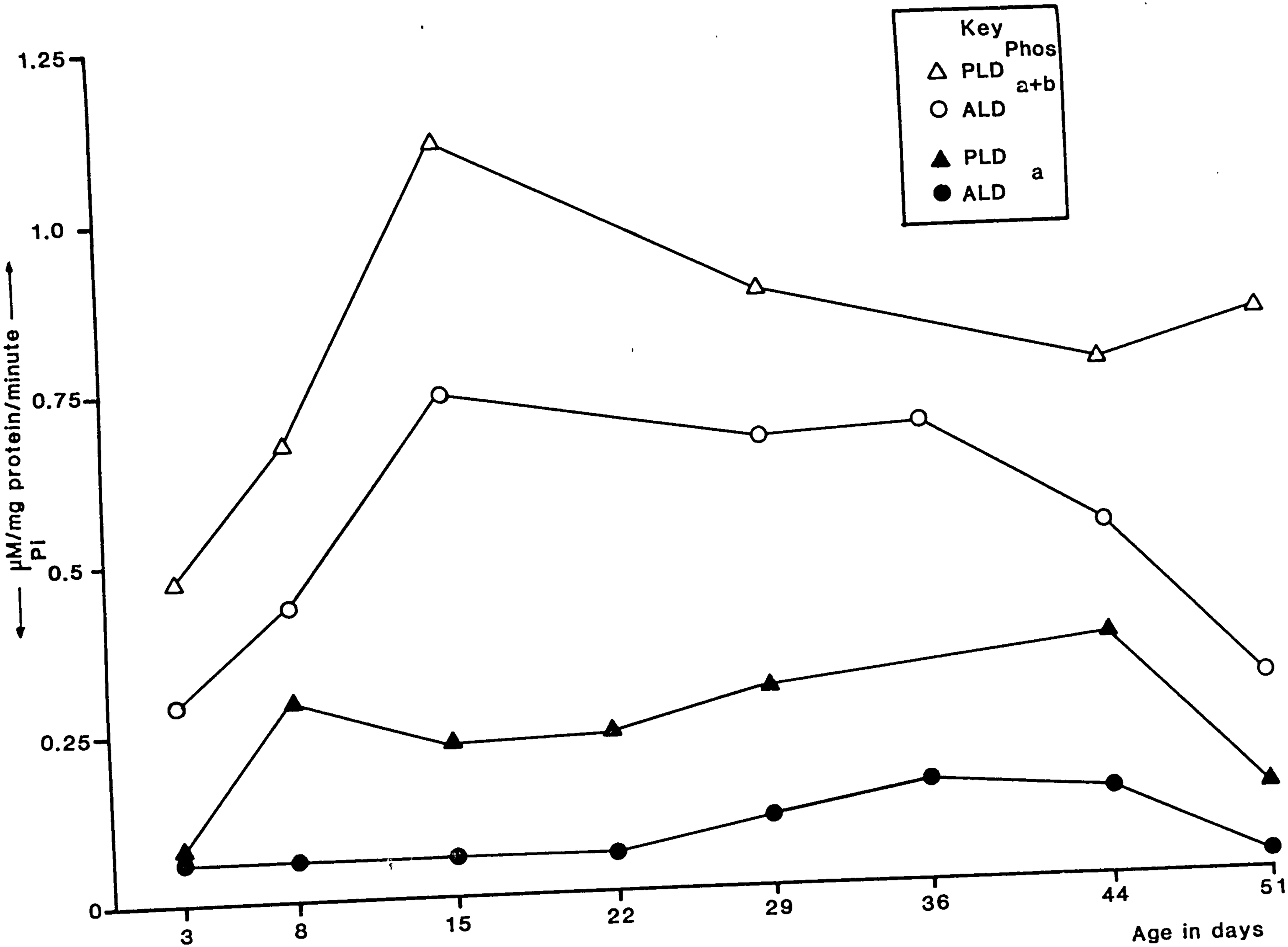
The first graphs on Fig. 4(xiii), Page 93, illustrates the changes in activity of phosphorylase a and total phosphorylase for the muscles

See overleaf for Figure and Figure Legend 4(xii).

Figure 4(xii)

Phosphorylase a and (a + b) activity for the control ALD and PLD muscles for the first 51 days ex ovo; incubated at 30°C, pH 6.6.

(Standard errors are only for the technique and are within the size of the point).


Key
Phos
PLD
a+b
ALD
PLD
a
ALD
1.25
1.0
0.75
0.5
0.25
0
μM/mg protein/minute
Pi
3
8
15
22
29
36
44
51
Age in days

See overleaf for Figure and Figure Legend 4(xiii).

Figure 4(xiii)

a) Phosphorylase a and (a + b) activity for the ALD experimental muscles in comparison with the controls for immobilisation in the resting position.

b) Phosphorylase a and (a + b) activity for the PLD experimental muscles in comparison with the controls for immobilisation in the resting position.

c) Phosphorylase a and (a + b) activity for the ALD experimental muscles in comparison with the controls for immobilisation in the shortened position.

d) Phosphorylase a and (a + b) activity for the PLD experimental muscles in comparison with the controls for immobilisation in the shortened position.

Incubated at 30°C, pH 6.6.

Standard errors for the technique are within the size of the point.

a)

b)

c)

d)

1.0

0.5

0

3 6 8 15 22 29

Key PLD Phos

Expems a+b

Controls a+b

Expems a

Controls a

Key ALD Phos

Expems a+b

Controls a+b

Expems a

Controls a

Recovery

µM/mg protein/minute

Pi

Age in days

See overleaf for Figure and Figure Legend 4(xiv).

Figure 4(xiv)

a) Phosphorylase a and (a + b) activity for the ALD contralateral muscles in comparison with the controls from immobilisation in the resting position.

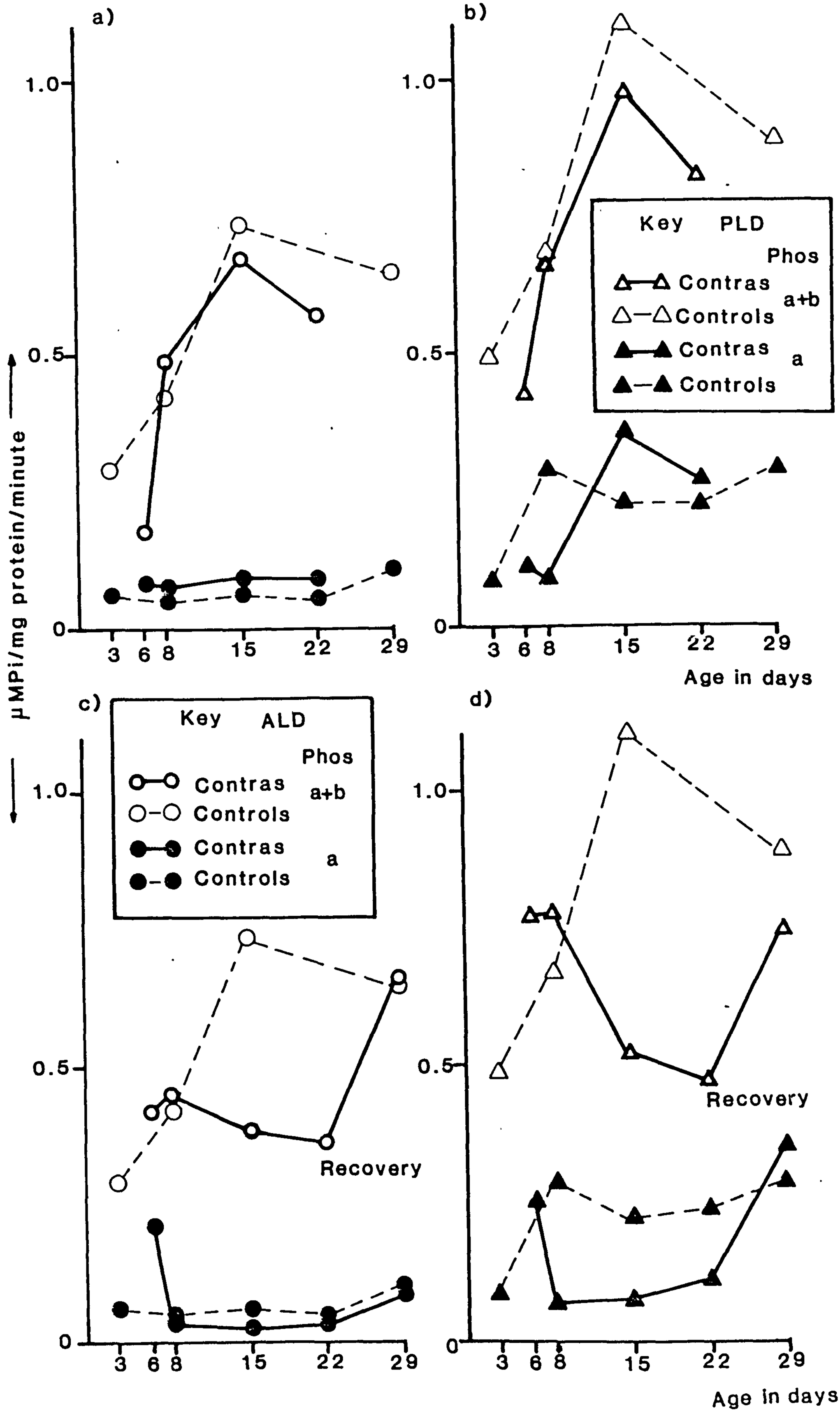
b) Phosphorylase a and (a + b) activity for the PLD contralateral muscles in comparison with the controls from immobilisation in the resting position.

c) Phosphorylase a and (a + b) activity for the ALD contralateral muscles in comparison with the controls from immobilisation in the shortened position.

d) Phosphorylase a and (a + b) activity for the PLD contralateral muscles in comparison with the controls from immobilisation in the shortened position.

Incubated at 30°C, pH 6.6.

Standard errors for the technique are within the size of the point.


a)
b)
c)
d)
1.0
0.5
0
μMP i/mg protein/minute
3 6 8 15 22 29
Age in days
Key PLD
Phos
Contras
Controls
a+b
Contras
Controls
a
Key ALD
Phos
Contras
Controls
a+b
Contras
Controls
a
Recovery

immobilised in the resting position. The overall trend of these plots is very similar to that of the control groups. The PLD showed a similar rise to the controls in total phosphorylase activity with the peak value of 0.979 μ MPi/mg protein/minute occurring after 6 days of immobilisation in the resting position. A peak was also shown for the ALD after this immobilised period although the activity in this case was only 0.53 μ MPi/mg protein/minute. This was similar to the activity exhibited at 8 days age for the controls but as a peak was lower than the peak of the controls which occured at 15 days age. Both muscles showed a slow decline in total phosphorylase activity after the peak had been reached. The ALD and PLD immobilised in the resting position for 4 and 6 days showed similar activities of phosphorylase a. After this similarity the activity increased for the PLD for the longer immobilised periods with the ALD's phosphorylase a activity remaining low.

The second graphs on Fig. 4(xiii), Page 93 , displays the total activity for phosphorylase and phosphorylase a alone for the ALD and PLD muslces immobilised in the shortened position. The overall pattern for both total activity of phosphorylase and phosphorylase a alone for the ALD and PLD with shortened immobilisation was similar. After 4 days immobilisation there was a drop in activity and then for the longer immobilised periods a steady increase showing peak values at the age of 22 days after 20 days immobilisation. Recovery for 7 days increased total phosphorylase activity for both the ALD and PLD muscles. The peak values were slightly lower than the peak values exhibited by the control groups but the difference is minimal. In all cases the activity of the phosphorylase whether total or a alone was higher for the PLD than the ALD.

Fig. 4(xiv),Page-94, shows the changes in total phosphorylase activity and phosphorylase a for the contralateral ALD and PLD immobilised in the two positions as compared with the control groups.

The first graphs show the enzyme changes with immobilisation in the resting position. The overall trends were very similar to those of the controls with a peak in total phosphorylase activity at 15 days followed by a slow decline.

The second graphs Fig. 4 (xiv), Page 94 , show the effect on phosphorylase activities for the contralaterals from immobilisation in the shortened position. The trends were not similar to the control groups but were similar to the experimentals from the right hand side of the birds, (See Fig. 4 (xiii), second graph, Page 93), particularly in the changes in phosphorylase a activity. The peaks in activity were lower than the controls although removal of the casts for 7 days showed a rapid increase in total phosphorylase activity for both muscles. The enzymatic changes exhibited by the contralaterals which obviously differ markedly from the control groups show further the importance of separate control groups for immobilisation studies of this nature particularly when a shortened position of inactivity is imposed.

Discussion

The activity of Mg^{2+}-activated myofibrillar ATPase and phosphorylase was measured in the ALD and PLD muscles for two main reasons. First, there are large differences both in terms of the rates of contraction and the energetics between the ALD and PLD and these differences have not been closely studied with respect to the biochemical activities of these two enzymes. The changes in activity with development should give more

insight into these differences from an enzymatic viewpoint. Secondly, the main object of this analysis has been to study development during the first 51 days with full and reduced activity of the muscles. During normal development, the changes occuring in the two muscles reflected by these enzymes has been shown to be affected by immobilisation and differentially in the two positions.

Bendall (1969), studied the myofibrillar ATPase of many animals, and stresses the need to use myofibrils in physiological conditions similar to the in vivo situation, in order to demonstrate more accurately the biochemistry of contraction. The pH, temperature and ionic constituents of the myofibrillar ATPase assay are all important in showing maximum activity of the enzyme. In muscle, contraction is not an isolated process and it is more accurate to consider it as a contraction-relaxation cycle. In the relaxed state the muscle contains ATP in the $MgATP^{-2}$ form with Ca^{2+} ions chelated away from the contractile proteins by the surrounding T-system. On contraction, calcium is needed and is released by the sarcoplasmic reticulum to a concentration of 10^{-5}M. The calcium triggers the cycling of the cross-bridges of myosin with the actin filaments. The presence of Ca^{2+} permits the structural repositioning of troponin from the actin-tropomyosin complex to allow attachment of the cross-bridge head, which requires the energy from ATP in the $MgATP^{-2}$ form. Using myofibrils in a buffer containing Ca^{2+} ions, the addition of ATP with $MgCl_2$ will stimulate the contraction part of the contraction-relaxation cycle and the ATPase activity of the muscle. (See Table 5, Page 98, for a summary of myofibrillar ATPase activities for various animals and muscles).

Muscles of large mammals split ATP at their body temperature ($37^{o}C$) at only half the rate shown by the "white" leg muscles of the rat and rabbit. The myofibrillar ATPase activity of chicken breast muscle is found

TABLE 5

Activation of Mg^{2+}-activated myofibrillar ATPase for various species.

(Adapted from Bendall (1969) p.57)

Animal	Temp °C	mols ATP split/mol. myosin/second
Rat or rabbit (leg muscle)	0.6	0.14
	18.5	3.80
	35.0	35.30
Bullock, pig sheep	0.6	0.14
	18.5	2.20
	35.0	16.70
Chicken (breast muscle)	0.6	0.13
	18.5	2.50
	35.0	29.60
Pigeon (breast muscle)	0.6	0.27
	18.5	2.30
	35.0	14.20
Chicken		
ALD	22.0	0.6
PLD		2.0

Results from this study 51 days of age

Actomyosin ATPase (Reasons and Hikida, (1973))

Chicken		μ moles Pi/mg actomyosin/min		mols ATP split /mol myosin/sec
ALD	22.0	0.265	equivalent to	1.961
PLD		0.371	assuming conversion factor 7.4	2.745

Phosphorylase Activity (Adapted from Newsholme and Start (1974) p.151)

Animal	Muscle	μ moles Pi/min/g fresh wt. at 25°C	* μ moles Pi/min/mg protein
Trout	Red	14.0	0.07
Pigeon	Pectoral (red)	18.0	0.09
Pheasant	Pectoral (white)	120.0	0.60
Rabbit	Semitendinosus (red)	8.0	0.04
	Adductor longus (white)	30.0	0.15
Rat	Heart	12.0	0.06
This Study			
Chicken 51 days old	ALD (slow tonic) at 30°C		0.29
	PLD (fast phasic) at 30°C		0.83

* conversion factor ÷ 1000 x 5 (Approximately 20% of muscle wet weight is protein).

to resemble the rat or rabbit, but pigeon breast muscle activity is nearer that of the large mammals. These results appear to indicate that "white" muscles such as some of the leg muscles of the rat or the breast of the chicken are always fast and that the redder muscles of large mammals and pigeon breast are slow. This association of redness with slowness is too naive and there are many exceptions to this generalisation. It is safer to regard the differing ATPase activities of the various species as adaptations to function. Animals would need to possess the genetic code necessary for producing a variety of iso-myosins, each adapted to a specific muscle function.

In the present study of Mg^{2+}-activated myofibrillar ATPase, an assay temperature of $22^{o}C$ was used to compare the results with those for the actomyosin ATPase found by Reasons and Hikida, (1973). The activity of the myofibrillar ATPase in the present study for the ALD and PLD was much reduced at this temperature but compared well with the results for chicken breast muscle shown by Bendall, (1969) at $18.5^{o}C$. (See Table 5 , Page 98).

Reasons and Hikida, (1973) have found that the PLD shows an actomyosin ATPase activity of only 1.4 times that of the ALD. In the present study the activity of the Mg^{2+}-activated myofibrillar ATPase at 51 days was 4 times greater in the PLD than the ALD. This difference reflects more accurately the 6 to 8 times faster speed of contraction shown by the PLD than the ALD (Page, 1969). The use of myofibrils instead of purified actomyosin may portray more accurately the <u>in vivo</u> activities of the different ATPases in these two muscles. However, the differences were still not as large as would have been expected from the different contraction speeds. The activity however, does appear to be positively proportionally related to contractile speed as are the results for myosin ATPase shown in Bárány's

findings, (1967).

The changes in the Mg^{2+}-activated myofibrillar ATPase activity throughout the first 22 days of ex ovo development in the ALD and PLD are very interesting. Both muscles were initially similar in activity and the contraction times for the muscles have also been found to be similar, although both slow. (Shear and Goldspink, 1971). During the next 20 days of development the muscles showed a peak in activity of the enzyme. This peak was also shown with the PLD muscle in the development with immobilisation, although the imposed activity appeared to retard the peak by a week to 10 days. It is possible that a new iso-enzyme of myosin or a different balance of those present is being produced during this developmental period. Foetal chicken ALD and PLD myosin has been shown to have a different balance of light chain iso-enzymes to the adult mysoin (Pette, Vrbová and Whalen, 1979). Perhaps there are further changes in the balance of such regulatory proteins during this developmental period.

The wings which are not initially used much, develop throughout the first week and "flapping" occurs much more frequently by 15 days of age. The changes from the premature ex ovo state to the young adult may require a stage of increased activity related to normal muscle growth. The activity peak was more marked in the fast contracting PLD muscle which is associated directly with a "flipping-back" of the wing. Furthermore this activity peak was displaced when inactivity was imposed, so that normal contractile shortening would seem to be important in triggering the onset of this peak.

Total phosphorylase activity, considered to reflect anaerobic capacity, has also been shown to vary between the ALD and PLD muscle across development and in absolute values. The PLD showed 3 times the activity of the

ALD at 51 days of age. (See Table 5 for a literature review of values). This was to be expected because the PLD is a fast anaerobic muscle and this high capacity for glycolytic energy production has also been shown with the histochemical analyses in this study (Chapter 3).

The total phosphorylase activity also showed a peak at 15 days of age, but this was not such a dramatic peak as that shown by the Mg^{2+}-activated myofibrillar ATPase. Immobilisation in the resting position did not affect the total phosphrylase activity to any great extent. Whereas, immobilisation in the shortened position led to an initial decline and subsequent increase until normal levels were reached at the end of the studied period.

The nutritional state of the bird may affect the amount of total phosphorylase present in these muscles. In muscle variable effects of fasting on the total phosphorylase activity have been reported; a decrease has been shown in frog muscles, (Cori, 1945); no change in rat, rectus femoris muscle, (Leonard, 1957); and an increase in rat leg muscle, (Lundback and Goranson, 1949). Skeletal muscle phosphorylase levels are not significantly affected by cold adaptation in rabbits. (Krebs and Fischer, 1955 (b)). There is no adaptive response of rat muscle phosphorylase levels to exercise. (Gould and Rawlinson, 1959). Neurotomy has been shown to cause a decrease in total muscle phosphorylase levels following a transient increase. (Humoller, Hatch and McIntyre, 1951). This is in contrast with the present effects of inactivity imposed by a shortened position of immobilisation. Cosmos, 1966, has studied the phosphorylase activity in chicken breast muscle with hereditary muscular dystrophy. She found that the total phosphorylase activity at 60 days age is 1/3 of the control value, 40 μ g Pi/Mg protein/15 minutes as opposed to

120, respectively.

A change in the relative proportions of phoshorylase a and b with muscle contraction is an attractive hypothesis to explain the correlation that exists between glycogenolysis and work performed by the tissue. Early studies indicated that resting muscle contained mostly phosphorylase a and that electrical stimulation led to the formation of b (Cori, 1945). It was found, later, that a phosphorylase b to a conversion can readily occur during the extraction process and that when this conversion is blocked the resting muscle yields phosphorylase predominantly in the b form. (Krebs and Fischer, 1955 (a)). More recent studies have shown that the phosphorylase a content of muscle increases with contraction, but prolonged stimulation to the point of fatigue can result in the formation of phosphorylase b (Cori and Gaebler, 1956).

The amounts of phosphorylase a and b present in muscle at any time are determined by the relative rates of the phosphorylase b kinase and phosphorylase phosphatase reactions. A better understanding of the phosphorylase system in reference to muscle function will develop as these interconverting enzymes themselves are more fully understood. Analysis of the total phosphorylase activity is therefore presently one of the best markers for the anaerobic capacity of skeletal muscle.

The phosphorylase a activity measured was similar for both the ALD and PLD. Its activity was expected to be about 65-70% of maximal which is shown with AMP present. (Cori and Green, 1943). The ALD showed a total phosphorylase activity (a + b) of about 2 times the activity of a alone whereas the PLD's total activity was nearer 4 times that of its phosphorylase a. This balance with a much higher amount of phosphorylase b present in the PLD could be important in the control of glycolysis. It is

difficult however, without a full understanding of the interconverting enzymes, to infer much from this difference.

The PLD has been shown to be more active anaerobically than the ALD and imposed inactivity in a shortened position causes an initial decrease in total phosphorylase activity followed by recovery, despite continued inactivity.

Gutmann and Syrový, (1967), have studied the basic energy characteristics, (levels of glycogen and aldolase activity) and protein metabolism (rate of proteosynthesis, proteolytic activity and content of ribonucleic acids) during the early stages of ontogenetic development in the ALD and PLD of chickens. Glycogen content and aldolase activity was found to be higher in the PLD than the ALD which was immediately observable on hatching. Ribonucleic acid content and proteolytic activity was higher in the ALD than the PLD. This meant that a higher turnover of proteins was assumed for the ALD and related to the function of slow muscles in the long term maintenance of tension. The high glycogen content and aldolase activity exhibited by the PLD over the ALD are in agreement with the higher total phosphorylase activity shown by the PLD in this study.

Bacou and Nouquès, (1980), have studied the changes in many metabolic enzymes during development of two chicken muscles in culture. They used myoblasts, from the adductor magnus considered a slow muscle, and from the pectoralis major considered a fast muscle. They found that both "red" (slow) and "white" (fast) myogenic cells started out similarly but differed after prolonged incubation (21 days). The cells from the pectoralis major showed an increase in activities of enzymes associated with the glycolytic chain at the 14 th day of incubation. The activities of the same enzymes of the adductor magnus cells remained similar to earlier incubation periods

and low in comparison with the fast pectoralis major. These results fit in with those obtained for total phosphorylase in this study. Both the ALD and PLD start low although at different levels of activity and increase to reach a steady but different total phosphorylase activity level. It is sometimes difficult to compare cultured muscle with the in vivo situation but overall patterns of change have some significant similarities within the same species and muscles.

The ALD and PLD muscles, therefore, show large differences in overall activity for both enzymes studied. The activity of the enzymes varied throughout development and was altered to differential degrees with imposed inactivity. The absolute values are in agreement with those in the literature and the developmental changes observed (e.g. activity peaks) could perhaps be explained by functional changes during development.

The changes in the total activity of phosphorylase with inactivity was less surprising than those of the Mg^{2+}-activated myofibrillar ATPase. Phosphorylase is a metabolic enzyme whose control is not fully understood but it is known to rely on many cascades of enzyme reactions and needs to be switched on and off quickly to provide energy. Fluctuations in the activity of the a or a + b form is perhaps more easily explained through this complex but adaptive system.

The Mg^{2+}-activated myofibrillar ATPase would be expected to be a more rigid enzyme and not to fluctuate in activity throughout development or imposed inactivity. The reason for this is not known, however it could be related to a period of functionally increased contraction necessary in the development of both muscles. Also it may possibly reflect changes in the types of myosin or type of regulatory protein. Further work is required to establish the reasons for these activity

changes involving protein isolation and iso-enzymatic studies of the regulatory proteins at various time points during development.

The original expectations that the PLD is a fast-contracting anaerobic muscle and the ALD a slow-contracting aerobic muscle have been reflected and supported by the differences in total phosphorylase and Mg^{2+}-activated myofibrillar ATPase activities shown.

CHAPTER 5

THE EFFECT OF THYROIDECTOMY IN EARLY ADULTHOOD ON THE DEVELOPMENT OF THE ALD, PLD AND SCAPULOTRICEPS MUSCLES OF THE CHICKEN

Introduction

The function of the thyroid gland was first clarified in the nineteenth century when external clinical observations were associated with malfunctions of the gland. Robert Graves in 1835, first categorised the symptoms of hyperthyroidism as a general acceleration of all bodily and mental processes in response to an increased rate of cellular oxidations due to elevated levels of thyroid hormone. In contrast, thyroidectomy was found to cause a decrease in oxygen consumption. Reseverdini and Cochi (1883) were first to point out the similarity between thyroidectomy and the symptoms of myxedema (Greene, 1970). The first effective treatment of hypothyroidism was reported by Murray (1891) who demonstrated

the restoration of normal bodily functions in response to the administration of sheep thyroid extract. In 1915, Kendall isolated "thyroxin" (thyroxine, T_4) from the thyroid gland and in 1952 the second thyroid hormone, triiodothyronine (T_3) was isolated by Gross and Pitt-Rivers. The thyroid gland primarily releases T_4 into the circulation in response to a number of stimuli. Other factors regulate the conversion of T_4 to T_3. The relative potencies of T_3 to T_4 in birds is difficult to determine because T_4 is converted peripherally to T_3 (Astier and Newcomer, 1978). Initial studies in domestic birds indicated that T_3 constituted approximately 40% and T_4 approximately 60% of the levels of circulating hormones. (Wentworth and Mellen, 1961). T_3 is accepted as the most active of the two hormones, acting on most tissues by increasing metabolic activity and protein synthesis. In birds and mammals thyroid hormones play a role in heat production and in the metabolism of carbohydrates, proteins and lipids. Thyroid hormone is therefore essential for normal growth and development and is known to be required during an early period of development of the central nervous system for cell maturation and interaction of neurons (Legrand, 1979).

The exact mechanism of action of thyroid hormones is controversial but it is considered to be probably similar to that of the steroid hormones. Thyroid hormone T_3 has been shown to cause stimulating effects on many enzymes including the plasma membrane enzyme, Na^+-K^+-ATPase ('sodium pump') (Edelman, 1974). Free T_3 (not bound to a protein carrier) has also been shown to either diffuse through the plasma membrane or to bind to a specific receptor in the plasma membrane and be transported into the cell where it is bound by a cytosol binding protein (CBP) (Sterling, 1979). Instead of being translocated into the nucleus the CBP-T_3 complex is in reversible equilibrium with a minute moiety of intracellular unbound T_3

that can react with high affinity, low capacity receptors in the mitochondria and nuclear chromatin. Adamson, (1970) also showed a direct effect of thyroid hormones in chick cell membranes. Addition of thyroid hormone to embryonic chick bone increased the intracellular amino acid incorporation. Dratman (1974) has proposed that T_3 and T_4 function as amino acid analogues of tyrosine, thereby modifying the protein and catecholamine pathways of this amino acid. Furthermore evidence has shown that iodothyronine may enter into the catecholamine biosynthetic pathway and act as precursors for alternate adrenergic neurotransmitters. Dratman, Crutchfield, Axelrod, Colbum and Thoa, (1976) have found T_3 concentrated in peripheral adenergic nerves and has found thyroid hormone localised and metabolised within synaptosomes. This is all supporting evidence that thyroid hormones may serve as neurotransmitters.

One of the better known effects of thyroid hormones is their effect on mitochondria. They have been suggested to cause uncoupling of oxidative phosphorylation (major process for ATP production) by stimulating extra-mitochondrial pathways consuming nutrients but producing few high energy bonds (Martins and Hess, 1951; Hoch and Lipman, 1954). An effect on mitochondrial energy metabolism and plasma membrane followed by an increase in the rate of transcription of genetic sequences with a concomitant increase in the rate of ATP-consuming processes is now considered to be the general view of the mechanism of thyroid hormones. These cumulative effects are fundamental and essential for normal growth, differentiation and cell maintenance.

The above summary describes the suggested mechanism for thyroid hormone action. This present study describes the changes that occurred in the ALD, PLD and Scapulotriceps muscles in birds at 70 days of age after 4 weeks of thyroidectomy (leads to reduced thyroid hormones). The analysis involved the enzymatic assays of one contractile enzyme, Mg^{2+}-activated myofibrillar

ATPase and one metabolic enzyme, phosphorylase. This was supported by a histochemical analysis with staining for these two enzymes. The body and muscle weight and muscle lengths and also the amount of T_3 in the plasma, were measured to compare more carefully the overall changes between thyroidectomized and control birds and the differences between tonic and phasic muscle response to reduced thyroid hormone.

