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CATHEPSIN B AND D ACTIVITY IN VARIOUS TISSUES
AND CULTURED CELLS FROM NORMAL AND DYSTROPHIC
MICE.

by



PRATHIMA GOPALAN

A thesis

submitted to the Faculty of Graduate Studies
through the Department of Biology in
Partial Fulfillment of the requirements for the Degree of
Master of Science

Windsor, Ontario, Canada.

1982

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779045

To
my mother

ABSTRACT

The levels of cathepsin B and D have been studied in various tissues of normal and dystrophic mice of strain 129 ReJ and the results have revealed differences in the level of protease activity among the various tissues. Extracts from muscle, lung and heart in dystrophic mice show an elevation in cathepsin B and D activity compared to control or normal tissues while extracts from liver and kidney from dystrophic mice do not show any differences. Autoactivation experiments carried out with extracts from muscle and heart suggest an imbalance in enzyme to inhibitor ratio in dystrophic tissue. The response of the muscle tissue extracts to exogenous inhibitors like leupeptin and Artemia protease inhibitor indicates that differences exist between the cathepsin B enzymes in normal and dystrophic mouse muscle. A study of cathepsin B and D activity in a differentiating myoblast cell line (L69/1) has also shown that protease activity in muscle cells increases markedly during differentiation. In fibroblast cells (LMTK) cathepsin B and D activity were found to be 5-fold and 2-fold lower, respectively, than in myoblasts at the post-fusion stage. However, cathepsin B activity in fibroblasts can be increased 2-fold by autoactivation unlike cathepsin B in myoblast cell line which is resistant to autoactivation. Autoactivation experiments indicate the presence of considerable cathepsin B and/or D inhibitor activity in myoblast but less in

fibroblast. This was further confirmed by mixing experiments with homogenates from a cell-cell hybrid formed between the rat myoblast (AG2) and mouse L-cell fibroblast (LMTK). The Rat x Mouse hybrid (RM-5) did not express cathepsin B and D activity compared to the parental cells. Autoactivation of the homogenates from primary cell cultures of dystrophic mice (strain 129 ReJ) shows an increase in the specific activity of cathepsin B compared to the control samples maintained at 0° C while cells cultured from normal mouse muscle do not show an increase in specific activity upon incubation at pH 4.5 and 30° C. These studies suggest the presence of a cathepsin B and/or D regulator in normal cells and tissues which may be either lacking or present in limited quantities in dystrophic animals. The consequences of an imbalance in the enzyme to inhibitor ratio in the onset of muscular dystrophy is discussed.

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TABLE OF CONTENTS

	PAGE
Dedication.....	iv
Abstract.....	v
Acknowledgements.....	vii
List of Figures.....	xi
List of Tables.....	xiii
I. INTRODUCTION.....	1
II. MATERIALS AND METHODS.....	13
A. Biochemicals.....	13
B. Established cell lines.....	13
C. Primary cell cultures.....	14
D. Tissues.....	14
E. Methods.....	14
1. Tissue sample preparation.....	14
2. Development of primary cell cultures from the skeletal muscles of normal and dystrophic mice.....	15
3. Harvesting of cells and preparation of cell homogenates.....	16
4. Sample preparation for activation experi- ment.....	17
5. Cathepsin B assay.....	17
A. Incubation buffer.....	18
B. Coupling reagent.....	18
6. Cathepsin D assay.....	19

III. RESULTS.....	21.
A. Cathepsin B and D activity in skeletal muscle tissue of normal and dystrophic mice.....	21
B. Cathepsin B and D activity in liver tissue of normal and dystrophic mice.....	29
C. Cathepsin B and D activity in cardiac tissue of normal and dystrophic mice.....	37
D. Cathepsin B and D activity in lung tissue of normal and dystrophic mice.....	43
E. Cathepsin B and D activity in kidney tissue of normal and dystrophic mice.....	46
F. Cathepsin B and D activity in differentiating rat myoblast cell line L69/1.....	46
G. Cathepsin B and D activity in mouse L-cell fibroblast cell line LMTK.....	55
H. Cathepsin B and D activity in a myoblast x fibroblast hybrid (RM-5).....	62
I. Cathepsin B activity in normal and dystrophic primary cell strains.....	69
IV. DISCUSSION.....	77
A. Cathepsin D activity in mouse muscle tissue..	79
B. Cathepsin B activity in mouse muscle tissue.	82
C. Changes in cathepsin B and D in mouse liver.....	84
D. Changes in cathepsin B and D in cardiac	

muscle tissue.....	87
E. Changes in cathepsin B and D in lung tissue..	88
F. Changes in cathepsin B and D in kidney tissue.	89
G. Cathepsin B and D activity in rat myoblast cell line, L69/1.....	91
H. Cathepsin B and D activity in mouse L-cell fibroblast cell line, LMTK.....	93
I. Activation experiment with cell line, RM-5....	94
J. Activation experiment with primary culture of normal and dystrophic cells.....	95
V. BIBLIOGRAPHY.....	98

List of Figures

Figures	Description	Page
1	Cathepsin B and D activity in skeletal muscle tissue (hind leg).....	23
2	Cathepsin B activity after activation of skeletal muscle tissue.....	28
3	Cathepsin B activity of skeletal muscle tissue in presence of leupeptin and acid protease inhibitor from Artemia sp.....	31
4	Cathepsin B and D activity in liver tissue...	34
5	Cathepsin B activity after activation of liver tissue.....	36
6	Cathepsin B and D activity in cardiac muscle tissue	39
7	Cathepsin B activity after activation of cardiac muscle tissue.....	42
8	Cathepsin B and D activity in lung tissue....	45
9	Cathepsin B activity after activation of kidney tissue.....	48
10	Stages in myogenesis	50
11	Cathepsin B and D activity in rat myoblast cell line L6-9/1.....	53
12	Cathepsin B activity after activation of rat myoblast cells, L6-9/1.....	57
13	Cathepsin B and D activity of mouse L-cell	

	fibroblast , LMTK.....	59
14	Cathepsin B activity after activation of homogenates of mouse L-cell fibroblasts, LMTK.....	61
15	Cathepsin B activity after activation of AG2 RM-5 and LMTK cell lines.....	65
16	Cathepsin D activity of homogenate mixtures of AG2, LMTK and RM-5 cell lines.....	68
17	Cathepsin B activity after activation of homogenates from normal and dystrophic cells.	72
18	Cathepsin D activity of homogenate mixtures of normal and dystrophic cells.....	74
19	Cathepsin D activity after activation of homogenate mixtures of normal and dystrophic cells.....	77

List of Tables

Table	Description	Page
1	Cathepsin B and D activity in normal and dystrophic mouse tissues along with the body weight and weight of individual tissues.....	24
2	Specific activities of cathepsin B and D in the cell-free extracts from whole muscle, differentiating mvoblast populations and fibroblasts.....	54
3	Specific activities of cathepsins B and D in cell lines AG2, LMTK and RM-5.....	63
4	Specific activities of cathepsin B in homogenates of normal and dystrophic primary strains at different stages.....	70

I. INTRODUCTION

The initial studies of proteinases of mammalian cells and tissues can be traced back to their origin in the German laboratories of physiological chemistry in about 1890. It was around this time that the proteolytic enzymes were first recognized for their involvement in biological control mechanisms. Kunitz reported that the trypsin catalysed activation of chymotrypsinogen occurred without detectable proteolytic degradation (Kunitz, 1935). The significance of this discovery was greatly extended by the work of Lorand who subsequently discovered that the catalytic effect of thrombin in blood clotting was due to limited proteolysis of the substrate fibrinogen (Lorand, 1951). It has since become clear that specific limited proteolysis is a general mechanism in many physiological processes. Limited proteolysis is a process in which a small and/or selected number of peptide bonds are cleaved. Processes which involve limited proteolysis are defence mechanisms such as blood clotting, complement fixation and repair of tissue injury, hormone production (conversion of pro-insulin to insulin), self assembly of viruses, digestion, fertilization, development and metamorphosis. In addition to the above processes several new ideas have emerged which implicate proteases

in the control of cell behaviour during malignant transformation. Limited proteolysis probably also occurs as an early step in intracellular protein-turnover (Goldberg et al., 1978).

Evidence that protein catabolism is important in physiology comes from the realization that the amount of amino acids produced each day by breakdown of tissue proteins in the human body is well in excess of that obtained by digestion in the alimentary tract in the same period. Clearly the degradation of intracellular and extracellular proteins is controlled precisely in the healthy state. In fact, one remarkable property of these dynamic systems is the exquisite regulation of proteases after their synthesis. Elucidation of the nature of this regulation is a tantalizing challenge for the future.

The term "protease" includes both exopeptidases and endopeptidases. Endopeptidases cleave bonds distant from the ends of polypeptide chains, whereas exopeptidases cleave bonds only near the ends. The chemical composition of the essential catalytic groups of the enzymes was used by Hartley to identify 4 classes of proteases namely, the serine, thiol, carboxyl and metalloproteinases (Hartley, 1960).

Proteases have been implicated in a number of diseases

in man, among the best known of these are the hereditary muscular dystrophies. In such states there could be an activation of enzymes which are normally latent or an activation of pre-existing enzyme by removal of a specific inhibitor/regulator which controls the activity of the enzyme under normal condition in situ. Increases in proteolytic activity may also occur in response to food deprivation which results in the decrease in the weight of muscles (Millward, 1970).

Wasting of muscles is a well known phenomenon which accompanies cancer at various sites in humans. However, the cause(s) of this loss in muscle mass is not clear. Protein turnover studies by Goodlad and Clark (1973) have provided evidence to show that the overall loss of proteins from muscles of rats with Walker 256 carcinomas is due to an accelerated catabolism of proteins with a concomitant decline in the synthetic rate. In other studies Holmes et al. (1974) have measured the activity of several peptide hydrolases in muscles bearing transplantable tumors. In the extensor digitorum longus muscle which showed wasting, the acid and alkaline proteinases showed elevated specific activities while that of cathepsin B decreased. In no case was there an increase in the total activity of any of the enzymes in the muscle.

There are other diseases such as pulmonary emphysema (Eriksson, 1978), glomerular nephritis (Davies et al., 1978), arthritis (Barrett, 1978), and myelin degeneration (Cammer et al., 1978) in which cellular proteinases are responsible for tissue damage. It could be that normal tissues are given considerable protection against inappropriate proteolysis by a variety of endogenous proteinase inhibitors.

In search for clues to the nature of hereditary muscular dystrophy, considerable work has been done in recent years on the metabolism of muscle proteins. Despite these efforts the mechanism by which protein degradation is regulated in skeletal muscles is poorly understood. However, there has been an increased appreciation of the regulatory role of proteases. Although many proteases exist in muscle it is not known which of these enzyme(s) actually degrade native protein in the pathological state. Both lysosomal and non-lysosomal proteases can hydrolyze muscle protein in cell-free preparations but the relative importance of lysosomal to non-lysosomal proteases in the dystrophic process remains unclear. One of the widely studied non-lysosomal proteases is the calcium activated neutral protease (CANP) found originally in pig skeletal muscle by Dayton et al. (1975), Kar and Pearson (1976),

Mokri and Engel (1975), Neerunjun and Dubowitz (1979), Bush et al. (1972) and Ishiura et al. (1978, 1979, 1980, 1981). Among these researchers Mokri and Engel (1975) have observed lesions in plasma membrane of human tissues affected with dystrophy. However, it should be noted that the level of CANP in muscle is very low and the concentration of calcium required for maximal activation is 1mM which is in excess of the physiological concentration of calcium in normal muscle. Thus for this enzyme to be activated in vivo local calcium concentrations must increase, at least temporarily, to a level sufficient to activate the CANP. It is quite possible that the lesions in the plasma membrane allow the calcium rich extracellular fluid into the fibre interior and thereby increase the concentration of intracellular calcium. Therefore failure to regulate the intracellular level of calcium could be a cause for the pathological state.

Another non-lysosomal protease studied extensively is a serine protease found in muscle. Sanada et al. (1978) have observed elevation of serine protease activity in mice with hereditary muscular dystrophy. On SDS-PAGE gels the muscle proteins from the dystrophic mice show the same pattern as those from the normal mice after incubation with

the above mentioned serine protease. Similar observations have been made by Katanuma et al., (1978) in human hereditary myotonic dystrophy.

The lysosomal proteases implicated in the onset and progression of dystrophy are cathepsins A,B,C and D. Increased level of these enzymes have been observed by many investigators. Kar and Pearson (1977) have shown increased cathepsin B and H activity in human skeletal muscles in dystrophy. Similar observations of elevated cathepsin B and D activity have been made by Schwartz and Bird (1977), Libby and Goldberg (1978) in rat skeletal muscles, Tappel et al. (1962) in mice and chicken, Iodice et al. (1972) in chicken and Stracher et al. (1978) and McGowan et al. (1976) in chicken muscle cells in culture.

Although these enzymes are reported to increase in activity in dystrophic animals as compared to the normal, it is not known whether the changes occurred in enzyme synthesis or in modifying factors such as inhibitors. Protease inhibitors in cells and tissues are probably concerned with the control of protease activity within the same cell or proteases produced by other types of cells in the same tissue. Clues to their function can be gained by selectively measuring the activity of proteases and their

inhibitors in various normal and pathological states. When proteinase-inhibitor interactions occur in vivo, the inhibitor could have a regulatory effect, increasing or decreasing the concentration of free protease. It is likely that endogenous inhibitors have a protective role such that where there is a large excess of inhibitor over enzyme the protease activity is effectively quenched by the inhibitor. However, it is important to know the localization of the protease and its inhibitor. If a protease leaks out of lysosomes, the cytosolic inhibitor will protect other cytoplasmic proteins from destruction. This may occur normally at a slow rate and at a faster pace under certain pathological conditions. Schwartz and Bird (1977) have demonstrated the presence of an inhibitor of cathepsin B in rat skeletal muscles and Lenney et al. (1979), have shown the presence of inhibitors in rat and human tissues. The role of these inhibitors in regulating the enzyme activity has been implicated from the use of exogenous inhibitors like pepstatin and leupeptin. Libby and Goldberg (1978) have carried out such studies on rat skeletal muscles. Others such as Iodice (1976) and McGowan et al., (1976) have conducted studies with skeletal muscles and cultured chick muscle cells. Hashida et al. (1980) have studied the activity of another cathepsin

B inhibitor, E-64, extracted from Aspergillus japonicus and Sugita et al. (1980) have tested the therapeutic effect of E-64 on CANP from dystrophic chickens. The results of these inhibitor studies suggest that protease inhibitors play a central role in the control of protein metabolism which when altered leads to the onset of various pathological conditions.

There is little reason to believe that the animal myopathies are identical with the human forms of dystrophy but it seems possible that our comprehension of human disorders will be advanced by acquiring an understanding of the derangements of animal myopathies. Many structural physiological, and biochemical changes have been reported for muscle from dystrophic mice (Coleman and Ashworth, 1959; Srivastava, 1967, 1968); chicken (Julian and Asmundson, 1963; Weinstock and Iodice, 1969).

The first information on the mode of inheritance of dystrophy came from an analysis of pedigree records of mice at Jackson laboratories in Maine. The incidence of dystrophy varied in different subcolonies of the 129/Re inbred strain, but no more than 25% of the animals in a single litter were affected (Michelson et al. 1955). Proof for a unit recessive inheritance was derived from transplantation of ovaries from dystrophic females to ovariectomized histocompatible (129/Re x DBA/2J) F1 normal females

(Stevens et al. 1962). Matings between these recipient F1 hybrids and normal 129/Re produced all normal offsprings in F1 generation , 20-25% dystrophic in F2 generation and 44-50% dystrophic in a backcross of F1 carrier males to females bearing implanted dystrophic ovaries. Since the dystrophic mice never breed , the colony was maintained by repeated ovarian transplantation in each generation. Tests of expression of the dystrophic genotype (dy/dy) on a variety of different genetic backgrounds including several heterogeneous hybrid populations and linkage crosses, showed that the proportion of dystrophics in each background was close to 25% and the clinical signs were identical to 129/Re dystrophy except for increased longevity (Loosli et al. 1961). By repeated cross-intercross matings to the inbred strain C57BL/6J, the dystrophy gene has been transferred to a genetically homogeneous background (Loosli et al. 1961). The best animals appear in (129Re dy/+ x C57BL/6J/+) F1 hybrids. These animals are essentially identical to their normal litter mates except for the genes at the dy-locus (Russell et al. 1962) . Further, Wolfe and Southard (1962) succeeded in producing all dystrophic litters by artificial insemination of dystrophic young adult female with sperm collected from dystrophic males. In this case 126 B6 F1-dy/dy females (129 Re/+ x C57B2/6/dy + are subjected to priming doses of pregnant mare serum and human chorio-


gonadotropic hormone, then after 10h they received dystrophic sperm by intrauterine injection.

Numerous biochemical studies have been done in this and other strains of mice. The dystrophic mouse, although small compared to most other models for muscular dystrophy, possesses sufficient similar characteristics of human disease and hence allows significant amounts of information to be gained.

Because of its characteristic morphological, biochemical and functional features, whole skeletal muscle and muscle cells in culture present ideal experimental systems for studies of cellular and molecular differentiation. However, little information about the genetic control of myogenesis has been obtained despite voluminous investigations of normal muscle in vitro and in vivo.

In the investigation described in this thesis, the activities of acid proteases (mainly cathepsins B and D) and protease inhibitors leupeptin and acid protease inhibitor from the brine shrimp, Artemia sp., have been studied using protein preparations from skeletal muscle, liver, lung, kidney and heart of normal and dystrophic mice (strain 129 ReJ). Also, the protease activity in extracts of established cell cultures of muscle such as L6 and AG2 and mouse L-cell fibroblasts, LMTK and (primary) cell cultures of normal and dystrophic mouse muscle were investigated.

Myoblast and fibroblast are the two major cell types of adult muscle which take their origin from the primitive mesoderm. These cell types contribute to the protein hydrolase activities in muscle homogenate in addition to those from the minor cell types like macrophages and mast cells. Macrophages have been shown to be rich in hydrolases (Schwabe, 1969). Since the total activities of many protein hydrolases found in muscle extracts tends to be rather low, the contribution from the various cell types could be relatively large. Moreover, in diseased muscle the proportion of some of these other cell types is often much higher than normal. Bois (1964) has shown that in diseased muscle from mouse and humans, there is a proliferation of connective tissue. This may be true of normal muscle also. Sweeney and Brown (1972) have observed intrastructural changes in the connective tissue and capillary beds which precede changes in muscle and nerve in cardiac muscular dystrophy. The same authors have also observed marked changes in the fibroblasts of selenium deficient ducklings (Sweeney and Brown, 1981). In view of the changes seen in fibroblasts of the ducklings it is reasonable to assume that the lesion may start with the inability of fibroblasts to synthesize proper collagen. Since the fibroblasts have



been implicated in dystrophy, it would be interesting to study the protease activity of fibroblasts in addition to myoblasts in order to estimate the contributions of each to the protease activity in whole muscle.

