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PURIFICATION AND CHARACTERIZATION OF A DEFECTIVE THIOL PROTEASE INHIBITOR FROM THE SKELETAL MUSCLE OF MICE WITH HEREDITARY MUSCULAR DYSTROPHY

by

PRATHIMA GOPALAN

A Dissertation
Submitted to the Faculty of Graduate Studies
through the Department of Biological Sciences in
Partial Fulfillment of the requirements for the Degree of
Doctor of Philosophy

Windsor, Ontario, Canada.
1986.

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To

my family who made everything possible.

ABSTRACT

The thiol protease inhibitor (TPI-d) from hindlimb skeletal muscle of 60 day old dystrophic male mice (strain 129/ReJ-dy) and thiol protease inhibitor (TPI-h) from the skeletal muscle of 9 months old carrier females, heterozygous for the disease, have been purified to apparent homogeneity and compared with the thiol protease inhibitor (TPI-n) from 60 day old normal male littermates and thiol protease inhibitor (TPI-c) from 9 month old normal retired breeders. While TPI-d, TPI-n, TPI-h and TPI-c all displayed identical properties on SDS-polyacrylamide gels (Mr 14,800), analytical isoelectric focusing gels (pI 4.5), and high performance liquid chromatography columns, TPI-d was unable to inhibit papain and cathepsin B after purification by isoelectric focusing. However, a component in the apparently purified TPI-d preparation with a pI of 4.9 masked the functional state of TPI-d when papain or cathepsins H and L were used as test proteases. This inhibitory component was also observed in the TPI-h preparation from carrier females. However, TPI-h inhibits cathepsin B activity to the extent observed with inhibitor preparations from normal muscle. The inhibitory component of pI 4.9 was absent from TPI-n and TPI-c preparations. Pure TPI-d was also unable to inhibit myosin hydrolysis _in vitro_ by cathepsin B, whereas TPI-n completely blocked cathepsin B catalyzed myosin hydrolysis. Given the central role of the thiol proteases, especially cathepsin B, in intracellular protein metabolism, and the possibility that uncontrolled thiol protease activity in muscle leads to muscle protein breakdown and dystrophy, this study suggests that the thiol protease inhibitor in dystrophic mouse muscle (TPI-d) may be (one of) the end-product(s) of the dystrophy gene in mice with the hereditary form of the disease.

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TABLE OF CONTENTS

Page

Dedication iv

Abstract v

Acknowledgements vii

List of Figures xi

List of Tables xiii

List of Abbreviations xiv

I. INTRODUCTION 1

A. Use of animal models to study the disease process 2

B. Murine muscular dystrophy 3

C. Comparison of dystrophy in mice and humans 10

D. Syrian hamster dystrophy 11

E. Comparison of dystrophy in hamsters and humans ... 14

F. Muscular dystrophy in the chickens 15

G. Comparison of dystrophy in chickens and humans ... 16

H. Summary of morphological changes in dystrophic animals compared to dystrophic humans 17

I. Biochemical characteristics of dystrophy 17

J. Integrity of plasma membrane in dystrophic animals 26

K. Turnover of proteins in dystrophic animals........ 28

II. MATERIALS AND METHODS 35

A. Animals and tissues............................... 35

B. Biochemicals 35

C. Methods 36
1. Tissue sample preparation 36
2. Preparation of mouse liver lysosomal thiol proteases 36
3. Partial purification of cathepsins B, H and L from mouse liver lysosomes 37
4. Assay for cathepsins B and H 38
5. Assay for cathepsin L 39
6. Assay of thiol protease inhibitor activity 40
7. Purity and molecular weight determinations of the TPIs 40
8. Analysis of TPI's on isoelectric focusing gels . 41
9. High performance liquid chromatography of TPI's 42
10. Assay for myosin hydrolysis by cathepsin B in the presence of TPI-n and TPI-d 43
11. Thymol sulfuric acid test for glycoproteins ... 43
12. Protein determination 44

III. RESULTS 45
A. Purification of a thiol protease inhibitor from normal and dystrophic mouse muscle 45
B. Physical protepties of the thiol protease inhibitor from normal and dystrophic hindlimb muscle of the mouse 50
C. Activity of TPI-n and TPI-d on various thiol proteases 53
D. Activity of TPI-n and TPI-d after isoelectric

focusing on polyacrylamide gels 62
E. Effect of TPI-n and TPI-d on the hydrolysis of myosin by cathepsin B 69
F. pH and thermal stability of TPI-n 72
G. Effect of trypsin treatment on TPI-n 72
H. Purification of TPI's from carrier mice heterozygous for muscular dystrophy (+/dy) and disease free normal retired breeders (+/+) 77
I. Physical properties of TPI-h and TPI-c 79
J. Effect of TPI-h and TPI-c on cathepsins B, H and L and papain 79
K. Analysis of TPI-h and TPI-c on isoelectric focusing gels 82
IV. DISCUSSION 89
V. REFERENCES 99
VI. VITA AUCTORIS 119

LIST OF FIGURES

Figures	Description	Page
1a.	Photograph of normal and dystrophic mice...	6
1b.	Photograph showing the hind leg muscles of normal and dystrophic mice..................	8
2.	Purification of TPI-n and TPI-d on gel filtration columns	48
3.	Comparison of TPI-n and TPI-d on SDS-polyacrylamide gels	52
4.	Molecular weight determination of thiol protease inhibitor protein	55
5.	High performance liquid chromatography of TPI-n and TPI-d..............................	57
6.	Isoelectric focusing gels containing TPI-n and TPI-d.............................	59
7.	Effect of TPI-n and TPI-d on the activity of various thiol proteases......................	61
8.	Analysis of TPI-n and TPI-d on analytical isoelectric focusing gels....................	66
9.	Determination of pI value of the thiol protease inhibitor protein.............................	68
10.	Effect of TPI-n and TPI-d on myosin hydrolysis by cathepsin B	71
11.	Stability of TPI-n at various pH	74
12.	Temperature stability of TPI-n	76
13.	Chromatography of TPI-c and TPI-h on HPLC column...	81

14. Effect of TPI-c and TPI-h on papain and cathepsins B, H and L........................ 84

15. Isoelectric focusing of TPI-c and TPI-h on a polyacrylamide gel support..................... 87

LIST OF TABLES

Table	Description	Page
1.	Summary of steps of purification of thiol protease inhibitor from normal and dystrophic mouse muscle	49
2.	Summary of thiol protease inhibitor activity from normal and dystrophic mouse muscle on various cathepsins	63
3.	Content and inhibitor activity of TPI-h and TPI-c in hindlimb muscle compared to TPI-n and TPI-d...	78
4.	Summary of thiol protease inhibitor activity from skeletal muscle of normal retired breeders (+/+) and carrier females heterozygous for the disease on various cathepsins and papain.................	85

LIST OF ABBREVIATIONS

ADPR	Adenosine diphospho ribose.
ATP	Adenosine triphosphate.
cAMP	Cyclic adenosine monophosphate.
BANA	N- -benzoyl-DL-arginine-2-naphthylamide.
DEAE	Diethylaminiethyl cellulose.
DTT	Dithiothreitol.
DMD	Duchenne muscular dystrophy.
EDTA	Ethylenediamine tetraacetate (disodium salt)
EF_2	Elongation factor 2.
HPLC	High performance liquid chromatography.
ID_{50}	Inhibitor dose which gives 50% inhibition of enzyme activity.
IU	Inhibitor unit.
M + L	Mitochondria and lysosomal extract.
NADPH	Nicotinamide adenine dinucleotide phosphate.
PAGE	Polyacrylamide gel electrophoresis.
pI	Isoelectric point.
pCMB	para-chloromercuribenzoate.
TFA	Trifluoroacetic acid.
SDS	Sodium dodecyl sulfate.
TPI-n	Thiol protease inhibitor from normal skeletal muscle.
TPI-d	Thiol protease inhibitor from dystrophic skeletal muscle.
TPI-h	Thiol protease inhibitor from carrier females heterozygous for hereditary muscular

	dystrophy.
TPI-c	Thiol protease inhibitor from normal retired female breeders.

I. INTRODUCTION

The metabolic disorders known as muscular dystrophies are a heterogeneous group of disorders characterised by muscle weakness and degeneration (Knudson, 1965). Muscular dystrophies are regarded as primary myopathies; the lesion(s) is(are) presumed to be present and expressed in the muscle itself (Perry, 1984). Dystrophic tissues exhibit a gradual deterioration of function associated with progressive necrosis and often, in the early stages of the disease, certain muscles which are characteristic of the type of dystrophy, are specifically affected. One of the most severe forms of the disease is the X-linked form known as Duchenne muscular dystrophy (DMD) which affects mainly young males. The DMD locus in humans is at position Xp 21 on the X-chromosome (Jacobs et al., 1981). In humans the disease is usually diagnosed clinically by about age 3-5, while in mice and chickens the expression of the disease begins around 2-3 weeks ex-utero (Walton and Nattras, 1954). Histological examinations of muscle in the early stages of Duchenne muscular dystrophy have revealed differences in the organization of the contractile elements compared to normal muscle. As the disease progresses many muscle fibres show extensive degeneration, the sarcolemma shows areas of discontinuity and there is a disproportionate increase in connective tissue and fat cells in severely affected muscle (Cazzato, 1968; Hunters, 1980). Numerous other morphological changes have been reported in the various forms of dystrophy

(Cullen and Mastaglia, 1980).

During the past 20 years several hypotheses have been advanced to explain the pathogenesis of muscle in laboratory animals and Duchenne muscular dystrophy patients. These include the vascular hypothesis (Sweeney and Brown, 1981), neurogenic theory (Desmos, 1961), membrane theory (McComas et al. 1978), myogenetic theory (Witkowski and Jones, 1981) and connective tissue theory (Lucy, 1980). While there are abundant experimental data to support each of these views, it is not yet clear whether the observations which led to these hypotheses are primary or secondary process(es) of the disease. The biochemical nature of muscular dystrophy is poorly understood. Thus the cause(s) of the myriad of biochemical alterations which occur(s) in dystrophic animals has(have) not been identified.

A. Use of animal models to study the disease process

A disease in experimental animals which simulates both morphologically and clinically a disease process in humans, does not indicate, a priori, that the etiology or pathology of the experimental animal and human disease are the same (Bajusz et al., 1966). The value of the experimental model in elucidating the etiology and pathogenesis of human diseases becomes apparent only when it can be shown that the responses to a variety of stimuli of animal/tissue under investigation are identical or similar to that in the human. Differences in tissue response per se indicate a difference in pathogenesis and warn the investigator of the degree of its possible existence (Bajusz et al., 1966). Therefore the greater the difference in response, both clinically and morphologically between the experimentally induced disease and the disease in humans, the greater the likelihood of etiological and pathogenetic differences. By the intelligent use of experimental models and a variety of experimental techniques, the possibility exists that greater insight into human disease processes may be obtained. Despite the fact that inherited forms of muscular dystrophy were first described in the mouse and chicken more than 4 decades ago, and in the hamster in 1962, the debate continues regarding their relevance to human forms of muscular dystrophy (Mendell et al., 1979; Watts and Reid, 1969; Bradley and Jenkinson, 1975; Johnson and Montgomery, 1976; Jasmin and Bokdawala, 1970). The controversy will probably continue until specific biochemical defects are identified which can be used as a basis for comparison between human and animal forms of muscular dystrophy.

B. Murine muscular dystrophy

Murine Muscular dystrophy was first observed as a spontaneous autosomal mutation in strain 129/ReJ (Michelson et al., 1955) causing muscular weakness, atrophy and reduced life span characteristic of myopathy. In mice there are a number of clinical, histological and pathological similarities to DMD in humans, except for the difference in inheritance, which is autosomal. Clinically, dystrophic mice can be identified by their abnormal behaviour at about 2 weeks of age. Clinical signs are muscular weakness, periodic dragging of the rear feet, clasping of the hindlimbs when the animal is suspended, and spasmodic gaping or nodding of the head (Russell, 1961). Also, the dystrophic mice are much smaller than their normal litter mates (see Fig 1a) and the leg muscles weigh about one half that of the normal mice (Gopalan et al., 1986a). Fig. 1b shows the leg musculature of normal and dystophic mice. In certain stocks, afflicted animals often show tetanic seizures which are not usually lethal. If dystrophic mice live long enough, they develop permanent paralysis of hind limbs and severe contractures. Very old dystrophic mice often develop and die of pneumonia (Russel and Meier, 1979).

Extensive investigations have defined structural details of muscle fibre breakdown in murine dystrophy (Platzer and Powell, 1975). According to Platzer and Powell the earliest identifiable ultrastructural change is seen in the sarcoplasmic reticulum which becomes dialated. This change is followed sequentially by the appearance of fragmented myofibrils which are out of register, and swollen spherical mitochondria which contain irregularities in the cristae and some electron dense areas in the matrix. With continued muscle fibre breakdown, the fibres show retraction clots, sparsity of cellular organelles, including the T-system, myofibrillar fragmentation and further sarcolemmal breakdown, leading finally to phagocytic infiltration.

Figure 1a. Photograph of normal and dystrophic mice. Note the reduced size of the dystrophic mouse (right).

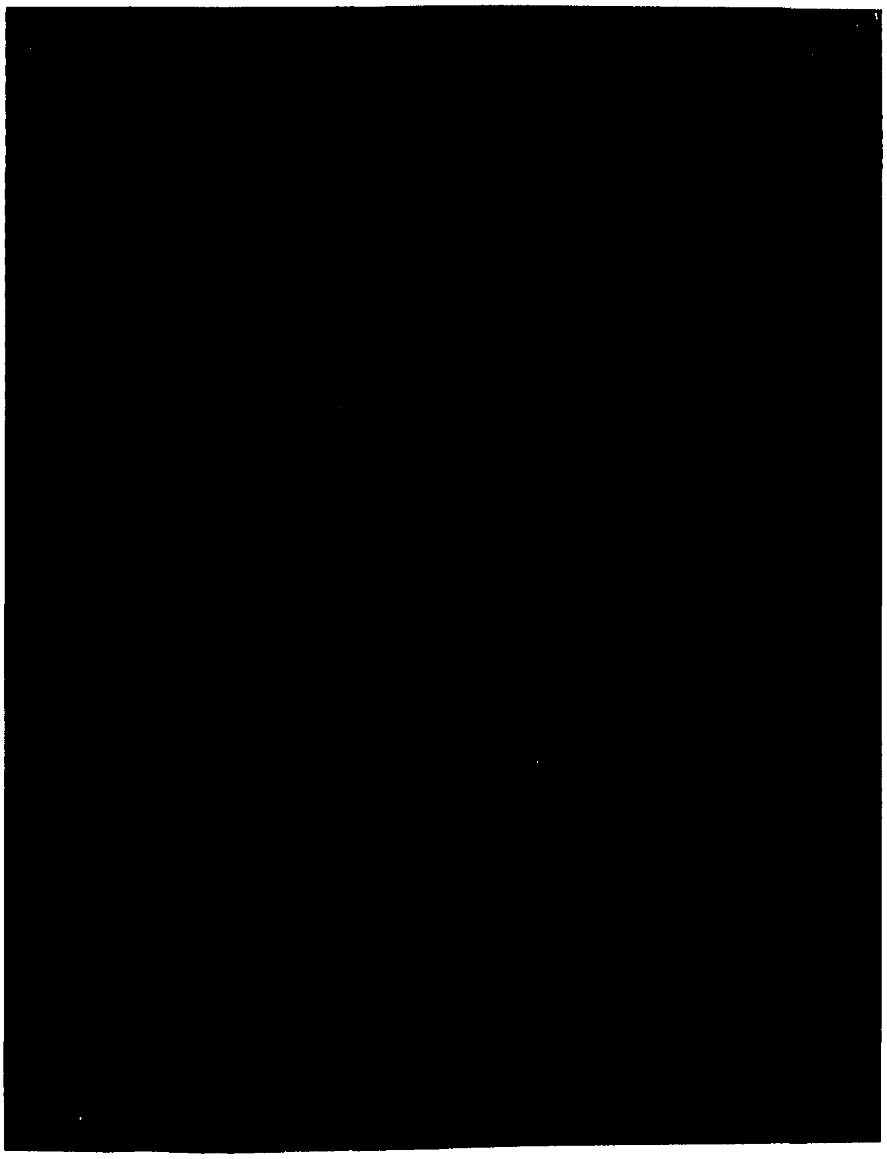


Figure 1b. Photograph showing the hind leg muscles of normal and dystrophic mice.

Top: Hind leg muscles from normal mouse.

Bottom: Hind leg muscles from dystrophic mouse.

In the mouse, the individual pattern of muscle fibre breakdown occurs in an entirely different setting than that seen in the hamster (Mendell et al., 1979). The degenerating fibres are usually randomly distributed as either isolated fibres or as two or three together. There are, however, areas of the muscle that display preferential involvement in the process. In the 129/ReJ dy/dy strain, the crown position of the anterior tibialis, composed of predominantly fast twitch glycolytic fibres, appears to be the site of primary expression of the disease. In C57BL/dy^{2J}/dy^{2J} strain, the target is believed to be the slow twitch oxidative fibres of the core portion of the anterior tibialis and soleus muscles.

The initial stage of muscle fibre breakdown appears to involve partial or complete segments of muscle fibres. These areas of the muscle fibre are then invaded by macrophages. Before phagocytosis is complete, evidence of muscle fibre regeneration ensues with the appearance of presumptive mononuclear myoblasts, which apparently fuse to form regenerating fibres (West and Murphy, 1960).

It is during the latter stage of regenerative activity that murine dystrophy shows singular differences from the hamster form of the disease. In association with the muscle fibre regeneration, proliferation of the endomysial connective tissue is quite prominent. West and Murphy (1960) describe this phenomenon as an "early sequela to regenerative activity" which they believe is the result of newly proliferated connective tissue rather than condensation of existing endomysium. The final stages of murine dystrophy are characterised by marked variability in muscle fibre size, and significant endomysial connective tissue proliferation, which surrounds smaller diameter fibres. Fatty replacement is not a prominent feature; however, it can be enhanced by prolonging the life span of the animal (Mendell et al., 1979).

C. Comparison of dystrophy in mice and humans

In humans, *in vitro* studies show that the early stages of muscle fibre breakdown are associated with extensive leakage of horseradish peroxidase into muscle fibres (Mendell et al., 1979). The small wedge-shaped pattern of horseradish peroxidase leakage observed in hamster and Duchenne human dystrophy is not seen in murine dystrophy. However, in the mouse, horseradish peroxidase infiltration is usually more extensive, suggesting that larger segments of the plasma membrane are affected during muscle fibre degeneration. This conclusion is consistent with previous ultrastructural reports on mouse dystrophy which describe alterations in sarcoplasmic organelles prior to breakdown of the cell membrane (Platzer and Powell, 1975). Accumulation of calcium deposits and other signs of cell damage support the conclusion that focal defects in the plasma membrane are probably not the initial events in cellular degeneration in murine dystrophy. However, identification of both horseradish peroxidase leakage and calcium influx during early cellular necrosis (prior to phagocytosis) may have relevance to the pathogenesis of muscle fibre breakdown in some types of human muscular dystrophies.