There have been several studies on the effect of thyroidectomy on the muscles in birds and mammals both during development and as adult animals. King and King, (1973; 1976) report a differential response between muscles to hypothyroidism in chickens. The weight of the gastrocnemius total crude fibrillar protein and DNA levels were reduced to a much larger extent than the same parameters for the sartorius muscle. Hypothyroidism for 8 weeks from2 days of age (ex ovo) had approximately 20% less effect on the sartorius muscle. Enzymatic changes have also been studied in chicken muscles with hypothyroidism induced in the embryo. Lippe, Gassman, Soltoff and King, (1977), have described the changes induced by hypothyroidism in the Lactate dehydrogenase (LDH) isozyme pattern. The pectoralis muscle from chick embryos, prior to hatching, contains five isozymes of LDH. It was observed that the LDH 4, and 5 isozymes were missing from 20-day embryos made hypothyroid with methimazole for 5 to 10 days. Bacou, Jallageas, Nouquès and Vigneron (1980) have also studied the effect of methimazole induced hypothyroidism on male and female chick embryo myogenesis. They studied the changes in fibre number and selected enzymes both histochemically and biochemically. Only the methimazole-treated males showed a significant increase in the total number of fibres in two muscles, tibialis posterior and flexor digitorum. Their histochemical results for succinate dehydrogenase and myosin ATPase showed that of the three fibre types (αR, αW and βR; same classification system as Ashmore and Doerr

(1971) see Chapter 3, Table 2) only the α fibres increased in number. The female chickens muscles did not exhibit this fibre increase. The adolase (glycolytic enzyme) and NADP isocitrate dehydrogenase (tricarboxylic acid cycle enzyme) activities measured on the adductor ("slow muscle") and pectoralis ("fast muscle") however showed a similar pattern in control and hypothyroid animals. These studies indicate that the thyroid hormones have many affects on chicken muscles both in the alteration of the activities of enzymes metabolically and from a contractile protein viewpoint.

Changes in enzyme activities and development with thyrpid hormone inbalance have also been reported for mammalian muscle. (Nicol and Johnston, 1981; Nwoye et al., 1982). Nwoye et al. has shown using rat soleus that hypothyroidism caused fast to slow changes in fibre type composition, ATPase activities (down 20-30%), myosin light chain pattern (54% less fast light chains) and LDH activity (down 11%). They found changes of similar magnitude but to the opposite direction induced by hyperthyroidism. Johnson et al. (1980) have also shown fast to slow conversions in rat soleus muscles induced by hypothyroidism. The muscles were also shown to have lower tetanic tension and longer, contraction time and time to half relaxation than control soleus muscles. (Johnson, Mastaglia, Montgomery, Pope and Weeds, 1980). Nicol and Johnston, 1981, studied the effects of hyperthyroidism and hypothyroidism on the rat soleus and extensor digitorum longus (EDL) in terms of their balance of metabolic enzymes. They found very little change in enzyme activities with the hypothyroid state but the excess of thyroid hormone T_3, induced marked alterations in the fibre type populations in both muscles. In the soleus there is a conversion of SO to FOG fibres, whereas in the EDL, FG fibres are converted to FOG fibres. The soleus alone however showed an increase in anaerobic glycolytic and aerobic metabolism enzymatic activities. Baldwin, Hooker, Campbell and Lewis (1978) have shown a great reduction in protein concen-

tration (-20%), citrate synthase (-50%), cytochrome C concentration (-50%), phosphofructokinase (-30%) and myofibrillar ATPase activity (-30%) in the rat quadriceps at 42 days muscle if made hypothyroid at birth.

From these studies on both birds and mammals it can be concluded that the presence of the thyroid hormone is essential in the normal development and continued growth of both fast and slow muscles. The affect of thyroidectomy or drug induced hypothyroidism appears to have differential responses in fast and slow muscle fibre types. It does seem however to cause a "slowing down" in terms of contractile enzymes and regulatory marker proteins and a change in the metabolic state resulting in reduced enzyme activity for both glycolytic and aerobic pathways. Hyperthyroidism or thyrotoxicosis however causes opposite effects with increased metabolic enzymes particularly those from the aerobic side of energy production. These changes link directly with the stimulating affect that the thyroid hormones have on the uncoupling of oxidative phosphorylation.

Materials and Methods

Birds

Males, breed, Thornber 404 (Light Sussex cross Rhode Island Red) were used, sacrificed with an overdose of pentobarbitone sodium (I.P.), at 10 weeks of age (70 days). The ALD, PLD and Scapulotriceps (ST) muscles were excised and used for histochemical and biochemical analyses for enzyme activities. The Scapulotriceps muscle was used for extra analysis due to its close proximity to the ALD and PLD and to act as a marker for another phasic muscle. (It has a mixed, twitch, histochemical profile). M.scapulotriceps arises from the inferolateral surface of the scapula and by a tough aponeurosis from the inferior margin of the posterior lip of the glenoid cavity (George and Berger, 1966). The Scapulotriceps is an upper

wing flexor and antagonistic to the biceps.

The experimental birds were surgically thyroidectomized under synacthin anaesthesia (I.M.) at 6 weeks of age (42 days). Control birds underwent sham thyroidectomy operations at the same age. Six birds were used for this study, individually, from each group.

Blood samples (1.5ml) were taken from the experimental and control birds before sacrifice. The blood plasma was stored, frozen, prior to the assay for thyroxine (T_4) and triiodothyronine (T_3). After use of the thyroidectomized birds the success of the surgery was confirmed by gross examination.

The birds were all weighed and the individual muscles weights and lengths recorded before the biochemical or histochemical analysis was carried out. The length of the muscles was measured when the wing was held in the "resting" position (See Chapter 2).

Measurement of Plasma T_3

Plasma triiodothyronine, T_3, was measured using a radioimmunoassay, modified from a method by Seth, Toft and Irvine (1978) by Klandorf (1982).

Histochemical Methods

The distal end of the ALD, PLD and ST muscles from the right-hand side of all the birds, were blocked, frozen and sectioned at 10 µm thickness,

FOOTNOTE:

The thyroidectomies and T_4 and T_3 assays were performed by Hillar Klandorf of the Wolfson Institute. The T_3 results only are presented. The T_4 data is of lesser importance physiologically and a delay in their measurement have prevented them from being included.

mounted on slides and stored at -20°C until stained.

The sections were stained for; Myosin ATPase with preincubations at pH 4.3 (for the ALD muscle), and pH 4.6 (for the PLD and ST muscle); and Phosphorylase (See Chapter 3 for details of these histochemical stains and procedures).

Biochemical Assays

The ALD, PLD and ST muscles were used with the right and left hand muscles pooled. These muscles from the six thyroidectomized and six sham-operated birds were individually assayed for Mg^{2+}-activated myofibrillar ATPase and Phosphorylase activity (both a + b and a alone). See Chapter 4 for the protocols for these assays.

Results

Statistical analyses were carried out using Student's-t-test, (Bailey, 1974), on the means of the individual control and experimental groups.

General Growth Characteristics

The individual birds' body weights and muscle weights are given in Fig. 5(i), Page 114, for both control (sham-operated) and experimental (thyroidectomized) birds. (A mean value is given for the right and left hand muscles).

The experimental birds were significantly lighter for overall body weight than the controls (DF 10, $t=2.947$, $p < 0.05$).

See overleaf for Figure and Figure Legend 5(i).

Figure 5(i)

Graphical illustration of the differences in individual whole body weight, muscle weights (ALD, PLD and ST) and amount of T_3 hormone between the thyroidectomized (experimental) and sham-operated (control) birds.

Body weight x10³ in g

1.0

0.5

0

Key

Expems

Controls

ALD

0.6

0.4

0.2

0

PLD

0.6

0.4

0.2

0

Wet weight of muscle in grammes

ST

2.0

1.0

0

T_3 ng/ml

2.0

1.0

0

Thyroid Tissue Remnant

Bird No. 1 2 3 4 5 6 7 8 9 10 11 12

The ALD muscles from the experimental birds showed the greatest weight reduction of all three muscles studied when compared with the control birds (DF 22, t=4.0344, $p < 0.001$). The PLD muscles from the experimental birds did not show a significant weight loss. The experimental ST muscles however were significantly reduced in weight (DF 22, t=2.445, $p < 0.05$) but the reduction was not as great as that for the experimental ALD muscles.

There was no significant difference between the experimental and control muscle lengths.

Fig. 5(i), Page 114, also displays the T_3 in ng/ml measured for each bird. As would be anticipated with a successful thyroidectomy, the experimentals showed a significant reduction in T_3 hormone. (DF 10, t=7.363, $p < 0.001$).

The effectiveness of the thyroidectomy was assessed after the bird was used for the above analyses. Bird, Nos. 4, 5 and 6 all showed minor thyroid tissue remnants but still showed low T_3 levels in comparison with Bird Nos. 1, 2 and 3. Some tissue regrowth is expected after 4 weeks after surgery. (Klandorf, unpublished observations). Despite the absence of thyroid tissue a small amount of thyroxine has been reported in the plasma of some thyroidectomized birds (Davison, Misson and Freeman, 1980).

Histochemical Characteristics

Staining the muscles for Myosin ATPase and Phosphorylase did not reveal any large activity between the experimental and control birds. Fig. 5(ii), Page 116, depicts plates for the stained sections from a typical thyroidectomized bird (No. 3) and sham-operated bird (No. 12). The fibre size for the thyroidectomized birds appears to be reduced, particularly for the ALD which showed a significant whole muscle weight

See overleaf for Figure and Figure Legend 5(ii).

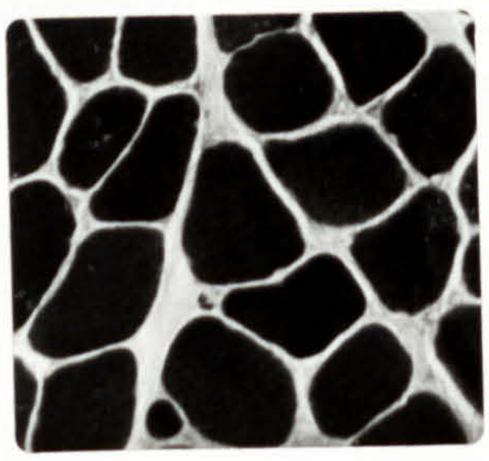
Figure 5(ii)

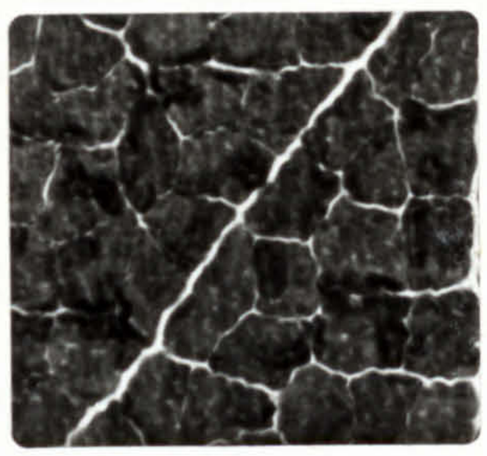
Plates to illustrate the staining for Myosin ATPase (preincubation pH 4.3 for the ALD and pH 4.6 for the PLD and ST) and Phosphorylase for these muscles from a typical thyroidectomized bird (No. 3) and a sham-operated bird (No. 12).

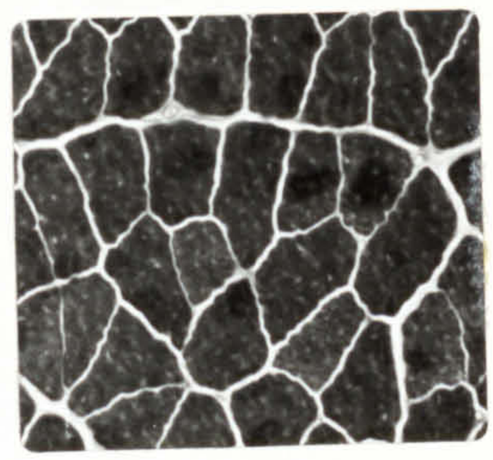
ALD PLD ST

Bird No. 3 Thyroidectomized

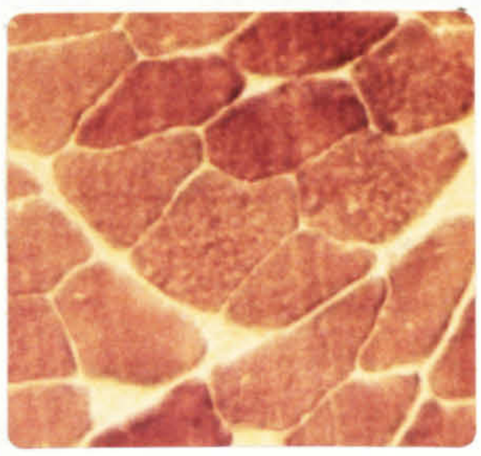
Myosin ATPase





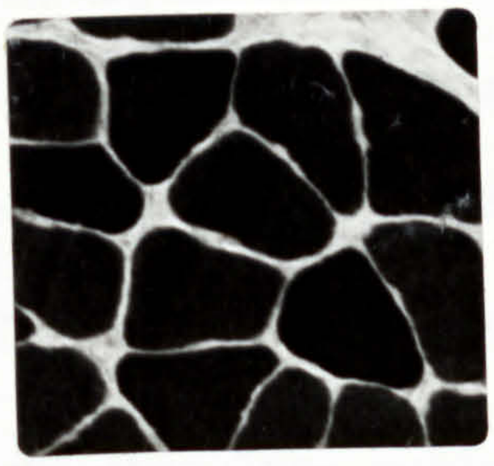


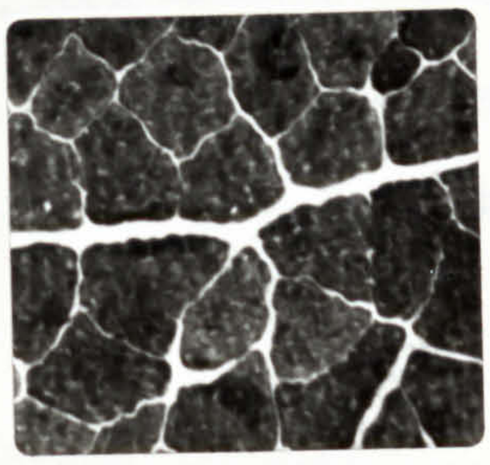
Phos – phorylase

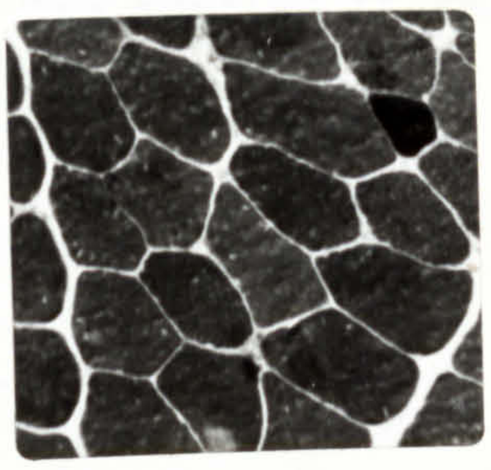


Bird No. 12 Sham-operated Control

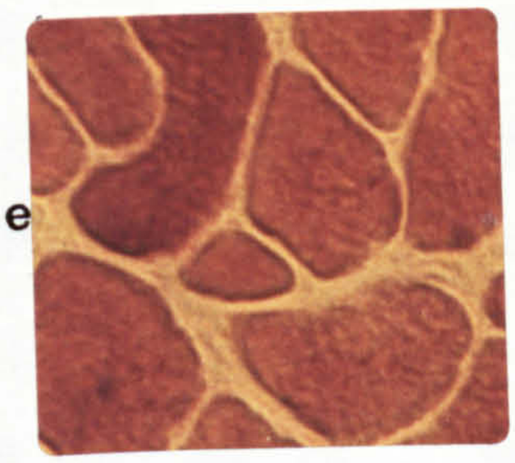
Myosin ATPase

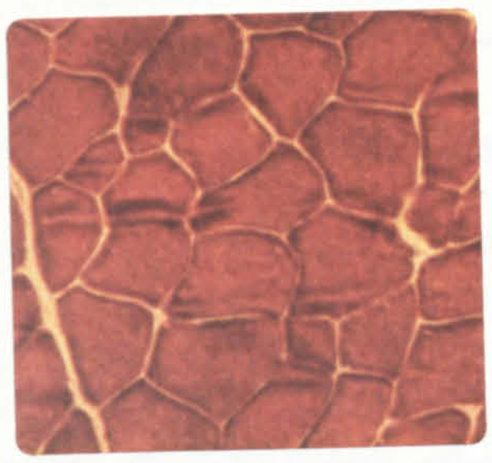


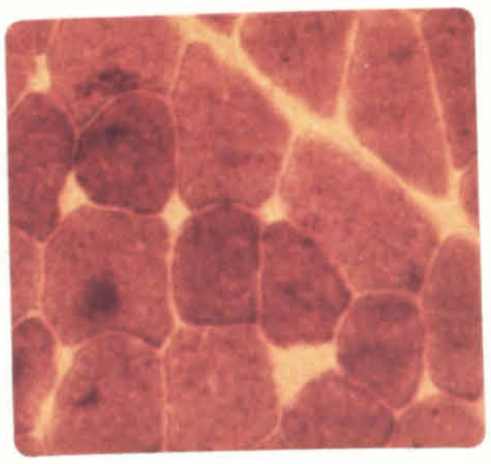




Phos – phorylase







Magnification 200 µm

reduction. The staining intensities for both enzymes were similar in all the birds.

Enzyme Assay Activities

The biochemical assays for phosphorylase (total activity a + b and a alone) and Mg^{2+}-activated myofibrillar ATPase did show up differences between the control and experimental birds. Figs. 5(iii) and 5(iv), Pages, 118 & 120 , depicts the activities for the above mentioned enzymes in the muscles for each individual bird.

The Mg^{2+}-activated myofibrillar ATPase activity of the ALD muscle, was not significantly different between the controls and the experimental birds. However it was shown to be significantly lower in the experimentals for the PLD muscles. (DF 10, t=6.3594, $p < 0.001$). It was also significantly lower in the experimentals for the ST muscles (DF 10, t=7.3498, $p < 0.001$). The muscles therefore showed a differential effect with the fast phasic PLD and ST both showed a significant reduction in activity whereas the slow tonic ALD was unaffected (See Fig. 5(iii), Page 118).

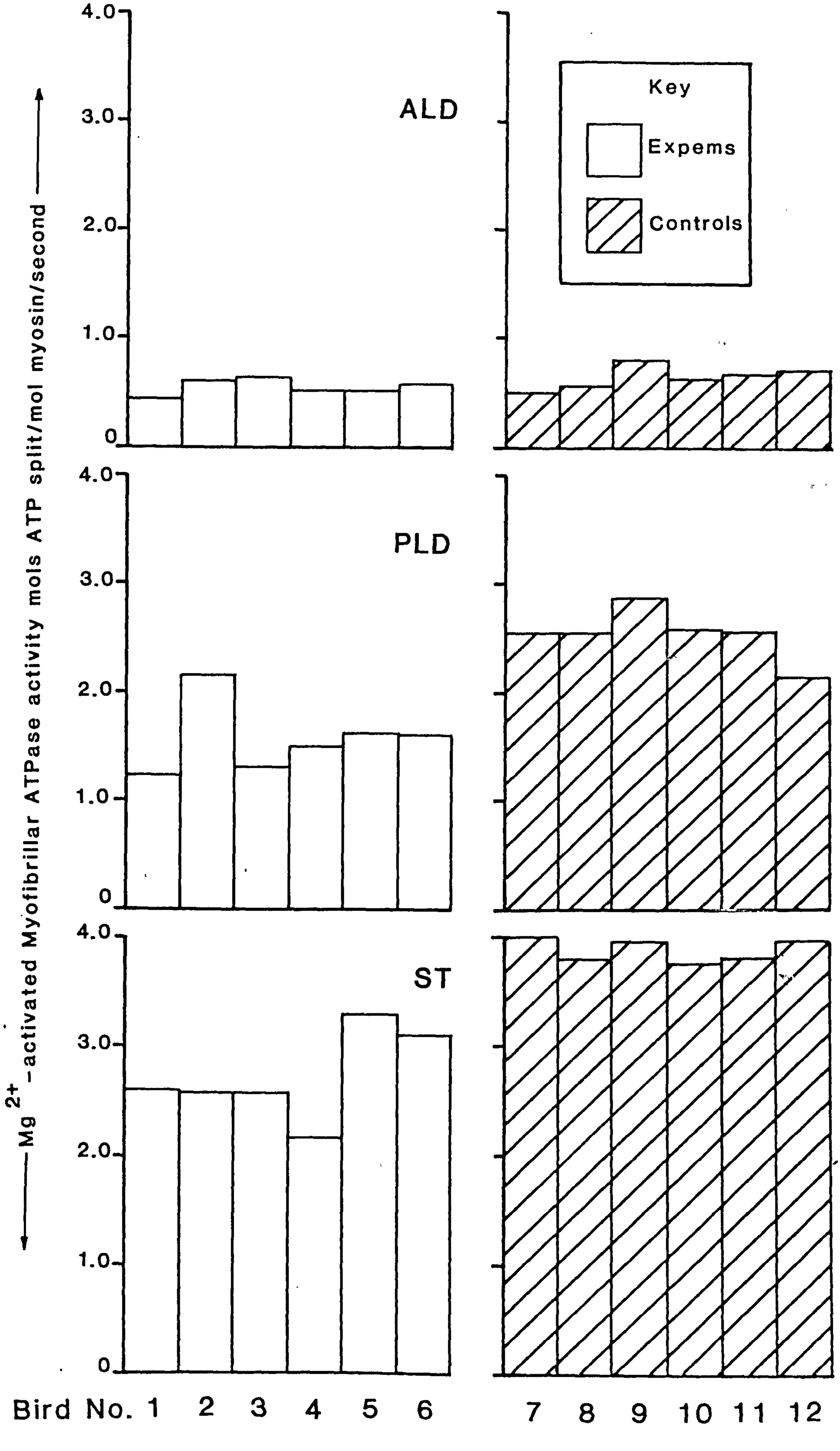
The phosphorylase activity (total a + b) was not as markedly reduced for the PLD and ST experimental muscles as the Mg^{2+}-activated myofibrillar ATPase activity. However, the PLD and ST did show significant reductions in the total activity (DF 10, t=2.8038, $p < 0.05$; DF 10, t=3.667, $p < 0.01$ for PLD and ST respectively). The ALD experimental muscles total phosphorylase activity showed no significant difference when compared with the control ALD muscles.

Phosphorylase a activity alone was not significantly different between the experimentals and controls for the ST and PLD or ALD muscles.

See overleaf for Figure and Figure Legend 5(iii).

Figure 5(iii)

Grpahical illustration of the differences in activity of Mg^{2+}-activated myofibrillar ATPase between the thyroid-ectomized and sham-operated birds. The values for individual ALD, PLD and ST muscles are displayed.


Key
Expems
Controls
ALD
PLD
ST
4.0
3.0
2.0
1.0
0
Mg 2+ -activated Myofibrillar ATPase activity mols ATP split/mol myosin/second
Bird No. 1 2 3 4 5 6 7 8 9 10 11 12

The following table summarises the means and significance levels for these studies.

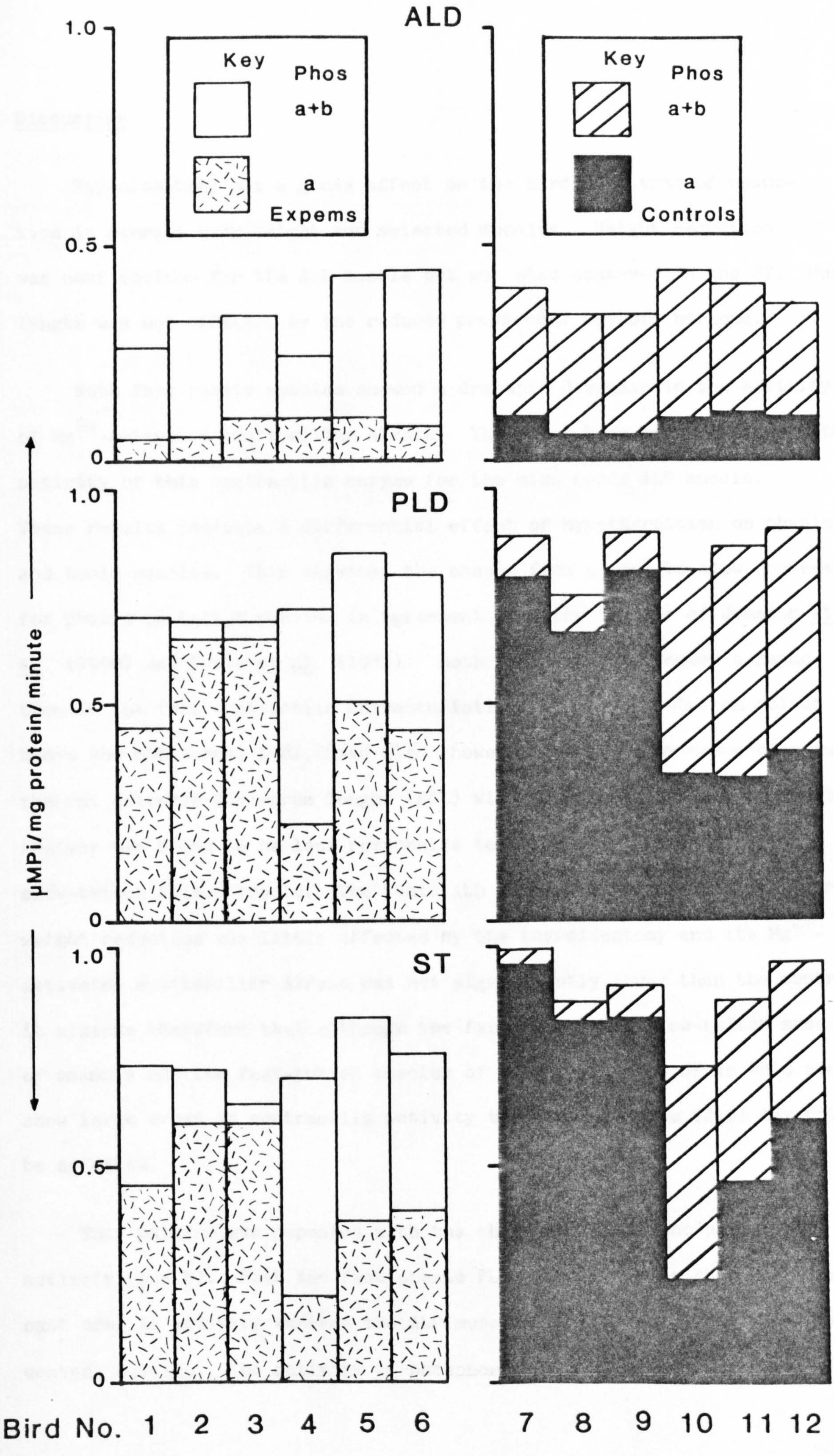
	Experimentals n=6 Thyroidectomised Birds	Controls n=6 Sham-Operated Birds	
Body Weights grammes	996.17	1148.33	*
T_3 ng/ml weights grammes	0.755	2.017	***
ALD	0.388	0.491	***
PLD	0.455	0.472	ns
ST	1.619	1.883	*
Lengths cm			
ALD	3.80	3.97	ns
PLD	3.92	4.11	ns
ST	7.57	7.83	ns
Mg^{2+}-activated myofibrillar ATPase mols ATP split/mol myosin/sec			
ALD	0.55	0.65	ns
PLD	1.58	2.54	***
ST	2.73	3.90	***
Total phosphorylase (a + b) μ MPi/mg protein/minute			
ALD	0.348	0.381	ns
PLD	0.749	0.850	*
ST	0.738	0.889	**
Phosphorylase a μ MPi/mg protein/minute			
ALD	0.079	0.094	ns
PLD	0.492	0.553	ns
ST	0.440	0.653	ns

$p < 0.05$	*
$p < 0.01$	**
$p < 0.001$	***
ns	not significant

See overleaf for Figure and Figure Legend 5(iv).

Figure 5(iv)

Graphical illustration of the differences in activity of total phosphorylase (a + b) and a alone between the thyroidectomized and sham-operated birds. The values for individual ALD, PLD and ST muscles are displayed.


ALD
1.0
0.5
0
Key
Phos
a+b
a
Expems
Key
Phos
a+b
a
Controls
PLD
1.0
0.5
0
ST
1.0
0.5
0
µMPi/mg protein/ minute
Bird No. 1 2 3 4 5 6 7 8 9 10 11 12

Discussion

Thyroidectomy has a gross affect on the birds in terms of reduction in overall body weight and selected muscles. Weight reduction was most obvious for the ALD muscle but was also observed in the ST. Muscle length was not affected by the reduced presenceof thyroid hormone.

Both fast phasic muscles showed a dramatic decrease in the activity of Mg^{2+}-activated myofibrillar ATPase. There was however no change in the activity of this contractile enzyme for the slow tonic ALD muscle. These results indicate a differential effect of hypothyroidism on phasic and tonic muscles. This stresses the change from a fast to slow conversion for phasic or twitch muscles in agreement with the results of Johnson et al. (1980) and Nwoye et al. (1982). Both these studies showed a reduction in the fast contractile characteristics for the slow-twitch soleus. Nwoye and Mommaerts, 1981, have also showed a fast to slow conversion in the rat extensor digirorum longus (EDL) with hypothyroidism. This muscle however was found to be less responsive to thyroidal influence than the slow-twitch soleus muscle. The tonic ALD muscle of the chicken apart from weight reduction was little affected by the thyroidectomy and its Mg^{2+}-activated myofibrillar ATPase was not significantly lower than the controls. It appears therefore that although the fast-twitch and slow-twitch muscles of mammals and the fast-twitch muscles of birds, (PLD and ST in this study) show large drops in contractile activity the tonic muscles (ALD) may not be affected.

