Age related changes and tissue distribution of parvalbumin in both normal and dystrophic 129 ReJ mice.

Douglas Standish. Greaves

University of Windsor

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AGE RELATED CHANGES AND TISSUE DISTRIBUTION OF PARVALBUMIN IN BOTH NORMAL AND DYSTROPHIC 129 ReJ MICE

by

Douglas Standish Greaves

A Thesis

Submitted to the Faculty of Graduate Studies and Research Through the Department of Biological Sciences

In Partial Fulfillment of the Requirements for the

Degree of Master of Science at the

University of Windsor

Windsor, Ontario, Canada

1989
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To Kathy and my family, who have endured much.
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Lastly, I wish to thank my colleagues for their friendship and help over the years.
ABSTRACT

In murine muscular dystrophy (strain 129 ReJ), hindlimb muscle contains a functionally defective thiol protease inhibitor (TPI) which has been implicated in the onset and progression of the disease in this strain of mice. More recently, and through the work of several researchers, this protease inhibitor has been identified as parvalbumin, a calcium binding protein. In this study, a polyclonal antibody raised in rabbits against normal mouse muscle parvalbumin (TPI) was used to study the concentration and tissue distribution of this protein in both normal and dystrophic male mice at various ages. Western blotting assays were used to screen extracts of hindlimb, forelimb, brain, heart, lung, liver and kidney in 60 day old normal and dystrophic male mice for parvalbumin content. Parvalbumin was detected in relatively high amounts in both hindlimb and forelimb muscle extracts while much lower concentrations were detected in brain of both normal and dystrophic animals. No parvalbumin was detected in lung, liver, heart or kidney preparations using the above antibody. With aging, the parvalbumin concentration in hindlimb muscle extracts of normal mice was found to remain fairly constant (on a percent protein basis) for 90 days whereupon the levels increased. In contrast, in dystrophic mice the parvalbumin concentration decreased steadily with age to about 22% of control animals at 120 days.
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<table>
<thead>
<tr>
<th>Ab</th>
<th>antibody</th>
</tr>
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<tbody>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>Ab-Ag</td>
<td>antibody-antigen complex</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolylphosphate-p-toluidine salt</td>
</tr>
<tr>
<td>BMD</td>
<td>Becker's muscular dystrophy</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne's muscular dystrophy</td>
</tr>
<tr>
<td>CANP</td>
<td>calcium activated neutral protease</td>
</tr>
<tr>
<td>CDNP</td>
<td>calcium dependent neutral protease</td>
</tr>
<tr>
<td>CDPI</td>
<td>calcium dependent protease inhibitor</td>
</tr>
<tr>
<td>DT'T</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetate (disodium salt)</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol bis(h-aminoethylether N,N'-tetraacetic acid</td>
</tr>
<tr>
<td>EMD</td>
<td>Emery-Dreifuss muscular dystrophy</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>MyD</td>
<td>myotonic muscular dystrophy</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium chloride salt</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PV</td>
<td>parvalbumin</td>
</tr>
<tr>
<td>TPI</td>
<td>thiol protease inhibitor</td>
</tr>
<tr>
<td>TPI-n</td>
<td>thiol protease inhibitor from normal mouse muscle extracts</td>
</tr>
<tr>
<td>TPI-d</td>
<td>thiol protease inhibitor from dystrophic mouse muscle extracts</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TTBS</td>
<td>tris buffered saline with 0.05% Tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>-N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>dH₂O</td>
<td>nanopure distilled water</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
</tbody>
</table>
INTRODUCTION

Muscular dystrophy can be described as the progressive loss of muscle tissue and its eventual replacement by fat and connective tissue. In humans, 17 different muscular dystrophies have been characterized and described by Gardner-Medwin (1980). "As a common feature, all muscular dystrophies are hereditary, involve voluntary muscles and tend towards a progressive deterioration of the muscles due to a gradual and piecemeal necrosis. All diseases present with muscle weakness, are insidious in their onset and continuously progressive".

In this introduction, a few of the human muscular diseases, namely the X-linked Duchenne (DMD), Beckers (BMD) and Emery-Dreifuss (EMD) forms of muscular dystrophy and a myotonic form of dystrophy will be discussed. Next, some hypotheses regarding the primary cause of the various myopathies will be discussed. In order to study the various human conditions, animal models were utilized and this paper will examine how murine models of dystrophy address both the genotypic and phenotypic expression of the human muscular dystrophy genes. The focus will then shift to the murine model used in this study, namely the 129 ReJ normal (+/+ ) and dystrophic (dy/dy) mouse. The 129 ReJ mouse has been used as a model to investigate the myolytic processes occurring in muscular dystrophy. Some of the biochemical differences between normal and dystrophic muscle will be discussed, and the paper will then show how studies on biochemical differences led to the discovery of a defective thiol protease inhibitor in dystrophic muscle of considerable relevance to this thesis (Gopalan et al., 1986-1987). Finally, the rationale behind the experiments reported here on the status of the protein, parvalbumin, in dystrophic muscle will be outlined.

1.1 Duchenne, Becker’s, Emery-Dreifuss and Myotonic Muscular Dystrophies.

The human Duchenne form of muscular dystrophy (DMD) affects one in every 3300 newborn males (Gardner-Medwin, 1980). Affected individuals first walk at 18 months of age. By 4 years they exhibit a waddling gait and demonstrate hypertrophy
of the calf muscles. Next, the lateral vasti of the quadriceps, deltooids, extensor muscles in the forearms and the temporal muscles are affected. Children with DMD are normally confined to a wheelchair by 12 years of age and death occurs commonly due to respiratory failure before the beginning of the third decade of life (Malhotra et al., 1988).

Cytological studies have demonstrated many differences between normal and dystrophic muscle. In humans, dystrophic muscle shows an early leakage, as demonstrated by the use of horse radish peroxidase, in a wedge shaped pattern in the muscle fibre prior to necrosis (Mokri and Engel, 1975). These "delta lesions" are found beneath the sarcolemma of the hypercontracted fibres and their base rests on the fibre surface (Morizumi et al., 1984). A contracted sarcomere is noted around the lesion (Morizumi et al., 1984). In the 129/ReJ dy/dy mouse, horse radish peroxidase leakage occurs in muscle prior to its phagocytosis (Mendell et al., 1979). However, this leakage does not occur as discrete delta lesions, but rather as extensive leakage across the entire cross-sectional diameter of the muscle fibre. Other observations have indicated that the myofibrils are sparse; the sarcoplasmic reticulum is dilated and mitochondria appear to be swollen in the human dystrophic muscle (Morizumi et al., 1984). From the literature, other features of DMD include: 1) elevated serum creatine kinase activity (Rowland, 1980; Gardner-Medwin, 1980; Perry, 1984; Dickson et al., 1989) which appears to reach peak levels (about 200 times normal) at 2 years of age (Perry, 1984), and 2) mental retardation which is associated with DMD in 30% of the cases. In these individuals, the mean IQ is between 70 and 85 (Gardner-Medwin, 1980).

Becker's muscular dystrophy (BMD) is much less severe than the Duchenne form and it has been described as "benign dystrophy" (Gardner-Medwin, 1980). Patients with BMD can usually walk until age 16 and generally have a lifespan of between 40 and 50 years (Malhotra et al., 1988). The BMD locus has been linked to the DMD locus on the human X chromosome and patients with BMD have deletions which can be detected using the DMD probes (Monaco and Kunkel,
1987, Witkowski, 1988; Nudel et al., 1988). It is now believed that the BMD mutations may represent in-frame translational deletions while the DMD mutations represent frame-shift deletions (Witkowski, 1988). In BMD individuals, this results in the production of a semi-functional protein product and helps to explain the delay in the onset and severity of this form of dystrophy (Witkowski, 1988).

Emery-Dreifuss muscular dystrophy (EMD) was first described in 1966 (Gardner-Medwin, 1980). It is characterized as "having an X-linked transmission, a benign myopathy with atrophy of the lower legs and upper arms, contractures of the neck, elbow and ankles (equinus contractures) and cardiomyopathy with conduction block". Also, a high incidence of "sudden death" due to heart failure has been reported to be associated with this form of dystrophy.

Myotonic muscular dystrophy (MyD) is an autosomally inherited disorder (found on human chromosome 19) producing myotonia, progressive weakness and eventual muscle atrophy (Hartwig et al., 1982). Many hypotheses exist as to the reason for the prolonged contractions which diminish with repeated muscle use and after twitches associated with this form of dystrophy. Work with the myotonic adr mouse mutant (arrested development of righting response) has demonstrated a decrease in the parvalbumin content in the adr muscle (Stuhlfauth et al., 1984; Jockusch et al., 1988; Kluxen et al., 1988), a decreased fast-myosin light chain 2 (LC-2f) phosphorylation (Jockusch et al., 1988) and a defective Ca$^{2+}$ uptake into the sarcoplasmic reticulum of the adr muscle (Leberer et al., 1988). All of these defects could lead to a decreased movement of Ca$^{2+}$ into the sarcoplasmic reticulum after the contraction and could lead to prolonged after-contractions (Zeeman and Sandrow, 1979, Leberer et al., 1988).

1.2 Hypotheses as to the Aetiology of Duchenne Muscular Dystrophy.

Many hypotheses as to the primary defect in Duchenne muscular dystrophy have emerged. A neurogenic hypothesis was proposed which stated that neurogenic factors are important in the genesis of human and animal dystrophies
(Appenzeller and Ogin, 1975). This hypothesis was based on the observation that a selective loss of motor units is seen in Duchenne, myotonic fasioscapulohumeral and limb-girdle muscular dystrophies. It has since been widely shown that a characteristic of dystrophic muscle is a decrease in the number of functional fast-twitch muscle fibres (Takagi et al., 1982; Lexell et al., 1983; Leberer et al., 1987). Early and frequent stimulation of immature muscle fibres destined to become fast twitch muscle by mature motor neurons could lead to the destruction of the immature fibres as they cannot handle the activity imposed upon them by the adult motor neurons (Vrbova, 1983). Confirmatory evidence supporting the above came from a later murine study performed by Wirtz and Loermans, 1983. This group of researchers found that immobilization of the dystrophic mouse hindlimb prevented muscle necrosis and concluded that contractile activity correlated with the observed muscle damage in the mouse muscle. Other observations such as denervation studies comparing normal and dystrophic fast and slow twitch muscle energy levels (Clow and Boegman, 1987) and their ability to incorporate Ca\(^{2+}\) into the sarcoplasmic reticulum (Mrak and Fleicher, 1982; Leberer et al., 1988) also support a neurogenic model.

A vascular hypothesis was proposed which suggested that muscular dystrophy is due to a decrease in the vascularization of dystrophic muscles (Appenzeller and Ogin, 1975). This phenomenon is now believed to be due to a shunting of blood around the diseased muscle and this hypothesis has now been generally discarded. An extension of this hypothesis is the sympathetic neurovascular hypothesis which states that muscle hypoactivity is due to decreased vasomotor tone of the sympathetic vasomotor neurons (Appenzeller and Ogin, 1975). This in turn leads to vasoconstriction; the blood moves away from the muscles and the resultant ischemia leads to the leakage of muscle enzymes. As stated previously, the vascular hypotheses are now believed to describe secondary events to the primary dystrophic lesion and the above hypotheses have been generally discarded.
The membrane hypothesis states that a genetic fault of DMD affects an enzyme or structural protein which is decreased or rendered functionally abnormal due to an altered amino acid sequence. This altered protein results in altered composition and function of the muscle membrane surface proteins (Rowland, 1980). There are many studies which have demonstrated defective membrane functions. For example, the Ca$^{2+}$-ATPase in the sarcoplasmic reticulum of dystrophic muscle has decreased activity (Leberer et al., 1988). Dystrophin (the protein product of the DMD locus) was initially believed to be associated with the terminal cisternae of the sarcoplasmic reticulum, invaginations of the plasma membrane and the myofilaments (Hoffman et al., 1987c). "These muscle triads provide a pathway whereby excitation of the muscle fibre surface can trigger contraction in normal muscle and the dystrophin protein is absent in dystrophic muscle" (Hoffman et al., 1987c). Using antibodies raised against fragments of adult mouse cardiac dystrophin, it has been demonstrated "that dystrophin has been localized to the inner surface of the plasma membrane and is likely to be associated with an integral membrane glycoprotein" (Monaco, 1988).

Another extension of the membrane hypothesis relates directly to the lipid composition of the dystrophic muscle membrane. An increased cholesterol:phospholipid ratio (Totsuka et al., 1981a; Totsuka et al., 1982) suggests that dystrophic muscle membranes may have an increased fluidity due to their elevated cholesterol content and this in turn may cause the lipid bilayers to move apart (Totsuka et al., 1982). Cornellio and Dones in 1984 showed the presence of albumin and C3 and C9 complement components (extracellular markers) in a significant number of necrotic fibres in 164 DMD muscle biopsies. They suggested that the massive muscle fibre necrosis is linked to the massive inflow of extracellular fluid through the permeable membranes and complement activation occurs. It has also been reported that fibroblasts associated with DMD muscle have altered lysosomal membranes (Gelman et al., 1981).
An extension of the membrane hypothesis is that some lysosomal proteolytic enzymes are not properly compartmentalized in lysosomes. Hence, there is leakage of these proteases into the muscle cell itself and these hydrolases increase the rate of intracellular proteolysis. Also, there is an increasing body of evidence which demonstrates an increase in the activity of calcium dependent neutral proteases (calpains) (Rowland, 1980; Klamut et al., 1983) as well as the lysosomal proteases; cathepsin B (Iodice et al., 1972; Hashida et al., 1981; Spanier and Bird, 1982; Komatsu et al., 1986), cathepsin L (Hashida et al., 1982; Kominami et al., 1984b; Komatsu et al., 1986) and cathepsin D (McGowan et al., 1976; Spanier and Bird, 1982) in dystrophic muscle as compared to age-matched normal muscle. Consequently, an overall net loss of muscle protein might be expected when the rate of protein degradation exceeds that of its synthesis. The importance of a shift in the equilibrium between synthesis and degradation of muscle protein in dystrophic animals will be examined later in this thesis.


"While perhaps no animal model can be expected to be identical in every way to any one human neuromuscular disease, each has features that are important in relationship to particular aspects of human muscle pathology" (Mendell et al., 1979). This statement holds true for the Duchenne, Becker and Emery-Dreifuss forms of muscular dystrophy. All of these are X-linked human myopathic diseases, but the X-linked murine model (the mdx mouse) does not show the progressive deterioration of the fast twitch muscle fibres seen in the human conditions (Tanabe et al., 1986). However, this X-linked dystrophic mouse model has revealed several important features regarding the genetics of several human myopathies. For this reason, some of the murine studies will now be discussed in more detail.

Kunkel and Worton spearheaded two approaches used to isolate and characterize the DMD locus. According to the review of Witkowski (1988), Worton et al set out to isolate and sequence the gene by studying autosomal translocations within the DMD gene. This group compared the X chromosomes from both normal and
dystrophic carrier females and they then identified regions of the X chromosome at the junction of the translocations. These sequences were cloned and used as probes for the DMD gene. Using these probes, the mRNA's produced from the DMD (and BMD) locus were isolated and studied. It was determined that the translational reading frames in DMD mRNA's were disrupted, whereas only 13 of the 29 BMD patients mRNA's had a disruption of the translational reading frame (Malhotra et al., 1988). They believed that the other 13 BMD mRNA's which were found to disrupt the reading frame were still capable of forming a smaller, semi-functional protein, possibly due to the occurrence of a new, in-frame translational start codon immediately downstream from the mutation site (Malhotra et al., 1988).