D. Syrian_hamster_dystrophy

Syrian hamsters suffering from hereditary muscular dystrophy develop acute cardiac muscle lesions in addition to skeletal muscle breakdown. The acquisition of knowledge concerning involvement of the heart in progressive muscular dystrophy, and in other forms of primary myopathies is important from several viewpoints. Ignorance of this subject may lead not only to incorrectly identified clinical symptoms, but also to inaccurate etiological diagnosis and treatment. From a pathophysiological viewpoint, cardiac involvement is of great interest in respect to both its haemodynamic effect per se and the underlying mechanisms of heart failure. Furthermore, studies of this type of cardiac condition provide an opportunity to gain information concerning differences in susceptibility between cardiac and skeletal muscles, since virtually nothing is known about the factors that determine which of these two muscle systems will undergo degeneration in any given set of pathological circumstances (Bajusz et al., 1966). From a biochemical viewpoint, an analysis of the altered myocardial metabolism may elucidate the primary biochemical lesion responsible for the disease in striated muscles. In view of these considerations, studies of cardiac muscle lesions which develop in 100% of the Syrian hamsters suffering from hereditary muscular dystrophy become important.

In a large percentage of these animals (ca. 90%), cardiac involvement progresses to congestive heart failure. The gross and histopathological abnormalities seen outside the skeletal and cardiac muscles in the myopathic hamsters are consistent with chronic passive venous congestion, and the progressive cardiovascular insufficiency may be regarded as the ultimate cause of death. Thus, the inbred line of Syrian hamsters provides not only an opportunity to study the involvement of the cardiovascular system in a genetically determined primary myopathy, but it also presents a useful and unique disease model for analysis of the underlying mechanism of congestive heart failure. The slow progression of the cardiovascular condition in the myopathic hamsters and high incidence of congestive heart failure within the homozygous line offers new possibilities for investigative cardiology.

The initial stages of individual skeletal muscle fibre breakdown are characterised by a series of changes in the myofilaments and mitochondria. Under the light microscope the mitochondria appear swollen and often clumped together, and the muscle fibres have lost their normal striations usually appearing hyalinized or hypercontracted. This stage is soon followed by the presence of macrophages surrounding and finally invading the fibre (Mendell et al., 1979). At the ultrastructural level, the mitochondria display a spectrum of changes accompanying the early events. The clumping of mitochondria seen in the light microscope is quite apparent. The mitochondria become spherical rather than elongated in appearance and they are often larger than normal. The cristae are indistinct or absent in some mitochondria, while in others they exhibit parallel and concentric arrays. Concommitent with histological changes, myofilaments ultimately become more fragmented and randomly oriented. Initially, the A bands are preferentially spared, while there is a complete absence of the Z bands. Accompanying the initial stages of muscle fibre breakdown, but preceeding phagocytosis, are intracellular accumulations of granular material seen with alizarin red stain for calcium (Mendell et al., 1979). Some fibres demonstrate granular deposits restricted to the subsarcolemmal regions while in others, calcium deposits are observed to be distributed throughout the cross sectional diameter of the fibre (Mendell et al., 1979).

Various stages of horseradish peroxidase leakage into muscle fibres were observed corresponding to the stages of muscle fibre breakdown. The earliest detectable change that preceded phagocytosis was leakage into the subsarcolemmal region, which could be seen in the light and electron microscope. The area of leakage was often restricted to a small wedge shaped area with the base toward the periphery or in the form of a restricted subsarcolemmal band of horseradish peroxidase. The area of wedge shaped leakage displayed a loss of overlying plasma membrane with preservation of the basement membrane. In advanced stages of muscle fibre breakdown, leakage of horseradish peroxidase extended further into the sarcoplasm (Mendell et al., 1979).

E. Comparison of dystrophy in hamsters and humans

The initial stages of skeletal muscle fibre breakdown in the hamster have certain similarities to Duchenne muscular dystrophy (Mokri and Engel, 1975; Bodensteiner and Engel, 1977). The early leakage of horseradish peroxidase in a wedge-shaped pattern in the muscle fibre is similar to that described by Mokri and Engel in humans (1975). This finding suggests that the plasma membrane is disrupted as one of the early morphological events in muscle fibre breakdown in the hamster. This occurence is different from that observed in Duchenne muscular dystrophy where the breakdown of the plasma membrane accompanies, rather than precedes, other alterations in the muscle fibre (Mendell et al., 1979). The early accumulation of calcium deposits in the muscle fibre is also similar to that found in Duchenne dystrophy (Bodensteiner and Engel, 1977). A significant distinguishing feature, however, is that groups of muscle fibres rich in calcium deposits are far more extensive in the hamster, and overt mineralization of the mitochondria is not a prominent feature of human dystrophy.

Several additional points of morphological differences from human dystrophy have been observed in the hamster. Groups of muscle fibres undergoing necrosis and regeneration have been seen in Duchenne muscular dystrophy (Karpati et al., 1974), but they never reach the extensive size seen in the hamster where they can often be visualized grossly (Mendell et al., 1979). Furthermore, the marked degree of phagocytosis in the hamster is a far more subtle feature in human dystrophy. In addition, the presence of intracristal plates in the mitochondria of degenerating fibres in the hamster are rarely seen in Duchenne dystrophy (Karpati et al., 1974). The final point, and one that more clearly distinguishes the dystrophic process in the hamster from that in humans (aside from the fact that dystrophy affects primarily the cardiac muscle in hamsters) is the absence of endomysial connective tissue proliferation and fat infiltration in the hamster. The degenerative process in the hamster simply does not seem to reach this stage (Mendell et al., 1979).

F. Muscular dystrophy in the chickens

Myopathy in chickens is characterized principally by a failure in the development of fast twitch glycolytic fibres in the pectoralis muscles shortly after hatching (Cosmos and Butler, 1967). This developmental failure may occur through an inability to respond to neurotropic influences mediated by a defective sarcolemma. However, evidence that the fetal spinal cord imprints a dystrophic character on the pectoralis muscle at a very early stage has also been obtained (Rathbone et al., 1975). A rapid reversal of metabolic behaviour to a fetal fibre type occurs during the first 2 weeks ex-ovo. Subsequently, the pectoralis displays a marked pseudohypertrophy, phagocytosis of individual fibres and an increase in connective adipose tissue (Owens, 1979).

Horseradish peroxidase studies in dystrophic chicken muscle showed that the vacuoles in the individual muscle fibres, studied by light and electron microscopy, communicate with the extracellular spaces. The limiting membrane, distinct in some places, usually could not be traced around the entire vacuole. In addition, the foci of the myofibrillar disarray begin rather abruptly. The Z-band material appears as an irregularly dispersed mass, but the Z-band streaming is not seen. The boundaries of A and I bands can be discerned, and the thick and thin filaments are completely disoriented. There are no obvious organelle changes to give a clue to the pathogenesis. Muscle fibres that demonstrate myofibrillar disarray do not show any evidence of horseradish peroxidase leakage across the membrane (Mendell et al., 1979).

G. Comparison of dystrophy in chickens and humans

The muscle fibres in dystrophic chickens contain a marked increase in lipid droplets. Several abnormalities like variation in muscle fibre size, muscle fibre splitting, increase in the number of internal nuclei, vacuolization of fibres and scattered fibres undergoing necrosis and phagocytosis are also seen (Mendell et al., 1979). In the chicken there is no evidence in support of a significant plasma membrane defect, either by horseradish peroxidase leakage or by accumulation of calcium deposits, as one of the causes for these known abnormalities. In this way, dystrophy in chicken muscle shows changes unlike those observed in the DMD disorder, where the influx of calcium apparently is the stimulus for the muscle fibre to undergo a series of morphological changes.

H. Summary of morphological changes in dystrophic animals compared to dystrophic humans

The hamster and mouse show changes of potential relevance toward understanding the pathogenesis of individual muscle fibre breakdown in human DMD. In both species, horseradish peroxidase leakages and calcium deposits accompany muscle fibre breakdown. The chicken, in contrast, offers the challenge of understanding the vast array of morphological changes in the absence of a significant plasma membrane defect as a step in the development of these changes. While perhaps no animal disease model can be expected to be identical in every way to a human disease, each has features that are important in relationship to particular aspects of human muscle pathology. Furthermore, the inherited form of muscular dystrophy in animals provides a unique opportunity to study sequential morphological changes in the development of this pathological process.

I. Biochemical characteristics of dystrophy

The primary biochemical lesions involved in muscular dystrophy are not known, although, a great deal of research has been done during the last few years. Changes in lipids (Hughes, 1972), carbohydrate (Johnson et al., 1979), purines (Thomson and Smith, 1978), and protein metabolism (Iodice et al., 1972; Strickland et al., 1979) have been implicated in the onset of dystrophy. Other workers have shown that both RNA content and synthesis are increased in dystrophic muscles of the mouse (Srivastava, 1968; 1967; Girkin et al., 1962; Fowler et al., 1977). However, the RNA content was found to be decreased relative to the DNA content (Hayashi et al., 1975). Fowler et al.(1977) observed an increase in DNA content in dystrophic muscle of the mouse. The significance of increased RNA and DNA levels in dystrophic muscle is difficult to understand. However, these increases may be due, in part, to muscle hypertrophy, which, in turn, reflects the compensatory attempts of dystrophic muscle.

Progressive muscular dystrophy is characterised by severe wasting of skeletal muscle and this is confirmed by the lower body and muscle weights of dystrophic animals (Hayashi et al., 1975; Watts and Reid, 1969); Kitchin and Watts, 1973). In view of the great importance of proteins in the structure and function of muscle, many laboratories have studied protein metabolism in dystrophic animals. Several studies have shown that the total nitrogen content is decreased in dystrophic muscle (Srivastava, 1968; Berilinguet and Srivastava, 1966; Srivastava and Berilinguet, 1964). Also, the rate of increase in body weight or in leg musculature of the dystrophic mouse is only about 50% of normal at 4-8 weeks of age (Gopalan et al., 1986a). However, it has been shown that non-collagen nitrogen per gram of muscle is not altered (Nihei and Filipenko, 1974; Fowler et al., 1977). Since the amount of DNA (per unit weight) in muscle is increased, it may be concluded that the relative reduction in proteins is due either to a decrease in protein synthesis or to an increase in protein degradation. Several studies designed to measure the rate of protein synthesis in normal and dystrophic muscle have shown that the rate of protein synthesis in intact dystrophic skeletal muscle is elevated markedly. In dystrophic hamsters, the rate of total protein synthesis _in vivo_ is nearly two-fold greater than in corresponding controls, and the same is true for myosin synthesis (Strickland et al., 1979). In dystrophic mice (129/ReJ) Garber et al.(1980a) observed that the rate of protein synthesis is nearly 5-fold greater than litter mate controls. When the identity of the protein synthesized was studied using SDS-PAGE they observed that the majority of the proteins migrating between heavy chain myosin and actin were synthesized in similar proportion in both normal and dystrophic muscle. Several differences in the relative rates of protein synthesis were observed in lower molecular weight proteins such as myosin light chains 1, 2 and 3 and troponin I and C which all showed a reduced rate of synthesis relative to actin and myosin, but still higher than in the controls. Using human muscle from Duchenne muscular dystrophy patients, Ionascescu et al.(1971) observed that protein synthesis in vitro is 5-6 times higher than normal controls. Their later work showing an increase in number of membrane bound ribosomes in preparations from human dystrophic muscle also support this view (Ionasescu et al., 1981b). However, a previous observation, contrary to these, had been made where no difference in the ribosomal content of normal and dystrophic muscle was noticed (Watts and Reid, 1969). According to these workers the "machinery" for protein synthesis appears to be normal, and any difference must be due to altered concentrations that result from changes in muscle volume. Yoshikawa et al. (1984) and Saleem and Nicholls (1979) observed an increase in the number of EF-2 molecules in the cytoplasm of dystrophic muscle which may account for the increase in protein synthesis activity observed in dystrophic chickens and hamsters. Other studies indicate a defective protein synthesis "apparatus". Nicholls et al.(1986) have shown that the ability to translate mRNA for tropomyosin in the dystrophic hamster muscle homogenate is decreased compared with the normal control. Srivastava (1969) showed that in dystrophic muscle, incorporation of radioactivity into low-molecular weight proteins like tropomyosin is higher, while that into high molecular weight proteins like myosin is lower than normal. Synthesis of actin was not affected. These findings suggest that the polyribosomal fraction from dystrophic muscle is less active, while the monoribosomal fraction is more active. Several laboratories have studied the synthesis of myosin in dystrophic muscle (Nihei and Filipenko, 1974; John et al., 1973; Monckton and Marusyk, 1975). Monckton and Marusyk (1975) used autoradiography and showed decreased myofibrillar synthesis in vivo in dystrophic muscle of humans. Nihei and Filipenko (1974) also noted a decreased in vivo synthesis of myosin in murine dystrophy. These workers demonstrated that polyribosomes from dystrophic muscle are more active in the synthesis of non-myosin proteins. A potassium chloride extract from normal polyribosomes was found to restore the myosin synthesis activity of dystrophic polyribosomes (Nihei and Filipenko, 1974). Thus, there is considerable evidence to suggest that the polyribosomal system from dystrophic muscle is deficient in a myosin specific factor(s) and thus may be responsible for the impaired synthesis of myosin.

In the past two decades several studies have focused on carbohydrate metabolism in dystrophic muscle to determine whether alterations in carbohydrate metabolism are the cause of muscle weakening in Duchenne muscular dystrophy patients (Strickland et al., 1979; Ellis, 1980). The published literature on this topic has revealed that several aspects of the glycolytic process are altered in dystrophic muscle. First, mammalian muscle has a reduced capacity for both the synthesis and utilization of glycogen. This lesion appears to be due to a marked reduction in glycogen, UDP-glycosyl transferease and glycogen phosphorylase activities (Garber et al., 1980a; Dreyfus, 1954; Ellis, 1980). Most other glycolytic enzymes show some reduction in activity in dystrophic muscle. As a result, dystrophic muscle has a reduced capacity to produce lactate and ATP (Dreyfus,1954). Also, the dystrophic muscle is capable of increased utilization of glucose via hexose monophosphate shunt, increased synthesis of NADPH and thus an increased synthesis of lipids in dystrophic muscle has been proposed by Ellis (1980). Garber and co-workers have also shown that dystophic muscle has a reduced responsiveness to insulin stimulation of glucose uptake and adenyl cyclase activation of epinephrine (1980b). However, since adenyl cyclase from dystrophic muscle membrane can be fully activated by sodium fluoride, these workers have suggested that the defect in the adenyl cyclase mechanism may be due to a defect in the adrenergic membrane receptor or receptor coupling to adenyl cyclase. In addition to this, increased amino acid release especially alanine and glutamine was observed by Garber et al. (1978). Amino acid formation and release seems to be modulated by the cyclic nucleotides in association with insulin and also with other agonists. Catecholamines acting through a B-adrenergic receptor and in association with increased intracellular levels of cAMP are potent inhibitors of alanine and glutamine formation and release in skeletal muscle (Garber et al., 1976; Garber, 1978). Serotonin (5-hydroxytryptamine) acting through D-serotonergic receptors and in association with increased levels of cAMP, inhibits alanine and glutamine formation and release from muscle (Garber, 1978). The increase in amino acid release appears to result, in part, from an increase in skeletal muscle protein degradation (Garber et al., 1980a). Increased cyclic GMP levels were found in dystrophic muscles as compared with normal muscle by the same workers, and these higher levels may lead to increased rates of muscle proteolysis and hence greater alanine and glutamine formation and release. The apparent inability of cAMP to inhibit amino acid output may be derived from the augmented cGMP level in dystrophic mouse muscle since antagonistic roles have been proposed for these cyclic nucleotides in other biological systems (Goldberg et al., 1973).

A symptom of muscular dystrophy of particular clinical relevance is the high level of activity of serum creatine kinase (Bradley and Fell, 1980). The dramatic elevation of serum creatine kinase activity is a diagnostic feature of muscular dystrophy in humans, and an increase, though less dramatic, occurs in each of the three main animal models of the disease. While the increase in serum creatine kinase activity is generally assumed to be due to leakage from diseased muscle fibres, it is by no means clear what sort or extent of cellular damage would be consistent with the increased serum activity. Kuby et al.(1977) made a remarkable observation regarding creatine kinase. They showed by direct isolation of creatine kinase that 3 isoenzymes of creatine kinase are present in the musculature of males affected by terminal Duchenne muscular dystrophy, whereas only the muscle type was to be found in the skeletal muscle of normal adult human males. In human brain tissue of both the normal and dystrophic animals, only a single isoenzyme could be detected and isolated. The creatine kinase isoenzymes from dystrophic muscle were compared electrophoretically and immunologically with isoenzyme preparations from normal muscle (Keutel et al., 1972) and identified as muscle type, hybrid type, and brain type. The unique isoenzyme distribution of the creatine kinases found in the atrophying muscle of patients with Duchenne muscular dystrophy resembles that of foetal muscle and this has led to the speculation that the major biochemical defect in Duchenne muscular dystrophy may lie in the inability to "switch off" the synthesis of foetal-like proteins (Schapira, 1967; Schapira et al., 1968; Eppenberger et al., 1964; Cao et al., 1968; Dawson et al., 1968, Kuby et al.(1977) This, in turn, might lead to a foetal-like complement of proteins in skeletal muscles, in the membrane of muscle, or even within the membrane of red cells. Thus , the so-called "leaky" or "fluid" membrane abnormalities described for the dystrophic red cell (Butterfield, 1977) might simply be another manifestation of a more generalised phenomenon, namely that of a foetal-type membrane composed of a foetal-like complement of proteins. Other proteins which show immature isoenzyme patterns are lactate dehydrogenase (Kaplan and Cahn, 1962; Emery, 1964; Tsvetanova and Ognianov, 1967), myosin (Obinata et al., 1980; Fitzsimons and Hoh, 1981) and tropomyosin (Takeda and Nonumura, 1980) in dystrophic muscle. The presence of foetal myosin in patients with Duchenne muscular dystrophy probably reflects a certain amount of muscle regeneration, and immaturity of some muscle cells. If the suggestion given by Kuby et al.(1977) bears merit, the X-linked Duchenne muscular dystrophy might be an inborn error in the final development of the muscle tissue in the human male organism such that there is a reversion back to foetal development, or that there is a reversion back to the foetal state in muscle prior to death.

Many other factors also seem to be impaired in dystrophy. Poly-ADPR synthetase decreases during normal differentiation of muscle cell (Caplan et al., 1979). A delayed decrease in the amount of poly-ADPR in dystrophic chickens suggests an abnormal developmental program (Yoshikawa and Masaki, 1985). Abnormal acetylcholine esterase content has been observed in dystrophic muscle. High levels of acetylcholine esterase are found throughout the fibre of normal and dystrophic embryo muscles. This activity is associated with several molecular forms separable on polyacrylamide gels. In the normal muscle, the enzyme levels decline after hatching, cytochemical staining decreases and low molecular weight forms of this enzyme can no longer be detected on polyacrylamide gels. However, in muscles afflicted with dystrophy the embryo-like forms are retained. Moreover, acetylcholine esterase levels are high, low molecular weight forms are present, (seen in polyacrylamide gels) and the acetylcholine staining is high in sarcoplasm outside motor end-plate regions (Wilson et al., 1970; Linkhart and Wilson, 1975 and Weidoff and Wilson, 1977). These observations support the idea that there is an error in in development and it involves processes mediated by nerve muscle interactions.