Also the study of protease activity of myoblasts at various stages of differentiation would help to elucidate the onset of enhanced protease activity in muscle during normal myogenesis.

Further study of the effect of inhibitors like leupeptin on protease activity may shed some light on the role of endogenous inhibitors in the regulation of protease activities in normal and dystrophic tissues. The project is also aimed at determining whether or not the acid protease inhibitor from embryos of the brine shrimp, Artemia sp., is an effective inhibitor of muscle cathepsin B and/or D and of potential importance as a protease regulator in mammalian system.

II. Materials and Methods.

A. Biochemicals.

Bovine serum albumin was purchased from Worthington Biochemicals (Montreal, Québec). Benzoyl arginine 2-naphthylamide and Fast garnet GBC salt were purchased from Sigma Chemicals Co. (St. Louis, Mo.). Gentamycin was purchased from GIBCO, (Ontario, Canada). Horse serum and Fetal calf serum were purchased from Flow Laboratories (Ontario, Canada). Leupeptin was a gift from Dr.M. Troll, New York Medical centre (New York). All other chemicals were of reagent grade.

The Artemia protease inhibitor was obtained from dormant encysted embryos (cysts) of the brine shrimp, Artemia sp. using a method developed in our laboratory (Warner, unpublished).

B. Established cell lines:

A clone of Yaffe's (1968) L6 rat skeletal myoblast line, designated L69/1 and its genetic variant AG2 (Dufresne, 1976) were used. Mouse L-cells (LMTK) generally regarded as fibroblasts (i.e.produce collagen) were obtained from Ontario Cancer Institute (Toronto, Canada). A clone of rat myoblast and Mouse L-cell fibroblast hybrids designated as RM-5 was developed by Dr. M. Dufresne (1976).

The established cell lines were routinely grown in 25 cm² Falcon culture flasks in 5 ml of regular MEM

(Modified Eagle's Minimal alpha medium), supplemented with 10% horse serum (HS) and 50 µg/ml gentamycin. The cells were incubated at 37° C in an atmosphere of 10% CO₂ and 90% air. All cell transfers were routinely made using 0.05% trypsin to detach the cells from the plates.

C. Primary cell strains:

The normal and dystrophic primary cell strains were prepared from the cells obtained at 35 days from normal mice and their dystrophic litter mates (strain 129 ReJ) purchased from Jackson Laboratories (Bar Harbour, Maine). The cells were grown in MEM medium supplemented with fetal calf serum (FCS) and 50 µg/ml gentamycin.

D. Tissues (muscle, liver, heart, lung and kidney).

For the enzyme assays the tissues from normal and dystrophic mice (strain 129 ReJ) which were perfused by a procedure routinely used in the lab by cardiac puncture with mammalian Ringer solution containing 0.1mM EDTA were obtained from animals maintained up to 60 days of age.

E. Methods

1. Tissue sample preparations for protease assays.

Extracts from various tissues were prepared by homogenizing the tissues in a glass homogenizer with 5 volumes of a solution containing 2% butanol, 0.15 M

KCl and 0.1 M EDTA. Tween 80 was added to the homogenate at 0.1% of the volume and the solution was kept in an ice bath for 30 min. After 30 min, the samples were centrifuged at 27,000 x g for 15 min in a Sorvall RC2-B centrifuge. The 27,000 x g supernatant was passed through miracloth and dialysed overnight against the homogenizing solution. The preparations were divided into aliquots of less than 1 ml and stored at -70° C. Since extracts from liver tissue were very labile to freezing and thawing, they were prepared fresh prior to each assay.

2. Development of primary cell strains from the skeletal muscles of normal and dystrophic mice.

Normal and dystrophic mice of age 35 days were sacrificed by cervical dislocation and immediately opened aseptically. The hind leg muscles were then taken in sterile phosphate buffered saline (PBS). A preliminary coarse mince of the tissue was done to wash out the red blood cells from the tissue and the buffer aspirated out. The moistened pieces of tissue were finely minced and the tissue suspended in 10 ml of 0.05% trypsin to dissociate the muscle cells. The solution was incubated at 37° C for 10-15 min with intermittent agitation. The samples were sucked up and down in a sterile syringe to maximize the dissociation of tissues into cells. The suspension at this point contained

a mixed population of single cells plus a few small pieces of connective tissue. The small pieces were eliminated by allowing them to settle in the tubes and transferring the supernatant to sterile tubes. About 1 ml of growth medium containing 10% fetal calf serum and 50µg/ml gentamycin was added to the supernatant and the cells were collected by centrifugation at 12,000 x g. Fibroblasts were selected by resuspending the muscle cells in MEM medium containing FCS (a medium which preferentially supports fibroblast growth), plated and incubated at 37°C. Fresh medium was subsequently added every two days.

3. Harvesting of cells and preparation of cell homogenates.

The cells, grown in 16 mm petri plates, were washed thoroughly with 0.05% citrate saline. The cells were released from the plate by gentle scraping with a rubber policeman and washed off the dishes with homogenizing solution into a centrifuge tube. The cells from all the plates were collected similarly and centrifuged at 12,000 x g for 10 min to pellet the cells. The supernatant was discarded and 1 ml of homogenizing solution was added to the pellet. The cells were broken by vortexing with glass beads and the solution allowed to stand for 5-10 min in an ice bath. The clear homogenates were pooled and the protein content determined by the Method of Lowry et al. (1951). This procedure was used to harvest all cell lines and primary cell strains.

4. Sample preparation for activation experiment.

The extract (1.2 ml) from the various tissues and the homogenates from the cell lines and cell strains (in homogenizing solution, pH 6.0), were treated with 0.05 ml of 0.2 M sodium formate, pH 2.5 to adjust the pH to 4.5. Two identical samples were prepared and one was kept in water bath at 30° C and the other was kept at 0° C (in an ice bath). At various times 0.3 ml of the sample was removed and 0.05 ml of 0.2 M K_2HPO_4 , pH 7.0 was added to neutralize the solution. The neutralized samples were centrifuged at 12,000 x g for 10 min and the supernatant was removed and stored at -70° C. After collecting all samples, the amount of cathepsin B activity in each sample was determined as described below.

5. Cathepsin B assay.

The method used to measure cathepsin B was the one developed by Barrett (1972) in which the liberation of 2-naphthylamine from the substrate, benzoyl arginine 2-naphthylamide (BANA), by the enzyme present in the extracts/homogenates was used as a measure of cathepsin B activity. To 0.25 ml of the sample to be assayed was added 0.75 ml of the incubation buffer containing 0.032% cysteine and the solution was incubated at 40° C for 10 min. / To this

solution 0.025 mls of BANA (40 mg/ml DMSO) was added to start the reaction. At the desired times 0.25 ml was removed from each incubation mixture and placed in an ice bath. After collecting all samples the presence of 2-naphthylamine in the reaction mixture was determined by adding 0.25 ml of coupling reagent to each tube followed by incubation at room temperature for 5-10 min to allow the color reaction to take place. Subsequently, 0.5 ml of n-butanol was added to each tube, mixed well and centrifuged. The intensity of the azo dye in the butanol phase was read at 520 nm in Beckmann Spectrophotometer (model 25). One unit of proteolytic activity is defined as that quantity of enzyme which gives rise to an absorbance of 0.010 in 1 min at a wavelength of 520 nanometers (nm).

A. Incubation Buffer:

The incubation buffer contained 0.495 gm Na_2EDTA , 12 gm KH_2PO_4 and 3.676 gm Na_2HPO_4 (7 H_2O) per litre, pH 6.0. The EDTA/phosphate buffer (0.10 M phosphate) was stable indefinitely at room temp. Prior to use, 32.4 mg cysteine was added to each 100 ml of buffer.

B. Coupling Reagent:

a) Chloromercuribenzoate (CMB) (3.57 gm) was dissolved in 120 ml of 0.5M NaOH , then 18.6 gm of Na_2EDTA was added

and the volume adjusted to 950 ml with water. When the EDTA had dissolved the pH was adjusted to 6.0 by the dropwise addition of HCl and the stock reagent diluted to 1000 ml with water and stored at room temp.

b) Fast garnet GBC (0.5 mg/ml) was dissolved in in 4% (w/v) Brij 35 and the solution was made fresh daily.

The coupling reagent was prepared by mixing equal volumes of reagents (a) and (b) just prior to use and the reagent was used within 30 min.

6. Cathepsin D assay

Cathepsin D activity was determined by measuring the amino groups liberated during proteolysis by the method developed by Fields (1971) with modifications of Nagainis and Warner (1978). The assay contained the following components.

Stock-substrate solution:

Dimethylated bovine serum albumin (Me_2 BSA) was prepared according to the method of Lin et al. (1969) from BSA fraction V (Worthington Biochemicals, Montreal), then dissolved in 0.4 M sodium acetate, pH 4.5 at a concentration of 16 mg/ml. All reaction vessels contained 4 mg/ml Me_2 BSA 0.1 M sodium acetate, pH 4.5, 0.1mM EDTA and 0.5 mM DTT and enzyme in a final volume of 0.4 ml. The reaction was started by the addition of the substrate/buffer and placing

the reaction vessels in a 37° C water bath. At various times aliquots (0.05 ml) were removed for the determination of amino groups as described below.

To 0.05 ml of the sample to be assayed for amino groups was added 0.95 ml of a solution containing 0.053 M $\text{Na}_2\text{B}_4\text{O}_7$ and 0.053 M NaOH (placed in an ice bath). Next, 0.025 ml of 0.22 M 2,4,6-trinitrobenzene sulfonic acid (TNBS) was added and the tubes were allowed to stand at room temperature for 15 min. Color development was stopped by the addition of 2 ml of a solution containing 0.1M NaH_2PO_4 and 1.5 mM Na_2SO_3 and the intensity of the color in each sample was determined immediately at 420 nm in a Beckmann Spectrophotometer (model 25).

III. Results

A. Cathepsin B and D activity in muscle tissue of normal and dystrophic mice.

Studies on the proteolytic activities of extracts from leg muscle of normal and dystrophic mice were undertaken to study the potential role of these enzymes in diseased muscle. The muscle tissue used was from a) a pool of 24 normal and, b) a pool of 24 dystrophic (litter mates) mice of average age 60 ± 1 days.

Fig.1 shows the kinetics of cathepsin B and D activity in extracts from normal and dystrophic mouse muscle. In addition to the finding that dystrophic tissue contains more measurable cathepsin B and D than normal muscle (see Table 1), it should be noted that the activity of cathepsin D is biphasic in dystrophic muscle extracts, whereas this was not observed with normal muscle. In the cathepsin D assay, the extract from normal muscle gave a linear curve with a specific activity of 0.061 units at the end of 8 h, whereas the extract from the dystrophic muscle gave an initial rate (at 2 h,) of 0.233 units which subsequently decreased to a rate of 0.071 units. The reason for the biphasic curve in Fig.1 (bottom panel) is not known but it may be due to release of an inhibitor of cathepsin D early in the reaction which alters the subsequent rate of dimethylated albumin hydrolysis in the assay mixture.

Fig. 1. Cathepsin B and D activity in skeletal muscle tissue.

Top panel: Cathepsin B activity. The assay was done as described in the Methods at 40°C in presence of benzoyl-arginine-2-naphthylamide as substrate. Normal, x—x (0.53 mg/ml); Dystrophic o—o (0.563 mg/ml).

Bottom panel: Cathepsin D activity. The assay was done as described in the Methods at 37°C in presence of dimethylated bovine serum albumin as substrate. Normal, x—x (0.394 mg/ml); Dystrophic, o—o (0.440 mg/ml).

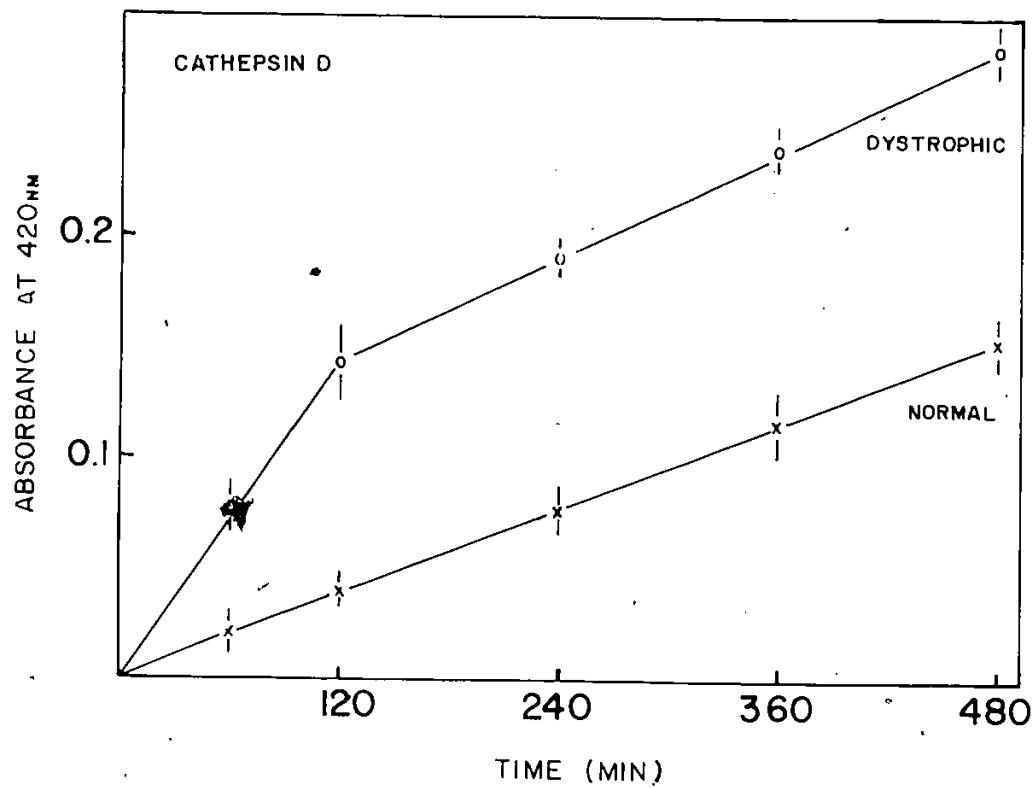
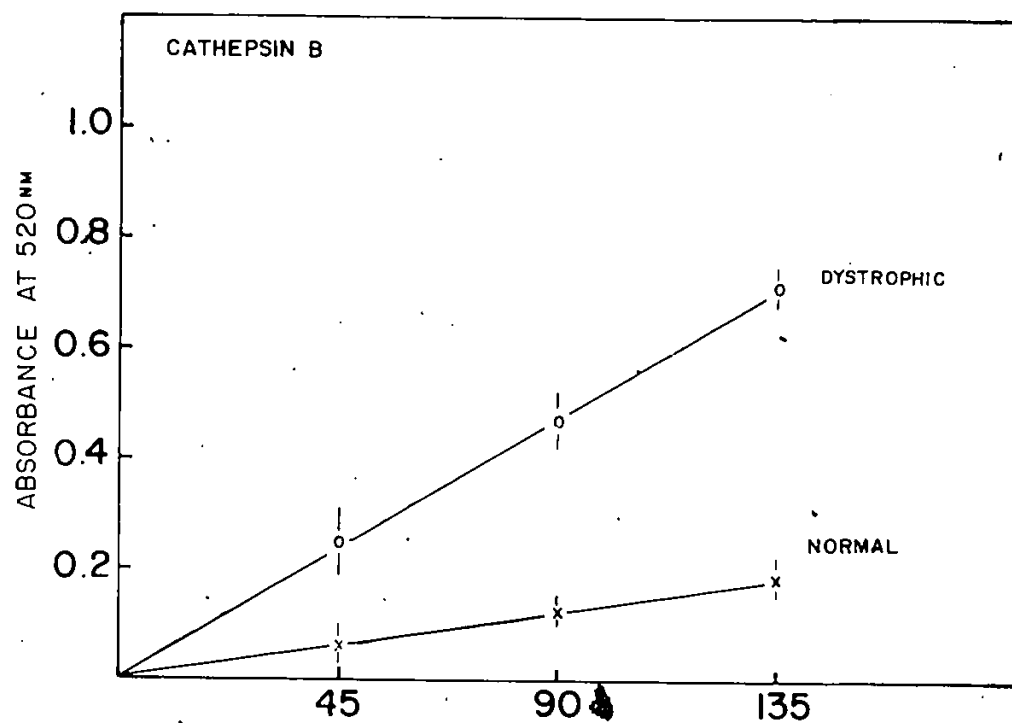


Table 1: Cathepsin B and D activities in normal and dystrophic mouse tissues along with the body weight and weight of individual tissues.

Tissue	No. of expts. conducted on each tissue	Wt. of body (gm)	Wt. of tissue per animal (gm)	Total soluble protein in tissue (mg)	Cathepsin B activity ^b		Cathepsin D activity ^c	
					Specific activity	Total enzyme units	Specific activity	Total enzyme units
Normal muscle Dystrophic muscle	8	26.1 ± 1.7	2.05 ± 0.10	26.8 ± 1.8	0.025 ± 0.002	0.67 ± 0.053	0.06 ± 0.007	1.63 ± 0.187
		13.9 ± 0.78	1.05 ± 0.40	15.5 ± 0.7	0.148 ± 0.009	2.29 ± 0.139	0.23 ± 0.004	3.56 ± 0.062
Normal liver Dystrophic liver	3	26.1 ± 1.7	1.50 ± 0.05	101.1 ± 21	1.33 ± 0.090	134.40 ± 9.090	0.84 ± 0.030	84.92 ± 3.030
		13.9 ± 0.78	1.30 ± 0.03	76.8 ± 12	1.67 ± 0.080	128.30 ± 6.140	1.15 ± 0.270	88.35 ± 20.700
Normal heart Dystrophic heart	2	26.1 ± 1.7	0.17 ± 0.01	5.3	0.04	0.221	0.23	1.21
		13.9 ± 0.78	0.13 ± 0.02	4.4	0.076	0.333	0.23	1.00
Normal kidney Dystrophic kidney	1	26.1 ± 1.7	0.30 ± 0.01	7.7	12.81	99.150	1.20	9.28
		13.9 ± 0.78	0.33 ± 0.03	9.6	12.22	116.950	1.27	12.15
Normal lung Dystrophic lung	1	26.1 ± 1.7	0.30 ± 0.01	3.65	0.265	0.967	0.545	1.98
		13.9 ± 0.78	0.20 ± 0.01	4.02	0.350	1.407	0.706	2.83

a) The tissues were from a pool of 24 animals of each type (normal and dystrophic) at 60 ± 1 days of age.

b) Specific activity of cathepsin B is defined as nanthalamine liberated in μ moles/h/mg protein.

c) Specific activity of cathepsin D is defined as enzyme units /mg protein.

Notwithstanding the difficulty of quantitating cathepsin D activity in the total soluble fraction due to its kinetic profile, the specific activity of cathepsin D in extracts from dystrophic muscle is 3.8 units (Table 1). Thus there is 2.2 times more cathepsin D activity in extracts from dystrophic muscle compared to extracts from control muscle. The reason for increased cathepsin B and D activity in dystrophic muscle is not known but it may relate to the level of endogenous inhibitors of cathepsin B and/or D in the muscle as reported by several workers (Schwartz and Bird, 1977; Kar and Pearson, 1976). Thus an imbalance in the enzyme to inhibitor ratio could be of importance in the onset of dystrophy.