Kunkel and coauthors approached the gene isolation by studying actual deletions in the male X-chromosome. Working with Kunkel, deMartinville et al. (1985) demonstrated that the DMD gene is located on the Xp21 arm of the X-chromosome near the markers Msp1, QTC, B24 and C7. In 1986, Monaco et al. (1986) isolated cDNA fragments within the DMD gene. This cloned gene (probe DSX164 or pERT 87) was then used to detect DNA deletions in unrelated DMD and BMD males to collect more sequence data. Kunkel then shared his pERT 87 probe with many researchers who then screened DMD and BMD carrier and patients DNA (Kunkel et al., 1986). These researchers determined that DMD deletions could be reliably detected 5% of the time using only the pERT 87 probe, suggesting that the the DMD locus is very large and that deletions in this locus are frequent (Kunkel et al., 1986).

The DMD gene was shown also to be missing on the mdx mouse X-chromosome (Bulfield et al., 1984, Brockdorff et al., 1987; Chamberlain et al., 1987; Heilig et al., 1987). The Chamberlain group further showed that the mdx gene position was near both the DMD(BMD) and EMD human gene markers and that both EMD and DMD may have originated from a common ancestral mdx gene.
In 1987, Koenig et al. isolated, cloned and sequenced the complete 14 Kb human DMD cDNA. This huge gene transcript was formed by 60 exons, all of which mapped to Xp21 (Koenig et al., 1987). The first half of the transcript is formed by a minimum of 33 exons spanning nearly 1000 Kb and the remainder is formed by 27 exons which may be spread over a similar distance (Koenig et al., 1987). They found that: the majority of deletions arose in a single genomic segment corresponding to only 2 Kb, that 1/3 of all DMD cases arise from new mutations, and that the genomic locus for DMD is nearly 200 times the size of the mRNA transcript (Koenig et al., 1987). These observations suggested that the DMD gene comprises nearly 0.1% of the total human genome (Koenig et al., 1987).

In the same year, the human DMD gene cDNA and mdx gene were compared by Hoffman et al. (1987a). A segment of the human DMD gene corresponding to 25% of the total gene transcript from skeletal muscle was sequenced and compared to the mdx locus. A comparison between human foetal skeletal muscle and adult mdx mouse heart DNAs demonstrated a nucleic acid sequence homology of 88% and a predicted amino acid sequence homology of 87% between the human and mdx transcripts (Hoffman et al., 1987b). In the mdx mouse, it was later shown that the DMD mRNA was detected in both skeletal and cardiac muscle, and at a 90% lower level in the brain (Chamberlain et al., 1988). Nudel and coworkers then examined rabbit, rat and mouse tissues to determine if the DMD gene was expressed. They found that the DMD gene was indeed expressed in rat and mouse myogenic cell lines, in rat and mouse striated muscle, in mouse smooth muscle and in rat, rabbit and mouse brain (Nudel et al., 1988).

The focus of research then shifted towards the identification of the protein product of the DMD locus. An early candidate protein was nebulin, a 550 Kd myofibrillar protein whose mRNA was 16 Kb in length (Wood et al., 1987). Nebulin is found at the junction of the A and I band and is missing in DMD patients' striated muscle (Wood et al., 1987). Hoffman et al. (1987b) argued that due to the tissue distribution of the DMD mRNA (DMD mRNA can be found in cardiac tissue but
nebulin cannot), and due to its abundance (nebulin comprises 3% of the total myofibrillar proteins; the DMD protein constitutes 0.002%), nebulin could not be the product of the DMD gene. They then isolated and identified dystrophin using polyclonal antibodies raised against fusion proteins containing two distinct regions of the mdx mouse mDMD cDNA. Dystrophin is a 400 Kd protein which comprises 0.002% of the total skeletal muscle protein and it is also present in smooth muscle. Since 30% of DMD patients exhibit mental retardation, they also checked for and detected small amounts of dystrophin in human brain.

Hoffman et al. (1987c) localized dystrophin to the muscle triads; intracellular structures in which there is a close association between the plasma membrane, the terminal cisternae of the sarcoplasmic reticulum and the myofilaments. The amino terminal 200 amino acids of dystrophin showed a striking similarity to the actin filament binding domain and thus, dystrophin probably interacts with the actin filaments in myofibers (Hoffman et al., 1987c). In this way, dystrophin may serve as an anchor for the triads by binding actin at its amino terminus. This group noted that mdx and DMD exhibit nearly identical patterns of myofibrillar degeneration and they concluded that this is probably a direct consequence of a dystrophin deficiency in the myofibres. It has since been demonstrated by Monaco (1988) that dystrophin is localized not in the muscle triads (as believed by Hoffman et al.) but rather on the inner surface of the plasma membrane in muscle cells and is probably associated with an integral membrane glycoprotein. This was demonstrated by the use of antibodies directed against fragments of mouse cardiac dystrophin. It has also been reported in other studies involving the use of specific anti-dystrophin antibodies that this protein is localized in the sarcolemma of human skeletal muscle (Zubrzycka-Gaarn et al., 1988) and is associated with the transverse tubules in rabbit skeletal muscle (Knudson et al., 1988).

In 1989, Dickson et al. reported the existence of two mRNA species in mouse skeletal muscle, an embryonic and an adult dystrophin mRNA. They demonstrated that the mRNA size in embryos increases to the adult form and they feel that this
indicates a developmentally regulated mRNA isoform switch of the DMD gene expression in skeletal muscle. This finding could help to explain some of the confusing clinical cases such as those reported by Zatz et al. in 1989. In the first study, they reported one DMD boy who walked until 19 years of age even though both of his brothers and seven other DMD relatives were confined to a wheelchair prior to their tenth birthdays (Zatz et al., 1989). They suggested that this boy may represent the case where the embryonic dystrophin mRNA was good but the adult dystrophin mRNA was defective. Their second case involved a boy whose creatine kinase activity at 2 years of age was highly elevated. The parents stopped treatment on the child and a follow-up was performed when the child turned 5 years old. The second testing revealed that the creatine kinase levels had returned to normal, and the child showed normal muscle strength and functional abilities. They suggested that this child represents the DMD population whose embryonic dystrophin mRNA is defective but whose adult dystrophin mRNA population is functional. Although very rare, it could explain the early myopathic processes in this child’s first two years of life which gradually disappeared when the adult mRNA isoform switch occurred. They concluded that the majority of DMD cases would have both embryonic and adult defective mRNAs.

From a genetic standpoint, it can be seen that the mdx mouse is a powerful animal model to describe some of the genetics behind human DMD. Unfortunately, the clinical course of the mdx murine dystrophy is very different from the human dystrophy. In the human, the muscle fibre necrosis is progressive and continuous. In human dystrophic muscle, there is an infiltration of fats and connective tissue into the necrotic fibres and it has even been suggested that this infiltration represents the primary lesion in DMD (Ionasescu et al., 1971; Sweeney and Brown, 1981). In the mdx mouse, 2 and 6 week old mouse anterior tibialis muscles show identical tension output, speeds of contraction and relaxation and overall muscle mass (Dangain and Vrbova, 1984). However, in the 3 to 4 week old mice, there are big decreases in mdx tibialis anterior muscles. A rapid synchronized necrosis of muscle clusters also occurs, but this is followed by complete muscle recovery (Dangain and Vrbova, 1984). The simultaneous necrosis at 30 days of age of clusters of
muscle fibres (containing groups of tens to hundreds) in an mdx mutant mouse strain in both the soleus and external digitorus longus muscles followed by a complete muscle recovery has also been reported by Tanabe et al. in 1986. The interstitial fibrosis and adipose tissue replacement were minimal, there was no apparent fibre loss and mdx mice developed no obvious muscle weakness (Tanabe et al., 1986). Since human DMD does not demonstrate this total muscle regeneration, other animal models are required to study the fiber necrosis and loss observed in the human myopathy.

1.4 Murine models of DMD: A Myopathic Approach.

Platzer and Powell (1975) described the cytological events in murine dystrophic breakdown in 129 ReJ dy/dy mice. "The first identifiable ultrastructural change is a dilation of the sarcoplasmic reticulum. Next, fragmented myofibrils appear and the mitochondria appear swollen and spherical and contain irregular cristae. As the muscle breakdown continues, the muscle fibres themselves break down, fewer cellular organelles (including the T system) are evident and myofibrillar fragmentation occurs. From here, further sarcolemmal breakdown occurs and necrosis and phagocytic infiltration follow" (Platzer and Powell, 1975). As stated previously, the horse radish peroxidase staining did not produce the discrete "delta lesions" in dystrophic 129 ReJ muscle as in the human DMD, but rather there was extensive leakage of horse radish peroxidase across the entire fibre (Mendell et al., 1979). However, this murine model resembles the pattern of muscle breakdown observed in human DMD in that this myopathy is progressive and distinct muscles are singled out for the myolysis. In the 129 ReJ dy/dy mouse, the affected muscles are the crown portion of the anterior tibialis (mostly fast-twitch glycolytic type 2B fibres), whereas the primary site of expression for the C57Bl/6J dy^{2J}/dy^{2J} mouse is in the slow twitch oxidative type 1 fibres of the core portion of the anterior tibialis and soleus muscle (Mendell et al., 1979). The differential targeting of affected muscles in these two murine dystrophic strains is characteristic of these particular strains of murine mutants.
The dy/dy and dy^{2J}/dy^{2J} strains of the C57Bl/6J mouse were both derived from crosses involving a C57Bl +/+ and a 129 ReJ dy/dy mutant (Parsons, 1974). With time, it became apparent that the dy/dy and dy^{2J}/dy^{2J} forms of dystrophy were different. A comparison of 129 ReJ dy/dy and C57Bl/6J dy^{2J}/dy^{2J} involving the quadriceps and triceps showed that the cultures derived from dy^{2J} muscle formed multinucleated myotubes, whereas dy/dy muscle cultures formed cell associations called "pseudostraps", but the myoblast aggregates did not fuse to form myotubes (Parsons, 1974). This suggested that the dy mutant causal lesion existed within the muscle cell itself, whereas the dy^{2J} causal lesion probably was not within the muscle cell (Parsons, 1974). Jasch et al later demonstrated that C57Bl/6J dy^{2J}/dy^{2J} mouse soleus (SOL, slow twitch) and extensor digitalis longus (EDL, fast twitch) both demonstrate a loss of differentiation in the dystrophic mice, whereas only the fast twitch EDL muscle was affected in the 129 ReJ dy/dy mouse (Jasch, 1982). This becomes a very important factor when examining the literature as many early reports failed to distinguish between the dy and dy^{2J} alleles.

The dy^{2J} mutant, C57Bl/6J dy^{2J}/dy^{2J} has been described as having a "benign dystrophy" due to the fact that they have a much longer lifespan and the muscle necrosis is not as severe as that found in the 129 ReJ dy/dy mutant (Ontell, 1981). One feature of the the dy^{2J} dystrophy is that the hindlimb fast-twitch muscles have a prolonged isometric twitch (Harris and Montgomery, 1975).

The probable cause of this prolonged twitch has been extensively studied. Klamut and coworkers showed calcium accumulation in dystrophic skeletal, heart and tongue muscles (Klamut et al., 1983). They suggested that an increased intracellular calcium concentration could stimulate calcium dependent neutral proteases (or calpains) and impair the oxidative phosphorylation in mitochondria, both of which could lead to muscle necrosis (Klamut et al., 1983). Cornelio and Dones (1984) found that the extracellular Ca^{2+} concentration in dystrophic muscle cells was approximately 200 times the level found in normal muscle cells. They noted that a calcium influx into the muscle fibre due to extracellular leakage would lead to
a severe energy deficit in the muscle fibre and could activate the calpains. In 1985 Klug et al. found that C57Bl/6J dy^{2J}/dy^{2J} dystrophic muscle had a decreased parvalbumin (PV) content (about 60% of normal muscle levels). This reduced level of parvalbumin in the dystrophic muscle was confirmed by Jasch et al. (1985). Earlier, Jasch et al. (1982) had reported an apparent loss of differentiation in both slow twitch (SOL) and fast twitch (EDL) in the dystrophic C57Bl/6J mice. Both groups attributed this decrease in parvalbumin to the loss of differentiation in these muscles. Edwards et al. (1986) also reported a decrease in the levels of parvalbumin in dystrophic muscle of mice, but they attributed this apparent loss of parvalbumin to its leakage out of the damaged dystrophic muscle fibres. In any event, it can be envisioned that a decrease in parvalbumin in the muscle fibre would slow the rate of contraction in that fibre as parvalbumin is associated with fast-twitch fibres (Celio and Heinzman, 1982), and its amount correlates to the relaxation speed of the muscle (Heinzman et al., 1982). Also, a muscle with a decreased parvalbumin content would also have more free calcium present in the myoplasm and this could have a role in the activation and/or duration of calpain stimulation within the muscle cell.

In 1988 Leberer et al. demonstrated that in C57Bl/6J dy^{2J}/dy^{2J} mice the dystrophic muscle sarcoplasmic reticulum contains a defective Ca^{2+}-ATPase which hydrolysed ATP at a rate which was about 50% of normal. They showed that this was probably due to a decreased ATP binding, that the defect in the sarcoplasmic reticulum pump was progressive (implying the importance of age in the disease aetiology) and that the dystrophic muscle contained a decreased calsequestrerin content in both fast and slow twitch muscles. Taken together, these findings suggest that the increased Ca^{2+} in the muscle cell causes the prolonged isometric twitch observed in this strain of mice and that the elevated calcium may be the primary cause of the dystrophy in this murine model.

In the 129 ReJ mouse the myopathy is much more severe. These dystrophic mice are much smaller, drag their hindlimbs and have a matted coat (Gopalan et
al., 1986). The first signs of illness are evident at two weeks of age (Tsuji and Matsushita, 1986) and more than one-half of the dystrophic mice will die prior to 90 days of age (personal observation). Unlike the dystrophy pattern seen in the mdx mutant, muscle fibre necrosis in the 129 ReJ dy/dy mouse does not occur in clusters of fibres (Tanabe et al., 1986). Instead, muscle fibre breakdown is extensive and involves parts or complete segments of the muscle fibre (Mendell et al., 1979). Before complete phagocytosis of the muscle fibres occurs by invading macrophages, there is ongoing muscle fibre regeneration with the appearance of mononuclear myoblasts which fuse to form regenerating fibres (Mendell et al., 1979). The appearance of these type 2C undifferentiated fibres in DMD muscle biopsies has been reported, but it is not known whether these type 2C fibre segments develop into fully differentiated functional fibre types (Nonaka et al., 1981). During muscle phagocytosis and fibre regeneration, a prominent proliferation of endomysial connective tissue occurs into the muscle fibre (Mendell et al., 1979). It is possible that this proliferation of connective tissue may impair dystrophic muscle recovery, and it was suggested earlier that changes in dystrophic connective tissue development may represent the primary lesion in muscular dystrophies (Sweeney and Brown, 1981). Other biochemical differences between normal and dystrophic muscles have been identified and some of these differences will now be presented.

1.5 Other Biochemical Aspects of Dystrophic Myolysis.

Many biochemical changes have been reported in dystrophic muscle. An article by Strickland reviews many of these changes as they pertain to differences in nucleic acid, protein, carbohydrate, lipid and mitochondrial metabolism although it is now believed that most of these effects are secondary consequences of the primary dystrophic defect (Strickland et al., 1979). Later, Aoyagi et al. (1981) demonstrated that 27 enzymes had increased activities in muscle and bone of dystrophic mice and that forelimb and hindlimb muscle pools showed similar increases in enzyme activity. Studies such as the previous two suggested that protein metabolism is affected in the dystrophic muscle.
The maintenance of a constant amount of functional protein will rely on an equilibrium between the synthesis of new proteins and the degradation of old (or defective) proteins. When this system is at equilibrium, the rate of protein synthesis will be identical to the rate of protein degradation and overall, there will be no net gain or loss of protein in this system. In the muscular dystrophies, there is a progressive loss of muscle proteins (Mendell et al., 1979, Gardner-Medwin et al., 1980). This loss can occur in one of two ways; either there is a decrease in the rate of new protein synthesis over protein degradation (Rennie, 1985), or there is an increase in protein degradation over that of protein synthesis (Simon et al., 1962, Warnes et al., 1981, Pearson and Kar, 1979).