J. Integrity of plasma membrane in dystrophic animals

The hypothesis that muscular dystrophy is primarily the result of a membrane defect has been supported by several observations among which are differences in ionic permeability of the sarcolemma, decrease in nucleotide and creatine phosphate levels, increase in serum levels of several intracellular enzymes, and alterations in the plasma membrane structures of several cell types.

One of the most striking features of Duchenne muscular dystrophy in humans (as indicated earlier) and a commonly used clinical indicator of the disease, is an elevation of serum creatine kinase which may reach as high as fifty times the normal plasma level in the early stages of the disease (Munstat et al., 1973 ; Rowland, 1980). The source of this enzyme is thought to be due to "leaky" skeletal muscle cells which are rich in creatine kinase. However, there is still controversy in the literature concerning the relevance of creatine kinase as an indicator of Duchenne muscular dystrophy since dystrophic human muscle cells in culture do not appear to leak creatine kinase into the medium although the concentration of creatine kinase is low in these cells. Also, the serum level of creatine kinase is not elevated in the commonly used strain (129/ReJ) of dystrophic mice (Ionasescu et al., 1981a; Lieberman et al., 1981). Despite apparent strain differences, muscle appears to be the source of plasma creatine kinase as well as serum aldolase in Duchenne muscular dystrophy patients (Strickland et al., 1979; Ionasescu et al., 1981a).

Other evidence which is consistent with the view that dystrophic cells are "leaky" comes from measurements of the electrolyte content of normal and dystrophic muscle cells. It has been shown that dystrophic muscle cells have an elevated concentration of sodium and chloride ions and a decreased concentration of potassium ions (Strickland et al., 1979; Duncan, 1978). However, since the literature contains conflicting reports on the integrity of plasma membrane ATPase activity in dystrophic tissues, it is still not clear whether the alterations in sodium and potassium levels in diseased muscle are due to defective Na^+/K^+ ATPase molecules in the plasma membrane or to enhanced "leakyness" of the membrane. The change in electrolyte distribution during the onset of dystrophy may also be due to the appearance of "holes" in the plasma membrane (Cullen and Mastaglia, 1980). Regardless of the cause of this defect, the possibility that an increase in intracellular calcium in dystrophic muscle promotes the activity of muscle proteases capable of degrading the myofibrillar proteins has been clearly noted (Kar and Pearson, 1976).

Another membrane defect which has been shown to occur in both erythrocytes and muscle of dystrophic humans and mice is depletion of transmembrane particles. Freeze fracture techniques have revealed that the plasma membrane of both erythrocyte and myotubes have 40-60% fewer transmembrane particles than normal cells (Cullen and Mastaglia, 1980; Munstat et al., 1973; Shivers and Atkinson, 1979). Since these membrane particles are involved in transport mechanism, the disappearance of these membrane particles in dystrophy implicates proteases in the removal of these particles and in disruption of transport phenomenon.

K. Turnover of proteins in dystrophic animals

The intracellular process which regulates the mechanism of protein turnover is a fundamental problem about which very little is known (Katanuma et al., 1976). In skeletal muscle the half-life of the proteins in the various muscles of adult organism varies from 4-40 days depending on the source of the muscle. However, the turnover rate of the various proteins is dependent on a number of factors including age, nutritional state, hormone balance and level of contractile activity of the animal/muscle under study (Millward, 1980). Unfortunately, numerous technical problems arise when one attempts to measure protein turnover in skeletal muscle (and other tissues) which make data assessment extremely difficult (Everett and Zak, 1980). Notwithstanding these difficulties, it appears certain that protein turnover in dystrophic muscle of humans, mice, hamsters, chickens, and cultured dystrophic cells is enhanced markedly compared with non-dystrophic controls (Young and Munro, 1980; Garber et al., 1980b; Li, 1980; Ettienne et al., 1980). Moreover, since the outcome of dystrophy is muscle wasting, there must be an accelerated rate of protein degradation over protein synthesis in vivo.

The onset and progression of muscular dystrophy in humans and animals is characterized by a marked increase in intracellular protease activity in skeletal muscle, leading to degradation of myofibrillar proteins, and muscle necrosis. The proteases showing the greatest increase in activity in dystrophic muscle are the thiol proteases, particularly cathepsin B (Kar and Pearson, 1977). Goldspink et al.(1977) and Rouke (1975) have indicated increased proteolysis in muscles in conditions such as muscular dystrophy and the important role of lysosomal proteases in degradation of myofibrills has been suggested by Weinstock and Iodice (1969). Since increased lysosomal enzyme activities are found in experimental myopathies and hereditary muscular dystrophy, these enzymes are frequently implicated as a major factor in the muscle wasting process. Consistent with this idea, increased levels of cathepsin B have been observed in muscle of patients with muscular dystrophy and related diseases (Kar and Pearson, 1977; Katanuma et al., 1977). Also, lysosomal (thiol) proteases such as cathepsins B and L were found to degrade purified myofibrillar proteins in vitro though in different ways (Schwartz and Bird, 1977; Noda et al., 1980; Matsukura et al., 1981). Given that the thiol proteases have been shown to degrade a wide variety of sarcoplasmic and myofibrillar proteins in vitro, it would appear that control of these enzymes in vivo is of vital importance in maintaining normal cell function.

In recent years considerable attention has focused on the physiological role of protease inhibitors in the process of protein metabolism (Lasowski et al., 1980). Since the time Frankenstadt (1957) first reported the presence of endogenous thiol protease inhibitors in rat liver, many workers have attempted to purify and characterize these inhibitors. The inhibition of proteases by protein inhibitors is an intriguing problem in studies of specific protein-protein interactions (Ruhlman et al., 1973; Mitsui et al., 1979). It has been reported that several cathepsins of lysosomal origin which play an important role in regulating the degradation of soluble and structural proteins in many tissues have specific endogenous inhibitors (Lenney, 1980). Several thiol proteinase inhibitors have been isolated from mammalian tissues (Udaka et al., 1965; Jarvinen, 1976; Lenney et al., 1979; Kopitar et al., 1978; Hibino et al., 1980; Kominami et al., 1982) which form reversible complexes either with a specific protease or its substrate. Those which affect metal containing proteases are usually larger proteins. In most tissues, these inhibitors are localised in the cytosol fraction of the cell, whereas the majority of the proteases are associated with organelles such as lysosomes, golgi or in other particulate fractions (Lenney, 1980). Furthermore, three low molecular weight thiol protease inhibitors were detected in human epidermis (Jarvinen, 1978). Hibino et al. (1980) indicated that a protease inhibitor purified from new born rat epidermis was synthesized in epidermal cells during keratinization. Ohitani et al. (1982) showed only subtle differences in inhibitory activity between inhibitor from new born rat and human epidermis towards several thiol proteases despite significant differences in sequence homology.

Of relevance is the finding of Schwartz and Bird (1977) who reported that rat muscle contains 2 inhibitors of cathepsin B activity which can be inactivated by incubation of muscle extracts at pH 4.5. These inhibitors have not been fully characterised but they have molecular weights of 12,500 and 62,000 daltons. It is presumed that the activity of cathepsin B in muscle is regulated by these endogenous inhibitors which are, in turn, regulated by hydrolases or some other mechanism (Schwartz and Bird, 1977). The importance of proteinase inhibitors which suppress the activity of cathepsin B and D and calcium activated neutral protease in normal muscle, and which may be lost in diseased muscle, has been suggested by the studies of Libby and Goldberg (1978) and Stracher et al. (1978). In this respect, the findings of Spanier and Bird (1982) are most interesting. They have shown that the gastronemius and masseter muscles from female guinea pigs maintained on a vitamin E deficient diet have reduced cathepsin B inhibitor activity compared to similar cytosol fractions prepared from guinea pigs on a normal diet. Furthermore, they suggested that the apparent increase in cathepsin B activity in muscle extract of vitamin E deficient animals, and perhaps in dystrophic muscles, may be attributable to a decreased level of endogenous protease inhibitor in these groups of animals. Of considerable significance to the above findings are the results of Stracher et al. (1978) who showed that leupeptin, an effective inhibitor of cathepsin B and calcium activated neutral protease, and pepstatin, an inhibitor of cathepsin D, inhibit both the rate of total protein degradation and cathepsin B activity in intact rat muscle in vitro. Moreover, Libby and Goldberg (1978) have reported that leupeptin and pepstatin are able to offer some protection to the general architecture of chick muscle and to reduce markedly the high level of serum creatine kinase in dystrophic chicken when injections are started one day after hatching. In other studies, Sher et al. (1981) have reported that leupeptin treatment (via I.P. injections) of 3 week old mice with genetic muscular dystrophy delays the appearance of myopathy for at least 24 weeks. Recently, Tsuji and Matsushita (1986) have reported successful control of depeptidyl peptidase activity in dystrophic mice with the protease inhibitor bestatin along with the disappearance of myopathological features characteristic of the disease. These studies, while incomplete, strongly suggest that the loss of control of protease activity in skeletal muscle is of fundamental importance in the onset and development of muscle wasting characteristic of various muscle diseases. While a potential role for protease inhibitors as modulators of intracellular proteolysis has been recognised, the intracellular function of the molecules involved are not very well understood. Since, the tissue proteins are subject to continuous turnover, a control mechanism must exist to ensure protein homeostasis and this control could also be performed by protease inhibitors. Thiol protease inhibitors assayed for activity against cathepsin H have been found in all organs of the rat (Kominami et al., 1982b). Immunodiffusion analysis with anti-liver thiol protease inhibitor serum indicated that the inhibitor in rat liver is immunologically identical with the inhibitor in other rat tissues (Kominami et al., 1982b). However, contrary results have been reported recently where anti-rat liver inhibitor serum did not react with anti-rat epidermis serum (Katanuma and Kominami, 1985). A comparison of the properties of the purified inhibitors showed that at least two different types of thiol proteinase inhibitors are present in the cell, the liver type and the epidermal type. The presence of inhibitors in various other tissues of mammals, including pigs, hamsters, rabbits, and humans has been reported (Lenney et al., 1979; Turk et al., 1982). Thus intracellular protease inhibitors seem to be ubiquitous in mammals. However, species specificity exists since, anti-rat protease inhibitor serum did not cross react with either human or rabbit protease inhibitor (Wakamatsu et al., 1982).

Since the wasting of muscles in the various forms of muscular dystrophy is obviously the consequence of breakdown occuring at a rate exceeding that of resynthesis, a natural approach to control this situation is to try and inhibit the proteases responsible for the excessive degradation. The finding that muscle tissue extracts contain inhibitory activity against thiol proteases (Schwartz and Bird, 1977) prompted us to examine whether these inhibitors might be involved in the modulation of protease activity in normal muscle and whether changes, if any, in inhibitor activity occur in dystrophic muscle which might be the cause for the increased protease activity found in the diseased muscle. This dissertation describes the purification and properties of a thiol protease inhibitor from normal and dystrophic hindlimb muscle of the mouse of strain 129/ReJ. This study, a part of which has been published (Gopalan et al., 1986b) also shows that the thiol protease inhibitor from dystrophic muscle is functionally defective in the control of cathepsin B activity compared with the thiol protease inhibitor protein from normal mouse littermates.

II. MATERIALS AND METHODS

A. Animals

Dystrophic male mice (strain 129/ReJ-dy) and their normal littermates were obtained from Jackson Laboratories (Bar Harbor, Maine) at about 30 days of age and maintained in the laboratory to age 60 days on standard laboratory chow (Purina). Also, female mice of strain 129/ReJ, heterozygous for the disease and normal retired breeders of the same strain of about 9 months of age were used.

B. Biochemicals

N-α- benzoyl-DL-arginine-2-naphthylamide (BANA), N-α-CBZ-L-arginyl-L-arginine-4-methoxy-B-naphthylamide, Fast Garnet GBC salt, azocaesin, rabbit myosin and pI markers for isoelectric focusing were purchased from Sigma Chemical Co (St Louis, Mo.). The protein molecular weight markers for electrophoresis were from Pharmacia (Montreal, P. Q.). The Chemopack C-18 HPLC column was prepared by Dr. F. L. Huang from material obtained from Chemco Scientific Co. (Osaka, Japan). Acetonitrile was of HPLC grade and the pI 3.5-10 ampholytes were from LKB (Fisher Scientific, Toronto, Ont.). Ultrafiltration Diaflo membranes were from Amicon Corp. (Danvers, Ma.). Acrylamide and SDS were from BioRad Laboratories (Mississuaga, Ont.). All other chemicals were of reagent grade or better.

C. Methods

1. Tissue sample preparation

The tissues for isolation of thiol proteases and thiol protease inhibitors (TPI's) were obtained by cervical dislocation of the animals and the tissues perfused in situ by pumping 40 ml of mammalian Ringers solution containing 0.1 mM EDTA through the animal at 20 ml per minute using direct cardiac puncture. The total hindlimb muscle and liver were excised and used either immediately or stored at -70 C.

2. Preparation of mouse liver lysosomal thiol proteases

Frozen livers from normal and dystrophic 60 day old male mice were thawed, cut into small pieces and ground with fine glass beads and 5 volumes of ice-cold 0.25 M sucrose in a mortar and pestle at 4 C. The homogenate was centrifuged at 600 x g for 10 minutes to remove intact cells and nuclei. The resulting supernatant was centrifuged at 27,000 x g for 20 minutes to pellet the mitochondria and lysosomes (M+L). The crude M+L pellet was washed with about 25 volumes of 0.25 M sucrose solution containing 0.1 mM EDTA and 0.03 M Tris-HCl, pH 7.0, and the M+L fraction was collected again by centrifugation as above. The resulting M+L pellet was suspended in 20mM sodium acetate, pH 5, containing 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 10% glycerol and homogenized thoroughly in a ground glass homogenizer. The homogenate was centrifuged at 27,000 x g and the resulting pellet re-homogenized 3-4 times. The 27,000 x g supernatant fractions were pooled and concentrated by ultrafiltration on a Diaflo YM-5 membrane and used as the source of lysosomal (total) thiol proteases.

3. Partial purification of cathepsins B, H and L from mouse liver lysosomes

Cathepsins B (EC 3.4.22.1), H (EC 3.4.22.16) and L (EC 3.4.22.15) were prepared from the crude lysosomal extract from mouse liver according to the procedure of Barrett and Kirschke (1981) with slight modifications. The total M + L extract was concentrated to about 2 ml by ultrafiltration as described above and passed through a Sephadex G-75 column (1 x 50cm) equilibrated with 20 mM sodium phosphate buffer pH 6, containing 1 mM DTT and 0.1 mM EDTA and 10% glycerol. The fractions giving a positive reaction to N-α-benzoyl-DL-arginine-2-naphthylamide (BANA) as substrate were pooled and concentrated on a Diaflo YM-5 membrane. The enzyme concentrate was applied to a DEAE-cellulose column (DE-32, 1 x 19 cm) equilibrated with 20 mM sodium phosphate, pH 6, containing 1 mM DTT, 0.1 mM EDTA, and 10% glycerol. The column was washed with the equilibrating buffer and cathepsin H (which does not bind to DEAE-cellulose) was collected in the wash and concentrated by ultrafiltration (YM-5). Cathepsins B and L were eluted from the column by stepwise elution with 0.2 M and 0.45 M NaCl, respectively, in the equilibrating buffer. Each protease fraction collected from the column was pooled and concentrated by ultrafiltration using a YM-5 filter.

Cathepsins B, H and L prepared as above were used as test thiol proteases in the inhibitor assays.

4. Assay for cathepsins B and H

The assay conditions for cathepsin B and H were the same except that BANA was used as substrate for cathepsin H and N-ℒ-CBZ-L-arginyl-L-arginine-4-methoxy-naphthylamide was used as the susbstrate for cathepsin B. The reaction mixture contained 0.05 ml of enzyme, 0.05 ml of incubation buffer containing 0.074 M potassium phosphate, 1 mM EDTA and 0.024% cysteine (freshly prepared), and 0.05 ml of water. The reaction mix was incubated at 40 C for 5 min and then 0.010 ml of BANA (40 mg/ml) or N-ℒ-CBZ-L-arginyl-arginine-4-methoxy-naphthylamide (10 mg/ml) was added to start the reaction. After 0, 15, and 30 minutes, 0.05 ml of the reaction mixture was removed into 0.025 ml of coupling reagent (pH 6.0) containing 0.36% chloromercuribenzoate (PCMB) and 1.86% EDTA in 0.05M NaOH, and kept on ice. Color development occurred upon addition of 0.025 ml of a fast garnet salt solution (0.005% fast garnet in 4% Brij 35). Butanol was added to extract the colored complex and the samples were centrifuged in a clinical centrifuge to separate the phases. The organic phase was removed and analyzed at 520 nm. One enzyme unit of cathepsin B or H is defined as the liberation of 1 nmole of naphthylamine per hour in a standard enzyme reaction mix.

5. Assay for cathepsin L

Azocaesin was used as substrate to determine the TPI activity on cathepsin L according to the procedure of Barrett and Kirschke (1981). Azocaesin is a derivative of caesin in which the tyrosine and histidine side chains have been coupled with diazotized sulfanilic acid or sulfanilamide in alkali. The azo-coupling confers an intense yellow color on the protein. Proteolytic degradation of azocaesin yields peptides soluble in dilute TCA solution, which may be quantified by their absorbance at 366 nm. The method is especially suited for the determination of proteolytic activity in crude samples, being very resistent to interference. The assay for cathepsin L is made very specific by the inclusion of 3M urea which enhances the activity of cathepsin L and depresses that of cathepsin B. Thus for the cathepsin L assay, 0.25 ml of enzyme preparation was mixed with 0.25 ml of an assay buffer containing 0.1 M sodium acetate, pH 5, and 1 mM EDTA and 40 mM cysteine (freshly prepared). After 5 minutes at room temperature, 0.5 ml of an azocaesin-urea mixture (10 ml of 6% azocaesin and 10.8 g of urea in a final volume of 30 ml assay buffer lacking cysteine) was added. After 0, 15, and 30 minutes, 1 ml of 3% TCA was added to the reaction vessels and the mixture centrifuged in a clinical centrifuge. The supernatant was removed and analyzed at 366 nm. One enzyme unit of cathepsin L is defined as the amount of enzyme which brings about a change of 0.01 A_{366} per minute.

6. Assays of thiol protease inhibitor activity

Determination of thiol protease inhibitor (TPI) activity during the purification procedure was carried out using the crude M+L extract as described above as the source of the total lysosomal thiol proteases. N-α-benzoyl-DL-arginine-2-naphthylamide (BANA) was used as the substrate. The extent of 2-naphthylamine liberation from BANA by thiol proteases, in the presence of various column fractions (see purification of inhibitor in Results), was measured using the procedure of Barrett (1972). Each reaction vessel contained 0.075 M potassium phosphate, pH 6, 1 mM EDTA, 0.024% cysteine, 1 mg/ml BANA, 40-50 milliunits of thiol protease activity, and 0.1 ml of various column fractions in 0.8 ml total volume. Aliquots of 0.25 ml were removed after an appropriate incubation period at 40 C (usually 30 minutes), and the percent inhibition of 2-naphthylamine liberation was determined.