Cathepsin B activity was studied with BANA as substrate at pH 6.0. The data in Fig.1 (top panel) illustrates the activity of cathepsin B in extracts from normal and dystrophic mouse muscle which, in contrast to cathepsin D, is linear with time. In these experiments extracts from the dystrophic muscle had a specific activity of 0.148 units, whereas the extracts from normal muscle had a value of 0.025 units. Thus there is a 6-fold increase in the specific activity of cathepsin B in the dystrophic muscle and nearly 3.5-fold more total activity compared to extracts from normal muscle (see Table 1).

To ascertain whether or not cathepsin B activity

is regulated by endogenous inhibitors in normal and dystrophic muscle, activation experiments were carried out as described by Schwartz and Bird (1977).

Control extracts maintained for up to 8 h, at 0°C showed only a slight increase in cathepsin B

activity, whereas the extracts incubated at 30°C and

pH 4.5 showed marked increases in activity in

both normal and dystrophic samples. These results

are shown in Fig. 2. It is noteworthy that the activity

of cathepsin B in dystrophic samples increased

nearly 1.8-fold while in extracts from normal

muscle, the extent of cathepsin B activation was

almost 2.5 fold after 2 h incubation. Of primary

importance is the observation that while the non-activated

extract from dystrophic muscle contains 2.63-fold more

cathepsin B activity than similar extracts from normal

muscle, the total cathepsin B activity in normal and

dystrophic extracts after 2 h activation is very

similar (2.41 units in normal and 2.79 units in dystrophic

extract). These observations suggest that extracts from normal

muscle contain more endogenous inhibitor(s) of cathepsin B

activity than extracts from dystrophic muscle. Alternatively,

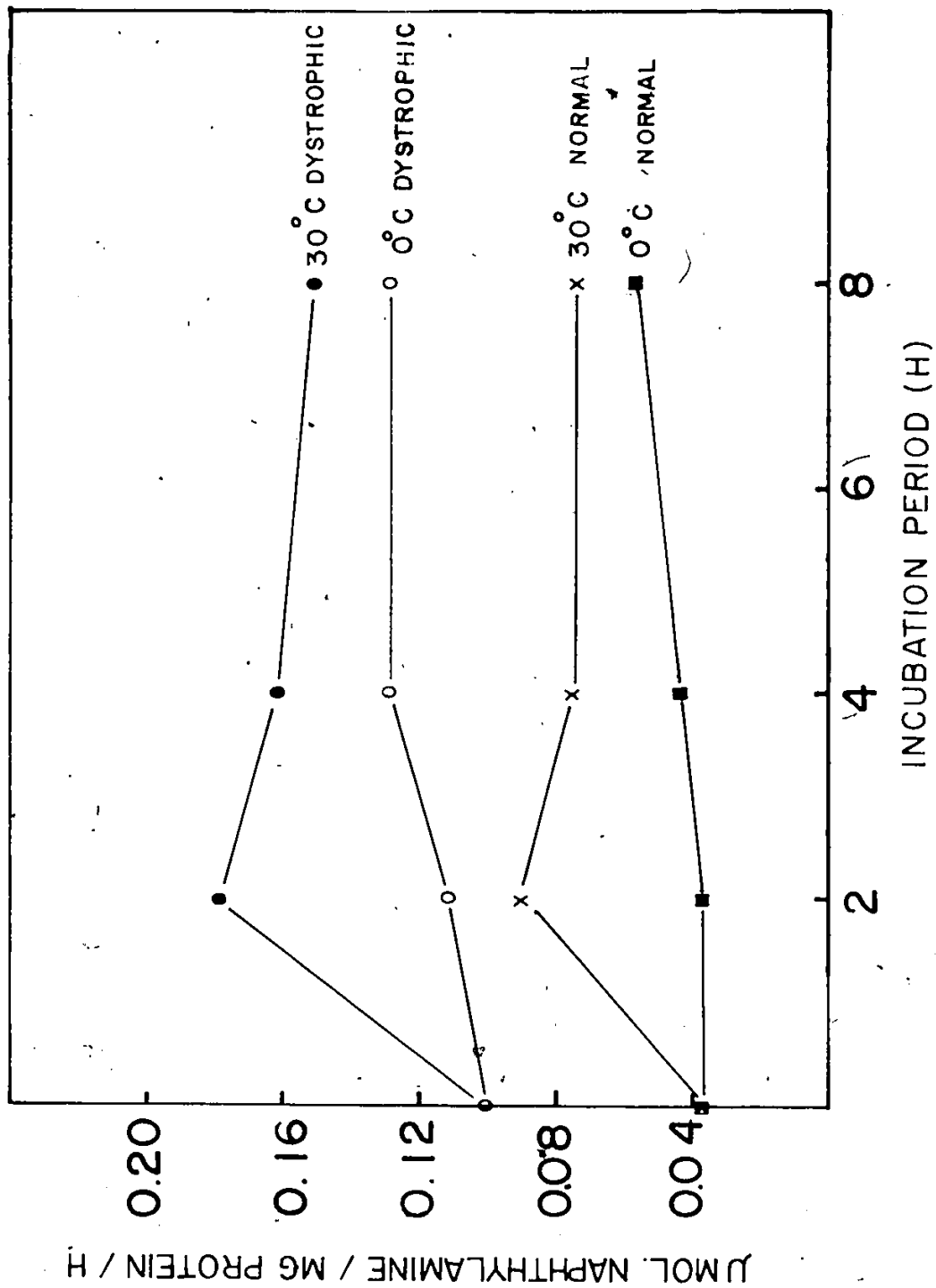
one may conclude that cathepsin B-like enzymes

in dystrophic muscle are different from those

in normal muscle and hence respond differently to

endogenous regulator molecules. To elucidate

Fig. 2. Cathepsin B activity after activation of skeletal muscle extract. The extracts were adjusted to pH 4.5 with 0.2 M sodium formate, pH 2.5, and incubated at 30°C in water a bath. The control samples were maintained at 0°C. Normal at 0°C, ■——■; Normal at 30°C, x——x; Dystrophic at 0°C, o——o; Dystrophic at 30°C, ●——●.



further this view, the effect of exogenous inhibitors on cathepsin B activity in normal and dystrophic extracts was studied. The data in Fig. 3 show the activity of cathepsin B in soluble extracts of normal and dystrophic muscle at three concentrations of leupeptin and Artemia protease inhibitor. Using extracts from normal hind leg muscle, these two protease inhibitors were found to be equally effective as inhibitors of cathepsin B activity (65-70% inhibition) when added to crude extracts. In contrast, extracts from dystrophic hind leg muscle responded differently to these two protease inhibitors. Eighty percent inhibition of cathepsin B occurred in the presence of 0.1 $\mu\text{g/ml}$ leupeptin whereas the Artemia protease inhibitor at 0.1 $\mu\text{g/ml}$ produced only 28% inhibition. When leupeptin and Artemia protease inhibitor were tested at 0.5 $\mu\text{g/ml}$ 79% and 60% inhibition, respectively, occurred. From the response of these tissue extracts to leupeptin and Artemia protease inhibitor it appears that the cathepsin B-like enzyme(s) in extracts of dystrophic muscle are not the same as those in extracts of normal muscle.

B. Cathepsin B and D activity in liver tissue from normal and dystrophic mice.

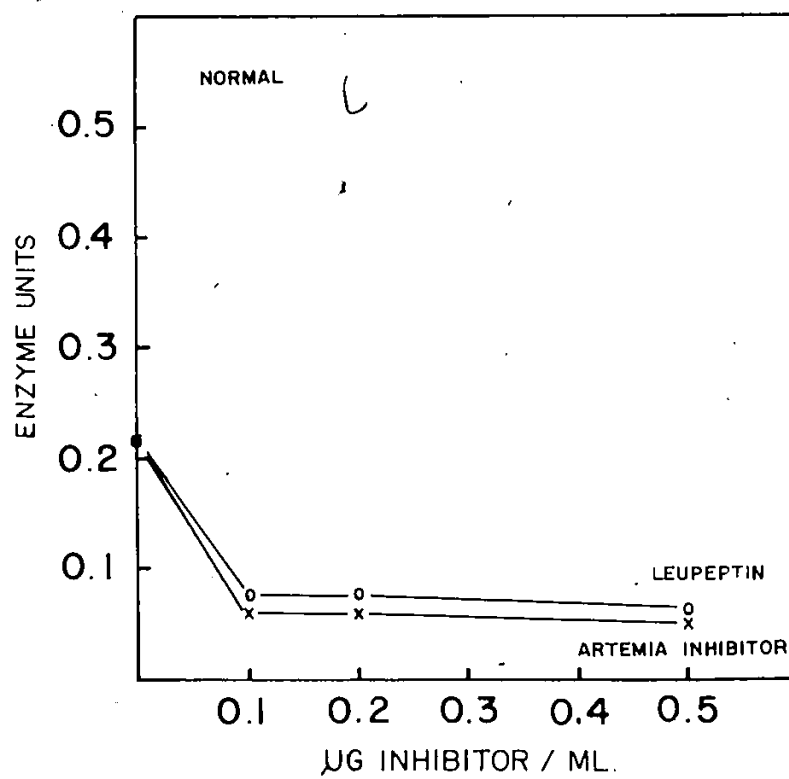
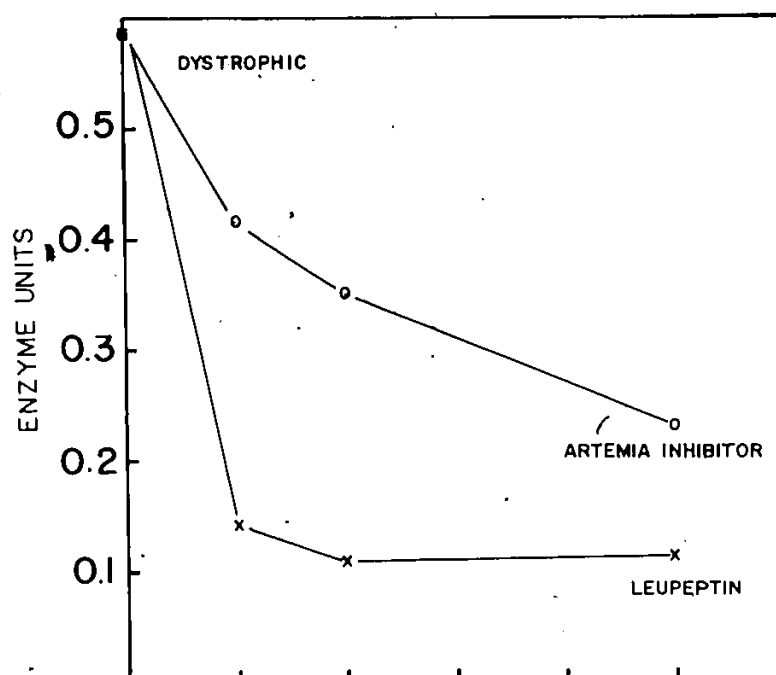
Proteolytic activity of cathepsins B and D

Fig. 3. Cathepsin B activity in skeletal muscle extract in presence of leupeptin and Artemia protease inhibitor. The assay for cathepsin B was as described in the Methods. The inhibitor stock solutions (leupeptin and protease inhibitor from Artemia sp.) were diluted so that the final concentration of the inhibitor in the reaction was 0.1, 0.2 and 0.5 $\mu\text{g/ml}$.

Top panel: Cathepsin B activity in Dystrophic muscle extract in presence of leupeptin and Artemia protease inhibitor.

Dyst + Leupeptin, x—x; Dyst + Artemia protease inhibitor, o—o.

Bottom panel: Cathepsin B activity in normal muscle extract in presence of leupeptin and Artemia protease inhibitor. Normal + leupeptin o—o; Normal + Artemia protease inhibitor, x—x



in soluble fractions of liver was studied to determine the effect of dystrophy on the liver protease activity. The results presented in Table 1 and Fig. 4 show that liver cathepsins B and D activity levels are affected only slightly by dystrophy. While the specific activities of cathepsins B and D are elevated slightly (25-30%) in dystrophic liver, the total activity of these enzymes compares favourably in both livers. Of interest, however, is the fact that the kinetics of cathepsin D are strikingly similar to that observed using extracts from normal and dystrophic leg muscle. In the case of cathepsin B, the kinetics are similar in both normal and dystrophic extracts (Fig. 4, bottom panel)

Attempts to activate enzymatically cathepsin B in extracts from normal liver by autoactivation at pH 4.5 and 30° C were without effect. However, extracts from dystrophic liver showed slight increases in cathepsin B activity following autoactivation. These results are shown in Fig. 5. It should be noted, however, that after 2 h at pH 4.5 and 30° C the cathepsin B activity in extracts from normal liver falls below the control level (0° C), whereas this is not the case using extracts from the dystrophic liver. Either cathepsin B is more resistant to enzymatic inactivation in dystrophic tissue or dystrophic tissue contains more

Fig. 4. Cathepsin B and D activity in mouse liver extracts.

Top panel: Cathepsin D activity. The assay was done as described in the Methods.

Normal, x—x (0.191 mg/ml); Dystrophic, o—o (0.25 mg/ml).

Bottom panel: Cathepsin B activity. The assay was done as described in the Methods.

Normal, x—x (0.373 mg/ml); Dystrophic, o—o (0.337 mg/ml).

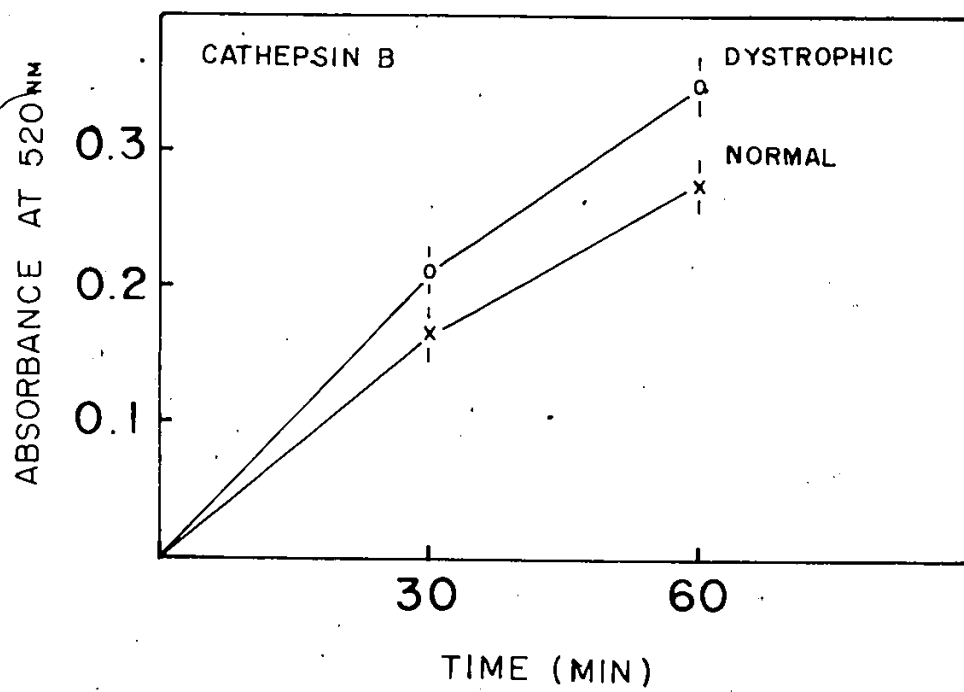
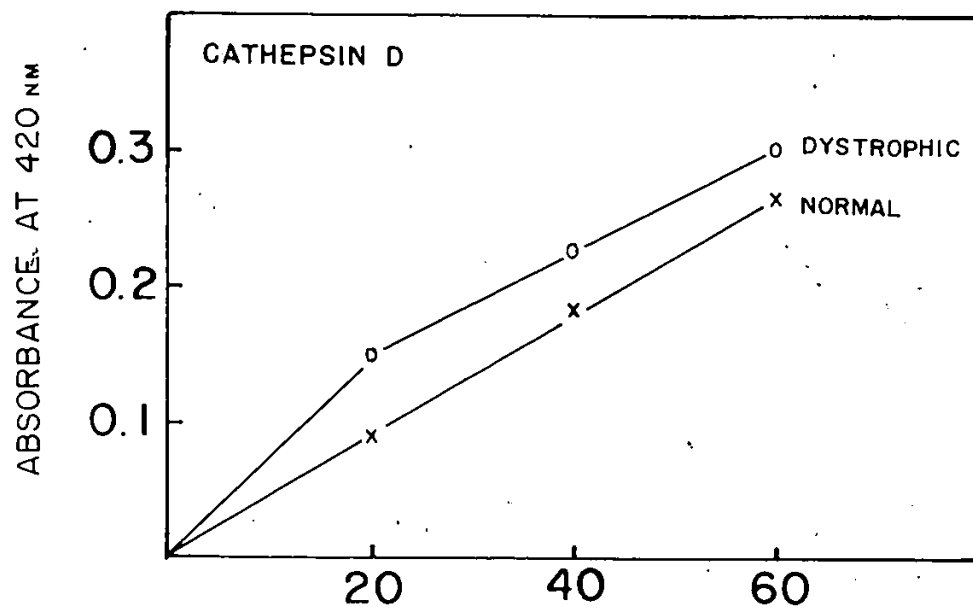
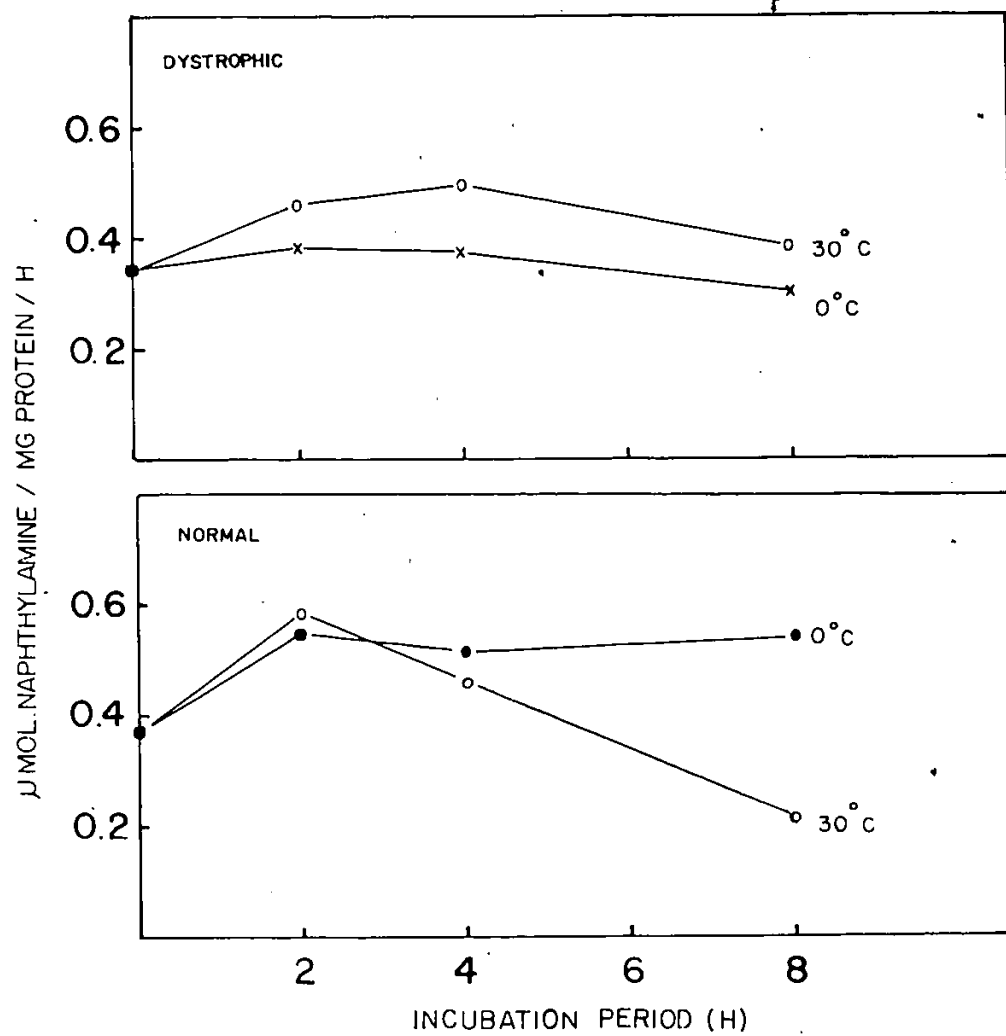


Fig. 5. Cathepsin B activity after activation of liver extracts. The "activation" procedure was done as in the muscles.