A review by Rennie (1985) suggested that muscle wasting in muscular dystrophy is due to "a failure in growth and maintenance (at least in the later stages) with a slowing down of muscle protein turnover as an adaptation to this". It was noted that in Duchenne muscular dystrophy, there is little evidence of a rapid loss of muscle during the onset of the disease, while in later chronic stages, muscle masses fall by no more than 4% per year (Rennie, 1979). He concluded that a decrease in protein synthesis accounts for the shift in the synthesis/degradation equilibrium leading to the observed loss of protein.

However, several other researchers have reported an increase in the rate of protein synthesis in muscular dystrophy (Ionasescu et al., 1971, Pearson and Kar, 1979, Garber et al., 1980). These researchers have concluded that the loss of skeletal muscle protein in dystrophic animals is due to an increase in the activity of proteolytic enzymes over and above the measured increase in protein synthesis observed in the dystrophic muscles.

There are three main pathways for the activation of muscle protein breakdown in skeletal muscle; a basal nonlysosomal pathway, a calcium dependent pathway involving proteases called calpains and a lysosomal process (Goldberg et al., 1986). Calpains have been extensively studied. There are two classes of calpains. One
class requires micromolar amounts of \( \text{Ca}^{2+} \) for its activation and this class is referred
to as class I calcium (activated or dependent) neutral proteases (or CANP-I's = CDNP-I's) (Kubota and Suzuki, 1982; Suzuki, 1987). The other class of calpains
require much higher \( \text{Ca}^{2+} \) concentrations for their activation (in the millimolar
range), and these are referred to as class II calcium (activatated or dependent)
neutral proteases (or CANP-II's = CDNP-II's) (Suzuki, 1987). Both calpains I and II
consist of two subunits; a large 80 Kd catalytic subunit with 4 domains which bind
calcium and a smaller 30 Kd subunit which has at least two \( \text{Ca}^{2+} \)-binding domains,
one of which has a high degree of sequence homology with calmodulin.

In the resting eukaryotic cell cytosolic [free] \( \text{Ca}^{2+} \) is present between 10\(^{-7}\) and
10\(^{-9}\)M (Kretzinger, 1980), and both calpain classes require much higher calcium
concentrations for their activation than are normally found in the cell (Suzuki, 1987).
Moreover, precursors of both CANP-I and CANP-II are partially proteolyzed in the
presence of the required calcium concentrations (Mellgren, 1987). This processing
event increases the \( \text{Ca}^{2+} \) affinity of the calpain and autoactivation occurs at the
\[ \text{Ca}^{2+} \] attainable within muscle (Mellgren, 1987). In bovine muscle, the CANP-II
small subunit undergoes three successive proteolytic steps (from a 26 to a 24 to a
22 to a 17 Kd small subunit size), while the 80 Kd subunit remains unchanged
(DeMartino et al., 1986). In other words, the 80/26 CANP-II complex is an inactive
precursor, the first cleavage activates the enzyme and the 80/17 CANP-II
heterodimer has a 25 fold reduced \( \text{Ca}^{2+} \) requirement for protease activity (De-
Martino et al., 1986). In this manner, the CANP-II is converted to a uCANP, although
uCANP and CANP-I are clearly different (Kubota and Suzuki, 1982).

Dayton et al. demonstrated that calpains were involved in myofibrillar protein
turnover (Dayton et al., 1976a; Dayton et al., 1976b). They further reasoned that
lysosomal cathepsins rarely work over \( \text{pH} 5.0 \) and that lysosomes do not engulf
intact myofibrils; therefore, the initial proteolytic disassembly of myofibrils was
thought not to be due to the cathepsins. Sugita et al. (1980) then showed that the
calpains degrade myosin heavy chain, troponin I, troponin C and release alpha-ac-
tinin from intact myofibrils in vitro. These findings suggested that the increased Ca²⁺ in the dystrophic muscle could be the primary cause of the myolysis, as the activated calpains can degrade intact myofibrils (Rowland; 1980). However, since no one protease can degrade proteins to amino acids, other proteases are involved in the breakdown of skeletal muscle proteins in dystrophy (Pearson and Kar, 1979).

Many early biochemical studies were directed towards a comparison of the activity of lysosomal enzymes in normal and dystrophic muscle. Tappel and coworkers (1962) showed large increases in lysosomal enzymes in dystrophic 129 ReJ mouse hindlimb muscle extracts compared to normal muscle, and they suggested that these lysosomal enzymes were the cause of the "hydrolytic and catabolic wasting processes" in muscular dystrophies. Simon et al. (1962) then demonstrated that liver proteins of normal and dystrophic mice showed no significant differences in protein turnover, but that there was an accelerated turnover of muscle proteins in dystrophic animals due to an increased rate of degradation. In 1980, Garber et al. demonstrated the increased protein synthesis in the dystrophic muscle (3 to 7 fold increased incorporation of alanine, leucine and glutamate in vitro). They thus concluded that in dystrophic muscle, the rates of protein degradation must be elevated to even higher levels in the proteolytic enzymes in order to offset this observed increase in protein synthesis in the muscle. In 1972, Kar and Pearson suggested that the cathepsins are involved in the muscle wasting process in human muscle diseases. They demonstrated that acid, neutral and alkaline cathepsins had increased activities in the skeletal muscles of DMD patient and that both muscle autolysis and cathepsin activity were most active at the same pH values, suggesting that the cathepsins are involved in the intracellular breakdown of skeletal muscle. Iodice et al. (1972) also demonstrated that cathepsin B activity increases first when the overall proteolytic activity in chicken skeletal muscle first begins to increase. This observation suggested to them that cathepsin B represents the rate limiting enzyme in the proteolysis of the muscle fibre, a view which was later supported by Pearson and Kar (1979). Noda et al. (1981) then showed that cathepsin B degrades myosin, troponin T and troponin I in vitro, and
when applied to intact myofibrils, causes the early disappearance of the Z band, followed by a loss of the M line and a later decrease in the A band density.

Combinations of cathepsins were also studied. In 1977, Schwartz and Bird showed that a mixture of rat liver cathepsins B and D could degrade rabbit or rat myosin and F-actin at pH 5.0 in vitro, showing that these two enzymes could have a role in cellular myoproteolysis. The ability of cathepsins B and D to degrade purified native and denatured myosin in vitro was confirmed by Spanier and Bird, (1982). Kominami et al. (1984c) showed that the increase in levels of cathepsins B and L in dystrophic hamsters was tissue specific as the increase occurred in the skeletal muscles but not in heart muscle of diseased animals. Komatsu et al. (1986a) later showed that the combined activities of these two cathepsins was significantly higher in the forelimb and hindlimb muscles of C57Bl/6J dy^{2J}/dy^{2J} mice than in normal mice and the combined activities (B + L) in hindlimb and forelimb muscle were similar. Overall, combinations of proteases are believed to be involved in abnormal proteolysis of dystrophic myofibres. Berlinquet and Srivastova (1966) demonstrated increases in both neutral (pH 7.5) and alkaline (pH 9.0) proteases in dystrophic 129 ReJ mouse muscle especially between 80 and 90 days of age. More recently, acid, neutral and alkaline protease involvement in dystrophic muscle was confirmed (Kar and Pearson, 1972, Obinata et al., 1981). This led researchers to investigate the cause of the increased lysosomal protease activity in dystrophic muscle.

1.6 **Protease Inhibitors and their Involvement in Muscular Dystrophy.**

Since an increase in protease activity was proposed to be the primary defect behind the dystrophic myolesion, researchers have examined the effect of various protease inhibitors towards controlling the dystrophic process.

In 1978, Libby and Goldberg showed that leupeptin (an inhibitor of thiol proteases including cathepsin B) decreased the in vitro protein degradation in both normal and dystrophic skeletal muscle in chickens without affecting the rate of
protein synthesis in these muscles. More recently, Hollenberg-Sher and coworkers (1981) confirmed the Libby study by showing that IP injections of leupeptin into C57Bl/6J dy^2J/dy^2J mice delayed the onset of muscular dystrophy in a significant number of mice on the basis of histological evidence.

McGowan and coworkers (1976) demonstrated that the combination of antipain (which inhibits cathepsins A and B), leupeptin and pepstatin (which inhibits cathepsin D) delayed the atrophy and degeneration of dystrophic muscle fibres markedly in culture. These studies suggested that protease inhibitors might be used clinically to combat the dystrophic process. In 1978, Stracher et al. demonstrated that injection of leupeptin and pepstatin (8 mg/kg body weight) into dystrophic chicken muscle inhibited muscle degeneration in vivo.

In 1981, Hashida et al. showed that E-64, a protease inhibitor isolated from cultures of Aspergillus japonicus TPR-64, specifically inhibited cathepsins B and L in vitro and in vivo. They suggested that this proteinase inhibitor entered the lysosomes (possibly by diffusion) and then inhibited the E-64 sensitive proteinases. Komatsu et al. (1986b) later showed that E-64 and E-64-d (a synthetic analogue) retarded the increase in locomotor activity and decreased the protease levels significantly in dystrophic mice and they lived longer, whereas these inhibitors had no effect on normal mice.

In 1982, Aoyagi et al. compared the in vivo effects of leupeptin and bestatin on catabolic enzymes in various tissues of dystrophic mice. They showed that many enzymes were affected in the forelimb, hindlimb and heart muscles (creatine kinase activity was decreased in all three muscle tissue extracts) and that there was probably a different metabolism occurring in the forelimb and hindlimb extracts as bestatin and foramphenicol affected forelimb and heart much more than they did the hindlimb muscle extract. In 1986, Tsuji and Matsushita showed that bestatin (a small immunomodifier originally isolated from Streptomyces olivoreticuli which inhibits dipeptidyl proteinases) "cured" half of the tested C57Bl/6J dy^2J/dy^2J mice
after 3 months of treatment. They also showed that cessation of treatment was not followed by a relapse which suggested to them that a critical time point in the aetiology of dystrophy in the muscle exists; once this has been accomplished, the muscle will develop normally. That same year, it was demonstrated that the serine protease inhibitor, chymostatin retarded the decrease in locomotor activity, decreased protease levels and increased the lifespan of the C57Bl/6J dy^2J/dy^2J mice (Komatsu et al., 1986c).

Since these studies demonstrated that artificial protease inhibitors had a beneficial effect against the dystrophic muscle breakdown, researchers began to search for and isolate naturally occurring protease inhibitors in various organs, tissues and body fluids of many animal species. The inhibitor of calpain classes I and II is known as calpastatin (or calcium dependent protease inhibitor (CDPI)) (Mellgren, 1987). It is believed that calpastatin is membrane bound and acts as a buffer to prevent the degradation of membrane proteins on a brief exposure to Ca^{2+}. There is evidence that the increase in CANP activity during skeletal muscle myotube formation is due to decreases in CDPI. When the CANP is purified from its inhibitor in dystrophic patients, it was noted that the enzyme's specific activity was significantly higher in both muscle and platelets from DMD samples than from normal patients (Rabbani et al., 1984). This study demonstrated the importance of CDPI levels in the maintenance of calpain activity in the normal tissues. Salvensen et al. (1986) later isolated and described a low molecular weight kininogen with three cystatin-like sequences rather than two, and whose second domain inhibited chicken calpain. The above studies all suggested the importance of calpain regulation in muscular dystrophy.

Spanier and Bird (1982) demonstrated that a similar regulatory mechanism against the thiol proteases exists. Although vitamin E deficient myopathic guinea pig muscle had more cathepsin B activity, it was demonstrated that these tissues also contained less inhibitor activity against cathepsin B than their normal muscle counterparts. When the cathepsin B activity was corrected for inhibitor activity, it
was shown that there was no difference between normal and myopathic muscle cathepsin B activities. This showed that the presence of protease inhibitors affects the enzyme activity measurements in these muscles, and suggests a regulatory role for the inhibitor in muscle protein metabolism.

Cystatins are members of a superfamily of low molecular mass cysteine protease inhibitors (Barrett, 1985). This superfamily is made up of three subgroups; the stefins, the cystatins and the kininogens (Sali and Turk, 1987). Muller-Esterl et al. (1985) presented a hypothesis which states that the three groups arise from an common ancestor. Stefins, cystatins and kininogens all share a region of sequence homology called the "homology box" which centres around the proposed reactive site. Stefins have one sequence (sequence A) shared with cystatins and kininogens; cystatins consist of this region plus another region (sequence B) to form an A-B sequence. The large molecular weight kininogens possess three cystatin-like regions (AB-AB-AB). Stefins (group 1) and cystatins (group 2) have molecular masses between 11 and 14 Kd while kininogens have a mass of between 50 and 120 Kd (Sali and Turk, 1987). One common feature among the cystatins is that they show considerable stability to alkaline and acidic environments and to high temperatures (Wood et al., 1985).

Barrett (1981) has described a purification protocol for a cystatin from chicken egg white. He showed that egg white cystatin inhibited ficin, papain, cathepsins B and C, had a molecular mass of approximately 13 Kd and was heat stable. In 1985 Korant et al. showed that egg white cystatin had two major forms, one of 108 amino acids with a pI of 5.6 and another of 116 amino acids with a pI of 6.5, the extra 8 amino acids being added to the amino terminus. This group showed that cystatins can enter cells in an active form and thus could be supplied externally as an inhibitor. This observation has implications for protease inhibitors as therapeutic agents (Korant et al., 1985).
Later, other extracellular fluid protease inhibitors were isolated. Cystatins were isolated from whole human saliva such as cystatin S, cystatin SN (Isemura et al., 1986) and cystatin SA (Isemura et al., 1987), all of which are effective inhibitors against cathepsin B. Cystatin SA has 90 and 87% sequence homology with cystatins S and SN (Isemura et al., 1987). Lenney and coworkers in 1982 isolated two plasma and nine serum low molecular mass thiol protease inhibitors, all of which are different from the low MW tissue inhibitors. In 1984, Ohkubo et al. isolated the cDNA for the high molecular mass thiol protease inhibitor from human plasma. The cDNA sequence suggests that both the human\(\alpha_1\)-TPI\(_2\) (heavy chain MW = 65 Kd, light chain 4.2 Kd) and \(\alpha_2\)-TPI\(_2\) (heavy chain MW = 66 Kd, light chain 4.2 Kd) forms are identical to low molecular mass kininogen (Ohkubo et al., 1984).