The inhibitor activity in each TPI preparation was measured using papain (EC 3.4.4.10) and cathepsins B, H and L as test proteases. With papain or cathepsins B as the test protease, the substrate was N-α-CBZ-L-arginyl-arginine-4-methoxy-naphthylamide while with cathepsin H the substrate was BANA. Azocaesin was the substrate for cathepsin L. One inhibitor unit is defined as the amount of protein required to reduce the thiol protease activity by one enzyme unit.

7. Purity and molecular weight determinations of the TPI's

The purity and molecular weight of the TPI from normal and dystrophic mouse muscle were analyzed on 7-18% gradients of polyacrylamide gels (1.5 x 100 x 135 mm) containing 0.1% sodium dodecyl sulfate (SDS) according to the procedure of Laemmli (1970). Protein was stained with Coomassie brilliant blue (R-250) in 7.5% acetic acid and 50% methanol, and the gel destained in a solution composed of 7.5% acetic acid and 5% methanol. The molecular weight of the TPI's was determined using the following standard molecular weight markers: phosphorylase b (Mr 94,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 43,000), carbonic anhydrase (Mr 30,000), soybean trypsin inhibitor (Mr 20,100) and α-lactalbumin (Mr 14,400).

8. Analysis of TPI's on isoelectric focusing gels

Isoelectric focusing of the purified TPI's was carried out in jacketed glass columns (4 x 100 mm) containing 6% polyacrylamide gels with 2% of 3.5-10 ampholytes according to the procedure of Blerkom (1970). Electrophoresis was carried out for at least 6 hours at 75 volts and 0.5 ma per column. At the end of the run, one gel from each set was cut into 2 mm sections, and each section was placed in a vial containing 1.2 ml of nanopure water. The vials were covered with parafilm and after three to four hours at room temperature, the pH of each gel extract was measured using a micro-pH electrode. A second gel was washed in ice-cold water overnight with at least 4 changes of fresh cold water, and then cut into 2 mm sections, and each section was placed in a vial containing 0.5 ml of 50 mM Tris-HCl and 0.2 M NaCl, pH 8. After incubation at 40 C for 30 minutes, the amount of thiol protease inhibitor in each gel extract was determined using either papain or cathepsin B as test protease as described above. A third gel was placed in 5% TCA for 3 hours and subsequently stained for protein with Coomassie blue as described above for the SDS-polyacrylamide gels. In some experiments standard proteins with known pI values were run along with the TPI's to provide an additional means of assessing the pI values of the TPI's. The standard protein mix for the latter assay contained amyloglucosidase (pI 3.55), methyl red (pI 3.75), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.13), carbonic anhydrase (pI 5.85), myoglobin (pI 7.16 and 6.76), and L-lactic dehydrogenase (pI 8.55).

9. High performance liquid chromatography of TPI's

Thiol protease inhibitor from normal and dystrophic muscle was analyzed on a C-18 reverse phase column (4.6 x 250 mm, Chemopack) driven by a Gilson HPLC system. The column was equilibrated with 12% acetonitrile containing 0.1% trifluoroacetic acid (TFA), and following sample injection, the protein was eluted at a flow rate of 1 ml/min using a linear gradient (40 minutes) of acetonitrile to 60% with 0.1% TFA. The column effluent was monitored at 214 nm using a Gilson Holochrome detector.

10. Assay for myosin hydrolysis by cathepsin B in the presence of TPI-n and TPI-d

The effect of TPI-n and TPI-d on the hydrolysis of rabbit myosin was analyzed on a 7-18% SDS-polyacrylamide gel as described above. Reaction vessels containing 20 mM sodium acetate pH 5, 1 mM EDTA, 1 mM DTT, 0.15 M KCl, 10 % glycerol, rabbit myosin (38 µg), cathepsin B (16.8 milli-units), and TPI-n (46 µg) or TPI-d (46 µg) in separate reaction vessels were incubated at 40 C for periods up to 1 hour. The reactions were stopped at the desired time by heating an aliquot of the reaction mixture at 95 C for 5 minutes in the presence of 1 % SDS. Samples were then applied to an SDS-polyacrylamide gel slab and electrophoresed at 130 volts for 3 hours.

11. Thymol sulfuric acid test for glycoproteins

The presence of glycoprotein in the TPI-n and TPI-d preparations was tested using the thymol-sulpfuric acid method outlined by Gander (1984). After electrophoresis the gels were washed with isopropanol-acetic acid-water (25:10:65) for at least 2 hours to fix the proteins and to remove the low molecular weight substances. After two additional washes the final wash was done for another 2 hours in the same solvent containing 0.2% thymol (w/v). After washing with thymol, the gel was transferred to a solution of concentrated sulfuric acid and absolute ethanol (80:20) and shaken at room temperature for a minimum of 2 hours or until the opalascent appearance of the gels just disappeared. Zones containing glycoproteins stained red, whereas the background remained yellow. Proteins lacking carbohydrate markers do not form red bands when treated in this manner.

The normal and dystrophic inhibitor proteins were run on a 7-18% SDS-polyacrylamide gel and stained for glycoproteins using an acid protease from *Artemia* embryos, a known glycoprotein, as a marker.

12. Protein determination

The protein content of each enzyme and inhibitor preparation was determined by the method of Lowry et al.(1951) using bovine serum albumin as protein standard.

III. RESULTS

A. Purification of a thiol protease inhibitor from normal and dystrophic mouse muscle

The low molecular weight thiol protease inhibitors from 60 day old normal (TPI-n) and dystrophic (TPI-d) mouse hindlimb muscle were purified to apparent homogeneity as described by Hirado et al. (1981) with slight modification. Approximately 4-5 grams of hindlimb muscle from 3 normal or 6 dystrophic mice were homogenised thoroughly with 5 volumes of 10 mM Tris-HCl, pH 8.0 containing 1 mM dithiothreitol, 0.2 M NaCl and 10% glycerol in a loose fitting ground-glass homogenizer. In each case the homogenate was centrifuged at 27,000 x g for 20 minutes and the supernatant fluid passed through miracloth to remove any floating material. The filtrate was adjusted to pH 5.0 with acetic acid and then stirred for 3-4 hours at 4 C. The precipitate that formed at pH 5 was removed by centrifugation and the crude extract was concentrated to about 2 ml by ultrafiltration using a Diaflo YM-5 (Amicon) membrane.

After concentration, the crude extracts from normal and dystrophic muscle were applied (separately) to a Sephadex G-50 column (1 x 50 cm, superfine) equilibrated with the homogenization buffer, and the protein was eluted with the same buffer. Each column fraction was assayed for UV absorbing material at 280 nm and for TPI activity using a crude thiol protease preparation from normal mouse liver and benzoyl arginine naphthylamide (BANA) as substrate at pH 6. The column fractions containing inhibitor activity were pooled and concentrated by ultrafiltration using a Diaflo YM-2 (Amicon) membrane.

The concentrated TPI preparation from the Sephadex G-50 column was applied to a Sephadex G-75 column (1 x 50 cm, superfine) equilibrated with homogenization buffer, and the protein was eluted and analyzed as described above for the first gel filtration step. The column fractions containing inhibitor activity were pooled and concentrated by ultrafiltration on a Diaflo YC-05 (Amicon) membrane. The TPI preparation was rechromatographed on a second Sephadex G-75 column and fractions containing inhibitor activity were pooled and concentrated as before. The final preparations, designated as TPI-n from normal mouse muscle and TPI-d from dystrophic mouse muscle were stored at -20 C.

The results in Figure 2 show the elution and activity profiles of the TPI's after each chromatographic step. The protein content and inhibitor activity after each step in the purification process are also summarized in Table 1. Two aspects of the data in Table 1 are notable. First, it is clear that crude extracts of dystrophic muscle contain about 75% less inhibitor activity than crude extracts from normal muscle, data which are consistent with that reported previously from this laboratory (Gopalan et al., 1986a). Since I did not attempt to inactivate the thiol protease

Figure 2. Purification of TPI-n and TPI-d on gel filtration columns. All columns (1 x 50 cm) were equilibrated with buffer containing 10 mM Tris-HCl, pH 8, 0.2 M NaCl, 1 mM DTT and 10% glycerol. The protein was eluted with the same buffer.

Panel A: Crude extract from hindlimb of normal mice on Sephadex G-50 (superfine) column

B: TPI-n activity on the first Sephadex G-75 (superfine) column from pool of Sephadex G-50 column material (A).

C: TPI-n activity on second Sephadex G-75 (superfine) column from pooled material from the first Sepahdex G-75 column (B)

D: Crude extract from hindlimb of dystrophic mice on Sephadex G-50 (superfine) column.

E: TPI-d activity on the first Sephadex G-75 (superfine column) from pool of Sephadex G-50 column (D).

F: TPI-d activity on the second Sephadex G-75 (superfine) column from pooled material from the first Sephadex G-75 column (E).

O---O, protein as measured by absorbance at A_{280}.

x---x, inhibitor activity (as percent inhibition) using total lysosomal thiol proteases with BANA as substrate.

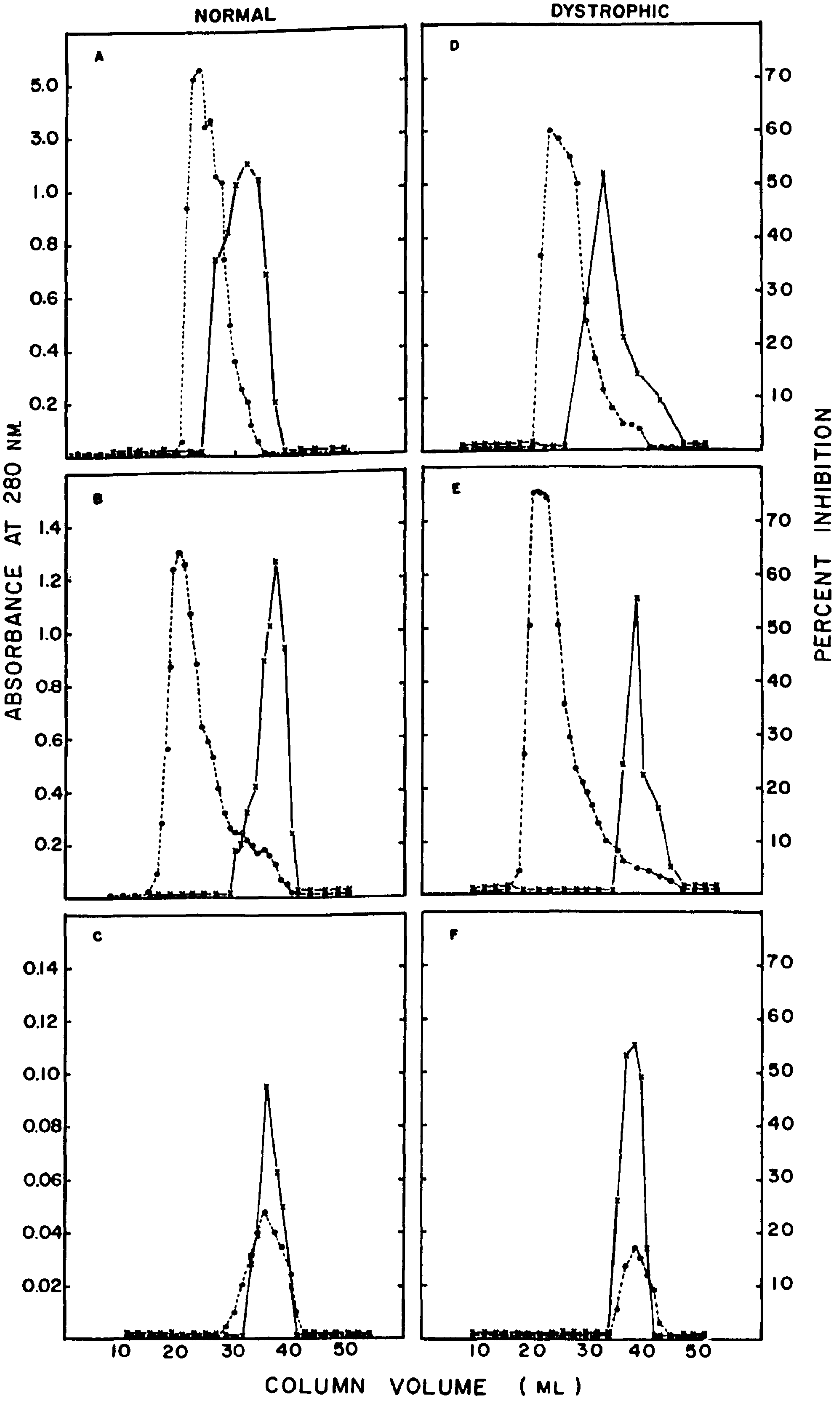

NORMAL
DYSTROPHIC
A
B
C
D
E
F
5.0
3.0
1.0
0.8
0.6
0.4
0.2
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.14
0.12
0.10
0.08
0.06
0.04
0.02
ABSORBANCE AT 280 NM
70
60
50
40
30
20
10
PERCENT INHIBITION
10 20 30 40 50
COLUMN VOLUME (ML)

Table 1. Summary of steps in the purification of thiol protease inhibitor from normal and dystrophic mouse muscle.

Fraction[a]	Total Protein (mg)	Total milli-IU	Specific Activity
		Normal muscle[b]	
Crude extract	36.6	760	21
Sephadex G-50	32.3	1160	36
1st Sephadex G-75	5.6	1388	248
2nd Sephadex G-75	2.4	1203	501
		Dystrophic muscle[b]	
Crude extract	43.8	198	5
Sephadex G-50	20.8	1285	62
1st Sephadex G-75	3.7	1012	274
2nd Sephadex G-75	2.7	891	330

[a] Each preparation was assayed using 41 milliunits of thiol protease activity in a standard reaction vessel.

[b] 4.5 grams of muscle were taken from normal mice and 4.6 grams were taken from dystrophic mice.

The data presented in this Table are representative of one of 15 experiments conducted.

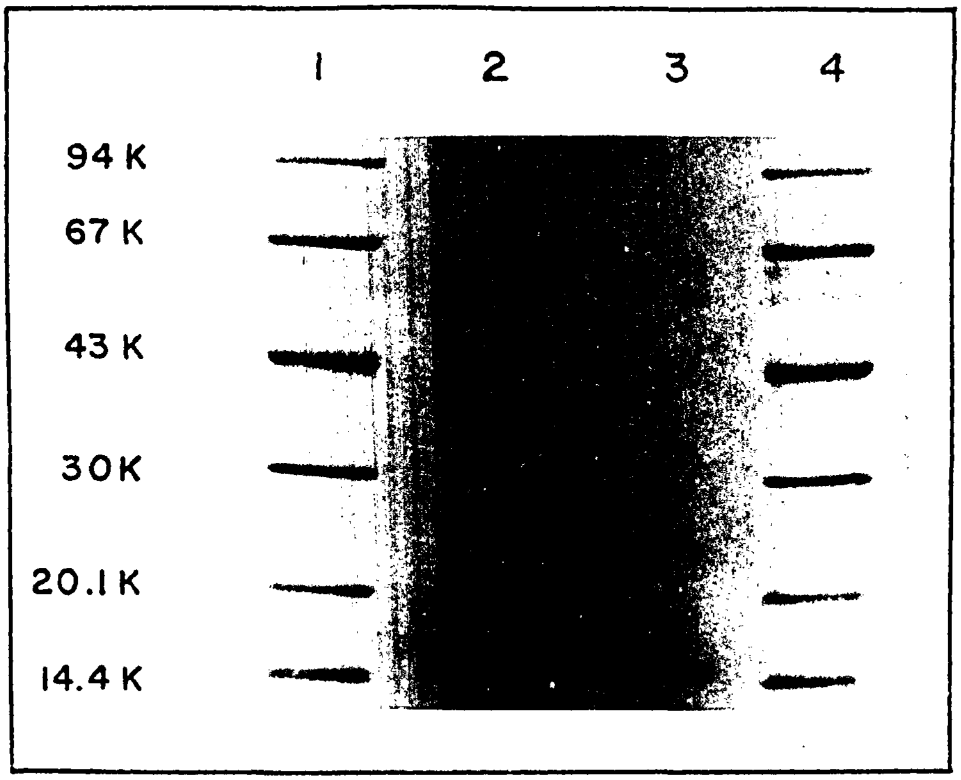
activity in the crude extracts, it is likely that the low inhibitor levels in these preparations are due to elevated amounts of these enzymes which lower the apparent inhibitor activity (Ohtani et. al., 1982). Second, the total TPI activity in the final preparation (after the 2nd Sephadex G-75 step) is about 35% higher in preparations from normal muscle than from dystrophic muscle. These differences are not significant for reasons described below.

In several other preliminary experiments, attempts to purify the protease inhibitor on CM-cellulose, DEAE-cellulose, CM-affigel blue, agarose blue, papain sepharose, and Con-A sepharose columns were made.. In all of these cases unsatisfactory yields of activity were obtained (compared with the gel filtration protocol used here) so further attempts to use these media to purify the inhibitor were abandoned.

B. Physical properties of the thiol protease inhibitor from normal and dystrophic hindlimb muscle of the mouse

Selected physical properties of TPI-n and TPI-d preparations after the second Sephadex G-75 column were analyzed using three analytical procedures: SDS-polyacrylamide gel electrophoresis, high performance liquid chromatography, and analytical isoelectric focusing. The migration property of the protein in each TPI preparation on a 7-18% gradient of polyacrylamide in the presence of 0.1% SDS is shown in Figure 3. These data show that only one polypeptide is present in each inhibitor preparation, and

Figure 3. Comparison of TPI-n and TPI-d after SDS-polyacrylamide gel electrophoresis. Samples of each thiol protease inhibitor preparation, after the second Sephadex G-75 step were electrophoresed on a 7-18% polyacrylamide gel in the presence of 0.1% SDS. Lanes 1 and 4 are standard molecular weight proteins. Lane 2 contains 20 µg TPI-n while lane 3 contains 20 µg TPI-d.


1
2
3
4
94 K
67 K
43 K
30K
20.1 K
14.4 K

based on migration distances of the TPI's compared with standard protein markers, the molecular weight of each TPI was calculated to be 14,800. The latter results are shown in Figure 4. The results in Figure 5 compare the elution profiles of TPI-n and TPI-d from an HPLC column (4.6 x 250 mm, Chemopack). The column was eluted with a linear gradient (40 minutes) of acetonitrile to 60% containing 0.1% TFA. Only one major protein peak was detected in each preparation. TPI-n and TPI-d also co-eluted from the HPLC column when present as a mixture (data not shown). Both TPI-n and TPI-d eluted from the HPLC column at 47% acetonitrile.

Data from the analytical isoelectric focusing experiments are shown in Figure 6. The only protein detectable on these gels after staining with Coomassie brilliant blue focused at pH 4.5. Mixtures of TPI-n and TPI-d also gave a single band upon focusing at pH 4.5. These data show that both TPI-n and TPI-d are apparently homogeneous after the second Sephadex G-75 step and that they have very similar, if not identical, physical properties.

C. Activity of TPI-n and TPI-d on various thiol proteases

The inhibitor activity of purified TPI-n and TPI-d was compared using papain and cathepsins B, H and L and the results are shown in Figure 7. When both TPI's were tested against papain, no differences in inhibitor activity were found, and both TPI's inhibited papain by 50% (ID_{50}) at 4 µg/ml. With normal mouse liver cathepsin B, TPI-d was

Figure 4. Molecular weight determination of the thiol protease inhibitor protein. Standard protein markers used were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000) soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). The arrow indicates the electrophoretic position of TPI-n on the gel compared to the standard proteins.