This pattern was repeated with the change in total phosphorylase activity (a + b). Both the fast phasic PLD and ST muscles showed a significant drop in activity whereas the ALD muscles were no different from the control muscles. The activity of phosphorylase a alone was not changed

in the PLD and ST muscles but was significnatly increased in the ALD muscle. This increase in the amount of phosphorylase a may be a reflection of the activity of the interconverting enzyme and it is difficult to assess the balance of the a and b forms without understanding in more detail the activities of both interconverting enzymes (See Chapter 4).

It must also be pointed out that the ST and PLD showed greater activities for both enzymes than the ALD and this is in agreement with the study in Chapter 4 and further stresses the faster and higher glycolytic capacity of the fast phasic muscles such as the ST and PLD in comparison with the slow tonic ALD.

This study therefore draws further attention to the large differences between the phasic PLD and tonic ALD. The thyroid hormones appear to have little affect on the ALD either metabolically or from a contractile viewpoint. The PLD and ST muscles did show large reductions in both enzymes' activity and were therefore affected by the hormone imbalance to a greater extent. It would be interesting to have studied an aerobic enzyme to further clarify the hormone's affect on the metabolic balance of anaerobic/aerobic metabolism. Further work should also include isozyme studies on the light chains of myosin to see if the slowing down of the fast phasic PLD and ST is reflected in the balance of slow and fast isozyme light chains.

CHAPTER 6

A STUDY OF THE ISOMETRIC-TENSION-pCA RELATIONSHIP IN SINGLE SKINNED FIBRES OF THE ALD AND PLD MUSCLES IN CHICKENS.

Introduction

Chapters 3 and 4 discuss the histochemistry and biochemistry of the different myosin ATPases of the ALD and PLD muscles. Biochemical and histochemical analyses of the two muscles has shown their differences in relation to the activity of their myosin ATPases. There have been no previous studies on the Ca^{2+} regulation of contraction in avian fast and slow muscles. In this chapter single skinned fibres wereisolated from the ALD and PLD of young adult chickens to analyse directly the relationship between tension generation and free calcium (pCa).

The relationship between the isometric tension and pCa in skinned or glycerinated fibres has been studied in various vertebrates; frog semitendinosus., (Gordon, Godt, Donaldson and Harris, 1973; Hellam and

Podolsky, 1969); rabbit psoas, (Wise, Rondinone and Briggs, 1971); and invertebrates, acorn barnacle, lateral depressor (Ashley and Moisescu, 1977). There have been no reports for the ALD and PLD muscle of chickens or indeed of studies on single tonic muscle fibres such as those found in the ALD.

The presence of the cell membrane, the sarcolemma, around the muscle fibre makes it methodologically difficult to alter the ionic composition of the sarcoplasm. There are three different preparations of muscle fibres that overcome this difficulty. First, physically skinning the fibre of its membrane. This is a delicate, skilled operation first carried out by Natori, (1954), and is normally only used with large fibres e.g. those of the frog semitendinosus. Although, Fabiato and Fabiato, (1978), have reported skinning cardiac cells of 10 µm in diameter. Secondly, the fibres can be glycerolated by soaking small bundles of fibres in 50% aqueous glycerol thereby causing the structure of the membrane to be damaged by osmotic shock. As a result the membrane becomes permeable and soluble proteins and metabolites diffuse out. (Varga, 1946; Szent-Györgyi, 1949). Thirdly, fibres can be treated with detergent e.g. Lubrol-WX (Julian, 1971) or EDTA, (Winegrad, 1971) or 1% Brij 58 (Altringham, 1981). These detergent preparations have the advantage of removing virtually all the sarcolemma (Fabiato, 1982).

The chemically skinned fibre can now be surrounded by a "relaxing" solution with ionic constituents similar to those of the sarcoplasm. In this solution the amount of ATP present can be controlled and regenerated by adding creatine phosphate (CP) and the enzyme creatine phospho-kinase (CPK). In muscle, <u>in vivo</u>, CPK catalyses an ATP regeneration reaction, known as the "Lohmann reaction". (Lohmann, 1934).

$$CP + ADP \xrightarrow{CPK} Creatine + ATP$$

This ensures that the ADP/ATP ratio, even at the centre of a contracting fibre cannot rise abnormally, thereby allowing the maximum efficiency of contraction to be maintained. The need for such an ATP regeneration system has been demonstrated by Ashley and Moisescu, (1977), and Godt, (1974).

Small amounts of Ca^{2+} ions can reverse the effect of the relaxing solution. However, a chelating agent such as Ethylene glycol bis - (amino ethyl ether) N, N' tetra acetate, (EGTA) can bind to any Ca^{2+} ions and replace the natural factor that causes relaxation. EGTA binds Ca^{2+} strongly and Mg^{2+} weakly, performing much the same function as the calcium pump of the sarcoplasmic reticulum in the intact fibres.

The levels of Ca^{2+} required for contraction are best studied using a mixture of Ca EGTA and free EGTA. An EGTA concentration of 5m$\underline{M}$ was chosen to avoid the delayed onset of force production and a reduced rate of tension rise found at lower concentrations by Ford and Podolsky (1972). Using a system such as the "activating solution" in this study it can be shown that the development of full tension in glycerinated or skinned muscle fibres requires about $10^{-5}\underline{M}$ Ca^{2+}. (Hellam and Podolsky, 1969). The manner in which Ca^{2+} ions stimulate muscle contraction has been extensively reviewed. (Ebashi, Maruyama and Endo, 1980; Murray and Weber, 1974).

Several Ca^{2+}-control systems have been described from <u>in vitro</u> experiments that are capable of regulating muscle contraction (Lehman and Szent-Györgyi, 1975). In addition in striated muscle other types of

Ca^{2+}-control protein systems have been implicated. (Bárány and Bárány, 1977). Four models for the Ca^{2+} control of muscle contraction have been proposed. Two of these are concerned with thin filament (actin) regulation and two involve thick filament (myosin)-linked regulation.

Those concerned with thin filament regulation were first proposed by Ebashi and colleagues, (1968) and (1978, Mikawa et al.). Perhaps the best known of these models is the troponin system, generally believed to be responsible for the Ca^{2+} regulation of striated vertebrate muscle. The interaction of Ca^{2+} ions with Troponin-C protein exposes sites on the actin filament used for cross-bridge attachement, thereby making them available for they myosin cross-bridges to cycle. This model is now under question particularly with reference to the steric blocking part of the model. New evidence suggests that exposition of the sites on the actin filament via a conformational change in the regulatory proteins whether troponin or the light chains of myosin cannot and need not physically occur (Chantler, 1982). The second system involves the regulatory protein leiotonin and has been proposed for the Ca^{2+} regulation of smooth muscle contraction. However, as mentioned below the light chains are possibly of more importance in smooth muscle control.

The first of the control systems involving thick filament (myosin) -linked regulation concerns the Ca^{2+}-regulated myosin light chains found in the mollusc (Scallop). These have also been shown to be present in some muscles regulated by a thin filament system (Lehman and Szent-Györgyi, 1975). Ca^{2+} is known to bind to the light chains of striated muscle, (Bagshaw, 1977) but their role in the in vivo regulation of muscle contraction is still unclear. (Lehman, 1978). The most recently discovered myosin-linked regulation system is the light chain Kinase-phosphorylase system proposed for the regulation of smooth muscle.

(Adelstein and Conti, 1975). This system is unique in that it involves the activation of a Ca^{2+}-sensitive light-chain kinase, which in turn phosphorylates myosin light chains resulting in activation of the contractile proteins. Relaxation is caused by dephosphorylation of the light chains by a phosphatase in the absence of Ca^{2+}. In addition, the light chain kinase-phosphatase system has been shown in vitro to be present in striated (Pires, Perry and Thomas, 1974) as well as in smooth muscle and to be implicated in the Ca^{2+} regulation of in vivo muscle contraction (Bárány and Bárány, 1977).

Magnesium ions are included as activating agents for the myosin ATPase. It has been shown by Lymn and Taylor, (1970), that ATP when acting as a substrate for hydrolysis in solution takes the form of a complex with the Mg^{2+} ion as Mg ATP^{2-}. At very low Mg ATP^{2-} concentrations, resting tension increases dramatically due to rigor complex formation. (Bremel and Weber, 1972). A decrease in Mg ATP^{2-} concentration from 2mM (through 0.1mM) to 20 M shifts the pCa to tension curve to a higher pCa but does not affect the overall maximum isometric tension. (Godt, 1974). Large changes in Mg ATP^{2-} concentration in the millimolar range do not significantly alter the pCa to tension relationship. (Ashley and Moisescu, 1977; Kerrick and Donaldson, 1972). Changes in the free Mg^{2+} concentration from 1-5 M shifts the curve for isometric tension with pCa, 0.7 log units to higher free Ca^{2+} concentration without affecting steepness. (Ashley and Moisescu, 1977). Decrease in pCa concentration will change the amount of free Mg^{2+} but within 0.1 of a milimolar and the effect is negligible and will not alter the relative positions of the curves from different fibre types.

The ionic strength of the activating and relaxing solution can be controlled with added potassium chloride (KCl). The effect of the KCl

concentration on the contraction kinetics has been studied by Thames, Teichholz and Podolsky, (1974). The maximum isometric tension was found to decrease continuously from 0 to 280mM of added KCl. Resting tension increased after the first activation cycle with a KCl concentration below 140mM. Gordon et al., 1973, suggest a value between 140-170 mM for the ionic strength will approximate to physiological conditions. A value of 180-185mM for the ionic strength used for these experiments would, using the data of Thames et al., (1974), produce a drop of < 2% in isometric tension, and a shift in the pCa-tension curve of < 0.04 pCa log units towards higher free Ca^{2+} concentration. These effects are considered to be within the limits of resolution of the experiments.

In this study the changes in isometric tensions alone were recorded but the mechanical apparatus described in this study has been designed for force-velocity experiments and pCa-isometric-tension studies. The force produced for both isometric and isotonic measurement is measured by an isometric strain guage. Further details of the apparatus are given in Materials and Methods.

Materials and Methods

Birds

Male chickens, breed Thornber 404; (Light Sussex cross Rhode Island Red) were used aged between 90-100 days. The chickens were killed with an overdose of pentobarbitone sodium (I.P.). Small strips of both the ALD and PLD muscle were taken, pinned to cork and kept in ice cold chicken Ringer. Bundles of 20-40 fibres were dissected from the original strip, care being taken to touch only the ends of the bundles. These bundles were then placed immediately in a 3mm deep glass trough containing silicone oil

at 5°C. A drop of the standard relaxing solution was then injected around the fibres within the oil.

Fibre skinning and attachment

Single PLD and ALD fibres were teased from this bundle of 20-40 fibres. The fibres were then chemically skinned and this procedure was followed for all dissected fibres before isometric tension experiments were performed. The single fibres were wrapped across the tips of fine forceps and rapidly transferred to the hooks of the apparatus. The ends were wrapped around the hooks and secured with a drip of plexiglass/ acetone glue. (See Fig. 6(i), Page 130).

The fibre was then immersed in the first incubation solution. The thin covering of silicone fluid helps to prevent dehydration with transference of the fibre usually complete within 30 seconds. The first incubation solution contains relaxing solution with 1% Brij 58 (polyoxyethylene 20 cetylether), a non-ionic detergent. A 30 minute incubation rendered the fibre membrane completely permeable to external solutions; with diffusion of the relaxing solution components to the centre of the fibre also complete within this period (Godt, 1974). The fibre was then transferred to relaxing solution without Brij 58 for 3 to 5 minutes. The fibres can then be activated by immersion in activating solutions of varying free Ca^{2+} concentrations.

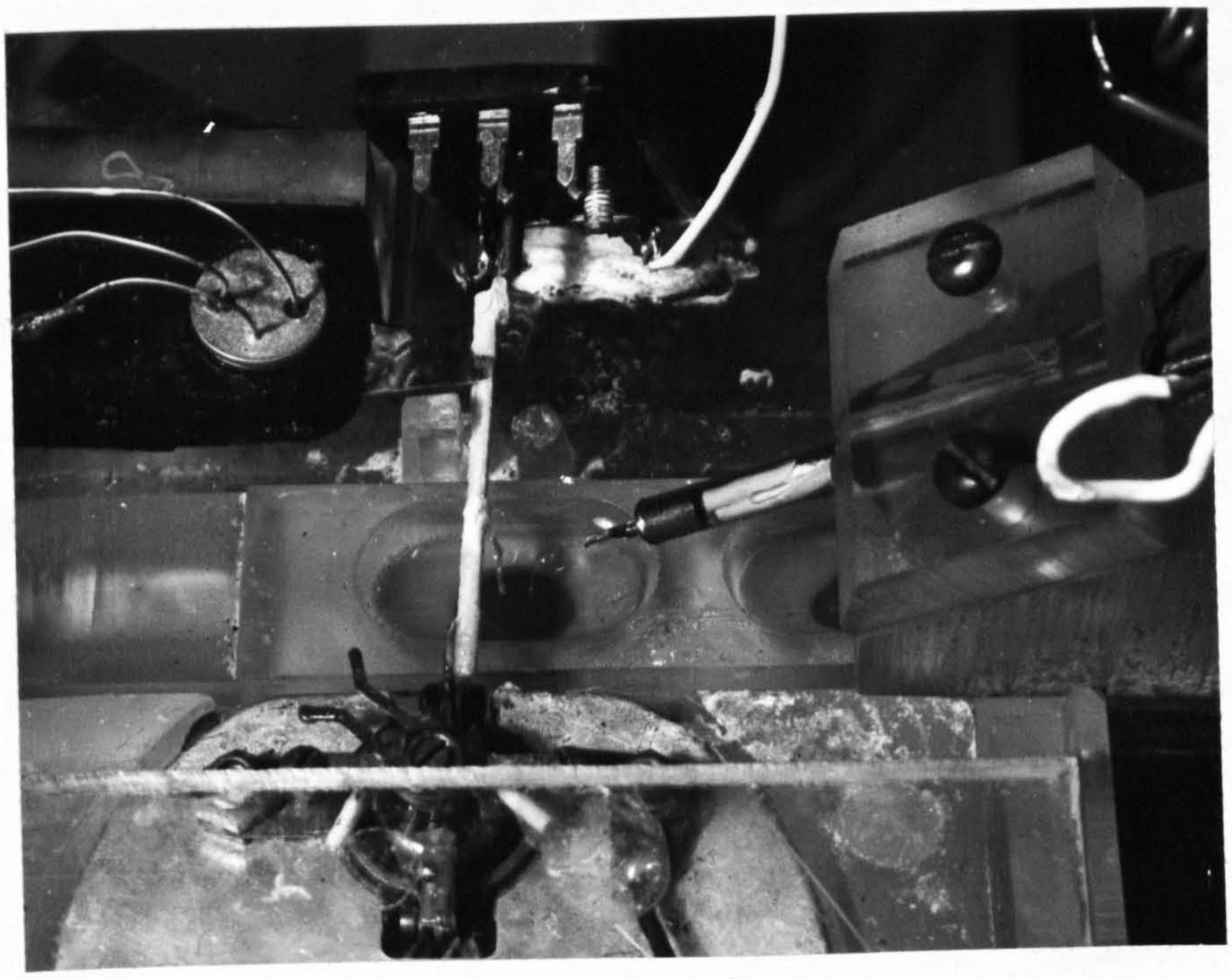
Immediately after attachment, the sarcomere length was set at 2.3 μm and the fibre diameter measured. After the first activation cycle, the sarcomere length may have decreased to 2.1 to 2.2 μm at the centre of the preparation, possibly due to damage and subsequent extension at the ends. For this reason, the sarcomere length was always checked after the first

See overleaf for Figure and Figure Legend 6(i).

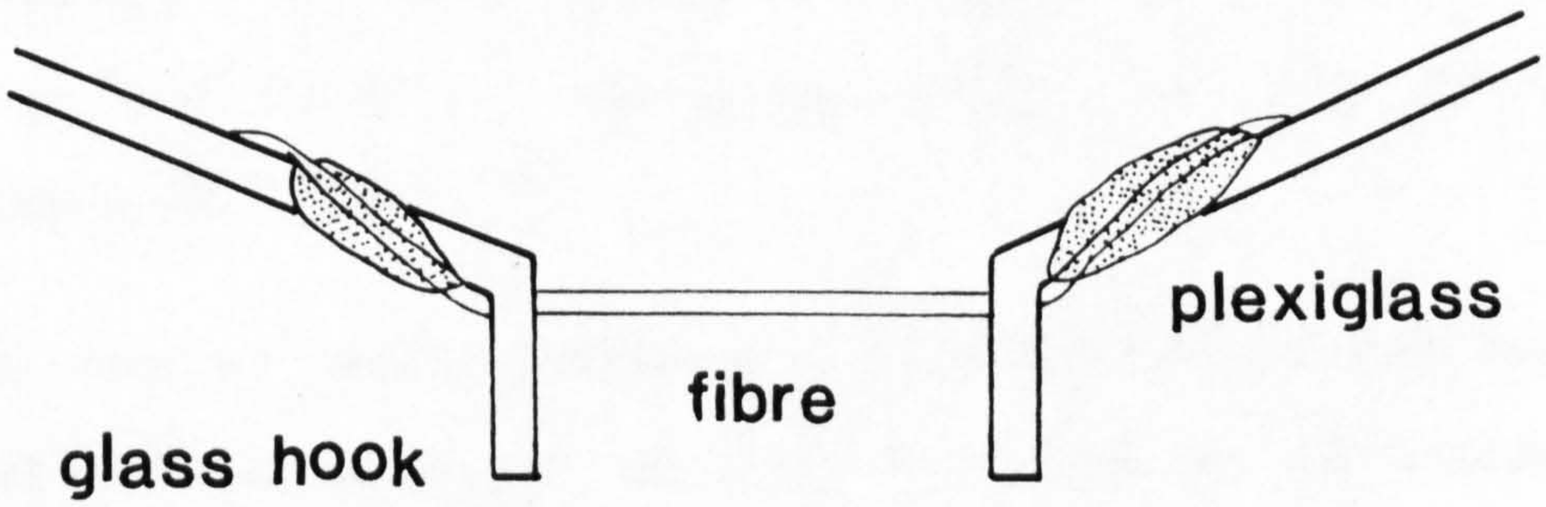
Figure 6(i)

a) Photograph of the hooks and incubation baths in situ.

b) Diagram to illustrate the method of fibre attachment.



a



b

activation and reset to 2.3 μm as necessary. Further shortening may occur, but was less than 0.1 μm. The fibre diameter of the ALD varied between 45-108 μm and the PLD between 23-75 μm.

Apparatus

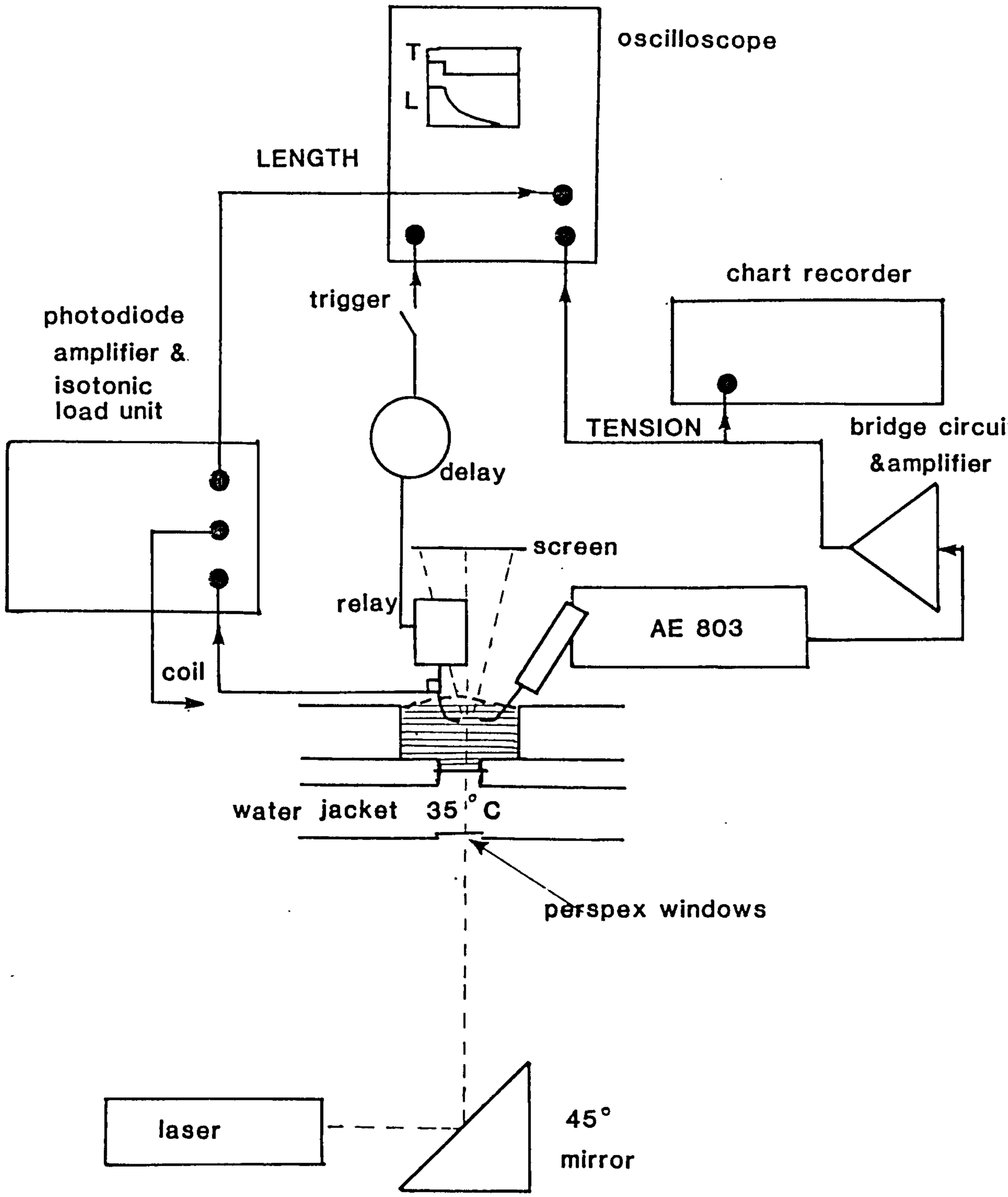
The apparatus developed by Dr. I. Johnston and Dr. J. Altringham allowed the independent measurement of tension or length. A block diagram is shown in Fig. 6(ii), Page 132, and details of the mechanical section in Fig. 6(iii), Page 133. A 1-2.5mm length of the fibre was glued between the two glass hooks. One hook was attached directly to the silicon beam of an AE 803 strain guage element (A.M.E. Horton, Norway). The time element was held rigidly in a screened, aluminium and perspex adaptor, mounted on a one way micromanipulator. This allowed the distance between the hooks to be adjusted. The output from the element was fed to a bridge circuit and amplifier unit. The sensitivity varied slightly with the element in use, a typical value being 3.5mVmg^{-1}. Noise was < 3mV and the drift < 1mVhr^{-1}. The other glass hook was attached to a 3.7cm long balsa wood lever. During the isometric contractions (those in this study), a brass pin, glued to the armature of a miniature relay, held the free end of the lever against a stop. The total compliance of the system was < 40 μmg^{-1}.

A block of perspex mounted on a 2-way micromanipulator slid in a channel beneath the hooks. The lower half contained circulating ethylene glycol/water from a Grant cooling system to regulate the temperature (from ± 0.5°C to 37°C). The upper half of this block had 3 chambers, each of 1.5ml capacity. The block could be raised to immerse the fibre in any of these 3 chambers. A change from one bath to another could be effected in ≤ 5 seconds. A thin perspex window set in the block allowed the beam

See overleaf for Figure and Figure Legend 6(ii).

Figure 6(ii)

A block diagram to illustrate the apparatus for force-velocity and isometric tension experiments.


T
L
oscilloscope
LENGTH
trigger
photodiode
amplifier &
isotonic
load unit
chart recorder
TENSION
bridge circuit
&amplifier
delay
screen
relay
AE 803
coil
water jacket 35°C
perspex windows
laser
45°
mirror

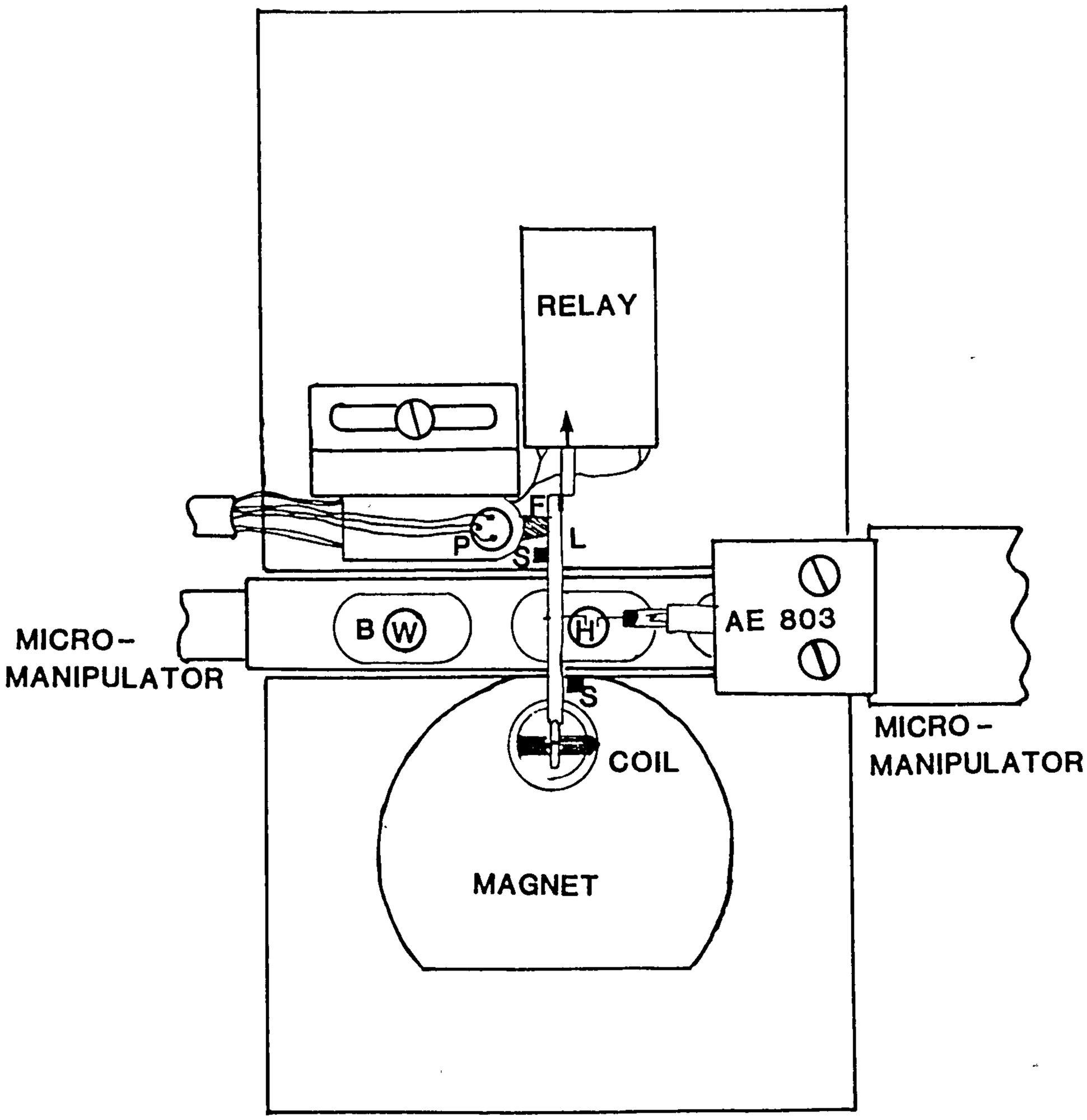
See overleaf for Figure and Figure Legend 6(iii).

Figure 6(iii)

A diagram to illustrate the details of the mechanical section of the apparatus.

KEY

B = incubation chamber
F = foil flag
H = glass hooks
L = balsa wood lever
P = photodiode assembly
S = stops



from a He-Ne laser to be passed through the fibre from below.

The diffraction pattern was viewed on a small translucent screen placed above the fibre. The screen was calibrated in sarcomere lengths, calculated from Bragg's equation for diffraction of light:

$$s = \frac{\lambda}{\sin \theta}$$

s = sarcomere length

λ = wavelength of laser (0.6328 μm)

θ = angle subtended by the zero and first order diffraction patterns.

The sarcomere length was adjusted by moving the tension transducer back and forth on its micromanipulator.

The length-tension curve for skinned fibres has been determined by Moss (1979) on frog anterior tibialis muscles. These results did not differ from those of Gordon, Huxley and Julian (1966) on intact fibres, with a tension plateau between 2.0-2.2 μm. Edman (1979) studied the contraction velocity in tetanically stimulated fibres isolated from frog muscles and found it to be independent of sarcomere length 1.65-2.7 μm.

The dissection and mounting of the fibres was facilitated with binocular microscopes. A calibrated graticule in one eye-piece was used to measure the fibre diameter in situ. The tension was continuously monitored on a Bryans 28,000 chart recorder. The strain guage plus hook was calibrated using standard weights with tension recordings made on the chart recorder. A new calibration was made each time the hook was reglued.

(See Appendix D, Page 201, for a specimen standard curve).

Solutions

See Appendix B, Page 192, for list of chemicals and suppliers.

Chick Ringer buffered at pH 7.2

NaCl	8.6 g	
KCl	0.31 g	
$CaCl_2$	0.20 g	(dihydrate)
K_2HPO_4	0.80 g	
KH_2PO_4	0.20 g	
$MgCl_2$	0.10 g	
Glucose	2.00 g	

Added to 1 litre of distilled water.

Basic Relaxing Solution pH 7.2 at 20°C

Imidazole	10mM
KCl	110mM
$MgCl_2$	3mM
EGTA	5mM
Phosphocreatine	10mM
ATP	2.5mM

Creatine phospho-kinase in solid form was added before each experiment to a final concentration of > 20 μ ml^{-1}. KOH and HCl were added to obtain the exact pH and the amount added was noted in each case.