At about the same time several reports appeared on the properties of intracellular inhibitors. In 1981, Hirado et al. isolated cytosolic inhibitor(s) from 17 different rat tissues. They observed a cytosolic inhibitor in all tissues tested of molecular mass around 12.4 Kd which caused noncompetitive inhibition of cathepsins B, B\(_2\), C, H, L and papain. The next year, Kominami et al. (1982a and 1982b) isolated TPI-\(\alpha\), a 12.5 Kd inhibitor of cathepsins B, H, L, C, papain and ficin from rat liver. Wakamatsu (1982) then compared liver TPI-\(\alpha\) to the known rat serum TPIs of molecular masses 90, 95 and 160 Kd and found that there was no cross-reactivity between these TPIs. In 1983, Takeda et al. isolated two TPI's from rat epidermis (MW's 12 and 13 Kd). That same year, Takio et al. (1983) deduced the amino acid sequence for the rat liver inhibitor, TPI-\(\alpha\), a 98 residue protein with the unique terminal sequence Ac-Met-Met-Cys. They then sequenced the rat epidermal TPI, namely TPI-\(\beta\). This 103 amino acid protein has a high sequence homology to rat liver TPI and is resistant to heat and extreme pH, thus, it is a cystatin (Takio et al., 1984). Kominami and coworkers (1984a) then detected these epidermal liver TPIs in rat serum. In the serum, the liver TPI was 47 times more abundant than epidermal TPI. Ritonja et al. reported the amino acid sequence of human liver cystatin B (Ritonja et al., 1985). In the same year, Wood and coworkers (1985) then isolated a low molecular mass (14 kD) cysteine proteinase inhibitor from chicken skeletal muscle; it was similar to other tissue inhibitors, had activity against papain and cathepsin L.
and it cross-reacted with chicken egg white cystatin. This study demonstrated that muscle tissue also contained TPIs.

Gopalan, Dufresne and Warner (1986) believed that the increased proteolysis observed in muscular dystrophy could be due to a reduced protease inhibitor activity in the diseased muscle. During their studies on the 129 ReJ dy/dy mouse, they isolated and identified a defective TPI in hindlimb muscle. Both the TPI-n (isolated from normal mouse muscle) and TPI-d (isolated from dystrophic muscle) inhibited papain equally and only a slight decrease in the activity of TPI-d against cathepsins H and L was observed. However, although the normal muscle TPI-n showed strong cathepsin B inhibition, the dystrophic muscle TPI-d had no inhibitory effect on cathepsin B. TPI-n and TPI-d both showed an apparent molecular mass of 14.4 Kd on SDS-PAGE analysis and had a pl value of 4.5. Both inhibitors eluted at 47% acetonitrile on the reverse phase HPLC column. The Gopalan study demonstrated that in the 129 ReJ dy/dy mouse, one form of the cathepsin B regulation is inoperative in the dystrophic condition. This lack of regulation on a catabolic enzyme could shift the equilibrium between muscle protein buildup and breakdown, thus leading to the progressive loss of muscle seen in the dystrophic process (Gopalan et al., 1986).

1.7 Properties of the Calcium Binding Protein Parvalbumin

Parvalbumin was first isolated and characterized in skeletal muscle of higher vertebrates (rabbit, turtle, chicken and man) by Lehky et al. in 1974. Since it was previously believed to be only present in fish and amphibians, these authors postulated that the conservation of parvalbumin throughout evolution suggested a physiologically important function in muscle. In 1976, Capony and coworkers isolated and sequenced a portion of the primary structure of rabbit muscle parvalbumin. They found that it had a pl of 4.47, an absorbance maximum at 259 nm and an approximate MW of 12 Kd. In 1977, Strehler et al. isolated parvalbumin from chicken skeletal muscles. This group also noted the evolutionary conservation of parvalbumin and they alluded to an undetected yet important functional role for
parvalbumin in muscle. They also showed that the parvalbumins are heat stable, bind 2 moles Ca\(^{2+}\)/mol parvalbumin and have a high phenylalanine to (tyrosine and tryptophan) content. In the same year, Pechere et al. (1977) suggested that parvalbumins constitute a non-interacting regulatory system of sarcoplasmic Ca\(^{2+}\) levels in fast twitch muscle. They also demonstrated that parvalbumin binds calcium equally at pH 8.2 or 6.5, thus demonstrating that the high pH associated with contraction did not affect calcium binding in muscle.

In 1979, Godman et al. proposed an evolutionary diversification scheme for all of the calcium binding proteins. They believed that all calcium binding proteins arose from a common ancestral protein. The proposed ancestor protein contained four domains (each domain consisting of a 40 amino acid sequence containing a mid-region 12 residue segment which binds Ca\(^{2+}\)) (Carafoli, 1985), and it is this ancestor which has evolved into the large superfamily of calcium binding proteins seen today (Godman et al., 1979). The Godman group noted that parvalbumin is closely related to the regulatory myosin light chains and evolutionarily distant from the intestinal Ca\(^{2+}\)-binding protein. They hypothesized that parvalbumin arose from the four domain ancestor. Through evolution, the ancestral calcium binding protein lost segment 1 while segment 2 calcium binding activity was inactivated; in domains 3 and 4, Ca\(^{2+}\) binding affinities increased, thus forming parvalbumin.

Parvalbumin was reported to contain 6 alpha-helical regions identified as A to F. The C-D and E-F loops bind calcium while the A-B loop contributes to the hydrophobic core of the molecule (Kretzinger, 1980). This review reported that parvalbumin (pCa\(^{2+}\) 7.0) in the resting cell has either Mg\(^{2+}\) or K\(^{+}\) bound at the calcium binding sites and that without Mg\(^{2+}\), the pKd (Ca\(^{2+}\)) was approximately 8.4 for one or both calcium sites. However, at physiological concentrations of Mg\(^{2+}\) (2.0 mM), the pKd (Ca\(^{2+}\)) was 6.5 for one or both sites. Kretzinger also reported that parvalbumin is present in smaller (10 to 35 um diameter) white muscle fibres but not in larger (35 to 60 um) glycogenolic fibres.
In 1981, Celio and Heinzman were the first to demonstrate that parvalbumin exists in tissues other than skeletal muscle. In their studies with rats, they detected parvalbumin in the Perkinje cells of the cerebellum, and noted that similarities existed between the tissue distribution of the parvalbumin positive neurons and those containing the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA) such as the stellate cells in the cerebellum, the basket cells of the hippocampus and the periglomerular cells in the olfactory bulbs. In 1982, rat parvalbumin was completely sequenced by Berchtold et al. The protein was shown to contain 109 amino acids and a high sequence homology with both rabbit and carp parvalbumin. It was also shown to contain a lysine triplet at positions 36-39 (Berchtold et al., 1982).

In 1982, Celio and Heinzman then showed that parvalbumin was associated with the fast contracting muscle fibres; that is, it is found in type 2 muscle (Celio and Heinzman, 1982). Later, the parvalbumin content of various fibre types was established. Type 1 (slow twitch) muscle contains virtually no parvalbumin, type 2B (fast twitch glycolytic fibre) muscle contains 5 mg parvalbumin/g muscle protein and type 2A (fast twitch oxidative) muscle contains less than 0.05 mg parvalbumin/g muscle, or less than 1% of the parvalbumin levels in type 2B muscle (Stuhlfauth et al., 1984).

While Celio and Heinzman were showing that parvalbumin was present in fast twitch muscles, Heinzman and coworkers (1984) showed that parvalbumin is present in the highest amounts in muscles which had the fastest rates of relaxation, and lowest in amount in muscles with the slowest relaxation speeds. This observation suggested that parvalbumin is directly involved in the relaxation process in vertebrate fast twitch muscle.

An interesting report by Permyakov et al. in 1982 suggested that ATP and ADP bound to parvalbumin molecules with affinities which allowed for complex formations at physiological concentrations of parvalbumin and adenine nucleotides. This group showed that ATP can bind Mg\(^{2+}\), causing Ca\(^{2+}\) release from the parval-
bumin and that parvalbumin binds GTP and cAMP as well. These findings suggested that a significant pool of parvalbumin-nucleotide complexes must exist in skeletal muscle. If the metal associated with the parvalbumin changes from Mg$^{2+}$ to Ca$^{2+}$, the affinity of parvalbumin for ATP and ADP may also change. These observations suggest a functional importance of nucleotide binding to the parvalbumins (Permyakov et al., 1982). This aspect of parvalbumin becomes of interest when another possible role for the parvalbumins is considered, namely that of a cytosolic thiol protease inhibitor (to be discussed later).

In 1985, Berchtold and Means identified 2 mRNA species of 700 and 1100 nucleotides and showed that these mRNAs were probably derived from a single gene copy. They believed that the mRNAs encoding for parvalbumin and calmodulin (another calcium binding protein found in the muscle fibres which binds 4 moles Ca$^{2+}$/mol protein) are developmentally regulated in a tissue specific manner.

Parvalbumin and its mRNA have been shown to be absent during early development (e.g. not present during oogenesis, fertilization, blastula formation and gastrulation in Xenopus embryos) (Kay, 1987). It is first detected in Xenopus embryos at stage 24, the stage at which the initiation of muscle development occurs, suggesting a role for parvalbumin in the development of muscle (Kay, 1987).

In 1986, Epstein et al. carried out a complete nucleotide sequence of the rat mRNAs. They concluded that both the 700 and 1100 nucleotide species are derived from the same single primary transcript, and provided support for the ancestral calcium binding protein model by noting a 32 base pair region of 81% sequence homology to chicken calmodulin cDNA. They further noted that bone and many endocrine glands also contain low amounts of parvalbumin.

Later, Berchtold et al. (1987) showed that the rat parvalbumin transcriptional unit is 15.5 kilobase pairs long with 4 introns. They also confirmed a tentative 4 domain parvalbumin ancestral model by comparing the parvalbumin gene organiza-
tion to other calcium binding protein genes; the crystal structures of domains 2 and 3 in parvalbumin were very similar to the carboxyl halves of calmodulin and troponin. Also, the parvalbumin gene in humans mapped to chromosome 22 (Berchtold et al., 1987); the murine parvalbumin gene (Pva) of C57Bl/6J mice was later mapped to chromosome 15 using RFLP’s (Zulke, 1988).

Muntener et al. (1985) demonstrated that the content of parvalbumin is neuronally controlled. After innervating a fast twitch muscle with a slow twitch nerve, the parvalbumin level decreased threefold after 9 weeks but returned to "normal" when the muscle was reinnervated with the proper fast twitch nerve. This group later showed that a slow twitch muscle innervated with a fast type neuron increased its parvalbumin complement (Muntener et al., 1987a), and that this increase was not due to thyroid hormone influences on muscle (Muntener et al., 1987b), thus supporting their theory that parvalbumin levels in muscle are controlled by neurological activity. This viewpoint is supported by Leberer and coworkers (1986) who noted that the onset of parvalbumin synthesis is correlated with the neonatal to adult transition in motor neuron activity imposed on that fibre. They also reported that the increase in parvalbumin levels can be suppressed by denervation.

1.8 Involvement of Parvalbumin in Muscular Dystrophy

Nonaka and coworkers (1981) reported that muscle from DMD patients contains many undifferentiated type 2C fibres (16.1%), but they were not sure if these fibres would develop into fully differentiated functional fibres. Fitzsimons and Hoh (1981) then demonstrated that skeletal muscle from DMD patients contains the fetal myosin isoform instead of the adult form of myosin. In 1982, Jasche and coworkers reported an apparent loss of differentiation in murine dystrophic muscle, both in the slow twitch soleus and fast twitch extensor digitorum longus muscles. Takagi and coworkers (1982) then showed that the fast twitch fibres in DMD muscle are more diverse in myosin light chain (MLC) composition than in normal muscle. The basic conclusion arrived at by these researchers was that the large number of undifferentiated fibres suggested that dystrophic muscles are undergoing extensive
regeneration. This regeneration thus elevates the number of immature fibers in the dystrophic muscle.

In 1985, Bandman reported that myosin heavy chain in adult human dystrophic skeletal muscle is the normal neonatal fibre type, thus "suggesting that muscular dystrophy inhibits myosin gene switching which normally occurs during muscle maturation". There are at least two ways to explain this lack of myosin switching during muscle maturation. The muscle fibres themselves could be immature, regenerating fibres as was suggested by Jasche et al. (1982) and Takagi et al. (1982) or it is possible that a functional innervation problem exists in mature muscle fibres affected by the dystrophy mechanism.

As early as 1976, it was recognized that myosin genes whose expression is regulated by nerve mediated factors are affected in murine muscular dystrophy (John, 1976). In 1986, Buckingham et al reported that innervation "may be the physiological factor responsible for the developmental transitions in actin and myosin expression, although this has not been proven conclusively".

Parry and Desypris (1983) demonstrated that dystrophic dy²J mouse hindlimb fast twitch muscles contain slow twitch myosin isoforms; this switch was attributed to changes in innervation of the muscle which slowed the twitch from fast to slow and the slow twitch myosin isoforms became expressed. Researchers then attempted to demonstrate that innervation changes affected protein expression in the muscles affected. Muntener et al. (1985) showed that innervation of a fast twitch muscle (extensor digitorum longus) with a slow twitch soleus nerve caused the muscle to become a slow twitch muscle; self-innervation of the extensor digitorum longus muscle lead to parvalbumin levels returning to a normal level after 9 weeks. Muntener's study (1985) supported the idea that "parvalbumin levels and other muscle protein expression are due to the neurological activity imposed on the muscle fibres". They then confirmed this hypothesis by innervating a slow twitch soleus muscle with a fast twitch extensor digitorum longus nerve thus producing a
slow-to-fast muscle transformation. The above studies demonstrated that a change in innervation to fast twitch muscles will cause them to transform from fast to slow twitch muscles, and this is a possible cause for the loss of differentiated muscle fibre (and the decrease in parvalbumin content of these muscles) observed in the dystrophic muscle.

In 1984, Heinzman stated that "parvalbumin is a small, water soluble protein which may be involved in the pathogenesis of muscular dystrophy as a calcium regulator". Later, it was demonstrated that dystrophic fast twitch muscles contain reduced parvalbumin (Jasche and Moase, 1985, Klug et al., 1985, Edwards et al., 1986). How can this lead to necrosis of the fibres observed in muscular dystrophy as simple denervation studies do not account for this muscle necrosis? To answer this, the two proposed functions for parvalbumin must be considered; 1) as a cytoplasmic Ca$^{2+}$ binding protein involved in muscle relaxation (Heinzman et al., 1982) and 2) as a potential thiol protease inhibitor whose activity against cathepsin B is lost in dystrophic 129 ReJ muscle (Gopalan et al., 1986).

Goldberg et al. (1986) stated that 3 proteolytic pathways exist which can be activated in disease states to enhance muscle protein breakdown. These are a nonlysosomal pathway, a Ca$^{2+}$-dependent cytosolic process and a lysosomal based process. In 1976, Dayton et al. isolated a calpain believed to be involved in the turnover of myofibrillar proteins. Because of its neutral pH requirement and the near neutral pH of the muscle cytosol, they suggested that the initial proteolytic disassembly of myofibres may be due to calpains and not to the lysosomal cathepsins. In 1983, Klamut and coworkers demonstrated that Ca$^{2+}$ accumulates in dystrophic skeletal, tongue and heart muscles. The increased Ca$^{2+}$ could "stimulate calpains, leading to proteolysis of intact myofibrils and impairment of oxidative phosphorylation in mitochondria, both leading to muscle fibre necrosis" (Klamut et al., 1983).
In 1977, Pechere et al. proposed that parvalbumins "may constitute a non-interacting regulatory system of sarcoplasmic Ca$^{2+}$ levels in fast twitch muscle". Klamut et al. (1983) demonstrated that calmodulin levels in dystrophic and normal muscle are the same. Calmodulin is a calcium binding cytosolic muscle protein which binds four Ca$^{2+}$ ions. It is believed that when calcium ions occupy the initial binding sites on the calmodulin, this affects the binding of subsequent Ca$^{2+}$ ions to unoccupied binding sites on the molecule (Carafoli and Penniston, 1985). Calmodulin has also been reported to affect enzymes which catalyze reactions involving phosphorylation (Carafoli and Penniston, 1985). Regardless, calmodulin does not appear to cause the increased Ca$^{2+}$ accumulation in dystrophic muscle as it is equivalent in both normal and dystrophic muscle (Klamut et al., 1983).