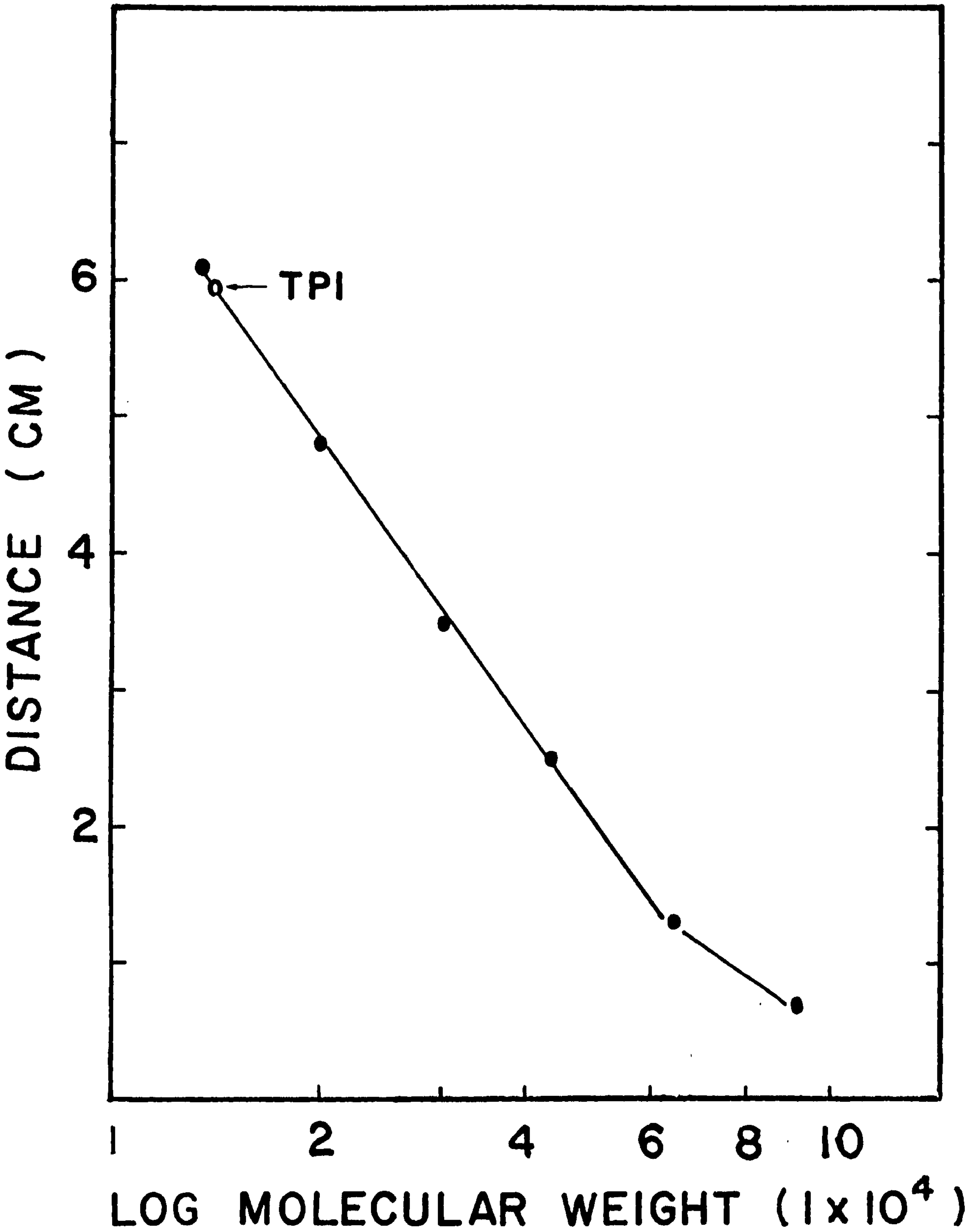

TPI
DISTANCE (CM)
2
4
6
1
2
4
6
8
10
LOG MOLECULAR WEIGHT (1x10^4)

Figure 5. High performance liquid chromatography of TPI-n and TPI-d. Samples of TPI-n (10 µg) and TPI-d (5 µg) were applied in separate runs to a C-18 reverse phase column (Chemopack, 4.6 x 250 mm) previously equilibrated with 12% acetonitrile containing 0.1% TFA. The columns were developed with a linear gradient (over 40 min duration) of acetonitrile to 60% containing 0.1% TFA. The actual tracing from the recorder is shown here including the column pressure curve (p) and zero voltage curve (v). The small vertical arrows indicate material present in the buffer as observed in a separate run (not shown).

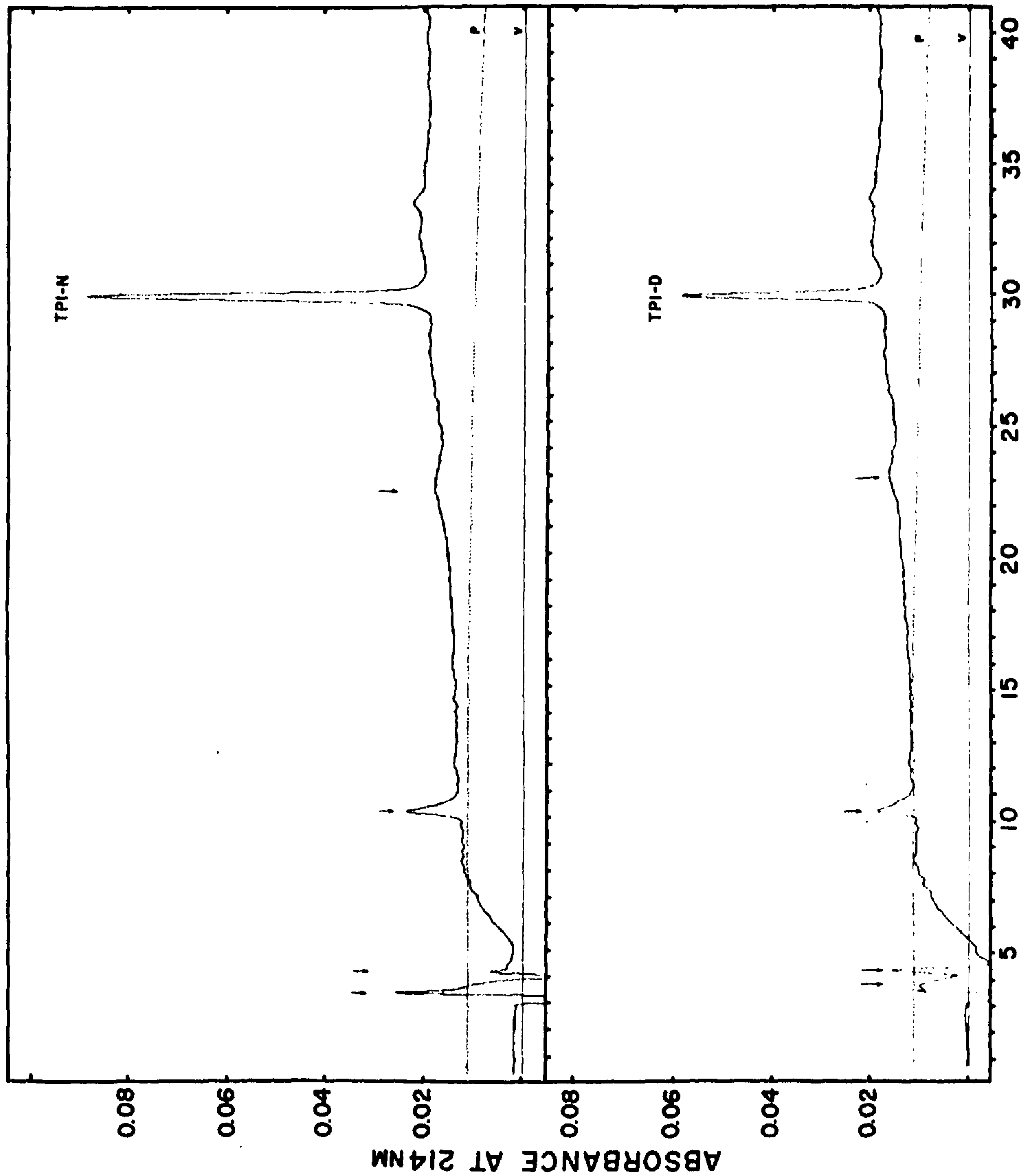

TPI-N
TPI-D
P
V
ABSORBANCE AT 214NM
0.08
0.06
0.04
0.02
VOLUME (ML)
5
10
15
20
25
30
35
40

Figure 6. Isoelectric focusing gels containing TPI-n and TPI-d and a mixture of TPI-n and TPI-d. The pI of the standard proteins are shown at the far left.

Lane 1. Standard protein mix together with TPI-n +TPI-d.

Lane 2. TPI-n only (20 µg).

Lane 3. TPI-d only (20 µg).

Lane 4. TPI-n + TPI-d (40 µg total).

The standard proteins used were: L-Lactic dehydrogenase (pI 8.55), myoglobin (pI 7.16 and 6.76), carbonic anhydrase (5.75), β-lactoglobulin A (pI 5.13), methyl red (3.75) and amyloglucosidase (pI 3.55).

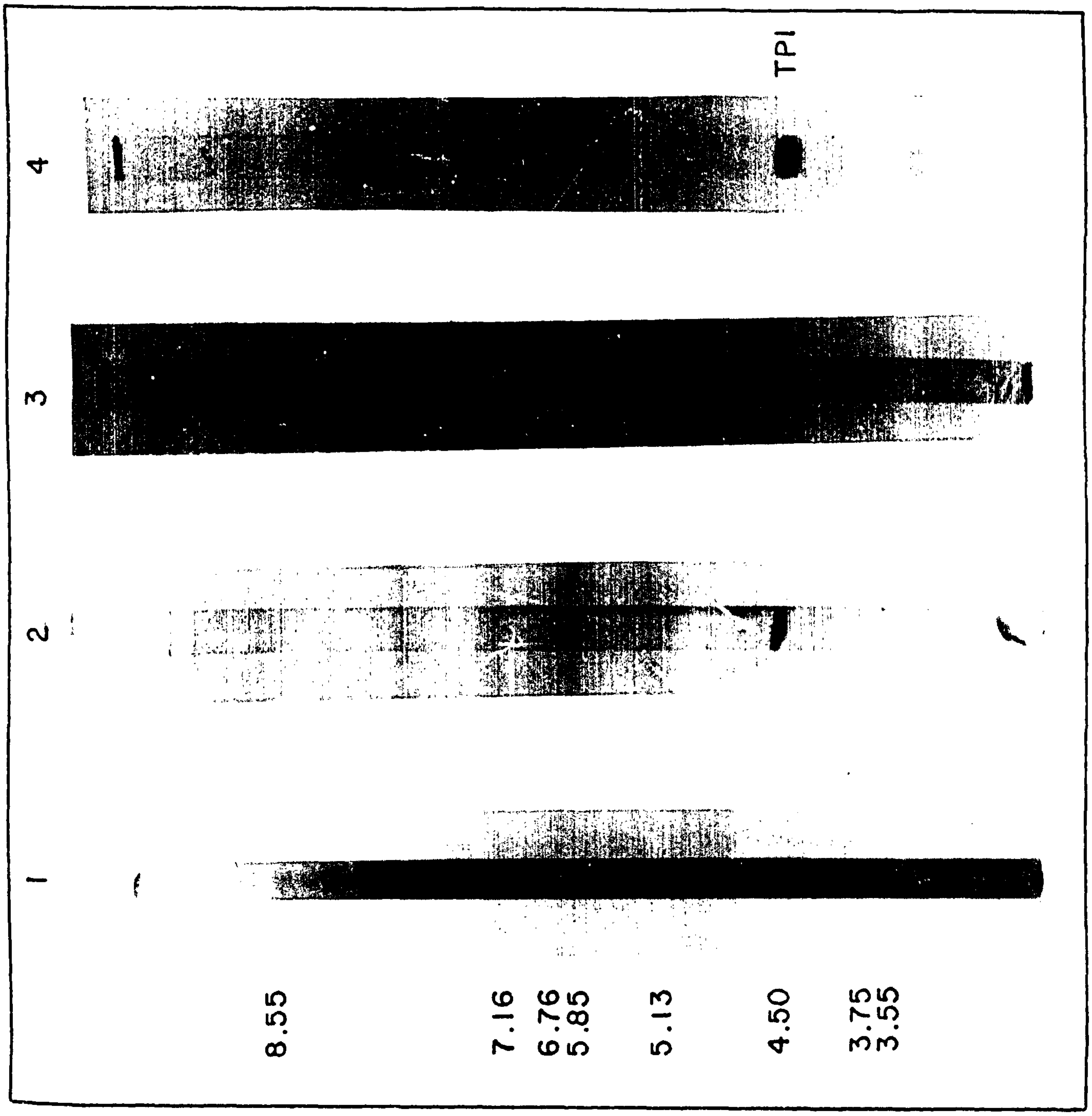
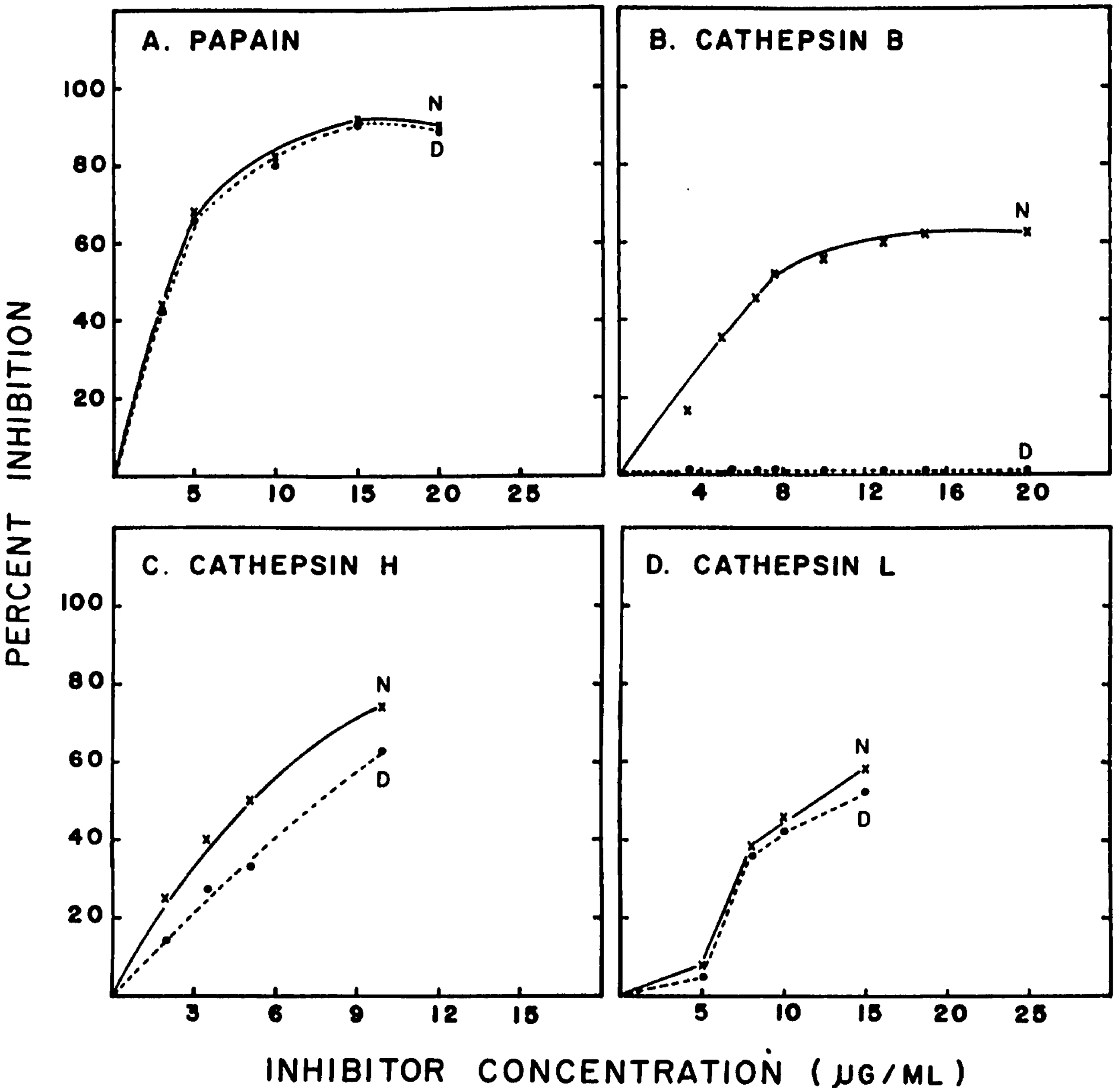

1
2
3
4
TPI
8.55
7.16
6.76
5.85
5.13
4.50
3.75
3.55

Figure 7. Effect of TPI-n and TPI-d on the activity of various thiol proteases. Varying amounts of inhibitor from normal (N) and dystrophic (D) muscle were added to a standard reaction vessel containing one of the thiol proteases indicated, and the percent inhibition was determined.

x --- x, indicates TPI from normal muscle.

■ --- ■, indicates TPI from dystrophic muscle.

Cathepsins B, H and L were from normal mouse liver.


A. PAPAIN
B. CATHEPSIN B
C. CATHEPSIN H
D. CATHEPSIN L
PERCENT INHIBITION
INHIBITOR CONCENTRATION (µG/ML)
100
80
60
40
20
5
10
15
20
25
4
8
12
16
20
3
6
9
12
15
N
D

inactive while TPI-n had an ID_{50} of 7.5 µg/ml. No significant differences in inhibitor activity were observed using cathepsin L, while with cathepsin H about 35% more TPI-d was required to reach the ID_{50} level. Similar findings were observed using cathepsin B, H and L isolated from livers of dystrophic mice. These findings are summarized in Table 2. Thus while the thiol protease inhibitor from normal mouse muscle (TPI-n) appears to be effective in the regulation of papain and homologous cathepsins B, H and L, the thiol protease inhibitor from dystrophic mouse muscle (TPI-d) appears to be inactive against cathepsin B, partially active when cathepsin H is used, and fully active when cathepsin L and papain are the test enzymes. The fact that TPI-d preparations are unable to regulate cathepsin B activity under conditions (in vitro) where TPI-n preparations are active is an important observation.

D. Activity of TPI-n and TPI-d after isoelectric focusing on polyacrylamide gels

To ascertain whether the inhibitor activity in each preparation is associated with the major protein observed on SDS-polyacrylamide and isoelectric focusing gels, 10-20 µg of each TPI preparation were electrophoresed on analytical isoelectric focusing gel columns, either separately or as a mixture. One gel was left intact and analyzed for protein by staining with Coomassie brilliant blue (R-250), while the other gels were cut into 2 mm sections. One of the latter was

Table 2. Summary of thiol protease inhibitor activity from normal and dystrophic mouse muscle on various cathepsins[a].