An iterative computer program (Perrin and Sayce, 1967) modified by White and Thorson (1972) was used to calculate the concentrations of the

various ionic species in the solutions. The affinity constants used in the programme are listed in the AppendixD, Page 201 .

Activating Solutions

Activating solutions were made by the addition of $CaCl_2 5H_2O$ from 0-5mM, to the basic relaxing solution. The concentrations of selected ionic species in the relaxing and activating solutions are listed in Table 6, Page 137.

Experimental procedure for the measurement of isometric tensions for various pCa concentrations.

Fibres from the ALD and PLD muscles in turn were skinned and left in the relaxing solution for 3-5 minutes. Eight different calcium concentrations pCa's from 7.41-5.31 were used in succession in the activating bath to give the maximum isometric tension (Po) and the intermediate tensions (P). Activating solutions were used randomly, with concentrations giving the maximum isometric tension at intervals throughout the experiments. Relaxation-contraction cycles were performed at high or low Ca^{2+} concentrations with little or no increase in resting tension. In most experiments on both muscle fibre types repeated activations at maximum isometric tension showed little or no decrease in Po over 10-20 cycles. A drop below 75% of the initial Po or an unclear diffraction pattern and the fibres were discarded. A drop within 25%, and the new lower Po was incorporated into the later results allowing for the decline. The maximum isometric tension (Po) and the rate of rise of tension were not increased by raising the concentation of $MgATP^{2-}$, creatine phospho-kinase orphosphocreatine. The fibre was replaced in the relaxing solution bath while the activating solutions were changed.

TABLE 6 THE CONCENTRATIONS OF SELECTED IONIC SPECIES IN THE RELAXING AND ACTIVATING SOLUTIONS.

SOLUTION	($CaCl_2$) (mM)	(Ca^{2+}) free (μ m)	pCa	(Mg^{2+}) free (mM)	($MgATP^{2-}$) (mM)	Ionic Strength (mM)
RELAXING	0	–	–	0.48	2.24	180.2
ACTIVATING	0.20	0.04	7.41	0.48	2.24	180.4
	0.80	0.18	6.75	0.49	2.24	181.0
	1.00	0.23	6.63	0.49	2.24	181.3
	1.50	0.40	6.40	0.50	2.25	181.9
	2.25	0.77	6.12	0.51	2.25	182.6
	2.50	0.94	6.03	0.51	2.25	182.9
	2.70	1.10	5.96	0.51	2.25	183.1
	4.20	4.88	5.31	0.53	2.25	184.6

At pH 6.95, 35°C.

The temperature of the incubation solutions was maintained at 35°C, the physiological temperature for chicken muscle.

Results

The results for a typical fibre preparation are shown on Fig. 6 (iv), Page 139. The fibre showed no residual tension with relaxation and the maintenance of the initial Po at the end of the experiments.

To study the decline of the initial Po on repeated activations an ALD fibre was repeatedly activated at a pCa giving maximum isometric tension and a new ALD fibre at a half maximal pCa value. These repeated activations are shown in values of relative tension $^{P}/_{Po}$ against the experiment length in Fig. 6 (v), Page 140. Only the repeated activations at the pCa equivalent to Po causes a decline in tension. The drop was below 75% of the original Po after 20 minutes and 6 maximal activations. Repeated activations at half maximal values of pCa did not cause a decline in tension.

pCa-isometric tension relationship

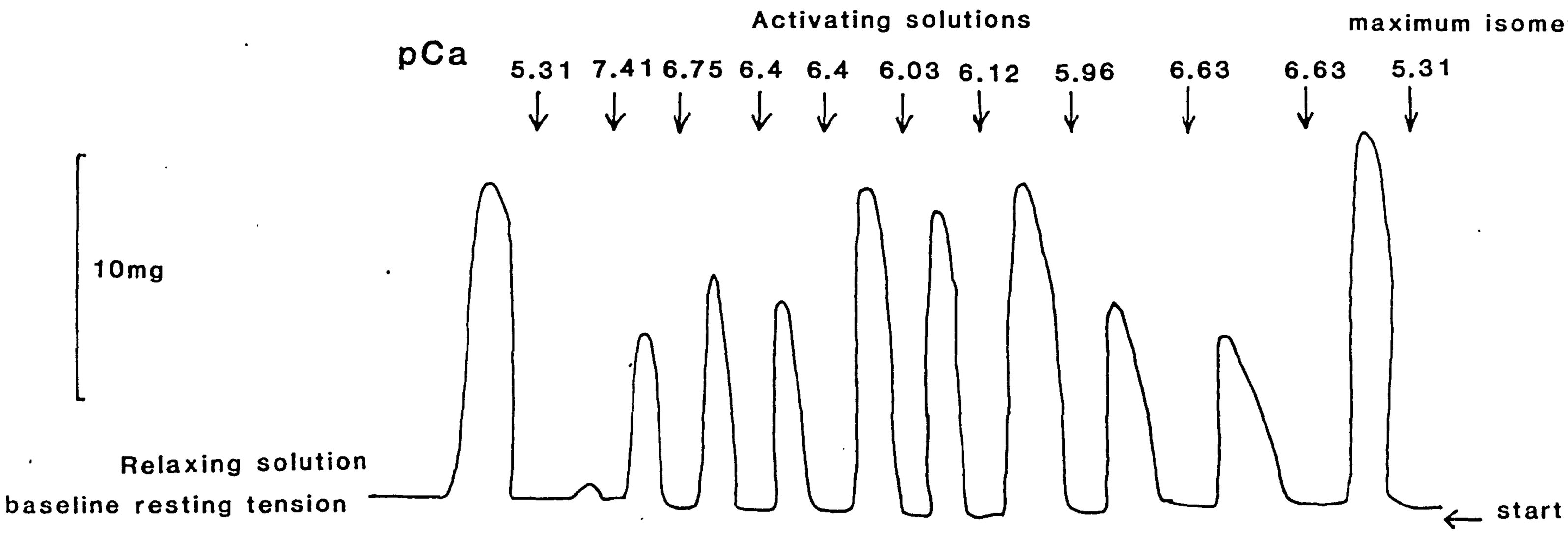
The pooled data for the pCa-tension relationship from 5 fast PLD and 5 slow ALD fibres are shown in Fig. 6(vi), Page142. The tension is expressed relative to the maximum isometric tension obtained from each fibre.

The threshold for tension generation for the ALD fibres was a pCa of 7.5 and for the PLD fibres a pCa of 6.63. Maximal tension was reached at a pCa of 5.7 for the ALD and 5.3 for the PLD (extrapolated from the sigmoidally fitted curves). A two-factor analysis of variance (ANOVA; muscle type against pCa) was conducted on the individual values for the single fibres for both muscles. The curves were significantly different. ($F(1,8)$ =

See overleaf for Figure and Figure Legend 6(iv).

Figure 6(iv)

Results from a typical fibre preparation.

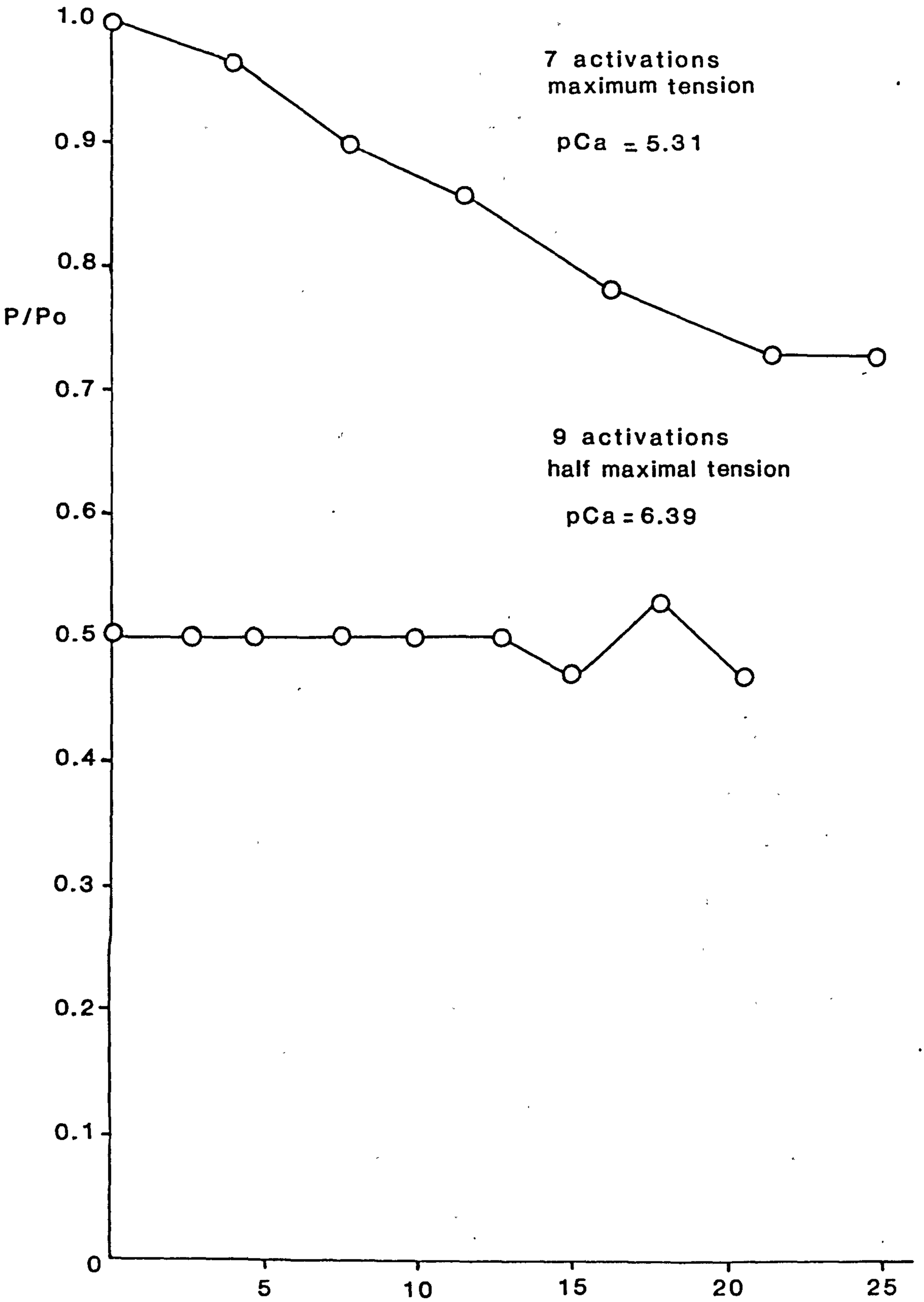


ALD fibre diameter 50μm

See overleaf for Figure and Figure Legend 6(v).

Figure 6(v)

A study of the drop in initial maximum isometric tension on repeated activations compared with repeated activations at half maximal tension for two separate ALD fibres of 72 μm and 50 μm diameter respectively.


1.0
0.9
0.8
P/Po
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
Time in minutes
7 activations
maximum tension
pCa = 5.31
9 activations
half maximal tension
pCa = 6.39

41.869, $p < 0.001$). They were, however, shown not to be significantly different at a pCa of 5.31 or 7.41. A 3rd degree polynomial equation provided the curve of best fit for the means of the results and this relationship plotted on Fig. 6 (vi), Page 142, was sigmoidal for both muscles; this indicates multiple Ca^{2+} binding sites. (See Appendix, D, Page 201, for data and statistical tests). The pCa-tension curves as shown on Fig. 6 (vii), Page 143, produced by the relationship between the free calcium and the steady isometric tension P/maximum isometric tension Po can be linearised according to the Hill equation.

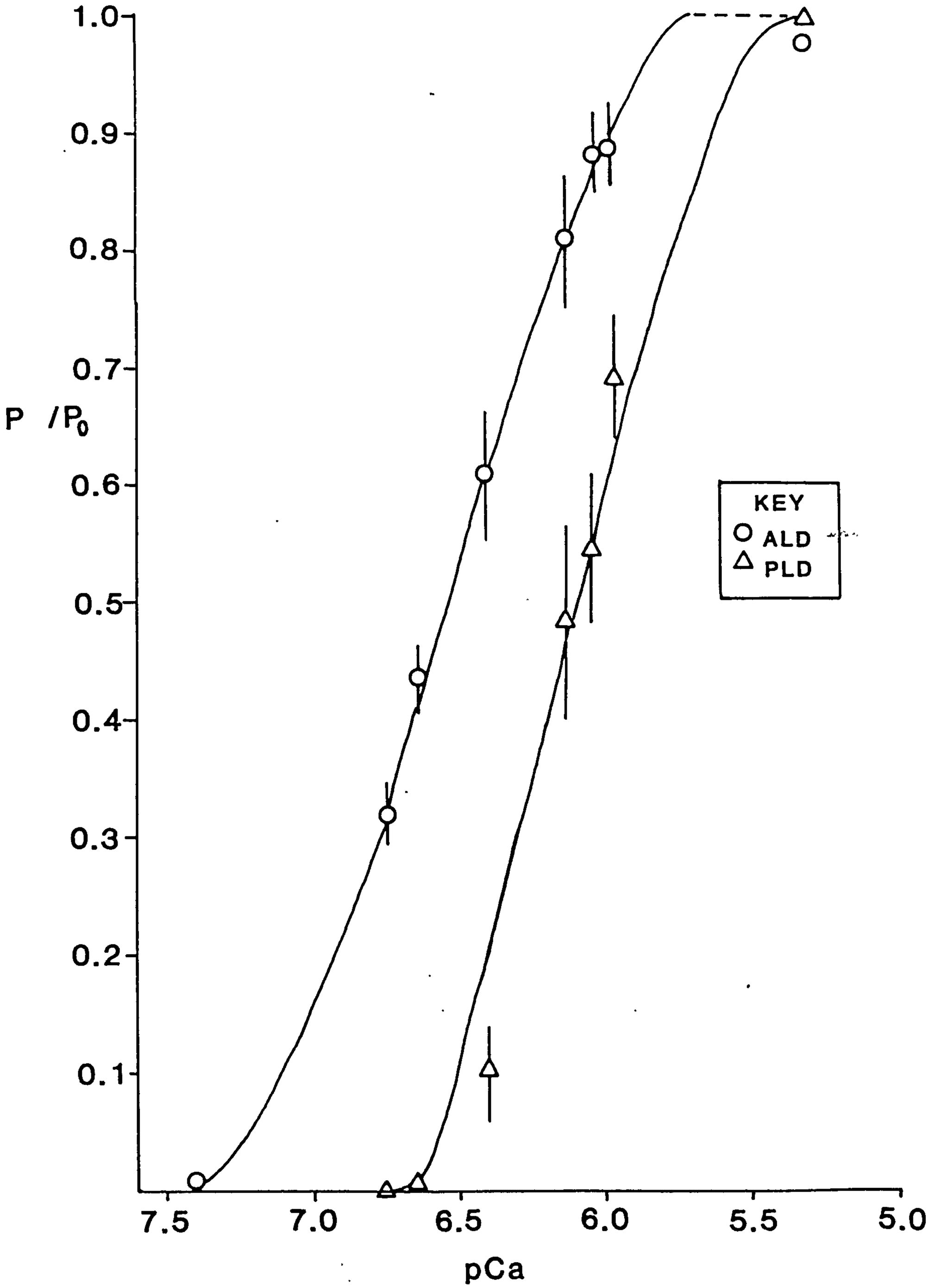
$$\log_{10} \frac{(P)}{Po-P} = n \log_{10} (Ca^{2+}) + h$$

where n and h are constants. A straight line is obtained by plotting $\log_{10}$ (P/Po-P) against pCa. When p = 0.5Po, then $\log_{10}$ (P/Po-P) = 0. The calcium concentration at this point is the concentration required to produce half maximal tension. The constant n in the Hill equation gives an estimate of the number of Ca^{2+} binding sites on the troponin C molecule. The shape of the pCa-tension curve is reflective of the number of sites and the degree of interaction between them. From a single kinetic analysis such as this it is difficult to distinguish between these factors, however, it can give information of their combined effects. The situation would be complicated further if the sites do not have identical Km's for Ca^{2+}, and if the amount of interaction varies between sites. Fig. 6 (vii), Page 143, shows the linearisation of the pCa-tensions curves using the Hill plot, $\log_{10}$ ($^{P}/_{Po-p}$) plotted against pCa. Linear regression lines were plotted against this data and the gradient of constant n was = 3.0 for the PLD fibres and = 1.5 for the ALD, indicating a minimum of 3 Ca^{2+} sites for the PLD and 1 or 2 for the ALD fibres. Calcium concentra-

See overleaf for Figure and Figure Legend 6(vi).

Figure 6(vi)

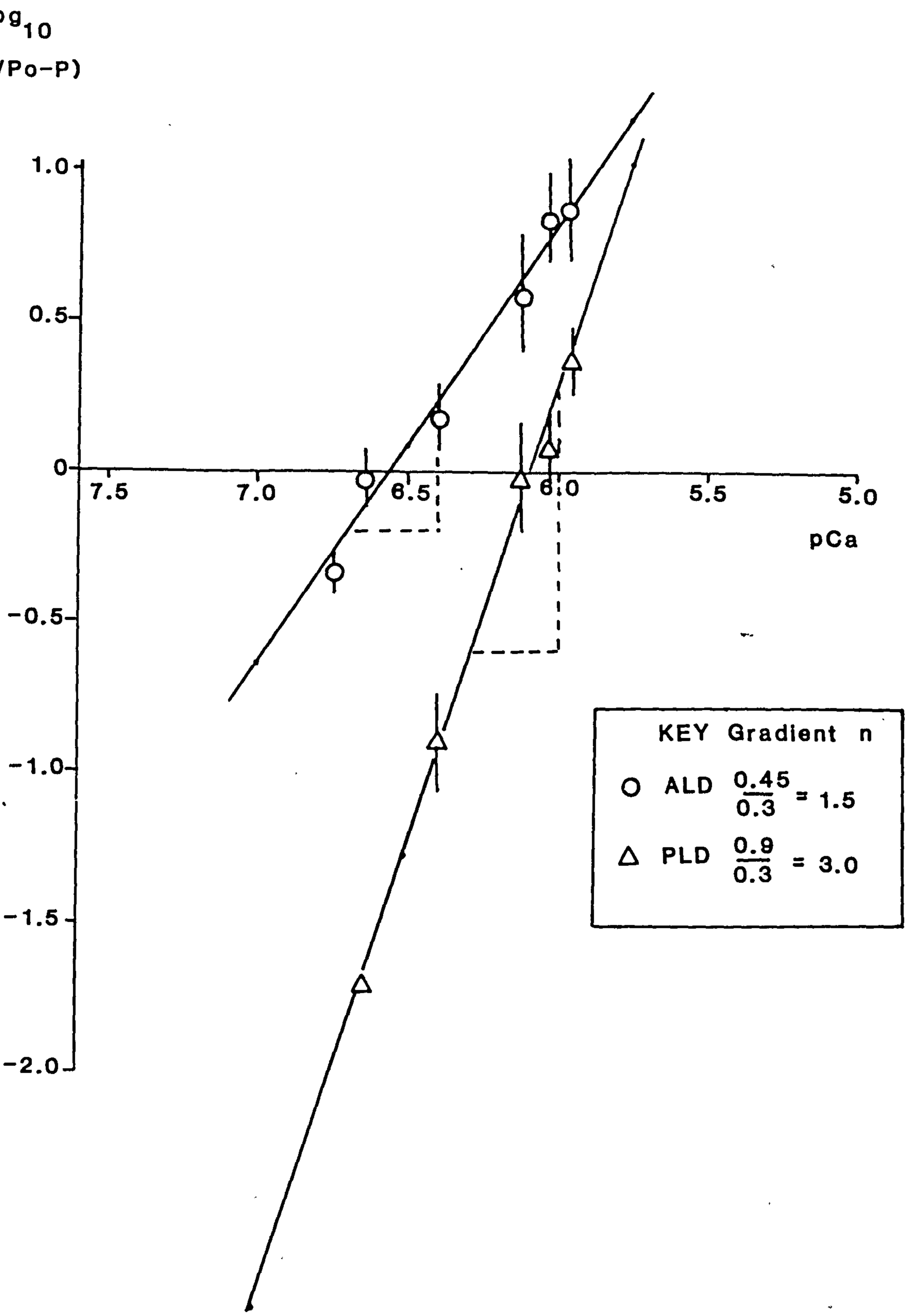
pCa - tension curves from 5 fibres for the ALD and PLD, with statistically fitted sigmoidal curves.


1.0
0.9
0.8
0.7
P /P0
0.6
0.5
0.4
0.3
0.2
0.1
7.5
7.0
6.5
6.0
5.5
5.0
pCa
KEY
ALD
PLD

See overleaf for Figure and Figure Legend 6(vii).

Figure 6(vii)

Hill plots for the data from the pCa-tension curves of Figure 6(vi) showing the fitted linear plots and the gradient values (n) for the ALD and PLD muscles.


log10 (P/Po-P)
1.0
0.5
0
-0.5
-1.0
-1.5
-2.0
7.5
7.0
6.5
6.0
5.5
5.0
pCa
KEY Gradient n
O ALD 0.45/0.3 = 1.5
Δ PLD 0.9/0.3 = 3.0

tion for half maximal tension was found to be 6.56 for the ALD and 6.08 for the PLD.

Maximum isometric tension

The force for skinned fibres has been shown to be proportional to cross-sectional area (Hellam and Podolsky, 1969; Wise et al., 1971). Cross-sectional area was calculated from the mean diameter assuming circularity of the fibres. The pooled results from all the fibres studied are shown below (see Appendix D for raw data).

	Maximum Isometric Tension		
	$Kgcm^{-2}$	Ncm^{-2}	Fibre No.
ALD	0.838 ± 0.136	8.221 ± 1.334	(n=9)
PLD	0.860 ± 0.163	8.437 ± 1.599	(n=11)

(All values $\pm$ S.E.)

Maximal isometric tensions were produced at sarcomere lengths between 2.2 and 2.3 µm.

Discussion

The maximum isometric tension, Po, observed for the fast PLD and slow ALD fibres was similar at 0.86 and 0.84 $Kgcm^{-2}$ respectively, and compared relatively well with those values for skinned muscle fibres found in the literature. The values were slightly low but this could be due to minor damage during dissection. There was a large range of values and further analysis of more fibres from each muscle would give a more accurate description of the absolute Po values.

Values of 1.4 and 1.8 $Kgcm^{-2}$ have been found for frog semitendonosus fibres by Hellam and Podolsky, (1969), and Gordon et al. (1973) respectively. Wise et al. (1971), found Po at 1.34 $Kgcm^{-2}$ for rabbit psoas muscle fibres. Live preparations of frog muscle fibres however, have given values of Po between 2-3 $Kgcm^{-2}$ (Ramsey and Street, 1940; Gordon et al., 1966). These differences may be due to the obvious differences between the bathing solution and the intracellular environment of an intact fibre.

A mechanically skinned fibre is known to swell by 15% in relaxing solution. (Matsubura and Elliot, 1972). This could be the reason for the lower values found for skinned fibres, through an overestimation of the fibre's cross-sectional area. A proportion of this swelling in mechanically skinned fibres is thought to be caused by the S.R. enlarging. (Taylor and Godt, 1976). Chemical skinning, however, disrupts both the S.R. and the sarcolemma (Altringham, 1981) and no swelling was observed for the fibres in this study. It is apparent that the variety of methods used to study single fibres will cause a degree of abnormality to the fibre's environment, and the lower tensions (Po) recorded may be related to this. (See Table 7, Page 146).

The Po values for the ALD and PLD muscles were very similar and it is suggested that the fractional volume occupied by the myofibrils must be similar. Altringham, 1981, found that the Po for the fast fibres of dogfish and cod were twice that for the slow fibres from the same fish. Although, in this case, there is thought to be a difference in the relative fractional myofibrillar volume (50% more myofibrils in fast fish fibres than slow), it still appeared that the Po/myofibril was greater in fast fibres than slow.

Although the Po for the ALD and PLD fibres was similar, the curve shown for the pCa-isometric tension relationship is shifted to lower

TABLE 7

Animal	Maximum Isometric Tension Po in $Kgcm^{-2}$ Fast Muscle	Slow Muscle	Temp.	Reference
Fish				
Cod (chemically skinned fibres)	1.9 ± 0.12	0.85 ± 0.10	0-5°C	Altringham, (1981).
Dogfish (chemically skinned fibres)	1.87 ± 0.09	0.84 ± 0.04		
Barnacle				
Balanus nubilus (mechanically skinned myofibril bundles)	3.4		20°C	Ashley and Moisescu, (1977).
Frog				
semitendonosus muscle (mechanically skinned fibres)	1.4-1.7		22°C	Hellam and Podolsky, (1969).
	1.8		22°C	Gordon *et al.*, (1973).
	1.5-2.0			Endo, (1967).
Mammals				
Rabbit psoas (glycerinated fibres)	1.34		19°C	Wise *et al.*, (1971).
Birds				
ALD, PLD Domestic Chicken (single chemically skinned fibres)	0.86 ± 0.163	0.838±0.136	35°C	*This study.*

free (Ca^{2+}) for the slow ALD fibres relative to the fast PLD fibres. This indicates a lower Km for Ca^{2+} binding for the slow ALD fibres than the fast PLD fibres. The data suggest that the PLD has a minimum of 3 Ca^{2+} binding sites and the ALD a minimum of 2 Ca^{2+} sites. The PLD muscle appears to have twice the Ca^{2+} binding site number than the ALD muscle. These results are similar to those of Kerrick, Secrist, Coby and Lucas, (1976), who found a n of 2.0 for the rabbit, fast, adductor magnus and 1.7 for the slow soleus. In this case, as with the results for cod fast and slow muscle found by Altringham, (1981), (n = 1.9 and 1.6 for fast and slow respectively) the contraction activation involves a minimum of 2 Ca^{2+} binding sites. It would appear that from the present study the PLD muscle contraction-activation involves a minimum of 3 sites for Ca^{2+} ions.

Both pCa/tension curves are sigmoidal which indicates the existence of multiple substrate-binding sites and of a co-operative effect whereby the binding of substrate at one site facilitates the binding of others at the adjacent site (homotrophic effect). The threshold for tension generation lies around pCa 7.0 and was at a lower free (Ca^{2+}) for the ALD than the PLD, but for both muscles lies within 0.2 μ M free (Ca^{2+}). The curves are steep and maximum isometric tension was achieved within 2pCa units at 5 μM free (Ca^{2+}).

As described in the introduction there are Ca^{2+}-binding sites on both the thick and thin filaments in skeletal muscle. Four sites have been identified by Collins, Potter, Horn, Wilshire and Jackman, (1973), on rabbit troponin C, and 2 low affinity and 2 high affinity sites by Potter and Gergely, (1975), (the latter sites also binding Mg^{2+}). The Ca^{2+} sensitive regulation contributed by the thick filaments is found on the 2 DTNB or LC_2 light chains of the myosin heads. (Kendrick-Jones, Szentkiralyi and Szent-Györgyi, 1976). Most muscle including those of

higher vertebrates contain both regulatory systems, although the actual importance of the light chain control is unclear.

There is therefore a total of 6 sites which may be involved in the activation of contraction, 4 on troponin C and 2 on the DTNB light chains. Thick filament control, as proposed for invertebrates, alone would only require $2Ca^{2+}$ ions. (Szent-Györgyi, 1975). However, a kinetic analysis of the isometric tension transients from frog skinned fibres by Moisescu, (1976), gave a minimum of 6 Ca^{2+} ions/functional unit in the frog. It may be that the tonic ALD muscle exhibits a control process determined by the thick filament light chain regulation requiring only 2 Ca^{2+} ions. In contrast, the phasic PLD muscle could show control via the thin filament regulation and require up to 4 Ca^{2+} ions for the activation via Troponin C. This simplified analysis is hypothetical and the control of activation in both muscles is probably more complex.

Troponin has been biochemically identified as 3 separate molecules, I, T, and C, and isozymes of these proteins have been found to vary through development and from muscle to muscle. (Perry, 1979). The differences between fast, slow and cardiac muscle in terms of their light chains has also been extensively researched. (Weeds, Hall and Spurway, 1975; Sarkar, Sreter and Gergely, 1971). The balance of the light chain isozymes in the slow (s) ALD ($LC1_s$ and $LC2_s$) and PLD fast (f) ($LC1_f$, $LC2_f$ and $LC3_f$) muscle have been found to vary between muscles and throughout development. (Pette, Vrbová and Whalen, 1979). However, Pette et al. (1979) also showed that the evolution of the overall contractile properties of the two muscles is independent of the myosin light chains. This implies an obsolescence of this thick filament system of regulation of contraction-activation in these two chicken muscles.

The differing threshold of free (Ca^{2+}) needed to produce tension for the slow ALD and fast PLD may reflect differences in the sarcoplasmic reticulum of the two muscles. The ALD muscle starts contracting at a free (Ca^{2+}) of 0.04 μM whereas the PLD muscle needs a concentration of 0.23 μM. The PLD muscle reaches maximum tetanic tension 10 times faster and relaxes 8 times faster than the ALD muscle (Canfield, 1971). The PLD needs to be relaxed even when a certain amount of Ca^{2+} is present and therefore only becomes activated above a free (Ca^{2+}) of 0.23 μM. It also has a more highly developed sarcoplasmic reticulum (SR) than the ALD (Ryan and Shafig, 1980) and this is consistent with a rapid withdrawal of calcium after contraction, to aid relaxation. The ALD however is a muscle that needs to be activated for long periods of time and therefore needs to contract at a low Ca^{2+} concentration and to remain activated to function normally. It has a reduced sarcoplasmic reticulum (SR) in comparison with the PLD muscle. (Page, 1969). These differences in their respective SR is in agreement with the differing thresholds of pCa needed for contraction-activation. The ALD has been shown to have a very efficient contractile system energetically (See Chapter 2, Table 1.). The calcium pump of the sarcoplasmic reticulum would require energy if the ions were continually being pumped out to lead to a state of maintained tonic contraction. It is therefore interesting to note that contraction at low calcium levels would also result in energy-saving for this muscle which is in keeping with its energetically efficient contractile system.