Parvalbumin was then showed to be reduced in dystrophic muscle (Jasche et al., 1985, Klug et al., 1985, Edwards et al., 1986, this thesis) and the large decrease in parvalbumin content suggests that this defect could be a contributing factor to the elevated sarcoplasmic free Ca$^{2+}$ levels in dystrophic muscle which could lead to the stimulation of the calpains (Klug et al., 1985). In other words, a decrease in parvalbumin content in dystrophic muscle could lead to elevated cytosolic Ca$^{2+}$ (free) and protein breakdown by Ca$^{2+}$-activated calpains.

1.9 Objectives of This Thesis

There were four main objectives to be satisfied during the course of this thesis. These were as follows;

- Using isolated and purified TPI from normal hindlimb muscle extracts, a polyclonal antibody system in rabbits was to be produced.
- These antibodies could then be used to compare normal mouse muscle TPI to rabbit, frog and carp parvalbumin in order to detect antigenic similarities between these proteins should the similarities exist.
- The same system was to be used to establish whether the TPI levels in hindlimb muscle from normal and dystrophic mice were age dependent.
- These antibodies were to be used to study the TPI levels in different normal and dystrophic mouse muscle and tissue extracts.
MATERIALS AND METHODS

2.1 Materials

Tris, NaCl, glycerol, Freund's Complete Adjuvant, sodium azide, glycine, polyoxyethylene-sorbitan monolaureate (Tween 20) and both rabbit and frog parvalbumin were purchased from Sigma Chemical Co., St. Louis, MO. Dithiothreitol, low molecular weight protein markers, sodium dodecyl sulfate, acrylamide, bis-acrylamide and BioRad protein staining kit (silver and dye) were purchased from BioRad Laboratories, Mississauga, ON. The G-50 and G-75 Sephadex beads were from Pharmacia Chemical Co., Dorval, PQ. The biotinylated goat-anti-rabbit antibody, streptavidin-alkaline phosphatase, nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoyl-phosphate-p-toluidine (BCIP) substrate kit and the 0.2 micron pore nitrocellulose sheets were purchased from BRL Laboratories, Burlington, ON. EGTA was from Lamont Laboratories, Dallas, TX. Acetonitrile was purchased from Fisher Scientific, Toronto, ON. and Noble's Special Agar was from Difco Laboratories, Detroit, MI. Trifluoroacetic acid (TFA) and Coomassie Blue (R-250) were purchased from Eastman Kodak Co., Rochester, NY. Instant skim milk powder was from Carnation Inc., Toronto, ON. All other materials were of reagent grade or better.

2.2 Animals and Tissue Preparation

Dystrophic and normal mice (strain 129 ReJ) were obtained from Jackson Laboratories, Bar Harbour, ME. New Zealand white rabbits were obtained from Rieman's Fur Ranches Ltd., Saint Agatha, ON.

For this study, mice at various ages were sacrificed via cervical dislocation and the tissues were perfused by direct cardiac puncture with chilled 0.93% NaCl containing 1mM EDTA in situ. The total hindlimb muscle, forelimb muscle, brain, heart, lung, liver and kidney were excised and stored at -70°C.
2.3 Purification of Mouse Muscle Parvalbumin

Purification of the muscle parvalbumin for antibody production was carried out using normal mouse muscle as described by Gopalan et al. (1986). Briefly, the total hindlimb muscle was homogenized in five volumes of homogenization buffer (Appendix A) and centrifuged at 10,000 x g for 20 minutes. The resulting supernatant was then centrifuged at 50,000 x g for 3 hours to obtain the postribosomal supernatant. The supernatant was first fractionated on successive gel filtration columns composed of G-50 and G-75 Sephadex, then on an HPLC column (C-18 reverse phase) driven by a Gilson HPLC system. The major protein peak (parvalbumin) eluted at 47% acetonitrile; it was collected, lyophilized and stored at -70°C.

2.4 Preparation of Polyclonal Antibody to Normal Mouse Muscle Parvalbumin

Initially, a pre-immune bleed was performed and 10 ml of blood were collected from the external marginal vein of the rabbit ear as described by Campbell et al. (1970). Lyophilized parvalbumin was resuspended to a final concentration of 100 µg/ml in 0.93% NaCl, heat inactivated at 70°C for 30 minutes and then mixed 1:1 (v:v) with Complete Freund's Adjuvant. One ml of this mixture (50 µg parvalbumin) was then injected intramuscularly into each of the right and left hip muscles of the New Zealand White rabbits as described by Campbell et al. (1970). Blood was sampled weekly from the ear and the serum was prepared by centrifugation of the clotted blood at 1,500 x g for 10 minutes. The serum from these samples was tested for the presence of antibody using a modified Ouchterlony system (Ouchterlony, 1949). Briefly, 5 ml of 1.5% agar in 0.93% NaCl solution with 0.002% sodium azide was overlaid onto a clean microscope slide. This was allowed to harden at room temperature for 15 minutes then stored at 4°C overnight. Wells were suctioned from the agar and these were filled with 25 µl of either test antibody or test antigen. The solutions were allowed to diffuse into the agar at room temperature for 2 hours. Once diffusion of fluid into the wells was complete (no solution evident in the wells), the wells were plugged using fresh agar and the plugs were then allowed to harden (approx. 5 minutes). The slides were placed in the cold room at
4°C and allowed to develop (usually between 2 to 5 days). Once the bands had developed, the Ouchterlony slides were photographed using a modified light scattering box developed by Dr. H. B. Fackrell (University of Windsor).

2.5 Quantification of Parvalbumin on Western Blots

All steps in the preparation of the tissue extracts were carried out at 4°C. The buffers used for this procedure are listed in Appendix A. The crude homogenate was obtained as described by Gopalan et al. (1987) and centrifuged at 50,000 x g for 3 hours to obtain the postribosomal fraction. The protein content of each tissue sample was determined using the BioRad microassay procedure modified after Bradford (1976).

For Western blot analyses, aliquots of each tissue extract were first separated on 1.0 mm thick SDS-PAGE minigel (7 to 15%) similar to that described by Laemmli et al. (1970). The stock solutions for the gels and the required buffers are given in Appendix A. Electrophoresis was carried out at a constant voltage of 130 V (the current should read near 45 mA) until the bromphenol blue tracking dye reached the bottom of the gel (normally occurred within 1 hour). The gel was then removed from the electrophoresis chamber, separated from the stacking gel, then equilibrated in the blotting buffer for 5 minutes. This treatment prevented gel shrinkage in the blotting chamber and the production of air bubbles which interfere with the transfer of protein bands to the nitrocellulose filter during blotting. The protein was then blotted onto a 0.2 micron pore size nitrocellulose filter for 2 hours at a constant current of 45 mA (approximately 10V) as described by Towbin et al. (1979). After the protein transfer was complete, the filter was placed in a 9% skim milk solution and incubated at room temperature for 4 hours with constant shaking to block any unreacted protein binding sites on the nitrocellulose filter. The filter was then rinsed twice with distilled water to remove excess milk and incubated overnight (with shaking) in a 1:40 dilution of the rabbit anti-parvalbumin antibody in TBS solution (Appendix A). The filter was washed for 15 minute intervals in TBS, TTBS, TTBS and TBS solutions (see Appendix A), then incubated overnight with shaking in 100 ml of a 1 μg/ml biotinylated goat-anti-rabbit antibody solution. The filter was washed
as before, then incubated for 45 minutes in 100 ml of a 1 μg/ml streptavidin-alkaline phosphatase solution. The filter was washed as above and the bands were allowed to develop by exposing the filter to 100 ml of NBT-BCIP substrate solution (refer to Appendix A) for approximately 30 minutes with slow shaking. When the desired visual intensity was reached, the reaction was stopped by immersing the filter in cold dH2O. The bands were then quantified using a Shimadzu Dual Wavelength TLC Scanning Densitometer at an absorption wavelength of 525 nm.

2.6 Staining of Gels for Proteins

Duplicate gels not used for the Western blotting assays were stained for proteins. The tissue samples were prepared and electrophoresed using the same techniques employed in the Western assay except that each lane was loaded with 10 μg of extract. The gels were stained for 2 hours using 0.01% Coomasie Blue (R-250) in 10% acetic acid and 40% methanol then destained overnight in a solution containing 5% methanol and 7.5% acetic acid. The Coomasie Blue stained gels were photographed and completely destained using 40% methanol and 10% acetic acid. These gels were then restained with the BioRad silver reagent using a modified procedure of Merril et al. (1981).

2.7 HPLC Analysis of Parvalbumin in Extracts of Hindlimb Muscle From 34 and 120 Day Old Normal and Dystrophic Mice

Crude extracts of normal and dystrophic hindlimb from 34 and 120 day old mice were collected as described earlier. Samples containing 50 μg protein were prepared in 100 μl of 15% acetonitrile and incubated at room temperature for 1 hour. These samples were then centrifuged for 5 minutes at 4°C in a Beckman Microfuge E centrifuge to remove any insoluble material. One hundred μl of the resultant supernatant were then loaded onto a 4.6 x 250 mm C-18 reverse phase column (Chemopack) driven by a Gilson HPLC system and eluted with a 12% to 60% acetonitrile linear gradient in 0.1% TFA. The protein in the effluent was detected with a Gilson Holochrome detector. Elution profiles for the samples and for a mouse parvalbumin standard were analysed to determine the quantity (as %) of each major protein in the preparation.
RESULTS

3.1 Production of Anti-Parvalbumin Antibodies

To develop an immunoassay to quantify parvalbumin in various tissues, an antibody preparation was obtained in rabbits using purified normal mouse muscle parvalbumin as the antigen. The time course of antibody production was assayed using the Ouchterlony immunodiffusion procedure (Ouchterlony, 1949).

Figure 1 shows a set of Ouchterlony plates containing serial 1:2 dilutions of serum around a central well containing purified antigen (2 μg mouse parvalbumin). All uppermost wells (i.e., at the 12 O’clock position) contained undiluted serum except for the plate in Panel C where the well at the 10 O’clock position contained the undiluted serum. The dilution series was set up in a clockwise direction from the undiluted well. In each panel, the left side dilution series is a duplicate of the right side dilution series. Pre-immune serum contains no detectable antibody as can be seen by the lack of precipitin bands in Figure 1A. Since no antibody was detected at 21 days following the first injection of the antigen (Fig. 1B), the rabbits were re-innected with an additional 100μg for each animal. Twenty-eight days after the first injection (Fig. 1C), antibody titres were detected in the undiluted serum only. These levels increased with time as demonstrated in Panels D-F of Figure 1 with higher dilutions yielding strong precipitin bands.

Since amino acid sequence analyses of the mouse hindlimb muscle protein with thiol protease inhibitor (TPI) indicated that this protein is either highly related to, or a modified form of parvalbumin (PV) (A.H.Warner, personal communication), protein (TPI) was compared to rabbit, frog and carp parvalbumin using both pre-immune (day 0) and immune (day 81) serum. The Ouchterlony procedure was used to study the relatedness between and among these proteins by comparing their antigenicity to a given antibody preparation (Campbell, 1970).
Figure 1

Production of Polyclonal Rabbit Antibodies Against Normal Mouse Muscle Parvalbumin.

Panel A is a photograph of a Ouchterlony slide in which duplicate serial 1:2 dilutions of pre-immune serum surround a center well containing 2 μg purified parvalbumin. In all panels, the uppermost wells contain undiluted serum except for panel C in which the 10 O’clock position contains the undiluted serum. Panels B-F are serial 1:2 dilutions of days 21, 28, 36, 49 and 81 day old serum, respectively. The right and left hand sides of each panel are duplicate runs for each series.
The data in Figure 2 shows that no precipitin bands were formed between the pre-immune serum and the mouse or parvalbumin samples from other species suggesting that no detectable antibody exists in the pre-immune serum against these proteins. However, strong precipitin bands formed with immune serum to both the mouse muscle protein (TPI) and rabbit parvalbumin (panels A-D). These precipitin bands show partial fusion (note the fused precipitin band with the spur continuing from the mouse parvalbumin band) demonstrating that these proteins share common surface antigenic determinants. Although not demonstrated by this series of photographs, the carp and frog parvalbumin samples also showed faint precipitin bands after two weeks of development (in panels C and D) and they also had lines of partial identity to the mouse protein (parvalbumin).

These results indicated that a polyclonal antibody system raised against a mouse muscle protein previously identified as a thiol protease inhibitor (TPI) by Gopalan et al (1986) also reacts with rabbit parvalbumin, and to a lesser extent with frog and carp parvalbumin. This antigenic similarity between all these samples supports the finding that the mouse muscle protein with TPI activity is also parvalbumin.

In order to test both normal and dystrophic tissues, it was important to demonstrate that the antibody preparation raised against a normal muscle protein (PV-n) would recognize and bind its dystrophic tissue counterpart. The results in Figure 3 compare the specificity of the antibody to parvalbumin purified from normal (PV-n) and dystrophic (PV-d) mice of strain 129 ReJ. These data show lines of perfect identity between the normal and dystrophic mouse muscle parvalbumin samples. Since the polyclonal antibody preparation recognized both the normal and dystrophic parvalbumin, it was used to screen the various tissue extracts of both normal and dystrophic mice for parvalbumin content. Moreover, Figure 3 also shows that the antigenicity of both the normal and dystrophic muscle parvalbumin is unaffected by heating at 70°C for 30 minutes. This finding was of interest as the extracts were heated in the buffer dye during electrophoresis. Thus, the antibody
Figure 2

A Comparison of Mouse Muscle Protein to Rabbit, Frog and Carp Parvalbumin Using an Ouchterlony Based Assay.

The diagrams on the left side of panels A-D indicate the contents of each well for the Ouchterlony plates on the right side. Symbols: M, 2 μg normal mouse muscle parvalbumin; R, 2 μg rabbit parvalbumin; F, 2 μg frog parvalbumin; C, 2 μg carp parvalbumin; 0, undiluted day 0 pre-immune (day 0) serum; 81, 1:4 dilution of immune (day 81) serum.
Figure 3

Immunoprecipitin Reactions between Normal and Dystrophic Mouse Muscle Protein (Parvalbumin).

Parvalbumin obtained from normal hindlimb extract of the mouse was placed in the upper well (native form). An aliquot of this parvalbumin was heat denatured (70°C for 30 minutes), then placed in the lower well. The right and left wells contained native and heat denatured parvalbumin from dystrophic mice, respectively. All samples surround a central well containing 50 μl of a 1:4 dilution of day 81 serum. The right and left setups are duplicate runs.
preparation could be used to quantitate parvalbumin from either normal or
dystrophic mice using a Western blot immunoassay.

3.2 Electrophoretic Analysis of Soluble Muscle Proteins

The results in Figure 4 compare the major proteins of hindlimb muscle extracts
from different aged normal and dystrophic mice. The SDS-PAGE gel in Panel A was
stained with Coomasie blue, photographed, destained and restained using the
BioRad silver stain to give the results in panel B. Small differences in the band pattern
were detected between normal and dystrophic mice of the same age, especially in
the 14,000 to 15,000 dalton ranges (see arrows in Fig 4). These gels demonstrate
that while minor differences are apparent in the protein patterns between normal
and dystrophic hindlimb muscle, the band corresponding to normal and dystrophic
parvalbumin cannot be quantified using one dimensional SDS-PAGE analysis since
the 14,000-15,000 dalton region contains a mixture of proteins of similar molecular
weights. Due to this inability to isolate only the parvalbumin band using the
one-dimensional SDS-PAGE, a quantitative Western assay was developed.