Source of enzymes	Source of Inhibitor	Cathepsin B	Cathepsin H	Cathepsin L
Normal liver[b]	normal muscle (TPI-n)	7.5	5.0	11.5
	dystrophic muscle (TPI-d)	>50	7.6	13.5
Dystrophic liver[b]	normal muscle (TPI-n)	6.5	3.5	11.5
	dystrophic muscle (TPI-d)	>50	5.6	12.0

[a] Inhibitor concentration (ug/ml) required to achieve 50% inhibition (ID_{50})

[b] 40-50 milliunits were used in each assay.

The values shown above are representative of one of the 15 experiments.

used to determine the pH gradient that formed during the electrophoretic run. From the other cut gels, protein was eluted with 50 mM Tris-HCl containing 0.2 M NaCl, pH 8. In one of these gels, protein from each section was extracted and assayed for inhibitor activity against papain, while in the other gel, extracts of sections were assayed for inhibitor activity against cathepsin B. Results of these experiments are shown in Figure 8.

As observed previously (see Figure 6), only one discrete protein band could be detected in either TPI-n or TPI-d preparations. The pH at which this protein focused (pI) was determined to be 4.5 by direct pH measurements of individual gel sections (as shown in Figure 8), and by the use of protein standards with known pI values as shown in Figure 9. Assays of protein eluted from the gel sections revealed that while the TPI-n of pI 4.5 is active against both papain and cathepsin B, the pI 4.5 protein in the TPI-d preparation is inactive against both of these proteases. However, the agent in the TPI-d preparation which inhibited papain in other experiments (see Figure 7) focused at pH 4.9. This inhibitor could not be detected with Coomassie blue stain, and it was clearly different from the major protein which focused at pH 4.5. When equal amounts of TPI-n and TPI-d preparations were combined, electrophoresed on an analytical isoelectric focusing gel column, and then analyzed for inhibitor activity as before, both inhibitor activities were detectable using papain as the test thiol protease (see Figure 8c). These

Figure 8. Analysis of TPI-n and TPI-d on analytical isoelectric focusing gels. Samples containing either TPI-n (20 µg), TPI-d (20 µg) or a mixture of both (TPI-n + TPI-d, 40 µg) were run on analytical isoelectric focusing gels, then analysed for protease activity using either papain (panel A, B, C) or cathepsin B (panel D, E, and F) as test proteases. The photograph at the top of each panel shows the Coomassie blue stained protein in gels run in parallel compared to the pH gradient (---) and inhibitor activity in separate gels containing TPI-n (x --- x) and TPI-d (■ ---■) or a mixture of inhibitor proteins.

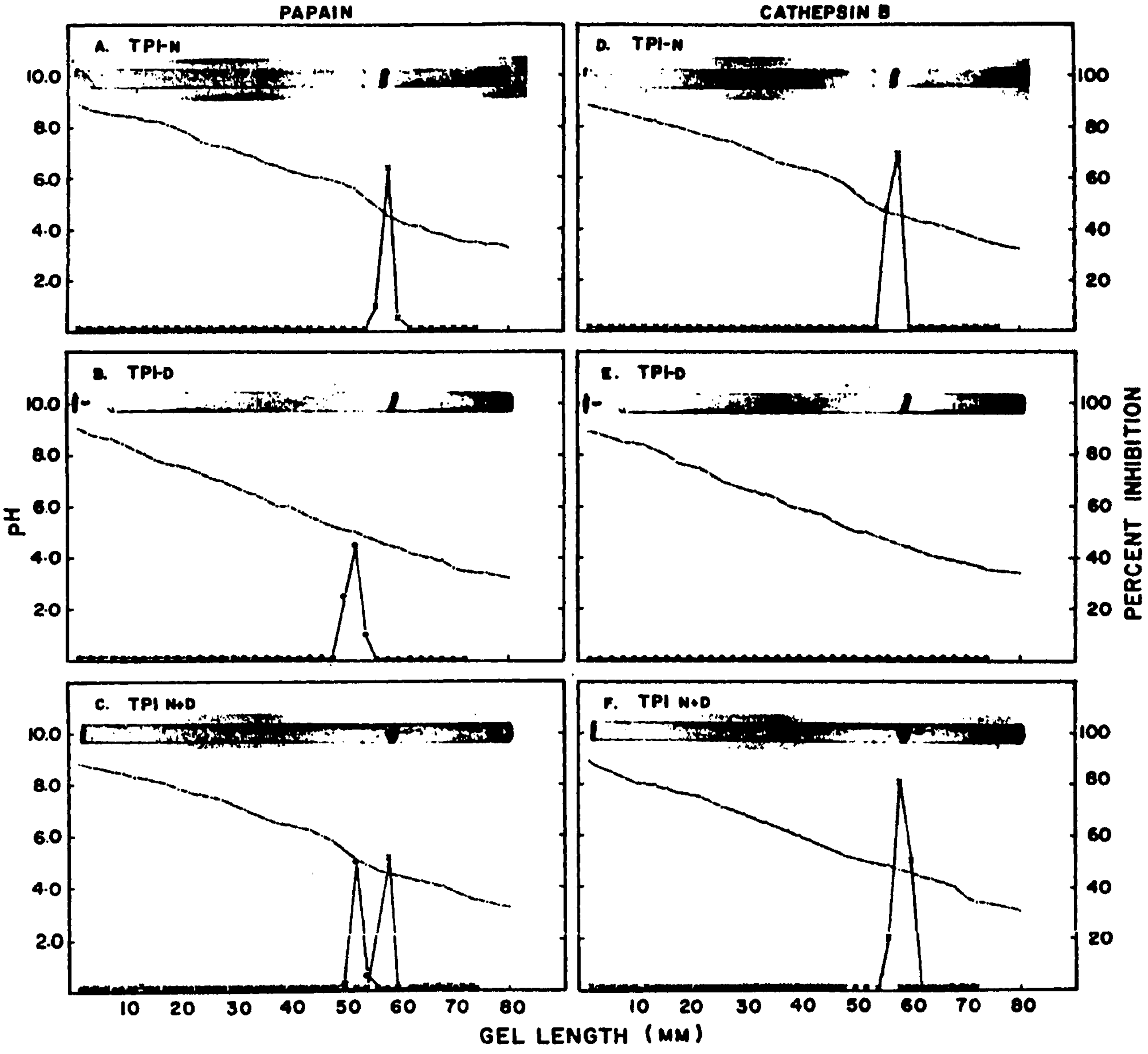
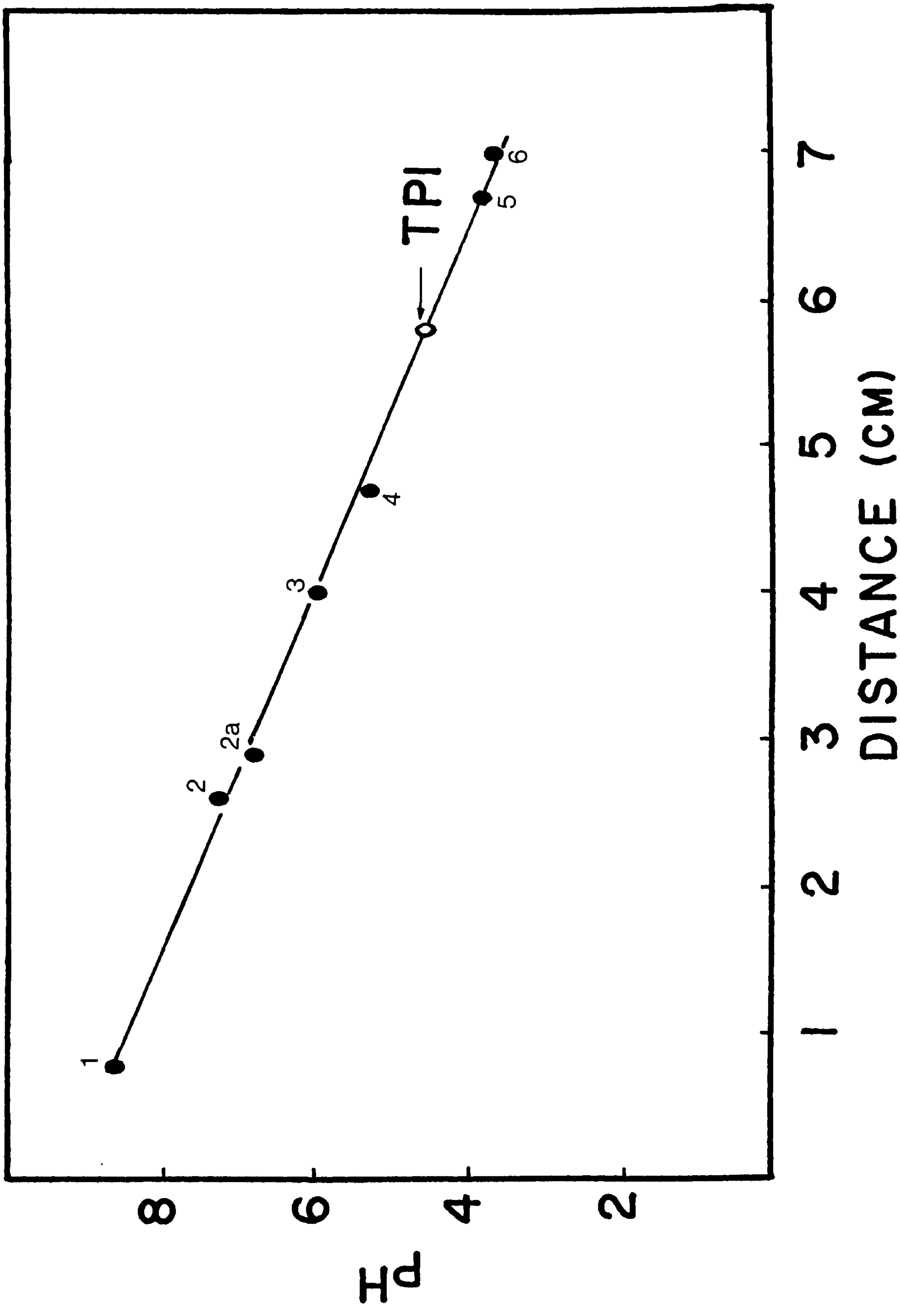

PAPAIN
CATHEPSIN B
A. TPI-N
B. TPI-D
C. TPI N+D
D. TPI-N
E. TPI-D
F. TPI N+D
pH
10.0
8.0
6.0
4.0
2.0
PERCENT INHIBITION
100
80
60
40
20
10 20 30 40 50 60 70 80
GEL LENGTH (MM)

Figure 9. Determination of pI values of the thiol protease inhibitor protein. Standard proteins with known pI values were run with normal mouse muscle TPI as shown in Figure 6.

Standard proteins used were 1)lactic dehydrogenase (pI 8.55), 2 and 2a) myoglobin (pI 7.16 and 6.76), 3) carbonic anhydrase (pI 5.85), 4) lactoglobulin (pI 5.13), 5) methyl red (pI 3.75), and 6) amyloglucosidase (pI 3.55). The arrow indicates the position of the inhibitor protein in the gel.


pH
8
6
4
2
1
2
3
4
5
6
7
DISTANCE (CM)
TPI
1
2
2a
3
4
5
6

results demonstrate that the TPI-d protein which focuses at pH 4.5 lacks inhibitor activity using either papain or cathepsin B as the test protease. Otherwise it is identical to TPI-n which is active on papain and cathepsin B. The inhibitory activity in the TPI-d preparations must be due to some other substance, either a protein in amounts too small to be detected by staining with Coomassie blue, or some other component.

E. Effect of TPI-n and TPI-d on the hydrolysis of myosin by cathepsin B

It had been demonstrated previously that cathepsin B hydrolyzes myofibrillar proteins (Schwartz and Bird, 1977). Therefore, the efficiency of TPI-n and TPI-d in blocking the hydrolysis of myosin by cathepsin B was examined *in vitro*. Rabbit myosin was incubated with mouse liver cathepsin B and TPI-n or TPI-d in separate reaction vessels. After incubation for one hour at pH 5 and 37 C, the reaction products were analyzed by electrophoresis on a 7-18% polyacrylamide gel in the presence of 0.1% SDS. The results of this experiment are shown in Figure 10. From these data it is clear that TPI-d is unable to prevent the hydrolysis of myosin by cathepsin B in contrast to TPI-n (compare columns 6 and 7 in Fig 10). The arrow in Fig 10 indicates the position of TPI-n and TPI-d.

Figure 10. Effect of TPI-n and TPI-d on myosin hydrolysis by cathepsin B. Reaction vessels containing rabbit myosin (38 µg), partially purified cathepsin B (16.8 milli-units), and different thiol protease inhibitors (46 µg of each) in a reaction volume of 115 ul were set up and incubated as shown below. Aliquots (40 µl) of each reaction were analysed on 7-18% polyacrylamide gel slab.

Lane 1. myosin only 60 min. 37 C.

Lane 2. myosin + cathepsin B, 5 min. 37 C.

Lane 3. myosin + cathepsin B + TPI-d, 0 min. 37 C

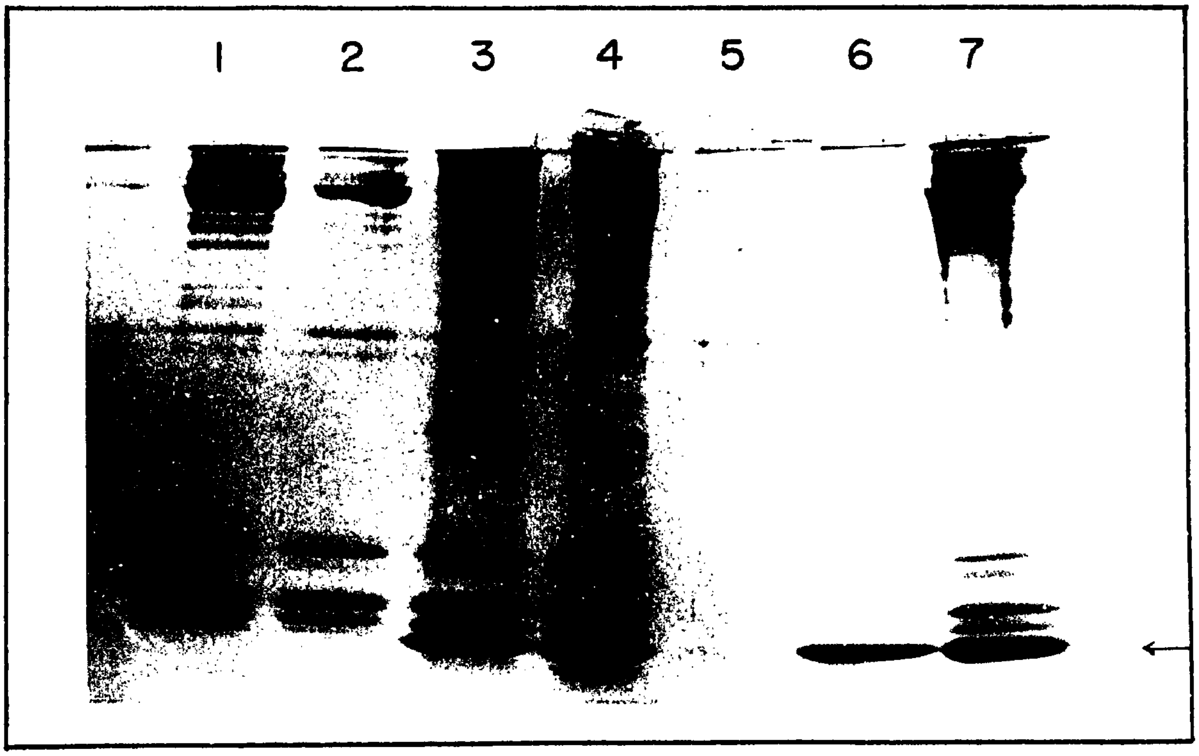
Lane 4. myosin + cathepsin B + TPI-n, 0 min. 37 C

Lane 5. myosin + cathepsin B, 60 min. 37 C.

Lane 6. myosin + cathepsin B + TPI-d, 60 min. 37C

Lane 7. myosin + cathepsin B + TPI-n, 60 min. 37C

The protein band identified by the arrow at the right is the TPI protein.


1
2
3
4
5
6
7

F. pH and thermal stability of TPI-n

The stability of TPI-n at different pH was studied using cathepsin B as the test protease. When the protein was incubated for 1 hour in buffers at pH values ranging between 3-10 (20 mM sodium acetate for pH range 3-5, and 20 mM sodium phosphate for the pH range 6-10) for 1 hour and then assayed at pH 6, the results shown in (Figure 11) were obtained. These results show that the normal muscle protease inhibitor (TPI-n) is most stable at pH 6 and is inactive below pH 5 or above pH 8.0.

To determine the stability of the normal protease inhibitor at different temperatures, purified TPI-n was incubated at varying temperatures ranging from 30 C to 95 C for 1 hour at pH 6. The inhibitor activity remaining after heat treatment was examined using cathepsin B as the test protease in the standard reaction vessel. The results of this experiment are presented in Figure 12 and show that the inhibitor activity (TPI-n) is stable at temperatures up to 65 C and inactivated above this temperature. Similar observations on pH and thermal stability have been made using rat epidermal thiol protease inhibitor by Takeda et al., 1983.

G. Effect of trypsin treatment on TPI-n

The effect of trypsin on TPI-n activity was determined by preincubating 12 units of normal thiol protease inhibitor for 2 hours with 10 and 20 units of trypsin in separate reaction vessels. After the incubation period, 10 and 20 units of

Figure 11. Stability of TPI-n at various pH. Purified TPI-n was incubated at various pH ranging from 3-10 for 1 hour at 40 C, and then assayed for inhibitor activity against cathepsin B at pH 6.0 as described in the Methods.