CHAPTER 7

DISCUSSION

A training in academic biology is intended to produce a critical, objective attitude, which should enable the biologist to analyse and study any problem and ascertain a clearer understanding quantitatively and qualitatively of the processes that build up a complicated living animal or tissue. In studying muscle function and control many approaches have been explored to clarify the specific details of the contractile process. Indeed, studies on the ultrastructure and mechanics of the individual muscle fibres have led to exciting hypotheses of the mechanism of contraction which appears to be basically similar for many species. Evolutionary based differences however have been identified with regard to the muscle regulatory proteins and these differences are most dramatic between phylogenetically distant species.

The different research areas sometimes appear to be too isolated with insufficient interaction between the various specialists. All the approaches are independently valid but if they could be integrated perhaps a more

accurate appreciation of the static or dynamic state of the muscle would emerge.

The work in this thesis has endeavoured to combine several biological techniques, histochemistry, biochemistry and mechanics in a study on two functionally different muscles. A further aim of the work was to study the development ex ovo of these two muscles.

The development of muscles and their individual fibres is complex and will vary from muscle to muscle within a species and also between species. The rôle of innervation in the eventual adult characteristics of the muscle fibres is in some dispute and although the importance of the rôle of the nerve in the triggering of muscle contraction is unequivocal; whether it is in overall control of the differentiation and development of the muscle fibres is not clear. The ALD muscle is thought to mature earlier than the PLD. The first signs of innervation of the two muscles are seen between the 12th and 14th day of incubation. (Gordon, Perry, Tuffery and Vrbová, 1974). However the initial development of enzymatic activities, ultra-structure, and amount of contractile proteins and ribosomes is more rapid in the ALD than the PLD. (Gordon, Perry, Srihari, and Vrbová, 1977; Gutmann, Hanzlíková and Holečková, 1969; Gordon, Perry, Spurway and Vrbová, 1975). The studies by Vrbová and colleagues have dealt with the embryonic growth of these muscles. The present study has addressed the developmental stages of these muscles that occur post-hatching until early adulthood.

The histochemical results shown in this study also display differences in the rate of development of the ALD and PLD muscles. At 3 days of age the ALD appears to be completely developed in terms of the two fibre types observed, both showing the same activity of phosphorylase and Myosin ATPase as in the later age groups. The PLD, however, exhibited a few small and

possibly foetal fibres during the first two weeks of ex ovo growth. These fibres were active for the Myosin ATPase stain over a wide range of preincubation pH. A histochemical feature of foetal fibres is that they cannot be differentiated by reciprocal acid and alkali Myosin ATPase preincubations. (Guth and Samaha, 1972).

The literature has generally reported the ALD as a pure slow tonic muscle and the PLD as a pure fast phasic muscle. The ALD has been shown histochemically to possess two main fibre types which are independent in their characteristics from the slow or fast phasic muscle fibres exhibited in other avian and mammalian muscles. They did not show reciprocality with the traditional Myosin ATPase stain but did stain at an acid preincubation with a division into two types. These two types were also observed when the muscle was stained for phosphorylase activity. It is concluded therefore, that tonic muscle fibres have a different Myosin ATPase activity from phasic muscle fibres and the ALD can be considered to have two types of tonic fibres both when compared from a contractile and metabolic viewpoint. The ALD muscle did not appear to be as active glycolytically as the PLD and this was reflected by the higher activity of phosphorylase exhibited in the PLD fibres' darker staining.

The fast phasic PLD showed a uniform staining after the traditional Myosin ATPase stain with little or no activity following high acid preincubations and high activity after the alkali preincubation. "Transient" fibre types were shown when the muscle was preincubated at pH 4.6. Several fibre types are shown when the muscle was stained for phosphorylase indicating the existence of metabolically different fibres within the muscle.

The histochemical analysis of the two muscles proved to be more complex than anticipated. The muscles were histochemically very different but

parallels have been drawn between mammalian phasic muscles and the fast phasic PLD muscle. It was difficult to draw many hard and fast conclusions from the histochemical results. Histochemically the ALD would appear to be quite distinct from both the PLD and phasic mammalian muscles, both fast or slow. The PLD was more similar to these mammalian muscles, but the absence of cross-correlation between the fibre types exhibited with the metabolic enzyme stain and the contractile enzyme stain indicates that they were not completely homologous.

Enzymatic assays of the Mg^{2+}-activated myofibrillar ATPase and phosphorylase activities were developed and carried out on the ALD and PLD for the same age groups as those for the histochemistry and histology. These biochemical assays require the use of the whole muscle and will therefore mask any individual fibre differences in activity. They are however more quantitative in assessing an actual activity of individual enzymes.

The activity of the contractile marker enzymes e.g. Myosin, Actomyosin or Myofibrillar ATPase have been proposed to be directly proportional in activity to the actual speed of contraction of the individual muscles. (Bárány, 1967). The PLD muscle is known to reach maximum tetanic tension 10 times faster and relax 8 times faster than the ALD muscle (Canfield, 1971). The PLD therefore was expected to show an overall greater activity for Mg^{2+}-activated myofibrillar ATPase than the ALD. At the end, age point of the study the PLD showed 4 times the Mg^{2+}-activated myofibrillar ATPase activity than for the ALD. Reasons and Hikida, (1973), however, showed only a 1.4 times difference between the PLD and ALD for the activity of actomyosin ATPase. This emphasises the need to obtain enzyme activities that represent the <u>in vivo</u> state most accurately. Myofibrillar ATPase activity may represent more accurately the <u>in vivo</u> state than isolated myosin or actomyosin.

The ALD and PLD muscle showed a similar, initial Mg^{2+}-activated myofibrillar ATPase activity. The ALD then showed a decrease in activity with development and the PLD an increase. On hatching both the ALD and PLD muscle contract slowly with the PLD showing an increase in contraction speed with age and the ALD remaining slow. (Shear and Goldspink, 1971). The actual activity of the Mg^{2+}-activated myofibrillar ATPase at 3 days age may not be totally reflective of the muscles' speed of shortening. The development of the associated systems, sarcoplasmic reticulum and regulatory proteins, may not be complete and therefore both muscles might only be able to contract slowly. The myofibrillar ATPase activity may not be the rate-limiting step at this stage; this may lie elsewhere in the system, certainly large differences in the Ca^{2+} sensitivity of the ALD fibres as compared with other types of fibres were revealed in the single skinned fibre studies. This indicates that the activation and contractile systems in the ALD may be quite different from most types of muscle.

Both muscles exhibited a peak in Mg^{2+}-activated myofibrillar ATPase activity at 15 days of age. The peak was more marked in the PLD than the ALD. This peak implies a change in the overall functional state of the muscle to require it to have a high activity during this growth period. It also points to the plasticity of all the enzymes in the muscle. Metabolic enzymes that are required to be switched on and off to meet spasmodic energy demands would be expected to fluctuate in activity but perhaps not the contractile enzymes whose function might be considered to be more constant.

Such a metabolic enzyme is phosphorylase which is known to occur in two forms a and b and be part of a larger enzyme cascade system on which the normal balance of breakdown or storage of glycogen depends. (Cori and

Cori, 1943; Cori and Green, 1943). The total activity of this enzyme reflects the glycolytic capacity of an organ or tissue and the enzyme assay has been used to determine both pathological and developmental states. The total activity of this enzyme (a + b) was also found to increase in both the ALD and PLD muscles during ex ovo development. At the end age point the PLD showed 3 times the activity of total phosphorylase than the ALD. Both muscles showed lower activities at 3 days than at 51 days. The PLD muscle, however always showed a higher activity of this enzyme reflecting its physiologically faster and higher anaerobic demands. The activity of phosphorylase also showed a peak around 15 days of age for both muscles, but the peak was not as dramatic as that exhibited by the Mg^{2+}-activated myofibrillar ATPase for the same age.

Immobilisation, which considerably reduces the activity of the muscle without damaging the nerve supply was also employed for various time periods over this first 50 day developmental study. This inactivity imposed on the muscles was intended to show the effects shown by developing muscle when its normal contractile function is prevented. The muscles of the experimental right-hand side and contralateral left-hand side were analysed for the activities of phosphorylase and Myosin ATPase histochemically and phosphorylase and Mg^{2+}-activated myofibrillar ATPase biochemically. These results were compared with the non-immobilised developmental control groups. Immobilisation, in the two different positions, "shortened" and "resting" resulted in enzymatic changes in both the right-handed experimental and left-handed contralateral muscles. The most dramatic changes were showed when the muscles were in the shortened position. It was also found that the contralaterals hypertrophied and exhibited enzyme changes and this stressed the need to use separate control groups for comparison when this and similar unilateral physiological constraints are studied.

The ALD and PLD muscle showed a reduction in both the stain intesities of Myosin ATPase and Phosphorylase activity when examined histochemically for both positions of immobilisation. The qualitative results of the histochemical studies led to the enzymatic assays for the two enzymes being employed for a closer analysis of the changes in the enzyme activities than appeared qualitatively. It was found that immobilisation caused a delay in the normal pattern of activities of the two enzymes. After the shorter time periods of inactivity the muscles showed a reduction in activity. Higher activities however were shown even after longer periods of immobilised inactivity. The peaks exhibited at 15 days in the normally developing muscles were displaced to a later age. Contractile activity is therefore important for the normal muscle growth patterns and activities of both metabolic and contractile enzymes. This study also shows the importance of muscle activity alone without association with the nerve supply. The actual use of the muscle has some direct effect on the growth of these muscles and the activities of their enzymes. The embryological development of the ALD and PLD may be almost complete at hatching, but normal growth was dependent on contractile activity. This study indicates that these muscles during this first 50 day period are very dynamic and could adapt to new physiological situations, although this might involve a time delay in development.

The considerations described above pose many questions that could lead to further experimental analysis. What is the balance of the regulatory proteins during the developmental peak? Do the light chain ratios vary? Can these developmental changes be altered by artificial changes in the nervous activities without immobilisation? Could artificial normal nervous activation of the immobilised muscles prevent the delay in the growth patterns? Pilot experiments using artificial stimulation of adult ALD muscles with a direct phasic stimulus after dennervation showed some

fibre changes, revealed by the Myosin ATPase stain. Despite alot of damaged fibres the stain showed a proportion of fibres with no stain at low acid preincubations of pH 4.3, possibly indicating phasic fast fibres. In conclusion the study on the deveopment of the ALD and PLD muscle with imposed immobilisation emphasised the plasticity of both muscles and the importance of normal contractile activity to produce normal growth patterns.

Hormonal control of muscle development and growth is an exciting new area and as new methods for hormone assays are being developed a more accurate description of the various hormonal states of an animal can now be reported. In the experiment as described in Chapter 5, chickens were thyroidectomized from 6 weeks of age for 4 weeks. This length of absence of thyroid hormone causes gross changes in the chickens and analysis of the activities of Mg^{2+}-activated myofibrillar ATPase and phosphorylase of three muscles (ALD, PLD and ST) both histochemically and biochemically displayed interesting changes in enzyme activities. It is difficult to state whether these changes were as a direct result of the absence of thyroid hormones or as secondary effects from other bodily changes shown by the absence of thyroid hormones (e.g. the overall weight reduction). There was however a more marked change in activity of the contractile enzyme marker, Mg^{2+}-activated myofibrillar ATPase than the metabolic enzyme phosphorylase. The phasic muscles, the PLD and ST, showed the largest reductions in enzyme activity in comparison with the tonic ALD which was only slightly affected. Thyroidectomy therefore has differential effects on tonic and phasic muscles and this in in agreement with other studies on hypothyroidism and its influence on the growth of fast and slow muscles. (Nicol and Johnston, 1981; Nwoye et al., 1982). Hormonal control on muscle growth is therefore of more importance than once appreciated. The

differential effects exhibited in different muscle types by the absence of the thyroid further complicates the understanding of muscle control and growth.

The balance of hormones, contractile function and nervous stimuli are all important to the control processes for growth and development of muscles. One process alone cannot be totally responsible for all the development and differentiation of the muscle fibres. The studies reported in this volume demonstrate the complexity of these effects and stresses the danger of over-simplification in any classification of the control of growth in a tissue such as muscle. The use of two different muscles with tonic and phasic fibres gives a complete spectrum of the types of effects possible and hopefully a clearer understanding of the complexity.

This thesis, also includes a separate analysis of skinned single fibres from adult ALD and PLD muscles and the difference in the response of the muscles measured in isometric tension values to free calcium concentration. The results showed that the slow tonic ALD muscle will become activated for contraction at very low free calcium concentrations. This is in agreement with its functional physiological role to be contracted for long periods. Relaxation of the muscle could only occur when practically no calcium was present. The PLD however was activated at higher free calcium and shows an activation similar to other phasic muscles. The PLD contracts rapidly and remains contracted for brief periods. It therefore needs a higher threshold so that it can remain relaxed despite some surrounding calcium. This study also gave information on the possible number of calcium binding sites (n) for each muscle. The ALD muscle appears to have only half the number of sites of the PLD. (1.5 to 3.0 respectively). The actual site for calcium binding in skeletal, striated muscle is thought to be on the thin-filaments via the troponin. This is thought to have up

to 4 possible calcium binding sites. Regulation of contraction via calcium has also been linked with the DTNB light chains on the myosin heads of the thick filaments. In this case there are two Ca^{2+} binding sites. It is suggested that perhaps the ALD is controlled via the DTNB light chains and the PLD via troponin. This suggestion is mere speculation but it opens up the possibility that both control processes could exist within a species and be used for different muscle types.

The work in this thesis has spanned several research areas and has drawn together ideas to look at the development of two chicken muscles. The muscles chosen the latissimus dorsii, provided a good model to study differences between the fast phasic twitch fibres and the slow tonic fibres from one species. The results have indicated a greater complexity and plasticity in the physiology and development of the two muscles than perhaps anticipated. However, enzyme activity analysis using several techniques has elucidated some definite differences between the two muscles both during development and when the muscle has matured. The use of parallel techniques has also displayed many of the problems of combining analyses across research areas. It is felt however that this is the better form of analysis for studying developmental muscle growth.

SUMMARY

1. The ALD and PLD muscles from the chicken were studied throughout ex ovo development from 3 days of age until early adulthood. These two muscles provided a model for studying phasic fast fibres and tonic slow fibres.

2. Development over the first 30 days was compared in control groups and groups immobilised in two positions, "resting" and shortened. Analysis included histochemically staining for Myosin ATPase, Phosphorylase, Succinic Dehydrogenase and histological staining for nuclei/cytoplasm and nerve endings. The ALD was shown to have 2 tonic fibre types described by both contractile and metabolic enzyme markers. The PLD was shown to have different fibre types when stained for phosphorylase activity. These types were not observed with the traditional Myosin ATPase stain. The PLD also exhibited a few, possibly foetal fibres during the first two weeks of ex ovo growth. Immobilisation in both positions caused the histochemically displayed activities of these enzymes to become reduced.

3. Biochemically assays for Mg^{2+}-activated myofibrillar ATPase (both a alone and total activity a + b) activity were developed for chicken muscle. These assays were used to study the changes in the ALD and PLD muscles across development and with immobilisation in both positions. Both enzyme's activities increased during normal development with peaks in activity shown at 15 days. The PLD was shown to have 4 times the Mg^{2+}-activated myofibrillar ATPase activity than the ALD at 51 days age. For the same age, total phosphorylase activity was 3 times higher in the PLD muscles than the ALD muscles. The higher activities

exhibited by the fast-phasic PLD muscle over the slow-tonic ALD muscle is in agreement with the theory that the PLD is a faster contracting,highly anaerobic,muscle and the ALD a slow contracting, aerobic muscle. Immobilisation in both positions showed an initial reduction in both enzyme's activities followed by a recovery despite longer periods of immobilisation. The growth peaks exhibited by these enzymes appeared to be displaced in time when inactivity through immobilisation was imposed.

4. Chickens were thyroidectomized from 6 weeks age for 4 weeks and the ALD, PLD and ST muscles were analysed for Mg^{2+}-activated myofibrillar ATPase and phosphorylase (a and a + b) activity. Sham-operated birds acted as controls. The muscles were also stained histochemically for Myosin ATPase and phosphorylase. Thyroidectomy caused a differential effect in the PLD and ST with the ALD in terms of changes in these enzymes' activities. The PLD and ST showed a significant reduction in activity of both main enzymes (phosphorylase a activity was unchanged). The ALD however, exhibited no change in activity of the two main enzymes. In conclusion the reduced presence of thyroid hormone slows down fast-phasic chicken muscles but has little effect on slow-tonic chicken muscles.

5. A study was made on the pCa-tension relationship for skinned single fibres from the ALD and PLD muscles from 9 week old chickens. The threshold for calcium activation of contraction was found to at a pCa of 7.5 for the ALD and of 6.63 for the PLD. The ALD showed a minimum calcium binding site number of 2 and the PLD of 3. The maximum isometric tension observed was approximately 8.3 Ncm^{-2} for

both muscles. The ALD therefore will contract at extremely low free calcium levels whereas the PLD needs almost 0.2 μM higher free calcium to contract. Possible explanations for this difference and the differences in physiology and function of the two muscles are presented.

ACKNOWLEDGEMENTS

First, I should like to thank Professor Geoff Goldspink for his kindness and supervision over the last three years. I must also thank all the members of the Muscle Research Unit for their friendship and helpful interest in many of the problems that occurred throughout this piece of work.

Special thanks go to Pam Williams for her complete support and help in reading preliminary drafts.

I am also indebted to Roland Wheeler-Osman for his help with photographs and graphics.

For personal encouragement and the painstaking typing of this manuscript I should like to say "Vielen Dank" to Denise Wyatt.

Acknowledgement and gratitude go to the SERC for awarding me travel money to work with Dr. Ian Johnston at St. Andrew's University; whose time and help was extremely valuable for the single fibre mechanics study.

For collaboration in the thyroid experiments thanks to Dr. Steve Harvey and Dr. Hillar Klandorf of the Wolfson Institute, Hull University.

Finally, I must thank Andy for his advice, help and friendship throughout.

This work was funded by a Ph.D. grant awarded by the SERC.

APPENDIX A

Raw Data Means and Statistical Results for Chapter 2

Chicken Body Weight - CONTROLS

Age in days	3	8	15	22	29	37	40	43	46
Mean body weight in grammes	42.6	72.6	152.7	220.5	343.8	478.9	541.5	570.2	632.7
Standard Error ±	0.566	1.243	3.72	6.85	10.81	9.78	10.27	11.24	11.72

Age in days	51	54	61	81	90	94	102	109	116
Mean body weight in grammes	766.42	848.3	1018.9	1550	1895	2006	2168.5	2426	2665.3
Standard Error ±	15.61	44.69	50.55	130.7	122.36	123.35	137.52	155.62	158.43

Statistical Test for Linearity of Growth Curve (All 120 Days)

Pearson's R	Slope B (YonX)	Intercept A (X=0)	ETA	F	DF
0.9686	22.7139	- 266.393	0.9825	12.4251	9,143
				p ≤ 0.001	
				not linear	
First 50 days:					
0.9789	14.9091	- 56.8395	0.9755	-2.5230	6,112
				ns	
				linear	
51-120 days:					
0.9467	29.1403	- 752.38	0.9127	-5.6756	2,30
				ns	
				linear	

APPENDIX A

Statistical comparisons of the weights and lengths of the ALD and PLD right and left-hand muscles for the controls using:

Two way anlaysis of variance tests

1. Left vs. Right Comparison of ALD Control Muscle Weights

CELL MEANS: FOR 12 ANIMALS/CELL

Age	(days)	Left ALD (B1)	Right ALD (B2)
(A1)	3	.0213333	.0195500
(A2)	8	.0289833	.0289167
(A3)	15	.0725000	.0743417
(A4)	22	.0982500	.1034167
(A5)	29	.1587500	.1530833
(A6)	51	.3916667	.3954167

SOURCE	SS	DF	MS	F
BETW SUBJS	2.371522	71		
A	2.313216	5	.4626431	523.6933
SUBJ W GPS	.0583060	66	.0008834	
WITHN SUBJ	.0086051	72		
B	.0000105	1	.0000105	.0852918
AB	.0004662	5	.0000932	.7570333
B SWG	.0081284	66	.0001232	
A AT B 1	1.147333	5	.2294665	455.9320
A AT B 2	1.1666349	5	.2332699	463.4890
SSWCELL	.0664344	132	.0005033	
B AT A 1	.0000191	1	.0000191	.1549360
B AT A 2	.0000000	1	.0000000	.0002153
B AT A 3	.0000204	1	.0000204	.1652401
B AT A 4	.0001602	1	.0001602	1.300471
B AT A 5	.0001927	1	.0001927	1.564381
B AT A 6	.0000844	1	.0000844	.6851777
SSBSWG	.0081284	66	.0001232	
F MAX SWG	169.4646			
F MAX BSWG	162.6388			

APPENDIX A

2. Left vs. Right Comparison of ALD Control Muscle Lengths

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Left ALD (B1)	Right ALD (B2)
(A1)	3	.7925833	.7480000
(A2)	8	1.048833	1.033167
(A3)	15	1.366250	1.365250
(A4)	22	2.166667	2.141667
(A5)	29	2.429167	2.358333
(A6)	51	3.266667	3.200000

SOURCE	SS	DF	MS	F
BETW SUBJS	107.0372	71		
A	104.4767	5	20.89533	538.5868
SUBJ W GPS	2.560575	66	.0387966	
WITHN SUBJ	.4334409	72		
B	.0500591	1	.0500591	9.189819
AB	.0238643	5	.0047729	.8761980
B SWG	.3595173	66	.0054472	
A AT B 1	53.23824	5	10.64765	481.3168
A AT B 2	51.26229	5	10.25246	463.4527
SSWCELL	2.920092	132	.0221219	
B AT A 1	.0119258	1	.0119258	2.189335
B AT A 2	.0014727	1	.0014727	.2703596
B AT A 3	.0000058	1	.0000058	.0010614
B AT A 4	.0037506	1	.0037506	.6885263
B AT A 5	.0301031	1	.0301031	5.526309
B AT A 6	.0266628	1	.0266628	4.894747
SSBSWG	.359173	66	.0054472	
F MAX SWG	12.34617			
F MAX BSWG	48.91643			

APPENDIX A

3. Left vs. Right Comparison of PLD Control Muscle Weights

CELL MEANS: FOR 12 ANIMALS/CELL

Age (Days)		Left PLD (B1)	Right PLD (B2)
(A1)	3	.0154167	.0112017
(A2)	8	.0330250	.0308583
(A3)	15	.0928417	.0867917
(A4)	22	.1201667	.1240833
(A5)	29	.1993750	.2055000
(A6)	51	.4483333	.4482500

SOURCE	SS	DF	MS	F
BETW SUBJS	3.150561	71		
A	3.089721	5	.6179441	670.3532
SUBJ W GPS	.0608400	66	.0009218	
WITHN SUBJ	.0085120	72		
B	.0000062	1	.0000062	.0524091
AB	.0006653	5	.0001331	1.120137
B SWG	.0078404	66	.0001188	
A AT B 1	1.528555	5	.3057111	587.5597
A AT B 2	1.561831	5	.3123661	600.3503
SSWCELL	.0686804	132	.0005203	
B AT A 1	.0001066	1	.0001066	.8973280
B AT A 2	.0000282	1	.0000282	.2371015
B AT A 3	.0002196	1	.0002196	1.848699
B AT A 4	.0000920	1	.0000920	.7747996
B AT A 5	.0002251	1	.0002251	1.894699
B AT A 6	.0000000	1	.0000000	.0004077
SSBSWG	.0078404	66	.0001188	
F MAX SWG	498.9276			
F MAX BSWG	63.47025			

APPENDIX A

4. Left vs. Right Comparison of PLD Control Muscle Lengths

CELL MEANS: FOR 12 ANIMALS/CELL

Age (Days)		Left PLD (B1)	Right PLD (B2)
(A1)	3	1.151083	1.111750
(A2)	8	1.376833	1.356167
(A3)	15	1.809167	1.836000
(A4)	22	2.383333	2.358333
(A5)	29	2.749167	2.720000
(A6)	51	3.608333	3.550000

SOURCE	SS	DF	MS	F
BETW SUBJS	102.4335	71		
A	100.5545	5	20.11090	706.3878
SUBJ W GPS	1.879023	66	.0284701	
WITHN SUBJ	.4482191	72		
B	.0212834	1	.0212834	3.487444
AB	.0241463	5	.0048293	.7913113
B SWG	.4027889	66	.0061029	
A AT B 1	50.96033	5	10.19207	589.5984
A AT B 2	49.61831	5	9.923663	574.0715
SSWCELL	2.281812	132	.0172865	
B AT A 1	.0092824	1	.0092824	1.520983
B AT A 2	.0025617	1	.0025617	.4197618
B AT A 3	.0043193	1	.0043193	.7077516
B AT A 4	.0037488	1	.0037488	.6142650
B AT A 5	.0051024	1	.0051024	.8360661
B AT A 6	.0204172	1	.0204172	3.345515
SSBSWG	.4027889	66	.0061029	
F MAX SWG	3.851544			
F MAX BSWG	52.25838			

APPENDIX A

Statistical comparisons of the chicken body weights of the controls and experimentals (immobilised in the resting and shortened positions) using:

Two way analysis of variance tests - (ANOVAS)

1. A comparison of the body weights of chickens immobilised in the resting position with the controls

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Experimentals (B1)	Controls (B2)
(A1)	8	88.64750	72.58333
(A2)	15	168.0833	152.7208
(A3)	22	278.1083	220.4750

SOURCE	SS	DF	MS	F
A	341751.7	2	170875.8	620.5700
B	15863.47	1	15863.47	57.61137
AB	7030.514	2	3515.257	12.76636
WITHN CELL	18173.30	66	275.3531	
TOTAL	382819.0	71		
A AT B 1	217243.8	2	108621.9	394.4823
A AT B 2	131538.4	2	65769.18	238.8540
SSWCELL	18173.30	66	275.3531	
B AT A 1	1548.344	1	1548.344	5.623123
B AT A 2	1416.039	1	1416.039	5.142631
B AT A 3	19929.60	1	19929.60	72.37833
SSWCELL	18173.30	66	275.3531	
F MAX	30.38758			

APPENDIX A

2. A comparison of the body weights of the chickens immobilised in the shortened position with the controls

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Experimentals (B1)	Controls (B2)
(A1)	8	87.23333	72.58333
(A2)	15	162.0833	152.7208
(A3)	22	248.1667	220.4750

SOURCE	SS	DF	MS	F
A	286120.0	2	143060.0	520.0842
B	5346.689	1	5346.689	19.43750
AB	1067.935	2	533.9675	1.941200
WITHN CELL	18154.68	66	275.0709	
TOTAL	310689.3	71		

A AT B 1	155649.6	2	77824.80	282.9264
A AT B 2	131538.4	2	65769.18	239.0991
SSWCELL	18154.68	66	275.0709	
B AT A 1	1287.734	1	1287.734	4.681464
B AT A 2	525.9346	1	525.9346	1.911997
B AT A 3	4600.968	1	4600.968	16.72648
SSWCELL	18154.68	66	275.0709	

F MAX 30.38758

APPENDIX A

3. One way analysis of variance test comparing the body weights of the controls at 29 days age with those chickens immobilised in the shortened position for 20 days plus 7 days recovery.