Top panel: Dystrophic liver extract at 0° and 30°C.
Dystrophic at 0° C, x—x; Dystrophic at 30°C,
o—o.

Bottom panel: Normal liver extract at 0° and 30°C.
Normal at 0° C, ●—●; Normal at 30° C, o—o.



cathepsin D inhibitor. Thus far these studies support the former interpretation. It should also be noted that the liver enzyme was very labile and much activity was lost on freezing and thawing. For this reason all assays were done with freshly prepared extracts.

C. Cathepsin B and D activity in cardiac tissue of normal and dystrophic mice.

The heart in normal and dystrophic 60 day old animals weighed about the same within the range of 0.13 to 0.17 gm although the soluble protein content was higher in dystrophic hearts compared to the normal hearts (Table 1). The results in Fig. 6 (bottom panel) show that cathepsin D from either normal or dystrophic hearts displays a biphasic curve. The initial rate (up to 30 min) is higher in both cases with 0.25 enzyme units and 0.366 enzyme units for the normal and dystrophic extracts, respectively. Subsequently (after 30 min) the rate of cathepsin D activity declines considerably in both cases. Whether this decline in activity is due to the release of inhibitory substance(s) after 30 min is not known. However, it is interesting to note that the enzyme kinetics in both cases is very similar to that observed in extracts from dystrophic skeletal muscle. Also, the total amount of enzyme units in extracts from dystrophic hearts are

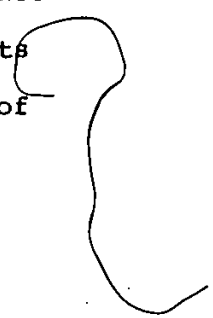
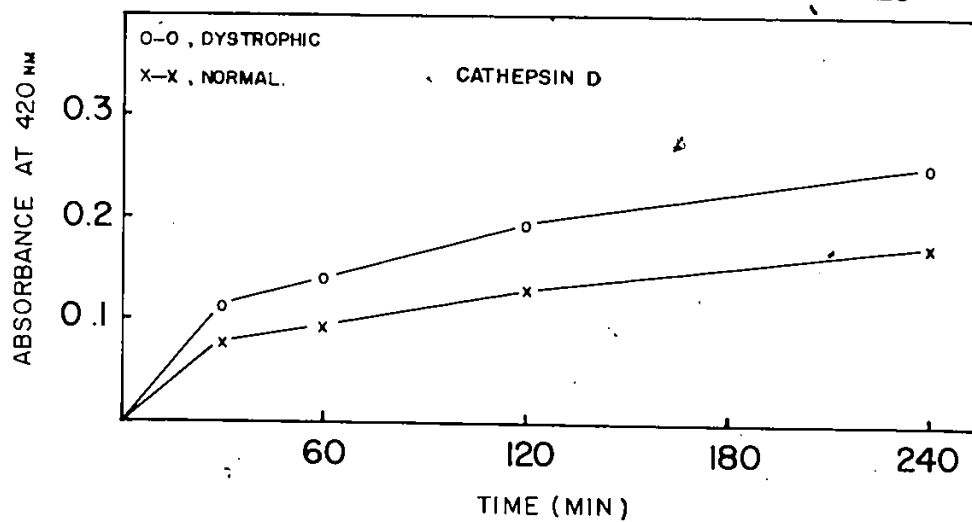
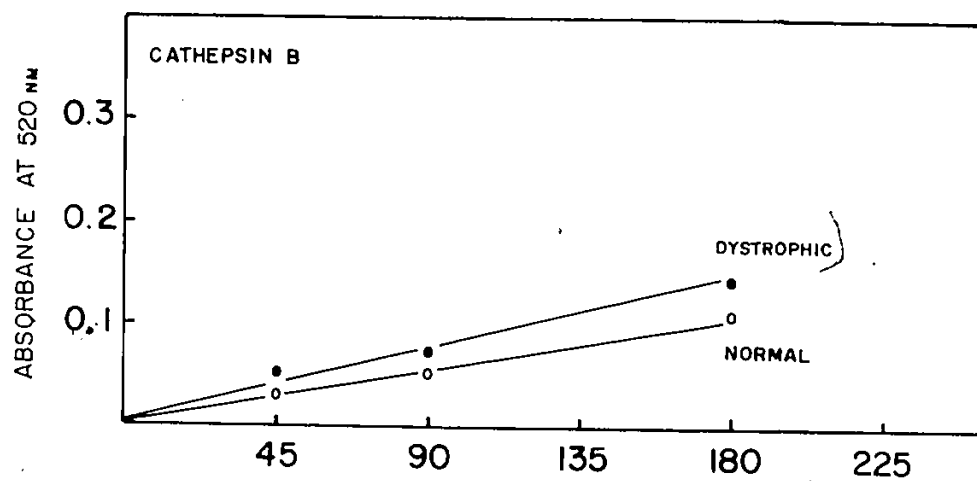


Fig. 6. Cathepsin B and D activity in cardiac muscle extract.

Top panel: Cathepsin B activity. The assay was done as described in methods. Normal, o—o (0.635 mg/ml); Dystrophic, ●—● (0.660 mg/ml).

Bottom panel: Cathepsin D activity. The assay was done as described in the Methods.

Normal, x—x (0.765 mg/ml); Dystrophic, o—o (0.765 mg/ml).



not significantly different from those in the normal extracts (Table 1).

In respect to cathepsin B activity, we observed this enzyme to be 1.8-fold higher in extracts from dystrophic hearts compared to extracts from normal hearts. Also the kinetics of cathepsin B activity are linear in both normal and dystrophic hearts but the total activity in dystrophic hearts is about 1.5-fold higher than in extracts from normal hearts (0.221 units). See Fig. 6 (top panel).

The autoactivation experiments done at pH 4.5 and 30° C show that the cathepsin B activity in extracts from normal cardiac muscle increases 1.5-fold after 2 hours of incubation and remains stable for at least 6 additional hours (Fig. 7). In non-activated extracts from dystrophic tissue, cathepsin B activity is about 2-fold higher than the non-activated normal heart extract. On incubation at 30° C for 2 h, the cathepsin B activity in dystrophic heart extracts increases 1.6-fold. But after 2 h the activity declines which is observed even in the control sample maintained at 0° C. Given that the activity at 8 hours is much higher than at 4 hours, it is difficult to conclude whether this decline in activity at 4 h incubation is real or an artifact, but each tissue (normal and dystrophic) shows about 1.4 to 1.6-fold increase in activity after 2 h incubation.


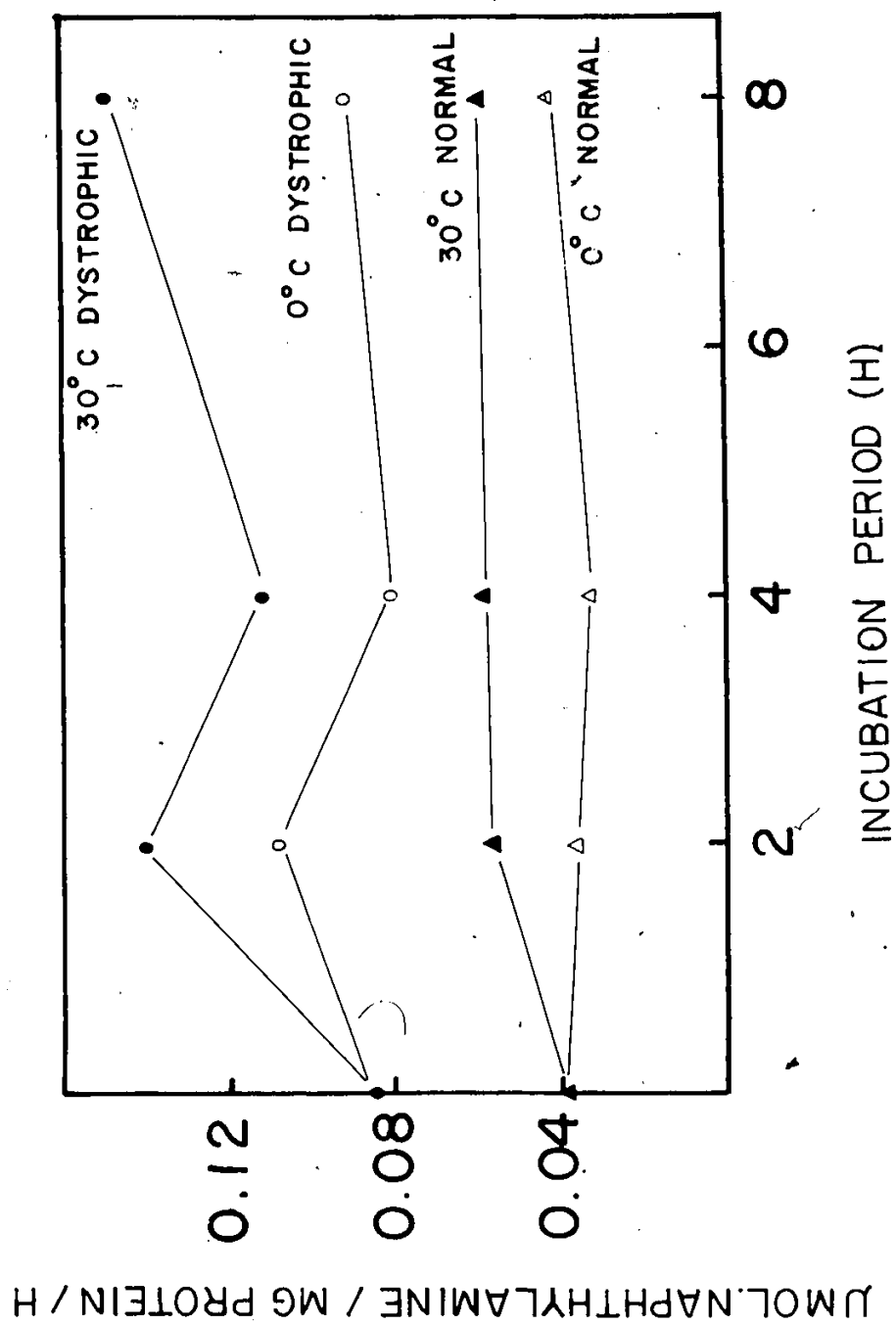


Fig 7. Cathepsin B activity after activation of cardiac muscle extract. The "activation" was done as described earlier. Normal at 0°C, Δ — Δ ; Normal at 30°C, \blacktriangle — \blacktriangle ; Dystrophic at 0°C, o—o; Dystrophic at 30°C, \bullet — \bullet .



Since the activity of the non-activated dystrophic extract is 2-fold higher than in the non-activated extract from normal heart, it is possible that the increase in activity is due to lesser amounts of inhibitor in the extract from dystrophic hearts.

D. Cathepsin B and D activity in lung tissue of normal and dystrophic mice.

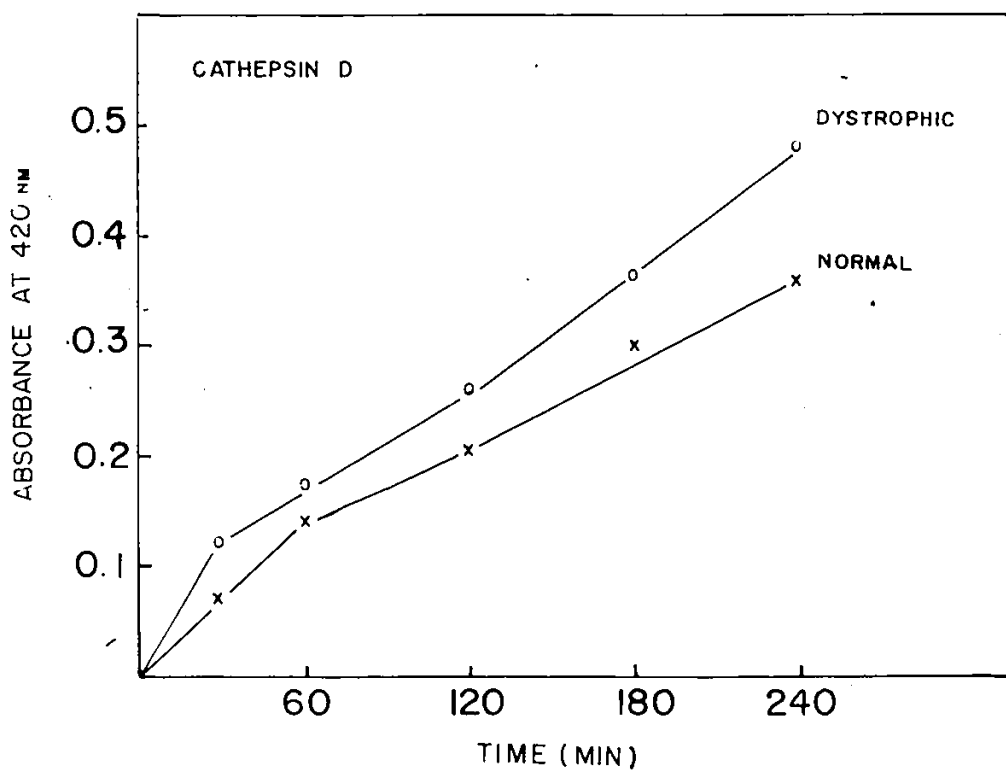
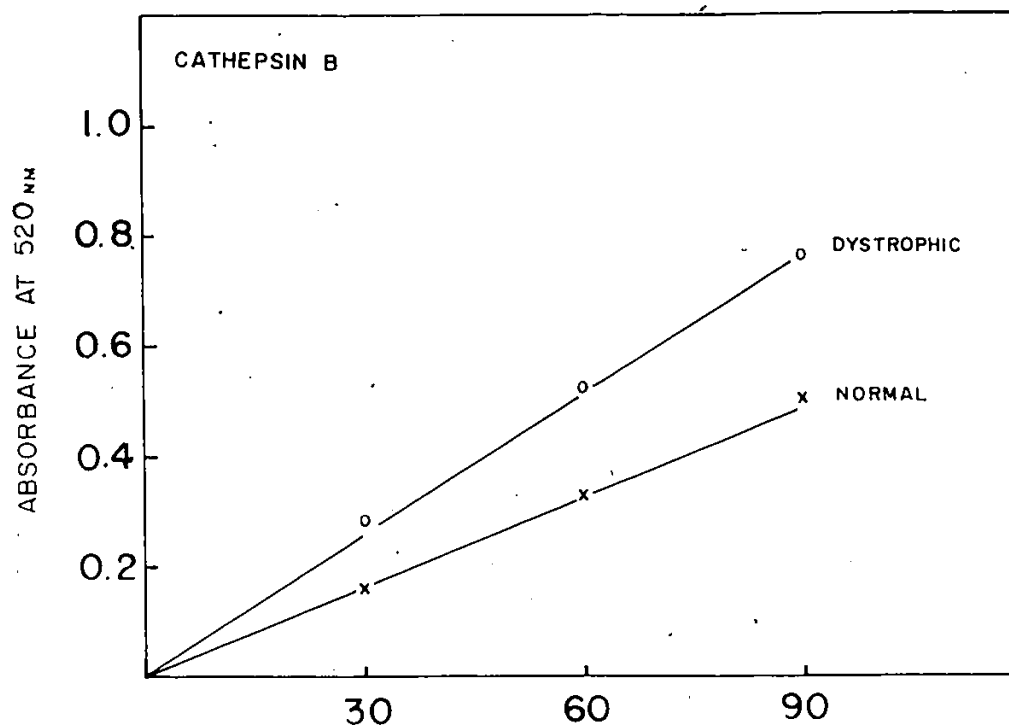
Cathepsin B and D activity in lung tissue was studied to observe whether or not the above proteases in the lung are affected in dystrophy. The assay for cathepsin D activity showed that the kinetics of protein hydrolysis by the enzyme is similar to that found in the skeletal muscles and heart (see Fig. 8, bottom panel). The specific activity of cathepsin D in crude extracts of normal lung was 0.545 units over a period of 4 h and 0.706 units in similar extracts from dystrophic lung. Moreover, the total cathepsin D activity in the dystrophic lung is 1.4-fold higher than normal lung (Table 1).

The kinetics of cathepsin B activity in extracts from lung is shown in Fig. 8 (top panel). Here too, the total activity of cathepsin B is 1.45-fold higher in dystrophic lung than in normal lung (Table 1).

Fig. 8. Cathepsin B and D activity in lung tissue.

Top panel: Cathepsin B activity. Normal, x—x (0.228 mg/ml); Dystrophic, o—o (0.241 mg/ml).

Bottom panel: Cathepsin D activity. Normal, x—x (0.365 mg/ml); Dystrophic, o—o (0.386 mg/ml).



E. Cathepsin B and D activity in kidney tissue of normal and dystrophic mice.

The kidneys seem to be affected least by the dystrophic condition. Kidneys from 60 day old normal and dystrophic mice weighed 0.30 to 0.33 gm. The specific activity of cathepsin B and D also appear to be similar in both normal and dystrophic mice with 1.20 units of cathepsin D in normal and 1.27 units in dystrophic. The cathepsin B specific activity is 12.81 units in normal and 12.22 units in dystrophic (Table 1).

Both normal and dystrophic kidneys also showed a slightly elevated response to autoactivation of cathepsin B at pH 4.5 and 30 C (Fig. 9). These results suggest that that this organ might not be affected by dystrophy.