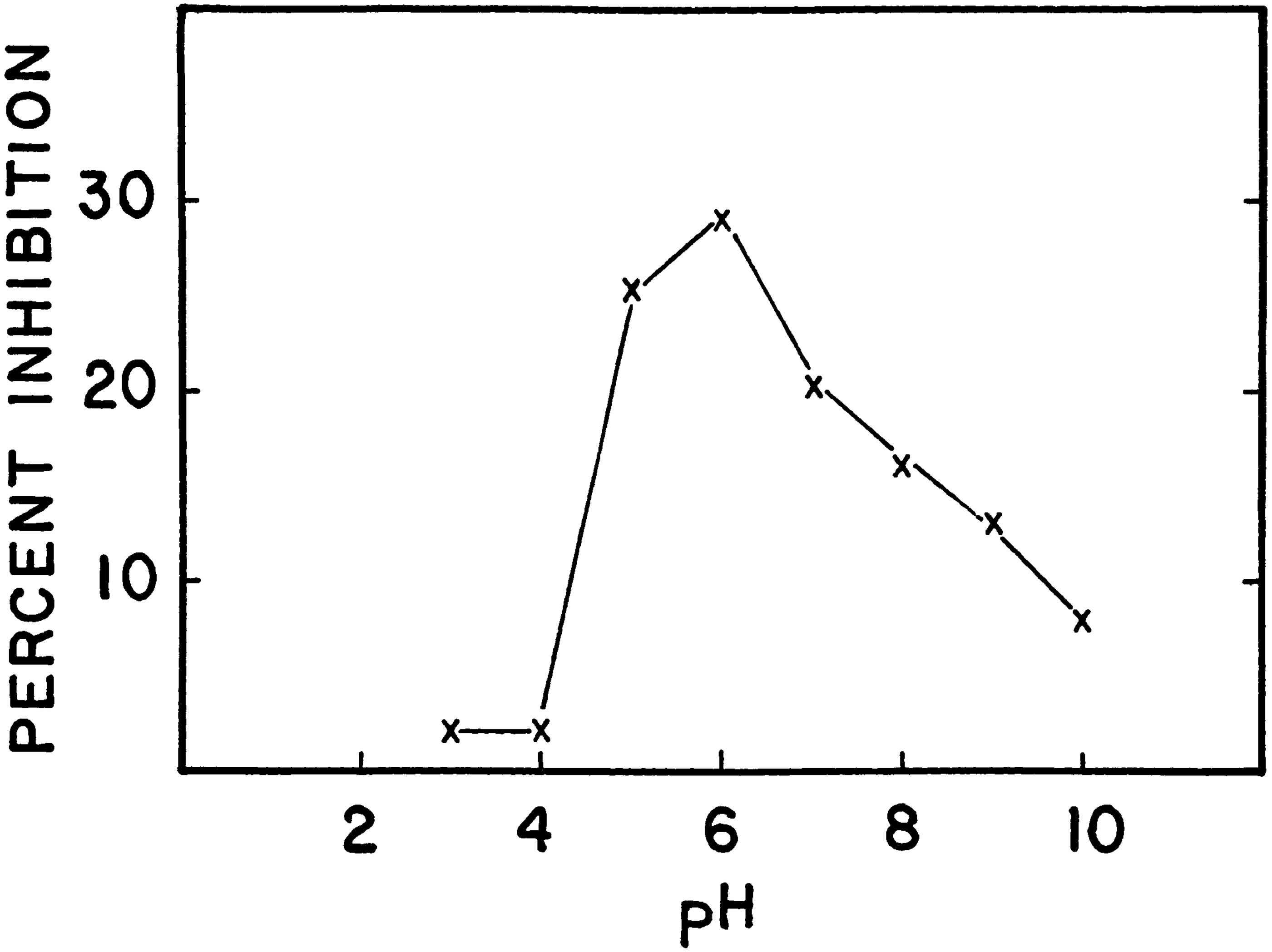
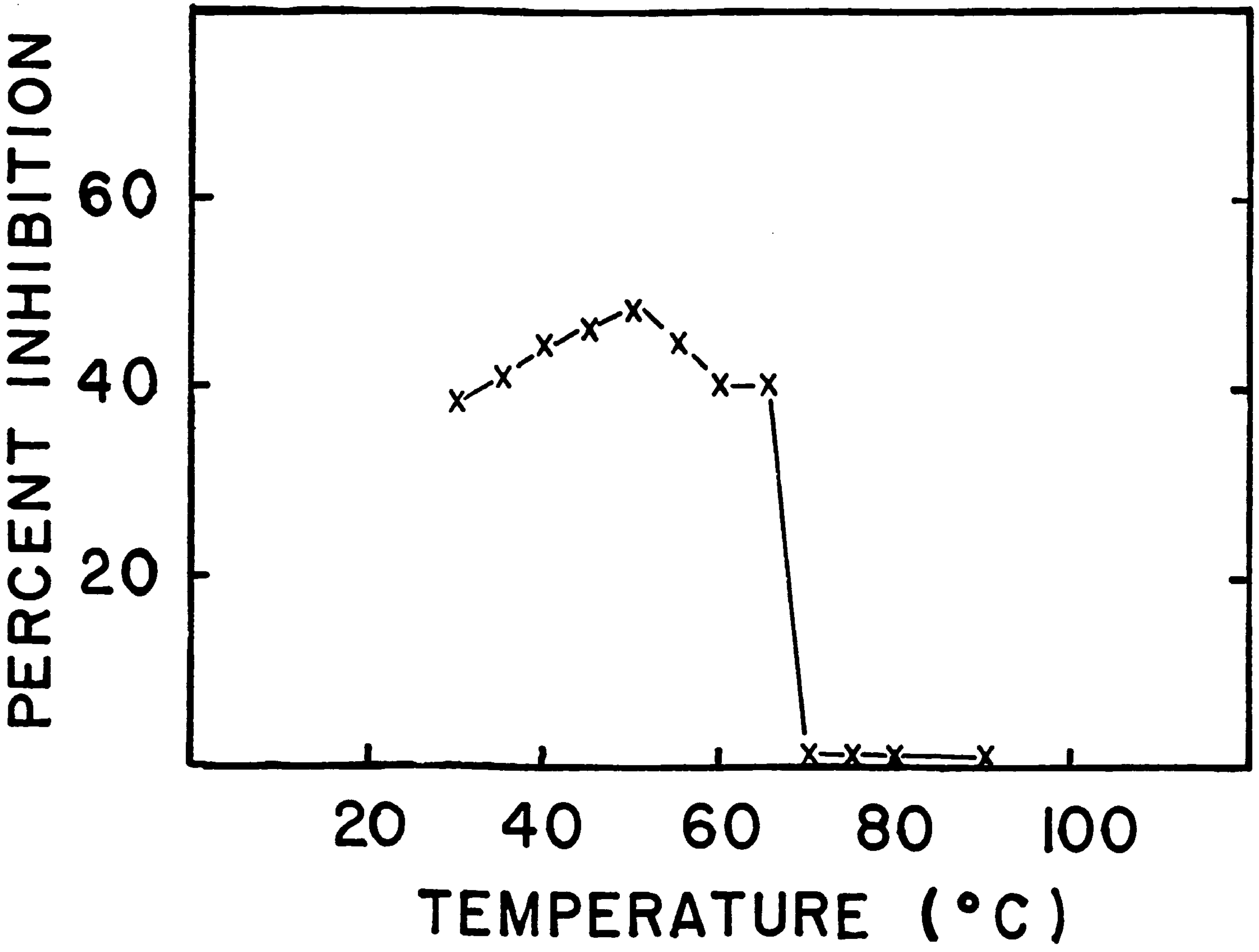

PERCENT INHIBITION
30
20
10
2
4
6
8
10
pH

Figure 12. Thermal stability of TPI-n. Purified TPI-n was incubated at varying temperatures ranging from 30 to 95 C for 1 hour at pH 6. The inhibitor activity remaining after the heat treatment was measured using cathepsin B as the test protease.


PERCENT INHIBITION
60
40
20
20
40
60
80
100
TEMPERATURE (°C)

soybean trypsin inhibitor were added to the reaction vessels to counteract the trypsin, and the remaining thiol protease inhibitor activity (if any) was assayed using cathepsin B as the test enzyme. Under these conditions no inhibitor activity was lost by the trypsin treatment compared to the control samples not treated with trypsin. Samples from similar reactions were analyzed using SDS-polyacrylamide gel electrophoresis and high performance liquid chromatography and the treated TPI-n was found to be unchanged (data not shown). These results show that normal mouse muscle thiol protease inhibitor is resistant to trypsin digestion and, in this respect, is similar to rat skin epidermal protease inhibitor (Takio et al., 1984).

H. Purification of TPIs from carrier mice heterozygous (+/dy) for muscular dystrophy and disease free normal retired breeders (+/+)

The low molecular weight thiol protease inhibitor from carrier females (TPI-h) heterozygous for the disease, and disease free retired females (TPI-c) (both approximately 9 months of age) was isolated using the same procedure as for the normal or dystrophic hindlimb muscle of younger mice. The results in Table 3 show the amount of thiol protease inhibitor activity in hindlimb muscle of heterozygous (+/dy) and disease free retired breeder mice (+/+) compared to the protease inhibitor activity in normal and dystrophic young males (2 months old). From these results it is clear that there is

Table 3 : Content and inhibitor activity of TPI-h and TPI-c in hindlimb muscle compared with TPI-n and TPI-d

Source of muscle inhibitor	Total protein content (mg)	Total inhibitor activity (milli-units)	Specific activity (units /mg protein)
Normal males (2 months old)	2.4	1203	501
Dystrophic males (2 months old)	2.7	891	330
Carrier females (9 months old)	3.4	2784	568
Normal breeders (9 months old)	6.2	4862	788

The above values were obtained from 5 grams wet weight of hindlimb.

3 to 4 fold more thiol protease inhibitor in the normal retired females than in the normal young males. The content of protease inhibitor in carrier females, heterozygous for the disease (+/dy), also showed more inhibitor activity than young dystrophic males, but less activity than normal, age matched females. These data suggest that the inhibitor concentration in mouse muscle increases with age.

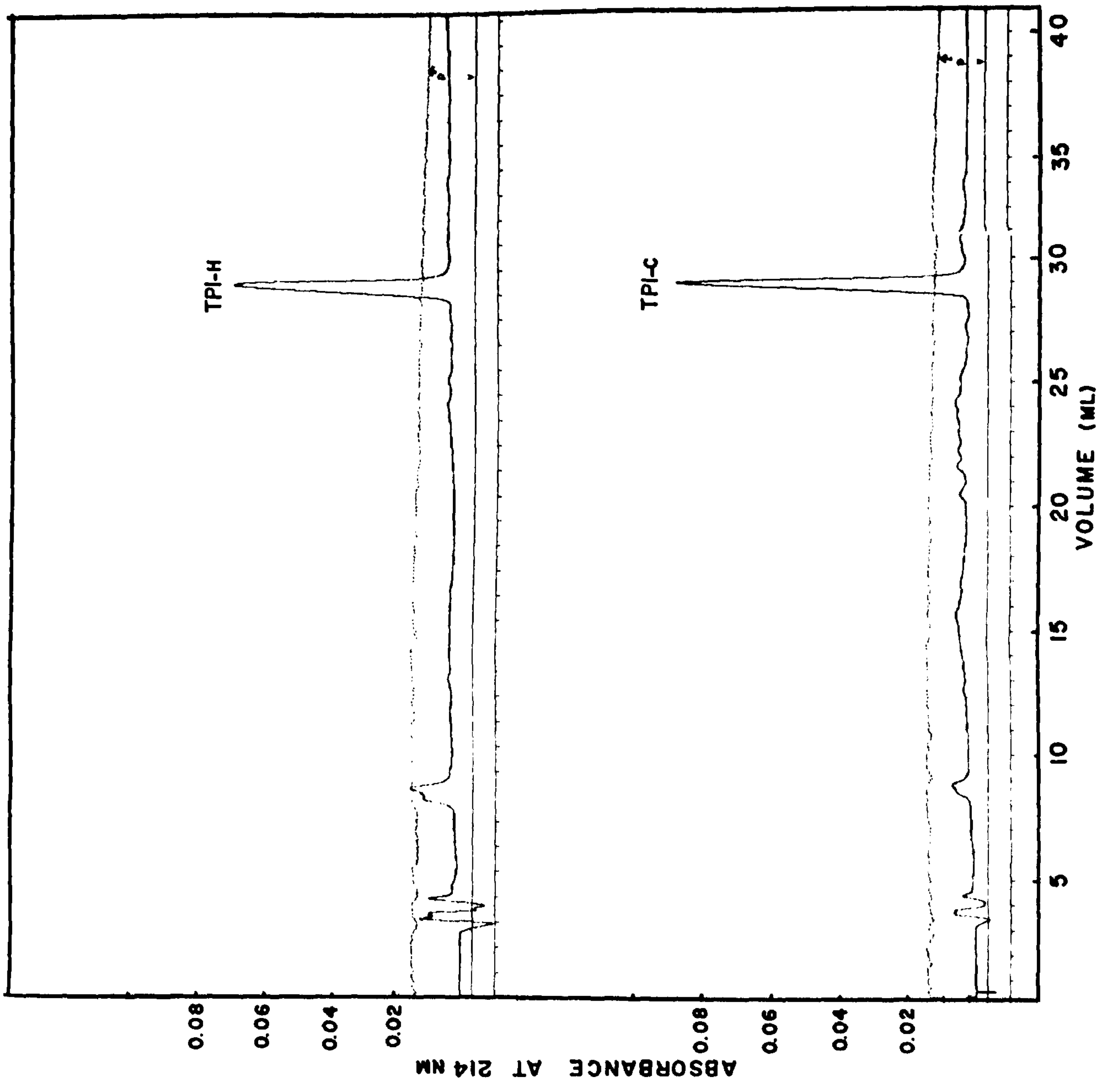
I. Physical properties of TPI-h and TPI-c

The TPI-h and TPI-c preparations after the second Sephadex G-75 column were analysed using SDS-polyacrylamide gel electrophoresis, isoelectric focusing and high performance liquid chromatography. The migration properties of TPI-h and TPI-c during electrophoresis on a 7-18% gradient of polyacrylamide in the presence of 0.1% SDS show that only one polypeptide is present in each inhibitor preparation. Based on the migration distance of the TPI's compared to standard protein markers, the molecular weight was determined to be 14,800 which is the same as that for TPI-n and TPI-d. The elution profile of TPI-h and TPI-c from an HPLC column is shown in Figure 13. Only one major protein peak was detected in each preparation, and this protein eluted at the same position as TPI-n and TPI-d (see Figure 5 also).

J. Effect of TPI-h and TPI-c on cathepsins B, H and L and papain

Effect of TPI-h and TPI-c on mouse liver cathepsin B, H

Figure 13. Chromatography of TPI-c and TPI-h on HPLC column. 100 µg of the TPI-h and TPI-c were applied separately to a C-18 reverse phase column (Chemopack, 4.6 x 250 mm) equilibrated with 12% acetonitrile and 0.1% TFA. The protein was eluted from the column with an acetonitrile gradient as described in the legend to Figure 5. The actual tracing from the recorder is shown here including the column pressure curve (p) and zero voltage curve (v).


TPI-H
TPI-C
0.08
0.06
0.04
0.02
0.08
0.06
0.04
0.02
ABSORBANCE AT 214 NM
5
10
15
20
25
30
35
40
VOLUME (ML)

and L and papain was tested exactly as for TPI-n and TPI-d (see Fig. 7 and Table 2). Figure 14 shows the results of these experiments and Table 4 shows the ID_{50} values of TPI-h and TPI-c for the different enzymes. In all cases the ID_{50} data were found to be similar to that obtained for TPI-n (compare with data in Table 2).

K. Analysis of TPI-h and TPI-c on isoelectric focusing gels

To ascertain whether the inhibitor activity is associated with the major protein on SDS-polyacrylamide and isoelectric focusing gels, 50 µg of TPI-h and TPI-c were electrophoresed separately on gel columns and the columns were then analyzed for pH, protein content and inhibitor activity as before.

With both TPI-h and TPI-c only one discrete protein band could be detected and these results are shown in Figure 15. The pH at which the inhibitor protein focused was 4.5. Assays of the inhibitor activity in the protein extracted from the gels showed that, in both cases, the protein which focused at pH 4.5 was active against cathepsin B. However, in the TPI-h preparation, apart from the peak of inhibitor activity at pH 4.5, inhibitor activity was also observed at pH 4.9 when papain was used as the test enzyme. This inhibitory agent, whatever its nature, appears to be the same component observed previously in muscle TPI-d preparations (see Fig. 8). Again, as with the protease inhibitor from dystrophic muscle, the inhibitor activity which focused at pH 4.9 could not be stained with Coomassie brilliant blue. TPI-c showed only one

Figure 14. Effect of TPI-c and TPI-h on papain, and cathepsins B, H and L. Varying amounts of inhibitor protein from carrier females (TPI-h) and normal female breeders (TPI-c) were added to separate standard reaction vessels containing the thiol proteases as indicated and the percent inhibition was determined.

■---■, TPI-c from normal female breeders.

x---x, TPI-h from carrier females.

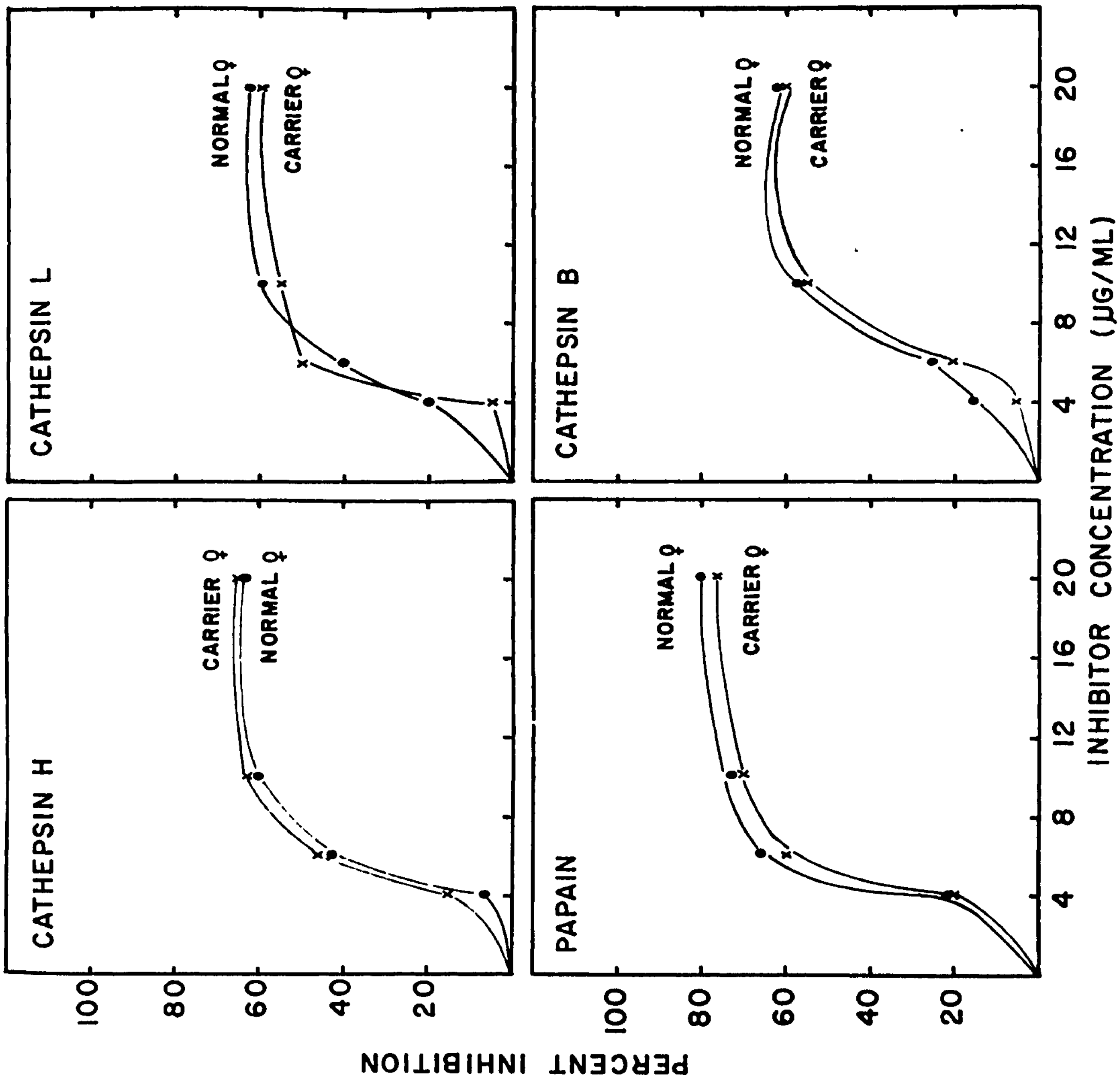

CATHEPSIN H
CARRIER ♀
NORMAL ♀
CATHEPSIN L
NORMAL ♀
CARRIER ♀
PAPAIN
NORMAL ♀
CARRIER ♀
CATHEPSIN B
NORMAL ♀
CARRIER ♀
100
80
60
40
20
PERCENT INHIBITION
4
8
12
16
20
INHIBITOR CONCENTRATION (µG/ML)