3.3 Development of a Quantitative Western Blot Assay for Parvalbumin

Using purified mouse muscle parvalbumin as a standard, the limits of the
Western/immunoblotting method were determined. The data in Figure 5 represent
a single run of a series of parvalbumin standards quantified using a Western
immunoassay. In Figure 5, this particular run of the parvalbumin standards on the
Western filter develops such that the band area and intensity products are linear
between 50 and 500ng. Overall, the stained parvalbumin bands in the double
antibody reaction are linear to at least 500 ng parvalbumin with an apparent lower
limit of detection of approximately 25 ng. Thus, during each Western analysis,
parvalbumin standards ranging between 25 and 500 ng were included in separate
lanes on every polyacrylamide filter used in the tissue analyses. It was not known
if the antibody preparation had different binding affinities between normal and
dystrophic parvalbumin. Therefore, normal and dystrophic tissue or organ extracts
were analysed on separate filters. Further, dystrophic tissues were quantified
Figure 4

SDS-PAGE Analysis of Soluble Hindlimb Muscle Proteins from Normal and Dystrophic Mice at Various Ages

Panel A contains a Coomassie Blue (R-250) stained gel and Panel B contains the same gel stained with the BioRad Silver reagent using a modified procedure of Merril et al (1981). Lane 1 contains the following standard molecular weight markers: phosphorylase B (97.4 Kda), bovine serum albumin (66.2 Kda), ovalbumin (42.7 Kda), carbonic anhydrase (31.0 Kda), soybean trypsin inhibitor (21.5 Kda) and lysozyme (14.4 Kda).

Lanes 2 and 3: 1 ug norm. and dyst. mouse
parvalbumin, resp.

Lanes 4 and 5: norm. and dyst. day 34 proteins.

Lanes 6 and 7: norm. and dyst. day 50 proteins.

Lanes 8 and 9: norm. and dyst. day 61 proteins.

Lanes 10 and 11: norm. and dyst. day 76 proteins.

Lanes 12 and 13: norm. and dyst. day 90 proteins.

Lanes 14 and 15: norm. and dyst. day 120 proteins.

The arrows show the migration position of mouse muscle parvalbumin. Each lane contains 10 ug crude extract.
Figure 5

Quantitative Western Standard Curve for Varying Parvalbumin Concentrations.

Purified parvalbumin samples of known concentrations were tested using the Western immunoblotting assay and the band intensity of each sample was measured using scanning densitometry. The area values (band area x band absorbance at 525 nm) were recorded and the data points were then analysed using linear regression analysis with one independent variable. The slope and y-intercept values were then used to construct a standard curve as shown (solid lines). The closed circles represent the actual data obtained from the densitometric scan on the Western filter.
using parvalbumin isolated from dystrophic hindlimb extracts and analyses of normal tissue were performed using parvalbumin standards from healthy mice. Figure 6 summarizes the results of a study of parvalbumin levels in hindlimb muscle of both normal and dystrophic mice at various ages. Each point on the curve was calculated from at least 5 measurements using the Western immunoassay procedure for each extract (Appendix B). In the normal hindlimb extracts, the level of parvalbumin remains relatively constant over 90 days after which time there appears to be an increase in the parvalbumin content of hindlimb muscle as a proportion of the total protein in the extract. This increase in parvalbumin content is of interest to us as Gopalan (1987) observed a 50% increase in the thiol protease inhibitor (TPI) activity in the PV fraction of normal breeder mice at 9 months of age. The apparent increase in PV content detected in hindlimb muscle of 120 day old normal mice is consistent with the increased TPI activity observed in hindlimb muscle of older mice. The dystrophic TPI is inactive against cathepsin B and thus the opposite of the above statement cannot be made. The data in Figure 6 also indicate that the parvalbumin content of hindlimb muscle of dystrophic mice is lower than in hindlimb muscle of age-matched normal muscle. In 34 day old dystrophic mice, the hindlimb muscle parvalbumin content was 66% of 34 day old normal muscle parvalbumin levels. Beyond 45 days of age, the parvalbumin content of the dystrophic muscle decreased, reaching 22% of normal in 120 day old animals. Edwards et al. (1986) reported that dystrophic muscle extracts contain less parvalbumin than normal muscle extracts. These data support the above findings. Moreover, they suggest that the decrease in parvalbumin content in muscle of dystrophic mice is age dependent in 129 ReJ mice.

3.4 Measurement of Parvalbumin Content by HPLC

Since a marked change in muscle parvalbumin content appeared with age in dystrophic mice as measured using the Western blot assays, we attempted to confirm these observations using another technique. The results in Figure 7 compare the soluble proteins in extracts from 34 and 120 day old normal and dystrophic muscle using HPLC. Panels A and D are profiles of 34 day old normal and dystrophic hindlimb muscle extracts, respectively, while Panels B and E are
Figure 6.

Quantification of Parvalbumin in both Normal and Dystrophic Hindlimb Muscle Extracts of Mice at Various Ages.

Three normal (closed box) or dystrophic (closed circle) mice at various ages were sacrificed and their hindlimb muscles were removed and pooled. Post-ribosomal supernatants of pooled hindlimb muscle extracts were prepared and the amount of parvalbumin in each extract was determined using a Western immunoblot assay. Each point represents an average of between 5 and 10 measurements of the parvalbumin content (as ng PV per μg total soluble protein) of the muscle extract post-ribosomal supernatants at the ages shown. Standard deviations for each set of samples are shown as vertical lines.
Figure 7.

HPLC Analysis of Hindlimb Muscle Extracts from 34 and 120 Day Old Normal and Dystrophic Mice.

Panels A through F represent the tracings of the actual HPLC profiles obtained for the various samples. The muscle extracts tested were 34 day old normal hindlimb (panel A), 120 day old normal hindlimb (panel B), 34 day old dystrophic hindlimb (panel D) and 120 day old dystrophic hindlimb muscle (panel E). 50 μg of crude protein from each sample were treated with acetonitrile as described in the Methods and Materials section, then applied to a C-18 reverse phase column. A linear gradient of acetonitrile between 12 and 60% in 0.1% TFA was run at a flow rate of 1 ml/min over 45 minutes. Partially purified mouse muscle parvalbumin was run as the standard in panels C and F. The arrows identify the parvalbumin peak in each panel.
comparative profiles of 120 day old normal and dystrophic mouse hindlimb muscle extracts, respectively. Panels C and F are profiles of a standard mouse muscle parvalbumin. The area under each column peak was determined by weighing paper cut-outs of all the profile peaks and the parvalbumin peak in order to assess the relative parvalbumin content (as a percent of total protein) present in the extract. These results are shown in Table 1.

These results indicate that there is a higher proportion of parvalbumin in skeletal muscle of 120 day old normal mice than in 34 day old normal animals. Similarly, the decrease in parvalbumin content in muscles of aging dystrophic animals observed in Western assays was also observed by HPLC analysis. Overall the HPLC data, while preliminary in nature, support the results obtained by the Western immunoblotting assay.

3.5 Parvalbumin Content of Other Tissues of Normal And Dystrophic Mice

The level of parvalbumin in other tissues and organs of normal and dystrophic mice was determined using the Western assay. The SDS-PAGE gels in Fig 8 (Panels A and B) compare protein profiles of hindlimb muscle, forelimb muscle, brain, heart, liver, lung and kidney extracts from both normal and dystrophic 60 day old animals. Based on protein staining, only minor differences in the protein profile were observed between the normal and dystrophic samples.

Each tissue extract was then analysed at least 5 times using the Western immunoassay as described previously and the values obtained for parvalbumin (if detectable) were averaged (Table 2). Of the seven tissues screened, only hindlimb muscle, forelimb muscle and brain (both normal and dystrophic) extracts of 60 day old animals were found to contain (detectable) parvalbumin. The 60 day old hindlimb muscle served as a control, and it can be seen that the values obtained for both the normal and dystrophic hindlimb muscle extracts agree well with the levels calculated in the age study (refer to Figure 6). These results demonstrate that
Table 1

Comparison of the Parvalbumin Measurements Using Western Blotting and HPLC Analyses.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Total Sample Applied (\textmu g)</th>
<th>PV Detected (\textmu g)</th>
<th>PV % of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Mouse Hindlimb Muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34(HPLC)</td>
<td>50</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>120(HPLC)</td>
<td>50</td>
<td>10.1</td>
<td>20</td>
</tr>
<tr>
<td>34(West)</td>
<td>2.5</td>
<td>0.325 ± 15</td>
<td>13</td>
</tr>
<tr>
<td>120(West)</td>
<td>2.5</td>
<td>0.406 ± 19</td>
<td>16</td>
</tr>
<tr>
<td>Dystrophic Mouse Hindlimb Muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34(HPLC)</td>
<td>50</td>
<td>7.9</td>
<td>16</td>
</tr>
<tr>
<td>120(HPLC)</td>
<td>50</td>
<td>4.8</td>
<td>10</td>
</tr>
<tr>
<td>34(West)</td>
<td>2.5</td>
<td>0.215 ± 40</td>
<td>9</td>
</tr>
<tr>
<td>120(West)</td>
<td>2.5</td>
<td>0.089 ± 6</td>
<td>4</td>
</tr>
</tbody>
</table>

\(\textsuperscript{a}\) The designation "HPLC" in brackets next to the age of the sample denotes that the data for these samples were obtained from single runs using an HPLC analysis. The "West" data were obtained using the Western immunoblotting assay. Only extracts from hindlimb muscle of 34 and 120 day old mice were analysed. The "PV Detected" values for the Western Samples represent the mean value for that particular sample obtained from the age study (Figure 6; n = 5).
Figure 8.

SDS-PAGE Analysis of the Soluble Proteins from Various Tissues of 60 Day Old Normal and Dystrophic Mice.

Panel A contains a Coomasie blue (R-250) stained gel while Panel B contains a gel which has been silver stained. The lanes contain the following:

Lane 1: molecular weight markers as described in Figure 4.

Lanes 2 and 3: norm. and dyst. day 60 hindlimb muscle

Lanes 4 and 5: norm. and dyst. day 60 forelimb muscle

Lanes 6 and 7: norm. and dyst. day 60 heart.

Lanes 8 and 9: norm. and dyst. day 60 brain.

Lanes 10 and 11: norm. and dyst. day 60 kidney.

Lanes 12 and 13: norm. and dyst. day 60 liver.

Lanes 14 and 15: norm. and dyst. day 60 lung.

The arrows show the relative position of parvalbumin. Each lane contains 10 μg crude extract except for lane 8 (normal brain) which appears to contain less protein.
Table 2.

Comparison of Parvalbumin Content of Extracts of Various Tissues from 60 Day Old Normal and Dystrophic Mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Protein/lane (ug)</th>
<th>PV Detected (ug)(^a)</th>
<th>PV as % of Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>N Hindlimb muscle</td>
<td>2.6</td>
<td>0.325 ± 25</td>
<td>12.5</td>
</tr>
<tr>
<td>D Hindlimb muscle</td>
<td>2.5</td>
<td>0.182 ± 16</td>
<td>7.3</td>
</tr>
<tr>
<td>N forelimb muscle</td>
<td>2.5</td>
<td>0.171 ± 38</td>
<td>6.8</td>
</tr>
<tr>
<td>D forelimb muscle</td>
<td>2.5</td>
<td>0.072 ± 11</td>
<td>2.9</td>
</tr>
<tr>
<td>N brain</td>
<td>23.2</td>
<td>0.068 ± 21</td>
<td>0.29</td>
</tr>
<tr>
<td>D brain</td>
<td>18.5</td>
<td>0.041 ± 16</td>
<td>0.22</td>
</tr>
<tr>
<td>N heart</td>
<td>33.8</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>D heart</td>
<td>23.8</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>N kidney</td>
<td>44.9</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>D kidney</td>
<td>45.8</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>N liver</td>
<td>61.3</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>D liver</td>
<td>54.9</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>N lung</td>
<td>45.1</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>D lung</td>
<td>28.6</td>
<td>nd</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Represents the mean from a minimum of 4 measurements.

nd, not detected or less that 25 ng or 0.08% of the protein in each lane.; N, normal and D, dystrophic
normal tissue contains higher levels of PV than their dystrophic counterparts in the Table 1 three tissues containing detectable parvalbumin. Specifically, dystrophic hindlimb muscle of 60 day old mice contains 58% of that found in normal muscle, while dystrophic forelimb contains 42% of normal. Dystrophic brain contains 76% of the normal parvalbumin complement. The decrease in parvalbumin level in the brain extracts may not accurately reflect the true tissue level of this protein since very large amounts of proteins in these extracts had to be loaded onto the SDS-PAGE gel for the Western immunoassay in order to yield detectable parvalbumin levels. Furthermore, the parvalbumin levels detected in both the normal and dystrophic brain extracts approach the limits of detection for the Western immunoassay.

3.6 **Parvalbumin Content of Normal and Dystrophic Tissues as a Function of Tissue Weight**

Dystrophic muscle is easily recognized microscopically due to the breakdown of the orderly arrangement of muscle fibres and their replacement by fat and connective tissue (Mendell, 1979). For this reason, the PV data were analyzed on a tissue wet weight basis, the results of which are summarized in Table 3.

In all cases, the postribosomal supernatant fraction of 60 day normal tissue contained more protein per gram wet weight of tissue than their dystrophic counterpart. When the parvalbumin content of each tissue was compared on a gram wet weight basis, it can be seen that tissues from 60 day old dystrophic mice contain even less parvalbumin on a dry weight basis than their normal counterpart. For example, hindlimb muscle extracts of dystrophic animals contain 49% of the level in normal hindlimb muscle compared to 58% on a dry weight basis. The dystrophic forelimb extracts contains only 24% of the normal forelimb extract parvalbumin content on a wet weight basis as opposed to 42% on a dry weight basis. These differences may explain the increased atrophy of forelimb muscle (biceps brachii and extensor carpi radialis longus) observed during the clinical course of murine muscular dystrophy (Rowe and Goldspink, 1969; Parry and Desypris, 1983). The extract from dystrophic brain was found to contain 33% of the normal brain
parvalbumin on a wet weight basis at 60 days of age compared to 76% on a dry weight basis. The effects of PV reduction in the brain of dystrophic mice are not yet clear.
### Table 3

**Comparisons of Parvalbumin Content of Normal and Dystrophic Tissues of 60 Day Old Mice**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Tissue Protein</th>
<th>Parvalbumin Contenta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg protein/g wet tissue</td>
<td>mg PV/g protein</td>
</tr>
<tr>
<td>Normal hindlimb muscle</td>
<td>38.9</td>
<td>125</td>
</tr>
<tr>
<td>Dystrophic hindlimb muscle</td>
<td>32.8 (84.3%)</td>
<td>72.8 (58%)</td>
</tr>
<tr>
<td>Normal forelimb muscle</td>
<td>50.8</td>
<td>68.4</td>
</tr>
<tr>
<td>Dystrophic forelimb muscle</td>
<td>28.9 (56.9%)</td>
<td>29 (42%)</td>
</tr>
<tr>
<td>Normal brain</td>
<td>63.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Dystrophic brain</td>
<td>27.8 (43.6%)</td>
<td>2.2 (76%)</td>
</tr>
</tbody>
</table>

a/ Calculations resulting in these data are shown in Appendix B. The numbers in parentheses represent percent of normal values for each tissue.
DISCUSSION

4.1 Analysis of Parvalbumin Tissue Distribution in the 129 ReJ Normal and Dystrophic Mouse

When this thesis research was initiated, the proteins isolated from mouse hindlimb muscle by Gopalan et al. (1986) showing thiol protease inhibitor activity had been characterized with respect to size (MW 14.4 Kd), pI (4.5), and elution position from a C-18 HPLC column (47% acetonitrile). The protein had been isolated from whole hindlimb muscle of both normal and dystrophic mice (129 ReJ) and assays using a variety of target proteases (cathepsins B, H and L) revealed that both forms of the inhibitor protein (TPI-n and TPI-d) were sensitive to heat and pH extremes. Therefore, it was concluded that the TPIs isolated by Gopalan were not cystatins as these low molecular weight protease inhibitors are characteristically stable to extremes of heat and pH (Sali and Turk, 1987). Thus, the initiation of a program to produce polyclonal antibodies against the protein isolated from normal mouse hindlimb muscle as described in this thesis was part of the overall program to further characterize the skeletal muscle thiol protease inhibitor.