GROUP		MEAN	SS	VARIANCE
CONTROLS	1	343.8667	15419.75	1401.795
EXPERIS	2	369.2917	3468.797	315.3452

12 ANIMALS

SOURCE	SS	DF	MS	F
TREATMENTS	3878.531	1	3878.531	4.517426
ERROR	18888.56	22	858.5710	
TOTAL	22767.09	23		

APPENDIX A

Statistical comparisons of the weights and lengths of the ALD and PLD muscles, controls and experimentals (immobilised in the resting and shortened positions) using:

Two way analysis of variance tests - (ANOVAS)

1. A comparison of the experimental ALD muscle weights for immobilisation in the resting position with the controls (right-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Experimentals (B1)	Controls (B2)
(A1)	8	.0269917	.0289167
(A2)	15	.0732333	.0743417
(A3)	22	.1276667	.1031667

SOURCE	SS	DF	MS	F
A	.0918670	2	.0459335	172.4250
B	.0009216	1	.0009216	3.459609
AB	.0027095	2	.0013547	5.085403
WITHN CELL	.0175822	66	.0002664	
TOTAL	.1130803	71		
A AT B 1	.0609469	2	.0304735	114.3912
A AT B 2	.0336295	2	.0168147	63.11915
SSWCELL	.0175822	66	.0002664	
B AT A 1	.0000222	1	.0000222	.0834604
B AT A 2	.0000074	1	.0000074	.0276691
B AT A 3	.0036015	1	.0036015	13.51928
SSWCELL	.0175822	66	.0002664	
F MAX	19.32005			

APPENDIX A

2. A comparison of the experimental ALD muscle lengths for immobilisation in the resting position with the controls (right-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Experimentals (B1)	Controls (B2)
(A1)	8	.9868333	1.033167
(A2)	15	1.354000	1.365250
(A3)	22	1.960833	2.141667

SOURCE	SS	DF	MS	F
A	13.47828	2	6.739141	422.3103
B	.1136922	1	.1136922	7.124558
AB	.0961517	2	.0480759	3.012688
WITHN CELL	1.053214	66	.0159578	
TOTAL	14.74134	71		
A AT B 1	5.806935	2	2.903468	181.9467
A AT B 2	7.767499	2	3.883749	243.3763
SSWCELL	1.053214	66	.0159578	
B AT A 1	.0128804	1	.0128804	.8071551
B AT A 2	.0007585	1	.0007585	.0475334
B AT A 3	.1962042	1	.1962042	12.29520
SSWCELL	1.053214	66	.0159578	
F MAX	13.79438			

APPENDIX A

3. A comparison of the experimental PLD muscle weights for immobilisation in the resting position with the controls (right-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Experimentals (B1)	Controls (B2)
(A1)	8	.0337250	.0313250
(A2)	15	.0792583	.0867917
(A3)	22	.1340250	.1240833

SOURCE	SS	DF	MS	F
A	.1118945	2	.0559473	354.8158
B	.0000462	1	.0000462	.2932493
AB	.0009218	2	.0004609	2.923170
WITHN CELL	.0104069	66	.0001577	
TOTAL	.1232695	71		
A AT B 1	.0605310	2	.0302655	191.9430
A AT B 2	.0522853	2	.0261427	165.7959
SSWCELL	.0104069	66	.0001577	
B AT A 1	.0000346	1	.0000346	.2191769
B AT A 2	.0003405	1	.0003405	2.159478
B AT A 3	.0005930	1	.0005930	3.760913
SSWCELL	.0104069	66	.0001577	
F MAX	20.12814			

APPENDIX A

4. A comparison of the experimental PLD muscle lengths for immobilisation in the resting position with the controls (right-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Experimentals (B1)	Controls (B2)
(A1)	8	1.364417	1.356167
(A2)	15	1.789583	1.836000
(A3)	22	2.409167	2.358333

SOURCE	SS	DF	MS	F
A	12.62573	2	6.312865	465.4428
B	.0003148	1	.0003148	.0232123
AB	.0285193	2	.0142596	1.051352
WITHN CELL	13.54973	71		
TOTAL				
A AT B 1	6.624609	2	3.312305	244.2138
A AT B 2	6.029641	2	3.014821	222.2805
SSWCELL	.8951670	66	.0135631	
B AT A 1	.0004082	1	.0004082	.0300987
B AT A 2	.0129260	1	.0129260	.9530231
B AT A 3	.0155027	1	.0155027	1.143002
SSWCELL	.8951670	66	.0135631	
F MAX	3.204772			

APPENDIX A

5. A comparison of the experimental ALD muscle weights for immobilisation in the shortened position with the controls (right-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Experimentals (B1)	Controls (B2)
(A1)	8	.0286250	.0289167
(A2)	15	.0709375	.0743417
(A3)	22	.1081417	.1034167

SOURCE	SS	DF	MS	F
A	.0716239	2	.0358119	284.4599
B	.0000021	1	.0000021	.0167621
AB	.0002019	2	.0001009	.8017897
WITHN CELL	.0083090	66	.0001259	
TOTAL	.0801369	71		
A AT B 1	.0379896	2	.0189948	150.8787
A AT B 2	.0338361	2	.0169181	134.3830
SSWCELL	.0083090	66	.0001259	
B AT A 1	.0000005	1	.0000005	.0040546
B AT A 2	.0000695	1	.0000695	.5522851
B AT A 3	.0001340	1	.0001340	1.064027
SSWCELL	.0083090	66	.0001259	
F MAX	26.47433			

APPENDIX A

6. A comparison of the experimental ALD muscle lengths for immobilisation in the shortened position with the controls (right-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Experimentals (B1)	Controls (B2)
(A1)	8	.9221667	1.033167
(A2)	15	1.301750	1.365250
(A3)	22	1.891667	2.141667

SOURCE	SS	DF	MS	F
A	13.38285	2	6.691425	449.5649
B	.3604092	1	.3604092	24.21417
AB	.1127099	2	.0563549	3.786219
WITHN CELL	.9823589	66	.0148842	
TOTAL	14.83833	71		
A AT B 1	5.728062	2	2.864031	192.4206
A AT B 2	7.767499	2	3.883749	260.9306
SSWCELL	.9823589	66	.0148842	
B AT A 1	.0739259	1	.0739259	4.966725
B AT A 2	.0241934	1	.0241934	1.625439
B AT A 3	.3750001	1	.3750001	25.19447
SSWCELL	.9823589	66	.0148842	
F MAX	13.79438			

APPENDIX A

7. A comparison of the experimental PLD muscle weights for immobilisation in the shortened position with the controls (right-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Experimentals (B1)	Controls (B2)
(A1)	8	.0359583	.0313250
(A2)	15	.0821667	.0867917
(A3)	22	.1250550	.1240833

SOURCE	SS	DF	MS	F
A	.0996758	2	.0498379	383.0255
B	.0000019	1	.0000019	.0149004
AB	.0002609	2	.0001304	1.002479
WITHN CELL	.0085877	66	.0001301	
TOTAL	.1085263	71		
A AT B 1	.0476513	2	.0238257	183.1105
A AT B 2	.0522853	2	.0261427	200.9175
SSWCELL	.0085877	66	.0001301	
B AT A 1	.0001288	1	.0001288	.9899340
B AT A 2	.0001283	1	.0001283	.9863620
B AT A 3	.0000057	1	.0000057	.0435254
SSWCELL	.0085877	66	.0001301	
F MAX.	20.12811			

APPENDIX A

8. A comparison of the experimental PLD muscle lengths for immobilisation in the shortened position with the controls (right-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Experimentals (B1)	Controls (B2)
(A1)	8	1.393250	1.356167
(A2)	15	1.800083	1.836000
(A3)	22	2.456667	2.365833

SOURCE	SS	DF	MS	F
A	12.98288	2	6.491438	574.3949
B	.0169300	1	.0169300	1.498050
AB	.0485631	2	.0242816	2.148554
WITHN CELL	.7458891	66	.0113013	
TOTAL	13.79426	71		
A AT B 1	6.909878	2	3.454939	305.7103
A AT B 2	6.121562	2	3.060781	270.8332
SSWCELL	.7458891	66	.0113013	
B AT A 1	.0082507	1	.0082507	.7300644
B AT A 2	.0077397	1	.0077397	.6848494
B AT A 3	.0495038	1	.0495038	4.380344
SSWCELL	.7458891	66	.0113013	
F MAX	3.564998			

APPENDIX A

9. A comparison of the contralateral ALD muscle weights from immobilisation in the resting position with the controls (left-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Contralaterals (B1)	Controls (B2)
(A1)	8	.0321750	.0289833
(A2)	15	.0785083	.0724167
(A3)	22	.1381667	.0982500

SOURCE	SS	DF	MS	F
A	.0921647	2	.0460824	252.1559
B	.0048413	1	.0048413	26.49074
AB	.0050025	2	.0025013	13.68658
WITHN CELL	.0120617	66	.0001828	
TOTAL	.1140703	71		
A AT B 1	.0677605	2	.0338803	185.3878
A AT B 2	.0294067	2	.0147034	80.45472
SSWCELL	.0120617	66	.0001828	
B AT A 1	.0000611	1	.0000611	.3344402
B AT A 2	.0002226	1	.0002226	1.218308
B AT A 3	.0095600	1	.0095600	52.31114
SSWCELL	.0120617	66	.0001828	
F MAX	29.73220			

APPENDIX A

10. A comparison of the contralateral ALD muscle lengths from immobilisation in the resting position with the controls (left-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Contralaterals (B1)	Controls (B2)
(A1)	8	1.043250	1.048833
(A2)	15	1.430000	1.366250
(A3)	22	2.195833	2.166667

SOURCE	SS	DF	MS	F
A	16.20755	2	8.103777	510.4519
B	.0152480	1	.0152480	.9604621
AB	.0144302	2	.0072151	.4544755
WITHN CELL	1.047796	66	.0158757	
TOTAL	17.28503	71		
A AT B 1	8.258097	2	4.129049	260.0862
A AT B 2	7.963886	2	3.981943	250.8201
SSWCELL	1.047796	66	.0158757	
B AT A 1	.0001870	1	.0001870	.0117796
B AT A 2	.0243843	1	.0243843	1.535953
B AT A 3	.0051042	1	.0051042	.3215094
SSWCELL	1.047796	66	.0158757	
F MAX	12.93855			

APPENDIX A

11. A comparison of the contralateral PLD muscle weights from immobilisation in the resting position with the controls (left-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Contralaterals(B1)	Controls (B2)
(A1)	8	.0415833	.0330250
(A2)	15	.0970167	.0928417
(A3)	22	.1625000	.1201667

SOURCE	SS	DF	MS	F
A	.1303684	2	.0651842	542.4558
B	.0060647	1	.0060647	50.46952
AB	.0052320	2	.0026160	21.77026
WITHN CELL	.0079309	66	.0001202	
TOTAL	.1495960	71		
A AT B 1	.0879270	2	.0439635	365.8595
A AT B 2	.0476734	2	.0238367	198.3665
SSWCELL	.0079309	66	.0001202	
B AT A 1	.0004395	1	.0004395	3.657222
B AT A 2	.0001046	1	.0001046	.8702979
B AT A 3	.0107527	1	.0107527	89.48250
SSWCELL	.0079309	66	.0001202	
F MAX	22.85015			

APPENDIX A

12. A comparison of the contralateral PLD muscle lengths from immobilisation in the resting position with the controls (left-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Contralaterals(B1)	Controls (B2)
(A1)	8	1.441000	1.376917
(A2)	15	1.942833	1.809167
(A3)	22	2.525000	2.383333

SOURCE	SS	DF	MS	F
A	13.15893	2	6.579466	429.6674
B	.2304200	1	.2304200	15.04742
AB	.0218357	2	.0109178	.7129821
WITHN CELL	1.010653	66	.0153129	
TOTAL	14.42184	71		
A AT B 1	7.063240	2	3.531620	230.6300
A AT B 2	6.117528	2	3.058764	199.7505
SSWCELL	1.010653	66	.0153129	
B AT A 1	.0246402	1	.0246402	1.609111
B AT A 2	.1071998	1	.1071998	7.000608
B AT A 3	.1204165	1	.1204165	7.863717
SSWCELL	1.010653	66	.0153129	
F MAX	2.047674			

APPENDIX A

13. A comparison of the contralateral ALD muscle weights from immobilisation in the shortened position with the controls (left-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Contralaterals (B1)	Controls (B2)
(A1)	8	.0389417	.0289833
(A2)	15	.0892500	.0724167
(A3)	22	.1448750	.0982500

SOURCE	SS	DF	MS	F
A	.0922360	2	.0461180	338.7631
B	.0107800	1	.0107800	79.18531
AB	.0045585	2	.0022793	16.74246
WITHN CELL	.0089850	66	.0001361	
TOTAL	.1165595	71		
A AT B 1	.0673878	2	.0336939	247.5009
A AT B 2	.0294067	2	.0147034	108.0047
SSWCELL	.0089850	66	.0001361	
B AT A 1	.0005950	1	.0005950	4.370692
B AT A 2	.0017002	1	.0017002	12.48870
B AT A 3	.0130433	1	.0130433	95.81079
SSWCELL	.0089850	66	.0001361	
F MAX	17.14369			

APPENDIX A

14. A comparison of the contralateral ALD muscle lengths from immobilisation in the shortened position with the controls (left-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Contralaterals (B1)	Controls (B2)
(A1)	8	1.067750	1.098833
(A2)	15	1.427000	1.364583
(A3)	22	2.205417	2.166667

SOURCE	SS	DF	MS	F
A	15.50566	2	7.752832	377.3304
B	.0098156	1	.0098156	.4777269
AB	.0283671	2	.0141836	.6903135
WITHN CELL	1.356071	66	.0205465	
TOTAL	16.89992	71		
A AT B 1	8.117114	2	4.058557	197.5300
A AT B 2	7.416915	2	3.708457	180.4906
SSWCELL	1.356071	66	.0205465	
B AT A 1	.0057969	1	.0057969	.2821371
B AT A 2	.0233749	1	.0233749	1.137657
B AT A 3	.0090100	1	.0090100	.4385147
SSWCELL	1.356071	66	.0205465	
F MAX	7.962066			

APPENDIX A

15. A comparison of the contralateral PLD muscle weights from immobilisation in the shortened position with the controls (left-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Contralaterals(B1)	Controls (B2)
(A1)	8	.0448083	.0330250
(A2)	15	.1033750	.0928417
(A3)	22	.1618500	.1201667

SOURCE	SS	DF	MS	F
A	.1261342	2	.0630671	443.1545
B	.0081920	1	.0081920	57.56285
AB	.0037318	2	.0018659	13.11112
WITHN CELL	..0093927	66	.0001423	
TOTAL	.1474507	71		
A AT B 1	.082.925	2	.0410963	288.7718
A AT B 2	.0476734	2	.0238367	167.4939
SSWCELL	.0093927	66	.0001423	
B AT A 1	.0008331	1	.0008331	5.853830
B AT A 2	.0006657	1	.0006657	4.677720
B AT A 3	.0104250	1	.0104250	73.25351
SSWCELL	.0093927	66	.0001423	
F MAX	22.85015			

APPENDIX A

16. A comparison of the contralateral PLD muscle lengths from immobilisation in the shortened position with the controls (left-hand side).

CELL MEANS: FOR 12 ANIMALS/CELL

Age (days)		Contralaterals (B1)	Controls (B2)
(A1)	8	1.513083	1.376833
(A2)	15	1.950500	1.809167
(A3)	22	2.664583	2.383333

SOURCE	SS	DF	MS	F
A	14.14604	2	7.073021	741.5658
B	.6245948	1	.6245948	65.48519
AB	.0812463	2	.0406231	4.259102
WITHN CELL	.6295050	66	.0095380	
TOTAL	15.48139	71		
A AT B 1	8.108803	2	4.054401	425.0808
A AT B 2	6.118485	2	3.059242	320.7440
SSWCELL	.6295050	66	.0095380	
B AT A 1	.1113836	1	.1113836	11.67793
B AT A 2	.1198498	1	.1198498	12.56557
B AT A 3	.4746081	1	.4746081	49.75994
SSWCELL	.6295050	66	.0095380	
F MAX	2.168709			

APPENDIX A

Statistical comparisons of the weights and lengths of the ALD and PLD from immobilisation in the shortened position with a weeks recovery using:

One way analysis of variance tests

1. A comparison of the experimental ALD muscle weights for the recovery group with the controls at 29 days age.

	GROUP	MEAN	SS	VARIANCE
CONTROLS	1	.1530833	.0038609	.0003510
EXPERIS	2	.1716667	.0061887	.0005626

SOURCE	SS	DF	MS	F
TREATMENTS	.0020720	1	.0020720	4.536006
ERROR	.0100496	22	.0004568	
TOTAL	.0121216	23		

2. A comparison of the experimental ALD muscle lengths for the recovery group with the controls at 29 days age.

	GROUP	MEAN	SS	VARIANCE
CONTROLS	1	2.358333	.2641668	.0240152
EXPERIS	2	2.558333	.1491671	.0135606

SOURCE	SS	DF	MS	F
TREATMENTS	.2399979	1	.2399979	12.77406
ERROR	.4133339	22	.0187879	
TOTAL	.6533318	23		

APPENDIX A

3. A comparison of the experimental PLD muscle weights for the recovery group with the controls at 29 days age.

GROUP		MEAN	SS	VARIANCE
CONTROLS	1	.2055000	.0139250	.0012659
EXPERIS	2	.1878333	.0061597	.0005600

SOURCE	SS	DF	MS	F
TREATMENTS	.0018727	1	.0018727	2.051244
ERROR	.0200846	22	.0009129	
TOTAL	.0219573	23		

4. A comparison of the experimental PLD muscle lengths for the recovery group with the controls at 29 days age.

GROUP		MEAN	SS	VARIANCE
CONTROLS	1	2.720000	.3228025	.0293457
EXPERIS	2	3.016667	.0616684	.0056062

SOURCE	SS	DF	MS	F
TREATMENTS	.5280628	1	.5280628	30.21647
ERROR	.3844719	22	.0174760	
TOTAL	.9125347	23		

APPENDIX A

5. A comparison of the contralateral ALD muscle weights for the recovery group with the controls at 29 days age.

	GROUP	MEAN	SS	VARIANCE
CONTROLS	1	.1587500	.0037482	.0003407
CONTRAS	2	.1825833	.0035729	.0003248

SOURCE	SS	DF	MS	F
TREATMENTS	.0034082	1	.0034082	10.24148
ERROR	.0073212	22	.0003328	
TOTAL	.0107293	23		

6. A comparison of the contralateral ALD muscle lengths for the recovery group with the controls at 29 days.

	GROUP	MEAN	SS	VARIANCE
CONTROLS	1	2.429167	.2472925	.0224811
CONTRAS	2	2.629167	.0622902	.0056627

SOURCE	SS	DF	MS	F
TREATMENTS	..2399998	1	.2399998	17.05515
ERROR	.3095837	22	.0140720	
TOTAL	.5495834	23		

APPENDIX A

7. A comparison of the contralateral PLD muscle weights for the recovery group with the controls at 29 days age.

	GROUP	MEAN	SS	VARIANCE
CONTROLS	1	.1993750	.0068716	.0006247
CONTRAS	2	.2271667	.0061797	.0005618

SOURCE	SS	DF	MS	F
TREATMENTS	.0046343	1	.0046343	7.811847
ERROR	.0130512	22	.0005932	
TOTAL	.0176855	23		

8. A comparison of the contralateral PLD muscle lengths for the recovery group with the controls at 29 days age.

	GROUP	MEAN	SS	VARIANCE
CONTROLS	1	2.748333	.3893652	.0353968
CONTRAS	2	3.136667	.1594639	.0144967

SOURCE	SS	DF	MS	F
TREATMENTS	.9048176	1	.9048176	36.26998
ERROR	.5488281	22	.0249467	
TOTAL	1.453646	23		

APPENDIX B

A List of Chemicals and Suppliers:

Tissue-Tek - Raymond A. Lamb, London NW10 6JL.

The following chemicals were supplied by Fisons (Loughborough, England).

Isopentane
Sodium succinate S.L.R.
Sodium hydroxide A.R.
Cobaltous chloride A.R.
Calcium chloride (dihydrate)
Ammonium sulphide
Sodium chloride A.R.
Acetic acid
Iodine A.R.
Potassium iodide A.R.
Tertiary Butyl Alcholol
Glycerol
Trichloroacetic acid (T.C.A.)
Glycine A.R.
Magnesium chloride A.R.
Sodium sulphite
Ammonium molybdate
Potassium di-hydrogen ortho phosphate

The following chemicals were supplied by Sigma Chemical Corporation.

ATP Adenosine 5'-Triphosphate, disodium Grade II (A3377)
AMP Adenosine 5'-Monophosphate, sodium salt Type II (A1752)
G-1-P Glucose-mono-phosphate, dipotassium, Grade I (G6875)
Glycogen Type II G8751
Insulin
Nitroblue Tetrazolium (NBT)
Acetylthiocholine Iodine
Bovine serum albumin Fraction V A4503
Tris-HCl AR
L-cysteine-HCl AR
Ethylene diaminetetraacetate (EDTA)

Creatine Phosphate

Creatine Phosphokinase

Imidazole AR

Ethylene glycol bis-amino ethyl ether N, N' tetra acetate (EGTA)

The following chemicals were supplied by B.D.H. Chemicals Ltd., (Poole, England).

L-Histidine monohydrochloride AR

Di-sodium hydrogen orthophosphate AR

Sodium dihydrogen orthophosphate AR

Glycerin jelly

Sodium acetate AR

Ammonium alum AR

Potassium permanganate AR

Sodium fluoride AR

Maleic acid AR

Sodium sulphate AR

Silver nitrate AR

Calcium carbonate AR

Quinol (hydroquinone)

Perchloric acid

The following chemicals were supplied by Hopkins and Williams.

Paraformaldehyde

Haematoxylin

Copper sulphate AR

Potassium ferrocyanide AR

Sodium thiosulphate AR

Ferrous sulphate AR

The following chemicals were supplied by Gurr Searle Diagnostic.

DPX

Eosin

Sodium pentobarbitone SAGATAL May & Baker Ltd.

Sodium glycerophosphate AR Kooh Light Laboratories.

APPENDIX C

Results of overall means for Mg^{2+}-activated myofibrillar ATPase

mol ATP split/mol myosin/sec

CONTROLS

AGE	ALD	± SD	± SE	PLD	± SD	± SE
3 days	2.2378	0.1066	0.06154	2.3798	0.2682	0.1548
8 days	0.9235	0.0615	0.0355	2.0957	0.3742	0.2160
11 days	1.563	0.0612	0.0353	3.126	0.123	0.0710
15 days	2.1312	0.1066	0.06154	4.3689	0.1846	0.1066
18 days	1.4917	0.1064	0.06143	3.1617	0.1626	0.0542
22 days	1.2076	0.0615	0.0355	1.9891	0.0615	0.0355
29 days	1.301	0.0615	0.0355	2.0246		
36 days	0.6749	0.0615	0.0355	1.7049	0.1846	0.1066
44 days	0.7814	0.2218	0.1281	2.3443	0.1066	0.0615
51 days	0.6749	0.0615	0.0355	1.9891	0.1628	0.09399

IMMOBILISED RESTING

EXPERIS

AGE	ALD	± SD	± SE	PLD	± SD	± SE
6 days	1.3853	0.1066	0.06154	1.6339	0.1230	0.0710
8 days	2.0602	0.1230	0.0710	1.9536	0.0615	0.0355
15 days	1.2787			2.6285	0.2682	0.1548
22 days	0.9945	0.0615	0.0355	3.3744	0.1628	0.09399

CONTRALATERALS

AGE	ALD	± SD	± SE	PLD	± SD	± SE
6 days	1.1722	0.1066	0.06154	1.6694	0.06149	0.0355
8 days	1.0656			1.8115	0.1845	0.1065
15 days	1.4918			3.1258	0.1230	0.0710
22 days	1.2727			3.1258	0.1230	0.0710

mol ATP split/mol myosin/sec

IMMOBILISED SHORTENED

EXPERIS

AGE	ALD	± SD	± SE	PLD	± SD	± SE
6 days	2.2	0.1212	0.06997	2.0593	0.1224	0.0704
8 days	0.8525	0.1845	0.1065	1.3853		
15 days	0.9945	0.1627	0.0939	1.030	0.0615	0.0355
22 days	1.2787	0.1066	0.0615	3.090		
29 days (Recovery)	0.9590	0.1628	0.9399	2.9837		

APPENDIX C

CONTRALATERALS

AGE	ALD	± SD	± SE	PLD	± SD	± SE
6 days	2.344	0.2135	0.1232	2.913	0.12297	0.07099
8 days	0.9235	0.2218	0.1281	1.5273	0.0615	0.0355
15 days	1.0656			1.5317	0.1572	0.09076
22 days	1.2787			2.5219	0.1628	0.09399
29 days (Recovery)	0.6039	0.0615	0.0355	2.0957	0.1231	0.0711

APPENDIX C

Results of Overall Means - Phosphorylase

μMPi/mg prot/min

CONTROLS

AGE	ALD	± SD	± SE	PLD	± SD	± SE
3 days a	0.06032	4.018 x 10-3	2.3197 x 10^{-3}	0.0789	0.0106	6.1199 x 10^{-3}
a + b	0.2923	0.0418	0.02413	0.4802	0.0121	6.956 x 10^{-3}
8 days a	0.058	4.018 x 10-3	2.3197 x 10^{-3}	0.2946	0.0106	6.1199 x 10^{-3}
a + b	0.4269	0.0224	0.01293	0.6728	0.0145	8.3716 x 10^{-3}
15 days	0.0603	4.018 x 10-3	2.3197 x 10^{-3}	0.2250	0.0106	6.1199 x 10^{-3}
a + b	0.7378	6.9 x 10-3	3.9837 x 10^{-3}	1.1113	0.0145	8.3716 x 10^{-3}
22 days a	0.05568	-	-	0.2343	3.983 x 10^{-3}	2.995 x 10^{-3}
a + b	inactive AMP		-	inactive AMP		-
29 days a	0.1044	-	-	0.2946	0.0408	0.023556
a + b	0.6589	0.0224	0.01293	0.8816	0.0704	0.04065
36 days a	0.1462	6.95 x 10^{-3}	3.9837 x 10^{-3}	not active soln		-
a + b	0.6728	0.04252	0.02454			
44 days a	0.1253	0.0121	6.986 x 10^{-3}	0.3527	4.041 x 10^{-3}	2.3331 x 10^{-3}
a + b	0.5174	0.0224	0.01293	0.754	0.0448	0.02587
51 days a	0.0232	0.0106	6.1199 x 10^{-3}	0.1299	8.025 x 10^{-3}	4.633 x 10^{-3}
a + b	0.2900	8.025 x10^{-3}	4.633 x 10^{-3}	0.8329	0.0201	0.01160

IMMOBILISED RESTING

EXPERIS

AGE	ALD	± SD	± SE	PLD	± SD	± SE
6 days a	0.0872	3.25 x10^{-3}	1.876 x 10^{-3}	0.1114	-	-
a + b	0.3573	0.02899	0.01674	0.6519	0.0224	0.01293
8 days a	0.0974	6.95 x 10^{-3}	3.9837 x 10^{-3}	0.1206	4.041 x 10^{-3}	2.3331 x 10^{-3}
a + b	0.5313	0.0106	6.1199 x 10^{-3}	0.9790	0.0244	0.01293

APPENDIX C

AGE	ALD	± SD	± SE	PLD	± SD	± SE
15 days a	0.05336	4.018 x 10^{-3}	2.3197 x 10^{-3}	0.2181	8.025 x 10-3	4.633 x 10^{-3}
a + b	0.4849	0.0263	0.01518	0.9628	0.0383	0.02211
22 days a	0.07424	8.037 x 10^{-3}	4.64 x 10^{-3}	0.2530	0.0145	8.3716 x 10^{-3}
a + b	0.4106	0.0121	6.986 x 10-3	0.7818	4.041 x 10-3	2.3331 x 10^{-3}
CONTRAS						
6 days a	0.08815	8.048 x 10-3	4.6465 x 10-3	0.1183	6.95 x 10-3	3.9837 x 10^{-3}
a + b	0.1844	9.2034 x 10^{-3}	5.3136 x 10^{-3}	0.4362	0.0408	0.023556
8 days a	0.07193	4.041 x 10-3	2.333 x 10-3	0.0974	-	-
a + b	0.4918	0.0459	0.02823	0.6728	0.0224	0.01293
15 days a	0.09973	4.0414 x 10^{-3}	2.333 x 10-3	0.3619	6.95 x 10-3	3.9837 x 10^{-3}
a + b	0.6798	0.0344	0.01986	0.9814	6.95 x 10-3	3.9837 x 10^{-3}
22 days a	0.0928	3.9837 x 10-3	2.2999 x 10-3	0.2714	0.0139	8.0252 x 10^{-3}
a + b	0.5730	4.041 x 10-3	2.333 x 10-3	0.8120	0.0175	0.01010

Overall Means - Phosphorylase

µMPi/mg prot/min

IMMOBILISED SHORTENED

EXPERIS

AGE	ALD	± SD	± SE	PLD	± SD	± SE
6 days a	0.1809	0.01395	8.054 x 10-3	0.1949	-	-
a + b	0.3945	0.0212	0.1223	0.5105	0.0426	0.0246
8 days a	0.0278	-	-	0.0371	4.04 x 10-3	2.333 x 10^{-3}
a + b	0.2320	8.025 x 10-3	4.633 x 10-3	0.3550	0.01395	8.054 x 10^{-3}
15 days a	0.0302	8.025 x 10-3	4.633 x 10-3	0.1786	0.0212	0.01223
a + b	0.3341	6.95 x 10-3	3.9837	0.638 x 10-3	3.984	2.300 x 10^{-3}

APPENDIX C

AGE	ALD	± SD	± SE	PLD	± SD	± SE
22 days a	0.0812	8.025 x 10^{-3}	4.633 x 10^{-3}	0.2111	0.0145	8.3716 x 10^{-3}
a + b	0.5035	0.0541	0.03123	0.8352	0.021	0.01223

CONTRAS

6 days a	0.2088	0.0139	8.054 x 10^{-3}	0.2599	0.0161	9.2953 x 10^{-3}
a + b	0.4222	8.025 x 10^{-3}	4.633 x 10^{-3}	0.7702	0.0289	0.01669
8 days a	0.04176	-	-	0.0766	0.012	6.928 x 10^{-3}
a + b	0.4501	0.0161	9.2953 x 10^{-3}	0.7772	8.025 x 10^{-3}	4.633 x 10^{-3}
15 days a	0.01857	4.04 x 10^{-3}	2.333 x 10^{-3}	0.0742	8.025 x 10^{-3}	4.633 x 10^{-3}
a + b	0.3294	0.0106	6.1199 x 10^{-3}	0.5104	0.0106	6.1199 x 10^{-3}
22 days a	0.0487	-	-	0.1183	6.95 x 10^{-3}	3.9837 x 10^{-3}
a + b	0.3619	0.0120	6.928 x 10^{-3}	0.4779	0.0145	8.3716 x 10^{-3}

RECOVERY

EXPERIS

AGE	ALD	± SD	± SE	PLD	± SD	± SE
29 days a	0.04175	6.95 x 10^{-3}	3.9837 x 10^{-3}	0.2482	0.0106	6.1199 x 10^{-3}
a + b	0.5568	7 x 10^{-3}	4.041 x 10^{-3}	0.9118	0.0368	0.02124