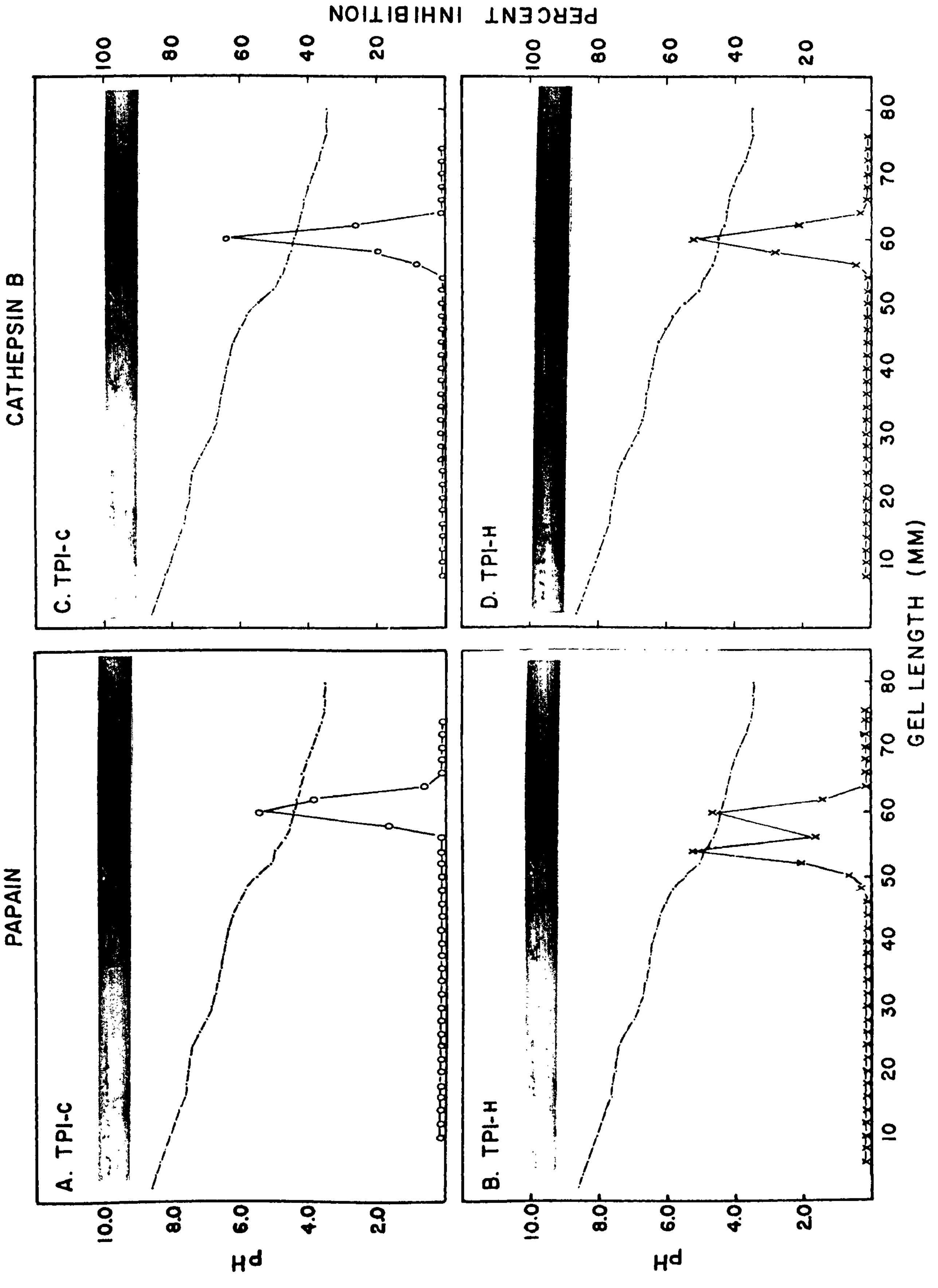
Table 4: Summary of thiol protease inhibitor activity from skeletal muscle of normal retired breeders and carrier females heterozygous for the disease on various cathepsins and papain

Source of skeletal muscle inhibitor	ID_{50} (µg/ml)			
	Cathepsin B	Cathepsin H	Cathepsin L	Papain
Carrier females	7.5 ± 0.5	8.6 ± 0.5	8.2 ± 0.2	5.9 ± 0.8
Normal breeders	7.2 ± 0.6	8.9 ± 0.5	5.8 ± 0.2	5.7 ± 0.6

The data shown are averages of 4 experiments showing sample standard deviations about the mean.

Figure 15. Isoelectric focusing of TPI-c and TPI-h on a polyacrylamide gel support.
Samples containing TPI-h (50 µg) or TPI-c (50 µg) were run on analytical isoelectric focusing gels and then analysed for protease inhibitor activity using papain (panel A and B) and cathepsin B (panel C and D) as test proteases. The photographs at the top of each panel show the Coomassie blue stained protein in a gel run in parallel compared with the pH gradient (.-.) and inhibitor activity in separate gels containing TPI-c (O ---O) and TPI-h (x---x).

Note: The photographs at the top each panel were trimmed on either side because they were 10% longer than the gel length as shown in the diagram


PAPAIN
CATHEPSIN B
A. TPI-C
B. TPI-H
C. TPI-C
D. TPI-H
pH
10.0
8.0
6.0
4.0
2.0
PERCENT INHIBITION
100
80
60
40
20
GEL LENGTH (MM)
10
20
30
40
50
60
70
80

peak of activity, and this material coincided with the protein band at pH 4.5.

IV. DISCUSSION

It has long been established that the onset and progression of muscular dystrophy is associated with increased activity levels of several proteases, but there has been little agreement among researchers in the field as to the biochemical lesion(s) responsible for the onset of the disease in mammals (Ayogi et al., 1981; Wada et al., 1981; Perry, 1984; Sweeny and Brown, 1981). One of the theories as to the cause of the disease is that proteases are responsible for the cytoarchitectural lesions since an increase in protease activity has been noticed in dystrophic muscles of numerous animals including chickens (Ettienne et al., 1980), mice (Garber et al., 1980a), hamsters (Li, 1980) and humans (Kar and Pearson, 1976). A concommitent increase in protease inhibitor activity has been reported in many tissues of diseased animals (Lenny, 1980), but the exact nature and source of these inhibitors has not been established.

In an earlier study we demonstrated that crude extracts from dystrophic mouse muscle (strain 129 ReJ/dy) contain a greater proportion of active to inactive thiol protease activity than crude extracts from normal muscle (Warner et al., 1983; Gopalan et al., 1986a). We suggested that enhanced thiol protease activity in dystrophic muscle could result from a lesion in one of the protease regulatory mechanisms which normally masks over 80% of thiol protease activity in skeletal muscle. Such a defect could account for the 4-6 fold increase in thiol protease activity observed in crude extracts of skeletal muscle in most dystrophic animals.

In the study presented here, a purification procedure based mainly on gel filtration columns and one which avoids a heat step, commonly used by other researchers was used. Since the latter procedure often gives rise to multiple molecular forms of inhibitor activity (Katanuma et al., 1983a). Ion-exchange and papain-sepharose affinity columns were not used in the purification protocol as established for TPI purification by other researchers because considerable inhibitor activity was lost using these media. It is also known that papain-sepharose affinity columns generate artifacts if not used carefully (Lenney et al., 1979; Hirado et al., 1981; Katanuma et al., 1983a). Also, since it is known that some low molecular weight thiol protease inhibitors are inactive in the absence of sulfhydryl agents, all buffers contained 1 mM DTT to minimize oxidation of thiol groups (if present) in the proteins (Kominami et al., 1982a). Thus by using only gel filtration chromatography, a thiol protease inhibitor protein has been obtained from the hindlimb muscle of dystrophic (TPI-d) and normal (TPI-n) males, carrier females heterozygous for the disease (TPI-h) and normal retired females (TPI-c). In all cases the purified protease inhibitor appears to be homogeneous by three criteria: SDS-polyacrylamide gel electrophoresis (Fig. 3), isoelectric focusing (Fig 6, 15) and high performance liquid chromoatography (Fig 5, 13). Furthermore, the inhibitor from normal muscle (TPI-n), carrier females (TPI-h) and normal females (TPI-c) all have an ID_{50} (6-9 ug/ml) similar to low molecular weight thiol protease inhibitors from other mammals (Hirado et al., 1981; Kominami et al., 1982a). The major difference between the thiol protease inhibitor from normal and dystrophic muscle of the mouse is that TPI-n is active in the regulation of cathepsin B activity, while TPI-d is lacking inhibitor activity at concentrations up to 50 ug/ml. Table 2 shows experiments using cathepsin B from both normal and dystrophic livers, and the results show that it is indeed the inhibitor which is altered in the dystrophic animal and not the thiol proteases because the ID_{50} values for TPI-n were found to be the same against cathepsins B, H and L regardless of the source of these enzymes. Also, these results indicate that cathepsin B, H and L are "normal" in skeletal muscles of dystrophic mice.

In the study reported here, the most pure preparations of TPI-d showed significant activity against papain and cathepsin H and L, whereas it was inactive against cathepsin B. The nature of the inhibitor substance in TPI-d preparations (measured using papain) has not been determined but evidence suggests that it might not be protein. Thus, while the "apparently" homogeneous TPI-d has inhibitor activity against papain, cathepsins H and L, isoelectric focusing shows this inhibitor to be separate from the TPI-d protein. The latter is inactive against cathepsin B and papain after purification on isoelectric focusing gels. Thus, unless isoelectric focusing is performed, the defect in function of TPI-d is not apparent except in the case where the cathepsin B is the test protease. In contrast to our findings with mice, Kominami and coworkers have found high levels of thiol protease inhibitor activity in extracts from dystrophic hamster muscle assayed using cathepsin H (Kominami et al., 1984). The apparent increases in TPI levels in muscle extracts of dystrophic hamsters may reflect a different biochemical lesion in hamsters than in mice, since the dystrophic hamsters (BIO-14.6) used in their experiments were from a cardiomyopathic line. Alternatively, the high level of TPI activity observed in protein preparations from these hamsters could be due to the use of cathepsin H as the test protease. In crude extracts of skeletal muscle of dystrophic hamsters of strain Bio. 14.6, the elevated thiol protease activity appears to be the result of enhanced protein synthesis (Katanuma et al., 1983b). Perhaps in dystrophic hamsters, muscle necrosis occurs by a different route than in dystrophic mice.

In muscle preparations from normal mice the protease inhibitor of pI 4.5 (TPI-n) was found to be functional against papain and cathepsin B, while in muscle preparations from dystrophic mice the protease "inhibitor" of pI 4.5 (TPI-d) was non-functional against these thiol proteases (see Table 2). An inhibitor activity at pI 4.9 was observed in the dystrophic muscle which becomes detectable after isoelectric focusing of TPI-d. This inhibitory activity was also detected in carrier female mice (+/dy) heterozygous for the disease but not in normal retired female breeders (+/+) of the same age (Fig. 15). The source of the pI 4.9 inhibitor activity in muscle which is active against papain (and perhaps cathepsin H and L) is not known. Whatever its source, if it is a protein it has a high specific inhibitor activity against papain. Attempts to trace the origin of this inhibitor have not been made, however, its source could be from macrophages because macrophage infiltration is observed widely in diseased tissues (Lenney, 1980). Recently Kominami et al. (1984) demonstrated the presence of protease inhibitors in macrophages from dystrophic hamsters. It is known that while the dystrophy gene is expressed initially in muscle, its effects are also detectable in non-muscle cells such as erythrocytes, fibroblasts and lymphoblasts (Sweeney and Brown, 1981; Davis et al., 1982; Pimplikar and Malhotra, 1983). Reasons why the product of dystrophy gene should be manifested to a greater extent in muscle than in other tissues are not known. Perhaps in non-target tissues the dystrophy gene is inactive, while another gene coding for a similar protein is active. However, since our knowledge of the regulation of intracellular protein breakdown is not as advanced as our overall knowledge of protein synthesis, the protein catabolic mechanism may be regulated differently in muscle than in other tissues where protein turnover rates differ and the dystrophy gene product is of greater importance (Millward et al., 1980; Garber et al., 1980a). While the function of TPI's in healthy individuals is not clearly understood (Dean, 1979), it is possible that TPI's normally function by protecting the cell from inappropriate intracellular proteolysis.

It is not known whether the thiol proteases that damage the structural and soluble components of the myotubes and other cells are cytosolic or lysosomal in origin, but at least one report indicates that lysosomes from dystrophic animals have altered membrane properties which may allow some lysosomal thiol proteases to leak into the cytosol in dystrophic but not normal individuals (Davis et al., 1982). Circulating macrophages and other protease rich cells (fibroblasts) in muscle may also contribute proteases to cells lacking functional TPI's compounding the problem for myoblasts and myotubes in dystrophic animals (Kar and Pearson, 1978). In this study it is assumed that the TPI isolated from the whole muscle is myocytic in origin. Evidence from other workers has shown that thiol protease inhibitors in muscle are located mainly in the cytosol of post-fusion myoblasts (myotubes) and not in fat or connective tissue cells (Lenny, 1980).

That muscular dystrophy in most animals appears to be closely linked to a defect in the protease regulatory mechanism is a view supported by several studies using protease inhibitors. Libby and Goldberg (1978) and Sher et al. (1981) have shown that leupeptin, a thiol protease inhibitor, significantly reduces the rate of protein degradation in mice with hereditary muscular dystrophy, while

McGowan et al. (1976) and Stracher et al. (1978; 1979) made similar observations using muscle and cells from dystrophic chickens. However, Hudecki et al. (1981) were unable to observe any beneficial effects of leupeptin on dystrophic chickens. Recently Tsuji and Matsushita (1986) have shown beneficial effects of bestatin, a diaminopeptidase inhibitor, on dystrophic mice with elevated levels of diaminopeptidases. (The exact role of diaminopeptidases in myofibrillar protein turnover is, however, not understood). Bestatin is a small molecular weight immunomodifier originally isolated from *Streptomyces olivoretivuli*. It is known to have a potent inhibitor effect on aminopeptidase B and leucine aminopeptidase existing exclusively in the cell membrane, and to attach to the cell membrane of macrophages, T-lymphocytes and other mammalian cells and modify their functions (Muller et al., 1982). If immunological deficiencies play a major role in the pathology of muscular dystrophy, then it may be that the immuno-potentiating action of bestatin is conducive to controlling the disease process. Thus despite certain equivocal data on the effectiveness of protease inhibitors in dystrophic animals, it appears that lysosomal proteases, especially the thiol proteases, play a key role in the onset and progression of muscle degeneration in dystrophic animals. The observations made in this study show that the TPI in dystrophic mouse muscle is functionally defective supports this conclusion.

The reason(s) for the loss of activity in the TPI protein of skeletal muscle of dystrophic mice is not yet known. It may be due to an amino acid substitution in TPI-n which affects the molecular conformation of the protein, but not its pI, or it may be due to a post-translational modification of one or more amino acids in the molecule. Recent data from an amino acid analyses of the TPI protein carried out in collaboration with Dr. M. Bloom at the University of Toronto have shown one less tyrosine residue and one or two fewer histidine residues in TPI-d compared to TPI-n. Also, there are two additional lysine residues in TPI-d compared to TPI-n. The N-terminal amino acids are blocked in both TPI-n and TPI-d. Since both TPI-n and TPI-d resist hydrolysis by trypsin at high enzyme to substrate ratios (1:20) and long incubation periods (48 hours at 37 C), comparative analysis of the tryptic fragments of the TPI's have not been carried out. Also, glycoprotein analyses were negative for both TPI-n and TPI-d which indicate that the differences in inhibitor activity are not due to differences in their carbohydrate moiety. Structural studies, including amino acid sequence analyses to identify the altered or missing amino acid(s) in the TPI of animals with hereditary muscular dystrophy remain to be done.

The appearance of inhibitor activity at pI 4.9 in extracts from skeletal muscles of carrier females heterozygous for the disease opens up the possibility for carrier detection, since carrier detection of Duchenne muscular dystrophy is still a significant problem (Garden-Medwin, 1983;

Dubowitz, 1982). Despite the introduction of new techniques with more or less success, a significant percent of carriers remain without diagnosis and, consequently, the disease goes on being transmitted. The introduction recently of a radioimmunoassay for serum myoglobin has opened up new perspectives for the detection of carriers with muscular dystrophy because "leaky" plasma membranes in patients and carriers would allow abundant loss of myoglobin from the skeletal muscles into serum. This assay has been used as an alternative to the use of creatine kinase for carrier detection (Sabria-Leal, 1986). However, this assay is not unique to muscular dystrophy because myoglobin release into serum has been observed also in myocardial infarction (Adornato et al., 1978). The findings described in this dissertation for dystrophic mice may serve as a model for human muscular diseases including Duchenne muscular dystrophy and if so, an effective assay may be developed to screen potential carriers of the disease for the presence of either the defective TPI or the pI 4.9 inhibitor component in muscle extracts.

Recent reports on the isolation of portions of Duchenne muscular dystrophy gene (Monaco et al., 1986; Worton et al., 1984) are also important and relevant to the problem at hand. Although the product(s) of the cloned gene fragments have not been identified, it will be interesting to see whether the defective gene in Duchenne muscular dystrophy codes for a protease inhibitor as suggested by the work in this dissertation.

As the amino acid sequence data of mouse muscle TPI-d become available, ^{32}P-labelled DNA probes can be constructed to detect and isolate the dystrophic and normal genes from restriction enzyme digests of genomic DNA. The desired restriction fragments can then be cloned in a suitable phage/plasmid system. The nucleotide sequence of the cloned (defective and normal) genes would help to identify the altered/missing nucleotide(s) in the dystrophy gene, and allow in situ hybridization to localize the TPI gene.

Muscular dystrophy in mice of strain 129/ReJ(dy/dy) is a disease transmitted by a single autosomal recessive factor (West and Murphy, 1960). However, in many of its clinical features it resembles the Duchenne form of muscular dystrophy (DMD) affecting human males. Thus if the TPI gene in dystrophic mice is functionally inactive or defective, the primary lesion causing the onset and progression of the Duchenne form of muscular dystrophy in humans might be (similar or identical) to the one in mice. The significance of the findings reported in this dissertation have enhanced our understanding of the biochemical basis of muscular dystrophy to the point where a cure for the disease may be in sight. Should future research on dystrophic muscle in humans reveal a defect in the TPI protein similar to that found in mice, then a major effort should be mounted to develop and test additional protease inhibitors in search of a treatment to ameliorate this debilitating disease until such time as the frequency of the disease in humans can be reduced to the lowest level possible or the disease eliminated completely.

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