F. Cathepsin B and D activity in the rat myoblast cell line L69/1

Myogenesis is a process in which the avian or mammalian myoblasts of clonal origin in primary culture or myoblasts of a permanent rat line are allowed to grow in in a suitable medium, they multiply rapidly, orient themselves in strings, and fuse to form multinucleated myotubes (Dufresne, 1976). This process of differentiation goes through three specific stages (Fig. 10). At confluence the cells are in active mitotic division and are closely packed but

Fig. 9. Cathepsin B activity after activation of extracts
from kidney.
Normal at 0°C, x—x; Normal at 30°C, ▲—▲
Dystrophic at 0°C, o—o; Dystrophic at 30°C, ●—●.

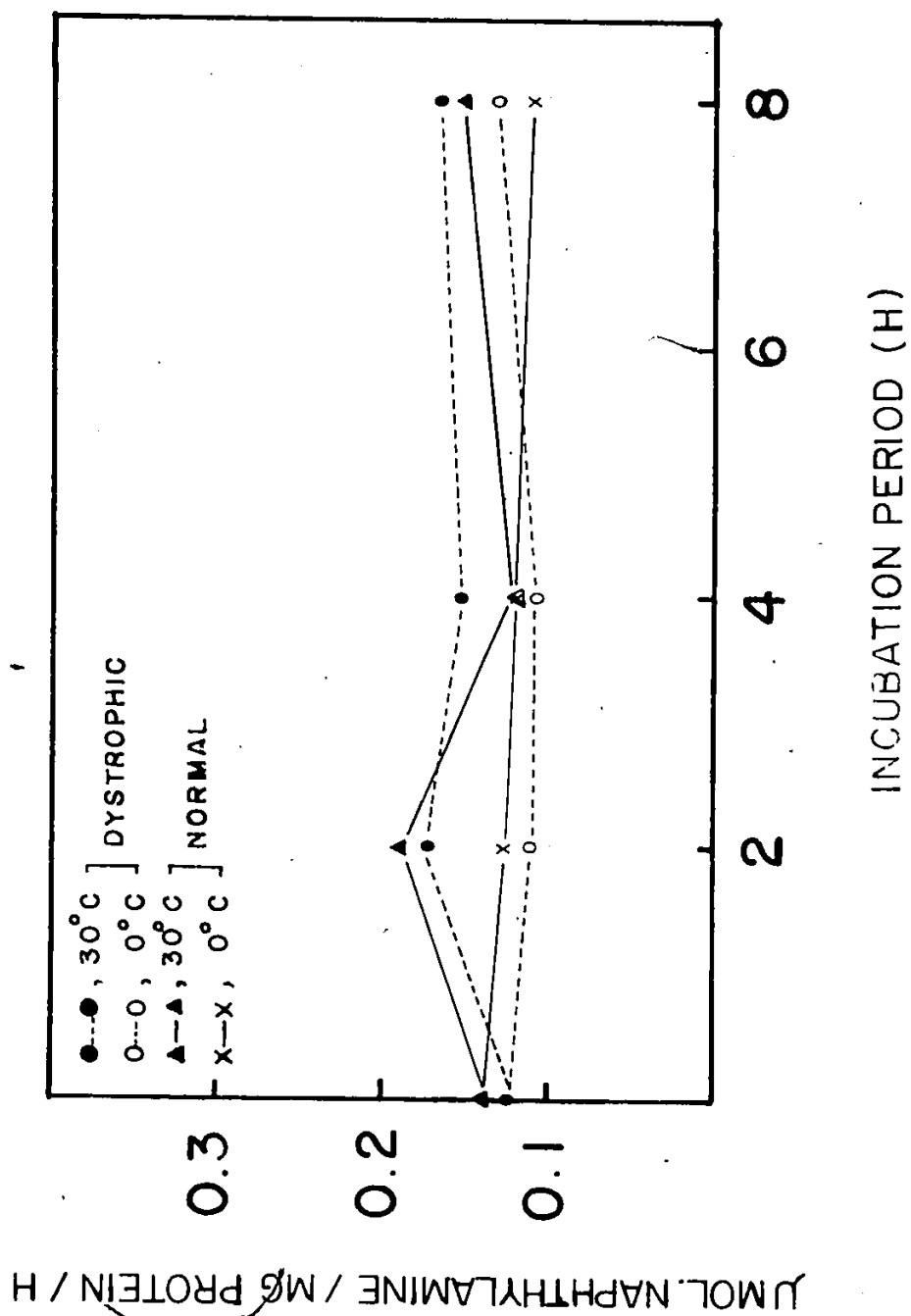
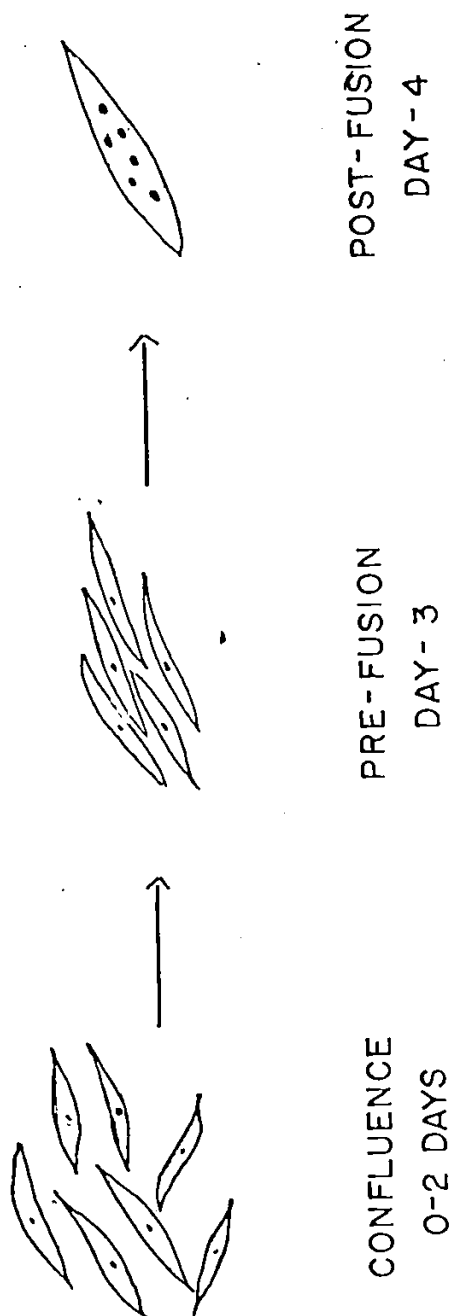


Fig. 10. Stages in myogenesis.



yet not close enough to fuse. At pre-fusion the cells arrange themselves parallel to each other and at post-fusion the the cells fuse to form multinucleated myotubes. Cathepsin B activity in differentiating myoblasts of clone L69/1 was studied to observe the levels of cathepsin B activity with myoblast differentiation and also to know the time of onset of enhanced protease activity in the myoblast cells. Cells from all three stages of differentiation, namely confluence, pre-fusion and post-fusion were studied. The results in Fig. 11 show that cathepsin B activity in homogenates from cultures myoblasts is linear for atleast 3 h incubation at the conditions studied. In addition, the data in Table 2 show that the cathepsin B activity increased markedly during the time that myoblasts are preparing to fuse. At confluence (before fusion) the specific activity was found to be 0.16 units whereas just prior to fusion the specific activity had increased to 0.569 units. Following fusion of the myoblast to give myotubes the specific activity of cathepsin B was determined to be 0.756 units. Thus the post-fusion myoblasts contain about 4.7 times more cathepsin B activity than their precursor cells at the stage of confluence. Similar observations have been made by Bird et al. (1980). The data in Table 2 also show that newly fused myoblasts contain about 50-fold more cathepsin B activity per unit protein than whole muscle (hind leg). In a similar

Fig. 11. Cathepsin B and D activity in rat myoblast cell line L6-9/1 at different stages of differentiation.

Top panel: Cathepsin B activity. The assay was done as described in the Methods.

Confluence, \square — \square (0.1 mg/ml); Pre-fusion, x — x (0.125 mg/ml); Post-fusion, o — o (0.125 mg/ml).

Bottom panel: Cathepsin D activity. The assay was done as described in the Methods. Confluence, \square — \square (0.1 mg/ml); Pre-fusion, x — x (0.125 mg/ml); Post-fusion, o — o (0.125 mg/ml).

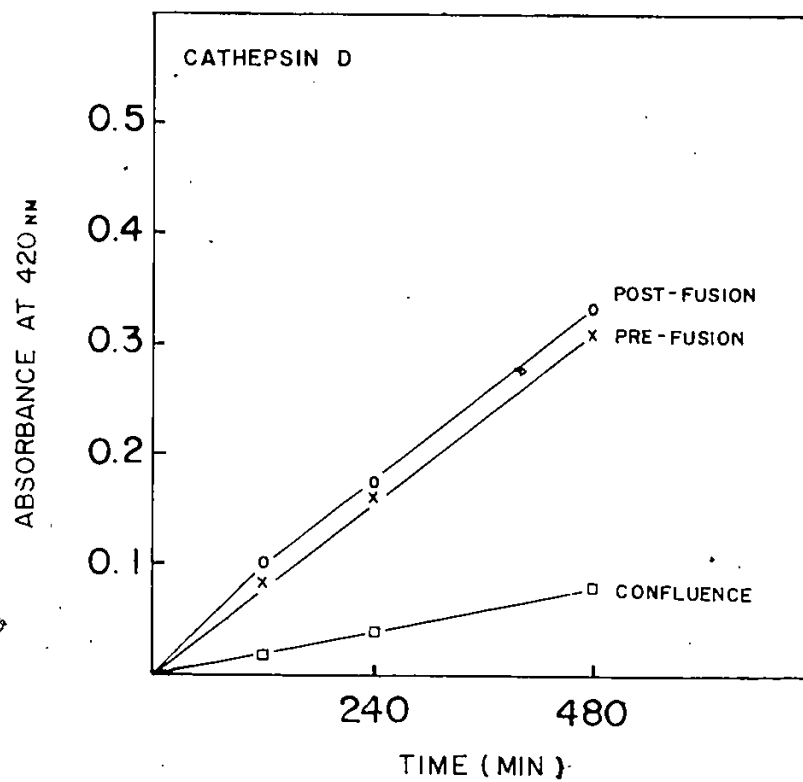
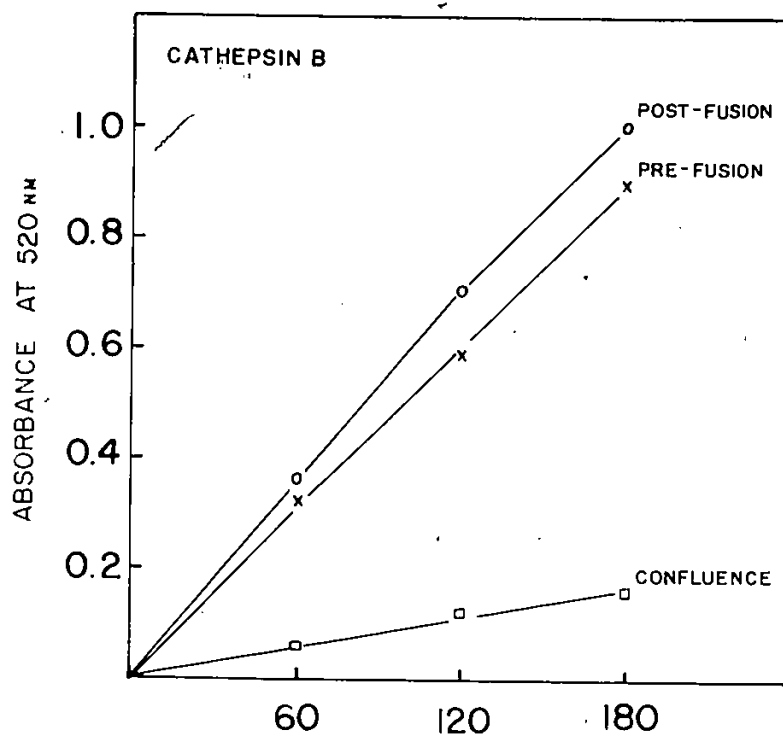


Table 2: Specific activities of Cathepsin B and D in the cell-free extract from whole muscle, differentiating myoblast cells and fibroblasts.

Tissue/cell line	Cathepsin D ^a	Cathepsin B ^b
Rat Myoblast-169/1		
Confluence	0.398	0.160
Pre-fusion	0.358	0.569
Post-fusion	0.403	0.756
Mouse L-Fibroblast (LMTK)	0.246	0.160
Whole leg muscle	0.06 ± 0.007	0.025 ± 0.002

- a) Specific activity of cathepsin D is defined as enzyme units / mg protein. These values were from one experiment except for the leg muscle which represents the results of 8 experiments.
- b) Specific activity of cathepsin B is defined as naphthylamine liberated in $\mu\text{moles / h / mg protein}$. These values were from one experiment except for the leg muscle which represents the results of 8 experiments.

study, Bird et al. (1980) observed a 90-fold increase in cathepsin B activity. Thus it is clear that homogenates of whole muscle cells contain considerably more cathepsin B activity than homogenates of whole muscle.

Attempts to activate-latent cathepsin B activity in homogenates from undifferentiated and differentiated myoblast cells in culture at pH 4.5 and 30° C have not been successful (Fig. 12). These findings suggest that either myoblasts contain a) an excess of protease inhibitor(s), b) insufficient acid protease activity, or (c) no precursor to cathepsin B. Since cathepsin D activity is high in cultured myoblasts compared to whole muscle and relatively constant at the three stages of myoblast development studied (0.36 to 0.40 enzyme units/mg protein) (Fig. 11 and Table 2), it is unlikely that failure to activate cathepsin B is due to a paucity of cathepsin D in myoblast homogenates. These findings may indicate non co-ordinate regulation of cathepsin B and D activity in the developing myoblasts.

G. Cathepsin B and D activity in mouse L-cell fibroblast cell line, LMTK.

Mouse L-cell fibroblasts of clone LMTK, were assayed for cathepsin B and D activity and the results are shown in Fig. 13. The activity of cathepsins B and D from fibroblasts compared to myoblasts is shown in Table 2.

Fig. 12. Cathepsin B activity after activation of the myoblast homogenates at different stages of differentiation. Control samples at 0°C, o—o; Samples at 30°C, x—x.

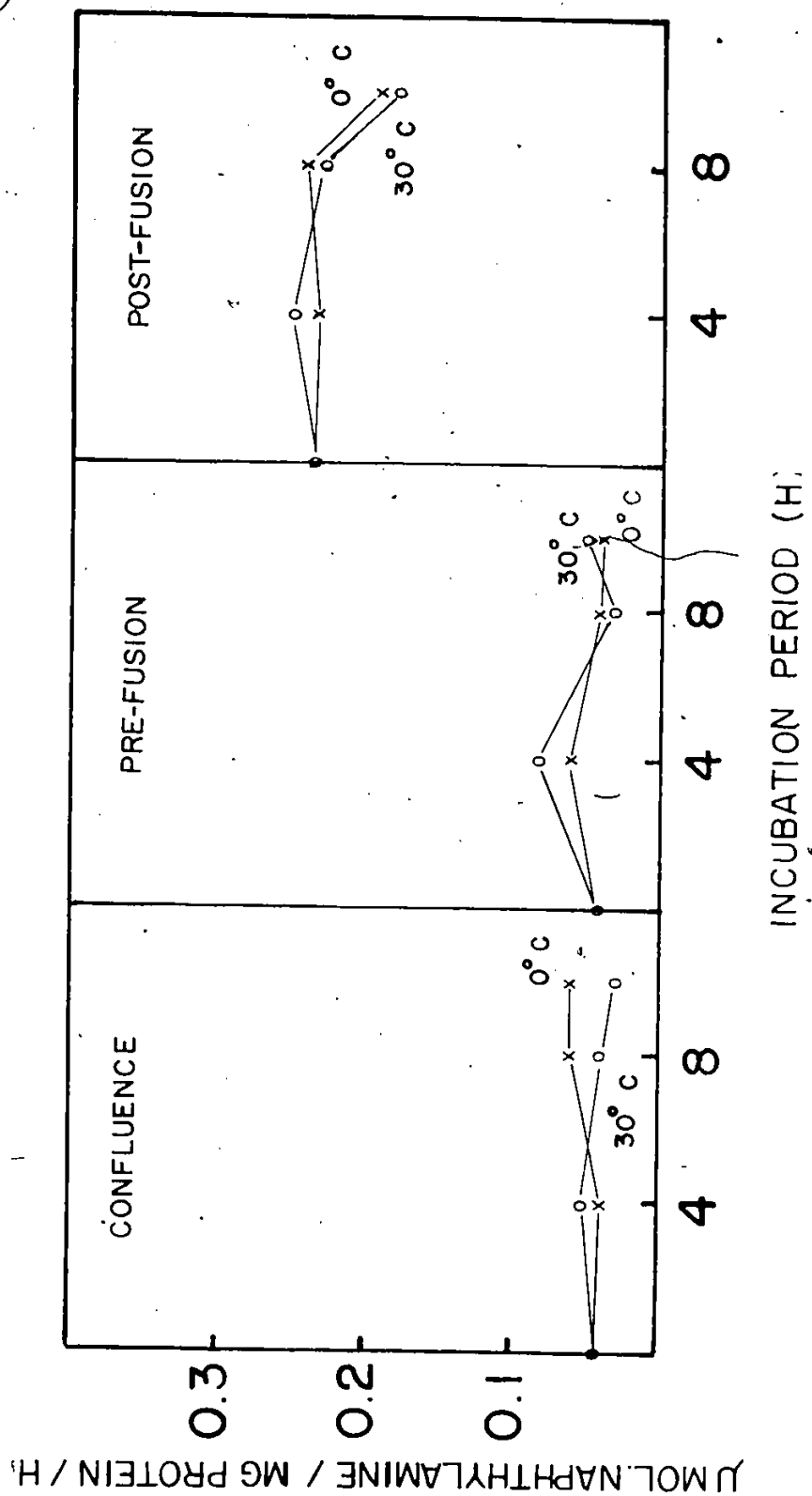


Fig. 13. Cathepsin B and D activity in mouse L-cell fibroblasts, LMTK.

Top panel: Cathepsin B activity.

Bottom panel: Cathepsin D activity.