CONTRAS

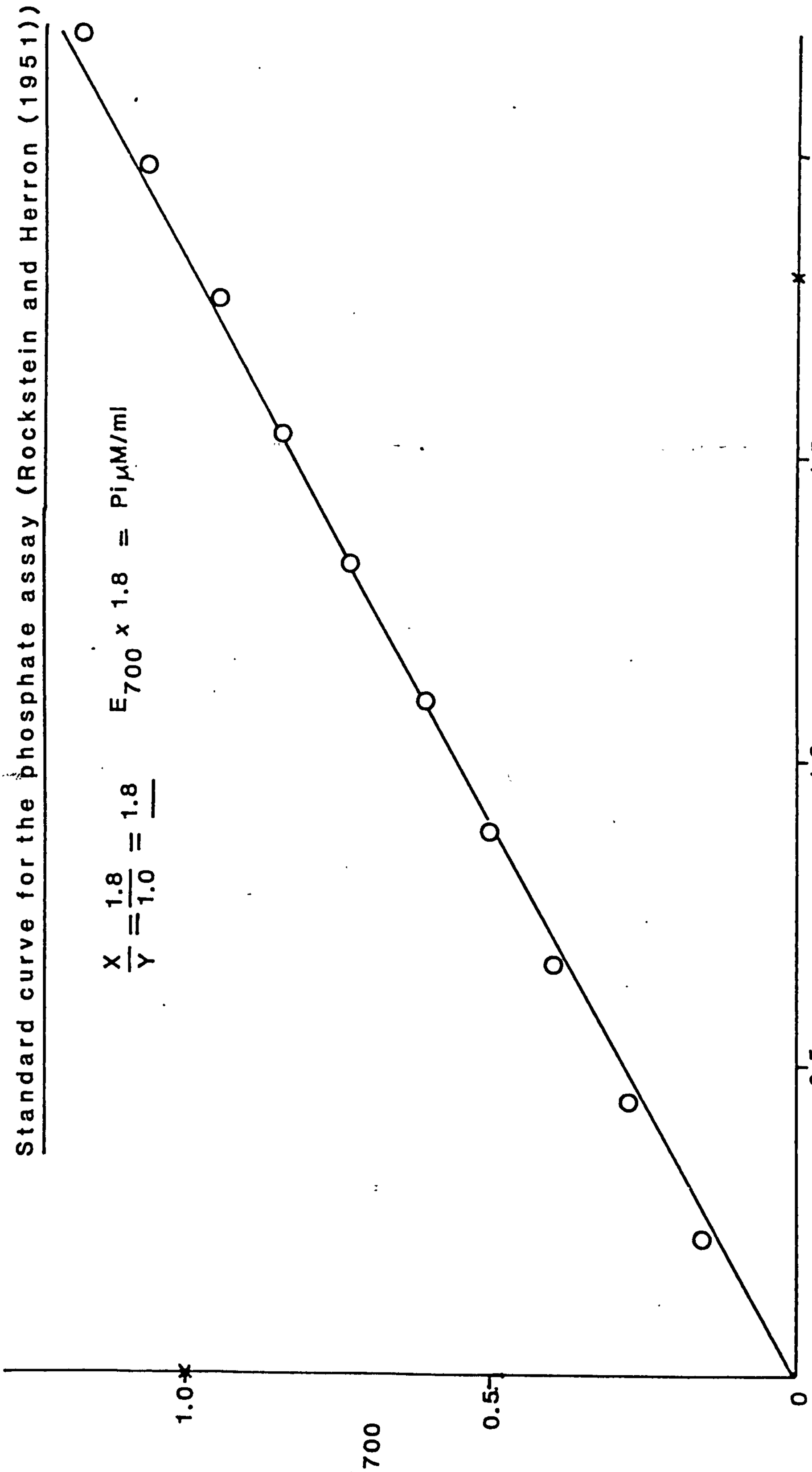
29 days a	0.0812	0.106	6.1199 x 10^{-3}	0.3503	4.041 x 10^{-3}	2.333 x 10^{-3}
a + b	0.6542	0.0279	0.01611	0.7540	8.025 x 10^{-3}	4.633 x 10^{-3}

Appendix C

Standard curve for the phosphate assay (Rockstein and Herron (1951))

$E_{700} \times 1.8 = Pi\ \mu M/ml$

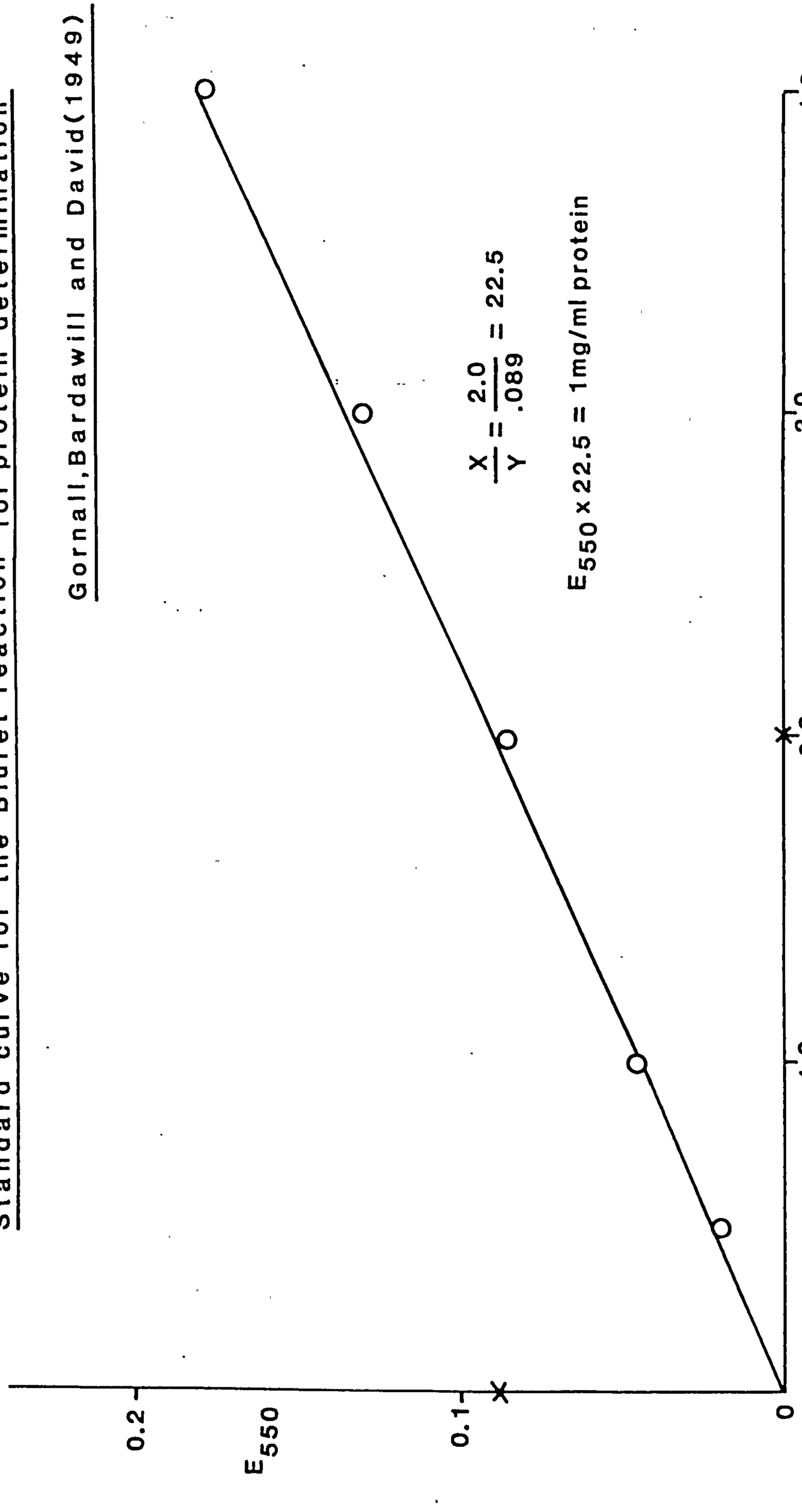
$\frac{X}{Y} = \frac{1.8}{1.0} = 1.8$



Appendix C

Standard curve for the Biuret reaction for protein determination

Gornall, Bardawill and David (1949)

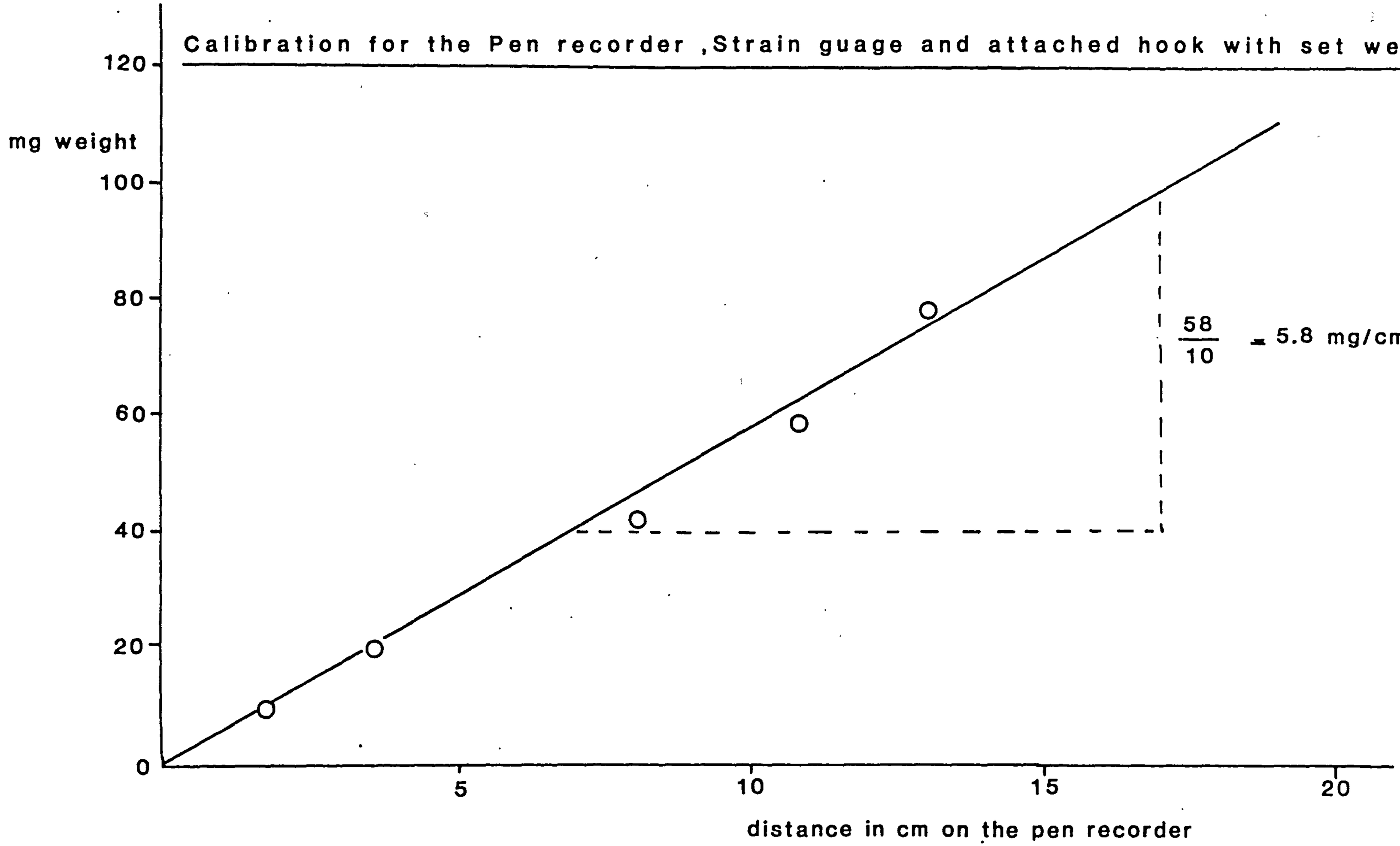


APPENDIX D

Species		$\log_{10}$ affinity constant
Imidazole - H*		7.09
EGTA	- H	9.43
	- 2H	18.28
	- 3H	20.96
	- 4H	23.04
	- Mg	5.21
	- MgH	12.79
	- Ca	10.42
	- CaH	14.75
	- K	0.96
	- Na	1.80
ATP	- H	6.95
	- 2H	10.99
	- K	0.90
	- Na	1.17
	- Mg	4.78
	- MgH	7.56
	- Ca	4.40
	- CaH	6.88
ADP	- H	6.35
	- 2H	10.34
	- Ca	2.78
	- Mg	3.11
	- MgH	7.87
	- K	1.15
	- Na	1.17
Pi	- H	11.89
	- 2H	18.59
	- 3H	20.69
	- KH	12.38
	- NaH	12.49
	- MgH	13.77
	- CaH	13.59
CP	- H	2.70
	- 2H	7.28
	- Mg	1.60
	- Ca	1.30

Affinity constants used in the iterative programme for solving ionic species binding equations.

Calibration for the Pen recorder ,Strain guage and attached hook with set weights



APPENDIX D

3. Statistical comparison of the pCa-tension (P/Po) plots was carried out for the mean data for 5 fibres from the ALD and PLD muscle using a 2-factor analysis of variance test:

P/Po Mean Tension

pCa		PLD (A1)	ALD (A2)
7.41	(B1)	0.0000000	0.0000000
6.75	(B2)	0.0000000	0.3220000
6.63	(B3)	0.0056120	0.4325400
6.40	(B4)	0.1020800	0.6097000
6.12	(B5)	0.4836200	0.8081200
6.43	(B6)	0.5480800	0.8853200
5.96	(B7)	0.6926800	0.8848600
5.31	(B8)	1.0000000	0.9736200

SOURCE	SS	DF	MS	F
BETW SUBJS	1.624429	9		
A	1.363839	1	1.363839	41.86936
SUBJ W GPS	.2605895	8	.0325737	
WITHN SUBJ	9.462565	70		
B	8.499146	7	1.214164	215.3053
AB	.6476203	7	.0925172	16.40589
B SWG	.3157989	56	.0056393	
A AT B 1	0.000000	1	0.000000	0.000000
A AT B 2	.2592100	1	.2592100	28.78170
A AT B 3	.4664045	1	.4664045	51.78780
A AT B 4	.6441952	1	.6441952	71.52901
A AT B 5	.2632506	1	.2632506	29.23036
A AT B 6	.2843271	1	.2843271	31.57061
A AT B 7	.0923329	1	.0923329	10.25230
A AT B 8	.0017398	1	.0017398	.1931775
SSWCELL	.5763884	64	.0090061	
B AT A 1	5.109792	7	.7299703	129.4442
B AT A 2	4.036974	7	.5767106	102.2670
SSBSWG	.3157989	56	.0056393	
F MAX SWG	1.088340			
F MAX BSWG	1.746723			

APPENDIX D

4. Statistical results to show that the 3rd degree polynomial equation provided the curve of best fit for the graphs of pCa-tension (P/Po) for both the data from the ALD and PLD muscle fibres. (Mean of 5 fibres).

Additional tables give the calculated values for the individual sigmoidal curves for the ALD and PLD.

MUSCLE TYPE ALD/SAMPLE NO. 6: MEAN OF 5 FIBRES/NO. OF VALUES 8.

INPUT PAIRS OF VALUES:	pCa	P/Po
	7.4100	.0000
	6.7500	.3220
	6.6300	.4375
	6.4000	.6097
	6.1200	.8081
	6.0300	.8853
	5.9600	.8849
	5.3100	.9736

ENTER HIGHEST DEGREE OF POLYNOMIAL TO BE FITTED 3
PCA RANGE FROM 5.31 TO 7.41

POLYNOMIAL DEGREE 3 COEFFICIENTS ARE
AO = -70.562676048902
A1 = 34.237403777581
A2 = -5.3653897153178
A3 = o.27395999315475

STANDARD ERROR OF ESTIMATE = 0.011517190989524

POLYNOMIAL DEGREE 2 COEFFICIENTS ARE
AO = -19854479934314
A1 = 1.3445871258881
A2 = -0.14630495894l1

STANDARD ERROR OF ESTIMATE = 0.063548128702s3

POLYNOMIAL DEGREE 1 COEFFICIENTS ARE
AO = 3.9017483431695
A1 = -0.51951959583797

STANDARD ERROR OF ESTIMATE = 0.090446430496s8

APPENDIX D

Using the co-efficients from the 3rd degree polynomial equation: the data points for the sigmoidal curve for the ALD muscle fibres were

pCa	P/Po
7.50000	-.00845
7.40000	.00054
7.30000	.02385
7.20000	.05985
7.10000	.10689
7.00000	.16333
6.90000	.22753
6.80000	.29784
6.70000	.37261
6.60000	.45022
6.50000	.52900
6.40000	.60731
6.30000	.68352
6.20000	.75598
6.10000	.82305
6.00000	.88308
5.90000	.93442
5.80000	.97544
5.70000	1.00449
5.60000	1.01992
5.50000	1.02010
5.40000	1.00338
5.30000	.96811
5.20000	.91265
5.10000	.83536
5.00000	.73460

APPENDIX D

MUSCLE TYPE PLD/SAMPLE NO. 6:. MEAN OF 5 FIBRES/NO. OF VALUES 8.

INPUT PAIRS OF VALUES:	pCa	P/Po
	7.4100	.0000
	6.7500	.0000
	6.6300	.0056
	6.4000	.1021
	6.1200	.4836
	6.0300	.5481
	5.9600	.6927
	5.3100	1.0000

ENTER HIGHEST DEGREE OF POLYNOMIAL TO BE FITTED 3
PCA RANGE FROM 5.31 TO 7.41

POLYNOMIAL DEGREE 3 COEFFICIENTS ARE
AO = -94.47048685562
A1 = 48.123419193631
A2 = -7.9594605615287
A3 = 0.42992549981389

STANDARD ERROR OF ESTIMATE = 0.04863414895594

POLYNOMIAL DEGREE 2 COEFFICIENTS ARE
AO = 13.14777779738
A1 = -3.4952878899947
A2 = 0.2308507193986 3

STANDARD ERROR OF ESTIMATE = 0.10947102987187

POLYNOMIAL DEGREE 1 COEFFICIENTS ARE
AO = 3.8585266374631
A1 = -0.55396327009898

STANDARD ERROR OF ESTIMATE = 0.14932065926531

APPENDIX D

Using the co-efficients from the 3rd degree polymonial equation:
the data points for the sigmoidal curve for the PLD muscle fibres were:

pCa	P/Po
7.50000	.11032
7.40000	-.00111
7.30000	-.08085
7.20000	-.13147
7.10000	-.15555
7.00000	-.15567
6.90000	-.13442
6.80000	-.09436
6.70000	-.03808
6.60000	.03184
6.50000	.11282
6.40000	.20228
6.30000	.29765
6.20000	.39633
6.10000	.49576
6.00000	.59336
5.90000	.68653
5.80000	.77272
5.70000	.84932
5.60000	.91377
5.50000	.96349
5.40000	.99590
5.30000	1.00841
5.20000	.99844
5.10000	.96343
5.00000	.90078

APPENDIX D

5.- Linear regression statistical anlysis for the pCa-Hill plots for the ALD and PLD muscle fibre means. (pooled from 5 fibres).

1. MUSCLE TYPE ALD
SAMPLE NO. 6 : MEAN OF 5 FIBRES
NO. OF VALUES 6

INPUT PAIRS OF VALUES

pCa	Hill Plot $\log_{10}(P/Po-P)$
6.7500	-.3318
6.6300	-.0055
6.4000	.1955
6.1200	.5839
6.0300	.8324
5.9600	.8454

POLYNOMIAL DEGREE 1 COEFFICIENTS ARE
AO = 9.466469801434
A1 = -1.4430942414519

STANDARD ERROR OF ESTIMATE = 0.05957100173869

DATA POINTS:

pCa	$\log_{10}(P/Po-P)$
6.5	0.087
5.75	1.16925
7.00	-0.6335

When $\log_{10}(P/Po-P) = 0$

$= 9.46665 + (-1.443 \times X)$

$X = 6.56$ = pCa at $\frac{1}{2}$ maximal tension

From graph:

Gradient n = $\frac{0.45}{0.3} = 1.5$

==========

APPENDIX D

2. MUSCLE TYPE PLD
SAMPLE NO. 6 : MEAN OF 5 FIBRES
NO. OF VALUES 5

INPUT PAIRS OF VALUES (pCa, HILL PLOT)

6.6300	-1.7020
6.4000	-.9222
6.1200	-.0396
6.0300	.0872
5.9600	.3724

POLYNOMIAL DEGREE 1 COEFFICIENTS ARE
AO = 18.520144626081
A1 = -3.0444711987932

STANDARD ERROR OF ESTIMATE = 0.052961459778887

DATA PONTS:

pCa	$\log_{10}(P/Po-P)$
6.5	-1.2706
5.75	1.01308
7.00	-2.7928

When $\log_{10}(P/Po-P) = 0$

$= 18.5201 + (-3.0445 \times X)$

$X = 6.08$ = pCa at $\frac{1}{2}$ maximal tension

From graph:

Gradient n = $\frac{0.9}{0.3} = 3.0$

==========

APPENDIX D

Raw data for:

Maximum Isometric Tensions

PLD	$Kgcm^{-2}$	µm Diameter	ALD	$Kgcm^{-2}$	µm Diameter
Fibre No 1	0.3703	48	Fibre No 1	0.4834	45
2	1.6545	30	2	0.6825	52
3	0.6455	60	3	0.9317	50
4	1.636	23	4	0.4296	40
5	0.2228	60	5	1.706	32
6	0.4132	62	6	0.6949	50
7	0.4105	93	7	1.0447	50
8	0.7467	75	8	1.0876	72
9	0.7423	62	9	0.4813	100
10	1.004	50			
11	1.611	32			
MEAN	0.8597			0.83797	
	± 0.163			± 0.136	

BIBLIOGRAPHY

ABBOTT, B.C. and LOWY, J. (1953)

Mechanical properties of Mytilus muscle.

J. Physiol. (Lond.) 120, 50P.

ADAMSON, L.F. (1970)

Stimulation by N^6, O^{21}-dibutryl adenosine 3'-5'-cyclic-phosphate of amino acid transport, protein synthesis, and chondroitin sulfate synthesis in embryonic bone in vitro, contrasted with the effects of thyroid hormones.

Biochem. Biophys. Acta. 201, 446-452.

ADELSTEIN, R.S. and CONTI, M.A. (1975)

Phosphorylation of smooth muscle myosin light chain kinase by the catalytic subunit of adenosine 3':5'-monophosphate-dependent protein kinase.

J. Biol. Chem. 253, 8347-8350.

ALTRINGHAM, J.D. (1981)

"The mechanical properties of fish myotomal muscle".

Ph.D. Thesis - Physiology Dept., University of St. Andrews, Scotland.

ARIANO, M.A., ARMSTRONG, R.B. and EDGERTON, V.R. (1973)

Hindlimb muscle fiber populations of five mammals.

J. Histochem. Cytochem. 21(1), 51-55.

ASHLEY, C.C. and MOISESCU, D.G. (1977)

The effect of changing the composition of the bathing solution upon the

isometric - pCa relationship in bundles of crustacean myofibrils.
J. Physiol. (Lond.) 270, 627-652.

ASHMORE, C.R. and DOERR, L. (1971)
Comparative aspects of muscle fiber types in different species.
Exp. Neurol. 31, 408-418.

ASHMORE, C.R., KIKUCHI, T. and DOERR, L. (1978)
Some observations on the innervation patterns of different fibre types of chick muscle.
Exp. Neurol. 58, 272-284.

ASIEDU, S. and SHAFIQ, S.A. (1972)
Actomyosin ATPase activity of the Anterior Latissimus dorsi muscle of the chicken.
Exp. Neurol. 35, 211-213.

ASTIER, H. and NEWCOMER, W.S. (1978)
Extrathyroidal conversion of thyroxine to triiodothyronine in a bird: the Peking duck:
Gen. Comp. Endocrinol. 35, 496-499.

ATSUMI, S. (1979)
The size distribution of motoneurons innervating fast and slow muscles in the chicken.
Integrative Control Functions of the Brain, 2, 165-167. (Eds. Masao,I. et al.)

BACOU, F. and NOUGUÈS, J. (1980)

Alterations of enzymatic activities during red and white muscle differentiation in vitro.

Exp. Cell Res. 129, 455-460.

BACOU, F., JALLAGEAS, M., NOUQUÈS, J. and VIGNERON, P. (1980)

Influence of experimental hypothyroidism on chick myogenesis.

Reprod. Nutr. Dévelop. 20(1B), 217-224.

BAGSHAW, C.R. (1977)

On location of the divalent metal binding sites and the light chain subunit of vertebrate myosin.

Biochem. 16, 59-67.

BAGUST, J., LEWIS, D.M. and WESTERMAN, R.A. (1973)

Polyneuronal innervation of kitten skeletal muscle.

J. Physiol. (Lond.) 229, 241-255.

BAILEY, N.T.J. (1974)

"Statistical Methods in Biology".

Unibooks English Universities Press.

BALDWIN, K.M., HOOKER, A.M., CAMPBELL, P.J. and LEWIS, R.E. (1978)

Enzyme changes in neonatal skeletal muscle: effect of thyroid deficiency.

Am. J. Physiol. 235, C97-C102.

BÁRÁNY, K. and BÁRÁNY, M. (1977)

Phosphorylation of the 18,000 dalton light chain of myosin during a

single tetanus of frog muscle.

J. Biol. Chem. 252, 4752-4754.

BÁRÁNY, M. (1967)

ATPase activity of myosin correlated with speed of muscle shortening.

J. Gen. Physiol. 50, 197-218.

BÁRÁNY, M. and CLOSE, R.I. (1971)

The transformation of myosin in cross-innervated rat muscles.

J. Physiol. (Lond.) 213, 455-474.

BARKA, T. and ANDERSON, P.J. (1963)

"Histochemistry".

Harper and Row, N. York.

BARNARD, R.J., EDGERTON, V.R., FURUKAWA, TETSUO. and PETER, J.B. (1971)

Histochemical, biochemical and contractile properties of red, white and intermediate fibers.

Am. J. Physiol. 220, 410-414.

BARNARD, E.A., LYLES, J.M. and PIZZEY, J.A. (1982)

Fibre types in chicken skeletal muscles and their changes in muscular dystrophy.

J. Physiol. (Lond.) October 1982, 331, In Press.

BENDALL, J.R. (1961)

A study of the kinetics of the fibrillar adenosine triphosphatase of rabbit skeletal muscle.

Biochem. J. 81, 520-535.

BENDALL, J.P. (1969)

In: Muscles, Molecules and Movement, p. 51.

Heinemann Publication.

BOOTH, F.W. (1977)

Time course of muscular atrophy during immobilisation of hind-limbs in rats.

J. Appl. Physiol. Resp. Environ. Exer. Physiol. 43, 656-661.

BOOTH, F.W. (1978)

Regrowth of atrophied skeletal muscle in adult rats after ending immobilisation.

J. Appl. Physiol. Resp. Environ. Exer. Physiol. 44(2), 225-230.

BREMEL, R.D. and WEBER, A. (1972)

Co-operation within actin filaments in vertebrate skeletal muscle.

Nature 238, 97-101.

BROOKE, M.H. and KAISER, K.K. (1974)

The use and abuse of muscle histochemistry. In: The Trophic Functions of the Neuron.

Ann., N.Y. Acad. Sci. 228, 121-144.

BROWN, M.D., COTTER, M.A., HUDLICKÁ, O. and VRBOVÁ, G. (1976)

The effects of different patterns of muscle activity on capillary density, mechanical properties and structure of slow and fast rabbit muscles.

Pflügers Arch. 361, 241-250.

BULLER, A.J. ECCLES, J.C. and ECCLES, R.M. (1960 (a))

Differentiation of fast and slow muscles in the cat hind limb.

J. Physiol. (Lond.) 150, 399-416.

BULLER, A.J., ECCLES, J.C. and ECCLES, R.H. (1960 (b))

Interactions between motoneurones and muscles in respect of the characteristic speeds of their responses.

J. Physiol. (Lond.) 150, 417-439.

BURKE, R.E., LEVINE, D.N., TSAIRIS, P. and ZAJAC, F.E. (1973)

Physiological types and histochemical profiles in motor units of the cat gastrocnemius.

J. Physiol. (Lond.) 234, 723-743.

BUTLER, J. and COSMOS, E. (1981)

Differentiation of the avian latissimus dorsi primordium: Analysis of fiber type expression using the myosin ATPase histochemical reaction.

J. Exp. Zool. 218, 219-232.

CANFIELD, S.P. (1971)

The mechanical properties and heat production of chicken latissimus dorsi muscles during tetanic contractions.

J. Physiol. (Lond.) 219, 281-302.

CHANTLER, P. (1982)

Retreats from the steric blocking of muscle contraction.

Nature 298, 120-121.

CLELAND, W.W. (1964)

Dithiothreitol, a new protective reagent for SH groups.

Biochem. 3, 480-482.

CLOSE, R. (1965)

The relation between intrinsic speed of shortening and duration of the active state of muscle.

J. Physiol. (Lond.) 180, 542-559.

COLLINS, J.H., POTTER, J.D., HORN, M.J., WILSHIRE, G. and JACKMAN, N. (1973)

The amino acid sequence of rabbit skeletal muscle troponin C : gene replication and homology with calcium-binding proteins from carp and hake muscle.

Febs Lett. 36, 208-272.

CORI, G.T. (1945)

The effect of stimulation and recovery on the phosphorylase a content of muscle.

J. Biol. Chem. 158, 333-339.

CORI, C.F. and CORI, G.T. (1936)

Mechanism of formation of hexosemonophosphate in muscle and isolation of a new phosphate ester.

Proc. Soc. Exp. Biol. Med. 34, 702-705.

CORI, G.T. and CORI, C.F. (1943)

Crytalline muscle phosphorylase IV formation of glycogen.

J. Biol. Chem. 158, 57-63.

CORI, C.F. in O.H. GAEBLER, (Ed.) (1956)

Enzymes: Units of Biological Structure and Function.

Acad. Press. N.Y., p.573.

CORI, G.T. and GREEN, A.A. (1943)

Crystalline muscle phosphorylase. II Prosthetic Group.

J. Biol. Chem. 151, 31-39.

COSMOS, E. (1966)

Enzymatic activity of differentiating muscle fibres. I. Development of phosphorylase in muscles of the domestic fowl.

Dev. Biol. 13, 163-181.

DAVIES, R.E. (1964)

Adenosine triphosphate breakdown during single muscle contractions.

Proc. R. Soc. Ser. B. 160, 480-484.

DAVISON, T.F., MISSON, B.H. and FREEMAN, B.M. (1980)

Some effects of thyroidectomy on growth, heat production and the thermoregulatory ability of the immature fowl. (Gallus domesticus).

J. therm. Biol. 5, 197-202.

DIXON, M. and WEBB, E.C. (1964)

"Enzymes".

2nd Edition, Longmans, London.

DRATMAN, M.B. (1974)

On the mechanism of action of thyroxin, an amino acid analog of tyrosine.