During the period of antibody production, evidence emerged which suggested that the TPI protein from mouse muscle is parvalbumin or a modified form of this protein. Initial amino acid sequence studies showed that the muscle protein contained the sequence "phe-his-ile-leu-asp", a sequence unique to the parvalbumin family (A.H. Warner, personal communications), and the protein showed almost 90% amino acid sequence homology to rabbit parvalbumin (Max Blum, University of Toronto, personal communications). Based on SDS-PAGE analysis of frog, carp, rat and rabbit parvalbumin, the mouse muscle protein was found to be slightly larger than these parvalbumins (estimated to be 132 amino acids long and 14.4 Kda) and thus, it was concluded that the mouse muscle protease inhibitor protein was either parvalbumin or a modified form of parvalbumin. Subsequent isolation and sequencing of a cDNA isolated from a cDNA library in λgt11 using an antibody screening technique provided further support for the identification of the
mouse protein as parvalbumin (A. Chatterjee, University of Windsor, personal communications). Certain other data suggest that the mouse muscle thiol protease inhibitor activity may not be inherent to parvalbumin, but to contaminant protein in very small quantities (less than 0.1%) which co-elutes with parvalbumin from the C-18 reverse phase column and which has the same pl and molecular mass as parvalbumin. This line of study is currently being investigated by other workers in our laboratory. Regardless, most evidence obtained on this protein suggests that the thiol protease inhibitor identified by Gopalan et al. in 1986 is parvalbumin.

Comparisons between the mouse muscle protein and rabbit, frog and carp parvalbumin samples shown in Figure 2 indicated that TPI shares antigenic determinants with rabbit parvalbumin. Frog and carp parvalbumin samples also demonstrated antigenic similarity to the mouse muscle protein, but the precipitin bands took much longer to develop and are not apparent in photographs of Ouchterlony plates. The highly antigenic nature of the mouse muscle protein (high titres developed as shown in Figure 1) also support its identification as parvalbumin as these proteins are highly antigenic (Gosselin-Rey and Gerday, 1977).

To compare parvalbumin obtained from normal and dystrophic mouse muscle, it was first neccessary to demonstrate that the antibody formed using normal mouse muscle protein as antigen would recognize parvalbumin from both normal and dystrophic animals. Parvalbumin isolated from both normal and dystrophic muscle was recognized by the antibody preparation (i.e., they shared common surface antigenic determinants) and denaturing these proteins did not affect their antigenicity. These data were consistent with previously published results which demonstrated that the removal of Ca$^{2+}$ from parvalbumin (denaturing the protein) did not affect the antigenicity of parvalbumin (Gosselin-Rey and Gerday, 1977), and indicated that the antibody preparation could be used to screen both normal and dystrophic animal tissue extracts for the presence (or absence) of parvalbumin. Furthermore, the tissue proteins could be separated by SDS-PAGE electrophoresis preparatory to the Western immunoblotting assay as denaturing the proteins in the sample (using SDS) does not destroy the antigenicity of parvalbumin.
Several immunodetection systems exist and these could have been used to quantitate the levels of parvalbumin present in the normal and dystrophic tissues. An enzyme-linked immunosorbent assay (ELISA) could have been developed but the method selected was the Western immunoblotting assay because the many different tissue extracts could be directly compared on one filter.

One limitation of the Western system was that it could not determine whether the antibody binding affinities for normal and dystrophic muscle parvalbumin were the same or different. If the antibody affinities were different, the quantification measurements would be inaccurate, depending on the parvalbumin source used as the standard. For this reason, normal and dystrophic tissue samples could not be compared on the same filter. Therefore, normal tissue extracts were analysed using normal parvalbumin as standards while dystrophic tissue samples were analysed using only dystrophic parvalbumin as standards on individual filters.

Once the immunoassay protocol was developed, known amounts of pure mouse muscle parvalbumin were applied to the first few wells of the stacking gel for the Western analysis. These samples would be used as standards to correlate band area and intensity of various extracts with parvalbumin concentrations. The remaining wells were then filled with the tissue samples to be tested and quantified. Standard concentration curves were generated for each Western Immunoblot filter. Powdered milk was used as a blocking agent as it blocked non-specific binding sites on the nitrocellulose filter much better than 7% enzyme grade bovine serum albumin and it is much cheaper. A full day incubation at 0°C was required for the antibody to recognize and bind large amounts of parvalbumin quantitatively and another full day incubation was required for the goat-anti-rabbit antibody to recognize and bind the rabbit antibody quantitatively. The substrate solution was also modified (as per Appendix A) to contain only enough substrate to cover the area of the immunoblot filter as increasing the volume of the concentrated substrate solution to cover and wash the filter caused high non-specific colour development and high background on the filter. Overall, the quantification curves generated on each Western immunoblot filter appear linear (usually between 25 and 500 ng).
Thus, the amount of parvalbumin in various tissues could be quantified by applying 5 to 7 known parvalbumin standards to the wells of the SDS-PAGE gel to generate the quantification curve for the rest of that filter. The rest of the wells were then used for the various tissue extracts to be tested.

Using a different technique, it had been reported previously that parvalbumin levels are decreased in dystrophic hindlimb tissue extracts as compared to their normal tissue counterparts (Jasche et al., 1985; Klug et al., 1985; Edwards et al., 1986. This observation was confirmed in this study (refer to Figure 6) and extended to include measurements throughout the lifespan of the dystrophic mouse. The loss of parvalbumin in the skeletal muscle of dystrophic mice had been attributed to either a loss of differentiation in the dystrophic muscle extracts (Jasche et al., 1985), an increased regeneration of immature type 2C fibres in dystrophic muscles (Nonaka et al., 1981) which would lower the relative parvalbumin in the overall extract (Klug et al., 1985), or due to parvalbumin leakage from the damaged fibres (Edwards et al., 1986).

Sweeny and Brown, (1981) and later Tsuji and Matsushita (1986) alluded to a time dependent phenomenon in which the regulation of an enzyme is critical for the development or maturation of normal muscle. The lack of this regulation could be the cause of the myopathic lesions occurring in muscular dystrophy. The data presented in this thesis (Figure 6 and Table 1) demonstrate a specific loss of parvalbumin over time in the dystrophic hindlimb muscle extracts as a function of total protein content. On the other hand, the amount of parvalbumin remains relatively constant in the normal hindlimb extracts when different age groups are compared. An apparent increase in parvalbumin levels in the 120 day old normal hindlimb extract was observed. This is similar to the observed increase in thiol protease inhibitory activity in aging normal hindlimb muscle extracts reported previously (Gopalan, 1987, dissertation.)
These data also show that the decrease in parvalbumin content in young dystrophic hindlimb muscle continues with age in the 129 ReJ mouse (refer to Figure 6). This suggests the importance of reporting the age of the animals used during such a study.

The mean values in Figure 6 are the average of 5-10 values selected from the total population of data points obtained for each tissue extract (refer to Appendix B for selection procedure justification and calculations). In all cases, these (selected) data points produced a mean value which was within +/- 0.5% of the entire population mean value. Thus, it is likely that the selected data set accurately represents the population as a whole. The larger population will have many possible sources of error inherent in their application in the acquisition of any single data point. Pipetting errors (5μl/well) probably represent the largest contributor to variance in this experiment (run-to-run). This type of analysis allows for these outlying values to be used in the determination of the mean without affecting the standard deviation to a great degree.

To confirm that changes were occurring in parvalbumin levels in hindlimb muscles of normal and dystrophic mice with age, an HPLC analysis of selected tissue extracts was performed. Using this method, a decrease in parvalbumin levels in 120 day old mice compared to 34 day old mice was demonstrated (on a percent protein basis, refer to Figure 7 and Table 1). The HPLC methods also demonstrated an increase in parvalbumin levels (on a percent protein basis) in normal hindlimb extracts with age. Although the differences detected with age using both the HPLC and immunoblotting methods were not the same for either the normal or dystrophic muscle, the HPLC data support the findings of the Western analyses which showed that the parvalbumin content of dystrophic muscle decreases with age (Table 1).

The other study performed in this thesis was a tissue analysis of both normal and dystrophic 60 day old 129 ReJ mice (refer to Tables 2 and 3). This analysis was performed for several reasons. Kominami and coworkers (1984c) demonstrated
that two TPIs (of epidermal and liver origin) showed different tissue expression and also very low levels of both of these inhibitors in muscle. These findings suggested that the muscle inhibitor isolated by Gopalan et al. (1986) was a different protein from that found in the other tissues; thus a tissue analysis was required. Second, it could be inferred that the tissues expressing the TPI gene were tissues which required PV to perform a certain function. Therefore, if the protease inhibitors in a certain tissue are defective (or lacking), that tissue would be affected by the dystrophy process more severely than other tissues which do not rely on that protease inhibitor for regulation. Third, tissue analysis should demonstrate whether differences exist in parvalbumin (TPI) levels in normal and dystrophic tissues. Fourth, it would be noted whether the parvalbumin decrease observed in the different tissues was identical (e.g., a consistant 20% decrease in the parvalbumin content from day 60 normal to dystrophic tissue), or if the levels differed in the different tissue types. Lastly, this study could be viewed as supportive evidence for the identification of parvalbumin as the mouse muscle TPI protein if the distribution of the protein detected during this analysis paralleled the tissue distribution of thiol protease inhibitors.

Of the seven 60 day old tissue extracts tested, heart, lung, liver and kidney extracts were all devoid of detectable parvalbumin even when large quantities of protein were analyzed (Table 2). Hindlimb muscle, forelimb muscle and brain all contained detectable parvalbumin and normal tissue consistently contained more parvalbumin than its dystrophic counterpart.

In the comparison of 60 day old normal and dystrophic mouse tissue, the dystrophic hindlimb muscle showed only a 16% decrease in wet weight protein from normal to dystrophic samples (refer to Table 3). Dystrophic forelimb muscle extract showed a 42% decrease in the protein content of dystrophic extract from normal extract levels and dystrophic brain extract a 58% decrease compared to normal samples on a wet weight basis. When comparing the parvalbumin levels in dystrophic tissue, it can be seen that the specific reduction of parvalbumin detected in the tissues from normal to dystrophic samples does not parallel the total protein
decrease for that tissue; i.e., there seems to be a selective loss of parvalbumin in the tissue. Parvalbumin losses in the dystrophic tissue range from a 67% reduction in brain extracts to a 76% reduction in the forelimb muscle extracts with hindlimb extracts having an intermediate level of reduction of 51% on a wet weight basis. These differences were somewhat less in each tissue when the data were expressed on the basis of protein content wet weight comparisons between normal and dystrophic samples (Table 3). These finding indicate that changes in parvalbumin levels in tissues are not uniform, and they tend to support the study of Berchtold and coworkers (1985) who reported that different amounts of parvalbumin mRNA detected in brain and muscle appear to be developmentally regulated in a tissue specific manner. Taken altogether, these data suggest that the expression of the parvalbumin gene in hindlimb muscle, forelimb muscle and brain is different and that in dystrophic mice, these tissues "lose" (degrade/synthesize?) their parvalbumin content at different rates. Parvalbumin has also been detected in bone and in many endocrine glands (Epstein et al., 1986), however these tissues were not tested in this thesis research.

It was noted previously that both forelimb muscles and hindlimb muscles are affected by muscular atrophy. In 1969, Rowe and Goldspink reported that forelimb fast twitch biceps brachii in 129 ReJ dy/dy mice show a greater extent of atrophy than hindlimb fast twitch extensor digitorum longus muscle of the dystrophic mice. In 1983, Parry and Desypris demonstrated that the extensor carpi radialis longus muscle (in forelimb) is more affected than the extensor digitorum longus muscle (in hindlimb) in C57Bl/6J dy^2J/dy^2J mice. Totsuka and Watanabe in 1981 showed similar atrophies in both forelimb and hindlimb muscle endurance at 3 weeks of age in the C57Bl/6J dy/dy mouse. The fact that both hindlimb and forelimb extracts of the 129 ReJ dy/dy mouse show comparable decreases in parvalbumin is puzzling and at first seems contradictory since clinically, the hindlimbs are much more affected than the forelimbs in dystrophic animals. Totsuka and Watanabe (1981a, 1982) proposed an interesting hypothesis to address this paradox. They suggest that the muscular dystrophy symptoms in murine models may be due to an age-related imbalance between growth-arrested muscles and bone growing almost
normally in length. They argue "that dystrophic muscle stops growing and as bone continues to grow, the muscle diameter to bone length ratio continues to decrease and the limb gets weaker and weaker". They also suggested that their "bone length" theory of muscular dystrophy explains why the "muscular dystrophy phenotype is latent in genotypically dystrophic dwarf dy/dy -dw/dw mice".

4.2 Parvalbumin as a Potential Protease Inhibitor in the 129 ReJ Mouse

As discussed earlier in this thesis, parvalbumin acts as a calcium binding protein and regulates free calcium levels within the fast twitch muscle cells. Evidence has been obtained to suggest that parvalbumin may also function as a thiol protease inhibitor in skeletal muscle (Gopalan et al., 1986). Cathepsin B has been implicated as one of the rate limiting enzymes in skeletal muscle fibre proteolysis (Pearson and Kar, 1979), and a mixture of cathepsins B and D have been shown to degrade rabbit and rat myosin and F-actin (Schwartz and Bird, 1977). Normal murine skeletal muscle contains parvalbumin which is capable of inhibiting cathepsin B while parvalbumin, prepared from the skeletal muscles of dystrophic mice, has no inhibitory activity against cathepsin B. Amino acid composition and sequence analyses performed by Dr. Max Blum, University of Toronto demonstrated only minor differences in the composition of parvalbumin between normal and dystrophic muscle. The study of Permyakov et al. (1982) suggested a functional significance for the interaction between parvalbumin and various nucleotides and perhaps minor amino acid substitutions affect one or more of these interactions. It has been reported previously that normal muscle parvalbumin loses all inhibitory activity against cathepsin B when heated to 70°C (pH 7.0), subjected to pH 2-3 or purified on an HPLC column (Gopalan et al., 1986). These observations suggest that parvalbumin may require a cofactor such as a nucleotide for its protease inhibitory activity. It is noted that the HPLC purified, inactive parvalbumin regains its protease inhibitory activity against cathepsins B and L secreted from cultured breast cancer cells (Yagel et al., 1989), suggesting that the parvalbumin- cofactor complex is restored in the culture medium used for maintaining the cell cultures. These observations suggest that further research involving reconstitution experiments may be useful to determine which cofactor-parvalbumin complexes have inhibitory
activity against cathepsin B/L and also to determine if the various parvalbumin-cofactor combinations show different levels of inhibitory activity.