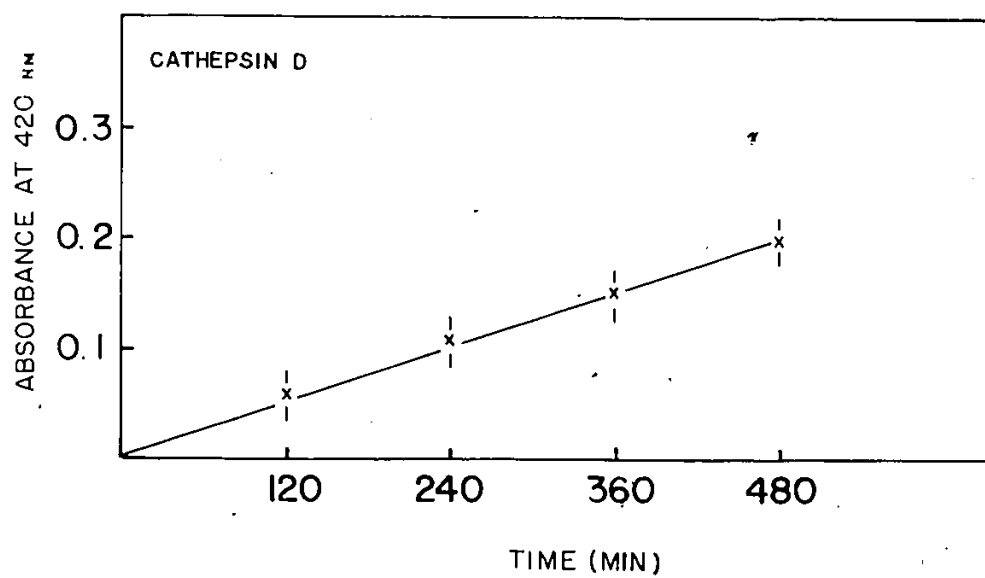
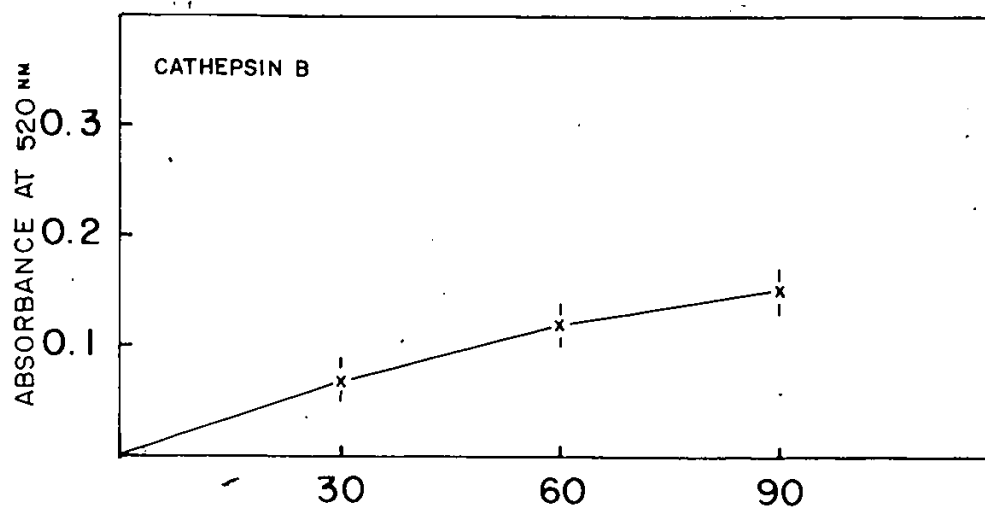
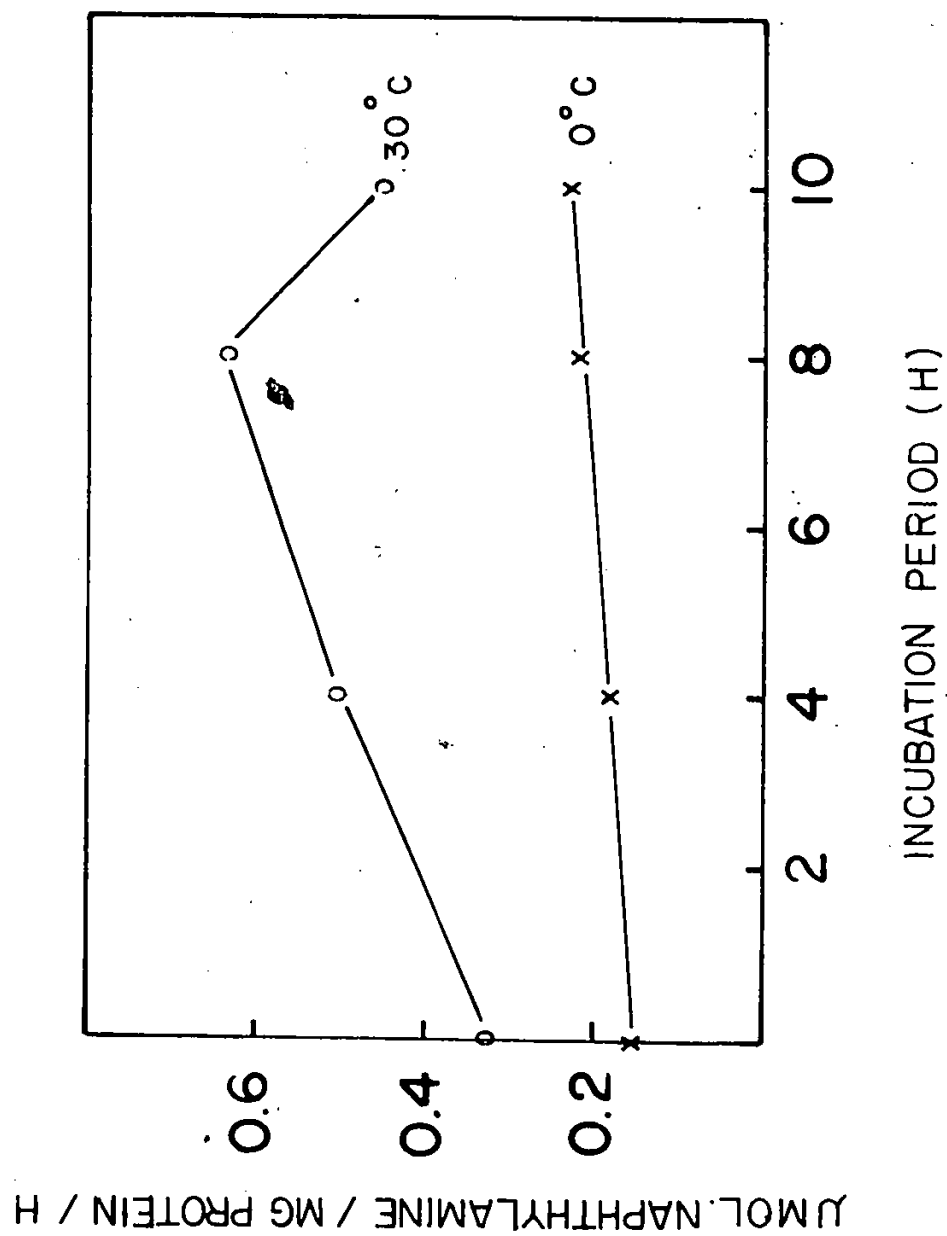


Fig. 14. Cathepsin B activity after activation of the
homogenates from fibroblast cell line, LMTK.
Control sample at 0°C, x—x;
Sample maintained at 30°C, o—o.



Here it is seen that the activity of cathepsins B and D are nearly 5-fold and 2-fold lower, respectively, than in fused myoblasts but 10-fold and 4-fold greater, respectively than cathepsin B and D activity of whole leg muscle.

However, the activity of cathepsin B in fibroblasts was similar to that found in confluent mvoblasts. Yet, and in contrast to myoblasts, cathepsin B activity in fibroblasts can be activated enzymatically as shown in Fig.14 suggesting that fibroblasts may contain a considerable amount of inactive cathepsin B which can be rendered functional by autolysis at pH 4.5.

H. Cathepsin B and D activity in a myoblast x fibroblast hybrid, RM-5.

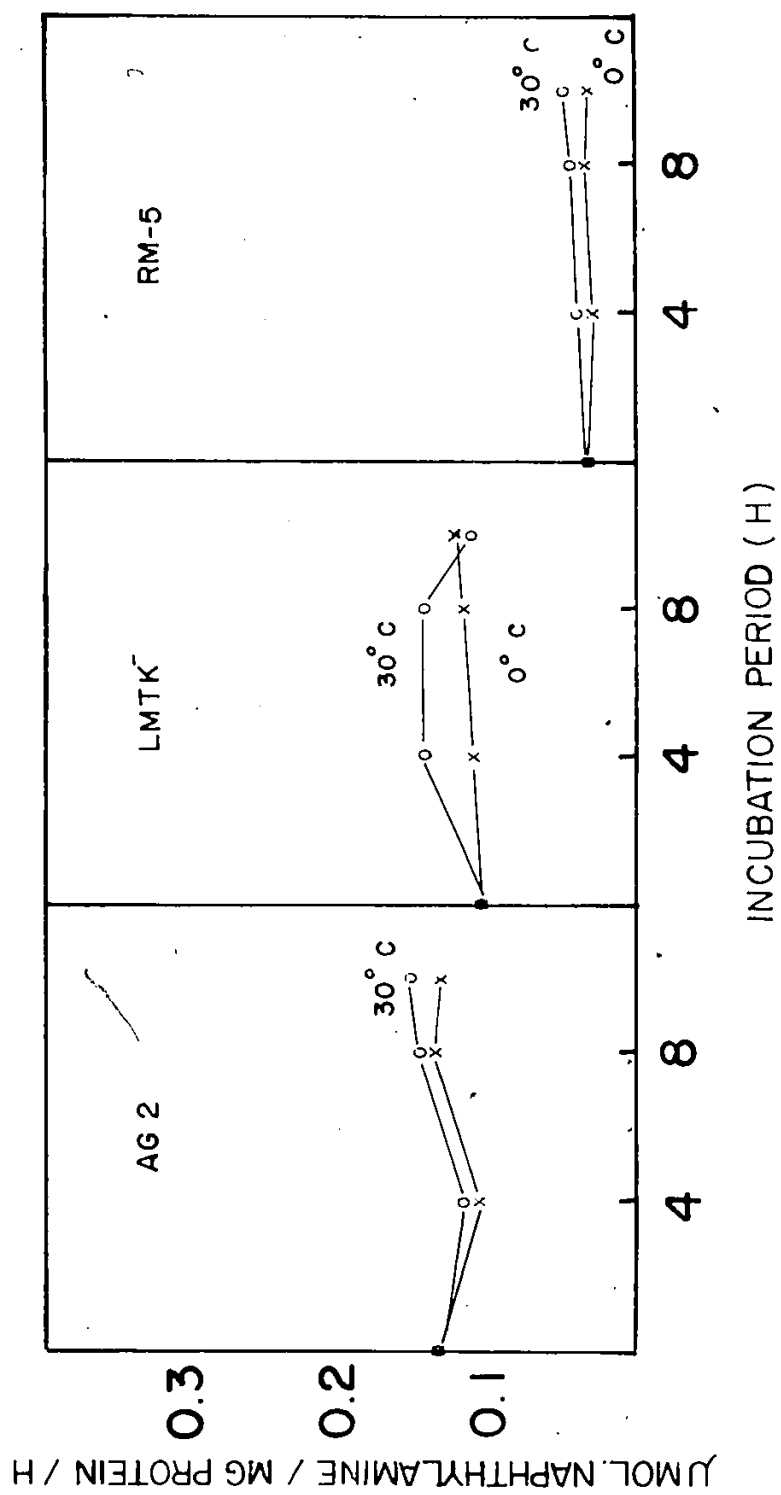
The cell-cell hybrid between rat myoblast variant AG2, and mouse L-cell fibroblast, LMTK, was assayed along with its parental cell lines AG2 and LMTK for cathepsin B and D activity to determine the effect of hybridization on the expression of these protease activities. Since the hybrid has the genetic complement of fibroblast and myoblast, its response to the cathepsin B and D activity would provide incite on the presence of regulator molecules (if any) in either of the parental cell lines. The data in Table 3 show that hybridization results in a marked decrease in the expression of cathepsin B and D activities. The mechanisms underlying this partial extinction of cathepsin B and D activities has not been elucidated but it may be

Table 3: Specific activities of cathepsin B and D in cell lines
AG2, LMTK and RM-5.

Cell line	Cathepsin D (specific activity)	Cathepsin B (specific activity)
AG2 (Myoblast)	0.428	0.127
LMTK (Fibroblast)	0.332	0.101
RM-5 (LMTK + AG2)	0.232	0.030

Fig. 15. Cathepsin B activity after activation of AG2,
LMTK and RM-5 cell lines.

Samples at 0°C, x—x; Samples at 30°C, o—o.



due to the production of cathepsin inhibitor(s) by the AG2 genome which suppresses the activity of these proteases. When we attempted to activate cathepsin B in the hybrid (RM-5) and parental cell lines, RM-5 did not show any activation whereas the fibroblast parent (LMTK) showed some activation (Fig. 15). Failure of homogenates of RM-5 to respond to enzymatic activation of cathepsin B may be due to the presence of a cathepsin inhibitor in the AG2 cell line which is refractory to enzymatic inactivation. However, further work is essential to confirm these observations.

To test for the presence of protease inhibitor(s) in AG2, the RM-5 homogenate was mixed in equal proportions with AG2 homogenate, and another sample of RM-5 was mixed with LMTK. The samples were preincubated at pH 4.5 and 30° C for 1 hour and the "activated" samples assayed for cathepsin D activity. The data in Fig. 15 show that RM-5 combined with AG2 resulted in a low level of activity with 0.059 enzyme units, whereas RM-5 combined with LMTK had 0.074 enzyme units. The latter is closer to the LMTK control of 0.083 enzyme units (Fig. 16, right panel). These findings suggest that AG2 contains at least one protease inhibitor which regulates the activity of cathepsin B and/or D in AG2 and the hybrid, (RM-5).

Fig. 16. Cathepsin D activity in AG2, LMTK, RM-5 and mixture of RM-5 with Ag2 and LMTK.

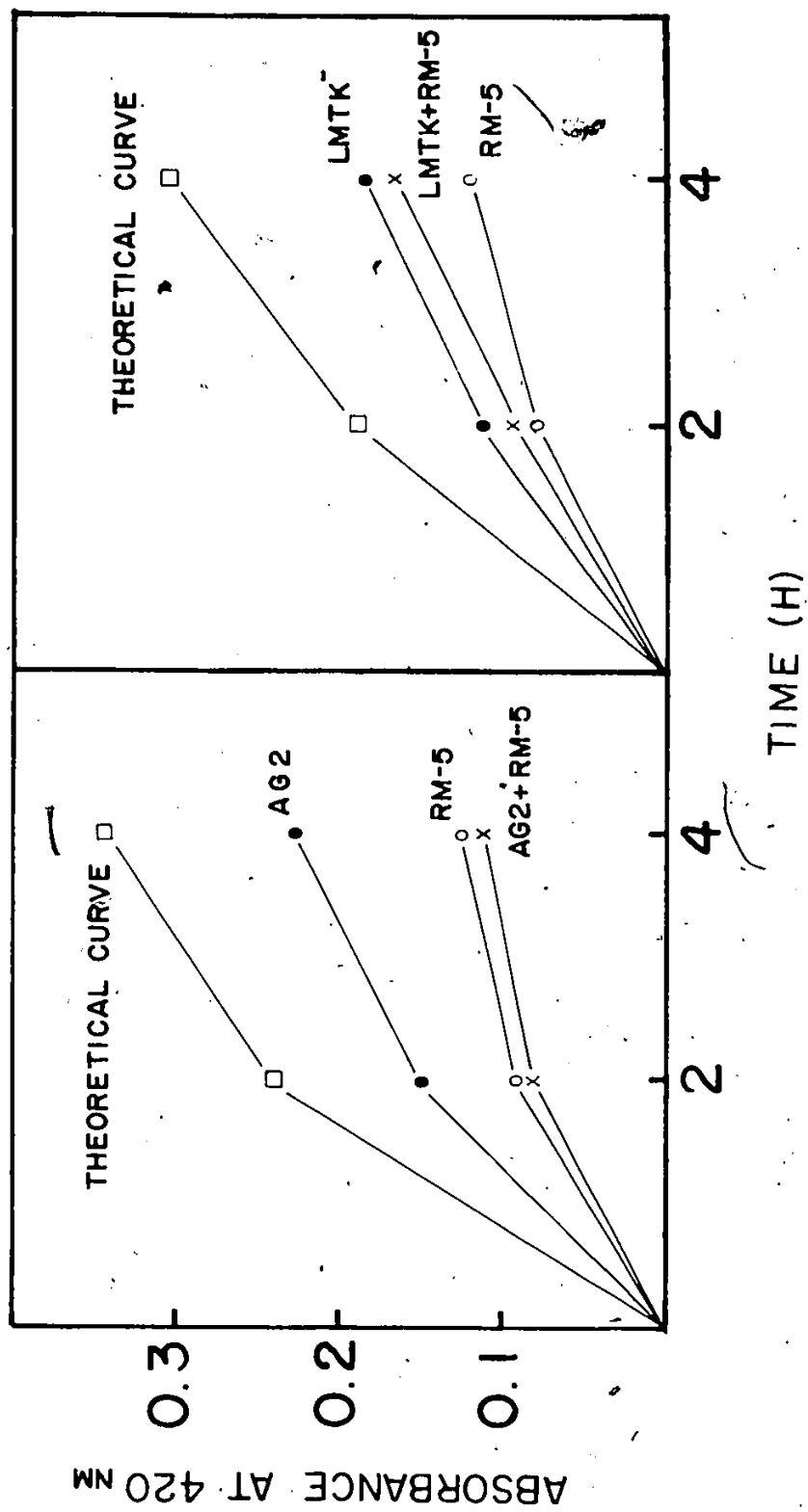
The homogenate from RM-5 was mixed with AG2/LMTK so that the protein content of the homogenate in the reaction mix was 2 mg/ml.

Left panel: AG2 + RM-5, x—x (2 mg/ml); AG2 alone, ●—● (1 mg/ml); RM-5 alone, o—o (1 mg/ml);

Theoretical curve, □—□

Right panel: LMTK + RM-5 x—x (2 mg/ml); LMTK alone, ●—● (1 mg/ml); RM-5 alone, o—o (1 mg/ml),

Theoretical curve, □—□



I. Cathepsin B activity in normal and dystrophic primary cell strains.

The activity of cathepsin B in homogenates from normal and dystrophic primary cells grown in culture was determined at various ages (3 months, 4.5 months and 6 months). The results shown in Table 4 demonstrate that the specific activity of cathepsin B is similar in the primary cell strains from normal and dystrophic animals after 3 and 6 months in culture but distinctly different after 4.5 months in culture. When these cells were treated for auto-activation of cathepsin B (pH 4.5, 30° C), the activity increased significantly in homogenates from dystrophic cells at various times in culture but not in homogenates from normal cells at any time in culture studied (Fig. 17). It was also observed that the extent of cathepsin B activation in dystrophic cells decreased with time in culture. Thus a culture maintained for 3 months showed greater activation than that observed at 6 months.