J. Theor. Biol. 46, 255-270.

DRATMAN, M.B., CRUTCHFIELD, F.L., AXELROD, J., COLBURN, R.W. and THOA, N. (1976)

Localization of triiodothyronine in nerve endings of rat brain.

Proc. Natl. Acad. Sci. U.S.A. 73, 941-944.

DUBOWITZ, V. and BROOKE, M.H. (1973)

Histological and histochemical stains and reactions. In: "Muscle Biopsy" - A Modern Approach, p.32, London, Philadelphia, Saunders.

DUBOWITZ, V. and PEARSE, A.G.E. (1961)

Enzymatic activity of normal and dystrophic human muscle :

A histochemical study.

J. Pathol. Bacteriol. 81, 365-378.

EBASHI, S., KODAMA, A. and EBASHI, F. (1968)

Troponin I. Preparation and physiological function.

J. Biochem. (Tokyo) 64, 465-477.

EBASHI, S., MARUYAMA, K. and ENDO, M. (1980) (Eds.)

Muscle contraction. Its regulatory mechanisms.

Japan Scientific Soc. Press.

EDELMAN, I.S. (1974)

Thyroid thermogenesis.

N. Engl. J. Med. 290, 1303-1308.

EDJTEHADI, G.D. and LEWIS, D.M. (1979)

Histochemical reactions of fibres in a fast twitch muscle of the cat.

J. Physiol. (Lond.) 287, 439-453.

EDMAN, K.A.P. (1979)

The velocity of unloaded shortening and its relation to sarcomere length and isometric force in vertebrate muscle fibres.

J. Physiol. (Lond.) 291, 143-159.

EDSTRÖM, L. and KUGELBERG, E. (1968)

Histochemical composition, distribution of fibres and fatiguability of single motor units. Anterior tibialis muscle of the rat.

J. Neurol. Neurosurg. Psychiat. 31, 424-433.

EISENBERG, E. and MOOS, C. (1968)

Adenosine triphosphate activity of acto-heavy meromyosin: a kinetic analysis of actin activation.

Biochem. 7, 1486-1489.

EISENBERG, E. and MOOS, C. (1970)

Actin activation of heavy-meromyosin adenosine triphosphatase.

J. Biol. Chem. 245, 2451-2456.

ENDO, M. (1967)

Regulation of Contraction - relaxation cycle of muscle (In Japanese).

Proc. XVII Gen. Ass. Japan Med. Cong. 1, 193-197.

ENGEL, W.K. (1962)

The essentiality of histo- and cytochemical studies in the investigation of neuromuscular disease.

Neurol. 12, 778-784.

ERÄNKÖ, O. and PALKAMA, A. (1961)

Improved localization of phosphorylase by the use of polyvinyl pyrrolidine and high substrate concentration.

J. Histochem. Cytochem. 9, p. 585.

FABIATO, A. (1982)

Skinned fibers from skeletal, cardiac and smooth muscle.

Fed. Proc. 41(7), 2223-2224.

FABIATO, A. and FABIATO, F. (1978)

Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles.

J. Physiol. (Lond.) 276, 233-255.

FENG, D.P., WANGYAN, W. and DAXING, L. (1981)

The occurrence of post-denervation hypertrophy in chick slow muscle fibres after complete elimination of stretch and tension.

Scientia Sinica 24(4), 554-562.

FISCHER, E.H. and KREBS, E.G. (1955 (a))

Conversion of phosphorylase b to phosphorylase a in muscle extracts.

J. Biol. Chem. 216, 121-132.

FISCHER, E.H. and KREBS, E.G. (1955 (b))

Phosphorylase activity of skeletal muscle extracts.

J. Biol. Chem. 216, 113-121.

FORD, L.E. and PODOLSKY, R.J. (1972)

Calcium uptake and force development by skinned muscle fibers in EGTA

buffered solutions.

J. Physiol. (Lond.) 223, 1-19.

GAUTHIER, G.F. (1969)

On the relationship of ultrastructural and cytochemical features to colour in mammalian skeletal muscle.

Z. Zellforsch. Mikroskop. Anat. 95, 462-482.

GEORGE, J.C. and BERGER, A.J. (1966)

Avian Myology.

Academic Press, N.Y. and London.

GINSBORG, B.L. (1960)

Some properties of avian skeletal muscle fibres with multiple neuromuscular junctions.

J. Physiol. (Lond.) 154, 581-598.

GINSBORG, B. and MACKAY, B. (1961)

A histochemical demonstration of two types of motor innervation in avian skeletal muscle.

Bibliotheca Anat. 2, 174-181.

GODFREY, J.E. and HARRINGTON, W.F. (1970)

Self-Association in the myosin system at high ionic strength. I. Sensitivity of the interaction to pH and ionic environment.

Biochem. 9, 886-893.

GODT, R.E. (1974)

Calcium-activated tension of skinned muscle fibers of the frog.

Dependence on the magnesium adenosine triphosphate concentration.

J. Gen. Physiol. 63, 722-739.

GOLDBERG, A. (1980)

The regulation of protein turnover by endocrine and nutritional factors.

In: Plasticity of Muscle, Ed. D. Pette, 469-493.

GOLDSPINK, G., LARSON, R.E. and DAVIES, R.E. (1970)

Thermodynamic efficiency and physiological characteristics of the chick anterior latissimus dorsi muscle.

Z. vergl. Physiologie 66, 379-388.

GOLDSPINK, G. and ROWE, R.W.D. (1968)

Surgically induced muscle fibre hypertrophy.

Anat. Records 161, 69-76.

GORDON, A.M., GODT, R.E., DONALDSON, S.K.B. and HARRIS, C.E. (1973)

Tension in skinned frog muscle fibres in solutions of varying ionic strength and neutral salt composition.

J. Gen. Physiol. 62, 550-574.

GORDON, A.M., HUXLEY, A.F. and JULIAN, F.J. (1966)

The variation in isometric tension with sarcomere length in vertebrate muscle fibres.

J. Physiol. (Lond.) 184, 170-192.

GORDON, T., PERRY, R., TUFFERY, A.R. and VRBOVÁ, G. (1974)

Possible mechanisms determining synapse formation in developing skeletal muscles of the chick.

Cell and Tissue Res. 155, 13-25.

GORDON, T., PERRY, R., SPURWAY, N.C. and VRBOVÁ, G. (1975)

Development of slow and fast muscles in chick embryos.

J. Physiol. (Lond.) 254, 24-25.

GORDON, T., PERRY, R., SRIHARI, T. and VRBOVÁ, G. (1977)

Differentiation of slow and fast muscles in chickens.

Cell and Tissue Res. 180, 211-222.

GORDON, T. and VRBOVÁ, G. (1975)

Influence of innervation on the differentiating tonic and twitch muscles of the chicken.

J. Physiol. (Lond.) 360, 199-218.

GORDON, T., VRBOVÁ, G. and WILCOCK, G. (1981)

Influence of innervation on differentiating tonic and twitch muscles of the chicken.

J. Physiol. (Lond.) 319, 261-269.

GORNALL, A.G., BARDAWILL, C.J. and DAVID, M.M. (1949)

Determination of serum proteins by means of the biuret reaction.

J. Biol. Chem. 177, 751-766.

GOULD, M.K. and RAWLINSON, W.A. (1959)

Biochemical adaptation as a response to exercise. I Effect of swimming on the levels of lactic dehydrogenase, malic dehydrogenase and phosphorylase in muscles of 8-, 11- and 15 week old rats.

Biochem. J. 73, 41-48.

GREENE, R. (1970)

The thyroid gland. In: "Human Hormone".

World University Library, Weidenfeld and Nicolsor, London.

GROSS, J. and PITT-RIVERS, R. (1952)

The identification of 3, 5, 3'-triiodothyronine in human plasma.

Lancet 1, 439-441.

GUTMANN, E. and SYROVÝ, I. (1967)

Metabolic differentiation of the anterior and posterior latissimus dorsi of the chicken during development.

Physiologia Bohemoslovaca 16, 232-243.

GUTH, L. and SAMAHA, F.J. (1969)

Qualitative differences between actomyosin ATPase of slow and fast mammalian muscle.

Exper. Neurol. 25, 138-152.

GUTH, L. and SAMAHA, F.J. (1972)

Erroneous interpretations which may result from application of the "Myofibrillar ATPase" Histochemical Procedure to Developing Muscle.

Exper. Neurol. 34, 465-475.

GUTMANN, E., HANZLÍKOVÁ, V. and HOLEČKOVÁ, E. (1969)

Development of fast and slow muscles of the chicken *in vivo* and their latent period in tissue culture.

Exp. Cell. Res. 56, 33-38.

HANSON, J. and LOWY, J. (1960)

Structure and function of the contractile apparatus in the muscles of invertebrate animals.

In: The Structure and Function of Muscle. Ed. G.H. Bourne, Acad. Press Inc., N.Y., 1, 312.

HELLAM, D.C. and PODOLSKY, R.J. (1969)

Force measurements in skinned muscle fibres.

J. Physiol. (Lond.) 200, 807-819.

HESS, A. (1961)

Structural differences of fast and slow extrafusal muscle fibres and nerve endings in chickens.

J. Physiol. (Lond.) 157, 221-231.

HESS, A. (1967)

The structure of vertebrate slow and twitch muscle fibres.

Invert. Ophthalmol. 6, 217-228.

HESS, A. (1970)

Vertebrate slow muscle fibers.

Physiol. Reviews. 50(1), 40-62.

HIKIDA, R.S. (1972)

Morphological transformation of slow to fast muscle fibres after tenotomy.

Exper. Neurol. 35, 265-273.

HIKIDA, R.S. (1973)

Avian fast and slow muscle: a comparison of their ultrastructure, histology, histochemistry and end-plates.

Anat. Records 175 (Abstract).

HIKIDA, R.S. (1976)

Regeneration of a tonic avian muscle: transformation to a twitch morphology.

Dev. Biol. 48, 67-79.

HIKIDA, R.S. and BOCK, W.J. (1972)

Effect of dennervation on pigeon slow skeletal muscle.

Z. Zellforsch. 128 (1), 1-18.

HIKIDA, R.S. and WANG, R.J. (1981)

Tenotomy of the avian ALD muscle. I. Effect of age on fibre-type transformation and regeneration from the stump in chickens.

Am. J. Anat. 160, 395-408.

HOCH, F.L. and LIPMAN, F. (1954)

The uncoupling of respiration and phosphorylation by thyroid hormones.

Proc. Natl. Acad. Sci., U.S.A., 40, 909-921.

HOLLY, R.G., BARNETT, J.G., ASHMORE, C.R., TAYLOR, R.G. and MOLÉ, P.A. (1980)

Stretch-induced growth in chicken wing muscles: a new model of stretch hypertrophy.

Am. J. Physiol. 238 (Cell Physiol. 7), C62-C71.

HUMOLLER, F.L., HATCH, D. and McINTYRE, A.R. (1951)

Effect of neurotomy on hexokinase and phosphorylase activities of rat muscle.

Am. J. Physiol. 167, 656-664.

IANUZZO, D., PATEL, P., CHEN, V. and O'BRIEN, P. (1980)

A possible thyroidal trophic influence on fast and slow skeletal muscle myosin.

In: Plasticity of Muscle. Ed. D. Pette, 593-605.

(N.Y., de Gruyter).

ILLINGWORTH, B. and CORI, G.T. (1953)

Crystalline muscle phosphorylase.

Biochem. Preps. 3, 1-9.

JANSSEN, J.W., HARDEVELD, C. van, KASSENAR, A.A.H. (1978)

Evidence for a different response of red and white skeletal muscle of the rat in different thyroid states.

Acta. Endocrinol. 87, 768-775.

JIRMANOVÁ, I. and ZELENA, J. (1975)

Ultrastructural transformation of fast chicken muscle fibres induced by nerve-cross union.

Z. Zellforsch. 146, 104-121.

JOHNSON, M.A., MASTAGLIA, F.L., MONTGOMERY, A.G., POPE, B. and WEEDS, A.G. (1980)

Changes in myosin light chains in the rat soleus after thyroidectomy.

Febs Lett. 110, 230-235.

JULIAN, F.J. (1971)

The effect of calcium on the force-velocity relation of briefly glycerinated frog muscle fibres.

J. Physiol. (Lond.) 218, 117-145.

KELLER, P.J. and CORI, G.T. (1953)

Enzymic conversion of phosphorylase a to phosphorylase b.

Biochem. et Biophys. Acta 12, 235-238.

KELLER, P.J. and CORI, G.T. (1955)

Purification and properties of the phosphorylase-rupturing enzyme.

J. Biol. Chem. 214, 127-134.

KENDALL, E.C. (1915)

The isolation in crystalline form of the compound containing iodine which occurs in the thyroid: its chemical nature and physiological activity.

Trans. Assoc. Am. Physicians 30, 420-449.

KENDRICK-JONES, J., SZENTKIRALYI, E.M. and SZENT-GYÖRGYI, A.G. (1976)

Regulatory light chains in myosin.

J. Mol. Biol. 104, 747-775.

KERRICK, W.G.L. and DONALDSON, S.K.B. (1972)

The effects of Mg^{2+} on submaximum Ca^{2+}-activated tension in skinned fibers of frog skeletal muscle.

Biochemica et Biophysica Acta 275, 117-122.

KERRICK, W.G.L., SECRIST, D., COBY, R. and LUCAS, S. (1976)

Development of difference between red and white muscle sensitivity to Ca^{2+} in the rabbit from embryo to adult.

Nature 260, 440-441.

KHAN, M.A. (1976)

Histochemical sub-types of three fiber-types of avian skeletal muscle.

Histochemie 50, 9-16.

KING, D.B. and KING, C.R. (1973)

Thyroidal influence on early muscle growth of chickens.

Gen. Comp. Endocrinol. 21, 517-529.

KING, D.B. and KING, C.R. (1976)

Thyroidal influence on gastrocnemius and sartorius muscle growth in young white Leghorn Cockerels.

Gen. Comp. Endocrinol. 29, 473-479.

KING, D.B., KING, C.R. and JACARUSO, R.B. (1980)

Avian muscular dystrophy: Thyroidal influence on pectoralis muscle growth and glucose-6-phosphate dehydrogenase activity.

Life Sciences 28, 577-585.

KLANDORF, H. (1982)

The regulation of plasma thyroid hormones in poultry.

Ph.D. Thesis, Dept. of Reproductive Physiology, Agricultural Research Council's Poultry Research Centre, Roslin, Midlothian, Scotland.

KÖENIG, J. and FARDEAU, M. (1973)

Étude histochimique des muscles grands dorsaux antérieur et postérieur du pulet et des modifications observees apres dénervation et réinnervation homologue ou croisee.

Arch. Anat. Microsc. 62, 249-267.

KREBS, E.G., GRAVES, D.J. and FISCHER, E.H. (1959)

Factors affecting the activity of muscle phosphorylase b kinase.

J. Biol. Chem. 234(2), 2867-2873.

KRÜGER, P. and GÜNTHER, P.G. (1955)

Fasern mit 'Fibrillenstruktur' und Fasern mit 'Felderstruktur' in der quergestreiften skeletmuskulatur der Sänger und des Menschen.

Z. Ges. Anat. 118, 313-323.

LEE, S.Y. (1971)

A histochemical study of twitch and tonus fibres.

J. Morph. 133, 253-271.

LEGRAND, J. (1979)

Morphogenetic actions of thyroid hormones.

Trends in Neurosciences, 234-236.

LEHMAN, W. (1978)

Thick-filament-linked calcium regulation in vertebrate striated muscle.

Nature 274, 80-81.

LEHMAN, W. and SZENT-GYÖRGYI, A.G. (1975)

Regulation of muscular contraction. Distribution of actin control and myosin control in the animal kingdom.

J. Gen. Physiol. 66, 1-30.

LEONARD, S.L. (1957)

Phosphorylase and glycogen levels in skeletal muscle of mice with hereditary myopathy.

Proc. Soc. Exp. Biol. Med. 96, 720-722.

LEONARD, S.L. (1957)

The effects of hormones on phosphorylase activity in skeletal muscle.

Endocrinology 60, 619-624.

LIPPE, M.S., GASSMAN, J.I., SOLTOFF, S.P. and KING, D.B. (1977)

Thyroidal influence on the lactate dehydrogenase isozyme pattern in pectoralis muscle of chick embryos.

Gen. Comp. Endocrinol. 33, 405-411.

LOHMANN, K. (1934)

Weitere Untersuchungen über das Co-Ferment der Milchsäurebildung.

Biochem. Z. 271, 278-279.

LOWEY, S., SLAYTER, H.S. and WEEDS, A.G. (1969)

Substructure of the myosin molecule.

J. Mol. Biol. 42, 1-29.

LUNDBAEK, K. and GORANSON, E.S. (1949)

The effect of fasting on muscle phosphorylase activity in the rat.

Acta. Physiol. Scand. 17, 280-288.

LYMN, R.W. and TAYLOR, E.W. (1970)

Transient state phosphate production in the hydrolysis of nucleoside triphosphates by myosin.

Biochem. 9, 2975-2983.

McNEIL ALEXANDER, R. and GOLDSPINK, G. (Eds.) (1977)

Mechanics and Energetics of Animal Locomotion.

London, Chapman and Hall.

MAGEE, B. (1979)

"Popper", p. 39.

Fontana Modern Masters.

MARTINS, C. and HESS, B. (1951)

The mode of action of thyroxine.

Arch. Biochem. Biophys. 33, 486-487.

MATSUBURA, I. and ELLIOT, G.F. (1972)

X-ray diffraction studies on skinned single fibres of frog skeletal muscle.

J. Mol. Biol. 72, 657-669.

MATSUMOTO, Y., HOEKMAN, T. and ABBOTT, B.C. (1973)

Heat measurements associated with isometric contraction in fast and slow muscles of the chicken.

Comp. Biochem. Physiol. 46A, 785-797.

MIKAWA, T., NONOMURA, Y., HIRATA, M., EBASHI, S. and KAKIUCHI, S.

(1978)

Involvement of an acidic protein in the regulation of smooth muscle contraction by the tropomyosin-leiotonin system.

J. Biochem. (Tokyo) 84, 1633-1636.

MOISESCU, D.G. (1976)

Kinetics of reaction in Ca^{2+}-activated skinned muscle fibres.

Nature 262, 610-613.

MOSS, R.L. (1979)

Sarcomere length-tension relations of frog skinned muscle fibres during calcium activation at short lengths.

J. Physiol. (Lond.) 292, 177-192.

MURRAY, G.R. (1891)

Note on the treatment of myxedema by hypodermic injections of an extract of the thyroid gland of a sheep.

Brit. Med. J. 2, 796-797.

MURRAY, J.H. and WEBER, A. (1974)

The co-operative action of muscle proteins.

Sci. Amer. 230(2), 58-71.

NACHLAS, M.M., TSOU, K.C., DE SOUZA, E., CHENG, C.S. and SELIGMAN, A.M. (1957)

Cytochemical demonstration of succinic dehydrogenase by the use of a new p-Nitrophenyl substituted Ditetrazole:

J. Histochem. Cytochem. 5, 420-437.

NATORI, R. (1954)

The property and contraction process of isolated myofibrils.

Jikeikai Med. J., 1, 119-126.

NEMETH, P.M., PETTE, D. and VRBOVÁ, G. (1980)

Malate dehydrogenase homogeneity of single fibres of the motor unit.

In: Plasticity of Muscle, Ed. D. Pette, 45-55.

NENE, R.V. and CHINOY, N.J. (1965)

Histochemical observations of the avian Mm. latissimus dorsi, anterior and posterior.

Pavo. 3, 29-34.

NEWSHOLME, E.A. and START, C. (1974)

Regulation in Metabolism, p.151, Wiley Publication.

NICOL, C.J.M. and JOHNSTON, I.A. (1981)

Energy metabolism of fast- and slow-twitch skeletal muscle in the rat: thyroid hormones induced changes.

J. Comp. Physiol. 142, 465-472.

NWOYE, L. and MOMMAERTS, W.F.H.M. (1981)

The effects of thyroid status on some properties of rat fast-twitch muscle.

J. Muscle Res. & Cell Motility 2, 307-320.

NWOYE, L., MOMMAERTS, W.F.H.M., SIMPSON, D.R., SERAYDARIAN, K. and MARUSICH, M. (1982)

Evidence for a direct action of thyroid hormone in specifying

muscle properties.

Am. J. Physiol. 242, R401-R408.

OGATA, T. (1958)

A histochemical study of the red and white muscle fibres. Part I. Activity of the succinooxydase system in muscle fibres.

Acta. Med. Okayama. 12, 216-227.

OGATA, T. and MORI, M. (1964)

Histochemical study of oxidative enzymes in vertebrate muscles.

J. Histochem. Cytochem. 12, 171-182.

OVALLE, W.K. (1978)

Histochemical dichotomy of extrafusal and intrafusal fibers in an avian slow muscle.

Am. J. Anat. 152(4), 587-598.

PADYKULA, H.A. and GAUTHIER, G.F. (1967)

Morphological and cytochemical characteristics of fiber types in normal mammalian skeletal muscle. In: Exploratory concepts in muscular dystrophy and related disorders. Ed. A.T. Milhorat, Amsterdam. Excepta. Med. Found., 117-128.

PADYKULA, H.A. and HERMAN, E. (1955 (a))

Factors affecting the activity of adenosine triphosphatase and other phosphatases as measured by histochemical techniques.

J. Histochem. Cytochem. 3, 161-169.

PADYKULA, H.A. and HERMAN, E. (1955 (b))

The specificity of the histochemical method for adenosine triphosphatase.

J. Histochem. Cytochem. 3, 170-195.

PAGE, S. (1969)

Structure and some contractile properties of fast and slow muscles of the chicken.

J. Physiol. (Lond.) 205, 131-145.

PAGE, S. and SLATER, C.R. (1965)

Observations on the fine structure and rate of contraction of some muscles from the chicken.

J. Physiol. (Lond.) 179, 58-59 P.

PENNEY, R.K. (1978)

Thermal acclimation and excitation - contraction coupling in the epaxial musculature of the goldfish (Carassius auratus, L.).

Ph.D. Thesis, Dept. Zoology, Univeristy of Hull.

PERRIN, D.D. and SAYCE, I.G. (1967)

Computer calculations of equilibrium concentrations in mixtures of metal ions and complex species.

Talanta 14, 833-842.

PERRY, S.V. (1979)

The regulation of contractile activity in muscle.

Biochem. Soc. Trans. 7, 593-617.

PERRY, S.V. and GREY, T.C. (1956)

Ethylenediamine tetra-acetate and the adenosine triphosphatase activity of actomyosin systems.

Biochem. J. 64, 5P.

PETER, J.B., BARNARD, R.J., EDGERTON, V.R., GILLESPIE, C.A. and STEMPEL, K.E. (1972)

Metabolic profiles of three fiber types of skeletal muscle in guinea pigs and rabbits.

Biochem. 11(14), 2627-2633.

PETTE, D., VRBOVÁ, G. and WHALEN, R.C. (1979)

Independent development of contractile properties and myosin light chains in embryonic chick fast and slow muscle.

Pflügers Arch. 378, 251-257.

PIRES, E., PERRY, S.V. and THOMAS, M.A.W. (1974)

Myosin light-chain kinase, a new enzyme from striated muscle.

Febs Lett. 41, 292-296.

POTTER, J.D. and GERGELY, J. (1975)

The calcium and magnesium binding sites on troponin and their role in the regulation of myofibrillar adenosine triphosphatase.

J. Biol. Chem. 250, 4628-4633.

RAMSEY, R.W. and STREET, S.F. (1940)

Isometric length-tension diagrams of isolated skeletal muscle fibres of the frog.

J. Cell. Comp. Physiol. 15, 11-34.

REASONS, R.H. and HIKIDA, R.S. (1973)

Biochemistry of adenosine triphosphatase activity of avian fast and slow muscle.

Exper. Neurol. 38(1), 27-32.

REITSMA, W. (1969)

Skeletal muscle hypertrophy after heavy exercise in rats with surgically reduced muscle function.

Am. J. Physical Med. 48(5), 237-258.

RITCHIE, J.M. and WILKIE, D.R. (1956)

Muscle: Physical Properties.

In: Handbook of Biological Data, W.S. Spector, Ed. W.B. Saunders Company, Philadelphia.

ROCKSTEIN, M. and HERRON, P.W. (1951)

Calorimetric determination of inorganic phosphate in microgram quantities.

Anal. Chem. 23, 1500-1501.

ROMANUL, F.C.A. (1964)

Enzymes in muscle. I. Histochemical studies of enzymes in individual muscle fibres.

Arch. Neurol. 11, 355-369.

RYAN, D.M. and SHAFIQ, S.A. (1980)

A freeze-fracture study of the anterior and posterior latissimus dorsi muscles of the chicken.

The Anat. Record 198, 147-161.

SARKAR, S., SRETER, F.A. and GERGELY, J. (1971)

Light chains of myosin from white, red and cardiac muscles.

Proc. Natl. Acad. Sci., U.S.A., 68, 946-950.

SETH, J., TOFT, A.D. and IRVINE, W.J. (1976)

Simple solid-phase radioimmunoassays for total triiodothyronine and thyroxine in serum and their clinical evaluation.

Clin. Chem. Acta. 68, 291-301.

SHEAR, C.R. (1978)

Cross-sectional myofibre and myofibril growth in immobilized developing skeletal muscle.

J. Cell Sci. 29, 297-312.

SHEAR, C.R. (1981)

Effects of disuse on growing and adult chick skeletal muscle.

H II Cell Sci. Article 806, 1-20.

SHEAR, C.R. and GOLDSPINK, G. (1971)

Structural and physiological changes associated with the growth of avian fast and slow muscle.

J. Morph. 135, 351-372.

STEIN, J.H. and PADYKULA, H.A. (1962)

Histochemical classification of individual skeletal muscle fibres of the rat.

Am. J. Anat. 110, 103-124.

STERLING, K. (1979)

Thyroid hormone action at the cell level.

N. Engl. J. Med. 300, 173-177.

SWANSON, M.A. (1948)

Studies on the structure of polysaccharides. IV. Relation on the iodine colour to the structure.

J. Biol. Chem. 172, 825-837.

SZENT-GYÖRGYI, A.G. (1949)

Free-energy relations and contraction of actomyosin.

Biol. Bull. 96, 140-161.

SZENT-GYÖRGYI, A.G. (1975)

Calcium regulation of muscle contraction.

Biophys. J. 15, 707-723.

TAKEUCHI, T. and KURIAKI, H. (1955)

Histochemical detection of phosphorylase in animal tissue.

J. Histochem. Cytochem. 3, 153-160.

TASSONI, J.P., MANTEL, L. and HARMAN, P.J. (1964)

Enzyme alterations in muscle cells from mice with hereditary dystrophy.

Exp. Cell. Res. 35, 219-229.

TAYLOR, S.R. and GODT, R.E. (1976)

Calcium release and contraction in vertebrate skeletal muscle.

S.E.B. Symposium, 361-380.

THAMES, M.P., TEICHOLZ, L.E. and PODOLSKY, R.J. (1974)

Ionic strength and the contraction kinetics of skinned muscle fibres.

J. Gen. Physiol. 63, 509-530.

TOOP, J. (1976)

A rapid method for demonstrating skeletal muscle motor innervation in frozen sections.

Stain Technology 51(1), 1-6.

TOUTANT, J.P., ROUAUD, T. and LE DOUARIN, G.H. (1981)

Histochemical properties of the biventer cervicis muscle of the chick: a relationship between multiple innervation and slow-tonic fibre types.

Histochem. J. 13, 481-493.

TOUTANT, J.P., TOUTANT, M.N., RENAUD, D. and LE. DOUARIN, G.H. (1979)

Enzymatic differentiation of muscle fibre types in embryonic latissimus dorsi of the chick: Effects of spinal cord stimulation.

Cell. Diff. 8, 375-382.

TUNELL, G.L. and HART, M.N. (1977)

Simultaneous determination of skeletal muscle fiber, types I, IIA, and IIB by histochemistry.

Arch. Neurol. 34, 171-173.

ULBRECHT, M. (1962)

Der Austauch und Abspaltung des ϒ-phosphate des Adenosin-triphosphates durch Sarkosomen und kleine Grana des Kaninchen-Muskles.

Biochem., Biophys., Acta. 57, 455-474.

VARGA, L. (1946)

Hung. Acta. Physiol. 1, 1.

VRBOVÁ, G., GORDON, T. and JONES, R. (1978)

"Nerve-muscle interaction"

London, Chapman and Hall.

WEEDS, A.G., HALL, R. and SPURWAY, N.C.S. (1975)

Characterisation of myosin light chains from histochemically identified fibres of rabbit psoas muscle.

Febs Lett. 49, 320-324.

WENTWORTH, B.C. and MELLEN, W.J. (1961)

Effect of thiouracil on plasma PBI^{131} in the fowl.

Poult. Sci. 40, 1275-1276.

WHITE, D.C.S. and THORSON, J. (1972)

Phosphate starvation and the non-linear dynamics of insect fibrillar flight muscle.

J. Gen. Physiol. 60, 307-336.

WHITE, D.C.S. and THORSON, J. (1975)

"The kinetics of muscle contraction"

Pergamon Studies in the Life Sciences.

WILLIAMS, P.E. and GOLDSPINK, G. (1973)

The effect of immobilisation on the longitudinal growth of striated muscle fibres.

J. Anat. 116(1), 45-55.

WILLIAMS, P.E. and GOLDSPINK, G. (1978)

Changes in sarcomere length and physiological properties in immobilised muscle.

J. Anat. 127(3), 459-468.

WINEGRAD, S. (1971)

Studies of cardiac muscle with a high permeability to calcium produced by treatment with Ethylene Diamine Tetraacetic Acid. (EDTA)

J. Gen. Physiol. 58, 71-93.

WISE, R.M., RONDINONE, J.F. and BRIGGS, F.N. (1971)

Effect of calcium on force-velocity characteristics of glycerinated skeletal muscle.

Am. J. Physiol. 221(4), 973-979.