A final observation links parvalbumin deficiency to muscular dystrophy. Karpati and Carpenter (1986) noted that small diameter skeletal muscle fibres (less than 20-25 um) do not undergo necrosis in DMD, mdx dystrophy or CHF-147 dystrophic hamster muscles. It had been noted previously that parvalbumin is present only in the smaller 10 to 35um diameter fibres and not in the larger 35-60 um diameter fibres (Kretzinger, 1980). This suggests that the smaller muscle fibres containing parvalbumin are not susceptible to the dystrophic myolesion. In total, it appears that the loss of parvalbumin in functional muscle implicates this protein in the necrosis seen in muscular dystrophy through its role as a Ca\(^{2+}\) binding protein in normal muscle and perhaps as an inhibitor of lysosomal proteases such as cathepsin B, H and L. One may now consider how this new information fits into what is known about the muscular dystrophies of mice and men, and also how this information may be used to suggest certain types of therapies for the treatment of muscle wasting diseases.

Proteases have been implicated in the pathogenesis of several maladies such as arthritis (Starkey et al., 1977), antifertility (Zanefeld et al., 1970), malignancy (Mort et al., 1983, Sloane et al., 1986) and muscular dystrophy. A review article by Barrett (1980) summarizes many of these studies. Due to protease involvement in many disease states, extensive studies have been performed on both the proteases and their inhibitors by many researchers. Research on proteases have included studies on calpains (Dayton et al., 1976a-b) as well as the lysosomal proteases such as cathepsin B (Katunuma and Kominami, 1983, Katunuma and Kominami, 1985, Howie et al., 1985, Kominami et al., 1985, Ritonja et al., 1985), cathepsin L (Lenney et al., 1980, Katunuma and Kominami, 1985, Bando et al., 1986, Dufour et al., 1987, Wada et al., 1987), cathepsin D (Lapresle et al., 1986), cathepsin H (Katunuma and Kominami, 1985, Kominami et al., 1985), cathepsin E (Lapresle et al., 1986) and cathepsin J (Liao and Lenney, 1984). The focus of these studies has been to learn
about the tissue distribution, function and possible regulation of these enzymes in protein metabolism *in vivo*.

In 1987, Katunuma stated: "It is now generally accepted as fact that in physiological conditions, alterations in protease activity are probably due to changes in the concentrations of inhibitors". Many researchers believe that these inhibitors can be used effectively as therapeutic agents to control many disease states (Barrett, 1980, Cosmos and Butler, 1980, Lenney *et al*., 1980, Warnes *et al*., 1981, Laslowski *et al*., 1983, Turk *et al*., 1983). Researchers have studied protease inhibitors and their effects on calpains (Barrett, 1980, Cosmos and Butler, 1980, Murakami and Ettlinger, 1986) and the thiol proteases (Aoyagi *et al*., 1982, Kominami *et al*., 1982a, Wakamatsu *et al*., 1982, Kominami *et al*., 1984a, Hirado *et al*., 1985, Ritonja *et al*., 1985b, Gopalan *et al*., 1986, Tsujii and Matsushita, 1986). Studies incorporating both proteases and their inhibitors have also been performed with the objective of designing protease inhibitors which can react specifically with the target protease (Lenney *et al*., 1980, Laslowski *et al*., 1983, Katunuma and Kominami, 1983-85). This thesis suggests that necrosis in skeletal muscle of the 129 ReJ dystrophic mice may be due to changes in the content of parvalbumin, a Ca\(^{2+}\) binding protein which may also have thiol protease inhibitor activity against proteases such as cathepsins B, H and L. This model may also describe the myopathy observed in Duchenne muscular dystrophy in humans. It is possible that human DMD (and BMD) dystrophies may soon be successfully managed. Early detection of DMD candidates is possible due to indirect assays such as by measuring creatine kinase levels (may be elevated up to 200x normal levels in dystrophic carriers and patients) (Perry, 1984, Griggs *et al*., 1985, Monaco *et al*., 1986, Scheuerbrandt *et al*., 1986, Monuko *et al*., 1987), the immunodetection of dystrophin in muscle culture (Miranda *et al*., 1989) or by direct genomic probing using such gene probes as pERT 87 (Kunkel *et al*., 1986, Witkowski, 1988) or by RFLP analyses (Muller *et al*., 1985, Davies *et al*., 1987, denDunnen *et al*., 1987, Darras *et al*., 1988, LeRay *et al*., 1988). All of these methods permit the early detection of individuals affected with the DMD (or BMD) myopathy.
Evidence of a time dependent event in the pathogenesis of muscular dystrophy has been discussed previously and molecular and/or biochemical screening procedures should allow for the detection of potentially affected individuals prior to the critical myopathic event.

Evidence suggesting that protease inhibitors delay the onset and severity of the dystrophic phenotype have been introduced earlier in this paper. In mice, "the introduction of a therapeutic agent prior to the critical event resulted in the suppression of the dystrophic genotype as the mice grew" (Tsuji and Matsushita, 1986). As the treated mice aged, they showed normal muscular endurance, grew to normal size and their lifespan was prolonged, even after treatment was stopped (Tsuji and Matsushita, 1986). The Tsuji study and the work of many researchers suggest that early introduction of thiol protease inhibitors into human dystrophic muscle prior to wholesale muscle necrosis which starts near age 4 in DMD patients may allow the muscle to develop beyond the "critical developmental time" and the muscle would thus develop normally.

In this study, a distinct change point in the parvalbumin levels which would indicate a critical period in the hindlimb extracts tested was not detected. This may be due in part to the ages tested and the fact that a mixture (pool) of muscles was used to prepare the extracts. The earliest age tested was 34 day old mice (the youngest age available after delivery of the mice). Tsuji and Matsushita first noted changes in the appearance of the dystrophic mice at or near 14 days of age (Tsuji and Matsushita, 1986). Thus, it is entirely possible that the mice used in this study had already passed the critical stage in muscle development, and this would explain why an initial parvalbumin loss in the dystrophic tissue was not detected in this study. However, a specific decrease in muscle parvalbumin content in dystrophic mice (as compared to normal) was demonstrated in this thesis. The difference in parvalbumin levels from normal to dystrophic muscle was also shown to increase with age. If the parvalbumin is acting as a thiol protease inhibitor as suggested by Gopalan et al. (1986), it is apparent that the dystrophic muscle will lack this form of cathepsin B regulation and proteolysis due to cathepsin B will increase. This
research further suggests that calcium ion management in the dystrophic muscle may be important in regulating calpain activity, as the decreased parvalbumin content in the dystrophic muscle may lead to increased free Ca$^{2+}$ levels in myofibres leading to activation of the calpains.
SUMMARY

The muscle-specific thiol protease inhibitor described by Gopalan et al. (1986) has been identified as either parvalbumin or a modified form of parvalbumin. This identification is based on its amino acid composition and its cross-reactivity to rabbit, frog and carp parvalbumins using an antibody system raised against the normal hindlimb inhibitor protein. Moreover, the level of parvalbumin appeared to remain constant in hindlimb muscle of normal mice (strain 129 ReJ +/+ ) (on a percent protein basis) up to about 90 days. By 120 days, this level had increased. In hindlimb muscle of dystrophic mice (strain 129 ReJ dy/dy), the parvalbumin content was found to be lower than in age matched normal counterparts, decreasing steadily throughout the lifespan of dystrophic mice to 22% of normal levels after 120 days. Parvalbumin was also detected in forelimb muscle and brains of 60 day old normal and dystrophic mice. The parvalbumin content of the tissues was always found to be lower in the dystrophic mice. No parvalbumin was detected (minimum level of sensitivity was 25 ng) in the heart, lung, liver or kidney extracts of either normal or dystrophic mice.
REFERENCES


APPENDIX A- Buffers

1) **Homogenization Buffer**: 0.05 mM EGTA, 10 mM Tris-HCl, pH 8.0, 1mM DDT, 0.2M NaCl and 10% glycerol.

2) **Antigen Innoculum**: 100 µg purified antigen (lyophilized parvalbumin) was dissolved in 1 ml of 0.93% NaCl, heat inactivated (70°C for 30 minutes) and mixed 1:1 with Freund’s Complete Adjuvant.

3) **TBS Buffer**: 0.1 M Tris containing 0.25 M NaCl, pH 7.4.

4) **TTS Buffer**: 0.1 M Tris containing 0.25 M NaCl, pH 7.4 and 0.05% Tween-20.

5) **SDS-PAGE Running Buffer**: 25mM Tris containing 188 mM glycine and 0.1% SDS, pH 8.3.

6) **Blotting Buffer**: 25 mM Tris, containing 188 mM glycine in 20% methanol, pH 8.3

7) **Stock Acrylamide**: 30 g acrylamide, 0.8 g bis-acrylamide and 1 ml 10% SDS were dissolved in autoclaved nanopure water to 100 ml, final volume.

8) **Upper Tris Buffer (4X)**: 6.06 g Tris and 4.0 ml 10% SDS were dissolved in autoclaved, nanopure dH2O, adjusted to pH 6.8 with conc. HCl, then brought to a final volume of 100 ml with H2O.

9) **Lower Tris Buffer (4X)**: 18.17 g Tris and 4.0 ml 10% SDS were dissolved in autoclaved, nanopure dH2O, adjusted to pH 8.8 with conc. HCl, then brought to a final volume of 100 ml using H2O.

10) **Buffer Dye**: 0.5 ml 100% glycerol, 0.25% B-mercaptoethanol, 0.25 ml Upper Tris 4X buffer, 0.1 g SDS (10% final volume) and 0.04 mg bromphenol Blue.
11) **Separating Gel Formulations**: for the SDS-PAGE analyses, the required gel concentration was prepared by adding the components listed under each concentration column.

<table>
<thead>
<tr>
<th>Required gel con</th>
<th>7%</th>
<th>10%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower Tris Buffer</td>
<td>2.53 ml</td>
<td>2.53 ml</td>
<td>2.53 ml</td>
</tr>
<tr>
<td>dH2O</td>
<td>5.13 ml</td>
<td>4.13 ml</td>
<td>-------</td>
</tr>
<tr>
<td>80% glycerol</td>
<td>-------</td>
<td>-------</td>
<td>1.46 ml (100%)</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>2.33 ml</td>
<td>3.33 ml</td>
<td>6.0 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>30 ul</td>
<td>30 ul</td>
<td>30 ul</td>
</tr>
<tr>
<td>TEMED</td>
<td>7.5 ul</td>
<td>7.5 ul</td>
<td>7.5 ul</td>
</tr>
<tr>
<td>final conc.</td>
<td>6.97%</td>
<td>9.97%</td>
<td>4.76%</td>
</tr>
</tbody>
</table>

12) **Stacking Gel**: 0.65 ml 30% stock acrylamide, 3.1 ml dH2O, 1.25 ml Upper Tris (4X) buffer, 30 ul 10% APS and 7.5 ul TEMED; final acrylamide is 3.96%.

13) **Ouchterlony Agar**: 15 g Noble’s Special Agar in 0.93% NaCl, 0.02% sodium azide and 0.02 mM EGTA. Solubilize by heating at 100°C for 15 minutes. 5 ml. of above is used to overlay slides.

14) **Western Immunoassay Reagents**: the anti-parvalbumin antibody, goat anti-rabbit antibody and streptavidin-alkaline phosphatase were all diluted to the appropriate concentration using the TBS reagent.

15) **Western Immunoassay Substrate Buffer**: for band development on filters, 44 ul NBT solution (50 mg/ml NBT in 70% dimethylformamide) were added to 100 ml of 0.01 M Tris-HCl solution (pH 9.5) containing 0.01 M NaCl and 5 mM MgCl₂. To this, 33 ul BCIP solution (75 mg/ml BCIP in 100% dimethylformamide) was added and the solution was gently mixed.
APPENDIX B- Calculations

As an example, I will process the data from a Western immunoblot assay study comparing normal hindlimb muscle extracts of various ages.

The first seven wells contained parvalbumin standards ranging from 25 to 500 ng and these points were used to construct the quantification curve for this filter (see Figure 9). Well 8 was left blank and wells 9 through 15 contained crude muscle extract from animals of various ages. The proteins in each well were separated on a 7 to 15% SDS-PAGE gel, blotted onto nitrocellulose and the filter was then developed using the protocol for the Western immunoassay. The band intensities were then measured and quantified using the scanning densitometer. The final quantification values for each band are dependent on the size of the band and the band absorption at a 525 nm wavelength. The parvalbumin standards (and their corresponding final quantification values) were then subjected to a one-dimensional linear regression Student-t test with one independent variable using the Sci-Stat program. From this analysis, the line of best fit was determined (m = 1.3, b = 13.8). The R² value for the curve generated by these seven parvalbumin standard points (0.993) shows that this filter data are significant (Rohlf and Sokal, 1981) and thus the curve was used to quantify the various hindlimb extracts. By extrapolating the densitometric final quantification value for the muscle extract from the quantification curve for that filter (refer to Figure 9), the amount of parvalbumin present in that particular crude muscle extract could be calculated.

Twenty replicate Western immunoblot assays were performed for both normal and dystrophic muscle extracts in order to compare the parvalbumin content in the muscle extracts with age. The calculated parvalbumin content values for each age group were then pooled and the mean values for these pools were calculated.

The largest source of error in this parvalbumin quantification was probably due to pipetting errors as only 2.5μl samples of the crude extract were syringed into
Western Immunoblot Analysis on Normal Hindlimb Extracts at Various Ages: One Particular Run

This particular figure depicts an immunoblot of normal hindlimb extracts and parvalbumin standards isolated from normal mouse muscle. The first seven lanes contained the following parvalbumin standards; lane 1, 25 ng PV; Lane 2, 50 ng PV; Lane 3, 75 ng PV; Lane 4, 125 ng PV; Lane 5, 250 ng PV; Lane 6, 375 ng PV; and Lane 7 contained 500 ng PV. Lane 8 was left blank. Lanes 9 through 15 contain the post-ribosomal supernatants of the following muscle extracts; Lane 9, 36 day normal; Lane 10, 50 day normal; Lane 11, 60 day normal; Lane 12, 76 day normal; Lane 13, 90 day normal; and Lane 14, 120 normal muscle extract. All extract lanes contain 2.5 ug total protein per well.
each well. Small inaccuracies in pipetting would lead to large variations in the
calculated parvalbumin content as these small differences in pipetting translate into
large differences of actual crude muscle protein applied onto each well. In this
experiment, I collected 5ul into a syringe (minimizes initial loading error into the
syringe) and divided this into two well's (2.5ul sample per gel). Pooling all values
obtained yields an accurate mean for the muscle extract parvalbumin content as
the pipetting errors will average out. To reduce the variance and standard deviation
for each muscle extract, 5 to 10 individual quantification values with values near the
pool mean were selected from each group. These points were then used as the
raw data for the hindlimb age analysis as represented by Figure 5 in this paper. It
is important to note here that the selected data point groupings all had calculated
means within 0.5% of the total group pooled mean values. Thus, it is believed that
these selected groups are actually reflective of the larger data pools for each muscle
extract.

For the tissue study, Western immunoblot assays were performed as per the
hindlimb muscle age study. The first seven lanes were filled with varying concentra-
tions of parvalbumin and the last seven lanes contained the tissues to be compared.
Either 2.5 or 2.6ug of 60 day old crude hindlimb or forelimb (as per listing in Table
2) were loaded into the wells of the SDS-PAGE stacking gel. Due to the low or
undetectable levels of parvalbumin present in the other five tissue extracts (brain, heart, lung, liver and kidney), the maximum levels of crude extract protein were
loaded into the wells of the stacking gel. The varying initial loads of these normal
and dystrophic tissue extracts reflects the different protein contents present in the
crude extract and can be found listed in Table 2. Parvalbumin was only detected in
the hindlimb, forelimb and brain extracts. The mean, variance and standard devia-
tion for each tissue were calculated and appear in Table 2.

The data in Table 3 were calculated from the information in Table 2. It can be seen that the dystrophic tissue consistently contains less protein than its normal
counterpart and the wet weight parvalbumin content calculations correct for the
initial tissue dilution in the homogenization buffer.
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