When homogenates from normal and dystrophic cells at 3 months in culture were mixed in equal proportions the cathepsin B activity in the dystrophic cell homogenate decreased 2-fold compared to the theoretical value had the enzyme activities been additive (Fig. 18). These results suggest that there are inhibitory substance(s) present in the homogenates from normal cells which, under

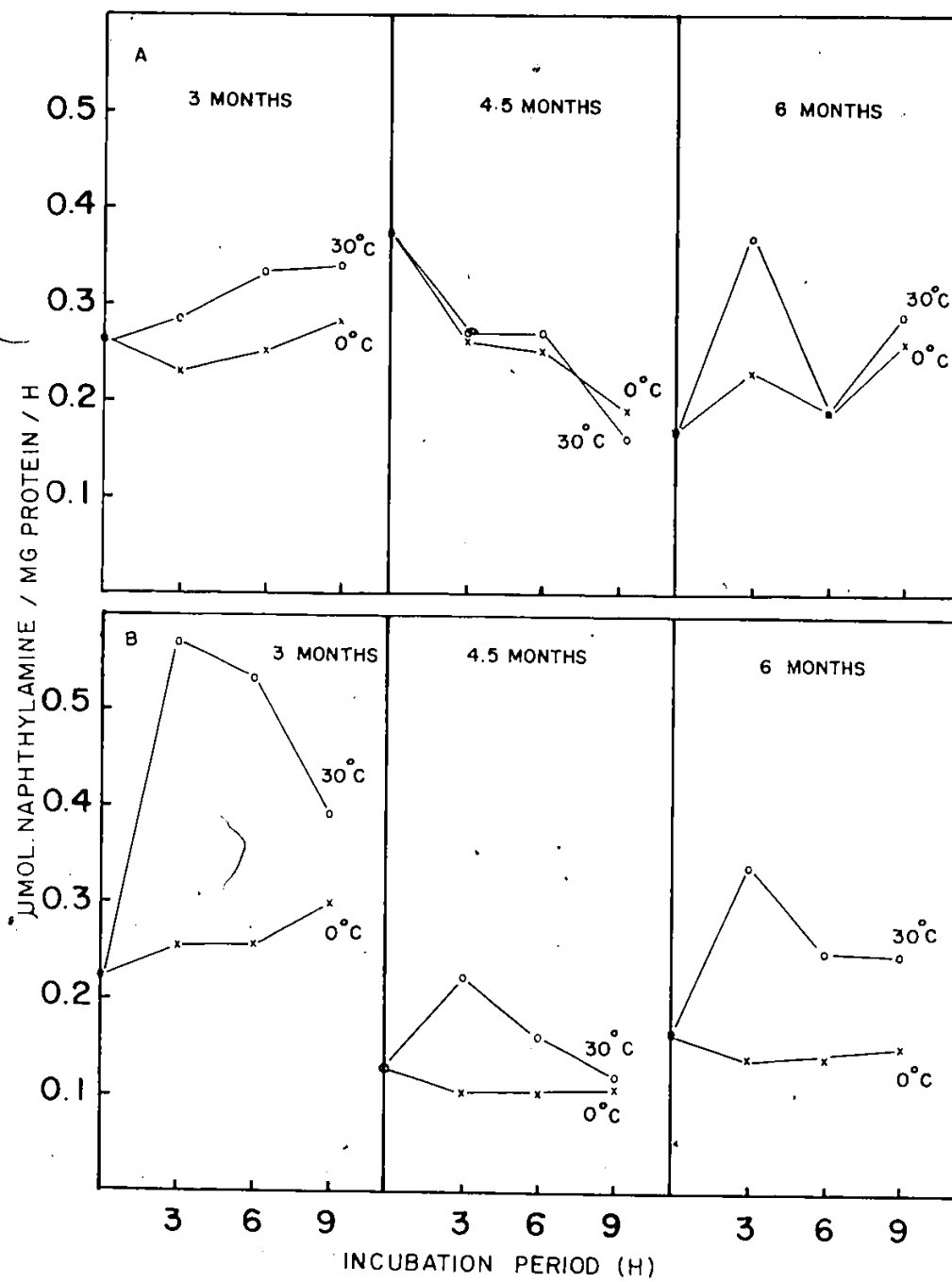
Table 4: Specific activities of cathepsin B in homogenates of normal and dystrophic cells at different stages

Age of culture	Normal	Dystrophic
3 months	0.26	0.22
4.5 months	0.37	0.13
6 months	0.16	0.17

Fig. 17. Cathepsin B activity after activation of homogenates from normal and dystrophic primary strains.

Top panel: Normal cell culture at different times in culture. Samples at 0°C, x—x; Samples at 30°C o—o.

Bottom panel: Dystrophic cell culture at different times in culture. Samples at 0°C, x—x ; Samples at 30°C, o—o.



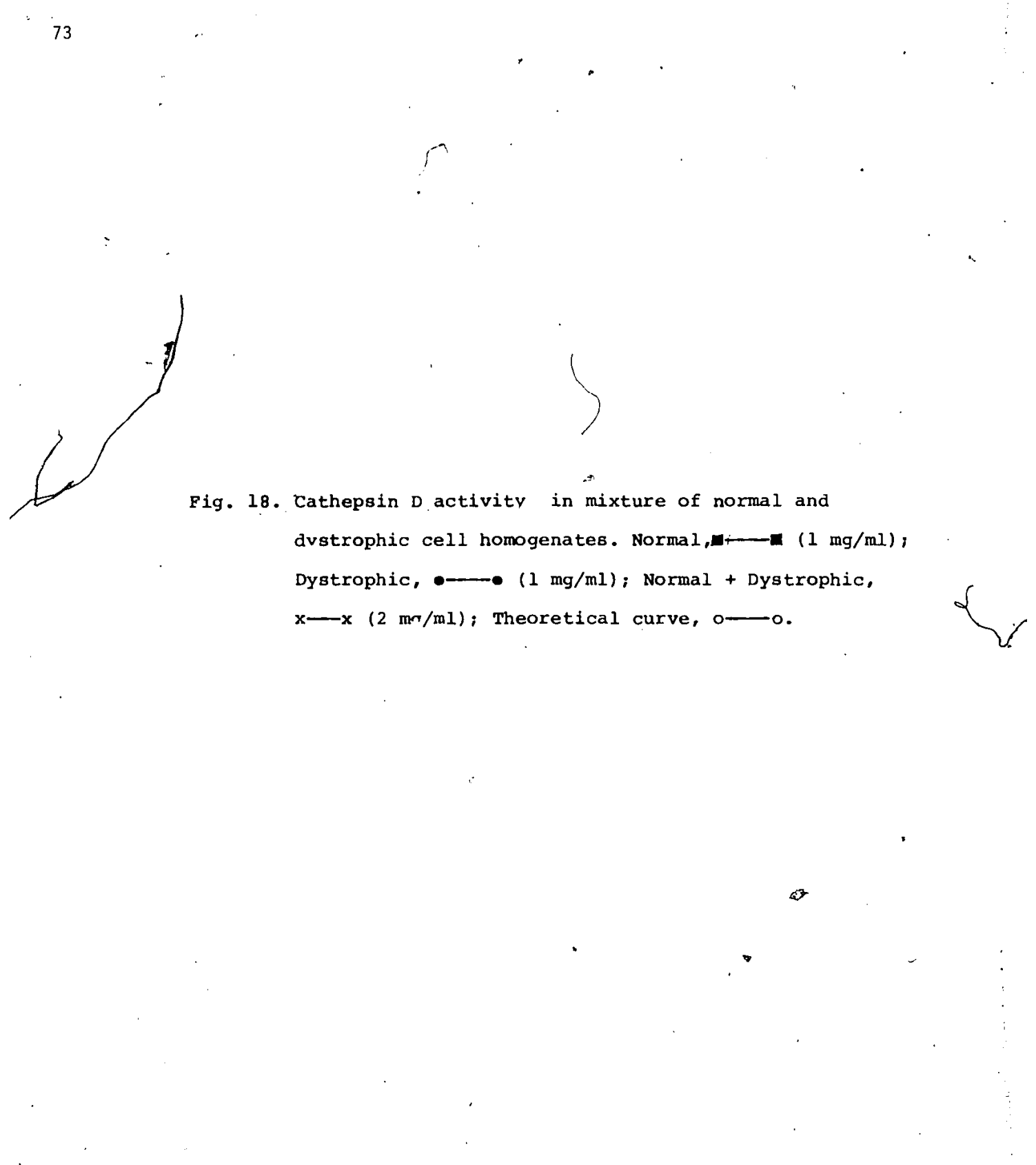
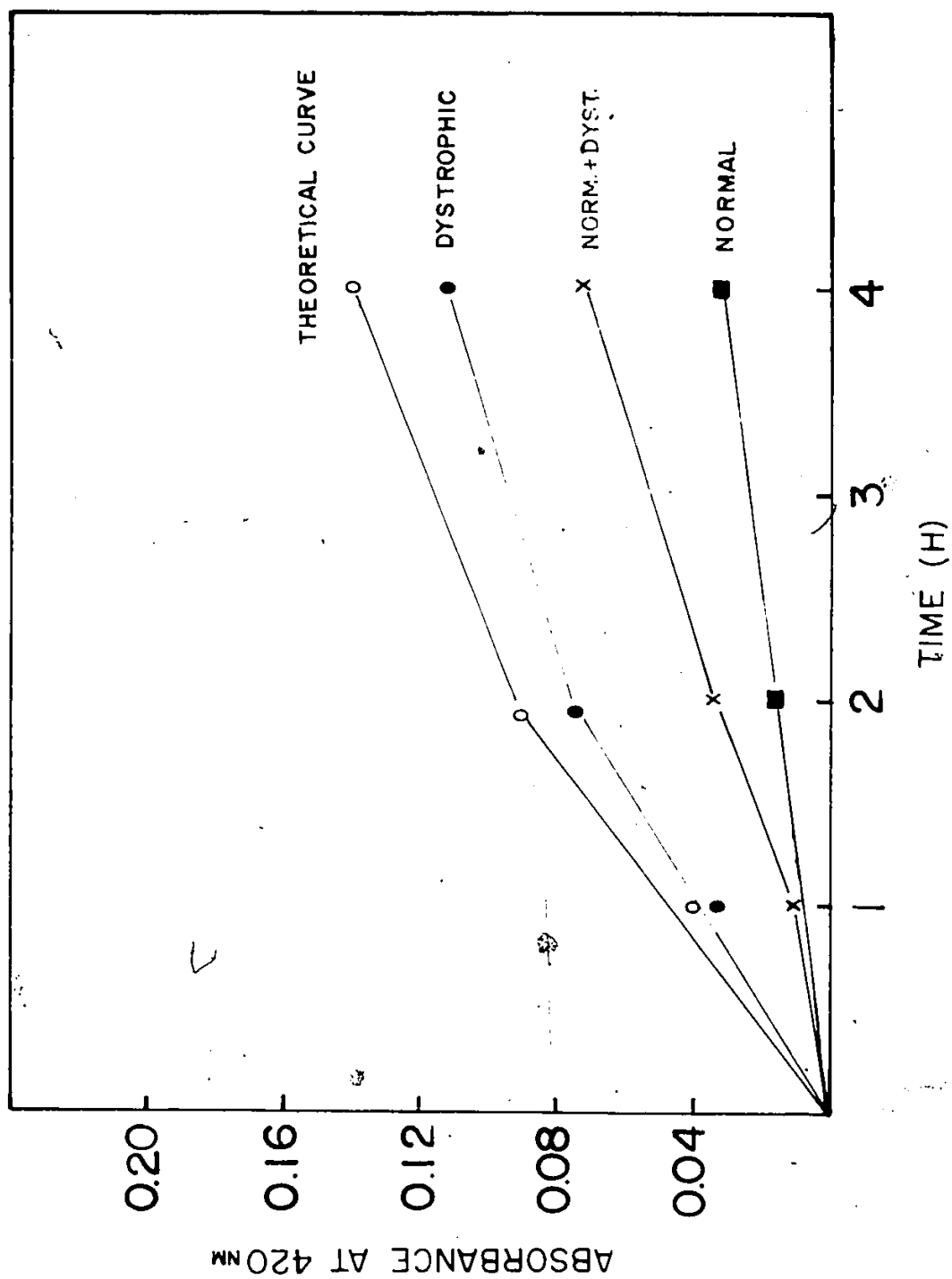


Fig. 18. Cathepsin D activity in mixture of normal and dystrophic cell homogenates. Normal, ■—■ (1 mg/ml); Dystrophic, ●—● (1 mg/ml); Normal + Dystrophic, x—x (2 mg/ml); Theoretical curve, o—o.



normal conditions, depress the expression of cathepsin D activity in these cells. Also, dystrophic cells appear to contain less inhibitor(s) of cathepsin D (and perhaps B) than normal cells.

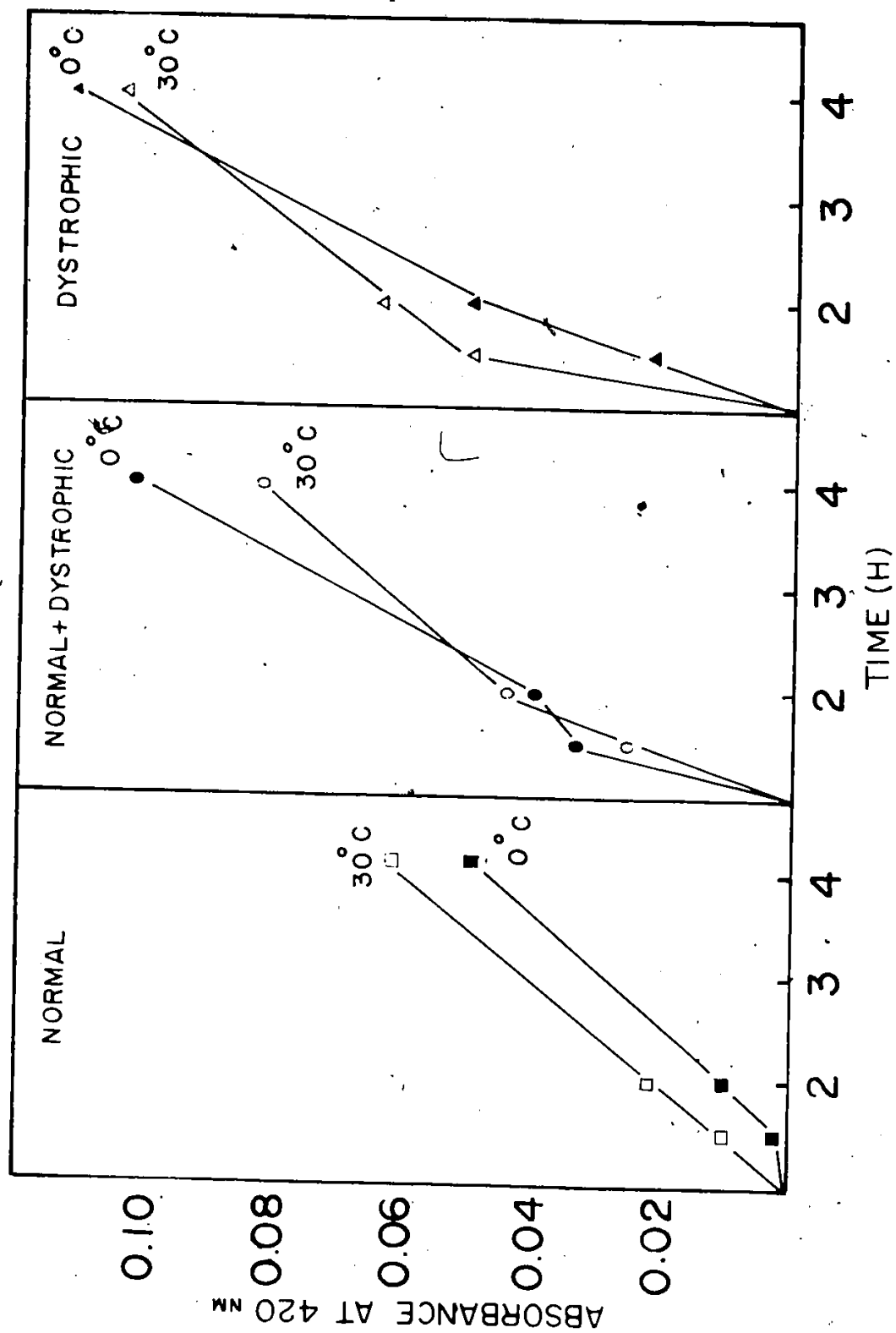
To further test for the presence of protease inhibitor(s), the mixed sample was preincubated in 0.8 M acetate buffer at pH 4.5 and 30° C for 1 hour and then assayed for cathepsin D activity. The results in Fig. 19 show that cathepsin D activity in the dystrophic cell homogenates (at 30° C) increased 1.4-fold but cathepsin D activity in normal cell homogenates did not. In fact, the preincubated sample had only 0.003' enzyme units whereas the control maintained at 0° C had 0.011 units. The mixed sample showed similar activity at both 0° C and 30° C and after 4 hours had a value of 0.026 enzyme units. However, the activity is much closer to the dystrophic than the normal cell homogenates. At this stage the results suggest the presence of a higher concentration of inhibitor(s) in normal cells than in dystrophic but the evidence is still not conclusive.

Fig. 19. Cathepsin D activity after activation of the homogenate mixtures of normal and dystrophic cells at 30°C for 1 h. Cathepsin D assay was done as described in the Methods.

Left panel: Normal at 0°C, ■ — ■ ; Normal at 30°C, □ — □

Middle panel: Normal + Dystrophic at 0°C, ● — ● ;
Normal + Dystrophic at 30°C, ○ — ○ ;

Right panel: Dystrophic at 0°C, ▲ — ▲ ; Dystrophic at 30°C, △ — △.



IV. DISCUSSION

In muscle as in other tissues, protein degradation as well as synthesis determines the concentration of cell protein (Goldberg and John, 1976). Therefore changes in the overall rate of protein catabolism can contribute to muscle growth or atrophy (Goldberg and John, 1976 ; Goldberg et al. 1974). Proteases serve a variety of functions aside from protein turnover. These include limited proteolysis in metabolic regulation and protein maturation, and degradation of extracellular proteins and peptide hormones (Neurath, 1975). Although many proteases exist in muscle which might degrade muscle protein it is not known which enzymes actually function in this way in various pathological states. Both lysosomal and non-lysosomal proteases can hydrolyse muscle protein (Kozalka and Miller, 1960; Reddy et al., 1975).

One of the salient features of muscular dystrophy, whether hereditary or nutritional in origin, is the failure of dystrophic muscle to maintain normal levels of protein. Since virtually all of the lysosomal acid hydrolases that have been measured in inherited and nutritional forms of muscular dystrophy show increased activity (reviewed by Weinstock and Iodice, 1969; Pennington, 1979; Strickland et al. 1979), these enzymes frequently have been implicated as a major factor in the muscle

wasting process. To gain further insight into the possible role of lysosomal acid proteases and of the rate controlling steps in the muscle wasting process of dystrophy (and in intracellular protein catabolism in general), a study of the levels of cathepsin B and D in normal and dystrophic cells and tissues has been carried out. The results of the present investigation have extended the current information on the involvement of hydrolases (lysosomal) in diseased conditions by demonstrating increased protease activity in dystrophic cells and tissues compared to normal controls.

A. Cathepsin D activity in mouse muscle.

It has been proposed that cathepsin D may be involved in a variety of pathological processes related to inflammation. Cochrane and Aiken (1966) investigated the enzyme basis of some abnormal skin reactions in rabbit. In these reactions, numerous polymorphonuclear leucocytes accumulate in the vessel walls and increase the permeability of the vessel. The increased permeability was attributed to the degradation of the vascular basement membrane by the lysates from the lysosomes which contains cathepsin D. There has been much interest in the idea that cathepsin D may be important in the degradation of the proteoglycans of the cartilage matrix in arthritis (Lucy et al., 1961 ; Dingle,

et al., 1971,1973).

Our findings on the activity of cathepsin D in dystrophic and normal skeletal muscle are in agreement with several other publications. Canonico and Bird (1970) have observed two different populations of lysosomes; one set associated with macrophages and leucocytes and the other associated with muscle cells. The non-myocytic cells are known to contain the highest concentration of lysosomal enzymes of any cell (Straus, 1967). Similar observations have been made by Canonico and Bird (1969). These observations emphasize the significant lysosomal contribution made by non-myocytic cells to the total acid hydrolase picture in the muscle tissue. The fragility of these lysosomes are said to increase in the pathological state. In the present investigation the increase in total cathepsin D activity of dystrophic muscle (about 2.2-fold) could be due to the increase in lysosomal activity of non-myocytic cells. The kinetics of proteolysis using extracts from normal and dystrophic muscle also differ considerably. Extracts from normal muscle show linear kinetics whereas extracts from dystrophic muscle show biphasic kinetics. The decrease in the rate of proteolysis after 2h could be due

to end product repression of the initial reaction or release of an inhibitor. Since multiple forms of cathepsin D have been observed in many tissues (Press et al., 1960; Barrett, 1970) this also might indicate a difference in cathepsin D enzyme between normal and dystrophic tissues. Whether the difference in kinetics of the two tissues is due to different isoenzyme composition remains to be determined.

Firfarova and Orekhovich (1971) have observed an inactive precursor of cathepsin D in chicken liver. Two components of the enzyme were isolated on a DEAE cellulose column, one with high proteolytic activity and another with low activity. On storage of the low activity fraction at pH 3.0, the activity increased to as much as that of the other component with high activity. In the course of this reaction an additional, highly active component and an inactive component of low molecular weight were formed. The role of the inactive component however, is not known. Unfortunately, there is no follow up of this work. A similar kind of precursor activation could be occurring in normal and dystrophic muscle preparations incubated at pH 4.5. Furthermore, it might be interesting to define the role of the component released from the protease precursor in the muscle.

This component could be the inhibitor controlling the activation. However, based on our present data it is difficult to speculate on the importance of such a reaction in the dystrophic organism.

B. Cathepsin B activity in mouse muscle tissue.

A comparison of cathepsin B activity in extracts from skeletal muscles of normal and dystrophic animals shows that dystrophic muscle has 3.4-fold more activity than normal muscle. Similar results have been obtained by Schwartz and Bird (1977): Consideration of the total enzyme units in the soluble fraction gives a clearer picture of the protease activity. Thus 2.05 gm of normal muscle which contained 26.8 mg/ml protein had 0.67 units of cathepsin B activity. Similarly, 1.05 gm of dystrophic leg muscle, which had 15.5 mg/ml protein had 2.29 units of cathepsin B activity. However, the actual amount of the enzyme present is not known because the assays were conducted on crude extracts which contain protease inhibitors.

Kar and Pearson (1976) reported that patients with muscular dystrophy and related disorders had muscle cathepsin B concentrations 2-6 times higher than normal. Since the extracts contained endogenous inhibitors of these enzymes, it would be

interesting to ascertain whether or not the enzyme levels are elevated in dystrophic muscle due to depressed inhibitor levels or increased number of enzyme molecules. When crude enzyme extracts of certain tissues were autolysed at pH 4.5, the activities of cathepsin B increased considerably. The optimum pH for activation of rat cathepsin H was 3.5-4.5 (Lenney et al., 1979). This phenomenon has been observed by many other workers (Schwartz and Bird, 1977; Barrett, 1977). The increase in enzyme activity during autolysis probably occurs due to a drop in inhibitor concentration resulting from proteolysis. In the present investigation the increase in cathepsin B activity resulting from activation is less in dystrophic muscle than in normal tissue. The increase in dystrophic tissue is 1.7-fold, whereas in normal muscle it is 2.5-fold. Also, the specific activity of the enzyme in dystrophic tissue prior to activation is slightly higher than the specific activity of extracts from the normal tissue after 2h of activation (compare 0.10 unit with 0.090 unit, Fig. 2). This suggests that there is some inhibitory substance in the normal tissue homogenate which is absent in dystrophic tissue. However, the dystrophic enzyme does show some activation at 30° C. This could mean that the inhibitor levels

are depressed in dystrophic homogenate compared to normal homogenates.

C. Changes in cathepsin B and D in mouse liver tissue.

The liver is an organ which is extremely active in the synthesis and degradation of proteins. In the present study we found that the specific activity of cathepsin B and D was elevated by only 1.3-fold in the liver from dystrophic mice compared to the normal. Since the total cathepsin B and D activity in the dystrophic mouse liver was the same as in the normal control liver, the slight increase in specific activity appears to be due to a reduction in weight and general protein content of the liver from the dystrophic mice. Moreover, since both normal and dystrophic livers were perfused in situ, it is unlikely that the slight increase in cathepsin B and D activity in dystrophic tissue is the result of this treatment (Motimore et al., 1973; Neeley et al., 1974). De Duve (1959) has summarized evidence indicating that liver hydrolytic enzymes play an important role in autolysis and necrosis. In comatose rats, considerable transfer of liver lysosomal enzymes from particulate to the soluble state was noticed (Beaufay et al., 1959). When liver lobes in rats were rendered ischemic by ligation, the progressive release of lysosomal hydrolases was found

to precede general necrosis (De Duve et al., 1959). Becker and Baron (1961) have related the opening of neuronal lysosomes to post-mortem autolysis and anoxic ischemic encephalopathy in rats. Whether lysosomal rupture is a part of early state of tissue injury or whether it is secondary to this damage, ie. involved as a type of scavenging mechanism, is still not understood.

Mice affected with muscular dystrophy are generally smaller than their normal litter mates (about 50% of normal body weight) (see Table 1). However, diminution in muscle mass is considerably greater than the differences in body weight. The liver weight is only slightly reduced in dystrophic mice compared to normal controls. Similar observations have been made by Simons et al. (1962). The weight of gastronemius muscle in dystrophic animal was 1.05 gm as compared to 2.05 gm in normal animal, but the weight of the liver in both cases was around 1.3-1.5 gm. Sandow and Brust (1958) have made similar observations where the weight of the muscle is only half that expected on the basis of body weight of the dystrophic mice. This observation suggests that the liver may not be involved in the dystrophic process. Furthermore, Simon et al., (1962) have also shown that there is no difference in

the protein-turnover pattern in liver between dystrophic and normal mice. Comparison of cathepsin B and D activities in liver with those of muscle shows that cathepsin B and D are several orders of magnitude greater in liver than that of muscle in both normal and dystrophic mice. Autolysis experiment at pH 4.5 and 30° C did not show any significant difference between the normal and dystrophic liver although both responded with a 1.2-fold increase in activity compared to controls at 0° C (Fig 5). Also, Schwartz and Bird (1977) have observed that the liver cathepsins B and D are less active in myosin degradation than muscle cathepsin B and D while the activities of both eluted within the same molecular weight range from a gel filtration column. Since different cathepsin B-like enzymes have been reported to occur in rat (Davidson and Poole, 1975; Towatari et al., 1976), a likely explanation for these results is that enzyme preparations from liver contain different isoenzymes of cathepsin B than that isolated from muscle.

Although the experiments conducted in this study do not show involvement of the liver catheptic enzymes with dystrophy, they certainly aid in understanding the significance of muscle hydrolytic enzymes in the pathophysiology of the disease.

7

D. Changes in cathepsin B and D in cardiac muscle tissue.

The mechanism by which protein degradation is regulated in heart is poorly understood. Proteolytic enzymes are undoubtedly involved, but the relative roles of lysosomal versus non-lysosomal processes and the relative importance of the specific types of proteases still remains unclear. A non-lysosomal protease with an alkaline pH optimum has been described to be active in rat heart (Sue et al., 1978). It has been suggested that this enzyme may play a causal role in cardiac myopathy. Marakami and Uchida (1978) have suggested the existence of a seryl proteolytic enzyme in the myofibrillar fraction of rat cardiac muscle, which degrades heart muscle myosin. Similar observations have been made by Kuo et al., (1981). The present investigation was undertaken to study the role of catheptic enzymes B and D on heart muscle of mice affected with muscular dystrophy.

Strobeck et al., (1979) have observed increased cathepsin D activity in late stages of cardiac myopathy when myocardial hypertrophy and cardiac failure are prominent. At one month of age they did not observe any change in specific activity of the diseased and control hearts but at 6 months they observed a 30%

increase in cathepsin D activity. Cardiac ischemia is associated with increased cathepsin D activity (Wildenthal, 1978). In the present investigation we observed no significant difference in specific activity of cathepsin B or D in dystrophic compared to control (normal) hearts. Since the mice were only 2 months old, it may have been too early to observe any changes. However, the results presented herein seem to confirm the observations made by others.

E. Changes on cathepsin B and D activity in lung tissue.

In general, normal lysosomal functions are concerned with adsorption, defence, differentiation and cell death. When considering the lysosomal function in the lung, an additional facet of their physiological role can be postulated, that being to transport specific lipids from the interior of the cell through the surface membrane to the outside. This special mechanism of exporting lipid materials raises a number of questions connected with the lamellar bodies. The function of other lysosomal enzymes associated with lamellar bodies is also quite uncertain, especially as they are likely to operate catabolically.

Moriyama and Takahashi (1980) have reported the presence of cathepsin D in a variety of primate lungs. Singh and Kalnitsky (1980) have observed two different enzymes, cathepsin B and BANA hydrolase, which are very

active in rabbit lung. However, the role of these proteases in normal physiology is not clearly known.

We report for the first time, the differences in catheptic enzymes B and D in normal and dystrophic lungs. The dystrophic lung has 1.4-fold higher activity of cathepsin B and D compared to normal lung. The significance of this difference is not known. Since respiratory failure is common in dystrophy, it is possible that death occurs or at least promoted by a reduction in the elasticity of the lung tissue caused by damage rendered by the catheptic enzymes. It could however, also be due to lack of, or inactivation of inhibitors which control protease activity. Based on our data, it is difficult to speculate on these hypotheses but it is certainly an area of research worthy of further exploration.

F. Changes in cathepsin B and D in kidneys tissue.

Kidneys are the most important organs in maintaining the homeostasis of the body. The vital conditions of fluid and electrolyte balance and acid-base balance are controlled by the kidneys. Kidneys are also the site of storage and breakdown of reabsorbed proteins. Lysosomes in the kidney cells are functionally important organelles under both physiological and pathological conditions and they participate in heterophagy as well as autophagy (Maunsbach, 1969).

The lysosomes of the proximal tubule cells have so far been investigated more intensively and in greater detail than the lysosomes in other renal cells. The enzymes identified by Straus in the first isolated lysosomal fraction from kidney were acid phosphatase, β glucosidase, cathepsin, acid ribonuclease and acid deoxyribonuclease. Later studies have shown several other hydrolytic enzymes such as arylsulfatase, β glucosidase, α -glucosidase (Shibko and Tappel, 1965), β aspartylglucosylamine amide hydrolase, β -N-acetylglucosaminidase (Mahadevan and Tappel, 1967a), sialidase (Mahadevan et al., 1967b), arylamidase (Mahadevan and Tappel, 1967c), glucocerebrosidase, galactocerebrosidase and sphingomyelinase (Weinreb et al., 1968). Thus kidney lysosomes appear to have the potential to degrade a large variety of substrates, ranging from proteins and carbohydrates to glycoproteins and sphingolipids.

In the present investigation cathepsin B and D activities in both normal and dystrophic kidney are very high as in the case of liver. But there is no significant difference in the activities of the normal and dystrophic extracts. On autolysis at pH 4.5 and 30° C, there is activation shown by both the tissues, but again the difference is not significant. These data show that kidneys are probably

not involved in the process of dystrophy.

G. Cathepsin B and D activity in rat myoblast cell line,
L69/1.

There now appears to be substantial evidence to support the hypothesis that the lysosomal apparatus of muscle cells are involved in the terminal degradation of the myofibrillar proteins in normal as well as in pathological tissues (Bird, 1975). In studies utilizing muscle homogenates it is difficult to assign a special role to a particular enzyme or group of proteases in the degradation of myofibrillar proteins because of the cellular heterogeneity of the muscle tissue (macrophages, fibroblasts, nerve fragments, epithelial cells in addition to myoblasts). Therefore there is no certainty that a specific enzyme (ie., cathepsin B or D) originates solely from myocytes. The present study was undertaken to determine the protease activity in developing cultured myoblast and fibroblast cells to circumvent this problem.

The striated muscle cell cultures show an extensive and early development of the lysosomal apparatus (Canonico and Bird, 1970). The significance of highly developed lysosomal apparatus in muscle cells is at present unclear (Bird et al., 1980). However, it is apparent that the lysosomes and its complement of acid hydrolases are intimately involved in the dramatic

morphological changes associated with differentiation of myotube. These changes probably involve the recycling of membrane elements of the cytoplasm during the fusion process, assembly of contractile apparatus, activation of enzymes required for muscle metabolism and development of an excitable membrane (Bird et al., 1980).

We compared the specific activities of lysosomal proteolytic enzymes, cathepsins B and D, in cultured myoblast cells at different stages of development with that of the adult mouse muscle extract. The specific activity of cathepsin B in post-fusion cells is 50-fold higher than the specific activity in normal adult muscle extracts. In similar experiments, Bird et al., (1981) observed a 90-fold increase. Cathepsin B activity is seen to increase throughout development. The lowest cathepsin B activity was seen in cells at the confluent stage and the highest in cells at post-fusion where it is 5-fold greater than in cells at confluence. The reason for the measurable increase in activity in cells following fusion is, as yet, unknown but it may reflect changes in the activities of protease inhibitors rather than the number of protease molecules. The specific activities of cathepsin B increased in parallel with the process of differentiation. These observations are in conformity with those of Bird et al., (1981).

Study of the autolysis of myoblast cell homogenates at pH 4.5 and 30° C did not show any activation of cathepsin B at any stage of myogenesis. This could be due either to the absence of pro-cathepsin B or the presence of large amounts of inhibitor(s) in myoblast cells. The presence of such inhibitors has been observed by Lenney (1976), Schwartz and Bird (1977), and Kar and Pearson (1976). A similar lack of response to autolysis was observed with cells of the AG2 line which is a fusing mutant of the L69/1 clone.

Further, the differences in cathepsin B and D activity at different stages of myoblast differentiation are significant. The specific activity of cathepsin D remains relatively constant between confluence and post-fusion stages (Table 2), whereas cathepsin B activity increases gradually. The functional implications of these differences in lysosomal enzymes remains to be demonstrated.

H. Cathepsin B activity in mouse L-cell fibroblast cell line, LMTK.

The mouse L-cell fibroblast cell line, LMTK shows cathepsin B activity which is 5-fold lower than the differentiated myoblast (Table 2). Autolysis of the cell homogenates at pH 4.5 and 30° C shows a 2-fold increase in activity at the end of 8h compared to controls at 0° C. This increase in activity could reflect the inactivation

of cathepsin B inhibitor at pH 4.5. An increase in the proteolytic activity of non-myocytic cells has been suggested as one of the causes in the pathological conditions (Libby and Goldberg, 1978) but our data do not support this view.

I. Activation experiment with the rat myoblast x mouse fibroblast hybrid, RM-5.

The autoactivation experiment with the hybrid, RM-5 (hybrid between LMTK and AG2) along with its parental controls LMTK and AG2 (which does not show activation) was done to ascertain the presence of inhibitors in AG2. We observed that the hybrid did not show any activation and further experiments carried out with homogenates of the hybrid pre-incubated in presence of a homogenate from AG2 also does not show any increase in activity. This certainly supports the presence of a inhibitor(s) in AG2 which may act by suppressing the activity of cathepsin B from LMTK in the hybrid. Depressed activities in hybrid cells is a well known phenomenon, but whether this is due to the inhibitory activity of one gene product on another- or to some other factor -remains to be seen. Furthermore, comparison of the protein bands on 2-dimensional gels of these cells, shows that in RM-5 many of the protein bands present in the parental cell lines are absent, preferentially those of LMTK (Dufresne, unpublished observations). More

work done in this direction should advance our understanding of the process of differentiation in myoblasts.

J. Activation experiment with primary cultures of normal and dystrophic cells.

Interesting observations have been made from the study of autolysis of normal and dystrophic primary cell strains. The study was to ascertain whether or not there was an inhibitor in the normal cells which controlled the activity of the cathepsins B and/or D. The normal primary fibroblast cells were studied at ages 3 months, 4.5 months and 6 months in culture. Parallel studies of cells from dystrophic muscle (i.e. times in culture) showed that the dystrophic cells respond to autolysis by increasing cathepsin B activity 2.5-fold whereas the normal cells do not respond to autoactivation (see Fig 16). The extent of activation of the dystrophic primary cell strain is about the same as that of the established fibroblast cell line, LMTK. The increase in specific activity in the whole muscle preparation on autolysis is 2 to 5 fold which is close to that observed in established fibroblast cell line. Thus the increase in activity seen in adult muscle on activation could be due to activation of cathepsin B precursors from fibroblast cells in the muscle. Since enhanced activities in fibroblasts have been implied in pathological conditions (Libby and Goldberg, 1978), the increase in cathepsin B activity

in dystrophic cell homogenates could be due to the lack of inhibitor(s) controlling the fibroblast activation. In fact mixing the two homogenates (dystrophic and normal) the activity of the mixture was decreased about 2 to 5-fold. These data support the view that inhibitors in the normal cell control the expression of various proteases while in pathological states such as muscular dystrophy, the low level of inhibitors is unable to meet the requirements of the cells in terms of protein-turnover regulation.

In summary, the observations reported in this thesis have revealed that there is a marked increase in the specific activity of cathepsin B and D in dystrophic cells and tissues compared to normal controls. Whether this increase is due to a decrease in regulator molecules or to increased synthesis of these proteases is not known. The observations made here strongly indicate the presence of an inhibitor/regulator in normal muscle and myoblast cells which appears to regulate the protease activity in normal tissues. In dystrophic cells and tissues there appears to be decreased levels of the same regulator(s). The response of normal and dystrophic muscle tissue extracts to Artemia protease inhibitor compared to the protease inhibitor leupeptin also indicates that differences may exist in the biochemical properties between normal and dystrophic

tissues. However, at this stage of study it is difficult to ascertain whether there is an imbalance in enzyme to inhibitor ratio or differences in the properties of the proteases from normal and dystrophic muscle which cause the disease.

However, these results emphasize the potential importance of protease activity and regulation in muscle dysfunction.

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